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NUCLEOTIDES AS ANTIVIRAL COMPOUNDS: ON THE FEASIBILITY OF AN ESTERASE-DEPENDENT PRODRUG STRATEGY FOR 2-5A

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ABSTRACT

UNIVERSITY OF TURKU
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KIURU, EMILIA: Nucleotides as Antiviral Compounds: On the Feasibility of an Esterase-dependent Prodrug Strategy for 2-5A

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Analogues of nucleotides and oligonucleotides have a significant role in the antiviral therapy. These compounds may inhibit specifically viral proteins or promote the innate immune system, where short 2´,5´-linked oligomers, called 2-5A, are the key players. The efficiency of nucleotide and oligonucleotide based drugs is largely dependent on the prodrug strategy used to enhance their cellular uptake. A common prodrug strategy includes masking of the negatively charged phosphate groups with biodegradable lipophilic protecting groups in order to facilitate permeation of the molecule through cell membrane. Inside the cell, upon unmasking by cellular enzymes, such as esterases, the prodrug is transformed into active drug.

In the present thesis, the feasibility of an esterase-triggered prodrug strategy for 2-5A trimer has been evaluated by synthesizing two different 2-5A prodrug candidates and studying the release of 2-5A by carboxyesterase. The protecting group scheme was based on esterase-labile 2,2-disubstituted acyloxypropyl groups for the phosphate protection and acyloxymethyl groups for the 3´-hydroxyl protection. The results demonstrated that deprotection of 2-5A, bearing esterase-labile protecting groups, became significantly slower upon accumulation of negative charge. Additionally, decomposition of the protecting groups produced electrophilic alkylating agents, which have been associated with potential toxicity. For these reasons, six different 2,2-disubstituted 4-acylthio-3-oxobutyl groups were developed as protecting groups for phosphodiesters. These groups are cleaved by esterases, but in addition they exhibit a novel feature being removed thermally, which is an advantage when the affinity of the enzyme to a negatively charged substrate is reduced. The hydrolytic and enzymatic stability of the protecting groups is easily adjustable to optimize the deprotection rate. The released protecting groups are not markedly alkylating, since no alkylation with glutathione was observed.

Key words: antiviral nucleotide, 2-5A, prodrug, esterase-labile, thermolabile, synthesis

TIIVISTELMÄ

TURUN YLIOPISTO
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KIURU, EMILIA: Nukleotidit antiviraalisina yhdisteinä: Esteraasiriippuvaisen aihiolääkestrategian soveltuvuudesta 2-5A:lle

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Nukleotidien ja oligonukleotidien analogeilla on merkittävä rooli virusten aiheuttamien tautien hoidossa. Tämän kaltaiset yhdisteet voivat estää spesifisesti virusten proteiineja tai aktivoida luontaista immuunijärjestelmää, jossa 2-5A:ksi kutsutut lyhyet 2´,5´-sitoutuneet oligomeerit ovat keskeisiä tekijöitä. Nukleotideihin ja oligonukleotideihin pohjautuvien lääkkeiden tehokkuus riippuu pääasiassa aihiolääkestrategiasta, jolla niiden sisäänottoa soluun tehostetaan. Tavanomaisessa aihiolääkestrategiassa negatiivisesti varautuneet fosfaattiryhmät suojataan rasvaliukoisilla biohajoavilla suojaryhmillä, jotta molekyyli läpäisee solukalvon helpommin. Solun sisällä aihiolääke muuttuu aktiiviseksi lääkeaineeksi, kun suojaryhmät irtoavat solun entsyymien, kuten esteraasien vaikutuksesta.

Väitöskirjassa arvioitiin esteraasin katalysoiman aihiolääkestrategian soveltuvuutta 2-5A-trimeerille syntetisoimalla kaksi erilaista 2-5A-aihiolääkekandidaattia ja tutkimalla 2-5A:n purkautumista karboksiesteraasi-entsyymin vaikutuksesta. Suojaryhmäsuunnitelma perustui esteraasilabiileihin 2,2-disubstituoituihin asyylioksipropyyliryhmiin ja asyylioksimetyyliryhmiin, joilla suojattiin trimeerien fosfaatti- ja 3´-hydroksyyliryhmät. Tulokset osoittivat, että esteraasilabiilien suojaryhmien irtoaminen 2-5A:sta hidastui merkittävästi, kun yhdisteeseen kertyi negatiivista varausta. Lisäksi suojaryhmien hajotessa muodostui elektrofiilisiä alkyloivia aineita, jotka ovat mahdollisesti toksisia. Näistä syistä johtuen kehitettiin kuusi uudenlaista 2,2,-disubstituoitua 4-asyylitio-3-oksobutyyliryhmää fosfodiestereiden suojaamiseksi. Suojaryhmät irtoavat sekä esteraasin katalysoimana, että lämpötilan vaikutuksesta. Tämä on hyödyllinen ominaisuus silloin, kun entsyymin affiniteetti negatiivisesti varattuun substraattiin heikkenee. Suojaryhmien hydrolyyttinen ja entsymaattinen stabiilisuus on helposti säädeltävissä, jotta suojauksen purkautumisen nopeus voidaan optimoida. Vapautuneet suojaryhmät eivät ole merkittävästi alkyloivia, sillä niiden ei havaittu alkyloivan glutationia.

Avainsanat: antiviraalinen nukleotidi, 2-5A, aihiolääke, esteraasilabiili, termolabiili, synteesi

PREFACE

This thesis is based on experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during the years 2007-2013. The financial support of Alios Biopharma, Graduate School of Organic Chemistry and Chemical Biology and University of Turku are gratefully acknowledged.

My deepest gratitude goes to Professor Harri Lönnberg for believing in me and giving me the opportunity to pursue a doctoral thesis under his guidance and explore the secrets of nucleic acid chemistry. I am truly impressed by his excellent knowledge of bioorganic chemistry and inspiring way of thinking. I am also thankful to my other supervisor Dr Mikko Ora for his kind support and interest in this work. He has always found time to answer my questions and have discussions, no matter what topic. His strong expertise in the field of reaction kinetics has been extremely important in solving all the confusing HPLC results.

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Turku, April 2015

Emilia Kiuru

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals:

I Ora, M., Taherpour, S., Linna, R., Leisvuori, A., Hietamäki, E., Poijärvi-Virta, P., Beigelman, L. and Lönnberg, H.: Biodegradable Protections for Nucleoside 5′-Monophosphates: Comparative Study on the Removal of *O*-Acetyl and *O*-Acetyloxymethyl Protected 3-Hydroxy-2,2-bis(ethoxycarbonyl)propyl Groups. *J. Org. Chem.* **2009**, *74*, 4992–5001.

II Kiuru, E., Ora, M., Beigelman, L., Blatt, L. and Lönnberg, H.: Synthesis and Enzymatic Deprotection of Biodegradably Protected Dinucleoside-2′,5′-monophosphates: 3-(Acetyloxy)-2,2-bis(ethoxycarbonyl)propyl Phosphoesters of 3′-*O*-(Acyloxymethyl)adenylyl-2′,5′-adenosines. *Chem. Biodiversity* **2011**, *8*, 266-286.

III Kiuru, E., Ora, M., Beigelman, L., Blatt, L. and Lönnberg, H.: Synthesis and Enzymatic Deprotection of Fully Protected 2′-5′ Oligoadenylates (2-5A): Towards a Prodrug Strategy for Short 2-5A. *Chem. Biodiversity* **2012**, *9*, 669-688.

IV Kiuru, E., Ahmed, Z., Lönnberg, H., Beigelman L. and Ora, M.: 2,2-Disubstituted 4-Acylthio-3-oxobutyl Groups as Esterase- and Thermolabile Protecting Groups of Phosphodiesters. *J. Org. Chem.* **2013**, *78*, 950-959.

V Kiuru, E., Lönnberg, H. and Ora, M.: 2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl Group: A Thermolabile Protecting Group for Phosphodiesters. *Helv. Chim. Acta* **2013**, *96*, 1997-2008.

ABBREVIATIONS

2′-PDE	2′-phosphodiesterase
2-5A	2′-5′oligoadenylate
Ac	acetyl
ACV	acyclovir
Ade	adenine
ATP	adenosine 5′-triphosphate
AZT	3′-azido-3′-deoxythymidine
CFC	chronic fatigue syndrome
Cordycepin	3′-deoxyadenosine
COSY	correlation spectroscopy
*Cyclo*Sal	cyclosaligenyl
DCM	dichloromethane
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EMCV	encephalomyocarditis virus
ESI	electrospray ionization
FDA	Food and Drug Administration
FU	5-fluorouridine
FUdR	2′-deoxy-5-fluorouridine
GSH	glutathione
HBV	hepatitis B virus
HCV	hepatitis C virus
HeLa cell	immortal cell line from cervical cancer cells
HEPES	*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HLE	hog liver carboxyesterase
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
HSV	herpes simplex virus
iBu	isobutyl
IFN	interferon
iPr	isopropyl
LC-MS	liquid chromatography mass spectrometry
Lev	levulinoyl

Me	methyl
MES	2-(*N*-morpholino)ethanesulfonic acid
MHC	major histocompatibility complex
MMTr	4-methoxytrityl
mRNA	messenger RNA
MS	mass spectrometry
NMR	nuclear magnetic resonance
Nuc	nucleoside
OAS	2′,5′-oligoadenylate synthetase
Piv	pivaloyl
POM	pivaloyloxymethyl
RNA	ribonucleic acid
RNase L	ribonuclease L
RP-HPLC	reversed-phase HPLC
RSV	respiratory synthical virus
SATE	*S*-acyl-2-thioethyl
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
*t*Bu	*tert*-butyl
TetH	1*H*-tetrazole
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thy	thymine
TMS	trimethylsilyl
Tubercidin	7-deazaadenosine
Ura	uracil
UV	ultraviolet
VSV	vesicular stomatitis virus
VV	vaccinia virus

1 INTRODUCTION

1.1 General

The greatly expanded knowledge of the structure and function of viruses has resulted in development of several antiviral drugs.[1,2,3,4] Majority of the current antiviral repertoire consists of small molecule drugs for the treatment of human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV), influenza viruses and herpes viruses. Researchers are continuously working to extend the range of antivirals for other viral infections or improve the existing antiviral compounds. The emergence of viral resistance to drugs and drug-related side effects are among the main reasons why the established treatments do not always stay effective or well tolerated. Vaccines have led to eradication of some important viral pathogens, but their use has not eliminated the need for effective antivirals, especially for emerging highly pathogenic RNA viruses, each having a specific replication strategy. Antiviral drug could, in principle, be targeted specifically at viral proteins without harming the host's cells. Alternatively, antiviral compounds targeted at cellular proteins might afford drugs, which could be used for treatment of a variety of viral infections, but with higher likelihood of toxicity. Development of these broad-spectrum drugs would be an economical option, as well as a solution to rapidly spreading fatal viruses causing epidemics.

1.2 Antiviral strategies

Antiviral strategies that interfere with the key events in the viral life cycle have provided a number of useful therapeutics.[1,2] The first antiviral drug, iodoxuridine used topically for treatment of the herpes simplex virus (HSV) infections of eye, was described in 1959.[5] Since then, several nucleoside and nucleotide analogues have been accepted as therapeutic agents against a number of viruses (Figure 1 and Figure 2).[6]

The first antiviral nucleosides were not safe for systemic administration, because of inadequate specificity for virus-infected cells.[7] Selective approach to attack virus infections was launched by the discovery of acyclovir (ACV) in 1977.[8] This acyclic guanine nucleoside inhibited selectively HSV and varicella-zoster virus devoid of toxic side-effects. Antiviral nucleosides must be phosphorylated to active triphosphates by cellular or viral kinases before they can interact with their target enzymes.[6] ACV is converted to 5´-monophosphate by a viral enzyme, virus-encoded thymidine kinase, but not with the corresponding enzyme of the

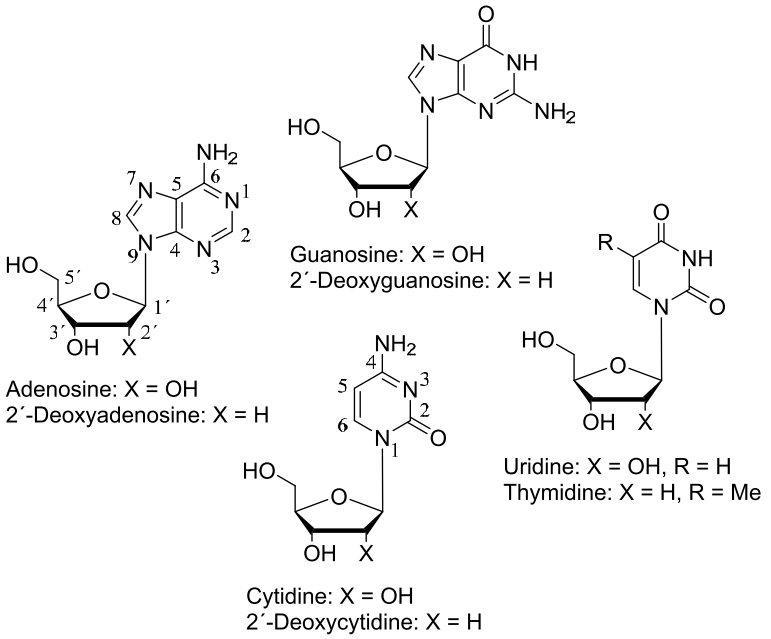


Figure 1 Structures of natural ribonucleosides (X = OH, R = H) and 2´-deoxyribonucleosides (X = H, R = Me) and the IUPAC numbering of the sugar and base moieties.

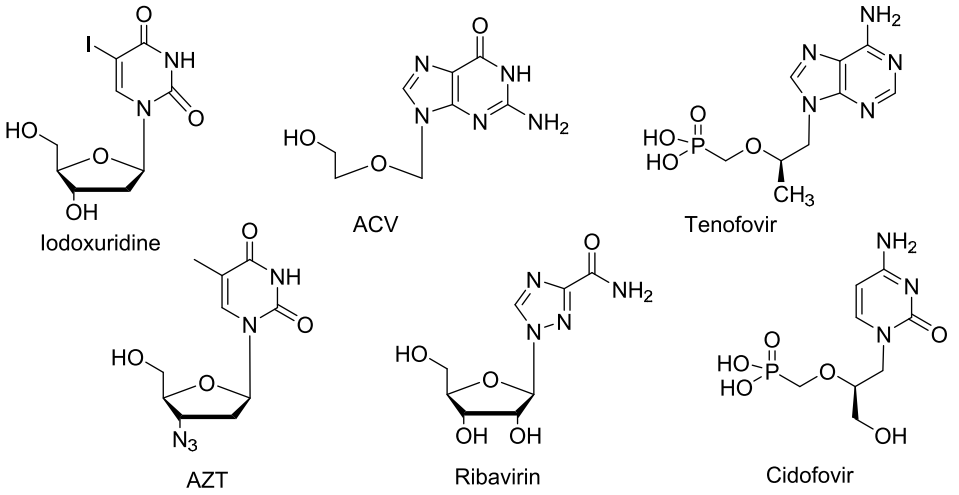


Figure 2 Examples of antiviral nucleoside analogues.

host cell. After subsequent conversion to 5´-triphosphate, the compound acts as an inhibitor of viral DNA polymerase by preventing incorporation of natural nucleotides into the growing DNA chain. Enzymes needed for viral DNA or

RNA synthesis have proven to be attractive targets for several other antivirals as well.[1,2] The first antiretrovirus agent, 2′,3′-dideoxynucleoside analogue, azidothymidine (AZT),[9] described soon after discovery of HIV, inhibits the HIV reverse transcriptase, that is responsible for the HIV DNA synthesis. Acyclic nucleotide analogues containing a phosphonate moiety have shown broad antiviral activity spectrum, since they are able to bypass the first phosphorylation step. Tenofovir[10] is active against both HIV and HBV reverse transcriptase and cidofovir[11] targets DNA polymerases. Search for compounds targeting RNA replicase (RNA-dependent RNA polymerase), an essential enzyme in the replication of HCV and other flaviviruses, has led to identification of several nucleosides having 2′-*C*-methyl pharmacophore.[2,4,12] Ribavirin, reported as a broad-spectrum antiviral,[13] inhibits almost all RNA viruses *in vitro*, albeit its clinical use is limited to only a few infections, including HCV and respiratory syncytial virus (RSV) and it is usually taken in combination with another medication.[4] The mechanism by which ribavirin exerts its antiviral activity is not specific and it varies depending on the virus.

Other viral enzymes, which have been favored as antiviral targets include proteases and neuraminidases. Oseltamivir (Tamiflu®) and other sialic acid analogues specifically interact with viral neuraminidases preventing the release of newly formed viruses from the cell.[14] These compounds have been used for the treatment of influenza viruses, although unfortunately resistance seems to emerge with extended use. Combinations of different antiviral drugs acting by different mechanisms may be a solution to prevent the drug resistance development. For the treatment of HIV, drug combination is already a vital strategy.[2] Antiviral strategies, not addressed here in detail, such as antisense oligonucleotides[15], RNA interference[16] and ribozymes[17] are targeted at selected viral messenger RNA (mRNA) preventing its translation into protein.

Another approach for fighting against viruses is based on promotion of the immune system. Knowledge gained from studies on interferon[18] (IFN) action has had a great impact on development of therapies against viral pathogens. IFNs are a family of cytokines that cells secrete in response to viruses and other harmful microbes.[19,20] They affect a number of processes including interference with viral replication, regulation of cell growth and modulation of inflammatory response. The antiviral actions of IFNs are mediated through IFN-stimulated genes. Protein kinase, that inhibits viral translation, and 2′,5′-oligoadenylate synthase (OAS), that mediates RNA degradation, are among the major IFN-induced proteins that affect virus multiplication within virus –infected cell. The IFN-stimulated genes do not have virus specificity and therefore interferons can function as broad-spectrum antivirals. Interferons have been used most widely in the treatment of chronic viral infections, such as HCV and HBV. Therapies combining interferons

with other antiviral drugs have also been successful. For example, combination of the polyethylene glycol conjugate of interferon with ribavirin is currently the standard care for HCV infection.[12,21] However, IFN-therapy is associated with severe side-effects, which is the downside of the nonspecific actions of interferons.

1.3 2-5A system

1.3.1 Mechanism of action

2-5A system is one of the principal pathways involved in the interferon antiviral response (Figure 3).[22,23,24] As a result of ongoing viral infection, IFN signaling activates genes encoding OAS[25] and ribonuclease L[26] (RNase L). Double-stranded RNA (dsRNA), derived from viral replication intermediates, binds and activates the OAS to generate unique short 2´,5´-linked oligomers, called 2-5A,[27] from adenosine triphosphate (ATP).[28] The trimeric form of 2-5A, $p_3(A2´p5´)_2A$, is the principal species formed in virus-infected cells. 2-5A binds to RNase L causing catalytically inactive RNase L monomers to form a potently active dimeric endoribonuclease.[29] RNase L cleaves viral and cellular single-stranded RNA in U-rich sequences, preferentially after UU and UA dinucleotides, resulting in inhibition of viral replication, leading sometimes to apoptosis.[30] RNase L activation has been shown to participate in the interferon antiviral effect also indirectly.[31] Some of the RNA cleavage products may contribute to the production of interferons.

RNase L is normally inactive within the cell. The activity is primarily regulated by 2-5A, the concentration of which, however, normally remains low since it is cleaved rapidly by nucleases within cells.[32] Thus, RNase L cannot degrade a significant amount of native RNA vital for normal cell function. Some viruses have evolved mechanism to down-regulate RNase L activity, by inducing expression of regulatory proteins called RNase L inhibitors. These inhibitors prevent 2-5A binding to RNase L.[33]

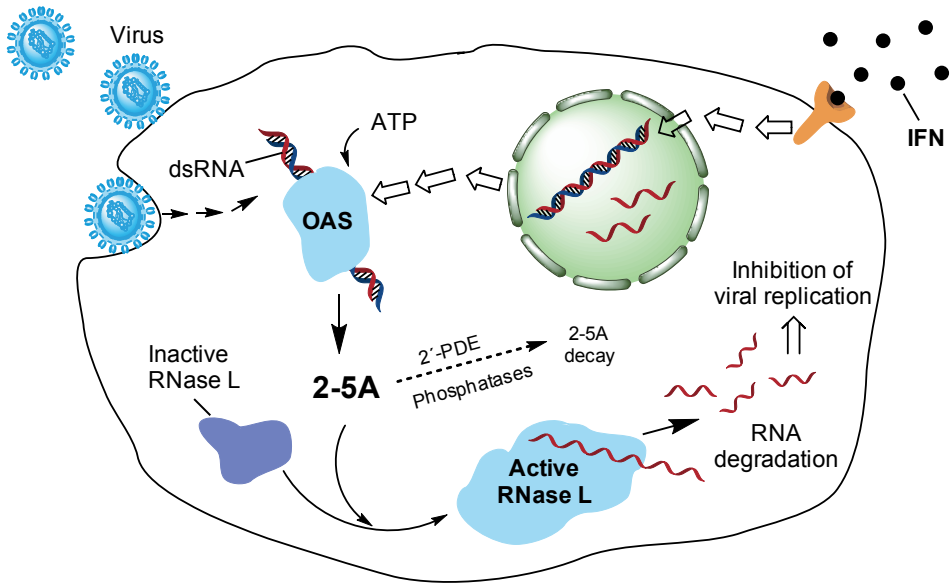


Figure 3 The principal components of the 2-5A system. 2´-PDE = 2´-phosphodiesterase.

1.3.2 Viral targets

The antiviral effects of the 2-5A system depend on the virus and cell type. Most RNA viruses are inhibited by the 2-5A system. The viral replication intermediates of encephalomyocarditis virus (EMCV) were shown to activate OAS in the IFN-treated cells, causing accumulation of 2-5A and ribosomal RNA cleavage products characteristic to RNase L activity.[34] Antiviral activity of 2-5A system was demonstrated by generating mice with a targeted disruption of the RNase L gene. Death due to EMCV infection occurred at a higher rate in mice lacking RNase L compared to wild-type mice, both in the absence or presence of prior interferon treatment, although IFN treatment extended the survival times.[35]

HCV was reported to activate the 2-5A system resulting in cleavage of HCV mRNA in HeLa cells. The susceptibility of HCV mRNA for cleavage by RNase L was shown to correlate with the sensitivity of HCV infections to interferon therapy. Interferon-sensitive HCV genotypes 2 and 3 mRNAs have much more RNase L cleavage sites (UU and UA dinucleotides), than relatively interferon-resistant HCV genotype 1 mRNA.[36]

During the initial state of HIV infection, human T cells were found to increase the levels of OASs, followed by a strong increase of 2-5A concentration and enhancement in the RNase L activity. RNase L was able to degrade HIV

transcripts, but after three days, the enzyme activities were decreased and cells began to release virus particles.[37] This results from mechanisms that HIV-1 has developed to overcome the antiviral pathways. HIV-1-trans-activating protein prevents OAS activation, by binding to the transactivation responsive region of HIV-1 mRNA required for OAS activation.[38] The activation of RNase L is, in turn, inhibited by overexpression of RNase L inhibitors.[39]

The 2-5A pathway has been reported to have antiviral effects also on replication of several other RNA viruses, such as RSV, coxsackievirus, mengovirus, vesicular stomatitis virus (VSV), West Nile virus and reovirus.[40,23] Most DNA viruses are resistant to the antiviral action of 2-5A system. Although some DNA viruses generate dsRNA that activate OAS to produce 2-5A, RNase L is only weakly activated. In cells infected with vaccinia virus (VV), HSV-1 or Simian virus 40, a mixture of 2-5A analogues, that were inhibitory to RNase L, were detected.[23] However, the exact structures of these 2-5A-related compounds have not been determined.

1.3.3 Other roles in innate immunity

In addition to RNA cleavage, 2-5A activation of RNase L has been reported to have broad range of biological roles, including stimulation of the expression of genes that have profound effect on viruses, inflammation and tumorgenesis.[41,26]

2-5A system has been suggested to be involved in tumor suppression because of its cell growth inhibitory and pro-apoptotic activities. Mutations of RNase L decreased the ability of the enzyme to dimerize in active form and reduced its ability to cause apoptosis.[42] These finding have supported the identification of the RNaseL gene as a candidate for the hereditary prostate cancer 1 gene. Also OAS polymorphism has been associated with prostate cancer.[43]

Dysfunctions in the 2-5A pathway were associated with chronic fatigue syndrome (CFS). The native 83-kDa RNase L is degraded by proteases into 37-kDa and 30 kDa fragments in peripheral blood mononuclear cells of CFC patients. An analysis of the size of 2-5A oligomers in the CFC cell extracts revealed that they contained inactive 2-5A dimers rather than higher oligomers, which likely resulted from a defect in OAS. The cells were incubated with 2-5A trimer (pA_3) and tetramer (pA_4), which led to protection of RNase L against the proteolytic cleavage. These results suggested that binding with 2-5A dimer targets the monomeric enzyme for proteolytic cleavage, while longer 2-5A oligomers induce homodimerization and activation of RNase L.[44]

2-5A system was recently shown to contribute also to antibacterial immunity. RNase L has a functional role in regulating the induction of proinflammatory cytokines and endolysosomal activities that suppress bacterial infections. However, the source of dsRNA needed for production of 2-5A to activate RNase L is still unknown.[45]

1.4 2-5A as therapeutics

The important role of 2-5A system in innate immunity provides a way to control virus and cell growth. RNase L may be recruited for therapeutic purposes by directly activating it with synthetic 2-5A. Viruses and disorders, that are potential targets of a 2-5A drug, have been discussed in sections 1.3.2 and 1.3.3.

