Design, Synthesis and Biological Evaluation of Some Novel Nucleotide Prodrugs as Potential Anticancer Agents

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A Thesis submitted to the University of Wales for the Degree of
PHILOSOPHIAE DOCTOR

The Welsh School of Pharmacy
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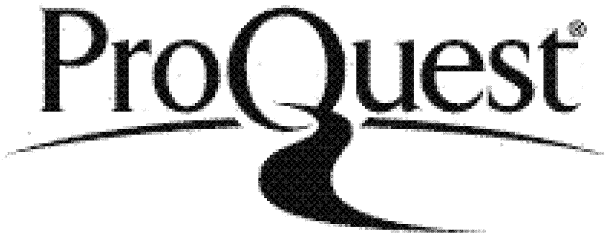
February 2006



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Abstract

The development of phosphoramidates as a new pronucleotide approach has shown to lead to a significant boost in activity for a series of antiviral nucleoside analogues. Given the importance of nucleoside analogues in anticancer therapy and the need of more potent drugs against what is considered the major cause of death in the developed world, the aim of the present thesis was to investigate phosphoramidates as new potential anticancer prodrugs.

A consistent part of the work presented is related to the further SAR investigation of Thymectacin, a BVdU phosphoramidate prodrug that has recently entered clinical trials against colon cancer. Herein, the use of naphthyl as a new phosphate masking group is reported for the first time.

The antileukaemic drug Cladribine was a new target for the application of our approach and two series of related phosphoramidates represent the second major contribution to this thesis.

Synthesis and cytostatic evaluation were also carried out for novel phosphoramidate derivatives of a different anticancer agent (Zebularine), a natural nucleoside (Adenosine) and an inactive nucleoside analogue ("IsoCladribine").

Through the combination of computational and NMR studies, the absolute stereochemistry of the phosphorus center of separated phosphoramidate diastereoisomers was suggested for the first time.

Eventually, a preliminary molecular modelling study was performed on the human Hint1 enzyme to investigate its possible role in the activation of phosphoramidates.

Acknowledgements

I would like to express my deepest gratitude to my supervisor Prof. Christopher McGuigan for the chance given, support and help.

Thanks to Helen for her constant assistance and huge patience.

Thanks to all the technical staff for their support and assistance.

A special thanks to the biological collaborators: Prof. Mason, Dr. Jiang and Dr. Mills from the University of Wales College of Medicine. My warmest thank you to Elisabeth Walsby for her precious work and prompt assistance.

Thanks to Dr. Brancale, as a scientist for his invaluable support and as Andrea for his encouragement and friendship.

I would like to thank all the colleagues and visitors I have worked with in the lab: who left (Antonella, Chris, Federica, Felice, Giovanna, Mary Rose, Olivier, Samantha, Sinead and Stephen), who stayed as a visitor (Anke, Birgit, Luca and Ranjith) and who are here at the moment (Annette, Kevin, Marco, Michaela, Monica, Plinio, Rina, Rita, Rocco and Youcef). In particular, I wish to express my gratitude to Rina and Kevin for their help in reviewing my writing. A special thank you to Giuseppe for his companionship.

Thanks to my parents and my brother for their love and for being always close to me.

Thanks to Laura for her immense love, generosity and understanding.

Publications

A part of the work presented in this Thesis has been published in the following journals:

Congiatu, C.; McGuigan, C; Jiang, W. G.; Davies, G.; Mason, M. D. Naphthyl phosphoramidate derivatives of BVdU as potential anticancer agents: design, synthesis and biological evaluation. *Nucleosides Nucleotides and Nucleic Acids* **2005**, 24, 485-489.

Congiatu, C.; Brancale, A.; Mason, M. D.; Jiang, W. G.; McGuigan, C. Novel potential anticancer naphthyl phosphoramidates of BVdU: separation of diastereoisomers and assignement of the absolute configuration of the phosphorus center. *J. Med. Chem.* **2006**, 49, 452-455.

The full articles are referred in the Appendix II.

Table of Contents

Abbreviations & Acronyms

ABC	Abacavir
Ac	Acetyl
ACV	Acyclovir
ADA	Adenosine deaminase
Ala	Alanine
AMP	Adenosine monophosphate
APL	Acute promyelocytic leukaemia
AraA	Vidarabine
AraC	Cytarabine
ATP	Adenosine triphosphate
5-aza-C	Azacytidine
5-aza-dC	Decitabine
AZT	3'-Azido-3'-deoxythymidine
BCNAs	Bicyclic pyrimidine nucleoside analogues
Bn	Benzyl
BTEA-Cl	Benzyltriethylammonium chloride
2Bu	2-Butyl
BVdU	Brivudin
CAFdA	Clofarabine
CDA	Cytidine deaminase
2CdA	Cladribine
CDK	Cyclin-dependent kinase
CLogP	Calculated LogP
CMP	Cytidine monophosphate
dA	Deoxyadenosine
d4A	2',3'-Dideoxy-2',3'-didehydroadenosine
dAMP	Deoxyadenosine monophosphate
DAPdR	2,6-Diaminopurine-2'-deoxyriboside
dC	Deoxycytidine

dCK	Deoxycytidine kinase
DCM	Dichloromethane
dCMP	Deoxycytidine monophosphate
ddA	2',3'-Dideoxyadenosine
ddC	Zalcitabine
ddI	Didanosine
ddNs	Dideoxy nucleosides
ddU	2',3'-Dideoxyuridine
ddUMP	2',3'-Dideoxyuridine monophosphate
dFdC	Gemcitabine
dGMP	Deoxyguanosine monophosphate
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
d4T	Stavudine
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
dZeb	2'-Deoxyzebularine
EC_{50}	effective concentration for 50% inhibition of cell proliferation
ECTA	Enzyme-catalysed therapeutic approach
eq	Equivalent
Et	Ethyl
5FdU	Fluorodeoxyuridine
Flu	Fludarabine
FTC	Emtricitabine
GA	Genetic algorithm
GCV	Ganciclovir
GMP	Guanosine monophosphate
HCMV	Human cytomegalovirus

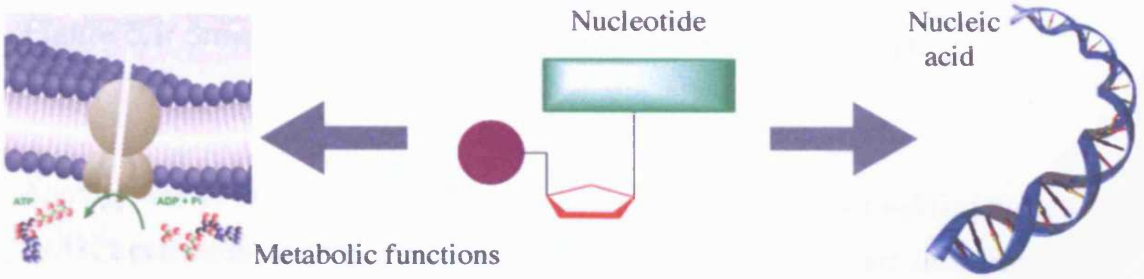
HIV	Human immuno-deficiency virus
HSV	Herpes virus
IC_{50}	Concentration causing 50% inhibition of cell proliferation
Ile	Isoleucine
i-Pr	Isopropyl
Me	Methyl
Me_2Gly	Dimethylglycine
Met	Methionine
MTS	Tetrazolium salt
MTT	Formazan derivative
NAD	Nicotinamide adenine dinucleotide
Naph	1-Naphthyl
NAs	Nucleosides analogues
NBS	N-bromosuccinimide
NDPK	Nucleoside diphosphate kinase
neoPent	Neopentyl
NMI	N-methylimidazole
NMPK	Nucleoside monophosphate kinase
N,N-DMA	N,N-Dimethylaniline
nPent	Normalpentyl
5'-NT	5'-Nucleotidase
Ph	Phenyl
Phe	Phenylalanine
Phgly	Phenylglycine
Pg-P	P-Glycoprotein
PMEA	9-(2-Phosphonylmethoxyethyl)-adenine
PMPA	9-(2-phosphonylmethoxypropyl)-adenine
Pro	Proline
ProTide	Nucleotide prodrug
p-TSA	para-Toluenesulfonic acid
Pyr	Pyridine

RA	Retinoic acid
RARα	Retinoic acid receptor α
Rf	Retention factor
RNA	Ribonucleic acid
rt	Room temperature
SAR	Structure activity relationship
TBA-NO_2	Tetrabutylammonium nitrite
tBu	Tert-buthyl
3TC	Lamivudine
TEA	Triethylamine
THF	Tetrahydrofuran
TK^-	Thymidine kinase deficient (cell line)
TK^+	Thymidine kinase competent (cell line)
TiPBS-Cl	2,4,6-Triisopropylbenzenesulfonyl chloride
Tos	Tosylate
TS	Thymidylate synthase
UMP	Uridine monophosphate
Val	Valine
VZV	Varicella zoster virus
Zeb	Zebularine

Chapter 1: Introduction

1.1 Nucleosides and Nucleoside Analogues

As building blocks of DNA and RNA, deoxyribonucleotides and ribonucleotides are generally identified as the fundamental units of genetic information. In addition, nucleotides have a variety of other functions such as energy carriers, components of enzyme cofactors, and chemical messengers. Therefore, among the cell metabolites, nucleotides may be considered one of the most important.[1]



As units of the genetic code, nucleotides play a crucial role in the life of a cell. Continuity of life is based on the unique ability of DNA to not only direct the synthesis of proteins but also to replicate itself. From an extreme point of view, a living cell could be considered as an artificial environment, created by DNA for the benefit of its own replication.[2] The accuracy of DNA replication is fundamental for the genetic stability of the cell. Although errors in DNA synthesis have a role in ageing and diseases like cancer, spontaneous mutations also provide the opportunity for genetic variation and are a primary basis for evolution.

Inhibition of deoxyribonucleotide synthesis, which results in mutagenic changes, cell growth arrest or cell death, can be achieved with compounds able to inhibit enzymes involved with the deoxyribonucleotide metabolism, leading to deoxyribonucleotide pool imbalances and thus impaired DNA replication (e.g., hydroxyurea, a ribonucleotide reductase inhibitor, and methotrexate, which inhibits dihydrofolate

reductase, thymidilate synthase and also the purine nucleotide de novo pathway, Figure 1.1).

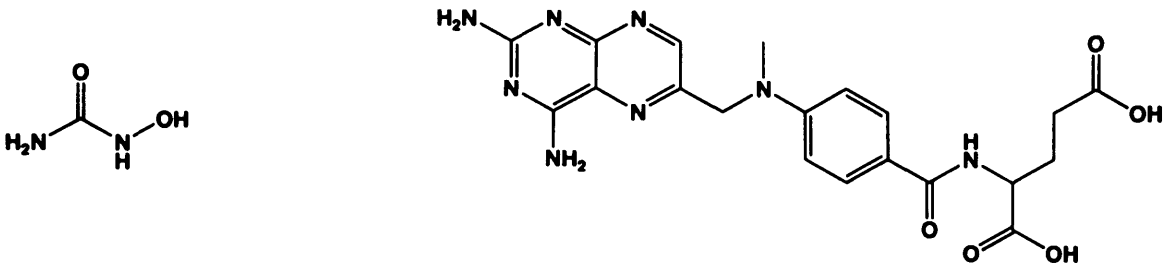


Figure 1.1: Structures of hydroxyurea (left) and methotrexate (right).

Another way to interfere with DNA synthesis is by structurally modified nucleosides (NAs, nucleoside analogues). NAs that lack the 3'-OH group are incorporated into DNA and act as chain terminators because no new deoxyribonucleotide can be attached to the growing DNA strand. NAs with an intact 3'-OH group are not absolute chain terminators, but there is evidence that they are incorporated into DNA and severely impair chain elongation.[3]

Therefore, by interfering with either synthesis or function of naturally occurring nucleotides, NAs could exert anticancer and/or antiviral activity. Anticancer NAs inhibit cellular DNA replication and repair while antiviral NAs inhibit replication of the viral genome.

Since the discovery of 3'-azido-3'-deoxythymidine (AZT; Zidovudine) as the first nucleoside drug for the treatment of AIDS, considerable efforts have been made to develop new nucleoside analogues that would be more active and less toxic inhibitors of the HIV reverse transcriptase, leading to new promising antiviral agents such as abacavir (ABC), didanosine (ddI), lamivudine (3TC), emtricitabine (FTC), stavudine (d4T), zalcitabine (ddC) (Figure 1.2).[4]

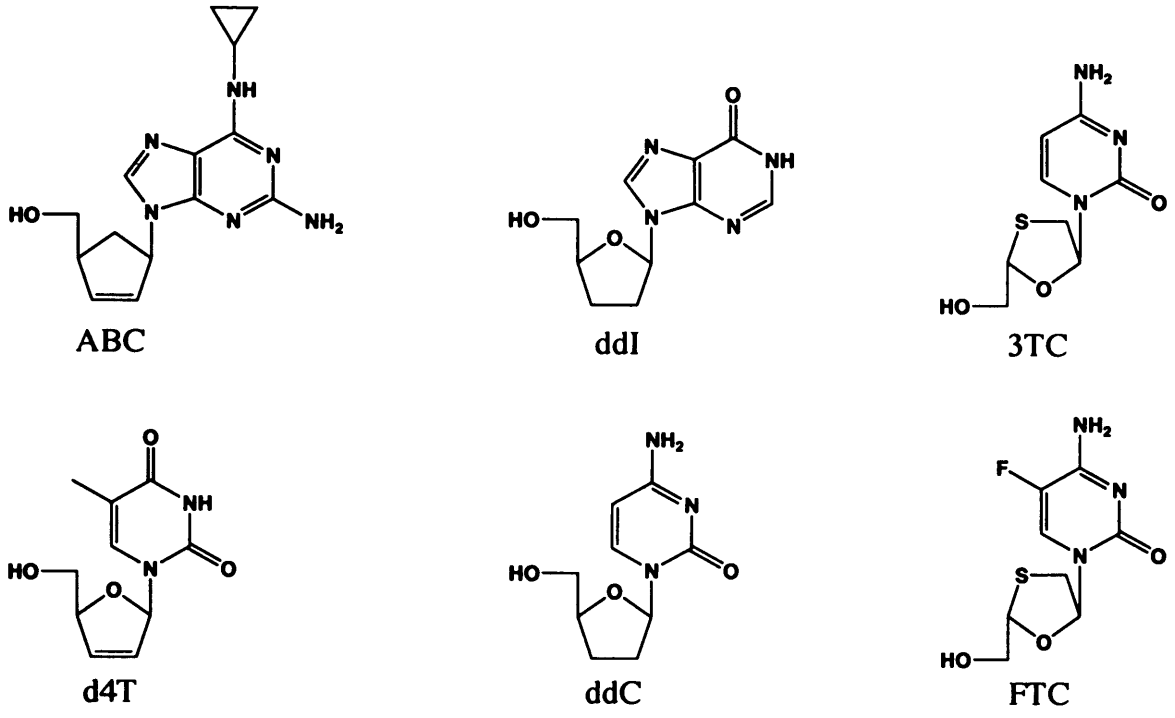


Figure 1.2: Structures of some antiviral NAs.

Moreover, cytotoxic nucleoside analogues were among the first chemotherapeutic agents to be introduced for the medical treatment of cancer. This family of compounds has grown to include a variety of purine and pyrimidine nucleoside derivatives such as fludarabine (Flu), cladribine (2CdA), clofarabine (CAFdA), cytarabine (araC), gemcitabine (dFdC), fluorodeoxyuridine (5FdU) (Figure 1.3). These display activity in both solid tumours and malignant disorders of the blood.

Nucleoside analogues are in essence prodrugs since they require an intracellular 5'-phosphorylation to form active nucleotides that can function as inhibitors of viral or cellular replication processes. Regardless the site of action, the majority of the NAs necessitates after cell penetration a kinase-mediated conversion to the corresponding mono-, di-, and triphosphate, which is in most cases the biologically active form. However, kinases have such a significant specificity that any deviation from the structure of the normal substrate can result in partial or complete loss of activity.

Consequently, the same structural changes that lead nucleoside analogues to show antiviral or anticancer activity are also responsible for their poor activation. Conversely, nucleotides themselves do not usually penetrate cells at a sufficient rate to show any significant chemotherapeutic effect.[5]

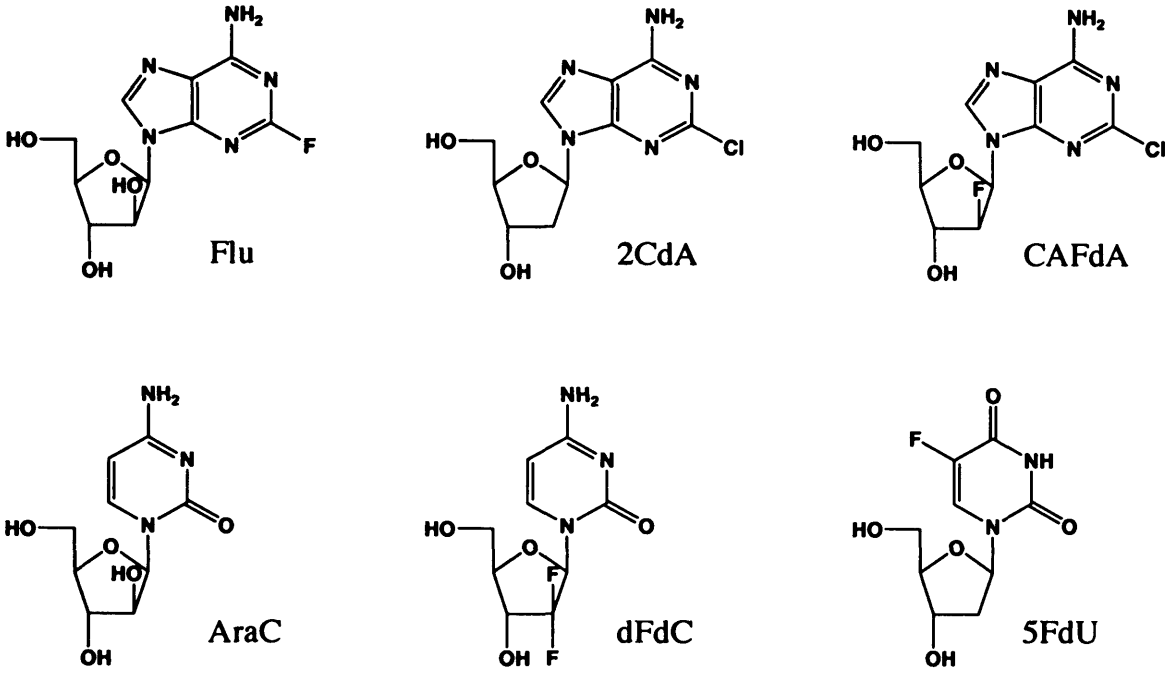
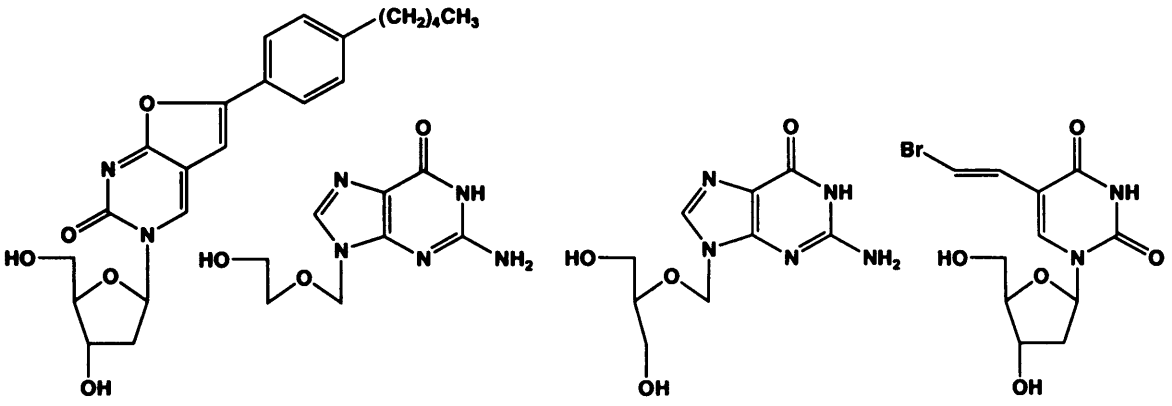


Figure 1.3: Structures of some anticancer NAs.

It would appear that kinase selectivity towards substrates would depend upon the distance between the point of insertion of the phosphate and the nucleoside analogue: although with few exceptions, several kinases can readily convert diphosphate on nucleoside analogues into triphosphate.[6]

Nevertheless, pyrimidine and purine analogues, such as brivudin (BVdU), acyclovir (ACV), ganciclovir (GCV) and our Cf1743[7] (Figure 1.4), are very poorly monophosphorylated by cellular deoxynucleoside kinases. This is the basis for the excellent properties of these NAs, as they are very good substrates for herpes virus kinases (HSV1-TK) which explains their selective antiviral action. However, as

reported for GCV, a prolonged therapy can cause mutations on the viral genetic sequences (UL97 in this case) responsible for the NA-activation and lead to insurgence of resistance.[8]



Cf1743 ACV GCV BVdU

Figure 1.4: Structures of some antiviral NAs selectively phosphorylated by viral kinases.

Typically, monophosphorylation represents the kinetic limiting step of the activation process while di- and triphosphorylation appear to be less substrate selective. One exception is given by AZT (Figure 1.5), where the rate limiting step is the conversion of mono- to diphosphate.[9]

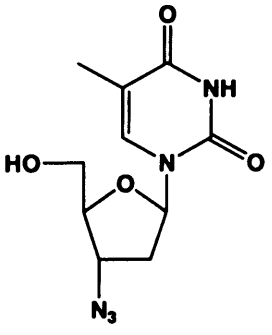
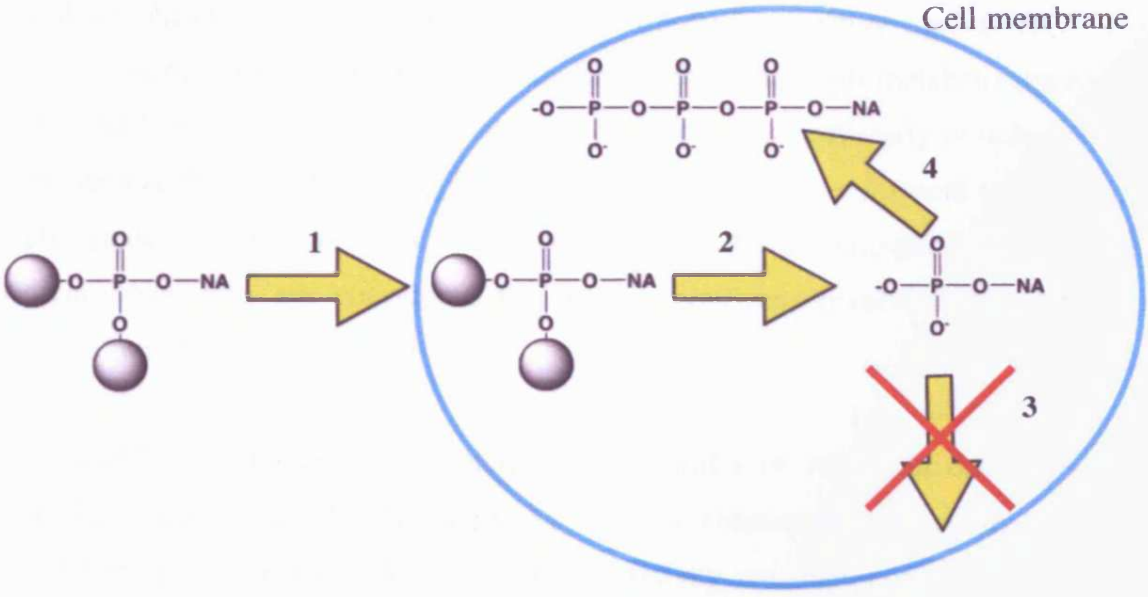


Figure 1.5: AZT.

1.2 From early studies to the first aryloxy phosphoramidate

Due to the poor membrane permeability of free nucleotides, a suitable approach to increase the potency of anticancer and antiviral NAs would involve masking the phosphate charges with lipophilic moieties. This would facilitate passive diffusion through the cell membrane which should be followed by an hydrolytic or enzymatic release of the nucleoside monophosphate. Due to its polarity, the NA monophosphate would be then trapped inside the cell. In some cases that would already be bioactive, in others it would then be converted to the active di- or triphosphate forms (Scheme 1.1).



Scheme 1.1: Ideal pronucleotide strategy: 1) passive diffusion through the membrane; 2) activation to NA monophosphate; 3) due to the high polarity profile, passive diffusion is now blocked; 4) phosphorylation to triphosphate.

In the pronucleotide field, several approaches have been developed in the attempt to optimise the activation of such prodrugs and deliver the target NA monophosphates intracellularly (for a comprehensive review about pronucleotide strategies see *Meier* 1998, *Wagner et al* 2000 and *Meier* 2004). This need drove our laboratories through

a series of studies which have eventually led McGuigan and coworkers to the development of the aryloxy phosphoramidate approach, a widely established pro-nucleotide strategy.[10-12]

Our first studies were focused on the development of alkyl and haloalkyl phosphate triesters of antiviral agents such as vidarabine (araA), AZT, ddC and of the anti-neoplastic agent araC (**a-d** Figure 1.6).
Although displaying some biological activity, dialkyl phosphate derivatives of araA and araC (**e**) failed to boost the activity of the parent NAs. Moreover, the biological data acquired were not sufficient to demonstrate whether the nucleotide, the nucleoside or both had been delivered inside cells.[13,14] In addition, simple AZT dialkyl phosphate derivatives (**f**) were shown to be inactive against HIV. This discouraging result was rationalised as a high stability towards metabolic conversion into the free 5'-monophosphate, which should occur intracellularly in order to exert the desired biological activity.[15] These findings led the way forward to new labile phosphate substituents. However, the kinetics of the phosphorylation to the diphosphate was not considered to have a significant involvement in the bio-activation of AZT.

5'-bis(2,2,2-trichloroethyl) phosphate pronucleotides of AZT and ddC were then synthesised and evaluated, leading to a significant enhancement in activity compared to the simple dialkyl derivatives. However, activity remained considerably reduced when compared to the corresponding NAs. Nevertheless, a stability study in biological medium suggested that P-O-alkyl bonds were preferentially cleaved in these conditions and that release of the nucleotide was favoured by the mechanism of action of these compounds.[16]
In addition, araA and araC haloalkyl derivatives (**g**) showed activities close or even slightly higher than the parent NAs, suggesting that such compounds might act as an intracellular source of free nucleotides.[17] This strategy was further investigated by exploring different degrees of halogenation on the alkyl substituents, but did not lead to relevant improvements.[18]

The first phosphoramidate structure was introduced on AZT pronucleotide derivatives in an attempt to investigate the possibility that an HIV protease might cleave the peptido mimetic P-N bond and free the nucleoside monophosphate only in HIV infected cells.[19] Unsymmetrical phosphates bearing one amino acid moiety displayed a marked activity (**h**), which was a significant improvement in comparison to the inactive simple dialkyl phosphate triesters of AZT. This discovery was a fundamental breakthrough in the development of our aryloxy phosphoramidate technology as the enzymatic cleavage of the P-N bond was conceived as a means to trigger the activation of our pronucleotides.

Supplementary studies concerning the nature of the amino acid and its chain length on the biological activity were investigated. L-Valine, glycine and L-phenylalanine were good substitutes for L-alanine while a substantial reduction in activity was observed with L-leucine and L-isoleucine, implying that the increasing steric bulk of the amino acid side chain would reduce the enzymatic cleavage of the amino acid moiety and by doing so, the activation of these pronucleotides.[20] Elongation of the amino acid chain (moving from α- to β-, γ- etc amino acid derivatives) led to a complete loss of anti-HIV activity, indicating that α-amino acids were markedly preferred.[21]

Given the improvements observed with the haloalkyl esters in the early studies, trichloro- and trifluoroethyl groups were also taken into account. This new series did not show any enhancement in antiviral activity versus the NA. However, among all the haloalkyl derivatives, the trichloroethyl methoxyalaninyl pronucleotide of AZT (**i**) was 50-fold more potent than its ethyl derivative and only 20-fold less potent than the NA.[22] At this point, the importance of a C-protected, N-linked α-amino acid building block was clear, and L-alanine was the most favoured.

Due to the remarkable effect of the amino acid moiety as a phosphate masking group, phosphorodiamidate derivatives of AZT were designed. These involved the use of two amino acids or two simple alkylamino groups (**l** and **m**). All the amino acid derivatives were particularly active while the non-amino acid had reduced efficacy.[23]

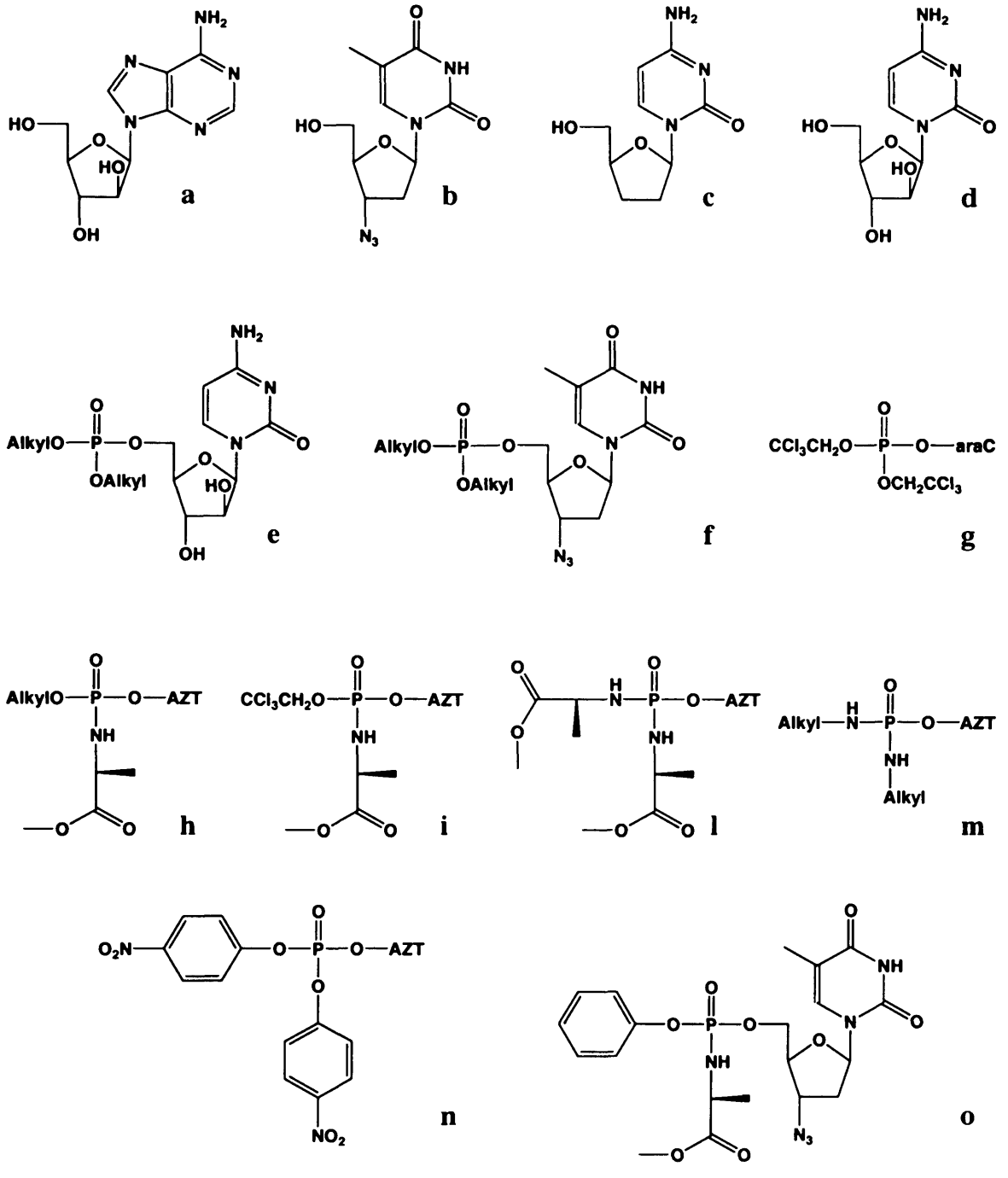


Figure 1.6: From alkyl phosphate esters to the first aryloxy phosphoramidate.

In order to establish the importance of the bridging nitrogen in the phosphoramidate structure, lactate and glycolate derivatives were synthesised as P-O variations of the alaninyl and glycinyl phosphoramidate compounds. Although these compounds showed biological activity, no enhancement in potency was observed compared to AZT.[24]

Another significant discovery was made when aryl moieties were considered as phosphate masking groups. In an investigation about the biological properties of diaryloxy phosphate derivatives, the activity of the bis(p-nitrophenyl) phosphate (**n**) exceeded that of AZT but also toxicity was significantly increased.[25] Due to AZT-insensitivity, JM cells were employed as a "kinase deficient" cell line and used as a test to verify kinase bypass of these compounds. Although more active than AZT (up to 10-fold), the bis(p-nitrophenyl) and other diaryl phosphates subsequently designed[26] did not lead to a significant antiviral effect on this kind of cells. At that time, JM cells were considered to be insensitive towards AZT treatment because of poor phosphorylation. However, it has emerged in more recent studies that an AZT-efflux pump was the real reason for the poor activity observed.[27] Nevertheless, when diaryl phosphates were tested on TK$^-$ CEM cells they lost activity. Now, we can assess these results as a confirmation that monophosphorylation bypass is not a crucial step in the attempt to boost AZT antiviral activity.

Among the aryloxy phosphates tested, there was a derivative which combined the structure of the previous phosphoramidates with a phenyloxy masking group on the phosphate (**o**). It was the only compound that displayed a potent antiviral activity in JM cells: the first aryloxy phosphoramidate synthesised in our laboratories.[25] Given the peculiar response of these cells towards AZT treatment, the retained activity was a sign of nucleotide delivery. Although the term "phosphoramidate" does not fully indicate the exact structure of the molecule, it is considered as an abbreviation for a nucleoside analogue 5'-[aryl-(C-esterified α-aminoacyl)]-phosphate and with this meaning it is used in the present Thesis.

1.3 Application of the phosphoramidate approach

The first aryloxy phosphoramidate was reported by McGuigan et al[25] in 1992 as a pronucleotide form of AZT, the best treatment against HIV known at the time. In a subsequent study, the range of AZT aryloxy phosphoramidate pronucleotides was extended and the compounds tested against HIV-1 and HIV-2 infected cells (CEM/0 and MT-4) as well as TK^- cells (CEM/TK^-). Once again, none of the phosphoramidates had a better antiviral activity than AZT in TK competent cell lines but, while AZT was completely inactive, they all retained marked antiviral activity in the kinase deficient cell line.[28]

From this point on, the application of our pronucleotide approach was directed towards NAs whose activity could be greatly improved by circumventing the first phosphorylation step in cells.
2',3'-Dideoxyuridine (ddU) is a NA essentially inactive against HIV (due to its poor phosphorylation) but application of our strategy led to a significant, selective antiviral effect.[29,30] This finding clearly shows that phosphorylated forms of inactive nucleoside analogues can display a relevant biological activity if they are delivered inside cells.

Therefore, a vast range of antiviral NAs was exploited (Figure 1.7), leading to significant improved antiviral activities in most of the cases.[31-38]

Apart from HIV, other viruses were targeted and particular attention was given to antiherpetic drugs such ACV. Although its pronucleotide showed an improvement against HCMV, it was not superior to acyclovir in the herpes simplex virus assay. In contrast to AZT, ACV monophosphorylation seems to be the key limiting step of its activation and the HCMV data would indicate a successful bypass of it.[39]

The role of the C-protection on the amino acid moiety had been conceived only as a tool to improve membrane passive diffusion but, as studies conducted on d4T phosphoramidates showed, its hydrolysis to the free carboxylic acid would appear to

be a crucial step for the activation of our pronucleotides.[40] Possibly, the aryloxy phosphoramidate of ACV was a poor substrate for the enzymes that initiate activation of our pronucleotides in the herpes virus infected cell lines tested.

The same explanation can be used to rationalise the data observed with 3TC pronucleotides. Although the corresponding aryloxy phosphoramidate showed a reduced anti-HIV activity of only 20-fold in kinase deficient cells compared to the 1,500-fold reduction of the parent NA in the same assay, its antiviral activity in kinase competent cells was much lower than 3TC itself.[41]

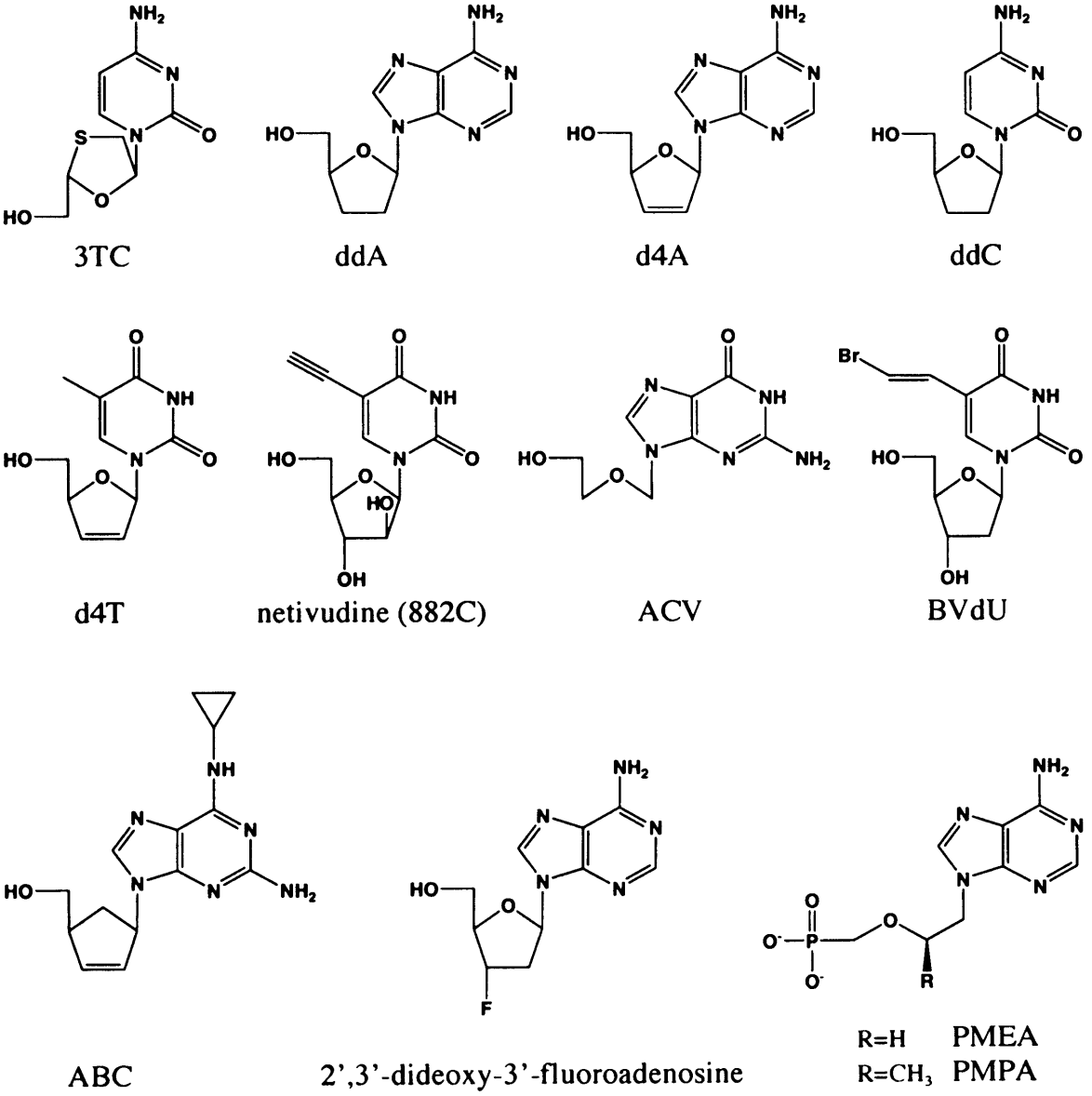


Figure 1.7: Some NAs the phosphoramidate approach has been applied to.

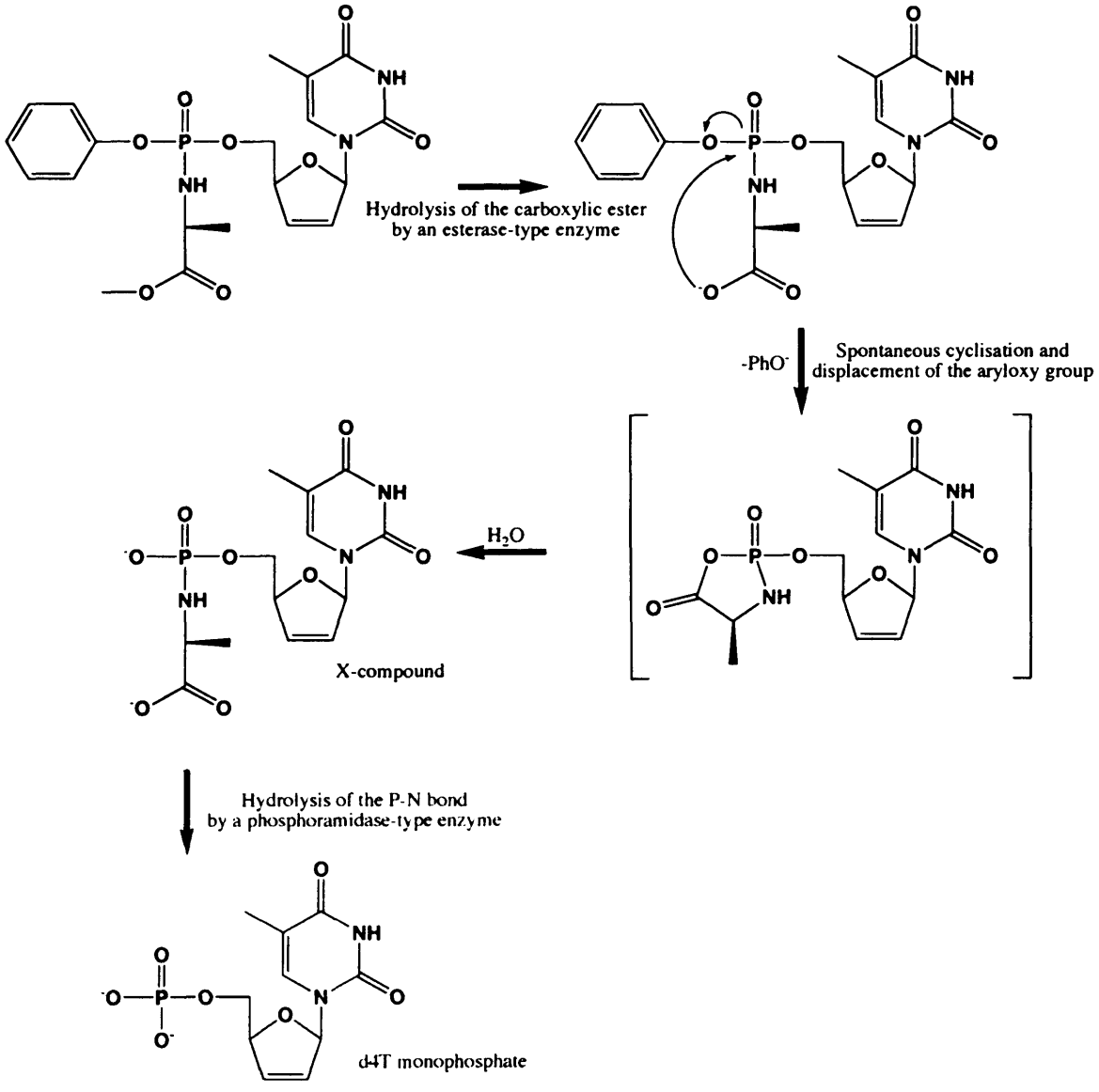
1.4 SARs and postulated mode of action of phosphoramidates

Knowledge held about the way these pronucleotides are able to release intracellular NA monophosphates (Scheme 1.2) and the phosphoramidate structure-activity relationships (Figure 1.8), was obtained through an extensive study conducted on d4T, which can count on over 250 derivatives synthesised to date.[42]

Aryloxy phosphoramidates are lipophilic pronucleotides that are supposed to permeate cell membranes through passive diffusion. Once inside cells, cleavage of the amino acid carboxylic ester moiety is a determinant, first step for the activation of phosphoramidates.[40,43-45] However, as the kinetics of hydrolysis does not linearly correlate with antiviral potency, no clear correlation was found between antiviral activity and stability towards the pig liver esterase assay developed in our laboratories.[45] Nevertheless, cleavage of the ester moiety is a requirement for the biological activity of these compounds since derivatives with an high degree of stability towards our enzymatic assay do not display antiviral activity. Although the nature of the enzyme that catalyses in human cells the hydrolysis of this moiety has not been identified, it must possess an esterase activity. Recently, aryl substituted phosphoramidate derivatives of d4T were found to undergo hydrolysis of the amino acid ester function in the presence of proteases.[46]

Data in TK^- versus TK^+ cells clearly demonstrate that the antiviral activity of d4T phosphoramidates is independent of thymidine kinase mediated phosphorylation.[47,48] The most obvious conclusion is that d4T monophosphate arises from phosphoramidates and it is further phosphorylated to the active triphosphate form. In order to test this hypothesis, ^{3}H-labeled d4T phosphoramidates were incubated in CEM and CEM-TK^- cells and the intracellular formation of the radiolabelled metabolites was studied by HPLC. Firm evidence that d4T phosphoramidates could give rise the corresponding d4T triphosphate was observed, by a mechanism completely independent of thymidine kinase, unlike d4T which could give rise to similar levels of triphosphate, but by an entirely thymidine kinase dependent process. Hydrolysis of d4T phosphoramidates to d4T could not play a major role in the

activation pathway otherwise the generation of d4T triphosphate (and its antiviral activity) would have been significantly reduced in thymidine kinase deficient cells. Furthermore, a different metabolite named 'X-compound' (d4T aminoacyl phosphate) was shown to markedly accumulate in treated cells and appeared to act as a depot of d4T monophosphate. Its structure was identified as the product of hydrolysis of the carboxylic and the aryloxy phosphate esters.[49,50]



Scheme 1.2: Postulated mechanism of intracellular activation of phosphoramidates.

It is our belief that such a metabolite would arise from the enzymatic cleavage of the carboxylic function of the amino acid moiety followed by displacement of the aryl masking group through intramolecular cyclisation. Then, the cyclic intermediate would be spontaneously hydrolysed to the X-compound. The final step would be the conversion of the X-compound to the free nucleotide, namely the cleavage of the P-N bond. Although the enzyme responsible for phosphoramidate hydrolysis has not been determined, direct evidence of intracellular P-N bond hydrolysis by a putative phosphoramidase has been demonstrated by studies of the intracellular metabolism of fluorodeoxyuridine phosphoramidates.[51,52] Recently, yeast Hint1, rabbit Hint1, and chicken Hint have been shown to be AMP-lysine hydrolases, suggesting that this class of enzymes may be responsible for nucleoside phosphoramidase activities.[53-55] A particular attention is given to the possible role of Hints as phosphoramidates activating enzymes in Chapter 8.

According to the postulated mechanism of activation of our pronucleotides, it is now possible to correlate the enormous amount of biological data we have collected to the phosphoramidate structure. SARs are summarised in Figure 1.8.

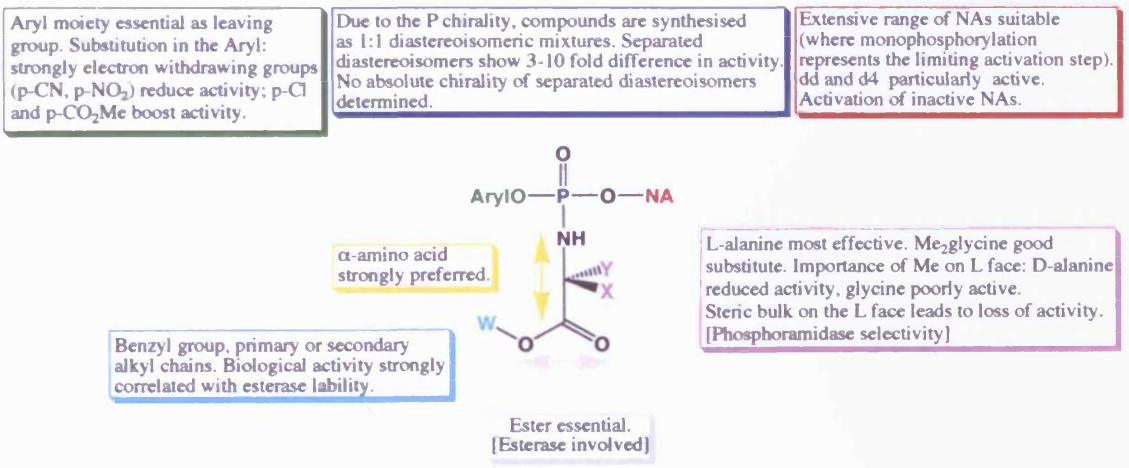


Figure 1.8: Structure-activity relationships of phosphoramidates.

1.5 Aim of work

The development of the phosphoramidate technology was the result of an extensive search for a new prodrug system able to deliver NA monophosphates inside cells. In many cases, our approach emerged as an extremely successful tool in the attempt to boost the biological activity of NAs. The enormous amount of SARs collected was mostly based on the application of our technology to antiviral NAs, d4T in particular.

The aim of the work carried out and presented in the following chapters was to apply our ProTide method to anticancer NAs, which had been only partly taken into consideration. Given the role of cancer as a major cause of death in the developed world[56] and the importance of NAs in the current anticancer therapy, the possible development of phosphoramidates as more potent anticancer agents than existing NA drugs would be particularly beneficial.

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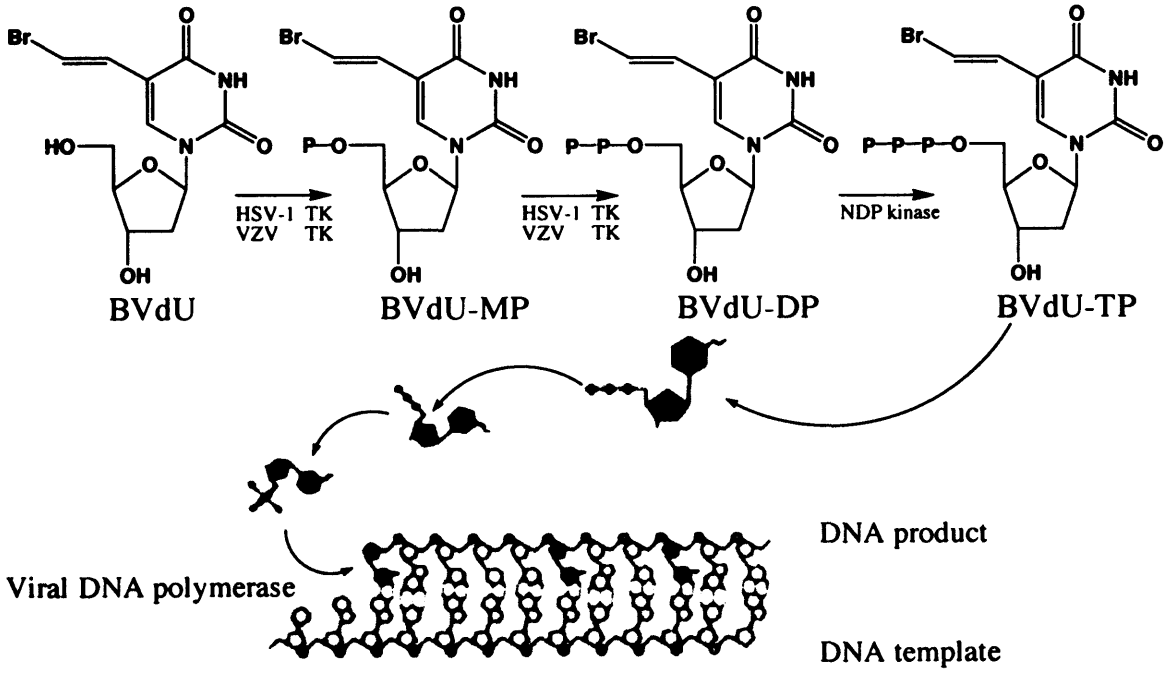
Chapter 2: BVdU naphthyloxy phosphoramidates

2.1 BVdU background

BVdU [(E)-5-(2-bromovinyl)-2'-deoxyuridine, brivudin] (Scheme 2.1) was originally synthesised in 1976 as a potential radiation-sensitizing agent, assuming that it would be incorporated into DNA. When its antiviral activity was discovered, BVdU proved more potent and more selective in its activity against HSV-1 than all other anti-herpes compounds and this is remained the case for twenty five years, until the advent of our bicyclic pyrimidine nucleoside analogues (BCNAs).[1-3] The mechanism of action of BVdU against HSV-1 and VZV depends on a specific phosphorylation by the virus encoded thymidine kinase, which converts BVdU to its 5'-monophosphate and 5'-diphosphate. Upon further phosphorylation by cellular kinases, BVdU triphosphate can then interact with the viral DNA polymerase, either as a competitive inhibitor with respect to the natural substrate, or as an alternative substrate, allowing the incorporation of BVdU triphosphate into the growing DNA chain, affecting both the stability and functioning of the DNA during the replication and transcription processes (Scheme 2.1).[4]



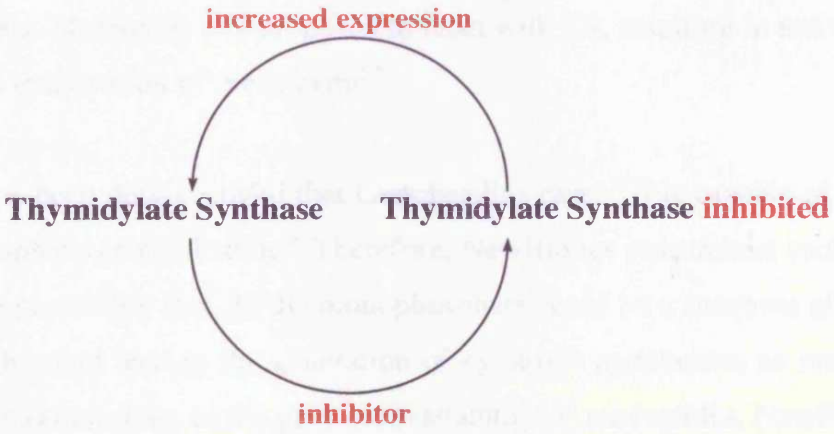
Scheme 2.1: Mechanism of action of BVdU in HSV-1 and VZV infected cells.

The predominant determinant in the antiviral activity of BVdU is the virus encoded thymidine kinase, which is responsible for the first and the second phosphorylation in cells. In fact, when we applied our phosphoramidate approach to BVdU no significant improvements in anti-HSV-1 and anti-VZV potency was observed (only one phosphoramidate showed a 200-fold decrease in antiviral activity in HSV-1 TK^- infected cells, which is quite marked in comparison to the 16,000 fold decrease for BVdU in the same assay).[5] BVdU phosphoramidates were not then further investigated as antivirals.

As mentioned in Chapter 1, the major disadvantage of NAs is their activation to phosphorylated forms. Thus, some NAs are inactive only in appearance and can display a significant biological activity when converted into pronucleotides (examples of this will be given in this Thesis). Unfortunately we only explored the antiviral activity of BVdU phosphoramidates and not other biological effects such as anticancer, as this was considered an unlikely target at that time.

2.2 NB1011: an intriguing BVdU phosphoramidate with anticancer activity

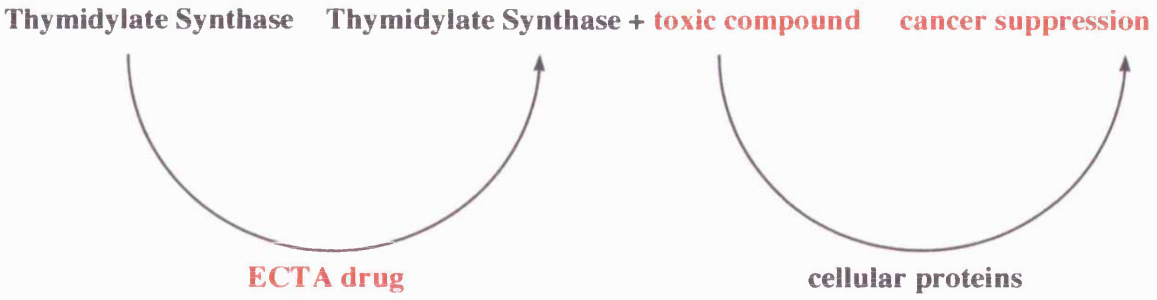
Thymidylate synthase (TS) is a key enzyme in *de novo* synthesis of thymidine. it catalyses the reductive methylation of dUMP to dTMP using 5,10-methylenetetrahydrofolate as a cofactor. TS is a critical enzyme for DNA synthesis in all organisms and is the target for both fluoropyrimidine and antifolate-based chemotherapies. Unfortunately, many tumour cells develop resistance towards these chemotherapies by increasing their TS levels; this is due to the loss of tumour suppressor gene functions, which consequently leads to an abnormal expression of TS. These increased TS levels render cancer cells less sensitive to inhibitors than normal cells; as a consequence, it is often impossible to reach an inhibitory concentration high enough to eradicate cancer cells without severely damaging normal cells. For decades, the focus of drug discovery has been directed to find more potent TS inhibitors. However, better TS inhibition results in even higher levels of TS expression in tumour cells, which further erodes an already poor therapeutic index (Scheme 2.2).



Scheme 2.2: Classical TS inhibition.

It would be particularly advantageous to capitalize on the elevated TS levels by administering a relatively non-toxic compound specifically designed to generate a toxic species as a result of the enzymatic processing. This enzyme-catalysed

therapeutic approach (ECTA) would provide ECTA drugs with a beneficial therapeutic index due to the differential TS levels between tumour (high) and normal cells (low) and this was the principal aim of the Californian company NewBiotics (Scheme 2.3).[6]



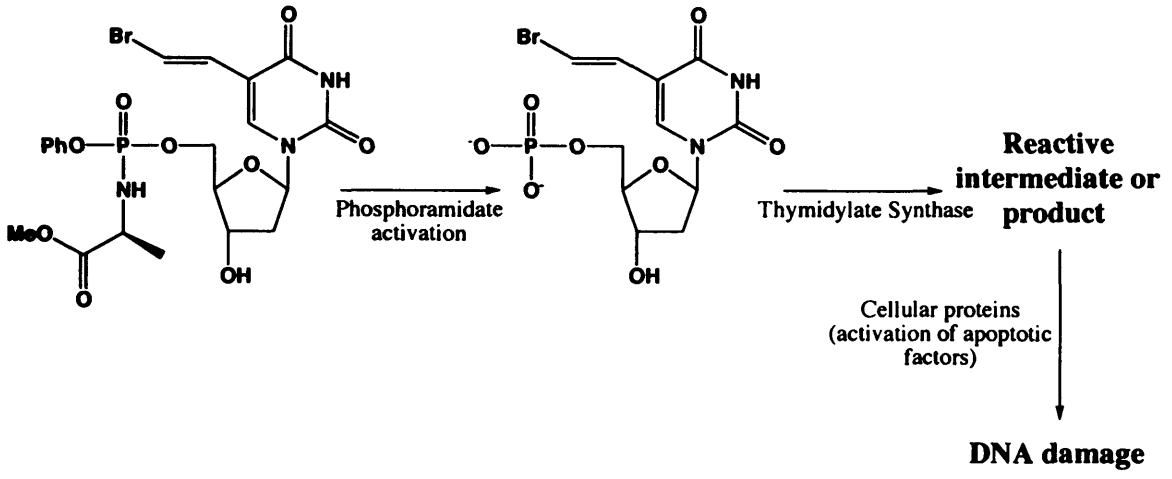
Scheme 2.3: ECTA method.

As described in the previous paragraph, BVdU has been extensively studied as an antiviral agent and its activation in human cells requires phosphorylation by herpes virus-encoded TKs. Moreover, it is proposed to react with TS, resulting in active site modification and inactivation of the enzyme.[7]

Despite this, it has been demonstrated that Lactobacillus casei TS is capable of using BVdU monophosphate as a substrate.[8] Therefore, NewBiotics researchers took into consideration the possibility that BVdU monophosphate could be a substrate also for human TS, which could lead to the generation of cytotoxic metabolites as result of the enzymatic activation. Due to the poor bioavailability of nucleotides, NewBiotics decided to apply our phosphoramidate technology to BVdU monophosphate synthesising the compound NB1011.

Although it has been not determined which TS catalysed products arise from NB1011, it has been found that NB1011 cytotoxicity is TS-dependent. Thus, increased overexpression of TS in tumour cells enhances their sensitivity to NB1011 and moreover its activity can be antagonised by TS inhibitors.[9] The suggested

mechanism of action for NB1011 is rather unusual for a nucleotide-based anticancer drug: inside cells, NB1011 would be converted into the corresponding monophosphate that would be activated by TS giving rise to a reactive intermediate or product. The reactive intermediate or product would covalently modify intracellular proteins and not DNA, but TS itself would appear to escape irreversible inactivation.[10] Nevertheless, NB1011 treatment results in activation of pro- and apoptotic factors which are responsible for the final DNA damage (Scheme 2.4).[11]



Scheme 2.4: NB1011 postulated mode of action.

According to NewBiotics results, NB1011 can be used for selectively targeting colorectal tumour cells overexpressing TS. Preclinical and phase I/II clinical results involving patients with colorectal cancer indicated that NB1011 is well tolerated at all dose levels tested, without significant haematology toxicity. Given intravenously, the half-life of NB10 11 is just under one hour. Data have not been fully disclosed to date.[12-13]

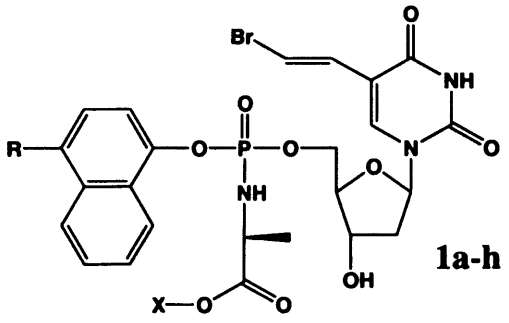
Based on the intriguing results obtained by NewBiotics, we decided to reconsider the application of our phosphoramidate approach to BVdU and, given our experience in this field, to target a boost in anticancer activity.

2.3 BVdU naphthyloxy phosphoramidates and their biological evaluation

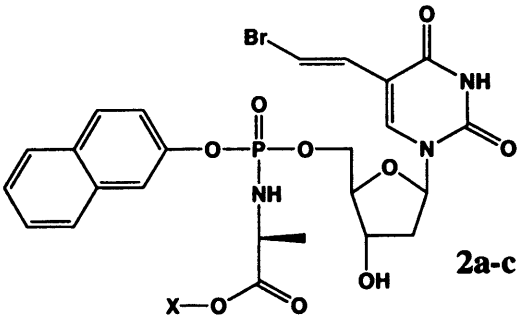
As part of our ongoing studies of BVdU protide SARs,[14] modifications to the aryl group on the phosphate region were planned. Since the aryloxy phosphoramidate approach was invented, phenyl (with a wide range of substitutions) was mostly considered. Nevertheless, a different aryl group might better tune the biological activity of phosphoramidates: firstly, in terms of overall lipophilicity of the molecule, it might facilitate passive diffusion. Secondly, according to the leaving group ability required by the activation of phosphoramidates in cells, it might result as a useful tool in an attempt to influence the kinetics of the spontaneous displacement of the aryl moiety.

Owing to these observations, a new series of naphthyl L-alaninyl phosphoramidates was designed, taking into consideration both α- and β-naphthyl and different ester chains (Table 2.1).

Cpd	R	X
1a	H	Me
1b	H	Et
1c	H	Bn
1d	MeO	Me
1e	MeO	Bn
1f	Cl	Me
1g	Cl	Bn
1h	H	tBu
2a	-	Me
2b	-	Et
2c	-	Bn



1a-h



2a-c

Table 2.1: Naphthyloxy BVdU phosphoramidates.

The phosphoramidates **1a-h** and **2a-c** were evaluated for their cytostatic activity against two different tumour cell lines in vitro: human breast cancer cell line MDA MB 231 and prostate cancer cell line PC-3 (Table 2.2). The method is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt, into an insoluble formazan precipitate that is dissolved and quantified by spectrophotometry.[15]

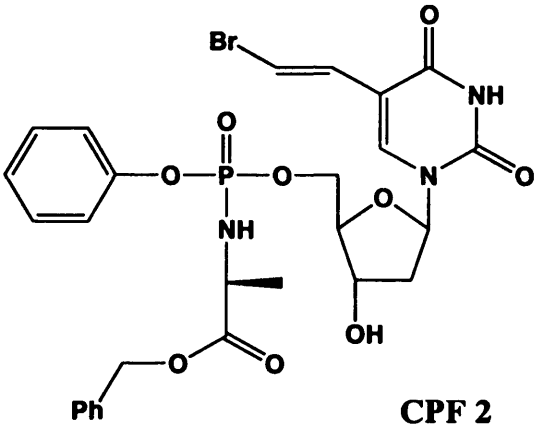
Cpd	Aryl	Ester	breast MDA MB 231	prostate PC-3
NB1011	Ph	Me	79	155
CPF 2	Ph	Bn	34	19
1a	1-Naph	Me	12.9	10.6
1b	1-Naph	Et	11.6	47
1c	1-Naph	Bn	15.2	1.7
1d	1-(4-MeO)-Naph	Me	6.3	12.4
1e	1-(4-MeO)-Naph	Bn	11.1	19.3
1f	1-(4-Cl)-Naph	Me	9	22.6
1g	1-(4-Cl)-Naph	Bn	12.8	20.5
1h	1-Naph	tBu	4.8	11
2a	2-Naph	Me	21	53.2
2b	2-Naph	Et	4.03	4.3
2c	2-Naph	Bn	14.1	17.7

Table 2.2: Naphthyloxy BVdU phosphoramidates, biological evaluation ($EC_{50}/\mu M$).

In our hands NB1011 is only moderately active in vitro, with EC_{50} values of 79-155 μM. However, by introducing naphthyl as new aryl moiety and slightly modifying the amino acid ester chain, the cytostatic activity was significantly increased. The simple substitution of phenyl with naphthyl (**1a** and **2a**) led to an

enhanced activity for both of the two cancer cell lines compared to NB1011, with α-naphthyl being generally more active than the β-isomer.

In the breast cancer cell line (MDA MB 231), EC_{50} values of the tested compounds were in such a small range (4-21 μM) that SARs were rather difficult to recognise. Significantly, compounds **1d**, **1f** and **2b** displayed a 10-fold boost in potency versus NB1011 and no benefit seemed to be given by the introduction of a benzylic ester chain (as a comparison, the L-alaninyl benzylic ester derivative of NB1011 is included in Table 2.2; **CPF 2**, Figure 2.1).



CPF 2

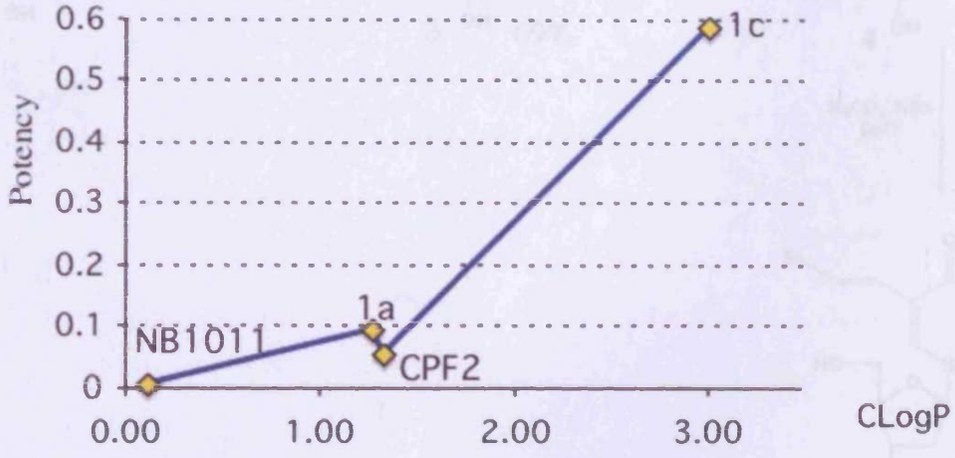
Figure 2.1: Structure of CPF 2.

Conversely, the prostate cancer cell line results (PC-3) showed a 6-fold increased activity for the benzyl ester of the unsubstituted α-naphthyl derivative (**1c**) compared to the methyl ester (**1a**). This trend reflected what we had already observed with the phenyl series (**CPF 2** versus **NB1011**) and it was also confirmed by the β-naphthyl derivatives (**2a** versus **2c**) although they displayed a reduced activity when compared to the α-naphthyls. Benzyl esters had shown to be generally more active than methyls in our anti-HIV d4T phosphoramidate SAR,[16] probably due to a higher stability towards extracellular metabolism, a more efficient activation by cellular carboxyl esterases and/or to a more effective passive diffusion through cell membranes.

The tert-butyl ester derivative (**1h**) showed interesting cytostatic activities against the two cancer cell lines. This was in contrast to our previous SARs conducted on d4T phosphoramidates, where the poor antiviral activity showed by the corresponding L-alaninyl tert-butyl ester derivative was explained with the extremely low esterase-mediated cleavage in our enzymatic tests.[17]

In general, introduction of an electron-donating (**1d,e**) and –withdrawing (**1f,g**) substituents on the naphthyl, did not lead to further improvements.

Interestingly, compound **1c** shows a 100-fold boost in potency versus NB1011 and 10-fold versus **CPF 2** in the prostate cancer cell line. The trend **1c > CPF 2 > NB1011** reproduces the lipophilic character of these compounds and it is in full agreement with the role of phosphoramidates as membrane soluble pronucleotides (Table 2.3).

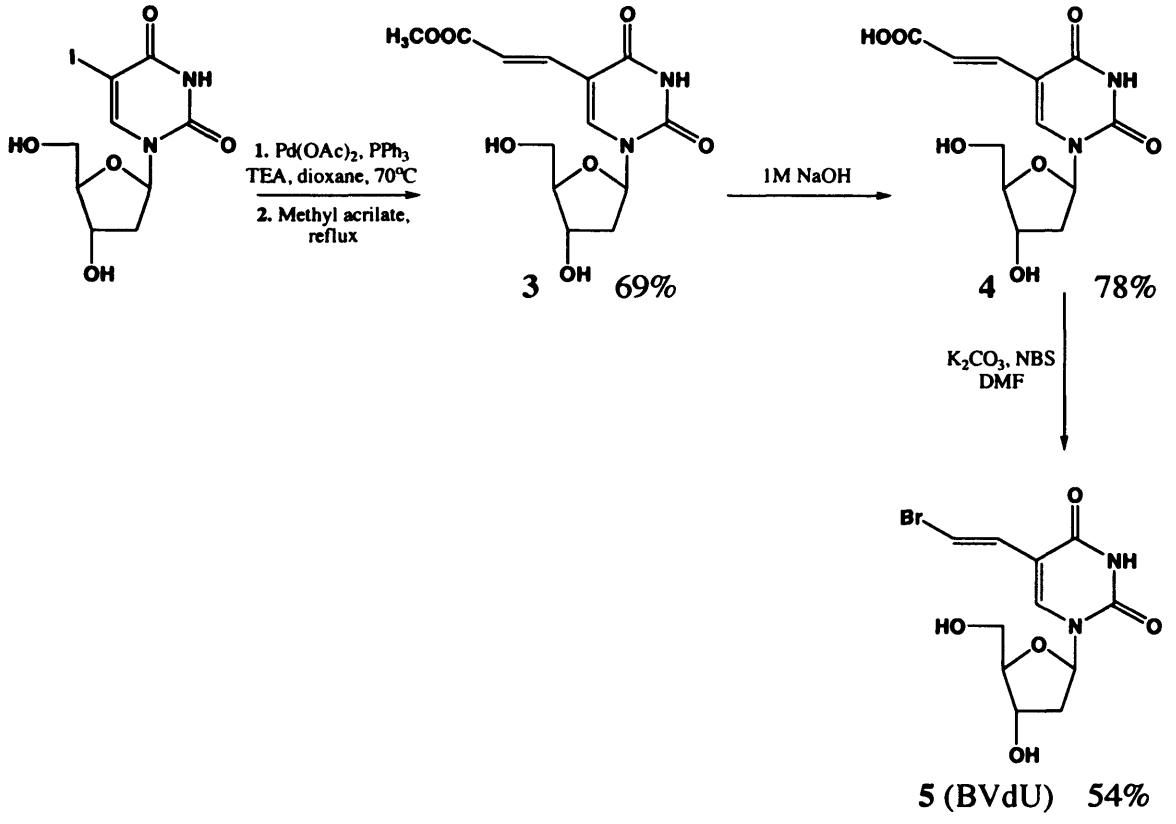


Cpd	CLogP	Potency ($1/EC_{50}$)
NB1011	0.11	0.006
CPF2	1.33	0.053
1a	1.28	0.094
1b	1.81	0.021
1c	3.00	0.588
1h	2.52	0.091

Table 2.3: ClogP and PC-3 potency data comparison.

