Cardiff School of Pharmacy and Pharmaceutical Science
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Design, Synthesis and Biological Evaluation of Nucleoside Phosphoramidates with potential Anticancer Activity

A thesis submitted for the degree of
Philosophiae Doctor in Cardiff University

by
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I wish to express my sincere appreciation to those who have contributed to this thesis and supported me in one way or the other during this amazing journey.

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A special thanks to my family and their encouragement during the times of struggle and success. Words cannot express my gratitude to Simon, with whom I shared the last part of this adventure, for his unconditional support friendship and love.

Thank you

One in seven approved anticancer drugs in the UK are nucleoside analogues (NA). However, frequent development of resistance and unpredictable toxicity are crucial drawbacks of these compounds. Some of the main resistance mechanisms against NAs include limited cellular permeability and decreased initial phosphorylation of the NAs, thus limiting the concentration of active NAs inside the target cells. The ProTide approach is a pronucleotide technology that successfully overcomes these drawbacks by releasing the monophosphorylated NA into the cell and has led to multiple clinical candidate drugs.

This work was focussed on the application of the ProTide approach to different anticancer NAs with the aim of improving their performance and pharmacological properties. These NAs were **2-chlorocordycepin**, **3'-ethynylnucleosides** and the novel NA **3-N^4-ethenogemcitabine**.

A new and efficient synthesis for **2-chlorocordycepin** was developed, and three synthetic routes to obtain its ProTides were explored. This family of compounds was then enlarged by developing ProTide-related nucleoside phosphorodiamidates and a nucleoside phosphonodiamidate of 2-chlorocordycepin.

The family of **3'-ethynylnucleosides** was explored next. The synthesis of 3'-ethynyluridine and 3'ethynylcytidine was optimised and implemented for 3'-ethynyl-5-methyluridine. In addition, a small family of novel ProTides for each of these 3'-ethynylnucleosides was prepared.

Finally, **3-N^4-ethenogemcitabine**, a novel NA originating from the fusion of gemcitabine and 3,N^4-deoxycytidine, was designed and synthesised. The ProTide approach was then applied to create a family of pronucleotides that was also expected to be fluorescent.

2-Chlorocordycepin and 3'-ethynyl-5-methyluridine ProTides showed a clear improvement of the *in vitro* anti-cancer activity compared to the respective parent nucleosides. Enzymatic assays and docking studies were performed to gain understanding of the pronucleotides' activation processes. Finally, the fluorescence

properties of the novel nucleoside analogue 3-N^4-Ethenogemcitabine and its ProTides were characterised.

2-ClCordy	2-Chlorocordycepin
4-ClBz	4-Chlorobenzoyl
4-ClBzCl	4-chlorobenzoyl chloride
5-FU	5-Fluorouracil 5-FU
5,10-CH2-THF	5,10-Methylenetetrahydrofolate
5'-NT	5'-Nucleotidase
aa-ester	Amino acid ester
ADA	Adenosine deaminases
AIBN	α,α'-Azobisisobutyronitrile
Ar	Aryl residues
ATP	Adenosine triphosphate
AZT	3'-azidothymidine
Benz or Bz	Benzyl
BOC	*tert*-Butyloxycarbonyl
BSA	bis(Trimethylsilyl)acetamide
CH≡CMgBr	Ethynyl magnesium bromide
cHex	Cyclohexyl
CNT	Concentrative nucleoside transporters
CSA	Camphor sulfonic acid
DBU	1,8-Diazabicycloundec-7-ene
DCM	Dichloromethane
ddCyd	2', 3'-Dideoxycytidine
dFdU	2',2'-Difluorodeoxyuridine
DIPEA	Diisopropylethylamine
DMAP	Dimethylamino)pyridine

DMF	*N,N*-dimethylformamide
DMP	Dess Martin Periodinane
DMTr	4,4'-Dimethoxytrityl
dUMP	Deoxyuridine monophosphate
EC50	Half maximal effective concentration
ECyd	3'-Ethynylcytidine
ECyd-TP	ECyd-5'-triphosphate
EMUrd	3'-Ethynyl 5-methyluridine
ENT	Equilibrative nucleoside transporters
EtMgBr	Ethylmagnesiumbromide
EUrd	3'-Ethynyl uridine
EUrd-TP	EUrd-5'-triphosphate
FDA	Food and drug Administration
FUDR	5-Fluoro deoxyribouracile
Gem-DP	Gemcitabine diphosphate
Gem-TP	Gemcitabine triphosphate
Hex	Hexyl
Hint	Human triad nucleotide-binding proteins
HMDS	Hexamethyldisilazane
IBX	2-Iodoxybenzoic acid
iPr	Isopropyl
KHMDS	Potassium hexamethyldisilazide
L-Ala	*L*-Alanine
NA	Nucleoside and Nucleotide analog
Naph	Naphthyl
Neop	Neopentyl

NMI	N-methylimidazole
NOESY	Nuclear Overhauser enhancement spectroscopy
NTP	Nucleotide triphosphate
Ph	Phenyl
POC	Diisopropyloxycarbonyloxymethyl
POM	Pivaloyloxymethyl
ppm	Parts per million
RR	Ribonucleotide diphosphate reductase
SAR	Structure-activity relationship
SATE	S-acyl-2-thioethyl
TBDMS	*tert*-Butyldimethylsilyl
*t*BuMgBr	*tert*-Butyl magnesium chloride
TdT	Terminal deoxynucleotidyl Transferases
TEA	Triethylamine
TEMPO	2,2,6,6-Tetramethylpiperidinyl-1-oxy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TI	Top Inhibition
TMP	Thymidine monophosphate
TMS	Trimethylsilyl
TMSTf	trimethylsilyl triflate
TP	5'-Triphosphate
TS	Thymidylate synthase
UCK	Uridine-cytidine kinase
WHO	World Health Organisation
αAIBBr	α-Acetoxyisobutyryl bromide

εC	3,N^4-Ethenocytidine
εdC	3,N^4-Ethenodeoxycytidine
εGem	3,N^4-Etheno-gemcitabine

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1. Introduction

Nucleotide analogues in the treatment of cancer

In 2013, cancer caused 29% of all deaths registered in England and Wales.[1] In the previous year, the World Health Organisation (WHO) estimated 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer within 5 years of diagnosis worldwide.

Cancer is a collective term used for a group of diseases characterised by the loss of control of the growth, division, and spread of a group of cells, which leads to a primary tumour that invades and destroys adjacent tissues. The primary tumour may also spread to other regions of the body through metastasis, which causes 90% of cancer associated deaths.[2] Major treatment modalities include surgery, radiotherapy and chemotherapy.[3] However, other new strategies such as immunotherapy, thermal therapy, photodynamic therapy, and stem cell transplants, have emerged recently.[4]

Nucleosides and nucleotides are endogenous compounds involved in several cellular processes. Their key role is DNA and RNA synthesis, cell signalling, enzyme regulation and metabolism.[5] They take part in essential processes, including energy provision (ATP), protein synthesis and cell replication.

Nucleoside and Nucleotide analogues (NA) are chemically modified compounds developed to mimic their physiological counterparts.[5] Currently their main applications exploit their antiviral and anti-cancer activity. The British National Formulary shows 14 NAs among the 92 approved drugs used in anti-cancer therapy (*Table 1.1*).[6] Some of these NA are core to cancer chemotherapy and considered crucial to modern human healthcare. Indeed, cytarabine (approved in 1969), is included in the WHO's List of Essential Medicines.[7]

Table 1.1 NA approved in the UK as anti-cancer agents

Drug	Indication
Azacytidine	Chronic myelomonocytic leukaemia and acute myeloid leukaemia.
Capecitabine	Adjuvant treatment following surgery for stage III (Dukes' C) colon cancer.
Cladribine	Hairy cell leukaemia and chronic lymphocytic leukaemia in patients who have failed to respond to standard regimens containing an alkylating agent.
Clofarabine	Acute lymphoblastic leukaemia.
Cytarabine	Induction of remission of acute myeloblastic leukaemia.
Decitabine	Newly diagnosed acute myeloid leukaemia.
Floxuridine	Gastrointestinal cancer that has spread to the liver.
Fludarabine	Advanced B-cell chronic lymphocytic leukaemia, or after first-line treatment in patients with sufficient bone-marrow reserves.
5-Fluorouracil	Solid tumours, including gastro-intestinal tract cancers and breast cancer.
Gemcitabine	Pancreatic and metastatic breast cancer.
Mercaptopurine	Acute leukaemia and chronic myeloid leukaemia.
Nelarabine	T-cell acute lymphoblastic leukaemia and T-cell lymphoblastic lymphoma.
Tegafur	Advanced gastric cancer.
Thioguanine	Acute leukaemia and chronic myeloid leukaemia.

Anti-cancer NAs are designed to interfere with the function of endogenous nucleosides by exploiting the same metabolic pathways. They can potentially target any of the involved enzymes or biochemical processes, from the cellular uptake through nucleoside transporters to the incorporation into nucleic acids, including inhibition of enzymes that participate in essential biosynthetic processes. This leads to a modification of the cell's normal function and often triggers apoptosis.[8] It should be noted that even if cell apoptosis is the usual target for NAs, each compound has its own metabolic and pharmacological properties and mechanism or mechanisms of action.[2]

Most often, anti-cancer NAs are inactive prodrugs that depend on intracellular phosphorylation to reach their nucleoside phosphate forms (nucleotides), most frequently triphosphates, which are the active species with biological effects.[9]

There are two processes for the cell to acquire nucleotides: the *de novo* synthesis pathway and the salvage pathway. The *de novo* pathway involves the synthesis of ribonucleotides from small molecules. Ribonucleotides can then be transformed into deoxyribonucleotides by reduction of the 2'-hydroxyl group by ribonucleotide reductase (RR). The salvage pathway is the transport of nucleosides into the cell and their subsequent phosphorylation (Figure 1.1). NAs exploit both pathways to exert their intracellular effects.

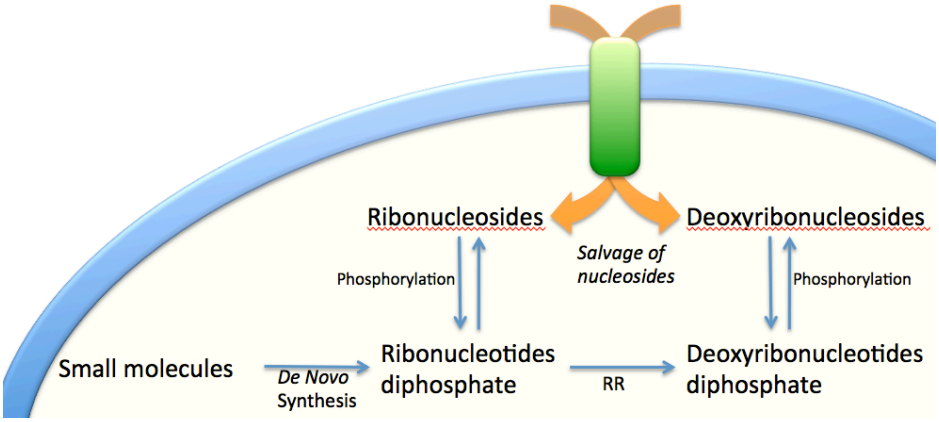


Figure 1.1 *Pathways by which nucleotides and deoxyribonucleotides inside the cell.*

The salvage pathway depends on the specific nucleoside transporters, specifically concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs). CNTs perform an energy dependant (active) transport of nucleosides into the cell. ENTs carry out a facilitated transport where nucleosides and nucleobases move along their concentration gradient between the inside and outside of the cell.[10]

Once the nucleosides are inside the cell they undergo a first phosphorylation step at position 5' of the sugar moiety which is catalysed by ribo- or deoxyribonucleoside kinases. These enzymes only catalyse the conversion of the nucleoside into the mono-phosphorylated nucleotide. This step is counteracted by 5' triphosphorylated nucleotides (the final metabolites) that exert a negative feedback

in the enzyme, and by 5' nucleotidase (5'-NT), which cleaves the monophosphate moiety, thus again releasing the free nucleoside.

Second and third nucleotide phosphorylations are reversible phosphorotransferase reactions performed by nucleotide monophosphate kinase and nucleotide diphosphate kinase, to obtain the active nucleotide triphosphate (NTP).

When NA are administrated, they compete with their endogenous counterparts for their activation to the triphosphate form (Figure 1.2). Triphosphate NAs exert their actions by being incorporated into nucleic acids in competition with the endogenous ones, by inhibiting the enzymes such as polymerases that catalyse the synthesis of DNA and RNA, or by negative feedback regulation of the enzymes involved in the synthesis of endogenous nucleotide triphosphates.

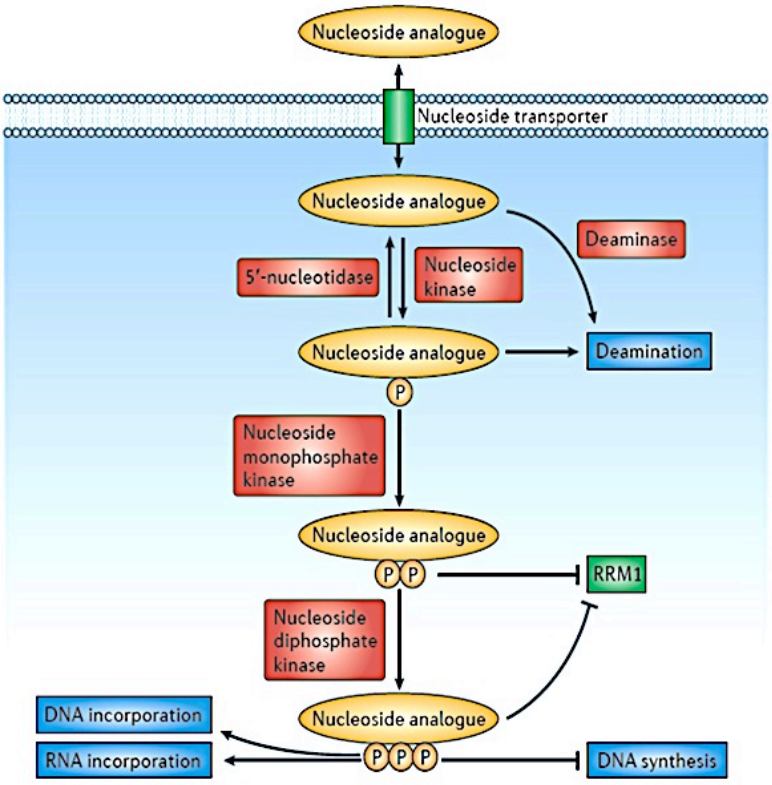


Figure 1.2 *Mechanism of activation of most NAs (Figure by Jordheim et al., 2013).*[5] *Reprinted by permission from Macmillan Publishers Ltd: Jordheim et al. Nat Rev Drug Discov. 2013, 12 (6) 447-64, copyright 2013.*

Weaknesses of NAs as anticancer drugs

Despite the numerous approved anti-cancer NAs, some of their pharmacological characteristics still need to be improved. The main issues are the development of resistance and side effects such as delayed unspecific toxicity.

Resistance to the treatment may involve different processes such as drug inactivation or not activation of the drug, drug target alteration (i.e point mutations), drug efflux, failure to sufficiently alter nucleic acid or NTP pools, DNA damage repair or defective induction to apoptosis.[9,11]

Resistance of NA therapy linked to insufficient intracellular concentration of the triphosphate species of the NA is largely reported. This is due to either inefficient cellular uptake of the NA, decreased activity levels of activating enzymes (kinases), increased catabolism (5'-NT or deaminases), or expansion of nucleoside triphosphate pools (retroregulation).[9]

As mentioned above, ENT or CNT transporters are responsible for the cellular uptake of NAs. These channels are generally substrate specific.[10] Some NAs are not recognised at all by these transporters while others may only be poorly transported. In addition, several studies postulate that resistance to some NA anticancer drugs is linked to a down-regulation of CNTs.[10]

NA phosphorylation, which is inhibited by a negative feedback loop via the final triphosphates as well as countermanded by 5'-NTs, is another rate limiting step, where NAs have to compete with the natural nucleosides and are potentially worse substrates for the enzymes than their natural counterparts. These enzymes are also substrate specific, so NAs may not even be recognised and the crucial initial mono phosphorylation step of the NA cannot take place. In addition, some cancer cells can down-regulate nucleoside kinases or up-regulate 5'-NTs as a resistance mechanism.[12]

Pronucleotide approach

The development of new NAs is being driven by the need to overcome resistance and efficacy issues. This means that the NA has to achieve sufficient concentration of its active form inside the cell to efficiently induce apoptosis. Some developed strategies to achieve this goal include the synthesis of new NAs with changes on their sugar or base moieties, liposomal formulations, orally administrated formulations, and nucleotide prodrugs: pronucleotides.[5]

Strategies aiming to increase the concentration of active 5'-NA triphosphates inside the cell seek to bypass the two major limiting steps: transport-dependent intracellular uptake, and the first phosphorylation step. Pronucleotides are designed to get inside of the cell through passive diffusion across the membrane, and, once inside, to release the monophosphorylated NA. Therefore, no transporters or activation by nucleoside kinases will be required as illustrated in Figure 1.3.

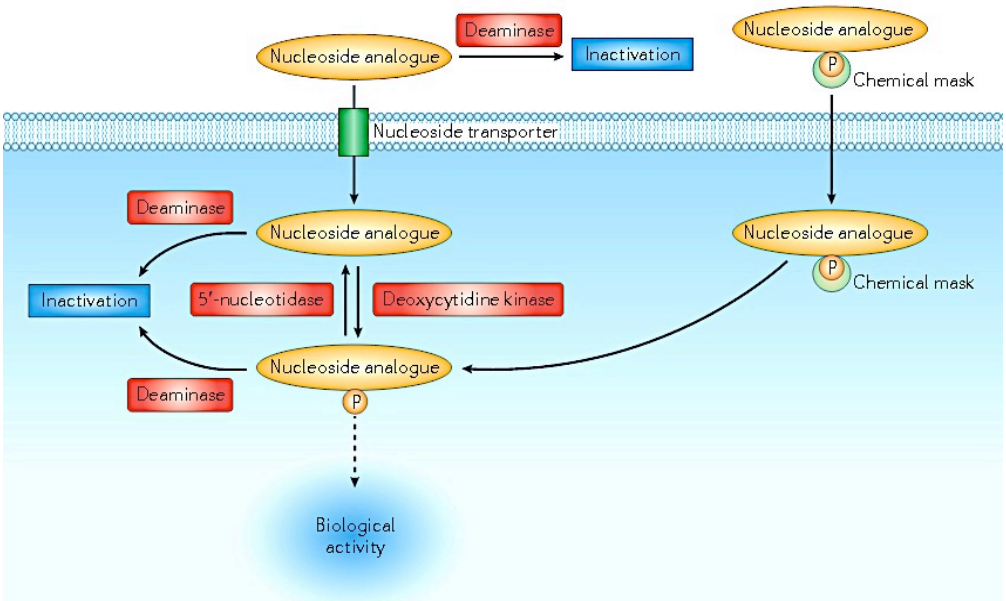
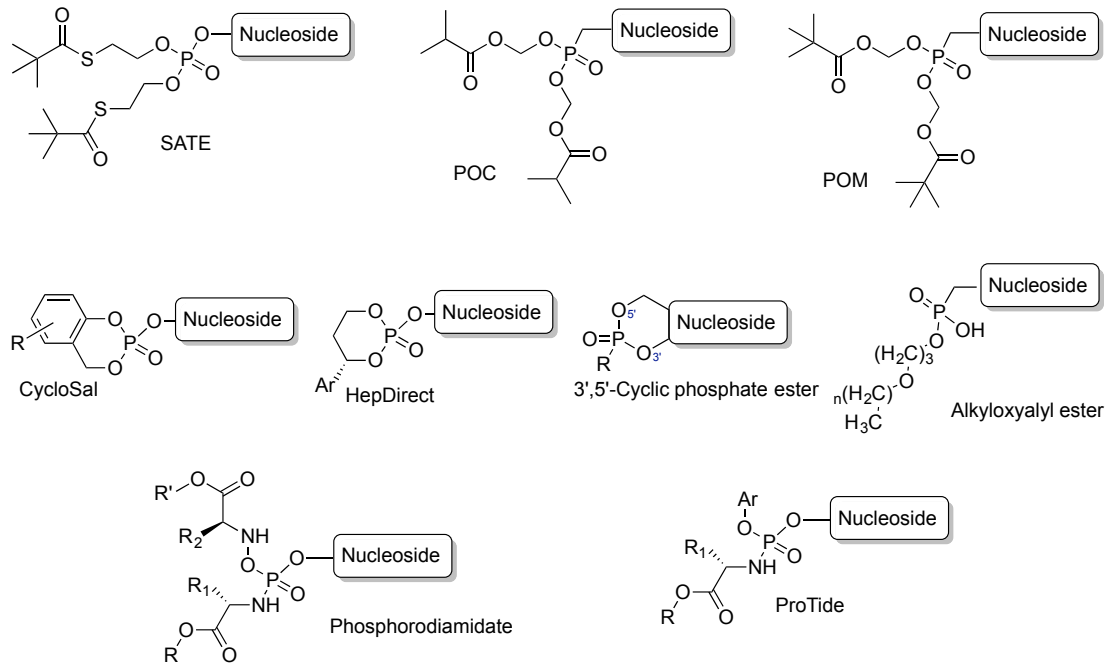


***Figure 1.3**: Pronucleotide approach. (Figure by Jordheim et al., 2013).[5] Reprinted by permission from Macmillan Publishers Ltd: Jordheim et al. Nat Rev Drug Discov. 2013, 12(6)447-64, copyright 2013.*

To achieve this capability for passive diffusion, pronucleotides need to mask the hydrophilicity of the NA monophosphate. Usually they bear groups attached to the hydroxyls of the phosphate moiety (which in its free form is deprotonated and thus highly polar at physiological pH). This chemical mask needs to be cleaved inside

the cell to release the monophosphorylated species.[5] Some of the pronucleotide strategies are described below (Scheme 1.1).

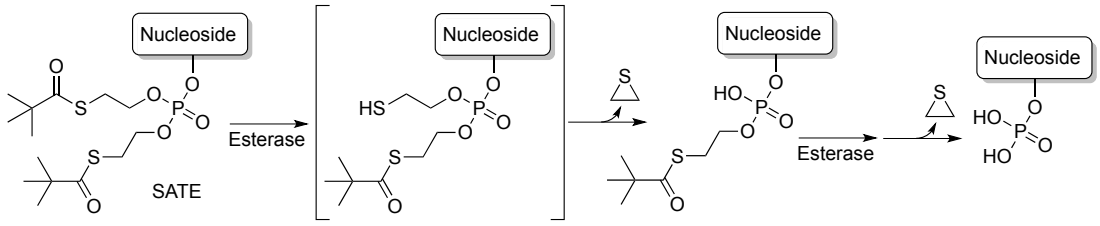


Scheme 1.1 *General structure of some important pronucleotides*

SATE

The SATE approach consists of symmetric pronucleotides bearing S-acyl-2-thioethyl (SATE) groups as esterase-labile phosphate protecting groups. This strategy has been applied to phosphate and phosphonate drugs, especially in antiviral therapy. Some studies for the treatment of hepatitis B virus with derivatives of acyclovir showed better results of the SATE analogue than the parent nucleoside[13]. In its phosphonate form it was applied to adefovir for treatment of HIV, leading to better activity than the parent nucleoside.[14]

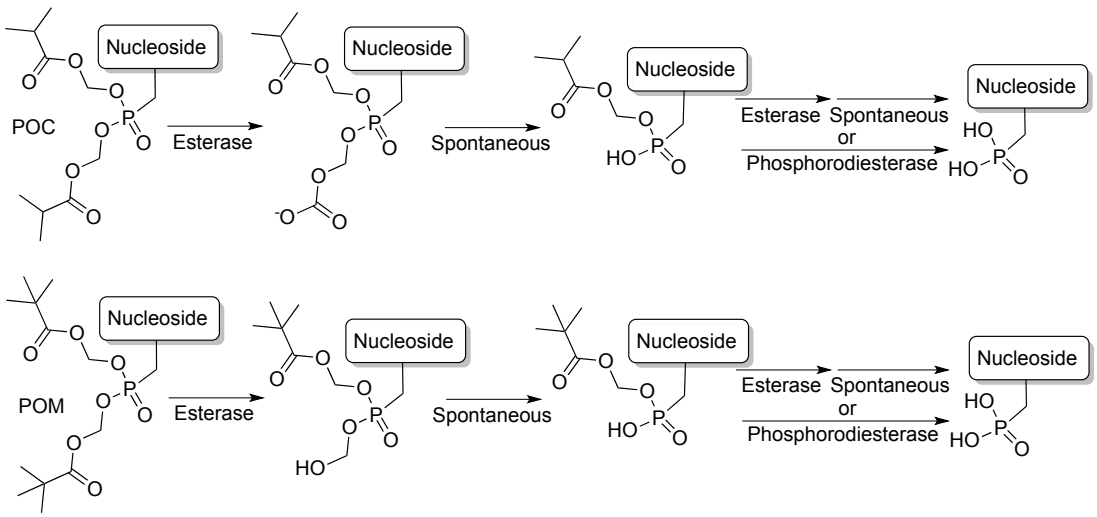
The moieties of SATE pronucleotides are hydrolysed by nonspecific esterases, leading to an unstable 2-thioethyl intermediate. This intermediate is then quickly cleaved, releasing the 2-thioethyl moiety as an episulfide as well as the mono SATE compound. The process is then repeated to release the monophosphorylated NA (Scheme 1.2).[15] However, episulfide, the byproduct formed in the activation process was proven to be toxic.[16]



Scheme 1.2 *Mechanism of activation of SATE pronucleotides*

Bis-POM and Bis-POC

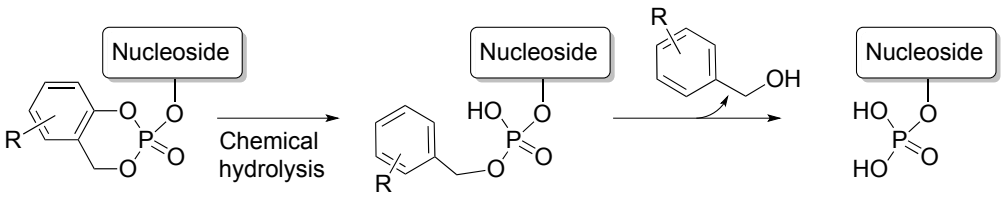
The POM and POC pronucleotide strategies use bis (pivaloyloxymethyl) ester (POM) or diisopropyloxycarbonyloxymethyl ester (POC) groups to mask the phosphate. This strategy was successfully applied to nucleoside phosphonate prodrugs, leading to the approved agents Hepsera (bis-POM of adefovir) and Viread (bis-POC of tenofovir), both of which are used for the treatment of hepatitis B infections. They get enzymatically activated by unspecific esterases. Despite the structural similarity Bis-POC and Bis-POM follow slightly different activation pathways. When bis-POC interacts with an esterase, the terminal isopropyl is cleaved, leading to a carboxylate intermediate that delivers the mono-POC compound by chemical rearrangement. In contrast, in the esterase-mediated activation of POM, the entire terminal ester is cleaved (pivalic acid), after which rearrangement delivers the mono-POM compound. The mono POC and mono POM compounds then either undergo an activation second cycle or are being cleaved by phosphodiesterase enzymes, leading to the delivery of the monophosphate compound (Scheme 1.3).[17]



Scheme 1.3 *Mechanism of activation of bis-POC pronucleotides*

CycloSal

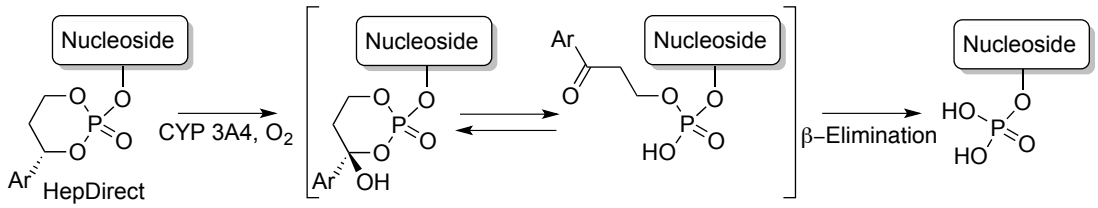
In the CycloSal approach, the phosphate is masked with a salicyl alcohol in a cyclic bifunctionality that integrates the two hydroxyl residues in the phosphate group. The release of the NA inside the cell is driven by pH: in acid conditions (pH < 7),[18] the P-O bond at 5' of the nucleoside is broken, opening the cycle and followed by spontaneous C-O cleavage of the salicyl alcohol (Scheme 1.4). [19] Changes in the aromatic ring have led to a second generation of the pronucleotide, cycloSal-triesters, which have potential applications as antiviral agents.[20] This approach was also applied to acyclic nucleoside phosphonates for targeting HIV.[14]



Scheme 1.4 *Mechanism of activation of CycloSal*

HepDirect

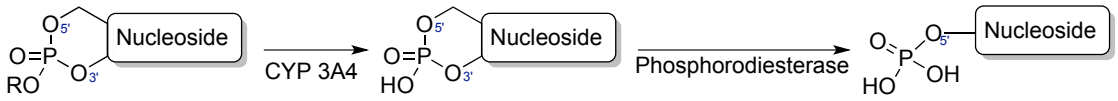
The HepDirect prodrug modifies the NA monophosphate by introducing cyclic 1,3-propanyl esters in the phosphate moiety. The activation of this prodrug is accomplished via cytochrome P450-catalyzed oxidation, resulting in the intracellular release of monophosphate NAs in hepatocytes (Scheme 1.5).[21] This approach was designed to specifically target hepatotropic virus infections. HepDirect of 2'-*C*-methylcytidine (a viral polymerase inhibitor) increased the concentration of the active inhibitory species in Hepatitis C virus infected hepatocytes.[22] The HepDirect approach was also applied to cytarabine, leading to MB-07133 for the treatment of hepatocellular carcinoma[23]. It recently completed phase I/II clinical trials,[24] and was given orphan drug status by the United States Food and Drug Administration (FDA).[25] This approach was also applied to the phosphonate drug adefovir leading to pradefovir, which underwent phase II clinical trials in 2012 for the treatment of hepatitis B infection but no results of the study are posted.[26]



Scheme 1.5 *Mechanism of activation of HepDirect pronucleotides*

3',5'-Cyclic phosphate ester

In the 3',5'-Cyclic phosphate ester strategy the phosphate group at 5' of the nucleotide is forming a 6-membered ring with the 3' hydroxyl group of the nucleotide. In this way, one of the hydroxyl groups in the phosphate group is being masked. The remaining hydroxyl group of the phosphate moiety is bound to an alkyl moiety. The prodrug is activated by dealkylation of the phosphate group via cytochrome P450. Then, a phosphodiesterase enzyme releases the hydroxyl group at position 3' (Scheme 1.6). This strategy led to PSI-352938 that underwent phase I clinical trials for the treatment of hepatitis C infections.[17]

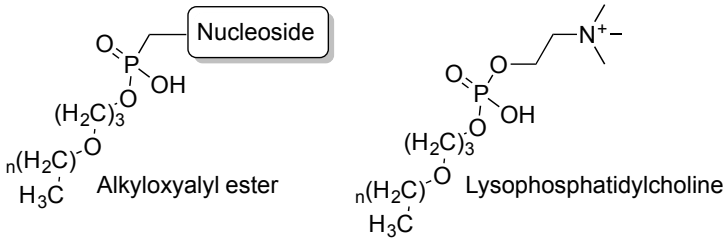


Scheme 1.6 *Mechanism of activation of 3', 5'-cyclyc phosphate ester pronucleotides*

Alkoxyalkyl phosphate mono ester

This strategy aims to introduce the NAs into the cell by mimicking lysophosphatidylcholine, replacing the choline of the natural substrate by the nucleotide analogue (Scheme 1.7). The prodrug is being intracellularly released as monophosphate NA by the action of lysophospholipase C.[17] Of note, unlike the previously reported approaches, only one hydroxyl group of the phosphate is masked. This approach led to CMX 157, a prodrug of tenofovir for the treatment of Hepatitis B which is currently undergoing phase 2 clinical studies,[27] and brincidofovir, a prodrug of cidovir that reached phase III clinical trials for the treatment of late

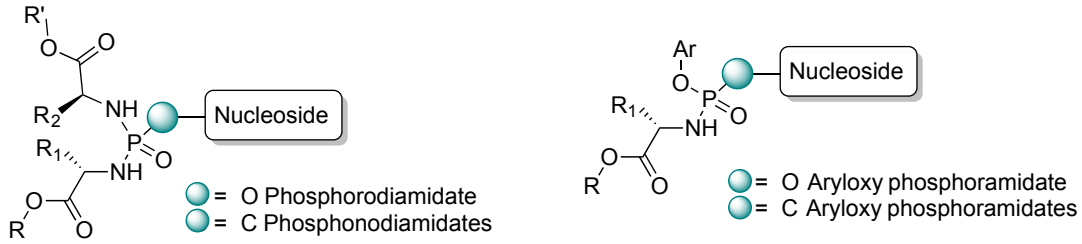
adenovirus infection. In December 2016 the trials were terminated due to ineffectiveness of the treatment.[28]



Scheme 1.7: *General structures of alkoxyalkyl phosphate mono ester pronucleotides and lisophosphatidylcholine*

Phosphoramidates

This category developed by Professor Chris McGuigan and co-workers is covered in detail in chapter 2. There are three main generations in the phosphoramidate family. The first two generations were developed in the early nineteen nineties. The phosphorodiamidates were the first of these compounds that were synthesised. They mask the phosphate hydroxyl groups with two amino acid ester groups.[29] In the second generation of compounds, one of the amino acid ester groups was changed to an aryloxy group, originating the aryloxy phosphoramidates or "ProTides".[30] More recently, the third generation was developed, comprising the phosphonamidates which link the phosphate group to the NA through a P-C bond (Scheme 1.8).[31] As will be discussed in chapter 2, this approach led to numerous new compounds that underwent clinical trials, and some other approved agents (Scheme 2.1), making from this approach the most successful pronucleotide approach to date.



Scheme 1.8 *General structure of Phosphorodiamidate and phosphonodiamidate (left), and of aryloxy phosphoramidates (ProTide) and aryloxy phosphonamidate (right).*

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2. Introduction to ProTide Approach

Phosphorodiamidates, ProTides and Phosphonoamidates

A ProTide is a prodrug of a monophosphorylated NA. The charge of the phosphate group (which is dissociated at physiological pH) is masked with an aryloxy group bound to the phosphate by a phosphoester bond, and an ester of an amino acid moiety (usually a natural *L*-amino acid) bound to the phosphate by a phosphoramidate bond. Those moieties improve the cellular uptake of the monophosphorylated NA by enabling efficient passive cell-membrane penetration and release of the free monophosphate NA inside of the cell. Variations of ProTides include nucleoside phosphorodiamidates and aryloxy phosphonodiamidates. [1,2]

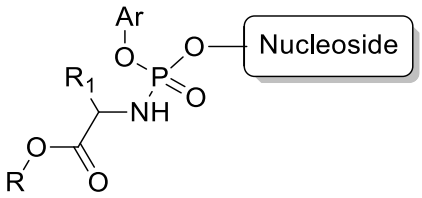


Figure 2.1: *General structure of a ProTide*

The history of the ProTides started in the early 1990s. Professor Chris McGuigan and co-workers were working on introducing monophosphorylated nucleotide analogues into the cells by masking the negative charges of the phosphate group with different groups. They aimed for groups that would allow the molecule to enter by passive diffusion, and that could be enzymatically cleaved to deliver the monophosphate NA. This was a prerequisite to bypass the "bottleneck" kinase activation of unphosphorylated NAs. Not only would this avoid a mechanism of acquired resistance of the target cells, but it would also allow more variety in the modifications of the nucleoside analogues, as they would not need to be recognised by the kinase.[3]

Their first approach was to synthesise alkyl and haloalkyl phosphate triester derivatives. Those compounds enhanced the activity of vidarabine (antiviral) and cytarabine (anti-neoplasic),[1] but when they applied this strategy to 3'-

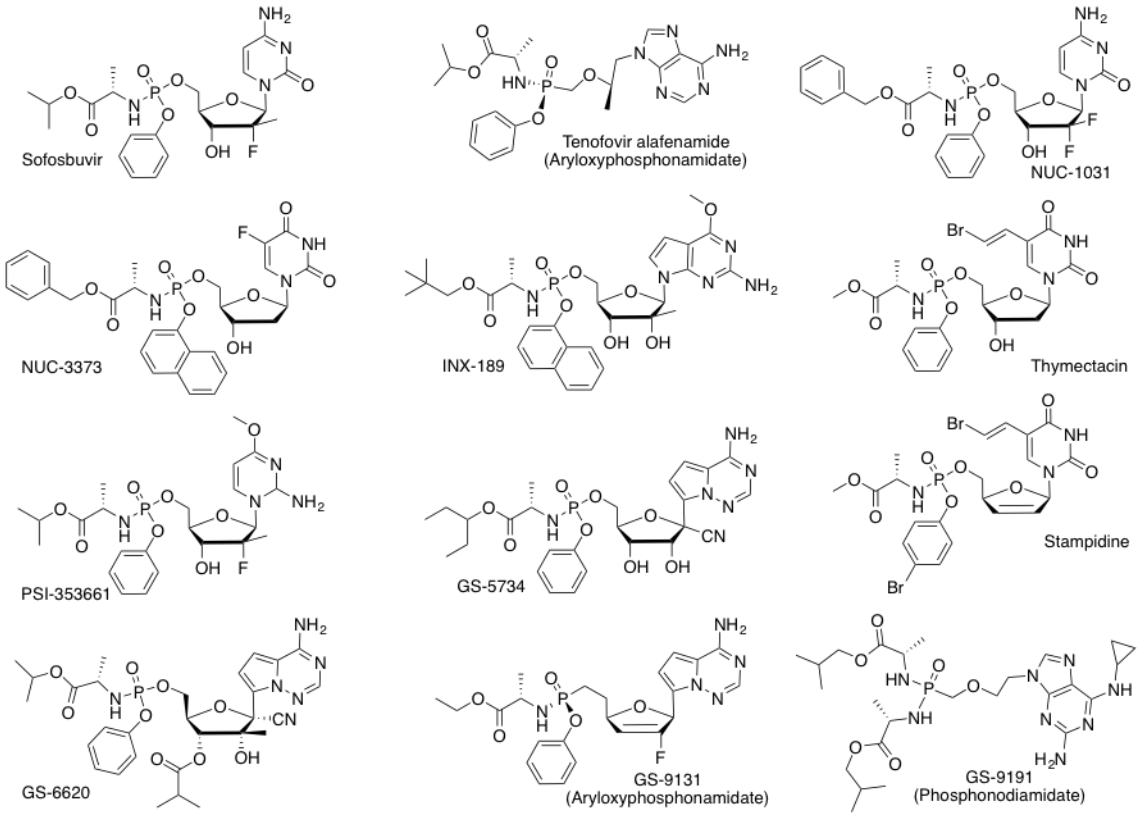
azidothymidine (AZT) and 2', 3'-dideoxycytidine (ddCyd), and despite numerous tested modifications very poor activity was observed compared to their parent nucleosides. [1,4]

By focusing on improving the activity of AZT, McGuigan and colleagues investigated alternative masking groups to further facilitate intracellular release of the nucleotides. They substituted one of the (halo) alkyl esters for an amino acid ester (aa-ester), creating the first alkyloxy phosphoramidates.[5] These new compound showed an increased activity compared to the previously explored dialkyl phosphate prodrugs of AZT. Over the next year, they introduced a second amino acid ester moiety instead of the alkyl residue, thereby generating the first nucleoside phosphorodiamidates of AZT,[3] and other 3'-modified nucleosides. This modification transformed previously inactive NAs into active antiviral compounds in AZT resistant strains of HIV virus.[6] This study provided evidence that while the NA by itself did not reach the triphosphate form and thus did not exert activity due to lack of initial phosphorylation, the phosphorodiamidate bypassed the kinase-dependent activation and exhibited high antiviral activity. Structure activity relationship studies on both alkyl phosphoramidate and phosphorodiamidate approaches showed the importance of the amino acid moiety. Small changes in the amino acid severely compromised the activity of the phosphoramidates. They postulated that an enzyme could be involved in the activation of these compounds and identified *L*-Alanine (*L*-Ala) as one of the most active amino acid moieties.[1]

At the same time, McGuigan and colleagues investigated the impact of substituting the alkyl ester groups of the alkyl phosphate triester with aryl moieties. Some of these diaryl phosphate prodrugs improved the anti-HIV activity of AZT for the first time.[7,8] However, despite their potency, these drugs did not bypass the phosphorylation step. They were prodrugs of AZT and delivered the nucleoside more efficiently into the cells, but they did not release the 5´-monophosphate form.[9]

Therefore, the high activity of aryloxy nucleosides was combined with the capability of the phosphoramidates to bypass kinase-dependent activation, thereby creating a family of aryloxy phosphoramidates of AZT: the first ProTides. These

compounds were active in cell lines where AZT had acquired resistance.[10] Those studies identified phenyl methoxyalanilyl substituents as those with the highest antiviral activity, performing 100 times better than AZT. This strategy has then been widely applied by McGuigan and co-workers and other groups to develop approved drugs such as Sofosbuvir for the treatment of hepatitis C,[11] and other nucleoside analogues with different therapeutic targets. Some of the ProTides that had reached a clinical development stage are shown in Scheme 2.1.



Scheme 2.1 *Examples of approved (Sofosbuvir and Tenofovir alafenamide) or clinically tested ProTides, phosphonates or phosphorodiamidates*

Another modification of the phosphoramidate approach was to apply it to acyclic nucleoside phosphonates as tenofovir or adefovir.[12] Nucleoside phosphonates are nucleotides linked to the phosphate group through a P-C bond. This strategy aims to deliver the monophosphate species into the cells and to provide resistance to enzymatic dephosphorylation. However, their main drawback is again cellular uptake because of the negatively charged phosphonate moiety. For Tenofovir, the first strategy was to mask the hydroxyl groups of the phosphonate using the bis-POC pronucleotide approach (see chapter 1) leading to the

development of the approved drug tenofovir disoproxil. However, due to high renal and osteo-toxic effects caused by high plasma levels of tenofovir, other pronucleotide forms were investigated and the aryloxy phosphonamidate approach was applied. Tenofovir alafenamide showed a 90% reduction in plasma tenofovir concentration and lower toxicity in clinical trials,[13] which led to its approval by the FDA in 2015.

The bulk of this thesis is focused on the ProTide approach and its anticancer application, except when promising biological results stimulated further research into other prodrug approaches as in the case of chapter 4.

ProTides as anticancer agents

To date, three ProTides have undergone clinical trials as anticancer agents: NUC-1031, NUC-3370 and Thymectacin.

NUC-1031 is a ProTide of gemcitabine. Gemcitabine is an anticancer agent used in the treatment of different solid tumours, and especially in the treatment of pancreatic cancer, where it is the current first line drug. It interferes with different cellular functions leading to apoptosis. The major mechanism for its anticancer activity is the incorporation into the DNA during the replication process where it replaces deoxycytidine, but other synergies are reported as well (chapter 6). All mechanisms described in the literature involve at the very least the conversion of the unphosphorylated drug to the monophosphorylated form. Resistance to the drug has been mainly attributed to the low plasma membrane permeability of the drug,[14] downregulation of deoxycytidine kinase[15] (which prevents the metabolism of the drug into the active monophosphate form), and overexpression of the catabolic enzymes that process gemcitabine into 2',2'-difluorodeoxyuridine (dFdU), a toxic metabolite.[16] The application of the ProTide approach on gemcitabine lead to a family of compounds which overcome all of these resistance mechanisms: 1) by entering the cell by passive diffusion into the cells without the need of hENT transporters, 2) by releasing the monophosphate species independently of kinases and 3) by showing resistance towards deoxycytidine deaminase, and therefore preventing the release of the toxic metabolite.[17] The ProTides were endowed with

higher activity *in vitro* and *in vivo* compared to the parent nucleoside. The selected lead NUC-1031 reached phase III clinical studies where high intracellular levels of gemcitabine triphosphate were confirmed. The drug was well tolerated and significant disease control was achieved in the majority of patients.[18,19]

Another important example of anticancer ProTides is NUC-3373, a ProTide of 5-fluoro deoxyribouracil (FUDR), an anticancer drug derived from 5-Fluorouracil (5-FU).[20] FUDR is the deoxyribonucleoside of 5-FU and was designed to facilitate the intracellular conversion into the active metabolite which is the monophosphorylated form 5-fluoro-2'-deoxyuridine-5'-monophosphate. They are largely used against colon and breast cancer The main mechanism of action of 5-FU and FUDR is the inhibition of thymidylate synthase (TS), and consequently the inhibition of DNA synthesis, which leads to apoptosis.[21]

TS catalyses the conversion of deoxyuridine monophosphate (dUMP) into thymidine monophosphate (TMP). This is the only *de novo* source of TMP. In the enzymatic process, a carbon atom of the cofactor 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) is transferred to the 5 position of the pyrimidine ring. The mechanism involves the formation of a ternary complex at position 5, and subsequent enzymatic catalysed abstraction of the H-5 proton (Figure 2.2, blue square).[22] 5-FU and FUDR inhibit TS in this step. Since the 5-H is substituted by a fluorine atom, its abstraction is no longer possible, and TS would be permanently bound to FUDR-MP.

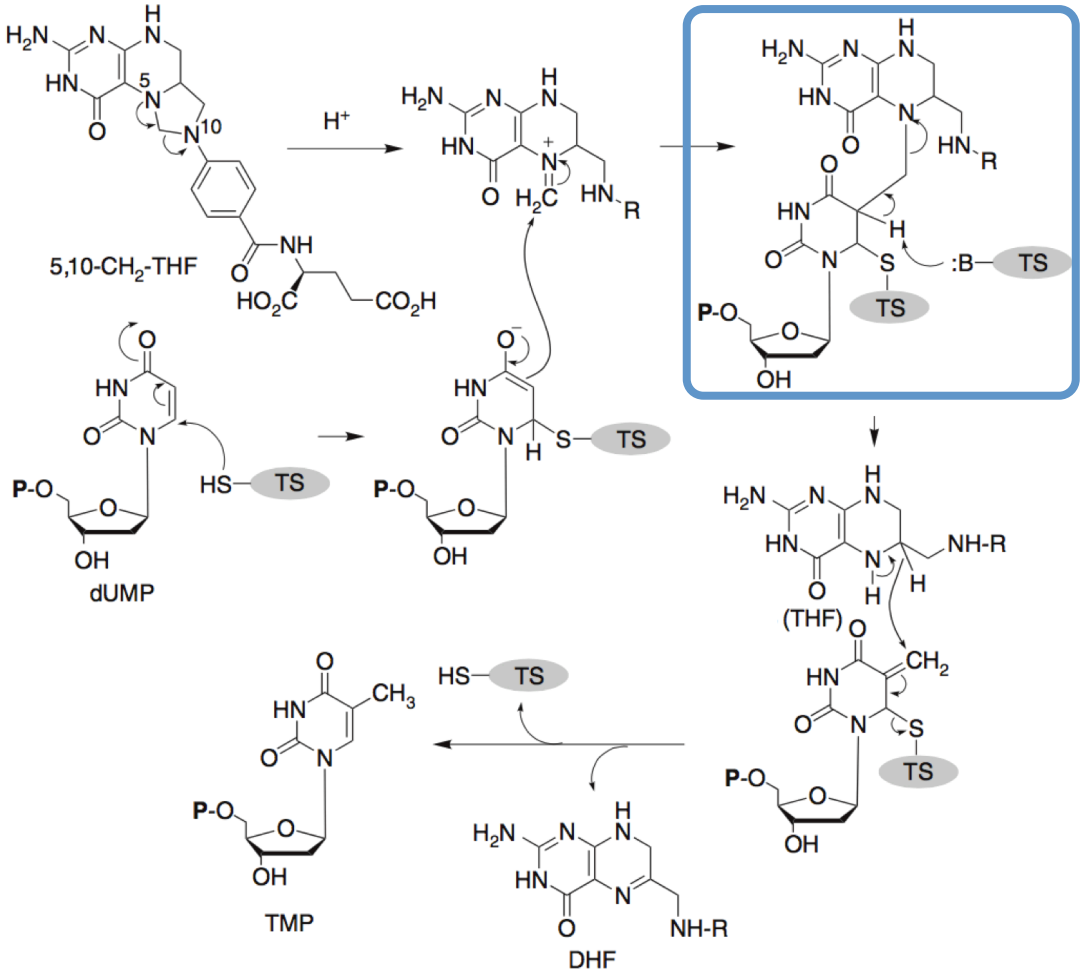


Figure 2.2 *Catalytic cycle of TS. The binding of deoxyuridine monophosphate (dUMP) to the receptor involves a change of the conformation in the nucleoside that induces the ring opening of the cofactor 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) which lies in an adjacent position. Then a cysteine residue in the active site undergoes a Michael addition with the unsaturated carbonyl system in dUMP to give intermediate enolate. The enolate attacks the cation of the cofactor forming a covalent ternary complex (blue square). Then the abstraction of the proton at position 5 of dUMP in the complex promotes a β-elimination of the cofactor, and generates a methylene intermediate of the nucleoside. The reduction of the methylene by transfer of a hydrogen atom from the cofactor and subsequent release of the enzyme, leads to thymidine monophosphate (TMP). Reprinted from Avedaño C. et al. Medicinal chemistry of anticancer drugs, chapter 2, with permission from Elservier copyright 2008.*[22]

Resistance and low activity have been observed in 5-FU and FUDR because of different factors, including reduced activity of the enzyme thymidine kinase, which is responsible for the first phosphorylation step, increased activity of thymidine phosphorylase (that cleaves the nucleobase from the sugar by introducing a phosphate at position 1'), and reduced transporter mediated cellular uptake.[21] The ProTide approach was rationally applied to this drug and proved not only to retain the high potency of FUDR *in vitro*, but also to significantly overcome the high dependence of the parent nucleoside on kinase activation and on cell transporters.[20]

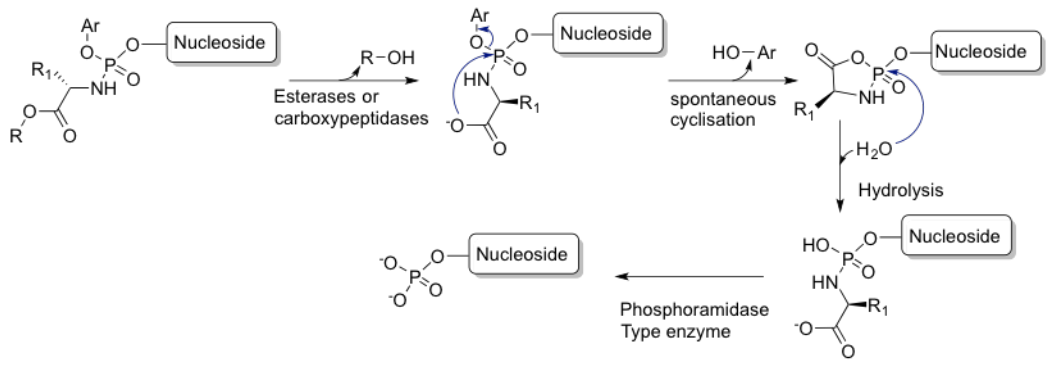
FUDR ProTides were also resistant to thymidine phosphorylase and they were stable in acidic and neutral pH, as well as in plasma; suggesting a mainly intracellular activation.[20] NUC-3373, the best ProTide in the family, is currently undergoing phase I clinical trials.[23]

Thymectacin is a ProTide of brivudine. Interestingly, despite the anticancer effects of thymectacin, brivudine is an approved oral treatment against severe herpes zoster infection. Its activity as antiviral therapeutic depends on its phosphorylation to the triphosphate form.[24] However, the ProTide form was also found to be useful for the treatment of advanced colorectal cancer with resistance to fluoropyrimidine drugs. It reached Phase I clinical trials for this indication in 2002, but since then no other updates on this drug were published.[25]

Mechanism of activation of ProTides

As mentioned before, due to the ability of the ProTides of masking the negatively charged phosphate group, they can enter into the cell through passive diffusion, in the same way as many other pronucleotides. However, what makes the ProTide approach so distinct is the mechanism by which the monophosphorylated NA is released (Scheme 2.2). Once the ProTide is inside the cell and the masking groups are cleaved to release the monophosphorylated NA, it cannot exit the cell anymore because there are no suitable channels for it and the negatively charged phosphate group (in addition to the general hydrophilicity of most NA) prevents diffusion across the membrane.[26] The first (rate limiting) phosphorylation step of the NA is no longer required, so the NA monophosphate can be converted directly into the 5'-di and triphosphate species by nucleoside mono and diphosphate kinases.

The activation of a ProTide is postulated to happen in four steps (Scheme 2.2). Two enzyme-mediated and two spontaneous reactions have being described. Nevertheless, some further studies are still needed for a full characterisation of the process.



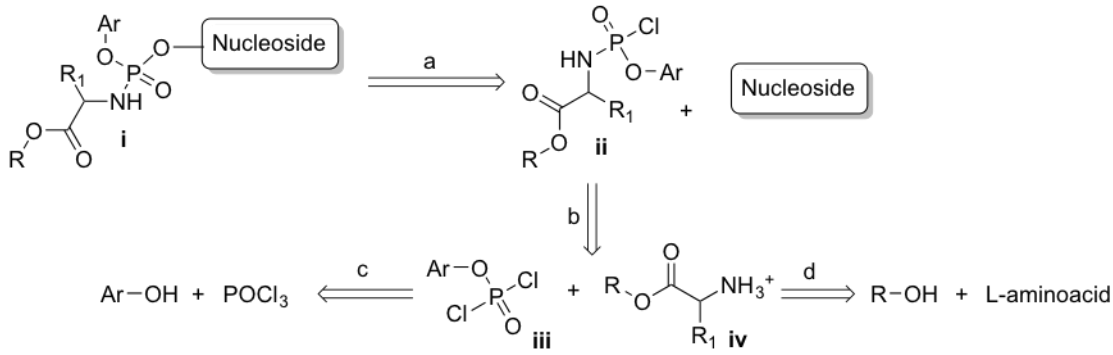
Scheme 2.2 *Postulated mechanism of ProTide activation*

The putative activation begins with the cleavage of the ester moiety by esterases or carboxypeptidase type enzymes.[27] Studies identified cathepsin A as the major enzyme involved in the reaction.[28] Then, a spontaneous cyclisation takes place via the attack of the carboxylate anion in the amino acid on the phosphate, releasing the aryloxy moiety as the best leaving group. The five-membered ring is then opened by spontaneous hydrolysis caused by the attack of a water molecule at the phosphorus centre.[27,29] Finally, a phosphoramidase type enzyme, putatively belonging to the HINT family, mediates a reaction to cleave the *L*-amino acid moiety from the molecule, and hence releases the monophosphorylated NA.[30]

The first activation step, which is principally performed by the intracellular enzyme Cathepsin A, could be a promising target for decreasing toxicity in non-tumour cells. Some studies showed particularly high concentrations of this enzyme in malignant transformations and metastases of certain cancer cells[7]. This difference in enzyme content may lead to compounds that specifically target cancer cells, and hence decrease the general toxicity of NAs. Moreover, since a variety of enzymes can catalyse this ester cleavage it does not provide an easy resistance path for the target cell. Cathepsin A was also considered to be chiefly responsible for the first activation step of phosphonates[28,31] and phosphorodiamidates.[32] In some cases (*e.g.* Sofosbuvir), the enzyme showed a preference for the activation of one of the diastereoisomers of the ProTide, which suggested the clinical development of the best processed diasteroisomer (with Sp configuration). However, other ProTides of other NA did not show this steric preference for their activation, and the mixture of diastereoisomers proceeded through clinical trials.[17,20]

Synthesis of ProTides

The McGuigan group described the general synthesis (Scheme 2.3) of ProTides (**i**).[33] The key reaction is the coupling of the NA and an appropriate amino phosphorochloridate (**ii**). These compounds are obtained by a reaction of an aryloxy phosphorodichloridate (**iii**) and an amino acid ester (**iv**). Most often these moieties need to be synthesised. The aryloxy phosphorodichloridate is made by reaction of an aryl alcohol with $POCl_3$. The amino acid ester salt is obtained by reaction of an *L*-amino acid and the chosen alcohol. Some of these intermediates are commercially available.

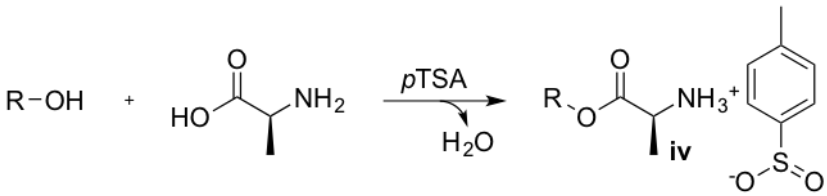


Scheme 2.3 *General synthesis of ProTides developed by the group of Professor Chris McGuigan. Reagents and conditions: (a) NMI (5 eq), THF, RT 20 h or tBuMgCl (2 eq), THF, RT, 20 h. (b) TEA, DCM, Ar atm, -78˚C, 1h; then RT, 3 h. (c) TEA, DEE, Ar atm, -78˚C 1h then RT 1 h (d) pTSA, Toluene, Reflux temperature; 20 h.*

The vast experience in the synthesis and evaluation of ProTides, has allowed identifying moieties which have repeatedly led to better results in the ProTide approach even if the behaviour of each NA is different.[34] The chosen moieties in this work correspond to these most successful ones: *L*-Alanine (*L*-Ala) as amino acid, naphthyl (Naph) and phenyl (Ph) as aryl residues (Ar), and neopentyl (Neop), benzyl (Benz or Bz), ethyl, hexyl (Hex), cyclohexyl (cHex), isopropyl (iPr) and octyl as ester moieties were selected.

Synthesis of amino acid esters

Five non-commercially available amino acid ester tosylated salts, bearing *L*-alanine as amino acid, and neopentyl, hexyl, cyclohexyl, isopropyl and octyl as ester moieties were synthesised.



Scheme 2.4 *Preparation of amino acid ester salts. Reagents and conditions: pTSA, Toluene, Reflux temperature; 20 h*

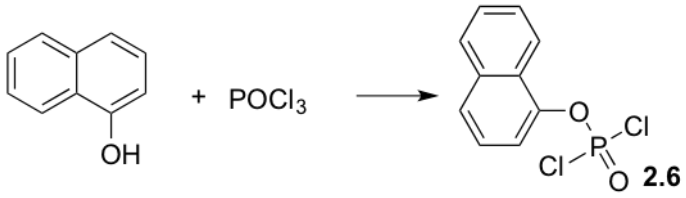
L-Alanine reacts with an excess of alcohol in the presence of *p*-toluene sulphonic acid at reflux temperature. The reaction water was eliminated via a Dean Stark apparatus. This was necessary to prevent the counter reaction. The performed reactions are summarised in *Table 2.1*.

Table 2.1 Summary of the performed reactions to obtain aa-ester tosylated salts

Compound	(R)	Amino Acid	Yield
2.1	Neopentyl	*L*-Alanine	77 %
2.2	Hexyl	*L*-Alanine	76 %
2.3	Cyclohexyl	*L*-Alanine	84 %
2.4	Isopropyl	*L*-Alanine	95 %
2.5	Octyl	*L*-Alanine	50 %

Synthesis of Aryl Phosphorodichloridate

Phenyl phosphorodichloridate is commercially available, so the designed ProTides only required the synthesis of 1-naphthyl phosphorodichloridate **2.6**. It was synthesised via the reaction of naphthol with phosphorus oxychloride in the presence of triethylamine (TEA) (Scheme 2.5).



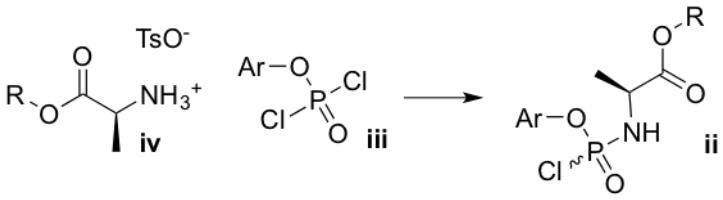
Scheme 2.5 *Synthesis of 1-naphthyloxy phosphorodichloridate. Reagents and conditions: TEA, diethyl ether, Ar atmosphere, -78 ˚C 1h, RT 1h*

Phosphorodichloridates are easily degraded and require inert atmosphere to avoid reactions that may form the free phosphate. Compound **2.6** was synthesised several times, yielding from 83% to 98%. Due to its instability, this product was used

for the following reactions without further purification. Characterization of this compound by ^{31}P NMR gave a singlet at 3.52 ppm

Synthesis of Phosphorochloridate

Synthesis of phosphorochloridates (**ii**) was performed by reaction of a phenyl or naphthyl phosphorodichloridate (**iii**) with an appropriate aa-ester as tosylated or chlorinated salt in the presence of triethylamine. This reaction required 2 equivalents of the base. The first equivalent replaces the salt. Then the lone pair of electrons of the amino group of the aa-ester attacks the phosphate at the phosphorus atom and displaces one chlorine atom. The second equivalent of triethylamine will accept the additional proton of the amino group. This is a non-stereoselective reaction and two diastereoisomers ($L_{Ala}R_P$) and ($L_{Ala}S_P$) will be formed. Therefore, the ^{31}P NMR spectrum of such compounds is characterised by two singlets (one for each isomer) at a ratio of ca 1:1 at a chemical shift of 7.50-8.50 ppm.



Scheme 2.6: *Synthesis of aminophosphorochloridates. Reagents and conditions: TEA, DCM, Ar atm, -78˚C 1h; then RT 3 h.*

The removal of the tosylated salt from the crude was achieved either by filtration of the crude with diethyl ether (in which the product is soluble but the salt is not) or by a rapid chromatographic column to retain the salt in the silica. The faster this process was performed, the better yields were observed.

Table 2.2: Summary of the synthesised phosphorochloridates showing the yields and ^{31}P NMR shifts in $CDCl_3$

Entry	R	Aryl (Ar)	Yield	^{31}P NMR
2.7	Neop	Naphthyl	63 %	8.28, 8.02 ppm
2.8	Benz	Naphthyl	63 %	8.33, 8.05 ppm
2.9	Ethyl	Naphthyl	53 %	8.33, 8.06 ppm
2.10	Hexyl	Naphthyl	90 %	8.25, 7.98 ppm
2.11	cHex	Naphthyl	77 %	8.17, 7.92 ppm

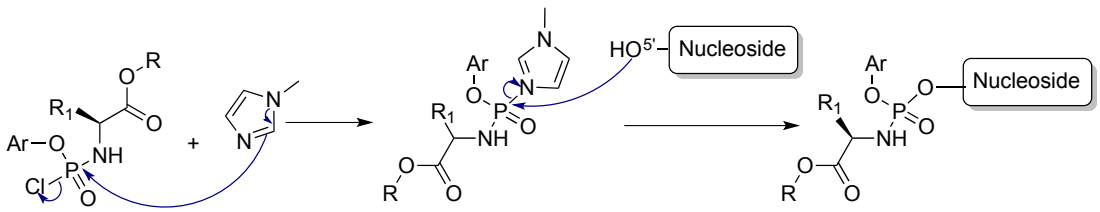
Entry	R	Aryl (Ar)	Yield	^{31}P NMR
2.12	Neop	Phenyl	77%	7.64, 7.98 ppm
2.13	Benz	Phenyl	88 %	7.89, 7.57 ppm
2.14	Ethyl	Phenyl	82 %	8.06, 8.33 ppm
2.15	Hexyl	Phenyl	77 %	8.03, 7.71 ppm
2.16	cHex	Phenyl	77 %	8.12, 7.75 ppm
2.17	iPr	Phenyl	53 %	8.06, 7.69 ppm
2.18	Octyl	Phenyl	70 %	8.02, 7.65 ppm

Coupling Reaction: Obtainment of the ProTide

Two main coupling reaction methods are described between the phosphorochloridate and the nucleoside analogue, which react in different ways.

Using NMI reagent

NMI is a mild base, and it is not strong enough to deprotonate the hydroxyl groups of the nucleoside analogue. On the other hand, it easily attacks the phosphorochloridate, forming a labile intermediate that is being attacked by the most acidic hydroxyl of the nucleoside, which is most often the 5' hydroxyl group (typically the only primary alcohol), to form the ProTide (Scheme 2.7). This method is preferred for the synthesis of ProTides bearing more than one hydroxyl group because it mainly delivers the desired 5'-ProTide.



Scheme 2.7: *Reaction mechanism of the synthesis of ProTides using NMI.*

Using *tert*-Butyl Magnesium Chloride.

The Grignard reagent *tert*-butyl magnesium chloride (*t*BuMgBr) is used as a strong base. It removes the proton of one hydroxyl group in the NA, usually at position 5' because it is the least hindered and most acidic. The generated anion attacks the phosphorus atom of the amino phosphorochloridate, cleaving the

chlorine from the molecule (Scheme 2.8). The Grignard reagent is more reactive than NMI and can easily deprotonate other hydroxyl groups in the molecule. It is often the preferred method for NA that have a free hydroxyl group only at the 5' position or if NMI achieves very poor yields. In the latter case, selective protection of the other hydroxyl groups is usually required.

Scheme 2.8 *Synthesis of ProTides using Grignard Reagent*

The details of the reaction for each NA used for this work are described in the corresponding chapter and in the experimental part.

Aim of the thesis

Due to the need for new therapeutic anti-cancer agents, this work aimed the synthesis new families of ProTides of novel and known anticancer NA in order to improve their potential therapeutic application

The targeted NAs were: 2-chlorocordycepin, 3'-ethynyl nucleosides (with focus on 3'-ethynyluridine, 3'-ethynylcytidine and 3'-ethynyl-5-methyluridine), and the new nucleoside 3,N^4-ethenogemcitabine.

The new compounds, along with their parent nucleosides, were then to be tested for their activity in an *in vitro* cell-based assay. Moreover, enzymatic assays and docking studies in some members of each family aimed to elucidate how the new ProTides were activated to the monophosphate species, and, in the case of 2-chlorocordycepin, to test the compound's resistance to deamination.

A second objective was sought for 3,N^4-ethenogemcitabine and its ProTides. As these compounds were expected to emit fluorescence, experiments to characterise their fluorescence properties and intracellular distribution were to be performed, in addition to the activity and enzymatic assays mentioned above.

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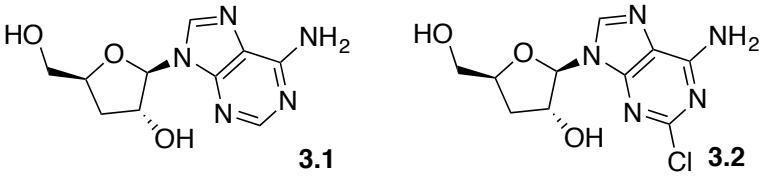
3. 2-Chlorocordycepin

Cordycepin and 2-Chlorocordycepin – Rationale behind the design



Scheme 3.1 *Cordycepin* ***3.1*** *and 2-Chlorocordycepin* ***3.2***

Cordycepin **3.1** is a nucleoside analogue of adenosine differing from the latter by the absence of hydroxyl group at the 3' position. It was first isolated as a biometabolite from *Cordyceps militaris* in the nineteen fifties,[1] and it is also present in *Cordyceps sinensis.*[2] *Cordyceps* is a caterpillar parasite fungus with medicinal properties widely used in traditional Chinese medicine. There it has broad applications because of its numerous attributed pharmacological properties.[3]

This caterpillar fungus grows in the Himalayan lands, on the Tibetan Plateau, above 3800 m above sea level. Demand of this mushroom due to its interesting properties has increased. However its availability is limited because of its confined geographic location. In addition, the Chinese government protects *Cordyceps sinensis* by listing it as the key wild species under second grade state protection and promulgating laws and regulations to protect the fungus' sustainable use, development and collection.[4] Therefore, acquisition of the desired metabolites of *Cordyceps sp*. is subject to artificial culture or chemical synthesis. Nowadays, an efficient method in organic synthesis allows access to cordycepin from adenosine in a two-step synthesis.[5]

The described properties of cordycepin cover such diverse claims like antifungal[6] and trypanocidal activity,[7,8] anti-inflammatory properties,[9] and inhibition of platelet aggregation.[10] However, the most reported ones are the anticancer activities of this compound: There are studies showing anti-tumour effects on mouse melanoma and lung carcinoma cells,[11] bladder cancer cells,[12] breast cancer cells,[13]

hepatocellular carcinoma cells[14] and leukaemia,[15] for which clinical trials were performed.[16]

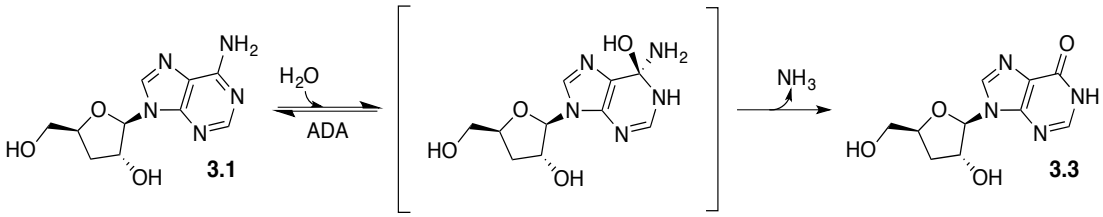
Cordycepin enters cancer cells using the equilibrative nucleoside transporter hENT3.[17] Once inside, it competes with adenosine for the nucleoside and nucleotide phosphorylating enzymes[18] to be converted into the 5'-mono, di- and triphosphate forms,[15,19] the latter being the toxic metabolite of cordycepin.[20]

The reported anticancer activity was first identified in the nineteen seventies as the ability of cordycepin triphosphate to inhibit the action of RNA polymerases (Poly(A) synthesis is preferentially affected).[21] Later, it was discovered that the synthesis of the DNA primer was also affected,[22] and more recently an inhibition of the action of terminal deoxynucleotidyl transferases (TdT) was also described.[15]

However the exact molecular modes of action of this drug remain unclear. Since cordycepin triphosphate is an adenosine triphosphate (ATP) analogue, the nucleotide can interfere with numerous cellular processes that require ATP binding or hydrolysis.[20] In the first attempt to elucidate the mechanism of action of cordycepin *in vitro* assays were performed to check if cordycepin could act as chain terminator due to the lack of the hydroxyl group at position 3'. Müller *et al*. (1977) proved that cordycepin triphosphate competes with ATP for the incorporation into the RNA chain, especially in enzyme systems containing nuclear poly (A) polymerase, which then leads to termination of the chain.[18] Later, Holbein *et al*. (2009) performed more exhaustive studies on the molecular basis of the activity of cordycepin.[20] Those studies agreed that the toxic species is cordycepin triphosphate, and that the activity is counteracted by the presence of ATP. They also proved that cordycepin triphosphate can act as a chain terminator, but found that the nuclear concentration of cordycepin triphosphate is too low to cause significant chain termination. However, it was sufficiently high to reduce efficiency of 3' end formation. Therefore they described that the ability of interfering in the formation of the 3' end was independent to the potential to terminate RNA chain, but chiefly responsible for the cytotoxic effect of cordycepin triphosphate.[20]

The efficacy of cordycepin is compromised by the rapid metabolic degradation by adenosine deaminase (ADA), which is ubiquitous in almost all human tissues.[23]

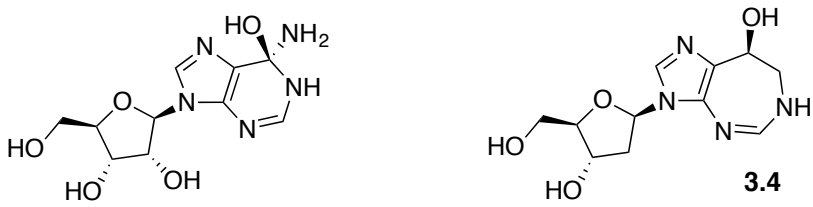
ADA is a major enzyme of purine metabolism responsible for conversion of adenosine and 2'-deoxyadenosine into their corresponding inosine derivatives. It was shown that ADA interacts with cordycepin **3.1** with similar kinetic parameters as adenosine,[24] and this interaction irreversibly leads to 3'-deoxyinosine **3.3** that lacks cytotoxic activity.[15] The catalytic mechanism proposed for this process is the same as that for their counterparts (Scheme 3.2).). An initial stereospecific addition of a hydroxyl group at position 6 and protonation of N-1 performed by the enzyme, leads to a putative tetrahedral transition-state, which is followed by ammonia elimination to form the inosine derivative.[23,25]



Scheme 3.2 *Mechanism of deamination of Cordycepin by ADA*

There are two approaches to endow cordycepin with resistance towards ADA. The first is the co-administration with an ADA inhibitor, and the second the modification of the molecule with groups that may provide resistance towards ADA without compromising its activity

As an example for the first approach, the combination of cordycepin with (8*R*)-2'-deoxycoformycin **3.4** (henceforth referred to as deoxycoformycin) was investigated. Deoxycoformicin **3.4** is a potent ADA inhibitor whose activity is attributed to an almost irreversible bind to the ADA active site by mimicking the transition state of adenosine (Scheme 3.3).[23]



Scheme 3.3 *Left: Chemical structure of the transitional state of adenosine in ADA. Right: Chemical structure of deoxycoformicin **3.4**.*

This combination enhanced the anticancer and trypanocidal activity of cordycepin **3.1** in cells and in mice.[8,26,27] However, inhibiting an enzyme such as ADA, which is ubiquitous in the body, may lead to unspecific toxicity and indeed, deoxycoformicin **3.4** is used as an anticancer agent in hairy leukaemia[28] and also exhibits teratogenic effects.[29] Because of this cytotoxicity, the dose needs to be monitored carefully. When the co-administration of cordycepin **3.1** and deoxycoformicin **3.4** was studied in Beagle dogs, it led to severe gastrointestinal toxicity and bone marrow toxicity, establishing the maximum tolerated dose of cordycepin in combination with deoxycoformicin in 8 mg/kg/day.[30] Those effects may limit the co-administration in human. Two Clinical trials administering cordycepin in combination with deoxycoformycin for treatment in patients with refractory TdT-Positive Leukaemia and with refractory acute lymphocytic or chronic myelogenous leukaemia were carried out however, to date, no study results have been posted.[16]

The second strategy to provide cordycepin with resistance to ADA deamination is to modify the molecule so that it is no longer an ADA substrate. Such modifications were explored in the course of this thesis, with focused interest in modifications at position 5' and position 2.

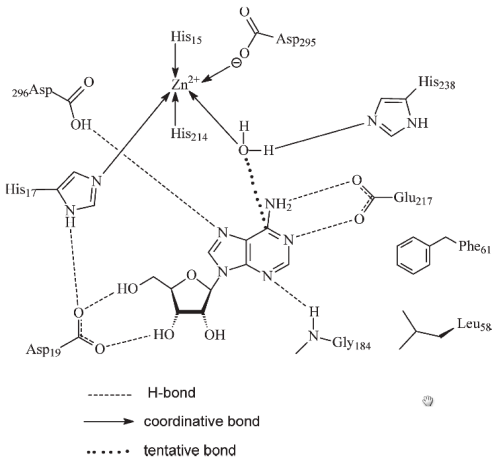


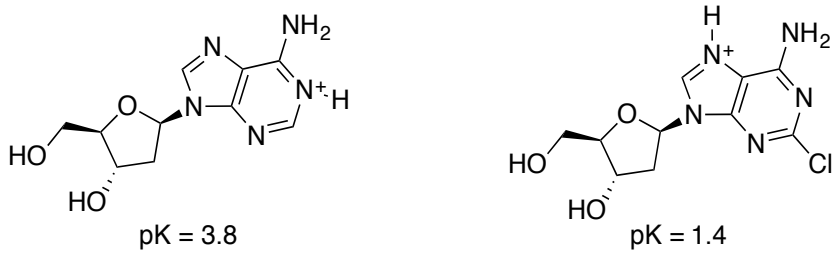
Figure 3.1 *Schematic representation of adenosine in ADA active-site, showing reported interactions. Reprinted with permission from Gillerman et al. J.Med.Chem.2011, 54 (1), 107-121. Copyright 2011, American Chemical Sociey.*

Some studies have shown the importance of the hydroxyl group at position 5' for the activity of ADA. Figure 3.1 shows the schematic pocket site of ADA and the interactions with adenosine, the natural substrate.[23]

When position 5' of cordycepin was acetylated, ADA totally lost its activity. It is postulated that the lack of activity is related to the inability to form interactions with His 17 and Asp 19.[31] The

ProTide approach would also prevent this interaction, since position 5' would be bond to the phosphoramidate moiety. Therefore the ProTide strategy could endow cordycepin with resistance towards deamination by ADA. Some ProTides of different NA have already also shown to be entirely resistant to deaminase degradation.[32]

The insertion of substituents at position 2 of some adenosine analogues led to both ADA deamination resistance, and inhibitors of ADA.[23] For example, 2-Chloroadenosine[24] and Cladribine (2-chloro-2'-deoxyadenosine, a potent anticancer nucleoside analogue)[33] were shown not be substrates of ADA. The postulated mechanism of resistance for C-2-halogenated adenosine nucleosides states that the protonation of those molecules would happen at N-7 instead of N-1 (Scheme 3.4). This new situation would not provide optimal conditions for hydroxylation at C-6 and final deamination.[34]



Scheme 3.4 *Theoretical protonation of 2'-deoxyadenosine (left) vs Cladribine (right) Kazimierczuk et al.*

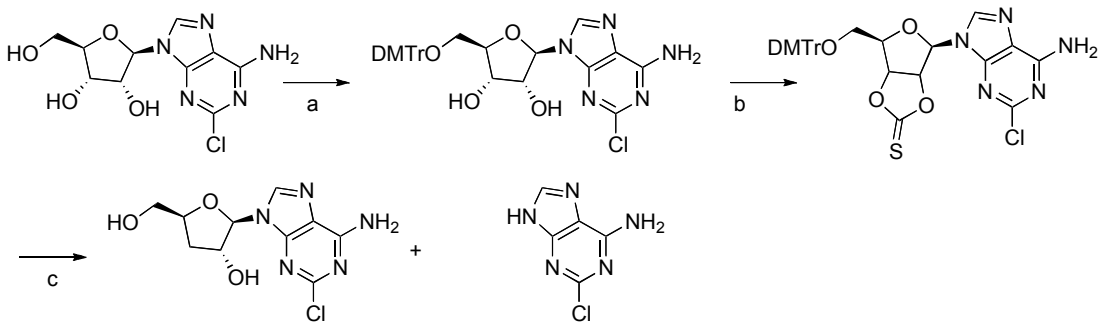
Following the considerations mentioned above, it was worth to investigate the implications of inserting a 2-chloro group in cordycepin. At present there is not much literature regarding 2-chlorocordycepin. It has been previously synthesised as an antiviral drug, where despite of some good activity results, the high cytotoxicity against human T and B lymphocytes and mouse fibroblasts discouraged further research.[35] It was also tested as an apoptotic agent in peripheral blood mononuclear cells, but only poor effects were observed compared to cladribine.[36] Finally, 2-chlorocordycepin was tested as an antibacterial agent, but the compound did not show activity.[37] Recently, the compound was further evaluated as a trypanocidal agent, but showed poor activity probably due to a reduced cellular uptake of the molecule through the required transporters.[38]

This work describes the synthesis of a 2-chlorocordycepin ProTide family that intends to improve the activity of cordycepin by providing resistance towards ADA metabolic degradation and by supplying high intracellular concentrations of the monophosphate derivative, independently of nucleoside transporters or kinases, thus circumventing competition with endogenous adenosine.

