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SYNTHESIS OF AMIDE-BACKBONE DNA ANALOGUES AND THEIR POLY(ETHYLENE GLYCOL) DERIVATIVES

by

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A thesis submitted to the Faculty of Graduate Studies
and Research of McGill University in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

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I have the conviction that excessive literary production is a social offence.

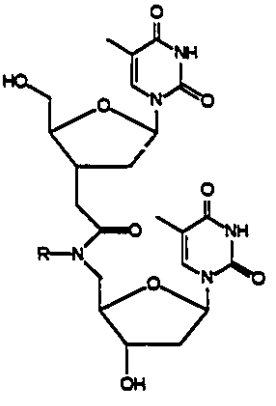
George Eliot

ABSTRACT

Thymidine dimers **16** and **18**, connected by amide or *N*-methylamide linkages, have been prepared. The dimers were incorporated into normal strands of DNA by solid phase synthesis. Thermal denaturation studies, using complementary single-stranded RNA, indicated that these modifications caused no destabilization of the DNA-RNA duplex.

The block synthesis of amide-linked homotetramer **30** is described. The synthesis of the corresponding octamer could not be verified because of lack of solubility. One by one homologation was found to be a suitable method for the preparation of *N*-methylamide analogues.

Poly(ethylene glycol), covalently attached to the 3' or 5' end of amide-backbone thymidine homopolymers, was found to greatly increase their solubility. The poly(ethylene glycol) simultaneously served as a soluble solid support for the homologation reactions.



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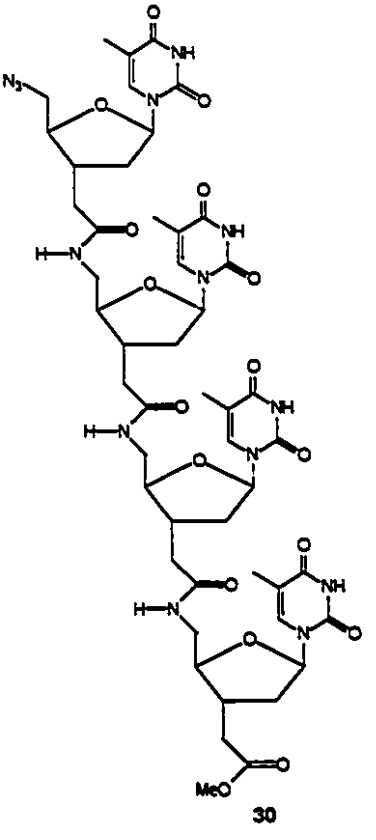
18 R = Me



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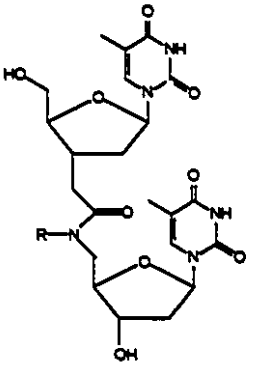
RESUME

Nous avons synthétisé les dimères de thymidine **16** and **18**, connectés par une liaison amide ou *N*-méthylamide. Ces dimères ont été incorporés dans un brin d'ADN par synthèse en phase solide. Les études de dénaturation thermique, utilisant un brin simple d'ARN complémentaire, ont montré que ces modifications ne provoquaient aucune déstabilisation du duplexe DNA-RNA correspondant.

La synthèse d'un homotétramère **30** connecté par des liens amides est decrite. La synthèse de l'octamère correspondant n'a pu être effectuée pour des raisons de solubilité. Nous avons montré qu'une homologation monomère par monomère était la méthode de choix pour la préparation des analogues *N*-méthylamides.

La polyéthylène glycol, lié de façon covalente au terminus 3' ou 5' des homopolymères de thymidine ayant des jonctions amides, augmente de beaucoup leur solubilité. Simultanément, nous avons utilisé le polyéthylène glycol comme support solide soluble pour les réactions d'homologation.



16 R = H
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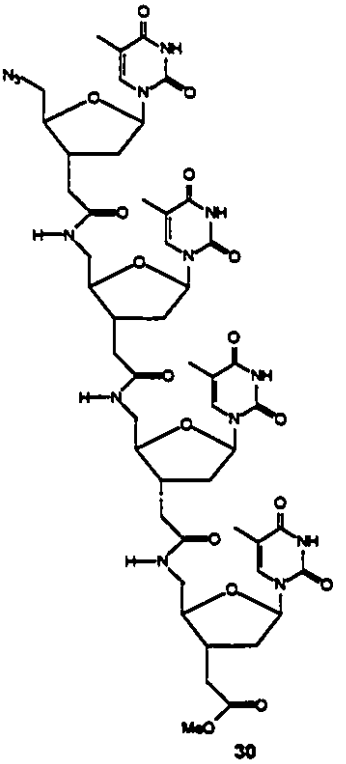

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Many people have contributed to the work presented in this thesis. I would like to thank my advisor, Prof. George Just, for his guidance and support throughout this work. I also thank Prof. Masad Damha for his advice and encouragement in the binding studies. I am grateful to Dr. J.-F. Lavallée for sharing his ideas and for his instruction in the lab. Special thanks must go to Prof. E.S. Idziak for his help and advice.

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GLOSSARY OF ABBREVIATIONS

A	adenine
Å	angstrom
ADH	alcohol dehydrogenase
AIBN	2,2'-azobisisobutyronitrile
AIDS	aquired immuno-deficiency syndrome
APT	attached proton test
B	base
BOP	benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
Bn	benzyl
Bu	butyl
Bz	benzoyl
C	cytosine
°C	Celsius
Cbz	benzyloxycarbonyl
CI	chemical ionization
COSY	correlation spectroscopy
CPG	controlled pore glass
d	deoxy
DCC	1,3-dicyclohexylcarbodiimide
DMAP	*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMTr	dimethoxytrityl
DNA	deoxyribonucleic acid

EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
Et	ethyl
eq	equivalents
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
g	gram(s)
G	guanine
h	hour(s)
HMQC	heteronuclear correlation
HPLC	high-pressure liquid chromatography
HRMS	high-resolution mass spectrometry (spectrum)
Hz	Hertz
imid	imidazole
i-pr	isopropyl
IR	infrared
l	liter(s)
LRMS	low-resolution mass spectrometry (spectrum)
M	molar(ity)
m	meter(s)
m	milli
μ	micro
MALDI TOF	matrix-assisted laser desorption ionization time-of-flight spectroscopy
Me	methyl
MHz	megaHertz
min	minute(s)
mol	mole(s)

m.p.	melting point
mPEG	poly(ethylene glycol) methyl ether
mRNA	messenger ribonucleic acid
Ms	mesyl or methanesulfonyl
n	nano
NBA	nitrobenzyl alcohol
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
OD	optical density
P	protecting group
p-	para
PDC	pyridinium dichromate
PEG	poly(ethylene glycol)
Ph	phenyl
PNA	peptide nucleic acid
ppm	parts per million
py	pyridine
R_f	retardation factor
RNA	ribonucleic acid
T	thymine
t-	tertiary
TaT	amide-linked thymidine dimer
TBAF	tetrabutylammonium fluoride
TBDMS	tertiary-butyldimethylsilyl
TBDPS	tertiary-butyldiphenylsilyl
TEA	triethylamine

TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
T_m	melting temperature
TmaT	methylamide-linked thymidine dimer
Ts	tosyl or *p*-toluenesulfonyl
U	uracil
UV	ultraviolet
vol	volume

TABLE OF CONTENTS

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Structure and role of nucleic acids.

" It is a strange model and embodies several unusual features. However, since DNA is an unusual substance, we are not hesitant in being bold."[1] Since James Watson wrote these words in 1953, an unprecedented amount of research has been conducted into the structure and the role of DNA. No single molecule is more important in the cellular processes of living organisms. All genetic information is stored, as well as expressed by DNA.

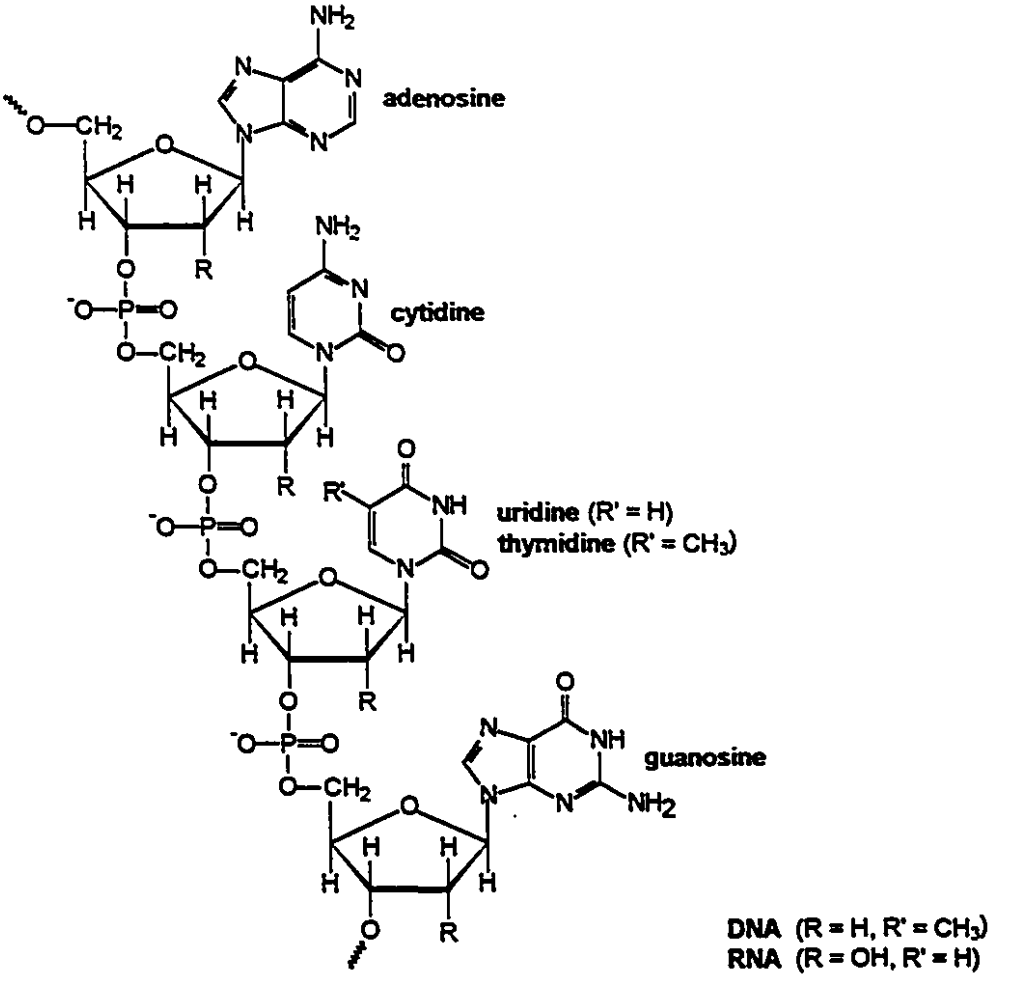

Figure 1. Schematic representation of the structure of DNA and RNA.

[1] Watson, J. *The Double Helix*, 1969, The New American Library of Canada Ltd., Toronto, Canada.

The chemical structure of DNA reflects the multifunctional nature of this biopolymer.(Figure 1) The ribose-phosphate backbone imparts flexibility and hydrophilicity, while the four nucleoside bases, adenine (A), guanine (G), thymine (T) and cytosine (C) ensure the fidelity of the genetic information transfer.

The familiar double helical shape of DNA is due to horizontal and vertical stabilizing forces, namely Watson-Crick hydrogen bonding in the plane of the bases (Figure 2) and forces perpendicular to the bases that are responsible for base stacking.

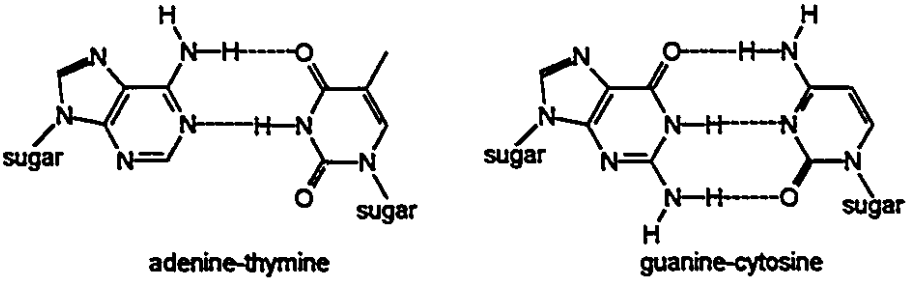

Figure 2. Watson-Crick binding of complementary nucleoside bases.

The stacking forces are the result of dipole interactions caused by the superimposition of carbonyl and exocyclic amino groups over the π electron system of the next base. These attractive forces are additive, but decrease sharply with distance.

Watson-Crick binding of two strands of DNA occurs only between the complementary purine-pyrimidine base pairs, adenine-thymine (A-T) and guanine-cytosine (G-C). For RNA the same principle applies, with the difference that uracil is substituted for thymine. It is this specificity that guarantees the correct transfer of information from the DNA, and ultimately leads to the synthesis of characteristic proteins, which affect biological functions at all levels. Although this chain of events is highly complex, it can be summarized in a few paragraphs.

Double stranded DNA, partially unwound by the action of RNA polymerase at the promotor region, serves as a template for the synthesis of RNA.

In eukaryotic cells, DNA is transcribed to preliminary RNA in the nucleus. At this stage the RNA contains regions called introns, the function of which is not known at this time. The introns are excised from the newly-formed pre-messenger RNA strand and the resulting sections, the exons, are spliced together. The shortened messenger RNA (mRNA) then leaves the nucleus and enters the cytoplasm. In this part of the cell, ribosomes recognize the leader region at the 5' end and form complexes with the mRNA, initiating protein synthesis in a process called translation. A schematic representation of these events by K. Kutterer[2] is shown in Figure 3.

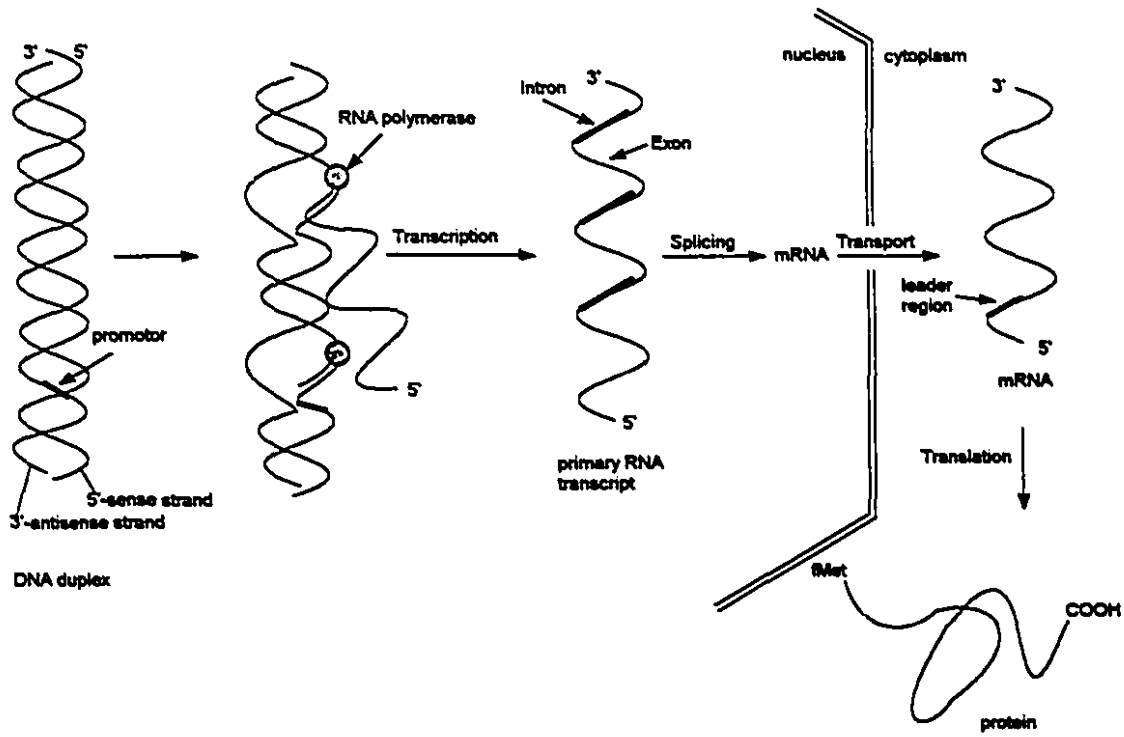

Figure 3. Eukaryotic gene expression.

Prokaryotic cells do not contain a nucleus. Both, the transcription and the translation take place in the cytoplasm.

The viral genome may contain DNA or RNA, which can both replicate.

[2] Kutterer, K.M.K. *Doctoral Thesis* 1995, McGill University.

Retroviruses use a host genetic material for their reproduction. This is achieved by reverse transcriptase-mediated DNA synthesis from the viral RNA, followed by integration of the DNA into the hosts' genome.

The viral DNA is smaller than the prokaryotic DNA, which, in turn, is smaller than eukaryotic DNA.

1.2 Antisense as a regulator of gene expression.

The search for new drugs has traditionally involved testing large numbers of natural and synthetic products for pharmacological activity. Rational drug design is an attractive alternative, but requires detailed understanding of the mode of action, as well as the targeted receptor sites. The shape and electronic environment of drug and receptor must be exactly matched in order to result in binding. The design of compounds that inhibit pathological actions at the protein (enzyme) level has had limited success thus far.

The control of gene expression for therapeutic purposes has certain advantages compared to intervention at the protein level. A single gene is transcribed to multiple copies of mRNA, each giving rise to large amounts of proteins. It should be more efficient to prevent the production of undesirable proteins, rather than inhibit their action.

Intervention at the genetic level is not a new concept. Classical anti-cancer agents, such as adriamycin and cisplatin, derive their cytotoxicity from interference at the DNA level by intercalation. The limited specificity of these drugs, however, leads to severe side effects. The ability of DNA and RNA to selectively bind to their complements provides a potential mechanism for artificial, specific regulation of gene expression. As early as 1978 Zamecnik and Stephenson[3] reported that a synthetic 13-mer strand of DNA, complementary to the terminal sequences of the Rous Sarcoma virus RNA, inhibited virus production. RNA thus provided the receptor site for sequence specific recognition. This important finding eventually generated a vast amount of research into the short regulatory strands of oligonucleotides, now known as antisense.

The antisense mode of action is based on the specificity of Watson-Crick binding to a known mRNA sequence, thus preventing translation to undesirable proteins. Potentially, antisense therapy could find application in the treatment of cancer, when the pertinent oncogene has been identified. Viral infections are obvious targets as they are

[3] Zamecnik, P.C.; Stephenson, M.L. *Proc. Natl. Acad. Sci.*, **1978**, *75*, 280.

difficult to treat by means of conventional drugs.