2.4 Synthesis of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU)

BVdU was synthesised according to a convenient procedure reported by Ashwell.[18] (E)-5-(2-Carbomethoxyvinyl)-2'-deoxyuridine (**3**) was obtained from 2'-deoxy-5-iodouridine by a palladium-catalysed coupling reaction with methyl acrylate. Subsequent hydrolysis of **3** under basic conditions yielded (E)-5-(2-carboxyvinyl)-2'-deoxyuridine (**4**), which was converted into the final product **5** by treatment with N-bromosuccinimide (Scheme 2.5).

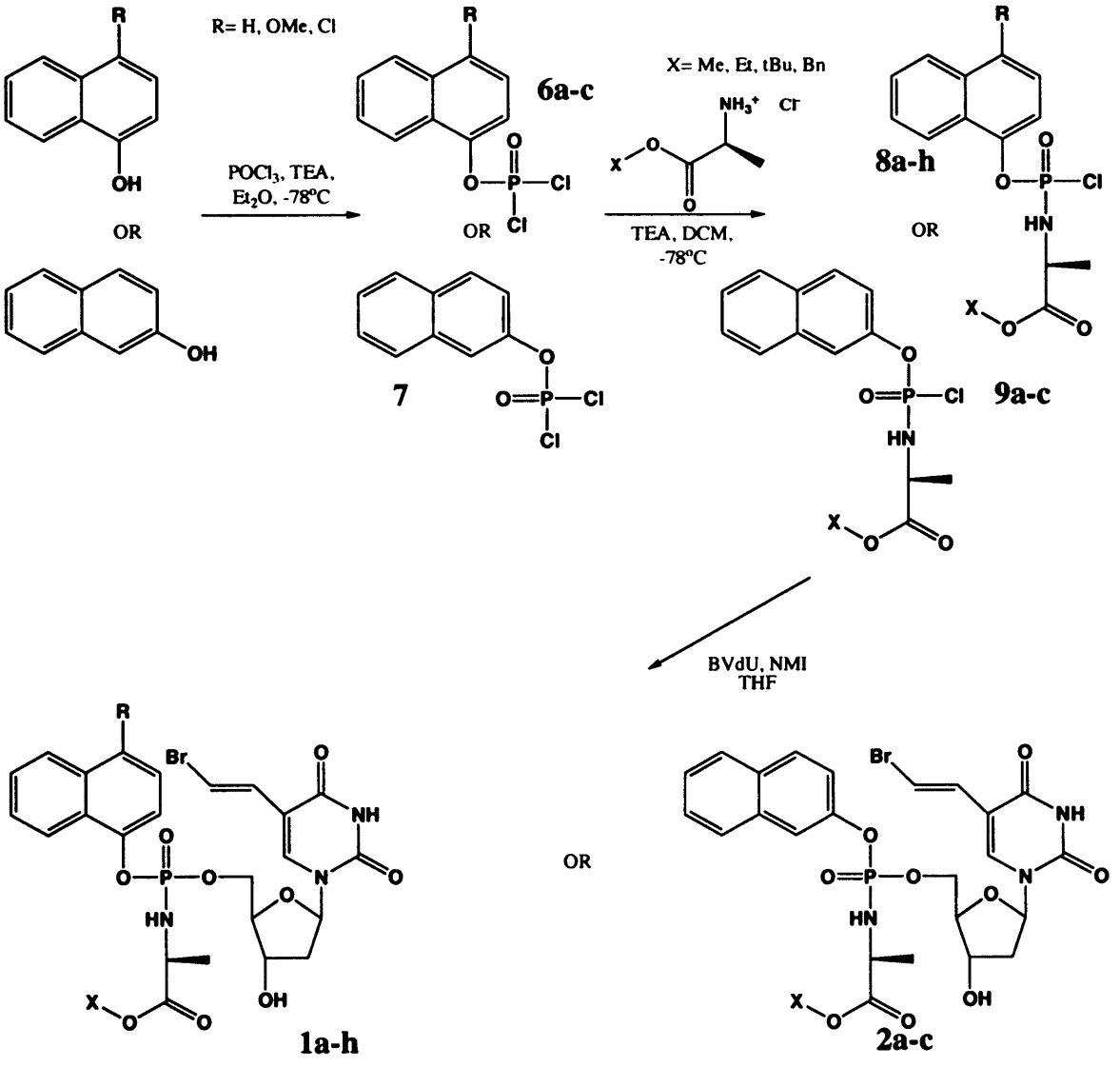


Scheme 2.5: Synthesis of BVdU.

^{1}H and ^{13}C NMRs of final compound **5** were consistent with the reported literature[13] and the assignment of the (E)-configuration is based upon the coupling constant of the vinylic protons (^{3}J=13.6 MHz). Details of the synthetic procedure are reported in the experimental section.

2.5 Synthesis of BVdU naphthyloxy phosphoramidates

Compounds **1a-h** and **2a-c** (Scheme 2.6) were synthesised according to the established phosphorochloridate chemistry reported in the literature by us.[14,19-21] Phosphorylation of the corresponding naphthol with phosphorus oxychloride at low temperature and in the presence of triethylamine (TEA), gave naphthyl dichlorophosphate (**6a-c** and **7**, Table 2.4). The product was obtained as a crude oil and always used in the next step without further purification.



Scheme 2.6: Synthesis of BVdU naphthyloxy phosphoramidates.

Cpd	R	Yield (%)	^{31}P-NMR ($CDCl_3$) δ
6a	H	84.5	5.07
6b	OMe	Quantitative	5.33
6c	Cl	Quantitative	5.15
7	-	90.0	5.01

Table 2.4: naphthyl dichlorophosphates.

Dichlorophosphates were then reacted under similar conditions with L-alanine ester salts to give the naphthyl(L-alaninyl ester) phosphorochloridates (**8a-h** and **9a-c**, Table 2.5), which were used in the final step either crude or purified by flash chromatography. The presence of two peaks on proton decoupled ^{31}P NMR was due to these compounds being a diastereoisomeric mixture at the phosphate.

Cpd	R	X	Yield (%)	^{31}P-NMR ($CDCl_3$) δ
8a	H	Me	57.2	9.62, 9.40
8b	H	Et	Quantitative	9.67, 9.46
8c	H	Bn	Quantitative	9.57, 9.40
8d	OMe	Me	27.9	10.28, 10.36
8e	OMe	Bn	49.3	10.16, 10.26
8f	Cl	Me	80.7	9.61, 9.37
8g	Cl	Bn	49.2	9.82
8h	H	tBu	23.7	9.88, 9.75
9a	-	Me	quantitative	9.29, 9.01
9b	-	Et	quantitative	9.76, 9.61
9c	-	Bn	84.7	9.25, 8.95

Table 2.5: naphthyl phosphorochloridates.

Derivative **8g** showed one singlet only. This was probably due to the low resolution of the NMR experiment recorded.
Phosphorochloridates **8d,e** and **8g,h** were purified through column chromatography and this was the reason for their lower yields compared to those used unpurified. However, purification of the intermediate phosphorochloridates showed to improve the yields of the final compounds (**1d,e** and **1g,h** respectively).

The final coupling between BVdU and the target phosphorochloridates was performed according to the Van Boom procedure[21] in the presence of 1-methylimidazole (NMI), which acts as coupling reagent as well as hydrochloric acid scavenger. Furthermore, NMI offered the advantage of an easy work up of the crude product through a mild acidic extraction.

Cpd	R	X	Yield (%)	^{31}P-NMR ($CDCl_3$) δ
1a	H	Me	10.3	4.88, 4.80
1b	H	Et	2.1	4.97, 4.89
1c	H	Bn	1.3	4.93, 4.76
1d	OMe	Me	37.5	5.30
1e	OMe	Bn	15.5	5.34, 5.21
1f	Cl	Me	4.3	4.85, 4.94
1g	Cl	Bn	36.8	4.90, 4.80
1h	H	tBu	25.0	5.86, 5.83
2a	-	Me	5.3	4.92, 4.62
2b	-	Et	13.4	4.99, 4.68
2c	-	Bn	9.1	4.91, 4.61

Table 2.6: Naphthyloxy phosphoramidates.

Due to the chirality of the phosphorus center, the final products (**1a-h** and **2a-c**, Table 2.6) were obtained as mixtures of two diastereoisomers in a general ratio 1:1. Although a single signal appears on ^{31}P NMR for derivative **1d**, the presence of a diastereoisomeric mixture could be clearly observed on ^{1}H NMR (e.g. two singlets for the methyl ester).

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Chapter 3: BVdU naphthyloxy phosphoramidates bearing amino acid variations

3.1 Amino acid variations

The preliminary biological evaluation of the L-alanine series would denote naphthyl to be an excellent aryl candidate as a masking group on the phosphate moiety. Thus, naphthyl derivatives containing modifications to the amino acid moiety (Figure 3.1) seemed promising targets in an attempt to identify any structure-activity relationships and further improve the cytostatic activity against the cancer cell lines employed. In fact, the nature of the amino acid group appeared from our previous SARs to be of fundamental importance for the biological activity of phosphoramidates. Based upon the postulated activation of phosphoramidates by esterase- and phosphoramidase-type enzymes, a different amino acid core would be responsible for different kinetics during the activation process and lead to a different degree of biological activity, as reported in the literature.[1-4]

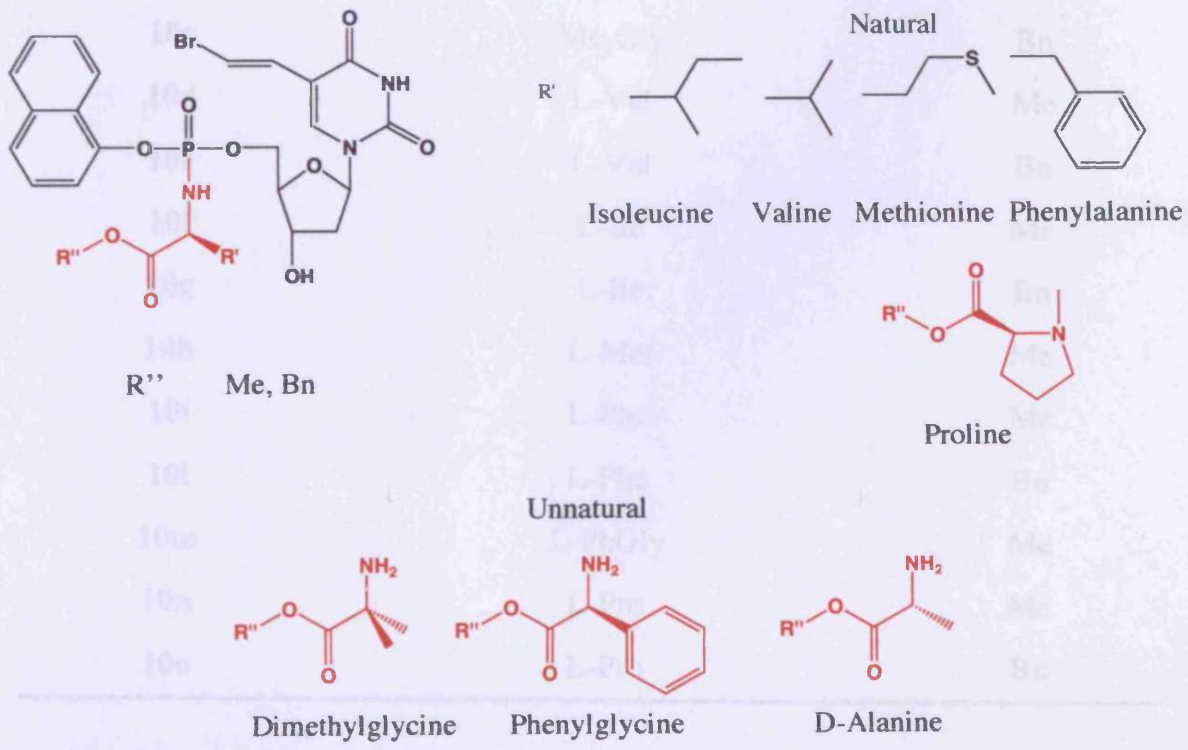


Figure 3.1: Amino acid variations to BVdU naphthyloxy phosphoramidates.

Furthermore, from our first study on BVdU phenyloxy phosphoramidates, dimethylglycine (unnatural amino acid) emerged as a possible substitute for L-alanine and would strongly indicate the amino acid moiety as a key region for further SAR investigations.[5]

In order to understand the role of the amino acid structure, natural and unnatural amino acids were considered (Table 3.1). The D-alanine derivative could enable us to understand whether the position of the methyl on the L face of alanine is necessary to display biological activity. Consequently, dimethylglycine can be considered as a combination of L- and D-alanine.

Cpd	Amino Acid	R''
10a	D-Ala	Bn
10b	Me_2Gly	Me
10c	Me_2Gly	Bn
10d	L-Val	Me
10e	L-Val	Bn
10f	L-Ile	Me
10g	L-Ile	Bn
10h	L-Met	Me
10i	L-Phe	Me
10l	L-Phe	Bn
10m	L-PhGly	Me
10n	L-Pro	Me
10o	L-Pro	Bn

Table 3.1: Synthesised BVdU naphthyloxy phosphoramidates bearing amino acid variations.

L-Valine, L-isoleucine and L-methionine derivatives were designed in order to explore the effect of the L-alanine side chain branching on biological activity. L-Phenylalanine is a sort of branched alanine derivative where one of the hydrogens of the methyl is substituted by a phenyl ring. At the same time, the presence of an aromatic ring on the side chain might involve interactions with hydrophobic residues of target enzymes. Therefore, L-phenylglycine was also designed.

L-Proline, possessing a five-member ring, is a unique derivative among natural amino acids which was considered by our SARs.

Generally, methyl and benzyl esterified amino acids were employed.

3.2 Biological evaluation

The phosphoramidates **10a-o** were evaluated for their cytostatic effect against a panel of tumour cell lines in vitro: human breast cancer cell line MDA MB 231, prostate cancer cell line PC-3 and bladder cancer cell line T24 (Table 3.2). In order to estimate the relevance of this new series of BVdU naphthyloxy phosphoramidates, the biological data of the lead compound NB1011 and compound **1c** (1-naphthyl and L-alanine benzyl ester as phosphate masking groups) are included in Table 3.2.

				breast	prostate	bladder
Cpd	**Aryl**	**Amino Acid**	**Ester**	**MDA MB 231**	**PC3**	**T24**
NB1011	Ph	L-Ala	Me	79	155	-
1a	1-Naph	L-Ala	Me	12.9	10.6	-
1c	1-Naph	L-Ala	Bn	15.2	1.7	-
10a	1-Naph	D-Ala	Bn	6.3	6.1	2.8
10b	1-Naph	Me_2Gly	Me	0.32	65.9	-
10c	1-Naph	Me_2Gly	Bn	2.7	1.5	-
10d	1-Naph	L-Val	Me	14.8	15.8	43.5
10e	1-Naph	L-Val	Bn	5.9	8.3	12.7
10f	1-Naph	L-Ile	Me	1.5	6.9	-
10g	1-Naph	L-Ile	Bn	130	1.4	-
10h	1-Naph	L-Met	Me	28.1	44.6	19.6
10i	1-Naph	L-Phe	Me	8.5	10.2	5.3
10l	1-Naph	L-Phe	Bn	1.96	5.8	269
10m	1-Naph	L-PhGly	Me	105	1.7	-
10n	1-Naph	L-Pro	Me	6.5	10.5	0.4
10o	1-Naph	L-Pro	Bn	-	-	-

Table 3.2: Compounds **10a-o**, biological evaluation ($EC_{50}/\mu M$).

Biological evaluations for compound **10o** are in progress. The evaluation against the bladder cancer cell line T24 became available only recently to our biological collaborators. Therefore, some of the compounds synthesised were not tested against this latter cell line.

For the prostate cancer cell line (PC3), lipophilicity (Figure 3.2) seemed to play a fundamental role for the biological activity of phosphoramidates: the activity of compounds bearing the same amino acid moiety (dimethylglycine: **10b,c**; L-valine: **10d,e**; L-isoleucine: **10f,g**) was boosted every time the ester group was changed from methyl to benzyl, leading to a 100-fold increase versus NB1011 (entries **10c** and **10g**).

Considering the data acquired for the d4T phenyloxy phosphoramidate series, after overnight incubation in rat liver enzyme preparation, the L-alanine benzyl ester derivative (higher antiviral activity) appeared to be mainly converted to the corresponding aminoacyl metabolite (92%), while the L-alanine methyl ester derivative (lower antiviral activity) was cleaved to the corresponding nucleoside monophosphate in 67%.[6] This would suggest that, apart from a different degree of passive diffusion, different kinetics for the phosphoramidate activation could also occur when the same amino acid bears different ester chains and also stability towards extracellular metabolism might be affected.

Lastly, the presence of a highly lipophilic amino acid such as phenylglycine led to a significant activity (**10m**).

Conversely, this trend (Bn>Me) was not applicable to the breast cancer cell line (MDA MB 231). Activation of phosphoramidates has been reported to be dependent on the action of carboxyl esterases and phosphoramidases;[7-9] it is possible that different cell lines might possess diverse enzymatic pools which could be responsible for the differences observed in the biological data.

Nevertheless, naphthyl phosphoramidates showed significant activities also against the breast cancer cell line, notably compounds **10c**, **10f** and **10l**, which displayed between 30- and 50-fold increase in potency versus NB1011.

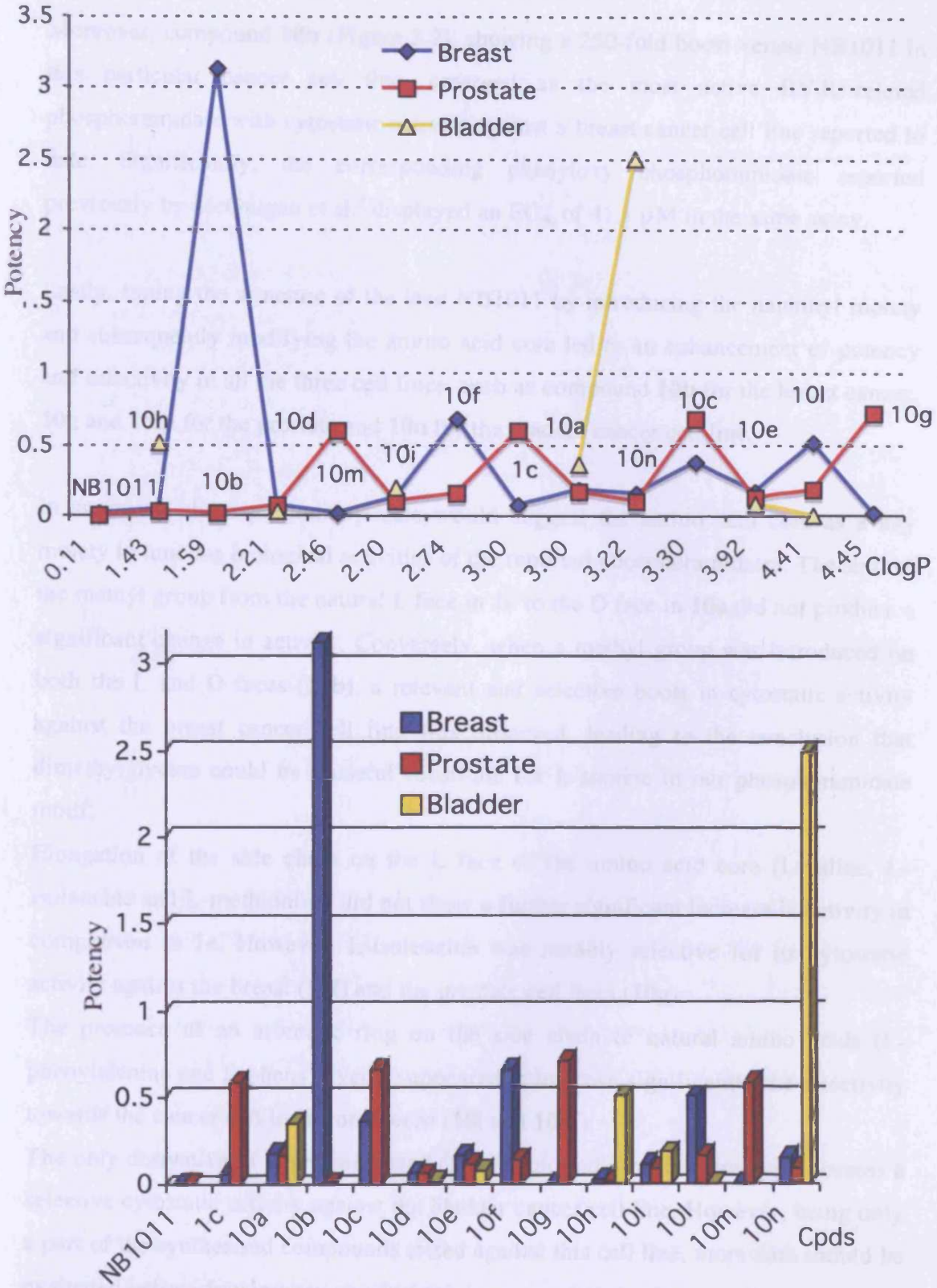


Figure 3.2: CLogP[10] and potency ($1/EC_{50}$) data plots for tested compounds.

Moreover, compound **10b** (Figure 3.2), showing a 250-fold boost versus NB1011 in this particular cancer cell line, emerged as the most active BVdU-related phosphoramidate with cytostatic activity against a breast cancer cell line reported to date. Significantly, the corresponding phenyloxy phosphoramidate reported previously by McGuigan et al.[5] displayed an EC_{50} of 41.1 μM in the same assay.

Lastly, tuning the structure of the lead NB1011 by introducing the naphthyl moiety and subsequently modifying the amino acid core led to an enhancement of potency and selectivity in all the three cell lines, such as compound **10b** for the breast cancer, **10g** and **10m** for the prostate and **10n** for the bladder cancer cell line.

In summary, these preliminary data would suggest the amino acid core as a key moiety to tune the biological activities of the reported phosphoramidates. The shift of the methyl group from the natural L face in **1c** to the D face in **10a** did not produce a significant change in activity. Conversely, when a methyl group was introduced on both the L and D faces (**10b**), a relevant and selective boost in cytostatic activity against the breast cancer cell line was observed, leading to the conclusion that dimethylglycine could be a useful substitute for L-alanine in our phosphoramidate motif.

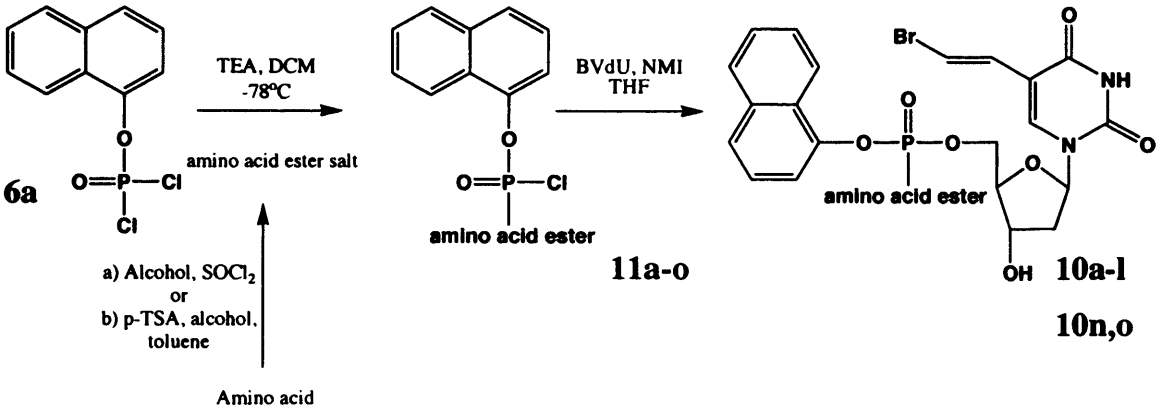
Elongation of the side chain on the L face of the amino acid core (L-valine, L-isoleucine and L-methionine) did not show a further significant increase in activity in comparison to **1c**. However, L-isoleucine was notably selective for its cytostatic activity against the breast (**10f**) and the prostate cell lines (**10g**).

The presence of an aromatic ring on the side chain of natural amino acids (L-phenylalanine and L-phenylglycine) appeared to improve significantly the selectivity towards the cancer cell lines considered (**10l** and **10m**).

The only derivative of L-proline tested (**10l**) would indicate this moiety to possess a selective cytostatic activity against the bladder cancer cell line. However, being only a part of the synthesised compounds tested against this cell line, more data should be evaluated before drawing any conclusions.

3.3 Synthesis of BVdU naphthyloxy phosphoramidates containing amino acid variations

The method used for the synthesis of compounds **10a-o** was based on the same phosphorochloridate chemistry previously described (Scheme 3.1). The amino acid esters employed were commercially available or readily prepared by reaction of the appropriate alcohol with either thionyl chloride ($SOCl_2$) or para-toluene sulfonic acid (p-TSA) in toluene using a Dean-Stark apparatus.



Scheme 3.1: Synthesis of BVdU naphthyloxy phosphoramidates.

1-Naphthyl dichlorophosphate (**6a**) was coupled to the corresponding amino acid ester salts under the standard procedure to give phosphorochloridates **11a-o** (Table 3.4). For the L-alanine phosphoramidate series described in Chapter 2, using purified phosphorochloridates were higher yielding in comparison to the reactions carried out with crude reagents. Therefore, column chromatography was systematically performed on the phosphorochloridates **11a-o**.

Apart from the enantiomeric mixtures **11b** and **11c** (being the phosphate the only chiral centre), all the phosphorochloridates were obtained as a mixture of two diastereoisomers. The presence of two diastereoisomers was confirmed by the presence of two signals on ^{31}P proton decoupled NMR (1:1 ratio).

Cpd	Amino Acid	Ester	Yield (%)	^{31}P-NMR ($CDCl_3$) δ
11a	D-Ala	Bn	23.6	9.65, 9.54
11b	Me_2Gly	Me	41.7	7.41
11c	Me_2Gly	Bn	32.1	7.32
11d	L-Val	Me	48.5	11.24, 10.72
11e	L-Val	Bn	90.0	11.32, 10.72
11f	L-Ile	Me	48.9	10.84, 10.39
11g	L-Ile	Bn	75.6	11.03, 10.75*, 10.45
11h	L-Met	Me	37.8	10.30, 10.11
11i	L-Phe	Me	61.5	9.67, 9.91
11l	L-Phe	Bn	82.3	9.37, 9.55, 9.22*
11m	L-PhGly	Me	34.3	9.13
11n	L-Pro	Me	75.3	9.38, 9.34
11o	L-Pro	Bn	67.5	9.49, 9.30

Table 3.4: Naphthyl phosphorochloridates **11a-o**.

Phosphorochloridates **11g** and **11l** showed a third, minor signal (asterisk in Table 3.4) in the same chemical shift area of the two diastereoisomers. Although not isolated in the present cases, from our experience, side reaction products arising from the double coupling of amino acids with phosphorodichloridates (naphthyl phosphorodiamidates) might be responsible for such signals.

Compound **11m** showed a single signal on ^{31}P NMR which can either be due to the presence of one single diastereoisomer or the overlapping of the two diastereoisomeric signals. However, the presence of a diastereoisomeric mixture could be clearly observed on ^{1}H NMR (two singlets for the methyl ester) and therefore, the two diastereoisomers must have been overlapping on the ^{31}P NMR spectrum recorded.

As already noted, coupling of BVdU with purified phosphorochloridates led generally to higher yields (Table 3.5). The low yields observed for **10g** and **10l** were due to an extensive purification process (repeated column chromatography and usage of preparative thin layer chromatography).

Compounds **10b** and **10o** were obtained after a single column chromatography. The bulky five-member ring of compound **10o** (L-proline) further hindered by a benzyl ester moiety, might have reduced the reactivity of this phosphorochloridate.

Cpd	Amino Acid	Ester	Yield (%)	^{31}P-NMR δ*
10a	D-Ala	Bn	37.6	6.18, 5.82
10b	Me_2Gly	Me	6.5	3.60, 3.50
10c	Me_2Gly	Bn	41.9	4.29, 4.22
10d	L-Val	Me	39.8	6.76, 6.54
10e	L-Val	Bn	27.0	6.82, 6.48
10f	L-Ile	Me	46.5	6.66, 6.40
10g	L-Ile	Bn	11.7	6.01, 5.58
10h	L-Met	Me	24.4	6.18, 5.82
10i	L-Phe	Me	41.7	5.77, 5.40
10l	L-Phe	Bn	12.9	4.59, 4.34
10m	L-PhGly	Me	6.8	5.53, 5.44
10n	L-Pro	Me	49.7	3.75, 3.40
10o	L-Pro	Bn	4.2	3.72, 3.42

Table 3.5: Naphthyl phosphoramidates **10a-o.*** ^{31}P-NMR samples were run in either $CDCl_3$ or MeOD, see experimental section for further details.

For compound **10m,** coupling with BVdU was performed in the presence of tBuMgCl instead of the usual NMI (see experimental section for further details) in an

attempt to verify whether a Grignard reagent could improve the yield. In this case it did not appear to be useful.

Lastly, ^{31}P NMRs of compound **10n** and **10o** did not show the usual 1:1 ratio between the two diastereoisomers, a typical behaviour for phosphoramidates containing proline (ratio 1:6) (Figure 3.3).

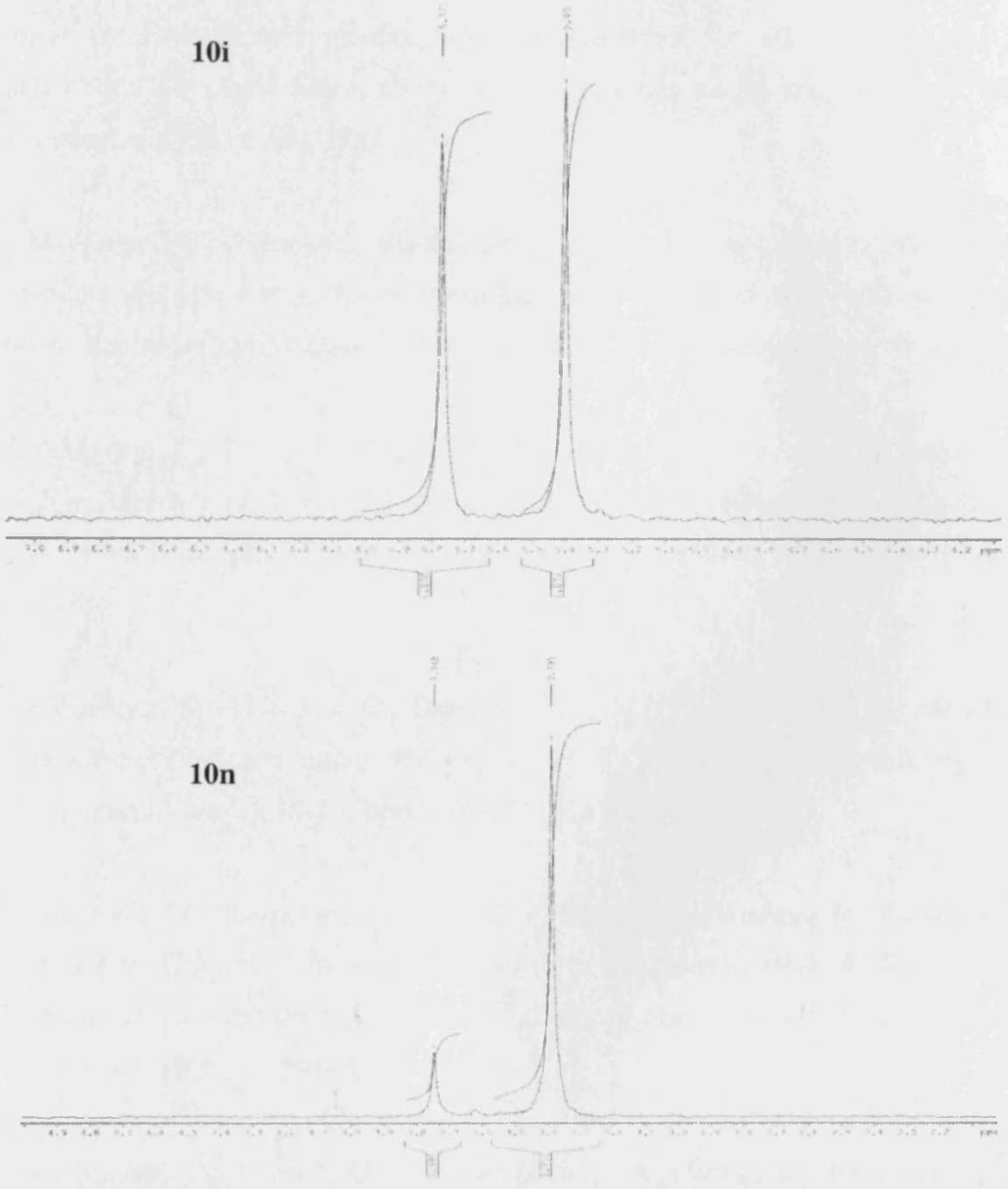


Figure 3.3: ^{31}P NMR of phosphoramidates: **10i** as an example of typical 1:1 diastereoisomeric ratio; **10n** shows the 1:6 ratio for proline based phosphoramidates.

Possibly, the steric hindrance generated by the cyclic chain would either induce a different reactivity for the two diastereoisomers constituting the corresponding phosphorochloridate, or a higher stability for one of the two diastereoisomers of the final phosphoramidate product.

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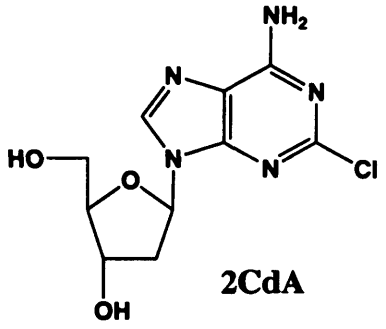
Chapter 4: Cladribine phosphoramidates

4.1 Cladribine background

Cladribine (2-chloro-2'-deoxyadenosine, 2CdA, Figure 4.1) is a potent anticancer and immunosuppressive purine NA with a preferential lymphocytotoxicity. The development of 2CdA derives from the understanding of the pathogenesis of



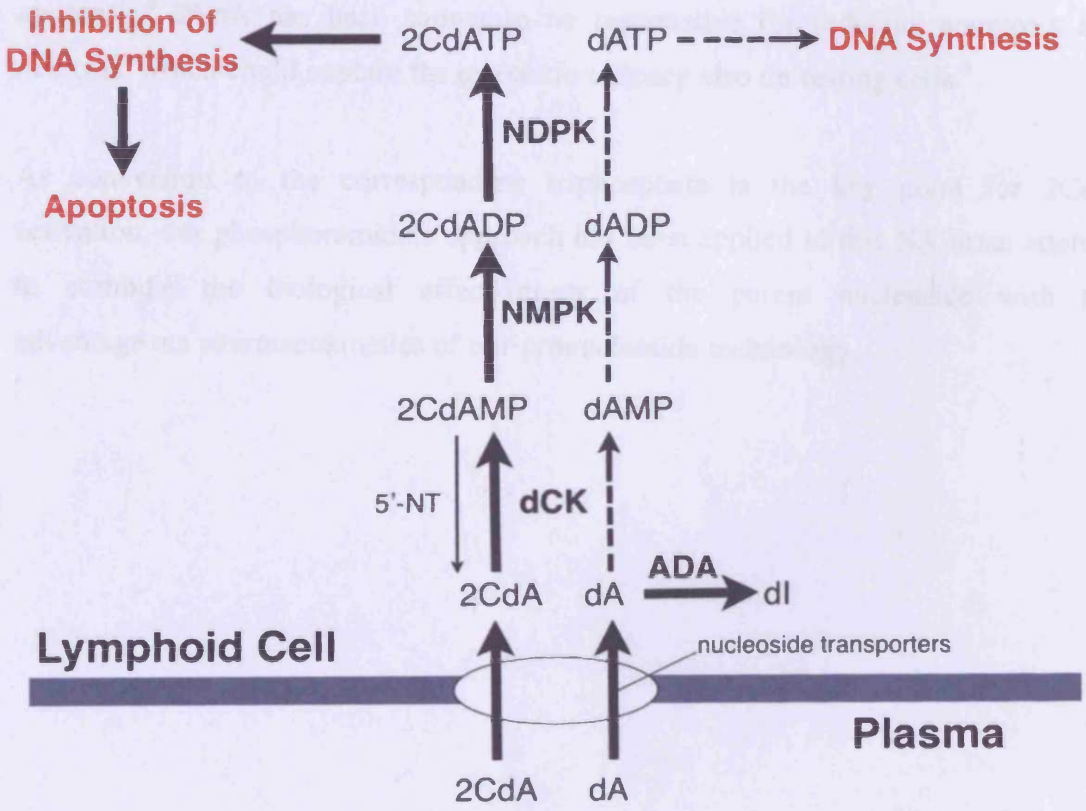
2CdA

Figure 4.1: Cladribine (2CdA).

adenosine deaminase (ADA) deficiency, a rare inborn error of metabolism which accounts for 20-30% of the cases of severe combined immunodeficiency.[1] The recognition that the accumulation of 2'-deoxyadenosine (resulting from the enzyme defect) was responsible for the profound, ultimately fatal lymphopenia in the affected patients led to the synthesis of 2'-deoxyadenosine analogues that were similarly lymphocytotoxic and that were not deaminated by ADA. Of several such analogues, 2CdA had the most favourable therapeutic ratio.[2]

Furthermore, lymphocytes contain unusually high concentrations of deoxycytidine kinase (dCK), the enzyme that phosphorylates deoxyadenosine (dA) to deoxyadenosine monophosphate (dAMP), whereas the activity of the 5'-nucleotidase (5'-NT) that dephosphorylates dAMP to deoxyadenosine is unusually low in the same cells.[3]

Deoxypurine nucleosides such dA or 2CdA are transported into cells through an efficient transport system.[3] Although deoxyadenosine concentrations are regulated by the enzyme ADA, 2CdA accumulates intracellularly owing to its resistance towards ADA activity and is phophorylated by deoxycytidine kinase to the corresponding monophosphate. Thus, cells rich in dCK and poor in 5'-NT activity (e.g. lymphocytes) accumulate 2CdA monophosphate, which is then further phosphorylated to di- and triphosphate by nucleoside mono- (NMPK) and diphosphate kinases (NDPK) respectively (Scheme 4.1).[1,3-5]



Scheme 4.1: Metabolism of 2'-deoxyadenosine (dA) and cladribine (2CdA) in lymphoid cells.

The mechanism by which 2CdA kills cells and the basis of its selectivity are not yet clearly understood and may differ according to cell type.

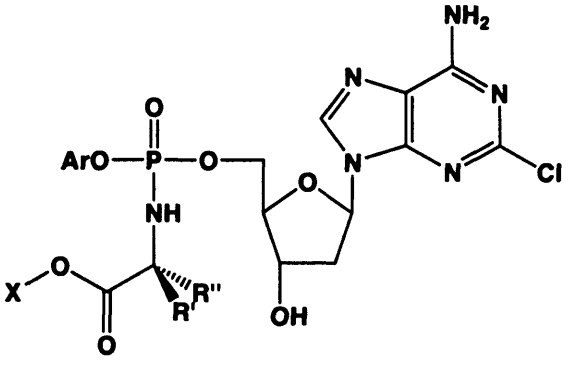
2CdATP inhibits DNA synthesis by incorporation into the A sites of the growing DNA strand, leading to death of dividing cells.[6] In fact, high concentrations of deoxynucleotides render cells unable to properly repair single-strand DNA breaks and the broken ends activate polymerase enzymes resulting in nicotinamide adenine dinucleotide (NAD) and ATP depletion and disruption of cellular metabolism.[7] However, this is not the only cause of cell death, because, in contrast to most antineoplastic agents, the ability of 2CdA to kill resting and dividing cells is the same.

It has been suggested that accumulation of deoxyribonucleotides may trigger apoptosis;[8] 2CdA has been shown to be responsible for inducing apoptosis and necrosis, which could explain the cytotoxic efficacy also on resting cells.[6]

As conversion to the corresponding triphosphate is the key point for 2CdA activation, our phosphoramidate approach has been applied to this NA in an attempt to combine the biological effectiveness of the parent nucleoside with the advantageous pharmacokinetics of our pronucleotide technology.

4.2 Cladribine phosphoramidates

As 2CdA was a completely new target of our pronucleotide strategy, a first series of phosphoramidates were designed taking into account our classical phenyloxy as well as the emerging naphthyloxy moiety as masking groups on the phosphate (Table 4.1).



Cpd	Ar	R'	R''	X
12a	Ph	Me	H	Me
12b	Ph	Me	H	Bn
12c	1-Naph	Me	H	Bn
12d	4-Cl-1-Naph	Me	H	Bn
12e	Ph	Me	Me	Bn
12f	1-Naph	Me	Me	Bn

Table 4.1: Cladribine phosphoramidates.

The first 2CdA phosphoramidates were planned considering L-alanine as the amino acid core (either methyl and benzyl esters) and phenyl as the aryl masking group on the phosphate according to our usual lead structure (**12a** and **12b**). Following the biological results observed with the BVdU phosphoramidate series, naphthyl (entries

12c and **12d**) was introduced as a different aryl group in an attempt to possibly further improve any activity displayed by the phenyl derivatives.

Lastly, given the favourable properties noticed in our previous SAR studies, dimethylglycine was also investigated as an amino acid moiety (**12e** and **12f**).

4.3 Biological evaluation

2CdA phosphoramidates were evaluated against a panel of leukaemic cell lines in vitro, applying MTS assays based on the reductive mitochondrial activity to detect and determine cell viability. The morphologic, karyotypic, and immunohistochemical features of each cell line were established and thus allowed testing of each 2CdA phosphoramidate against cells that had known, specific molecular abnormalities and/or belonged to different subgroups of leukaemia.

All the 2CdA pronucleotides displayed a higher cytostatic activity in comparison to cladribine, with individual responses versus each type of cell lines (IC_{50} values in Table 4.2; potency data in Figure 4.2).

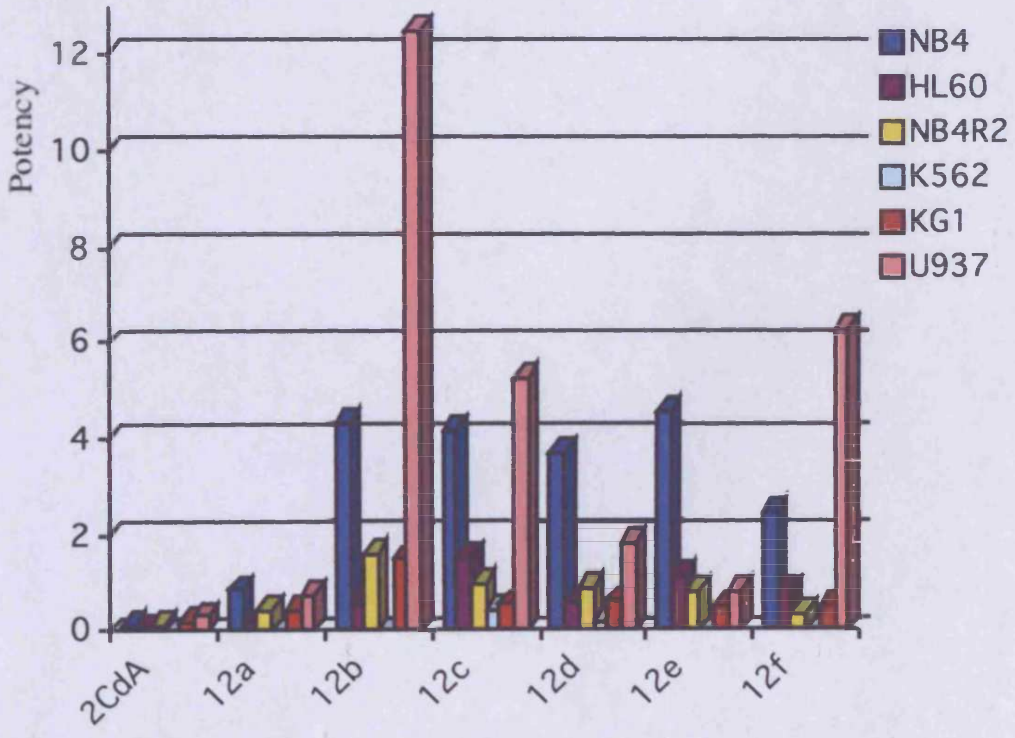


Figure 4.2: Potency ($1/IC_{50}$) data plot for 2CdA and compounds **12a-f**.

Cpd	NB4	HL60	NB4R2	K562	KG1	U937
2CdA	9.39	10.28	7.81	>10	5.48	3.08
12a	1.16	6.23	2.27	>10	2.24	1.29
12b	0.23	1.75	0.63	>10	0.67	0.08
12c	0.24	0.65	1.03	2.36	1.80	0.19
12d	0.27	1.75	1.09	4.41	1.56	0.55
12e	0.22	0.87	1.21	>10	1.89	1.20
12f	0.41	1.17	2.59	>10	1.90	0.16

Table 4.2: IC_{50} (μM) of cladribine and its phosphoramidates.

NB4 is a permanent cell line characterised by chromosomal translocation t(15;17) established from the leukaemic cells of a patient with acute promyelocytic leukaemia (APL). This specific translocation is the hallmark for APL as it causes rearrangements of the retinoic acid receptors (RARα) and several promyelocytic genes leading to an uncontrolled cell proliferation and maturation blockade. Once treated with retinoic acid (RA), APL cells respond by a rapid growth arrest as well as morphological and functional maturation suggesting that such a chromosomal translocation is tightly implicated in RA signal transduction.[9,10]

For this cell line, all the phosphoramidates showed a boost in activity versus 2CdA (Figure 4.3). The phenyloxy phosphoramidate **12a** was the only derivative bearing a methyl as an amino acid ester chain and showed a 10-fold boost in potency against 2CdA. Significantly, the presence of a benzyl ester instead of the methyl, further increased the activity from 20- up to 40-fold in comparison to the NA (**12b-f**). Tuning of the aryl moiety caused a more accentuated change in biological activity for the dimethylglycine derivatives (**12e,f**) when compared to the L-alanine phosphoramidates (**12b-d**). The phenyloxy dimethylglycine phosphoramidate **12e** resulted equipotent to the L-alanine counterpart **12b**, which led to the conclusion that

the unnatural amino acid could be a good substitute for L-alanine against this leukaemic cell line.

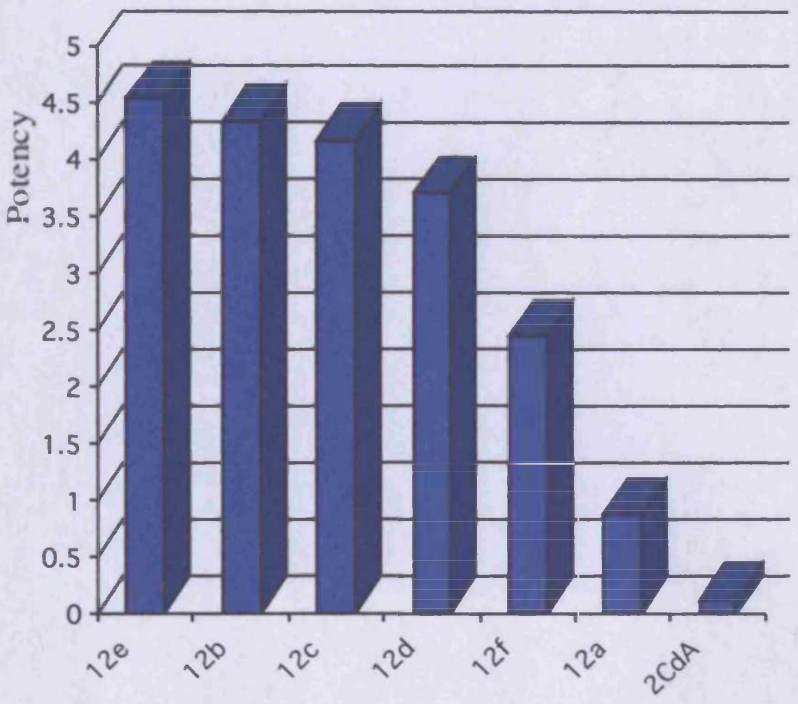


Figure 4.3: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for NB4 cells.

The HL60 cell line, derived from a patient with APL, possesses unusual properties among human myeloid leukaemias. While most fresh myeloid leukaemic cells cultured in liquid suspension undergo a limited number of cell divisions prior to growth arrest and cell death, HL60 cells continuously proliferate with a doubling time of 36-48 hours and can be induced to differentiate to a number of different cell types in vitro. One possible explanation for such limited in vitro proliferation for most of leukaemic cells is that certain growth factors sustaining leukaemic cell division in the patients are absent from the in vitro culture environment. Therefore, HL60 appears to be a factor-independent immortal cell line.[11]

Also for this cell line, all the 2CdA phosphoramidates resulted more active than the parent NA. In particular, our basic phenyloxy phosphoramidate **12a** was only slightly more active than 2CdA. As noted in our previous SARs and for all the cell lines

employed in this study, the cytostatic activity increased when the methyl ester (**12a**) was replaced by a benzyl ester (**12b**) on the amino acid moiety (L-alanine), which led to a 10-fold increase versus 2CdA (Figure 4.4). The replacement of the phenyloxy (**12b**) with a naphthyloxy group (**12c**) further increased the activity to an overall 20-fold boost versus the NA. The 4-chloro substituted naphthyl (**12d**) did not further improve the activity of L-alanine containing derivatives.

Notably, the naphthyloxy L-alanine benzyl ester derivative (**12c**) had a similar cytostatic activity to the phenyloxy dimethylglycine benzyl ester derivative (**12e**), whereas the introduction of the naphthyl moiety on this latter phosphoramidate did not lead to a further boost in activity (**12f**), as also observed for the NB4 cell line.

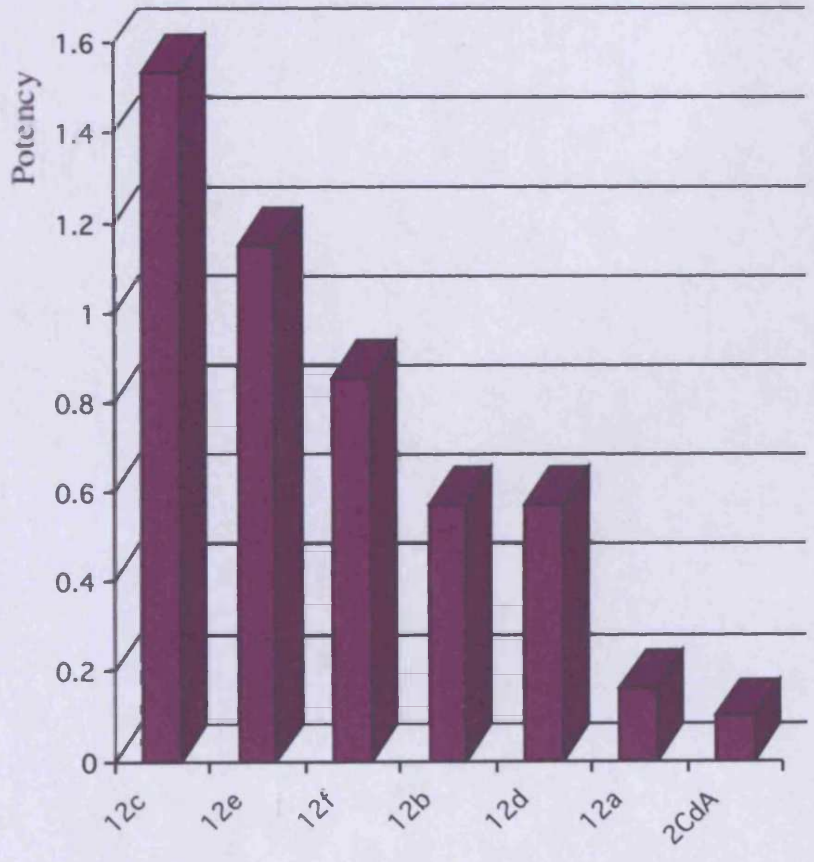


Figure 4.4: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for HL60 cells.

NB4R2 is a cell line derived from NB4 cells, which have a mutation in the ligand binding domain of RARα that disrupts retinoid signalling.[12,13] Therefore, NB4R2 cells are resistant to RA.

Compound **12b** appeared to be the most effective phosphoramidate against this RA resistant cell line displaying a 10-fold increase in cytostatic activity versus 2CdA (Figure 4.5). Within this series, the introduction of either a naphthyloxy moiety on the phosphate or dimethylglycine as the amino acid core did not show to be beneficial modifications to further improve the activity of 2CdA phosphoramidates.

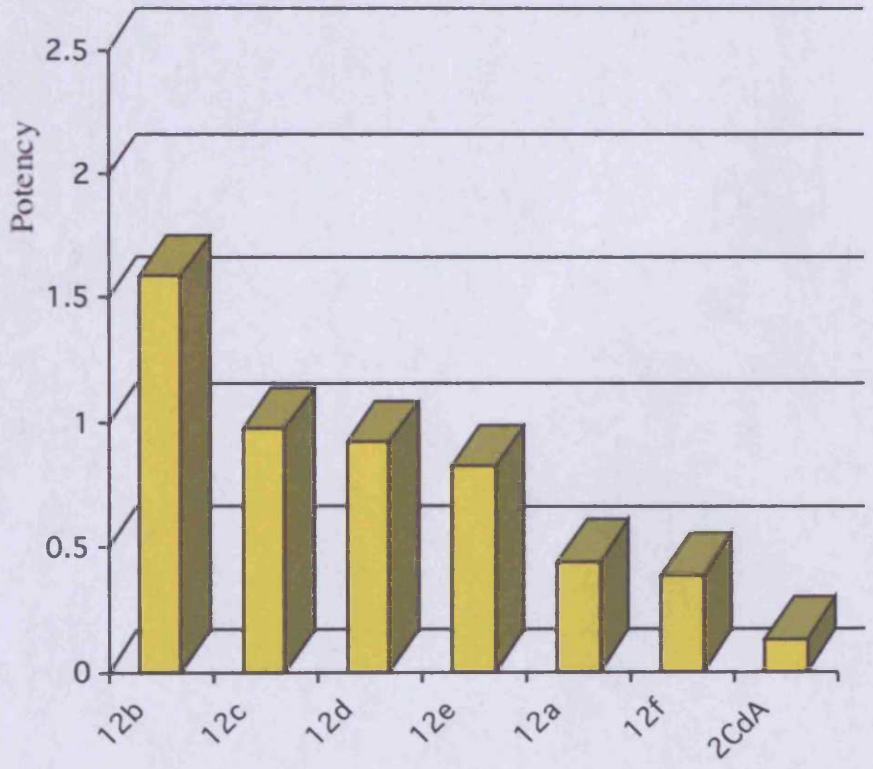


Figure 4.5: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for NB4R2 cells.

K562 is a cell line established in vitro from a pleural effusion of a patient with chronic myeloid leukaemia in blast crisis (advanced phase of chronic myelogenous leukaemia characterised by an increase in the number of immature white blood

cells).[14] The K562 is a human myeloid leukaemic, morphologically and cytochemically undifferentiated blast cell line.[15]

2CdA and its phosphoramidates appeared to be poorly active against this particular cell line (Figure 4.5). Derivatives **12c** and **12d** were the only 2CdA phosphoramidates displaying an IC_{50} value under the top concentration tested (10 μM) as the other compounds in Table 4.2 never succeeded in killing 50% of the K562 cells. Notably, L-alanine appeared to be preferred to dimethylglycine as well as naphthyl to phenyl on the phosphate moiety.

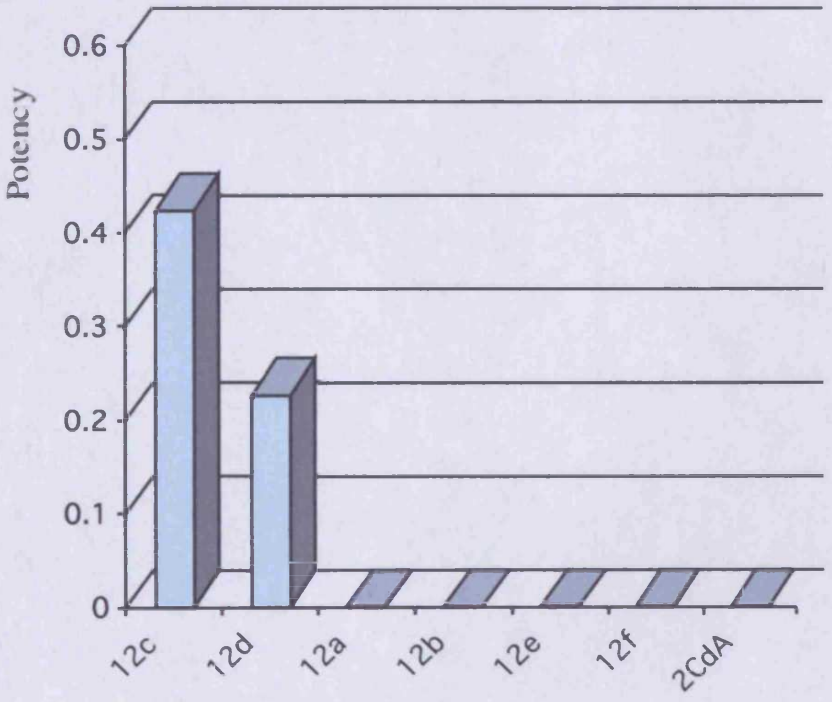


Figure 4.5: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for K562 cells.

KG1 is a myeloid cell line, derived from the bone marrow of a man with erythroleukemia, used as a tool for the study of human erythroid differentiation and globin gene expression.[16]

Compounds **12a-f** showed for this cell line a trend similar to that observed for NB4R2: the phenyloxy L-alanine benzyl ester phosphoramidate (**12b**) emerged as

the most active with no particular benefits arising from the introduction of naphthyl as the aryloxy group nor from dimethylglycine as a different amino acid core (Figure 4.6).

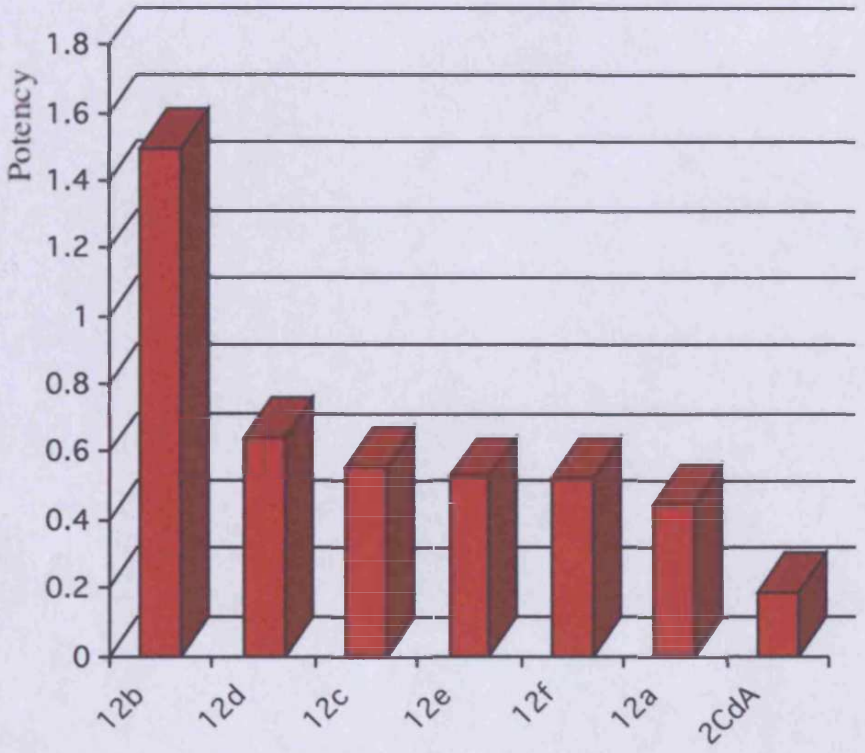


Figure 4.6: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for KG1 cells.

U937 is a histiocytic lymphoma cell line with monoblast-like characteristics.[17,18] Cladribine phosphoramidates **12a-f** showed a significant boost in cytostatic activity against the last leukemic cell line (Table 4.2 and Figure 4.7). Derivative **12b** (phenyloxy L-alanine benzyl ester) displayed a 40-fold increase in activity versus 2CdA and emerged as the most effective of the series. The corresponding dimethylglycine derivative **12e** transpired as one of the less active, substantially equipotent to **12a**, the L-alanine methyl ester. However, dimethylglycine appeared to be a better amino acid core than L-alanine when the naphthyl was used as the aryl masking group on the phosphate (**12f** versus **12c,d**).

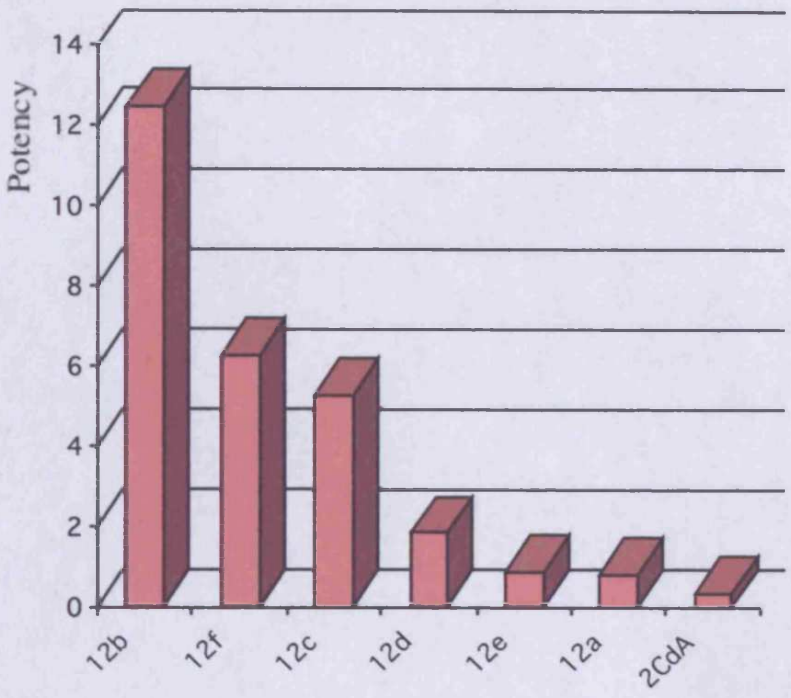
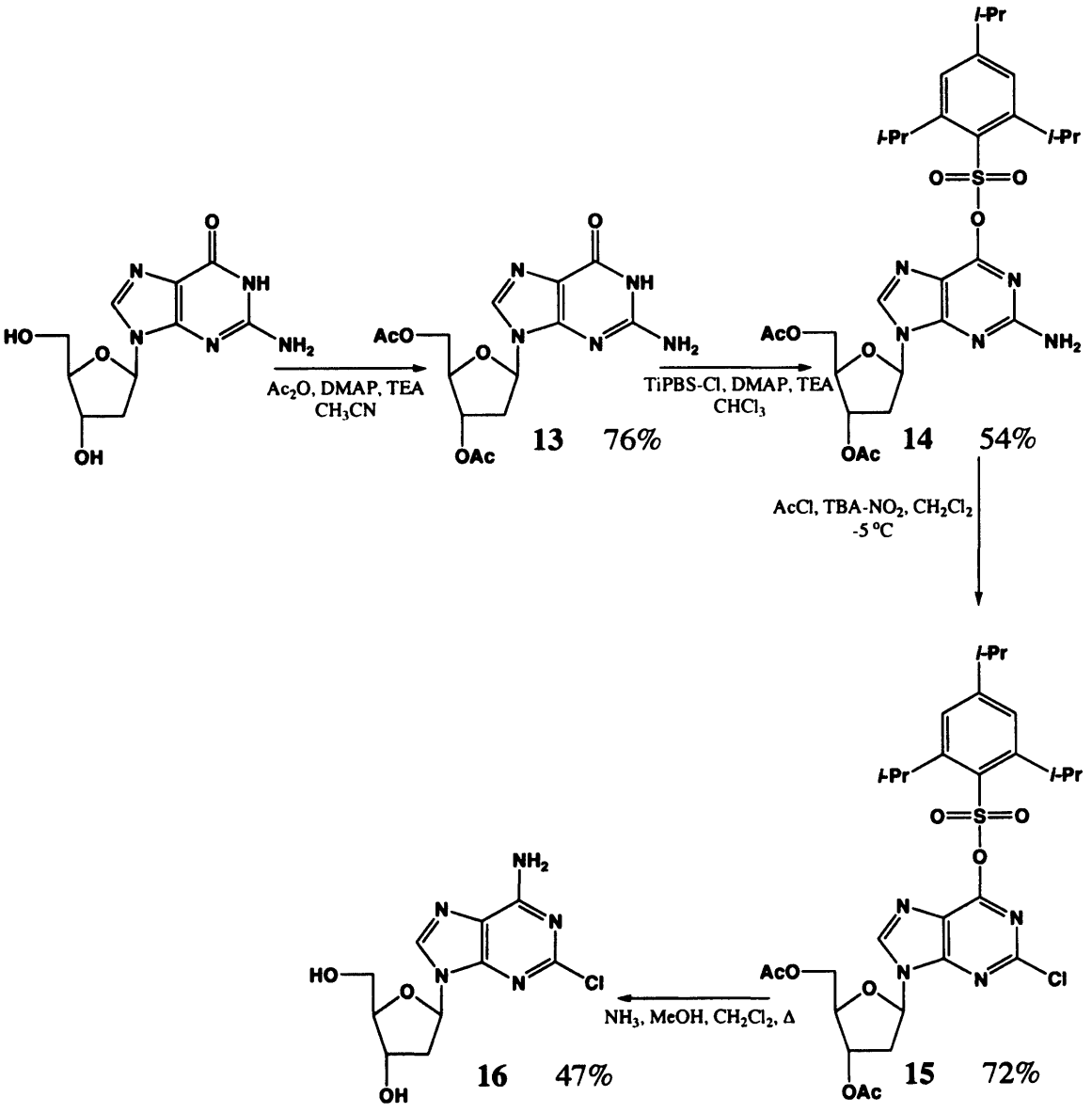


Figure 4.7: Potency ($1/IC_{50}$) of 2CdA and compounds **12a-f** for U937 cells.

In conclusion, the application of our phosphoramidate approach led to an evident boost in activity versus 2CdA against all the different leukaemia cell lines tested. Significantly, all the phosphoramidates synthesised appeared to be more active than the parent nucleoside. The phenyloxy phosphoramidate bearing the L-alanine benzyl ester motif (**12b**) was the most active against the NB4, NB4R2, KG1 and U937 leukaemia cell lines. The replacement of L-alanine with dimethylglycine or phenyl with naphthyl on the phosphoramidate structure led to individual modifications in activity according to the each leukaemic cell type.

4.4 Synthesis of 2-chloro-2'-deoxyadenosine (2CdA, cladribine)

2CdA (**16**) was synthesised according to an efficient method reported by Janeba et al (Scheme 4.2).[19] The hydroxyl groups of 2'-deoxyguanosine were selectively protected by a 4-dimethylaminopyridine (DMAP) catalysed acetylation to give **13**. Transformation of the 6-oxo function into a good leaving group was achieved through the treatment of **13** with 2,4,6-triisopropylbenzene-sulfonyl chloride (TiPBS-Cl).



Scheme 4.2: Synthesis of 2CdA.

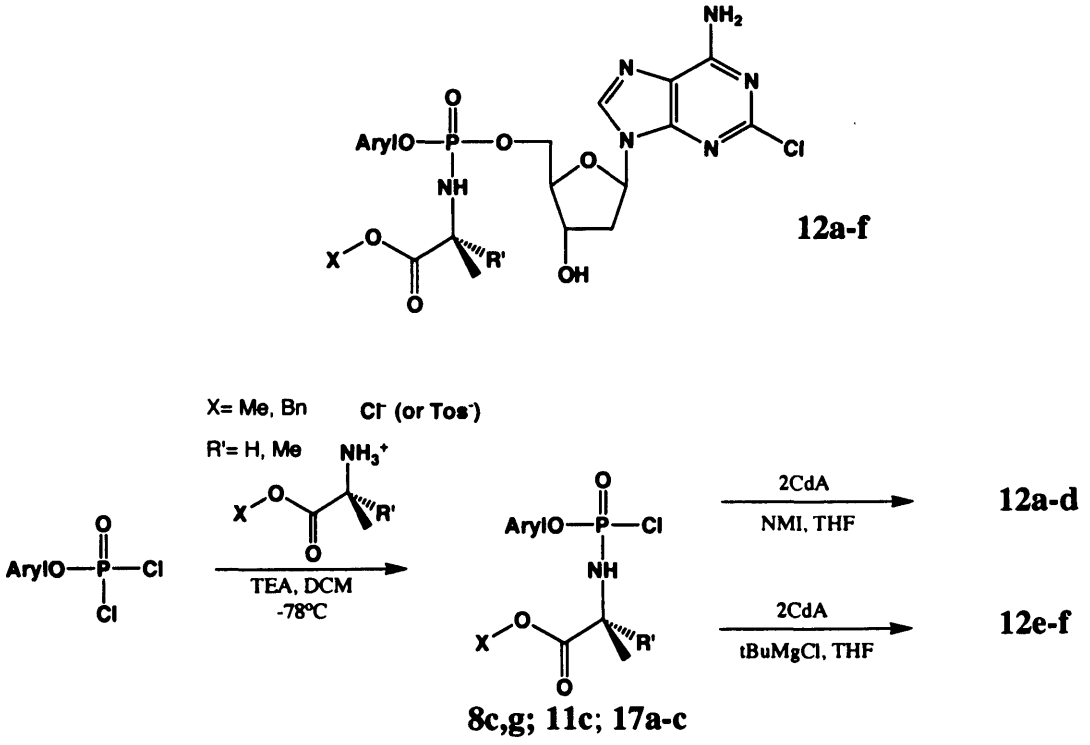
Nonaqueous diazotization/chloro dediazoniation of **14** led to the 2-chloro derivative **15**. Lastly, displacement of the hindered arylsulfonate at C-6 with the accompanying cleavage of the sugar esters was achieved through ammonolysis, leading to 2CdA (**16**).

4.5 Synthesis of 2CdA phosphoramidates

The phenyl phosphorochloridate intermediates (**17a-c**, Scheme 4.3) were obtained through coupling of the commercially available phenyl dichlorophosphate to the corresponding amino acid ester salts (Table 4.4). Synthesis of the naphthyl phosphorochloridates **8c,g** and **11c** is described in Chapters 2 and 3 respectively.