Synthesis of 2-chlorocordycepin

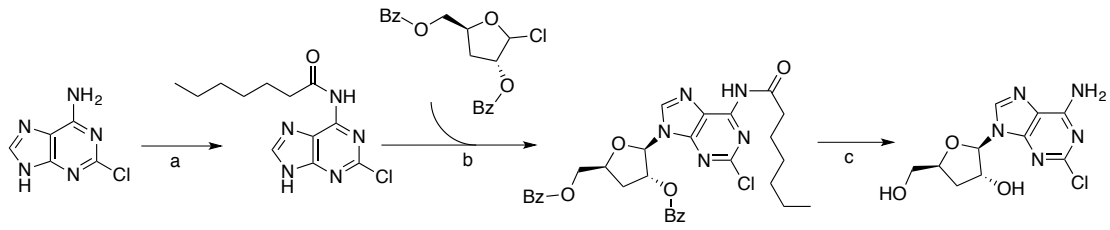
Several published procedures for the synthesis of 2-chlorocordycepin are available. They were scrutinized for applicability and feasibility for the purpose of this work.

Synthesis of 2-chlorocordycepin was first reported by Rosowsky *et al*. in 1989.[35] Starting from commercially available 2-chloroadenosine, a three step synthesis lead to a mixture containing 2-chlorocordycepin, with an overall yield of 15 % after purification (Scheme 3.5). The synthesis involves the protection of the hydroxyl group at position 5' of 2-chloroadenosine with a 4,4'-dimethoxytrityl (DMTr) group, followed by the condensation with 1,1'-thiocarbonyldiimidazole in acetonitrile, giving the 2',3'-O-thiocarbonyl derivate. Then, treatment with n-Bu_3SnH in toluene in the presence of α,α'-azobisisobutyronitrile (AIBN) results in a mixture of 2-chloroadenine and 2-chlorocordycepin. Low priority was given to this synthetic pathway because of the low yield.



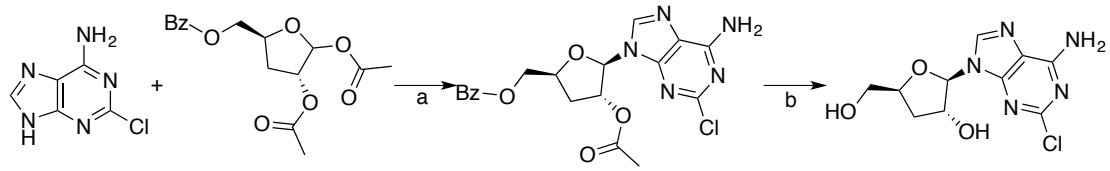
Scheme 3.5 *Synthesis of 2-chlorocordycepin reported by Rosowsky et al.[35] Reagents: (a) 4,4'-DMTr chloride in pyridine; (b) 1,1'-thiocarbonyldiimidazole in CH_3CN; (c) n-Bu_3SnH in toluene and AIBN.*

A patent by Gupta *et al*. in 2004[39] described another method to furnish the desired nucleoside from 2-chloroadenine (Scheme 3.6). In this synthesis the amino group of 2-chloroadenine is protected with heptanoic anhydride to be condensed with 1-chloro-2,5-di-*O*-benzoyl-3-deoxy-*D*-ribose using potassium hexamethyldisilazide (KHMDS). A deprotection of the amino and hydroxyl groups with sodium methoxide and methanol (CH_3ONa-MeOH) leads to 2-chlorocordycepin in 21 % overall yield.



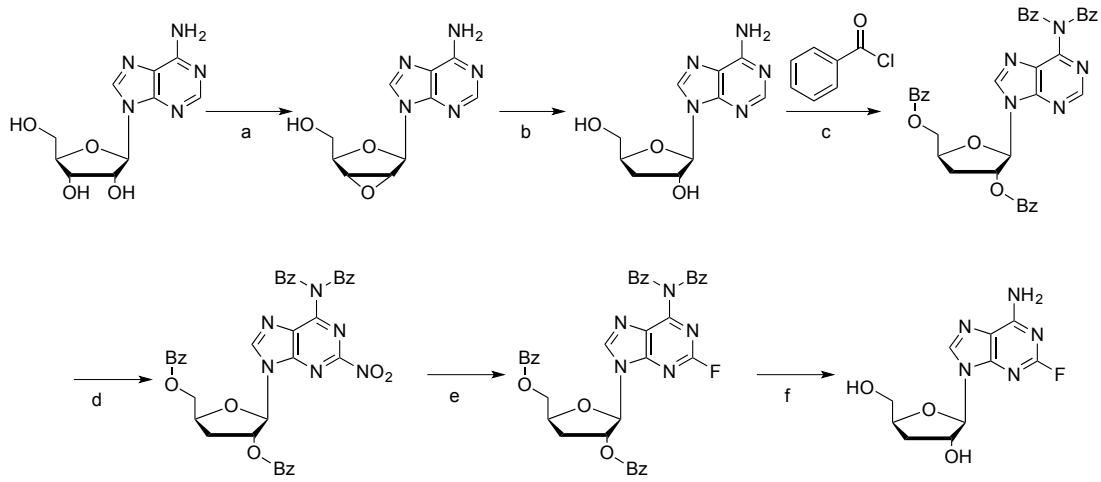
Scheme 3.6 *Synthesis of 2-chlorocordycepin patented by Gupta et al.[39] Reagents: (a) NMI, heptanoic anhydride (72 %); (b) KHMDS (41 %); (c) CH_3ONa-MeOH (72 %).*

Recently Vodnala *et al*. designed a new 2 step pathway for the synthesis of 2-fluoro and 2-chlorocordycepin.[38] Starting with the condensation of 2-chloroadenine and 5-*O*-benzoyl-1,2-di-*O*-acetyl-3-deoxy-*D*-ribose, using bis(trimethylsilyl)acetamide (BSA) and trimethylsilyl triflate (TMSTf), followed by deprotection of the hydroxyl groups with methanolic ammonia. The overall yield was over 35 % (Scheme 3.7). This strategy was dismissed because of the expensive starting materials.[40]



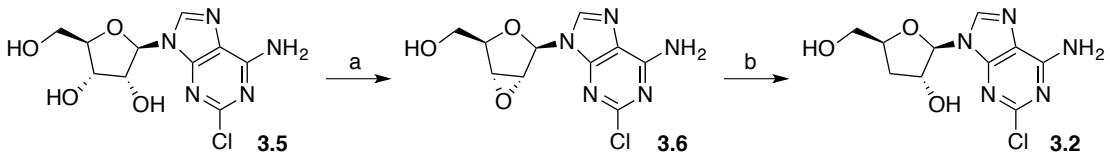
Scheme 3.7 *Synthesis of 2-chlorocordycepin reported by Vodnala et al.[38] Conditions and reagents: (a) BSA, TMSTf (93%); (b) NH_3, CH_3OH (38 %).*

A method to synthesise 2-halogenated cordycepin analogues from adenosine was also reviewed in case it was suitable to apply it for the synthesis of the 2-chloro analogue. First steps consist of the synthesis of cordycepin by applying the method reported by Robins *et al*.[5] The hydroxyl groups are then protected, and a selective nitration at position 2 is performed. Next a halogen atom (fluorine) substitutes the nitro group and finally the protecting groups are cleaved from the molecule (Scheme 3.8). This method has been applied to the synthesis of 2-fluorocordycepin with 2 % overall yield.[38] Adapting the method to the synthesis of 2-chloroadenosine was dismissed due to the poor achieved yield for the fluoro-analogue.



Scheme 3.8 *Reported synthesis of 2-fluorocordycepin by Vodnala et al.[38] Conditions and reagents: (a) 2-acetoxyisobutiryl bromide, Amberlite 400, OH^-, CH_3CN (62 %); (b) $(C_2H_5)_3LiH$, DMSO (53 %); (c) Pyridine (47 %); (d) TBAN, TFAA, (45 %); (e) TBAF, CH_3CN (59 %); NH_3, CH_3OH (59 %).*

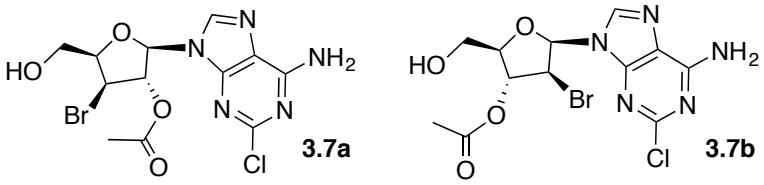
Because of the inadequacy of these reported procedures a novel route to obtain 2-chlorocordycepin was designed. This new pathway based on the cordycepin synthesis by Robins *et al*. [5] aimed for a two-step synthesis starting at 2-chloroadenosine **3.5**, which is commercially available (Scheme 3.9). Even though no evidence for previous attempts of this synthetic pathway was found in the literature, this method was chosen because it is short, inexpensive and delivered only one product.



Scheme 3.9 *Novel designed pathway to synthesise 2-chlorocordycepin. Reagents and conditions: (a) αAIBBr in CH_3CN/H_2O, RT, 2h; Amberlite IRN 78 (OH-) in MeOH RT 5h; (b) $LiEt_3BH$ in THF/DMSO 4˚ C, 1h, RT 15 h, Ar atm.*

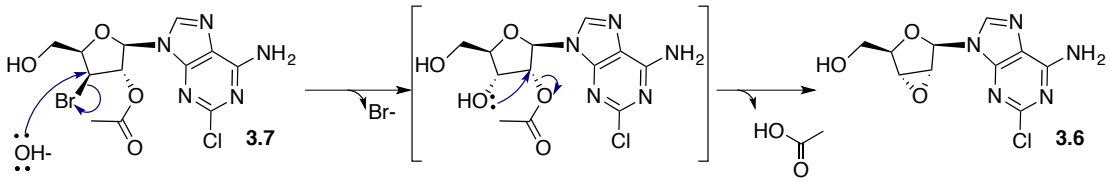
The first step involved the conversion of 2-chloroadenosine **3.5** into 2-chloroadenosine-2',3'-riboepoxide **3.6**. 2-Chloroadenosine **3.5** was treated with α-acetoxyisobutyryl bromide (αAIBBr) in dry acetonitrile with 1 equivalent of water (moist acetonitrile).[5] The proposed mechanism of reaction of α-acetoxyisobutyryl halides with cis diols[41] and adenosine[42] was described in 1973 by Moffat *et al*. They proved that the reaction with adenosine led to an intermediate that only exhibited alpha configuration of the acetoxy moiety on either 2' or 3' position. They postulated

that the stereoselectivity was due to sterical impediments. The reaction with 2-chloroadenosine **3.5** was expected to act in the same way, so only two intermediates would be formed **3.7a** and **3.7b** and would acquire alpha configuration for the acetoxy moiety and beta for the bromine, as it is shown in the Scheme 3.10. The intermediates were not isolated and the next step was done without further purification.



Scheme 3.10 *Trans-bromo acetate derivatives intermediate putatively formed in the reaction of 2-chloroadenosine with αAIBBr.*

The reaction was then treated with amberlite IRN 78 $(OH)^-$. It is postulated that the OH^- groups in this resin would attack intermediate **3.7** on the activated carbon bearing the bromine substituent (C2' or C3'). The bromine atom is a good leaving group and it can be cleaved. Then, the lone pair of electrons of the hydroxyl group attacks the carbon bond to the acetoxy moiety, to form 2-chloroadenosine-2',3'-riboepoxide **3.6**, the desired intermediate (Scheme 3.11). The reaction was filtered and washed with methanol to recover the product from the amberlite IRN 78 (OH^-) resin.



Scheme 3.11 *Final step to obtain of the 2-chloroadenosine-2',3'-riboepoxide **3.6***

The identity of 2-chloroadenosine-2',3'-riboepoxide **3.6** was established by the mass spectrum with 284 $[M + H^+]$ and 306 $[M + Na^+]$ (observed), and from its ^1H NMR spectrum in deuterated DMSO. Figure 3.2 shows the comparison between the spectra of 2-chloroadenosine **3.5** and 2-chloroadenosine-2',3'-riboepoxide **3.6**. Some key differences are noted: two doublet signals corresponding to the proton in the hydroxyl groups at 2' and 3' position disappear in the epoxide. The signal for H-1'

appears as a singlet not showing any coupling with the vicinal H-2'. This is due to the change of the conformation when the sugar is bearing an epoxide instead of an alcohol: H-1' and H-2' adopt a torsion angle very close to 90 degrees.[43] According to the Karplus equation in these conditions the magnitude of *J* coupling constants between vicinal protons are generally the smallest, and they may collapse into a singlet. Therefore the proton at position 2' appears like a doublet, only coupling with the proton at position 3'.

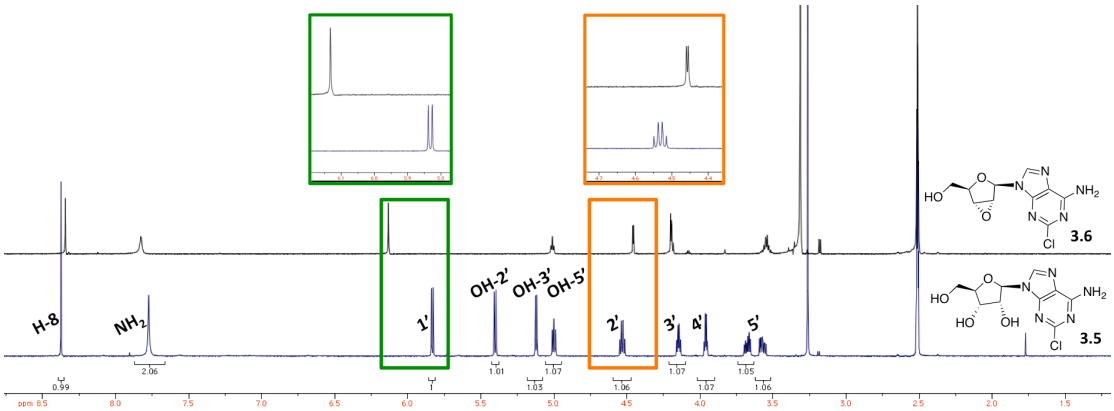


Figure 3.2 *Comparison of the ^{1}H NMR Spectra in d6-DMSO of 2-chloroadenosine **3.5** and 2-chloroadenosine-2',3'-riboepoxide **3.6**.*

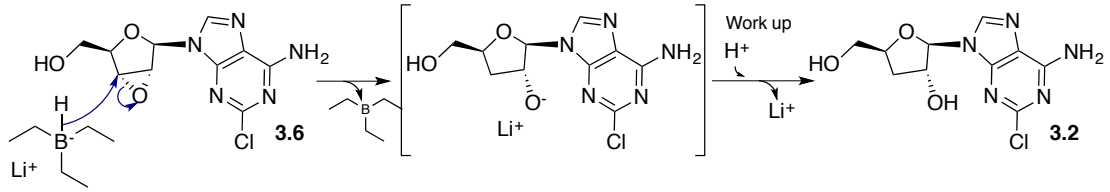
The conditions tested in order to optimise yields are summarized in *Table 3.1*. The best conditions were obtained using 10.0 g of starting material. It was observed that the yield increased with the amount of 2-chloroadenosine **3.5**.

Table 3.1 Summary of the quantity of 2-chloroadenosine **3.5** and reaction times for steps 1 (1) and 2 (2) to achieve the 1,2-riboepoxide **3.6** in different yields.

Compound	Attempt	3.5	Time	Yield
3.6	A	0.2 g	(1) 2 h, (2) 4 h	33 %
3.6	B	1.0 g	(1) 2 h, (2) 20 h	54 %
3.6	C	3.8 g	(1) 2 h, (2) 23 h	41 %
3.6	D	5.0 g	(1) 2 h, (2) 3 h	60 %
3.6	E	10 g	(1) 2 h, (2) 3 h	65 %

The next reaction was the reduction of the epoxide to 2-chlorocordycepin **3.2** using lithium triethylborohydride. In 1980 Brown *et al*. described the regio and stereoselectivity of the process that takes place.[44] Under these conditions, the reaction yields exclusively the Markovnikov alcohol: the reducing agent attacks the

less hindered position of the epoxide **3.6**, position 3', releasing the alcohol at position 2', and therefore giving one molecule of 2-chlorocordycepin **3.2** (Scheme 3.12).



Scheme 3.12 *Proposed mechanism of reaction of lithium triethylborohydride and 2-chloroadenosine-2', 3'-riboepoxide* ***3.1*** *leading to 2-chlorocordycepin* ***3.2****.*

In the literature this reaction is described to yield about 90 % for non-chlorinated analogues.[5] Here, when the reaction was performed with the chlorinated species, the best achieved yield for 2-chlorocordycepin **3.2** was 69 %, observing nonetheless that the yield improved by increasing the quantity of **3.6**. In the first batch of 2-chlorocordycepin **3.2B**, a salt from the lithium hydride was observed after the work up of the product. Low reactivity was noticed when **3.2B** was used to generate ProTides (compounds **3.9A**, **3.9B**, **3.10**, **3.11**, and **3.12**, (*Table 3.3*). In the following reactions, **3.2** was purified by filtration on silica gel for three times, to keep the salt was trapped in the silica. After this process an improvement in reactivity was seen (compounds **3.9C**, **3.13**, **3.14** and **3.15**, *Table 3.4*). The filtration on silica gel was not enough to remove the salt when the reaction was performed in a bigger scale. Purification by recrystallization in methanol achieved the pure compound (**3.2E**). *Table 3.2* summarises the different conditions tested to achieve 2-chlorocordycepin.

Table 3.2: Summary of the quantity of starting material (SM) and time used to achieve 2-chlorocordycepin.

Compound	Attempt	Conditions	Purification	Yield
3.2	A	**3.6** = 64 mg; 4 eq $LiEt_3BH$ 24 h	-	No reaction
3.2	B	**3.6** = 800 mg; 10 eq $LiEt_3BH$ 20 h	-	22 %
3.2	C	**3.6** = 2180 mg; 4 eq $LiEt_3BH$ 3 h	Filtration on silica gel	55 %
3.2	D	**3.6** = 1655 mg; 4 eq $LiEt_3BH$ 20 h	Filtration on silica gel	69 %
3.2	E	**3.6** = 6112 mg; 4 eq $LiEt_3BH$ 20 h	Recrystallisation	62 %

The identity of 2-chlorocordycepin was established by mass spectroscopy and from its ^{1}H NMR and ^{13}C NMR spectra in deuterated methanol (CD_3OD). COSY and

HSQC NMR experiments correlated each proton and carbon in the molecule, proving the formation of the hydroxyl group at position 2'. Figure 3.3 shows the spectra of 2-chloroadenosine **3.5** and 2-chlorocordycepin **3.2**. The formation of two H at position 3' is observed. As the conformation of the sugar changes again, a narrow doublet corresponding to the proton at position 1' is perceived, instead of the singlet in the epoxide, or the triplet that according to the literature shows for the isomer 2'-deoxy-2-chloroadenosine (Cladribine).[45] Only one singlet at 8.41 ppm (in CD_3OD) for the proton at position 8 in the base means that position 2 was substituted.

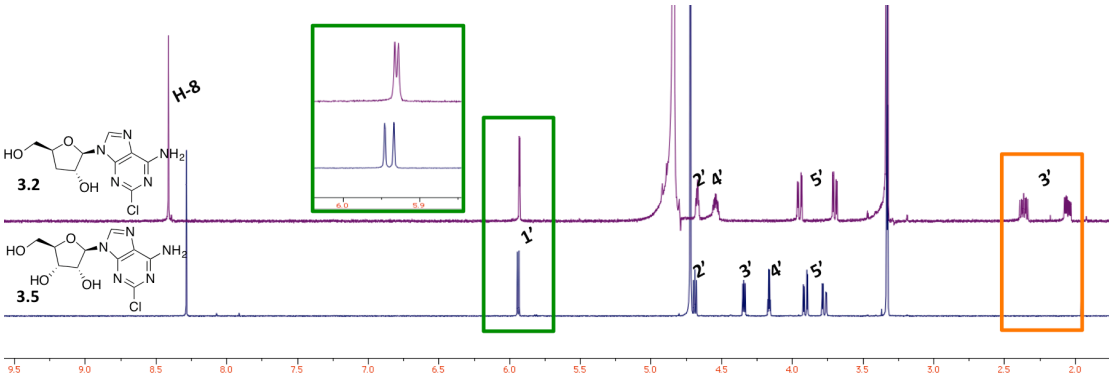


Figure 3.3 *Comparison of the ^{1}H NMR Spectra in CD_3OD of 2-Chloroadenosine and 2-Chlorocordycepin* ***3.2***

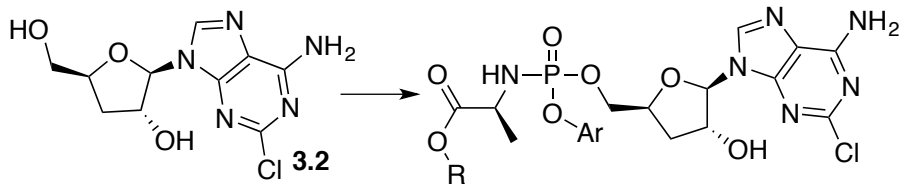
In the mass spectrum, two main peaks at 286 [M+H$^+$] and 308 [M+Na$^+$] showed that the nucleoside bears a chlorine atom. Additional peaks at 288 [M+H$^+$] and 310 [M+Na$^+$] indicate the presence of the other chlorine isotope (^{37}Cl). Chlorine has two main isotopes whose abundance are 75.78% for ^{35}Cl and 24.22% for ^{37}Cl. A similar pattern of signals was found in the mass spectra of all other the synthesised chloro-compounds.

Synthesis of 2-chlorocordycepin ProTides

Three strategies were used to synthesise 2-chlorocordycepin ProTides: 1) using NMI reagent, 2) using Grignard reagent and 3) using Grignard reagent with selectively protected 2-chlorocordycepin. These strategies are described in the following sections.

Using NMI reagent

The first approach was to use NMI reagent since it is the preferred method for NA with several hydroxyl groups (Scheme 3.13). The first attempts for ProTiding 2-chlorocordycepin (**3.2**) using N-methylimidazole (NMI), summarised in *Table 3.3*, resulted in either no product formation or very low yields. From those attempts only one product was isolated to perform biological tests.



	R	Ar
3.9	Neop	Naph
3.10	Benz	Naph
3.11	Ethyl	Naph
3.12	Hex	Naph
3.13	Neop	Ph
3.14	Hex	Ph
3.15	Octyl	Ph

Scheme 3.13 *First approach of the synthesis of 2-Chlorocordycepin ProTides. Reagents and conditions: NMI (5 eq), appropriate phosphorochloridate (3 eq), anhydrous THF, RT, 20 h.*

Table 3.3: Summary of the coupling reactions performed using NMI reagent and the first batch of 2-chlorocordycepin

Comp.	3.2	Yield	^{31}P NMR Shift	EI/MS [M+H$^+$]
3.9 A	100 mg	-	4.30, 4.15 ppm	633.2
3.9 B	150 mg	1 %	4.30, 4.15 ppm	633.2
3.10	100 mg	3 %	4.39, 4.12 ppm	653.2
3.11	90 mg	3 %	4.31, 4.21 ppm	591.1
3.12	150 mg	Traces	-	647.2

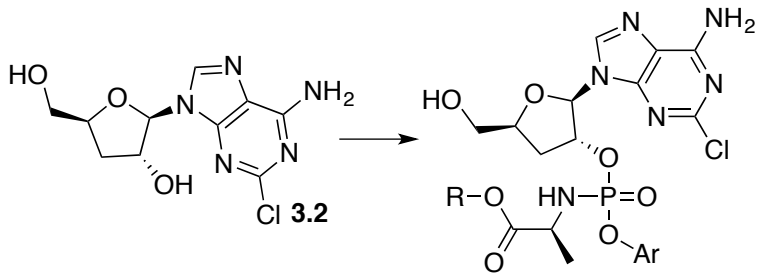
The following reactions were performed with repurified batches of **3.2** to improve the reactivity of the coupling reaction. Also increased amount of **3.2** was used in the reactions. Those changes significantly improved the yield of the reaction that increased over 5-10 fold from 3 % to 15-34 % as shown in *Table 3.4*.

Table 3.4: Summary of the coupling reactions performed using NMI reagent and the second repurified batch of 2-chlorocordycepin.

Comp.	3.2	Yield	^{31}P NMR Shift
3.9 C	350 mg	34 %	4.35, 4.20 ppm
3.13	350 mg	25 %	3.93, 3.72 ppm
3.14	288 mg	15 %	3.94, 3.74 ppm
3.15	339 mg	28 %	3.98, 3.76 ppm

Using Grignard Reagent

Considering the obtained results with the NMI reagent and aiming to achieve better yields with lower amount of starting material, the use of a stronger base to enhance the reactivity was explored. Therefore, the reaction was performed using the Grignard reagent *t*BuMgCl (Scheme 3.14).



	R	Ar
3.16	Benz	Naph
3.17	Benz	Ph

Scheme 3.14 *Second approach of the synthesis of 2-Chlorocordycepin ProTides. Reagents and conditions Appropriate phosphorochloridate (2 eq), tBuMgCl (1 eq), THF, RT, 20 h.*

Under these conditions, the reactions went to completion in a shorter amount of time, however in no case was a disappearance of the whole amount of starting material observed (*Table 3.5*).

Table 3.5: Summary of the coupling reactions performed using Grignard reagent. Only 2' ProTides were achieved.

Entry	3.2	Yield	Observations	EI/MS [M+H$^+$]	^{31}P NMR
3.16	200 mg	7 %	2' ProTide achieved	653.2	3.93, 3.72 ppm
3.17	211 mg	7 %	2' ProTide achieved	603.1	3.01, 2.41 ppm

^{13}C NMR and HSQC analysis proved that the 2' regio isomer was formed instead of the desired 5'-ProTide. Figure 3.4 shows the ^{13}C NMR of compound **3.16**. A doublet signal (one doublet for each diastereoisomer) for C-2' showed the P-O-C coupling and for C-1' the long-range P-O-C-C coupling with the phosphorus atom of

the ProTide. It was also noted that compared to the 5'-Protides obtained using the NMI reagent, the ^{31}P NMR signals for 2'-Protides were shifted upfield.

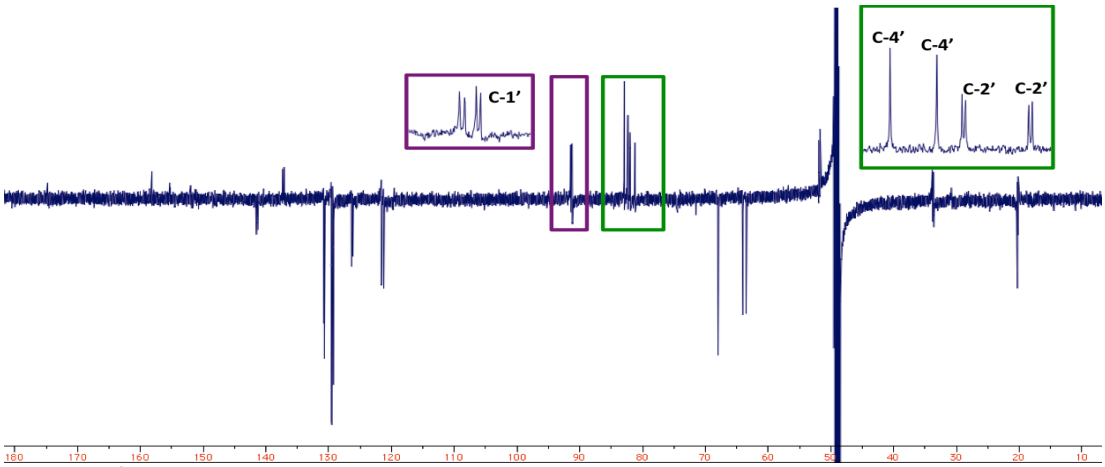
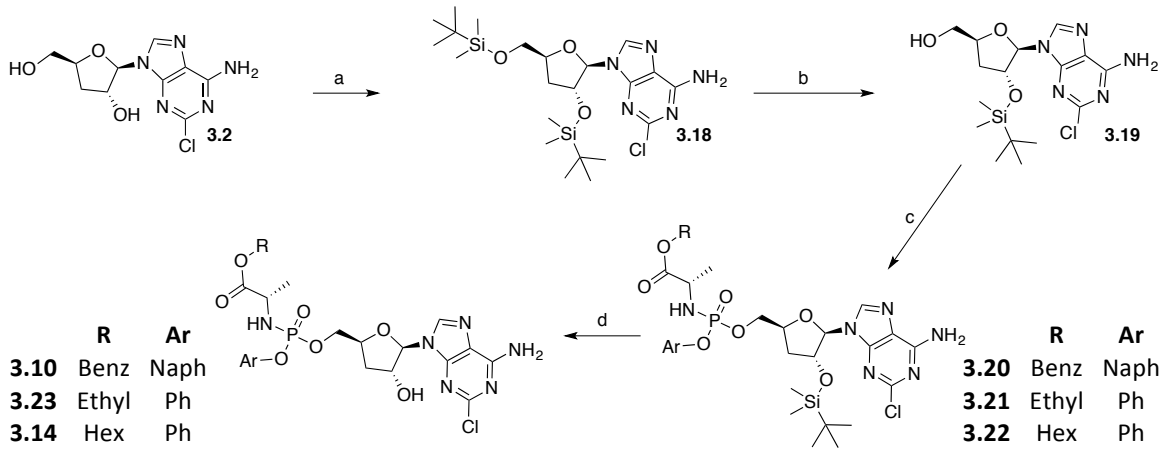


Figure 3.4 *^{13}C NMR spectra of compound **3.16**. Detail of doublet signals formed for C-1' and C-2' because of the long range coupling with the phosphorus atom*

Since those species would not deliver the nucleoside 5'-monophospate into the cells to mimic the natural counterparts, they were not tested for biological assays and a different approach, using selectively protected 2-chlorocordycepin was explored.

Using Grignard Reagent on selective protected 2-chlorocordycepin:

This strategy was designed to afford 5'-ProTides by using a Grignard Reagent on selectively protected 2-chlorocordycepin (Scheme 3.15). It first required the protection of both hydroxyl groups in 2-chlorocordycepin **3.2** with *tert*butyldimethylsilyl (TBDMS) groups delivering the bis-silylated compound **3.18**, followed by the selective deprotection of the hydroxyl at position 5' (compound **3.19**). Next, a coupling reaction using *t*BuMgCl were to deliver the protected 5' ProTide. A final deprotection step would then give the desired final product. The TBDMS group was chosen because it is easily introduced with different reagents, it is stable to a variety of organic reactions especially towards bases, and it can be selectively removed under conditions that would not affect other functional groups.[46,47]



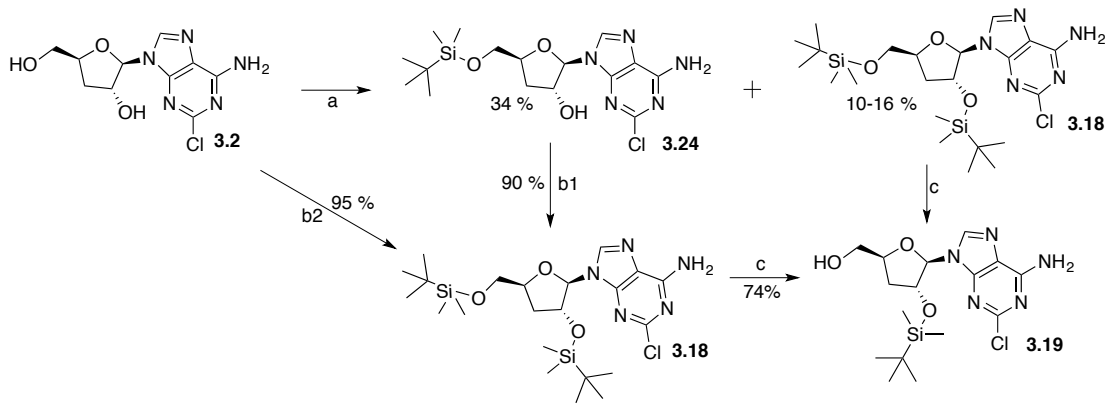
	R	Ar
3.10	Benz	Naph
3.23	Ethyl	Ph
3.14	Hex	Ph

	R	Ar
3.20	Benz	Naph
3.21	Ethyl	Ph
3.22	Hex	Ph

Scheme 3.15: *Synthesis of 2-Chlorocordycepin ProTides by selectively protecting position 2' and performing the coupling reaction with a Grignard reagent as a base. Reagents and conditions: (a) TBDMS Cl, Pyridine, RT; (b) TFA-H_2O-THF (1:1:4) 0 ˚C; (c) Phosphorochloridate, tBuMgCl, THF (d) TFA-H_2O-THF (1:1:1) RT.*

The attempts to achieve compound **3.18** are summarised in Scheme 3.16. The protection of both hydroxyl groups with TBDMSCl was first performed following a protocol by Halligan *et al*. [46] where pyridine was used as a base and solvent during the reaction (a). Under these conditions the bis-silylated compound **3.18** was achieved in 10-16% yield. The main product in this reaction was the 5'-monosilylated derivative **3.24,** which was achieved in a 34 % yield. Silylation at position 5' was proved due to the ^{1}H NMR spectrum in $CDCl_3$ showing the free proton of the hydroxyl group at position 2'. The preferential formation of the 5' protected derivative instead of the 2' or bis-silylated species can be explained by the low reactivity of the secondary alcohol at position 2' as a nucleophile. In the reaction 5'-hydroxyl group would react with the silicon atom, and pyridine would accept the proton formed in the reaction.

Further investigations were done to set optimal conditions. It was found in the literature that a solution of imidazole in *N,N*-dimethylformamide (DMF) should deliver the best yields for adenosine deoxynucleosides in this reaction.[47,48] DMF, as an aprotic solvent, facilitates reactions that follow polar mechanisms, such as SN2. Imidazole reacts with TBDMSCl, and then both hydroxyl groups can displace the imidazole from the intermediate. A first try under these conditions were performed with the 5'-monosilylated compound **3.24** achieving 90 % yield for compound **3.18** (b1). The actual reaction with 2-chlorocordycepin **3.2** (b2) achieved the bis-silylated compound in quantitative yield.



Scheme 3.16: *Methods used for the synthesis of 2',5'-bis silylated 2-chlorocordycepin. Reagents and conditions: (a)TBDMSCl, Pyridine, 20 h; (b) TBDMSCl, Imidazole, DMF, 20 h (c) TFA/H_2O/THF (1:1:4) 0 ˚C, 3h.*

Selective deprotection of the hydroxyl group at position 5' was then performed. It had been reported that best condition to selectively desilylate the 5' position in a 2', 3', 5' silylated adenosine molecule is dissolving the starting material in a solution of trifluoroacetic acid (TFA), water and THF in a ratio of (1:1:4) at 0 ˚C for 3 hours.[49] Those conditions were applied to 2',5'-bis-silylated 2-chlorocordycepin **3.18** resulting in a successful reaction yielding 72 – 74 % of the 2' monosilylated species **3.19 (**Scheme 3.16, (c)).

Coupling reaction using *t*BuMgCl and three different phosphorochloridates afforded three protected 5'-ProTides yielding 28-51 % as shown in *Table 3.6*.

Table 3.6: Summary of the coupling reactions performed on 2' protected 2-chlorocordycepin **3.19** using Grignard reagent.

Compound	3.19	Yield
3.20	91 mg	51 %
3.21	204 mg	28 %
3.22	204 mg	48 %

Total desilylation of the achieved ProTides was then performed using stronger conditions than those to selectively deprotect 2-chlorocordycepin. The silylated ProTides were dissolved in 1mL of THF and a solution of TFA-H_2O (5.5:4.5) was added at 0 ˚C and was slowly allowed to reach RT. After 24 h, the reactions showed no further progression even if TLC showed there was still some starting material left. The yields of these reactions were lower than expected at 52 – 66 %, delivering an overall yield of 13 – 21 % (*Table 3.7*). Therefore this method was

considered to be less successful than the second strategy using NMI, because it afforded lower yields and is a longer process.

Table 3.7: Summary of the deprotection of the silylated ProTides. Overall yield through all the process.

Compound	Starting Material	Yield	Overall Yield
3.10	**3.20** = 90 mg	52 %	18 %
3.23	**3.21** = 93 mg	66 %	13 %
3.14	**3.22** = 180 mg	62 %	21 %

Cell viability assays

Compounds **3.9**, **3.10**, **3.13**, **3.14**, **3.15**, **3.23**, the parent nucleoside **3.2** (2-ClCordy), and a diamidate compound from the next chapter **4.1** were selected for a initial screening on cell viability performed by WuXi AppTec to assess the cytotoxicity of these prodrugs in comparison to 2-chlorocordycepin (2-ClCordy), using paclitaxel as a standard control (Table 3.8).

Table 3.8: Compounds tested in a cell viability screening performed by WuXi AppTec. Log P values generated by a computer-based predictive algorithm using ChemDraw Professional 15.0.

Compound	Moieties	Log P
2-ClCordy		0.22
3.9	Neop-*L*-Ala-Naph	5.27
3.10	Bz-*L*-Ala-Naph	5.33
3.13	Neop-*L*-Ala-Ph	4.27
3.14	Hexyl-*L*-Ala-Ph	4.55
3.15	Octyl-*L*-Ala-Ph	5.34
3.23	Ethyl-*L*-Ala-Ph	2.89
4.1	Bz-*L*-Ala	4.25

The cytotoxicity of the compounds was tested against a diverse sample of blood cancer cells measuring the cell viability of leukemic (the main indication of cordycepin), lymphoma, and myeloma cell lines, when they were exposed to the compounds. Also, a broader screening including pancreas, colon, liver, breast, head and neck, and bladder cancer cells was performed. (Table 3.9)

Table 3.9: Cell lines used for the *in vitro* cell viability assay.

Cell Line	Malignancy	
CCRF-CEM	Leukaemia	Acute Lymphoblastic Leukaemia
MOLT-4	Leukaemia	Acute Lymphoblastic Leukaemia
K562	Leukaemia	Chronic Myelogenous Leukaemia
HEL92.1.7	Leukaemia	Erythroleukaemia
KG-1	Leukaemia	Acute Myelogenous Leukaemia
MV4-11	Leukaemia	Biphenotypic B Myelomonocytic Leukaemia
HL-60	Leukaemia	Acute Promyelocytic Leukaemia
Thp-1	Leukaemia	Acute Monocytic Leukaemia
Z-138	Leukaemia	Mantle Cell Lymphoma
RL	Myeloma	Non-Hodgkin's Lymphoma
Jurkat	Myeloma	Acute T Cell Leukaemia
Hs-445	Myeloma	Hodgkin's Lymphoma
RPMI-8226	Myeloma	Peripheral Blood Plasmacytoma
NCI-H929	Myeloma	Bone Marrow Plasmacytoma
Mia-Pa-Ca-2	Pancreas	Pancreas Carcinoma
BxPC-3-Luc	Pancreas	Pancreas Adenocarcinoma
HT29	Colon	Colorectal Adenocarcinoma
SW620	Colon	Colorectal Adenocarcinoma. Derived From Metastatic - Lymph Node Dukes' Type C
HepG2	Liver	Hepatocellular Carcinoma
MCF-7	Breast	Breast Adenocarcinoma. Derived From Metastatic Site: Pleural Effusion Epithelial -Mammary Gland
Cal27	Head and Neck	Squamous cell carcinoma

9 serial doses of the compounds using DMSO as a solvent were prepared. The highest concentration was 198 µM and the lowest concentration was 0.0199 µM. The selected cell lines were incubated with the different doses of the compounds over 72 hours, and then the cell viability was measured. The resulting dose-response curve was analysed using XL-fit software. The half maximal effective concentration (EC_{50}) and Top Inhibition percentage (percentage of non-viable cells at the highest concentration of tested for compound, TI %) were stated for each compound for every cell line as shown in Table 3.10.

Table 3.10: Cytotoxic activity in μM of 2-chlorocordycepin and prodrugs against haematological cancer cell lines

	Leukaemia													
	CCRF-CEM		MOLT-4		K562		HEL92.1.7		KG-1		MV4-11		HL-60	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
2-ClCordy	>198	38	>198	57	>198	39	82	56.0	>198	-0.2	65	98	104	62
3.9	15	99	9	100	18	99	6	104	25	100	5	100	13	100
3.10	9	100	10	100	24	101	8	100	30	99	6	101	14	102
3.13	67	98	45	99	81	82	29	96	64	90	1	100	66	95
3.14	80	94	36	99	>198	7	89	82	-	-	-	-	190	53
3.15	86	83	52	99	>198	22	107	58	-	-	-	-	100	70
3.23	43	98	58	124	57	87	74	92	75	71	9	100	95	68
4.1	72	87	42	99	>198	50	59	74	-	-	-	-	116	70
Paclitaxel	0.006	97	0.006	99	0.02	83	0.1	75	-	-	-	-	0.01	98

	Leukaemia				Lymphoma						Myeloma			
	Thp-1		Z-138		RL		Jurkat		Hs-445		RPMI-8226		NCI-H929	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%
2-ClCordy	>198	8	>198	42	>198	39	>198	3	>198	<50	>198	36	103	68
3.9	31	99	8	100	12	100	26	101	27	102	33	100	19	99
3.10	63	97	60	90	11	100	-	-	40	106	29	100	7	102
3.13	84	90	39	97	49	85	100	67	>198	<50	83	99	74	77
3.14	-	-	189	55	176	54	-	-	>198	28	79	97	-	-
3.15	-	-	101	67	>198	53	-	-	>198	27	>198	50	-	-
3.23	97	72	62	78	40	85	>198	36	198	<50	176	51	60	77
4.1	-	-	28	103	94	79	-	-	87	82	6	95	-	-
Paclitaxel	-	-	0.004	99	0.01	79	-	-	0.005	89	0.003	96	-	-

2-Chlorocordycepin **3.2** was inactive in most of the blood cancer cell lines, where the EC_{50} value was higher than the highest concentration tested (198 μM). Moderate activity was found in HEL92.1.7, HL-60, and NCI-H929 cell lines where 2-chlorocordycepin at the highest concentration inhibited the viability of the cells from 56 to 67 % and the EC_{50} was found to lie between 82 – 103 μM. 2-Chlorocordycepin exhibited a different behaviour in the cell line MV4-11. This was the only case where top inhibition reached 100 %. The EC_{50} for this cell line was 65 μM, the lowest for 2-chlorocordycepin among all tested cell lines.

A general boost of the activity of the ProTides compared to the parent nucleosides was observed. The most active compounds characterised by the lowest

EC_{50} in the series were **3.9** and **3.10**. The EC_{50} of **3.10** ranges from 5.73-63 μM and the EC_{50} of **3.9** ranged from 4.77-33 μM. The activity of the diamidate compound **4.1** was comparable to **3.10** and **3.9** in the cell line Z-138, while it was slightly more active and in the cell line RPMI-8226. Compounds bearing a long aliphatic chain **3.14** (hexyl) and **3.15** (octyl) were generally the ProTides with the lowest measured activity.

In terms of improving the activity of the parent nucleoside, ProTides **3.9** and **3.10** generally were also the most active compounds. **3.9** improved the activity of 2-chlorocordycepin 25-fold in Z-138, 23-fold in MOLT-4 cells and 16-fold in RL cells. **3.10** improved the activity 21-fold for CCRF-CEM cells, 19-fold for MOLT-4 cells, and 18-fold for RL cells. It is worth pointing out that **3.13,** which in most of the cell lines showed intermediate activity, was found to be the most active tested compound in the cell line MV4-11 (especially sensitive to this series of compounds) improving the activity of the parent nucleoside 45 times. The diamidate compound **4.1** was found to be the most active in the cell line RPMI, improving the activity 31-fold compared to 2-chlorocordycepin.

2-Chlorocordycepin and the tested compounds followed a similar trend of activity when they were tested in solid tumours (Table 3.11).

Table 3.11: Cytotoxic activity in μM of 2-chlorocordycepin and prodrugs against solid tumour cancer cell lines

	Pancreas Mia-Pa-Ca-2		Pancreas BxPC-3-Luc		Colon HT29		Colon SW620		Liver HepG2		Breast MCF-7		H&N Cal 27	
	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%	EC_{50}	TI%
2-ClCordy	>198	4	>198	-0.5	>198	3	>198	4	>198	10.2	>198	2.4	>198	-2
3.9	35	99	79	98	69	98	40	101	66	98	33	101	54	99
3.10	31	98	71	82	72	88	37	92	56	85	31	101	-	-
3.13	140	56	>198	9	>198	33	155	57	>198	42	174	58	114	57
3.14	95	75		-	>198	52	-	-	>198	39	127	67	177	53
3.15	103	84	-	-	126	60	-	-	114	66	93	81	155	57
3.23	>198	50	>198	13	>198	15	>198	44	>198	55	>198	42	>198	25
4.1	16	93	-	-	>198	47	-	-	168	55	91	58	146	60
Paclitaxel	0.004	83	-	-	0.004	75	-	-	0.09	51	0.004	79	0.002	94

The parent nucleoside was found to be totally inactive. The percentage of the Top inhibition ranged -1.8 % to 10.2 %, and the estimated EC_{50} value always exceeded the highest concentration 198 μM.

As in the case of haematological malignancies, all ProTides increased the activity of the parent nucleoside in the solid tumour cells. The best compounds in the series, characterised by the lowest EC_{50}, were again **3.9** and **3.10** whose EC_{50} ranged between 31 and 79 μM, with high values (~100 %) of top inhibition. Compound **3.9** improved the activity of the parent nucleoside 6-fold for MCF-7 cells and 5-fold for Mia-Pa-Ca-2 cells. Similarly compound **3.10** improved the activity 6-fold both in MCF-7 and Mia-Pa-Ca-2 cells. It is noted that the diamidate compound **4.1** showed the highest activity in the Mia-Pa-Ca-2 cell line, improving the activity of 2-chlorocordycepin 12 times.

The potency of **3.9** in the different cell line was found as follows in descending order: MV4-11 > HEL92.1.7 > Z-138 > MOLT-4 > RL>HL-60 > CCRF-CEM > K562 > NCI-H929 > KG-1 > Jurkat > Hs-445 > Thp-1 > RPMI-8226 > MCF-7 > Mia-Pa-Ca-2 > SW620 > Cal27 > HepG2 > HT29 > BxPC-3-Luc.

The potency of **3.10** in the different cell line was found as follows in descending order: MV4-11 > NCI-H929 > HEL92.1.7 > CCRF-CEM > MOLT-4 > RL > HL-60 > K562 > RPMI-8226 > KG-1 > MCF-7 > Mia-Pa-Ca-2 > SW620 > Hs-445 > HepG2 > Z-138 > Thp-1 > BxPC-3-Luc > HT29 > Jurkat.

These data suggest a preference in the activity of the compounds for haematological malignancies.

The compounds with the highest activity, **3.9** and **3.10**, share some similarities. They are the only ones in the series bearing a naphthyl moiety, and have similar lipophilicity according to the predicted Log P, algorithmically calculated using ChemDraw Professional 15.0 software (**3.9** LogP 5.27; **3.10** LogP 5.33). However the fact that compound **3.15**, one of the compounds which shows the lowest activity, has a similar theoretical lipophilicity (LogP 5.34) suggests that the naphthyl residue is more important for the activity than the lipophilicity of the compound.

Enzymatic assays

Carboxypeptidase Y

After the cell viability assays, ProTide **3.9** with high activity, ProTide **3.13** with medium activity and ProTide **3.14** with low activity were selected to be tested in a carboxypeptidase Y enzymatic assay (*Table 3.12*). This assay aims to analyse if the different performance of the ProTides is related to any difference in the first activation step of the prodrug. This is achieved by determining whether the 2-chlorocordycepin ProTides can be processed by the enzyme that was identified as being chiefly responsible for this first activation towards the monophosphorylated species.

Table 3.12*:* Compounds tested in Carboxypeptidase Y enzymatic assay

Compound	Moieties
3.9	Neop-*L*-Ala-Naph
3.13	Neop-*L*-Ala-Ph
3.14	Hexyl-*L*-Ala-Ph

As described in the second chapter, a carboxypeptidase enzyme (mainly Cathepsin A) initiates the intracellular release process of the nucleotide analogue. As human Cathepsin A is not commercially available, carboxypeptidase Y was chosen as a model enzyme. Both enzymes belong to the same family of C-type carboxypeptidases, and exhibit a high degree of structural homology around the catalytic site.[50]

The experiment measures the ^{31}P NMR shift of selected compounds in deuterated acetone and TRIZMA buffer at 25 ˚C. The first experiment provides the blank reference at time 0. Then after the addition of the enzyme, a ^{31}P NMR spectra is recorded every 15 minutes for 13 hours. Shifts in the signal indicate the formation of new species.

Two intermediate compounds are often observed in this experiment: the phosphoramidate after the cleavage of the ester moiety, and the aminoacyl

phosphoramidate intermediate after the subsequent cleavage of the aryloxy moiety. The aminoacyl phosphoramidate would then require another enzyme, a phosphoramidase, to finally release the monophosphorylated NA.

This experiment was first performed with 7 mg of **3.9**. In the blank sample containing the mixture of diastereoisomers, two signals are observed at 3.85, 3.62 ppm (A). After addition of the enzyme at minute 7, the formation of two small signals downfield (B, C) was noted, while one of the diastereoisomers slowly disappeared. Signal B corresponds to the hydrolysed intermediate (4.77 ppm), which quickly converted into the aminoacyl intermediate C (6.99 ppm) as seen in Figure 3.5. Interestingly it was observed that one of the diastereoisomers is more stable than the other, not showing any apparent metabolism by Carboxypeptidase Y after 13 h.

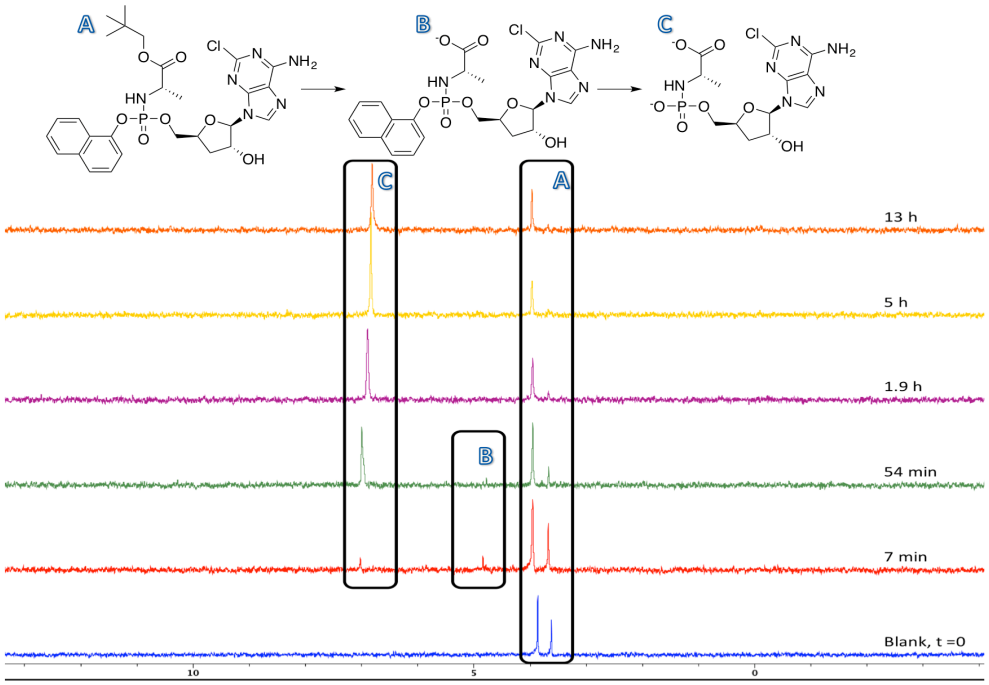


Figure 3.5: *^{31}P NMR enzymatic experiment of Compound **3.9***

The following experiment was performed with 3 mg of compound **3.13**. This experiment was performed with lower resolution and thus only shows the total conversion of the ProTide (3.85, 3.60 ppm) into the aminoacyl phosphoramidate (7.06) but not the hydrolysed intermediate. (Figure 3.6) Even though the enzyme

eventually processed both diastereoisomers of the ProTide, it was still observed that one of the diastereoisomers was more stable than the other.

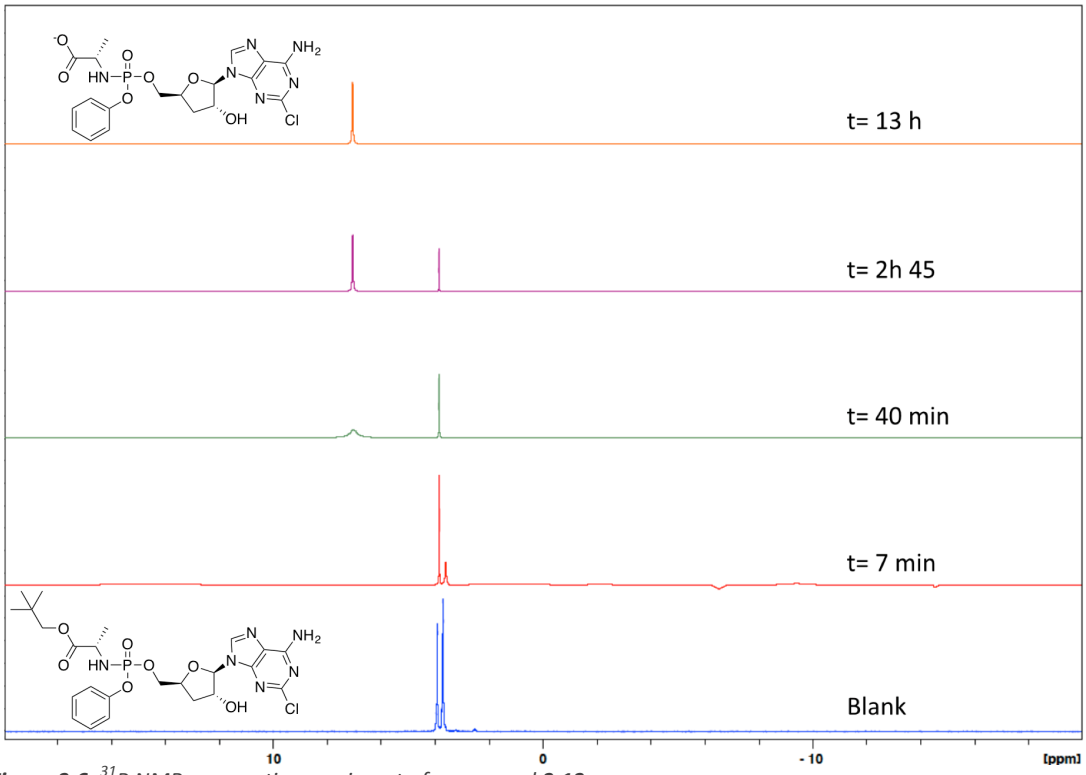


Figure 3.6: *^{31}P NMR enzymatic experiment of compound* ***3.13***

A final carboxypeptidase experiment was performed using 5 mg of compound **3.14**. Here, the metabolism of the two diastereoisomers of the ProTide (signal A, 3.84, 3.66 ppm) was observed without any apparent sterical preference, leading to the formation of the hydrolysed intermediate B (4.77 ppm), and the quick conversion into the aminoacyl intermediate C (7.07 ppm), (Figure 3.7).

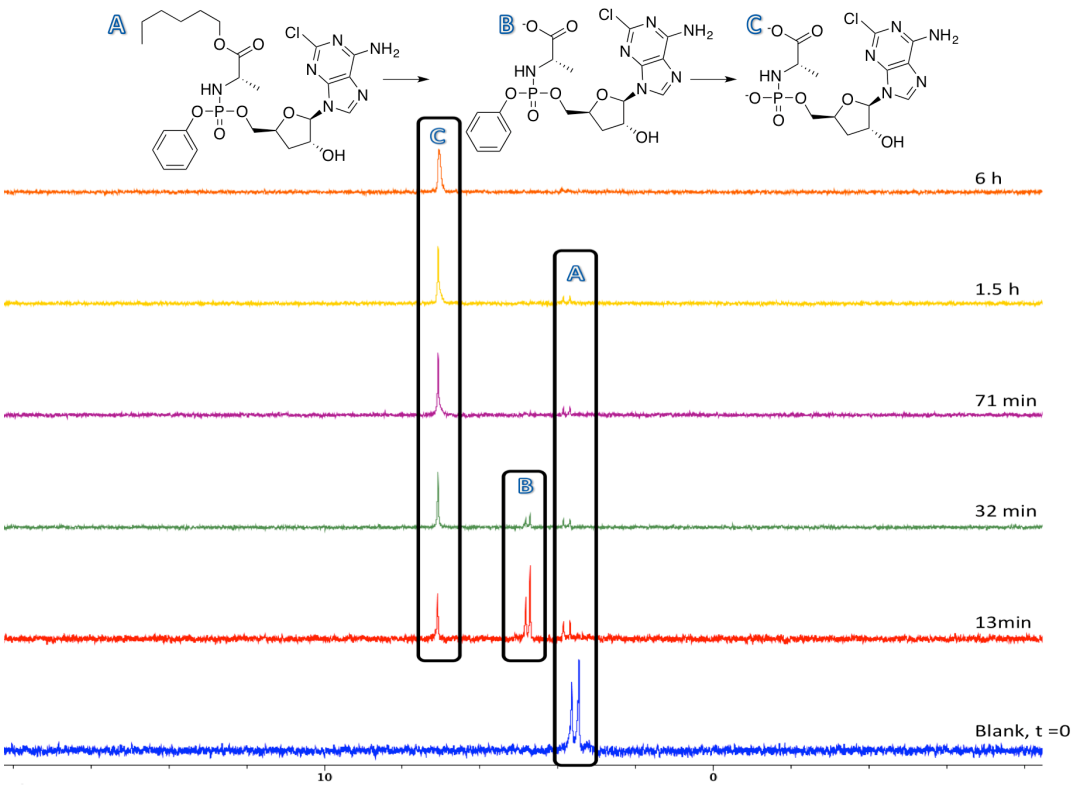


Figure 3.7: *^{31}P NMR enzymatic experiment of compound* ***3.14***

In the compounds **3.9** and **3.13** bearing a neopentyl moiety, a preferential metabolism for one of the diastereoisomers was observed, whereas the compound **3.14** bearing the hexyl moiety did not show any discrimination for either the Rp or Sp diastereoisomer. That may be due to the flexibility of the hexyl substituent in comparison to the neopentyl. The experiments showed that all three compounds were processed by the enzyme, except for one diastereoisomer of **3.9**, which surprisingly is also the compound that performed the best in the cell viability assay. This suggests two possibilities: The first one being that compound **3.9** would follow the pattern of compound **3.13** and that the more stable diastereoisomer would eventually be converted by the enzyme if the experiment was prolonged or more enzyme would be added. The second possibility is that one of the diastereoisomers does not get activated at all by carboxypeptidase Y. Then either other enzymes in the cell might perform the activation, or if this is not the case, the single activated isomer alone already leads to increased potency for this compound.

The selectivity of the carboxypeptidase enzyme towards one diastereoisomer of the ProTide mixture had already been described. The most remarkable example is

Sofosbuvir. This anti HVC ProTide contains only one diastereoiromer with an Sp configuration of the phosphorus centre. During the development of the drug, it was noted that the Sp diastereoisomer was more than 10-fold more active than the Rp analogue.[51] Studies to elucidate the mechanism of activation discovered that cathepsin A processed the Sp diastereoisomer 18-fold more efficiently than the Rp-diastereoisomer.[52] However, in our laboratory the synthesised ProTide compounds usually exhibited complete hydrolysis independently of their stereochemical configuration, as exemplified by NUC-3373,[53] NUC-1031.[32]

Adenosine deaminase assay

The resistance of 2-chlorocordycepin and **3.9** (one of the most active ProTides in the cell viability assay) against ADA metabolism was tested using cordycepin (synthesized following the method by Rosowsky *et al.*[35]) as a reference compound.

In the literature it is well established that cordycepin undergoes degradation by ADA to 3'-deoxyinosine.[24,38] One of the aims of this work is to bypass this drawback by inserting a substituent at position 2 and applying the ProTide approach. This assay will establish if those modifications were successful.

During the assay a UV spectrophotometer recorded the spectra of 1 ml of a 40 µM solution of each compound in phosphate buffer pH 7.5 before the addition of the enzyme. Then 0.0027 U of human ADA were added and spectra were recorded in one-minute intervals for 3 hours and then every 15 minutes for 24 hours.

The first experiment aimed to validate the assay and to be used as a reference for the subsequent experiments. For this, cordycepin (**3.1**) was incubated with ADA to create a reference experiment. The results in Figure 3.8 show that the maximum absorbance in the UV spectrum shifts from 259.0 nm to 249.0 nm. This shift of 10 nm when the compound was exposed to the enzyme showed the formation of a new species. This is in accordance with the literature[24,38] where it was established that the newly forming species is the corresponding inosine **3.3**. The shift started after one hour of incubation of cordycepin with the enzyme and it was

stabilized at 249.0 nm after 8 hours, when all starting material was converted to 3'-deoxyinosine **3.3**.

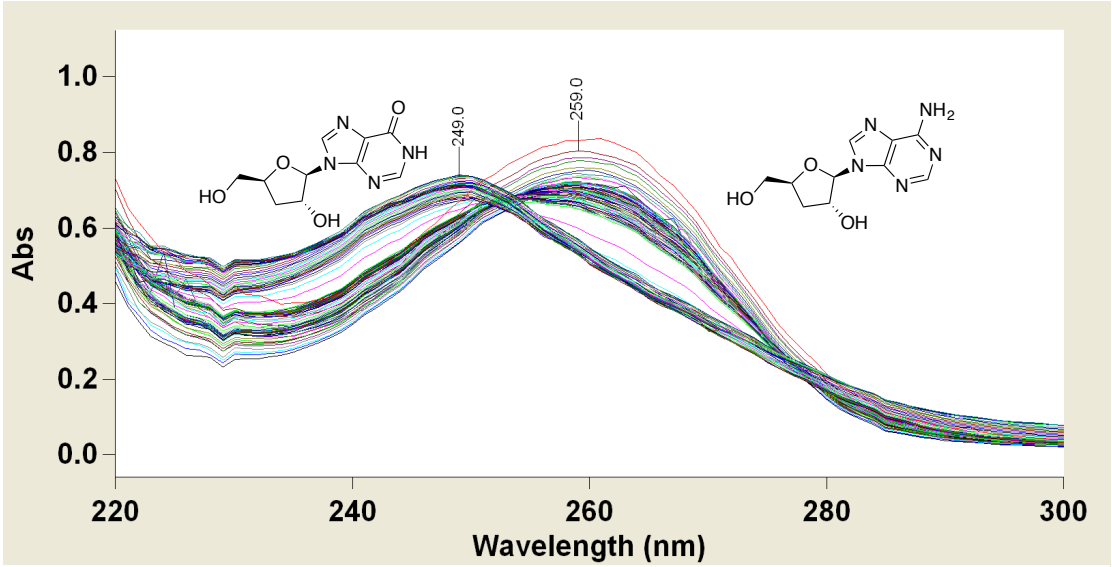


Figure 3.8: *Spectral change observed by UV spectrophotometer recording every minute for one hour and then every 15 minutes for 12 h using 1 ml of cordycepin 40 µM in phosphate buffer pH 7.5 at RT. At time 0 0.0027 U of human ADA are added.*

In the second experiment 2-chlorocordycepin **3.2** was incubated using the same conditions (Figure 3.9). The maximum wavelength for the compound was 265.0 nm. No spectral change observed in UV spectrophotometer records after 24 h indicating a stability of the substrate to the ADA metabolism. This result was expected as other 2-halogenated purines such as Cladribine (2-chloro-2'-deoxyadenosine)[33] or 2-fluorocordycepin[38] showed this ADA resistance in the past.

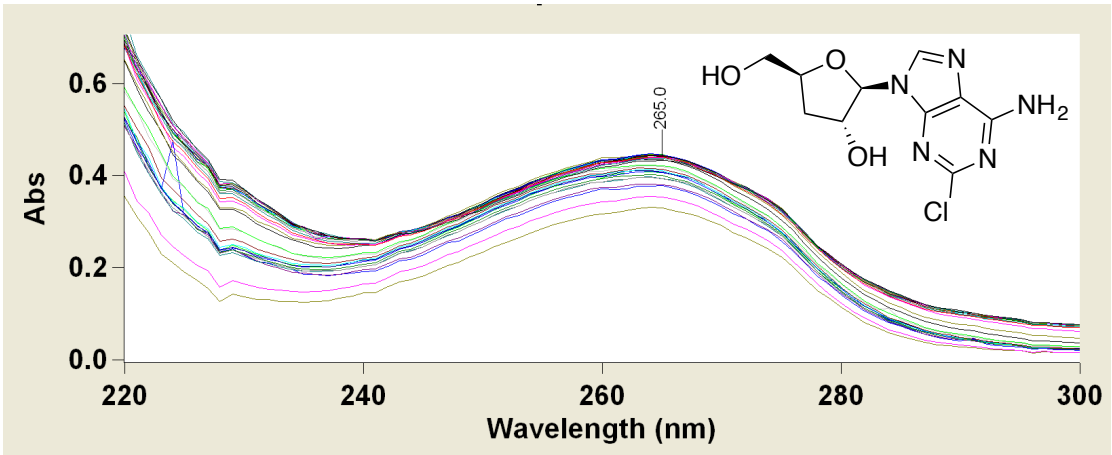


Figure 3.9: *No spectral change observed by UV spectrophotometer recording every minute for one hour and then every 15 minutes for 12 h using 1 ml of 2-chlorocordycepin 40 µM in phosphate buffer pH 7.5 at RT. At time 0 0.0027 U of human ADA were added.*

In the third experiment ProTide **3.9** was analysed in the same conditions (Figure 3.10). The ProTide showed a maximum wavelength of 265.9 nm, almost the same as the parent nucleoside. No spectral changes in UV spectrophotometer records over 24 h of incubation with ADA were observed, indicating the stability of the substrate to ADA metabolism.

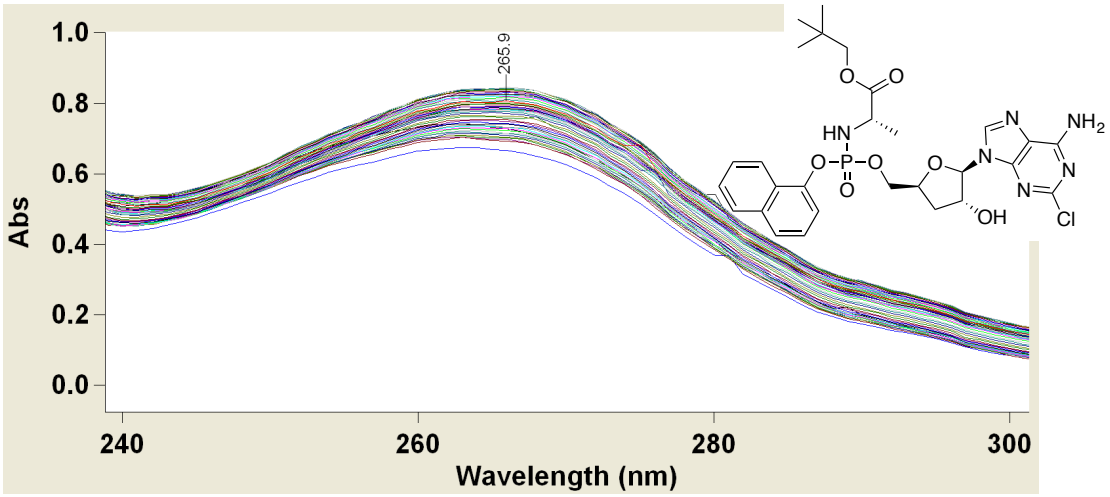


Figure 3.10: *No spectral change observed by UV spectrophotometer recording every 30 minutes for 24 h using 1 ml of ProTide **3.9** 40 µM in phosphate buffer pH 7.5 at RT. At time 0 0.0027 U of human ADA are added.*

These results show that the chlorination of cordycepin at position 2 and the ProTide approach provided the molecule with the expected resistance towards ADA degradation. Interestingly, even if 2-chlorocordycepin bypasses the major drawback of cordycepin, which is deamination, it was still inactive in most of the tested cell lines. This suggested that indeed the ProTide approach was necessary not only to provide additional resistance against deamination but especially to deliver the monophosphate into the cells.

Molecular Modelling

These experiments were performed in collaboration with Doctor Salvatore Ferla.

Molecular modelling studies were performed to gain understanding on the ester hydrolysis of the 2-chlorocordycepin ProTides in the first activation step, and to predict the conversion of the aminoacyl intermediate into 2-chlorocordycepin-5'-monohosphate by Hint-1 enzyme

Docking with Carboxypeptidase Y

During the carboxypeptidase Y enzymatic assay differences in the metabolism between the diastereoisomers of the ProTide **3.9** were observed. To better understand the enzymatic results, molecular modelling studies using the crystal structure of carboxypeptidase Y available in the protein data bank (PDB 1YSC)[54] were performed. According to Jung *et al.* (1999) the ester hydrolysis process, requires a suitable position of the carbonyl moiety in the enzyme pocket for the nucleophile attack from the catalytic residue Ser146. Gly52 and Gly53 need to be correctly placed to stabilize the tetrahedral intermediate formed (Figure 3.11).[54]

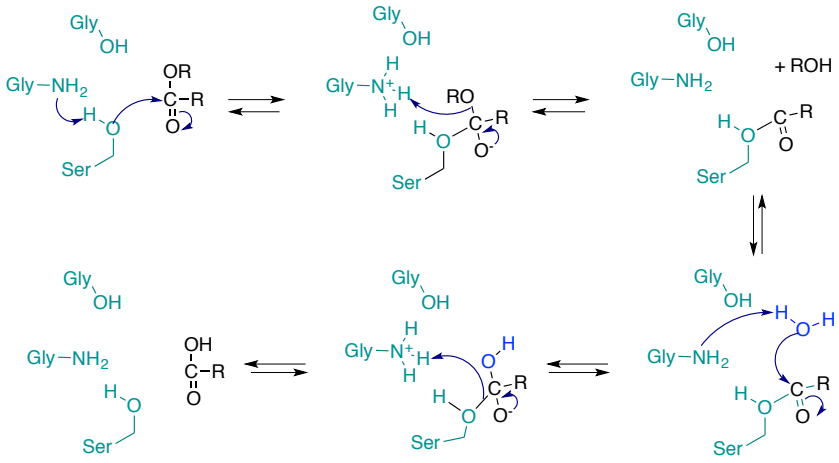


Figure 3.11 *Steps of decarboxylation of ProTides by carboxypeptidase Y.[55] The amino acid residues of the enzyme are coloured in green.*

The R diastereoisomer of ProTide **3.9** in the best docking pose did not place the carbonyl group in proximity to the Ser146 residue to allow nucleophilic attack. In addition it fitted tightly in the catalytic site (Figure 3.12).

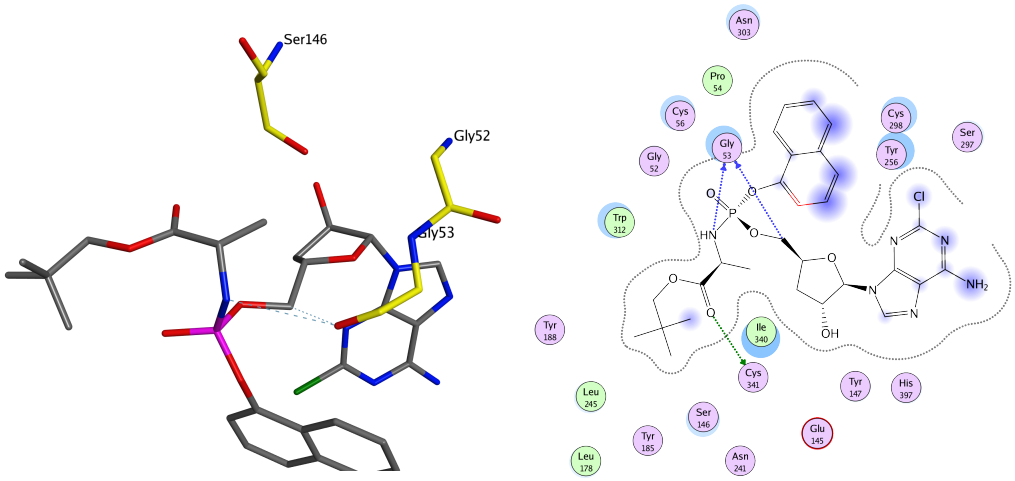


Figure 3.12 *Left: Ser146, Gly52 and Gly52 residues (yellow) in the site of action of Carboxypeptidase Y interacting with the R diastereoisomer of ProTide* ***3.9*** *(grey). Right: Interactions of R diastereoisomer of compound* ***3.9*** *in the pocket site of carboxypeptidase Y*

The S Diastereoisomer of ProTide **3.9** occupied the binding pocket with the carbonyl group in proximity of the Ser146, in an orientation that could allow the nucleophile attack (Figure 3.13)

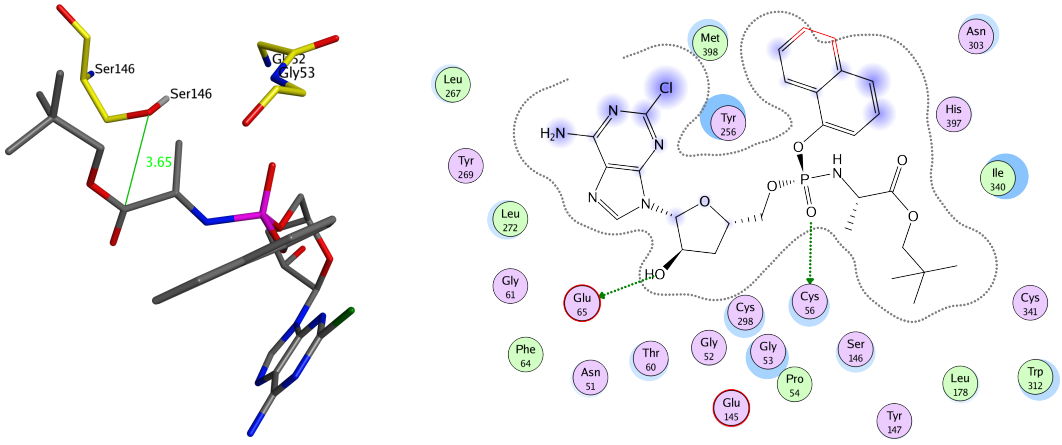


Figure 3.13 *Left: Ser146, Gly52 and Gly52 residues (yellow) in the site of action of Carboxypeptidase Y interacting with the S- diastereoisomer of ProTide* ***3.9*** *(grey). Right: Interactions of S diastereoisomer of compound* ***3.9*** *in the pocket site of carboxypeptidase Y*

Docking results taken along with the enzymatic experiments would also suggest a better interaction of one of the diastereoisomers with carboxypeptidase Y. The S diastereoisomer seems a potential good substrate for this enzyme, whereas the R diastereoisomer does not show a favourable docking pose for the proper interaction with the catalytic site of the enzyme. These results however, are not

conclusive because of the limitations of docking studies to considerate other potential factors (i. e. rate of processing, stability etc.) that may be involved in the ester hydrolysis process.