Figure 4 illustrates at which points an antisense strand might successfully interfere with the genetic information transfer in eukaryotic cells.[2]

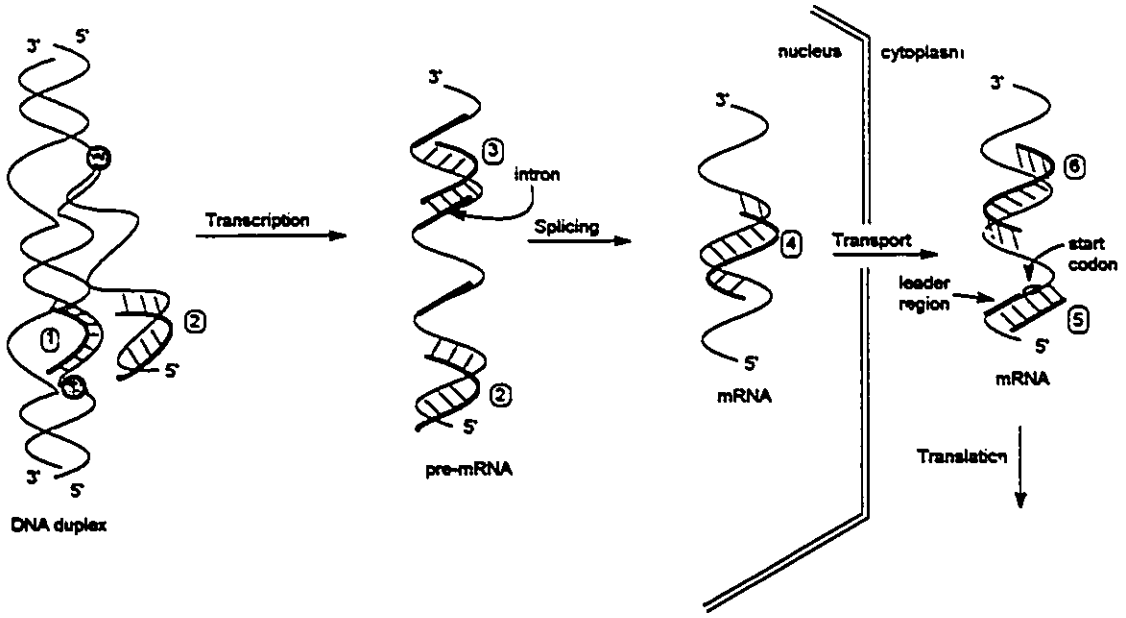

Figure 4. Potential receptor sites for antisense binding in eukaryotic cells.. Possible mode of action: (1) Attachment to the partially unwound DNA double helix prevents RNA polymerase from binding. (2) and (3) Interference in the formation of mRNA from pre-mRNA. (4) Impeding mRNA's transport through the nuclear membrane. (5) and (6) Interference in the translation process.[4]

The genetic material of viruses is often stored in single-stranded RNA, an obvious target for antisense therapy.

Antisense nucleotides, in order to be effective, must meet certain requirements. The specificity of antisense binding depends on the sequence, as well as the length of the strand. Statistically, a specific 17-mer occurs only once in the human genome, while the same principle applies for an 11-mer in prokaryotes and a 9-mer in viruses. It follows that the size of an antisense strand should be in the range of ten to twenty nucleoside bases,

[4] (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543. (and references therein)
(b) Hélène, C.; Toulmé, J,-J. *Biochem. Biophys. Acta*, **1990**, *1049*, 99.

depending on the target. There is no apparent advantage in the use of larger sequences.

To reach their target inside the cell, the antisense must be able to penetrate the cell wall and, possibly, the nuclear membrane. It is therefore prudent to keep the antisense size to a minimum.

Finally, the antisense half life *in vivo* must be long enough to achieve the desired effects. Natural oligonucleotide strands are subject to nuclease-mediated hydrolysis of the phosphodiester backbone and thus are not suitable. In order to acquire the necessary nuclease resistance, they must be chemically modified . Also, the ionic nature of the phosphate backbone may not be conducive to penetration of the lipophilic cell membrane. Therefore, reducing the electronic charge on the backbone may be advantageous. Of course, structural changes must not affect the strength and the specificity of hybridization.

This need for stable, more lipophilic nucleoside analogues gave rise to a variety of modifications, a few of which will be discussed here.

The earliest modifications include the replacement of one of the phosphate non-bridge oxygens with a sulfur atom.[5] The resulting phosphorothioate **a** (Figure 5) was shown to have increased nuclease resistance. Miller and Ts'O replaced the natural phosphate by a methylphosphonate, thus eliminating the ionic charge to improve cellular uptake.[6] A drawback of these modifications is that they introduce chirality, resulting in the formation of multiple diastereomers, which in turn cause a decrease in binding affinity.

Researchers initially concentrated on easily accessible modifications that did not require a major synthetic effort. The first generation of designer antisense molecules **a**, **b**, **c** and **d** (shown in Figure 5) still retained a phophorus atom in the backbone. They all showed increased resistance to degradation by nucleases, but a decreased affinity for the target RNA.[7] On the other hand, phosphoramidate **e**, which was synthesized more

---

[5] Frey, P.A.; Sammons, R.D. *Science* **1985**, *228*, 541.
[6] Ts'O, P.O.P.; Miller, P.S.; Aurelian, L.; Murakami, A.; Agris, C.; Blake, K.R.; Lin, S.-B.; Lee, B.L.; Smith, C.C. *Ann. N.Y. Acad. Sci.* **1988**, *507*, 220.
[7] De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H.E. *Acc. Chem. Res.* **1995**, *28*, 366.

recently, was reported to exhibit enhanced binding.[8]

The latest phosphorus-retaining backbone modification, **f**, was reported by Saha *et al.*[9] Although the introduction of a 5' methyl function resulted in the formation diastereomers, this did not adversely affect the hybridization properties. These results are in contrast to those reported for the phosphorothioates **a** and the methylphosphonates **b**.

The second column in Figure 5 depicts a sample of non-phosphorus backbones,

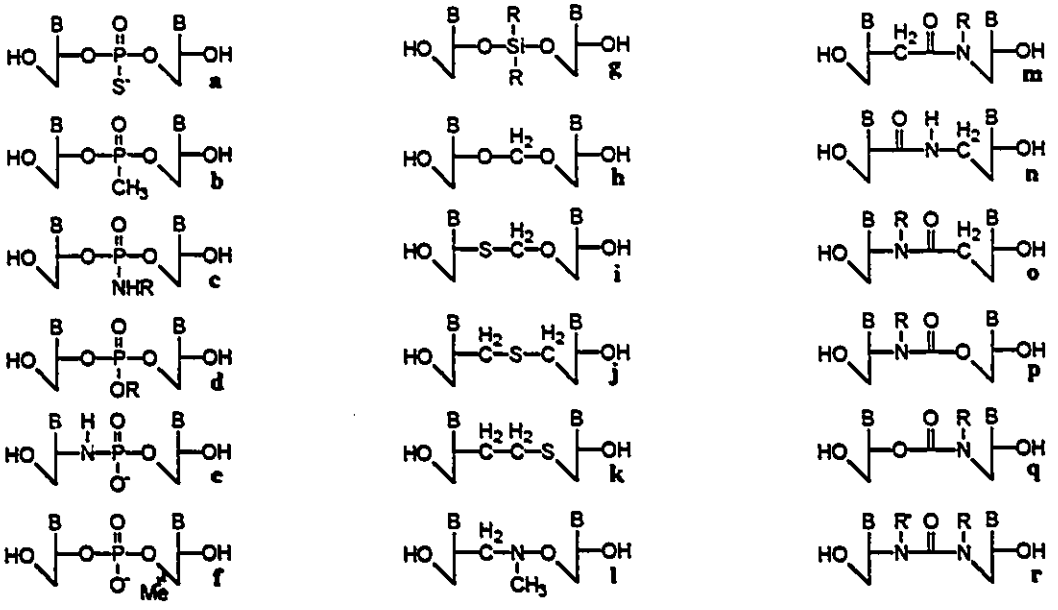

Figure 5. Structures of a selection of DNA backbone modifications.

which are more recent contributions to the field of antisense research. Most of the modifications **g** to **l** involve other hetero bridging atoms and are all non-ionic. These syntheses are in general more demanding and are not easy to adapt for solid phase polymer production. Rather, they are mostly prepared as dimer analogues, which are subsequently incorporated into natural DNA strands. This approach results in a reduction

[8] Gryaznov, S.; Chen, J.K. *J. Am. Chem. Soc.* **1994**, *116*, 3143.
[9] Saha, A.K.; Caulfield, T.J.; Hobbs, C. Upson, D.A.; Waychunas, C.; Yawman, A.M. *J. Org. Chem.* **1995**, *60*, 788.

in the ionic charge of the antisense strand, depending on the number of substitutions, while still maintaining water solubility.

In 1985 Ogilvie and Cormier reported the synthesis of thymidine dimer **g**, containing a diphenylsilyl linker.[10] The acid-base lability was later improved by substituting *iso*-propyl for the phenyl groups.[11] The lack of water solubility of the silyl-linked homopolymers precluded the conducting of hybridization studies at that time.

The synthesis of formacetal linkages of type **h** was independently reported in 1990 by Matteucci[12a] and van Boom and co-workers.[12b] The formacetal substitutions did not destabilize the RNA-DNA duplex, but led to lower melting temperatures for DNA-DNA hybridization. The 3'-thioacetal analogue **i** also exhibited satisfactory antisense properties, but the inverse configuration, the 5'-thioacetal, showed reduced binding.[13] It was proposed that steric interaction between the large sulfur atom at the 5' position and, either the ribose oxygen, or the thymine H6 might be responsible for the destabilization.

Positional thioether isomers **k**, made in our laboratory[14a], and **j**, prepared by Benner's group[14b] exhibited decreased binding. Hydroxylamine **l**, on the other hand, did not destabilize duplex formation.[15]

Another class of backbones is shown in the right-hand column of Figure 5. They all contain various combinations of nitrogen and carbonyl groups. This type of moiety introduces a reduced rotation in the backbone, which may be thermodynamically advantageous for binding. A stiffer backbone leads to less loss of entropy in the process of

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[10] Ogilvie, K. K.; Cormier, J.F. *Tetrahedron Lett.* **1985**, *26*, 4159.
[11] Cormier, J.F.; Ogilvie, K.K. *Nucleic Acids Res.* **1988**, *16*, 4583.
[12] (a) Matteucci, M. *Tetrahedron Lett.* **1990**, *31*, 2385.
(b) Veeneman, G.H.; van der Marel, G.A.; van der Elst, ; van Boom, J.H. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 449.
[13] Jones, R.J.; Lin, K.-Y.; Milligan, J.F.; Wadwani, S.; Matteucci, M.D. *J. Org. Chem.* **1993**, *58*, 2983.
[14] (a) Kawai, S.H.; Just, G. *Nucleosides Nucleotides*, **1991** *10*, 1485.
(b) Huang, Z.; Schneider, K.C.; Benner, S.A. *J. Org. Chem.* **1991**, *56*, 3869.
[15] Vasseur, J.J,; Debart, F.; Sanghvi, Y.S.; Cook, P.D. *J. Am. Chem. Soc.* **1992**, *114*, 4006.

hybridization. The right geometry, of course, remains a prerequisite.

Amides **m**[16], carbamates **p** and ureas **r**[17] were prepared in our laboratory. The amides **m** hybridized strongly to RNA and DNA, while the carbamates and the ureas caused some destabilization of the duplexes, but less for DNA than RNA. Researchers at ISIS Pharmaceuticals and at CIBA-GEIGY independently synthesized the same compounds. Their studies confirmed our results. Isosteric amides **n** and **o**, made at CIBA-GEIGY, were found to have poor binding properties.[18]

Finally, the total replacement of the ribose and phosphate moieties by suitably spaced amides has been surprisingly successful.[19] The resulting compounds (Figure 6),

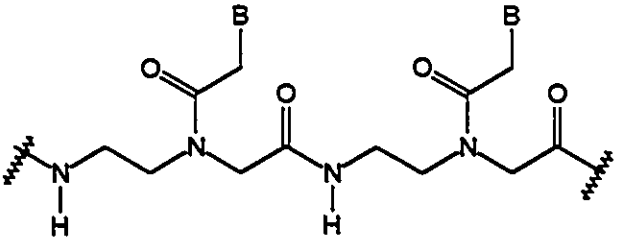


Figure 6. Nielsen's peptide nucleic acids.

referred to by the authors as peptide nucleic acids (PNA's) bind much stronger than natural DNA. Molecular modeling predicted that a sequence of monomeric units with six bonds, and a side chain spacer of two bonds carrying the base, should fulfill the requirements for binding to DNA in the B form. Analogous investigations conducted by Weller and his group led to the same conclusions.[20]

---

[16] This study.

[17] Kutterer, K.M.K.; Just, G. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 435.

[18] Lebreton, J.; de Mesmaeker, A.; Waldner, A.; Fritsch, V.;Wolf, R.M.; Freier, S.M. *Tetrahedron Lett.* **1993**, *34*, 6383.

[19] Egholm, M.; Buchardt, O.; Nielsen, P.E.; Berg, R.H. *J. Am. Chem. Soc.* **1992**, *114*, 1895.

[20] Weller, D.D.; Daly, D.T.; Olsen, W.K.; Summerton, J.E. *J. Org. Chem.* **1991**, *56*, 6000.

This overview of chemically modified nucleosides provides only a sample of what has been accomplished in this field of research.

Clinical studies are currently under way to test the efficacy of phosphorothioates and methylphosphonates as anti-viral and anti-tumor agents.[21] These studies will provide much needed information on the benefits and potential problems of antisense therapy.

[21](a) Ma, D.D.F.; Le Doan, T. *Ann. Intern. Med.* **1994**, *120*, 161.(and references therein)
(b) Boado, R.J. *Adv. Drug Delivery Rev.* **1995**, *15*, 73.
(c) Brysch, W.; Schlingensiepen, K.-H. *Cell. Mol. Neurobiol.* **1994**, *14*, 557.

2. RESULTS AND DISCUSSION

2.1 Synthetic strategy.

When the work towards this thesis was started, the elegant total 9-step synthesis of 3'-deoxy-3'-carbomethoxymethylthymidine **8** had just been accomplished by J.-F. Lavallée in our laboratory.[22] At that time our group's research was beginning to focus on the synthesis of backbone-modified DNA and RNA as potential antisense reagents. J.-F. Lavallée first introduced the concept of substituting the DNA phosphate linkage by an amide, keeping the number of atoms that separate the sugars the same. The amide moiety has the advantage that it is non-ionic and much less polar than the phosphate. This could be an important factor in improving cell penetration. The amide linkage is also achiral and, in contrast to methyl phosphonate and phosphorothioate modifications, it does not lead to a complicated mixture of diastereomers.

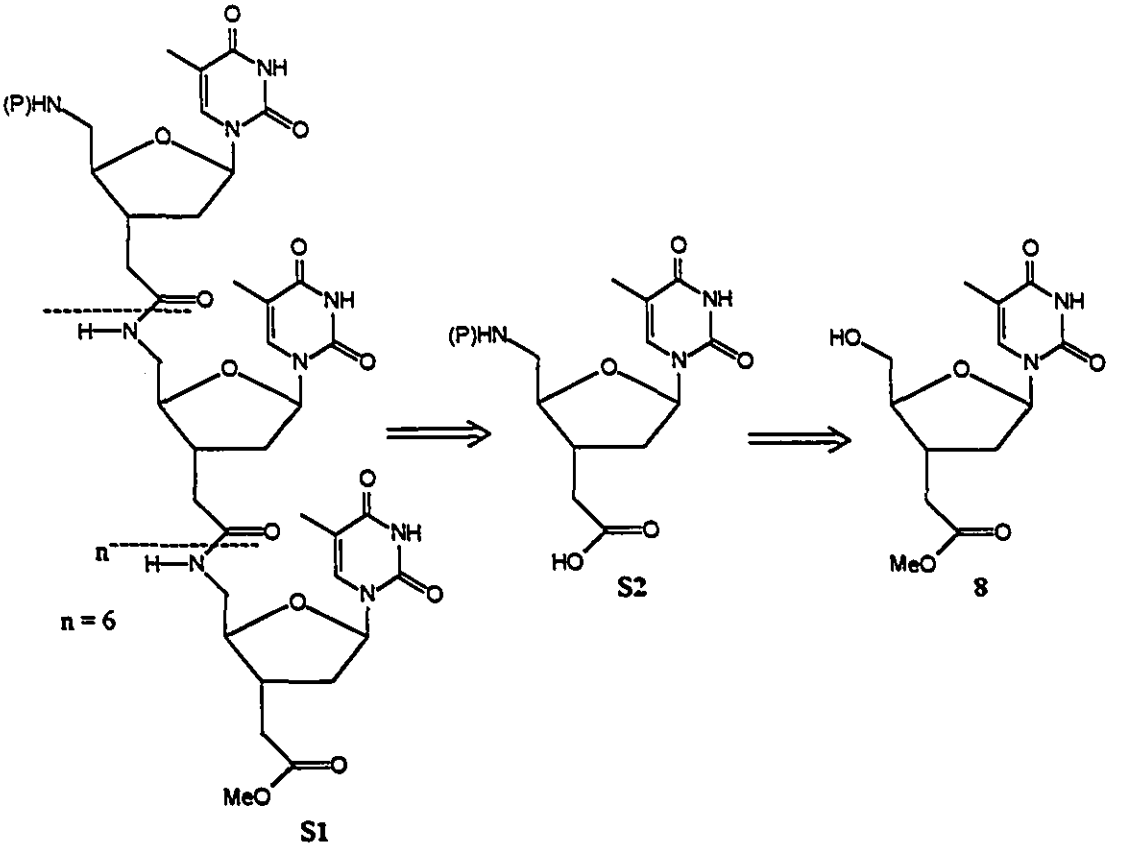
Modified thymidine **8** presented itself as a convenient precursor to homopolymer **S1**, via monomeric unit **S2**. (Scheme R1) The preliminary work on the coupling of the monomers held promise[23] and thus it was decided to further investigate the possibilities and scope of amide-backbone modified DNA.

Our first goal was to test if some of the phosphate linkages in DNA could be successfuly substituted by amide linkages, without compromising DNA's selective hybridization properties. In order to achieve this we wanted to prepare amide-linked nucleoside dimers that could be incorporated into natural DNA by solid phase synthesis. We wanted to follow established protocol for the solid phase synthesis.[24] This required the preparation of dimers with the 5' hydroxyl end group protected by the the dimethoxytrityl

[22] Lavallée, J.-F.; Just, G. *Tetrahedron Lett.* **1991**, *32*, 3469.
[23] Lavallée, J.-F.; Unpublished results.
[24] McBride, L.J.; Caruthers, M.H. *Tetrahedron Lett.* **1983**, *24*, 245.

group and the 3' hydroxyl end functionalized as a phosphoramidite, as shown in Scheme R2.