Cpd	R'	X	Yield (%)	^{31}P-NMR ($CDCl_3$), δ*
17a	H	Me	43.4	9.41,9.26
17b	H	Bn	95.5	9.33,9.13
17c	Me	Bn	79.9	5.55

Table 4.4: Phenyl phosphorochloridates **17a-c**. *See experimental section for deuterated solvents and field frequency used.



Scheme 4.3: Synthesis of 2CdA phosphoramidates.

Compounds **12a-d** were synthesised according to the NMI coupling procedure applied to the BVdU phosphoramidates (Scheme 4.3 and Table 4.5).
Investigation of the biological properties of 2CdA phosphoramidates was prioritised over the optimisation of the synthetic procedure. However, removal of NMI from the crude mixture could not be performed through mild acidic extractions without loss of the unreacted 2CdA. Moreover, in the presence of NMI, purification of the crude mixture required repetitive application of column chromatography and, in some cases, reverse phase HPLC.

Tert-butyl-magnesium chloride (tBuMgCl) was reported by Uchiyama[20] as an effective hydroxyl activator for a facile O-selective phosphorylation of nucleosides without any N-protections and was used for the synthesis of **12e,f** (Scheme 4.3 and Table 4.5). The use of a Grignard base allowed an easy purification of the crude mixture with recovery of the unreacted nucleoside analogue. In contrast to the NMI method, tBuMgCl did not show selectivity towards primary hydroxyl groups and formation of by-products of phosphorylation in 3' and 3',5' diphosphorylation was observed. However, these by-products showed such different Rfs from the desired 5'-derivatives to allow an easy separation by column chromatography.

Cpd	Aryl	R'	X	Yield (%)	^{31}P-NMR δ*
12a	Ph	H	Me	2.2	4.56,4.21
12b	Ph	H	Bn	3.6	5.11,4.81
12c	1-Naph	H	Bn	8.6	3.64,3.23
12d	4-Cl-1-Naph	H	Bn	3.4	3.45,3.26
12e	Ph	Me	Bn	6.3	1.73,1.62
12f	1-Naph	Me	Bn	11.6	1.97,1.93

Table 4.5: 2CdA phosphoramidates. *See experimental section for deuterated solvents and field frequency used.

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Chapter 5: Cladribine phosphoramidates, a second series

5.1 New SARs of 2CdA phosphoramidates

The preliminary biological evaluation of the first phosphoramidates of 2CdA described in Chapter 4 (see also Chapter 6 for the biological activity of separated diastereoisomers) showed an improvement in activity versus 2CdA. Following these data, new modifications to the amino acid motif in the phosphoramidate structure were planned to investigate new structure activity relationships and further improve the cytostatic activity against the leukaemic cell lines employed.

Firstly, new natural (isoleucine, valine and phenylalanine) and unnatural (D-alanine) amino acids were considered (Figure 5.1). Benzyl was chosen as the ester chain on the amino acid moiety within these derivatives.

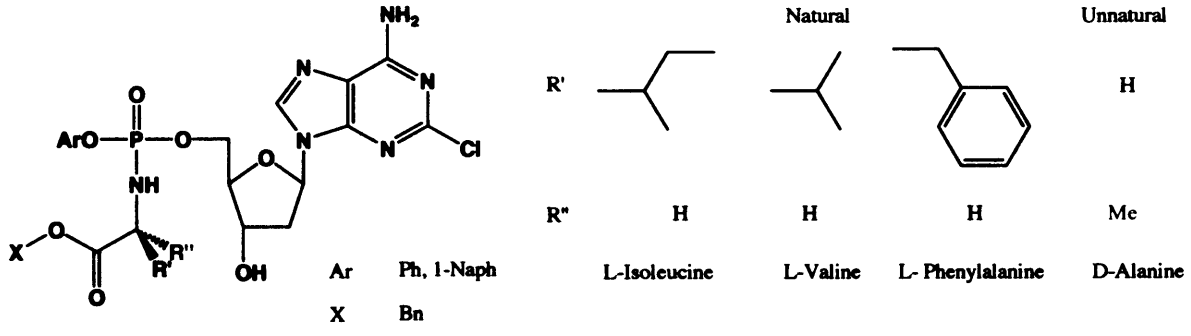


Figure 5.1: Variations on the amino acid core.

Secondly, given the established importance of L-alanine in our previous phenyloxy phosphoramidate SARs,[1] new esters on this amino acid moiety were considered (tert-butyl, ethyl, isopropyl and 2-butyl) in order to investigate if such variations could significantly affect the biological activity (Figure 5.2).

In Table 5.1 all the compounds synthesised are reported. Notably, apart from the usual and desired 5'-, also derivatives of phosphorylation in 3'- and 3',5'-diphosphorylation were isolated, purified and tested.

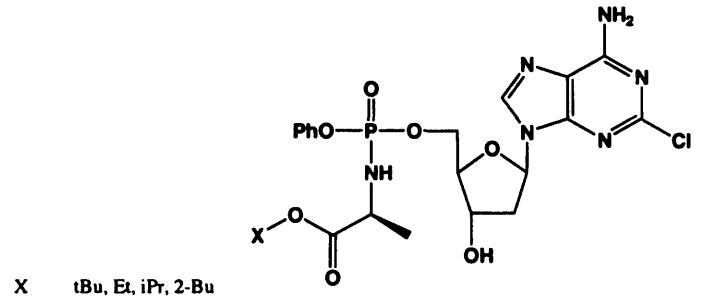


Figure 5.2: New L-alanine ester derivatives.

Cpd	Aryl	Amino Acid	X	Phosphate
18a	Ph	L-Phenylalanine	Bn	5'
18b	Ph	L-Phenylalanine	Bn	3'
18c	1-Naph	L-Phenylalanine	Bn	5'
18d	1-Naph	L-Phenylalanine	Bn	3'
18e	Ph	L-Valine	Bn	5'
18f	Ph	L-Valine	Bn	3'
18g	Ph	L-Isoleucine	Bn	5'
18h	Ph	D-Alanine	Bn	5'
18i	Ph	L-Alanine	Et	5'
18l	Ph	L-Alanine	iPr	5'
18m	Ph	L-Alanine	iPr	3'
18n	Ph	L-Alanine	iPr	3',5'
18o	Ph	L-Alanine	tBu	5'
18p	Ph	L-Alanine	2-Bu	5'
18q	Ph	L-Alanine	2-Bu	3'
18r	Ph	L-Alanine	2-Bu	3',5'

Table 5.1: 2CdA phosphoramidates, compounds synthesised.

For convenience, the biological evaluation of compounds **18a-r** is discussed in three separate sections. Phosphoramidates bearing an amino acid core different from L-alanine (**18a-h**), are firstly described (paragraph 5.2). Following the unexpected activity of some 3'-protides, a study concerning the investigation of their possible mode of action is then introduced (paragraph 5.3). Lastly, derivatives **18i-r** are described in the paragraph 5.4 as an extension of the L-alanine series initially investigated in Chapter 4.

5.2 Variation on the amino acid region: biological evaluation

Compounds bearing modifications on the amino acid moiety (**18a-h**) were evaluated against the same panel of leukaemic cell lines previously used for the first series of 2CdA phosphoramidates described in Chapter 4 (Table 5.2).

Cpd	AA	X	Phosp.	NB4	HL60	NB4R2	K562	KG1	U937
2CdA	-	-	-	9.39	10.28	7.81	>10	5.48	3.08
12b	L-Ala	Bn	5'	0.23	1.75	0.63	>10	0.67	0.08
12e	Me_2Gly	Bn	5'	0.22	0.87	1.21	>10	1.89	1.20
18a	L-Phe	Bn	5'	0.22	1.22	1.57	6.24	2.17	0.88
18b	L-Phe	Bn	3'	0.96	1.91	2.26	10.41	4.12	1.68
18c*	L-Phe	Bn	5'	0.26	1.09	1.06	8.67	1.28	0.73
18d*	L-Phe	Bn	3'	0.33	1.14	0.76	7.10	1.08	0.68
18e	L-Val	Bn	5'	0.34	6.25	3.36	>10	5.32	0.36
18f	L-Val	Bn	3'	0.78	2.48	2.25	>10	1.87	0.44
18g	L-Ile	Bn	5'	1.54	9.81	5.02	>10	8.58	0.62
18h	D-Ala	Bn	5'	>10	>10	>10	>10	>10	9.19

Table 5.2: IC_{50} (μM) of cladribine phosphoramidates. * Naphthyloxy derivatives.

The phenyloxy phosphoramidates (5'-phosphorylated) bearing the natural amino acids phenylalanine, valine and isoleucine (**18a,e,g**) displayed a higher cytostatic activity in comparison to 2CdA versus each cell line considered. However, only L-phenylalanine (**18a**) transpired as a possible substitute for L-alanine by showing IC_{50} values close to the corresponding L-alaninyl benzyl ester derivative (**12b**) for all the leukaemic cell lines.

The use of naphthyl as aryloxy moiety (**18c**) did not lead to a significant change in biological activity compared to the classic phenyloxy phosphoramidate (**18a**). This

was in agreement with the biological results of the previous 2CdA phosphoramidate series reported in Chapter 4 but in contrast to the data of the BVdU phosphoramidates presented earlier in this Thesis.

In spite of the fact that D-alanine did not significantly change the biological properties of BVdU phosphoramidates in comparison to the natural L-derivative, it appeared to lead to a significant loss of cytostatic activity within the 2CdA phosphoramidate series (**18h**) (potency data plot for the whole range of amino acid variation in Figure 5.3).

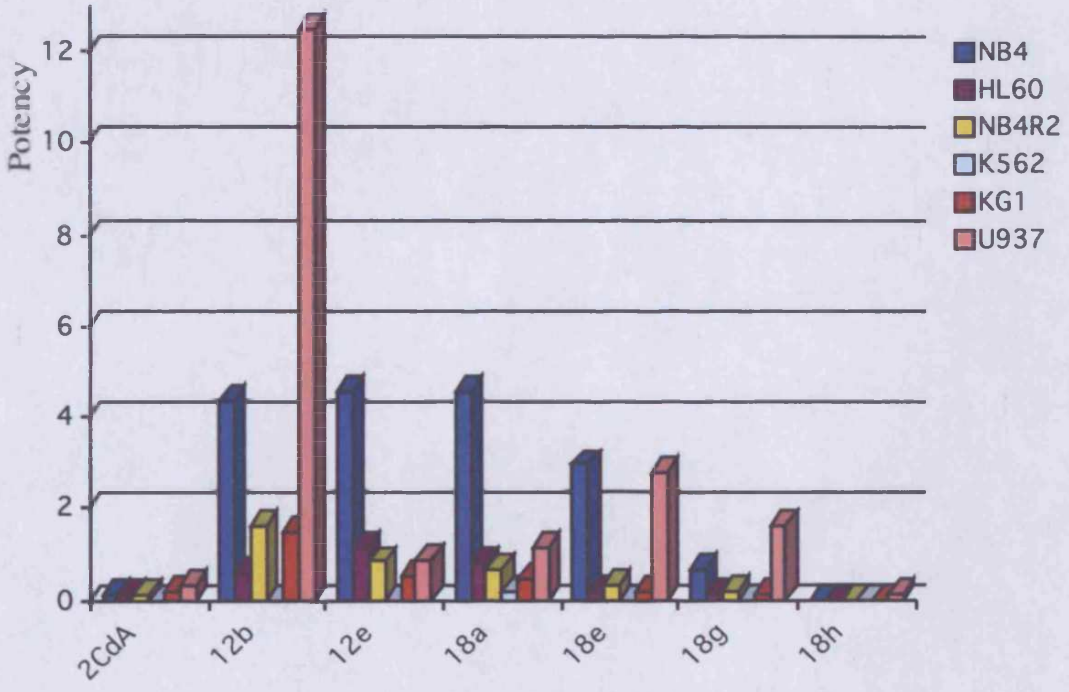


Figure 5.3: Potency ($1/IC_{50}$) data plot for amino acid variation of 2CdA phosphoramidates: L-alanine (**12b**), dimethylglycine (**12b**), L-phenylalanine (**18a**), L-valine (**18e**), L-isoleucine (**18g**) and D-alanine (**18h**).

The amino acid stereochemical requirements were extensively studied in our d4T phosphoramidate SARs leading to the conclusion that D-compounds were found to

be less effective than the L-parents.[2] Moreover, we also reported that enzymatic stability, cell permeability and also P-gp mediated efflux appeared to be stereoselective.[3,4]

Therefore, it was reasonable to obtain relevant biological differences between the L- and D-alanine 2CdA phosphoramidates.
An elevated biological activity for D-alanine derivatives (as in the BVdU series) might preferentially occur in cells where the enzymes involved in their activation possess a higher expression and/or a lower selectivity, with the metabolic pathway being unable to discriminate between the natural and the unnatural amino acid.
Unfortunately, we do not have experimental data to support these hypothesises.

Compounds **18b,d,f** were characterised as 3'-nucleoside phosphoramidates. This kind of derivative had been previously isolated. However, considering our 5'-target structure, they had always been discarded as side-reaction products. To our surprise, the 3'-phosphoramidates showed close and in some cases, even higher potency (Table 5.2; potency data plot in Figure 5.4) in comparison to the corresponding 5'-derivatives (**18a,c,e**).
Indeed by displaying an IC_{50} value of 0.76 μM, 3'-phosphoramidate **18d** became one of the most potent 2CdA phosphoramidates against the resistant cell line NB4R2, second only to **12b**.

Following these surprising results, a possible explanation for the observed biological activity for the 3'-phosphoramidates of 2CdA was investigated.

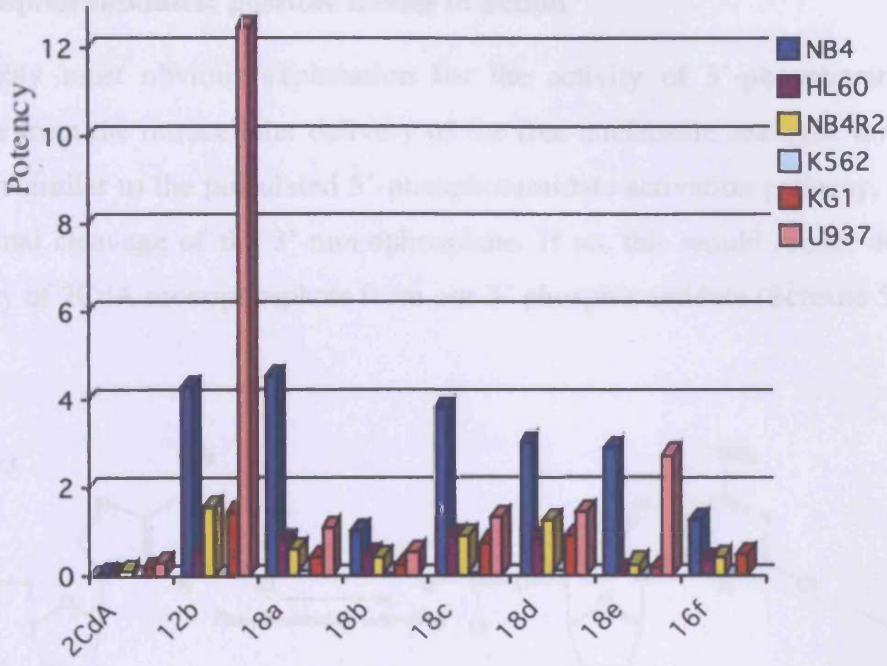
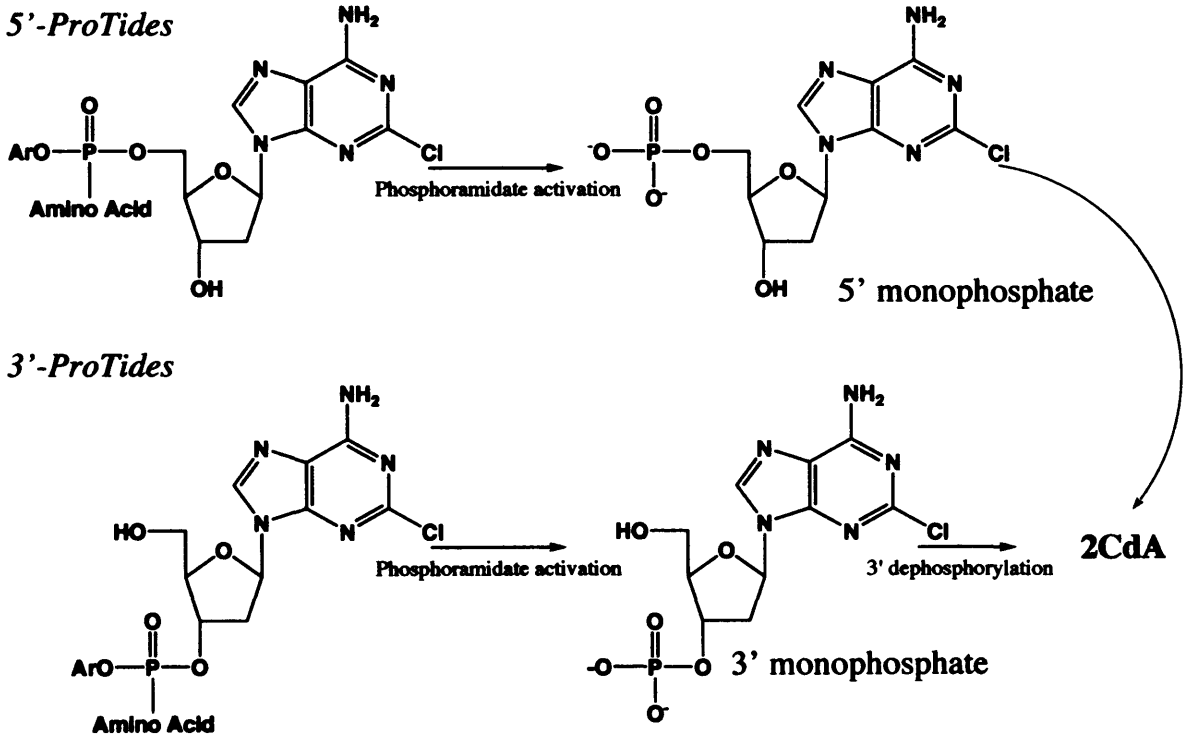


Figure 5.4: Potency ($1/IC_{50}$) data plot for 3'-phosphoramidates **18b,d,f** and corresponding 5'-derivatives **18a,c,e**.

5.3 3'-Phosphoramidates: possible modes of action

The probably most obvious explanation for the activity of 3'-phosphoramidates might arise from the intracellular delivery of the free nucleoside analogue through a mechanism similar to the postulated 5'-phosphoramidate activation pathway, ending with the final cleavage of the 3'-monophosphate. If so, this would render doubtful the delivery of 2CdA monophosphate from our 5' phosphoramidate (Scheme 5.1).



Scheme 5.1: First postulated mechanism of action of 3'-phosphoramidates: delivery of 2CdA.

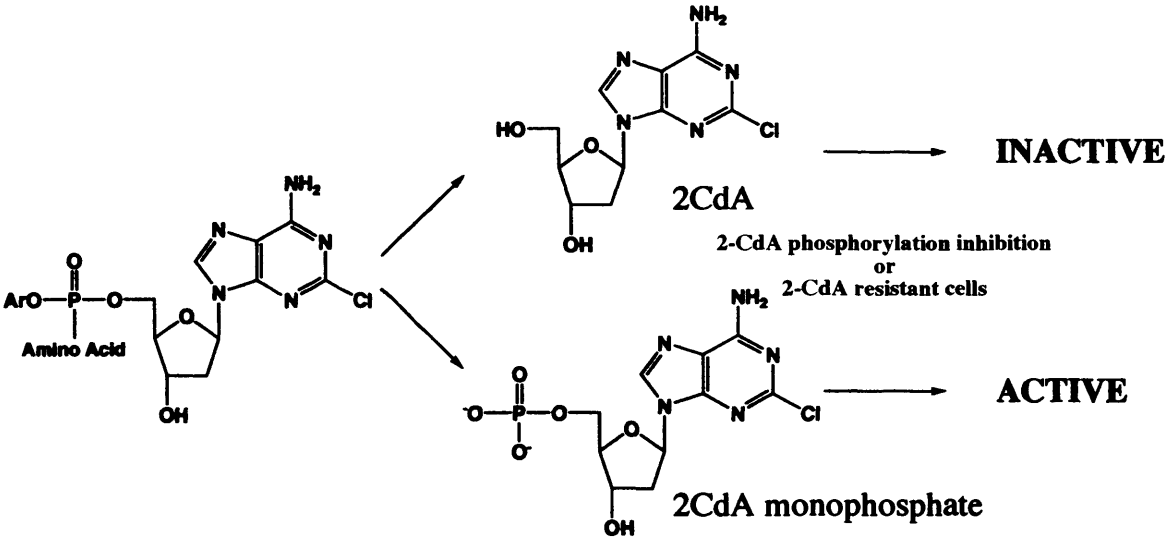
Therefore, we needed to prove the effectiveness of our approach in delivering the 5'-monophosphate in the first instance.

We found in the literature that the cytotoxic effect of 2CdA could be completely reversed by the simultaneous administration of the natural dCK substrate, 2'-

deoxycytidine (dC).[5] If the activity of our 5'-phosphoramidates would be retained in this situation, it would support their kinase by-pass ability.

Moreover, exposure to gradual increasingly concentrations of 2CdA was reported in the literature to be an efficient method to obtain CCRF-CEM human T-cell line resistant to 2CdA.[6] If the activity of our 5'-phosphoramidates was retained, it would further support the notion that our 5'-derivatives did not act as a simple depot of free nucleoside analogue in this case.

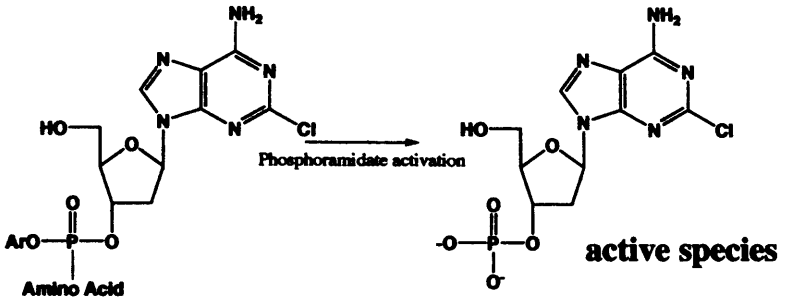
These two different assays (dC co-administration and use of 2CdA resistant cells, Figure 5.2) are in progress at the moment of writing.



Scheme 5.2: Biological confirmation of 5'-monophosphate delivery.

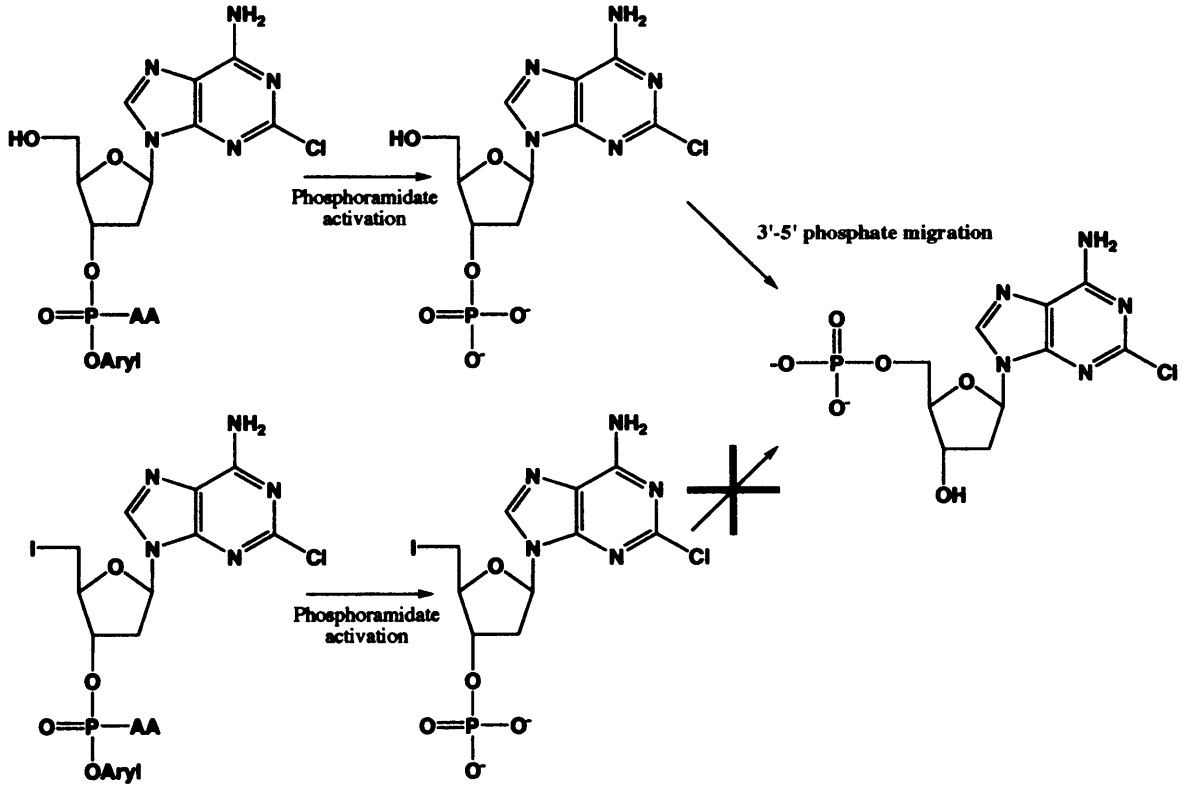
If the mode of action of 3'-phosphoramidates was indeed the delivery of 2CdA inside cells, they should loose their activity in the assays described.

Alternatively, 3'-phosphoramidate derivatives could act via an independent mode of action from the 5'-derivatives. Possibly, the 3'-(2CdA)-monophosphate could exert a cytostatic effect through a mechanism that does not include release of the free nucleoside analogue (Scheme 5.3). If confirmed, this would lead the way forward to a new potential class of cytostatic drugs.



Scheme 5.3: Second postulated mechanism of action of 3'-phosphoramidates: delivery of a 3'monophosphate active species.

Lastly, a third explanation was hypothesised for the observed activity of the 3'-phosphoramidates of 2CdA. In theory, the 3'-monophosphate arising from the activation of 3'-phosphoramidates (**18a,c,e**) could be converted into the 5'-monophosphate through an unknown intracellular process and be the real species responsible for the observed activity (Scheme 5.4).



Scheme 5.4: Third postulated mechanism of action of 3'-phosphoramidates: conversion of the 3'-monophosphate to the 5'-monophosphate.

Although such mechanism could not be readily elucidated, the substitution of the 5' hydroxy group from 3'-(2CdA)-phosphoramidates was suggested as a tool to prevent the postulated 3'-5' phosphate migration and to clarify whether it could play a role for the biological activity of these compounds or not. As a preliminary study, iodine was chosen as a substitute for the 5'-hydroxy moiety on the free nucleoside (**19a**) and the corresponding phenyloxy (**19b**) and naphtyloxy (**19c**) L-phenylanine benzy lester phosphoramidates were synthesised (Figure 5.5).

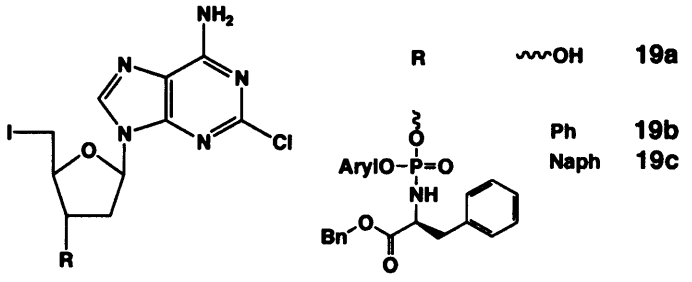


Figure 5.5: 5'-Iodo-3'phosphoramidate derivatives of 2CdA.

The 5'-iodo nucleoside analogue **19a** appeared from our biological evaluation to have IC_{50} values far over the 10 μM top concentration used against the leukaemic cell lines considered (Table 5.3). Although the 5'-iodo-3'-phosphoramidates **19b,c** showed a reduced activity in comparison to the corresponding 2CdA derivatives (**18b,c**), they displayed similar cytotoxic activities to cladribine in most of the cell lines in Table 5.3.

These unexpected results would indicate a mode of action independent from 5' phosphorylation for compounds **19b,c**. However, conversion to 2CdA could not be excluded for these compounds. This might occur chemically, due to the good leaving group ability of the iodine in the presence of nucleophiles (e.g. water), or enzymatically (e.g. cytochrome P450-mediated dehalogenation). The evaluation of these compounds against cladribine resistant cells might partially clarify this aspect.

In fact, the cytostatic activity observed might also arise from direct alkylation of macromolecules residues (nucleic acids, proteins, etc).

Cpd	AA	X	5'	NB4	HL60	NB4R2	K562	KG1	U937
2CdA	-	-	OH	9.39	10.28	7.81	>10	5.48	3.08
18b	L-Phe	Bn	OH	0.96	1.91	2.26	10.41	4.12	1.68
18d*	L-Phe	Bn	OH	0.33	1.14	0.76	7.10	1.08	0.68
18f	L-Val	Bn	OH	0.78	2.48	2.25	>10	1.87	0.44
19a	-	-	I	>10	>10	>10	>10	>10	>10
19b	L-Phe	Bn	I	8.86	10.12	12.53	>10	11.3	14.67
19c*	L-Phe	Bn	I	10.00	5.40	9.49	>10	7.82	13.39

Table 5.3: IC_{50} (μM) of cladribine, its 3'-phosphoramidates and the 5'-Iodo derivatives **19a-c**. * Naphthyloxy derivatives.

In order to better assess the biological activity of 3'-phosphoramidates, the 5' hydroxy group should be replaced by an unreactive group (e.g. an hydrogen atom or a methyl group) or converted into an ether function. Further studies are ongoing in our laboratories.

5.4 Variation on the L-alanine ester chain: biological evaluation

Phosphoramidates are believed to undergo partial hydrolysis by carboxyesterase enzymes to the aminoacyl NA monophosphate, which acts as an intracellular depot of NA monophosphate.[7,8] Although the enzymatic systems responsible for this activation remain to be identified, it is apparent that a carboxyesterase-type activity may play a key initial role. Purified pig liver carboxyesterase was reported as a simple model for the first esterase-mediated cleavage. In fact, inability of esterase to perform this activation in certain phosphoramidates correlated well with the poor biological activity of such analogues.[9]

According to the esterase lability of some d4T L-alaninyl phenyloxy phosphoramidates after 21h incubation,[10] benzyl esters emerged as a better substrate for esterase with 60% of conversion. Linear alkyl chains (nPent) were less labile towards the enzymatic cleavage (13%) but more quickly cleaved than the methyl ester derivative (2%). Notably, the branched tBu ester emerged as the most stable derivative on this assay (0%) but the insertion of a methylene spacer (neopenthyl) increased the lability of this derivative (7%). For these derivatives, esterase lability resulted in a predictive tool for the in vitro antiviral activity (Figure 5.6).

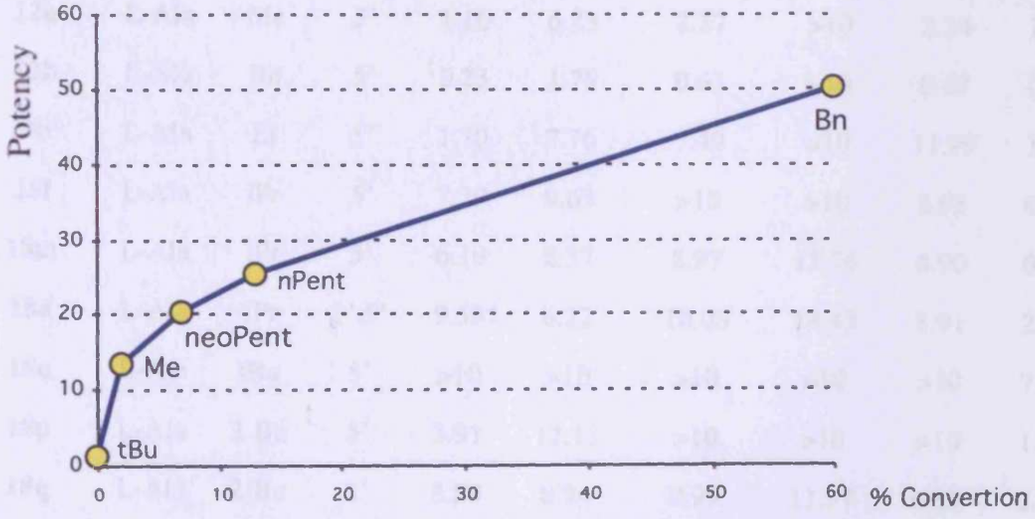


Figure 5.6: Potency ($1/EC_{50}$) and esterase lability (after 21h) of d4T L-alaninyl phenyloxy phosphoramidates bearing different ester chains.

From these data, benzyl ester emerged as the preferred ester moiety due to its higher biological activity and lability towards esterase cleavage.

Half-life investigation on a different class of phosphoramidates (structures and biological activities are currently covered by confidentiality) performed on Monkey plasma substantially confirmed that benzyl ester was more rapidly metabolised (10 h) than the tert-butyl derivative (114 h). The ethyl analogue, displaying a half-life of 33 h, showed a better pharmacokinetic profile. These data suggested that benzyl ester might be too labile in plasma with possible hydrolysis of the prodrug before it could reach the intracellular environment. Conversely, branched esters (tBu) showed an considerable stability towards metabolism which could also be the reason for their poor biological activity in vitro.

Therefore, we investigated different ester chains for L-alaninyl 2CdA phenyloxy phosphoramidates in an attempt to combine the higher biological activity of benzyl esters with the better predicted pharmacokinetic profile of alkyl esters (Table 5.4).

Cpd	AA	X	Phos.	NB4	HL60	NB4R2	K562	KG1	U937
2CdA	-	-	-	9.39	10.28	7.81	>10	5.48	3.08
12a	L-Ala	Me	5'	1.16	6.23	2.27	>10	2.24	1.29
12b	L-Ala	Bn	5'	0.23	1.75	0.63	>10	0.67	0.08
18i	L-Ala	Et	5'	1.70	7.76	7.49	>10	11.99	1.41
18l	L-Ala	iPr	5'	7.29	9.63	>10	>10	8.95	0.67
18m	L-Ala	iPr	3'	6.19	2.37	8.97	11.76	4.90	0.37
18n	L-Ala	iPr	3',5'	9.59	6.22	10.08	14.43	8.91	2.30
18o	L-Ala	tBu	5'	>10	>10	>10	>10	>10	7.96
18p	L-Ala	2-Bu	5'	3.91	12.13	>10	>10	>10	1.64
18q	L-Ala	2-Bu	3'	8.89	8.34	9.95	11.97	6.55	2.21
18r	L-Ala	2-Bu	3',5'	3.79	4.50	5.75	>10	8.77	3.45

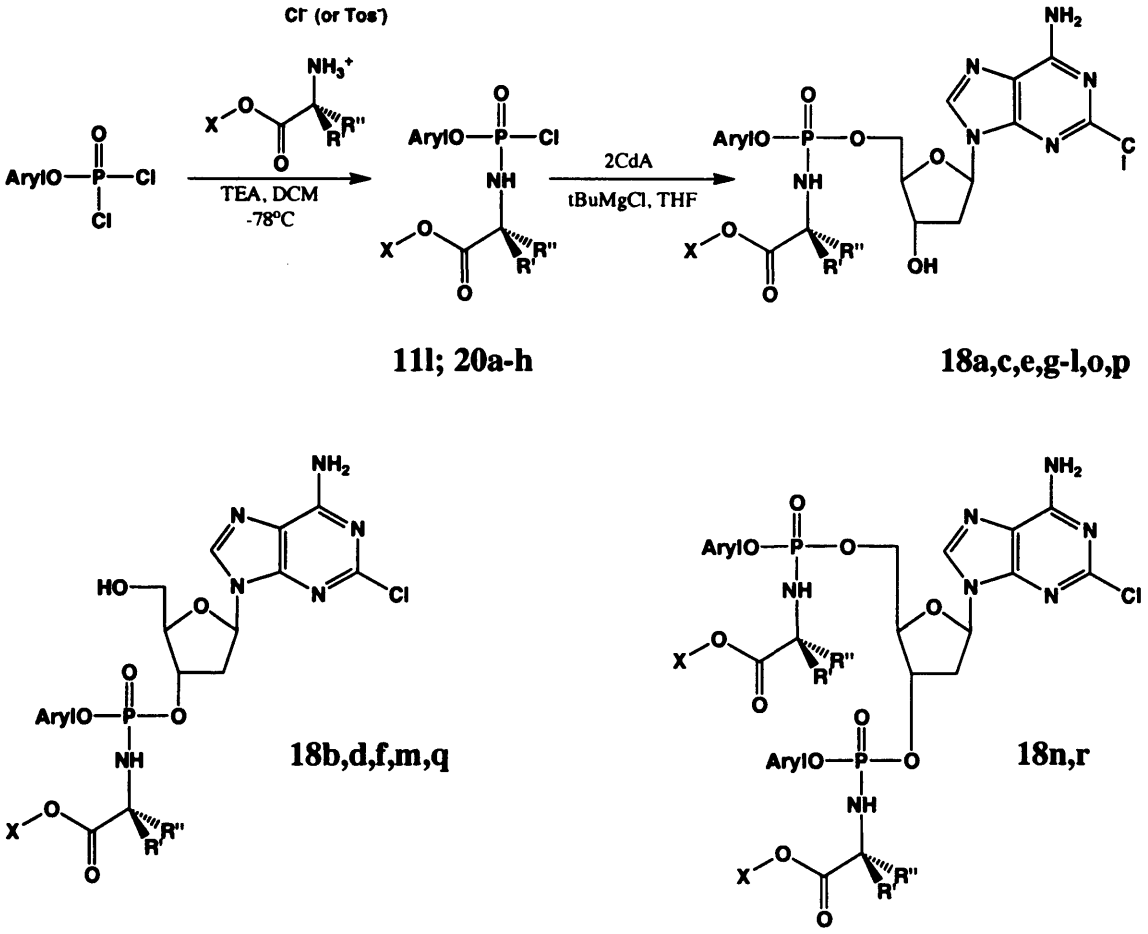
Table 5.4: L-alaninyl 2CdA phenyloxy phosphoramidate ester variation, $IC_{50}/\mu M$.

Our in vitro biological evaluation confirmed the benzyl ester (**12b**) as the most active derivative against the whole range of leukaemic cell lines used and the substantial inactivity of tert-butyl (**18o**). This would support the notion of esterase-type cleavage of the ester as a rate limiting step in the activation of phosphoramidates. Although less active, methyl (**12a**), ethyl (**18i**), and 2-buthyl analogues (**18p**) might be used as benzyl-substitutes against the NB4 cell line. Among the different alkyl chains, methyl (**12a**) remained the closest derivative to the benzyl ester analogue also against the NB4R2 and KG1 cells. Significantly, the isopropyl derivative (**18l**) showed an IC_{50} lower than 1 μM against the cell line U937.
Apart from the standard 5'-phosphoramidates, the 3'-phosphoramidate bearing isopropyl as the ester chain (**18m**) emerged as active against HL60 and particularly U937 cells.

In conclusion, although benzyl ester derivatives display higher activities in vitro, they might not be the best choices for in vivo models. Tuning of the ester chain could be used as a tool to selectively target individual cell lines and improve the pharmacokinetic properties of the prodrug.

5.5 Synthesis of 2CdA phosphoramidates

Phosphoramidates **18a-r** were synthesised by coupling of the corresponding phosphorochloridates (**11l**, napthyl phosphorochloridate; **20a-h**, phenyl phosphorochloridates) with 2CdA in the presence of tBuMgCl as described for compounds **12e,f** in Chapter 4 (Scheme 5.5, general synthetic pathway).



Scheme 5.5: Synthesis of compounds **18a-r**.

The phenyl phosphorochloridate intermediates (**20a-h**) were obtained through coupling of the commercially available phenyl dichlorophosphate to the corresponding amino acid ester salts and purified by flash chromatography before the

final coupling with 2CdA (Table 5.5). Synthesis of the naphthyl phosphorochloridate **11l** is described in Chapter 3.

Cpd	R'	R''	X	Yield (%)	^{31}P-NMR ($CDCl_3$), δ*
20a	$PhCH_2$	H	Bn	63.1	7.87,7.81
20b	$CH(Me)_2$	H	Bn	81.9	9.60,9.01
20c	CH(Me)Et	H	Bn	72.0	9.10,8.65
20d	H	Me	Bn	83.2	7.20,7.02
20e	Me	H	Et	59.2	8.03,7.72
20f	Me	H	iPr	71.4	8.14,7.87
20g	Me	H	tBu	59.1	8.21,7.85
20h	Me	H	2-Bu	31.8	8.12,7.84

Table 5.5: Phenyl phosphorochloridates **20a-h**. *See experimental section for field frequency used.

As described in Chapter 4, the use of a Grignard base offered the advantage of an easier purification of the final products but, at the same time, the coupling with the NA lost the selectivity for the 5' hydroxy group. In Table 5.6 the final products **18a-r** are reported, which represent the usual 5'-phosphoramidates along with the 3'-phosphoramidates and the 3',5'-diphosphoramidates.

Although the crude mixture arising from the final coupling with 2CdA was rendered more complex by the presence of three kinds of phosphorylated compounds, they could be separated by classical column chromatography. In fact, the 3',5'-diphosphoramidates (**18n,r**) were characterised by a higher lipophilicity in comparison to the monophosphorylated derivatives in 3' and 5' and were easily separated.

The derivatives of monophosphorylation in 3' and 5' (**18a,b**; **18c,d**; **18e,f**; **18l,m**; **18p,q**) showed closer retention times but still differed enough to be individually collected.

Cpd	R'	R''	X	Phosph.	Yield (%)	^{31}P-NMR δ#
18a	$PhCH_2$	H	Bn	5'	14.6Ω	3.33,3.07
18b	$PhCH_2$	H	Bn	3'	9.4Ω	1.42,1.23
18c*	$PhCH_2$	H	Bn	5'	8.9Ω	3.69,3.46
18d*	$PhCH_2$	H	Bn	3'	3.2Ω	2.09,1.78
18e	$CH(Me)_2$	H	Bn	5'	4.8	4.19,3.92
18f	$CH(Me)_2$	H	Bn	3'	0.8	2.40,2.20
18g	CH(Me)Et	H	Bn	5'	2.2	3.88,3.71
18h	H	Me	Bn	5'	6.9	3.33,2.93
18i	Me	H	Et	5'	6.3Ω	3.27,3.03
18l	Me	H	iPr	5'	5.1	3.28,3.12
18m	Me	H	iPr	3'	5.6	1.54,1.34
18n	Me	H	iPr	3',5'	1.8	2.97,2.94,2.83,2.77,1.94,1.89 ,1.84,1.82
18o	Me	H	tBu	5'	24Ω	3.36,3.19
18p	Me	H	2-Bu	5'	6.8	3.29,3.10
18q	Me	H	2-Bu	3'	6.5	1.62,1.60,1.41Ψ
18r	Me	H	2-Bu	3',5'	1.8	2.97,2.94,2.82,2.76,1.92,1.91 ,1.85,1.82,1.81Ψ

Table 5.6: 2CdA phosphoramidates. # See experimental section for deuterated solvents and field frequency used. * Naphthyloxy derivatives. Ω Calculated on reacted starting material. Ψ Number of signals increased by the use of 2-butyl as R,S mixture.

In Figure 5.7, the HPLC chromatogram of the mixture **18p-r** is reported as an example of the different retention for products of monophosphorylation (in 3' and 5') and diphosphorylation.

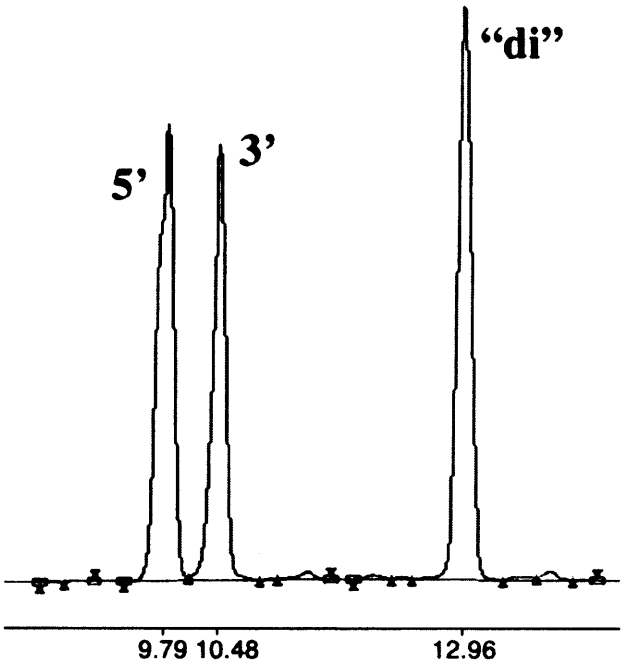


Figure 5.7: HPLC chromatogram (H_2O/CH_3CN, from 100/0 to 0/100 in 15 min) of the mixture **18p-r**.

3',5' Diphosphoramidates **18n,r** were readily distinguished from the derivatives of monophosphorylation by mass spectroscopy, ^{31}P NMR (eight signals due to the presence of four diastereoisomers) and integration of the corresponding ^{1}H NMR spectra.

Conversely, mass spectroscopy and ^{1}H NMR integration could not be used as tools to discriminate the 3'-phosphoramidates from the 5'-phosphoramidates. Although they showed significant chemical shift changes on ^{31}P, ^{1}H and ^{13}C NMR, these data could not be used to definitely confirm their structures. Splitting of the carbon signals due to the coupling with phosphorus was a useful means to identify the 5' phosphorylated derivatives (coupling of the C-5' with the P atom) and the 3'

phosphorylated derivatives (coupling of the C-3' with the P atom) as shown in Figure 5.8 for compounds **18a** and **18b**.

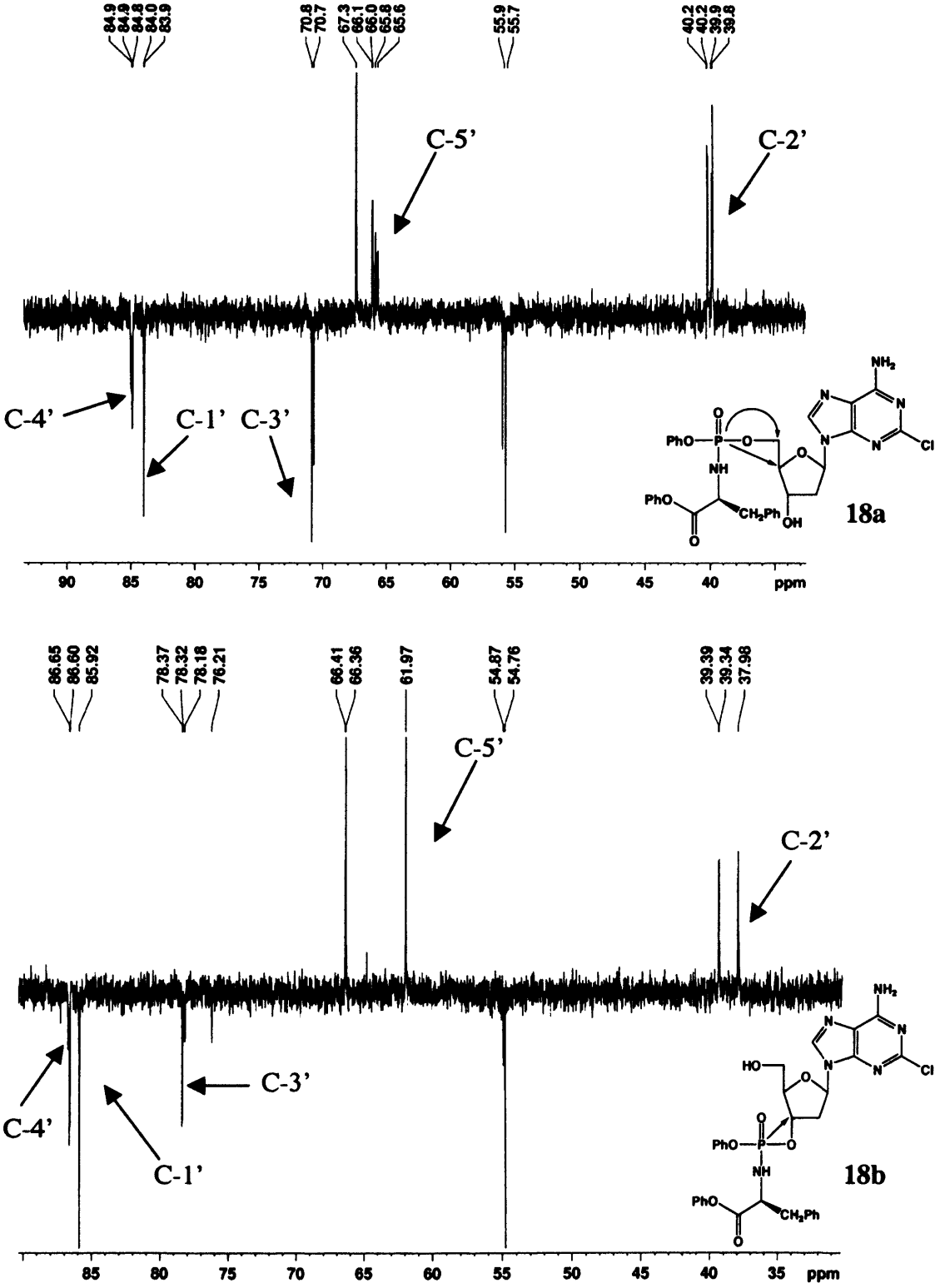


Figure 5.8: Expansions of ^{13}C NMR spectra ($CDCl_3$) of compounds **18a** and **18b**.

For compound **18a**, the effect of the P-coupling was clearly observed for C-5' (four peaks) and C-4' (three peaks) while the remaining carbons of the sugar (C-3', C-2' and C-1') showed only two peaks due to the presence of diastereoisomers.
Conversely, the multiplicity of C-5' was drastically reduced (one peak) in **18b** while C-3' showed the expected increase (3 peaks). Although C-2' and C-4' did not appear to show an extra multiplicity due to the P-coupling (probably due to an insufficient number of scans), the ^{13}C-NMR showed an expected series of chemical shift changes (eg, down-field for C-3', up-field for C-5'). For a better interpretation, the ^{13}C-NMR of 2CdA is shown in Figure 5.9 (N.B. in this case DMSO was used as deuterated solvent).

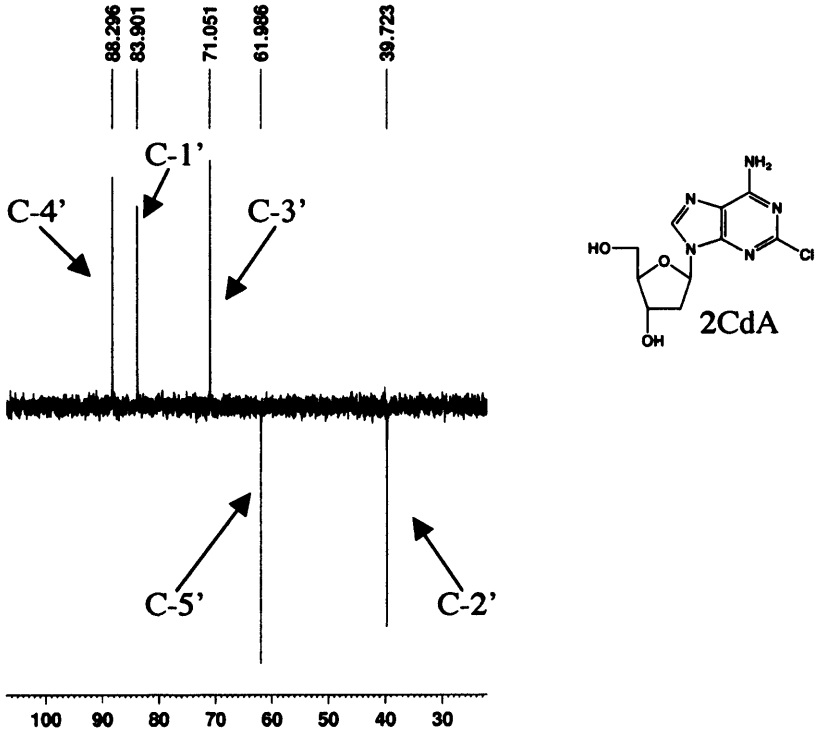
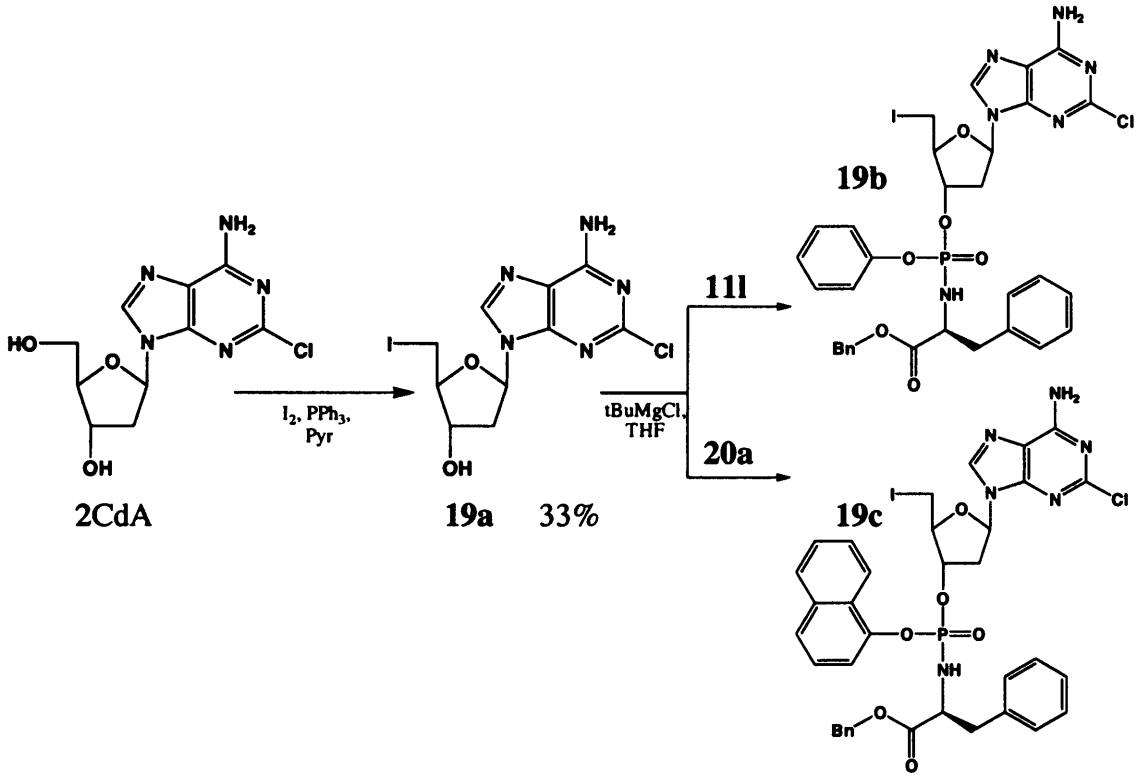


Figure 5.9: ^{13}C NMR expansion (DMSO) of 2CdA.

5.6 Synthesis of 5'-deoxy-5'-iodo-2CdA and its phosphoramidates

Unprotected 2CdA was selectively iodinated at 5'[11] through treatment with iodine and triphenylphoshine in pyridine to give **19a**. The 5'-iodinated nucleoside analogue was then coupled to the corresponding phosphorochloridates in the presence of tBuMgCl to yield the final products **19b** and **19c** (Scheme 5.6).



Scheme 5.6: Synthesis of **19a** and the corresponding phosphoramidates **19b,c**.

Use of a larger excess of phosphorylating agent **20a** (2.5 equivalents) led to an increased yield of the final derivative **19c** in comparison to **19b**, which was treated with 1.3 equivalents of **11l** (Table 5.7).

Cpd	Yield (%)$^{\Omega}$	^{31}P-NMR δ*
19b	13.4	2.63,2.37
19c	80.4	3.21,2.83

Table 5.7: 5'-I-2'-deoxy-2-chloroadenosine phosphoramidates. * See experimental section for deuterated solvents and field frequency used. $^{\Omega}$ Calculated on reacted starting material.

[1] Cahard, D.; McGuigan, C.; Balzarini, J. Aryloxy phosphoramidate triesters as pro-tides. *Mini Rev. Med. Chem.* **2004**, 4, 371-482.

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[8] Balzarini, J.; Egberink, H.; Hartmann, K.; Cahard, D.; Thormar, H.; De Clercq, E.; McGuigan, C. Anti-retrovirus specificity and intracellular metabolism of 2',3'-didehydro-dideoxythymidine (Stavudine) and its 5'-monophosphate triester prodrug So324. *Mol. Pharmacol.* **1996**, 50, 1207-1213.

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Chapter 6: Assignment of the absolute configuration of the phosphorus centre for isolated diastereoisomers

6.1 Introduction

Due to the chirality at the phosphorus centre, the final compound isolated from the coupling of a phosphorochloridate with a nucleoside analogue is a mixture of two diastereoisomers. The presence of two diastereoisomers is confirmed by HPLC, ^{1}H, ^{13}C and ^{31}P NMR. In particular, ^{31}P NMR is an extremely useful tool in this work as it offers a first indication about the formation and the purity of our samples. Regardless of the coupling reagent used (NMI or tBuMgCl), the final compounds are synthesised as mixtures of two phosphate diastereoisomers in a ca. 1:1 ratio. Phosphorochloridates containing proline represent the only notable exception as their coupling to nucleoside analogues leads to an imbalanced ratio between the final phosphoramidate diastereoisomers (typically 1:6).

From the synthesis of our first phosphoramidate in 1992,[1] separation and testing of isolated diastereoisomers has been pursued in order to verify possible biological differences between the two phosphate diastereoisomers.
The phosphorus stereochemistry has been shown to affect biological activity of phosphoramidates.[2] In some cases,[3] one diastereoisomer can be more active than the other one (up to a 10-fold difference) and the biological activity displayed by the 1:1 mixture results an average value between those arising from its single components.
In addition to biological activity, membrane permeability, chemical and enzymatic stability have been shown to be stereoselective,[4,5] suggesting that in vivo circulating levels of intact pronucleotide could exert a profoundly different activity or toxicity due to preferential distribution and metabolism of one diastereoisomeric form.

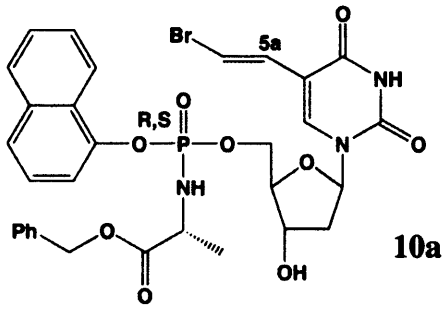
Although laborious, in some cases separation of diastereoisomers can be achieved by reverse phase preparative HPLC or repeated application of column chromatography over silica. Furthermore, when single diastereoisomers have been isolated,

identification of the corresponding absolute stereochemistry on the phosphorus centre has never been clarified, leaving HPLC retention time and ^{31}P NMR chemical shift as the only parameters to discriminate between the two isolated diastereoisomers.

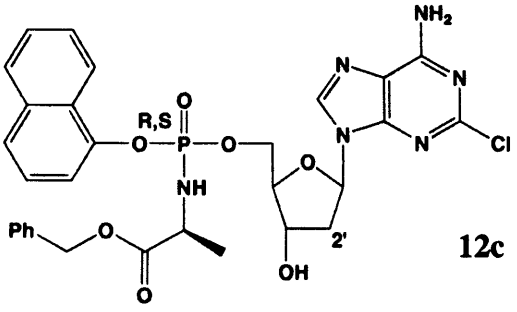
The present work describes how, through a combination of NMR and conformational modelling studies, the absolute stereochemistry of the phosphorus centre for some isolated diastereoisomers was suggested for the first time.

6.2 ^{1}H NMR of separated diastereoisomers

Within the phosphoramidates presented in this Thesis, partial separation of the corresponding diastereoisomers was achieved with compounds **10a** and **12c** (Figure 6.1) through preparative reverse phase HPLC.



10a



12c

Figure 6.1: Structures of **10a** and **12c**, diastereoisomeric mixtures.

As shown in Figure 6.2, the number of signals in the ^{1}H NMRs of our final compound mixtures (top spectra) clearly showed the presence of two diastereoisomers. In fact, the vinylic H-5a of **10a** gave a doublet for every separated diastereoisomer (bottom and middle spectra; NB: separation was not complete) resulting in two doublets in the mixture. The H-2' in the separated diastereoisomers of **12c** gave one (bottom spectrum) and two multiplets (middle spectrum) resulting in an extended multiplet in the mixture.

In general, signals in the mixtures were doubled compared to that expected for a diastereoisomerically pure compound and "double signals" were usually observed for almost every single hydrogen in the molecule.

Although signals related to the same proton (e.g. H-5a of **10a**) could have a chemical shift difference ($\Delta\delta$) of 0.2 ppm for the two diastereoisomers, it was hard to correlate the differences observed to the stereochemistry of the phosphorus centre.

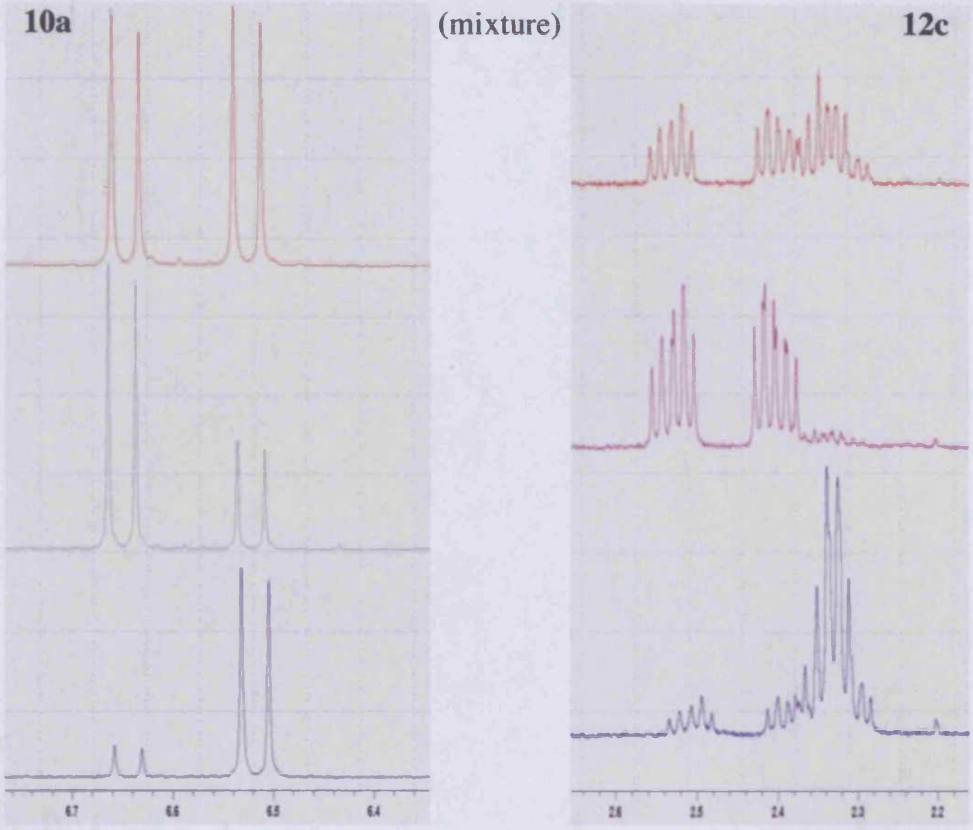


Figure 6.2: ^{1}H NMR ($CDCl_3$) expansions of diastereoisomeric mixtures (top spectra) and isolated diastereoisomers (middle and bottom spectra) of **10a** (vinylic H-5a shown) and **12c** (H-2' shown).

However, a striking result appeared when the ^{1}H NMRs of the methylene hydrogens of the benzylic ester were analysed (Figure 6.3). These two diastereotopic protons constitute an AB system and therefore, four doublets were expected to be displayed on the ^{1}H NMR of the mixture (an AB system for each diastereoisomer). Although an AB system was shown in the mixture (Figure 6.3, top spectra), it was related to only one of the two separated diastereoisomers while the remaining showed either a doublet or a singlet.

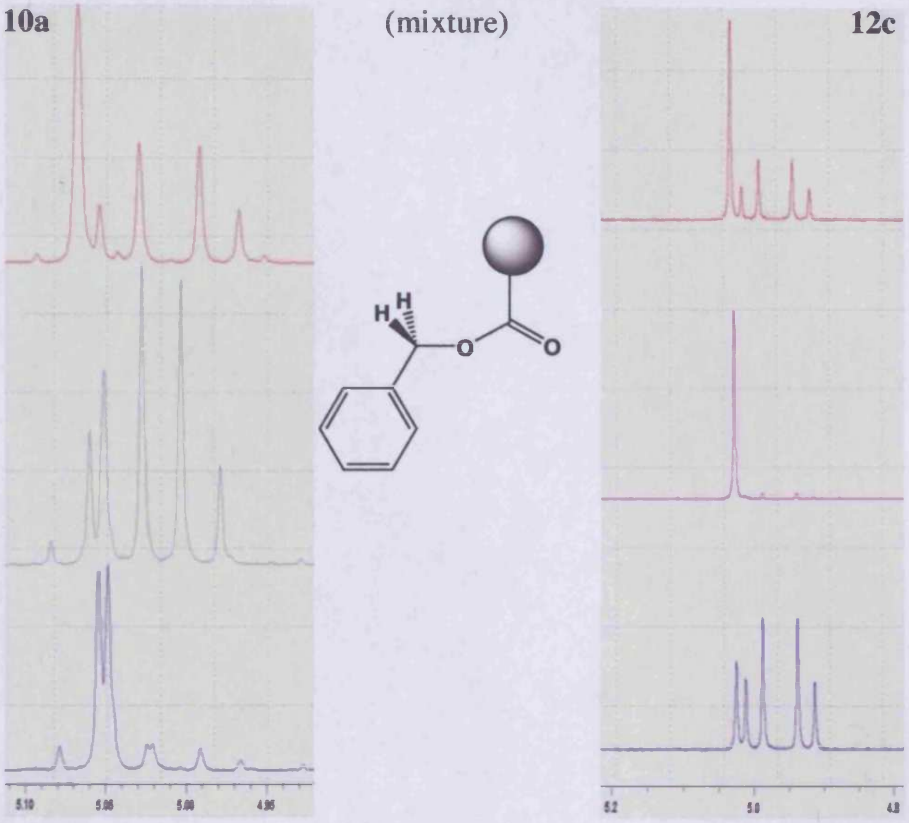


Figure 6.3: ^{1}H NMR ($CDCl_3$) expansions of the benzylic ester methylene protons of diastereoisomeric mixtures (top spectra) and isolated diastereoisomers (middle and bottom spectra) of **10a** and **12c**.

Possibly, the singlet and the doublet observed were apparent, in a sense that they were an AB system that collapsed to give a simplified spectrum. Given these observations, it was supposed that the different magnetic environment that led to such diversity on ^{1}H NMR could have been a consequence of a relevant conformational difference between the two phosphate diastereoisomers.

Moreover, if a correlation could be found between the conformations and the corresponding ^{1}H NMRs of the separated diastereoisomers, we could in theory suggest for the first time the absolute phosphorus configuration for each of the two phosphate diastereoisomers.

6.3 Conformational studies

Conformational simulations of the R and S phosphate diastereoisomers of **10a** and **12c** (Figure 6.4) were performed using the Sybyl 7.0 package. Two methods were used: Genetic Algorithm (GA) search and Random search. The GA search was performed using a number of generations of 5000 and a population of 100 with a dielectric constant of 4.8 ($CHCl_3$). The Random search was performed using a dielectric constant of 4.8 ($CHCl_3$) and minimisation of the generated conformers was performed using the MMFF94s Force Field to a gradient of 0.05 kcal mol^{-1} $Å^{-1}$; the conformations within 5 kcal mol^{-1} from the lowest energy conformation generated were stored and analysed. Results obtained from both the GA and the Random search were comparable.

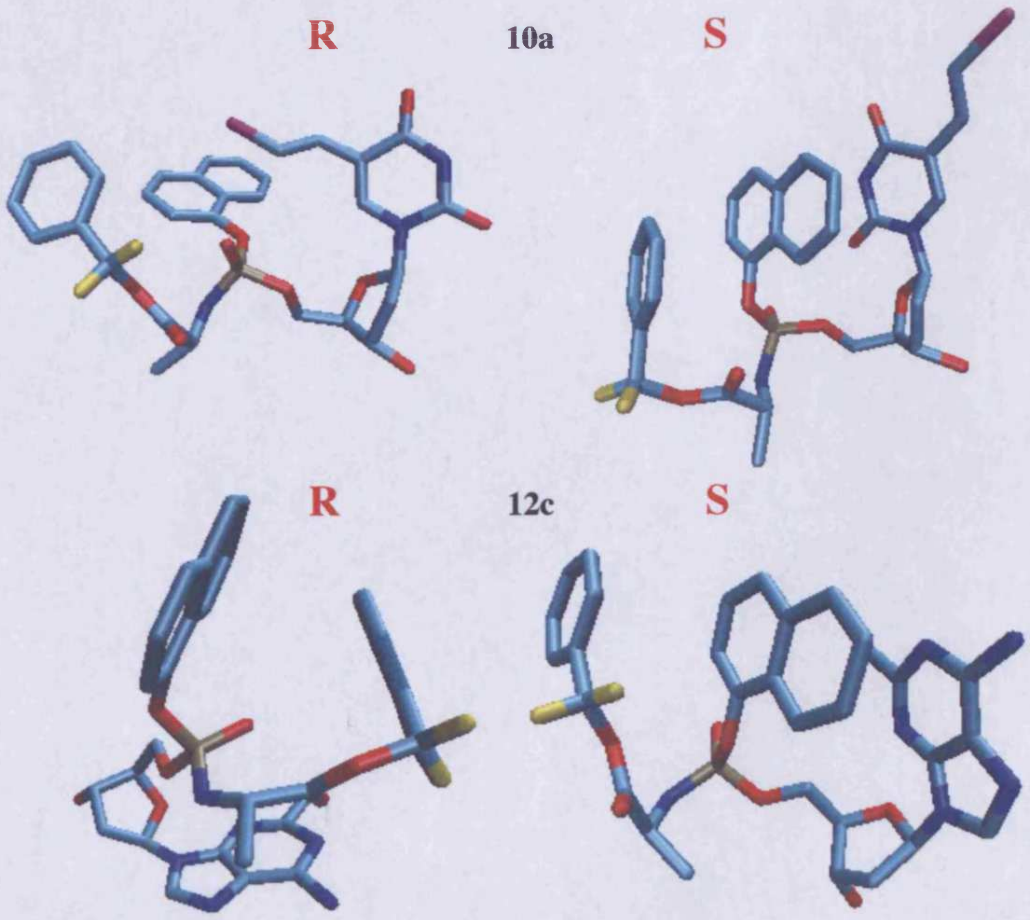


Figure 6.4: Lowest energy conformations for the diastereoisomers of **10a** and **12c**.

For the S diastereoisomers (for clarity, diastereoisomers were named after the absolute stereochemistry of the corresponding phosphorus centre) of compounds **10a** and **12c** (Figure 6.4; S phosphorus configuration on the right, R phosphorus configuration on the left), the three aromatic moieties constituting each molecule were stacked in pi-pi interactions where the naphthyl laid between the benzyl group and the nucleoside base. The apparent rigidity of this conformation, conferred by the observed aromatic interactions, could accentuate the magnetic difference between the methylene hydrogens of the benzylic ester (yellow atoms), justifying the AB system pattern where a double doublet was displayed (Figure 6.5).