Docking with Hint-1 enzyme

The final step to release of the monophosphorylated NA inside the cells is catalysed by Human triad nucleotide-binding proteins (Hint).[55] They are responsible for breaking the P-N bond between the aminoacyl residue and the phosphate by interaction of histidine residues in the active site. Residues His112, His 114 and Ser 107 are important for the activity.[56]

Docking studies with aminoacyl phosphoramidate compound of 2-chlorocordycepin were carried out on human Hint-1 available in the protein data bank (PDB 1KPF) co-crystallised with a molecule of AMP (yellow). The docking results show the phosphate moiety of the aminoacyl intermediate in a suitable position for the cleavage of the P-N bond. Moreover, the phosphate and the adenosine lie in the binding pocket in a similar manner of the co-crystallised AMP (Figure 3.14)

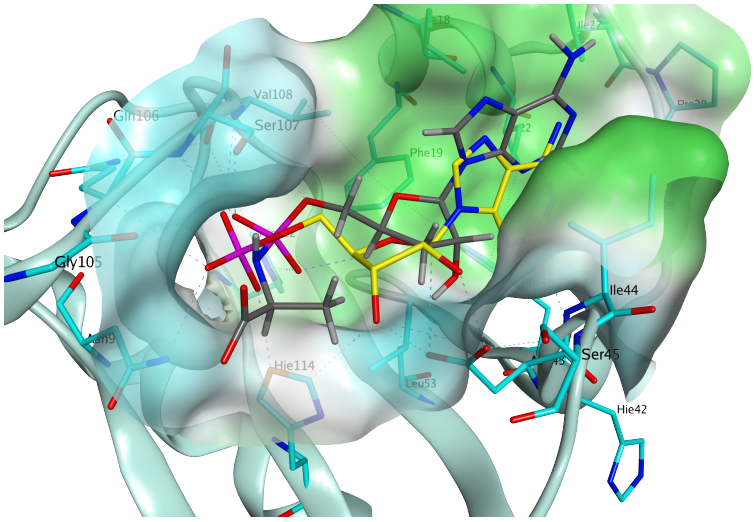


Figure 3.14 *L-alanilyl-2-chlorocordycepin phosphate (grey) and AMP (yellow) in the active site of Hint-1*

Figure 3.15 shows the interaction of the phosphate moiety with His112, and Ser107, and it is not far from His114, all of them responsible of the metabolism, suggesting that the last step would release 2-chlorocordycepin-5'-monophosphate.

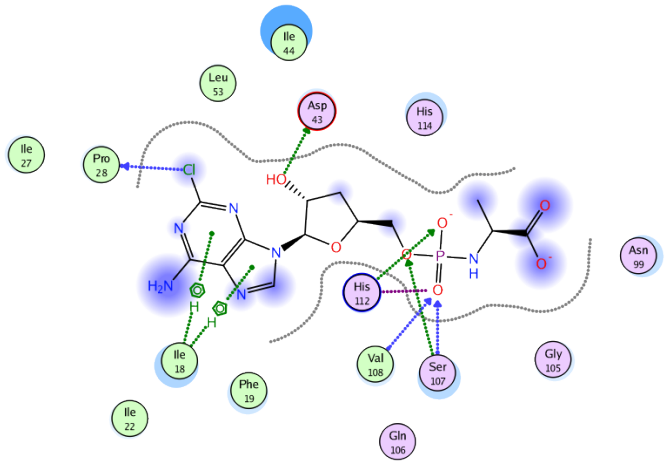


Figure 3.15 *Interactions of L-alanilyl-2-chlorocordycepin phosphate with the amino acid residues in the active site of Hint-1*

Conclusion

In this chapter, a new method for the synthesis of 2-chlorocordycepin was developed. This method improved the cost effectiveness of the methods reported previously in the literature. A small family of ProTides was synthesized from 2-chlorocordycepin using three different approaches. The NMI approach using re-purified cordycepin was found to provide the best yields in the overall process. These ProTides as well as the parent nucleoside 2-chlorocordycepin were then biologically evaluated against different cancer cell lines. Cell viability assays showed a general boost of the activity of the ProTides in comparison to the parent compound 2-chlorocordycepin. ProTides **3.9** and **3.10** were found to be the most active compounds, especially in leukemic cell lines CCRF-CEM, MOLT-4 and Z-138 (EC_{50} ~ 9 µM). It appears that the naphthyl residue is important for the activity of the compounds.

Enzymatic assays performed on compounds **3.9**, **3.13** and **3.14** showed that 2-chlorocordycepin ProTides are substrates for carboxypeptidase Y, and therefore are being activated to the monophosphate species. It was noted that for compounds **3.9**, **3.13** one of their respective diastereoisomers is more resistant against the activation. Docking studies suggested that the S diastereoisomer of ProTide **3.9** could be the best processed by the enzyme.

To elucidate the resistance of the compounds against deamination, one of the major aims of the modifications to the parent compound cordycepin, ADA essays were performed on cordycepin, 2-chlorocordycepin and compound **3.9**. The experiments determined that the modifications performed in the parent nucleoside by inserting a chlorine substituent at position 2 and by ProTiding position 5' endowed the compounds with resistance against deamination. However, the resistance innate to 2-chlorocordycepin was not enough to exert anticancer activity *in vitro*, suggesting that the introduction of the ProTide approach was required for this activity.

Overall, a variety of active compounds were synthesised, which undergo the first activation step to the monophosphate species and which are resistant to degradation by the ADA enzyme, indicating the successful completion of this part of the work.

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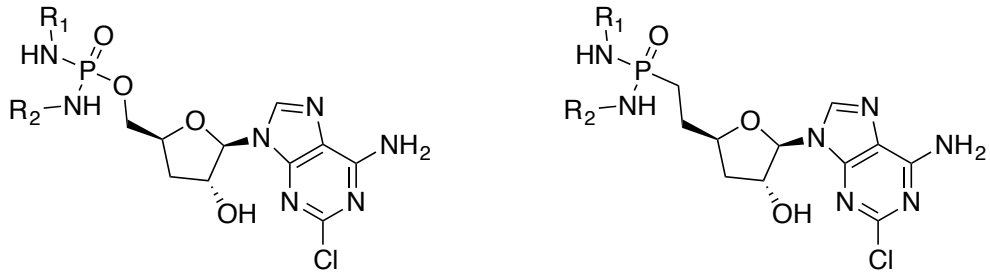
4. Other 2-chlorocordycepin prodrugs: phosphoramidate and phosphonodiamidate

2-Chlorocordycepin phosphorodiamidate and phosphonodiamidate – Rationale behind the design.



Scheme 4.1 *General structure of a phosphorodiamidate (left) and phosphonodiamidate (right) of 2-chlorocordycepin*

As previously discussed in Chapter 3, ProTiding 2-chlorocordycepin delivered new compounds that enhanced the activity of the parent nucleoside from inactive to micromolar activity. In addition, the new compounds were resistant to deamination, the major drawback of cordycepin, and it was proved that they were activated to the monophosphate species by carboxypeptidase Y.

It was worth exploring other forms of prodrugs to investigate if they would improve even further on the activity 2-chlorocordycepin and the ProTides.

The first explored modification was the phosphorodiamidate approach. This modification of the ProTide approach substitutes the aryl moiety for another amino acid ester moiety.

The second modification aimed to improve the biological activity and the stability of the compound by changing the P-O bond between the nucleoside and the phosphate group with a P-C bond. These alterations aimed to make the compound more stable towards dephosphorylation and to improve pharmacokinetics by increasing lipophilicity.

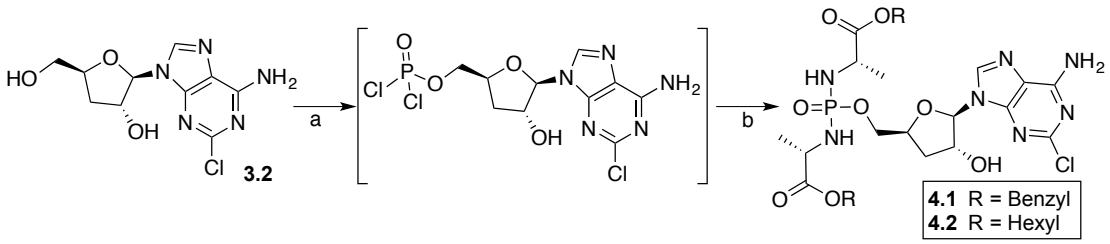
Synthesis and biological activity of phosphorodiamidates

This strategy, designed in 1991 by McGuigan *et al.*, has been widely applied on different nucleoside analogues, improving their activity.[1] However, despite promising *in vitro* activity results, none of them has reached clinical trials yet.

In most cases nucleoside phosphorodiamidates bear the same amino acid ester moieties, which prevents the formation of a chiral centre in the phosphorus atom. This provides an advantage in comparison to the ProTide because only one final compound is formed instead of a mixture of diasteroisomers.

Synthesis of Phosphorodiamidates

Two different methods are reported for the synthesis of symmetrical phosphorodiamidates.[2] Both of them involve the treatment of the nucleoside analogue with phosphorus oxychloride to obtain a nucleoside phosphorodichloridate intermediate. This is typically monitored by ^{31}P NMR and gives a signal between 7-8 ppm, but it is not isolated because of its instability. The phosphorodichloridate intermediate then reacts with tosylated amino acid salts to form the P-N bond, leading to a phosphorodiamidate. The main difference between the two approaches is the reagents used to initiate the process (Scheme 4.2).



Scheme 4.2 *General synthesis of symmetrical phosphorodiamidates. Reagents and conditions:* Approach 1. *(a1) TEA (1.2 eq), $POCl_3$ (1.2 eq), THF, -78 ˚C, 30 minutes, then RT, 1 h. (b1) Appropriate aa-ester salt (5 eq), TEA (10 eq), DCM -78 ˚C, 30 minutes, then RT, 20 h.* Approach 2. *(a2) $POCl_3$ (1 eq) in $(CH_3O)_3PO$, -5 ˚C, 30 minutes, then RT, 1 h. (b1) Appropriate aa-ester (5 eq), DIPEA (10 eq), DCM, -78 ˚C, 30 minutes, then RT, 20 h.*

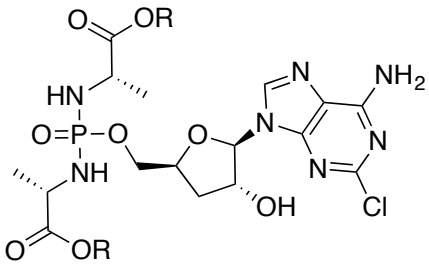
The first approach uses triethylamine in both steps a1 and b1. Similarly to the synthesis of aryl phosphorodichloridates in Chapter 2, the alcohol at position 5' (the most nucleophilic) reacts with phosphorus oxychloride by nucleophilic substitution displacing one of the chlorine atoms (a1). In the second step (b1), the lone pair of electrons in the amino group of the aa-ester reacts with the phosphorus via nucleophilic substitution, leading to the mono-phosphoroamidate. The role of the

triethylamine in both steps is to accept the additional protons. A second nucleophilic substitution with a second equivalent of the aa-ester then leads to the final compound.[1] However, it was reported that this approach is sometimes not successful, either by not achieving reactivity, or by generating tetra-phosphoramidates because of the reaction with the other hydroxyl groups and/or amino groups in the NA.[2] In this chapter, phosphorodiamidate **4.1** was obtained by this method in 8 % yield.

The second approach relies on the observations by Yoshiwaka *et al*. that the reaction between a deprotected nucleoside and phosphorus oxychloride to form the dichloridate intermediate would be accelerated by the addition of trialkyl phosphate as solvent (trimethyl phosphate being the most favourable).[3] The aa-ester was added to this intermediate using diisopropylethylamine (DIPEA) as a base whose reactivity is very similar to TEA. This method then furnishes the phosphorodiamidate by nucleophilic substitution as in the first approach. This strategy has been used since the first nucleoside phosphoramidates were reported.[4] Phosphoramidate **4.2** was obtained following this method in 13 % yield. *Table 4.1* compares the yields in approach 1 and 2, approach 2 giving a better yielding route.

Table 4.1 Comparison between the yields obtained in the synthesis of nucleoside Phosphorodiamidates using approach 1 and approach 2.

Compound	Approach	Yield
4.1 R = Benzyl	1	8 %
4.2 R = Neop	2	13 %



Biological activity of phosphorodiamidate 4.1

Compound **4.1** was tested in cell viability assays. Detailed results can be found in Chapter 3. Figure 4.1 shows a graphical representation of the data. The cell lines in which **4.1** was tested were CCRF-CEM, MOLT-4, K562, HEL92.1.7, HL-60, Z-138, RL, Hs-445, RPMI-8226, Mia-Pa-Ca-2, HT29, HepG2, MCF-7 and Cal27. It was found that **4.1** exhibited enhanced activity compared to 2-chlorocordycepin. However, it was generally less active than the other synthesised ProTides of 2-chlorocordycepin (see Chapter 3), except in the RPMI and the Mia-Pa-Ca cell lines. In these cell lines **4.1** showed the highest activity of the series. In the RPMI cell line the compound improved the activity of the parent nucleoside 31-fold (top inhibition 95.09 % and EC_{50} 6.38 μM) and in Mia-Pa-Ca cells the activity was improved 12-fold (top inhibition 92.8 % and EC_{50} 16.16 μM). However, very poor activity was achieved in the cell lines K562 and HT29, where at the highest tested concentration (198 μM), less than 50% of the cells were inhibited. In the other cell lines, **4.1** was active with an EC_{50} range around 60 μM for haematological malignancies and 100 μM for solid tumours.

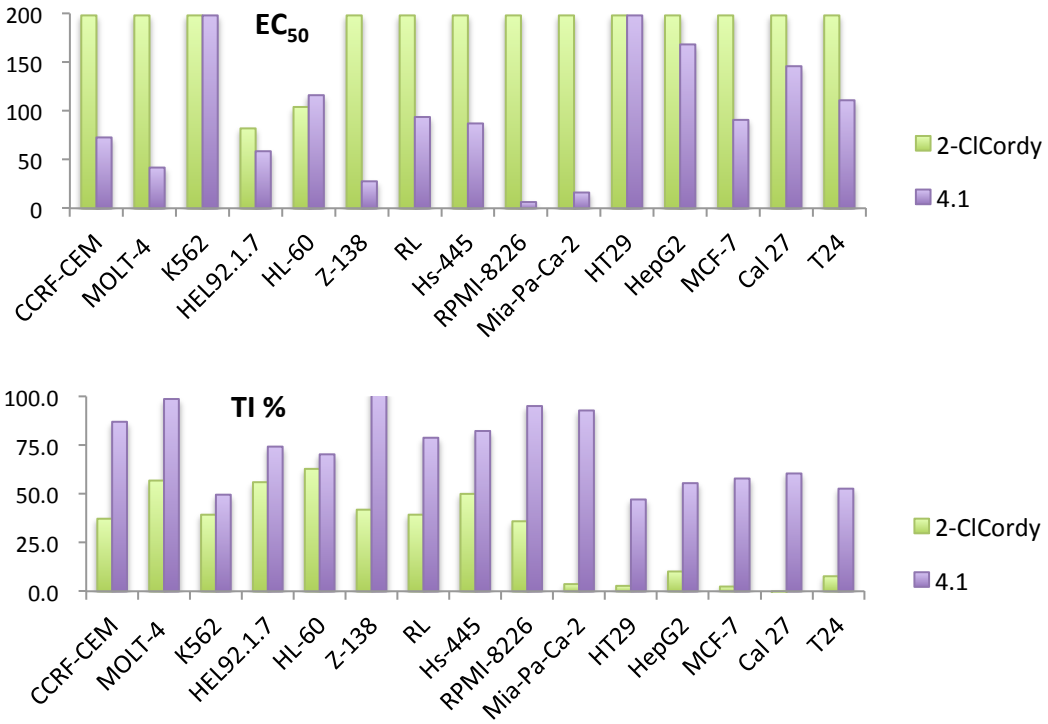
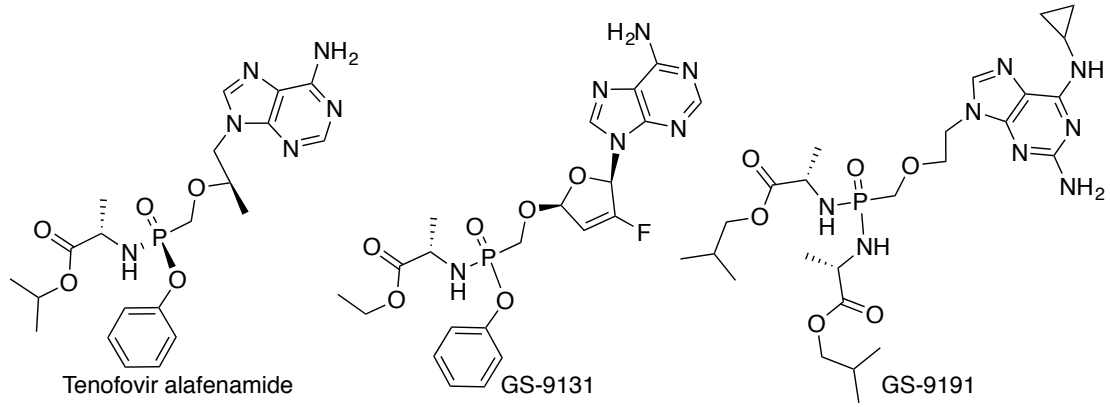


Figure 4.1 *Graphical representation of data in chapter 3 comparing the EC_{50} and top inhibition (TI %) of 2-chlorocordycepin (2-ClCordy) and compound* ***4.1***

Synthesis of 2-chlorocordycepin phosphonodiamidate

The phosphonodiamidate strategy aims to increase the stability of the activated monophosphate species towards enzymatic dephosphorylation by inserting a P-C bond between the nucleoside analogue and the phosphate group. At the same time, phosphonodiamidates keep the prodrug moieties of a phosphorodiamidates that allow bypassing cell resistance mechanisms such as decreased cellular uptake and limited first phosphorylation. Following this strategy, some phosphonodiamidates and aryl phosphonamidates have reached clinical trials or have been approved (Scheme 4.3).



Scheme 4.3 *Examples of other phosphonate approaches: Tenofovir alafenamide an acyclic aryl phosphonamidate approved by the FDA. GS-9131 cyclic aryl phosphonamidate. GS-9191 as a phosphonodiamidate.*

As it is shown in the previous Scheme 4.3, traditional phosphonates link the phosphorus atom with the sugar through a P-C-O bond which is isopolar and isosteric with the phosphate group P-O-C.[5] The McGuigan group is actively investigating the influence of different phosphonate linkers in the ProTide and phosphoramidate approach. The pipeline of this project include the synthesis of 5'-methylene phosphonate, 5'-(E)-vinylphosphonate, 5'-fluoromethylene phosphonate and 5'-difluoromethylene phosphonate. The project is on an early stage of development where the methylene bridge between the phosphorus atom and the sugar is being explored.

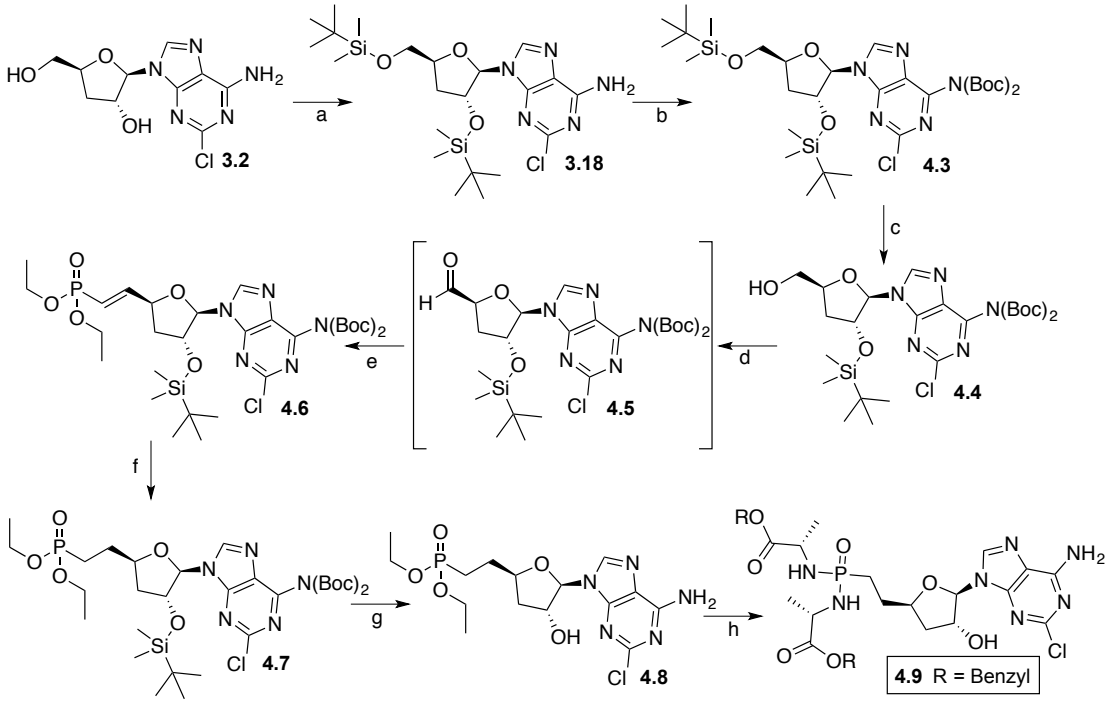
In the literature the application of the methylene bridge to mimic the phosphate bond in ribavirin 5'-monophosphate is described. The new compound retained some of the inhibitory activity against inosine 5'-monophosphate

dehydrogenase and it was considerably more stable to dephosphorylation. In this article Wang *et al.* encouraged further invenstigations.[6]

This part of the chapter is focused on the synthesis of a phosphonodiamidate of 2-chlorocordycepin, aiming to extrapolate the synthesis to the aryl phosphonamidates if it were to show interesting activity after future biological testing.

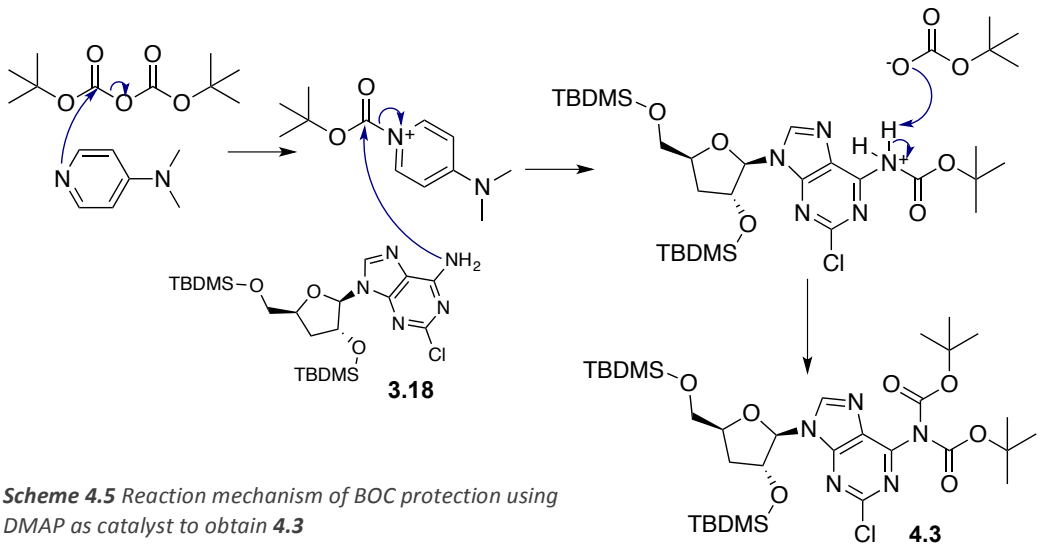
The strategy used for the synthesis of phosphonamidates of NA has already been applied in acyclic[7] and cyclic nucleosides, the latter was recently improved in the McGuigan laboratory. In this chapter the technique is applied to 2-chlorocordycepin, and the conditions are adapted to this particular nucleoside.

For the development of this method (summarised in Scheme 4.4), it was first required to selectively protect the amino group and 2'-hydroxyl group of nucleoside analogue **3.2**, leading to compound **4.4**. Then, ethenyl phosphonate formation and subsequent reduction of the double bond were performed following the procedure by Seamon *et al*: first, the alcohol was oxidised to aldehyde **4.5**; then, the alkene **4.6** was formed by reaction with a tetraethyl methylene bis phosphonate carbanion. The alkene **4.6** was then reduced by catalytic hydrogenation to form **4.7**.[8] Finally, without liberating the phosphate group of the alkyl moieties, and following the procedure by Pertusati *et al*. for one pot reaction of acyclic nucleotides,[7] the P-N bonds of the *L*-alanin-N-yl ester and phosphorus atom were formed and delivered the phophonodiamidate nucleotide **4.9**.



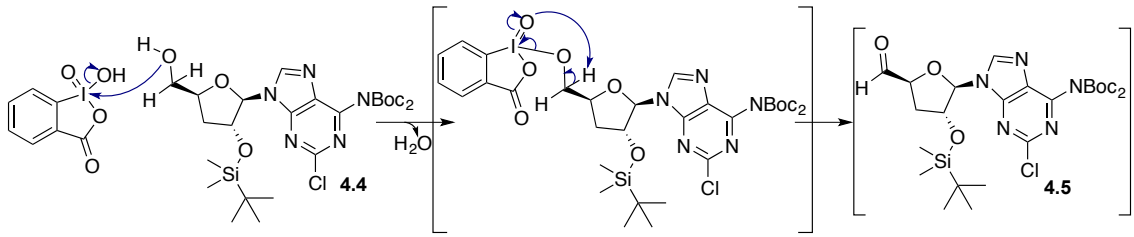
Scheme 4.4 *General synthesis of 2-chlorocordycepin phosphonates. Reagents and conditions: (a) TBDMSCl, Imidazole/DMF, RT, 20 h; (b) Boc_2O, DMAP, THF, Ar atm RT, 20 h; (c) CSA, MeOH, 0 ˚C, 1 h; (d) IBX, CH_3CN, 80 ˚C, Ar atm 90 min; (e) $(EtO_2PO)CH_2$, NaH, THF, 0 ˚C to RT, Ar atm 24 h; (f) H_2, Pd/C 10%, MeOH, RT, 24h; (g) $THF/HCO_2H/H_2O$ 1:2:1, RT, 72 h; (h)* 1. *TMSBr, CH_3CN, 2,6-lutidine;* 2. *Pyr, Et_3N, Alditriol, Ph_3P, appropriate aa-ester, 50 ˚C, 3 h.*

Protection of the hydroxyl groups with TBDMSCl to obtain compound **3.18** was performed following the best yielding method described in Chapter 3 using imidazole and DMF. As it was previously discussed, the TBDMS group was selected because it can be selectively cleaved from primary alcohols without affecting other functionalities. Then, a bis-*tert*-butyloxycarbonyl (BOC) protection of the amino group was performed using DMAP as catalyst, delivering compound **4.3** in quantitative yield. The lone electron pair of the nitrogen in the pyridine ring attacks one of the carbonyl groups of the BOC_2O, forming an intermediate with a good leaving group that can then be further attacked by the primary amine in the nucleoside. The mono-BOC compound is then attacked by the BOC-O anion delivering the bis-BOC protected compound **4.3,** as Scheme 4.5 illustrates.[9]



Scheme 4.5 *Reaction mechanism of BOC protection using DMAP as catalyst to obtain* ***4.3***

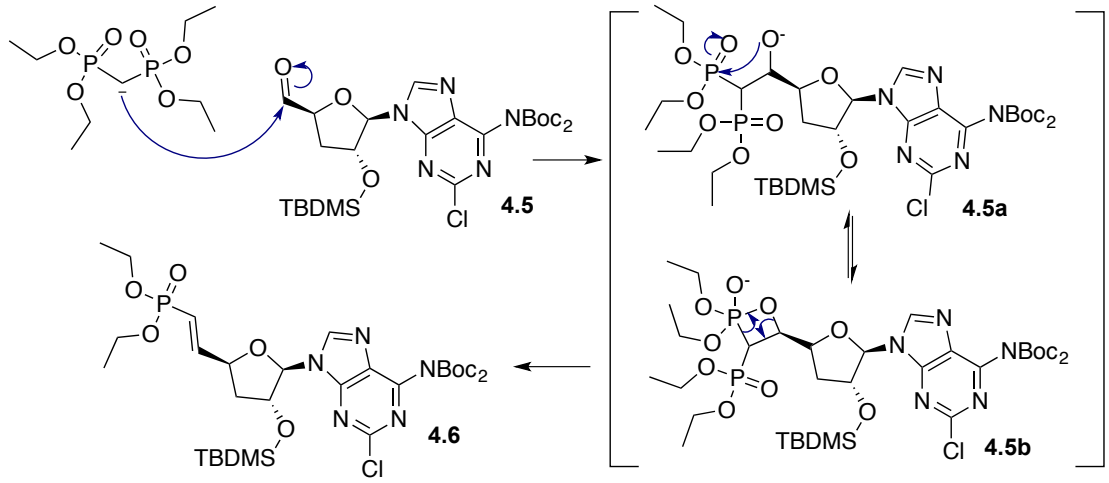
A selective deprotection of the hydroxyl group at 5' using camphor sulfonic acid (CSA) delivered compound **4.4** in quantitative yields. Compound **4.4** was suitable to undergo the procedure by Seamon *et al*. In the first step, treatment of **4.4** with freshly prepared 2-Iodoxybenzoic acid (IBX) (prepared according with the protocol described by Frigerio et al.[10]) at 80 ˚C led to oxidation of the 5' hydroxyl group into the aldehyde.[11] The proposed reaction mechanism is described in Scheme 4.6 and is based on the investigations by Su et al.[12]



Scheme 4.6 *Mechanism of oxidation of the 5'-hydroxyl group into an aldehyde.*

This compound was very unstable, and the formation was monitored by MS. The sample needed to be dissolved in ACN and all settings of the instrument needed to use the same solvent to avoid hemiacetal formation in the presence of methanol. Next, a Horner-Wadsworth-Emmons reaction formed the diethyl ethenyl phosphonate **4.6**. The postulated mechanism for this reaction[13] starts with the formation of the tetraethyl methyl bisphosphonate carbanion with NaH, which was added to the aldehyde **4.5** by nucleophilic addition, producing two interconvertible intermediate species **4.5a** and **4.5b**. **4.5b** forms a 4-membered ring which is sterically

unstable and leads to the formation of **4.6** via elimination of the oxygen (Scheme 4.7). Overall yield from **4.4** to **4.6** was 52 %.

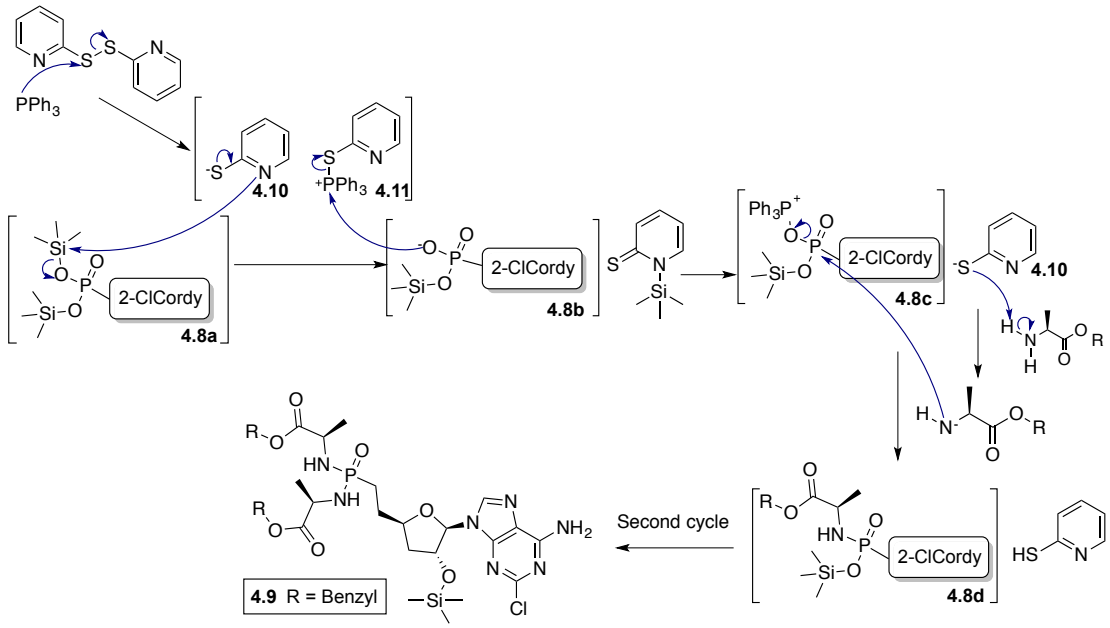


Scheme 4.7 *Postulated mechanism of reaction for the formation of* ***4.6*** *through a Horner-Wadsworth-Emmons reaction.* [13]

Catalytic hydrogenation using 10 % Pd/C then reduced the phosphonate double bond of compound **4.6,** leading to compound **4.7**. This reaction yielded 65 % after purification by column chromatography. Next, the protecting groups of the amine at 4 and hydroxyl at 2' were removed by applying a mixture THF, H_2O and formic acid (1:1:2) and stirring for 72 h at 30 ˚C, to furnish the unprotected compound **4.8** in 58 % yield.

Finally, the one pot reaction to obtain the final compound was initiated. In the first step, reaction of **4.8** with TMSBr delivered the bis-silylated phosphonate ester **4.8a**. 2,6-Lutidine was used as a mild base to prevent degradation of the product during the reaction because of the bromhidric acid forming during the silylation. The crude of the reaction was evaporated and dissolved in pyridine and triethylamine. The second part of the reaction took place following a reaction mechanism that was elucidated by Mukaiyama *et al.* in 1971.[14] In a separate flask aldrithiol-2 and triphenylphosphine (Ph_3P) were dissolved. The phosphorus attacks one of the sulphur atoms of aldrithiol-2, leading to an anion of 2-mercaptopyridine **4.10** and a cation of triphenylphosphine 2-mercaptopyridine **4.11**. The sulphur atom **4.10** attacks one of the silyl groups of **4.8a** leading to its cleavage forming intermediate **4.8b**. Intermediate **4.8b** then reacts with **4.11** leading to intermediate

4.8c. Meanwhile compound **4.10** reacts with the aa-ester deprotonating it. The negatively charged amine of the aa-ester reacts with the phosphorus of intermediate **4.8c** leading to a monophosphonamidate compound. Finally the amino group of the aa-ester will attack the phosphorus of intermediate **4.8c** leading to the desired phosphonodiamidate compound **4.8d**. This process repeats to create compound **4.9** with 3 % overall yield. (Scheme 4.8). Optimisation of the last reaction would be required if a large family of phosphonates of 2-chlorocordycepin were to be considered for future synthesis.



Scheme 4.8 *Putative reaction mechanism for the formation of the phosphonodiamidate.*

Conclusion

In addition to the ProTides discussed in chapter 3, other prodrug derivatives of 2-chlorocordycepin were explored. The first strategy was to apply the phosphorodiamidate approach, which is a variation of the ProTide approach. Two compounds **4.1** and **4.2** were synthesised using two different synthetic methods. Compound **4.1** was tested against a wide sample of cancer cells proving that it enhanced the activity of the parent nucleoside. Indeed, it showed better activity than the ProTides in RPMI and Mia-Pa-Ca cell lines. These findings encouraged further modifications on the prodrug.

The second investigated prodrug strategy was explored in the synthesis of a phosphonodiamidate derivative of 2-chlorocordycepin. A synthetic route leading to phosphonodiamidate **4.9** was developed. However, this seven-step synthesis will require optimisation in the last step, which only delivered 3 % yield. On the other hand, the other synthetic steps delivered their corresponding products either quantitatively or at yields or over 50 %. Studies to investigate the biological activity of these 2-chlorocordycepin phosphonodiamidates are going to be performed in the future.

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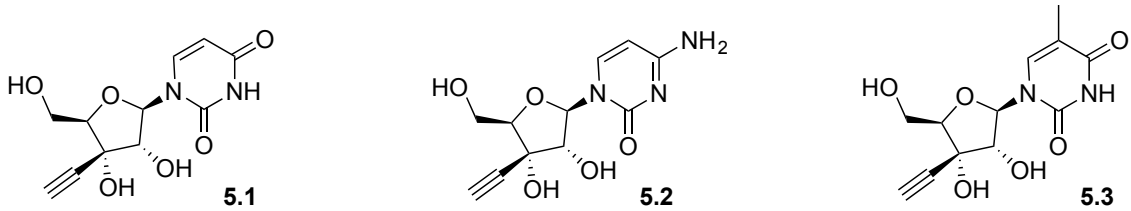
5. 3'-Ethynyl Nucleosides

3'-Ethynyl nucleoside analogues – Rationale behind the design.



Scheme 5.1 *3'-Ethynyluridine* ***5.1*** *(EUrd), 3'-ethynylcytidine* ***5.2*** *(ECyd), and 3'-ethynyl-5-methyl-uridine* ***5.3*** *(EMUrd).*

In 1996, Matsuda *et al.* aimed to synthesise a NA with antitumour activity specially addressed against slow-growing solid tumours, in which drugs have fewer chances to encounter S-phase (where DNA synthesis occurs). They aimed to inhibit both DNA and RNA synthesis, and although the inhibition of RNA synthesis would not be a major mechanism of tumour cell death, it would contribute to the antitumour activity against solid tumours.[1,2]

To design RNA synthesis antimetabolites, they decided to keep the riboconfiguration with both hydroxyls at the 2' and 3' positions, so the NA could be recognised by the RNA synthetic enzymes to either exert inhibition or to undergo metabolic transformations such as phosphorylation, since they considered that the triphosphate form would be required to inhibit RNA Polymerases.[1,2]

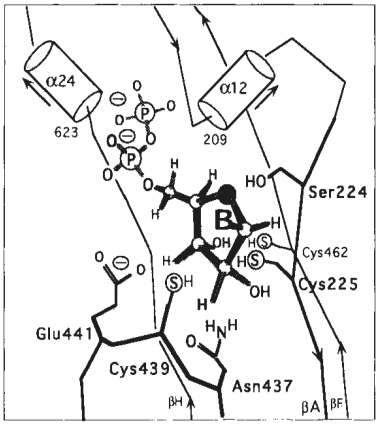


Figure 5.1*: Uhlin et al (1994). Schematic drawing of the active site region of subunit R1 of ribonucleotide reductase, with a model derived plausible position for a substrate molecule. The proximity between Cys439 and H at 3' of the ribonucleotide can be clearly seen.*[4] *Reprinted with permission from Macmillan Publishers Ltd: Uhlin et al. Nature. 1994, 370, 533-539, copyright 1994*

To inhibit DNA synthesis, they chose to target Ribonucleoside diphosphate reductase (RR), which catalyses the conversion of ribonucleoside 5'-diphosphate to their corresponding 2'-deoxyriborucleoside 5'-diphosphates in the *de novo* pathway.[1,2] Stubbe *et al*. (1980) postulated that the mechanism of this reaction would be initiated by the abstraction of a tyrosyl radical in subunit R2 stabilised

by a μ-oxo bridged binuclear iron (III) centre to 3' hydrogen atom of the ribonucleoside 5'-diphosphates which would be in the active site of subunit R1.[3] Eventually, the cysteine residue Cys 439 in subunit R1, which is suggested to be near the 3' hydrogen,[4] would play an important role in the re-abstraction of the radical.[3]

Matsuda *et al.* (1996) speculated that an acetylene group at position 3' of the diphosphate nucleoside could form a bond with the thiol of Cys 439 in an alkylthiovinyl sulphide through a radical reaction, and therefore inactivate the enzyme.[1,2]

Following this reasoning they first synthesised 3'-ethynyl uridine (EUrd) **5.1**, and, after achieving submicromolar EC_{50} values in *in vitro* cytotoxicity tests,[1] they enlarged the family by synthesising 3'-ethynylcytidine (ECyd) **5.2**, 3'-ethynyl 5-methyluridine (EMUrd) **5.3**, 3'-ethynyl-5-fluoro-cytidine, 3'-ethynyl-5-fluoro-uridine, 3'-ethynyl-adenosine and 3'-ethynyl-guanosine.[2] ECyd and EUrd achieved the most promising *in vitro* results in various solid tumour cell lines and experiments on human tumour xenografts in nude mice showed promising results for ECyd and EUrd[3] that led to ECyd being brought forward to clinical trials.[5]

Aiming to investigate the structure-activity relationship (SAR) of these drugs, and in order to examine if the drugs could be further improved, through the years, Matsuda and his group designed different analogues of EUrd and ECyd.[6,7] They first explored the length required between C3' and the ethynyl group. Therefore, they prepared ECyd and EUrd analogues bearing other 3' substituents: 1-Propynyl, 2-propynyl, 1-butynyl, ethenyl, ethyl and cyclopropyl. 1-propynyl and ethenyl substituents displayed some activity, but it was 1500 to 520-fold lower than ECyd or EUrd.[8] Then, the role of 3' hydroxyl was investigated and 3'-deoxy-ECyd and 3'-deoxy-EUrd were synthesised but the compounds lacked activity.[8] They next questioned the importance of the configuration at position 3' by inserting an ethynyl and 2-propynyl substituent in the α-position of the ribo- and 3' deoxyribo configuration of uridine and cytidine. Again, those substitutions delivered inactive compounds.[8] They also explored shifting the ethynyl substituent to position 2' and position 4'. Inserting an ethynyl moiety in α and β of cytosine lead to inactive

compounds,[8] but the introduction of the ethynyl group at 4' of cytidine and 2'-deoxycytidine delivered compounds with low cytotoxicity which were active against HIV.[6] Finally they explored the importance of the 4'-O functionality by replacing the oxygen with a sulphur atom. 4'-thio-3'-ethynylcytidine showed no activity.[7] A Danish group inspired by Matsuda's work reviewed and synthesised other ECyd and EUrd analogues with modifications in their pyrimidine nucleobases. The best tolerated modifications were C-5 halogen substitutions with iodine and fluorine but activity was 20 − 50 times lower than that of the parent nucleosides. They also noted that nucleosides with 5-hydroxyuridine and 3-deazauridine bases, which showed no activity, had a better cytotoxic profile without the 3'-ethynyl substituent.[9] SAR studies showed that the ethynyl group has the adequate length and that it has to be positioned in the β-configuration at the 3' position for the best fit and thus elicitation of activity. The presence of the hydroxyl groups at 2' and 3' was found to be also important for the activity, since the compounds are mainly RNA inhibitors and need to be recognised by the enzymes in charge of RNA synthesis, as RNA polymerases. The substitution of the oxygen at position 4' by a sulphur atom was not well tolerated for the anticancer activity, but it delivered compounds with antiviral activity. Cytidine and Uridine were the best tolerated nucleobases for these compounds

Studies on the mechanism of action of ECyd and EUrd revealed that their biological activity depends on their phosphorylation. The active form of those NA was identified to be ECyd-5'-triphosphate (ECyd-TP) and EUrd-5'-triphosphate (EUrd-TP), and the enzyme responsible for their phosphorylation is uridine-cytidine kinase (UCK).[10] Two isoforms of UCK were identified: UCK1 and UCK2. It was found that ECyd and EUrd might be preferentially phosphorylated by UCK2.[11,12] The phosphorylation by UCK was found to be upregulated in tumours, which phosphorylated ECyd 20-30-fold more than in non-cancerous tissues,[13] probably because of higher expression of UCK in tumour tissues than in non-neoplastic tissues.[14] Although the phosphorylation of both ECyd and EUrd happens very efficiently they can compete with endogenous cytidine and uridine.[10] However,

ECyd-TP and EUrd-TP are not efficiently dephosphorylated, so they overcome one important catabolic process that endows them with a long half life.[10]

Interestingly, cells treated with EUrd accumulated both EUrd-TP and ECyd-TP. This suggested an efficient conversion inside the cell, probably by cytidine-TP synthase.[15] However, very small amounts of EUrd-TP were found in cells treated with ECyd, indicating some resistance of ECyd towards deamination. This was an important in the consideration to choose ECyd over EUrd as the drug to proceed (Figure 5.2).[15]

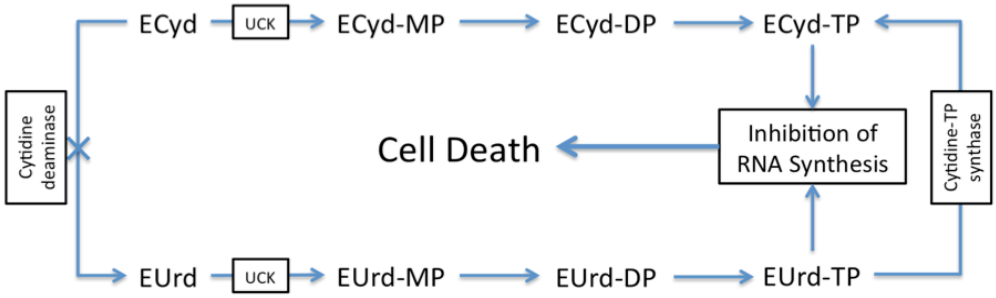


Figure 5.2 *Metabolism and cytotoxic mechanism of ECyd and EUrd*[15]

Furthermore, it was revealed that the diphosphate of ECyd or EUrd did not inhibit RR,[13] and that the biosynthesis of endogenous nucleoside triphosphate was not affected.[10]

However, ECyd-TP and EUrd-TP proved to be potent RNA inhibitors, acting as non-selective competitive inhibitors of RNA polymerases I, II, and III during RNA transcription.[10] ECyd-TP binds to RNA polymerase with 380 times higher affinity than cytidine-TP,[15] and through that inhibited over 80-90 % of the RNA synthesis in tumour cells,[15] resulting in cell death.[16] Some inhibition of DNA synthesis was also observed and it was probably secondary to the potent inhibition of RNA synthesis. ECyd inhibits cell growth in cells by the inhibition of RNA synthesis.[10] Other secondary mechanism of actions that were recently discovered for these compounds, specifically ECyd, involve the induction of G2/M arrest that leads to apoptosis,[17] and the downregulation of TIGAR (an apoptotic regulator).[17]

ECyd and EUrd have also shown activity towards cancer cells which had developed resistance to other anticancer agents: nasopharyngeal cancer cells

resistant to cisplatin[17] and pancreatic Mia-Pa-Ca-2 cells resistant to gemcitabine[18] were sensitive to the treatment with ECyd, and EUrd has been identified as inhibitor against temozolomide-resistant glioblastoma initiating cells.[19]

Combination of radiotherapy and ECyd has shown promising results *in vitro*. ECyd down-regulated the expression of BRCA2 after X-ray irradiation. BRCA2 was identified as responsible for repairing damaged DNA. Although the precise mechanism still remains unknown, this suppression enhanced the radiosensitivity of the tumour cells and may therefore result in a better effectivity of radiotherapy for solid tumours.[20] In addition, ECyd also proved to be a radiosensitiser by enhancing the proton-induced cell death in proton beam therapy. The involved mechanisms remain unclear, but seem to differ from those observed for X-ray irradiation.[21]

As mentioned before, ECyd was eventually carried forward to being studied in preclinical and clinical trials. Preclinical trials in nude rat models bearing human tumours showed strong antitumour activity without serious toxicity. Furthermore, ECyd-TP was retained in tumour tissue at high intracellular concentrations for prolonged periods, but not in non-tumour tissue. Those results encouraged clinical trials.[22]

Phase I Trials of ECyd in monotherapy identified peripheral neuropathy, neurotoxicity, neutropenia and thrombocytopenia at high doses as possible adverse events.[23,24] ECyd proceeded to Phase II to treat head and neck cancer, squamous cell cancer and nasopharyngeal cancer – but despite the promising preclinical studies, no anti-tumour efficacy was observed. Either disease progression or intolerable adverse event occurred in treated patients.[25] Furthermore, a combination of ECyd and carboplatin entered in phase I trials and showed to be well tolerated. However, even though the patients did achieve neither complete nor partial response, this combination will proceed to phase II.[26]

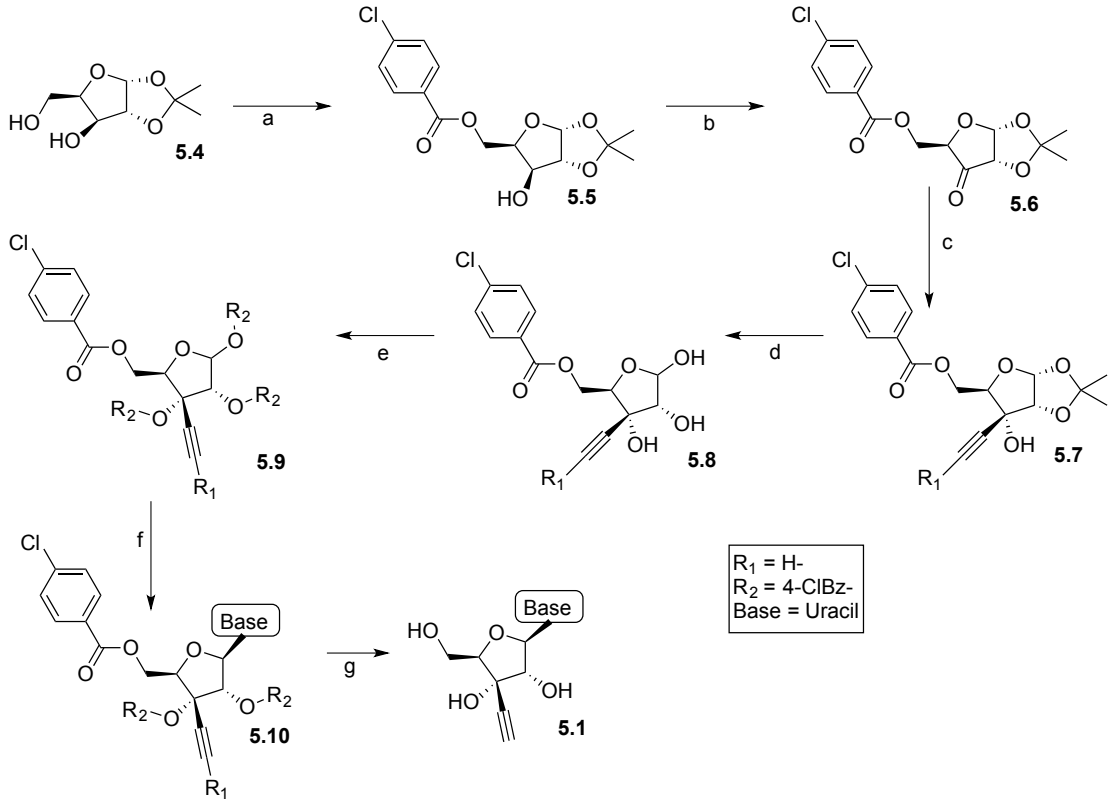
The low efficacy in clinical trials was explained by a deficiency to achieve sufficient intracellular levels of ECyd-TP.[12] The efficacy of ECyd and EUrd depends on UCK,[10] and it is a well known cancer cell mechanism of resistance to downregulate the expression of kinase enzymes.[27] A decrease of kinase expression was postulated

to be the main mechanism of resistance, but altered cell permeability (increasing efflux and decreasing uptake) might also have played a role.[10,12] *In vitro* data anticipated that the ECyd and EUrd resistant cells lacked UCK,[10,12] in particular isoform UCK2.[17] *In vitro* testing further identified that resistant cells to ECyd and EUrd had modifications in the genes encoding UCK2, and that while its expression was decreased, UCK1 encoding genes and expression remained unaltered in the same resistant cells.[17]

The objective in this chapter was the synthesis of a small families of ECyd, EUrd and EMUrd ProTides with anti-cancer properties that may overcome the resistance mechanisms directed against their parent nucleosides, since they would not require UCK for initial phosphorylation. While this project was in progress, a patent was filed in China exemplifying two ECyd ProTides, bearing isopropyl-*L*-Ala-Phenyl and Benzyl-*L*-Ala-Phenyl moieties.[28] Shortly after that, Idenix Pharmaceuticals, Inc. exemplified generic EUrd and ECyd ProTides bearing *D* amino acids.[29] This work was aimed at synthesising *L* amino acid ProTides, therefore the projects do not overlap. With regards to the first patent, it was decided to proceed with the synthesis of the ProTides, but with less focus on ECyd.

Synthesis of 3'-Ethynyl Nucleosides

Among the reported syntheses of 3'-ethynyl nucleosides, the procedure by Nomura *et al*. (2001) was chosen. They reported an optimized pathway for the synthesis of ECyd and EUrd, exploring different conditions that aimed for good yields and crystalline intermediate compounds.[30] Scheme 5.2 shows the selected seven-step synthesis.



Scheme 5.2: *3'E-Nucleoside synthesis selected from the different conditions reported by Nomura et al. to afford EUrd and ECyd. Reagents and conditions: (a) 4-ClBzCl, Et_3N, 0 ˚C; (b) TEMPO, 0 ˚C; (c) EthynylMgBr, 0 ˚C; (d) HCl/ MeOH, Reflux; (e) 4-ClBzCl, DMAP, Et_3N; (f) TMS_2 Cytosine or TMS Uracil, $SnCl_4$ (g) DBU, MeOH.* [30]

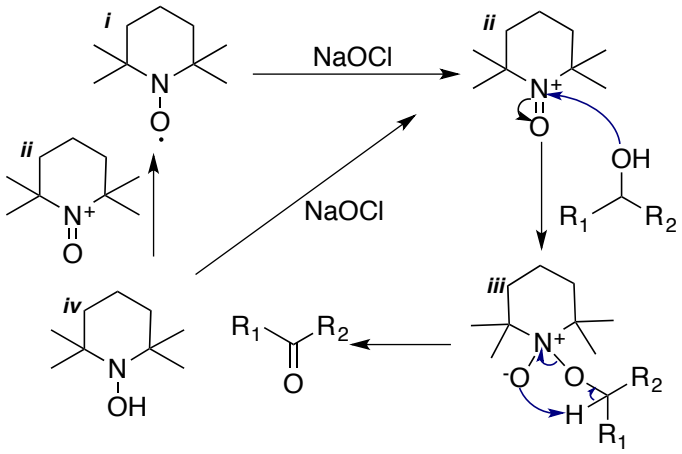
The synthesis used 1,2-*O*-isopropylidene-*D*-xylofuranose **5.4** as starting material, which is commercially available. In the first step, the hydroxyl group at 5' would be protected (a). Among the reported protecting groups, 4-Chlorobenzoyl (4-ClBz) was selected because the reaction was reported to yield 73-80 % and the product **5.5** was going to be obtained as crystals. Next, an oxidation of the hydroxyl at 3' position would take place to achieve compound **5.6** (b). Nomura *et al*. observed that the best yields were obtained using TEMPO as oxidising agent. The use of this reagent also prevented the need for the use of CrO_3 (a toxic reagent), which was

previously used to perform this reaction. Then, the ethynyl group at 3' position in β-conformation would be introduced via Grignard form using ethynyl magnesium bromide (CH≡CMgBr) to furnish compound **5.7** (c), and the isopropylidene protection would be removed from the hydroxyl groups at position 1' and 2'(compound **5.8**) to insert individual protecting groups for the 1', 2' and 3' hydroxyl groups (d, e). Here, the chosen protecting group was once again 4-ClBz because it was reported to deliver compound **5.9** as a precipitate that could be collected by filtration. A Vorbrüggen reaction for the coupling of the sugar **5.9** and the base would be performed with $SnCl_4$ (a Lewis acid) using previously bis-silylated Uracil (e). Finally, a deprotection of all protecting groups from **5.10** would lead to the desired compound EUrd **5.1**. Once optimised, this methodology was planned to be applied for the synthesis of ECyd and EMUrd.

First approach: R_1 H; R_2 4-ClBz

The protection of 1, 2-*O*-isopropylidene-D-xylofuranose **5.4** with 4-Cl-Bz-Cl achieved compound **5.5** in 47 % yield. The hydroxyl group at position 5' in the sugar would react with the carbonyl group in a nucleophilic substitution, cleaving the chlorine.

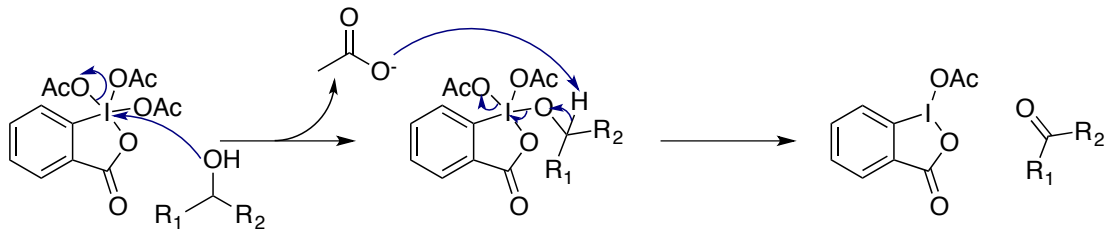
Optimisation of the oxidation of the 3'-hydroxyl group to obtain compound **5.6** was required. In the first attempt 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) was used as a catalyst and NaOCl as a co-oxidant. As shown in Scheme 5.3, during the process, a catalytic amount of NaOCl oxidises the TEMPO radical (i) to the N-oxoammonium ion (ii), which then oxidises the secondary alcohol at 3' giving a molecule of the corresponding hydroxylamine (iv). This intermediate can either be directly oxidised by NaOCl to the N-oxammonium ion (ii) or undergo a syn proportionation with a molecule of N-oxammonium (ii) to regenerate two molecules of TEMPO (i).[31,32]



Scheme 5.3: *TEMPO oxidation of a secondary alcohol.*[31]

Unfortunately, in this work, this reaction did not deliver compound **5.6** and other mechanisms of oxidation were investigated.

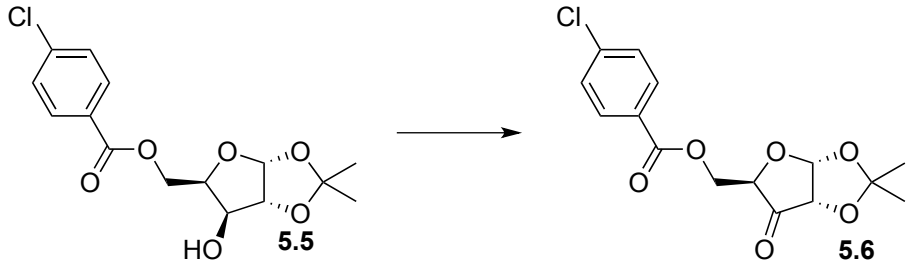
Dess Martin Periodinane (DMP) is a hypervalent iodine compound that performs a selective and mild oxidation of alcohols to aldehydes or ketones. The alcohol attacks the iodine atom, which is very electropositive due to the surrounding environment of electron-withdrawing groups. One of the acetoxy groups then leaves the molecule as a good leaving group. Then, the negatively charged oxygen of the acetoxy group accepts the hydrogen from the CH in the secondary alcohol. Electrons relocate, the ketone is formed and another acetoxy group is removed from the molecule (Scheme 5.4).[33]



Scheme 5.4: *DMP oxidation of secondary alcohol.* [33]

This oxidation (Scheme 5.5) was performed several times with varying results, yielding from 40 to 96 % (*Table 5.1*). When lower yields were achieved, unreacted starting material was still present in the reaction crude after several hours of reaction. This reaction benefited in form of acceleration and better yields when using water-saturated dichloromethane (DCM) (**5.6C**-**5.6F**), in accordance to the literature

that stated that the oxidation can be accelerated by addition of 1 μL of water per mL of DCM.[34] However, yields decreased when scaling up the reaction.



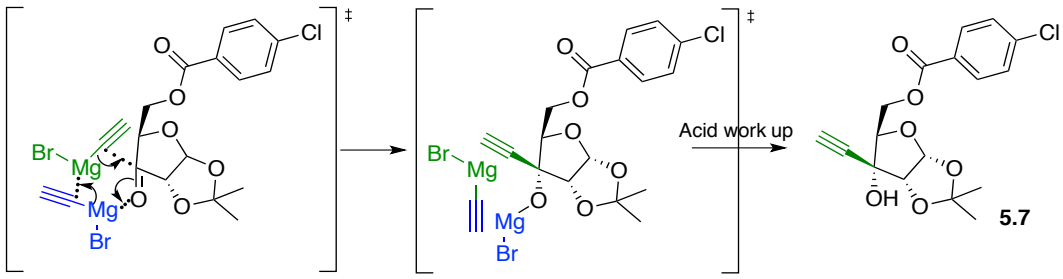
Scheme 5.5 *Oxidation of the hydroxyl group at 3' in* ***5.5*** *with DMP at 0 ˚C led compound* ***5.6***

Table 5.1: Summary of the tested conditions to oxidise the alcohol at position 3'. CC = column chromatography

Comp.	Attempt	Reagent	5.5 (g)	Yield	Purification
5.6	A	TEMPO (0.05 eq)	1.0	Compound not detected by MS	-
5.6	B	DMP	0.407	40 %	CC
5.6	C	DMP	2.0	96 %	Precipitation
5.6	D	DMP	6.5	88 %	Precipitation
5.6	E	DMP	10.53	54 %	CC
5.6	F	DMP	11.0	41 %	CC

A long range H-H coupling of the proton at 2' with the proton at 4' J = 1 Hz was observed in compound **5.6**. Other similar structures found in the literature, such as 5-*O*-*tert*butyldimethylsilyl-1,2-*O*-isopropylidene-*D*-*erythro*-pentofuranose-3-urose also present this small coupling constant for the coupling of the respective protons.[35] It was found that $^{4}J_{H\text{-}H}$ couplings can be observed in saturated systems if there is a proper orbital alignment between C-H bonds and intervening C-C bonds. The most favourable alignment is a "W" arrangement of the connecting bonds. Therefore, it is expected that the conformation acquired of 5-*O*-(4-chlorobenzoyl)-1,2-*O*-isopropylidene-*D*-*erythro*-pentofuranose-3-urose (**5.6**) would be close to a "W" arrangement.

The introduction of an ethynyl group in β-configuration to obtain compound **5.7** was achieved using CH≡CMgBr in THF at 0 ˚C. The Grignard reaction with the ketone is postulated to happen through a six membered ring transition state of the ketone with two molecules of CH≡CMgBr (Scheme 5.6).[36]



Scheme 5.6: *Putative mechanism of reaction for the introduction of ethynyl group at position 3'*

It is reported that this reaction delivers exclusively the 3'-β-ethynyl-3'-α-hydroxyl product.[30] Indeed, the reaction delivered only one product whose ^{1}H and ^{13}C NMR spectra (Figure 5.3) matched the reported ones for structurally similar compounds with a β-configured ethynyl group.

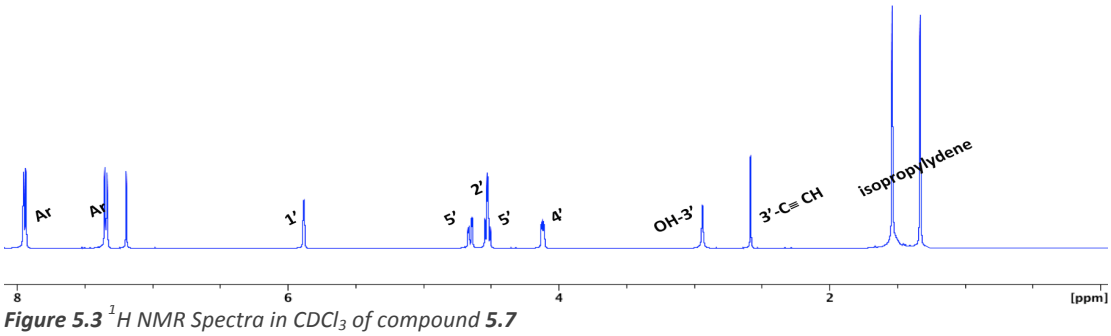


Figure 5.3 *^{1}H NMR Spectra in $CDCl_3$ of compound **5.7***

The stereochemistry of **5.7** was confirmed by 2D nuclear Overhauser enhancement spectroscopy (NOESY) experiment. The most relevant region of the NOESY spectrum is expanded in Figure 5.4. The correlation between the 3'-OH and 4'-H indicated their spatial proximity, and thus, that both protons were on the same face of the sugar. The α configuration of the 4'-H was establised, and therefore, it confirmed the α configuration of the 3'-OH. Thus, insertion of the ethynyl group had to have been performed in β configuration.

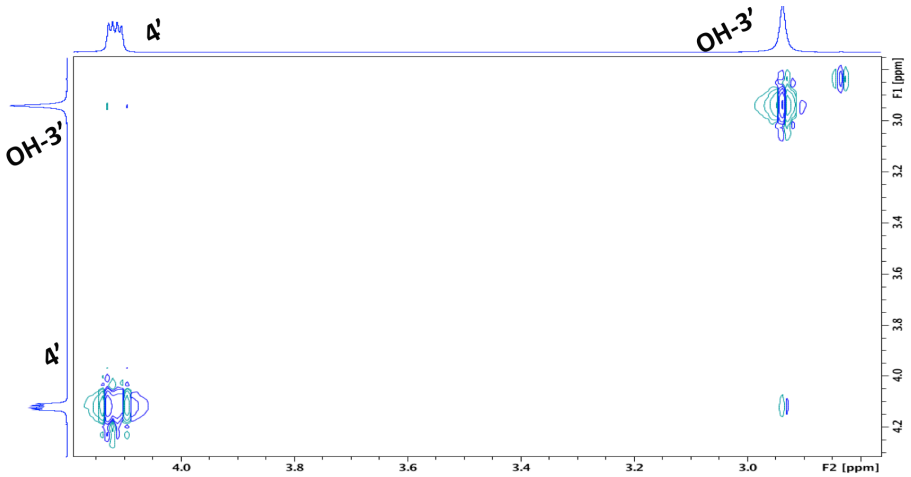
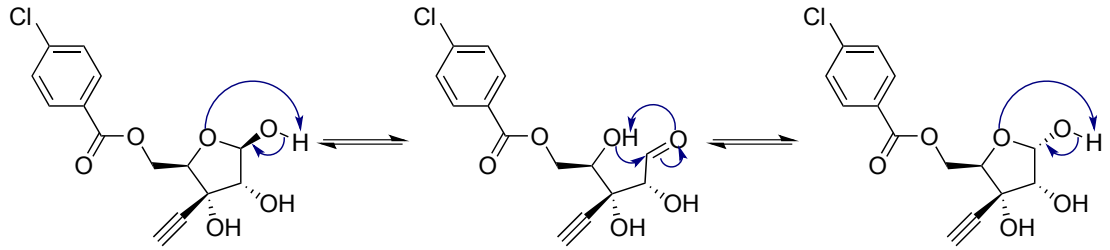


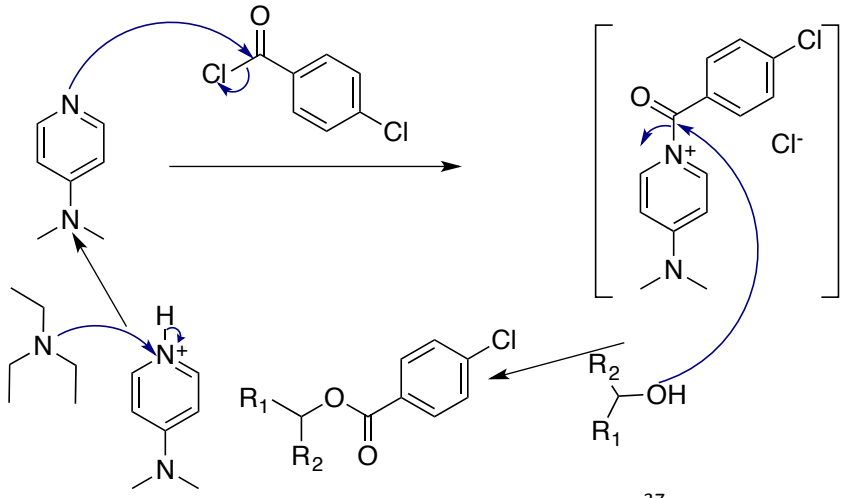
Figure 5.4 *Selected region of the 2D NOESY spectrum of **5.7***

Deprotection of the hemiacetal group in acidic conditions in compound **5.7** was performed using a mixture of formic acid (HCOOH) and water 1:1 at reflux temperature. The resulting compound **5.8** is a mixture of isomers where the hydroxyl group at position 1' is either in α or β-configuration. This is due to the equilibrium between the cyclic and linear forms of the ribose ring (Scheme 5.7).



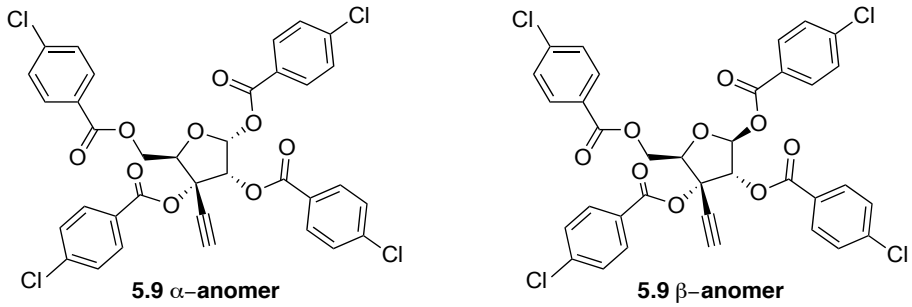
Scheme 5.7: *Equilibrium between the cyclic and linear forms of the ribose ring of compound* ***5.8.***

The hydroxyl groups of compound **5.8** were individually protected by 4-chlorobenzoyl chloride (4-ClBzCl) to achieve compound **5.9**. 4-(Dimethylamino)pyridine (DMAP) was used as a catalyst for the reaction and Et_3N was used as an auxiliary base to regenerate the activated form of DMAP. During the process DMAP reacts with 4-ClBzCl in an equilibrium forming an acylpyridinium cation as intermediate. The oxygen in the hydroxyl group reacts with this intermediate, releasing the protonated catalyst that accepted the hydrogen in the hydroxyl group. TEA then regenerates DMAP by its deprotonation.[37]



Scheme 5.8 *Mechanism of DMAP catalysed acetylation (Xu et al.)* [37]

During the acetylation, α and β isomers of compound **5.9** formed in a ratio of 7:3. (Scheme 5.9)



Scheme 5.9 *α and β anomers generated for compound* ***5.9***

The characterization of the individual isomers was made by comparing the reported spectra from the literature [30] and the spectra of the synthesised compounds (Figure 5.5). The main difference between the anomers was found in the signals for the hydrogen atoms at 1' and 2' of the sugar. The β conformation gives closer signals and a smaller coupling constant (*J* = 1 Hz) which is consistent with the Karplus equation because those protons should adopt a torsion angle close to 90˚. The α conformation gives a *J* coupling constant 4.5 Hz. Due to the reaction mechanism of the Vorbrüggen reaction (see below), both products were valid for the next step of glycosylation.

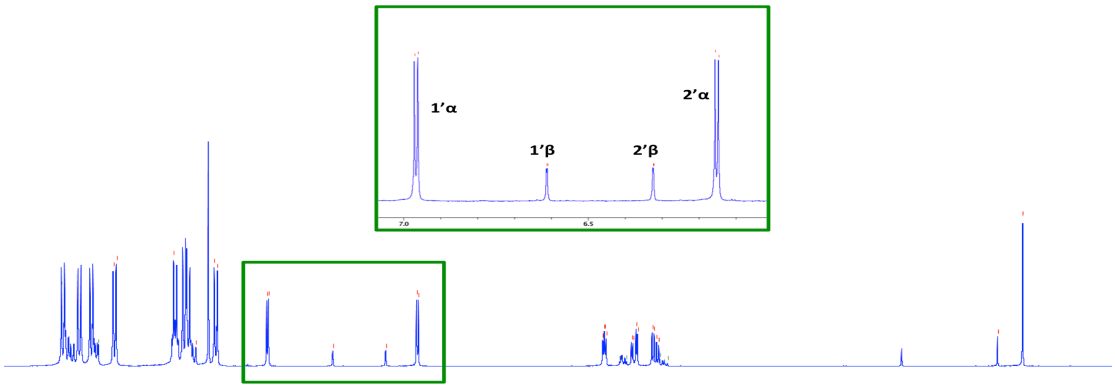
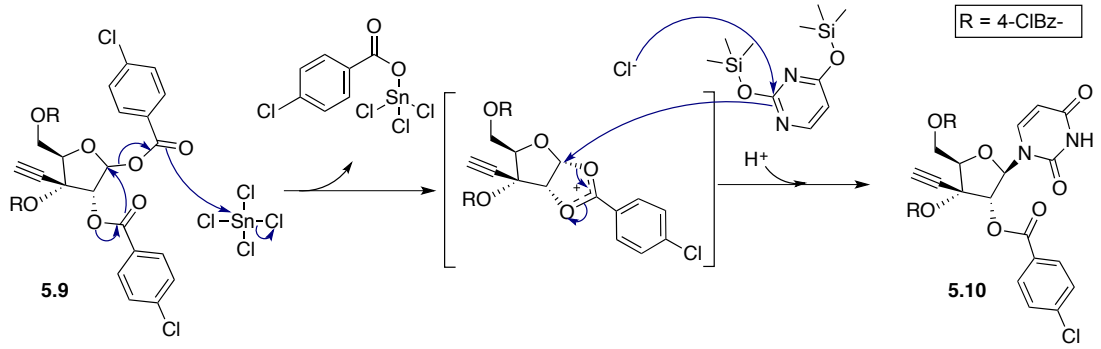


Figure 5.5: *^{1}H NMR Spectra of α and β isomers of 1,2,3-Tetra-O-(4-Chlorobenzoyl)-3-C-D-ribo-pentofuranose* ***5.9****. Focus on signals for 1'-H and 2'H*

This reaction yielded between 15 and 32 %. Column chromatography in CH_2Cl_2 allowed the isolation of the pure α isomer for its characterization. However, the following reactions were performed with the 7:3 mixture of the isomers.

The formation of the N glycosidic bond was performed via Vorbrüggen procedure.[38] This rate-limiting reaction required a silylated base at position 2 and 3, which was achieved with hexamethyldisilazane (HMDS) and a catalytic amount of ammonium sulphate. Scheme 5.10 illustrates the putative mechanism of action. The protected sugar **5.9** reacts in the presence of $SnCl_4$ by forming a cyclic intermediate of a 1,2-acyloxonium salt. The resulting stereochemistry of the intermediate compound depends on the stereochemistry of the C-2', delivering the 1,2-acyloxonium cation in the α-side of the sugar. This reaction is favoured because of the electrophilicity of C-1', with two neighbour oxygen atoms. Next, the released chlorine anion attacks the silylated base at position 2 (the most electropositive), which simultaneously attacks the sugar cation at position 1' (the most electrophilic) from the beta side of the sugar to form the nucleoside in the opposite side The remaining TMS group is then being released by the acidic work-up.[39]



Scheme 5.10: *Vorbrüggen mechanism of N-glycosylation delivering only the β isomer.*

Due to the reaction mechanism, only the β-nucleoside is being formed: the 1,2-acyloxonium ion is formed in the α side, because it depends on the conformation of the hydroxyl group at position 2'. Therefore, the base only can approach to the β side of the ring. The reaction achieved compound **5.10** in 7-20 % yield.

Finally, the deprotection of the 2', 3' and 5' hydroxyl groups with 1,8-diazabicycloundec-7-ene (DBU), a non-nucleophilic base, removed the protecting groups from the **5.10** without affecting other functional groups in the molecule.[40] After acidic work-up, EUrd (**5.1**) was obtained in 87 % yield.

