Cardiff School of Pharmacy and Pharmaceutical Sciences
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Design, Synthesis and Biological Evaluation of Nucleotide Pro-drugs Centred on Clinically Active Anticancer Nucleosides

A thesis submitted to Cardiff University in accordance with the requirements for the degree of Philosophiæ Doctor

by

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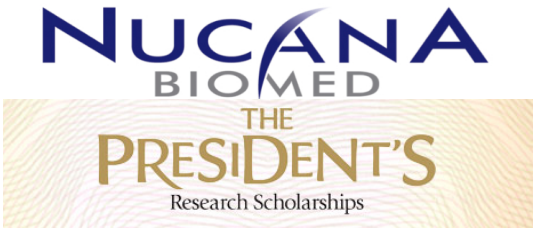
In collaboration with



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Abstract

Cancer is one the on the leading causes of mortality in world, causing 8.2 million deaths in 2012. In light of these statistics, the battle against cancer is ongoing. Nucleoside analogues are a major force in cancer chemotherapy. However, one problem accompanying nucleoside-based therapy is drug resistance, due to the abrogation of mechanisms that are crucial to their transformation to their bioactive metabolites (nucleoside phosphates). The ProTide technology was designed to overcome the limitations associated with nucleoside analogues. The technology enables the delivery of the nucleoside monophosphate into the cell by passive diffusion. Work in this thesis details the application of the aryloxyphosphoramidate and phosphorodiamidate pronucleotide approaches on potent anticancer purine and pyrimidine nucleoside analogues.

The work presented in this thesis shows that: I. ProTides of 5-fluorouracil-2'deoxyuridine (FUDR), the deoxyribonucleoside derivative of 5-fluorouracil (5-FU), were able to overcome several important cancer resistance mechanisms, including active transport and nucleoside kinase mediated activation, illustrated by a potent cytotoxic action in different cancer cell lines. Eight potential candidates were synthesised in large-scale and underwent a comprehensive lead selection, identifying NUC3373 for clinical trials, to start in 2015; II. The successful application of ProTide and phosphorodiamidate technologies to 6-thioinosine and 6-thioguanosine did not improve their activity nor did it help in clarifying their mechanism of action. 6-S-Methyl-thioinosine and its ProTides exhibited far greater efficacy compared to 6-thioinosine; III. Application of the ProTide technology on cladribine provided

proof of the enhanced potencies of 3'-ProTide derivatives over their 5'-counterparts; IV. 2'-deoxy-5-azacytidine (Decitabine) ProTides did not exhibit an improvement in activity compared to the parent nucleoside in different cell models of cancer; V. The bioactivation mechanisms of ProTides using enzymatic assays were successful.

Based on these findings, potential avenues to further explore are the cladribine and 6-S-methyl-thioinosine ProTide families, with the hope to identify new clinical candidates.

Publications

McGuigan, C.; Derudas, M.; **Gönczy, B.**; Hinsinger, K.; Kandil, S.; Pertusati, F.; Serpi, M.; Snoeck, R.; Andrei, G.; Balzarini, J.; McHugh, D. T.; Maitra, A.; Akorli, E.; Evangelopoulos, D.; Bhakta, S. ProTides of N-(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide as potential anti-tubercular and anti-viral agents. *Bioorg. Med. Chem.* **2014**, *22*, 2816-2824.

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Abbreviations and Acronyms

5-AzaC	5-azacytidine
5'-NT	5' nucleotides
5-Fu	5-fluorouracil
6-MP	6-mercaptopurine
6-TG	6-thioguanine
FUDR	5-fluoro-2'deoxyuridine
FdUMP	5-fluoro-2'deoxyuridine monophosphate
A	Adenine
AA	Amino acid
Ac	Acetyl
ACN	Acetonitrile
ADA	Adenosine deaminase
AIBN	Azobisisobutyronitrile
AML	Acute myelogenous leukaemia
AMP	Adenosine monophosphate
Ar	Aryl
ATP	Adenosine triphosphate
B	Nucleobase
Boc	*tert*-Butyloxycarbonyl
Bn	Benzyl
Bz	Benzoyl
BTEA-Cl	Benzyltriethylammonium chloride
BzCl	Benzoyl chloride
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CatA	Cathepsin A, carboxypeptidase A
CDA	Cytidine deaminase
cHex	Cyclohexyl
Cld	Cladribine, 2-chloro-2'deoxyadenosine
ClogP	Calculated logP
Cpd	Compound

*C*ycloSal	Cyclosaligenyl triester
CPY	Cytochrome P_{450}
DBU	1,8-diazabicyclo(5.4.0)undec-7-ene
d5AzaC	Decitabine, 5-aza-2'deoxycytidine
DCC	*N,N*-dicyclohexylcarbodiimide
dCK	Deoxycytidine kinase
DCM	Dichloromethane
DIPEA	Disopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Concentration of drug that causes 50% response
Et_3N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol
FDA	Food and Drug Administration
FUDR	5-fluoro-2'-deoxyuridine
G	Guanine
Gly	Glycine
HCL	Hairy cell leukaemia
hCNT	Human concentrative nucleoside transporter
hENT	Human equilibrative nucleoside transporter
HINT1	Human histidine triad nucleoside binding protein
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IC_{50}	Concentration of drug that causes 50% inhibition
IMPDH	Inosine-5'-monophosphate dehydrogenase
*i*Pr	Isopropyl
L-Ala	L-alanine
L-Ile	L-isoleucine
L-Leu	L-leucine

L-Met	L-methionine
L-Phe	L-phenylalanine
LR	Lawesson's reagent
L-Val	L-valine
LC-MS	Liquid Chromatography-Mass Spectroscopy
Me	Methyl
MeOH	Methanol
Me_2Gly	Dimethylglycine
min	Minutes
MDS	Myelodysplastic syndromes
MS	Mass Spectroscopy
NA	Nucleoside analogue
NMI	*N*-Methylimidazole
Naph	1-naphthyl
NMR	Nuclear magnetic resonance
NT	Nucleoside transporter
Nuc	Nucleoside
OMP	Orotidine monophosphate
P	Phosphate
PAOB	*para*-acyloxybenzyl
Ph	Phenyl
POM	Pivaloxymethyl
ProTide	Pronucleotide
*p*TSA	*para*-toluenesulfonic acid monohydrate
RNA	Ribonucleic acid
rt	Room temperature
SATE	S-acyl-2-thioethyl
T	Thymidine
TP	Triphosphate
TBDMS	*tert*-Butyldimethylsilyl
TBDMSCl	*tert*-Butyldimethylsilyl chloride
*t*BuMgCl	*tert*-Butyl magnesium chloride
$tBuCH_2$	Neopentyl

*t*BuCH$_2$CH$_2$	Neohexyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPDSCl	1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane
TK	Thimidine kinase
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TPMT	Thiopurine s-methyltransferase
TRIZMA	2-Amino-2-hydroxymethylpropane
U	Uracil

1 Introduction

Cancer is one of the leading causes of mortality in the world and according to the world health organisation 8.2 million deaths were attributed to cancer in 2012.[1] By 2030, death by cancer has been projected to rise above 11 million globally.[1] Cancer causes 25% of all deaths in the United Kingdom (UK), and within this subpopulation, 75% of these deaths occur in people aged 65 and over.[2] In order to reduce mortality, there is an urgent need for the development of new highly efficacious and minimally toxic treatments for all cancer types.

1.1 Cancer

Cancer is a disease caused by abnormal cells that undergo uncontrollable mitosis and are able to invade tissue either in situ or in remote areas of the body forming secondary focal proliferative lesions (malignant neoplasms) by a processes called metastasis.[3] Some neoplasms do not form a solid mass and this is true in the case of leukaemia[4-5] and a form of cancer known as carcinoma *in situ*.[6-8] The word tumour is often used to describe the physical appearance of a neoplasm and in general, can either be solid or cystic.[9-11]

1.1.1 Cancer classification

Cancer is classified according to the presumed origin of the neoplasm for example, carcinomas are epithelial of origin, the most common cancers such as those that form in vital organs and breast tissue, fall under this category.[12-13] Cancers arising from haematopoietic cells are called leukaemia or lymphoma depending on whether they

matured in blood or lymph nodes.[14-16] Other classes include, blastoma, sarcoma and germ cell tumours.[17-20]

1.1.2 Factors that cause cancer

There are many causes and risk factors for cancer, agents that cause cancer are referred to as carcinogens, and are usually categorised as biological, chemical or physical.[21-22] Carcinogens are highly capable of disrupting genetic homeostasis, these include biological entities such as bacteria, viruses and parasites, ionising radiation, and chemical entities that have the ability to destabilise DNA replication and repair mechanisms causing genetic mutations. Prolonged hormone stimulation, inherited and inborn genetic events and aneuploidy can cause cancer.[21-22] Of all cancer-related deaths, infectious agents cause approximately 15-20% of these deaths, 25-30% are due to tobacco use, 30-35% are related to diet, 10% are related to environmental carcinogens including radiation, and 5-10% are due to genetic aberrations.[23-26] Everyday lifestyle choices can be risk factors for cancer, with smoking, poor diet and alcohol misuse being major risk factors amongst many others.[3, 21, 23]

1.1.3 Cancer development and progression

Cancer is a heterogeneous disease capable of changing overtime.[21, 27] Cancer progression at the macroscopic level is shown in figure 1.1. The genetic paradigm features heavily in cancer research supported by many papers analysing the genetics of neoplastic cells destined to exhibit a malignant phenotype.[21, 27] Cancer progression is driven by chromosomal defects, mutations in proto-oncogenes and tumour suppressor genes.[3] Mutations in the coding or promoter region of a gene can render it non-functional and lead to a reduction in its expression, respectively. An imbalance

in normally functioning *proto-oncogenes* that are responsible for coding proteins that are responsible for regulating cell growth and division, and tumour suppressor genes that code proteins that repress the cell cycle, can lead to cancer.[28] The concept of cancer cell heterogeneity was first identified using histology where changes in cell morphology and phenotypic appearance were described moreover, cancer cell heterogeneity also extends to cell-to-cell genetic diversity, and diversity in biochemical and molecular biology properties.[3, 12, 21, 29] Cellular heterogeneity is not exclusive to abnormally dividing cells destined to form neoplasia, but is ever present in normally dividing cells too.[30-33] Cells with amassing chromosomal alterations, unregulated mitotic potential and mutational rates contributing to progressive genomic instability, have been identified in advanced cancer malignancies such as granulocyte leukaemia and Burkitt's lymphoma.[34-36]

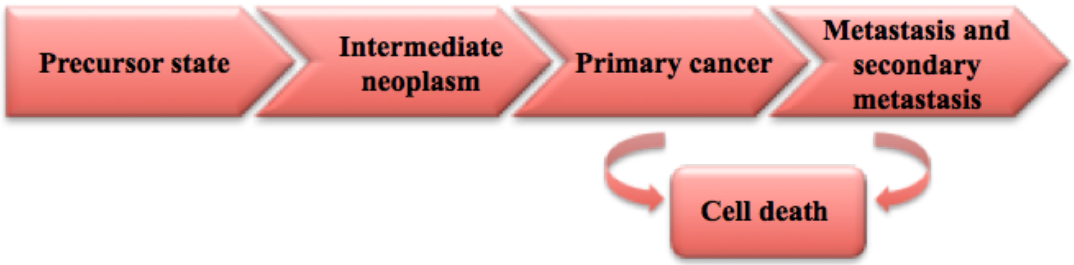


Figure 1.1 The process of cancer progression from its early precursor state to its advance metastatic phenotype. Figure adapted from Clark.[3]

1.1.3.1 Features of cancer

The Hallmarks of cancer is a series of reviews authored by Douglas Hanahan and Robert Weinberg, where they describe six distinctive and complimentary feature that favours neoplasm growth and metastatic potential.[21] The six hallmarks of cancer are: the ability to sustain proliferation; the ability to bypass growth suppressors such as the protein p53; the ability to invade local tissue and to metastasise; characteristic

unlimited replicative potential by upregulating the enzyme telomerase, thus stabilising the genome during repeated cell cycles; the ability to induce new blood vessel growth (angiogenesis); the ability to resist cell death processes such as apoptosis, autophagy and necrosis.[37-40] The six hallmarks of cancer are common to neoplasms undergoing malignant transformation and growth, and although some of the features stated are also phenotypic of benign masses, benign neoplasms do not have the capacity to invade local tissue and metastasise.[41-43]

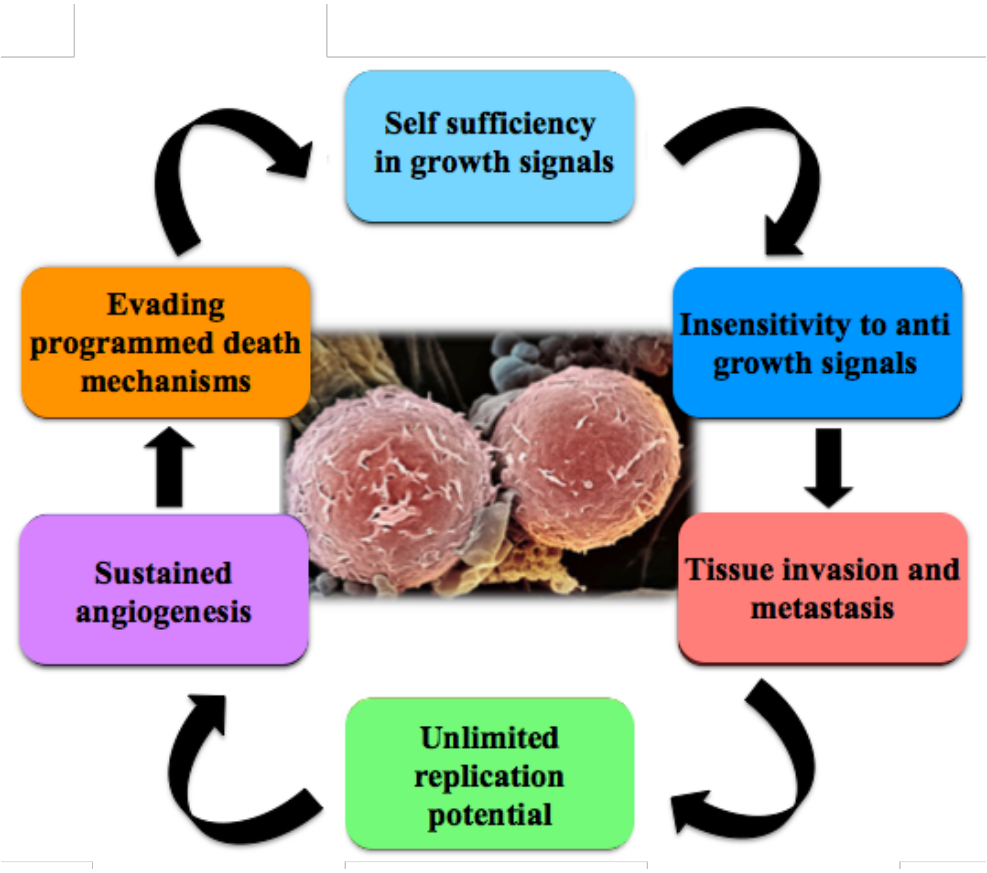


Figure 1.2 The hallmarks of cancer: processes that underly neoplastic cell proliferation. Figure by Hanahan and Weinberg's seminal review.[21]

An important feature that is common to malignant neoplastic masses is that they are highly glycolytic. A high rate adenosine 5'-triphosphate (ATP) turnover does not

occur exclusively in malignancies, as the need to utilise ATP derived from glycolysis and mitochondrial oxidative phosphorylation, is required by all cells.[44] However it is the high rate of glycolysis derived from lactate production, which is unique to cancer cells.[45] By nature, glycolysis is an inefficient route for ATP synthesis therefore in order to maintain ATP levels to satisfy the metabolic demand of neoplastic mass, the rate of glycolysis must surpass that normally observed in normal healthy tissues; glycolysis generates two moles of ATP for every mole of glucose whereas oxidative phosphorylation by comparison, produces 36 mol of ATP per mole of glucose.[46-47]

1.1.4 Thymidine kinase and cancer

Thymidine kinase (TK) is a highly conserved and ubiquitous enzyme in the pyrimidine salvage pathway that is present in organisms and viruses, and has a crucial role in DNA synthesis and cellular mitosis.[48-52] TK essentially functions as its names suggest, as a phosphotransferase enzyme that converts thymidine nucleoside to (deoxy)thymidine monophosphate, which is an essential nucleotide found in 2'-deoxyribonucleic acid (DNA).[48-52] The triphosphate component of deoxythymidine that is formed by the enzymatic action of nucleoside 5'-diphosphate kinase (NDK or UDP) is incorporated into DNA.[53-55] In higher organisms, TK exists as two distinct isoforms, type-1 (TK1) and type-2 (TK2) localised in different cellular compartments.[48, 50-52, 56-59] Taylor and colleagues provided comprehensive evidence to suggest there are two molecular forms of thymidine kinase with differing relative expression in human fetal and adult cells. Fetal thymidine kinase commonly referred to now as TK1 was shown to be more sensitive to high incubation temperature, and pH and exhibited different electrophoretic properties, compared to so-called adult thymidine kinase (TK2).[56] Work done by Hideo Masui and Leornard Garren on the

mechanism of action of adrenocorticotropic hormone and work done by Arnold J Berk and fellow scientists identified the subcellular location of different forms of TK.[48, 57, 60] Although both groups when discussing their contributions to understanding the distribution of TK in mammalian cells did not provide specific comment or use the term 'isoforms,' their research provided the first evidence for there being two distinct forms of TK. This work was later supported by Elsevier and coworkers and Willecke and colleagues, respectively they identified chromosomes 17 and 16 as the stores of the genetic information that encodes human cytoplasmic TK1 and human mitochondrial specific TK2.[61-62] The human TK1 gene was successfully cloned by Harvey D. Bradshaw in 1983.[49]

TK not only plays a key role in DNA synthesis but also plays a primary role in regulating thymidine pools during the cell cycle.[58-59, 63] TK activity although inconsistent is marked in proliferating cells particularly at the S-phase of the cell cycle, but stalls at daughter cell formation due to presumed enzymatic degradation (M phase; cytokinesis).[59] Kauffman and Kelly's data suggests the existence of TK degradation processes during specific phases of the cell cycle.[58-59] The upregulation of TK1 is prevalent in cells that are in S-phase, and this factor makes TK1 an attractive diagnostic marker in many human malignancies.[64-67] TK1 activity is elevated in the serum of patients presenting with different types of cancer ranging from acute forms of leukaemia, lymphoma, breast cancer and others, and is highly indicative of disease progression and severity especially when measured in conjunction with other cancer specific markers such as progesterone receptor (breast) and folate receptor over-expression (cervical).[65-74] TK2 is involved in the synthesis of mitochondrial DNA precursors, however it plays no role nor does its

activity correlate well with cell proliferation therefore making it an unsuitable diagnostic tool for cancer.[75-76]

1.1.5 Modelling cancer using immortalised cell lines

Human and rodent immortalised cell lines have significantly enhanced our understanding of how cancer develops and progresses, and have given us insights into the receptor-mediated and intracellular metabolic signalling pathways that allow cancer cells to increase metabolic demand and adapt to environmental changes in order thrive.[77-82] Furthermore, they have allowed us to identify the molecular genetics involved in many different types of cancer.[77-82] These immortalised cells not only provide us with invaluable information about human cancer biology but all represent excellent models for characterising the pharmacology of old and new chemical compounds that could have considerable therapeutic potential.

1.2 Treating cancer

Cancer is often treated by multiple strategies. Mainstay treatment still involves surgical removal of solid malignant neoplasm, however non-solid malignancies require alternative treatment.[83] Radiation therapy and chemotherapy are gold standard methods for stalling malignant cell proliferation or inducing cell death, combining both therapies can markedly improve clinical outcomes over that of mono-therapy.[84] Other approaches such as photodynamic therapy, radiofrequency ablation therapy, cryotherapy, monoclonal antibody therapy, inhibition of angiogenesis, kinase inhibition, vaccine related methods and gene therapy are all being investigated or utilised.[84] The induction of drug resistance is a major issue in

cancer chemotherapy since up to 50% of all malignant neoplasms have either *de novo* drug resistance or develop resistance after first treatment.[85-86] Radiation therapy and chemotherapy are discussed below.

1.2.1 Radiotherapy

Radiotherapy is one of the most important treatment modalities in the treatment of cancer malignancies with approximately 50% of all cancer patients receiving this form of cancer therapy.[87] Cancers exhibit different sensitivities to radiotherapy and the response of the neoplastic mass to radiation dose is inversely proportional to size thus large neoplasms respond less well than small neoplasms.[87] Most solid neoplasms excluding renal cell carcinoma and melanoma, respond moderately to radiotherapy characterised by a reduction in mass size.[87] Although blood-borne cancers are often highly sensitive to radiotherapy treatment using this method for these types of cancer is difficult and often not employed unless the cancer is localised to an area of the body.[87] Standard radiotherapy uses rectangular treatment fields however technological advances have led to the development of conformal radiotherapy, which improves dose delivery to the target neoplasm and markedly reduces the risk of damaging healthy tissue in close proximity.[88-89] Intensity modulated radiotherapy, image guided radiotherapy, three-dimensional conformal radiotherapy and charged particle radiotherapy are new advances. These inventions have improved neoplasm targeting, dose delivery and cumulative exposure, thus reducing toxicity to the patient and as consequence improving clinical outlook.[88-89]

1.2.2 Chemotherapy

Chemotherapy involves using chemical compounds to treat cancer that is at risk of metastasising or has metastasised.[90] Chemotherapy can either be curative, controlling or palliative (in advance malignancies) to improve symptoms and quality of life.[90] Chemotherapy is a variably effective treatment for a variety of cancers and these include testicular cancer, pre- and postmenopausal breast cancer, ovarian cancer, gastric cancer, trophoblastic disease, acute myeloid and lymphoblastic leukaemia, Hodgkin's and non-Hodgkin's lymphoma and paediatric solid neoplasms.[91-99] Low molecular weight compounds are often used in chemotherapy for the treatment of cancer.[84] The advantage of such an approach is that low molecular weight compounds can circulate around the body and gain easy access to almost all tissues, so therefore can kill neoplastic cells at primary local and sites in other regions of the body.[84] Anti-cancer chemical agents are subcategorised as cytotoxic agents under the main category "malignant disease and immunosuppression" in the British National Formulary.[100] The BNF lists chemotherapy agents under the following classes: alkylating drugs; anthracyclines and cytotoxic antibiotics; antimetabolites; vinca alkaloids and etoposides; other antineoplastic drugs.[100] Examples of these agents amongst others are given in Table 1.1, while Table 1.2 summarise some of the recently approved cancer therapies.

Table 1.1 Anticancer drug classes

Drug classes	
DNA interactive agents	
• Alkylating agents • Cross linking agents • Intercalating agents • Topoisomerase inhibitors • DNA cleaving agents • Other alkylating agents	Dacarbazine,Temozolomide Nitrogen mustards, nitrosoureas, platinum complexes Anthracyclines: Doxorubicin, Daunorubicin, Topotecan, Irinotecan Etoposide, Teniposide Bleomycins, Enediynes Procarbazine, Dacarbazine Altretamine, Cisplatin
Antimetabolites	
• DHFR inhibitors • Purine antagonists • Pyrimidine antagonists • Thymidylate synthase inhibition • Adenosine Deaminase inhibition • Ribonucleotide Reductase inhibition	Methotrexate Mercaptopurine (6-MP) Thioguanine (6-TG) Fludarabine Phosphate Cladribine Pentostatin Fluorouracil (5-FU) Cytarabine (ARA-C) Azacitidine Gemcitabine Capecitabin Tegafur Raltitrexed Pentostatin Hydroxycarbamide
Antitubulin agents	
• Vinca alkaloids • The Taxanes	Vinblastine (Velban) Vincristine (Oncovin) Paclitaxel (Taxol) Docetaxel (Taxotere)
Hormonal agents	
	Tamoxifen (Nolvadex) Flutamide (Eulexin) Gonadotropin-Releasing Hormone Agonists (Leuprolide and Goserelin (Zoladex)) Aromatase Inhibitors
Miscellaneous anticancer drugs	
	Amsacrine Hydroxyurea Asparaginase

Table 1.2 Recently approved cancer therapies

Generic Name	Trade Name	Type	Class	Target
Tipiracil	Lonsurf	Small Molecule	Antineoplastic	DNA Synthesis (colorectal cancer)
Rituximab	Mabthera	Monoclonal antibody	Biologics	CD20 antigen on B cells (non-Hodgkin's lymphoma)
Trastuzumab	Herceptin	Monoclonal antibody	Biologics	HER-2 (Breast cancer)
Gemtuzumab	Myolotarg	Monoclonal antibody	Biologics	CD33 (Myeloid Leukaemia
Brentuximab vedotin	Adcetris	Monoclonal antibody	Biologics	CD30 (Hodgkin lymphoma; anaplastic large cell lymphoma)
Bevacizumab	Avastin	Monoclonal Antibody	Biologics	Vascular Endothelial Growth Factor A Angiogenesis
Ramucirumab	Cyramza	Monoclonal Antibody	Biologics	Vascular Endothelial Growth Factor Receptor 2 (gastric adenocarcinoma)
Resminostat		Small Molecule	Histone deacetylase inhibitor	HDAC (cutaneous T-cell lymphoma)
NV1020		Oncolytic virus	Biologics (oncolytic virus)	Tumour cells (metastatic colorectal cancer)
Cabozantinib	Cabometyx	Small Molecule	Tyrosine Kinase inhibitor	Receptor Tyrosine Kinase (renal cell carcinoma)
Nivolumab	Opdivo	Monoclonal Antibody	Biologics	PD-1 receptor on T cells (Hodgkin lymphoma)
Atezolizumab	Tecentriq	Small molecule	Programmed death inducer	Programmed death-ligand (PD-L1) (urothelial carcinoma)
Venetoclax	Venclexta	Small molecule	BCL-2 inhibitor	BCL-2 inhibition and caspase activation (lymphocytic leukaemia)
Talimogene laherparepvec	Imlygic	Oncolytic virus	Biologics	Induce the production of the immune stimulatory protein GM-CSF (Unresectable recurrent melanoma)

Chemotherapy kills cancer cells by directly or indirectly targeting ribonucleic acid (RNA) and 2'-deoxyribonucleic acid (DNA), perturbing synthesis and damage response mechanisms and as a consequence, blunts uncontrolled cell division and induces cell death.[101] The propensity for chemotherapeutic agents to target nucleic acid synthesis underpins the unwanted toxicity associated with these agents even at therapeutic doses.[102] Common acute toxicities associated with cancer chemotherapy are cardio- and pulmonary toxicity, nephrotoxicity and haemorrhagic cystitis, hepatoxicity, gastrointestinal toxicity and dermatologic toxicity.[102] Subsequent sections will focus on a specific class of nucleic acid perturbing agents known as the antimetabolites, which over many decades have shown some therapeutic success. Despite this, efforts have been made over the years to improve the delivery, efficacy and unwanted toxic side effect profile associated with antimetabolite driven chemotherapy.[103-104]

1.3 Nucleotides

Nucleotides are the building blocks for nucleic acid synthesis, monomers that are comprised of a sugar moiety either ribose (in RNA) or 2'-deoxyribose (in DNA), a hetrocyclic nucleobase (nitrogenous base) attached to the 1' position on the sugar, and at least one phosphate group attached to the 5' position on the sugar.[105] The phosphate on the 5'carbon attaches to the 3' carbon on the next sugar as the nucleic acid chain grows.[105] In the absence of a phosphate group, the unit is known as a nucleoside.[105] There are five common nucleobases used in the construction of nucleotides: the purines adenine (A) and guanine (G) and the pyrimidines cytosine (C), thymine (T) and uracil (U), with the latter forming a base pair with adenine in

RNA.[106-108] Nucleotides exist in mono-, di- and triphosphate forms, with the triphosphate having an important role in enzymatic reactions and cellular metabolism. Nucleotide triphosphates form the repeating units of DNA and RNA, and are important second messengers in many intracellular signalling processes and examples of these molecules are adenosine- and guanosine 3',5'-cyclic monophosphate (cAMP and cGMP).[109]

1.3.1 Ribonucleic acid (RNA)

RNA is involved in coding, decoding and regulating gene and protein expression in mammalian cells and some viruses use an RNA genome to encode their genetic information.[110-111] The hydrogen bond pairing of ribose containing nucleotides (Watson-Crick canonical base-pairs) harbouring the bases, guanine, cytosine, adenine and uracil forms RNA.[105, 108] Non-canonical base pairing can occur in nature forming bulges and hairpin loops and these for example can feature in ribosomal (rRNA) and telomerase RNA (TERC) due to adenine-adenine and adenine-guanine base pairing.[112-114] RNA mostly functions as a single stranded molecule however its form can change by complementary intra-strand base pairing forming a double helix (TERC).[115] RNA is less stable than DNA as it contains ribose that has a hydroxyl group on the 2'carbon of the sugar making it more susceptible to backbone breakage because the hydroxyl group can act as a nucleophile and chemically attack the adjacent phosphodiester bond.[116] The hydroxyl group in the 2' position also ensures that RNA adopts the A-form geometry and not the B-form geometrical form commonly seen in DNA.[117] See figure 1.3 for the structural constituents of RNA

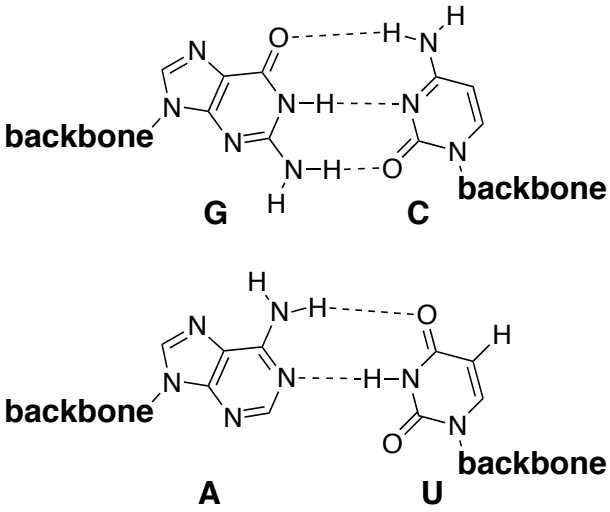


Figure 1.3 Nucleotide base pairing in RNA: Guanine (G); Cytosine (C); Adenine (A); Uracil (U)

There are different forms of RNA, messenger RNA (mRNA) that carries the genetic information from DNA to the ribosomes to translate the amino-acid sequence for protein synthesis, transfer RNA (tRNA) that transfers specific amino acids into the growing polypeptide chain, rRNA the catalytic component of ribosomes catalysing peptide bond formation, small nuclear RNA (snRNA) that is responsible for processing pre-mRNA and non-coding RNA (ncRNA).[118]

RNA is synthesised (transcription) by an enzyme called RNA polymerase that transcribes RNA using a DNA derived template. Transcription begins when RNA polymerase binds to the promoter sequence of DNA, and following unwinding of the double helix by DNA helicase, proceeds along (3' to 5') the template strand synthesising a complimentary RNA strand with extension occurring in the 5' to 3' direction.[119-122] Termination of RNA synthesis is dictated by the DNA sequence.[119-122]

1.3.2 2'-Deoxyribonucleic acid (DNA)

DNA is the main constituent of chromosomes and carries genetic information that is important for the function of all living organisms and viruses (excluding RNA viruses) however in humans only 2% of DNA carries genetic information.[123-124] Friedrich Meischer first isolated DNA in 1871, and then Watson and Crick description of the double helical structure of DNA was published in 1953.[105, 125-126] DNA is formed by nucleotides containing the nucleobases adenine, guanine, cytosine and thymine attached at the 1' position of the sugar 2'-deoxyribose and phosphate group at the 5' position. Unlike ribose, 2'-deoxyribose does not harbour a hydroxyl group at the 2'-carbon thus the backbone of DNA is not susceptible to nucleophilic attack, making it a very stable molecule furthermore nucleobase stacking ensures DNA integrity.[105] DNA in mammalian cells exists as a double helix formed from nucleotide repeats held together by hydrogen bonds and phosphodiester bonds along its backbone.[105] The asymmetric ends of DNA, 5' (terminal phosphate) and 3' (terminal hydroxyl), run antiparallel as the two DNA strands entwine.[105] Mitochondrial DNA and telomere repeats form displacement loop triple stranded and quadruple stranded DNA respectively.[127-131] See figure 1.4 for the structural constituents of DNA.

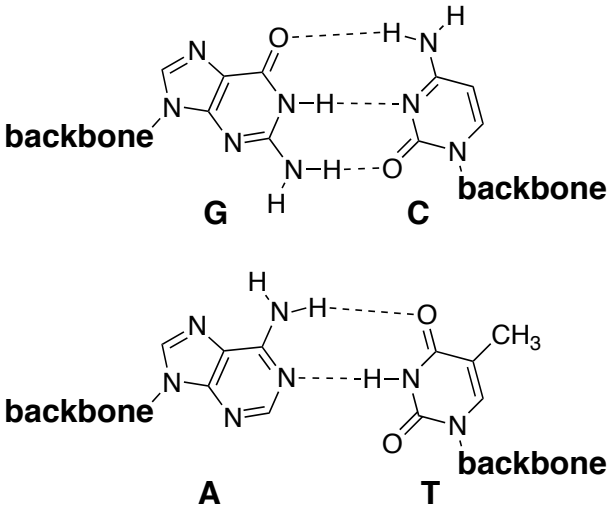


Figure 1.4 Nucleotide base pairing in DNA: Guanine (G); Cytosine (C); Adenine (A); Thymine (T)

DNA must be replicated in order to pass on genetic information during each cell cycle, the precision of the genetic material being passed on is crucial in ensuring that abnormalities are not inherited which can lead to disease and cell death.[132] During the S-phase of the cell cycle DNA is unwound by helicase and topoisomerase revealing two strands, complimentary DNA sequences are then synthesised by DNA polymerases (5' to 3' direction) called a leading- and lagging strand copy. The leading strand DNA replication proceeds continuously whereas lagging strand DNA replication continues in a stepwise manner with the addition of short discontinuous nucleotide sequences called Okazaki fragments that are joined together to form a complete strand by DNA ligase.[132]

1.4 Targeting polynucleotide synthesis: anticancer purine and pyrimidine nucleobase and nucleoside analogues

The halogenated pyrimidines 5-bromo, 5-chloro and 5-iodouracil were synthesised as antimalarial agents however, with the realisation that nucleic acid replication is

pivotal in neoplastic cell proliferation these compounds were targeted towards treating malignant neoplasms, with the hope that they would be incorporated into growing RNA and DNA chain, cause strand breaks and as a consequence cause genome rearrangement and cell death.[133-136] 2', 3'-dideoxynucleosides were developed as natural competitors to endogenous 2'-deoxynucleosides and 2'-deoxynucleoside-5'-triphosphates. 2',3'-dideoxynucleosides are phosphorylated to their corresponding 5'-triphosphate and incorporated in DNA by DNA polymerase causing DNA chain termination.[137] Well-known pyrimidine cytotoxic analogues are 5-fluorouracil, cytosine arabinoside, 5-azacytidine and 2', 2'-difluoro-2'-deoxycytidine.[138] The development of pyrimidine analogues of uracil was fuelled by the observation that uracil was more rapidly incorporated into preneoplastic rat liver than healthy liver, which encouraged Dushinsky and Heidelberger to synthesise 5-fluorouracil and its related fluorinated pyrimidine analogues.[138] The synthesis of purine analogues centred on the replacement of oxygen, nitrogen or carbon in the purine ring, with carbon-nitrogen or oxygen-nitrogen substitutions producing 8-azaguanine and 2,6-diaminopurine.[139] 6-Mercaptopurine is a clinically useful agent particularly in the treatment of leukaemia furthermore the purine analogues acyclovir and ganciclovir are used routinely to treat infections caused by herpes simplex virus, varicella zoster virus and cytomegalovirus.[140-142] According to the BNF, there are 13 purine and pyrimidine analogues used as antimetabolites in the treatment of solid and non-solid malignant neoplasms in the United Kingdom.[100] Purine and pyrimidine analogues relevant to this present work are analysed in far greater detail in Chapters 3, 4, 5 and 6. See Figure 1.5 for the structures of different purine and pyrimidine analogues.

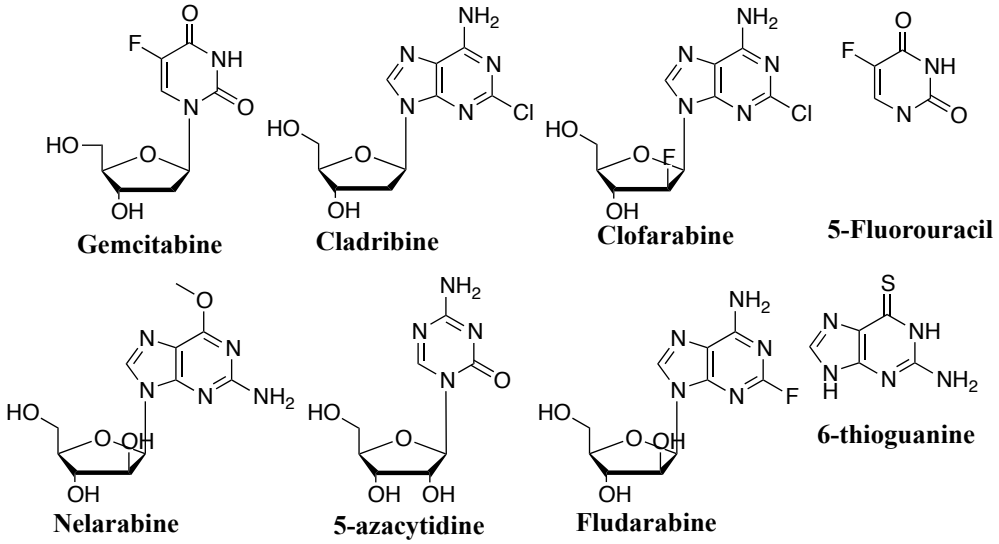


Figure 1.5 The structures of different anticancer purine and pyrimidine analogues

1.4.1 Membrane permeation of nucleobases and nucleosides

Cells rely on extracellular nucleosides and nucleobases to build nucleotides because de novo synthesis of purine and pyrimidine nucleotides is inefficient and high energy phosphate consuming.[143-147] Due to the hydrophilic nature of these molecules their movement across the membrane needs to be facilitated by specialised transporter proteins.[143-147] There are different specialised proteins that allow nucleosides to permeate biological membranes and these include equilibrium nucleoside transporter (ENT), concentrating nucleoside transporter (CNT), organic anionic transporter (OAT), organic cationic transporter (OCT) and ATP-binding cassette (ABC) transporter.[148-150] Antiviral nucleoside analogues can cross biological membranes via the ENT, CNT, OAT and OCT.[148-150] Anticancer nucleoside analogues appear to mainly enter cells through ENT and CNT.[151-152] ABC transporter and MRP are involved in antimetabolite drug resistance (see section 1.4.2). ENTs allow passive diffusion of nucleosides and their analogues.[153] There are four human ENTs encoded by the *SLC29* gene designated hENT1, hENT2, hENT3 and hENT4. hENT is more

abundant in the cell membrane however, it is also found in the membranes of subcellular organelles such as the endoplasmic reticulum and mitochondrion.[154-155] hENT1 and hENT2 are responsible for transporting most purine and pyrimidine nucleosbases and nucleosides and their analogues. CNT allows the unidirectional passage of nucleosides across the plasma membrane against the concentration gradient, in a sodium dependent manner thus distinguishing it from ENT.[153] The SLC28 gene encodes the CNT and there are five human forms, hCNT1, hCNT2, hCNT3, hCNT4 and hCNT5.[156] hCNT1-3 are distributed in similar fashion to hENT.[156] The characterisation of hCNT4 and hCNT5 is still in its infancy so their ability to transport nucleosides and nucleoside analogues is currently not known.[150] hCNT1 has a high affinity for pyrimidine nucleosides whereas purine nucleosides and uridine are transported by hCNT2. hCNT3 non-selectively transports both purine and pyrimidine nucleosides and respective synthetic analogues.[156] There are 10 OATs encoded by the *SLC22* gene, and their ability to transport a specific nucleoside is heavily dependent on the nucleoside's structure.[157-159] Many antiviral nucleoside analogues are substrates for OAT1 and OAT3 and these include acyclovir, didanosine, tenofovir, zidovudine and others.[160] OCT is a plasma membrane spanning protein encoded by the *SLC22* gene, which transports synthetic nucleosides (lamivudine and zalcitabine) but is incapable of transporting naturally occurring nucleosides.[161] There are three members of the OCT family; OCT1 and OCT2 are expressed in the liver and kidney, and OCT3 is expressed throughout the body.[161]

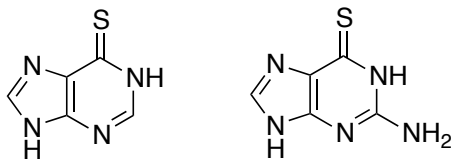
1.4.2 Nucleoside transport resistance mechanisms

Neoplastic cells can acquire resistance to nucleoside-based therapy due to the action of export transporters.[162]The ABC transporter family form the largest family of proteins in the body that mediate the bidirectional ATP-dependent transport of compounds.[162] They are important clearing compounds in alleviating unwanted toxicity in healthy tissue furthermore, they also have a central role in making malignant neoplasms resistant to nucleoside-based chemotherapy.[162] Multidrug resistance proteins (MRP1-9) are ABC transporters involved in drug efflux. These transporters can only clear monophosphorylated nucleosides from the intracellular environment. The *ABCC4* gene that encodes MRP4 is selectively amplified in human T-lymphoblastic leukaemia cells (CEM) conferring resistance to nucleoside analogues such as adefovir and azidothymidine.[163] Adenoviral induced overexpression of MRP4 in CEM cells induces resistance to thiopurine therapy (see section 1.5.1).[164] These studies prove that MRP4 is a genuine drug resistance protein. MRP5 has been shown to export the monophosphate metabolite of 5-fluorodeoxyuridine.[165] Cells engineered to overexpress MRP5, MRP7 and MRP8, have been shown to extrude thiopurine, gemcitabine, and fluoropyrimidine metabolites.[165-170]

1.5 Purine nucleobases relevant to the present work

1.5.1 6-Mercaptopurine and 6-Thioguanine

Azathioprine (AZA; Imuran; prodrug), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (see figure 1.6 for structures) collectively comprise a family of molecules known as thiopurines, which have been shown historically to exhibit remarkable anti-cancer, and anti-inflammatory properties.[140, 171-173] The immunosuppressant properties of AZA have improved post-operative graft survival and reduced solid-organ transplant rejection in the clinic, moreover both 6-MP and 6-TG not only have been shown to exert powerful dampening effects against the ensuing inflammation seen in disease states such as ulcerative colitis and Crohn's disease, psoriasis and rheumatoid arthritis, but have been shown in early studies by Clarke and others as potent inhibitors of neoplastic proliferation.[174-185] Following on from bench success, 6-MP was rapidly entered into clinical trials for the treatment of acute lymphoblastic leukaemia, the most common malignancy seen in children, which resulted in drastic improvement in survival from a cancer that otherwise had extremely poor prognosis.[186-188] 6-MP and its prodrug AZP were granted approval for use in clinical practice in 1953 and 1968 respectively by the US Food and Drug administration (FDA).[140]



6-mercaptopurine **6-thioguanine**

Figure 1.6 Structures of 6-mercaptopurine and 6-thioguanine

1.5.1.1 6-Mercaptopurine and 6-Thioguanine metabolism

Thiopurines require conversion to active compounds to produce their desired therapeutic effects, however according to Peter Karran the precise roles of different active metabolites generated through various chemical reactions and/or due to the actions of local enzymes have not been fully elucidated.[140] The metabolism of AZA alongside 6-MP and 6-TG will now be discussed. In brief, the prodrug AZA is activated by a non-enzymatic reaction involving glutathione that allows the removal of the substituted imidazole ring causing the release of active 6-MP.[189] 6-MP in turn is rapidly transported across the cell membrane where it enters the purine salvage pathway.[189] The first enzymatic step in the purine salvage pathway involves hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), which catalyses the addition of a ribose 5-phosphate generating thioguanosine monophosphate (TGMP) and thioinosine monophosphate (TIMP) from 6-TG and 6-MP respectively.[140] Thiopurine S-methyltransferase (TPMT) converts TIMP to methylated TIMP, which is a potent inhibitor of purine synthesis, a property not exhibited by the methylated form of TGMP.[140] The methylation of thiopurines is a process of degradation and inactivation, and therefore an important process of regulating thioguanine nucleotide synthesis.[190-191]

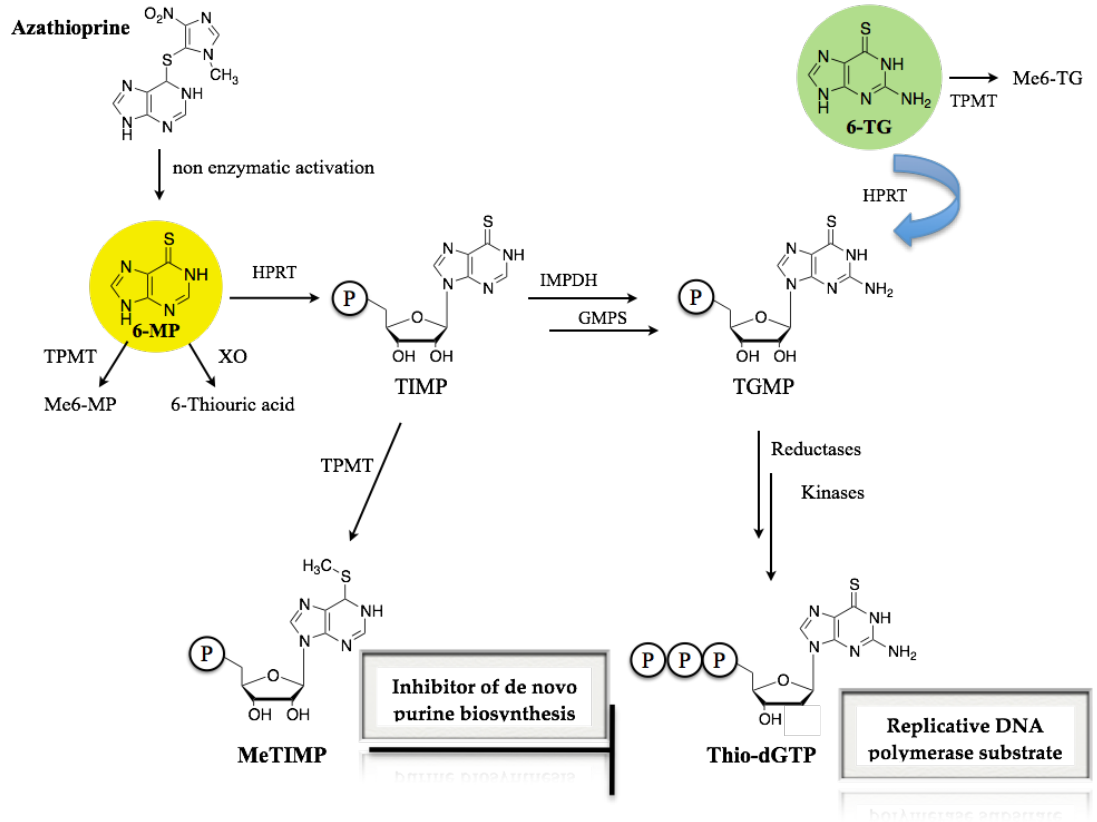


Figure 1.7 Thiopurines and their metabolism.
HPRT: hypoxanthine-guanine phosphoribosyltransferase; TIMP: thioinosine monophosphate; TGMP: thioguanosine monophosphate; TPMT: thiopurine S-methyltransferase; XO: xanthine oxidase; MeTIMP: methylated TIMP; IMPDH: inosine monophosphate dehydrogenase; GMPS: guanine monophosphate synthetase; Thio-dGTP; 2'-deoxy-6-thioguanosine triphosphate

1.5.1.2 6-Mercaptopurine and 6-Thioguanine and cell death

The dual action of deoxynucleoside kinase (dNK) and reductase enzymes on TGMP produces the metabolite thio deoxyguanosine triphosphate that is incorporated into RNA and DNA by their respective polymerase enzymes.[192-193] Under normal circumstances low level DNA substitution by 6-TG (approx 0.01-0.1% of guanine bases are replaced by TG) does not impact greatly on cellular genomics, however the generation of much higher levels induces chromosomal damage and apoptosis.[173, 194-197] A single mutation in the gene coding for HPRT1 can result in neoplastic cell populations that are resistant to 6-TG, allowing them to escape cell death during

thiopurine therapy.[140] Another mechanism or contributing factor that is thought to kill cancer cells during regimented thiopurine therapy is purine starvation, a process that is dependent on 6-MP formation from AZP.[140, 198-202] Methylated TIMP as mentioned earlier potently inhibits phosphoribosyl pyrophosphate amidotransferase (PPAT), the initial enzyme in *de novo* purine biosynthesis providing the building blocks for RNA, DNA, high-energy phosphate (ATP) and other phosphate species.[140, 198-202]

1.5.1.3 Human polymorphisms limiting therapeutic use of thiopurines

Polymorphisms of TMPT can result an enzyme that is less efficient at purine breakdown. One in every three hundred patients are homozygous for the sub-functional TPMT alleles, TPMT*3A and 3C, resulting in reduced thiopurine methylation.[140, 198-202] Excessive free purine in the body can lead to haematologic toxicity that involves myelosuppression and accompanied by massive decreases in white blood cell counts.[201, 203] Pre-therapy screening is therefore required to identify individual patients that are at risk of serious bone marrow toxicity before proceeding with any course of thiopurine therapy.[203]

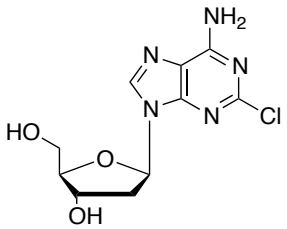
1.6 Purine nucleosides relevant to the present work

1.6.1 Cladribine

Adenosine deaminase (ADA) deficiency causes lymphospecific cytoxicity and accumulation of deoxypurine nucleotides.[204] ADA is a key effector of purine metabolism.[204] There are two isoforms, ADA1 and ADA2, of which the former is ubiquitously expressed in the body.[205] Although ADA2 is moderately expressed in

the body, its activity is increased in breast cancer and leukaemia.[205-207] For this reason adenosine analogues that are highly susceptible to deamination are not useful for treating cancer unless used in combination therapy with an ADA inhibitor such as pentostatin.[208] 2-chloro-2'-deoxyadenosine (cladribine; see figure 1.7 for structure) is a potent anticancer and immunosuppresive adenosine deaminase-resistant nucleoside analogue, indicated for the treatment of symptomatic hairy cell leukaemia.[209-210] Cladribine is resistant to deamination-induced inactivation, due to its chlorinated purine ring.[211-212] Cladribine was synthesised alongside several other halogenated deoxyadenosine derivatives in the late 70s and early 80s.[213-214] Beutler and collegues first used cladribine in 1981 to treat acute myeloid leukaemia.[215] Cladribine is used in combination therapy with pentostatin and rituximab for the treatment of hairy cell leukaemia after disease recurrence.[216-217]

Merck withdrew cladribine (in tablet form), from the market in 2011 for the treatment of multiple sclerosis following failure to get the drug approved by the FDA and the European medicines agency.[218]



Cladribine

Figure 1.8 The structure of 2-chloro-2'-deoxyadenosine (Cladribine)

1.6.1.1 Cladribine metabolism and resistance mechanisms

Cladribine is transported into cells by hENT1 and hENT2 where it is acted on by deoxycytidine kinase and converted to its 5'-monophosphate derivative.[211-212] Further phosphorylation steps by adenosine monophosphate kinase and nucleoside

diphosphate kinase produce the diphosphate and triphosphate metabolites respectively.[189-190] The latter is incorporated into DNA, causing inhibition of DNA methylation and synthesis. Cladribine depletes nucleoside triphosphate pools and also induces sustained expression of the cellular tumour suppressor antigen p53, thus inhibiting cancer progression.[219]

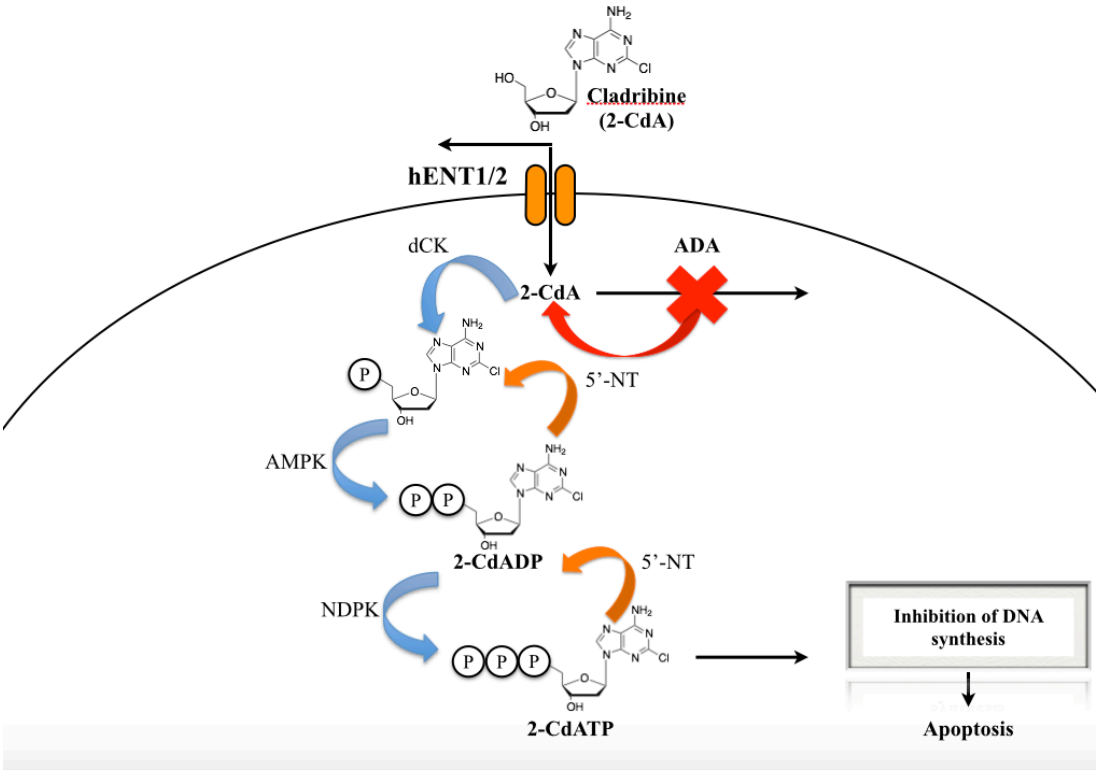


Figure 1.9 Intracellular metabolism of Cladribine
ADA: adenosine deaminase; 5'-NT: 5' nucleotidase, dCK: deoxycytidine kinase; AMPK: adenosinemonophosphate kinase; NDPK: nucleoside diphosphate kinase; P: phosphate.

5'-Nucleotidase dephosphorylates the 5'-monophosphate metabolite of cladribine preventing the accumulation of the cytotoxic cladribine-triphosphate metabolite, and this may present as a mechanism of resistance; the cytostatic action of cladribine is heavily dependent on the relative ratio between deoxycytidine kinase and 5'-nucleotidase.[220] Other reported mechanisms of resistance are decreased nucleoside

transport and the down regulation of programmed death pathways like apoptosis.[211-212, 221]

1.6.1.2 Cladribine toxicity

Lymphocytes have high levels of deoxcytidine kinase making them very susceptible to cladribine toxicity.[222] In the clinic, the most common serious toxicity associated with cladribine therapy is myelosupression.[223-225]

1.7 Pyrimidine nucleobases relevant to the present work

1.7.1 5-fluorouracil and 5-fluoro-2'-deoxyuridine

5-Fluorouracil (5-FU) was synthesised and patented by Charles Heidelberger in 1957.[138] 5-FU was shown to have potent anti-neoplastic effects particularly against colorectal and pancreatic cancer.[226] 5-FU and its cytotoxic metabolite 5-fluoro-2'-deoxyuridine (FUDR) (see figure 1.8 for structures) as part of the FOLFIRI regimen with leucovorin and irinotecan, are used in second line chemotherapy for patients with advanced small bowel adenocarcinoma.[227]

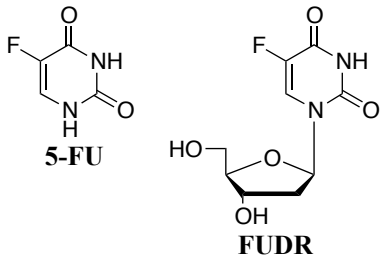


Figure 1.10 The structures of 5-Fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FUDR)

1.7.1.1 5-Fluorouracil metabolism

5-FU is trafficked across the cell membrane by facilitated transport systems, where it is converted by thymidine phosphorylase (TP) to FUDR.[228] Phosphorylation of the

deoxynucleoside by the phosphorotransferase TK generates the cytotoxic nucleotide 5'-fluoro-2'deoxy-monophosphate (5-FdUMP).[229] 5-FdUMP inhibits thymidylate synthase (TS) in the presence of a reduced folate (5, 10-methylenetetrahydrofolate), which reduces the ability of TS to remove fluorine from the five position on the molecule, therefore depleting (deoxythimidine triphosphate) dTTP which is a unit required for DNA synthesis. 5-FdUMP forms a dead end complex with TS reducing the synthesis of dTTP.[228-229] Another route for 5-FdUMP synthesis, is the conversion of 5-FUDP by ribonucleotide reductase (RNR) to fluorodeoxyuridine diphosphate (5-FdUDP).[230] Fluoropyrimidines compete with both biological substrates causing inhibition of RNR therefore augmenting DNA and RNA damage.[231-233] Other routes of DNA and RNA damage are the conversion of FUDR to 5-FdUTP and the conversion of 5FU to FUTP, perturbing DNA and RNA synthesis respectively.[231-233] This damaging effect on nucleic acid synthesis causes inhibition of the cell cycle at s-phase, and induces apoptotic cell death.[36]

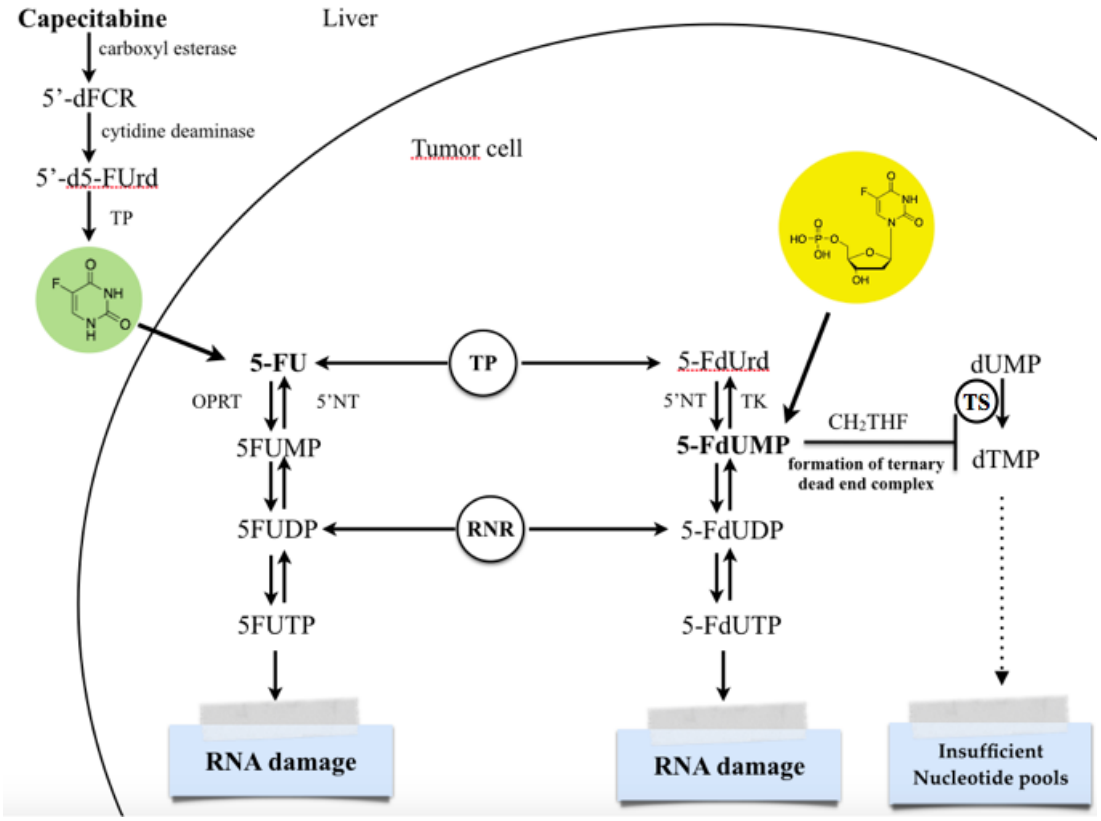


Figure 1.11 Metabolic pathway of Capecitabine, 5-FU and 5-FUDR
FdUMP: fluorodeoxyuridine monophosphate; FdUTP: fluorodeoxyuridine triphosphate; FUMP: fluorouridine monophosphate; FUTP: fluorouridine triphosphate; OPRT: orotate phosphoribosyltransferase; RNR: ribonucleotide reductase; TK: thymidine kinase; 5'NT: 5' nucleotidase; TS: thymidylate synthase; 5'-d5-FUrd: 5'deoxy-5-fluorouridine; 5'-dFCR: 5'deoxy-5-fluorocytidine.

1.7.1.2 Resistance associated with 5-fluorouracil therapy

Prolonged treatment with 5-FU compounds can generate a resistance phenotype in cancer forming cells, due to mass upregulation of TS, and this could be a reason for resistance to these types of drug in patients presenting with different malignancies.[234-236] Other postulated factors that can cause resistance: deletion of 5-FU transport mechanisms; deletion of TK; overproduction of TS; rapid phosphorolytic cleavage of FUDR to 5-FU by TP.[237-238] There are various

disadvantages associated with 5-FU therapy for example, toxicity due to lack of selectivity of the drug, most notably cardio- and neurotoxicity.[239-240]

1.7.1.3 5-Fluoro-2'-deoxyuridine therapy

FUDR, the prodrug of 5-FU, was approved by the FDA 1970, and has been used since to treat different malignant neoplasms of the breast, gastrointestinal tract and the ovaries.[241-251] The prodrug is particularly useful in the treatment of hepatic arterial metastasis over that of 5-FU because it is metablised in the liver more efficiently.[243] Furthermore, in cell models of cancer, FUDR exhibited remarkable anti-proliferation effects that far exceed 5-FU.[252] As its monophosphate FUDR is one of the potent TS inhibitors, however more accurately it is enzymatic production of 5-FdUMP from FUDR that is responsible for TS inhibition.[229] TS is involved in the de novo synthesis of thymidine. The conversion of 2'-deoxyuridine-5'monophosphate (dUMP) to thymidine-5' monophosphate (dTMP) by reductive methylation is catalysed by TS. Due to the loss of tumour suppressor gene function, many cancer cells can show resistance against fluorouracil metabolite chemotherapy, due to supraphysiological levels of TS.[253]

1.8 Pyrimidine nucleosides relevant to the present work

1.8.1 Decitabine and 5-azacytidine

5-Aza-2'-deoxycytidine (decitabine) and 5-azacytidine are pyrimidine nucleoside analogues that were first synthesised in the 1960s (see figure 1.9 for structures).[254-255] Decitabine is an analogue of 2'-deoxycytidine whereby the fifth carbon is exchanged for nitrogen in the pyrimidine ring.[254-255] 5-Azacytidine differs from

decitabine by having a hydroxyl group at the 2' position on the furanose[254-255]. Sorm and Vesely first demonstrated the anti leukaemia action of decitabine in AKR mice, which suffer from spontaneous lymphocytic leukaemia.[256] Decitabine was noted to have a more potent anti-leukaemia action than structurally similar pyrimidine analogue cytarabine.[257-258] Historically 5-azacytidine was demonstrated to have a potent anti-leukaemia action however since 2004 it is used in the treatment of myelodysplastic syndromes (MDS) a blood disorder characterised by a significant drop in the number of healthy erythrocytes, leukocytes and platelets.[256, 259-260] MDS disorders include refractory anaemia, refractory cytopenia and refractory anaemia with excess myoblasts in the bone marrow.[260] The FDA approved decitabine in 2006 for the treatment of MDS.[261] The national institute for health and care excellence (NICE, UK), recommended 5-azacytidine for the treatment of adult patients presenting with MDS, chronic myelomoncytic leukaemia and acute myeloid leukaemia in 2011.[262]

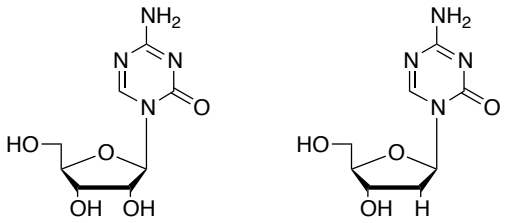


Figure 1.12 The structures of 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine)

1.8.2 Metabolism of decitabine and 5-azacytidine

Following transporter-mediated uptake into cells, decitabine is acted on by the phosphotransferase enzyme deoxycytidine kinase leading to the formation of 5-aza-2'-deoxycitidine-5'-monophosphate (rate limiting step).[263]

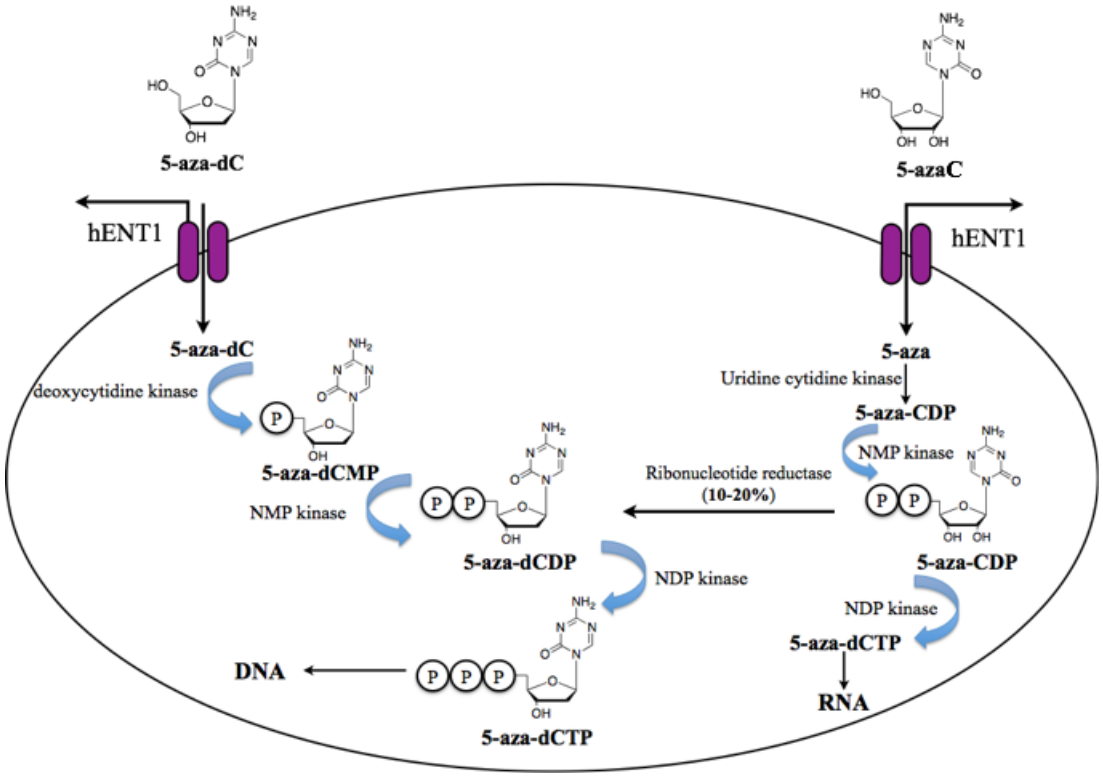


Figure 1.13 The uptake and intracellular anabolism of 5-azaC and 5-aza-dC (decitabine) hENT1: human equilibrative transporter 1; NMP: nucleoside monophosphate; NDP: nucleoside diphosphate.

Diphosphate and triphosphate forms are produced in subsequent steps by the actions of deoxcytidine monophosphate kinase and nucleoside diphosphate kinase.[263] 5-aza-2'-deoxycitidine-5'-triphosphate is then incorporated into the growing DNA chain, causing inhibition of DNA methylation.[263-264] In malignant neoplastic cells, the promoter region of DNA is hypermethylated, and as a consequence causes gene silencing aiding the progression of cancer.[265] 5-Azacytidine is phosphorylated by

uridine-cytidine kinase leading to the formation of 5-azacytidine monophosphate (rate limiting step).[263] Pyrimidine monophosphate kinase and Pyrimidine diphosphate kinase convert 5-azacytidine monophosphate into its di- and triphosphate derivative respectively.[263] 5-azacytidine-triphosphate is incorporated into RNA causing disassembly of ribosomes and inhibition of protein synthesis. 5-Azacytidine diphosphate can be converted to 5-aza-2'-deoxycytidine-5'-diphosphate by ribonuclease reductase, thus 5-azacytidine is capable of inhibiting DNA methylation. 5-Azacytidine covalently binds DNA methyl-transferase 1 causing inhibition of the enzyme. Consequently DNA is left in a hypomethylated state and this favours the re-expression genes that were once silent.[266-267]

1.8.3 Mechanisms of resistance: decitabine and 5-azacytidine therapy

Cytosine nucleoside analogues like decitabine and 5-azacytidine are transported across the plasma membrane by the hENT1 and hENT2, where they are processed by kinase enzymes (see section 1.8.2). The phosphorylated metabolites of decitabine and 5-azacytidine are potential substrates for the enzyme cytidine deaminase, which inactivates cytidine and uridine and their deoxy-derivatives. A deficit in functional deoxycytidine kinase due to *DCK* gene mutation is a major mechanism of resistant to cytidine and related nucleoside analogues, and this mechanism has been reported in mice with gemcitabine sensitive and -resistant solid neoplasms, in patients with acute myeloid leukaemia that is resistant to cytarabine therapy and other cytidine nucleoside resistant malignancies.[268-273] Interestingly, cytidine deaminase activity was markedly enhanced in males with MDS treated with decitabine and 5-azacytidine compared to females.[274] Overall clinical outcomes and survival were worse as a result.[274] This studies shows that upregulated cytidine deaminase activity

is a major mechanism that is responsible for compromising the efficacy of nucleoside analogues in the clinic.

1.8.4 Toxicity associated with decitabine and 5-azacytidine

Despite decitabine and 5-azacytidine improving clinical remissions and increasing overall survival in patients with MDS, severe adverse effects are associated with this therapy.[275-276] Patients receiving decitabine therapy for MDS frequently present with myelosuppression and haematological toxicities including neutropenia (low neutrophil granulocytes), thrombocytopenia (low platelet levels), neutropenia, febrile neutropenia and anaemia.[275-276] Due to its nature, MDS itself is likely to be responsible for some of the adverse drug toxicity affects seen with decitabine and 5-azacytidine.[277] 5-azacytidine produces the same adverse drug reactions to decitabine and as already mentioned, and these are the serious adverse effects that lead to discontinuation of this line of therapy.[278]

1.9 Drug discovery process

The drug discovery process starts with establishing the need for new therapies for a particular disease, and examining the therapeutic effectiveness of existing therapies. Using all the information collated using current knowledge about the target disease, hypotheses can established aimed at finding ways of improving current therapy or novel modes of talking the disease. Moreover, many factors need to be considered also and these include drug delivery, efficacy and safety or how mechanistically novel improvements will advance drug treatment in patients with the disease of interest. Following the establishment of a hypothesis specific aims and objectives are set for the task at hand. Chemical candidates can be selected from chemical libraries or novel analogues of appropriate established therapeutic agents can undergo testing in relevant biological assays in order to find biological activity (a hit) that helps support the initial hypothesis. The process involves *in vitro* and *in vivo* testing to characterize the biological efficacy of the chosen compounds. This is followed by process of producing structural analogues to see if chemical modification can enhance efficacy and limit any non-specific toxicities. Eventually a single is compound is chosen as a 'candidate' drug for further development, and testing in preclinical (*in vivo* studies) and eventually clinical investigation in human subjects, whilst adhering to ethical standards and government laws. Clinical investigations are done in normal human volunteers to establish toleration to drug treatment (Phase I), efficacy and dose range (Phase II), and large trials in thousands of criteria selected patients to gather a large database efficacy and safety profiles. All new drugs are assessed through an expert paneled government body (for example NICE, FDA) prior to be granted approval. Once approval is given the drug can be offered to

doctors and their patients to treat the disease at hand. The Drug discovery process is illustrated in Figure 1.14.

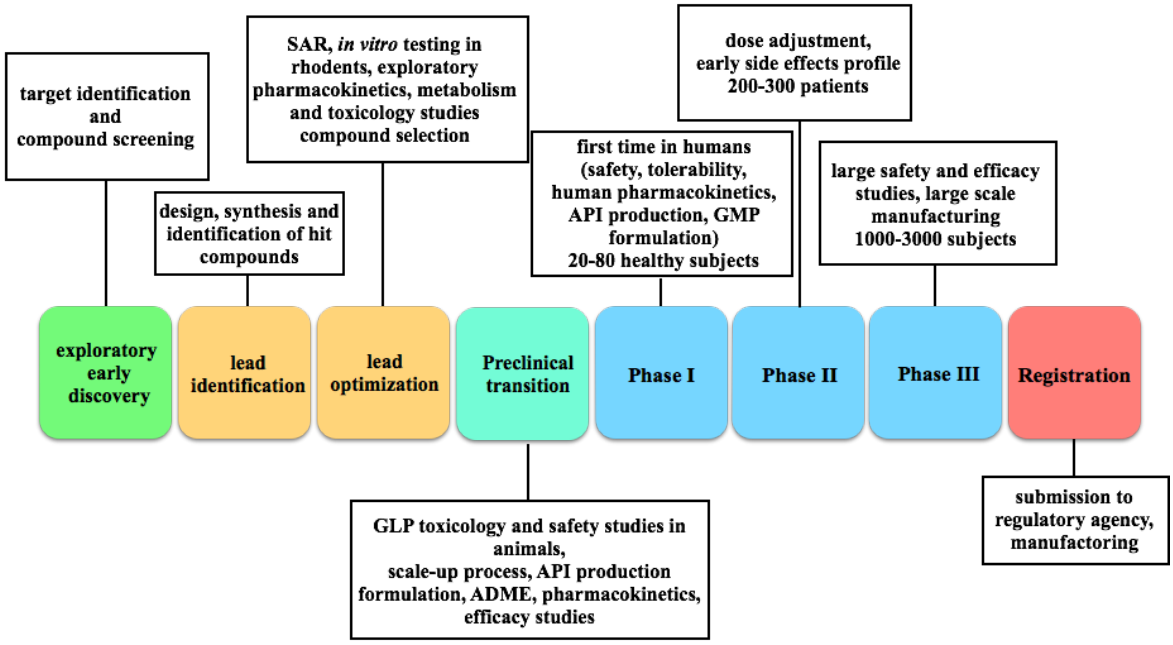


Figure 1.14 Drug discovery process

1.10 The advantages and shortcomings of 2D immortalised cell lines

Cancer cell lines used as models of experimental cancer have many advantages. In general they are easy to manipulate providing they are kept under optimal incubator conditions that allow them to replicate and grow moreover, their genetics can be easily manipulated chemically using demethylating and cytostatic agents, and biologically using small interfering RNA and expression vectors. Cancer cell lines have bypassed cellular senescence and thus can undergo continuous cell division,

making them ideal for modelling cancer progression experimentally, and amenable for the testing of anti-cancer agents. However, following each culture passage, the potential for the genetic makeup of these cells to change overtime increases. Tumour heterogeneity and ever changing morphological and phenotypic profiles of cells comprising a cancer tumour can be difficulty to model in-vitro, unless co-culture systems are used. Nevertheless, in cancer cell lines that are highly homogeneous can provide insights to the origin of cancers by the presence of precursor cells or cancer stem cells, can be used to examine established and putative biological signalling pathways that are crucial for cancer development and progression (until metastasis), and can be used to biologically screen a variety of drugs (anti-cancer drug testing) in relatively quick fashion using multi-well plates and microarrays. Table 1.3, outlines the advantages and disadvantages of the use of cancer cell lines to model cancer.

Table 1.3 Advantages and shortcomings of using cancer cell lines as a model for cancer study

Advantages of the use of cancer cell lines	Disadvantages of the use of cancer cell lines
Easy to handle and manipulate	Cross contamination with HeLa cells
High homogeneity	Loss of heterogeneity
High degree of similarity with the initial tumor	Genomic instability
High variety available	Possibility of modifying the caracteristics of the cells
Immediate asseccibility	Mycoplasma infection
Unlimited auto-replicative source	Difficulty in the establishment of long-term cancer cell lines
Easy substitution	Different tumor environment
Offers the reproducibility of results	

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2 Nucleoside and nucleotide prodrugs

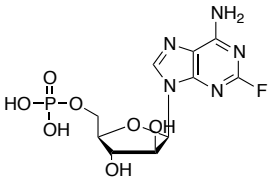
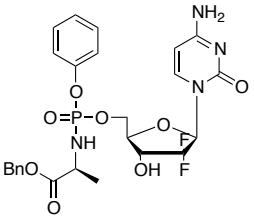
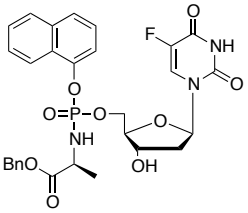
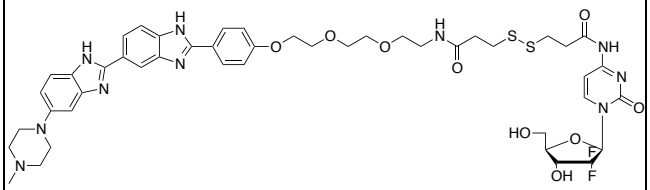
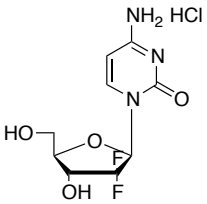
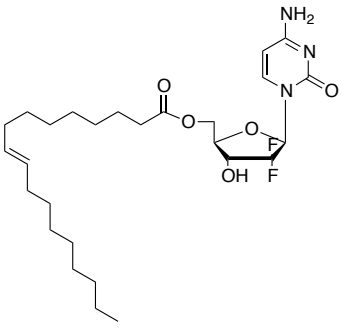
A prodrug is any compound that undergoes biotransformation before exhibiting its pharmacological effects.[1] Prodrugs are often developed to improve the absorption, distribution, metabolism, excretion (ADME) and the bioavailability of the parent compound and increase the selectivity of the compound for its intended target thus reducing undesirable side effects.[1] Prodrugs are precursor molecules containing specialised non-toxic protective groups used to alter or eliminate undesirable properties (poor ADME) in the parent molecule, which can affect its passage across the cell membrane.[2-3] Once transported across the cell membrane, the prodrug is metabolised usually during a series of chemical or enzymatic steps, forming an active metabolite. These types of compounds are categorised as type 1 prodrugs because they are bioactivated intracellularly.[4-8] A good prodrug should meet the following criteria: be less toxic than the parent drug; devoid of any activity until converted to the intended active drug; conversion to the active drug should be efficient and effective; any intermediary metabolites released upon conversion to the active drug should be non-toxic; markedly improved ADME and bioavailability.[9]

2.1 Delivering nucleoside monophosphates

The successful delivery of a nucleoside 5'-monophosphate is advantageous especially in cancer chemotherapy because nucleoside transporters (see chapter 1) responsible for facilitating the movement of nucleosides analogues into neoplastic cells, and enzymes like dCK, HPRT1, TK and UCK that are involved in the first phosphorylation step in the bioactivation of purine and pyrimidine nucleoside analogues may be significantly downregulated.[10-12] Then again, the highly charged

and lipophilic nature of nucleoside 5'-monophosphates under physiological conditions means they are not able to efficiently penetrate the cell membrane, which can limit their therapeutic potential.[9] Montgomery and co-workers in 1961 devised a solution in order to overcome the poor cell penetration of a nucleoside 5'-monophosphate, one could synthesise an ester of the nucleotide.[13] A nucleoside 5'-monophosphate prodrug will allow direct delivery of a nucleoside monophosphate, bypassing nucleoside transporter mechanisms and rate-limiting phosphorylation (first step).[12] A nucleoside monophosphate prodrug may be achieved by masking the negative charges on the phosphate group and as a consequence, this will potentially enable passive diffusion of the drug into the cell.[9, 12] Furthermore, this will potentially allow the prodrug to pass the gastrointestinal barrier more efficiently than the parent compound, which will lead to improved bioavailability and hopefully reduced toxicity.[12] Interest has been generated around ProTide technology that allows 5'-monophosphate nucleosides to efficiently cross biological membranes, which could have positive implications for cancer chemotherapy.[12] See table 2.1 for experimental anticancer prodrugs and are currently in clinical trials.

Table 2.1 Anticancer prodrugs

Prodrug name	**Structure**	**Status and cancer indication**
Fludara Fludarabine phosphate		2008 Chronic lymphocytic leukaemia
Acelarin Gemcitabine phosphoramidate ester		Phase 2/3. broad range of solid neoplasms
NUC-3373 FUDR phosphoramidate ester		Phase 1. broad range of solid neoplasms
H-gemcitabine extracellular DNA binding moiety (Hoechst) attached to the cytosine base		Due to enter Phase 1. experimental colon cancer
LY2334737 orally active prodrug		Phase 1. broad range of solid neoplasms including: pancreas, colon and ovarian
CO-101 Gemcitabine elaidate		Phase 1. Solid tumor, non-small-cell-lung cancer, lung cancer

2.2 Nucleotide prodrug technologies

There is a range of nucleotide prodrug strategies that allow masking of the hydrophilic charges on the parent compound using lipophilic moieties.[12] This may facilitate oral administration and improve cellular uptake. More complicated strategies involve enzymatic conversion or pH modification. Strategies involving enzymatic conversion and activation are usually more successful than those based on chemical conversion.[12] Different pronucleotide strategies that rely on enzymatic steps to release the active nucleoside 5'-monophosphate metabolite from the prodrug are given below.

2.2.1 *Cyclo*saligenyl (cycloSal) technology

The *cyclo*saligenyl (*cyclo*Sal) phosphotriester approach was employed for the potential delivery of clinically relevant anticancer and antiviral nucleoside analogues, including 5-FUDR, acyclovir, dideoxyadenosine, inosine and their fluorinated derivatives.[14-15] The *cyclo*Sal approach allows for the pronucleotide to be cleaved by selective hydrolysis of the phenolic ester bond, releasing the nucleotide metabolite. The following nucleotides can be released using the *cyclo*Sal approach: nucleoside 5'-di- and triphosphates; nucleoside mono- and diphosphate sugars; dinucleoside polyphosphates.[14-15] There are four generations of *cyclo*Sal pronucleotide compounds: 1. *cyclo*Sal pronucleotides, which break down into a highly reactive intermediate following chemical hydrolysis of the P-O bond ; 2. 'lock-in concept,' *cyclo*Sal pronucleotides, which contain an ester moiety attached the aromatic ring but is separated by a C_2-spacer to avoid chemical hydrolysis; 3. Enzymatically activated *cyclo*Sal pronucleotides, that contain lipophilic substituents

with electron donating or weak electron withdrawing properties attached to the *cyclo*Sal masking group; 4. 'high-loaded' pronucleotides (see Figure 2.1 and 2.2).[14-15] The first generation *cyclo*Sal approach used bis(phenyl) and bis(benzyl) esters in combination as part of a cyclic bifunctional masking unit of the pronucleotide.[14-15] Salicyl alcohol, another component of the pronucleotide is attached through the phenyl and the benzyl ester bond and the nucleoside is attached through an alkyl ester bond; this allows for good discrimination during the hydrolysis process. *Cyclo*Sal technology was applied to 2',3'-dideoxy-2',3'-didihydrothymidine (d4T) 5'-monophosphate (MP; d4TMP).[14-15] *Cyclo*Sal- d4TMP is synthesised by reacting salicyl alcohol with phosphorus trichloride to give a chlorophosphite, which is then treated with d4T in the presence of diisopropylethylamine (Hünig's base) to give rise to the cyclic phosphite triester.[14-15] The triester is then oxidised with *tert*-butylhydroperoxide or dimethyldioxirane in a one-pot reaction to give a mixture of *cyclo*Sal- d4TMP stereoisomers (See Figure 2.1).

The mechanism of d4TMP release from *cyclo*Sal- d4TMP is as follows (first generation): following the nucleophilic attack of hydroxide at the phosphorous atom, displacement of the phenolate occurs in a S_NP reaction that leads to the formation of a 2-hydroxybenzylphosphate diester.[14-15] As a consequence, the *ortho* substituent of the benzyl ester switches from a weak electron-donating group to a strong electron-donating group.[14-15] This change, activates the remaining masking group and causes spontaneous C-O bond cleavage and splitting of the diester, to give d4TMP and salicyl alcohol (see Figure 2.1). Another route to releasing d4TMP could begin with the cleavage of the benzyl ester bond by an S_N1-type C_{benzyl}-O bond break, which leads to formation of an intermediate with a benzyl cation and an anionic phosphate ester group (see Figure 2.1).[14-15] A phenylphosphate diester is formed by the addition

of water. The step that allows the release of free d4TMP is unknown, although it has been postulated to be enzyme-mediated.[14-15]

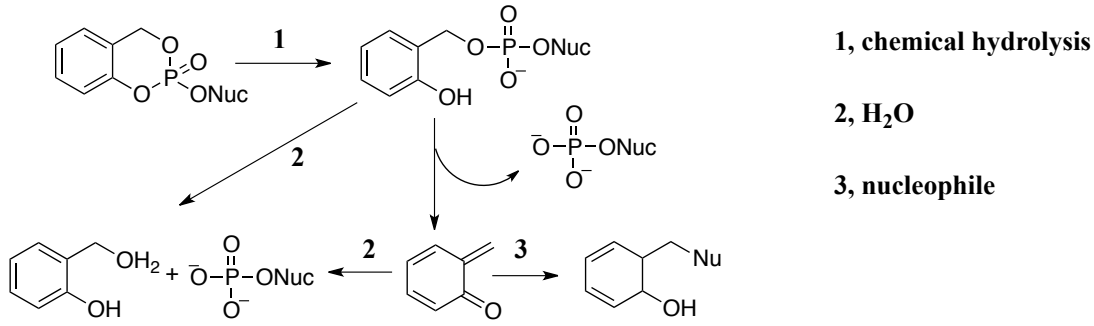


Figure 2.1 Mechanism of activation of first generation cycloSal nucleotide prodrugs

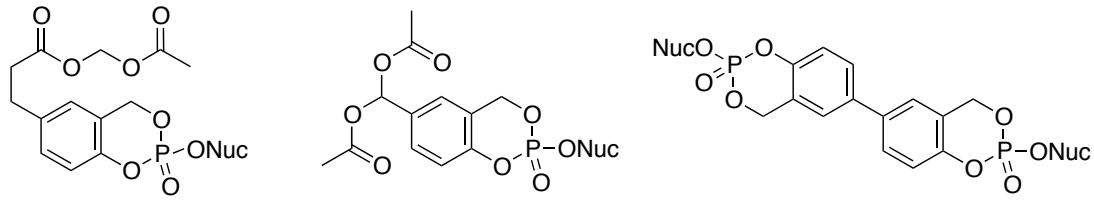


Figure 2.2 General structures of second, third and fourth generation of cycloSal nucleotide prodrugs

2.2.2 S-acyl-2-thioethyl (SATE) technology

The SATE approach was first described by Përigaud and colleagues, and led to the synthesis of pronucleotides with S-acyl-2-thioethyl (SATE) phosphate protections (biolable protecting group).[16] This technology has been applied to aryl phosphotriesters and phosphoramidate diester analogues of common antivirals. Bis(SATE) protected nucleotides can be synthesised using three strategies (see Figure 2.3) and in order to proceed with each reaction, S-acyl-2-thioethanol or a phosphoramidate needs to be prepared. In strategy one, the nucleoside 5'-monophosphate is reacted with S-acyl-2-thioethanol reagent in pyridine in the presence of mesitylene sulfonyl nitrotriazole.[16] Strategy two involves condensing hydrogen-phosphonate nucleoside with S-acyl-2-thioethanol reagent in the presence

of pivaloyl chloride catalyst to yield a phosphite intermediate, which is then oxidised.[16] In strategy three, the nucleoside is condensed with the phosphoramidite agent in tetrahydrofuran and in the presence of tetrazole.[16] Oxidation of the phosphite intermediate yields the bis(SATE) phosphotriester.[16]

The decomposition pathway of bis(SATE) triesters is triggered by carboxyesterase mediated hydrolysis of the thioester on the SATE group to form an unstable *O*-2-mercaptoethylphosphotriester intermediate.[17] The thiol produced, attacks the methylene carbon atom, releasing ethylene sulphide and the monoSATE phosphodiester. Phosphodiesterase mediated hydrolysis releases the nucleoside monophosphate and S-acyl-thioethanol.[17] Another route involves carboxyesterase thioester hydrolysis and an ethylene sulphide step to produce the nucleoside 5'-monophosphate (see Figure 2.3).[17]

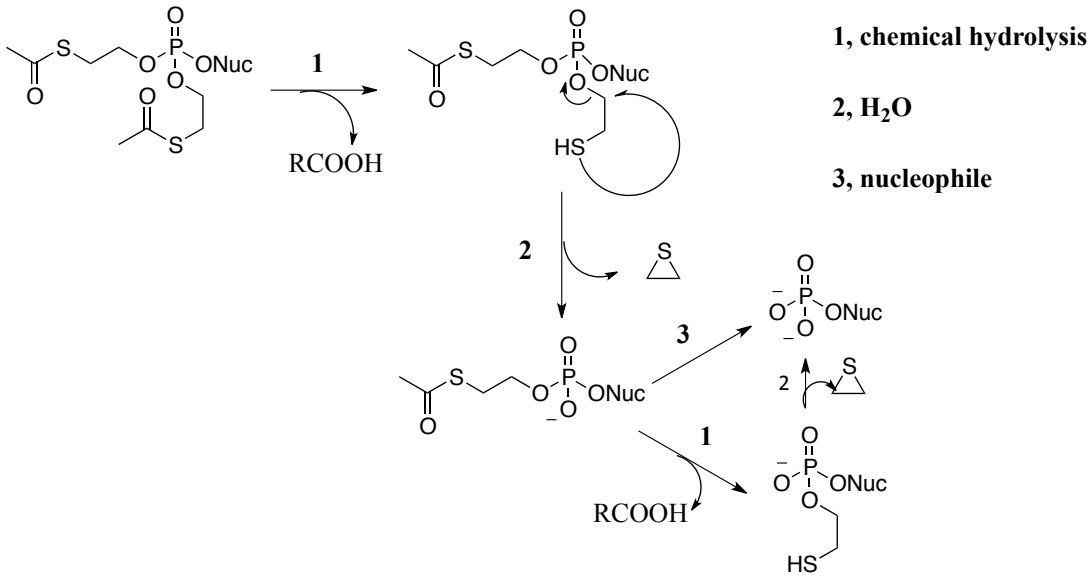


Figure 2.3 Mechanism of activation of bis(SATE) nucleotide prodrugs

2.2.3 Pivaloyloxymethyl (POM) technology

The pivaloyloxymethyl (POM) phosphoester approach (see Figure 2.4) has been used to generate bis(POM) triesters of 5-FUDR, 2',3'dideoxyuridine (ddU) and

zidovudine (AZT). Farquhar and co-workers found that mono-, di- and triphosphate metabolites of ddU and AZT were markedly increased in thymidine kinase deficient CEM cells following treatment with bis(POM) triesters of these nucleosides.[18-19] Bis(POM) triesters of AZT are less active than AZT in treating HIV-1 infections.[19] Bis(POM) triesters of nucleoside monophosphates, can be prepared by reacting the nucleoside 5-monophosphate (bis(triethyl or tributyl) ammonium salt) in anhydrous *N*-methylpyrrolidinone and triethylamine, with chloromethylpivalate. Subsequent steps with ethyl acetate and column chromatography isolates the desired bis(POM) phosphotriester.[20]

The breakdown of bis(POM) phosphotriester derivatives involves carboxyesterase-mediated hydrolysis which produces the unstable *O*-2-hydroxyethyl phosphotriester intermediate (see Figure 2.4). The *O*-2-hydroxyethyl phosphotriester then undergoes nucleophilic displacement to produce the monoPOM phosphodiester and formaldehyde as a by-product (see Figure 2.4).[21] The monoPOM phosphodiester is acted on by a carboxyesterase or phosphodiesterase to liberate the nucleoside monophosphate (see Figure 2.4).[21]

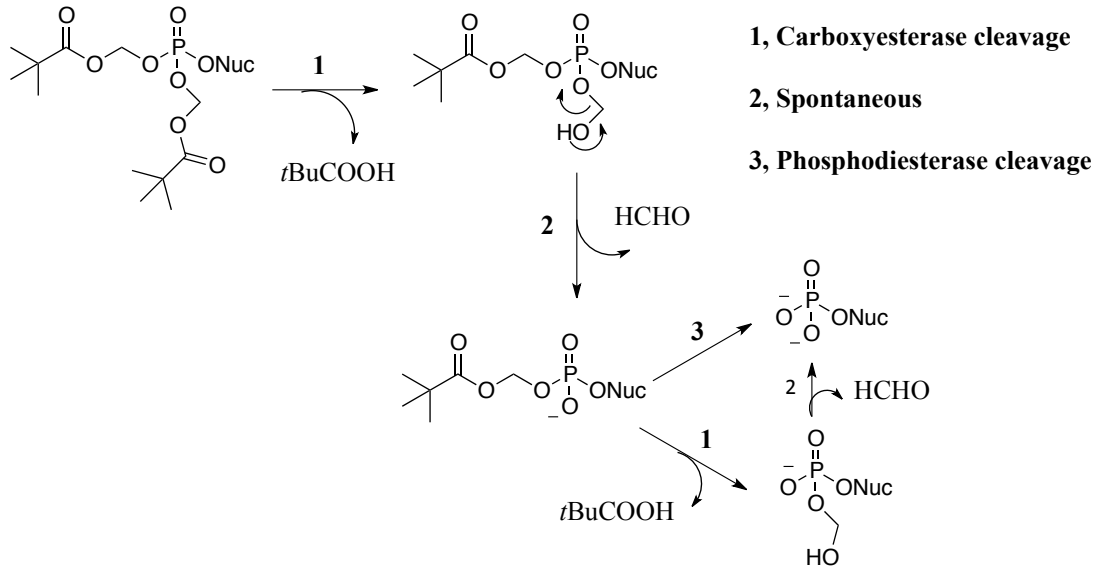


Figure 2.4 Mechanism of activation of bis(POM) nucleotide prodrugs

2.2.4 *Para*-acyloxybenzyl (PAOB) technology

The *para*-acyloxybenzyl (PAOB) approach is another route to the development of phosphotriester prodrugs of nucleoside analogues. Bis(PAOB) phosphotriesters of AZT, including phosphotriester homodimers and heterodimers have been investigated.[22-24] The bis(PAOB) phosphotriester requires esterase-mediated hydrolysis to generate the unstable *p*-hydroxybenzyl phosphotriester metabolite. Following *p*-hydroxybenzyl carbonium ion formation, the monoPAOB phosphodiester forms, which upon esterase mediated hydrolysis releases the nucleoside monophosphate (see Figure 2.5).[25] The *p*-hydroxybenzyl carbonium ion has been hypothesised to interact with DNA, proteins and cellular nucleophiles, thus highlighting potential toxicity issues associated with this form of nucleoside monophosphate delivery system.[25]

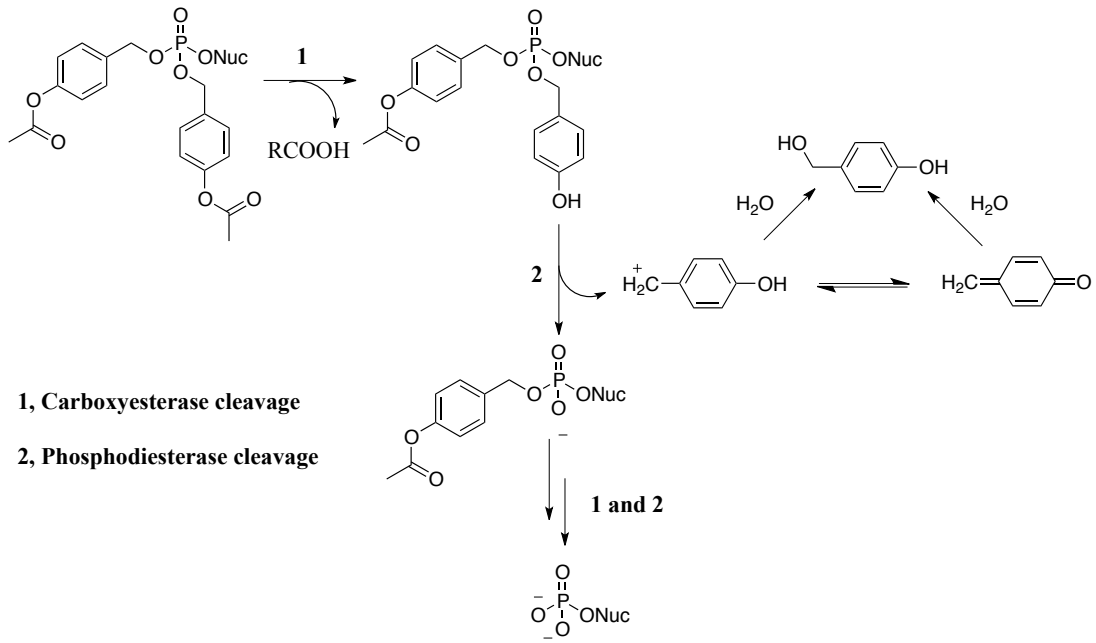


Figure 2.5 Mechanism of activation of bis(PAOB) nucleotide prodrugs

2.2.5 Phosphorodiamidates

Phosphorodiamidate prodrugs were looked into by McGuigan and co-workers over 20 years ago, focused on delivering diamidate derivatives of AZT 5'-monophosphate. These compounds had a marked inhibitory effect on human immunodeficiency virus type 1 (HIV-1) replication in a human lymphoblastoid cell line, compared to the parent AZT.[26] The major advantage of this approach is that the phosphorus in the symmetrical diamidate is achiral, thus negating the forming of diastereoisomer mixtures that commonly occur with other ProTides.[27] Another feature of this approach is that two amino groups are attached to the phosphate moiety to mask the negative charges.[27] This technology has been successfully applied to 2'-C-methylguanosine and related analogues, with some candidates exhibiting a promising inhibitory action against hepatitis C virus (HCV) replication.[28] Moreover this approach has been used to develop the fructose 1,6 biphosphatase inhibitor CS-917, which is a potential candidate to treat type-2 diabetes.[29-30]

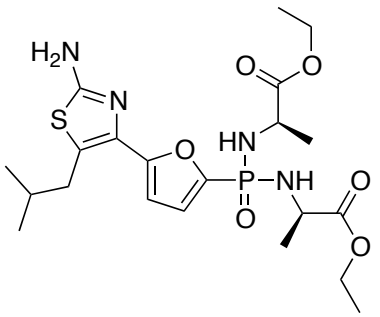


Figure 2.6 Structure of phosphonic diamidate prodrug against type 2 diabetes

2.2.6 Aryloxy and alkyloxy phosphoramidate (ProTide) technology

Aryloxy and alkyloxy phosphoramidates (ProTides) are a class of nucleotide prodrugs designed and synthesised to deliver the nucleoside monophosphate to the cell without the need for active uptake or phosphorylation by specific kinases.[12, 31] The technology has been utilised to generate different antiviral and anticancer compounds that have been introduced into preclinical development and or have entered clinic trials.[32-33]

2.2.6.1 Phosphoramidate monoesters and diesters

McGuigan and colleagues first developed bioactive phosphoramidate based prodrugs of AZT in 1992.[34-36] These derivatives were synthesised using phosphochloridate chemistry.[34-36] First generation ProTides were the alkyl and haloalkyl phosphotriesters, the incorporation of an amino acid ester in place of the alkyl and haloalkyl chain led to the development of alkyloxy phosphoramidates (ProTide technology).[34-36] There are several examples of ProTides being more active than the parent nucleoside; this technology has transformed once inactive nucleosides into highly potent compounds.[37-38] ProTide technology has been applied to different anticancer and antiviral nucleoside analogues with good success, and these include AZT, d4T, ddU, 2',3'dideoxyuridine (ddA), 9-(2-phosphonylmethoxyethyl) adenine (PMEA), (*R*)-9-(2-phosphonylmethoxyethyl) adenine (PMPA), gemcitabine, FUDR and more.[27-28, 32-37, 39]

The initial step in the activation of an aryloxyphosphoramidate (see Figure 2.7) involves hydrolysis of the amino acid moiety by a carboxyesterase or

carboxypeptidase A (cathepsin A), which hydrolyses the ester of the amino acid moiety.[32, 39-40] Interestingly carboxypeptidase A is upregulated in malignant neoplastic cells compared to normal proliferating cells, therefore ProTides are likely to be more efficacious than the parent nucleoside in killing cancer cells.[41] The nucleophilic carboxylic acid moiety is then thought to attack (intramolecularly) the phosphorus atom leading to the formation of an unstable cyclic intermediate following the release of the aryloxy moiety (see Figure 2.7). This unstable intermediate then undergoes further hydrolysis by a phosphoramidase-type enzyme, which results in the cleavage of the P-N bond and release of the nucleoside 5'-monophosphate (see Figure 2.7).[28, 32-33, 39-40] Histidine triad-nucleotide binding protein 1 (Hint-1) phosphoramidase, which belongs to the histidine triad (HIT) superfamily of proteins, is thought to be the enzyme responsible for releasing the nucleoside 5'-monophosphate.[42] Purine analogues have a higher affinity for the catalytic domain on Hint-1 although pyrimidine-derived phosphoramidates are also accepted as lower affinity substrates.[43]

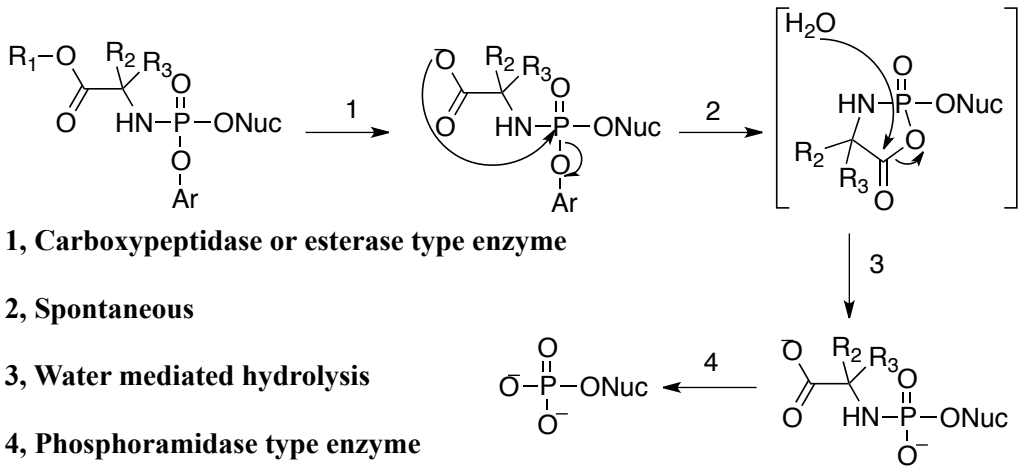


Figure 2.7 Putative mechanism of action of aryloxyphosphoramidates (ProTides)

Phosphoramidate monoesters contains an amino ester that masks one of the charges on the phosphate, and as result eliminates the chirality problem at the phosphate centre.[44] Phosphoramidate monoesters exhibit good water solubility and are stable in human plasma.[44] The nucleoside monophosphate is liberated in sequential steps, where the final step involves phosphoramidase-mediated P-N bond cleavage and release of the nucleoside monophosphate.[44]

2.2.6.2 Comparison of nucleotide prodrug approaches

Table 2.2 reports the summary of the different nucleotide prodrug approaches discussed in this chapter, highlighting their main attributes such the formed by-products during the their bioactivation. From the above mentioned technologies Bis(POM), HepDirect, ProTide and phosphorodiamidate technologies reached clinical trials in human.

Table 2.2 Comparison of nucleotide prodrug technologies

Prodrug approach	Prodrug class	Bioactivation	By-product
Phosphotriester	Bis (POM) Bis(SATE)/Bis(DTE) HepDirect Cyclosal	Esterase Esterase Cytochrome P_{450} Chemical	Formaldehyde Episulfide Aryl vinyl ketone Quinone methide
Phosphoramidate	Aryloxyphosphoramidate (ProTide) Phosphoramidate monoester (Wagner)	Esterase, amidase Amidase	Phenol, naphtol, amino acid Amino acid
Phosphorodiamidate	Phosphorodiamidate	Esterase, amidase	Amino acid

2.2.6.2 Structure activity relationships

The three main components making up the phosphoramidate determine its lipophilicity and pharmacokinetic properties, and these are the aryl ring, the amino acid and the ester (Figure 2.8). A study performed on 4'-azidouridine triphosphate phosphoramidate and other nucleotide analogues, allowed the optimisation of techniques that could be employed to modify the aforementioned moieties.[12, 32-33, 37-40] Since then the ProTide approach was successfully applied on a wide range of nucleoside analogs, showing significant improvement in the biological effect of the parent nucleoside due to increased lipophilicity, therefore enabling passive diffusion. A wide ranging structure activity relationship study took place in order to pursue the three most preferred, most effective masking moieties, namely the amino acid, the ester and the aryl groups.

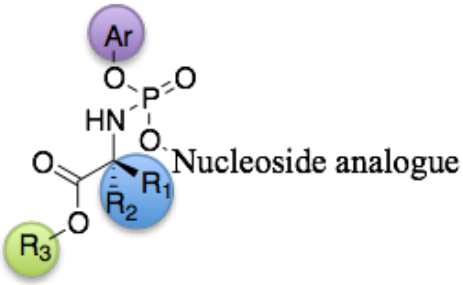


Figure 2.8 The three main modification sites of the phosphoramidate moiety, the amino acid (R_1, R_2), the ester (R_3) and the aryl ring (Ar)

Amino acid (R_1, R_2)

L-alanine aryloxy phosphoramidates commonly exhibits the best potencies regarding the majority of the ProTide projects carried out in the McGuigan group. Swapping L-alanine for the unnatural D-alanine form led to significant decrease in both anticancer and antiviral activity. Lacking substitutions at the α carbon (e.g. glycine) in general resulted in significant decrease in efficacy with the exception of the 8-

chloro adenosine ProTide project, where the lead compound was identified as the glycine-benzyl ester. Disubstitution at the α carbon in the contrary (e.g. dimethylglycine) retained the potency. The bulky L-phenylalanine, L-leucine, L-isoleucine and L-valine containing ProTides often exhibit poor potencies, due to impairment of an enzymatic cleavage step, thus affecting bioactivation of the prodrug. In contradiction regarding the FUDR ProTide project the L-leucine-pentyl derivative **3.1q** was considered as one of the main lead analogue, until the final decision of the clinical candidate.

Ester (R_3)

Ester lability greatly alters the biological activity of ProTides. Primary, secondary tertiary, alkyl and benzyl, linear and branch chain esters were evaluated. Amongst all benzyl ester demonstrated the most outstanding activity, whereas t-butyl ester reduced potency, mainly due to its probable poor susceptibility to esterase enzyme.

Aryl

The aryl moiety is considered as an essential leaving group. The phenyl group as an aryl masking moiety, has been given a lot of attention. Weak electron withdrawing substituents (e.g. *p*-Cl, *p*-COOMe) on the phenyl ring have been shown to improve the potency of the parent derivatives.[45-47] Recent evaluation of BVDU phosphoramidates by Congiato revealed, that derivatives bearing 1-naphthol aryl masking moieties are more potent upon compared to their phenyl counterparts, owing to their possibly better ability as a leaving group or their increased ClogP value.

2.2.6.3 Stereochemistry of aryloxyphosphoramidates

Due to the chirality of the phosphorus center Protide analogues were isolated as a mixture of two diastereoisomers, which fact is considered as a limitation of approach. Chirality is one of the big challenges in medicinal chemistry. Since cellular targets are chiral for instance a receptor or an enzyme, one diastereoisomer may fit to the target, while the other isomer may not.[48]

The diastereoisomeric ratio is usually 1:1, however due to the extensive purification this ratio can be altered. In some cases when the two phosphorus isomers can be separated a difference in activity has been observed. One diastereoisomer can be effectively processed while the cleavage is the other can be slow.[49] As phosphorus chirality is lost in the process of bioactivation even diastereoisomeric mixtures of ProTides can be highly potent and further progress into clinical trials like the anti-HCV Protide BMS-986094, previously synthesised in the McGuigan group.[50] Separation of the two diastereoisomers may not be essential.

2.3 Synthesis

Phosphoramidates were synthesised in a coupling reaction between the nucleoside analogue and the appropriate aryl amino acid phosphorochloridate following the synthetic procedure, developed in the McGuigan group utilising the nucleoside phosphorylation procedure reported by van Boom and colleagues.[51] The three-step synthetic pathway initiates with the preparation of the appropriate amino acid esters **2.2** from the amino acid and the appropriate alcohol, followed by its coupling with the appropriate aryl phosphorodichloridate resulting in the formation of the phosphorochloridate **2.3**, which used in the final coupling step with the choosen nucleoside analogue to provide the final phosphoramidate. Based on the nucleoside and base used during the final coupling step three different regioisomers can be isolated: the 3', the 5' and the 3'5'-bis-phosphoramidate species. Each ProTide appears as a mixture of diastereoisomers, due to their chiral phosphate centre.

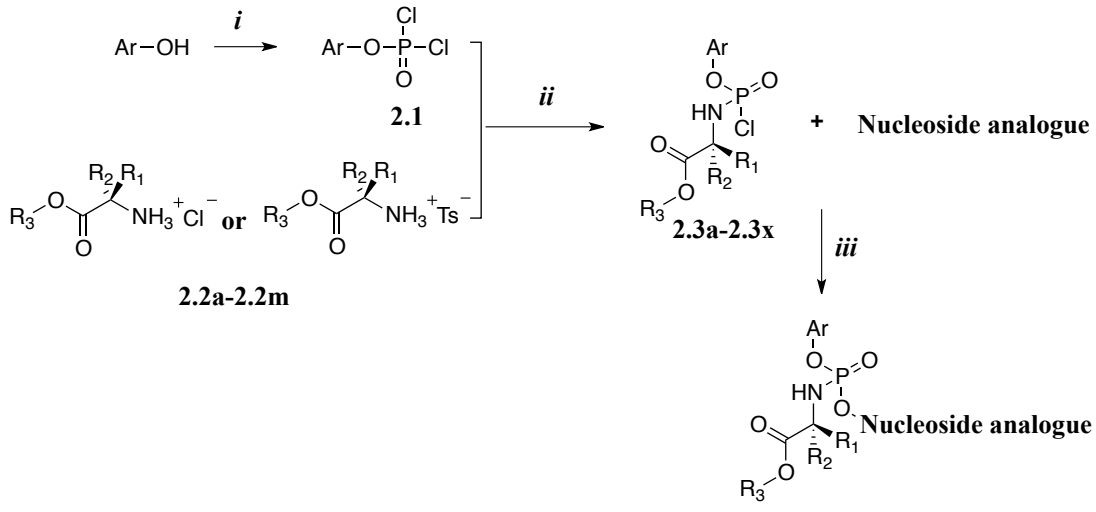


Figure 2.9 General synthetic pathway of aryloxyphosphoramidates
Reagents and Conditions: i, $POCl_3$, Et_3N, Et_2O, -78°C to rt, 2 hrs; *ii,* Et_3N, DCM, -78°C to rt, 1.5 hrs; *iii, t*BuMgCl or NMI, THF, rt, 16 hrs

2.3.1 Synthesis of amino acid esters

There are three synthetic routes available for the esterification reaction of those amino acid esters, which are not commercially available, depending of the nature of the chosen alcohol. In case of low boiling alcohols the thionyl chloride method was applied, where the amino acid and the appropriate alcohol were heated in the presence of thionyl chloride at 75°C overnight (see Figure 2.10). The pure amino acid esters were obtained as chloride salts.

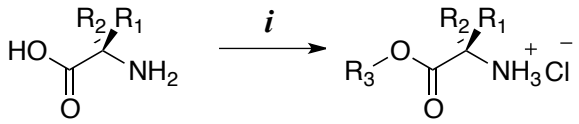


Figure 2.10 Amino acid ester synthesis using thionyl chloride method
Reagents and conditions: *i,* alcohol, $SOCl_2$, 75°C, 16 hours

For the synthesis of amino acid esters derived from high boiling alcohols the second method was applied, where the amino acid and the appropriate alcohol were suspended in toluene and heated at reflux overnight in the presence of para-toluene sulfonic acid monohydrate (pTSA), using Dean Stark apparatus (see Figure 2.11). The pure amino acid esters were obtained as tosylate salts.

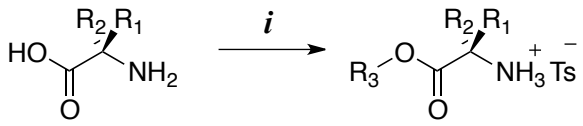


Figure 2.11 amino acid ester synthesis using *p*TSA method
Reagents and conditions: i, alcohol, *p*TSA, toluene, reflux, 16 hours

In case of the third method developed for the synthesis of sterically demanding amino acid esters, the solution of the Boc-protected amino acid in the presence of N,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in the

appropriate alcohol was allowed to stir at ambient temperature overnight.[52] Boc-deprotection was carried out on the formed Boc-protected amino acid ester in the presence of pTSA in ethyl acetate (see Figure 2.12). The pure amino acid esters were obtained as tosylate salts.

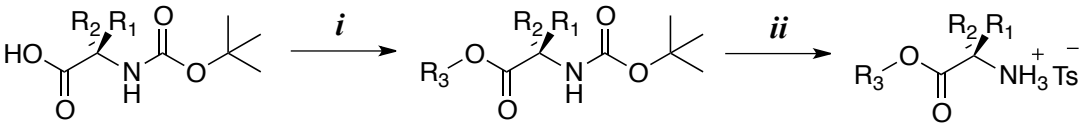


Figure 2.12 Amino acid ester synthesis using Boc protected amino acids
Reagents and conditions: i, alcohol, DCC, DMAP, rt, 16 hours; *ii, p*TSA, EtOAc, 65°C, 16 hours

Table 2.3 The list of amino acid esters synthesised, stating the isolated yields and the methods used.*Yields over two synthetic steps.