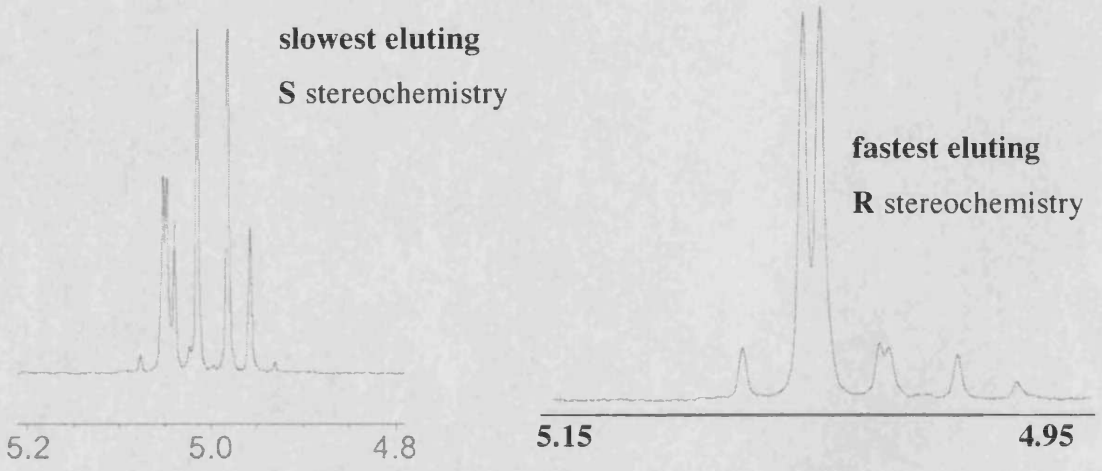


Figure 6.5: Assignment of the phosphorus stereochemistry to the separated diastereoisomers of **10a**.

The R conformations did not show pi-pi interactions. Therefore, the greater flexibility around the benzylic moiety could reduce the magnetic difference between the methylene hydrogens of the benzylic ester (yellow atoms) leading to a collapsed AB system (Figure 6.5).

Therefore, combining the NMR and the conformational data (Figure 6.5; **10a** was chosen as example), we proposed the S phosphorus absolute configuration to the diastereoisomers of **10a** and **12c** that displayed a double doublet for the benzylic methylene hydrogens (they are the slowest eluting on reverse phase HPLC), and the R configuration to the diastereoisomers displaying a doublet (**10a**) or a singlet (**12c**) (fastest eluting on reverse phase HPLC).

In order to confirm the assignment of the phosphorus configuration, several NMR experiments, including NOESY, ROESY and a series of NOEs, were performed but the results were unclear and inconclusive. The extensive overlapping in the aromatic region did not facilitate the interpretation of these spectra.

6.4 Biological activity of separated diastereoisomers

The separated phosphate diastereoisomers of **10a** and **12c** were evaluated against a breast cancer cell line and a wide panel of myeloid cell lines respectively (Table 6.1).

	breast	Leukaemic					
Cpd	**MDA MB 231**	**NB4**	**HL60**	**NB4R2**	**K562**	**KG1**	**U937**
10a	6.3	-	-	-	-	-	-
-S	0.5	-	-	-	-	-	-
-R	7.4	-	-	-	-	-	-
12c	-	0.24	0.65	1.03	2.36	1.80	0.19
-S	-	0.23	1.78	1.01	5.80	1.54	0.14
-R	-	0.43	1.66	1.18	2.28	2.63	0.28

Table 6.1: Biological evaluation of mixtures and separated diastereoisomers of **10a** and **12c**, $EC_{50}/\mu M$.

According to our existing data, the activity of one diastereoisomer generally differs from the activity of the other. The S diastereoisomer of **10a** appeared to be 10-fold more active than the R derivative, with the mixture showing an EC_{50} value included in this range. Although with a smaller magnitude, also separated diastereoisomers of **12c** showed differences in biological effects.

These data further demonstrated that separation of diastereoisomers could boost the activity of phosphoramidates. The suggested NMR-modelling combined approach can be used, in some cases, as a preliminary tool to predict the absolute configuration of the phosphate centre.

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[4] Siccardi, D.; Kandalaft, L. E.; Gumbleton, M.; McGuigan, C. Stereoselective and concentration-dependent polarized epithelial permeability of a series of phosphoramidate triesters prodrugs of d4T: an in vitro study in Caco-2 and Madin-Darby canine kidney cell monolayer. *J. Pharmacol. Exp. Ther.* **2003**, 3, 1112-1119.

[5] Siccardi, D.; Gumbleton, M.; Omidi, Y.; McGuigan, C. Stereospecific chemical and enzymatic stability of phosphoramidate triester prodrugs of d4T in vitro. *Eur. J. Pharm. Sci.* **2004**, 22, 25-31.

Chapter 7: Application of the phosphoramidate approach to active and inactive nucleosides

7.1 Isocladribine and DAPdR phosphoramidates

The application of the phosphoramidate approach to BVdU and Cladribine described in this Thesis has confirmed the effectiveness of our technology in boosting the properties of NAs, as extensively reported in the literature.[1-4] In the ProTide field, different strategies[5-7] have been investigated with the main intent to increase the biological activity of existing nucleoside analogue drugs. NAs with a high biological activity constitute the usual target for the application of such ProTide methods, since the intracellular delivery of the corresponding NA-monophosphates often leads to a further improved activity.

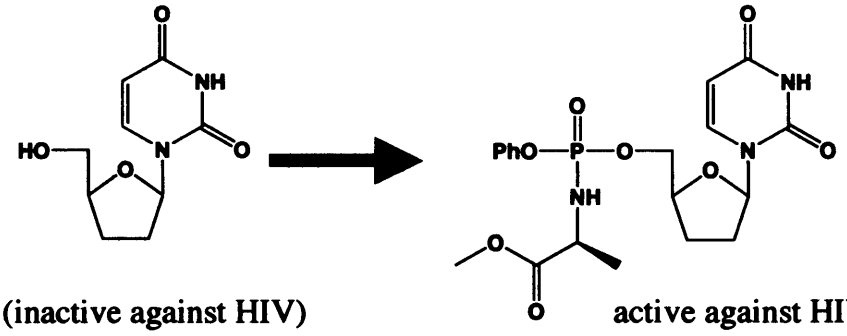
The activity of NAs is related to their phosphorylated forms but, in most of the cases, NAs are only poor substrates for kinases. When a NA is not a substrate for kinases, there is no biological correlation between the parent nucleoside and its monophosphate form. In these cases, the inactivity of the parent nucleoside does not necessarily constitute a biological evidence for the prediction of the biological properties of its phosphorylated species. It is possible for a NA to have in its structure the potential to be highly toxic towards cancer or virus-infected cells but because of the same structural differences from the natural nucleoside, is not recognised as a substrate by kinases and not converted to the phosphorylated active species.

Dideoxyuridine (ddU) and BVdU are two typical examples (Scheme 7.1).
DdU failed to show any anti-HIV activity in an antiviral screening of dideoxy-nucleosides (ddNs).[8] However, our basic aryloxy phosphoramidate derivative emerged as a potent anti-HIV agent, the activity of which was attributed to its ability to deliver the ddUMP inside infected cells.[9]

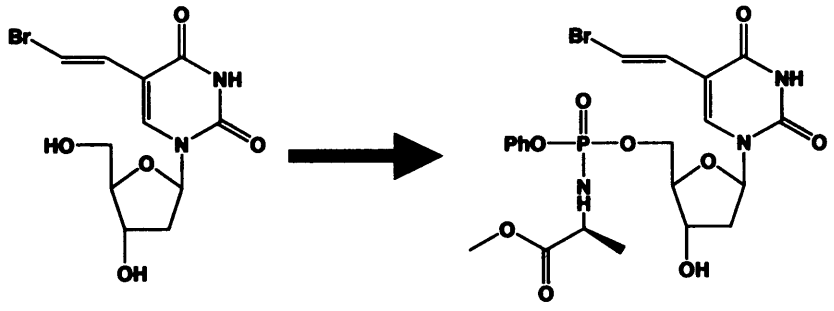
Moreover, as described in Chapter 2, the application of our approach to BVdU conferred unexpected anticancer properties on the phosphoramidate derivatives of an anti-herpetic nucleoside analogue.

The aim of our work was to explore the full potential of our technology. Therefore, we applied our technology to inactive nucleoside analogues to investigate whether their phosphoramidate derivatives maintained the substantial biological inactivity of their parent NAs or showed biological effects.

Inactive NAs ≠ Inactive ProTides



ddU (inactive against HIV) active against HIV



BVdU (anti-herpetic) Thymectacin (anti-cancer)

Scheme 7.1: Modifying the biological properties of NAs.

The synthesis of Cladribine (2CdA) offered access to some other purine-modified 2'-deoxy-ribonucleosides such as 2-amino-6-chloro-2'-deoxyriboside (for simplicity named Isocladribine, IsoClad) and 2,6-diaminopurine-2'-deoxyriboside (DAPdR) (Figure 7.1).

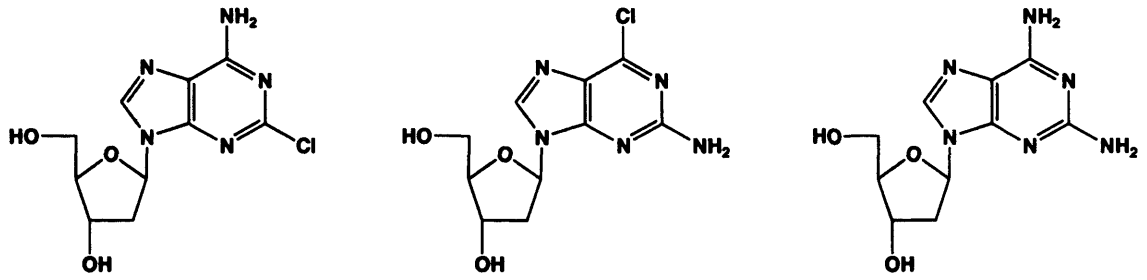


Figure 7.1: purine-modified deoxyribonucleosides.

To our knowledge, IsoClad is unknown as a cytostatic compound in the literature whereas DAPdR had been reported as moderately active against CCRF-CEM cells (IC_{50} 13 μM).[10] Therefore, IsoClad represented an ideal inactive nucleoside analogue for the application of our phosphoramidate approach (Figure 7.2). In addition, it was an available side product from 2CdA synthesis.

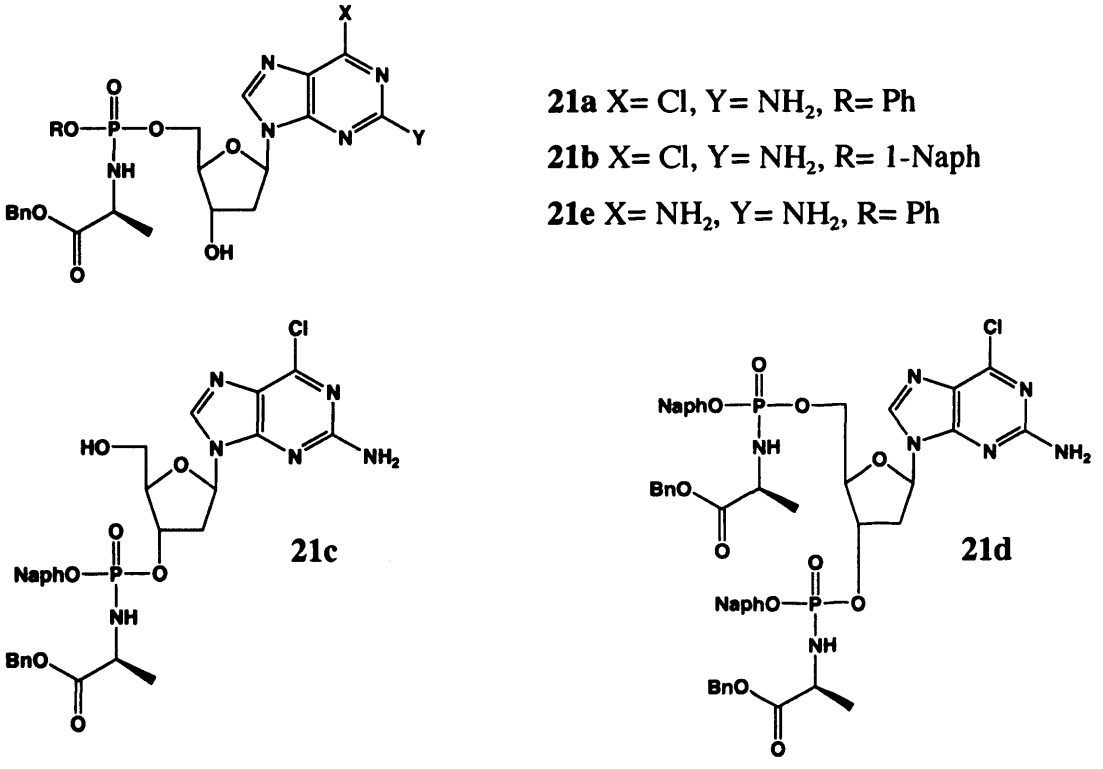


Figure 7.2: IsoClad and DAPdR phosphoramidates synthesised.

As expected, IsoClad did not show a relevant cytostatic activity against the panel of leukaemic cell lines used in our in vitro assays (Figure 7.3 and Table 7.1). Nevertheless, the IsoClad phenyloxy phosphoramidate **21a** displayed a significant and selective activity against KG1 cells. The introduction of a naphthyl as the aryl moiety on the standard 5'-phosphate structure (**21b**) did not appear to significantly enhance the activity observed for the latter compound. The derivative of phosphorylation in 3' (**21c**) and the 3',5'- diphosphorylated (**21d**) gained cytostatic effects against NB4, HL60, and U937 cell lines in comparison to **21a**. However the activity against KG1 appeared to be lost.

Cpd	Phosphate	NB4	HL60	NB4R2	K562	KG1	U937
IsoClad	-	>10	>10	>10	>10	>10	>10
21a	5'-IsoClad	>10	>10	>10	>10	2.64	>10
21b	5'-IsoClad	8.51	>10	>10	>10	>10	11.1
21c	3'-IsoClad	7.15	6.43	>10	>10	>10	4.83
21d	3',5'-IsoClad	5.07	5.66	11.05	>10	>10	4.63
DAPdR	-	>10	>10	>10	6.91	>10	4.80
21e	5'-DAPdR	9.92	8.56	>10	>10	>10	0.96

Table 7.1: IsoClad and DAPdR phosphoramidates activity (IC_{50} μM).

The moderate cytostatic activity reported in the literature for DAPdR was confirmed in our in vitro evaluation for the K562 and U937 leukaemic cell lines. The DAPdR phosphoramidate derivative **21e** showed an increased cytostatic activity against NB4 and HL60 in comparison to DAPdR. In the case of U937 cells, the cytostatic activity was significantly potentiated under 1 μM. Notably, **21e** lost the effect of the parent NA against the K562 cell line.

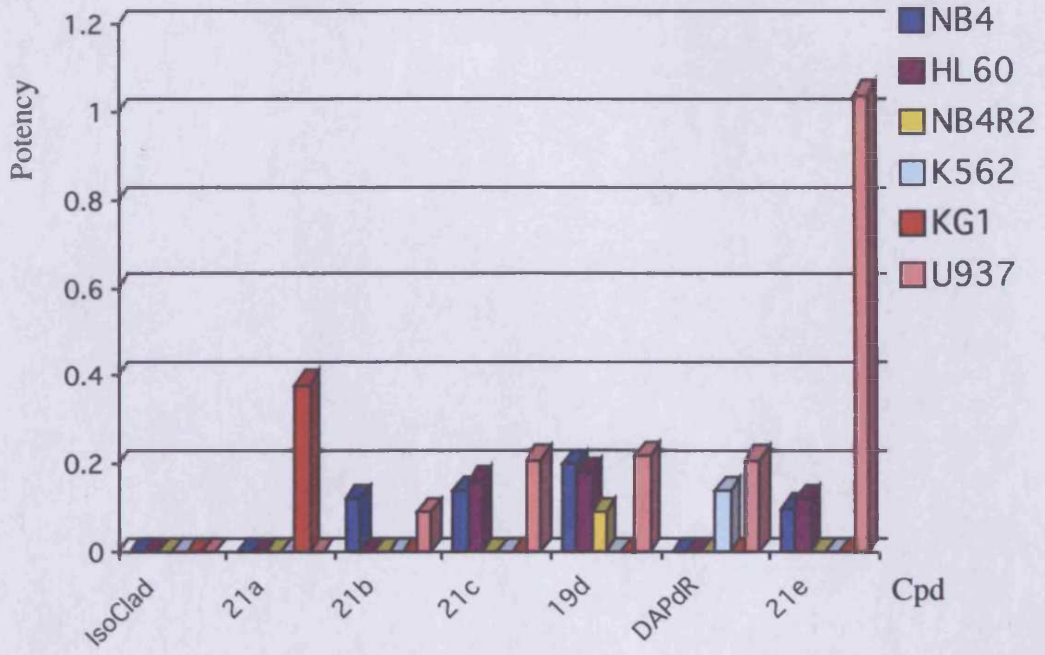
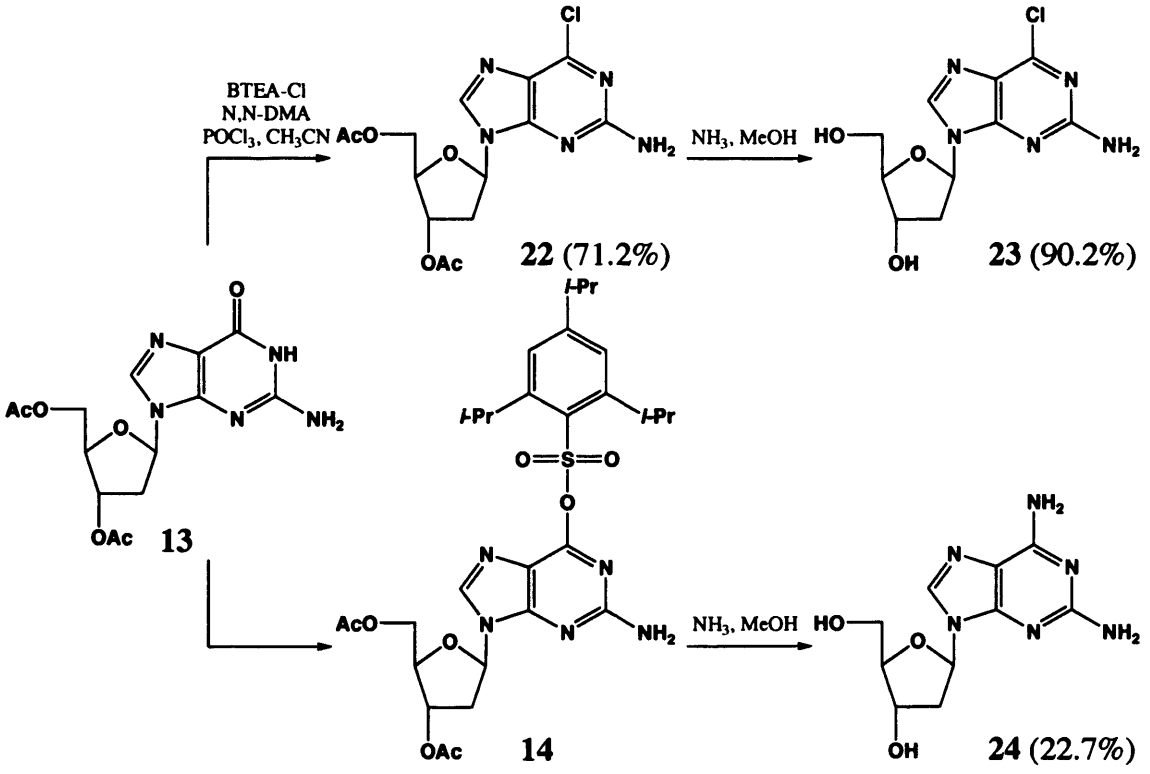


Figure 7.3: Potency ($1/IC_{50}$) of IsoClad, DAPdR and their phosphoramidates **21a-e** in leukaemic cells.

In particular, the biological profile of **21a** proved that the application of our phosphoramidate approach to the inactive nucleoside analogue IsoClad, could lead to a significant and specific cytostatic activity against the leukemic cell line KG1. Notably, IsoClad was known in the literature only as a synthetic precursor for the synthesis of modified deoxyadenosines. This evidence, along with the biological data for the DAPdR phosphoramidate **21e**, confirmed that biological activity of NAs is not a necessary requisite for the successful application of our ProTide technology.

7.2 Synthesis of IsoClad, DAPdR and related phosphoramidates

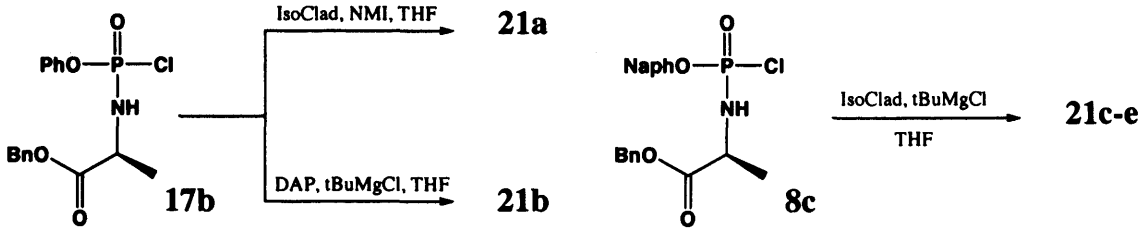
IsoClad (**23**) and DAPdR (**24**) were synthesised through the deprotection of **22** and **14** respectively, which were reported in the literature as intermediates for the synthesis of 2-CdA (Scheme 7.2).[11] Deoxychlorination of **13** with $POCl_3$, N,N-dimethylaniline (N,N-DMA) and benzyltriethylammonium chloride (BTEA-Cl) in acetonitrile gave **22**, which was converted to **23** through ammonolysis.
DAPdR (**24**) was obtained via ammonolysis of **14**, synthesis of which was previously described in Chapter 5.



Scheme 7.2: Synthesis of IsoClad (**23**) and DAPdR (**24**).

Phosphoramidates **21a-e** were synthesised through the coupling of the corresponding NA with the appropriate phosphorochloridate as shown in Scheme 7.3. Derivative **21a** was obtained through the use of NMI as a coupling reagent. Compounds **21b-e**

were synthesised by using tBuMgCl. Final yields and ^{31}P NMR chemical shifts are shown in Table 7.2.



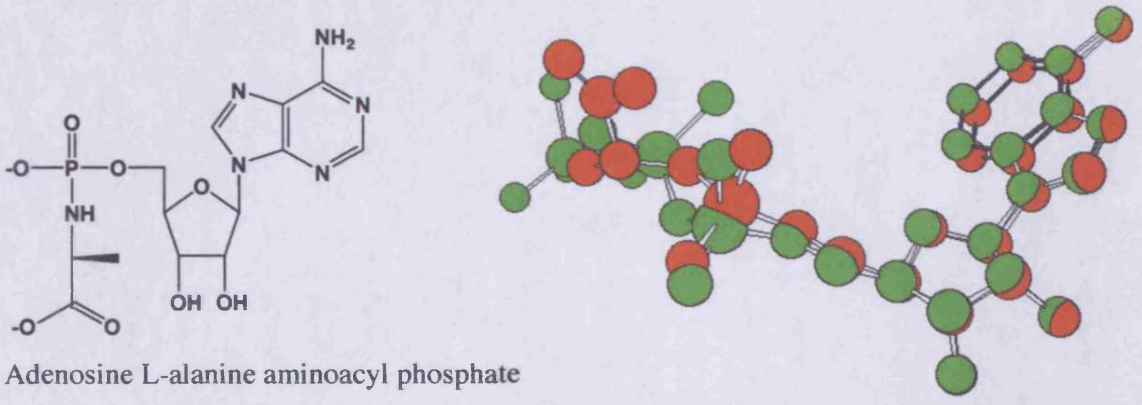
Scheme 7.3: Synthesis of phosphoramidates **21a-e.**

Cpd	Yield (%)	^{31}P-NMR δ*
21a	27.0	5.33,5.00
21b	22.4$^{\Omega}$	3.87,3.47
21c$^{\infty}$	6.6$^{\Omega}$	2.04,1.88
21d	62.7$^{\Omega}$	3.20,3.16,2.59,2.56,1.33,1.29,1.28,1.24
21e	10.7$^{\Omega}$	3.99,3.44

Table 7.2: Phosphoramidates **21a-e.** * See experimental section for deuterated solvents and field frequency used. $^{\Omega}$ Calculated on reacted starting material. $^{\infty}$ 3',5' bisphosphate.

7.3 Adenosine phosphoramidates

Adenosine triphosphate (ATP) is a ubiquitous cell metabolite that takes part in a wide variety of metabolic functions. In the present case, our interest was focused on the role of ATP in the regulation of the cyclin-dependent kinases (CDK), enzymes that play a fundamental role in the cellular cycle regulation. The L-alanine aminoacyl phosphate derivative of adenosine (Figure 7.4) had been previously designed by us as an ATP-mimic and found able to inhibit the cyclin-dependent kinase CDK2/A at 30 μM.



Adenosine L-alanine aminoacyl phosphate

Figure 7.4: 3-Dimensional alignment of ATP (green) with the adenosine L-alanine aminoacyl phosphate (orange).

Since aminoacyl phosphate derivatives were considered to be the main metabolic derivatives arising from the cellular activation of phosphoramidates, adenosine phosphoramidates could be strategically used to deliver their aminoacyl phosphates inside cells and alter the cellular cycle regulation through CDK inhibition, leading to cytotoxic effects.

On this basis, we planned the synthesis and cytostatic evaluation of new adenosine phosphoramidates (**25a-d**) focusing on the use of a phenyl masking group on the phosphate and methyl as the amino acid ester chain, according to our classical phosphoramidate structure (Figure 7.5). Although also the synthesis of the

corresponding aminoacyl phosphate derivatives was planned, only the L-methionine derivative (**25e**) was obtained (Figure 7.5).

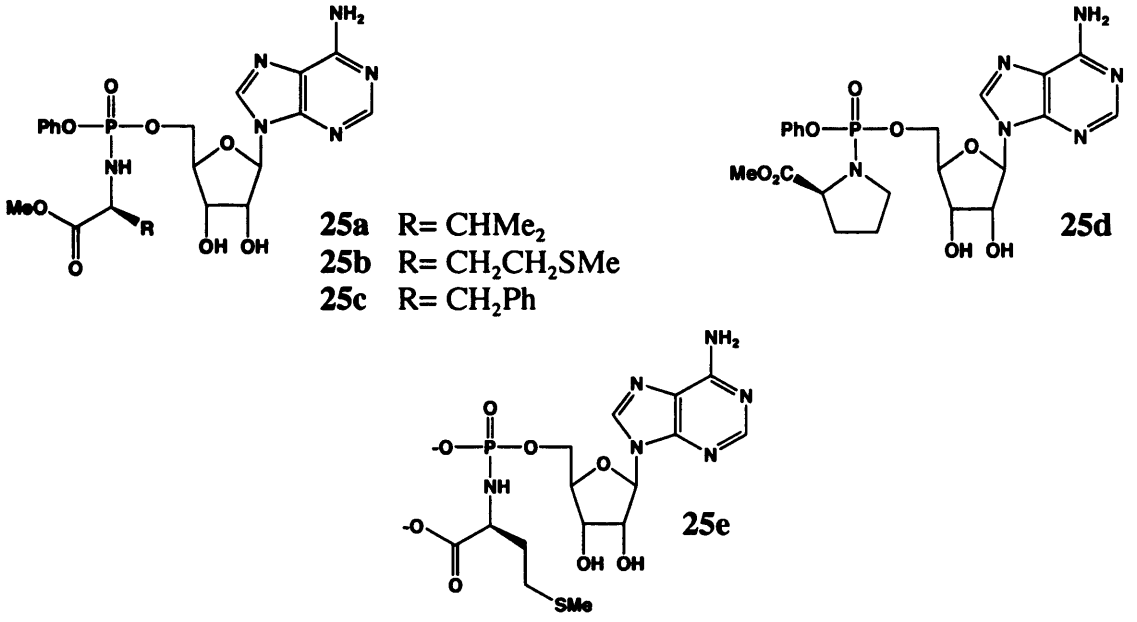


Figure 7.5: 25a-d adenosine phosphoramidates; **25e** adenosine L-methioninyl phosphate.

Protection of the 2' and 3' hydroxy groups of adenosine should be considered as a useful tool for the further development of this project. A protective group such acetonide (but also different ketals might be taken into account) could be used as a means to both improve the solubility of the nucleoside and reduced the formation of phosphorylated side products. In fact, purification of phosphoramidates was performed through repetitive application of column and preparative thin layer chromatography, which further reduced the final yields. Recently in our labs, deprotection of such groups was shown to occur in acidic conditions without damaging the phosphate moiety.

Hydrolysis was carried out on **25b** and **25c** by using a TEA/H_2O mixture. Given the difficulties experienced with the purification of **25e** on regular column chromatography, a reverse phase column chromatography was attempted on the

crude of the **25c** hydrolysis product (TEA/MeOH gradient elution). However, such purification was not successful.

Porcine liver esterases were used for the hydrolysis of **25d** in TRIZMA buffer at 37 °C for 27 h. However, hydrolysis appeared to be incomplete by ^{1}H NMR as the phenyl moiety was still attached to the phosphate.

Biological evaluation of test compounds **25a-e** was conducted on MDA MB 231 and PC-3 cancer cell lines (Table 7.3).

Adenosine phosphoramidates **25a-c** showed a significant and selective cytotoxicity towards the MDA MB 231 cancer cell line. Derivative **25c** emerged as the most potent of the series, with an IC_{50} value of 0.5 μM.
The phosphoramidate **25d** and the aminoacyl phosphate derivative **25e** showed only moderate biological activity against the two cancer cell lines considered. The lower activity observed for **25e** in comparison to its parent phosphoramidate **25b** can be explained with its reduced lipophilic character, which may impede passive diffusion through cell membranes.

Cpd	Amino Acid	Breast MDA MB 231	Prostate PC-3
25a	L-valine	18.3	97.6
25b	L-methionine	5.0	>200
25c	L-phenylalanine	0.5	>200
25d	L-proline	94	114.2
25e*	L-methionine	87.8	138.9

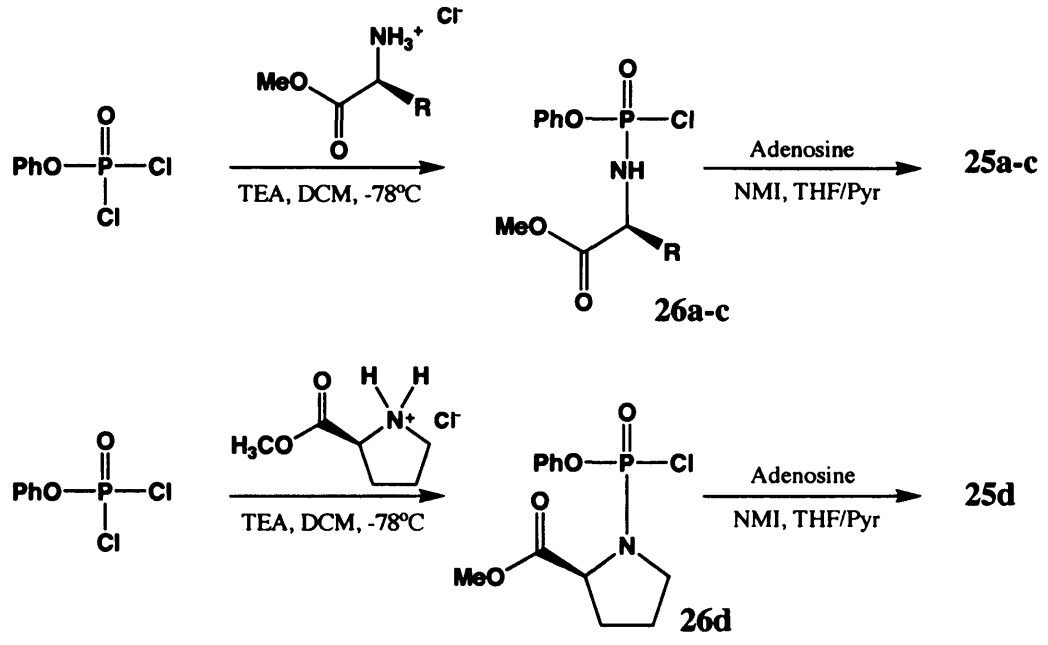
Table 7.3: Biological evaluation (IC_{50}/μM) for compounds **25a-e**. * Aminoacyl phosphate.

These surprising data might suggest that a selective cytotoxic activity can be achieved by the application of the phosphoramidate approach to natural nucleosides. Although the cytostatic mode of action of these adenosine phosphoramidates has not been clarified, previous data indicate a possible role as CDK inhibitors.

Alternatively, the intracellular delivery of adenosine monophosphate by administration of adenosine phosphoramidates could be considered as a means to cause ATP metabolic disruption. Complete intracellular activation of our phosphoramidates may cause an imbalanced level of the natural nucleoside monophosphate with consequent disruption of its metabolism leading to the observed cytostatic effects.

7.4 Synthesis of adenosine phosphoramidates

The phenyl phosphorochloridate intermediates (**26a-d**, Scheme 7.4) used for the synthesis of the adenosine phosphoramidates were obtained through the coupling of the commercially available phenyl dichlorophosphate to the corresponding amino acid ester salts following the standard conditions. The intermediate **26b** was used as crude material without further purification whereas all the other phosphorochloridates were purified through by flash chromatography (Table 7.4).



Scheme 7.4: Synthesis of adenosine phenyloxy phosphoramidates.

Cpd	R	Yield (%)	^{31}P-NMR δ($CDCl_3$)
26a	$CHMe_2$	39.9	11.01,10.40
26b	$CH_2CH_2SCH_3$	97.4	10.03,9.77
26c	CH_2Ph	59.4	9.53,9.31
26d	-	67.5	9.21,8.99

Table 7.4: Phenyl phosphorochloridates **26a-d**.

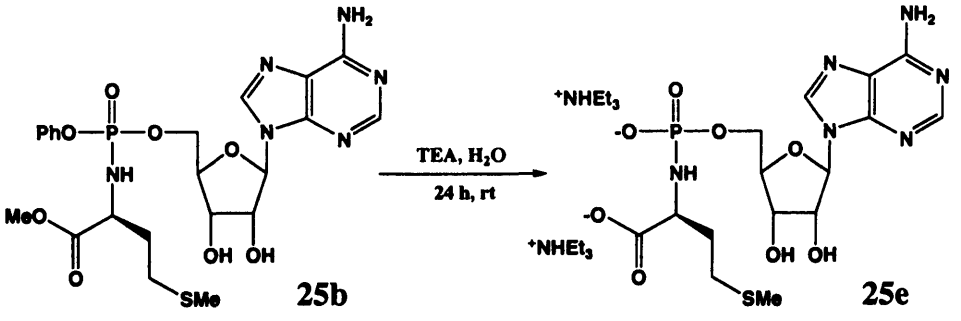
Phosphoramidates **25a-d** were synthesised according to the NMI coupling procedure in order to favour the phosphorylation at the primary hydroxy group at 5'. Pyridine was used as a co-solvent to enhance the solubility of adenosine in THF. Removal of NMI was performed through repeated application of column chromatography, which resulted in reduction of the final yields (Table 7.5).

Cpd	R	Yield (%)	^{31}P-NMR δ(MeOD)
25a	$CHMe_2$	13.3	5.88,5.79
25b	$CH_2CH_2SCH_3$	8.0	5.31,5.09
25c	CH_2Ph	2.5	4.96,4.60
25d	-	24.5	3.06,2.92

Table 7.5: Adenosine phenyloxy phosphoramidates **25a-d**.

Interestingly, the L-prolinyl phosphoramidate **25d** showed on ^{31}P NMR the same imbalanced ratio between the two diastereoisomers as already observed with other NAs. The approximate ratio was 3:1 versus the usual 1;1 ratio.

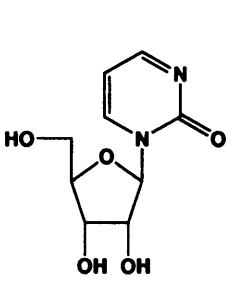
Compound **25e** was obtained through partial hydrolysis of **25b** in a triethylamine/water mixture (Scheme 7.5) and purified by preparative thin layer chromatography.

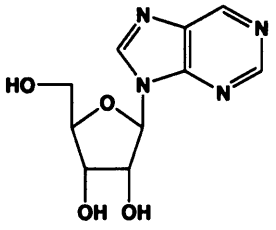


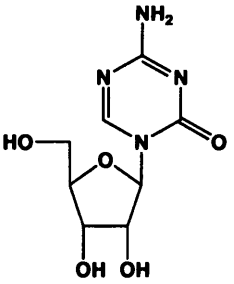
Scheme 7.5: Synthesis of **25e**.

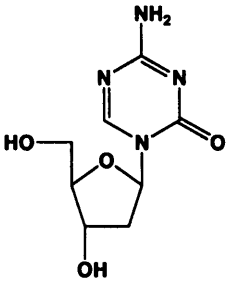
7.5 Zebularine phosphoramidates

Zebularine (Zeb) is a cytidine analogue containing a 2-(1H)-pyrimidinone ring, substantially the pyrimidine counterpart of the purine natural product nebularine, which is known as an adenosine deaminase (ADA) inhibitor. Zeb was first described in the literature as a potent inhibitor of bacterial growth[12] and subsequently reported as a potent inhibitor of cytidine deaminase (CDA).[11-14] However, more recent findings suggested Zeb as a new potential candidate for the therapy of epigenetic cancer due to its ability to inhibit DNA methyltransferase (DNMT).[16]


Zebularine


Nebularine


Azacytidine

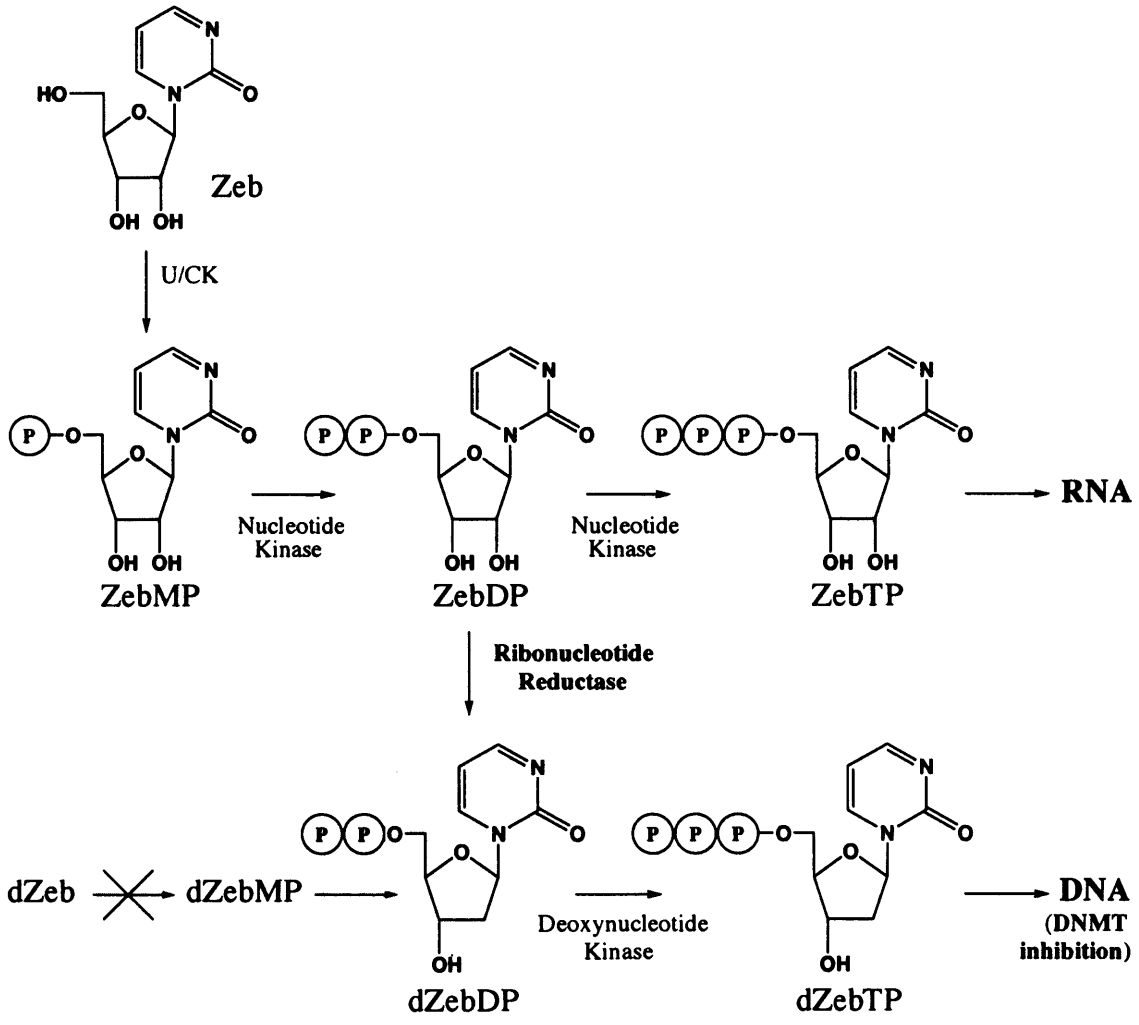

Decitabine

In tumorogenesis, tumor suppressor and cancer-related genes can be silenced by aberrant DNA methylation in their promoter regions. This event is potentially reversible and reactivation of these genes in cancer cells can result in suppression of cell growth, differentiation or increased sensitivities to existing therapies.[17] Although not as potent as the U.S. Food and Drug Administration approved nucleoside analogues 5-azacyticine (azacytidine, 5-aza-C) and 5-aza-2'-deoxycytidine (decitabine, 5-aza-dC), Zeb is an extremely attractive candidate for cancer therapy as an orally administered agent because of its chemical stability,[18] bioavailability[19] and low cytotoxicity.[20]

DNMT inhibition appears to result from the formation of a covalent complex between the enzyme and the 2'-deoxyzebularine-substituted DNA.[15,20] Therefore, incorporation into the DNA requires the phosphorylation of Zeb to the mono- (ZebMP) and diphosphate (ZebDP). A further phosphorylation to the triphosphate is

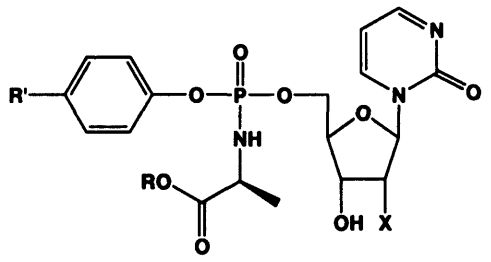
associated with RNA incorporation, which lowers the efficacy of zebularine as a DNA methylation inhibitor.

The rate limiting-step for the incorporation into the DNA is the postulated ribonucleotide reductase-catalysed conversion of ZebDP to the corresponding 2'-deoxyribonucleotide (dZebDP).[21] This may explain the weaker potency of zebularine in comparison to the other NAs DNMT inhibitors. Conversely, the direct use of dZeb did not overcome this drawback as it resulted in complete inactivity, perhaps due to the lack of recognition by the activating enzyme dCK.[18] The final phosphorylation to the triphosphate form allows the incorporation into the DNA (Scheme 7.6).

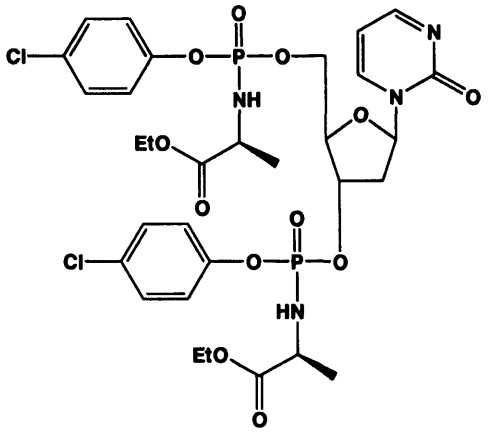


Scheme 7.6: Zebularine postulated mode of action.

In collaboration with Dr. Marquez and the National Cancer Institute-Frederick, we decided to apply our ProTide strategy to Zeb and dZeb with the purpose of potentiating the biological activities of these NAs (Figure 7.6).



27a X= OH, R= Bn, R'= H
27b X= H, R= Bn, R'= H
27c X= H, R= Et, R'= Cl



27d

Figure 7.6: Zeb and dZeb phosphoramidates.

The phenyloxy L-alaninyl phosphoramidates **27a** and **27b** were planned first. A benzyl moiety was used as the amino acid ester chain. In addition to this basic phosphoramidate motif, **27c** was designed with the object of tuning the rate of the ester cleavage by replacing the benzyl ester moiety with an alkyl chain and the spontaneous displacement of the aryloxy moiety by increasing the leaving group ability of the aromatic ring on the phosphate. Derivative **27d** was isolated as a diphosphorylation product of compound **27c**.

The cytostatic activity of compounds **27a-d** was tested against T24 bladder and HCT15 colon cancer cells using Zeb as a control. Although preliminary results showed growth inhibition, compounds **27a-d** appeared to be less active than Zeb itself.

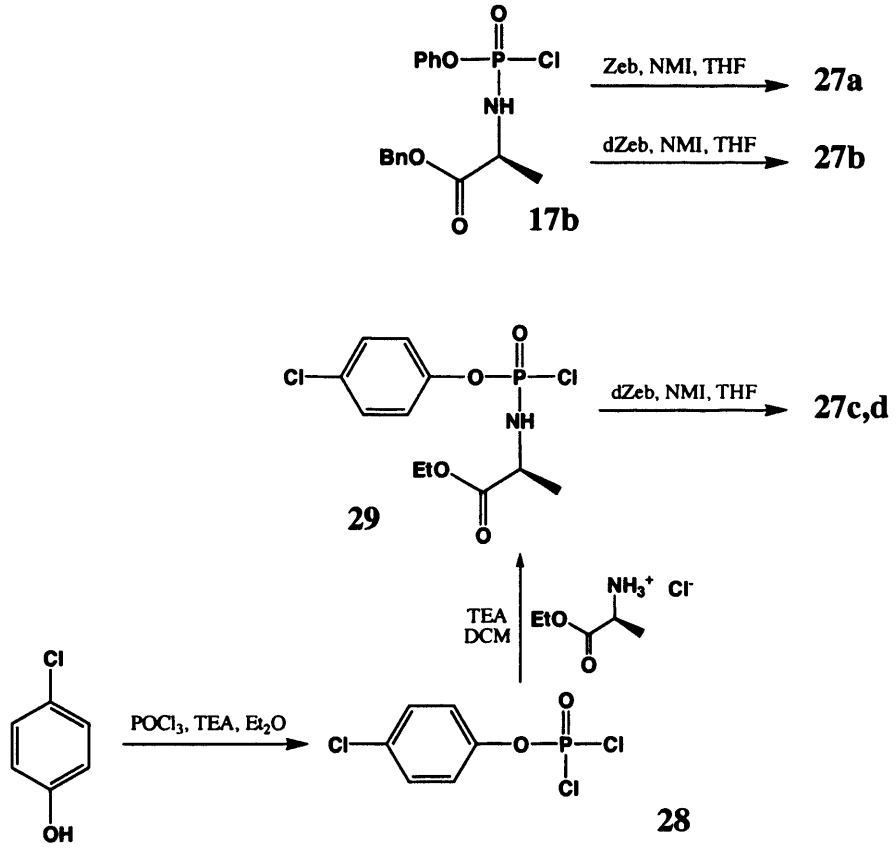
According to the suggested mode of action, the intracellular delivery of ZebMP via the phosphoramidate technology may not be sufficient to facilitate the activation of Zeb, probably being the conversion to the 2'-deoxy diphosphate the crucial limiting step.

Conversely, the application of our ProTide approach to dZeb did not lead to any benefits in terms of cytostatic potency. Possibly, the monophosphorylation of dZeb did not represent the major obstacle against the activation of this NA.

Surprisingly, a diphosphorylated phosphoramidate of dZeb has recently appeared to be more potent than Zeb in the same assays. Further SARs are in progress.

7.6 Synthesis of Zebularine phosphoramidates

Phosphoramidates **27a-d** were synthesised according to the NMI coupling procedure in order to favour the phosphorylation at the primary hydroxy group in 5' (Scheme 7.7). NMI was easily removed from the crude reaction product through a mild acidic extraction.



Scheme 7.7: Synthesis of phosphoramidates **27a-d**.

Phosphoramidates **27a,b** were obtained through the coupling of the corresponding NA with the phosphochloridate **17b**, synthesis of which was discussed in Chapter 5. Phosphochloridate **28** was synthesised from 4-chloro-phenyl dichlorophosphate according to the standard procedure and used for the synthesis of **27c**.

Compounds **27d** (3',5' diphosphorylated) was isolated from the **27c** crude reaction mixture and purified. Final yields and ^{31}P NMR chemical shifts are shown in Table 7.6.

Cpd	Yield (%)	^{31}P-NMR δ ($CDCl_3$)
27a	5.1	4.07,3.99
27b	14.1	4.28,4.09
27c	20.0	3.32,3.23
27d	9.1	3.11,3.09,3.04,2.47,2.42,1.89,1.84

Table 7.6: Phenyl phosphoramidates **27a-d.**

[1] Gudmundsson, K. S.; Daluge, S. M.; Johnson, L. C.; Jansen, R.; Hazen, R.; Condreay, L. D.; McGuigan, C. Phosphoramidate protides of 2',3'-dideoxy-3'-fluoroadenosine and related nucleosides with potent activity against HIV and HBV. *Nucleosides Nucleotides Nucleic Acids* **2003**, 22, 1953-1961.

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Chapter 8: Human Hint involvement in the activation of phosphoramidates, a first model

8.1 Introduction

The intracellular activation of phosphoramidates is considered to be based upon two enzymatic cleavages: the hydrolysis of the amino acid ester moiety as the trigger of the process, and the P-N bond cleavage as the final step that would release the corresponding nucleoside analogue monophosphate (Figure 8.1).[1] The described activation process is supported by the HPLC analysis of the products arising from the cellular metabolism of certain radiolabelled phosphoramidates.[2-3]

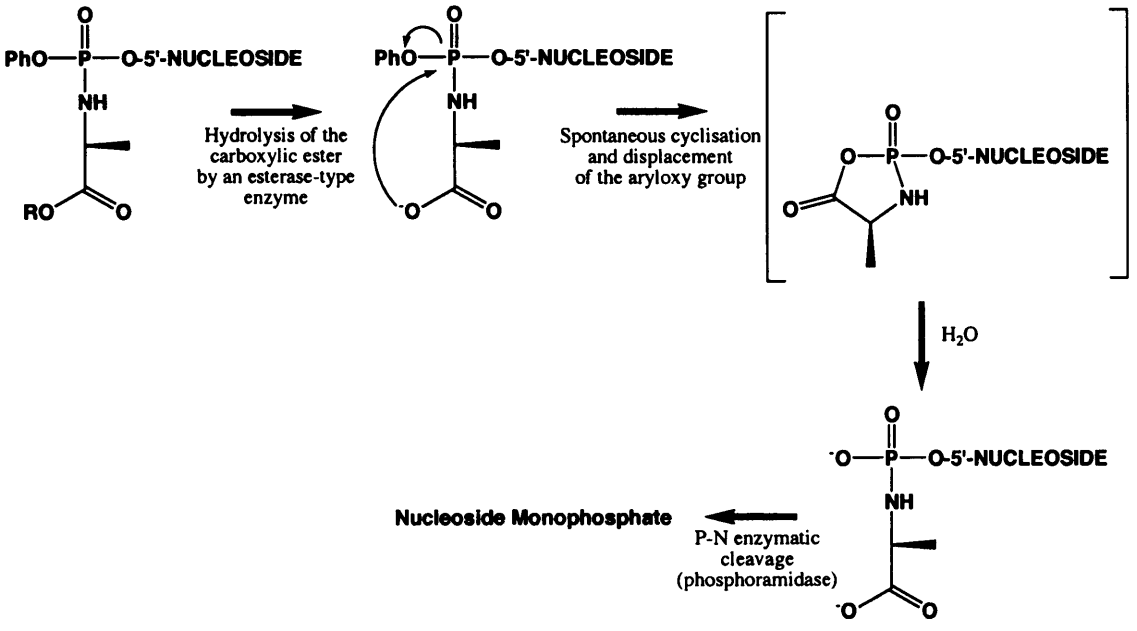


Figure 8.1: Postulated activation phosphoramidate pathway.

Although the suggested activation pathway has not been completely proved, a correlation between the carboxyl esterase lability of our compounds and their biological activity was found.[4] In fact, a porcine liver carboxyl esterase assay was

developed by us as a predictive test for the biological activity of phosphoramidates.[5] These data would strongly indicate an esterase-type enzyme to be involved with the first step of the postulated activation pathway.

Conversely, little information is available about the P-N enzymatic cleavage of phosphoramidates as well as the substrate structural requirements. However, the existence of enzymes able to cleave phosphoramido bonds is well documented and reported in the literature. In particular HIT (histidine triad) proteins, a superfamily of nucleotide hydrolases and tranferases that act on the α-phosphate of a ribonucleotide linked to an amino group, a nucleotide, or a phosphorylating sugar.[6] On the basis of sequence, substrate specificity, structure, evolution, and mechanism, HIT proteins can be classified into the Hint branch (adenosine 5'-phosphoramido hydrolases), the FHIT branch (diadenosine polyphosphate hydrolases), and the GalT branch (nucleoside monophosphate transferases) (Figure 8.2).[6]

HIT *Superfamily*

Hint FHIT GalT

Figure 8.2: Classification of HIT proteins.

Hints, the first branch of the superfamily, were first discovered in rabbits and subsequently shown to have homologues in all forms of life.[7] By screening a large number of compounds, adenosine monophosphoramidates AMP-NH_2, AMP-N-ε-(N-α-acetyl lysine methyl ester), and AMP-para-nitroaniline were identified as rabbit Hint and yeast Hint1 substrates (Figure 8.3).[8] Notably, AMP-N-alanine methyl ester was also reported as a substrate for the same enzymes, which might indicate their role in the activation of our phosphoramidates.[8]

In humans, the only characterized HIT proteins are Hint and FHIT enzymes.[9-11] Human Hints identified so far include Hint1/PKCI, Hint2, Hint3, Aprataxin and the scavenger mRNA decapping enzyme DcpS.[10-14]

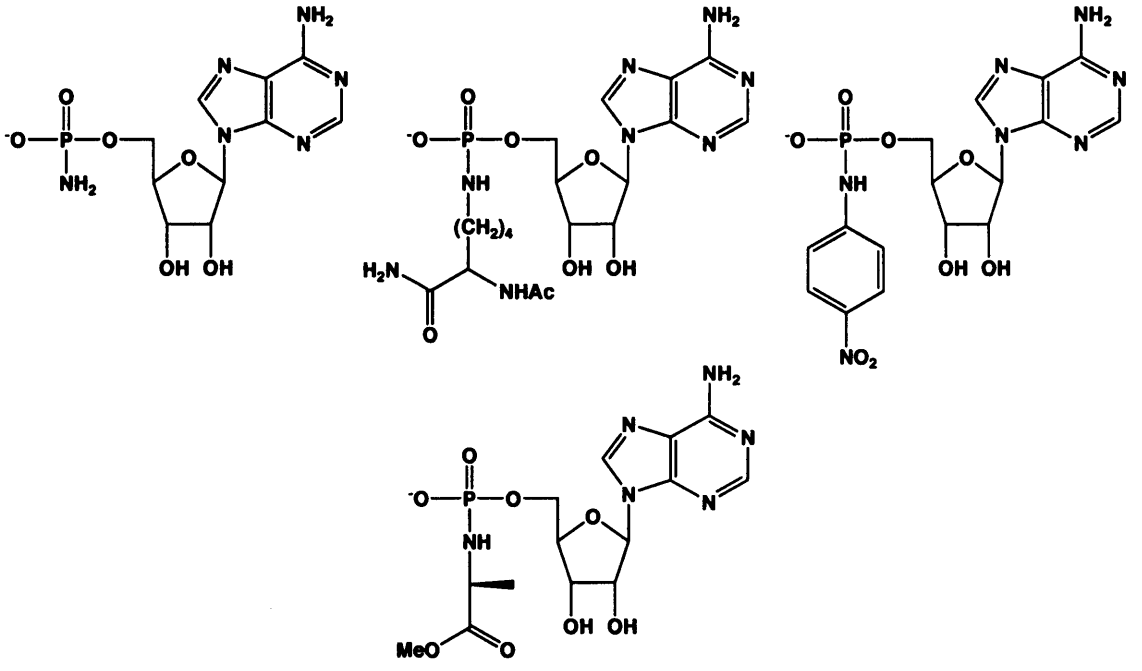
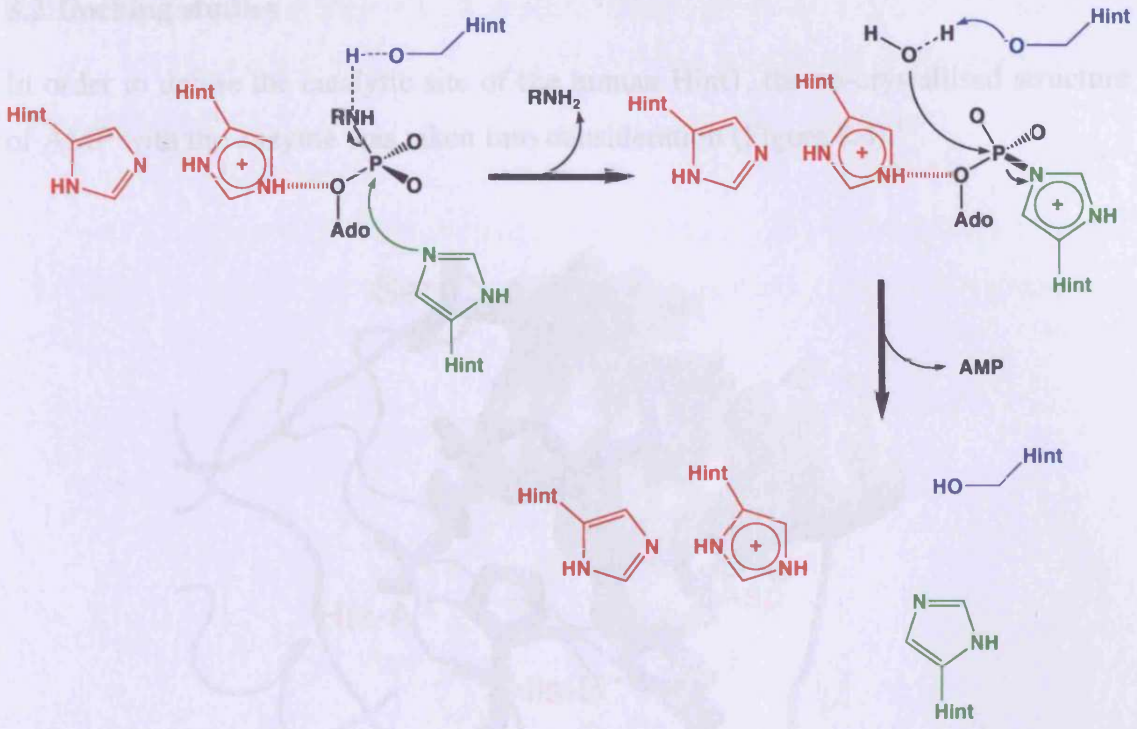


Figure 8.3: rabbit Hint and yeast Hint1 substrates.

The rabbit Hint/PKCI homologue was shown to bind selectively purine monophosphate nucleotides[15] and a similar behaviour was confirmed by a strong preference for the human Hint1 towards adenine base substrates.[16]

Three histidines have a fundamental role for the catalytic activity of Hints (Scheme 8.1).[6] The attack of the green His in Scheme 8.1 to the phosphorus center of AMP-NHR would release the amino moiety, being the cleavage of the P-N bond favoured by the transfer of a hydrogen from a serine (blue residue) to the N atom of the amino acid. The AMP-His bond would be subsequently cleaved by the attack of a molecule of water, which would lead to the release of AMP and the restoration of the Hint active site.[8]



Scheme 8.1: Proposed mechanism for Hint catalysed hydrolysis of AMP-N bonds.

Given these bases, the human Hint1 would appear to be a suitable enzyme for the activation of phosphoramidates. Therefore, a molecular modelling investigation based on docking and molecular dynamics studies was planned to evaluate this possibility.

8.2 Docking studies

In order to define the catalytic site of the human Hint1, the co-crystallised structure of AMP with the enzyme was taken into consideration (Figure 8.4).[17]

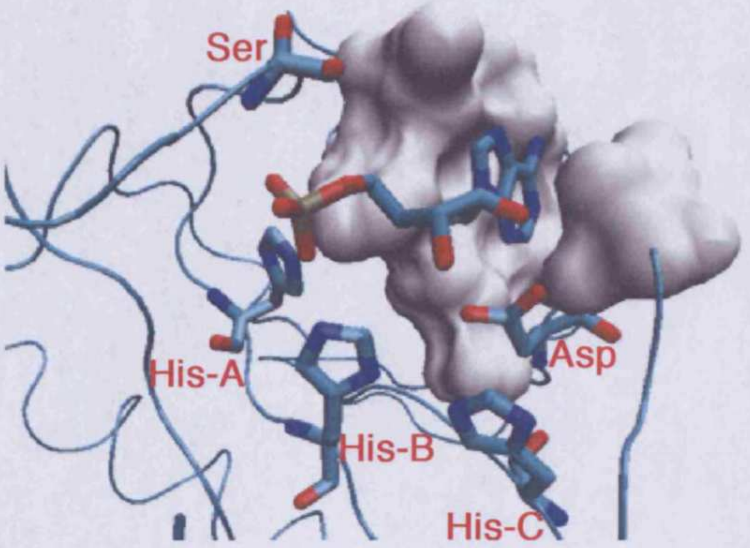


Figure 8.4: Human Hint1 (Hint1/PKCI) and AMP co-crystallised structure.

The three histidines (His-A, His-B and His-C) appear to be in proximity of the phosphate moiety with His-B in the centre of the triad interacting via hydrogen bonding with the phosphate. His-A might represent the one that attacks the phosphate and, by doing so, triggers the P-N cleavage. As described for the postulated mechanism of action in Scheme 8.1, a serine would favour such displacement and this sort of residue (Ser) can be clearly observed on the top of the phosphate. Moreover, the two hydroxy groups of the ribo-sugar moiety are involved in hydrogen bonding with an aspartic residue (Asp), enhancing the stability of the enzyme-substrate interactions. Furthermore, the purine ring fits in a hydrophobic pocket generated by the side chains of several enzymatic residues (four isoleucines, two phenylalanines and one alanine).

These data were in agreement with the information about the postulated mechanism of action of Hints described in Scheme 8.1, and were used to define the catalytic core of the human Hint1.

In Figure 8.5, the result of the docking of the enzyme with the postulated substrate adenosine L-alaninyl phosphate is shown. The docked compound appeared to

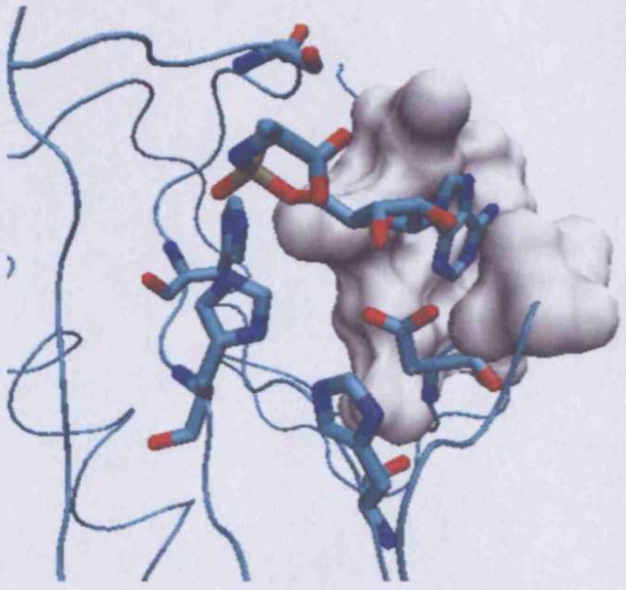


Figure 8.5: Docking of adenosine L-alaninyl phosphate.

be able to assume a conformation that would allow the interactions of the phosphate moiety with the key residues in the catalytic site (histidine triad and serine residue). Notably, also the sugar hydroxyls and the purine base maintained the same interactions previously described for AMP.

Interestingly, an identical pattern was found also for the natural nucleotides GMP, dGMP, and dAMP. Although the hydrogen bonding ability of the sugar moiety appeared to be reduced for the dNs, the hydroxyl at 3' was still able to interact with the aspartic residue and keep this moiety in a similar conformation to the ribo-counterparts. Notably, in the case of dAMP the purine base appeared to partially slide away from the hydrophobic pocket.

Given these encouraging data, L-alaninyl phosphate derivatives of some adenosine analogue nucleosides (ddA, d4A and 2CdA) were then taken into consideration to evaluate the hypothesis that the human hint could be involved with the metabolism of the corresponding phosphoramidates.

For the 2CdA derivative (red structure in Figure 8.6) the three main portions of the molecule (phosphate, sugar and base) fitted in the enzymatic core. Also in this case, the presence of a deoxyribose led to a partial movement of the base out of the hydrophobic pocket.

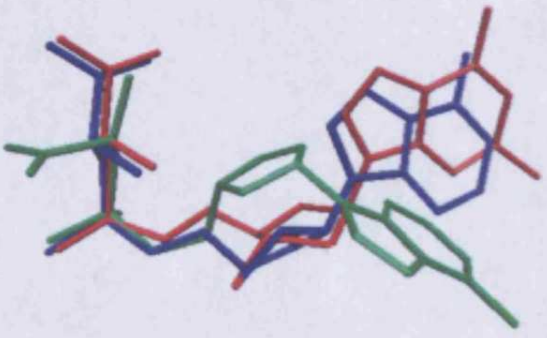


Figure 8.6: Docking of d4A (green), ddA (blue) and 2CdA (red) L-alaninyl phosphates.

The absence of hydroxyls on the sugar moiety caused a small perturbation in the conformation of the ddA derivative (blue) in comparison to AMP. Such deviation appeared to be critical in the case of the d4A derivative (green), where the presence of the C2'-C3' double bond led both the sugar moiety and the base to flip upside down. It would seem that the hydrogen bonding ability of the sugar moiety was a necessary requisite for the proper orientation of these moieties in the enzymatic pocket.

Surprisingly, such distortion did not affect the orientation of the phosphate group, which appeared to occupy a suitable position for the catalytic cleavage of the P-N bond. However, the lack of binding of the purine base may lead to reduced affinity in the d4A case.

Pyrimidine based nucleotides were subsequently taken into account. UMP, CMP and dCMP showed a comparable conformational behaviour: although both the sugar and the phosphate moiety appeared to interact with the correct Hint residues, the base was always placed outside the hydrophobic pocket and pointing outside the enzymatic core. In the case of dTMP, only the phosphate retained a suitable position while the rest of the molecule was placed outside the enzymatic cavity (Figure 8.7).

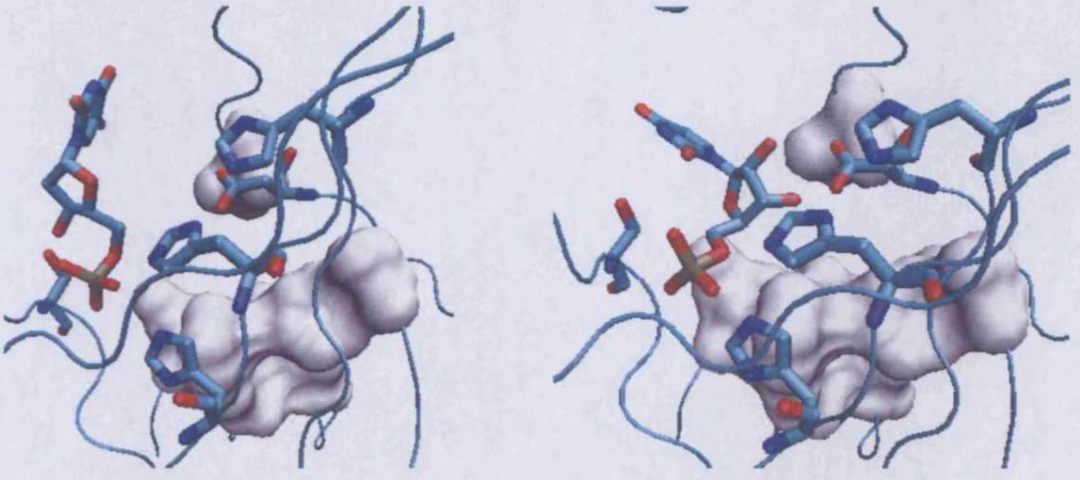


Figure 8.7: Docking of dTMP (left picture) and UMP (right picture).

Not surprisingly, the L-alaninyl phosphates of ddU and d4T showed similar features to the pyrimidine nucleotides by displaying the phosphate in the appropriate location while the sugar and the base were either twisted (ddU) or moved out of the enzymatic cavity (d4T).

The data collected so far would confirm the notion that Hint1 may show an higher selectivity towards purine based derivatives and that the presence of a ribo-sugar was preferred to 2'-deoxy, dd-, and especially d4-derivatives. Conversely, pyrimidine based compounds displayed an evident conformational distortion that would suggest a minor affinity towards this enzyme. However, pyrimidine based derivatives appeared to be able to locate their phosphate moieties in the proper site for the P-N catalysed cleavage rendering difficult to predict whether they could act as substrates or not.

Therefore, a series of BVdU aminoacyl phosphates were considered to evaluate how the nature of the amino acid could affect the binding of the phosphate in the Hint1 catalytic site.

As previously observed for other pyrimidine based derivatives, all the compounds showed the base moiety placed outside the hydrophobic pocket in the catalytic core of Hint1. However, L-alanine, dimethylglycine, and L-methionine emerged as able to orientate the phosphate in a suitable position for the enzymatic catalysis. For these amino acids, the sugar hydroxyls were shown to properly interact with the aspartic residue (L-alanine derivative, yellow structure in Figure 8.8).

When the methyl was moved to the D face of the amino acid (D-alanine), only few conformations predicted the phosphate to be placed in a suitable position while in most of the cases the molecule did not fit correctly in the catalytic core. A similar behaviour was observed also for L-phenylalanine.

For branched or bulky side chain amino acids such as L-valine, L-isoleucine, and L-phenylalanine none of the conformations were shown to occupy a suitable position for the enzymatic catalysis (L-valine derivative, green structure in Figure 8.8). In the case of L-proline, the phosphate was placed in proximity of the histidine triad but it caused an improper torsion of the molecular structure that led the cyclic side chain to

occupy the position usually related to the sugar moiety (L-proline derivative, blue structure in Figure 8.8).

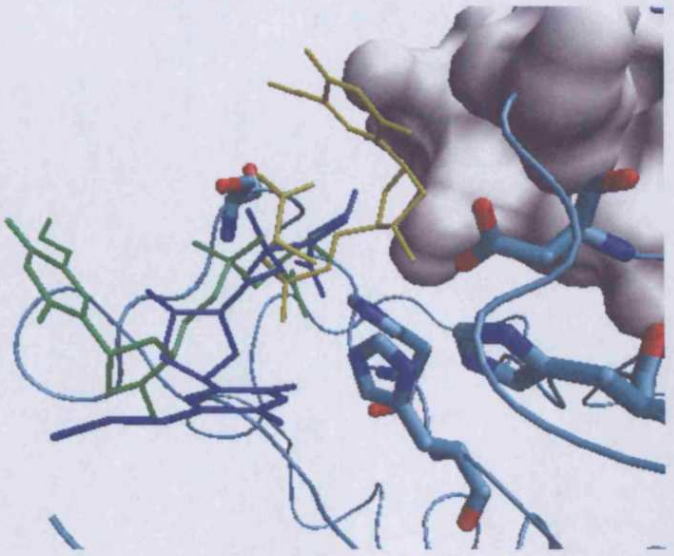


Figure 8.8: Docking of BVdU aminoacyl phosphates: L-Ala (yellow), L-val (green) and L-Pro (blue).