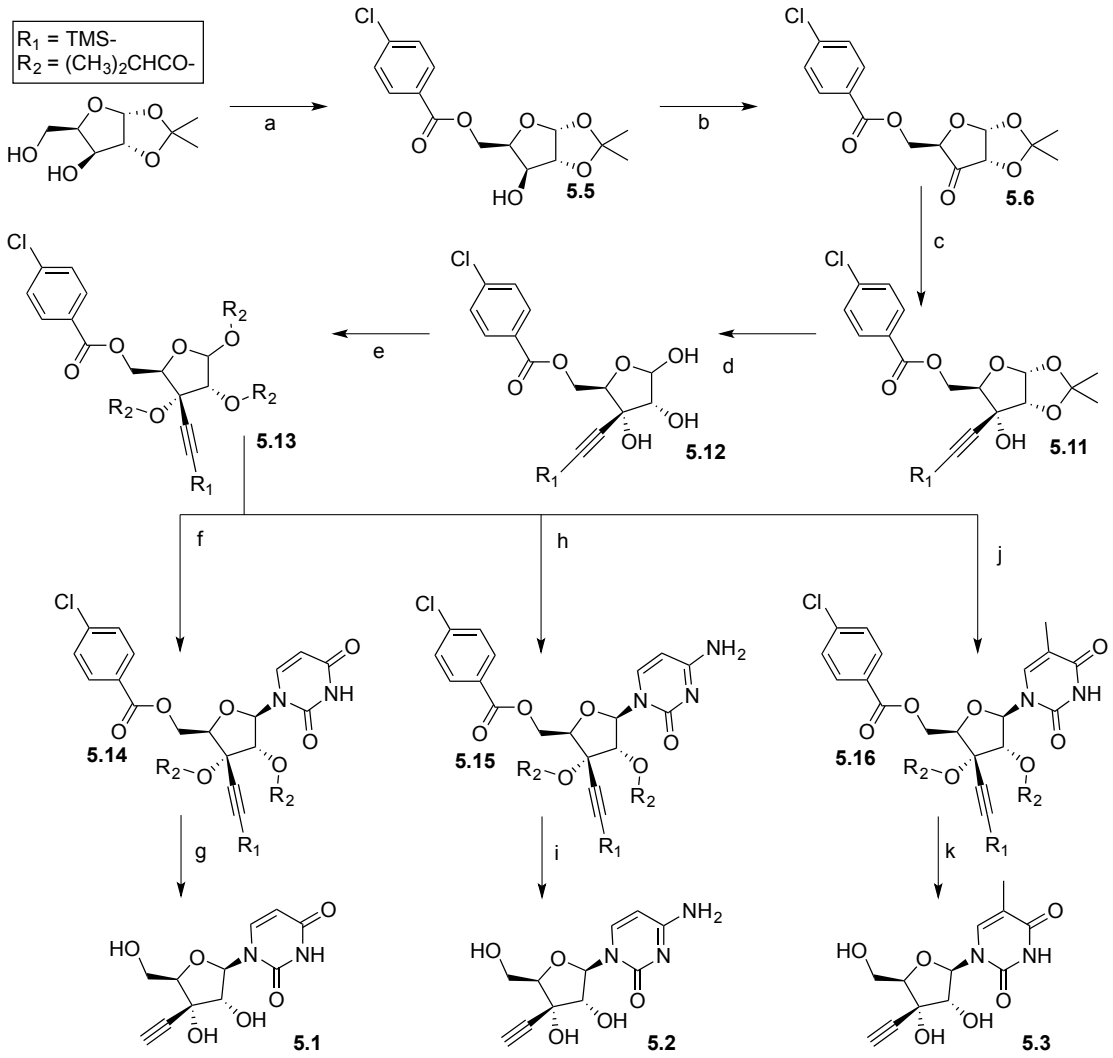
The selected strategy delivered a yield lower than expected (overall yield for the best performing reactions was 2.2 %). Changes on the synthetic approach were

designed to improve the synthesis. The glycosylation step of the ethynyl sugar was reviewed with the sugar bearing isobutyryloxy protecting groups at the hydroxyls in positions 1', 2', and 3' instead of the previously used 4-ClBzCl together with the addition of a trimethylsilyl moiety at the ethynyl group delivered the best yields.[30]

Second approach: R_1 TMS; R_2 Isobutyryloxy.

This strategy (Scheme 5.11) diverges from the former one (Scheme 5.2) in the introduction of the ethynyl group, which is inserted on compound **5.6** by the Grignard reagent TMS-C≡CMgBr. This reagent was generated *in situ* by reacting trimethylsilylacetylene with ethylmagnesiumbromide (EtMgBr). A six membered ring transition state leads to the introduction of the TMS-ethynyl group in beta configuration.[36] The formation of the resulting compound **5.11** was achieved with 50-69 % yield. Deprotection of the alcohols was performed similarly to the first approach. It gave a mixture of α and β isomers of compound **5.12** in a proportion of 85:15, yielding 84 %. Next, protection of the hydroxyl groups in 1', 2' and 3' position with isobutyryl chloride, DMAP and Et_3N, followed the same mechanism of reaction as the protection with 4-ClBzCl and gave quantitative yields of compound **5.13**. Isomers α and β were also obtained in an 8:2 proportion and were used for the next step without further separation. As explained above, assignment of the signals corresponding to the α and β isomers was possible by comparison with literature data. Indeed, the main characteristics remained the same as for the 4-ClBz protected sugar.

The glycosylation reaction with silylated uracil led to compound **5.14** and yielded 50-61 %, which was a more than 3-fold increase compared to the 1st approach to obtain compound **5.10**. After deprotection with DBU to cleave the protecting groups, including the TMS residue from the 3'-ethynyl group (48 % yield), the overall yield to obtain EUrd **5.1** with this approach was 8.6 %.



Scheme 5.11 *Synthesis of 3'E-Nucleoside Synthesis : EUrd, ECyd and EMUrd. Reagents and conditions: (a) 4-ClBzCl, Et_3N 0 ˚C; (b) DMP, 0 ˚C; (c) TMS-C≡CMgBr, 0 ˚C; (d) HCl/ MeOH, Reflux; (e) 4-ClBzCl, DMAP, Et_3N; (f) TMS_2 Uracil, $SnCl_4$ (h) TMS_2 Cytosine, $SnCl_4$ or (j) TMS_2 Thymine, $SnCl_4$ (g), (i), (k) DBU, MeOH.*

Table 5.2 shows the comparison of the best yields achieved in every step between the first and second approach. In most of the steps, the second approach delivered better yields, resulting in an overall yield that was 4 times greater for the second approach than for the first approach. Therefore, the second strategy was selected for pursuing ECyd and EMUrd.

Table 5.2 Comparison between the best yields achieved of every step in the first and second approach. The step letter correspond to the letters in ***Scheme 5.2*** and ***Scheme 5.11***. Steps A and B are the same for both approaches.

	Step a +b	Step c	Step d	Step e	Step f	Step e	Overall
First approach Scheme 5.2	45 %	65 %	95 %	45 %	20 %	87 %	2.2 %
Second approach Scheme 5.11	45 %	69 %	95 %	100 %	61 %	48 %	8.6 %

Synthesis of 3'-Ethynylcytidine and 3'-Ethynyl-5-methyluridine

Once the method to synthesise EUrd had been optimised, the synthesis of the other pyridine base nucleosides was performed as shown in Scheme 5.11.

A Vorbrüggen reaction to couple intermediate **5.13** with either cytosine or thymidine was performed (steps h and j) followed by deprotection with DBU (steps i and k). Vorbrüggen reaction delivered protected 3'-ethynylcytidine **5.15** in a 45 % yield. Deprotection with DBU then afforded the desired compound **5.2** with a 42 % yield. Vorbrüggen reaction with thymine delivered protected 3'-ethynyl-5-methyluridine **5.16** in a 48 % yield. Deprotection with DBU afforded desired compound **5.3** with an 89 % yield. Using this optimised pathway, similar reactivity for the Vorbrüggen reactions was observed for the three pyrimidine bases, delivering similar yields.

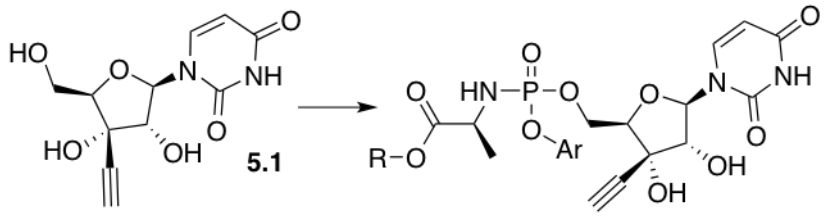
Table 5.3 *Summary of the Vorbrüggen reactions performed with uracil, cytosine and thymine using intermediate 5.13 as precursor*

Compound	Base	Yield
5.14	Uracil	50-61 %
5.15	Cytosine	45 %
5.16	Thymine	48 %

Synthesis of 3'-ethynylnucleoside ProTides

The preferred method for the synthesis of ProTides of 3'-ethynylnucleosides was to use the NMI approach to avoid coupling reactions on the other available hydroxyl groups.

3'-Ethynyluridine ProTides



	R	Ar
5.17	Neop	Naph
5.18	Neop	Ph
5.19	Benz	Ph
5.20	Ethyl	Ph
5.21	Hex	Ph
5.22	iPr	Ph

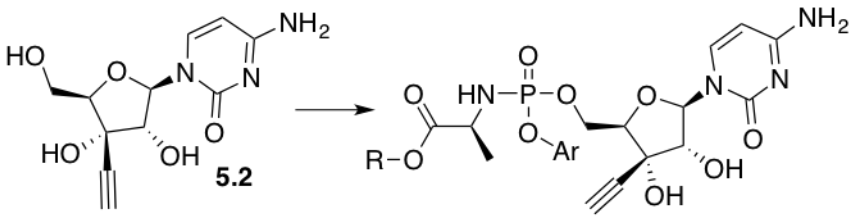
Scheme 5.12 *Synthesis of 3'-EUrd ProTides. Reagents and conditions: NMI (5 eq), appropriate phosphorochloridate (3 eq), anhydrous THF, rt, 20 h.*

3'EUrd **5.1** was found to have poor solubility in THF. Therefore, the reaction was performed at 30 ˚C, and no precipitate was observed. The reactions yielded between 7 and 13 %, as shown in *Table 5.4*. The first performed reaction was for compound **5.20,** using 56 mg of the starting material **5.1**. Conversion of 5.1 into the ProTide was observed by monitoring the reaction via TLC. However, after proceeding with the usual work-up with 0.5 M HCl to remove NMI, the entire compound went to the aqueous phase, owing to its hydrophilic nature. It was not possible to recover the compound. Thus, the compounds were directly purified by flash column chromatography. Remaining NMI was removed by purifying the compound two times using preparative TLC.

Table 5.4: Summary of the coupling reactions performed on EUrd **5.1**. ^{31}P NMR spectra recorded in MeOD.

Compound	5.1	Yield	^{31}PNMR (ppm)
5.17	158 mg	7 %	4.07, 3.72
5.18	100 mg	13 %	3.63, 3.41
5.19	100 mg	11 %	4.35, 4.20
5.20	56 mg	Traces	-
5.21	100 mg	7 %	3.61, 3.33
5.22	143 mg	9 %	3.63, 3.41

3'-Ethynylcytidine ProTides



	R	Ar
5.23	Benz	Ph
5.24	Hex	Ph
5.25	iPr	Ph

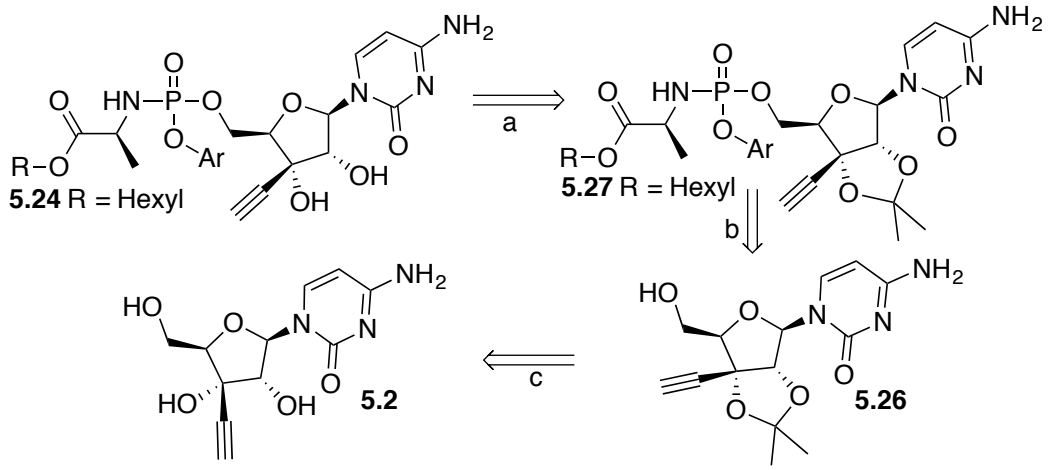
Scheme 5.13 *Synthesis of 3'-ECyd ProTides. Reagents and conditions: NMI (5 eq), appropriate phosphorochloridate (3 eq), anhydrous THF, rt, 20 h.*

When the same conditions were applied to **5.2** to synthesise ECyd ProTides, only very low reactivity was observed (*Table 5.5*), varying from no reaction at all (compound **5.25**) to 1-3 % yield. Compounds **5.23** and **5.24** were isolated for biological testing. Very low solubility of compound **5.2** in THF was observed, even after warming the reaction mixture to 30 ˚C. The tendency of cytidine to form salts might have negatively influenced the reactivity.

Table 5.5 Summary of the coupling reactions performed on ECyd **5.2** ^{31}P NMR spectra recorded in MeOD.

Compound	5.2	Yield	^{31}PNMR (ppm)
5.23	383 mg	1 %	3.27, 3.42
5.24	184 mg	3 %	3.72, 3.52
5.25	90 mg	No reaction	-

Therefore, a different approach was designed to synthesise ECyd ProTides. To improve reactivity, *t*BuMgCl was considered as stronger base for the coupling reaction. To avoid side reactions forming bis or tri ProTides and/or 2', or 3' ProTides, the hydroxyl groups at 2' and 3' were protected through a hemiacetal bond with acetone (Scheme 5.14).

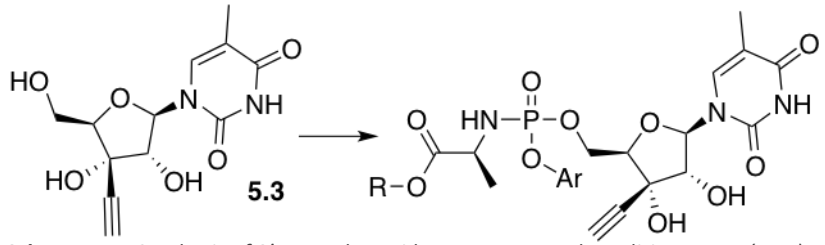


Scheme 5.14 *Retrosynthetic scheme of the planned synthesis of 3'-ECyd ProTides by selectively protecting hydroxyl groups at position 2' and 3', and performing the coupling reaction with a Grignard reagent as a base. Reagents and conditions: (a) HCOOH 60%, RT, overnight; (b) Appropriate phosphorochloridate (2 eq), tBuMgCl (1 eq), THF; (c) dry acetone (20 eq), H_2SO_4, 0 ˚C.*

The protection of the hydroxyl groups at 2' and 3' positions was performed with 500 mg of **5.2,** observing very poor solubility of the compound in acetone. Compound **5.26** was obtained in 26 % yield. Next, the coupling reaction tBuMgCl and hexyl-*L*-alaninyl-phenyl phosphorochloridate was attempted. However, the formation of the desired product **5.27** was not observed either by MS or ^{31}PNMR, even after prolonging the reaction for a further 36 h. Therefore, this synthetic route was no longer pursued.

Since compounds **5.23** and **5.24** had been obtained in enough quantity to be tested for biological activity, the optimisation of the synthesis of ECyd ProTides was put on hold awaiting the biological results of the other two compounds.

3'-Ethynyl-5-methyluridine ProTides



	R	Ar
5.28	Neop	Ph
5.29	Benz	Ph
5.30	Hex	Ph
5.31	iPr	Ph
5.32	cHex	Ph

Scheme 5.15 *Synthesis of 3'-EMUrd ProTides. Reagents and conditions: NMI (5 eq), appropriate phosphorochloridate (3 eq), anhydrous THF, rt, 20 h.*

EMUrd ProTides were synthesised using the same conditions as EUrd ProTides. *Table 5.6* shows the summary of the performed coupling reactions, which yielded from 2

to 15 %. In general, the yields were lower than for the 3'EUrd analogues even if more quantity of starting material was used. However, enough quantity of the compounds was obtained to perform biological tests.

Table 5.6 Summary of the coupling reactions performed on EMUrd **5.3** ^{31}P NMR spectra recorded in MeOD.

Compound	5.3	Yield	^{31}PNMR (ppm)
5.28	345 mg	2 %	3.60, 3.36
5.29	192 mg	9 %	3.53, 3.26
5.30	345 mg	9 %	3.63, 3.32
5.31	240 mg	15 %	3.64, 3.35
5.32	200 mg	6 %	4.04, 3.77

Cell viability assay

ProTides of EUrd **5.17**, **5.18**, **5.19**, **5.21**, **5.22**, and the parent nucleoside **5.1**; ProTides of ECyd **5.23**, **5.24**, and the parent nucleoside **5.2**; and ProTides on EMUrd **5.28**, **5.29**, **5.30**, **5.31** and the parent nucleoside **5.3** were selected for a cell viability assay. It was performed by WuXi AppTec to assess the cytotoxic activity of the prodrugs in comparison to their respective parent nucleosides, using paclitaxel as a control compound (*Table 5.7*).

Table 5.7: Compounds tested in a cell viability screening performed by WuXi AppTec. Log P values generated algorithmically by a computer-based predictive algorithm using ChemDraw Professional 15.0.

Compound	Moieties	LogP
EUrd	-	-2.18
5.17	Neop-*L*-Ala-Naph	3.34
5.18	Neop-*L*-Ala-Ph	2.34
5.19	Benz-*L*-Ala-Ph	2.40
5.21	Hexyl-*L*-Ala-Ph	2.62
5.22	Isoprop-*L*-Ala-Ph	1.38
ECyd	-	-1.94
5.24	Hexyl-*L*-Ala-Ph	2.96
5.23	Benz-*L*-Ala-Ph	2.18
EMUrd	-	-1.83
5.28	Benz-*L*-Ala-Ph	2.68
5.29	Hexyl-*L*-Ala-Ph	2.90
5.30	Neop-*L*-Ala-Ph	2.62
5.31	Isoprop-*L*-Ala-Ph	1.65

EUrd and ECyd had previously undergone exhaustive cytotoxicity screenings.[2] Therefore, basing our study on previous results by Hattori *et al*. the selected cell lines for this study come from the same tissue where EUrd and ECyd showed the highest cytotoxic activity. The chosen tissues were from leukaemic, pancreas, colon, liver, breast, head and neck, and gliobliastoma cancer cells. Whenever it was available in the laboratories of WuXi AppTec the same cell line as was used in Hattori *et al*. studies was selected (*Table 5.8*).

Table 5.8 Cell lines used for the in vitro cell viability assay.

Cell Line	**Malignancy**	
CCRF-CEM	Leukaemia	Acute Lymphoblastic Leukaemia
MOLT-4	Leukaemia	Acute Lymphoblastic Leukaemia
Mia-Pa-Ca-2	Pancreas	Pancreas Carcinoma
HT29	Colon	Colorectal Adenocarcinoma
HepG2	Liver	Hepatocellular Carcinoma
MCF-7	Breast	Breast Adenocarcinoma. Derived From Metastatic Site: Pleural Effusion Epithelial -Mammary Gland
Cal27	Head and Neck	Squamous cell carcinoma
T98G	Brain	Glioblastoma multiforme

The assays were performed as described in Chapter 3 and in the experimental part. Half maximal effective concentration (EC_{50}) and Top Inhibition percentage (percentage of non viable cells at the highest concentration, TI %) were stated for each compound for every cell line in *Table 5.9* and *Table 5.11* to *Table 5.13*.*Table 5.9* shows the results obtained for NA EUrd, ECyd and EMUrd.

Table 5.9: Cytostatic activity of EUrd, ECyd and EMUrd against selected cancer cell lines. EC_{50} values in μM.

	Leukaemia MOLT-4		Pancreas Mia-Pa-Ca-2		Colon HT29		Liver HepG2		Breast MCF-7		H&N Cal 27		Glioblastoma T98G	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
EUrd	<0.1	99	<0.1	99	0.1	83	0.3	80	0.9	92	0.5	101	1.8	86
ECyd	<0.1	99	<0.1	99	0.1	84	0.1	80	<0.1	90	0.1	99	0.7	84
EMUrd	>198	-9	>198	35	>198	35	>198	29	>198	-2	>198	9	>198	6

EMUrd was totally inactive in the tested cell lines. At the maximum concentration (198 μM), it inhibited a maximum of 35 % of the cells in pancreas and colon. The inactivity was also translated in the predicted EC_{50} values, which were higher than the maximum tested concentration. This was expected, as this compound did not show any inhibitory effect in the past when it was tested in other cell lines.[2]. It was suggested that EMUrd was not recognised by the putative activating enzymes such as uridine/cytidine kinase, because of the bulky substituent at position 5.[2] However, this issue was supposed to be overcome by the ProTide approach described above. Another reason for the inactivity of EMUrd could be due

to the mechanism of action. The activity of EUrd and ECyd was attributed to their ability to act as competitive inhibitors of RNA polymerase.[10] Therefore, since EMUrd bears a thymine base, it might not be recognised.

In contrast, EUrd and ECyd were found to be active. They reached high top inhibition percentages (above 80 %), and the activity characterised by EC_{50} was found to be sub-micromolar (ranging from 0.02 μM to 0.95 μM), except for the activity of EUrd on T98G that reached 1.79 μM. Both showed similar activity in most cell lines, with some exceptions where ECyd was more active than EUrd (5 times more active in Cal 27 and 9 times more active in MCF-7) (Figure 5.6).

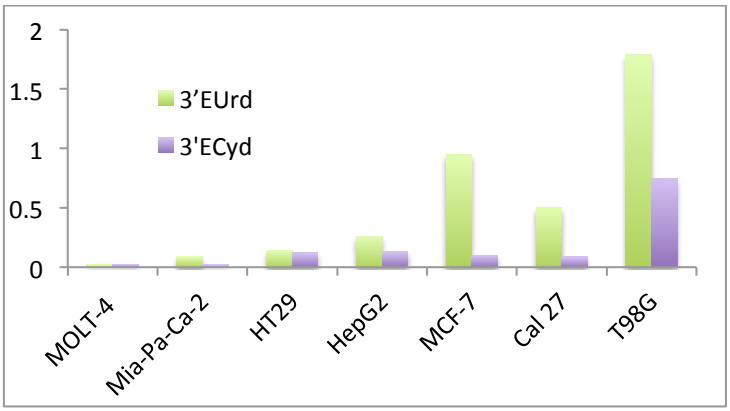


Figure 5.6 *EC_{50} of ECyd and EUrd in the selected cell lines*

Table 5.10 compares the EC_{50} values obtained in this assay with the values reported in the literature.[2,22] The values between them were very similar, which highlighted the reliability of the experiment.

Table 5.10: *Comparison of the values obtained in the cell viability assay performed in WuXi AppTec and the values reported by Hattori et al.[2] and Shimamoto et al.[22]* EC_{50} values in μM.

	Pancreas Mia-Pa-Ca-2		Colon HT29		Breast MCF-7	
	Experimental EC_{50}	Literature EC_{50}[2]	Experimental EC_{50}	Literature EC_{50}[22]	Experimental EC_{50}	Literature EC_{50}[2]
EUrd	<0.1	0.054	0.1	-	0.9	0.20
ECyd	<0.1	0.015	0.1	0.06	<0.1	0.069

Table 5.11 shows the results of EUrd and the synthesised ProTides **5.17**, **5.18**, **5.19**, **5.21** and **5.22**, compared to the positive control paclitaxel.

Table 5.11: Cytostatic activity of EUrd and ProTides against selected cancer cell lines. EC_{50} values in μM.

	Leukaemia MOLT-4		Pancreas Mia-Pa-Ca-2		Colon HT29		Liver HepG2		Breast MCF-7		H&N Cal 27		Glioblastoma T98G	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
EUrd	<0.1	99	<0.1	98	0.1	83	0.2	80	0.9	92	0.5	101	1.8	86
5.17	2.9	102	0.2	99	1.2	97	0.7	98	0.8	96	1.1	101	5.3	91
5.18	0.3	100	1.8	102	1.7	91	6.2	92	7.4	97	7.1	103	-	-
5.19	0.4	100	1.2	100	1.0	88	3.3	88	3.0	95	2.2	102	-	-
5.21	0.3	99	0.7	102	0.8	86	1.8	85	2.9	95	2.7	102	-	-
5.22	59.8	94	8.8	98	10.9	92	9.8	94	29.9	85	28.9	97	75.0	71.2
Paclitaxel	0.01	99	0.004	84	0.004	74	0.029	58	0.003	80	0.002	94	0.004	87

High top inhibition percentage was also observed in the ProTides. The most active ProTides were found to be **5.17** (Neop-*L*-Ala-Naph, LogP = 3.34) and **5.21** (Hexyl-*L*-Ala-Ph, LogP = 2.62). They bear very different moieties, but they exhibit higher lipophilicity in the range of the series. The cell lines where **5.17** was found to be active was as follows, in descending order: Mia-Pa-Ca-2 > HepG2 > HT29 > Cal 27 > MCF-7 > MOLT-4 >T98G. The the cell lines where **5.21** was found to be active was as follows in descending order: MOLT-4 > Mia-Pa-Ca-2 > HT29 > HepG2 > Cal 27 > MCF-7 > T98G.

However, these ProTides did not boost the activity of the parent nucleoside. In the best case, the activity was equal to the parent nucleoside as for **5.17** in cell line MCF-7, but the EC_{50} values were generally higher by one order of magnitude.

Special attention is drawn to **5.22** (iPr-*L*-Ala-Ph, LogP = 1.65), which performed much worse than the others, possibly because of the stability of the ester moiety, or because of the low LogP that may result in low cell permeability.

Table 5.12 shows the results of ECyd and its ProTides **5.24** and **5.23** compared to the positive control paclitaxel.

Table 5.12: Cytostatic activity of 3'ECyd and ProTides against selected cancer cell lines. EC_{50} values in μM.

	MOLT-4		Mia-Pa-Ca-2		HT29		HepG2		MCF-7		Cal 27		T98G	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
ECyd	<0.1	99	<0.1	99	0.1	84	0.1	80	<0.1	90	<0.1	99	0.7	84
5.23	57.8	-1	3.3	97	7.2	84	4.3	94	4.5	96	4.5	100	87.0	60
5.24	7.3	1	0.3	100	0.8	100	0.8	100	0.5	100	0.7	100	10.9	99
Paclitaxel	0.006	-5.8	0.003	85	0.004	80	0.075	54	0.003	81	0.002	97.75	0.008	87

The ProTides were not active in the leukaemic cell line MOLT-4, where inhibition at the top concentration did not reach 2 %. Interestingly, this was one of the cell lines where the parent nucleoside showed the highest activity. In the other cell lines, the ProTides moderately inhibited cell viability, especially **5.24** whose EC_{50} was submicromolar (~ 0.6 μM), and which showed the highest activity in the Mia-Pa-Ca-2 cell line; except for cell line T98G, where EC_{50} was 10.9 μM. Interestingly, **5.23** showed activity one order of magnitude worse than **5.24**. The cell lines where the ProTides showed highest activity was as follows, in descending order: Mia-Pa-Ca-2 > MCF-7 > Cal 27 > HepG2 > HT29 > MOLT-4 > T98G.

Table 5.13 shows the results of EMUrd and the synthesised ProTides **5.28**, **5.29**, **5.30** and **5.31** compared to the positive control paclitaxel.

Table 5.13: Cytostatic activity of 3'ECyd and ProTides against selected cancer cell lines. EC_{50} values in μM

	MOLT-4		Mia-Pa-Ca-2		HT29		HepG2		MCF-7		Cal 27		T98G	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
EMUrd	>198	-9	>198	35	>198	36	>198	29	>198	-2	>198	9	>198	6
5.28	>198	1	>198	38	>198	13	>198	4	>198	24	>198	18	>198	3
5.29	32.1	<1	7.0	94	11.2	75	16.5	65	22.8	72	19.5	93	>198	42
5.30	81.5	2	21.4	69	182.2	58	>198	50	61.2	68	72.7	70	>198	31
5.31	>198	5	>198	15	>198	10	>198	8	>198	19	>198	17	>198	9
Paclitaxel	0.006	-6	0.003	85	0.004	80	0.075	54	0.003	81	0.002	98	0.008	87

Here, the ProTides were found to be not as active as in the previously discussed families. In most cases, they did not reach high levels of top inhibition. The ProTides **5.28** and **5.31** totally lacked activity. Poor activity was displayed by **5.30**, which in most of the cell lines reached an inhibition of more than 50 % of cell viability at 198 μM concentration. **5.29** was found to be the most active of the group with the highest activity elicited on Mia-Pa-Ca-2 cells (EC_{50} = 7.03 μM) and Cal27 cells (EC_{50} = 19.46 μM). However, since the parent nucleoside was totally inactive, this still constitutes an enhancement in activity.

The best compounds of each family in comparison with their respective parent nucleoside are displayed in *Table 5.14*. Interestingly, they all bear hexyl-*L*-Ala-phenyl moieties (**5.21**, **5.24** and **5.29**) and are also among the most lipophilic. It will require further investigation to analyse if the activity is related to the specificity of the substituent or to the physicochemical characteristics.

Table 5.14: Comparison of the EC_{50} (μM) of the best performing ProTides of each family, and their parent nucleotide

	MOLT-4	Mia-Pa-Ca-2	HT29	HepG2	MCF-7	Cal 27	T98G
3'EUrd	<0.1	<0.1	0.1	0.3	0.9	0.5	-
5.21	0.3	0.7	0.8	1.8	3.0	2.7	-
3'ECyd	<0.1	<0.1	0.1	0.1	0.1	<0.1	0.7
5.24	7.3	0.3	0.8	0.8	0.5	0.7	10.9
3'EMUrd	>198	>198	>198	>198	>198	>198	>198
5.29	32.1	7.00	11.2	16.5	22.8	19.5	>198

Only in the case of the EMUrd family the best performing synthesised ProTide **5.29** enhanced the activity of the parent nucleoside. Of note, the parent nucleoside was completely inactive, and, therefore, a moderately active compound was derived from a non-active nucleoside analogue. **5.21** and **5.24** showed a similar activity as their parent nucleoside in cell lines HT29, HepG2 and MCF-7. However, even if they did not enhance the activity of their two very active parent nucleosides, **5.21** and **5.24** were the most active among all the investigated families. **5.21** was found to have the highest activity in MOLT-4 cells. In HT29 cells, the activity of the two

ProTides was similar and for the rest of the cells the activity of **5.24** was higher. (Figure 5.7)

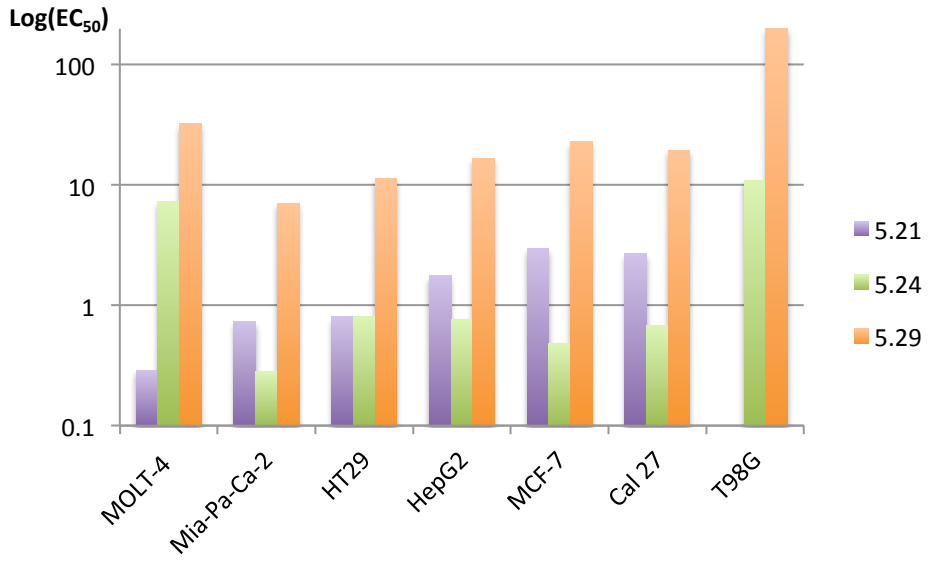


Figure 5.7 *Comparison of the EC_{50} (μM) of the best performing ProTides of each family, all bearing hexyl-L-Ala-Benzyl moieties.* ***5.21*** *derived from EUrd,* ***5.24*** *derived from ECyd,* ***5.29*** *derived from EMUrd.*

It is worth noting here that even if ECyd had a very high *in vitro* activity, it performed very poorly in clinical trials.[25] As mentioned before, those results were attributed to the resistance of cancer cells towards 3'-ethynyl nucleosides because of a decrease in membrane transport and uridine cytidine kinase activity.[8] Both of these processes are aimed to be overcome by ProTides. Therefore, a similar activity of the ProTides and parent nucleoside in this assay, as shown by **5.21** and **5.24** in cell lines HT29, HepG2, and MCF-7 may already constitute a promising result. It would be worth to test those compounds against colon, liver, and breast cell lines, which are resistant to their parent nucleosides.

Glioblastoma multiforme cell line T98G line was chosen for testing because of a recent publication that identified EUrd in a broad screening to be active against temozolidine-resistant-glioblastoma-initiating cells.[19] It was not possible to test our compounds in that specific cell line, so T98G was selected for the test. EUrd showed an EC_{50} of 1.79 μM and ECyd showed an EC_{50} of 0.75 μM for this cell line. EUrd ProTide **5.17** showed an EC_{50} of 5.32 μM, which was comparable to the parent nucleoside. Unfortunately, this information was published after the shipment of the

first batch of compounds to WuXi AppTec, so only a few compounds were tested to obtain preliminary results. Since the activity of the parent nucleoside and the ProTide was comparable, it could be possible that the activity could also be similar for temozolomide-resistant-glioblastoma-initiating cells. Further studies on the activity of the EUrd ProTides on glioblastoma cell lines may be desirable.

Enzymatic assays

Carboxypeptidase Y

After the cell viability assays, the EUrd ProTide **5.18** with medium activity, the EUrd ProTide **5.22** with low activity and the most active EMUrd ProTide **5.29** were tested in a carboxypeptidase Y enzymatic assay (*Table 5.15*). This assay aimed to analyse if the different performance of the ProTides is related to any difference in the first activation step of the prodrug. The assay also investigated whether the 3'-ethynyl ProTides can be processed by the enzyme that was identified as being chiefly responsible for this first activation towards the monophosphorylated species.

Table 5.15: Compounds tested in Carboxypeptidase Y enzymatic assay

Compound	Parent nucleoside	Moieties
5.18	EUrd	Neop-*L*-Ala-Ph
5.22	EUrd	iPr-*L*-Ala-Naph
5.29	EMUrd	Hexyl-*L*-Ala-Ph

The experiment was performed as described in Chapter 3 and the experimental chapter, by dissolving the compound in deuterated acetone and recording the spectrum to set the blank reference. After the addition of the Carboxypeptidase to the sample in TRIZMA buffer at pH 7.6 and 25 ˚C, mutiple ^{31}P NMR spectra were recorded periodically for 13 hours. Shifts in the signal indicated the formation of new species.

Two intermediate compounds are often observed in this experiment: The phosphoramidate after the cleavage of the ester moiety (usually showing two signals around 4-5 ppm), and the aminoacyl phosphoramidate intermediate after the subsequent cleavage of the aryloxy moiety (showing one signal usually around 6-7 ppm). The aminoacyl phosphoramidate would then require another enzyme, a phosphoramidase, to finally release the monophosphorylated NA.

This experiment was first performed with 7 mg of **5.22** (Figure 5.8). In the blank sample containing the mixture of the two diastereoisomers of the ProTide, the ^{31}P NMR spectrum showed two signals at 3.46 and 2.91 ppm (A). After 13 hours no

new signal was observed, so the sample was left incubating at 25 ˚C for a longer period. After 48 h, a small conversion of the compound was seen by the emergence of a small new signal at 6.00 ppm. The sample was incubated up to 20 days and the small signal at 6.00 ppm did not increase, showing stabilization in the activation. More enzyme was added to the sample and the spectra was recorded again after 24 hours. The signal at 6.00 ppm had grown, but the major signals still corresponded to remaining **5.22**. The sample was incubated up to 48 hours after the second addition of the enzyme, but the process seemed to have come to a halt again.

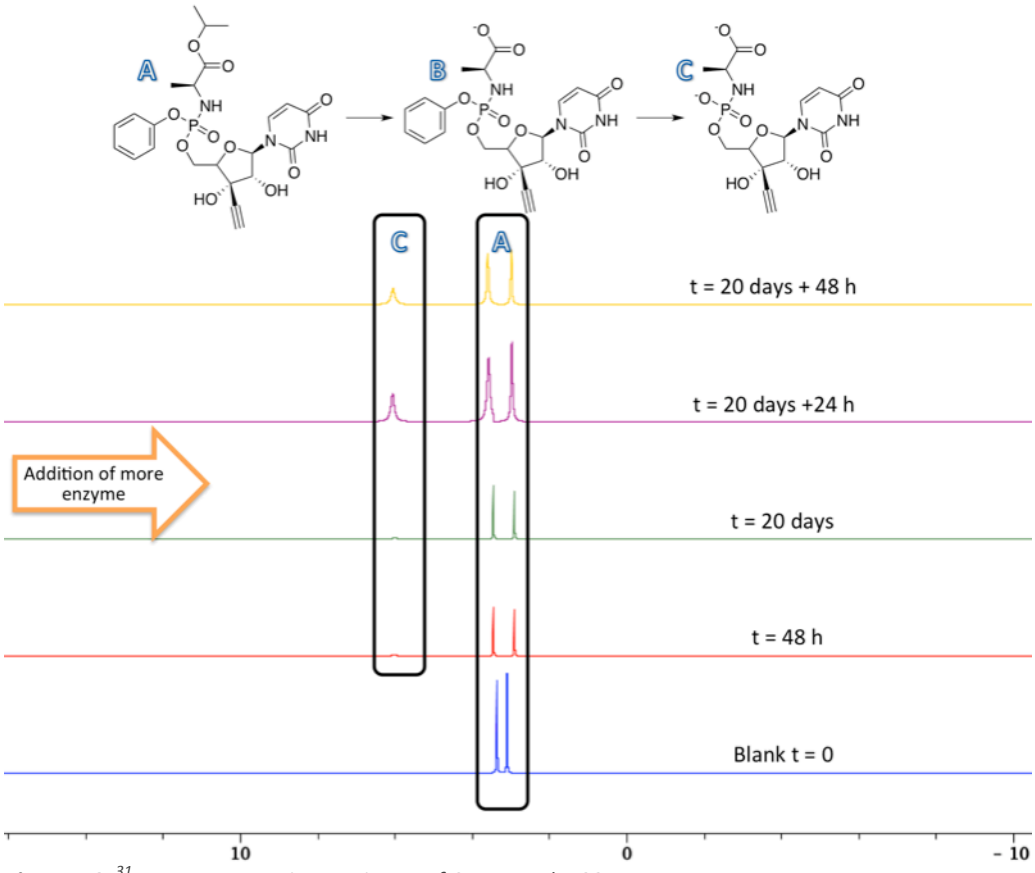


Figure 5.8: *^{31}P NMR enzymatic experiment of Compound* ***5.22***

Compound **5.22** showed very poor biological activity. The results of the enzymatic NMR assay therefore indicated that this might be due to **5.22** being very resistant to enzymatic activation. Following this, the experiment was performed for another EUrd ProTide, using 5 mg of compound **5.18** which had shown moderate activity in the cell viability assay. Figure 5.9 shows the main changes in the ^{31}P NMR spectra. The blank sample shows the signals for the mixture of diastereoisomers for the compound (two signals observed at 3.44, 2.86 ppm (A)). However, as for

compound **5.22**, no change occurred after 48 hours or 20 days after the addition of the enzyme (kept at 25 ˚C). After 20 days, more enzyme was again added to the sample and the spectrum was recorded again after 24 hours. A signal at 6.08 ppm appeared, but there was still a large amount of remaining ProTide **5.18**. The sample was then incubated for up to 48 hours after the second addition of the enzyme, but the compound seemed to undergo some degradation, as indicated by the appearance of a signal at -5.57 ppm (D)

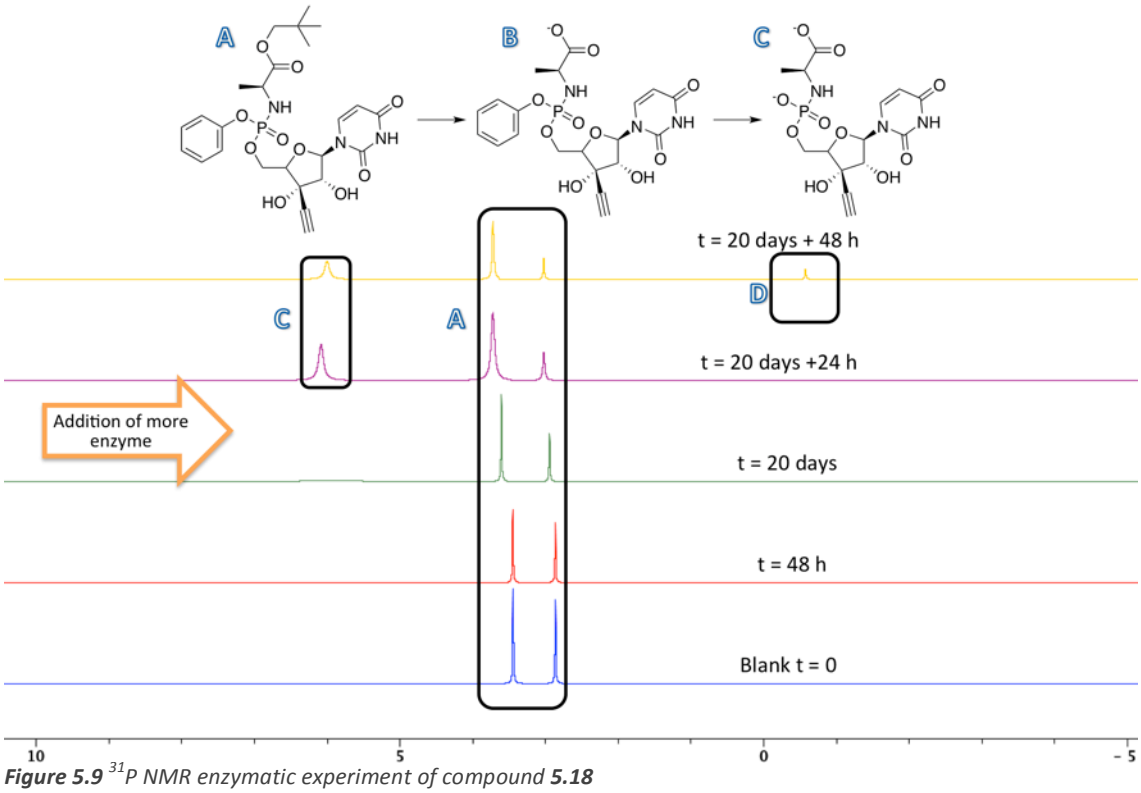


Figure 5.9 *^{31}P NMR enzymatic experiment of compound **5.18***

These results suggested that EUrd ProTides are very resistant to the activation by carboxypeptidase.

To verify if this characteristic is a particularity of other 3'-ethynyl-nucleosides, the experiment was performed on 7 mg of the most active EMUrd ProTide, **5.29** (Figure 5.10). The blank experiment showed the signal of the 2 diastereoisomers at 2.75 and 3.37 ppm. In the next recorded experiment, 7 minutes after the addition of the enzyme, the appearance of 2 small signals at 3.37 and 3.84 ppm (B) was observed, which corresponded to the phosphoramidate after the cleavage of the

ester moiety, which in turn was transformed into the aminoacyl phosphoramidate from minute 23 onwards, showing a new signal at 6.04 ppm (C). After 70 minutes the only observed signals belonged to ProTide **5.29** and its aminoacyl phosphoramidate. No more transformation was observed and the reaction seemed stable after 13 hours.

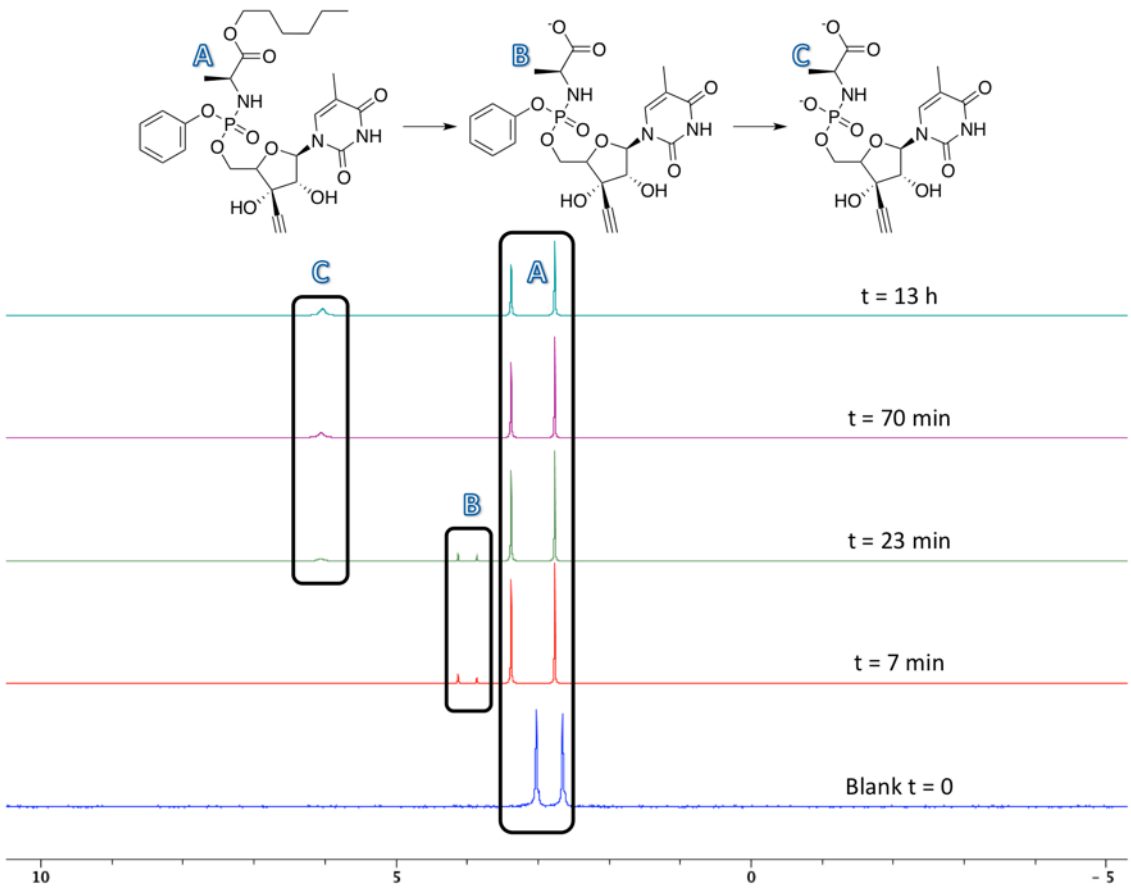


Figure 5.10 *^{31}P NMR enzymatic experiment of compound* ***5.29***

There is no evident explanation for the fact that the EUrd compounds were not processed at all after the first addition of the carboxypeptidase – apart from potential experimental error of course, but that seemed unlikely as the same effects were observed in two independent experiments. However, after the second addition of the enzyme, some quantity of compounds **5.18** and **5.22** underwent a slow activation. That activation eventually stopped and the rest of the ProTide remained unprocessed. No differences in the activation process were observed between the

ProTide **5.18** with medium activity in the cell viability assay and the ProTide **5.22** with low activity.

The experiment with the EMUrd ProTide **5.29** showed the same metabolism pattern as the EUrd ProTides after the second addition of the enzyme. As the experiment was recorded every 15.7 minutes, the slow transformation via intermediates until reaching the final aminoacyl phosphoramidate could be observed. However, similarly to EUrd ProTides, after a small quantity of ProTide had been processed, no more conversion was seen and the reaction had seemingly come to a halt.

This very slow conversion may explain why EUrd ProTides did not boost the activity with regards to their parent nucleosides after 72 hours of incubation with the cells, as perhaps a longer period is required for their activation. This characteristic could be useful to use the ProTides as reservoirs of the NA. Another possibility is that other enzymes perform the release of the monophosphate species.

Molecular Modelling

These experiments were performed in collaboration with Doctor Salvatore Ferla.

Molecular modelling studies were performed to gain understanding on the ester hydrolysis of the 3'-ethynyl-nucleoside-ProTides in the first activation step, and to predict the conversion of the aminoacyl intermediate into 3'ethynyl-nucleoside-5'-monohosphate by Hint-1 enzyme

Docking with cathepsin A

During the carboxypeptidase Y enzymatic assay a slow hydrolysis of the 3'-ethynyl-nucleoside-ProTides was observed. To better understand the enzymatic results, molecular modelling studies of EUrd ProTide **5.22** using crystal structure of carboxypeptidase Y available in the protein data bank (PDB 1YSC)[41] were performed. The ester hydrolysis process reported by Jung *et al.* (1999) is illustrated in chapter 3. It requires an interaction of the compound with Ser146, and proximity to Gly52 and Gly53.

Due to the sterical hindrance of the 3' ethynyl group none of the docking poses fitted into the receptor in an adequate manner. Figure 5.11 shows the best docking pose of the R diastereoisomer of ProTide **5.22**, which placed the carboxyl group in proximity to the Ser146 residue, and allowed its interaction with the Gly52 residue. However, in that orientation, the structure acquired a forced conformation and clashed in the catalytic site.

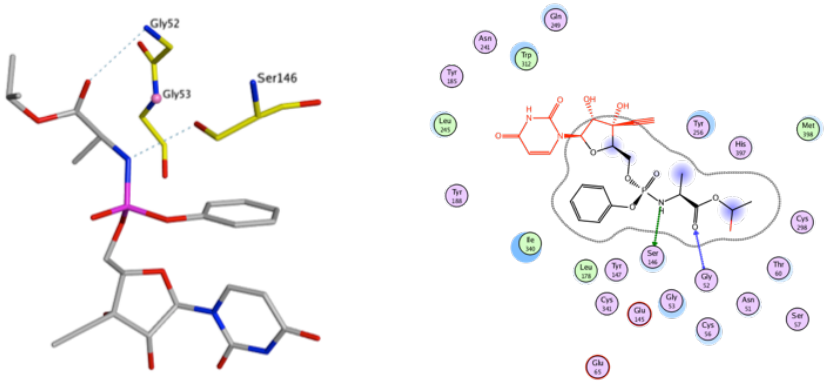


Figure 5.11*Left: Ser146, Gly52 and Gly52 residues (yellow) in the site of action of Carboxypeptidase Y interacting with the R diastereoisomer of ProTide* ***5.22*** *(grey). Right: Interactions of R diastereoisomer of compound* ***5.22*** *in the pocket site of carboxypeptidase Y*

Docking results suggested that EUrd ProTides are not good substrates for the carboxypeptidase Y enzyme. These findings may explain the slow process of these compounds during the carboxypeptidase experiments and low activity compared to their parent nucleosides.

Docking with Hint-1 enzyme

The final step to release of the monophosphorylated NA inside the cells is catalysed by Human triad nucleotide-binding proteins (Hint). They are responsible for breaking the P-N bond between the aminoacyl residue and the phosphate by interaction of histidine residues in the active site. Residues His112, His114 and Ser107 are responsible for the activity.[42]

Docking studies with the aminoacyl phosphoramidate analogue of EUrd, ECyd and EMUrd were carried out on human Hint-1, available in the protein data bank (PDB 1KPF)[43] co-crystallised with a molecule of AMP (yellow). The docking results showed the phosphate moiety of the aminoacyl intermediate of EUrd in a suitable position for the cleavage of the P-N bond. Moreover, the phosphate and the pyridine base lie in the binding pocket in a similar manner to the phosphate and adenine of the co-crystallised AMP. The best docking pose of the aminoacyl intermediates of ECyd and EMUrd do not lie in a similar position to AMP as EUrd, but the phosphate group lies in proximity of the histidine residues responsible for the activity. The interaction of the phosphate moiety of each compound with His112, His114, and Ser107 suggested that the last step would release the 5'-monophosphate species of each compound (Figure 5.12, Figure 5.13, Figure 5.14).

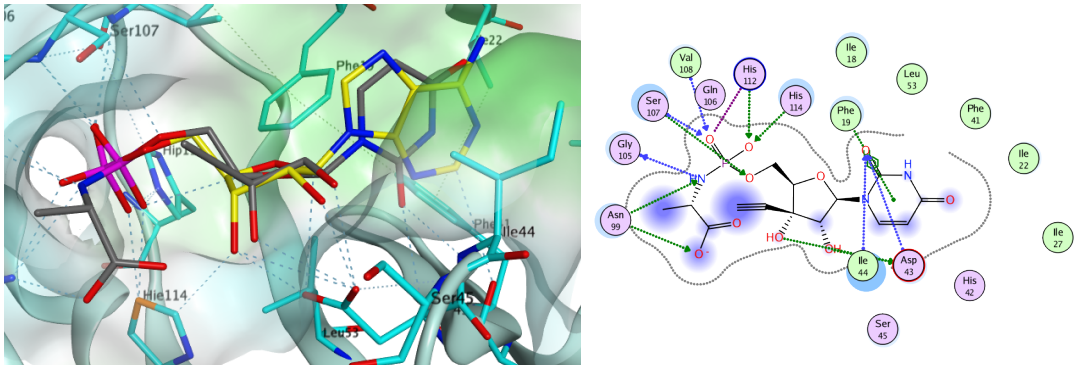


Figure 5.12 *Left: L-alanilyl-3'-ethynylcytidine phosphate (grey) and AMP (yellow) in the active site of Hint-1. Right: Interactions of L-alanilyl-3-ethynylcytidine phosphate with the amino acid residues in the active site of Hint-1*

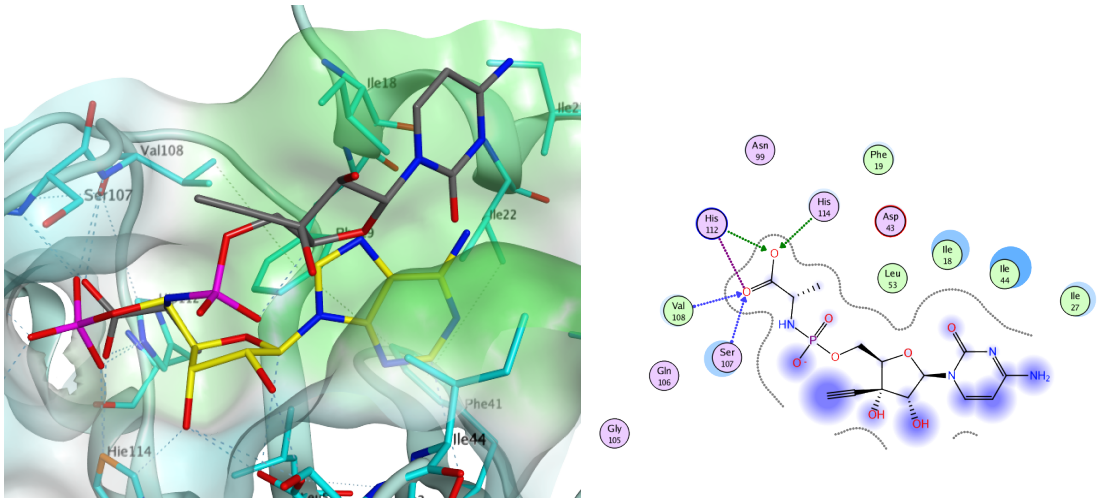


Figure 5.13 *Left: L-alanilyl-3'-ethynylcytidine phosphate (grey) and AMP (yellow) in the active site of Hint-1. Right: Interactions of L-alanilyl-3-ethynylcytidine phosphate with the amino acid residues in the active site of Hint-1*

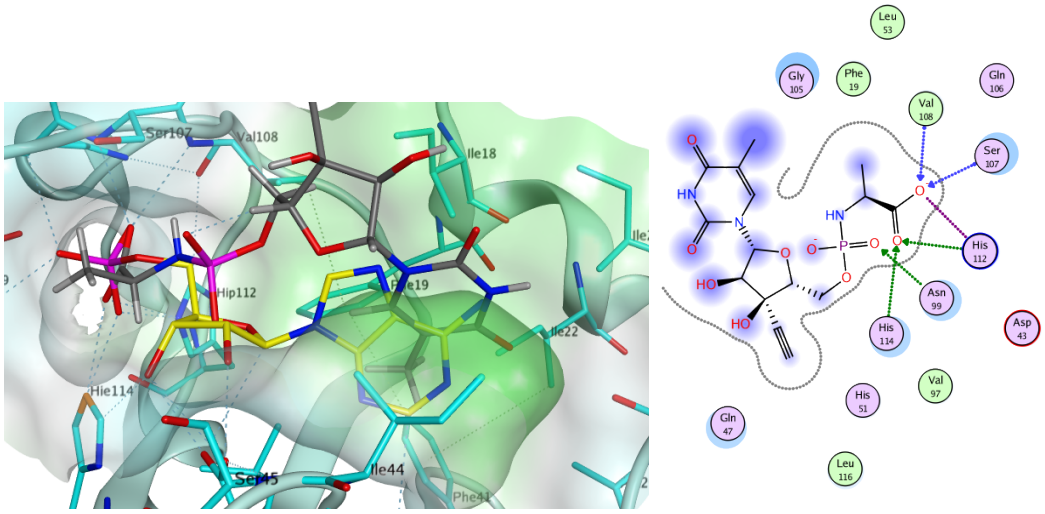


Figure 5.14 *Left: L-alanilyl-3'-ethynyl-5-methyluridine phosphate (grey) and AMP (yellow) in the active site of Hint-1. Right: Interactions of L-alanilyl-3-'ethynyl-5-methyluridine phosphate with the amino acid residues in the active site of Hint-1*

Conclusion

Two pathways for the synthesis of 3'-ethynyl nucleosides were explored, and optimisation of the reported synthesis by Nomura *et al*. (2002) was achieved, specifically, by changing the oxidising conditions by using DMP to obtain compound **5.6**. The second pathway explored delivered EUrd in a 3-fold increased yield in comparison to the first one. ECyd was also synthesised with the optimised conditions. Furthermore, EMUrd was synthesised for the first time using intermediate **5.13**.

A small family of EUrd, ECyd, and EMUrd ProTides was then synthesised using the NMI approach, although only low reactivity of the coupling reaction was observed. In particular, ECyd coupling reaction delivered yields of 1 – 2%. A different method to synthesise ECyd ProTides using *t*BuMgBr did not prove successful.

The synthesised parent nucleosides and ProTides were tested for their activity on cancer cell lines. In agreement with the literature, ECyd and EUrd showed submicromolar activity. The ProTides of these NAs did not enhance the activity. **5.21** and **5.24** were the most active ProTides in the EUrd and ECyd family respectively and showed similar activity than their parent nucleosides in cell lines HT29, HepG2 and MCF-7. EMUrd was not active, as expected from the literature. Most of the ProTides of this compound were inactive as well, except for ProTide **5.29** which transformed the inactive parent nucleoside into a partially active compound. The most active ProTides in each family bore hexyl-*L*-Ala-phenyl substituents and showed a high degree of lipophilicity.

Carboxypeptidase assays performed on compounds **5.18**, **5.22** and **5.29** showed very low activation by this enzyme. These results may correspond to their low observed *in vitro* activity compared with the activity of the parent nucleosides. However, it is possible that *in vitro* and *in vivo*, other enzymes could act in the first activation step.

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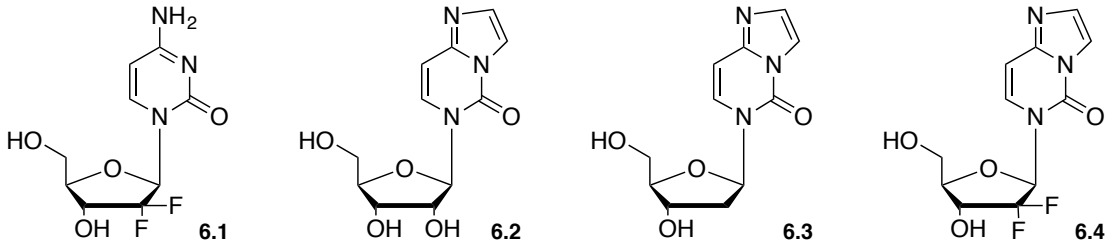
6. 3,N^4-Etheno-gemcitabine

3,N^4-Etheno-gemcitabine ProTides – Rationale behind the design

This novel nucleoside analogue is a fusion between the anticancer drug gemcitabine **6.1**[1] and 3,N^4-ethenodeoxycytidine **6.3**, a fluorescent nucleoside analogue,[2] which forms a highly mutagenic DNA adduct.[3] It has not been reported that 3,N^4-etheno-gemcitabine **6.4** was synthesised previously.



Scheme 6.1 *Structures of gemcitabine* ***6.1****, 3,N^4-ethenocytidine (****ε****C)* ***6.2****, 3,N^4-ethenodeoxycytidine (****ε****dC)* ***6.3*** *and 3,N^4-ethenogemcitabine (εGem)* ***6.4****.*

Gemcitabine **6.1** is broadly used as an anticancer agent. It is the first line treatment for pancreatic adenocarcinoma, but it is also used for the treatment of breast, bladder and non-small cell lung cancers.[4] Gemcitabine **6.1** acts as a prodrug that needs to reach its diphosphate (Gem-DP) or triphosphate (Gem-TP) forms in order to exert its biological actions. The major mechanism of action of gemcitabine is the inhibition of DNA synthesis by acting as a masked chain terminator. Once gemcitabine is incorporated, only one more nucleotide can be added. This 3' penultimate position incapacitates the polymerases and halts chain elongation, and also prevents removal of gemcitabine by DNA repair enzymes.[5]

Gemcitabine **6.1** also acts via self-potentiation. It inhibits enzymes related to deoxynucleotide catabolism such as deoxycytidylate deaminase. High concentrations of Gem-TP interact directly with the enzyme, partially avoiding its catabolism. Gem-DP also plays an important role: by directly inhibiting ribonucleoside reductase it inhibits the conversion from nucleotide diphosphate to deoxynucleotide diphosphate. This action results in the reduction of the deoxynucleotide

triphosphate pool, which downregulates deoxycytidylate deaminase. This reduction of the deoxynucleotide triphosphate pool also upregulates phosphorylation of gemcitabine to gemcitabine triphosphate. Both actions combined increase the ratio Gem-TP/deoxycytidine triphosphate, making Gem-TP more likely to be incorporated into the DNA where it again exerts its main activity.[1]

Resistance to gemcitabine has been widely studied. It is mainly caused by the low permeability of the drug, which can be either due to a lowly vascularised environment of the tumour, like in the case of pancreatic cancer, or due to a reduced expression of the nucleoside transporter protein hENT1.[1] Furthermore, the first phosphorylation step by deoxycytidine kinase is rate limiting, and it has been found that deoxycytidine kinases can be downregulated by cancer cells.[6] Overexpression of the catabolic enzymes that process gemcitabine is also a known phenomenon in the resistance of cancer cells to gemcitabine. The increase of cytidine deaminase is especially noteworthy because they transform gemcitabine into 2',2'-difluorodeoxyuridine (dFdU), a toxic metabolite that acts by inhibiting thymidylate synthase.[7] Finally, 5'-nucleotidases can be overexpressed as well. These enzymes cleave the phosphate from the 5' monophosphate end of the sugar moiety of gemcitabine[1].

Most of these mechanisms are overcome by the application of the ProTide approach on gemcitabine that led to the phase III drug NUC-1031.[8] Being a ProTide, it enters by passive diffusion into the cells without the need for hENT transporters. Inside of the cell it releases the monophosphate species independently of kinases. In addition, the ProTide NUC-1031 is not a substrate for deoxycytidine deaminase, and thus does not release the toxic metabolite.[9] Clinical trials are showing response to the treatment with NUC-1031 in patients who exhibit low expression of hENT1 and deoxycytidine kinase, and high expression of cytidine deaminase. Treatment with NUC-1031 led to disease control in patients with refractory to relapsed cancers on prior chemotherapy, including gemcitabine.[10]

3,N^4-Ethenocytidine (εC) **6.2** and 3,N^4-Ethenodeoxycytidine (εdC) **6.3** are modified nucleoside analogues characterised by bearing an etheno bridge between

the amino group at position 4 and the nitrogen atom at position 3. They are well known for their promutagenic effects. Certain endogenous compounds formed as lipid peroxidation byproducts, and some chemical carcinogens like vinyl chloride or ethyl carbamate (metabolised to 2-chloracetaldehyde and vinyl carbamate respectively) can act as nucleic acid adducts generating etheno bases in both RNA and DNA. Etheno adducts are among the most abundant DNA adducts found in the human genome. [3, 11]

When εdC **6.3** is incorporated into DNA it causes DNA lesions which may cause mutagenic transitions and initiate malignant transformations.[2] εdC **6.3** causes specific miscoding by pairing with thymine instead of guanine.[12] Cells have mechanisms for self-repair and some of the εdC **6.3** can be exised from the DNA, probably by thymine-DNA-glycosylase.[3] Mutations occurring in the RNA can also have serious implications: if they are present in mRNA, they can lead to misincorporation of amino acids during the translation process and to ribosomal arrest, which may compromise the viability of the cell. Finally, if the mutation occurs in non-coding RNAs involved in regulatory processes, the metabolism of the cell can be deeply affected.[13]

εC **6.2** was first designed as an RNA probe to analyse the ternary structure of tRNA, as it was predicted that it would emit fluorescence.[14] Since then, both εC **6.2** and εdC became of great value for probing biochemical and biophysical properties of nucleosides, nucleotides and nucleic acids.[15,16] εdC **6.3** in methanol shows a maximum emission wavelength at 335 nm and a maximum excitation wavelength of 272 nm. In these conditions, the fluorescence quantum yield is 0.055.[16] εdC **6.3** shows a maximum emission wavelength at 347 nm and a maximum excitation wavelength of 300 nm.[14] It was discovered that the fluorescence emission intensity of these compounds varies depending on the pH, having a greater intensity at lower pH values.[14] Indeed, studies shown the need for the nitrogen at position 1 to be protonated in order to produced fluorescence. Figure 6.1 shows the intensity of the fluorescence of εC **6.2** at different values of pH as measured by Barrio *et al*. (1976). Fluorescence intensity decreased progressively from pH 3.0 to pH 5.8, where the fluorescence intensity was about 1 % of the initial one at pH below 3.0.[17]

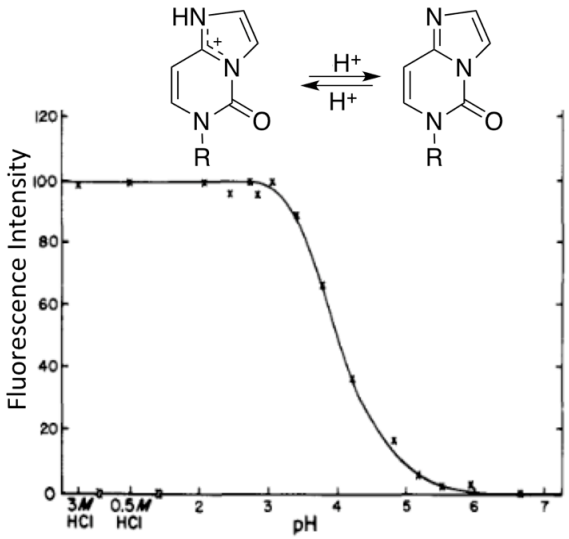
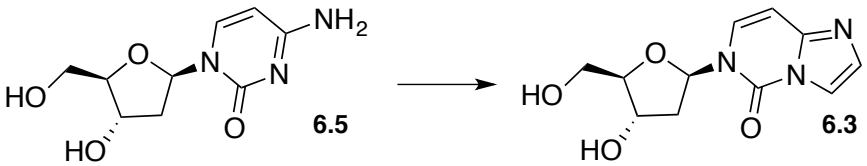


Figure 6.1 *Relative fluorescence quantum yield at different pH of εC (**6.2**)* [17] *R = Ribosyl. Adapted with permission from Barrio et al. J.Am.Chem.Soc. 1976, 98 (23), 7408-7413. Copyright 1976, American Chemical Sociey.*

The objective of this chapter was to create a new nucleoside analogue by combining the sugar moiety of gemcitabine **6.1** and 3,N^4-ethenocytosine as a base leading to 3,N^4-ethenogemcitabine **6.4**. These two compounds both interact with the DNA in cancer cells triggering apoptosis, and their combination may lead to an interesting compound with anticancer activity that may emit fluorescence. Applying the ProTide technology should enhance the interaction of the new compound with the cancer cell, providing it with an easier way to enter into the cell without the need for recognition by transporters and kinase enzymes, as it has been shown in the case of gemcitabine with NUC 1031.[9] ProTides are expected to retain the fluorescence of the parent nucleoside. This physical characteristic could be useful to observe the distribution of the drugs inside the cells.

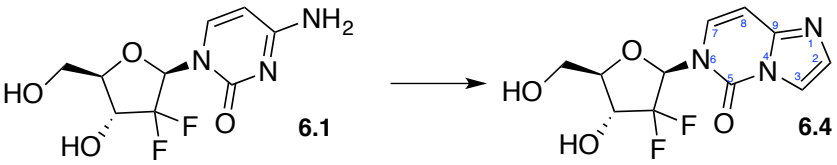
Synthesis of 3,N^4-etheno-gemcitabine

3,N^4-Etheno-gemcitabine **6.4** is a new compound, so there was no reported synthesis. The desired compound is expected to react similarly to εdC **6.3**, so the synthesis of εdC **6.3** by Li *et al*. (2002) was chosen as a reference point (Scheme 6.2),[16] aiming to extrapolate the method to synthesise 3,N^4-etheno-gemcitabine **6.4**. It is reported that the sugar has little influence on the reactivity of this reaction.[18] Therefore, it was reasonable to assume that the gemcitabine sugar would not influence the etheno-bridge formation. This synthesis is based on the introduction of the etheno bridge at positions 3 and N-4 of 1-N-methylcytosine derivatives designed by Kochetkov *et al*.[19] and it is a modification of the synthesis of εC by Barrio *et al*.[14]



Scheme 6.2 *Synthesis of 3,N^4-ethenodeoxycytidine **6.3**. Reagents and conditions: Chloracetaldehyde 2 M, 37 ˚C, pH 4.5, 20h.*

The replication of the reported reaction achieved compound **6.3** in 72 % yield after rapid purification via flash column chromatography. The same conditions were applied to synthesise 3,N^4-etheno-gemcitabine **6.4** using gemcitabine **6.1** as starting material (Scheme 6.3).



Scheme 6.3 *Synthesis of 3,N^4-etheno-gemcitabine **6.4**. Numbering for reference of ^{1}H NMR assignments (see main text). Reagents and conditions: Chloracetaldehyde 2 M, 37 ˚C, pH 4.5, 20h.*

This reaction delivered the desired product in 95 % yield. The presence of an etheno bridge between N-1 and N-4 of gemcitabine **6.1** was confirmed via ^{1}H NMR of **6.4** by two one-proton doublets at 7.85 ppm (H-3) and 7.42 ppm (H-2) and their vicinal coupling constant of 1.5 Hz. The assignment of the low-field signal to H-3 is based on the inductive effect of N-4 (enhanced by the conjugation with the carbonyl group) and the through-space deshielding effect of the carbonyl group.[20] A triplet

signal for H-1' was also observed, showing coupling of H-1' with the 2 fluorine atoms at position 2' (Figure 6.2).

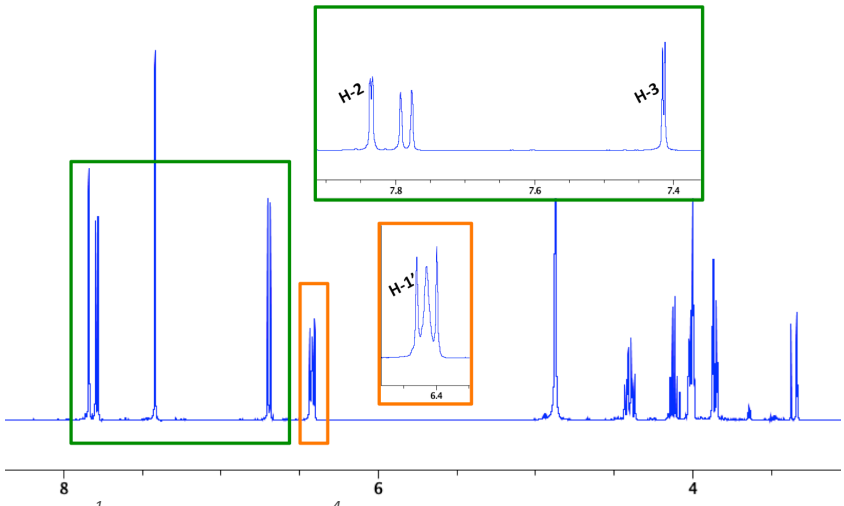
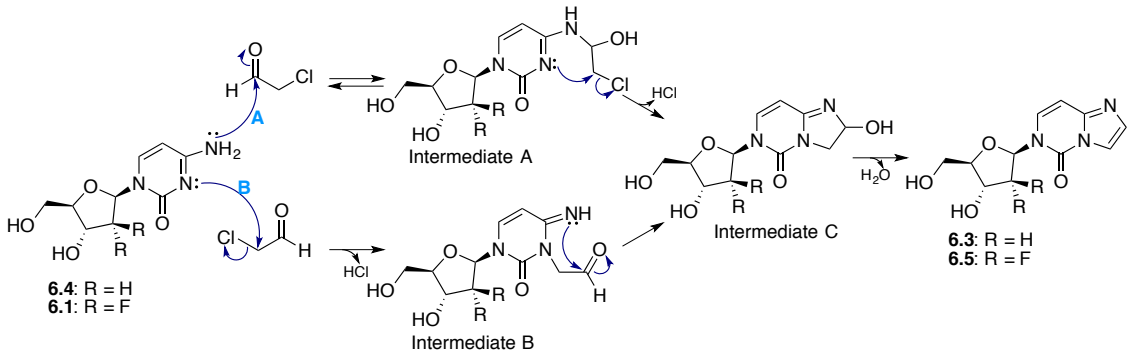


Figure 6.2 *^{1}H NMR spectrum of 3,N^{4}-etheno-gemcitabine* ***6.4****. Detail for H-2, H-3 and H-1'*

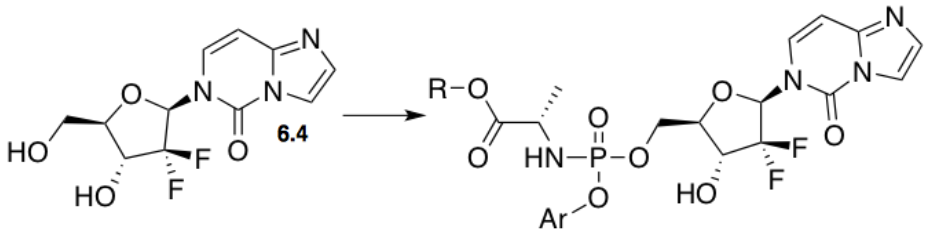
Scheme 6.4 summarises the mechanism for this reaction. Reported investigations on the formation of the etheno-bridge resulted in the isolation of intermediate C.[21] Therefore, the primary amine at position 4 reacts with the carbonyl group and the lone electron pair of N-1 attacks the chloromethylene moiety. However, there is some controversy regarding the order in which this reaction occurs. It was postulated that depending on the specific reagent used, either N-1 or the amine at position 4, would react first. Halohaldehyde reagents such as chloracetaldehyde supposedly react preferentially first with the amine, according to pathway A in Scheme 6.4.[18] A final dehydration of intermediate C then delivers the etheno-compound. It was stated that the optimal pH for the dehydration lies between 4.5-5.0.[21]



Scheme 6.4 *Putative mechanism for the formation of the etheno-bridge. A: Aldehyde reaction as first step. B: Alkylation of N-3 as first step.*[18]

Synthesis of 3,N^4-etheno-gemcitabine ProTides

Due to the availability of a free hydroxyl group at position 3', the NMI method was preferred to synthesise a small family of six ProTides of 3,N^4-etheno-gemcitabine, aiming to achieve 5'-ProTides only (Scheme 6.5). The use of a Grignard reagent would require the selective protection of position 3´ as established in the protocol followed for the synthesis of NUC-1031 and its analogues.[9] *Table 6.1* summarises the achieved ProTides using this method and the yield of the reactions.



	R	Ar
6.6	Neop	Naph
6.7	Neop	Ph
6.8	Benz	Ph
6.9	Ethyl	Ph
6.10	Hex	Ph
6.11	cHex	Ph

Scheme 6.5 *Synthesis of 3,N^4-etheno-gemcitabine ProTides. Reagents and conditions: NMI (5 eq), appropriate phosphorochloridate (3 eq), anhydrous THF, rt, 20 h.*

Table 6.1: Sumary of the coupling reactions performed on 3,N^4-etheno-gemcitabine

Compound	Yield	^{31}PNMR (ppm)
6.6	5 % (18 mg)	3.66, 3.34
6.7	32 % (99 mg)	3.85, 3.73
6.8	20 % (68 mg)	3.85, 3.65
6.9	39 % (105 mg)	3.89, 3.76
6.10	18 % (57 mg)	3.84, 3.72
6.11	11 %; 6 % after separation of isomers	3.88, 3.77

Compound **6.11** delivered a mixture of four isomers corresponding to two diastereoisomers for *L*-Alanine and two diastereoisomers for *D*-Alanine. This could be caused by a corrupted batch of *L*-Alanine. Separation of the *D* and *L*-Alanine ProTides was performed by using preparative HPLC (Analyt X Select C SH C18 (150 x 4.6 mm) column and flow of 20 ml/min) and by monitoring the purity of the mixture by ^{31}P NMR (Figure 6.3).