Cpd	Amino acid	Ester	Salt	Yield %	Method
2.2a	L-Ala	CH_2tBu	Ts	83	2
2.2b	L-Ala	Pnt	HCl	82	1
2.2c	L-Ala	Hex	Ts	79*	3
2.2d	L-Ala	cHex	Ts	95	2
2.2e	L-Ala	CH_2CH_2tBu	Ts	62*	3
2.2f	Me_2Gly	Bn	HCl	67	1
2.2g	Me_2Gly	CH_2tBu	Ts	66	2
2.2h	L-Ile	Pnt	HCl	53	1
2.2i	L-Leu	Pnt	HCl	94	1
2.2j	L-Met	Bn	Ts	87	3
2.2k	L-Met	iPr	HCl	81	1
2.2l	L-Phe	Pnt	HCl	85	1
2.2m	L-Val	Pnt	Ts	69	2

2.3.2 Synthesis of phosphorochloridates

2.3.2.1 Synthesis of aryl phosphorodichloridate

Phenyl phosphorodichloridate is commercially available, however the naphtyl analogue needs to be prepared from 1-naphthol and phosphorus oxychloride in the presence of anhydrous triethylamine. The quick reaction easily purified from the forming triethylamine hydrochloride salt by filtration, producing therefore the dichloridate **2.1** as a thick yellowish oil, indicated as a single isomer around 3.60 ppm in the ^{31}P NMR spectrum.[53]

2.3.2.2 Synthesis of phosphorochloridates

Phosphorochloridates were synthesised in a coupling reaction between either the amino acid ester hydrochlorides or tosylate salts and the appropriate phosphorodichloridates in the presence of triethylamine at low temperature (-78^{o}C) (see Figure 2.9 step ***i*** and ***ii***). Completion of reaction was monitored by ^{31}P-NMR, and this was indicated by the disappearance of the $POCl_3$ signal. Purification of the phosphorochloridates derived from amino acid ester hydrochlorides required only quick filtration, while the phosphorochloridate analogues derived from tosylate salts were purified by column chromatography. Phosphorochloridates, which were prepared from chiral amino acids appeared as two peaks at the ^{31}P NMR spectrum, corresponding to the presence of two different diastereoisomers. Phosphochloridates of achiral amino acids such as 2,2-dimethylglycine or glycine were obtained as mixture of enantiomers, hence gave a single peak in the ^{31}P NMR spectrum. For instance the 2,2 dimethylglycine-benzyl ester phosphochloridate derivative **2.3j** indicated as a single peak at 5.86 ppm**,** while similarly the glycine cyclohexyl

naphthyl derivative **2.3n**, appeared as one peak at 8.99 ppm in the ^{31}P NMR spectrum.

Table 2.4 The list of phosphorochloridates synthesised for the present work, stating the ^{31}P NMR chemical shifts and the isolated yields

Cpd	Amino acid	Ester	Aryl	^{31}P NMR	Yield %
2.3a	L-Ala	Me	Ph	7.88, 7.54	55
2.3b	L-Ala	Bn	Ph	7.85, 7.51	73
2.3c	L-Ala	Bn	1-Naph	8.13, 7.85	65
2.3d	L-Ala	Bu	1-Naph	8.41, 8.24	87
2.3e	L-Ala	CH_2tBu	Ph	8.17, 7.75	65
2.3f	L-Ala	CH_2tBu	1-Naph	8.23, 7.93	58
2.3g	L-Ala	Hex	1-Naph	8.21, 7.88	68
2.3h	L-Ala	cHex	1-Naph	8.30, 7.94	89
2.3i	L-Ala	CH_2CH_2tBu	1-Naph	8.24, 7.92	87
2.3j	Me_2Gly	Bn	1-Naph	5.86*	85
2.3k	Me_2Gly	Me	1-Naph	5.84*	94
2.3l	Me_2Gly	Et	1-Naph	5.90*	73
2.3m	Me_2Gly	CH_2tBu	1-Naph	5.49*	58
2.3n	Gly	cHex	1-Naph	8.99*	59
2.3o	Gly	iPr	1-Naph	9.04*	54
2.3p	L-Ile	Pnt	1-Naph	9.46, 9.05	76
2.3q	L-Leu	Bn	Ph	8.29, 8.06	83
2.3r	L-Leu	Bn	1-Naph	8.76, 8.40	72
2.3s	L-Leu	Pnt	1-Naph	8.78, 8.50	77
2.3t	L-Met	Bn	Ph	8.15, 8.08	56
2.3u	L-Met	Bn	1-Naph	8.71, 8.59	61
2.3v	L-Val	Et	Ph	9.51, 9.03	75
2.3w	L-Val	Pnt	Ph	9.78, 9.34	73
2.3x	L-Val	Pnt	1-Naph	9.78, 9.33	67

***** No diastereomeric splitting present as the compound is a mixture of enantiomers.

2.3.2.3 Synthesis of phosphoramidates

Phosphoramidates were obtained by coupling of the nucleoside analogues with phosphochloridates in the presence of either the tert-butylmagnesium chloride (*t*BuMgCl, Grignard reagent) or N-methylimidazole (NMI) at room temperature in anhydrous tetrahydrofuran. The Grignard reagent was first revealed by Uchiyama

and colleagues as a good activator for O-selective phosphorylation of nucleosides.[54] Grignard reagent is a very strong base and does not show selectivity towards primary hydroxyl groups. It has the propensity to attack hindered 2' and 3' hydroxyl groups beside the primary hydroxyl group in the 5' position. Therefore treating nucleoside analogues with Grignard reagents bearing more than one hydroxyl group often result in the undesired phosphoramidate isomers like the 3', 5' and the 3', 5'-bis ProTide species. Upon deprotonation, the 5'-alkoxide, which is much more nucleophilic than the 5'-hydroxy group attacks the phosphorochloridate in order to form the nucleoside phosphoramidate (Figure 2.13). However the Grignard method has its own advantages, so is favoured regardless of the lack of selectivity. Coupling reactions mediated by Grignard reagent are reasonably yielding, therefore making the purification steps easier.

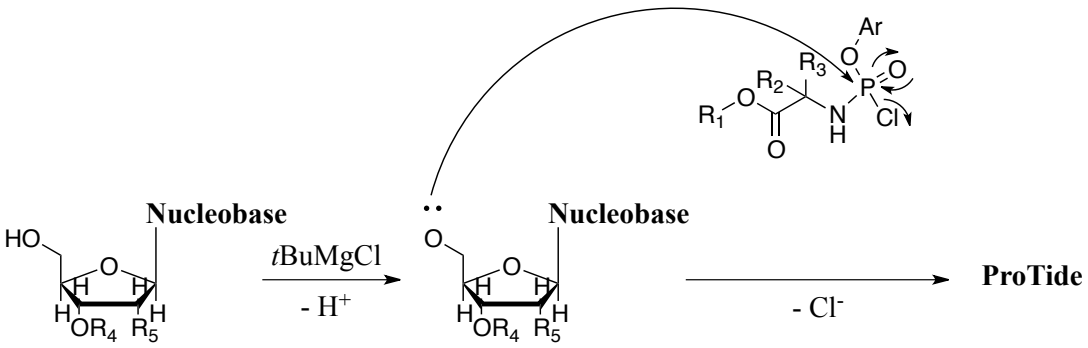


Figure 2.13 ProTide synthesis using *tert*-butyl magnesium chloride as a strong base
R_1 = alkyl; R_2, R_3 = H, alkyl; R_4 = H or protecting group; R_5 = H, OH or O-protecting group; Ar = aryl.

The NMI procedure shows selectivity towards primary hydroxyl groups, therefore in cases where only one free hydroxyl group of the nucleoside is present, the weak base NMI is preferred.[55] The pKA of the conjugated acid of NMI is 7, while the pKA of the nucleoside hydroxyls are around 15, therefore the base is not strong enough to deprotonate the hydroxyl groups. As NMI being a good nucleophile it can attack the phosphorochloridate, thereby displacing the chloride. The positively charged moiety is a good leaving group, hence the reactivity towards nucleophiles is increased. Furthermore steric hindrance of the phosphorus atom provided by the NMI moiety maybe one reason why reaction with the less hindered 5'-hydroxyl group is preferred. The main disadvantage of the procedure is that the obtained crude mixture requires acidic extraction to abolish the excess reagent. Despite its selectivity, the coupling reactions are poor yielding, making the purification process extremely problematic.

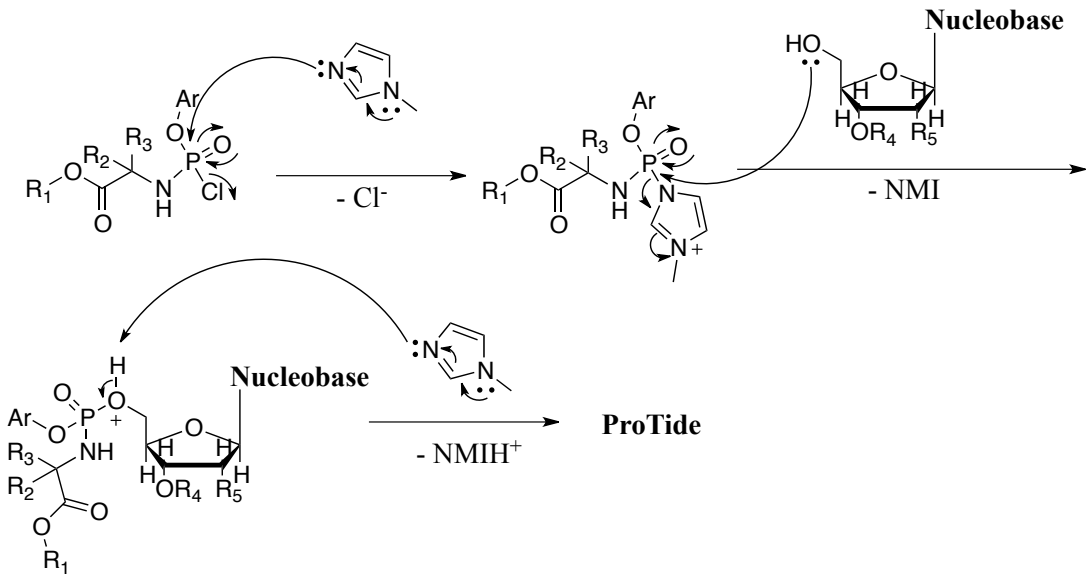


Figure 2.14 ProTide synthesis using *N*-methylimidazole to activate the phosphochloridate R_1 = alkyl; R_2, R_3 = H, alkyl; R_4 = H or protecting group; R_5 = H, OH or O-protecting group; Ar = aryl.

2.4 Aims and scope of thesis

Nucleoside analogues are used in treatment of cancer by perturbing nucleic acid and protein synthesis and consequently, limiting the proliferative potential of malignant neoplastic cells and cancer progression. However, nucleoside analogues have low aqueous solubility, their activity heavily depends on their metabolism to the 5'-monophosphate and they may be poor substrates for specific enzymes involved in their bioactivation, and this often limits their clinical response.

The focus of this thesis has been limited to the synthesis of pronucleotide (ProTide) derivatives of different purine and pyrimidine nucleoside analogues (see below) using phosphoramidate and phosphorodiamidate technology (Chapter 3-6). The cytostatic potential of each ProTide compared to the parent nucleoside was assessed in different wild-type and mutant cell lines, used to model different pathologies such as leukaemia, lymphoma, colorectal and pancreatic cancer (Chapter 3-6). Further experimental methods used to disseminate the identity, purity, bioactivation and efficacy of each ProTide are stated below.

2.4.1 Major aims of the thesis

- To synthesise ProTide derivatives of different anticancer nucleoside analogues such as 5-fluoro-2'-deoxyuridine, 6-thioinosine, 6-thioguanine, 6-S-methyl-thioinosine, cladribine and decitabine using phosphoramidate and phosphorodiamidate technology
- By varying the three main masking moieties and the nucleobase another aim is to develop ProTides that have efficacy and potency that exceeds that of the parent nucleoside

- To employ NMR spectroscopy, mass spectrometry and HPLC to identify and prove the purity of each compound
- To do molecular modeling and enzymatic studies to characterise the bioactivation of ProTides
- To test these compounds in different cancer cell lines to assess their ability to kill or significantly reduce cancer cell proliferation and to further progress them for clinical candidate selection.

2.4.2 Overarching hypothesis

The application of phosphoramidate and phosphorodiamidate technology will help overcome the limiting resistance mechanisms associated with nucleoside therapy in the treatment of cancer.

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3 Clinical candidate selection of 5-FUDR ProTides

5-Fluoro-2'-deoxyuridine (5-FUDR) is an organofluorine containing antimetabolite pyrimidine analogue, which is in major use for the chemotherapeutic treatment of many solid tumours including colon, gastric, breast and ovarian carcinoma.[1-4] The mechanism of action of 5-FUDR is shown in chapter 1 (Chapter 1.7.1.1).

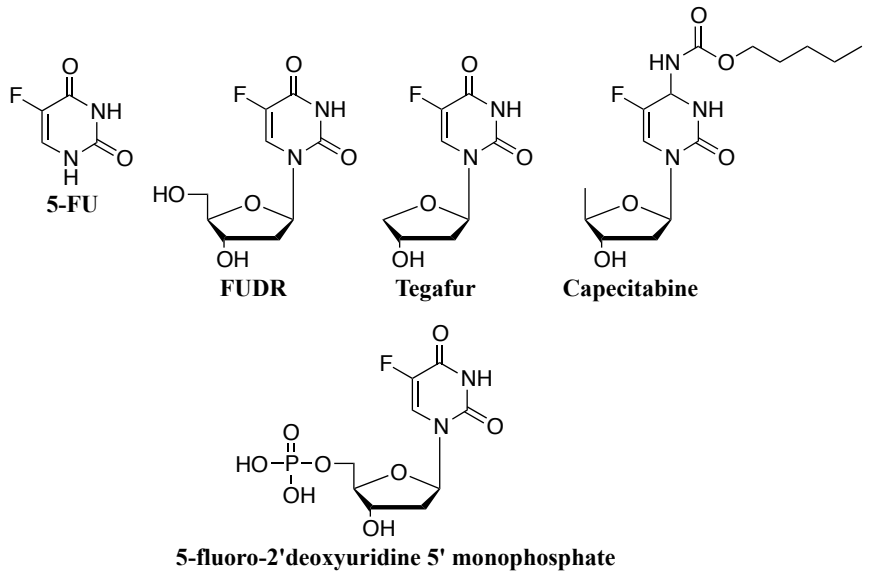

Figure 3.1 Fluorinated pyrimidines

3.1 5-FUDR Phosphoramidates

Application of the ProTide approach to 5-FUDR can bypass the resistance mechanisms associated with 5-FUDR therapy and these include: reduced levels of the enzyme thymidine kinase, which is important in the bioactivation of 5-FUDR; overexpression of thymidylate synthase, which results in off-targeting and toxicity; increased degradative cleavage by thymidine phosphorylase; reduced transporter mediated uptake into cells (Chapter 1.7.1.1.).[5]

Preliminary studies were performed by Paola Murziani and Magdalena Slusarczyk on 5-FUDR ProTides. The main leads discovered, were the L-alanine benzyl naphthyl **3.1b** and L-alanine pentyl naphthyl **3.1e** derivatives (Figure 3.2).

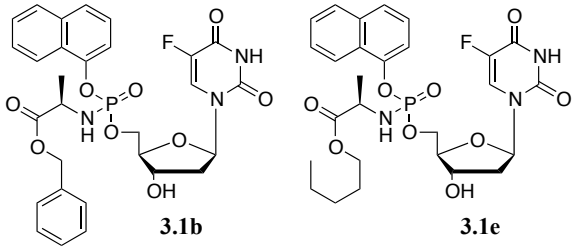

Figure 3.2 Two lead compounds identified at preliminary stage

Table 3.1 Cytostatic activity of 5-FU, 5-FUDR, and prodrugs **3.1b** and **3.1e** against wild type L1210/0, TK-deficient L1210, CEM/0, TK-deficient CEM, HeLa/0, TK-deficient HeLa cells. Data by Prof Balzarini, Rega Institute.
IC_{50} (μM): 50% inhibitory concentration or compound concentration required to reduce cell proliferation by 50%.

Cpd	L1210	L1210/TK$^-$	CEM	CEM/TK$^-$	HeLa	HeLa/TK$^-$
5-Fu	0.33	0.32	18	12	0.54	0.23
5-FUDR	0.0011	3.0	0.022	3.0	0.050	1.4
3.1b	0.0011	0.045	0.068	0.31	0.065	2.5
3.1e	0.0028	0.13	0.015	0.28	0.029	0.44

5-FU, 5-FUDR and FUDR 5' Protides were evaluated against a panel of thymidine kinase (TK) competent and TK-deficient leukaemia cell lines. The cytostatic assays were determined in L1210 murine lymphoblast cell line, derived from mouse lymphocytic leukaemia, human T-lymphoblast CEM cell line, derived from the blood of a patient suffering from acute lymphoblastic leukaemia and HeLa cell line as a model of human epithelial cervical adenocarninoma cell line.

At the preliminary stage mainly L-alanine amino acid ester derivatives were prepared by varying the aryl masking moiety between phenyl and 1-naphthyl aryl units (Figure 3.3).[6-9] Based on this fundamental study, naphthyl derivatives showed improved potency compared to phenyl derivatives in different wild type and thymidine kinase deficient mutant leukaemia cell lines.[9] The in vitro biological data showed that 5-FUDR phosphoramidate derivatives did not improve the cytostatic activity of the parent nucleoside, however while 5-FUDR lost 28 - 2727 fold (IC_{50} = 1.4 - 3 μM) activity against the TK-deficient tumor cells tested, ProTide **3.1b** (IC_{50} = 0.045 – 2.5 μM) and **3.1e** (IC_{50} = 0.13 – 0.44 μM) partially bypass the high dependence of the parent nucleoside on kinase-mediated activation.

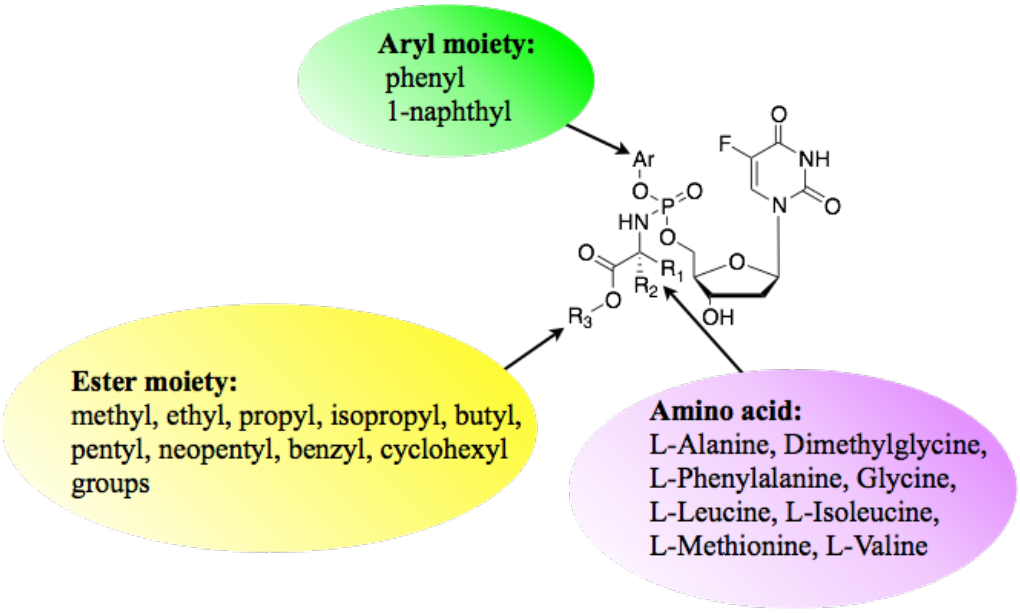

Figure 3.3 Design of 5-FUDR 5'ProTides

The design of the second generation of 5-FUDR ProTides study focused on the investigation of new amino acid esters with modified aryl masking moieties, with the aim to explore the potential of a structure activity relationship. Previously 3', 5' bis-, and 3'-phosphoramidates, isolated as by-products, showed negligible efficacy in

leukaemia and solid tumor cell lines, therefore isolation of these derivatives was not part of my research focus.[9]

3.2 Synthesis of 5-FUDR ProTides

To overcome the biological disadvantages of 5-Fu and 5-FUDR described in Chapter 1.7.1.1, the ProTide technology has been applied to 5-FUDR (Figure 3.3). In this thesis the second generation of 5-FUDR ProTides as potential anticancer agents are reported. The main objectives of this study were to build the SAR with the 5-FUDR phosphoramidates **3.1a - 3.1y** and enhance the cytostatic potential of previously synthesised lead compounds **3.1b** and **3.1e** (Table 3.1). Design of the 5-FUDR ProTides centered mainly around the use of L-alanine amino acid core due as its often appers to be beneficial. Besides a great variety of other amino acids were introduced namely dimethylglycine, L-Phenylalanine, L-Glycine, L-Leucine, L-Isoleucine, L-Methionine, L-Valine. In our SAR studies a wide range of esters of amino acids were introduced to investigate their lability towards the esterase and to improve their biological activity. The use of linear methyl, ethyl, propyl, butyl, pentyl, branched as isopropyl, neopentyl and cyclised cyclohexyl ester groups were considered besides the highly preferred benzyl ester. The aryl esters of the phosphate unit investigated included phenyl and 1-naphthyl.

Based on previous work done in the McGuigan group, 5-FUDR ProTides were synthesised following the Grignard reagent-based method using 1.1 equivalent of *t*BuMgCl.[10] Application of the NMI method resulted in poor yielding final products, and this was due to the acidic conditions of the workup, which was required for the removal of the reagent excess. 3', 5' bis-, and 3'-ProTides were not isolated based on

the results of the previous *in vitro* biological evaluation, where these derivatives greatly reduced the potency of the 5'-derivatives. The general synthetic scheme is shown in Figure 3.4.

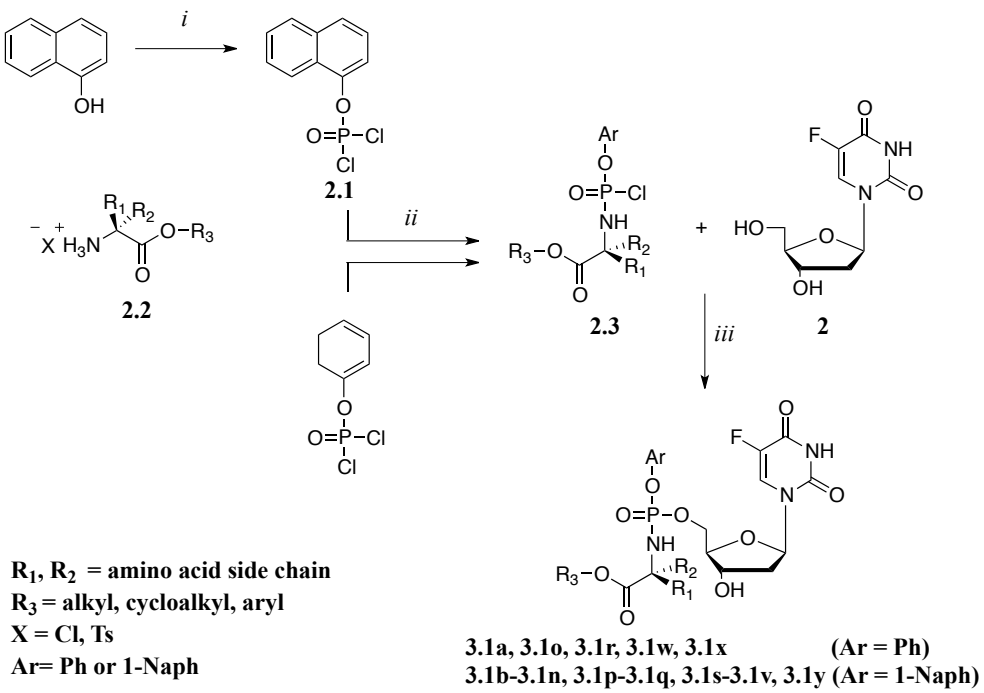

Figure 3.4 Synthesis of 5-FUDR 5'-ProTides
Reagents and Conditions: i, $POCl_3$, Et_3N, anhydrous Et_2O, -78 ^oC, 1hr, to rt, 1hr; *ii*, phenyl or 1-naphthyl phosphorodichloridate, Et_3N, anhydrous DCM, -78 ^oC, 1-2 hrs; *iii*, *t*BuMgCl or NMI, anhydrous THF, rt, overnight.

Conversion of the nucleoside 5-FUDR into 5' phosphate ProTides **3.1a-3.1y** was carried out by coupling of **2** (commercially available) with a range of aryl phosphorochloridates **2.3** in the presence of a strong base such as the Grignard reagent (*t*BuMgCl) or alternatively 1-methylimidazole (NMI) (Figure 3.4). The purification of 5' ProTides was extensive, they all required a second preparative purification by preparative TLC. Coupling reactions were low yielding in all cases mainly due to the formation of the dominant 3', 5' bis-phosphoramidate by-product alongside the 3'-phosphoramidate derivatives. All products were obtained as a

mixture of diastoisomers, confirmed by the presence of two peaks by ^{31}P NMR, generally in a ratio of 1:1. Final compounds were isolated in very low yields (1-7%) due to the repeated purification process. All 5' phosphoramidates prepared are reported in Table 3.2.

The L-leucine pentyl naphthyl phosphoramidate derivative **3.1q** gave two peaks at 4.48 ppm and 4.97 ppm in the ^{31}P NMR spectrum, corresponding to two diastereoisomers. The desired 5'-phosphoramidate regioisomer was determined by ^{13}C NMR spectrum, where C-5' gave two doublets, one for each diastereoisomers, due to their coupling to the phosphorus centre at 67.90 ppm ($^{2}J_{C\text{-}P}$ = 4.45 Hz) and 67.86 ppm ($^{2}J_{C\text{-}P}$ = 4.45 Hz). Regarding the distance between the C-3' and the phosphorus atom, coupling to phosphorus could not be seen, therefore C-3' appeared as two peaks at 72.23 and 72.20 ppm indicating the presence of two diastoisomers. HPLC analysis showed only one peak indicating the presence the two diastereoisomers, with an elution time of 12.52 minutes; which merging could be due to small size of the HPLC column used during the analysis. Structures, yields and key spectral a data of 5-FUDR ProTides are summarised in Table 3.2.

Table 3.2 Structures, calculated lipophilicity, ^{31}P chemical shifts and yields of 5-FUDR 5' ProTides. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0.
Ala: L-alanine; Me_2Gly: 2,2-Dimethylglycine; Gly: L-glycine; Val: L-valine; Phe: L-phenylalanine; Met: L-methionine; Leu: L-leucine; Ile: L-isoleucine

Cpd	AA	Ester	Aryl	ClogP	^{31}P NMR	Yields %
3.1a	L-Ala	Me	Ph	-0.47	3.79, 4.09	2
3.1b	L-Ala	Bn	1-Naph	2.40	4.58, 4.25	5
3.1c	L-Ala	Bu	1-Naph	2.28	4.52, 4.35	1
3.1d	L-Ala	CH_2tBu	1-Naph	2.55	4.56, 4.33	1
3.1e	L-Ala	Pnt	1-Naph	2.81	4.43, 4.29	3
3.1f	L-Ala	Hex	1-Naph	3.34	4.43, 4.28	7
3.1g	L-Ala	CH_2CH_2tBu	1-Naph	3.08	4.18, 3.86	4
3.1h	Me_2Gly	Bn	1-Naph	2.71	2.89, 3.05	4
3.1i	Me_2Gly	Me	1-Naph	1.00	2.98, 2.87	1
3.1j	Me_2Gly	Et	1-Naph	1.53	2.97, 2.85	2
3.1k	Me_2Gly	CH_2tBu	1-Naph	2.86	2.94, 2.82	2
3.1l	Gly	cHex	1-Naph	2.60	5.71, 5.60	2
3.1m	Gly	iPr	1-Naph	1.41	5.75, 5.63	2
3.1n	L-Ile	Pnt	1-Naph	4.26	5.32, 5.06	2
3.1o	L-Leu	Bn	Ph	2.69	4.43, 3.91	3
3.1p	L-Leu	Bn	1-Naph	3.86	4.40	2
3.1q	L-Leu	Pnt	1-Naph	4.26	4.48, 4.97	2
3.1r	L-Met	Bn	Ph	1.38	4.34, 3.94	1
3.1s	L-Met	Bn	1-Naph	2.55	4.95, 4.39	2
3.1t	L-Met	iPr	1-Naph	1.68	4.93, 4.56	2
3.1u	L-Phe	Bn	1-Naph	3.82	4.27, 4.14	1
3.1v	L-Phe	Pnt	1-Naph	4.23	4.39, 4.07	2
3.1w	L-Val	Et	Ph	0.97	4.96, 4.69	1
3.1x	L-Val	Pnt	Ph	2.56	4.95, 4.65	1
3.1y	L-Val	Pnt	1-Naph	3.74	5.39, 5.28	2

Twenty-two novel ProTides of 5-FUDR were prepared, some of which comprise a list of 39 new analogues that were published in 2011.[5] Eight compounds listed in Table 3.2. were scaled-up for clinical candidate selection. They have all been characterised by multinuclear ^{19}F, ^{31}P, ^{1}H, ^{13}C NMR spectroscopy, HPLC and mass spectrometry. Each ProTide was tested in different cancer cell lines to determine

their *in vitro* activity, and six derivatives were selected for *in vivo* biological evaluation.

3.2.1 Scale-up synthesis of 5-FUDR lead phosphoramidates

Six lead derivatives **3.1b**, **3.1e**, **3.1f**, **3.1g**, **3.1h** and **3.1q** has been resynthesized for *in vivo* testing according in order to isolate the final compounds in a 300 – 500 mg scale. To meet the tight time schedule of the shipments of these lead derivatives the best temporary solution seem to be using the well established Grignard method starting the synthetic method from 1200-1500mg starting material FUDR. Purification of the ProTide derivatives required repeated column prurification and it was also found that dividing the big batch of reaction mixture into two or three smaller batches increased the isolated yield of these derivatives (Table 3.3).

Table 3.3 Scaled-up FUDR lead Protides for clinical candidate selection

Cpd	Amino acid	Ester	Aryl
3.1h*	Me_2Gly	OBn	1-Naph
3.1q	L-leu	OPnt	1-Naph
3.1g*	L-Ala	OCH_2CH_2tBu	1-Naph
3.1f*	L-Ala	OHex	1-Naph
3.1e*	L-Ala	Pnt	1-Naph
3.1b*	L-Ala	Bn	1-Naph

Compounds with * were first synthesised by Slusarczyk and Murziani.

In the meantime although in order to improve the overall yield of synthesis of 5-FUDR ProTide lead analogues an alternative synthetic methodology was investigated based on protection-deprotection strategy, which besides having the advantage of leading only towards the 5'-phosphorylated ProTide analogues it might

allow the separation of the two diastereoisomers *R*p and *S*p at the level of the silylated phosphoramidate mixtures (Figure 3.4).

To a stirred solution of 5-FUDR, imidazole and DMAP in anhydrous DMF TBDMSCl (2.2 mmol) was added at 0^{o}C and the mixture was allowed to reach ambient temperature and was allowed to stirr for six hours. The reaction mixture was quenched with saturated aqueous solution of ammonium chloride, and the mixture was extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under vacuum to give a crude product 3',5'-disilylated 5-FUDR (**1**) as an oil, which was used for the second step without further purification (Figure 3.4).

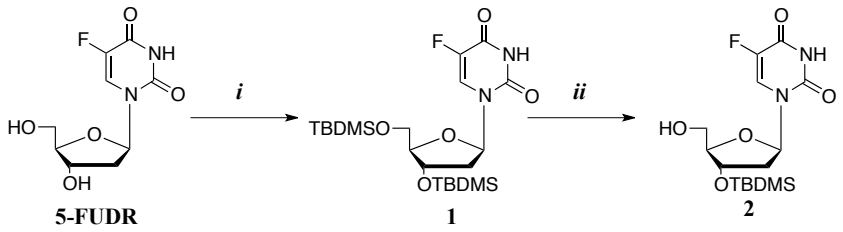

Figure 3.4 Selective 5'-desylilation of 3', 5'-*di*-O-TBDMS-5-FUDR performed by Slusarczyk.
Reagents and Conditions: i, imidazole, TBDMS, DMAP, DMF, rt. to 50^{o}C, 12 hours, 98%; *ii*, 80% acetic acid/THF 4:1, 60^{o}C, 12 hours, 31%.

The selective deprotection step with acetic acid performed by Slusarczyk suffered from long reaction time and it also required high temperature of 60^{o}C furthermore it was low yielding (31%). Therefore it was decided to further investigate the selective deprotection conditions in order to improve the yield. It is well known that primary silyloxy groups are cleaved under acidic conditions more easily than secondary ones, therefore we aim to different acidic conditions were applied onto the 3', 5'-*di*-O-

TBDMS-5-FUDR. An efficient method using TFA/H_2O/THF in a ratio of 1:1:4 at 0^oC were reported to be optimal for the selective removal of 5' TBDMS analogues by Zhu *et al.*[11] was first applied to the 3', 5'-*di*-O-TBDMS-5-FUDR (1) and the formation of the desired compound **2** was monitored by TLC plate.

3',5'-disilylated 5-FUDR (**1**) was dissolved in the 1 : 1 : 4 ratio of TFA/H_2O/THF at -5^oC, then was stirred for 4 hrs at -20^oC. After this period only the disubstituted 5-FUDR derivative, was present in the reaction mixture. Therefore the temperature was increased to -10^oC, and allowed to stir for two additional hours. At this timepoint the 3',5'-disilylated 5-FUDR, the 5' and the 3' monosilylated 5-FUDR derivative and 5-FUDR were present on the TLC plate in the following ratio: 2:1:1:0.5. In fact treating compound **1** under these conditions, there was no selectivity observed towards the formation of the desired 3' monosilylated 5-FUDR furthermore degradation of the starting material **1** to 5-FUDR could be also observed (Figure 3.5).

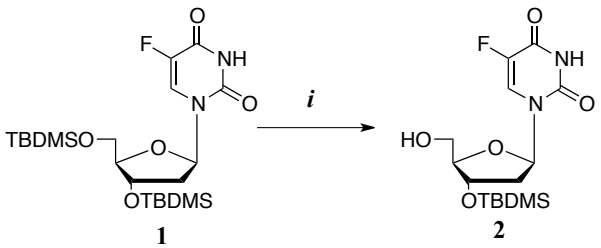

Figure 3.5 Selective 5'-desylilation of 3', 5'-*di*-O-TBDMS-5-FUDR monitored by TLC plate. *Reagents and Conditions: i,* TFA/H_2O/THF (1:1:4), -20^oC to -10^oC, 6 hrs.

Table 3.4 Acidic conditions to try for further investigation

Reagent	Solvent
p-Toluenesulfonic acid (0.5 – 1 eq.)[12]	MeOH/CH_2Cl_2
10-camphorsulfonic acid (0.5 – 1 eq.)[13]	MeOH/CH_2Cl_2
Oxalic acid (1 eq.)[13]	MeOH
Dichloroacetic acid (DCA) (1 eq.)	MeOH

Due to the lack of time remaining did not allow to further explore other acidic conditions. Table 3.4 summarise alternative acidic conditions, which are awaiting for further investigation.

3.3 Biological evaluation of 5-FUDR ProTides

3.3.1 Examining the role of TK

The 5-FUDR ProTides described above were tested for their cytostatic activity against some established tumor cell lines as presented in Table 3.5a and Table 3.5b. Compounds **1** and **2** were included as positive controls. In particular we studied the compounds in wild type murine leukaemia L1210, T lymphocyte CEM and the human cervical adenocarninoma cell line HeLa cells. In each case the thymidine kinase deficient (TK^-) mutant of the parent cell lines were also added in order to study the efficacy of compounds in thymidine kinase deficiency and their degree to bypass their dependence.

If was found that in two out three cell lines L1210 and HeLa, that 5-FU exhibited an IC_{50} of approximately 30-0.60 μM furthermore, 5-FU was found to be poorly active

against CEM cells (IC_{50} = 18 μM), although it did retain activity in the case of the TK^- cell lines tested. This is probably due to phosphoribosylation by the enzyme orotate phosphoribosyl transferase (OPRT). 5-FUDR was more potent in the wild type cell lines with IC_{50} = 1.1 - 50 nM, therefore having a 10-800 fold potency boost over 5-FU. On the other hand 5-FUDR proved to be extremely dependent on the expression of TK, in fact its cytostatic activity was almost 4000 fold lower in TK deficient L1210 and CEM and 30 fold lower in TK deficient HeLa tumor cell lines. This assay could be considered to mirror the clinical circumstance of kinase-deficiency leading to poor activity of nucleosides.

The effect of 5-FUDR ProTides on L1210, CEM and HeLa cell proliferation is shown in Table 3.5a and 3.5b.[14-18] Variability in efficacy and potency is seen amongst the ProTide family, which appears to be dependent on TK (Chapter 1.1.4). The concentration of ProTides excluding the lead compounds that caused 50% reduction in cancer cell proliferation significantly increased in the absence of TK. Not one of the ProTides shown in Table 3.5a and 3.5b exhibited potency that exceeded that of the parent nucleoside 5-FUDR, however the L-alanine pentyl naphthyl **3.1e**, L-alanine hexyl naphthyl **3.1f** and dimethylglycine benzyl naphthyl **3.1h** motifs potency was comparably closest to 5-FUDR's in all three cell lines with intact TK and they retained reasonable activity in TK deficient cell lines with the exception of **3.1f** (80 fold activity loss in CEM/TK^- and almost 50 fold decrease in cytostatic activity against HeLa/TK^-).

Table 3.5a The effect of 5-FUDR ProTides on inhibiting the proliferation of L1210 and CEM cells with or devoid of TK. Data shown are concentrations (μM) of compounds that caused 50% inhibition of cell proliferation (IC_{50}). Data are Mean ± SEM (n ≥ 3). Data were done in Prof Jan Balzarini's lab, Leuven, Belgium.

Cpd	L1210	L1210/ TK^-	CEM	CEM/ TK^-
5-FU	0.33 ± 0.17	0.32 ± 0.31	18 ± 5	12 ± 1
FUDR	0.0011 ± 0.0002	3.0 ± 0.1	0.022 ± 0.006	3.0 ± 0.4
3.1a	0.022 ± 0.007	41 ± 3	0.70 ± 0.37	35 ± 12
3.1b*	0.011 ± 0.007	0.045 ± 0.027	0.068 ± 0.035	0.31 ± 0.06
3.1c	0.022 ± 0.004	0.11 ± 0.06	0.064 ± 0.007	0.84 ± 0.60
3.1d	0.27 ± 0.11	1.2 ± 0.7	0.49 ± 0.05	6.7 ± 1.0
3.1e	0.0028 ± 0.0010	0.13 ± 0.13	0.015 ± 0.006	0.28 ± 0.04
3.1f	0.0072 ± 0.0000	0.076 ± 0.0015	0.0080 ± 0.0020	0.65 ± 0.34
3.1g	0.016 ± 0.006	0.0062 ± 0.009	0.053 ± 0.021	0.19 ± 0.04
3.1h	0.011 ± 0.005	0.13 ± 0.04	0.16 ± 0.02	2.4 ± 0.8
3.1n	0.22 ± 0.12	12 ± 2	0.46 ± 0.11	17 ± 1
3.1o	0.044 ± 0.025	2.0 ± 0.3	0.24 ± 0.04	16 ± 1
3.1p	0.028 ± 0.004	1.5 ± 0.6	0.13 ± 0.00	30 ± 6
3.1q	0.017 ± 0.001	1.2 ± 0.4	0.071 ± 0.008	15 ± 4
3.1r	0.073 ± 0.035	4.1 ± 1.2	0.28 ± 0.03	25 ± 0
3.1s	0.072 ± 0.001	1.9 ± 0.2	0.19 ± 0.10	11 ± 1
3.1u	0.012 ± 0.007	5.6 ± 1.3	0.10 ± 0.03	7.2 ± 0.1
3.1v	0.026 ± 0.001	2.9 ± 1.2	0.10 ± 0.00	8.3 ± 1.0
3.1w	0.16 ± 0.05	42 ± 2	1.0 ± 0.1	> 250

The L-alanine benzyl naphthyl **3.1b** and L-alanine pentyl naphthyl **3.1f** were amongst the most potent derivatives exhibiting IC_{50} = 1.1 and 2.8 nM, therefore 2.5 and 6.5 fold less active than the parent nucleoside and 30 and 100 times more potent than 5-FU. Notably **3.1b** retained significant cytostatic potency in L1210/TK^- (IC_{50} = 0.045 μM) versus wild type L1210/0 cells (IC_{50} = 0.011 μM) with approximately 70-fold increase over the cytostatic activity of the parent nucleoside.

Table 3.5b The effect of 5-FUDR ProTides on inhibiting the proliferation of HeLa cells with or devoid of TK. Data shown are concentrations (μM) of compounds that caused 50% inhibition of cell proliferation (IC_{50}). Data are Mean ± SEM ($n \geq 3$). Data were done in Prof Jan Balzarini's lab, Leuven, Belgium.

Cpd	HeLa	HeLa/ TK^-
5-Fu	0.54 ± 0.12	0.23 ± 0.01
FUDR	0.050 ± 0.011	1.4 ± 0.4
3.1a	0.28 ± 0.14	4.7 ± 0.4
3.1b	0.065 ± 0.013	2.5 ± 1.3
3.1c	0.12 ± 0.02	2.7 ± 1.5
3.1d	0.70 ± 0.11	32 ± 26
3.1e	0.029 ± 0.023	0.44 ± 0.35
3.1f	0.039 ± 0.018	1.8 ± 0.1
3.1g	0.078 ± 0.018	1.3 ± 0.9
3.1h	0.078 ± 0.020	3.1 ± 0.6
3.1n	0.30 ± 0.02	11 ± 1
3.1o	0.067 ± 0.042	5.6 ± 0.3
3.1p	0.080 ± 0.022	9.4 ± 1.4
3.1q	0.039 ± 0.014	7.5 ± 0.4
3.1r	0.15 ± 0.02	11 ± 7
3.1s	0.087 ± 0.017	8.3 ± 0.0
3.1u	0.16 ± 0.08	6.8 ± 1.5
3.1v	0.040 ± 0.000	6.6 ± 0.5
3.1w	1.2 ± 0.3	27 ± 7

Wild type HeLa cell line confirmed a lower cytostatic activity for ProTide **3.1b** (IC_{50} = 0.065 μM) and shown to be more dependent of TK for its cytostatic activity (IC_{50} = 2.5 μM) in Hela/TK^- cell line with an almost 40 fold loss of activity. Overall, it appears that TK is important in bioactivation of 5-FUDR ProTides, although some motifs are able to display a significant retention of activity in TK deficient cell lines.

3.3.2 Biological results on Mycoplasma hyorhinis infection

Several cancer cells were reported to be associated with Mycoplasma infections as Mycoplasma has the potential to cause chromosomal changes in normally dividing mammalian cell populations, promoting malignant transformation and

oncogenesis.[19-20] Furthermore through the expression of mycoplasma derived enzymes such as thymidine phosphorylase, the bacteria is able to greatly decrease the accumulated active metabolites of pyrimidine nucleoside analogues causing dramatic loss of their activity. FUDR is well known for being a subject to TP-mediated deactivation. In Mycoplasma hyorhinis infected MCF-7 breast cancer cell line (MCF/HYOR) 5-FUDR showed 20-150 fold activity loss due to mycoplasma-induced catabolic degradation.[21-22] A study on mycoplasma infection in human carcinomas reported that 40–56% of gastric, colon, oesophageal, lung and breast cancers were infected with mycoplasma compared to non-tumourigenic tissue.[19]

The biological assay was performed to evaluate the cytostatic activity of the compounds presented within this thesis, were conducted by Prof Jan Balzarini, Rega Institute. The cytostatic activity of 5-FUDR and 15 FUDR ProTide derivative was investigated in wild type murine leukaemia cell line L1210/0 and its Mycoplasma Hyorhinis infected mutant. The parent compound displayed a remarkable decrease in cytostatic activity by 378 fold (IC_{50} = 0.34 μM), while in contrast the ProTide analogues kept a significant cytostatic activity under the same experimental conditions losing 2-4 fold antiproliferative activity with the exeption of **3.1r**. (13 fold activity loss) in Mycoplasma infected L1210 cell line.

Table 3.6 The effect of 5-FUDR ProTides on inhibiting mycoplasma hyorhinis infected L1210 cell proliferation. Preliminary data shown are concentrations (μM) of compounds that caused 50% inhibition of cell proliferation (IC_{50}). This study was done in Prof. Jan Balzarini's lab. Data are mean ± SD.

Cpd	L1210	L1210/hyor	IC_{50}L1210: IC_{50}L1210/hyor
FUDR	0.0009 ± 0.0003	0.34 ± 0.13	378
3.1n	0.42 ± 0.021	0.70 ± 0.074	1.67
3.1p	0.029 ± 0.0021	0.048 ± 0.020	1.7
3.1q	0.031 ± 0.0020	0.035 ± 0.010	1.13
3.1r	0.058 ± 0.035	0.76 ± 0.18	13
3.1o	0.054 ± 0.021	0.17 ± 0.047	3.2
3.1u	0.021 ± 0.0061	0.021 ± 0.078	11
3.1s	0.054 ± 0.013	0.020 ± 0.098	3.7
3.1v	0.030 ± 0.0039	0.14 ± 0.007	4.67
3.1c	0.0095 ±0.0021	0.0210 ± 0.0071	2.2
3.1b	0.011 ± 0.009	0.025 ± 0.01	2.27
3.1f	0.0032 ± 0.00035	0.0022 ± 0.00028	0.69
3.1g	0.012 ± 0.0018	0.032 ± 0.0088	2.7
3.1h	0.019 ± 0.004	0.045 ± 0.004	2.4
3.1e	0.0021 ± 0.00007	0.006 ± 0.0014	2.9

The data demonstrate that 5-FUDR ProTides are resistant to mycoplasma encoded phosphorolytic activity of thymidine phophorylase, which may result in a therapeutic benefit as tumors often show an increased TPase activity allowing therefore better angiogenesis in the tumor tissue[23]. The parent nucleoside is a known substrate for TPase and based on this study the majority of the synthesised prodrugs are resistant for this phosphorolytic cleavage. Figure 3.6 visually illustrates the most potent prodrug motifs of this family, highlighting their remarkably retained cytostatic activity compared to the parent nucleoside in Mycoplasma infected L1210 cell line.

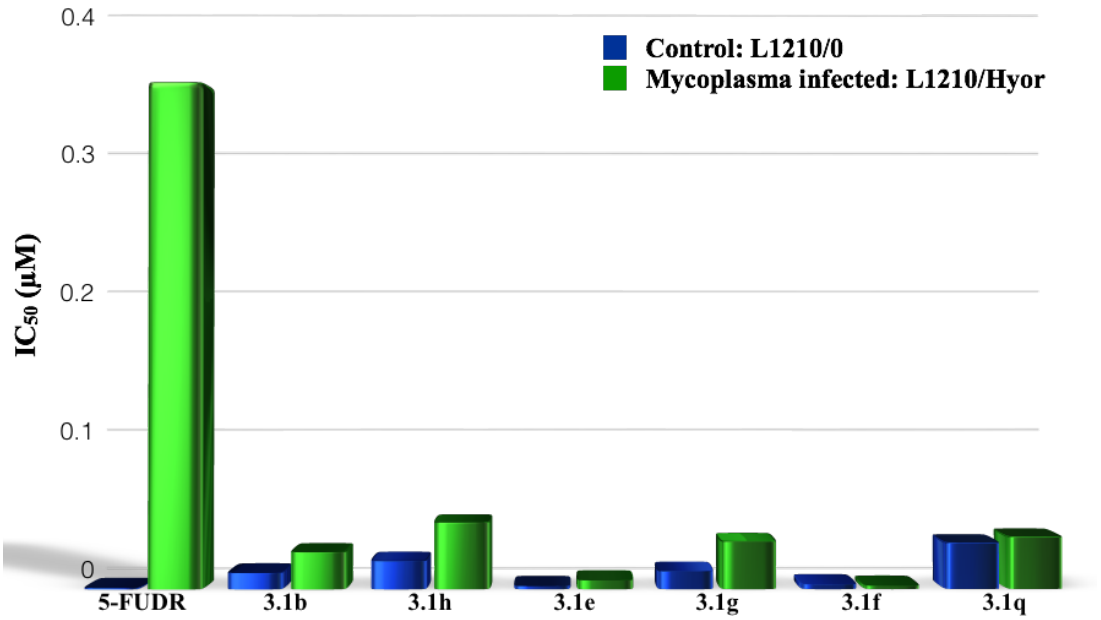

Figure 3.6 Cytostaic activity of 5-FUDR and 5-FUDR prodrugs: **3.1b, 3.1h, 3.1e, 3.1g, 3.1f, 3.1q** against wild type L1210 and Mycoplasma hyorhinis infected L1210 cell line

3.3.3 Xenograft study

Numerous murine models are available to study human cancer investigating the invasion, metastasis and the response to therapy. One of the most widely used transplantation model is the human tumor xenograft, which propagates tumor tissues for *in vivo* studies. In this method human cancer cells transplanted into the host immune-compromised mice, therefore rejection of the foreign cells can likely be excluded. The main advantage of the study, that the cancerous tissue carries human genetic information, although on the other hand due to host's impaired immune system may not be a good model of human cancer.

The biological study performed to evaluate the effect of 5-FU and 5-FUDR ProTides: **3.1b**, **3.1q**, **3.1h**, **3.1f**, **3.1g**, **3.1e** in the presence of vehicle control on body weight and tumor volume (mm^3), carried out by WuXiAppTec, China.

HT29 colorectal adenocarcinoma cell lines were injected into nude immune-compromised mice. Mice were treated with vehicle, 5-FU or 5-FUDR ProTides: **3.1b**, **3.1q**, **3.1h**, **3.1f**, **3.1g**, **3.1e** every three days intraperitoneally. Over 17 days, tumor volume was lower in mice treated with 5-FUDR ProTides and 5-FU compared to vehicle control. 5-FUDR ProTides were more effective at inhibiting tumour growth compared to the parent nucleobase 5-FU and amongst these, **3.1q** (147mg/kg IP) showed the greatest activity. However, despite **3.1q** having the best inhibitory effect on reducing the tumour volume, it exhibited the greatest toxicity illustrated by a significant reduction in the body weight of mice over 17 days (12% reduction in the body weight). Notably **3.1b** the L-alanine benzyl phenyl Protide motif also performed well in this assay by inhibiting tumor growth with (140mg/kg IP) slightly lower than of **3.1q**, but proved to be less toxic compared to **3.1q**, causing approximately 5% reduction in body weight (Figure 3.7a, 3.7b).

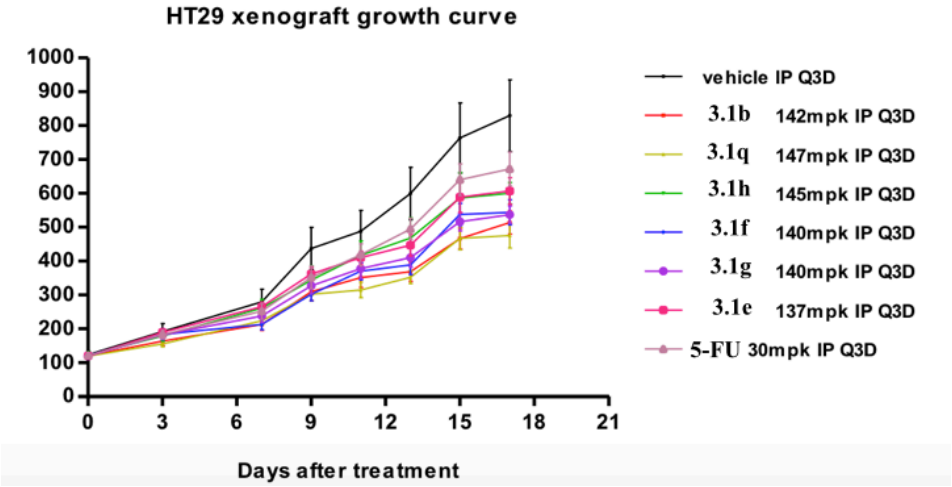

Figure 3.7a The effect of vehicle, 5-FU and 5-FUDR ProTides: **3.1b**, **3.1q**, **3.1h**, **3.1f**, **3.1g**, **3.1e** on tumor volume (mm^3, % change). Data are Mean ± SEM ($n \geq 3$).

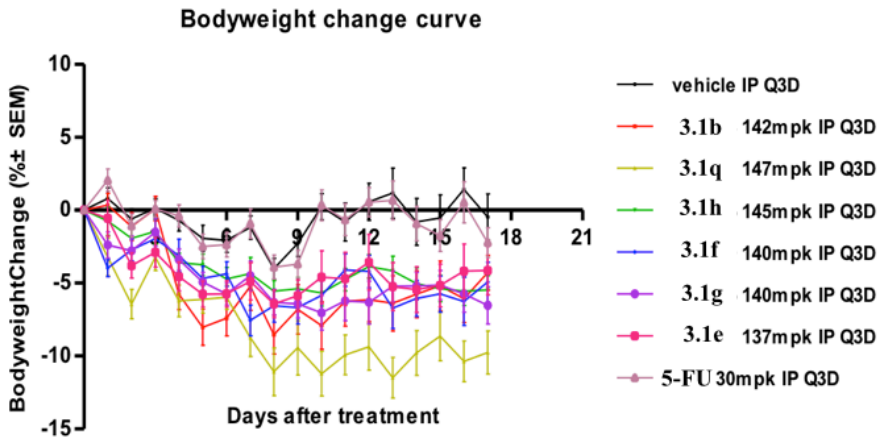

Figure 3.7b The effect of vehicle, 5-FU and 5-FUDR ProTides: **3.1b**, **3.1q**, **3.1h**, **3.1f**, **3.1g**, **3.1e** on body weight (% change). Data are Mean ± SEM (n ≥ 3).

3.4 5-FUDR ProTides and the acute myeloid leukaemia (AML) stem cell model

A pilot study of gemcitabine ProTides indicated some unexpected selectivity for the leukaemic stem cell compartment, which led us to look into the potential of 5-FUDR ProTides whether they are able to preferentially target leukaemic stem cells (LSC). To show this, KG1a acute myeloid leukaemia cell line was used as it manifests a minor stem cell like compartment with a specific immunophenotype ($CD34^+/CD38^-/CD123^+$). In order to compare the cytotoxicity and the stem cell targeting properties of the ProTide analogues, 5-FUDR ProTides were evaluated for their LD_{50} value and their effect was investigated on the leukaemic stem cell compartment ($CD34^+/CD38^-/CD123^+$).

3.4.1. Identification of leukaemic stem cell compartment and biological evaluation

KG1a cells were grown in the presence of the LD_{50} concentration of each 5-FUDR ProTide for 72hrs. Cells were then sampled and labeled with the antibodies CD34-FITC, CD38-PE and CD123 PERCP-cy5. The subpopulation ($CD34^+/CD38^-/CD123^+$) of each culture was expressed as a proportion of the total culture. The effect of each FUDR ProTide on the stem cell compartment was examined and compared with untreated cultures labelled with the same antibodies.

A total of five 5-FUDR phosphoramidate besides 5-FU and 5-FUDR as positive controls were evaluated. 5-FU (LD_{50} = 2.08 μM) and 5-FUDR (LD_{50} = 1.34 μM) displayed low micromolar cytotoxic activity in KG1-a cell line, however 5-FUDR

ProTides showed a stricking 2 – 57 fold enhanced *in vitro* cytotoxity (LD_{50} = 0.036 – 0.92 μM) when compared 5-FU (LD_{50} = 2.08 μM) and 1.5 – 37 fold increase in cytotoxic efficacy when compared to their parental nucleoside 5-FUDR (LD_{50} = 1.34 μM). In terms of stem cell targeting 5-FU and 5-FUDR did not target the specified stem cell compartment (stem cell selectivity = 4.3 and 4.6 %, control = 4%), while the L-alanine benzyl naphthyl derivative **3.1b** (stem cell selectivity = 2.2%, control = 4%), and **3.1e** (stem cell selectivity = 2.0%, control = 4%) showed increased selectivity towards this stem cell like compartment, suggesting stem cell specific action (Table 3.8). The rest of the 5-FUDR phosphoramidate analogues **3.f**, **3.1g** and **3.1q** did not display any selectivity towards the leukaemic stem cell compartment.

Table 3.8 Comparative cytotoxicity of 5-FUDR lead ProTides, 5-FU and their respective parental nucleoside 5-FUDR in KG1a cell line.
LD_{50}: concentration of compound required to kill 50% of test polulation in μM.
LD_{50} values are the mean of 3 separate experiments. Study was performed by Prof. Pepper.

Cpd	**LD_{50} value (μM)**	**Stem cell%** Control: 4%
5-FU	2.08	4.3
5-FUDR	1.34	4.6
3.1f	0.094	4.0
3.1e	0.036	2.0
3.1b	0.063	2.2
3.1q	0.70	5.2
3.1g	0.92	4.4

3.5 Putative metabolism mechanism assays

The putative mechanism of activation of phosphoramidate prodrugs requires an initial hydrolysis of the ester unit by esterase or carboxypeptidase type enzyme. Subsequently the nucleophilic attack of the carbonyl group on the phosphorus centre resulting in the spontaneous cyclisation and elimination of the aryloxy masking moiety. The presumably formed five membered ring, is very unstable and undergoes opening to give the diacid metabolite. The last step in the bioactivation pathway involves the hydrolysis of the P-N bond mediated by phosphoramidase type enzyme relying on the activity of the human histidine triad nucleotide binding protein HINT1 in order to obtain the desired monophosphate. The resulting monophosphate then undergoes subsequent phosphorylation by specific kinases to form di- and triphosphates. Birkus *et al*.[24] discovered that one of the lysosomal associated enzymes namely carboxypeptidase A (cathepsin A) is responsible for the ester cleavage of the nucleotide amidate prodrugs. It is a multifunctional enzyme with esterase, deaminase and carboxypeptidase catalytic activity furthermore shows high affinity for hydrophobic and basic amino acids[25] and demonstrate high level of enzyme expression in kidney, liver and lung tissues.[25, 26]

To establish the influence of different esters on potency and anticancer activity of 5-FUDR aryloxyphosphoramidates, due to being better substrate for the activating enzymes, Carboxypeptidase Y assay was utilized to demonstrate the rate of the ester hydrolysis as they share high degree of structural homology.

3.5.1 Carboxypeptidase Y study

Human Cathepsin A (human Carboxypeptidase Y) is not commercially available, however yeast derived Carboxypeptidase Y is available on the market, and has similar sequence homology around the catalytic unit (Ser146, Asp338 and His397). Carboxypeptidase Y cleaves the amino acid esters of the ProTides at physiological conditions (pH= 7.6) at room temperature.[27-28] Enzymatic studies have been carried out using carboxypeptidase Y assay to evidently show the putative mechanism of activation of the 5-FUDR phosphoramidates.[27] The first activation step is critical in conferring potency of our ProTides. The enzymatic assay was developed within the McGuigan group and run at ambient temperature optimal for the hydrolytic activity of Carboxypeptidase Y at pH = 7.6 followed by ^{31}P NMR.

The L-alanine pentyl naphthyl derivative **3.1e** was dissolved in deuterated acetone-*d6*, Trizma buffer and the blank ^{31}P NMR recorded. Thereafter compound **3.1e** was incubated with Carboxypeptidase Y enzyme and monitored at seven minutes intervals (128 scans) overnight. The ^{31}P NMR spectrum showed rapid hydrolysis of the phosphoramidate **3.1e** represented as two signals indicating the presence of two diastereoisomers at δ 4.03 and 4.31 ppm to the first rate limiting metabolite **3.1e*i*** lacking the ester moiety at δ 4.99 - 5.13 ppm. At the seven minute time point a single peak formed corresponding to the final aminoacyl derivative at δ 6.82 ppm. The half-life of the compound is approximately three minutes (the compound is well processed), which is consistent with the good activity found for this compound (see Table 3.1 and Figure 3.9).

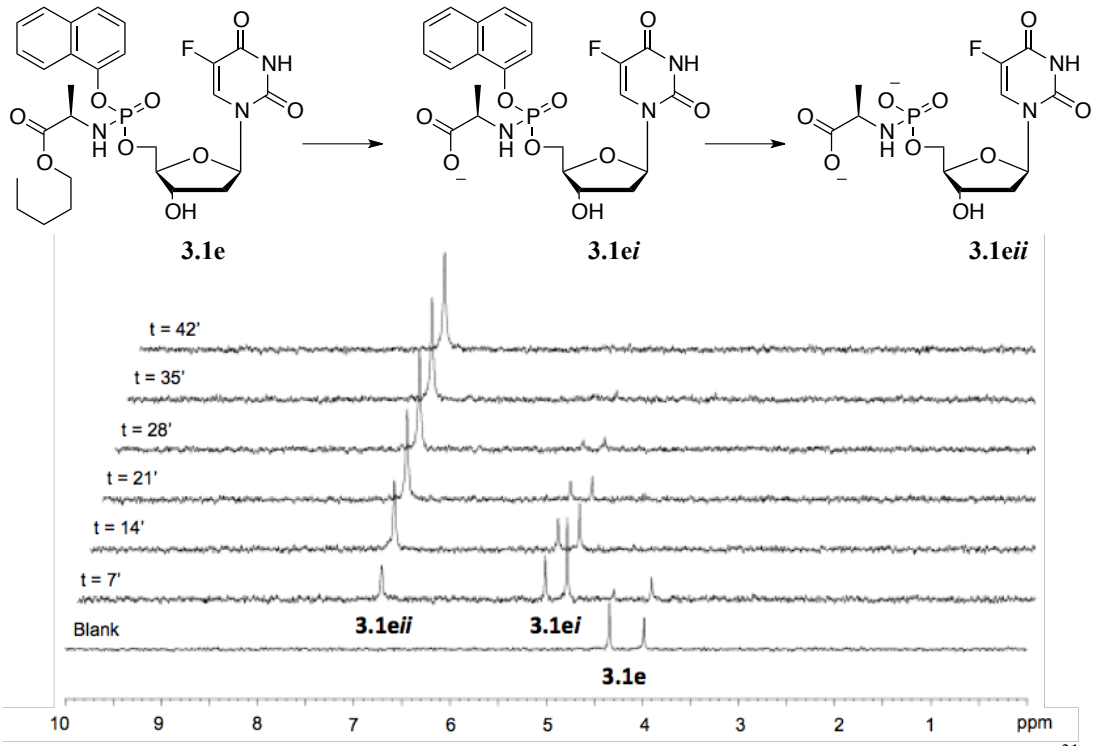

Figure 3.8 Carboxypeptidase Y mediated activation of compound **3.1e,** monitored by ^{31}P NMR. Enzymatic experiment performed by Slusarczyk.

3.5.2 Stability in human serum assay

The stability towards hydrolysis in human serum was investigated using in situ ^{31}P NMR. The aim of the experiment was to identify the formation of any metabolites of the ProTide derivative used. The assay was carried out on the L-alanine ethyl naphthyl derivative compound **3.1z** synthesized by Slusarczyk. ProTide **3.1z** dissolved in DMSO-*d6* and D_2O and the blank ^{31}P NMR was recorded at 37^{o}C. The NMR sample was incubated with human serum (0.3 ml) and subjected to further ^{31}P NMR experiment at 37^{o}C, acquiring scans at regular intervals of 15 minutes over 14 hours. In order to improve visualization of the results due to the the excess noise and poor shimming profiles raising from the presence of the biological media, these spectras needed further processing by Lorentz-Gauss deconvolution method (Figure 3.10).

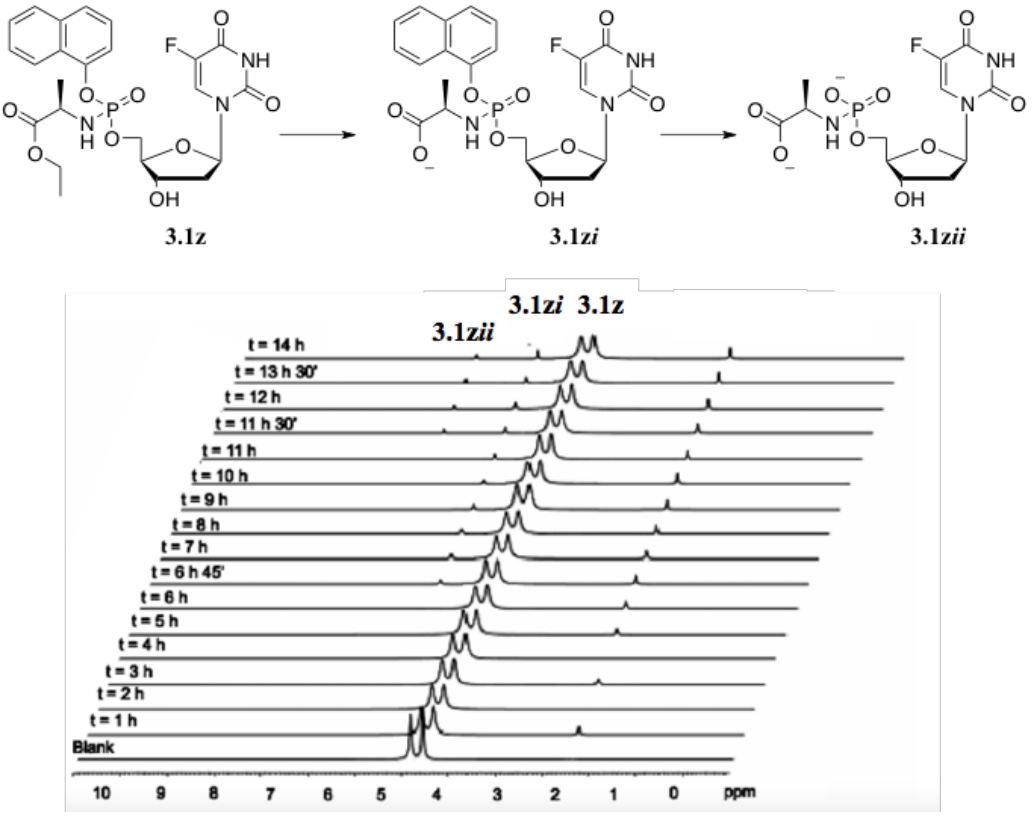

Figure 3.9 Human serum assay applied on L-Alanine ethyl naphtyl ProTide **3.1z,** monitored by ^{31}P NMR at 37^{o}C. Human serum experiment were carried out by Slusarczyk.

Human serum was represented as a single peak at δ 2 ppm next to compound **3.1z**, where two signals indicative of two diastereoisomers at δ 4.59 and 4.84 ppm are present. After 7 hours in the presence of the human serum intermediate, **3.1z*i*** appeared at δ 4.59 ppm, which partly hydrolysed to a singlet peak downfield at δ 7.09 ppm arising from the achiral phosphate metabolite **3.1z*ii*** after 11 hours. At the end of the experiment, the enzymatic mixture contained the parent ProTide **3.1z** with intermediates **3.1z*i*** and **3.1z*ii*** in a ratio of 96:3:1%. The hydrolysis proceeded slowly until the end of the experiment, which demonstrated that phosphoramidate is highly stable in human serum, with approximately 96% recovery after 14 hours.

3.5.3. Computational docking of FUDR phosphoramidate monoester with Hint1 enzyme

Preliminary information on ProTide mechanism, stability and their activation in different environments, together with biological results, suggested us to conduct molecular modeling studies using docking techniques. This tool was applied in order to understand if 5-fluoro-2'deoxyuridine L-alanine phosphate, as the aminoacyl derivative intermediate obtained during the enzymatic experiment, could be a good substrate for the human Hint1 enzyme in order to generate the free monophosphate. As mentioned in Chapter 2.2.7.1, Figure 2.7 the last step of the bioactivation of our ProTides involves hydrolysis of the P-N bond by a phosphoramidase-type enzyme [29-30]. Human Hint1 enzyme, which belongs to the HIT superfamily, could be responsible for the cleavage of the phosphorus-nitrogen bond. The proposed mechanism of action of Hint1 enzyme is showed in Figure 3.10.[29]

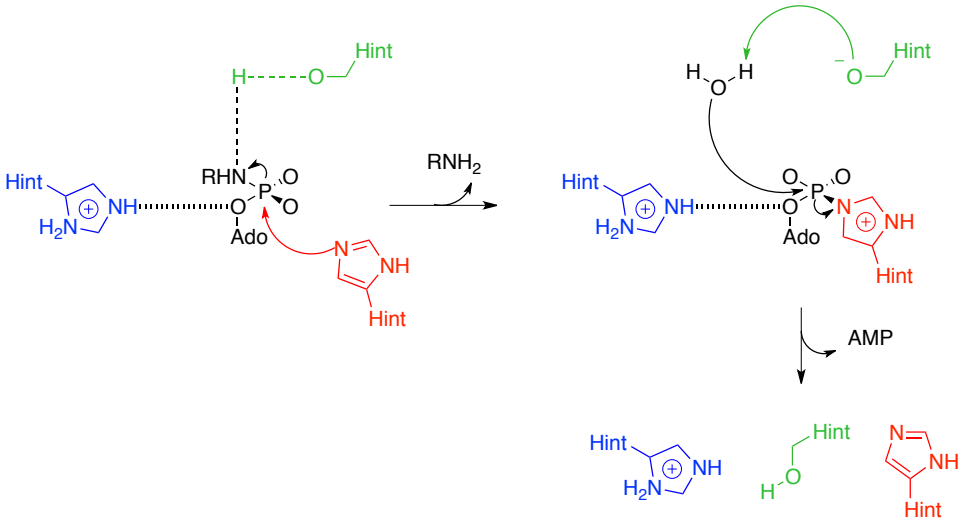

Figure 3.10 The proposed mechanism of action of Hint1 enzyme

The attack of the histidine (red) to the phosphorus centre would release the amino acid through the cleavage of the N-P bond, which was mediated by the proton

transfer from the Serine 107 (green). Subsequently a molecule of water could attack the phosphorus centre with the release of the AMP, histidine (red) and regenerating the Hint1 active site.

Preliminary docking studies were performed using co-crystallised structure of adenosine monophosphate (AMP) with the enzyme Hint1 in order to identify the catalytic site (Figure 3.11).

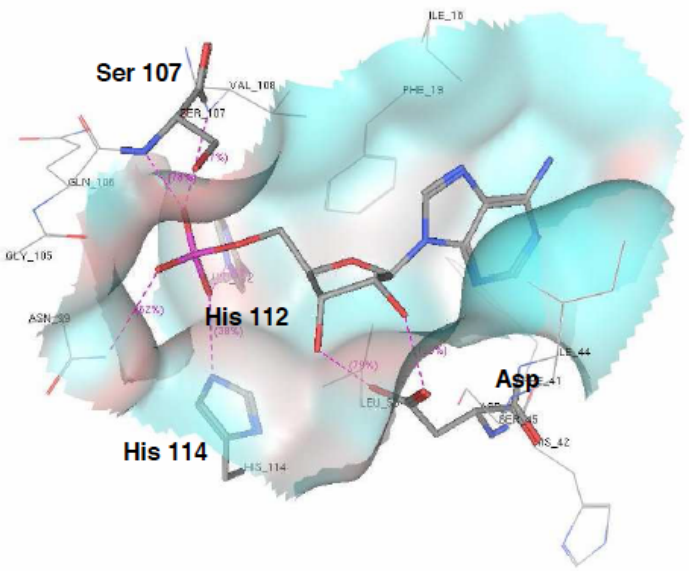

Figure 3.11 Co-crystallised structure of adenosine monophosphate (AMP) in the active site of Hint1 enzyme.

The phosphate group of adenosine monophosphate (AMP) is positioned in the small pocket between the serine (Ser 107) and histidine (His 112 and His 114) residues of the active site of Hint1 enzyme. The hydroxyl groups of the sugar moiety are involved in the hydrogen bonding with an aspartic residue (Asp). Furthermore the purine base fits in the narrow hydrophobic pocket generated by the side chains of different residues.

In the following docking studies of FUDR L-alanine phosphate was investigated in order to investigate whether it is a possible pubstrate for the Hint1 enzyme.

Figure 3.12 illustrates the interaction between 5 FUDR L-alanine phosphate and the amino acid residues involved in the hydrolysis of the P-N bond. The phosphate centre is involved in bonding with Ser 107 and both, His 112 His 114 residues of the active site, while the hydroxyl groups of the sugar moiety interacts with the aspartic residue.

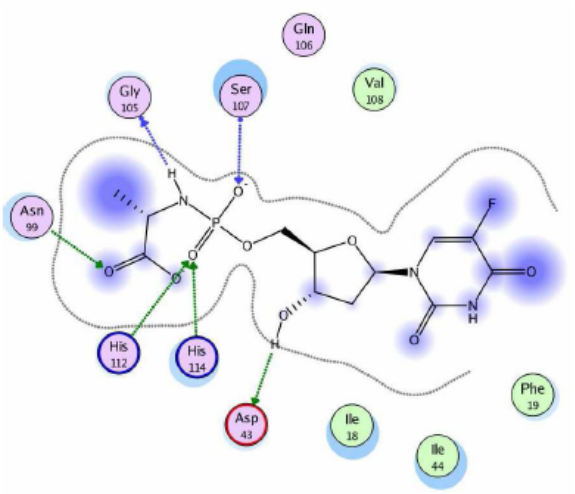

Figure 3.12 Interaction of FUDR L-alanine phosphate within the catalytic site of human Hint1 enzyme.

Molecular docking studies were carried out using a co-crystallised structure of adenosine monophosphate (AMP), which was serving as a template with the phosphoramidase type enzyme Hint1 in order to identify the catalytic site. Two lead derivatives **3.1e*i*** (FUDR L-alanine phosphate) and **3.1q*i*** (FUDR L-leucine phosphate), with the most promising biological data, were docked within the active site of human Hint1 enzyme in order to investigate their binding properties to the active site of the enzyme (Figure 3.13). From the studies, it appears that the

phosphate moiety of **3.1e*i***, indicated in pink, fits well with the enzyme pocket, the phosphorus lies in a suitable position for the P-N bond cleavage in comparison with AMP. The sugar unit of derivative **3.1e*i***, showed similar orientation of AMP sugar moiety. However the pyrimidine base displayed poor level of interaction with the hydrophobic pocket and a disfavoured spatial orientation. Consequently the pyrimidine base was pushed outside of the hydrophobic pocket in comparison with the AMP purine moiety. While in case of docking **3.1q*i***, it appears, that the FUDR L-leucine phosphate derivative is not able to interact with the catalytic site of the enzyme, in fact the phosphate moiety did not lie in the best position for the P-N bond cleavage. This outcome suggests that the last step of the bioactivation of FUDR ProTides in particular L-alanine derivatives, may proceed well, supporting the anticancer activity found for compound **3.1e*i*.**

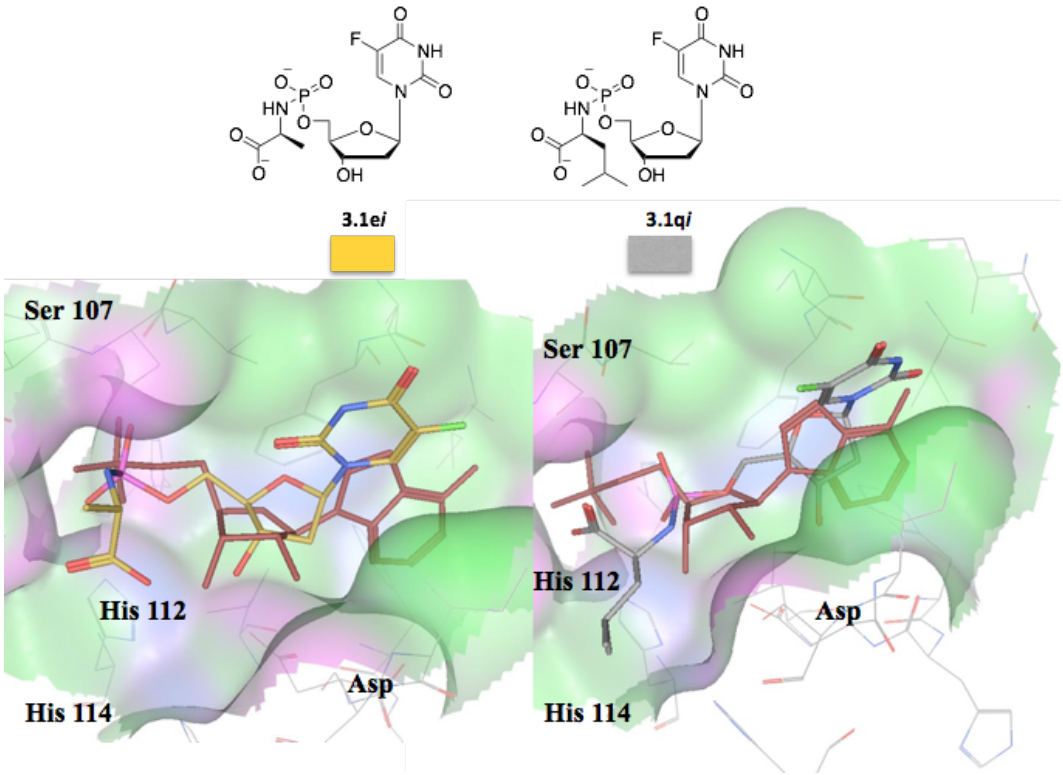

Figure 3.13 Docking of compound **3.1e*i*** (in yellow) and **3.1q*i*** (in grey) with human Hint-1 enzyme.

3.6 Conclusion

These series of studies demonstrate that 5-FUDR ProTides are able to overcome several of the resistance mechanisms associated with 5-FUDR anticancer therapy in the clinic. Several ProTides were able to retain the high potency of 5-FUDR in vitro and partially bypass the high dependence of the parent nucleoside on TK rate-limiting phosphorylation and TP mediated degradation.

The lead analogues displayed similar efficacy to the parent nucleoside, and do not show significant loss of activity upon Mycoplasma infection as displayed by the parent. Furthermore, *in vivo* xenograft data suggests that the lead compounds inhibited tumour growth with marginal treatment associated toxicity.

5-FUDR ProTide analogues were found to be stable in plasma and activated by intracellular carboxypeptidase. Molecular modeling showed that the moderate activity of **3.1q** in comparison with **3.1b** could emerge from the inefficient cleavage of the P-N bond in the last step of the bioactivation to release the 5-FUDR monophosphate inside the cell. 5-FUDR phosphoramidates **3.1b** and **3.1e** exhibited selectivity towards KG1a stem-like cell compartment, which results may be clinically relevant, as cancer stem cells have the potential to form new neoplasms following metastasis. Furthermore relapse from conventional chemotherapy that targets differentiating or differentiated cells, may be prevented by targeting the cancer stem cell compartments of solid neoplasms.[31-32]

The final selection for the lead candidates **3.1b**, **3.1e**, **3.1f**, **3.1g**, **3.1h and 3.1q** was

made based on data collated from all *in vitro*, *in vivo* experiments and depending of their ability of selectivity targeting the KG1a stem cell compartment

On this basis 5-FUDR ProTides proved to be potential anticancer drugs. In particular compound **3.1b** was emerged as top candidate and this is not surprising considering that the Acelarin ProTide of gemcitabine that entered phase 2/3 clinical trials in 2014, synthesized in the McGuigan group, also harbours the same L-alanine benzyl ester moiety.[33]

3.7 References

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4 ProTides of Thiopurine analogues

The principal therapeutic targets of thiopurines are cancer, autoimmune disorders (Crohn's disease and Rheumatoid Arthritis) and immunosuppression.[1] The pioneering use of 6-mercaptopurine is most common in childhood acute lymphoblastic leukaemia, which has contributed to considerable improvements in therapy.[1-2] One of the biggest achievements of 6-mercaptopurine as an immunosupressant is the increased survival rate after unrelated organ transplant.[1-2] Their pharmacological mode of action is described in detail in Chapter 1.5. The successful application of the research ideas of collaborating pharmaceutical chemists, later Nobel Laureates (1988), Gertrude Belle Elion and George Hitchings was exemplified in 1950 by 6-thioguanine, the purine analogue, which could treat leukemia by stopping cancerous white blood cells from proliferating.[3] During 1950-51 their research culminated in the development of the highly active immunosuppressive drug 6-mercaptopurine.[1, 4-6] An important part of their discovery, was that sulphonamides were capable of enhancing the chemotherapeutic effect of other antileukaemic agents. They are highly valued drugs in combination therapy to this day.[7-9] for an illustration of the metabolism of thiopurines[1] (Chapter 1, Figure 1.7).

4.1 The first series

A new series of 6-thioinosine and 6-thioguanosine ProTides were designed with the application of both phosphoramidate and phosphorodiamidate technologies for the first time in the McGuigan group, in order to investigate their true therapeutic potential using *in vitro* models of leukaemia and non-Hodgkin's lymphoma.[6, 10-13]

The series consisted of ten ProTide analogues and six diamidates for each nucleoside. In the case of the ProTides, eight analogues contained L-alanine as natural amino acid and two contained the unnatural L-leucine, with variation of the ester functionality through linear, branched and cyclic esters, together with the variation of the aryl moiety from phenyl to naphthyl. In the case of the diamidate series, four out of five analogues contained L-Alanine amino acid and one L-leucine.

It was hypothesised that the application of phosphoramidate and phosphorodiamidate technologies will help overcome the rate limiting and resistance mechanisms associated with thiopurine therapy in the treatment of blood borne cancers.

4.2 Synthesis

4.2.1 The first synthetic route towards 6-thioinosine

6-Mercaptopurine riboside was first synthesizsed by Elion and Hitchings in 1951 by condensing the silver salt of 6-benzylmercaptopurine with tetraacetylglucopyranosyl chloride followed by deacetylation then debenzylation.[14] There were many subsequent approaches developed in order to optimise the conditions leading towards the formation of the desired compound.[15-16] Some of the synthetic pathways involved the reaction between chloromercurie derivatives of 6-chloropurine and triacetyl ribofuranosyl chloride, followed by deacetylation then further reaction with sodium hydrogen sulphide in methanol to gave rise to the final product.[17] The reaction between 2',3',5'-tribenzoylated inosine and phosphorus pentasulphide, followed by deprotection and the reaction of 6-chloro-9-O-acetyl inosine with nucleophilic thiating agent, where the chlorine atom is replaced by a mercapto group.[18] The nucleoside is now commercially available, although expensive. The

first attempt towards the synthesis of 6-thioinosine was done according to Gupte and Buolamwini (Figure 4.1)[19].

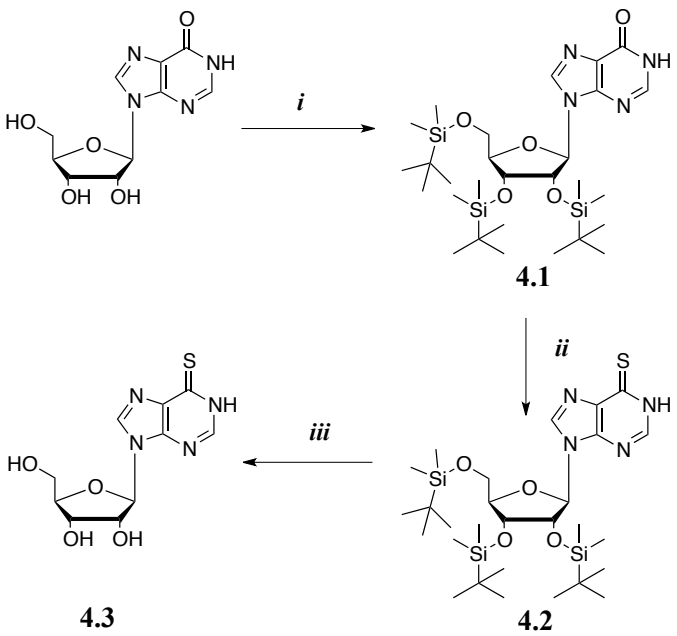

Figure 4.1 The first synthetic route towards 6-thioinosine
Reagents and Conditions: i, TBDMSCl, imidazole, DMF, rt., 16 hours; *ii,* Lawesson's reagent, toluene, reflux, 110°C, 2 hours; *iii,* TBAF, THF, 16 hours

In the first step of the synthesis inosine was subjected to TBDMS protection. To a solution of inosine and imidazole in anhydrous DMF TBDMSCl was added and stirred at room temperature under inert gas for 24 hrs. The reaction mixture was poured into a one to one ratio of ethyl acetate-water mixture then the organic layer was separated, and dried over $MgSO_4$ then evaporated. After purification on flash column chromatography, the desired compound **4.1** was isolated in 85% yield.

The second step of the synthetic route converted 2', 3', 5'-O-(tert-butyldimethylsilyloxy) inosine to the triprotected 6-thioinosine, by treatment with Lawesson's reagent (Figure 4.1).[19-20] Lawesson's reagent was added to the dry solution of **4.1** in toluene, and the mixture was stirred at 110°C for 2-2.5 hours until the starting material was consumed. It was found that the reaction did not succeed in all cases owing to the degradative nature of the reagent itself. After numerous attempts to remove the excess Lawesson's reagent, including multiple column chromatographies, this synthetic route was abandoned. Lawesson's reagent is the most widely used reagent for the transformation of a carbonyl functional group into a thio-carbonyl, although it has disadvantages when used in particular reactions. As the usual method of thionation (Figure 4.2) was performed in refluxing toluene or xylene; under these conditions the reagent could undergo dissociation equilibriums, and the formed decomposition products could then interact with carbonyl functional groups forming four-membered rings, which decompose to form thioketones (Figure 4.3). Many research groups have isolated p-methoxy phenyl metathiophosphonate, a side product of the Lawesson's reagent giving evidence for the mechanism.

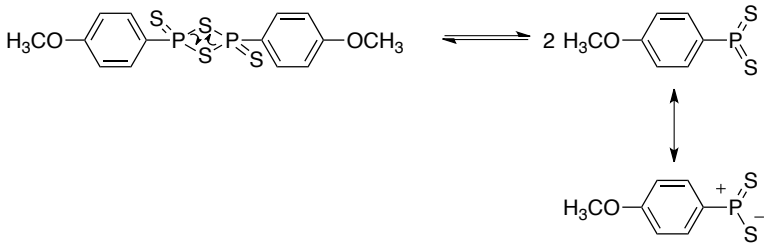

Figure 4.2 Dissociation mechanism of Lawesson's reagent

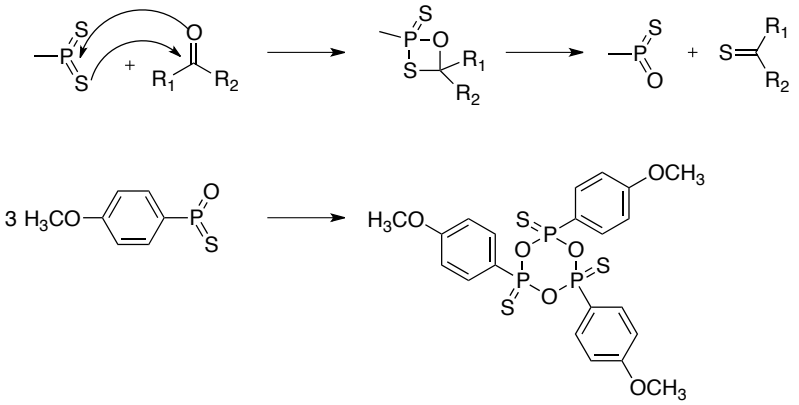

Figure 4.3 Thionation mechanism and formation of the side product

4.2.2 The second synthetic route towards 6-thioinosine

Acetylation of inosine was accomplished in anhydrous acetonitrile by added triethylamine in the presence of DMAP (Figure 4.4). To the stirred mixture acetic anhydride was added dropwise and was allowed to stir at room temperature overnight. Reaction was quenched with MeOH, then diethyl ether was added, and the mixture was left on ice for 1 hour. The desired product **4.4**, as white precipitate was filtered off and used without further purification resulting in 68% yield.

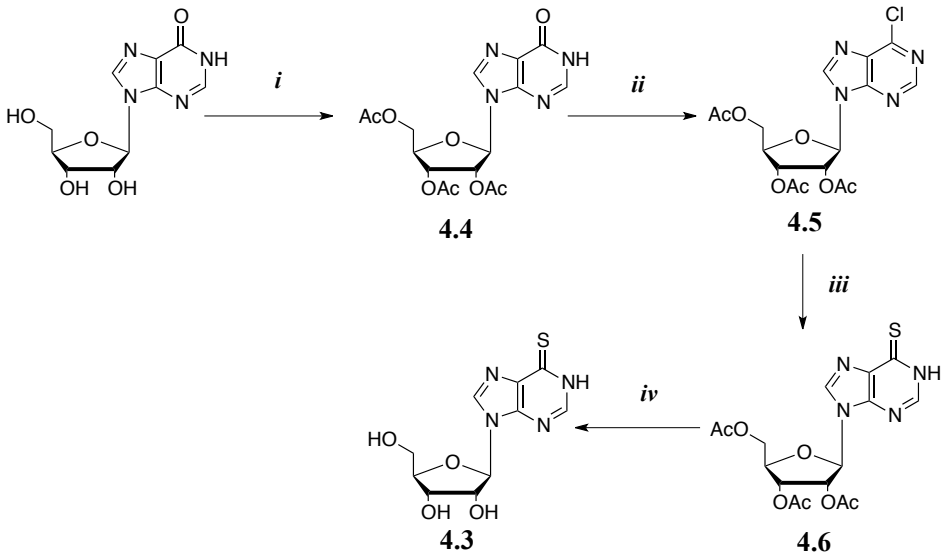

Figure 4.4 The second synthetic route towards 6-thioinosine
Reagents and Conditions: *i,* TEA, DMAP, acetic anhydride, rt, 16 hours; *ii*, BTEA-Cl, *N,N*-dimethylaniline, $POCl_3$, acetonitrile, reflux, 3 hours; *iii*, thiourea or sodium thiosulphate pentahydrate, EtOH, reflux, 2-6 hours; *iv,* NaOMe, MeOH, 2-3 hours

The following chlorination of the 2', 3', 5'-O-acetylated inosine was accomplished by refluxing the protected nucleoside with benzyltriethylammonium chloride (BTEA-Cl), *N,N*-dimethylaniline and phosphorus oxychloride ($POCl_3$) in anhydrous acetonitrile at 95°C for 2-3 hrs. The resulting mixture was carefully evaporated and stirred in the presence of crushed ice for 30 minutes, before the two interface were

separated. The organic layer was separated and washed with ice-cold water and neutralised with 5% $NaHCO_3$. The combined organic layer was dried over $MgSO_4$ and was evaporated to give a yellow oil **4.5,** in approximately 89% yield.

The third step was the rapid formation of thiocarbonyl group from the fully acetylated 6-chloropurine riboside in refluxing ethanol with thiourea at 90°C. After stirring for 10 minutes a white precipitate started to form and allowed to reflux for 1-2 hours. After cooling the white precipitate was collected and washed with hot water resulting **4.6** in 55% yield.[21] Formation of the thiocarbonyl group was also achieved by refluxing aqueous solution of sodium thiosulphate pentahydrate in hot ethanol. Although the transformation was not as rapid as in the case of thiourea, it required 6-7 hours to complete and resulted in the unprotected 6-thioguanosine in low yield (15-20%).[22]

In the final step the intermediate was subjected to deprotection of the acetyl protecting groups with sodium methoxide in anhydrous methanol. The solution was stirred for 2-3 hours, until TLC monitoring showed the consumption of the starting material. The mixture was neutralised with amberlite and subjected to column purification in order to give the desired compound **4.3** in a 23% yield.

4.2.3 The third synthetic route towards 6-thioinosine

Following the establishment of a successful although poor yielding and multiple step synthetic route, a new path was explored aiming for improved yields and possibly fewer chemical steps, in order to aid the scaling up process of 6-thioinosine. In this new synthetic pathway the only difference was that acetyl group was used as a protecting group instead of tert butyl dimethylsilyl (Figure 4.5). Using this method, the first synthetic route towards the thiopurine riboside was revisited. Acetyl protection of inosine was accomplished based on the procedure described in Section 4.2.2. Lawesson's reagent was added to the dry solution of **4.4** in toluene, and the mixture was stirred at 110°C for 2 - 2.5 hours until the consumption of the starting material.

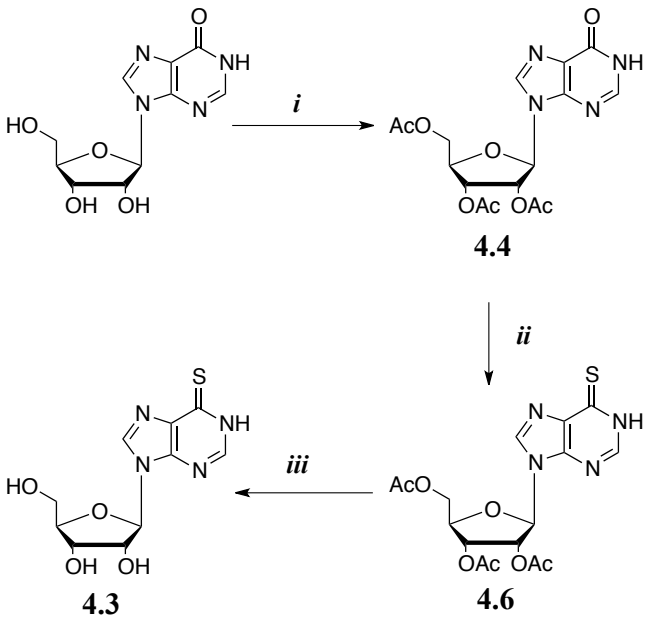

Figure 4.5 The third synthetic route leading to the formation of 6-thioinosine
Reagents and Conditions: *i,* TEA, DMAP, acetic anhydride, rt, 16 hours; *ii*, LR, toluene, reflux, 2 hours; *iii,* 36% NH_4OH, CH_3COOH

After this period of time the reaction mixture was allowed to slowly cool down, thus allowing the majority of reagent to crystallise and to be removed easily

therefore, facilitating the purification process. The reaction mixture was evaporated and subjected to column chromatography. The pure fractions were evaporated and dissolved in a tiny volume of 36% ammonia solution then adjusted to neutral pH with acetic acid and left at -5 to 0°C for 3-5 days, until the appearance of yellowish crystals as the desired compound **4.3** isolated at an overall 48% yield. The crystals were filtered off and used in the next step without further purification.

4.2.4 Synthesis of phosphoramidates

Ten 6-thioinosine ProTides were synthesised from 2',3'-*O*-isopropylidene-6-thioinosine **4.10** prepared by dissolving the nucleoside in dry acetone with catalytic amounts of aqueous perchloric acid (Figure 4.6). After two hours, the reaction was quenched with saturated solution of NH_4OH and evaporated, then subjected to quick column purification and isolated with 78% yield.

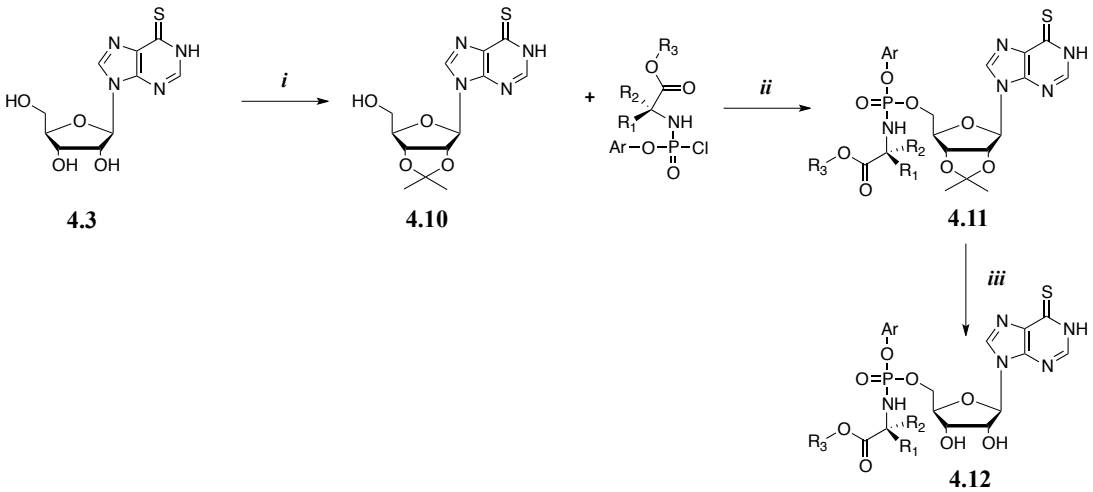

Figure 4.6 General synthetic pathway of 6-thioinosine phosphoramidates.
Reagents and Conditions: i, $HClO_4$, acetone, rt, 16 hours; *ii, t*BuMgCl, THF, rt, 16 hours; *iii*, 60% acetic acid, 65°C, 10-16 hours

All ProTides was synthesized from using a small excess of *t*BuMgCl (1.2 equivalent) as described in Chapter 2.3.2.3. The coupling reaction between the appropriate phosphorochloridate and 2',3'-*O*-isopropylidene-6-thioinosine was low yielding although easy to purify reaching a general 25-64% yield after column chromatography with $CHCl_3$/MeOH (98:2-92:8). The deprotection of the 2',3' hydroxy groups were carried out in 60% aqueous acetic acid at 65°C and was complete after being stirred overnight, with a slight breakdown evident as a baseline spot on the TLC plate. All the final compounds **4.12a - 4.12j** were purified by column chromatography and isolated with 3-36% yield. ProTides were characterised by most or all of the following techniques: ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, MS and HPLC (Table 4.1).

Table 4.1 Summary of synthesised 6-thioinosine ProTides, structures, their calculated lipophilicity and key spectral data. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	AA	Ester	Aryl	ClogP	^{31}P NMR
4.3	-	-	-	-3.54	-
4.12a	L-Ala	cHex	Naph	0.58	4.18, 4.14
4.12b	L-Ala	OCH_2CH_2tBu	Naph	0.93	4.07, 4.05
4.12c	Me_2Gly	Bn	Naph	0.57	2.61, 2.56
4.12d	L-Ala	Bn	Naph	0.26	4.19, 4.02
4.12e	L-Leu	Pnt	Naph	2.12	4.45, 4.23
4.12f	L-Ala	CH_2tBu	Naph	0.40	4.19, 4.09
4.12g	L-Ala	Pnt	Naph	0.66	4.12, 4.09
4.12h	L-Ala	Bn	Ph	-0.90	3.94, 3.66
4.12i	L-Ala	cHex	Ph	-0.58	3.98, 3.81
4.12j	L-Ala	Hex	Naph	1.19	4.17, 4.13
4.17a	L-Ala	OBn	-	1.06	13.71
4.17b	L-Ala	OcPnt	-	0.58	13.86
4.17c	L-Ala	OCH_2tBu	-	1.35	13.84
4.17d	L-Ala	OcHexyl	-	1.70	13.87
4.17e	L-Ala	OCH_2CH_2tBu	-	2.41	13.82
4.17f	L-Ala	OPnt	-	1.87	13.81

4.2.5 Synthesis of 6-thioinosine phosphorodiamidates

Six 6-thioinosine diamidates were synthesized in a one pot reaction from the free nucleoside **4.3** prepared by suspending the nucleoside in anhydrous trimethylphosphate at -5°C followed by the dropwise addition of phosphorus oxychloride to give the 5'-phosphorylated intermediate **4.16** (Figure 4.7). The disappearance of the $POCl_3$ ^{31}P signal and the formation of the new peak ~ 7 ppm, indicating the formed phosphorylated intermediate **4.16** was followed by ^{31}P NMR. The phosphorylation step found to be critical for the formation of phosphorodiamidates. Formation of the new intermediate took place in the 4-5 hours, if the reaction time was prolonged degradation or hydrolysis of **4.16** could be observed. After the appearance of the intermediate **4.16** the next component of the reaction could be added at -78°C in the form of the solution of the appropriate amino acid in anhydrous dichloromethane followed by the dropwise addition of diisopropylethylamine.

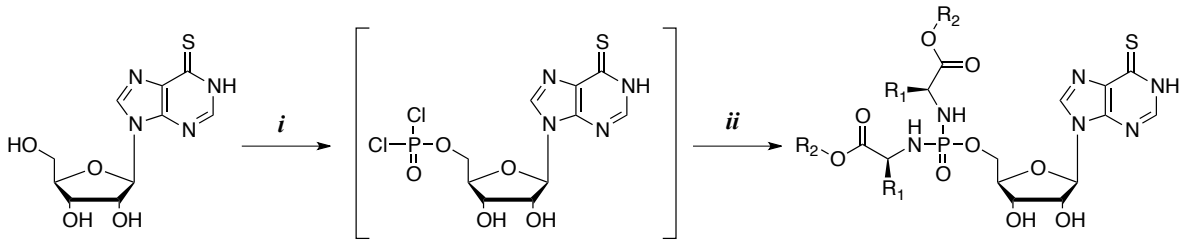

Figure 4.7 General synthetic pathway of 6-thioinosine symmetrical phosphorodiamidates. *Reagents and Conditions: i*, $POCl_3$, TMP, -5°C to rt. 4-5 hrs; *ii*, appropriate amino acid ester, DIPEA, $CHCl_3$, -78°C to rt., 16 hours.

The reaction mixture was left to stir for 30 minutes at -78^{o}C, then allowed to reach room temperature and stirred overnight and the progress of the reaction was monitored by phosphorus NMR. Depending on the amino acid ester used the appearance of the single peak at ~13-17 ppm indicated the formation of the final product **4.17.** The purification carried out by column chromatography and followed by preparative TLC plates resulted in the final phosphorodiamidates **4.17a**-**4.17f** in moderate to good yields (12-26%).

4.3. Biological evaluation

4.3.1 Evaluation of 6-mercaptopurine and 6-thioguanine in a KG1a cell line model of acute myeloid leukaemia

6-Mercaptopurine and 6-Thioguanine purine nucleobase analogues alongside with the nucleoside 6-thioinosine (**4.3)** were selected for analysis based on two criteria: 1. Potency compared to the parent nucleoside; 2. Selectively targets leukaemic stem cells (LSCs) (Chapter 3.4.1).

6-Mercaptopurine and 6-Thioguanine both displayed LD_{50} values in the low micromolar range (LD_{50} = 28 μM and 36 μM) respectively, both exhibited comparable cytotoxic effect in KG1a cell line. It can also be stated, that 6-Mercaptopurine and its parent nucleoside 6-thioinosine displayed similar killing effect effect (LD_{50} = 28 μM and 40 μM), with the latter **4.3** loosing a fold in cytotoxic affect compared to its parent nucleobase. The stem cell compartment was identified as $Lin^-/CD34^+/CD38^-/CD123^+$ cells that comprised approximately 3.4% of the entire KG1-a cell population. From the data it is clear, that none of the thiopurine analogues targeted selectively the specified KG1a stem cell compartment, thus are not selectively lethal to KG1a stem cells. (Table 4.2).

Table 4.2 Evaluation of 6-mercaptopurine, 6-thioguanine and 6-thioinosine in KG1a cell line model of acute myeloid leukaemia
LD_{50}: concentration of compound required to kill 50% of test polulation in μM.
Data by Prof. Pepper.

Cpd	LD_{50} (μM)	Stem cell % Control: 3.4 %
6-mercaptopurine	28	5.5
6-thioguanosine	36	3.4
6-thioinosine	40	5.2

4.3.2 Evaluation of 6-thioinosine ProTides in a KG1a cell line model of acute myeloid leukaemia

6-thioinosine **4.3** and four new ProTide analogues **4.12d**, **4.12f**, **4.12h**, **4.12i** of the parent nucleoside were evaluated for their potency and selectivity towards leukaemic stem cells (Chapter 3.4.1). (Table 4.3)

Table 4.3 Evaluation of 6-thioninoisne ProTides in KG1a cells
LD_{50}: concentration of compound required to kill 50% of test polulation in μM
Data by Prof. Pepper. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	ClogP	Aryl	Ester	AA	LD_{50} (μM)	Stem cell% Control:3.3%
4.3	-3.54	-	-	-	40	5.2
4.12d	0.26	Naph	Bn	L-Ala	210	4.3
4.12f	0.40	Naph	CH_2tBu	L-Ala	2200	5
4.12h	-0.90	Ph	Bn	L-Ala	1500	4.6
4.12i	-0.58	Ph	cHex	L-Ala	600	4.6

6-thioinosine **4.3** found to be active in a low micromolar scale and displayed 1.4 fold reduction in potency upon compared to its parent nucleobase 6-mercaptopurine (LD_{50} = 40 μM). Moreover 6-thioinosine ProTide analogues exhibited 5 to 55-fold decreases in activity (LD_{50} = 210 μM and 2200 μM) when compared to their parent nucleoside **4.3** (Table 4.3). The L-alanine benzyl naphthyl **4.12d** emerged as the best analogue of this series, while loosing 5 fold activity compared to the parent (LD_{50} = 210), it gained a 7-fold potency boost over its phenyl counterpart **4.12h** (LD_{50} = 1500 μM). This effect could not be explained with the higher lipophilic value. The naphthyl derivative **4.12f** bearing L-alanine neopentyl ester lost 55-fold cytotoxic activity compared to the parent nucleoside. Moreover no stem selectivity was observed either in the case of the parent nucleoside or its ProTide derivatives (Table

4.3). These data suggested that this family is poorly active against this cell line. Following the common trend concerning the findings of the McGuigan group, the best ProTide motif was the L-Ala Bn derivative.

4.3.3 Evaluation of 6-thioinosine ProTides in blood-borne cancer cell lines

Nine out of ten ProTide derivatives **4.12a-4.12h** and two out of six diamidate analogues **4.17a**, **4.17c** alongside with the parent nucleoside 6-thioinosine were tested in two human blood borne cancer cell lines, CCRF-CEM as a model of acute lymphoblastic leukaemia and MOLT-4 as a model of acute T lymphoblastic leukaemia. 6-thioinosine exhibited submicromolar activity in the case of both cell lines (IC_{50} = 0.638 and 0.851μM).

Table 4.4 Evaluation of 6-thioinosine and 6-thioinosine ProTides in CCRF-CEM and MOLT-4 cell lines and their calculated lipophilicity. IC_{50} (μM): 50% inhibitory concentration or compound concentration required to reduce cell proliferation by 50%. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	AA	Ester	Aryl	ClogP	CCRF-CEM	MOLT-4
4.3	-	-	-	-1.19	0.638	0.851
4.12a	L-Ala	cHex	Naph	0.58	13.919	14.278
4.12b	L-Ala	OCH_2CH_2tBu	Naph	0.93	3.129	3.082
4.12c	Me_2Gly	Bn	Naph	0.57	35.777	43.434
4.12d	L-Ala	Bn	Naph	0.26	1.852	2.118
4.12e	L-Leu	Pnt	Naph	2.12	12.668	7.887
4.12f	L-Ala	OCH_2tBu	Naph	0.40	16.859	13.878
4.12g	L-Ala	Pnt	Naph	0.66	0.476	0.531
4.12h	L-Ala	Bn	Ph	-0.90	1.858	2.391
4.12i	L-Ala	cHex	Ph	-0.58	12.874	20.608
4.17a	L-Ala	Bn	-	1.06	1.867	2.691
4.17c	L-Ala	OCH_2tBu	-	1.35	24.081	36.968

Amongst 6-thioinosine ProTides and diamidates tested, **4.12g** bearing the L-alanine pentyl naphthyl masking moieties stood out according to its efficacy as it retained

the activity of the parent nucleoside in both cell lines (IC_{50} = 0.476 and 0.531 μM). Remarkably **4.12d**, **4.12h** bearing L-alanine benzyl naphthyl and phenyl moieties and the diamidate **4.17a** containing the L-alanine benzyl masking group all had similar potencies, with only a 3-fold loss in activity compared to 6-thioinosine (IC_{50} = 1.852-1.867 μM in CCRF-CEM cell line and IC_{50} = 2.118 - 2.691 μM in MOLT-4 cell lines). Furthermore it can be concluded from this preliminary assay that the naphthyl derivatives did not show any improvement in the cytostatic activity as **4.12d**, **4.12h, 4.17a** showed comparable potencies across the two cell lines tested.

None of the other ProTides in this series showed activity or comparable potency to the parent nucleoside in CCRF-CEM and in MOLT-4 cell line with similar pattern in term of activity loss. Interestingly the L-alanine cyclohexyl naphthyl derivative **4.12a** and its phenyl analogue **4.12i** also exhibited comparable cytostatic affect in CCRF-CEM and MOLT-4 cell lines (IC_{50} = 13.919 and 12.874 μM), while loosing 20-fold in activity compared to the parent. Upon lengthening the branched ester moiety from neopentyl to neohexyl ester found to be a favourable modification as compound **4.12b**, bearing L-alanine neohexyl ester showed a 5 fold improvement in efficacy compaired to the L-alanine neopentyl naphthyl derivative **4.12f** (IC_{50} = 3.129 and 16.859 μM). (Table 4.4)

4.4 ProTides of 6-thioguanosine

4.4.1 Series one

A new series of 6-thioguanosine ProTides were designed with the application of both phosphoramidate and phosphorodiamidate technologies for the first time in the McGuigan group as described in chapter 4.1 in order to investigate their therapeutic potential compared to the 6-thioinosine series using in vitro models of leukaemia and non-Hodgkin lymphoma.

4.4.2 Synthesis of 6-thioguanosine

After the establishment of the successful synthetic route towards 6-thioinosine the same synthetic strategy was applied on guanosine (Chapter 4.2.3). The free hydroxyl groups of the nucleoside was fully acetylated to give compound **4.7** in 56% yield, then it was subjected the thiocarbonylation with Lawesson's reagent, resulted in the isolation of **4.8** with 48% yield (Figure 4.8).

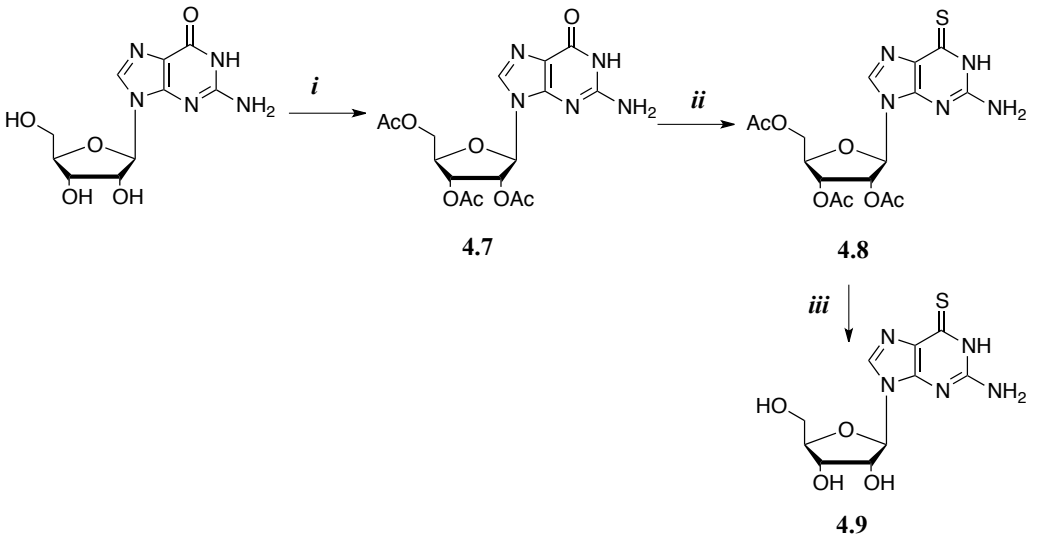

Figure 4.8 The general synthetic pathway of 6-thioguanosine
Reagents and Conditions: *i,* TEA, DMAP, acetic anhydride, rt, 16 hours; *ii*, LR, toluene, reflux, 2 hours; *iii,* 36% NH_4OH, CH_3COOH

In the final step of the synthesis the fully acetylated 6-thioguanosine was subjected to deprotection in the presence of 36% ammonia solution and acetic acid in order to give the desired compound 6-thioguanosine **4.9,** in 51 % yield.

4.4.3 Synthesis of 6-thioguanosine phosphoramidates

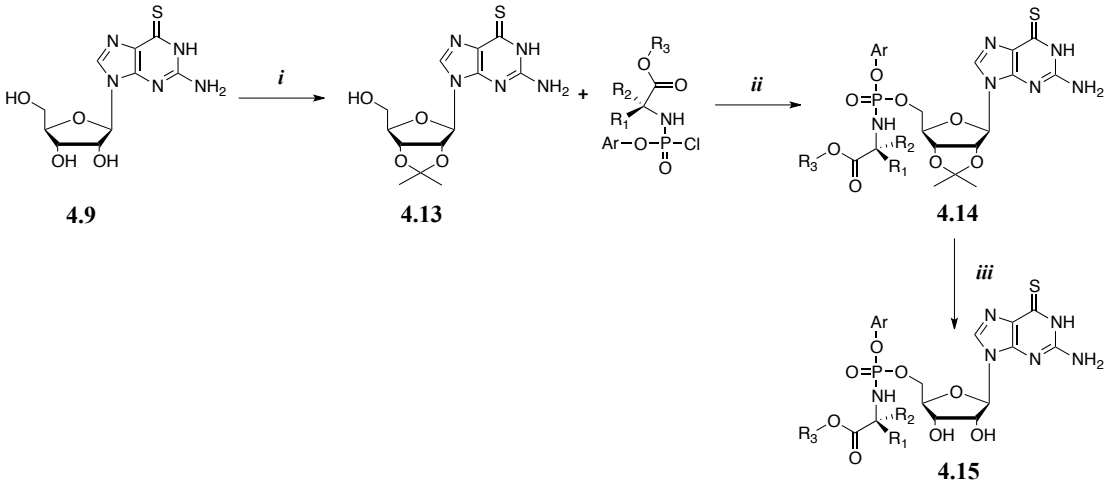

Figure 4.9 General synthetic method of 6-thioinosine phosphoramidates.
Reagents and Conditions: i, $HClO_4$, acetone, rt, 16 hours; *ii, t*BuMgCl, THF, rt, 16 hours; *iii*, 60% acetic acid, 65 ^{o}C, 10-16 hours.

Ten 6-thioguanosine ProTide derivatives were synthesised from 2',3'-*O*-isopropylidene-6-thioguanosine **4.13** with approximately 23-45% yield, then subjected to deprotection from the isopropylidene protecting groups and purified by column chromatography with 5-18 % yield over the second step (Figure 4.9) as described in Chapter 4.2.4. All 6-thioguanosine ProTides were characterised by the following techniques: ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, MS, HPLC. The structures, yields and key spectral data of the ProTides are shown below.

Table 4.5 Summary of 6-thioguanosine ProTides, their calculated lipophilicity and ^{31}P chemical shifts. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	AA	Ester	Aryl	ClogP	^{31}P NMR
4.9	-	-	-	-1.11	-
4.15a	L-Ala	Bn	Naph	0.17	4.41, 4.22
4.15b	L-Ala	CH_2CH_2tBu	Naph	0.84	4.33, 4.30
4.15c	L-Ala	CH_2tBu	Naph	0.31	4.11, 4.08
4.15d	L-Leu	Pnt	Naph	2.03	4.68, 4.41
4.15e	L-Ala	cHex	Naph	0.49	4.34, 4.29
4.15f	Me_2Gly	Bn	Naph	0.48	4.34, 4.29
4.15g	L-Ala	Pnt	Naph	0.57	4.33, 4.27
4.15h	L-Ala	Bn	Ph	-0.99	4.09, 3.81
4.15i	L-Ala	cHex	Ph	-0.67	4.08, 3.91
4.15j	L-Ala	Hex	Naph	1.10	4.34, 4.28
4.19a	L-Ala	Bn	-	0.97	13.92
4.19b	L-Ala	cHex	-	1.61	13.94
4.19c	L-Ala	CH_2tBu	-	1.26	13.83
4.19d	L-Ala	Hex	-	2.84	13.87
4.19e	L-Leu	Pnt	-	4.70	13.88

4.4.4 Synthesis of 6-thioguanosine phosphorodiamidates

Five 6-thioinosine diamidates were synthesized in a one pot reaction as previously described regarding the 6-thioinosine phosphorodiamidates (4.2.5). The free nucleoside **4.9** suspended in anhydrous trimethylphosphate at -5^{o}C followed by the dropwise addition of phosphorus oxychloride to give the 5'-phosphorylated intermediate **4.18** (Figure 4.10). The disappearance of the $POCl_3$ ^{31}P signal and the formation of the new peak ~ 7 ppm, indicated the formation of the phosphorylated

intermediate **4.18** monitored by ^{31}P NMR. Formation of the new intermediate took place in 4-5 hours and found to be the critical stage in the phosphorodiamiate synthesis. After the appearance of the intermediate **4.18** the next component of the reaction was added at -78°C as the solution of the appropriate amino acid in anhydrous chloroform followed by the dropwise addition of diisopropylethylamine.