Although preliminary, most of these data were consistent with the phosphoramidate SARs described in Chapter 1. Encouraging was the fact that dimethylglycine could be a good substitute for L-alanine and that the movement of the methyl group to the D face (D-alanine) would reduce the interactions with the suitable residues in the catalytic core. Moreover, a steric bulk on the side chain of natural amino acids would cause a decrease in the substrate affinity for the enzyme; this being entirely consistent with in vitro antiviral SARs as presented earlier.

However, these relationships might depend upon cellular specificity and also on the fact that different human Hint isoforms could be involved.

8.3 Molecular dynamics

In order to have a quantitative assessment of the enzyme/substrate interactions arising from the docking studies, molecular dynamics were taken into consideration. The program Gromacs was chosen for this purpose as it is able to calculate the potential binding energy for an enzyme/substrate complex. In Figure 8.9 the result of molecular dynamics studies applied to the Hint1/adenosine L-alaninyl phosphate complex is shown.

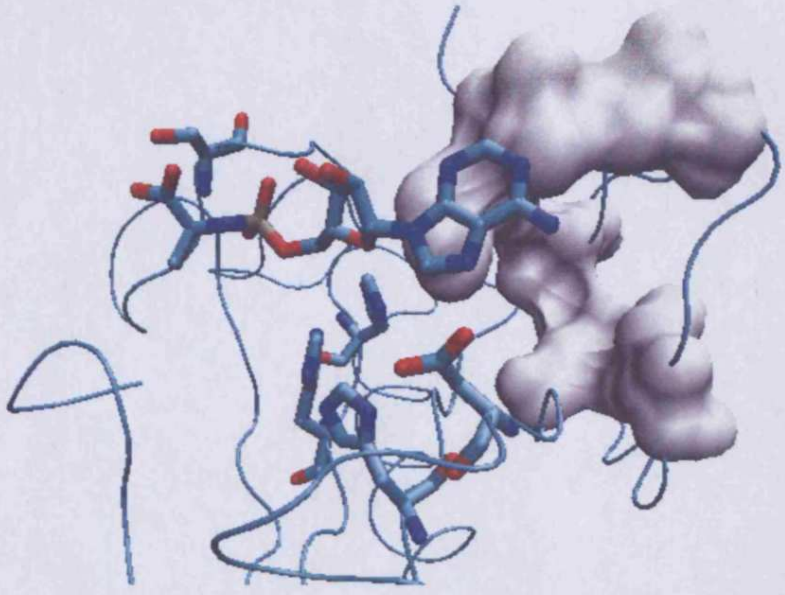


Figure 8.9: Hint1/adenosine L-alaninyl phosphate molecular dynamics.

The phosphoramidate ligand was not shown to be able to interact with the enzymatic residues observed in the previous docking studies. In particular, the substrate appeared not to be maintained in the catalytic core, leading to its movement outside the enzymatic cavity. Neither the base nor the sugar moieties were able to interact to the appropriate residues in the catalytic core. Importantly, also the phosphate group was shown to slide away from the histidine triad and the serine residue. A similar trend was revealed also for AMP.

One problem that might have occurred during our investigations was probably related to the parameters defining the substrate (topology parameters). They were generated by the Dundee PRODRG2 Server[18] in the case of adenosine L-alaninyl phosphate and subsequently adjusted with respect to the topology parameters given by Gromacs for the two components AMP and L-alanine. Probably, the lack of recognition of the suitable enzyme/substrate interactions was due to an inappropriate estimation of these parameters. It would be beneficial to try to further optimise such parameters, which was not possible in the course of this Thesis.

Moreover, Gromacs is based on the use of a force field that is highly specific for proteins. Therefore, the program might not be suitable for the application on structures where a P-N bond is involved. It would be useful to investigate a different platform (e.g. Tripos) where less specific force fields are available (MMFF94, Amber, etc) and phosphoramidate structures might be better recognised.

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Chapter 9: Experimental procedures

9.1 List of compounds Synthesised

Aryl phosphorochloridates

9.2 General experimental details

Solvents and reagents

The following anhydrous solvents and reagents were bought from Aldrich with sureseal stoppers: chloroform ($CHCl_3$), dichloromethane (DCM), diethyl ether (Et_2O), 1,4-dioxane, N,N-dimethylformamide (DMF), N-methylimidazole (NMI), tetrahydrofuran (THF). All the reagents commercially available were used without further purification.

Thin Layer Chromatography

Precoated, aluminium backed plates (60 F-254, 0.2 mm thickness; Merck) were used with a variety of mobile phases. TLC plates were visualised under both short (254 nm) and long wave (365 nm) ultraviolet light. Preparative TLC plates (20 X 20 cm, 500-2000 μm) were purchased from Merk.

Column Chromatography

Column chromatography procedures were carried out using silica gel supplied by Fluka (silica gel 60, 35-70 μM). Glass columns were slurry packed using the appropriate eluent and samples were applied either as a concentrated solution in the same eluent or pre-adsorbed on silica gel.

HPLC

Analitycal and semi-preparative procedures were run on a Varian ProStar intrument (LC work station, Varian Prostar 355 LC detector) using a Polaris C18-A 10u column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient.

NMR Spectroscopy

Proton (^1H), carbon (^{13}C) and phosphorus (^{31}P) NMR spectra were recorded on a Bruker Avance DPX300 or a Bruker Avance 500 specrometer at 25 °C. Spectra were auto-calibrated to the deuterated solvent peak and all ^{13}C NMR and ^{31}P NMR were

proton-decoupled. Chemical shifts are quoted in parts per million (ppm) and coupling constants (J) are given in Hertz (Hz). The following abbreviations are used to describe the multiplicity of peaks: s (singlet), d (douplet), t (triplet), q (quartet), m (multiplet), bs (broad signal), dd (douplet of douplets).

Mass Specrometry

Low resolution mass spectra were run on a microTOFLC Bruker Daltonics spectrometer in electropositive mode.

Elemental Analysis

Elemental analysis data were recorded on a Carlo-Erba EA 1108 elemental analyser, PC based data system, Eager 200 for Windows and a Sartorius Ultra Micro balance 4504MP8.

Molecular Modelling

All molecular modelling studies were performed on a RM Innovator Pentium IV 2.4GHz running either Linux Fedora Core 3 or Windows XP using Molecular Operating Environment (MOE) 2004.03[1] and FlexX module in SYBYL 7.0[2] molecular modelling software. Ligands were docked within the active site of the homology model using the FlexX docking programme of SYBYL, performed with the default values. The active site was defined by all the amino acid residues within a 6.5 Å distance from the co-crystallised ligand.

Molecular dynamics simulations were performed with GROMACS 3.2[3,4] and the Gromacs force field in a NVT (canonical) environment. Individual ligand/protein complexes obtained from the docking results were soaked in a triclinic water box and minimised using a steepest descent algorithm to remove unfavorable van der Waals contacts. The system was then equilibrated via a 20 ps MD simulation at 300°K with restrained ligand/protein complex atoms. Finally, a 500 ps simulation was performed at 300°K with a time step of 2 fs and hydrogen atoms constrained with a LINCS algorithm. Visualisation of the dynamics trajectories was performed with the VMD software package, version 1.8.3.[5]

9.3 Standard Procedures and synthesis

For convenience, standard procedures have been given, as similar procedures were employed for the synthesis of phosphoramidates and their precursors. Individual variations from these procedures are described in the main text.

Standard Procedure A: synthesis of dichlorophosphates

Phosphorus oxychloride (1.0 mol eq.) and the appropriate substituted phenol or naphthol (1.0 mol eq.) were stirred in anhydrous Et_2O under argon atmosphere. Anhydrous TEA (1.0 mol eq.) was added dropwise at -78 °C. Following the addiction, the reaction mixture was allowed to slowly warm to room temperature and stirred overnight. Formation of the desired compound was monitored by ^{31}P NMR. The mixture was filtered under nitrogen and the corresponding filtrate reduced to dryness to give the crude product as an oil, which was used without further purification in the next step.

Standard Procedure B: synthesis of phosphorochloridates

Anhydrous TEA (2.0 mol eq.) was added dropwise at -78 °C to a stirred solution of the appropriate aryl dichlorophosphate (1.0 mol eq.) and the appropriate amino acid ester salt (1.0 mol eq.) in anhydrous DCM under argon atmosphere. Following the addiction, the reaction mixture was allowed to slowly warm to room temperature. Formation of the desired compound was monitored by ^{31}P NMR. The solvent was removed under reduced pressure. The residue was resuspended in anhydrous Et_2O and filtered under nitrogen. The filtrate was reduced to dryness to give the crude product as an oil, which was in some cases used without further purification in the next step. Most of the aryl phosphorochloridates synthesised were purified by flash column chromatography (ethyl acetate/hexane 7/3).

Standard Procedure C: synthesis of phosphoramidates (NMI method)

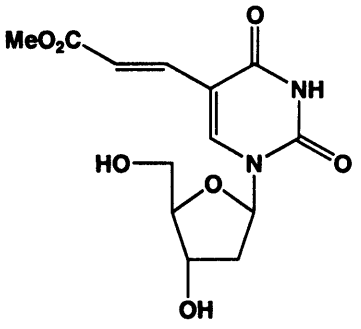
To a stirring solution of the appropriate nucleoside analogue (or natural nucleoside) (1.0 mol eq.) and the appropriate phosphorochloridate (3.0 mol eq.) in anhydrous THF at –78 °C was added dropwise over 1 min NMI (5.0 mol eq.). After 15 min the reaction was let to rise to room temperature and stirred overnight. MeOH was added to quench the reaction. The solvent was removed under reduced pressure and the oil obtained was dissolved in DCM, washed with HCl 0.5 M and water. The organic layer was dried over $MgSO_4$, filtered, reduced to dryness and purified by column chromatography (DCM/MeOH in different proportions).

Standard Procedure D: synthesis of phosphoramidates (^{t}BuMgCl method)

To a stirring suspension of the appropriate nucleoside analogue (1.0 mol eq.) in anhydrous THF a 1.0 M solution of ^{t}BuMgCl in anhydrous THF (1.0-1.2 mol eq.) was added dropwise. The addition of ^{t}BuMgCl was carried out either at room temperature or at -17 °C. After 30 min a solution of the appropriate phosphorochloridate (1.0-1.2 mol eq.) in anhydrous THF was added dropwise. The reaction was stirred overnight at room temperature. The solvent was removed under reduced pressure and the crude residue was purified by column chromatography (DCM/MeOH in different proportions).

Synthesis of BVdU

3 (E)-5-(2-Carbomethoxyvinyl)-2'-deoxyuridine[6]

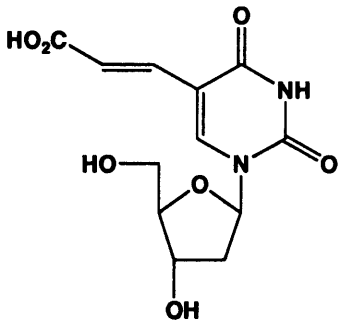


$C_{13}H_{16}N_2O_7$
Mol Wt.: 312.28

A mixture of palladium (II) acetate (0.32 g, 1.41 mmol), triphenylphosphine (0.74 g, 2.82 mmol) and TEA (5 mL) in dioxane (50 mL) was stirred at 70 ^{0}C until an intense red colour developed. To this solution, 5-iodo-2'-deoxyuridine (10 g, 28.24 mmol) and methyl acrylate (4.86 g, 56.48 mmol, 5.08 mL) were added and the mixture was stirred under reflux for 30 min. The suspension was filtered while still hot and the filtrate cooled overnight at 4 °C. The resulting pale yellow precipitate was filtered, washed with DCM and dried in vacuo to give the product as a white solid (6.05 g, 68.7%).

^{1}H-NMR (DMSO, 300 MHz): δ 11.32 (1H, bs, H-3'), 8.41 (1H, s, H-6), 7.37 (1H, d, vinylic H, ^{3}J=15.9 Hz), 6.84 (1H, d, vinylic H, ^{3}J=15.9 Hz), 6.12 (1H, t, H-1'), 5.22 (2H, bs, OH-3', OH-5'), 4.29 (1H, m, H-3'), 3.80 (1H, m, H-4'), 3.65 (3H, s, OCH_3), 2.19 (2H, m, H-2').

4 (E)-5-(2-Carboxyvinyl)-2'-deoxyuridine[6]

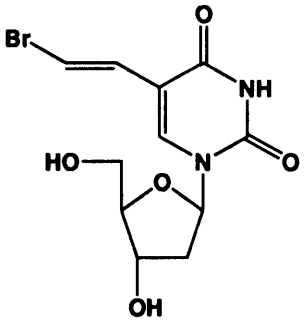


$C_{12}H_{14}N_2O_7$
Mol Wt.: 312.28

(E)-5-(2-Carbomethoxyvinyl)-2'-deoxyuridine (**3**) (6.00 g, 19.33 mmol) was dissolved in aqueous 1M NaOH (300 mL) and the mixture stirred at room temperature for 3 h. The reaction mixture was filtered and the clear filtrate adjusted to pH 2 with 1M HCl. On cooling at 4 °C a white precipitate formed. This was filtered and washed with water, acetone and dried to give the product as a white solid (4.50 g, 78.0%).

^{1}H-NMR (DMSO, 300 MHz): δ 8.39 (1H, s, H-6), 7.30 (1H, d, vinylic H, ^{3}J=15.8 Hz), 6.78 (1H, d, vinylic H, ^{3}J=15.8 Hz), 6.14 (1H, t, H-1'), 5.27 (2H, bs, OH-3', OH-5'), 4.27-4.25 (1H, m, H-3'), 3.80-3.79 (1H, m, H-4'), 3.69-3.55 (2H, m, H-5'), 2.22-2.13 (2H, m, H-2').

5 (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU)[6]



$C_{11}H_{13}BrN_2O_5$
Mol Wt.: 333.14

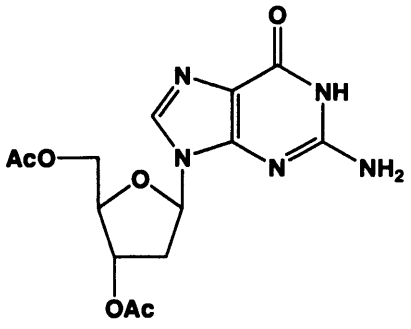
To a solution of (E)-5-(2-carboxyvinyl)-2'-deoxyuridine (**4**) (4.00 g, 13.41 mmol) in DMF (20 mL) potassium carbonate (4.08 g, 29.50 mmol) was added and the suspension stirred at room temperature for 15 min. A solution of N-bromosuccinimide (2.53 g, 14.21 mmol) in DMF (20 mL) was added dropwise over 30 min at room temperature. The resulting suspension was filtered and the solid washed with DMF. The combined filtrate and washings were evaporated to dryness in vacuo. The resulting residue was purified by column chromatography (DCM/MeOH 9/1) to give the product as a white solid (2.40 g, 53.9%). Crystallisation from water gave white needles.

^{1}H-NMR (DMSO, 300 MHz): δ 8.08 (1H, s, H-6), 7.25 (1H, d, ^{3}J=13.6 Hz, H-5b), 6.85 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.13 (1H, t, ^{3}J=6.5 Hz, H-1'), 5.27-5.26 (1H, bs, OH-3'), 5.10 (1H, bs, OH-5'), 4.25 (1H, m, H-3'), 3.80-3.78 (1H, m, H-4'), 3.66-3.55 (2H, m, H-5'), 2.14 (2H, m, H-2').

^{13}C-NMR (DMSO, 75 MHz): δ 40.1 (C-2'), 61.3 (C-5'), 70.3 (C-3'), 84.9 (C-1'), 87.9 (C-4'), 106.9 (C-5b), 110.0 (C-5), 130.2 (C-5a), 139.8 (C-6), 149.6 (C-4), 162.0 (C-2).

Synthesis of 2CdA

13 3',5'-Diacetyl-2'-deoxyguanosine[7]

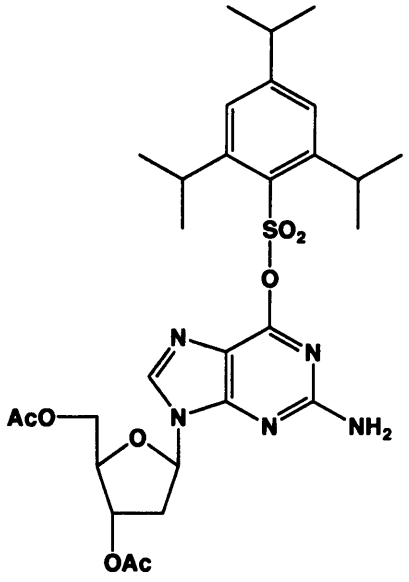


$C_{14}H_{17}N_5O_6$
Mol Wt.: 351.31

To a suspension of 2'-deoxyguanosine (5.00 g, 18.71 mmol), TEA (5.00 g, 49.39 mmol, 6.88 mL), DMAP (0.18 g, 1.50 mmol) in acetonitrile (250 mL) acetyc anhydride was added dropwise (4.58 g, 44.90 mmol, 4.24 mL). The reaction was stirred at room temperature for 2 h. Methanol was added to quench the reaction and the mixture was filtered. The resulting solid was crystallised from methanol, washed with Et_2O and dried (5.00 g, 76.1 %).

^{1}H-NMR (DMSO, 300 MHz): δ 10.81-10.61 (1H, bs, NH-1), 7.92 (1H, s, H-8), 6.62-6.40 (1H, bs, NH_2), 6.17-6.11 (1H, m, H-1'), 5.33-5.22 (1H, m, H-3'), 4.32-4.10 (3H, m, H-4', H-5'), 3.00-2.85 (1H, m, one of H-2'), 2.54-2.40 (1H, m, one of H-2'), 2.09,2.04 (6H, 2s, Ac).

14 3',5'-Diacetyl-2-amino-6-O-(2,4,6-triisopropylbenzenesulfonyl)-2'-deoxyguanosine[7]



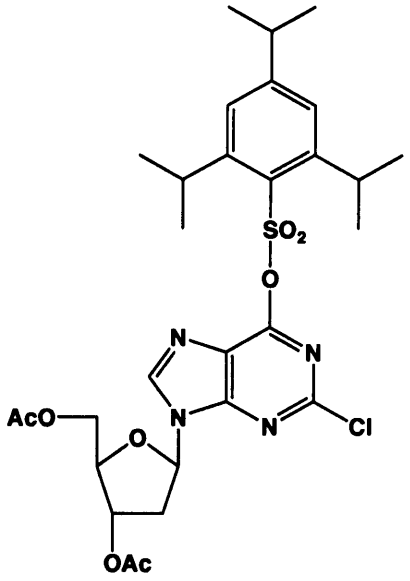
$C_{29}H_{39}N_5O_8S$

Mol Wt.: 617.71

TEA (2.65 g, 26.22 mmol, 3.64 mL) was added dropwise to a stirred solution of 3',5'-diacetyl-2'-deoxyguanosine (**13**) (4.85 g, 13.80 mmol), TiPBS-Cl (7.94 g, 26.22 mmol) and DMAP (0.22 g, 1.79 mmol) in dry $CHCl_3$ (200 mL) at –5°C under Argon atmosphere. The reaction was stirred for 24 h and volatiles were evaporated. The residue was purified by column chromatography (DCM/MeOH 97/3) to give the product as a yellow foamy solid (4.56 g, 54.0% yield).

^{1}H-NMR (MeOD, 300 MHz): δ 8.02 (1H, s, H-8), 7.22 (2H, s, H-3 and H-5 Ph), 6.24 (1H, H-1', ^{3}J=7.0 Hz), 5.41-5.35 (1H, m, H-3'), 4.32-4.11 (5H, m, H-4', H-5', two of *CH*$(CH_3)_2$), 3.05-2.80 (2H, m, one of *CH*$(CH_3)_2$, one of H-2'), 2.55-2.44 (1H, m, one of H-2'), 2.01,1.91 (6H, 2s, AcO), 1.22-1.19 (18H, m, CH(*CH*$_3)_2$).

15 3',5'-Diacetyl-2-chloro-6-O-(2,4,6-triisopropylbenzenesulfonyl)purine-2'-deoxyriboside[7]



$C_{29}H_{37}ClN_4O_8S$

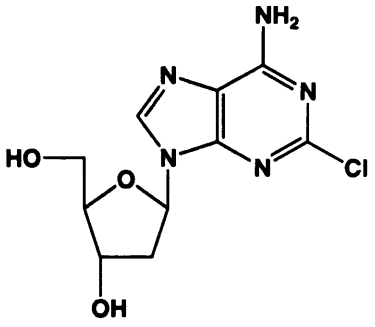
Mol Wt.: 637.14

A solution of AcCl (0.71 g, 9.07 mmol, 0.65 mL) in anhydrous DCM (40 mL) under nitrogen atmosphere was chilled in a NaCl/ice/H_2O bath for 15 min. TBA-NO_2 (2.06 g, 7.13 mmol) was dissolved in anhydrous DCM (25 mL), and this solution was immediately added dropwise to the cold, stirred solution of AcCl/CH_2Cl_2. A solution of **14** (1.00 g, 1.62 mmol) in anhydrous DCM (15 mL) was then added dropwise to the cold solution and stirring was continued for 5 min (TLC, DCM/MeOH 95/5, showed complete conversion of **14** in a single product). The reaction mixture was added dropwise at a rapid rate to a cold (ice/H_2O bath) vigorously stirred mixture of a saturated $NaHCO_3$/H_2O/CH_2Cl_2. The layers were separated and the organic phase was washed with cold H_2O and dried on $MgSO_4$. Volatiles were evaporated and the residue was purified by column chromathography (DCM/MeOH 99/1). The collected material was crystallised from MeOH to give the product as a white solid (0.75 g, 72.0% yield).

^{1}H-NMR (DMSO, 300 MHz): δ 8.97 (1H, s, H-8), 7.39 (2H, s, H-3 and H-5 Ph), 6.41 (1H, H-1', ^{3}J=7.0 Hz), 5.44-5.38 (1H, m, H-4'), 4.33-4.10 (5H, m, H-3', H-5',

two of $CH(CH_3)_2$), 3.12-2.93 (2H, m, one of $CH(CH_3)_2$, one of H-2'), 2.77-2.56 (1H, m, one of H-2'), 2.09,1.96 (6H, 2s, AcO), 1.26-1.20 (18H, m, $CH(CH_3)_2$).

16 2-chloro-2'-deoxyadenosine (2CdA)[7]

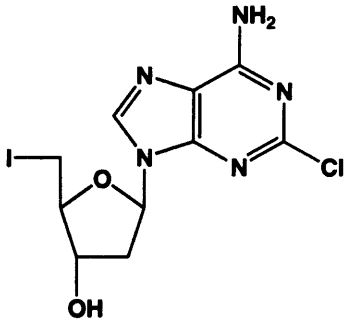


$C_{10}H_{12}ClN_5O_3$
Mol Wt.: 285.69

NH_3/MeOH (75 mL, saturated at 0°C) was added to a solution of **15** (1.00 g, 1.57 mmol) in DCM (50 mL) in an autoclave. The autoclave was sealed and immediately immersed in an oil bath preheated to 80 °C. Heating was continued for 7 h. Volatiles were evaporated and the resulting residue was purified by column chromathography (DCM/MeOH 9/1). The material collected was reduced to dryness and dissolved in H_2O/MeOH. The solution was stirred in the presence of Amberlite (HCO_3^-), filtered and reduced to dryness to give the product as a yellow solid (0.21 g, 46.7% yield).
^{1}H-NMR (DMSO, 300 MHz): δ 8.37 (1H, s, H-8), 7.83 (2H, bs, NH_2), 6.27 (1H, t, ^{3}J=6.8 Hz, H-1'), 5.34 (1H, d, OH-3'), 4.98 (1H, t, OH-5'), 4.40 (1H, m, H-3'), 3.88-3.85 (1H, m, H-4'), 3.64-3.52 (2H, m, H-5'), 2.67-2.61 (1H, m, one of H-2'), 2.32-2.25 (1H, m, one of H-2').
^{13}C-NMR (DMSO, 75 MHz): δ 39.7 (C-2'), 62.0 (C-5'), 71.0 (C-3'), 83.9 (C-1'), 88.3 (C-4'), 118.5 (C-5), 140.2 (C-8), 150.4 (C-6), 153.3 (C-2), 157.1 (C-4).

Synthesis of other nucleoside analogues

19a 5'-Deoxy-5'-iodo-2CdA



$C_{10}H_{11}ClIN_5O_2$
Mol Wt.: 395.58

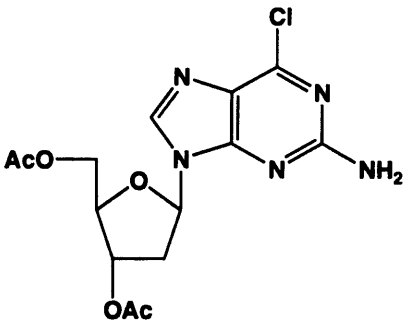
Triphenylphosphine (1.11 g, 4.25 mmol), and iodine (1.08 g, 4.25 mmol) were added to a solution of 2CdA (0.81 g, 2.83 mmol) in anhydrous pyridine (15 mL). The reaction was monitored by TLC (DCM/MeOH 95/5) and stirring was continued until complete conversion of the starting material in a single product (20 h). Methanol was added, and then volatiles were evaporated. The crude was purified by column chromathography (DCM/MeOH 95/5). The mateial collected was washed with methanol to give the product as a white solid (0.36 g, 32.4% yield).

^{1}H-NMR (DMSO, 500 MHz): δ 8.38 (1H, s, H-8), 7.83 (2H, bs, NH_2), 6.32 (1H, t, ^{3}J=7.0 Hz, H-1'), 5.56 (1H, d, OH-3'), 4.44-4.39 (1H, m, H-3'), 3.99-3.96 (1H, m, H-4'), 3.58-3.54 (1H, dd, one of H-5'), 3.47-3.43 (1H, dd, one of H-5'), 2.90-2.85 (1H, m, one of H-2'), 2.37-2.32 (1H, m, one of H-2').

^{13}C-NMR (DMSO, 125 MHz): δ 7.8 (C-5'), 38.0 (C-2'), 73.1 (C-3'), 83.6 (C-1'), 86.5 (C-4'), 118.2 (C-5), 140.1 (C-8), 150.1 (C-6), 153.0 (C-2), 156.8 (C-4).

Anal. Calcd for $C_{10}H_{11}ClIN_5O_2$: C, 30.38; H, 2.78; N, 17.72. Found: C, 30.43; H, 2.84; N, 17.42.

22 3',5'-diacetyl-6-chloro-2'-deoxyguanosine[7]

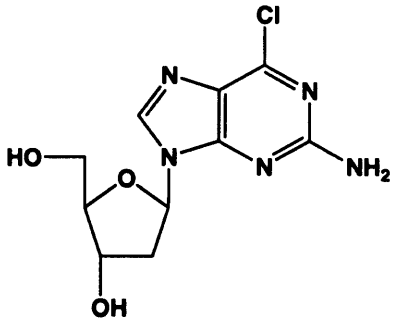


$C_{14}H_{16}ClN_5O_5$
Mol Wt.: 369.76

A mixture of **13** (1.00 g, 2.85 mmol), BTEA-Cl (1.30 g, 5.70 mmol), N,N-dimethylaniline (0.38 g, 3.15 mmol, 0.40 mL) and $POCl_3$ (2.18 g, 14.25 mmol, 1.33 mL) in acetonitrile (12 mL) was stirred in a preheated oil bath at 85 °C for 20 min. Volatiles were evaporated immediately. The yellow foam was dissolved in $CHCl_3$ (30 mL) and vigorously stirred with crushed ice for 30 min. The layers were separated and the aqueous phase was extracted with $CHCl_3$. Crushed ice was added to the combined organic phase, which was washed [ice/H_2O (3 times), 5% $NaCO_3/H_2O$ (once)] and dried on $MgSO_4$. The residue was purified by column cromathography (DCM/MeOH 95/5) to give **22** (0.75 g, 71.2% yield).

^{1}H-NMR (DMSO, 300 MHz): δ 8.44 (1H, s, H-8), 7.02 (2H, bs, NH_2), 6.30-6.22 (1H, 2d, H-1'), 5.36-5.29 (1H, m, H-4'), 4.30-4.15 (3H, m, H-3', H-5'), 3.10-2.99 (1H, m, one of H-2'), 2.77-2.50 (1H, m, one of H-2'), 2.10,2.02 (6H, 2s, AcO).

23 2-amino-6-chloropurine-2'-deoxyriboside (IsoClad)



$C_{10}H_{12}ClN_5O_3$

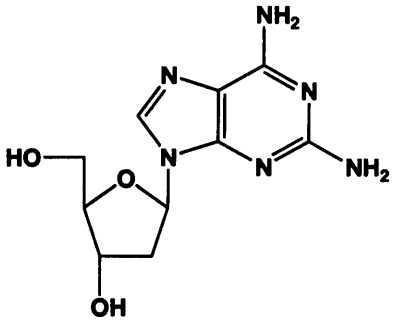
Mol Wt.: 285.69

Compound **22** (0.70 g, 1.90 mmol) was dissolved in NH_3/MeOH (20 mL, saturated at 0 °C) and stirred for 4 h in a sealed tube at room temperature. Volatiles were evaporated. The residue was purified by column chromatography (DCM/MeOH 98/2) to give the product as a white solid (0.49 g, 90.2% yield).

^{1}H-NMR (DMSO, 300 MHz): δ 8.36 (1H, s, H-8), 7.00 (2H, bs, NH_2), 6.23 (1H, t, ^{3}J=6.8 Hz, H-1'), 5.32 (1H, d, OH-3'), 4.96 (1H, t, OH-5'), 4.37 (1H, m, H-3'), 3.87-3.81 (1H, m, H-4'), 3.61-3.49 (2H, m, H-5'), 2.78-2.58 (1H, m, one of H-2'), 2.30-2.20 (1H, m, one of H-2').

^{13}C-NMR (DMSO, 75 MHz): δ 39.6 (C-2'), 61.9 (C-5'), 70.9 (C-3'), 83.3 (C-1'), 88.1 (C-4'), 123.9 (C-5), 141.4 (C-8), 149.8 (C-6), 154.0 (C-2), 160.1 (C-4).

24 2,6-diaminopurine-2'-deoxyriboside (DAP)



$C_{10}H_{14}N_6O_3$
Mol Wt.: 266.26

NH_3/MeOH (25 mL, saturated at 0 °C) was added to **14** (1.00 g, 1.62 mmol) and stirred overnight in a sealed tube at room temperature. Volatiles were evaporated. The residue was purified by column chromatography (DCM/MeOH, from 95/5 to 9/1). The material collected was reduced to dryness and dissolved in H_2O/MeOH. The solution was stirred in the presence of Amberlite (HCO_3^-), filtered and reduced to dryness to give the product as a white solid (0.98 g, 22.7% yield).

^{1}H-NMR (DMSO, 500 MHz): δ 7.92 (1H, s, H-8), 6.72 (2H, bs, NH_2), 6.18,6.16 (1H, 2d, ^{3}J=6.0 Hz, H-1'), 5.71 (2H, bs, NH_2), 5.27-5.24 (2H, m, OH-3', OH-5'), 4.37-4.36 (1H, m, H-3'), 3.84-3.83 (1H, m, H-4'), 3.61-3.57 (1H, m, one of H-5'), 3.54-3.52 (1H, m, one of H-5'), 2.60-2.59 (1H, m, one of H-2'), 2.19-2.17 (1H, m, one of H-2').

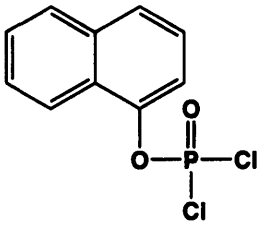
^{13}C-NMR (DMSO; 125 MHz): δ 39.5 (C-2'), 62.0 (C-5'), 71.0 (C-3'), 83.1 (C-1'), 87.6 (C-4'), 113.4 (C-5), 135.8 (C-8), 151.2,156.2 (C-2, C-6), 160.0 (C-4).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 4.21 min.

ESI MS m/z (positive) 267 [M + H].

Aryl dichlorophosphates

6a 1-Naphthyl dichlorophosphate



$C_{10}H_7Cl_2O_2P$

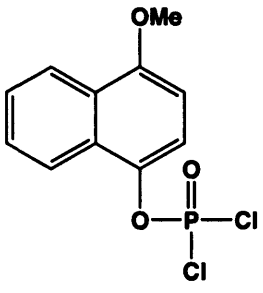
Mol. Wt.: 261.04

Prepared according to Standard Procedure A, from 1-naphthol (3.00 g, 20.81 mmol), $POCl_3$ (3.19 g, 20.81 mmol, 1.94 mL), TEA (2.11g, 20.81 mmol, 2.9 mL) and anhydrous Et_2O (70 mL). After 1 h at -78 °C the reaction was left to rise to room temperature and stirred for 3 h. The crude product was obtained as an oil (4.59 g, 84.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.07.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.19-8.16 (1H, m, H-8), 7.98-7.95 (1H, m, H-5), 7.89-7.86 (1H, m, H-4), 7.71-7.52 (4H, m, H-2, H-3, H-6, H-7).

6b 4-Methoxy-1-naphthyl dichlorophosphate



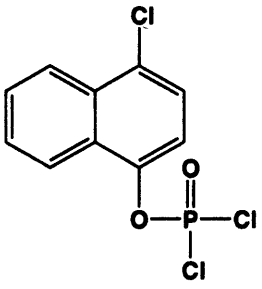
$C_{11}H_9Cl_2O_3P$

Mol Wt.: 291.07

Prepared according to Standard Procedure A, from 4-methoxy-1-naphthol (1.50 g, 8.61 mmol), $POCl_3$ (1.32 g, 8.61 mmol, 0.80 mL), TEA (0.87 g, 8.61 mmol, 1.20 mL) and anhydrous Et_2O (15 mL) to give the product as an oil (2.51 g, quantitative yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.33.

6c 4-Chloro-1-naphthyl dichlorophosphate



$C_{10}H_6Cl_3O_2P$

Mol Wt.: 295.49

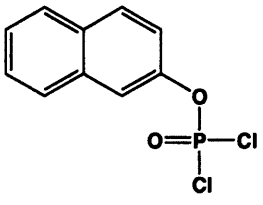
Prepared according to Standard Procedure A, from 4-chloro-1-naphthol (1.00 g, 5.60 mmol), $POCl_3$ (0.86 g, 5.60 mmol, 0.52 mL), TEA (0.62 g, 6.16 mmol, 0.85 mL) and anhydrous Et_2O (20 mL) to give the product as an oil (1.37 g, quantitative yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.15.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.33-8.12 (2H, m, H-8, H-5), 7.72-7.49 (4H, m, H-2, H-3, H-6, H-7).

^{13}C-NMR ($CDCl_3$; 75 MHz): δ 116.5,116.7 (C-2), 122.2,125.4,125.8,125.9,127.2, 127.3,128.3,128.4,128.5,128.7,128.8,131.1,132.4 (C-3, C-4, C-4a, C-5, C-6, C-7, C-8, C-8a), 145.2,145.3 (C-1).

7 2-Naphthyl dichlorophosphate



$C_{10}H_7Cl_2O_2P$

Mol. Wt.: 261.04

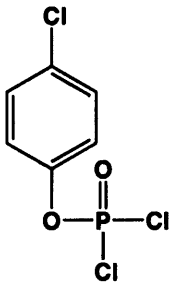
Prepared according to Standard Procedure A, from 2-naphthol (1.50 g, 10.40 mmol), $POCl_3$ (1.59 g, 10.40 mmol, 0.97 mL), TEA (1.05 g, 10.40 mmol, 1.45 mL) and anhydrous Et_2O (35 mL). After 1 h the reaction was left to rise to room temperature and stirred for 3 h. The crude product was obtained as an oil (2.44 g, 90.0% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.01.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.96 (1H, d, ^{3}J=9.5 Hz, H-4), 7.94-7.89 (2H, m, H-5, H-8), 7.84 (1H, s, H-1), 7.62-7.56 (2H, m, H-6, H-7), 7.47 (1H, d, ^{3}J=9.5 Hz, H-3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 118.3,119.8,119.9 (C-1, C-3), 127.1,127.7,127.9, 128.1,128.2,128.4,131.1,131.2 (C-4, C-5, C-6, C-7, C-8), 132.2,134.0 (C-4a, C-8a), 147.6,147.8 (C-2).

28 4-Chloro-phenyl dichlorophosphate



$C_6H_4Cl_3O_2P$

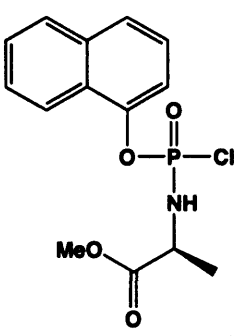
Mol Wt.: 245.43

Prepared according to Standard Procedure A, from 4-chloro-phenol (1.00 g, 7.78 mmol), $POCl_3$ (1.43 g, 9.34 mmol, 0.87 mL), TEA (0.95 g, 9.34 mmol, 1.30 mL) and anhydrous Et_2O (3o mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (1.95 g, quantitative yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.18.

Aryl phosphorochloridates

8a 1-Naphthyl(methoxy-L-alaninyl) phosphorochloridate



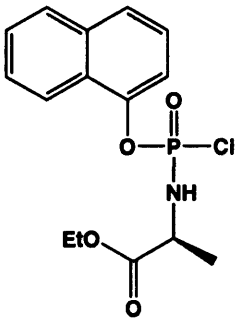
$C_{14}H_{15}ClNO_4P$

Mol Wt.: 327.70

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (1.88g, 7.20 mmol), L-alanine methyl ester hydrochloride (1.00 g, 7.16 mmol), TEA (1.46 g, 14.43 mmol, 2.01 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred for 2 h. The crude product was obtained as an oil (1.35 g, 57.2% yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.62, 9.40.

8b 1-Naphthyl(ethoxy-L-alaninyl) phosphorochloridate



$C_{15}H_{17}ClNO_4P$

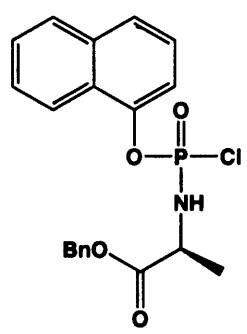
Mol Wt.: 341.73

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (1.77g, 6.78 mmol), L-alanine ethyl ester hydrochloride (1.04 g, 6.78 mmol), TEA (1.37 g, 13.56 mmol, 1.89 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (2.87 g, quantitative yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.67, 9.46.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.04-7.25 (7H, m, Naph), 4.10 (1H, q, ^3J=7.1 Hz, CHCH$_3$), 4.04 (2H, q, ^3J=7.3 Hz, OCH_2CH$_3$), 1.43 (3H, d, ^3J=7.1 Hz, CHCH_3), 1.18 (3H, t, ^3J=7.3 Hz, OCH$_2CH_3$).

8c 1-Naphthyl(benzoxy-L-alaninyl) phosphorochloridate



$C_{20}H_{19}ClNO_4P$

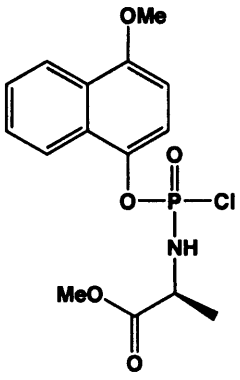
Mol Wt.: 403.80

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00g, 7.66 mmol), L-alanine benzyl ester hydrochloride (1.65 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 1.14 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (3.45 g, quantitative yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.57, 9.40.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.15-7.33 (12H, m, Naph, Ph), 5.29-5.23 (2H, m, OCH_2Ph), 4.28-4.21 (1H, m, $CHCH_3$), 1.46-1.36 (3H, m, $CHCH_3$).

8d 4-Methoxy-1-naphthyl(methoxy-L-alaninyl) phosphorochloridate



$C_{15}H_{17}ClNO_5P$

Mol Wt.: 357.73

Prepared according to Standard Procedure B, from 4-methoxy-1-naphthyl dichlorophosphate (2.51 g, 8.61 mmol), L-alanine methyl ester hydrochloride (1.20 g, 8.61 mmol), TEA (1.67 g, 17.22 mmol, 2.30 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred for 6 h. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.86 g, 27.9% yield).

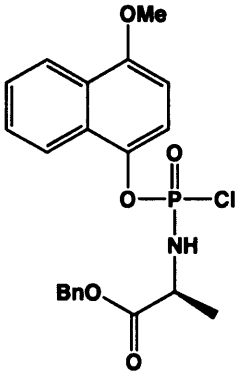
^{31}P-NMR ($CDCl_3$, 121 MHz): δ 10.28,10.36.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.32-8.08 (2H, m, H-5, H-8), 7.62-7.53 (3H, m, H-2, H-6, H-7), 6.72-6.69 (1H, m, H-3), 5.34-5.27 (1H, m, $CHNH$), 4.42-4.32 (1H, m,

CHCH_3), 3.96 (3H, s, OCH_3 Naph), 3.82,3.77 (3H, 2s, OCH_3), 1.61-1.57 (3H, m, $CHCH_3$).

^{13}C-NMR ($CDCl_3$; 75 MHz): δ 20.7,20.8,20.9 ($CHCH_3$), 51.0,51.2, 53.1,53.2 (*CH*CH_3, OCH_3), 56.1 (OCH_3 Naph), 103.2 (C-3), 116.5,116.6,116.7 (C-2), 121.6, 121.7,122.7,122.8,126.5,126.8,127.3,127.4,127.6,127.7 (C-4a, C-5, C-6, C-7, C-8, C-8a), 139.7,139.8,139.9 (C-1), 153.7 (C-4), 173.6,173.7,173.8,173.8 (*C*$OOCH_3$).

8e 4-Methoxy-1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate



$C_{21}H_{21}ClNO_5P$

Mol Wt.: 433.82

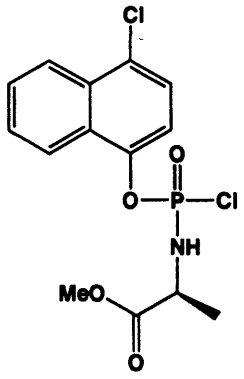
Prepared according to Standard Procedure B, from 4-methoxy-1-naphthyl dichlorophosphate (2.40 g, 8.24 mmol), L-alanine benzyl ester hydrochloride (1.78 g, 8.24 mmol), TEA (1.67 g, 16.48 mmol, 2.30 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred for 3 hr. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.76 g, 49.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 10.16,10.26.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.36-8.08 (2H, m, H-5 Naph, H-8 Naph), 7.65-7.57 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.45-7.37 (5H, m, Ph), 6.73 (1H, d, H-3 Naph), 5.29-5.24 (2H, m, CH_2Ph), 5.14-5.07 (1H, m, CH*NH*), 4.49-4.40 (1H, m, *CH*CH_3), 4.01 (3H, s, OCH_3), 1.65-1.61 (1H, m, $CHCH_3$).

^{13}C-NMR ($CDCl_3$; 75 MHz): δ 20.8,20.9,21.0 ($CHCH_3$), 51.2,51.4 (*CH*CH_3), 56.2 (OCH_3), 67.9,68.1 (CH_2Ph), 103.2 (C-3), 116.6,116.7 (C-2), 121.6,121.7, 122.8,122.8,126.5,126.8,127.4,127.7,128.7,128.8,129.0,129.1 (C-4a, C-5, C-6, C-7, C-8, C-8a, CH_2Ph), 135.6 ('ipso' CH_2Ph), 139.8 (C-1), 153.8 (C-4), 173.0,173.1,173.2 (*C*$OOCH_2$Ph).

8f 4-Chloro-1-naphthyl(methoxy-L-alaninyl) phosphorochloridate



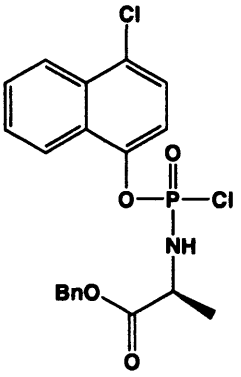
$C_{14}H_{14}Cl_2NO_4P$

Mol Wt.: 362.15

Prepared according to Standard Procedure B, from 4-chloro-1-naphthyl dichlorophosphate (1.37 g, 5.58 mmol), L-alanine methyl ester hydrochloride (0.78 g, 5.58 mmol), TEA (1.13 g, 11.16 mmol, 1.56 mL) and anhydrous DCM (18 mL). After 15 min the reaction was left to rise to room temperature and stirred for 2 h. The crude product was obtained as an oil (1.63 g, 80.7% yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.37,9.58,9.61.

8g 4-Chloro-1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate



$C_{20}H_{18}Cl_2NO_4P$

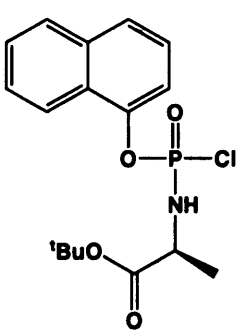
Mol Wt.: 438.24

Prepared according to Standard Procedure B, from 4-chloro-1-naphthyl dichorophosphate (2.06 g, 8.40 mmol), L-alanine benzyl ester hydrochloride (1.81 g, 8.40 mmol), TEA (1.70 g, 16.80 mmol, 2.34 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred for 1.5 h. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.81 g, 49.2% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.82.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.28-8.13 (2H, m, H-8 Naph, H-5 Naph), 7.66-7.47 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.38-7.34 (5H, m, Ph), 5.27-5.20 (2H, m, CH_2Ph), 4.45-4.39 (1H, m, *CH*CH_3), 1.62-1.58 (3H, m, CH*CH*$_3$).

8h 1-Naphthyl(tert-butoxy-L-alaninyl) phosphorochloridate



$C_{17}H_{21}ClNO_4P$

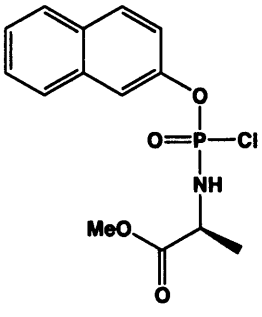
Mol Wt.: 369.78

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00 g, 7.66 mmol), L-alanine tertbutoxy ester hydrochloride (1.39 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 2.14 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.67 g, 23.7% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.88,9.75.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.20,8.10 (1H, m, H-8 Naph), 7.90-7.83 (1H, m, H-5 Naph), 7.76-7.64 (1H, m, H-4 Naph, H-2 Naph), 7.60-7.37 (3H, m, H-3 Naph, H-6 Naph, H-7 Naph), 5.20-4.91 (1H, bs, *NH*CH), 4.27-4.17 (1H, m, *CH*NH), 1.51,1.47 (9H, 2s, tertbutyl), 1.31-1.24 (3H, m, CH_3CH).

9a 2-Naphthyl(methoxy-L-alaninyl) phosphorochloridate



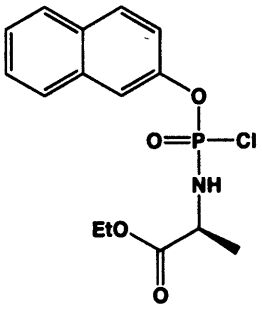
$C_{14}H_{15}ClNO_4P$

Mol Wt.: 327.70

Prepared according to Standard Procedure B, from 2-naphthyl dichlorophosphate (0.94 g, 3.60 mmol), L-alanine methyl ester hydrochloride (0.50 g, 3.60 mmol), TEA (0.37 g, 7.20 mmol, 1.00 mL) and anhydrous DCM (15 mL). After 15 min the reaction was left to rise to room temperature and stirred for 2 h. The crude product was obtained as an oil (1.18 g, quantitative yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.29,9.01.

9b 2-Naphthyl(ethoxy-L-alaninyl) phosphorochloridate



$C_{15}H_{17}ClNO_4P$

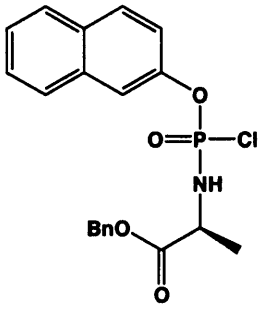
Mol Wt.: 341.73

Prepared according to Standard Procedure B, using 2-naphthyl dichlorophosphate (1.89 g, 7.24 mmol), L-alanine ethyl ester hydrochloride (1.11 g, 7.24 mmol), TEA (1.47 g, 14.48 mmol, 2.02 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (2.81 g, quantitative yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.76,9.61.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.84-7.42 (7H, m, Naph), 5.18 (1H, bs, CH*NH*), 4.30-4.22 (3H, 2q, *CH*CH_3, O*CH*$_2$$CH_3$), 1.58,1.57 (3H, 2d, ^{3}J=7.0 Hz, CH*CH*$_3$), 1.34-1.22 (3H, m, OCH_2*CH*$_3$).

9c 2-Naphthyl(benzoxy-L-alaninyl) phosphorochloridate



$C_{20}H_{19}ClNO_4P$

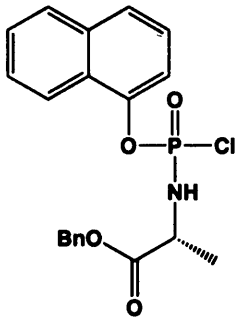
Mol Wt.: 403.80

Prepared according to Standard Procedure B, from 2-naphthyl dichlorophosphate (2.28 g, 8.73 mmol), L-alanine benzyl ester hydrochloride (1.88 g, 8.72 mmol), TEA (1.76 g, 17.46 mmol, 2.40 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (2.87 g, 84.7% yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.25,8.95.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.91-7.30 (12 H, m, Naph, Ph), 5.33-5.15 (2H, m, *CH*$_2$Ph), 4.45-4.18 (1H, m, *CH*CH_3), 1.64-1.38 (3H, m, CH*CH*$_3$).

11a 1-Naphthyl(benzoxy-D-alaninyl) phosphorochloridate



$C_{20}H_{19}ClNO_4P$

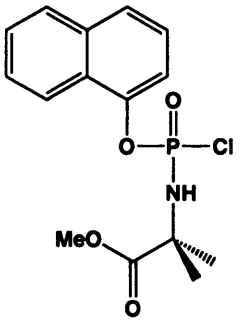
Mol Wt.: 403.80.

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00 g, 7.66 mmol), D-alanine benzoxy ester tosylate salt (2.69 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 2.14 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.73 g, 23.6 % yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.65,9.54.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.17,8.13 (1H, m, H-8 Naph), 7.92-7.89 (1H, m, H-5 Naph), 7.61 (1H, d, ^{3}J=8.2 Hz, H-4 Naph), 7.70-7.66 (1H, m, H-2 Naph), 7.61-7.32 (8H, m, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*), 5.29-5.16 (2H, m, CH_2Ph), 5.10-4.99 (1H, m, NH), 4.47-4.38 (1H, m, *CH*NH), 1.62-1.58 (3H, m, CH_3).

11b 1-Naphthyl(methoxy-dimethylglycinyl) phosphorochloridate



$C_{15}H_{17}ClNO_4P$

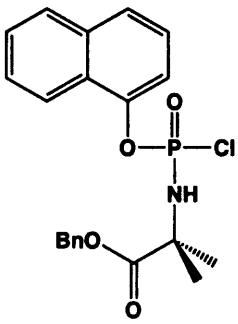
Mol Wt.: 341.73

Prepared according to Standard Procedure B, from 1-naphthyl dichlophosphate (3.00 g, 11.49 mmol), dimethylglycine methyl ester hydrochloride (1.76 g, 11.49 mmol), TEA (2.33 g, 22.98 mmol, 3.2 mL) and anhydrous DCM (45 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.64 g, 41.7% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 7.41.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.18 (1H, m, H-8), 7.88 (1H, d, H-5), 7.74 (1H, d, H-4), 7.66 (1H, d, H-2), 7.59-7.44 (3H, m, H-3, H-6, H-7), 5.15-5.12 (1H, bs, NH), 3.81 (3H, s, OCH_3), 1.78,1.75 (6H, 2s, $C(CH_3)_2$).

11c 1-Naphthyl(benzoxy-dimethylglycinyl) phosphorochloridate



$C_{21}H_{21}ClNO_4P$

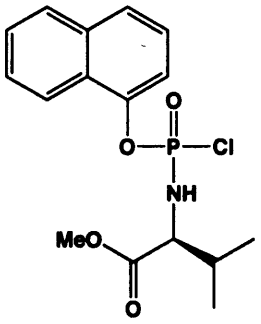
Mol Wt.: 417.82

Prepared according to the Standard Procedure B, using 1-naphthyl dichlorophosphate (1.50 g, 5.74 mmol), dimethylglycine benzyl ester tosylate (2.10 g, 5.74 mmol), TEA (1.16 g, 11.48 mmol, 1.60 mL) and anhydrous DCM (50 mL). The reaction was carried out at room and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.77 g, 32.1% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 7.32.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.19-7.34 (12H, m, Naph, Ph), 5.25 (1H, s, CH_2Ph), 1.80,1.77 (6H, 2s, $C(CH_3)_2$).

11d 1-Naphthyl(methoxy-L-valinyl) phosphorochloridate



$C_{16}H_{19}ClNO_4P$

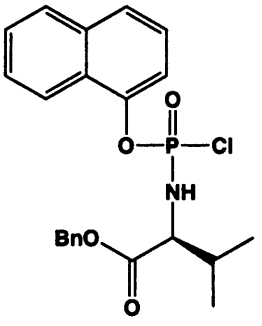
Mol Wt.: 355.75

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (1.00 g, 3.83 mmol), L-valine methoxy ester hydrochloride (0.64 g, 3.83 mmol), TEA (0.78 g, 7.66 mmol, 1.07 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.66 g, 48.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 11.24,10.72.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.20-8.13 (1H, m, H-8 Naph), 7.94-7.90 (1H, m, H-5 Naph), 7.78 (1H, d, ^{3}J= 8.2 Hz, H-4 Naph), 7.70-7.42 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 4.81-4.70 (1H, m, *NH*CH), 4.16-4.09 (1H, m, *CH*NH), 3.82,3.79 (3H, 2s, CH_3O), 2.29-2.19 (1H, m, *CH*$(CH_3)_2$), 1.11-1.00 (6H, m, CH(*CH_3*)$_2$).

11e 1-Naphthyl(benzoxy-L-valinyl) phosphorochloridate



$C_{22}H_{23}ClNO_4P$

Mol Wt.: 431.85

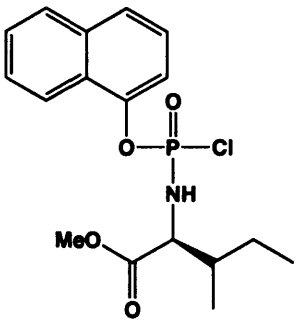
Prepared according to Standard Procedure B, using 1-naphthyl dichlorophosphate (2.08 g, 7.98 mmol), L-isoleucine benzyl ester tosylate (3.10 g, 7.98 mmol), TEA (1.61 g, 15.96 mmol, 2.22 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash

chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (2.69 g, 75.6% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 11.32,10.72.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.17-8.12 (1H, m, H-8 Naph), 7.90-7.85 (1H, m, H-5 Naph), 7.76 (1H, d, ^{3}J= 8.2 Hz, H-4 Naph), 7.69-7.32 (9H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*), 5.26-5.15 (2H, m, CH_2Ph), 5.00-4.88 (1H, bs, NH), 4.21-4.11 (1H, m, NH*CH*), 2.29-2.13 (1H, m, $CH(CH_3)_2$), 1.09-0.96 (6H, m, $CH(CH_3)_2$).

11f 1-Naphthyl(methoxy-L-isoleucinyl) phosphorochloridate



$C_{17}H_{21}ClNO_4P$

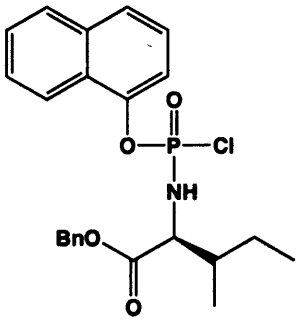
Mol Wt.: 369.78

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (1.31 g, 5.00 mmol), L-isoleucine methyl ester hydrochloride (0.91 g, 5.00 mmol), TEA (1.01 g, 10.00 mmol, 1.39 mL) and anhydrous DCM (20 mL). After 15 min the reaction was left to rise to room temperature and stirred for 6 h. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.91 g, 48.9% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 10.84,10.39.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.17-8.13 (1H, m, H-8), 7.93-7.90 (1H, m, H-5), 7.77 (1H, d, H-4), 7.69-7.64 (1H, m, H-2), 7.63-7.45 (3H, m, H-3, H-6, H-7), 4.64-4.60 (1H, bs, NH), 4.21-4.13 (1H, m, NH*CH*), 3.83,3.79 (3H, 2s, OCH_3), 1.99-1.95 (1H, m, NHCH*CH*), 1.60-1.52 (1H, m, one of CH_2), 1.32-1.23 (1H, m, one of CH_2), 1.06-0.94 (6H, m, $CHCH_3$, CH_2CH_3).

11g 1-Naphthyl(benzoxy-L-isoleucinyl) phosphorochloridate



$C_{23}H_{25}ClNO_4P$

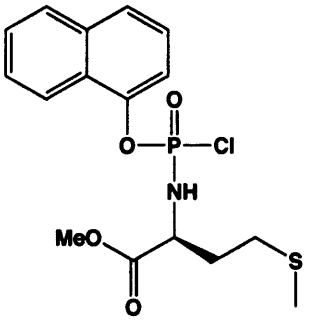
Mol Wt.: 445.88

Prepared according to Standard Procedure B, using 1-naphthyl dichlorophosphate (2.08 g, 7.98 mmol), L-isoleucine benzyl ester tosylate (3.10 g, 7.98 mmol), TEA (1.61 g, 15.96 mmol, 2.22 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (2.69 g, 75.6% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 11.03,10.75,10.46.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.20-8.10 (1H, m, H-8 Naph), 7.91-7.85 (1H, m, H-5 Naph), 7.77 (1H, d, H-4 Naph), 7.70-7.30 (9H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*), 5.01-5.32 (2H, m, CH_2Ph), 4.29-4.09 (1H, m, NH*CH*), 1.90-1.08 (1H, m, NHCH*CH*), 1.62-1.48 (1H, m, one of CH_2CH_3), 1.30-1.18 (1H, m, one of CH_2CH_3), 1.09-0.90 (6H, m, CHCH_3, CH_2CH_3).

11h 1-Naphthyl(methoxy-L-methioninyl) phosphorochloridate



$C_{16}H_{19}ClNO_4PS$

Mol Wt.: 387.82

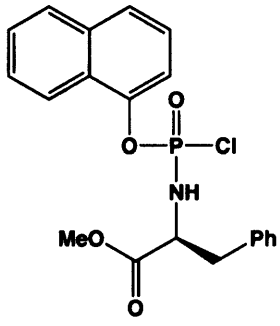
Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (1.05 g, 4.01 mmol), L-methonine methyl ester hydrochloride (0.80 g, 4.01 mmol), TEA (0.81 g, 8.02 mmol, 1.12 mL) ans anhydrous DCM (15 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was

purified by flash chromatography (ethyl acetate/hexane 7/3) to give an oil as product (0.59 g, 37.8% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 10.30,10.11.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.17,8.15 (1H, 2d, ^{3}J=8.0 Hz, H-8 Naph), 7.91-7.89 (1H, m, H-5 Naph), 7.76 (1H, d, ^{3}J=8.2 Hz, H-4 Naph), 7.68-7.43 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 5.20-5.00 (1H, bs, *NH*CH), 4.48-4.40 (1H, m, NH*CH*), 3.84,3.78 (3H, 2s, OCH_3), 2.67-2.59 (2H, m, CH_2S), 2.25-2.11 (5H, m, CH_2CH_2S, SCH_3).

11i 1-Naphthyl(methoxy-L-phenylalaninyl) phosphorochloridate



$C_{20}H_{19}ClNO_4P$

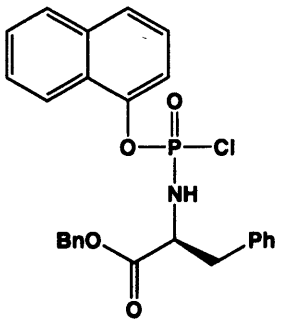
Mol Wt.: 403.80

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00 g, 7.66 mmol), L-phenylalanine methoxy ester hydrochloride (1.65 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 2.14 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.90 g, 61.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.67,9.91.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.16-8.10 (1H, m, H-8 Naph), 7.96-7.90 (1H, m, H-5 Naph), 7.79-7.74 (1H, m, H-4 Naph), 7.63-7.10 (9H, m, H-2 Naph H-3 Naph, H-6 Naph, H-7 Naph, *Ph*), 4.83-4.50 (2H, m, *CHNH*), 3.80,3.75 (3H, 2s, CH_3O), 3.22-3.17 (2H, m, CH_2Ph).

11l 1-Naphthyl(benzoxy-L-phenylalaninyl) phosphorochloridate



$C_{26}H_{23}ClNO_4P$

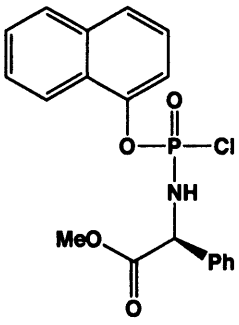
Mol Wt.: 479.89

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.12 g, 8.12 mmol), L-phenylalanine benzoxy ester hydrochloride (2.37 g, 8.12 mmol), TEA (1.64 g, 16.24 mmol, 2.26 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (3.21 g, 82.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.37,9.55,9.22.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.20-7.10 (17H, m, Naph, $CHCH_2$*Ph*, CO_2CH_2*Ph*), 5.26-5.01 (2H, m, CO_2*C*H_2Ph), 4.71-4.37-4.50 (2H, m, *CHNH*), 3.30-2.93 (2H, m, CH*C*H_2Ph).

11m 1-Naphthyl(methoxy-L-phenylglycinyl) phosphorochloridate



$C_{19}H_{17}ClNO_4P$

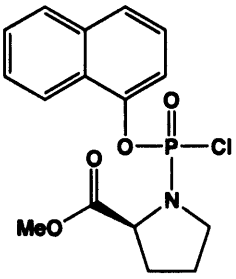
Mol Wt.: 389.77

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (3.50 g, 13.40 mmol), L-phenylglycine methyl ester hydrochloride (2.70 g, 13.40 mmol), TEA (2.71 g, 26.80 mmol, 3.74 mL) and anhydrous DCM (40 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.79 g, 34.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.13.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.09-7.31 (12H, m, Naph, Ph), 5.88-5.78 (1H, m, NH), 5.40-5.24 (1H, m, NH*CH*), 3.73,3.70 (3H, 2s, OCH_3).

11n 1-Naphthyl(methoxy-L-prolinyl) phosphorochloridate



$C_{16}H_{17}ClNO_4P$

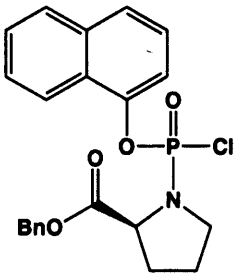
Mol Wt.: 353.74

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00 g, 7.66 mmol), L-proline methyl ester hydrochloride (2.27 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 2.14 mL) and anhydrous DCM (25 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (2.04 g, 75.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.38,9.34.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.13,8.08 (1H, 2d, ^{3}J=8.0 Hz, H-8 Naph) , 7.83 (1H, d, ^{3}J=7.6 Hz, H-5 Naph), 7.69-7.60 (2H, m, H-4 Naph, H-2 Naph), 7.57-7.37 (3H, m, H-3 Naph, H-6 Naph, H-7 Naph), 4.57-4.52 (1H, m, H-2 *prol*), 3.73,3.67 (3H, 2s, OCH_3), 3.65-3.53 (2H, m, H-5 *prol*), 2.25-2.04 (4H, m, H-3 *prol*, H-4 *prol*).

11o 1-Naphthyl(benzoxy-L-prolinyl) phosphorochloridate



$C_{22}H_{11}ClNO_4P$

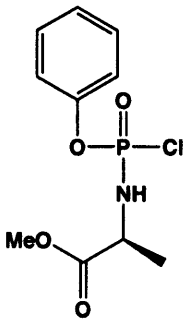
Mol Wt.: 429.83

Prepared according to Standard Procedure B, from 1-naphthyl dichlorophosphate (2.00 g, 7.66 mmol), L-proline benzoxy ester hydrochloride (1.85 g, 7.66 mmol), TEA (1.55 g, 15.32 mmol, 2.14 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (2.22 g, 67.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.49,9.30.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.22-8.11 (1H, m, H-8 Naph), 7.97-7.90 (1H, m, H-5 Naph), 7.80-7.68 (1H, m, H-2 Naph, H-4 Naph), 7.60-7.30 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 5.30-5.12 (2H, m, *CH*$_2$Ph), 4.73-4.61 (1H, m, *CH*NH), 3.76-3.68 (2H, m, H-5 *prol*), 2.35-2.11 (4H, m, H-3 *prol*, H-4 *prol*).

17a Phenyl(methoxy-L-alaninyl) phosphochloridate



$C_{10}H_{13}ClNO_4P$

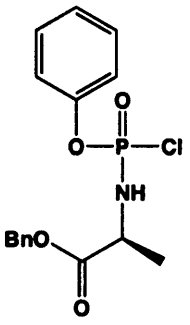
Mol Wt.: 277.64.

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (1.51 g, 7.16 mmol, 1.07 mL), L-alanine methyl ester hydrochloride (1.00 g, 7.16 mmol), TEA (1.45 g, 14.32 mmol, 1.99 mL) and anhydrous DCM (30 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.86 g, 43.4% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.41,9.26.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.30-7.09 (5H, m, Ph), 4.86-4.69 (1H, m, NH), 4.18-4.03 (1H, m, *CH*NH), 3.69,3.66 (3H, 2s, CH_3O), 1.45-1.40 (3H, m, CH_3).

17b Phenyl(benzoxy-L-alaninyl) phosphochloridate



$C_{16}H_{17}ClNO_4P$

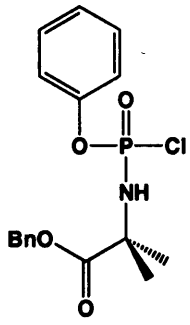
Mol Wt.: 353.74.

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (2.93 g, 13.91 mmol, 2.08 mL), L-alanine benzyl ester hydrochloride (3.00 g, 13.91 mmol), TEA (2.82 g, 27.82 mmol, 3.88 mL) and anhydrous DCM (60 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (4.70 g, 95.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.33,9.13.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.41-7.26 (10H, m, PhO, *Ph*CH_2), 5.24,5.23 (2H, 2s, Ph*C*H_2), 4.89-4.74 (1H, m, CH*NH*), 4.32-4.21 (1H, m, *CH*NH), 1.60-1.56 (3H, m, CH_3).

17c Phenyl(methoxy-dimethylglycinyl) phosphochloridate



$C_{17}H_{19}ClNO_4P$

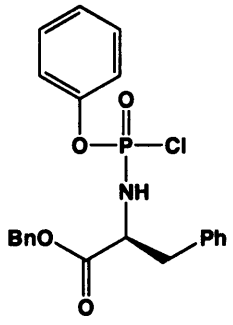
Mol Wt.: 367.76

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.886 g, 4.20 mmol, 0.63 mL), dimethylglycine benzyl ester tosylate (1.53 g, 4.20 mmol), TEA (0.85 g, 8.40 mmol, 1.17 mL) and anhydrous DCM (30 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.23 g, 79.9% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 5.55.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.30-7.10 (10H, m, PhO, *Ph*CH_2), 5.12 (2H, s, Ph*CH_2*), 4.71,4.69 (1H, 2bs, NH), 1.63,1.60 (6H, 2s, C(*CH_3*)$_2$).

20a Phenyl(benzoxy-L-phenylalaninyl) phosphorochloridate



$C_{22}H_{21}ClNO_4P$

Mol Wt.: 429.83

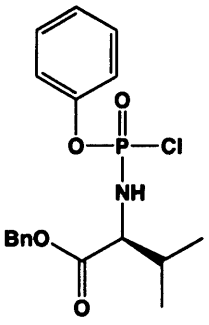
Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.58 g, 2.74 mmol, 0.41 mL), L-phenylalanine benzyl ester hydrochloride (0.80 g, 2.74 mmol), TEA (0.55 g, 5.48 mmol, 0.41 mL) and anhydrous DCM (20 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.74 g, 63.1% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 7.87,7.81.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.29-6.94 (15H, m, PhO, $CHCH_2$*Ph*, CO_2CH_2*Ph*), 5.09-5.04 (2H, m, CO_2*C*H_2Ph), 4.43-4.34 (1H, m, *CH*NH), 4.13-4.00 (1H, m, CH*NH*), 3.11-3.02 (2H, m, CH*C*H_2Ph).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 39.9,40.0 (CH*C*H_2Ph), 55.4,55.9 (CHNH), 67.6,67.7 (CO_2*C*H_2Ph), 120.5,120.6,126.0,127.3,127.4,128.6,128.7,128.8,129.5,129.6,129.7, 129.9 (PhO, $CHCH_2$*Ph*, CO_2CH_2*Ph*), 134.7,134.8 ('ipso' PhO, $CHCH_2$*Ph*, CO_2CH_2*Ph*), 170.9,171.0,171.1 (*C*$OOCH_2$Ph).

20b Phenyl(benzoxy-L-valininyl) phosphorochloridate



$C_{18}H_{21}ClNO_4P$

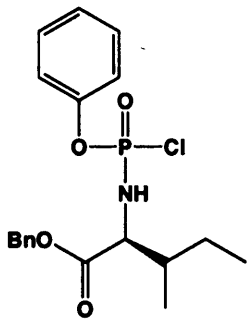
Mol Wt.: 381.79

Prepared according to Standard Procedure B, using phenyl dichlorophosphate (0.58 g, 2.74 mmol, 0.41 mL), L-valine benzyl ester tosylate (1.04 g, 2.74 mmol), TEA (0.55 g, 5.48 mmol, 0.76 mL) and anhydrous DCM (20 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.86 g, 81.9% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 9.79,9.01.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.28-7.11 (10H, m, PhO, CH_2*Ph*), 5.12,5.10 (2H, 2s, *C*H_2Ph), 4.50-4.46 (1H, m, NH), 3.92-3.87 (1H, m, NH*CH*), 2.11-2.06 (1H, m, *CH*$(CH_3)_2$), 0.92,0.90 (3H, 2d, ^3J=6.8 Hz, CH*C*H_3), 0.83,0.81 (3H, 2d, ^3J=6.6 Hz, CH*C*H_3).

20c Phenyl(benzoxy-L-isoleucinyl) phosphorochloridate



$C_{19}H_{23}ClNO_4P$

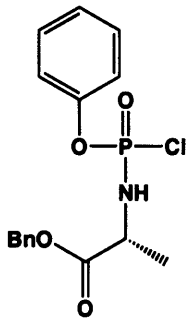
Mol Wt.: 395.82

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.54 g, 2.54 mmol, 0.38 mL), L-isoleucine benzyl ester tosylate (1.00 g, 2.54 mmol), TEA (0.51 g, 5.08 mmol, 0.71 mL) and anhydrous DCM (20 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to the product as an oil (0.72 g, 72.0% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 9.10,8.65.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.29-7.13 (10H, m, PhO, CH_2*Ph*), 5.14-5.11 (2H, m, *CH_2*Ph), 4.24-4.13 (1H, m, NH), 4.01-3.95 (1H, m, NH*CH*), 1.85-1.79 (1H, m, NHCH*CH*), 1.40-1.33 (1H, m, one of *CH_2*CH_3), 1.15-1.06 (1H, m, one of *CH_2*CH_3), 0.89,0.87 (3H, 2d, ^{3}J=6.9 Hz, CH*CH_3*), 0.81-0.78 (3H, m, CH_2*CH_3*).