In order to use 2-5A for therapeutic applications, biological barriers that affect the delivery of these compounds to cells, have to be taken into consideration. The native 2-5A has short biological half-life due to nuclease activities within cells and in serum. Even though 2′,5′-bond is resistant to most known RNases, cleavage of the internucleosidic phosphodiester bonds by 2′-phosphodiesterases (2′-PDEs) leads to complete inactivation of 2-5A. The ability of 2-5A to activate RNase L is also inhibited by 5′-phosphatases that catalyze the cleavage of the 5′-phosphate groups.[32] Another major limitation of 2-5A molecule is its polyanionic nature. The negatively charged 2-5A will not easily penetrate the cell membrane, which leads to inability of these compounds to achieve high concentrations in the cell. In addition, direct introduction of 2-5A into cells may cause toxic side effects, since activated RNase L has been reported to cleave cellular RNA substrates in addition to viral substrates.[46]

Numerous modifications have been made to enhance the nuclease stability and reduce the negative charge of 2-5A. In HIV-1-infected cells, 2-5A analogues were able to restore the down-regulated antiviral pathway by activation of RNase L leading to inhibition of viral RNA synthesis and production of infectious HIV-1 particles.[47,48,49.] RNase L activation by phosphorothioate analogues of 2-5A was reported to lead apoptosis in metastatic prostate tumor cell lines.[42]

To limit non-specific global RNA degradation, 2-5A molecule has been covalently attached to an antisense oligonucleotide containing complementary sequence to a target RNA. The antisense region of the chimera guides the activated RNase L to cleave selectively the targeted RNA.[50,51] Several applications of 2-5A-antisense against cancers have been described.[51] The efficacy of RNase L activation by 2-5A-antisense in suppression of RSV infections was demonstrated in African green monkeys.[52] 2-5A was attached to

an oligonucleotide complementary to RSV repetitive gene-start sequences and administered to the monkeys by the intranasal route. This 2-5A antisense was shown to cleave RSV genomic RNA and reduce effectively viral replication.

Some chemical modifications to improve the therapeutic potential of 2-5A are introduced in sections 1.4.1.2-1.4.1.4.

1.4.1 Synthetic analogues of 2-5A

1.4.1.1 Structural requirements

Studies with 2-5A analogues containing modifications on bases, riboses, internucleotide linkages or 5′-phosphoryl groups have provided a considerable amount of information about the structural requirements necessary for RNase L binding and activation.

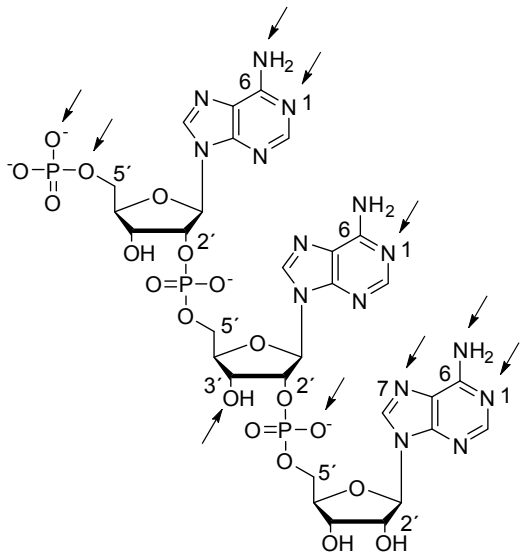


Figure 4 The structure of 2-5A trimer (having one 5′-phosphoryl group). The atoms or groups of atoms that interact with RNase L through hydrogen bonds or salt bridges are indicated with arrows.

The 2′,5′-phosphodiester bonds are the key requirements for the biological activity of 2-5A.[27] Replacing either one or both of the 2′,5′ linkages with a 3′,5′ linkage weakens drastically the binding interactions between 2-5A and RNase L (Figure 4).[53] Another critical feature is the length of the oligoadenylate chain,

which has to be trimer or longer.[54] 2-5A trimer and tetramer have been shown to activate RNase L equally well, whereas 2-5A dimer fails to activate the enzyme. Additionally, 2-5A has to have at least one 5′-phosphoryl group to optimally activate human RNase L. The 5′-dephosphorylated core species has been reported to have significantly reduced activity. The role of ribose moieties has been examined, since riboses are attractive targets for modifications in order to improve the resistance towards nucleases. Only the 3′-hydroxyl group of the intervening adenosine of 2-5A trimer is required for efficient activation of RNase L, since this hydroxyl group may form a hydrogen bond with an acceptor in RNase L.[22] Activation of RNase L also depends on the nature of the bases. The N1 and N6 atoms of the 5′-terminal adenine are required for RNase L binding and the N1 and N6 of the 2′-terminal adenine are required for activation.[22,53] In addition, *syn* conformation of the 2′-terminal adenosine increases binding activity. The intervening adenine is recognized only by a single hydrogen bond. Accordingly, this base moiety appears to play a less important role in the RNase L activation.

1.4.1.2 Modifications of the ribose-phosphate backbone

Modification of the 2′-terminal ribose of 2-5A is a convenient way to enhance the stability against enzymatic degradation, since 2′,5′-phosphodiesterase has been reported to require free 2′- and 3′-terminal hydroxyl groups for its activity. Even large chemical modification may be possible without reducing the biological activity of 2-5A.[55] Moreover, various conjugate groups have been attached at the 2′-end to improve the cellular uptake. These have been discussed in section 1.4.2.

Nuclease resistance has been enhanced by replacing the 2′-terminal adenosine of 2-5A trimer with cordycepin[56] (3′-deoxyadenosine), 3′-*O*-methyladenosine[57,58], 3′-fluoro-3′-deoxyadenosine[59] or 3′-amino-3′-deoxyadenosine[60] (Figure 5). The 3′-*O*-methylated analogues of 2-5A were shown to activate RNase L in Hela cell extracts and inhibit protein synthesis in human fibroblasts more efficiently than the natural 2-5A trimer.[58] 3′-Fluoro-3′-deoxyadenosine substitution increased the binding affinity to RNase L over three times in mouse L cells compared to natural 2-5A trimer.[59]

The 5′-terminal adenosine may also be replaced with a 3′-modified adenosine, without losing the ability to activate RNase L, but the 3′-hydoxyl group of the intervening adenosine is important for RNase L activation.[61,56] For example, when one adenosine moiety was replaced at each position of the 2-5A trimer with cordycepin, a notable increase in binding and activation was observed on

replacing the 2´-terminal adenosine unit, while the replacement of the intervening unit resulted in dramatic decrease of RNase L activation.[56] The triphosphates of cordycepin trimer and tetramer were not able to inhibit translation of EMCV, nor activate RNase L in cell-free extracts of mouse L-cells. However, these analogues were able to bind RNase L and antagonize the protein synthesis inhibitory effect of 2-5A.[62]

2-5A core analogues of cordycepin[47], *xylo*-adenosine[63] and 3´-*O*-methyladenosine[64], lacking the 5´-phosphate groups have also been reported to exhibit antiviral activities, by inhibiting HIV-1, HSV and VV production, respectively, but by a mechanism different from RNase L activation.

Acyclonucleosides, in which the ribose moiety is replaced with an acyclic chain, were incorporated into 2-5A tetramers instead of adenosines to increase the resistance towards nucleases. An analogue containing this modification at the third position from the 5´-end was found to be as an RNase L activator only 1.7-fold less potent than the native 2-5A tetramer.[65]

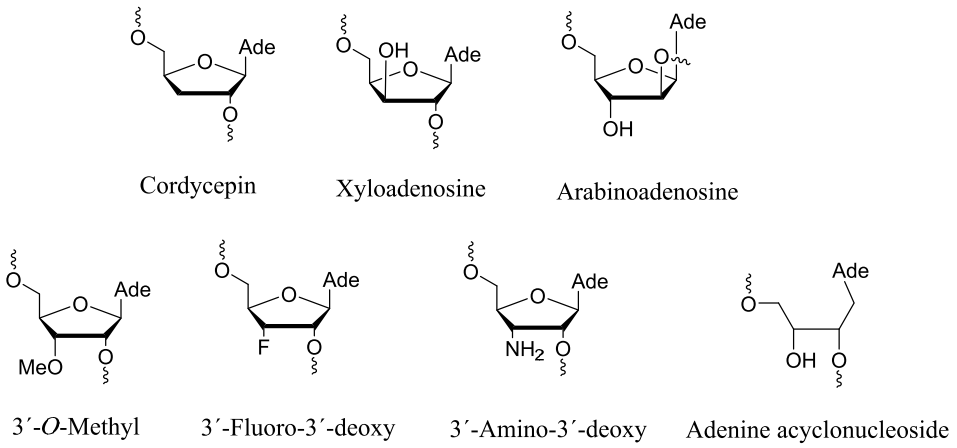
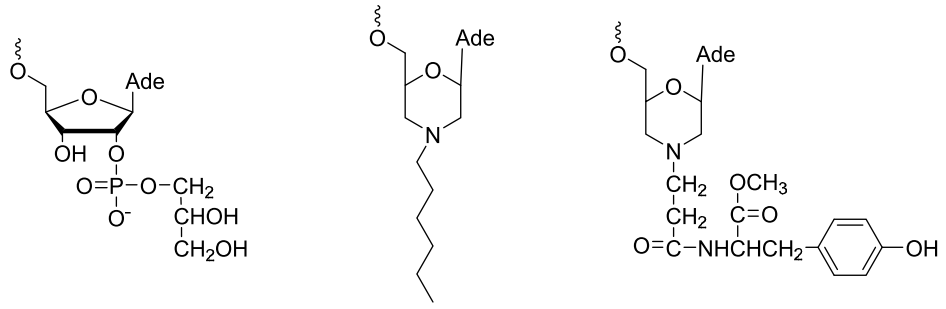


Figure 5 Modifications of the ribose moieties. Ade = adenine

2-5A analogues containing a large modification at the 2´-end are able to bind and activate RNase L. 2´-*O*-Phosphoglyceryl derivatives of 2-5A trimer[66] and an *N*-substituted morpholine modified 2-5A tetramer[40] were shown to inhibit the growth of VSV, when microinjected into the cytoplasm of HeLa cells (Figure 6). In addition, the tailed analogue inhibited translation of EMCV in cell-free extract of mouse L-cells.[55] β-Alanyltyrosine derivative of 2-5A tetramer inhibited protein synthesis in mouse L cells more efficiently than the corresponding native 2-5A.[67] The tyrosine residue can be easily labelled and used as a probe in biological applications.



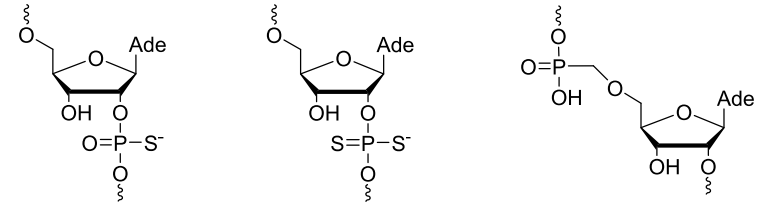
2′-*O*-Phosphoglyceryl *N*-Hexylmorpholine β-Alanyltyrosine

Figure 6 Modifications of the 2′-terminus.

Another way to enhance the stability of 2-5A against enzymatic degradation is to replace the 2′,5′-internucleotide linkages with phosphorothioates (Figure 7).[68] Full phosphorothioate modification may also increase cellular uptake of the compounds, because of more efficient binding to proteins on cell surfaces, yet also nonspecific protein binding is increased.[69] The replacement of oxygen with sulfur introduces chirality into the phosphorus and changes ionic properties of 2-5A. Studies with four diastereomeric 2′,5′-phosphorothioate trimer core analogues and their 5′-monophosphates showed that the stereochemical configuration did not significantly effect on binding to RNase L. Marked differences were, however, obtained in the activation of RNase L between the isomers.[68] Three of the four sulfur substituted cores (R_pR_p, S_pR_p and R_pS_p) were able to activate RNAse L although 1000-fold less efficiently than the corresponding 5′-monophosphates. The analogues with S_p configuration in all of the phosphodiester linkages were antagonists of RNase L activation. Especially, R_p configuration at the 2′-terminus of a core trimer is important for allosteric interaction with RNase L. ApA(R_p)A, the core analogue of 2-5A, was found to inhibit HIV-1 replication almost completely in infected cells.[48] In addition, enhanced uptake of phosphorothioate/phosphodiester 2-5A core analogues in HIV-1 infected cells was observed. To avoid formation of diastereomers, the internucleotide phosphodiester linkages of 2-5A trimer were replaced with phophorodithioates.[70] This analogue was ten-fold less efficient in binding and activation of human recombinant RNase L than the natural 2-5A.

Modifications at the 5′-phosphate of 2-5A may enhance the stability towards phosphatases. Replacement of the 5′-monophoshate group with 5′-thiophosphate gave compounds that were resistant against phosphatases and retained the activation ability of RNase L.[71]

Incorporation of 5′-*O*-phosphonomethyl *ribo*, *arabino* or *xylo* unit in the 2′-terminal end of 2-5A trimer gave compounds that were stable towards exonucleases and were able to activate human RNase L as efficiently as the native 2-5A.[72] The sugar configuration of the 5′-*O*-phosphonomethyl unit did not have a marked effect on the activation ability, whereas *ribo* configuration was best tolerated when the 5′-*O*-phosphonomethyl modification was introduced into intrachain positions within a tetramer. Total resistance against phosphomonoesterases was obtained when the 5′-terminal phosphate was replaced with a phosphonomethyl group. This modified tetramer was almost as efficient activator of RNase L as the natural compound. However, replacement of two or more phosphates for phosphonates had a negative effect on the activation ability.



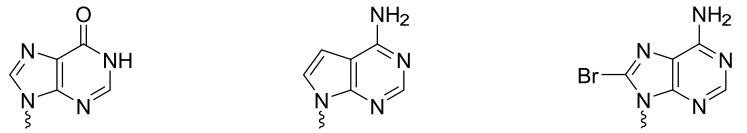
Phosphorothioate Phosphorodithioate 5′-*O*-Phosphonomethyl

Figure 7 Modifications of the phosphodiester backbone and 5′-phosphates.

1.4.1.3 Modifications of the base moieties

Several studies have demonstrated the importance of the N1 and N6 atoms of the 5′-and 2′-terminal adenine bases of 2′,5′-oligoadenylate for the binding and activation process (Figure 4). Replacement of one these adenine moieties with hypoxanthine (Figure 8) or uracil has been shown to have detrimental effect on the binding and activation of RNase L, while replacement of the intervening adenine, has resulted in only moderate loss of the biological activity.[73,74] The roles of other purine atoms in the activation process have also been evaluated. According to a crystal structure analysis, the N7 atom of the third adenine ring interacts with RNase L.[53] Introduction of tubercidin (7-deazaadenosine) into the 5′- or 2′-terminus of 2-5A trimer decreased the ability to activate RNase L, while this ability was retained when the intervening adenosine was replaced.[75] In contrast, introduction of 3-deazaadenosine was found to have little or no effect on activation of RNase L.[76]

Substitution of C8 of the purine ring by bromine in one or more adenosine moieties was reported to have a pronounced effect on the binding and activation of RNase L. Introduction of 8-bromoadenosine into the 5′-terminus or the intervening position decreased the activation ability drastically, while introduction of this modification into the 2′-terminus increased the activation efficiency 10-fold.[77] Similar kind of results were obtained with 8-methyladenosine modified 2′,5′-oligoadenylates.[78] These effects have been attributed to changes in the conformation. Bromine or methyl substitution at the 2′-terminal adenine ring forces this adenosine to adopt *syn* conformation around the base-sugar glycosidic bond influencing positively on RNase L activation. Additionally, this conformation appears to reduce the requirement for the 5′-phosphate group.[79] 2-5A tetramer containing 8-methyladenosine at the 2′-end and its 5′-dephosphorylated core analogue were able to activate human RNase L equally well and fourfold more efficiently than the parent monophosphorylated 2-5A.[80] Furhermore, bromo- and methyl-modifications enhanced the stability towards phosphodiesterases. Ionic character and size of the substituent also have effect on the biological properties of 2-5A. The trimer and tetramer 2-5A modified at the 2′-end by 8-azidoadenosine were shown to activate RNase L 10-fold less efficiently than the parent 2-5A tetramer, while the corresponding 8-aminoadenosine analogues could not activate RNase L at all.[81]



Hypoxanthine 7-Deazaadenine 8-Bromoadenine

Figure 8 Modifications of the adenine moiety.

1.4.1.4 2-5A analogues containing multiple modifications

Acyclo adenosine, containing a 2-hydroxyethoxymethyl group in the place of the ribose moiety, was attached via a phosphodiester linkage to the 2′-terminal hydroxyl function of a cordycepin core trimer and the 5′-terminal adenine was N^6-benzylated (Compound **A**, Figure 9) to provide nonpolar groups for increased cellular uptake and stability against nucleases.[49] In contrast to previous results with cordycepin core trimers, these analogues were found to upregulate the RNase L antiviral pathway of HIV-1 infected cells. The compounds were taken up intact by T cells in culture at concentrations required for activation of RNase L. No evidence of cytotoxicity was observed.

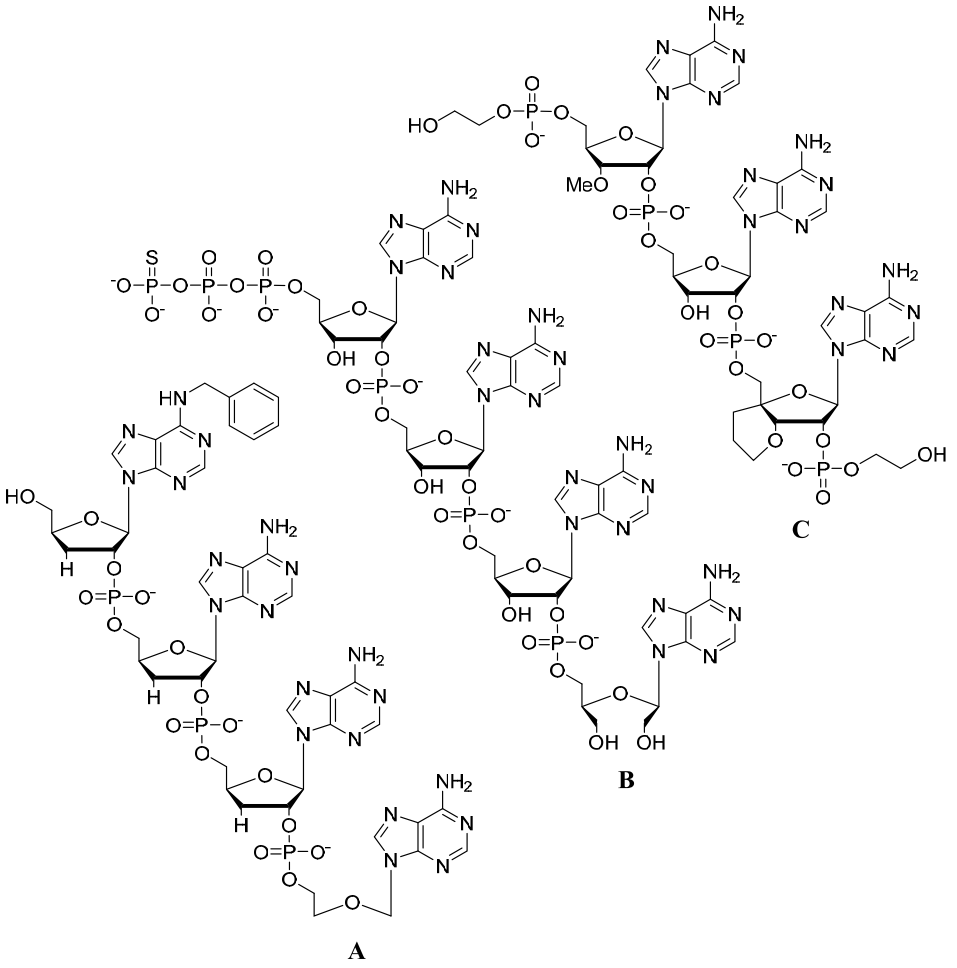


Figure 9 Potent RNase L activator analogues of 2-5A containing multiple modifications.

5′-γ-Phosphorothioate analogue of 2-5A tetramer triphosphate containing 2′,3′-*seco*-adenosine at the 2′-end (Compound **B**) was found to be 16 times more stable towards enzymatic degradation in HeLa cell extract, than the unprotected core.[82] This analogue was bound to RNase L as efficiently as the native 2-5A and it inhibited VSV growth almost completely in HeLa cells.

2-5A analogues containing three types of 3′-*O*,4′-*C*-bridged adenosines were examined.[61] Compound **C** having 3′-*O*-Me group at the 5′-end and 3′-*O*,4′-*C*-propylene adenosine at the 2′-end was the most potent RNase L agonist with as high RNase L activity as native 2-5A and high resistance to enzymatic degradation. Addition of a 2-hydroxyethylphosphate group at the 2′-and 5′-end brought drastically increased stability against exonucleases and phosphatases.

1.4.2 Cell delivery

Most of the modifications of 2-5A are aimed at enhancing its enzymatic stability. Cellular uptake, however, still remains as a threshold to overcome, since the polar nature of 2-5A prevents diffusion through the cell membrane. Several techniques have been tested to facilitate the cell delivery. 2-5A has been successfully introduced into intact cells *in vitro* by membrane permeabilization in hypertonic medium[83], by calcium phosphate precipitation[84] and by microinjection with micropipettes[85,66]. However, these techniques are quite drastic and, hence, not applicable for therapeutic purposes.

Encapsulation in liposomes offered the first delivery method that could be used *in vivo*. The specific delivery of 2-5A and its derivatives into mouse leukemia cells was achieved with the aid of monoclonal antibodies targeted against cell surface proteins.[86] The compounds were released from liposomes to cytosol via endocytosis. 2-5A analogues inhibited VSV replication at 10-fold lower concentration than the corresponding derivatives, which were directly microinjected to cytoplasm.

Ways to dispense with the negative charge of the 2-5A, while still retaining the ability to activate RNase L, have been studied to facilitate the passage through cell membrane.[87] 2-5A tetraphosphates capped with adenosine, A5′pppp5′A2′(p5′A)$_n$ (n = 2-3), were found to be stable in human serum, but were rapidly degraded to 2-5A by the enzymes of human cell extract. These analogues bound to RNase L and inhibited translation of EMCV in mouse L cell extract as efficiently as native 2-5A. Capped 2-5A di- and triphosphates did not, in turn, give 2-5A as a cleavage product and had a diminished ability to inhibit the protein synthesis.

Chemical conjugation of the polyanionic 2-5A to a synthetic polypeptide membrane carrier has been shown to enable efficient intracellular transport.[88] Additionally, conjugation at the 2′-end enhances resistance towards phosphodiesterases. 2-5A tetramer conjugated to positively charged poly(L-lysine) was introduced to murine leukemic cells. This conjugate inhibited protein synthesis in intact cells by promoting activation of RNase L, while a mixture of unbound 2-5A and poly(L-lysine) had no effect on protein synthesis. Additionally, an inhibition of virus growth was observed in VSV infected cells. 2-5A conjugated to a cell penetrating trans-activating-peptide was able to activate RNase L in intact HeLa cells.[89] This conjugate has expected to provide a solution for targeted destruction of HIV RNA *in vivo*.

Conjugation with lipophilic groups has been examined to facilitate interactions with substituents and receptors in the hydrophobic membrane. Cordycepin trimer

cores conjugated with cholesterol, vitamins or lipids at the 2´- and 5´-end have given promising results by exhibiting highly increased HIV-1 activities.[90,91,92] This could be attributed in part to the activation of RNase L. These conjugates were attached via ester linkages using carbonate, succinate or 6-aminohexanoyl linkers, which are cleaved off from the conjugates by intracellular esterases.

1.5 Prodrug strategies

Development of a prodrug strategy offers an alternative approach to improve cell permeability and enzymatic stability of 2-5A. The term prodrug refers to a chemical derivative of a drug molecule that requires one or two enzymatic or chemical transformation steps *in vivo* to release the parent drug.[93] A viable prodrug should have sufficient metabolic stability to survive in the gastrointestinal tract and systematic circulation, until distributed into cells. At the site of action, the prodrug should be converted to its active form at an efficacious rate. Moreover, the prodrug and metabolized promoieties should not cause excess toxicity.[94,95,96]

A number of prodrug strategies have been developed for analogues of nucleoside monophosphates and some of them have been approved for pharmaceutical use.[6,94,95,96,97,98] A few of the approaches have been further applied to oligonucleotides.[98] The nucleoside analogues are themselves prodrugs, since they are phosphorylated to mono- di- and triphosphates by intracellular or viral kinases to achieve the biological activity. The first phosphorylation is the rate-limiting step in human cells. Sometimes mutations in viral kinases (a mechanism of antiviral resistance) may result in prevention of this phosphorylation step entirely.[99] Direct administration of nucleoside as its monophosphate circumvents these limitations, but due to the ionic structure and degradation by phosphatases, these compounds are not able to achieve high concentrations at the target site.

A common prodrug strategy for nucleotides and oligonucleotides is protection of the negatively charged phosphate groups with biodegradable lipophilic protecting groups. The hydrophobic pronucleotides and pro-oligonucleotides are expected to permeate through lipid cell membrane into cytoplasm, where enzymes trigger chemical cleavage of the protecting groups releasing the intact parent nucleotide/ oligonucleotide drug (Figure 10).