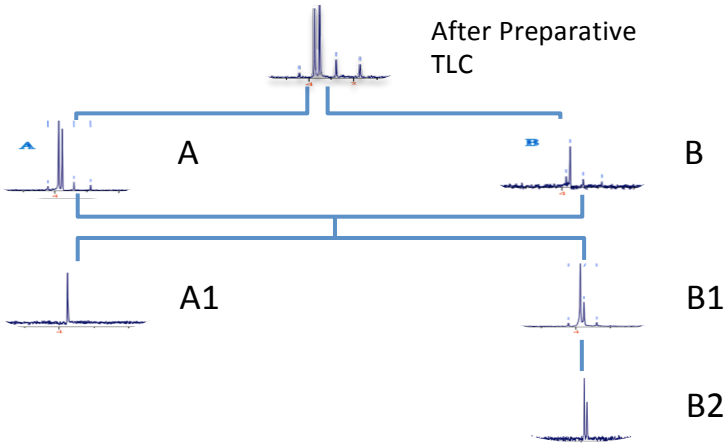


Figure 6.3 *Summary of the fractions isolated from the mixture of isomers generated during the synthesis of compound* ***6.11*** *for the isolation of the L-Alanine diastereoisomers.*

^{31}P NMR spectra for the mixture after purification by flash column chromatography and preparative TLC (Figure 6.4) showed 5 signals: 3.88 and 3.77 ppm are the singlets for the 2 diastereoisomers of the predominant *L*-Alanine ProTide. 4.22 and 3.40 ppm are the singlets for the 2 diastereoisomers of the *D*-Alanine ProTide. 2.88 ppm is an undetermined impurity.

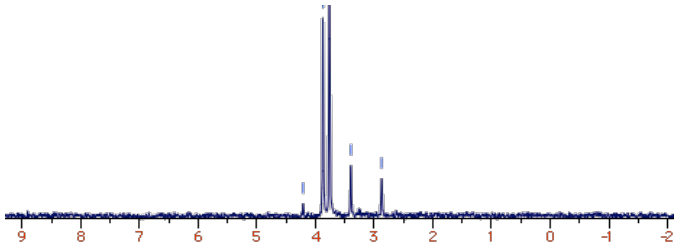


Figure 6.4 *^{31}P NMR Spectra of compound* ***6.11*** *before HPLC separation*

The first Preparative HPLC separation (Eluent system H_2O/ACN from 90:10 to 0:100 in 30 min) gave two different fractions (A and B) where separation was not achieved (Figure 6.5).

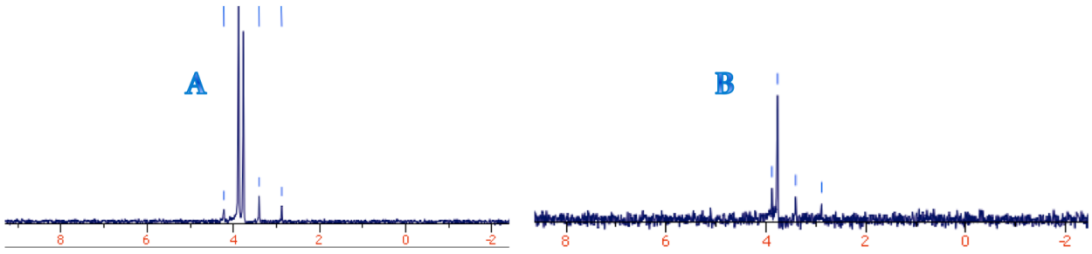


Figure 6.5 *^{31}P NMR Spectra of compound* ***6.11*** *after HPLC separation using eluent system H_2O/ACN from 90:10 to 0:100 in 30 min showing the two isolated fractions A and B.*

Fractions A and B were combined and a different eluent system (H_2O/ACN 40:60 isocratic) was used. This system achieved the isolation of the *L*-Alanine ProTide (3.77 ppm) in fraction A1. However, fraction B1 still contained the mixture of *L* and *D*-Alanine ProTides (Figure 6.6).

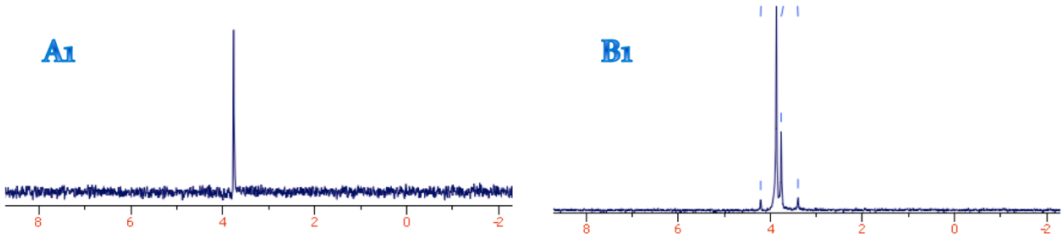


Figure 6.6 *: ^{31}P NMR Spectra of compound **6.11** after HPLC separation using an isocratic eluent system of H_2O/ACN 40:60, showing the two isolated fractions A1 and B1.*

Fraction B1 was subjected to chromatography again in a more hydrophilic isocratic gradient (H_2O/ACN 60:40 isocratic) and, isolation of the desired species was achieved in fraction B2 (Figure 6.7).

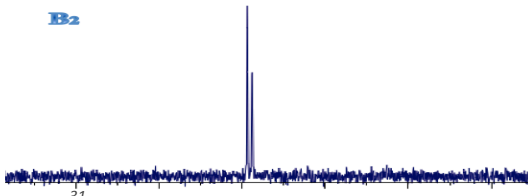
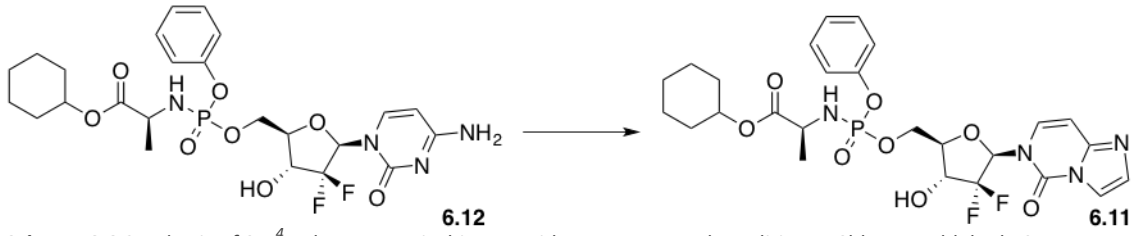


Figure 6.7*: ^{31}P NMR Spectra of compound **6.11** after HPLC separation using an isocratic eluent system of H_2O/ACN 60:40.*

In conclusion, conditions for the separation of L-/D-amino acid diastereosiomers of gemcitabine ProTides were optimised. This constitutes valuable information for a possible future combined synthesis of D-/L-ProTides.

Building a 3,N^4-etheno bridge in a gemcitabine ProTides

Ethenonucleosides are generally not very stable. They may suffer ring opening in strong basic conditions[22] and indeed, to build a ProTide basic conditions have to be used. This may result in lower yields due to degradation. A different method to avoid ring opening is therefore to build the etheno-bridge directly onto a gemcitabine ProTide. Since the reaction conditions to synthesise 3,N^4-etheno-gemcitabine are mild and the reaction delivered high yields, exploring the possibility to apply this reaction to already synthesised gemcitabine ProTides was studied (Scheme 6.5).



Scheme 6.6 *Synthesis of 3,N^4-etheno-gemcitabine ProTide. Reagents and conditions: Chloracetaldehyde 2 M, anhydrous THF, 37 ˚C, pH 5.0, 24 h.*

Doctor Magdalena Slusarczyk kindly provided 85 mg of 2'-Deoxy-2',2'-difluoro-*D*-cytidine-5'-*O*-[phenyl(cyclohexyloxy-*L*-alanine)] phosphate (compound **6.12**) to this project. The ProTide was not soluble in the chloracetaldehyde solution so THF was added as a co-solvent to mix the reagents. The same conditions as for the synthesis of 3,N^4-etheno-gemcitabine were then applied, while the pH of the reaction was slightly raised to 5. This reaction yielded 61 %, which demonstrated that this was a much more effective way to build 3,N^4-etheno-gemcitabines whenever the initial gemcitabine ProTide is available.

Cell viability assay

ProTides **6.7**, **6.9**, **6.11**, **6.6**, **6.8**, **6.10**, and the parent nucleoside 3,N^4-etheno-gemcitabine **6.4** (εGem) (*Table 6.2*), were selected for a cell viability assay performed by WuXi AppTec to assess the cytotoxic activity of the Prodrugs in comparison to their parent nucleoside, using paclitaxel as a standard control.

Table 6.2: Compounds tested in a cell viability screening performed by WuXi AppTec. Log P values generated algorithmically by a computer-based predictive algorithm using ChemDraw Professional 15.0.

Compound	Moieties	LogP
εGem (6.4)	-	-0.39
6.7	Neop-*L*-Ala-Ph	4.26
6.9	Ethyl-*L*-Ala-Ph	2.88
6.11	cHexyl-*L*-Ala-Ph	4.13
6.6	Neop-*L*-Ala-Naph	5.26
6.8	Bz-*L*-Ala-Ph	4.32
6.10	Hexyl-*L*-Ala-Ph	4.54

The cytotoxicity of the compounds was tested by measuring the cell viability of a diverse sample of cell lines from leukaemic, pancreas, colon, liver, breast, and head and neck cancer cells after the exposure to the compounds (*Table 6.3*).

Table 6.3: Cell lines used for the *in vitro* cell viability assay.

Cell Line	Malignancy	
MOLT-4	Leukaemia	Acute Lymphoblastic Leukaemia
Mia-Pa-Ca-2	Pancreas	Pancreas Carcinoma
HT29	Colon	Colorectal Adenocarcinoma
HepG2	Liver	Hepatocellular Carcinoma
MCF-7	Breast	Breast Adenocarcinoma. Derived From Metastatic Site: Pleural Effusion Epithelial -Mammary Gland
Cal27	Head and Neck	Squamous cell carcinoma

The assays were performed as described in chapter 3 and in the experimental chapter. Half maximal effective concentration (EC_{50}) and Top Inhibition percentage (percentage of non viable cells at the highest concentration TI %) were stated for each compound for every cell line as shown in *Table 6.4* and *Table 6.5*. The values for

gemcitabine **6.1** shown corresponded to the values obtained previously by the research group of Professor Chris McGuigan in the same kind of test for a different project (unpublished data, not shown). Unfortunately values for MOLT-4 and Cal 27 were missing.

Table 6.4: Comparison between the cytotoxicity of gemcitabine and the new nucleoside analogue 3,N4-etheno-gemcitabine (εGem).

	Leukaemia MOLT-4		Pancreas Mia-Pa-Ca-2		Colon HT29		Liver HepG2		Breast MCF-7		H&N Cal 27	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
εGem	1.1	87	3.2	98	15.3	90	5.7	88	4.8	76	5.1	94
Gemcitabine	-	-	<0.1	80	2.2	57	52.0	50	>198	51	-	

3,N^4-Etheno-Gemcitabine **6.4** showed high top inhibition. Inhibition values at 198 µM were above 85 % except for MCF-7 where it only achieved 76.25 % cytotoxicity. The EC_{50} values were below 6 µM except for HT29 where the EC_{50} reached 15.34 µM. 3,N^4-etheno-gemcitabine **6.4** showed higher activity than Gemcitabine **6.1** in cell lines HepG2 (10 times improvement) and MCF-7 where gemcitabine **6.1** displayed low activity and 3,N^4-etheno-gemcitabine **6.4** is moderately active. In Mia-Pa-Ca-2 and HT29 cell lines gemcitabine **6.1** (first line drug for the treatment of advanced (non-resectable Stage II or Stage III) or metastatic (Stage IV) adenocarcinoma of the pancreas)[4] showed a better activity profile.

Table 6.5: Cytotoxic activity of 3,N^4-etheno-gemcitabine and ProTides against selected cancer cell lines

	Leukaemia MOLT-4		Pancreas Mia-Pa-Ca-2		Colon HT29		Liver HepG2		Breast MCF-7		H&N Cal 27	
	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %	EC_{50}	TI %
εGem	1.1	87	3.2	98	15.3	90	5.7	88	4.8	76	5.1	94
6.7	18.7	107	14.2	100	40.7	98	24.8	98	20.4	99	3.8	89
6.9	41.6	94	59.2	90	77.6	78	52.2	93	56.6	83	64.4	91
6.11	18.8	110	27.7	100	53.8	103	37.5	99	34.0	100	18.7	99
6.6	0.5	79	2.6	76	65.3	91	7.9	56	2.1	61	1.1	78
6.8	5.7	101	4.7	100	45.4	92	7.8	79	8.5	97	3.4	89
6.10	10.7	106	21.5	99	83.3	93	43.2	88	29.9	93	6.3	84
Paclitaxel	0.01	99	0.004	84	0.004	74	0.03	58	0.003	80	0.002	94

ProTides exhibited high top inhibition percentages, most often above 85%. Surprisingly compound **6.6** (Neop-*L*-Ala-Naph, LogP = 5.26) showed the lowest top inhibition values, but was the most active compound in terms of EC_{50} along with **6.8** (Bz-*L*-Ala-Ph, LogP = 4.32). Both ProTides bear very different moieties, but they are among the most lipophilic of the series. The order of the cell lines where **6.6** was found to be active was as follows, in descending order: MOLT-4 > Cal 27 > MCF-7 > Mia-Pa-Ca-2 > HepG2 > HT29. The order of the cell lines where **6.8** was found to be active was as follows, in descending order: Cal 27 > Mia-Pa-Ca-2 > MOLT-4 > HepG2 > MCF-7 > HT29. Low activity of all compounds in the cell line HT29 was observed, as they were more than 10 times less active than in the other cell lines.

ProTides **6.6** and **6.8** retained the activity of the parent nucleoside in most of the tested cell lines. The rest of ProTides displayed an activity that was around one order of magnitude lower than the parent compound.

The ProTide approach does not show any advantage to the parent nucleoside **6.4**. This can be due to different factors. The first is a possible slow activation of the ProTide. In that case 3,N^4-Etheno-Gemcitabine **6.4** could reach the monophosphate form using the kinase enzymes more efficiently than the ProTides though their activation pathway. 3,N^4-Etheno-Gemcitabine **6.4** is a new NA, and therefore, it the mechanism of action is not studied yet. It is possible that to exert its actions it does not require the phosphorylated form, and therefore to apply the ProTide approach would not be a wise strategy. Also it is possible that 3,N^4-Etheno-Gemcitabine **6.4** acts in a totally NA unrelated pathway. More studies to elucidate its cytotoxic mechanism would be required.

Enzymatic assays

Carboxypeptidase Y

After the cell viability assays, ProTide **6.7** with medium activity and ProTide **6.8** with high activity in the series were tested in a carboxypeptidase Y enzymatic assay (*Table 6.6*). This assay aimed to analyse if the different performance of the ProTides is related to any difference in the first activation step of the prodrug and if 3,N^4-etheno-gemcitabine ProTides can be processed by the enzyme that was identified as being chiefly responsible for this first activation towards the monophosphorylated species.

Table 6.6: Compounds tested in Carboxypeptidase Y enzymatic assay

Compound	Moieties
6.7	Neop-*L*-Ala-Naph
6.8	Benzyl-*L*-Ala-Ph

The experiment was performed as described in Chapter 3 and the experimental section, by dissolving the compound in deuterated acetone and recording the blank reference spectrum. After the addition of the enzyme to the sample in TRIZMA buffer at pH 7.6 and 25 ˚C ^{31}P NMR spectra was recorded periodically for 13 hours. Shifts in the signal indicate the formation of new species.

As previously mentioned, two intermediate compounds are often observed in this experiment: The phosphoramidate after the cleavage of the ester moiety (usually shows two signals at around 4-5 ppm), and the aminoacyl phosphoramidate intermediate after the subsequent cleavage of the aryloxy moiety (shows one signal usually at around 6-7 ppm). The aminoacyl phosphoramidate then requires another enzyme, a phosphoramidase, to finally release the monophosphorylated NA.

This experiment was first performed with 5 mg of **6.7** (Figure 6.8). In the blank sample containing the mixture of diastereoisomers the ^{31}P NMR spectrum showed two signals at 3.55 and 3.30 ppm. After 13 hours no new signals were observed, so the sample was left incubating at 25 ˚C for a longer period. There was

still no observable change after 48 h of incubation there was still no observable change, so more enzyme was added to the sample, and spectra were recorded after 24h of the addition. This process was repeated two more times. 5 days after the first addition of the enzyme, the spectrum still showed the two unaltered signals of the two diastereoisomers of the ProTide. The experiment was repeated but still showed the same result.

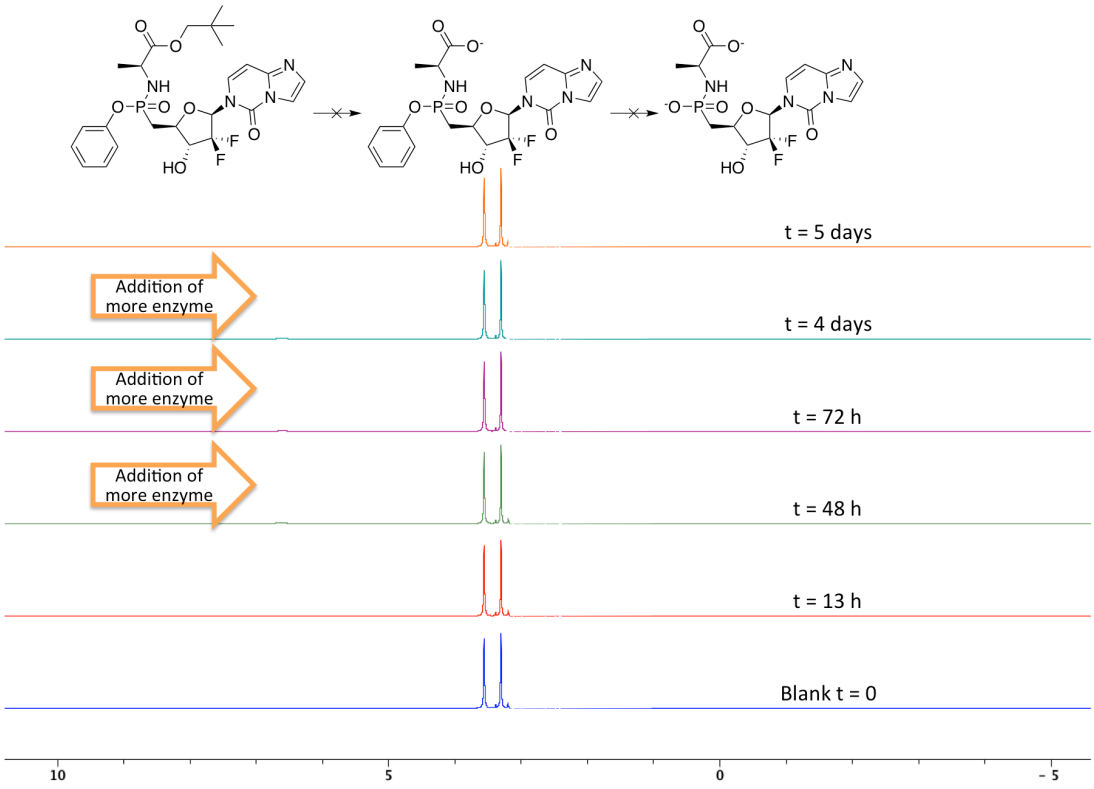


Figure 6.8 *^{31}P NMR enzymatic experiment of compound* ***6.7***

The same experiment was then performed using 5 mg of compound **6.8** (Figure 6.9) in order to determine if the behaviour of **6.7** was shared among the family of 3,N^4-etheno-gemcitabine ProTides. The first ^{31}P NMR experiment showed two signals in the spectrum at 3.51 and 3.31 ppm corresponding to the ProTide. Similarly to **6.7**, no change was seen after incubating the compound **6.8** with the enzyme for 48 h and after subsequent enzyme additions and incubations.

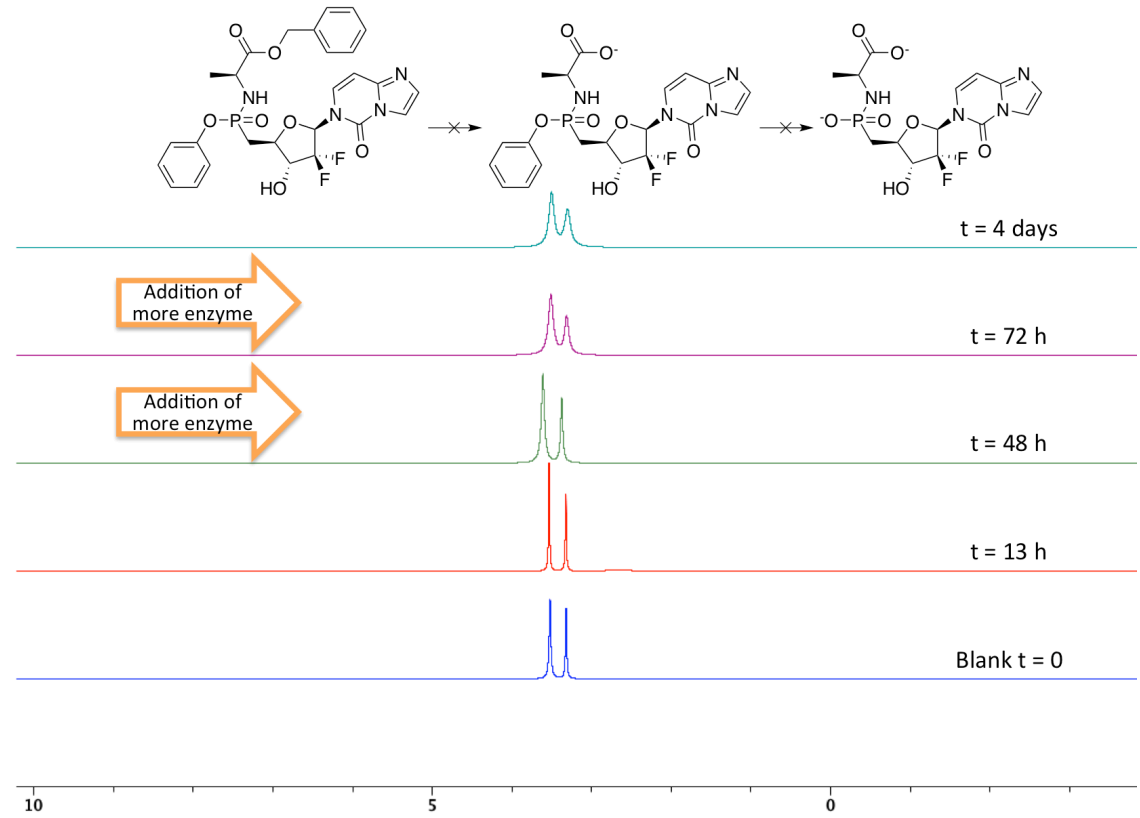


Figure 6.9 *^{31}P NMR enzymatic experiment of compound* ***6.8***

This behaviour was most unexpected, and no explanation or similarities in this behaviour was found in the literature for any other ProTides. In order to dismiss a possible experimental error, poor manipulation or poor quality of the reagents, the same experiment was performed on 10 mg of ProTide NUC 1031, which was kindly donated by Doctor Magdalena Slusarczyk, reproducing the exact same conditions and in parallel to the experiment with compound **6.8**. NUC 1031 is known to be processed in this experiment.[9] A ^{31}P NMR spectrum was recorded for the blank sample, after 15 minutes of the addition of the enzyme and 20 hours after the addition of the enzyme. Full conversion of the ProTide A (3.53, 3.29 ppm) into the aminoacyl intermediate C (6.76 ppm) via intermediate B (4.16 ppm) was observed in accordance with the literature. (Figure 6.10) This validated the previously used methods and that the observed inertness of the ProTides **6.7** and **6.8** is likely to be a feature of this class of compounds, and may also explain the lack of activity in the cell viability assays.

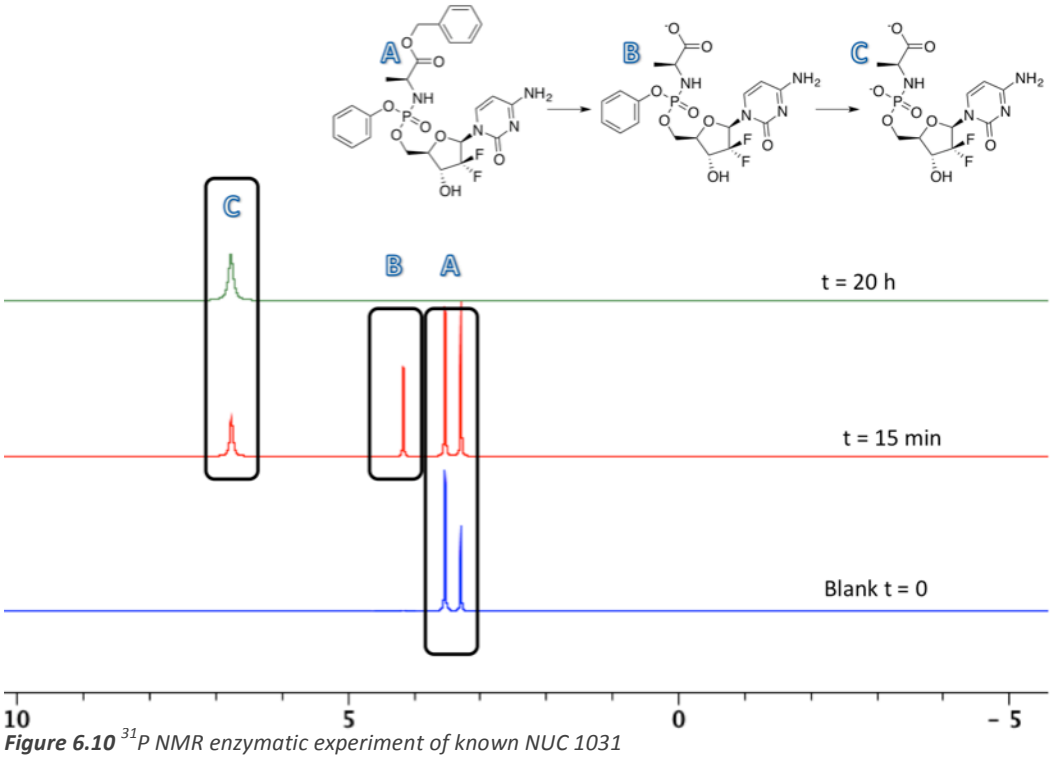


Figure 6.10 *^{31}P NMR enzymatic experiment of known NUC 1031*

Intrinsic fluorescence of the compounds

Fluorecence spectra

The synthesized compounds were expected to be fluorescent. The first assay to characterise their fluorescence was to record the fluorescence spectra of the parent nucleoside **6.4** and ProTide **6.10**. In order to do so, the compounds were dissolved in methanol at a concentration of 1 mg/mL. Fluorescence was then recorded by exciting the samples at different wavelengths until the maximum excitation and emission wavelengths were identified using an Agilent Cary Eclipse Fluorescence Spectrophotometer.

Figure 6.11 shows the absorption and emission spectra of the parent nucleoside **6.4**. The absorption maximum was 243 nm. When the compound was excited at this wavelength, the emission maximum was 485 nm.

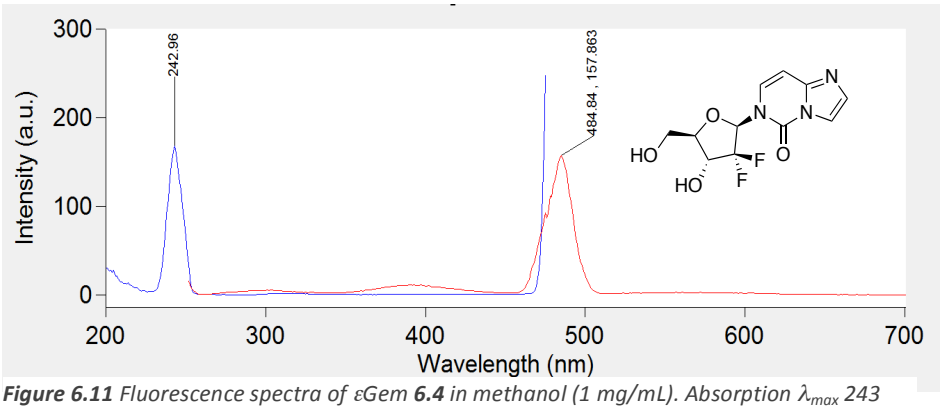


Figure 6.11 *Fluorescence spectra of εGem **6.4** in methanol (1 mg/mL). Absorption λ_{max} 243 nm (blue trace). Emission λ_{max} 485 nm (red trace).*

Figure 6.12 shows the absorption and emission spectra of ProTide **6.10**. The absorption maximum was 244 nm. When the compound was exited at that wavelength, the emission maximum was 488 nm.

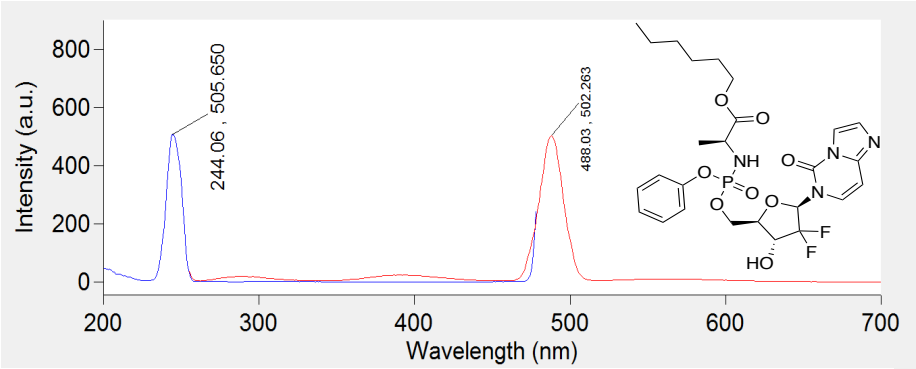


Figure 6.12 *Fluorescence spectra of εGem **6.10** in methanol (1 mg/mL). Absorption λ_{max} 244 nm (blue trace). Emission λ_{max} 488 nm (red trace).*

The very similar fluorescence profiles are probably due to the fact that the fluorescence of these molecules probably stems mostly from the bicycle ring of the base, which is unaltered across the series. Because of the low ("blue") absorption wavelength maximum, these substances are not ideal to be used in fluorescence investigations, because the techniques would require a minimal excitation wavelength below 350 nm (which is usually the lowest available wavelength for the excitation filters / lasers of common research microscopes). However these values could change when the molecule interacts with the environment inside the cell. Indeed, fluorophores are usually very sensitive to changes in the environment and to guest molecules and can reflect those changes through measurable differences in their fluorescence spectra.[16]

Epifluorescence microscopy studies of the solid compounds

These experiments were preformed in collaboration with Professor Arwyn Tomos Jones and Doctor Jennifer Mary Wymant.

Before incubating cells with the synthesised compounds, it was worth checking if the confocal microscope could detect the fluorescence of the solid compounds when they were excited at 350 nm. Therefore, the parent nucleoside 3,N^4-etheno-gemcitabine **6.4** and one of its ProTides, compound **6.10,** were observed on a Leica DMIRB inverted epifluorescence microscope. These experiments revealed some dim fluorescence (Figure 6.13 and Figure 6.14).

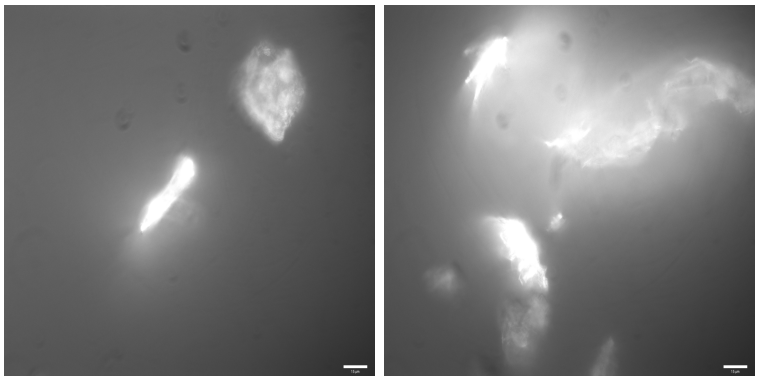


Figure 6.13 *Representative Images of a precipitate of compound* ***6.4***

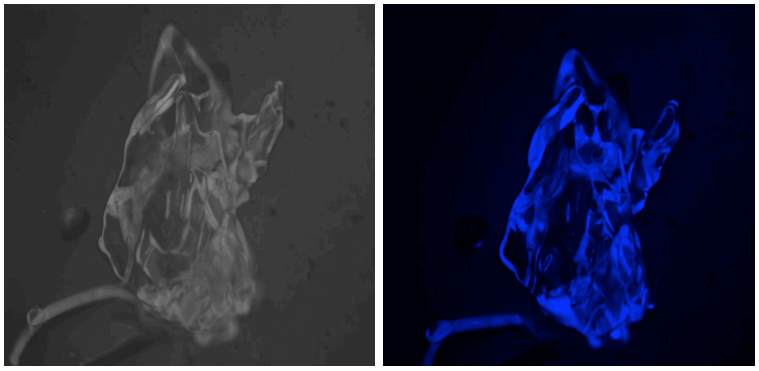


Figure 6.14 *Representative images of a precipitate of compound* ***6.10.*** *The Blue colour emulates the microscope filter in which the fluorescence of the compounds was observed.*

Fluorescence of HeLa incubated with the compounds

Epifluorescence microscopy studies of HeLa cells incubated with 3,N4-etheno-gemcitabine and some of its ProTides

These experiments were preformed in collaboration with Doctor Jennifer Mary Wymant.

To monitor the fluorescence of the compounds in living cells, HeLa cells (cervical cancer) were incubated with the parent nucleoside **6.4** and ProTides **6.7**, **6.8** and **6.10** at 20 μM for 15 or 30 minutes at 37 ˚C prior to washing and directly visualised on a Leica DMIRB inverted epifluorescence microscope.

Figure 6.15 shows representative images from these experiments. The control sample shows HeLa cells without being exposed to any of the compounds, but grown at the same time and in the same conditions as the rest of the cells. They show the intrinsic autofluorescence of the cells. The comparison between the autofluorescence of the control cells and the fluorescence of the cells incubated with the selected compounds was then used to determine if the compound emitted fluorescence inside the HeLa cells.

Two plates of HeLa cells were incubated with 3,N^4-etheno-gemcitabine **6.4**. The first was visualised after 15 minutes and the second after 30 minutes. Images for 15 minutes incubation with ProTides **6.10**, **6.7** and **6.8** and 30 minutes incubation with **6.8** are shown. In summary, the cells that were incubated with the compounds might have emitedt slightly more fluorescence in comparison to the control cells, but the difference wasnot clear enough to be attributed to the compound or to variations in autofluorescence of each sample. No difference in fluorescence was observed between the parent nucleoside and the ProTides, or among the different ProTides. No clear difference was also observed when incubating the cells with the compounds for a longer period. In conclusion, this experiment suggests that the fluorescence elicited by the different compounds, if existent, is not intense enough to monitor the distribution of the compounds inside the cells.

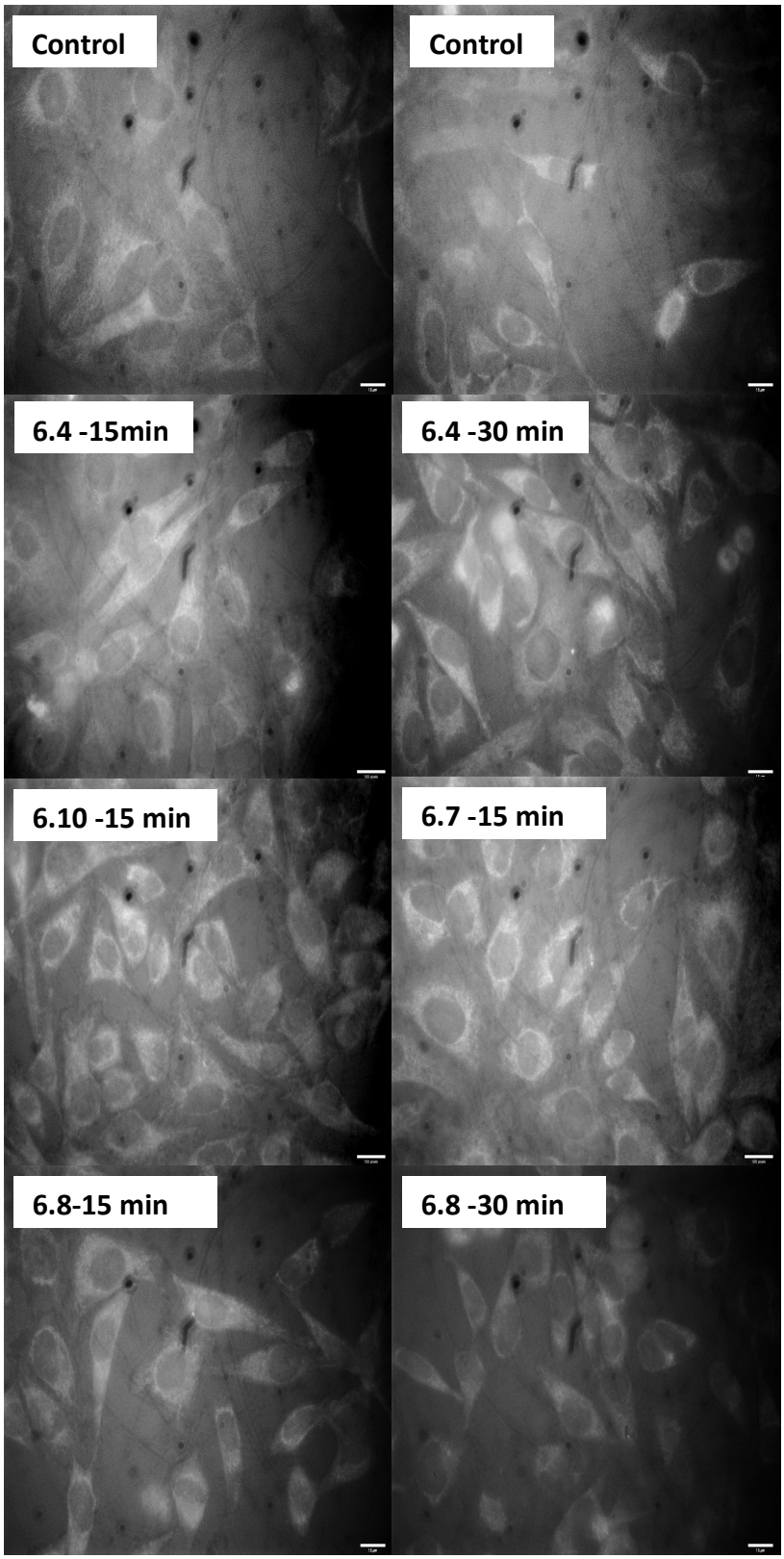


Figure 6.15 *Fluorescence mages from HeLa cells incubated with compounds **6.4**, **6.10**, **6.7** and **6.8**. ProTides.. The two upper pictures show control HeLa cells (no incubation with any compound). Picture **6.4**-15 min shows the cells after incubation with compound **6.4** for 15 min. Picture **6.4**-30 min shows the cells after incubation with compound **6.4** for 30 min. Picture **6.10**-15 min shows the cells after incubation with compound **6.10** for 15 min. Picture **6.7**-15 min shows the cells after incubation with compound **6.7** for 15 min, Picture **6.8**-15 min shows the cells after incubation with compound **6.8** for 15 min, Pictures **6.8**-30 min shows the cells after incubation with compound **6.8** for 30 min. No clear difference between the autofluorescence and the cells incubated with the new compounds was observed.*

Conclusion

The synthetic pathway of the new compound 3,N^4-etheno-gemcitabine was designed and synthesis was achieved in a quick process with high yield. A family of 3,N^4-etheno-gemcitabine ProTides was synthesised using the NMI approach for the synthesis of ProTides. A second pathway to obtain 3,N^4-etheno-gemcitabine ProTides was developed and proved to deliver much better yields when using gemcitabine ProTides directly as starting material. This result suggests the possibility to perform not only this, but also other reactions using mild conditions on already assembled ProTides.

Cell viability assays showed that this new nucleoside was moderately active as an anticancer agent, showing higher activity than Gemcitabine in cell lines HepG2 and MCF-7. ProTides **6.6** and **6.8** tended to retain the activity of the parent nucleoside. However, no advantage on the use of the ProTide approach was observed. Slow activation as a possible explanation for the poor activity of the 3,N^4-etheno-gemcitabine ProTides was proved by Carboxypeptidase Y assays. The experiment performed in compounds **6.7** and **6.8** showed that the enzyme could not remove the ester moiety to initiate the activation process. Other explanation for the activity observations is that the 3,N^4-etheno-gemcitabine **6.4** can exert cytotoxicity independently of its phosphate form. It is also possible that it exerts cytotoxicity in a different pathway not nucleotide related. More studies to elucidate the cytotoxic mechanism of 3,N^4-etheno-gemcitabine **6.4** would be desirable.

The fluorescent spectra of the parent nucleoside **6.4** and ProTide **6.10** were recorded, showing absorption maxima at 242 and 243 nm and emission maxima at 485 and 488 nm respectively. Fluorescence microscopy experiments on compounds **6.4** and **6.10** revealed that these compounds emit some dim fluorescence. Incubation of HeLa cells with compounds **6.4**, **6.7**, **6.8** and **6.10** did not show an effect of the tested compounds over that of the intrinsic cell fluorescence. Therefore, this family of compounds would probably not be suitable to monitor their intracellular distribution via their own fluorescence.

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7. Conclusion

Design, synthesis, and evaluation of different families of ProTides were reported in this thesis. They were applied to 2-chlorocordycepin, to 3'ethynyl-nucleosides (ECyd, EUrd and EMUrd), and to 3,N4-etheno-gemcitabine.

The synthesis of these ProTide families aimed to improve the anticancer activity of the parent nucleosides, and 2-chlorocordycepin and 3'-ethynyl-5-methyluridine ProTides showed a clear improvement in activity compared to their respective parent nucleosides. These encouraging results for 2-chlorocordycepin ProTides led to the exploration of other ProTide-related prodrug forms: phosphorodiamidate and phosphonodiamidate analogues were synthesised, and are awaiting in vitro activity testing in the future.

It was reported that the main hindrance for the activity of EUrd and ECyd is downregulation of the first phosphorylation step. Therefore, the lower anticancer activity of their ProTides in the in vitro activity assay was unexpected, as the ProTides were supposed to bypass this first phosphorylation step. Enzymatic assays and docking studies then suggested that their low activity was the consequence of these ProTides' poor interaction with carboxypeptidase Y, leading to a poor rate of activation.

The new synthesised nucleoside 3,N4-etheno-gemcitabine showed anticancer activity. The synthesised ProTides displayed lower or similar activity compared to their parent nucleoside. Here, no activation by carboxypeptidase Y was observed. Because this is a novel NA, the mechanism of its cytotoxic activity remains yet to be explored. Multiple explanations are possible for this behaviour, including the proven non-activation of the ProTide by the usual activating enzymes, the possibility that the monophosphorylated species is not responsible for the activity, or a non-nucleoside related mode of action. No significant fluorescence of the parent nucleoside or the ProTides was observed when incubated with HeLa cells.

In conclusion, these results provide clear evidence for the applicability of the ProTide approach to different NAs for improving their value in anti-cancer therapy. However, they also stress the point that applying the ProTide approach does not automatically lead to more active compounds and that continued studies like this one are essential for furthering the understanding of the mechanisms and activities of NAs.

Future work should benefit from the recent discoveries in this thesis in order to develop safer drugs with better activity and to elucidate their mechanisms of bio activation or reasons for non-activation.

The encouraging results of the 2-chlorocordycepin ProTides warrant further biological testing and lead optimisation. Future in vitro testing of the phosphorodiamidate and phosphonodiamidate compounds of 2-chlorocordycepin will then unveil if those new strategies can successfully deliver more resistant monophosphorylated species, and more active prodrugs.

Further studies on the slow activation of the 3'-ethynyl nucleoside family should deliver useful knowledge on the practicability for applying the ProTide approach. The exploration of other pronucleotide forms of this family than ProTides, which successfully release the monophosphorylated NA, would be of interest to enhance the activity of ECyd and EUrd, since their ProTides did not boost their activity.

The synthesis of the hydrochloride salt of 3,N4-etheno-gemcitabine and the subsequent synthesis of its ProTides should be investigated in order to see if their fluorescence can be enhanced and therefore make the visualisation of drug traffic via its own fluorescence possible. Studies on the mechanism of action of 3,N4-etheno-gemcitabine would be desirable to better understand its cytotoxic modes of actions.

8. Experimental procedures

General experimental methods

Solvent and reagents

All solvents were anhydrous and used as supplied by Sigma-Aldrich. All commercially available were used without further purification and bought from Carbosynth or Sigma-Aldrich. All nucleosides and solid reagents were dried under high vacuum for several hours prior to use.

All glassware was dried in the oven at 130 ˚C before use.

Thin Layer Chromatography (TLC) was performed on commercially available aluminium backed plates from Merck, (Gel 60-F_{254} thickness 0.2 mm) and separated fractions were visualized using ultra-violet light (254 nm)

Preparative TLC was performed on 20 x 20 cm, 500 to 2000 μm silica gel plates from Merck.

Column chromatography was performed in a manual way using silica gel (60 Å, 30-70 μm as stationary phase. Samples were applied as concentrated solutions in the same eluent or pre absorbed onto silica gel.

Flash column chromatography was performed on a Biotage Isolera One with 200-400 nm variable detector.

High performance Liquid Chromatography (HPLC) analytical and semi-preparative experiments were carried out on a Varian Prostar (LC Workstation-Varian Prostar 335 LC detector) using Analyt X Select C SH C18 (150 x 4.6 mm) as an analytic column and Varian Pursuit XRs 5C18 (150 x 21.22 mm) as semi-preparative column. Software used was Galaxie Chromatography Data System.

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 with respective

frequencies of 500, 125, 202, and 470 MHz. Spectra were auto-calibrated to the deuterated solvent peak and all ^{13}C NMR and ^{31}P NMR were proton-decoupled. Chemical shifts (δ) are given in parts per million (ppm) and coupling constant (*J*) in Hertz (Hz). The NMR signals are assigned for each molecule using the following abbreviations: singlet), br s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet) m, (multiplet)

Low Resolution Mass spectroscopy (MS) experiments were performed on a Bruker Daltonics micro Tof-LC system (atmospheric pressure ionization, electron spry mass spectroscopy) in positive mode MS (ES+)

The UV experiments were run on Varian 50 Bio UV-visible spectrophotometer and recorded by the WinUV version 3.1 software.

Fluorescence spectra of the compounds were run on Agilent Cary Eclipse

Fluorescence Spectrophotometer and recorded by the WinUV version 3.1 software.

Enzymatic assays

Carboxypeptidase Y

Before use carboxypeptidase Y assay was aliquoted in a concentration of 50 units of enzyme 0.15 mL of TRIZMA buffer and stored at -20 ˚C.The experiments were performed by dissolving 3-7 mg of the appropriate ProTide in 200 µl of deuterated acetone and 400 µl of TRIZMA buffer (pH 7.6). A ^{31}P NMR experiment was conducted as a reference t = 0. To this mixture 0.3 mg of Carboxypeptidase dissolved in 200 µl of TRIZMA buffer was added. ^{31}P NMR (128 scans) experiments of the mixture were conducted every 15 minutes for 13 hours.

Adenosine deaminase UV assay

The experiments were conducted in UV transparent cuvettes using Envision microplate reader (Perkin Elmer) Samples contained 1 mL of substrate in 40 µM solution in phosphate buffer pH 7.5. To them 20 µl of human adenosine deaminase

(ac 0.0027 U) were added and spectra were recorded in one-minute intervals for 3 hours and then every 15 minutes for 24 hours.

Cell viability assays

Cell viability assays were performed by WuXi AppTec. All the compounds were diluted by DMSO to their stock concentrations, and kept in -80 degree freezer. The compounds concentration starts at the calculated concentrations, 3.16-fold dilution in DMSO, 9 doses. For the compounds, the high concentration was 198 µM, the low concentration was 0.0199 µM. While for the reference compound (Paclitaxel) the high concentration was 0.5 µM, the low concentration was 0.0001 µM. Data was analysed using XL-fit software (Supplier: ID Business Solutions Ltd., Software version: XL fit 5.0), Inhibition% = (Max-Sample value)/Max*100.

The assay was performed to assess the effect of the selected compounds on cell viability in the selected cell lines over 72 hours using MTS cell viability assay. The tests were performed with treatment of compounds at 9 points in 96 well plates over ~72 hours. The data with the absolute EC_{50} determination and Top inhibition of each assay was provided by NuCana as shown and analysed in every chapter.

Molecular Modelling Experimental

All molecular modelling studies were performed on a Viglen Genie Intel®Core™ i7-3770 vPro CPU@ 3.40 GHz x 8 running Ubuntu 14.04. Molecular Operating Environment (MOE) 2015.10[1] and PLANTS[2] were used as molecular modelling software. The human HINT-1 and the monomeric serine carboxypeptidase from Saccharomyces cerevisiae strucures were downloaded from the PDB data bank (http://www.rcsb.org/; PDB code 1KPF and 1YSC). Hydrogen atoms were added to the proteins, using the Protonate 3D routine of the Molecular Operating Environment (MOE). Ligand structures were built with MOE and minimized using the MMFF94x force field until a RMSD gradient of 0.05 kcal $mol^{-1}/Å^{-1}$ was reached. The docking simulations were performed using PLANTS applying the following

parameters: search algorithm: aco_ants 20, aco_evap 0.15, aco_sigma 2.0; binding site: bindingsite_center [10.77 11.16 13.79-for Hint 1 protein; 47.758 -2.019 35.82-for carboxypeptidase], binding site_radius 12; cluster algorithm: cluster_rmsd 2.0, cluster_strucures 10; scoring function: chemplp. The best 10 docking poses for each compound docked, obtained as mol2 file, were then visually inspected for their potential binding in MOE 2015.10.

Fluorescence assays

1.2 x 105 cells were plated on to 35 mm glass- bottomed culture dishes and incubated under tissue culture for 16-20 h. The cells were then washed two times with D-MEM phenol red-free medium (clear medium) and incubated for 15-30 minutes at 37 ˚C with clear medium containing 20 μM solution of the compound to test. The medium was removed and the cells washed two times in clear medium prior to adding 1mL of clear medium containing 20 mM HEPES buffer, pH 7.4. The fluorescence of the compound in live cells was then immediately analysed on Leica DMIRB inverted fluorescent microscope equipped with a 340-380 nm band pass filter and 40 x oil objective. Images were captured with QIMAGING RETIGA 1300 camera and processed using Improvision Openlab 5.0.2 software, either as grey scale or as blue images.

General synthetic procedures

Synthesis of amino acid esters

A mixture of the appropriate amino acid (1 eq), the appropriate alcohol (5-15 eq) and *p*-Toluene sulphonic acid monohydrate (1 eq) are dissolved in Toluene. The stirred solution is heated at reflux temperature overnight using Dean Stark apparatus. Then allow to slowly reach RT. Solvent is evaporated under reduced pressure. The solid purified by washing the precipitate with diethyl ether to give us the amino acid ester tosylated salt.

Synthesis of Phosphorodichloridates

1 eq of the appropriate amino acid ester is dissolved in dichloromethane in anhydrous conditions and under Ar atmosphere. 1 eq of the appropriate phosphorodichloridate is carefully added to the stirring solution. Then the temperature of the mixture is lowered at -78 °C and 2 eq of triethylamine are added dropwise. The solution is stirring at -78 °C for one hour. Then the reaction is allowed to slowly reach RT. The desired product is formed in 2-4 h and the reaction is monitored by ^{31}P NMR. Solvent is evaporated under reduced pressure. Purification of the compound is achieved by either dissolving in diethyl ether and filtrating, or by a quick chromatographic column (CC) using hexane/ethylacetate 1: 1 as eluent system. Solvent is evaporated under reduced pressure, to give us the amino phosphorochloridate as a coloured oil.

Synthesis of ProTides

General Procedure A: Using NMI

To 1 eq of the appropriate nucleoside analogue dissolved in THF in anhydrous conditions and under Ar atmosphere, 5 eq of NMI are added dropwise. Some times the mixture is warmed at 30 ˚C to improve the solubility. After 30 minutes the appropriate phosphorochloridate is added to the mixture dissolved in THF. The reaction is kept between 15 h to 72 h monitoring the formation of the desired specie by TLC. Solvent is evaporated under reduced pressure. When specified, the crude is partitioned between dichloromethane and HCl 0.5 M aqueous, dried over $NaSO_4$, filtered and solvent is evaporated by reduced pressure. The crude is purified by flash chromatography followed by preparative TLC and/or preparative HPLC.

General Procedure B: Using Grignard Reagent

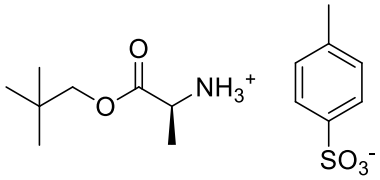
1 eq of the nucleoside analogue is dissolved in THF in anhydrous conditions and under Ar atmosphere. 1 eq of *tert*-butyl magnesium chloride is added to the stirred solution. After 30 minutes the appropriate phosphorochloridate is added to the mixture dissolved in THF. The reaction is kept between 24 and 28 hours, monitoring the formation of the desired specie by TLC. Solvent is evaporated under

reduced pressure. Then the crude is purified by flash chromatography followed by preparative TLC or preparative HPLC.

Experimental details Chapter 2

Synthesis of amino acid esters

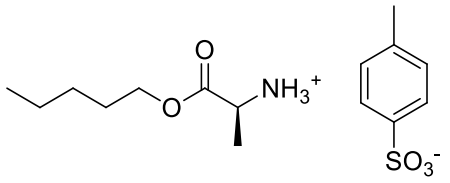
L-Alanine neopentyl ester *p*-toluenesulphonate salt (2.1)



The compound was prepared according the general procedure, using 5 g of *L*-Alanine (56.1 mmol); 30 g of neopentyl alcohol (340.3mmol) and 10.67 g (56.1 mmol) of *p*-Toluene sulphonic acid monohydrate suspended in 50 ml of toluene. The reaction was kept at reflux temperature for 16 hours. 14.36 g of a fine white powder were obtained. (Yield 77 %)

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.73 (d, *J* = 8.5 Hz, 2H, H-Ar), 7.25 (d, *J* =8 Hz, 2H, H-Ar), 4.16 (q, *J* = 7.5 Hz, 1H, C***H***CH_3), 4.04 (d, *J* = 10.5 Hz, 1H C***H***$HC(CH_3)_3$), 3.93 (d, *J* = 10.5 Hz, 1H, CH***H***$C(CH_3)_3$), 2.39 (s, 3H, CH_3-TsO^-), 1.58 (d, *J* = 7.5 Hz, 3H, $CHC\boldsymbol{H}_3$), 1.01 (s, 9H, $CH_2C(C\boldsymbol{H}_3)_3$),.

L-Alaninie hexyl ester *p*-toluenesulphonate salt (2.2)

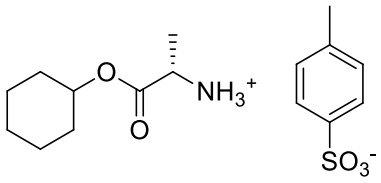


The compound was prepared according the general procedure, using 5.05 g of *L*-Alanine (56.7 mmol); 31.27 mL of hexanol (850.8 mmol) and 10.79 g (56.7 mmol) of *p*-Toluene sulphonic acid monohydrate suspended in 60 ml of toluene. The reaction was kept at reflux temperature for 18 hours. 14.17 g of a fine white powder were obtained. (Yield 75.8 %)

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.73 (d, *J* = 8.5 Hz, 2H, H-Ar), 7.25 (d, *J* = 8 Hz, 2H, H-Ar), 4.22-4.31 (m, 2H, OC***H***$_2(CH_2)_4CH_3$), 4.10 (q, *J* = 7.5 Hz, 1H, C***H***CH_3), 2.39 (s, 3H, C***H***$_3$-TsO^-), 1.69-1.74 (m, 2H, OCH_2C***H***$_2CH_2CH_2CH_2CH_3$), 1.55 (d, *J* = 7.5 Hz, 3H, CHC***H***$_3$), 1.35-1.45 (m, 6H, OCH_2CH_2C***H***$_2C$***H***$_2C$***H***$_2CH_3$), 0.94 (t, *J* =7 Hz, 3H, $O(CH_2)_5C$***H***$_3$).

^{13}C NMR (126 MHz, CD_3OD): δ_C 171.10 (C=O), 143.64 (***C***-Ar), 141.65 (***C***-Ar), 129.80 (***C***H-Ar x 2), 126.99 (***C***H-Ar x 2), 67.63 (O***C***$H_2(CH_2)_4CH_3$), 49.88 (***C***HCH_3), 32.54, 29.55, 26.54, 23.58 (OCH_2(***C***$H_2)_4CH_3$), 21.31 (***C***H_3-TsO^-), 16.26 (CH***C***H_3), 14.31($O(CH_2)_5$***C***H_3).

_L_-Alanine cyclohexyl ester p-toluenesulphonate salt (2.3)

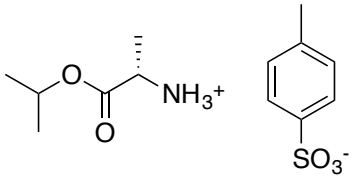


The compound was prepared according the general procedure, using 4.89 g of *L*-Alanine (54.9 mmol); 40 mL of cyclohexanol (384.3mmol) and 10.43 g (54.9 mmol) of *p*-Toluene sulphonic acid monohydrate suspending in 60 ml of toluene. The reaction was kept at reflux temperature for 20 hours. 15.80 g of a fine white powder were obtained. (Yield 84 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.20 (br s, 3H, NH_3), 7.79 (d, *J* = 8 Hz, 2H, H-Ar), 7.16 (d, *J* = 8 Hz, 2H, ***H***-Ar), 4.74-4.79 (m, 1H, OC***H***$(CH_2)_5$), 3.96 (q, *J*=7.5 Hz, 1H, C***H***CH_3), 2.37 (s, 3H, C***H***$_3$Ph), 1.47 (d, *J* = 7.5 Hz, 3H, CHC***H***$_3$), 1.20-1.79 (m, 10H, OCH(C***H***$_2)_5$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 169.27 (C=O), 141.51 (***C***-Ar), 140.34 (***C***-Ar), 128.82 (***C***H-Ar x 2), 126.11 (***C***H-Ar x 2), 75.00 (O***C***$H(CH_2)_5$), 49.18 (***C***HCH_3), 31.08, 25.16, 23.44, (OCH(***C***$H_2)_5$), 21.34 (***C***H_3-TsO^-), 16.03 (CH***C***H_3).

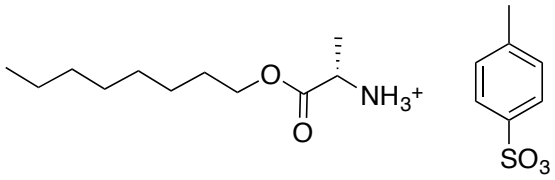
_L_-Alanine isopropyl ester p-toluenesulphonate salt (2.4)



The compound was prepared according the general procedure, using 10.0 g of *L*-Alanine (112 mmol); 128 mL of 2-isopropanol (1.68 mol) and 21.3 g (112 mmol) of *p*-Toluene sulphonic acid monohydrate suspending in 100 ml of toluene. The reaction was kept at reflux temperature for 20 hours. 32.42 g of a fine white powder were obtained. (Yield 95 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.17 (br s, 3H, N***H***$_3$), 7.78 (d, *J* = 8 Hz, 2H, ***H***-Ar), 7.16 (d, *J* = 8 Hz, 2H, ***H***-Ar), 4.96-5.02 (m, 1H, OC***H***$(CH_3)_2$), 3.95 (q, *J* = 7.2 Hz, 1H, C***H***CH_3), 2.37 (s, 3H, C***H***$_3$Ph), 1.45 (d, *J* = 7.2 Hz, 3H, CHC***H***$_3$), 1.19 (t, 6H, OCH(C***H***$_3)_2$).

_L_-Alanine octyl ester p-toluenesulphonate salt (2.5)



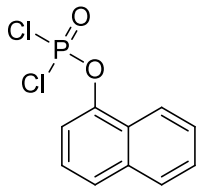
The compound was prepared according the general procedure, using 5.0 g of *L*-Alanine (56.12 mmol); 44.34 mL of 1-octanol (280 mmol) and 10.7 g (56.12 mmol) of *p*-Toluene sulphonic acid monohydrate suspending in 50 ml of toluene. The reaction was kept at reflux temperature for 18 hours. Then the solvent was evaporated under vacuum, and water was added to extract the compound from the octanol layer. The process was repeated for ten times, and then water was evaporated under high pressure to give 3.2 g of a fine white powder were obtained. (Yield 15 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.17 (br s 3H, NH_3), 7.76 (d, *J* = 8.2 Hz, 2H, ***H***-Ar), 7.16 (d, *J* = 8.2 Hz, 2H, ***H***-Ar), 4.08 (q, *J* = 7.2 Hz, 1H, C***H***CH_3), 4.00 (q, *J* = 7.1 Hz 2H, OC***H***$_2(CH_2)_6CH_3$), 2.37 (s, 3H, C***H***$_3$-TsO^-), 1.53-1.61 (m, 2H, OCH_2C***H***$_2(CH_2)_5CH_3$), 1.46

(d, *J* = 7.2 Hz, 3H, CHC***H***$_3$), 1.34-1.25 (m, 10H, OCH$_2$CH$_2$(C***H***$_2$)$_5$CH$_3$), 0.90 (t, *J* =7.1 Hz, 3H, O(CH$_2$)$_7$C***H***$_3$).

Synthesis of phosphorodichloridates

Naphtyl Phosphorodichloridate (2.6)



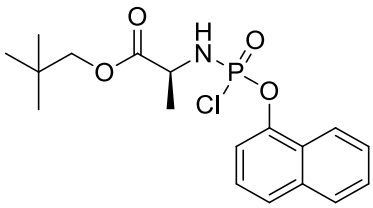
Naphtol (5 g, 34.68 mmol) was dissolved in 20 mL of diethylether in anhydrous conditions and under Ar atmosphere. To the stirring solution $POCl_3$ (3.23 ml, 34.68 mmol) was added dropwise. The temperature of the mixture was lowered at -78 °C. After ten minutes, 4.8 mL of triethylamine (34.68 mmol) were added dropwise. The solution was stirring at -78 °C for one hour. Then let the reaction to slowly reach RT. It was monitored by ^{31}P NMR. After 90 minutes stirring at RT the formation of the product was observed. The mixture was filtered and the solvent was evaporated under reduced pressure and Argon atmosphere to give us 8.90 g of brown oil (34.09 mmol, 98 %). The product was used in the following reactions without further purification.

^{31}P NMR (202 MHz, $CDCl_3$): δ_H 3.52.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.11 (d, *J* = 8.4 Hz, 1H, ***H***-Ar), 7.89 (d, *J* = 7.5 Hz, 1H, ***H***-Ar), 7.81 (d, *J* = 8.3 Hz, 1H, ***H***-Ar), 7.65-7.54 (m, 3H, ***H***-Ar), 7.45 (t, *J* = 8.0 Hz, 1H, ***H***-Ar),

Synthesis of phosphorodichloridates

Neopentyl-L-alaninyl-naphtyl phosphorochloridate (2.7)

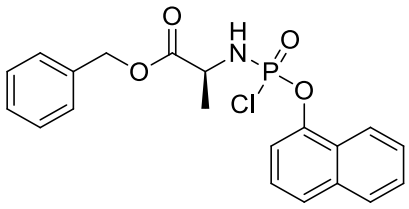


The compound was prepared according the general procedure, using 3.02 g of *L*-alanine neopentyl ester *p*-toluenesulphonate salt **2.1** (9.1 mmol) dissolved in 25 mL of anhydrous dichloromethane, 2.38 g of naphtyl phosphorodichloridate **2.6** (9.1 mmol) dissolved in 5 mL (2 x 2.5 ml) of anhydrous dichloromethane, and 2.5 mL of triethylamine (18.2 mmol). The desired product was formed after 1 hour at RT. Purification was made using CC, to give us 2.21 g of a brown oil. (Yield 63 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.02, 8.28.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.41-8.09 (m, 7H, Naph*H*) 4.50-4.61 (m, 1H, N*H*), 4.30-4.36 (m, 1H, C*H*CH_3), 3.83-3.95 (m, 2H, *CH*$_2$C(CH_3)$_3$), 1.55-1.59 (m, 3H, CHC*H*$_3$), 0.96 (s, 3.5H, $CH_2C(CH_3)_3$), 0.94 (s, 1.01, 3.5H, $CH_2C(CH_3)_3$).

Benzyl-L-alaninyl-naphtyl phosphorochloridate (2.8)

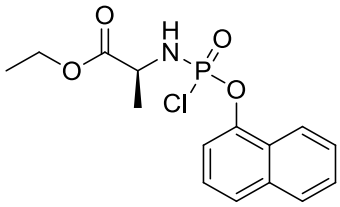


The compound was prepared according the general procedure, using 5 g of benzyl-L-alanine ester tosylated salt (14 mmol) dissolved in 20 mL of anhydrous dichloromethane, 3.71 g of naphtyl phosphorodichloridate **2.6** (14 mmol) dissolved in 4 mL (2 x 2.5 ml) of anhydrous dichloromethane, and 3.9 mL of triethylamine (28 mmol). The desired product was formed after 3 hours at RT. Purification was made using CC, to give us 3.63 g of a yellow oil. (Yield 63 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.05, 8.33.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.24-8.06 (m, 12H, Naph*H*-Ph*H*), 5.13-5.24 (m, 2H OC*H*$_2$Ph), 4.46-4.57 (m, 1H, N*H*), 4.31-4.37 (m, 1H, C*H*CH_3), 1.53-1.56 (m, 3H, CHC*H*$_3$).

Synthesis of Ethyl-L-alaninyl-naphtyl phosphorochloridate (2.9)

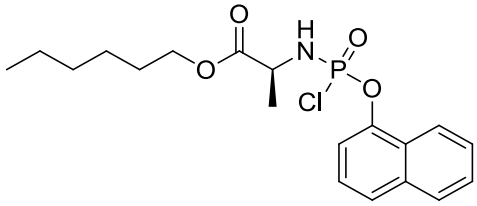


The compound was prepared according the general procedure, using 2 g of ethyl-L-alanine ester chlorinated salt (13 mmol) dissolved in 12 mL of anhydrous dichloromethane, 3.40 g of naphtyl phosphorodichloridate **2.6** (13 mmol) dissolved in 4 mL (2 x 2 ml) of anhydrous dichloromethane, and 3.6 mL of triethylamine (26 mmol). The desired product was formed after 2 hours at RT. Purification was made using CC, to give us 2.37 g of a yellow oil. (Yield 53 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.06, 8.33.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.40-7.88 (m, 7H, Naph*H*), 4.41-4.53 (m, 1H, N*H*), 4.24-4.30 (m, 2H OC*H*$_2$CH$_3$), 4.19-4.23 (m, 1H, C*H*CH_3), 1.53-1.57 (m, 3H, CHC*H*$_3$), 1.24-1.7 (m, 3H, OCH$_2$C*H*$_3$).

Synthesis of Hexyl-L-alaninyl-naphtyl phosphorochloridate (2.10)



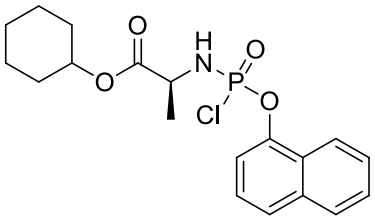
The compound was prepared according the general procedure, using 1.81 g of *L*-Alaninie hexyl ester *p*-toluenesulphonate salt **2.2** (8.7 mmol) dissolved in 25 mL of

anhydrous dichloromethane, 2.26 g of naphtyl phosphorodichloridate **2.6** (8.7 mmol) dissolved in 5 mL (2 x 2.5 ml) of anhydrous dichloromethane, and 2.46 mL of triethylamine (17.6 mmol). The desired product was formed after 3 hour at RT. Purification was made by dissolving the crude in diethylether and filtering it. 3.07 g of a beige oil were obtained. (Yield 90 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 7.98, 8.25.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.31-8.09 (m, 7H, Naph*H*), 4.60 (br s, 1H, N*H*), 4.11-4.20 (m, 1H, C*H*CH_3), 3.97-4.10 (m, 2H, O*C*$H_2(CH_2)_4CH_3$), 1.60-1.69 (m, 2H, $OCH_2CH_2(CH_2)_3CH_3$), 1.52-1.55 (m, 3H, CHC*H*$_3$), 1.24-1.40 (m, 6H, $OCH_2CH_2(CH_2)_3CH_3$), 0.82-0.90 (m, 3H, OCH_2 $(CH_2)_4CH_3$).

Synthesis of Cyclohexyl-L-alaninyl-naphtyl phosphorochloridate (2.11)

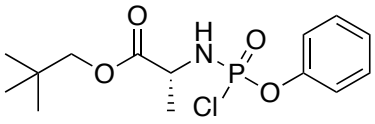


The compound was prepared according the general procedure, using 5.15 g of *L*-alanine cyclohexyl ester p-toluenesulphonate salt **2.3** (15 mmol) dissolved in 20 mL of anhydrous dichloromethane, 3.92 g of naphtyl phosphorodichloridate **2.6**(15 mmol) dissolved in 5 mL (2 x 2.5 ml) of anhydrous dichloromethane, and 4.2 mL of triethylamine (30 mmol). The desired product was formed after 3 hours at RT. Purification was made using CC, to give us 4.58 g of a yellow oil. (Yield 77 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 7.92, 8.17.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.38-7.94 (m, 7H, Naph*H*), 4.83-4.90 (m, 1H, N*H*), 4.76-4.82 (m, 1H, O*C*$H(CH_2)_5$), 3.98-4.06 (m, 1H, C*H*CH_3), 1.54-1.58 (m, 3H, CHC*H*$_3$), 1.22-1.90 (m, 10H, $OCH(CH_2)_5$).

Neopentyl-L-alaninyl-benzyl phosphorochloridate (2.12)

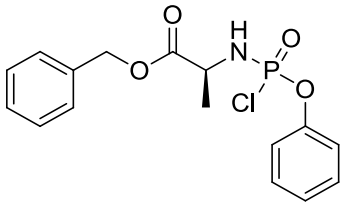


The compound was prepared according the general procedure, using 5 g of *L*-alanine neopentyl ester *p*-toluenesulphonate salt **2.1** (15.1 mmol) dissolved in 40 mL of anhydrous dichloromethane, 2.26 mL of phenyl dichlorophosphate (15.1 mmol) and 4.2 mL of triethylamine (30.2 mmol). The desired product was formed after 4 hours at RT. Compound was purified by chromatographic column according to the protocol. 3.9g of a colourless oil were obtained. (Yield 77 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 7.64, 7.98.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.24-7.42 (m, 5H, Ar-*H*), 4.20-4.30 (m, 1H, C*H*CH_3), 3.85-3.98 (m, 2H O*CH*$_2$C$(CH_3)_3$), 1.55, 1.58 (m, 3H, CHC*H*$_3$), 0.99 (s, 4.5H, $OCH_2C(CH_3)_3$), 0.98 (s, 4.5H, $OCH_2C(CH_3)_3$).

Benzyl-L-alaninyl-benzyl phosphorochloridate (2.13)

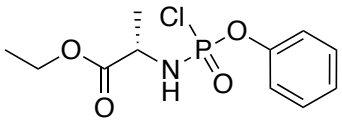


The compound was prepared according the general procedure, using 3 g of benzyl-L-alanine ester tosylated salt (8.5 mmol) dissolved in 20 mL of anhydrous dichloromethane, 1.27 mL of phenyl dichlorophosphate (8.5 mmol) and 2.34 mL of triethylamine (17 mmol). The desired product was formed after 1 hour at RT. Purification was made by chromatographic column according to the protocol. 2.54 g of colourless oil were obtained. (Yield 88 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 7.89, 7.57.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.36-7.40 (m, 7H, Ar-*H*), 7.24-7.28 (m, 3H, Ar-*H*), 5.22-5.24 (m, 2H OCH_2Ph), 4.21-4.30 (m, 1H, $CHCH_3$), 1.53-1.55 (m, 3H, $CHCH_3$).

Synthesis of Ethyl-L-alaninyl-benzyl phosphorochloridate (2.14)

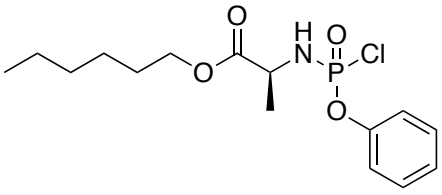


The compound was prepared according the general procedure, using 5 g of ethyl-L-alanine ester chlorinated salt (13 mmol) dissolved in 20 mL of anhydrous dichloromethane, 4.8 mL of phenyl dichlorophosphate (31.9 mmol) and 8.9 mL of triethylamine (63.8 mmol). The desired product was formed after 3 hours at RT. Purification was made by filtration of the crude mixture with diethylether. 8.93 g of the desired product were obtained as a yellow oil. (Yield 82 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.06, 8.33.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.36-7.40 (m, 2H, Ar-*H*), 7.23-7.29 (m, 3H, Ar-*H*), 4.50 (br s, 1H, N*H*), 4.22-4.29 (m, 2H OCH_2CH_3), 4.13-4.22 (m, 1H, $CHCH_3$), 1.52-1.54 (m, 3H, $CHCH_3$), 1.29-1.34 (m, 3H, OCH_2CH_3).

Hexyl-L-alaninyl-benzyl phosphorochloridate (2.15)



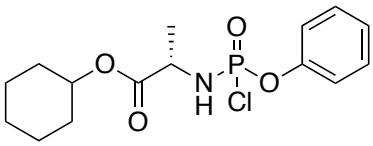
The compound was prepared according the general procedure, using 6.85 g of *L*-Alaninie hexyl ester *p*-toluenesulphonate salt **2.2** salt (20.7 mmol) dissolved in 60 mL of anhydrous dichloromethane, 3.1 mL of phenyl dichlorophosphate (20.7 mmol) and 5.8 mL of triethylamine (41.4 mmol). The desired product was formed after 4

hours at RT. Compound was purified by chromatographic column according to the protocol. 5.6 g of colourless oil was obtained. (Yield 77 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.03, 7.71.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.23-7.40 (m, 5H, Ar-*H*), 4.15-4.24 (m, 3H, C*H*CH_3; O*C*$H_2(CH_2)_4CH_3$), 1.64-1.71 (m, 2H, $OCH_2CH_2(CH_2)_3CH_3$), 1.52-1.54 (m, 3H, CHCH_3), 1.27-1.41 (m, 6H, $OCH_2CH_2(CH_2)_3CH_3$), 0.89-0.93 (m, 3H, $O(CH_2)_5CH_3$).

Cyclohexyl-L-alanine-phenyl phosphorochloridate (2.16)

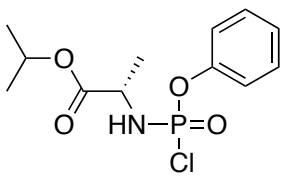


The compound was prepared according the general procedure, using 5.06 g (14.7 mmol) of *L*-alanine cyclohexyl ester p-toluenesulphonate salt **2.3** and 2.20 mL g of phenyl dichlorophosphate (14.7 mmol) and 4.11 mL (29.5 mmol) of triethylamine dissolved in 50 mL of anhydrous dichloromethane. The desired product was formed after 3 hours at RT. Purification was made using chromatographic column, to give us 3.48 g (77 %)of the desires product as a yellow oil.

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.12, 7.75.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.23-7.45 (m, 5H, H-Ar), 4.82-4.94 (m, 1H, N*H*), 4.28-4.46 (m, 1H, O*C*$H(CH_2)_5$), 4.09-4.24 (m, 1H, C*H*CH_3), 1.17-2.10 (m, 13H, CHCH_3, $OCH(CH_2)_5$).

Isopropyl-L-alaninyl-benzyl phosphorochloridate (2.17)

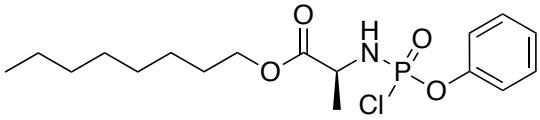


The compound was prepared according the general procedure, using 6.0 g of *L*-Alanine isopropyl ester p-toluenesulphonate salt **2.4** (19.7 mmol) dissolved in 50 mL of anhydrous dichloromethane, 2.9 mL of phenyl dichlorophosphate (19.7 mmol) and 5.5 mL of triethylamine (39.5 mmol). The desired product was formed after 4 hours at RT. Compound was purified by chromatographic column according to the protocol. 3.3 g of a colourless oil was obtained. (Yield 53 %).

^{31}P NMR (202 MHz, CD_3OD): δ_H 8.06, 7.69.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.28-7.34 (m, 2H, Ar-*H*), 7.14-7.20 (m, 3H, Ar-*H*), 4.97-5.04 (m, 1H OCH_2CH_3), 4.96-5.02 (m, 1H, $OCH(CH_3)_2$), 3.99-4.12 (m, 1H, $CHCH_3$), 1.43 (d, *J* = 2.75 Hz, 1.5H, $CHCH_3$), 1.41 (d, *J* = 2.65 Hz, 1.5H, $CHCH_3$), 1.22-1.18 (m, 6H, $OCH(CH_3)_2$).