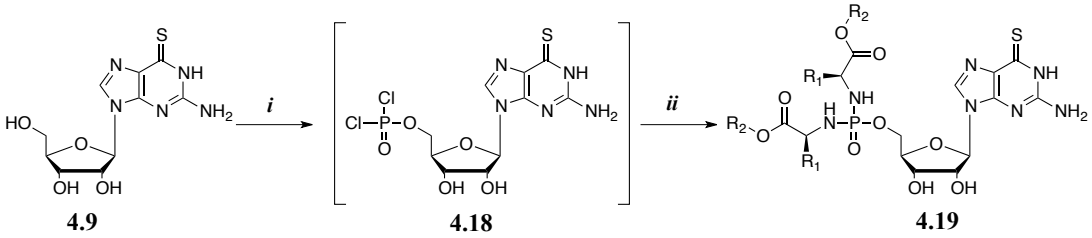

Figure 4.10. General synthetic pathway of 6-thioguanosine symmetrical phosphorodiamidates. *Reagents and Conditions: i*, $POCl_3$, TMP, -5°C to rt. 4-5 hrs; *ii*, appropriate amino acid ester, DIPEA, $CHCl_3$, -78°C to rt., 16 hours.

The reaction mixture was left to stir for 30 minutes at -78°C, then left to reach room temperature and stirred overnight as the progress of the reaction was monitored by phosphorus NMR. Depending on the amino acid ester used the appearance of the single peak at ~13-17 ppm indicated the formation of the final product **4.19.** The purification step was carried out by column chromatography, followed by preparative TLC, which resulted in the isolation of the final phosphorodiamidates **4.19a** - **4.19e** in moderate to good yields (1-21%).

4.5 Biological evaluation

4.5.1 Evaluation of 6-thioguanosine ProTides in a KG1a cell line model of acute myeloid leukaemia

6-thioguanosine and four of its ProTide derivatives was analysed in the leukaemic stem cell compartment (LSCs) (Chapter 3.4.1).

As shown in Table 4.6 the parent nucleoside 6-thioguanosine exhibited LD_{50} = 1μM cytotoxic activity in KG1-a cell lines, **4.15a** the L-Alanine benzyl naphthyl derivative was equipotent with the parent nucleoside (LD_{50} = 1.1 μM), while **4.15h** its phenyl partner, found to be slightly more active than both (LD_{50} = 0.81 μM). However the L-alanine neopentyl naphthyl **4.15c** and L-alanine cyclohexyl phenyl **4.15i** motifs exhibited far less potencies, they lost cytotoxic activity on 14 to 500-fold scale (LD_{50} = 14 and 500 μM). Amongst this small family of compounds slight stem cell selectivity could be observed. The most potent compound of this series **4.15h** not only exhibited the lowest LD_{50} in KG1-a cell line, but targeted the previously defined stem cell compartment in the most selective way (**4.15h** = 2.3%, 6-thioguanosine = 3.5%, control: 4%) amongst the ProTide motifs.

Table 4.6 Biological evaluations of 6-thioguanosine ProTides in a KG1a cell line model of acute myeloid leukaemia. LD_{50}: concentration of compound required to kill 50% of test polulation in μM. Data by Prof. Pepper.

Cpd	Aryl	Ester	AA	LD_{50} (μM)	Stem cell % Control 4 %
4.9	-	-	-	1	3.5
4.15a	Naph	Bn	L-Ala	1.1	3
4.15c	Naph	Neopnt	L-Ala	14	2.5
4.15h	Ph	Bn	L-Ala	0.81	2.3
4.15i	Ph	cHexyl	L-Ala	500	3.5

Nine ProTide derivatives and two diamidate analogues of the 6-thioguanosine series were tested in HL-60 human promyelocytic leukaemia, KG-1 bone marrow myelogenous leukaemia and K562 as chronic mylogenous leukaemia cell lines.[23-27] In case of HL-60 cell line the nucleoside exhibited submicromolar IC_{50} (IC_{50} = 0.489 μM), while none of the ProTides retained the activity of the parent, moreover from 7- to 156-fold higher concentrations of compounds were needed to produce the same cytostatic effect as the parent control (IC_{50} = 3.261 and 76.56 μM). The L-alanine benzyl motif showed the best potencies amongst this family of ProTides. The phosphorodiamidate derivative **4.19a** exhibited the highest cytostatic activity against HL-60 cell line with (IC_{50} = 3.261 μM), although displayed approximately 7-fold activity loss compared to the parent nucleoside. ProTides **4.15a** and **4.15h** did not show any improvement of activity compared to the parent, however the L-alanine benzyl naphythyl derivative **4.15a** (IC_{50} = 3.889 μM), found to be almost 3-fold more active, than its phenyl counterpart **4.15h** (IC_{50} = 5.493 μM), which can be explained with its slightly increased ClogP value of 0.17 over its phenyl derivative **4.15h** (ClogP = -0.99), therefore enhancing its cellular permeability.

In case of the KG-1 cell line, 6-thioguanosine exhibited cytostatic activity (IC_{50} = 0.981 μM). Regarding other myelogenous leukaemia cell lines this family were slightly more active, ProTide derivatives were about 1-26 fold less active than the parent **4.9** (IC_{50} = 1.001 – 24.28 μM). **4.15h** bearing L-Alanine benzyl phenyl masking moiety in parallel with the findings of the Pepper report (Table 4.6) retained the activity of **4.9** (IC_{50} = 1.001 μM), while **4.19a** the diamidate bearing L-Alanine benzyl motif was even slightly more potent, required slightly lower concentration to produce the same cytostatic effect as the parent control (IC_{50} = 0.793 μM). No relationship between lipophilicity and activity were found for these compounds.

Table 4.7 Biological evaluation of 6-thioguanosine ProTides in HL-60, KG-1, K562 cell line models of acute myeloid leukaemia. IC_{50} (μM): 50% inhibitory concentration or compound concentration required to reduce cell proliferation by 50%. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0.

Cpd	AA	Ester	Aryl	ClogP	HL-60	KG-1	K 562
4.9	-	-	-	-1.11	0.489	0.938	1.052
4.15a	L-Ala	OBn	ONaph	0.17	3.889	2.299	9.044
4.15b	L-Ala	OCH_2CH_2tBu	ONaph	0.84	12.039	2.997	25.44
4.15c	L-Ala	OCH_2tBu	ONaph	0.31	27.529	24.28	37.628
4.15d	L-Leu	Pnt	ONaph	2.03	76.56	19.657	88.297
4.15e	L-Ala	cHex	ONaph	0.49	26.142	5.625	40.104
4.15f	DMG	Bn	ONaph	0.48	16.495	7.652	32.645
4.15g	L-Ala	Pnt	ONaph	0.57	11.487	4.09	0.064
4.15h	L-Ala	Bn	OPh	-0.99	5.493	1.001	8.85
4.15i	L-Ala	cHex	OPh	-0.67	32.395	6.682	99.542
4.19a	L-Ala	Bn	-	0.97	3.261	0.793	9.795
4.19b	L-Ala	CH_2tBu		1.26	29.614	6.999	95.856

In the K562 cell line none of the compounds retained the low micromolar activity of the parent nucleoside (IC_{50} = 1.052 μM), 8 to 95-fold higher concentration of the phosphoramidate and phosphorodiamidate derivatives were needed in order to exhibit the same cytostatic effect as the parent **4.9** respectively (IC_{50} = 8.85 – 95.856) μM). Suprisingly **4.15g**, bearing L-Alanine pentyl naphthyl masking groups exhibited submicromolar activity (IC_{50} = 0.064 μM) and 16-fold lower concentration was required to produce show the same cytostatic effect as the parent nucleoside. This marked enhancement cannot be explained with the increased ClogP value of **4.15g** (ClogP = 0.57) as the L-alanine cyclohexyl naphthyl derivative **4.15e** had markedly reduced cytostatic activity (approximately 40-fold higher concentration; IC_{50} = 40.104 μM) compared to the parent while having comparable ClogP value (0.57) to that of compound **4.15g.**

4.6 6-S-methyl-thioinosine ProTides

4.6.1 The second series

Based on the previous biological result of the thiopurine ProTide and diamidate series our attention turned towards new potential modifications of these nucleosides hoping for pharmacologically more active analogues. Karran stated, that methylated 6-S-methyl-thioinosine monophosphate is an effective inhibitor of de novo purine biosynthesis.[1] 6-thioinosine that escapes catabolism by thiopurine S-methyl transferase can be metabolised to thioguanosine monophosphate, via the sequential actions of deoxynucleoside kinases and reductases leading to the formation of 6-thio 2'-deoxyguanosine triphosphate, which is a replicative DNA polymerase substrate.[1] The nucleoside 6-S-methyl mercaptopurine was synthesised and a small family of 4

ProTide analogues were designed in order to enhance the biological activity of the nucleoside 6-thioinosine and its ProTide analogues. In the first instance L-alanine motif was retained with varied ester functionality between the highly preferred benzyl, the branched neopentyl ester alongside with the L-leucine pentyl naphthyl derivative. Regarding the L-alanine benzyl derivatives the phenyl unit was replaced by 1-naphthyl, while in the case of the L-alanine neopentyl ester only the more lipophilic naphthyl group was utilised.

4.7 Synthesis

4.7.1 Synthesis of 6-S-methyl-thioinosine

The influence of thiomethoxy substitution of the oxygen atom at the 6-position of the guanine base was investigated. The synthetic route consisted of 1,2,3,5-tetra-O-benzoyl-β-D-ribofuranose **4.20** synthesis followed by the glycosylation step with 6-chloropurine. The coupling reaction was carried out in dry acetonitrile in the presence of 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) and TMS triflate at 70^{o}C for 5-6 hours (Figure 4.11). The pure compound **4.21** was obtained by crystallization from methanol with 43% yield. Replacement of the oxygen atom to the 6-position of the hypoxanthine base by thiomethoxy group proved to produce clinically effective agents. In the presence of 15% aqueous solution of sodium methanothiolate in anhydrous dimethylformamide 2,3,5-tetra-O-benzoyl-6-chloro-9-β-D-ribofuranosyl purine **4.21** was converted to 6-S-methyl-thioinosine **4.22**, while deprotection of the benzoyl protecting groups took place under reaction conditions. The reaction mixture was stirred for 2-3 hours at ambient temperature, then diluted with water and

extracted with ethyl acetate. Column chromatography provided the desired compound in 38% yield.

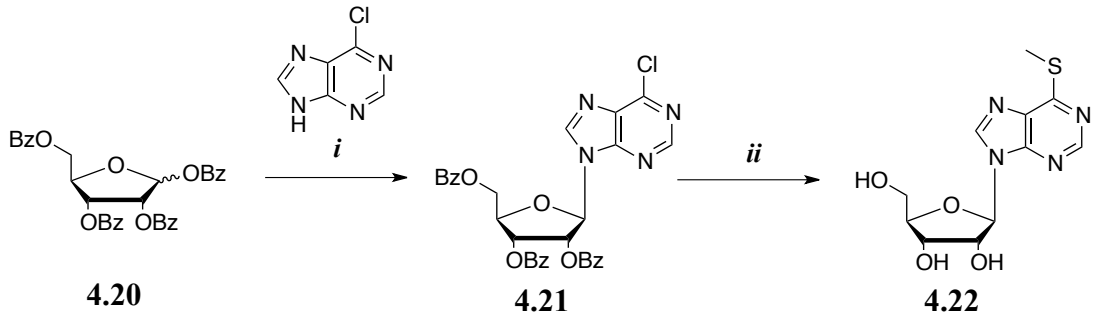

Figure 4.11 General synthetic pathway of 6-S-methyl-thioinosine
Reagents and Conditions: i, DBU, TMSOTf, dry ACN, 70^{o}C, 5-6 hours *ii*, NaSMe/H_2O, DMF, rt, 2 hours.

4.7.2 Synthesis of 6-S-methyl-thioinosine phosphoramidates

Four 6-S-methyl-thioinosine ProTide derivatives were prepared by suspending the nucleoside in dry tetrahydrofuran using a small excess of *t*BuMgCl (1.1 eq) as shown in Figure 4.12. The coupling reaction between the appropriate phosphorochloridate was very low yielding, however easy to purify by coloumn chromatography reaching a general 1-2% yield. ProTides were characterised by ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, MS, HPLC. The structures, yields and key spectral data of 6-S-methyl-ProTides are shown in (Table 4.8). The variation of amino acid, ester and aryl moieties lead to the formation of compounds with a ClogP values between 1.63 and 4.40 (Table 4.8).

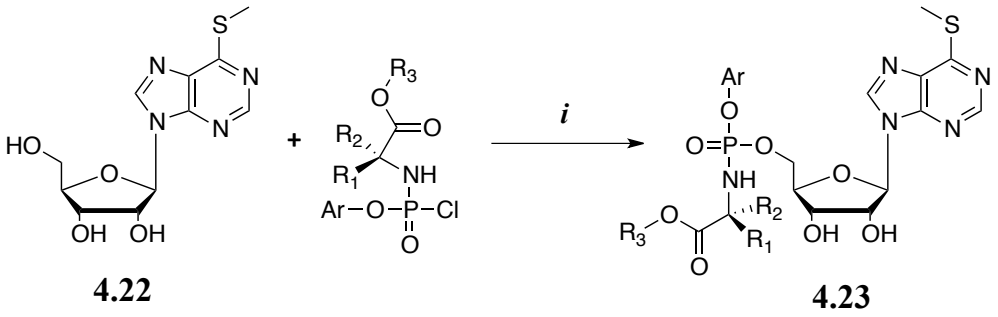

Figure 4.12 General method for the synthesis of 6-S-methyl-thioinosine phosphoramidates, Reagents and conditions: *i, t*BuMgCl, THF, rt, 16 hours.

Table 4.8 Summary of the synthesised 6-S-methyl-thioinosine phosphoramidates, their calculated lipophilicity, ^{31}P chemical shifts and isolated yields. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0.

Cpd	AA	Ester	Aryl	ClogP	^{31}P NMR	Yields
4.22	-	-	-	-1.00	-	38%
4.23a	L-Ala	OBn	ONaph	2.80	4.16, 3.99	1%
4.23b	L-Ala	OBn	OPh	1.63	3.89, 3.63	2%
4.23c	L-Ala	OCH_2tBu	ONaph	2.94	4.16, 4.03	2%
4.23d	L-Leu	OPnt	ONaph	4.40	4.46, 4.24	1%

4.8 Biological evaluation

4.8.1 Evaluation of 6-S-methyl-thioinosine ProTides in a KG1a cell line model of acute myeloid leukaemia

6-S-methyl-thioinosine **4.22** and four of its ProTide derivatives were studied in a leukaemic stem cell model (LSCs) (Chapter 3.4.1).

As is evident in Table 4.9 6-S-methyl thioinosine **4.22** exhibited low micromolar activity and it showed 11-fold enhancement in cytotoxic efficacy (LD_{50} = 3.8 μM), compared to 6-thioinosine (LD_{50} = 40 μM, Table 4.3), thus the replacement of the sulphur atom to thiomethoxy group greatly increased the potency. Although the synthesised nucleoside **4.22** and ProTide analogues **4.23a** – **4.23d** did not show any evidence of stem cell selectivity in this model (Stem cell selectivity = 4.5 - 6%, control = 4%), according to their displayed LD_{50} values, they are considered as the most promising compounds synthesised for the thiopurine project.

Table 4.9 Biological evaluations of 6-S-methyl-thioinosine ProTides in a KG1-a cell line model of acute myeloid leukaemia. LD_{50}: concentration of compound required to kill 50% of test polulation in μM. Data by Pepper. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0.

Cpd	ClogP	Aryl	Ester	AA	LD_{50} (μM)	Stem cell % Control: 4%
4.22	-1.00	-	-	-	3.8	4
4.23a	2.80	Naph	Bn	L-Ala	2.5	6
4.23b	1.63	Ph	Bn	L-Ala	1.7	5.5
4.23c	2.94	Naph	Neopnt	L-Ala	1.9	4.5
4.23d	4.40	Naph	Pent	L-Leu	7.4	5

Three out of four compounds **4.23a**, **4.23b** and **4.23c**, retained the activity of the parent nucleoside with an approximate 1.5 to 2 fold increased lethal activity over the parent **4.22** (LD_{50} = 1.7 -2.5 μM). While the 6-thioinosine family lost its lethal activity on a 5-55 scale (LD_{50} = 210 - 2200 μM, Table 4.3), compounds of the 6-S-methyl-thioinosine series maintained their overall efficacy and displayed only 2-fold activity loss in case of the L-leucine pentyl naphthyl derivative **4.23d** (LD_{50} = 7.4 μM).

4.8.2 In vitro results of 6-S-methyl-thioinosine ProTides in leukaemia cell line

Based on the WuxiAppTec data (Table 4.10), which was generated in MOLT-4 leukaemia cell line 6-S-methyl-thioinosine **4.22** showed 37-fold potency boost (IC_{50} = 0.023 μM) over 6-thioinosine **4.3** (IC_{50} = 0.851 μM) and although none of the 6-S-methyl-thioinosine derivatives **4.23a** – **4.23c** retained the cytostatic efficacy of the parent nucleoside **4.22**, they all displayed low submicromolar activity in MOLT-4 cell line (IC_{50} = 0.102 – 0.249 μM). In the case of the L-alanine benzyl naphthyl derived ProTides of both nucleosides **4.3** and **4.22**, it can be concluded, that **4.23a** (IC_{50} = 0.102 μM) had an 18-fold potency boost over **4.12d** (IC_{50} = 2.118 μM).

Table 4.10 Biological evaluations of 6-S-methyl-thioinosine ProTides in a MOLT-4 cell line model of acute myeloid leukaemia. LD_{50}: concentration of compound required to kill 50% of test polulation in μM. Data by Pepper.

CPF	ClogP	AA	Ester	Aryl	MOLT-4
4.22	-1.00	-	-	-	0.023
4.23a	2.80	L-Ala	OBn	ONaph	0.102
4.23b	1.63	L-Ala	OBn	OPh	0.249
4.23c	2.94	L-Ala	OCH_2tBu	ONaph	0.23

In terms of its phenyl motif with similar pattern **4.23b** (IC_{50} = 0.249 μM) had 10-fold potency boost over **4.12h** (IC_{50} = 2.391 μM) in MOLT-4 cells (Table 4.4 and Table 4.10). Therefore 6-S-methyl-thioinosine phosphoramidate analogues present as an important family for future development.

4.9. 2'-deoxy-6-thioguanosine

4.9.1 Synthesis of 2' deoxy-6-thioguanosine

Oligonucleotides containing a thio-substituted base, such as 6-thioguanine and 4-thiouracil have been widely used for various biological purposes. The synthesis of 2'-deoxy-6-thioguanosine was already reported, both approaches utilized protecting groups, which are easy to remove under mild basic conditions in order to protect the 6-thiocarbonyl group such as cyanoethyl or the 2,4-dinitrophenyl groups another approach reported the 6-carbonyl group transformation into a labile halide or arylsulfonyloxy leaving group followed by final displacement with 2-cyanoethanethiol. In order to eradicate the downside of both approaches using highly odorous agents, such as 2-cyanoethanethiol or hydrogen sulfide, another synthetic route was chosen.

Synthesis of 2'-deoxy-6-thioguanosine was carried out based on a new odorless way reported by K. Onizuka et al. 2'-deoxy-guanosine was protected with tert-butyldimethylsilyl groups with 89% yields (Figure 4.13). The protected deoxy-guanosine **4.24** was subjected to a one-flask, two-step synthetic procedure, where it was reacted with 2-mesitylenesulfonyl chloride, so the formed intermediate could activate the 6-position of guanine, which was treated with 2-ethylhexyl 3-mercaptopropionate afterwards in the presence of N-methylpyrrolidine to give the desired compound **4.25** with moderate yields of 39% after purification by column chromatography. The reaction could also be easily performed on a large-scale synthesis.

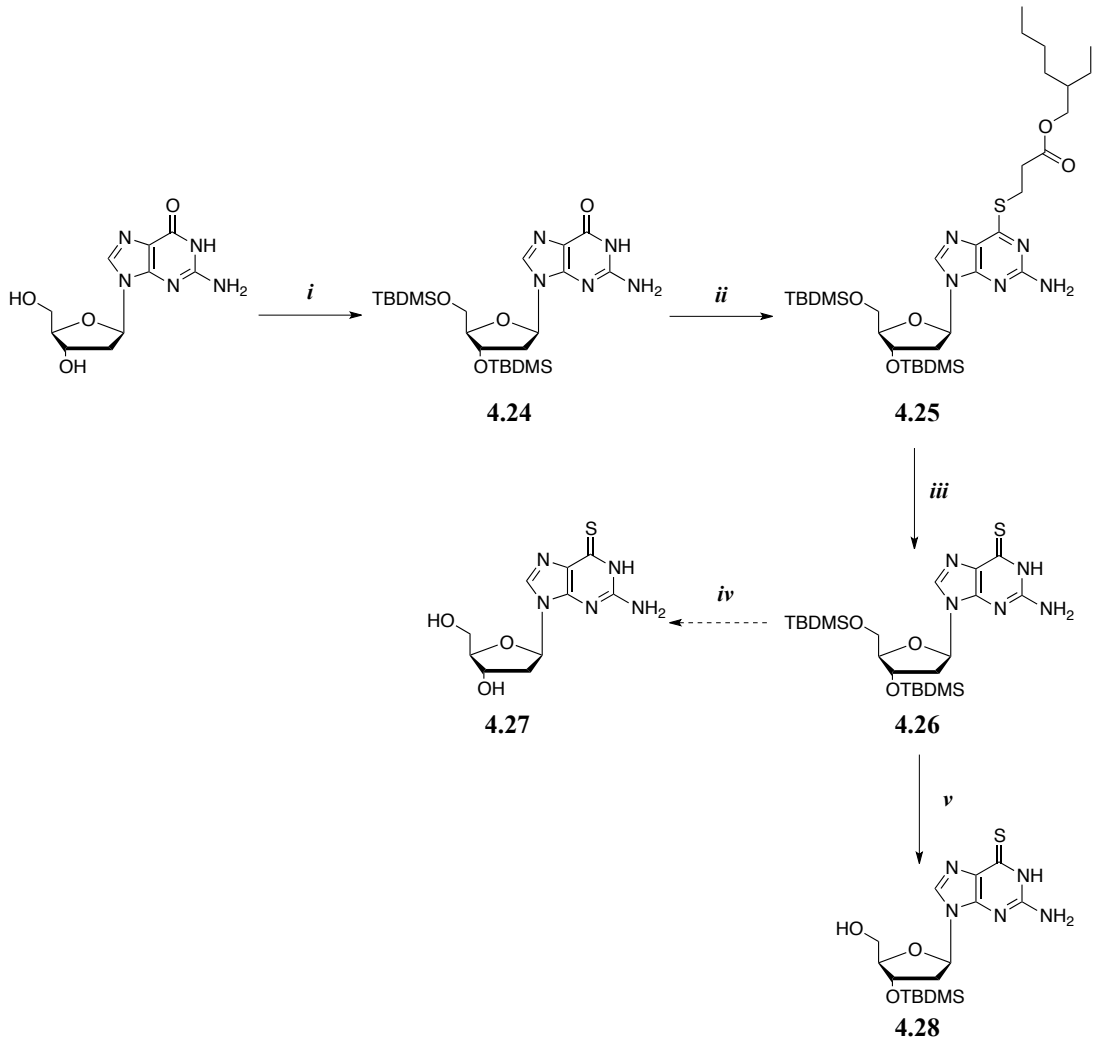

Figure 4.13 Synthetic scheme for 2' deoxy-6-thioguanosine
Reagents and Conditions: i, TBDMSCl, imidazole, DMF, rt, 5-6 hours; *ii*, 2-mesitylenesulfonylchloride, TEA, DMAP, CH_2Cl_2, 0°C - rt, *N*-methylpyrrolidine, 2-ethylhexyl-3-mercaptopropionate, 0°C - rt; *iii*, 1M DBU, ACN *iv*, TBAF, THF, rt, *v,* dry THF : TFA : H_2O (4:1:1), rt., 2hrs.

6-S-2-((2-ethylhexyl)oxycarbonyl)ethyl))-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxy-6-thioguanosine **4.25** was subjected to deprotection from the 2-ethylhexylpropionate group in the presence of 1M DBU in anhydrous acetonitrile to provide the 2'-deoxy-3',5'-*O*-bis(tert-butyldimethylsilyl)-6-thioguanosine **4.26** after coloumn purification in 74% yield.

One attempt was made to fully deprotect the 3',5'-O-bis(tert-butyldimethylsilyl-protected nucleoside **4.26** in the presence of TBAF in anhydrous THF. Due to the low scale of the reaction, the desired product could not be isolated and the scaling up of the above mentioned synthetic route could not be completed during the timescale of the this work in order to reattempt the isolation of the desired product **4.28** (Figure 4.13).

In the final step intermediate **4.26** was dissolved in anhydrous THF and treated with 50% TFA in water at 0^{o}C for 2 hours. After neutralization and column purification 3'-O-(tert-butyldimethylsilyl)-2'-deoxy-6-thioguanosine **4.28** was isolated in 31% yield (Figure 4.13).

4.9.2 Synthesis of 2'-deoxy-6-thioguanosine phosphoramidates

The first attempt to synthesise 2'-deoxy-6-thioguanosine ProTides was performed by coupling the 3'-O-(tert-butyldimethylsilyl)-2'-deoxy-6-thioguanosine **4.28** in the presence of *t*BuMgCl in anhydrous tetrahydrofuran. The reaction was stirred at room temperature overnight. Unfortunately, the desired product was not detected by ^{31}P NMR, which could be partially due to the low amount of starting material available. Due to the lack of time remaining did not allow to perform further studies on the reaction conditions (Figure 4.14).

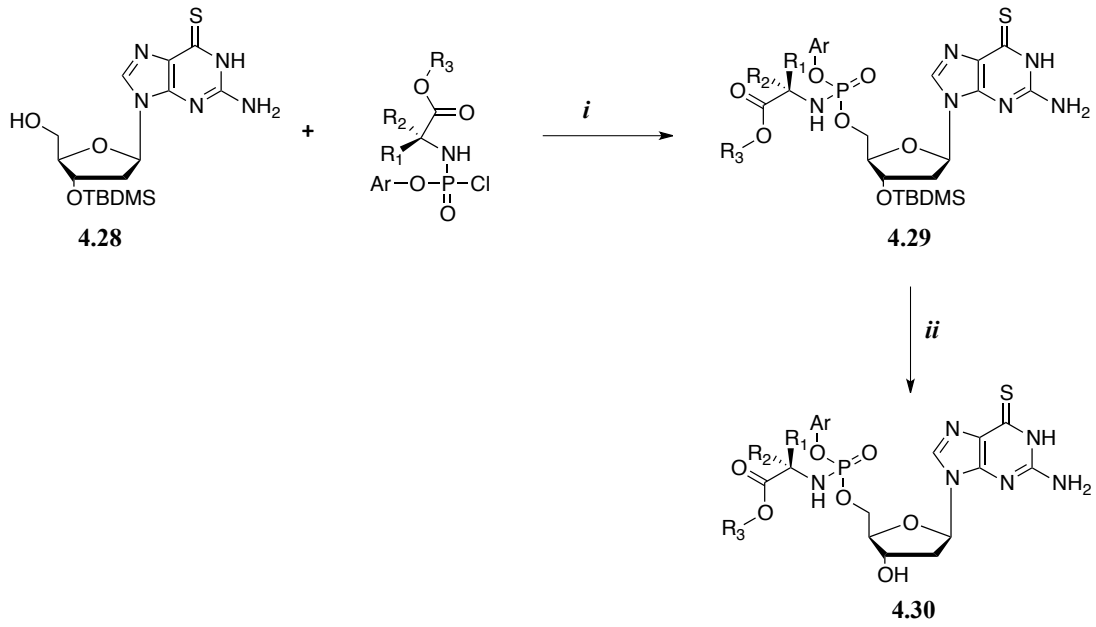

Figure 4.14 Proposed synthetic scheme for 2'-deoxy-6-thioguanosine phosphoramidates *Reagents and Conditions: i*, anhydrous THF, *t*BuMgCl (1.1 eq), rt., 16 hrs; *ii*, dry THF : TFA : H_2O (4:1:1), rt., 2-3hrs.

4.10 Mechanistic studies

4.10.1 Putative mechanism of activation of symmetrical diamidates

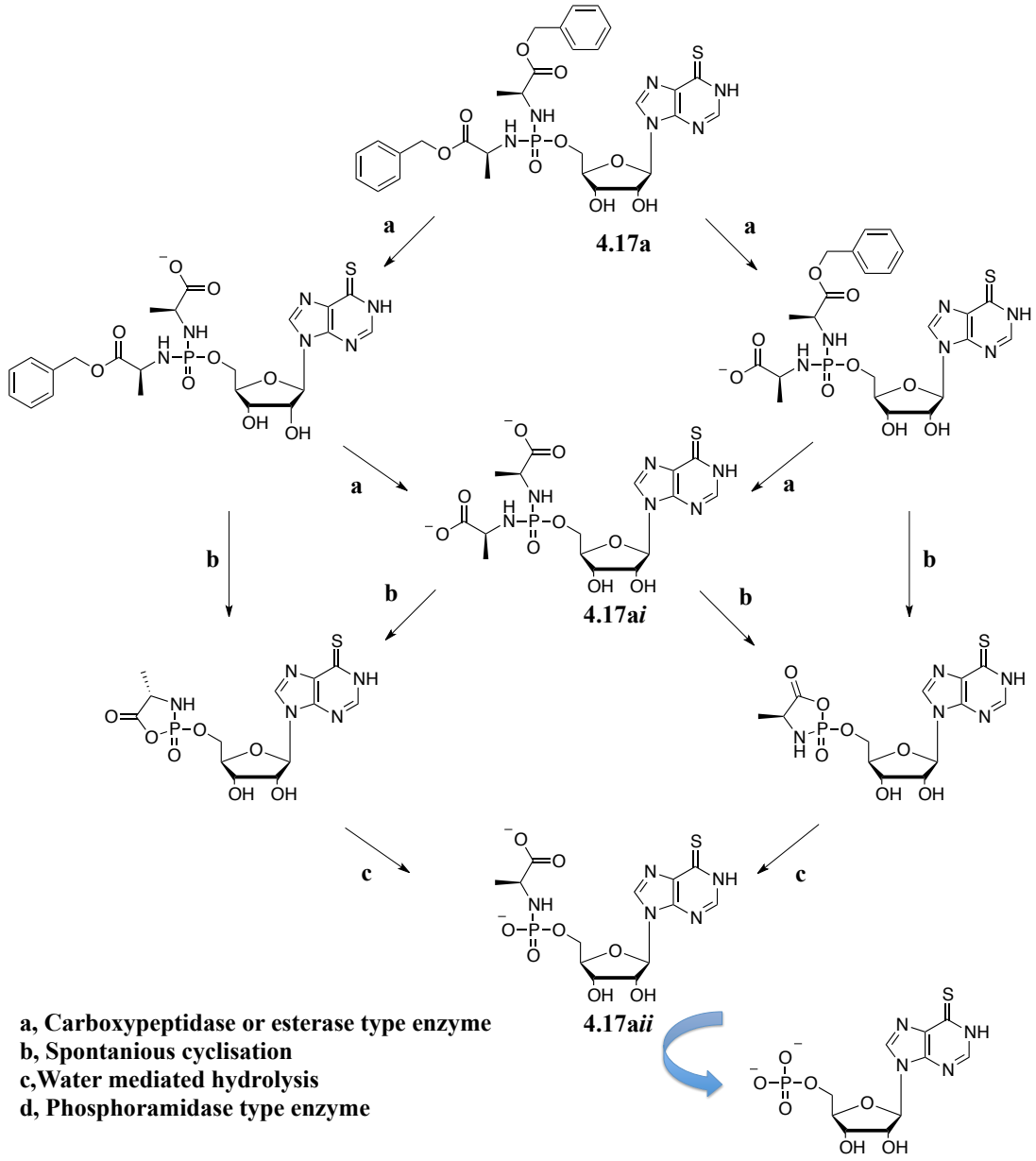

Figure 4.15 Putative mechanism of activation of phosphorodiamidate **4.17a**

The proposed mechanism of activation of phosphorodiamidate prodrugs involves the ester cleavage of one of the masking units (Figure 4.15), which is followed by the intramolecular attack of the amino acid carboxylate anion onto the phosphorus centre with a subsequent cyclisation and elimination of the second amino acid. It is unclear

whether both esters arehydrolysed in the same time or only one of them. Thereafter the unstable five-membered ring would undergo hydrolysis resulting in the formation of the final aminoacyl intermediate **4.17a*ii***. Release of the monophosphate is believed to rely on human Hint enzyme and it could undergo further phosphorylation in order to be converted to its 5' triphosphate form.

4.10.2 Carboxypeptidase Y study on symmetrical diamidate 4.17a

The suggested initial step of activation of diamidates involves an enzyme-mediated hydrolysis of the amino acid ester moiety. Therefore to demonstrate this activation pathway an enzymatic assay were performed in the presence of Carboxypeptidase Y enzyme, well known of being capable of the *in vitro* ester cleavage were performed and monitored by ^{31}P NMR. The assay was conducted on the L-alanine benzyl ester derivative **4.17a.**

In the blank ^{31}P spectrum a single peak at 14.06 ppm stands for the 6-thioinosine symmetrical phosphorodiamidate **4.17a**. After addition of the enzyme slow hydrolysis of the ester unit was observed within three hours, without the disappearance of the starting material.

The chemical structures depicted in Figure 4.16 represent the possible pathways leading towards the aminoacyl derivative **4.17a*ii***. The appearance of intermediate **4.17a*i*** after 3 hours incubation, with a ^{31}P chemical shift more downfield at 14.35 ppm could correspond to the structure lacking one ester moiety or both ester groups being cleaved. During the simultaneous formation of **4.17a*i*** the final metabolite **4.17a*ii*** appeared at 6.71 ppm. After 24 hours the parent diamidate prodrug **4.17a** has

been transformed to the free diacid intermediate **4.17a*ii*.** In this case could be concluded, that the slow rate of carboxypeptidase Y processing could correlate directly with the anticancer potency, however other steps, besides initial esterase activation, are also necessary for eventual biological activity.

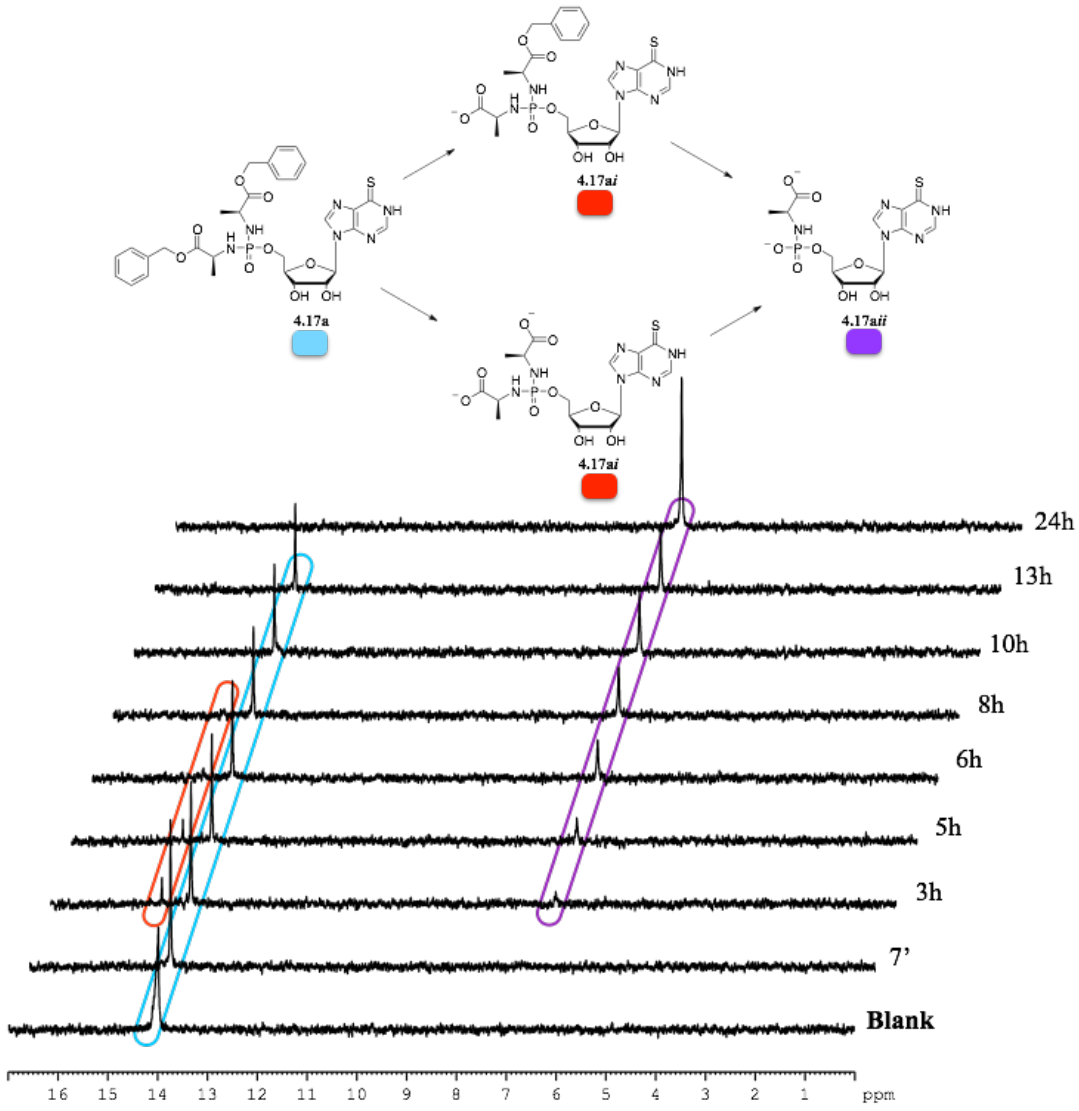

Figure 4.16 Carboxypeptidase Y mediated hydrolysis of **4.17a**, followed by ^{31}P NMR with the proposed metabolite structures **4.17a*i*** and **4.17a*ii***.

4.10.3 Carboxypeptidase Y study on 6-thioguanosine phosphoramidate 4.15a

The enzymatic experiment was performed with the L-alanine benzyl naphthyl phosphoramidate derivative of 6-thioguanosine **4.15a**. In the blank ^{31}P NMR, two diastoisomers (1:1 ratio) signals were observed at 4.41 and 4.22 ppm. Once the enzyme was added **4.15a** was slowly hydrolysed to the intermediate **4.15a*i*** within two and a half hours, which appeared in a 1:1 ratio at 4.87 and 5.01 ppm and simultaneously transformed to the peak corresponding to the final aminoacyl derivative **4.15a*ii*** at 6.99 ppm.

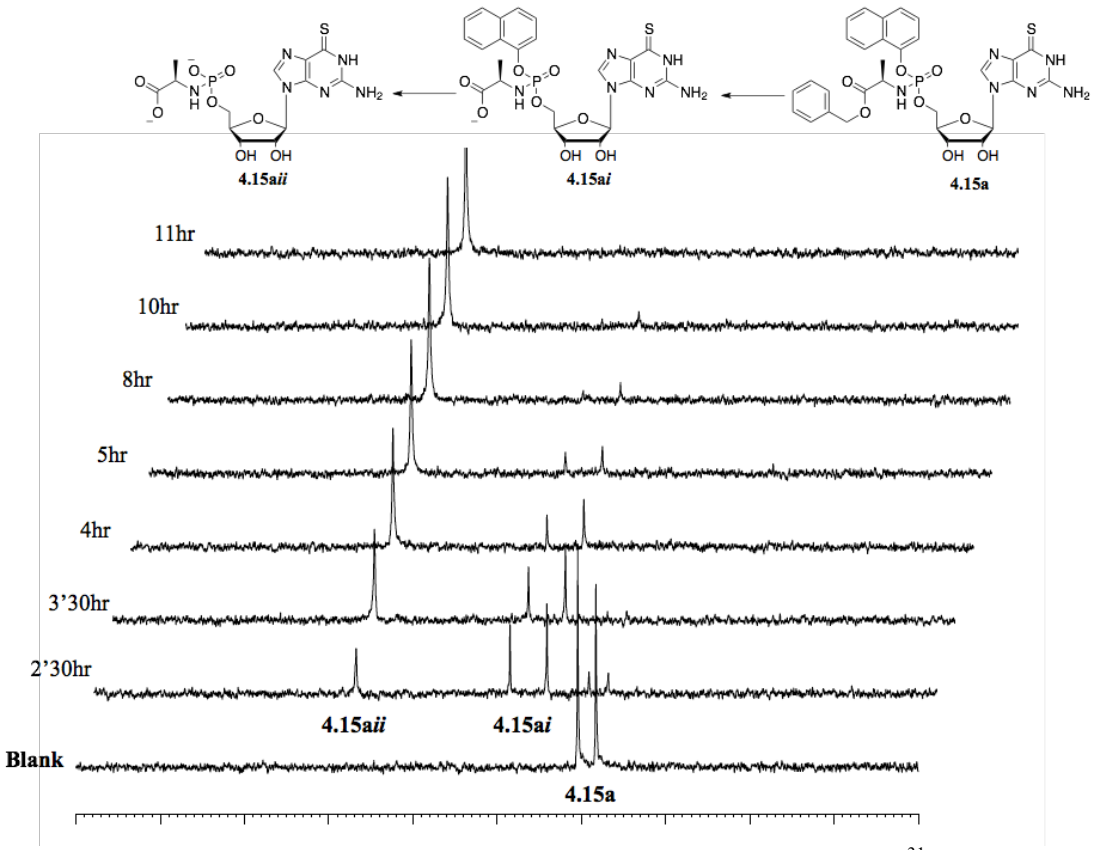

Figure 4.17 Carboxypeptidase Y mediated cleavage of **4.15a**, followed by ^{31}P NMR with the proposed metabolites **4.15a*i*** and **4.15a*ii***.

4.11 Conclusion

A series of thiopurine ProTides were synthesised to examine their lethality and cellular proliferation inhibitory action on human leukaemia cell lines. By applying ProTide technology to different purine nucleobase analogues, 6-thioinosine, 6-thioguanosine and 6-S-methyl-mercaptopurine, the aim of these studies was to examine whether ProTide technology could enhance the anticancer activity of the aforementioned nucleoside analogues. The first series of studies examined the lethality of thiopurine ProTide, in a leukaemia stem cell model of cancer, using the KG1-a cell line. The mean lethal dose (LD_{50}) of thiopurine ProTides, and KG1-a stem population percentages, were determined by Pepper. The L-alanine benzyl phenyl ProTide analogue of 6-thioguanosine (**4.15h**) retained the activity of the parent nucleoside (LD_{50} = 1.1 μM), furthermore 6-S-methyl-mercaptopurine phosphoramidate analogues bearing L-alanine benzyl phenyl **4.23b**, L-alanine neopentyl naphthyl **4.23c** and L-alanine benzyl naphthyl **4.23a** maskings groups were more active than their corresponding parent nucleoside (LD_{50} = 1.7, 1.9 and 2.5 μM). 6-Thioinosine ProTides were poorly active, comparatively. Only 6-thioguanosine ProTides had the ability to target KG1-a stem cells, no selectivity was seen with 6-thioinosine or 6-S-methyl-mercaptopurine ProTides. Structural activity relationships were examined through the modification of ester, aryl and amino acid moieties. In general, L-alanine benzyl ester motif of thiopurine ProTides were the most lethal exhibiting low or sub-micromolar LD_{50}s. In a second series of experiments done in collaboration with WuxiChemApp, the ability of thiopurine ProTides and diamidates to inhibit leukaemia cell proliferation was examined. In nearly all cases, ProTides of 6-thionosine, 6-thioguanosine and 6-S-methyl-

mercaptopurine were poorly active exhibiting medium to high micromolar IC_{50}s, which was in marked contrast to parent control (sub micromolar IC_{50}s). Amongst the 6-thioinosine Protides and diamidates tested **4.12g**, bearing L-alanine benzyl naphthyl derivative stood out, exhibiting activity that exceeded that of the parent, in CCRF-CEM and MOLT4 cells (IC_{50} = 0.476 and 0.531 μM). In the 6-thioguanosine prodrug family phosphoramidate 4.15g, bearing L-alanine pentyl naphthyl derivative displayed submicromolar activity in K 562 cell line (IC_{50} = 0.064 μM), while the L-alanine benzyl phenyl derivative **4.15h** and diamidate **4.19a** bearing L-alanine benzyl ester groups retained the activity of the parent nucleoside in KG-1 cell line. Although none of the phosphoramidates synthesised in the 6-S-methyl ProTide family was able to retain the activity of the parent nucleoside, they all displayed low submicromolar activites in MOLT-4 cell line.

4.12 References

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5 ProTides of Cladribine analogues

Cladribine or as known by its chemical name 2-chloro-2'-deoxyadenosine, was reported to induce long lasting complete remission in patients with Hairy cell leukemia (HCL) by investigators at Scripps Clinic.[1] It was used to treat multiple sclerosis until Merck withdrew all its marketing applications from Russia and Australia in 2011.[2-4] Furthermore, Cladribine showed impressive activity among lymphoproliferative disorders.[5] Its specific biochemical interactions are given in chapter 1.6.1. (Figure 1.9)

5.1 Phosphoramidates of Cladribine

Cladribine phosphoramidates were specifically designed in order to overcome the two main points of resistance to cladribine, namely the hENT 1/2 nucleoside transporter and dCK deficiency.[6-7] The monophosphate of cladribine maybe used to treat malignancies of other cellular lineages that have low dCK activity but may suffer from poor cell permeability and stability.[8] The synthesis and mechanism of action of cladribine ProTides was first characterised by Costantino Congiatu and Rocco Valente in the McGuigan laboratory.[6-7] Following the synthesis of different Cladribine ProTide derivatives the L-alanine benzyl ester motifs with phenyl or L-naphthyl aromatic groups were shown to exhibit the best potencies. In addition, L-leucine, 2,2-dimethylglycine and glycine derivatives displayed good activity in vitro. Due to the inferiority of the phenyl motif compared to the 1-naphthyl, the latter was solely used to prepare ProTides containing L-Alanine amino acids.[6-7, 9] The surprising activity of the 3'-regioisomer being more potent in leukaemia based assays than the 5'-regioisomer, warranted further investigation.

5.2 Synthesis

5.2.1 Synthesis of Cladribine

Cladribine was first isolated as a byproduct of Fischer-Helferich 2'-deoxyinosine synthesis by Harry Venner in 1960 upon the coupling reaction between 2,8-dicholoroadenosine and 1,3,5-triacetyl-2-deoxy-B-D-ribofuranose.[10] Robins has driven research into discovering efficient routes for the synthesis of this clinical agent, which led to a three step synthetic route using the starting material 3',5'-*O*-diacetyl or benzoyl-2'-deoxyguanosine.[11] Upon gaining the protected 6-O-arylsulfonyl derivative, diazotisation with chloro dediazoniation in the presence of benzyltriethylammonium nitrite and acetyl chloride, gave rise to the 2-chloropurine derivative. The final step involved the selective ammonolysis and concomitant deprotection of the sugar moiety. This synthetic route was slightly modified in the McGuigan group by utilising tetrabutylammonium nitrite instead of benzyltriethylammonium nitrite.[7] Following on from the work done by Congiatu, Valente, Murziani and Thomson, focus was only given to synthesising prodrugs of cladribine in order to corroborate the striking and unprecedented potency of the 3'-regioisomer over the 5'-regioisomer.

5.2.2 Synthesis of Cladribine phosphoramidates

In order to overcome the biological disadvantages of Cladribine described in Section 1.6.1.1, the ProTide technology has been applied to commercially available 2-chloro-2'-deoxyadenosine (Figure 5.1). In this thesis the fourth generation of Cladribine ProTides as potential anticancer agents is reported. The main objectives of this chapter were to build a family of Protides to enhance the cytostatic potency of

previous generations, investigate the SAR of the newly synthesised Cladribine phosphoramidates **5.1a,b – 5.7a,b** and to confirm, whether the enhanced potency boost of the 3'-ProTides over their 5'-counterparts is still present in the study therefore corroborates with the previous findings.

Design of the Cladribine ProTides centred mainly on the use of L-alanine as amino acid core due to its potent activity as reported previously by Murziani and Thomsen. The new series also contained one example of the L-leucine amino acid unit. As esters of amino acids benzyl, pentyl, neopentyl, cyclohexyl groups were introduced to investigate their lability towards the esterase, and for each amino acid ester derivative, both phenyl and 1-naphthyl aryl esters were applied.

Cladribine aryloxyphosphoramidates were synthesised by coupling the unprotected commercially available nucleoside 2-chloro-2'-deoxyadenosine with a range of aryl phosphorochloridates (**2.3b**, **2.3c**, **2.3e**, **2.3f**, **2.3h**, **2.3s**) following the Grignard-reagent based method using 0.9 - 1.1 equivalent *t*BuMgCl. (Figure 5.1). The design of the synthesis focused on the isolation of both 3'- and 5'-regioisomer ProTides, which was successfully achieved in all cases with the only exeption of the L-alanine hexyl naphthyl derivative, in which case the 3'-regioisomer due to the extremely low yielding reaction conditions and extensive purification process, could not be isolated and therefore compared to its 5'-counterpart.

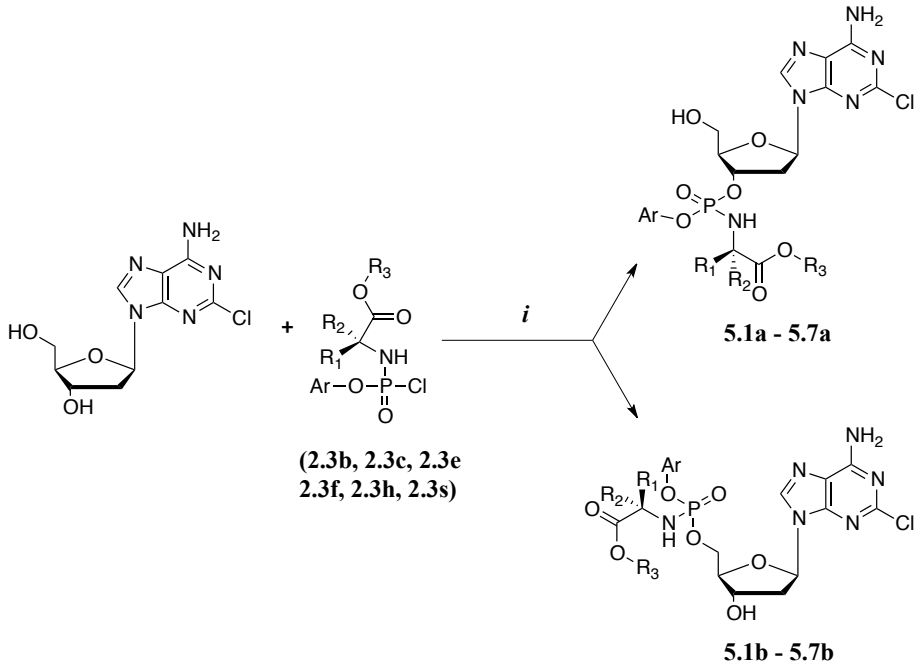

Figure 5.1 A general method for the synthesis of 3'- and 5'-aryloxyphosphoramidates of Cladribine using *t*BuMgCl. *Reagents and Conditions: i,* appropriate phosphorochloridate, *t*BuMgCl, dry THF, -20 ^{o}C, 5-6 hours, to rt, overnight. Phosphorochloridate available from the laboratory: phenyl-(cyclohexoxy-L-alaninyl).

In order to successfully isolate both the 3'- and 5'-regioisomer ProTide pairs in the highest possible yield, the reaction temperature needed to be reduced to -20 ^{o}C and kept for five – six hours. Furthermore it was also found that minimal amount (0.9 - 1.1 equivalent) of *t*BuMgCl reagent reduced the formation of the possible sideproducts, therefore allowing better separation of the species in slightly better yields without using preparative HPLC. Using this method to synthesise each ProTide, it was possible to obtain both regioisomers in almost all cases. 5' phosphate and 3' phosphate cladribine phosphoramidates were obtained as mixtures of two diastereoisomers, showed a total diastomeric ratio of 1:1 by ^{31}P NMR. Purification of these compounds was done by column chromatography, followed by preparative TLC in order to ensure the high degree of purity (<95%).

All the phosphoramidates prepared are reported in Table 5.1. Despite their low yield (1-3%), which was not critical at this stage, sufficient quantities of these compounds were obtained in order to evaluate and ensure their biological activity.

Table 5.1 Structures and specific spectral information of Cladribine ProTides
AA: amino acid; ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0.

Cpd	position	AA	Ester	Aryl	ClogP	^{31}P NMR
Cld	**-**	-	-	-	-0.90	-
5.1a	**3'**	L-Ala	OBn	ONaph	3.09	3.32, 2.58
5.1b	**5'**	L-Ala	OBn	ONaph	2.90	3.89, 3.58
5.2a	**3'**	L-Ala	OBn	OPh	1.92	3.67, 3.09
5.2b	**5'**	L-Ala	OBn	OPh	1.73	4.24, 4.00
5.3a	**3'**	L-Ala	OCH_2tBu	ONaph	3.24	3.68, 3.27
5.3b	**5'**	L-Ala	OCH_2tBu	ONaph	3.05	4.25, 4.10
5.4a	**3'**	L-Leu	OPnt	ONaph	4.76	4.02, 3.48
5.4b	**5'**	L-Leu	OPnt	ONaph	4.76	4.58, 4.25
5.5b	**5'**	L-Ala	OcHex	ONaph	3.22	4.26, 4.12
5.6a	**3'**	L-Ala	OcHex	OPh	2.24	3.34, 2.80
5.6b	**5'**	L-Ala	OcHex	OPh	2.05	3.92, 3.67
5.7a	**3'**	L-Ala	OCH_2tBu	OPh	2.06	3.30, 2.76
5.7b	**5'**	L-Ala	OCH_2tBu	OPh	1.87	3.89, 3.64
5.8	**-**	L-Ala	OCH_2tBu	-	3.70	13.77

The 3' phosphoramidates **5.1a,b – 5.7a,b** could not be distinguished from the 5' phosphoramidates by either mass spectroscopy or ^{1}H NMR integration. Although they showed significant chemical shift changes on the basis of ^{31}P NMR and ^{1}H NMR spectras, these data could not provide sufficient confirmation of their structures.^{13}C NMR splitting patterns due to the carbon-phosphorus interactions were a useful tool in order to identify the 3'- and 5'-phosphorylated derivatives. The ^{13}C NMR spectrum derived from the 3'- and 5'-phosphoramidate structure is able to specify whether the splitting of the phosphoramidate structure occur to the carbon, linked to the phosphate with a two-bond coupling constant ($^{2}J_{C\text{-}P}$) or to the vicinal

carbons with a three-bond constant ($^3J_{C\text{-}P}$). The effect of the P-C coupling in case of the synthesised derivatives was clearly observed (Table 5.2).

The 3' phosphoramidate derivative **5.2a** appeared as four peaks, two for each diastereoisomers for C-3' ($^2J_{C\text{-}P}$ = 5.50 Hz and 5.50 Hz) at 78.64 and 78.43 ppm, C-4' ($^3J_{C\text{-}P}$ = 5.0 Hz, 6.1 Hz) at 88.82 and 88.62 ppm and C-2' ($^3J_{C\text{-}P}$ = 3.8 Hz, 3.8 Hz), at 38.66 and 38.33 ppm, while the remaining carbons of the sugar C-1' (two signals) and C-5' (two signals) did not show any multiplicity due to the C-P coupling (Table 5.2).

The 5' phosphoramidate derivative **5.2b** appeared as four peaks, two for each diastereoisomer for 5' ($^2J_{C\text{-}P}$ = 5.0 Hz and 5.0 Hz) at 66.50 and 66.28 ppm and for the vicinal C-4' ($^3J_{C\text{-}P}$ = 7.90 Hz, the second P-C coupling constant was not calculated due to the partial overlapping of the signals rised from C-1') at 85.49 and 85.44, while the remaining carbons of the sugars C-1', C-2' and C-3'appeared as two peaks regarding the presence of two diastoisomers. Each compound was analysed by ^{31}P, ^{1}H, ^{13}C NMR.

Taking as an example of the L-alanine neopentyl naphthyl derivative **5.3b** appears as two peaks in the ^{31}P NMR spectrum (4.25, 4.10 ppm), which is indicative of the presence of diastereoisomers. The two diastereoisomers generated signals at 70.89 and 70.80 ppm in the ^{13}C NMR, corresponding to the C of the 3'- postion of the sugar moiety, while approximately 4 ppm upfield at 66.55 and 66.42 ppm there were two doublets due to the coupling of the phosphorus atom, confirming the presence of the 5'-derivative with the splitting value of J = 5.0 Hz and 6.3 Hz. The ^{31}P NMR

spectra corresponding to the 3'-regioisomer **5.3a** gave two peaks at 3.68 and 3.27 ppm, further upfield compared to the 5'-derivative **5.3b**.

Table 5.2 Key spectral data and yields of 3'- and 5'-aryloxyphosphoramidates of Cladribine.

Cpd	P	^{31}P NMR	^{13}C-NMR (C-3')	^{13}C-NMR (C-5')	Yield (%)
5.1a	**3'**	3.32, 2.58	80.12, 79.91 (2xd, *J* =5.0Hz)	63.22, 63.15	1
5.1b	**5'**	3.89, 3.58	72.35	67.93	1
5.2a	**3'**	3.67, 3.09	78.64, 78.43 (2xd, *J* =5.5Hz)	63.23, 63.17	2
5.2b	**5'**	4.24, 4.00	78.05	66.50, 66.28 (2xd, *J* = 5.0Hz)	3
5.3a	**3'**	3.68, 3.27	78.61, 78.42 (2xd, *J* = 5.0Hz)	61.80, 61.60	1
5.3b	**5'**	4.25, 4.10	70.89, 70.80	66.55, 66.42 (2xd, *J* = 5.0, 6.3Hz)	2
5.4a	**3'**	4.02, 3.48	80.20, 79.86 (2xd, *J* = 5.25Hz)	63.24, 63.12	1
5.4b	**5'**	4.58, 4.25	72.42, 72.27	68.07, 67.93 (2xd, *J* = 5.5Hz)	3
5.5b	**5'**	4.26, 4.12	72.29, 72.26	68.00, 67.79 (2xd, *J* = 5.25Hz)	3
5.6a	**3'**	3.34, 2.80	79.88, 79.61 (2xd, *J* = 5.5Hz)	63.27, 63.17	2
5.6b	**5'**	3.92, 3.67	74.94, 74.92	67.75, 67.43 (2xd, *J* = 5.5Hz)	3
5.7a	**3'**	3.30, 2.76	79.87, 79.65 (2xd, *J* = 5.0Hz)	63.28, 63.14	2
5.7b	**5'**	3.89, 3.64	72.32, 72.25	67.80, 67.54 (2xd, *J* = 5.0Hz)	3

H-3' and H-5' protons of compounds **5.1a** and **5.1b** looked at together in order to distinguish the species. In the case of the H-3' protons **5.1a** (a multiplet at 5.31 - 5.27 ppm) was significantly deshielded compared to **5.1b** (a multiplet at 4.60 - 4.56 ppm), while the opposite pattern was observed in the case of the H-5' protons, where

5.1a appeared more shielded (a multiplet at 3.84 – 3.73 ppm) compared to **5.1b** (a multiplet at 4.35 – 4.25 ppm) (Table 5.2).

5.2.3 Synthesis of Cladribine symmetrical phosphorodiamidate

The L-alanine neopentyl diamidate analogue of Cladribine were synthesized in a one pot reaction from the free nucleoside prepared by suspending the nucleoside in anhydrous trimethylphosphate at -5^{o}C followed by the dropwise addition of phosphorus oxychloride to give the 5'-phosphorylated intermediate **5.8a*i*** (Figure 4.8). The disappearance of the $POCl_3$ ^{31}P signal and the formation of the new peak ~ 7 ppm, indicating the formed phosphorylated intermediate **5.8a*i*** was followed by ^{31}P NMR. The phosphorylation step found to be critical for the formation of phosphorodiamidates. Formation of the new intermediate took place in the 4-5 hours, if the reaction time was prolonged degradation or hydrolysis of **5.8a*i*** could be observed. After the appearance of the intermediate **5.8a*i*** the next component of the reaction could be added at -78^{o}C in the form of the solution of the appropriate amino acid in anhydrous dichloromethane followed by the dropwise addition of diisopropylethylamine. The appearance of a new signal at 13.77 ppm in the ^{31}P NMR spectrum confirmed the formation of the desired product. The crude mixture was purified by column chromatography underwent second purification by preparative TLC in order to give **5.8a** with 6% yield (Figure 5.2).

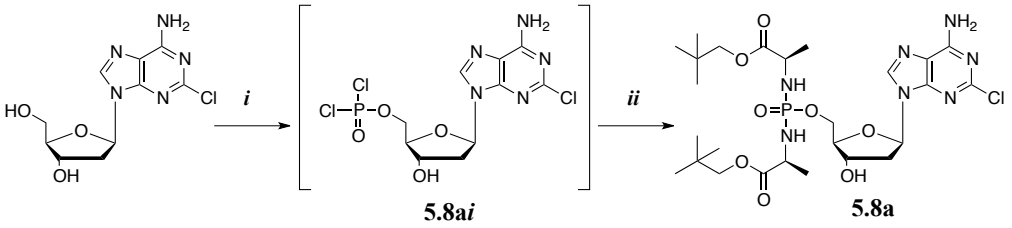

Figure 5.2 General synthetic pathway of Cladribine symmetrical phosphorodiamidates **5.8**. *Reagents and Conditions: i*, $POCl_3$, TMP, -5°C to rt. 4-5 hrs; *ii*, L-alanine neopentyl ester, DIPEA, $CHCl_3$, -78°C to rt., 16 hours.

5.3 Biological evaluation

5.3.1 Cladribine ProTides in solid tumor cell lines

Cladribine ProTides were previously tested in cell lines derived from solid tumors of major organs. The following human cancer cell lines were used: LoVo (colon); MCF7 (breast); PC-3 (prostate); A549 (lung). LoVo cells were taken from a metastatic tumor in the left supraclavicular lymphnode from a 56 year old male patient with adenocarcinoma of the colon.[12-14] MCF7 is a human Caucasian breast adenocarcinoma cell line established from a 69 year old female.[15-16] PC-3 is a human Caucasian prostate adenocarcinoma derived from a 62 year old male.[17-18] A549 is a human Caucasian lung carcinoma taken from a 58 year old male patient.[19] Cladribine showed good cytostatic activity against the panel of solid tumor cell lines tested (Table 5.3) with an $IC_{50} = 0.01 - 0.5$ μM, excluding MCF7 cell line. Whereas the Cladribine ProTides displayed low micromolar potency in Lovo cells, they were poorly active in MCF7, PC3 and A549 cells. Expect from MCF7 cell line, all cell lines showed selectivity to the lead ProTide derivatives, furthermore there was no significant difference in potency between the 5'-ProTide **5.1b** and its 3'-counterpart **5.1a.**

Table 5.3 Cytostatic activity of Cladribine and prodrugs 5' lead **5.1b** and 3' lead Protide derivatives **5.1a**, **5.2a** against LoVo, MCF7, PC3 and A549 cell lines.
Data by Guy, Cardiff University. IC_{50} data in μM = 50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

Cpd	Regio isomer	ClogP	LoVo	MCF7	PC3	A549
Cld	**-**	-0.90	0.01	>100	0.05	0.5
5.1a	**3'**	3.00	0.6	50	0.8	2
5.1b	**5'**	2.90	0.6	>10	1	5
5.2a	**3'**	1.92	<1	>100	0.9	57

5.3.2 Cladribine ProTides in leukaemia cell lines

Four generations of ProTide families produced by Congiatu, Valente, Murziani and Thomsen were tested against a panel of leukaemia cell lines by Walsby, leading to the unexpected identification of the main leads for the project. Six leukaemia cell lines were chosen for the pharmacological assessment and these include: KG-1 (acute myelogenous leukaemia), U937 (histiocytic lymphoma), K562 (chronic myelogenous leukaemia), NB4 (acute promyelocytic leukaemia), NB4R2 (an all trans retinoic acid insensitive subline derived from NB4 cells), HL60 (promyelotic leukaemia).[20-26]

Table 5.4 In vitro data from Leukaemia cell lines. IC_{50} data in μM of 5'- and 3'-ProTide pairs as identified leads from four generations of Cladribine ProTides, originally synthesised by Murziani and Thomsen. Data by Walsby, Cardiff University. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	Regio isomer	ClogP	KG1	U937	K562	NB4R2	NB4	HL60
5.1a	**3'**	3.09	0.3	0.01	5	1	0.1	0.4
5.1b	**5'**	2.90	2	0.2	2	1	0.2	0.7
5.2a	**3'**	1.92	0.4	0.03	>10	0.1	0.2	0.1
5.2b	**5'**	1.73	0.7	0.1	8	0.6	0.2	2

The previously obtained lead compounds **5.1a**, **5.1b**, **5.2a**, **5.2b** were compared in order to establish how superior potency of the 3'-ProTide was compared to its 5' analogue (Table 5.4).

Unlike what was observed with previous biological evaluations of ProTides in solid tumor cell lines, 3'-ProTides were more potent than their corresponding 5'-derivatives in various leukaemia cell lines (Table 5.4).

In the acute myelogenous leukaemia cell line KG1 **5.1a** (IC_{50} = 0.3 μM) has an almost 7 fold potency boost over its 5'-derivative **5.1b** (IC_{50} = 2.0 μM), however its potency found to be more striking in case of the U937 histiocytic lymphoma cell line, where **5.1a** (IC_{50} = 0.1 μM) exhibited approximately 20-fold potency boost over **5.1b** (IC_{50} = 2.0 μM). ProTides did not show much activity in the chronic myelogenous leukaemia cell line K562. **5.2a** maintained similar activity in most cell lines tested compared to its 5'-derivative. However **5.2a** (IC_{50} = 0.3 μM) exhibited an approximate 20-fold activity boost over **5.2b** (IC_{50} = 2.0 μM) in HL-60 promyelotic leukaemia cell line (Table 5.4).

This is a surprising tendency because one would not expect the 3'-ProTide to have superior biologically activity, as 5'-nucleotides are naturally incorporated into DNA chains. In order to exclude confusion surrounding these data, it was decided to extend this study to corroborate these findings. Therefore the previous lead derivatives were resynthesized and a small ProTide family was introduced containing both of 5'- and 3'-derivatives and evaluated to assess their biological activity.

Table 5.5 In vitro data from Leukaemia cell lines. IC_{50} data in μM of Cladribine (Cld) and 5'- and 3'-Cladribine ProTide pairs. Experiments were conducted and data collated by Wuxi AppTec, China. ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Cpd	Regio isomer	ClogP	KG-1	Hel92.1.7	MV-4-11	RL	Z138
Cld	**-**	-0.90	0.068	0.009	0.011	0.044	1.313
5.1a	**3'**	3.09	0.606	0.19	0.07	0.46	0.595 **<2.20**
5.1b	**5'**	2.90	1.938	0.371<2	0.064	0.357	0.734 **<1.79**
5.2a	**3'**	1.92	0.405	0.151	0.074	0.408	0.014 **<93.78**
5.2b	**5'**	1.73	1.867	0.396<3	0.069	0.471	2.013

Four new leukaemia cell lines in addition to the KG-1 linaege were used: Hel92.1.7 (erythroleukaemia); MV4-11 (myelomonocytic leukaemia); RL (non-Hodgkin lymphoma); Z138 (mantle cell lymphoma, identified as a very rare form of B lymphoma) (Table 5.5).[27-31]

The nucleoside Cladribine showed good ability to reach the 50% inhibition with submicromolar activity in KG1, Hel92.1.7, MV-4-11 and RL cell lines IC_{50} (0.009-0.068 μM) and demonstrated IC_{50} (1.313 μM) against Z138 cell line. None of the lead ProTides 5.1a, 5.1b, 5.2a, 5.2b exhibited potency that exceeded that of the parent nucleoside Cladribine. The Protides were approximately 5-30 fold less active then the parent nucleoside in KG1, 16-44 fold less active in Hel92.1.7 cell lines. Furthermore 6-fold activity loss could be seen in the MV-4-11 and 10-fold activity loss in RL cell lines. Regarding the Z-138 cell line, all lead derivatives displayed a marked retention of activity, whereas 5.2a (IC_{50} = 0.014 μM) was found to be approximately 94-fold more active than Cladribine (IC_{50} = 1.313 μM). Furthermore

the 3' ProTide derivatives were shown to have a slight increase (2-3 fold) in activity against their 5' counterparts in the KG1 and Hel92.1.7 cell lines. In case of MV-4-11 and RL cell lines, 3'-ProTides did not show distinct advantage over the 5'-ProTides. In Z138 cell lines however 5.2a exhibited a striking 143-fold activity enhancement in activity compared to its 5'-derivative (Table 5.5).

In order to optimize the Cladribine ProTide motif 9 new ProTide derivate were synthesised. In the first instance alongside with the phenyl and naphthyl unit, the L-alanine motif was retained and L-leucine pentyl motif was also designed as it appeared to be a highly preferred ProTide motif in the FUDR project. The ester moiety was branched to neopentyl, cyclized to cyclohexyl and lengthened to pentyl. The newly synthesised compounds were tested for their biological evaluation against a panel of leukaemia cell lines. Six leukaemia cell lines were chosen, two of which were new, the HL-60 (human promyelocytic leukaemia cell line) and MCF-7 (human breast adenocarcinoma cell line) alongside with KG1, Hel92.1.7, Rl and Z-138 cell lines for the pharmacological assessment. The nucleoside Cladribine showed submicromolar activity in HL-60 cell line (IC_{50} = 0.043 μM) and an IC_{50} of 1.27 μM in MCF-7 cell line. In fact the new series of Cladribine ProTides displayed a lower cytostatic activity than the parent nucleoside in HL-60 and KG-1 cell lines. In case of the Z-138 and Hel.92.1.7 cell lines **5.6a** the 3'-L-alanine cyclohexyl phenyl and **5.7a** 3'-L-alanine neopentyl derivatives showed similar activity that of Cladribine (Table 5.6).

Remarkably **5.6** (IC_{50} = 0.7 μM) lead to an approximately 7 fold enhancement in activity when compared to the their 5'-counterpart **5.6b** (IC_{50} = 4.8 μM), while **5.7a**

(IC_{50} = 0.59 μM) exhibited cytostatic potency 10 fold greater than **5.7b** (IC_{50} = 5.8 μM) in KG-1 cell line. The new ProTide motifs did not deliver any improvement in potency in the MCF-7 cell lines, MCF-7 cells are proved to be resistant to The ProTide family. There was somewhat similar profile observed, when compared the phenyl derivatives with their naphthyl analogues, furthermore **5.4a** and **5.4b** exhibited lower cytostatic activity then the L-alanine derivatives (Table 5.6).

Table 5.6 In vitro data from leukaemia and lymphoma cell lines. IC_{50} data in μM of cladribine (Cld) and 5'- and 3'-Cladribine ProTide pairs. Experiments were conducted and data collated by Wuxi AppTec, China.

Cpd	ClogP	HL-60	KG-1	Z-138	HEL92.1.7	RL	MCF-7
Cld	-0.90	0.043	0.12	0.031	0.039	0.93	1.27
5.3a	3.24	0.18	1.05	1.75	0.07	0.25	6.55
5.3b	3.05	0.18	1.60	0.19	0.11	0.21	8.41
5.4a	4.76	0.26	1.36	1.03	0.07	0.41	4.9
5.4b	4.76	0.39	2.1	0.23	0.13	0.35	5.87
5.5b	3.22	0.62	4.4	0.38	0.18	0.57	27
5.6a	2.24	0.11	0.7	0.071	0.046	0.12	19
5.6b	2.05	0.39	4.8	0.21	0.14	0.34	31
5.7a	2.06	0.11	0.59	0.07	0.043	0.12	20
5.7b	1.87	0.67	5.8	0.34	0.32	0.35	26

ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

5.3.3 Evaluation of Cladribine ProTides in a KG1a cell line model of acute myeloid leukaemia

The most promising Cladribine ProTide candidates were selected for analysis based on two criteria: 1. A potency boost compared to the parent nucleoside; 2. Their selective action against leukaemic stem cells (LSCs). In order to establish stem cell selectivity, acute myeloid leukaemic cell line KG1a was used as it harbours a minor stem cell-like compartment with a specific immunophenotype $Lin^-/CD34^+/CD38^-/CD123^+$. The effect of the four ProTide derivatives of cladribine were evaluated over an extended dose range (0.01-10μM) and the effect of each ProTide on the stem cell compartment were evaluated across the whole dose range.

KG1a cell culture, apoptosis assessments (Annexin V/propidium iodide) and leukaemic stem cell compartment identification, were all performed in the laboratory of Professor Chris Pepper, Cardiff University.

KG1a cells were cultured in the presence of ProTides **5.1a**, **5.1b**, **5.2a**, **5.2b** for 72 hours. Cells were then harvested and probed with a mixture of anti-lineage antibodies (PE-cy7), anti-CD34 (FITC), anti- CD38 (PE) and anti-CD123 (PERCP cy5) in order to visually identify the stem cell compartment. Leukaemic stem cell populations were identified and expressed as a percentage of all living cells in the culture. The percentages of stems cells remaining were then plotted on a dose-response graph and the effect of the cladribine ProTides were compared with the parent. Data are expressed as mean +/- the standard deviation (SD). Raw data were compared for statistical significance using one-way analysis of variance (ANOVA).

Differences between means, were considered when $P<0.05$. Statistical analyses were done using Graphpad Prism 6.0 software.

The stem cell compartment identified as $Lin^-/CD34^+/CD38^-/CD123^+$ comprised approximately 4.2% of the entire KG1a cell population. The **median lethal dose**, **LD_{50}** (lethal dose, 50%), is a measure of the dose required to kill half the members of a tested population after a specified, test duration. Although the lead derivatives displayed 2- to 10-fold lower lethal activity (LD_{50} = 0.27 – 1.7 μM) than the parent nucleoside (LD_{50} = 0.18 μM), all ProTides selectively targeted the KG1a stem cell compartment. **5.1a** (4.5%), **5.1b** (3.5%), **5.2a** (3.3%) and **5.2b** (2.4%) at the concentrations tested, caused a reduction in the stem cell compartment compared to cladribine control (5.9%) (Table 5.7 and Figure 5.2). In particular **5.2b** is outstanding in this regard as although it exhibited the lowest LD_{50} values it displayed the highest stem cell selectivity. In agreement with earlier data, the stem cell data also confirmed that the 3'-derivatives were more potent than the 5' regioisomers. Intriguingly, 5'-regioisomers were more stem cell selective than their 3' counterpart.

Table 5.7 Cladribine ProTide stem cell selectivity. Experiments were conducted and data collated in the laboratory of Professor Chris Pepper, Cardiff University.
LD_{50} is a measure of the lethal dose required to kill half the members of a tested population after a specified, test duration. (μM)

Cpd	Regio isomer	AA	Ester	Aryl	LD_{50} (μM)	Stem cell% Control: 4.2%
Cld	-	-	-	-	0.18	5.9
5.1a	3'	L-Ala	Bn	Naph	0.27	4.5
5.1b	5'	L-Ala	Bn	Naph	0.82	3.5
5.2a	3'	L-Ala	Bn	Ph	0.83	3.3
5.2b	5'	L-Ala	Bn	Ph	1.7	2.4

There is a growing belief that cancers are dependent on a small population of stem cells in order to grow and maintain viability. The leukaemia stem cell was the first kind of cancer stem cell to be discovered.[32] Stem cells are considered to play an important role in cancer survival due to their ability to regenerate and differentiate. So in cases where targeted anticancer treatment kills the differentiated cancer cell population, the elusive cancer stem cell population remains intact and is therefore able to re-establish cancer growth and progression.[32-34] These notions form part of the of the cancer stem cell hypothesis.[35-36] The cancer stem cell compartment appears to represents an attractive target in anticancer therapy.

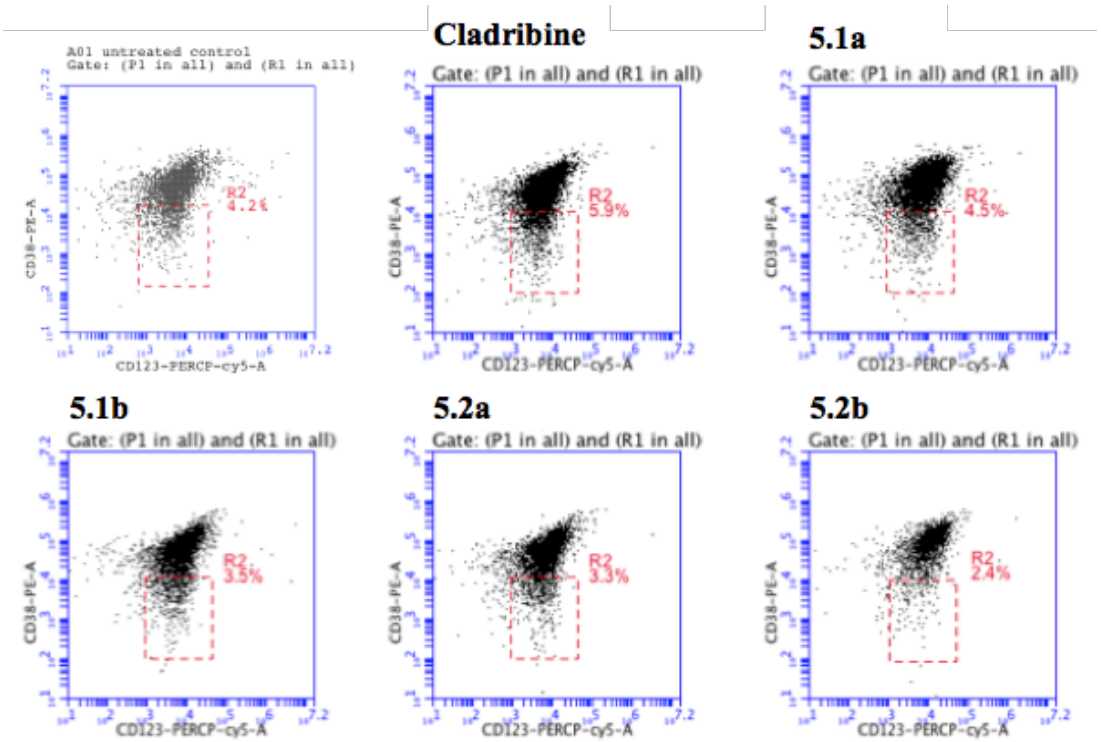

Figure 5.2 A gating strategy to define the LSC sub-population in the KG1a cell line.

5.4 The 3'-ProTide conundrum

According to convention the 5'-ProTides are anticipated to deliver the 5'-monophosphate, which subsequently undergoes further phosphorylation to the diphosphate and eventually the bioactive 5'-triphosphate form. Therefore it is puzzling that 3'-ProTides that are expected to deliver the 3'-monophosphate into cells in many cases exhibit better potency than the comparative 5'-derivative. Valente, who then synthesised 5'-deoxycladribine and its 3'-ProTide to find a solution to this puzzle, first addressed these unusual findings. Both compounds tested negative towards NB4 and HL60 cell lines, suggesting that the active species is a 5'-phosphate. Moreover, this indicates that 3'-cladribine ProTide may undergo some of molecular rearrangement, which leads to the formation of a 5'-phosphate. Speculatively, the 3'-ProTide could potentially be processed in different pathway to that of the 5'-ProTide, which could potentially favour the greater membrane penetration and intracellular metabolism, which may lead to more active 5'-triphosphate metabolite produced (Figure 5.3). Finally, the possibility of the ProTide breakdown to cladribine at any point is unlikely, as proved by the Carboxypeptidase Y and lysate processing assays previously performed by Thomsen. The reasons for this surprising activity of 3'-ProTide derivatives are yet to be determined.

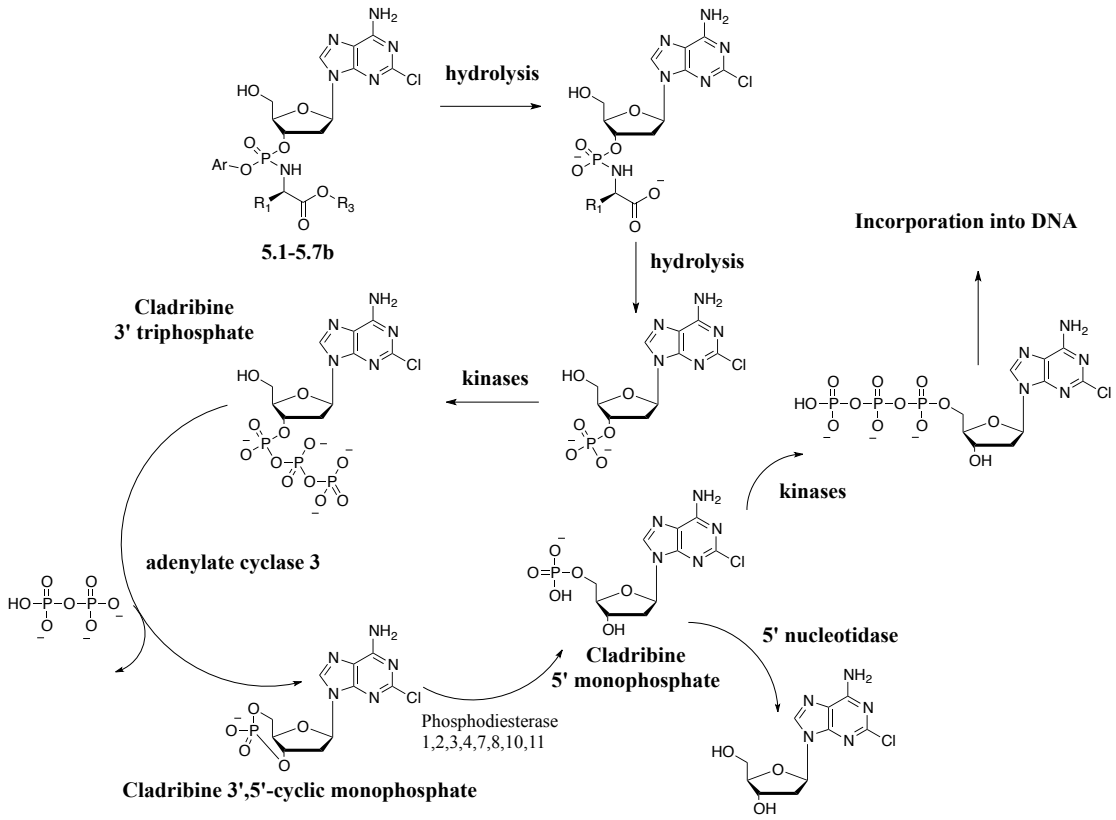

Figure 5.3 Postulated molecular rearrangement of a cladribine 3'-ProTide

5.5 Conclusion

The fifth generation of Cladribine ProTides, described in this chapter were designed in order to confirm the previously observed tendency of the 3'-ProTide regioisomers to being more potent than the 5'-regioisomer analogues, identified in the first and second generation of Cladribine ProTides. The whole project covers a range of ProTide motifs, but the original lead compounds the phenyl **5.2a**, **5.2b** and naphthyl L-alanine benzyl ester derivatives **5.1a**, **5.1b** remained as the most potent of all tested. However they do not show increased potency compared to their parent nucleoside. Enzymatic ester hydrolysis assay were performed by Thomsen to investigate this unique phenomenon, showing apparently small differences in hydrolysis rate. Furthermore it would be interesting to perform wide spectrum phosphodiesterase or lysate based enzymatic studies in order to find proof of the assumed molecular rearrangement of the phosphoramidate or phosphate moiety between from the 3' position to 5'. In vivo metabolic evaluation could be informative, as it could lead to some interesting findings about the different behaviour of these unique 3' and 5' ProTide derivatives.

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6 ProTides of Decitabine analogues

Epigenetic modifications such as DNA methylation are very important in gene expression both in normal developing processes and in carcinogenesis too.[1-2] Abnormal DNA hypermethylation of the promoter regions of tumor-suppressor genes are distinctive to AML and chronic leukaemias.[1-2] The genetic changes associated with carcinogenesis representing such aberrant reversible epigenetic modifications, hypermethylations, led to the clinical development of the hypomethylating agent azacytidine and decitabine.[3-4] The first DNA methylation inhibitor approved by the US FDA in May 2004 was azacytidine (Vidaza, Pharmion), its 2' deoxy derivative 5-aza-2'-deoxycytidine (decitabine) also purported to exert good therapeutic potency (Dacogen, MGI Pharma) was approved in 2006 for the treatment of myelodysplastic syndromes.[5] (Chapter 1.8.)

As previously described in Chapter 1.8, Decitabine causes hypomethylation in the promoter region of the tumour suppressor gene p15 in MDS patients.[6] Hypermethylation and critical silencing of p15 encoded genes have a leading role in cancer cell proliferation, differentiation and disease progression.[2, 6-9]

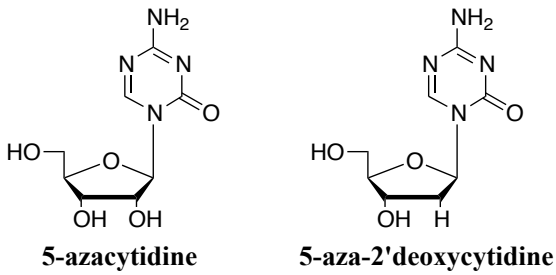
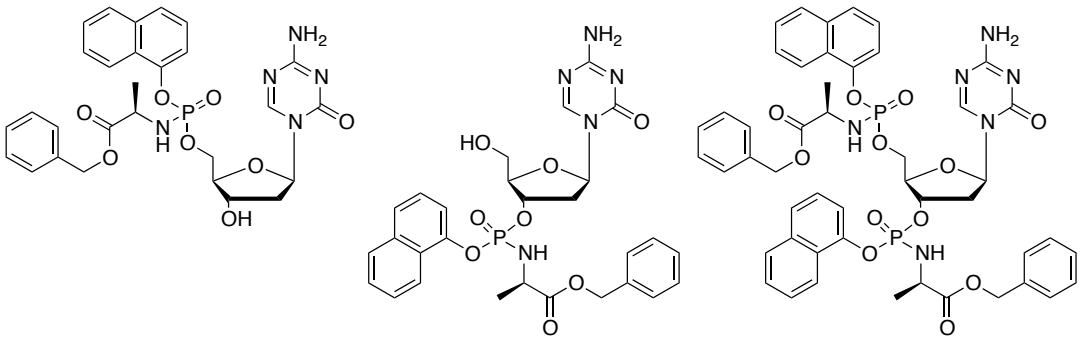


Figure 6.1 DNA methyltransferase inhibitors 5-azacitidine and 5-aza-2'deoxycitidine (Decitabine).[5]

Decitabine is a cytidine analogue that was first synthesised in the early 1960s by Pliml and Sorm.[10] Although similar in structure to cytidine it contains a nitrogen atom that replaces the carbon atom at the 5 position of the pyrimidine ring.[10] This natural nucleoside 2'-deoxycytidine analogue is cytotoxic at high doses just as many cytidine analogues, but it also has the ability to inhibit DNA methyltransferase following phosphorylation and direct incorporation into the DNA.[11-13] Overall in term of haematological improvement azacitidine and decitabine represent the most active single agents for AML patients, causing haematological improvement and enhancing quality of life.[14-16]

6.1 Decitabine phosphoramidates

Three ProTide analogues of decitabine have been previously prepared in the McGuigan group by Congiatu and tested for their anticancer activity (Figure 6.2 and Table 6.1) against a small selection of leukaemia cell lines.[17]



6.8*a* **6.8*ai*** **6.8a*ii***

Figure 6.2 Decitabine aryloxyphosphoramidates **6.8**, **6.8a*i***, **6.8a*ii*** have previously been synthesized by Congiatu in the McGuigan group.

The first generation of Decitabine ProTides **6.8**, **6.8a*i***, **6.8a*ii*** were tested for their cytostatic activity across a panel of leukaemia cell lines including acute promyelocytic leakaemia cell lines NB4 and NB4R2, acute promyelotic leukaemia

cell line HL60, chronic myelogenous leukaemia cell line K562, acute myeloid leukaemia cell line KG1 and finally histiocytic lymphoma cell line U937.[18-24] (Table 6.1)

Table 6.1 Evaluation of decitabine and ProTide **6.8**, **6.8a*i***, **6.8a*ii*** in leukaemia cell lines. Data are IC_{50} (μM). Experiments done by Mills.

Cpd	ClogP	NB4	HL60	NB4R2	K562	KG1	U937
Decitabine	-1.9	0.3	35	0.4	116	2	83
6.8a	1.9	3	96	9	88	53	85
6.8a*i*	2.1	8	96	6	80	>100	135
6.8a*ii*	6.6	3	33	3	16	15	39

The parent nucleoside exhibited moderate or poor activity against this panel of blood borne cancer cell lines (IC_{50} = 0.5 μM - 83 μM). Compounds **6.8**, **6.8a*i***, **6.8a*ii*** displayed low cytostatic activity, however **6.8**, **6.8a*i*** showed reasonable potency in two out of six cell lines (NB4 and NB4R2 cells). Compound **6.8a and 6.8a*i*** (IC_{50} = 88 μM and 80 μM)**,** the 3' and 5'-ProTide L-alanine benzyl naphthyl derivatives retained the activity of the parent nucleoside (IC_{50} = 116 μM) in the K562 cell line. However the biggest highlight from these sets of data is the activity of compound **6.8a*ii***, the 3',5'-bisProTide in the HL-60, K562 and U937 cell lines. In terms of acute myeloid leukaemia K562 cell line all analogues show improvement in potency, moreover **6.8a*ii***, the most lipophilic derivative, increased its potency by over 7 fold (IC_{50} = 16 μM). In the histiocytic lymphoma cell line U937 compound **6.8a*ii*** retained the activity of the parent as it showed a 2-fold enhancement in activity over the nucleoside (IC_{50} = 39 μM). The potency of **6.8a*ii*** may correlate with its marked enhancement in lipophilicity, although other factors cannot be ruled out.

Furthermore these ProTide analogues have been tested against a broad spectrum of solid tumour cell lines including MCF7 breast cancer, Lovo and HT29 colon cancer, PC3 prostate, MIA-Pa-Ca2 pancreatic and A2780 and A2780R ovarian cancer cell lines.[25-34] (Table 6.2).

Table 6.2 Evaluation of Decitabine and Decitabine ProTide analogues **6.8**, **6.8a*i***, **6.8a*ii*** in solid tumour cell lines. Data are IC_{50} (μM). Experiments done by Mills.

Cpd	ClogP	MCF7	Lovo	PC3	HT29	MIA-Pa-Ca2	A2780	A2780R
Decitabine	-1.9	0.2	0.3	0.3	28	53	0.4	0.7
6.8a	1.9	4	3	4	57	47	2	7
6.8a*i*	2.1	23	11	38	>100	>100	6	32
6.8a*ii*	6.6	8	5	9	37	42	4	12

Despite the parent nucleoside exhibiting higher IC_{50} in the HT29 and MIA-Pa-Ca2 cell lines, which may be indicative of nucleoside resistance, none of the ProTides were more potent than decitabine. **6.8a*i*** (IC_{50} = 23 - >100 μM) lost 15 to greater than a 100-fold potency across the spectrum of cell lines. In comparison compounds **6.8a** (IC_{50} = 3 - 57 μM) and **6.8a*ii*** (IC_{50} = 4 - 37 μM) were 5 to greater than 20-fold less active compared to the parent control. Therefore it seems that based on Table 6.1 and 6.2, decitabine ProTide analogues perform better in blood borne cancer cell lines compare to cell lines representative of solid tumours.

Studies centred on applying the ProTide approach to decitabine, having a primary insight into their activity in various cancer cell lines were further continued.

6.1.1 New series

A new series of decitabine ProTides were designed in order to truly establish the efficacy of this ProTide family and to establish SARs. The series consists of four analogues; two of those bearing L-alanine-benzyl ester, replacing the phenyl unit with 1-naphthyl as aryl functionality and two with the L-leucine pentyl ester moieties. In the case of L-leucine analogues both a 3'- and 5'-regioisomers were isolated.

6.2. Synthesis

6.2.1. Synthesis of decitabine

The FDA approved the use of decitabine for the treatment of myelodysplastic syndromes, in May 2006.[35-36] The first route to d5azaC from 5azaC for L-nucleosides was described by Gaubert and colleagues in 2000, with the overall yield of 42%.[37] The long synthetic route involving four steps, was previously applied to decitabine in the McGuigan group by Contagiou with the overall yield falling to 18%, primarily because of the introduction of the phenoxy thiocarbonyl protecting group.[17] Decitabine was patented by Ionescu and Blumbergs in 2004, where they described a method for synthesising the nucleoside utilising a Vorbrüggen coupling method between silylated 5-azacytosine and peracetylated ribose.[38]

6.2.2 Markiewicz protection of 5-azacytidine

The published route, which is based on the classic Barton McCombie deoxygenation reaction, was followed with a couple of small modifications (Figure 6.3). In order to deoxygenate the 2'-position of 5-azacytidine, simultaneous protection of 3′-OH and

5′-OH groups is required, which was accomplished with the suitable bidentate 1,3-dichloro-1,1,3,3-tetraalkyldisiloxane protecting group (TIPDSCl), introduced by Markiewicz in 1979, in the presence of anhydrous pyridine at 0°C.[39] To purify the crude product from that large amount of excess silane the combination of aqueous workup and column chromatography did not prove to be efficient. The crude mixture was purified by flash column chromatography with 89 % yield (Figure 6.3).

6.2.3 Barton McCombie elimination

For the reduction of the 2'-OH group the classic Barton-McCombie elimination has been applied onto the silylated nucleoside as described by Gaubert *et al.* (Figure 6.4). The free hydroxyl group acts as a radical leaving group by the reaction with phenyl chlorothionoformate.[37]

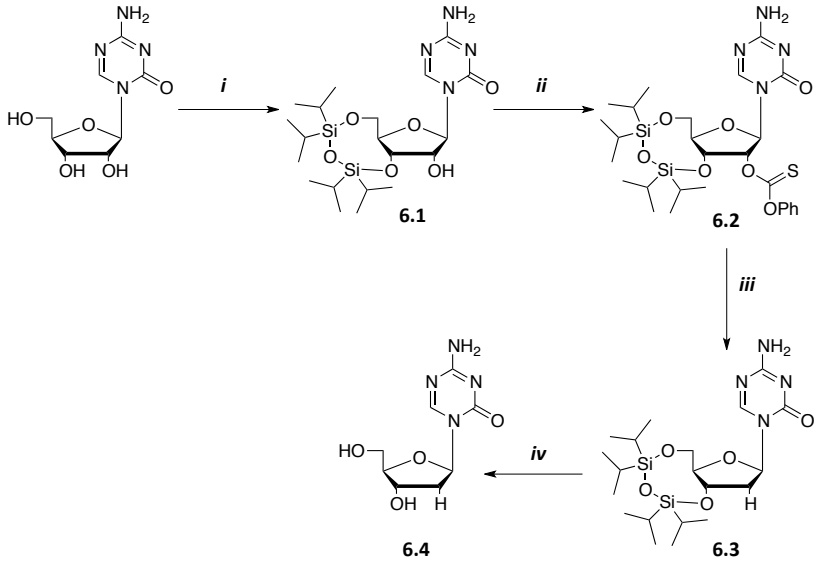


Figure 6.3 General synthetic route leading to the formation of decitabine (**6.4**) from 5-azacytidine
Reagents and Conditions: i, dry pyridine, 1,3-dichloro-1,1,3,3-tetra-isopropyldisiloxane, 0°C to rt., 16 hrs; *ii,* dry acetonitrile, phenoxythiocarbonyl chloride, rt., 4hrs; *iii,* dry toluene, tributyltin hydride, AIBN, 100°C, 2hrs; *iv,* dry THF, TBAF 1.0M in THF, rt, 16 hrs.

The 2'-hydroxy group is reduced in a subsequent radical reaction upon reacting with phenyl chlorothionoformate in the presence of DMAP and acetonitrile. The nucleoside was synthesised beforehand by Congiatu in the McGuigan lab based on a published procedure by Gaubert et al.[37] The disclosed method obtained the desired compound after aqueous work-up, whereas Congiatu's method required purification on flash chromatography with a disappointing yield (27%) compared to the published method.[17] Several attempts of slightly modified methods have been made by Thomsen in order to improve the yield of the reaction based on the findings of Chu and co-workers in the case of thymidine.[40-41] Phenyl chlorothionoformate was added to a cooled and extremely anhydrous solution of TIPS protected nucleoside and a mixture of DCM and pyridine yielding the desired product with a convincing but still moderate yield of 39% followed by aqueous work up and flash column chromatography. According to Thomsen, flushing of the column with pure ethyl acetate during the purification process improved the yield to 79%, however this yield was not always reproducible during my synthetic work (60-65% yield). On a larger scale of 3-4 g, the average best yield achieved was 27%. One possible explanation for the low yielding step could be due to the interaction between the thionoformate group and the slightly acidic nature of the silica gel. This suspected interaction could lead to the compound being retained on the column during the purification process. The binding ability of the thionoformate group towards protons is evident also in the case of the H-4', which is deshielded with an appearance of a singlet in the ^{1}H NMR spectrum. (5.78 ppm for **6.2** compared to 4.01 ppm for **6.1**).

The third step of the elimination process involves the reduction of the 2'OH of the

nucleoside in the presence of tributyltin hydride and azobisisobutyronitrile (AIBN). The reaction requires extremely anhydrous conditions, which was achieved by bubbling argon through the reaction mixture for 30 minutes. The desired 2'-deoxy-analogue was purified by a quick flash column chromatography in order to obtain the pure product in 85% yield. (Figure 6.4).

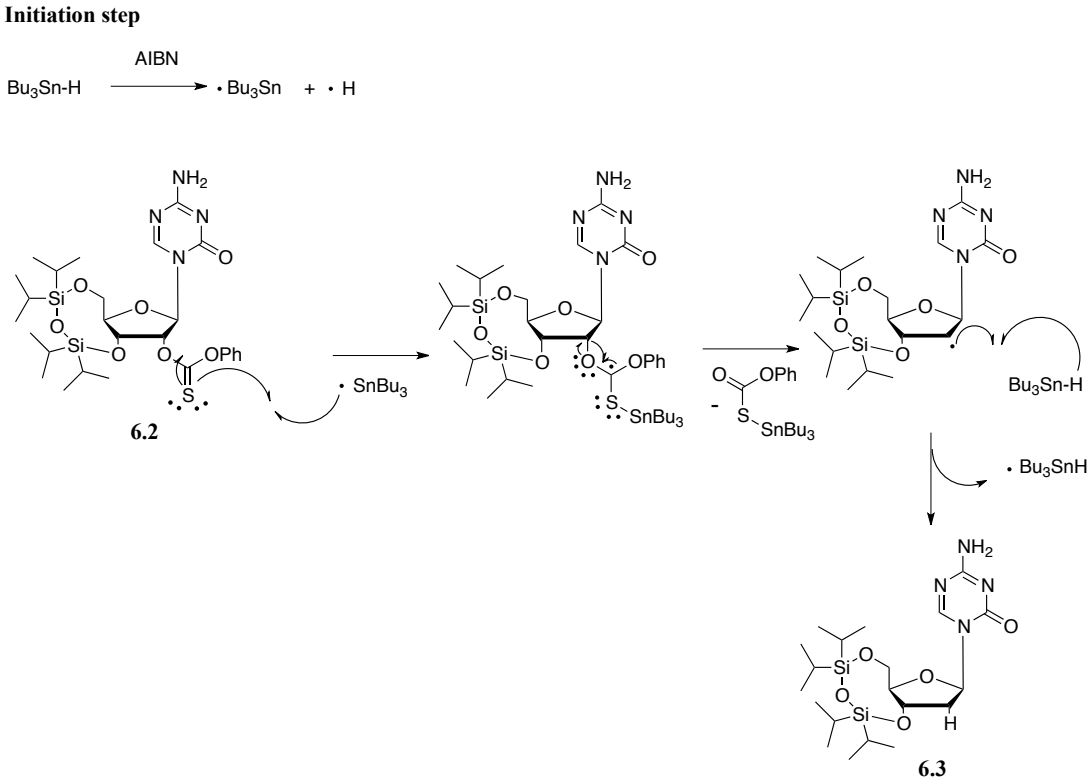


Figure 6.4 Barton-McCombie deoxygenation reaction mechanism

6.2.4 Desilylation

The final and most challenging step of the synthetic route towards decitabine is the removal of the Markiewicz protecting group. Removal of the silyl protecting group via fluoride addition is the most common approach based on the strong and selective silane-fluorine bond formation. Removal of the TIPS protecting group offers a wide repertoire of reagents from which, one is the most commonly used is

tetrabutylammonium fluoride (TBAF). Following on the procedure by *Gaubert et al.*, the protected nucleoside was treated with 1M TBAF in dry tetrahydrofuran and was stirred at room temperature for 8 hours.[37] Monitoring the reaction progress by TLC during this period of time, there was no sign of the desired product, therefore it the mixture was left to stir overnight. After this time, several new polar side-products appeared without a significant change in the amount of starting material. The desired nucleoside was expected to crystallise out after evaporation from the oily material in a small amount of methanol. Although the purification step was successful, it required a longer progression time of 24-48 hours at 0°C and only provided the desired nucleoside at yield of 3%. Analysing the residue over by ^{1}H NMR, it was found that the majority of the mixture was TBAF residue with traces of the deoxygenated nucleoside. Therefore a slightly modified deprotection method with solid supported TBAF has been applied onto the protected nucleoside. The potential advantages of using solid supported TBAF is excluding the need of excessive amount of reagent, required for the deprotection step and easier purification. Using TBAF on solid support also negates the repelling property of the deprotected compound being highly water soluble, whilst the removal of the reagent required aqueous phase extraction. However despite all these known benefits, the use of this reagent was found to be very demanding. The nucleoside, in its purest state is very polar, with a calculated ClogP value of -1.9, therefore making it very difficult to filter off from the solid support without actually dissolving some of the TBAF residue from the support or the tetrabutylammonium salt. Regardless of the choice of solvents (tetrahydrofuran or methanol) used at cold or ambient temperature, or on a small or on large scale, is was not possible to isolate the pure compound in comparable fashion to Gaubert and colleagues.[37]

6.2.5 Selective 5'-end cleavage of 3',5'-TIPDS protected decitabine

Investigating alternative approaches to solve the case of the problematic last step led to the design and preparation of the selective 5' end deprotected 3' TIPDS protected d5AzaC. This increases the solubilty of the nucleoside allowing positive impact on the coupling reaction with phosphorochloridates and providing improved yields of the final ProTides. A paramount feature of this elegant protecting group is its ability to be partially cleaved at the less sterically hindered site upon being hydrolysed under acidic conditions. Previous pursuits were done over the last two decades in order to find the right conditions avoiding the 3' end cleavage just as well as the full deprotection of TIPDS. Conventional methods with mineral acidic conditions like 0.2 M HCl in dioxane-water mixture in the ratio of 4:1, 1 M HCl in dioxane or HF-pyridine complexes found to achieve this goal only with partial selectivity, which resulted in the desired 5'-desilylated products in very low yields. Xue-Feng Zhu *et al.* demonstrated that the mixture of TFA-H_2O-THF in the ratio of 1:1:4 could lead to an efficient 5'-desilylation of multisilylated nucleoside derivatives with quantitative transformation (Figure 6.5).[42] Such a deprotecting system was applied multiple times as a successful selective desilylation method for TBDMS protecting groups in the McGuigan group and was investigated for the first time in the case of the partial cleavage of TIPDS (Figure 6.5).

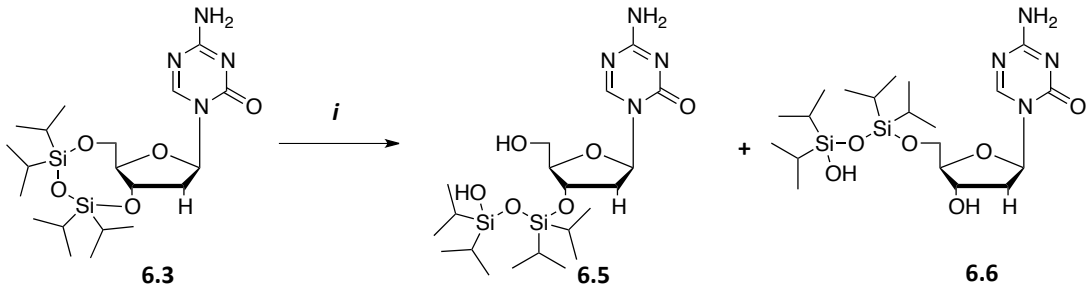


Figure 6.5 Selective 5' and 3' desilylation method from 3', 5' TIPDS protected decitabine by of Xue-Feng Zhu *et al.*[42]
Reagents and Conditions: i, THF, TFA-H_2O (1:1), 2 hours, rt.

The intention of the experimental method was to find the optimal time point to stop the reaction and isolate the desired derivative with the best yields possible. To the cooled solution of 3', 5'-TIPDS protected decitabine in THF, an aqueous solution of TFA was added dropwise and allowed to stir for 50-90 minutes at 0^oC under rigorous monitoring. After this time interval, the formation of the 3'-desilylated derivative started to appear alongside the desired 5' desilylated compound, which gave rise about 85-89% of the latter. Based on previous experiences related to the cladribine ProTide project, whereas the 3'-ProTide derivative showed unexpected activity improvement over the 5'-derivative, it was highly desirable to collect the 3'-desilylated nucleoside from consecutive reactions. The reaction mixture was neutralised with saturated solution of $NaHCO_3$, diluted with ethyl acetate and washed with water and brine, dried over $NaSO_4$ and evaporated under reduced pressure. The residue was purified by flash column chromatography and followed by preparative TLC to provide the pure 5'-desilylated and 3'-desilylated products as white crystals in 67% and 31% yields.

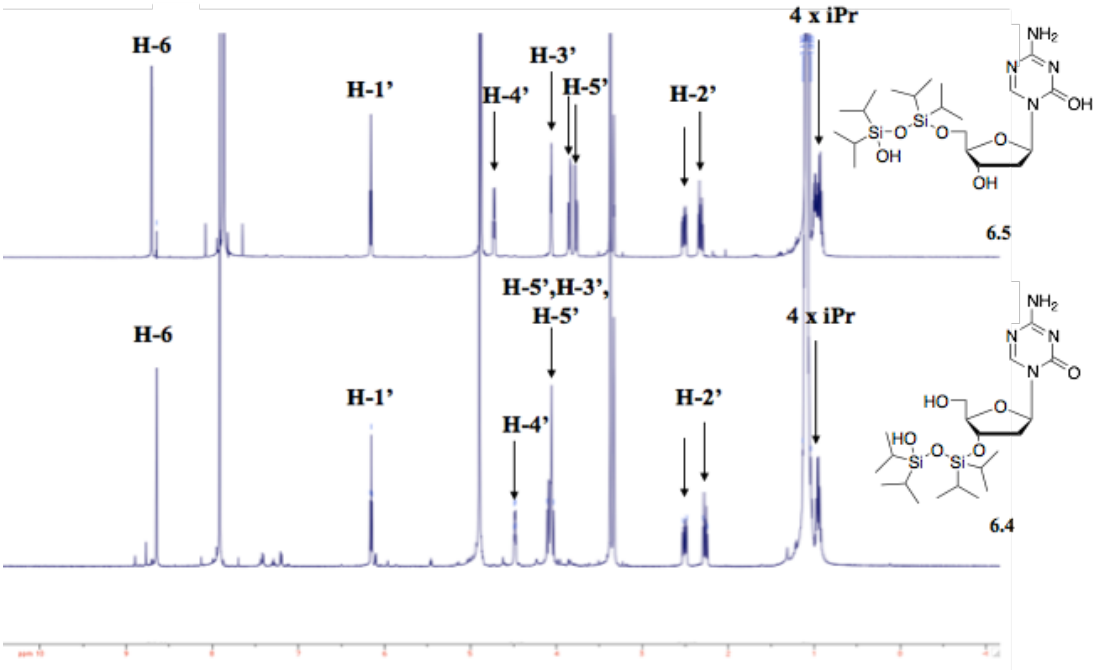


Figure 6.6 ^{1}H NMR spectrum of 3', 5' TIPDS protected decitabine

6.3 Synthesis of decitabine phosphoramidates

Decitabine ProTides have been synthesized via the Grignard method. The 3'-TIPDS protected decitabine was dissolved in dry THF followed by the dropwise addition of 1.1 equivalent of *t*BuMgCl reagent and the appropriate phosphorochloridate and allowed to stir overnight at room temperature. The reaction mixture was evaporated and subjected to flash column chromatography. The pure compound was present as white crystals with a conversion of an average 44-67% yield. Thereafter the protected ProTide derivatives were subjected to deprotection. The deprotection of 3' TIPDS protected ProTides were carried out in 3 ml of dry THF then the solution was cooled down to -5-0^{o}C and 1.5 ml of TFA-H_2O mixture in a ratio of 1:1 was added dropwise and allowed to stir at the same temperature under rigorous monitoring. After allowing the reaction to proceed for 18 hours at -5 - 0^{o}C, no product formation or degradation of the starting material were observed according to TLC and LC MS.

It was decided to let the reaction continue at room temperature, where after 28 hours the formation of the product was in 1:1 ratio with the starting material. The reaction mixture was evaporated without neutralisation, which led to the cleavage of the glycosidic bond of the ProTide. A new batch of the product was progressed to deprotection using the same reaction conditions, but instead allowing the reaction to stir for 48 hours at room temperature followed by the neutralisation of the reaction by saturated $NaHCO_3$ and evaporation, followed by extraction in EtOAc from H_2O. In regards to the small-scale reaction, the final purification took place on preparative TLC plates. The overall yield over two steps for the entire series was an average of 40-43%. ProTides were characterised by ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, MS and HPLC.

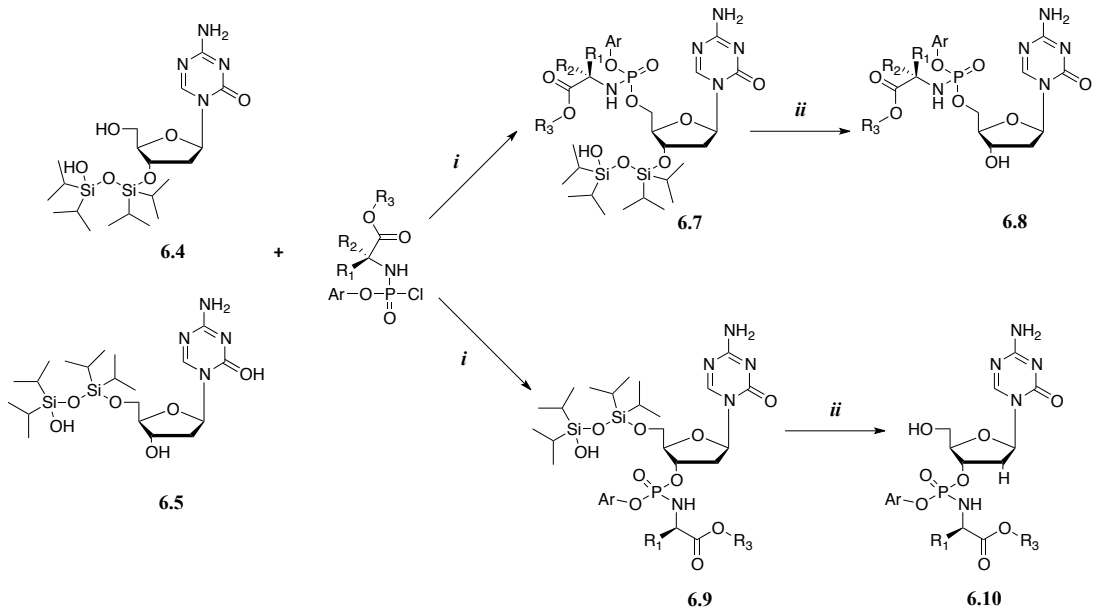


Figure 6.7 A general synthetic pathway towards decitabine 5' and 3'-ProTides
Reagents and Conditions: i, appropriate phosphorochloridate: **2.3b**, **2.3c**, **2.3s**; *t*BuMgCl, dry THF, rt., 16 hours.; *ii,* THF, TFA-H_2O (1:1), 2 hours, rt.

The 5'TIPDS protected decitabine was collected from three consecutive reactions done previously and the same ProTide approach was applied, followed by

deprotection in order to isolate a 3'-ProTide. Figure 6.7 shows the general synthetic scheme of the decitabine ProTide approach, while Table 6.3 contains the newest ProTide derivatives.

Table 6.3 Second generation Decitabine ProTides, their structures, yields, calculated lipophilicity and key spectral data.

Cpd	Regio isomer	ClogP	AA	Ester	Aryl	^{31}P NMR	Yields %
6.4	**-**	-2.03	**-**	**-**	**-**	**-**	
6.8a	**5'**	1.91	L-Ala	Bn	Naph	4.63, 4.24	12
6.8b	**5'**	0.73	L-Ala	Bn	Ph	4.16, 3.71	5
6.8c	**5'**	3.77	L-Leu	Pnt	Naph	5.02, 4.55	23
6.10a	**3'**	3.77	L-Leu	Pnt	Naph	4.17, 3.47	19

ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

6.4 Biological evaluation

6.4.1 Evaluation of decitabine ProTides in a KG1-a leukaemic stem cell model

The new family of decitabine ProTide candidates were selected for analysis based on two criteria, an improvement in potency and selective targeting of leukaemic stem cells (LSCs). (Chapter 3.4.1).

The stem cell compartment identified as $Lin^-/CD34^+/CD38^-/CD123^+$ comprised approximately 3.4% of the entire KG1-a cell population. Decitabine ProTides were far less potent (LD_{50} = 1.2 – 19 μM) then the parent nucleoside (LD_{50} = 0.085 μM), they required 14-223 fold higher concentration then decitabine control to produce the same cytotoxic effect (Table 6.4). Furthermore decitabine prodrugs **6.8a**, **6.8b**, **6.8c**

did not target selectively the KG1-a stem cell compartment either, while the parent nucleoside decitabine showed stem cell insensitivity (stem cell selectivity = 5% compared; control = 3.4%) compared to its ProTide analogues (stem cell selectivity = 3.45 – 3.6%, control = 3.4%). The 3' -L-leucine pentyl naphthyl derivative **6.10a** appeared to be 4-fold more potent (LD_{50} = 5.1 μM), than the 5'-derivative **6.8c** (LD_{50} = 19 μM), although it still displayed a 60-fold loss in cytotoxic activity compared to decitabine (Table 6.4).