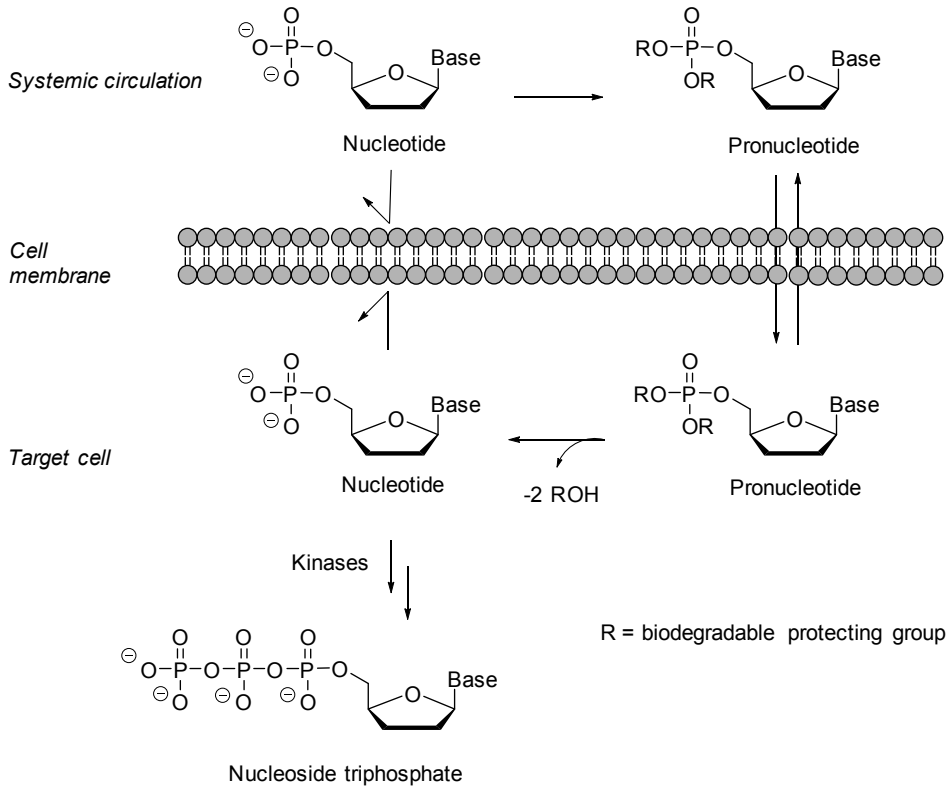


Figure 10 Representative illustration of the pronucleotide concept.

1.5.1 Esterase-labile protecting groups

The biodegradable protecting groups may be divided into different categories depending on by which mechanism they are cleaved. The enzymes responsible for prodrug activation include esterases, reductases, oxidase (cytochrome P_{450}), phospholipase C and phosphoramidase.[100] For example, reductases cleave the disulfide bond of 2-(2-hydroxyethyldisulfanyl)ethyl group[101] followed by spontaneous removal of the remnants of the protecting group and cytochrome P_{450} catalyses the oxidative cleavage of 1-arylpropan-1,3-diyl group[102]. In addition, a few chemically removable protecting groups, which do not require enzymatic activation, have been developed. These have been discussed in section 1.5.2.

Most of the enzyme-triggered prodrug strategies are dependent on esterase-mediated deacylation. The esters are rapidly hydrolyzed by carboxyesterases and other esterase enzymes present in tissues at high levels.[95] Breakdown may, however, occur to some extent before the absorption, since esterases are also

present in small intestine. The ester group is usually connected to the phosphate group through a linker, which is spontaneously removed after deacylation. The aim of the linker group is to accelerate the rate of hydrolysis. Unfortunately, these groups often have toxic side-effects.

Under physiological conditions, the nucleoside 5´-monophosphate carries two negative charges, which both should be masked in order to convert polar monoester into neutral lipophilic triester. The first protecting group is usually cleaved from the neutral triester quite smoothly. However, the nucleoside phosphate diester carrying a negative charge is a poor substrate for esterases, due to the short distance between the enzyme cleavage site and the negative charge.[103] Therefore, the cleavage of the second esterase-labile protecting group may be catalyzed by another enzyme, such as phosphodiesterase, if the esterase-catalysed deprotection becomes too slow.[104] Extensively investigated aryl phosphoramidate prodrugs, omitted from this discussion, are activated by esterases, but the conversion of the phosphoramidate to phosphate monoester is dependent on the action of phosphoramidase.[105,95] Examples of the esterase-labile protecting groups will be addressed in the following sections 1.5.1.1-1.5.1.5 in more detail.

1.5.1.1 Acyloxymethyl and alkoxycarbonyloxymethyl

Acyloxymethyl esters constitute one of the most commonly used prodrug-categories of nucleotides. Esterase enzymes catalyse the cleavage of the acyl group from the neutral nucleoside phosphate triester followed by rapid chemical departure of the hydroxymethyl function as formaldehyde.[104,106] The cleavage of the second acyloxymethyl group from the monoanionic phosphate diester, to yield the nucleotide drug may take place similarly, but more likely the hydrolysis is catalysed by phosphodiesterases (Figure 11).

Phenyl phosphate was used as a model compound to investigate enzymatic and chemical stability of bis(acyloxymethyl) phosphates.[103] The study indicated that the rate of hydrolysis was dependent on the nature of the acyl group. Bulkier alkyl substituents increased the stability of the phosphotriesters. The rate of hydrolysis decreased in the order acetyloxymethyl > isobutyryloxymethyl > pivaloyloxymethyl (POM).

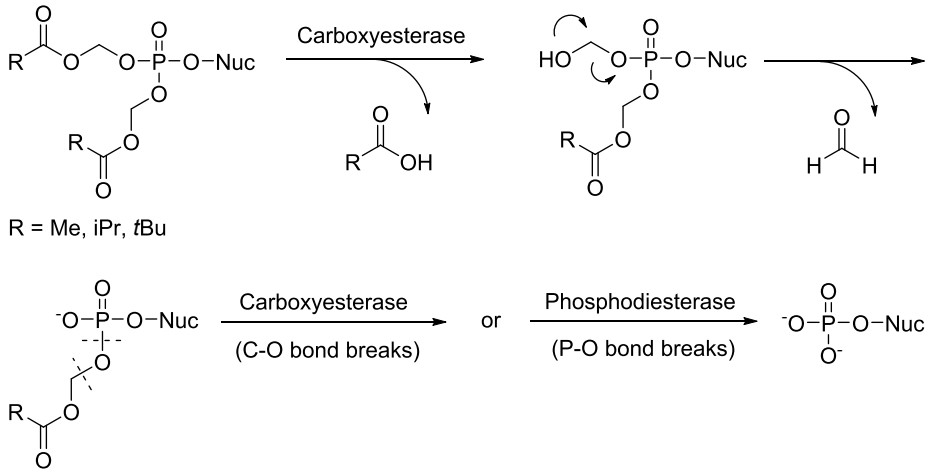


Figure 11 Biodegradation of bis(acyloxymethyl) nucleoside monophosphate prodrugs.

The prodrug strategy using POM group has been afterwards successfully applied to a number of nucleoside 5′-monophosphates to allow their penetration into cells by passive diffusion. The compounds studied include anti-HIV agents 2′,3′-dideoxyuridine[107] and 3′-azido-3′-deoxythymidine (AZT)[104] and anticancer agents 2′-deoxy-5-fluorouridine (FUdR)[106,108], 8-aza- and 8-bromo-2′-deoxyadenosine[109], and thymidine[110]. Applications to antiviral nucleoside phosphonates have also been reported. Bis(POM)adefovir[111,112] demonstrated significantly improved oral bioavailabilities in rats and was approved by the Food and Drug Administration (FDA) in 2002 for the treatment of hepatitis B infection (Hepsera®). Additionally, the applicability of acyloxymethyl group in pro-oligonucleotide approach has been evaluated.[113,114] The internucleosidic linkages of dinucleoside phosphorothioates were masked with acyloxymethyl group. The compounds were converted to the parent dinucleotides when exposed to serum and porcine liver esterases, although partial desulfurization took place.

The acyloxyalkyl prodrugs generate aldehyde, typically formaldehyde and carboxylic acid, in case of pivaloyloxymethyl group pivalic acid, upon cleavage the esters.[106,95] In chronic use, these by-products may have toxic effects. Exposure to high doses of pivalic acid has been connected with carnitine depletion.[115] Another drawback of this strategy is that these nucleotide prodrugs are degraded by plasma enzymes, which limits their systemic application.

To avoid formation of pivalic acid, the potential of alkoxycarbonyloxymethyl group as promoiety was investigated.[116] Carboxyesterases catalyse the cleavage of the carboxylate ester releasing alcohol and carbon dioxide as by-products. The

remnant of the protecting group is degraded spontaneously as with acyloxymethyl nucleotides. The conversion of the nucleotide diester to monoester may proceed by similar mechanism or is dependent on phosphodiesterase activity (Figure 12).

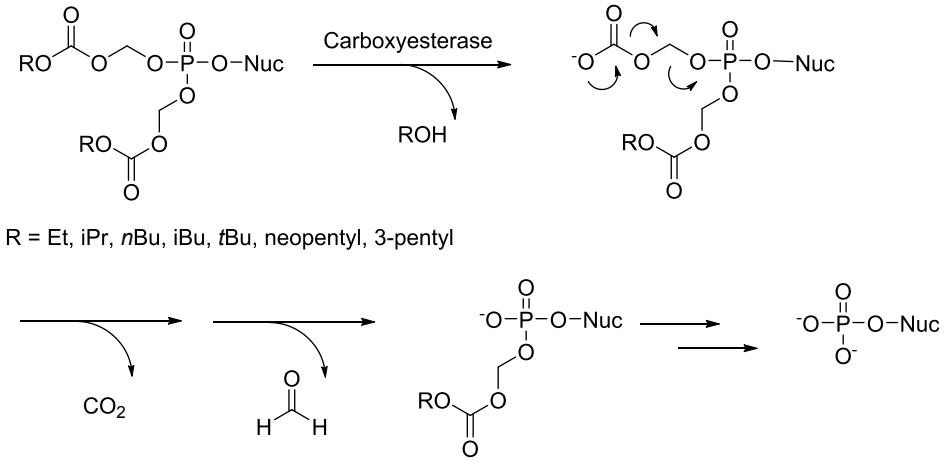


Figure 12 Biodegradation of bis(alkoxycarbonyloxymethyl) nucleoside monophosphate prodrugs.

A series of carbonate esters were tested *in vitro* and *in vivo* as prodrugs of tenofovir.[116,117] The compounds had reasonable chemical stabilities especially at lower pH, but they were rapidly hydrolyzed to the corresponding monoesters in the presence of plasma. Based on its good oral bioavailability (30 %) in dogs and low toxicity, bis(isopropyloxycarbonyloxymethyl)tenofovir was chosen as a clinical candidate. The compound was approved by the FDA in 2001 for the treatment of HIV-1 virus (Viread®) and later on in 2008 for the treatment of hepatitis B.

1.5.1.2 S-*Acyl-2-thioethyl*

S-Acyl-2-thioethyl (SATE) prodrugs of nucleotides are also dependent on esterase-mediated activation. Hydrolysis of the acyl group from the bis(SATE) phosphotriester yields an unstable 2-thioethyl intermediate, which then decomposes to episulfide and nucleoside phosphodiester.[118,119] The removal of the second SATE group follows the same mechanism. In some cases, the transformation to phosphomonoester may proceed through phosphodiesterases (Figure 13).

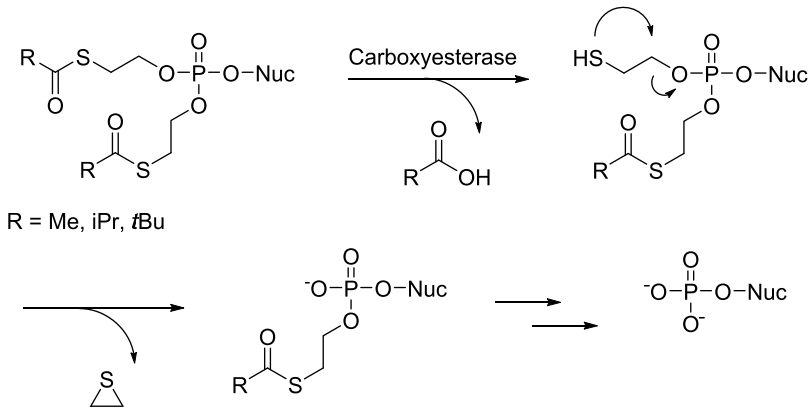


Figure 13 Biodegradation of bis(SATE) nucleoside monophosphate prodrugs.

The SATE prodrugs have been applied to nucleoside monophophosphates and phosphonates including dideoxyuridine[118], AZT, acyclovir and adefovir[120] and these compounds have demonstrated enhanced antiviral activities *in vitro*, compared to the parent nucleotides.[96,121] As with acyloxymethyl prodrugs, the stability of the SATE prodrugs may be adjusted by varying the size of the alkyl group in the ester moiety. When the stabilities and antiviral activities of bis(POM) and bis(SATE) derivatives of adefovir were evaluated, the bis(*t*-Bu-SATE) derivative proved to be the most promising having antiviral potency comparable to bis(POM) adefovir, but markedly greater chemical and enzymatic stability.[120] In addition to monomeric nucleotides, SATE approach has been extended to oligonucleotides.[122] Fully masked Me-SATE pro-dodecathymidylates were hydrolyzed to corresponding oligonucleotides in cell extract having a half-life about 21 hours.[123] The pro-oligonucleotides were also efficiently taken up by HeLa cells.[124]

Although SATE approach has shown promising results *in vitro* studies, the prodrug strategy has not been accepted for drug development, since episulfide, released as a by-product, is a strongly alkylating agent.[121,95]

1.5.1.3 4-Acyloxybenzyl

4-Acyloxybenzyl group was explored as an esterase-labile protecting group of nucleotide phosphates in order to increase the rate of removal of the second masking group from negatively charged nucleotide phosphodiesters.[125,126] The idea of this approach was to use benzyl group as a spacer to keep a considerable distance between the phosphate and the cleavage site of the carboxyesterase.[127] After enzymatic activation, the resulting 4-hydroxybenzyl phosphotriester

undergoes spontaneous decomposition to phosphodiester and a resonance-stabilized 4-hydroxybenzyl carbenium ion, which is presumably hydrolyzed to yield 4-hydroxybenzylalcohol. The same process is then repeated to give the phosphomonoester (Figure 14).

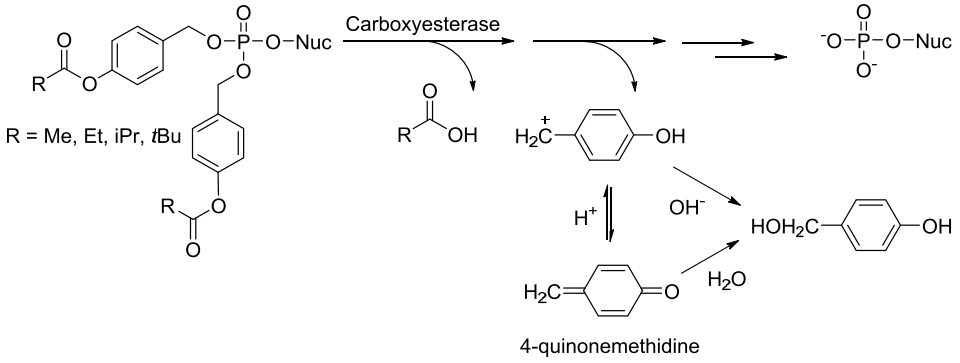


Figure 14 Biodegradation of bis(acyloxybenzyl) nucleoside monophosphate prodrugs.

The 4-acyloxybenzyl approach has been applied for the delivery of 5´-monophosphate of AZT[126] and 5,5´-nucleotide dimers containing AZT[128]. These triesters displayed anti-HIV activities comparable and superior to AZT. Studies on acyloxybenzyl prodrugs of oligonucleotides have also been reported.[129] Short half-life in serum and lipophilic structure however, makes systemic applications of 4-acyloxybenzyl prodrugs problematic. Furthermore, the carbenium ion generated by the fragmentation of the hydroxybenzyl intermediate, may interact with cellular nucleophiles and is associated with acute toxicity.[127]

1.5.1.4 2,2-Bis(substituted)-3-acyloxypropyl

2,2-Bis(substituted)-3-acyloxypropyl groups were studied as biodegradable protecting groups of phosphodiester bonds. Deacylation by carboxyesterases enables base-catalyzed retro aldol condensation that gives the desired phosphodiester (Figure 15). The stability after enzymatic deacylation is dependent on the nature of the 2-substituents.[130,131] The half-life for the non-enzymatic deprotection on using 2,2-bis(ethoxycarbonyl) substitutions was 1.5 hours at pH 7 and 25 °C, while the half-life for 2,2-dicyano derivative was only 0.3 seconds.

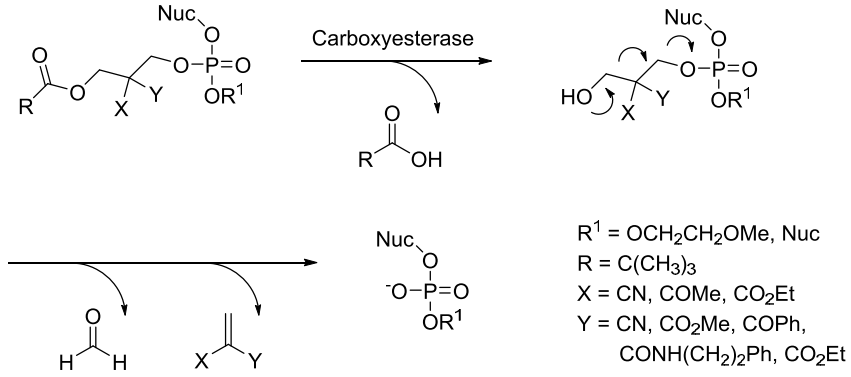


Figure 15 Biodegradation of 2,2-bis(substituted)-3-acyloxypropyl protected nucleoside phosphodiesters.

The hydrolytic stabilities of thymidylyl-(3′,5′)-thymidine phosphorothioates[132] and oligothymidylate pro-oligonucleotides[133] bearing one or two 2,2-bis(ethoxycarbonyl)-3-pivaloyloxy)propyl groups were studied. The protecting group was stable under physiological conditions but it was readily removed upon hydrolysis of the pivaloyl group by porcine liver esterase. The enzymatic hydrolysis was however markedly retarded with oligomers containing two of these protecting groups and partial degradation of the phosphorothioate backbone took place. It was suggested that hydrophobic interactions between the protecting groups may hinder the action of carboxyesterase.[98]

This prodrug approach shares the same safety concern as acyloxymethyl compounds, potentially toxic formaldehyde and carboxylic acid are liberated upon activation of the produgs. In addition, the released enone is potentially an alkylating agent.

1.5.1.5 1-Acyloxypropan-1,3-diyl

Surprisingly little attention has been paid on the fact, that the ability of esterases to catalyze the second deacylation of a negatively charged phosphate is severely retarded. The number of prodrugs of monophosphates dependent on only one enzymatic step is very limited. Nucleoside 5′-monophosphates containing cyclic 1,3-propylene ester substituted with an acyloxy group were designed to undergo carboxyesterase mediated deacylation to 4-hydroxy analogue, which should penetrate cells by passive diffusion.[134] The prodrug cleavage was suggested to proceed via ring-opening to an aldehyde, which would then be converted to the free nucleotide by spontaneous elimination of acrolein (Figure 16).

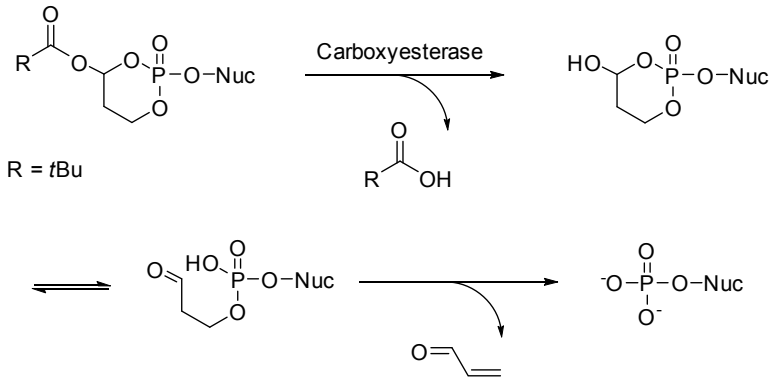


Figure 16 Biodegradation of 1-acyloxypropan-1,3-diyl nucleoside monophosphate prodrugs.

The prodrug strategy was applied to 5′-monophosphate of FUdR and its hydrolytic stability was studied. The prodrug was moderately stable in aqueous media, at physiological pH the half-life was 31 hours. In the presence carboxyesterase or plasma, the compound was readily degraded to FUdR. The prodrug inhibited the growth of Chinese hamster ovary cells in culture and prolonged the life spans of mice bearing leukemia as effectively as 5′-fluorouridine (FU).

A probable drawback of the strategy is the formation of chemically reactive acrolein upon biodegradation of the prodrug. This by-product has been connected with toxicity at high drug doses.

1.5.2 Thermolabile protecting groups

The number of prodrug strategies independent of enzymatic activation is so far quite limited. The major difficulty of this approach is optimization of the degradation rate in a way that the prodrug has sufficient extracellular stability, but is released rapidly inside the cell.

Cyclosaligenyl (*cyclo*Sal) group, which is degraded by an entirely pH-driven chemical hydrolysis mechanism, has been employed for protection of nucleoside monophosphates.[135] Nucleophilic attack of a hydroxide ion on the phosphorus atom results in cleavage of the phenyl ester bond and subsequent cleavage of the benzyl ester yields the nucleotide and salicyl alcohol. The stability of the pronucleotides may be tuned by varying the substituents on the aromatic ring. The electron-withdrawing substituents were found to increase the rate of hydrolysis. In order to increase the intracellular concentrations, “lock-in”-

*cyclo*Sal-pronucleotides bearing an esterase cleavable group attached to the aromatic ring has been developed.[136,137,138] Enzymatic cleavage yields a polar intermediate, trapping the pronucleotide inside the cell. The release of nucleotide from carboxylic acid intermediate was too slow, but incorporation of diacetoxymethyl[138] (Figure 18) or 1-acetoxyvinyl[136] group at the 5-position of a 3-alkyl-substituted cyclosal ring strongly accelerated the chemical hydrolysis. Bis-*cyclo*Sal prodrugs, in turn, were designed to deliver two active nucleotides for each prodrug molecule, displaying a mask-drug ratio of 1:2.[139]

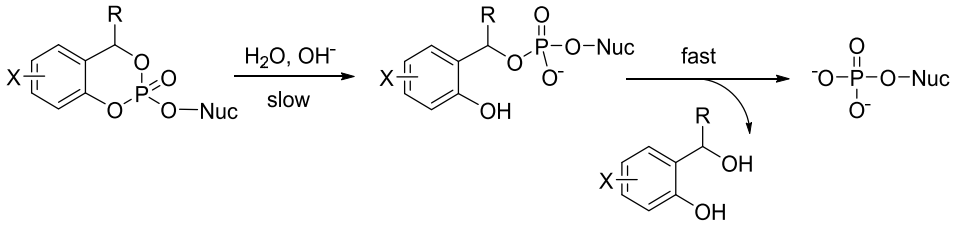


Figure 17 Biodegradation of *cyclo*Sal nucleoside monophosphate prodrugs.

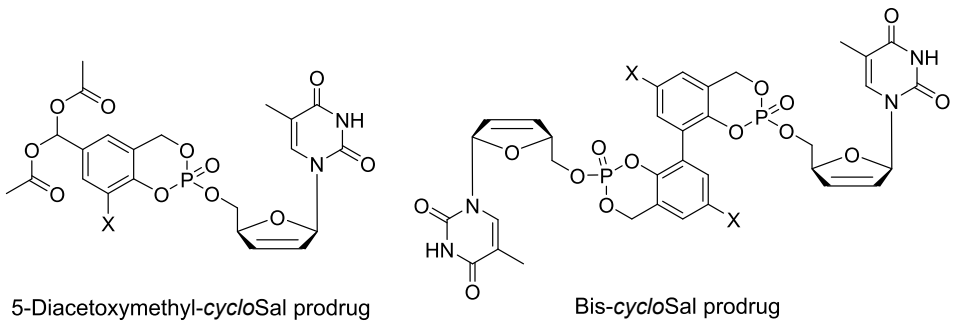


Figure 18 *Cyclo*Sal prodrugs of 2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphate.

As with 4-acyloxybenzyl prodrugs, the potential toxicity of released quinone methidine, which is further hydrolysed to salicylic alcohol, evokes a concern, although salicylic alcohol showed no toxicity when evaluated for cytotoxicity in mice. Another limitation of the strategy is that *cyclo*Sal pronucleotides are potential human butyrylcholinesterase inhibitors. Bulkier substituents on the aromatic ring were shown to decrease the inhibition activity.[135]

Purely thermolabile thiophosphate protecting groups (*N*-formyl-*N*-methyl)-2-aminoethyl[140], 4-hydroxybutyl[141] and *ω*-(alkylthio)alkyl[142] groups, have been studied for the protection of immunostimulatory phosphoromonothioate oligomers. The protecting groups are released by cyclization in an aqueous

environment at 37 °C. For example, the half-life for the deprotection of 4-(methoxymethylthio)butyl protected dinucleoside phosphorothioate triester was 6.5 h, while the half-life for the corresponding (*N*-formyl-*N*-methyl)-2-aminoethyl protected compound was 72 h.