Octyl-L-alaninyl-benzyl phosphorochloridate (2.18)



The compound was prepared according the general procedure, using 1.256 g of *L*-Alaninie octyl ester *p*-toluenesulphonate salt **2.5** salt (3.36 mmol) dissolved in 5 mL of anhydrous dichloromethane, 0.5 mL of phenyl dichlorophosphate (3.36 mmol) and 0.94 mL of triethylamine (6.72 mmol). The desired product was formed after 2 hours at RT. Compound was purified by chromatographic column according to the protocol. 0.89 g (2.37 mmol) of a colourless oil was obtained. (Yield 70 %).

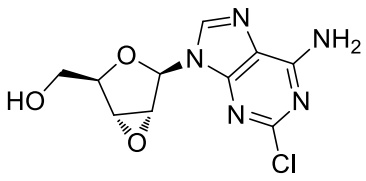
^{31}P NMR (202 MHz, CD_3OD): δ_H 8.02, 7.65.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.28-7.32 (m, 2H, Ar-*H*), 7.17-7.20 (m, 3H, Ar-*H*), 4.21-4.29 (m, 1H, $CHCH_3$), 4.08-4.13 (m, 2H, $OCH_2(CH_2)_6CH_3$), 1.53-1.61 (m, 2H, $OCH_2CH_2(CH_2)_5CH_3$), 1.43-1.46 (m, 3H, $CHCH_3$), 1.17-1.32 (m, 10H, $OCH_2CH_2(CH_2)_5CH_3$), 0.79-0.82 (m, 3H, $O(CH_2)_7CH_3$).

Experimental details Chapter 3

Synthesis of 2-chlorocordycepin

2-Chloroadenosine-2',3'-riboepoxide (3.6)



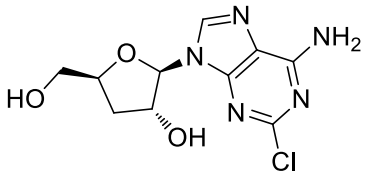
5 g of 2-Chloroadenosine **3.5** (16.6 mmol) where dissolved in 38 mL of acetonitrile in anhydrous conditions and under argon atmosphere. 2.98 mL of a solution acetonitile/water 9:1 (16.6 mmol of water) were added to the stirring solution. 9.7 ml of α-acetoxyisobutiryl bromide were added dropwise to the stirring solution. The reaction was monitored by TLC (CH_2Cl_2/CH_3OH 9:1). After 2 h the reaction was stopped by adding 90 ml of an aqueous saturated solution of $NaHCO_3$ to reach pH 7. The mixture was partionated and washed with ethyl acetate (4 x 40 ml). The organic phase was gathered and washed with 100 mL of brine (2 x 50 ml), then dried with Na_2SO_4 and filtered. Solvent was evaporated under reduced pressure, to give us 7.54 g of an intermediate product as a yellow foam. The intermediate product was hen dissolved in 300 ml of dry methanol, and 150 ml of IRN 78 (OH^-) were added. The mixture was stirring for 3 hours and then filtered and redissolving in methanol and filtrate again to recover the final product. Two filtrations were needed. Solvent was evaporated under reduced pressure to obtain 3.03 g of the desired product as a white fine powder. (Yield 60 %)

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.34 (s, 1H, ***H***-8), 7.82 (2H, br s, N***H$_2$***), 6.13 (s, 1H, ***H***-1'), 5.01 (t, *J* = 5, 1H, O***H***-5'), 4.46 (d, *J* = 2.5, 1H, ***H***-2'), 4.18-4.20 (m, 2H, ***H***-3', ***H***-4'), 3.49-3.58 (m, 2H, ***HH***-5').

^{13}C NMR (126 MHz, DMSO): δ_C 156.77 (C-6), 153.11 (C-2), 150.23 (C-4), 139.88 (CH-8), 117.75 (C-5), 81.93 (CH-1'), 81.28 (CH-4'), 60.81 (CH_2-5'), 58.51 (CH-3'), 57.62 (CH-2').

MS (ES+) m/z: Found: 284.1 [M + H^+], 306.1 [M + Na^+] $C_{10}H_{12}ClN_5O_3$ $C_{10}H_{10}ClN_5O_3$ required: 283.1 [M].

2-Chlorocordycepin (3.2)



2.18 mg of **3.6** (7.68 mmol) were suspended in 3 ml of DMSO and 5 ml of THF in anhydrous conditions and under Ar atmosphere. The temperature of the stirring solution was lowered at 0 °C ant 30.7 ml of lithium triethylborohydrade 1 M in THF (30.7 mmol) were added. Then the reaction slowly reached RT. The formation of the desired product was monitored by TLC (CH_2Cl_2/CH_3OH 9:1). After 3 h N_2 was purged inside the reaction for 30 minutes and then it was neutralized by adding 20 ml of an aqueous solution of acetic acid 10 %. A yellow mixture was formed. Solvent was co-evaporated with toluene under high pressure to give us a pale yellow solid. Then the solid was suspended in 500 ml of diethylether and stirred overnight. Solvent was removed by decantation, and the solid was dried evaporating under reduced pressure. A salt was formed. It was purified by filtering twice in silica in chromatographic columns (CH_2Cl_2/CH_3OH 9:1) to obtain the 1.202 g of the desired product as of a white fine powder.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.41 (s, 1H, H-8), 5.93 (d, *J* = 2.5, 1H, H-1'), 4.66-4.68 (m, 1H, H-2'), 4.52-4.56 (m, 1H, H-4'), 3.95 (dd, *J* = 3, 12.5 Hz, 1H, H-5'), 3.70 (dd, *J* = 3, 12.5 Hz, 1H, H-5'), 2.33-2.39 (m, 1H, H-3'), 2.03-2.08 (m, 1H, H-3').

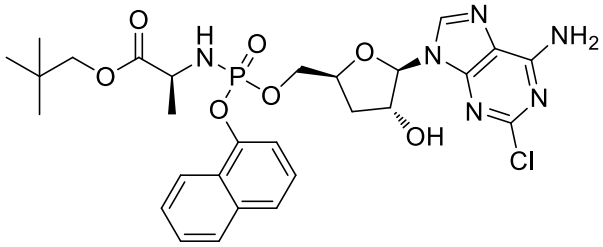
^{13}C NMR (126 MHz, CD_3OD): δC 158.14 (C-6), 155.19 (C-2), 151.15 (C-4), 141.30 (CH-8), 119.56 (C-5), 93.58 (CH-1'), 82.80 (CH-4'), 76.81 (CH-2'), 64.01 (CH_2-5'), 34.33 (CH_2-3').

MS (ES+) m/z: Found: 286.1 [M + H^+], $C_{10}H_{12}ClN_5O_3$ required: 652.16 [M];

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 259 nm, t_R 10.05 min

1st Strategy for the synthesis of 2-Chlorocordycepine ProTides

2-Chloro-3'deoxyadenosine5'-O-[1-naphtyl(2,2-dimethylpropoxy-L-alaninilyl)] phosphate (3.9)

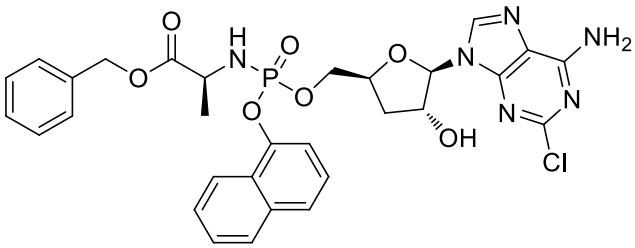


The compound was prepared according the general procedure A, using 150 mg of **3.2** (0.52 mmol) dissolved in 10 ml of THF, 0.2 ml of NMI (2.62 mmol) and 604 mg of naphtyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.7** (1.57 mmol) dissolved in 5 ml of THF (2 x 2.5 ml). The desired product was formed after 4 days stirring at RT. It was detected by MS and ^{31}P NMR. After flash chromatography and preparative TLC, 3 mg of a mixture containing the desired compound were obtained, but further purification was unable.

^{31}P NMR (202 MHz, CD_3OD): δ_H 4.30, 4.15.

MS (ES+) m/z: 633.2 [$M+H^+$], 655.2 [$M+Na^+$]; $C_{28}H_{34}ClN_6O_7P$

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate (3.10) (NMI)



Compound was prepared according to the general procedure A from 2-chloro-3'-deoxyadenosine **3.2** (100 mg, 0.35 mmol), N-methylimidazole (140 μL, 1.75 mmol) and naphtyl(benzoxy-L-alaninyl) phosphorochloridate **2.8** (424 mg, 1.05 mmol).

Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 0:100 to 5:95) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded the title compound as a white solid (7 mg, 3 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.39, 4.12.

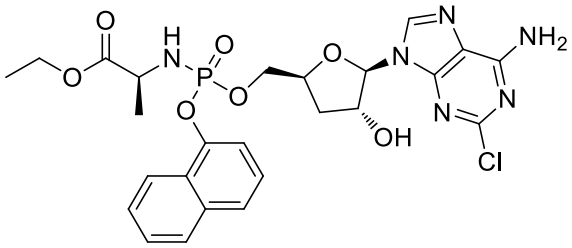
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.10 (s, 0.5H, H8), 8.07 (s, 0.5H, H8), 7.97-8.02 (m, 3H, Ar), 7.14-7.43 (m, 9H, Ar), 5.81-5.80 (m, 1H, H1'), 4.97-4.89 (m, 2H, O*C*H_2Ph) 4.53-4.49 (m, 2H, H4', H2'), 4.30-4.35 (m, 1H, H5'), 4.15-4.21 (m, 1H, H5'), 3.87-3.95 (m, 1H, C*H*CH_3), 2.12-2.23 (m, 1H, H3'), 1.86-1.93 (m, 1H, H3'), 1.14-1.17 (m, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 174.85 (d $^{3}J_{CP}$ = 4.0 Hz, C=O), 174.55 (d $^{3}J_{CP}$ = 4.3 Hz, C=O), 158.07, 158.04 (C6), 155.31, 155.28 (C2), 151.34, 151.31 (C4), 149.69 (C-Ar), 147.96 (d $^{2}J_{CP}$ = 7.25 Hz, C-*ipso* Naph), 147.90 (d $^{2}J_{CP}$= 7.0 Hz, C-*ipso* Naph), 140.70 (C8), 137.21, 137.16 (C-*ipso* OCH_2Ph), 136.26 (C-Ar), 130.92, 130.80 (CH-Ar), 129.56, 129.53 (CH-Ar), 129.31 (CH-Ar), 129.27, 129.25 (CH-Ar), 128.88, 128.81 (CH-Ar), 127.78 (d $^{3}J_{CP}$ = 4.7 Hz, CH-Ar), 127.50 (d $^{3}J_{CP}$ = 6.2 Hz, CH-Ar), 126.48 (CH-Ar), 126.02, 125.97 (CH-Ar), 119.46, 119.42 (C5), 116.33 (d, $^{4}J_{CP}$ = 3.0, CH-Ar), 116.16 (d, $^{4}J_{CP}$ = 3.4, CH-Ar), 93.30, 93.27 (C1'), 80.56 (d ^{3}J = 8.3 Hz, C4'), 80.51 (d ^{3}J = 8.4 Hz, C4'), 76.61, 76.54 (C2'), 68.74 (d $^{2}J_{CP}$= 5.3 Hz, C5'), 68.54 (d $^{2}J_{CP}$= 5.1 Hz, C5'), 67.93, 67.90 (*C*H_2Ph), 51.81, 51.70 (*C*HCH$_3$), 34.79, 34.53 (C3'), 20.42 (d $^{3}J_{CP}$ = 6.5 Hz, CH*C*H$_3$), 20.23 (d $^{3}J_{CP}$ = 7.7 Hz, CH*C*H$_3$).

MS (ES+) m/z: Found: 653 [M + H^+], 675 [M + Na^+] $C_{30}H_{30}ClN_6O_7P$ required: 652.16 [M];

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1ml/min, λ = 254 nm, t_R 18.03 min

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (ethyl-L-alaninyl)] phosphate (3.11)



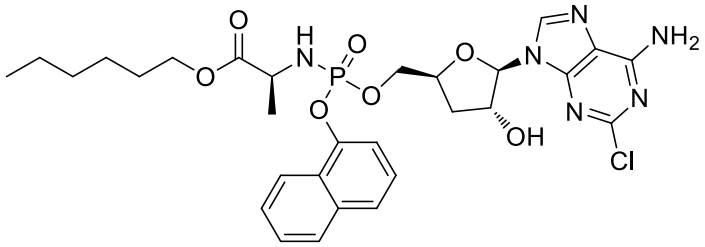
The compound was prepared according the general procedure A, using 90 mg of **3.6** (0.31mmol) dissolved in 10 ml of THF, 0.12 ml of NMI (1.57 mmol) and 323 mg of phosphorochloridate **2.9** (0.94 mmol) dissolved in 5 ml of THF (2 x 2 ml). The desired product was formed after 5 days stirring at RT. After flash chromatography and preparative TLC, 5 mg of a mixture containing the desired compound were obtained, but further purification was unable.

^{1}H NMR (500 MHz, CD_3OD): δ_H 1.03-1.06 (m, 3H, $OCH_2C\boldsymbol{H}_3$), 1.48-1.53 (m, 3H, $CHC\boldsymbol{H}_3$), 2.52-2.57 (m, 1H, ***H***H-3'), 2.66-2.77 (m, 1H, H***H***-3'), 3.57-3.561(m, 1H, ***H***H-5'), 3.78-3.81 (m, 1H, H***H***-5'), 3.91-3.94 (m, 1H, $C\boldsymbol{H}CH_3$), 4.43-4.45 (m, 1H, ***H***-4'), 4.57-4.60 (m, 1H, ***H***-2'), 5.23-5.25 (m, 2H $OC\boldsymbol{H}_2CH_3$), 5.82-5.84 (m, 1H, ***H***-1'), 7.25-7.46 (m, 7H, Naph***H***), 8.09-8.11 (m, 1H ***H***-8).

^{31}P NMR (202 MHz, CD_3OD): δ_H 4.31 (s), 4.21 (s).

MS (ES+) m/z: 591.1 [$M+H^+$]; 613.1 [$M+Na^+$]; $C_{25}H_{28}ClN_6O_7P$, required 590.1 [M]

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (hexyl-L-alaninyl)] phosphate (3.12)

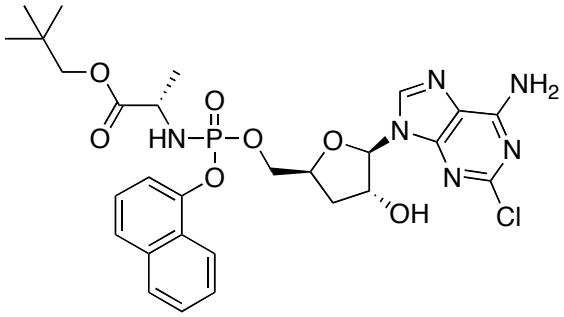


The compound was prepared according the general procedure A, using 150 mg of **3.2** (0.52 mmol) dissolved in 10 ml of THF, 2.1 ml of NMI (26.2 mmol) and 620 mg of

phosphorochloridate **2.10** (1.57 mmol) dissolved in 5 ml of THF (2 x 2.5 ml). The desired product was formed after 7 days stirring at RT. It was detected by MS. After flash chromatography 72 mg of a complex mixture was obtained. MS showed only traces of the desired compound, that could not be identified further.

MS (ES+) m/z: Found: 647.2 [M + H^+), $C_{29}H_{36}ClN_6O_7P$ required: 646.2 [M];

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (2,2-dimethylpropoxy-L-alaninilyl)] phosphate (3.9)



Compound **3.9** was prepared according to the general procedure A using 2-chloro-3'-deoxyadenosine **3.2** (350 mg, 1.25 mmol), N-methylimidazole (490 μL, 6.15 mmol) and naphtyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.7** (1416 mg, 3.69 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 1:99 to 8:92 over 15 CV) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 4/96) afforded the title compound as a white solid (264 mg, 34 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.35, 4.20.

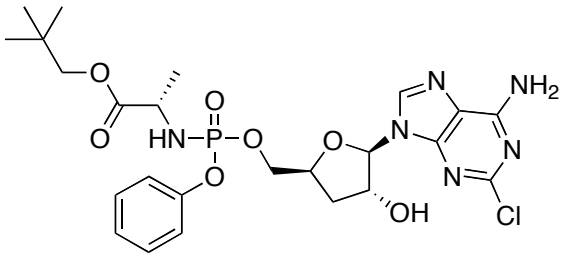
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.23 (s, 0.5H, H8), 8.21 (s, 0.5H, H8), 8.11-8.16 (m, 1H, Naph), 7.86-7.89 (m, 1H, Naph), 7.69-7.70 (m, 1H, Naph), 7.54-7.46 (m, 3H, Naph), 7.37-7.41 (m, 1H, Naph), 5.95 (d *J* = 2, 0.5H, H1'), 5.94 (d *J* = 1.5, 0.5H, H1'), 4.67-4.73 (m, 2H, H4'and H2'), 4.34-4.55 (m, 2H, H5'), 4.00-4.08 (m, 1H, C*H*CH_3), 3.66-3.81 (m, 2 H OC*H*$_2$C(CH_3)$_3$), 2.28-2.41 (m, 1H, H3'), 2.03-2.10 (m, 1H, H3'), 1.31-1.34 (m, 3H, CHC*H*$_3$), 0.90 (s, 4.5 H $OCH_2C(CH_3)_3$), 0.89 (s, 4.5 H $CH_2(CH_3)_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 175.11 (d $^3J_{CP}$ = 4.1 Hz, C=O), 174.85 (d $^3J_{CP}$ = 5.0 Hz, C=O), 158.10, 158.04 (C6), 155.32, 155.30 (C2), 151.33 (C4), 147.96 (d $^2J_{CP}$ = 7.25 Hz, C-*ipso* Naph), 147.93 (d $^2J_{CP}$= 7.25 Hz, C-*ipso* Naph), 140.84, 140.76 (C8), 136.29 (C-Ar), 128.87, 128.82 (CH-Ar), 127.85 (C-Ar), 127.77, 127.74, 127.48, 127.45, 126.47, 125.99, 125.96, 122.74, 122.66 (CH-Ar), 119.47 (C5), 116.29 (d $^3J_{CP}$ = 3.4 Hz, CH-Ar), 116.17 (d $^3J_{CP}$ = 2.9 Hz, CH-Ar), 93.42, 93.34 (C1'), 80.57 (d $^3J_{CP}$ = 8.1 Hz, C4'), 80.53 (d $^3J_{CP}$ = 5.1 Hz, C4'), 76.61, 76.53 (C2'), 75.41, 75.38 ($OCH_2C(CH_3)_3$), 68.95 (d $^2J_{CP}$= 5.3 Hz, C5'), 68.82 (d $^2J_{CP}$= 5.2 Hz, C5'), 51.84, 51.73 ($CHCH_3$), 35.04, 34.75 (C3'), 32.29 ($OCH_2C(CH_3)_3$), 26.70 ($OCH_2C(CH_3)_3$), 20.76 (d $^3J_{CP}$ = 6.4 Hz, $CHCH_3$), 20.55 (d $^3J_{CP}$ = 7.2 Hz, $CHCH_3$).

MS (ES+) m/z: Found: 633 [M + H^+], 655 [M + Na^+] $C_{28}H_{34}ClN_6O_7P$ required: 652.16 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 254 nm, $\mathbf{t_R}$ 19.16 min.

2-Chloro-3'deoxyadenosine 5'-O-[1-phenyl (2,2-dimethylpropoxy-L-alaninyl)] phosphate (3.13)



Compound **3.13** was prepared according to the general procedure A using 2-chloro-3'-deoxyadenosine **3.2** (350 mg, 1.25 mmol), N-methylimidazole (490 μL, 6.15 mmol) and phenyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.12** (1231 mg, 3.69 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 2:98 to 20:80 over 14 CV) and preparative TLC (1000 μm, eluent

system CH_3OH/CH_2Cl_2 4/96) afforded the title compound as a white solid (181 mg, 25 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.93, 3.72.

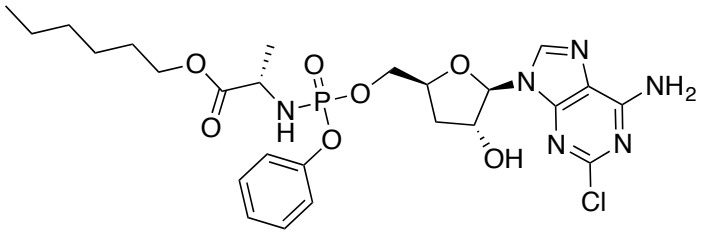
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.12 (s, 0.5H, H8), 8.10 (s, 0.5H, H8), 7.19-7.23 (m, 2H, Ph), 7.03-7.12 (m, 3H, Ph), 5.84 (d *J* = 2, 0.5H, H1'), 5.83 (d *J* =2, 0.5H, H1'), 4.54-4.60 (m, 2H, H4'and H2'), 4.34-4.38 (m, 0.5H, H5'), 4.27-4.31 (m, 0.5H, H5'), 4.16-4.23 (m, 1H, H5'), 3.80-3.90 (m, 1H, C*H*CH_3), 3.57-3.73 (m, 2 H OC*H*$_2$C$(CH_3)_3$), 2.18-2.28 (m, 1H, H3'), 1.94-1.99 (m, 1H, H3'), 1.20-1.24 (m, 3H, CHC*H*$_3$), 0.81 (s, 4.5 H $OCH_2(CH_3)_3$), 0.79 (s, 4.5 H $OCH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 175.09 (d $^3J_{CP}$ = 4.75 Hz, C=O), 174.90 (d $^3J_{CP}$ = 5.37 Hz, C=O), 158.10, (C6), 155.31, 155.28 (C2), 152.14 (d $^2J_{CP}$= 6.37 Hz, C-*ipso* Ph), 152.13 (d $^2J_{CP}$= 6.25 Hz, C-*ipso* Ph), 151.33, 151.30 (C4), 140.87, 140.76 (C8), 130.78, 130.77 (CH-Ar), 126.17, 126.42 (CH-Ar), 121.45 (d $^3J_{CP}$ = 11.75 Hz, CH-Ar), 121.41 (d $^3J_{CP}$ = 11.75 Hz, CH-Ar), 119.52, 119.48 (C5), 93.49, 93.35 (C1'), 80.67 (d ^3J = 8.62 Hz, C4'), 80.65 (d ^3J = 8.25 Hz, C4'), 76.70, 76.67 (C2'), 75.43, (O*C*$H_2C(CH_3)_3$), 68.68 (d $^2J_{CP}$= 5.12 Hz, C5'), 68.42 (d $^2J_{CP}$= 5.12 Hz, C5'), 51.77, 51.60 (*C*HCH_3), 34.94, 34.67 (C3'), 32.36, 32.32 (OCH_2***C***$(CH_3)_3$), 26.78, 26.76 ($OCH_2C(CH_3)_3$), 20.83 (d $^3J_{CP}$ = 6.25 Hz, CH*C*H_3), 20.61 (d $^3J_{CP}$ = 7.12 Hz, CH*C*H_3).

MS (ES+) m/z: Found: 583 [M + H^+], 605 [M + Na^+] $C_{24}H_{32}ClN_6O_7P$ required: 582.18 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 258 nm, $\mathbf{t}_R$ 16.37, 16.55 min

2-Chloro-3'deoxyadenosine-5'-O-[1-phenyl(hexyl-L-alaninyl)] phosphate (3.14)



The title compound was prepared according to the general procedure A using 2-chloro-3'-deoxyadenosine **3.2** (288 mg, 1.01 mmol), N-methylimidazole (0.4 mL, 5.05 mmol) and hexyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.15** (702.5 mg, 2.02 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 4:96 to 16:84 over 10 CV) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 4/96) afforded the title compound as a white solid (185 mg, 15 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.94, 3.74

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.24 (s, 0.5H, H-8), 8.23 (s, 0.5H, H-8), 7.30-7.35 (m, 2H, Ar), 7.20-7.24 (m, 2H, Ar), 7.14-7.18 (m, 1H, Ar), 5.98 (d, *J* = 1.4 0.5H, H1'), 5.97 (d, *J* = 1.3 0.5H, H1'), 4.66-4.76 (m, 2H, H2', 4'), 4.48-4.52 (m, 0.5H, H5'), 4.40-4.44 (m, 0.5H, H5'), 4.29-4.37 (m, 1H, H5'), 3.88-4.08 (m, 3H, OC***H***CH_3, OC***H***$_2(CH_2)_4CH_3$), 2.29-2.37 (m, 1H, H3'), 2.03-2.11 (m, 1H, H3'), 1.52-1.61 (m, 2H, OCH_2C***H***$_2(CH_2)_3CH_3$), 1.24-1.34 (m, 9H, CHC*H*$_3$, $OCH_2CH_2(C$***H***$_2)_3CH_3$), 0.85-0.89 (m, 3H, $OCH_2CH_2(CH_2)_3C$***H***$_3$).

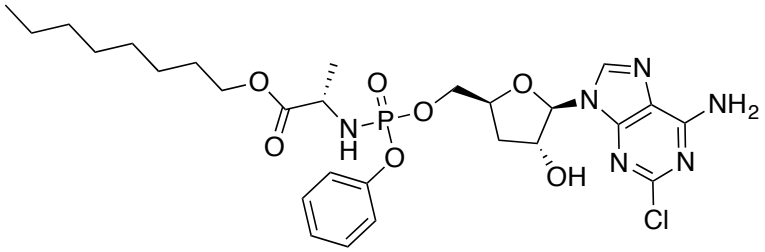
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.77 (d $^3J_{CP}$ = 4.4 Hz, C=O), 173.58 (d $^3J_{CP}$ = 5.3 Hz, C=O), 156.78, 156.66 (C6), 153.86, 153.82 (C2), 150.69 (d $^2J_{CP}$= 6.3 Hz, C-*ipso* Ph), 150.68 (d $^2J_{CP}$= 6.3 Hz, C-*ipso* Ph), 149.82, 149.77 (C4), 139.32, 139.28 (C8), 129.38, 129.35 (CH-Ar x 2), 124.76, 124.74 (CH-Ar), 120.07 (d $^3J_{CP}$ = 4.81 Hz, CH-Ar), 119.98 (d $^3J_{CP}$ = 4.66 Hz, CH-Ar), 118.08, 118.04 (C5), 92.06, 91.90 (C1'), 79.33, 79.26 (C4'), 75.35, 75.32 (C2'), 67.17 (d $^2J_{CP}$= 5.16 Hz, C5'), 66.79 (d $^2J_{CP}$= 5.06 Hz, C5'), 65.07 (O***C***$H_2(CH_2)_4CH_3$), 50.25, 50.11 (*C*HCH_3), 33.36, 33.15 (C3'), 31.18, 28.26, 25.22, 22.21 ($OCH_2($***C***$H_2)_4CH_3$), 19.33 (d $^3J_{CP}$ = 6.39 Hz, CH*C*H_3), 19.15 (d $^3J_{CP}$ = 7.11 Hz, CH*C*H_3),

13.06 ($O(CH_2)_5$***C***H_3).

MS (ES+) m/z: Found: 597.2 [M + H^+], 619.2 [M + Na^+] $C_{25}H_{34}ClN_6O_7P$ required: 596.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 264 nm, t_R 17.64 min

2-Chloro-3'deoxyadenosine-5'-O-[1-phenyl(octyl-L-alaninyl)] phosphate (3.15)



The title compound was prepared according to the general procedure A using 2-chloro-3'-deoxyadenosine **3.2** (339 mg, 1.18 mmol), N-methylimidazole (0.47 mL, 5.9 mmol) and octyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.18** (891 mg, 2.32 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 1:99 to 12:88 over 10 CV) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 3/97) afforded the title compound as a white solid (185 mg, 15 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.98, 3.76

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.25 (s, 0.5H, H-8), 8.24 (s, 0.5H, H-8), 7.31-7.36 (m, 2H, Ar), 7.17-7.24 (m, 3H, Ar), 5.97 (d, *J* = 1.35 0.5H, H1'), 5.96 (d, *J* = 1.25 0.5H, H1'), 4.69-4.72 (m, 2H, H2', H4'), 4.47-4.51 (m, 0.5H, H5'), 4.41-4.45 (m, 0.5H, H5'), 4.29-4.34 (m, 1H, H5'), 3.99-4.08 (m, 2H, OC***H***$_2(CH_2)_6CH_3$), 3.91-3.96 (m, 0.5H OC***H***CH_3), 3.86-3.91 (m, 0.5H OC***H***CH_3), 2.31-2.38 (m, 1H, H3'), 2.07-2.11 (m, 1H, H3'), 1.53-1.61 (m, 2H, OCH_2C***H***$_2(CH_2)_3CH_3$), 1.18-1.33 (m, 13H, CHC***H***$_3$, $OCH_2CH_2(C$***H***$_2)_5CH_3$), 0.88-0.90 (m, 3H, $OCH_2CH_2(CH_2)_5C$***H***$_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.75 (d $^3J_{CP}$ = 4.6 Hz, C=O), 173.57 (d $^3J_{CP}$ = 5.8 Hz, C=O), 156.81, 156.67 (C6), 153.88, 153.80 (C2), 150.69 (d $^2J_{CP}$= 6.0 Hz, C-*ipso* Ph),

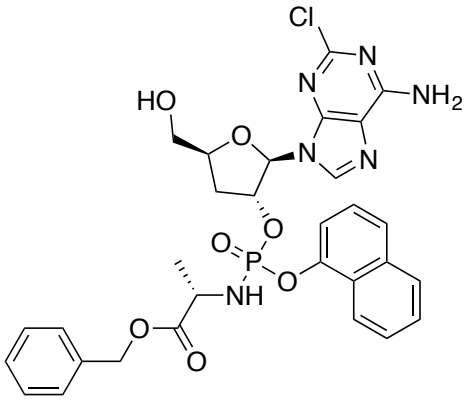
150.67 (d $^{2}J_{CP}$= 6.1 Hz, C-*ipso* Ph), 149.85, 149.80 (C4), 139.31, 139.27 (C8), 129.41, 129.38 (CH-Ar x 2), 124.77, 124.75 (CH-Ar), 119.80 (d $^{3}J_{CP}$ = 4.70 Hz, CH-Ar), 119.70 (d $^{3}J_{CP}$ = 4.76 Hz, CH-Ar), 118.07, 118.03 (C5), 92.10, 92.02 (C1'), 79.34, 79.28 (C4'), 75.36, 75.33 (C2'), 67.17 (d $^{2}J_{CP}$= 5.20 Hz, C5'), 66.79 (d $^{2}J_{CP}$= 5.10 Hz, C5'), 65.10 (O***C***$H_2(CH_2)_6CH_3$), 50.22, 50.13 (***C***HCH_3), 33.34, 33.22 (C3'), 31.62, 29.26, 26.21, 25.22, 22.43, 21.58 (OCH_2(***C***$H_2)_6CH_3$), 19.23 (d $^{3}J_{CP}$ = 6.41 Hz, CH***C***H_3), 19.15 (d $^{3}J_{CP}$ = 7.01 Hz, CH***C***H_3), 12.86 ($O(CH_2)_7$***C***H_3).

MS (ES+) m/z: Found: 726.1 [M + H^+], 748.1 [M + Na^+] $C_{27}H_{38}ClN_6O_7P$ required: 625.06 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 264 nm, t_R 20.32 min

2nd Strategy for the synthesis of 2-Chlorocordycepine ProTides

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate (3.16)



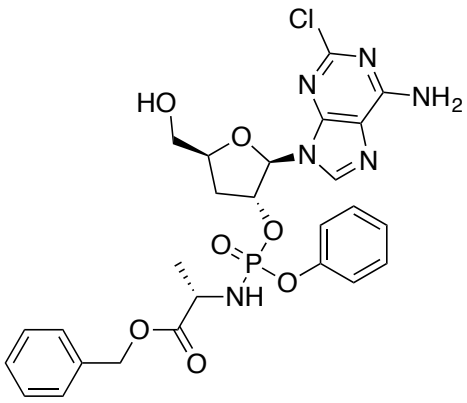
Compound was prepared according to the general procedure B using 2-chlorocordycepin **3.2** (200 mg 0.7 mmol), *tert*-butyl magnesium chloride 1M (0.7 ml, 0.74 mmol) and Phenyl(isopropoxy-L-alaninyl) phosphorochloridate **2.8** (565 mg 1.40 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 2:98 to 10:90 over 15 CV) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 4:96) afforded the title compound as a light brown precipitate (12 mg, 3 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.01, 2.41.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.12 (s, 0.5H, H-8), 8.10 (s, 0.5H, H-8), 7.00-7.78 (m, 12H, Ar), 5.86 (d *J* = 1.6, 0.5H, H-1'), 5.84 (d *J* = 1.5, 0.5H, H-1'), 4.58-4.60 (m, 2H, H-4'and H-2'), 4.41-4.46 (m, 1H, C*H*CH_3), 4.28-4.38 (m, 1H, H-5'), 4.16-4.23 (m, 1H, H-5'), 3.61-3.88 (m, 2H OC*H*$_2$Ph), 1.95-2.28 (m, 2H, H-3') 1.02-1.04 (m, 3H, CHC*H*$_3$).

MS (ES+) m/z: Found: 653.2 [M + H^+], $C_{30}H_{30}ClN_6O_7P$ required: 652.2 [M].

2-Chloro-3'deoxyadenosine 5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate (3.17)



Compound was prepared according to the general procedure B using 2-chlorocordycepin **3.2** (211 mg 0.74 mmol), *tert*-butyl magnesium chloride 1M (0.74 ml, 0.74 mmol) and Naphtyl(isopropoxy-L-alaninyl) phosphorochloridate **2.13** (495 mg 1.47 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 2:98 to 10:90 over 15 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 4:96) afforded the title compound as a light brown precipitate (12 mg, 3 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.01, 2.41.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.37 (s, 0.5H, H-8), 8.26 (s, 0.5H, H-8), 7.09-7.39 (m, 10H, Ar), 6.20 (d, *J* = 1.8, 0.5H, H-1'), 6.02 (d *J* = 3.1, 0.5H, H-1'), 5.39-5.44 (m, 1H, H-2'), 5.17 (dd, *J* = 6.55, 12.25, 1H OC*H*$_2$Ph), 4.49-5.08 (m, 1H OC*H*$_2$Ph), 4.40-4.45 (m, 1H, H-4'), 4.02-4.07 (m, 1H, C*H*CH_3), 3.86-3.94 (m, 1H, H-5'), 3.60-3.68 (m, 1H, H-5'), 2.42-2.55 (m, 1H, H-3'), 2.19-2.27 (m, 1H, H-3') 1.30-1.36 (m, 3H, CHC*H*$_3$).

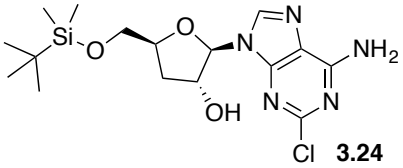
^{13}C NMR (125 MHz, CD_3OD): δ_C 174.78 (d $^3J_{CP}$ = 4.6 Hz, C=O), 174.54 (d $^3J_{CP}$ = 5.0 Hz, C=O), 158.15 (C6), 155.31, 155.25 (C2), 151.95 (d $^2J_{CP}$ = 10.0 Hz, C-*ipso* Ph), 151.89 (d $^2J_{CP}$= 10.0 Hz, C-*ipso* Ph), 151.27, 151.13 (C4), 141.51, 141.31 (C8), 137.32, 137.09 (C-*ipso* Bz), 130.79, 130.71 (CH-Ph x 2), 129.59, 129.50 (CH-Bz x 2), 129.37, 129.24 (CH-Bz x 2), 129.36, 129.28 (CH-Bz), 126.36, 126.16 (CH-Ph), 121.59 (d $^3J_{CP}$ = 4.5 Hz, CH-Bz), 121.23 (d $^3J_{CP}$ = 4.7 Hz, CH-Bz), 119.58, 119.54 (C5), 91.39, 31.19 (d $^3J_{CP}$ = 8.2 Hz, C1'), 91.91 (d $^3J_{CP}$ = 6.7 Hz, C1'), 82.89, 82.31 (C4'), 81.98 (d $^2J_{CP}$= 5.1 Hz, CH-2'), 81.16 (d $^2J_{CP}$= 5.4 Hz, CH-2'), 67.97, 67.94 (OCH_2Bz), 64.01, 63.46 (CH_2-5'), 51.89, 51.70 (*C*HCH_3), 33.77 (d $^3J_{CP}$ = 4.6 Hz, C3'), 33.59 (d $^3J_{CP}$ = 4.6 Hz, C3'), 20.26 (d $^3J_{CP}$ = 7.1 Hz, CH*C*H_3), 20.21 (d $^3J_{CP}$ = 7.5 Hz, CH*C*H_3).

MS (ES+) m/z: Found: 603.1 [M + H^+], 625.1 [M+Na^+] $C_{30}H_{30}ClN_6O_7P$ required: 602.1 [M].

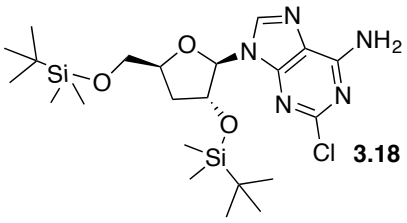
HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 265 nm, $\mathbf{t_R}$ 24.52 min.

3rd Strategy for the synthesis of 2-Chlorocordycepine ProTides

5'-*O*-(*tert*-butyldimethylsilyl)-2-chlorocordycepin (3.24) and 2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-2-chlorocordycepin (3.18)



3.24



3.18

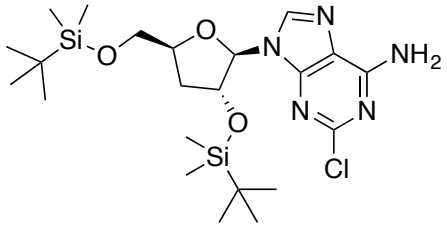
1.11 g (3.88 mmol) of 2-Chlorocordycepin **3.2** and 1.74 g (11.64 mmol) of TBDMSCl were dissolved in 10 ml of pyridine in anhydrous conditions under Ar atmosphere. The reaction was stirred for 18 h at RT. Solvent was evaporated under reduced pressure. The crude mixture was dissolved in 7 ml of chloroform and washed seven times with a saturated aqueous solution of NH_4Cl. The organic phase was dried on $NaSO_4$ and solvent was evaporated under reduced pressure. Product was purified by

flash chromatography using gradient of Hexane / AcOEt from 88:12 to 0:100 % in 10 CV to obtain 530 mg of compound **3.24** and 320 mg of compound **3.18** as a white powder.

Compound **3.24**

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.16 (s, 1H, H-8), 5.83 (d, *J* = 2.5, 1H, H-1'), 5.72 (br s, 2H, N***H***$_2$), 4.61-4.64 (m, 1H, H-2'), 4.50-4.54 (m, 1H, H-4'), 3.95 (dd, *J* = 2.8, 11.5 Hz, 1H, H-5'), 3.83 (d, J = 2.6, 1H, OH-2'), 3.66 (dd, *J* = 2.8, 11.4 Hz, 1H, H-5'), 2.25-2.30 (m, 1H, H-3'), 2.00-2.05 (m, 1H, H-3'), 0.80 (s, 9H, (C***H***$_3$)$_3$-C-SiO-(CH$_3$)$_2$), 0.01 (s, 3H, (CH$_3$)$_3$-C-SiO-(C***H***$_3$)$_2$), 0.00 (s, 6H, (CH$_3$)$_3$-C-SiO-(C***H***$_3$)$_2$).

2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-2-chlorocordycepin (3.18)



530 mg (1.32 mmol) of **3.24**, 398 mg (2.64 mmol) of TBDMSCl and 359 mg (5.28 mmol) of imidazole were dissolved in 2 ml of DMF under Argon atmosphere. The solution was stirring for 20 h. Then reaction was stopped by quenching 10 ml of saturated aqueous solution of NH_4Cl. Product was extracted with 20 x 5 mL of AcOEt. The organic phase was dried on $NaSO_4$ and solvent was evaporated under reduced pressure. Product was purified by flash chromatography using gradient of Hexane / AcOEt from 80:12 to 0:100 % in 10 CV to obtain 459 mg of compound **3.18** as a white powder.

343 mg (0.66 mmol) of 2-chlorocordycepin **3.2**, 328 (2.18 mmol) of TBDMSCl and 297 (4.36 mmol) of imidazole were dissolved in 1.5 ml of DMF under Argon atmosphere. The solution was stirring for 20 h. Then reaction was stopped by quenching 10 ml of saturated aqueous solution of NH_4Cl. Product was extracted with 20 x 5 mL of AcOEt. The organic phase was dried on $NaSO_4$ and solvent was evaporated under reduced

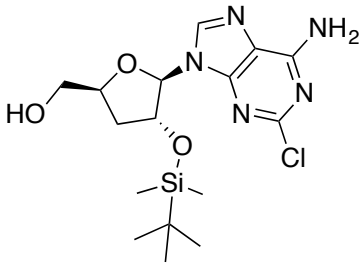
pressure. Product was purified by flash chromatography using gradient of Hexane / AcOEt from 80:12 to 0:100 % in 10 CV to obtain 453 mg of compound **3.18** as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.18 (s, 1H, H-8), 6.03 (br s, 2H, N***H***$_2$), 5.81 (d, *J* = 1, 1H, H-1'), 4.48-4.49 (m, 1H, H-2'), 4.22-4.46 (m, 1H, H-4'), 4.01 (dd, *J* = 2.5, 11.5 Hz, 1H, H-5'), 3.65 (dd, *J* = 2.5, 11.5 Hz, 1H, H-5'), 2.05-2.10 (m, 1H, H-3'), 1.68-1.72 (m, 1H, H-3'), 0.82 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 0.79 (s, 9H, (C***H***$_3$)$_3$-C-SiO(5')-(CH$_3$)$_2$), 0.06 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), 0.01 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), 0.00 (s, 6H, (CH$_3$)$_3$-C-SiO(5')-(C***H***$_3$)$_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 156.01 (C-6), 153.87 (C-2), 150.20 (C-4), 139.42 (CH-8), 118.95 (C-5), 92.16 (CH-1'), 81.59 (CH-4'), 77.45 (CH-2'), 63.54 (CH$_2$-5'), 33.40 (CH$_2$-3'), 26.03 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 25.74 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(5')-(CH$_3$)$_2$), 18.57 (C, (CH$_3$)$_3$-***C***-SiO(3')-(CH$_3$)$_2$), 17.99 (C, (CH$_3$)$_3$-***C***-SiO(5')-(CH$_3$)$_2$), -4.70, -5.09, -5.31, -5.43 (CH$_3$, (CH$_3$)$_3$-C-SiO(2', 5')-(***C***H$_3$)$_2$).

MS (ES+) m/z: Found: 514.3 [M + H$^+$] $C_{22}H_{40}ClN_5O_3Si_2$ required: 513.3 [M].

2'-*O*-(*tert*-butyldimethylsilyl)-2-chlorocordycepin (3.19)



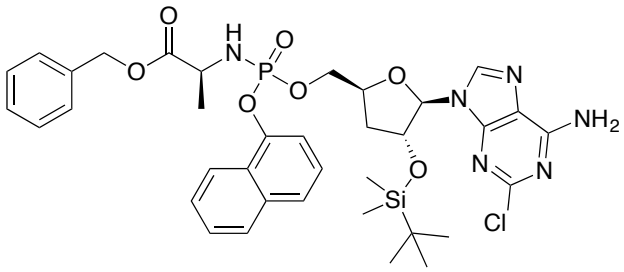
970 mg (1.89) mmol of compound **3.18** was dissolved in 12 ml of THF at 0 ˚C. Then 6 ml of a solution H_2O/TFA 1:1 were added. The reaction was left stirring at 0 ˚C monitoring the progress by TLC. After 4 h the solution was carefully neutralised with a saturated aqueous solution of $NaCO_3$. The mixture was extracted three times with 15 ml of AcOEt. Then the organic phase was washed with brine. The organic phase was dried on $NaSO_4$ and solvent was evaporated under reduced pressure. Product

was purified by flash chromatography using gradient of CH2Cl2 / MeOH from 100:0 to 88:12 % in 11 CV to obtain 544 mg of the desired compound as a white precipitate.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.91 (s, 1H, H-8), 6.21 (br s, 2H, N***H***$_2$), 5.68 (d, *J* = 1, 1H, H-1'), 4.65-5.01 (m, 2H, H-2', OH-5'), 4.57-4.59 (m, 1H, H-4'), 4.07-4.11 (m, 1H, H-5'), 3.63-3.68m (m, 1H, H-5'), 2.56-2.60 (m, 1H, H-3'), 2.18-2.24 (m, 1H, H-3'), 0.87 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 0.00 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), -0.11 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 156.15 (C-6), 154.06 (C-2), 150.28 (C-4), 140.77 (CH-8), 119.87 (C-5), 93.59 (CH-1'), 80.55 (CH-4'), 77.33 (CH-2'), 64.78 (CH$_2$-5'), 34.63 (CH$_2$-3'), 25.57 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO-(CH$_3$)$_2$), 19.88 (C, (CH$_3$)$_3$-***C***-SiO-(CH$_3$)$_2$), -5.03 (CH$_3$, (CH$_3$)$_3$-C-SiO-(***C***H$_3$)$_2$).

2'-*O*-(*tert*-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate (3.20)



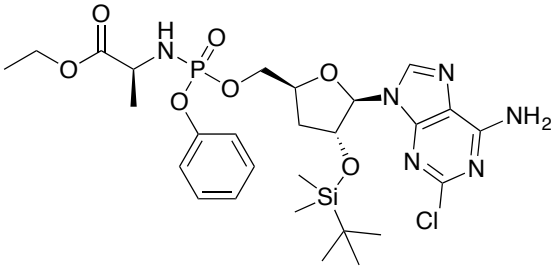
Compound was prepared according to the general procedure B using 2'-*O*-(*tert*-butyldimethylsilyl)-2-chloro-3'-deoxyadenosine **3.19** (91 mg 0.23 mmol), *tert*-butyl magnesium chloride 1M (0.23 ml, 0.23 mmol) and Naphtyl(benzyloxy-L-alaninyl) phosphorochloridate **2.8** (183 mg 0.45 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 2:98 to 8:92 over 15 CV) afforded the title compound as a white precipitate (12 mg, 3 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.44, 4.11.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.14 (s, 0.5H, H-8), 8.11 (s, 0.5H, H-8), 8.02-8.07 (m, 1H, Ar), 7.75-7.79 (m, 1H, Ar), 7.58-7.61 (m, 1H, Ar), 7.34-7.44 (m, 3H, Ar), 7.26-7.30 (m, 1H, Ar), 7.15-7.20 (m, 5H, Ar), 5.77 (d, *J* = 2.6, 0.5H, H1'), 5.76 (d, *J* = 2.1, 0.5H, H1'), 4.93-5.00 (m, 2H, OCH_2Ph) 4.67-4.70 (m, 1H, H2'), 4.49-4.54 (m, 1H, H4'), 4.34-4.39 (m, 1H, H5'), 4.18-4.24 (m, 1H, H5'), 3.94-4.01 (m, 1H, CHCH$_3$), 2.02-2.12 (m, 1H, H3'), 1.80-1.86 (m, 1H, H3'), 1.20-1.22 (m, 3H, CHCH_3), 0.81 (s, 9H, (C$\boldsymbol{H}_3$)$_3$-C-SiO-(CH$_3$)$_2$), 0.00 (m, 6H, (CH$_3$)$_3$-C-SiO(2')-(C$\boldsymbol{H}_3$)$_2$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 174.86 (d $^3J_{CP}$ = 4.2 Hz, C=O), 174.55 (d $^3J_{CP}$ = 4.8 Hz, C=O), 158.04, 158.02 (C6), 155.41, 155.40 (C2), 151.39 (C4), 149.35 (C-Ar), 147.94 (d $^2J_{CP}$ = 6.67 Hz, C-*ipso* Naph), 147.91 (d $^2J_{CP}$= 6.6 Hz, C-*ipso* Naph), 140.70 (C8), 137.19, 137.14 (C-*ipso* OCH$_2$Ph), 136.28, 136.26 (C-Ar), 129.54, 129.52 (CH-Ar x 2), 129.28, 129.24 (CH-Ar), 129.22, 129.18 (CH-Ar x 2), 128.90, 128.82 (CH-Ar), 127.93, 127.88 (C-Ar), 127.76 (d $^3J_{CP}$ = 5.8 Hz, CH-Ar), 127.48 (d $^3J_{CP}$ = 8 Hz, CH-Ar), 126.50 (CH-Ar), 126.00 (CH-Ar), 122.78, 122.63 (CH-Ar), 119.46, 119.44 (C5), 116.36 (d, $^4J_{CP}$ = 2.9, CH-Ar), 116.19 (d, $^4J_{CP}$ = 3.0, CH-Ar), 93.32 (CH-1'), 80.32, 80.26 (CH-4'), 77.82, 77.76 (CH-2'), 68.79 (d $^2J_{CP}$= 5.3 Hz, C5'), 68.65 (d $^2J_{CP}$= 5.2 Hz, C5'), 67.95, 67.91 (*C*H$_2$Ph), 51.87, 51.74 (*C*HCH$_3$), 35.56, 35.29 (CH$_2$-3'), 26.23 (CH$_3$ x 3 (C$\boldsymbol{H}_3$)$_3$-C-SiO-(CH$_3$)$_2$), 20.43 (d $^3J_{CP}$ = 6.4 Hz, CH*C*H$_3$), 20.25 (d $^3J_{CP}$ = 7.5 Hz, CH*C*H$_3$), 18.82 (C, (CH$_3$)$_3$-***C***-SiO(3')-(CH$_3$)$_2$), -4.66, -4.78 (CH$_3$, (CH$_3$)$_3$-C-SiO-(C$\boldsymbol{H}_3$)$_2$).

2'-*O*-(*tert*-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-phenyl(ethyl-L-alaninyl)] phosphate (3.21)



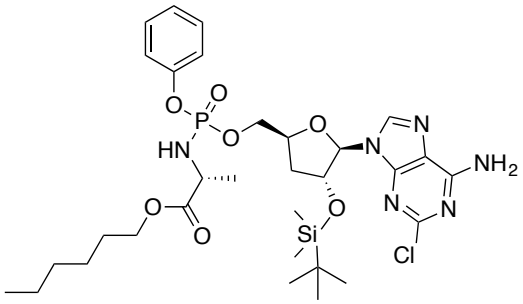
Compound was prepared according to the general procedure B using 2'-*O*-(*tert*-butyldimethylsilyl)-2-chloro-3'-deoxyadenosine **3.19** (204 mg 0.51 mmol), *tert*-butyl

magnesium chloride 1M (0.51 ml, 0.51 mmol) and Phenyl(benzyloxy-L-alaninyl) phosphorochloridate **2.14** (348 mg 1.02 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 0:100 to 8:92 over 15 CV) afforded the title compound as a white precipitate (93 mg, 28 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.09, 3.85.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.16 (s, 0.5H, H-8), 8.13 (s, 0.5H, H-8), 7.03-7.24 (m, 5H, Ar), 5.79 (m, 1H, H1'), 4.71-4.75 (m, 1H, H2'), 4.52-4.59 (m, 1H, H4'), 4.36-4.40 (m, 0.5H, H5'), 4.30-4.34 (m, 0.5H, H5'), 4.17-4.23 (m, 1H, H5'), 3.93-4.02 (m, 2H, OCH_2CH_3), 3.76-3.84 (m, 1H, $CHCH_3$), 2.10-2.19 (m, 1H, H3'), 1.88-1.94 (m, 1H, H3'), 1.20 (d, J = 7.1, 1.5H, $CHCH_3$), 1.17 (d, J = 7.1, 1.5H, $CHCH_3$), 1.06-1.11 (m, 3H, OCH_2CH_3) 0.79 (s, 9H, $(CH_3)_3$-C-SiO-$(CH_3)_2$), 0.00 (m, 6H, $(CH_3)_3$-C-SiO-$(CH_3)_2$).

2'-*O*-(*tert*-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-phenyl(hexyl-L-alaninyl)] phosphate (3.22)



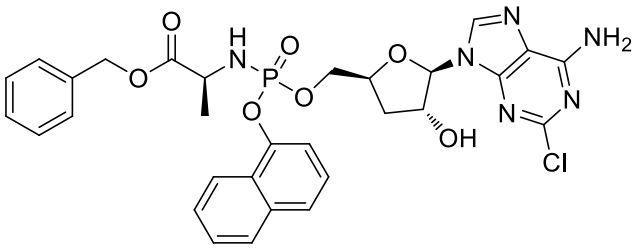
Compound was prepared according to the general procedure B using compound **3.19** (204 mg 0.52 mmol), *tert*-butyl magnesium chloride 1M (0.52 ml, 0.52 mmol) and phosphorochloridate **2.15** (348 mg 1.02 mmol). Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 1:99 to 8:92 over 14 CV) afforded the title compound as a white precipitate (180 mg, 48 %). Further purification by preparative TLC (2000 μm, eluent system CH_2Cl_2/MeOH 93:7), or preparative HPLC (reverse phase eluent system H_2O/ACN 0-100 in 30 min) did not gave any separation of the isomers.

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.07, 3.79

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.16 (s, 0.5H, H-8), 8.14 (s, 0.5H, H-8), 7.04-7.25 (m, 5H, Ar), 5.77-5.81 (m, 1H, H1'), 4.69-4.75 (m, 1H, H2'), 4.53-4.60 (m, 1H, H4'), 4.31-4.41 (m, 1H, H5'), 4.12-4.26 (m, 1H, H5'), 3.77-3.98 (m, 3H, OC***H***CH_3, OC***H***$_2(CH_2)_4CH_3$), 2.04-2.22 (m, 1H, H3'), 1.49-1.96 (m, 1H, H3'), 1.36-1.49 (m, 2H, OCH_2C***H***$_2(CH_2)_3CH_3$), 1.15-1.21 (m, 9H, $CHCH_3$, $OCH_2CH_2(C$***H***$_2)_3CH_3$), 0.79-0.80 (m, 9H, (C***H***$_3)_3$-C-SiO-$(CH_3)_2$), 0.74-0.78 (m, 3H, $OCH_2CH_2(CH_2)_3C$***H***$_3$), 0.00-0.15 (m, 6H, $(CH_3)_3$-C-SiO-(C***H***$_3)_2$).

MS (ES+) m/z: Found: 711.3 [M + H^+], 733.3 [M + Na^+] $C_{31}H_{48}ClN_6O_7PSi$ required: 710.3 [M].

2-Chloro-3'deoxyadenosine-5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate (3.10)



The title compound was obtained by dissolving 2'-O-(tert-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-naphtyl (benzyl-L-alaninyl)] phosphate **3.20** (90 mg, 0.12 mmol) in 2 ml of an aqueous solution of THF/H_2O/TFA 1/1/1 at 0 ˚C. The mixture was stirred at RT for 25 h. Solvent was evaporated under reduced pressure. Purification by preparative TLC (eluent system CH_2Cl_2/MeOH 92:8) afforded the 41 mg (18 %) of the desired compound a white solid.

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.39, 4.12.

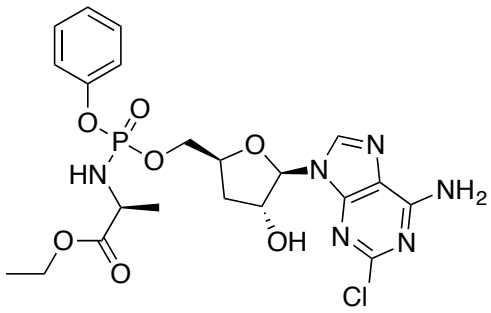
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.10 (s, 0.5H, H8), 8.07 (s, 0.5H, H8), 7.97-8.02 (m, 3H, Ar), 7.14-7.43 (m, 9H, Ar), 5.81-5.80 (m, 1H, H1'), 4.97-4.89 (m, 2H, OCH_2Ph) 4.53-4.49 (m, 2H, H4', H2'), 4.30-4.35 (m, 1H, H5'), 4.15-4.21 (m, 1H, H5'), 3.87-3.95 (m, 1H, $CHCH_3$), 2.12-2.23 (m, 1H, H3'), 1.86-1.93 (m, 1H, H3'), 1.14-1.17 (m, 3H, $CHCH_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 174.85 (d $^3J_{CP}$ = 4.0 Hz, C=O), 174.55 (d $^3J_{CP}$ = 4.3 Hz, C=O), 158.07, 158.04 (C6), 155.31, 155.28 (C2), 151.34, 151.31 (C4), 149.69 (C-Ar), 147.96 (d $^2J_{CP}$ = 7.25 Hz, C-*ipso* Naph), 147.90 (d $^2J_{CP}$= 7.0 Hz, C-*ipso* Naph), 140.70 (C8), 137.21, 137.16 (C-*ipso* OCH_2Ph), 136.26 (C-Ar), 130.92, 130.80 (CH-Ar), 129.56, 129.53 (CH-Ar), 129.31 (CH-Ar), 129.27, 129.25 (CH-Ar), 128.88, 128.81 (CH-Ar), 127.78 (d $^3J_{CP}$ = 4.7 Hz, CH-Ar), 127.50 (d $^3J_{CP}$ = 6.2 Hz, CH-Ar), 126.48 (CH-Ar), 126.02, 125.97 (CH-Ar), 119.46, 119.42 (C5), 116.33 (d, $^4J_{CP}$ = 3.0, CH-Ar), 116.16 (d, $^4J_{CP}$ = 3.4, CH-Ar), 93.30, 93.27 (C1'), 80.56 (d ^3J = 8.3 Hz, C4'), 80.51 (d ^3J = 8.4 Hz, C4'), 76.61, 76.54 (C2'), 68.74 (d $^2J_{CP}$= 5.3 Hz, C5'), 68.54 (d $^2J_{CP}$= 5.1 Hz, C5'), 67.93, 67.90 (*C*H_2Ph), 51.81, 51.70 (*C*HCH$_3$), 34.79, 34.53 (C3'), 20.42 (d $^3J_{CP}$ = 6.5 Hz, CH*C*H$_3$), 20.23 (d $^3J_{CP}$ = 7.7 Hz, CH*C*H$_3$).

MS (ES+) m/z: Found: 653 [M + H^+], 675 [M + Na^+] $C_{30}H_{30}ClN_6O_7P$ required: 652.16 [M];

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1ml/min, λ = 254 nm, t_R 18.03 min

2-Chloro-3'deoxyadenosine-5'-O-[1-Phenyl(ethyl-L-alaninyl)] phosphate (3.23)



The title compound was obtained by dissolving 2'-O-(tert-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-phenyl(ethyl-L-alaninyl)]phosphate **3.21** (93 mg, 0.14 mmol) in 2 ml of an aqueous solution of THF/H_2O/TFA 1/1/1 at 0 ˚C. The mixture was stirred at RT for 25 h. Solvent was evaporated under reduced pressure. Purification by preparative TLC (eluent system CH_2Cl_2/MeOH 98:2) afforded the 50 mg (66 %) of the desired compound as a white solid.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.93, 3.72.

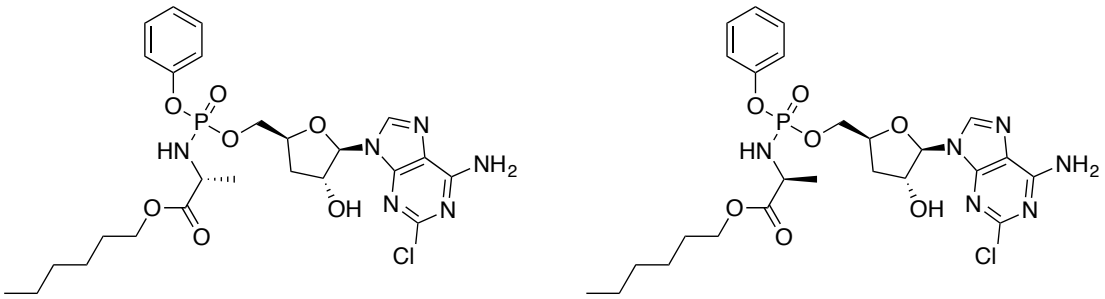
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.12 (s, 0.5H, H8), 8.11 (s, 0.5H, H8), 7.18-7.23 (m, 2H, Ph), 7.03-7.12 (m, 3H, Ph), 5.85 (d *J* =1.5, 0.5H, H1'), 5.84 (d *J* =2, 0.5H, H1'), 4.55-4.62 (m, 2H, H4'and H2'), 4.34-4.38 (m, 0.5H, H5'), 4.28-4.32 (m, 0.5H, H5'), 4.16-4.22 (m, 1H, H5'), 3.93-4.03 (m, 2H, OCH_2CH_3), 3.70-3.84 (m, 1H, $CHCH_3$), 2.20-2.28 (m, 1H, H3'), 1.95-1.99 (m, 1H, H3'), 1.15-1.21 (m, 3H, $CHCH_3$), 1.06-1.11 (m, 3H, OCH_2CH_3).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.66 (d $^{3}J_{CP}$ = 4.5 Hz, C=O), 173.65 (d $^{3}J_{CP}$ = 5.3 Hz, C=O), 156.68, 156.70 (C6), 153.93, 153.88 (C2), 150.72 (d $^{2}J_{CP}$= 6.7 Hz, C-*ipso* Ph), 150.71 (d $^{2}J_{CP}$= 6.5 Hz, C-*ipso* Ph), 149.89, 149.94 (C4), 139.41, 139.35 (C8), 129.33 (CH-Ar x 2), 124.74, 124.73 (CH-Ar), 120.03 (d $^{3}J_{CP}$ = 4.75 Hz, CH-Ar), 119.97 (d $^{3}J_{CP}$ = 4.87 Hz, CH-Ar), 118.07, 118.03 (C5), 92.02, 91.88 (C1'), 79.26, 79.19 (C4'), 75.26, 75.24 (C2'), 67.18 (d $^{2}J_{CP}$= 5.25 Hz, C5'), 66.81 (d $^{2}J_{CP}$= 5.12 Hz, C5'), 60.96 (OCH_2CH_3), 50.23, 50.12 ($CHCH_3$), 33.46, 33.21 (C3'), 19.16 (d $^{3}J_{CP}$ = 6.3 Hz, $CHCH_3$), 18.97 (d $^{3}J_{CP}$ = 7.2 Hz, $CHCH_3$), 13.10, 13.07 (OCH_2CH_3).

MS (ES+) m/z: Found: 541 [M + H^+], 563 [M + Na^+] $C_{21}H_{26}ClN_6O_7P$ required: 540 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 254 nm, t_R 12.41, 12.83 min

2-Chloro-3'deoxyadenosine-5'-O-[1-phenyl(ethyl-L-alaninyl)] phosphate and 2-Chloro-3'deoxyadenosine-5'-O-[1-phenyl(ethyl-D-alaninyl)] phosphate (3.14)



The title compound was obtained by dissolving 2'-O-(tert-butyldimethylsilyl)-2-chloro-3'deoxyadenosine-5'-O-[1-phenyl(hexyl-L,D-alaninyl)]phosphate **3.22** (180 mg, 0.14 mmol) in 4 ml of an aqueous solution of THF/H_2O/TFA 1/1/1 at 0 ˚C. The mixture was stirred at RT for 25 h. Solvent was evaporated under reduced pressure. Purification by flash chromatography (eluent system from CH_2Cl_2/MeOH 100:0 to 92:8 over 15 CV) afforded the 94 mg (62 %) of the desired compound as a white solid.

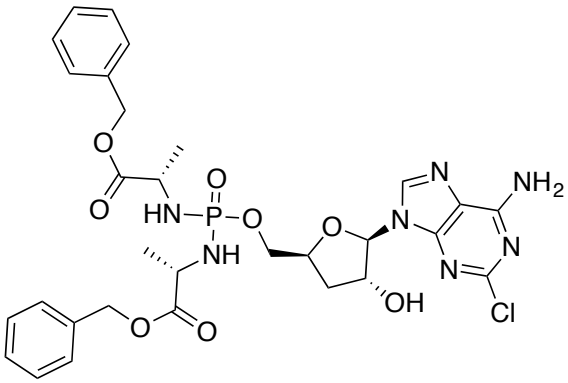
^{31}P NMR (202 MHz, CD_3OD): δ_P 3.93, 3.73

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.17 (s, 0.5H, H-8), 8.16 (s, 0.5H, H-8), 7.08-7.28 (m, 5H, Ar), 5.88-5.91 (m, 1H, H1'), 4.59-4.66 (m, 2H, H2', 4'), 4.33-4.43 (m, 1H, H5'), 4.17-4.30 (m, 1H, H5'), 3.78-4.03 (m, 3H, OC***H***CH_3, OC***H***$_2(CH_2)_4CH_3$), 2.18-2.36 (m, 1H, H3'), 1.93-2.07 (m, 1H, H3'), 1.42-1.53 (m, 2H, OCH_2C***H***$_2(CH_2)_3CH_3$), 1.16-1.26 (m, 9H, $CHCH_3$, $OCH_2CH_2(C$***H***$_2)_3CH_3$), 0.79-0.82 (m, 3H, $OCH_2CH_2(CH_2)_3C$***H***$_3$).

Experimental details Chapter 4

Synthesis of diamidates

2-Chloro-3'-deoxyadenosine-5'-*O*-bis (benzyloxy-L-alaninyl) phosphate (4.1)



To a stirring suspension of compound **3.2** (102 mg, 0.36 mmol) and triethyl amine (0.06 ml, 0.43 mmol) in anhydrous THF (5 ml), $POCl_3$ (0.04 ml 0.43 mmol) was added dropwise at -78 ˚C. The mixture was allowed to slowly reach RT and it was stirred for one hour. The formation of the intermediate was monitored by ^{31}P NMR. A suspension of *L*-Alanine benzyl ester *p*-toluenesulphonate salt (632 mg, 1.8 mmol) in DCM (5 ml) was added to the stirring mixture. Temperature of the reaction was lowered at -78 ˚C and TEA (0.50 ml, 3.6 mmol) was added dropwise to the mixture. After stirring for 20 h at RT H_2O (3ml) was added to the mixture and the desired compound was extracted with DCM. The organic was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography (eluent system from CH_2Cl_2/MeOH 100:0 to 88:12 over 12 CV) and afforded the 20 mg (8 %) of the desired compound as a white solid.

^{31}P NMR (202 MHz, CD_3OD): δ_P 13.69.

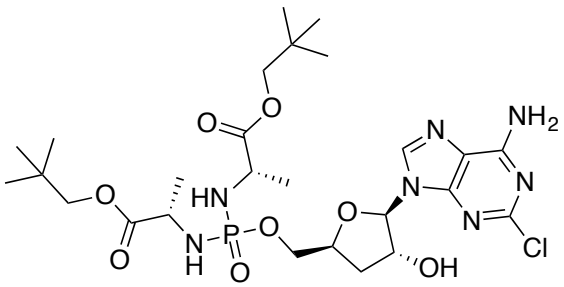
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.12 (s, 1H, *H*-8), 7.26 –7.15 (m, 10H, *H*-Ph), 5.81 (d, *J* = 1.0 Hz, 1H, *H*-1'), 5.00 – 4.95 (m, 4H, C*H*$_2$Ph), 4.53 – 4.52 (m, 1H, *H*-2'), 4.49 – 4.45 (m, 1H, *H*-4'), 4.12 – 4.08 (m, 1H, *H*-5'), 3.99– 3.94 (m, 1H, H5'), 3.85 – 3.77 (m, 2H, 2 x NHC*H*CH_3), 2.16 – 2.11 (m, 1H, *H*-3'), 1.91 – 1.87 (m, 1H, *H*-3'), 1.22 – 1.18 (m, 6H, 2 x NHCHC*H*$_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 174.00 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, C=O), 173.94 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C=O), 156.67 (C6), 153.90 (C2), 150.21 (C4), 139.33 (C8), 135.89 (C-*ipso* OCH_2Ph), 135.85 (C-*ipso* OCH_2Ph), 128.22 (CH-Ar), 128.19 (CH-Ar), 128.15 (CH-Ar), 127.98 (CH-Ar), 127.94 (CH-Ar), 127.89 (CH-Ar), 127.87 (CH-Ar), 127.81 (CH-Ar), 119.73 (C5), 91.81 (C1'), 79.50 (d, $^{3}J_{C\text{-}P}$ = 8.2 Hz, C4'), 75.24 (C2'), 66.70 (d, $^{2}J_{C\text{-}P}$ = 5.3 Hz, C5'), 67.46 (*C*H_2Ph), 49.68 (d, $^{2}J_{C\text{-}P}$ = 2.0 Hz, ***C***HCH_3), 49.50 (d, $^{2}J_{C\text{-}P}$ = 1.8 Hz, ***C***HCH_3), 33.42 (C3'), 19.33 (d, $^{3}J_{C\text{-}P}$ = 5.5 Hz, CH***C***H_3), 19.15 (d, $^{3}J_{C\text{-}P}$ = 6.4 Hz, CH***C***H_3)

MS (ES+) m/z: Found 688.2 [M + H^+], 710.2 [M + Na^+], $C_{30}H_{35}ClN_7O_8P$ required 687.20 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 260 nm, t_R 16.81 min.

2-Chloro-3'-deoxyadenosine-5'-O-bis (neopentyloxy-L-alaninyl] phosphate (4.2)



To a stirring suspension of compound **3.2** (121 mg, 0.42 mmol) in trimethylphosphate (1 ml), $POCl_3$ (0.04 ml 0.43 mmol) was added dropwise at -5 ˚C. The mixture was allowed to slowly reach RT and it was stirred for four hours. The formation of the intermediate was monitored by ^{31}P NMR. A suspension of *L*-Alanine neopentyl ester *p*-toluenesulphonate salt (695 mg, 2.1 mmol) in DCM (5 ml) was added to the stirring mixture. Temperature of the reaction was lowered at -78 ˚C and DIPEA (0.73 ml, 4.2 mmol) was added dropwise to the mixture. After stirring for 48 h at RT solvent was removed under reduced pressure and the mixture was redisolved in H_2O (3ml) was added to the mixture and the desired compound was extracted with DCM. The organic was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography (eluent system from

CH_2Cl_2/MeOH 99:1 to 90:10 over 10 CV) and afforded the 36 mg (13 %) of the desired compound as a white solid.

^{31}P NMR (202 MHz, CD_3OD): δ_P 12.47.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.15 (s, 1H, *H*-8), 5.87 (d, *J* = 1.0 Hz, 1H, *H*-1'), 4.67-4.70 (m, 1H, *H*-2'), 4.59-4.64 (m, 1H, *H*-4'), 4.18-4.23 (m, 1H, *H*-5'), 4.08-4.13 (m, 1H, H5'), 3.88-3.94 (m, 2H, 2 x NHC*H*CH_3), 3.76-3.81 (m, 4H, 2x CH_2C$(CH_3)_3$) 2.22 – 2.11 (m, 2H, *H*-3'), 1.28-1.32 (m, 6H, 2 x NHCHCH_3), 0.84 (s, 9H, CH_2C(C$H_3)_3$), 0.83 (s, 9H, CH_2C(C$H_3)_3$).

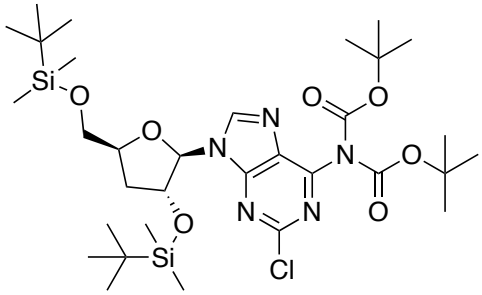
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.98 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, C=O), 173.93 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, C=O), 156.67 (C6), 153.89 (C2), 150.32 (C4), 139.33 (C8), 119.73 (C5), 91.81 (C1'), 79.50 (d, $^3J_{C\text{-}P}$ = 8.2 Hz, C4'), 75.23 (C2'), 66.74 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, C5'), 65.42, (O*C*H_2C$(CH_3)_3$), 65.40, (O*C*H_2C$(CH_3)_3$), 49.62 (d, $^2J_{C\text{-}P}$ = 2.2 Hz, ***C***HCH_3), 49.41 (d, $^2J_{C\text{-}P}$ = 2.0 Hz, ***C***HCH_3), 33.42 (C3'), 31.36, 31.32 (OCH_2***C***$(CH_3)_3$), 25.78, 25.76 (OCH_2C(***C***$H_3)_3$), 19.33 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, CH***C***H_3), 19.15 (d, $^3J_{C\text{-}P}$ = 7.4 Hz, CH***C***H_3)

MS (ES+) m/z: Found 648.3 [M + H^+], 670.3 [M + Na^+], $C_{26}H_{43}ClN_7O_8P$ required 647.3 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 260 nm, t_R 16.81 min.

Synthesis of phosphonates

2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-bis-N^6,N^6-tert-butylcarbonyl-2-chlorocordycepin (4.3)



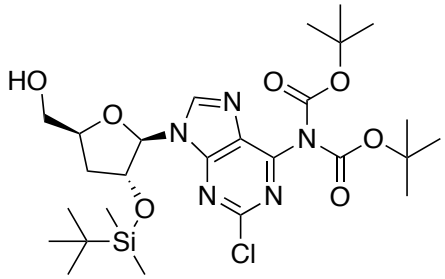
To an anhydrous solution of **3.18** (1.79 g, 3.48 mmol) and DMAP (1.27 g, 10.44 mmol) in THF (20 ml) under argon atmosphere, a solution of BOC_2O (4.55 g, 20.88 mmol) in THF (10 ml) was added dropwise. The mixture was stirring for 20 h at RT. The mixture was then diluted with hexane (30 ml), washed twice with brine (2 x 10 ml) and dried over $MgSO_4$, filtered and solvent was evaporated under reduced pressure and purification by flash chromatography (eluent system from Hexane/AcOEt 95:5 to 40:60 over 12 CV) and afforded 2.49 g t100 %) of the title compound an oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.71 (s, 1H, H-8), 5.90 (d, *J* = 1.6, 1H, H-1'), 4.62-4.63 (m, 1H, H-2'), 4.43-4.46 (m, 1H, H-4'), 4.02 (dd, *J* = 2.4, 11.7 Hz, 1H, H-5'), 3.71 (dd, *J* = 2.5, 11.7 Hz, 1H, H-5'), 2.15-2.20 (m, 1H, H-3'), 1.81-1.86 (m, 1H, H-3'), 1.29 (s, 18H, N(COOC(C***H***$_3$)$_3$)$_2$) 0.81 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 0.79 (s, 9H, (C***H***$_3$)$_3$-C-SiO(5')-(CH$_3$)$_2$), 0.01 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), 0.00 (s, 6H, (CH$_3$)$_3$-C-SiO(5')-(C***H***$_3$)$_2$), -0.01 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 153.83 (C-6), 152.32 (N(***C***OOC(CH$_3$)$_3$), 150.68 (N(***C***OOC(CH$_3$)$_3$), 149.83 (C-2), 149.82 (C-4), 144.83 (CH-8), 128.01 (C-5), 92.21 (CH-1'), 84.24 (N(COO***C***(CH$_3$)$_3$)$_2$), 81.76 (CH-4'), 77.35 (CH-2'), 63.48 (CH$_2$-5'), 33.19 (CH$_2$-3'), 26.67 (N(COOC(***C***H$_3$)$_3$)$_2$) 25.28 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(5')-(CH$_3$)$_2$), 24.93 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 18.09 (C, (CH$_3$)$_3$-***C***-SiO(5')-(CH$_3$)$_2$), 17.51 (C, (CH$_3$)$_3$-***C***-SiO(2')-(CH$_3$)$_2$), -5.86, -6.02, -6.44, -6.48 (CH$_3$, (CH$_3$)$_3$-C-SiO(2', 5')-(***C***H$_3$)$_2$).

MS (ES+) m/z: Found: 714.3 [M + H^+], 736.3 [M + Na^+] $C_{32}H_{56}ClN_5O_7Si_2$ required: 713.3 [M].