20d Phenyl(benzoxy-D-alaninyl) phosphorochloridate



$C_{16}H_{17}ClNO_4P$

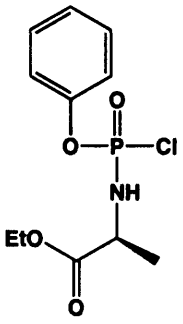
Mol Wt.: 353.74

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.60 g, 2.85 mmol, 0.43 mL), D-alanine benzyl ester tosylate (1.00 g, 2.85 mmol), TEA (0.58 g, 5.70 mmol, 0.79 mL) and anhydrous DCM (25 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.84 g, 83.2% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 7.20,7.02.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.26-7.05 (10H, m, PhO, CH_2*Ph*), 5.13,5.11 (2H, 2s, *CH_2*Ph), 4.80-4.76 (1H, m, NH), 4.18-4.14 (1H, m, NH*CH*), 1.44,1.43 (3H, 2d, ^3J=7.0 Hz, CH*CH_3*).

20e Phenyl(ethoxy-L-alaninyl) phosphorochloridate



$C_{11}H_{15}ClNO_4P$

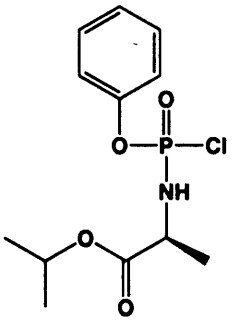
Mol Wt.: 291.67

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.55 g, 2.60 mmol, 0.39 mL), L-alanine ethyl ester hydrochloride (0.40 g, 2.60 mmol), TEA (0.53 g, 5.20 mmol, 0.72 mL) and anhydrous DCM (15 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.45 g, 59.2% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.03,7.72.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.32-7.14 (5H, m, PhO), 4.42-4.32 (1H, m, NH), 4.17,4.15 (2H, 2q, ^3J=7.1 Hz, O*CH_2*CH_3), 4.13-4.07 (1H, m, NH*CH*), 1.44,1.43 (3H, 2d, ^3J=7.0 Hz, CH*CH_3*), 1.23,1.22 (3H, 2t, ^3J=7.1 Hz, OCH_2*CH_3*).

20f Phenyl(iso-propoxy-L-alaninyl) phosphorochloridate



$C_{12}H_{17}ClNO_4P$

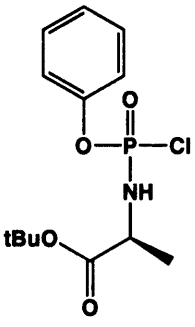
Mol Wt.: 305.69

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.63 g, 2.98 mmol, 0.45 mL), L-alanine iso-propyl ester hydrochloride (0.50 g, 2.98 mmol), TEA (0.60 g, 5.96 mmol, 0.83 mL) and anhydrous DCM (20 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.65 g, 71.4% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.14,7.87.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.29-7.15 (5H, m, PhO), 5.01,4.99 (1H, 2 septets, ^{3}J=6.2 Hz, O*CH*$(CH_3)_2$), 4.54-4.50 (1H, m, NH), 4.10-4.03 (1H, m, NH*CH*), 1.41,1.40 (3H, 2d, ^{3}J=7.0 Hz, CHCH_3), 1.23,1.22 (6H, 2d, ^{3}J=6.2 Hz, OCH$(CH_3)_2$).

20g Phenyl(tert-butoxy-L-alaninyl) phosphorochloridate



$C_{13}H_{19}ClNO_4P$

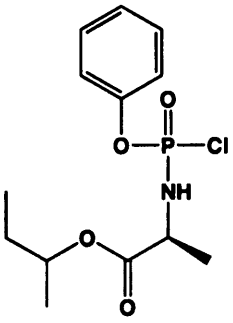
Mol Wt.: 319.72

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.58 g, 2.75 mmol, 0.41 mL), L-alanine tert-butyl ester hydrochloride (0.50 g, 2.75 mmol), TEA (0.56 g, 5.50 mmol, 0.77 mL) and anhydrous DCM (15 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.52 g, 59.1% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.21,7.85.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.29-7.23 (5H, m, PhO), 4.43-4.33 (1H, m, NH), 4.11-4.02 (1H, m, NH*CH*), 1.51-1.49 (12H, m, CHCH_3, tBu).

20h Phenyl(2-butoxy-L-alaninyl) phosphorochloridate



$C_{13}H_{19}ClNO_4P$

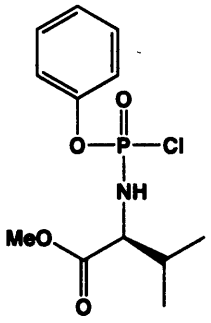
Mol Wt.: 319.72

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (0.58 g, 2.75 mmol, 0.41 mL), L-alanine 2-butyl ester hydrochloride (0.50 g, 2.75 mmol), TEA (0.56 g, 5.50 mmol, 0.77 mL) and anhydrous DCM (20 mL). After 15 min the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.28 g, 31.8% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.12,7.50.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.37-7.24 (5H, m, PhO), 4.96-4.90 (1H, m, O*CH*(CH_3)CH_2CH_3), 4.57 (1H, bs, NH), 4.20-4.10 (1H, m, NH*CH*), 1.65-1.56 (2H, m, OCH(CH_3)*CH_2*CH_3), 1.54-1.50 (3H, m, NHCH*CH_3*), 1.29-1.24 (3H, m, OCH(*CH_3*)CH_2CH_3), 0.94-0.87 (3H, m, OCH(CH_3)CH_2*CH_3*).

26a Phenyl(methoxy-L-valinyl) phosphorochloridate



$C_{12}H_{17}ClNO_4P$

Mol Wt.: 305.69

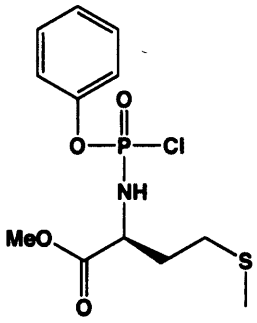
Prepared according to Standard Procedure B, from phenyl dichlorophosphate (2.11 g, 10.00 mmol, 1.49 mL), L-valine methyl ester hydrochloride (1.65 g, 10.00 mmol), TEA (2.02 g, 20.00 mmol, 2.79 mL) and anhydrous DCM (30 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (1.22 g, 39.87% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 11.01,10.40.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.35-7.12 (5H, m, Ph), 4.70 (1H, m, NH), 3.93-3.79 (1H, m, *CH*NH), 3.68 (3H, 2s, OCH_3), 2.09 (1H, m, *CH*$(CH_3)_2$), 0.96-0.86 (6H, m, CH(*CH*$_3)_2$).

^{13}C-NMR ($CDCl_3$; 75 MHz): δ 17.8,17.9,19.3,19.4 (CH(*CH*$_3)_2$), 32.3,32.4,32.5 (*CH*$(CH_3)_2$), 52.8,52.9 (OCH_3), 60.4,60.8,61.0 (CHNH), 120.9,121.0,126.3,130.0, 130.3 (Ph), 150.1,150.2,150.3,150.4 ('ipso', Ph), 172.5,172.6,172.8,172.9 (*C*$OOCH_3$).

26b Phenyl(methoxy-L-methioninyl) phosphorochloridate



$C_{12}H_{17}ClNO_4PS$

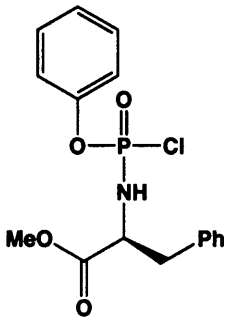
Mol Wt.: 337.76

Prepared according to Standard Procedure B, using phenyl dichlorophosphate (1.69 g, 8.01 mmol, 1.20 mL), L-methionine methyl ester hydrochloride (1.60 g, 8.01 mmol), TEA (1.62 g, 16.02 mmol, 2.23 mL) and anhydrous DCM (25 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (2.64 g, 97.4% yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 10.03,9.77.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.41-7.18 (5H, m, Ph), 5.12-5.02 (1H, m, NH), 4.31-4.25 (1H, m, *CH*NH), 3.81,3.79 (3H, 2s, OCH_3), 2.65-2.60 (2H, m, CH_2S), 2.23-1.95 (5H, m, CH_2CH_2S, SCH_3).

26c Phenyl(methoxy-L-phenylalaninyl) phosphorochloridate



$C_{16}H_{17}ClNO_4P$

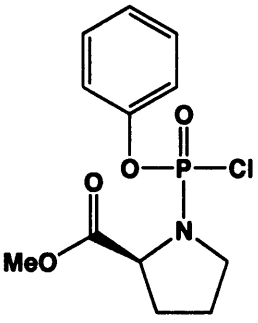
Mol Wt.: 353.74

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (3.37 g, 16.00 mmol, 2.39 mL), L-phenylalanine methyl ester hydrochloride (3.45 g, 16.00 mmol), TEA (3.24 g, 32.00 mmol, 4.46 mL) in anhydrous DCM (50 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (3.36 g, 59.4% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.53,9.31.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.43-7.12 (10H, m, PhO, CH_2*Ph*), 4.75-4.64 (1H, m, NH), 4.54-4.39 (1H, m, *CH*NH), 3.77,3.75 (3H, 2 s, OCH_3), 3.18 (2H, d, *CH_2*Ph).

26d Phenyl(methoxy-L-prolinyl) phosphorochloridate



$C_{12}H_{17}ClNO_4P$

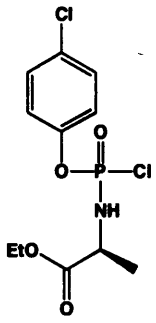
Mol Wt.: 303.68

Prepared according to Standard Procedure B, from phenyl dichlorophosphate (1.27 g, 6.04 mmol, 0.90 mL), L-proline methyl ester hydrochloride (1.00 g, 6.04 mmol), TEA (1.22 g, 12.08 mmol, 1.68 mL) and anhydrous DCM (20 mL). After 1 h the reaction was left to rise to room temperature and stirred overnight. The crude product was obtained as an oil (1.12 g, 61.0% yield), which was used without further purification.

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 9.20,8.98.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.32-7.11 (5H, m, PhO), 4.52-4.43 (1H, m, H-2 *prol*), 3.62 (3H, s, OCH_3), 3.50-3.30 (2H, m, H-5 *prol*), 2.21-1.86 (4H, m, H-3 *prol*, H-4 *prol*).

29 4-chloro-phenyl(ethoxy-L-alaninyl) phosphorochloridate



$C_{11}H_{14}Cl_2NO_4P$

Mol Wt.: 326.11

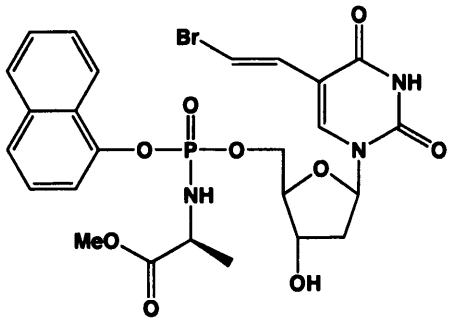
Prepared according to Standard Procedure B, using 4-chloro-phenyl phosphodichloridate (1.91 g, 7.77 mmol), L-alanine ethyl ester hydrochloride (1.20 g, 7.77 mmol), TEA (1.57 g, 15.56 mmol, 2.17 mL) and anhydrous DCM (30 mL). After 15 min the reaction was left to rise to room temperature and stirred for 5 h. The crude was purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil (0.82 g, 32.4% yield).

^{31}P-NMR ($CDCl_3$, 250 MHz): δ 8.53,8.33.

^{1}H-NMR ($CDCl_3$, 250 MHz): δ 7.18 (4H, AB system, pCl-Ph), 4.81-4.70 (1H, m, CH*NH*), 4.21-3.99 (3H, m, *CH*NH, OCH_2CH_3), 1.41,1.40 (3H, 2d, ^{3}J=7.0 Hz, $CHCH_3$), 1.22-1.11 (3H, m, OCH_2CH_3).

Phosphoramidates

1a BVdU-5'-[1-naphthyl(methoxy-L-alaninyl)] phosphate



$C_{25}H_{27}BrN_3O_9P$
Mol Wt.: 624.37

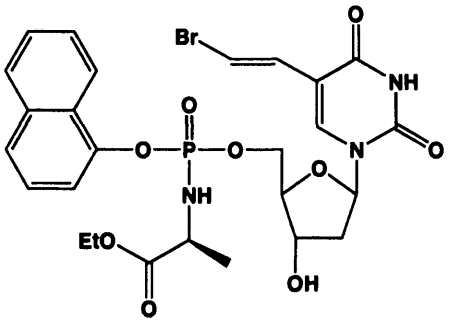
Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 1-naphthyl(methoxy-L-alaninyl) phosphorochloridate (**8a**) (0.62 g, 1.89 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL). The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 95/5) to give the pure product as a white foamy solid (48 mg, 10.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.88, 4.80.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 10.01 (1H, bs, H-3), 8.13 (1H, m, H-8 Naph), 7.89 (1H, m, H-5 Naph), 7.71 (1H, d, H-4 Naph), 7.65-7.53 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.47-7.36 (2H, m, H-6, ^{3}J=13.6 Hz, H-5b), 6.72,6.65 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.31-6.22 (1H, m, H-1'), 4.65-4.34 (5H, m, H-3', H-4', H-5', CH*NH*), 4.22-4.10 (2H, m, OH-3', *CH*CH_3), 3.72,3.67 (3H, 2s, OCH_3), 2.42-2.40 (1H, m, one of H-2'), 1.97-1.93 (1H, m, one of H-2'), 1.41,1.39 (3H, d, ^{3}J=6.9 Hz, CH*CH_3*).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 21.2,21.3 (*CH_3*CH), 40.6,40.7 (C-2'), 50.8,50.9 (*CH*CH_3), 53.1 (CH_3O), 66.6 (C-5'), 71.0,71.3 (C-3'), 85.5,85.8,86.1 (C-1', C-4'), 110.4,110.5 (C-5b), 111.9 (C-5), 115.6, 115.8 (C-2 Naph), 121.5,125.7,125.9,126.0, 127.7,126.8,127.0,127.1, 127.2, 127.3,128.4,128.8(C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 135.2 (C-4a Naph), 137.8,138.0 (C-6), 146.5,146.6,146.7 ('ipso' Naph), 149.6 (C-4), 161.8 (C-2), 174.4,174.5,174.6 (*C*$OOCH_3$).

1b BVdU-5'-[1-naphthyl(ethoxy-L-alaninyl)] phosphate



$C_{26}H_{29}BrN_3O_9P$

Mol Wt.: 638.40

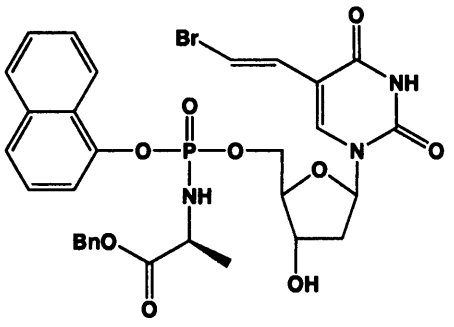
Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 1-naphthy(ethoxy-L-alaninyl) phosphorochloridate (**8b**) (0.64 g, 1.89 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL) for 6h. The crude was purified by column chromatography (DCM/MeOH 97/3) to give the pure product as a white foamy solid (10 mg, 2.1% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.97, 4.89.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.03-8.00 (1H, m, H-8 Naph), 7.79-7.76 (1H, m, H-5 Naph), 7.61-7.59 (1H, m, H-4 Naph), 7.52-7.42 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.35-7.28 (2H, m, H-5b, H-6), 6.61,6.54 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.23-6.18 (1H, m, H-1'), 4.43-4.26 (4H, m, H-3', H-4', H-5'), 4.18-3.95 (5H, m, *CH*CH_3, O*CH*$_2$$CH_3$, OH-3', CH*NH*), 2.38-2.35 (1H, m, one of H-2'), 1.95-1.86 (1H, m, one of H-2'), 1.43-1.20 (6H, m, CH*CH*$_3$, OCH_2*CH*$_3$).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 13.0 (*CH*$_3$$CH_2O$), 19.8,19.9 (*CH*$_3$CH), 39.1,39.3 (C-2'), 49.4,49.5 (*CH*CH_3), 60.8,60.9 (CH_3*CH*$_2$O), 65.2 (C-5'), 69.6,69.9 (C-3'), 84.0, 84.1,84.2,84.4,84.6 (C-1', C-4'), 109.0 (C-5b), 110.5 (C-5), 114.1,114.2,114.3 (C-2 Naph), 120.1,124.2,124.5,125.2,125.3,125.5,125.6,125.7,125.8,127.0,127.3,127.4 (C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 133.7 (C-4a Naph), 136.4, 136.5 (C-6), 145.1, 145.2, 145.3 ('ipso' Naph), 148.3 (C-4), 160.4, 160.5 (C-2), 172.5, 172.6, 172.7 (*C*OOCH_2CH_3).

1c BVdU-5'-[1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{31}H_{31}BrN_3O_9P$

Mol Wt.: 700.47

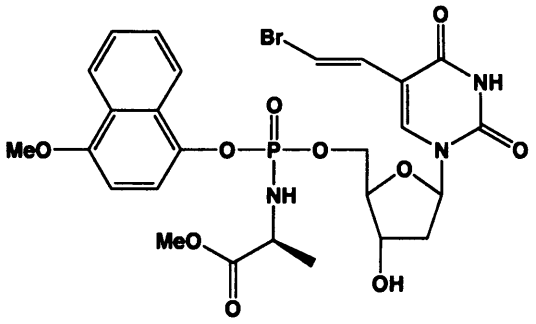
Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8c**) (0.73 g, 1.89 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL). The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 95/5) to give the pure product as a white foamy solid (18 mg, 3.4% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.93, 4.76.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.65 (1H, bs, H-3), 8.17-814 (1H, m, H-8 Naph), 7.94-7.90 (1H, m, H-5 Naph), 7.77-7.73 (1H, m, H-4 Naph), 7.64-7.57 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.50-7.34 (7H, m, H-5b, H-6, Ph), 6.76,6.70 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.32-6.22 (1H, 2t, ^{3}J=6.6 Hz, H-1'), 5.22-5.08 (2H, m, PhCH_2), 4.56-4.18 (7H, m, H-3', H-4', H-5', CHCH$_3$, OH-3', CH*NH*), 2.44-2.37 (1H, m, one of H-2'), 2.01-1.86 (1H, m, one of H-2'), 1.47-1.43 (3H, m, CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 19.7,19.8 (CH_3CH), 39.1,39.2 (C-2'), 49.5,49.6 (CHCH$_3$), 65.1 (C-5'), 66.4 (CH_2Ph), 69.5,69.8 (C-3'), 83.9,84.0,84.1,84.4,84.6 (C-1', C-4'), 109.0, 109.1 (C-5b), 110.5 (C-5), 114.2,114.3 (C-2 Naph), 120.1,124.3, 124.4,124.5,125.3,125.6,125.7,125.8,127.0,127.2,127.4,127.5,127.6,127.7 (C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, CH$_2$*Ph*), 133.7,134.0 (C-4aNaph,'ipso' Ph), 136.5 (C-6), 145.0,145.1,145.2 ('ipso' Naph), 148.1(C-4), 160.3 (C-2), 172.3,172.4,172.5 (*C*OOCH$_2$Ph).

1d BVdU-5'-[4-methoxy-1-naphthyl(methoxy-L-alaninyl)] phosphate



$C_{26}H_{29}BrN_3O_{10}P$

Mol Wt.: 654.40

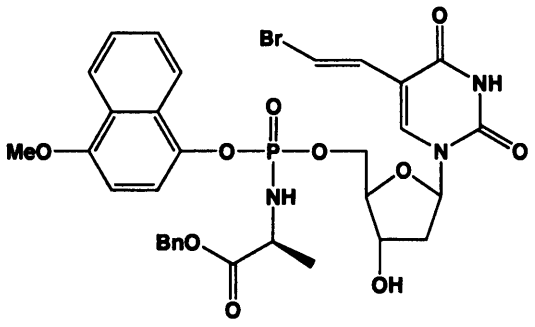
Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 4-methoxy-1-naphthyl(methoxy-L-alaninyl) phosphorochloridate (**8d**) (0.86 g, 2.40 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (6.5 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.15 g, 37.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.30.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 10.30 (1H, bs, H-3), 8.25-8.01 (2H, m, H-5 Naph, H-8 Naph), 7.64-7.36 (5H, m, H-2 Naph, H-6 Naph, H-7 Naph, H-6, H-5b), 6.73-6.61 (2H, m, H-5a, H-3 Naph), 6.29-6.21 (1H, m, H-1'), 4.68-4.04 (7H, m, H-3', H-4', H-5', *CH*CH_3, CH*NH*, OH), 3.95 (3H, s, OCH_3 Naph), 3.68,3.62 (3H, 2s, CO_2CH_3), 2.38 (1H, m, one of H-2'), 1.96-1.89 (1H, m, one of H-2'), 1.37-1.33 (3H, m, CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 21.2 (CHCH_3), 40.6 (C-2'), 50.8 (*CH*CH_3), 53.0,53.1 (CO_2CH_3), 56.2 (OCH_3 Naph), 66.9 (C-5'), 71.2,72.4 (C-3'), 85.7,86.0,86.2 (C-1', C-4'), 103.2 (C-3 Naph), 110.1,110.2 (C-5b), 111.8,111.9 (C-5), 115.5,115.7 (C-2 Naph), 121.4,122.8,126.5,126.7,127.5,128.9,129.0 (C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 138.1 (C-6), 140.0,140.1,140.2 ('ipso' Naph), 149.9 (C-4), 153.2 (C-4 Naph), 174.5,174.6,174.7 ($COOCH_3$).

1e BVdU-5'-[4-methoxy-1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{32}H_{33}BrN_3O_{10}P$

Mol Wt.: 654.40

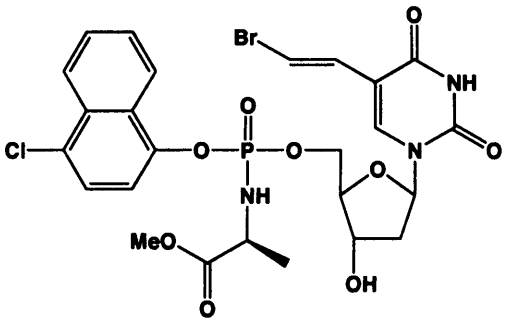
Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 4-methoxy-1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8e**) (1.30 g, 3.00 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (68 mg, 15.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 5.34,5.21.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.66 (1H, bs, H-3), 8.32-8.04 (2H, m, H-5 Naph, H-8 Naph), 7.64-7.32 (10H, m, H-2 Naph, H-6 Naph, H-7 Naph, H-5b, H-6, Ph), 6.79-6.68 (2H, m, ^{3}J=13.9 Hz, H-5a, H-3 Naph), 6.28,6.20 (1H, 2t, ^{3}J=6.5 Hz, H-1'), 5.19-5.05 (2H, AB system, CH_2Ph), 4.55-4.11 (7H, m, H-3', H-4', H-5', CHCH$_3$, OH, CH*NH*), 4.02,4.01 (3H, 2s, OCH_3), 2.44-2.33 (1H, m, one of H-2'), 2.03-1.82 (1H, m, one of H-2'), 1.44-1.35 (3H, m, CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 19.7,19.8 (CHCH_3), 39.1 (C-2'), 49.5,49.6 (CHCH$_3$), 54.7 (OCH_3), 65.2 (C-5'), 66.4 (CH_2Ph,), 69.6,66.9 (C-3'), 84.1,84.2,84.4,84.6 (C-1', C-4'), 101.7,101.8 (C-3 Naph), 108.8,109.0 (C-5b), 110.4,110.5 (C-5b), 114.1,114.3 (C-5), 119.9,121.4,125.0,125.1,125.2,125.9,126.0,126.1,127.1,127.2,127.3,127.4, 127.6 (C-2 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, CH_2Ph, C-5a), 134.1 ('ipso' Ph), 136.4,136.6 (C-6), 138.5,138.6 ('ispo' Naph), 148.2,148.3 (C-4), 151.8 (C-4 Naph), 160.4,160.5 (C-2), 172.4,172.5, 172.6 (COOCH$_2$Ph).

1f BVdU-5'-[4-chloro-1-naphthyl(methoxy-L-alaninyl)] phosphate



$C_{25}H_{26}BrClN_3O_9P$

Mol Wt.: 658.82

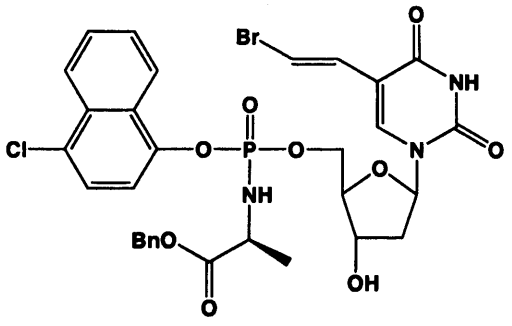
Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 4-chloro-1-naphthyl(methoxy-L-alaninyl) phosphorochloridate (**8f**) (1.63 g, 4.50 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (6.5 mL) for 20 h. The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (17 mg, 4.3% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.85,4.94.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.28-8.12 (2H, m, H-8 Naph, H-5 Naph), 7.69-7.51 (5H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-6), 7.41-7.35 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.72-6.60 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.26-6.21 (1H, m, H-1'), 4.54-4.08 (7H, m, H-3', H-4', H-5', *CH*CH_3, OH, CH*NH*), 3.71,3.65 (3H, 2s, OCH_3), 2.45-2.42 (1H, m, one of H-2'), 2.07-2.01 (1H, m, one of H-2'), 1.39-1.32 (3H, m, CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 21.2,21.3 (CHCH_3), 40.7 (C-2'), 50.8,50.9 (*CH*CH_3), 53.1,53.2 (OCH_3), 66.8 (C-5'), 71.1-71.3 (C-3'), 85.5,86.0,86.3 (C-1',C-4'), 110.5 (C-5b), 112.0 (C-5), 122.1 (C-2 Naph), 125.3,126.0,127.7,127.8,128.3,128.6,128.7, 128.9,132.1 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 137.8,138.0 (C-6), 145.5,145.6,145.7 ('ipso' Naph), 149.7 (C-4), 161.7,161.8 (C-2), 174.3, 174.4,174.5,174.6 (*C*$OOCH_3$).

1g BVdU-5'-[4-chloro-1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{31}H_{30}BrClN_3O_9P$

Mol Wt.: 734.92

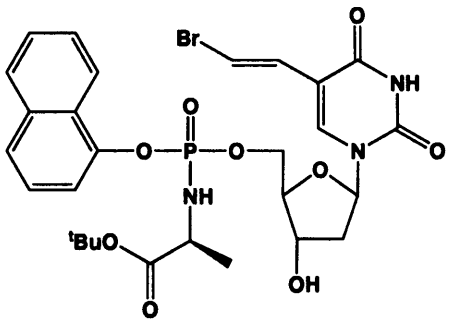
Prepared according to Standard Procedure C, from BVdU (0.31 g, 0.93 mmol), 4-chloro-1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8g**) (1.81 g, 4.14 mmol), NMI (0.37 g, 4.55 mmol, 0.36 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 97/3) to give the pure product as a white foamy solid (0.25 g, 36.8% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.90,4.80.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.25-8.10 (2H, m, H-5 Naph, H-8 Naph), 7.66-7.47 (5H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-6), 7.41-7.26 (6H, m, H-5b, Ph), 6.68,6.60 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.28-6.18 (1H, 2t, H-1'), 5.14-4.99 (2H, AB system, CH_2Ph), 4.73-4.12 (7H, m, H-3', H-4', H-5', CHCH$_3$, OH, CH*NH*), 2.42-2.39 (1H, m, one of H-2'), 2.02-1.94 (1H, m, one of H-2'), 1.39 (3H, d, ^{3}J=7.0 Hz, CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 21.2 (CHCH_3), 40.6 (C-2'), 50.9 (CHCH$_3$), 67.0,67.9 (CH_2Ph, C-5'), 71.2-71.4 (C-3'), 85.5,86.0,86.2 (C-1',C-4'), 110.4 (C-5b), 111.9,112.0 (C-5), 115.7 (C-2 Naph), 122.1,125.2,126.0,127.8,128.3,128.6,128.7, 128.8,128.9,129.0,129.1 132.0 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, CH$_2$*Ph*), 138.0 (C-6), 145.5 ('ipso' Naph), 149.8 (C-4), 162.0 (C-2), 173.9 (*C*OOCH$_2$Ph).

1h BVdU-5'-[1-naphthyl(tert-butoxy-L-alaninyl)] phosphate



$C_{24}H_{24}BrN_3O_9P$
Mol Wt.: 666.45

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(tert-butoxy-L-alaninyl) phosphorochloridate (**8h**) (0.67 g, 1.8 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.10 g, 25.0% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 5.86,5.83.

^{1}H-NMR (MeOD, 300 MHz): δ 8.16-8.12 (1H, m, H-8 Naph), 7.89-7.84 (1H, m, H-5 Naph), 7.71-7.66 (2H, m, H-4 Naph, H-6), 7.57-7.45 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.30,7.28 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.70 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.20,6.16,1.12,6.10 (1H, 4d, ^{3}J=7.8 Hz, H-1'), 4.45-4.28 (3H, m, H-3', H-5'), 4.17-4.10 (1H, m, H-4'), 3.96-3.88 (1H, m, *CH*NH), 2.23-2.12 (1H, m, one of H-2'), 1.89-1.64 (1H, m, one of H-2'), 1.44,1.40 (9H, 2s, tertbutyl), 3.31-3.36 (3H, m, CH*CH$_3$*)

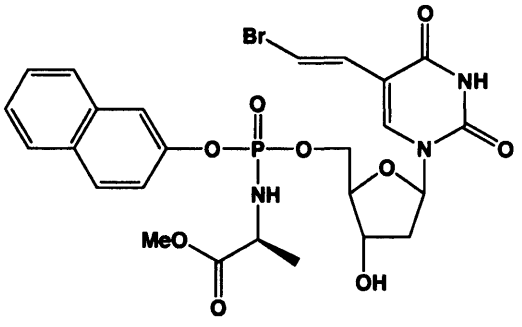
^{13}C-NMR (MeOD, 75 MHz): δ 21.1,21.2 (CH*CH$_3$*), 28.6 ($CO_2C(CH_3)_3$), 41.5,41.7 (C-2'), 52.8 (NHCH), 68.3,68.4 (C-5'), 72.7 (C-3'), 83.1,83.2 ($CO_2C(CH_3)_3$), 87.2,87.3,87.6 (C-1', C-4'), 109.6,109.7 (C-5b), 112.6 (C-5), 116.7,117.0 (C-2 Naph), 123.0,123.1,126.6, 126.7,126.9,127.9,128.3,129.3,129.4,130.8 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a), 136.7 (C-4a Naph), 139.9,140.0 (C-6), 148.2,148.4 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 174.6,174.8 (*C*OOCHCH$_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 11.56 min.

ESI MS m/z (positive) 666 [M + H], 668 [M(^{81}Br) + H], 688 [M + Na], 690 [M(^{81}Br) + Na].

Anal. Calcd for $C_{28}H_{33}BrN_3O_9P$: C, 50.45; H, 4.95; N, 6.30. Found: C, 50.23; H, 4.62; N, 6.05.

2a BVdU-5'-[2-naphthyl(methoxy-L-alaninyl)] phosphate



$C_{25}H_{27}BrN_3O_9P$

Mol Wt.: 624.37

Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 2-naphthyl(methoxy-L-alaninyl) phosphorochloridate (**9a**) (0.62 g, 1.89 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL). The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 95/5) to give the pure product as a white foamy solid (25 mg, 5.3% yield).

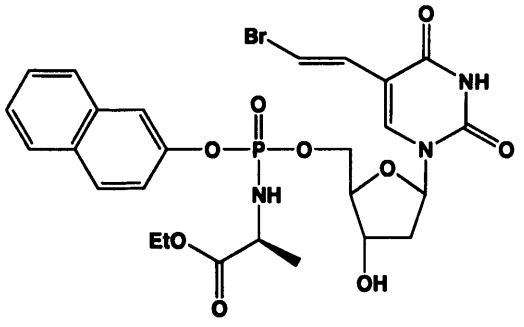
^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.92,4.62.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.21 (1H, bs, H-3), 7.88-7.38 (8H, m, *Naph*, H-6), 7.51,7.45 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.76 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.31-6.22 (1H, 2t, H-1'), 4.63-4.00 (6H, m, H-3', H-4', H-5', *CH*CH_3, CH*NH*), 3.72,3.71 (3H, 2s, OCH_3, OH-3'), 2.51-2.39 (1H, m, one of H-2'), 2.14-2.08 (1H, m, one of H-2'), 1.43 (3H, d, ^{3}J=6.5 Hz CH*CH$_3$*).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 19.8 (*CH$_3$*CH), 39.2,39.4 (C-2'), 49.3,49.4 (*CH*CH_3), 51.7 (CH_3O), 65.0 (C-5'), 69.1,69.6 (C-3'), 84.0,84.2,84.6 (C-1', C-4'), 109.0,109.1 (C-5b), 110.5 (C-5), 115.7,115.8,118.8,119.0 (C-1 Naph, C-3 Naph), 124.7,125.9, 126.4,126.5,126.7, 127.4,129.1 (C-4 Naph, C-5 Naph, C-5a, C-6 Naph, C-7 Naph, C-

8 Naph), 130.0,132.7 (C-4a Naph, C-8a Naph), 136.4,136.5 (C-6), 146.7,146.8 ('ipso' Naph), 148.0 (C-4), 160.2 (C-2), 173.0,17316 (COOCH$_3$).

2b BVdU-5'-[2-naphthyl(ethoxy-L-alaninyl)] phosphate



$C_{26}H_{29}BrN_3O_9P$
Mol Wt.: 638.40

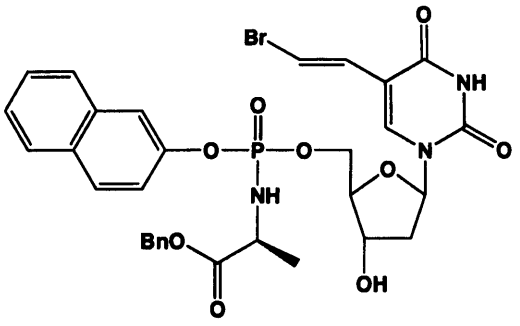
Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 2-naphthyl(ethoxy-L-alaninyl) phosphorochloridate (**9b**) (0.96 g, 2.83 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL) for 19 h. The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (64 mg, 13.4% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.99,4.68.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.87-7.34 (8H, m, *Naph*, H-6), 7.49,7.44 (1H, 2d, ^{3}J=14.0 Hz, H-5b), 6.75 (1H, t, ^{3}J=14.0 Hz, H-5a), 6.32-6.23 (1H, m, ^{3}J=6.6 Hz, H-1'), 4.63-4.02 (9H, m, H-3', H-4', H-5', OCH_2CH$_3$, CHCH$_3$, CH*NH*, OH), 2.48-2.37 (1H, m, one of H-2'), 2.14-2.05 (1H, m, one of H-2'), 1.42 (3H, d, ^{3}J=6.6 Hz, CHCH_3), 1.25,1.24 (3H, 2t, ^{3}J=7.1 Hz, OCH$_2CH_3$).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 14.46 (OCH$_2CH_3$), 21.29 (CH_3CH), 40.7,40.8 (C-2'), 50.8,50.9 (CHCH$_3$), 62.0,62.2,62.3 (OCH_2CH$_3$), 66.4,66.7 (C-5'), 70.9,71.3 (C-3'), 85.6,86.1 (C-1', C-4'), 110.3 (C-5b), 111.9 (C-5), 117.1,117.2,117.3,120.5 (C-1 Naph, C-3 Naph), 125.8,126.1,127.3,127.8,127.9,128.1,128.9,130.5 (C-4 Naph, C-5 Naph, C-5a, C-6 Naph, C-7 Naph, C-8 Naph, Ph), 131.4,134.2 (C-4a Naph, C-8a Naph), 138.1 (C-6), 148.2,148.3,149.9 ('ipso' Naph, C-4), 162.1,162.2 (C-2), 174.1,174.2 (COOCH$_2$CH$_3$).

2c BVdU-5'-[2-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{31}H_{31}BrN_3O_9P$

Mol Wt.: 700.47

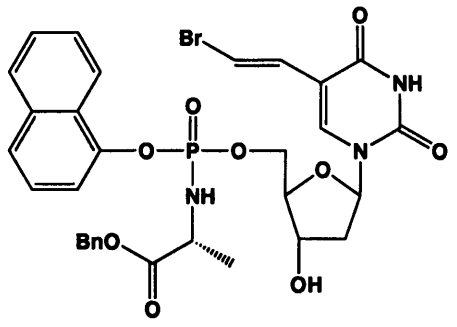
Prepared according to Standard Procedure C, from BVdU (0.25 g, 0.75 mmol), 2-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**9c**) (1.25 g, 3.22 mmol), NMI (0.31 g, 3.75 mmol, 0.30 mL) and anhydrous THF (6.3 mL) for 21 h. The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the pure product as a white foamy solid (48 mg, 9.1% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.91,4.61.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.82-7.27 (14H, m, Naph, Ph, H-6, H-5b), 6.76 (1H, m, ^{3}J=13.8 Hz, H-5a), 6.76,6.22 (1H, 2t, H-1'), 5.18-5.06 (2H, m, PhCH_2), 4.60-4.08 (6H, m, H-3', H-4', H-5', *CH*CH_3, CH*NH*), 2.44-2.39 (1H, m, one of H-2'), 2.07-2.02 (1H, m, one of H-2'), 1.41 (3H, d, ^{3}J=7.0 Hz CHCH_3).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 21.2 (CHCH_3), 40.8 (C-2'), 50.9,51.0 (*CH*CH_3), 66.4,66.6 (C-5'), 67.8 (*CH*$_2$Ph), 70.8,71.2 (C-3'), 85.7 (C-1',C-4'), 110.3 (C-5b), 111.9 (C-5), 117.2,120.3,120.4 (C-2 Naph, C-3 Naph), 134.4,130.5,129.1,129.0, 128.9,128.6,128.2,27.9, 27.3,26.1 (C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-5a, CH_2*Ph*), 134.2,135.5 (C-4a Naph, C-8a Naph), 138.0 (C-6), 148.2, 148.3,149.8 ('ipso' Naph, C-4), 162.0 (C-2), 173.9 (*C*OOCH_2Ph).

10a BVdU-5'-[1-naphthyl(benzoxy-D-alaninyl)] phosphate



$C_{31}H_{31}BrN_3O_9P$
Mol Wt.: 700.47

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(benzoxy-D-alaninyl) phosphorochloridate (**11a**) (1.25 g, 3.22 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.16 g, 37.6% yield). The diastereoisomeric mixture was separated through the application of preparative reverse phase HPLC, using water/acetonitrile 67/33 as an isocratic eluting system.

R,S-mixture

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.91,3.47.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.90 (1H, bs, H-3), 7.99-7.95 (1H, m, H-8 Naph), 7.76-7.73 (1H, m, H-5 Naph), 7.58,7.57 (1H, 2d, ^{3}J=8.2 Hz, H-4 Naph), 7.52 (0.5H, s, H-6 of one diastereoisomer), 7.45-7.17 (10.5H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*, H-5b, H-6 of one diastereoisomer), 6.62 (0.5H, d, ^{3}J=13.6 Hz, H-5a of one diastereoisomer), 6.50 (0.5H, d, ^{3}J=13.6 Hz, H-5a of one diastereoisomer), 6.09 (1H, m, H-1'), 5.05 (1H, s, CH_2Ph, of one diastereoisomer), 5.02,4.96 (1H, AB system, ^{2}J=12.3 Hz, CH_2Ph of one diastereoisomer), 4.35-3.70 (7H, m, H-3', H-4', H-5', OH-3', *CH*NH, CH*NH*), 2.23-2.15 (1H, m, one of H-2'), 1.83-1.78 (0.5H, m, one of H-2' of one diastereoisomer), 1.73-1.67 (0.5H, m, one of H-2' of one diastereoisomer), 1.27 (3H, m, CHCH_3).

^{13}C-NMR (MeOD, 75 MHz): δ 20.7,20.8 (CH_3), 41.6,41.7 (C-2'), 52.2,52.3 (NHCH), 68.1,68.2,68.3,68.4,68.5,68.6 (C-5', CH_2Ph), 72.5,72.7 (C-3'), 87.2,87.4, 87.5 (C-1', C-4'), 109.6,109.7 (C-5b), 112.6 (C-5), 116.5 (C-2 Naph), 122.7,123.1, 126.6,126.9,127.9,128.1,128.3,129.3,129.4,129.7,129.8,129.9,130.0,130.9 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a, Ph), 136.7 (C-4a Naph), 137.5 ('ipso' Ph), 139.8,140.0 (C-6), 148.1,148.2 ('ipso' Naph), 151.3 (C-4), 163.8,163.9 (C-2), 174.9,175.0, 175.4,175.5 (*C*OOCH$_2$Ph).
HPLC (67/33 water/acetonitrile v/v, isocratic): Rt 29.01, 31.49 min.
ESI MS (positive): 700 [M + H], 722 [M + Na].

R diastereoisomer

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 4.01,**3.53** (int. 1.0/8.2).
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.35 (1H, bs, H-3), 7.98-7.96 (1H, m, H-8 Naph), 7.77-7.75 (1H, m, H-5 Naph), 7.59 (1H, d, ^{3}J=8.3 Hz, H-4 Naph), 7.46-7.25 (11H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*, H-5b, H-6), 6.64 (0.1H, d, ^{3}J=13.6Hz, H-5a of the minor diastereoisomer), 6.52 (0.9H, d, ^{3}J=13.6 Hz, H-5a), 6.08 (1H, t, ^{3}J=6.5 Hz, H-1'), 5.05 (1.8H, s, CH_2Ph), 5.07,5.04 (0.2H, AB system, ^{2}J=12.2 Hz, CH_2Ph of the minor diastereoisomer), 4.35-3.83 (6H, m, H-3', H-4', H-5', *CH*NH, CH*NH*), 2.82 (1H, bs, OH-3'), 2.20-2.16 (1H, m, one of H-2'), 1.76-1.70 (1H, m, one of H-2'), 1.27 (3H, d, ^{3}J=7.0 Hz, CHCH_3).
HPLC (67/33 water/acetonitrile v/v, isocratic): Rt29.01 min.
ESI MS (positive): 700 [M + H], 722 [M + Na].

S diastereoisomer

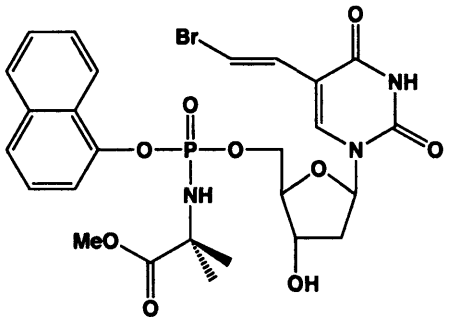
^{31}P-NMR ($CDCl_3$, 202 MHz): δ **3.97**,3.51 (int. 2.8/1.0).
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.64 (1H, bs, H-3), 7.99-7.96 (1H, m, H-8 Naph), 7.77-7.74 (1H, m, H-5 Naph), 7.58 (1H, d, ^{3}J=8.2 Hz, H-4 Naph), 7.52 (1H, s, H-6), 7.46-7.17 (10H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, *Ph*, H-5b), 6.64 (0.7H, d, ^{3}J=13.6 Hz, H-5a), 6.51 (0.3H, d, ^{3}J=13.6 Hz, H-5a of the minor

diastereoisomer), 6.10-6.06 (1H, m, H-1'), 5.05 (0.6H, s, CH_2Ph of the minor diastereoisomer), 5.03,4.97 (1.4H, AB system, ^{2}J=12.2 Hz, CH_2Ph), 4.33-3.94 (5H, m, H-3', H-4', H-5', *CH*NH), 3.62 (1H, bs, CH*NH*), 3.13 (1H, bs, OH-3'), 2.25-2.20 (0.7H, m, one of H-2'), 2.19-2.16 (0.3H, m, one of H-2' of the minor diastereoisomer), 1.86-1.80 (0.7H, m, one of H-2'), 1.75-1.69 (0.3H, m, one of H-2' of the minor diastereoisomer), 1.26 (3H, d, ^{3}J=7.0 Hz, CHCH_3).

HPLC (67/33 water/acetonitrile v/v, isocratic): Rt 31.49 min.

ESI MS (positive): 700 [M + H], 722 [M + Na].

10bBVdU-5'-[1-naphthyl(methoxy-dimethylglycinyl)] phosphate



$C_{26}H_{29}BrN_3O_9P$

Mol Wt.: 638.40

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-dimethylglycinyl) phosphorochloridate (**11b**) (0.62 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (25 mg, 6.5% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 3.60, 3.50.

^{1}H-NMR (MeOD, 300 MHz): δ 8.15-8.12 (1H, m, H-8 Naph), 7.83-7.79 (1H, m, H-5 Naph), 7.64 (1H, s, H-6), 7.61 (1H, d, H-4 Naph), 7.51-7.42 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.38-7.32 (1H, m, H-3 Naph), 7.25,7.22 (1H, 2d, ^3J=13.6 Hz, H-5b), 6.66,6.60 (1H, 2d, ^3J=13.6 Hz, H-5a), 6.17-6.08 (1H, m, H-1'), 4.38-4.29 (3H, m, H-3', H-5'), 4.09-4.05 (1H, m, H-4'), 3.64,3.63 (3H, 2s, OCH_3), 2.18-2.09 (1H, m, one of H-2'), 1.93-1.80 (1H, m, one of H-2'), 1.46-1.45 (6H, 2s, $C(CH_3)_2$).

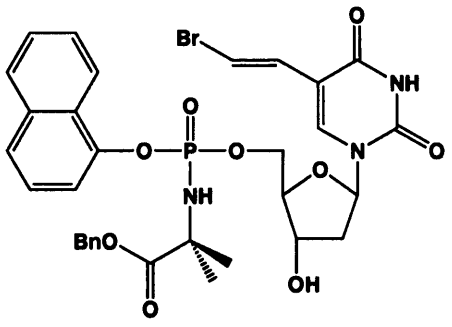
^{13}C-NMR (MeOD, 75 MHz): δ 27.9,28.0,28.3,28.4,28.5 (C(CH_3)$_2$), 41.4,41.6 (C-2'), 53.5 (CH_3O), 58.7 (C(CH_3)$_2$), 68.3,68.4 (C-5'), 72.6,72.7 (C-3'), 87.2,87.3,87.5 (C-1', C-4'), 109.5,109.6 (C-5b), 112.6 (C-5), 116.8,117.2 (C-2 Naph), 123.3,126.4, 126.5,126.8,126.9,127.8,128.2,128.3,128.4,129.3,130.8,130.9 (C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.7 (C-4a Naph), 140.1 (C-6), 148.2,148.3,148.4,148.5 ('ipso' Naph), 151.3 (C-4), 163.9,164.0 (C-2), 177.7,177.8 (COOCH$_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.57 min.

ESI MS (positive): 640 [M + H], 662 [M + Na].

Anal. Calcd for $C_{26}H_{29}BrN_3O_9P$: C, 48.91; H, 4.95; N, 6.58. Found: C, 49.14; H, 4.24; N, 6.35.

10c BVdU-5'-[1-naphthyl(benzoxy-dimethylglycinyl)] phosphate



$C_{32}H_{33}BrN_3O_9P$

Mol Wt.: 714.50

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(benzoxy-dimethylglycinyl) phosphorochloridate (**11c**) (0.77 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.18 g, 41.9% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 4.29, 4.22.

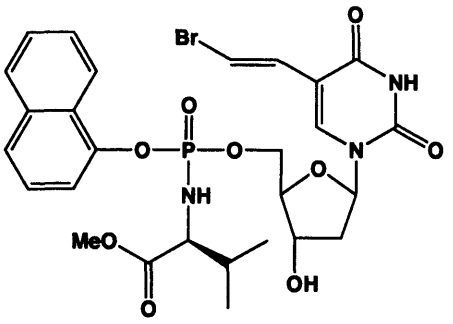
^{1}H-NMR (MeOD, 300 MHz): δ 8.19-8.15 (1H, m, H-8 Naph), 7.90-7.81 (1H, m, H-5 Naph), 7.69-7.61 (2H, m, H-4 Naph, H-6), 7.56-7.25 (10H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b, *Ph*), 6.72,6.65 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.20-6.11 (1H, m, H-1'), 5.20-5.09 (2H, m, CH_2Ph), 4.39-4.24 (3H, m, H-3', H-5'), 4.08-4.02 (1H, m, H-4'), 2.20-2.11 (1H, m, one of H-2'), 1.92-1.73 (1H, m, one of H-2'), 1.52-1.50 (6H, 2s, $C(CH_3)_2$).

^{13}C-NMR (MeOD, 75 MHz): δ 28.0,28.4,28.5,28.6 ($C(CH_3)_2$), 41.5,41.6 (C-2'), 58.7 ($C(CH_3)_2$), 68.3,68.4,68.8,69.3 (C-5', CH_2Ph), 72.5,72.6 (C-3'), 87.1,87.2,87.4,87.5 (C-1', C-4'), 109.8 (C-5b), 112.6 (C-5), 116.8,117.2 (C-2 Naph), 123.3,126.5,126.6, 127.0,127.9,128.2,128.3,129.4,129.7,130.0,130.8,130.9 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a, *Ph*), 136.6 (C-4a Naph), 137.6 ('ipso' Ph), 140.1 (C-6), 148.35 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 177.0,177.1 ($COOCH_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 13.04 min.

ESI MS (positive): 714 [M + H], 736 [M + Na].

10d BVdU-5'-[1-naphthyl(methoxy-L-valinyl)] phosphate



$C_{27}H_{31}BrN_3O_9P$

Mol Wt.: 652.43

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-L-valinyl) phosphorochloridate (**11d**) (0.65 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.16 g, 39.8% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 6.76,6.54.

^{1}H-NMR (MeOD, 300 MHz): δ 8.19-8.11 (1H, m, H-8 Naph), 7.87-7.81 (1H, m, H-5 Naph), 7.70-7.64 (2H, m, H-4 Naph, H-6), 7.54-7.37 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.31,7.29 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.70 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.21-6.11 (1H, m, H-1'), 4.48-4.28 (3H, m, H-3', H-5'), 4.17-4.10 (1H, m, H-4'), 3.81-3.71 (1H, m, *CH*NH), 4.61,4.59 (3H, 2s, CH_3O), 2.28-2.17 (1H, m, *CH*(CH_3)$_2$), 2.10-1.79 (2H, m, H-2'), 0.91-0.82 (6H, m, CH(*CH_3*)$_2$).

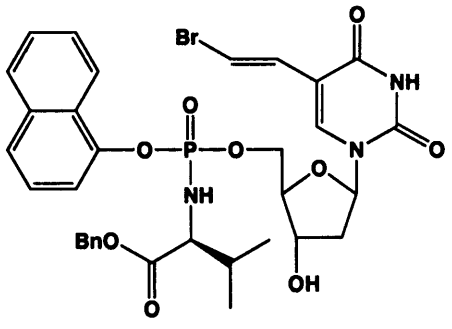
^{13}C-NMR (MeOD, 75 MHz): δ 18.5,18.8,19.9,20.0 (CH(*CH_3*)$_2$), 33.3,33.4,33.6,33.7 (*CH*(CH_3)$_2$), 41.5,41.7 (C-2'), 52.9 (CH_3O), 62.4 (NH*CH*), 68.4 (C-5'), 72.5,72.6 (C-3'), 87.1,87.2,87.3,87.4,87.5 (C-1', C-4'), 109.6,109.7 (C-5b), 112.6 (C-5), 116.8,116.9 (C-2 Naph), 123.0,123.1,126.6,126.9,127.9,128.2,128.3,129.3,129.4, 130.8 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a), 136.7 (C-4a Naph), 140.0 (C-6), 148.1,148.3 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 174.9,175.2 (*CO_2CH_3*).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.91 min.

ESI MS (positive): 652 [M + H], 674 [M + Na].

Anal. Calcd for $C_{27}H_{31}BrN_3O_9P$: C, 49.70; H, 4.75; N, 6.44. Found: C, 50.00; H, 4.62; N, 6.25.

10e BVdU-5'-[1-naphthyl(benzoxy-L-valinyl)] phosphate



$C_{33}H_{35}BrN_3O_9P$
Mol Wt.: 728.52

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(benzoxy-L-valinyl) phosphorochloridate (**11e**) (0.78 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.12 g, 27.0% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 6.82, 6.48.

^{1}H-NMR (MeOD, 300 MHz): δ 8.19-8.12 (1H, m, H-8 Naph), 7.89-7.85 (1H, m, H-5 Naph), 7.70 (1H, d, ^{3}J=7.7 Hz, H-4 Naph), 7.66 (1H, s, H-6), 7.54-7.35 (5H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b), 7.32-7.27 (5H, m, Ph), 6.71,6.70 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.21-6.10 (1H, m, H-1'), 5.14-5.02 (2H, AB system, ^{2}J=12.2 Hz, CH_2Ph), 4.37-4.21 (3H, m, H-3', H-5'), 4.11-4.08 (1H, m, H-4'), 3.85-3.75 (1H, m, *CH*NH), 2.23-1.71 (3H, m, H-2', *CH*(CH_3)$_2$), 0.89-0.80 (6H, m, CH(*CH_3*)$_2$).

^{13}C-NMR (MeOD, 75 MHz): δ 18.5,18.9,19.9,20.0 (CH(*CH_3*)$_2$), 33.3,33.4,33.6,33.7 (*CH*(CH_3)$_2$), 41.5,41.7 (C-2'), 62.5,62.6 (NHCH), 68.3,68.4 (C-5', CH_2Ph), 72.5,72.6 (C-3'), 87.1,87.2,87.4,87.5 (C-1', C-4'), 109.6,109.9 (C-5b), 112.6 (C-5), 116.9 (C-2 Naph), 123.0,123.1,126.6,126.8,126.9,128.0,128.2,128.3,129.3,129.4,129.8,129.9, 130.0, 130.1 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a

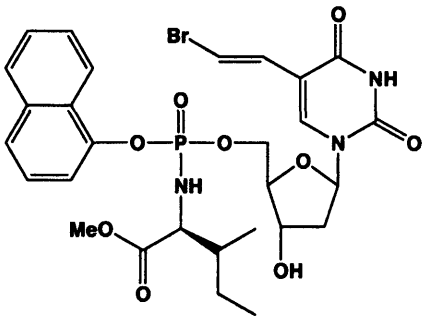
Naph, C-5a, *Ph*), 136.7 (C-4a Naph), 137.4 ('ipso' Ph), 139.9 (C-6), 148.1,148.3 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 174.2 (*C*OOCH$_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 12.31 min.

ESI MS (positive): 728 [M + H], 750 [M + Na], 766 [M + K].

Anal. Calcd for $C_{33}H_{35}BrN_3O_9P1.5H_2O$: C, 52.46; H, 5.07; N, 5.56. Found: C, 52.48; H, 4.41; N, 5.33.

10f BVdU-5'-[1-naphthyl(methoxy-L-isoleucinyl)] phosphate



$C_{28}H_{33}BrN_3O_9P$

Mol Wt.: 666.45

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-L-isoleucinyl) phosphorochloridate (**11f**) (0.67 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.19 g, 46.5% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 6.66,6.40.

^{1}H-NMR (MeOD, 300 MHz): δ 8.20-8.14 (1H, m, H-8 Naph), 7.89-7.86 (1H, m, H-5 Naph), 7.72-7.68 (2H, m, H-6, H-4 Naph), 7.57-7.28 (5H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b), 6.72 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.24-6.14 (1H, m, H-1'), 4.47-4.28 (3H, m, H-5', H-3'), 4.17-4.12 (1H, m, H-4'), 3.85-3.78 (1H, m, NH*CH*), 3.61,3.59 (3H, 2s, OCH_3), 2.25-2.18 (1H, m, one of H-2'), 1.98-1.70 (2H, m, one of H-2', NHCH*CH*), 1.51-1.37 (1H, m, one of CH_2CH_3), 1.18-1.09 (1H, m, one of CH_2CH_3), 0.89-0.78 (6H, m, $CHCH_3$, CH_2CH_3).

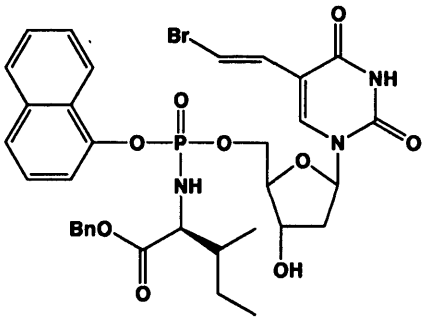
^{13}C-NMR (MeOD, 75 MHz): δ 12.0,12.1 (CH_2CH_3), 16.2,16.4 ($CHCH_3$), 26.3,26.4 (CH_2CH_3), 40.3 (NHCH*CH*), 41.5,41.7 (C-2'), 52.9 (CH_3O), 61.3,61.4 (NH*CH*), 68.3,68.4 (C-5'), 72.5,72.6 (C-3'), 87.1,87.2,87.5 (C-1', C-4'), 109.6,109.7 (C-5b), 112.6 (C-5), 116.9 (C-2 Naph), 123.0,123.1,126.6,126.9,127.9,128.3,129.3,130.8 (C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.7 (C-4a Naph), 140.0 (C-6), 148.2,148.3,148.4,148.5 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 175.5,175.6 ($COOCH_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 11.48 min.

ESI MS (positive): 666 [M + H], 688 [M + Na], 704 [M + K].

Anal. Calcd for $C_{28}H_{33}BrN_3O_9P$: C, 50.45 H, 4.95; N, 6.31. Found: C, 50.52; H, 4.69; N, 6.11.

10g BVdU-5'-[1-naphthyl(benzoxy-L-isoleucinyl)] phosphate



$C_{34}H_{37}BrN_3O_9P$

Mol Wt.: 742.55

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(benzoxy-L-isoleucinyl) phosphorochloridate (**11g**) (0.80 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 97/3) to give the pure product as a white foamy solid (52 mg, 11.7% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 6.01,5.58.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.08-8.03 (1H, m, H-8 Naph), 7.84-7.81 (1H, m, H-5 Naph), 7.67-7.62 (1H, m, H-4 Naph), 7.55-7.25 (11H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b, H-6, *Ph*), 6.67-6.62 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.21-6.12

(1H, m, H-1'), 5.14-4.93 (2H, m, CH_2Ph), 4.44-4.23 (2H, m, H-3', one of H-5'), 4.22-4.18 (1H, m, one of H-5'), 4.08-4.06 (1H, m, H-4'), 3.98-3.92 (1H, m, NH*CH*), 2.31-2.26 (1H, m, one of H-2'), 1.81-1.73 (2H, m, one of H-2', NHCH*CH*), 1.34-1.28 (1H, m, one of CH_2CH_3), 1.15-0.90 (1H, m, one of CH_2CH_3), 0.82-0.74 (6H, m, CHCH_3, CH_2CH_3).

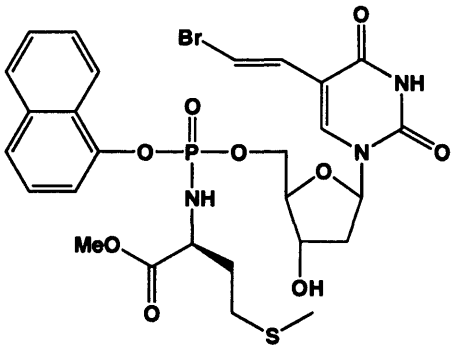
^{13}C-NMR (MeOD, 75 MHz): δ 11.8 (CH_2CH_3), 15.7,15.8 (CHCH_3), 24.8,25.0 (CH_2CH_3), 39.2,39.3,39.5 (NHCH*CH*), 40.6,40.7 (C-2'), 59.6,59.8,60.2 (NH*CH*), 66.6 (C-5'), 67.4,67.7 (CH_2Ph), 71.0,71.4 (C-3'), 85.4,85.5,85.6,85.7,85.9 (C-1', C-4'), 110.5,110.6 (C-5b), 111.9 (C-5), 115.8 (C-2 Naph), 121.5,121.6,125.6,125.7, 125.9,126.0, 126.7,126.9,127.2,128.4,128.7,128.9,129.0,129.1 (C-5a, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, *Ph*), 135.1,135.5 (C-4a Naph, 'ipso' Ph), 137.9 (C-6), 146.5,146.6,146.7 ('ipso' Naph), 149.6 (C-4), 161.8 (C-2), 173.1,173.2 (*C*OOCH$_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 12.96 min.

ESI MS (positive): 742 [M + H], 764 [M + Na], 780 [M + K].

Anal. Calcd for $C_{34}H_{37}BrN_3O_9P$: C, 54.99 H, 4.98; N, 5.66. Found: C, 54.81; H, 4.78; N, 5.52.

10h BVdU-5'-[1-naphthyl(methoxy-L-methioninyl)] phosphate



$C_{27}H_{31}BrN_3O_9PS$

Mol Wt.: 684.49

Prepared according to Standard Procedure C, from BVdU (0.19 g, 0.60 mmol), 1-naphthyl(methoxy-L-methioninyl) phosphorochloridate (**11h**) (0.59 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.10 mg, 24.4% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 6.18,5.82.

^{1}H-NMR (MeOD, 300 MHz): δ 8.19-8.12 (1H, m, H-8 Naph), 7.89-7.86 (1H, m, H-5 Naph), 7.72-7.68 (2H, m, H-4 Naph, H-6), 7.56-7.37 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.33,7.31 (1H, 2d, H-5b), 6.78-6.69 (1H, m, H-5a), 6.20-6.13 (1H, m, H-1'), 4.48-4.33 (3H, m, H-3', H-5'), 4.19-4.10 (2H, m, H-4', *CH*NH), 3.65,3.64 (3H, 2s, OCH_3), 2.47-2.33 (2H, m, CH_2S), 2.23-1.17 (1H, m, one of H-2'), 2.02-1.94 (5H, m, CH_2CH_2S, SCH_3), 1.91-1.80 (1H, m, one of H-2').

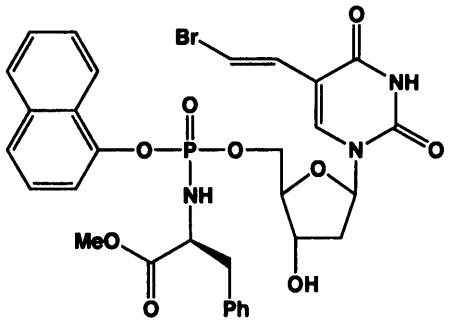
^{13}C-NMR (MeOD, 75 MHz): δ 15.5,15.6 (SCH_3), 31.3 (CH_2S), 34.0,34.1,34.5,34.6 (CH_2CH_2S), 41.6,41.7 (C-2'), 53.3 (OCH_3), 55.3,55.4 (NHCH), 68.4 (C-5'), 72.5,72.8 (C-3'), 87.1,87.2,87.4,87.5,87.6 (C-1', C-4'), 109.7 (C-5b), 112.6 (C-5), 116.9 (C-2 Naph), 123.0,126.7,126.9,128.0,128.2,128.3,129.4,130.8,130.9 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a), 136.7 (C-4a Naph), 140.0,140.1 (C-6), 148.2 ('ipso' Naph), 151.3 (C-4), 164.0 (C-2), 175.0,175.5 ($COOCH_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.73 min.

ESI MS (positive): 684 [M + H], 706 [M + Na], 722 [M + K].

Anal. Calcd for $C_{27}H_{31}BrN_3O_9PS$: C, 47.37 H, 4.52; N, 6.13. Found: C, 47.63; H, 4.38; N, 5.93.

10i BVdU-5'-[1-naphthyl(methoxy-L-phenylalaninyl)] phosphate



$C_{31}H_{31}BrN_3O_9P$
Mol Wt.: 700.47

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-L-phenylalaninyl) phosphorochloridate (**11i**) (0.73 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.18 g, 41.7% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 5.77,5.40.

^{1}H-NMR (MeOD, 300 MHz): δ 8.02-7.94 (1H, m, H-8 Naph), 7.79-7.75 (1H, m, H-5 Naph), 7.57 (1H, d, H-4 Naph), 7.50,7.51 (1H, 2s, H-6), 7.45-7.07 (10H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b, Ph), 6.57 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.12,6.09,6.03,6.01 (1H, 4d, ^{3}J=5.9 Hz, H-1'), 4.26-3.71 (5H, m, H-3', H-4', H-5', *CH*NH), 3.48,3.52 (3H, 2s, OCH_3), 3.09-2.98 (1H, m, one of CH_2Ph), 2.81-2.70 (1H, m, one of CH_2Ph), 2.13-2.01 (1H, m, one of H-2'), 1.80-1.68 (1H, m, one of H-2').

^{13}C-NMR (MeOD, 75 MHz): δ 41.2,41.3,41.5,41.8 (C-2', CH_2Ph), 53.2,54.2 (CH_3O), 58.3,58.5 (NHCH), 67.9,68.0,68.1 (C-5'), 72.6 (C-3'), 87.1,87.2,87.4,87.5 (C-1', C-4'), 109.6,109.8 (C-5b), 112.6 (C-5), 116.4,117.0 (C-2 Naph), 122.9,123.1,126.4, 126.6,126.9,127.9,128.2,128.4,129.3,130.0,130.8,131.0 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a, Ph), 136.6 (C-4a Naph),

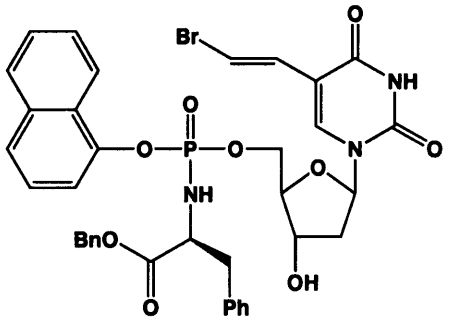
138.6 ('ipso' Ph), 139.8 (C-6), 148.2 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 174.8,175.0 ($COOCH_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 11.48 min.

ESI MS (positive): 700 [M + H], 722 [M + Na].

Anal. Calcd for $C_{31}H_{31}BrN_3O_9P$: C, 53.15; H, 4.43; N, 5.99. Found: C, 53.23; H, 4.31; N, 5.87.

10l BVdU-5'-[1-naphthyl(benzoxy-L-phenylalaninyl)] phosphate



$C_{37}H_{35}BrN_3O_9P$

Mol Wt.: 776.57

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(benzoxy-L-phenylalaninyl) phosphorochloridate (**11l**) (0.84 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (60 mg, 12.9% yield).

^{31}P-NMR (MeOD, 202 MHz): δ 4.59,4.34.

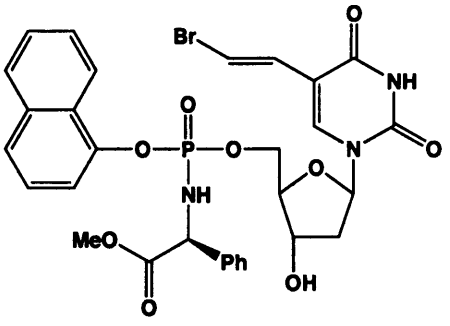
^{1}H-NMR (MeOD, 500 MHz): δ 8.07,8.03 (1H, 2d, H-8 Naph), 7.87-7.83 (1H, m, H-5 Naph), 7.66 (1H, d, H-4 Naph), 7.57 (1H, s, H-6), 7.52-7.43 (2H, m, H-6 Naph, H-7 Naph), 7.47-7.11 (13H, m, H-2 Naph, H-3 Naph, $CHCH_2Ph$, CO_2CH_2Ph, H-5b), 6.66,6.64 (1H, 2d, ^{3}J=13.6 Hz, H-5a), 6.17,6.09 (1H, 2t, H-1'), 5.09-5.00 (2H, m, CO_2CH_2Ph), 4.30-4.21 (2H, m, H-3', $CHCH_2Ph$), 4.19-3.69 (3H, m, H-4', H-5'), 3.08-3.14 (1H, m, one of $CHCH_2Ph$), 2.91-2.81 (1H, m, one of $CHCH_2Ph$), 2.18-2.09 (1H, m, one of H-2'), 1.81-1.60 (1H, m, one of H-2').

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 12.87 min.

ESI MS (positive): 776 [M + H], 798 [M + Na], 814 [M + K].

Anal. Calcd for $C_{37}H_{35}BrN_3O_9P$: C, 57.21; H, 4.51; N, 5.41. Found: C, 56.87; H, 4.49; N, 5.15.

10m BVdU-5'-[1-naphthyl(methoxy-L-phenylglycinyl)] phosphate



$C_{36}H_{33}BrN_3O_9P$

Mol Wt.: 762.54

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-L-phenylglycinyl) phosphorochloridate (**11m**) (0.70 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (28 mg, 6.8% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 5.53, 5.44.

^{1}H-NMR (MeOD, 300 MHz): δ 8.06-8.01 (1H, m, H-8 Naph), 7.88-7.83 (1H, m, H-5 Naph), 7.72-7.66 (2H, m, H-4 Naph, H-6), 7.55-7.22 (10H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b, *Ph*), 6.72 (1H, d, ^{3}J=13.7 Hz, H-5a), 6.23-6.15 (1H, 2t, H-1'), 5.12-5.06 (1H, m, *CH*NH), 4.46-4.07 (4H, m, H-3', H-4', H-5'), 3.63,3.61 (3H, 2s, OCH_3), 2.30-2.15 (1H, m, one of H-2'), 2.05-1.76 (1H, m, one of H-2').

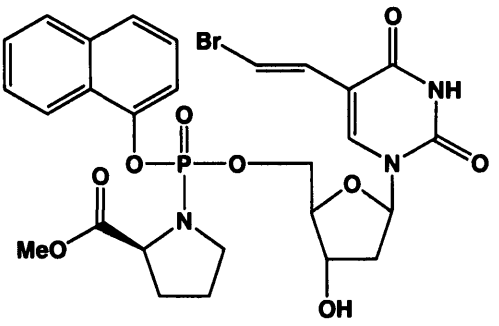
^{13}C-NMR (MeOD, 75 MHz): δ 41.6 (C-2'), 53.5,53.6 (OCH_3), 60.2 (NHCH), 68.4 (C-5'), 72.6 (C-3'), 87.1,87.2,87.5 (C-1', C-4'), 109.6 (C-5b), 112.6 (C-5), 116.8,116.9 (C-2 Naph), 123.0,126.6,126.9,127.9,128.2,128.5,128.7,129.3,129.8, 130.2,130.9 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a, *Ph*), 136.7 (C-4a Naph), 139.6,139.9 (C-6), 148.0,148.1 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 173.4,173.5 (*C*$OOCH_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 11.11 min.

ESI MS (positive): 686 [M + H], 708 [M + Na], 724 [M + K].

Anal. Calcd for $C_{30}H_{29}BrN_3O_9P \cdot 1Et_2O$: C, 53.69; H, 5.17; N, 5.52. Found: C, 53.82; H, 4.09; N, 5.41.

10n BVdU-5'-[1-naphthyl(methoxy-L-prolinyl)] phosphate



$C_{27}H_{29}BrN_3O_9P$

Mol Wt.: 650.41

Prepared according to Standard Procedure C, from BVdU (0.20 g, 0.60 mmol), 1-naphthyl(methoxy-L-prolinyl) phosphorochloridate (**11n**) (0.64 g, 1.80 mmol), NMI (0.25 g, 3.00 mmol, 0.24 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (0.19 g, 49.7% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 3.75, 3.40.

^{1}H-NMR (MeOD, 300 MHz): δ 8.13-8.03 (1H, m, H-8 Naph), 7.86-7.83 (1H, m, H-5 Naph), 7.70 (1H, s, H-6), 7.67-7.63 (1H, m, H-4 Naph), 7.53-7.2 (4H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph), 7.27,7.25 (1H, 2d, ^{3}J=13.6 Hz, H-5b), 6.69 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.15,6.12,6.08,6.06 (1H, 4d, ^{3}J=7.8 Hz, H-1'), 4.54-4.25 (4H, m, H-3', H-4', H-5'), 4.15-4.04 (1H, m, *CH*NH), 3.67 (3H, s, OCH_3), 3.49-3.32 (2H, m, H-5 *prol*), 2.22-1.71 (6H, m, H-3 *prol*, H-4 *prol*, H-2').