The L-alanine benzyl phenyl motif **6.8a** performed better in this assay compared to its phenyl pair **6.8b**, comparatively, **6.8b** (LD_{50} = 4.3 μM) required 3.6-fold higher concentration of compound, than its naphthyl pair **6.8a** (LD_{50} = 1.2 μM). The cytotoxic effect does not seem to correlate with the lipophilicity of the ProTides, because **6.8c**, being the most lipophilic compound of the series required considerably higher concentration in order to produce the same pharmacological effect and found to be 223 fold less active (LD_{50} = 19 μM), than any other members of this small series (Table 6.4).

5-azacytidine nucleoside, **6.xa** and **6.xb** was evaluated in this assay in order to compare the efficacy of the nucleoside and the prodrug analogues, with the only difference in structure of bearing 2' hydroxyl group on the sugar moiety, to 5aza-2'-deoxycytidine and their equal motifs. 5-azacytidine performed poorly in this study, it exhibited approximately 32-fold loss (LD_{50} = 2.7 μM) in activity upon compared to decitabine (LD_{50} = 0.085 μM). The L-alanine benzyl naphthyl derivative **6.xa** (LD_{50} = 33 μM) and the L-alanine benzyl phenyl motif **6.xb** (LD_{50} = 3500 μM) of 5-azacytidine compared to the analogues of decitabine were found to be far less potent

also. **6.xa** presented a 27-fold loss in cytotoxic activity compared to **6.8a**, while **6.xb** activity significantly reduced upon compared to **6.8b**, by approximately 820-fold. Effective concentrations were exhibited from high micromolar to low millimolar, thus highlighting their poor activity (Table 6.5).

Table 6.4 Evaluation of decitabine and ProTides in KG1a cells

Cpd	ClogP	Regio-isomer	Amino acid	Ester	Aryl	LD_{50} µM	Stem cell% Control: 3.4%
Decitabine	-2.03	-	-	-	-	0.085	5
6.8a	1.91	5’	L-Ala	OBn	ONaph	1.2	3.45
6.8b	0.73	5’	L-Ala	OBn	OPh	4.3	3.6
6.8c	3.77	5’	L-Leu	OPnt	ONaph	19	3.6
6.10a	3.77	3’	L-Leu	OPnt	ONaph	5.1	3.5

ClogP values generated algorithmically by computer-based predictive program Chem Office ultra 11.0

Table 6.5 Comparative evaluation of decitabine, 5-azacytidine and their ProTide derivatives in KG1a cell line

Cpd	LD_{50} µM	stem cell % Control: 3.3	Cpd	LD_{50} µM	stem cell % Control: 3.3
Dectiabine	0.085	5	**Azacytidine**	2.7	4
6.8a	1.2	3.5	**6.xa**	33	4.2
6.8b	4.3	3.6	**6.xb**	3500	4

6.4.2 Evaluation of decitabine ProTides in leukaemia cell lines

Five new leukaemia cell lines in addition to the KG-1 linaege were used: THP-1 (acute monocytic leukaemia); RL (non-Hodgkin lymphoma); NCI-H929 (Myeloma); MV4-11 (acute myelocytic leukaemia); K 562 (myelogenous leukaemia); HL-60 (promyelocytic leukaemia); HEL92.1.7 (erythroleukaemia).[27, 43-46]

Decitabine (**6.4**) showed poor ability to reach 50% cytotoxic effect on a high micromolar scale (LD_{50} = 10 - 46 μM). The efficacy of L-alanine benzyl naphthyl **6.8a** and phenyl **6.8b** derivatives on causing 50% killing effect was comparable across the whole panel of cell lines (LD_{50} = 7.26 - 28 μM).

Surprisingly, decitabine was less potent than **6.8a** and **6.8b** in six out of seven cell lines, and this could relate to solubility issues resulting from the precipitation of the parent nucleoside in the solvent media reported by Wuxi AppTec, China. Only in the case of the myelogenous leukaemia cell line K 562, where the nucleoside and its prodrug derivatives exhibited comparable potencies (LD_{50} = 28 μM).

Table 6.6 Evaluation of d5AzaC (**6.4**) and ProTides in leukaemia cell lines

Cpd	ClogP	THP-1	RL	NCI-H929	MV4-11	K 562	HL-60	Hel92.1.7
6.4	-2.03	68	10	46	55	28	17	30
6.8a	1.91	27	7.88	27	8.96	28	11	14
6.8b	0.73	27	7.26	26	6.06	22	9.11	11

6.5 Mechanistic studies

6.5.1 Carboxypeptidase Y ester hydrolysis study

An enzymatic assay using carboxypeptidase Y was performed in order to investigate the relative ease of hydrolysis of decitabine ProTides. The analysis was applied to the L-leucine pentyl naphthyl 5' phosphoramidate **6.8c.** Carboxypeptidase Y, Trizma buffer (pH= 7.6) was dissolved in acetone-*d6* and the enzymatic analysis followed by ^{31}P NMR with spectra acquired at regular intervals at 25^{o}C over 14 hours. (Figure 6.8). The spectra showed that the parent compound **6.8c** displayed as two diastoisomers with chemical shifts at δ 5.02 and 4.55 ppm was rapidly metabolised to intermediate **6.8c*iii*** at δ 7.00 ppm, with completion within 35 minutes. After seven minutes the spectrum showed the chiral phosphorus species **6.8c*ii*** as two signals appeared at δ 5.73 and 4.16, which therefore shown to be consistant with the proposed activation pathway and confirms that the initial activation step of compound **6.8** is sufficiently efficient. As far as it can be observed, both diastereoisomers appear to be metabolised at similar rates in this assay with a half-life of less than seven minutes.

As no other enzyme existed in this assay, it was surprising to observe further degradation of the phosphoramidate monoester. Following compound **6.8**'s complete conversion to the phosphoramidate monoester after 11 hours of monitoring an unknown species started to appear with similar ^{31}P chemical shift and after 24 hours the breakdown species presented as the main species in the solution (Section 6.5.2).

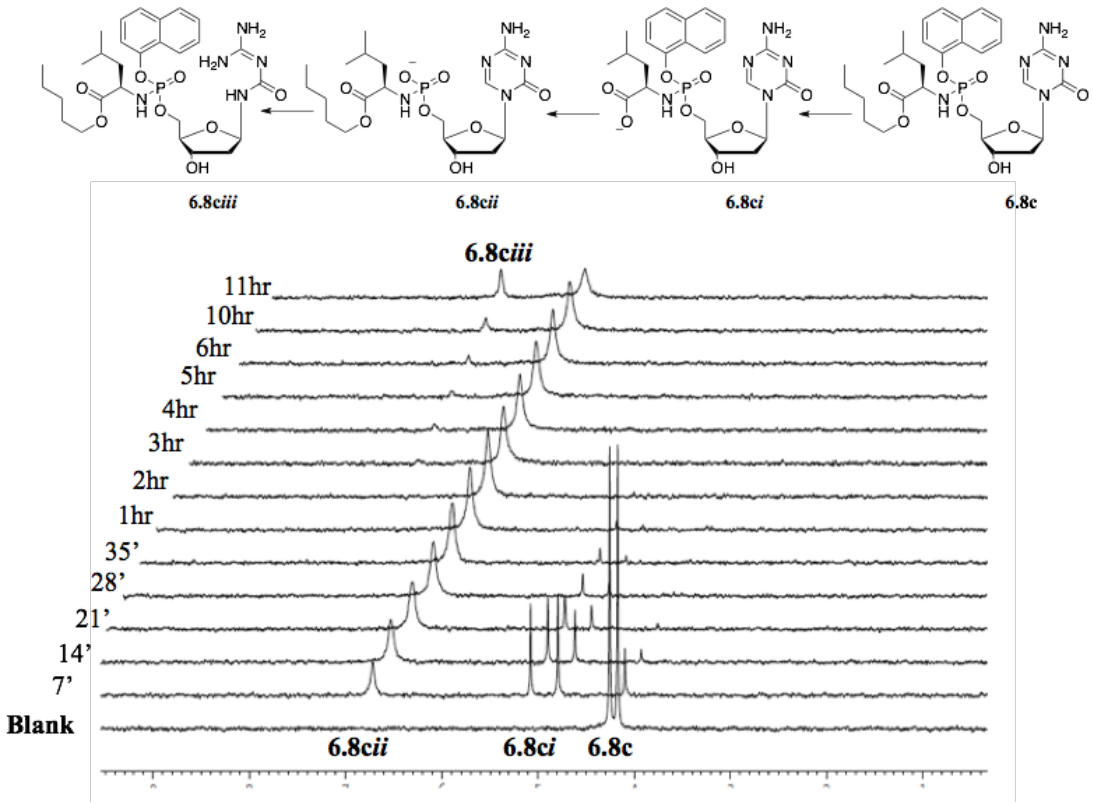


Figure 6.8 Carboxypeptidase Y mediated assay applied on **6.8c** monitored by ^{31}P NMR. The enzymatic mixture also contained the breakdown species **6.8c*iii***, confirmed by LC-MS.

6.5.2 Unmasking of the breakdown species

The breakdown compound of 5-azacytidine nucleoside level was first studied and confirmed by Thomsen. 5-azacytidine and 5-aza-2'deoxycytidine is known to be unstable in aqueous solution therefore it could be possible that the phosphoramidate monoester would further increase the instability of the nucleoside structure. The double bond between N5 and C6 in d5AzaC makes C6 very electrophilic and hence prone to attack by nucleophiles such as water. In neutral or basic aqueous solution, the main route of degradation is by ring-opening of the nucleobase to give the highlighted compound in Figure 6.9, while under acidic conditions the main breakdown route of decitabine is the glycosidic bond cleavage and deamination.

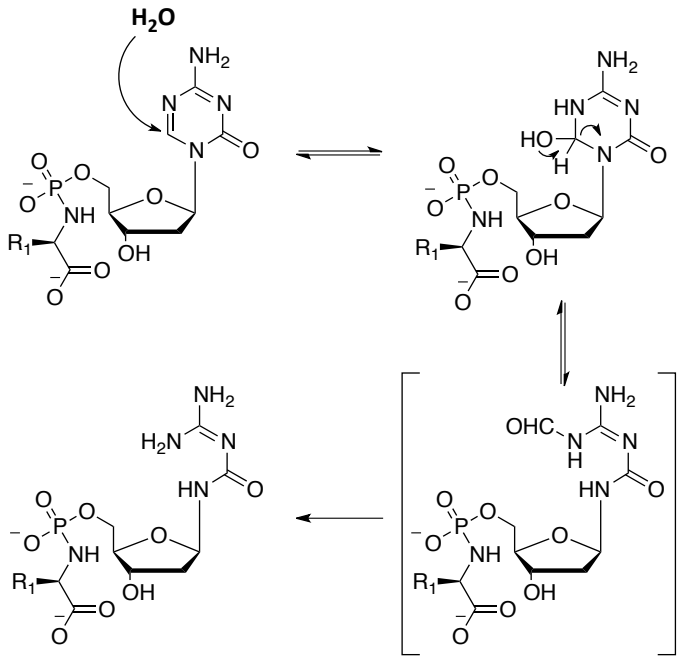


Figure 6.9 Proposed mechanism of ring opening of decitabine ProTides in neutral or basic conditions

Based on the ^{31}P chemical shift of the breakdown product it was assumed, that the forming compound could not be the phosphoramidate monoester, as the ^{31}P NMR peaks appear around 1 ppm, but instead, a change in the nucleoside structure could relate to the similar chemical shift of the phosphorus atom. To further study the stability of decitabine, ProTide **6.8a** was tested without Carboxypeptidase Y under the same conditions used in the assay, then monitored over time using ^{31}P NMR.

The L-alanine benzyl naphthyl derivative **6.8a** appeared with chemical shifts δ 4.63, and 4.24 ppm in the blank assay. A new compound was formed as a broad peak with a chemical shift of δ 6.43 ppm after 15 hours and the ratio between the parent compound **6.8a** and the breakdown derivative **6.8a*ii*** increased after 48 hours. The mixture of the enzymatic assay was analysed by LC-MS and confirmed the presence of the breakdown compound in the mixture (m/z = 586.20). Due to the fact, that only a small amount of ProTide **6.8a** was available, and as its conversion was also

partially done by preparative HPLC, separation of compounds **6.8a** and **6.8a*iii*** was not possible at this stage.

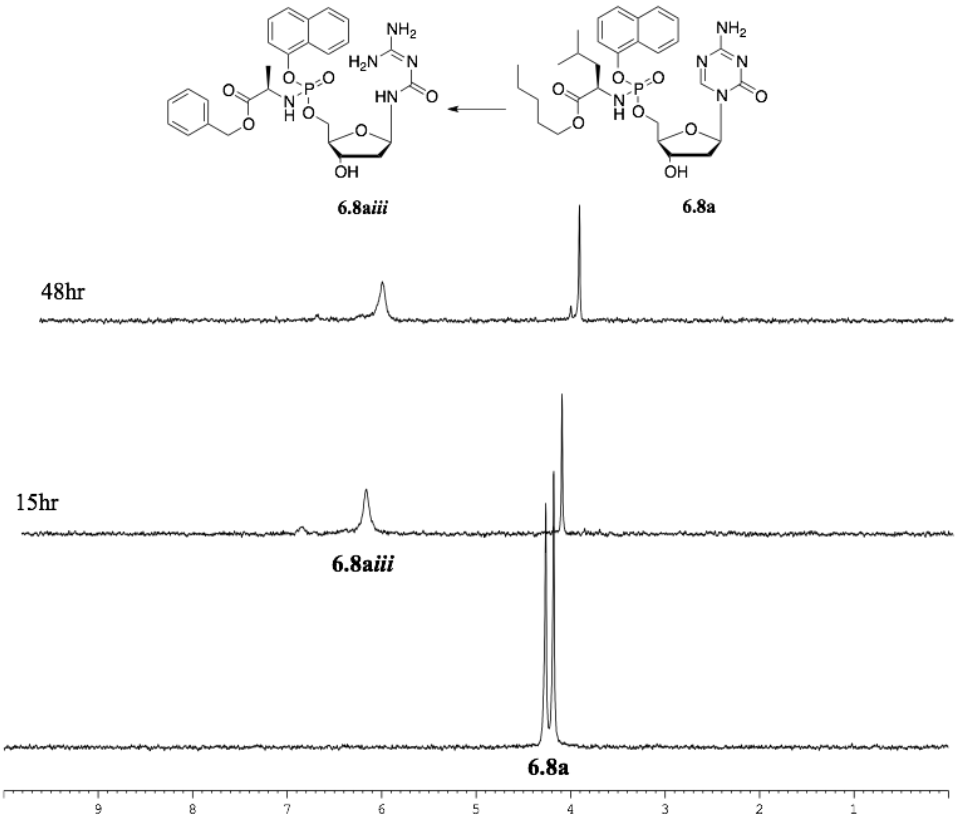


Figure 6.10 Enzymatic assay of **6.8a** in Trizma buffer (pH= 7.6) and acetone-*d6* followed by ^{31}P NMR at 25^{o}C over 48 hours.

6.6 Conclusion

A new series of decitabine ProTides was considered. The use of L-alanine as amino acid was probed in combination with the phenyl and naphthyl moieties as masking groups. A new series was designed to have higher lipophilicity therefore L-leucine pentyl naphthyl motif was included in this series both as 3' and 5' phophoramidate analogues, as during the synthesis of the nucleoside the Markiewicz protection could be selectively cleaved from the 3' and 5' hydroxyl groups of the sugar moiety therefore resulting in the isolation of decitabine ProTides of the 3' and 5' phosphates. All decitabine Protides performed better across the panel of leukaemia cell lines tested, then the nucleoside itself. However they exhibited IC_{50} values on a high micromolar to millimolar scale, which is suprising as only the L-alanine benzyl naphthyl derivative **6.8a** exhibited comparable cytotoxic potency to decitabine, while ProTide motifs **6.8b**, **6.8c, 6.10a** required 14-223 fold higher concentration then decitabine control to produce the same cytotoxic effect. Decitabine prodrugs did not show stem cell selectively towards the KG1-a stem cell compartment.

Interestingly the 3' L-leucine pentyl naphthyl derivative **6.10a** appeared to be 4-fold more potent than the 5'-derivative therefore the synthesis of new 3'-ProTide derivatives is highly considered as they might help optimizing the ProTide motif.
Unfortunately due to the lack of time remaining did not allow further studies on the isolation of the breakdown compound, which could include the resynthesis of **6.8a** on a larger scale, therefore the separation of the species could be performed by preparative HPLC.

6.7 References

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7 Main conclusion and perspectives

Each project was concluded in the relevant chapter. In the following, the main conclusions are summarised.

Design, synthesis and biological evaluation of 5-FUDR ProTides proved that the application of the ProTide technology to 5-FUDR could lead to the isolation of potent anticancer compounds, suitable for further preclinical development. Several compounds retained the high potency of 5-FUDR *in vitro*, and their action was partially independent of TK, the activating enzyme required to phosphorylate 5-FUDR. These compounds were shown to be substrates for carboxypeptidase Y and were stable in human plasma. Moreover most of the compounds retained their activities in mycoplasma infected cancer cell cultures. Based on these early results six lead compounds were selected for large scale synthesis and their activities were evaluated in a mouse *in vivo* model of colorectal cancer and tested in the KG1a stem cell model of cancer. All data from these experiments were considered when identifying the 5-fluoro-2'-deoxyuridine 1-naphthyl-L-alanine-*O*-benzyl derivative as the clinical candidate **NUC-3373**. **NUC-3373** is currently in the phase I clinical development.

A novel series of thiopurine aryloxyphosphoramidate and diamidate prodrugs were synthesised, however neither of these approaches produced analogues that were able to enhance the anticancer activity of the parent, but instead these compounds had markedly lower ability to inhibit leukaemia cell proliferation compared to their moderately potent parent nucleosides, 6-thioinosine and 6-thioguanosine. ProTide

derivatives were evaluated for their biological activities in blood borne cancer cell lines, and results from these experiments showed that they were less potent then their corresponding parent nucleosides, with concentrations ranging in the moderate to high micromolar range. Thiopurine phosphoramidates and symmetrical phosphorodiamidate analogues were both activated by carboxypeptidase Y. The replacement of the sulphur atom to thiomethoxy group in case of the 6-S-methyl thioinosine greatly increased its potency by 11-fold compared to 6-thioinosine. Furthermore 6-S-methyl thioinosine ProTides also displayed cytotoxic activities comparable to their corresponding nucleoside. Based on the promising preliminary biological data on the 6-S-methyl-thioinosine series, investigation exploring this particular family should be carried out alongside with the application of the ProTide technology to 2'deoxy-6-thioguanosine.

The fifth generation of Cladribine ProTides was evaluated in order to confirm the previously observed tendency of 3'-ProTide regioisomers to be more active than their 5'-regioisomer analogues. Biological data clearly showed, that the 3'-isomers were either more active in various leukaemia cell lines, than their 5'counterparts or retained their activity, while 5'-regioisomers were found to be slightly more stem cell selective in in the KG1a stem cell model. Further investigation of this family of compounds would be required in order to understand the assumed molecular rearrangements.

Additionally a novel family of 2'-deoxy-5-azacytidine (Decitabine) ProTides were synthesised including both the 3' and 5' phosphoramidate analogues. The performed

enzymatic and lysate studies clearly showed evidence for the great lack of activity due to the enhanced aqueous instability of the phosphoramidate monoester.

CHAPTER 8

EXPERIMENTAL PROCEDURES

8.1. General Experimental Details

Solvents and reagents

All solvents and reagents commercially available were used without any further purification.

Thin layer chromatography

The reactions were analysed on Thin Layer Chromatography (TLC) on commercially available Merck Kieselgel plates. The separated components were visualized using ultraviolet light (245 and 366 nm).

Column chromatography (CC)

Column chromatography was performed using Silica gel 35-70 μm, 60 A8 Fluka) as stationery phase. Glass columns were packed in the appropriate eluent system under gravity. Samples were applied as a concentrated solution in the same eluent or pre-absorbed onto silica gel. The fractions, which containined the product were analised by TLC, then combined together and the solvent removed under vacuum.

Nuclear magnetic resonance (NMR)

^{1}H, ^{13}C, ^{31}P were recorded on a Brucker Avance 500 spectrometer (500, 125, 202 MHz). ^{31}P-NMRs are reported in units of δ relative to 85% phosphoric acid as external standard. In ^{13}C-NMR, ppm shifts rounded to one decimal place.

The following abbreviations are used in the NMR signals assignment: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Coupling constants (*J)* are measured in Hertz.

High Performance Liquid Chromatography (HPLC)

Analytical and semi preparative experiments were ran on a Varian ProStar (LC Work Station – Varian Prostar 335LC detector, Varian fraction collector – model 701, Prostar 201 delivery system, using Varian Pursuit XRs 5C18 (150 × 4.6 mm) as an analytical column and Varian Pursuit XRs 5C18 (150 × 4.6 mm) as semi preparative column. Used software was Galaxie Chromatography Data System. Elution was performed using mobile phase water/acetonitrile in gradient

System 1 = (H_2O/ACN : 0% to 100% of ACN in 45 minutes).

System 2 = (H_2O/ACN : 0% to 100% of ACN in 30 minutes).

Mass Spectroscopy (MS)

Low resolution mass spectroscopy was performed on my compounds as a service by Cardiff University using electrospray.

Enzymatic assays

Carboxypeptidase Y assay

5mg of the appropriate phosphoramidate was dissolved in 150 µl of deuterated acetone and 300 µl of TRIZMA buffer (pH=7.6) was added thereto. ^{31}P-NMR was recorded as a reference. 0.1mg of Carboxypeptidase Y enzyme (purchased from Sigma, > 50 unit/mg EC 3.4.16.1) was dissolved in 150 µl of TRIZMA buffer and

added to the latter mixture. ^{31}P-NMR was conducted of the reaction mixture every 7 minutes for 14 hours at room temperature.

^{31}P NMR stability experiments in human serum

The stability has been studied towards hydrolysis by human serum using in situ ^{31}P NMR (202 MHz). Each experiment was carried out by dissolving the appropriate phosphoramidate (2.5 - 5mg) in DMSO (0.05 ml) and D_2O (0.15 ml). Thereafter the sample was inserted into the NMR chamber, which was warmed up to 37 °C and blank spectrum was recorded. Thereafter 0.3 ml human serum was added to the sample and NMR experiments were monitored every 15 min for 14 hours. Because of excess noise and poorshimming profiles individual spectras were deconvoluted (Lorentz-Gauss deconvolution) to improve visualization of the results. Data recorded were processed and analysed with BrukerTopspin 2.1 program. Human serum is commercially available and was purchased from Sigma-Aldrich.

Biological testing

Biological testing was carried out by Prof Jan Balzarini‘s group, Rega Institute Katholieke Universiteit; WuXi AppTec, China and by Prof Chris Pepper’s lab, School of Medicine, Cardiff University

8.2. Standard Procedures

Standard procedure 1 a : Preparation of amino acid ester hydrochloride salts

To a stirred solution of the appropriate alcohol (15.0 mol eq) at 0°C under nitrogen atmosphere, thionyl chloride (2.0 mol eq) was added. The reaction mixture was stirred at 0°C for 30 minutes and after that time slowly allowed to warm up to room temperature. The appropriate amino acid (1.0 mol eq) was added and the mixture was heated at 70 °C overnight. The solvents were removed under vacuum and the last traces of solvents were removed by co–evaporation. After precipitation from diethyl ether, product was obtained as the white solid of hydrochloride salt.

Standard procedure 1 b : Preparation of amino acid ester sulfonate salts

To the amino acid (1 mol eq) in toluene, was added the alcohol (5 to 15 mol eq) and para-toluene sulfonic acid (1.1 mol eq). The mixture was heated at reflux overnight using Dean Stark apparatus. After the solvent was removed under reduced pressure, the amino acid ester was precipitated either from diethyl ether or ethyl acetate forming the white solid of *p*- toluene sulfonate salt.

Standard procedure 1 c : Preparation of Boc amino acid esters

To the Boc-protected amino acid (1 mol eq) in DCM (20ml/g of amino acid) the alcohol (1.2 to 2 eq) DCC (1 mol eq) and DMAP (0.1 mol e.q.) were added at room temperature. After being stirred overnight, the solvent was removed under reduce

pressure and the residue was purified on silica gel (Hexane /AcOEt 9:1) to afford the pure Boc amino acid ester.

Standard procedure 1 d : Deprotection of Boc amino acid esters

To the Boc amino acid ester (1 mol eq) in AcOEt (35 ml/ g of Boc amino acid ester), pTSA (1 mol eq) was added. The mixture was stirred for 2 hours at 65°C and the solvent was removed under reduce pressure to afford the pure amino ester pTSA salt. The latter can crystallize in AcOEt at 0°C or from MeOH / Et_2O.

Standard procedure 2 : Synthesis of aryl phosphorodichloridate

Phophorus oxychloride (6.46 ml, 1.0 mol eq) was added to a stirred solution of the appropriate phenol or naphthol (10 g, 69.39 mmol, 1.0 mol eq) in dry ether (100 mL). Then the solution was stirred at -78°and anhydrous triethylamine (9.67 ml, 1.0 eq) was added dropwise. After 1 hr the reaction was left to rise to room temperature and stirred for another 2 hours, monitoring the formation of the desired compound by ^{31}P-NMR. The triethylamine hydrochloride salt was filtered off and the filtrate reduced to dryness to give a crude oil that was used without further purification for the next step.

Standard procedure 3: Synthesis of aryl phosphorochloridate

Anhydrous triethylamine (2.0 mol eq) was added dropwise at -78° to a stirred solution of the appropriate phosphorodichloridate (1.0 eq) and the appropriate amino acid salt (1.0 mol eq) in anhydrous dichloromethane (10 ml). After 1 hour the reaction was allowed to slowly warm to room temperature. The formation of the desired compound was monitored by ^{31}P-NMR. After 1 hour the solvent was removed under reduced pressure and the crude residue was purified by a short coloumn (hexane ethyl acetate/ hexane 7/3 in the case of tosylate salt. The HCl salt was dissolved in diethyl ether and removed by filtration. After being purified, the crude gave the product as an oil.

Standard procedure 4: Synthesis of phosphoramidates (*t*BuMgCl method)

Tert-butylmagnesium chloride (1M solution THF, 1.1 mol eq) was added to a stirring suspension of the appropriate nucleoside (1.0 mol eq) in dry THF (10 ml) under Argon atmosphere. The appropriate phosphorochloridate (1.2 mol eq) dissolved in dry THF (3-5 ml) was added dropwise and the reaction was left stirring overnight volatiles were evaporated under vacuum and the residue was purified by flash chromatography (CH_2Cl_2/CH_3OH) to give the desired product.

Standard procedure 5: Synthesis of aryl phosphoroamidates (NMI method)

N-Methylimidazole (5.0 mol eq) was added to a stirring suspension of the appropriate nucleoside (1.0 mol eq) in dry THF (10 ml), ander Argon atmosphere.

The appropriate phosphochloridate (3 mol eq) dissolved in THF (3-5 ml) was added dropwise and the reaction left stiring overnight. Volatiles were evaporated under vacuum the residue was dissolved in CH_2Cl_2 and washed with aqueous HCL 0.5M. the organic layer was dried over $MgSO_4$, filtered, reduced to dryness and purified by flash chromatography (CH_2Cl_2 /CH_3OH).

Standard procedure 6a: Synthesis of 2', 3'-isopropylidene protected 6-thioinosine ProTides

To a solution of 6-thioinosine (0.95g, 3.34 mmol) in anhydrous acetone (50 ml), at room temperature, a catalytic amount of perchloric acid (60 % in aqueous solution, 0.50 ml) was added dropwise under Argon atmosphere and stirred for 2 hrs. Then a saturated solution of NH_4OH was added drop by drop to achieve a neutral pH. The solvent was removed under reduced pressure and the product was sometimes used as a crude, or was purified by coloumn chromatography ($CHCl_3$/ MeOH 95:5) if needed to yield the protected nucleoside with 92% average yield.

Standard procedure 6b: Preparation of 2', 3'- isopropylidene protected 6-thioguanosine ProTides

To a solution of 6-thioguanosine (0.50 g, 1.67 mmol) in anhydrous acetone (50 ml), at room temperature, a catalytic amount of perchloric acid (60 % in aqueous solution, 0.50 ml) was added dropwise under Argon atmosphere and stirred for 2 hrs. Then a saturated solution of NH_4OH was added drop by drop to achieve a neutral pH. The solvent was removed under reduced pressure and the product was

sometimes used as a crude, or was purified by coloumn chromatography ($CHCl_3$/ MeOH 95:5) if needed to yield the protected nucleoside with 90 % average yield.

Standard procedure 7: Deprotection of 2', 3'-isopropylidene protected 6-thioinosine and 6-thioguanosine ProTides

The appropriate 2', 3'-isopropylidene protected phosphoramidates was dissolved in 60% acetic acid in water(~10ml/ ~ 100mg) and heated at 50-60°C for 24-36 hrs. The solvent was removed under reduced pressure and was co-evaporated with toluene. The residue was purified by column chromatography using an eluent system (DCM/MeOH 9:1) to give the desired compound.

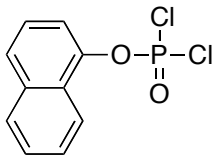
Standard procedure 8: Preparation of symmetrical phosphorodiamidates

The appropriate nucleoside (1.0 mol equivalent) was suspended in dry tetrahydrofuran, then triethylamine (1.0 mol equivalent) was added and was allowed to stirr for 30 minutes at ambient temperature. To this mixture phosphorus oxychloride was added dropwise at -78^{o}C and was allowed to stirr for 30 minutes, before it was slowly allowed to warm up to room temperature. Reaction progress was followed by ^{31}P NMR. After reaction completion, anhydrous dichloromethane was added, followed by amino acid ester (3-5 equivalent) and triethylamine (5-10 mol equivalent) at -78^{o}C. The reaction mixture was allowed to stirr at room temperature for 16-20 hours, then evaporated to dryness and the resulting residue

was purified by silica gel coloumn chromatography. Using as eluent a gradient of methanol in chloroform.

8.3. Experimental procedures – Chapter 2

Synthesis of 1-naphthyl phosphorodichloridate (2.1).

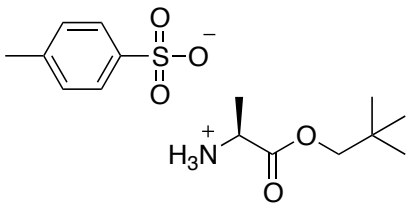


Prepared according to standard procedure 1, from α-naphthol (69.36 mmol, 10.00 g), phosphorus oxychloride (69.36 mmol, 6.46 ml) and triethylamine (69.36 mmol, 9.67 ml) in 50 ml anhydrous diethyl ether. The triethylamine hydrochloride salt was filtered off and the filtrate reduced to dryness to give a crude product as a thick yellow oil (16.2 mg, 90%).

^{31}P-NMR ($CDCl_3$, 202 MHz) δ 3.73

^{1}H-NMR (MeOD, 500 MHz) δ 8.12 (d, *J* = 8.0 Hz, 1H, H_8) 7.92 (d, *J* = 7.5 Hz, 1H, H_5), 7.83 (d, *J* = 8.0 Hz, 1H, H_4), 7.65 - 7.57 (m, 3H, H_2, H_6, H_7) 7.43 (t, *J* = 8.0 Hz, 1H, H_3).

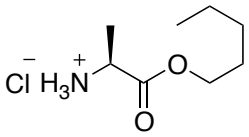
Synthesis of L-alanine 2,2 dimethylpropyl ester *p*-toluene sulfonate salt (2.2a).



Prepared according to standard procedure 2b, from L-alanine (5.00 g, 56.12 mmol), neopentyl alcohol (36.55 ml, 33.66 mol) and *p*TSA (11.74g, 61.7 mmol) in 130 ml of toluene. The product was obtained as a white powder. (15.43 g, 83%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.30 (m, 3H, NH_3^+), 7.50 (d, *J* = 8.0 Hz, 2H, H-Ar), 7.13 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.14 (q, *J* = 7.3 Hz, 1H, C*H*CH_3), 3.92, 3.82 (AB, J_{AB} = 10.5 Hz, 2H, OCH_2C$(CH_3)_2$), 2.28 (s, 3H, CH_3, Ts), 1.39 (d, *J* = 7.5 Hz, 3H, CHCH_3), 0.92 (s, 9H, $OCH_2C(CH_3)_3$).

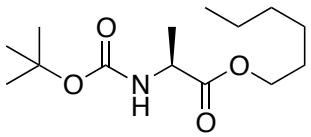
Synthesis of L-alanine pentyl ester hydrochloride salt (2.2b).



Prepared according to standard procedure 2a, from L-alanine (8.00 g, 89.78 mmol), pentanol (97 ml, 89.78 mol) and thionyl chloride (13.04 ml, 17.95 mmol). The product was obtained as white solid. (14.58 g, 83%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.20 (bs, 3H, NH_3^+), 7.77 (d, *J* = 8.0 Hz, 2H, H-Ar), 7.17 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.11 – 4.04 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 4.01 – 3.97 (m, 1H, $CHCH_3$), 2.37 (s, 3H, CH_3-Ts), 1.60 – 1.55 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.48 (d, *J* = 7.0 Hz, 3H, $CHCH_3$), 1.31 – 1.24 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.88 (t, *J* = 7.0 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_2CH_3$).

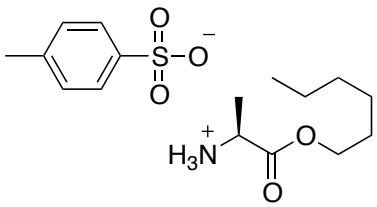
Synthesis of Boc-L-alanine hexyl ester (2.2c').



Prepared according to standard procedure 2c, from Boc-L-alanine (7 g, 36.99 mmol), hexanol (9.29 ml, 73.98 mmol) and DCC (7.63 g, 36.99 mmol), DMAP (0.45 g, 36.99 mmol) in 80 ml of DCM. The product was obtained as an oil. (8.80 g, 87%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.28 (s, 1H, NH), 4.16 – 4.10 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 3.47 – 3.46 (d, *J* = 5.5 Hz, 1H, $CHCH_3$), 1.65 – 1.60 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 1.44 (s, 9H, 3 x CH_3-Boc), 1.38 – 1.29 (m, 9H, $CHCH_3$, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 0.88 (t, *J* = 7.0 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_2CH_3$).

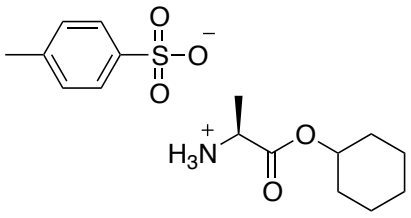
Synthesis of L-alanine hexyl ester *p*-toluene sulfonate salt (2.2c).



Prepared according to standard procedure 2d, from Boc-L-alanine hexyl ester (8.80 g, 32.19 mmol) and *p*TSA (6.12 g, 32.19 mmol) in 175 ml of ethyl alcohol. The product was obtained as white solid. (10.12 g, 91%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.18 (bs, 3H, NH_3^+), 7.77 (d, *J* = 8.5 Hz, 2H, H-Ar), 7.15 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.10 – 4.07 (m, 1H, C*H*CH_3), 4.03 - 3.98 (m, 2H, O*C*$H_2$$CH_2CH_2CH_2CH_2CH_3$), 2.36 (s, 3H, C$H_3$-Ts), 2.19 (s, 3H, CHC$H_3$), 1.56 - 1.53 (m, 2H, OCH_2C$H_2$$CH_2CH_2CH_2CH_3$), 1.45 (m, 2H, OCH_2CH_2C$H_2$$CH_2CH_2CH_3$), 1.30 – 1.23 (m, 4H, $OCH_2CH_2CH_2$CH_2C$H_2$$CH_3$), 0.88 (t, *J* = 7.0 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_2$CH_3).

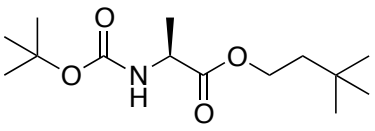
Synthesis of L-alanine cyclohexyl ester *p*-toluene sulfonate salt (2.2d).



Prepared according to standard procedure 2b, from L-alanine (5.00 g, 56.11 mmol), cyclohexanol (33.72 ml, 33.66 mol) and *p*TSA (11.74g, 61.72 mmol) in 50 ml of toluene. The product was obtained as a white powder. (18.30 g, 95%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.18 (bs, 3H, NH_3^+), 7.78 (d, *J* = 8.5 Hz, 2H, H-Ar), 7.15 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.76 – 4.72 (m, 1H, C*H*CH_3), 5.15 (m, 1H, OC*H*-ester), 2.36 (s, 3H, CH_3-Ts), 1.76 – 1.65 (m, 4H, 2 x CH_2-ester), 1.52– 1.49 (m, 1H, CH_2-ester), 1.46 (d, *J* = 7.5 Hz, 3H, CHCH_3), 1.52– 1.49 (m, 1H, CH_2-ester), 1.41– 1.21 (m, 5H, CH_2-ester).

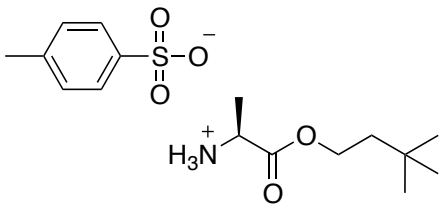
Synthesis of Boc-L-alanine 3,3 dimethyl-1-butyl ester (2.2e').



Prepared according to standard procedure 2c, from Boc-L-alanine (4 g, 21.14 mmol), 2,2 dimethyl-1-butanol (5.11 ml, 42.28 mmol) and DCC (4.36 g, 21.14 mmol), DMAP (0.26 g, 2.11 mmol) in 80 ml of DCM. The product was obtained as an oil. (5.65 g, 98%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.03 (bs, 1H, NH), 4.32 – 4.24 (m, 1H, C*H*CH_3), 4.21 – 4.15 (m, 2H, OC*H*$_2$$CH_2C(CH_3)_3$), 1.59 (t, *J* = 6.5 Hz, 2H, OCH_2CH_2C(CH$_3$)$_3$), 1.47 (s, 9H, 3 x CH_3 Boc), 1.36 (d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 0.95 (s, 9H, $OCH_2CH_2C(CH_3)_3$).

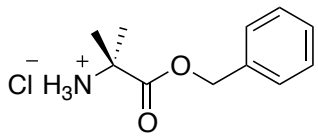
Synthesis of L-alanine 3,3 dimethyl-1-butyl ester *p*-toluene sulfonate salt (2.2e).



Prepared according to standard procedure 2d, from Boc-L-alanine 3,3 dimethyl-1-butyl ester (5.65 g, 20.66 mmol) and *p*TSA (3.93 g, 20.66 mmol) in 175 ml of AcOEt. The product was obtained as white solid. (5.28 g, 74%).

^{1}H-NMR ($CDCl_3$, 500 MHz) 8.21 (bs, 3H, NH_3^+), 7.79 (d, *J* = 8.0 Hz, 2H, H-Ar), 7.17 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.19 – 4.06 (m, 2H, OC*H*$_2$$CH_2C(CH_3)_3$), 4.00 – 3.96 (m, 1H, C*H*CH_3), 2.37 (s, 3H, C*H*$_3$-Ts), 1.51 – 1.46 (m, 5H, CHC*H*$_3$, OCH_2C*H*$_2$C(CH$_3$)$_3$), 0.89 (s, 9H, $OCH_2CH_2C(CH_3)_3$).

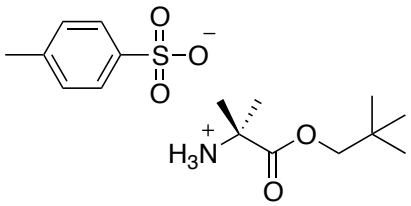
Synthesis of dimethylglycine benzyl ester hydrochloride salt (2.2f).



Prepared according to standard procedure 2a, from dimethylglycine (4.00 g, 38.78 mmol), benzyl alcohol (60.27 ml, 58.18 mol) and thionyl chloride (5.63 ml, 77.58 mmol). The product was obtained as white solid. (5.96 g, 67%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.32 (bs, 3H, NH_3^+), 7.76 (d, *J* = 8.0 Hz, 2H, H-Ar), 7.28 (s, 5H, H-Ar), 7.12 (d, *J* = 8.0 Hz, 2H, H-Ar), 5.15 (s, 2H, OC*H*$_2$Ph), 2.35 (s, 3H, C*H*$_3$-Ts), 1.57 (s, 6H, 2 x C*H*$_3$).

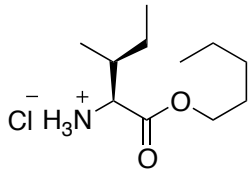
Synthesis of dimethylglycine 2,2 dimethylpropyl ester *p*-toluene sulfonate salt (2.2g).



Prepared according to standard procedure 2b, from dimethylglycine (4.00 g, 38.78 mmol), neopentyl alcohol (63.32 ml, 58.18 mol) and *p*TSA (8.11g, 42.66 mmol) in 50 ml of toluene. The product was obtained as a white powder. (6.22 g, 66%).

^{1}H-NMR ($CDCl_3$, 500 MHz) 8.34 (bs, 3H, NH_3^+), 7.76 (d, *J* = 7.5 Hz, 2H, H-Ar), 7.18 (d, *J* = 7.5 Hz, 2H, H-Ar), 3.83 (s, 2H, OC*H*$_2$C(CH_3)$_3$), 2.39 (s, 3H, C*H*$_3$-Ts), 1.59 (s, 6H, C(C*H*$_3$)$_2$), 0.92 (s, 9H, CH_2C(C*H*$_3$)$_3$).

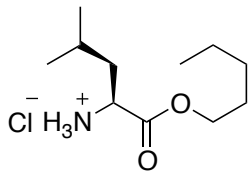
Synthesis of L-isoleucine pentyl ester hydrochloride salt (2.2h).



Prepared according to standard procedure 2a, from L-isoleucine (4.00 g, 3.49 mmol), pentanol (49.51 ml, 45.75 mol) and thionyl chloride (4.43 ml, 6.98 mmol). The product was obtained as white solid. (3.25 g, 53%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.74 (bs, 3H, NH_3^+), 4.17 – 4.08 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 3.85 (d, J = 8.0 Hz, $CHCH(CH_3)CH_2CH_3$), 2.01 – 1.98 (m, 1H, $CHCH(CH_3)CH_2CH_3$), 1.63 – 1.57 (m, 2H, $CHCH(CH_3)CH_2CH_3$), 1.49 – 1.42 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.34 – 1.26 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.91 – 0.86 (m, 9H, $CHCH(CH_3)CH_2CH_3$), $OCH_2CH_2CH_2CH_2CH_3$).

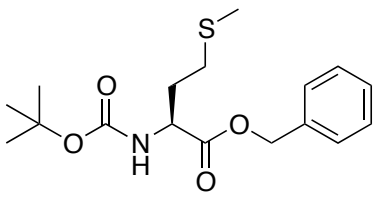
Synthesis of L-leucine pentyl ester hydrochloride salt (2.2i).



Prepared according to standard procedure 2a, from L-leucine (4.00 g, 30.49 mmol), pentanol (33.00 ml, 30.49 mol) and thionyl chloride (4.43 ml, 6.98 mmol). The product was obtained as white solid. (6.69 g, 94%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.76 (bs, 3H, NH_3^+), 4.22 – 4.21 (m, 1H, $CHCH_2CH(CH_3)_2$), 4.07 – 4.03 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 2.00 – 1.95 (m, 2H, $CHCH_2CH(CH_3)_2$), 1.75 – 1.73 (m, 3H, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.37 – 1.34 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.03 – 1.01 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 0.93 (s, 9H, $OCH_2CH_2CH_2CH_2CH_3$, $CHCH_2CH(CH_3)_2$).

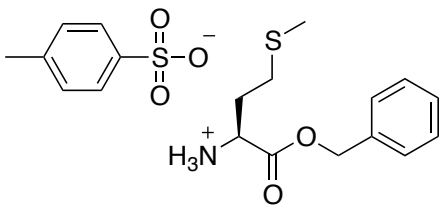
Synthesis of Boc-L-methionine benzyl ester (2.2j').



Prepared according to standard procedure 2c, from Boc-L-methionine (4.00 g, 16.04 mmol), benzyl alcohol (2.49 ml, 24.00 mmol) and DCC (3.30 g, 16.04 mmol), DMAP (0.19 g, 16.04 mmol) in 100 ml of DCM. The product was obtained as an oil. (3.49 g, 58%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.36 -7.34 (m, 5H, H-Ar), 5.18, 5.11 (AB, J_{AB} = 13.0 Hz, 2H, OC*H*$_2$Ph), 4.17 – 4.15 (m, 1H, C*H*$CH_2CH_2SCH_3$), 2.51 - 2.48 (m, 2H, $CHCH_2$C*H*$_2SCH_3$), 2.01 (s, 3H, $CHCH_2CH_2$SC*H*$_3$), 1.92 – 1.87 (m, 2H, CHC*H*$_2CH_2SCH_3$), 1.38 (s, 9H, 3 x C*H*$_3$-Boc).

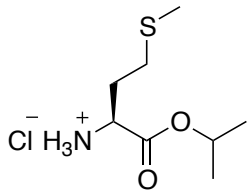
Synthesis of L-methionine benzyl ester *p*-toluene sulfonate salt (2.2j).



Prepared according to standard procedure 2d, from Boc-L-methionine methyl ester (3.49 g, 9.34 mmol) and *p*TSA (1.77 g, 9.34 mmol) in 200ml of AcOEt. The product was obtained as white solid. (1.69 g, 76%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.33 (m, 3H, NH_3^+), 7.49 (d, *J* = 7.5 Hz, 2H, H-Ar), 7.43 – 7.38 (m, 5H, H-Ar), 7.12 (d, *J* = 7.5 Hz, 2H, H-Ar), 5.26 (AB, J_{AB} = 13.5 Hz, 2H, OC*H*$_2$Ph), 4.21 – 4.20 (t, *J* = 6.0 Hz, 1H, C*H*$CH_2CH_2SCH_3$), 2.62 - 2.36 (m, 1H, $CHCH_2$C*H*$_2SCH_3$), 2.52 - 2.49 (m, 1H, $CHCH_2$C*H*$_2SCH_3$), 2.29 (s, 3H, C*H*$_3$-Ts), 2.08 – 2.04 (m, 2H, CHC*H*$_2CH_2SCH_3$), 2.01 (s, 3H, $CHCH_2CH_2$SC*H*$_3$).

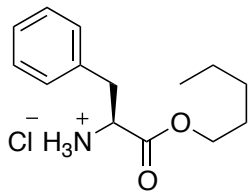
Synthesis of L-methionine isopropyl ester hydrochloride salt (2.2k).



Prepared according to standard procedure 2a, from L-methionine (4.00 g, 26.80 mmol), isopropanol (30.74 ml, 40.20 mol) and thionyl chloride (4.66 ml, 53.60 mmol). The product was obtained as white solid. (4.94g, 81%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.96 (bs, 3H, NH_3^+), 5.18 – 5.13 (m, 1H, OC*H*$(CH_3)_2$), 4.24 - 4.22 (m, 1H, C*H*CH_3), 2.86 – 2.74 (2 x m, 2H, CHC*H*$_2$$CH_2SCH_3$), 2.39 - 2.36 (m, 2H, $CHCH_2$C*H*$_2$$SCH_3$), 1.34, 1.33 (dd, *J* = 3.0 Hz, 6H, $OCH(CH_3)_2$), 0.95 (s, 3H, $CHCH_2CH_2SCH_3$).

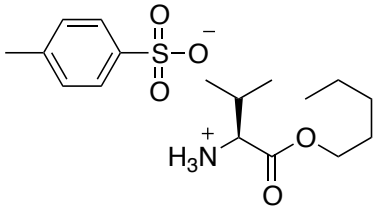
Synthesis of L-phenylalanine pentyl ester hydrochloride salt (2.2l).



Prepared according to standard procedure 2a, from L-phenylalanine (4.00 g, 24.21 mmol), pentanol (38.96 ml, 36.32 mol) and thionyl chloride (3.48 ml, 48.42 mmol). The product was obtained as white solid. (4.74 g, 85 %).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.79 (bs, 3H, NH_3^+), 7.34 – 7.31 (m, 2H, H-Ar), 7.27-7.25 (m, 3H, H-Ar), 4.21 – 4.19 (m, 1H, C*H*CH_3), 4.01 (t, *J* = 6.5 Hz, 2H, OC*H*$_2$$CH_2CH_2CH_2CH_3$), 3.27, 3.25 (AB, J_{AB} = 8.0 Hz, 1H, CHC*H*$_2$Ph), 3.08 – 3.03 (m, 1H, CHC*H*$_2$Ph), 1.45 – 1.40 (m, 2H, OCH_2C*H*$_2$$CH_2CH_2CH_3$), 1.24 – 1.20 (m, 2H, OCH_2CH_2C*H*$_2$$CH_2CH_3$), 1.15 – 1.10 (m, 2H, $OCH_2CH_2CH_2$C*H*$_2$$CH_3$), 0.83 (t, *J* = 7.5 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_3$).

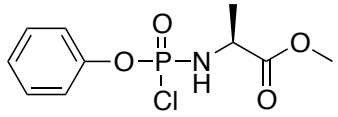
Synthesis of L-valine pentyl ester *p*-toluene sulfonate salt (2.2m).



Prepared according to standard procedure 2b, from L valine (3.00 g, 25.60 mmol), pentanol (27.71 ml, 58.18 mmol) and *p*TSA (5.35 g, 28.16 mol) in 50 ml of toluene. The product was obtained as a white powder. (6.35 g, 69%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.24 (bs, 3H, NH_3^+), 7.49 (d, *J* = 8.0 Hz, 2H, H-Ar), 7.11 (d, *J* = 8.0 Hz, 2H, H-Ar), 4.22 – 4.13 (m, 2H, OC*H*$_2$$CH_2CH_2CH_2CH_3$), 3.92 (d, *J* = 5.5 Hz, 1H, C*H*$CH(CH_3)_2$), 2.29 (s, 3H, C*H*$_3$-Ts), 2.17 - 2.13 (m, 1H, $CHCH(CH_3)_2$), 1.62 – 1.61 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.34 – 1.31 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.98, 0.96 (dd, *J* = 5.5 Hz, 6H, $CHCH(CH_3)_2$), 0.88 (t, *J* = 7.0 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_3$).

Synthesis of phenyl-(methoxy-L-alaninyl) phosphorochloridate (2.3a).

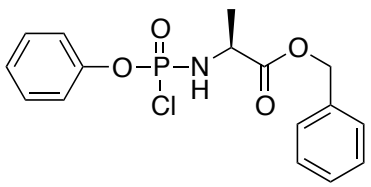


Prepared according to standard procedure 3, from L-alanine methyl ester hydrochloride salt (1.50 g, 10.75 mmol), phenyl phosphorodichloridate (2.27 g, 10.75 mmol) and Et_3N (2.99 ml, 21.50 mmol) in 15 ml of anhydrous DCM. The final product isolated as a thick, yellowish oil. (1.58 g, 55%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 7.88, 7.54

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.64 (bs, 1H, NH), 7.41 – 7.20 (m, 5H, H-Ar), 3.83, 3.81 (2 x s, 3H, OC*H*$_3$), 3.16 – 3.10 (m, 1H, C*H*CH_3), 1.67, 1.54 (2 x d, *J* = 7.5 Hz, 3H, CHC*H*$_3$).

Synthesis of phenyl-(benzoxy-L-alaninyl) phosphorochloridate (2.3b).

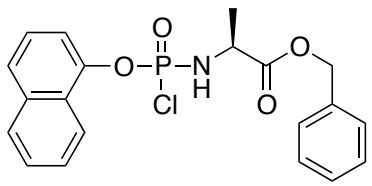


Prepared according to standard procedure 3, from L-alanine benzyl ester tosylate salt (2.50 g, 7.11 mmol), phenyl phosphorodichloridate (1.5 g, 7.11 mmol) and Et_3N (1.98 ml, 14.22 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.82 g, 73%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 7.85, 7.51

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 – 7.36 (m, 6H, H-Ar), 7.26 – 7.24 (m, 4H, H-Ar), 5.24, 5.23 (2 x s, OC*H*$_2$Ph), 4.26 – 4.22 (m, 1H, C*H*CH_3), 1.80 (bs, 1H, NH), 1.55 (2 x d, *J* = 7.0 Hz, 3H, C*H*CH_3).

Synthesis of α-naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (2.3c).

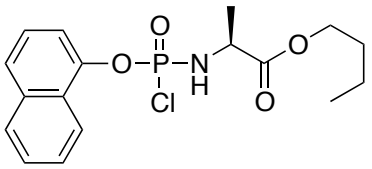


Prepared according to standard procedure 3, from L-alanine methyl ester hydrochloride salt (2.00 g, 5.7 mmol), α-naphthyl phosphorodichloridate (1.48 g, 5.7 mmol) and Et_3N (1.58 ml, 11.38 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.50 g, 65%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.13, 7.85

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.86 – 7.83 (m, 1H, H-Ar), 7.72 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.60 – 7.50 (m, 3H, H-Ar), 7.42 - 7.31 (m, 6H, H-Ar), 5.22 – 5.12 (m, 2H, OC*H*$_2$Ph), 4.39 (bs, 1H, NH), 4.37 – 4.32 (m, 1H, C*H*CH_3), 1.56 – 1.53 (m, 3H, CHC*H*$_3$).

Synthesis of α-naphthyl-(butoxy-L-alaninyl) phosphorochloridate (2.3d).

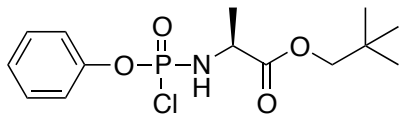


Prepared according to standard procedure 3, from L-alanine buthyl ester hydrochloride salt (3.00 g, 16.51 mmol), α-naphthyl phosphorodichloridate (4.30 g, 16.51 mmol) and Et_3N (4.6 ml, 33.02 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (5.31 g, 87%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.41, 8.24

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13, 8.11 (t, *J* = 8.0 Hz, 1H, H-Ar), 7.86 (d, *J* = 6.0 Hz, 1H, H-Ar), 7.71 (d, *J* = 7.5 Hz, 1H, H-Ar), 7.62 (d, *J* = 6.0 Hz, 1H, H-Ar), 7.59 – 7.48 (m, 2H, H-Ar), 7.42 (t, *J* = 7.0 Hz, 1H, H-Ar), 5.00, 4.30 (2 x bs, 1H, NH), 4.20, 4.14 (2 x q, *J* = 7.5 Hz, 2H, OC*H*$_2$CH$_2$CH$_2$CH$_3$), 3.49 – 3.48 (m, 1H, C*H*CH$_3$), 1.65 – 1.61 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_3$), 1.40 – 1.36 (m, 2H, OCH$_2$CH$_2$C*H*$_2$CH$_3$), 1.23, 1.21 (2 x d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 0.94, 0.90 (2 x t, *J* = 7.5 Hz, 3H, OCH$_2$CH$_2$CH$_2$C*H*$_3$).

Synthesis of phenyl-(2,2-dimethylpropoxy -L-alaninyl) phosphorochloridate (2.3e).

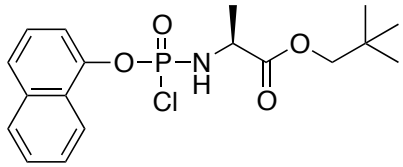


Prepared according to standard procedure 3, from L-alanine 2,2-dimethylpropyl ester tosylate salt (1.00 g, 3.01 mmol), phenyl phosphorodichloridate (0.63 g, 3.01 mmol) and Et_3N (0.84 ml, 6.03 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (0.65 g, 65%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.17, 7.75

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13, 8.11 (dt, *J* = 8.0 Hz, 2H, H-Ar), 7.32 – 7.24 (m, 3H, H-Ar), 4.28, 4.20, 4.18, 4.13 (2 x AB, J_{AB} = 11.0 Hz, 2H, OC*H*$_2$C(CH$_3$)$_3$), 3.15 – 3.09 (m, 1H, C*H*CH$_3$), 1.52, 1.51 (2 x s, 9H, OCH$_2$C(C*H*$_3$)$_3$), 1.49,1.47 (2 x d, *J* = 6.5 Hz, CHC*H*$_3$).

Synthesis of α-naphthyl-(2,2-dimethylpropoxy -L-alaninyl) phosphorochloridate (2.3f).

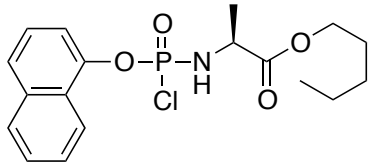


Prepared according to standard procedure 3, from L-alanine 2,2-dimethylpropyl ester tosylate salt (1.00 g, 3.01 mmol), α-naphthyl phosphorodichloridate (0.79 g, 3.01 mmol) and Et_3N (0.84 ml, 6.03 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (0.67 g, 58%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.23, 7.93

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10 (d, J = 7.5 Hz, 1H, H-Ar), 7.91 – 7.89 (m, 1H, H-Ar), 7.76 (d, J = 8.0 Hz, 1H, H-Ar), 7.64 – 7.55 (m, 3H, H-Ar), 7.46 - 7.45 (m, 1H, H-Ar), 4.38 – 4.32 (m, 1H, C*H*CH_3), 3.97, 3.94, 3.91, 3.86 (2 x AB, J_{AB} = 10.5 Hz, 2H, OC*H*$_2$C$(CH_3)_3$), 1.61, 1.59 (2 x d, J = 7.0 Hz, 3H, CHC*H*$_3$), 1.00, 0.98 (2 x s, 9H, $OCH_2C(CH_3)_3$).

Synthesis of α-naphthyl-(pentoxy-L-alaninyl) phosphorochloridate (2.3g).

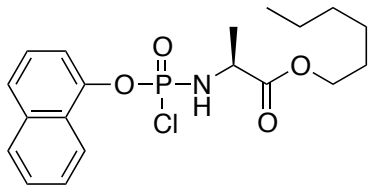


Prepared according to standard procedure 3, from L-alanine pentyl ester tosylate salt (4.00g, 12.00 mmol), α-naphthyl phosphorodichloridate (3.15g, 12.00 mmol) and Et_3N (3.36 ml, 24.00 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (4.41 g, 92%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.21, 7.90

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (d, J = 8.0 Hz, 1H, H-Ar), 7.91 (m, 1H, H-Ar), 7.76 (d, J = 8.0 Hz, 1H, H-Ar), 7.62 – 7.41 (m, 4H, H-Ar), 4.36 – 4.30 (m, 1H, C*H*CH_3), 4.23 – 4.17 (m, 2H, OC*H*$_2$$CH_2CH_2CH_2CH_3$), 1.65 – 1.61 (m, 2H, OCH_2C*H*$_2$$CH_2CH_2CH_3$), 1.42 – 1.29 (m, 7H, CHC*H*$_3$, OCH_2CH_2C*H*$_2$C*H*$_2$$CH_3$), 0.93 (s, 3H, CHC*H*$_3$).

Synthesis of α-naphthyl-(hexoxy-L-alaninyl) phosphorochloridate (2.3h).

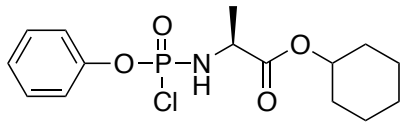


Prepared according to standard procedure 3, from L-alanine hexyl ester tosylate salt (4.00g, 11.58 mmol), α-naphthyl phosphorodichloridate (3.02 g, 11.58 mmol) and Et_3N (3.22 ml, 23.16 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (3.12g, 68%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.21, 7.88

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 – 8.08 (m, 1H, H-Ar), 7.91 – 7.84 (m, 1H, H-Ar), 7.63 – 7.39 (m, 5H, H-Ar), 4.22 – 4.02 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 3.60 – 3.56 (m, 1H, $CHCH_3$), 1.60 – 1.56 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 1.44 – 1.29 (m, 9H, $OCH_2CH_2CH_2CH_2CH_2CH_3$, $CHCH_3$), 0.90 – 0.88 (m, 3H, $OCH_2CH_2CH_2CH_2CH_2CH_3$).

Synthesis of phenyl-(cyclohexoxy-L-alaninyl) phosphorochloridate (2.3i).

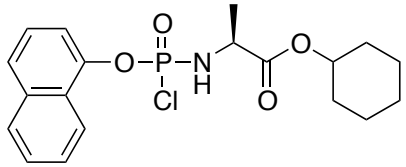


Prepared according to standard procedure 3, from L-alanine cyclohexyl ester tosylate salt (3.48 g, 10.13 mmol), phenyl phosphorodichloridate (2.14 g, 10.13 mmol) and Et_3N (2.82 ml, 20.26 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (3.22 g, 92%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 7.77, 8.13

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 – 7.35 (m, 2H, H-Ar), 7.34 - 7.21 (m, 3H, H-Ar), 4.46 – 4.27 (m, 1H, C*H*-ester), 4.24 – 4.10 (m, 1H, $CHCH_3$), 1.93-1.69 (m, 4H, 2 x CH_2-ester), 1.61, 1.57 (2 x d, J = 7.5 Hz, 3H, $CHCH_3$), 1.43 – 1.28 (m, 12H, 6 x CH_2-ester).

Synthesis of α-naphthyl-(cyclohexoxy-L-alaninyl) phosphorochloridate (2.3j).

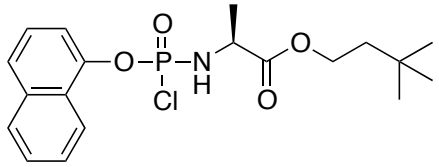


Prepared according to standard procedure 3, from L-alanine cyclohexyl ester tosylate salt (2.00 g, 5.8 mmol), α-naphthyl phosphorodichloridate (1.50 g, 5.8 mmol) and Et_3N (1.62 ml, 11.64 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.04 g, 89%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.30, 7.94

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12, 8.10 (2 x d, *J* = 6.5 Hz, 1H, H-Ar), 7.92 – 7.89 (m, 1H, H-Ar), 7.75 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.65 – 7.55 (m, 3H, H-Ar), 7.46, 7.45 (m, 1H, H-Ar), 4.92 – 4.84 (m, 1H, C*H*-ester), 4.34 - 4.22 (m, 2H, NH, C*H*CH_3), 1.92 - 1.84 (m, 2H, CH_2-ester), 1.80 - 1.72 (m, 2H, CH_2-ester), 1.57, 1.55 (2 x d, *J* = 7.0 Hz, 3H, CHCH_3), 1.51 – 1.33 (m, 12H, 6 x CH_2-ester).

Synthesis of α-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl) phosphorochloridate (2.3k).



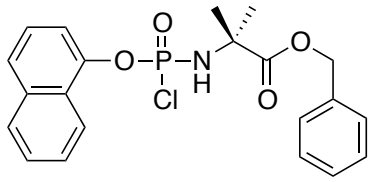
Prepared according to standard procedure 3, from L-alanine 3,3-dimethyl-1-butyl ester tosylate salt (4.35 g, 12.6 mmol), α-naphthyl phosphorodichloridate (3.28 g, 12.6 mmol) and Et_3N (3.51 ml, 25.2 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (3.8 g, 87%).

^{31}P NMR (202 MHz, $CDCl_3$): δ 8.21, 7.86

^{1}H NMR (500 MHz, $CDCl_3$): 8.11, 8.09 (2 x d, *J* = 7.5 Hz, 1H, H-Ar), 7.90 - 7.89 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.76 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.63 - 7.57 (m, 3H, H-Ar), 7.45 (t, *J* = 7.5 Hz, 1H, H-Ar), 4.44 - 4.40 (m, 1H, NH), 4.32 - 4.27 (m, 2H, OC$H_2$$CH_2C(CH_3)_3$), 4.24 - 4.21 (m, 1H, C*H*CH_3), 1.64 - 1.61 (m, 2H,

$OCH_2CH_2C(CH_3)_3$), 1.58, 1.55 (2 x d, *J* = 7.0 Hz, 3H, $CHCH_3$), 0.98, 0.96 (2 x s, 9H, $OCH_2CH_2C(CH_3)_3$).

Synthesis of α-naphthyl-(benzoxy-dimethylglicynyl) phosphorochloridate (2.3l).

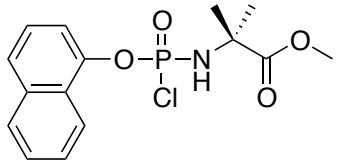


Prepared according to standard procedure 3, from L-alanine dimethylglycine benzyl ester tosylate salt (2.99 g, 8.18 mmol), α-naphthyl phosphorodichloridate (2.13 g, 8.18 mmol) and Et_3N (2.28 ml, 16.36 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.90 g, 85%).

^{31}P NMR (202 MHz, $CDCl_3$): δ 5.86

^{1}H NMR (500 MHz, $CDCl_3$): 8.13 (d, *J* = 7.0 Hz, 1H, H-Ar), 7.89 (d, *J* = 7.0 Hz, 1H, H-Ar), 7.74 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.58 - 7.55 (m, 3H, H-Ar), 7.45 (t, *J* = 8.0 Hz, 1H, H-Ar), 7.39 - 7.36 (m, 5H, OCH_2Ph), 5.25 (s, 2H, OCH_2Ph), 4.89 (bs, 1H, NH), 1.79 (s, 3H, CH_3-Me_2Gly), 1.76 (s, 3H, CH_3-Me_2Gly).

Synthesis of α-naphthyl-(methoxy-dimethylglicynyl) phosphorochloridate (2.3m).

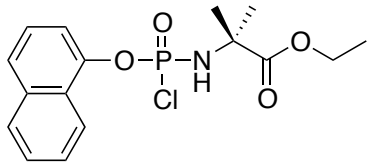


Prepared according to standard procedure 3, from L-alanine dimethylglycine methyl ester tosylate salt (1.50 g, 9.76 mmol), α-naphthyl phosphorodichloridate (2.55 g, 9.76 mmol) and Et_3N (2.72 ml, 19.53 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (3.13 g, 94%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 5.84

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13 (d, *J* = 8.0 Hz, 1H, Ar), 7.88 (d, *J* = 7.5 Hz, 1H, Ar), 7.74 (d, *J* = 8.5 Hz, 1H, Ar), 7.62 – 7.54 (m, 3H, H-Ar), 7.45 (t, *J* = 8.0 Hz, 1H, H-Ar), 3.83 (s, 3H, CH_3-ester), 1.78, 1.73 (2 x s, 6H, CH_3-Me_2Gly).

Synthesis of α-naphthyl-(ethoxy-dimethylglicynyl) phosphorochloridate (2.3n).

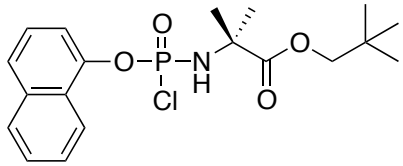


Prepared according to standard procedure 3, from L-alanine dimethylglycine ethyl ester tosylate salt (1.50 g, 8.95 mmol), α-naphthyl phosphorodichloridate (2.33 g, 8.95 mmol) and Et_3N (2.49 ml, 17.89 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.32 g, 73%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 5.90

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 (bs, 1H, NH), 8.13 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.88 (d, *J* = 7.5 Hz, 1H, H-Ar), 7.74 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.62 – 7.54 (m, 3H, H-Ar), 7.45 (t, *J* = 8.0 Hz, 1H, H-Ar), 4.28 (q, 2H, OCH_2CH_3), 1.77 (s, 3H, CH_3-Me_2Gly), 1.73 (s, 3H, CH_3-Me_2Gly), 1.33 (t, *J* = 7.5 Hz, 3H, OCH_2CH_3).

Synthesis of α-naphthyl-(2,2-dimethylpropoxy-dimethylglicynyl) phosphorochloridate (2.3o).

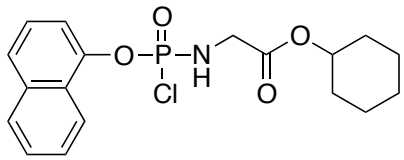


Prepared according to standard procedure 3, from L-alanine dimethylglycine ethyl ester tosylate salt (1.50 g, 6.16 mmol), α-naphthyl phosphorodichloridate (1.60 g, 6.16 mmol) and Et_3N (1.72 ml, 12.33 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.41 g, 58%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 5.49

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 - 7.23 (m, 7H, H-Ar), 4.01 - 3.98 (m, 2H, $OCH_2C(CH_3)_3$), 1.76 (s, 3H, CH_3-Me_2Gly), 1.72 (s, 3H, CH_3-Me_2Gly), 0.99 (s, 9H, $OCH_2C(CH_3)_3$).

Synthesis of α-naphthyl-(cyclohexoxy-glicynyl) phosphorochloridate (2.3p).

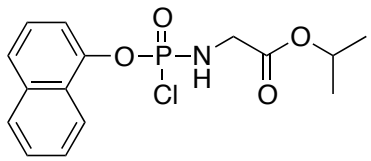


Prepared according to standard procedure 3, from glycine cyclohexyl ethyl ester hydrochloride salt (1.50 g, 7.99 mmol), α-naphthyl phosphorodichloridate (2.08 g, 7.99 mmol) and Et_3N (2.23 ml, 16.00 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.79 g, 59%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.99

^{1}H NMR (500 MHz, CDCl3) δ 8.10 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.90 (d, *J* = 7.0 Hz, 1H, H-Ar), 7.76 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.63 – 7.57 (m, 3H, H-Ar), 7.46 (t, *J* = 8.0 Hz, 1H, H-Ar), 4.94 - 4.89 (m, 1H, C*H*-ester), 4.31 (bs, 1H, NH), 4.02 – 3.98, 3.86 – 3.83 (2 x m, 2H, CH_2-Gly), 1.94 - 1.89 (m, 2H, CH_2-ester), 1.80 - 1.73 (m, 2H, CH_2-ester), 1.50 – 1.27 (m, 12H, CH_2-ester).

Synthesis of α-naphthyl-(isopropoxy-glicynyl) phosphorochloridate (2.3q).

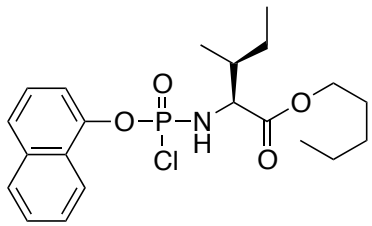


Prepared according to standard procedure 3, from glycine isopropyl ester hydrochloride salt (1.20 g, 7.81 mmol), α-naphthyl phosphorodichloridate (2.04 g, 7.81 mmol) and Et_3N (2.18 ml, 15.62 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.43 g, 54%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 9.04

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.90 (d, *J* = 7.0 Hz, 1H, H-Ar), 7.77 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.63 – 7.55 (m, 3H, H-Ar), 7.46 (t, *J* = 8.0 Hz, 1H, H-Ar), 5.18 – 5.13 (m, 1H, OC*H*(CH_3)$_2$), 4.37 (bs, 1H, NH), 4.03 – 3.97, 3.84 – 3.81 (2 x m, 2H, CH_2-Gly), 1.31 (d, *J* = 6.0 Hz, 6H, OCH(CH_3)$_2$).

Synthesis of α-naphthyl-(pentoxy-L-isoleucinyl) phosphorochloridate (2.3r).

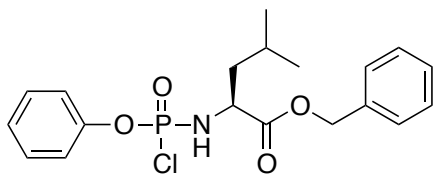


Prepared according to standard procedure 3, from L-alanine pentyl ester hydrochloride salt (2.00 g, 8.41 mmol), α-naphthyl phosphorodichloridate (2.19 g, 8.41 mmol) and Et_3N (2.34 ml, 16.82 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.72 g, 76%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 9.46, 9.05

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.91 (bs, 1H, NH), 8.07 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.85 (d, *J* = 7.0 Hz, 1H, H-Ar), 7.71 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.59 – 7.48 (m, 3H, H-Ar), 7.41 (t, *J* = 8.0 Hz, 1H, H-Ar), 4.17 – 4.04 (m, 2H, OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 4.03 – 3.88 (m, 1H, C*H*CH(CH$_3$)CH$_2$CH$_3$), 1.92 – 1.87 (m, 1H, CHC*H*(CH$_3$)CH$_2$CH$_3$), 1.67 – 1.59 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.56 -1.45 (m, 2H, CHCH(CH$_3$)C*H*$_2$CH$_3$), 1.32 - 1.21 (m, 4H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$), 0.96 (d, *J* = 6.5 Hz, 3H, CHCH(C*H*$_3$)CH$_2$CH$_3$), 0.91 (t, *J* = 7.5 Hz, 3H, CHCH(CH$_3$)CH$_2$C*H*$_3$), 0.88 – 0.82 (m, 3H, OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$).

Synthesis of phenyl-(benzoxy-L-leucinyl) phosphorochloridate (2.3s).



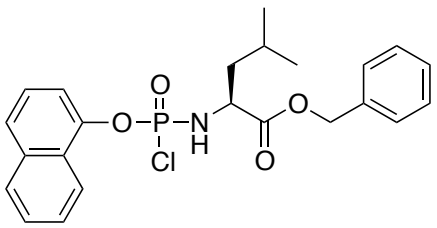
Prepared according to standard procedure 3, from L-leucine benzyl ester tosylate salt (3.00 g, 7.62 mmol), phenyl phosphorodichloridate (1.60 g, 7.62 mmol) and Et_3N (2.12 ml, 15.24 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.50 g, 83%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.29, 8.06

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.13 (2 x m, 10H, H-Ar), 5.26 – 5.17 (m, 2H, OC*H*$_2$Ph), 4.23 – 4.12 (m, 2H, NH, C*H*CH$_2$CH(CH$_3$)$_2$), 1.87 - 1.77 (2 x m, 1H, CHCH$_2$C*H*(CH$_3$)$_2$), 1.68 – 1.60 (m, 2H, CHC*H*$_2$CH(CH$_3$)$_2$), 0.97 – 0.94 (m, 6H,

$CHCH_2CH(CH_3)_2$).

Synthesis of α-naphthyl-(benzoxy-L-leucinyl) phosphorochloridate (2.3t).

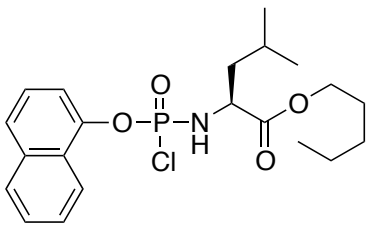


Prepared according to standard procedure 3, from L-leucine benzyl ester tosylate salt (3.00 g, 7.62 mmol), α-naphthyl phosphorodichloridate (1.99 g, 7.62 mmol) and Et_3N (2.12 ml, 15.24 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.44 g, 72%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.76, 8.40

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10 – 7.27 (m, 12H, H-Ar), 5.27 - 5.17 (m, 2H, CH_2Ph), 4.30 - 4.18 (m, 2H, $CHCH_2CH(CH_3)_2$), 1.86 - 1.80 (2 × m, 1H, $CHCH_2CH(CH_3)_2$), 1.87 – 1.60 (m, 3H, $CHCH_2CH(CH_3)_2$, $CHCH_2CH(CH_3)_2$), 0.99 - 0.94 (m, 6H, $CHCH_2CH(CH_3)_2$).

Synthesis of α-naphthyl-(pentoxy-L-leucinyl) phosphorochloridate (2.3u).



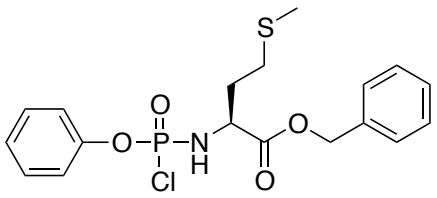
Prepared according to standard procedure 3, from L-leucine pentyl ester hydrochloride salt (4.00 g, 16.8 mmol), α-naphthyl phosphorodichloridate (4.39 g, 16.8 mmol) and Et_3N (4.68 ml, 33.64 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (5.51 g, 77%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.78, 8.50

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (d, J = 7.5 Hz, 1H, H-Ar), 7.87 (d, J = 7.0 Hz, 1H, H-Ar), 7.75 (d, J = 7.5 Hz, 1H, H-Ar), 7.63 -7.52 (m, 3H, H-Ar), 7.46 – 7.37 (m, 1H, H-Ar), 4.24 - 4.12 (m, 3H, N*H*, $OCH_2CH_2CH_2CH_2CH_3$), 4.04 – 3.84 (m, 1H, $CHCH_2CH(CH_3)_2$), 1.70 – 1.52 (m, 4H, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$),

1.34 – 1.22 (m, 5H, $OCH_2CH_2C\mathit{H_2}C\mathit{H_2}CH_3$, $CHCH_2C\mathit{H}(CH_3)_2$), 0.97 – 0.87 (m, 9H, $OCH_2CH_2CH_2CH_2C\mathit{H_3}$, $CHCH_2CH(C\mathit{H_3})_2$).

Synthesis of phenyl-(benzoxy-L-methioninyl) phosphorochloridate (2.3v).

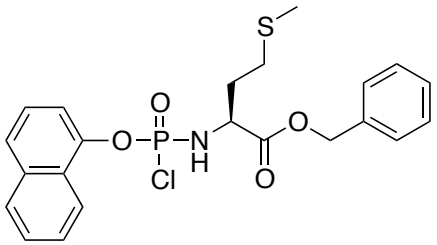


Prepared according to standard procedure 3, from L-methionine benzyl ester tosylate salt (1.00 g, 2.43 mmol), phenyl phosphorodichloridate (0.36 g, 2.43 mmol) and Et_3N (0.67 ml, 4.86 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (0.56 g, 56%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.15, 8.08

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 – 7.37 (m, 6H, H-Ar), 7.30 – 7.25 (m, 4H, H-Ar), 5.26 – 5.23 (AB, J_{AB} = 9 Hz, 2H, $OC\mathit{H_2}Ph$), 4.37 – 4.31 (m, 1H, $C\mathit{H}CH_2CH_2SCH_3$), 2.65 – 2.51 (m, 2H, $CHCH_2C\mathit{H_2}SCH_3$), 2.20 – 2.14 (m, 2H, $CHC\mathit{H_2}CH_2SCH_3$), 2.07, 2.06 (2 x s, 3H, $CHCH_2CH_2SC\mathit{H_3}$).

Synthesis of α-naphthyl-(benzoxy-L-methioninyl) phosphorochloridate (2.3w).



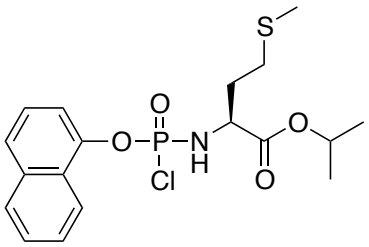
Prepared according to standard procedure 3, from L-methionine benzyl ester tosylate salt (0.80 g, 2.10 mmol), α-naphthyl phosphorodichloridate (0.55 g, 2.10 mmol) and Et_3N (0.58 ml, 4.21 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (0.59 g, 61%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.71, 8.59

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 – 8.08 (m, 1H, H-Ar), 7.91 – 7.88 (m, 1H, H-Ar), 7.76 (d, J = 8.5 Hz, 1H, H-Ar), 7.63 – 7.55 (m, 3H, H-Ar), 7.42 (t, J = 8.0 Hz, 1H, H-Ar), 7.39 – 7.28 (m, 5H, H-Ar), 5.25 – 5.23 (AB, J_{AB} = 8.5 Hz, 2H, $OC\mathit{H_2}Ph$),

4.48 – 4.44 (m, 1H, C*H*$CH_2CH_2SCH_3$), 2.64 – 2.52 (m, 2H, $CHCH_2CH_2SCH_3$), 2.26 – 2.20 (m, 2H, $CHCH_2CH_2SCH_3$), 2.03, 2.01 (2 x s, 3H, $CHCH_2CH_2SCH_3$).

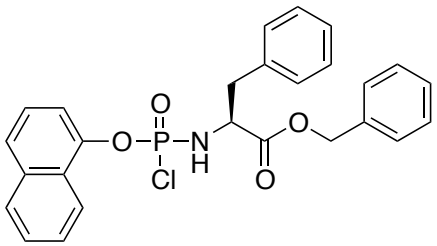
Synthesis of α-naphthyl-(isopropoxy-L-methioninyl) phosphorochloridate (2.3x).



Prepared according to standard procedure 3, from L-methionine benzyl ester hydrochloride salt (1.50 g, 6.58 mmol), α-naphthyl phosphorodichloridate (1.72 g, 6.58 mmol) and Et_3N (1.84 ml, 13.17 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.45 g, 53%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.65, 8.60

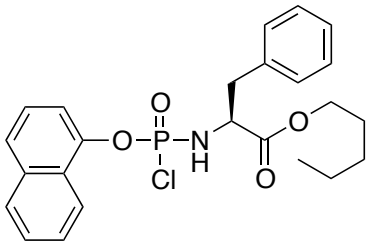
Synthesis of α-naphthyl-(benzoxy-L-phenylalaninyl) phosphorochloridate (2.3y).



Prepared according to standard procedure 3, from L-phenylalanine benzyl ester hydrochloride salt (1.20 g, 4.12 mmol), α-naphthyl phosphorodichloridate (1.07 g, 4.12 mmol) and Et_3N (1.14 ml, 8.24 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.93 g, 98%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 7.75, 7.64

Synthesis of α-naphthyl-(pentoxy-L-phenylalaninyl) phosphorochloridate (2.3z).

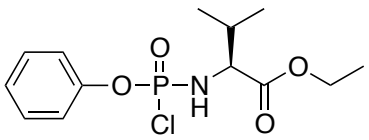


Prepared according to standard procedure 3, from L-phenylalanine pentyl ester hydrochloride salt (2.50 g, 9.2 mmol), α-naphthyl phosphorodichloridate (2.40 g, 4.12 mmol) and Et_3N (2.57 ml, 18.42 mmol) in 15 ml of anhydrous DCM. The final product produced was a

thick, yellowish oil. (4.73 g, 97%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 8.40, 8.23

Synthesis of phenyl-(ethoxy-L-valinyl) phosphorochloridate (2.4a).

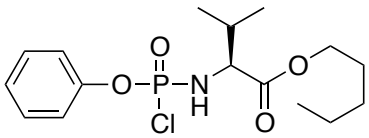


Prepared according to standard procedure 3, from L-valine ethyl ester hydrochloride salt (1.50 g, 8.26 mmol), phenyl phosphorodichloridate (1.74 g, 8.26 mmol) and Et_3N (2.30 ml, 16.51 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.98 g, 75%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 9.51, 9.03

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 – 7.37 (m, 2H, H-Ar), 7.29 – 7.25 (m, 3H, H-Ar), 4.28 – 4.20 (m, 3H, N*H*, OC*H*$_2$CH$_3$), 4.01 – 3.90 (m, 1H, C*H*CH(CH$_3$)$_2$), 2.22 – 2.16 (m, 1H, CHC*H*(CH$_3$)$_2$), 1.33, 1.30 (2 x s, 3H, OCH$_2$C*H*$_3$), 1.06, 1.03 (2 x d, *J* = 6.5 Hz, 3H, CHCH(C*H*$_3$)$_2$), 0.97, 0.95 (2 x d, *J* = 6.5 Hz, 3H, CHCH(C*H*$_3$)$_2$).

Synthesis of phenyl-(pentoxy-L-valinyl) phosphorochloridate (2.4b).

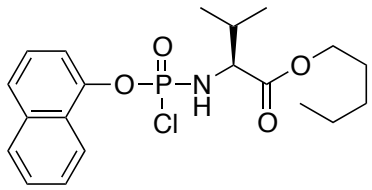


Prepared according to standard procedure 3, from L-valine pentyl ester hydrochloride salt (1.50 g, 6.7 mmol), phenyl phosphorodichloridate (0.99 g, 6.7 mmol) and Et_3N (1.86 ml, 13.4 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (2.37 g, 73%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 9.78, 9.34

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 – 7.40 (m, 5H, H-Ar), 4.28 – 4.12 (m, 3H, N*H*, OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 4.09 – 3.98 (m, 1H, C*H*CH(CH$_3$)$_2$), 2.24 – 2.17 (m, 1H, CHC*H*(CH$_3$)$_2$), 1.71 – 1.63 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.37 – 1.31 (m, 4H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$), 1.28, 1.26 (2 x s, 3H, OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$), 0.99, 0.95 (2 x d, *J* = 6.5 Hz, 3H, CHCH$_2$CH(C*H*$_3$)$_2$), 0.92, 0.89 (2 x d, *J* = 6.5 Hz, 3H, CHCH$_2$CH(C*H*$_3$)$_2$).

Synthesis of α-naphthyl-(pentoxy-L-valinyl) phosphorochloridate (2.4c).



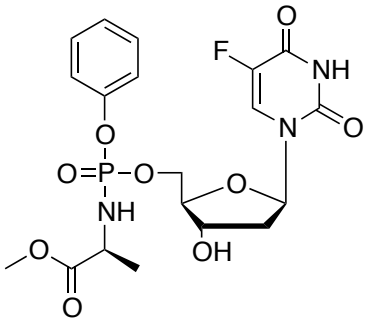
Prepared according to standard procedure 3, from L-valine pentyl ester hydrochloride salt (1.50 g, 6.7 mmol), α-naphthyl phosphorodichloridate (1.75 g, 6.7 mmol) and Et_3N (1.86 ml, 13.4 mmol) in 15 ml of anhydrous DCM. The final product produced was a thick, yellowish oil. (1.84 g, 67%).

^{31}P NMR (202 MHz, $CDCl_3$) δ 9.78, 9.33

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 – 8.08 (2 x d, *J* = 7.5 Hz, 1H, H-Ar), 7.88 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.75 (d, *J* = 8.5 Hz, 1H, H-Ar), 7.64 – 7.54 (m, 3H, H-Ar), 7.45 (t, *J* = 8.0 Hz, 1H, H-Ar), 4.33 – 4.25 (m, 1H, NH), 4.25 – 4.04 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 3.51 – 3.47 (m, 1H, $CHCH(CH_3)_2$), 1.70 – 1.64 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.37 – 1.29 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 1.23 - 1.21 (m, 1H, $CHCH(CH_3)_2$), 1.08 – 0.88 (m, 9H, $OCH_2CH_2CH_2CH_2CH_3$, $CHCH(CH_3)_2$).

8.4 Experimental section – Chapter 3

Synthesis of 5-Fluoro-2'-deoxyuridine-5'-*O*-[Phenyl-(methoxy-L-alaninyl)]phosphate (3.1a).



Prepared according to the standard procedure **4** from 5-Fluoro-2'-deoxyuridine (0.40 g, 1.62 mmol), *t*BuMgCl (1.78 ml, 1.78 mmol) and phenyl-(methoxy-L-alaninyl)-phosphorochloridate (**2.3a**, 0.90 g, 3.25 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1a** as a yellowish solid (0.008 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 3.79, 4.09

^{19}F-NMR (MeOD, 470 MHz) δ -167.78, -167.72

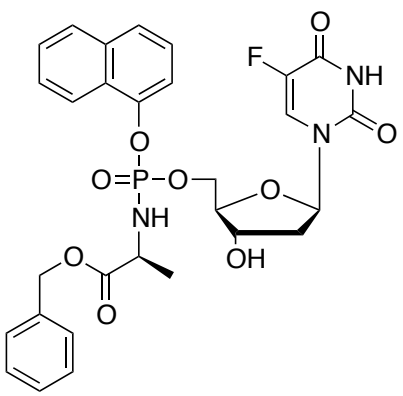
^{1}H-NMR (MeOD, 500 MHz) δ 7.85 (d, 1H, $^{3}J_{H\text{-}F}$ = 6.4 Hz, H-6), 7.35 – 7.40 (m, 2H, H-Ar), 7.18 – 7.28 (m, 3H, H-Ar), 6.20 - 6.29 (m, 1H, H-1'), 4.27 - 4.45 (m, 3H, C*H*$_2$OPh, H-3'), 4.08 – 4.13 (m, 1H, H-4'), 3.93 – 4.02 (m, 1H, C*H*CH$_3$), 3.70 (s, 3H, C*H*$_3$-ester, one diast.), 3.69 (s, 3H, C*H*$_3$-ester, one diast.), 2.25 - 2.34 (m, 1H, H-2'), 2.02 - 2.16 (m, 1H, H-2'), 1.36 (d, *J* = 7.1 Hz, 3H, CHC*H*$_3$, one diast.), 1.34 (d, *J* = 7.1 Hz, 3H, CHC*H*$_3$, one diast.).

^{13}C (MeOD, 125 MHz) δ 175.5 (d, $^{3}J_{C\text{-}F}$ = 3.7 Hz, C=O, ester), 175.2 (d, $^{3}J_{C\text{-}F}$ = 4.8 Hz, C=O, ester), 159.4 (d, $^{2}J_{C\text{-}F}$ = 26 Hz, C=O, base), 152.2 (d, $^{2}J_{C\text{-}P}$ = 6.8 Hz, C=O, base), 152.1 (d, $^{2}J_{C\text{-}P}$ = 6.8 Hz, C-Ar, Ph), 150.6 (d, $^{4}J_{C\text{-}F}$ = 3.6 Hz, C-Ar, Ph), 141.7 (d, $^{1}J_{C\text{-}F}$ = 233.9 Hz, CF-base), 141.6 (d, $^{1}J_{C\text{-}F}$ = 233.8 Hz, CF-base), 130.9, 130.8, 126.2 (*C*H-Ar), 125.9 (d, $^{5}J_{C\text{-}P}$ = 2.9 Hz, *C*H-Ar), 125.6 (d, $^{5}J_{C\text{-}P}$ = 2.9 Hz, *C*H-Ar), 121.4 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, *C*H), 121.2 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, *C*H), 87.02, 86.9 (*C*H), 86.8 (d,$^{3}J_{C\text{-}P}$ = 8.2 Hz, *C*H), 86.7 (d, $^{3}J_{C\text{-}P}$ = 8.2 Hz, *C*H), 72.10, 72.0 (*C*H), 67.5 (d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, *C*H$_2$), 52.8 (*C*H), 52.7 (*C*H), 51.6 (CH$_3$), 51.5 (CH$_3$), 40.9 (CH$_2$), 40.8 (CH$_2$), 20.5 (d, $^{3}J_{C\text{-}P}$ = 6.7 Hz, *C*H$_3$), 20.2 (d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, *C*H$_3$).

HPLC (System 1) t_R = 23.14, 24.08 min.

(ES+) m/z, found: ($M+Na^+$) 510.12. $C_{19}H_{23}N_3O_9FNaP$ required: (M^+) 487.12.

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]) phosphate (3.1b).



Prepared according to the standard procedure **4** from 5-Fluoro-2'-deoxyuridine (0.15 g, 0.67 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) and α-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (**2.3c**, 0.49 g, 1.22 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1b** as a yellowish solid (0.018 g, 5%).

^{31}P-NMR (202 MHz, MeOD) δ 4.61, 4.25

^{19}F NMR (470 MHz, MeOD) δ -167.45, -167.25

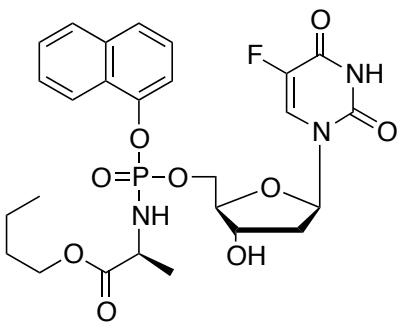
^{1}H NMR (500 MHz, MeOD) δ 8.18, 8.12 (m, 1H, H-Ar), 7.90 - 7.86 (m, 1H, H-Ar), 7.72 - 7.67 (m, 2H, H-Ar, H-6), 7.55 - 7.47 (m, 3H, H-Ar), 7.45 - 7.27 (m, 6H, H-Ar), 6.16 - 6.06 (m, 1H, H-1'), 5.13, 5.08 (2 x AB, J_{AB} = 12.0 Hz, 2H, OC*H*$_2$Ph), 4.36 - 4.24 (m, 3H, H-5', H-5', H-3'), 4.15 - 4.03 (m, 2H, C*H*CH_3, H-4'), 2.17 - 2.08 (m, 1H, H-2'), 1.79 - 1.67 (m, 1H, H-2'), 1.38 - 1.34 (m, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 174.9 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, C=O, ester), 174.6 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, C=O, ester), 159.3 (d, $^2J_{C\text{-}F}$ = 26.10 Hz, C=O, base), 150.5 (d, $^4J_{C\text{-}F}$ = 4.0 Hz, C=O, base), 147.9 (d, $^2J_{C\text{-}P}$ = 7.4 Hz, C-Ar, Naph), 147.8 (d, $^2J_{C\text{-}P}$ = 7.7 Hz, *C*-Ar, Naph), 141.7, 141.6 (2 x d, $^1J_{C\text{-}F}$ = 234.0 Hz, CF-base), 137.2, 137.1, 136.2 (*C*-Ar), 129.7, 129.6, 129.5, 129.4, 129.0, 128.9, 128.1, 128.0 (*C*H-Ar), 127.9, 127.8 (*C*-Ar), 127.7, 127.6, 126.6, 126.5, 126.2 (*C*H-Ar), 125.6, 125.5 (2 x d, $^2J_{C\text{-}F}$ = 34.0 Hz, *C*H-base), 122.6 (*C*H-Ar), 116.5, 116.2 (2 x d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*H-Ar), 87.0, 86.9 (*C*-1'), 86.8, 86.7 (2 x d, $^3J_{C\text{-}P}$ = 8.1 Hz, *C*-4'), 72.1, 72.0 (*C*-3'), 68.1, 68.0 (*C*H$_2$Ph), 67.8, 67.6 (2 x d, $^2J_{C\text{-}P}$ = 5.2 Hz, *C*-5'), 51.9, 51.8 (*C*HCH$_3$), 40.9, 40.8 (*C*-2'), 20.5 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, CH*C*H$_3$), 20.3 (d, $^3J_{C\text{-}P}$ = 7.6 Hz, CH*C*H$_3$).

HPLC (System 1) t_R = 34.21, 34.57 min.

(ES+) m/z, found: ($M+Na^+$) 636.15. $C_{29}H_{29}N_3O_9FNaP$ required: (M^+) 613.15.

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphthyl-(butoxy-L-alaninyl)] phosphate (3.1c).



Prepared according to the standard procedure **5** from 5-Fluoro-2'-deoxyuridine (0.25 g, 1.01 mmol), NMI (0.41 g, 5.07 mmol, 0.40 ml) and α-naphthyl-(butoxy-L-alaninyl) phosphorochloridate (**2.3d**, 0.75 g, 2.03 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1c** as a yellowish solid (0.005g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.52, 4.35

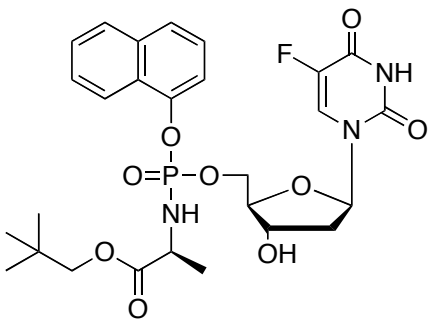
^{19}F-NMR (MeOD, 470 MHz) δ – 167.36, – 167.49

^{1}H-NMR (MeOD, 500 MHz) δ 8.19 – 8.16 (m, 1H, H-Ar, Naph), 7.93 – 7.89 (m, 1H, H-Ar, Naph), 7.75 – 7.72 (m, 2H, H-Ar, Naph), 7.58 – 7.51 (m, 3H, 2 x H-Ar, H-base), 7.46 – 7.41 (m, 1H, H-Ar), 6.18 – 6.11 (m, 1H, H-1'), 4.42 - 4.40 (m, 1H, H-5'), 4.37 - 4.32 (m, 2H, H-5', H-3'), 4.12 – 4.01 (m, 4H, H-4', C*H*CH_3, OC*H*$_2CH_2CH_2CH_3$), 2.20 – 2.12 (m, 1H, H-2'), 1.85 – 1.73 (m, 1H, H-2'), 1.61 – 1.54 (m, 2H, $OCH_2CH_2CH_2CH_3$), 1.39 – 1.31 (m, 5H, $OCH_2CH_2CH_2CH_3$, $CHCH_3$), 0.93 – 0.89 (m, 3H, $OCH_2CH_2CH_2CH_3$).

HPLC (System 1) t_R = HPLC data lost

(ES+) m/z, found: ($M+Na^+$) 602.20 $C_{26}H_{31}N_3O_9FNaP$ required: (M^+), 579.18.

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (3.1d).



Prepared according to the standard procedure **5** from 5-Fluoro-2'-deoxyuridine (0.25 g, 1.01 mmol), NMI (0.41 g, 5.07 mmol, 0.40 ml) and α-naphthyl-(2,2-dimethylpropoxy -L-alaninyl) phosphorochloridate (**2.3f**, 0.78 g, 2.03 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1d** as a white solid (0.006g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.56, 4.33

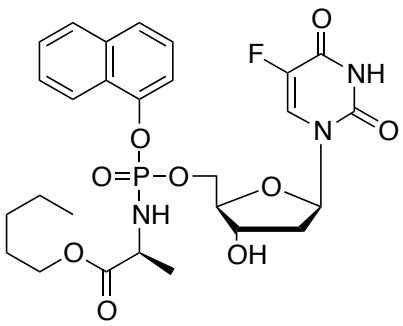
^{19}F-NMR (MeOD, 470 MHz) δ – 167.32, – 167.43

^{1}H-NMR (MeOD, 500 MHz) δ 8.19 – 8.16 (m, 1H, H-Ar, Ar), 7.91 – 7.89 (m, 1H, H-Ar), 7.74 – 7.71 (m, 2H, H-Ar), 7.57 - 7.51 (m, 3H, 2 x H-Ar, *H*-base), 7.46 – 7.41 (m, 1H, H-Ar), 6.17 – 6.10 (m, 1H, H-1'), 4.42 – 4.30 (m, 3H, H-3', H-5', H-5'), 4.13 - 4.07 (m, 2H, H-4', C*H*CH_3), 3.86, 3.75 (2AB, J_{AB} = 10.50 Hz, 2H, C*H*$_2$C$(CH_3)_3$), 2.18 – 2.10 (m, 1H, H-2'), 1.81 – 1.70 (m, 1H, H-2'), 1.41 – 1.38 (m, 3H, CHC*H*$_3$), 0.95, 0.94 (2 x s, 9H, CH_2C(C*H*$_3$)$_3$).

HPLC (System 1) t_R = HPLC data lost

(ES+) m/z, found: (M+Na$^+$) 616.20 $C_{27}H_{33}N_3O_9FNaP$ required: (M$^+$) 593.19.

Synthesis of 5-Fluoro-2'-deoxyuridine-5'-O-[1-naphthyl (pentoxy-alaninyl)] phosphate (3.1e).



Prepared according to the standard procedure **4** from 5-Fluoro-2'-deoxyuridine (0.30 g, 1.22 mmol), tBuMgCl (1.46 mmol, 1.46 ml) and α-naphthyl-(pentoxy-L-alaninyl) phosphorochloridate (**2.3g**, 0.78 g, 2.03 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1e** as a white solid (0.021g, 3%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.48, 4.32;

^{19}F-NMR (MeOD, 470 MHz) δ –167.18, –167.29;

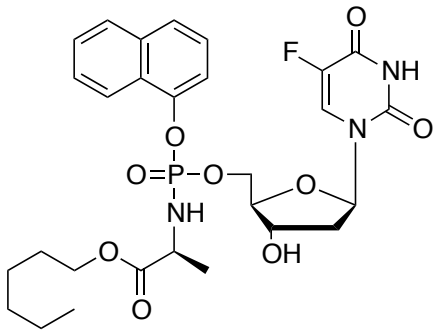
1H-NMR (MeOD, 500 MHz) δ 8.25 – 8.17 (m, 1H, H-Ar), 8.05 – 7.95 (m, 2H, H-Ar), 7.85 – 7.60 (m, 2H, H-Ar, H-base), 7.65 – 7.48 (m, 3H, H-Ar), 6.30 – 6.18 (m, 1H, H-1'), 4.60 – 4.37 (m, 3H, H-3', H-5', H-5'), 4.28 – 4.00 (m, 4H, H-4', C*H*CH_3, O*C*$H_2$$CH_2CH_2CH_2CH_3$), 2.32 – 2.12 (m, 1H, H-2'), 1.95 – 1.75 (m, 1H, H-2'), 1.70 –1.55 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.50 – 1.28 (m, 7H, OCH_2CH_2C*H*$_2$*C*H_2CH_3, $CHCH_3$), 0.83, 0.82 (2 x d, *J* = 7.9 Hz, 3H, $OCH_2CH_2CH_2CH_2C$*H*$_3$).

^{13}C-NMR (MeOD, 125 MHz) δ 175.22, 174.91 (C=O, ester), 159.5 (C=O, base), 150.54 (C=O, base), 147.90, 147.88 (C-Ar), 141.75 (d, $^1J_{C\text{-}F}$ = 225 Hz, CF-base), 136.37 (C-Ar), 128.95, 127.90, 127.56, 126.55, 126.19 (*C*H-Ar), 125.64, 125.53 (2 x d, $^2J_{C\text{-}F}$ = 34.0 Hz, *C*H-base), 122.65 (*C*H-Ar), 116.51, 116.21 (*C*H-Ar), 87.03, 86.96 (C-1'), 86.85, 86.74 (C-4'), 72.16, 72.05 (C-3'), 67.87 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, C-5'), 66.54 (O*C*$H_2CH_2CH_2CH_2CH_3$), 51.87, 51.81 (d, $^2J_{C\text{-}P}$ = 7.5 Hz, *C*HCH_3), 40.87, 40.80 (C-2'), 29.35, 29.10 (*C*H_2-ester), 23.33 (*C*H_2-ester), 20.60, 20.43 (2 x d, $^3J_{C\text{-}P}$ = 6.5 Hz, *C*HCH_3), 14.28 ($OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 15.57 min.

(ES+) m/z, found: ($M+Na^+$) 616.20 $C_{27}H_{33}N_3O_9FNaP$ required: (M^+) 593.19.

Synthesis of 5-Fluoro-2'-deoxyuridine-5'-O-[1-naphthyl(hexoxy-L-alaninyl)] phosphate-(3.1f).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (1.20 g, 4.87 mmol), *t*BuMgCl (5.36ml, 5.46 mmol) and α-naphthyl-(hexoxy-L-alaninyl) phosphorochloridate (**2.3h**, 3.87 g, 9.75 mmol) in THF (15 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1f** as a white solid (0.120 g, 7%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.48, 4.33;

^{19}F-NMR (MeOD, 470 MHz) δ – 167.09, – 167.15;

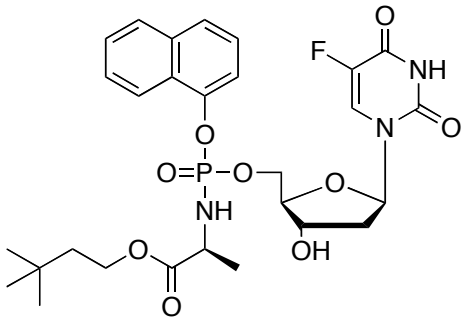
^{1}H-NMR (MeOD, 500 MHz) δ 8.19 – 8.16 (m, 1H, H-Ar), 7.89 – 7.87 (m, 1H, H-Ar), 7.73 – 7.70 (m, 2H, H-Ar), 7.57 – 7.51 (m, 3H, 2 x H-Ar, H-base), 7.45 – 7.40 (m, 1H, H-Ar), 6.18 – 6.12 (m, 1H, H-1'), 4.41 – 4.31 (m, 3H, 2 x H-5', H-3'), 4.12 – 4.00 (m, 4H, *H*-4', C*H*CH_3, O$CH_2CH_2CH_2CH_2CH_2CH_3$), 2.20 – 2.12 (m, 1H, *H*-2'), 1.84 – 1.63 (m, 1H, H-2'), 1.60 – 1.54 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 1.36 –1.25 (m, 9H, $OCH_2CH_2CH_2CH_2CH_2CH_3$, $CHCH_3$), 0.93 – 0.89 (m, 3H, $OCH_2CH_2CH_2CH_2CH_2CH_3$).

^{13}C-NMR (MeOD, 125 MHz) δ 175.22, 174.90 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C=O, ester), 159.50 (d, $^{2}J_{C\text{-}F}$ = 26.2 Hz, C=O, base), 150.67 (C=O, base), 148.03, 147.88 (2 x d, $^{2}J_{C\text{-}P}$ = 7.5 Hz, O*C*-Ar), 141.81, 141.70 (2 x d, $^{1}J_{C\text{-}F}$ = 232.5 Hz, *C*F-base), 136.30 (*C*-Ar), 128.95 (d, $^{3}J_{C\text{-}P}$ = 3.5 Hz, *C*H-Ar), 127.91, 127.88, 127.84, 127.80, 127.58, 127.55, 126.56, 126.55, 126.53, 126.19, 126.15 (*C*H-Ar), 125.60, 125.50 (2 x d, $^{2}J_{C\text{-}F}$ = 33.5 Hz, *C*H-base), 122.67, 122.63 (*C*H-Ar), 116.51, 116.22 (2 x d, $^{4}J_{C\text{-}P}$ = 3.7 Hz, *C*H-Ar), 87.04, 86.96 (C-1'), 86.88, 86.70 (2 x d, $^{3}J_{C\text{-}P}$ = 8.7 Hz, *C*-4'), 72.16, 72.06 (C-3'), 67.83, 67.78 (2 d, $^{2}J_{C\text{-}P}$ = 5.3 Hz, C-5'), 66.56, 66.53 ($OCH_2CH_2CH_2CH_2CH_2CH_3$), 51.85 (d, $^{2}J_{C\text{-}P}$ = 6.3 Hz, $CHCH_3$), 40.90, 40.82 (C-2'), 32.56, 32.54, 29.69, 29.63, 26.65, 26.61, 23.60, 23.56 (CH_2-ester), 20.58, 20.44 (2 x d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, $CHCH_3$), 14.35 ($OCH_2CH_2CH_2CH_2CH_2CH_3$).

HPLC (System 1) t_R = 24.21 min.

(ES+) m/z, found: ($M+Na^+$) 630.20, $C_{28}H_{35}N_3O_9FNaP$ required: : (M^+) 607.56

5-Fluoro-2'-deoxyuridine-5'-O-[1-naphthyl(3,3-dimethyl-1-butoxy-L-alaninyl)] phosphate (3.1g).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (1.00g, 4.06 mmol), *t*BuMgCl (4.46 ml, 4.46 mmol) and α-naphthyl-(hexoxy-L-alaninyl) phosphorochloridate (**2.3k**, 3.23g, 8.12 mmol) in THF (13 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1g** as a white solid (0.098 g, 4%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.48, 4.33;

^{19}F-NMR (MeOD, 470 MHz) δ – 167.30, – 167.47;

^{1}H-NMR (MeOD, 500 MHz) δ 8.20 – 8.17 (m, 1H, *H*-Ar), 7.91 – 7.89 (m, 1H, *H*-Ar), 7.77 – 7.72 (m, 2H, *H*-Ar), 7.58 – 7.51 (m, 3H, *H*-base, 2H-Ar), 7.46 – 7.41 (2 x t, 1H, *J* = 7.8 Hz, H-Ar), 6.19 – 6.13 (m, 1H, H-1'), 4.42 – 4.40 (m, 1H, H-5'), 4.38 - 4.32 (m, 2H, H-3', H-5'), 4.14 – 4.00 (m, 4H, H-4', C*H*CH_3, O$CH_2$$CH_2(CH_3)_3$), 2.21 – 2.13 (m, 1H, H-2'), 1.91 – 1.76 (m, 1H, H-2'), 1.52 – 1.48 (m, 2H, $OCH_2C$$H_2(CH_3)_3$), 1.37 – 1.35 (m, 3H, CHCH_3), 0.92, 0.91 (2 x s, 9H, $OCH_2CH_2(C$$H_3)_3$).

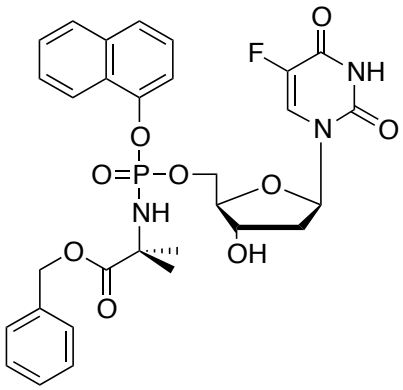
^{13}C-NMR (MeOD, 125 MHz) δ 175.16, 174.84 (2 d, $^3J_{C\text{-}P}$ = 4.75 Hz, C=O, ester), 159.56, 159.35 (C=O, base), 150.61 (C=O, base), 148.00, 147.86 (2 x d, $^2J_{C\text{-}P}$ = 6.25 Hz, *C*-Ar), 141.78, 141.73 (2 x d, $^1J_{C\text{-}F}$ = 232 Hz, *C*F-base), 136.28 (C-Ar), 128.98, 128.95, 127.92, 127.90, 127.58, 126.57, 126.20, 126.14 (*C*H-Ar), 125.63, 125.55 (2 d, $^2J_{C\text{-}F}$ = 34 Hz, *C*H-base), 122.65, 122.63 (*C*H-Ar), 116.48, 116.15 (2 x d, $^3J_{C\text{-}P}$ = 3.0 Hz, *C*H-Ar), 87.01, 86.94 (C-1'), 86.73, 86.68 (d, $^3J_{C\text{-}P}$ = 7.75 Hz, C-4'), 72.18, 72.07(*C*-3'), 67.87, 67.85 (2 x d, $^2J_{C\text{-}P}$ = 5.0 Hz, C-5'), 64.08, 64.05 (O*C*$H_2CH_2(CH_3)_3$), 51.86 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, *C*HCH_3), 42.74 (OCH_2*C*$H_2(CH_3)_3$),

40.91, 40.83 (C-2'), 29.96 ($OCH_2CH_2(CH_3)_3$), 20.50, 20.34 (2 x d, $^3J_{C\text{-}P}$ = 6.5 Hz, $CHCH_3$).

HPLC (System 1) t_R = 18.89 min

(ES+) m/z, found: ($M+Na^+$) 630, $C_{28}H_{35}N_3O_9FNaP$ required: : (M^+) 607.56

Synthesis of 5-Fluoro-2'-deoxyuridine-5'-O-[1-naphthyl (benzoxy-dimethyl-glycinyl)] phosphate (3.1h).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (1.00g, 4.06 mmol), *t*BuMgCl (4.46 ml, 4.46 mmol) and α-naphthyl-(benzoxy-dimethylglicynyl) phosphorochloridate (**2.3l**, 3.39 g, 4.80 mmol) in THF (13 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1h** as a white solid (0.110g, 4%).

^{31}P-NMR (202 MHz, MeOD) δ 2.87, 3.03;

^{19}F-NMR (470 MHz, MeOD) δ -167.95, -167.13;

^{1}H-NMR (500 MHz, MeOD) δ 8.22 – 8.17 (m, 1H, H-Ar), 7.90 – 7.86 (m, 1H, H-Ar), 7.70, 7.62 (2d, $^3J_{H\text{-}F}$ = 6.4 Hz, 1H, H_6), 7.55 – 7.48 (m, 3H, H-Ar), 7.40 – 7.28 (m, 7H, H-Ar), 6.15 – 6.05 (m, 1H, H-1'), 5.19 – 5.13 (m, 2H, OCH_2Ph), 4.35 – 4.19 (m, 3H, H-3', H-5', H-5'), 4.04 – 3.98 (m, 1H, H-4'), 2.15 – 2.06 (m, 1H, H-2'), 1.87 – 1.79, 1.69 – 1.61 (2 x m, 1H, H-2'), 1.42 – 1.37 (m, 6H, $C(CH_3)_2$).

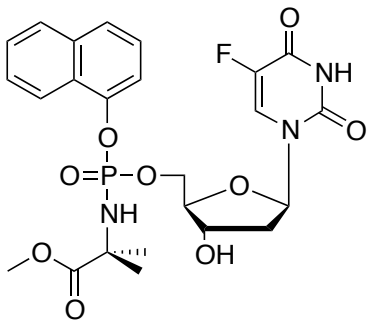
^{13}C-NMR (125 MHz, MeOD) δ 176.6, 176.5 (*C*=O, ester), 159.6, 159.5 (2 x d, $^2J_{C\text{-}F}$ = 25.8 Hz, *C*=O, base), 150.7 (d, $^4J_{C\text{-}F}$ = 3.7 Hz, *C*=O, base), 148.0, 147.9 (2d, $^3J_{C\text{-}P}$ = 7.7 Hz, O*C*-Ar), 141.8 (d, $^1J_{C\text{-}F}$ = 234.4 Hz, *C*F-base), 137.3, 136.2 (*C*-Ar), 129.6, 129.4, 129.3, 128.9, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 126.5, 126.4, 126.1, 126.0 (CH), 125.6, 125.4 (2 x d, $^2J_{C\text{-}F}$ = 25.0 Hz, *C*H-base), 122.9, 122.8 (*C*H-Ar), 116.7, 116.3 (2 x d, $^3J_{C\text{-}P}$ = 2.9 Hz, *C*H-Ar), 86.9 (*C*-1'), 86.8, 86.6 (2d, $^3J_{C\text{-}P}$ = 8.2 Hz, *C*-4'), 72.1, 72.0 (*C*-3'), 68.30 (OCH_2Ph), 67.80 (d, $^2J_{C\text{-}P}$ = 6.5 Hz, *C*-5'), 62.2

($C(CH_3)_2$), 40.8, 40.7 (*C*-2'), 28.0, 27.9 (2 x d, $^3J_{C\text{-}P}$ = 6.0 Hz, $C(CH_3)_2$), 27.5 (d, $^3J_{C\text{-}P}$ = 6.0 Hz, $C(CH_3)_2$).

HPLC (System 1) t_R = 20.80, 21.00 min

(ES+) m/z, found: ($M+Na^+$) 650.17, $C_{30}H_{31}N_3O_9NaPF$ required: (M^+) 627.55

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(metoxy-dimethylglycinyl)phosphate (3.1i).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) and α-naphthyl-(methoxy-dimethylglicynyl) phosphorochloridate (**2.3m**, 0.42 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1i** as a white solid (0.003 g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 2.98, 2.87

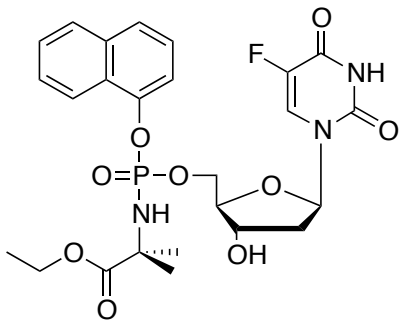
^{19}F-NMR (MeOD, 470 MHz) δ -167.99, 167.54

^{1}H-NMR (MeOD, 500 MHz) δ 8.26 - 8.20 (m, 1H, H-Ar), 7.97- 7.87 (m, 1H, H-Ar), 7.77-7.67 (m, 2H, H-Ar), 7.58-7.52 (m, 3H, H-Ar), 7.47 - 7.42 (m, 1H, H-Ar), 6.18 - 6.12 (m, 1H, H-1'), 4.42 – 4.28 (m, 3H, H-3', H-5', H-5'), 4.11 - 4.08 (m, 1H, H-4'), 3.75– 3.66 (m, 3H, OCH_3), 2.23 – 1.71 (4 × m, 2H, H-2'), 1.56 - 1.48 (m, 6H, $NH(CH_3)_2$).