1.5.3 Special requirements of ribonucleotide protection

Protecting of negatively charged phosphate esters of nucleosides and oligonucleotides turns the phosphomonoesters and phosphodiesters into phosphotriesters. Ribonucleotides and oligoribonucleotides have a free hydroxyl group neighbouring the phophotriester, which makes the use of a prodrug strategy problematic.

Studies with dialkyl esters of 5′-*O*-protected uridine 2′- and 3′-monophosphates have shown that an unprotected hydroxyl group attacks in aqueous solutions immediately on the adjacent phosphotriester leading to migration of the phosphate group between the 2′- and 3′-hydroxy functions and cleavage of the alkyl ester linkages to yield 2′- and 3′-alkylphosphates and a 2′,3′-cyclic phosphate.[143,144] At physiological pH, the isomerization and cleavage reactions are hydroxide ion catalysed. Deprotonated hydroxyl group attacks the neutral phosphotriester giving monoanionic phosphorane. (Figure 19) Pseudorotation of the phosphorane intermediate results in departure of the 2′- or 3′-oxyanions. Alternatively, the alkoxy group is cleaved. Isomerization is much (several orders of magnitude) faster than the cleavage, since endocyclic cleavage of the phosphorane intermediate occurs more readily than the exocyclic cleavage. The reaction rates were accelerated when the electronegativity of the alkyl substituents were increased. The electronegative substituents diminish the electron density at phosphorous atom and facilitate the attack of neighbouring hydroxyl group.[145]

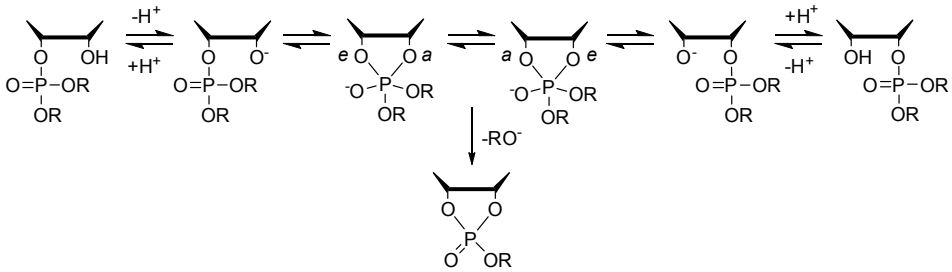


Figure 19 Hydroxide ion catalyzed isomerization and cleavage of phosphotriesters.

Accordingly, when designing a prodrug-strategy for an oligoribonucleotide such as 2-5A, the hydroxyl groups neighbouring the phosphotriesters should be protected. Additionally, these hydroxyl groups should not be exposed before the protecting groups of the phosphodiester bonds are cleaved.

2 AIMS OF THE THESIS

Analogues of nucleotides and oligonucleotides constitute a promising class of therapeutic agents. The efficiency of phosphate based drugs, however, largely depends on the prodrug strategy used to enhance their cellular uptake. The basic idea with most of the prodrug strategies is that the negative charges of the phosphate groups are kept masked in plasma and restored by an enzyme-triggered removal of the protecting groups inside the cell. The primary aim of the thesis was to evaluate the feasibility of a prodrug strategy, based on esterase-labile protecting groups, for the short oligoribonucleotide, 2-5A trimer.

Under physiological conditions, the 2-5A trimer bears four negative charges, which all should be masked to improve the internalization (Figure 20). In addition, the 3´-hydroxyl groups adjacent the phosphodiester groups must be kept protected as long as the phosphodiester bonds are protected. Otherwise the attack of the unprotected 3´-hydroxyl group on the neighboring phosphotriester would result in isomerization to a 3´,5´- linkage and cleavage of the p-O5´bond.

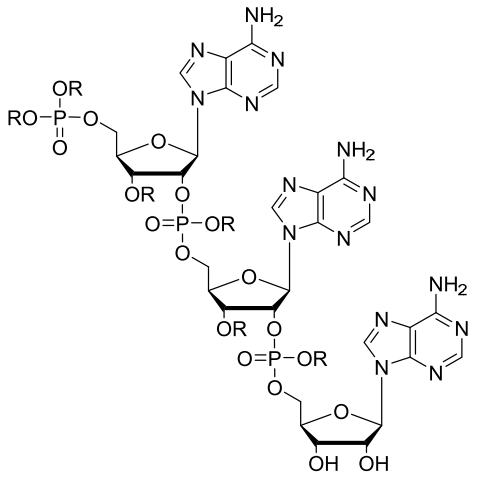


Figure 20 Structure of the protected 2-5A trimer studied in the thesis. R = esterase-labile protecting group.

In spite of the success of the esterase-dependent prodrug strategies, they still suffer from one major shortcoming. The enzymatic deacylation is markedly retarded upon accumulation of negative charge on the substrate, which is the case with molecules having a monophosphate function or several phosphodiester linkages. Another general problem is that biodegradation of the prodrugs often

releases potentially toxic by-products. For example, formaldehyde and alkylating enone structure are formed upon breakdown of the 3-acyloxy-2,2-bis(ethoxycarbonyl)propyl group. For these reasons, development of protecting groups, which are not dependent on enzymatic activation and do not liberate electrophilic alkylating agents or other toxic compounds, appeared highly desirable.

The aims of the thesis, studied in publications I-V, may be summarized as follows:

i. To optimize the structures of esterase-labile protecting groups of 2-5A by synthesizing protected dinucleoside-2′,5′-monophosphates and nucleoside 5′-monophosphates as model compounds and studying the enzyme-triggered deprotection reactions in buffered solutions of carboxyesterases (Papers I and II).

ii. To synthesize fully protected 2′-5′-oligoadenylate trimers as prodrug candidates for 2-5A and investigate their potential as therapeutic agents by following multistep release of 2-5A by carboxyesterases (Paper III).

iii. To develop esterase-labile phosphate protecting groups that additionally are thermolabile and study the rate of the enzymatic and non-enzymatic removal of such groups from phosphotriesters. (Papers IV and V)

3 RESULTS

3.1 2-5A prodrug candidate I (1)

As a first step towards a viable 2-5A pro-drug, trimer **1** bearing esterase-labile protecting groups was prepared.[III] The negatively charged phosphate groups were protected with 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl groups, since these groups had been shown to be stable under physiological conditions, but to be cleaved by retro-aldol condensation after enzymatic deacylation.[130,131] The 3´-hydroxyl groups adjacent to the phosphodiester bonds were protected with pivaloyloxymethyl groups. The enzymatic deacylation is susceptible to steric properties of the acyl group and the bulky pivaloyl group was aimed at decelerating the deacylation to prevent exposure of the 3´-hydroxyl group before removal of the protecting group from the neighboring phosphodiester linkage. A dimeric model compound (**2**) was first synthesized to assess the compatibility of the 3´-*O* and phosphate protecting groups.[II]

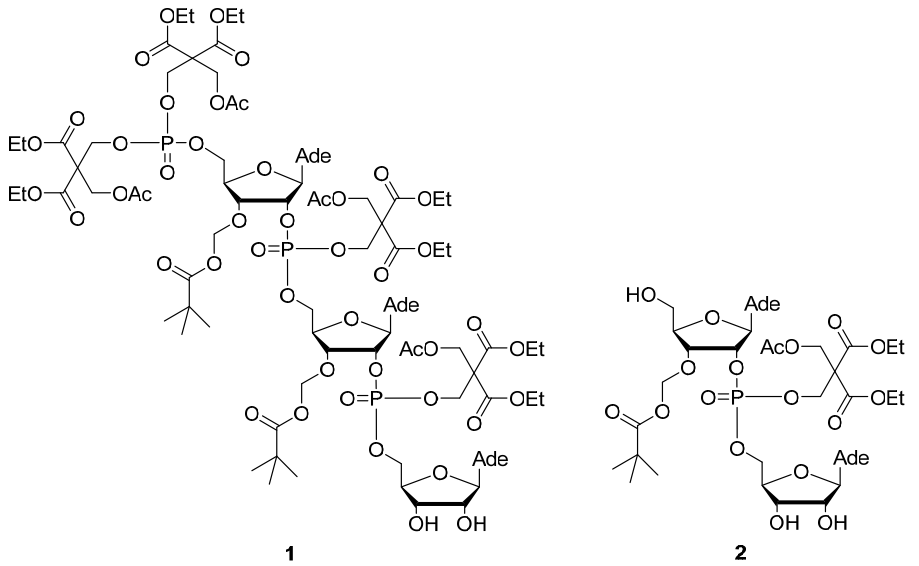


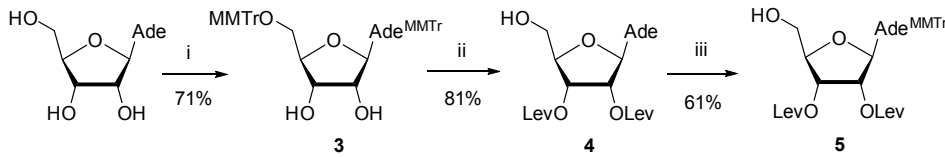
Figure 21 Structures of 2-5A trimer **1** (prodrug candidate I) and adenylyl-2´,5´-adenosine **2** protected with esterase-labile protecting groups.

3.1.1 Syntheses

3.1.1.1 Nucleosidic building blocks (5, 9, 10)

Synthesis of the protected 2′-terminal nucleoside 2′,3′-di-*O*-levylinoyl-N^6-(4-methoxytrityl)adenosine (**5**), is depicted in Scheme 1.[II] The 4-methoxytrityl group was introduced to the 5′-hydroxyl and N^6-amino functions of adenosine,[146] the 2′- and 3′-hydroxyl groups were protected with levulinoyl groups and the fully protected compound was detritylated to obtain **4**. 4-Methoxytrityl group was re-introduced at the amino function using trimethylsilyl group for the transient protection[147] of the 5′-hydroxyl.

Scheme 1[a]

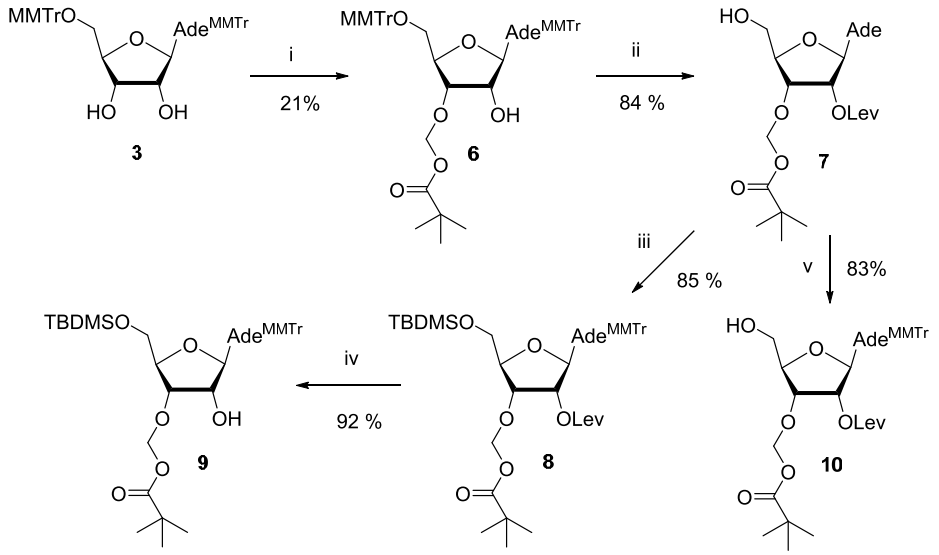


[a]*Conditions: (i) MMTrCl, pyridine; (ii) (1) $LevO_2$, dioxane, pyridine, (2) 80 % AcOH; (iii) (1) Me_3SiCl, pyridine, (2) MMTrCl, pyridine, (3) TBAF, AcOH, THF*

Synthesis of the intervening nucleoside 5′-*O*-(*tert*-butyldimethylsilyl)-N^6-(4-methoxytrityl)-3′-*O*-pivaloyloxymethyladenosine[II] (**9**), and the 5′-terminal nucleoside, 2′-*O*-levulinoyl-N^6-(4-methoxytrityl)-3′-*O*-pivaloyloxymethyladenosine[III] (**10**), is outlined in Scheme 2. Pivaloyloxymethyl protection was introduced to the 3′-hydroxyl group of the N^6,O^5-bis(monomethoxytrityl) adenosine (**3**) by deprotonating the hydroxyl group with 1 equiv. of sodium hydride in THF and then alkylating the nucleoside with pivaloyloxymethyl chloride in the presence of a catalytic amount of sodium iodide. A mixture of 3′-*O*- and 2′-*O*-(pivaloyloxymethyl) derivatives in 21 % and 7% yields, respectively, was obtained. Additionally, 2′-*O*- and 3′-*O*-pivalates were formed as side-products, possibly by intramolecular attack of the free hydroxyl group on the adjacent carbonyl function, which explains the low yield of the desired products. Protection of the 2′-hydroxy function of the 3′-*O*-(pivaloyloxymethyl) isomer **6** with levulinoyl group and subsequent acidolytic removal of the monomethoxytrityl groups afforded **7**, which was used as a starting material for both **9** and **10**. For this purpose, the 5′-hydroxyl group of **7** was protected with a *tert*-butyldimethylsilyl group and the adenine moiety with a 4-methoxytrityl group to yield **8**. Removal of the levulinoyl group completed the

synthesis of the intervening nucleoside (**9**). The 5´-terminal nucleoside **10** was, in turn, obtained by temporary silylation of the 5´-hydroxyl group of **7**, followed by 4-methoxytritylation of the 6-amino group.

Scheme 2[a]

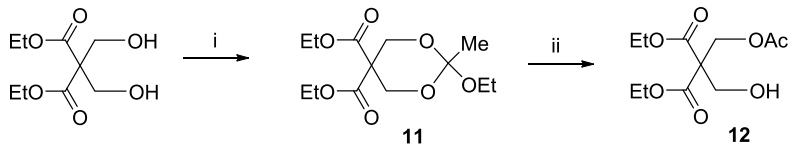


[a]*Conditions: (i) PivOCH₂Cl, NaH, NaI, THF; (ii) (1) LevO₂, dioxane, pyridine, (2) 80% AcOH; (iii) (1) TBDMSCl, pyridine, (2) MMTrCl, pyridine; (iv) NH₂NH₂·H₂O, AcOH, pyridine; (v) (1) TMSCl, pyridine, (2) MMTrCl, pyridine, (3) Bu₄NF, AcOH, THF*

3.1.1.2 Assembly of the 2´-terminal dimer (2)

The phosphate protecting group reagent was prepared from the commercially available diethyl 2,2-bis(hydroxymethyl)malonate (Scheme 3).[I] The compound was converted to orthoacetate **11** and then treated with acetic acid to obtain **12**.[148]

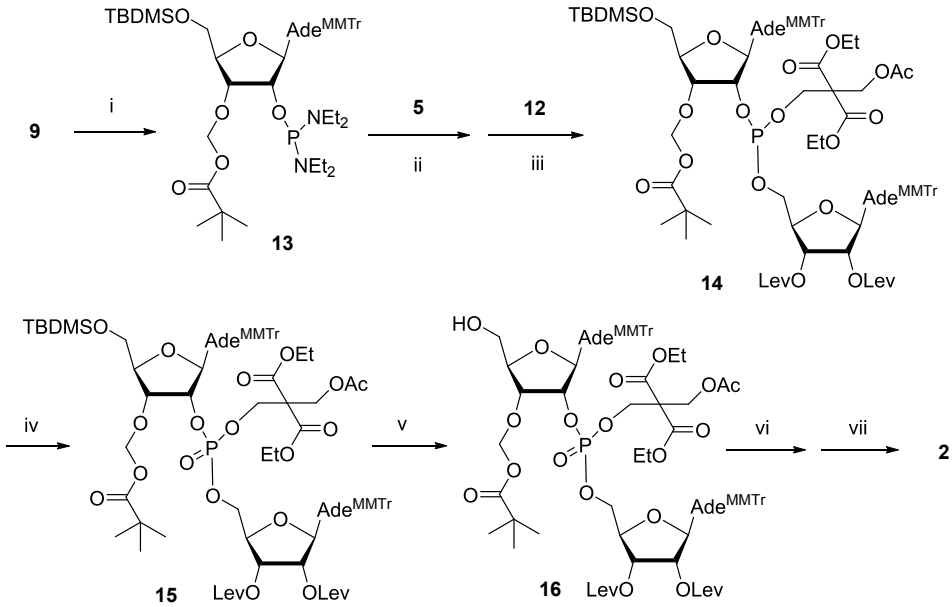
Scheme 3[a]



[a]*Conditions: (i) (EtO)₃CMe, H₂SO₄, THF; (ii) 80% AcOH*

The 2´-terminal dimer (**2**) was prepared from the appropriately protected nucleosides **5** and **9** as depicted in Scheme 4.[II] The 2´-hydroxy function of nucleoside **9** was first phosphitylated with 1-chloro-*N*,*N*,*N*´,*N*´-tetraethylphosphanediamine in the presence of Et_3N giving **13**. The diethylamino ligands were then stepwise replaced by nucleoside **5** and diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**12**) using 1*H*-tetrazole as an activator. The resulted phosphite triester **14** was oxidized to phosphate triester **15**.

Scheme 4[a]



[a]*Conditions: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) TetH, MeCN; (iii) TetH, MeCN; (iv) I_2, THF, H_2O, 2,6-lutidine; (v) Bu_4NF, AcOH, THF; (vi) $NH_2NH_2{\cdot}H_2O$, AcOH, pyridine, (vii) 80% AcOH*

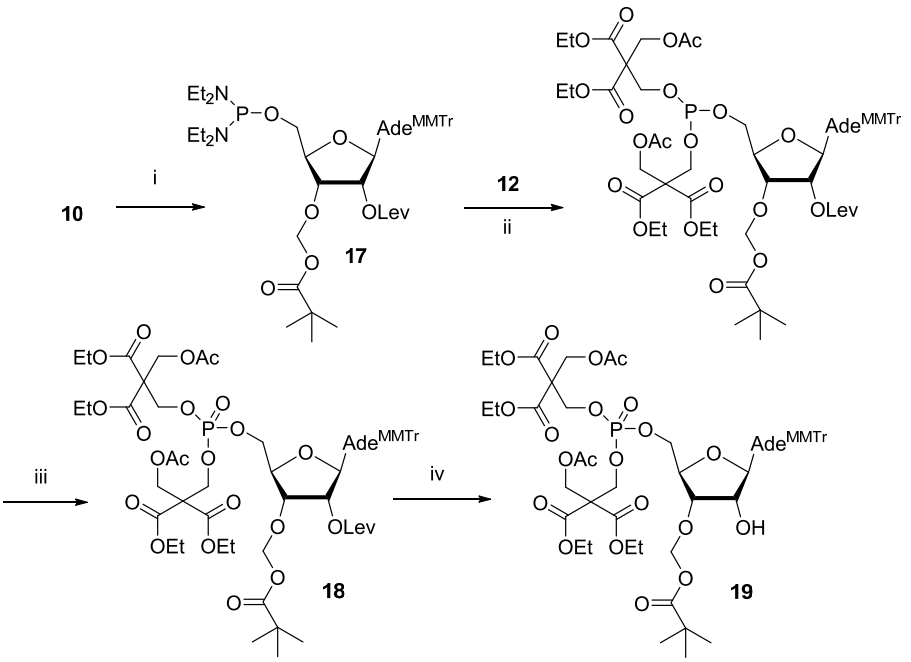
The course of the phosphitylation, coupling and oxidation reactions was followed by ^{31}P NMR spectroscopy. The formation of *N*,*N*,*N*´,*N*´-tetraethylphosphorodiamidite (**13**) was accompanied by appearance of a ^{31}P NMR resonance at 137.2 ppm. Conversion to phosphite triester (**14**) gave ^{31}P NMR signals at 140.6 ppm and 140.5 ppm [(R_P)- and (S_P)-diastereomers)] and oxidation to the corresponding phosphate triester (**15**) moved the ^{31}P NMR resonances to -2.2 ppm and -2.7 ppm. Finally, the 5´-*O*- silyl group was removed with Bu_4NF in THF in the presence of AcOH to obtain **16**. Acid was used for decreasing the basicity of the fluoride anion. The levulinoyl groups were

removed by treatment with 0.5 M hydrazine in pyridine-acetic acid (4:1 v/v) and finally the compound was subjected to detritylation to afford a diastereomeric mixture of **2**.

3.1.1.3 Conversion of the 5′-terminal nucleosidic building block (10) *to protected 5′-monophosphate* (19)

Fully protected adenosine 5′-monophosphate (**19**) was prepared as outlined in Scheme 5.[III] Phosphitylation of the 5′-hydroxy function of 2′-*O*-levulinoyl-N^6-(4-methoxytrityl)-3′-*O*-(pivaloyloxymethyl)adenosine (**10**) with $(Et_2N)_2PCl$ in the presence of Et_3N to obtain **17**, followed by tetrazole-aided displacement of the diethylamino ligands with diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**12**) gave after oxidation phosphate triester **18**.

Scheme 5[a]



[a]*Conditions: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O, lutidine; (iv) $NH_2NH_2 \cdot H_2O$, AcOH, pyridine*

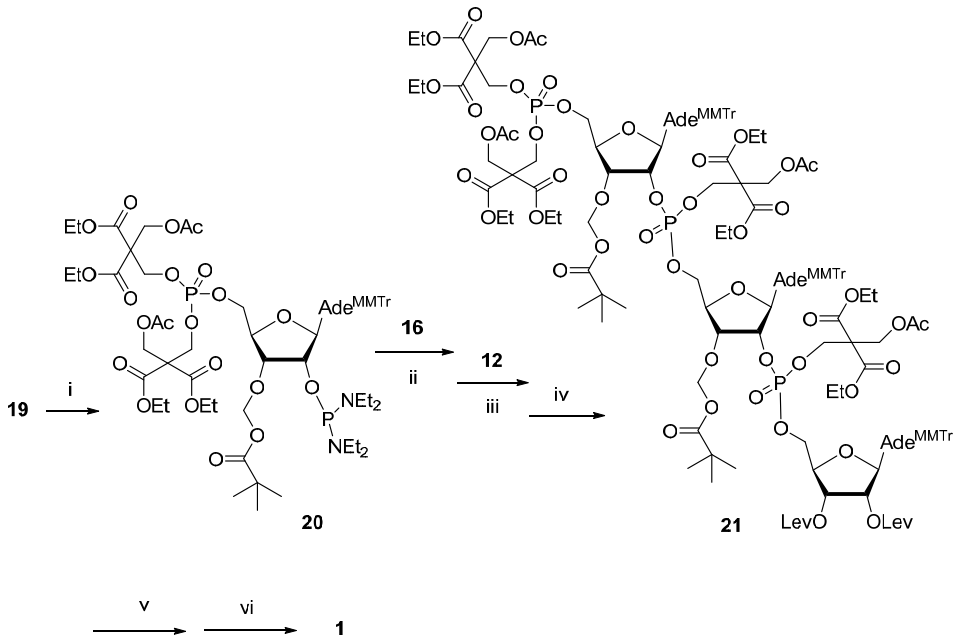
^{31}P NMR monitoring verified the formation of the *N,N,N′N′*-tetraethylphosphorodiamidite (**17**) by appearance of a ^{31}P NMR resonance at 133.3 ppm. The phosphite triester resonated at 138.8 ppm and oxidation moved

the ^{31}P NMR resonance to -2.6 ppm (**18**). Removal of the levulinoyl group by treatment with 0.5 M hydrazine in pyridine-acetic acid (4:1 v/v) yielded **19**.

3.1.1.4 Assembly of the 2-5A trimer (1)

The assembly of trimer **1** was analogous to the preparation of dimer **2** (Scheme 6).[III] The 2´-hydroxy function of the protected adenosine 5´-monophosphate (**19**) was phosphitylated with $(Et_2N)_2PCl$. The diethylamino ligands were stepwise replaced with dimer **16** (see Scheme 4) and diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**12**) by a 1*H*-tetrazole –promoted reaction and the resulted phosphite triester was subjected to oxidation to yield **21**.

Scheme 6[a]



[a]*Conditions: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) TetH, MeCN; (iii) TetH, MeCN; (iv) I_2, THF, H_2O, lutidine; (v) $NH_2NH_2{\cdot}H_2O$, AcOH, pyridine; (vi) 80% AcOH*

The course of the phosphitylation, coupling and oxidation reactions was followed by ^{31}P NMR spectroscopy. The formation of the *N,N,N´,N´*-tetraethylphosphorodiamidite (**20**) was verified by appearance of a ^{31}P NMR resonance at 137.4 ppm. The phosphite triester resonated at 140.5 and 140.4 ppm [(R_P)- and (S_P)-diastereomers] and the phosphate triesters (**21**) at -1.5-(-3.0) ppm.

The levulinoyl groups and the trityl groups were finally removed to obtain the biodegradably protected trimer **1**. One of the diastereomers was separated by reversed-phase HPLC for the enzymatic studies.

3.1.2 Enzymatic deprotection

The enzymatic hydrolysis of the protected adenylyl-2′,5′-adenosine (**2**) and 2-5A trimer (**1**) was studied with hog liver carboxyesterase (HLE; 2.6 units ml^{-1}) in a HEPES buffer at pH 7.5 and 37 °C. Aliquots were taken at suitable time intervals from the reaction mixture and determined by HPLC. The products and intermediates were characterized by mass spectrometric analysis (HPLC/ESI-MS) or by spiking with authentic samples.