Scheme R1.

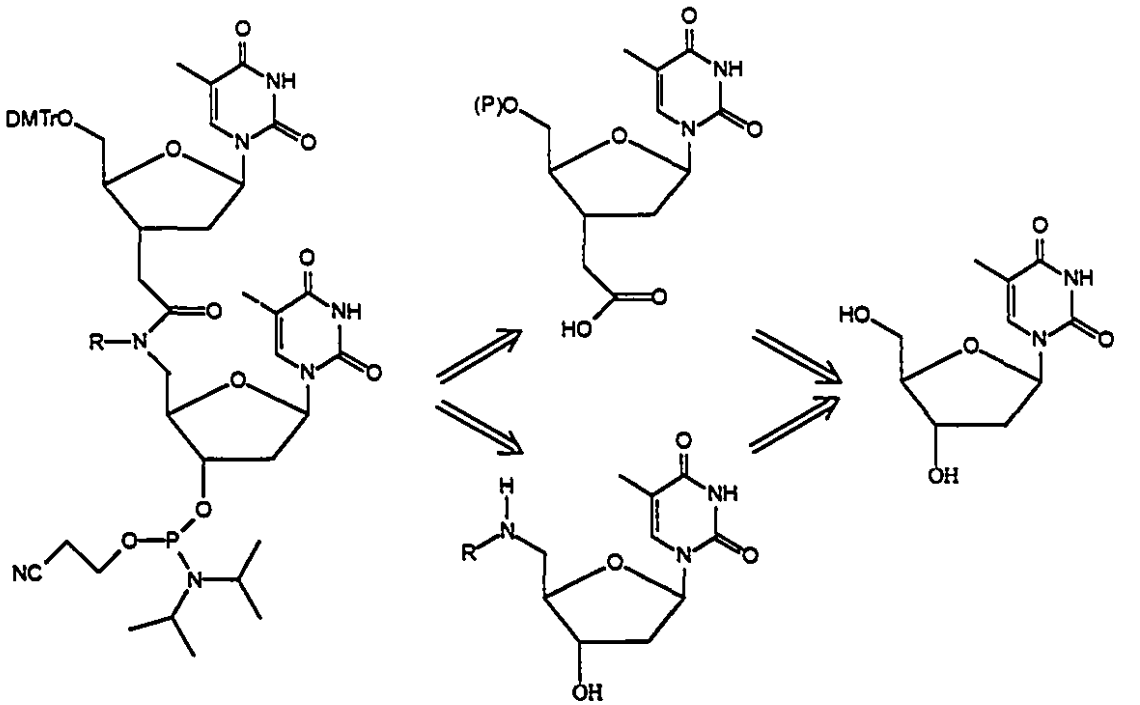


We also wanted to decrease the number of steps that were required for the synthesis of the 3' carboxylic acid monomer, by using commercially available thymidine as starting material. Chu *et al.* had previously described the deoxygenation and radical introduction of an allyl group in the 3' position of thymidine in order to obtain a possible AZT analogue.[25] Although this compound tested negative for AIDS virus inhibition, the synthetically versatile allyl group made it a potentially useful precursor for 3' carbon chain nucleosides. The oxidation of the allylic double bond was expected to yield the desired carboxylic acid.

The amine component of the dimer is represented by 5'-amino-5'-deoxythymidine,

[25] Chu, C.K.; Doboszewski, B.; Schmidt, W.;Ullas, G.V.; Van Roey, P. *J. Org. Chem.* **1989**, *54*, 2767.

Scheme R2



R = H, Me

a known compound, prepared in three steps from thymidine. We did not foresee any need to protect the 3' hydroxyl group, as the primary amino group was expected to be far more nucleophilic than the secondary hydroxyl group.

As was mentioned, one of the reasons for amide backbone substitution was to increase the lipophilicity of the modified DNA strand in order to facilitate cell wall penetration. For this purpose an alkyl-substituted tertiary amide should be even more effective than a secondary amide. We did not want to introduce a large alkyl group in the backbone for fear that its bulk would interfere with hybridization. It was therefore decided to make a methylamide, as well as an H-amide. This required the synthesis of 5'-methylamino-5'-deoxythymidine, which was not expected to pose a problem.

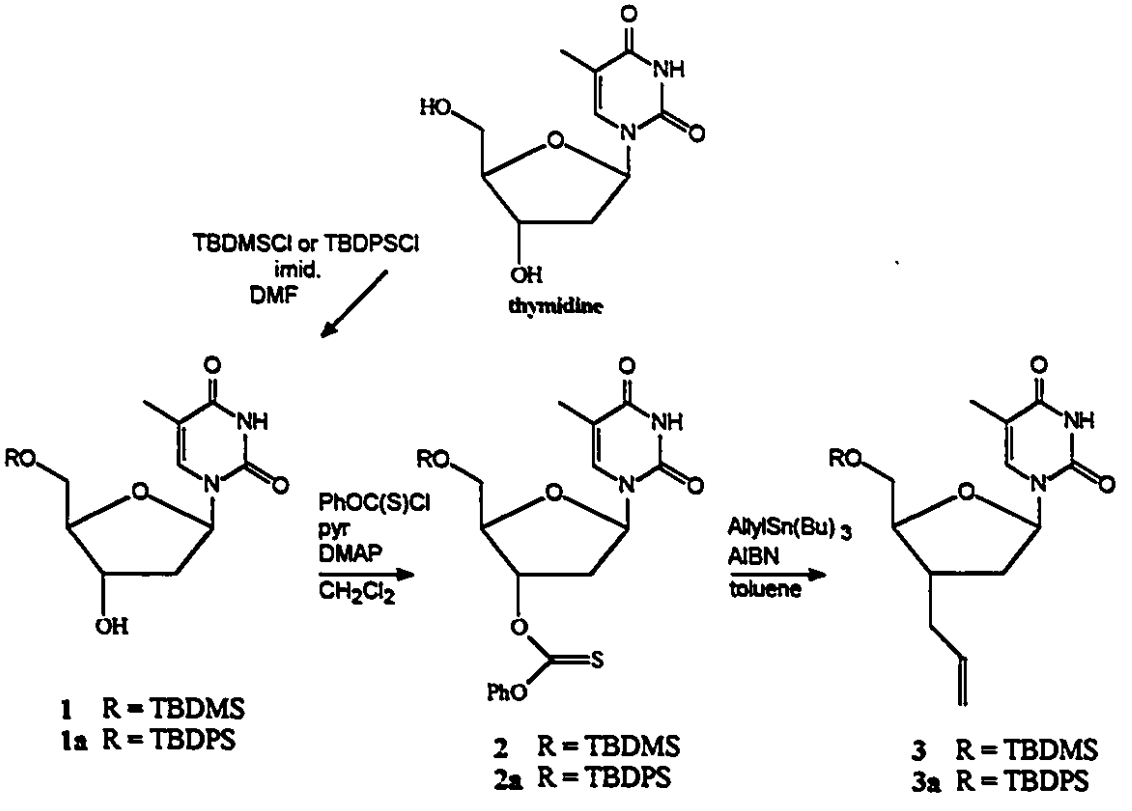
A wide choice of peptide coupling reagents is available to mediate the condensation of the acid and amino components. Mostly these are mild reagents that are compatible with nucleoside chemistry. Alternatively, a 3' activated ester, such as a succinimidyl or pentachlorophenyl ester, could be used as precursor.

We were also interested in continuing the work on the homopolymer synthesis, according to Scheme R1. In order to conduct significant binding studies, the homopolymer would have to be at least eight units long. We found it an interesting concept that in principle it should be possible to make use of solid phase peptide synthesis to prepare a modified nucleotide. However, it probably would be easier to follow solution peptide chemistry methods.

2.2 Synthesis of the carboxylic acid monomer.

Our first objective was to design a short and direct route for the synthesis of the carboxylic acid component of the targeted dimer, shown in Scheme R2. 3'-Allyl-3'-deoxythymidine was prepared according to the procedure described by Chu *et al.*[25], starting from commercially obtained thymidine. (Scheme 1)

Scheme 1



The 5' hydroxyl group was first protected as the *t*-butyldimethylsilyl ether, following standard procedures.[26] Subsequently the 3'-phenylthionocarbonate was formed in preparation for radical deoxygenation and introduction of the alllyl group at the 3' position.This reaction is totally stereospecific and leads exclusively to the formation of the

[26] Hakimelahi, G.H.; Proba, Z.A.; Ogilvie, K.K. *Can. J. Chem.* **1982**, *60*, 1106.

3' α product.

We first attempted to oxidize the double bond to the carboxylic acid in a one-step reaction. The use of sodium periodate in conjunction with ruthenium tetraoxide under Sharpless conditions[27] led to a mixture of non-UV active products. It was since found that attempts to make 5' carboxylic acid derivatives of nucleosides using the same conditions, are successful only for the purines, but result in the complete loss of the chromophore in the case of the pyrimidines.[28] Similarly, the application of potassium permanganate with sodium periodate in a two-phase system of water and ether[29] proved to be too strong to specifically react with the allyl double bond and resulted in the destruction of the molecule. It was thus decided to do the oxidation in two steps, keeping conditions mild.

For the synthesis of aldehyde **4** we followed the literature procedure of Fiandor and Tam.[30] 3'-Allyl-3'-deoxythymidine **3** was reacted with sodium periodate and a catalytic amount of osmium tetroxide in a two-phase system of water and ether.(Scheme 2) The reaction was followed by TLC, with ethyl acetate / hexanes, 3 / 2, as eluent. As the reaction progressed, two new spots appeared, the more intense one having an R_f value of 0.25, while the lesser spot showed just above the baseline. The intensity of this spot was estimated to be approximately 20% of that of the main product. The R_f value of the more polar compound was later compared to that of carboxylic acid **6** and was found to be identical. We were unable to isolate this compound from the reaction mixture for further identification. The formation of the by-product accounted for the relatively poor yield (60%) of this reaction.

Aldehyde **4** was further oxidized under mild neutral conditions, with pyridinium

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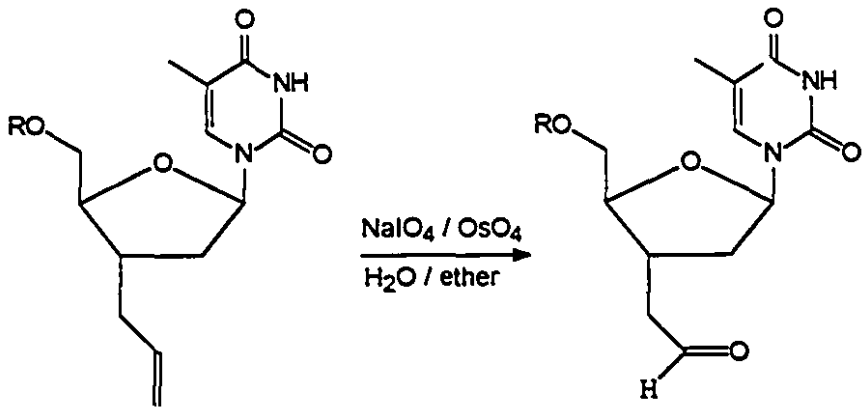
[27] Carlsen, P.H.J.; Katsuki, T.; Martin, V.S.; Sharpless, K.B. *J. Org. Chem.* **1981**, *19*, 3938.
[28] Singh, A.K.; Varma, R.S. Tetrahedron Lett. **1992**, *33*, 2307.
[29] Whitesell, J.K.; Nabona, K.; Deyo, D. *J. Org. Chem.* **1989**, *54*, 2258.
[30] Fiandor, J.; Tam, S.Y. *Tetrahedron Lett.* **1990**, *31*, 597.

dichromate in DMF. Only one reaction product was observed by TLC (R_f = 0.38,

Scheme 2



3 R = TBDMS
3a R = TBDPS

4 R = TBDMS
4a R = TBDPS

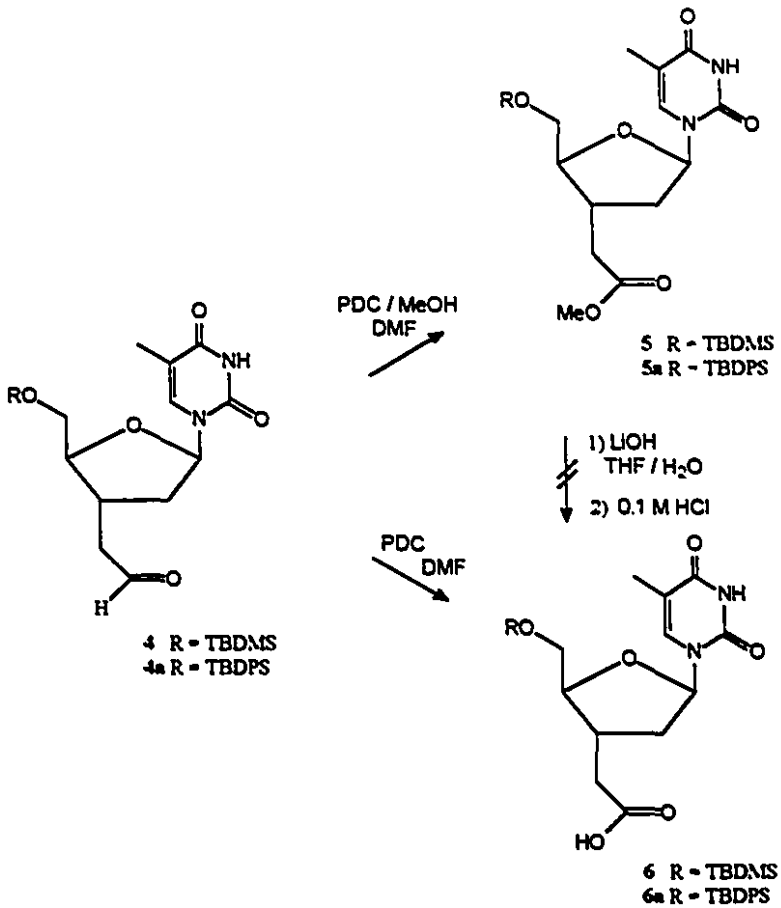
methylene chloride / methanol, 5 / 1). The work-up of this reaction, however, gave problems. Chromatographic purification was unsuccessful, because the acid strongly adhered to the silica gel and increasingly polar eluents failed to elute the desired compound. Dilution of the reaction mixture with water resulted in an oily suspension, from which the targeted product could not be cleanly extracted with organic solvents. In order to facilitate the work-up, it was decided to oxidize the aldehyde to the methyl ester 5 instead, followed by alkaline hydrolysis. (Scheme 3)

After following literature procedure[31] (simultaneous addition of methanol and PDC to a solution of aldehyde in DMF), pure methyl ester 5 was isolated in 60 % yield. However, if a DMF solution of the aldehyde plus methanol was stirred for 0.5 h before the addition of PDC, the yield of the methylester was increased to 95 - 98 %. This difference in yield can be explained in terms of the mechanism of this reaction, described by Corey *et al.* for the oxidative formation of *t*-butyl esters from aldehydes.[32] They

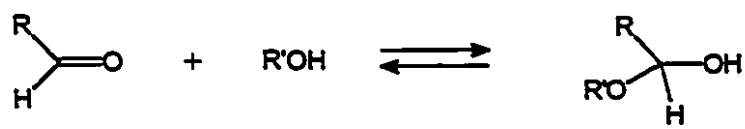
[31] O'Connor, B.; Just, G. *Tetrahedron Lett.* **1987**, *28*, 3235.
[32] Corey, E.J.; Samuelsson, B. *J. Org. Chem.* **1984**, *49*, 4735.

proposed as the first, and rate determining, step in this oxidation reaction the nucleophilic addition of the alcohol to the aldehyde carbonyl group to form the hemiacetal.(Equation 1)

Scheme 3



Allowing sufficient time for this type of reaction to reach an equilibrium minimizes the direct oxidation from aldehyde to carboxylic acid as a competing reaction. Our results also suggest that the equilibrium of the reaction lies to the right of the equation.

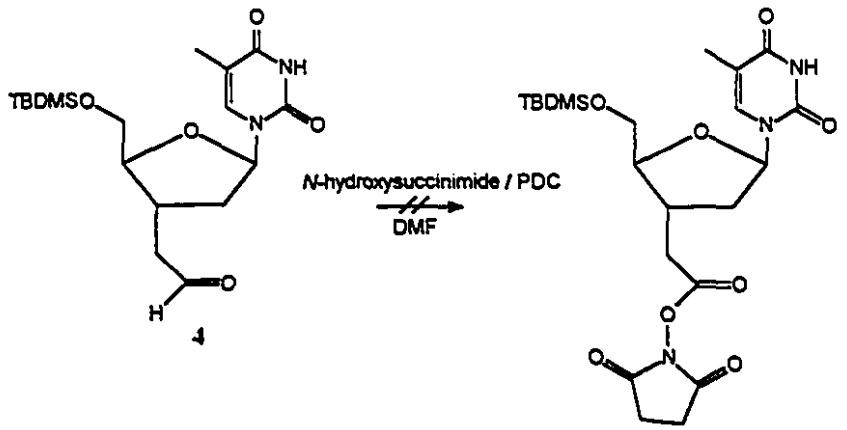


Equation 1

The work-up of this reaction was very simple: the methyl ester was isolated by diluting the reaction mixture with ethyl acetate and filtering it through a pad of silica gel. No further purification was necessary.

It occurred to us that it would be very convenient if it were possible to oxidize aldehyde **4** in the presence of *N*-hydroxysuccinimide and form an activated ester, according to Scheme 4.

Scheme 4

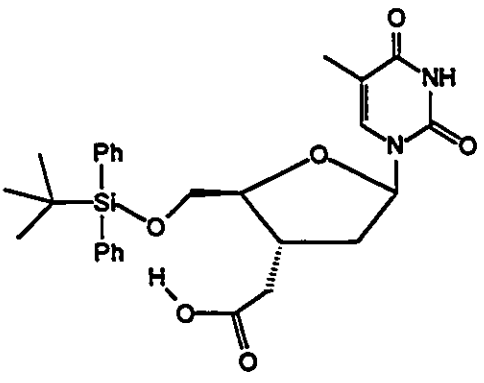


Probably, the *N*-hydroxysuccinimide was either not sufficiently nucleophilic, or too bulky to form the necessary hemiacetal, for the sole product of the reaction was the acid **6** instead of the activated ester.