HPLC (System 1) = HPLC data lost

(ES+) m/z, found: ($M+Na^+$) 574.20, $C_{24}H_{27}FN_3O_9NaPF$ required: (M^+) 551.15

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(etoxy-dimethylglycinyl)]phosphate (3.1j).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(ethoxy-dimethylglicynyl) phosphorochloridate (**2.3n**, 0.43 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1j** as a white solid (0.006 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 2.97, 2.85

^{19}F-NMR (MeOD, 470 MHz) δ -167.92, 167.61

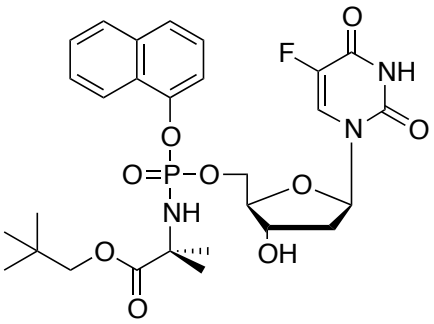
^{1}H-NMR (MeOD, 500 MHz) δ 8.25- 8.20 (m, 1H, H-Ar), 7.92 - 7.87 (m, 1H, H-Ar), 7.76 - 7.66 (m, 2H, H-Ar), 7.59 - 7.51 (m, 3H, H-Ar), 7.46 - 7.41 (m, 1H, H-Ar), 6.19 – 6.13 (m, 1H, H-1'), 4.41 - 4.28 (m, 3H, H-3', H-5', H-5'), 4.19 – 4.13 (m, 2H, OC*H*$_2$CH$_3$), 4.10 - 4.07 (m, 1H, H-4'), 2.15 - 1.68 (4 × m, 2H, H-2'), 1.57 - 1.50 (m, 6H, NH(C*H*$_3$)$_2$).

^{13}C (MeOD, 125 MHz) δ 176.88, 176.82 (C=O, ester), 160.12 (2 × d, $^{2}J_{C\text{-}F}$ = 22.46 Hz, C-F, base), 151.16 (C=O, base), 148.17, 148.01(2 × d, $^{2}J_{C\text{-}F}$ = 20.49Hz, C=O), 142.76, 140.90 (C-Ar), 127.98, 127.93, 127.90, 127.83, 127.79, 127.45, 127.39, 126.01, 125.79, 122.95, 122.84 (CH - Ar) 116.67, 116.32 (2 × d, $^{4}J_{C\text{-}P}$ = 2.62 Hz, CH-Ar), 86.90, 86.80 (C-1'), 86.69, 86.63 (2 × d, $^{3}J_{C\text{-}P}$ = 7.85 Hz, C-4'), 72.13, 71.06 (C-3'), 67.90, 67.83 (OCH$_2$C*H*$_3$), 62.65, 62.64 (CH(C*H*$_3$)$_2$, 40.80, 40.77 (C-2'), 27.92, 27.54 (OCH$_2$(*C*H$_3$).

HPLC (System 1) t_R = HPLC data lost

(ES+) m/z, found: (M+Na$^+$) 588.22, $C_{25}H_{29}N_3O_9NaPF$ required: (M$^+$) 565.16

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(2,2-dimethylpropoxy-dimethylglycinyl)] phosphate (3.1k).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(2,2-dimethylpropoxy-dimethylglicynyl) phosphorochloridate (**2.3o**, 0.48 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1k** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 2.94, 2.82

^{19}F-NMR (MeOD, 470 MHz) δ - 167.27

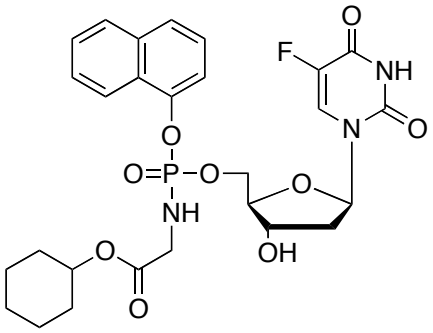
^{1}H-NMR (MeOD, 500 MHz) δ 8.22 – 8.11 (m, 1H, H-Ar), 7.89 – 7.88 (m, 1H, H-Ar), 7.75 – 7.67 (m, 2H, H-Ar), 7.57 - 7.53 (m, 3H, H-Ar), 7.45 - 7.41 (m, 1H, H-Ar, Ar), 6.16 – 6.13 (m, 1H, H-1'), 4.39 – 4.36 (m, 3H, H-3', H-5', H-5'), 4.07 (s, 1H, H-4'), 3.83 (s, 2H, OC*H*$_2$(CH$_3$)$_3$), 2.3 – 2.25, 2.09 – 2.07, 1.92 – 1.89, 1.72 – 1.69 (4 x m, 2H, H-2'), 1.57 – 1.55 (m, 6H,C(C*H*$_3$)$_2$), 0.98 – 0.95 (m, 9H, CH$_2$(C*H*$_3$)$_3$).

^{13}C (MeOD, 125 MHz) δ 176.31 (C=O, base), 155.65 (C=O, base), 151.03 (C=O, base), 147.97, 147.52 (CO-Ar), 141.82 (2 × d, $^{1}J_{C\text{-}F}$ = 245.74 Hz, C-F, base), 136.29 (CH-Ar), 128.90, 127.84, 127.79, 127.46, 127.40, 126.51, 126.03 (CH-Ar), 125.52 (d, $^{2}J_{C\text{-}F}$ = 33.58 Hz, C-H, base), 122.91 (CH-Ar), 116.64, 116.30 (CH-Ar), 86.96 (C-1'), 86.70, 86.64 (C-4'), 75.78 (OCH$_2$(CH$_3$)$_3$), 72.11, 72.04 (C-3'), 67.93, 67.88 (d, $^{2}J_{C\text{-}P}$ = 6.0 Hz, C-5'), 49.87 (C*H*CH$_3$), 40.75, 28.00, 27.62 (*C*H$_3$)$_2$-Me$_2$Gly), 26.78 (OCH$_2$C(*C*H$_3$)$_3$).

HPLC (System 1) t_R = 16.28 min

(ES+) m/z, found: (M+Na$^+$) 630, $C_{28}H_{35}N_3O_9NaPF$ required: (M$^+$) 607.21

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(cyclohexoxy-L-glycinyl)] phosphate (3.1l).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(cyclohexoxy-glycinyl) phosphorochloridate (**2.3p**, 0.46 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1l** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 5.71, 5.60

^{19}F-NMR (MeOD, 470 MHz) δ -167.27

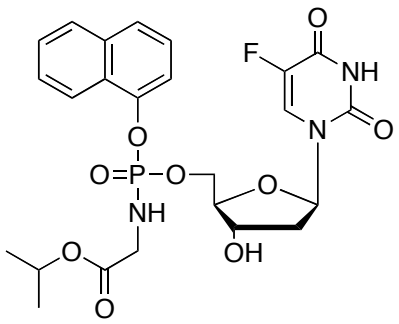
^{1}H-NMR (MeOD, 500 MHz) δ 8.18 - 8.17 (m, 1H, H-Ar), 7.90 – 7.89 (m, 1H, H-Ar), 7.77 – 7.68 (m, 2H, H-Ar), 7.57 - 7.50 (m, 3H, H-Ar), 7.46 - 7.41 (m, 2H, H-Ar), 6.18 – 6.15 (m, 1H, H-1'), 4.78 – 4.74 (m, 1H, C*H*-cHexyl), 4.47 – 4.43 (m, 3H, H-3', H-5', H-5'), 4.11 – 4.10 (m, 1H, H-4'), 3.85 – 3.80 (m, 2H, 2 × $NHCH_2$), 2.20 – 2.17, 2.12 – 2.09, 1.87 – 1.82, 1.73 – 1.68, 1.56 – 1.53, 1.45 – 1.33, 1.30 – 1.26 (7 × m, 12 H, 2 × H-2', 10H-cHexyl).

^{13}C (MeOD, 125 MHz) 172.00 (C=O ester), 160.07, 159.87 (2 × d, $^{2}J_{C\text{-}F}$ = 25.8 Hz, C=O-base), 151.05 (C=O, base), 147.93, 17.88 (2 × d, $^{2}J_{C\text{-}P}$ = 20.2 Hz, C-F, base), 141.85, 141.82 (2 × d, $^{1}J_{C\text{-}F}$ = 233 Hz, C-F, base), 136.31(C-Ar), 128.95, 128.94, 127.94, 127.88, 127.64, 127.56, 126.56, 126.23, 126.19 (CH-Ar), 116.34, 116.31 (2 × d, $^{4}J_{C\text{-}P}$= 2.76 Hz, CH-Ar), 87.21, 87.06 (C-1'), 86.88, 86.81 (2 × d, $^{3}J_{C\text{-}P}$ = 9.21, C-4'), 75.18, 75.16 (C=O, cHexyl), 72.24, 72.18 (C-3'), 67.95, 67.91 (2 × d, $^{2}J_{C\text{-}P}$= 11.98, C-5'), 44.2, 44.14 ($NHCH_2$), 40.90, 40.83 (*C*H_2-cHexyl), 32.53, 26.38, 24.69 (*C*H_2-cHexyl).

HPLC (System 1) t_R = 13.61 min.

(ES+) m/z, found: ($M+H^+$) 592, $C_{27}H_{31}N_3O_9HPF$ required: (M^+) 591.18

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(isopropoxy-glycinyl)] phosphate (3.1m).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(isopropoxy-glicynyl) phosphorochloridate (**2.3q**, 0.41 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1m** as a white solid (0.006 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 5.75, 5.63

^{19}F-NMR (MeOD, 470 MHz) δ -167.48

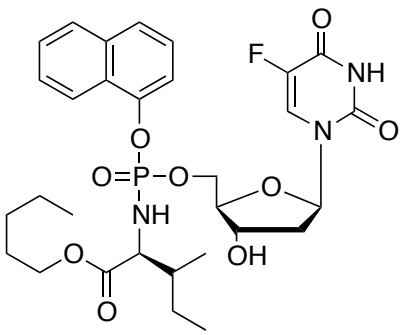
^{1}H-NMR (MeOD, 500 MHz) δ 8.18 - 8.17 (m, 1H, H-Ar), 7.90 - 7.89 (m, 1H, H-Ar), 7.78 - 7.70 (m, 2H, H-Ar), 7.55 - 7.49 (m, 3H, H-Ar), 7.45 – 7.43 (m, 1H, H-Ar), 6.16 – 6.15 (m, 1H, H-1'), 5.03 – 5.00 (m, 2H, C*H*$(CH_3)_2$), 4.44 – 4.38 (m, 3H, H-3', H-5', H-5'), 4.11 - 4.10 (m, 1H, H-4'), 3.82 – 3.77 (m, 2H, NHCH_2) 2.21 – 2.18, 2.13 – 2.08, 1.89 – 1.84, 1.71 – 1.65 (4 × m, 2H, H-2'), 1.24 – 1.19 (m, 6H, $(CH_3)_2$ iPr).

^{13}C (MeOD, 125 MHz) δ 172.05 (C=O, base), 159.59 (C=O, base), 150.57 (C=O, base), 148.05, 147.87 (CO-Ar), 141.73 (d, $^{1}J_{C\text{-}F}$ = 234.3 Hz, C-F, base), 136.31 (C-Ar), 128.94, 127.93, 127.87, 127.62, 127.55, 126.55, 126.23, 126.19 (CH-Ar), 125.72, 125.52 (2 × d, $^{2}J_{C\text{-}F}$ = 33.78 Hz, C-H, base), 122.57 (CH-Ar), 116.35, 116.33 (2 × d, $^{4}J_{C\text{-}P}$ = 41.27 Hz, CH-Ar), 87.02, 86.84 (C-1'), 86.85, 86.79 (C-4'), 72.24, 72.18 (C-3'), 70.30 (CH-iPr), 67.99, 67.87 (C-5'), 44.89, 44.81 (d, $^{2}J_{C\text{-}P}$= 9.82 Hz, CH_2 - α), 40.89, 40.81 (C-2'), 30.70 $(CHCH_3)_2$).

HPLC (System 2) t_R = 11.15 min

(ES-) m/z, found: ($M+Cl^-$) 586, $C_{24}H_{27}FN_3O_9PClF$ required: (M^+) 551.15

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(penthoxy-L-isoleucinyl)]phosphate (3.1n).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(pentoxy-L-isoleucinyl) phosphorochloridate (**2.3r**, 0.52 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1n** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 5.32, 5.06

^{19}F-NMR (MeOD, 470 MHz) δ -168.16

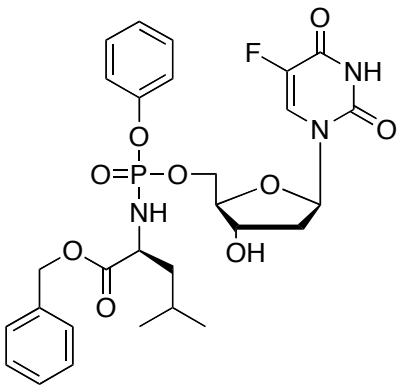
^{1}H-NMR (MeOD, 500 MHz) δ 8.27 – 8.18 (m, 1H, H-Ar), 7.90 - 7.88 (m, 1H, H-Ar), 7.76 - 7.70 (m, 2H, H-Ar), 7.56 – 7.50 (m, 2H, H-Ar), 7.50 - 7.45 (m, 1H, H-Ar), 7.45 - 7.40 (m, 1H, H-Ar), 6.16 – 6.13 (m, 1H, H-1'), 4.40 – 4.30 (m, 3H, H-3', H-5', H-5'), 4.10 – 4.06 (m, 1H, H-4'), 4.06 – 3.97 (m, 2H, O*C*$H_2$$CH_2CH_2CH_2CH_3$) 3.84 – 3.80 (m, 1H, NHCH), 2.25 – 2.21 (m, 1H, H-2'), 1.85 – 1.66 (m, 1H, H-2') 1.59 - 1.54, 1.54 - 1.45, 1.33 – 1.29, 1.22 – 1.14 (m, 18H, ($CH_2CH(CH_3)_2$), (CH_2)$_3$$CH_3$).

^{13}C (MeOD, 125 MHz) δ 174.37, 174.02 (C=O, -ester), 150.57 (C=O, -base), 148.99, 147.93 (OC-Ar), 141.75 (d, $^{1}J_{C\text{-}F}$ = 233Hz, CF-base), 136.30 (C-Ar), 132.37, 128.93, 127.89, 127.86, 127.83, 127.81, 127.57, 127.49, 126.52, 126.50, 126.17, 126.11 (C-Ar), 125.69, 125.51 (2 × d, $^{2}J_{C\text{-}F}$ = 34Hz, CH-base), 122.72 (CH-Ar), 116.38, 116.35 (CH-Ar), 86.96, 86.89 (C-1'), 86.67, 86.61 (C-4'), 72.16, 71.9 (C-3'), 67.94, 67.90 (2 × d, $^{2}J_{C\text{-}P}$ = 4.45Hz, C-5'), 66.3 (OCH_2(CH_2)$_3$$CH_3$), 61.08, 60.09 (d, $^{2}J_{C\text{-}P}$ = 17.5Hz, *CH*CH_3), 40.86, 40.78 (C-2'), 40.05, 39.99 (*C*H-isoLeu), 29.35, 29.18 (*C*H_2-ester), 26.04, 25.99 (*C*H_2-isoLeu), 23.30 (CH_2-ester), 15.89, 14.25, 11.52 (NHCHCH(*C*H_3)CH_2*C*H_3).

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: (M+Na^+) 658, $C_{30}H_{39}FN_3O_9PNaF$ required: (M^+) 635.24

5-Fluoro-2'-deoxyuridine-5'-*O*-[phenyl-(benzoxy-L-leucinyl)] phosphate (3.1o).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) phenyl-(benzoxy-L-leucinyl) phosphorochloridate (**2.3s**, 0.48 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1o** as a white solid (0.011 g, 3%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.43, 3.91

^{19}F-NMR (MeOD, 470 MHz) δ -167.20

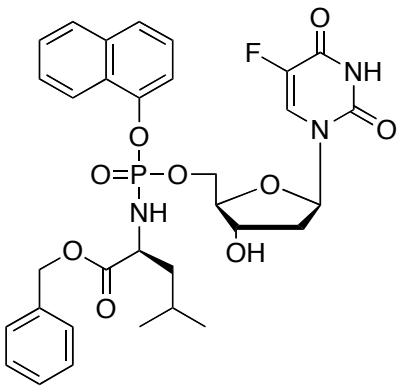
^{1}H-NMR (MeOD, 500 MHz) δ 7.83 – 7.80 (m, 1H, H-Ar), 7.37 – 7.32 (m, 7H, H-Ar), 7.23 - 7.19 (m, 3H, H-Ar), 6.23– 6.18 (m, 1H, H-1'), 5.21 – 5.19 (m, 2H, C*H*$_2$Ph), 4.37 – 4.20 (m, 3H, H-3', H-5', H-5'), 4.06 – 4.05 (m, 1H, H-4'), 3.97 – 3.94 (m, 1H, NHC*H*CH$_2$CH(CH$_3$)$_2$), 2.29 – 2.22, 2.04 – 1.96 (2 × m, 2H, H-2') 1.74-1.68 NHCHCH$_2$C*H*(CH$_3$)$_2$), 1.60 – 1.55 (m, 2H, NHCHC*H*$_2$CH(CH$_3$)$_2$), 0.95 – 0.85 (m, 6H, 2 × CHCH$_2$CH(C*H*$_3$)$_2$).

^{13}C-NMR (MeOD, 125 MHz) δ 175.2, 174.7 (C=O, -ester), 160.0 (d, $^{2}J_{C\text{-}F}$ = 26 Hz, *C*-O, -base), 152.2 (d, $^{4}J_{C\text{-}F}$ = 8.8 Hz, *C*=O, -base), 152.2, 152.1 (2 × d, $^{4}J_{C\text{-}F}$ = 8.8 Hz, *C*=O, base), 141.0 (d, $^{1}J_{C\text{-}F}$ = 230 Hz, C-F, base), 137.2 (*C*-Ar), 129.7, 129.6, 129.5, 129.4, 128.3, 128.0, 126.3, 126.2 (*C*H-Ar), 125.6 (d, $^{2}J_{C\text{-}F}$ = 35.3 Hz, *C*-H, base), 121.5, 121.3 (*C*H-Ar), 86.9 (*C*-1'), 86.7 (d, $^{3}J_{C\text{-}P}$ = 8.8 Hz, *C*-4'), 72.1, 71.9 (*C*-3'), 67.9 (*CH*$_2$Ph), 67.7 (d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, *C*-5'), 53.5 (*C*HCH$_2$CH(CH$_3$)$_2$), 45.0 (CH*CH*$_2$CH(CH$_3$)$_2$), 41.0 (*C*-2'), 25.8 (*C*HCH$_2$*C*H(CH$_3$)$_2$), 23.2, 22.5 (CHCH$_2$CH*(C*H$_3$)$_2$.

HPLC (System 2) t_R = 14.29 min

(ES+) m/z, found: (M+Na$^+$) 628, $C_{28}H_{33}N_3O_9PNaF$ required: (M$^+$) 605.19

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(benzoxy-L-leucinyl)] phosphate (3.1p).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(benzoxy-L-leucinyl) phosphorochloridate (**2.3t**, 0.54 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1o** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.40

^{19}F-NMR (MeOD, 470 MHz) δ - 167.1, - 167.01

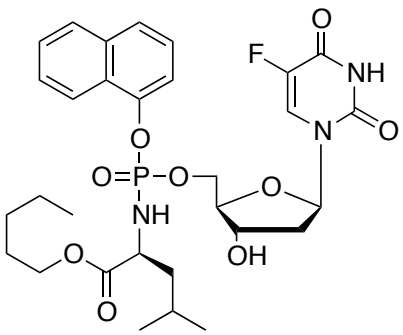
^{1}H-NMR (MeOD, 500 MHz) δ 8.15 (m, 1H, *H*-Ar), 7.88 (m, 1H, H-Ar), 7.72 – 7.67 (m, 2H, H-Ar), 7.54 – 7.47 (m, 3H, H-Ar), 7.41 – 7.36 (m, 1H, H-Ar), 7.34 – 7.27 (m, 5H, H-Ar), 6.12 – 6.16 (2 × t, *J* = 6.6 Hz, 1H, H-1'), 5.15 – 5.04 (m, 2H, C*H*$_2$Ph), 4.37 – 4.21 (m, 3H, H-3', H-5', H-5'), 4.05 (m, 1H, H-4'), 4.00 (m, 1H, *CH*CH$_2$CH(CH$_3$)$_2$), 2.157 (m, 1H, *H*-2'), 1.76 (m, 1H, H-2'), 1.63 (m, 1H, CHCH$_2$*CH*(CH$_3$)$_2$), 1.57 - 1.46 (m, 2H, CHC*H*$_2$CH(CH$_3$)$_2$), 0.82 (d, *J* = 6.3 Hz, 3H, CH$_3$), 0.76 (d, *J* = 6.6 Hz , 3H, CH$_3$).

^{13}C (MeOD, 125 MHz) δ 174.68, 174.66 (C=O, ester), 159.38 (d, $^{2}J_{C\text{-}F}$ = 26.94 Hz, *C*= O, base), 150.53 (*C*= O, base), 147.92, 147.86 (2 × d, $^{2}J_{C\text{-}P}$ = 7.02 Hz, *C*-O), 141.71 (d, $^{1}J_{C\text{-}F}$ = 234.58 Hz, C-F, base), 137.09, 136.30 (C-Ar), 127.82, 127.77, 129,6, 129.4, 128.97, 127.88, 127.56, 126.55, 126.13 (C-Ar), 125.40 (d, $^{2}J_{C\text{-}F}$ = 34.7 Hz, C-H, base), 122.70, 122.63 (CH-Ar), 116.35, 116.32 (CH-Ar), 86.9 (C-1'), 86.63, 86.57 (C-4'), 72.29, 71.93 (C-3'), 67.96 (*C*H$_2$Ph), 67.85 (d, $^{2}J_{C\text{-}P}$ = 5.6 Hz, C-5'), 55.0 (*CH*CH$_2$CH(CH$_3$)$_2$), 44.99 (d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, CH*CH*$_2$CH(CH$_3$)$_2$), 40.90 (C-2'), 25.64, 25.38 (CHCH$_2$*CH*(CH$_3$)$_2$), 23.12, 22.95, 21.94, 14.5 (CHCH$_2$CH(*C*H$_3$)$_2$).

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: (M+Na$^+$) 678, $C_{32}H_{35}N_3O_9PNaF$ required: (M$^+$) 655.21

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(penthoxy-L-leucinyl)] phosphate (3.1q).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.45 g, 1.83 mmol), *t*BuMgCl (2.00 ml, 2.00 mmol) α-naphthyl-(pentoxy-L-leucinyl) phosphorochloridate (**2.3u**, 1.55 g, 3.65 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1q** as a white solid (0.034 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.48, 4.97

^{19}F-NMR (MeOD, 470 MHz) δ -167.17

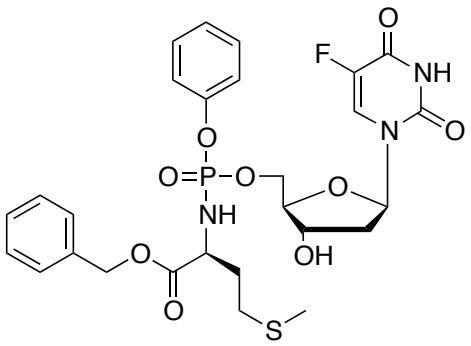
^{1}H-NMR (MeOD, 500 MHz) δ 8.20 – 8.18 (m, 1H, H-Ar), 7.90 – 7.88 (m, 1H, H-Ar), 7.75 – 7.70 (m, 2H, H-Ar), 7.55 – 7.51 (m, 3H, H-Ar), 7.44 – 7.40 (m, 1H, H-Ar), 6.18 – 6.13 (m, 1H, H-1'), 4.41 – 4.30 (m, 3H, H-3', H-5', H-5'), 4.12 – 4.04 (m, 1H, H-4'), 4.04 – 4.02 (m, 2H, $OCH_2(CH_2)_3CH_3$), 3.96 – 3.93 (m, 1H, $NHCHCH_2CH(CH_3)_2$), 2.20 – 2.18 (m, 1H, H-2'), 1.85 – 1.66 (m, 1H, H-2'), 1.67 – 1.58 (m, 5H, OCH_2CH_2, $CHCH_2CHCH_3$, $NHCHCH_2CH(CH_3)_2$), 1.31 – 1.28 (m, 4H, 2 × CH_2, ester).

^{13}C -NMR (MeOD, 125 MHz) 175.04, 174.98 (C=O, ester), 159.2 (C=O, base), 150.5 (d, $^{4}J_{C\text{-}F}$ = 7.29 Hz, C=O, base), 147.91, 147.90 (OC-Ar), 141.72 (d, $^{1}J_{C\text{-}F}$ = 236 Hz, C-F, base), 136.37 (C-Ar), 130.8, 128.95, 128.91, 127.87, 127.57, 127.5, 126.53, 126.19 (CH-Ar), 125.65, 125.56 (2 × d, $^{2}J_{C\text{-}F}$ = 35Hz, *C*-H, base), 122.70, 122.68 (CH-Ar), 116.42, 116.30 (CH-Ar), 86.95, 86.91 (C-1'), 86.69, 86.63 (C-4'), 72.23, 72.00 (C-3'), 67.90, 67.86 (d, $^{2}J_{C\text{-}P}$ = 4.45Hz, C-5'), 66.4 ($OCH_2(CH_2)_3CH_3$), 54.87, 54.80 (d, $^{2}J_{C\text{-}P}$ = 7.78Hz, $CHCH_3$), 44.16 ($NHCHCH_2CH(CH_3)_2$), 40.87, 40.80 (C-2'), 29.34, 29.14 ($CH_2(CH_2)_3CH_3$), 25.60 (CH_2-ester), 23.02, 21.82 ($CH(CH_3)_2$), 14.26 ($CH_2(CH_2)_3CH_3$).

HPLC (System 2) t_R = 19.32 min

(ES+) m/z, found: ($M+Na^+$) 658, $C_{30}H_{39}N_3O_9PNaF$ required: (M^+) 635.24

5-Fluoro-2'-deoxyuridine-5'-*O*-[phenyl-(benzoxy-L-methyonyl)]) phosphate (3.1r).



Prepared according to the standard procedure **5** from 5-fluoro-2'-deoxyuridine (0.20 g, 0.81 mmol), NMI (0.32 ml, 4.06 mmol) phenyl-(benzoxy-L-methioninyl) phosphorochloridate (**2.3v**, 0.67 g, 1.62 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1r** as a white solid (0.003 g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.34, 3.94

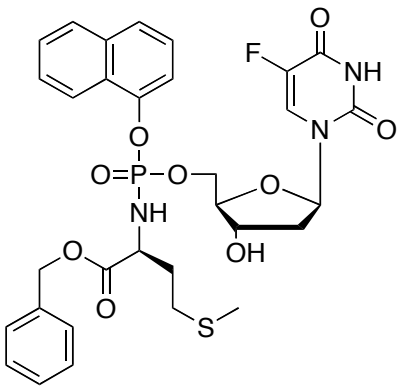
^{19}F-NMR (MeOD, 470 MHz) δ – 167.40, – 167.69

^{1}H-NMR (MeOD, 500 MHz) δ 7.83 – 7.80 (m, 1H, H-Ar), 7.74 – 7.72 (m, 1H, H-Ar), 7.64 – 7.62 (m, 1H, H-Ar), 7.37 – 7.32 (m, 6H, H-Ar, H-base), 7.26 – 7.17 (m, 2H, H-Ar), 6.25 – 6.17 (m, 1H, H-1'), 5.18, 5.13 (2AB, J_{AB} = 12.0 Hz, 2H, CH_2Ph), 4.40 – 4.35 (m, 1H, H-3'), 4.32 – 4.22 (m, 2H, H-5'), 4.16 – 4.03 (m, 2H, NHC*H*, *H*-4'), 2.44, 2.36 (2 x t, *J* = 7.50 Hz, CH_2S), 2.16 – 2.08 (m, 1H, 1 x *H*-2'), 1.98 – 1.82 (m, 6H, 1 x *H*-2', NHCHCH_2CH_2SCH_3).

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: (M+Na$^+$) 646, $C_{27}H_{31}FN_3O_9PNaS$ required: (M$^+$) 623.15

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(benzoxy-L-methyonyl)] phosphate (3.1s).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(benzoxy-L-methioninyl) phosphorochloridate (**2.3w**, 0.56 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1s** as a white solid (0.008 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.95, 4.39

^{19}F-NMR (MeOD, 470 MHz) δ -167.28,

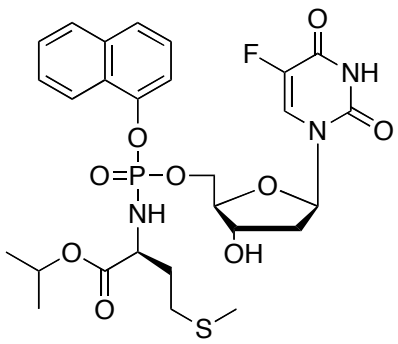
^{1}H-NMR (MeOD, 500 MHz) δ 8.19 – 8.15 (m, 1H, H-Ar), 7.90 – 7.88 (m, 1H, *H*-Ar), 7.75 - 7.70 (m, 2H, H-Ar), 7.56 - 7.50 (m, 3H, H-Ar), 7.44 - 7.28 (m, 6H, H-Ar), 6.15 – 6.10 (m, 1H, H-1'), 5.15 - 5.07 (m, 2H, OC*H*$_2$Ph), 4.36 - 4.15 (m, 4H, H-3', H-5', H-5', C*H*CH$_2$CH$_2$SCH$_3$), 4.07 - 4.03 (m, 1H, H-4'), 2.45 – 1.64 (m, 9H, 2 × H-2', CHC*H*$_2$C*H*$_2$SC*H*$_3$).

^{13}C-NMR (MeOD, 125 MHz) δ 173.92, 174.89 (*C*=O, ester), 157.53 (*C*=O, base), 150.66 (*C*=O, base), 147.85 (OC-Ar), 141.72 (d, $^{1}J_{C\text{-}F}$ = 244 Hz, CF-base), 136.76 (*C*-Ar), 129.62, 129.50, 129.45, 129.41,128.33, 127.88, 127.79,127.63, 127.56, 127.52, 126.52, 126.24, 126.19 (*C*H-Ar), 125.73, 125.46 (2 × d, $^{2}J_{C\text{-}F}$ = 35 Hz, CH-base), 122.71, 122.63 (CH-Ar), 116.44, 116.41 (CH-Ar), 86.98, 86.92 (C-1'), 86.64, 86.57 (C-4'), 72.22, 71.96 (C-3'), 68.15 (O*C*H$_2$Ph), 67.94 (d, $^{2}J_{C\text{-}F}$ = 6.0 Hz, C-5'), 55.14 (*C*HCH$_2$CH$_2$SCH$_3$), 40.87 (C-2'), 34.2, 33.79 (*C*HCH$_2$*C*H$_2$SCH$_3$), 30.83, 30.75 (*C*H$_2$CH$_2$SCH$_3$), 15.05, 14.93 (*C*H$_3$S).

HPLC (System 2) t_R = 12.13 min

(ES+) m/z, found: (M+Na$^+$) 696, $C_{31}H_{33}FN_3O_9PNaS$ required: (M$^+$) 673.17

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(isopropoxy-L-methyonyl)] phosphate (3.1t).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(isopropoxy-L-methioninyl) phosphorochloridate (**2.3x**, 0.50 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1t** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.93, 4.56

^{19}F-NMR (MeOD, 470 MHz) δ -167.32

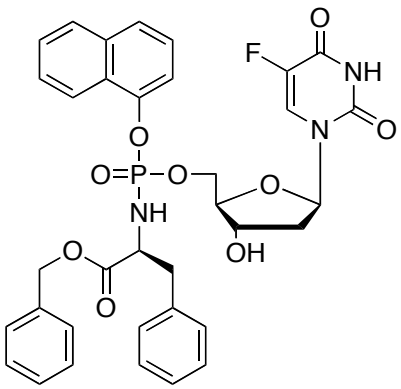
^{1}H-NMR (MeOD, 500 MHz) δ 8.20 – 8.17 (m, 1H, H-Ar), 7.89 – 7.87 (m, 1H, H-Ar), 7.76 – 7.69 (m, 2H, H-Ar), 7.56 – 7.52 (m, 3H, H-Ar), 7.45 - 7.40 (m, 1H, H-Ar), 6.15 – 6.13 (m, 1H, H-1') 5.01-4.94 (m, 1H, C*H*$(CH_3)_2$), 4.49 – 4.31 (m, 3H, H-3', H-5', H-5'), 4.14 – 4.07 (m, 2H, NHCHC*H*$_2$$CH_2SCH_3$), 2.52– 2.37 (m, 2H, NHCHCH_2C*H*$_2$$SCH_3$), 2.21 – 2.17, 2.12 – 2.07 (m, 1H, H-2'), 2.16 (s, 3H, NHCHCH_2CH_2SC*H*$_3$), 1.93 – 1.87, 1.85 – 1.79 (m, 1H, H-2'), 1.23 – 1.19 (m, 6H, CH(C*H*$_3$)$_2$).

^{13}C (MeOD, 125 MHz) δ 174.12, 173.71 (C=O, ester), 159.66, 159.45 88 (2 × d, $^{2}J_{C\text{-}F}$ = 27.53 Hz, C=O, base), 150.70 (C=O, base), 147.96, 147.90 (2 × d, $^{2}J_{C\text{-}P}$ =17.58 Hz, C=O, base), 141.78 (d, $^{1}J_{C\text{-}F}$ =322.5 Hz, C-F, base), 136.30 (C-Ar), 128.98 (CH-Ar), 127.94, 127.93, 127.65, 127.58, 126.58, 126.27, 126.22 (CH-Ar), 125.66, 125.60 (2 × d, $^{2}J_{C\text{-}F}$ = 34.71 Hz, C-H, base), 122.74, 122.67 (CH-Ar), 116.45, 116.39 (d, $^{4}J_{C\text{-}P}$ = 3.7 Hz, CH-Ar), 87.02, 86.96 (C-1'), 86.64, 86.54 (C-4'), 72.28, 71.99 (C-3'), 70.52, 70.49 (*C*H$(CH_3)_2$), 68.03, 67.94 (C-5'), 55.26, 55.16 (NH*C*H$CH_2CH_2SCH_3$), 40.87, 40.85 (C-2'), 34.35, 33.89 (NHCH*C*H$_2$$CH_2SCH_3$), 30.98, 30.88 (NHCH$CH_2$*C*H$_2$$SCH_3$), 22.05, 21.99 (NHCH$CH_2CH_2$S*C*H$_3$), 15.22, 15.10 (CH(*C*H$_3$)$_2$).

HPLC (System 2) t_R = 14.36 min

(ES+) m/z, found: ($M+Na^+$) 648, $C_{27}H_{33}FN_3O_9PNaS$ required: (M^+) 625.17

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphthyl-(benzoxy-L-phenylalaninyl)] phosphate (3.1u).



Prepared according to the standard procedure **5** from 5-fluoro-2'-deoxyuridine (0.25 g, 1.01 mmol), NMI (0.41 g, 5.07 mmol, 0.40 ml) α-naphthyl-(benzoxy-L-phenylalaninyl) phosphorochloridate (**2.3y**, 0.45 g, 2.00 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1u** as a white solid (0.007 g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.27, 4.14

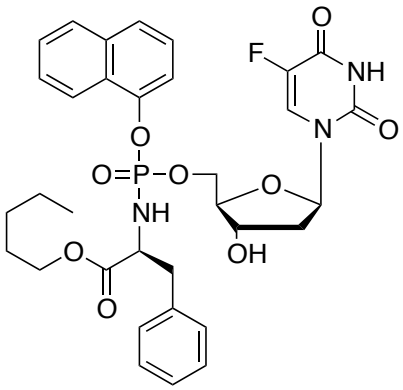
^{19}F-NMR (MeOD, 470 MHz) δ – 166.99, – 167.18

^{1}H-NMR (MeOD, 500 MHz) δ 8.11 – 8.00 (m, 1H, H-Ar), 7.89 – 7.85 (m, 1H, H-Ar), 7.69 – 7.67 (m, 1H, H-Ar), 7.60 – 7.49 (m, 3H, 2 x H-Ar, H-base), 7.37 – 7.33 (m, 2H, H-Ar), 7.25 – 7.12 (m, 10H, H-Ar), 6.09 – 6.04 (m, 1H, H-1'), 5.11 – 5.01 (m, 2H, C*H*$_2$Bn), 4.29 – 4.18 (m, 1H, NHC*H*CH$_2$Bn), 4.15 – 4.08 (m, 1H, H-3'), 4.02 – 3.95 (m, 2H, H-5'), 3.86 – 3.67 (m, 1H, H-4'), 3.14 – 3.10 (m, 1H, NHCHC*H*$_2$Bn), 2.91 – 2.82 (m, 1H, NHCHC*H*$_2$Bn), 2.12 – 2.06, 2.00 – 1.95 (2 x m, 1H, H-2'), 1.68 – 1.62, 1.42 – 1.36 (2 x m, 1H, H-2').

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: ($M+Na^+$) 662, $C_{31}H_{31}FN_3O_9PNa$ required: (M^+) 639.18

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(penthoxy-L-phenylalaninyl)]phosphate (3.1v).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(pentoxy-L-phenylalaninyl) phosphorochloridate (**2.3z**, 0.56 g, 1.21 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1v** as a white solid (0.008 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.39, 4.07

^{19}F-NMR (MeOD, 470 MHz) δ -167.19

^{1}H-NMR (MeOD, 500 MHz) δ 8.14 – 8.06 (2 × m, 1H, H-Ar), 7.88 – 7.86 (m, 1H, H-Ar), 7.69 – 7.68 (m, 2H, H-Ar), 7.56 - 7.51 (m, 3H, H-Ar), 7.41 - 7.35 (m, 2H, H-Ar), 7.27 – 7.18 (m, 5H, H-Ar), 6.10 – 6.06 (m, 1H, H-1'), 4.25 – 4.11 (2 × m, 3H, H-3', H-5', H-5'), 4.04 – 3.94 (m, 2H, H-4',OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$) 3.89 – 3.86, 3.69 – 3.66 (2 × m, 1H, NHC*H*CH$_2$Bn), 3.12 – 3.09, 2.92 – 2.84 (2 × m, 2H, NHCHC*H*$_2$Bn), 2.16 – 2.11, 2.00 – 1.97 (2 × m, 2H, H-2'), 1.73 – 1.67, 1.43 – 1.36 (2 × m, 2H, H-2'), 1.54 – 1.47, 1.28 – 1.20, 1.84 – 1.82 (3 × m, 9H, OCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_3$).

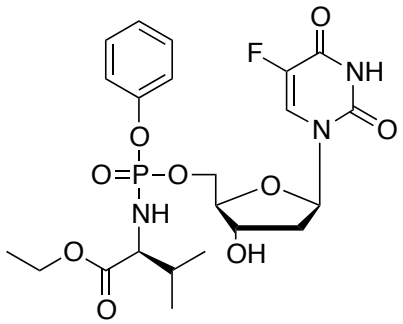
^{13}C (MeOD, 125 MHz) δ 174.21, 174.07 (C=O, ester), 159.38, 159.32 (2 × d, $^{2}J_{C\text{-}F}$ = 8.16 Hz, C=O), 150.53, 150.45 (C=O, base), 147.91, 147.85 (2 × d, $^{1}J_{C\text{-}F}$ = 233Hz, C-F, base), 138.39, 138.22 (C-Ar), 136.27(C-Ar), 130.64, 130.55, 129.69, 129.60, 128.94, 128.06, 127.98, 127.92, 127.85, 127.81, 127.68, 1272, 127.57, 127.50, 126.55, 126.17, 126.09 (*C*H-Ar), 125.54, 125.30 (2 × d, $^{2}J_{C\text{-}F}$ = 34Hz, *C*H-base), 122.74, 122.49 (*C*H-Ar), 116.0, 116.17 (2 × d, $^{4}J_{C\text{-}P}$ = 68Hz, *C*H-Ar), 86.98, 86.89 (C-1'), 72.26, 71.09 (C-3'), 67.50, 67.46 (2 × d, $^{2}J_{C\text{-}P}$ = 4.58 Hz, C-5'), 66.59, 66.51 (OCH$_2$), 58.32, 57.96 (NH*C*HCH$_2$Bn), 41.07, 41.02 (NHCH*C*H$_2$Bn), 40.94, 40.80 (C-2'), 29.32, 29.28, 29.08 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 23.31(OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$),

14.25 ($OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: ($M+Na^+$) 692, $C_{33}H_{37}FN_3O_9PNa$ required: (M^+) 669.23

5-Fluoro-2'-deoxyuridine-5'-*O*-[phenyl-(ethoxy-L-valinyl)] phosphate (3.1w).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.25 g, 1.01 mmol), *t*BuMgCl (1.34 ml, 1.34 mmol) phenyl-(ethoxy-L-valinyl) phosphorochloridate (**2.4a**, 0.65 g, 2.43 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1w** as a white solid (0.005 g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.96, 4.69

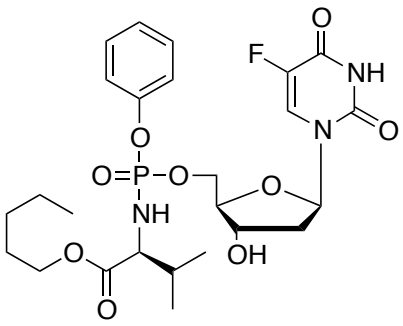
^{19}F-NMR (MeOD, 470 MHz) δ -167.54

^{1}H-NMR (MeOD, 500 MHz) δ 7.86 – 7.80 (m, 1H, H-Ar), 7.39 – 7.32 (m, 2H, H-Ar), 7.28 – 7.22 (m, 2H, H-Ar), 7.22 – 7.16 (m, 2H, H-Ar), 6.25 – 6.20 (t, *J* = 6.4 Hz, 1H, H-1'), 4.43-4.38 (m, 6H, O*CH*$_2$CH$_3$, H-3', H-4', H-5'), 3.73 – 3.63 (t, *J* = 8.3 Hz, 1H, NHCH*CH*$(CH_3)_2$), 2.34 – 2.22 (m, 1H, H-2'), 2.15 – 1.97 (m, 2H, H-2', NHC*HC*H$(CH_3)_2$), 1.32 – 1.21 (m, CH*CH*$(CH_3)_2$, OCH$_2$*CH*$_3$), 1.00 – 0.86 (m, 6H, CHCH*(CH*$_3$*)*$_2$).

HPLC (System 2) t_R = 11.77 min

(ES-) m/E, found: ($M+Cl^-$) 564, $C_{22}H_{29}FN_3O_9PNa$ required: (M^+) 529.16

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-phenyl-(pentoxy-valinyl)] phosphate (3.1x).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) phenyl-(pentoxy-L-valinyl) phosphorochloridate (**2.4b**, 0.44 g, 1.22 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1x** as a white solid (0.003 g, 1%).

^{31}P-NMR (MeOD, 202 MHz) δ 4.95, 4.65

^{19}F-NMR (MeOD, 470 MHz) δ -167.40

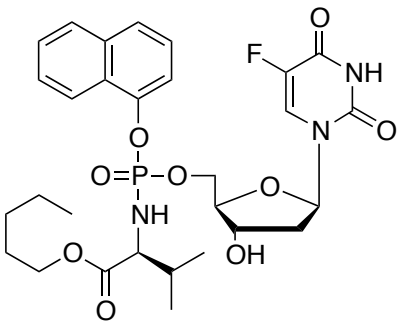
^{1}H-NMR (MeOD, 500 MHz) δ 7.87 - 7.80 (m, 1H, H-Ar) , 7.39 - 7.31 (m, 2H, H-Ar), 7.27 - 7.23 (m, 2H, H-Ar), 7.22 - 7.16 (m, 1H, H-Ar), 6.27 - 6.19 (m, 1H, H-1'), 4.50 - 4.28 (m, 3H, H-3',H-5', H-5'), 4.14 – 4.01(m, 3H, H-4', $OCH_2CH_2CH_2CH_2CH_3$), 3.71 - 3.65 (m, 1H, $NHCHCH(CH_3)_2$), 2.14-1.98 (m, 2H, H-2'), 1.60 - 1.57, 1.40 – 1.26, 1.14 - 1.08 (3 × m, 17H, $NHCHCH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$,$CH(CH_3)_2$.

^{13}C (MeOD, 125 MHz) δ 174.10, 174.09 (C=O, ester), 152.10 (2 × d, $^{2}J_{C\text{-}F}$ = 7.56 Hz, C-F, base), 150.69 (C=O, base), 142.76, 140.91 (2 × d, $^{2}J_{C\text{-}F}$ = 7.68 Hz, C=O), 130.79 (C-Ar), 126.32, 126.23, 125.94, 125.89, 125.63 (2 × d, $^{2}J_{C\text{-}F}$ = 33.36 Hz, C-H), 86.96, 86.85 (C-1'), 86.67, 86.60 (2 × d, $^{2}J_{C\text{-}P}$= 7.33 Hz, C-4'), 72.13, 71.93 (C-3'), 67.85, 67.57 (2 × d, $^{2}J_{C\text{-}P}$ = 6.11 Hz, C-5'), 66.33 ($OCH_2CH_2CH_2CH_2CH_3$), 62.04 ($NHCHCH(CH_3)_2$), 40.93, 40.90 (C-2'), 33.29, 33.24 ($NHCHCH(CH_3)_2$), 29.41, 29.20, 23.33 ($OCH_2CH_2CH_2CH_2CH_3$), 19.55, 19.06, 18.40, 18.10, 14.29 ($OCH_2CH_2CH_2CH_2CH_3$, $NHCHCH(CH_3)_2$).

HPLC (System 2) t_R = HPLC data lost

(ES+) m/z, found: ($M+Na^+$) 594, $C_{25}H_{35}FN_3O_9PNa$ required: (M^+) 571.21

5-Fluoro-2'-deoxyuridine-5'-*O*-[1-naphtyl-(pentoxy-L-valinyl)] phosphate (3.1y).



Prepared according to the standard procedure **4** from 5-fluoro-2'-deoxyuridine (0.15 g, 0.61 mmol), *t*BuMgCl (0.67 ml, 0.67 mmol) α-naphthyl-(pentoxy-L-valinyl) phosphorochloridate (**2.4c**, 0.50 g, 1.22 mmol) in THF (10 ml). The crude mixture was purified by column chromatography, using CH_2Cl_2/MeOH (1 to 5% gradient) as an eluent system, followed by preparative purification to give the pure product **3.1y** as a white solid (0.007 g, 2%).

^{31}P-NMR (MeOD, 202 MHz) δ 5.39, 5.20

^{19}F-NMR (MeOD, 470 MHz) δ -167.21

^{1}H-NMR (MeOD, 500 MHz) δ 8.20 - 8.18 (m, 1H, H-Ar), 7.87 (m, 1H, H-Ar), 7.75 – 7.68 (m, 2H, H-Ar), 7.54 - 7.50 (m, 3H, H-Ar), 7.42 - 7.40 (m, 1H, H-Ar), 6.16 – 6.15 (m, 1H, H-1') 4.43 – 4.30 (m, 3H, H-3', H-5', H-5'), 4.14 – 3.99 (m, 3H, H-4', OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 3.75 – 3.74 (m, 1H, NHC*H*CH(CH$_3$)$_2$), 2.26 – 1.60 (m, 3H, H-2', NHCHC*H*(CH$_3$)$_2$), 1.60 – 1.50, 1.42 – 1.21, 1.02– 0.78 (3 × m, 15H, OCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_3$, CH(C*H*$_3$)$_2$).

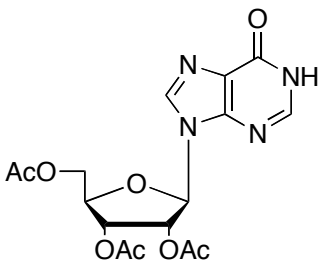
^{13}C (MeOD, 125 MHz) δ 174.45, 174.14 (C=O-ester), 159.51 (2 × d, $^{2}J_{C\text{-}F}$ = 26.78 Hz, C=O, base), 150.54 (C=O, base), 148.00, 147.94 (2 × d, $^{2}J_{C\text{-}F}$ = 7.30 Hz, C=O), 136.27 (C-Ar), 128.97, 127.93, 127.89, 127.86, 127.83, 127.76, 127.61, 127.54, 126.58, 126.19, 126.16 (*C*H-Ar), 125.70 (d, $^{2}J_{C\text{-}F}$ = 34.09 Hz, *C*H-base), 122.74, 122.69 (*C*H-Ar), 116.40, 116.37 (2 × d, $^{4}J_{C\text{-}P}$ = 2.50 Hz, *C*H-Ar), 87.02, 86.98 (C-1'), 86.79, 86.74 (2 × d, $^{3}J_{C\text{-}P}$ = 7.27 Hz, C-4'), 72.19, 71.98 (C-3'), 67.98, 67.94 (2 × d, $^{2}J_{C\text{-}P}$ = 5.45 Hz, C-5'), 66.38, 66.23 (OCH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 62.24, 62.15 (CH-α), 40.87, 40.80 (C-2'), 33.19, 33.13 (NHCH*C*H(CH$_3$)$_2$), 29.34, 29.25, 23.35, 23.33(OCH$_2$*C*H$_2$*C*H$_2$*C*H$_2$CH$_3$), 19.64, 19.20, 18.47, 18.33, 18.08, 14.38, (OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_3$, NHCHCH(*C*H$_3$)$_2$).

HPLC (System 2) t_R = 17.04 min

(ES+) m/z, found: (M+Na$^+$) 644, $C_{29}H_{37}FN_3O_9P$ required: (M$^+$) 621.23

8.5 Experimental section – Chapter 4

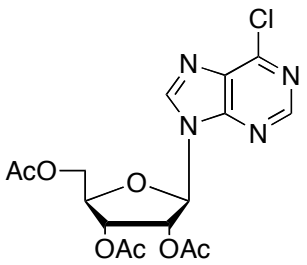
Synthesis of 2',3',5'-tri-*O*-acetyl-inosine (4.4).



To a suspension of commercially available inosine (3.00 g, 11.18 mmol) DMAP (0.23 g, 1.90 mmol) and Et_3N (6.23 ml, 44.73 mmol) in anhydrous ACN (115 ml) acetic anhydride was added dropwise (3.80 ml, 40.26 mmol). The reaction mixture was allowed to stir at ambient temperature overnight. Anhydrous MeOH (25 ml) was added to quench the reaction and the pure product **4.4** (3.0 g, 68%) precipitated as a white solid after the addition of Et_2O (300 ml).

^{1}H NMR (500 MHz, MeOD) δ 8.22 (s, 1H, H-8), 8.10 (s, 1H, H-2), 6.24 (d, 1H, *J* = 5 Hz, 1H, H-1'), 5.99 (t, 1H, *J* = 6 Hz, 1H, H-2'), 5.69 (t, 1H, *J* = 5.5 Hz, 1H, H-3'), 4.48 – 4.43 (m, 2H, H-4', H-5'), 4.38 (dd, 1H, *J* = 11.5 Hz, 4.5 Hz, H-5'), 2.15 (s, 3H, CH_3 - acetyl), 2.09, 2.07 (2 x s, 6H, 2 x CH_3 - acetyl).
^{13}C NMR (125 MHz, MeOD) δ 172.20, 171,37, 171.14 (*C*=O, acetyl), 158.85 (C=O, base), 149.82 (C-4), 147.10 (C-2), 141.01 (C-8), 126.20 (C-5), 88.34 (C-1'), 81.71 (C-2'), 74.54 (C-3'), 71.95 (C-4'), 64.19 (C-5'), 20.63, 20.43, 20.25 (3 x *C*H_3 - acetyl).

Synthesis of 2',3',5'-tri-*O*-acetyl-6-chloro-inosine (4.5).

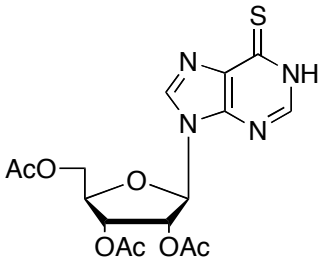


The suspension of 2',3',5'-tri-*O*-acetyl-inosine (**4.4,** 1.0 g, 2.54 mmol), BTEA-Cl (1.15 g, 5.07 mmol), N, N-dimethylaniline (0.35 ml, 2.79 mmol) and $POCl_3$ (1.18 ml, 12.68 mmol) in anhydrous ACN (65 ml) were heated under reflux at 85^{o}C for 3 hrs. After that time volatiles were evaporated under reduced pressure to give the crude mixture as a yellow oil. The crude oil was dissolved in $CHCl_3$ and it was allowed to stir in the presence of crushed ice at ambient temperature for 30 minutes. The two layers were separated and the aqueous layer was extracted with $CHCl_3$. The combined organic layers were

washed with cold water and 5% aqueous solution of $NaHCO_3$, dried over $MgSO_4$ and evaporated under reduced pressure to give the title compound **4.5** (0.93 g, 89%).

^{1}H NMR (500 MHz, MeOD) δ 8.74 (s, 1H, H-8), 8.66 (s, 1H, H-2), 6.35 (d, 1H, *J* = 5 Hz, 1H, H-1'), 6.05 (t, 1H, *J* = 5.5 Hz, 1H, H-2'), 5.73 (t, 1H, *J* = 5.5 Hz, 1H, H-3'), 4.48 (q, *J* = 3.5 Hz, 1H, H-4'), 4.44 (dd, *J* = 12.5 Hz, 3.5 Hz, 1H, H-5'), 4.38 (dd, *J* = 12.5 Hz, 4.5 Hz, 1H, H-5'), 2.14 (s, 3H, C*H*$_3$ - acetyl), 2.07, 2.06 (2 x s, 6H, 2 x C*H*$_3$ - acetyl).
^{13}C NMR (125 MHz, MeOD) δ 172.15, 171,37, 171.19 (*C*=O, acetyl), 153.33 (C-2), 152.67 (C-4), 151.79 (C-Cl), 146.98 (C-8), 133.15 (C-5), 88.67 (C-1'), 81.85 (C-2'), 74.40 (C-3'), 71.89 (C-4'), 64.17 (C-5'), 20.86, 20.65, 20.47 (3 x *C*H$_3$ - acetyl).

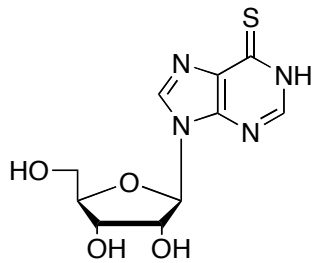
Synthesis of 2',3',5'-tri-*O*-acetyl-6-thioinosine (4.6).



The suspension of 2',3',5'-tri-*O*-acetyl-inosine (**4.4,** 1.0 g, 2.54 mmol) and Lawesson's reagent (2.26 g, 5.58 mmol) in anhydrous toluene (60 ml) were heated under reflux at 110^{o}C for 3 hrs until the consumption of the starting material. The reaction mixture was allowed to slowly cool down and the majority of the precipitated Lawesson's reagent was removed by filtration. The reaction mixture was evaporated under reduced pressure and purified by column chromatography, using $CHCl_3$/MeOH (2 to 5% gradient) as an eluent system to give **4.6** as a yellow solid (0.57 g, 55%).

^{1}H NMR (500 MHz, MeOD) δ 8.34 (s, 1H, H-2), 8.16 (s, 1H, H-8), 6.25 (d, 1H, *J* = 5, 1H, H-1'), 5.99 (t, 1H, *J* = 5.5, 1H, H-2'), 5.68 (t, 1H, *J* = 5.5, 1H, H-3'), 4.48 (q, *J* = 3.5, 1H, H-4'), 4.49 – 4.40 (m, 2H, H-4', H-5'), 4.38 (dd, *J* =12, 4.5, 1H, H-5'), 2.15 (s, 3H, CH_3 - acetyl), 2.09, 2.08 (2 x s, 6H, 2 x CH_3 - acetyl).
^{13}C NMR (125 MHz, MeOD) δ 178.70 (C=S), 172.19, 171,36, 171.14 (*C*=O - acetyl), 146.65 (C-2), 144.77 (C-4), 142.73 (C-8), 137.30 (C-5), 88.44 (C-1'), 81.79 (C-2'), 74.50 (C-3'), 71.93 (C-4'), 64.15 (C-5'), 20.63, 20.42, 20.24 (3 x *C*H$_3$ - acetyl).

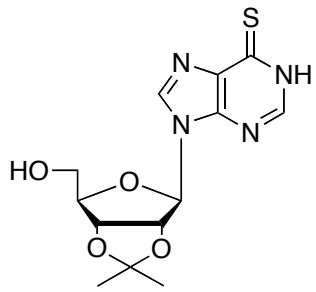
Synthesis of 6-thioinosine (4.3).



2',3',5'-tri-*O*-acetyl-6-thioinosine (**4.6,** 0.5 g, 1.22 mmol) was dissolved in saturated solution of NH_4OH (0.1 ml), then adjusted to neutral pH with 0.1M acetic acid and kept in the fridge at -5°C for 3-5 days until the appearance of the yellowish crystals. The crystals were filtered off and used in the next step without further purification to give the title compound **4.3** (0.17 g, 48%).

^{1}H NMR (500 MHz, MeOD) δ 8.49 (s, 1H, H-2), 8.14 (s, 1H, H-8), 6.05 (d, 1H, *J* = 5 Hz, 1H, H-1'), 4.63 (t, 1H, *J* = 5 Hz, 1H, H-2'), 4.35 (t, 1H, *J* = 3.5 Hz, 1H, H-3'), 4.16 (q, *J* = 3.5 Hz, 1H, H-4'), 3.88 (dd, *J* = 12.5 Hz, 3 Hz, 1H, H-5'), 3.77 (dd, *J* = 12.5 Hz, 3 Hz, 1H, H-5').
^{13}C NMR (125 MHz, MeOD) δ 178.63 (*C*=S), 146.49 (C-2), 144.80 (C-4), 142.73 (C-8), 137.21 (C-5), 90.59 (C-1'), 87.56 (C-2'), 76.22 (C-3'), 72.10 (C-4'), 62.91 (C-5').

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine (4.10).



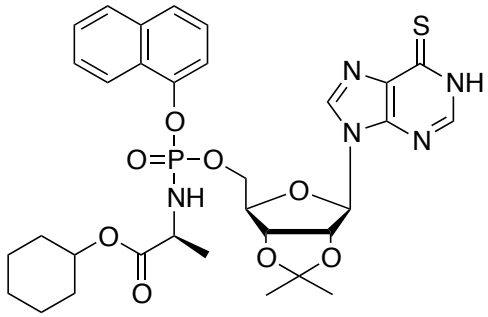
To the solution of 6-thioinosine (**4.3**, 1.40 g, 4.92 mmol) in anhydrous acetone (50 ml) 60% aqueous solution of perchloric acid (0.90 ml) was added dropwise and stirred overnight under inert atmosphere at ambient temperature. Saturated solution of NH_4OH was added dropwise in order to reach neutral pH. The reaction mixture was evaporated and the resulting white solid was purified by column chromatography ($CHCl_3$/MeOH, 7:3) to give the protected nucleoside **4.10** (1.24 g, 78%).

^{1}H NMR (500 MHz, MeOD) δ 8.46 (s, 1H, H-2), 8.14 (s, 1H, H-8), 6.22 (d, 1H, *J* = 3 Hz, 1H, H-1'), 5.30 (dd, *J* = 6 Hz, 2.5 Hz, 1H, H-2'), 5.01 (dd, *J* = 6 Hz, 2.5 Hz, 1H, H-3'), 4.37 (q, *J* = 4 Hz, 1H, H-4'), 3.77 (dd, *J* = 12 Hz, 4 Hz, 1H, H-5'), 3.72

(dd, *J* = 12 Hz, 4 Hz, 1H, H-5'), 1.62 (s, 3H, CH_3 - isopropylidene), 1.40 (s, 3H, CH_3 - isopropylidene).

^{13}C NMR (125 MHz, MeOD) δ 178.12 (*C*=S), 146.33 (C-2), 144.40 (C-4), 142.80 (C-8), 137.04 (C-5), 123.42 (*C*$(CH_3)_2$ - isopropylidene), 92.54 (C-1'), 88.78 (C-2'), 86.05 (C-3'), 82.93 (C-4'), 63.29 (C-5'), 27.54 (*C*H_3 - isopropylidene), 25.53 (*C*H_3 - isopropylidene).

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.11a).

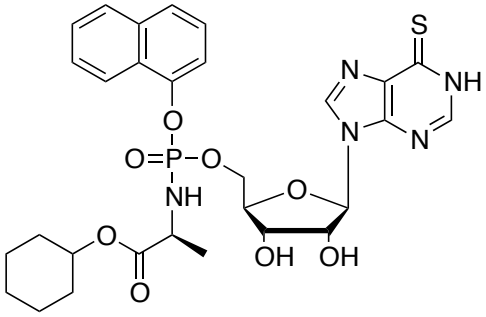


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.168 g, 0.518 mmol) *t*BuMgCl (1.0 M in THF, 1.03 ml, 1.03 mmol) and 1-naphthyl-(cyclohexoxy-L-alaninyl)phosphorochloridate (**2.3j**, 0.48 g, 1.23 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent sytem to give the pure product **4.11a** as a yellow foam (0.088 g, 25%).

^{31}P NMR (202 MHz, MeOD) δ 4.24, 3.96

^{1}H NMR (500 MHz, MeOD) δ 8.27, 8.26 (2 x s, 1H, H-2), 8.11 - 8.09 (m, 0.6H, H-Ar), 8.03, 7.99 (2 x s, 1H, H-8), 7.97 (d, *J* = 8 Hz, 0.4H, H-Ar), 7.85 – 7.8 (m, 0.6H, H-Ar), 7.81 (d, *J* = 7.5 Hz, 0.4H, H-Ar), 7.67 – 7.64 (m, 1H, H-Ar), 7.52 – 7.33 (m, 4H, H-Ar), 6.16, 6.07 (2 x dd, *J* = 2.5 Hz, 1H, H-1'), 5.17, 5.03 (2 x dd, *J* = 2.5 Hz, 1H, H-2'), 4.81, 4.74 (2 x dd, *J* = 2.5 Hz, 1H, H-3'), 4.70 – 4.65 (m, 1H, C*H*-ester), 4.53 – 4.51 (m, 0.4H, H-4'), 4.46 – 4.44 (m, 0.6H, H-4'), 4.41 – 4.28 (m, 2H, H-5', H-5'), 4.01 – 3.93 (m, 1H, C*H*CH_3), 1.77 – 1.66 (m, 4H, 2 x CH_2-ester), 1.57, 1.55, 1.35, 1.34 (4s, 6H, 2 x CH_3 isopropylidene), 1.31, 1.29 (2 x d, ^{3}J = 7.0 Hz, 3H, CHC*H*$_3$), 1-28 – 1.21 (m, 6H, 3 x CH_2-ester).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.12a).



Prepared according to standard procedure **7** from, 2', 3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (**4.11a**, 0.088 g, 0.128 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, to give the pure product **4.12a** as a yellow foam (0.017 g, 21%).

^{31}P NMR (202 MHz, MeOD) δ 4.18, 4.14

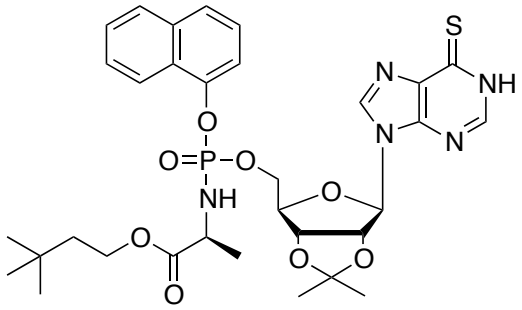
^{1}H NMR (500 MHz, MeOD) δ 8.36, 8.35 (2 x s, 1H, H-2), 8.12 - 8.07 (m, 1H, H-Ar), 8.08, 8.02 (2 x s, 1H, H-8), 7.84 – 7.80 (m, 1H, H-Ar), 7.66 (t, *J* = 7.0 Hz, 1H, H-Ar), 7.48 – 7.43 (m, 3H, H-Ar), 7.34, 7.33 (dt, *J* = 8.0 Hz, 1H, H-Ar), 6.16, 6.07 (2 x d, *J* = 5.0 Hz, 1H, H-1'), 4.66 (t, *J* = 5.0 Hz, 1H, H-2'), 4.64 – 4.60 (m, 1H, C*H*-ester), 4.51 – 4.42 (m, 3H, H-3', H-5', H-5'), 4.31 – 4.29 (m, 1H, H-4'), 4.02 – 3.92 (m, 1H, C*H*CH_3), 1.70 – 1.65 (m, 4H, 2 x CH_2-ester), 1.28, 1.27 (2 x d, ^{3}J = 7.0 Hz, 3H, CHC*H*$_3$), 1.24 – 1.19 (m, 6H, 3 x CH_2-ester).

^{13}C NMR (125 MHz, MeOD) δ 178.08, 178.07 (C=S), 174.57, 174.30 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.25 Hz, 4.37 Hz, C=O), 147.92, 147.87 (C-2), 144.94 (C-Ar), 142.87, 142.78 (C-4), 140.46, 140.37 (C-8), 137.01, 136.97 (C-5), 128.92, 128.87, 127.80, 127.49, 126.47, 126.42, 125.99, 122.67, 122.58, 116.16, 116.14 (C-Ar), 90.71, 90.59 (C-1'), 84.69, 84.57 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 75.28, 75.22 (C-2'), 75.01 (*C*H-ester), 71.56, 71.47 (C-3'), 67.78, 67.51 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ =5.25, C-5'), 51.87, 51.82 (*C*HCH_3), 32.39, 32.33 (CH_2-ester), 26.37 (CH_2-ester), 24.64, 24.60 (CH_2-ester), 20.68, 20.47 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.36, 17.54 min

(ES+) m/z, found: (M+Na^+) 666.20, $C_{29}H_{34}N_5O_8PS$ required: (M^+) 643.19

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl)]-phosphate (4.11b).

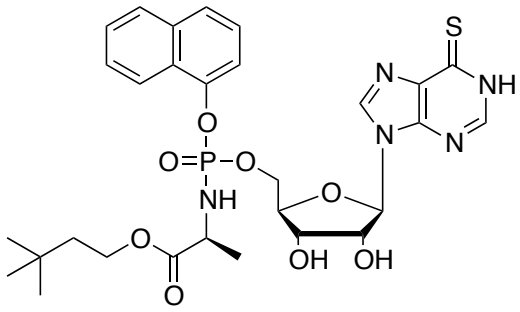


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.164 g, 0.50 mmol) *t*BuMgCl (1.0 M in THF, 1.01 ml, 1.01 mmol) and 1-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl)phosphorochloridate (**2.3k**, 0.40 g, 1.01 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11b** as a yellow foam (0.148 g, 43%).

^{31}P NMR (202 MHz, MeOD) δ 4.19, 3.97

^{1}H NMR (500 MHz, MeOD) δ 8.52 – 8.48 (m, 0.4H, H-Ar), 8.29, 8.28 (2 x s, 1H, H-2), 8.11 - 8.09 (m, 0.6H, H-Ar), 8.04, 7.99 (2 x s, 1H, H-8), 7.84 – 7.79 (m, 1H, H-Ar), 7.65 – 7.59 (m, 1H, H-Ar), 7.51 – 7.33 (m, 4H, H-Ar), 6.16, 6.07 (2 x dd, J = 2.5 Hz, 1H, H-1'), 5.17, 5.03 (2 x dd, J = 2.5 Hz, 1H, H-2'), 4.84, 4.79 (2 x dd, J = 2.5 Hz, 1H, H-3'), 4.53 – 4.45 (m, 1H, H-4'), 4.40 – 4.29 (m, 2H, H-5', H-5'), 4.10 – 3.95 (m, 3H, C*H*CH_3, O$CH_2$$CH_2C(CH_3)_3$), 1.57, 1.54, 1.34, 1.33 (4s, 6H, 2 x CH_3-isopropylidene), 1.47 -1.43 (m, 2H, OCH_2CH_2C$(CH_3)_3$), 1.32 – 1.26 (2 x d, J = 7.0 Hz, 3H, CHCH_3), 0.88, 0.87 (2 x s, 9H, O$CH_2$$CH_2C(CH_3)_3$).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butyl-L-alaninyl)]-phosphate (4.12b).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butyl-L-alaninyl)]-phosphate (**4.11b**, 0.148 g, 0.216 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, to give the pure product **4.12b** as a yellow foam (0.016 g, 12%).

^{31}P NMR (202 MHz, MeOD) δ 4.07, 4.05

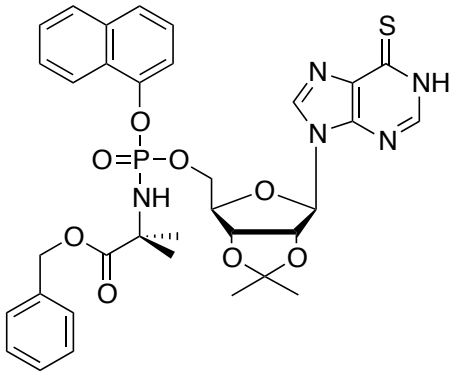
^{1}H NMR (500 MHz, MeOD) δ 8.36 (s, 1 H, H-2), 8.15 - 8.09 (m, 1H, H-Ar), 8.02 (s, 1H, H-8), 7.89 – 7.85 (m, 1H, H-Ar), 7.68 (t, *J* = 7.5 Hz, 1H, H-Ar), 7.53 – 7.45 (m, 3H, H-Ar), 7.39 - 7.35 (m, 1H, H-Ar), 6.02 (t, *J* = 5.0 Hz, 1H, H-1'), 4.67 – 4.65 (m, 1H, H-2'), 4.49 – 4.42 (m, 3H, H-3', H-5', H-5'), 4.31 – 4.29 (m, 1H, H-4'), 4.07 – 3.91 (m, 3H, C*H*CH_3, OC*H*$_2$$CH_2C(CH_3)_3$), 1.46 – 1.40 (m, 2H, OCH_2C*H*$_2$$C(CH_3)_3$), 1.30, 1.26 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$), 0.88, 0.87 (2 x s, 9H, $OCH_2CH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.12 (C=S), 174.57, 174.30 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.37 Hz, 4 Hz, C=O), 147.93, 147.89 (C-2), 144.92 (C-Ar), 142.86, 142.71 (C-4), 136.25, 136.21, 128.93, 128.88, 127.80, 127.49, 126.46, 126.42, 125.99, 125.95, 122.66, 122.60, 116.11, 116.09 (C-Ar), 90.75, 90.57 (C-1'), 84.70, 84.58 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 75.30, 75.24 (C-2'), 71.58, 71.45 (C-3'), 67.73, 67.42 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 64.01, 64.01 (O*C*$H_2CH_2C(CH_3)_3$), 51.72 (*C*HCH_3), 42.72, 42.68 (OCH_2*C*$H_2C(CH_3)_3$), 30.69 (OCH_2CH_2C(*C*H_3)$_3$), 29.94, 29.91(OCH_2CH_2*C*$(CH_3)_3$), 20.48, 20.27 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.76, 17.42 min

(ES+) m/z, found: (M+Na^+) 668.10, $C_{29}H_{36}N_5O_8PNaS$ required: (M^+) 645.20

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (4.11c).

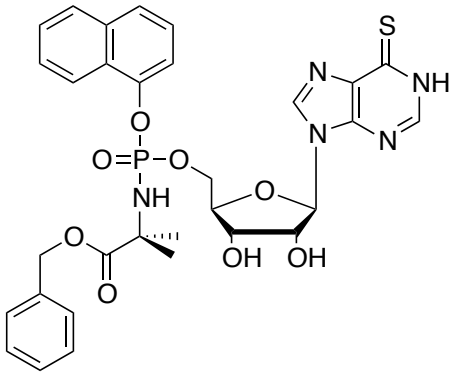


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.20 g, 0.61 mmol) *t*BuMgCl (1.0 M in THF, 1.23 ml, 1.23 mmol) and 1-naphthyl-(benzoxy-dimethylglicinyl)phosphorochloridate (**2.3l**, 0.50 g, 1.23 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11c** as a yellow foam (0.14 g, 32%).

^{31}P NMR (202 MHz, MeOD) δ 2.70, 2.56

^{1}H NMR (500 MHz, MeOD) δ 8.26, 8.21 (2 x s, 1H, H-2), 8.01, 7.95 (2 x s, 1H, H-8), 7.83 – 7.77 (m, 1H, H-Ar), 7.63 – 7.55 (m, 1H, H-Ar), 7.48 – 7.24 (m, 10H, H-Ar), 6.15, 6.04 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.18 – 4.98 (m, 3H, H-2', C*H*$_2$Bn), 4.78 – 4.76 (m ,1H, H-3'), 4.41 -4.40 (m, 1H, H-4'), 4.29 – 4.27 (m, 2H, H-5', H-5'), 1.55, 1.53, 1.33, 1.28 (4s, 6H, 2 x CH_3-isopropylidene), 1.51, 1.47 (2 x d, *J* = 7.0 Hz, 6H, 2 x CHC*H*$_3$), 1.46, 1.45 (2 x s, CHC*H*$_3$).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (4.12c).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (**4.11c**, 0.14 g, 0.197 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, to give the pure product **4.12c** as a yellow foam (0.030 g, 23%).

^{31}P NMR (202 MHz, MeOD) δ 2.61, 2.56

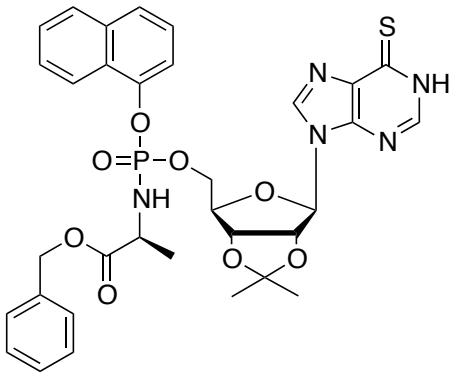
^{1}H NMR (500 MHz, MeOD) δ 8.33, 8.28 (2 x s, 1H, H-2), 8.14 - 8.10 (m, 1H, H-Ar), 8.02, 7.95 (2 x s, 1H, H-8), 7.83 – 7.78 (m, 1H, H-Ar), 7.63 – 7.59 (m, 1H, H-Ar), 7.47 – 7.40 (m, 3H, H-Ar), 7.32 - 7.21 (m, 6H, H-Ar), 6.01, 5.97 (2 x d, J = 5.0 Hz, 1H, H-1'), 5.13 – 5.05 (m, 2H, CH_2Bn), 4.64, 4.60 (2 x t, J = 5.0 Hz, 1H, H-2'), 4.45 – 4.37 (m, 2H, H-5', H-5', 0.5H, H-3'), 4.34 (m, 0.5H, H-3'), 4.28 – 4.24 (m, 1H, H-4'), 1.47 (s, 6H, CHCH_3).

^{13}C NMR (125 MHz, MeOD) δ 178.14 (C=S), 176.50, 176.43 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.25 Hz, 4.25 Hz, C=O), 157.81, 153.13, 150.49, 147.96, 147.90, 145.62, 145.58, 137.25, 137.23, 136.20, 136.15, 133.12, 133.07, 129.52, 129.20, 128.83, 128.78, 127.69, 127.67, 127.31, 127.28, 126.38, 126.35, 125.84, 125.79, 122.82, 116.30 (C-Ar), 90.76, 90.66 (C-1'), 84.68, 84.55 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.25 Hz, C-4'), 75.06, 74.95 (C-2'), 71.61, 71.51 (C-3'), 68.28 (m, CH$_2$Bn), 67.79, 67.67 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.75 Hz, C-5'), 58.22 (NHC(CH$_3$)$_2$), 27.77, 27.50 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 3.6 Hz, NHC(CH$_3$)$_2$).

HPLC (System 2) t_R = 16.83, 17.16 min

(ES+) m/z, found: (M+Na$^+$) 688.20, $C_{31}H_{32}N_5O_8PNaS$ required: (M$^+$) 665.17

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (4.11d).

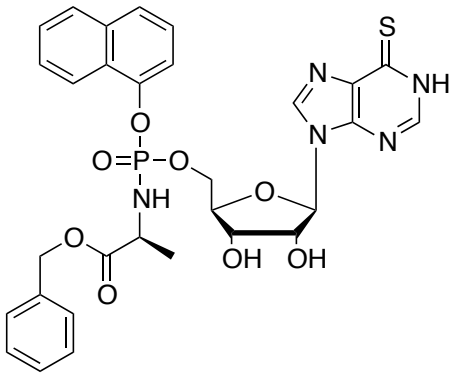


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.15 g, 0.46 mmol) *t*BuMgCl (1.0 M in THF, 0.92 ml, 0.92 mmol) and 1-naphthyl-(benzoxy-L-alaninyl)phosphorochloridate (**2.3c**, 0.36 g, 0.92 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11d** as a yellow foam (0.108 g, 34%).

^{31}P NMR (202 MHz, MeOD) δ 4.19, 3.78

^{1}H NMR (500 MHz, MeOD) δ 8.24 (s, 1H, H-2), 8.08 – 8.06 (m, 0.6H, H-Ar), 8.04, 8.00 (2 x s, 1H, H-8), 7.95 – 7.92 (m, 0.4H, H-Ar), 7.79 – 7.75 (m, 1H, H-Ar), 7.60 – 7.59 (m, 1H, H-Ar), 7.45 – 7.37 (m, 3H, H-Ar), 7.31 – 7.21 (m, 6H, H-Ar), 6.09, 5.99 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.09 – 4.95 (m, 3H, C*H*$_2$Bn, H-2', 0.6H, H-2'), 4.61, 4.60 (dd, *J* = 2.5 Hz, 0.4H, H-3'), 4.44 -4.40 (2 x m, 1H, H-4'), 4.35 – 4.22 (2 x m, 2H, H-5', H-5'), 4.11 – 4.04 (m, 1H, C*H*CH$_3$), 1.55, 1.52, 1.30, 1.26 (4s, 6H, 2 x CH_3-isopropylidene), 1.31 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (4.12d).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (**4.11d**, 0.108 g, 0.157 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, to give the pure product **4.12d** as a yellow foam (0.026 g, 26%).

^{31}P NMR (202 MHz, MeOD) δ 4.19, 4.02

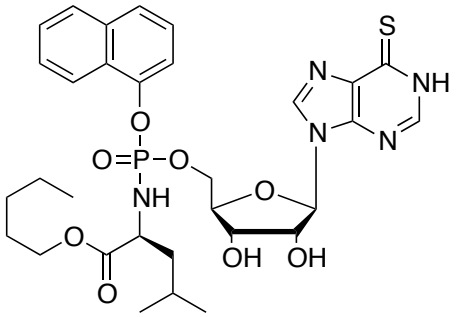
^{1}H NMR (500 MHz, MeOD) δ 8.34, 8.28 (d, *J* = 6.0 Hz, 1H, H-2), 8.10 (t, *J* = 9.0 Hz, 1H, H-Ar), 8.05, 8.00 (2 x s, 1H, H-8), 7.86 (t, *J* = 7.5 Hz, 1H, H-Ar), 7.66 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.51 – 7.42 (m, 3H, H-Ar), 7.35 - 7.23 (m, 6H, H-Ar), 5.99 (d, *J* = 5.0 Hz, 1H, H-1'), 5.08 – 5.00 (m, 2H, C*H*$_2$Bn), 4.64 - 4.60 (m, 1H, H-2'), 4.46 – 4.36 (m, 3H, H-3', H-5', H-5'), 4.25 – 4.24 (m, 1H, H-4'), 4.07 – 4.00 (m, 1H, C*H*CH_3), 1.29, 1.25 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.13 (C=S), 174.87, 174.58 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 4.25 Hz, C=O), 147.88, 147.86 (ipso, C-Naph), 144.88 (C-4), 142.85, 142.71 (C-8), 137.17, 137.12 (C-5), 136.20, 1136.17 (C-Naph), 129.53, 129.50, 129.27, 129.23, 128.90, 128.85, 127.79, 127.52, 126.50, 126.43, 126.00, 122.69, 122.55, 116.33, 116.28, 116.18, 116.16 (C-Ar), 90.69, 90.53 (C-1'), 84.63, 84.53 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.62 Hz, C-4'), 75.29, 75.22 (C-2'), 71.54, 71.40 (C-3'), 68.01, 67.98 (*C*H$_2$Bn), 67.76, 67.38 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.37 Hz, C-5'), 51.78, 51.72 (*C*HCH_3), 20.50, 20.29 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.37 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 16.11, 16.47 min

(ES+) m/z, found: (M+Na$^+$) 674.20, $C_{30}H_{30}N_5O_8PNaS$ required: (M$^+$) 651.16

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (4.12e).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (**4.11e**, 0.04 g, 0.128 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, to give the pure product **4.12e** as a yellowish foam (0.002 g, 7%).

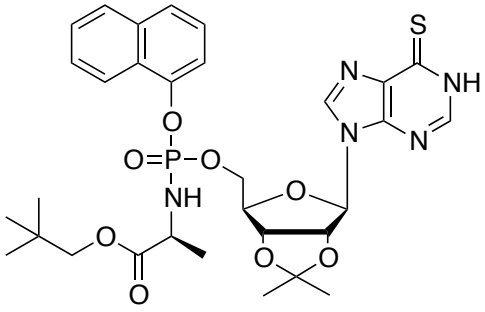
^{31}P NMR (202 MHz, MeOD) δ 4.45, 4.23

^{1}H NMR (500 MHz, MeOD) δ 8.57 (d, *J* = 4.0 Hz, 0.3H, H-2), 8.38 (s, 0.7H, H-2), 8.15 - 8.10 (2 x d, *J* = 8.0 Hz, 1H, H-8, 1H, H-Ar), 7.88 – 7.85 (m, 1H, H-Ar), 7.69 – 7.66 (m, 1H, H-Ar), 7.52 – 7.44 (m, 3H, H-Ar), 7.38 – 7.35 (m, 1H, H-Ar), 7.31 – 7.21 (m, 6H, H-Ar), 6.11, 6.01 (2 x d, *J* = 4.5 Hz, 1H, H-1'), 4.73, 4.64 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.50 – 4.46 (m, 1H, H-5', 0.3H, H-3'), 4.43 – 4.38 (m, 1H, H-5', 0.7H, H-3'), 4.33 - 4.27 (m, 1H, H-4'), 3.97 – 3.88 (m, 3H, $OCH_2CH_2CH_2CH_2CH_2$, $NHCHCH_3$), 1.66 -1.60 (m, 1H, $NHCHCH_2CH(CH_3)_2$), 1.52 – 1.46 (m, 4H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.26 – 1.24 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.86 - 0.76 (m, 9H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 22.73, 23.11 min

(ES+) m/z, found: $(M+H^+)$ 674.20, $C_{31}H_{40}N_5O_8PS$ required: (M^+) 673.23

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (4.11f).

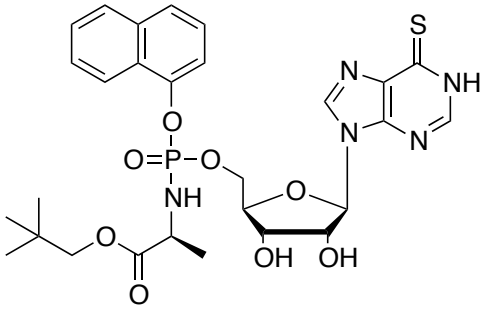


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.130 g, 0.40 mmol) *t*BuMgCl (1.0 M in THF, 0.80 ml, 0.80 mmol) and 1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)phosphorochloridate (**2.3f**, 0.31 g, 0.80 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11f** as a yellow foam (0.137 g, 51%).

^{31}P NMR (202 MHz, MeOD) δ 4.22, 3.90

^{1}H NMR (500 MHz, MeOD) δ 8.29 (d, *J* = 5.0 Hz, 1H, H-2), 8.10 – 8.09 (m, 0.6H, H-Ar), 8.07, 8.03 (2 x s, 1H, H-Ar), 7.96 – 7.94 (m, 0.4H, H-Ar), 7.80 – 7.75 (m, 1H, H-Ar), 7.62 – 7.60 (m, 1H, H-Ar), 7.47 – 7.40 (m, 3H, H-Ar), 7.34 – 7.30 (m, 1H, H-Ar), 6.14, 6.05 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.14, 5.01 (2 x dd, *J* = 2.5 Hz, 6.0 Hz, 1H, H-2'), 4.81, 4.73 (2 x dd, *J* = 2.5 Hz, 6.0 Hz, 1H, H-3'), 4.52 – 4.50 (m, 0.4H, H-4'), 4.46 – 4.43 (m, 0.6H, H-4'), 4.41- 4.29 (m, 2H, H-5', H-5'), 4.10 – 4.03 (m, 1H, C*H*CH_3), 5.14, 5.01 (2 x dd, *J* = 2.5 Hz, 6.0 Hz, 1H, H-2'), 3.82, 3.80, 3.71, 3.69 (2AB, J_{AB} = 10.5 Hz, OCH_2C$(CH_3)_3$), 1.55, 1.52, 1.32, 1.28 (4s, 6H, 2 x CH_3-isopropylidene), 1.37, 1.33 (2 x d, *J* = 7.0 Hz, 3H, CHCH_3), 0.89, 0.88 (2 x s, 9H, $OCH_2C(CH_3)_3$).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (4.12f).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (**4.11f,** 0.137 g, 0.20 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent to give the pure product **4.12f** as a yellow foam (0.019 g, 15%).

^{31}P NMR (202 MHz, MeOD) δ 4.19, 4.09

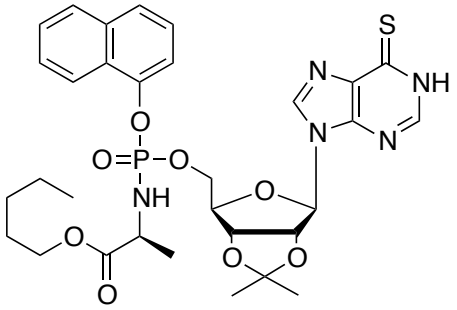
^{1}H NMR (500 MHz, MeOD) δ 8.36 (s, 1H, H-2), 8.14 – 8.11 (m, 0.7H, H-Ar), 8.10 (s, 0.3H, H-8), 8.03 (s, 0.3H, H-Ar), 7.88 – 7.84 (m, 1H, H-Ar), 7.67 (t, *J* = 8.0 Hz, 1H, H-Ar), 7.51 – 7.44 (m, 3H, H-Ar), 7.38 – 7.33 (m, 1H, H-Ar), 6.01 (t, *J* = 5.0 Hz, 1H, H-1'), 4.66 – 4.62 (m, 1H, H-2'), 4.50 - 4.40 (m, 3H, H-5', H-5', H-3'), 4.31 – 4.27 (m, 1H, H-4'), 4.05 – 4.03 (m, 1H, C*H*CH_3), 3.80, 3.78, 3.70, 3.68 (2AB, J_{AB} = 10.5 Hz, 4.5 OCH_2C$(CH_3)_3$), 1.34, 1.31 (2 x d, *J* = 7.0 Hz, 3H, CHCH_3), 0.89 (s, 9H, $OCH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.89 (C=S), 175.16, 174.90 (2 x d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 4.5 Hz, C=O), 147.88, 147.82 (ipso, C-Naph), 144.93 (C-4), 136.22, 136.12, 128.94, 128.87, 127.82, 127.50, 126.50, 126.42, 126.02, 122.65, 122.54, 116.26, 116.22, 116.19, 116.15 (C-Ar), 90.74, 90.65 (C-1'), 84.78, 84.60 (d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 75.50, 75.46 (C-2'), 71.55 (C-3'), 67.83, 67.66 (2 x d, $^2J_{C\text{-}O\text{-}P}$ = 5.12 Hz, C-5'), 51.84, 51.74 (*C*HCH_3), 32.30 ($OCH_2C(CH_3)_3$), 26.75 ($OCH_2C(CH_3)_3$), 20.80, 20.58 (2 x d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 7.25 Hz, CH*C*H_3).

HPLC (System 2) t_R = 16.87, 17.13 min

(ES+) m/z, found: ($M+H^+$) 634, $C_{28}H_{34}N_5O_8PS$ required: (M^+) 631.19

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (4.11g).

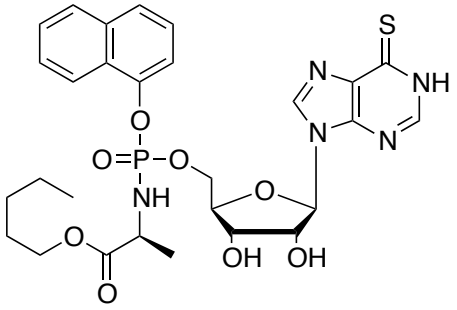


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.10 g, 0.3 mmol) *t*BuMgCl (1.0 M in THF, 0.6 ml, 0.6 mmol) and 1-naphthyl-(pentoxy-L-alaninyl)phosphorochloridate (provided by Slusarczyk, 0.236 g, 0.60 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11g** as a yellowish foam (0.132 g, 64%).

^{31}P NMR (202 MHz, MeOD) δ 4.15, 3.88

^{1}H NMR (500 MHz, MeOD) δ 8.31, 8.30 (2 x s, 1H, H-2), 8.11, 8.07 (2 x s, 1H, H-8), 8.09 - 8.08 (m, 0.7H, H-Ar), 7.95 – 7.93 (m, 0.3H, H-Ar), 7.76 – 7.73 (m, 1H, H-Ar), 7.60 – 7.58 (m, 1H, H-Ar), 7.46 – 7.39 (m, 3H, H-Ar), 7.33 – 7.30 (m, 1H, H-Ar), 6.13, 6.03 (2 x d, J = 2.5 Hz, 1H, H-1'), 5.14, 5.01 (2 x dd, J = 2.5 Hz, 6.0 Hz, 1H, H-2'), 4.79, 4.68 (2 x dd, J = 2.5 Hz, 6.0 Hz, 1H, H-3'), 4.52 – 4.51 (m, 0.3H, H-4'), 4.45 (m, 0.7H, H-4'), 4.41- 4.30 (m, 2H, H-5', H-5'), 4.05 – 3.94 (m, 3H, C*H*CH_3, O$CH_2$$CH_2$$CH_2$$CH_2$$CH_3$), 1.55, 1.52, 1.32, 1.30 (4s, 6H, 2 x CH_3-isopropylidene), 1.50 – 1.48 (m, 2H, OCH_2C$H_2$$CH_2$$CH_2$$CH_3$), 1.27 – 1.22 (m, 7H, O$CH_2$$CH_2CH_2CH_2$$CH_3$, CHC$H_3$), 0.83, 0.80 (2 x t, J = 7.5 Hz, 3H, O$CH_2$$CH_2$$CH_2$$CH_2CH_3$).

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (4.12g).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (**4.11g,** 0.132 g, 0.19 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.12g** as a yellow foam (0.044 g, 36%).

^{31}P NMR (202 MHz, MeOD) δ 4.12, 4.09

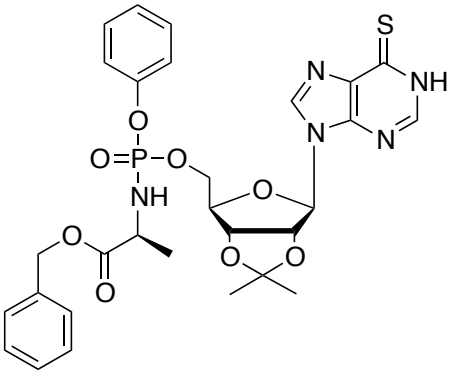
^{1}H NMR (500 MHz, MeOD) δ 8.33 (s, 1H, H-2), 8.13 – 8.08 (m, 1H, H-Ar), 8.07, 8.01 (2 x s, 1H, H-8), 7.84 – 7.80 (m, 1H, H-Ar), 7.65 – 7.62 (m, 1H, H-Ar), 7.49 - 7.44 (m, 3H, H-Ar), 7.36 – 7.33 (m, 1H, H-Ar), 6.02, 6.01 (2 x d, *J* = 5.0 Hz, 1H, H-1'), 4.65 (t, *J* = 5.0 Hz, 1H, H-2'), 4.51 - 4.40 (m, 3H, H-5', H-5', H-3'), 4.32 – 4.31 (m, 1H, H-4'), 4.04 – 3.92 (m, 3H, C*H*CH_3, OC*H*$_2$$CH_2CH_2CH_2CH_3$), 1.52 – 1.48 (m, 2H, OCH_2C*H*$_2$$CH_2CH_2CH_3$), 1.32 – 1.21 (m, 7H, OCH_2CH_2C*H*$_2$C*H*$_2$$CH_3$, CHC*H*$_3$), 0.85, 0.82 (2 x t, *J* = 7.0 Hz, 3H, $OCH_2CH_2CH_2CH_2$C*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.08 (C=S), 175.20, 174.94 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.25 Hz, C=O), 147.92, 147.86 (ipso, C-Naph), 146.30, 146.24 (C-2), 144.87, 144.84 (C-4), 142.85, 142.75 (C-8), 137.16, 137.10, 136.19, 136.16, 128.95, 128.91, 127.83, 127.78, 127.73, 127.55, 126.53, 126.50, 126.05, 122.70, 122.63, 116.25, 116.20, 116.17 (C-Ar), 90.70, 90.53 (C-1'), 84.62, 84.53 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.12 Hz, C-4'), 75.42, 75.38 (C-2'), 71.59, 71.47 (C-3'), 67.79, 67.51 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 66.62, 66.58 (O*C*$H_2CH_2CH_2CH_2CH_3$), 51.78, 51.73 (*C*HCH_3), 30.98 (OCH_2*C*$H_2CH_2CH_2CH_3$), 29.35, 29.12 (OCH_2CH_2*C*$H_2CH_2CH_3$), 23.39, 23.36 ($OCH_2CH_2CH_2$*C*H_2CH_3), 20.88, 20.69 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.37 Hz, CH*C*H_3), 14.47, 14.44 ($OCH_2CH_2CH_2CH_2$*C*H_3).

HPLC (System 2) t_R = 17.64, 17.91 min

(ES+) m/z, found: ($M+Na^+$) 654, $C_{28}H_{34}N_5O_8PNaS$ required: (M^+) 631.19

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (4.11h).

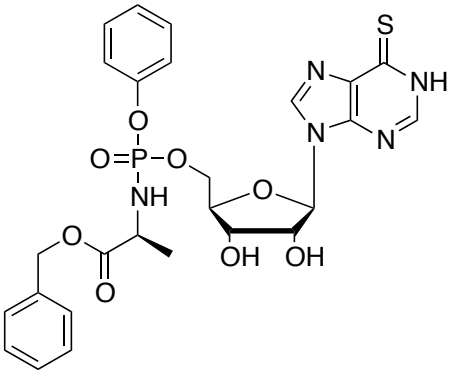


Prepared according to standard procedure **6a** from, 2',3'-*O,O*-isopropylidene-6-thioinosine (0.10 g, 0.30 mmol) *t*BuMgCl (1.0 M in THF, 0.61 ml, 0.61 mmol) and phenyl-(benzoxy-L-alaninyl)phosphorochloridate (**2.3b**, 0.218 g, 0.61 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.11h** as a yellow foam (0.083 g, 42%).

^{31}P NMR (202 MHz, MeOD) δ 3.77, 3.41

^{1}H NMR (500 MHz, MeOD) δ 8.58, 8.57 (2 x s, 0.2H, H-2), 8.53 (s, 0.2H, H-8), 8.33, 8.32 (2 x s, 0.8H, H-2), 8.15 (2 x s, 0.8H, H-8), 7.29 – 7.06 (m, 10H, H-Ar), 6.26, 6.23 (2 x d, *J* = 2.5 Hz, 0.2H, H-1'), 6.18, 6.15 (2 x d, *J* = 2.5 Hz, 0.8H, H-1'), 5.36, 5.35 (2 x dd, *J* = 2.0 Hz, 6 Hz, 0.1H, H-2'), 5.29, 5.28 (2 x dd, *J* = 2.0 Hz, 6.0 Hz, 0.6H, H-2'), 5.11 – 5.02 (m, 3H, OC*H*$_2$Bn, H-3'), 4.99, 4.97 (2 x dd, *J* = 2.0 Hz, 6.0 Hz, 0.3H, H-2'), 4.50 – 4.42 (2 x m, 1H, H-4'), 4.31- 4.20 (m, 2H, H-5', H-5'), 3.99 – 3.93 (m, 1H, C*H*CH_3), 1.58, 1.57, 1.31, 1.29 (4s, 6H, 2 x CH_3-isopropylidene), 1.36 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

Synthesis of 6-thioinosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (4.12h).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (**4.11h,** 0.08 g, 0.128 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.12h** as a yellowish foam (0.002 g, 3%).

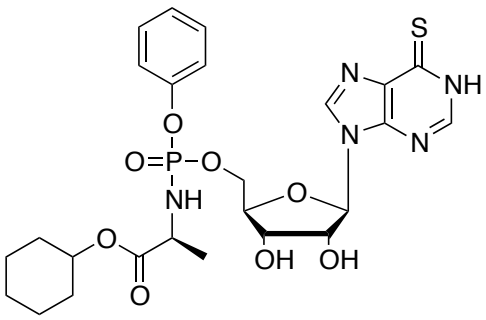
^{31}P NMR (202 MHz, MeOD) δ 3.94, 3.66

^{1}H NMR (500 MHz, MeOD) δ 8.38, 8.36 (2 x s, 1H, H-2), 8.14, 8.13 (2 x s, 1H, H-8), 7.35 – 7.28 (m, 7H, H-Ar), 7.20 – 7.16 (m, 3H, H-Ar), 6.03 (t, *J* = 5.5 Hz, 1H, H-1'), 5.13 – 5.08 (m, 2H, OC*H*$_2$Bn), 4.66, 4.63 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.40 – 4.29 (m, 3H, H-5', H-5', H-3'), 4.27 – 4.23 (m, 1H, H-4'), 4.00 – 3.94 (m, 1H, C*H*CH_3), 1.33, 1.28 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

HPLC (System 2) t_R = 15.31, 15.57 min

(ES+) m/z, found: ($M+Na^+$) 624, $C_{26}H_{28}N_5O_8PS$ required: (M^+) 601.14

Synthesis of 6-thioinosine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.12i).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)]-phosphate (**4.11i,** 0.037 g, 0.058 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.12i** as a yellow foam (0.038g, 11%).

^{31}P NMR (202 MHz, MeOD) δ 3.82, 3.58

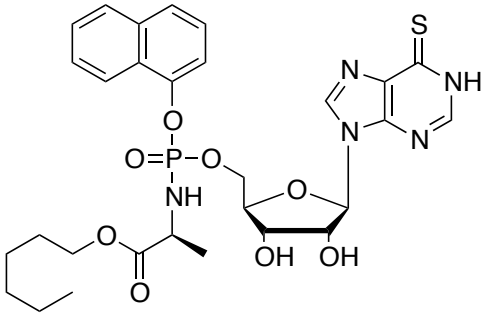
^{1}H NMR (500 MHz, MeOD) δ 8.37 (s, 1H, H-8), 8.15, 8.12 (2 x s, 1H, H-2), 7.35 – 7.30 (m, 2H, H-Ar), 7.24 – 7.16 (m, 3H, H-Ar), 6.04 (t, *J* = 4.0 Hz, 1H, H-1'), 4.73 – 4.65 (m, 2H, OC*H*-ester, H-2'), 4.44 – 4.36 (m, 3H, H-5', H-5', H-3'), 4.30 – 4.27 (m, 1H, H-4'), 3.92 – 3.87 (m, 1H, C*H*CH_3), 1.80 – 1.70 (m, 4H, 2 x CH_2-ester), 1.55 – 1.35 (m, 6H, 3 x CH_2-ester), 1.32, 1.29 (2 x d, ^{3}J = 7.0 Hz, 3H, CHCH_3).

^{13}C NMR (125 MHz, MeOD) δ 178.17 (C=S), 175.53, 174.31 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.12 Hz, C=O), 152.15, 152.08 (ipso, C-Ph), 144.99 (C-2), 142.69 (C-8), 136.98, 130.80, 130.78, 126.19, 121.44, 121.41, 121.37, (C-Ar), 90.51 (C-1'), 84.70, 84.58 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.25 Hz, C-4'), 75.51 (*C*H-ester), 75.00, 74.96 (C-2'), 71.63, 71.58 (C-3'), 67.55, 67.21 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.35 Hz, C-5'), 51.84, 51.70 (*C*HCH_3), 32.48, 32.41 (*C*H_2-ester), 26.41 (*C*H_2-ester), 24.66, 24.60 (*C*H_2-ester), 20.68, 20.47 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, CH*C*H_3).

HPLC (System 2) t_R = 15.26, 15.50 min

(ES+) m/z, found: ($M+Na^+$) 616.20, $C_{25}H_{32}N_5O_8PNaS$ required: (M^+) 593.17

Synthesis of 6-thioinosine 5'-*O*-[1-naphthyl-(hexoxy-L-alaninyl)]-phosphate (4.12j).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioinosine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)]-phosphate (**4.11j,** 0.041 g, 0.06 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.12j** as a yellowish foam (0.03 g, 8%).

^{31}P NMR (202 MHz, MeOD) δ 4.17, 4.13

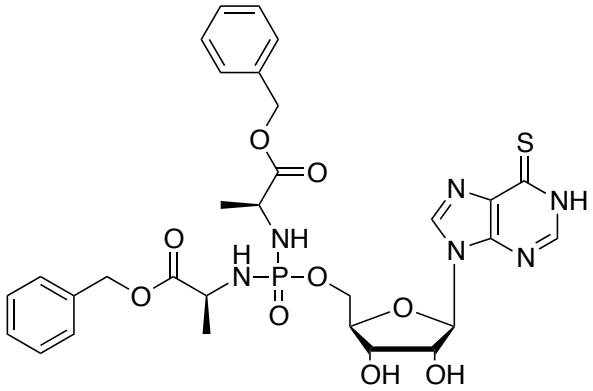
^{1}H NMR (500 MHz, MeOD) δ 8.36 (s, 1H, H-2), 8.15 – 8.10 (m, 1H, H-Ar), 8.08, 8.02 (2 x s, 1H, H-8), 7.89 (t, *J* = 9.0 Hz, 1H, H-Ar), 7.69 (t, *J* = 7.0 Hz, 1H, H-Ar), 7.53 – 7.45 (m, 3H, H-Ar), 7.38, 7.37 (2 x t, *J* = 8.0 Hz, 1H, H-Ar), 6.01 (t, *J* = 5.5 Hz, 1H, H-1'), 4.67 – 4.63 (m, 1H, H-2'), 4.48 – 4.42 (m, 3H, H-5', H-5', H-3'), 4.30 – 4.28 (m, 1H, H-4'), 4.01 - 3.93 (m, 3H, O*C*$H_2$$CH_2CH_2CH_2CH_3$, C*H*$CH_3$), 1.53 – 1.49 (m, 2H, OCH_2C*H*$_2CH_2CH_2CH_2CH_3$), 1.31 – 1.22 (m, 9H, OCH_2CH_2C*H*$_2$*C*H_2*C*H_2*C*H_3), 0.86, 0.85 (d, *J* = 6.5 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.10, 178.07 (C=S), 175.17, 174.87 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 4.6 Hz, C=O), 147.93, 147.88 (C-2), 144.92, 144.89 (C-4), 142.91, 142.75 (C-8), 136.24, 136.21, 128.92, 128.87, 127.80, 127.49, 126.47, 126.43, 125.99, 122.69, 122.59, 116.20, 116.18, 116.15, 116.12 (C-Ar), 90.71, 90.52 (C-1'), 84.63, 84.58 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.12 Hz, C-4'), 75.28, 75.20 (C-2'), 71.56, 71.40 (C-3'), 67.72, 67.35 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.12 Hz, C-5'), 66.50, 66.46 (O*C*$H_2CH_2CH_2CH_2CH_2CH_3$), 51.69 (*C*HCH_3), 32.58 ($OCH_2CH_2CH_2$*C*$H_2CH_2CH_3$), 29.62, 29.60 (OCH_2*C*$H_2CH_2CH_2CH_2CH_3$), 26.60, 26.58 (OCH_2CH_2*C*$H_2CH_2CH_2CH_3$), 23.57 ($OCH_2CH_2CH_2CH_2$*C*H_2CH_3), 20.58, 20.38 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.75 Hz, CH*C*H_3), 14.36 (CH*C*H_3).

HPLC (System 2) t_R = 21.35, 21.63 min

(ES+) m/z, found: ($M+H^+$) 646, $C_{29}H_{36}N_5O_8PNaS$ required: (M^+) 645.20

Synthesis of 6-thioinosine 5'-*O*-bis(benzoxy-L-alaninyl)-phosphate (4.17a).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.50 ml, 0.53 mmol), L-alanine benzyl ester tosylate salt (0.88 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17a** as a white foam (0.043 g, 12%).

^{31}P NMR (202 MHz, MeOD) δ 13.71

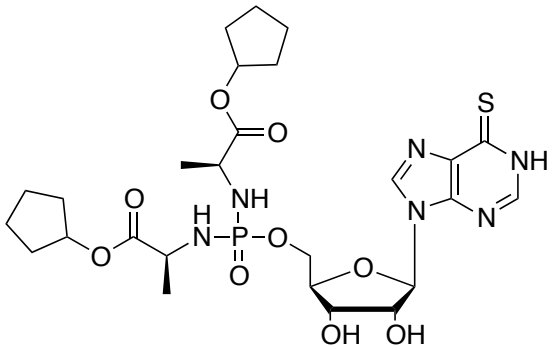
^{1}H NMR (500 MHz, MeOD) δ 8.40 (bs, 1H, H-2), 8.15 (bs, 1H, H-8), 7.35 – 7.29 (m, 10H, H-Ar), 6.01 (d, J = 4.5 Hz, 1H, H-1'), 5.15, 5.13, 5.10, 5.07 (2 x AB, J_{AB} = 12.5 Hz, 4.5 Hz, 4H, 2 x OCH_2Ph), 4.70 (t, J = 5.0 Hz, 1H, H-2'), 4.40 (t, J = 4.5 Hz, 1H, H-3'), 4.25 – 4.14 (m, 3H, H-4', H-5', H-5'), 3.94 – 3.91 (m, 2H, 2 x CHCH$_3$), 1.32, 1.29 (2 x d, J = 7.0 Hz, 2 x CHCH_3).

^{13}C NMR (125 MHz, MeOD) δ 178.27 (C=S), 175.44, 175.38 (C=O), 145.03 (C-2), 142.80 (C-4), 142.80 (C-8), 137.31, 137.27 (ipso C-Ph), 137.05 (C-5), 129.58, 129.57, 129.31, 129.30, 129.17 (C-Ar), 90.42 (C-1'), 84.74, 84.70 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.2 Hz, C-4'), 75.53 (C-2'), 71.57 (C-3'), 67.99, 67.96 (OCH$_2$Ph), 66.22, 66.18 (C-5'), 51.14, 51.10 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 6.25 Hz, CHCH$_3$), 20.85, 20.62 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, CHCH$_3$).

HPLC (System 2) t_R = 16.71 min

(ES+) m/z, found: (M+H$^+$) 687, $C_{30}H_{35}N_6O_9PS$ required: (M$^+$) 686.19

Synthesis of 6-thioinosine 5'-*O*-bis(cyclopentoxy-L-alaninyl)-phosphate (4.17b).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.05 ml, 0.53 mmol), L-alanine cyclopentyl ester tosylate salt (provided by Madela, 0.86 g, 2.64 mmol) in dry $CHCl_3$ and DIPEA (0.92 ml, 5.27 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17b** as a white foam (0.067 g, 20%).

^{31}P NMR (202 MHz, MeOD) δ 13.86

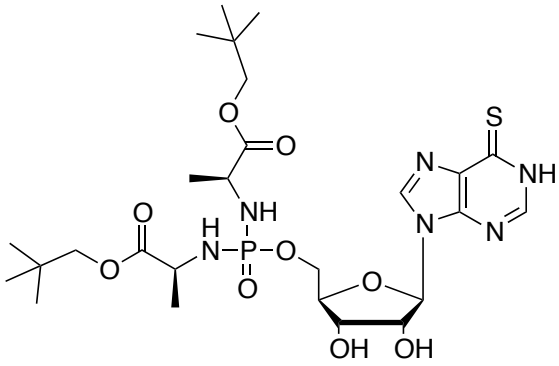
^{1}H NMR (500 MHz, MeOD) δ 8.41 (bs, 1H, H-2), 8.19 (bs, 1H, H-8), 6.05 (d, *J* = 5.0 Hz, H-1'), 5.15 - 5.10 (m, 2H, 2 x OC*H*-ester), 4.72 (t, *J* = 5.5 Hz, 1H, H-2'), 4.43 (t, *J* = 5.0 Hz, 1H, H-3'), 4.29 – 4.19 (m, 3H, H-4', H-5', H-5'), 3.86 – 3.82 (m, 2H, 2 x C*H*CH_3), 1.90 – 1.83 (m, 4H, 2 x CH_2-ester), 1.74 – 1.60 (m, 12H, 2 x CH_2-ester), 1.32 (d, *J* = 7.0 Hz, 6H, CHCH_3).

^{13}C NMR (125 MHz, MeOD) δ 178.31 (C=S), 175.47, 175.42 (C=O), 146.32 (C-2), 144.99 (C-4), 142.84 (C-8), 137.15 (ipso *C*-Ph), 90.45 (C-1'), 84.84, 84.78 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 79.47 (O*C*H-ester), 75.33 (C-2'), 71.62 (C-3'), 66.34, 66.29 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 6.25 Hz, C-5'), 51.15, 51.09 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 6.25 Hz, *C*HCH_3), 33.67, 33.49 (CH_2-ester), 24.70 (CH_2-ester), 20.96, 20.78 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.6 Hz, CH*C*H_3).

HPLC (System 2) t_R = 15.12 min

(ES+) m/z, found: ($M+H^+$) 643, $C_{26}H_{39}N_6O_9PS$ required: (M^+) 642.66

Synthesis of 6-thioinosine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl)-phosphate (4.17c).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.05 ml, 0.53 mmol), L-alanine 2,2-dimethylpropyl ester tosylate salt (**2.2a**, 0.91 g, 2.64 mmol) in dry $CHCl_3$ and DIPEA (0.92 ml, 5.27 mmol).The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17c** as a white foam (0.064 g, 19%).

^{31}P NMR (202 MHz, MeOD) δ 13.84

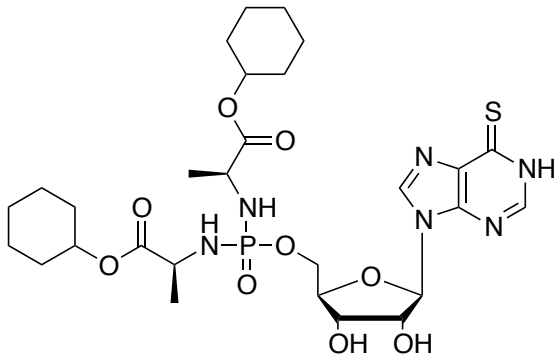
^{1}H NMR (500 MHz, MeOD) δ 8.43 (bs, 1H, H-2), 8.19 (bs, 1H, H-8), 6.04 (d, J = 5.0 Hz, H-1'), 4.72 (t, J = 5.5 Hz, 1H, H-2'), 4.43 (t, J = 5.0 Hz, 1H, H-3'), 4.30 – 4.21 (m, 3H, H-4', H-5', H-5'), 3.97 – 3.93 (m, 2H, 2 x C*H*CH_3), 3.88, 3.86, 3.76, 3.74 (2AB, J_{AB} = 10.5 Hz, 4.5 Hz, 4H, 2 x C*H*$_2$C$(CH_3)_3$), 1.39 (d, J = 7.0 Hz, 6H, 2 x CHC*H*$_3$), 0.95 (s, 18H, 2 x CH_2C(C*H*$_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.44 (C=S), 175.07, 175.02 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, 2 x C=O), 146.47 (C-2), 145.03 (C-4), 142.76 (C-8), 137.19 (C-5), 90.44 (C-1'), 84.82, 84.76 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 7.75 Hz, C-4'), 79.47 (O*C*H_2C$(CH_3)_3$), 74.89 (C-2'), 71.58 (C-3'), 66.27, 66.23 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 51.19, 51.09 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 6.0 Hz, *C*HCH_3), 32.53, 32.50, 32.46 (2 x OCH_2*C*$(CH_3)_3$), 30.73, 26.44, 24.67 (2 x OCH_2C(*C*$H_3)_3$), 21.13, 21.89 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.6 Hz, CH*C*H_3).

HPLC (System 2) t_R = 18.48 min

(ES+) m/z, found: (M+Na^+) 669, $C_{26}H_{43}N_6O_9PNaS$ required: (M^+) 646.25

Synthesis of 6-thioinosine 5'-*O*-bis(cyclohexoxy-L-alaninyl)-phosphate (4.17d).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.05 ml, 0.53 mmol), L-alanine cyclohexyl ester tosylate salt (**2.2d**, 0.90 g, 2.64 mmol) in dry $CHCl_3$ and DIPEA (0.92 ml, 5.27 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17d** as a white foam (0.081 g, 23%).

^{31}P NMR (202 MHz, MeOD) δ 13.87

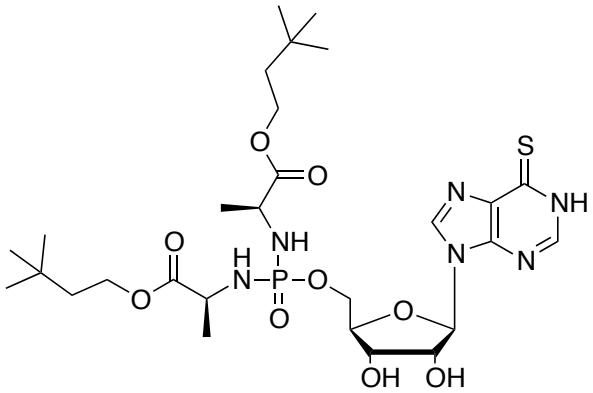
^{1}H NMR (500 MHz, MeOD) δ 8.43 (bs, 1H, H-2), 8.25 (bs, 1H, H-8), 6.06 (d, *J* = 4.5 Hz, 1H, H-1'), 4.74 (t, *J* = 5.0 Hz, 1H, H-2', 2 x ipso C*H*-ester), 4.46 (t, *J* = 4.5 Hz, 1H, H-3'), 4.31 – 4.22 (m, 3H, H-4', H-5', H-5'), 3.89 – 3.86 (m, 2H, 2 x C*H*CH_3), 1.82 – 1.81 (m, 4H, 2 x CH_2-ester), 1.74 – 1.72 (m, 4H, 2 x CH_2-ester), 1.56 – 1.55 (m, 2H, CH_2-ester), 1.46 – 1.30 (m, 16 H, 5 x CH_2-ester, 2 x CH(CH_3)$_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.14 (C=S), 175.16, 175.07 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.12 Hz, 2 x C=O), 145.03 (C-2), 142.85 (C-4), 137.00 (C-8), 90.53 (C-1'), 84.84, 84.75 (C-4'), 79.50 (2 x ipso C*H*-ester), 75.34 (C-2'), 71.60 (C-3'), 66.30, 66.26 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 4.62 Hz, C-5'), 51.19, 51.09 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 12.5 Hz, *C*HCH_3), 32.55, 32.52, 32.48 (4 x *C*H_2-ester), 26.46, 24.74, 24.72 (4 x *C*H_2-ester), 21.14, 21.09 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 16.22 min

(ES+) m/z, found: ($M+H^+$) 671, $C_{28}H_{43}N_6O_9PS$ required: (M^+) 670.25

6-thioinosine 5'-*O*-bis(3,3-dimethyl-1-butoxy-L-alaninyl)-phosphate (4.17e).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.155 g, 0.54 mmol) in anhydrous TMP, $POCl_3$ (0.05 ml, 0.54 mmol), L-alanine 3,3 dimethyl-1-butyl ester tosylate salt (**2.2e**, 0.94 g, 2.73 mmol) in dry $CHCl_3$ and DIPEA (0.95 ml, 5.45 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17e** as a white foam (0.047 g, 13%).