2'-*O*-(*tert*-butyldimethylsilyl)-bis-N^6,N^6-tert-butylcarbonyl-2-chlorocordycepin (4.4)



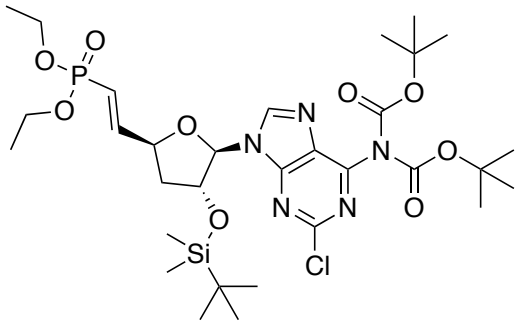
To a solution of **4.3** (672 mg, 0.94 mmol) in 30 ml of MeOH at 0 ˚C, CSA (1.1 mg, 0.56 mmol) were added. The formation of the product was monitored by TLC. After one hour stirring at 0 ˚C, $NaHCO_3$ was added dropwise until reachin pH 7 in the mixture. Solvent was evaporated and the residue was dissolved in DCM (30 ml) and washed with water (2 x 10 ml). The organic layer was dried on $MgSO_4$, filtered and solvent was evaporated under reduced pressure and purification by flash chromatography (eluent system from Hexane/AcOEt 95:5 to 50:50 over 10 CV) and afforded 2.49 g (100 %) of the title compound white solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.28 (s, 1H, H-8), 5.79 (d, *J* = 4.4, 1H, H-1'), 4.87-4.90 (m, 1H, H-2'), 4.59-4.62 (m, 1H, H-4'), 4.09-4.12 (m, 1H, H-5'), 3.88-3.90 (m, 1H, OH-5'), 3.66-3.71 (m, 1H, H-5'), 2.47-2.52 (m, 1H, H-3'), 2.11-2.17 (m, 1H, H-3'), 1.48 (s, 18H, N(COOC(C***H***$_3$)$_3$)$_2$) 0.86 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 0.00 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), -0.09 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 153.92 (C-6), 152.37 (N(***C***OOC(CH$_3$)$_3$), 150.37 (N(***C***OOC(CH$_3$)$_3$), 149.83 (C-2), 149.83 (C-4), 145.30 (CH-8), 128.02 (C-5), 92.31 (CH-1'), 84.34 (N(COO***C***(CH$_3$)$_3$)$_2$), 81.34 (CH-4'), 76.97 (CH-2'), 62.06 (CH$_2$-5'), 33.51 (CH$_2$-3'), 26.57 (N(COOC(***C***H$_3$)$_3$)$_2$) 24.82 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 17.45 (C, (CH$_3$)$_3$-***C***-SiO(2')-(CH$_3$)$_2$), -6.04, -6.17, (CH$_3$, (CH$_3$)$_3$-C-SiO(2')-(***C***H$_3$)$_2$).

MS (ES+) m/z: Found: 600.3 [M + H^+], 622.3 [M + Na^+] $C_{26}H_{42}ClN_5O_7Si_1$ required: 599.3 [M].

9-[5',6'-Vinyl-6'-(bis-ethylphosphinyl)-2'-*O*-(*tert*-butyldimethylsilyl)-D-ribohexofuranosyl] bis-N^6,N^6-tert-butylcarbonyl-2-chlorocordycepin (4.6)



To a solution of **4.4** (245 mg, 0.40 mmol) in 10 ml of ACN and argon atmosphere, 2-iodoxtbenzoic acid (246 mg, 0.88 mmol) was added. The mixture was stirring for 90 minutes at 80 ˚C. Then the temperature of the reaction was lowered at 0˚C, was stirred for 30 minutes, and solvent evaporated. The residue was dissolved in THF and filtrated. The precipitate was dried under high vacuum for an hour generating the aldehyde **4.5** which formation was monitored by ^{31}P NMR. The aldehyde was suspended without further purification in 10 ml of THF at 0 ˚C under argon atmosphere. To this, a solution of NaH (24.96 mg, 1.04 mmol) and tetraethyl methylene diphosphonate (0.26 ml, 1.04 mmol) in THF (6 ml) was added dropwise. The solution was allowed to slowly reach RT and was stirring for 24 h. Then 10 mL of AcOEt were added and the mixture was washed with H_2O (3 ml) NH_4Cl (3 ml and H_2O (3 ml). The organic layer was dried over Na_2SO_4, filtered, solvent was evaporated and the residue was purified by flash chromatography (eluent system from Hexane/AcOEt 50:50 to 0:100 over 8 CV) and afforded 153 mg (52 %) of the title compound white solid.

^{31}P NMR (202 MHz, $CDCl_3$): δ_P 16.79

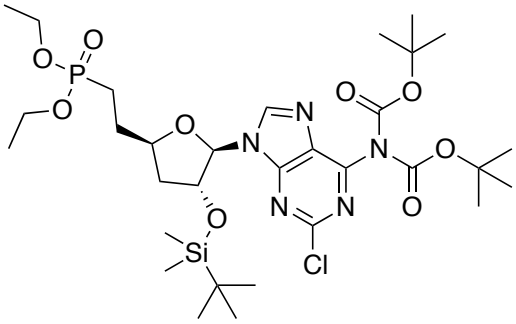
^1H NMR (500 MHz, $CDCl_3$): δ_H 7.90 (s, 1H, H-8), 6.77 (ddd, J_{HP}= 22.03, J_{HH} = 17.3, 5.7, H-5'), 5.92 (ddd, J_{HP}= 18.84, J_{HH} = 17.3, 1.5, H-6'), 5.85 (d, J = 0.5, 1H, H-1'), 4.91-4.95

(m, 1H, H-4'), 4.62-4.63 (m, 1H, H-2'), 3.94-4.01 (m, 4H, OC***H***$_2$CH$_3$ x 2), 2.02-2.06 (m, 1H, H-3'), 1.86-1.93 (m, 1H, H-3'), 1.41 (s, 18H, N(COOC(C***H***$_3$)$_3$)$_2$) 1.21 (t, *J* = 7.25, 6H, OCH$_2$C***H***$_3$ x 2), 0.78 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 0.04 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), 0.00 (s, 3H, (CH$_3$)$_3$-C-SiO(2')-(C***H***$_3$)$_2$).

^{13}C NMR (126 MHz, CDCl$_3$): δ_C 154.10 (C-6), 151.65 (N(***C***OOC(CH$_3$)$_3$), 150.64 (N(***C***OOC(CH$_3$)$_3$), 149.36 (C-2), 149.83 (C-4), 148.67 (d, $^2J_{CP}$ = 5.4 C-5'), 140.55 (CH-8), 121.41 (C-5), 118.63 (d, $^1J_{CP}$ = 188 C-6'), 92.91 (CH-1'), 82.62 (N(COO***C***(CH$_3$)$_3$)$_2$), 80.43 (d, $^3J_{CP}$ = 23.10, CH-4'), 76.59 (CH-2'), 62.02 (d, $^2J_{CP}$ = 6.4 O***C***H$_2$CH$_3$), 61.97 (d, $^2J_{CP}$ = 6.6 O***C***H$_2$CH$_3$), 38.76 (CH$_2$-3'), 28.08 (N(COOC(***C***H$_3$)$_3$)$_2$) 25.65 (CH$_3$ x 3 (***C***H$_3$)$_3$-C-SiO(2')-(CH$_3$)$_2$), 17.91 (C, (CH$_3$)$_3$-***C***-SiO(2')-(CH$_3$)$_2$), 16.38 (d, $^3J_{CP}$ = 5.52 OCH$_2$***C***H$_3$), 16.34 (d, $^3J_{CP}$ = 5.16 OCH$_2$***C***H$_3$) -4.75, -5.02, (CH$_3$, (CH$_3$)$_3$-C-SiO(2')-(***C***H$_3$)$_2$).

MS (ES+) m/z: Found: 732.3 [M + H$^+$], 754.3 [M + Na$^+$] $C_{31}H_{51}ClN_5O_9PSi$ required: 731.3 [M].

9-[5',6'-dideoxy-6'-(bis-ethylphosphinyl)-2'-*O*-(*tert*-butyldimethylsilyl)-D-ribohexofuranosyl] bis-N^6,N^6-tert-butylcarbonyl-2-chlorocordycepin (4.7)



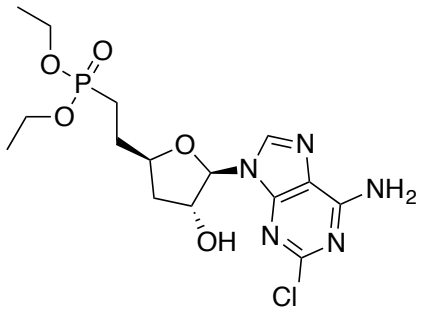
To solution of **4.6** (272 mg, 0.37 mmol) in MeOH (10 ml) under argon atmosphere, 30 mg of Pd/C were added. Then the mixture was set under vaccum and H_2 atmosphere was added. The mixture was stirring for 2 h, filtrated on a short pad of celite and concentrated under vacuum. The mixture was purified by flash chromatography (eluent system from Hexane/AcOEt 50:50 to 0:100 over 10 CV) and afforded 176 mg (65 %) of the title compound white solid.

^{31}P NMR (202 MHz, $CDCl_3$): δ_P 30.90

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.15 (s, 1H, H-8), 5.91 (s, 1H, H-1'), 4.78 (d, *J* = 4.5, 1H, H-2'), 4.50-4.56 (m, 1H, H-4'), 4.13 (q, *J* = 7.5, 2H, OC***H***$_2$$CH_3$), 4.12 (q, *J* = 7.6, 2H, OC***H***$_2$$CH_3$), 1.81-2.08 (m, 6H, HH-6', HH5', HH-3'), 1.48 (s, 18H, N(COOC(C***H***$_3$)$_3$)$_2$), 1.35 (t, *J* = 7.0, 3H, OCH_2C***H***$_3$), 1.34 (t, *J* = 7.1, 3H, OCH_2C***H***$_3$), 0.92 (s, 9H, (C***H***$_3$)$_3$-C-SiO(2')-(CH_3)$_2$), 0.17 (s, 3H, (CH_3)$_3$-C-SiO(2')-(C***H***$_3$)$_2$), 0.13 (s, 3H, (CH_3)$_3$-C-SiO(2')-(C***H***$_3$)$_2$).

MS (ES+) m/z: Found: 734.3 [M + H^+], 756.3 [M + Na^+] $C_{31}H_{53}ClN_5O_9PSi$ required: 733.3 [M].

9-[5',6'-dideoxy-6'-(bis-ethylphosphinyl)-D-ribohexofuranosyl]-2-chlorocordycepin (4.8)



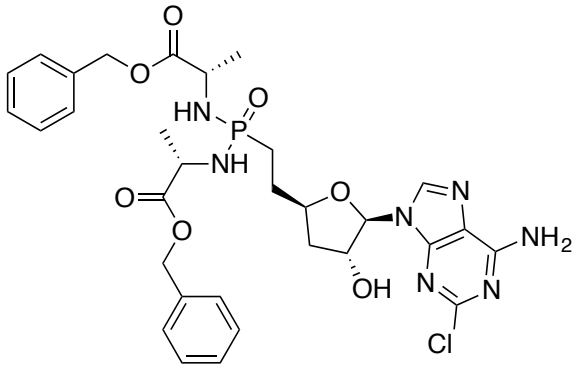
Compound **4.7** (1.76 mg, 0.24 mmol) was dissolved in 15 ml of a solution H2O/HCOOH/THF 1:2:1. The solution was stirring for 72 h at RT. The solution was evaporated under vacuum and the residue was purified by flash chromatography (eluent system from CH_2Cl_2/MeOH 95:5 to 85:15 over 10 CV) and afforded 59 mg (58 %) of the title compound white solid.

^{31}P NMR (202 MHz, $CDCl_3$): δ_P 31.06

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.94 (s, 1H, H-8), 5.87 (d, *J* = 1.9, 1H, H-1'), 4.76-4.79 (m, 1H, H-2'), 4.52-4.56 (m, 1H, H-4'), 4.11-4.15 (m, 4H, OC***H***$_2$$CH_3$ x 2), 1.81-2.08 (m, 6H, HH-6', HH5', HH-3'), 1.35 (t, *J* = 3.5, 3H, OCH_2C***H***$_3$), 1.34 (t, *J* = 7.0, 3H, OCH_2C***H***$_3$).

MS (ES+) m/z: Found: 442.1 [M + Na^+] $C_{15}H_{23}ClN_5O_5P$ required: 419.1 [M].

2-Chloro-9-[5',6'-dideoxy-6'-(bis-(benzyloxy-L-alaninyl)-phosphinyl-3'-deoxy)-D-ribohexofuranosyl] adenine (4.9)



To a solution of 4.8 (63 mg, 0.15 mmol) in ACN (2 ml) at 0 ˚C, TMSBr (0.06 ml, 0.45 mmol) and 2,6-lutidine (0.05 ml, 0.45 mmol) were added dropwise. The solution was stirring for 4h at 0 ˚C. After this time, the solvent was evaporated and the residue, under argon atmosphere, was redisolved in anhydrous triethylamine (0.25 ml) and pyridine (1 ml). To this mixture *L*-Alanine benzyl ester *p*-toluenesulphonate salt (198 mg, 0.9 mmol) was added. In a separated flask, Ph_3P (236 mg (0.9 mmol) and aldrithiol-2 (158 mg, 0.45 mmol) were dissolved in anhydrous pyridine (1.2 ml), and this solution was immediately added to the reaction mixture, which was stirred at 50 ˚C for 20 h. After this time, the mixture was cooled at RT, solvent evaporated under vacuum and the crude was purified by flash chromatography (eluent system from CH_2Cl_2/MeOH 100:0 to 90:10 over 15 CV), and preparative HPLC (20 ml/min, H_2O/CH_3CN 55:45 and afforded 3 mg (3 %) of the title compound white solid.

^{31}P NMR (202 MHz, $CDCl_3$): δ_P 31.63

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.90 (s, 1H, H-8), 7.25-7.29 (m, 10H, H-Ar), 5.79 (d, *J* = 1.0, 1H, H-1'), 4.99-5.07 (m, 4H, OC***H***$_2$Ph), 4.54-4.58 (m, 1H, H-2'), 4.38-4.46 (m, 1H, H-4'), 3.54-3.61 (m, 2H, C***H***CH_3 x 2) 1.83-2.09 (m, 6H, HH-6', HH5', HH-3'), 1.30-1.32 (m, 6H, CHC***H***$_3$)

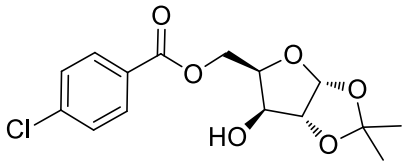
MS (ES+) m/z: Found: 686.2 [M + H^+], 708.2 [M + Na^+] $C_{15}H_{23}ClN_5O_5P$ required: 685.2 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 264 nm, t_R 15.37 min.

Experimental details Chapter 5

1st Route to synthesise 3'-ethynyluridine

5-*O*-(4-Chlorobenzoyl)-1,2-*O*-isopropylidene-D-xylofuranose (5.5)



To 500 mg (2.63 mmol) of 1,2-*O*-isopropylidene-D-xylofuranose **5.4** dissolved dichloromethane in anhydrous conditions and Ar atmosphere, 0.99 ml (7.15 mmol) of triethylamine were added. The temperature of the mixture was lowered at 0 °C. Then 0.37 ml (2.89 mmol) of 4-chlorobenzoylchloride were added dropwise to the stirring mixture. The reaction was stirring at 0 °C for two hours. The formation of the product was monitored by TLC. 16 ml of a saturated aqueous $NaHCO_3$ solution were added, and the resulting mixture was partitioned. The organic layer was washed twice with 16 ml of brine, dried over $MgSO_4$ and filtrated. Solvent was evaporated by reduced pressure and the mixture was crystallized using hexane and chloroform to give us a 0.408 g (47 %) of the titled compound as a white precipitate.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.01 (d, *J* = 8.5 Hz, 2H H-Ar), 7.46 (d, *J* = 8.5 Hz, 2H H-Ar), 5.98 (d, *J* = 3.5 Hz, 1H, H-1'), 4.81 (dd, *J* = 9.5, 13.5 Hz, 1H, H-5'), 4.61 (d, *J* = 3.5 Hz, 1H, H-2'), 4.38-4.44 (m, 2H, H-5', H-4'), 4.20 (d, *J* = 2.5 Hz, 1H, H-3'), 1.54 (s, 3H, $C(CH_3)_2$), 1.35 (s, 3H, $C(CH_3)_2$).

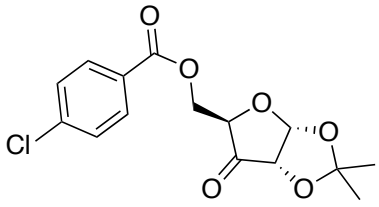
^{13}C NMR (126 MHz, $CDCl_3$): δ_C 166.47 (C=O), 140.09 (C-Ar), 131.30 (CH x 2 Ar), 128.88 (CH x 2 Ar) 112.54 (C-Ar), 111.92, ($\boldsymbol{C}(CH_3)_2$), 104.79 (CH-1'), 85.07 (CH-2'), 78.46 (CH-4'), 74.47 (CH-3'), 61.79 (CH_2-5'), 28.81 ($C(\boldsymbol{C}H_3)_2$), 26.16 ($C(\boldsymbol{C}H_3)_2$).

MS (ES+) m/z: Found: 351.1 [M + Na^+], $C_{15}H_{17}ClO_6$ required: 328.1 [M].

5-*O*-(4-Chlorobenzoyl)-1,2-*O*-isopropylidene-*D-erythro*-pentofuranose-3-urose (TEMPO) (5.6)

A mixture of **5.5** (1.0 g, 3.04 mmol) and TEMPO (24 mg, 0.15 mmol) was dissolved in a solution of NaClO (1.72 ml, 25.6 mmol), $NaHCO_3$ (574 mg, 6.8 mmol) and H_2O (9.74 mL, 0.54 mmol) at 0 ˚C. Progress of the reaction was monitored every 30 minutes for 3 h and then after 15 h. The reaction was stopped by adding 2-propanol to the mixture and stirring for 10 min at RT. The mixture was partitioned, the organic phase washed with H_2O, dried over $MgSO_4$ and filtered. Solvent was evaporated under reduced pressure, to give 0.910 g of yellow oil. Neither the oil nor any of the extracted phases contained the desired product.

5-*O*-(4-Chlorobenzoyl)-1,2-*O*-isopropylidene-*D-erythro*-pentofuranose-3-urose (DMP) (5.6)



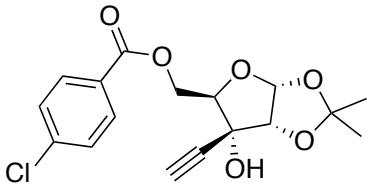
To an anhydrous solution of **5.5** (2.0 g, 6.08 mmol) in 20 ml of CH_2Cl_2, DMP (3.63 g, 8.51 mmol) was added at 0 ˚C, The reaction was stirring at 0 ˚C for 15 minutes and then it was allowed to slowly reach RT. After 24 hours a second addition of DMP (1.55 g, 3.65 mmol) was made at 0 ˚C. 4 hours later the reaction was stopped by the addition of 40 ml of diethylether. The mixture was washed with 20 ml of a 10 % $Na_2S_2O_3$ solution in saturated $NaHCO_3$. Then the organic phase was washed with brine and the aqueous phase extracted with diethylether. The organic layers were combined dried over Na_2SO_4 and filtered. Solvent was evaporated under reduced pressure. The mixture was crystallized using hexane and chloroform to give us a 1.91 g (96 %) of a white precipitate.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.81 (d, *J* = 8.5 Hz, 2H H-Ar), 7.34 (d, *J* = 8.5 Hz, 2H H-Ar), 6.04 (d, *J* = 4 Hz, 1H, H-1'), 4.63-4.60 (m, 2H, H-5', H-4'), 4.39 (dd, *J* = 4.5, 13 Hz, 1H, H-5'), 4.34 (dd, *J* = 1, 4.5 Hz, 1H, H-2'), 1.44 (s, 3H, $C(CH_3)_2$), 1.36 (s, 3H, $C(CH_3)_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 207.23 (C=O-3'), 165.02 (C=O), 139.96 (C-Ar), 130.97 (CH x 2 Ar), 128.92 (CH x 2 Ar), 127.79 (C-Ar), 114.52 ($\boldsymbol{C}(CH_3)_2$), 103.03 (CH-1'), 76.84 (CH-4'), 76.11 (CH-2'), 65.46 (CH_2-5'), 27.42 ($C(\boldsymbol{C}H_3)_2$), 27.05 ($C(\boldsymbol{C}H_3)_2$).

MS (ES+) m/z: Found: 327.1 [M + H^+],349.1 [M + Na^+] $C_{15}H_{15}ClO_6$ required: 326.1 [M].

5-*O*-(4-Chlorobenzoyl)-3-*C*-ethynyl-1,2-*O*-isopropylidene-*D*-*ribo*-pentofuranose (5.7)



To a solution of **5.6** (1.91 g, 5.8 mmol) in 35 ml of THF in anhydrous conditions and under Ar atmosphere CH≡CMgBr was added dropwise at 0 ˚C. After 1.5 h full conversion of the mixture was observed. Reaction was stopped by adding 4.8 ml of NH_4Cl aqueous solution 15 %, then, the mixture was partitioned and the organic layer was washed with 4.8 ml of NaCl 25 % aqueous solution. Solvent was evaporated under reduced pressure giving us a red residue. Purification by flash chromatography (eluent system from Hexane/AcOEt 88:12 to 0:100 over 10 CV) afforded 1.33 g (65 %) of the desired compound as a white solid.

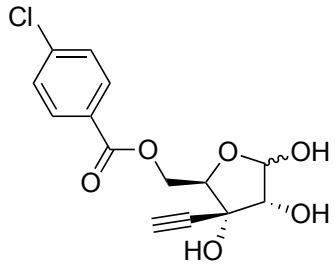
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.94 (d, *J* = 8.5 Hz, 2H H-Ar), 7.34 (d, *J* = 8.5 Hz, 2H H-Ar), 5.88 (d, *J* = 4 Hz, 1H, H-1'), 4.65 (dd, *J* = 3.5, 12 Hz, 1H, H-5'), 4.52 (d, *J* = 4 Hz, 1H, H-2'), 4.50-4.54 (m, 1H, H-5'), 4.11 (dd, *J* = 4, 8 Hz, 1H, H-4'), 2.92 (br s, 1H, OH-C3'), 2.58 (s, 1H, CH≡C-C3'), 1.54 (s, 3H, $C(CH_3)_2$), 1.33 (s, 3H, $C(CH_3)_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 165.55 (C=O), 139.63 (C-Ar), 131.26 (CH x 2 Ar), 128.73 (CH x 2 Ar), 128.25 (C-Ar), 113.62 ($\boldsymbol{C}(CH_3)_2$), 104.26 (CH-1'), 83.51 (CH-2'),

74.84 (CH-4'), 79.31 (CH≡***C***-C3'), 77.27 (***C***H≡C-C3'), 74.89 (C-3'), 64.08 (CH_2-5'), 26.76 (C(***C***$H_3)_2$), 26.54 (C(***C***$H_3)_2$).

MS (ES+) m/z: Found: 375.1 [M + Na^+], $C_{17}H_{17}ClO_6$ required: 352.1 [M].

5-*O*-(4-Chlorobenzoyl)-3-*C*-ethynyl-*D*-*ribo*-pentofuranose (5.8)



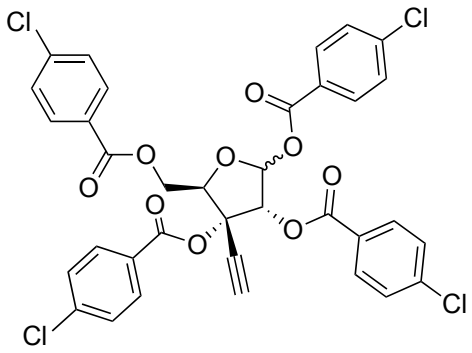
A suspension of **5.7** (510 mg, 1.44 mmol) in 5.8 ml of a mixture of HCOOH/H_2O 1:1 was heated at reflux temperature 100-120 ˚C for 2 h. Reaction was cooled at RT and 2.9 ml of H_2O were added. Reaction was stirring for 1 h. Solvent was evaporated under reduced pressure giving us a yellow residue. Crystallisation with $CHCl_3$, filtration washing with H_2O, $NaHCO_3$ and H_2O afforded 473 mg (95 %) of the desired compound, the mixture of α and β anomers (1:1) as a white solid.

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.96 (d, *J* = 8.5 Hz, 1H H-Ar), 7.92 (d, *J* = 8.5 Hz, 1H H-Ar), 7.40 (d, *J* = 8.5 Hz, 1H H-Ar), 7.39 (d, *J* = 8.5 Hz, 1H H-Ar), 5.29 (d, *J* = 4 Hz, 0.5H, H-1'), 5.06 (d, *J* = 4.5 Hz, 0.5H, H-1'), 4.56-4.50 (m, 2H, H-5'), 4.24-4.26 (m, 0.5H, H-4'), 4.10-4.12 (m, 0.5H, H-4'), 3.96 (d, *J* = 4 Hz, 0.5H H-2'), 3.87 (d, *J* = 4.5 Hz, 0.5H H-2'), 2.99 (s, 0.5H, CH≡C-C3'), 2.93 (s, 0.5H, CH≡C-C3').

^{13}C NMR (126 MHz, CD_3OH): δ_C 166.87 (C=O), 140.66, 140.60 (C-Ar), 132.37, 132.28 (CH x 2 Ar), 130.08 (C-Ar), 129.91, 129.88 (CH x 2 Ar), 103.00, 97.98 (CH-1'),84.14, 82.34 (CH-4'), 82.98, 78.20 (CH-2'), 83.05, 82.99 (CH≡***C***-C3'), 76.95, 76.64 (***C***H≡C-C3'), 74.31 (C-3'), 66.87, 66.50 (CH_2-5').

MS (ES+) m/z: Found: 335.0 [M + Na^+], $C_{14}H_{13}ClO_6$ required: 312.0 [M].

1,2,3,5-Tetra-*O*-(4-Chlorobenzoyl)-3-*C*-ethynyl-*D*-*ribo*-pentofuranose (5.9)



To a suspension of **5.8** (421 mg, 1.35 mmol) and DMAP (5 mg, 0.04 mmol) in 7 ml of CH_2Cl_2, 0.75 ml (5.4 mmol) of Et_3N were added at 0 ˚C to give us a clear solution. Then 945 mg (5.4 mmol) of 4-ClBzCl were added. The reaction was stirring for 17 h at RT. Then 7 ml of MeOH and 7 ml of CH_2Cl_2 were added. Solution was partitioned and organic layer washed with 10 ml of H_2O, 5 ml of aqueous solution of NH_4Cl 15 % and 5 mL of aqueous solution of NaCl 25 %. The organic layer was dried over Na_2SO_4 and filtered. Solvent was evaporated under reduced pressure. The residue contained a mixture of α and β isomers in a 7:3 ratio. Isomer α was isolated by column chromatography using CH_2Cl_2 100 % as eluent system, affording 348 mg of pure isomer α and 95 mg of a mixture of α and β. (443 mg, 45 %)

Isomer α:

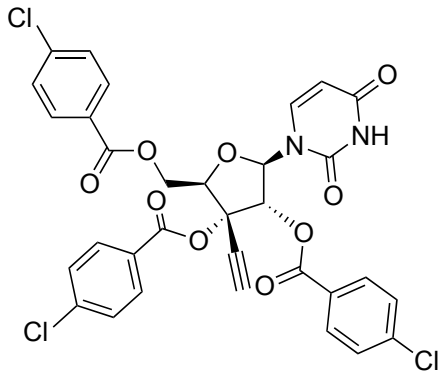
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.07 (d, *J* = 8.5 Hz, 2H H-Ar), 7.99 (d, *J* = 8.5 Hz, 2H H-Ar), 7.92 (d, *J* = 8.5 Hz, 2H H-Ar), 7.79 (d, *J* = 8.5 Hz, 2H H-Ar), 7.46 (d, *J* = 8.5 Hz, 2H H-Ar), 7.38-7.47 (m, 4H H-Ar), 7.24 (d, *J* = 8.5 Hz, 2H H-Ar), 6.96 (d, *J* = 4.5 Hz, 1H, H-1'), 6.15 (d, *J* = 4.5 Hz, 1H, H-2'), 5.13 (dd, *J* = 3.7, 5.4 Hz, 1H, H-4'), 4.97 (dd, *J* = 3.7, 12.1 Hz, 1H, H-5'), 4.86 (dd, *J* = 5.6, 12.1 Hz, 1H, H-5'), 2.86 (s, 1H, CH≡C-C3').

Mixture isomer α and β 85:15.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.88-8.08 (m, 8H, H-Ar), 7.35-7.47 (m, 6.3H, H-Ar), 7.23 (d, *J* = 8.6 Hz, 1.7H, H-Arα), 6.96 (d, *J* = 4.5 Hz, 0.85H, H-1'α), 6.61 (d, *J* = 1 Hz, 0.15H, H-1'β), 6.32 (d, *J* = 1 Hz, 0.15H, H-2'β), 6.15 (d, *J* = 4.5 Hz, 1H, H-2'α), 5.13 (dd, *J* = 3.7, 5.5 Hz, 0.85H, H-4'α), 5.01-5.05 (m, 0.3H, H4'β+H5'β), 4.97 (dd, *J* = 3.7, 12.2 Hz,

0.85H, H-5α'), 4.86 (dd, *J* = 5.6, 12.2 Hz, 0.85H, H-5'α), 4.79-4.83 (m, 0.15H, H-5'β), 2.99 (s, 0.15H, CH≡C-C3'β), 2.86 (s, 1H, CH≡C-C3'α).

1-[2,3,5-Tri-*O*-(4-chlorobenzoyl)-3-*C*-2-ethynyl-β-*D*-*ribo*-pentofuranosyl]uracil (5.10)



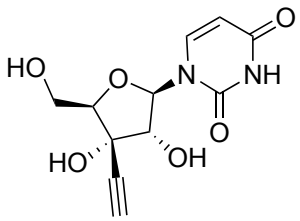
A mixture of uracil (264 mg, 2.36 mmol) and $(NH_4)_2SO_4$ (10 mg, 0.08 mmol) was suspended in 4.7 ml (22.42 mmol) of HMDS and solution was warmed at reflux temperature (120-130 ˚C) in anhydrous conditions and under argon atmosphere for 2 h. Solvent was evaporated and then dissolved in 2 mL of ACN. To this solution compound **5.9** (859 mg, 1.18 mmol) was added dissolved in 6 ml of ACN. $SnCl_4$ (0.17 ml, 1.44 mmol) was added to the stirring mixture at 0 ˚C. Next the temperature was raised at 30 ˚C and the reaction was kept for 20 h. Solvent was evaporated and the residue was partitioned between toluene (50 ml) and HCl 3M (50 ml). The organic layer was washed with saturated Na_2CO_3 (50 ml), dried over Na_2SO_2 and evaporated. Purification by chromatographic column (eluent system Hexane/ AcOEt from 90:10 to 50:50 afforded 161 mg (20 %) of the desired product as an oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 9.06 (br s, 1H, NH), 8.05 (d, *J* = 8.5 Hz, 2H H-Ar), 7.93 (d, *J* = 8.6 Hz, 2H H-Ar), 7.82 (d, *J* = 8.6 Hz, 2H H-Ar), 7.68 (d, *J* = 8.5 Hz, 1H H-6), 7.46 (d, *J* = 8.5 Hz, 2H H-Ar), 7.42 (d, *J* = 8.6 Hz, 2H H-Ar), 7.31 (d, *J* = 8.5 Hz, 2H H-Ar), 6.34 (d, *J* = 4.7 Hz, 1H, H-1'), 5.97 (d, *J* = 4.7 Hz, 1H, H-2'), 5.80 (d, *J* = 8.1 Hz, 1H H-5), 4.82-4.98 (m, 3H, H-4', H-5'), 2.96 (s, 1H, CH≡C-C3').

^{13}C NMR (126 MHz, CD_3OH): δ_C 165.30 (C=O, C-4), 163.58, 163.13, 162.60 (C=O, C-4ClBzO x 3), 150.09 (C=O, C-2), 140.87, 140.66, 140.22 (C, 4ClBzO x 3), 138.94 (CH-6)

131.23, 131.20, 131.15 (CH x 2, 4ClBzO x 3), 129.15, 129.05, 128.96 (CH x 2, 4ClBzO x 3), 127.69, 126.79, 126.45 (C, 4ClBzO x 3), 103.90 (CH-5), 86.97 (CH-1'), 80.66 (CH-4'), 80.65 (***C***H≡C-C3'), 78.18 (CH-2'), 76.20 (CH≡***C***-C3'), 75.63 (C-3'), 63.61 (CH_2-5').

3'-Ethynyluridine (5.1)



To a solution of compound **5.10** (160 mg, 0.23 mmol) in anhydrous MeOH (5 ml), DBU (0.14 ml, 0.94 mmol) was added. The mixture was stirring for 1.5 h at 40 ˚C. 0.14 ml of AcOH glacial were added, then solvent was evaporated. Purification by CC (eluent system $CHCl_3$/MeOH from 97:3 to 90:10) afforded the nucleoside as a white precipitate in a 87 % yield.

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.97 (d, *J* = 8.1 Hz, 1H H-6), 5.86 (d, *J* = 6.5 Hz, 1H, H-1'), 5.62 (d, *J* = 8.1 Hz, 1H H-5), 5.62 (d, *J* = 6.5 Hz, 1H, H-2'), 3.93-3.94 (m, 1H, H-4'), 3.84 (dd, *J* = 4.3, 12.1 Hz, H-5'), 3.73 (dd, *J* = 2.8, 12.1 Hz, H-5'), 3.00 (s, 1H, CH≡C-C3').

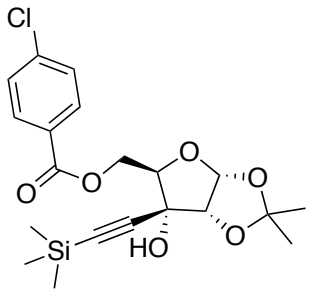
^{13}C NMR (126 MHz, CD_3OH): δ_C 166.04 (C=O, C-4), 152.68 (C=O, C-2), 142.90 (CH-6), 102.96 (CH-5), 89.07 (CH-1'), 88.35 (CH-4'), 80.29 (CH-2', ***C***H≡C-C3'), 77.17 (CH≡***C***-C3'), 74.26 (C-3'), 63.10 (CH_2-5').

MS (ES+) m/z: Found: 269.1 [M + H^+], 291.1 [M + Na^+] $C_{17}H_{17}ClO_6$ required: 268.1 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 208 nm, t_R 6.4 min

2nd Route to synthesise 3'-ethynyluridine

5-*O*-(4-Chlorobenzoyl)-1,2-*O*-isopropylidene-3-*C*-[2-(trimethylsilyl)ethynyl]-*D*-*ribo*-pentofuranose (5.11)



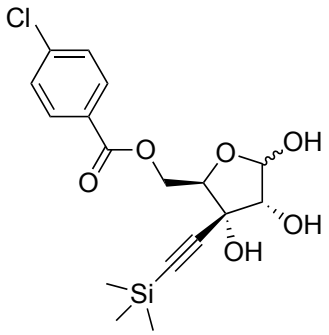
Trimethylsililacetilene (2.1 ml, 15.0 mmol) was added dropwise at 0 ˚C to a solution of EtMgBr 1M in THF (13.0 ml, 13.0 mmol). This mixture was transferred in anhydrous conditions to a solution of compound **5.6** (3.3 g, 10.0 mmol) in 30 ml of THF Ar atmosphere. The solution became dark orange. The reaction was stirring for 45 minutes at 0 ˚C and for 1 h at RT. Reaction was stopped by adding 6.6 ml of NH_4Cl aqueous solution 15 %. Mixture was partitioned and THF was washed with 6.6 ml of NaCl 25 % aqueous solution. Solvent was evaporated under reduced pressure giving us a red residue. Purification by flash chromatography (eluent system Hexane/AcOEt from 93:7 to 40:60 over 10CV) afforded 2.75 g (69 %) of the desired compound as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.82 (d, *J* = 8.5 Hz, 2H H-Ar), 7.21 (d, *J* = 8.5 Hz, 2H H-Ar), 5.75 (d, *J* = 3.5 Hz, 1H, H-1'), 4.54 (dd, *J* = 3.5, 12 Hz, 1H, H-5'), 4.32-4.37 (m, 2H, 2', 5') 3.98 (dd, *J* = 3.5, 8 Hz, 1H, H-4'), 2.73 (s, 1H, OH-C3'), 1.40 (s, 3H, $C(CH_3)_2$), 1.20 (s, 3H, $C(CH_3)_2$), 0.00 (s, 9H, $(CH_3)_3SiC{\equiv}C$-C3').

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 165.84 (C=O), 139.89 (C-Ar), 131.57 (CH x 2 Ar), 129.04 (CH x 2 Ar), 128.63 (C-Ar), 113.81 ($\boldsymbol{C}(CH_3)_2$), 104.71 (CH-1'), 100.39 ($(CH_3)_3SiC{\equiv}\boldsymbol{C}$-C3'), 94.93 ($(CH_3)_3Si\boldsymbol{C}{\equiv}$C-C3'), 83.93 (CH-2'), 80.37 (CH-4'), 75.47 (C-3'), 64.68 (CH_2-5'), 27.08 ($C(\boldsymbol{C}H_3)_2$), 26.90 ($C(\boldsymbol{C}H_3)_2$), 0.00 ($(\boldsymbol{C}H_3)_3SiC{\equiv}$C-C3').

MS (ES+) m/z: Found: 447.1 [M + Na^+], $C_{20}H_{25}ClO_6Si$ required: 352.1 [M].

5-*O*-(4-Chlorobenzoyl)- 3-*C*-[2-(trimethylsilyl)ethynyl]-*D-ribo*-pentofuranose (5.12)

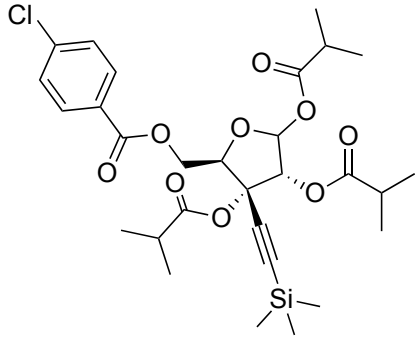


A suspension of **5.11** (10.63 mg, 25 mmol) in 100 ml of a mixture of HCOOH/H_2O 1:1 was heated at reflux temperature 100-120 ˚C for 45 min. 50 ml of H_2O were added and reaction was cooled at RT and kept stirring for 15 h. Precipitates formed were washed with 25 ml of H_2O, 25 ml of $NaHCO_3$ and 25 ml of H_2O. this resulted in 473 mg (95 %) of the desired compound, the mixture of α and β anomers (85:15) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.89 (d, *J* = 8.6 Hz, 0.3H H-Ar), 7.85 (d, *J* = 8.5 Hz, 1.7H H-Ar), 7.30 (d, *J* = 8.5 Hz, 2H H-Ar), 5.34 (d, *J* = 4.5 Hz, 0.85H, H-1'), 5.16 (d, *J* = 3.1 Hz, 0.15H, H-1'), 4.44-4.55 (m, 0.3H, H-5'), 4.48 (dd, *J* = 5.5, 14 Hz, 0.85H, H-5'), 4.34-4.38 (m, 1.7H, H-4', H5'), 4.20-4.22 (m, 0.15H, H-4'), 4.11 (d, *J* = 4.5 Hz, 0.85H, H-2'), 4.03 (d, *J* = 3.1 Hz, 0.15H, H-2'), 0.02 (s, 1.35H, $(CH_3)_3$SiC ≡C-C3'), 0.0 (s, 7.65H, $(CH_3)_3$SiC ≡C-C3').

MS (ES+) m/z: Found: 385.1 [M + H^+], 407.1 [M + Na^+] $C_{17}H_{21}ClO_6Si$ required: 384.1 [M].

5-*O*-(4-Chlorobenzoyl)-3-*C*-ethynyl-1,2,3-tri-*O*-isobutyryl-*D*-*ribo*-pentofuranose (5.13)



To a suspension of compound **5.12** (8.15 g, 21.2 mmol) and DMAP (80 mg, 0.66 mmol) in 80 ml of CH_2Cl_2, 11.8 ml (84.8 mmol) of Et_3N were added at 0 ˚C to give us a clear solution. Then 8.9 ml (84.8 mmol) of 4-ClBzCl were added. The reaction was stirring for 5 h at RT. Then 20 ml of MeOH and 20 ml of CH_2Cl_2 were added. Solution was partitioned and organic layer washed with 20 ml of H_2O, 10 ml of aqueous solution of NH_4Cl 15 % and 10 mL of aqueous solution of NaCl 25 %. The organic layer was dried over Na_2SO_4 and filtered. Solvent was evaporated under reduced pressure to give us a brown oil that was purified isolated by column chromatography using CH_2Cl_2 100 % as eluent system. 12.7 g (100 %) of the titled compound were afforded containing a mixture of isomers α and β (80:20).

Mixture isomer α and β 80:20

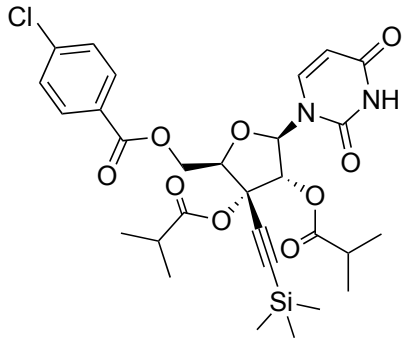
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.91-7.95 (m, 2H, H-Ar), 7.32-7.36 (m, 2H, H-Ar), 6.42 (d, *J* = 4.5 Hz, 0.8H, H-1'α), 6.01 (d, *J* = 1.1 Hz, 0.2H, H-1'β), 5.65 (d, *J* = 1.1 Hz, 0.2H, H-2'β), 5.61 (d, *J* = 4.4 Hz, 0.8H, H-2'α), 4.68 (dd, *J* = 4.1, 11.5 Hz, 0.2H, H-5'β), 4.61-4.65 (m, 1.6H, H4'α+H5'α), 4.52-4.56 (m, 1H, H-5'α, H-4'β), 4.48 (dd, *J* = 7.1, 11.5 Hz, 0.2H, H-5'β), 2.41-2.55 (m, 3H, OCOC***H***$(CH_3)_2$ x 3), 1.12-1.07 (m, 18H, OCOCH(C***H***$_3)_2$ x 3), 0.05 (s, 1.8H, $(CH_3)_3$SiC ≡C-C3'), 0.00 (s, 7.2H, $(CH_3)_3$SiC ≡C-C3').

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 182.58 (C=O, C-4ClBz), 175.90, 175.37, 174.99 (C=O, Cα-O***C***OCH$(CH_3)_2$ x 3), 175.64, 175.29, 174.60 (C=O, Cβ-O***C***OCH$(CH_3)_2$ x 3), 165.84 (Cα-Ar), 165.81 (Cβ-Ar), 131.76 (CH$\alpha\beta$ x 2 Ar), 129.39 (CHα x 2 Ar), 129.32 (CHβ x 2 Ar), 128.82 (Cα-Ar), 128.80 (Cβ-Ar), 99.12 (CHβ-1'), 94.09 (CHα-1') 83.21 (CHβ-4'),

82.6 (CHα-4'), 79.30 (CHβ-2'), 76.98 (Cβ-3'), 76.48 (Cα-3'), 75.87 (CHα-2'), 65.88 (CH_2β-5'), 64.74 (CH_2α-5'), 34.70, 34.64, 34.44, (CHα, OCO***C***H$(CH_3)_2$ x 3), 34.58, 34.21, 34.43, (CHβ, OCO***C***H$(CH_3)_2$ x 3), 19.46, 19.37, 19.35, 19.33, 19.22., 19.17, 19.12, 19.06 26.90 (CHαβ, OCOCH(***C***$H_3)_2$ x 3), 0.04 (CH_3β, (***C***$H_3)_3$SiC≡C-C3'), 0.00 (CH_3α, (***C***$H_3)_3$SiC≡C-C3').

MS (ES+) m/z: Found: 595.2 [M + H^+], 617.2. [M + Na^+] $C_{29}H_{39}ClO_9Si$ required: 594.2 [M].

1-[5-*O*-(4-chlorobenzoyl)-2,3,di-*O*-isobutyryl-3-*C*-2-ethynyl-β-*D*-*ribo*-pentofuranosyl]uracil (5.14)



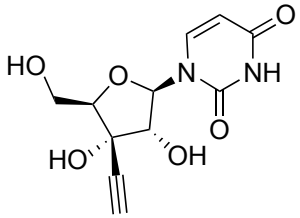
A mixture of uracil (1.13 g, 10.1 mmol) and $(NH_4)_2SO_4$ (92 mg, 0.7 mmol) was suspended in 20.1 (95 mmol) ml of HMDS and solution was warmed at reflux temperature (120 ˚C) in anhydrous conditions and under argon atmosphere for 1 h. Solvent was evaporated. To this mixture compound **5.13** (3.0 g, 5.0 mmol) was added dissolved in 10 ml of ACN. $SnCl_4$ (0.17 ml, 1.44 mmol) was added to the stirring mixture at 0 ˚C. Next the temperature was raised at 30 ˚C and the reaction was kept for 24 h. Solvent was evaporated and the residue was partitioned between toluene and HCl 3M. The organic layer was washed with saturated $NaHCO_3$, dried over Na_2SO_2 and evaporated. Purification by flash chromatography (eluent system Hexane/ AcOEt from 90:10 to 0:100 over 15 CV) afforded 1.92 g (61 %) of the desired product as an oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 9.14 (br s, 1H, NH), 7.87 (d, *J* = 8.6 Hz, 2H H-Ar), 7.53 (d, *J* = 8.1 Hz, 1H H-6), 7.29 (d, *J* = 8.6 Hz, 2H H-Ar), 5.95 (d, *J* = 4.3 Hz, 1H, H-1'), 5.56 (d, *J* = 8.1 Hz, 1H H-5), 5.45 (d, *J* = 4.3 Hz, 1H, H-2'), 4.62 (dd, *J* =3.0, 12.2 Hz, 1H H-5'), 4.54 (dd, *J* =6.5, 12.2 Hz, 1H H-5'), 4.35 (dd, *J* =3.1, 6.5 Hz, 1H H-4'), 2.39-2.50 (m, 2H, OCOC***H***$(CH_3)_2$ x 2.) 1.00-1.05 (m, 12H, OCOCH(C***H***$_3)_2$ x2), 0.00 (s, 9H, $(CH_3)_3$SiC ≡C-C3').

^{13}C NMR (126 MHz, CD_3OH): δ_C 175.49, 174.69 (C=O O***C***OCH$(CH_3)_2$ x 2) 165.88 (C=O, C-4), 163.54 (C=O, C-4ClBzO), 150.82 (C=O, C-2), 140.61 (C, 4ClBzO), 139.94 (CH-6) 131.76 (CH x 2, 4ClBzO), 129.57 (CH x 2, 4ClBzO), 128.47 (C, 4ClBzO), 104.18 (CH-5), 97.93 (C, $(CH_3)_3$SiC≡***C***-C3'), 97.25 (C, $(CH_3)_3$Si***C***≡C-C3'), 87.81 (CH-1'), 80.92 (CH-4'), 78.18 (CH-2'), 75.59 (C-3'), 64.46 (CH_2-5'), 34.42, 34.33 (CH, OCO***C***H$(CH_3)_2$ x 2), 19.36, 19.33, 19.28, 19.21 (CH_3, OCOCH(***C***$H_3)_2$ x 2), 0.00 (CH_3, (***C***$H_3)_3$SiC≡C-C3').

MS (ES+) m/z: Found: 619.2 [M + H^+], 641.2 [M + Na^+] $C_{29}H_{35}ClN_2O_9Si$ required: 618.2 [M].

3'-Ethynyluridine (5.1)



To a solution of compound **5.14** (510 mg, 0.93 mmol) in anhydrous MeOH (8 ml), DBU (0.56 ml, 3.73 mmol) was added in three portions. The mixture was stirring for 2.5 h at 40 ˚C. 0.48 ml of AcOH glacial were added, then, solvent was evaporated. Purification by CC (eluent system $CHCl_3$/MeOH from 95:5 to 90:10) afforded 0.12 (48 %) of the nucleoside analogue as a white precipitate.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.97 (d, *J* = 8.1 Hz, 1H H-6), 5.86 (d, *J* = 6.5 Hz, 1H, H-1'), 5.62 (d, *J* = 8.1 Hz, 1H H-5), 5.62 (d, *J* = 6.5 Hz, 1H, H-2'), 3.93-3.94 (m, 1H, H-4'),

3.84 (dd, *J* = 4.3, 12.1 Hz, H-5'), 3.73 (dd, *J* = 2.8, 12.1 Hz, H-5'), 3.00 (s, 1H, CH≡C-C3').

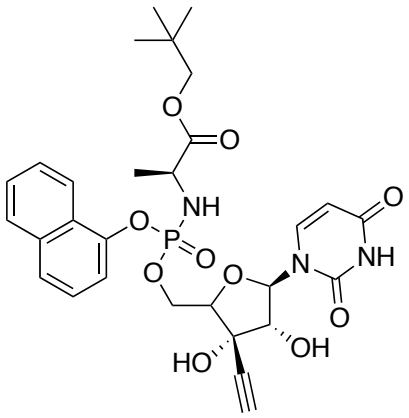
^{13}C NMR (126 MHz, CD_3OD): δ_C 166.04 (C=O, C-4), 152.68 (C=O, C-2), 142.90 (CH-6), 102.96 (CH-5), 89.07 (CH-1'), 88.35 (CH-4'), 80.29 (CH-2', ***C***H≡C-C3'), 77.17 (CH≡***C***-C3'), 74.26 (C-3'), 63.10 (CH_2-5').

MS (ES+) m/z: Found: 269.1 [M + H^+], 291.1 [M + Na^+] $C_{17}H_{17}ClO_6$ required: 268.1 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, t_R 6.4 min

Synthesis of 3'-ethynyluridine ProTides

3'-Ethynyluridine-5'-O-[1-naphtyl (2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.17)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.1** (158 mg, 0.59 mmol), N-methylimidazole (0.23 mL, 2.95 mmol) and naphtyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.7** (456 mg, 1.37 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (80 g column ZIP-KP-SIL eluent system CH_3OH/CH_2Cl_2 0:100 to 10:90 over 10 CV), and two preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 10:90

and 1000 μm, eluent system CH_3OH/CH_2Cl_2 7:93) afforded the titled compound as a white solid (23 mg, 7 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.07, 3.72.

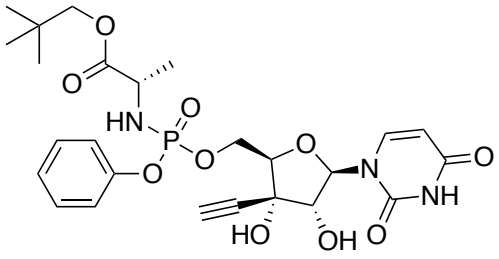
^{1}H NMR (500 MHz, CD_3OD): δ_H 8.06-8.10 (m, 1H, Naph), 7.77-7.80 (m, 1H, Naph), 7.59-7.63 (m, 1H, Naph), 7.55 (d, *J* = 8.1 Hz, 0.5H H-6), 7.42-7.53 (m, 4.5H, H-6, Naph), 7.30-7.35 (m, 1H, Naph), 5.82 (d, *J* = 6.3 Hz, 0.5H, H-1'), 5.80 (d, *J* = 5.9 Hz, 0.5H, H-1'), 5.40 (d, *J* = 8.0 Hz, 0.5H H-5), 5.39 (d, *J* = 8.0 Hz, 0.5H H-5), 4.32-4.48 (m, 2H, H-5'), 4.15-4.17 (m, 0.5H, H-4'), 4.10-4.12 (m, 0.5H, H-4'), 4.07 (d, *J* = 5.9 Hz, 0.5H, H-2'), 4.04 (d, *J* = 6.3 Hz, 0.5H, H-2'), 3.94-4.00 (m, 1H, C***H***CH_3), 3.65-3.74 (m, 2H, OC***H***$_2C(CH_3)_3$), 3.08 (s, 0.5H, CH≡C-C3'), 3.07 (s, 0.5H, CH≡C-C3'), 1.26-1.29 (m, 3H, CHC*H*$_3$), 0.81, (4.5H, OC***H***$_2C(CH_3)_3$), 0.80 (4.5H, OC***H***$_2C(CH_3)_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.64 (d $^3J_{CP}$ = 4.8 Hz, ***C***$OOCH_2C(CH_3)_3$), 173.42 (d $^3J_{CP}$ = 5.6 Hz, ***C***$OOCH_2C(CH_3)_3$), 166.06 (C=O, C-4), 151.11, 151.02 (C=O, C-2), 147.96 (d $^2J_{CP}$ = 6.3 Hz, C-*ipso* Naph), 147.93 (d $^2J_{CP}$= 6.1 Hz, C-*ipso* Naph), 140.73, 140.58 (CH-6), 136.29 (C-Ar), 128.87, 128.82 (CH-Ar), 127.83 (C-Ar) 127.77, 127.74, 127.48, 127.45, 126.47, 125.99, 125.96, 122.47, 122.67 (CH-Ar), 116.29 (d, $^3J_{CP}$ = 3.4, CH Ar), 116.17 (d, $^3J_{CP}$ = 2.9, CH Ph x 2), 101.75, 101.57 (CH-5), 88.03, 87.56 (CH-1'), 84.16 (d ^3J = 9.0 Hz, C4'), 84.02 (d ^3J = 8.3 Hz, C4'), 80.78, 80.65 (CH≡***C***-C3'), 78.64, 78.58 (CH-2'), 76.77, 76.51 (***C***H≡C-C3'), 74.06, 73.98 (COO*C*$H_2C(CH_3)_3$), 72.56, 72.22 (CH-3'), 66.92 (d $^2J_{CP}$= 5.3 Hz, CH_2-5'), 66.70 (d $^2J_{CP}$= 5.1 Hz, CH_2-5'), 50.32, 50.16 (***C***HCH_3), 32.30, 32.13 ($COOCH_2$***C***$(CH_3)_3$), 25.31, 25.30 ($COOCH_2C$(***C***$H_3)_3$), 19.29 (d $^3J_{CP}$ = 6.1 Hz, CH***C***H_3), 19.08 (d $^3J_{CP}$ = 7.0 Hz, CH***C***H_3).

MS (ES+) m/z: Found: 616.2 [M + H^+], 638.2 [M + Na^+] $C_{29}H_{34}N_3O_{10}P$ required: 615.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, t_R 17.2 min

3'-Ethynyluridine-5'-O-[1-phenyl (2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.18)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.1** (100 mg, 0.37 mmol), N-methylimidazole (0.15 mL, 1.85 mmol) and phenyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.12** (370 mg, 1.11 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 0:100 to 18:82 over 15 CV) and two preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 10:90 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 7:93) afforded the title compound as a white solid (29 mg, 13 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.63, 3.41.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.68 (d, *J* = 8.1 Hz, 0.5H H-6), 7.65 (d, *J* = 8.1 Hz, 0.5H H-6), 7.25-7.30 (m, 2H, Ar), 7.08-7.18 (m, 3H, Ar), 5.88 (d, *J* = 6.3 Hz, 0.5H, H-1'), 5.84 (d, *J* = 5.8 Hz, 0.5H, H-1'), 5.61 (d, *J* = 8.1 Hz, 0.5H H-5), 5.58 (d, *J* = 8.1 Hz, 0.5H H-5), 4.28-4.43 (m, 2H, H-5'), 4.15-4.18 (m, 0.5H, H-4'), 4.08-4.13 (m, 1.5H, H-4', H-2'), 3.88-3.96 (m, 1H, C***H***CH_3), 3.66-3.78 (m, 2H, OC***H***$_2C(CH_3)_3$), 3.10 (s, 0.5H, CH≡C-C3'), 3.09 (s, 0.5H, CH≡C-C3'), 1.27-1.32 (m, 3H, CHC*H*$_3$), 0.85, (4.5H, OC***H***$_2C(CH_3)_3$), 0.85 (4.5H, OC***H***$_2C(CH_3)_3$).

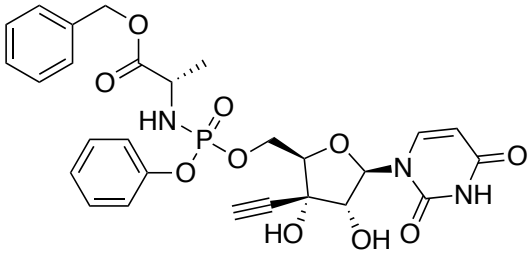
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.60 (d $^3J_{CP}$ = 4.8 Hz, ***C***$OOCH_2C(CH_3)_3$), 173.43 (d $^3J_{CP}$ = 5.6 Hz, ***C***$OOCH_2C(CH_3)_3$), 164.54 (C=O, C-4), 151.11, 151.02 (C=O, C-2), 150.73 (d $^2J_{CP}$ = 6.3 Hz, C-*ipso* Ph), 150.70 (d $^2J_{CP}$= 6.1 Hz, C-*ipso* Ph), 140.89, 140.82 (CH-6), 129.46, 129.40 (CH-Ar x 2), 124.87, 124.81 (CH-Ar), 120.08 (d, $^3J_{CP}$ = 5.0, CH Ph x 2), 120.01 (d, $^3J_{CP}$ = 4.9, CH Ph x 2), 101.81, 101.66 (CH-5), 88.09, 87.62 (CH-1'), 84.25 (d ^3J = 8.9 Hz, C4'), 84.04 (d ^3J = 8.2 Hz, C4'), 80.78, 80.65 (CH≡***C***-C3'), 78.69, 78.62 (CH-

2'), 76.77, 76.51 (***C***H≡C-C3'), 74.05, 74.02 (COO*C*H_2C(CH_3)$_3$), 72.32, 72.08 (CH-3'), 66.66 (d $^2J_{CP}$= 5.3 Hz, CH_2-5'), 66.54 (d $^2J_{CP}$= 5.1 Hz, CH_2-5'), 50.37, 50.19 (***C***HCH_3), 30.94, 30.93 (COOCH_2***C***(CH_3)$_3$), 25.34, (COOCH_2C(***C***H_3)$_3$), 19.37 (d $^3J_{CP}$ = 6.1 Hz, CH***C***H_3), 19.20 (d $^3J_{CP}$ = 7.0 Hz, CH***C***H_3).

MS (ES+) m/z: Found: 566.2 [M + H^+], 588.2 [M + Na^+] $C_{25}H_{32}N_3O_{10}P$ required: 565.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t_R}$ 16.56 min.

3'-Ethynyluridine-5'-O-[1-phenyl (benzyloxy-L-alaninyl)] phosphate (5.19)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.1** (100 mg, 0.37 mmol), N-methylimidazole (0.15 mL, 1.85 mmol) and phenyl(benzoxy-L-alaninyl) phosphorochloridate **2.13** (392 mg, 1.11 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 1:99 to 18:82 over 15 CV) and two preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 10:90 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 7:93) afforded the title compound as a white solid (24 mg, 11 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.35, 4.20.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.65 (d, *J* = 8.1 Hz, 0.5H H-6), 7.61 (d, *J* = 8.1 Hz, 0.5H H-6), 7.20-7.27 (m, 7H, Ar), 7.07-7.15 (m, 3H, Ar), 5.87 (d, *J* = 6.4 Hz, 0.5H, H-1'), 5.82 (d, *J* = 5.9 Hz, 0.5H, H-1'), 5.59 (d, *J* = 8.1 Hz, 0.5H H-5), 5.55 (d, *J* = 8.1 Hz, 0.5H H-5), 5.02-5.07 (m, 2H, OC*H*$_2$Ph) 4.26-4.38 (m, 2H, H-5'), 4.11-4.13 (m, 0.5H, H-4'), 4.08-

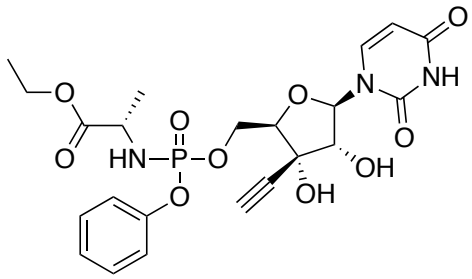
4.10 (m, 1H, H-4', H-2'), 4.06 (d, *J* = 6.4 Hz, 0.5H, H-2'), 3.89-3.98 (m, 1H, C***H***CH_3), 3.07 (s, 0.5H, CH≡C-C3'), 3.06 (s, 0.5H, CH≡C-C3'), 1.25-1.29 (m, 3H, CHCH_3).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.35 (d $^3J_{CP}$ = 4.4 Hz, C=O Bz), 173.17 (d $^3J_{CP}$ = 5.9 Hz, C=O Bz), 164.50 (C=O, C-4), 151.09, 151.00 (C=O, C-2), 150.68 (d $^2J_{CP}$ = 7.1 Hz, C-*ipso* Ph), 150.67 (d $^2J_{CP}$= 7.2 Hz, C-*ipso* Ph), 140.81, 140.73 (CH-6), 135.83, 135.82 (C-*ipso* Bz), 129.45, 129.40, 128.22, 128.20, 127.97, 127.93, 127.84 (CH-Ar x 7), 124.86, 124.80 (CH-Ar), 120.07 (d, $^3J_{CP}$ = 4.5, CH Ph x 2), 119.98 (d, $^3J_{CP}$ = 4.61, CH Ph x 2), 101.84, 101.66 (CH-5), 88.00, 87.48 (CH-1'), 84.30 (d ^3J = 8.7 Hz, C4'), 84.06 (d ^3J = 8.2 Hz, C4'), 80.77, 80.64 (CH≡***C***-C3'), 78.66, 78.57 (CH-2'), 76.74, 76.63 (***C***H≡C-C3'), 72.37, 72.11 (CH-3'), 66.61 (d $^2J_{CP}$= 7.2 Hz, CH_2-5'), 67.93, 67.90 (*C*H_2Ph), 66.46 (d $^2J_{CP}$= 5.2 Hz, CH_2-5'), 50.36, 50.19 (***C***HCH_3), 19.10 (d $^3J_{CP}$ = 6.2 Hz, CH***C***H_3), 18.95 (d $^3J_{CP}$ = 7.0 Hz, CH***C***H_3).

MS (ES+) m/z: Found: 586.1 [M + H^+], 608.1 [M + Na^+] $C_{27}H_{28}N_3O_{10}P$ required: 585.1 [M].

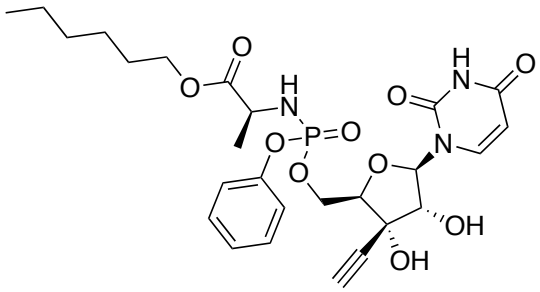
HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 16.07 min.

3'-Ethynyluridine-5'-O-[1-phenyl(ethyl-L-alaninyl)] phosphate (5.20)



Compound was prepared according to the general procedure A using 3'ehynyluridine **5.1** (56 mg, 0.21 mmol) in 5 ml of TFH, NMI (0.1 ml, 1.2 mmol) and phosphorochloridate **2.14** (604 mg, 1.57 mmol) dissolved in 3 ml of THF Solvent was evaporated and the residue was partitioned between CH_2Cl_2 and HCl 0.5M. After the acidic work-up compound was detected in the aqueous layer. All the phases were combined, solvent evaporated and separation by TLC was aimed. However the compound was no longer detected.

3'-Ethynyluridine-5'-O-[1-phenyl(hexyloxy-L-alaninyl)] phosphate (5.21)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.1** (100 mg, 0.37 mmol), N-methylimidazole (0.15 mL, 1.86 mmol) and naphthyl(hexyloxy-L-alaninyl) phosphorochloridate **2.15** (389 mg, 1.12 mmol). The reaction was stirring for 20 h. Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 from 3:97 to 10:90) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 6:94 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded the titled compound as a white solid (15 mg, 7 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.61, 3.33.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.66 (d, *J* = 8.1 Hz, 0.5H H-6), 7.63 (d, *J* = 8.1 Hz, 0.5H H-6), 7.22-7.27 (m, 2H, Ar), 7.06-7.16 (m, 3H, Ar), 5.87 (d, *J* = 6.3 Hz, 0.5H, H-1'), 5.83 (d, *J* = 5.8 Hz, 0.5H, H-1'), 5.61 (d, *J* = 8.1 Hz, 0.5H H-5), 5.56 (d, *J* = 8.1 Hz, 0.5H H-5), 4.27-4.41 (m, 2H, H-5'), 4.14-4.17 (m, 0.5H, H-4'), 4.09-4.17 (m, 1H, H-4', H-2'), 4.07 (d, *J* = 6.4 Hz, 0.5H, H-2'), 3.93-4.02 (m, 2H, $OC\boldsymbol{H}_2(CH_2)_4CH_3$), 3.82-3.90 (m, 1H, $C\boldsymbol{H}CH_3$), 3.07 (s, 0.5H, CH≡C-C3'), 3.06 (s, 0.5H, CH≡C-C3'), 1.47-1.53(m, 2H, $OCH_2C\boldsymbol{H}_2(CH_2)_3CH_3$), 1.23-1.27 (m, 3H, $CHC\boldsymbol{H}_3$), 1.16-1.22 (m, 6H, $OCH_2CH_2(C\boldsymbol{H}_2)_3CH_3$), 0.77-0.80 ($OCH_2CH_2(CH_2)_3C\boldsymbol{H}_3$).

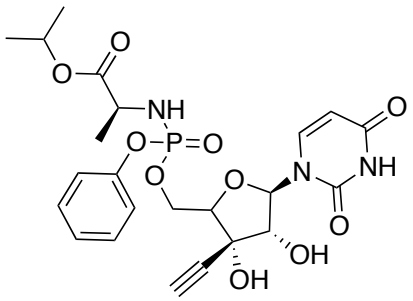
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.66 (d $^3J_{CP}$ = 4.6 Hz, $\boldsymbol{C}OOCH_2(CH_2)_4CH_3$), 173.49 (d $^3J_{CP}$ = 5.6 Hz, $\boldsymbol{C}OOCH_2(CH_2)_4CH_3$), 164.51 (C=O, C-4), 151.09, 151.01 (C=O, C-2), 150.72 (d $^2J_{CP}$ = 7.1 Hz, C-*ipso* Ph), 150.71 (d $^2J_{CP}$= 7.1 Hz, C-*ipso* Ph), 140.87, 140.79 (CH-6), 129.44, 129.40 (CH-Ar x 2), 124.86, 124.80 (CH-Ar), 120.08 (d, $^3J_{CP}$ = 4.6, CH Ar x 2), 119.98 (d, $^3J_{CP}$ = 4.9, CH Ar x 2), 101.82, 101.64 (CH-5), 88.07, 87.56 (CH-1'), 84.30 (d ^3J = 8.9 Hz, C4'), 84.07 (d ^3J = 8.4 Hz, C4'), 80.78, 80.66 (CH≡$\boldsymbol{C}$-C3'), 78.68, 78.61 (CH-2'), 76.75, 76.74 ($\boldsymbol{C}$H≡C-C3'), 72.36, 72.12 (CH-3'), 66.61 (d $^2J_{CP}$= 5.3 Hz,

CH_2-5'), 66.48 (d $^2J_{CP}$= 5.2 Hz, CH_2-5'), 65.09, 65.06 (COO***C***$H_2(CH_2)_4CH_3$), 50.32, 50.15 (***C***HCH_3), 31.18, 31.17, 28.26, 28.24, 25.22, 22.18 ($COOCH_2$(***CH$_2$***)$_4CH_3$), 19.20 (d $^3J_{CP}$ = 6.0 Hz, CH***C***H_3), 19.07 (d $^3J_{CP}$ = 7.1 Hz, CH***C***H_3), 13.01, 12.98 ($COO(CH_2)_5$***C***H_3),

MS (ES+) m/z: Found: 580.2 [M + H^+], 602.2 [M + Na^+] $C_{26}H_{34}N_3O_{10}P$ required: 579.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 16.56 min.

3'-Ethynyluridine-5'-O-[1-phenyl(isopropyloxy-L-alaninyl)] phosphate (5.22)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.1** (143 mg, 0.53 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and naphtyl(isopropyloxy-L-alaninyl) phosphorochloridate **2.17** (486.04 mg, 1.59 mmol). The reaction was stirring for 20 h. Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 from 0:100 to 10:90) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded 28 mg of yellow oil containing the titled compound that requires further purification.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.63, 3.41.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.67 (d, *J* = 8.1 Hz, 0.5H H-6), 7.63 (d, *J* = 8.1 Hz, 0.5H H-6), 7.22-7.29 (m, 2H, Ar), 7.06-7.17 (m, 3H, Ar), 5.86 (d, *J* = 6.4 Hz, 0.5H, H-1'), 5.82 (d, *J* = 5.9 Hz, 0.5H, H-1'), 5.58 (d, *J* = 8.1 Hz, 0.5H H-5), 5.56 (d, *J* = 8.1 Hz, 0.5H H-5), 4.84-4.89 (m, 1H, OC***H*** $(CH_3)_2$), 4.27-4.41 (m, 2H, H-5'), 4.14-4.16 (m, 0.5H, H-4'), 4.09-4.12 (m, 1H, H-4', H-2'), 4.07 (d, *J* = 6.4 Hz, 0.5H, H-2'), 3.79-3.84 (m, 1H, C***H***CH_3), 3.09 (s, 0.5H, CH≡C-C3'), 3.08 (s, 0.5H, CH≡C-C3'), 1.22-1.25 (m, 3H, CHC***H***$_3$), 1.11-1.13 ($OCH(C\mathbf{H}_3)_2$).

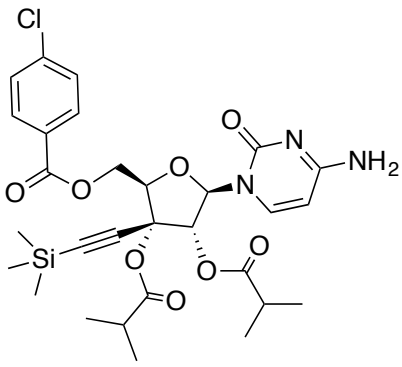
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.16 (d $^3J_{CP}$ = 5.0 Hz, ***C***OOCH$(CH_3)_2$), 172.97 (d $^3J_{CP}$ = 6.0 Hz, ***C***OOCH$(CH_3)_2$), 164.53 (C=O, C-4), 151.12, 151.05 (C=O, C-2), 150.74 (d $^2J_{CP}$ = 1.9 Hz, C-*ipso* Ph), 150.69 (d $^2J_{CP}$= 2.5 Hz, C-*ipso* Ph), 140.87, 140.80 (CH-6), 129.49, 129.43 (CH-Ar x 2), 124.89, 124.83 (CH-Ar), 120.10 (d, $^3J_{CP}$ = 4.7, CH Ar x 2), 120.01 (d, $^3J_{CP}$ = 5.1, CH Ar x 2), 101.88, 101.72 (CH-5), 88.01, 87.58 (CH-1'), 84.28 (d ^3J = 8.9 Hz, C4'), 84.08 (d ^3J = 8.2 Hz, C4'), 80.84, 80.70 (CH≡***C***-C3'), 78.65, 78.61 (CH-2'), 76.82, 76.74 (***C***H≡C-C3'), 72.38, 72.18 (C-3'), 69.82, 68.83 (COO***C***H$(CH_3)_2$), 66.33 (d $^2J_{CP}$= 5.3 Hz, CH_2-5'), 66.51 (d $^2J_{CP}$= 5.0 Hz, CH_2-5'), 50.41, 50.25 (***C***HCH_3), 20.63, 20.56 (COO***C***H$(CH_3)_2$), 19.26 (d $^3J_{CP}$ = 5.8 Hz, CH***C***H_3), 19.12 (d $^3J_{CP}$ = 7.1 Hz, CH***C***H_3), (COO(***CH₃***)$_2$).

MS (ES+) m/z: Found: 538.2 [M + H^+], 560.2 [M + Na^+] $C_{23}H_{38}N_3O_{10}P$ required: 537.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 12.28 min.

Synthesis of 3'-ethynylcytosine

1-[5-*O*-(4-chlorobenzoyl)-2,3,di-*O*-isobutyryl-3-*C*-2-ethynyl-β-*D*-*ribo*-pentofuranosyl]cytosine (5.15)



A mixture of cytosine (2.955 g, 26.6 mmol) and $(NH_4)_2SO_4$ (263 mg, 1.99 mmol) was suspended in 42 ml (120 mmol) ml of HMDS and solution was warmed at reflux temperature (120 ˚C) in anhydrous conditions and under argon atmosphere for 2 h. Solvent was evaporated. To this mixture compound **5.13** (7.91 g, 13.30 mmol) was

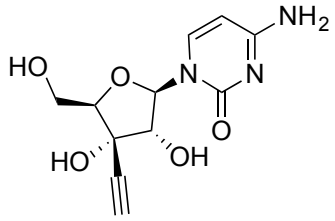
added dissolved in 20 ml of ACN. $SnCl_4$ (4.7 ml, 40 mmol) was added to the stirring mixture at 0 ˚C. Next the temperature was raised at 30 ˚C and the reaction was kept for 24 h. Solvent was evaporated and the residue was partitioned between toluene and HCl 3M. The organic layer was washed with saturated $NaHCO_3$, dried over Na_2SO_2 and evaporated. Purification by column chromatography (eluent system CH_2Cl_2/ MeOH from 98:2 to 90:10) afforded 3.71 g (45 %) of the desired product as an oil.

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.01 (d, *J* = 7.8 Hz, 1H H-6), 7.96 (d, *J* = 8.5 Hz, 2H H-Ar), 7.44 (d, *J* = 8.5 Hz, 2H H-Ar), 6.05 (d, *J* = 7.8 Hz, 1H, H-5), 5.99 (d, *J* = 4.7 Hz, 1H, H-1'), 5.71 (d, *J* = 4.8 Hz, 1H, H-2'), 4.72-4.79 (m, 2H, H-5', H-4'), 4.66 (dd, *J* = 3.3, 11.8 Hz, 1H H-5'), 2.54-2.59 (m, 2H, OCOC***H***$(CH_3)_2$ x 2) 1.08-1.11 (m, 12H, OCOCH(C***H***$_3)_2$ x2), 0.00 (s, 9H, $(CH_3)_3$SiC ≡C-C3').

^{13}C NMR (126 MHz, CD_3OD): δ_C 175.19, 174.31 (C=O O***C***OCH$(CH_3)_2$ x 2) 165.04 (C=O, C-4), 160.19 (C=O, C-4ClBzO), 147.60 (C=O, C-2), 143.44 (CH-6), 139.66 (C, 4ClBzO), 130.96 (CH x 2, 4ClBzO), 128.71 (CH x 2, 4ClBzO), 128.13 (C, 4ClBzO), 96.79 (C, $(CH_3)_3$SiC≡***C***-C3'), 96.32 (C, $(CH_3)_3$Si***C***≡C-C3'), 94.44 (CH-5), 87.83 (CH-1'), 81.69 (CH-4'), 77.78 (CH-2'), 75.78 (C-3'), 63.59 (CH_2-5'), 33.79, 33.65 (CH, OCO***C***H$(CH_3)_2$ x 2), 17.87, 17.71, 17.67, 17.51 (CH_3, OCOCH(***C***H$_3)_2$ x 2), -2.02 (CH_3, (***C***H$_3)_3$SiC≡C-C3').

MS (ES+) m/z: Found: 618.2 [M + H^+], 640.2 [M + Na^+] $C_{29}H_{36}ClN_3O_8Si$ required: 617.2 [M].

3'-Ethynylcytosine (5.2)



To a solution of compound **5.15** (493 mg, 0.80 mmol) in anhydrous MeOH (10 ml), DBU (0.48 ml, 3.19 mmol) was added in three portions. The mixture was stirring for 3 h at 40 ˚C. 0.47 ml of AcOH glacial were added, then, solvent was evaporated.

Purification by crystallisation in Toluene/MeOH afforded 90 mg (42 %) of the desired product.

^{1}H NMR (500 MHz, CD_3OH): δ_H = 7.88 (d, *J* = 7.5 Hz, 1H H-6), 5.81-5.84 (m, 2H, H-5, H-1'), 4.19 (d, *J* = 5.6 Hz, 1H, H-2'), 3.97 (dd, *J* = 2.8, 5.1 Hz, 1H, H-4'), 3.85 (dd, *J* = 5.1 , 12.1 Hz, H-5'), 3.76 (dd, *J* = 2.8, 12.1 Hz, H-5'), 2.98 (s, 1H, CH≡C-C3').

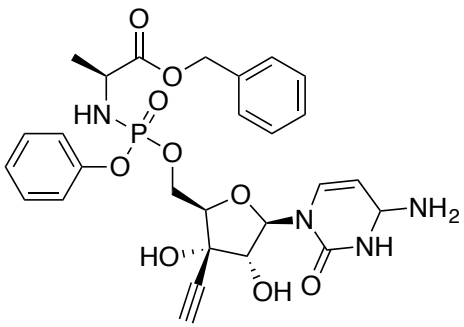
^{13}C NMR (126 MHz, CD_3OD): δ_C = 166.24 (C-NH_2, C-4), 157.24 (C=O, C-2), 142.06 (CH-6), 94.66 (CH-5), 89.70 (CH-1'), 86.40 (CH-4'), 81.39 (CH≡***C***-C3'), 79.47 (CH-2'), 75.96 (***C***H≡C-C3'), 72.70 (C-3'), 61.78 (CH_2-5').

MS (ES+) m/z: Found: 268.1 [M + H^+], 290.1 [M + Na^+] $C_{11}H_{13}N_3O_5$ required: 267.1 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 270 nm, $\mathbf{t_R}$ 2.9 min.

Synthesis of 3'-ethynylcytidine ProTides

3'-Ethynylcytidine-5'-O-[1-phenyl (benzyloxy-L-alaninyl)] phosphate (5.23)



Compound **5.23** was prepared according to the general procedure A using 3'ethynylcytidine **5.2** (383 mg, 1.43 mmol), N-methylimidazole (0.57 mL, 7.15 mmol) and phenyl(benzoxy-L-alaninyl) phosphorochloridate **2.13** (1.01 g, 2.86 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 3:97 to 18:82 over 10 CV) and two preparative TLC (2000 μm,

eluent system CH_3OH/CH_2Cl_2 10:90 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 7:93) afforded the title compound as a white solid (10 mg, 1 %).