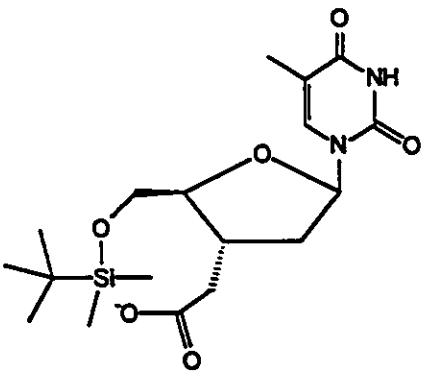
In the meantime we subjected methyl ester **5** to alkaline hydrolysis, under the mildest possible conditions (lithium hydroxide, 0.5 M in water / THF, 3 / 7), and subsequent acidification with 0.1 M HCl to pH ~3. Unfortunately this reaction resulted in the total loss of the *t*-butyldimethylsilyl protecting group. The characteristic smell of the liberated silyl group was noticed even before acidification. It was thus necessary to decide on a new, more stable, protecting group. Chu *et al.*[25] had used the *t*-butyldimethylsilyl group for the synthesis of 3'-allyl-3'-deoxythymidine and we did not want to jeopardize the outcome of the synthesis by making major changes. We therefore changed the protection to the *t*-butyldiphenylsilyl group, which we expected to be sufficiently stable under these conditions. The reactions were done according to schemes 1, 2, and 3. The conditions were kept the same as for the *t*-butyldimethylsilyl-protected compounds. The reaction times and the yields were not significantly different.

Unexpectedly, carboxylic acid **6a** still suffered a partial loss of the *t*-butyldiphenylsilyl group after the necessary hydrolysis of ester **5a**. However, no silyl group smell was noticed during treatment with lithium hydroxide.

A possible explanation for these phenomena is that the 3' carboxylic acid function acts as an intramolecular catalyst for the acid or base hydrolysis of the 5' silyl group. In order to determine whether the 3' substituent indeed influenced the outcome of the hydrolysis reactions of esters **5** and **5a**, we subjected 5'-*O*-TBDMS-thymidine **1** and 5'-*O*-TBDPS-thymidine **1a** to the same alkaline hydrolysis and acidification conditions. Compound **1a** was recovered unchanged after this treatment, while **1** lost its protecting group only after being left at pH ~ 3 for 0.5 h. Thus our experimental results do not support a thymine-assisted loss of the silyl group. Rather, our findings establish the involvement of the 3' carboxyl group of **6** in the loss of the TBDMS under alkaline conditions. They also show that the carboxyl group plays a role in the acid-mediated instability of the TBDPS group of **6a**. If an 8-membered ring geometry exists between the 3' and the 5' carbons (Figure 1), the carboxylic acid proton of compound **6a** is in the right position to destabilize the oxygen-silyl bond. Similarly, under alkaline conditions the negative charge of the carboxylate of compound **6** may cause the TBDMS group to migrate from the 5' position, forming the unstable 3' silyl ester.



6a



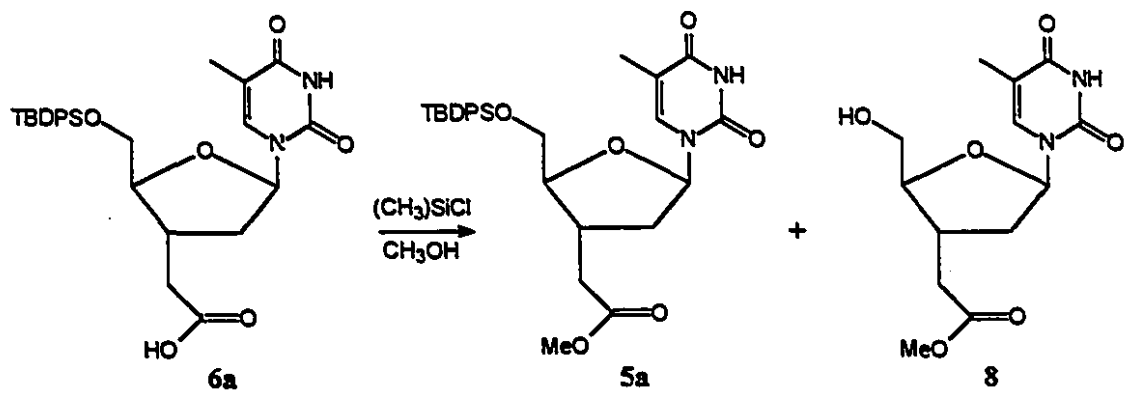
6

Figure 1. Intramolecularly assisted loss of silyl groups.

After these unsuccessful attempts to obtain the 5' protected carboxylic acid from the methyl ester, we went back to our initial method of PDC mediated one-step aldehyde to acid formation, but with *t*-butyldiphenylsilyl instead of *t*-butyldimethylsilyl protection. The acid precipitated out as a beige-coloured solid upon dilution of the reaction mixture with water. It was now possible to isolate the desired compound by simple filtration, in 80 % yield or better. We were not able to obtain a well resolved NMR spectrum of the acid, perhaps because of hydrogen bonding and, or, residual chromium contamination. The mass spectrum, however, confirmed the correct molecular mass.

In order to properly characterize the compound it was decided to derivatize the carboxyl group to the corresponding methyl ester. This was easily accomplished using Chan's method (Scheme 5).[33]

Scheme 5



Methyl ester **5a** was isolated in 50% yield; the NMR data were identical to those obtained from the ester made by the methanol-PDC aldehyde oxidation method, described above. A second, more polar compound was also formed during the reaction and was identified as the corresponding 5' desilylated ester. This partial loss of the

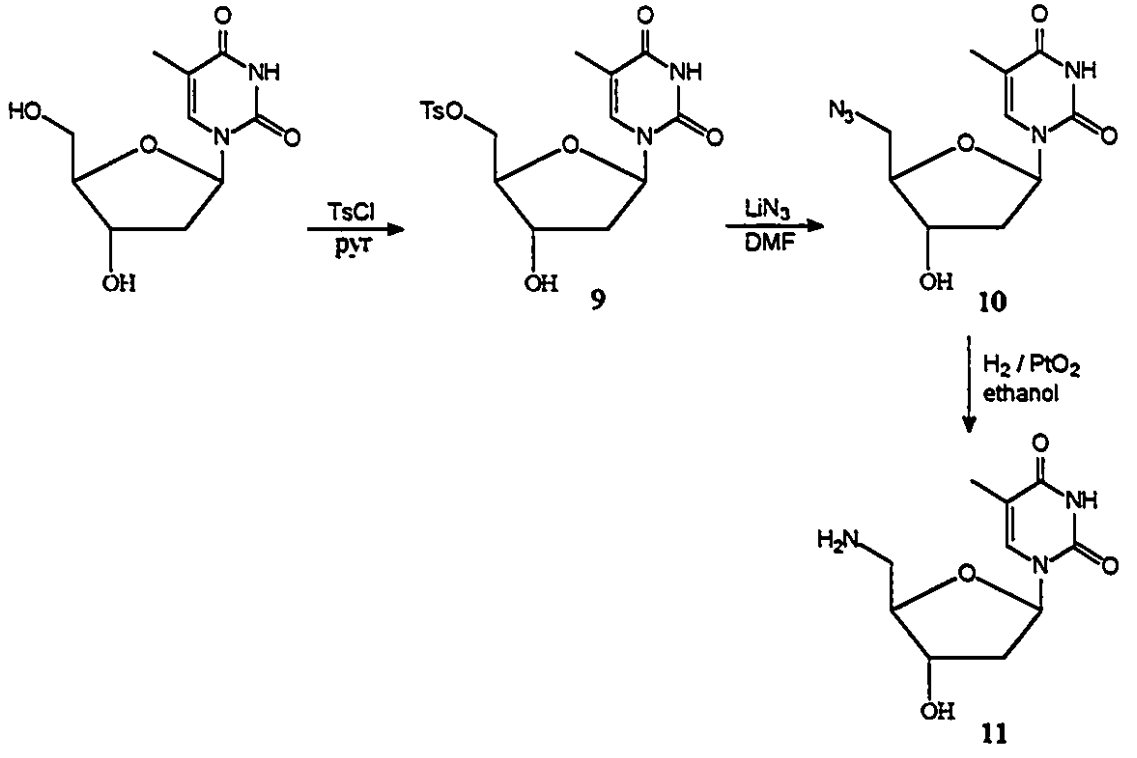
[33]Brooks, M.A.; Chan, T.H. *Synthesis*, **1983**, 201.

5' *t*-butyldiphenylsilyl group was to be expected, given the acidic conditions of the reaction.

2.3 Synthesis of the 5'-amino building blocks.

The synthesis of 5'-amino-5'-deoxythymidine **11** was accomplished in three steps from commercially obtained thymidine, partially following literature procedure.[34] (Scheme 6)

Scheme 6



We found that 5'-tosylthymidine could be quickly and conveniently prepared in the following manner. A pyridine solution of thymidine was treated at 0° C with a 4-fold to 6-fold excess of tosyl chloride, which was added over a span of ten minutes. After half an hour the TLC indicated that ~ 95 % of the starting material had been consumed. The reaction product was mostly 5'-tosylthymidine, but a small amoumt of 5',3'-ditosylthymidine (~ 5 %) was produced. There was no evidence of the formation or 3'-tosylthymidine.

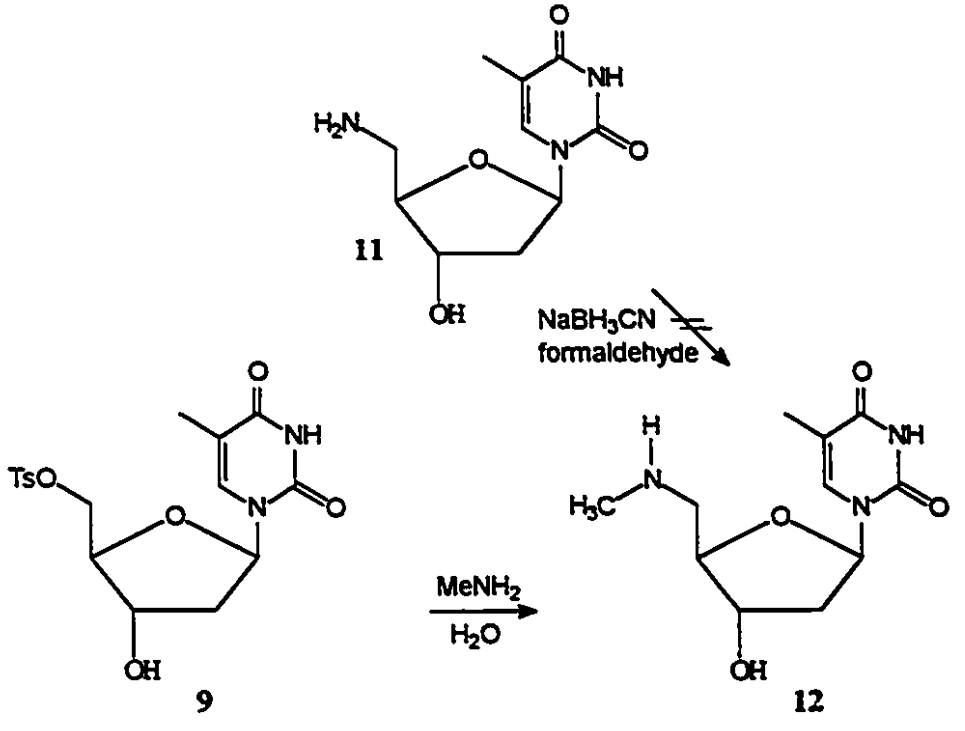
[34]Horwitz, J.; Tomson, A.; Urbanski, J.; Chua, J. *J. Org. Chem.* **1962**, *27*, 3045.

At that point the reaction mixture was poured into ether and stirred till the sticky precipitate hardened. The excess tosyl chloride and the ditosylthymidine stayed in solution. The crude tosylthymidine was then recrystallized from methanol. The convenience of this method and the fact that it was the first step of a longer synthesis compensated for the relatively poor yield (50 to 60 % after crystallization).

The tosyl group was subsequently displaced by the azide ion to give 5'-azido-5'-deoxythymidine **10**, which was hydrogenated in the presence of platinum(IV) oxide. Pure 5'-amino-5'-deoxythymidine **11** was isolated after filtration of the catalyst.

We also needed 5'-*N*-methylamino-5'-deoxythymidine **12** in order to make our second target compound, the *N*-methyl amide thymidine dimer. Since we had easy access to the primary amine, we decided to subject it to reductive amination using sodium cyanoborohydride and formaldehyde. (Scheme 7)

Scheme 7



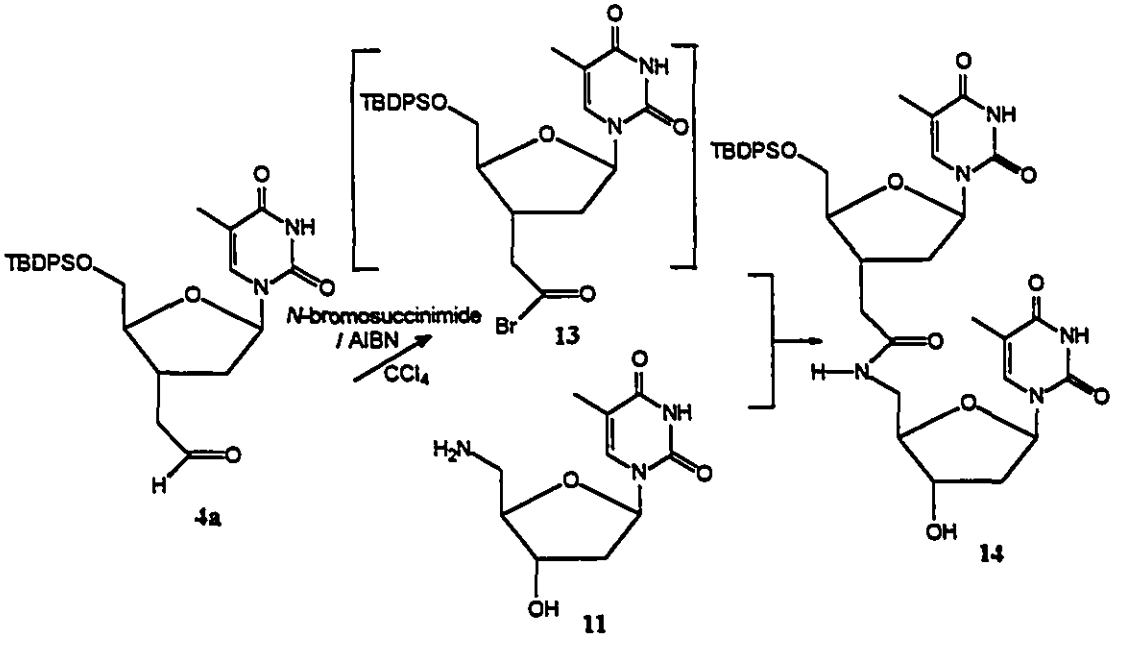
This reaction resulted in an inseparable mixture of products. NMR data of the crude product indicated a mixture of mono- and di-alkylated amine (two singlet signals for the *N*-methyl protons around 2.4 ppm).

We abandoned this approach and instead directly displaced the tosyl group in 5'-tosylthymidine with methylamine. Heating a solution of the tosyl thymidine in 40% aqueous methylamine at 55° C in an open vessel gave the tosylate salt of the desired product. Finally, silicagel column chromatography yielded the 5'-*N*-methylamino-5'-deoxythymidine **12**. (Scheme 7)

2.4 Attempts at dimer formation.

While we were still trying to find a suitable way to synthesize and isolate the 5' protected carboxylic acid component of the projected dimer, we considered several other options. We could, for instance, try and find a way to bypass the necessity of making the troublesome acid. One attractive idea was the oxidative coupling of aldehyde **4a** with amine **11** or **12**, following the method reported by Markó and Mekhalfia.[35] They described a two-step, one-pot formation of amides from the corresponding aldehydes and amines. The aldehydes were first oxidized by *N*-bromosuccinimide to the acid bromides, with a catalytic amount of AIBN to initiate the reaction. The acid bromide did not need to be isolated, and was reacted *in situ* with the appropriate amine, to give the amide in good yield. We felt that the neutral conditions of this radical oxidation reaction should be

Scheme 8



[35] Markó, I.E.; Mekhalfia, A. *Tetrahedron Lett.* **1990**, *31*, 7237.

compatible with our nucleosides. (Scheme 8)

We managed to isolate the targeted dimer **14**, but the yield was very low (~ 3 mg, 10 %). A reason for this poor result could be the moisture sensitivity of the acid bromide intermediate. The reaction was done on a very small scale (0.04 mmol). While reasonable care was taken to exclude moisture from the flask, even a very small amount of water would have a large influence on the outcome of a reaction on this scale. It would have been necessary to repeat this reaction on a much larger scale to get a conclusive result, but we did not consider this practical at that point.

After this rather unsatisfactory reaction, we looked at another way of making the dimer, following Scheme 9.

Scheme 9

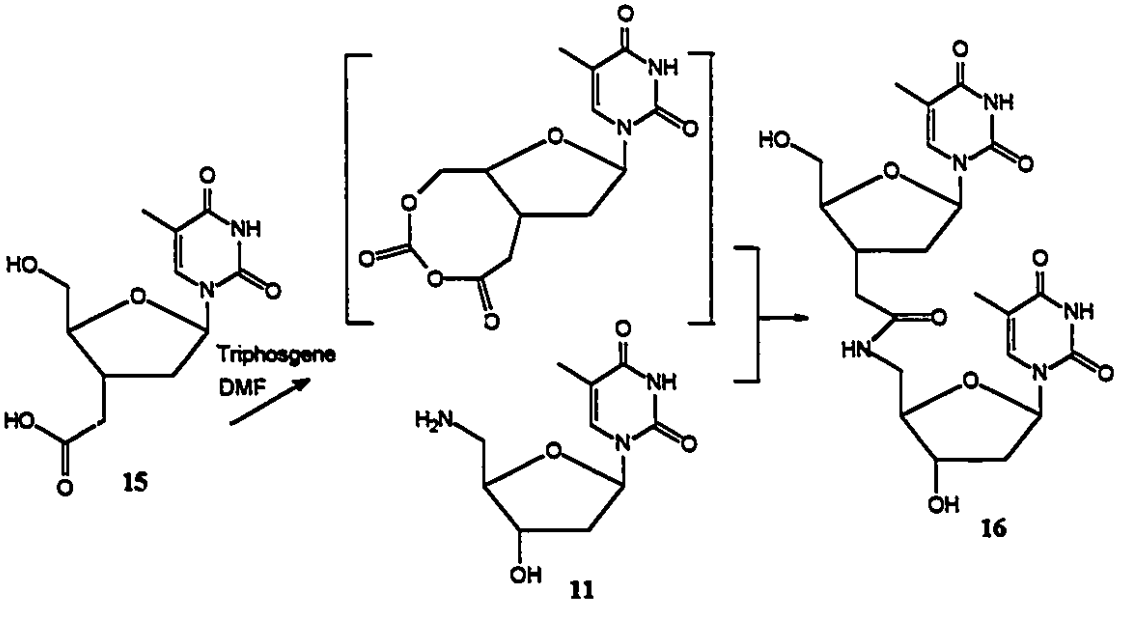


Figure 1 on page 21 shows an 8-membered ring formed by bringing the 3' and 5' functionalities in close proximity. This suggested to us the possibility of forming a mixed anhydride of the same geometry, starting with unprotected carboxylic acid **15**, by treating it with triphosgene. Each molecule of triphosgene, conveniently and safely, provides three carbonyl groups to connect the hydroxyl and carboxyl functions. A nucleophilic attack by

the amino group of 5'-amino-5'-deoxythymidine **11** on the carbonyl group closest to the 3' carbon could be expected to open up the ring, and result in the formation of the desired dimer **16**. An attack on the carbonate carbonyl would be less favoured, because of the greater electron density around this site, caused by the resonance contribution of the two neighbouring oxygen atoms, versus only one oxygen next to the other carbonyl.