^{31}P NMR (202 MHz, MeOD) δ 13.82

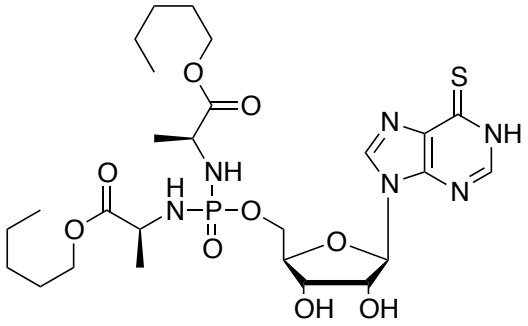
^{1}H NMR (500 MHz, MeOD) δ 8.48 (bs, 1H, H-2), 8.21 (bs, 1H, H-8), 6.06 (d, *J* = 5.0 Hz, 1H, H-1'), 4.72 (t, *J* = 5.5 Hz, 1H, H-2'), 4.46 (t, *J* = 5.0 Hz, 1H, H-3'), 4.30 – 4.11 (m, 7H, H-4', H-5', H-5', 2 x OC*H*$_2$CH$_2$C(CH$_3$)$_3$), 3.90 (q, *J* = 7.5 Hz, 2H, 2 x C*H*CH$_3$), 1.58 (t, *J* = 7.0 Hz, 8H, 2 x OCH$_2$C*H*$_2$C(CH$_3$)$_3$), 1.34 (d, *J* = 6.5 Hz, 2H, 2 x CHC*H*$_3$), 0.95 (s, 9H, OCH$_2$CH$_2$C(C*H*$_3$)$_3$), 0.94 (s, 9H, OCH$_2$CH$_2$C(C*H*$_3$)$_3$).

^{13}C NMR (125 MHz, MeOD) δ 178.26 (C=S), 175.16, 175.07 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, 2 x C=O), 146.41 (C-2), 144.94 (C-4), 142.87 (C-8), 137.11 (C-5), 90.50 (C-1'), 84.82 (d, $^{3}J_{C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 79.53 (C-2'), 75.43 (C-3'), 66.15 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 65.24 (O*C*H$_2$CH$_2$*C*(*C*H$_3$)$_3$), 64.08 (O*C*H$_2$CH$_2$*C*(*C*H$_3$)$_3$), 51.07 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 8.25 Hz, *C*HCH$_3$), 42.89 (OCH$_2$*C*H$_2$*C*(*C*H$_3$)$_3$), 42.87 (OCH$_2$*C*H$_2$*C*(*C*H$_3$)$_3$), 30.60 (OCH$_2$CH$_2$*C*(*C*H$_3$)$_3$), 30.07 (2 x OCH$_2$*C*H$_2$C(*C*H$_3$)$_3$), 29.97 (OCH$_2$CH$_2$*C*(*C*H$_3$)$_3$), 20.96, 20.73 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.6 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 18.85 min

(ES+) m/z, found: (M+Na$^+$) 697, $C_{28}H_{47}N_6O_9PS$ required: (M$^+$) 674.29

Synthesis of 6-thioinosine 5'-*O*-bis(pentoxy -L-alaninyl)-phosphate (4.17f).



Prepared according to standard procedure **8** from, 6-thioinosine (**4.3**, 0.155 g, 0.54 mmol) in anhydrous TMP, $POCl_3$ (0.05 ml, 0.54 mmol), L-alanine pentyl ester hydrochloride salt (**2.2b**, 0.53 g, 2.73 mmol) in dry $CHCl_3$ and DIPEA (0.95 ml, 5.45 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.17f** as a white foam (0.091 g, 26%).

^{31}P NMR (202 MHz, MeOD) δ 13.81

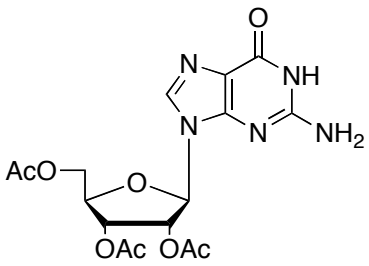
^{1}H NMR (500 MHz, MeOD) δ 8.69 (bs, 1H, H-2), 8.65 (bs, 1H, H-8), 6.15 (d, *J* = 5.0 Hz, 1H, H-1'), 4.56 (t, *J* = 5.5 Hz, 1H, H-2'), 4.82 (t, *J* = 5.0 Hz, 1H, H-3'), 4.47 (t, *J* = 5.0 Hz, 1H, H-3'), 4.31 – 4.21 (m, 3H, H-4', H-5', H-5'), 4.14 – 4.03 (m, 4H, 2 x OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 3.91 – 3.86 (m, 2H, 2 x C*H*CH$_3$), 1.65 - 1.62 (m, 4H, 2 x OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.35 – 1.31(m, 14H, 2 x OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$, CHC*H*$_3$), 0.93 – 0.90 (t, *J* = 7.0 Hz, 6H, 2 x OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 175.73, 175.69 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.5 Hz, 2 x C=O), 157.83 (C-2), 153.33 (C-4), 150.68 (C-8), 145.68 (C-5), 90.52 (C-1'), 84.75 (d, $^{3}J_{C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 75.11 (C-2'), 71.59 (C-3'), 66.49 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 2.75 Hz, C-5'), 65.21, 66.17 (2 x O*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 51.08 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 9.5 Hz, *C*HCH$_3$), 29.40 (2 x OCH$_2$*C*H$_2$CH$_2$CH$_2$CH$_3$), 29.20 (2 x OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 23.41 (2 x OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$), 21.08, 20.87 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 8.0 Hz, CH*C*H$_3$), 14.39 (2 x OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_3$).

HPLC (System 2) t_R = 20.47 min

(ES+) m/z, found: (M+Na$^+$) 669, $C_{26}H_{43}N_6O_9PS$ required: (M$^+$) 646.25

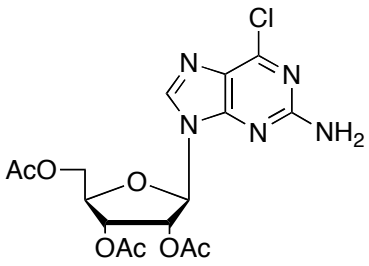
2',3',5'-tri-*O*-acetyl-guanosine (4.7).



To a suspension of commercially available guanosine (5.00 g, 17.65 mmol) DMAP (0.37 g, 3.00 mmol) and Et_3N (9.84 ml, 70.61 mmol) in anhydrous ACN (130 ml) acetic anhydride was added dropwise (5.90 ml, 63.5 mmol). The reaction mixture was allowed to stir at ambient temperature overnight. Anhydrous MeOH (25 ml) was added to quench the reaction and the pure product **4.7** (4.03 g, 56%) precipitated as a white solid after the addition of Et_2O (300 ml).

^{1}H NMR (500 MHz, MeOD) δ 7.86 (s, 1H, H-8), 6.07 (d, *J* = 5.0 Hz, 1H, H-1'), 5.95 (t, *J* = 6.0 Hz, 1H, H-2'), 5.69 (t, *J* = 5.5 Hz, 1H, H-3'), 4.47 – 4.37 (m, 3H, H-4', H-5', H-5'), 2.15 (s, 3H, CH_3-acetyl), 2.09, 2.08 (2 x s, 6H, 2 x CH_3-acetyl).
^{13}C NMR (125 MHz, MeOD) δ 170.04, 169.39, 169.22 (*C*=O-acetyl), 156.58 (C=O-base), 153.85 (C-2), 151.07 (C-4), 135.61 (C-8), 116.83 (C-5), 84.42 (C-1'), 79.53 (C-2'), 72.04 (C-3'), 70.30 (C-4'), 63.06 (C-5'), 20.50, 20.36, 20.17 (3 x *C*H_3-acetyl).

2',3',5'-tri-*O*-acetyl-6-chloro-guanosine

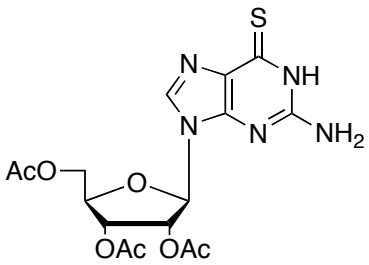


The suspension of 2',3',5'-tri-*O*-acetyl-guanosine (**4.7,** 2.28 g, 5.56 mmol), BTEA-Cl (2.53 g, 11.14 mmol), N, N-dimethylaniline (0.77 ml, 6.13 mmol) and $POCl_3$ (2.59 ml, 27.85 mmol) in anhydrous ACN (80 ml) were heated under reflux at 85^{o}C for 3 hrs. After that time volatiles were evaporated under reduced pressure to give the crude mixture as a yellow oil. The crude oil was dissolved in $CHCl_3$ and it was allowed to stir in the presence of crushed ice at ambient temperature for 30 minutes. The two layers were separated and the aqueous layer was extracted with $CHCl_3$. The combined organic layers were washed with cold water and 5% aqueous solution of $NaHCO_3$, dried over $MgSO_4$ and evaporated under reduced pressure to give the title compound (1.78 g, 75%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (s, 1H, H-8), 7.04 (s, 1H, NH_2), 6.11 (d, *J* = 6.0 Hz, 1H, H-1'), 5.88 (t, *J* = 6.0 Hz, 1H, H-2'), 5.55 (t, *J* = 4.5 Hz, 1H, H-3'), 4.40 (dd, *J* = 12.0 Hz, 4.0 Hz, 1H, H-5'), 4.36 (q, *J* = 4.0 Hz, 1H, H-4'), 4.29 (dd, *J* = 11.5 Hz, 6.0 Hz, 1H, H-5'), 2.12 (s, 3H, CH_3-acetyl), 2.04, 2.03 (2 x s, 6H, 2 x CH_3-acetyl).

^{13}C NMR (125 MHz, MeOD) δ 170.07, 169.40, 169.24 (*C*=O-acetyl), 159.85 (C-2), 153.63 (C-Cl), 149.87 (C-4), 141.26 (C-8), 130.93 (C-5), 84.93 (C-1'), 79.68 (C-2'), 71.88 (C-3'), 70.22 (C-4'), 62.92 (C-5'), 20.48, 20.34, 20.16 (3 x *C*H_3-acetyl).

2',3',5'-tri-*O*-acetyl-6-thioguanosine (4.8).

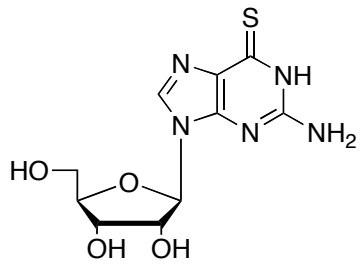


The suspension of 2',3',5'-tri-*O*-acetyl-guanosine (**4.7,** 1.0 g, 2.44 mmol) and Lawesson's reagent (2.17 g, 5.37 mmol) in anhydrous toluene (60 ml) were heated under reflux at 110^{o}C for 3 hrs until the consumption of the starting material. The reaction mixture was allowed to slowly cool down and the majority of the precipitated Lawesson's reagent was removed by filtration. The reaction mixture was evaporated under reduced pressure and purified by column chromatography, using $CHCl_3$/MeOH (2 to 5% gradient) as an eluent system to give **4.8** as a yellow solid (0.49 g, 48%).

^{1}H NMR (500 MHz, MeOD) δ 8.00 (s, 1H, H-8), 6.06 (d, *J* = 5.0 Hz, 1H, H-1'), 5.93 (t, *J* = 5.5 Hz, 1H, H-2'), 5.66 (t, *J* = 5.5 Hz, 1H, H-3'), 4.46 – 4.36 (m, 3H, H-4', H-5', H-5'), 2.14 (s, 3H, CH_3-acetyl), 2.09, 2.08 (2 x s, 6H, 2 x CH_3-acetyl).

^{13}C NMR (125 MHz, MeOD) δ 177.58 (C=S), 172.34, 171.50, 171.22 (*C*=O, acetyl), 154.82 (C-2), 148.58 (C-4), 140.46 (C-8), 130.18 (C-5), 87.98 (C-1'), 81.44 (C-2'), 74.28 (C-3'), 72.02 (C-4'), 64.25 (C-5'), 20.76, 20.58, 20.42 (3 x *C*H_3, acetyl).

6-thioguanosine (4.9).

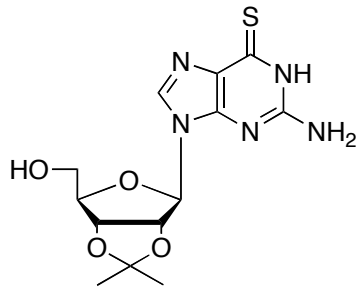


2',3',5'-tri-*O*-acetyl-6-thioguanosine (**4.8,** 0.5 g, 1.67 mmol) was dissolved in saturated solution of NH_4OH (0.1 ml), then adjusted to neutral pH with 0.1M acetic acid and kept in the fridge at -5°C for 3-5 days until the appearance of the yellowish crystals. The crystals were filtered off and used in the next step without further purification to give the title compound **4.9** (0.18 g, 51%).

^{1}H NMR (500 MHz, DMSO) δ 11.94 (bs, NH-base), 8.13 (s, 1H, H-8), 6.80 (s, 2H, NH_2-base), 5.69 (d, *J* = 5.5 Hz, 1H, H-1'), 5.42 (d, *J* = 4.5 Hz, 1H, 2'OH), 5.14 (d, *J* = 4.5 Hz, 1H, 3'OH), 5.04 (t, *J* = 5.5 Hz, 1H, 5'OH), 4.40 (q, *J* = 5.5 Hz, 1H, H-2'), 4.10 (q, *J* = 4.5 Hz, 1H, H-3'), 3.89 (q, *J* = 4.0 Hz, 1H, H-4'), 3.65 – 3.61 (m, 1H, H-5'), 3.56 – 3.52 (m, 1H, H-5').

^{13}C NMR (125 MHz, MeOD) δ 175.06 (*C*=S), 153.01 (C-2), 147.86 (C-4), 138.40 (C-8), 128.30 (C-5), 86.41 (C-1'), 85.26 (C-2'), 73.74 (C-3'), 70.25 (C-4'), 61.23 (C-5').

2',3'-*O*,*O*-isopropylidene - 6 – thioguanosine (4.13).



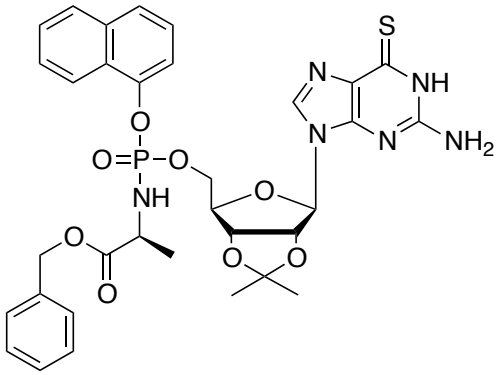
To the solution of 6-thioguanosine (**4.9**, 0.50 g, 1.67 mmol) in anhydrous acetone (40 ml) 60% aqueous solution of perchloric acid (0.90 ml) was added dropwise and stirred overnight under inert atmosphere at ambient temperature. Saturated solution of NH_4OH was added dropwise in order to reach neutral pH. The reaction mixture was evaporated and the resulting white solid was purified by column chromatography ($CHCl_3$/MeOH, 7:3) to give the protected nucleoside **4.13** (0.47 g, 83%).

^{1}H NMR (500 MHz, MeOD) δ 8.09 (s, 1H, H-8), 6.04 (d, *J* = 3.0 Hz, 1H, H-1'), 5.25 (dd, *J* = 6.0 Hz, 2.5 Hz, 1H, H-2'), 5.03 (dd, *J* = 6.0 Hz, 2.5 Hz, 1H, H-3'),

4.31 (q, *J* = 4.0 Hz, 1H, H-4'), 3.76 (dd, *J* = 12.0 Hz, 4.0 Hz, 1H, H-5'), 3.71 (dd, *J* = 12.0 Hz, 4.5 Hz, 1H, H-5'), 1.60 (s, 3H, CH_3-isopropylidene), 1.39 (s, 3H, CH_3-isopropylidene).

^{13}C NMR (125 MHz, MeOD) δ 174.52 (*C*=S), 153.99 (C-2), 148.29 (C-4), 140.72 (C-8), 130.06 (C-5), 115.19 (*C*$(CH_3)_2$-isopropylidene), 91.92 (C-1'), 88.66 (C-2'), 85.69 (C-3'), 82.94 (C-4'), 63.40 (C-5'), 27.57 (*C*H_3-isopropylidene), 25.58 (*C*H_3-isopropylidene).

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (4.14a).

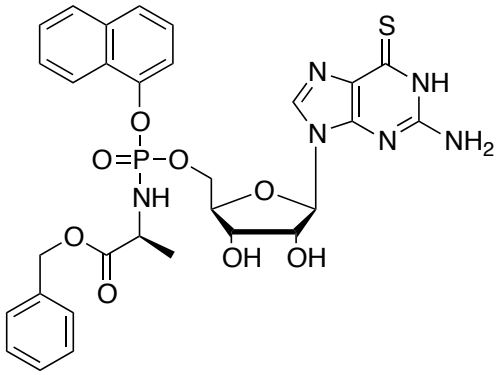


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.13 g, 0.38 mmol) *t*BuMgCl (1.0 M in THF, 0.76 ml, 0.76 mmol) and 1-naphthyl-(benzoxy-L-alaninyl)phosphorochloridate (**2.3c**, 0.30 g, 0.76 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14a** as a yellow foam (0.091 g, 34%).

^{31}P NMR (202 MHz, MeOD) δ 4.39, 4.19

^{1}H NMR (500 MHz, MeOD) δ 8.13 – 7.96 (m, 1H, H-Ar), 7.92 (s, 1H, H-8), 7.81 – 7.82 (m, 1H, H-Ar), 7.68 – 7.64 (m, 1H, H-Ar), 7.51 – 7.39 (m, 3H, H-Ar), 7.36 – 7.24 (m, 6H, H-Ar), 6.00, 5.91 (2 x s, 1H, H-1'), 5.12 -5.05 (m, 3H, H-2', OCH_2Bn), 4.89 – 4.87 (m, 0.5H, H-3'), 4.75 – 4.73 (m, 0.5H, H-3'), 4.47 – 4.34 (m, 2H, H-5', H-5'), 4.31 – 4.27 (m, 0.5H, H-4'), 4.20 – 4.16 (m, 0.5H, H-4'), 4.11 – 4.03 (m, 1H, C*H*CH_3), 1.54, 1.53, 1.32, 1.30 (4s, 6H, 2 x CH_3-isopropylidene), 1.32, 1.30 (2 x d, ^{3}J = 6.5 Hz, CHCH_3).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (4.15a).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (**4.14a**, 0.091 g, 0.128 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15a** as a yellow foam (0.009 g, 11%).

^{31}P NMR (202 MHz, MeOD) δ 4.41, 4.22

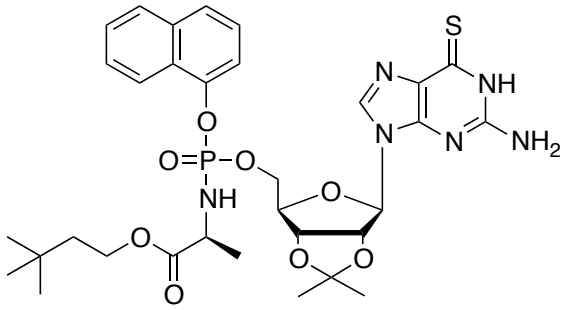
^{1}H NMR (500 MHz, MeOD) δ 8.14 - 8.07 (m, 1H, H-Ar), 7.98 (s, 1H, H-8), 7.86 – 7.82 (m, 1H, H-Ar), 7.68 – 7.64 (m, 1H, H-Ar), 7.51 – 7.43 (m, 3H, H-Ar), 7.36 – 7.25 (m, 6H, H-Ar), 5.84, 5.83 (2 x d, *J* = 5.0 Hz, 1H, H-1'), 5.07 -5.01 (m, 2H, OC*H*$_2$Bn), 4.61 – 4.56 (m, 1H, H-2'), 4.44 – 4.34 (m, 3H, H-4', H-5', H-5'), 4.25 – 4.22 (m, 1H, H-4'), 4.09 – 4.04 (m, 1H, C*H*CH_3), 1.29 (d, *J* = 7.0 Hz, CHC*H*$_3$).

^{13}C NMR (125 MHz, DMSO) δ 175.16 (C=S), 173.05, 172.93 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.0 Hz, C=O), 153.08 (C-2), 147.95, 147.90 (C-4), 146.43, 146.38 (ipso C-Naph), 138.24, 138.18 (C-8), 135,80, 134.22, 134.20, 128.36, 128.35, 127.98, 127.77, 127.67, 127.63, 126.66, 126.32, 126.26, 126.01, 125.96, 125.63, 125.57, 124.21, 124.18, 121.56, 121.49, 114.80, 114.77 (C-Ar), 86.58, 86.42 (C-1'), 82.61, 82.51 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.8 Hz, C-4'), 73.18, 73.08 (C-2'), 70.01, 70.05 (C-3'), 66.31, 66.14 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 65.99 (O*C*H_2Bn), 49.98, 49.89 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 6.25 Hz, *C*HCH_3), 19.78, 19.66 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.35 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.72, 18.11 min

(ES+) m/z, found: ($M+H^+$) 667.20, $C_{30}H_{31}N_6O_8PS$ required: (M^+) 666.17

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl)]-phosphate (4.14b).

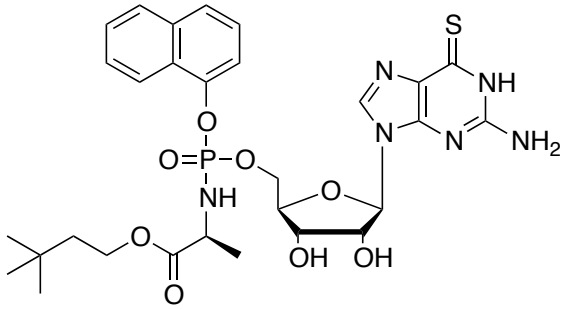


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.23 g, 0.67 mmol) *t*BuMgCl (1.0 M in THF, 1.34 ml, 1.34 mmol) and 1-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl)phosphorochloridate (**2.3i**, 0.53 g, 1.34 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent, followed by preparative purification to give the pure product **4.14b** as a yellow foam (0.21 g, 45%).

^{31}P NMR (202 MHz, MeOD) δ 4.34, 4.22

^{1}H NMR (500 MHz, MeOD) δ 8.14 – 8.12 (m, 0.5H, H-Ar), 7.98 – 7.97 (m, 0.5H, H-Ar), 7.96, 7.95 (2 x s, 1H, H-8), 7.86 -7.81 (m, 1H, H-Ar), 7.68 – 7.64 (m, 1H, H-Ar), 7.52 – 7.32 (m, 4H, H-Ar), 7.36 – 7.24 (m, 6H, H-Ar), 6.02, 5.94 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.16 - 5.12 (m, 1H, H-2'), 4.94, 4.92 (dd, *J* = 2.5 Hz, 0.5H, H-5'), 4.79, 4.78 (dd, *J* = 2.5 Hz, 0.5H, H-5'), 4.51 – 4.47 (m, 1H, H-5', 0.5H, H-3'), 4.42 – 4.40 (m, 0.5H, H-3'), 4.37 – 4.35 (m, 0.5H, H-4'), 4.29 – 4.24 (m, 0.5H, H-4'), 4.09 – 3.97 (m, 3H, OC*H*$_2$CH$_2$C(CH$_3$)$_3$, C*H*CH$_3$), 1.55, 1.54, 1.35, 1.30 (4s, 6H, 2 x CH$_3$-isopropylidene), 1.46 -1.43 (m, 2H, OCH$_2$C*H*$_2$C(CH$_3$)$_3$), 1.35 – 1.30 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$), 0.87, 0.86 (2 x s, 9H, OCH$_2$CH$_2$C(C*H*$_3$)$_3$).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butyl-L-alaninyl)]-phosphate (4.15b).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(3,3-dimethyl-1-butyl-L-alaninyl)]-phosphate (**4.14b**, 0.21 g, 0.30 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15b** as a yellow foam (0.011 g, 6%).

^{31}P NMR (202 MHz, MeOD) δ 4.31, 4.19

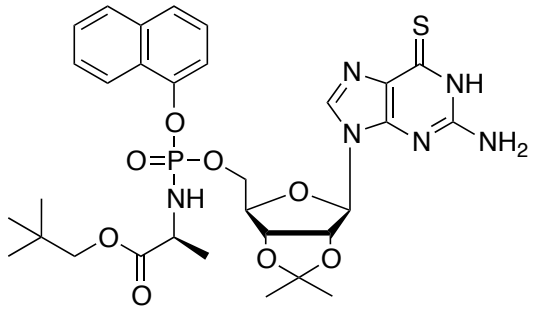
^{1}H NMR (500 MHz, MeOD) δ 8.15 - 8.13 (m, 0.5H, H-Ar), 8.09 - 8.08 (m, 0.5H, H-Ar), 7.98, 7.97 (2 x s, 1H, H-8), 7.86 – 7.84 (m, 1H, H-Ar), 7.68 – 7.66 (m, 1H, H-Ar), 7.52 – 7.43 (m, 3H, H-Ar), 7.39 – 7.29 (m, 1H, H-Ar), 5.85, 5.84 (2 x d, *J* = 5.0 Hz, 1H, H-1'), 4.62 – 4.57 (m, 1H, H-2'), 4.50 – 4.47 (m, 1.4H, H-3', H-5'), 4.42 – 4.39 (m, 1.6H, H-3', H-5'), 4.31 – 4.30 (m, 0.4H, H-4'), 4.27 – 4.26 (m, 0.6H, H-4'), 4.07 – 3.97 (m, 3H, $OCH_2CH_2C(CH_3)_3$, $CHCH_3$), 1.45 – 1.40 (m, 2H, $OCH_2CH_2C(CH_3)_3$), 1.31 (d, *J* = 7.0 Hz, $CHCH_3$), 0.87, 0.86 (2 x s, 9H, $OCH_2CH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.21, 177.13 (C=S), 175.22, 174.95 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.5 Hz, C=O), 154.64, 154.55 (C-2), 148.76, 148.73 (C-4), 147.93, 147.87 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 3.75 Hz, ipso C-Naph), 140.61, 140.55 (C-8), 136.23, 136.14 (C-5 NapH), 136.23, 136.14 (C-5), 130.39, 130.32, 128.94, 128.87, 127.81, 127.56, 127.52, 126.54, 126.40, 126.07, 126.02, 122.73, 122.68, 116.22, 116.19 (C-Ar), 90.25, 90.16 (C-1'), 84.58, 84.36 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.25 Hz, C-4'), 74.90 (C-2'), 71.30 (C-3'), 67.91, 67.80 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 64.19, 64.15 ($OCH_2CH_2C(CH_3)_3$), 51.80, 51.72 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 9.0 Hz, $CHCH_3$), 42.74, 42.71 ($OCH_2CH_2C(CH_3)_3$), 30.56, 30.53 ($OCH_2CH_2C(CH_3)_3$), 30.12, 30.10 ($OCH_2CH_2C(CH_3)_3$), 20.79, 20.61 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.25 Hz, $CHCH_3$).

HPLC (System 2) t_R = 17.83, 18.10 min

(ES+) m/z, found: ($M+H^+$) 661.30, $C_{29}H_{37}N_6O_8PS$ required: (M^+) 660.21

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (4.14c).

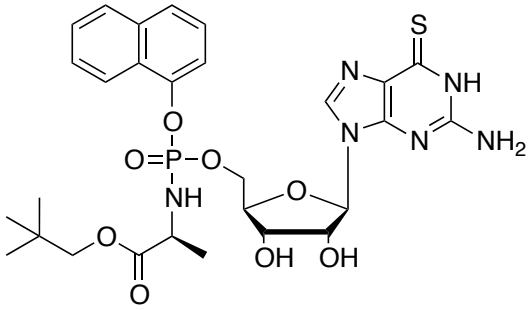


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.100 g, 0.29 mmol) *t*BuMgCl (1.0 M in THF, 0.59 ml, 0.59 mmol) and 1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)phosphorochloridate (**2.3f**, 0.225 g, 0.59 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14c** as a yellow foam (0.054 g, 27%).

^{31}P NMR (202 MHz, MeOD) δ 4.14, 4.11

^{1}H NMR (500 MHz, MeOD) δ 8.12 – 8.10 (m, 0.6H, H-Ar), 8.00, 7.98 (2 x s, 1H, H-8), 7.98 – 7.96 (m, 0.4H, H-Ar), 7.81 - 7.80 (m, 1H, H-Ar), 7.64 – 7.59 (m, 1H, H-Ar), 7.49 – 7.41 (m, 3H, H-Ar), 7.36 – 7.30 (m, 1H, H-Ar), 5.98, 5.89 (2 x d, J = 2.0 Hz, 1H, H-1'), 5.12 - 5.09 (m, 1H, H-2'), 4.91, 4.89 (dd, J = 2.5 Hz, 0.5H, H-5'), 4.72, 4.70 (dd, J = 2.5 Hz, 0.5H, H-5'), 4.54 – 4.47 (m, 1H, H-5', 0.5H, H-3'), 4.41 – 4.40 (m, 0.5H, H-3'), 4.38 – 4.37 (m, 0.5H, H-4'), 4.27 – 4.23 (m, 0.5H, H-4'), 4.14 – 4.05 (m, 1H, C*H*CH_3), 3.81, 3.79, 3.70, 3.68 (2AB, J_{AB} = 10.5 Hz, OCH_2C$(CH_3)_3$), 1.53, 1.52, 1.30, 1.28 (4s, 6H, 2 x CH_3-isopropylidene), 1.40 -1.36 (2 x d, J = 7.0 Hz, 3H, CHCH_3), 0.88, 0.87 (2 x s, 9H, $OCH_2CH_2C(CH_3)_3$).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (4.15c).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (**4.14c,** 0.054 g, 0.078 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15c** as a yellowish foam (0.006 g, 12%).

^{31}P NMR (202 MHz, MeOD) δ 4.11, 4.08

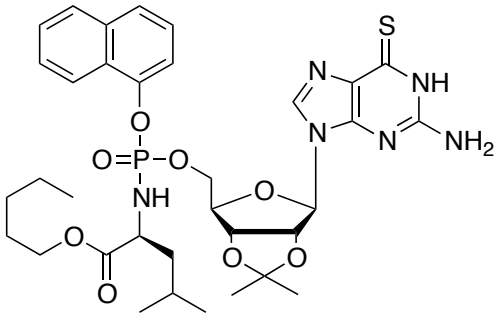
^{1}H NMR (500 MHz, MeOD) δ 8.16 - 8.09 (m, 1H, H-Ar), 7.98, 7.97 (2 x s, 1H, H-8), 7.97, 7.96 (2 x s, 1H, H-8), 7.84 – 7.83 (m, 1H, H-Ar), 7.70 – 7.65 (m, 1H, H-Ar), 7.53, 7.31 (2 x t, *J* = 7.0 Hz, 6.5, 1H, H-Ar), 5.83 (d, *J* = 5.0 Hz, 1H, H-1'), 4.60 – 4.57 (m, 1H, H-2'), 4.49 – 4.36 (m, 3H, H-3', H-5', H-5'), 4.29, 4.24 (2 x d, 1H, H-4'), 4.09 – 4.03 (m, 1H, C*H*CH_3), 3.81, 3.79, 3.71, 3.69 (2AB, J_{AB} = 10.5 Hz, OCH_2C$(CH_3)_3$), 1.35 (d, *J* = 7.0 Hz, CHCH_3), 0.90, 0.89 (2 x s, 9H, $OCH_2CH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.32, 177.22 (C=S), 175.16, 174.86 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 4.5 Hz, C=O), 154.69, 154.58 (C-2), 148.74 (C-4), 147.91, 147.85 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 3.75 Hz, ipso C-Naph), 140.62, 140.53 (C-8), 136.26, 136.18 (C-5 Naph), 130.35, 130.27, 128.92, 128.82, 127.80, 127.53, 127.47, 126.50, 126.35, 126.02, 122.72, 122.65, 116.38, 116.34, 116.24, 116.22 (C-Ar), 90.19, 90.12 (C-1'), 84.68, 84.42 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.6 Hz, C-4'), 75.51, 75.48 (C-2'), 74.80 (O*C*H_2C$(CH_3)_3$), 71.84, 71.73 (C-3'), 67.97, 67.90 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 51.76, 51.73 (*C*HCH_3), 42.74, 42.71 ($OCH_2CH_2C(CH_3)_3$), 32.35 ($OCH_2C(CH_3)_3$), 26.80 ($OCH_2C(CH_3)_3$), 20.86, 20.67 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.12 Hz, CH*C*H_3).

HPLC (System 2) t_R = 16.08, 16.41 min

(ES+) m/z, found: ($M+H^+$) 647, $C_{28}H_{35}N_6O_8PS$ required: (M^+) 646.20

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (4.14d).

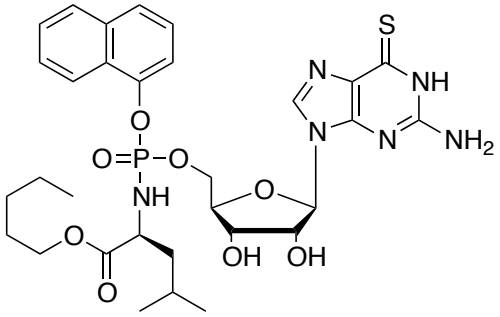


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.10 g, 0.29 mmol) *t*BuMgCl (1.0 M in THF, 0.59 ml, 0.59 mmol) and 1-naphthyl-(pentoxy-L-leucinyl)phosphorochloridate (**2.3s**, 0.25 g, 0.59 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14d** as a yellow foam (0.072 g, 34%).

^{31}P NMR (202 MHz, MeOD) δ 4.58, 4.42

^{1}H NMR (500 MHz, MeOD) δ 8.13 – 8.11 (m, 0.5H, H-Ar), 7.99 – 7.98 (m, 0.5H, H-Ar), 7.98, 7.96 (2 x s, 1H, H-8), 7.82 – 7.80 (m, 1H, H-Ar), 7.65 – 7.60 (m, 1H, H-Ar), 7.49 – 7.41 (m, 3H, H-Ar), 7.37 – 7.29 (m, 1H, H-Ar), 5.99, 5.92 (2 x d, *J* = 2.0 Hz, 1H, H-1'), 5.11 (m, 1H, H-2'), 4.94, 4.93 (dd, *J* = 2.5 Hz, 0.5H, H-5'), 4.79, 4.78 (dd, *J* = 2.5 Hz, 0.5H, H-5'), 4.55 – 4.48 (m, 1H, H-5', 0.5 H, H-3'), 4.40 – 4.34 (m, 0.5 H, H-3', 0.5H, H-4'), 4.25 – 4.21 (m, 0.5H, H-4'), 4.00 – 3.93 (m, 3H, C*H*CH_3, O$CH_2$$CH_2$$CH_2$$CH_2$$CH_3$), 1.52, 1.51, 1.30, 1.29 (4s, 6H, 2 x CH_3-isopropylidene), 1.50 -1.47 (m, 4H, NHCH$CH_2$$CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.26 – 1.22 (m, 5H, $OCH_2CH_2CH_2CH_2CH_3$, $NHCHCH_2CH(CH_3)_2$), 0.83, 0.81 (2 x s, 6H, $NHCHCH_2CH(CH_3)_2$), 0.78 (t, *J* = 6.0 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_3$).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (4.15d).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (**4.14d**, 0.072 g, 0.098 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent to give the pure product **4.15d** as a yellow foam (0.010g, 14%).

^{31}P NMR (202 MHz, MeOD) δ 4.68, 4.41

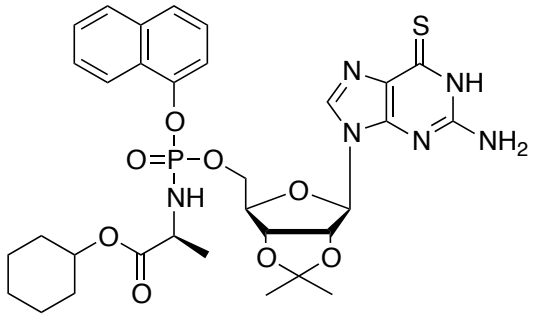
^{1}H NMR (500 MHz, MeOD) δ 8.16, 8.12 (2 x d, *J* = 7.0 Hz, 5.5 Hz, 1H, H-Ar), 7.98, 7.97 (2 x s, 1H, H-8), 7.90 - 7.85 (m, 1H, H-8), 7.71 – 7.66 (m, 1H, H-Ar), 7.55 – 7.44 (m, 3H, H-Ar), 7.39, 7.31 (2 x t, *J* = 8 Hz, 1H, H-Ar), 5.84 (d, *J* = 5.0 Hz, 1H, H-1'), 4.61 – 4.58 (m, 1H, H-2'), 4.52 – 4.45 (m, 1H, 0.5H, H-5'), 4.38 – 4.35 (0.5H, H-5', 1H, H-3'), 4.30 – 4.22 (m, 1H, H-4'), 4.02 – 3.89 ($OCH_2CH_2CH_2CH_2CH_3$, $CHCH_3$), 1.54 -1.46 (m, 4H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.28 – 1.25 (m, 5H, $OCH_2CH_2CH_2CH_2CH_3$, $NHCHCH_2CH(CH_3)_2$), 0.83, 0.81 (3 x s, 9H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.40, 177.38 (C=S), 175.16, 174.86 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 3.5 Hz, C=O), 148.84, 147.79 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 3.75 Hz, 2.5 Hz, ipso C-Naph), 140.62, 140.53 (C-8), 148.77, 148.73 (C-4), 140.55, 140.47 (C-8), 136.31, 136.21, 128.90, 128.81, 127.80, 127.76, 127.49, 127.44, 1276.48, 126.31, 126.00, 125.96, 122.76, 122.67, 116.43, 116.40, 116.04, 116.02 (C-Ar), 90.13, 90.10 (C-1'), 84.73, 84.40 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.5 Hz, C-4'), 74.80, 74.76 (C-2'), 71.86, 71.71 (C-3'), 67.93, 67.89 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.4 Hz, C-5'), 66.39 ($OCH_2CH_2CH_2CH_2CH_3$), 54.78, 54.72 ($CHCH_3$), 42.74, 42.71 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 8.3 Hz, $OCH_2CH_2C(CH_3)_3$), 29.33, 29.32 ($OCH_2CH_2CH_2CH_2CH_3$), 25.69, 25.49 ($OCH_2CH_2CH_2CH_2CH_3$), 23.32 ($NHCHCH_2CH(CH_3)_2$), 23.15, 23.00 ($OCH_2CH_2CH_2CH_2CH_3$), 22.02, 21.74, ($NHCHCH_2CH(CH_3)_2$), 14.28 ($OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 18.83, 19.12 min

(ES+) m/z, found: ($M+Na^+$) 711, $C_{31}H_{41}N_6O_8PS$ required: (M^+) 688.24

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.14e).

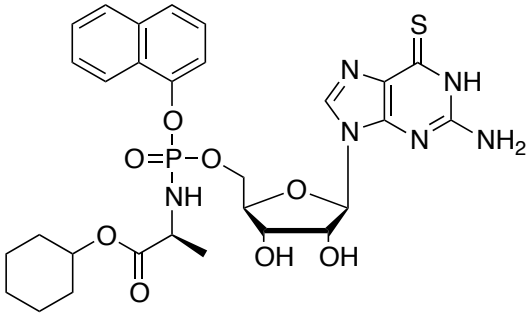


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.11 g, 0.32 mmol) *t*BuMgCl (1.0 M in THF, 0.65 ml, 0.65 mmol) and 1-naphthyl-(cyclohexoxy-L-alaninyl)phosphorochloridate (**2.3h**, 0.26 g, 0.65 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14e** as a yellow foam (0.063 g, 28%).

^{31}P NMR (202 MHz, MeOD) δ 4.39, 4.29

^{1}H NMR (500 MHz, MeOD) δ 8.13 – 8.12 (m, 0.5H, H-Ar), 7.98 – 7.96 (m, 0.5H, H-Ar), 7.97, 7.96 (2 x s, 1H, H-8), 7.83 – 7.79 (m, 1H, H-Ar), 7.66 – 7.62 (m, 1H, H-Ar), 7.50 – 7.41 (m, 3H, H-Ar), 7.38 – 7.33 (m, 1H, H-Ar), 6.01, 5.92 (2 x d, *J* = 2.0 Hz, 1H, H-1'), 5.14 – 5.13 (m, 1H, H-2'), 4.91, 4.90 (dd, *J* = 2.5 Hz, 0.5H, H-5'), 4.66 - 4.61 (m, 0.5H, H-5'), 4.53 – 4.48 (m, 1H, H-5', 0.5H, H-3'), 4.40 (t, *J* = 6.0 Hz, 0.5H, H-3',), 4.36 – 4.34 (m, 0.5H, H-4'), 4.26 – 4.23 (m, 0.5H, H-4'), 4.03 – 3.96 (m, 1H, C*H*CH_3), 1.71 – 1.64 (m, 4H, 2 x CH_2-ester), 1.53, 1.52, 1.36, 1.35 (4s, 6H, 2 x CH_3-isopropylidene), 1.32 - 1.29 (m, 9H, CHC*H*$_3$, 3 x CH_2-ester).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.15e).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)]-phosphate (**4.14e**, 0.063 g, 0.090 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15e** as a yellow foam (0.003 g, 9%).

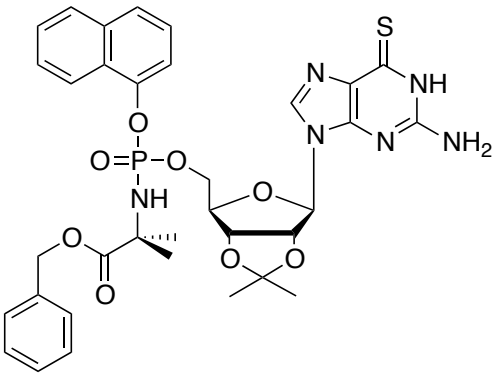
^{31}P NMR (202 MHz, MeOD) δ 4.34, 4.29

^{1}H NMR (500 MHz, MeOD) δ 8.16 - 8.14 (m, 0.5H, H-Ar), 8.11 - 8.09 (d, *J* = 7.5 Hz, 0.5H, H-Ar), 7.98, 7.97 (2 x s, 1H, H-8), 7.88 - 7.83 (m, 1H, H-8), 7.70 – 7.6 (m, 1H, H-Ar), 7.53 – 7.44 (m, 3H, H-Ar), 7.38, 7.32 (2 x t, *J* = 10.0 Hz, 5.0 Hz, 1H, H-Ar), 5.84 (d, *J* = 5.0 Hz, 1H, H-1'), 4.61, 4.58 (2 x t, *J* = 6.0 Hz, 5.0 Hz, 1H, H-2'), 4.49 – 4.45 (m, 1H, 0.5H, H-5'), 4.42 – 4.39 (0.5H, H-5', 1H, H-3'), 4.30 – 4.23 (2 x m, 1H, H-4'), 4.02 – 3.96 (m, 1H, C*H*CH_3), 1.75 - 1.65 (m, 4H, 2 x CH_2-ester), 1.32 - 1.30 (m, 9H, CHC*H*$_3$, 3 x CH_2-ester).

HPLC (System 2) t_R = 17.54, 17.72 min

(ES+) m/z, found: ($M+H^+$) 659.20, $C_{29}H_{35}N_6O_8PS$ required: (M^+) 658.20

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (4.14f).

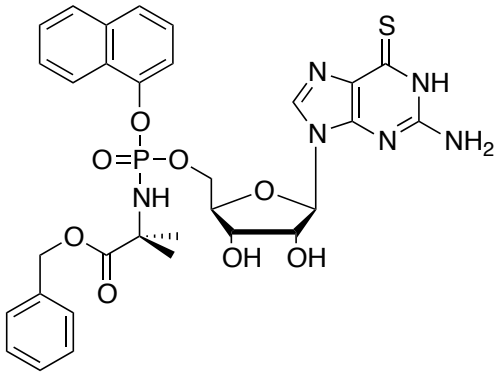


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.10 g, 0.29 mmol) *t*BuMgCl (1.0 M in THF, 0.59 ml, 0.59 mmol) and 1-naphthyl-(benzoxy-dimethylglicinyl)phosphorochloridate (**2.3j**, 0.24 g, 0.59 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14f** as a yellow foam (0.074 g, 36 %).

^{31}P NMR (202 MHz, MeOD) δ 2.86, 2.82

^{1}H NMR (500 MHz, MeOD) δ 8.15 – 8.02 (m, 1H, H-Ar), 7.97, 7.92 (2 x s, 1H, H-8), 7.83 – 7.79 (m, 1H, H-Ar), 7.82 – 7.77 (m, 1H, H-Ar), 7.64 – 7.59 (m, 1H, H-Ar), 7.48 – 7.39 (m, 3H, H-Ar), 7.33 – 7.20 (m, 5H, H-Ar), 5.99, 5.89 (2 x d, *J* = 2.5 Hz, 2.0 Hz, 1H, H-1'), 5.14 – 5.06 (m, 2H, CH_2Bn, 0.5H, H-2'), 4.78, 4.76 (2 x d, *J* = 2.5 Hz, 2.0 Hz, 0.5H, H-2'), 4.45 - 4.34 (m, 3H, H-3', H-5', H-5'), 4.27 – 4.24 (m, 1H, H-4'), 1.51 (s, 6H, 2 x CHC*H*$_3$), 1.47, 1.44, 1.29, 1.26 (4s, 6H, 2 x CH_3-isopropylidene).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (4.15f).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(benzoxy-dimethylglicinyl)]-phosphate (**4.14f**, 0.074 g, 0.10 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15f** as a yellowish foam (0.005 g, 8%).

^{31}P NMR (202 MHz, MeOD) δ 4.34, 4.29

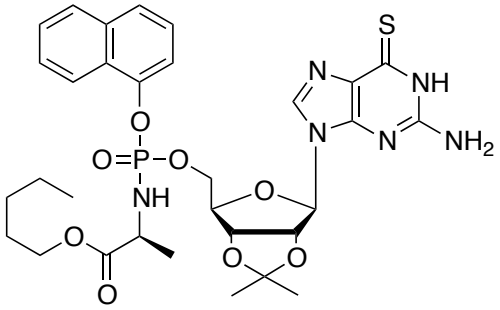
^{1}H NMR (500 MHz, MeOD) δ 8.17, 8.12 (2 x d, *J* = 9.5 Hz, 8.5 Hz, 1H, H-Ar), 7.98, 7.93 (2 x s, 1H, H-8), 7.87, 7.84 (2 x d, *J* = 7.0 Hz, 8.0 Hz, 1H, H-Ar), 7.69, 7.64 (2 x d, *J* = 8.0 Hz, 8.5 Hz, 1H, H-Ar), 7.53 – 7.40 (m, 3H, H-Ar), 7.36 - 7.22 (m, 6H, H-Ar), 5.84, 5.80 (2 x d, *J* = 5.0 Hz, 5.5 Hz, 1H, H-1'), 5.17 – 5.07 (m, 2H, C*H*$_2$Bn), 4.61, 4.58 (2 x t, *J* = 6.0 Hz, 5.0 Hz, 1H, H-2'), 4.44 – 4.32 (m, 2H, H-5', 0.5H, H-3'), 4.26 – 4.20 (0.5H, H-3', 1H, H-4'), 1.51, 1.48 (2 x d, *J* = 6.5 Hz, 4.0 Hz, 6H, 2 x C(CH$_3$)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 177.22, 176.62 (C=S), 148.74, 148.63 (C-Ar), 148.03, 147.97 (C-4), 140.71, 140.55 (C-8), 137.30, 137.25, 136.25, 136.13, 130.29, 130.13, 129.56, 129.53, 129.31, 129.27, 129.20, 128.83, 128.77, 127.85, 127.81, 127.71, 127.66, 127.36, 127.27, 126.45, 126.26, 125.87, 125.81, 122.90, 116.50, 116.37(C-Ar), 90.21, 90.07 (C-1'), 84.71, 84.42 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.75 Hz, C-4'), 74.76, 74.59 (C-2'), 71.79 (C-3'), 68.29, 67.83 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 4.6 Hz, C-5'), 27.84, 27.77, 27.58, 27.49 (4 x d, *J* = 4.5 Hz, C(*C*H$_3$)$_2$).

HPLC (System 2) t_R = 16.69, 16.95 min

(ES+) m/z, found: (M+H$^+$) 681.20, $C_{31}H_{33}N_6O_8PS$ required: (M$^+$) 680.18

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (4.14g).

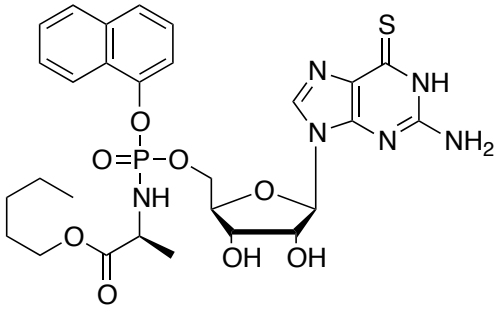


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.213 g, 0.627 mmol) *t*BuMgCl (1.0 M in THF, 1.25 ml, 1.25 mmol) and 1-naphthyl-(pentoxy-L-alaninyl)phosphorochloridate (provided by Slusarczyk, 0.48 g, 1.25 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.14g** as a yellow foam (0.133 g, 31%).

^{31}P NMR (202 MHz, MeOD) δ 4.40, 4.31

^{1}H NMR (500 MHz, MeOD) δ 8.14 – 8.12 (m, 0.3H, H-Ar), 7.98 - 7.95 (m, 0.7H, H-Ar), 7.94, 7.93 (2 x s, 1H, H-8), 7.84 – 7.82 (m, 1H, H-Ar), 7.69 – 7.64 (m, 1H, H-Ar), 7.54 – 7.33 (m, 4H, H-Ar), 6.02, 5.93 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.14 – 5.13 (m, 1H, H-5', 0.4 H, H-2'), 4.91, 4.90 (dd, *J* = 2.5 Hz, 0.3H, H-2'), 4.73, 4.72 (dd, *J* = 2.5 Hz, 0.3H, H-2'), 4.53 - 4.46 (m, 1H, H-5', 0.3H, H-3'), 4.40 (t, *J* = 6.0 Hz, 0.7H, H-3'), 4.37 -4.32 (m, 0.3H, H-4'), 4.27 – 4.22 (m, 0.7H, H-4'), 4.05 – 3.96 (m, 3H, C*H*CH_3, O$CH_2CH_2CH_2CH_2CH_3$), 1.55, 1.54, 1.33, 1.32 (4s, 6H, 2 x CH_3-isopropylidene), 1.52 – 1.51 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 1.37 – 1.24 (m, 7H, $OCH_2CH_2CH_2CH_2CH_3$, $CHCH_3$), 0.85, 0.84 (2 x t, *J* = 7.5 Hz, 3H, $OCH_2CH_2CH_2CH_2CH_3$).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (4.15g).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(pentoxy-L-alaninyl)]-phosphate (**4.14g,** 0.133 g, 0.19 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15 g** as a yellow foam (0.011 g, 9%).

^{31}P NMR (202 MHz, MeOD) δ 4.33, 4.27

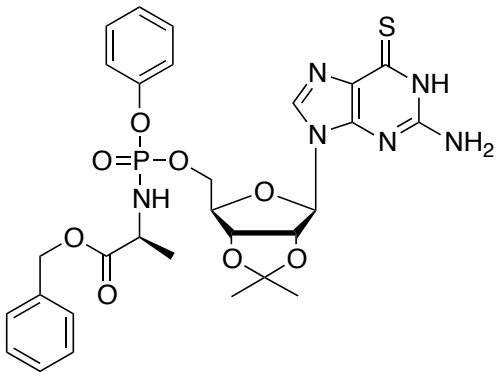
^{1}H NMR (500 MHz, MeOD) δ 8.17 - 8.10 (m, 1H, H-Ar), 7.97, 7.96 (2 x s, 1H, H-8), 7.91 - 7.85 (m, 1H, H-Ar), 7.72 - 7.66 (m, 1H, H-Ar), 7.55 – 7.44 (m, 3H, H-Ar), 7.40, 7.33 (2 x t, *J* = 7.5 Hz, 1H, H-Ar), 5.85 (d, *J* = 5.0 Hz, 1H, H-1'), 4.62 – 4.58 (m, 1H, H-2'), 4.48 – 4.41 (m, 1H, H-5', 0.3H, H-3'), 4.41 – 4.35 (m, 1H, H-5', 0.7H, H-3'), 4.24 – 4.23 (m, 1H, H-4'), 4.03 - 3.97 (m, 3H, OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$, C*H*CH$_3$), 1.55 – 1.52 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.31 – 1.26 (m, 7H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$, CHC*H*$_3$), 0.86, 0.85 (2 x t, *J* = 7.5 Hz, 3H, OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.34, 177.31(C=S), 175.20, 174.93 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 4.25 Hz, C=O), 148.73, 148.71 (C-4), 147.96, 147.91 (2 x d, *J* = 2.5 Hz, ipso Naph), 140.52, 140.48 (C-8), 136.29, 136.20, 130.20, 130.18, 128.89, 188.81, 127.86, 127.81, 127.79, 127.76, 127.50, 127.45, 126.48, 126.35, 126.01, 125.98, 122.71, 122.65, 116.29, 116.27, 116.22, 116.19 (C-Ar), 90.17, 90.10 (C-1'), 84.62, 84.43 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.5 Hz, C-4'), 74.77, 74.73 (C-2'), 71.73 (C-3'), 67.84, 67.71 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 66.52, 66.48 (O*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 51.75, 51.69 (C*H*CH$_3$), 29.33 (OCH$_2$*C*H$_2$CH$_2$CH$_2$CH$_3$), 29.09 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 23.34, 23.33 (OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$), 20.55, 20.40 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.5 Hz, CH*C*H$_3$), 14.28, 14.26 (OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_3$).

HPLC (System 2) t_R = 18.91, 19, 23 min

(ES+) m/z, found: ($M+Na^+$) 669, $C_{28}H_{35}N_6O_8PS$ required: (M^+) 646.19

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (4.14h).

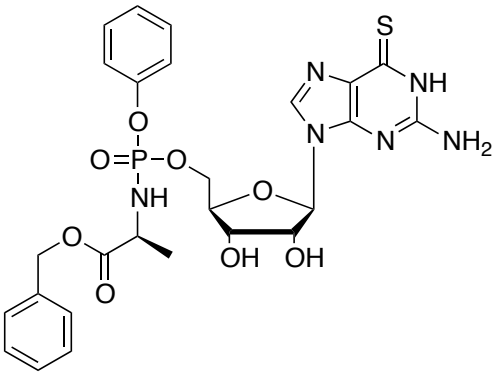


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.100 g, 0.295 mmol) *t*BuMgCl (1.0 M in THF, 0.59 ml, 0.59 mmol) and phenyl-(benzoxy-L-alaninyl)phosphorochloridate (**2.3b**, 0.20 g, 0.59 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as an eluent to give the pure product **4.14h** as a yellow foam (0.047 g, 24%).

^{31}P NMR (202 MHz, MeOD) δ 3.90, 3.81

^{1}H NMR (500 MHz, MeOD) δ 8.02, 8.01 (2 x s, 1H, H-8), 7.32 - 7.06 (m, 10H, H-Ar), 6.03, 5.99 (2 x d, *J* = 2.0 Hz, 1H, H-1'), 5.22, 5.21 (dd, *J* = 2.0 Hz, 0.4H, H-2'), 5.13, 5.11 (dd, *J* = 2.0 Hz, 0.6H, H-2'), 5.11 (d, *J* = 2.5 Hz, 1H, H-5'), 5.08 - 5.03 (m, 2H, C*H*$_2$Bn), 4.44 – 4.34 (m, 2H, H-5', H-3'), 4.28 – 4.24 (m, 0.6H, H-4'), 4.18 – 4.13 (m, 0.4H, H-4'), 4.02 – 3.93 (m, 1H, C*H*CH_3), 1.55, 1.33 (2 x s, 6H, 2 x CH_3-isopropylidene), 1.31, 1.29 (2 x d, *J* = 4.5 Hz, 3H, CHC*H*$_3$).

Synthesis of 6-thioguanosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (4.15h).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (**4.14h,** 0.047 g, 0.007 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15h** as a yellowish foam (0.008 g, 18%).

^{31}P NMR (202 MHz, MeOD) δ 4.09, 3.81

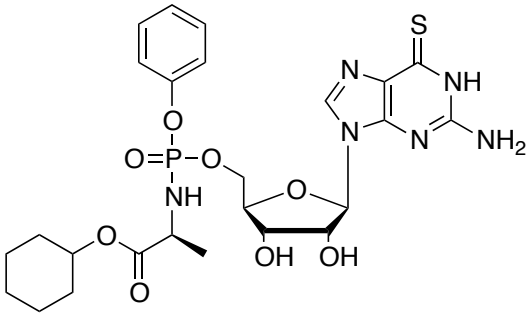
^{1}H NMR (500 MHz, MeOD) δ 8.02, 8.00 (2 x s, 1H, H-8), 7.34 – 7.25 (m, 7H, H-Ar), 7.21 - 7.12 (m, 3H, H-Ar), 5.88, 5.86 (2 x d, J = 5.0 Hz, 1H, H-1'), 5.15, 5.13, 5.10, 5.06 (2AB, J_{AB} = 12.5 Hz, 5.5 Hz, CH_2Bn), 4.64 – 4.60 (m, 1H, H-2'), 4.38 – 4.28 (m, 3H, H-5', H-5', H-3'), 4.23 – 4.20 (m, 1H, H-4'), 4.03 – 3.93 (m, 1H, CHCH$_3$), 1.31, 1.29 (2 x d, ^{3}J = 7.0 Hz, 3H, CHCH_3).

^{13}C NMR (125 MHz, MeOD) δ 177.31 (C=S), 174.90, 174.63 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.25 Hz, 4.0 Hz, C=O), 154.78 (*C*NH$_2$), 152.07, 152.01 (2 x d, J = 6.5 Hz, ipso C-phenyl), 148.84, 148.79 (C-4), 140.46, 140.37 (C-8), 137.28, 137.20 (ipso C-benzyl), 130.78, 130.71 (C-5), 129.57, 129.55, 129.33, 129.30, 129.28, 126.18 (C-Ar), 121.48, 121.40 (2 x d, J = 4.6 Hz, orto C-phenyl), 89.99, 89.88 (C-1'), 84.45, 84.33 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.5 Hz, C-4'), 70.04, 74.88 (C-2'), 71.73 (C-3'), 68.02 (CH_2Bn), 67.67, 67.34 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.25 Hz, C-5'), 51.74, 51.57 (*C*HCH$_3$), 20.39, 20.23 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 7.5 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 14.55, 14.86 min

(ES+) m/z, found: (M+Na$^+$) 639.70, $C_{26}H_{29}N_6O_8PS$ required: (M$^+$) 616.58

Synthesis of 6-thioguanosine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)]-phosphate (4.15i).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)]-phosphate (**4.14i,** 0.039 g, 0.061 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15i** as a yellow foam (0.002 g, 5%).

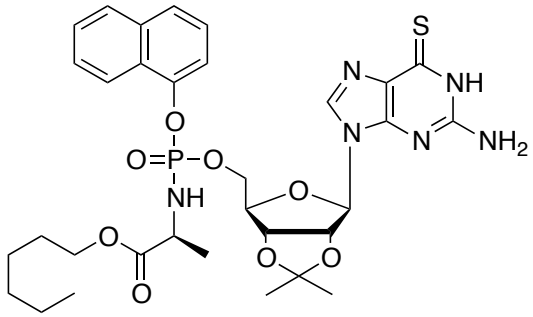
^{31}P NMR (202 MHz, MeOD) δ 4.08, 3.91

^{1}H NMR (500 MHz, MeOD) δ 8.05, 8.03 (2 x s, 1H, H-8), 7.35 – 7.13 (m, 5H, H-Ar), 5.89, 5.87 (2 x d, *J* = 5.0 Hz, 1H, H-1'), 4.73 – 4.63 (m, 2H, OC*H*-ester, H-2'), 4.45 – 4.33 (m, 3H, H-5', H-5', H-3'), 4.28 – 4.24 (m, 1H, H-4'), 3.95 – 3.89 (m, 1H, C*H*CH_3), 1.77 – 1.69 (m, 4H, 2 x CH_2-ester), 1.52 – 1.35 (m, 6H, 3 x CH_2-ester), 1.32, 1.29 (2 x d, ^{3}J = 7.5 Hz, 3H, CHCH_3).

HPLC (System 2) t_R = 14.26, 14.50 min

(ES+) m/z, found: ($M+H^+$) 594.20, $C_{25}H_{32}N_5O_8PS$ required: (M^+) 593.17

Synthesis of 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(hexoxy-L-alaninyl)]-phosphate (4.14j).

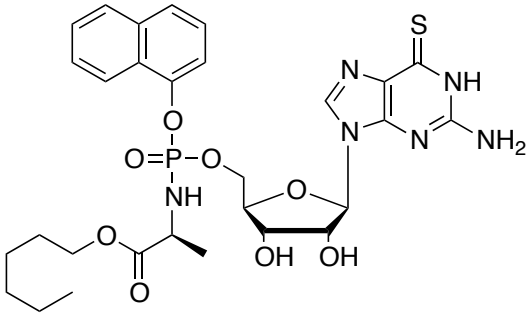


Prepared according to standard procedure **6b** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine (0.133 g, 0.40 mmol) *t*BuMgCl (1.0 M in THF, 0.78 ml, 0.78 mmol) and 1-naphthyl-(hexoxy-L-alaninyl)phosphorochloridate (**2.3g**, 0.31 g, 0.78 mmol). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-3%, gradient) as eluent to give the pure product **4.14j** as a yellow foam (0.063g, 23%).

^{31}P NMR (202 MHz, MeOD) δ 3.90, 3.81

^{1}H NMR (500 MHz, MeOD) δ 8.15 – 8.06 (2 x m, 1H, H-Ar), 7.95, 7.94 (2 x s, 1H, H-8), 7.89 – 7.84 (m, 1H, H-Ar), 7.70 – 7.66 (m, 1H, H-Ar), 7.54 – 7.34 (m, 4H, H-Ar), 6.04, 5.94 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.17 - 5.15 (m, 1H, H-5'), 4.99, 4.95 (dd, *J* = 2.0 Hz, 0.4H, H-2'), 4.76, 4.75 (dd, *J* = 2.5 Hz, 0.6H, H-2'), 4.52 – 4.39 (m, 2H, H-5', H-3'), 4.36 – 4.23 (m, 1H, H-4'), 4.05 – 3.98 (m, 3H, C*H*CH_3, O$CH_2$$CH_2CH_2CH_2CH_2CH_3$), 1.56, 1.55, 1.34, 1.31 (2 x s, 6H, 2 x CH_3-isopropylidene), 1.53 – 1.50 (m, 2H, $OCH_2CH_2$$CH_2CH_2CH_2CH_3$), 1.29 - 1.21 (m, 9H, $OCH_2CH_2CH_2$$CH_2CH_2CH_3$), 0.87, 0.86 (2 x t, *J* = 3.0 Hz, 3H, $CHCH_3$).

Synthesis of 6-thioguanosine 5'-*O*-[1-naphthyl-(hexoxy-L-alaninyl)]-phosphate (4.15j).



Prepared according to standard procedure **7** from, 2',3'-*O,O*-isopropylidene-6-thioguanosine 5'-*O*-[1-naphthyl-(hexoxy-L-alaninyl)]-phosphate (**4.14j,** 0.063 g, 0.089 mmol), in 10 ml of 60 % CH_3COOH in water at 65°C overnight. The crude mixture was purified by column chromatography using $CHCl_3$/MeOH (9:1, gradient) as eluent, followed by preparative purification to give the pure product **4.15j** as a yellow foam (0.013 g, 15%).

^{31}P NMR (202 MHz, MeOD) δ 4.34, 4.28

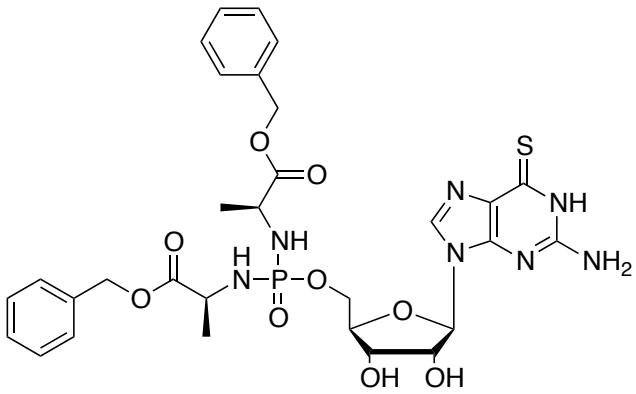
^{1}H NMR (500 MHz, MeOD) δ 8.16 - 8.09 (m, 1H, H-Ar), 7.99 (s, 1H, H-8), 7.89 - 7.84 (m, 1H, H-Ar), 7.71 - 7.66 (m, 1H, H-Ar), 7.54 – 7.31 (m, 4H, H-Ar), 7.39, 7.32 (2 x t, *J* = 8.0 Hz, 1H, H-Ar), 5.85 (d, *J* = 5.0 Hz, 1H, H-1'), 4.61 – 4.58 (m, 1H, H-2'), 4.48 – 4.44 (m, 1H, H-5', 0.6H, H-3'), 4.41 – 4.36 (m, 1H, H-5', 0.4H, H-3'), 4.29 – 4.27 (m, 0.6H, H-4'), 4.25 – 4.23 (m, 0.4H, H-4'), 4.03 - 3.96 (m, 3H, OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$, C*H*CH$_3$), 1.55 – 1.52 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.31 – 1.26 (m, 7H, OC*H*$_2$CH$_2$CH$_2$CH$_2$ CH$_2$CH$_3$, CHC*H*$_3$), 1.53 - 1.50 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.30 - 1.21 (m, 9H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_3$), 0.87, 0.85 (2 x t, *J* = 3.5 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.17, 177.10 (C=S), 175.25, 174.95 (2 x d, $^{3}J_{\text{C-C-N-P}}$ = 5.0 Hz, C=O), 148.67 (C-4), 147.93 (C-Ar), 140.55, 140.48 (C-8), 136.27, 136.19, 128.90, 128.82, 127.79, 127.77, 127.51, 127.46, 126.50, 126.36, 26.02, 125.95, 122.71, 122.65, 116.30, 116.21(C-Ar), 90.18, 90.11 (C-1'), 84.62, 84.44 (d, $^{3}J_{\text{C-C-O-P}}$ = 8.5 Hz, C-4'), 74.88 (C-2'), 71.74 (C-3'), 67.88, 67.73 (2 x d, $^{2}J_{\text{C-O-P}}$ = 5.15 Hz, C-5'), 66.55, 66.51 (O*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 51.75, 51.69 (*C*HCH$_3$), 32.58 (OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 29.62 (OCH$_2$*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 26.60 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_2$CH$_3$), 23.59 (OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$), 20.55, 20.40 (2 x d, $^{3}J_{\text{C-C-N-P}}$ = 7.5 Hz, CH*C*H$_3$), 14.38 (CH*C*H$_3$).

HPLC (System 2) t_R = 21.02, 21.29 min

(ES+) m/z, found: ($M+H^+$) 661.20, $C_{29}H_{37}N_6O_8PS$ required: 660.68.

Synthesis of 6-thioguanosine 5'-*O*-bis(benzoxy-L-alaninyl)-phosphate (4.19a).



Prepared according to standard procedure **8** from, 6-thioguanosine (**4.9**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.047 ml, 0.50 mmol), L-alanine benzyl ester tosylate salt (0.88 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.19a** as a white foam (0.049 g, 17%).

^{31}P NMR (202 MHz, MeOD) δ 13.92

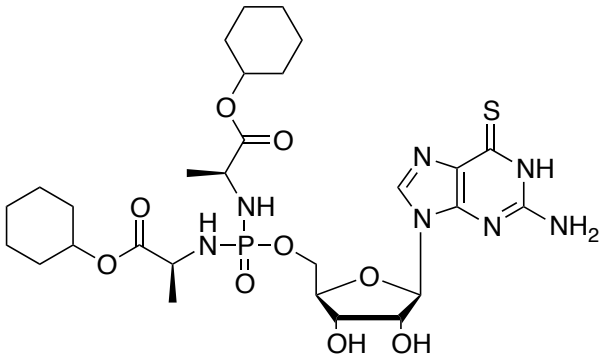
^{1}H NMR (500 MHz, MeOD) δ 8.02 (bs, 1H, H-8), 7.35 – 7.30 (m, 10H, 2 x H-Ar), 6.01 (d, *J* = 5.0 Hz, 1H, H-1'), 5.16 – 5.06 (m, 4H, 2 x OC*H*$_2$Ph), 4.68 (t, *J* = 5.5 Hz, 1H, H-2'), 3.68 (t, *J* = 4.5 Hz, 1H, H-3'), 4.24 – 4.20 (m, 1H, H-5'), 4.16 – 4.14 (m, 2H, H-4', H-5'), 3.97 – 3.91 (m, 2H, 2 x C*H*CH$_3$), 1.32, 1.30 (2 x d, *J* = 7.0 Hz, 6H, 2 x CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.12 (C=S), 175.44, 175.38 (2 x d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, 2 x C=O), 163.19 (C-2), 161.34 (C-4), 151.47 (C-8), 140.49 (C-5), 137.25, 137.20 (ipso *C*-Ph), 129.61, 129.37, 129.34, 129.32 (C-Ar), 90.02 (C-1'), 84.49, 84.41 (d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 10.25 Hz, C-4'), 74.63 (C-2'), 71.69 (C-3'), 68.07 (O*C*H$_2$Ph), 66.38, 66.32 (d, $^2J_{C\text{-}O\text{-}P}$ = 7.5 Hz, C-5'), 51.14, 51.08 (d, $^2J_{C\text{-}N\text{-}P}$ = 3.75 Hz, *C*HCH$_3$), 20.85, 20.62 (2 x d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 5.75 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 16.60 min

(ES+) m/z, found: ($M+H^+$) 702.20, $C_{30}H_{36}N_7O_9PS$ required: (M^+) 701.19

Synthesis of 6-thioguanosine 5'-*O*-bis(cyclohexoxy-L-alaninyl)-phosphate (4.19b).



Prepared according to standard procedure **8** from, 6-thioguanosine (**4.9**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.047 ml, 0.50 mmol), L-alanine cyclohexyl ester tosylate salt (**2.2d**, 0.86 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.19b** as a white foam (0.072 g, 21%).

^{31}P NMR (202 MHz, MeOD) δ 13.94

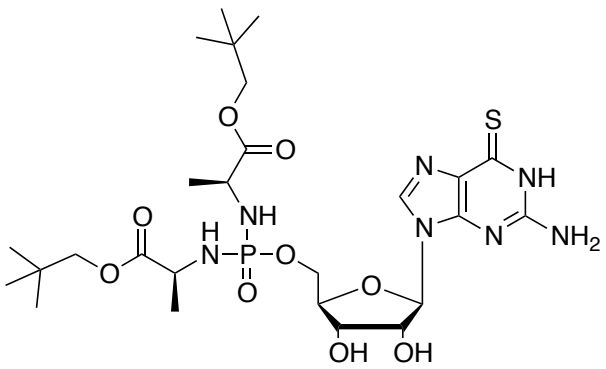
^{1}H NMR (500 MHz, MeOD) δ 8.04 (bs, 1H, H-8), 5.86 (d, *J* = 5.0 Hz, 1H, H-1'), 4.76 – 4.69 (m, 3H, H-2', 2 x ipso C*H*-ester), 4.39 (t, *J* = 4.5 Hz, 1H, H-3'), 4.31 – 4.26 (m, 1H, H-5'), 4.23 – 4.18 (m, 2H, H-4', H-5'), 3.91 – 3.85 (m, 2H, 2 x C*H*CH_3), 1.83 – 1.82 (m, 4H, 2 x CH_2-ester), 1.75 – 1.73 (m, 4H, 2 x CH_2-ester), 1.57 – 154 (m, 2H, CH_2-ester), 1.47 – 1.30 (m, 16 H, 5 x CH_2-ester, 2 x CH(CH_3)$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.33 (C=S), 175.16, 175.07 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, 2 x C=O), 148.82 (C-4), 142.85 (C-8), 130.24 (C-5), 90.12 (C-1'), 84.66, 84.60 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 7.5 Hz, C-4'), 74.90 (2 x ipso *C*H_2-ester), 74.70 (C-2'), 71.77 (C-3'), 66.45, 66.41 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 51.19, 51.09 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 8.75 Hz, *C*HCH_3), 32.53, 32.51, 32.48, 32.46 (*C*H_2-ester), 26.45 (*C*H_2-ester), 24.70 (*C*H_2-ester), 21.07, 20.85 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.8 Hz, CH*C*H_3).

HPLC (System 2) t_R = 16.28 min

(ES+) m/z, found: (M+Na^+) 708.4, $C_{28}H_{44}N_7O_9PS$ required: (M^+) 685.27

Synthesis of 6-thioguanosine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl)-phosphate (4.19c).



Prepared according to standard procedure 8 from, 6-thioguanosine (**4.9**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.047 ml, 0.50 mmol), L-alanine 2,2-dimethylpropyl ester tosylate salt (**2.2a**, 0.86 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.19c** as a white foam (0.029 g, 9%).

^{31}P NMR (202 MHz, MeOD) δ 13.83

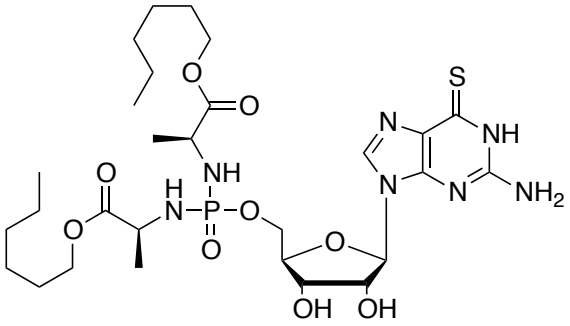
^{1}H NMR (500 MHz, MeOD) δ 8.05 (s, 1H, H-8), 5.87 (d, *J* = 5.0 Hz, 1H, H-1'), 4.71 (t, *J* = 5.0 Hz, 1H, H-2'), 4.41 (t, *J* = 5.0 Hz, 1H, H-3'), 4.30 – 4.19 (m, 3H, H-4', H-5', H-5'), 4.00 - 3.95 (m, 2H, 2 x C*H*CH_3), 3.88, 3.86, 3.76, 3.71 (2AB, J_{AB} = 10.5 Hz, 5.5 Hz, 4H, 2 x C*H*$_2$C(CH_3)$_3$), 1.38 (d, *J* = 7.0 Hz, 6H, 2 x CHC*H*$_3$), 0.94 (s, 18H, 2 x CH_2C(C*H*$_3$)$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.31 (C=S), 175.66, 175.58 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.8 Hz, 2 x C=O), 148.82 (C-4), 140.71 (C-8), 130.28 (C-5), 90.11 (C-1'), 84.70, 84.64 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 7.5 Hz, C-4'), 75.44 (O*C*H_2C(CH_3)$_3$), 75.41 (C-2'), 74.71 (C-3'), 66.56, 66.50 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 7.75 Hz, C-5'), 51.17, 51.09 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 10.5 Hz, *C*HCH_3), 32.38, 32.35 (2 x OCH_2*C*(CH_3)$_3$), 26.79 (OCH_2C(*C*H_3)$_3$), 21.12, 21.92 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.6 Hz,CH*C*H_3).

HPLC (System 2) t_R = 19.83 min

(ES+) m/z, found: (M+Na$^+$) 684.30, $C_{26}H_{44}N_7O_9PS$ required: (M^+) 661.27

Synthesis of 6-thioguanosine 5'-*O*-bis(hexoxy-L-alaninyl)-phosphate (4.19d).



Prepared according to standard procedure 8 from, 6-thioguanosine (**4.9**, 0.155 g, 0.52 mmol) in anhydrous TMP, $POCl_3$ (0.048 ml, 0.52 mmol), L-alanine hexyl ester hydrochloride salt (provided by Madela, 0.53 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.19d** as a white foam (0.057 g, 16%).

^{31}P NMR (202 MHz, MeOD) δ 13.87

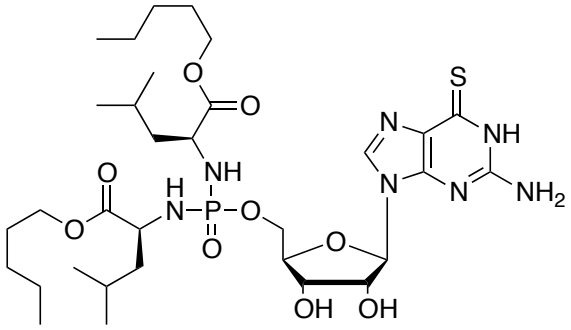
^{1}H NMR (500 MHz, MeOD) δ 8.04 (s, 1H, H-8), 5.86 (d, *J* = 5.0 Hz, 1H, H-1'), 4.70 (t, *J* = 5.5 Hz, 1H, H-2'), 4.39 (t, *J* = 4.5 Hz, 1H, H-3'), 4.28 – 4.18 (m, 3H, H-4', H-5', H-5'), 4.14 - 4.02 (m, 4H, 2 x OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 3.92 – 3.88 (m, 2H, C*H*CH$_3$), 1.66 – 1.61 (m, 4H, 2 x OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.36 – 1.33 (m, 18H, 2 x OCH$_2$CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_3$, 2 x CHC*H*$_3$), 0.92, 9.07 (2 x s, 6H, 2 x OCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 177.33 (C=S), 175.71, 175.67 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.0 Hz, 2 x C=O), 154.80 (C-2), 148.82 (C-4), 140.71 (C-8), 130.23 (C-5), 90.07 (C-1'), 84.64, 84.57 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.25 Hz, C-4'), 74.71 (C-2'), 71.73 (C-3'), 66.32, 66.29 (d, $^{2}J_{C\text{-}O\text{-}P}$ = 7.25 Hz, C-5'), 64.05 (O*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 51.10, 51.01 (d, $^{2}J_{C\text{-}N\text{-}P}$ = 11.0 Hz, *C*HCH$_3$), 42.85 (OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 30.75 (OCH$_2$*C*H$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 30.57 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_2$CH$_3$), 30.03 (OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$), 20.95, 20.65 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 5.6 Hz, CH*C*H$_3$), 15.10 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_2$*C*H$_3$).

HPLC (System 2) t_R = 18.97 min

(ES+) m/z, found: (M+Na$^+$) 712.30, $C_{28}H_{48}N_7O_9PS$ required: (M$^+$) 689.30

Synthesis of 6-thioguanosine 5'-*O*-bis(pentoxy-L-leucinyl)-phosphate (4.19e).



Prepared according to standard procedure 8 from, 6-thioguanosine (**4.9**, 0.150 g, 0.50 mmol) in anhydrous TMP, $POCl_3$ (0.047 ml, 0.50 mmol), L-leucine pentyl ester hydrochloride salt (**2.2i**, 0.60 g, 2.50 mmol) in dry $CHCl_3$ and DIPEA (0.87 ml, 5.01 mmol). The crude mixture was purified by column chromatography (6% MeOH/ $CHCl_3$, gradient) and followed by preparative purification to give the pure product **4.19e** as a white foam (0.003 g, 1%).

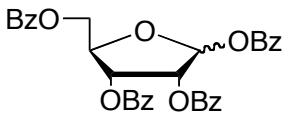
^{31}P NMR (202 MHz, MeOD) δ 13.88

^{1}H NMR (500 MHz, MeOD) δ 8.05 (s, 1H, H-8), 5.85 (d, 1H, *J* = 5.0 Hz, H-1'), 4.70 (t, *J* = 5.0 Hz, 1H, H-2'), 4.38 (t, *J* = 4.5 Hz, 1H, H-3'), 4.29 – 4.26 (m, 1H, H-5'), 4.23 – 4.19 (m, 2H, H-4', H-5'), 4.14 - 4.04 (m, 4H, 2 x OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 3.89 – 3.80 (m, 2H, C*H*CH$_2$CH(CH$_3$)$_2$), 1.66 – 1.62 (m, 4H, 2 x OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.54 - 1.48 (m, 4H, 2 x CHC*H*$_2$CH(CH$_3$)$_2$), 1.36 – 1.34 (m, 8H, 2 x OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$), 0.95 – 0.89 (m, 20H, 2 x CHCH$_2$C*H*(CH$_3$)$_2$), 2 x CHCH$_2$CH(C*H*$_3$)$_2$), 2 x OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$).

HPLC (System 2) t_R = 20.60 min

(ES+) m/z, found: ($M+Na^+$) 768.40, $C_{32}H_{56}N_7O_9PS$ required: (M^+) 745.36

Synthesis of 1,2,3,5-Tetra-*O*-benzoyl-D-ribofuranoside (4.20).

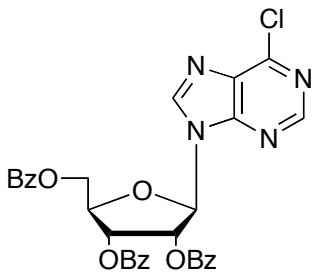


To a cooled solution (0^{o}C) of 1,3,5-Tri-*O*-benzoyl-α-D-ribofuranose (2.00 g, 4.32 mmol), 4-dimethylaminopyridine (0.05 g, 0.43 mmol) and triethylamine (3.0 ml, 0.11 mmol) in anhydrous THF (25 ml), benzoyl chloride (1.0 ml, 8.65mmol) was added dropwise and stirred for 16 hrs under inert atmosphere. The reaction mixture was

quenched with ice cold water and saturated aqueous solution of $NaHCO_3$, then THF was evaporated under vacuum and the remaining aqueous mixture was extracted with EtOAc. The organic layers were combined and washed with water and brine, then dried over $MgSO_4$ followed by evaporation resulting in a thick oil. This crude mixture was dissolved in the mixture of tert-butyl methyl ether (50 ml), heptane (50 ml) and water (1ml) and stirred for 2-3 hours to provide white cystalls, which were vacuum filtered and dried over high vacuum to give the desired pure product. (2.4 g, 70 %).

^{1}H NMR (500 MHz, MeOD) δ 8.15 – 8.07 (m, 6H, Bz), 7.89 – 7.88 (m, 2H, Bz), 7.65 – 7.58 (m, 3H, Bz), 7.54 – 7.50 (m, 3H, Bz), 7.42 – 7.37 (m, 4H, Bz), 7.32 - 7.30 (m, 2H, Bz), 6.98, 6.97 (2 x s, 1H, H_1), 5.94, 5.93 (dd, J = 2.5 Hz, 1H, H_3), 5.94, 5.93 (dd, J = 2.5 Hz, 1H, H_2), 4.94 (m, 1H, H_4), 4.81, 4.79 (dd, J = 12 Hz, 3.5 Hz, 1H, H_5), 4.71, 4.69 (dd, J = 12 Hz, 3.5 Hz, 1H, H_5).

Synthesis of 2,3,5-Tri-*O*-benzoyl-6-chloro-β-D-ribofuranoside (4.21).



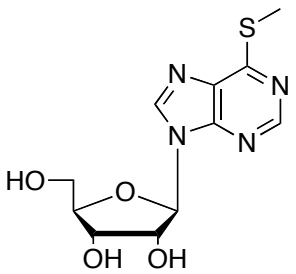
To a precooled (0^{o}C) solution of 1,2,3,5-Tetra-*O*-benzoyl-D-ribofuranoside (**4.20**, 1.70 g, 3.00 mmol), 6-chloropurine (0.51g, 3.3 mmol) 1,8-Diazabicycloundec-7-ene (1.34 ml, 9.00mmol) in 30ml of anhydrous acetonitrile, trimethylsilyl trifluoromethanesulfonate (2.17 ml, 12.00 mmol) was added and stirred for 6 hours at 65^{o}C. The crude mixture was allowed to cool down to room temperature then it was poured into saturated aqueous solution of $NaHCO_3$ and extracted with $CHCl_3$. The organic phase were collected, dried over $MgSO_4$ and purified by column chromatography (50% EtOAc/Hexane) to provide the desired compound as white crystals (0.77 g, 43%).

^{1}H NMR (500 MHz, MeOD) δ 8.99 (s, 1H, H-8), 8.65 (s, 1H, H-2), 7.98 (t, J = 7.0 Hz, 4H, Bz), 7.91 (d, J = 8.0 Hz, 2H, Bz), 7.64 – 7.59 (m, 3H, Bz), 7.48 – 7.40 (m,

6H, Bz), 6.79 (d, *J* = 4.5 Hz, 1H, H-1'), 6.57 (t, *J* = 5.5 Hz, 1H, H-2'), 6.35 (t, *J* = 6.0 Hz, 1H, H-3'), 4.98 – 4.96 (m, 1H, H-4'), 4.90, 4.88 (dd, *J* = 3.5 Hz, 12.5 Hz, 1H, H-5'), 4.76, 4.74 (dd, *J* = 3.5 Hz, 12.5 Hz, 1H, H-5').

^{13}C NMR (125 MHz, MeOD) δ 165.37 (C=O), 164.66 (C=O), 164.51 (C=O), 151.67 (C-2), 151.20 (C-6), 149.73 (C-4), 146.60 (C-8), 133.84, 133.76, 133.35, 129.34, 129.21, 128.62, 128.54 (*C*H-Bz), 86.95 (C-1'), 79.64 (C-4'), 73.18 (C-3'), 70.71 (C-2'), 63.13 (C-5').

Synthesis of 6-*S*-methyl-thioinosine (4.22).



To the solution of 2,3,5-Tri-*O*-benzoyl-6-chloro-β-D-ribofuranoside (**4.21**, 0.70 g, 1.168 mmol) in 10ml of anhydrous DMF, aqueous solution of $NaSCH_3$ (0.70g in 3.5ml H_2O, 10.00 mmol) was added dropwise. The reaction mixture was stirred for 2 hours at ambient temperature then the reaction was diluted with H_2O and extracted with EtOAc (3x10ml). The combined organic phases were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (3% MeOH/$CHCl_3$) to provide the desired compound as white solid (0.132 g, 38%).

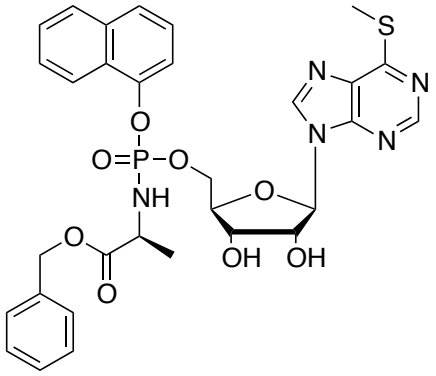
^{1}H NMR (500 MHz, MeOD) δ 8.70 (s, 1H, H-2), 8.59 (s, 1H, H-8), 6.09 (d, *J* = 6.0 Hz, 1H, H-1'), 4.75 (t, *J* = 5.5 Hz, 1H, H-2'), 4.38 (q, *J* = 3.5 Hz, 1H, H-3'), 4.19 (q, *J* = 3.0 Hz, 1H, H-4'), 3.92, 3.90 (dd, *J* = 3.0 Hz, 12.5 Hz, 1H, H-5'), 3.80, 3.78 (dd, *J* = 3.0 Hz, 12.5 Hz, 1H, H-5'), 2.74 (s, 3H, SC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 153.08 (C-6), 144.42 (C-2), 134.41(C-4), 130.53 (C-8), 129.63 (C-5), 91.12 (C-1'), 83.76 (C-4'), 74.78 (C-3'), 71.87 (C-2'), 64.89 (C-5'), 11.77 (S*C*H$_3$).

HPLC (System 2) t_R = 9.76 min

(ES+) m/z, found: (M+Na$^+$) 321.10, $C_{11}H_{14}N_4O_4S$ required: 298.07

Synthesis of 6-*S*-methyl-thioinosine 5'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)]-phosphate (4.23a).



Prepared according to standard procedure **4** from, 6-*S*-methyl-thioinosine (**4.22**, 0.16 g, 0.54 mmol), *t*BuMgCl (1.0 M, 1.07 ml, 1.07 mmol) and 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (**2.3c**, 0.43 g, 1.07 mmol) in anhydrous THF (10 ml). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-4%, gradient) as eluent, followed by preparative purification to give the pure product **4.23a** as a white solid (0.005 g, 1%).

^{31}P NMR (202 MHz, MeOD) δ 4.16, 3.99

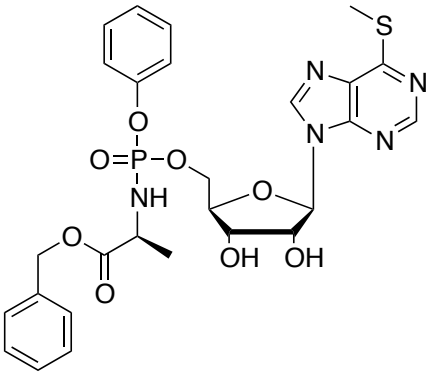
^{1}H NMR (500 MHz, MeOD) δ 8.63, 8.60 (2 x s, 1H, H-2), 8.43, 8.42 (2 x s, 1H, H-8), 8.08 (t, *J* = 7.0 Hz, 1H, H-Ar), 7.87 – 7.84 (m, 1H, H-Ar), 7.68 – 7.66 (m, 1H, H-Ar), 7.52 – 7.49 (m, 1H, H-Ar), 7.46 – 7.41 (m, 2H, H-Ar), 7.36 – 7.26 (m, 6H, H-Ar), 6.08 (t, *J* = 5.0 Hz, 1H, H-1'), 5.06 - 4.99 (m, 2H, C*H*$_2$Bn), 4.76, 4.72 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.48 – 4.36 (m, 3H, H-5', H-5', H-3'), 4.29 – 4.27 (m, 1H, H-4'), 4.06 – 3.97 (m, 1H, C*H*CH$_3$), 2.67 (s, 3H, SC*H*$_3$), 1.29, 1.22 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 174.81, 178.57 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 3.75 Hz, C=O), 163.01, 162.99 (C-6), 153.14, 153.10 (C-2), 149.15, 149.13 (C-4), 147.85, 147.80 (ipso C-Naph), 144.13, 144.01 (C-8), 137.18, 137.13, 136.22, 132.59, 132.52, 129.53, 129.51, 129.27, 129.23, 129.20, 128.88, 128.82, 127.75, 127.45, 126.43, 125.97, 125.95, 122.71, 122.57, 116.17, 116.10 (C-Ar), 90.54, 90.46 (C-1'), 84.51, 84.45 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 7.6 Hz, C-4'), 75.00, 74.91 (C-2'), 71.55, 71.38 (C-3'), 67.95, 67.89 (*C*H$_2$Bn), 67.70, 67.30 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 51.70 (*C*HCH$_3$), 20.41, 20.21 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.35 Hz, CH*C*H$_3$), 11.82 (S*C*H$_3$).

HPLC (System 2) t_R = 18.12, 18.45 min

(ES+) m/z, found: (M+Na$^+$) 688.20, $C_{31}H_{32}N_5O_8PS$ required: (M$^+$) 665.17

Synthesis of 6-*S*-methyl-thioinosine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)]-phosphate (4.23b).



Prepared according to standard procedure **4** from, 6-*S*-methyl-thioinosine (**4.22**, 0.16 g, 0.54 mmol), *t*BuMgCl (1.0 M, 1.07 ml, 1.07 mmol) and phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (**2.3b**, 0.38 g, 1.07 mmol).) in anhydrous THF (10 ml). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-4%, gradient) as eluent, followed by preparative purification to give the pure product **4.23b** as a white solid (0.007 g, 2%).

^{31}P NMR (202 MHz, MeOD) δ 3.89, 3.63

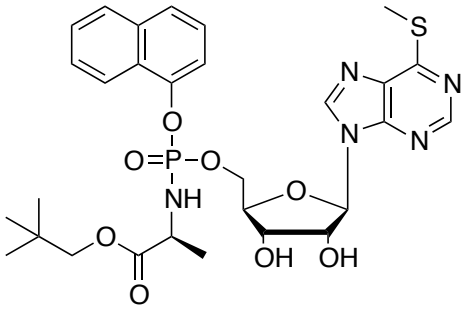
^{1}H NMR (500 MHz, MeOD) δ 8.68, 8.67 (2 x s, 1H, H-2), 8.47, 8.45 (2 x s, 1H, H-8), 7.32 – 7.28 (m, 7H, H-Ar), 7.19 – 7.15 (m, 3H, H-Ar), 6.11 (t, *J* = 5.0 Hz, 1H, H-1'), 5.11 – 5.03 (m, 2H, C*H*$_2$Bn), 4.76, 4.73 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.46, 4.45 (dt, *J* = 5.0 Hz, 2.0 Hz, 1H, H-3'), 4.42 – 4.38 (m, 1H, H-5'), 4.36 – 4.30 (m, 1H, H-5'), 4.27 – 4.25 (m, 1H, H-4'), 4.00 – 3.91 (m, 1H, C*H*CH$_3$), 2.70 (s, 3H, SC*H*$_3$), 1.31, 1.26 (2 x d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 174.80, 178.56 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 3.5 Hz, C=O), 163.03, 163.01 (C-6), 153.21, 153.17 (C-2), 143.95, 143.91 (C-8), 137.27, 130.74, 129.56, 129.54, 129.29, 129.25, 126.18, 121.45, 121.41, 121.39, 121.35 (C-Ar), 90.35, 90.34 (C-1'), 84.55, 84.42 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.5 Hz, C-4'), 75.21, 75.18 (C-2'), 71.60, 71.48 (C-3'), 67.94 (*C*H$_2$Bn), 67.51, 67.06 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.12 Hz, C-5'), 51.68, 51.56 (*C*HCH$_3$), 20.38, 20.21 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.6 Hz, CH*C*H$_3$), 11.80 (S*C*H$_3$).

HPLC (System 2) t_R = 16.52, 16.91min

(ES+) m/z, found: (M+Na$^+$) 638.10, $C_{27}H_{30}N_5O_8PS$ required: (M$^+$) 615.16

Synthesis of 6-*S*-methyl-thioinosine 5'-*O*-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (4.23c).



Prepared according to standard procedure **4** from, 6-*S*-methyl-thioinosine (**4.22**, 0.16 g, 0.54 mmol), *t*BuMgCl (1.0 M, 1.07 mL, 1.07 mmol) and 1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)-phosphorochloridate (**2.3f**, 0.41 g, 1.07 mmol) in anhydrous THF (10 ml). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-4%, gradient) as eluent, followed by preparative purification to give the pure product **4.23c** as a white solid (0.006 g, 2%).

^{31}P NMR (202 MHz, MeOD) δ 4.16, 4.03

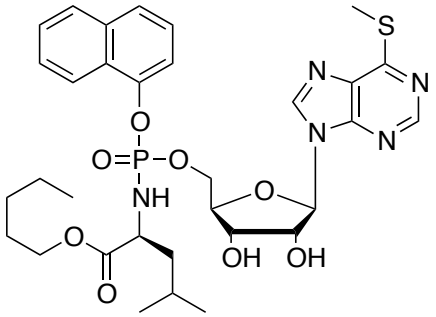
^{1}H NMR (500 MHz, MeOD) δ 8.65, 8.61 (2 x s, 1H, H-2), 8.46, 8.45 (2 x s, 1H, H-8), 8.10 (t, *J* = 9.0 Hz, 1H, H-Ar), 7.81 (t, *J* = 6.5 Hz, 1H, H-Ar), 7.69 – 7.67 (m, 1H, H-Ar), 7.52 – 7.44 (m, 3H, H-Ar), 7.38 – 7.34 (m, 1H, H-Ar), 6.10, 6.09 (2 x d, *J* = 2.5 Hz, 1H, H-1'), 5.11 – 5.03 (m, 2H, C*H*$_2$Bn), 4.77, 4.73 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.52 - 4.40 (m, 3H, H-3', H-5', H-5'), 4.33 – 4.28 (m, 1H, H-4'), 4.05 – 3.99 (m, 1H, C*H*CH$_3$), 3.79, 3.77, 3.68, 3.65 (2AB, J_{AB} = 10.5 Hz, OC*H*$_2$C(CH$_3$)$_3$), 2.69 (s, 3H, SC*H*$_3$), 1.32, 1.28 (2 x d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 0.89, 0.88 (2 x s, 9H, OCH$_2$C(C*H*$_3$)$_3$).

^{13}C NMR (125 MHz, MeOD) δ 175.03, 174.80 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 4.62 Hz, C=O), 163.05, 162.99 (C-6), 153.17, 153.11 (C-2), 149.19, 149.16 (C-4), 147.89, 147.83 (ipso C-Naph), 144.12, 144.07 (C-8), 136.24, 132.60, 132.55, 128.88, 128.83, 127.74, 127.43, 126.42, 125.95, 122.67, 122.58, 116.12, 116.10, (C-Ar), 90.50, 90.48 (C-1'), 84.67, 84.52 (d, $^{3}J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 75.40, 75.35 (O*C*H$_2$C(CH$_3$)$_3$), 75.01, 74.93 (C-2'), 71.57, 71.53 (C-3'), 67.77, 67.53 (2 x d, $^{2}J_{C\text{-}O\text{-}P}$ = 5.35 Hz, C-5'), 51.76, 51.71 (*C*HCH$_3$), 32.30 (OCH$_2$*C*(CH$_3$)$_3$), 26.70 (OCH$_2$C(*C*H$_3$)$_3$), 20.70, 20.50 (2 x d, $^{3}J_{C\text{-}C\text{-}N\text{-}P}$ = 6.5 Hz, CH*C*H$_3$), 11.80 (S*C*H$_3$).

HPLC (System 2) t_R = 17.64, 17.93 min

(ES+) m/z, found: (M+Na$^+$) 668.20, $C_{29}H_{36}N_5O_8PS$ required: (M$^+$) 645.20

Synthesis of 6-*S*-methyl-thioinosine 5'-*O*-[1-naphthyl-(pentoxy-L-leucinyl)]-phosphate (4.23d).



Prepared according to standard procedure **4** from, 6-*S*-methyl-thioinosine (**4.22**, 0.16 g, 0.54 mmol), *t*BuMgCl (1.0 M, 1.07 ml, 1.07 mmol) and 1-naphthyl-(pentoxy-L-leucinyl)-phosphorochloridate (**2.3s**, 0.46 g, 1.07 mmol) in anhydrous THF (10 ml). The crude mixture was purified by column chromatography, using $CHCl_3$/MeOH (1-4%, gradient) as eluent, followed by preparative purification to give the pure product **4.23d** as a white solid (0.003g, 1%).

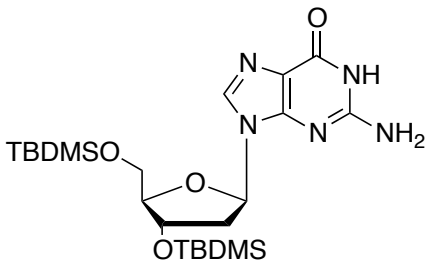
^{31}P NMR (202 MHz, MeOD) δ 4.46, 4.24

^{1}H NMR (500 MHz, MeOD) δ 8.64, 8.60 (2 x s, 1H, H-2), 8.48, 8.46 (2 x s, 1H, H-8), 8.12 – 8.08 (m, 1H, H-Ar), 7.87 – 7.84 (m, 1H, H-Ar), 7.68 – 7.66 (m, 1H, H-Ar), 7.53 – 7.44 (m, 3H, H-Ar), 7.36 (t, *J* = 8.0 Hz, 1H, H-Ar), 6.11, 6.10 (d, *J* = 5.0 Hz, 1H, H-1'), 4.79, 4.74 (2 x t, *J* = 5.0 Hz, 1H, H-2'), 4.53 - 4.43 (m, 2H, H-5', H-5', 0.3H, H-3'), 4.43 – 4.39 (m, 0.7H, H-3'), 4.36 – 4.34 (m, 0.3H, H-4'), 4.32 – 4.29 (m, 0.7H, H-4'), 3.96 – 3.85 (m, 3H, $OCH_2CH_2CH_2CH_2CH_2$, $NHCHCH_3$), 2.68 (s, 3H, SCH_3), 1.65 -1.59 (m, 1H, $NHCHCH_2CH(CH_3)_2$), 1.50 – 1.40 (m, 4H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.25 – 1.23 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.86 - 0.76 (m, 9H, $NHCHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 21.03, 21.45 min

(ES+) m/z, found: ($M+Na^+$) 710.30, $C_{32}H_{42}N_5O_8PS$ required: (M^+) 687.25

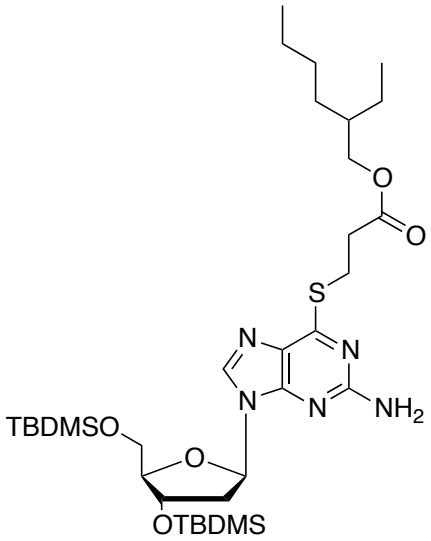
Synthesis of 3',5'-*O*-bis(tert-butyldimethylsilyl)-2-deoxy-guanosine (4.24).



To a cooled (0°C) suspension of 2'-deoxyguanosine (5.00g, 18.70 mmol) in anhydrous dimethylformamide (150 ml) *tert*-Butyldimethylsilyl chloride (9.30 g, 61.74 mmol) and imidazole (8.40 g, 0.123 mol) were added and was allowed to stir overnight at ambient temperature. The reaction mixture was evaporated and dissolved in EtOAc then washed with H_2O, saturated aqueous solution of $NaHCO_3$ and saturated aquous solution of NH_4Cl. The organic phase were collected and dried over $MgSO_4$. The residue was purified by column chromatography (5% MeOH, $CHCl_3$) to give the pure product as white crystalls (8.25 g, 89%).

^{1}H NMR (500 MHz, MeOD) δ 15.48 (bs, 1H, NH), 11.27 (s, 1H, H-8), 9.72 (t, *J* = 6.5 Hz, 1H, H-1'), 9.57 (bs, 2H, NH_2), 8.07 – 8.04 (m, 1H, H-3'), 8.07 – 8.04 (m, 1H, H-3'), 7.46 – 7.43 (m, 1H, H-4'), 7.29, 7.26 (dd, *J* = 3.5 Hz, 1H, H-5'), 7.25, 7.23 (dd, *J* = 3.0 Hz, 1H, H-5'), 6.02 – 5.97 (m, 1H, H-2'), 5.86 – 5.81 (m, 1H, H-2'), 4.39 (s, 18H, 2 x SiC(CH_3)$_3$), 3.58, 3.56 (2 x s, 12H, Si(CH_3)$_2$).
^{13}C NMR (125 MHz, MeOD) δ 159.25 (C=O), 153.47 (C-2), 151.50 (C-4), 135.82 (C-8), 117.48 (C-5), 87.73 (C-1'), 83.63 (C-4'), 71.83 (C-3'), 62.79 (C-5'), 41.11 (C-2'), 26.00, 25.80 (2 x Si(CH_3)$_2$), 18.46, 18.04 (2 x Si*C*(CH_3)$_3$).

Synthesis of 6-*S*-(2-(2-ethylhexyl)oxycarbonyl]ethyl))-3',5'-*O*-bis(tert -butyldimethylsilyl)-2-deoxy-6-thioguanosine (4.25).

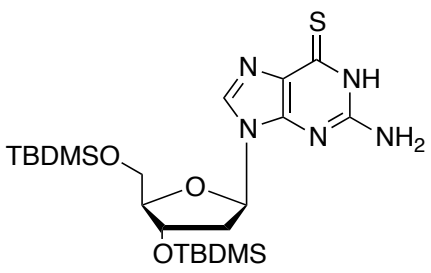


To the cooled suspension of 3',5'-*O*-bis(tert-butyldimethylsilyl)-2-deoxy-6-guanosine (**4.24**, 1.00 g, 2.0 mmol) in anhydrous CH_2Cl_2 (60 ml) under inert atmosphere, Et_3N (1.1 ml, 7.9 mmol), 2-mesitylenesulfonyl chloride (530 mg, 2.4 mmol), and DMAP (12.3 mg, 0.010 mmol) were added dropwise and it was allowed to stir for 12 hours at room temperature. Thereafter the reaction mixture was cooled to 0°C and N-methylpyrrolidine (2.1 ml, 20 mmol) and 2-ethylhexyl-3-mercaptopropionate (4.6 mL, 20 mmol) were added and stirred for further 6 hours at ambient temperature. The crude mixture was diluted with CH_2Cl_2 (40 ml) and washed with 1M KH_2PO_4 (3 x 30 ml), then the combined organic layers were dried over $MgSO_4$ and evaporated. The crude residue was purified by column chromatography (20% EtOAc/ Hexane) to give the pure product **4.25** as a yellow solid (0.54 g, 39%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (s, 1H, H-8), 6.32 (t, *J* = 6.5 Hz, 1H, H-1'), 4.92 (bs, 2H, NH_2), 4.61 - 4.59 (m, 1H, H-4'), 4.06 - 4.05 (m, 2H, $SCH_2CH_2(C{=}O)OC\mathit{H_2}CHCH_2CH_3$), 4.00 – 3.98 (q, *J* = 3.5 Hz, 1H, H-3'), 3.81 (dd, 1H, *J* = 11 Hz, 4.5 Hz, H-5'), 3.76 (dd, 1H, *J* = 11.5 Hz, 4.0 Hz, H-5'), 3.54 (t, *J* = 7.0 Hz, 2H, $SC\mathit{H_2}CH_2(C{=}O)OCH_2CHCH_2CH_3$), 2.84 (t, *J* = 7.5 Hz, $SCH_2C\mathit{H_2}(C{=}O)OCH_2CHCH_2CH_3$), 2.59 (ddd, *J* = 13.0 Hz, 6.0 Hz, 3.5 Hz, 1H, H-2'), 2.36 (ddd, *J* = 13.0 Hz, 6.0 Hz, 3.5 Hz, 1H, H-2'), 1.57 - 1.52 (m, 1H, $SCH_2CH_2(C{=}O)OCH_2C\mathit{H}CH_2CH_3$), 1.33 (q, 2H, *J* = 7.3 Hz, $SCH_2CH_2(C{=}O)OCH_2CHC\mathit{H_2}CH_3$), 1.30 - 1.20 (m, 6H, $SCH_2CH_2(C{=}O)OCH_2CHC\mathit{H_2}C\mathit{H_2}C\mathit{H_2}CH_3$), 0.93 (s, 9H, $SiC(C\mathit{H_3})_3$), 0.92 (s, 9H, $SiC(C\mathit{H_3})_3$), 0.89 (t, *J* = 7.5 Hz, 6H, $SCH_2CH_2(C{=}O)OCH_2CHCH_2C\mathit{H_3}$, $SCH_2CH_2(C{=}O)OCH_2CHCH_2CH_2CH_2C\mathit{H_3}$), 0.12 (s, 6H, $Si(C\mathit{H_3})_2$), 0.09 (s, 6H, $Si(C\mathit{H_3})_2$).

(ES+) m/z, found: ($M+Na^+$) 718.30, $C_{33}H_{61}N_5O_5SSi_2$ required: (M^+) 695.39

Synthesis of 3',5'-*O*-bis(tert-butyldimethylsilyl)-2-deoxy-6-thioguanosine (4.26).

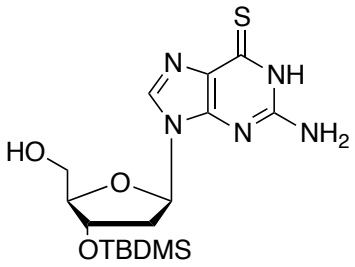


To the cooled solution of 6-*S*-[2-((2-ethylhexyl)oxycarbonyl]ethyl)]-3',5'-*O*-bis(tert-butyldimethylsilyl)-2-deoxy-6-thioguanosine (**4.25**, 0.28g, 0.4mmol) in anhydrous acetonitrile (8.5 ml), 1,8-diazabicycloundec-7-ene (1M, 1.5 ml) was added dropwise and let it stir for 30 minutes before it was slowly allowed to reach room temperature and stirred for additional 5 hours. The crude mixture was evaporated to dryness and purified by column chromatography to give the pure product **4.26** as a yellow solid (0.083g, 74%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (s, 1H, H-8), 6.43 (bs, 2H, NH_2), 6.27 (t, J = 6.5 Hz, 1H, H-1'), 4.59 (q, J = 3.5 Hz, 1H, H-3'), 4.00 (m, 1H, H-4'), 3.77 (dt, J = 3.5 Hz, 1H, H-5'), 3.61 – 2.56 (m, 1H, H-2'), 2.42 – 2.37 (m, 1H, H-2'), 0.92 (s, 9H, SiC(CH_3)$_3$), 0.87 (s, 9H, SiC(CH_3)$_3$), 0.12 (s, 6H, SiC(CH_3)$_2$), 0.07 (s, 6H, Si(CH_3)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 175.01 (C=S), 152.98 (C-2), 147.54 (C-4), 140.13 (C-8), 129.39 (C-5), 87.98 (C-1'), 83.67 (C-4'), 72.10 (C-3'), 63.05 (C-5'), 40.47 (C-2'), 29.40, 29.02 (2 x SiC(CH_3)$_3$), 18.42, 18.01 (2 x SiC(CH$_3$)$_3$), -4.60, -4.69, -5.27, -5.37 (2 x Si(CH$_3$)$_2$).

Synthesis of 3'-*O*-(tert-butyldimethylsilyl)-2-deoxy-6-thioguanosine (4.28).



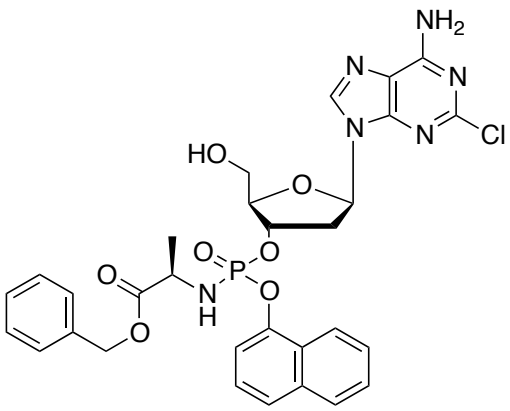
To the cooled solution of 3',5'-*O*-bis(tert-butyldimethylsilyl)-2-deoxy-6-thioguanosine (**4.26**, 0.083 g, 1.62 mmol) in anhydrous tetrahydrofuran (4 ml), 2 ml of 50% TFA in water was added dropwise and stirred for 2 hours at 0°C. The reaction mixture was neutralized with saturated aqueous solution of $NaHCO_3$ and diluted with EtOAc. After separation of the layers, the organic phase was washed with H_2O (10 ml) and brine (10 ml), then dried over $MgSO_4$ and purified by column chromatography (4% MeOH/ $CHCl_3$) to give the pure compound **4.28** as a white foam (0.019g, 31%).

^{1}H NMR (500 MHz, MeOD) δ 8.13 (s, 1H, H-8), 6.27 (t, *J* = 6.5 Hz, 1H, H-1'), 4.64 – 4.62 (m, 1H, H-3'), 3.99 (q, *J* = 4.0 Hz, 1H, H-4'), 3.77 (dd, 1H, *J* = 12 Hz, 4 Hz, H-5'), 3.72 (dd, 1H, *J* = 12.0 Hz, 4.0 Hz, H-5'), 2.68 (ddd, *J* =13.5 Hz, 8.0 Hz, 6.0 Hz, 1H, H-2'), 2.34 (ddd, *J* = 13.5 Hz, 6.5 Hz, 3.0 Hz, 1H, H-2'), 0.96 (s, 9H, SiC(C*H*$_3$)$_3$), 0.16 (s, 6H, SiC(C*H*$_3$)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 175.00 (C=S), 151.92 (C-2), 145.32 (C-4), 141.63 (C-8), 129.73 (C-5), 89.95 (C-1'), 85.74 (C-4'), 74.10 (C-3'), 63.05 (C-5'), 41.93 (C-2'), 30.71 (Si*C*(CH$_3$)$_3$), 26.27 (SiC(*C*H$_3$)$_3$), -1.88 (SiC(*C*H$_3$)$_2$).

8.6 Experimental section – Chapter 5

Synthesis of Cladribine 3'-*O*-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (5.1a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3c**, 0.56 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.1a** as a white solid (0.003 g, 1%).

^{31}P NMR (202 MHz, MeOD) δ 3.32, 2.58

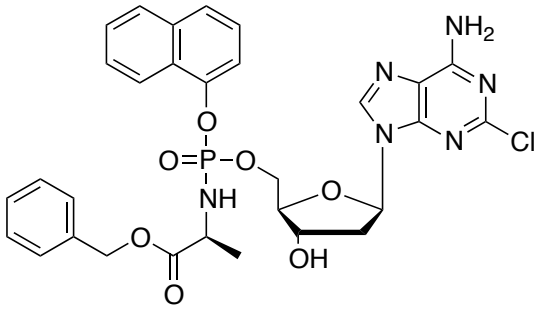
^{1}H NMR (500 MHz, MeOD) δ 8.27, 8.22 (2 x s, 1H, H-8), 7.40 – 7.22 (m, 12 H, H-Ar), 6.36, 6.25 (2 x dd, J_A = 5.8 Hz, 8.6 Hz, J_B = 5.9 Hz, 5.8 Hz, 1H, H-1'), 5.31 – 5.27 (m, 1H, H-3'), 5.22 – 5.16 (m, 2H, C*H*$_2$Ph), 4.26 – 4.19, 4.14 – 4.07 (2 x m, 2H, H-4', $CHCH_3$), 3.84 – 3.73 (m, 2H, H-5', H-5'), 2.89 – 2.81 (m, 1H, H-2'), 2.68 - 2.59 (2 x m, 1H, H-2'), 1.41, 1.39 (2 x d, *J* = 7.2 Hz, 7.1 Hz, 3H, CHC*H*$_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.94, 173.62 (2 x d, J_{C-P} = 4.0 Hz, 4.7 Hz, C=O), 158.1 (C-6), 153.77 (C-2), 155.2 (C-2), 151.1 (C-4), 147.78 146.48, 146.43 (d, J_{C-P} = 6.6 Hz, CO(Naph), 141.63 (C-4), 137.23, 137.15, 136.23, 136.21, 129.54, 129.5, 129.3, 129.2, 129.1, 128.9, 127.9, 127.7, 127.6, 126.65, 126.56, 126.34, 126.25, 122.7, 122.6, 119.7, 116.7, 116.68, 116.61, 116.56 (C-Ar), 88.3, 88.2 (2 x d, J_{C-P} = 6.3, C-4'), 86.67, 86.63 (C-1'), 80.12, 79.91 (2 x d, $^2J_{C-P}$ = 5.0 Hz, C-3'), 68.2 (d, J_{C-P} = 4.0, CH_2Bn), 63.22, 63.15 (C-5'), 52.15, 50.10 (*C*HCH_3), 40.01, 40.0 (C-2'), 19.98,19.93 (2 x d, $^3J_{C-P}$ = 7.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 18.53, 18.56 min

MS (ES^+): 675 ($M+Na^+$), 653 ($M+H^+$) $C_{30}H_{30}ClN_6O_7P$ required: (M^+) 652.16

Synthesis of Cladribine 5'-*O*-[1-naphthyl(benzoxy-L-alaninyl)] phosphate (5.1b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3c**, 0.56 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title compound **5.1b** as a white solid (0.004 g, 1%).