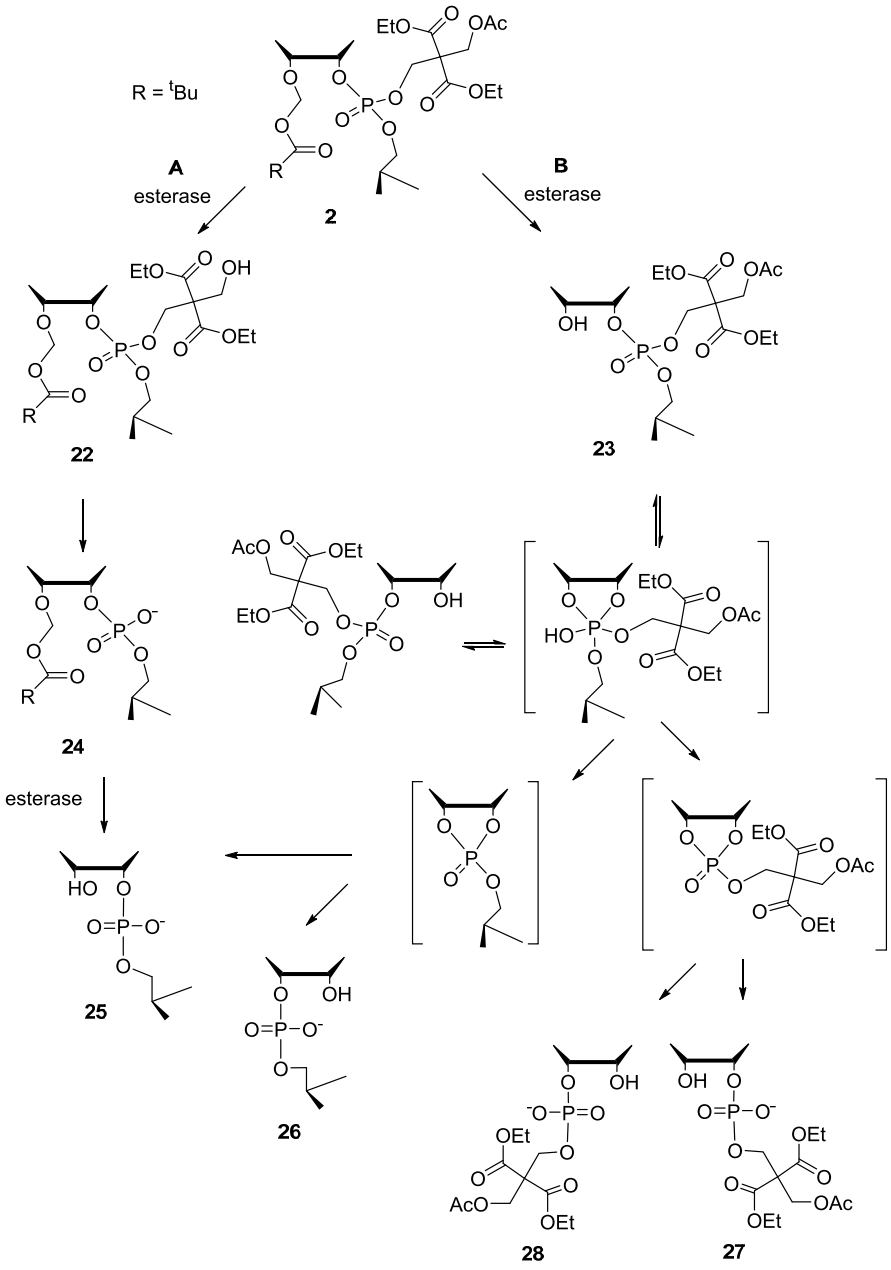
3.1.2.1 Esterase-triggered hydrolysis of the dimer (2)

Figure 22 shows the HPLC traces referring to the progress of the HLE-catalyzed hydrolysis of the diastereomers of **2**.[II] The compound undergoes two parallel carboxyesterase-catalyzed reactions: deacetylation of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group to **22** followed by hydroxide ion catalyzed loss of formaldehyde and concomitant elimination of diethyl 2-methylenemalonate to yield **24** (route A, Scheme 7) and deacylation of 3′-*O*-pivaloyloxymethyl group followed by rapid half-acetal hydrolysis of the exposed hydroxymethyl moiety to give **23** (route B). Among these products, esterase-triggered deacylation of **24** results in formation of 2′,5′-ApA (**25**) as the sole product. The competing route via **23** also yields **25**, but additionally its 3′,5′-isomer (**26**) and adenosine 2′-*O*- and 3′-*O*-[3-(acetyloxy),2,2-bis(ethoxycarbonyl)propyl] phosphates **27** and **28** are formed. The proposed mechanism for the formation of these compounds involves an intramolecular nucleophilic attack of the 3′-hydroxy group of **23** on the phosphorus atom. The obtained phosphorane intermediate is decomposed to acyclic 2′,5′- and 3′,5′-phosphotriesters and to two different 2′,3′-cyclic phosphotriesters. Finally, the cyclic esters are hydrolysed to acyclic diesters **25-28**.

As seen from Figure 22, 2′,5′-ApA (**25**) was the main product of the enzymatic deprotection. The exposure of the 3′-hydroxyl function (route B) was actually twice as rapid as the exposure of the phosphodiester bond (route A), but both routes resulted in formation of 2′,5′-ApA (**25**), Route A as a sole and Route B as a partial product. After 24 hours, the starting material **2** had disappeared and the proportion of 2′,5′-ApA (**25**) was 20 % and the proportion of side-products **26-28** was 32 %. Since the conversion of **24** to **25** was very slow, 24 % was still

present as **24**. During prolonged 96 hours treatment with HLE, the proportion of 2´,5´-ApA was increased to 37 %.

Scheme 7



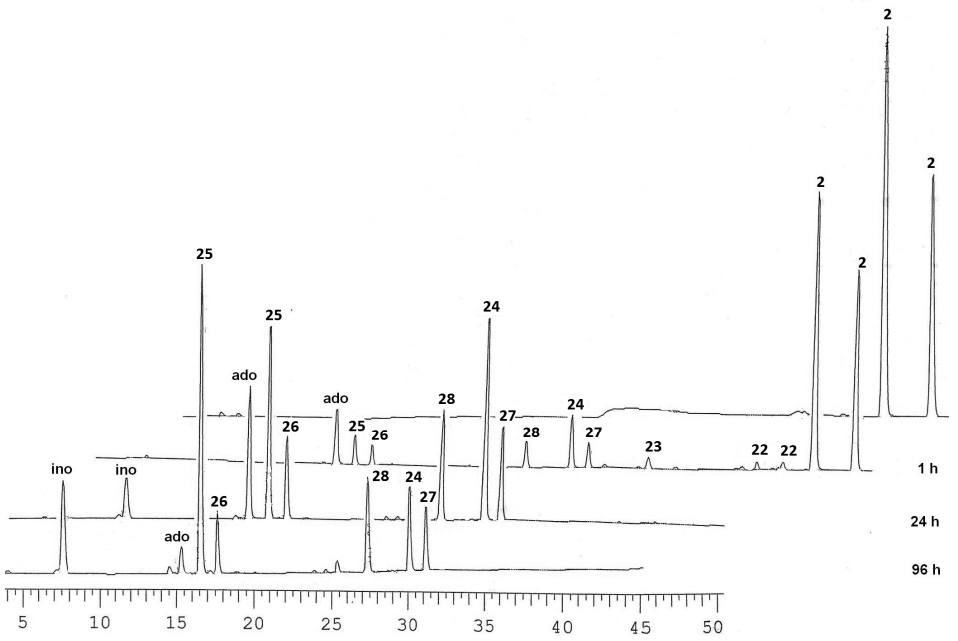


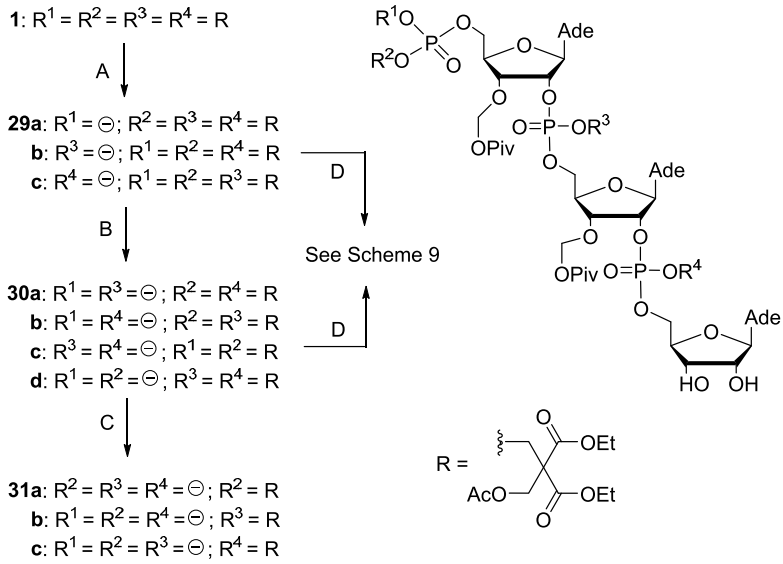
Figure 22 RP-HPLC traces for the HLE-catalyzed deprotection of [3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl [5′-adenosinyl] [3′-*O*-(pivaloyloxymethyl)-2′-adenosinyl] phosphate (**2**) at pH 7.5 and 37 °C (*I* = 0.1mol l^{-1} with NaCl). For the structures, see Scheme 7.

3.1.2.2 Esterase-triggered hydrolysis of the 2-5A trimer (1)

The HPLC traces referring to the HLE-triggered deprotection of one diastereomer of trimer **1** at two and eight days are shown in Figure 23.[III] During the first two days, only one phosphate protecting group was cleaved (Reaction A in Scheme 8). Enzymatic deacetylation of **1** triggers removal of the protecting group by retro-aldol condensation yielding one of the three possible diesters **29a**-**29c**. The departure of the second phosphate protecting group resulted in formation of three of the four possible dianions **30a**-**30d** (Reaction B). Since a phosphate diester carrying a negative charge is a poor substrate for esterases, the two major chromatographic signals most likely refer to **30a** and **30b** having one of the internucleosidic phosphodiester linkages deprotected in addition to the terminal phosphate. The minor signal may then represent a trimer bearing either deprotected internucleosidic phosphates (**30c**) or fully deprotected 5′-phosphate (**30d**). The departure of the next phosphate protecting group took place very slowly (Reaction C). From the three possible trianions **31a**-**31c**, only two were observed by HPLC- analysis. Even after 2 weeks treatment with HLE, the third

phosphate protecting group was removed only partially and the fully deprotected 2-5A never appeared.

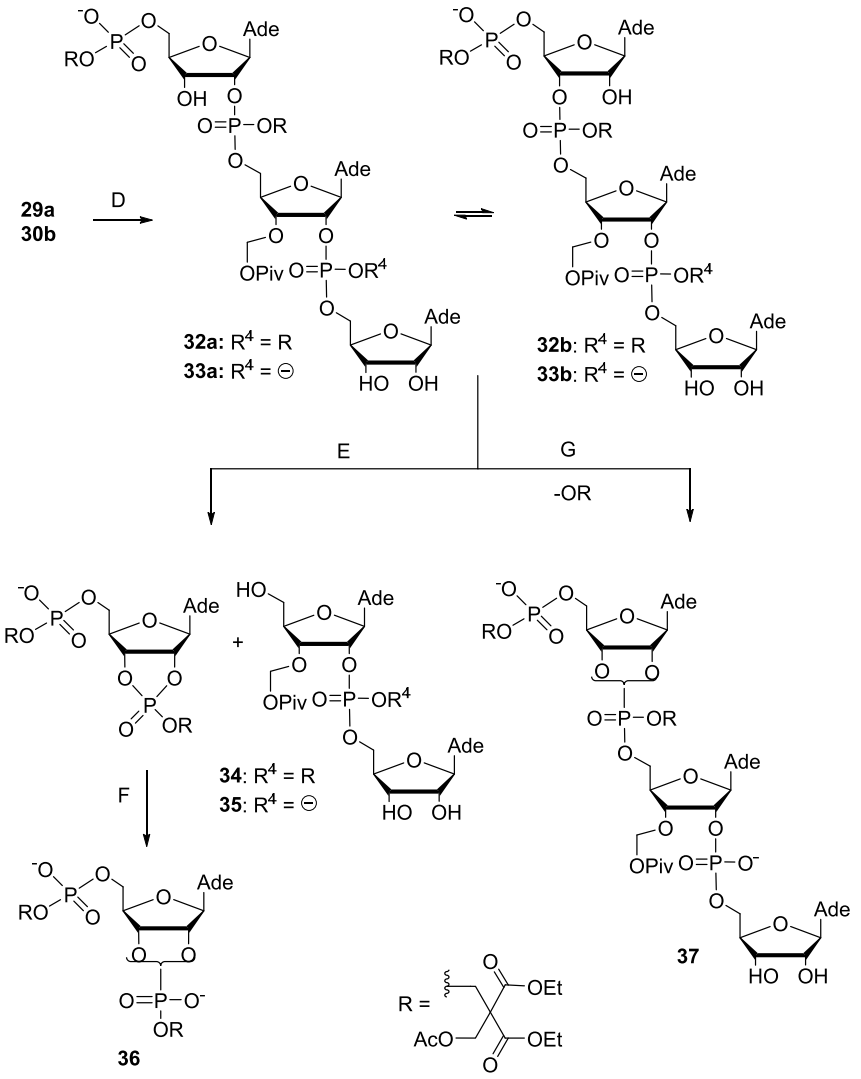
Scheme 8[a]



[a]*HLE-catalyzed deprotection of 2-5A trimer* ***1***

According to the product distribution, the 3′-*O*-(pivaloyloxy)methyl protection of the 5′-terminal nucleotide, seemed to be removed to a minor extent before the exposure of the adjacent phosphodiester linkage (Reaction D, Scheme 9). The attack of the unprotected 3′-hydroxyl group on the protected phosphate group leads to 2′,5-′ to 3′,5′-isomerization and cleavage of dimer **34** and **35** (Reaction E). The cyclic triester formed is then rapidly hydrolysed to phosphodiesters **36** (Reaction F). Cleavage of the lower phosphate protecting group from **32** and **33** gives isomeric phosphodiesters **37** (Reaction G).

Scheme 9[a]



[a]*Side reactions taking place during the HLE-catalyzed deprotection of 2-5A trimer* ***1***

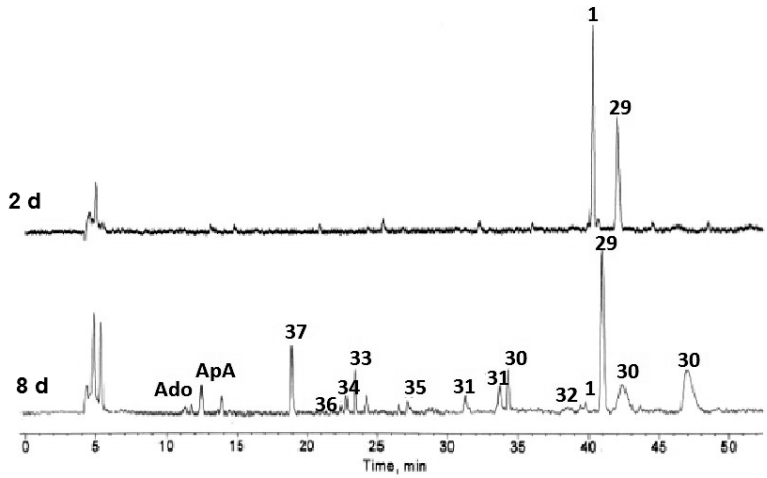


Figure 23 RP-HPLC traces for the HLE-catalyzed deprotection of 2-5A trimer **1** at pH 7.5 and 37.0 °C (I = 0.1 mol l^{-1} with NaCl). For the structures, see Schemes 8 and 9.

3.2 2-5A prodrug candidate II (38)

The first prodrug-strategy developed for 2-5A did not give satisfactory results, since the enzymatic deprotection turned out to be far too slow and the fully deprotected 2-5A trimer was never released.

The most severe problem was the removal of the second protecting group from the 5´-phosphate group. Carboxyesterases were unable to catalyze the deacylation of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group of the diester monoanion. To facilitate the enzymatic reaction, the distance between the acyl function and the phosphate moiety was increased by replacing the 3-acetyloxy group with 3-acetyloxymethoxy group. The removal of the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group from thymidine 5´-monophosphate **39b** was studied and the results were compared to those obtained with the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl protected counterpart **39a**.[I]

Another major problem was that, the removal of pivaloyloxymethyl protection from the 5´-terminal nucleotide was too fast compared to the exposure of the neighboring phosphate. Accordingly, the 3´-hydroxyl group of this nucleotide was protected permanently as methyl ether, since it had been shown[61] that this substitution did not prevent the activation of RNase L. The 3´-hydroxyl group of the intervening adenosine is, in turn, important for the activation of RNase L, and hence, an esterase-labile protection had still to be used for this functionality. To

accelerate the deprotection, pivaloyloxymethyl group was replaced with more labile acetyloxymethyl group. The HLE-triggered removal of the latter group was first studied with a dimeric model compound (**40**).[II] Finally, the second 2-5A prodrug candidate (**38)** was prepared and the multistep release of 2-5A was investigated.[III]

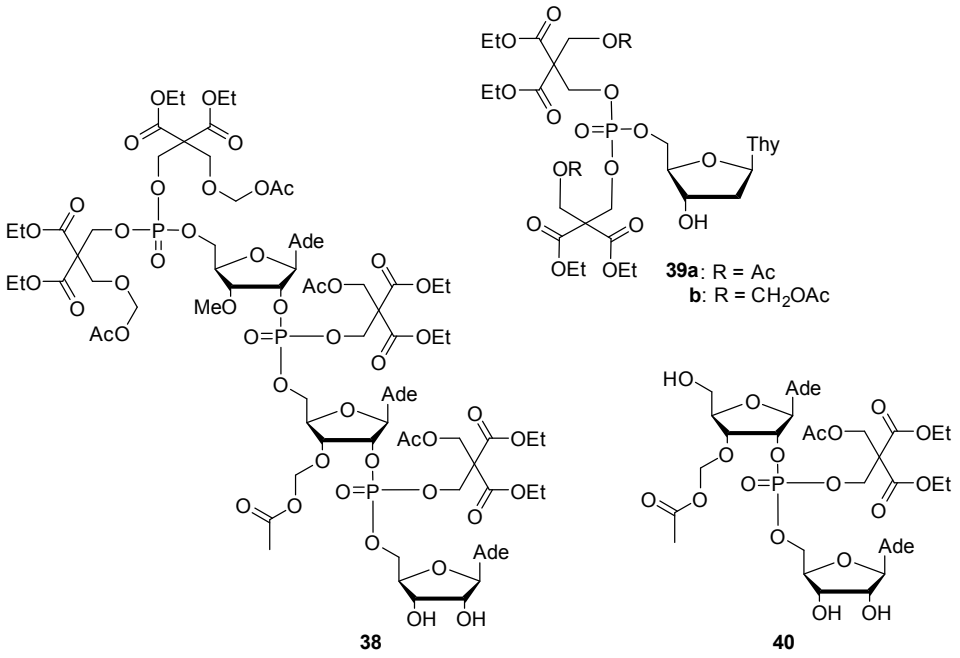


Figure 24 Structures of 2-5A trimer **38** (prodrug candidate II), adenylyl-2′,5′-adenosine **40** and thymidine 5′-monophosphates **39a** and **39b** protected with esterase-labile protecting groups.

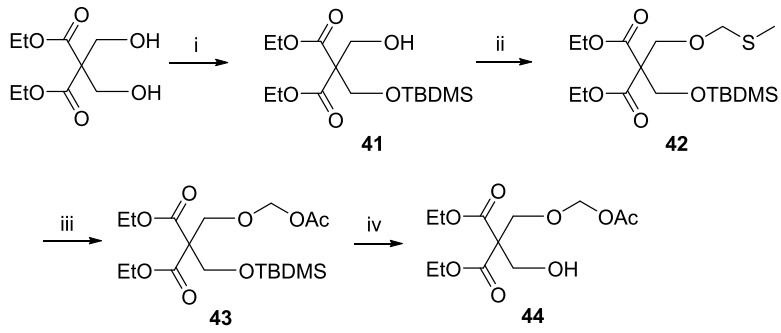
3.2.1 Comparison of *O*-acetyl and *O*-acetyloxymethyl protected 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl groups as protecting groups for 5′-terminal monophosphate

3.2.1.1 Syntheses of protected thymidine 5′-monophosphates (39a,b)

The preparation of protected thymidine 5′-monophosphates **39a** and **39b** was analogous to that of the protected adenosine 5′-monophosphate (**19**) (Scheme 5).[I] In other words, the compounds were obtained by stepwise alcoholysis of $(Et_2N)_2PCl$ first with 1 equiv. of 3′-*O*-levulinoylthymidine and then with either

diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**12**, Scheme 3) or diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**44**, Scheme 10) followed by oxidation to phosphate triester and finally removal of the levulinoyl group. The preparation of protecting group reagent **44** is outlined in Scheme 10. One of the hydroxyl groups of diethyl 2,2-bis(hydroxymethyl)malonate was protected with *tert*-butyldimethylsilyl group (**41**) and the other one was converted to methylthiomethyl ether[149] (**42**). The methylthio group was displaced with acetate ion (**43**) and the silyl group was removed cautiously with $Et_3N{\cdot}3HF$ to keep the acetyloxymethyl function intact.[150]

Scheme 10[a]



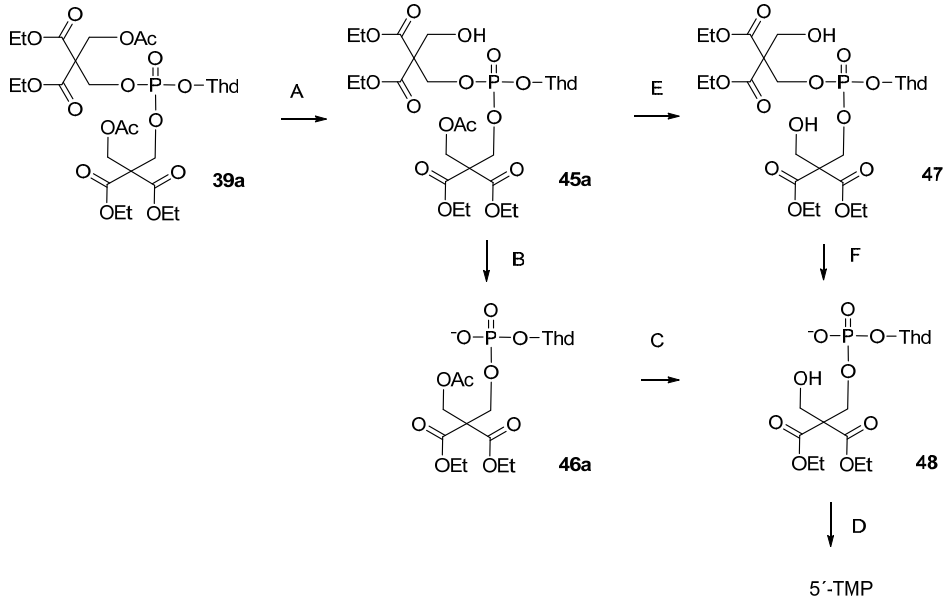
[a]*Conditions: (i) TBDMSCl, Py; (ii) DMSO, AcOH, Ac_2O; (iii) SO_2Cl_2, AcOK, DCM, 18-crown-6; (iv) $Et_3N{\cdot}3HF$, THF*

3.2.1.2 Enzymatic deprotection

The kinetics of the HLE-triggered deprotection of thymidine 5′-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39a**) and 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39b**) were followed in a HEPES buffer at pH 7.5 by HPLC.[I] The products and intermediates were identified by mass spectrometric analysis (HPLC/ESI-MS).

Figure 25 shows the time-dependent product distribution for the treatment of **39a** with HLE (26.0 units ml^{-1}) at 25 °C. HLE-triggered deacetylation of the starting material **39a** yielded **45a** (reaction A in Scheme 11), which underwent two parallel reactions: second deacetylation to triester **47** (reaction E) and cleavage of the deacetylated protecting group by retro-aldol condensation to yield diester **46a** (reaction B). The subsequent retro-aldol condensation of **47** (reaction F), and deacylation of **46a** (reaction C) yielded diester **48**, which finally released the monophosphate by retro-aldol condensation (reaction D).