This reaction yielded a small amount of dimer **16**, as viewed on TLC (R_f = 0.18 with methylene chloride / methanol, 100 / 15, as eluent). The R_f value was later compared to that of the fully characterized dimer and found to be identical.

In the meantime we had managed to isolate the *t*-butyldiphenylsilyl protected acid **6a** cleanly and in good yield and so abandoned the attempts at dimer formation that we just described.

2.5 Formation of the thymidine amide-linked dimers.

With the carboxylic acid **6a** and amines **11** and **12** in hand, we were now ready to proceed with the amide coupling . An overwhelming amount of literature,describing peptide chemistry methodology is available. Since we intended to only make a dipeptide, we were interested in solution, rather than solid phase chemistry, but we wanted to leave open the possibility of eventual solid phase synthesis of longer homopolymers. For both purposes, either the use of one of many coupling reagents, or of an activated ester would be suitable.

Our attempts to make the activated *N*-hydroxysuccinimidyl ester in a one-step aldehyde to ester oxidation had failed. We decided to concentrate instead on reagent mediated coupling. The use of the two reagents that we had on hand, dicyclohexylcarbodiimide (DCC) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), gave unsatisfactory results. The reactions were slow and did not go to completion. On the other hand, the use of benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[36] was highly effective in the coupling of carboxylic acid **6a** with amine **11**, or with methylamine **12**. (Scheme 10) The reactions were fast and yielded dimers **14** and **17** consistently in 80 - 85% yield, after chromatographic purification. As expected, no coupling between the carboxylic acid and the 3' hydroxyl group of the amines was observed.

Subsequent removal of the silyl protecting group by tetra-n-butylammonium fluoride in THF afforded amide-linked nucleoside dimers TaT (H-amide-linked thymidine dimer) **16** and TmaT (methylamide-linked thymidine dimer) **18**. The TmaT NMR spectra (Appendix 1, 2) indicated a small amount of tetra-n-butylammonium ion contamination, which could only be separated with considerable loss of the main product.

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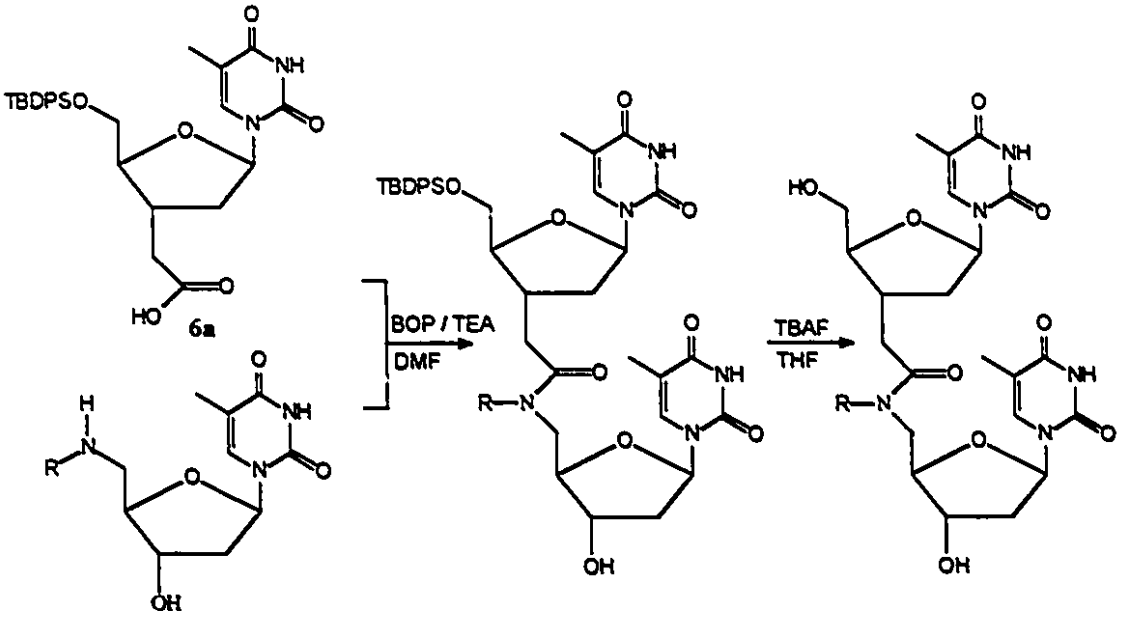
[36]Castro, B.; Dormoy, J.R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219.

Scheme 10



11 R = H
12 R = Me

14 R = H
17 R = Me

16 R = H
18 R = Me

2.6 Structure and conformational analysis of the TaT and TmaT dimers.

The NMR analysis of the TmaT dimer **18** structure was complicated by the fact that the proton and the carbon spectra (Appendix 1, 2) showed double signals for most of the nuclei. It was verified by HPLC that this was not due to an impurity. We concluded therefore that the TmaT dimer exists in solution as a mixture of two rotamers, in a cis and a trans conformation about the amide bond. (Figure 7)

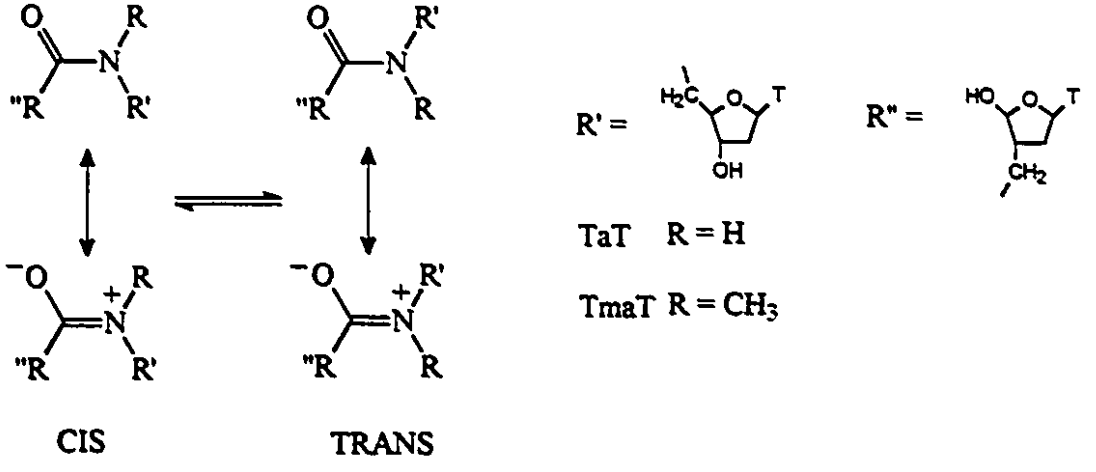


Figure 7. Amide cis - trans equilibrium.

It is well known that the amide bond has a partial double bond character, due to its mesomeric nature. This raises the rotation barrier to ~ 90 kJ per mole, which is sufficiently high to hinder rotation at room temperature. The interconversion of this bond is slow enough on the NMR time scale that, if present, both rotamers can be detected on a spectrum. Secondary amides strongly prefer a trans configuration for steric reasons. The energy of the cis conformation is approximately 9 kJ higher than the trans, which translates into a cis population of only 3 %.[37]

The picture is not as clear for tertiary amides. Much depends on the steric bulk of

[37] Dale, J. *Stereochemistry and conformational analysis*, Universitetsvorlaget, Oslo, 1978, p.82.

the substituents, but electronic factors can also play a role. The difference in energy for cis and trans conformers of *N*-dialkyl substituted amides is diminished, resulting in two more equally populated states.

The TaT spectrum (Appendix 4) indicated the presence of only one isomer. Since this compound is a *N*-monoalkyl substituted amide, this was exactly as expected. Even if the theoretical 3 % of cis conformer was present, it might not have been a sufficient amount for detection by NMR.

As was mentioned above, the TmaT spectrum clearly showed two rotamers. The integration of non-ambiguous, easily assigned proton NMR signals, such as the H6 characteristic fine doublets between 7 and 8 ppm, and the anomeric protons around 6 ppm, indicated a ratio of two to one.(Figure 8) The equilibrium constant of this system

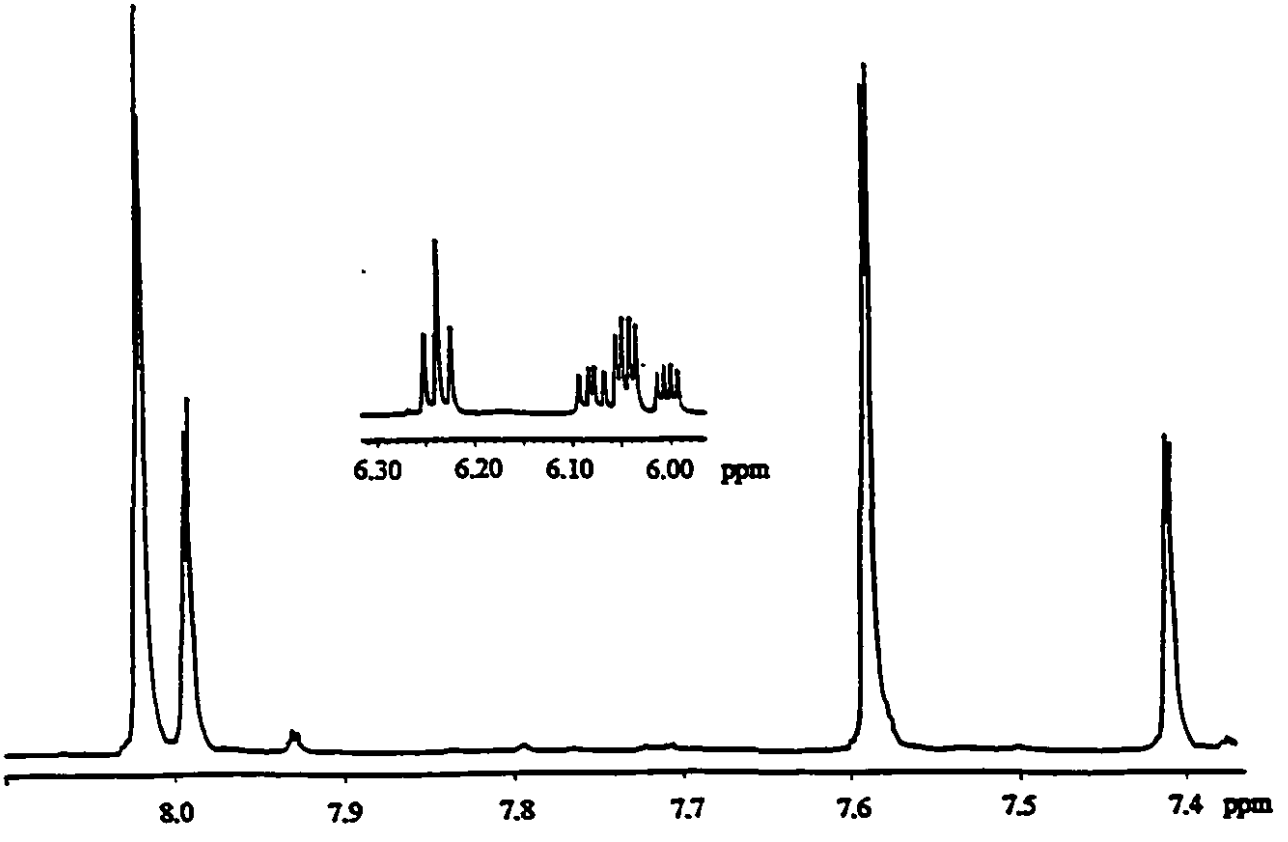


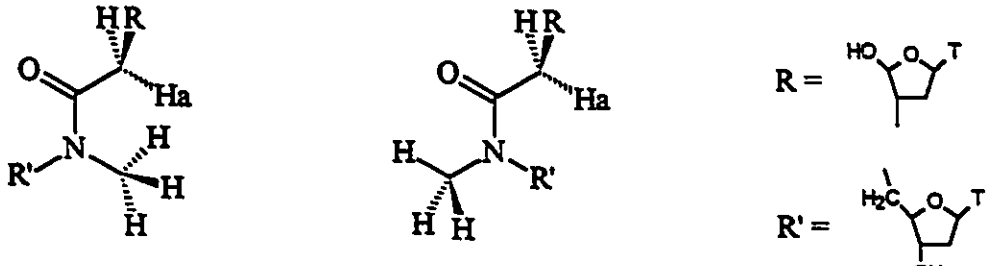
Figure 8. Parts of ^{1}H NMR spectrum of *N*-methylamide-linked thymidine dimer **18**, showing H6 protons and H1' protons (inset).

thus has a value of 2. Calculated from $\Delta G = -RT \ln K$, the energy difference between the cis and trans rotamers equals only 1.7 kJ per mole.

Interestingly, the spectra of the 5' *t*-butyldiphenylsilyl and the 5' dimethoxytrityl protected TmaT showed a rotamer ratio of three to one and four to one, respectively. This could be the result of the increased bulk of the ' top' unit. Another possibility is a stacking interaction between the amide carbonyl group and the 5' phenyl rings, which further stabilizes the trans conformation.

In order to determine which of the TmaT conformations was predominant, we decided to look for an NOE between the 3" bridge methylene protons and the *N*-methyl protons. The trans rotamer can be expected to show such an effect for Ha, while the cis should not, as is shown in Figure 9.

The *N*-methyl signals were easily recognizable as singlets at 2.98 ppm (minor) and 3.11 ppm (major), in a one to two ratio. A detailed analysis of the COSY spectrum (Appendix 3), however, was required for the assignment of the 3" methylene protons. Starting from the four anomeric proton signals at 6.00 - 6.24 ppm we systematically



Trans TmaT Cis TmaT

Figure 9. Position of the *N*-methyl protons in relation to the 3" methylene bridge protons.

followed each correlation pattern. This led to the unambiguous assignment of the 3" signal, an ABX system at 2.49 - 2.64 ppm. It also enabled us to determine which of the two nucleoside units, the 5' or the 3' end residue gave rise to a particular signal. The

superscripted numbers 5 or 3, before the H, are used to indicate to which unit the proton belongs.

The NOESY spectrum indicated a NOE between the 3" methylene Ha signal and the major *N*-methyl peak. No such relationship was found for the minor *N*-methyl signal. This clearly established that the major TmaT conformation is trans about the amide bond. The 2 / 1, trans / cis ratio reflects the difference in size of the *N*-substituents. The NOESY spectrum further revealed a correlation between ^{5}H6 and ^{5}H3' and also between ^{5}H6 and ^{5}H2' for both rotamers. This finding is consistent with an anti position of the thymine base and a C3' endo configuration of the deoxyribose ring. It was not possible to obtain all the coupling constants needed for the sugar ring conformational analysis, due to extensive overlap of the H2', H3' and the H3" bridge methylene protons.

A comparison between the TaT and the TmaT proton spectra showed greater similarity of chemical shifts and coupling constants for the TaT and the major (trans) TmaT, than for the TaT and the minor (cis) TmaT. The H6 - H2' and H6 - H3' NOE relationships were found to be the same for the TaT as for the TmaT. Since the solvent used for the TaT spectra was either CD_3OD or D_2O, it was not possible to test for a NOE between the 3" bridge methylene protons and the amide N-H, as this is an exchangeable proton and thus not observed in protic solvents. CD_3OD was used for the TmaT spectra. It would have been preferable to use D_2O to give a more realistic structural model, but unfortunately TmaT was insoluble in D_2O. TaT was only slightly soluble in both D_2O and CD_3OD and easily precipitated out of, even dilute, solution. The quality of the TaT spectrum (Appendix 4), which is slightly out of phase, reflects this.

Shortly after we published our TaT and TmaT synthesis and binding results, a group of Ciba - Geigy scientists independently reported the same findings.[38] In addition they had performed molecular modeling studies, which were of great interest to us.

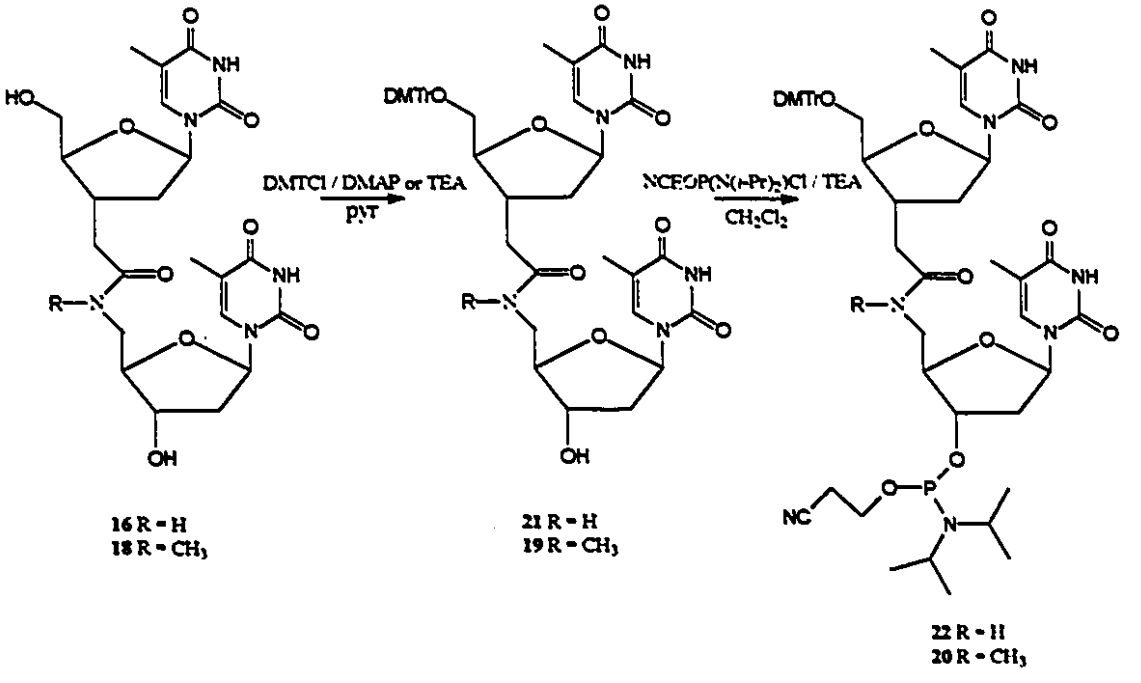
[38] De Mesmaeker, A.; Waldner, A.; Lebreton, J.; Hoffmann, P.; Fritsch, V.; Wolf, R.; Freier, S. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 226.