^{31}P NMR (202 MHz, MeOD) δ 3.89, 3.58

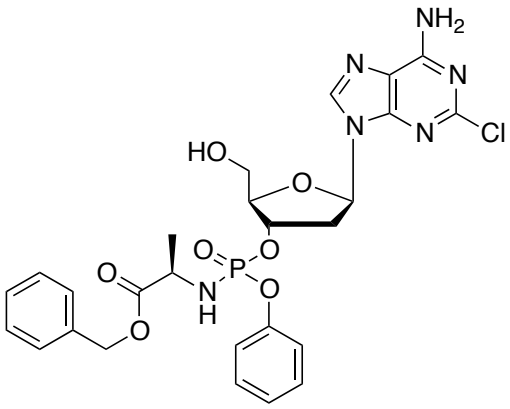
^{1}H NMR (500 MHz, MeOD) δ 8.22 – 8.19 (d, *J* = 6.5 Hz, 1H, H-Ar), 7.34 – 7.28 (m, 6.5H, H-Ar), 7.22 – 7.15 (m, 3.5H, H-Ar), 6.35 (t, *J* = 6.5 Hz, 1H, H-1'), 5.12 – 5.06 (m, 2H, C*H*$_2$Bn), 4.60 - 4.56 (m, 1H, H-3'), 4.35 – 4.25 (m, 2H, H-5'), 4.15 – 4.14 (m, 1H, H-4'), 4.01 – 3.95 (m, 1H, C*H*CH_3), 2.76 – 2.66 (m, 1H, H-2'), 2.48 – 2.45 (m, 1H, H-2'), 1.33 – 1.27 (m, 3H, $CHCH_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.9, 173.6 (C=O), 158.10 (C-6), 157.77, 155.3, 151.31, 147.82 (C-2, C-4, C-6, C-O (Naph)), 141.1, 140.8 (C-8), 137.13, 137.10, 136.25, 136.20, 129.64, 129.54, 129.37, 129.20, 128.91, 128.90, 127.91, 127.62, 127.53, 126.63, 126.50, 126.25, 122.7, 122.6, 119.7, 116.56 (C-Ar), 86.8, 86.7 (C-4'), 85.9 (C-1'), 72.35 (C-3'), 68.90 (CH_2Bn), 67.93 (C-5'), 51.32 (*C*HCH_3), 40.01, 40.0 (C-2'), 20.40, 20.30 (2 x d, $^{3}J_{C\text{-}P}$ = 6.3, 7.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.16, 17.18 min

MS (ES^+): 675 ($M+Na^+$), 653 ($M+H^+$) $C_{30}H_{30}ClN_6O_7P$ required: (M^+) 652.16

Synthesis of Cladribine 3'-*O*-[phenyl-(benzoxy-L-alaninyl)] phosphate (5.2a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3b**, 0.29 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.2a** as a white solid (0.008 g, 2%).

^{31}P NMR (202 MHz, MeOD) δ 3.67, 3.09

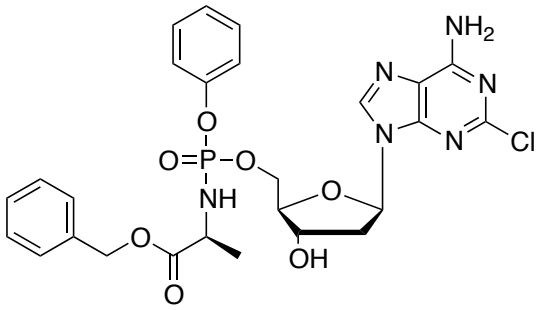
^{1}H NMR (500 MHz, MeOD) δ 8.25, 8.17 (2 x s, 1H, H-8), 7.58 – 7.22 (m, 10 H, H-Ar), 6.31, 6.17 (2 x dd, J_A = 5.8 Hz, 8.5 Hz, J_B = 5.8 Hz, 8.6 Hz, 1H, H-1'), 5.35,5.29 (2 x m, 1H, H-3'), 5.16, 5.12 (2 x s, 2H, CH_2Bn), 4.26 (m, 1H, H-4'), 3.82 – 3.67 (m, 2H, H-5'), 2.84, 2.68, 2.53 (3 x m, 2H, H-2'), 1.41, 1.35 (2 x dd, $^{3}J_{H\text{-}P}$ = 7.1 Hz, $^{4}J_{H\text{-}P}$ = 0.8 Hz, 3H, CH_3).

^{13}C NMR (126 MHz, MeOD) δ 173.54, 173.26 (2 x d, $J_{C\text{-}P}$ = 5.0 Hz, 3.9 Hz, C=O), 156.77 (C-6), 153.77 (C-2), 149.78 146.48, 146.43 (2 x d, $J_{C\text{-}P}$ = 1.6 Hz, 2.5 Hz, CO(Ph), 141.73 (C-4), 140.16 (C-8), 135.8, 137.5 (*C*H_2Bn), 130.92, 130.88, 128.16, 127.82, 127.53, 126.45, 126.22, 125.11, 124.77, 121.41, 121.27 (2 x Ph), 115.2 (C-5), 88.82, 88.62 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0, 6.3, C-4'), 85.26, 85.17 (C-1'), 78.64, 78.43 (2 x d, $^{2}J_{C\text{-}P}$ = 5.0, C-3'), 68.07, 68.05 (CH_2Bn), 63.23, 63.17 (C-5'), 50.43, 50.17 (*C*HCH_3), 38.66, 38.33 (2 x d, $^{3}J_{C\text{-}P}$ = 3.8, C-2'), 19.07,18.84 (2 x d, $^{3}J_{C\text{-}P}$ = 6.5 Hz, 7.5 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.43, 17.46 min

MS (ES^+): 625 ($M+Na^+$), 603 ($M+H^+$) $C_{26}H_{28}ClN_6O_7P$ required: (M^+) 602.14

Synthesis of Cladribine 5'-*O*-[phenyl-(benzoxy-L-alaninyl)] phosphate (5.2b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3b**, 0.29 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.2b** as a white solid (0.012 g, 3%).

^{31}P NMR (202 MHz, MeOD) δ 4.24, 4.00

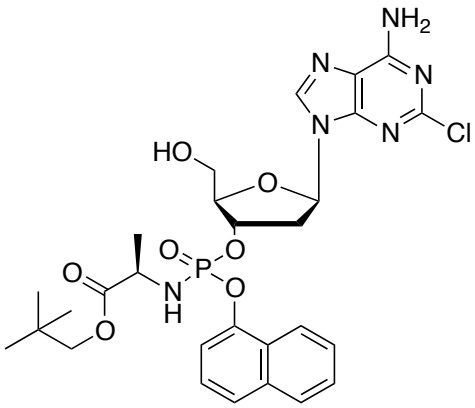
^{1}H NMR (500 MHz, MeOD) δ 8.10 – 8.06 (d, 2H, *J* = 6.5 Hz, Ar), 7.84 – 7.82 (m, 1H, H-Ar), 7.67 -7.63 (m, 1H, H-Ar), 7.50 – 7.42 (m, 3H, H-Ar), 7.35 – 7.25 (m, 4H, H-Ar), 6.31 (t, *J* = 6.5 Hz, 1H, H-1'), 5.08 – 5.01 (m, 2H,C*H*$_2$Bn), 4.58 - 4.53 (m, 1H, H-3'), 4.42 – 4.29 (m, H-5', H-5'), 4.15 – 4.13 (m, 1H, H-4'), 4.08 – 4.01 (m, 1H, C*H*CH_3), 2.54 – 2.44 (m, 1H, H-2'), 2.37 – 2.36 (m, 1H, H-2'), 1.32 – 1.27 (m, 3H, CHC*H*$_3$).

^{13}C NMR (126 MHz, MeOD) δ 173.41, 173.14 (C=O), 156.54, 153.87, 149.97, 146.38 (C-2, C-4, C-6, COPh), 139.56, 139.43 (C-8), 135.67, 134.77, 128.17, 127.86, 127.41, 127.35, 126.31, 126.04, 125.01, 124.53, 121.17 (C-Ar), 117.98 (C-5), 114.80 (C-Ar), 85.49, 85.44 (C-4'), 84.42, 84.43 (C-1'), 78.05 (C-3'), 70.77 (*C*H_2 Bn), 66.50, 66.28 (2 x d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, C-5'), 50.31 (*C*HCH_3), 39.44, 39.37 (C-2'), 19.05, 18.90 (2 x d, $^{3}J_{C\text{-}P}$ = 6.3, 7.6, CH*C*H_3).

HPLC (System 2) t_R = 15.91, 15.93 min

MS (ES^+): 625 ($M+Na^+$), 603 ($M+H^+$) $C_{26}H_{28}ClN_6O_7P$ required: (M^+) 602.14

Synthesis of Cladribine 3'-*O*-[1-naphthyl-(2,2 dimethylpropoxy -L-alaninyl)] phosphate (5.3a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl-(2,2 dimethylpropoxy-L-alaninyl)-phosphorochloridate (**2.3f**, 0.54 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.3a** as a white solid (0.004 g, 1%).

^{31}P NMR (202 MHz, MeOD) δ 3.68, 3.27

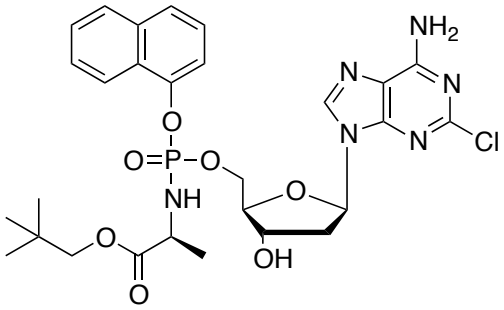
^{1}H NMR (500 MHz, MeOD) δ 8.28 – 8.20 (m, 2H, H-Ar), 7.92 (d, *J* = 8.0 Hz, 1H, H-Ar), 7.75 (d, *J* = 8.2 Hz, 1H, H-Ar), 7.63 - 7.47 (m, 4H, H-Ar), 6.38 - 6.21 (2 x m, 1H, H-1'), 5.39 - 5.33 (2 x m, 1H, H-3'), 4.32 - 4.23 (2 x m, 1H, H-4'), 4.17 - 4.11 (m, 1H, C*H*CH_3), 3.89 – 3.71 (m, 4H, H-5', H-5', OCH_2C$(CH_3)_3$), 2.96 – 2.78, 2.58 – 2.55 (2 x m, 2H, H-2'), 1.43, 1.38 (2 x d, *J* = 7.25 Hz, 3H, CHCH_3), 0.95, 0.93 (2 x s, 9H, OCH_2C(C$H_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 173.83, 173.47 (2 x d, $J_{C\text{-}P}$ = 3.80 Hz, C=O), 156.78, 153.77, 149.80, (C-2, C-4, C-6), 146.50 (d, $J_{C\text{-}P}$ = 7.6 Hz, CONaph), 140.18 (C-8), 134.93, 127.53, 127.50, 126.46, 126.20, 126.15, 125.20, 125.10, 124.77, 121.41, 121.30 (C-Ar), 118.24 (C-5), 115.2, 115.1 (2 x d, $J_{C\text{-}P}$ = 3.8 Hz, 2.5 Hz, C-Ar), 86.8, 86.7 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, 6.3 Hz, C-4'), 85.26, 85.17 (C-1'), 78.61, 78.42 (2 x d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, C-3'), 74.0, 73.9 (O*C*H_2C$(CH_3)_3$), 61.8, 61.6 (C-5'), 50.5, 50.4 (*C*HCH_3), 38.66 (d, $^{3}J_{C\text{-}P}$ = 7.6 Hz, C-2'), 25.32, 25.30 (O*C*H_2C(*C*$H_3)_3$), 19.27, 19.05 (2 x d, $^{3}J_{C\text{-}P}$ = 6.3, 5.0, CH*C*H_3).

HPLC (System 2) t_R = 20.47, 20.50 min

MS (ES^+): 656 ($M+Na^+$), 634 ($M+H^+$) $C_{28}H_{34}ClN_6O_7P$ required: (M^+) 633.03

Synthesis of Cladribine 5'-*O*-[1-naphthyl-(2,2 dimethylpropoxy -L-alaninyl)] phosphate (5.3b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl-(2,2 dimethylpropoxy -L-alaninyl)-phosphorochloridate (**2.3f**, 0.54 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.3b** as a white solid (0.008 g, 2%)

^{31}P NMR (202 MHz, MeOD) δ 4.25, 4.10

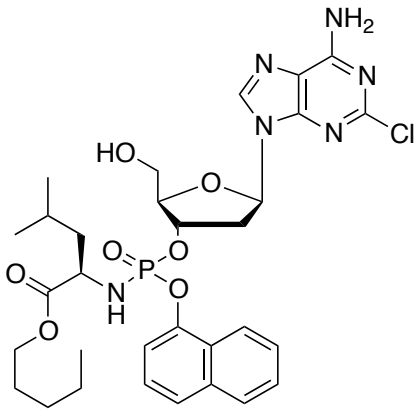
^{1}H NMR (500 MHz, MeOD) δ 8.14 – 8.09 (m, 2H, H-Ar), 7.87 (m, 1H, H-Ar), 7.68 (m, 1H, H-Ar), 7.51 – 7.46 (m, 3H, H-Ar), 7.39 -7.35 (m, 1H, H-Ar), 6.34 - 6.33 (m, 1H, H-1'), 4.61, 4.56 (2 x m, 1H, H-3'), 4.43 – 4.36 (m, 2H, H-5', H-5'), 4.20, 4.17 (2 x m, 1H, H-4'), 4.10, 4.06 (2 x m, 1H, C*H*CH_3), 3.82, 3.67 (m, 2H, C*H*$_2$C$(CH_3)_3$), 2.55- 2.53, 2.52 – 2.38 (2 x m, 2H, H-2', H-2'), 1.37, 1.33 (m, 3H, CHC*H*$_3$), 0.91 (s, 9H, CH_2C(C*H*$_3$)$_3$).

^{13}C NMR (126 MHz, MeOD) δ 173.68, 173.43 (2 x d, $J_{C\text{-}P}$ = 5.0 Hz, C=O), 156.58, 153.91, 153.88, 150.01, 149.95, 147.91, 146.57, 146.49, 146.41, 141.6, 139.68, 139.54, 134.81, 127.43, 127.38, 126.33, 126.04, 125.04, 124.57, 121.20 (C-2, C-4, C-6, H-Ar), 117.96 (C-5), 114.82, 114.77 (H-Ar), 85.60, 85.44 (2 x d, $^{3}J_{C\text{-}P}$ = 7.60 Hz, C-4'), 84.48, 84.39 (C-1'), 73.98, 73.94 (*C*H_2C$(CH_3)_3$), 70.89, 70.80 (C-3'), 66.55, 66.42 (2 x d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, 6.3 Hz, C-5'), 50.43 (d, $^{2}J_{C\text{-}P}$ = 8.80 Hz, *C*HCH_3), 39.41, 39.36 (C-2'), 25.28 (CH_2C(C*H*$_3$)$_3$), 19.28, 19.11 (2 x d, $^{3}J_{C\text{-}P}$ = 6.30 Hz, 7.60 Hz, CH*C*H_3).

HPLC (System 2) t_R = 18.88, 18.91 min

MS (ES^+): 656 ($M+Na^+$), 634 ($M+H^+$) $C_{28}H_{34}ClN_6O_7P$ required: (M^+) 633.03

Synthesis of Cladribine 3'-*O*-[1-naphthyl-(pentoxy -L-leucinyl)] phosphate (5.4a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl-(pentoxy-L-leucinyl)-phosphorochloridate (**2.3s**, 0.59 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.4a** as a white solid (0.003 g, 1%).

^{31}P NMR (202 MHz, MeOD) δ 4.02, 3.48

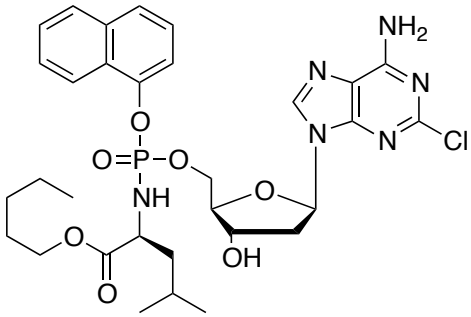
^{1}H NMR (500 MHz, MeOD) δ 8.24, 8.21 (2 x s, 1H, H-8), 7.94, 7.92 (2 x s, 1H, H-Ar), 7.76, 7.53 (2 x s, 1H, H-Ar), 7.63 – 7.55 (m, 3H, H-Ar), 7.52, 7.47 (2 x s, 1H, H-Ar), 6.40 – 6.33 (m, 0.2H, H-1'), 6.23 – 6.20 (m, 0.8H, H-1'), 5.40 – 5.38 (m, 0.2H, H-3'), 5.32 – 5.29 (m, 0.8H, H-3'), 4.34 – 4.33 (m, 0.8H, H-4'), 4.26 – 4.24 (m, 0.2H, H-4'), 4.08 – 4.05 (m, 2H, H-5', H-5'), 4.01 – 3.97 (m, 1H, C*H*$CH_2CH(CH_3)_2$), 3.88 - 3.80 (m, 2H, CHC*H*$_2CH(CH_3)_2$), 3.00 – 2.95 (m, 0.2H, H-2'), 2.89 – 2.79 (m, 1.8H, H-2'), 2.58 – 2.54 (m, 0.8H, H-2'), 1.75 – 1.69 (m, 1H, $CHCH_2CH(CH_3)_2$), 1.63 – 1.55 (m, 4H, CHC*H*$_2CH(CH_3)_2$), $OCH_2CH_2CH_2CH_2CH_3$), 1.35 – 1.28 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 0.91 – 0.82 (m, 9H, $OCH_2CH_2CH_2CH_2CH_3$, $CHCH_2CH(CH_3)_2$).

^{13}C NMR (125 MHz, MeOD) δ 175.08 (C=O), 158.23 (C-6), 155.24 (C-2), 151.26, (C-4), 147.97 (C-O, ipso Naph), 141.64, 141.57 (C-8), 136.55, 136.39, 128.99, 127.91, 127.63, 126.64, 126.25, 126.16, 122.88, 122.74, 119.70, 119.67, 116.75, 116.72 (C-Ar), 88.29, 88.17 (2 x d, $^{3}J_{C\text{-}P}$ = 6.25 Hz, C-4'), 86.70, 86.59 (C-1'), 80.20, 79.86 (2 x d, $^{2}J_{C\text{-}P}$ = 5.25 Hz, C-3'), 66.42 ($OCH_2CH_2CH_2CH_2CH_3$), 63.24, 63.12 (C-5'), 66.36 ($OCH_2CH_2CH_2CH_2CH_3$), 54.92, 54.80 ($CHCH_2CH(CH_3)_2$), 44.12, 44.05 (2 x d, $^{2}J_{C\text{-}P}$ = 8.25 Hz, $CHCH_2CH(CH_3)_2$), 40.20, 40.18 (C-2'), 29.40, 29.19 ($OCH_2CH_2CH_2CH_2CH_3$), 25.76, 25.48 ($CHCH_2CH(CH_3)_2$), 23.36, 23.22 ($OCH_2CH_2CH_2CH_2CH_3$), 23.09 ($OCH_2CH_2CH_2CH_2CH_3$), 21.96, 21.66 ($CHCH_2CH(CH_3)_2$), 14.29 ($OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 24.41, 24.44 min

MS (ES^+): 697 ($M+Na^+$), 675 ($M+H^+$), $C_{31}H_{40}ClN_6O_7P$ required: (M^+) 674.24

Synthesis of Cladribine 5'-*O*-[1-naphthyl-(pentoxy -L-leucinyl)] phosphate (5.4b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl-(pentoxy-L-leucinyl)-phosphorochloridate (**2.3s**, 0.59 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.4b** as a white solid (0.014 g, 3%).

^{31}P NMR (202 MHz, MeOD) δ 4.58, 4.25

^{1}H NMR (500 MHz, MeOD) δ 8.15 - 8.09 (m, 2H, H-8, H-Ar), 7.85 - 7.84 (m, 1H, H-Ar), 7.68 - 7.65 (m, 1H, H-Ar), 7.51 – 7.46 (m, 3H, H-Ar), 7.37 – 7.36 (m, 1H, H-Ar), 6.35 – 6.32 (m, 1H, H-1'), 4.60 – 4.56 (m, 1H, H-3'), 4.45 – 4.42 (m, 1.3H, H-5'), 4.36 – 4.33 (m, 0.7H, H-5'), 4.23 – 4.18 (m, 1H, H-4'), 3.99 – 3.90 (m, 3H, OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_3$, C*H*CH$_2$CH(CH$_3$)$_2$), 2.56 – 2.48 (m, 1H, H-2'), 2.44 – 2.36 (m, 1H, H-2'), 1.67 – 1.62 (m, 1H, CHCH$_2$C*H*(CH$_3$)$_2$), 1.53 – 1.44 (m, 4H, CHC*H*$_2$CH(CH$_3$)$_2$), OCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 1.27 – 1.24 (m, 4H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$), 0.84 – 0.71 (m, 9H, OCH$_2$C*H*$_2$CH$_2$CH$_2$C*H*$_3$, CHC*H*$_2$CH(C*H*$_3$)$_2$).

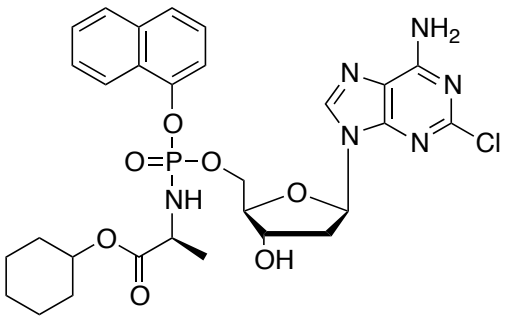
^{13}C NMR (126 MHz, MeOD) δ 175.44, 1745.06 (2 x d, $^3J_{C\text{-}P}$ = 4.5 Hz, C=O), 158.02 (C-6), 155.33 (C-2), 151.44, 151.39 (C-4), 147.93, 147.87 (2 x d, $^2J_{C\text{-}P}$ = 7.4 Hz, C-O Naph), 141.02, 140.93 (C-8), 136.25, 128.88, 128.82, 127.78, 127.75, 127.46, 126.49, 126.02, 125.91, 122.67, 122.70, 122.66, 116.33, 116.30, 116.07, 116.05 (C - Ar), 87.14, 86.91 (2 x d, $^3J_{C\text{-}P}$ = 8.12 Hz, C-4'), 85.93, 85.82 (C-1'), 72.42, 72.27

(C-3'), 68.07, 67.93 (2 x d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, C-5'), 66.36 (O*C*H_2C*H*$_2$CH$_2$CH$_2$C*H*$_3$), 54.80, 54.72 (*C*HCH$_2$CH(CH$_3$)$_2$), 44.20, 44.93 (2 x d, $^{2}J_{C\text{-}P}$ = 8.0 Hz, CH*C*H$_2$CH(CH$_3$)$_2$), 40.93, 40.89 (C-2'), 29.33, 29.13 (OCH$_2$*CH*$_2$CH$_2$CH$_2$C*H*$_3$), 25.69, 25.45 (CHCH$_2$*C*H(CH$_3$)$_2$), 23.34, 23.32 (OCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_3$), 23.16, 23.05 (OCH$_2$CH$_2$CH$_2$*C*H$_2$CH$_3$),), 22.03, 21.74 (CHCH$_2$CH(*C*H$_3$)$_2$), 14.31 (OCH$_2$CH$_2$CH$_2$CH$_2$*C*H$_3$).

HPLC (System 2) t_R = 21.55, 21.57 min

MS (ES$^+$): 697 (M+Na$^+$), 675 (M+H$^+$), $C_{31}H_{40}ClN_6O_7P$ required: (M$^+$) 674.24

Synthesis of Cladribine 5'-*O*-[1-naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (5.5b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and 1-naphthyl-(cyclohexoxy-L-alaninyl)-phosphorochloridate (**2.3h**, 0.55 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using CHCl$_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.5b** as a white solid (0.013 g, 3%).

^{31}P NMR (202 MHz, MeOD) δ 4.26, 4.12

^{1}H NMR (500 MHz, MeOD) δ 8.13 - 8.11 (m, 2H, H-8, H-Ar), 7.86 - 7.83 (m, 1H, H-Ar), 7.67 – 7.65 (m, 1H, H-Ar), 7.51 – 7.45 (m, 3H, H-Ar), 7.39 – 7.34 (m, 1H, H-Ar), 6.33 (t, *J* = 6.5 Hz, 1H, H-1'), 4.66 – 4.55 (m, 2H, H-3', OC*H*-ester), 4.45 – 4.36 (m, 2H, H-5', H-5'), 4.20 – 4.18 (m, 1H, H-4'), 4.01 – 3.93 (m, 1H, C*H*CH$_3$), 2.57 – 2.46 (m, 1H, H-2'), 2.43 – 2.35 (m, 1H, H-2'), 1.75 – 1.66 (m, 4H, 2 x CH_2-ester), 1.38 – 1.27 (m, 9H, 3 x CH_2-ester, CHCH_3).

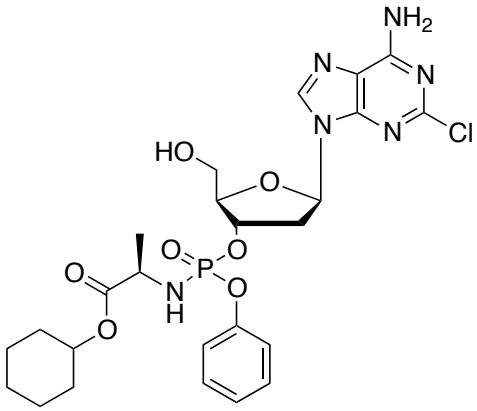
^{13}C NMR (125 MHz, MeOD) δ 175.0, 174.7 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C=O), 158.02 (C-6), 155.35, 155.32 (C-2), 151.44, 151.39 (C-4), 147.94, 147.86 (2 x d, $^{2}J_{C\text{-}P}$ =

7.5 Hz, C-O Naph), 141.03, 140.94 (C-8), 136.24, 128.88, 128.86, 128.83, 127.78, 127.47, 126.50, 125.98, 122.67, 122.64, 119.42, 116.25, 116.23, 116.18, 116.14 (C-Ar), 86.98, 86.86 (2 x d, $^{3}J_{C\text{-}P}$ = 8.0 Hz, C-4'), 85.91, 85.85 (C-1'), 74.98 (*C*H-ester), 72.29, 72.26 (C-3'), 68.00, 67.79 (2 x d, $^{2}J_{C\text{-}P}$ = 5.25 Hz, C-5'), 40.88, 40.78 (C-2'), 32.42, 32.35 (*C*H_2-ester), 26.39 (*C*H_2-ester), 24.62 (*C*H_2-ester), 20.67, 20.57 (2 x d, $^{3}J_{C\text{-}P}$ = 6.75 Hz, CH*C*H_3).

HPLC (System 2) t_R = 18.67, 18.71 min

MS (ES^+): 667 ($M+Na^+$), 645 ($M+H^+$), $C_{29}H_{34}ClN_6O_7P$ required: (M^+) 644.19

Synthesis of Cladribine 3'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)] phosphate (5.6a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and phenyl-(cyclohexoxy-L-alaninyl)-phosphorochloridate (provided by Gibbs, 0.48 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.6a** as a white solid (0.008 g, 2%).

^{31}P NMR (202 MHz, MeOD) δ 3.34, 2.80

^{1}H NMR (500 MHz, MeOD) δ 8.32 - 8.28 (m, 1H, H-8, H-Ar), 7.43 – 7.37 (m, 2H, H-Ar), 7.31 - 7.22 (m, 3H, H-Ar), 6.44 - 6.41 (m, 0.3H, H-1'), 6.36 – 6.29 (m, 0.7H, H-1'), 5.37 - 5.34 (m, 0.3H, H-3'), 5.30 – 5.27 (m, 0.7H, H-3'), 4.81 – 4.74 (m, 1H, C*H*-ester), 4.33 – 4.25 (m, 1H, H-4'), 4.01 – 3.92 (m, 1H, C*H*CH_3), 3.87 – 3.79 (m, 2H, H-5', H-5'), 3.01 – 2.89 (m, 1H, H-2'), 2.82 – 2.77 (m, 0.7H, H-2'), 2.67 – 2.63

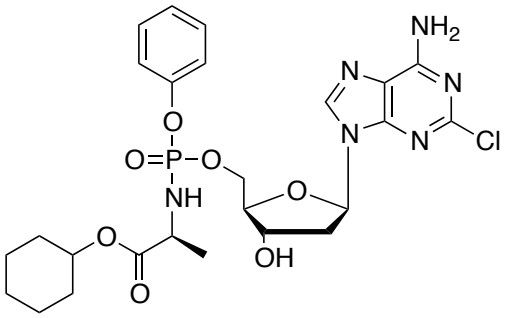
(m, 0.3H, H-2'), 1.86 – 1.74 (m, 4H, 2 x CH_2-ester), 1.57 – 1.30 (m, 9H, 3 x CH_2-ester, CHCH_3).

^{13}C NMR (126 MHz, MeOD) δ 174.62, 174.38 (2 x d, $^3J_{C\text{-}P}$ = 6.0 Hz, C=O), 158.23 (C-6), 155.22 (C-2), 152.12 (C-4), 147.66, 147.37 (2 x d, $^2J_{C\text{-}P}$ = 6.75 Hz, C-O Naph), 141.73, 141.41 (C-8), 130.96, 130.93, 130.87, 130.75, 126.32, 126.17, 121.64, 121.61, 121.56, 121.52 (C-Ar), 88.27, 88.16 (2 x d, $^3J_{C\text{-}P}$ = 7.0 Hz, C-4'), 86.76, 86.64 (C-1'), 79.88, 79.61 (2 x d, $^2J_{C\text{-}P}$ = 5.5 Hz, C-3'), 79.51 (*C*H-ester), 63.27, 63.17 (C-5'), 51.98, 51.81 (*C*HCH$_3$), 40.11, 40.05 (2 x d, $^3J_{C\text{-}P}$ = 5.0 Hz, C-2'), 32.54, 32.46 (*C*H$_2$-ester), 26.45 (*C*H$_2$-ester), 24.65 (*C*H$_2$-ester), 20.66, 20.50 (2 x d, $^3J_{C\text{-}P}$ = 7.0 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 19.95, 19.98 min

MS (ES$^+$): 617 (M+Na$^+$), 595 (M+H$^+$), $C_{25}H_{32}ClN_6O_7P$ required: (M$^+$) 594.18

Synthesis of Cladribine 5'-*O*-[phenyl-(cyclohexoxy-L-alaninyl)] phosphate (5.6b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and phenyl-(cyclohexoxy-L-alaninyl)-phosphorochloridate (provided by Gibbs, 0.48 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.6b** as a white solid (0.012 g, 3%).

^{31}P NMR (202 MHz, MeOD) δ 3.92, 3.67

^{1}H NMR (500 MHz, MeOD) δ 8.23, 8.22 (2 x s, 1H, H-8, H-Ar), 7.34 – 7.31 (m, 2H, H-Ar), 7.23 – 7.16 (m, 3H, H-Ar), 6.40 – 6.36 (m, 1H, H-1'), 5.37 – 5.34 (m, 0.3H, H-3'), 4.72 – 4.67 (m, 1H, C*H*-ester), 4.63 – 4.61 (m, 1H, H-3'), 4.42 – 4.29 (m, 2H, H-5', H-5'), 4.20 – 4.17 (m, 1H, H-4'), 3.93 – 3.82 (m, 1H, C*H*CH$_3$), 3.01 – 2.89 (m,

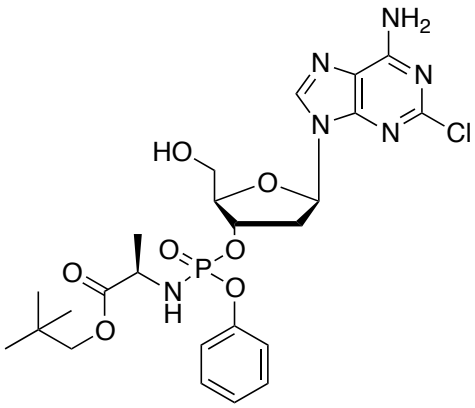
1H, H-2'), 2.82 – 2.77 (m, 0.7H, H-2'), 2.74 – 2.69 (m, 0.3H, H-2'), 2.51 – 2.44 (m, 1H, H-2'), 1.81 – 1.69 (m, 4H, 2 x C*H*$_2$-ester), 1.43 – 1.27 (m, 9H, 3 x C*H*$_2$-ester, CHC*H*$_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.49, 174.28 (2 x d, $^3J_{C\text{-}P}$ = 5.25 Hz, C=O), 158.09 (C-6), 155.40, 155.37 (C-2), 151.54, 151.47 (C-4), 143.49, 143.45, 143.37 (C-Ar), 141.19, 141.16 (C-8), 130.76, 126.17, 126.14, 121.45, 121.42, 121.40, 121.37, (C -Ar), 86.98, 86.85 (2 x d, $^3J_{C\text{-}P}$ = 8.5 Hz, C-4'), 85.93, 85.91 (C-1'), 79.50 (*C*H-ester), 74.94, 74.92 (C-3'), 67.75, 67.43 (2 x d, $^2J_{C\text{-}P}$ = 5.5 Hz, C-5'), 51.80, 51.67 (*C*HCH$_3$), 40.87, 40.81 (C-2'), 32.47, 32.40, 30.72, 26.39, 26.41, 24.65, 24.61 (*C*H$_2$-ester), 20.63, 20.44 (2 x d, $^3J_{C\text{-}P}$ = 6.25 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 17.76, 17.79 min

MS (ES$^+$): 617 (M+Na$^+$), 595 (M+H$^+$), $C_{25}H_{32}ClN_6O_7P$ required: (M$^+$) 594.18

Synthesis of Cladribine 3'-*O*-[phenyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.7a).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and phenyl-(2,2-dimethylpropoxy-L-alaninyl)-phosphorochloridate (**2.3e**, 0.46 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.7a** as a white solid (0.008 g, 2%).

^{31}P NMR (202 MHz, MeOD) δ 3.30, 2.76

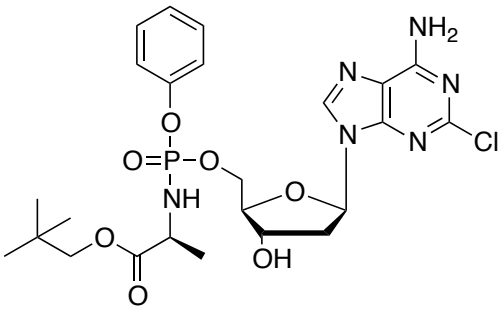
^{1}H NMR (500 MHz, MeOD) δ 8.32, 8.27 (2 x s, 0.7H, H-8), 8.23, 8.07 (2 x s, 0.3H, H-8), 7.57 - 7.52 (m, 0.3H, H-Ar), 7.43 - 7.38 (m, 2H, H-Ar), 7.31 – 7.22 (m, 2.7H, H-Ar), 6.44 – 6.41 (m, 0.3H, H-1'), 6.36 - 6.30 (m, 0.7H, H-1'), 5.38 - 5.36 (m, 0.3H, H-3'), 5.30 - 5.27 (m, 0.7H, H-3'), 4.32 - 4.30 (m, 0.7H, H-4'), 4.27 - 4.25 (m, 0.3H, H-4'), 4.15 - 4.12 (m, 0.3H, C*H*CH_3), 4.08 - 4.04 (m, 0.7H, C*H*CH_3), 3.87 - 3.79 (m, 2H, H-5', H-5'), 3.92 - 3.80 (m, 1H, H-5', 0.7H, H-5', 2H, OC*H*$_2$C$(CH_3)_3$), 3.68, 3.58 (2 x dd, *J* = 12.0 Hz, 5.0 Hz, 0.3H, H-5'), 3.01 - 2.89 (m, 0.7H, H-2'), 2.81 - 2.64 (m, 1H, H-2'), 2.35 - 2.29 (m, 0.3H, H-2'), 1.44, 1.39 (2 x d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 0.98, 0.96 (2 x s, 9H, $OCH_2CH(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 175.24, 174.95 (2 x d, $J_{C\text{-}P}$ = 3.8 Hz, C=O), 158.23, 155.23, 152.17, 152.14, 152.12, 152.08, 151.28 (C-2, C-4, C-6), 147.9, (d, $J_{C\text{-}P}$ = 7.6 Hz, ipso Naph), 141.72, 141.68 (C-8), 130.93, 130.89, 130.75, 126.34, 126.14, 121.65, 121.61, 121.57, 121.53, (C-Ar), 88.30, 88.21 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, 6.25 Hz, C-4'), 86.7, 86.6 (C-1'), 79.87, 79.65 (2 x d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, C-3'), 75.48, 75.44 ($OCH_2CH(CH_3)_3$), 63.28, 63.14 (C-5'), 51.88, 51.75 (*C*HCH_3), 40.13 (d, $^{3}J_{C\text{-}P}$ = 8.6 Hz, C-2'), 26.77, 26.75 ($OCH_2CH(CH_3)_3$), 20.7, 20.5 (2 x d, $^{3}J_{C\text{-}P}$ = 6.5 Hz, 5.0 Hz, $CHCH_3$).

HPLC (System 2) t_R = 19.23, 19.25 min

MS (ES^+): 605 ($M+Na^+$), 583 ($M+H^+$), $C_{24}H_{32}ClN_6O_7P$ required: (M^+) 582.18

Synthesis of Cladribine 5'-*O*-[phenyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.7b).



Prepared according to the standard procedure **4** from, cladribine (0.20 g, 0.70 mmol), *t*BuMgCl (0.84 ml, 0.84 mmol) and phenyl-(2,2-dimethylpropoxy-L-alaninyl)-phosphorochloridate (**2.3e**, 0.46 g, 1.40 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1 to 5% slow gradient), which was followed by preparative purification to give the title product **5.7b** as a white solid (0.012 g, 3%).

^{31}P NMR (202 MHz, MeOD) δ 3.89, 3.64

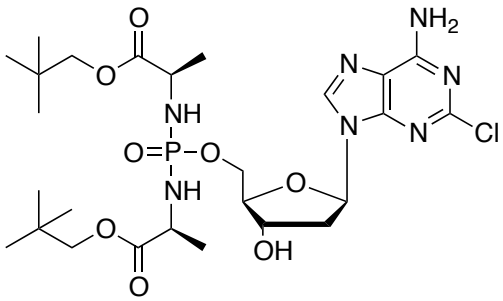
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23, 8.22 (2 x s, 1H, H-8, H-Ar), 7.34 – 7.31 (m, 2H, H-Ar), 7.23 – 7.16 (m, 3H, H-Ar), 6.40 – 6.36 (m, 1H, H-1'), 4.64 – 4.61 (m, 1H, H-3'), 4.42 – 4.29 (m, 2H, H-5', H-5'), 4.20 – 4.15 (m, 1H, H-4'), 4.06 – 3.93 (2 x m, 1H, C*H*CH_3), 3.83, 3.81, 3.77, 3.75 (2AB, J_{AB}= 10 Hz, 2H, O*C*H_2C$(CH_3)_3$), 2.73 – 2.70 (m, 1H, H-2'), 2.51 – 2.44 (m, 1H, H-2'), 1.35, 1.32 (2 x d, J = 7.5 Hz, CHC*H*$_3$), 0.94 , 0.92 (2 x s, 9H, $OCH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, MeOD) δ 175.09 (d, $J_{C\text{-}C\text{-}N\text{-}P}$ = 5.10 Hz, C=O), 174.88 (d, $J_{C\text{-}C\text{-}N\text{-}P}$ = 5.10 Hz, C=O), 158.08 (C-6), 155.40, 155.36 (C-2), 152.15, 152, 09 (C-4), 141.23, 141.15 (C-8), 130.76, 126.20, 126.15 (C-Ar), 121.46, 121.40 (2 x d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 4.50 Hz, *C*2-Ph), 86.76, 86.87 (2 x d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4'), 85.94, 85.89 (C-1'), 75.40 (O*C*H_2C$(CH_3)_3$), 72.32, 72.25 (C-3'), 67.80, 67.54 (2 x d, $^3J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 51.74, 51.1 (*C*HCH_3), 40.83 (C-2'), 32.36 ($OCH_2C(CH_3)_3$), 26.74, 26.72 ($OCH_2C(CH_3)_3$), 20.71, 20.52 (2 x d, $^3J_{C\text{-}O\text{-}P}$ = 6.50 Hz, CH*C*H_3).

HPLC (System 2) t_R = 17.47, 14.49 min

MS (ES^+): 605 ($M+Na^+$), 583 ($M+H^+$), $C_{24}H_{32}ClN_6O_7P$ required: 582.18

Synthesis of Cladribine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl)-phosphate (5.8a).



Prepared according to standard procedure **8**, from, cladribine (0.20 g, 0.70 mmol), $POCl_3$ (0.065 ml, 0.70 mmol), L-alanine 2,2-dimethylpropyl ester tosylate salt (**2.2a**, 1.16 g, 3.5 mmol) in dry $CHCl_3$, trimethylphosphate and DIPEA (1.22 ml, 0.70 mmol). The crude mixture was purified by column chromatography in gradient ($CHCl_3$/MeOH 0 to 6%) and followed by preparative purification in order to give the title product **5.8a** as a white solid (0.027 g, 6%).

^{31}P NMR (202 MHz, MeOD) δ 13.77

^{1}H NMR (500 MHz, MeOD) δ 8.29 (s, 1H, H-8, H-Ar), 4.66 – 4.63 (m, 1H, H-4'), 4.24 – 4.13 (m, 3H, H-5', H-5', H-3'), 3.99 – 3.89 (m, 1H, 2 x C*H*CH_3), 3.87, 3.85, 3.75, 3.73 (2AB, J_{AB} = 10.0 Hz, 4.0 Hz, 2 x OC*H*$_2$C$(CH_3)_3$), 2.83 - 2.78 (m, 1H, H-2'), 2.53 - 2.48 (m, 1H, H-2'), 1.37, 1.36 (d, *J* = 7.5 Hz, 6H, 2 x CHC*H*$_3$), 0.95, 0.94 (2 x s, 18H, $OCH_2C(CH_3)_3$).

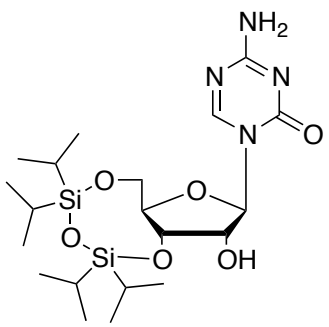
^{13}C NMR (126 MHz, MeOD) δ 175.58 (d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 3.8 Hz, C=O), 158.11 (C-6), 155.41 (C-2), 151.61 (C-4), 141.29 (C-8), 119.45 (C-5) 87.02 (d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 8.5 Hz, C-4'), 85.60 (C-1'), 75.42 (O*C*$H_2C(CH_3)_3$), 72.20 (C-3'), 66.51 (d, $^3J_{C\text{-}O\text{-}P}$ = 5.0 Hz, C-5'), 51.07 (d, $^2J_{C\text{-}N\text{-}P}$ = 7.5 Hz, *C*HCH_3), 40.76 (C-2'), 32.32 ($OCH_2C(CH_3)_3$), 30.72 ($OCH_2C(CH_3)_3$), 21.13, 20.99 (2 x d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 6.25 Hz, $CHCH_3$).

HPLC (System 2) t_R = 17.36 min

MS (ES^-): 682.24 ($M+Cl^-$), $C_{26}H_{43}ClN_7O_8P$ required: (M^+) 647.26

8.7 Experimental section – Chapter 6

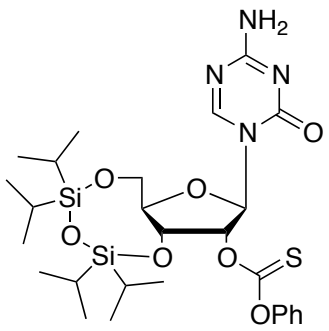
Synthesis of 3',5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-5-azacytidine (6.1).



5-Azacytidine (3.00g, 12.28 mmol) was suspended in anhydrous pyridine (100 ml) under inert atmosphere and cooled down to 0 °C. To this cooled suspension $TIPDSCl_2$ (1.2 eq, 4.71 mL, 14.74 mmol) was added dropwise and the mixture was stirred at ambient temperature for 16 hrs. Thereafter the solvent was evaporated in vacuo and the residue was purified by column chromatography (5% MeOH/$CHCl_3$) to give the title product **6.1** as a white foam (5.32 g, 89%).

^{1}H NMR (500 MHz, DMSO) δ 8.31, 8.27 (2 x s, 1H, H-6), 7.52 (d, *J* = 15.0 Hz, NH_2), 5.56 (d, *J* = 5.0 Hz, 1H, H-1'), 5.52 (s, 2'OH), 4.23 (q, *J* = 4.5 Hz, 1H, H-2'), 4.16 - 4.13 (m, 2H, H-3', H-5'), 4.01, 4.00 (2 x t, *J* = 2.0 Hz, 1H, H-4'), 3.93, 3.91 (2 x d, *J* = 2.5 Hz, 1H, H-5'), 1.06 – 1.01 (m, 28H, 4 x SiC*H*($C*H_3$)$_2$).
^{13}C NMR (125 MHz, MeOD) δ 165.95 (C=O), 155.01 (C-6), 152.88 (C-4), 90.96 (C-1'), 80.74 (C-4'), 73.47 (C-3'), 68.55 (C-2'), 60.07 (C-5'), 17.34, 17.24, 17.17, 17.14, 16.96, 16.88, 16.84, 16.79 (8 x SiCH($C*H_3$)$_2$), 12.71, 12.36, 12.13, 11.96 (4 x Si*C*H($C*H_3$)$_2$).
MS (ES^+): 509 ($M+Na^+$), 487 ($M+H^+$) $C_{20}H_{38}N_4O_6Si_2$ required: (M^+) 486.71

Synthesis of 2'-*O*-(phenoxythiocarbonyl)-3',5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-5-azacytidine (6.2).



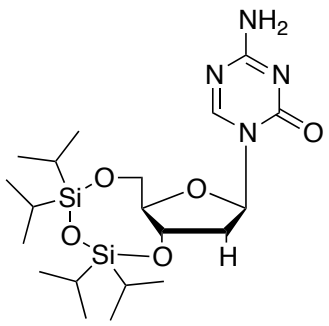
The protected nucleoside (**6.1**, 3.00 g, 6.16 mmol) was suspended in anydrous acetonitrile (70 ml) under inert atmosphere. To this mixture PhOC(S)Cl (2 eq, 1.70 ml, 12.33 mmol) was added dropwise in the presence of DMAP (5 eq, 3.765 g, 30.82 mmol). The reaction mixture was stirred at room temperature for 16-36 hrs. The crude residue was dissolved in $CHCl_3$ and washed with water, 0.1 M HCl then water. The aqeous phase were extracted with $CHCl_3$ and the combined organic phases were dried over $MgSO_4$ and evaporated to dryness. The crude mixture was purified by column chromatography (1-3% MeOH/$CHCl_3$) to give the title product **6.2** as a white foam (1.03 g, 27%).

1H NMR (500 MHz, CDCl3) δ 8.32 (s, 1H, H-6) 7.68 (bs, 2H, NH_2), 7.50 (t, *J* = 7.5 Hz, 1H, Ar), 7.35 (t, *J* = 7.0 Hz, 1H, H-Ar), 7.15 – 7.13 (m, 2H, H-Ar), 6.18 (d, *J* = 5.5 Hz, 1H, H-1'), 5.78 (s, 1H, H-4') 4.09 (dd, *J* = 9.3 Hz, *J* = 5 Hz, 1H, H-5'), 3.99 (dd, *J* = 9.3 Hz, *J* = 5.0 Hz, 1H, H-5'), 3.90 – 3.86 (m, 1H, H-3'), 1.08 – 1.03 (m, 28H, 4 × SiC*H*($C*H_3$)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 193.46 (C=S), 166.00 (C=O), 157.13 (C-4), 152.85 (C-6), 129.83, 126.75, 121.45, 120.79 (C-Ar), 89.63 (C-1'), 83.81 (C-4'), 81.40 (C-2'), 69.53 (C-3'), 60.81 (C-5'), 17.28, 17.20, 17.12, 17.02, 16.95, 16.89, 16.84 (8 x SiCH($C*H_3$)$_2$), 12.65, 12.37, 12.27, 12.08 (4 x Si*C*H($C*H_3$)$_2$).

MS (ES^+): 645 ($M+Na^+$), 623 ($M+H^+$) $C_{27}H_{42}N_4O_7SSi_2$ required: (M^+) 622.23

Synthesis of 3',5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (6.3).



Protected nucleoside (**6.2**, 0.96 g, 1.54 mmol) was dissolved in anhydrous toluene (15 ml) and the solution was degassed with argon for 30 mins. Thereafter 0.2 M AIBN solution in toluene (1.93 ml, 0.38 mmol) and Bu3SnH (0.83 mL, 3.08 mmol) was added dropwise and the mixture was heated up for 100 °C and stirred for 2 - 3hrs. The residue was evaporated to dryness and purified by column chromatography (1-3% MeOH/$CHCl_3$) to give the product **6.3** as a white foam (1.17 g, 85%).

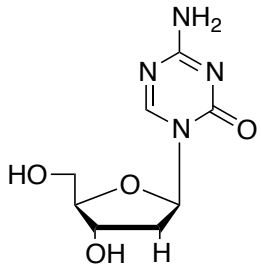
^{1}H NMR (500 MHz, MeOD) δ 8.42 (s, 1H, H-6), 5.96 (dd, *J* = 7.5 Hz, 2.5 Hz, 1H, H-1'), 4.63 (q, *J* = 8.5 Hz, 1H, H-3'), 4.08 (2 × dd, *J* = 2.5 Hz, 12.8 Hz, 2H, H-5'), 3.87 – 3.84 (m, 1H, H-4'), 2.56 – 2.44 (m, 2H, H-2'), 1.14 – 1.06 (m, 28H, 4 × SiC*H*($C*H_3$)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 168.00 (C=O), 157.04 (C-4), 156.1 (C-6), 87.07 (C-1'), 86.69 (C-4'), 70.02 (C-3'), 62.32 (C-5'), 40.78 (C-2'), 18.07, 18.02, 17.98, 17.92, 17.70, 17.59, 17.56, 17.49 (8 × SiCH($C*H_3$)$_2$), 14.68, 14.34, 14.10, 13.84 (4 x Si*C*H(CH_3)$_2$).

MS (ES^+): 493 ($M+Na^+$), 471 ($M+H^+$), $C_{20}H_{38}N_4O_5Si_2$ required: (M^+) 470.24

Synthesis of 2'-Deoxy-5-azacytidine (d5AzaC) (6.4).

Method 1:



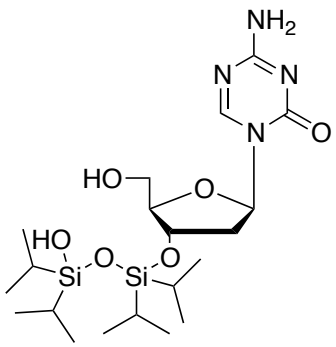
Protected nucleoside (**6.3**, 0.230 g, 0.48 mmol) was dissolved in anhydrous THF (10 ml) and therto solid supported TBAF (~1.5 mmol F-/g resin, 0.69 g = 1.05 mmol) was added. The mixture was stirred at ambient temperature overnight. The yellowish residue was filtered off and the crude mixture was evaporated. Only traces of the desired product was observed by TLC.

Method 2:

To the solution of **6.3** (0.23g, 0.48 mmol) in anhydrous THF, 1M TBAF in anhydrous THF (0.97 mmol, 0.97 ml) was added dropwise. The reaction mixture was stirred at room temperature overnight under inert atmosphere. After evaporation the yellowish oil was crystallised in the mixture of $CHCl_3$ and MeOH (0.003 g, 3%).

^{1}H NMR (500 MHz, DMSO) δ 8.58 (s, 1H, H-6), 7.52, 7.49 (2 × bs, NH_2), 6.03 (t, *J* = 6.5 Hz, 1H, H-1'), 5.25 (bs, 2'OH), 5.06 (t, *J* = 5.0 Hz, 5'OH), 4.25 – 4.22 (m, 1H, 3'OH), 3.31 (q, *J* = 3.5 Hz, 1H, H-4'), 3.61, 3.55 (2 × dd, *J* = 12.0 Hz, 3.5 Hz, 2H, H-5'), 2.22 – 2.18 (m, 1H, H-2'), 2.16 – 2.11 (m, 1H, H-2').
MS (ES^+): 251 ($M+Na^+$), 229 ($M+H^+$), $C_8H_{12}N_4O_4$ required: (M^+) 228.09

Synthesis of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (6.5).



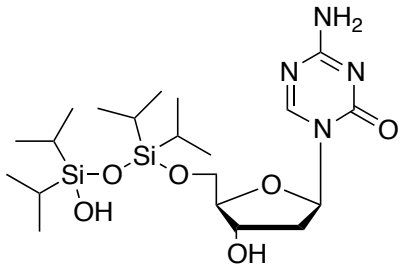
To the cooled solution of the protected nucleoside (**6.3**, 0.10g, 0.21mmol) in anhydrous THF (2ml), a mixture of TFA : H_2O (1ml) in the ratio of 1:1 was added dropwise. After approximately 90 minutes the reaction mixture was neutralized with saturated solution of $NaHCO_3$ and diluted with EtOAc. Separation the organic phase thereafter was washed with H_2O (10 ml), brine (10 ml) and the residue was dried over $MgSO_4$ (0.061g, 67%).

^{1}H NMR (500 MHz, MeOD) δ 8.64 (s, 1H, H-6), 6.15 (t, *J* = 3.0 Hz, 1H, H-1'), 6.03 (q, *J* = 4.5 Hz, 1H, H-4'), 4.11 – 4.03 (m, 3H, H-3', H-5', H-5'), 2.52 – 2.48 (m, 1H, H-2'), 2.29 – 2.24 (m, 1H, H-2'), 1.13 – 1.03 (m, 24H, CH(C*H*$_3$)$_2$), 0.97 – 0.91 (m, 4H, C*H*(CH$_3$)$_2$).

^{13}C NMR (125 MHz, DMSO) δ 167.93 (C=O), 157.00 (C-4), 156.35 (C-6), 89.17 (C-1'), 87.94 (C-4'), 79.49 (C-3'), 71.47 (C-5'), 63.45 (C-2'). 42.87, 17.93, 17.90, 17.88, 17.83 (8 x CH(*C*H$_3$)$_2$), 14.76, 14.73, 14.20, 13.99 (4 x *C*H(CH$_3$)$_2$).

MS (ES$^+$): 511 (M+Na$^+$), 489 (M+H$^+$) $C_{20}H_{40}N_4O_6Si_2$ required: (M$^+$) 488.25

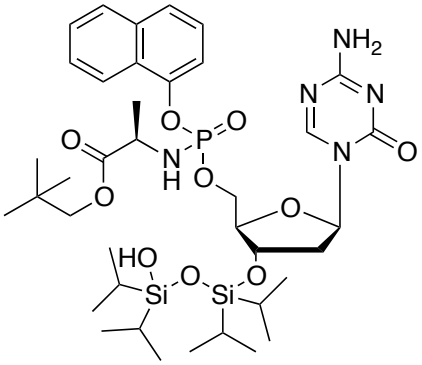
Synthesis of 5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (6.6).



To the cooled solution of the protected nucleoside (**6.3**, 0.10g, 0.21mmol) in anhydrous THF (2ml), a mixture of TFA : H_2O (1ml) in the ratio of 1:1 was added dropwise. After approximately 90 minutes the reaction mixture was neutralized with saturated solution of $NaHCO_3$ and diluted with EtOAc. Separation the organic phase thereafter was washed with H_2O (10 ml), brine (10 ml) and the residue was dried over $MgSO_4$ (0.031g, 31%).

^{1}H NMR (500 MHz, MeOD) δ 8.70 (s, 1H, H-6), 6.16 (t, *J* = 6.0 Hz, 1H, H-1'), 4.74 - 4.71 (m, 1H, H-4'), 4.06 (q, *J* = 3.0 Hz, 1H, H-3'), 3.86, 3.83 (2 x dd, *J* = 12.0 Hz, *J* = 3 Hz, 2H, H-5', H-5'), 3.39 – 3.32 (m, 1H, H-3'), 2.53 – 2.49 (m, 1H, H-2'), 2.34 - 2.29 (m, 1H, H-2'), 1.09 - 1.06 (m, 24H, CH(C*H*)$_3$), 1.00 – 0.91 (m, 4H, C*H*(CH)$_3$). MS (ES$^+$): 511 (M+Na$^+$), 489 (M+H$^+$) $C_{20}H_{40}N_4O_6Si_2$ required: (M$^+$) 488.25

Synthesis of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphate (6.7d).



Prepared according to standard procedure **4**, from 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (**6.5**, 0.080 g, 0.16 mmol), *t*BuMgCl (1.0 M, 0.49 mL, 0.49 mmol) and 1-naphthyl(2,2-dimethylpropoxy-L-alaninyl)-phosphorochloridate (**2.3f**, 0.18 g, 0.49 mmol) in THF (5 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1-3% gradient), followed by preparative purification to give the title product **6.7d** as a white foam (0.060 g, 44%).

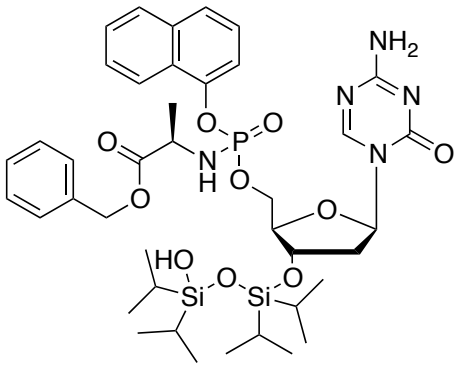
^{31}P NMR (202 MHz, MeOD) δ 4.59, 4.30

^{1}H NMR (500 MHz, MeOD) δ 8.38, 8.33 (2 x bs, 1H, H-6), 8.13 – 8.11 (m, 1H, H-Ar), 7.89 – 7.87 (m, 1H, H-Ar), 7.72 – 7.70 (m, 1H, H-Ar), 7.54 – 7.49 (m, 3H, H-Ar), 7.44 – 7.41 (m, 1H, H-Ar), 6.05 – 6.02 (m, 1H, H-1'), 4.56 – 4.55 (m, 1H, H-3'), 4.43 – 4.41 (m, 2H, H-5', H-5'), 4.26 – 4.25 (m, 1H, H-4'), 4.14 – 4.08 (m, 1H, C*H*CH_3), 3.85 (d, *J* = 10.5 Hz, 1H, C*H*$_2$C$(CH_3)_3$), 3.72 (d, *J* = 10.5 Hz, 1H, C*H*$_2$C$(CH_3)_3$), 2.29 - 2.25 (m, 1H, H-2'), 1.63 – 1.58 (m, 1H, H-2'), 1.42, 1.40 (dd, *J* = 7.5 Hz, CHC*H*$_3$), 1.39 – 1.33 (m, 24H, 4 x SiCH(*CH*$_3$)$_2$), 0.93 (s, 9H, CH_2C(C*H*$_3$)$_3$), 0.92 – 0.87 (m, 4H, 4 x SiC*H*(*C*H_3)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 175.21, 15.13 (2 x d, $^{3}J_{C\text{-}P}$ = 6.25 Hz, C=O, ester), 167.92, 167.81 (C=O, base), 156.69, 156.62 (C-4), 156.13, 156.06 (C-6), 148.01, 147.93, 136.30, 128.96, 127.96, 127.79, 126.59, 126.23, 122.60, 116.39, 116.34 (C-Ar), 88.35, 88.33 (C-1'), 88.15, 88.09 (2 x d, $J_{C\text{-}P}$ = 8.5 Hz, C-4'), 79.51, 79.48 (*C*H_2C$(CH_3)_3$), 73.83, 73.80 (C-3'), 67.81, 67.74 (2 x d, $J_{C\text{-}P}$ = 5.25 Hz, C-5'), 52.01, 51.86 (*C*H(CH_3), 43.04, 42.90 (C-2'), 32.45, 32.38 (CH_2*C*$(CH_3)_3$), 26.82, 26.80 (CH_2C(*C*H_3)$_3$), 20.53, 20.37 (CH_2C(*C*H_3)$_3$), 20.66, 20.60 (Si*C*H$(CH_3)_2$), 17.99, 17.86 (2 x d, $^{3}J_{C\text{-}P}$ = 6.25 Hz, CH(*C*H_3), 14.79, 14.77, 14.18 (SiCH(*C*H_3)$_2$).

MS (ES$^+$): 859 (M+Na$^+$), 836 (M+H$^+$) $C_{38}H_{62}N_5O_{10}PSi_2$ required: (M$^+$) 835.38

Synthesis of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(benzoxy-L-alaninyl) phosphate (6.7a).



Prepared according to standard procedure **4**, from 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (**6.5**, 0.16 g, 0.32 mmol), *t*BuMgCl (1.0 M, 0.96 ml, 0.96 mmol) and 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3c**, 0.26 g, 0.64 mmol) in THF (5 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1-3% gradient) to give the pure product **6.7a** as a white foam (0.160 g, 58%).

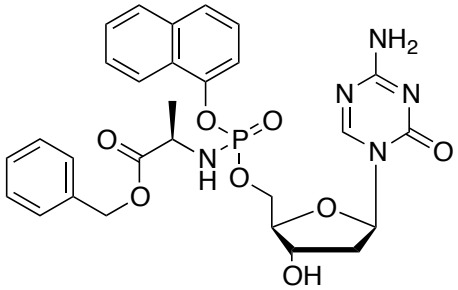
^{31}P NMR (202 MHz, MeOD) δ 4.62, 4.25

^{1}H NMR (500 MHz, MeOD) δ 8.38, 8.35 (2 x bs, 1H, H-6), 8.15 – 8.09 (m, 1H, H-Ar), 7.91 – 7.87 (m, 1H, H-Ar), 7.72 – 7.69 (m, 1H, H-Ar), 7.55 – 7.46 (m, 3H, H-Ar), 7.42 – 7.29 (m, 6H, H-Ar), 6.01 (t, *J* = 5.5 Hz, 1H, H-1'), 5.15 – 5.06 (m, 2H, C*H*$_2$Bn), 4.59 – 4.58, 4.53 – 4.52 (2 x m, 1H, H-3'), 4.38 – 4.33 (m, 1.5H, H-4', H-5'), 4.30 – 4.26 (m, 0.5H, H-4'), 4.20 – 4.19 (m, 1H, H-5'), 4.16 – 4.08 (m, 1H, C*H*(CH$_3$), 2.34, 2.26 (2 x ddd, *J* =13.5 Hz, 5.5 Hz, 2.5 Hz, 1H, H-2'), 1.70 – 1.65 (m, 0.5H, H-2'), 1.59 – 1.53 (m, 0.5H, H-2'), 1.39 – 1.33 (m, 3H, CH(*CH$_3$*), 1.06 – 1.03 (m, 24H, 4 x CH(C*H$_3$*)$_2$), 0.94 – 0.88 (m, 4H, 4 x C*H*(CH$_3$)$_2$).

^{13}C NMR (125 MHz, MeOD) δ 174.93, 174.47 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C=O, ester), 167.86, 167.83 (C=O, base), 156.72, 156.70 (C-4), 156.07, 156.05 (C-6), 147.95, 147.81 (2 x d, $^{2}J_{C\text{-}P}$ = 6.5 Hz, C-Naph), 137.19, 136.27, 129.60, 129.38, 129.32, 128.95, 127.90, 127.73, 126.55, 126.21, 122.59, 116.52, 116.38 (C-Ar), 88.31, 88.29 (C-1'), 88.09, 88.03 (2 x d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, C-4'), 73.76, 73.58 (C-3'), 68.07, 68.04 (*C*H$_2$Bn), 67.84, 67.67 (2 x d, $^{2}J_{C\text{-}P}$ = 6.0 Hz, C-5'), 51.98, 51.84 (*C*HCH$_3$), 42.88, 42.75 (C-2'), 20.53, 20.37 (2 x d, $^{3}J_{C\text{-}P}$ = 6.25 Hz, CH*C*H$_3$), 17.94, 17.89 (4 x CH(*C*H$_3$)$_2$), 14.76, 14.73, 14.15, 14.13 (4 x *C*H(CH$_3$)$_2$).

MS (ES$^+$): 878 (M+Na$^+$), $C_{40}H_{58}N_5O_{10}PSi_2$ required: (M$^+$) 855.35

Synthesis of 2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(benzoxy-L-alaninyl) phosphate (6.8a).



To the solution of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(benzoxy-L-alaninyl) phosphate (**6.7a**, 0.06 g, 0.07 mmol) in anhydrous THF (3ml) at 0°C, 50% aqueous solution of TFA (1.5 ml) was added dropwise and stirred for 48 to 72 hours at ambient temperature. The crude mixture was purified by preparative TLC plate using $CHCl_3$/MeOH (9:1) as eluent, to give the pure product **6.8a** as a white solid (0.013 g, 12%).

^{31}P NMR (202 MHz, MeOD) δ 4.63, 4.24

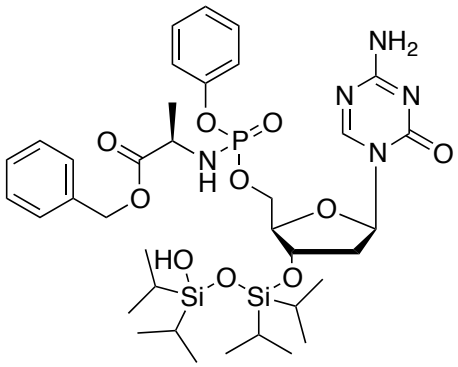
^{1}H NMR (500 MHz, MeOD) δ 8.34, 8.31 (2 x bs, 1H, H-6), 8.16 – 8.11 (m, 1H, H-Ar), 7.90 – 7.87 (m, 1H, H-Ar), 7.71 – 7.69 (m, 1H, H-Ar), 7.55 – 7.47 (m, 3H, H-Ar), 7.44 – 7.30 (m, 6H, H-Ar), 6.01 (m, 1H, H-1'), 5.15 – 5.07 (m, 2H, C*H*$_2$Bn), 4.59 – 4.58, 4.53 – 4.52 (2 x m, 1H, H-3'), 4.38 – 4.33 (m, 1.5H, H-4', H-5'), 4.36 – 4.31 (m, 1.5H, H-3', H-5'), 4.29 – 4.26 (m, 1H, H-4'), 4.23 – 4.21 (m, 0.5H, C*H*(CH$_3$), 4.14 – 4.10 (m, 2H, H-5', C*H*CH$_3$), 2.29 – 2.21 (m, 1H, H-2'), 1.75 – 1.65 (m, 1H, H-2'), 1.37, 1.36 (d, *J* = 7.0 Hz, 3H, CH(C*H*$_3$).

^{13}C NMR (125 MHz, MeOD) δ 174.95, 174.56 (2 x d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C=O, ester), 167.88, 167.85 (C=O, base), 156,79, 156.76 (C-6), 156.09 (C-4), 147.94, 147.78 (2 x d, $^{2}J_{C\text{-}P}$ = 7.5 Hz, C-Naph), 137.22, 136.28, 129.61, 129.59, 128.95, 128.90, 127.91, 127.87, 127.72, 127.64, 126.58, 126.53, 126.19, 122.63, 116.56 (C-Ar), 88.15, 88.13 (C-1'), 87.31, 87.08 (2 x d, $^{3}J_{C\text{-}P}$ = 8.75 Hz, C-4'), 79.51 (C-3'), 72.20, 72.01 (*C*H$_2$Bn), 68.06, 67.76 (2 x d, $^{2}J_{C\text{-}P}$ = 5.25 Hz, C-5'), 51.97, 51.82 (*C*HCH$_3$), 42.04, 42.97 (C-2'), 20.43, 20.26 (2 x d, $^{3}J_{C\text{-}P}$ = 6.25 Hz, CH*C*H$_3$).

HPLC (System 2) t_R = 14.53, 14.58 min

MS (ES$^+$): 618 (M+Na$^+$), 596 (M+H$^+$), $C_{28}H_{30}N_5O_8P$ required: (M$^+$) 595.18

Synthesis of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[phenyl-(benzoxy-L-alaninyl) phosphate (6.7b).



Prepared according to standard procedure **4**, from 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (**6.5**, 0.10 g, 0.20 mmol), *t*BuMgCl (1.0 M, 0.61 ml, 0.61 mmol) and phenyl(benzoxy-L-alaninyl)-phosphorochloridate (**2.3b**, 0.22 g, 0.61 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1-3% gradient) to give the pure product **6.7b** as a white foam (0.08 g, 48%).

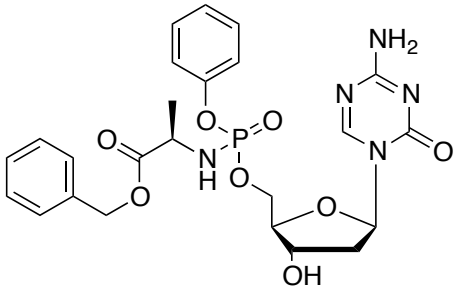
^{31}P NMR (202 MHz, MeOD) δ 4.14, 3.70

^{1}H NMR (500 MHz, MeOD) δ 8.44, 8.43 (2 x bs, 1H, H-6), 7.35 – 7.31 (m, 7H, H-Ar), 7.25 – 7.24 (m, 1H, H-Ar), 7.20– 7.18 (m, 2H, H-Ar), 6.13 – 6.08 (m, 1H, H-1'), 5.18 – 5.10 (m, 2H, C*H*$_2$Bn), 4.72 – 4.69 (m, 1H, H-4'), 4.35 – 4.24 (m, 3H, H-3', H-5', H-5'), 4.04 – 3.98 (m, 1H, C*H*CH_3), 2.51 – 2.41 (m, 1H, H-2'), 2.04 – 1.88 (m, 1H, H-2'), 1.36 (d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 1.08 – 1.07 (m, 24H, 4 x CH(C*H*$_3$)$_2$), 1.01 – 0.90 (m, 4H, 4 x C*H*$(CH_3)_2$).

^{13}C NMR (125 MHz, MeOD) δ 174.83, 174.44 (2 x d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, C=O, ester), 167.89 (C=O, base), 156.89, 156.85 (C-4), 156.14, 156.13 (C-6), 152.13, 152.09, 152.04, 137.28, 137.23, 137.21, 130.85, 129.63, 129.38, 126.34, 126.28, 121.45(C-Ar), 88.44, 88.36 (C-1'), 88.01, 87.84 (2 x d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, C-4'), 73.79, 73.63 (C-3'), 68.89 (*C*H$_2$Bn), 68.01, 67.74 (2 x d, $^{2}J_{C\text{-}P}$ = 8.75 Hz, C-5'), 51.87, 51.65 (*C*HCH$_3$), 43.10, 42.98 (C-2'), 20.61, 20.43 (2 x d, $^{3}J_{C\text{-}P}$ = 7.25 Hz, CH*C*H$_3$), 18.02, 17.89, 17.86, 17.84 (4 x *C*H$(CH_3)_2$), 14.83, 14.78, 14.27, 14.25 (4 x CH(*C*H$_3$)$_2$).

MS (ES$^+$): 828 (M+Na$^+$), 806 (M+H$^+$), $C_{36}H_{56}N_5O_{10}PSi_2$ required: (M$^+$) 805.33

Synthesis of 2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(benzoxy-L-alaninyl) phosphate (6.8b).



To the solution of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[phenyl-(benzoxy-L-alaninyl) phosphate (**6.7b**, 0.08 g, 0.10 mmol) in dry THF (4 ml) at 0^{o}C, 50% aqueous solution of TFA (2 ml) was added dropwise and stirred for for 48 to 72 hours at ambient temperature. The crude mixture was purified by preparative TLC plate using $CHCl_3$/MeOH (9:1) as eluent, to give the pure product **6.8b** as a white solid (0.003 g, 5%).

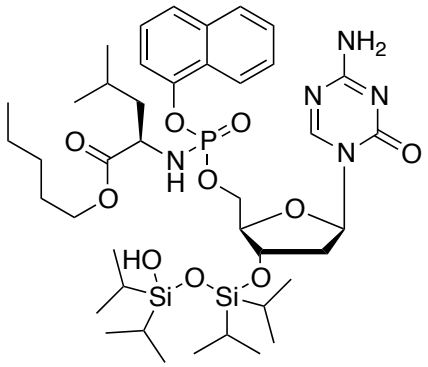
^{31}P NMR (202 MHz, MeOD) δ 4.16, 3.71

^{1}H NMR (500 MHz, MeOD) δ 8.41, 8.40 (2 x bs, 1H, H-6), 7.36 – 7.32 (m, 7H, H-Ar), 7.25 – 7.24 (m, 1H, H-Ar), 7.20– 7.19 (m, 2H, H-Ar), 6.11 – 6.06 (m, 1H, H-1'), 5.18 – 5.11 (m, 2H, C*H*$_2$Bn), 4.37 – 4.21 (m, 3H, H-3', H-5', H-5'), 4.12 – 4.11 (m, 1H, H-4'), 4.06 – 4.00 (m, 1H, C*H*CH$_3$), 2.43 – 2.35 (m, 1H, H-2'), 2.07 – 1.93 (m, 1H, H-2'), 1.37, 1.35 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$).

HPLC (System 2) t_R = 13.67, 13.72 min

MS (ES$^+$): 568 (M+Na$^+$), 546 (M+H$^+$), $C_{24}H_{28}N_5O_8P$ required: (M$^+$) 545.15

Synthesis of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (6.7c).



Prepared according to standard procedure **4**, from 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (**6.5**, 0.10 g, 0.20 mmol), *t*BuMgCl (1.0 M, 0.61 mL, 0.61 mmol) and 1-naphthyl(pentoxy-L-leucinyl)-phosphorochloridate (**2.3s**, 0.26 g, 0.61 mmol) in THF (10 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1-3% gradient) to give the pure product as a white foam (0.08 g, 45%).

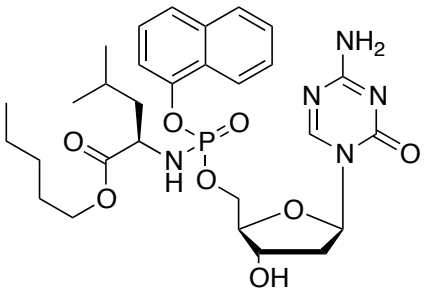
^{31}P NMR (202 MHz, MeOD) δ 5.07, 4.52

^{1}H NMR (500 MHz, MeOD) δ 8.42, 8.41 (2 x bs, 1H, H-6), 8.17 – 8.14 (m, 1H, H-Ar), 7.91 – 7.88 (m, 1H, H-Ar), 7.73 – 7.70 (m, 1H, H-Ar), 7.56 – 7.53 (m, 2H, H-Ar), 7.51 – 7.49 (2 x bs, 1H, H-Ar), 7.44 – 7.38 (m, 1H, H-Ar), 6.07 – 6.02 (m, 1H, H-1'), 4.61 – 4.60 (m, 1H, H-3'), 4.44 – 4.30 (m, 2H, H-5', H-5'), 4.27 – 4.24 (m, 1H, H-4'), 4.09 – 3.94 (m, 3H, C*H*$CH_2CH(CH_3)_2$, OC*H*$_2CH_2CH_2CH_2CH_3$), 2.36 – 2.28 (m, 1H, H-2'), 1.72 – 1.65 (m, 2H, H-2', $CHCH_2CH(CH_3)_2$), 1.58 - 1.52 (m, 4H, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.31 - 1.27 (m, 4H, $OCH_2CH_2CH_2CH_2CH_3$), 1.06 – 1.03 (m, 24H, 4 x $SiCH(CH_3)_2$), 0.90 – 0.86 (m, 9H, $CHCH_2CH(CH_3)_2$), $OCH_2CH_2CH_2CH_2CH_3$, 4 x $SiCH(CH_3)_2$)

^{13}C NMR (125 MHz, MeOD) δ 175.05, 174.95 (2 x d, $^{3}J_{C\text{-}P}$ = 4.0 Hz, C=O, ester), 167.85, 167.81 (C=O, base), 156.84, 156.73 (C-4), 147.95, 147.82 (2 x d, $^{2}J_{C\text{-}P}$ = 7.5 Hz, C_1-Naph), 136.30, 128.99, 128.93, 127.90, 127.77, 127.63, 126.55, 126.27, 126.13, 122.72, 122.66, 116.56, 116.36 (C-Ar), 88.35, 88.29 (C-1'), 88.09, 87.87 (2 x d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, C-4'), 73.89, 73.72 (C-2'), 67.96, 67.78 (2 x d, $^{2}J_{C\text{-}P}$ = 6.25 Hz, C-5'), 66.44, 66.41 (C-3'), 54.92, 54.80 ($OCH_2CH_2CH_2CH_2CH_3$), 44.18, 43.81 (2 x d, $^{3}J_{C\text{-}P}$ = 8.5 Hz, $CHCH_2CHCH_3$), 42.92, 42.80 ($CHCH_2CH(CH_3)_2$), 29.41, 29.37($OCH_2CH_2CH_2CH_2CH_3$), 29.18, 29.17 ($OCH_2CH_2CH_2CH_2CH_3$), 25.75, 25.52 ($CHCH_2CH(CH_3)_2$), 23.36, 23.31, 23.14 ($SiCH(CH_3)_2$), 22.12, 21.67 ($CHCH_2CH(CH_3)_2$), 17.99, 17.94, 17.83($SiCH(CH_3)_2$), 14.36, 14.17($SiCH(CH_3)_2$).

MS (ES^+): 878 ($M+H^+$), $C_{41}H_{68}N_5O_{10}PSi_2$ required: (M^+) 877.48

Synthesis of 2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (6.8c).



To the solution of 3'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (**6.7c**, 0.08 g, 0.10 mmol) in anhydrous THF (4 ml) at 0°C, 50% aqueous solution of TFA (2 ml) was added dropwise and stirred for 48 to 72 hours at ambient temperature. The crude mixture was purified by preparative TLC plate using $CHCl_3$/MeOH (9:1) as eluent, to give the pure product **6.8c** as a white solid (0.013 g, 23%).

^{31}P NMR (202 MHz, MeOD) δ 5.02, 4.55

^{1}H NMR (500 MHz, MeOD) δ 8.39, 8.35 (2 x bs, 1H, H-6), 8.18 – 8.15 (m, 1H, H-Ar), 7.90 – 7.89 (m, 1H, H-Ar), 7.74 – 7.70 (m, 1H, H-Ar), 7.59 – 7.54 (m, 2H, H-Ar), 7.51 – 7.49 (m, 1H, H-Ar), 7.46 – 7.40 (m, 1H, H-Ar), 6.06 – 6.02 (2 x t, J = 6.5 Hz, 7 Hz, 1H, H-1'), 4.42 – 4.37 (m, 1.5H, H-3', H-5'), 4.35 – 4.29 (m, 1.5H, H-3', H-5'), 4.18 – 4.13 (m, 1H, H-4'), 4.07 – 3.93 (m, 3H, C*H*$CH_2CH(CH_3)_2$), O*C*$H_2CH_2CH_2CH_2CH_3$), 2.32 – 2.24 (m, 1H, H-2'), 1.80 – 1.68 (m, 1H, H-2'), 1.60 – 1.49 (m, 4H, CHC$H_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 1.32 - 1.27 (m, 5H, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2CH_2CH_3$), 0.89 – 0.81 (m, 9H, $CHCH_2CH(CH_3)_2$).

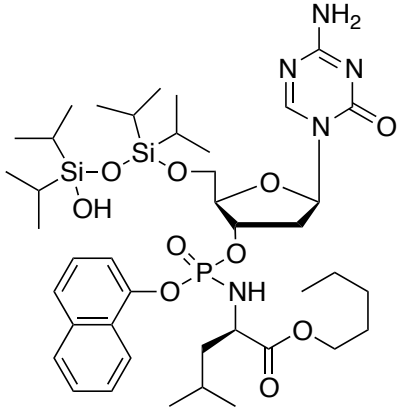
^{13}C NMR (125 MHz, MeOD) δ 175.50, 175.03 (2 x d, $^3J_{C\text{-}P}$ = 4.5 Hz, C=O, ester), 167.86 (C=O, base), 156,77 (C-6), 156.09 (C-4), 147.95, 147.82 (2 x d, $^2J_{C\text{-}P}$ = 7.0 Hz, C_1-Naph), 136.31, 128.95, 128.90, 127.91, 127.89, 127.87, 127.71, 127.59, 126.56, 126.52, 126.20, 126.09, 122.69 (C-Ar), 116.51, 116.37 (2 x d, $^2J_{C\text{-}P}$ = 3.0 Hz, C_2-Naph), 88.14, 88.13 (C-1'), 87.32, 87.07 (2 x d, $^3J_{C\text{-}P}$ = 8.25 Hz, C-4'), 72.28, 72.04 (C-3'), 68.82, 67.77 (2 x d, $^2J_{C\text{-}P}$ = 5.75 Hz, C-5'), 66.43, 66.39 ($OCH_2CH_2CH_2CH_2CH_3$), 44.16, 43.81 (2 x d, J = 8.75 Hz, $CHCH_2CH(CH_3)_2$), 42.07, 42.03($CHCH_2CH(CH_3)_2$), 35.82, 35.72 (C-2'), 32.80, 30.20 ($OCH_2CH_2CH_2CH_2CH_3$), 29.37, 29.16 ($OCH_2CH_2CH_2CH_2CH_3$), 25.73, 25.48

($CHCH_2CH(CH_3)_2$) 23.75, 23.36, 23.25, 23.12 ($CHCH_2CH(CH_3)_2$), 21.99, 21.63 ($OCH_2CH_2CH_2CH_2CH_3$),14.49, 14.32 ($OCH_2CH_2CH_2CH_2CH_3$).

HPLC (System 2) t_R = 19.83, 19.88 min

MS (ES^+): 640 ($M+Na^+$), $C_{29}H_{40}N_5O_8P$ required: (M^+) 617.26

Synthesis of 5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 3'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (6.9a).



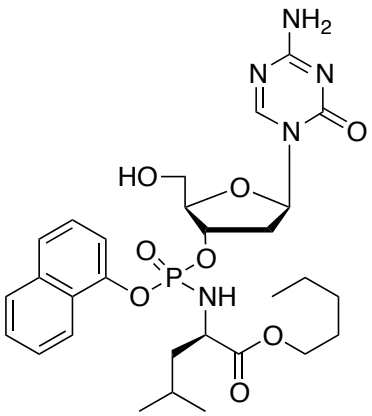
Prepared according to standard procedure **4**, from 5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine (**6.6**, 0.05 g, 0.10 mmol), *t*BuMgCl (1.0 M, 0.30 mL, 0.30 mmol) and 1-naphthyl(pentoxy-L-leucinyl)-phosphorochloridate (**2.3s**, 0.13 g, 0.30 mmol) in THF (5 ml). The crude mixture was purified by column chromatography using $CHCl_3$/MeOH eluent system (1-3% gradient) to give the pure product **6.9a** as a white foam (0.060 g, 67%).

^{31}P NMR (202 MHz, MeOD) δ 4.07, 3.19

^1H NMR (500 MHz, MeOD) δ 8.57, 8.53 (2 x bs, 1H, H-6), 8.18 (m, 1H, H-Ar), 7.91 – 7.90 (m, 1H, H-Ar), 7.74 – 7.72 (m, 1H, H-Ar), 7.60 – 7.43 (m, 5H, H-Ar), 6.19 – 6.13 (m, 1H, H-1'), 5.28 - 5.26 (m, 0.3H, H-3'), 5.23 - 5.20 (m, 0.7 H, H-3'), 4.47 – 4.45 (m, 0.7 H, H-4'), 4.29 – 4.28 (m, 0.3 H, H-4'), 4.1 – 3.91 (m, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2\,CH_2CH_3$, H-5', H-5'), 2.95 – 2.90 (m, 0.3H, H-2'), 2.79 – 2.75 (m, 0.7H, H-2'), 2.49 – 2.43 (m, 0.3H, H-2'), 2.34 – 2.28 (m, 0.7H, H-2'), 1.69 – 1.64 (m, 0.7H, $CHCH_2CH(CH_3)_2$), 1.58- 1.49 (m, 5.3H, $CHCH_2CH(CH_3)_2$, $CHCH_2CH(CH_3)_2$, $OCH_2CH_2CH_2\,CH_2CH_3$, 1.31 - 1.27 (m, 4H, $OCH_2CH_2CH_2\,CH_2CH_3$), 1.07 – 1.00 (m, 28H, 4 x $SiCH(CH_3)_2$, 4 x $SiCH(CH_3)_2$), 0.90 – 0.86 (m, 9H, $CHCH_2CH(CH_3)_2$), $OCH_2CH_2CH_2\,CH_2CH_3$).

MS (ES^+): 900 ($M+Na^+$), 878 ($M+H^+$), $C_{41}H_{68}N_5O_{10}PSi_2$ required: (M^+) 877.42

Synthesis of 2'-deoxy-5-azacytidine 3'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (6.10a).



To the solution of 5'-*O*-(1,1,3,3-Tetraisopropyldisilox-1,3-diyl)-2'-deoxy-5-azacytidine 5'-O-[1-naphthyl-(pentoxy-L-leucinyl) phosphate (**6.9a**, 0.06 g, 0.07 mmol) in dry THF (4 ml) at 0°C, 50% aqueous solution of TFA (2ml) was added dropwise and stirred for 48 to 72 hours at ambient temperature. The crude mixture was purified by preparative TLC plate using $CHCl_3$/MeOH (9:1) as eluent, to give the pure product **6.10a** as a white solid (0.008 g, 19%).

^{31}P NMR (202 MHz, MeOD) δ 4.17, 3.47

^{1}H NMR (500 MHz, MeOD) δ 8.39, 8.35 (2 x bs, 1H, H-6), 8.18 – 8.15 (m, 1H, H-Ar), 7.91 – 7.89 (m, 1H, H-Ar), 7.74 – 7.70 (m, 1H, H-Ar), 7.59 – 7.54 (m, 2H, H-Ar), 7.51 – 7.49 (m, 1H, H-Ar), 7.46 – 7.40 (m, 1H, H-Ar), 6.18 – 6.16 (t, *J* = 5.0 Hz, 0.3H, H-1'), 6.14 – 6.11 (t, *J* = 7.5 Hz, 0.7H, H-1'), 5.26 - 5.23 (m, 0.3H, H-3'), 5.20 - 5.17 (m, 0.7 H, H-3'), 4.32 (q, *J* = 3.0 Hz, H-4'), 4.19 (q, *J* = 3.0 Hz, H-4'), 4.05 – 4.02 (m, 2H, OC*H*$_2$CH$_2$CH$_2$ CH$_2$CH$_3$), 3.69 – 3.91 (m, 1H, C*H*CH$_2$CH(CH$_3$)$_2$), 3.83 – 3.68 (m, 2H, H-5'), 2.84 – 2.80 (m, 0.3H, H-2'), 2.69 – 2.64 (m, 0.7H, H-2'), 2.54 – 2.49 (m, 0.3H, H-2'), 2.43 – 2.38 (m, 0.7H, H-2'), 1.71 – 1.65 (m, 0.7H, CHCH$_2$C*H*(CH$_3$)$_2$), 1.59 - 1.48 (m, 4.3H, CHCH$_2$C*H*(CH$_3$)$_2$, OCH$_2$C*H*$_2$C*H*$_2$ C*H*$_2$CH$_3$), 1.32 - 1.28 (m, 5H, CHCH$_2$C*H*(CH$_3$)$_2$, OCH$_2$CH$_2$C*H*$_2$ C*H*$_2$CH$_3$), 0.89 – 0.80 (m, 9H, CHCH$_2$CH(C*H*$_3$)$_2$, OCH$_2$CH$_2$CH$_2$ CH$_2$C*H*$_3$).

HPLC (System 2) t_R = 18.52, 18.56 min

MS (ES$^+$): 618 (M+H$^+$), Accurate mass: $C_{29}H_{40}N_5O_8P$ required: (M$^+$) 617.26

Appendix I

1. McGuigan, C.; Derudas, M.; Gönczy, B.; Hinsinger, K.; Kandil, S.; Pertusati, F.; Serpi, M.; Snoeck, R.; Andrei, G.; Balzarini, J.; McHugh, D. T.; Maitra, A.; Akorli, E.; Evangelopoulos, D.; Bhakta, S. ProTides of N-(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide as potential anti-tubercular and anti-viral agents. *Bioorg. Med. Chem.* **2014**, *22*, 2816-2824.

2. McGuigan, C.; Murziani, P.; Slusarczyk, M.; Gönczy, B.; Vande Voorde, J.', Liekens, S.; Balzarini, J. Phosphoramidate ProTides of the anticancer agent FUDR successfully deliver the preformed bioactive monophosphate in cells and confer advantage over the parent nucleoside. *J. Med. Chem.* **2011**, *54*, 7247-7258.

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ProTides of *N*-(3-(5-(2′-deoxyuridine))prop-2-ynyl)octanamide as potential anti-tubercular and anti-viral agents

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ABSTRACT

The flavin-dependent thymidylate synthase X (ThyX), rare in eukaryotes and completely absent in humans, is crucial in the metabolism of thymidine (a DNA precursor) in many microorganisms including several human pathogens. Conserved in mycobacteria, including *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, it represents a prospective anti-mycobacterial therapeutic target. In a *M. tuberculosis* ThyX-enzyme inhibition assay, *N*-(3-(5-(2′-deoxyuridine-5′-phosphate))prop-2-ynyl)octanamide was reported to be the most potent and selective 5-substituted 2′-deoxyuridine monophosphate analogue. In this study, we masked the two charges at the phosphate moiety of this compound using our ProTide technology in order to increase its lipophilicity and then allow permeation through the complex mycobacterial cell wall. A series of *N*-(3-(5-(2′-deoxyuridine))prop-2-ynyl)octanamide phosphoroamidates were chemically synthesized and their biological activity as potential anti-tuberculars was evaluated. In addition to mycobacteria, several DNA viruses depend on ThyX for their DNA biosynthesis, thus these prodrugs were also screened for their antiviral properties.

1. Introduction

Tuberculosis (TB) is an infectious disease, caused by an extremely slow growing bacterial pathogen belonging to the *Mycobacterium tuberculosis* complex.[1] Among them, *M. tuberculosis* is responsible for the majority of human deaths.[2] It is estimated that at least one-third of the world's population is latently infected with the bacteria that causes TB, while in 2012, WHO reported 8.6 million new cases of the disease and 1.3 million deaths due to it, worldwide.[3] Lengthy treatment regimens have as a consequence, the rise of drug resistant TB-causing strains. This has set the alarm for controlling the disease, which necessitates the discovery of new therapeutic targets and synthesis of novel inhibitors.

In order to specifically inhibit mycobacterial growth, alternative metabolic pathways, exclusive to the bacteria, need to be targeted for an efficient anti-TB drug development programme.[4] In this regard, we focused our interest on the genome of *M. tuberculosis*, which has been reported to contain a *thyX* gene (Rv2754c), encoding the ThyX protein, a flavin-dependent thymidylate synthase responsible for the *de novo* synthesis of 2′-deoxythymidine 5′-monophosphate (dTMP), a key precursor of DNA.[5] ThyX, present primarily in prokaryotes and viruses, is rare in eukaryotes and absent in humans, where the corresponding metabolic function is carried out by the conventional ThyA protein. Both the enzymes catalyze the reductive methylation of 2′-deoxy-uridine-5′-monophosphate (dUMP) to dTMP in the presence of methylenetetrahydrofolate; however, based on their structural and catalytic dissimilarities, different enzymatic mechanisms have been suggested for each.[6] In contrast to ThyA, where methylenetetrahydrofolate acts both as the carbon donor and reducing agent, ThyX uses methylenetetrahydrofolate as the one-carbon donor molecule and reduced flavin adenine dinucleotide ($FADH_2$) as a cofactor that serves as the hydride donor.[7]

Micro-organisms contain either ThyA or ThyX; however, mycobacteria encode both ThyA and ThyX. However, a sequence similarity search yielded no significant matches between ThyX of mycobacteria to the ThyA of other eukaryotic cells. Recently,

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0968-0896/

functional studies, devoted to investigate the biological role of both of these enzymes, showed that ThyX is essential for the survival of *M. tuberculosis* even in the presence of ThyA and exogenous thymidine.[8,9] *M. tuberculosis* cannot utilize exogenous thymidine sources because it lacks thymidine kinase, the essential enzyme for the conversion of thymidine into dTMP.[5] All these factors support the existence of ThyX enzyme as a prospective therapeutic target for the development of a new selective anti-tubercular drug treatment.[10]

The chemical class of C5-alkynyl substituted 2′-deoxyuridine-5′-monophosphate (dUMP) has been reported as selective inhibitor of ThyX in *M. tuberculosis*.[11] In this study, among several compounds, *N*-(3-(5-(2′-deoxyuridine-5′-monophosphate))prop-2-ynyl)octanamide **1** (Fig. 1) was identified as the most potent and selective analogue with an IC_{50} value of 0.91 μM versus recombinant ThyX from *M. tuberculosis*. However, the polarity of this compound constitutes a major obstacle for its penetration through the complex mycobacterial cell wall. Indeed one of the major challenges for chemotherapeutic drugs is associated with difficulties in crossing the thick, lipid-rich cell wall of the mycobacteria, which prevents their permeation, with a consequential poor biological response. In particular, the permeation of hydrophilic compounds is not very efficient due to the presence of low numbers and the exceptional length of porins, water-filled open channels, responsible for mediating the diffusion of hydrophilic nutrients.[12] Moreover, the existence of active drug efflux pumps reduces the concentration of active molecules inside the bacterial cell and their up-regulation has been suggested to contribute to the emergence of drug resistance.[13] To the contrary more lipophilic drugs are more likely to diffuse through the lipid-rich environment of the mycobacterial cell wall. In addition to high polarity, the instability of **1** in biological media, a well-known drawback for free nucleoside phosphates, may also limit its therapeutic potential, with dephosphorylation likely exceeding cell permeation.

To improve stability, permeability and therefore antibacterial activity, lipophilic prodrugs of **1** are thus required. Among several strategies developed to overcome these issues, our phosphoramidate ProTide approach was selected for this study.[14,15] This technology consists of masking the negative charges of the phosphate group with an aromatic moiety and an amino acid ester. To date, this approach has been widely applied mainly to antiviral[16] and anticancer nucleoside analogues[17] and more recently also to *N*-acetyl glucosamine to treat osteoarthritis.[18] Typically, two enzymatic cleavages are involved in the cellular bio-activation of antiviral and anticancer ProTides, either in the viral infected or human cancer cell.[19] Firstly, a carboxypeptidase-type enzyme may mediate the cleavage of the ester moiety. This step is followed by a spontaneous intra-molecular cyclisation with the subsequent release of the aryl moiety and formation of an unstable mixed cyclic anhydride which undergoes ring-opening mediated by water to release a mono ester phosphate prodrug. In the last step, a phosphoramidase-type enzyme, most probably a human Hint-1, may be responsible for the cleavage of the phosphorus–nitrogen bond with the consequent release of the monophosphate.

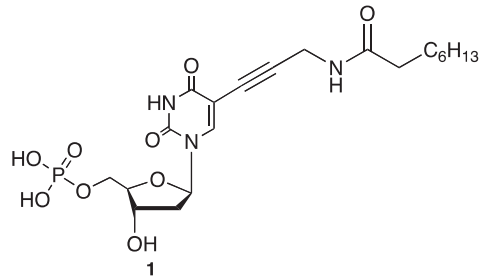


Figure 1. *N*-(3-(5-(2′-Deoxyuridine-5′-monophosphate))prop-2-ynyl)octanamide **1**.

Since we wanted to investigate whether phosphoramidates of **1** are capable of crossing the cell wall of mycobacteria and to be bioconverted into the monophosphate once inside, herein we report the synthesis of several derivatives and their biological evaluation against the TB vaccine strain, *M. bovis* BCG and the virulent TB causing lab strain, *M. tuberculosis* H37Rv, using HT-SPOTi, a rapid but gold standard whole-cell phenotypic assay. This is the first time that the application of the ProTide technology has been reported for improving the antimicrobial activity of inhibitors of a protein target of *M. tuberculosis*.

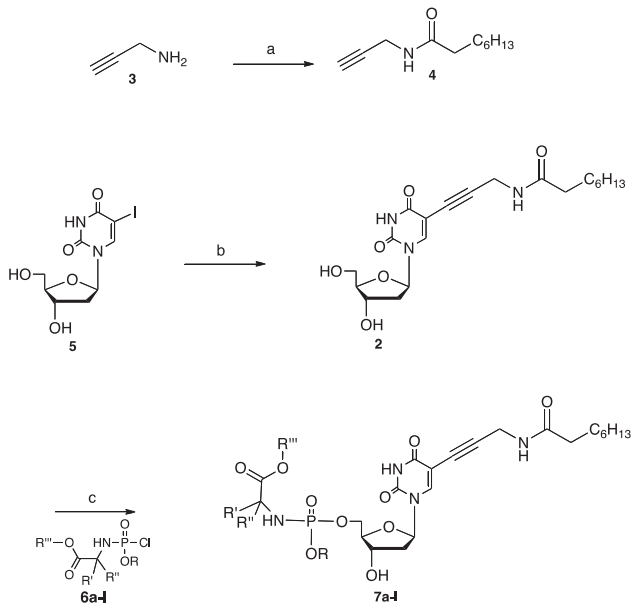
The Prodrugs were prepared by chemical modification of nucleoside **2** rather than directly modifying compound **1** (see Scheme 1). As ThyX proteins are also found in several double-stranded DNA viruses[5] and considering that some 5-alkynyl-2′-deoxyuridine analogues, in addition to their anti-TB activity[20–22] showed significant antiviral activity[23] we also report the antiviral evaluation of the parent nucleoside of **2** and its prodrug monophosphate derivatives.

2. Results and discussion

2.1. Chemistry

The phosphoramidates **7a–l** described in this study are shown in Table 1 with the reaction sequence for their synthesis summarized in Scheme 1.

Our synthetic efforts started with the synthesis of the propargyloctylamide **4** from propargylamine **3** and octanoyl chloride.[11] Then, Sonogashira cross coupling[24] of the commercially available 5-iodo-2′-deoxyuridine **5** with alkyne **4** afforded the desired nucleoside **2**, isolated in excellent yield (91%) without column chromatography purification. It is noteworthy to mention that our synthetic procedure to the preparation of **2** was made less time-consuming than the previously reported procedure, with great improvement in terms of yield achieved.[11] Following the general synthetic procedure for compounds **7a–l**, nucleoside **2** was reacted with the appropriate phosphorochloridate **6a–l** using *N*-methylimidazole as



Scheme 1. Synthetic method to obtain compounds **7a–l**. Reagents and conditions: (a) octanoyl chloride, DIPEA, anhydrous CH_2Cl_2, 0–20 °C, 2 h; (b) propargyloctanamide (**3**), $Pd(Ph_3)_4$, CuI, DIPEA, anhydrous DMF, 20 °C; (c) phosphorochlorides **6a–l** (for R, R′, R″ and R‴ see Table 1), NMI, anhydrous THF, 20 °C, 12 h.

Table 1
Substituent pattern, yields and ^{31}P NMR shifts of phosphoramidates **7a–l**

Compounds	R	R′	R″	AA	R‴	Yield (%)	^{31}P NMR[a] (ppm)
7a	Naph	H	CH_3	L-Ala	CH_3	20	4.36; 4.07
7b	Naph	H	CH_3	L-Ala	CH_2CH_3	5	4.36; 4.09
7c	Naph	H	CH_3	L-Ala	$CH_2CH(CH_3)_2$	39	4.34; 4.07
7d	Naph	H	CH_3	L-Ala	CH_2Ph	14	4.36; 3.98
7e	Ph	H	CH_3	L-Ala	CH_3	16	3.98; 3.57
7f	Ph	H	CH_3	L-Ala	CH_2CH_3	29	3.99; 3.58
7g	Ph	H	CH_3	L-Ala	$(CH_2)_2CH_3$	12	4.00; 3.59
7h	Ph	H	CH_3	L-Ala	CH_2Ph	12	4.01; 3.51
7i	Ph	CH_3	H	D-Ala	CH_2Ph	16	3.98; 3.50
7j	Ph	H	H	Gly	CH_2Ph	29	5.02; 4.93
7k	Ph	CH_3	CH_3	DMG	CH_2Ph	9	2.39; 2.10
7l	Ph	H	$CH(CH_3)_2$	L-Val	CH_2Ph	14	4.95; 4.30

[a] Recorded at 202 MHz in MeOH-d_4 with 85% H_3PO_4 as reference.

activator, according to a previously described method.[25] The desired phosphoroamidates **7a–l** were obtained from 5% to 39% yield. Yield optimization was beyond the scope of the work as we were in the early stages of establishing biological activity and potential lead molecules.

2.2. Biological activities

2.2.1. Antimycobacterial specificity

All synthesized compounds **7a–l** and nucleoside **2** were evaluated in vitro against *M. bovis* BCG and, *M. tuberculosis* H37Rv using the HT-SPOTi assay[26] at 1–250 mg/L concentrations. This whole-cell assay was indicative toward inherent resistance, such as cell wall impermeability. In order to evaluate a specific endogenous mechanism of anti-mycobacterial action for these compounds via ThyX inhibition, we also tested their biological activity against *Escherichia coli*, a Gram-ve bacterium where a functional ThyX homologue is missing from the genome. The minimum inhibitory concentrations (MIC) of compounds that completely inhibited growth of both mycobacterial strains are shown in Table 2.

The highly hydrophilic nucleoside **2** (*C*log*P* = −0.21) did not exhibit antimycobacterial activity with a MIC >250 mg/L (Table 2). For the first series of phosphoroamidates bearing a naphthyl group as an aromatic part (**7a–d**), a structure–activity relationship (SAR) was found with the activity depending on the size of the ester group of the L-alanine amino acid moiety. Methyl and ethyl derivatives (**7a** *C*log*P* = 1.89; **7b** *C*log*P* = 2.42) showed better inhibitory activity (**7a** MIC = 62.5 mg/L; **7b** MIC = 125 mg/L), whereas more hindered and lipophilic benzyl analogue (**7d** *C*log*P* = 3.60) was found to be significantly less inhibitory (MIC = 250 mg/L). A possible reason for this finding could be due to the higher size of compound **7d** that may prevent its entry inside the bacteria.

The second series of compounds bearing a phenyl aromatic moiety (**7e–l**) showed a different SAR. The smallest alkyl esters (**7e** *C*log*P* = 0.71; **7f** *C*log*P* = 1.24) did not inhibit mycobacteria growth, while propyl and benzyl derivatives (**7g** *C*log*P* = 1.77; **7h** *C*log*P* = 2.43) were slightly active (MIC = 125 mg/L). Further investigation on the amino acid part highlighted a certain preference toward alanine with the D-alanine analogue (**7i**) showing a MIC of 62.5 mg/L.

Glycine derivative **7j**, showed no activity against *M. bovis* BCG, whereas dimethylglycine or L-valyl derivatives (**7k** and **7l**) in the same assay were slightly active. (MIC = 125 mg/L). However, results for **7i** and **7l** differed in the case of *M. tuberculosis* H37Rv as can be inferred from the table.

Therefore, we postulate that the bioactivity against *M. bovis* BCG and *M. tuberculosis* H37Rv of phosphoroamidates **7a**, **7b**, **7c, 7g**, **7h, 7i** and **7l** might result from better permeation through the mycobacterial cell wall due to an improved balance between their lipophilicity and molecular size. Potencies are moderate in this first series of compounds and subsequent work is required to optimize their biological activity; however, there is a clear antimycobacterial effect for some of these molecules. Notably, the parent nucleoside **2** was devoid of activity showing the crucial importance of the ProTide motif.

No homologues with significant sequence similarity with the protein ThyX of *M. tuberculosis* were found in *E. coli*. This could be a possible explanation for the inactivity of the compounds towards this organism.

Table 2
Antimycobacterial specificity of nucleoside **2** and phosphoroamidates **7a–l**

Compounds	*C*log*P*[a]	MIC^{BCG} (mg/L)	MIC^{H37Rv} (mg/L)
2	−0.21	>250	>250
7a	1.89	62.5	62.5
7b	2.42	125	125
7c	3.74	125	62.5
7d	3.6	250	250
7e	0.71	>250	>250
7f	1.24	250	250
7g	1.77	125	125
7h	2.43	125	125
7i	2.43	62.5	125
7j	2.31	>250	>250
7k	2.73	125	>125
7l	3.35	125	31.25

[a] *C*log*P* values were calculated using CambridgeSoft ChemDraw® software. MICBCG and MICH37Rv state the minimum inhibitory concentration of the compounds tested against *M. bovis* BCG and *M. tuberculosis* H37Rv, respectively.

2.2.2. Antiviral activity

Parent nucleoside **2** and all synthesized phosphoramidates were also evaluated for their antiviral activity against varicella zoster virus (VZV), herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), and human cytomegalovirus (HCMV), vaccinia virus (VV) according to previously described methods.[27] Phosphoroamidate **7h** exhibited anti-VZV and anti-HSV activities in the low micromolar range (Table 3).

However, although quite potent against a thymidine kinase positive (TK^+) strain of VZV, **7h** was not active versus the thymidine kinase-deficient (TK^-) strain. This showed the importance of this enzyme in the bioactivation of this class of molecules to its active species. Moreover, the lead compound, the L-alanine benzyloxy ester phosphoramidate with a phenyl aromatic group **7h**, presented similar activity (EC_{50} = 2.0 μM) to acyclovir (EC_{50} = 2.6 μM) against VZV. Compound **7h** was also found to be active against HSV-1 and HSV-2 (EC_{50} of 2 and 4 μM) but not against a TK^- strain of HSV-1. In the case of **7a**, **7c**, **7d**, the antiviral activity observed against VZV may be rather due to their underlying cytotoxicity. The compounds

Table 3
Antiviral activity and cytotoxicity of phosphoramidates **7a**, **7c**, **7d** and **7h**

Compounds	EC_{50}[a] (μM)					CC_{50}[b] (μM)	MCC[c] (μM)
	VZV		HSV–1		HSV–2		
	TK^+ OKA	TK^- 07-1	TK^+ KOS	TK^-KOS	G		
2	>100	>100	>100	>100	>100	>100	–
7a	37	46	>100	>100	>100	64	>100
7c	20	>20	>100	>100	>100	15	>100
7d	8.6	>20	>100	>100	>100	39	>100
7h	2.0	>20	2	>100	4	48	>100
Acyclovir	2.6	140	0.2	50	0.1	440	>440

[a] Effective concentration required to reduce virus plaque formation by 50%. VZV, HSV-1 and HSV-2 represent EC_{50} values for varicella zoster virus, herpes simplex virus strains, respectively.
[b] Cytotoxic concentration required to reduce cell growth by 50%.
[c] Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

were not active against HCMV and VV (data not shown) in HEL cell cultures.

3. Conclusions

A phosphoroamidate prodrug (ProTide) approach was used to seek to deliver the pre-formed bioactive monophosphate of a new compound recently reported to be a potent and selective inhibitor of thymidylate synthase X, an enzyme essential for the survival of *M. tuberculosis*. The lipophilic ProTide motif was designed to improve permeability trough the mycobacterial cell wall. We have successfully synthesized twelve phosphoroamidate derivatives of *N*-(3-(5-(2′-deoxyuridine-5′-monophosphate))prop-2-ynyl)octanamide **1**, a potent in vitro ThyX inhibitors. Biological tests of our prodrugs showed antimycobacterial activity against *M. tuberculosis* H37Rv and *M. bovis* BCG in contrast to inactivity from the parent nucleoside. We considered that the increased lipophilicity together with the correct molecular size of phosphoroamidate derivatives **7a**, **7b**, **7c 7g**, **7h 7i** and **7l** allowed them to penetrate through the mycobacterial cell wall liberating the monophosphate intracellulary and targeting their proposed biological target, ThyX protein.

Moreover, we found interesting activities against VZV for some of our compounds, showing the necessity of the pro-moiety to help transport of the drug into cells. We identified the L-alanine benzyl ester phosphoramidate with the phenyl aromatic group **7h** as a potent antiviral agent, showing similar activity to acyclovir against VZV in this assay.

The results obtained highlight the possibility that the ProTide methodology could be used for the development of active molecules against TB and reveal the importance of improving lipophilicity to efficiently pass the mycobacteria wall barrier. To the best of our knowledge this is the first application of the ProTide technology to anti-mycobacterial agents. Further work is currently underway to enhance the potency of the new agents we herein report for the first time.

4. Experimental

4.1. Synthesis

Solvents and reagents: The following anhydrous solvents were purchased from Sigma-Aldrich: dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), dimethylformamide (DMF), and any other reagents used. Amino acid esters commercially available were purchased from Novabiochem. All reagents commercially available were used without further purification. Propargylamide and octanoyl chloride were purchased from Aldrich whereas 5-iodo-2′-deoxyuridine from Berry & Associates.

Thin layer chromatography (TLC): Precoated aluminum backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short and long wave ultraviolet light (254 and 366 nm) or by burning using the following TLC indicators: (i) molybdate ammonium cerium sulfate; (ii) potassium permanganate solution. Preparative TLC plates (20 cm × 20 cm, 500–2000 μm) were purchased from Merck.

Flash column chromatography: Flash column chromatography was carried out using silica gel supplied by Fisher (60A, 35–70 μm). Glass columns were slurry packed using the appropriate eluent with the sample being loaded as a concentrated solution in the same eluent or preadsorbed onto silica gel. Fractions containing the product were identified by TLC, and pooled and the solvent was removed in vacuo.

High performance liquid chromatography (HPLC): The purity of the final compounds was verified to be >95% by HPLC analysis using either I) ThermoSCIENTIFIC, SPECTRA SYSTEM P4000, detector SPECTRA SYSTEM UV2000, Varian Pursuit XRs 5 C18, 150 × 4.6 mm (as an analytic column) or II) Varian Prostar (LC Workstation-Varian Prostar 335 LC detector), Thermo SCIENTIFIC Hypersil Gold C18, 5 μ, 150 × 4.6 mm (as an analytic column). For the method of elution see the Section 4.

Nuclear Magnetic Resonance (NMR): ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and ^{31}P NMR (202 MHz) were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Chemical shifts (δ) are quoted in parts per million (ppm) relative to internal MeOH-d_4 (δ 3.34 ^{1}H NMR, δ 49.86 ^{13}C NMR) and $CHCl_3$-d (δ 7.26 ^{1}H NMR, δ 77.36 ^{13}C NMR) or external 85% H_3PO_4 (δ 0.00 ^{31}P NMR). Coupling constants (J) are measured in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br s (broad singlet), dd (doublet of doublet), dt (doublet of triplet), app (apparent). The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC, HMBC, PENDANT).

Mass spectrometry (MS): Low resolution mass spectra were performed on Bruker Daltonics microTof-LC, (atmospheric pressure ionization, electron spray mass spectroscopy) in positive mode.

4.1.1. Synthesis of *N*-(3-(5-(2′-deoxyuridine))prop-2-ynyl) octanamide 2

*Propargyloctanamide (**4**):* Propargylamine **3** (2.5 mL, 45.38 mmol) was dissolved in anhydrous CH_2Cl_2 (113 mL) DIPEA (10.4 mL, 59.46 mmol) was added and the solution was cooled to 0 °C. Octanoyl chloride (8.52 mL, 49.92 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. The reaction was quenched through dropwise addition of H_2O (5 mL) then diluted with CH_2Cl_2 and washed with saturated solution of $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to

obtain **4** as an orange solid (99%, 8.3 g) which was used in the subsequent Sonogashira-coupling step without further purification. ^{1}H NMR (500 MHz, $CHCl_3$-*d*): δ_H 5.68 (br s, 1H, NH) 4.04 (dd, 2H, *J* = 7.5 and 2.5 Hz, $CH_2C{\equiv}C$), 2.22 (t, 1H, *J* = 2.8 Hz, $HC{\equiv}C$), 2.20 (t, 2H, *J* = 8.0 Hz, CH_2CO), 1.69–1.61 (m, 2H, CH_2CH_2CO), 1.31–1.28 (m, 8H, 4× CH_2), 0.88 (t, 3H, *J* = 6.8 Hz, CH_3).

4.1.2. *N*-(3-(5-(2′-Deoxyuridine))prop-2-ynyl)octanamide (2)

A solution of 5-iodo-2′-deoxyuridine **5** (4.00 g, 11.29 mmol), alkyne **4** (6.14 g, 33.89 mmol), tetrakis Pd(PPh_3)$_4$ (1.30 g, 1.13 mmol), Cu(I)I (0.43 g, 2.26 mmol) and anhydrous DIPEA (3.93 mL, 22.58 mmol) in anhydrous DMF (75 mL) was stirred under an argon atmosphere at room temperature overnight. After this period, the solvent was removed under reduced pressure and the residue was suspended in CH_2Cl_2 and stirred at room temperature for 2 h. The suspension was filtered and the solid was washed with CH_2Cl_2 to give the desired compound as a light brown solid (91%, 4.20 g). For biological testing, the compound **2** was purified by preparative TLC using CH_2Cl_2/MeOH 9:1 as eluent. ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 11.59 (s, 1H, N*H*), 8.26 (t, 1H, *J* = 5.4 Hz, N*H* amide), 8.15 (s, 1H, *H*-6), 6.12 (t, 1H, *J* = 6.7 Hz, *H*-1′), 5.23 (d, 1H, *J* = 4.3 Hz, 3′-O*H*), 5.07 (t, 1H, *J* = 5.0 Hz, 5′-O*H*), 4.25–4.22 (m, 1H, *H*-3′), 4.06 (d, 2H, *J* = 5.5 Hz, $NHCH_2$), 3.80 (q, 1H, *J* = 3.3 Hz, *H*-4′), 3.62–3.56 (m, 2H, *H*-5′), 2.13–2.07 (m, 4H, *H*-2′, $COCH_2$), 1.52–1.46 (m, 2H, $COCH_2CH_2$), 1.28–1.21 (m, 8H, 4× CH_2), 0.86 (t, 3H, *J* = 6.9 Hz, CH_3). ^{13}C (125 MHz, DMSO-d_6): δ_C 171.83 (CONH), 161.57 (*C*-4), 149.39 (*C*-2), 143.57 (*C*-6), 98.14 (*C*-5), 89.76 ($CH_2C{\equiv}C$), 87.61 (*C*-4′), 84.69 (*C*-1′), 74.18 ($CH_2C{\equiv}C$), 70.23 (*C*-3′), 61.02 (*C*-5′), 40.06 (*C*-2′), 35.06 ($COCH_2$), 31.12, 28.58, 28.47, 28.39, 25.09, 22.01 (6× CH_2), 13.89 (CH_2CH_3). MS (ES$^-$) *m*/*z* 442 (M+Cl$^-$, 100%), 406 (M−H$^+$, 39%). Reverse-phase HPLC, eluting with H_2O/MeOH from 90:10 to 0:100 in 40 min, flow = 1 mL/min, λ = 280 nm, t_R = 25.71 min.