Scheme 11



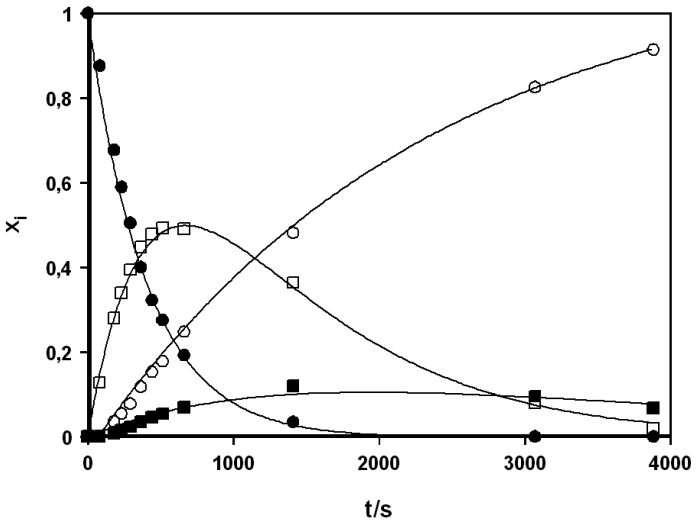


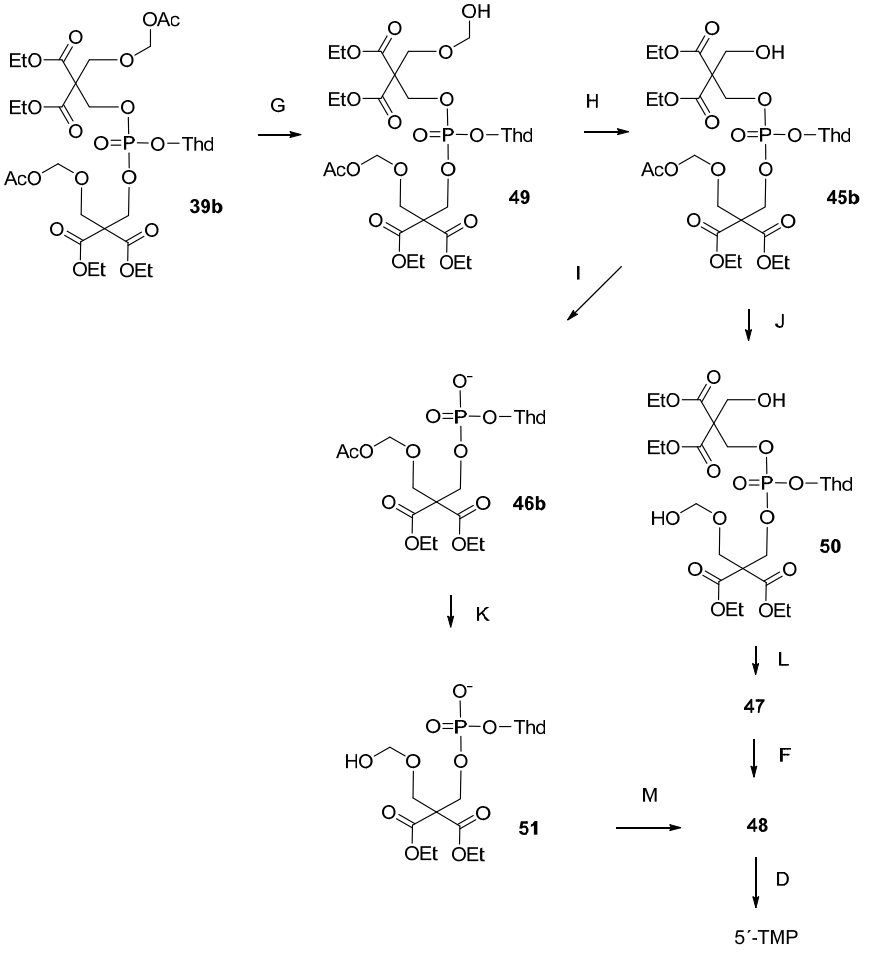
Figure 25 Time-dependent product distribution for the HLE-triggered hydrolysis of thymidine 5´-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)]propyl]phosphate (**39a**) at pH 7.5 and 25.0 °C (I = 0.1 mol l^{-1} with NaCl). Notation: (●) **39a**; (□) **45a**; (■) **47**; and (○) mixture of **46a** and **48**. For the structures, see Scheme 11.

As expected on the basis of the earlier results obtained with the 2-5A trimer **1**, the deacetylation of the negatively charged diester **46a** was extremely slow. While the half-life for the first deacetylation of **39a** to **45a** (reaction A) was 4.3 min and for the subsequent reaction to **47** (reaction E) 31 min, the half-life for the deacylation of **46a** to **48** (reaction C) was 190 h at 25 °C (Table 1). The cleavage of the 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl group from the diester **48** was slightly faster. The half-lives for the reactions B and D are 31 minutes and 23 hours, respectively. At the high HLE concentrations employed, **45a** was converted to the diesters **46a** and **48** in equimolar amounts. Since reaction E is enzyme catalysed and reaction B enzyme independent, at lower HLE concentration (4.3 units ml^{-1}), **46a** was accumulated as the main product. Consequently, deacylation of diester **46a** most likely is the rate limiting step for the release of monophosphate under physiological conditions. After 2 weeks, the proportion of acetylated diester **46a** was still 20 %. The proportion of released thymidine 5′-monophosphate (5′-TMP) was 15 % at maximum (at t = 5d). During a prolonged hydrolysis 5′-TMP was largely dephosphorylated to thymidine. This was probably caused by contamination with phosphomonoesterase.

To find out whether replacement of the acetyloxy group with acetyloxymethoxy group would facilitate the enzymatic deprotection, 5′-bis[3-acetyloxymethoxy-2,2-bis-(ethoxycarbonyl)propyl]phosphate (**39b**) was treated with HLE (26.0 units ml^{-1}) at 37 °C and the departure of the protecting groups was studied. As shown by the time-dependent product distribution in Figure 26, the disappearance of the starting material is accompanied by formation of triesters **49**, **45b**, **50** and **47**, which are subsequently decomposed to phosphodiesters **46b**, **51** and **48** releasing finally the 5′-TMP (Scheme 12).

Enzymatic deacylation of **39b** to **49** (Reaction G) is followed by rapid cleavage of the remaining hydroxymethyl function to yield triester **45b** (Reaction H). This triester then decomposes by two parallel reactions: retro-aldol condensation yields diester **46b** (Reaction I) and deacylation gives triester **50** (Reaction J). **46b** then undergoes deacylation (Reaction K) and concomitant loss of formaldehyde (Reaction M) to produce **48**. Cleavage of the hydroxymethyl group from triester **50** (Reaction L) and subsequent retro-aldol condensation (Reaction F) also yields diester **48**, which finally produces the desired 5′-TMP by retro-aldol condensation (Reaction D). Partial breakdown of 5′-TMP to thymidine occurred upon prolonged treatment with HLE.

Scheme 12


OAc
EtO
EtO
O=P–O–Thd
AcO
OEt OEt
39b
G
OH
49
H
45b
I
J
46b
K
50
L
47
F
HO
51
M
48
D
5´-TMP

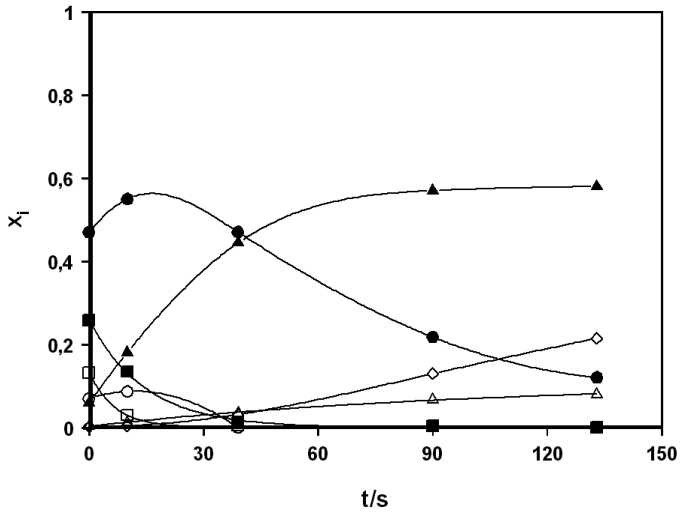


Figure 26 Time-dependent product distribution for the HLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propyl]phosphate (**39b**) at pH 7.5 and 37.0 °C (I = 0.1 mol l^{-1} with NaCl). Notation: (□) **39b**; (■) (**49**); (●) (**45b**); (○) (**50**); (▲) (**47**), (Δ) (**46b**), (◊) (**48**). For the structures, see Scheme 12.

In other words, the deacylation was markedly accelerated upon insertion of the extra $–CH_2O-$ fragment in the structure. The half-lives for the initial deacylation of the starting materials **39b** and **39a** were 10s and 194 s at 37 °C, respectively (Table 1). The half-lives for the disappearance of diesters **46b** and **46a** were 3.6 h and 150 h at the same temperature. Consequently, the first deacylation is accelerated 20-fold and the second 40-fold. At the high HLE concentrations employed, the conversion of **45b** to deacetylated triester **50** was faster than conversion to diester **46b**. At lower HLE concentrations, the route via **46b** predominates and the deacylation of the diester becomes rate-limiting. Finally, the half-life for the release of 5′-TMP from **48** by retro-aldol condensation was 6 h at 37 °C. The results clearly demonstrate that the 3-acetyloxymethoxy-2,2-bis-(ethoxycarbonyl)propyl group is more applicable for the protection nucleoside 5′-monophosphates than the 3-acetyloxy derivative.

Table 1 First-order rate constants for the partial reactions involved in the HLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39a**) and thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39b**) to 5′-TMP at pH 7.5 (I = 0.1 mol l^{-1} with NaCl).

Reaction[a]	T / °C	k / $10^{-4}s^{-1}$	$t_{1/2}$ / min
39a → **45a** (A)	25.0	27	4.3
	37.0	36	3.2
45a → **46a** (B)	25.0	3.8	31
45a → **47** (E)	25.0	3.8	31
46a →**48** (C)	25.0	0.010	11500
	37.0	0.013	9025
47 → **48** (F)	25.0	≈ 4	≈ 30
48 → 5′-TMP (D)	25.0	0.085	1360
39b → **49** (G)	37.0	680	0.17
49 → **45b** (H)	37.0	≈ 800	≈ 0.14
45b → **46b** (I)	37.0	11	10.4
45b → **50** (J)	37.0	130	0.90
46b → **51** (K)	37.0	0.54	210
50 → **47** (L)	37.0	≈ 800	≈ 0.14
47 → **48** (F)	37.0	≈ 10	≈ 10
51 → **48** (M)	37.0	fast	fast
48 → 5′-TMP (D)	37.0	0.31	370

[a]For the reactions, see Schemes 11 and 12. [HLE] = 26 units ml^{-1}.

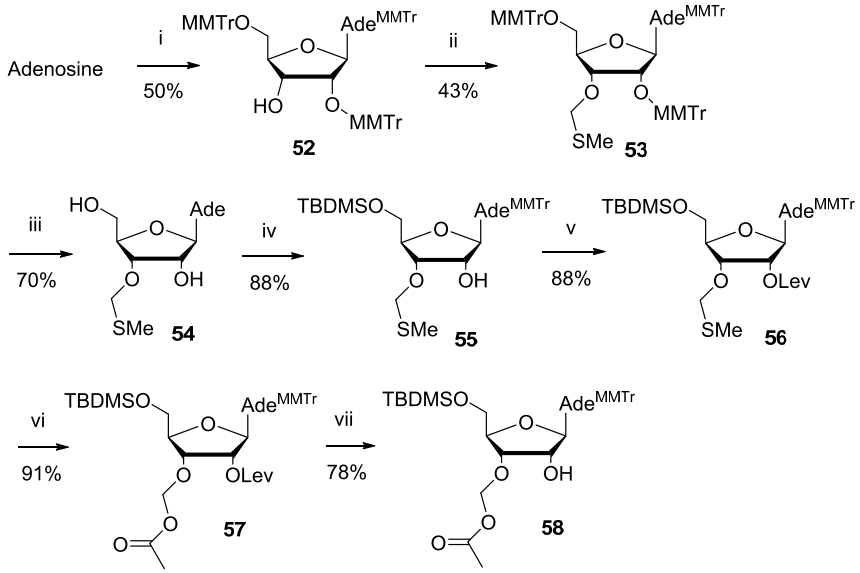
3.2.2 Syntheses

3.2.2.1 Nucleosidic building blocks (58, 61)

Synthesis of the intervening nucleoside 3′-*O*-(acetyloxymethyl)-5′-*O*-(*tert*-butyldimethylsilyl)-N^6-(4-methoxytrityl)adenosine (**58**) is depicted in Scheme 13.[II] The introduction of the 3′-*O*-acetyloxymethyl protection turned out to be intractable, since direct alkylation with acetyloxymethyl halides failed.

Fortunately, a two-step route via a methylthiomethyl intermediate gave the desired product: N^6,$O^{2'}$,$O^{5'}$-tris(monomethoxy)tritylated adenosine (**52**) was deprotonated with sodium hydride and treated with methylthiomethyl chloride to give derivative **53**. The compound was then subjected to detritylation to obtain **54**. Introduction of *tert*-butyldimethylsilyl and 4-methoxytrityl group to the 5′-hydroxyl and 6-amino functions, respectively, afforded **55**. The 2′-hydroxyl group was then protected with levulinoyl group to obtain **56**. The methylthiomethyl ether was converted to chloromethyl ether by sulfuryl chloride treatment, and the chloro substituent was displaced with acetate ion in the presence of dibenzo-18-crown-6, giving **57**.[150] Finally the levulinoyl group was removed with hydrazine acetate in a mixture of MeOH and DCM to obtain **58**. These conditions turned out to be sufficiently mild to leave the acetyloxymethyl group intact.[151]

Scheme 13[a]



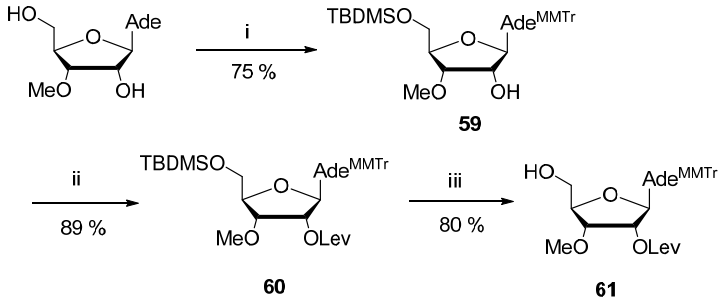
[a]*Conditions: (i) MMTrCl, pyridine; (ii) (1) NaH, DMF, (2) $MeSCH_2Cl$, DMF; (iii) 80% AcOH; (iv) (1) TBDMSCl, pyridine, (2) MMTrCl, pyridine, (v) Lev_2O, dioxane, pyridine; (vi) (1) SO_2Cl_2, DCM, (2) KOAc, dibenzo-18-crown-6, DCM; (vii) H_2NNH_3OAc, DCM, MeOH.*

Preparation of the 5′-terminal nucleoside, 2′-*O*-levulinoyl-N^6-(4-methoxytrityl)-3′-*O*-methyladenosine (**61**), is outlined in Scheme 14.[III] The 5′-hydroxyl group of the commercially available 3′-*O*-methyladenosine was protected with *tert*-butyldimethylsilyl group and the adenine moiety with 4-methoxytrityl group to have

59. The 2´-hydroxyl group was levulinoylated to obtain **60** and removal of the *tert*-butyldimethylsilyl protection completed the synthesis of **61**.

The synthesis of the 2´-terminal nucleoside, 2´,3´-di-*O*-levulinoyl-N^6-(4-methoxytrityl)adenosine (**5**) has been depicted above in Scheme 1.[II]

Scheme 14[a]

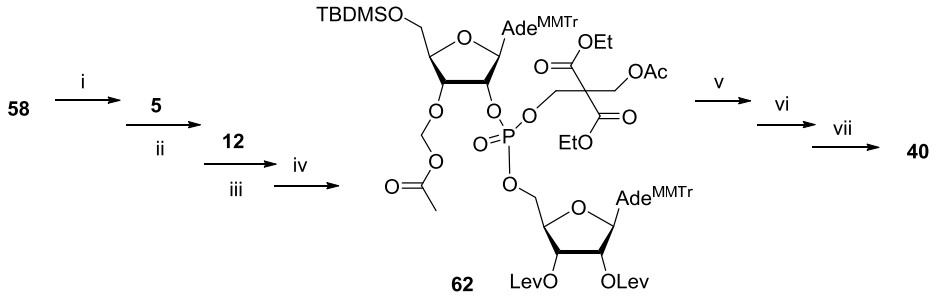


[a]*Conditions: (i) (1) TBDMSCl, pyridine, (2) MMTrCl, pyridine; (ii) Lev_2O, DMAP, dioxane, pyridine; (iii) Bu_4NF, AcOH, THF.*

3.2.2.2 Assembly of the 2´-terminal dimer (40) *and the 2-5A trimer* (38)

The 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl ester of 3-(acetyloxymethyl)adenylyl-2´,5´-adenosine (**40**) (Figure 24) was prepared from the nucleosidic building blocks **58** and **5** and the protecting group reagent **12** (Scheme 15), as described previously for dimer **2**.[II]

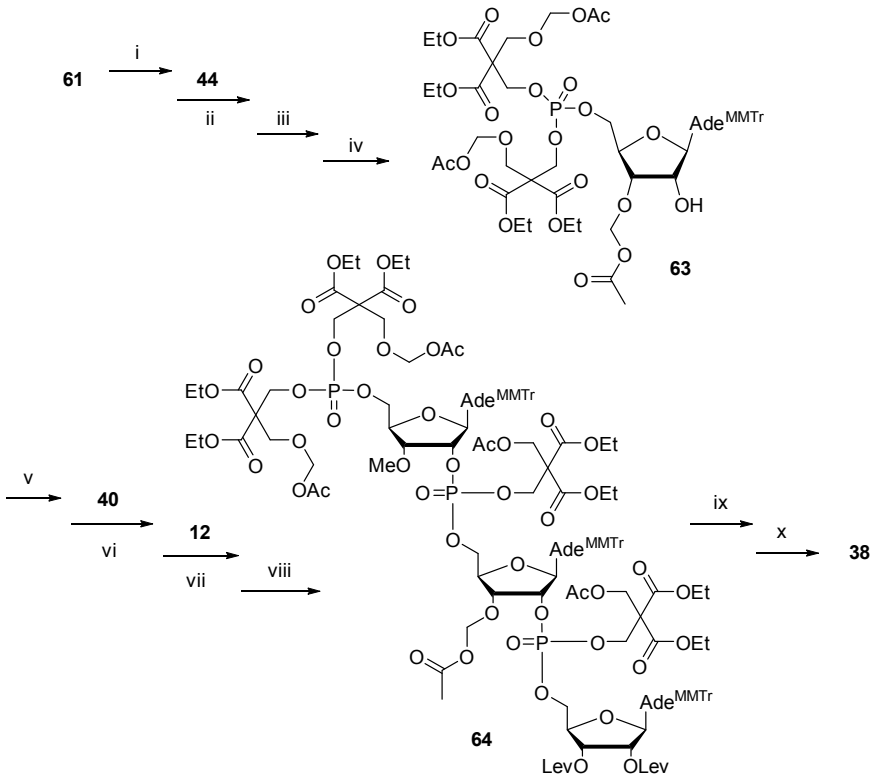
Scheme 15[a]



[a]*Conditions: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) TetH, MeCN; (iii) TetH, MeCN; (iv) I_2, THF, H_2O, 2,6-lutidine; (v) $Et_3N{\cdot}3HF$, THF; (vi) NH_2NH_3OAc, DCM, MeOH; (vii) 80% AcOH.*

Trimer **38** was then assembled from the bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl] protected adenosine 5′-monophopshate (**63**), dimer **40** and protecting group reagent **12** (Scheme 16) in an analogous manner to trimer **1**.[III] To keep the base-sensitive acetyloxymethyl functions intact, the *tert*-butyldimethylsilyl and levulinoyl groups were removed under milder conditions than previously, *viz.* with $Et_3N{\cdot}3HF$ in THF and hydrazinium acetate in a mixture of DCM and MeOH, respectively.

Scheme 16[a]



[a]*Conditions: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O, lutidine; (iv) NH_2NH_3OAc, DCM, THF (v) $(Et_2N)_2PCl$, Et_3N, DCM; (vi) TetH, MeCN; (vii) TetH, MeCN; (viii) I_2, THF, H_2O, 2,6-lutidine; (ix) NH_2NH_3OAc, DCM, MeOH; (x) 80% AcOH.*

3.2.3 Enzymatic deprotection

Enzyme-triggered removal of the esterase-labile protecting groups from protected adenylyl-2′,5′-adenosine (**40**) and 2-5A trimer (**38**) was studied with

hog liver carboxyesterase (HLE; 2.6 units ml^{-1}) in a HEPES buffer at pH 7.5 and 37 °C. Aliquots were taken at suitable time intervals from the reaction mixture and their composition was determined by HPLC. The products and intermediates were characterized by mass spectrometric analysis (HPLC/ESI-MS) or by spiking with authentic samples.

3.2.3.1 Esterase-triggered hydrolysis of the dimer (40)

The HPLC-traces referring to the HLE-catalyzed hydrolysis of the diastereomers of **40** at different stages are shown in Figure 27.[II] The compound was hydrolysed along the pathways described above for dimer **2** (Scheme 7, in the present case R = Me).

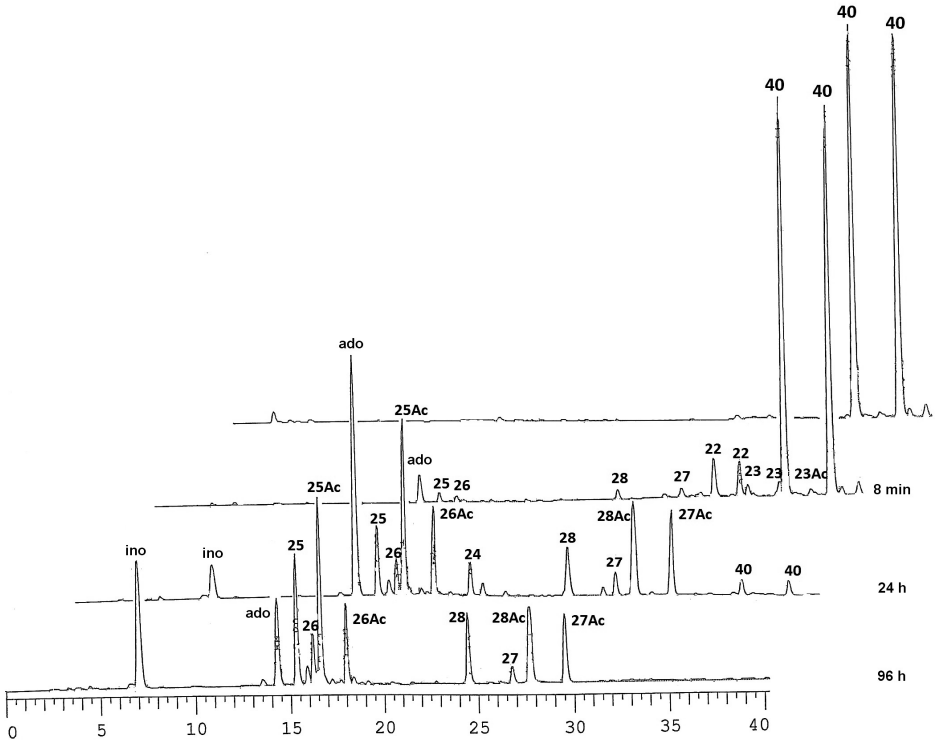


Figure 27 RP-HPLC traces for the HLE-catalyzed deprotection of [3′-*O*-(acetyloxymethyl)-2′-adenosinyl] [3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl] [5′-adenosinyl] phosphate (**40**) at pH 7.5 and 37 °C (I = 0.1mol l^{-1} with NaCl). For the structures, see Scheme 7.

During the initial stages of deprotection, the formation of deacetylated phosphotriester **22** and phosphate-protected adenylyl-2′,5′-adenosine **23** was accompanied by formation of monoacetylated derivative of **23**. Most likely, the acetyl group of the acetyloxymethyl moiety migrated to the 5′-hydroxyl group. This 5′-*O*-acetyl derivative **23-Ac** was then decomposed along the route B. Accordingly, acetylated derivatives of products **25-28** were accumulated in a considerable amount and 2′,5′-ApA (**25**) was released only as a minor product.

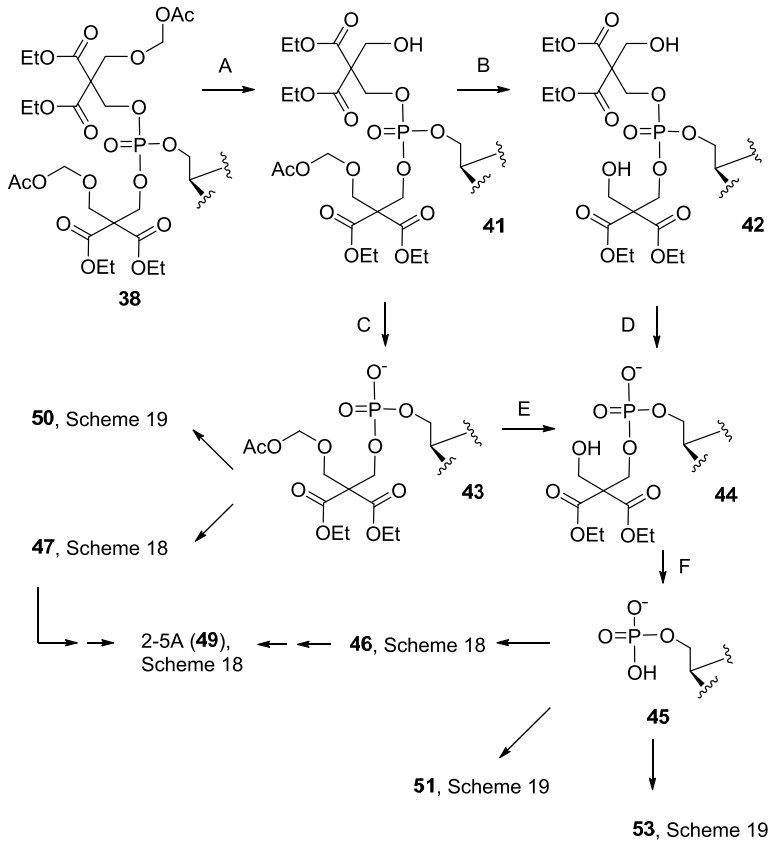
As seen from Figure 27, after 24 hours, 95 % of the starting material **40** had disappeared and the main products were adenosine (25 %), acetylated derivatives of 2′,5′-ApA (8%) and 3′,5′-ApA (15%) and acetylated diesters **27** and **28** (23 %). The proportion of 2′,5′-ApA (**25**) was only 6 %. Acetyl migration from the 3′-acetyloxymethyl group to the 5′-hydroxyl group is not however possible with 2-5A.