They took an octamer RNA-DNA duplex, r(GA_6G)-d(CTTTaTTTC), and allowed this double helix to find its energy minimum, using an AMBER all atom force field. The TaT dimer structure was taken from this optimized octamer duplex and analyzed. Their results basically confirmed what we had observed. The base angle χ at -157° is consistent with the anti position, while the sugar ring exhibited C3' endo puckering, indicative of DNA in an A form helix.

2.7 Protection and functionalization of the TaT and TmaT dimers.

With the synthesis of the TaT **16** and TmaT **18** dimers completed, we now needed to protect the 5' hydroxyl groups and functionalize the 3' hydroxyl groups (Scheme 11) in preparation for the solid phase synthesis of the modified DNA sequence.

Scheme 11



The protocols for these reactions, involving unmodified nucleoside monomers, are well established. We initially followed the literature procedure[39] for the 5' dimethoxytritylation of TmaT **18** (DMTrCl, pyridine, room temperature, after co-evaporation from pyridine) and found this reaction to be extremely slow. While the same reaction for thymidine was complete in 0.5 h, the TmaT reaction needed two days. We found, however, that we could speed up the reaction by the addition of a catalytic amount of dimethylaminopyridine and at the same time optimize our yield of DMTrTmaT **19** to 90 %. The time required for this reaction to go to completion varied from one to three

[39] Gait, M.J., Ed. *Oligonucleotide Synthesis, A Practical Approach*; IRL Press; Oxford, 1984.

hours.

The same strategy did not work as well for the TaT dimer **16**, but if triethylamine was substituted for the dimethylaminopyridine, the reaction time for the formation of DMTrTaT **21** was shortened to one hour and the yield was also optimized to 90 %.

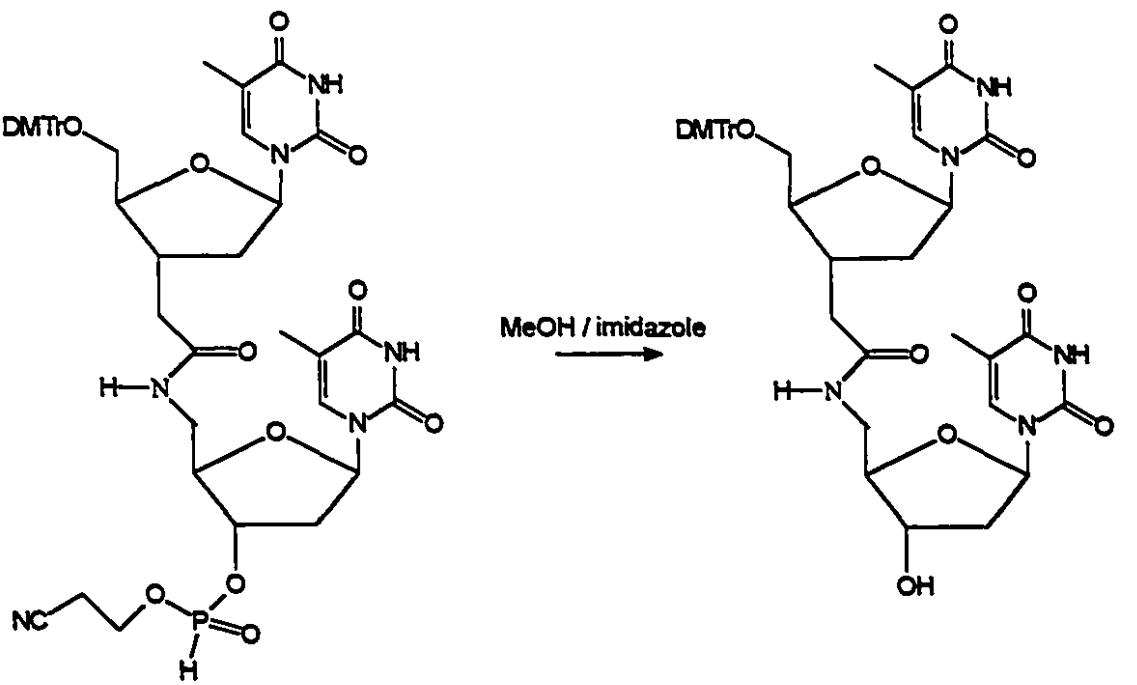
The 3' functionalization of the DMTrTmaT **19** with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite went smoothly and yielded the desired product **20** in 94 % yield as a 50 - 50 mixture of diastereomers. The ^{31}P NMR spectrum indicated that, in solution, the diasteromers were further split into two rotamers each, in a ratio of three to one. The FAB mass spectrum showed a molecular ion of the expected molecular weight. In addition it indicated the presence of a complex of phosphoramidite **20** with triethylamine. This same phenomenon was previously observed in our laboratory for a similarly protected and functionalized sulfur-linked thymidine dimer.

The 3' phosphitylation of the DMTrTaT dimer **21** proved to be a temperamental reaction. We initially made TaTphosphoramidite **22** in 94 % yield. The presence of the phosphoramidite function was confirmed by the ^{31}P NMR spectrum which showed two peaks at 150.6 and 151.0 ppm, the expected frequencies for phosphoramidites. The FAB mass spectrum confirmed the molecular weight of compound **22**, and also that of the triethylamine complex, in analogy with the TmaTphosphoramidite **20**. The TaTphosphoramidite **22** was subsequently used for solid phase DNA synthesis with excellent results. However, when we tried to repeat this reaction several months later, only phosphorus peaks, characteristic of phosphonates, were observed from the ^{31}P NMR spectrum, at 14.3 and 14.4 ppm. The only difference in the reaction conditions appeared to be some contamination of the DMTrTaT by triethylamine salt, which co-eluted with the DMTrTaT during chromatographic purification. This likely was due to atmospheric moisture which may have condensed on the eluents. The acidity of the triethylamine salt,

plus traces of residual moisture could account for this unwanted reaction.[40] Afterwards we were extra cautious to exclude moisture from this reaction, but we still sometimes obtained the undesired phosphonate.

Fortunately we were able to partially regenerate the DMTrTaT by freeing the 3'-hydroxy function from the cyanoethyl-H-phosphonate group, following the method described by Brill.[41](Scheme 12) This was basically a transesterification reaction in methanol, catalyzed by imidazole. After allowing this solution to stand for a week, we managed to recover 60 % of the DMTrTaT.

Scheme 12



[40] a) Marugg, J.E.; Burik, A.; Tromp, M.; van der Marel, G.A.; van Boom, J.A. *Tetrahedron Lett.* 1986, *27*, 2271.
b) Froneman, M.; Modro, T.A. *Synthesis* **1991**, 201.
[41] Brill, W.K.-D. *Tetrahedron Lett.* **1994**, *35*, 3041.

2.8 Solid phase synthesis of amide-modified DNA sequences.

The DNA sequence that we proposed to synthesize was designed to test binding, rather than a desire to inhibit a particular gene expression. At this stage of our research we were merely interested in determining the physical effects of replacement of the phosphate moiety by amides. Most importantly, we wanted to know if the amide substitution would cause a destabilization of the DNA-RNA or DNA-DNA duplex formation. Thus we decided to synthesize the same dodecamer sequence for our binding studies as the one that was previously used in our laboratory to test sulfide backbone-modified antisense, but with three amide instead of sulfide insertions.[42]

The solid phase synthesis of the methylamide-modified DNA strand **20a** and the binding studies were performed at Erindale College of the University of Toronto, under the guidance of Dr. M.H. Damha.(Table 1) Mr. P.A. Giannaris prepared the required normal DNA and RNA sequences **A, B, C** and **D** and gathered the hybridization data for **20a**. The H-amide DNA sequence **22a** was prepared at a later time by Mr. R. Hudson, also at Erindale College. Complementary RNA strand **D** contains one mismatched base

Table 1. Synthesized oligonucleotide strands.

Oligomer	Sequence
20a	d(5'-GpCpGpTmaTpTmaTpTmaTpGpCpT-3')
22a	d(5'-GpCpGpTaTpTaTpTaTpGpCpT-3')
A	d(5'-GpCpGpTpTpTpTpTpTpGpCpT-3')
B	d(3'-CpGpCpApApApApApApCpGpA-5')
C	(3'-CpGpCpApApApApApApCpGpA-5')
D	(3'-CpGpCpApApApUpApApCpGpA-5')

[42] Kawai, S.H.; Wang, D.; Giannaris, P.A.; Damha, M.J.; Just, G. *Nucleic Acids Res.* **1993**, *21*, 1493.

(uridine) in the middle. We designed this sequence to test the specificity of the binding.

Before we attempted the solid phase synthesis of **20a** and **22a**, we had made sure that the amide bond was stable to the base deprotecting conditions. We incubated the non-phosphitylated TaT and TmaT dimers for six hours at 55° C in a 29 % ammonium hydroxyde solution and found no evidence of hydrolysis.

Oligonucleotides **20a** and **22a** were prepared on a 0.2 μmol scale, using an Applied Biosystems DNA / RNA synthesizer, model 381 A, following standard solid support methodology. A few minor modifications were made to the protocols. A fifth bottle, containing 0.15 M of the protected and phosphitylated dimer **20** or **22** solution in acetonitrile, was attached to the system. Also, the coupling time was increased to 70 seconds, from the normal 60 seconds, for the couplings that involved the dimers.

Deoxyribonucleosides with exocyclic dimethylformamidine protecting groups were used in preference over benzoyl-protected ones, as the deprotection time for amidine groups is considerably less than for benzoyl groups (6 hours, versus 12 - 18 hours in concentrated aqueous ammonia at 55° C). We did not want to expose our nucleotides to unnecessarily harsh conditions.

The coupling efficiencies were monitored by measuring the UV absorption of the highly coloured dimethoxytrityl ion after it was liberated from the growing nucleotide chain. It was found that the coupling efficiency of the dimers, at greater than 98 %, was on par with that of the monomer units.

The final cleavage of the oligomers from the controlled pore glass supports was effected by treatment with aqueous concentrated ammonia for six hours at 55° C, which at the same time removed the exocyclic protecting groups.

We used the convenient 'trityl on' method of solid phase synthesis, meaning that the last dimethoxytrityl group on the completed sequence was left in place. This facilitated the final purification of the oligomers by making the completed strands less polar and thus allowing them to be separated chromatographically from any incomplete sequences, which

had been capped as 5'-acetates, and therefore were present as free alcohols after the ammonia treatment. After reverse phase chromatography and the removal of the dimethoxytrityl end group, using oligonucleotide purification cartridges, the purity of the dodecamers was proven by subjecting them to electrophoresis on 16 % non-denaturing polyacrylamide gel. Both sequences, H-amide DNA **22a** in lane 4 and methylamide DNA **20a** in lane 6, showed a single band.(Figure 10)

It is interesting to note that the normal DNA sequences **A** and **B** (lanes 8 and 10, respectively) traveled faster on the gel than the modified strands **20a** and **22a**. This reflects the difference in the ionic nature of the backbones.

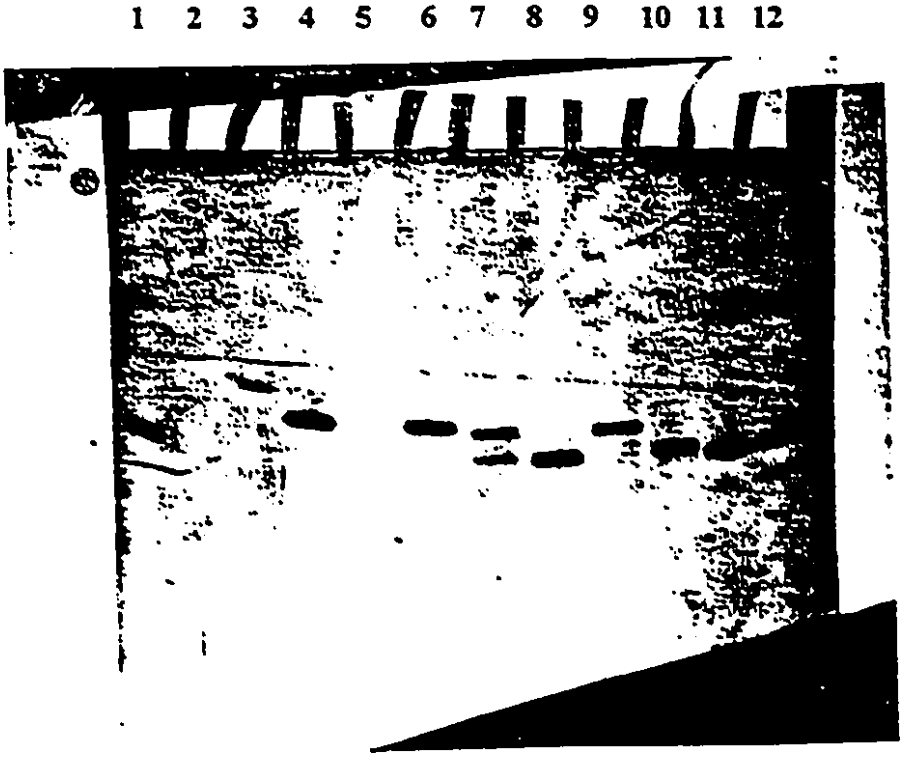


Figure 10. Polyacrylamide gel electrophoresis of modified oligomers **20a**, **22a**, and normal DNA **A** and **B**. Lanes 1, 11 and 12: Bromophenol blue dye; lane 4: **22a**; lane 6: **20a**; lane 8: **A**; lane 10: **B**.

2.9 Hybridization studies.

Duplex formation between complementary strands of DNA, or DNA and complementary RNA is routinely determined from the change in U.V. absorption as the temperature is varied. A duplex structure has a lower extinction coefficient than the single oligomer strands, due to hydrogen bonding between the complementary bases. Increasing temperature causes the strands to separate, resulting in higher UV absorbance. The stability of the duplex is expressed as its melting temperature (T_m), defined as the temperature at which half the duplex has unwound into single strands.

For our binding studies we prepared solutions of equimolar amounts of modified DNA and normal, complementary DNA or RNA in a 10 mM phosphate buffer, containing 1.0 M NaCl, at a pH of 6.9. The extinction coefficients were calculated using the nearest neighbour approximation as described by Tinoco et al.[43] These values were used to prepare the nucleotide solutions at concentrations of 2.5 μM for each strand.

The solutions were allowed to equilibrate at 40° C for 10 minutes, before being cooled down to 20° C over a period of 20 minutes. The temperature was then increased to 90° C at a rate of 0.5° C per minute. Absorbance versus temperature readings were recorded every minute. The thermal denaturation curves, depicted in Figure 11, are derived from these data. The strongly sigmoidal, steep slopes, as well as the high hypochromicity, which varied from 14 % to 18 %, are indicative of well-defined binding. The points of inflection of the thermal denaturation curves represent the melting temperatures, which are shown in Table 2.

The results of these studies clearly establish that modified dodecamer sequence **22a**, containing three TaT dimers, binds to complementary RNA as strongly as does normal DNA. Methylamide sequence **20a** also anneals well with RNA. The three

[43]Puglisi, J.D.; Tinoco, I. *Methods in Enzymology* **1989**, *180*, 304-324.

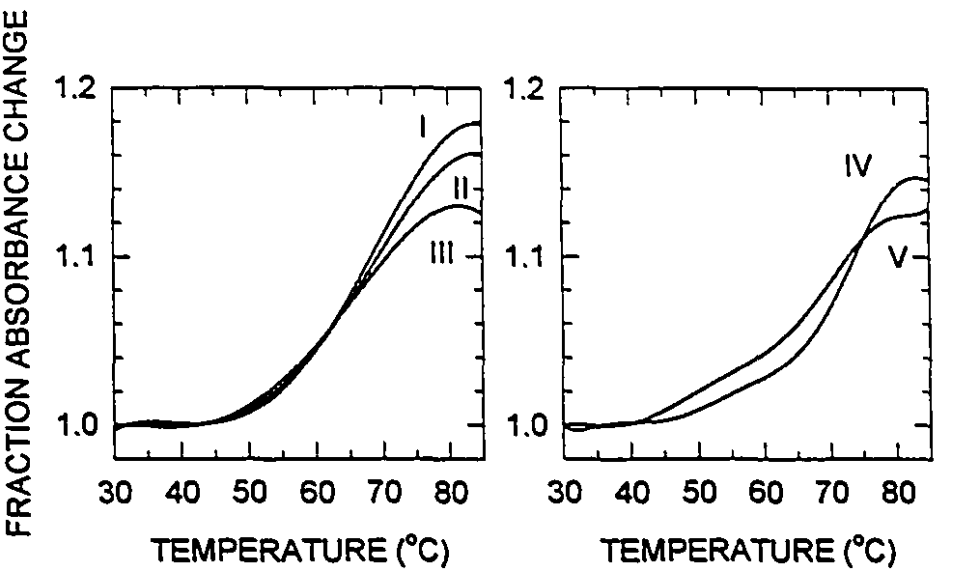


Figure 11. Melting curves of (I) **A** (normal DNA)- **C** (RNA); (II) **20a** (TmaT)- **C** (RNA); (III) **20a** (TmaT)- **B** (DNA); (IV) **22a** (TaT)- **C** (RNA); (V) **22a** (TaT)- **B** (DNA) hybrids.

methylamide insertions caused a lowering of the T_m by only 2° C. This slight destabilization by the methyl groups is not significant in view of the lipophilicity they are expected to impart. Both, H-amide sequence **20a**, and methylamide sequence **22a**, were found to be capable of forming duplexes with complementary DNA at only slightly lower temperatures than the normal DNA sequence **A**.

Single strands of **20a** or **22a** showed no hypochromicity under identical denaturation conditions. Both curves were linear with less than a 1 % rise over a 20° to 90° C temperature range.

As was mentioned previously, we also wanted to test whether the amide substitutions affected the Watson-Crick base pair specificity. Complementary RNA sequence **D**, containing one uridine instead of adenosine in the middle, was used to determine the extent of destabilization caused by the mismatch. We observed a 10° C lowering of the melting temperature for the normal DNA-RNA duplex, which was as

expected. The decrease in melting temperature for the TaT-modified sequence was 7° C, while the TmaT oligomer was destabilized by 5° C. The pertinent T_m values are shown in Table 2.

Table 2. Hybridization data.

Sequences	T_m (°C)[a] (DNA **B**)	T_m (°C)[a] (RNA **C**)	T_m (°C)[a] (mismatched RNA **D**)
A (normal DNA)	74.2	67.9	57.1
20a (TmaT)	62.8	65.8	60.4
22a (TaT)	65.7	66.8	60.0

a) experimental error is ± 0.5° C.

Both sequences **20a**, and **22a** exhibited stronger binding to RNA than to DNA and this is consistent with the results of the modeling studies that were performed at Ciba-Geigy (Section 2.6). They had found the lowest energy conformation of the amide-linked fragment, taken from the DNA-RNA duplex, to be in the A form. It is well known that RNA in DNA-RNA hybrids exist in the right-handed helical A form, due to conformational restrictions imposed by the 2' hydroxyl group. It follows that the similarity in geometry between RNA and the amide sequences should be conducive to binding. The small loss in hybridization strength to DNA is harder to explain. The interplay between base stacking, hydrogen bonding and conformational mobility is complicated. Given the relative stiffness of the amide backbone, the onus is on the sugar and the base to adopt the right geometry for binding to DNA. Any deviation could negatively affect the stability of the duplex.