4.1.3. Synthesis of phosphoramidates 7a–l

General procedure: To a solution of nucleoside **5** (1 mol/equiv) and the appropriate phosphorochloridate **6a–l** (3–5 mol/equiv) in anhydrous THF, anhydrous NMI (5 mol/equiv) was added dropwise and the reaction mixture was stirred at room temperature overnight. After this period, the solvent was removed under reduced pressure and the residue dissolved in CH_2Cl_2. The organic phase was washed with 0.5 M aqueous solution of citric acid, water and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated. The crude was purified by column chromatography using different eluting systems. Some of the compounds were further purified by preparative TLC using different eluting systems. All the compounds were recovered as a mixture of R_P and S_P diastereoisomers.

4.1.3.1. *N*-{3-[5-(2′-Deoxy-5′-*O*-(1-naphthyl(methyloxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7a).

Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and naphthyl(methyloxy-L-alaninyl)phosphorochloridate **6a** (0.80 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 95:5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95:5 to give **7a** as a white solid (20%, 0.07 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.19 (d *J* = 7.5 Hz, 1H, Naph), 7.95 (s, 0.5H of one diastereoisomer, *H*-6), 7.93 (s, 0.5H of one diastereoisomer, *H*-6), 7.92–7.89 (m, 1H, Naph), 7.84 (d, *J* = 7.5 Hz, 0.5H of one diastereoisomer, Naph), 7.74 (d, *J* = 7.5 Hz, 0.5H of one diastereoisomer, Naph), 7.60–7.53 (m, 3H, Naph), 7.47–7.43 (m, 1H, Naph), 6.17 (t, *J* = 5.5 Hz, 0.5H of one diastereoisomer *H*-1′), 6.15 (t, *J* = 6.0 Hz, 0.5H of one diastereoisomer *H*-1′), 4.44–4.33 (m, 3H, *H*-3′, *H*-5′), 4.16–4.07 (m, 2H, *H*-4′ and $CHCH_3$), 3.97, 3.96, 3.93 (3s, 2H, $NHCH_2C{\equiv}$), 3.68, 3.67 (s, 3H, OCH_3), 2.22 (ddd, *J* = 14.0, 6.5 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2′), 2.16 (ddd, *J* = 14.0, 6.0 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2′), 2.13 (t, *J* = 7.5 Hz, 1H, $COCH_2CH_2$), 2.09 (t, *J* = 7.5 Hz, 1H, $COCH_2CH_2$), 1.89–1.83 (m, 0.5H of one diastereoisomer, *H*-2′), 1.80–1.76 (m, 0.5 H of one diastereoisomer, *H*-2′) 1.57-.151 (m, 2H, $COCH_2CH_2$–), 1.39 (d, *J* = 8.5 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.38 (d, *J* = 8.5 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.31–1.21 (m, 8H, 4× CH_2), 0.89 (t *J* = 7.0 Hz, 3H, CH_3). ^{13}C (125 MHz, MeOH-d_4): δ_C 175.71, 175.68 (*C*ONH), 175.23 (d, $^3J_{C-P}$ = 4.6 Hz, *C*OOCH$_3$), 175.10 (d, $^3J_{C-P}$ = 3.7 Hz, *C*OOCH$_3$), 164.46 (*C*-4), 151.96, 15141 (*C*-2), 148.04 (d, $^2J_{C-P}$ = 8.0 Hz, '*ipso*' *Ph*O), 144.59 (*C*-6), 136.32, 137.30, 128.98, 128.95, 127.92, 127.72, 127.62, 126.63, 126.56, 126.25, 126.19, 122.72, 122.66 (C-1 '*ipso*' Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.47 (d, $^3J_{C-P}$ = 2.9 Hz, C-2 Naph), 116.41 (d, $^3J_{C-P}$ = 3.4 Hz, C-2 Naph), 100.26 (*C*-5), 90.36, 90.32 ($CH_2C{\equiv}C$), 87.44, 87.36 (*C*-1′), 87.16 (d, $^3J_{C-P}$ = 8.2 Hz, *C*-4′), 86.95 (d, $^3J_{C-P}$ = 8.2 Hz, *C*-4′), 75.42, 75.37 ($CH_2C{\equiv}C$), 72.24, 72.13 (*C*-3′), 67.85 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5′), 52.85 (OCH_3), 51,80, 51.69 ($CHCH_3$), 41.34, 41.27 (*C*-2′), 36.90, 36.87 ($COCH_2$), 32.91, 30.54, 30.48, 30.26, 30.13, 26.84, 26.82, 23.67 (CH_2NHCO, 6× CH_2), 20.59 (d, $^3J_{C-P}$ = 6.1 Hz, $CHCH_3$), 20.45 (d, $^3J_{C-P}$ = 7.2 Hz, $CHCH_3$), 14.42 (CH_3). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.36, 4.07. MS (ES+) *m*/*z*: 721 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H_2O/ACN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 17.86 min.

4.1.3.2. *N*-{3-[5-(2′-Deoxy-5′-*O*-(1-naphthyl(ethyloxy-L-alaninyl) phosphate-uridine))]prop-2-ynyl)octanamide (7b).

Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and naphthyl(ethyloxy-L-alaninyl)phosphorochloridate **6b** (0.84 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of ethyl acetate/MeOH = 100:0 to 98:2. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95:5 to give **7b** as a white solid (5%, 0.02 g). ^{1}H NMR (500 MHz; MeOH-d_4): δ_H 8.20–7.42 (m, 8H, Naph, H-6), 6.17–6.14 (m, 1H, H-1′), 4.45–4.32 (m, 3H, H-3′, H-5′), 4.17–4.07 (m, 4H, H-4′, $COOCH_2CH_3$, $CHCH_3$), 3.97, 3.93, 3.91 (3s, 2H, $NHCH_2C{\equiv}C$), 2.23–2.17 (m, 1H, H-2′), 2.16–2.08 (m, 2H, $COCH_2CH_2$), 1.87–1.74 (m, 1H, H-2′), 1.58–1.51 (m, 2H, $COCH_2CH_2$), 1.43–1.21 (m, 14H, 4× CH_2, $COOCH_2CH_3$, $CHCH_3$), 0.89–0.84 (t, 3H, *J* = 6.7 Hz, $CH_2CH_2CH_3$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.69, 175.68 (CH_2CO), 175.05, 174.73 ($COOCH_2CH_3$), 164.36 (*C*-4), 151.05, 150.93 (*C*-2), 148.00 (d, $^2J_{C-P}$ = 7.4 Hz, C-1 '*ipso*' Naph), 147.97 (d, $^2J_{C-P}$ = 7.2 Hz, C-1 '*ipso*' Naph), 144.55 (*C*-6), 136.29, 136.28, 128.95, 128.92, 127.91, 127.90, 127.84, 127.68, 127.58, 126.61, 126.60, 126.53, 126.52, 126.22, 126.17, 126.53, 126.52 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.42 (d, $^3J_{C-P}$ = 3.4 Hz, C-2 Naph), 116.37 (d, $^3J_{C-P}$ = 3.2 Hz, C-2 Naph), 100.24 (*C*-5), 90.33 ($CH_2C{\equiv}C$), 87.40, 87.37 (*C*-1′), 87.15 (d, $^3J_{C-P}$ = 8.4 Hz, *C*-4′), 86.91 (d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 75.40 ($CH_2C{\equiv}C$), 72.22, 72.11 (*C*-3′), 67.86 (d, $^2J_{C-P}$ = 5.4 Hz, *C*-5′), 62.47 ($COOCH_2CH_3$), 51.87, 51.77 ($CHCH_3$), 41.33, 41.25 (*C*-2′), 36.87, 36.84 ($COCH_2$), 32.85, 30.53, 30.44, 30.23, 30.09, 26.80, 23.64 (CH_2NHCO, 6× CH_2), 20.61 (d, $^3J_{C-P}$ = 6.6 Hz, $CHCH_3$), 20.47 (d, $^3J_{C-P}$ = 7.7 Hz, $CHCH_3$), 14.46, 14.39 ($COOCH_2CH_3$, CH_3). ^{31}P NMR (202 MHz; MeOD-d_4): δ_P 4.36, 4.09. MS (ES$^+$) *m*/*z*: 735 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H_2O/ MeOH from 90:10 to 0:100 in 40 min, flow = 1 mL/min, λ = 280 nm, t_R = 34.49 min (99.05%).

4.1.3.3. *N*-{3-[5-(2′-Deoxy-5′-*O*-(1-naphthyl(neopentyloxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7c).

Prepared according to standard procedure from nucleoside **2** (0.20 g,

0.49 mmol) and naphthyl(neopentyloxy-L-alaninyl)phosphorochloridate **6c** (0.94 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 95:5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH = 95:5 to give **7c** as a white solid (39%, 0.13 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.22–8.18 (m, 1H, Naph), 7.93 (s, 0.65H of one diastereoisomer, *H*-6), 7.92 (s, 0.35H of one diastereoisomer, *H*-6), 7.90–7.88 (m, 1H, Naph), 7.72 (d *J* = 8.5 Hz, 0.35H of one diastereoisomer, Naph) 7.71 (d, *J* = 8.5 Hz, 0.65H of one diastereoisomer, Naph) 7.58–7.52 (m, 3H, Naph), 7.46–7.42 (m, 1H, Naph), 6.16–6.12 (m, 1H, *H*-1′), 4.45–4.32 (m, 3H, *H*-3′, *H*-5′), 4.16–4.07 (m, 2H, *H*-4′, C*H*CH$_3$), 3.99, 3.97, 3.95, 3.94 (4s, 2H, NHCH_2C≡), 3.87, 3.86, 3.76, 3.75 (2× AB, 4H, J_{AB} = 10.5 Hz, 2× OCH_2C(CH$_3$)$_3$), 2.21–2.14 (m, 1H, *H*-2′), 2.13 (t, *J* = 7.5 Hz, 1H, COCH_2CH$_2$), 2.08 (t, *J* = 7.5 Hz, 1H, COCH_2-CH$_2$), 1.83–1.73 (m, 1H, *H*-2′), 1.56–1.51 (m, 2H, COCH$_2$CH_2), 1.43 (d, *J* = 7.0 Hz, 3H, CHCH_3), 1.29–1.19 (m, 8H, 4× CH_2), 0.94, 0.93 (2s, 9H, OCH_2C(CH$_3$)$_3$), 0.87 (t, *J* = 6.5 Hz, 3H, CH_3). ^{13}C (125 MHz, MeOH-d_4): δ_C 175.68, 175.64 (*C*ONH), 175.16 (d, $^3J_{C-P}$ = 5.0 Hz, *C*O$_2$-CH$_2$C(CH$_3$)$_3$), 174.83 (d, $^3J_{C-P}$ = 4.7 Hz, *C*O$_2$CH$_2$C(CH$_3$)$_3$), 164.42 (*C*-4), 151.00, 150.97 (*C*-2), 148.00 (d, $^2J_{C-P}$ = 7.3 Hz, C-1 '*ipso*' Naph), 147.90 (d $^2J_{C-P}$ = 7.2 Hz, C-1 '*ipso*' Naph), 144.64, 144.60 (*C*-6), 136.32, 136.30, 128.99, 128.97, 127.93, 127.73, 127.63, 126.67, 126.58, 126.28, 126.21, 122.77, 122.69 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.48 (d, $^3J_{C-P}$ = 5.7 Hz, C-2 Naph), 100.30, 100.27 (*C*-5), 90.36, 90.33 (CH$_2$C≡*C*), 87.43, 87.41 (*C*-1′), 87.19, (d, $^3J_{C-P}$ = 8.5 Hz, *C*-4′), 86.95 (d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 75.55 (OCH$_2$C(CH$_3$)$_3$), 75.10 (CH$_2$-*C*≡C), 72.30, 72.14 (*C*-3′), 67.97 (d, $^2J_{C-P}$ = 5.0 Hz, *C*-5′), 67.92 (d, $^2J_{C-P}$ = 5.0 Hz, *C*-5′), 52.03, 51.89 (*C*HCH$_3$), 41.36, 41.31 (*C*-2′), 36.94, 36.89 (COCH_2), 32.90 (CH_2), 32.39 (*C*(CH$_3$)$_3$), 30.65, 30.55, 30.30, 30.15, 26.86, 26.85 (CH_2NHCO, 4× CH_2), 26.81 (OCH$_2$-C(*C*H$_3$)$_3$), 26.75, 23.69 (*C*H$_2$), 20.90 (d, $^3J_{C-P}$ = 5.8 Hz, CH*C*H$_3$), 20.70 (d, $^3J_{C-P}$ = 7.6 Hz, CH*C*H$_3$), 14.48 (*C*H$_3$). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.34, 4.07. MS (ES+) *m*/*z*: 777 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 22.51, 22.68 min.

4.1.3.4. *N*-{3-[5-(2′-Deoxy-5′-*O*-(1-naphthyl(benzyloxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7d). Prepared according to standard procedure from nucleoside **2** (0.30 g, 0.75 mmol) and naphthyl(benzyloxy-L-alaninyl)phosphorochloridate **6d** (0.91 g, 2.25 mmol) in anhydrous THF (25 mL) and anhydrous NMI (0.30 mL, 3.80 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 95:5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95:5 to give **7d** as a white solid (14%, 0.08 g). ^{1}H NMR (500 MHz; MeOD-d_4) δ_H 8.19–8.17 (m, 1H, Naph), 7.93–7.90 (m, 2H, Naph, H-6), 7.74–7.69 (m, 1H, Naph), 7.58–7.52 (m, 3H, Naph), 7.40–7.30 (m, 6H, Naph, Ph), 6.13–6.10 (m, 1H, *H*-1′), 5.18–5.10 (m, 2H, COOCH_2Ph), 4.39–4.32 (m, 1H, H-5′), 4.31- 4.25 (m, 2H, H-3′, *H*-5′), 4.18–4.07 (m, 2H, H-4′, C*H*CH$_3$), 3.92 (s, 2H, NHCH_2C≡), 2.19–2.09 (m, 3H, H-2′, COCH_2-CH$_2$), 1.80–1.75 (m, 1H, H-2′), 1.59–1.50 (m, 2H, COCH$_2$CH_2), 1.40 (d, *J* = 7.0 Hz, 3H, CHCH_3), 1.36–1.21 (m, 8H, 4× CH_2), 0.89–0.87 (m, 3H, CH_3). ^{13}C NMR (125 MHz; MeOH-d_4): δ_C 175.23 (NHCO), 174.57 (*C*OOCH$_2$Ph), 164.32 (*C*-4), 150.87 (*C*-2), 148.60 (d, $^2J_{C-P}$ = 7.3 Hz, C-1 '*ipso*' Naph), 144.57, 144.52 (*C*-6), 137.14, 136.29 ('*ipso*' OCH$_2$-*Ph*), 129.80, 129.74, 129.64, 129.61, 129.42, 129.37, 128.97, 127.91, 127.72, 127.62, 126.64, 126.55, 126.27, 126.18, 122.74, 122.65 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, COOCH$_2$*Ph*), 116.43 (d, $^3J_{C-P}$ = 2.5 Hz, C-2 Naph), 100.26 (*C*-5), 90.40 (CH$_2$C≡*C*), 87.32, 87.19 (*C*-1′), 86.91 (d, $^3J_{C-P}$ = 8.8 Hz, *C*-4′), 75.43 (CH$_2$C≡C), 72.25, 72.07 (*C*-3′), 68.14, 68.11 (*C*H$_2$OPh), 67.87 (d, $^2J_{C-P}$ = 5.0 Hz, *C*-5′), 52.00, 51.85 (*C*HCH$_3$), 41.33, 41.29 (*C*-2′), 36.90 (COCH_2), 32.86, 30.56, 30.48, 30.25, 30.11, 26.83, 23.66 (CH_2NHCO, 6× CH_2), 20.55 (d, $^3J_{C-P}$ = 6.25 Hz, CH*C*H$_3$), 14.48, 14.41 (*C*H$_3$). ^{31}P NMR (202 MHz, MeOD-d_4): δ_P 4.36, 3.98. MS (ES+) *m*/*z*: 797 (M+Na$^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 16.63 min.

4.1.3.5. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(methoxy-L-alaninyl) phosphate-uridine))]prop-2-ynyl)octanamide (7e). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(methoxy-L-alaninyl)phosphorochloridate **6e** (0.68 g, 2.45 mmol) in anhydrous THF (18 mL) and with NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 94:6 to give **7e** as a white solid (16%, 0.05 g). ^{1}H NMR (500 MHz, MeOH-d_4) δ_H 7.99, 7.96 (2s, 1H, H-6), 7.40–7.36 (m, 2H, Ph), 7.30–7.27 (m, 2H, Ph), 7.23–7.20 (m, 1H, Ph), 6.25–6.20 (m, 1H, H-1′), 4.44–4.41 (m, 1H, H-3′), 4.39–4.37 (m, 1H, H-5′), 4.34–4.31 (m, 1H, H-5′), 4.15–4.12 (m, 1H, H-4′), 4.09 (s, 1H, NHCH_2), 4.06–4.01 (m, 2H, C*H*CH$_3$, NHCH_2), 3.71, 3.69 (2s, 3H, COOCH$_3$), 2.34 (ddd, *J* = 13.7, 6.0, 3.1 Hz, 0.5H of one diastereoisomer), H-2′, 2.27 (ddd, *J* = 13.7, 6.0, 2.9 Hz, 0.5H of one diastereoisomer, H-2′), 2.19–2.15 (m, 2H, COCH$_2$–), 2.14–2.08 (m, 0.5H of one diastereoisomer, H-2′), 2.01–1.95 (m, 0.5H of one diastereoisomer, H-2′), 1.60–1.56 (m, 2H, COCH$_2$CH_2–), 1.38 (d, *J* = 7.1 Hz, 3H, CHCH_3), 1.34–1.25 (m, 8H, 4× CH$_2$), 0.91 (t, *J* = 6.9 Hz, 3H, –CH$_2$-CH$_3$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.75, 175.55, 175.27, 175.23 (*C*OOCH$_3$, *C*ONH), 164.43 (C-4), 152.15 (C-2), 151.03 ('ipso' *Ph*), 144.72, 144.68 (C-6), 130.91, 130.89, 126.38, 126.32, 121.53, 121.49 (*Ph*O), 100.23 (C-5), 90.46, 90.42 (CH$_2$C≡*C*), 87.47, 87.26 (C-1′), 87.13 (d, $^3J_{C-P}$ = 8.1 Hz, C-4′), 87.13 (d, $^3J_{C-P}$ = 8.3 Hz, C-4′), 75.42, 75.37 (CH$_2$C≡C), 72.23, 72.14 (C-3′), 67.69 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5′), 67.57 (d, $^2J_{C-P}$ = 5.3 Hz, *C*-5′), 51.70, 51.53 (COO*C*H$_3$), 50.22 (*C*HCH$_3$), 41.38, 41.32 (C-2′), 36.92 (COCH_2), 32.89, 30.56, 30.53, 30.27, 30.13, 26.85, 23.67 (NHCH_2CO, 6× CH_2), 20.62 (d, $^3J_{C-P}$ = 6.0 Hz, CH*C*H$_3$), 20.44 (d, $^3J_{C-P}$ = 7.1 Hz, CH*C*H$_3$), 14.43 (*C*H$_3$). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 3.98, 3.57. MS (EI) *m*/*z*: 671 (M+Na$^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 100:0 to 0:100 in 30 min, flow = 1 mL/min, λ = 254 nm, t_R = 18.29 min.

4.1.3.6. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(ethoxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7f). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(ethoxy-L-alaninyl)phosphorochloridate **6f** (0.71 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH = 98:2 to 94:6 to give **7f** as a white solid (29%, 0.09 g). ^{1}H NMR (500 MHz, MeOH-d_4) δ_H 7.99, 7.96 (2s, 1H, H-6), 7.40–7.36 (m, 2H, Ph), 7.30–7.27 (m, 2H, Ph), 7.22–7.19 (m, 1H, Ph), 6.25–6.20 (m, 1H, H-1′), 4.44–4.41 (m, 1H, H-3′), 4.39–4.37 (m, 1H, H-5′), 4.35–4.30 (m, 1H, H-5′), 4.20–4.12 (m, 3H, H-4′, COOCH_2CH$_3$), 4.10, 4.05 (2s, 2H, NHCH_2), 4.04–3.97 (m, 1H, C*H*CH$_3$), 2.34 (m, *J* = 13.7, 6.0, 3.1 Hz, 0.5H of one diastereoisomer, H-2′), 2.27 (m, *J* = 13.8, 6.0, 3.0 Hz, 0.5H of one diastereoisomer, H-2′), 2.19–2.15 (m, 2H, COCH$_2$–), 2.13–2.07 (m, 0.5H of one diastereoisomer, H-2′), 1.99–1.94 (m, 0.5H of one diastereoisomer, H-2′), 1.62–1.56 (m, 2H, COCH$_2$CH_2–), 1.38 (d, *J* = 7.2 Hz, 3H, CHCH_3), 1.34–1.24 (m, 11H, 4× CH$_2$, COOCH$_2$CH_3), 0.90 (t, *J* = 6.9 Hz, 3H, –CH$_2$CH$_3$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.76, 175.11, 175.07 (*C*OOCH$_2$-CH$_3$, *C*ONH), 164.39 (C-4), 152.20, 152.14 (C-2), 151.02 ('ipso' *Ph*), 144.71, 144.67 (C-6), 130.92, 130.90, 126.40, 126.33, 121.53, 121.50 (*Ph*O), 100.28 (C-5), 90.48, 90.45 (CH$_2$C≡*C*), 87.43, 87.24 (C-1′), 87.12 (d, $^3J_{C-P}$ = 8.3 Hz, C-4′), 86.94 (d, $^3J_{C-P}$ = 8.2 Hz, C-4′), 75.47, 75.43 (CH$_2$C≡C), 72.21, 72.12 (C-3′), 67.71 (d, $^2J_{C-}$

$_P$ = 5.5 Hz, *C*-5′), 67.59 (d, $^2J_{C-P}$ = 5.4 Hz, *C*-5′), 62.52, 62.51 ($COOCH_2CH_3$), 51.81, 51.63 ($CHCH_3$), 41.42, 41.37 (C-2′), 36.96 ($COCH_2$), 32.90, 30.63, 30.59, 30.30, 30.15, 26.87, 23.69 ($NHCH_2CO$, 6× CH_2), 20.74 (d, $^3J_{C-P}$ = 6.2 Hz, $CHCH_3$), 20.55 (d, $^3J_{C-P}$ = 7.1 Hz, $CHCH_3$), 14.57, 14.55, 14.47 ($COOCH_2CH_3$, CH_3). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 3.99, 3.58. MS (EI) *m*/*z*: 685 ($M+Na^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 100:0 to 0:100 in 30 min, flow = 1 mL/min, λ = 254 nm, t_R = 19.10 min.

4.1.3.7. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(propyloxy-L-alaninyl) phosphate-uridine))]prop-2-ynyl)octanamide (7g). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(propyloxy-L-alaninyl)phosphorochloridate **6g** (0.71 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 94:6 to give **7g** as a white solid. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.99, 7.97 (2s, 1H, *CH*-6), 7.40–7.35 (m, 2H, *Ph*), 7.31–7.26 (m, 2H, *Ph*), 7.23–7.19 (m, 1H, *Ph*), 6.24–6.19 (m, 1H, *H*-1′), 4.39–4.35 (m, 1H, *H*-3′), 4.34–4.20 (m, 2H, *H*-5′), 4.15–3.99 (m, 6H, *H*-4′, OCH_2, $NHCH_2C{\equiv}C$, $CHCH_3$), 2.34 (ddd, *J* = 14.0, 6.0 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2′), 2.25 (ddd, *J* = 13.5, 6.0 and 2.5 Hz, 0.5H of one diastereoisomer, *H*-2′), 2.19–2.14 (m, 2H, *H*-2′, $COCH_2CH_2$), 2.12–2.00 (m, 1H of one diastereoisomer, *H*-2′), 199–1.96 (m, 1H of one diastereoisomer, *H*-2′), 1.70–1.63 (m, 2H, OCH_2CH_2), 1.62–1.56 (m, 2H, $COCH_2CH_2$), 1.41–1.37 (d, *J* = 7.5 Hz, 3H, $CHCH_3$), 1.34–1.26 (m, 8H, 4× CH_2), 0.96, 0.95 (2t *J* = 7.5 Hz, 3H, OCH_2CH_3), 0.90 (t, *J* = 7.0 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.75, 175.72 (CONH), 175.16 (d, $^3J_{C-P}$ = 4.5 Hz, CO_2CH_2), 174.88 (d, *J* = 5.4 Hz, CO_2CH_2), 164.40, 164.42 (*C*-4), 152.19 (d, $^2J_{C-P}$ = 7.1 Hz 'ipso' *Ph*O), 152.19 (d, $^2J_{C-P}$ = 7.1 Hz 'ipso' *Ph*O), 152.13 (d, $^2J_{C-P}$ = 6.5 Hz 'ipso' *Ph*O), 151.04 (*C*-2), 144.70, 144.74, (*C*-6), 130.89, 130.87, 126.36, 126.29, (*Ph*O), 121.52 (d, $^2J_{C-P}$ = 7.1 Hz, *Ph*O), 100.25, 100.28 (*C*-5), 90.46, 90.44 ($CH_2C{\equiv}C$), 87.48, 87.37 (*C*-1′), 86.97 (d, $^3J_{C-P}$ = 8.2 Hz, *C*-4′), 86.16 (d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 75.41, 75.34 ($CH_2C{\equiv}C$), 72.21, 72.12 (*C*-3′), 68.06, 68.05 (OCH_2), 67.75 (d, $^2J_{C-P}$ = 5.6 Hz, *C*-5′), 67.63 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5′), 51.88, 51.72 ($CHCO_2$), 41.38, 41.35 (*C*-2′), 36.96 ($COCH_2$), 32.90, 30.59, 30.56, 30.27, 30.09, 26.87, 23.05, 23.03 (–CH_2–), 20.58 (d, $^3J_{C-P}$ = 7.0 Hz, $CHCH_3$), 20.77 (d, $^3J_{C-P}$ = 6.1 Hz, $CHCH_3$), 14.37 (CH_3), 10.64 (OCH_2CH_3); ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.00, 3.59. MS (EI) *m*/*z*: 711 ($M+Cl^-$, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 35 min, flow = 1 mL/min, λ = 280 nm, t_R= 17.77 min.

4.1.3.8. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl-(benzyloxy-L-alaninyl) phosphate-uridine))]prop-2-ynyl)octanamide (7h). Prepared according to standard procedure nucleoside **2** (0.30 g, 0.75 mmol) and phenyl(benzyloxy-L-alaninyl)phosphorochloridate **6h** (0.80 g, 2.25 mmol) in anhydrous THF (25 mL) and anhydrous NMI (0.30 mL, 3.80 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH = 98:2 to 95:5. The residue was further purified by preparative TLC, eluting with CH_2Cl_2/MeOH 95:5 to give **7h** as a white solid (11%, 0.06 g). ^{1}H NMR (500 MHz; MeOH-d_4) δ_H 7.97–7.93 (m, 1H, H-6), 7.91–7.87 (m, 1H, *Ph*), 7.41–7.32 (m, 6H, *Ph*), 7.29–7.17 (m, 3H, Ph), 6.23–6.17 (m, 1H, H-1′), 5.20–5.11 (m, 2H, OCH_2Ph), 4.41–4.34 (m, 1H, H-3′), 4.33–4.29 (m, 2H, H-5′), 4.11–4.01 (m, 2H, H-4′, $CHCH_3$), 3.31 (s, 2H, $NHCH_2C{\equiv}$), 2.32–2.21 (m, 3H, *H*-2′, $COCH_2CH_2$), 1.93–1.88 (m, 1H, *H*-2′), 1.63–1.51 (m, 2H, $COCH_2CH_2$), 1.41 (d, *J* = 7.0 Hz, 3H, $CHCH_3$), 1.37–1.21 (m, 8H, 4× CH_2), 0.92–0.88 (m, 3H, CH_3). ^{13}C NMR (125 MHz; MeOD-d_4): δ_C 174.93 (NHCO), 171.69 (CO_2CH_2Ph), 164.37 (C-4), 152.25 (d, $^3J_{C-P}$ = 7.4 Hz '*ipso*' O*Ph*), 151.17 (C-2), 144.84, 144.70 (C-6), 137.21, 137.18 ('*ipso*' OCH_2Ph), 131.25, 130.90, 129.80, 129.76, 129.73, 129.65, 129.64, 129.61, 129.44, 129.40, 129.38, 129.18, 126.99, 126.39, 126.30, 121.55, 121.52, 121.47 (*Ph*, OCH_2Ph), 100.22 (C-5), 90.50 ($CH_2C{\equiv}C$), 87.36, 87.32 (*C*-1′), 87.14, 87.07 (d, $^3J_{C-P}$ = 8.8 Hz, *C*-4′), 75.42 ($CH_2C{\equiv}C$), 72.24, 72.06 (*C*-3′), 68.13, 68.09 ($PhCH_2$), 67.65 (d, $^2J_{C-P}$ = 5.8 Hz, *C*-5′), 51.89, 51.69 ($CHCH_3$), 41.37, 40.99 (C-2′), 36.99, 36.93 ($COCH_2$), 32.89, 30.58, 30.54, 30.45, 30.28, 26.85, 23.67 ($NHCH_2CO$, 6× CH_2), 20.61, 20.56 ($CHCH_3$), 14.43 (CH_3). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.01, 3.51. MS (ES+) *m*/*z*: 747 ($M+Na^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 16.22 min.

4.1.3.9. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(benzyloxy-D-alaninyl) phosphate-uridine))]prop-2-ynyl)octanamide (7i). Prepared according to standard procedure from nucleoside **2** (0.25 g, 0.61 mmol) and phenyl(benzyloxy-D-alaninyl)phosphorochloridate **6i** (1.10 g, 3.05 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.24 mL, 3.05 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of ethyl acetate/MeOH from 100:0 to 98:2. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 90:10 to give **7i** as a white solid (16%, 0.07 g). ^{1}H NMR (500 MHz; MeOH-d_4): δ_H 7.99, 7.82 (2s, 1H, H-6), 7.38–7.20 (m, 10H, PhO, $COOCH_2Ph$), 6.21 (dd, *J* = 7.4 and 6.1 Hz, 0.5H of one diastereoisomer, H-1′), 6.16 (dd, *J* = 7.4 Hz and 6.1 Hz, 0.5H of one diastereoisomer, H-1′), 5.15 (2H, m, OCH_2Ph), 4.41–4.25 (m, 3H, H-3′, H-5′), 4.16–4.01 (m, 4H, H-4′, $NHCH_2C{\equiv}C$, $CHCH_3$), 2.29 (ddd, *J* = 13.7, 6.0, 3.0 Hz, 0.5H of one diastereoisomer, H-2′), 2.18–2.06 (m, 3H, H-2′, $COCH_2CH_2$), 1.70 (m, 0.5H of one diastereoisomer, H-2′), 1.60–1.57 (m, 2H, $COCH_2CH_2$), 1.43 (d *J* = 7.1 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.36 (d, *J* = 7.1 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.35–1.26 (m, 8H, 4× CH_2), 0.90 (t, 3H, *J* = 7.0 Hz, CH_3). ^{13}C NMR (125 MHz; MeOH-d_4): δ_C 175.69 (CH_2CO), 174.87 (d, $^3J_{C-P}$ = 3.9 Hz, $COOCH_2Ph$), 174.80 (d, $^3J_{C-P}$ = 4.3 Hz, $COOCH_2Ph$), 164.37 (*C*-4), 152.20 (d, $^2J_{C-P}$ = 6.6 Hz, '*ipso*' *Ph*O), 152.01 (d, $^2J_{C-P}$ = 6.7 Hz, '*ipso*' *Ph*O), 150.85 (*C*-2), 144.79, 144.41 (*C*-6), 137.18, 137.04 ('*ipso*' $COOCH_2Ph$), 130.93, 130.83, 129.59, 129.56, 129.46, 129.41, 129.37, 129.35, 126.33, 121.60, 121.56, 121.27, 121.23 (*Ph*O, $COOCH_2Ph$), 100.21, 100.12, (C-5), 90.78, 90.26 ($CH_2C{\equiv}C$), 87.24, 86.94 (*C*-1′), 87.05 (d, $^3J_{C-P}$ = 8.6 Hz, *C*-4′), 86.90 (d, $^3J_{C-P}$ = 8.7 Hz, *C*-4′), 75.48, 75.44 ($CH_2C{\equiv}C$), 72.14, 72.03 (*C*-3′), 68.13, 68.07 (OCH_2Ph), 67.70 (d, $^2J_{C-P}$ = 5.5 Hz, C-5′), 67.10 (d, $^2J_{C-P}$ = 4.8 Hz, C-5′), 51.77, 51.56 ($CHCH_3$), 41.36 (*C*-2′), 36.90 (CH_2CO), 32.85, 30.56, 30.47, 30.25, 30.10, 26.82, 23.65 (CH_2NHCO, 6× CH_2), 20.43 (d, $^3J_{C-P}$ = 6.8 Hz, $CHCH_3$), 20.39 (d, $^3J_{C-P}$ = 7.6 Hz, $CHCH_3$), 14.39 (CH_3). ^{31}P NMR (202 MHz; MeOH-d_4): δ_P 3.98, 3.50. MS (ES^+) *m*/*z*: 747 ($M+Na^+$, 100%). Reverse-phase HPLC, eluting with H_2O/ACN from 90:10 to 0:100 in 20 min, flow = 1 mL/min, λ = 280 nm, t_R = 14.48 min, 14.86 min.

4.1.3.10. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(benzyloxy-glycinyl) phosphate-uridine))]prop-2-ynyl)octanamide (7j). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(benzyloxy-glycinyl)phosphorochloridate **6j** (0.87 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 95:5 to give **7j** as a white solid (29%, 0.10 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.09, 7.89 (2s, 1H, *H*-6), 7.38–7.19 (m, 10H, P*h*, OCH_2Ph), 6.24–6.18 (m, 1H, *H*-1′), 5.23–5.13 (m, 2H, OCH_2Ph), 4.36–4.30 (m, 3H, *H*-3′, *H*-5′), 4.13–4.08 (m, 1H, *H*-4′), 4.08, 4.07, 4.02 (3s, 2H, $NHCH_2C{\equiv}C$), 3.91–3.85 (m, 2H, CH_2CO_2Bn), 2.30 (ddd *J* = 14.0, 6.5 and 3.5 Hz, 0.5H of one diastereoisomer, *H*-2′), 2.18–2.09 (m, 3H, *H*-2′, $COCH_2CH_2$), 1.80–1.74 (m, 0.5H of one diastereoisomer, *H*-2′), 1.59–1.55 (m, 2H, $COCH_2CH_2$), 1.30–1.27 (m, 8H, 4× CH_2), 0.91-0.86 (t, *J* = 6.7 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.79, 175.77 (CONH), 172.42, 172.38

(d, $^3J_{C-P}$ = 4.0 Hz, *C*OOBn), 164.38 (*C*-4), 152.20 (d $^2J_{C-P}$ = 7.0 Hz, '*ipso*' *Ph*O), 151.04, 150.97 (*C*-2), 144.84, 144.60 (*C*-6), 137.10, 137.14 ('*ipso*' OCH_2*Ph*), 130.97, 130.90, 129.63, 129.60, 129.50, 129.44, 126.43, 126.37 (*Ph*O, OCH_2*Ph*), 121.55 (d, $^3J_{C-P}$ = 4.5 Hz, *Ph*O), 121.37 (d, $^3J_{C-P}$ = 4.6 Hz, *Ph*O), 100.20, 100.17 (*C*-5), 90.78, 90.44 (*C*≡C), 87.32 (*C*-1′), 87.20 (d, $^3J_{C-P}$ = 8.2 Hz, *C*-4′) 87.10 (*C*-1′), 87.03 (d, $^3J_{C-P}$ = 8.2 Hz, *C*-4′), 75.41, 75.34 (CH_2C≡C), 72.28, 72.20 (*C*-3′), 68.08 (OCH_2Ph) 67.79 (d, $^2J_{C-P}$ = 5.7 Hz, *C*-5′), 67.49 (d, $^2J_{C-P}$ = 5.1 Hz, *C*-5′), 44.03, 43.96 (CH_2CO_2), 41.43, 41.40 (*C*-2′), 36.94 ($COCH_2$), 32.89, 32.88, 30.53, 30.48, 30.29, 30.14, 26.85, 23.68 (CH_2NHCO, 6× CH_2), 14.43 (CH_3). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 5.02, 4.93. MS (ES+) *m*/*z*: 733 (M+Na^+, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 18.78, 19.15 min.

4.1.3.11. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(benzyloxy-dimethylglycine)phosphate-uridine))]prop-2-ynyl)octanamide (7k). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(benzyloxy-L-dimethylglycine)phosphorochloridate **6k** (0.86 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 94:6 to give **7k** as a white solid. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.95 (s, 0.3H of one diastereoisomer, *H*-6), 7.86 (s, 0.7H of one diastereoisomer, *H*-6), 7.40–7.17 (m, 10H, P*h*, OCH_2P*h*), 6.21–6.15 (m, 1H, *H*-1′), 5.20–5.12 (m, 2H,OCH_2Ph), 4.32–4.21(m, 3H, *H*-3′, *H*-5′), 4.09–3.99 (m, 3H, *H*-4′, NHCH_2C≡), 2.32–2.24 (m, 0.7H of one diastereoisomer, *H*-2′), 2.20–2.15 (m, 2.3H, *H*-2′, one diastereoisomer, NHCOCH_2), 2.13–2.05 (m, 0.70H, H-2′), 1.77–1.72 (m, 0.30H of one diastereoisomer, *H*-2′), 1.61–1.48 (m, 6H, $NHCOCH_2CH_2$, $C(CH_3)_2$), 1.36–1.23 (m, 8H, 4× CH_2), 0.95–0.85 (t, *J* = 6.0 Hz, 3H, CH_2CH_3). ^{13}C NMR (125 MHz, MeOH-d_4): δ_c 176.75 (d, $^3J_{C-P}$ = 2.5 Hz, $COCH_2$), 176.55, 176.53 (CONH), 164.52 (*C*-4), 152.35 (d, $^2J_{C-P}$ = 6.9 Hz 'ipso' *Ph*O), 152.22 (d, $^2J_{C-P}$ = 6.8 Hz 'ipso' *Ph*O), 151.11 (*C*-2), 144.86, 144.62 (*C*-6), 137.32 ('ipso' OCH_2*Ph*), 130.90, 130.81, 129.63, 129.54, 129.35, 129.30, 129.20, 126.31, 126.22 (*Ph*O, OCH_2*Ph*), 121.70 (d, $^3J_{C-P}$ = 5.0 Hz, *Ph*O), 121.56 (d, $^3J_{C-P}$ = 4.6 Hz, *Ph*O), 90.75, 90.60 (CH_2C≡*C*), 87.00 (d, $^3J_{C-P}$ = 10.6 Hz, *C*-4′), 86.79 (d, $^3J_{C-P}$ = 10.6 Hz, *C*-4′), 86.26 (*C*-1′), 75.49, 75.38 (CH_2C≡C), 72.07, 71.99 (*C*-3′), 68.36, 68.35 (OCH_2), 67.76 (d, $^2J_{C-P}$ = 5.62 Hz, *C*-5′), 67.53 (d, $^2J_{C-P}$ = 5.78 Hz, *C*-5′), 41.27, 41.24 (*C*-2′), 36.99 (NHCOCH_2), 32.92, 30.61, 30.59, 30.17, 30.12 (CH_2), 27.93 (d, $^3J_{C-P}$ = 6.4 Hz, CH_3), 27.85 (d, $^3J_{C-P}$ = 6.4 Hz, CH_3), 27.67 (d, $^3J_{C-P}$ = 4.2 Hz, CH_3), 27.57 (d, $^3J_{C-P}$ = 4.2 Hz, CH_3), 23.71, 23.69, 26.90 (CH_2), 14.37 (CH_2CH_3); ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 2.39, 2.1. MS (EI) *m*/*z*: 773 (M+Cl^-, 100%). Reverse-phase HPLC, eluting with H_2O/ CH_3CN from 90:10 to 0:100 in 35 min, flow = 1 mL/min, λ = 280 nm, t_R = 19.24 min.

4.1.3.12. *N*-{3-[5-(2′-Deoxy-5′-*O*-(phenyl(benzyloxy-L-valinyl) phosphate-uridine))]prop-2-ynyl)octanamide (7l). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(benzyloxy-L-valinyl)phosphorochloridate **6l** (0.93 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98:2 to 95:5. The residue was further purified by preparative TLC, eluting with CH_2Cl_2/MeOH 95:5 to give **7l** as a white solid (14%, 0.05 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.95 (s, 0.35H of one diastereoisomer, *H*-6), 7.91 (s, 0.65H of one diastereoisomer, *H*-6), 7.40–7.18 (m, 10H, P*h*, OCH_2P*h*), 6.21–6.17 (m, 1H, *H*-1′), 5.21–5.09 (m, 2H, OCH_2Ph), 4.39–4.37 (m, 1H, *H*-3′), 4.34–4.22 (m, 2H, *H*-5′), 4.11–4.06 (m, 3H, *H*-4′, NHCH_2C≡), 3.81 (dd, *J* = 9.5, 5.5 Hz, 0.65H of one diastereoisomer, *CH*CH$(CH_3)_2$), 3.76 (dd, *J* = 10.0, 6.0 Hz, 0.35H of one diastereoisomer, *CH*CH$(CH_3)_2$)), 2.30 (ddd, *J* = 13.5, 6.5 and 3.0 Hz, 0.35H of one diastereoisomer, *H*-2′), 2.22 (ddd, *J* = 13.5, 6.0 and 3.0 Hz, 0.65H of one diastereoisomer, *H*-2′), 2.18–2.04 (m, 3.35H, *H*-2′ of one diastereoisomer, $COCH_2CH_2$, $CH(CH_3)_2$), 1.90–1.64 (m, 0.65H of one diastereoisomer, *H*-2′), 1.61–1.65 (m, 2H, $COCH_2CH_2$), 1.33–1.29 (m, 8H, 4× CH_2), 0.92-0.86 (m, 9H, –CH_2CH_3, $CH(CH_3)_2$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.72, 175.69 (CONH), 174.09 (d, $^3J_{C-P}$ = 2.7 Hz, CO_2-Bn), 173.77 (d, $^3J_{C-P}$ = 3.7 Hz, CO_2Bn), 164.46 (*C*-4), 152.25 (d, $^2J_{C-P}$ = 7.2 Hz, '*ipso*' *Ph*O), 150.99 (C-2), 144.71, 144.68 (*C*-6), 137.20, 137.13 ('*ipso*' OCH_2*Ph*), 130.91, 130.87, 129.73, 129.67, 129.65, 129.51, 126.44, 126.29 (*Ph*, OCH_2*Ph*), 121.68 (d, $^3J_{C-P}$ = 4.5 Hz, *Ph*O), 121.52 (d, $^3J_{C-P}$ = 5.5 Hz, *Ph*O), 100.28, 100.26 (*C*-5), 90.54, 90.51 (C≡C), 87.42 87.27 (*C*-1′), 87.15, 86.85 (d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 75.59, 75.53 (CH_2C≡C), 72.28, 72.04 (*C*-3′), 68.04, 67.96 (OCH_2-Ph) 67.87 (d, $^2J_{C-P}$ = 5.7 Hz, *C*-5′), 67.65 (d, $^2J_{C-P}$ = 5.4 Hz, *C*-5′), 58.38 (*C*HCH$(CH_3)_2$), 41.40, 41.38 (*C*-2′), 36.97, 36.96 (CH_2CO), 32.91, 30.68, 30.64, 30.32, 30.17, 26.89, 26.87, 23.70 (CH_2NHCO, 6× CH_2), 19.70, 19,64, 18.49, 18,43, 18.06, 14.46 (CH(CH_3)$_2$, CH_3).^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.95, 4.30. MS (ES+) *m*/*z*: 775 (M+Na^+, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 min, flow = 1 mL/min, λ = 280 nm, t_R = 21.23, 21.49 min.

4.2. Biological evaluation

4.2.1. Assay for antimycobacterial activity

M. tuberculosis H37Rv (ATCC27294) was grown in Middlebrook 7H9 broth supplemented with 0.02% (v/v) glycerol, 0.05% (v/v) tween-80 and 10% oleic acid, albumin, dextrose and catalase (OADC; BD Biosciences) as a stand culture at 37 °C. *M. bovis* BCG (ATCC35734) was grown in Middlebrook 7H9 broth supplemented with 0.02% (v/v) glycerol, 0.05% (v/v) tween-80 and 10% albumin, dextrose and catalase (ADC; BD Biosciences) in a roller bottle at 2 rpm at 37 °C. The antimycobacterial activities of the compounds were tested following the HT-SPOTi.[28,29] The high throughput growth inhibition assay was conducted in a semi-automated 96 well plate format as described previously.[26] Briefly, compounds dissolved in DMSO at a final concentration of 250 mg/L were serially diluted and dispensed in a volume of 2 μL into each well of a 96 well plate to which 200 μL of Middlebrook 7H10 agar medium kept at 55 °C supplemented with 0.05% (v/v) glycerol and 10% (*v*/*v*) OADC was added. A well with no compounds (DMSO only) and isoniazid were used as experimental controls. To all the plates, a drop (2 μL) of *mycobacterial culture* containing 2 × 10^3 colony-forming units (CFUs) was spotted in the middle of each well and the plates were incubated at 37 °C for up to two weeks. The minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of the compound investigated where mycobacterial growth was completely inhibited by the presence of the compound.

4.2.2. Assay for antibacterial activity

E. coli DH5α (ATCC53868) was grown in Luria–Bertani broth (Oxoid, Thermoscientific) at 180 rpm as shaking culture with an incubation temperature of 37 °C. The antibacterial activities of the compounds were tested using the HT-SPOTi as mentioned earlier, using appropriate culture medium and growth conditions. The plates were incubated overnight following which observations were recorded.

4.2.3. Assay for antiviral activity

Cells: Human embryonic lung (HEL) fibroblasts were grown in minimum essential medium (MEM) supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 0.3% sodium bicarbonate.

Viruses: The laboratory wild-type varicella zoster virus (VZV) strain Oka, the thymidine kinase-deficient VZV strain 07-1, herpes

simplex virus (HSV-1, KOS), (HSV-2, G), the thymidine kinase-deficient (ACV^R) HSV-1 strain B-2006, human cytomegalovirus (HCMV) strains Davis and AD-169, and vaccinia virus (VV) were used in the virus inhibition assays.

Confluent HEL cell cultures grown in 96-well microtiter plates were inoculated with VZV at an input of 20 plaque forming units (PFU) per well or with HCMV at an input of 100 PFU per well. Confluent HEL cell cultures were inoculated with HSV at 100 $CCID_{50}$ (50% cell culture infective dose) per well. After a 1–2 h incubation period, residual virus was removed and the infected cells were further incubated with MEM (supplemented with 2% inactivated FCS, 2 mM L-glutamine, and 0.3% sodium bicarbonate) containing varying concentrations of the compounds. Antiviral activity was expressed as EC_{50} (50% effective concentration), or compound concentration required to reduce viral plaque formation after 5 days (varicella zoster virus (VZV)) or virus-induced cytopathicity (HCMV after 7 days and HSV, VV after 3 days) by 50% compared to the untreated control.

4.2.4. Assay for eukaryotic cell toxicity

Confluent monolayers of HEL cells as well as growing HEL cells in 96-well microtiter plates were treated with different concentrations of the experimental drugs. Cell cultures were incubated for 3 (growing cells) or 5 (confluent cells) days. At the indicated time, the cells were trypsinized, and the cell number was determined using a Coulter counter (Beckman, Analis, Suarlée, Belgium). The 50% cytostatic concentration (CC_{50}) was defined as the compound concentration required to reduce the cell number by 50%.

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Journal of Medicinal Chemistry

ARTICLE

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Phosphoramidate ProTides of the Anticancer Agent FUDR Successfully Deliver the Preformed Bioactive Monophosphate in Cells and Confer Advantage over the Parent Nucleoside

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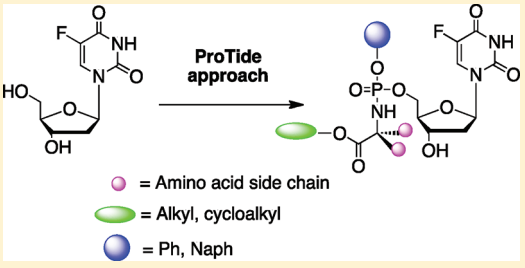
Supporting Information

ABSTRACT: The fluorinated pyrimidine family of nucleosides continues to represent major current chemotherapeutic agents for treating solid tumors. We herein report their phosphate prodrugs, ProTides, as promising new derivatives, which partially bypass the dependence of the current drugs on active transport and nucleoside kinase-mediated activation. They are also resistant to metabolic deactivation by phosphorolytic enzymes. We report 39 ProTides of the fluorinated pyrimidine FUDR with variation in the aryl, ester, and amino acid regions. Notably, only certain ProTide motifs are successful in delivering the nucleoside monophosphate into intact cells. We also find that the ProTides retain activity in mycoplasma infected cells, unlike FUDR. Data suggest these compounds to be worthy of further progression.



INTRODUCTION

Chemotherapeutic agents, based largely on nucleoside analogues, continue to make a major contribution to the current chemotherapy of cancer. One of the first developed derivatives, which is still of major use, is the fluorinated pyrimidine 5-fluorouracil **1** (5-FU) (Figure 1). This drug was first introduced in 1957 by Heidelberger[1] and remains of major value in the treatment of ovary, breast, and gastrointestinal tumors in particular.[2] Besides the free base **1** the agent is also used as its 2′-deoxynucleoside FUDR (**2**) and prodrug capecitabine **3**. The 2′-deoxynucleoside **2** appears to be of particular value against liver metastases, as it is well metabolized in the liver.[3] Capecitabine has an improved ease of administration and may cause less systemic toxicity.[4]

By several metabolic routes each of these agents (**1**−**3**) leads to the generation of the corresponding nucleoside 5′-monophosphate **4** (FdUMP), which is considered to be the primary bioactive entity in this class. FdUMP acts as a potent suicide-type inhibitor of thymidylate synthase, a key enzyme in DNA synthesis, and this leads to a potent toxic event in the cell.[5]

Poor activity of this family of agents in vitro, which has sometimes been observed, and innate or acquired drug resistance in the clinic have been ascribed to several parameters, including reduced levels of the activating enzyme (i.e., thymidine kinase), required to phosphorylate **2** to **4**; overexpression of thymidylate synthase, the target of antitumor action of **4**; increased degradative cleavage of **2** to **1** by thymidine phosphorylase; and reduced transporter-mediated entry of **1** or **2** into cells.[6]

One approach to overcoming the imperative dependence of bioactive nucleoside analogues on kinase-mediated activation is to consider the preformed nucleotide as a clinical entity. However, in general this is not a useful solution because such polar nucleotides are poorly membrane soluble and subject to dephosphorylation.

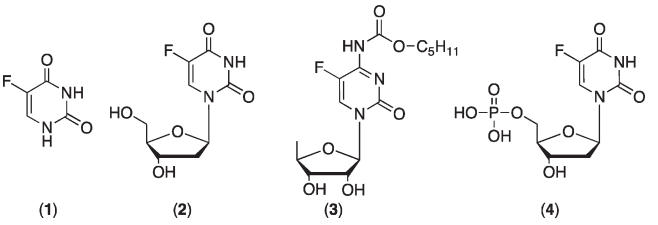


Figure 1. Some fluorinated pyrimidines.

A more successful approach is to mask the monophosphate creating a phosphate prodrug. Several methods exist to achieve this, and they have been reviewed.[7]

We have reported a phosphate prodrug ("ProTide") method, based on aryloxy phosphoramidates.[8] We initially applied this method to the anti-HIV agent d4T[9] and then to several other antivirals including abacavir[10] and more recently some anti hepatitis C virus agents.[11] We[12] and others[13] have also applied the method to the antiherpetic agent BVDU and revealed the interesting introduction of anticancer action of this antiviral compound upon ProTide formation. We have also reported the application of nucleoside ProTides to the antileukemic agent cladribine.[14]

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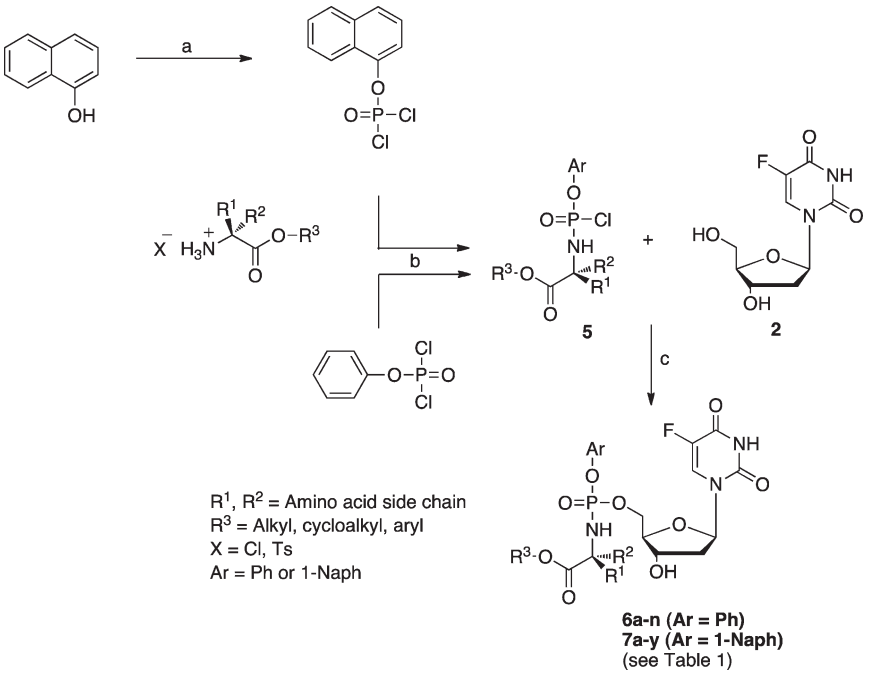
Scheme 1. General Synthesis of FUDR ProTides[a]



[a] Reagent and conditions: (a) $POCl_3$, Et_3N, anhydrous Et_2O, −78 °C for 1 h, then room temp for 1 h; (b) phenyl or 1-naphthyl phosphorodichloridate, Et_3N, anhydrous DCM, −78 °C, 1−3 h; (c) *tert*-BuMgCl (or) NMI, anhydrous THF, room temp, 16−18 h.

Thus, it was of interest to apply the ProTide method to the leading fluorinated pyrimidine family and **2** in particular. On the basis of prior art, we believed that ProTides of **2** could bypass their dependence on cell transporters and also upon thymidine kinase and that the ProTide should be resistant to the degradative pathway from **2** to **1**. We herein report the notable success of this enterprise.

CHEMISTRY

The target ProTides of **2** were prepared using phosphorochloridate chemistry, as we have extensively reported.[15]

One component of the ProTides is an aryl unit, in this case either phenol or 1-naphthol. In the case of phenol, commercial phenyl phosphorodichloridate was used and its purity was checked by ^{31}P NMR prior to use. For the naphthyl analogue, 1-naphthol was allowed to react with phosphoryl chloride in dry diethyl ether in the presence of triethylamine at low temperature to give the required dichloridate (Scheme 1). The second component of the ProTide motif is an esterified amino acid. In some cases these are commercially available, but in most cases they are not and were prepared by esterification of the amino acids using standard methods.[16]

The key reagent to prepare ProTides is the arylaminoacyl phosphorochloridate **5**. These were prepared by allowing the aryl phosphorodichloridate to react with the amino acid ester in dichloromethane at low temperature (Scheme 1). The formation of the key phosphorochloridate was monitored and confirmed by ^{31}P NMR. In some cases the reagent was used crude, and in others it was subjected to rapid silica gel chromatography. Each of the compounds derived from a chiral amino acid was generated as a pair of diastereoisomers at the phosphate center, in roughly 1:1 ratio, as revealed by two closely spaced peaks by ^{31}P NMR.

Finally, each of the phosphorochloridates **5** were allowed to react with FUDR to generate the target ProTides **6a**−**n** (Ar = Ph) and **7a**−**y** (Ar = 1-Naph) in one step as presented in Scheme 1. Two sets of conditions were variously used for this coupling reaction: *N*-methylimidazole in THF or *tert*-butylmagnesium chloride in THF, both at room temperature for 16−18 h. In many cases byproducts with dual phosphorylation at the 3′- and 5′-hydroxyl groups were formed, and in some cases the 3′-mono phosphorylated species was also observed. This required extensive and repeated chromatographic purification of ProTides (**6**, **7**), leading to modest isolated yields. These were not optimized in the present report, since the primary goal was to establish biological activity at this stage.

As noted in Table 1, we varied the aryl unit from phenyl to 1-naphthyl, the amino acid from L-alanine to glycine, valine, leucine, isoleucine, phenylalanine, methionine, proline, and α,α-dimethylglycine and the ester rather extensively. In total 39 ProTides were prepared, purified, and fully characterized. In every case, multiple peaks in ^{31}P and ^{13}C NMR and HPLC confirmed the presence of phosphate diastereoisomers. These were not routinely separated in this study and were tested as mixtures of isomers, since chiral ProTide isomers frequently show rather similar biological profiles. In the great majority of cases, such ProTides progressed to the clinic as mixed diastereomers.[17]

BIOLOGICAL ACTIVITY IN VITRO

The ProTides **6** and **7** described above were tested for their cytostatic activity against several established tumor cell lines, as presented in Table 1. Compounds **1** and **2** were included as positive controls. In particular, we first studied the compounds versus wild type L1210, CEM, and HeLa cells. In each case we also included a thymidine kinase deficient (TK^-) mutant of the parent cell line to probe the effect of TK deficiency on the

Table 1. Cytostatic Activity of 5-FU, FUDR, and FUDR Prodrugs against Tumor Cell Lines

				IC_{50}[a] (μM)					
compd	aryl	ester	AA	L1210/0	L1210/TK$^-$	Cem/0	Cem/TK$^-$	HeLa	HeLa/TK$^-$
1				0.33 ± 0.17	0.32 ± 0.31	18 ± 5	12 ± 1	0.54 ± 0.12	0.23 ± 0.01
2				0.0011 ± 0.0002	3.0 ± 0.1	0.022 ± 0.006	3.0 ± 0.4	0.050 ± 0.011	1.4 ± 0.4
6a	Ph	Me	Ala	0.022 ± 0.007	41 ± 3	0.70 ± 0.37	35 ± 12	0.28 ± 0.14	4.7 ± 0.4
6b	Ph	Et	Ala	0.13 ± 0.04	0.94 ± 0.18	0.92 ± 0.11	14 ± 0	0.48 ± 0.19	9.8 ± 1.4
6c	Ph	*i*-Pr	Ala	0.076 ± 0.022	1.1 ± 0.1	1.0 ± 0.1	30 ± 10	0.71 ± 0.15	25 ± 11
6d	Ph	*c*-Hex	Ala	0.039 ± 0.001	0.14 ± 0.02	0.17 ± 0.07	1.2 ± 0.01	0.18 ± 0.05	5.9 ± 0.4
6e	Ph	Bn	Ala	0.028 ± 0.007	13 ± 8	0.18 ± 0.03	22 ± 7	0.13 ± 0.01	19 ± 2
6f	Ph	Et	Val	0.16 ± 0.05	42 ± 2	1.0 ± 0.1	>250	1.2 ± 0.3	27 ± 7
6g	Ph	Bn	Leu	0.044 ± 0.025	2.0 ± 0.3	0.24 ± 0.04	16 ± 1	0.067 ± 0.042	5.6 ± 0.3
6h	Ph	Bn	Ile	0.076 ± 0.022	1.1 ± 0.1	1.0 ± 0.1	30 ± 10	0.71 ± 0.15	25 ± 11
6i	Ph	Bn	Phe	0.036 ± 0.010	39 ± 4	0.25 ± 0.02	11 ± 3	0.014 ± 0.007	12 ± 2
6j	Ph	Pnt	Met	0.11 ± 0.06	2.2 ± 0.5	0.35 ± 0.13	13 ± 1	0.15 ± 0.00	7.1 ± 1.2
6k	Ph	Bn	Met	0.073 ± 0.035	4.1 ± 1.2	0.28 ± 0.03	25 ± 0	0.15 ± 0.02	11 ± 7
6l	Ph	Bn	Pro	0.35 ± 0.07	31 ± 5	0.98 ± 0.40	28 ± 8	1.1 ± 0.4	20 ± 11
6m	Ph	Et	DMG	0.039 ± 0.001	4.6 ± 0.0	0.65 ± 0.16	22 ± 1	0.59 ± 0.09	17 ± 2
6n	Ph	Bn	DMG	0.017 ± 0.003	0.18 ± 0.05	0.23 ± 0.04	4.8 ± 0.7	0.24 ± 0.07	3.7 ± 0.1
7a	Nap	Et	Ala	0.031 ± 0.005	0.36 ± 0.01	0.25 ± 0.04	1.6 ± 0.2	0.22 ± 0.04	2.8 ± 0.0
7b	Nap	Pr	Ala	0.021 ± 0.012	0.16 ± 0.07	0.14 ± 0.01	1.1 ± 0.2	0.11 ± 0.03	2.5 ± 0.1
7c	Nap	butyl	Ala	0.022 ± 0.004	0.11 ± 0.06	0.064 ± 0.007	0.84 ± 0.60	0.12 ± 0.02	2.7 ± 1.5
7d	Nap	Pnt	Ala	0.0028 ± 0.0010	0.13 ± 0.13	0.015 ± 0.006	0.28 ± 0.04	0.029 ± 0.023	0.44 ± 0.35
7e	Nap	Hex	Ala	0.0072 ± 0.0000	0.076 ± 0.015	0.080 ± 0.020	0.65 ± 0.34	0.039 ± 0.018	1.8 ± 0.1
7f	Nap	*c*-Bu	Ala	0.014 ± 0.003	0.088 ± 0.038	0.073 ± 0.018	1.5 ± 0.3	0.069 ± 0.003	1.5 ± 0.6
7g	Nap	*c*-Pnt	Ala	0.031 ± 0.010	0.13 ± 0.02	0.035 ± 0.025	0.92 ± 0.007	0.071 ± 0.036	2.2 ± 1.3
7h	Nap	*c*-Hex	Ala	0.043 ± 0.023	0.15 ± 0.00	0.057 ± 0.055	1.0 ± 0.1	0.090 ± 0.014	ND
7i	Nap	CH_2-*t*-Bu	Ala	0.27 ± 0.11	1.2 ± 0.7	0.49 ± 0.05	6.7 ± 1.0	0.70 ± 0.11	32 ± 26
7j	Nap	CH_2CH_2-*t*-Bu	Ala	0.016 ± 0.006	0.062 ± 0.009	0.053 ± 0.021	0.19 ± 0.04	0.078 ± 0.018	1.3 ± 0.9
7k	Nap	CH_2-*c*-Pr	Ala	0.017 ± 0.007	0.12 ± 0.06	0.059 ± 0.017	1.1 ± 0.2	0.068 ± 0.001	1.4 ± 0.4
7l	Nap	2-Ind	Ala	0.021 ± 0.002	40 ± 0	0.079 ± 0.018	1.0 ± 0.2	0.10 ± 0.06	7.1 ± 2.1
7m	Nap	Bn	Ala	0.011 ± 0.007	0.045 ± 0.027	0.068 ± 0.035	0.31 ± 0.06	0.065 ± 0.013	2.5 ± 1.3
7n	Nap	THP	Ala	0.038 ± 0.014	27 ± 6	0.11 ± 0.02	43 ± 12	0.13 ± 0.04	15 ± 7
7o	Nap	*c*-Hex	Val	1.1 ± 0.5	35 ± 8	0.80 ± 0.28	46 ± 14	0.67 ± 0.03	27 ± 6
7p	Nap	Pnt	Leu	0.017 ± 0.001	1.2 ± 0.4	0.071 ± 0.008	15 ± 4	0.039 ± 0.014	7.5 ± 0.4
7q	Nap	Bn	Leu	0.028 ± 0.004	1.5 ± 0.6	0.13 ± 0.00	30 ± 6	0.080 ± 0.022	9.4 ± 1.4
7r	Nap	Pnt	Ile	0.22 ± 0.12	12 ± 2	0.46 ± 0.11	17 ± 1	0.30 ± 0.02	11 ± 1
7s	Nap	Pnt	Phe	0.026 ± 0.001	2.9 ± 1.2	0.10 ± 0.00	8.3 ± 1.0	0.040 ± 0.000	6.6 ± 0.5
7t	Nap	Bn	Phe	0.012 ± 0.007	5.6 ± 1.3	0.10 ± 0.03	7.2 ± 0.1	0.16 ± 0.08	6.8 ± 1.5
7u	Nap	Bn	Met	0.072 ± 0.001	1.9 ± 0.2	0.19 ± 0.10	11 ± 1	0.087 ± 0.017	8.3 ± 0.0
7v	Nap	Bn	Pro	0.21 ± 0.08	25 ± 8	0.89 ± 0.35	32 ± 9	1.2 ± 0.0	26 ± 1
7w	Nap	Et	DMG	0.064 ± 0.008	0.82 ± 0.16	0.36 ± 0.05	6.9 ± 1.8	0.20 ± 0.12	3.2 ± 0.0
7x	Nap	Pnt	DMG	0.037 ± 0.010	0.30 ± 0.13	0.14 ± 0.00	5.4 ± 1.1	0.12 ± 0.03	2.3 ± 0.1
7y	Nap	Bn	DMG	0.011 ± 0.005	0.13 ± 0.04	0.16 ± 0.02	2.4 ± 0.8	0.078 ± 0.020	3.1 ± 0.6

[a] IC_{50} or compound concentration required to inhibit tumour cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

cytostatic activity of **1** and **2** and the degree to which the ProTides could bypass this dependence. Previous examples of this type of study have revealed ProTides to be highly efficient at bypassing the dependence on nucleoside kinases.[8,9]

Thus, in 2 of the 3 cell lines studied (i.e., L1210 and HeLa) 5-FU showed activity at ~0.5 μM, being rather poorly active against CEM cells (IC_{50} = 18 μM). Compound **1** largely retained activity in the TK$^-$ cells, indicating that it must be primarily activated to **4** by other metabolic routes such as phosphoribosylation. Activation of 5-FU by phosphoribosylation is catalyzed by the enzyme orotate phosphoribosyl transferase (OPRT) responsible for the direct conversion of the nucleobase to the nucleoside monophosphate.[18,19] On the other hand **2** was more active in the wild-type cell lines, being active at 1−50 nM and thus 10−800 times more potent than 5-FU. But **2** is highly dependent on TK activity, being 30- to 3000-fold less active in the TK-deficient tumor cells than in the parent cell lines. The L1210 cells were particularly striking in this regard. These data clearly show the presence of TK as a prerequisite for **2** to exert

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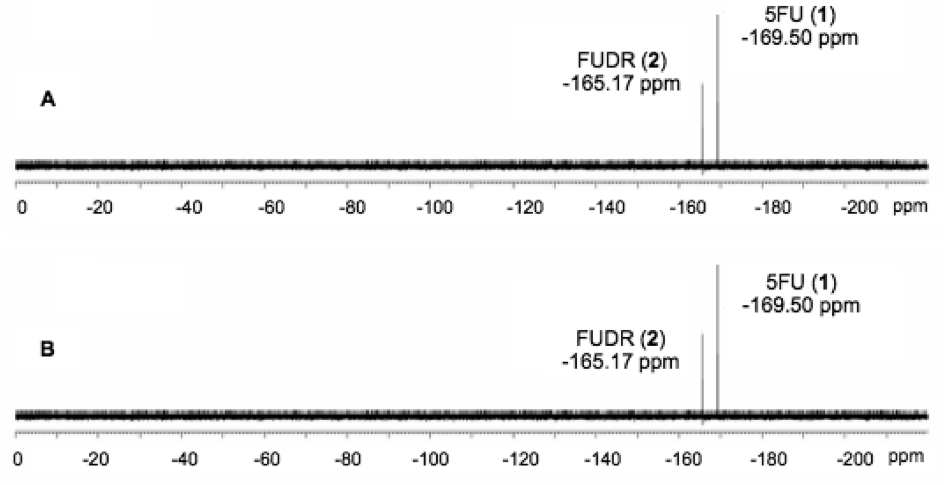


Figure 2. ^{19}F NMR spectra: (A) FUDR and 5-FU in methanol-d_4, 25 °C; (B) FUDR submitted to the thymidine phosphorylase assay, 25 °C, 5 min.

cytostatic activity. The first ProTide prepared, the L-Ala-OMe phenyl derivative **6a**, is approximately 5- to 30-fold less active than the nucleoside **2** against the parent cell lines (Table 1). Moreover and in notable contrast to our prior ProTide work,[8−12] **6a** was very significantly reduced in cytostatic activity against the TK-deficient cells: 2000-fold difference in cytostatic activity against the L1210 wild-type and TK-deficient cells, for example. Although the cytostatic reduction was less in the HeLa cells, the agent still lost significant activity in the absence of thymidine kinase (∼15-fold). These data clearly show that **6a** requires TK to exert biological activity, most probably through efficient liberation of **2** as liberation of **1** should lead to TK-independence according to the data for **1** in Table 1. The notable success of the ProTide approach on other deoxypyrimidine nucleosides such as BVDU[12] makes this especially surprising. However, we have earlier observed the need to optimize the ProTide motif for each nucleoside we have studied,[10,11] and so we varied the ProTide motifs of **6a**. In the first instance we retained the phenyl unit and the L-alanine motif, as the latter in particular often appears to be beneficial.[8] Thus, the methyl ester in **6a** was lengthened to ethyl (**6b**), branched to isopropyl (**6c**), and cyclized to cyclohexyl (**6d**). We also prepared the benzyl analogue (**6e**), which has often been found by us to be a highly preferred ProTide motif.[8] In general each of these esters maintained similar potency in the parent cell lines compared to **6a**. Compound **6d** was also the compound that retained the highest potency in the TK-deficient cells, particularly in L1210/TK$^-$ where it was only 3-fold reduced and thus 21-fold more active than FUDR. Thus, among the family of phenyl FUDR ProTides, **6d** emerged as the most successful compound in our study to date. Interestingly, the "preferred" benzyl compound (**6e**) hardly retained activity in the TK$^-$ cells and thus demonstrated a very low degree of effectiveness as a phosphate prodrug. This highlights the need to optimize and tune the ProTide motif for every nucleoside, as already mentioned above.

We next studied amino acid variation. L-Val-OEt (**6f**), which showed a somewhat similar profile compared to the L-alanine analogues (**6b**), was in general less active as a cytostatic agent. Also, the L-Leu-OBn (**6g**) and L-Ile-OBn (**6h**) were similar to the L-Ala-OBn analogue (**6e**). Reasonably similar data were noted for L-phenylalanine (**6i**), L-methionine (**6j**, **6k**), and L-proline (**6l**) derivatives. In general, the compounds were active at submicromolar concentrations in TK-competent cells but significantly less active in the TK-deficient cells, as FUDR. All of these data demonstrated a poor degree of effectiveness as ProTides. Notably, our generally observed preference for L-alanine was not apparent in this series, and indeed little amino acid SAR could be discerned in contrast to our prior work.[20] Finally in this series we prepared analogues of the actual unnatural amino acid α, α-dimethylglycine as its −OEt (**6m**) and −OBn (**6n**) esters, but these derivatives showed no distinct advantage over earlier analogues.

We have recently reported that in some cases we can achieve a modest potency boost for some ProTides on replacing the phenyl unit by 1-naphthyl.[11,21] Thus, we prepared a series of 25 naphthyl ProTides **7a**−**y** with variation in the amino acid and ester moieties. In general, each of the naphthyl analogues was more potent than its phenyl equivalent across the range of cell lines. However, by comparison to the phenyl series **6a**−**n**, the naphthyl family tended to display a more significant retention of activity in the TK$^-$ panel of tumor cell lines. Compounds emerging as most potent in L1210/TK$^-$, for example, were the L-Ala-OHex (**7e**) and L-Ala-OBn (**7m**) and also an extended L-alanine ester (**7j**). By comparison and in marked contrast to the phenyl series here, the usual amino acid SARs emerged with L-alanine strongly preferred. Naphthyl ProTides of other amino acids were all significantly more dependent on TK for their cytostatic activity as demonstrated in L1210/TK$^-$ cells. Thus, a number of naphthyl L-Ala ProTides with a variety of esters emerged as reasonably effective in bypassing the TK dependence of FUDR. The L-Ala-OBn (**7m**) and L-Ala-OPnt (**7d**) derivatives were among the most potent, being active at an IC_{50} of 11 and 2.8 nM in L1210, respectively. Thus, compounds **7m** and **7d** were only 10-fold and 2.5-fold less active than the parent FUDR but 30 times and 100 times more active than 5-FU, respectively. In L1210/TK$^-$, compound **7m** retained significant cytostatic potency, being only 5-fold reduced, versus FUDR which was 3000-fold diminished, whereas compound **7d** has shown a 40-fold reduction of cytostatic activity in L1210/TK$^-$. The data were less dramatic for CEM/0 versus CEM/TK$^-$ cells but conveyed the same message. These data are in marked contrast to our prior phenyl/naphthyl comparisons where the replacement only caused modest increases in potency.

Although the data on the cytostatic activity of several ProTides of FUDR against a variety of tumor cell lines look interesting, one should be aware that all data were obtained from in vitro testing

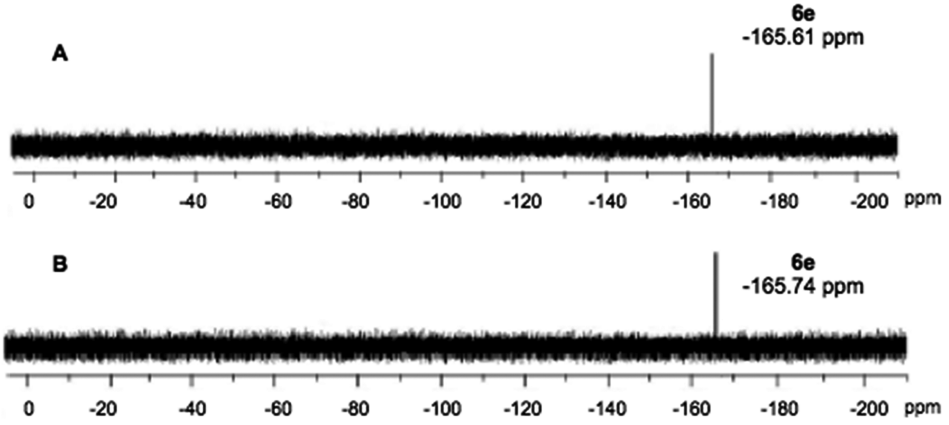


Figure 3. ^{19}F NMR spectra of compound **6e** in phosphorylase assay: (A) **6e** in the absence of the enzyme (TP), 25 °C; (B) **6e** submitted to the action of thymidine phosphorylase (TP), spectra recorded after 72 h, 25 °C.

assays. Whereas the parent drug FUDR has a proven efficacy in in vivo animal models and in cancer patients, the in vivo efficacy of the FUDR ProTides need still to be established . In this respect, phosphoramidate prodrugs of acyclic nucleoside phosphonates have been shown to be quite effective in vivo. Therefore, such types of prodrug may well be efficacious in vivo when applied on FUDR as well. Experiments to demonstrate efficacy of the FUDR ProTides in a mouse model are under consideration.

As was previously noted, FUDR can be degraded to its nucleobase 5-fluorouracil in a phosphorolytic reaction catalyzed by thymidine phosphorylase (TP). This breakdown has been suggested to be one of the reasons for the limited therapeutic effectiveness of FUDR.[22] Therefore, in order to investigate the susceptibility of our FUDR ProTides to phosphorolysis, we have performed an enzymatic phosphorylase assay. This assay was carried out for comparison for both FUDR and one of our first synthesized model compound **6e**, using TP (purified from *Escherichia coli*) in the presence of potassium phosphate buffer (300 mM solution, pH 7.4). A potential phosphorolysis reaction and hence potential formation of 5-FU were monitored by in situ ^{19}F NMR experiments. Thus, we first recorded the individual ^{19}F NMR spectra of FUDR and 5-FU (spectra not shown) and the additional ^{19}F NMR spectra of compounds **2** and **1** together (Figure 2A). The single peak at δ_F −165.17 is assigned to the nucleoside **2**, whereas the single peak at δ_F −169.50 ppm is assigned to the nucleobase **1**. The phosphorylase assay was then carried out by dissolving **2** in methanol-d_4 in the presence of potassium phosphate buffer and recording the blank ^{19}F NMR spectrum prior to addition of the enzyme (spectra not shown). A single peak at δ_F −165.17 ppm was observed. After 5 min from the addition of thymidine phosphorylase (20.7 UNI), the ^{19}F NMR spectrum (Figure 2B) revealed the appearance of an additional peak at δ_F −169.50 ppm due to the release of 5-FU from the FUDR. Two single peaks with the same chemical shifts as have been observed for the first experiment were found; therefore, these data might confirm that the signal at δ_F −169.50 ppm can be assigned to 5-FU which was formed upon phosphorolytic action of the enzyme in the assay.

The ^{19}F NMR spectrum of compound **6e** under conditions of the phosphorolysis assay (Figure 3B) was recorded after 5 min, 14 h, and 72 h and did not show any evidence of phosphorolysis at these three time points. These experiments confirmed that, in contrast to nucleoside **2**, the ProTide **6e** is at best a very poor, if any, substrate for thymidine phosphorylase.

We have recently reported that mycoplasma infection of cells can significantly alter the metabolism of nucleoside analogues, partly through the expression of mycoplasma-derived enzymes such as TP.[23] FUDR is known to be subject to TP-mediated deactivation, and we thus expected that **2** would be less cytostatic in the presence of mycoplasmas. If ProTides were able to deliver bioactive **4** directly and were resistant to TP, then they may also be less subject to mycoplasma-induced catabolic degradation. We present the data from such a study in Table 2. Interestingly, whereas the cytostatic activity of FUDR was heavily compromised in mycoplasma infected cells (a drop of cytostatic activity by 378-fold was observed), the prodrugs generally kept a significant cytostatic activity under similar experimental conditions. In general, the 1-naphthyl prodrug derivatives (**7**) markedly kept their cytostatic potential in the mycoplasma-infected tumor cell cultures. They often lost only 2- to 3-fold antiproliferative activity (Table 2).

Thus, we herein demonstrate that the ProTides of FUDR, in contrast with the parent nucleoside, are resistant to the phosphorolytic activity of mycoplasma-encoded thymidine phosphorylase but also cellular phosphorylases. This property may give the ProTides of FUDR a therapeutic edge when exposed not only to mycoplasma-infected tumor tissue but also to any TP-expressing tumor in general. It is indeed well-known that tumors often show an increased TPase activity to allow a better angiogenesis in the tumor tissue. Such activity should result in an increased rate of hydrolysis (inactivation) of parent FUDR that is a known substrate for TPase[23] but should not affect the FUDR ProTides, shown to be resistant to this phosphorolytic cleavage.

The eventual cytostatic activity of FUDR also highly depends on its efficient transport into the tumor cells. Both FUDR and FdUMP show a 60- to 70-fold decreased cytostatic activity against CEM cells that lack the hENT1 transporter (designated Cem/hEnt-0) (Table 3). Importantly, the FUDR prodrugs proved to be less dependent on the presence of the hENT1 transporter, since they lost only 7- to 15-fold antiproliferative activity against the hENT1-deficient CEM cells. These observations are in agreement with an only 2- to 7-fold decreased cytostatic activity of the ProTides in the presence of transport inhibitors (i.e., dipyridamole and NBMPR), compared to a 20- to 60-fold loss of antiproliferative activity of FUDR and FdUMP under similar experimental conditions.

With the aim of investigating the chemical and enzymatic stability of FUDR ProTides to ester hydrolysis under biologically relevant conditions, we performed several stability studies at

Table 2. Cytostatic Activity of FUDR and FUDR Prodrugs in Wild Type Murine Leukemia L1210 Cell Cultures (L1210/0) and L1210 Cell Cultures, Infected with *Mycoplasma hyorhinis* (L1210.Hyor)

				IC_{50}[a] (μM)		
compd	aryl	ester	AA	L1210/0	L1210.Hyor	IC_{50}(L1210.Hyor)/IC_{50}(L1210/0)
2				0.0009 ± 0.0003	0.34 ± 0.13	378
6a	Ph	Me	Ala	0.040 ± 0.016	0.87 ± 0.28	22
6b	Ph	Et	Ala	0.11 ± 0.0021	0.54 ± 0.12	5
6c	Ph	*i*-Pr	Ala	0.050 ± 0.013	0.70 ± 0.10	14
6d	Ph	*c*-Hex	Ala	0.032 ± 0.0050	0.040 ± 0.016	1.25
6e	Ph	Bn	Ala	0.026 ± 0.008	0.15 ± 0.006	5.8
6f	Ph	Et	Val	0.20 ± 0.033	4.4 ± 1.1	22
6g	Ph	Bn	Leu	0.054 ± 0.0021	0.17 ± 0.047	3.2
6h	Ph	Bn	Ile	0.98 ± 0.39	2.2 ± 0.031	2.2
6i	Ph	Bn	Phe	0.016 ± 0.0014	0.56 ± 0.023	35
6j	Ph	Pnt	Met	0.13 ± 0.0078	0.41 ± 0.21	3.2
6k	Ph	Bn	Met	0.058 ± 0.035	0.76 ± 0.18	13
6l	Ph	Bn	Pro	0.35 ± 0.022	18 ± 0.71	51
6m	Ph	Et	DMG	0.030 ± 0.0005	0.26 ± 0.01	8.7
6n	Ph	Bn	DMG	0.029 ± 0.001	0.02 ± 0.002	0.69
7a	Naph	Et	Ala	0.028 ± 0.0021	0.095 ± 0.0028	3.4
7b	Naph	Pr	Ala	0.030 ± 0.00035	0.036 ± 0.0064	1.2
7c	Naph	butyl	Ala	0.0095 ± 0.0021	0.021 ± 0.0071	2.2
7d	Naph	Pnt	Ala	0.0021 ± 0.00007	0.006 ± 0.0014	2.9
7e	Naph	Hex	Ala	0.0032 ± 0.00035	0.0022 ± 0.00028	0.69
7f	Naph	*c*-Bu	Ala	0.011 ± 0.0014	0.024 ± 0.00014	2.2
7g	Naph	*c*-Pnt	Ala	0.016 ± 0.0007	0.024 ± 0.005	1.5
7h	Naph	*c*-Hex	Ala	0.036 ± 0.017	0.049 ± 0.004	1.4
7i	Naph	CH_2-*t*-Bu	Ala	0.093 ± 0.033	0.18 ± 0.069	1.9
7j	Naph	CH_2CH_2-*t*-Bu	Ala	0.012 ± 0.0018	0.032 ± 0.0088	2.7
7k	Naph	CH_2-*c*-Pr	Ala	0.014 ± 0.0042	0.031 ± 0.0064	2.2
7l	Naph	2-Ind	Ala	0.039 ± 0.019	0.042 ± 0.040	1.08
7m	Naph	Bn	Ala	0.011 ± 0.009	0.025 ± 0.01	2.27
7n	Naph	THP	Ala	0.041 ± 0.0028	0.48 ± 0.11	11.7
7o	Naph	*c*-Hex	Val	1.2 ± 0.17	1.29 ± 0.29	1.08
7p	Naph	Pnt	Leu	0.031 ± 0.0020	0.035 ± 0.010	1.13
7q	Naph	Bn	Leu	0.029 ± 0.0021	0.048 ± 0.020	1.7
7r	Naph	Pnt	Ile	0.42 ± 0.021	0.70 ± 0.074	1.67
7s	Naph	Pnt	Phe	0.030 ± 0.0039	0.14 ± 0.007	4.67
7t	Naph	Bn	Phe	0.021 ± 0.0061	0.23 ± 0.078	11
7u	Naph	Bn	Met	0.054 ± 0.013	0.20 ± 0.098	3.7
7v	Naph	Bn	Pro	0.26 ± 0.055	0.65 ± 0.070	2.5
7w	Naph	Et	DMG	0.056 ± 0.04	0.17 ± 0.03	3
7x	Naph	Pnt	DMG	0.045 ± 0.0021	0.019 ± 0.0028	0.42
7y	Naph	Bn	DMG	0.019 ± 0.004	0.045 ± 0.004	2.4

[a] IC_{50} or compound concentration required to inhibit tumor cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

different pH values, in the presence of human serum and carboxypeptidase Y.

A chemical hydrolysis of L-Ala-OBn phenyl ProTide **6e** was evaluated under experimental conditions at pH 1 and pH 8 and monitored by ^{31}P NMR. During the assay (14 h) under acidic conditions (pH 1) only two peaks representing two diastereoisomers of **6e** were recorded (Figure 4). Lack of formation of new signals in the ^{31}P NMR spectrum indicates that the tested compound **6e** is highly stable in acidic medium. The same result was observed when the ProTide **6e** was subjected to the assay under mild basic conditions (pH 8; data not shown).

In order to explore whether FUDR ProTides can be activated via our putative mechanism,[24,25] we carried out an enzymatic study using a carboxypeptidase Y assay following the protocol already described.[26] As depicted in Figure 5, the mechanism of activation of phosphoramidates begins with the hydrolysis of the carboxylic ester moiety (a) hypothesized to be mediated by a carboxyesterase-type enzyme to give the intermediate **8**. In the

Table 3. Cytostatic Activity of FUDR and Several FUDR Prodrugs in CEM Cell Cultures Containing (Cem/hEnt-1) or Lacking (Cem/hEnt-0) the hEnt1 Transporter

				IC_{50} [a] (μM)			
compd	aryl	ester	AA	Cem/hEnt-1	Cem/hEnt-0	Cem/hEnt-1 + dipyridamole	Cem/hEnt-1 + NBMPR
5-FdUMP				0.05 ± 0.02	3.6 ± 0.69	1.74	1.06
2				0.04 ± 0.02	2.5 ± 0.65	1.36	0.80
6e	Ph	Bn	Ala	0.13 ± 0.05	1.4 ± 0.65	0.66	0.72
6m	Ph	Et	DMG	0.37 ± 0.14	5.8 ± 0.50	2.35	2.56
6n	Ph	Bn	DMG	0.17 ± 0.06	1.2 ± 0.11	0.26	0.61
7m	Naph	Bn	Ala	0.05 ± 0.02	0.6 ± 0.11	0.13	0.26
7w	Naph	Et	DMG	0.21 ± 0.07	1.4 ± 0.20	0.52	0.62
7y	Naph	Bn	DMG	0.05 ± 0.03	0.4 ± 0.13	0.16	0.28

[a] IC_{50} or compound concentration required to inhibit tumor cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

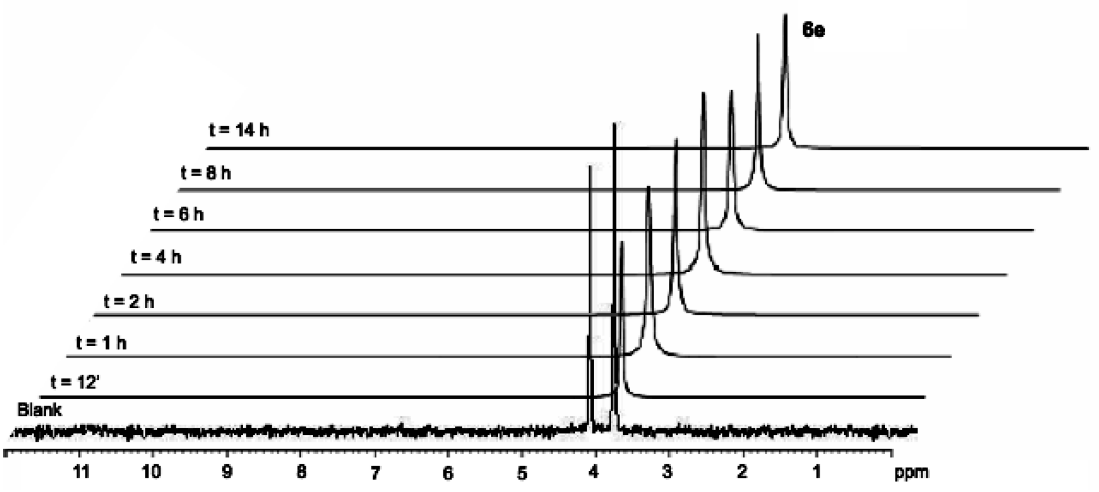


Figure 4. Convoluted and deconvoluted ^{31}P NMR specta of phosphoramidate **6e** (buffer, pH 1).

second step (b) a spontaneous cyclization occurs through an internal nucleophilic attack of the carboxylate residue on the phosphorus center following a displacement of the aryl moiety to yield **9**. The third step (c) is the opening of the unstable cyclic mixed anhydride mediated by water with the release of the intermediate **10**, which upon the cleavage of the P−N bond (d) mediated by a hypothesized phosphoramidase-type enzyme gives the corresponding monophosphate **4**. Among our large family of FUDR ProTides, the enzymatic assay was applied to one of our lead compounds **7d**. Thus, the L-Ala-OPnt naphthyl derivative **7d**, carboxypeptidase Y, and Trizma buffer (pH 7.6) were dissolved in acetone-d_6 and ^{31}P NMR (202 MHz) spectra were recorded at regular intervals (every 7 min) over 14 h (Figure 6). According to the results, the parent ProTide **7d** (represented as two signals at δ_P 4.03 and 4.31 ppm) was rapidly hydrolyzed to the first metabolite **8** lacking the ester moiety shown in the ^{31}P NMR spectrum at δ_P 4.99 and 5.13 ppm. Noteworthy, both diastereoisomers of **7d** were processed at roughly similar rate. A further processing of **8** led to the formation of metabolite **10** shown as a single peak at δ_P 6.82 ppm. During the enzymatic process, compound **7d** was fully converted to the metabolite **10** within approximately 45 min with an estimated half-life of less than 5 min. In fact, this assay showed that the rate of the initial activation step might be considered in general as one of requirements for good biological activity of phosphoramidates. In order to support the proposed putative mechanism and the results from the enzymatic assay, the intermediate **10** was prepared via a synthetic route (Scheme 2). Therefore, chemical hydrolysis of compound **7m** in the presence of triethylamine and water was performed. Product **10′** obtained as a diammonium salt was then added to the final assay sample **7m** (containing only the enzymatic metabolite **10** in Trizma). Its ^{31}P NMR spectrum has exclusively shown one peak at δ_P 6.85 ppm, strongly supporting this part of the metabolic pathway and activation of the ProTides.

The stability of the prodrug **7a** in the presence of human serum was investigated using in situ ^{31}P NMR. The aim of this experiment was to identify the formation of any metabolites of **7a** (Figure 5), which would appear as new peaks in the ^{31}P NMR spectrum. Thus, after the first (control) ^{31}P NMR data of **7a** in DMSO and D_2O were recorded, the NMR sample was treated with human serum (0.3 mL) and immediately subjected to further ^{31}P NMR experiments at 37 °C. The ^{31}P NMR data were recorded every 15 min over 14 h and are reported in Figure 7. In order to improve a visualization of results, all the spectra were further processed using the Lorentz−Gauss deconvolution method. The spectra displayed a single peak inherent to the human serum at ~δ_P 2.00 ppm and two

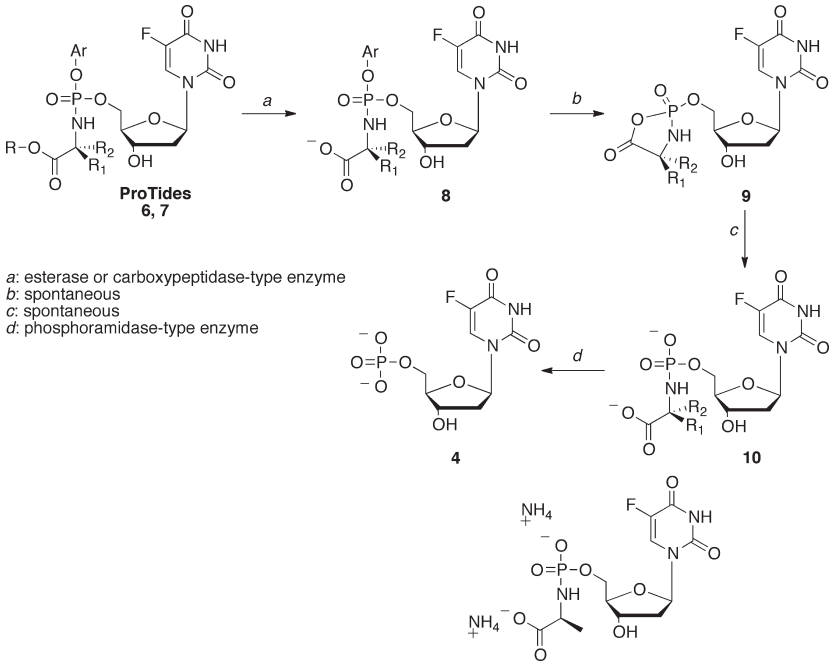


Figure 5. Proposed activation pathway of FUDR ProTides.

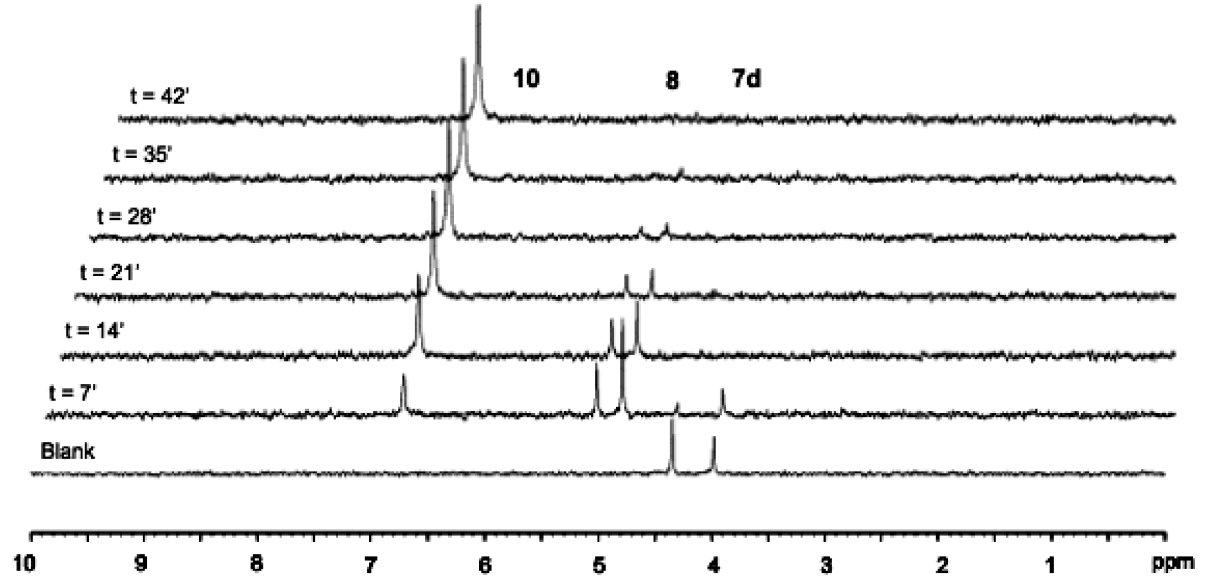
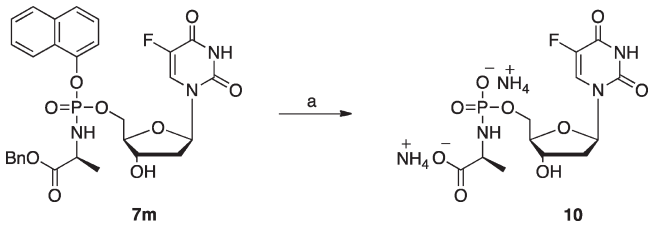


Figure 6. Carboxypeptidase Y assay applied on ProTide **7d** and monitored by ^{31}P NMR, 25 °C.

Scheme 2. Synthesis of Intermediate 10[a]



[a] Reagents and conditions: (a) Et_3N/H_2O (1:1), 35 °C, 16 h.

peaks corresponding to **7a** at ~δ_P 4.59 and 4.84 ppm. After about 6 h and 45 min the compound was hydrolyzed partly to the intermediate **8** shown as a single peak at δ_P 5.79 ppm. After 11 h and 30 min, the formation of the second metabolite **10** shown as a single peak at δ_P 7.09 ppm was observed. After 13 h and 30 min the reaction mixture contained 96% of the parent compound **7a** together with the proposed metabolites **8** (3%) and **10** (1%).

CONCLUSIONS

We herein report the successful application of ProTide technology to the anticancer agent FUDR. Several ProTides emerged that

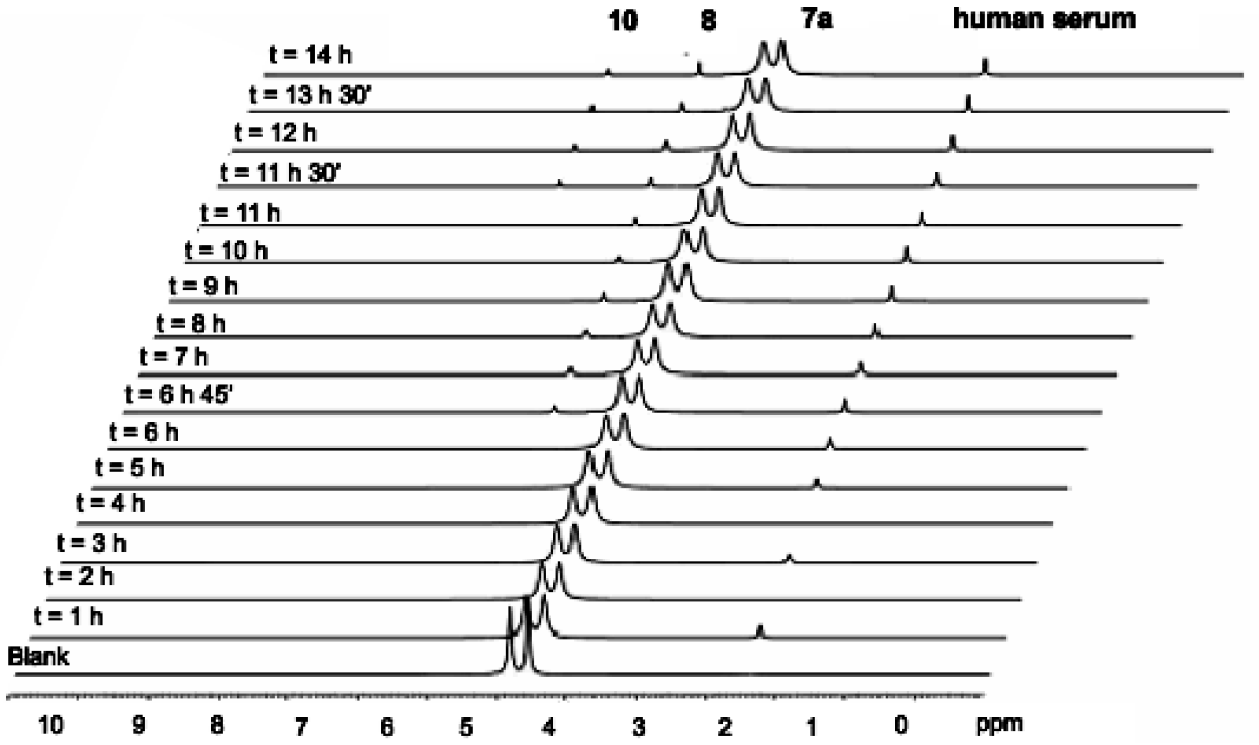


Figure 7. Human serum assay applied on ProTide **7a** and monitored by ^{31}P NMR, 37 °C.

retain the high potency of FUDR in vitro and in addition partially bypass the high dependence of the parent nucleoside on kinase-mediated activation and on cell transporter-mediated uptake.

The compounds are also resistant to thymidine phosphorylase and do not show significant loss of activity as displayed by FUDR upon mycoplasma infection of the tumor cell cultures. The ProTides are stable in acid and at neutral pH and in plasma but are activated by intracellular carboxypeptidase. The ability of the ProTides to overcome several of the sources of resistance of FUDR in the clinic suggests that these agents should be further progressed to (pre)clinical trials.

EXPERIMENTAL SECTION

Cell Cultures and Cytostatic Assays. Murine leukemia L1210/0, human T-lymphocyte CEM/0, and human cervix carcinoma HeLa/0 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Thymidine kinase deficient CEM/TK$^-$ cells were a kind gift from Prof. S. Eriksson (currently at Uppsala University, Uppsala, Sweden) and Prof. A. Karlsson (Karolinska Institute, Stockholm, Sweden), and CEM/hENT-0 samples were obtained from Prof. Cass (Cross Cancer Institute, Edmonton, Alberta, Canada). Thymidine kinase deficient L1210/TK$^-$ and HeLa/TK$^-$ cells were derived from L1210/0 and HeLa/0 cells, respectively, after selection for resistance against 5-bromo-2′-dUrd. The HeLa/TK$^-$ cells were kindly provided by Prof. Y.-C. Cheng, Yale University, New Haven, CT. Infection of the cell lines with *Mycoplasma hyorhinis* (ATCC) resulted in chronically infected cell lines further referred to as L1210.Hyor. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), 10 mM Hepes, and 1 mM sodium pyruvate (Invitrogen). Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO_2.

Monolayer cells (HeLa/0 and HeLa/TK$^-$) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark) at 10 000 cells/well. After 24 h, an equal volume of fresh medium containing the test compounds was added. On day 5, cells were trypsinized and counted in a Coulter counter (Analis, Suarlée, Belgium). Suspension cells (L1210/0, L1210/TK$^-$, L1210.Hyor, CEM/0, CEM/TK$^-$, CEM/hEnt-1, CEM/hEnt-0) were seeded in 96-well microtiter plates (Nunc) at 60 000 cells/well in the presence of a given amount of the test compounds. The cells were allowed to proliferate for 48 h (L1210) or 72 h (CEM) and were then counted in a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce cell proliferation by 50%. In the nucleoside transporter inhibition experiments, dipyridamole (10 μM) and NBMPR (10 μM) were added to the CEM/hEnt-1 cells in the presence of different concentrations of the test compounds. The cytostatic activity of the compounds was determined after 3 days, as outlined above.

Phosphorylase Assay Using Thymidine Phosphorylase Purified from *Escherichia coli*. The experiment was carried out by dissolving FUDR ProTide **6e** (6.0 mg) in methanol-d_4 (0.05 mL), followed by addition of 300 mM potassium phosphate buffer (pH 7.4, 0.45 mL). The resulting cloudy solution was submitted to the ^{19}F NMR experiment at 25 °C, and the data were recorded as a control. Then to that sample was added thymidine phosphorylase (17 μL). The resulting sample was submitted for ^{19}F NMR experiment. Additional ^{19}F NMR experiments for the same sample was repeated after 72 h.

^{31}P NMR Stability Experiments in Acidic and Basic pH. *Buffer pH 1.* The stability assay toward hydrolysis by aqueous buffer at pH 1 was conducted using in situ ^{31}P NMR (202 MHz). The experiment was carried out by dissolving FUDR ProTide **6e** (2.6 mg) in methanol-d_4 (0.10 mL) and then adding buffer, pH 1 (prepared from equal parts of 0.2 M HCl and 0.2 M KCl). Next, the sample was subjected to ^{31}P NMR experiments at 37 °C and the spectra were recorded every 12 min over 14 h.

Buffer pH 8. The stability assay toward hydrolysis by aqueous buffer at pH 8 was conducted using in situ ^{31}P NMR (202 MHz). The experiment was carried out by dissolving FUDR ProTide **6e** (4.5 mg) in methanol-d_4 (0.10 mL) and then adding buffer, pH 8 (prepared from solution of 0.1 M Na_2HPO_4 and adjusted to the appropriate pH using 0.1 M HCl).

Next, the sample was subjected to ^{31}P NMR experiments at 37 °C and the spectra were recorded every 12 min over 14 h.

Carboxypeptidase Y (EC 3.4.16.1) Assay. The experiment was carried out by dissolving FUDR ProTide **7d** (3.0 mg) in acetone-d_6 (0.15 mL) and by adding 0.30 mL of Trizma buffer (pH 7.6). After the ^{31}P NMR data were recorded at 25 °C as a control, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 mL of Trizma) was added to the sample. Next, the sample was submitted to ^{31}P NMR experiments (at 25 °C) and the spectra were recorded every 7 min over 14 h. ^{31}P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

Stability Assay in Human Serum. The experiment was carried out by dissolving FUDR ProTide **7a** (5.0 mg) in DMSO (0.050 mL) and D_2O (0.15 mL). After the ^{31}P NMR data were recorded at 37 °C as a control, a previously defrosted human serum (0.30 mL) was added to the sample. Next, the sample was submitted to ^{31}P NMR experiments at 37 °C and the spectra were recorded every 15 min over 14 h. ^{31}P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

Chemistry. General. Anhydrous solvents were obtained from Aldrich and used without further purification. Amino acid esters were purchased from Carbosynth. Carboxypeptidase Y, human serum, and buffers were from Sigma-Aldrich. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35−70 μM). Proton (^{1}H), carbon (^{13}C), phosphorus (^{31}P), and fluorine (^{19}F) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all ^{13}C NMR and ^{31}P NMR were proton-decoupled. The purity of final compounds was verified to be >95% by HPLC analysis using Varian Polaris C18-A (10 μM) as an analytic column with a gradient elution of H_2O/MeOH from 100/0 to 0/100 in 45 min (method 1) and with a gradient elution of H_2O/CH_3CN from 100/0 to 0/100 in 35 min (method 2). The HPLC analysis was conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector). Low and high resolution mass spectra were performed as a service by Birmingham University, Birmingham, U.K., using electrospray mass spectrometry (ESMS). CHN microanalysis was performed as a service by MEDAC Ltd., Surrey, U.K.

General Method for the Preparation of Phosphorochloridates (5). Anhydrous triethylamine (2.0 mol equiv) was added dropwise at −78 °C to a stirred solution of the appropriate aryl dichlorophosphate (1.0 mol equiv) and an appropriate amino acid ester (1.0 mol equiv) in anhydrous DCM under argon atmosphere. Following the addition, the reaction mixture was allowed to slowly warm to room temperature and was stirred for 1−2 h. The formation of desired compound was monitored by ^{31}P NMR. After the reaction was completed, the solvent was evaporated under reduced pressure and the resulting residue was redissolved in anhydrous Et_2O and filtered. The filtrate was reduced to dryness to give a crude product as an oil, which was in some cases used without further purification in the next step. Most of aryl phosphorochloridates, in particular those obtained from the amino acid tosylate salt, were purified by flash column chromatography using EtOAc/hexane (7:3) as an eluent.

1-Naphthyl (Benzyl-L-alaninyl)phosphorochloridate (5m). Yellowish oil; yield, 47% (1.82 g). R_f = 0.90 (hexane−EtOAc, 7:3). ^{31}P NMR (202 MHz, $CDCl_3$, mixture of diastereoisomers): δ_P 7.92, 8.14 (int, 1.00:1.00). ^{1}H NMR (500 MHz, $CDCl_3$, mixture of diastereoisomers with a ratio of 1:1): δ_H 8.12−7.97 (m, 1H, Ar*H*), 7.73−7.09 (m, 11H, Ar*H*), 5.09 (s, 2H, OCH_2Ph), 4.81−4.78 (m, 1H, N*H*), 4.23−4.20 (m, 1H, C*H*CH$_3$), 1.45−1.43 (m, 3H, CHCH_3).

General Method for the Preparation of FUDR ProTides (6a−n and 7a−y). To a solution of 5-fluoro-2′-deoxyuridine (0.25 g, 1.01 mmol) in dry THF (10 mL) at 0 °C under argon atmosphere was added dropwise NMI (0.40 mL, 5.07 mmol). The reaction mixture was allowed to stir for 30 min, and then a solution of appropriate phosphorochloridate (**5**) (3.04 mmol) dissolved in anhydrous THF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16−18 h and then evaporated in vacuo to give a residue that was redissolved in CH_2Cl_2 and washed twice with 0.5 M HCl (2 × 5 mL). The organic phase was purified by column chromatography on silica gel, eluting with CH_2Cl_2−MeOH as a gradient (0−5% MeOH) to afford the products as white solid.

5-Fluoro-2′-deoxyuridine 5′-*O*-[1-Naphthyl(benzyl-L-alaninyl)] phosphate (7m). **7m** was obtained from 5-fluoro-2′-deoxyuridine and **5m** as a white solid. Yield, 8% (47.0 mg). R_f = 0.19 (CH_2Cl_2−MeOH, 95:5). (ES+) *m*/*z*, found: (M + Na$^+$) 636.1520. $C_{29}H_{29}N_3O_9FNaP$ required: (M$^+$), 613.15. Mixture of diastereoisomers (43%, 57%). ^{31}P NMR (202 MHz, MeOD): δ_P 4.61, 4.25. ^{19}F NMR (470 MHz, MeOD): δ_F −167.45, −167.25. ^{1}H NMR (500 MHz, MeOD): δ_H 8.18−8.12 (m, 1H, Ar*H*), 7.90−7.86 (m, 1H, Ar*H*), 7.72−7.67 (m, 2H, Ar*H*, *H*-6), 7.55−7.47 (m, 3H, Ar*H*), 7.45−7.27 (m, 6H, Ar*H*), 6.16−6.06 (m, 1H, *H*-1′), 5.13, 5.08 (2 × AB system, 2H, *J* = 12.0 Hz, OCH_2Ph), 4.36−4.24 (m, 3H, 2 × *H*-5′, *H*-3′), 4.15−4.03 (m, 2H, C*H*CH$_3$, *H*-4′), 2.17−2.08 (m, 1H, *H*-2′), 1.79−1.67 (m, 1H, *H*-2′), 1.38−1.34 (m, 3H, CHCH_3). ^{13}C NMR (125 MHz, MeOD): δ_C 174.9 (d, $^3J_{C-P}$ = 4.3 Hz, *C*═O, ester), 174.6 (d, $^3J_{C-P}$ = 5.0 Hz, *C*═O, ester), 159.3 (d, $^2J_{C-F}$ = 26.1 Hz, *C*═O, base), 150.5 (d, $^4J_{C-F}$ = 4.0 Hz, *C*═O, base), 147.9 (d, $^2J_{C-P}$ = 7.4 Hz, *C*-Ar, Naph), 147.8 (d, $^2J_{C-P}$ = 7.7 Hz, OC-Naph), 141.7, 141.6 (2d, $^1J_{C-F}$ = 234.0 Hz, *C*F-base), 137.2, 137.1, 136.2 (*C*-Ar), 129.7, 129.6, 129.5, 129.4, 129.0, 128.9, 128.1, 128.0 (*C*H-Ar), 127.9, 127.8 (*C*-Ar), 127.7, 127.6, 126.6, 126.5, 126.2 (*C*H-Ar), 125.6, 125.5 (2*d*, $^2J_{C-F}$ = 34.0 Hz, *C*H-base), 122.6 (*C*H-Ar), 116.5, 116.2 (2d, $^3J_{C-P}$ = 3.5 Hz, *C*H-Ar), 87.0, 86.9 (*C*-1′), 86.8, 86.7 (2d, $^3J_{C-P}$ = 8.1 Hz, *C*-4′), 72.1, 72.0 (*C*-3′), 68.1, 68.0 (CH_2Ph), 67.8, 67.6 (2d, $^2J_{C-P}$ = 5.2 Hz, *C*-5′), 51.9, 51.8 (CHCH$_3$), 40.9, 40.8 (*C*-2′), 20.5 (d, $^3J_{C-P}$ = 6.5 Hz, CHCH$_3$), 20.3 (d, $^3J_{C-P}$ = 7.6 Hz, CHCH$_3$). Reverse HPLC, eluting with H_2O/MeOH from 100/0 to 0/100 in 45 min, showed two peaks of the diastereoisomers with t_R = 34.23 min and t_R = 34.59 min (47%, 51%). Anal. Calcd for $C_{29}H_{29}FN_3O_9P$: C, 56.77; H, 4.76; N, 6.85. Found: C, 56.57; H, 5.06; N, 6.72. UV (0.05 M phosphate buffer, pH 7.4) λ_{max} = 271 nm (ε_{max} = 7050). log *P* measured: 1.74.

5-Fluoro-2′deoxyuridine 5′-*O*-[(L-Alaninyl)]phosphate Ammonium Salt (10′). 5-Fluoro-2′deoxyuridine 5′-*O*-[1-naphthyl(benzyl-L-alaninyl)]phosphate (**7m**) (0.08 g, 0.130 mmol) was dissolved in a solution of triethylamine (5 mL) and water (5 mL). The reaction mixture was stirred at 35 °C for 16 h, and then the solvents were removed under reduced pressure. The residue was treated with water and extracted with dichloromethane. The aqueous layer was concentrated and evaporated under reduced pressure. Then the resulting crude material was purified by column chromatography on silica, eluting with 2-propanol−H_2O−NH_3 (8:1:1) to afford the title compound **10′** as a white solid. Yield, 30% (15.0 mg). R_f = 0.04 (2-propanol−H_2O−NH_3 (8:1:1)). ^{31}P NMR (202 MHz, D_2O): δ_P 7.13. ^{19}F NMR (470 MHz, D_2O): δ_F −168.00. ^{1}H NMR (500 MHz, D_2O): δ_H 7.93 (d, 1H, $^3J_{H-F}$ = 6.1 Hz, *H*-6), 6.30−6.25 (m, 1H, *H*-1′), 4.49−4.44 (m, 1H, *H*-3′), 4.11−4.06 (m, 1H, *H*-4′), 3.94−3.83 (m, 2H, *H*-5′), 3.53 (q, 1H, *J* = 7.5 Hz, C*H*CH$_3$), 2.37−2.28 (m, 2H, *H*-2′), 1.25−1.19 (m, 3H, CHCH_3). ^{13}C NMR (125 MHz, MeOD): δ_C 174.8 (d, $^3J_{C-P}$ = 4.6 Hz, *C*═O), 159.2 (d, $^2J_{C-F}$ = 26.2 Hz, *C*═O, base), 150.3 (d, $^4J_{C-F}$ = 4.0 Hz, *C*═O, base), 141.8 (d, $^1J_{C-F}$ = 233.8 Hz, *C*F-base), 125.6 (d, $^2J_{C-F}$ = 34.0 Hz, *C*H-base), 87.0 (*C*-1′), 86.7 (d, $^3J_{C-P}$ = 7.5 Hz, *C*-4′), 71.1 (*C*-3′), 67.2 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5′), 51.0 (CHCH$_3$), 40.2 (*C*-2′), 20.3 (d, $^3J_{C-P}$ = 7.2 Hz, CHCH$_3$). *m*/*z* (ES) 396.1 (M − 2 NH$_4^-$ + H]$^-$, 100%). Reverse-phase HPLC, eluting with H_2O/MeOH from 100/0 to 0/100 in 45 min, 1 mL/min, λ = 275 nm, showed a peak of the diastereoisomer with t_R = 3.65 min (95%).

■ ASSOCIATED CONTENT

Supporting Information. Preparative methods and spectroscopic and analytical data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

TLC, thin layer chromatography; FUDR, 2′-deoxy-5-fluorouridine; TP, thymidine phosphorylase; TK^-, thymidine kinase deficient; OPRT, orotate phosphoribosyl transferase; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CEM, human T-lymphocyte; NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine; ESMS, electrospray mass spectrometry; HPLC, high performance liquid chromatography; ClogP, calculated logarithm of the octanol/water partition

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