3.2.3.2 Esterase-triggered hydrolysis of the 2-5A trimer (38)

The HPLC traces referring to the HLE-triggered deprotection of one diastereomer of trimer **38** at various times are shown in Figure 28.[III] According to the product distribution, there are two major pathways for the release of 2-5A. Firstly, the exposure of 5′-phosphate, which takes place mechanistically analogously with the hydrolysis of the thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis-(ethoxycarbonyl)propyl]phosphate (**39b**), produced monoester **45** (Scheme 17) and subsequent exposure of the 2′-terminal phosphodiester linkage yielded **46** (Reaction G in Scheme 18). After removal of the last phosphate protecting group (Reaction I) and the 3′-*O*-acetyloxymethyl group (Reaction J), the fully deprotected trimer **49** was formed without any side reactions.

The second major pathway consisted of cleavage of only one of the 5′-phosphate protecting group before the exposure of the 2′-terminal phosphodiester bond (**38**→**43**→**47**) (Scheme 17). Removal of the other 5′-terminal phosphate protection (Reaction H in Scheme 18), consequently yielded the trimer **49**.

Scheme 17[a]



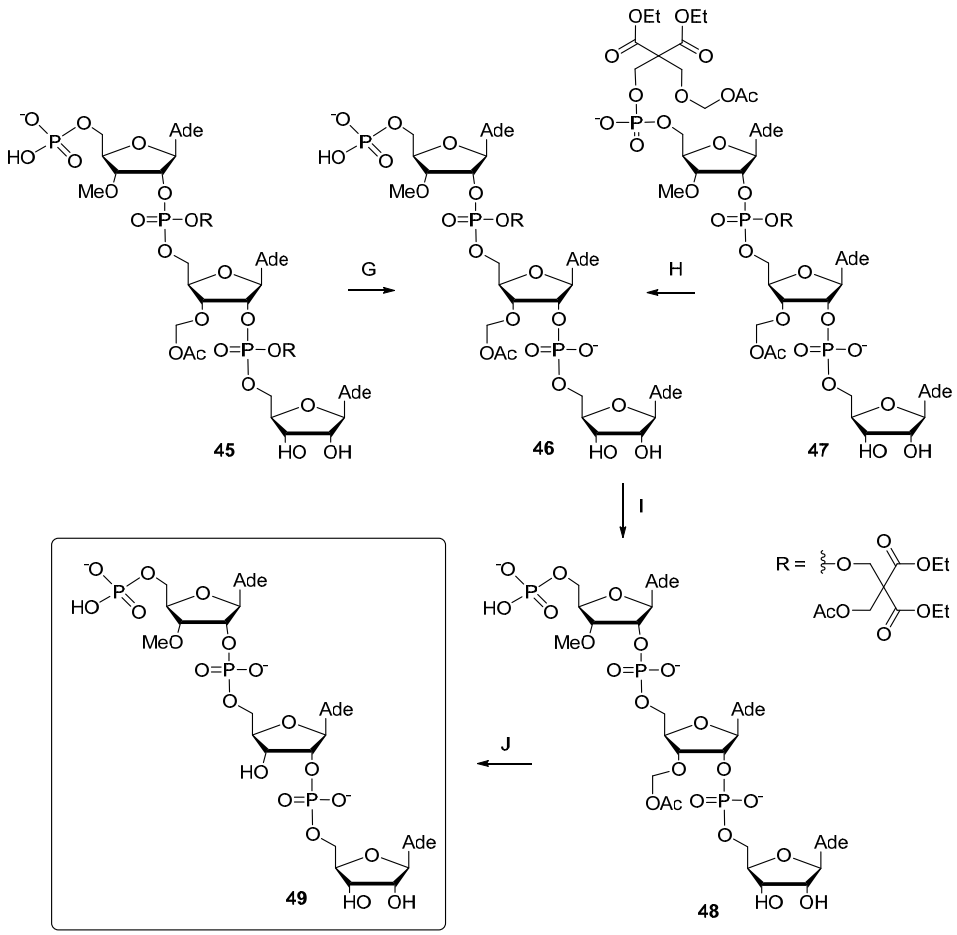
[a]*Early stages of HLE-catalyzed deprotection of 2-5A trimer* ***38***

Unfortunately, several side reactions took place. Exposure of the intervening phosphodiester bond competed with the exposure of the 2′-terminal phosphodiester and compounds **50** and **51**, still having the 2′-terminal phosphodiester linkages protected, were formed (Scheme 19). In case the remaining internucleosidic phosphodiester protecting group was removed before the 3′-*O*-acetyloxymethyl group, the desired 2-5A was finally released (Reaction L). However, if the 3′-*O*-protecting group was removed prior to the exposure of the adjacent phosphodiester bond (reaction M), the attack of the 3′-hydroxyl group on the adjacent phosphotriester took place leading to isomerization and cleavage of the 2′-terminal phosphoester and trimers **49** and **56**, dimers **59** and **60** and adenosine were released.

Finally, the 3′-*O*-acetyloxymethyl group seems to be removed to a minor extent before either of the internucleosidic phosphate protecting groups (Reaction N)

leading also to 2′→3′-phosphate migration and release of adenosine upon formation of products **54**, **55**, **57** and **58**.

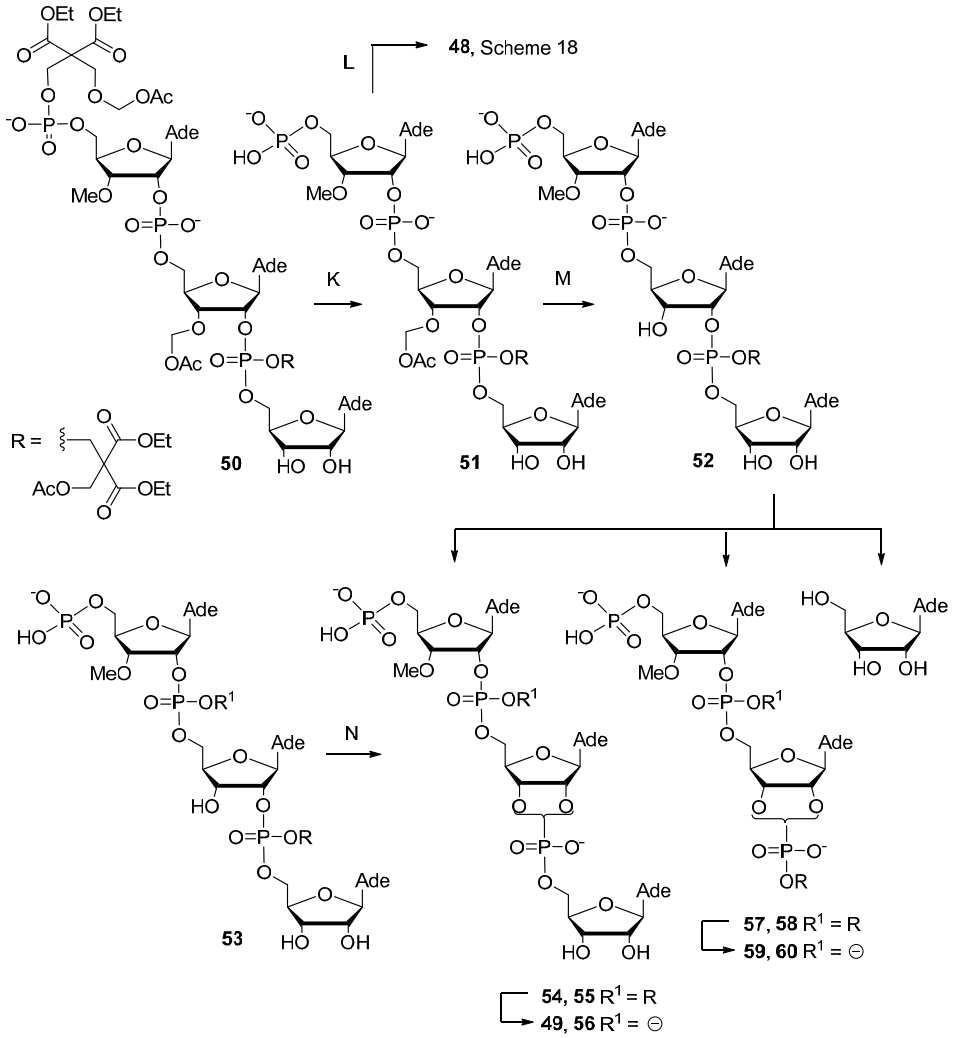
Scheme 18[a]



[a]*Final stages of HLE-catalyzed deprotection of 2-5A trimer* ***38***

As seen from Figure 28, the fully deprotected 2-5A (**49**) and several intermediates that eventually will give 2-5A (**45**-**48**, **50**, **51**) are formed. However, the prodrug strategy still severely suffers from formation of side-products. The main reason is too sluggish removal of the 2′-terminal phosphate protecting group compared to the removal of the adjacent 3′-hydroxyl protecting group.

Scheme 19[a]



[a]*Side reactions taking place during the HLE-catalyzed deprotection of 2-5A trimer* ***38***

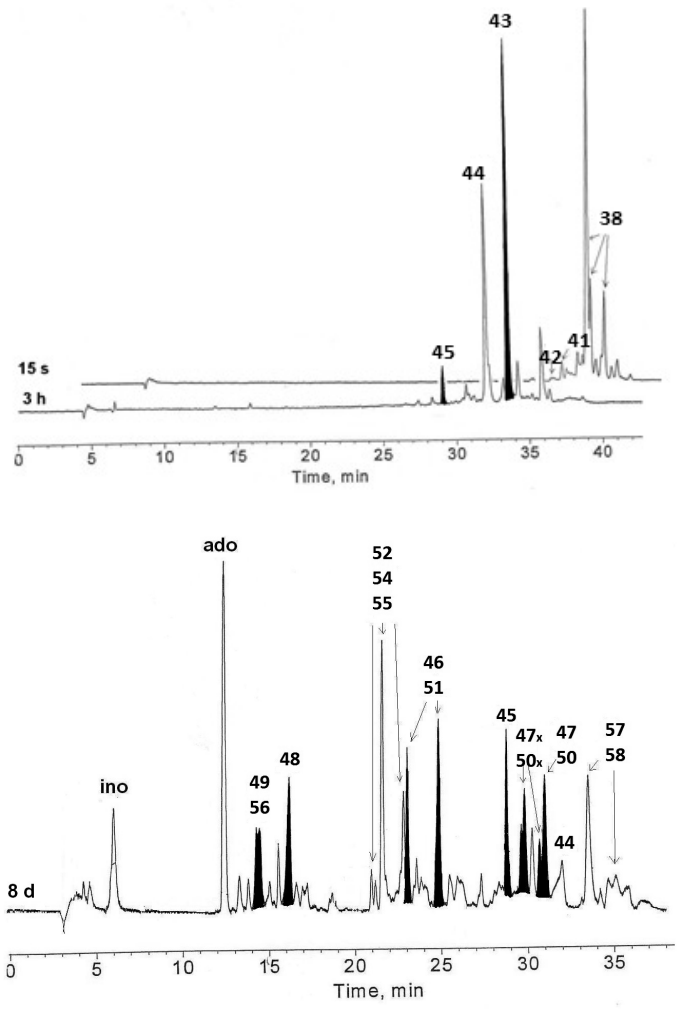


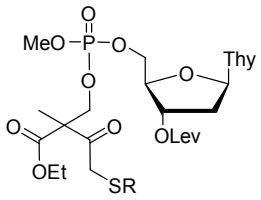
Figure 28 RP-HPLC traces for the HLE-catalyzed deprotection of 2-5A trimer **38** at pH 7.5 and 37.0 °C (I = 0.1 mol l^{-1} with NaCl). Signals marked with x refer to deacetylated 5´-diesters of compounds **47** and **50**. The black signals refer to those products and intermediates finally giving the fully deprotected trimer **49**. For the structures, see Schemes 17-19.

3.3 Esterase- and thermolabile protecting groups

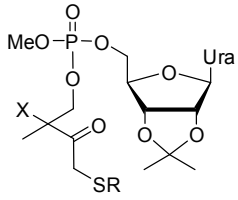
The approaches discussed above did not lead to a fully workable prodrug-strategy for 2-5A. The major shortcoming was that the removal of the phosphate protecting groups was markedly decelerated upon accumulation of negative charge. In addition, release of the phosphate protecting groups produced alkylating enones, which have been shown to form covalent adducts with

glutathione[152]. Protecting groups, which would not only be enzymatically labile, but also thermally labile, were thought to show promise for the protection of compounds containing more than one phosphodiester linkages, such as the short oligonucleotide 2-5A.

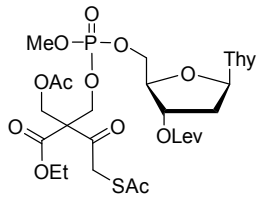
The potential of novel 2,2-disubstituted 4-acylthio-3-oxobutyl groups as esterase- and thermolabile protecting groups for phosphodiester bonds was studied.[IV,V] The original idea was that an intramolecular sulfur nucleophile would release the groups by cyclization, avoiding formation of alkylating by-products. Appropriately protected nucleoside 5′-methyl phosphates **61-63** were prepared as model compounds and the removal of the protecting groups was followed in the presence and in the absence of enzyme at physiological pH.



61a: R = Ac
b: R = Bz



62a: R = Ac; X = Me
b: R = Bz; X = Me
c: R = Piv; X = CO_2Et



63

Figure 29 Structures of nucleoside 5′-methylphosphates protected with thermolytically removable esterase labile protecting groups.

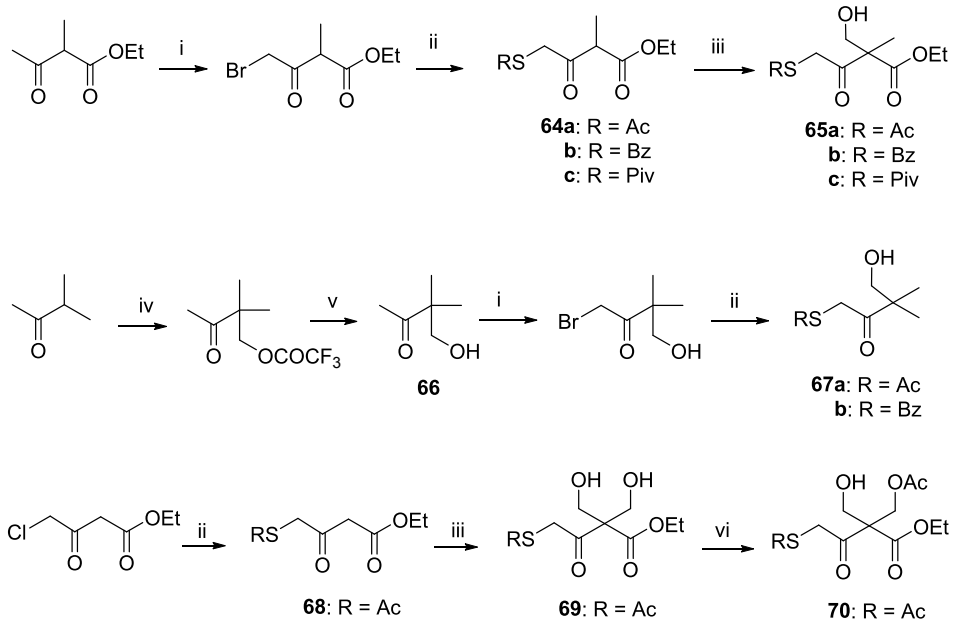
3.3.1 Syntheses

3.3.1.1 2,2-Disubstituted 4-acylthio-3-oxobutyl groups

The preparation of various protecting groups as alcohols is depicted in Scheme 20.[IV,V] Bromination of the commercially available ethyl 2-methyl-3-oxobutanoate[153] and subsequent treatment with thioacetic, thiobenzoic or thiopivalic acid in the presence of triethylamine yielded thioesters **64a-c**, which were then converted to the desired ethyl-4-acylthio-2-hydroxymethyl-2-methyl-3-oxobutanoates **65a-c** by triethylamine-promoted hydroxymethylation[154]. Treatment of 3-methylbutan-2-one with trifluoroacetic acid, followed by alkaline hydrolysis gave 4-hydroxy-3,3-dimethylbutan-2-one[155] (**66**), which was subjected to bromination and then to thioacetic or thiobenzoic acid treatment to obtain *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) thioacetate (**67a**) and thiobenzoate (**67b**). The chloro substituent of ethyl 4-chloroacetoacetate was replaced with thioacetic

acid and the compound **68** was bis-hydroxymethylated to have diol **69**. Conversion to orthoacetate[148] and subsequent hydrolysis finally afforded ethyl 2-(acetyloxymethyl)-4-(acetylthio)-2-(hydroxymethyl)-3-oxobutanoate **70**.

Scheme 20[a]

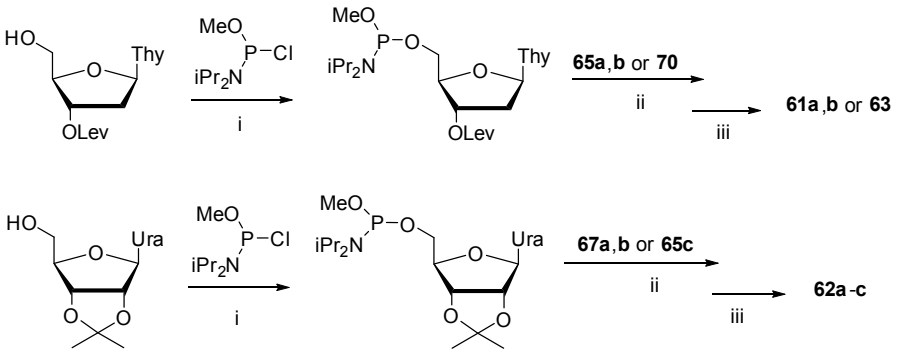


[a]*Conditions: (i) Br_2, DCM; (ii) RSH, Et_3N, Et_2O; (iii) H_2CO, Et_3N, dioxane; (iv) H_2CO, TFA, reflux; (v) 15% aq $NaHCO_3$; vi) 1. $(EtO)_3CCH_3$, H_2SO_4, THF; 2. CC (SiO_2; DCM/MeOH).*

3.3.1.2 Protected phosphotriesters (61-63)

The preparation of phosphotriesters **61a-b**, **62a-c** and **63** is outlined in Scheme 21.[IV,V] 3′-*O*-Levulinoylthymidine[I] or 2′,3′-*O*-isopropylideneuridine[156,157] was phosphitylated with 1-chloro-*N*,*N*-diisopropyl-1-methoxyphosphanamine in the presence of triethylamine. The diisopropylamino ligand was replaced by the appropriate alcohol and the resulting phosphite triester was oxidized to phosphate triester.

Scheme 21[a]

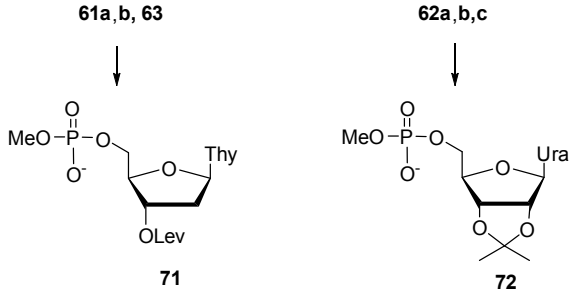


[a]*Conditions: (i) Et_3N, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O, 2,6-lutidine.*

3.3.2 Hydrolytic stability of phosphotriesters

Hydrolytic stability of the phosphotriesters **61-63** was studied in a HEPES buffer at pH 7.5 at 37.0 ± 0.1 °C by analysing the composition of the aliquots withdrawn at suitable intervals from the reaction mixture by RP-HPLC.[IV,V] The products were characterized by mass-spectrometric analysis (HPLC/ESI-MS). Diastereomeric mixtures of **61-62** and the slowest-eluted diastereomer pair of **63** were used as starting materials for the measurements. The removal of the protecting groups gave 3′-*O*-levulinoylthymidine 5′-methylphosphate (**71**) from the phosphotriesters **61a**,**b** and **63**, and 2′,3′-*O*-isopropylideneuridine 5′-methylphosphate (**72**) from **62a-c** as the main products (Scheme 22). Only **61a** and **61b** underwent side reactions in 15% and 5 % yields, respectively, giving demethylated phosphodiesters still bearing the protecting group. The rate constants and half-lives for the cleavage of the protecting groups are shown in Table 2. The half-lives for the nonenzymatic deprotection varied from 0.23 to 35 h depending on the size of the acylthio group. Replacement of the acetyl group with bulkier pivaloyl or benzoyl group decreased the rate of non-enzymatic deprotection. The stability was also susceptible to the electronegativity of the 2-substituents. The 2,2-dimethyl substituted protecting groups were observed to be more stable compared to their 2-ethoxycarbonyl counterparts.

Scheme 22[a]



[a]*Conditions: HEPES buffer, pH 7.5*

Table 2 First-order rate constants and half-lives for the hydrolysis of phosphotriesters **61-63** in HEPES buffer at pH 7.5 and 37 °C (I = 0.1 mol l^{-1} with NaCl).

Group RS	2-substituents	Triester	k^a / 10^{-6} s^{-1}	$t_{1/2}$ / h
AcS	Me, COOEt	**61a**	338	0.57
BzS	Me, COOEt	**61b**	121	1.59
PivS	Me, COOEt	**62c**	14.9[b]	12.9
BzS	Me, Me	**62b**	5.45[c]	35.3
AcS	Me, Me	**62a**	17.2	11.2
AcS	Me, Me	**62a**	2.42[d]	79.6
AcS	CH_2OAc, COOEt	**63**	844	0.23

[a]Mean of the rate constants of various diastereomers, differed by less than 5%. [b,c]Contains side reactions not giving the desired diester. [d]At 20 °C.

3.3.3 Enzymatic deprotection of phosphotriesters

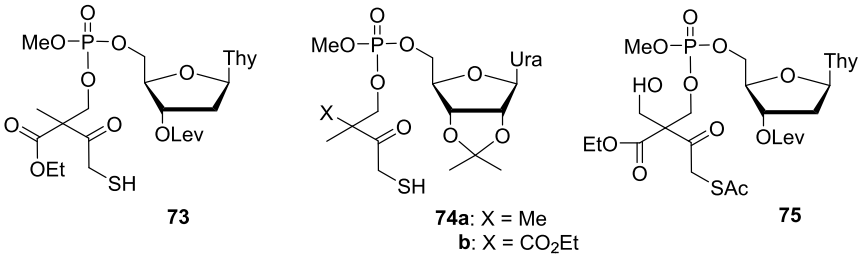
Enzymatic deprotection of diastereomeric phosphotriesters **61-62** and the fastest eluted diastereomer pair of **63** was studied in a HEPES buffer at pH 7.5 and 37 °C in the presence of hog liver carboxyesterase (HLE; 2.6 units ml^{-1}).[IV,V] Under these conditions, the triesters were transformed to the corresponding diesters **71** and **72**. Upon deprotection, triesters bearing deacylated mercapto group (**73**, **74a**) or hydroxyl group (**75**) were observed to be formed as intermediates (Figure 30). With **62b** and **62a**, the maximal accumulation of the intermediate **74a** was 80%

and 25 %, respectively. With compounds **61a**,**b** and **62c**, the accumulation of the mercapto intermediate **73** remained at low level (< 2 %) and **74b** was not detected, resulting from slow deacylation of 4-pivaloylthio analogue and at the same time, fast departure of 2-ethoxycarbonyl-4-mercapto-2-methyl-3-oxobutyl group. With **63**, the accumulation level of deacetylated phosphotriester **75** was up to 6 % of the products. Additionally, small amounts of delevulinoylated products were detected. The rate constants and half-lives for the esterase-catalyzed deacylation and chemical removal of the deacylated protecting groups varied from 0.17 to 40 min (Table 3). The rate could be tuned by the nature of the 4-acylthio substituent, the benzoyl group and acetyl groups being removed 50 and 5 times as fast as the pivaloyl group. The 2-(acetyloxymethyl)-4-(acetylthio)-2-ethoxycarbonyl)-3-oxobutyl group turned out to be unexpectedly thermolabile. More than 70 % of the deprotection of **63** was nonenzymatic.

Table 3 First-order rate constants and half-lives for the esterase-catalyzed deacylation and chemical removal of deacylated protecting groups of phosphodiesters **61**-**63** in HEPES buffer at pH 7.5 and 37 °C (I = 0.1 mol l^{-1}) containing HLE 2.6 units ml^{-1}.

Group RS	2-substituents	Triester	Deacylation k / 10^{-3} s^{-1}	$t_{1/2}$ / min	Removal of deacyl. p.g. k^a / 10^{-3} s^{-1}	$t_{1/2}$ / min
AcS	Me, COOEt	**61a**	4.05	2.9	-	-
BzS	Me, COOEt	**61b**	39.1	0.3	-	-
PivS	Me, COOEt	**62c**	0.81	14	-	-
BzS	Me, Me	**62b**	67.4	0.17	7.2	1.6
AcS	Me, Me	**62a**	2.45	4.7	9.6	1.2
AcS	CH_2OAc, COOEt	**63**	0.29	40	-	-

[a]Could not be determined for compounds **61a-c** and **63**, since the accumulation of the deacylated intermediates remained at low level or was not detected.