The planned mismatch does not destabilize the modified duplexes quite to the same extent as the normal one. This may be caused by the rigidity of the amide bond, which

prevents the effects of the mismatch from further contorting the backbone. To determine whether hybridization specificity is affected, it would be necessary to conduct further binding studies with longer RNA oligomers, containing the right sequence, as well as the mismatch.

Concerning the structure of the TmaT oligomer, the same, previously mentioned, Ciba-Geigy study indicated that there was indeed sufficient room around the backbone to accomodate the methyl group, or even the bulkier isopropyl group, without changing the shape of the helix. It is not known at this time whether the methylamides are totally in the trans conformation when they are part of a RNA-DNA duplex. Our crude modeling results suggest that the cis conformation also leads to the right binding geometry. It is also conceivable that the free energy change due to the base stacking interactions is enough to overcome the energy difference between the cis and trans rotamers.[44]

[44]Chen, S.-M.; Mohan, V.; Kiely, J.S.; Griffith, M.C.; Griffey, R.H. *Tetrahedron Lett.* **1994**, *35*, 5105.

2.10 Preparation of amide-linked DNA homopolymers.

After establishing the excellent hybridization properties exhibited by mixed phosphate- and amide-linked DNA, we were interested in the synthesis of amide-linked thymidine homopolymers. The preliminary work on the synthesis of a homo thymidine dimer had been done by J.-F. Lavallée in our laboratory and is shown in Figure 12. The momomeric units were prepared by total synthesis.[22] We intended to synthesize the monomer precursors according to Schemes 1, 2 and 3, outlined in Section 2.2.

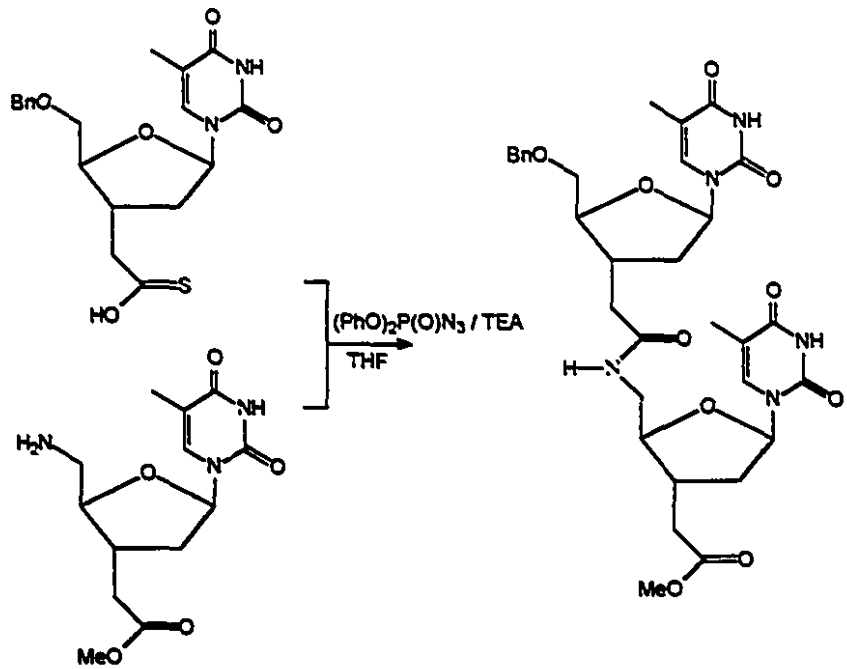
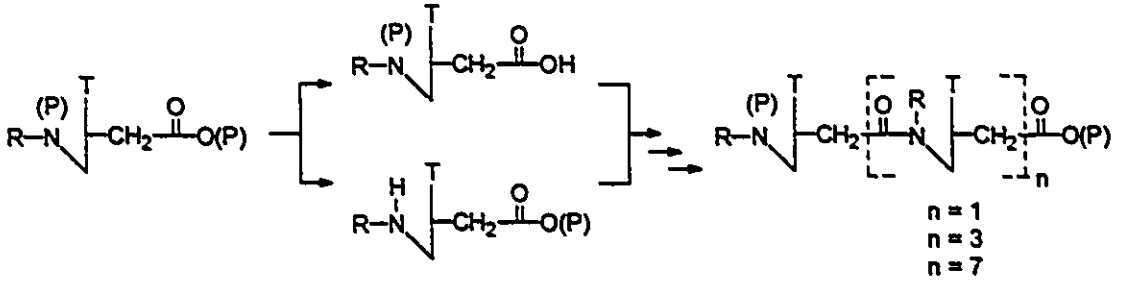


Figure 12. Lavallée synthesis of amide-linked thymidine dimer.

Before we started the homopolymer synthesis, we carefully examined our goals and options. Although we were planning to make a DNA analogue, the coupling and protection methods involved fall in the realm of peptide chemistry. It was not unreasonable to consider making an amide-linked DNA polymer by solid phase peptide synthesis.[45] The advantages of this method, such as the ease of purification and higher yields, are well known. Solution chemistry on the other hand is often preferred for smaller

peptides. It was previously determined that a minimum of an octamer was needed to conduct significant binding studies.[46] Since this was a relatively small polymer, we felt it did not warrent the use of solid phase synthesis. Instead, we decided that the shortest, most convenient way, shown in Scheme 13, would be a block synthesis, going from monomer to dimer, couple the dimers to make the tetramer, etc.

Scheme 13



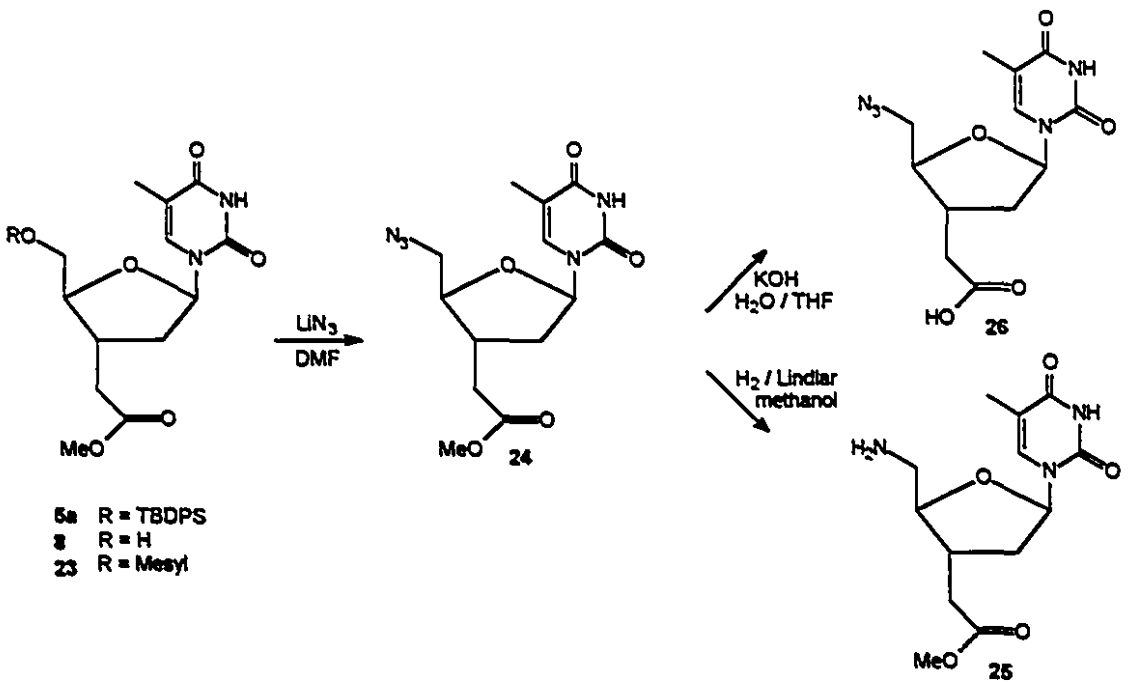
This approach required the use of 5' amino and 3' carboxyl protecting groups that could be selectively removed. For the preparation of the H-amide sequence the 5'-azido-3'-carbomethoxymethyl-5'-3'-dideoxythymidine **24** (Scheme 14) was a suitable monomeric unit. The azide moiety can be selectively reduced to the amine and is not affected by alkaline hydrolysis. The synthesis of the methylamide monomer and the choice of protecting groups proved to be more complicated, and will be discussed later.

As starting material for the H-amide we could make use of 5' silyl-protected 3'-carbomethoxymethyl-3'-deoxythymidine **5a**, made according to the methods described in Section 2.2, and further derivatize the 5' position by partially following J.-F. Lavallée's methods. The first step, as depicted in scheme 14, was the removal of the 5' silyl group from compound **5a** by tetrabutylammonium fluoride in THF, which yielded 3'-carbomethoxymethyl-3'-deoxythymidine **8**. This was followed by the mesylation of the

[45]Merrifield, B. *Science* **1985**, *232*, 341.
[46] Sugimoto, N.; Sasaki, M. *Nucleosides Nucleotides* **1992**, *11*, 515.

freed 5' hydroxyl group, in preparation for displacement by the azide ion, giving monomer **24** in excellent yield.[47]

Scheme 14



Azide ester **24** was hydrogenated in methanol solution at atmospheric pressure in the presence of Lindlar catalyst, giving amine **25** in quantitative yield. An equal amount of compound **24** was subjected to alkaline hydrolysis in a mixture of 10 % aqueous KOH and THF. Acid **26** was obtained by extraction with methylene chloride, after the reaction mixture was acidified to pH~3.

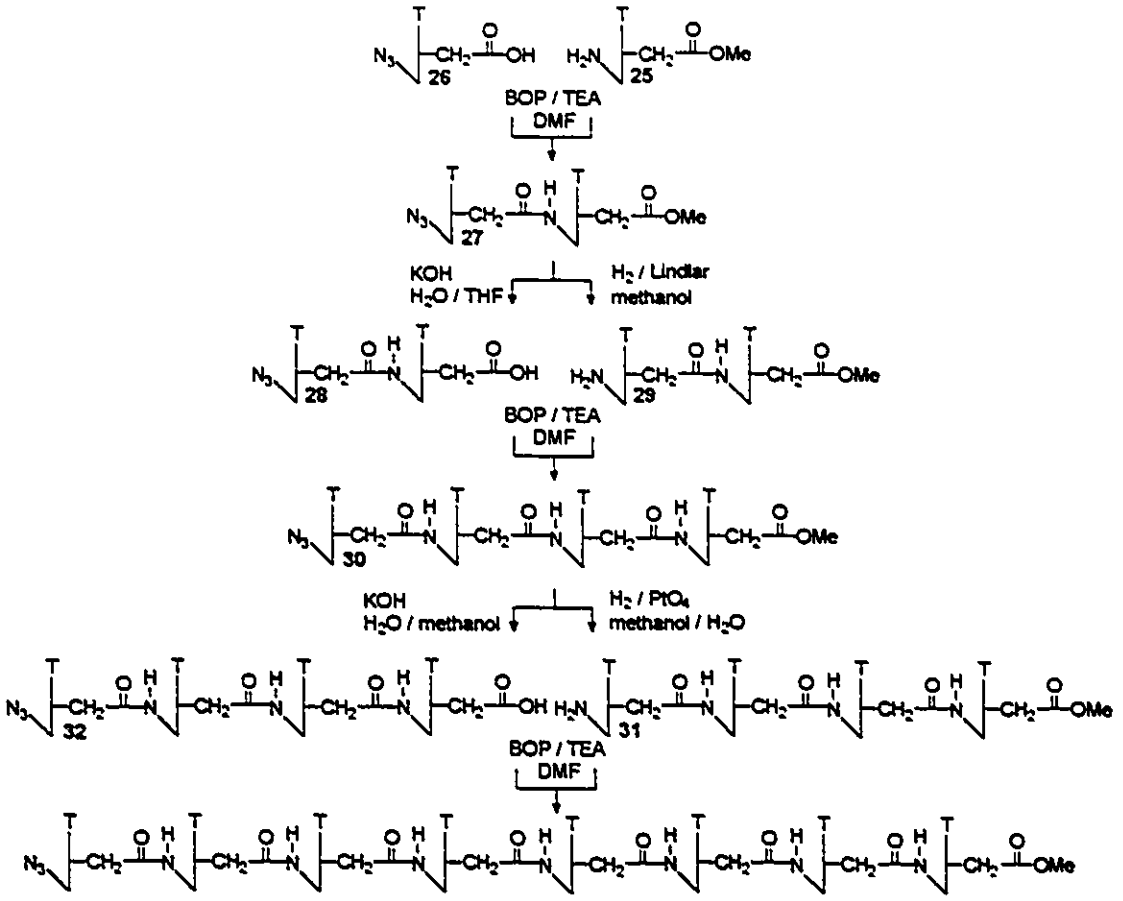
The attempted octamer synthesis is shown in full in Scheme 15.

The condensation of acid **26** and amine **25** was effected with BOP coupling reagent in DMF. The same procedure was followed as for the formation of the TaT **16** and TmaT **18** dimers. Homo dimer **27** was isolated after chromatographic purification in 87 % yield.

The hydrogenation and hydrolysis steps were repeated for dimer **27**. The

[47] Compounds **23**, **24** and **25** were prepared by J.-F. Lavallée's methods.

Scheme 15



hydrogenation conditions and reaction time were exactly the same as for the monomer. The reaction was monitored by TLC for the disappearence of the starting material and the formation of a new, more polar spot which tested positive to ninhydrin.

Dimer acid **28**, which was generated the same way as monomer acid **26**, could not be extracted from the aqueous hydrolysis solution. Therefore, after adjusting the pH to approximately 6, the reaction mixture was evaporated to dryness and the dimer acid was used without further purification for the next coupling step.

The reaction conditions for the tetramer formation were the same as those for the dimer synthesis. The extra salt concentration from the crude dimer acid did not adversely affect the coupling. Tetramer **30** could be chromatographically purified, although it was

evident from the NMR spectrum that a small amount of triethylamine salt co-eluted with the tetramer.

The monomer, dimer and tetramer were all characterized by ^{1}H, ^{13}C and HMQC 500 MHz NMR experiments. Depicted in Figure 13 are the low-field regions of the proton spectra. They clearly show the presence of single, double and quadruple signals for the monomer, dimer and tetramer H6 protons, respectively. The number of anomeric protons is also easily determined from the spectra. There is a difference in shift between the H1', belonging to the 'bottom' unit and the 'top' H1' protons. The 'bottom' one is more deshielded than the other ones, which completely overlap for the tetramer. The integration of the anomeric protons is consistent with the proposed structures. The very crowded high-field region shows overlapping signals for the sugar protons, unfortunately preventing us from analyzing the sugar conformations. An absolute comparison of the chemical shifts was also not possible because of the necessity to use different solvents. Interestingly, the solvent mixture used for the tetramer (methylene chloride / methanol, 5 / 1) was one that was often effective in dissolving very polar nucleoside analogues, that were neither soluble in methylene chloride, nor in methanol, but only in high-boiling solvents, such as DMF and DMSO.

Unlike monomer **24** and dimer **27**, tetramer **30**, as we mentioned, was not soluble in methanol, so a different solvent was required for the hydrogenation of this compound. A mixture of water and methanol (1 / 1) was found to be a suitable solvent system for the dissolution and the hydrogenation of the tetramer, but the product of this reaction precipitated out of solution, which made separation from the catalyst impossible. We felt that protonation of the 5' amino group would make this compound more soluble. However, the addition of an equimolar amount of 0.1 M HCl after the hydrogenation was finished, resulted in the decomposition of the calcium carbonate based Lindlar catalyst. This complicated the work-up of the reaction.

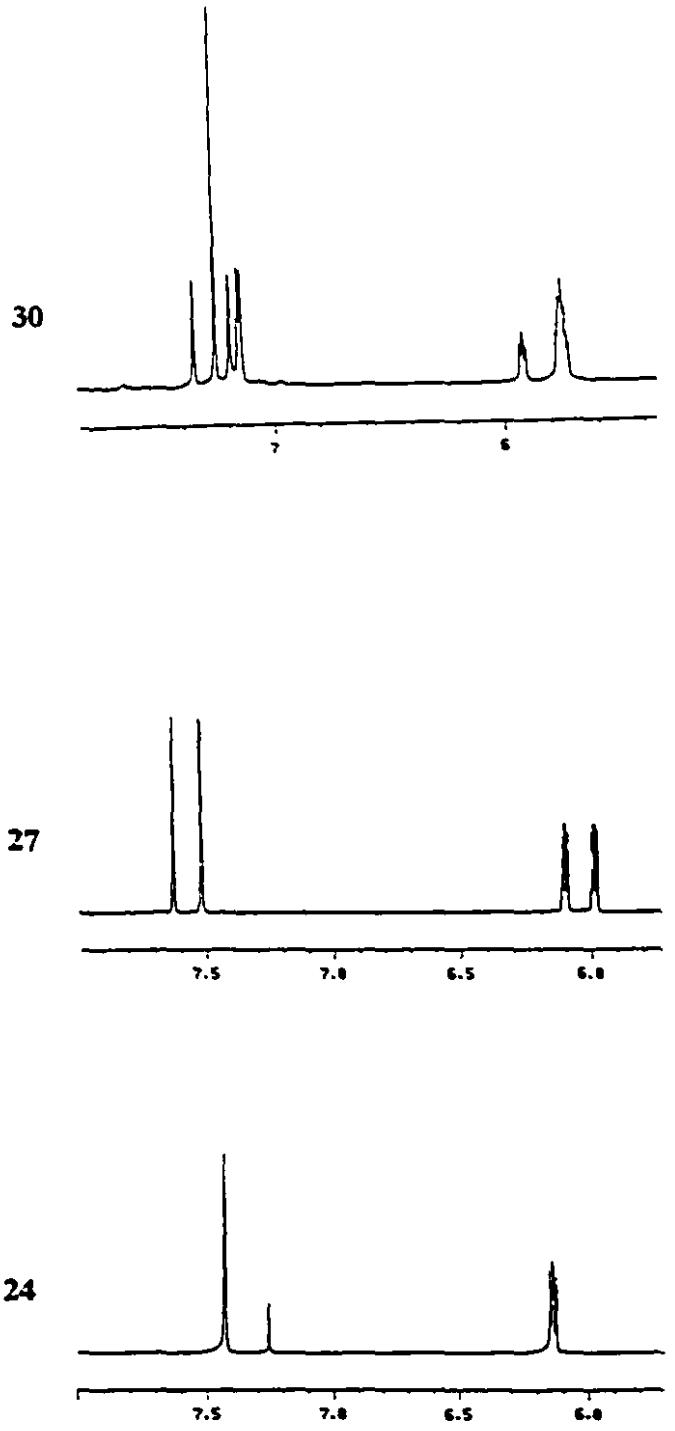


Figure 13. Parts of the ^1H NMR spectra of monomer **24** ($CDCl_3$), dimer **27** (CD_3OD), and tetramer **30** ($CDCl_3$ + CD_3OD), showing the signals for H1' (around 6 ppm) and H6 (between 7 and 8 ppm).