^{13}C-NMR (MeOD, 75 MHz): δ 26.5,26.6,26.7 (C-4 *prol*), 32.5,32.6,32.9 (C-3 *prol*), 41.6,41.8 (C-2'), 48.6,48.7 (C-5 *prol*), 53.4 (OCH_3), 62.6,62.7 (NHCH), 68.6,68.7 (C-5'), 72.4,72.8 (C-3'), 87.2,87.5,87.6,87.7 (C-1', C-4'), 109.7 (C-5b), 112.5 (C-5), 116.1,116.2 (C-2 Naph), 122.5,122.9,126.7,127.0,128.0,128.2,128.3,128.4,129.5,

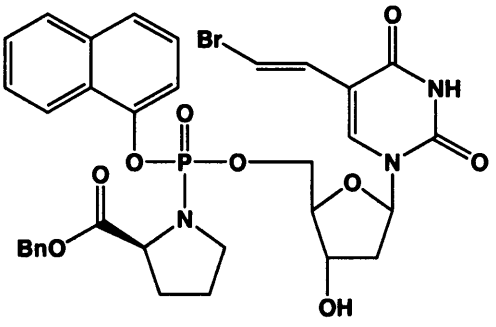
130.8,130.9 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, C-5a), 136.7 (C-4a Naph), 140.0 (C-6), 148.1,148.2 ('ipso' Naph), 151.3 (C-4), 163.9 (C-2), 175.7,176.0 ($COOCH_3$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.87 min.

ESI MS (positive): 650 [M + H], 672 [M + Na], 688 [M + K].

Anal. Calcd for $C_{27}H_{29}BrN_3O_9P$: C, 49.85; H, 4.46; N, 6.46. Found: C, 49.85; H, 4.27; N, 6.16.

10o BVdU-5'-[1-naphthyl(benzoxy-L-prolinyl)] phosphate



$C_{27}H_{29}BrN_3O_9P$

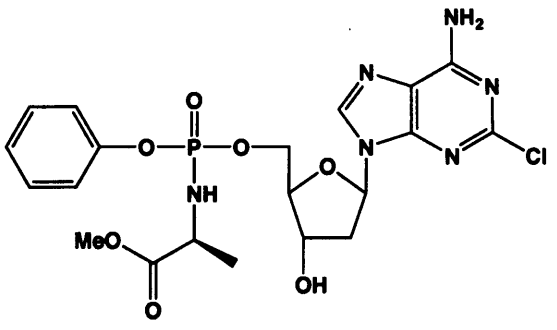
Mol Wt.: 650.41

Prepared according to Standard Procedure C, from BVdU (0.13 g, 0.39 mmol), 1-naphthyl(benzoxy-L-prolinyl) phosphorochloridate (**11o**) (0.50 g, 1.17 mmol), NMI (0.16 g, 1.95 mmol, 0.15 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (12 mg, 4.2% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 3.72,3.42.

^{1}H-NMR (MeOD, 300 MHz): δ 8.10-8.06 (1H, m, H-8 Naph), 7.90-7.85 (1H, m, H-5 Naph), 7.74-7.70 (2H, m, H-4 Naph, H-6), 7.54-7.20 (H, m, H-2 Naph, H-3 Naph, H-6 Naph, H-7 Naph, H-5b), 6.71 (1H, d, ^{3}J=13.6 Hz, H-5a), 6.11,6.13 (1H, 2d, ^{3}J=7.9 Hz, H-1'), 5.20,5.09 (2H, AB system, ^{3}J=12.3 Hz, CH_2Ph), 4.50-4.28 (4H, m, H-3', H-4', H-5'), 4.12-4.09 (1H, m, *CH*NH), 3.51-3.40 (2H, m, H-5 *prol*), 2.21-1.73 (6H, m, H-3 *prol*, H-4 *prol*, H-2').

12a 2CdA-5'-[phenyl(methoxy-L-alaninyl)] phosphate



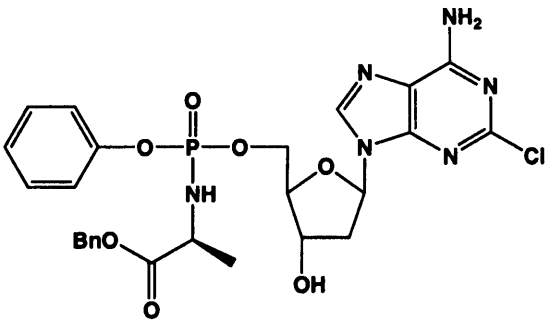
$C_{20}H_{24}ClN_6O_7P$
Mol Wt.: 526.87

NMI (0.29 g, 3.50 mmol, 0.29 mL) was added to a stirring suspension of 2CdA (0.20 g, 0.70 mmol) in dry THF (10 mL). A solution of phenyl(methoxy-L-alaninyl) phosphorochloridate (**17a**) (0.58 g, 2.10 mmol) in dry THF was added dropwise at – 78 °C. After 15 min the reaction was let to rise to room temperature. The reaction was followed by TLC (DCM/MeOH 95/5). After 4 h, further **17a** (0.28 g, 1.0 mmol) was added and the reaction was stirred overnight. MeOH was added to quench the reaction. Volatiles were evaporated and the residue was purified by column chromatography (DCM/MeOH from 100/0 to 95/5) and preparative TLC (DCM/MeOH 96/4) to give the product as a white foamy solid (8 mg, 2.2 % yield; 2CdA recovered 0.15 g).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.56,4.21.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.14,8.07 (1H, 2s, H-8), 7.52-7.29 (5H, m, Ph), 6.56-6.50 (1H, m, H-1'), 6.18-6.01 (2H, bs, NH_2), 4.90-4.77 (1H, m, H-3'), 4.46-4.56 (2H, m, H-5'), 4.32-4.26 (1H, m, H-4'), 4.22-4.02 (1H, m, *CH*NH), 3.85,3.83 (3H, 2s, CH_3O), 2.92-2.61 (2H, m, H-2'), 1.56-1.44 (3H, m, CH_3CH).

12b 2CdA-5'-[phenyl(benzoxy-L-alaninyl)] phosphate



$C_{26}H_{28}ClN_6O_7P$
Mol Wt.: 602.96

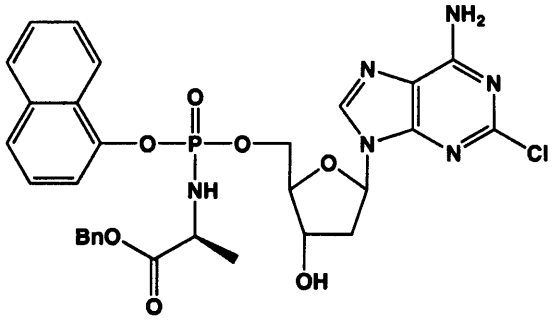
NMI (0.29 g, 3.50 mmol, 0.29 mL) was added to a stirring suspension of 2CdA (0.20 g, 0.70 mmol) in dry THF (10 mL). A solution of phenyl(benzoxy-L-alaninyl) phosphorochloridate (**17b**) (0.74 g, 2.10 mmol) in dry THF was added dropwise at – 78 °C. After 15 min the reaction was let to rise to room temperature and stirred overnight. MeOH was added to quench the reaction. Volatiles were evaporated and the residue was purified by column chromatography (DCM/MeOH from 100/0 to 95/5) and preparative TLC (DCM/MeOH 96/4) to give the product as a white foamy solid (15 mg, 3.6 % yield).

^{31}P-NMR (MeOH, 121 MHz): δ 5.11,4.81.

^{1}H-NMR (MeOH, 300 MHz): δ 8.12,8.10 (1H, 2s, H-8), 7.23-7.04 (10H, m, PhO, *Ph*CH_2), 6.30-6.24 (1H, m, H-1'), 5.04-5.00 (2H, 2 AB systems, Ph*CH_2*), 4.52-4.48 (1H, m, H-3'), 4.29-4.15 (1H, m, H-5'), 4.08-4.04 (1H, m, H-4'), 3.91-3.81 (1H, m, *CH*NH), 2.63-2.54 (1H, m, one of H-2'), 2.41-2.33 (1H, m, one of H-2'), 1.24-1.17 (3H, m, CH*CH_3*).

^{13}C-NMR (MeOD, 75 MHz): δ 20.6,20.8 (CH_3), 41.2 (C-2'), 52.0,52.1 (*CH*CH_3), 67.7,68.1,68.2,68.3 (C-5', *CH_2*Ph), 72.6 (C-3'), 86.2,86.4 (C-1'), 87.2,87.3 (C-4'), 121.7,121.8,126.5,129.6,129.7,129.9,131.1 (*Ph*CH_2, *Ph*O), 137.6 ('ipso' *Ph*CH_2), 141.5 (C-8), 151.8,151.9 (C-6), 152.4,152.5 ('ipso' PhO), 155.7,155.8 (C-2), 158.4 (C-4), 175.0,175.2 (*C*OOCH_2Ph).

12c 2CdA-5'-[1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{30}H_{30}ClN_6O_7P$
Mol Wt.: 653.02

NMI (0.29 g, 3.50 mmol, 0.29 mL) was added to a stirring suspension of 2CdA (0.20 g, 0.70 mmol) in dry THF (6 mL). A solution of 1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8c**) (0.85 g, 2.10 mmol) in dry THF (4 mL) was added dropwise at –78 °C. After 15 min the reaction was let to rise to room temperature and stirred overnight. MeOH was added to quench the reaction. Volatiles were evaporated and the residue was purified by column chromatography (DCM/MeOH from 100/0 to 95/5) and preparative reverse phase HPLC (H_2O/CH_3CN 60/40, isocratic elution) to give the product as a white foamy solid (39 mg, 8.6 % yield).

R,S-mixture

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.64,3.23.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.00-7.98 (1H, m, H-8 Naph), 7.86 (0.5 H, s, H-8 of one diastereisomer), 7.77 (1H, d, H-5 Naph, ^{3}J=7.4 Hz), 7.75 (0.5 H, s, H-8 of one diastereisomer), 7.56 (1H, d, H-4 Naph, ^{3}J=8.3 Hz), 7.44-7.40 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.31-7.15 (6H, m, H-3 Naph, *Ph*), 6.25-6.21 (1H, m, H-1'), 5.73 (2H, bs, NH_2), 5.04 (1H, s, CH_2Ph of one diastereoisomer), 4.95 (1H, AB system, ^{2}J=12.2 Hz, CH_2Ph of one diastereoisomer), 4.57-4.54 (0.5H, m, H-3'of one diastereoisomer), 4.49-4.46 (0.5H, m, H-3'of one diastereoisomer), 4.34-4.23 (2H, m, H-5'), 4.11-4.00 (2H, m, H-4', *CH*NH), 3.91 (0.5H, CH*NH* of one diastereoisomer), 3.90 (0.5H, CH*NH* of one diastereoisomer), 3.13 (0.5H, OH of one diastereoisomer), 3.01 (0.5H, OH of one diastereoisomer), 2.56-2.51 (0.5H, m, one

H-2'of one diastereoisomer), 2.43-2.29 (1.5H, m, three H-2'), 1.27-1.24 (3H, 2d, CH*CH*$_3$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 19.7,19.8 (CH_3), 38.8 (C-2'), 49.4,49.5 (*CH*CH_3), 64.8,66.2,66.3 (C-5',*CH*$_2$Ph), 69.8 (C-3'), 83.0 (C-1'), 83.9,84.0 (C-4'), 113.9,114.0 (C-2 Naph), 117.6,120.2,120.3,123.9,124.5,125.2,125.4,125.5,125.7,126.8,127.1, 127.4,127.5,127.6 (C-5, C-8, *Ph*CH_2, C-5a Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 133.6,133.7,134.1 ('ipso' *Ph*CH_2, C-4a Naph), 145.3 ('ipso' Naph), 149.3 (C-6), 153.0 (C-2), 155.13 (C-4), 172.4 (*C*OOCH_2Ph).

HPLC (H_2O/CH_3CN 40/60): Rt 8.92, 9.59 min.

R diastereoisomer

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.64,3.25 (int.: **1.00**,**28.15**).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.99-7.97 (1H, dd, H-8 Naph), 7.87 (1H, s, H-8), 7.77-7.74 (1H, m, H-5 Naph), 7.57 (1H, d, H-4 Naph, ^{3}J=8.3 Hz), 7.44-7.40 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.29-7.20 (6H, m, H-3 Naph, *Ph*), 6.23 (1H, m, H-1'), 5.81 (2H, bs, NH_2), 5.03 (2H, s, *CH*$_2$Ph), 5.00,4.92 (AB system, ^{2}J=12.3 Hz, *CH*$_2$Ph of minor diastereisomer), 4.58-4.55 (1H, m, H-3'), 4.49,4.48 (m, H-3' of minor diastereoisomer), 4.34-4.23 (2H, m, H-5'), 4.08-3.99 (3H, m, H-4', *CH*NH, CH*NH*), 3.78 (CH*NH* of minor diastereoisomer), 3.31 (1H, bs, OH), 2.56-2.50 (1H, m, one H-2'), 2.42-2.38 (1H, m, one H-2'), 2.37-2.31 (m, H-2' of minor diastereoisomer), 1.26 (3H, 2d, CH*CH*$_3$).

HPLC (H_2O/CH_3CN 40/60): Rt 9.59-10.92 min.

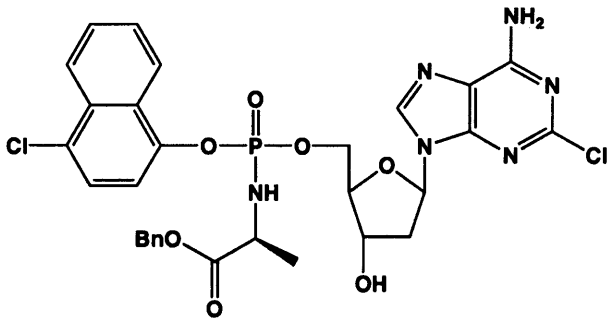
S diastereoisomer

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.60,3.22 (int.: **4.87**,**1.00**).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.03 (1H, d, ^{3}J=7.5 Hz, H-8 Naph), 7.92 (0.2H, s, one of H-8 of minor diastereoisomer), 7.80 (0.8H, s, H-8), 7.79 (1H, d, ^{3}J=7.4 Hz, H-5 Naph), 7.61 (1H, d, H-4 Naph, ^{3}J=8.3 Hz), 7.47-7.44 (3H, m, H-2 Naph, H-6 Naph,

H-7 Naph), 7.35-7.12 (6H, m, H-3 Naph, *Ph*), 6.29-6.26 (1H, m, H-1'), 5.88 (2H, bs, NH_2), 5.08 (0.4H, s, CH_2Ph of minor diastereoisomer), 5.05,4.97 (1.6H, AB system, ^{2}J=12.2 Hz, CH_2Ph), 4.61-4.58 (0.2H, m, H-3' of minor diastereoisomer), 4.54-4.51 (0.8H, m, H-3'), 4.36-4.32 (2H, m, H-5'), 4.12-4.06 (2.2H, m, H-4', *CH*NH, CH*NH* of minor diastereoisomer), 3.82 (0.8H, CH*NH*), 3.32 (0.2H, OH of minor diastereoisomer), 3.25 (0.8H, OH), 2.58-2.53 (0.2H, m, one H-2'of minor diastereoisomer), 2.46-2.42 (0.2H, m, one H-2'of minor diastereoisomer), 2.41-2.33 (1.6H, m, H-2'), 1.31-1.29 (3H, 2d, CHCH_3).
HPLC (H_2O/CH_3CN 40/60): Rt 7.59-8.92 min.

12d 2CdA-5'-[4-chloro-1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{30}H_{29}Cl_2N_6O_7P$
Mol Wt.: 687.47

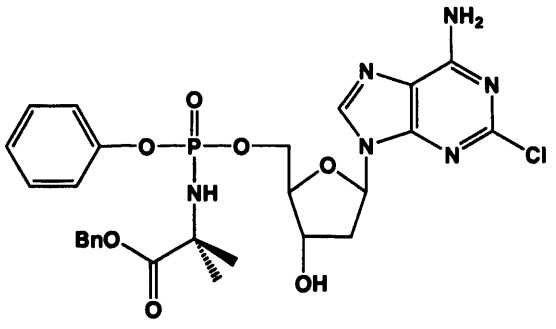
NMI (0.26 g, 3.20 mmol, 0.25 mL) was added to a stirring suspension of 2CdA (0.18 g, 0.64 mmol) in dry THF (10 mL). A solution of 4-chloro-1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8g**) (0.87 g, 1.92 mmol) in dry THF was added dropwise at –78 °C. After 15 min the reaction was let to rise to room temperature and stirred overnight. MeOH was added to quench the reaction. Volatiles were evaporated and the residue was purified by column chromatography (DCM/MeOH from 100/0 to 95/5) and preparative reverse phase HPLC (H_2O/CH_3CN 60/40, isocratic elution) to give the product as a white foamy solid (15 mg, 3.4 % yield).
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.45,3.26.
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.14-8.10 (1H, m, H-5 Naph), 7.99-7.96 (1H, m, H-8 Naph), 7.88,7.82 (1H, 2s, H-8), 7.54-7.49 (1H, m, H-6 Naph), 7.47-7.40 (1H, m, H-7

Naph), 7.32-7.14 (7H, m, H-2 Naph, H-3 Naph, Ph), 6.25-6.22 (1H, m, H-1'), 6.04 (2H, bs, NH_2), 5.00-4.89 (2H, m, Ph*CH_2*), 4.59-4.54 (1H, m, H-3'), 4.33-4.19 (3H, m, H-5', CH*NH*), 4.08-3.96 (2H, m, H-4', *CH*NH), 3.61,3.57 (1H, 2 bs, OH-3'), 2.59-2.34 (2H, m, H-2'), 1.25-1.23 (3H, m, *CH_3*CH).

^{13}C-NMR ($CDCl_3$; 125 MHz): δ 20.8 (CH_3), 39.7 (C-2'), 50.5 (*CH*CH_3), 66.2,66.3 (C-5'), 67.3,67.4 (*CH_2*Ph), 80.0 (C-3'), 84.1 (C-1'), 84.9,85.0,85.1 (C-4'), 114.8, 114.9,115.1,118.8,121.8,124.7,125.5,127.2,127.8,128.1,128.3,128.5,128.6,128.7 (C-2, C-5, Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, *Ph*CH_2), 131.6 (C-4a Naph), 135.0 ('ipso' *Ph*CH_2), 139.3,139.4 (C-8), 145.3,145.4 ('ipso' Naph), 150.4 (C-6), 154.1 (C-2), 156.1 (C-4), 173.3(*C*$OOCH_2$Ph).

HPLC (H_2O/CH_3CN 40/60): Rt 7.05,8.13 min.

12e 2CdA-5'-[phenyl(benzoxy-dimethylglycinyl)] phosphate



$C_{27}H_{30}ClN_6O_7P$

Mol Wt.: 616.99

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M THF solution, 0.84 mL, 0.84 mmol), phenyl(benzoxy-dimethylglycinyl) phosphorochloridate (**17c**) (0.31 g, 0.84 mmol) for 4 h. The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (27 mg, 6.3 % yield).

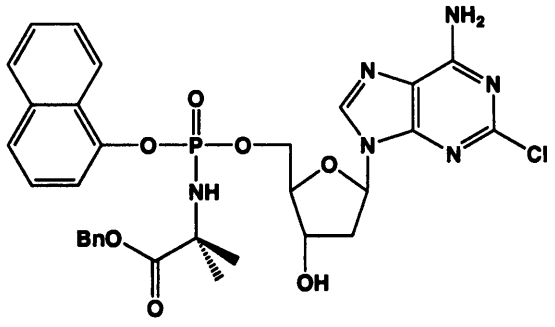
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 1.73,1.62.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.93,7.82 (1H, 2s, H-8), 7.25-7.00 (10H, m, PhO, *Ph*CH_2), 6.47,6.42 (2H, 2bs, NH_2), 6.27-6.25 (1H, m, H-1'), 5.05,5.03 (2H, 2s, Ph*C*H_2), 4.61-4.51 (1H, m, H-3'), 4.40-4.20 (2H, m, H-5'), 4.15 (1H, bs, OH-3'), 4.13-4.07 (1H, m, $C(CH_3)_2$*NH*), 4.05-4.02 (1H, m, H-4'), 2.61-2.32 (2H, m, H-2'), 1.46-1.42 (6H, m, $C(CH_3)_2$).

^{13}C-NMR ($CDCl_3$; 125 MHz): δ 26.8 ($C(CH_3)_2$), 39.8,39.9 (C-2'), 57.2 ($C(CH_3)_2$), 66.0,66.3,66.4 (C-5'), 67.6 (CH_2Ph), 70.8,71.0 (C-3'), 83.9 (C-1'), 85.0,85.1 (C-4'), 118.8,120.1,125.0,125.1,127.9,128.0,128.4,128.5,128.6,129.7, (C-5, PhO, *Ph*CH_2), 135.3 ('ipso' PhCH_2), 139.0 (C-8), 150.4,150.6 (C-6,'ipso' PhO), 154.1 (C-2), 156.3 (C-4), 175.1,175.2,(*C*OOCH_2Ph).

ESI MS m/z (positive) 639 [M + Na], 655 [M + K].

12f 2CdA-5'-[1-naphthyl(benzoxy-dimethylglycinyl)] phosphate



$C_{31}H_{32}ClN_6O_7P$

Mol Wt.: 667.05

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M THF solution, 0.84 mL, 0.84 mmol), 1-naphthyl(benzoxy-dimethylglycinyl) phosphorochloridate (**11c**) (0.35 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (54 mg, 11.6 % yield).

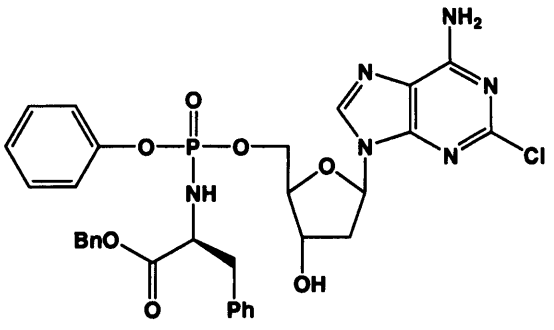
^{31}P-NMR ($CDCl_3$, 121 MHz): δ 1.97,1.93.

^{1}H-NMR ($CDCl_3$, 500MHz): δ 8.00-7.93 (1H, m, H-8 Naph), 7.97,7.89 (1H, 2s, H-8), 7.73-7.71 (1H, m, H-5 Naph), 7.53-7.49 (1H, 2d, ^{3}J=8.0 Hz, H-4 Naph), 7.41-7.33 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.27-7.16 (6H, m, H-3 Naph, *Ph*CH_2), 6.33,6.31 (2H, 2s, NH_2), 6.24-6.20 (1H, 2t, ^{3}J=6.0 Hz, H-1'), 5.04,5.02 (2H, 2s, Ph*CH_2*), 4.59-4.54,4.45-4.42 (1H, 2m, H-3'), 4.31-4.26 (2H, m, H-5'), 4.21-4.18 (1H, m, $C(CH_3)_2$*NH*), 4.6-3.99 (1H, m, H-4'), 3.91,3.86 (1H, 2bs, OH-3'), 2.55-2.29 (2H, m, H-2'), 1.48-1.43 (6H, m, $C(CH_3)_2$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 29.9 ($C(CH_3)_2$), 38.7 (C-2'), 56.3 ($C(CH_3)_2$), 65.4,66.6 (C-5',CH_2Ph), 69.9,70.0 (C-3'), 82.9 (C-1'), 83.9,84.0 (C-4'), 114.0 (C-2 Naph), 117.8,120.5,123.8,124.4,124.5,125.4,125.6,126.8,126.9,127.0,127.4,127.6 (C-5, *Ph*CH_2, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph) 133.7,134.3 ("ipso" *Ph*CH_2, C-4a Naph), 138.4 (C-8), 145.5 ("ipso" Naph), 149.4 (C-6), 153.1 (C-2), 155.2 (C-4), 1742 (COOCH$_2$Ph).

ESI MS m/z (positive) 689 [M + Na], 691 [M(^{37}Cl) + Na].

18a 2CdA-5'-[phenyl(benzoxy-L-phenylalaninyl)] phosphate



$C_{32}H_{32}ClN_6O_7P$

Mol Wt.: 679.06

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M THF solution, 0.84 mL, 0.84 mmol), phenyl(benzoxy-L-phenylalaninyl) phosphorochloridate (**20a**) (0.36 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative reverse phase HPLC (H_2O/CH_3CN 60/40, isocratic elution) to give the product as a white foamy solid (25 mg, 14.6 % yield of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.33,3.07.

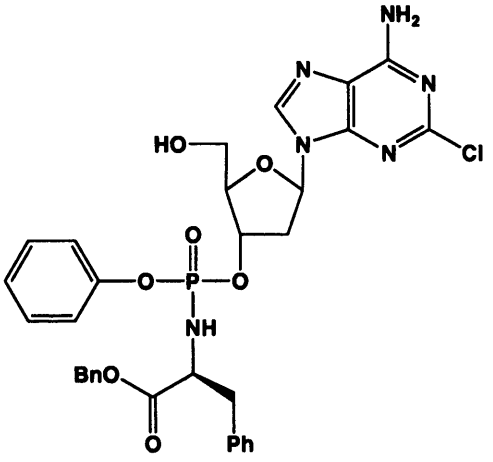
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.86,7.80 (1H, 2s, H-8), 7.21-6.88 (15 H, m, PhO, $CHCH_2$*Ph*, OCH_2*Ph*), 6.42,6.39 (2H, 2bs, NH_2), 6.25-6.21 (1H, m, H-1'), 4.97,4.95 (2H, AB system, OCH_2Ph), 4.47-4.43 (1H, m, H-3'), 4.21-3.95 (6H, m, H-4', H-5', OH-3', CH*NH*, *CH*CH_2), 2.89-2.85 (2H, m, $CHCH_2$Ph), 2.49-2.36 (2H, m, H-2').

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 38.8,39.9 (C-2'), 40.2,40.3 ($CHCH_2$Ph), 55.7,56.0 (*CH*CH_2), 65.6,66.1 (C-5'), 67.4 (OCH_2Ph), 70.7,70.9 (C-3'), 83.9,84.0 (C-1'), 84.9,85.0 (C-4'), 118.7,120.0,125.1,125.2,127.1,128.4,128.5,128.6,129.4,129.5, 129.8 (C-5, PhO, *Ph*CH_2CH, *Ph*CH_2O), 134.9,135.0 ('ipso' $CHCH_2$*Ph*, 'ipso' OCH_2*Ph*), 139.8 (C-8), 150.3 (C-6, 'ipso' PhO), 154.1 (C-2), 156.2 (C-4), 172.4 (*C*$OOCH_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 8.69.

ESI MS m/z (positive) 679 [M + H], 681 [M(^{37}Cl) + H], 701 [M + Na], 703 [M(^{37}Cl) + Na].

18b 2CdA-3'-[phenyl(benzoxy-L-phenylalaninyl)] phosphate



$C_{32}H_{32}ClN_6O_7P$

Mol Wt.: 679.06

Collected as the slowest eluted fraction from the preparative HPLC purification of **18a** (16 mg, 9.4% of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 1.42,1.23.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.69,7.55 (1H, 2s, H-8), 7.28-6.92 (15H, m, PhO, *Ph*CH_2CH, *Ph*CH_2O), 6.11 (2H, bs, NH_2), 6.01-5.86 (1H, m, H-1'), 5.30-5.02 (4H, m, H-3', OH-3', O*C*H_2Ph), 4.31-4.15 (2H, m, H-4', *C*HCH_2Ph), 3.86-3.65 (3H, m, H-5', CH*NH*), 2.98-2.81 (3H, m, CH*C*H_2Ph, one of H-2'), 2.39-2.31 (1H, m, one H-2').

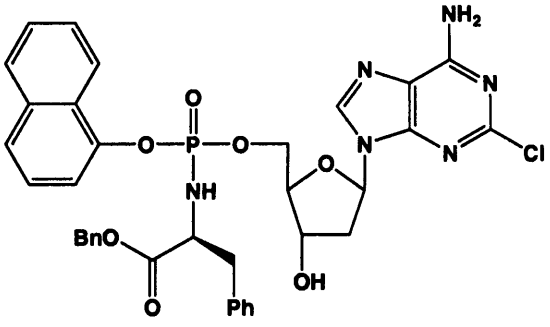
^{13}C-NMR ($CDCl_3$; 125 MHz): δ 38.0 (C-2'), 39.3,39.4 (CH*C*H_2Ph), 54.8,54.9 (*C*HCH_2Ph), 62.0 (C-5'), 66.4 (O*C*H_2Ph), 78.1,78.2,78.3,78.4 (C-3'), 85.9 (C-1'), 86.6,86.7 (C-4'), 118.9,119.0,119.1,119.2,124.2,126.2,126.3,127.5,127.6,127.7, 128.4,128.5,128.8(C-5, PhO, $CHCH_2$*Ph*, OCH_2*Ph*), 133.9,134.2,134.4 ('ipso' $CHCH_2$*Ph*, 'ipso' OCH_2*Ph*), 139.5 (C-8), 148.8,1494,149.5 (C-6, 'ipso' PhO), 152.9 (C-2), 155.4 (C-4), 171.0,171.1 (*C*$OOCH_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 9.19 min.

ESI MS m/z (positive) 679 [M + H], 681 [M(^{37}Cl) + H], 701 [M + Na], 703 [M(^{37}Cl) + Na], 717 [M + K], 719 [M(^{37}Cl) + K].

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 9.19 min.

18c 2CdA-5'-[1-naphthyl(benzoxy-L-phenylalaninyl)] phosphate



$C_{36}H_{34}ClN_6O_7P$
Mol Wt.: 729.12

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M THF solution, 0.84 mL, 0.84 mmol), 1-naphthyl(benzoxy-L-phenylalaninyl) phosphorochloridate (**11l**) (0.40 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the product as a white foamy solid (17 mg, 8.9 % yield of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.69,3.46.

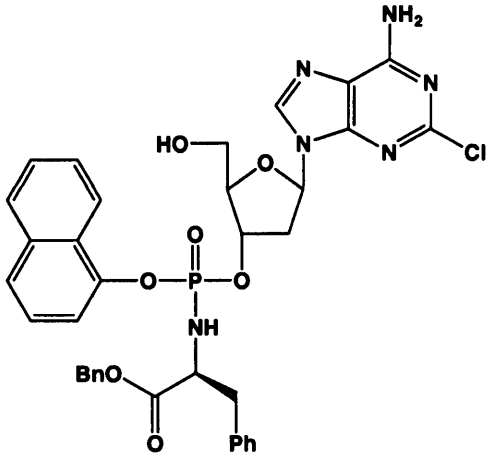
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.92-7.90 (1H, m, ^{3}J=8.0 Hz, H-8 Naph), 7.84,7.74 (1H, 2s, H-8), 7.71-7.69 (1H, m, H-5 Naph), 7.50 (1H, d, ^{3}J=8.2 Hz, H-4 Naph), 7.38-7.30 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.21-7.17 (4 H, m, H-3 Naph, Ph), 7.09-7.08 (2H, m, Ph), 7.02-7.97 (3H, m, Ph), 6.81-6.72 (2H, m, Ph), 6.35 (2H, bs, NH_2), 6.21-6.18 (1H, m, H-1'), 4.92-6.83 (2H, s, AB system, ^{2}J=12.1 Hz, O*C*H_2Ph), 4.44-4.42 (1H, m, H-3'), 4.33-4.31 (1H, m, CH*NH*), 4.25-4.13 (3H, m, H-5', *CH*NH), 4.003.90 (2H, m, H-4', OH-3'), 2.87-2.77 (2H, m, CH*C*H_2Ph), 2.40-2.27 (2H, m, H-2').

^{13}C-NMR ($CDCl_3$; 125 MHz): δ 39.8 (C-2'), 40.2,40.3 (CH*C*H_2Ph), 55.8,56.0 (*C*HCH_2), 65.8,65.9,66.2,66.3,67.4,67.9 (C-5', O*C*H_2Ph), 70.8,71.0 (C-3'), 83.9 (C-1'), 84.9,85.0 (C-4'), 114.9,115.1,118.7,121.3,125.0,125.1,125.5,126.2,126.3,126.5, 126.6,126.7,127.1,127.9,128.4,128.5,128.6,129.4 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 134.7,134.9,135.3 ('ipso' CHCH$_2$*Ph*, 'ipso' OCH$_2$*Ph*), 139.1,139.3 (C-8), 146.3 ('ipso' Naph), 150.3 (C-6), 154.1 (C-2), 156.2 (C-4), 172.3,172.4 (*C*OOCH$_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 9.99 min.

ESI MS m/z (positive) 729 [M + H], 731 [M(^{37}Cl) + H], 751 [M + Na], 753 [M(^{37}Cl) + Na].

18d 2CdA-3'-[1-naphthyl(benzoxy-L-phenylalaninyl)] phosphate



$C_{36}H_{34}ClN_6O_7P$
Mol Wt.: 729.12

Isolated through column chromatography purification of **18c** crude reaction mixture (6 mg, 3.2% yield of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 2.09,1.78.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.02-7.91 (1H, m, H-8 Naph), 7.81-7.79 (1H, m, H-5 Naph), 7.65-7.60 (1H, m, H-4 Naph), 7.49-7.41 (4H, m, H-8, H-2 Naph, H-6 Naph, H-7 Naph), 7.33-6.75 (11H, m, H-3 Naph, OCH_2*Ph*, $CHCH_2$*Ph*), 6.00 (2H, bs, NH_2), 5.92-5.62 (1H, 2m, H-1'), 5.28-5.13 (2H, m, H-3', OH-3'), 5.05-4.89 (2H, m, O*C*H_2Ph), 4.34-4.27 (1H, m, *C*HCH_2Ph), 4.18-4.12 (1H, 2m, H-4'), 3.83-3.61 (3H, m, H-5', CH*NH*), 2.95-2.88 (2H, m, CH*C*H_2Ph), 2.84-2.79 (1H, m, one of H-2'), 2.30-2.14 (1H, 2m, one H-2').

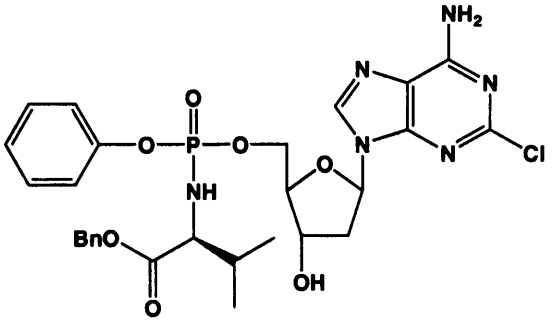
^{13}C-NMR ($CDCl_3$; 125 MHz): δ 39.1 (C-2'), 40.4 (CH*C*H_2Ph), 55.8,56.0 (*C*HCH_2Ph), 63.0 (C-5'), 67.4 (O*C*H_2Ph), 79.4,79.7 (C-3'), 86.9 (C-1'), 86.6,86.7, (C-4'), 115.4,115.6,115.7,119.9,121.4,125.2,125.7,126.4,126.6,126.8,127.3,128.0, 128.5,128.6,128.7,129.4,129.5 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 134.8,134.9,135.1,135.2

('ipso' $CHCH_2Ph$, 'ipso' OCH_2Ph), 140.5 (C-8), 146.3 ('ipso' Naph), 149.7 (C-6), 153.9 (C-2), 156.3 (C-4), 172.0 ($COOCH_2Ph$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 10.33 min.

ESI MS m/z (positive) 729 [M + H], 751 [M + Na], 767 [M + K].

18e 2CdA-5'-[phenyl(benzoxy-L-valinyl)] phosphate



$C_{28}H_{32}ClN_6O_7P$

Mol Wt.: 631.02

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.74 mL, 0.74 mmol), phenyl(benzoxy-L-valinyl) phosphorochloridate (**20b**) (0.32 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (21 mg, 4.8% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 4.19,3.92.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.93,7.86 (1H, 2s, H-8), 7.24-7.02 (10H, m, PhO, $PhCH_2$), 6.38 (2H, bs, NH_2), 6.27-6.24 (1H, t, ^{3}J=6.1 Hz, H-1'), 5.04-4.98 (2H, AB system, ^{3}J=12.2 Hz, $PhCH_2$), 4.56-4.51 (1H, m, H-3'), 4.17-4.27 (1H, m, H-5'), 4.11-3.82 (3H, m, H-4', CH*NH*, OH-3'), 3.77-3.68 (1H, m, *CH*NH), 2.58-2.53 (1H, m, one of H-2'), 2.47-2.40 (1H, m, one of H-2'), 0.77 (3H, d, ^{3}J=6.7 Hz, one of CH_3), 0.71 (3H, d, ^{2}J=6.7 Hz, one of CH_3)

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 16.7,16.8,18.4 ($CH(CH_3)_2$), 31.5,31.6 ($CH(CH_3)_2$), 39.3 (C-2'), 59.6,59.7 (*CH*NH), 65.3,65.7 (C-5'), 66.6 (CH_2Ph), 70.3 (C-3'), 83.5, 84.4 (C-1', C-4'), 118.2,119.4,119.5,124.5,127.8,128.0,129.2 (C-5, OPh, CH_2Ph),

134.6 ('ipso' *Ph*CH_2), 138.8 (C-8), 149.8,149.9,150.0 (C-6, 'ipso' PhO), 153.5 (C-2), 155.7 (C-4), 172.3 (*C*$OOCH_2Ph$).

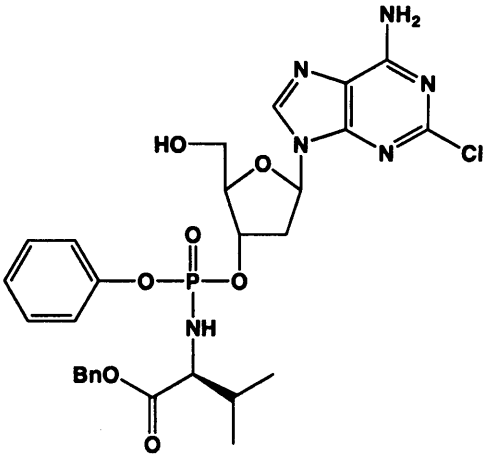
HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): Rt 8.95 min.

ESI MS m/z (positive) 631 [M + H], 633 [M(^{37}Cl) + H], 653 [M + Na].

18f 2CdA-3'-[phenyl(benzoxy-L-valinyl)] phosphate

$C_{28}H_{32}ClN_6O_7P$

Mol Wt.: 631.02

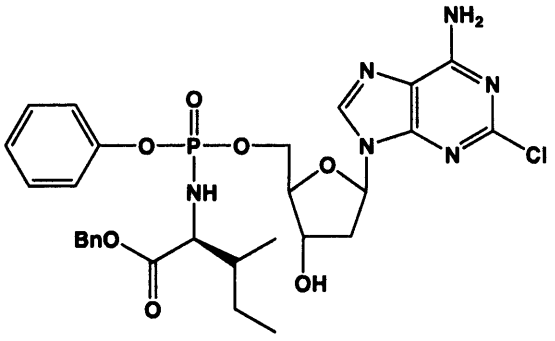


Isolated and partly purified through column chromatography purification of **18e** crude reaction mixture (3.6 mg).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 2.40,2.20 (major signals).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.74,7.58 (1H, 2s, H-8), 7.30-7.09 (10H, m, PhO, *Ph*CH_2), 5.91 (2H, bs, NH_2), 6.13-6.11,6.27-6.24 (1H, 2m, H-1'), 5.27-5.25 (1H, m, H-3'), 5.10-5.04 (2H, m, Ph*C*H_2), 4.25-4.21 (1H, m, H-4'), 3.86-3.62 (4H, m, H-5', CH*NH*, *CH*NH), 2.95-2.90 (1H, m, one of H-2'), 2.45-2.37 (1H, m, one of H-2'), 2.02-1.99 (1H, m, *CH*$(CH_3)_2$), 0.88-0.77 (6H, m , CH$(CH_3)_2$).

18g 2CdA-5'-[phenyl(benzoxy-L-isoleucinyl)] phosphate



$C_{29}H_{34}ClN_6O_7P$

Mol Wt.: 645.04

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.74 mL, 0.74 mmol), phenyl(benzoxy-L-isoleucinyl) phosphorochloridate (**20c**) (0.33 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (10 mg, 2.2 %).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.88,3.71.

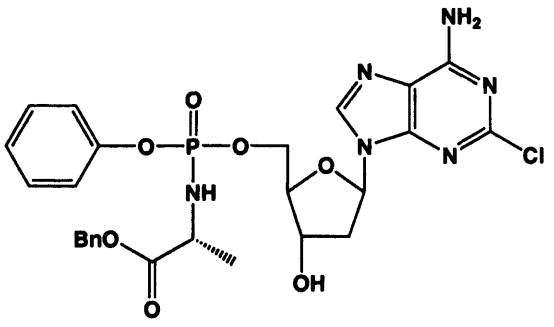
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.91 (1H, s, H-8), 7.27-7.04 (10H, m, PhO, CH_2*Ph*), 6.25 (1H, t, ^{3}J=6.2 Hz, H-1'), 6.03 (2H, bs, NH_2), 5.05-5.01 (2H, AB system, ^{2}J=12.2 Hz, Ph*C*H_2), 4.58-4.54 (1H, m, H-3'), 4.23-4.18 (2H, m, H-5'), 4.02-3.99 (1H, m, H-4'), 3.87-3.83 (1H, m, CH*NH*), 3.80-3.75 (1H, m, *CH*NH), 3.58 (1H, bs, OH-3'), 2.59-2.55 (1H, m, one of H-2'), 2.46-2.41 (1H, m, one of H-2'), 1.71-1.65 (1H, m, NHCH*CH*), 1.28-1.22 (1H, m, one of *C*$H_2$$CH_3$), 1.02-0.97 (1H, m, one of *C*$H_2$$CH_3$), 0.76-0.70 (6H, m, CH_3).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 11.4,15.4 (2CH_3), 24.5 (*C*$H_2$$CH_3$), 39.0 (NHCH*CH*), 39.8 (C-2'), 59.2 (NHCH), 66.1 (C-5'), 67.2 (*C*H_2Ph), 71.0 (C-3'), 84.0,84.9 (C-1', C-4'), 119.9,120.0,125.1,128.4,128.5,128.6,129.7 (C-5, PhO, CH_2*Ph*), 135.1 ('ipso' *Ph*CH_2), 139.4 (C-8), 150.0 (C-6, 'ipso' PhO), 154.1 (C-2), 156.1 (C-4), 172.5 (*C*$OOCH_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.60,11.07 min.

ESI MS m/z (positive) 645 [M + H], 647 [M(^{37}Cl) + H], 667 [M + Na].

18h 2CdA-5'-[phenyl(benzoxy-D-alaninyl)] phosphate



$C_{26}H_{28}ClN_6O_7P$

Mol Wt.: 602.96

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.70 mL, 0.70 mmol), phenyl(benzoxy-D-alaninyl) phosphorochloridate (**20d**) (0.25 g, 0.70 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (29 mg, 6.9%).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.33,2.93.

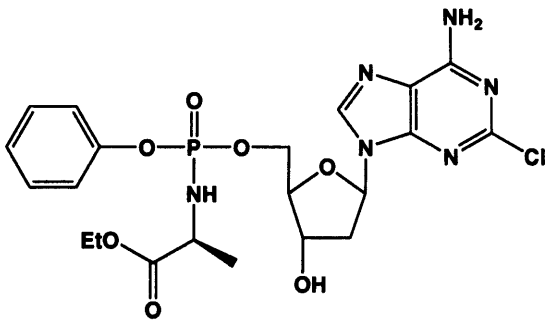
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.97,7.86 (1H, 2s, H-8), 7.24-7.03 (10H, m, PhO, CH_2*Ph*), 6.34 (2H, bs, NH_2), 6.28-6.24 (1H, m, H-1'), 5.07-5.00 (2H, m, *CH_2*Ph), 4.65-4.61,4.51-4.54 (1H, 2m, H-3'), 4.29-4.20 (2H, m, H-5'), 4.07-3.90 (4H, m, H-4', CH*NH*, *CH*NH, OH-3'), 2.61-2.42 (2H, m, H-2'), 1.27-1.24 (3H, m, CH*CH_3*).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.7 (CH*CH_3*), 39.8 (C-2'), 50.3,50.5 (*CH*NH), 65.9,66.1,66.2 (C-5'), 67.3,67.4 (*CH_2*Ph), 70.8 (C-3'), 84.0 (C-1'), 85.0 (C-4'), 118.7,120.0,120.1,125.2,128.1,128.2,128.5,128.6,129.7,129.8 (C-5, PhO, CH_2*Ph*), 135.2 ('ipso' CH_2*Ph*), 139.5 (C-8), 150.4 (C-6, 'ipso' PhO), 154.1 (C-2), 156.2 (C-4), 172.5 (*C*OOCH_2Ph).

ESI MS m/z (positive) 604 [M + H], 626 [M + Na].

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 8.75 min.

18i 2CdA-5'-[phenyl(ethoxy-L-alaninyl)] phosphate



$C_{21}H_{26}ClN_6O_7P$

Mol Wt.: 540.89

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.74 mL, 0.74 mmol), phenyl(ethoxy-L-alaninyl) phosphorochloridate (**20e**) (0.25 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (12 mg, 6.3% yield of reacted starting material).

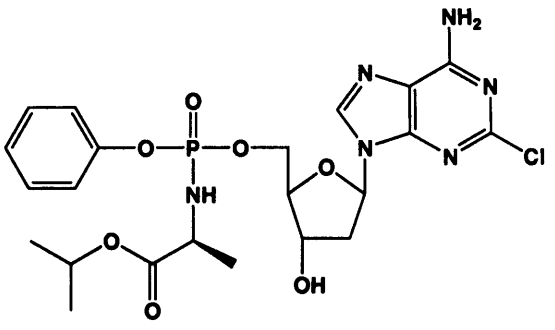
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.27,3.03.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.94,7.89 (1H, 2s, H-8), 7.25-7.12 (5H, m, PhO), 6.31-6.27 (1H, m, H-1'), 6.18 (2H, bs, NH_2), 5.55,5.43 (1H, 2bs, OH-3'), 4.65-4.58 (1H, m, H-3'), 4.31-4.26 (2H, m, H-5'), 4.10-4.02 (3H, m, H-4', CH*NH*, *CH*NH), 3.92-3.86 (2H, m, CH_2CH_3), 2.65-2.60 (1H, m, one of H-2'), 2.53-2.43 (1H, m, one of H-2'), 1.31-1.14 (6H, m, $CHCH_3$, CH_2CH_3).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 8.32,8.53 min.

ESI MS m/z (positive) 542 [M + H], 544 [M(^{37}Cl) + H], 564 [M + Na], 566 [M(^{37}Cl) + Na].

18l 2CdA-5'-[phenyl(iso-propoxy-L-alaninyl)] phosphate



$C_{22}H_{28}ClN_6O_7P$
Mol Wt.: 554.92

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.70 mL, 0.70 mmol), phenyl(iso-propiloxy-L-alaninyl) phosphochloridate (**20f**) (0.21 g, 0.70 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the product as a white foamy solid (20 mg, 5.1% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.28,3.12.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.96,7.90 (1H, 2s, H-8), 7.23-7.02 (5H, m, Ph), 6.46 (2H, bs, NH_2), 6.31-6.27 (1H, 2d, ^{3}J=6.3 Hz, H-1'), 4.90 (1H, septet, O*CH*$(CH_3)_2$, ^{3}J=3.2 Hz), 4.63-4.56 (1H, m, H-3'), 4.32-4.06 (5H, m, H-5', H-4', CH*NH*, OH-3'), 2.61-2.57 (0.5H, m, H-2'), 2.51-2.39 (1.5H, m, H-2'), 1.27-1.23 (3H, m, NHCH*CH_3*), 1.14-1.10 (6H, m, OCH*$(CH_3)_2$*).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.8 (OCH*$(CH_3)_2$*), 21.6,21.7 (NHCH*CH_3*), 39.9 (C-2'), 50.4,50.5 (CHNH), 66.1,66.2 (C-5'), 69.4,69.5 (O*CH*$(CH_3)_2$), 70.8,70.9 (C-3'), 84.1,84.2 (C-1'), 85.0,85.1,85.2 (C-4'), 118.6,118.7 (C-5), 120.0,120.1,125.1,125.2, 129.8 (Ph), 139.3,139.4 (C-8), 150.3,150.5 (C-6, 'ipso' Ph), 154.1 (C-2), 156.3 (C-4), 173.1,173.2 (*C*OOCH$(CH_3)_2$).

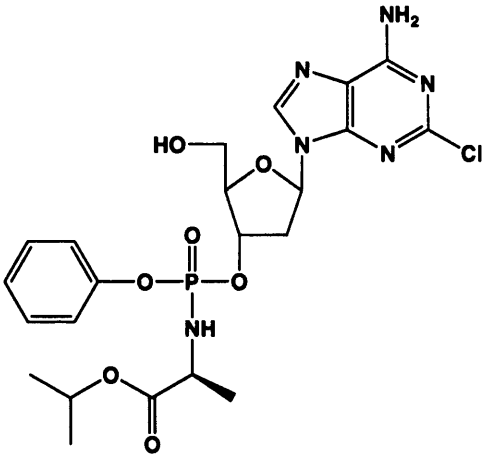
HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 8.92 min.

ESI MS m/z (positive) 555 [M + H], 556 [M(^{13}C) + H], 557 [M(^{37}Cl) + H], 558 [M(^{37}Cl,^{13}C) + H], 577 [M + Na], 578 [M(^{13}C) + Na], 579 [M(^{37}Cl) + Na], 580 [M(^{37}Cl,^{13}C) + Na].

18m 2CdA-3'-[phenyl(iso-propoxy-L-alaninyl)] phosphate

$C_{22}H_{28}ClN_6O_7P$

Mol Wt.: 554.92



Isolated through column chromatography purification of **18l** crude reaction mixture (7 mg, 1.8% yield).

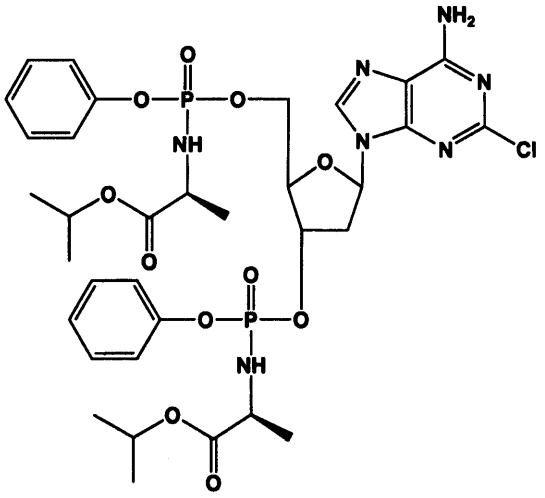
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 1.54,1.34.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.79,7.71 (1H, 2s, H-8), 7.31-7.12 (5H, m, Ph), 6.21-6.17 (0.5H, m, H-1'), 6.05-6.01 (0.5H, m, H-1'), 5.70 (2H, bs, NH_2), 5.31-5.26 (1H, m, H-3'), 4.99-4.93 (1H, m, O*CH*$(CH_3)_2$), 4.44-4.42 (1H, m, H-4'), 3.98-3.87 (2H, m, *CH*CH_3, OH-5'), 3.82-3.74 (1H, m, *NH*CH), 3.67-3.53 (3H, m, H-5', NH*CH*), 3.08-2.97 (1H, m, H-2'), 2.62-2.49 (1H, m, H-2'), 1.34,1.30 (3H, 2d, ^{3}J=7.0 Hz, NHCH*CH_3*), 1.22-1.19 (6H, m, OCH*$(CH_3)_2$*).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 9.92 min.

ESI MS m/z (positive) 555 [M + H], 556 [M(^{13}C) + H], 557 [M(^{37}Cl) + H], 558 [M(^{37}Cl,^{13}C) + H], 577 [M + Na], 578 [M(^{13}C) + Na], 579 [M(^{37}Cl) + Na], 580 [M(^{37}Cl,^{13}C) + Na].

18n 2CdA-3',5'-bis-[phenyl(iso-propoxy-L-alaninyl)] phosphate



$C_{34}H_{44}ClN_7O_{11}P_2$
Mol Wt.: 824.15

Isolated through column chromatography purification of **18l** crude reaction mixture (22 mg, 5.6% yield).

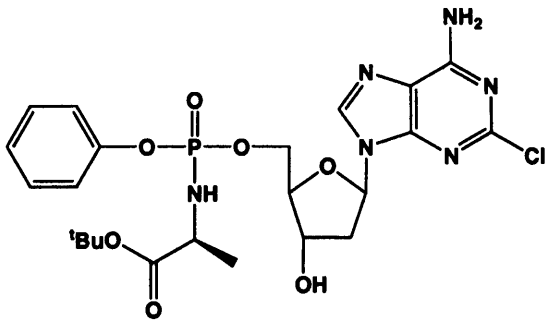
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 2.97,2.94,2.83,2.77,1.94,1.89,1.84,1.82.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.00,7.97 (1H, 2s, H-8), 7.27-7.03 (10H, m, 2 Ph), 6.34-6.22 (1H, m, H-1'), 6.18 (2H, bs, NH_2), 5.33-5.22 (1H, m, H-3'), 5.01-4.88 (2H, m, 2 O*CH*$(CH_3)_2$), 4.38-4.27 (3H, m, H-4', H-5'), 4.03-3.87 (4H, m, 2 *NH*CH, 2 NH*CH*CH_3), 2.73-2.55 (2H, m, H-2'), 1.32-1.15 (18H, m, 2 NHCH*CH_3*, 2 OCH*$(CH_3)_2$*).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 12.05 min.

ESI MS m/z (positive) 824 [M + H], 825 [M(^{13}C) + H], 826 [M(^{37}Cl) + H], 827 [M(^{37}Cl,^{13}C) + H], 846 [M + Na], 847 [M(^{13}C) + Na], 848 [M(^{37}Cl) + Na], 849 [M(^{37}Cl,^{13}C) + Na].

18o 2CdA-5'-[phenyl(tert-butoxy-L-alaninyl)] phosphate



$C_{23}H_{30}ClN_6O_7P$

Mol Wt.: 568.95

Prepared according to Standard Procedure D, from 2CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.74 mL, 0.74 mmol), phenyl(tert-butoxy-L-alaninyl) phosphorochloridate (**20g**) (0.27 g, 0.84 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (38 mg, 24% yield of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.36,3.19.

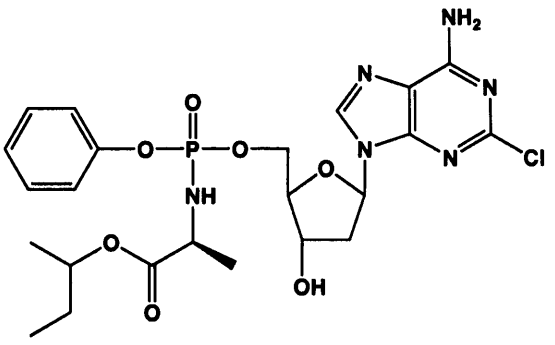
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.95-7.90 (1H, 2s, H-8), 7.22-7.01 (5H, m, Ph), 6.52 (2H, bs, NH_2), 6.31-6.27 (1H, m, H-1'), 5.64-4.58 (1H, m, H-3'), 4.33-4.02 (5H, m, H-5', H-4', CH*NH*, OH-3'), 3.82-3.77 (1H, m, *CH*NH), 2.61-2.40 (2H, m, H-2'), 1.32,1.33 (9H, 2s, $C(CH_3)_3$), 1.25-1.22 (3H, m, $CHCH_3$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.9,21.0 ($CHCH_3$), 27.9 ($C(CH_3)_3$), 39.9,40.0 (C-2'), 50.9 (*CH*NH), 66.3 (C-5'), 70.9,71.1 (C-3'), 82.2 ($C(CH_3)_3$), 84.0,84.2,85.0 (C-1', C-4'), 118.8,120.1,125.1,129.7 (C-5, OPh), 139.3,139.4 (C-8), 150.3,150.5,150.6 (C-6, 'ipso' PhO), 154.1 (C-2), 156.3 (C-4), 172.8 ($COOC(CH_3)_2$).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 9.48 min.

ESI MS m/z (positive) 570 [M + H], 572 [M(^{37}Cl) + H], 592 [M + Na], 594 [M(^{37}Cl) + Na].

18p 2CdA-5'-[phenyl(2-butoxy-L-alaninyl)] phosphate



$C_{23}H_{30}ClN_6O_7P$
Mol Wt.: 568.95

Prepared according to Standard Procedure D, from 2-CdA (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.70 mL, 0.70 mmol), phenyl(2-butoxy-L-alaninyl) phosphorochloridate (**20h**) (0.22 g, 0.70 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the product as a white foamy solid (27 mg, 6.8% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.29,3.10.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.95,7.90 (1H, 2s, H-8), 7.23-7.02 (5H, m, Ph), 6.44,6.42 (2H, bs, NH_2), 6.31-6.28 (1H, m, H-1'), 4.74 (1H, sestet, ^{3}J=6.3 Hz, O*CH*(CH_3)CH_2CH_3), 4.64-4.58 (1H, m, H-3'), 4.30-4.19 (3H, m, H-5'), 4.10-3.99 (2H, m, H-4', OH-3'), 3.93-3.87 (1H, m, NH*CH*CH_3), 2.63-2.58 (0.5H, m, H-2'), 2.52-2.40 (1.5H, m, H-2'), 1.51-1.39 (2H, m, OCH(CH_3)*CH_2*CH_3), 1.29-1.24 (3H, m, NHCH*CH_3*), 1.10-1.07 (3H, m, OCH(*CH_3*)CH_2CH_3), 0.80-0.74 (3H, m, OCH(CH_3)CH_2*CH_3*).

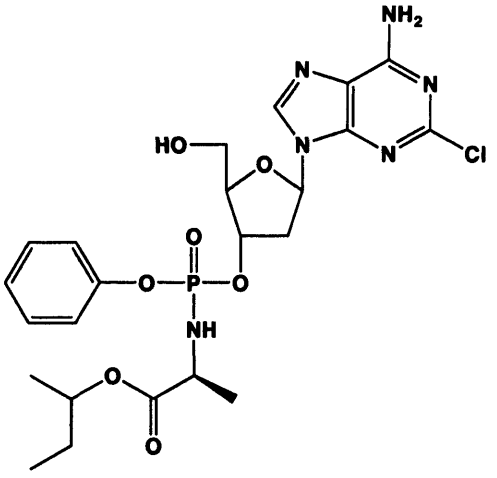
^{13}C-NMR ($CDCl_3$, 125 MHz): δ 9.5,9.6 (OCH(CH_3)CH_2*CH_3*), 19.2,19.4 (OCH(*CH_3*)CH_2CH_3), 20.8,20.9,21.0 (NHCH*CH_3*), 28.6 (OCH(CH_3)*CH_2*CH_3), 39.9 (C-2'), 50.4,50.5 (CHNH), 65.9,66.2 (C-5'), 70.4,71.0 (C-3'), 74.0 (O*CH*(CH_3)CH_2CH_3), 84.0,84.1 (C-1'), 85.0,85.1 (C-4'), 118.8 (C-5), 120.0,120.1, 125.1,125.2,129.8 (Ph), 139.4 (C-8), 150.4,150.5 (C-6,'ipso' Ph), 154.1 (C-2), 156.3 (C-4), 173.3 (*C*OOCH(CH_3)CH_2 CH_3).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 9.79 min.

ESI MS m/z (positive) 569 [M + H], 570 [M(^{13}C) + H], 571 [M(^{37}Cl) + H], 572 [M(^{37}Cl,^{13}C) + H], 591 [M + Na], 592 [M(^{13}C) + Na], 593 [M(^{37}Cl) + Na], 594 [M(^{37}Cl,^{13}C) + Na].

18q 2CdA-3'-[phenyl(2-butoxy-L-alaninyl)] phosphate

$C_{23}H_{30}ClN_6O_7P$
Mol Wt.: 568.95



Isolated through column chromatography purification of **18p** crude reaction mixture (2.8 mg, 1.8% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 1.62,1.60,1.41.

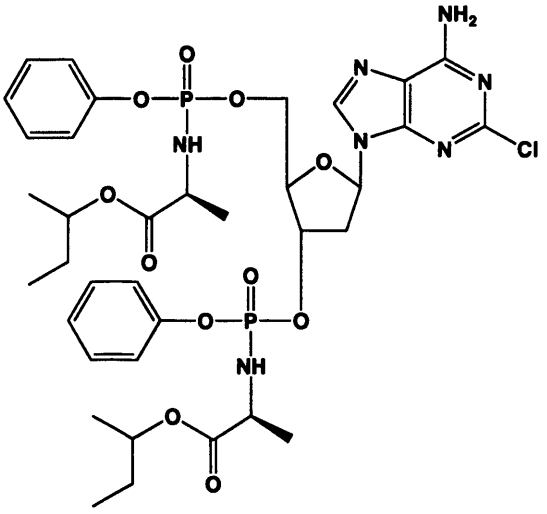
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.81,7.73 (1H, 2s, H-8), 7.30-7.11 (5H, m, Ph), 6.21-6.03 (3H, m, H-1', NH_2), 5.32-5.23 (2H, m, H-3', *NH*CH), 4.85-4.76 (1H, m, O*CH*(CH_3)CH_2CH_3), 4.34-4.31 (1H, m, H-4'), 3.95-3.75 (4H, m, NH*CH*CH_3, H-5', OH-5'), 3.07-2.95 (1H, m, H-2'), 2.62-2.48 (1H, m, H-2'), 1.55-1.47 (2H, m, OCH(CH_3)*CH_2*CH_3), 1.37-1.31 (3H, m, NHCH*CH_3*), 1.19-1.09 (3H, m, OCH(*CH_3*)CH_2CH_3), 0.85-0.79 (3H, m, OCH(CH_3)CH_2*CH_3*).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 9.7 (OCH(CH_3)CH_2*CH_3*), 19.2,19.3,19.4,19.5 (OCH(*CH_3*)CH_2CH_3), 21.1,21.2,21.3 (NHCH*CH_3*), 27.9,28.0 (OCH(CH_3)*CH_2*CH_3), 39.2 (C-2'), 50.5,50.6 (CHNH), 63.1 (C-5'), 74.0,74.1 (O*CH*(CH_3)CH_2CH_3), 79.1,79.2 (C-3'), 87.0,87.1 (C-1'), 87.7,87.8,87.9 (C-4'), 119.9 (C-5), 120.2,120.3, 125.2,129.8 (Ph), 140.4,140.5 (C-8), 149.9 (C-6), 150.4,150.5,150.6 ('ipso' Ph), 153.9 (C-2), 156.4 (C-4), 173.0,173.1 (*C*OOCH(CH_3)CH_2 CH_3).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.48 min.

ESI MS m/z (positive) 569 [M + H], 570 [M(^{13}C) + H], 571 [M(^{37}Cl) + H], 572 [M(^{37}Cl,^{13}C) + H], 591 [M + Na], 592 [M(^{13}C) + Na], 593 [M(^{37}Cl) + Na], 594 [M(^{37}Cl,^{13}C) + Na], 607 [M + K], 608 [M(^{13}C) + K], 609 [M(^{37}Cl) + K].

18r 2CdA-3',5'-bis-[phenyl(2-butoxy-L-alaninyl)] phosphate



$C_{36}H_{48}ClN_7O_{11}P_2$

Mol Wt.: 852.21

Isolated through column chromatography purification of **18p** crude reaction mixture (26 mg, 6.5% yield).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 2.97,2.94,2.82,2.76,1.92,1.91,1.85,1.82,1.81.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.00,7.97 (1H, 2s, H-8), 7.29-7.04 (10H, m, 2 Ph), 6.34-6.21 (1H, m, H-1'), 6.08 (2H, bs, NH_2), 5.30-5.23 (1H, m, H-3'), 4.83-4.73 (2H, m, 2 O*CH*(CH_3)CH_2CH_3), 4.37-4.26 (3H, m, H-4', H-5'), 3.99-3.80 (4H, m, 2 *NH*CH, 2 NH*CH*CH_3), 2.69-2.53 (2H, m, H-2'), 1.60-1.41 (4H, m 2 OCH(CH_3)*CH_2*CH_3), 1.37-1.07 (12H, m, 2 NHCH*CH_3*, 2 OCH(*CH_3*)CH_2CH_3), 0.84-0.75 (6H, m, OCH(CH_3)CH_2*CH_3*).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 9.7 (OCH(CH_3)CH_2*CH_3*), 19.2,19.3,19.4,19.5 (OCH(*CH_3*)CH_2CH_3), 21.0,21.1 (NHCH*CH_3*), 28.6 (OCH(CH_3)*CH_2*CH_3), 39.1 (C-2'), 50.4,50.5 (CHNH), 65.7 (C-5'), 73.8,73.9,74.0 (O*CH*(CH_3)CH_2CH_3), 77.0 (C-3'), 83.9,84.1,84.2 (C-1',C-4'), 119.9 (C-5), 120.2,125.1,125.2,129.7,129.8 (Ph),

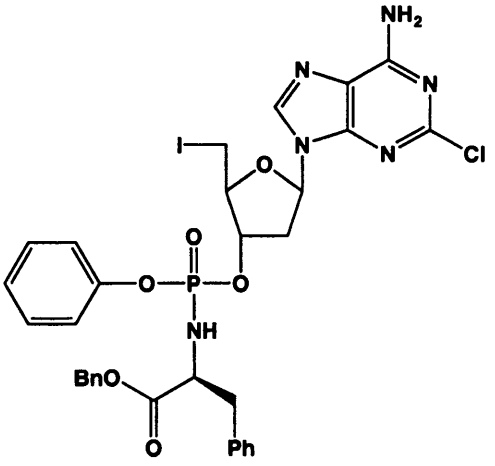
140.4,140.5 (C-8), 150.5 (C-6, 'ipso' Ph), 154.4 (C-2), 156.2 (C-4), 173.0 (*C*OOCH(CH_3)CH_2 CH_3).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 12.96 min.

ESI MS m/z (positive) 852 [M + H], 853 [M(^{13}C) + H], 854 [M(^{37}Cl) + H], 855 [M(^{37}Cl,^{13}C) + H], 874 [M + Na], 875 [M(^{13}C) + Na], 876 [M(^{37}Cl) + Na], 877 [M(^{37}Cl,^{13}C) + Na].

19b 5'-Deoxy-5'-iodo-2CdA-3'-[phenyl(benzoxy-L-phenylalaninyl)] phosphate

$C_{32}H_{31}ClIN_6O_6P$
Mol Wt.: 788.96



Prepared according to Standard Procedure D, from 5'-deoxy-5'-iodo-2CdA (**19a**) (0.15 g, 0.38 mmol) in THF (6 mL), ^{t}BuMgCl (1M solution THF, 0.38 mL, 0.38 mmol), phenyl(benzoxy-L-phenylalaninyl) phosphorochloridate (**20a**) (0.20 g, 0.47 mmol) in THF (1 mL) at -17 °C. The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 97/3) to give the product as a white foamy solid (19 mg, 13.4% yield of reacted starting material).

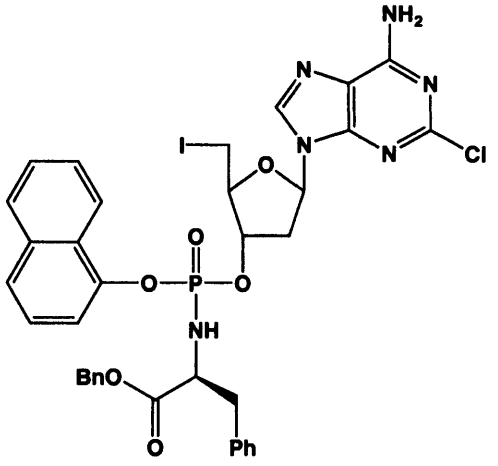
^{31}P-NMR (MeOD, 202 MHz): δ 2.63,2.37.

^{1}H-NMR (MeOD, 500 MHz): δ 8.19,8.16 (1H, 2s, H-8), 7.34-7.08 (15H, m, PhO, *Ph*CH_2CH, *Ph*CH_2O), 6.22,6.21,6.20,6.19 (3H, 4d, ^{3}J=6.3 Hz, H-1'), 5.19-5.10 (2H, m, Ph*C*H_2O), 4.96-4.93 (1H, m, H-3'), 4.28-4.16 (1H, m, PhCH_2*CH*), 4.14-4.11 (1H, m, H-4'), 3.53-3.41 (2H, m, H-5'), 3.15-2.83 (3H, m, Ph*C*H_2CH, one of H-2'), 2.47-2.35 (1H, m, one of H-2').

^{13}C-NMR (MeOD, 125 MHz): δ 5.2,5.0 (C-5'), 38.0 (C-2'), 40.8,40.9,41.1 (Ph*C*H_2CH), 58.0,58.2 (PhCH_2*C*H), 68.0,68.1,68.2 (Ph*C*H_2O), 80.6,80.9,81.0 (C-3'), 86.0 (C-1'), 86.3 (C-4'), 121.4,121.5,126.2,126.3,128.0,128.1,129.4,129.5,129.6, 129.7,130.4,130.7,130.8 (C-5, PhO, *Ph*CH_2O, *Ph*CH_2CH), 137.1 ('ipso' *Ph*CH_2O), 138.1,138.3 ('ipso' *Ph*CH_2CH), 141.7 (C-8), 151.5,152.1 (C-6, 'ipso' PhO), 155.4 (C-2), 158.2 (C-4), 174.1 (*C*O_2CH_2Ph).

19c 5'-Deoxy-5'-iodo-2CdA-3'-[1-naphthyl(benzoxy-L-phenylalaninyl)] phosphate

$C_{36}H_{33}ClIN_6O_6P$
Mol Wt.: 839.02



Prepared according to Standard Procedure D, from 5'-deoxy-5'-iodo-2CdA (**19a**) (0.15 g, 0.38 mmol) in THF (6 mL), ^{t}BuMgCl (1M solution THF, 0.46 mL, 0.46 mmol), 1-naphthyl(benzoxy-L-phenylalaninyl) phosphorochloridate (**11l**) (0.45 g, 0.95 mmol) in THF (2 mL) at -17 °C and stirred for 3 h at room temperature. The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 97/3) to give the product as a white foamy solid (74 mg, 80.4% yield of reacted starting material).

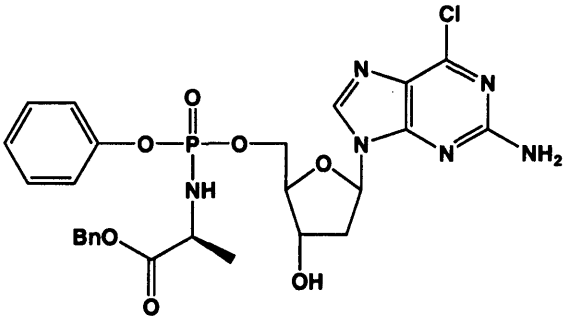
^{31}P-NMR (MeOD, 202 MHz): δ 3.11,2.83.

^{1}H-NMR (MeOD, 500 MHz): δ 8.16,8.07 (2H, m, H-8 Naph, H-8), 7.89-7.88 (1H, m, H-5 Naph), 7.72-7.70 (1H, m, H-4 Naph), 7.56-7.54 (2H, m, H-6 Naph, H-7 Naph), 7.41-7.37 (2H, m, H-3 Naph, H-2 Naph), 7.28-7.16 (10H, m, *Ph*CH_2CH, *Ph*CH_2O), 6.16,6.14,6.11,6.10 (1H, 4d, ^{3}J=6.3 Hz, ^{3}J=6.1 Hz, H-1'), 5.09 (2H, AB

system, ^{2}J=12.2 Hz, Ph*CH*$_2$O), 4.96-4.89 (1H, m, H-3'), 4.34-4.05 (1H, m, H-4'), 3.45-3.35 (2H, m, H-5'), 3.17-3.09 (1H, m, one of Ph*CH*$_2$CH), 2.95-2.78 (2H, m, one of Ph*CH*$_2$CH, one of H-2'), 2.41-2.34 (1H, m, one of H-2').