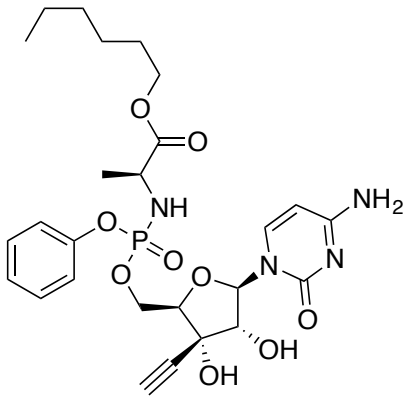
^{31}P NMR (202 MHz, CD_3OD): δ_P 3.27, 3.42.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.87 (d, *J* = 7.7 Hz, 0.5H H-6), 7.80 (d, *J* = 7.5 Hz, 0.5H H-6), 7.33-7.41 (m, 10H, Ar), 6.11 (d, *J* = 4.9 Hz, 0.5H, H-1'), 6.05 (d, *J* = 4.2 Hz, 0.5H, H-1'), 5.91 (d, *J* = 7.6 Hz, 0.5H H-5), 5.85 (d, *J* = 7.5 Hz, 0.5H H-5), 5.02-5.07 (m, 2H, OCH_2Ph) 4.27-4.48 (m, 2H, H-5'), 4.05-4.26 (m, 2H, H-2'), 4.3-4.05 (m, 1H, H-2'), 3.89-4.00 (m, 1H, C***H***CH_3), 3.07 (s, 0.5H, CH≡C-C3'), 3.06 (s, 0.5H, CH≡C-C3'), 1.25-1.29 (m, 3H, CHC***H***$_3$).

MS (ES+) m/z: Found: 609.2 [M + Na^+] $C_{27}H_{31}N_4O_9P$ required: 586.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 240 nm, $\mathbf{t}_R$ 15.71 min.

3'-Ethynylcytidine-5'-O-[1-phenyl(hexyloxy-L-alaninyl)] phosphate (5.24)



Compound **5.24** was prepared according to the general procedure A using 3'ethynylcytidine **5.2** (184 mg, 0.69 mmol), N-methylimidazole (0.27 mL, 3.49 mmol) and benzyl(hexyloxy-L-alaninyl) phosphorochloridate **2.15** (517 mg, 1.38 mmol). The reaction was stirring for 20 h. Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 from 3:97 to 10:90) and preparative TLC (1000 μm, eluent system CH_3OH/CH_2Cl_2 5:95 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 7:93) afforded the titled compound as a white solid (14 mg, 3 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.72, 3.52

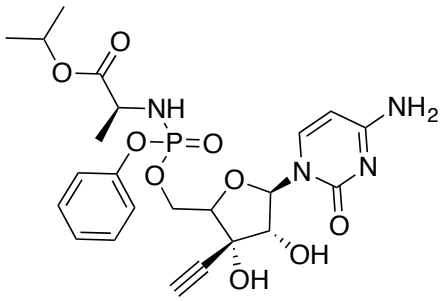
^{1}H NMR (500 MHz, CD_3OD): δ_H 7.85 (d, *J* = 7.2 Hz, 0.5H H-6), 7.81 (d, *J* = 7.0 Hz, 0.5H H-6), 7.03-7.26 (m, 5H, Ar), 6.07-6.16 (m, 1H, H-5), 5.78-5.87 (m, 1H, H-1'), 4.30-4.51 (m, 2H, H-5'), 4.13-4.22 (m, 2H, H-4', H-2'), 3.88-4.02 (m, 2H, $OCH_2(CH_2)_4CH_3$), 3.83-3.88 (m, 1H, $CHCH_3$), 3.07 (s, 0.5H, CH≡C-C3'), 3.06 (s, 0.5H, CH≡C-C3'), 1.41-1.53(m, 2H, $OCH_2CH_2(CH_2)_3CH_3$), 1.25-1.31 (m, 3H, $CHCH_3$), 1.14-1.24 (m, 6H, $OCH_2CH_2(CH_2)_3CH_3$), 0.77-0.82 ($OCH_2CH_2(CH_2)_3CH_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.88 (d $^{3}J_{CP}$ = 4.5 Hz, $COOCH_2(CH_2)_4CH_3$), 173.49 (d $^{3}J_{CP}$ = 5.7 Hz, $COOCH_2(CH_2)_4CH_3$), 165.96 (C=O, C-4), 157.24, 157.22 (C=O, C-2), 150.73 (d $^{2}J_{CP}$ = 6.2 Hz, C-*ipso* Ph), 150.68 (d $^{2}J_{CP}$= 6.2 Hz, C-*ipso* Ph), 142.06, 141.98 (CH-6), 129.45, 129.40 (CH-Ar x 2), 124.87, 124.80 (CH-Ar), 120.08 (d, $^{3}J_{CP}$ = 4.7, CH Ar), 120.04 (d, $^{3}J_{CP}$ = 4.7, CH Ar), 97.30, 97.28 (CH-5), 89.76, 89.18 (CH-1'), 84.15 (d ^{3}J = 9.1 Hz, C4'), 83.97 (d ^{3}J = 8.2 Hz, C4'), 80.72, 80.61 (CH≡***C***-C3'), 79.58, 79.39 (CH-2'), 76.75, 76.74 (***C***H≡C-C3'), 72.21, 72.06 (CH-3'), 66.52 (d $^{2}J_{CP}$= 5.3 Hz, CH_2-5'), 66.48 (d $^{2}J_{CP}$= 5.2 Hz, CH_2-5'), 64.95, 64.92 ($COO\boldsymbol{C}H_2(CH_2)_4CH_3$), 50.16, 50.10 (***C***$HCH_3$), 31.18, 31.17, 28.26, 28.24, 25.23, 22.20 ($COOCH_2(\boldsymbol{CH_2})_4CH_3$), 19.21 (d $^{3}J_{CP}$ = 6.0 Hz, $CH\boldsymbol{C}H_3$), 19.11 (d $^{3}J_{CP}$ = 7.4 Hz, $CH\boldsymbol{C}H_3$), 12.96, 12.93 ($COO(CH_2)_5\boldsymbol{C}H_3$).

MS (ES+) m/z: Found: 579.2 [M + H^+], 601.2 [M + Na^+] $C_{26}H_{35}N_4O_9P$ required: 578.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 240 nm, $\mathbf{t}_R$ 12.37 min.

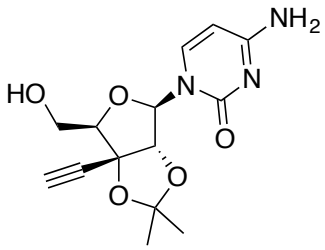
3'-Ethynylcytidine-5'-O-[1-phenyl(isopropyloxy-L-alaninyl)] phosphate (5.25)



Compound was prepared according to the general procedure A using 3'ethynylcytosine **5.2** (90 mg, 0.34 mmol), N-methylimidazole (0.13 mL, 1.7 mmol) and naphtyl(isopropyloxy-L-alaninyl) phosphorochloridate **2.17** (311 mg, 1.02 mmol). The reaction was stirring for 20 h. Then solvent was evaporated and reaction monitored by ^{31}PNMR and TLC. No formation of the product was observed.

Alternative route for the synthesis of 3'-ethynylcytidine ProTides

2',3'-*O*-Isopropylidene-3'-ethynylcytidine (5.26)

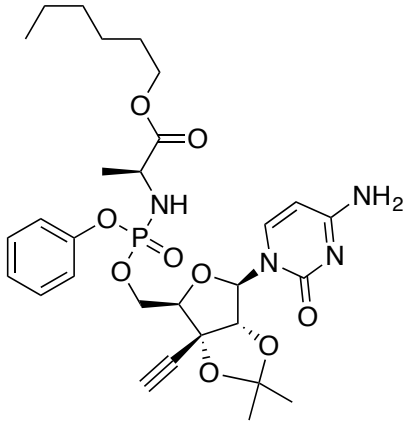


Compound **5.2** (500 mg, 1.87 mmol) was dissolved in dry acetone (30 ml). The temperature of the stirring mixture was lowered to 0 ˚C and concentrated solution of H_2SO_4 (0.2 ml) was added dropwise. The reaction was stirring overnight at room temperature. Then solvent was removed under reduced pressure. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 7:93 to 18:82 over 10 CV) afforded compound **5.26** as a white solid. (148 mg, 26 %)

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.81 (d, *J* = 7.5 Hz, 1H H-6), 6.07 (d, *J* = 2.2, 1H, H-1'), 6.03 (d, *J* = 7.5 1H, H-5), 4.83 (d, *J* = 2.2 Hz, 1H, H-2'), 4.15 (dd, *J* = 3.8, 7.1 Hz, 1H, H-4'), 3.85-3.95 (m, HH-5'), 2.93 (s, 1H, CH≡C-C3'), 1.59 (s, 3H, $(CH_3)_2CO_2$), 1.51 (s, 3H, $(CH_3)_2CO_2$).

MS (ES+) m/z: Found: 330.1 [M + Na^+] $C_{25}H_{32}N_3O_{10}P$ required: 307.1 [M].

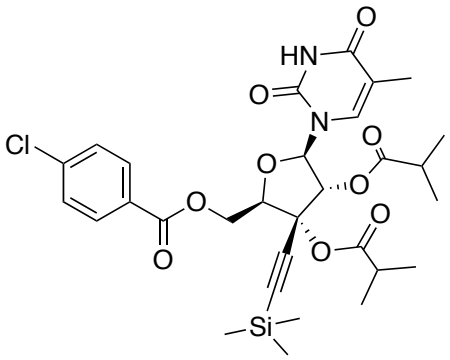
2',3'-*O*-Isopropylidene-3'-ethynylcytidine-5'-O-[1-phenyl(hexyloxy-L-alaninyl)] phosphate (5.27)



Compound **5.27** was prepared according to the general procedure B compound **5.26** (148 mg, 0.48 mmol), *tert*-butyl magnesium chloride 1M (0.48 ml, 0.48 mmol) and Phenyl(hexyloxy-L-alaninyl) phosphorochloridate **2.15** (327 mg 0.96 mmol). Then solvent was evaporated and reaction monitored by ^{31}PNMR, TLC and MS. No formation of the product was observed.

Synthesis of 3'-ethynyl-5-methyluridine

1-[5-*O*-(4-chlorobenzoyl)-2,3,di-*O*-isobutyryl-3-*C*-2-ethynyl-β-*D*-*ribo*-pentofuranosyl]-5-methyl-uridine (5.16)



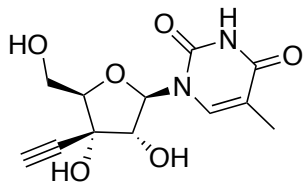
A mixture of thymine (445 mg, 3.52 mmol) and $(NH_4)_2SO_4$ (48 mg, 0.3 mmol) was suspended in 9 (43 mmol) ml of HMDS and solution was warmed at reflux temperature (120 ˚C) in anhydrous conditions and under argon atmosphere for 2.5 h. Solvent was evaporated. To this mixture compound **5.13** (1.04 g, 1.7 mmol) was added dissolved in 5 ml of ACN. $SnCl_4$ (0.62 ml, 5.28 mmol) was added to the stirring mixture at 0 ˚C. Next the temperature was raised at 30 ˚C and the reaction was kept for 24 h. Solvent was evaporated and the residue was partitioned between toluene and HCl 3M. The organic layer was washed with saturated $NaHCO_3$, dried over $MgSO_2$ and evaporated. Purification by flash chromatography (eluent system Hexane/ AcOEt from 95:5 to 60:40 over 10 CV) afforded 532 mg (48 %) of the desired product as an oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 9.54 (br s, 1H, NH), 7.91 (d, *J* = 8.6 Hz, 2H H-Ar), 7.32 (d, *J* = 8.6 Hz, 2H H-Ar), 7.26 (d, *J* = 1.1 Hz, 1H H-6), 6.03 (d, *J* = 5.4 Hz, 1H, H-1'), 5.53 (d, *J* = 5.4 Hz, 1H, H-2'), 4.64 (dd, *J* = 3.2, 12.3 Hz, 1H H-5'), 4.59 (dd, *J* =5.6, 12.3 Hz, 1H H-5'), 4.42 (dd, *J* =3.1, 5.5 Hz, 1H, H-4'), 2.43-2.54 (m, 2H, OCOC***H***$(CH_3)_2$ x 2), 1.62 (d, *J* = 0.9, 3H, CH_3-C-5) 1.08 (d, *J* = 2.9, 3H, OCOCH(C***H***$_3)_2$), 1.07 (d, *J* = 2.9, 3H, OCOCH(C***H***$_3)_2$), 1.04 (d, *J* = 2.5, 3H, OCOCH(C***H***$_3)_2$), 1.03 (d, *J* = 2.5, 3H, OCOCH(C***H***$_3)_2$), 0.00 (s, 9H, $(CH_3)_3$SiC ≡C-C3').

^{13}C NMR (126 MHz, $CDCl_3$): δ_C 175.64, 174.76 (C=O O***C***OCH$(CH_3)_2$ x 2) 165.77 (C=O, C-4), 164.15 (C=O, C-4ClBzO), 151.14 (C=O, C-2), 140.59 (C, 4ClBzO), 135.18 (CH-6) 131.65, (CH x 2, 4ClBzO), 129.57 (CH x 2, 4ClBzO), 128.42 (C, 4ClBzO), 112.79 ($(CH_3)_3$SiC≡***C***-C3'), 97.78 (C-5), 96.59 (C, $(CH_3)_3$Si***C***≡C-C3'), 87.72 (CH-1'), 80.90 (CH-4'), 77.44 (CH-2'), 75.96 (C-3'), 64.52 (CH_2-5'), 34.43, 34.28 (CH, OCO***C***H$(CH_3)_2$ x 2), 19.34, 19.24, 19.23, 19.10, (CH_3, OCOCH(***C***$H_3)_2$ x 2), 12.95 (***C***H_3-C-5) 0.00 (CH_3, (***C***$H_3)_3$SiC≡C-C3').

MS (ES+) m/z: Found: 633.2 [M + H^+], 655.2 [M + Na^+] $C_{30}H_{37}ClN_2O_9Si$ required: 632.2 [M].

3'-Ethynyl-5-methyluridine (5.3)



To a solution of compound **5.16** (532 mg, 0.84 mmol) in anhydrous MeOH (8 ml), DBU (0.50 ml, 3.36 mmol) was added in three portions. The mixture was stirring for 2.5 h at 40 ˚C. Then, 0.6 ml of AcOH glacial were added, and solvent was evaporated. Purification by CC (eluent system CH_2Cl_2/MeOH from 100:0 to 81:19) afforded 742 mg (89 %) of the nucleoside analogue as a white precipitate.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.84 (s, 1H, H-6), 5.90 (d, *J* = 6.8 Hz, 1H, H-1'), 4.26 (d, *J* = 6.8 Hz, 1H, H-2'), 3.95 (t, *J* = 3.8 Hz, 1H, H-4'), 3.88 (dd, *J* = 4.1, 12.0 Hz, H-5'), 3.77 (dd, *J* = 2.8, 12.0 Hz, H-5'), 3.03 (s, 1H, CH≡C-C3'), 1.81 (s, 3H, CH_3-C-5).

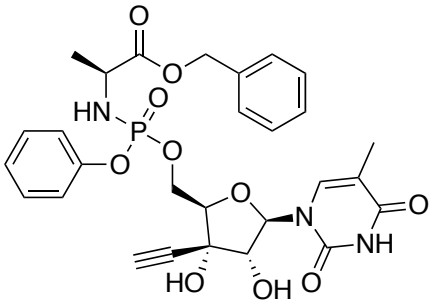
^{13}C NMR (126 MHz, CD_3OD): δ_C 164.91 (C=O, C-4), 151.48 (C=O, C-2), 137.17 (CH-6), 110.32 (C-5), 87.52 (CH≡***C***-C-3'), 89.30 (CH-1'), 88.85 (CH-4'), 81.37 (C-3'), 78.58 (CH-2'), 75.59 (***C***H≡C-C3'), 61.70 (CH_2-5'), 11.02 (***C***H_3-C-5).

MS (ES+) m/z: Found: 283.1 [M + H^+], 305.1 [M + Na^+] $C_{12}H_{14}N_2O_6$ required: 282.1 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 208 nm, t_R 6.82 min

Synthesis of 3'-ethynyl-5-methyluridine ProTides

3'-Ethynyl-5-methyluridine-5'-O-[1-phenyl (benzyloxy-L-alaninyl)] phosphate (5.28)



Compound was prepared according to the general procedure A using 3'ethynyluridine **5.3** (211 mg, 0.37 mmol), N-methylimidazole (0.30 mL, 3.75 mmol) and phenyl(benzoxy-L-alaninyl) phosphorochloridate **2.13** (508 mg, 1.50 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 1:99 to 8:92 over 10 CV) and two preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 15:95 afforded the title compound as a white solid (40 mg, 9 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.60, 3.36.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.85 (s, 0.5H H-6), 7.81 (s, 0.5H H-6), 7.14-7.48 (m, 10H, Ar), 6.03 (d, *J* = 6.9 Hz, 0.5H, H-1'), 5.98 (d, *J* = 6.4 Hz, 0.5H, H-1'), 5.11-5.18 (m, 2H, OC*H*$_2$Ph) 4.38-4.50 (m, 2H, H-5'), 4.20-4.26 (m, 2H, H-4', H-2'), 4.01-4.08 (m, 1H, C***H***CH_3), 3.17 (s, 0.5H, CH≡C-C3'), 3.19 (s, 0.5H, CH≡C-C3'), 1.25-1.29 (m, 3H, CHC*H*$_3$), 1.84 (s, 1.5H, CH_3-C-5), 1.82 (s, 1.5H, CH_3-C-5).

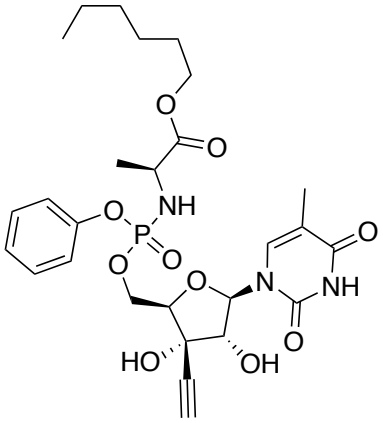
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.30 (d $^3J_{CP}$ = 4.6 Hz, C=O Bz), 173.19 (d $^3J_{CP}$ = 5.3 Hz, C=O Bz), 164.79 (C=O, C-4), 151.48, 151.45 (C=O, C-2), 151.35 (d $^2J_{CP}$ = 7.4 Hz, C-*ipso* Ph), 151.63 (d $^2J_{CP}$= 7.3 Hz, C-*ipso* Ph), 136.35, 136.17 (CH-6), 135.80, 135.78 (C-*ipso* Bz), 129.45, 129.37, 128.23, 128.21, 128.00, 127.96, 127.84 (CH-Ar x 7), 124.87, 124.81 (CH-Ar), 120.08 (d, $^3J_{CP}$ = 4.4, CH Ph), 120.05 (d, $^3J_{CP}$ = 4.5, CH Ph), 110.78, 110.63 (C-5), 87.47, 86.90 (CH-1'), 84.25 (d $^3J_{CP}$ = 8.9 Hz, C4'), 84.02 (d $^3J_{CP}$ = 8.4 Hz,

C4'), 80.93, 80.79 (CH≡***C***-C3'), 78.38, 78.33 (CH-2'), 72.43, 72.19 (***C***H≡C-C3'), 72.37, 72.11 (C-3'), 66.61, 66.62, 66.59, 66.56 (CH_2-5' *C*H_2Ph), 50.29, 50.16 (***C***HCH_3), 19.12 (d $^3J_{CP}$ = 6.2 Hz, CH***C***H_3), 19.07 (d $^3J_{CP}$ = 7.0 Hz, CH***C***H_3), 11.02 (***C***H_3-C-5).

MS (ES+) m/z: Found: 600.2 [M + H^+], 622.2 [M + Na^+] $C_{28}H_{30}N_3O_{10}P$ required: 599.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 17.33 min.

3'-Ethynyl-5-methyluridine-5'-O-[1-phenyl(hexyloxy-L-alaninyl)] phosphate (5.29)



Compound **5.29** was prepared according to the general procedure A using 3'ethynyl-5-methyluridine **5.3** (192 mg, 0.79 mmol), N-methylimidazole (0.32 mL, 3.95 mmol) and naphthyl(hexyloxy-L-alaninyl) phosphorochloridate **2.15** (549 mg, 1.58 mmol). The reaction was stirring for 20 h. Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 from 0:100 to 10:90 over 20 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 6:94 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded the titled compound as a white solid (38 mg, 9 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.00, 3.76.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.46 (m, 1H H-6), 7.23-7.26 (m, 2H, Ar), 7.07-7.14 (m, 3H, Ar), 6.14-6.20 (m, 1H, H-1'), 4.28-4.36 (m, 2H, H-5'), 4.14-4.21 (m, 1H, H-4'), 3.93-4.02 (m, 3H, H-2', OC***H***$_2(CH_2)_4CH_3$), 3.81-3.87 (m, 1H, C***H***CH_3), 3.07 (s, 0.5H, CH≡C-C3'), 3.06 (s, 0.5H, CH≡C-C3'), 2.10-2.17 (m, 2H, OCH_2C***H***$_2(CH_2)_3CH_3$), 1.73-1.74 (m,

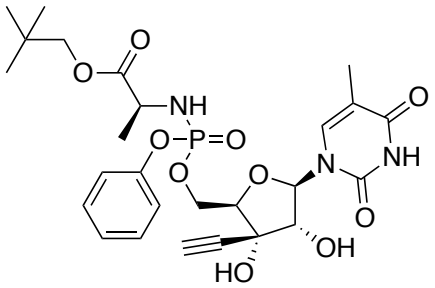
3H, CH_3-C-5), 1.47-1.53 (m, 2H, $OCH_2CH_2(C\boldsymbol{H}_2)_3CH_3$), 47-1.53 (m, 7H, $CHC\boldsymbol{H}_3$ $OCH_2CH_2(C\boldsymbol{H}_2)_3CH_3$), 0.77-0.80 (m, 3H, $OCH_2CH_2(CH_2)_3C\boldsymbol{H}_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.76 (d $^3J_{CP}$ = 3.8 Hz, $\boldsymbol{C}OOCH_2(CH_2)_4CH_3$), 173.51 (d $^3J_{CP}$ = 5.6 Hz, $\boldsymbol{C}OOCH_2(CH_2)_4CH_3$), 164.92 (C=O, C-4), 151.05, 151.01 (C=O, C-2), 150.88 (d $^2J_{CP}$ = 6.8 Hz, C-*ipso* Ph), 150.73 (d $^2J_{CP}$= 7.2 Hz, C-*ipso* Ph), 136.21, 136.15 (CH-6), 129.44, 129.43 (CH-Ar x 2), 124.90, 124.88 (CH-Ar), 120.04 (d, $^3J_{CP}$ = 4.4, CH Ar x 2), 120.03 (d, $^3J_{CP}$ = 4.4, CH Ar x 2), 110.66, 110.43 (C-5), 85.15 (d ^3J = 8.3 Hz, C4'), 85.04 (d ^3J = 7.0 Hz, C4'), 85.07, 84.98 (CH-1'), 80.78, 80.66 (CH≡$\boldsymbol{C}$-C3'), 78.68, 78.61 (CH-2'), 76.75, 76.74 ($\boldsymbol{C}$H≡C-C3'), 70.82, 70.78 (CH-3'), 66.37 (d $^2J_{CP}$= 5.5 Hz, CH_2-5'), 66.28 (d $^2J_{CP}$= 5.5 Hz, CH_2-5'), 65.09, 65.07 ($COO\boldsymbol{C}H_2(CH_2)_4CH_3$), 50.36, 50.20 ($\boldsymbol{C}HCH_3$), 31.18, 31.17, 28.27, 28.24, 25.22, 22.18 ($COOCH_2(\boldsymbol{CH_2})_4CH_3$), 19.16 (d $^3J_{CP}$ = 6.7 Hz, $CH\boldsymbol{C}H_3$), 19.03 (d $^3J_{CP}$ = 7.3 Hz, $CH\boldsymbol{C}H_3$), 12.94 ($COO(CH_2)_5\boldsymbol{C}H_3$), 11.23, 11.15 ($\boldsymbol{C}H_3$-C-5).

MS (ES+) m/z: Found: 594.2 [M + H^+], 616.2 [M + Na^+] $C_{25}H_{32}N_3O_{10}P$ required: 593.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 16.63 min.

3'-Ethynyl-5-methyluridine-5'-O-[1-phenyl (2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.30)



Compound was prepared according to the general procedure A using 3'ethynyl-5-methyluridine **5.3** (3.45 mg, 1.22 mmol), N-methylimidazole (0.51 mL, 6.45 mmol) and phenyl(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **2.12** (529 mg, 6.45

mmol). The reaction was stirring for 20 h. Purification by flash column chromatography (eluent system CH_3OH/CH_2Cl_2 2:98 to 16:84 over 10 CV) and two preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 8:92 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded the title compound as a white solid (58 mg, 9 %).

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.63, 3.32.

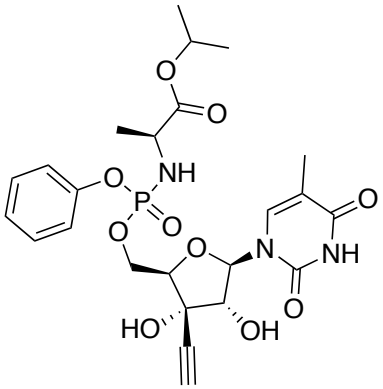
^{1}H NMR (500 MHz, CD_3OD): δ_H 7.85 (s, 0.5H H-6), 7.83 (s, 0.5H H-6), 7.34-7.38 (m, 2H, Ar), 7.18-7.28 (m, 3H, Ar), 6.03 (d, *J* = 6.8 Hz, 0.5H, H-1'), 5.99 (d, *J* = 6.2 Hz, 0.5H, H-1'), 4.40-4.55 (m, 2H, H-5'), 4.21-4.28 (m, 2H, H-4', H-2'), 4.00-4.06 (m, 1H, C***H***CH_3), 3.85-3.87 (m, 1H, OC***H***$_2C(CH_3)_3$), 3.74-3.78 (m, 1H, OC***H***$_2C(CH_3)_3$), 3.22 (s, 0.5H, CH≡C-C3'), 3.23 (s, 0.5H, CH≡C-C3'), 1.84 (s, 1.5H, CH_3-C-5), 1.82 (s, 1.5H, CH_3-C-5), 1.39-1.41 (m, 3H, CHC*H*$_3$), 0.95, (4.5H, OC***H***$_2C(CH_3)_3$), 0.94 (4.5H, OC***H***$_2C(CH_3)_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.60 (d $^3J_{CP}$ = 4.8 Hz, ***C***$OOCH_2C(CH_3)_3$), 173.43 (d $^3J_{CP}$ = 5.6 Hz, ***C***$OOCH_2C(CH_3)_3$), 164.54 (C=O, C-4), 151.11, 151.02 (C=O, C-2), 150.73 (d $^2J_{CP}$ = 6.3 Hz, C-*ipso* Ph), 150.70 (d $^2J_{CP}$= 6.1 Hz, C-*ipso* Ph), 137.17 (CH-6), 129.46, 129.40 (CH-Ar x 2), 124.87, 124.81 (CH-Ar), 120.08 (d, $^3J_{CP}$ = 5.0, CH Ph x 2), 120.01 (d, $^3J_{CP}$ = 4.9, CH Ph x 2), 110.32 (C-5), 88.09, 87.62 (CH-1'), 88.85 (d $^3J_{CP}$ = 8.9 Hz, C4'), 88.74 (d $^3J_{CP}$ = 8.2 Hz, C4'), 80.78, 80.65 (CH≡***C***-C3'), 78.69, 78.62 (CH-2'), 76.77, 76.51 (***C***H≡C-C3'), 74.05, 74.02 (COO*C*$H_2C(CH_3)_3$), 72.32, 72.08 (C-3'), 66.66 (d $^2J_{CP}$= 5.3 Hz, CH_2-5'), 66.54 (d $^2J_{CP}$= 5.1 Hz, CH_2-5'), 50.37, 50.19 (***C***HCH_3), 30.94, 30.93 ($COOCH_2$***C***$(CH_3)_3$), 25.34, ($COOCH_2C$(***C***$H_3)_3$), 19.37 (d $^3J_{CP}$ = 6.1 Hz, CH***C***H_3), 19.20 (d $^3J_{CP}$ = 7.0 Hz, CH***C***H_3), 11.04, 11.01 (***C***H_3-C-5).

MS (ES+) m/z: Found: 580.2 [M + H^+], 602.2 [M + Na^+] $C_{26}H_{34}N_3O_{10}P$ required: 579.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 265 nm, $\mathbf{t}_R$ 16.91 min.

3'-Ethynyl-5-methyluridine-5'-O-[1-phenyl(isopropyloxy-L-alaninyl)] phosphate (5.31)



Compound was prepared according to the general procedure A using 3'ethynyl-5-methyluridine **5.3** (240 mg, 0.85 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and phosphorochloridate **2.17** (486.04 mg, 1.59 mmol). The reaction was stirring for 20 h. Purification by column chromatography (eluent system CH_3OH/CH_2Cl_2 from 0:100 to 8:92) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 5:95 and 1000 μm, eluent system CH_3OH/CH_2Cl_2 3:97) afforded 71 mg (6 %) of the titled compound as a white solid

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.64, 3.35.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.85 (s, 0.5H H-6), 7.83 (s, 0.5H H-6), 7.33-7.41 (m, 2H, Ar), 7.19-7.30 (m, 3H, Ar), 5.86 (d, *J* = 6.4 Hz, 0.5H, H-1'), 6.04 (d, *J* = 6.0 Hz, 0.5H, H-1'), 4.47-4.55 (m, 1H, OC***H*** $(CH_3)_2$), 4.28-4.39 (m, 2H, H-5'), 4.19-4.25 (m, 2H, H-4', H-2'), 3.91-3.97 (m, 1H, C***H***CH_3), 3.10 (s, 0.5H, CH≡C-C3'), 3.08 (s, 0.5H, CH≡C-C3'), 1.86 (s, 1.5H, CH_3-C-5), 1.84 (s, 1.5H, CH_3-C-5), 1.33-1.37 (m, 3H, CHC***H***$_3$), 1.20-1.24 (OCH(C***H***$_3)_2$).

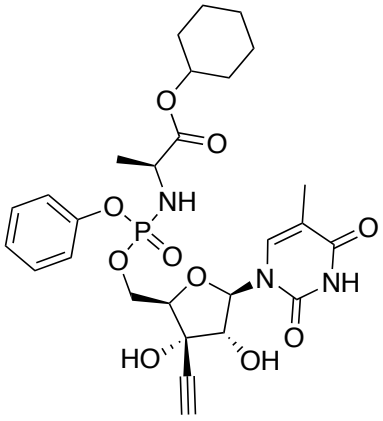
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.60 (d $^3J_{CP}$ = 5.5 Hz, ***C***$OOCH(CH_3)_2$), 173.42 (d $^3J_{CP}$ = 6.0 Hz, ***C***$OOCH(CH_3)_2$), 164.53 (C=O, C-4), 151.12, 151.05 (C=O, C-2), 150.74 (d $^2J_{CP}$ = 1.9 Hz, C-*ipso* Ph), 150.69 (d $^2J_{CP}$= 2.5 Hz, C-*ipso* Ph), 139.87, 139.80 (CH-6), 129.49, 129.43 (CH-Ar x 2), 124.89, 124.83 (CH-Ar), 120.10 (d, $^3J_{CP}$ = 4.7, CH Ar x 2), 120.01 (d, $^3J_{CP}$ = 5.1, CH Ar x 2), 110.72 (C-5), 88.01, 87.58 (CH-1'), 87.28 (d $^2J_{CP}$ = 8.7 Hz, C4'), 87.08 (d $^2J_{CP}$ = 8.2 Hz, C4'), 80.84, 80.70 (CH≡***C***-C3'), 78.65, 78.61 (CH-2'), 76.82,

76.74 (***C***H≡C-C3'), 72.38, 72.18 (C-3'), 69.82, 68.83 (COO***C***H(CH_3)$_2$), 66.33 (d $^3J_{CP}$= 5.3 Hz, CH_2-5'), 66.51 (d $^3J_{CP}$= 5.0 Hz, CH_2-5'), 50.41, 50.25 (***C***HCH_3), 20.63, 20.56 (COO***C***H(CH_3)$_2$), 19.37 (d $^3J_{CP}$ = 5.8 Hz, CH***C***H$_3$), 19.20 (d $^3J_{CP}$ = 7.1 Hz, CH***C***H$_3$), (COO(***CH$_3$***)$_2$), 11.23, 11.15 (***C***H$_3$-C-5).

MS (ES+) m/z: Found: 552.2 [M + H^+], 574.2 [M + Na^+] $C_{24}H_{30}N_3O_{10}P$ required: 551.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 265 nm, $\mathbf{t}_R$ 12.53 min.

3'-Ethynyl-5-methyluridine-5'-O-[1-phenyl(cuclohexyloxy-L-alaninyl)] phosphate (5.32)



Compound **5.32** was prepared according to the general procedure A using 3'-ethynyl-5-methyluridine **6.3** (200 mg, 0.83 mmol), N-methylimidazole (0.33 mL, 4.15 mmol) and phenyl(cyclohexyloxy-*L*-alaninyl) phosphorochloridate **2.16** (614.07 mg, 1.77 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2 0:100 to 10:90 over 20 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded of the titled product as a white solid 26 mg (6 %)

^{31}P NMR (202 MHz, CD_3OD): δ_P 4.04, 3.77.

^{1}H NMR (500 MHz, CD_3OD): δ_H 7.74 (s, 0.5H H-6), 7.71 (s, 0.5H H-6), 7.23-7.28 (m, 2H, Ar), 7.07-7.14 (m, 3H, Ar), 6.20 (d, *J* = 6.9 Hz, 0.5H, H-1'), 6.14 (d, *J* = 6.7 Hz, 0.5H, H-1'), 4.70-4.78 (m, 1H, OC***H***$(CH_2)_5$), 4.28-4.39 (m, 2H, H-5'), 4.19-4.25 (m, 2H, H-4', H-2'), 3.91-3.97 (m, 1H, C***H***CH_3), 3.10 (s, 0.5H, CH≡C-C3'), 3.08 (s, 0.5H, CH≡C-C3'), 1.84 (s, 1.5H, CH_3-C-5), 1.82 (s, 1.5H, CH_3-C-5), 1.78-1.84 (m, 2H, OCH(C***H***$_2)_5$), 1.68-1.75 (m, 2H, OCH(C***H***$_2)_5$), 1.50-1.57 (m, 1H, OCH(C***H***$_2)_5$), 1.35-1.39 (m, 3H, CHC***H***$_3$), 1.27-1.49 (m, 5H, OCH(C***H***$_2)_5$).

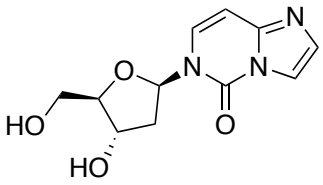
MS (ES+) m/z: Found 592.2 [M + H^+], 614.2 [M + Na^+], $C_{27}H_{34}N_3O_{10}P$ required 591.2 [M].

HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, λ = 261 nm, $\mathbf{t}_R$ 16.24 min.

Experimental details Chapter 6

Synthesis of 3,N^4-ethenogemcitabine:

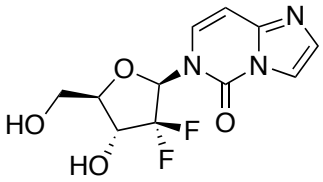
3,N^4-ethenodeoxycytidine (6.3)



To a solution of 2'-deoxy-cytidine (237 mg, 1.04 mmol) in 5 ml of H_2O at 37 ˚C Chloracetaldehyde 2 M was carefully added. pH was controlled by adding aqueous solution of HCl 0.5 M and saturated aqueous solution of $NaHCO_3$ dropwise to reach pH 4.5-5.0. The solution was stirring for 20 h. Purification by flash column chromatography (eluent system CH_2Cl_2/MeOH 80:20) afforded 190 mg (72 %) of the titled product.

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.72 (d, *J* = 7.9 Hz, 1H H-7), 7.66 (d, *J* = 1.0 Hz, 1H H-3), 7.25 (d, *J* = 1.3 Hz, 1H H-2), 6.53 (d, *J* = 7.9 Hz, 1H H-8), 6.42 (t, *J* = 6.8 Hz, 1H H-1'), 4.33-4.35 (m, 1H, H-3'), 3.88 (dd, *J* = 3.5, 6.9 Hz, 1H-H4'), 3.72 (dd, *J* = 3.5, 12.0 Hz, 1H-H5'), 3.66 (dd, *J* = 3.5, 12.0 Hz, 1H-H5'), 2.26-2.31 (m, 1H, H-3'), 2.17-2.22 (m, 1H, H-3').

3,N^4-ethenogemcitabine (6.4)



To a solution of 2'-deoxy-2',2'-di-fluoro-cytidine **6.1** (3.0 g, 11.4 mmol) in 8 ml of H_2O Chloracetaldehyde 2 M was carefully added at 0 ˚C. pH was controlled by adding aqueous solution of HCl 0.5 M and saturated aqueous solution of $NaHCO_3$ dropwise to reach pH 4.5-5.0. The solution was stirring for 20 h at 37 ˚C. Purification by flash

column chromatography (eluent system AcOEt/MeOH 90:10) afforded 3.13 g (95 %) of the titled product.

^{19}F NMR (470 MHz, CD_3OH): δ_F 119.05 (d, *J* = 241.1Hz), -119.75 (d, *J* = 242.9 Hz),

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.85 (d, *J* = 1.35 Hz, 1H H-3), 7.79 (d, *J* = 8.0 Hz, 1H H-7), 7.42 (d, *J* = 1.6 Hz, 1H H-2), 6.70 (d, *J* = 8.0 Hz, 1H H-8), 6.42 (t, J_{H-F} = 7.6 Hz, 1H H-1'), 4.36-4.42 (m, 1H, H-3'), 3.98-4.04 (m, 2H, H-4', 5'), 3.83-3.87 (m, 1H, H-5').

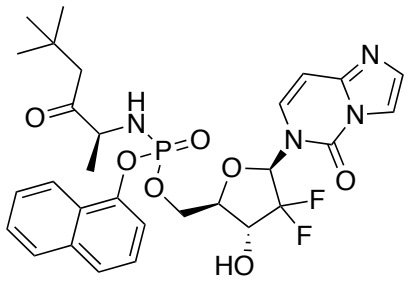
^{13}C NMR (126 MHz, CD_3OD): δ_C 145.18 (C=O, C-5), 138.26 (C-9), 131.61 (CH-2), 128.44 (CH-7), 120.01 (CF_2-2'), 112.94 (CH-3), 97.65 (CH-8), 84.83 (CH-1'), 81.50 (CH-4'), 68.87 (CH-3'), 58.94 (CH_2-5').

MS (ES+) m/z: Found 288.1 [M + H^+], 310.1 [M + Na^+], $C_{11}H_{11}F_2N_3O_4$ required 287.1 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 8.71 min.

Synthesis of 1,N6-Ethenogemcitabine ProTides

1,N^6-Ethenogemcitabine-5'-*O*-[1-naphthyl(2,2-di-methylpropoxy-*L*-alaninyl)] phosphate (6.6)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (154 mg, 0.54 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and naphthyl(2,2-dimethylpropoxy-*L*-alaninyl) phosphorochloridate **2.7** (397.23 mg, 1.08 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2

0:100 to 10:90 over 20 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 10:90) afforded a yellow oil that was dissolved in CH_2Cl_2 and washed with an aqueous solution of HCl 0.5 M. Then evaporated under reduced pressure to afford the titled compound in a 5 % yield (18 mg).

^{19}F NMR (470 MHz, CD_3OH): δ_F -115.18 (d, *J* = 240.4 Hz), -115.70 (d, *J* = 240.4 Hz), 1F; -116.18 (br s), -116.70 (br s), 1F.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.66, 3.34.

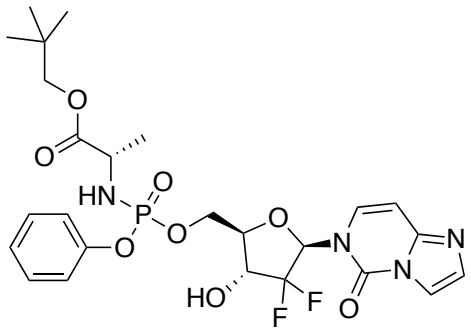
^{1}H NMR (**500 MHz, CD_3OH): δ_H** 8.05-8.09 (m, 1H, Ar), 7.0 (s, 1H H-3), 7.61-7.78 (m, 2H, Ar), 7.48 (d, *J* = 8.1 Hz, 0.5H H-7), 7.43 (d, *J* = 7.4 Hz, 0.5H H-7), 7.31-7.47 (m, 4H, Ar), 7.46 (s, 1H H-2), 6.65 (d, *J* = 8.1 Hz, 0.5H H-8), 6.55 (d, *J* = 7.8 Hz, 0.5H H-8), 6.41-6.45 (m, 1H H-1'), 4.40-4.59 (m, 2H, H-5'), 4.30-4.35 (m, 1H, H-3'), 4.15-4.20 (m, 1H, H-4'), 4.02-4.08 (m, 1H, C*H*CH_3), 3.62-3.72 (m, 2 H OC*H*$_2$C$(CH_3)_3$), 1.39-1.43 (m, 3H, CHC*H*$_3$), 0.95 (s, 4.5 H $OCH_2(CH_3)_3$), 0.94 (s, 4.5 H $OCH_2C(CH_3)_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 174.11 (d $^3J_{CP}$ = 4.1 Hz, C=O, ester), 173.85 (d $^3J_{CP}$ = 5.0 Hz, C=O, ester), 150.72, 150.68 (C=O, C-5), 147.95 (d $^2J_{CP}$= 7.25 Hz, C-*ipso* Naph), 174.93 (d $^2J_{CP}$= 7.25 Hz, C-*ipso* Naph), 131.74 (CH-2), 128.87, 128.82 (CH-Ar x 2), 128.77, 128.68 (CH-7), 127.85 (C-Ar), 127.77, 127.74, 127.48, 127.45, 126.47, 125.99, 125.96 (CH-Ar), 124.30, 124.27 (C-9), 122.74, 122.66 (CH-Ar), 122.36, 122.32 (CF_2-2'), 116.29 (d $^3J_{CP}$ = 3.4 Hz, CH-Ar), 116.17 (d $^3J_{CP}$ = 2.9 Hz, CH-Ar), 113.31 (CH-3), 98.05, 98.03 (CH-8), 84.91 (br s, CH-1'), 79.32 (br s, CH-4'), 74.12 (O***C***$H_2C(CH_3)_3$), 69.60 (br s, CH-3'), 64.32 (d $^2J_{CP}$= 5.0 Hz, CH_2-5'), 64.11 (d $^2J_{CP}$= 4.6 Hz, CH_2-5'), 51.22, 51.01 (***C***HCH_3), 31.06, 30.91 (OCH_2***C***$(CH_3)_3$), 25.94, 25.91 (OCH_2C(***C***$H_3)_3$), 19.34 (d, $^3J_{CP}$ = 6.7 Hz, CH*C*H_3), 19.16 (d $^3J_{CP}$ = 7.5 Hz, CH*C*H_3).

MS (ES+) m/z: Found 635.2 [M + H^+], 657.2 [M + Na^+], $C_{29}H_{33}F_2N_4O_8P$ required 634.2 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 18.82 min.

1,N^6-Ethenogemcitabine-5'-*O*-[phenyl(2,2-di-methylpropoxy-*L*-alaninyl)] phosphate (6.7)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (150 mg, 0.52 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and phenyl(2,2-dimethylpropoxy-*L*-alaninyl) phosphorochloridate **2.12** (520.65 mg, 1.56 mmol). The reaction was stirring for 22 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2 0:100 to 10:90 over 20 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 10:90) afforded the title compound as a white solid (99 mg, 32 %).

^{19}F NMR (470 MHz, CD_3OH): δ_F -117.99 (d, *J* = 240.4 Hz), -118.16 (d, *J* = 240.4 Hz), 1F; -119.00 (br s), -119.51 (br s), 1F.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.85, 3.73.

^{1}H NMR (**500 MHz, CD_3OH): δ_H** 7.84 (s, 1H H-3), 7.50 (d, *J* = 8.0 Hz, 0.5H H-7), 7.45 (d, *J* = 7.5 Hz, 0.5H H-7), 7.19-7.38 (m, 5H, Ph), 7.46 (s, 1H H-2), 6.67 (d, *J* = 8.0 Hz, 0.5H H-8), 6.60 (d, *J* = 8.0 Hz, 0.5H H-8), 6.41-6.46 (m, 1H H-1'), 4.42-4.61 (m, 2H, H-5'), 4.34-4.40 (m, 1H, H-3'), 4.18-4.22 (m, 1H, H-4'), 4.02-4.08 (m, 1H, C*H*CH_3), 3.74-3.88 (m, 2 H OC*H*$_2$C$(CH_3)_3$), 1.39-1.43 (m, 3H, CHC*H*$_3$), 0.94 (s, 4.5 H $OCH_2(CH_3)_3$), 0.93 (s, 4.5 H $OCH_2C(CH_3)_3$).

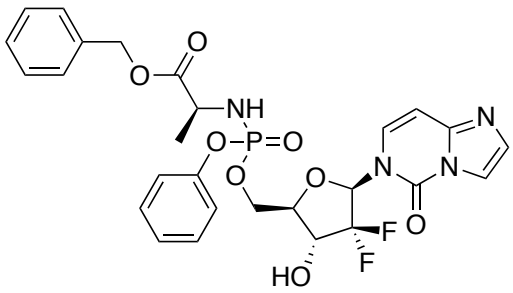
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.68 (d $^3J_{CP}$ = 4.5 Hz, C=O, ester), 173.43 (d $^3J_{CP}$ = 5.3 Hz, C=O, ester), 150.76, 150.71 (C=O, C-5), 145.60 (d $^2J_{CP}$= 2.9 Hz, C-*ipso* Ph), 145.02 (d $^2J_{CP}$= 3.6 Hz, C-*ipso* Ph), 131.70 (CH-2), 129.51, 129.49 (CH-Ar x 2), 128.57, 128.38 (CH-7), 124.90 (CH-Ar), 124.30, 124.26 (C-9), 122.24, 122.20 (CF_2-2'), 120.03 (d $^3J_{CP}$ = 4.5 Hz, CH-Ar x 2), 120.00 (d $^3J_{CP}$ = 4.5 Hz, CH-Ar x 2), 113.11 (CH-3), 98.11,

98.03 (CH-8), 85.10 (br s, CH-1'), 79.44 (br s, CH-4'), 74.05 (O***C***$H_2C(CH_3)_3$), 69.57 (br s, CH-3'), 64.34 (d $^2J_{CP}$= 5.1 Hz, CH_2-5'), 64.11 (d $^2J_{CP}$= 4.8 Hz, CH_2-5'), 50.41, 50.26 (***C***HCH_3), 30.94, 30.91 (OCH_2***C***$(CH_3)_3$), 25.34, 25.33 (OCH_2C(***C***$H_3)_3$), 19.37 (d, $^3J_{CP}$ = 6.4 Hz, CH***C***H_3), 19.19 (d $^3J_{CP}$ = 7.3 Hz, CH***C***H_3).

MS (ES+) m/z: Found 585.2 [M + H^+], 607.2 [M + Na^+], $C_{25}H_{31}F_2N_4O_8P$ required 584.2 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 17.88 min.

1,N^6-Ethenogemcitabine-5'-*O*-[phenyl(benzyloxy-*L*-alaninyl)] phosphate (6.8)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (61 mg, 0.56 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and phenyl(benzoxy-*L*-alaninyl) phosphorochloridate **2.13** (427.7 mg, 1.47 mmol). The reaction was stirring for 18 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 45 g column (eluent system CH_3OH/CH_2Cl_2 1:99 to 10:90 over 10 CV) afforded a yellow oil that was dissolved in CH_2Cl_2 and washed with an aqueous solution of HCl 0.5 M. Then evaporated under reduced pressure and repurified by preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 6:94) afforded the title compound as a white solid (68 mg, 20 %)

^{19}F NMR (470 MHz, CD_3OH): δ_F -118.05 (d, *J* = 240.4 Hz), -118.24 (d, *J* = 240.4 Hz), 1F; -119.10 (br s), -119.56 (br s), 1F.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.85, 3.66.

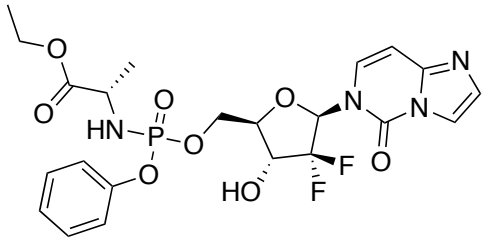
^{1}H NMR (500 MHz, CD_3OH): δ_H 7.81 (d, *J* = 1.55, 0.5H, H-3), 7.80 (d, *J* = 1.55, 0.5H, H-3), 7.45 (d, *J* = 8.0, 0.5H, H-7), 7.40-7.42 (m, 0.5H, H-7), 7.40 (d, *J* = 1.75, 0.5H, H-2), 7.39 (d, *J* = 1.60, 0.5H, H-2), 7.17-7.37 (m, 10H, Ar), 6.62 (d, *J* = 8.0 Hz, 0.5H H-8), 6.56 (d, *J* = 8.0 Hz, 0.5H H-8), 6.38-6.43 (m, 1H, H-1'), 5.13-5.15 (m, 2H, OC***H***$_2$-Ph), 4.48-4.55 (m, 1H, H-5'), 4.44-4.45 (m, 1H, H-5'), 4.43-4.44 (m, 1H, H-3'), 4.14-4.17 (m, 1H, H-4'), 4.03-4.09 (m, 1H, C*H*CH_3), 1.36-1.40 (m, 3H, CHC*H*$_3$).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.42 (d $^3J_{CP}$ = 3.9 Hz, C=O, ester), 173.19 (d $^3J_{CP}$ = 4.9 Hz, C=O, ester), 150.73, 150.68 (C=O, C-5), 145.59 (d $^2J_{CP}$= 3.8 Hz, C-*ipso* Ph), 145.03 (d $^2J_{CP}$= 4.1 Hz, C-*ipso* Ph), 135.81, 135.78 (C-*ipso* Bz), 131.65, 131.63 (CH-2), 129.47, 129.46, (CH-Ar x 2), 128.53, 128.39 (CH-7), 128.22, 128.20, 127.97, 127.93, 127.84, 124.88 (CH-Ar), 124.27, 124.24 (C-9), 122.22, 122.17 (CF_2-2'), 120.01 (d, $^3J_{CP}$ = 4.5, CH Ph x 2), 119.97 (d, $^3J_{CP}$ = 4.6, CH-Ar), 113.05 (CH-3), 98.04, 97.96 (CH-8), 85.09 (br s, CH-1'), 79.37 (br s, CH-4'), 69.48 (br s, CH-3'), 66.59, 66.56 (*C*H_2Ph), 64.30 (d $^3J_{CP}$= 5.1 Hz, CH_2-5'), 63.99 (d $^3J_{CP}$= 4.7 Hz, CH_2-5'), 50.40, 50.24 (***C***HCH_3), 19.03 (d, $^3J_{CP}$ = 6.7 Hz, CH***C***H_3), 18.86 (d $^3J_{CP}$ = 7.4 Hz, CH***C***H_3),

MS (ES+) m/z: Found 605.1 [M + H^+], 627.1 [M + Na^+], $C_{27}H_{27}F_2N_4O_8P$ required 604.1 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 19.88 min.

1,N^6-Ethenogemcitabine-5'-*O*-[phenyl(ethoxy-*L*-alaninyl)] phosphate (6.9)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (140 mg, 0.49 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and phenyl(ethoxy-*L*-alaninyl) phosphorochloridate **2.14** (427.7 mg, 1.47 mmol). The

reaction was stirring for 18 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2 2:98 to 10:90 over 10 CV) afforded the title compound as a white solid (105 mg, 39 %).

^{19}F NMR (470 MHz, CD_3OH): δ_F -118.19 (d, *J* = 241.77 Hz), -118.35 (d, *J* = 241.33 Hz), 1F; -119.07 (br s), -119.60 (br s), 1F.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.89, 3.76.

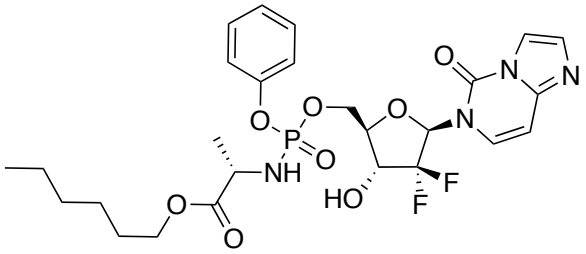
^{1}H NMR (**500 MHz, CD_3OH): δ_H** 7.84 (s, 1H H-3), 7.51 (d, *J* = 8.03 Hz, 0.5H H-7), 7.45 (d, *J* = 8.03 Hz, 0.5H H-7), 7.42 (s, 1H H-2), 7.19-7.39 (m, 5H, Ph), 6.68 (d, *J* = 8.0 Hz, 0.5H H-8), 6.60 (d, *J* = 8.0 Hz, 0.5H H-8), 6.41-6.46 (m, 1H H-1'), 4.57-4.61 (m, 0.5, H-5'), 4.51-4.55 (m, 0.5H, H-5'), 4.43-4.50 (m, 2H, H-5'), 4.34-4.42 (m, 1H, H-3'), 4.19-4.23 (m, 1H, H-4'), 4.13-4.18 (m, 2H, OCH_2CH_3), 3.96-4.02 (m, 1H, $CHCH_3$), 1.39 (d, *J* = 7.21, 1.5H, $CHCH_3$), 1.36 (d, 1.5H, *J* = 7.16, 1.5H, $CHCH_3$), 1.22-1.26 (m, 3H, OCH_2CH_3).

^{13}C NMR (125 MHz, CD_3OD): δ_C 173.65 (d $^3J_{CP}$ = 4.5 Hz, C=O, ester), 173.48 (d $^3J_{CP}$ = 5.2 Hz, C=O, ester), 150.77, 150.71 (C=O, C-5), 145.60 (d $^2J_{CP}$= 1.8 Hz, C-*ipso* Ph), 145.03 (d $^2J_{CP}$= 3.2 Hz, C-*ipso* Ph), 131.65 (CH-2), 129.48, (CH-Ar x 2), 128.53, 128.39 (CH-7), 124.89 (CH-Ar), 124.36, 124.43 (C-9), 122.27, 122.19 (CF_2-2'), 119.99 (d $^3J_{CP}$ = 4.7 Hz, CH-Ar x 2), 119.98 (d $^3J_{CP}$ = 4.7 Hz, CH-Ar x 2), 113.06 (CH-3), 98.03, 97.92 (CH-8), 84.81 (br s, CH-1'), 79.34 (br s, CH-4'), 69.38 (br s, CH-3'), 64.18 (d $^3J_{CP}$= 5.3 Hz, CH_2-5'), 64.00 (d $^3J_{CP}$= 5.07 Hz, CH_2-5'), 61.00 ($O\boldsymbol{C}H_2CH_3$), 50.32, 50.17 ($\boldsymbol{C}HCH_3$), 19.13 (d, $^3J_{CP}$ = 7.5 Hz, $CHCH_3$), 18.93 (d $^3J_{CP}$ = 6.8 Hz, $CHCH_3$), 13.07, 13.05 ($OCH_2\boldsymbol{C}H_3$).

MS (ES+) m/z: Found 543.1 [M + H^+], 565.2 [M + Na^+], $C_{22}H_{25}F_2N_4O_8P$ required 542.1 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 15.93 min.

1, N^6-Ethenogemcitabine-5'-*O*-[phenyl(hexyloxy-*L*-alanine)] phosphate (6.10)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (152 mg, 0.53 mmol), N-methylimidazole (0.2 mL, 2.6 mmol) and phenyl(hexyloxy-*L*-alaninyl) phosphorochloridate **2.15** (427.7 mg, 1.47 mmol). The reaction was stirring for 32 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2 2:98 to 5:95 over 10 CV) afforded a yellow oil that was dissolved in CH_2Cl_2 and washed with an aqueous solution of HCl 0.5 M. Then evaporated under reduced pressure and repurified by preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 3:97) afforded the title compound as a white solid (57 mg, 18 %)

^{19}F NMR (470 MHz, CD_3OH): δ_F -118.18 (d, *J* = 240.4 Hz), -118.33 (d, *J* = 240.6 Hz), 1F; -119.10 (br s), -119.62 (br s), 1F.

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.85, 3.73.

^{1}H NMR (**500 MHz, CD_3OH): δ_H** 7.84-7.85 (m, 1H H-3), 7.51 (d, *J* = 8.0 Hz, 0.5H H-7), 7.46 (d, *J* = 8.0 Hz, 0.5H H-7), 7.42 (s, 1H H-2), 7.19-7.40 (m, 5H, Ph), 6.68 (d, *J* = 8.0 Hz, 0.5H H-8), 6.61 (d, *J* = 8.0 Hz, 0.5H H-8), 6.42-6.46 (m, 1H H-1'), 4.56-4.60 (m, 0.5, H-5'), 4.50-4.54 (m, 0.5H, H-5'), 4.41-4.49 (m, 1H, H-5'), 4.34-4.40 (m, 1H, H-3'), 4.16-4.22 (m, 1H, H-4'), 4.07-4.13 (m, 2H, $OC\boldsymbol{H}_2(CH_2)_4CH_3$), 3.97-4.02 (m, 1H, $CHCH_3$), 1.57-1.65 (m, 2H, $OCH_2C\boldsymbol{H}_2(CH_2)_3CH_3$), 1.35-1.39 (m, 3H, $CHCH_3$), 1.26-1.39 (m, 6H, $OCH_2CH_2(C\boldsymbol{H}_2)_3CH_3$), 0.87-0.90 (m, 3H, $OCH_2CH_2(CH_2)_3C\boldsymbol{H}_3$).

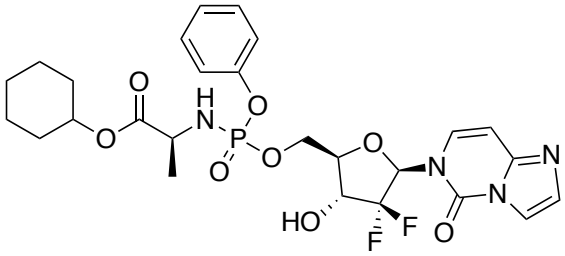
^{13}C NMR (125 MHz, CD_3OD): δ_C 173.73 (d $^3J_{CP}$ = 4.6 Hz, C=O, ester), 173.49 (d $^3J_{CP}$ = 5.4 Hz, C=O, ester), 150.77, 150.72 (C=O, C-5), 145.65 (d $^2J_{CP}$= 2.7 Hz, C-*ipso* Ph), 145.03 (d $^2J_{CP}$= 3.7 Hz, C-*ipso* Ph), 131.68 (CH-2), 129.48, 129.46 (CH-Ar x 2), 128.53, 128.39 (CH-7), 124.89 (CH-Ar), 124.29, 124.25 (C-9), 122.23, 122.19 (CF_2-2'), 119.99

(d $^3J_{CP}$ = 4.7 Hz, CH-Ar x 2), 119.98 (d $^3J_{CP}$ = 4.7 Hz, CH-Ar x 2), 113.11 (CH-3), 98.06, 97.98 (CH-8), 85.13 (br s, CH-1'), 79.34 (br s, CH-4'), 69.53 (br s, CH-3'), 65.08 (O***C***$H_2(CH_2)_4CH_3$), 64.25 (d $^3J_{CP}$= 4.7 Hz, CH_2-5'), 64.03 (d $^3J_{CP}$= 4.7 Hz, CH_2-5'), 50.36, 50.21 (***C***HCH_3), 31.14, 28.25, 28.21, 25.18, 22.15 (OCH_2(***C***$H_2)_4CH_3$), 19.15 (d, $^3J_{CP}$ = 6.6 Hz, CH***C***H_3), 18.99 (d $^3J_{CP}$ = 7.4 Hz, CH***C***H_3), 12.91 ($O(CH_2)_5$***C***H_3).

MS (ES+) m/z: Found 599.2 [M + H^+], 621.2 [M + Na^+], $C_{26}H_{33}F_2N_4O_8P$ required 598.2 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 20.13 min.

1, N^6-Ethenogemcitabine-5'-*O*-[phenyl(cyclohexyloxy-*L*-alanine)] phosphate (6.11)



Compound was prepared according to the general procedure A using 1, N^6-ethenogemcitabine **6.4** (170 mg, 0.59 mmol), N-methylimidazole (0.24 mL, 2.9 mmol) and phenyl(cyclohexyloxy-*L*-alaninyl) phosphorochloridate **2.16** (614.07 mg, 1.77 mmol). The reaction was stirring for 20 h. Purification by flash column chromatography using Biotage ZIP KP-Sil 80 g column (eluent system CH_3OH/CH_2Cl_2 0:100 to 10:90 over 20 CV) and preparative TLC (2000 μm, eluent system CH_3OH/CH_2Cl_2 5:95) afforded a mixture of *D* and *L* ProTides. Preparative HPLC (H2O/ACN 40:60 isocratic) and a second Preparative HPLC (H2O/ACN 60:40 isocratic) isolated a mixture of diasteroisomers *R* and *S* of *L* Alanine, and a single *L* Alanine diasteroisomer.

To a solution of 2'-Deoxy-2',2'-difluoro-*D*-cytidine-5'-*O*-[phenyl(cyclohexyloxy-*L*-alanine)] phosphate (85 mg, 0.15 mmol) in 1 mL of THF, 0.3 ml of chloracetaldehyde 2 M was carefully added at 0 ˚C. pH was controlled by adding aqueous solution of

HCl 0.5 M and saturated aqueous solution of $NaHCO_3$ dropwise to reach pH 4.5-5.0. The solution was stirring for 20 h at 37 ˚C. Purification by preparative TLC (eluent system CH_3OH/CH_2Cl_2 5:95) afforded 55 mg (61 %) of the titled product.

One diasteroisomer

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.77.

^{1}H NMR (500 MHz, CD_3OH): δ_H 7.84 (d, *J* = 1.3 Hz, 1H H-3), 7.46 (d, *J* = 8.0 Hz, 1H H-7), 7.42 (d, *J* = 1.3 Hz, 1H H-2), 7.38-7.41 (m, 2H, Ph), 7.28-7.30 (m, 2H, Ph), 7.21-7.24 (m, 1H, Ph), 6.61 (d, *J* = 8.0 Hz, 0.5H H-8), 6.43 (t, J_{H-F} = 8.5 Hz, 1H H-1'), 4.70-4.75 (m, 1H, OC***H***$(CH_2)_5$), 4.50-4.54 (m, 1H, H-5'), 4.41-4.46 (m, 1H, H-5'), 4.34-4.39 (m, 1H, H-3'), 4.17-4.20 (m, 1H, H-4'), 3.94-4.00 (m, 1H, C***H***CH_3), 1.77-1.84 (m, 2H, OCH(C***H***$_2)_5$), 1.68-1.75 (m, 2H, OCH(C***H***$_2)_5$), 1.50-1.56 (m, 1H, OCH(C***H***$_2)_5$), 1.37-1.39 (m, 3H, CHC***H***$_3$), 1.27-1.39 (m, 5H, OCH(C***H***$_2)_5$).

Two diasteroisomers

^{31}P NMR (202 MHz, CD_3OD): δ_P 3.88, 3.77.

^{1}H NMR (**500 MHz, CD_3OH): δ_H** 7.84 (br s, 1H H-3), 7.51 (d, *J* = 8.0 Hz, 0.5H H-7), 7.46 (d, *J* = 8.0 Hz, 0.5H H-7), 7.42 (br s, 1H H-2), 7.36-7.41 (m, 2H, Ph), 7.19-7.30 (m, 3H, Ph), 6.67 (d, *J* = 8.0 Hz, 0.5H H-8), 6.61 (d, *J* = 8.0 Hz, 0.5H H-8), 6.41-6.46 (m, 1H H-1'), 4.70-4.78 (m, 1H, OC***H***$(CH_2)_5$), 4.42-4.61 (m, 2H, H-5'), 4.33-4.41 (m, 1H, H-3'), 4.17-4.23 (m, 1H, H-4'), 3.94-4.00 (m, 1H, C*H*CH_3), 1.78-1.84 (m, 2H, OCH(C***H***$_2)_5$), 1.68-1.75 (m, 2H, OCH(C***H***$_2)_5$), 1.50-1.57 (m, 1H, OCH(C***H***$_2)_5$), 1.35-1.39 (m, 3H, CHC***H***$_3$), 1.27-1.49 (m, 5H, OCH(C***H***$_2)_5$).

MS (ES+) m/z: Found 597.2 [M + H^+], 619.2 [M + Na^+], $C_{26}H_{31}F_2N_4O_8P$ required 596.2 [M].

HPLC Reverse-phase eluting with H_2O/CH_3CN from 90:10 to 0:100 in 30 minutes, F = 1 mL/min, λ = 270 nm, t_R 19.43 min.

REFERENCES

(1) Molecular Operating Environment (MOE 2015.10); Chemical Computing Group, Inc.: Montreal, Quebec, Canada; URL http://www.chemcomp.com (2015).

(2) Korb, O.; Stützle, T.; Exner, T. E. PLANTS: Application of Ant Colony Optimization to Structure-Based Drug Design. In *Ant Colony Optimization and Swarm Intelligence*; Springer, Berlin, Heidelberg, 2006; pp 247–258.

APPENDIX

Procedure for cell panel screening by WuXi AppTec

Compounds preparation:

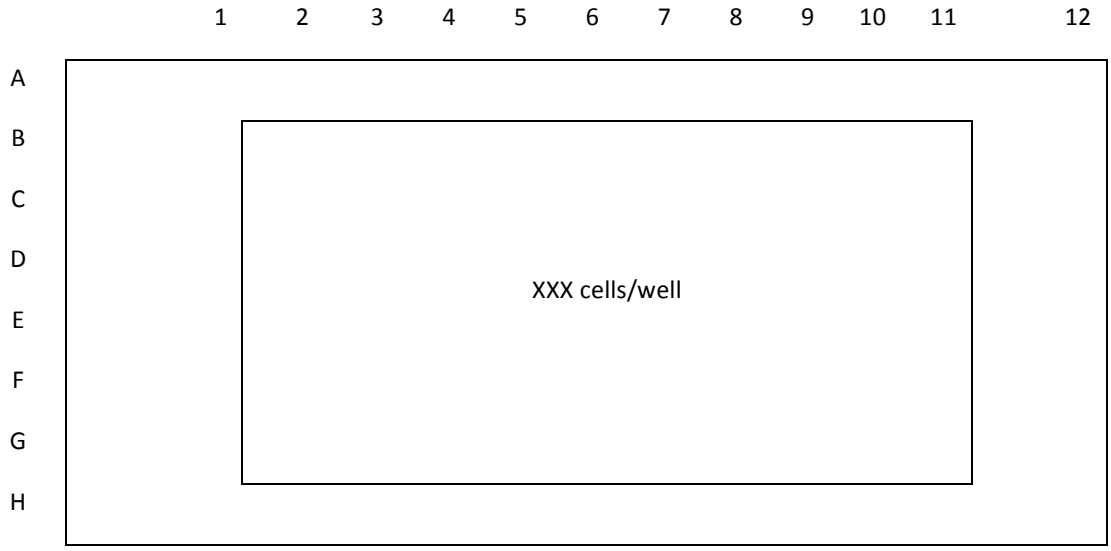
All the compounds are diluted by DMSO to their stock concentrations, and kept in -80 degree freezer.

Procedure

Observe the compounds solubility while dissolving in DMSO.

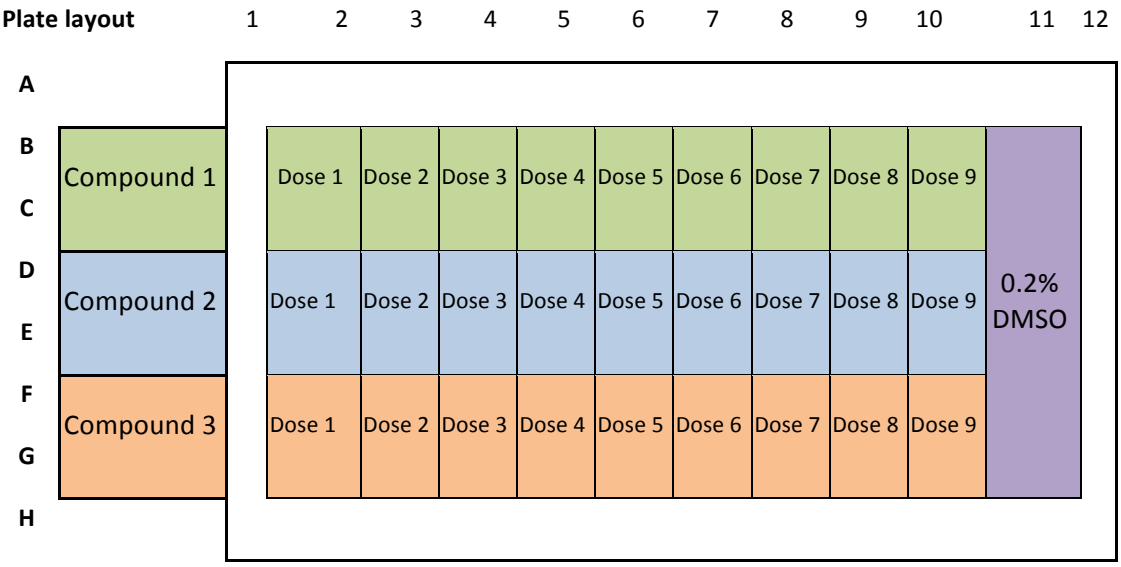
Day -1:

1. Adjust the cell density to the recommended information.
2. Add 100 µL cell suspension to the assay plate(96w) as the following platemap.
3. Add 100 µL PBS in the untested edge well.



Day 0:

4. Set compounds adding procedure on Tecan HP D300e digital dispenser.
5. Add compounds to wells using Tecan D300e.
6. Observe the assay plate under the microscope, record the compounds precipitation in the sheet "work plan".



Compounds test start with 198 μM concentration, 3.16 fold dilution, 9 dose points

The reference Paclitaxel start with 0.5 μM concentration, 3.16 fold dilution, 9 dose points

Note: Some compounds with lower stock concentration will start with a different top concentration, details refer to the sheet "work plan".

7. For Day 0 plate, if needed, add 50 μL CellTiter Glo reagent and detect.

Day 3 (72h)**:**

8. Equilibrate the assay plate to the room temperature for nearly 30 minutes. Observe the compounds solubility and cells status before adding CTG.
9. Add 50 μL CellTiter Glo reagent to each well
10. Detect the plate after 10 mins by EnVision (LμM inescence)

Data analysis:

Data was analyzed using XL-fit software (Supplier: ID Business Solutions Ltd., Software version: XL fit 5.0), Inhibition% = (Max-Sample value)/Max*100.

Example of analyse data:
Mia-pa-ca-2 cell line

Avg DMSO Control	**92223**
Stdev	**2619**
CV(%)	**2.84**

Raw Data

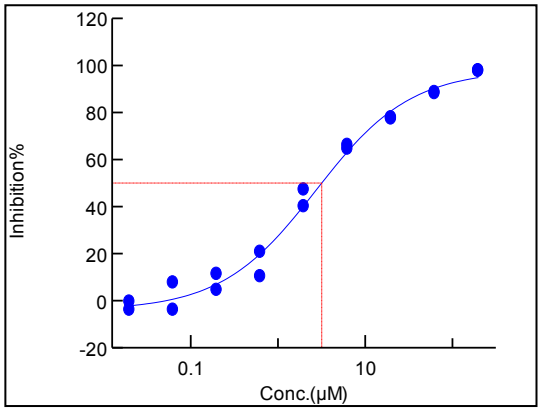
	Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
	A											**Max**	
εGem	B		2240	10980	20940	32800	48780	82800	81860	85220	92760	91040	
	C		2020	10560	20480	31280	55340	73200	88140	95940	95940	96860	
6.7	D		340	21340	40160	54540	78700	82900	89420	90760	94080	90480	
	E		420	22200	40640	69960	85040	85960	94180	105760	94920	89540	
6.9	F		13540	43740	91720	93500	90740	92080	102300	99000	98260	93300	
	G		13400	42860	84200	92180	99940	91740	101460	102940	95520	92120	
	H												

Inhibition %

	Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
	A											**Max**	
εGem	B		97.57	88.09	77.29	64.43	47.11	10.22	11.24	7.59	-0.58	1.28	
	C		97.81	88.55	77.79	66.08	39.99	20.63	4.43	-4.03	-4.03	-5.03	
6.7	D		99.63	76.86	56.45	40.86	14.66	10.11	3.04	1.59	-2.01	1.89	
	E		99.54	75.93	55.93	24.14	7.79	6.79	-2.12	-14.68	-2.92	2.91	
6.9	F		85.32	52.57	0.55	-1.38	1.61	0.16	-10.93	-7.35	-6.55	-1.17	
	G		85.47	53.53	8.70	0.05	-8.37	0.52	-10.02	-11.62	-3.57	0.11	
	H												

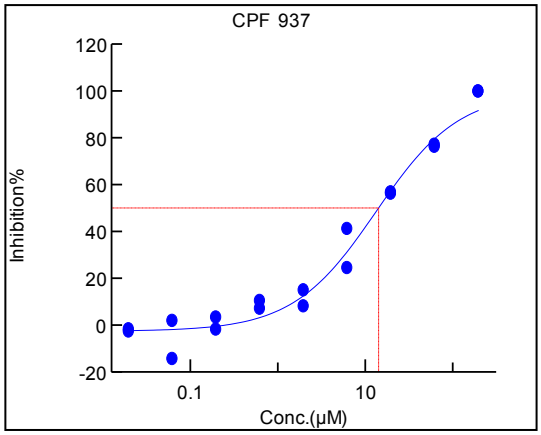
Plate 1 Curve fitting

εGem		
Conc.(μM)	**Inhibition%**	
198.00	97.57	97.81
62.66	88.09	88.55
19.83	77.29	77.79
6.27	64.43	66.08
1.99	47.11	39.99
0.63	10.22	20.63
0.20	11.24	4.43
0.063	7.59	-4.03
0.020	-0.58	-4.03



Absolute EC_{50} (μM)	3.17
Relative EC_{50} (μM)	2.73
Top(%)	98.04
Bottom(%)	-4.15
Slope	0.80
Y-Max	120
Y-Min	-20
Step	20

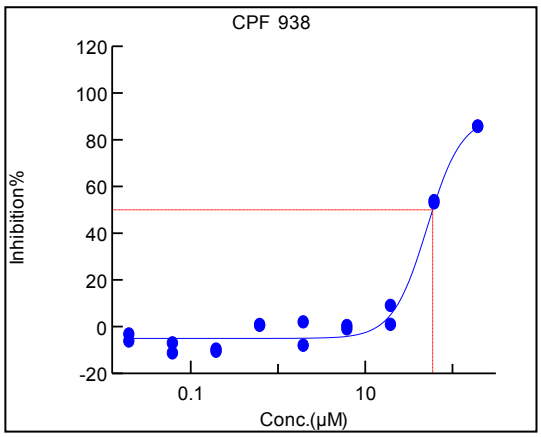
Compound **6.7**		
Conc.(μM)	**Inhibition%**	
198.00	99.63	99.54
62.66	76.86	75.93
19.83	56.45	55.93
6.27	40.86	24.14
1.99	14.66	7.79
0.63	10.11	6.79
0.20	3.04	-2.12
0.063	1.59	-14.68
0.020	-2.01	-2.92



Absolute EC_{50} (μM)	14.21
Relative EC_{50} (μM)	13.32
Top(%)	99.63
Bottom(%)	-2.65
Slope	0.92
Y-Max	120
Y-Min	-20
Step	20

Compound **6.9**		
Conc.(μM)	**Inhibition%**	
198.00	85.32	85.47
62.66	52.57	53.53
19.83	0.55	8.70
6.27	-1.38	0.05
1.99	1.61	-8.37
0.63	0.16	0.52
0.20	-10.93	-10.02
0.063	-7.35	-11.62
0.020	-6.55	-3.57

Absolute EC_{50} (μM)	59.20
Relative EC_{50} (μM)	51.24
Top(%)	90.06
Bottom(%)	-5.06
Slope	2.20
Y-Max	120
Y-Min	-20
Step	20



Cell Viability Assay Summary in MIA PaCa-2

Compound ID	Ab EC_{50} (μM)	Re EC_{50} (μM)	Top inhibition%	Bottom inhibition%	Slope	High Conc. (μM)
εGem	3.17	2.73	98.04	-4.15	0.80	198
6.7	14.21	13.32	99.63	-2.65	0.92	198
6.9	59.20	51.24	90.06	-5.06	2.20	198