73 **74a**: X = Me **b**: X = CO_2Et **75**

Figure 30 The deacylated intermediates formed during the esterase-triggered deprotection.

3.3.4 Deprotection in the presence of glutathione

Triesters **62b** and **63** were additionally treated with HLE in the presence of glutathione in a MES buffer at pH 6.1 to evaluate the potential of the released protecting groups as alkylating agents.[IV,V] In neither case, any alkylation of glutathione was detected.

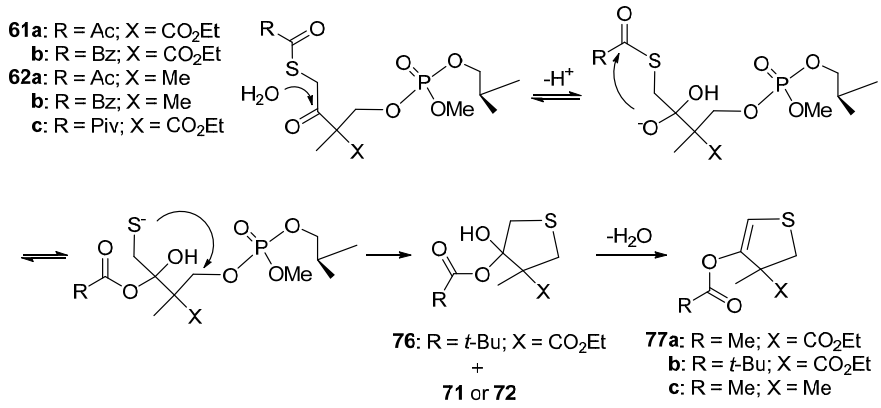
3.3.5 Mechanisms

3.3.5.1 Nonenzymatic removal of the protecting groups

It has been reported, that thioesters and oxoesters have similar reactivity towards hydroxide ion.[158] The half-life for the hydroxide ion catalyzed deacylation of thymidine 5′-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39a**) is 20 days (k = 4.0 x $10^{-7}s^{-1}$) at pH 7.5 and 37 °C.[I] Consequently, the nonenzymatic departure of the protecting groups from **61**-**63** is too fast to be initiated by hydroxide ion catalyzed hydrolysis of thioester or oxoester linkage.[IV,V] Migration of the acyl group from thiomethyl or hydroxymethyl to the neighboring 3-oxo group seems more likely. It may proceed by initial hydration of the keto group (Scheme 23 and Scheme 24) or alternatively by enolization of the starting material. The first option appears more attractive, since ESI^+-MS signal of **76** was observed during the deprotection of **62c**. The exposed mercapto group of phosphotriesters **61**-**62** attacks on the phosphate-bound carbon and releases phosphodiesters **71** and **72** and remnants of the protecting group undergo cyclization (Scheme 23). Dehydration of the intermediate **76** then yields 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophene **77** as the final product. Migration of the oxygen-bound acetyl group of **63**, however, triggers *retro*-aldol condensation with loss of formaldehyde releasing the phosphodiester **71** and the

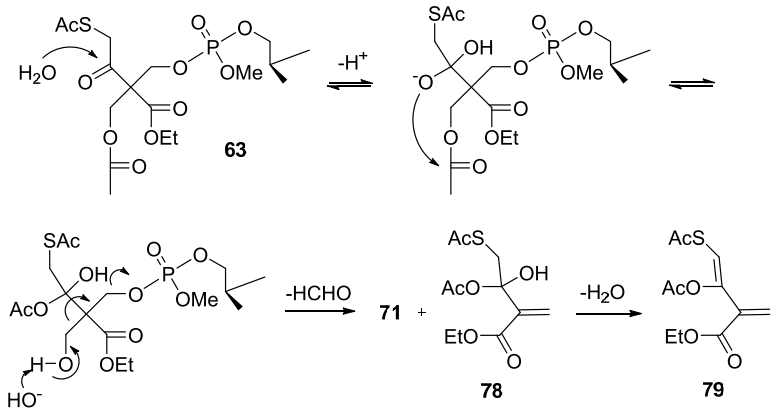
protecting group as enone **78**, which is then dehydrated to ethyl 3-(acetyloxy)-4-(acetylsulfanyl)-2-methylenebut-3-enoate **79** (Scheme 24). The formation of compounds **77a**, **77c** and **79** was observed by MS analysis. Additionally, the course of removal of the protecting groups from **61a** and **63** was followed by NMR spectroscopy in a mixture of D_2O and CD_3CN buffered with potassium phosphate (pH 7.0). The ^1H chemical shifts assigned as **77a** and **79** (Figure 31) are shown in Table 4 and Table 5, respectively. Keto-enol tautomerism caused rapid deuteration of the methylene protons between the acetylthio and keto groups and thus these protons could not be detected.

Scheme 23[a]



[a]*Nonenzymatic removal of the phosphate protecting groups from **61-62***

Scheme 24[a]

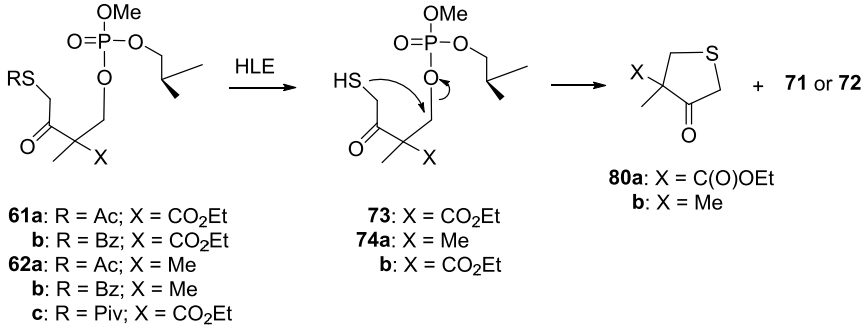


[a]*Nonenzymatic removal of the phosphate protecting group from **63***

3.3.5.2 Esterase-triggered removal of the protecting groups

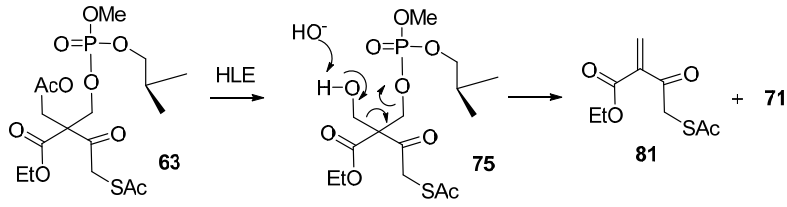
The enzymatic removal of the protecting groups from **61-62** is initiated by carboxyesterase-catalyzed deacylation of the mercapto group to yield **73** and **74** (Scheme 25).[IV,V] The remnants of the protecting groups are released from phosphodiesters **71** and **72** by cyclization to 4,4-disubstituted dihydrothiophen-3(2*H*)-ones **80**. In contrast, enzymatic removal of the protecting group from **63** results in formation of an enone structure instead of a cyclic structure (Scheme 26). The reason is probably too slow deacylation of the mercapto function compared to the deacylation of the hydroxyl group. The *O*-deacetylated compound **75** then undergoes *retro*-aldol condensation giving phosphodiester **71** by concomitant release of ethyl 4-(acetylsulfanyl)-2-methylidene-3-oxobutanoate **81**. The formation of compounds **80a** and **81** was verified by MS analysis.

Scheme 25[a]



[a]*Enzymatic removal of the phosphate protecting groups from* ***61-62***

Scheme 26[a]



[a]*Enzymatic removal of the phosphate protecting groups from* ***63***

Additionally, the departure of the protecting groups from **61a**, **62b** and **63** was followed by NMR spectrometry in the presence of HLE in a mixture of D_2O and CD_3CN buffered with potassium phosphate (pH 7.0). ^1H chemical shifts shown

in Table 4 were consistent with the assumed structure **80a** (Figure 31). During the deprotection of **63**, ^{1}H NMR signals referring to compounds **81** and **79** were both observed, since the nonenzymatic hydrolysis competed with the enzymatic one (Table 5). The methylene protons between the acetylthio and keto groups could not be detected, because of rapid deuteration under the experimental conditions. However, all the ring protons of the structure **80b** were observed. ^{1}H NMR resonance signals at 3.39 and 2.98 ppm referred to C2 protons and 2.49 and 2.34 ppm to C4 protons.

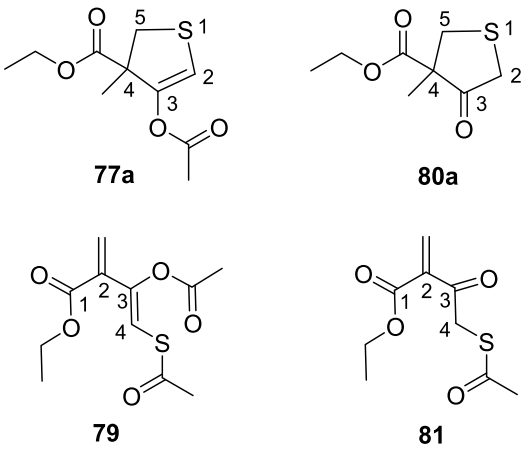


Figure 31 Numbering of atoms used in the NMR interpretation of the compounds **77a**, **79**, **80a** and **81**.

Table 4 ^{1}H chemical shifts in ppm and ^{1}H-^{1}H coupling constants in Hz for the compounds **77a** and **80a** (Figure 30) recorded during the deprotection of **61a** in the absence and presence of HLE, respectively.

Position	**77a**: δ ^{1}H (ppm)/ (^{1}H multiplicity)	$J_{H,H}$ (Hz)	**80a**: δ ^{1}H (ppm)/ (^{1}H multiplicity)	$J_{H,H}$ (Hz)
2	Not detected	-	Not detected	-
5	3.13 (d), 3.65 (d)	12.0	2.89 (d), 3.33 (d)	11.5
3-OAc	2.06 (s)	-	-	-
4-C*H*$_3$	1.54 (s)	-	1.25	-
4-CO$_2$C*H*$_2$CH$_3$	4.35 (q)	7.0	3.77 (q)	7.0
4-CO$_2$CH$_2$C*H*$_3$	1.36 (t)	7.0	1.28 (t)	7.0

Table 5 ^{1}H chemical shifts in ppm and ^{1}H-^{1}H coupling constants in Hz for the compounds **79** and **81** (Figure 30) recorded during the deprotection of **63** in the absence and presence of HLE, respectively.

Position	**79**: δ ^{1}H (ppm)/ (^{1}H multiplicity)	$J_{H,H}$ (Hz)	**81**: δ ^{1}H (ppm)/ (^{1}H multiplicity)	$J_{H,H}$ (Hz)
1-OC*H*$_2$CH$_3$	4.38 (q)	7.0	4.44-4.50 (m)	-
1-OCH$_2$C*H*$_3$	1.42 (t)	7.0	1.51 (t)	7.0
2=C*H*$_2$	6.54 (s), 6.13 (s)	-	6.62 (s), 6.21 (s)	-
3-OAc	2.24 (s)	-	-	-
4	not detected	-	not detected	-
4-SAc	2.52 (s)	-	2.59 (s)	-

4 DISCUSSION

4.1 Comparison of the 2-5A prodrug candidates I and II

Two different oligoadenylate trimers **1** and **38**, protected with esterase-labile protecting groups, were synthesized as prodrug candidates for the short oligoribonucleotide 2-5A and the multistep release of 2-5A by carboxyesterases was studied. In addition, measurements were made with protected adenylyl-2′,5′-adenosines **2** and **40**, and thymidine 5′-monophosphates **39a** and **39b** to optimize the structures of esterase-labile protecting groups.

The protecting group scheme of the prodrug candidate I (**1**) was based on 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group for the protection of the phosphate groups and pivaloyloxymethyl group for the protection of 3′-hydroxyl groups adjacent the phosphodiester linkages. The enzymatic deprotection of the trimer turned out to be extremely slow. After 8 days treatment with carboxyesterase, the product mixture consisted mainly of compounds having one or two of the phosphate protecting groups cleaved. Additionally minor chromatographic signals indicating to a loss of third protecting group were detected. Studies with 5′-bis-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**39a**) showed that enzymatic deacetylation of the negatively charged phosphodiester is very slow ($t_{½}$ = 9025 min) compared to deacetylation of the corresponding phosphotriester structure ($t_{½}$ = 3.2 min) at 37 °C in the presence of 26 units of HLE per ml. In turn, the initial deacetylation of protected 2′,5′-ApA dimer (**2**) at a lower HLE concentration (2.6 units ml^{-1}) was slower ($t_{½}$ = 395 min at 37 °C) than that of phosphotriester **39a**. Consequently, the first protecting group was most likely cleaved from 5′-terminal phosphate and the internucleosidic linkages were exposed after. In addition, small amounts of side products resulting from removal of the 3′-O-(pivaloyloxy)methyl protection of the 5′-terminal nucleotide prior to the exposure of the adjacent phosphodiester linkage, were observed. Even after a prolonged treatment, the conversion to trianionic diester form, still bearing one 5′-terminal phosphate protecting group was not complete and fully deprotected 2-5A never appeared. It seemed obvious, that carboxyesterases were unable to catalyze deacylation of the second 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group of the 5′-terminal phosphate.

Measurements with 5′-bis-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl] phosphate (**39b**) indicated that the enzymatic deacylation could be markedly accelerated by insertion of an additional hydroxymethyl group between the acyl

function and the 2,2-disubstituted propyl group. By this structural modification, the deacylation of the protected phosphate triester was accelerated 20-fold and the deacylation of the diester, obtained by departure of the first protecting group, was accelerated 40- fold. Consequently, the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl was selected for the protection of the 5′-terminal monophosphate group of the prodrug candidate II (**38**). The internucleosidic phosphodiester linkages were still protected with 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, permanent 3′-*O*-methyl protection was used for the 5′-terminal adenosine and more labile 3′-*O*-acetyloxymethyl protection for the intervening adenosine. During the carboxyesterase-catalyzed hydrolysis the fully deprotected 2-5A and several intermediates, eventually giving 2-5A, were formed. The prodrug strategy still severely suffered from formation of several by-products and less than half of the starting material was finally converted to 2-5A. The main reason was too sluggish removal of the 2′-terminal phosphate protecting group compared to the removal of the adjacent 3′-*O*-acetyloxymethyl group, which led to undesired isomerization and degradation of the phosphate backbone. The situation could have been improved by protecting this phosphodiester linkage with the more labile 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group.

4.2 The advantages of thermolability

As demonstrated above, the cleaving activity of esterases is reduced upon accumulation of negative charge on the substrate. Another issue, with the presented pro-drug strategy based on 2,2-disubstituted acyloxypropyl groups, is that decomposition of the protecting groups produces enone structures, which have been shown to alkylate glutathione. For these reasons, six esterase-labile 2,2-disubstituted 4-acylthio-3-oxobutyl groups were developed as protecting groups for phosphodiesters. The novelty of these protecting groups is their thermolabile feature. These protecting groups will be cleaved even when the enzymatic reaction becomes decelerated. The branched structure allows tuning of the deprotection rate by varying the structure of 2-substituents. The developed protecting groups are expected to be useful as protecting groups of oligonucleotides and compounds containing several phosphodiester linkages.

The hydrolytic stability of protected nucleoside 5′-methyl phosphates **61**-**63** was determined in the presence and in the absence of a carboxyesterase. The half-lives for the enzymatic deprotection varied from 0.17 to 40 min on using 2.6 units per ml at 37 °C. The rate could be tuned by the nature of the 4-acylthio substituent, the benzoyl group and acetyl groups being removed 50 and 5 times as fast as the pivaloyl group. In contrast to the 2-methyl substituted protecting

groups, the 2-(acetyloxymethyl)-4-(acetylthio)-2-(ethoxycarbonyl)-3-oxobutyl group was unexpectedly thermolabile. The enzymatic deprotection rate of phosphotriester **63** was only one third of that of the nonenzymatic deprotection at pH 7.5 in the presence of 2.6 units of HLE per ml. The enzymatic removal of the protecting groups is initiated by deacylation that triggers chemical removal of the remnants of the protecting groups from **61** and **62** by cyclization to 4,4-disubstituted dihydrothiophen-3(2*H*)-one **80** and from **63** by *retro*-aldol condensation to ethyl 4-(acetylsulfanyl)-2-methylidene-3-oxobutanoate **81**. The reason for the different mechanism of the latter reaction was probably too slow deacylation of the mercapto function compared to the deacylation of the hydroxyl group. The released protecting groups did not appear to form adducts with glutathione and thus were not markedly alkylating, even though the degradation of **63** resulted in formation of an enone.

The half-lives for the nonenzymatic deprotection (pH 7.5, 37 °C) varied from 0.23 to 35 h depending on the size of the acylthio group and the electronegativity of the 2-substituents. Smaller acyl groups migrate easier, hence replacement of the acetyl group with bulkier pivaloyl or benzoyl group decreased the rate of non-enzymatic deprotection. At the same time, by increasing the electronegativity of the 2-substituents, the nucleophilic attack of mercapto group on C1 carbon is facilitated. Therefore the 2,2-dimethyl substituted protecting groups were more stable compared to their 2-ethoxycarbonyl counterparts. The nonenzymatic departure of the protecting groups was too fast to be initiated by hydroxide ion catalyzed hydrolysis of thioester or oxoester linkage. Evidently, the acyl group migrated from thiomethyl or hydroxymethyl to the neighboring *gem*-diol obtained by hydration of the keto group and subsequently the protecting groups were then released from **61**-**62** as 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophene **77** and from **63** as ethyl 3-(acetyloxy)-4-(acetylsulfanyl)-2-methylidenebut-3-enoate **79**.

4.3 Is there a feasible esterase-dependent prodrug strategy for 2-5A?

The results demonstrated that the deprotection of 2-5A, bearing four esterase-labile protecting groups, became significantly slower upon accumulation of negative charge. The 3-acetyloxy-2,2-bis(ethoxycarbonyl) propyl groups, shown previously to be a viable esterase-labile protecting group of phosphodiesters, did not function as protecting groups for nucleoside 5´-monophosphates and the 2-5A prodrug candidate I (**1**) was never converted to the desired 2-5A. The situation was markedly improved when the distance between the enzyme

cleavage site and the negative charge was increased and a considerable proportion of 2-5A having a bis(3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl) propyl) protected 5´-terminal phosphate, was released from the 2-5A prodrug candidate II (**38**). The removal of the protecting groups still seemed to be far too slow, leading to formation of several by-products. For the reasons, a feasible protecting group scheme for 2-5A could be found on the introduced esterase-labile protecting groups, that additionally are thermolabile.

The applicability of the 4-acetylthio-2,2-dimethyl-3-oxobutyl group, studied in the present thesis as a protecting group of nucleoside 5´-methyl phosphate (**62a**), has also been employed for the protection of oligomeric phosphodiesters.[159] Oligothymidylates bearing three of these groups were converted into unprotected phosphodiesters in the presence and absence of carboxyesterase at pH 7.5 and 37 °C. After 4 days 70 % of the starting material was fully deprotected. The deprotection could be still too slow for an efficient pro-drug strategy of 2-5A. By replacement of the 2-methyl substituent with a more electronegative ethoxycarbonyl group, the thermolability may however be increased. The half-life for the non-enzymatic hydrolysis of **62a** was 11.2 h, at pH 7.5 and 37 °C, while the half-life for corresponding reaction of **61a** was 0.57 h. The rate acceleration is thus 20-fold. The 4-acetylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl group could be explored as a protecting group for the intervening phosphates of a third 2-5A prodrug candidate (Figure 32).

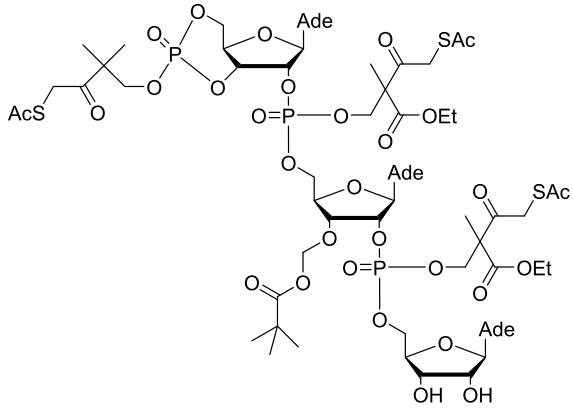


Figure 32 The structure of a potential 2-5A prodrug candidate III

For the 5´-terminal phosphate, however, a protection, the release of which is dependent on only one esterase-mediated step, appears an attractive option. 4-Acetylthio-2,2-dimethyl-3-oxobutyl group was recently studied for the protection of nucleoside 3´,5´-cyclic phosphate triester.[160] The half-lives for the removal of the protecting group in the presence and absence of carboxyesterase (2.6 units

ml^{-1}) were 6 min and 9.3 h, respectively. The 3′,5′-cyclic phosphate is then expected to undergo phosphodiesterase-catalyzed ring opening to give the desired nucleoside 5′-monophosphate. The cyclic protection would be a convenient option also, because 3′-hydroxyl protecting group would not be needed at the 5′-terminal adenosine.

It was shown, that 3′-*O*-acetyloxymethyl group was partly cleaved prior to the adjacent phosphate protecting group. For the reason, pivaloyloxymethyl group, being more stable than acetyloxymethyl group, appears more appropriate for the protection of 3′-hydroxyl group of the 2′-terminal nucleoside.

5 EXPERIMENTAL

5.1 Methods

The synthetic methods discussed in the thesis are described in the original publications I-V except for the synthesis of 2´,3´-*O*-isopropylidene-2´-*C*-methyluridine[156,157], which has been reported previously.

The compounds have been characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and MS techniques when applicable. 2D-NMR methods: COSY, HSQC and HMBC have been employed for the assignments of the NMR signals. NMR spectra were acquired on a Bruker Avance 500 and 400 spectrometer equipped with BBO-5mm-Zgrad or BBI-5mm-Zgrad-ATM probe operating at 500 MHz and 400 MHz for ^{1}H, 126 MHz and 101 MHz for ^{13}C and 202 MHz and 162 MHz for ^{31}P, respectively. Spectra were recorded at 298 K or 310 K using $CDCl_3$, CD_3CN, CD_3OD and D_2O as solvents. The chemical shifts are given in ppm. The coupling constants are given in Hz. The HR-MS analysis was performed on Bruker Daltonics micrOTOF-Q instrument and LC-MS on Perkin-Elmer Sciex-API-365 Triple Quadrupole. RP-HPLC analysis was performed using Merck Hitachi LaChrom D7000 with L-7455 UV-detector and L-7100 pump.

5.2 Kinetic measurements

The reactions were carried out in sealed tubes immersed in a thermostated water bath (± 0.1 °C). The hydronium ion concentration of the reaction solutions (3.0 ml) was adjusted with sodium hydroxide and N-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES) buffer. The ionic strength of the solutions was adjusted to 0.1 mol l^{-1}. The hydronium ion concentrations were calculated with the aid of the known pK_a values of the buffer acid under the experimental conditions. The initial substrate concentrations varied from 0.15 mmol l^{-1} to 0.3 mmol l^{-1}.

The enzymatic and non-enzymatic hydrolysis was carried out at pH 7.5 in a HEPES buffer (0.036/0.024 mol l^{-1}). The acyl group was removed with hog liver esterase (2.6 units ml^{-1}). Aliquots (200 µl) taken at suitable intervals were made acidic with 1 mol l^{-1} aqueous HCl (pH 2) or 1 mol l^{-1} AcOH (pH 4) to inactivate the enzyme and quench the hydrolysis, cooled in an ice-bath and filtered with RC4 syringe filters (0.2 µm). The composition of aliquots was analysed by RP-HPLC on an ODS Hypersil C_{18} column (4 x 250 mm, 5 µm, flow rate 1 ml min^{-}

[1]). The signals were recorded on a UV-detector at a wavelength of 260 nm and 267 nm. A detailed description of the chromatographic conditions has been given in the original publications I-V. The identity of the hydrolysis products was verified by LC-MS on a Phenomenex Gemini C18 column (2.0 x 150 mm 5 µm, flow rate 0.4 ml min-1).

The enzymatic deacylations obeyed first-order kinetics at the HLE concentrations employed. The pseudo-first-order rate constants for the reactions were calculated by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material. The rate-constants were then bisected to the rate constants of the parallel first order reactions on the basis of product distribution at the early stages of the reactions. More detailed description of the calculations has been given in publication I.

5.3 Enzymatic deprotection in the presence of glutathione

The enzymatic deprotection of **62b** and **63** was carried out in the presence of glutathione (GSH, 5.0 mmol l^{-1}) and hog liver carboxyesterase (2.6 units ml^{-1}) in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.005/0.005 mol l^{-1}) at pH 6.1 and 37 °C. The initial substrate concentration was ca. 0.5 mmol l^{-1}. Glutathione was present in the reaction mixture from the beginning or it was added 20 minutes after addition of the starting material. The aliquots (250 µl) withdrawn at suitable intervals were treated as described above for the kinetic measurements and the reaction products were identified by LC-MS. The signals were recorded on a UV-detector at a wavelength of 220 nm and 260 nm. The chromatographic conditions have been given in publication IV.

5.4 Follow-up of the deprotection reactions by NMR

The enzymatic and non-enzymatic hydrolysis **61a**, **62b** and **63** was recorded by ^{1}H NMR in a mixture of D_2O/CD_3CN buffered with potassium phosphate (5 mmol l^{-1} or 10 mmol l^{-1}, pH 7.0) in the presence or in the absence of HLE (26 units ml^{-1}) at 37 °C. The products and intermediates were identified on the basis of HSQC and HMBC correlations.

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