^{13}C-NMR (MeOD, 125 MHz): δ 5.2,5.0 (C-5'), 38.0 (C-2'), 40.8,40.9,41.0 (Ph*CH*$_2$CH), 58.2,58.4 (PhCH$_2$*CH*), 68.1,68.2 (Ph*CH*$_2$O), 80.8,81.1,81.2 (C-3'), 86.0 (C-1'), 86.2,86.3,86.4 (C-4'), 116.4,116.5,122.8,122.9,126.2,127.6,127.8,127.9, 128.0,128.1,128.9,129.3,129.4,129.5,129.6129.7130.6,130.7 (C-5, NaphO, *Ph*CH$_2$O, *Ph*CH$_2$CH), 136.9,137.0 ('ipso' *Ph*CH$_2$O), 138.0,138.3 ('ipso' *Ph*CH$_2$CH), 141.6,141.7 (C-8), 147.8 ('ipso' Naph), 151.4 (C-6), 155.4 (C-2), 158.2 (C-4), 174.1 (*C*O$_2$CH$_2$Ph).

21a IsoClad-5'-[phenyl(benzoxy-L-alaninyl)] phosphate



$C_{26}H_{28}ClN_6O_7P$
Mol Wt.: 602.96

Prepared according to Standard Procedure C, from IsoClad (**23**) (0.25 g, 0.88 mmol), phenyl(benzoxy-L-alaninyl) phosphorochlori date (**17b**) (0.93 g, 2.64 mmol), NMI (0.36 g, 4.40 mmol, 0.35 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH from 100/0 to 96/4) and preparative TLC (DCM/MeOH 97/3) to give the pure product as a white foamy solid (0.14 g, 27.0 % yield).

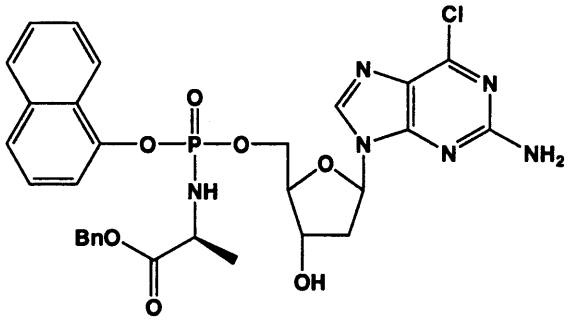
^{31}P-NMR (MeOD, 121 MHz): δ 5.33,5.00.

^{1}H-NMR (MeOD, 300 MHz): δ 8.17,8.16 (1H, 2s, H-8), 7.32-7.13 (10H, m, PhO, *Ph*CH$_2$), 6.37,6.32 (1H, 2d, ^{3}J=6.3 Hz, H-1'), 5.11-5.06 (2H, m, ^{2}J= 12.2 Hz, Ph*CH*$_2$), 4.61-4.56 (1H, m, H-3'), 4.41-4.20 (1H, m, H-5'), 4.18-4.08 (1H, m, H-4'), 4.00-

3.89 (1H, m, *CH*NH), 2.84-2.68 (1H, m, one of H-2'), 2.41-2.30 (1H, m, one of H-2'), 1.30-1.24 (3H, m, CH*CH*$_3$).

^{13}C-NMR (MeOD, 75 MHz): δ 20.6,20.7,20.8 (CH_3), 40.4,40.7 (C-2'), 51.9,52.1 (*CH*CH_3), 67.8,68.1,68.4 (C-5', *CH*$_2$Ph), 72.8 (C-3'), 86.4 (C-1'), 87.1,87.3,87.4 (C-4'), 121.7,121.8, 125.8,126.5,129.7,129.9,131.1 (*Ph*CH_2, *Ph*O), 137.5,137.6 ('ipso' *Ph*CH_2), 143.2,143.3 (C-8), 152.0 (C-6), 152.4,152.5 ('ipso' PhO), 155.0,155.1 (C-2), 161.9 (C-4), 175.0,175.2 (*C*OOCH$_2$Ph).

21b IsoClad-5'-[1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{30}H_{30}ClN_6O_7P$
Mol Wt.: 653.02

Prepared according to Standard Procedure D, from IsoClad (**23**) (0.20 g, 0.70 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.70 mL, 0.70 mmol), 1-naphthyl(benzoxy-L-alaninyl) phosphorochloridate (**8c**) (0.28 g, 0.70 mmol). The crude was purified by column chromatography (DCM/MeOH 97/3) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (51 mg, 22.4% yield of reacted starting material).

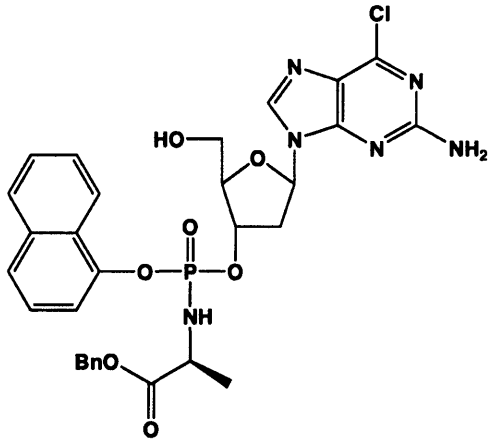
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.87,3.47.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.99-7.92 (1H, m, H-8 Naph), 7.77-7.69 (1H, m, H-8, H-5 Naph), 7.55-7.50 (1H, m, H-4 Naph), 7.41-7.33 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.27-7.14 (6H, m, H-3 Naph, Ph), 6.08 (1H, t, ^{3}J=6.5 Hz, H-1'), 5.50,5.37 (2H, 2bs, NH_2), 5.02-4.90 (2H, m, ^{2}J=12.2 Hz, *CH*$_2$Ph), 5.62-4.57 (1H, m, H-3'), 4.41-4.38 (1H, m, H-4'), 4.29-3.98 (4H, m, H-5', *CH*NH, CH*NH*), 3.75 (1H, bs, OH-3'), 2.62-2.24 (2H, m, H-2'), 1.24-1.21 (3H, m, CH*CH*$_3$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.6,20.7 (CH*C*H_3), 38.8,39.1 (C-2'), 50.4,50.6 (*C*HNH), 66.2,66.3 (C-5'), 67.4 (*C*H_2Ph), 71.5 (C-3'), 84.6,84.7 (C-1'), 85.0,85.1,85.2,85.3 (C-4'), 115.0.115.1,121.3,125.1,125.3,125.5,125.7,126.3,126.5, 126.8,127.8,127.9,128.1,128.2,128.5,128.6,134.7,135.1 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, Ph, 'ipso' CH_2Ph), 140.9,141.0 (C-8), 146.3,146.4 ('ipso' Naph), 151.3,151.4,152.9, 153.0 (C-2, C-6), 159.2 (C-4), 173.3,173.6 (*C*OOCH_2Ph).
HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.68 min.
ESI MS m/z (positive) 654 [M + H], 656 [M(^{37}Cl) + H], 676 [M + Na], 678 [M(^{37}Cl) + Na], 692 [M + K].

21c IsoClad-3'-[1-naphthyl(benzoxy-L-alaninyl)] phosphate

$C_{30}H_{30}ClN_6O_7P$
Mol Wt.: 653.02



Isolated through column chromatography purification of **21b** crude reaction mixture (15 mg, 6.6% yield of reacted starting material).
^{31}P-NMR ($CDCl_3$, 202 MHz): δ 2.04,1.88 (int. 0.2,0.8).
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.05-8.03 (1H, m, H-8 Naph), 7.82-7.80 (1H, m, H-5 Naph), 7.67 (0.2H, s, H-8), 7.61 (1H, d, ^{3}J=8.2 Hz, H-4 Naph), 7.49-7.45 (3H, m, H-2 Naph, H-6 Naph, H-7 Naph), 7.39 (0.8H, s, H-8), 7.35-7.18 (6H, m, H-3 Naph, Ph), 5.99-5.96 (0.2H, m, H-1'), 5.60,5.57 (1H, 2bs, OH-5'), 5.56-5.53 (0.8H, m, H-1'), 5.30 (0.2H, m, H-3'), 5.25 (0.8H, m, H-3'), 5.20,5.19 (2H, 2bs, NH_2), 5.09

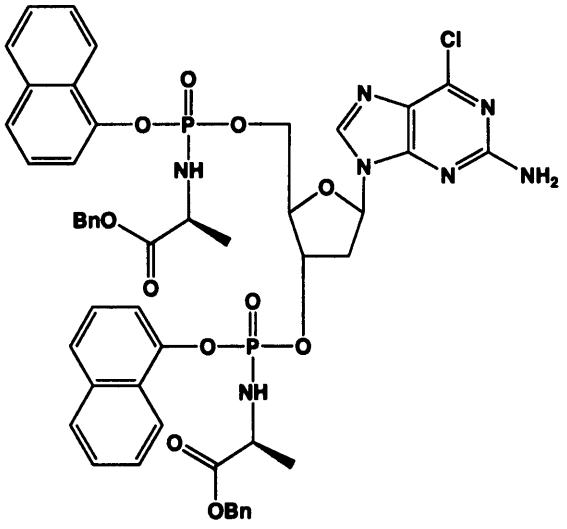
(1.6H, AB system, ^{2}J=12.2 Hz, *CH*$_2$Ph), 5.02,4.97 (0.4H, AB system, ^{2}J=12.2 Hz, *CH*$_2$Ph), 4.24-4.07 (2H, m, *CH*CH$_3$, H-4'), 3.91-3.87 (1H, m, *NH*CH), 3.78-3.59 (2H, m, H-5'), 3.00-3.92 (0.2H, m, H-2'), 2.85-2.79 (0.8H, m, H-2'), 2.49-2.45 (0.2H, m, H-2'), 2.11-2.08 (0.8H, m, H-2'), 1.37-1.35 (3H, m, CH*CH*$_3$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.9,21.0 (CH*CH*$_3$), 38.6 (C-2'), 50.5 (CHNH), 66.3.0 (C-5'), 67.4 (*CH*$_2$Ph), 79.4,79.5 (C-3'), 86.9,87.0 (C-1'), 87.3,87.4 (C-4'), 115.5,115.8,115.9,121.3,121.4,125.2,125.7,126.5,126.6,126.7,126.8,128.0,128.1,128 .2,128.5,128.6,128.7,134.8,135.2 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, 'ipso' CH_2Ph), 141.6,141.7 (C-8), 146.0 ('ipso' Naph), 152.7 (C-6), 155.0 (C-2), 158.3 (C-4), 173.2 (*C*OOCH$_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 11.31 min.

ESI MS m/z (positive) 654 [M + H], 656 [M(^{37}Cl) + H], 676 [M + Na], 678 [M(^{37}Cl) + Na].

21d IsoClad-3',5'-bis-[1-naphthyl(benzoxy-L-alaninyl)] phosphate



$C_{50}H_{48}ClN_7O_{11}P_2$
Mol Wt.: 1020.36

Isolated through column chromatography purification of **21b** crude reaction mixture (0.14 g, 62.7% yield of reacted starting material).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.20,3.16,2.59,2.56,1.33,1.29,1.28,1.24.

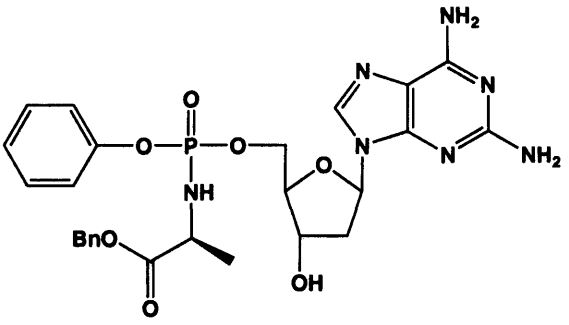
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.27-6.31 (25H, m, H-8, 2 Naph, 2 Ph), 5.23-5.17 (0.5H, m, H-1'), 5.97-5.92 (0.5H, m, H-1'), 4.63-4.50 (3H, m, H-3', NH_2), 4.25-4.04 (4H, m, 2 CH_2Ph), 3.79-3.09 (7H, m, H-4', H-5', 2 *CH*NH, 2 *NH*CH), 2.12-2.00 (1H, m, H-2'), 1.70-1.62 (0.5H, m, H-2'), 1.49-1.41 (0.5H, m, H-2'), 0.51-0.33 (6H, m, 2 CHCH_3).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.0,20.1 (2 CHCH_3), 36.2 (C-2'), 49.6,49.8,49.9 (2 CHNH), 64.6,64.8,64.9 (C-5'), 66.5,66.6 (2 CH_2Ph), 77.1 (C-3'), 82.8,82.9,83.0 (C-4'), 84.2,84.3,84.5 (C-1'), 114.3,114.4,114.6,114.7,114.8,120.6,120.7,124.1,124.2, 124.4,124.6,124.8,125.2,125.6,125.7,125.8,125.9,126.0,127.0,127.1,127.2,127.3, 127.4,127.6,127.7,127.8,127.9,133.9,134.0,134.4 (C-5, 2 Naph, 2 Ph), 140.3,140.5 (C-8), 145.6 (2 'ipso' Naph), 150.9 (C-6), 152.0,152.1,152.2 (C-2), 158.3 (C-4), 172.4,172.6 (2 *C*OOCH$_2$Ph).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 14.40 min.

ESI MS m/z (positive) 1022 [M + H], 1023 [M(^{13}C) + H], 1024 [M(^{37}Cl) + H], 1044 [M + Na], 1045 [M(^{13}C) + Na], 1046 [M(^{37}Cl) + Na].

21e DAPdR-5'-[phenyl(benzoxy-L-alaninyl)] phosphate



$C_{26}H_{30}N_7O_7P$

Mol Wt.: 583.53

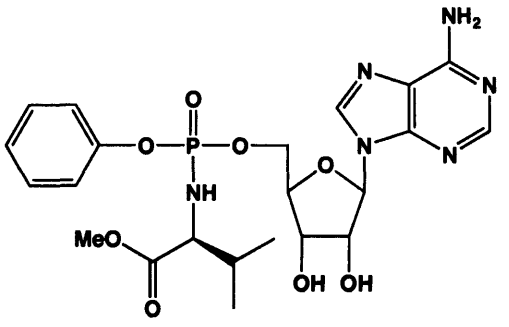
Prepared according to Standard Procedure D, from DAP (**24**) (0.10 g, 0.38 mmol) in THF (10 mL), ^{t}BuMgCl (1M solution THF, 0.38 mL, 0.38 mmol), phenyl(benzoxy-L-alaninyl) phosphorochloridate (**17b**) (0.13 g, 0.38 mmol). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (15 mg, 10.7% yield of reacted starting material).

^{31}P-NMR (MeOD, 202 MHz): δ 3.99,3.44.

^{1}H-NMR (MeOD, 500 MHz): δ 8.24,8.17 (1H, 2s, H-8), 7.33-7.28 (10H, m, PhO, CH_2*Ph*), 6.38,6.34 (1H, 2t, ^{3}J=6.6 Hz, H-1'), 5.14-5.02 (2H, m, *CH_2*Ph), 4.64-4.61,4.56-4.53 (1H, 2m, H-3'), 4.35-4.28 (2H, m, H-5'), 4.17-4.14,4.12-4.11 (H, 2m, H-4'), 4.01-3.90 (1H, m, *CH*NH), 2.73-2.40 (2H, m, H-2'), 1.32-1.28 (3H, m, CH*CH_3*).

HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 15 min): Rt 10.01 min.

25a Adenosine-5'-[phenyl(methoxy-L-valinyl)] phosphate



$C_{22}H_{29}N_6O_8P$
Mol Wt.: 536.47

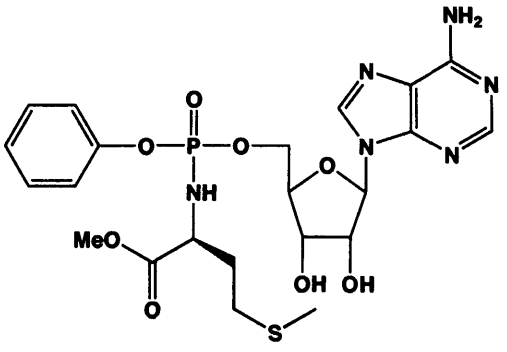
To a suspension of adenosine (0.43 g, 1.60 mmol) in anhydrous THF/Pyridine (4/3, 16 mL) and NMI (0.66 g, 8.00 mmol, 0.64 mL) a solution of phenyl(methoxy-L-valinyl) (**26a**) phosphorochloridate (1.22 g, 3.99 mmol) in anhydrous THF (1.22 mL) was added dropwise at -78 °C. After 15 min the reaction was let to rise to room temperature and stirred for 24 h. The crude was ripetitively purified by column chromatography (DCM/MeOH 9/1, 95/5, 97/3) to give the product as a white foamy solid (0.11 g, 13.3 %yield). A portion of the product (20 mg) was further purified by preparative TLC (DCM/MeOH 95/5) and used for the biological evaluation (9 mg).

^{31}P-NMR (MeOD, 121 MHz): δ 5.88,5.79.

^{1}H-NMR (MeOD, 300 MHz): δ 8.28,8.26 (1H, 2s, H-8), 8.20 (1H, s, H-2), 7.35-7.14 (5H, m, Ph), 6.08,6.06 (1H, 2d, H-1'), 4.70-4.66 (1H, m, H-2'), 4.46-4.40 (3H, m, H-3', H-5'), 4.31-4.29 (1H, m, H-4'), 3.82-3.73 (1H, m, *CH*NH), 3.62,3.59 (3H, 2s, OCH_3), 2.02-1.95 (1H, m, *CH*$(CH_3)_2$), 0.93-0.79 (6H, m, CH(*CH*$_3)_2$).

^{13}C-NMR (MeOD, 75 MHz): δ 18.6,18.8,19.0,19.8,19.9 (CH(*CH*$_3)_2$), 33.3,33.4,33.5, 33.6 (*CH*$(CH_3)_2$), 52.9 (CH_3O), 62.2,62.3 (*CH*NH), 67.6,67.7,68.1 (C-5'), 71.9,72.0 (C-3'), 75.8 (C-2'), 84.7,84.8,84.9 (C-4'), 90.3,90.4 (C-1'), 120.9 (C-5), 121.7,121.8, 124.1,126.6,130.5,131.1 (Ph), 141.4 (C-8), 151.1 (C-6), 152.4,152.5,152.6 ('ipso' Ph), 154.4 (C-2), 157.7 (C-4), 174.9,175.2 (*C*$OOCH_3$).

25b Adenosine-5'-[phenyl(methoxy-L-methioninyl)] phosphate



$C_{22}H_{29}N_6O_8P$

Mol Wt.: 536.47

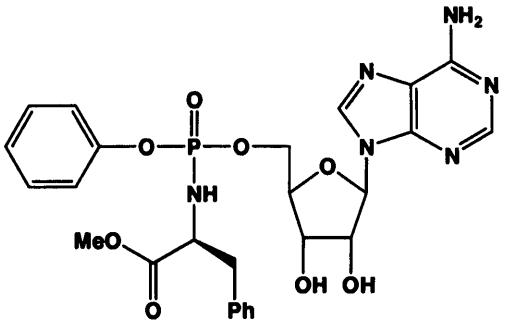
To a suspension of adenosine (0.53 g, 1.96 mmol) in anhydrous THF/Pyridine (4/3, 16 mL) and NMI (0.81 g, 9.80 mmol, 0.77 mL) a solution of phenyl(methoxy-L-methioninyl) phosphorochloridate (**26b**) (2.64 g, 7.82 mmol) in anhydrous THF (2.6 mL) was added dropwise at -78 °C. After 15 min the reaction was let to rise to room temperature and stirred for 21 h. The crude was ripetitively purified by column chromatography (DCM/MeOH 9/1, 95/5, 97/3) to give the product as a white foamy solid (90 mg, 13,3 %yield). A portion of the product (19 mg) was further purified by preparative TLC (DCM/MeOH 93/7) and used for the biological evaluation (14 mg).

^{31}P-NMR (MeOD, 121 MHz): δ 5.31,5.09.

^{1}H-NMR (MeOD, 300 MHz): δ 8.31,8.26 (1H, 2s, H-8), 8.22 (1H, s, H-2), 7.37-7.18 (5H, m, Ph), 6.06 (1H, 2d, H-1'), 4.69-4.66 (1H, m, H-2'), 4.46-4.32 (4H, m, H-3', H-4', H-5'), 4.02 (1H, m, *CH*NH), 3.68-3.65 (3H, 2s, OCH_3), 2.48-2.44 (1H, m, one of CH_2S), 2.35 (1H, m, one of CH_2S), 1.99,1.97 (3H, 2s, SCH_3), 1.61 (2H, m, $CH_2CH_2SCH_3$).

^{13}C-NMR (MeOD, 75 MHz): δ 15.5 (SCH_3), 31.1,31.2 (CH_2S), 34.3,34.4 (CH_2CH_2S), 53.2 (OCH_3), 55.1,55.2 (*CH*NH), 67.7 (C-5'), 71.9 (C-3'), 75.7,75.8 (C-2'), 84.8,84.9 (C-4'), 90.3 (C-1'), 120.9 (C-5), 121.7,121.8,126.6,131.2 (Ph), 141.4 (C-8), 151.0 (C-6), 153.0 ('ipso' Ph), 154.4 (C-2), 157.7 (C-4), 175.3 ($COOCH_3$).

25c Adenosine-5'-[phenyl(methoxy-L-phenylalaninyl)] phosphate



$C_{26}H_{29}N_6O_8P$

Mol Wt.: 584.52

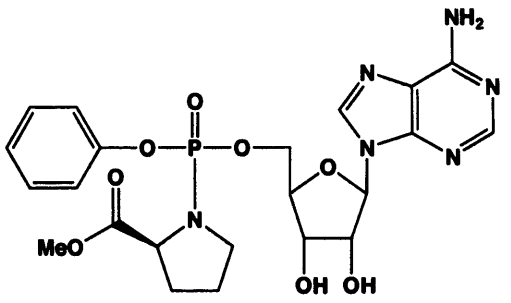
To a suspension of adenosine (0.85 g, 3.17 mmol) in anhydrous THF/Pyridine (4/3, 26 mL) and NMI (1.30 g, 15.85 mmol, 1.26 mL) a solution of phenyl(methoxy-L-phenylalaninyl) phosphorochloridate (**26c**) (3.36 g, 9.51 mmol) in anhydrous THF (3.4 mL) was added dropwise at -78 °C. After 15 min the reaction was let to rise to room temperature and stirred for 15 h. The crude was ripetitively purified by column chromatography (DCM/MeOH 95/5 and 97/3) and preparative TLC (DCM/MeOH 95/5) to give the product as a white foamy solid (47 mg, 2.5 %yield).

^{31}P-NMR (MeOD, 121 MHz): δ 4.96,4.60.

^{1}H-NMR (MeOD, 300 MHz): δ 8.2-8.19 (2H, m, H-8, H-2), 7.29-7.07 (10H, m, PhO, CH_2*Ph*), 6.05-6.01 (1H, 2d, H-1'), 4.61-4.60 (1H, m, H-2'), 4.30 (1H, m, H-3'), 4.20-3.90 (4H, m, H-4', H-5', *CH*NH), 3.62,3.59 (3H, 2s, OCH_3), 3.20-3.00 (1H, m, one of *CH*$_2$Ph), 2.87-2.80 (1H, m, one of *CH*$_2$Ph).

^{13}C-NMR (MeOD, 75 MHz): δ 41.3 (*CH*$_2$Ph), 53.0 (OCH_3), 58.1 (CHNH), 67.3 (C-5'), 71.8 (C-3'), 75.7 (C-2'), 84.8 (C-4'), 90.2 (C-1'), 120.9 (C-5), 121.5,121.6,121.8,126.4,128.3,129.9,130.9,131.9 (OPh, CH_2*Ph*), 138.4 ('ipso' CH_2*Ph*), 141.3 (C-8), 151.1 (C-6), 152.9 ('ipso' OPh), 154.4 (C-2), 157.7 (C-4), 175.3 (*C*$OOCH_3$).

25d Adenosine-5'-[phenyl(methoxy-L-prolinyl)] phosphate



$C_{22}H_{27}N_6O_8P$

Mol Wt.: 534.46

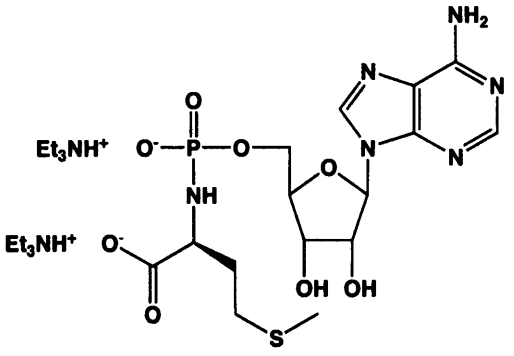
To a suspension of adenosine (0.35 g, 1.30 mmol) in anhydrous THF/Pyridine (4/3, 14 mL) and NMI (0.53 g, 6.5 mmol, 0.52 mL) a solution of phenyl(methoxy-L-prolinyl) phosphorochloridate (**26d**) (1.12 g, 3.68 mmol) in anhydrous THF (1.5 mL) was added dropwise at -78 °C. After 15 min the reaction was let to rise to room temperature and stirred for 18 h. The crude was ripetitively purified by column chromatography (DCM/MeOH 9/1 and 97/3) and preparative TLC (DCM/MeOH 92/8) to give the product as a white foamy solid (0.11 mg, 24.5 %yield).

^{31}P-NMR (MeOD, 121 MHz): δ 3.06,2.92.

^{1}H-NMR (MeOD, 300 MHz): δ 8.32-8.21 (2H, m, H-8, H-2), 7.39-7.17 (5H, m, PhO), 6.10-6.04 (1H, 2d, H-1'), 4.70-4.20 (6H, m, H-2', H-3', H-4', H-5', H-2 *prol*), 3.74,3.72 (3H, 2s, OCH_3), 3.38-2.83 (2H, m, H-5 *prol*), 2.21-1.71 (4H, m, H-3 *prol*, H-4 *prol*).

^{13}C-NMR (MeOD, 75 MHz): δ 26.4,26.5,26.6 (C-4 *prol*), 31.3,32.4,32.5,32.6,32.7 (C-3 *prol*), 47.9,48.4,49.1 (C-5 *prol*), 53.1,53.2 (OCH_3), 62.4,62.5 (C-2 *prol*), 67.4,67.5 (C-5'), 71.8,71.9 (C-3'), 75.6,76.0 (C-2'), 84.6,84.7 (C-4'), 90.3,90.6 (C-1'), 120.8 (C-5), 121.4,121.5,121.7,121.8,126.7,131.2,131.4 (OPh,), 141.3,141.5 (C-8), 151.0,151.1 (C-6), 152.3,152.4,152.5 ('ipso' OPh), 154.4 (C-2), 157.7 (C-4), 175.6,175.9,176.3 ($COOCH_3$).

25e Adenosine-5'-(L-methioninyl) phosphate



$C_{27}H_{53}N_8O_8PS$

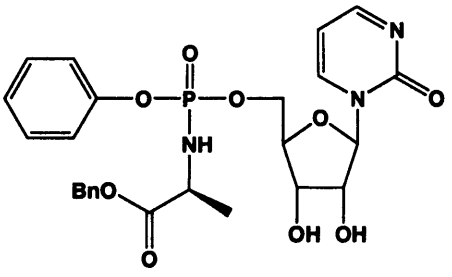
Mol Wt.: 680.80

Adenosine-5'-[phenyl(methoxy-L-methioninyl)] phosphate (**25b**) (65 mg, 0.11 mmol) was suspended in TEA/H_2O (4/1, 1.7 mL). The reaction was stirred at room temperature for 24 h. The crude was reduced to dryness and purified by preparative TLC (DCM/MeOH 7/3) to give the pure product as white foamy solid (39 mg, 52% yield).

^{31}P-NMR (MeOD, 121 MHz): δ 8.57.

^{1}H-NMR (MeOD, 300 MHz): δ 8.62 (1H, s, H-8), 8.20 (1H, s, H-2), 6.11 (1H, d, ^{3}J=5.9 Hz, H-1'), 4.79 (1H, t, H-2'), 4.42-4.39 (1H, m, H-3'), 4.22 (1H, m, H-4'), 4.05 (2H, m, H-5'), 3.73-3.70 (1H, m, *CH*NH), 3.12 (q, $(CH_3CH_2)_3NH^+$), 2.63-2.65 (2H, m, CH_2S), 2.03 (3H, s, SCH_3), 2.00-1.90 (2H, m, CH_2CH_2S), 1.28 (t, $(CH_3CH_2)_3NH^+$).

27a Zebularine-5'-[phenyl(benzoxy-L-alaninyl)] phosphate



$C_{25}H_{28}N_3O_9P$

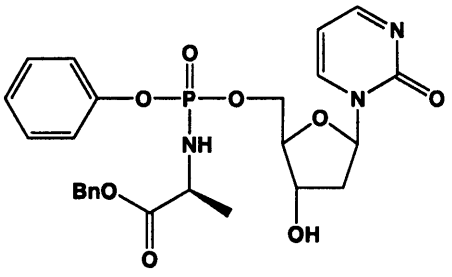
Mol Wt.: 545.48

Prepared according to Standard Procedure C, from Zeb (0.20 g, 0.87 mmol), phenyl(benzoxy-L-alaninyl) phosphorochloridate (**17b**) (0.92 g, 2.61 mmol), NMI (0.36 g, 4.35 mmol, 0.35 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) and preparative TLC (DCM/MeOH 95/5) to give the pure product as a white foamy solid (24 mg, 5.1% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.07,3.99.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.58-8.53 (1H, m, H-6), 8.35-8.29 (1H, m, H-4), 7.39-7.17 (10H, m, PhO, *Ph*CH_2), 6.43-6.40 (0.5H, m, one of H-5), 6.33-6.30 (0.5H, m, one of H-5), 5.89,5.84 (1H, 2d, H-1'), 5.19-5.14 (2H, m, Ph*CH_2*), 4.53-4.01 (8H, m, H-3', H-4', H-5', OH-2', OH-3', *CH*NH, CH*NH*), 1.44-1.39 (3H, m, CH*CH_3*).

27b Deoxyzebularine-5'-[phenyl(benzoxy-L-alaninyl)] phosphate



$C_{25}H_{28}N_3O_8P$

Mol Wt.: 529.48

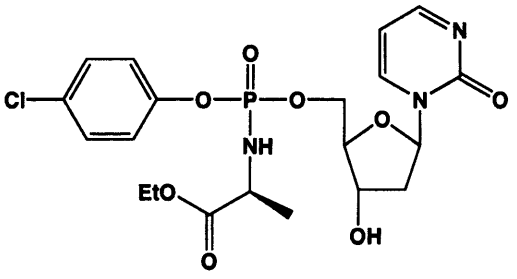
Prepared according to Standard Procedure C, from dZeb (0.20 g, 0.94 mmol), phenyl(benzoxy-L-alaninyl) phosphorochloridate (**17b**) (1.00 g, 2.83 mmol), NMI (0.39 g, 4.70 mmol, 0.37 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 96/4) and preparative TLC (DCM/MeOH 96/4) to give the pure product as a white foamy solid (70 mg, 14.1% yield).

^{31}P-NMR ($CDCl_3$, 121 MHz): δ 4.28,4.09.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.54-8.46 (1H, m, H-6), 8.25-8.21 (1H, m, H-4), 7.38-7.20 (10H, m, PhO, *Ph*CH_2), 6.32-6.18 (2H, m, H-5, H-1'), 5.22-5.14 (2H, m, Ph*CH_2*), 4.48-4.03 (7H, m, H-3', H-4', H-5', OH-3', *CH*NH, CH*NH*), 2.78-2.69 (1H, m, one of H-2'), 2.12-1.99 (1H, m, one of H-2'), 1.45-1.42 (3H, m, CH*CH_3*).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ 19.6,19.7,19.8 (CH_3), 40.2 (C-2'), 40.3,40.4,40.5 (*CH*CH_3), 64.6,64.7,64.8 (C-5'), 66.2,66.3 (*CH_2*Ph), 68.8,68.9 (C-3'), 84.4,84.5,84.6,84.7 (C-4'), 86.6 (C-1'), 103.3 (C-5), 119.0,119.1,119.3,124.2,127.2, 127.5,127.6,127.7,128.7,128.8 (OPh,), 134.2 ('ispo' CH_2Ph), 142.5,142.6 (C-4), 149.4,149.5 ('ipso' OPh), 164.6,164.8 (C-6), 172.3,172.4 (*C*OOCH_2Ph).

27c Deoxyzebularine-5'-[4-chloro-phenyl(ethoxy-L-alaninyl)] phosphate



$C_{20}H_{25}ClN_3O_8P$

Mol Wt.: 501.85

Prepared according to Standard Procedure C, from dZeb (0.19 g, 0.88 mmol), 4-chloro-phenyl(ethoxy-L-alaninyl) phosphorochloridate (**29**) (0.86 g, 2.64 mmol), NMI (0.36 g, 4.40 mmol, 0.35 mL) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 95/5) to give the pure product as a white foamy solid (88 mg, 20.0% yield).

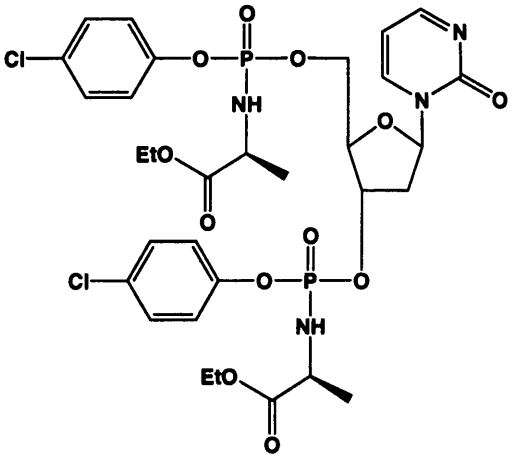
^{31}P-NMR ($CDCl_3$, 250 MHz): δ 3.32,3.23.

^{1}H-NMR ($CDCl_3$, 250 MHz): δ 8.53-8.45 (1H, m, H-6), 8.19-8.09 (1H, m, H-4), 7.22-7.13 (4H, m, pCl-Ph), 6.30-6.10 (2H, m, H-5, H-1'), 4.40-3.83 (9H, m, H-3', H-4', H-5', OH-3', *CH*NH, CH*NH*, OCH_2CH_3), 2.73-2.61 (1H, m, one of H-2'), 2.09-1.99 (1H, m, one of H-2'), 1.42-1.39 (3H, m, $CHCH_3$), 1.20-1.11 (3H, m, OCH_2CH_3).

^{13}C-NMR ($CDCl_3$, 250 MHz): δ 14.1 (CH_3CH_2O), 20.8 (CH_3CH), 41.2 (C-2'), 50.3,50.4 ($CHCH_3$), 61.8 (CH_3CH_2O), 65.9 (C-5'), 69.8,70.0 (C-3'), 85.4,85.5 (C-4'), 87.7 (C-1'), 121.4,121.5,121.6,129.8 (C-5, pCl-Ph), 130.5 (C-2), 143.6 (C-4), 149.0 (C-4 pCl-Ph), 155.5 (C-1 pCl-Ph), 165.7 (C-6), 173.6 (CO_2Et).

HPLC (H_2O/CH_3CN from 70/30 to 0/100 in 10 min): Rt 4.61 min.

27d Deoxyzebularine-3',5'-bis-[phenyl(ethoxy-L-alaninyl)] phosphate



$C_{31}H_{38}Cl_2N_4O_{12}P_2$
Mol Wt.: 791.51

Isolated through column chromatography purification of **27c** crude reaction mixture and purified by preparative reverse phase HPLC (40 mg, 9.1% yield).

^{31}P-NMR ($CDCl_3$, 250 MHz): δ 3.11,3.09,3.04,2.47,2.42,1.89,1.84.

^{1}H-NMR ($CDCl_3$, 250 MHz): δ 8.51-8.42 (1H, m, H-6), 8.18-8.03 (1H, m, H-4), 7.28-7.01 (8H, m, 2 pCl-Ph), 6.30-6.03 (2H, m, H-5, H-1'), 5.21-4.99 (1H, m, H-3'), 4.42-3.82 (9H, m, H-4', H-5', 2 *CH*NH, 2 CH*NH*, 2 OCH_2CH_3), 2.97-2.78 (1H, m, one of H-2'), 2.14-1.98 (1H, m, one of H-2'), 1.37-1.11 (12H, m, 2 $CHCH_3$ 2 OCH_2CH_3).

^{13}C-NMR ($CDCl_3$, 250 MHz): δ 14.1 (2 CH_3CH_2O), 20.6,20.8,20.9 (2 CH_3CH), 39.8,39.9, 40.0 (C-2'), 50.2,50.3,50.4,50.5 (2 $CHCH_3$), 61.8,61.9 (2 CH_3CH_2O), 65.6,65.7,66.8,66.9 (C-5'), 76.7,77.0,77.1 (C-3'), 84.2,84.3 (C-4'), 87.3,87.4 (C-1'), 121.3,121.5,121.6,129.7,129.8 (C-5, 2 pCl-Ph), 130.7 (C-2), 143.8,144.0,144.1 (C-4), 149.1,149.2 (2 C-4 pCl-Ph), 155.3,155.4 (2 C-1 pCl-Ph), 165.5,165.6 (C-6), 173.4,173.5,173.7 (CO_2Et).

HPLC (H_2O/CH_3CN from 70/30 to 0/100 in 10 min): Rt 7.21 min.

[1] Molecular Operating Environment 2004.03 (MOE) Chemical Computing Group Inc Montreal Quebec Canada http://www.chemcomp.com

[2] Tripos SYBYL 7.0, Tripos Inc 1699 South Hanley Rd St Louis Missouri 63144 USA http://www.tripos.com

[3] Berendsen, H.J.C.; Van der Spoel, D.; Van Drunen, R. GROMACS: A message-passing parallel molecular dynamics implementation. *Comp Phys Comm* **1995**, 91, 43-56.

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[5] Humphrey, W.; Dalke, A.; Schulten, K. VMD - Visual Molecular Dynamics. *J Molecular Graphics* **1996**, 14, 33-38.

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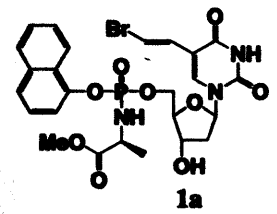
[7] Janeba, Z.; Francom, P.; Morris, J. R. Efficient synthesis of 2-chloro-2'-deoxyadenosine (cladribine) from 2'-deoxyguanosine. *J. Org. Chem.* **2002**, 68, 989-992.

Appendix I: Structures of final compounds



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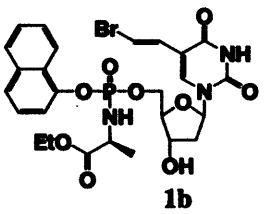

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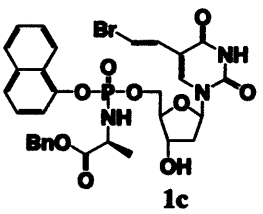

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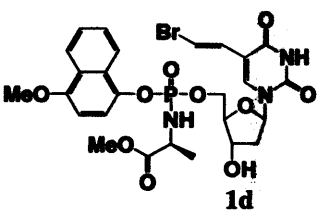

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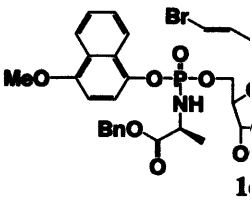

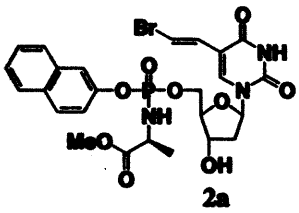

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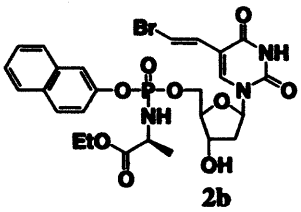

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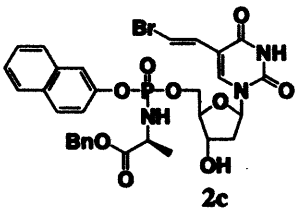

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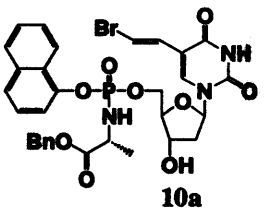

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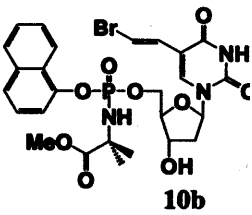

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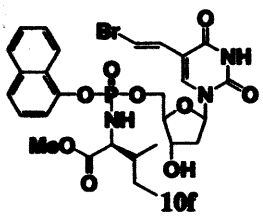

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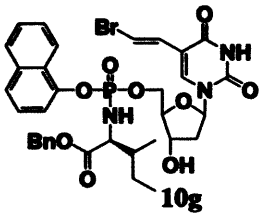

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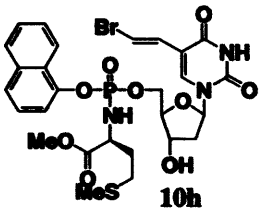

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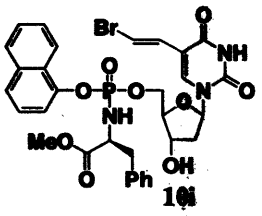

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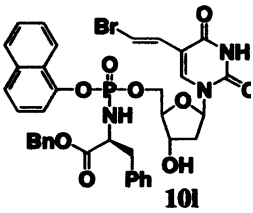

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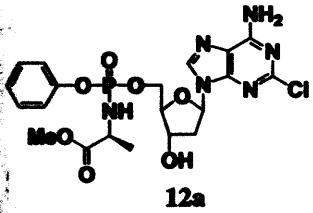

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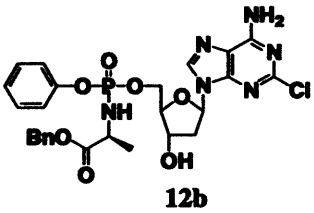

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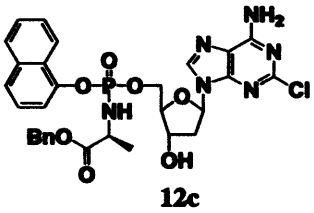

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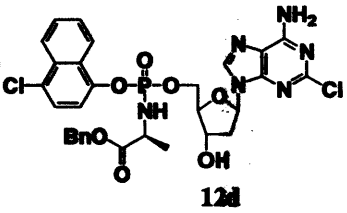

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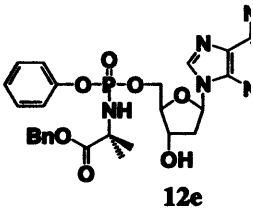

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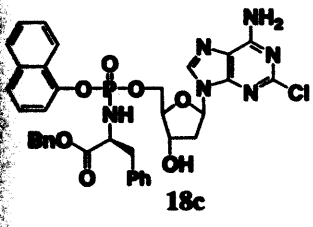

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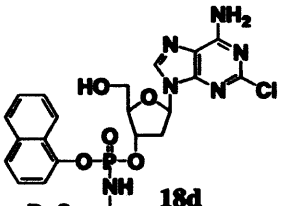

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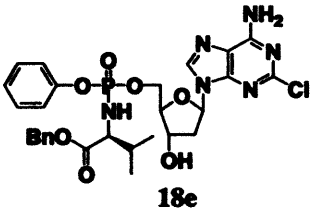

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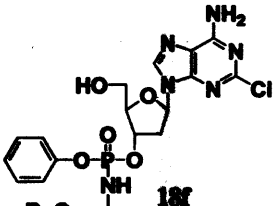

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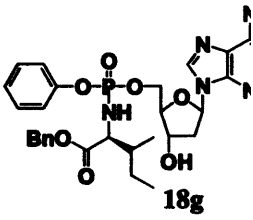

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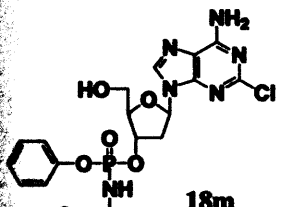

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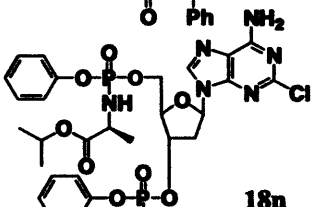

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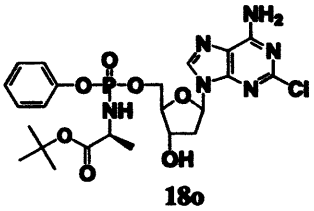

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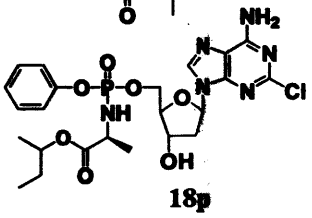

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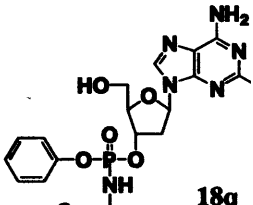

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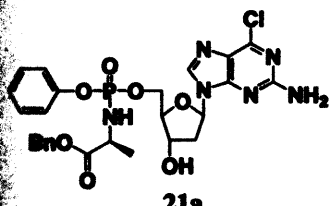

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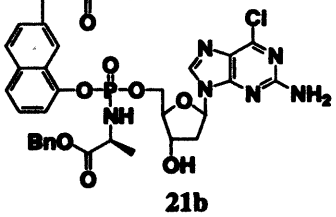

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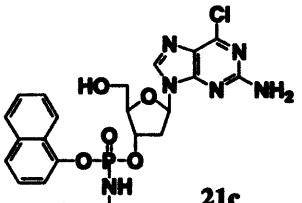

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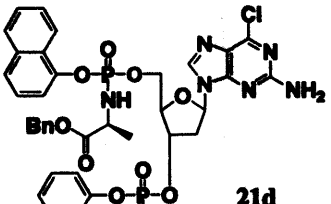

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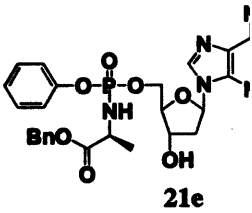

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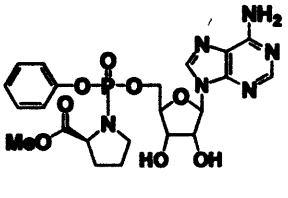

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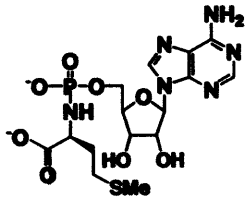

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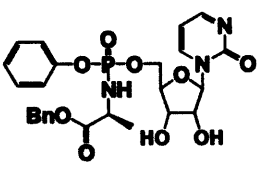

27a

Appendix II: Publications

Congiatu, C.; McGuigan, C; Jiang, W. G.; Davies, G.; Mason, M. D. Naphthyl phosphoramidate derivatives of BVdU as potential anticancer agents: design, synthesis and biological evaluation. *Nucleosides Nucleotides and Nucleic Acids* **2005**, 24, 485-489.

Congiatu, C.; Brancale, A.; Mason, M. D.; Jiang, W. G.; McGuigan, C. Novel potential anticancer naphthyl phosphoramidates of BVdU: separation of diastereoisomers and assignement of the absolute configuration of the phosphorus center. *J. Med. Chem.* **2006**, 49, 452-455.

Nucleosides, Nucleotides, and Nucleic Acids, 24 (5–7):485–489, (2005)

ISSN: 1525-7770 print/ 1532-2335 online
DOI: 10.1081/NCN-200061774

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NAPHTHYL PHOSPHORAMIDATE DERIVATIVES OF BVdU AS POTENTIAL ANTICANCER AGENTS: DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION

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W. G. Jiang and G. Davies ▫ *Metastasis Research Group, University of Wales College of Medicine, Cardiff, UK*

M. D. Mason ▫ *Section of Oncology, University of Wales College of Medicine, Cardiff, UK*

▫ *The phosphoramidate technology we have developed has been recently applied to BVdU, leading to NB1011 (NewBiotics Inc., California), a novel potential anticancer compound recently entered into phase 2 of the clinical trials for colon cancer. We report in this work a new series of derivatives containing naphthol as aryl masking group on the phosphate moiety, which has shown a significant increase in anticancer activity in preliminary biological evaluations.*

Keywords Phosphoramidate, Protide, Anticancer

INTRODUCTION

The phosphoramidate approach was conceived as a means to improve cellular penetration of nucleotides and to bypass the first step of kinase-mediated activation of nucleosides. It has been observed that the ability of phosphoramidate protides to deliver the monophosphate derivative can lead to an impressive boost in activity compared with the corresponding nucleoside.[1] NewBiotics discovered a surprising anticancer activity in a phosphoramidate derivative of the anti-herpetic (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU)[2,3] that we synthesized and tested only as potential antiviral prodrug[4] (Figure 1).

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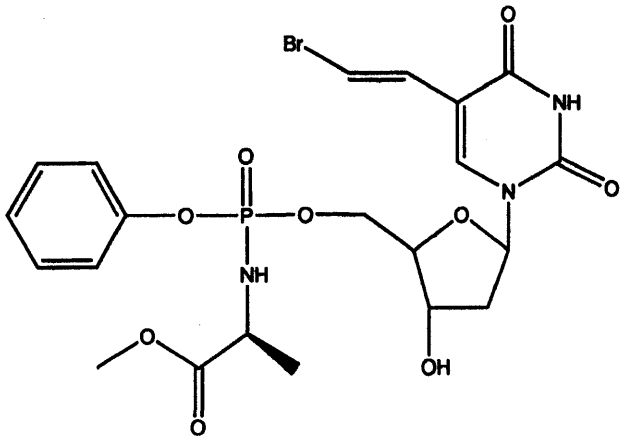


FIGURE 1 NB1011.

Following this, a new series of BVdU phosphoramidates was designed considering naphthol as a new aromatic moiety, and the effect of an electron withdrawing and a donating substituent has also been investigated (Table 1 and Figure 2).

Furthermore, in order to identify structure-activity relationshi ps, different amino acids containing alternative ester moieties have been investigated (Table 2 and Figure 3).

The synthesis of BVdU-5′-phosphoramidate compounds follows the procedure of Van Boom et al.,[5] further developed by McGuigan.[1] Phosphorylation of the corresponding naphthol derivatives with phosphorus oxychloride, followed by coupling with different esterified amino acid salts, gave aryloxy-phosphochloridates, which were generally purified by flash chromatography and then coupled with BVdU in the presence of 1-methylimidazole (NMI). Due to the chirality of the phosphorus center, the final products were obtained as mixtures of two diastereoisomers. In the following scheme, CPF 98 has been chosen to show the standard procedure used for the synthesis of naphthyl-phosphoramidates (Scheme 1).

TABLE 1 BVdU-Phosphoramidates

Compound	α/β Naphthol	X	R
CPF 96	α	H	Me
CPF 97	α	H	Et
CPF 98	α	H	Bn
CPF 99	β	–	Me
CPF 100	β	–	Et
CPF 101	β	–	Bn
CPF 102	α	Cl	Me
CPF 104	α	MeO	Me

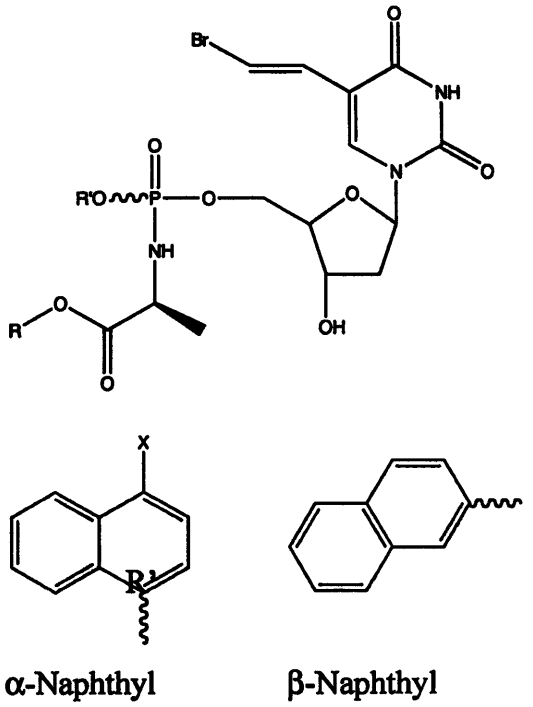
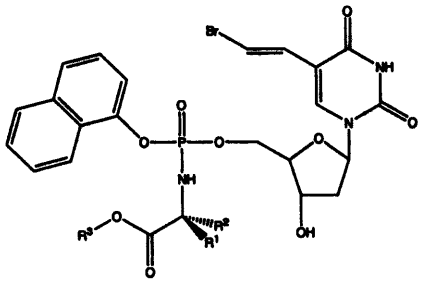


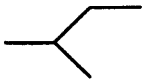
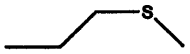
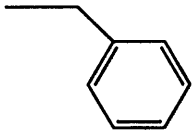
FIGURE 2 BVdU-phosphoramidates.

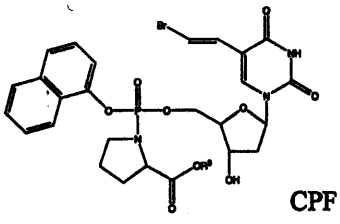
Compounds CPF 96-102 and 104 have been tested against two different tumor cell lines, MCF7 (breast cancer) and PC-3 (prostate cancer), showing a general increase in activity over the lead compound NB1011 (Table 3). In particular, this preliminary evaluation reveals a 100-fold increase in potency against prostate cancer achieved with the compound **CPF 98**.

TABLE 2 BVdU-Phosphoramidates, Amino Acid Moiety Tuning

Compound	Amino acid	R^3
CPF 127	Dimethylglycine	Me
CPF 156	L-Isoleucine	Me
CPF 146	L-Isoleucine	Bn
CPF 147	Dimethylglycine	Bn
CPF 149	L-Phenylglycine	Me
CPF 165	L-Valine	Bn
CPF 166	L-Proline	Me
CPF 167	L-Methionine	Me
CPF 168	D-Alanine	Bn
CPF 169	L-Phenylalanine	Me
CPF 170	L-Phenylalanine	Bn
CPF 171	L-Alanine	tBu
CPF 172	L-Valine	Me

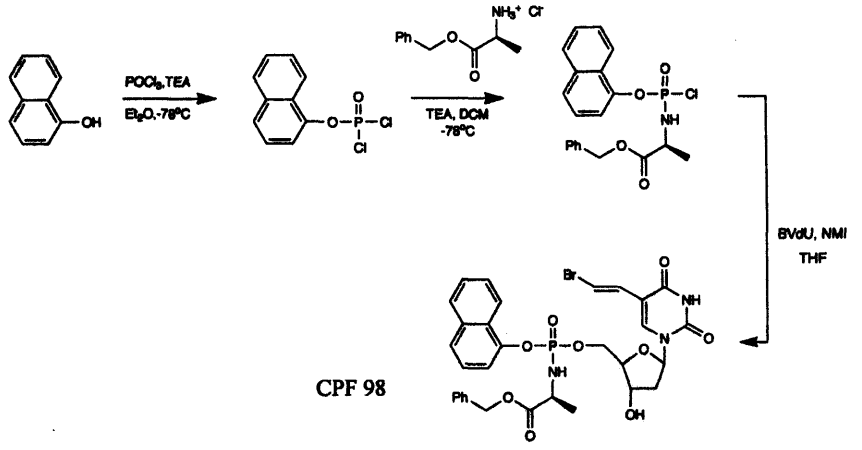


R^1	R^2	Amino acid
	H	L-Isoleucine
	H	L-Valine
	H	L-Methionine
	H	L-Phenylalanine
CH_3	CH_3	Dimethylglycine
Ph	H	L-Phenylglycine
H	CH_3	D-Alanine



CPF 166

FIGURE 3 BVdU-phosphoramidates, amino acid moiety tuning.



SCHEME 1 Synthesis of phosphoramidates.

TABLE 3 Preliminary Biological Evaluations

Compound	Breast cancer (MCF7) EC_{50} (μM)[a]	Prostate cancer (PC-3) EC_{50} (μM)[a]
NB1011	200	155
CPF 96	78	90
CPF 97	46	18
CPF 98	24	1.6
CPF 99	80	70
CPF 100	22	17
CPF 101	14	3.5
CPF 102	22	13
CPF 104	22	26

[a]EC_{50}: effective concentration required to cause 50% inhibition of cell growth.

Following this, derivatives containing modifications to the amino acid moiety are appealing targets in an attempt to further improve anticancer activity and these are currently under biological evaluation.

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Novel Potential Anticancer Naphthyl Phosphoramidates of BVdU: Separation of Diastereoisomers and Assignment of the Absolute Configuration of the Phosphorus Center

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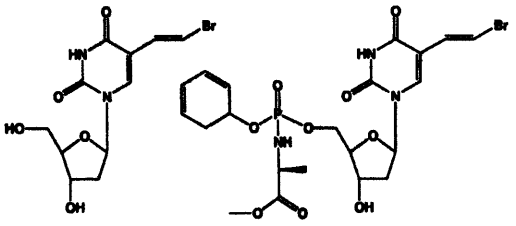
Received October 5, 2005

Abstract: We have previously reported our SAR optimization of the anticancer agent thymectacin. Tuning of the parent ProTide structure initially involved the amino acid and, subsequently, the aromatic masking group on the phosphate moiety. Herein, derivatives bearing the combined modifications are reported and biological evaluation is described. Moreover, separation of the diastereoisomeric final product mixture shows a different cytostatic activity for the two diastereoisomers. Through computational and NMR studies, the absolute stereochemistry of the phosphorus center of the two diastereoisomers has been suggested.

Nucleoside analogues represent an extremely effective tool for the treatment of cancer and viral infections. For these compounds, the action of kinases is required to convert the inactive nucleoside into biologically active nucleotide (mono-, di-, and triphosphate). Unfortunately, the dependence on kinases significantly limits the biological profile of nucleoside analogues because of their high specificity toward substrates and, no less important, a lower expression of these enzymes often leads to emergence of resistance to the nucleoside treatment. Due to the polarity of nucleotides themselves, circumventing kinase activation problems cannot be achieved with direct administration of the preformed free phosphate, as the resulting cell penetration would be too poor to show any significant therapeutic effect.

The phosphoramidate approach was introduced by McGuigan et al. in 1992[1] as a means to improve cellular penetration of nucleotides and to bypass the first step of kinase-mediated activation of nucleosides. Our method has been applied by both our lab and others to a wide variety of nucleosides and, nowadays, is recognized as one of the most successful approaches for the delivery of nucleoside monophosphates inside cells.[2]

Our technology has recently led NewBiotics to NB1011[3] (thymectacin), an aryloxy phosphoramidate derivative of BVdU (brivudin), which entered clinical evaluation against colon cancer.[4] On the basis of our experience of widespread phosphoramidate modifications, we first prepared a new series of phenyl phosphoramidates related to thymectacin, tuning the phenyl, ester, and amino acid regions, observing significant enhancements in activity versus three different tumor cell lines.[5] Because lipophilicity might play a crucial role for the delivery of ProTides inside cells,[6] new analogues have been designed focusing on the introduction of naphthyl as new aromatic masking group on the phosphate moiety, which has led to a further increase in cytostatic activity against a panel of cancer cell lines.[7a,b]



BVDU NB1011

The present work describes the synthesis and biological evaluation of a new family of BVdU ProTides, combining the modifications on the ester and amino acid moieties with the use of the new aryl group on the phosphate, naphthyl. The main objectives of this study were (i) to enhance the cytostatic potency and (ii) to further investigate the structure−activity relationship within BVdU phosphoramidates.

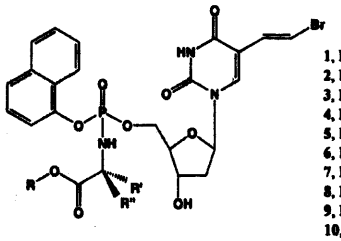
The compounds involved with our study are shown in Scheme 1. The target ProTides were all prepared in one step from BVdU using the phosphorochloridate chemistry we have extensively described.[8,9]

The synthesis involves phosphorylation of 1-naphthol with phosphorus oxychloride, followed by the coupling with different esterified amino acid salts to give naphthyloxy-phosphorochloridates, which were generally purified by flash chromatography and then coupled with BVdU in the presence of 1-methylimidazole (NMI). Yield of the final purified compounds resulted in a 6.5−49.7% range. The general synthetic route is reported in Scheme 2.

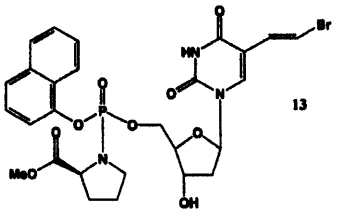
Due to the stereochemistry at the phosphorus center, the final compounds isolated from the coupling to BVdU are diastereoisomers. The confirmation of the presence of two diastereoisomers is shown by P-31 (two signals, 1:1 ratio), H-1, and C-13 NMR.

Compounds **1**−**13** were evaluated for their cytostatic activity against a panel of different tumor cell lines in vitro: MDA

Scheme 1. Structures of **1**−**13**



1, R=Me, R'=Me, R"=Me
2, R=Bn, R'=Me, R"=Me
3, R=Me, R'=H, R"=CHMeEt
4, R=Bn, R'=H, R"=CHMeEt
5, R=Me, R'=H, R"=Ph
6, R=Me, R'=H, R"=$CH(Me)_2$
7, R=Bn, R'=H, R"=$CH(Me)_2$
8, R=Me, R'=H, R"=CH_2Ph
9, R=Bn, R'=H, R"=CH_2Ph
10, R=Bn, R'=Me, R"=H
11, R=Me, R'=H, R"=$CH_2CH_2SCH_3$
12, R=tBu, R'=H, R"=Me



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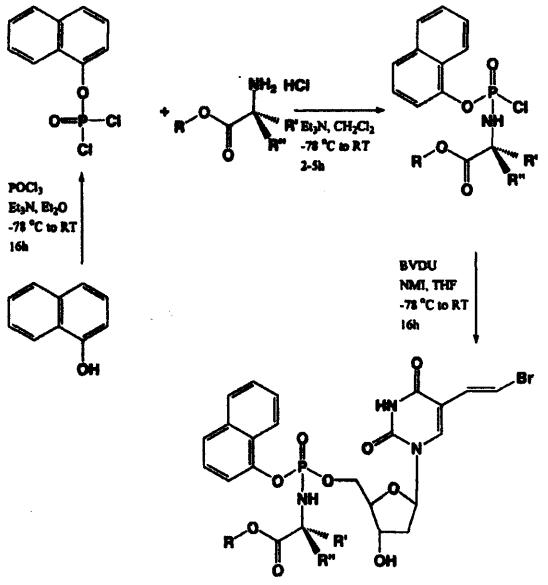
10.1021/jm0509896 CCC: $33.50
Published on Web 12/17/2005

Scheme 2. Synthesis of Naphthyl Phosphoramidates



MB231 (breast cancer) and PC-3 (prostate cancer) for entries **1**−**5** and thymectacin; T24 (bladder cancer) was also considered for entries **6**−**13**. Data are reported in Table 1.

According to the data shown in Table 1, most of the naphthyl phosphoramidates synthesized appear to be significantly more active than thymectacin, which displays only a moderate activity in our in vitro evaluations. Moreover, the L-alaninyl benzyl ester naphthyloxy-phosphoramidate (CPF98) which has been previously reported by us has been added to Table 1.[7a-b]

For the prostate cancer cell line, lipophilicity seems to play a fundamental role for the biological activity of these ProTides: the activity of compounds bearing the same amino acid moiety (dimethylglycine: **1**, **2**; L-isoleucine **3**, **4**; L-valine: **6**, **7**) is boosted every time the ester group is changed from methyl to benzyl, leading to a 100-fold increase versus thymectacin (entries **2** and **4**). Not surprisingly, also the presence of a highly lipophilic amino acid as phenylglycine leads to a significant activity (compound **5**).

On the other hand, this trend is not applicable to the breast cancer cell line. Activation of phosphoramidates has been reported to be dependent on the action of esterases and phosphoramidase activities;[8-10] it is possible that a different enzymatic activity in the two cell lines is responsible for the differences observed in the biological data. Further assays are underway to probe this aspect. Nevertheless, naphthyl phosphoramidates did show noteworthy activities also versus the breast cancer cell line, in particular compounds **2**, **3**, and **9** have achieved between 30- and 50-fold increase in potency versus thymectacin. Moreover, compound **1**, showing a 250-fold boost versus this particular cancer cell line, becomes the most active BVdU-related phosphoramidate with cytostatic activity against a breast cancer cell line reported. Significantly, the corresponding 'phenyl' ProTide reported previously by us displays an EC_{50} of 41.1 μM in the same assay.[5]

Last, tuning the structure of the lead ProTide by introducing the naphthyl moiety and subsequently modifying the amino acid core has led to an enhancement of potency in all the three cell lines, such as compound **1** for the breast cancer, **4** and **5** for the prostate, and **13** for the bladder cancer cell line.

As formerly mentioned, all the compounds have been synthesized and tested as mixtures of two phosphate diastereoisomers (1:1 ratio). Previous work carried out by Saboulard et al.[11] in 1999 indicates that carboxyl ester cleavage is a fundamental step for the activation of phosphoramidates. Enzymatic stability in the extracellular environment (i.e. plasma) and in different cellular preparations was found to be stereospecific with large and unpredictable differences in stereoselective metabolic rate noted by Siccardi et al.[12] Separation of phosphoramidate diastereoisomers by column chromatography has been shown to be problematic and even by using HPLC preparative methods, it remains a hard task to achieve.[13] Furthermore, when single diastereoisomers have been isolated, identification of the corresponding absolute stereochemistry has never been elucidated, leaving HPLC retention time and ^{31}P NMR chemical shift as the only parameters to discriminate between the two isomers.

Table 1. Cytostatic Effect of Test Compounds ($EC_{50}/\mu M$)

compound	amino	ester	breast MDAMB231	prostate PC-3	bladder T24
NB1011	L-Ala	Me	79	155	–
CPF98	L-Ala	Bn	15.2	1.7	–
1	Me_2Gly	Me	0.32	65.9	–
2	Me_2Gly	Bn	2.7	1.5	–
3	L-Ile	Me	1.5	6.9	–
4	L-Ile	Bn	130	1.4	–
5	L-PhGly	Me	105	1.7	–
6	L-Val	Me	14.8	15.8	43.5
7	L-Val	Bn	5.9	8.3	12.7
8	L-Phe	Me	8.5	10.2	5.3
9	L-Phe	Bn	1.96	5.8	269
10	D-Ala	Bn	6.3	6.1	2.8
11	L-Met	Me	28.1	44.6	19.6
12	L-Ala	tBu	4.8	11	4
13	L-Pro	Me	6.5	10.5	0.4

In the case of compound **10**, the mixture has been reasonably separated on reverse phase and the two diastereoisomers were tested against the MDA MB231 cell line (breast cancer). The fast eluting diastereoisomer (**fe**) emerged as slightly less active than the mixture (compd **10**), with an EC_{50} of 7.4 μM. On the other hand, the slow eluting diastereoisomer (**se**) is about 10 times more active than the mixture, showing an EC_{50} of 0.5 μM. The higher activity could be due to a better diffusion through cell membranes,[14] the slow eluting diastereoisomer being the more lipophilic, or to a more efficient stereoselective metabolism of the **se** diastereoisomer. Biological evaluation against the other different cancer cell lines is in progress.

Given this striking result, our major interest has been to find out a method to attribute the corresponding absolute stereochemistry to each of the two diastereoisomers.

The slow eluting (**se**) diastereoisomer shows downfield shifts (between 0.1 and 0.2 ppm) on H NMR for the H-5b, H-6, and H-2′ protons compared to the fast eluting (**fe**) isomer. Owing to the presence of three aromatic systems (nucleoside analogue base, phenyl and naphthyl), the former protons' chemical shifts might be perturbed by an anisotropic effect. The methylene protons of the benzylic ester display a more striking difference: for the **fe** diastereoisomer, a double doublet results as the main feature of their signal (traces of the other isomer are present) while, for the **se** diastereoisomer, the two protons couple with each other and show an AB-system (Figure 1).

Conformational studies were performed using the Sybyl 7.0 software package,[15] which allowed the identification of a series of distinct low energy conformations. The lowest energy conformation found for each diastereoisomer is shown in Figure 2. Interestingly, when the computational and NMR data were compared, particular features were recognized.

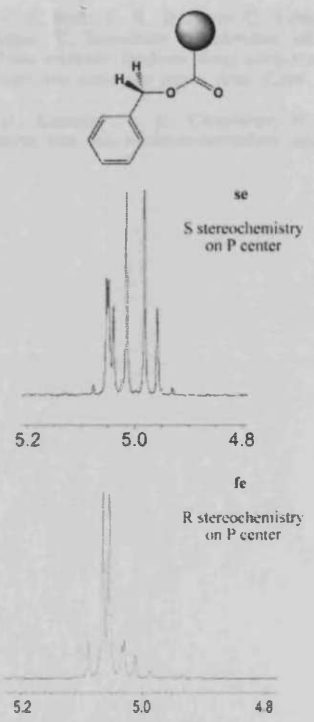


Figure 1. H NMR spectra of the methylene protons of the benzylic ester moiety of the isolated diastereoisomers of compound **10**.

For the *S* diastereoisomer (for clarity the two diastereoisomers are named after the absolute stereochemistry of the corresponding phosphorus center) the three aromatic moieties are stacked in pi-pi interactions where the naphthyl lays between the phenyl group and the nucleoside base. In this case, chemical shift changes are reasonable to appear for the protons closer to such an extended pi-electron cloud (e.g. H-5b, H-6 and H-2′). Furthermore, the apparent rigidity of this conformation, conferred by the described aromatic interactions, justifies the observed NMR pattern for the methylene hydrogens of the benzylic ester, which became nonequivalent.

The *R* conformation does not show any pi-pi interaction among the aromatic rings as in the case of the *S* diastereoisomer, and the greater flexibility around the methylene group of the ester moiety reduces the magnetic differences between the two diastereotopic protons. Therefore, by combining the NMR and the conformational data, we can propose the *S* phosphorus absolute configuration to the slow eluting (more lipophilic) diastereoisomer and, consequently, the *R* configuration to the fast eluting one.

The lowest energy conformation values generated by the Genetic Algorithm search used are -10.55 kcal mol^{-1} for the **se** diastereoisomer (suggested *S* phosphorus configuration) and -6.45 kcal mol^{-1} for the **fe** diastereoisomer (suggested *R* phosphorus configuration).

In summary, a new series of naphthyl phosphoramidates of BVdU has led to a significant improvement in the cytostatic activity of the parent lead compound thymectacin, against a panel of different cancer cell lines. Separation of the diastereoisomeric mixture of compound **10** has shown to be a useful approach to further enhance the anticancer effect of phosphoramidate ProTides.

Moreover, we have been able to suggest the absolute stereochemistry of the phosphorus center for a potential anticancer ProTide for the first time, by a combination of NMR and conformational studies.

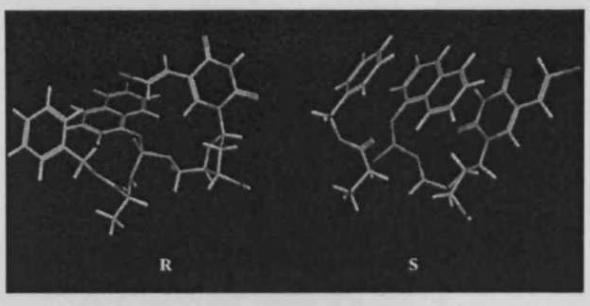


Figure 2. Lowest energy conformations of the two diastereoisomers of compound **10**.

Supporting Information Available: Synthesis, NMR, HPLC, low resolution mass, elemental analysis data, and conformational and biological evaluation methods' descriptions. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0509896