The solution to this problem was a change of catalyst, which, in turn, necessitated a change in the other hydrogenation conditions. The tetramer solution was thus shaken with platinum(IV) oxide, under 40 lbs hydrogen pressure, for two hours, while the reaction was monitored by TLC, in the same way as for the dimer hydrogenation reaction. The subsequent acidification brought the tetramer amine **31** into solution, and it could then be cleanly separated from the catalyst.

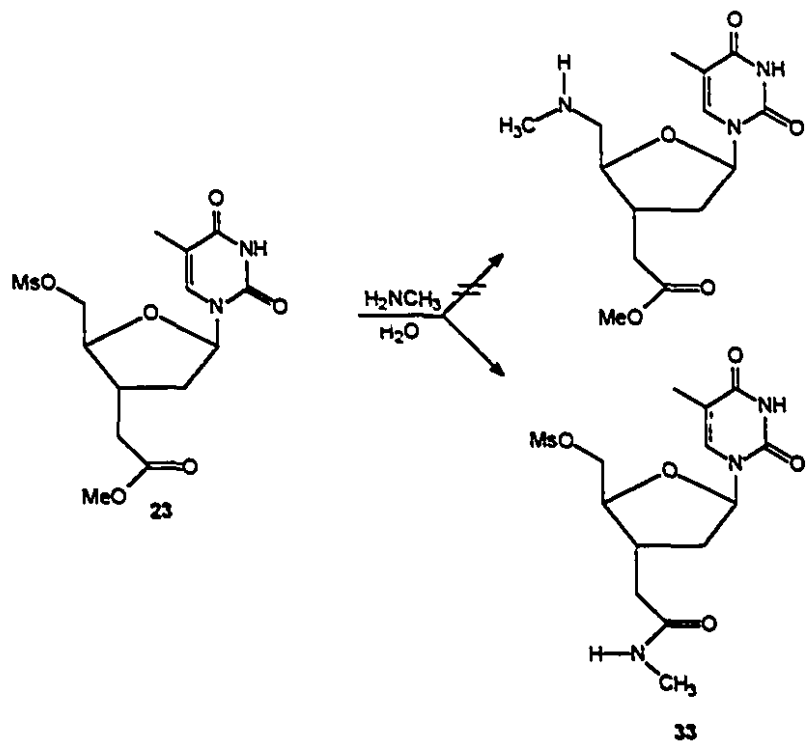
The hydrolysis procedure for the tetramer was similar to that for the dimer, with a few minor modifications. Methanol was sustituted for THF to help bring the tetramer in solution and, furthermore, the reaction required a substantially longer time to go to completion. Like the dimer acid, tetramer acid **32** could also not be extracted from aqueous solution and was used for coupling without further purification.

The reaction conditions for the final condensation of tetramer amine **31** with tetramer acid **32** were the same as for the previously described monomer and dimer amide couplings. The possible formation of the desired octamer was observed on TLC, but could not be confirmed spectroscopically, as this compound proved to be insoluble in a wide range of solvents. The lack of solubility unfortunately precluded us from conducting binding studies.

Simultaneously we had worked on designing a scheme for the synthesis of the methylamide-linked thymidine homopolymer. We wanted to follow the same dimer to tetramer to octamer strategy, since that was the shortest synthetic pathway. As mentioned before, this required us to find protecting groups for the amino and carboxylic acid functionalities that could be selectively removed under conditions that were compatible with nucleosides. Our first choice was benzyloxycarbonyl (Cbz) protection for the amino group, frequently used in peptide synthesis. The Cbz protecting group is removable by catalytic hydrogenation and resistant to alkaline hydrolysis. This last property would enable us to selectively hydrolyze the methyl ester in the 3' position.

Before we considered protection, however, we needed to introduce the methylamino group in the 5' position. The easiest way would be a direct displacement of the 5' mesyl group of compound **23**, which we had in hand, with methylamine, in analogy to the method we used for the preparation of 5'-methylamino-5'-deoxythymidine **12**. We were not really surprised when this reaction turned out to be the aminolysis of the 3' methyl ester instead of the desired 5' mesyl displacement. (Scheme 16)

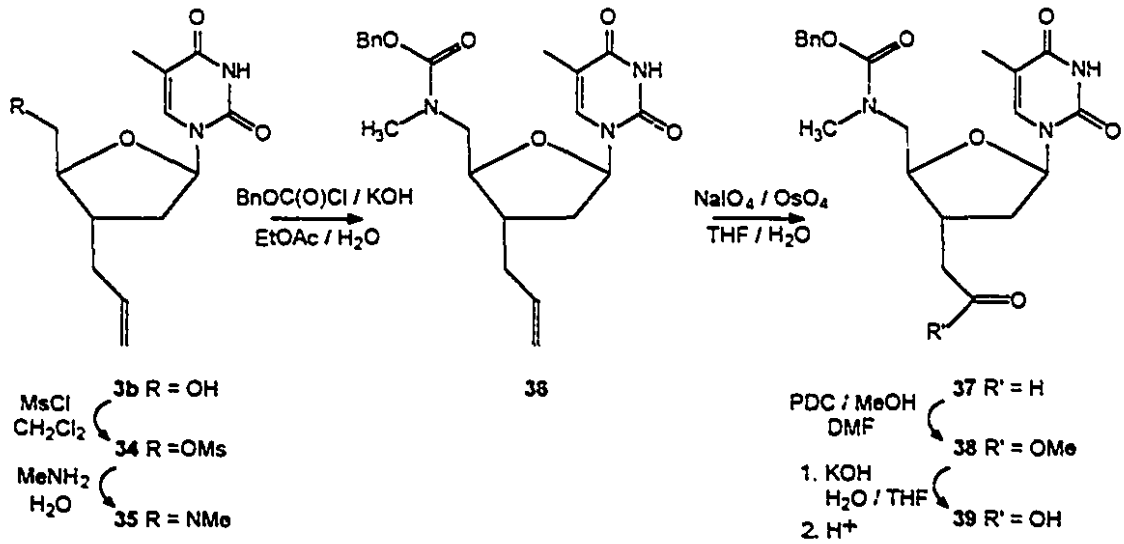
Scheme 16



It was obvious that the 5' methylamino group had to be in place before the introduction of the 3' methyl ester functionality. That meant going back to 3'-allyl-3'-deoxythymidine **3b** as starting material. (Scheme 17)

The mesylation of the 5' hydroxyl group of compound **3b** was a clean reaction and pure 5' mesylate **34** was obtained in 94 % yield. Heating the mesylate in 40 % aqueous methylamine for six hours gave 5'-methylamino-3'-allyl-5',3'-dideoxythymidine **35** after chromatographic purification.

Scheme 17



The reaction time was considerably longer than for the formation of 5'-methylamino-5'-deoxythymidine **12**.

Methylamine **35** was then reacted with benzyl chloroformate under Schotten-Baumann conditions to give Cbz-protected methylamine **36**. This reaction was followed by oxidation of the allyl moiety to the aldehyde by sodium periodate in the presence of a catalytic amount of osmium tetraoxide in a two-phase system. This reaction was similar to the oxidation of 5'-*O*-TBDPS-3'-allyl-3'-deoxythymidine **3a**, but required a different solvent system, since methylamine **36** was not soluble in ether. THF / water was successfully substituted for ether / water. This reaction was five times faster than the one which involved compound **3a** and also gave a higher yield. A better contact between the reacting species could be the reason for this improvement. Aldehyde **37** was further oxidized to methyl ester **38**, under the same conditions used for the preparation of methylester **5**.

Cbz-protected compounds **36**, **37** and **38** all existed in solution as a three to one mixture of rotamers, due to the hindered rotation of the 5' carbamate bond.

With the synthesis of methyl ester **38** completed, we had in hand the protected monomeric unit from which we thought to make the targeted methylamide-linked DNA octamer.

The next two steps were the alkaline hydrolysis of the ester function, while an equal amount of compound **38** was subjected to catalytic hydrogenation. The hydrolysis and subsequent acidification with weakly acidic resin yielded acid **39** in quantitative yield. The palladium catalyzed hydrogenation, however, was not a clean reaction. The yield of pure product was only 58 %. We did not find this acceptable in view of the fact that the hydrogenation step would have to be repeated for the dimer and the tetramer.

Our search for more suitable protection for the amino group led us to consider the 9-fluorenylmethoxycarbonyl (Fmoc) group.[48] It has the advantage that it is very easy to remove under mild alkaline conditions. It is extensively used in solid phase peptide synthesis for that very reason, but also because the fluorene chromophore that is released upon deprotection is highly UV-active. This provides a means of monitoring peptide coupling efficiencies.[49]

We expected the Fmoc chemistry to be compatible with our nucleosides, but we also had to consider the deprotection of the 3' carboxylic acid functionality. The alkaline hydrolysis of the 3' ester would likely result in partial or total loss of the Fmoc group.Therefore we looked at alternative methods for the ester deprotection. Apart from the alkaline lability of the Fmoc due to the acidic 9-fluorenyl proton, there was the carbamate functionality to consider. Most non-hydrolytic ester cleaving reagents, such as trimethylsilyl iodide[50], are known to cleave esters under non-alkaline conditions, but they also attack carbamate bonds. Thus we needed a reagent that was chemoselective towards esters in the presence of carbamates.

Bis(tributyltin) oxide (BBTO) has recently been successfully used to cleave esters

[48] Carpino, L.A.; Han, G.Y. *J. Org. Chem.* **1972**, *37*, 3404.
[49] Angell, Y.M.; García-Escheverría, C.; Rich, D.H. *Tetrahedron lett.* **1994**, *35*, 5981.
[50] Olah, G.A.; Narang, S.C. *Tetrahedron* **1982**, *38*, 2225.

and was reported to be tolerant of a wide range of other reactive functional groups.[51] Although carbamates were not mentioned, we felt that BBTO might be selective enough to suit our purpose.

Mata and Mascaretti proposed the following mechanism for the ester cleavage.[52] (Figure 12)

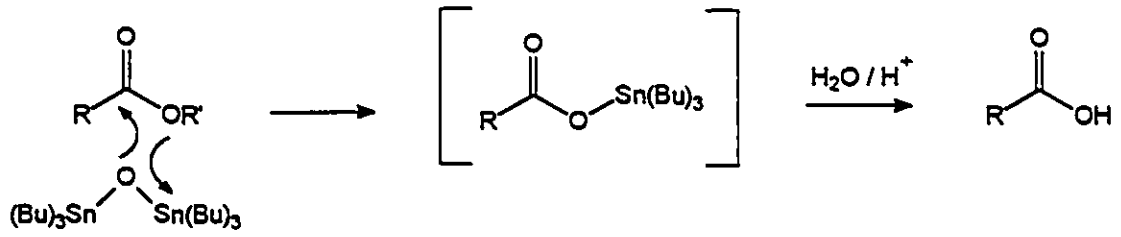
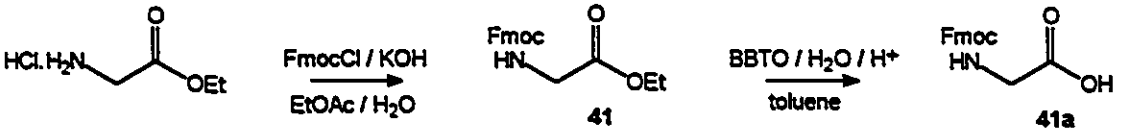


Figure 12. Mechanism for bis(tributyltin) oxide mediated ester cleavage.

The oxygen, its nucleophilicity enhanced by the electron-rich tributyltin moieties, attacks the ester carbonyl group to form the unstable organo-tin ester intermediate. The free carboxylic acid is liberated during the mildly acidic work-up. Addition of AIBN did not increase the reaction rate, therefore it was concluded that no radical mechanism was involved.

Nothing in this reaction mechanism contraindicated its use in connection with nucleosides. We felt it looked promising enough to warrant a small model study to determine if an ester could be selectively deprotected in the presence of a Fmoc group.

Scheme 18



The ethyl ester of glycine.HCl was first reacted with Fmoc chloride under Schotten-Baumann conditions, to yield the corresponding *N*-Fmoc-protected ethyl

[51] Salomon, C.J.; Mata, E.G.; Mascaretti, O.A. *Tetrahedron Lett.* **1991**, *32*, 4239.
[52] Mata, E.G.; Mascaretti, O.A. *Tetrahedron Lett.* **1988**, *29*, 6893.

glycinate **41**. (Scheme 18) A toluene solution of this compound was subsequently heated under reflux with BBTO for 6 hours. After acidification with 0.5 M HCl and the usual extraction-purification procedures, the free carboxylic acid **41a** was isolated in 70 % yield. This yield was not optimized. The reaction was monitored by TLC for the loss of the Fmoc group, which, if present, shows as an intense blue fluorescent spot. No loss of Fmoc was observed.

After we obtained this positive result, we went ahead with the synthesis of the Fmoc protected monomer **44**. This compound was generated under the same conditions as the Cbz-protected monomer **38**. (Scheme 17) The syntheses of the *N*-Fmoc protected aldehyde **43** and of the *N*-Fmoc ester **44** gave higher yields than the corresponding Cbz-protected compounds (75 % versus 80 %, and 87 % versus 98 %). After the introduction of the Fmoc group, all compounds existed in solution as rotamers in a two to one ratio.

The next two steps were the removal of the Fmoc group and the selective ester deprotection, followed by the formation of the targeted methylamide homo dimer. (Scheme 19)

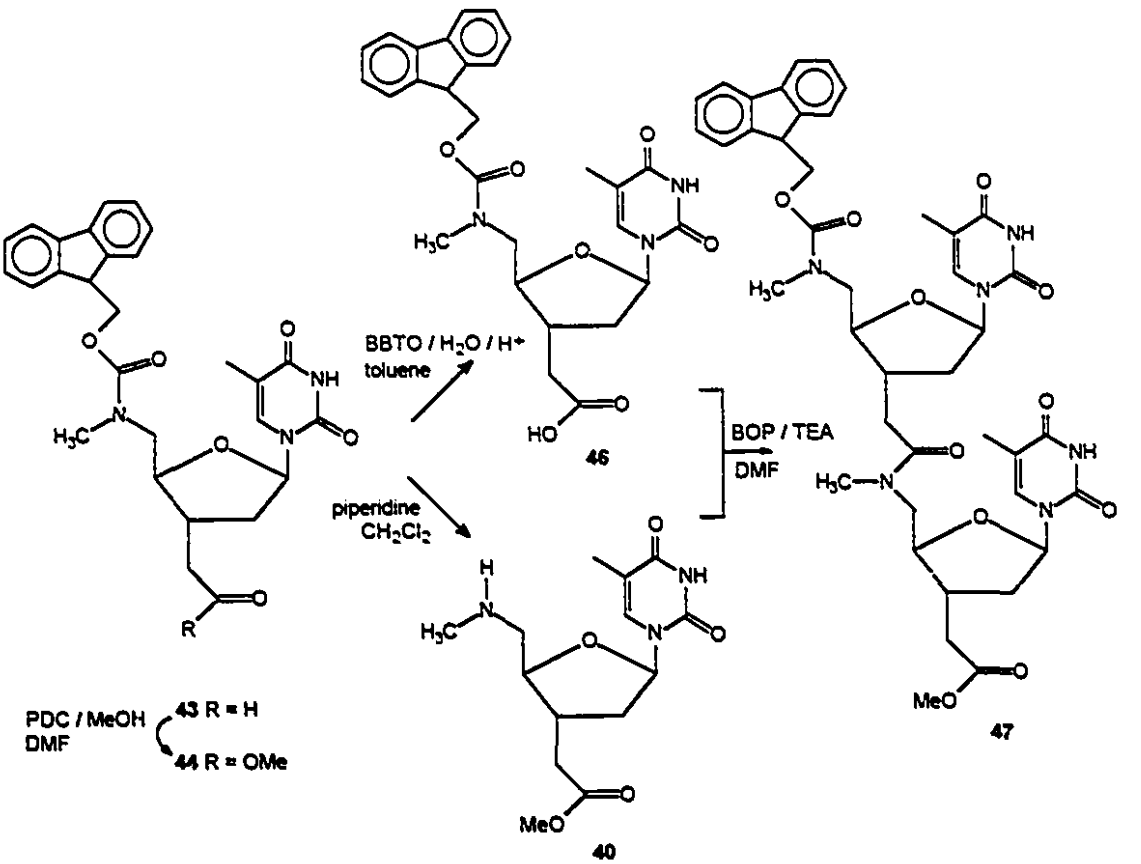
The usual reagents for the Fmoc deblocking reaction are unhindered secondary amines, for instance piperidine, which can form an adduct with the initially generated dibenzofulvene. This adduct is soluble in a variety of solvents and easily removed during the purification work-up of the reaction, whereas the dibenzofulvene by itself polymerizes easily and becomes insoluble.[53]

N-Fmoc ester was stirred for 10 minutes in a methylene chloride / piperidine (4 / 1) mixture, after which the solvent and the piperidine were removed under reduced pressure. Chromatographic purification yielded 80 % of the free methylamine **40**, as a hard foam.

The 3' ester functionality could indeed be selectively deprotected by reacting

[53] Carpino, L.A. *Acc. Chem. Res.* **1987**, *20*, 401.

Scheme 19



ester 44 with BBTO in toluene solution at 90° C for 10 hours. The reaction mixture was acidified with 0.5 M HCl, causing a white preciptate to form, which was subsequently dissolved in ethyl acetate. This solution was extracted with saturated sodium bicarbonate, the aqueous layer was reacidified and the resulting white precipitate filtered off to give carboxylic acid 46 in 50 % yield. The yield could be optimized to 68 % by further extracting the aqueous layer with ethyl acetate, but a small amount of impurities was co-extracted. No loss of Fmoc was observed.

The methylamide homodimer 47 was obtained from methylamine 40 and carboxylic acid 46 in a BOP- mediated coupling reaction in a manner analogous to the formation of the H-amide homo dimer 27. The ^{1}H NMR was highly complex with multiple overlapping signals for the two anomeric protons. This indicated that there were the

expected four rotamers in solution. The molecular weight was confirmed by high resolution mass spectrometry.

At this stage of our work, the lack of solubility of the H-amide octamer had become apparent and we decided to focus our attention on solving this problem, rather than continue the synthesis of the methylamide homopolymer sequence.

2.11 Increasing the homopolymer solubility.

The poor solubility of certain modified non-phosphate, non-ionic backbone oligonucleosides was previously reported by other research teams. In 1988 Ogilvie and Cormier had prepared a diisopropylsilyl-bridged thymidine hexamer.[54] (Figure 14) It was not possible to determine the binding properties of this hexamer because it was not sufficiently soluble in water.

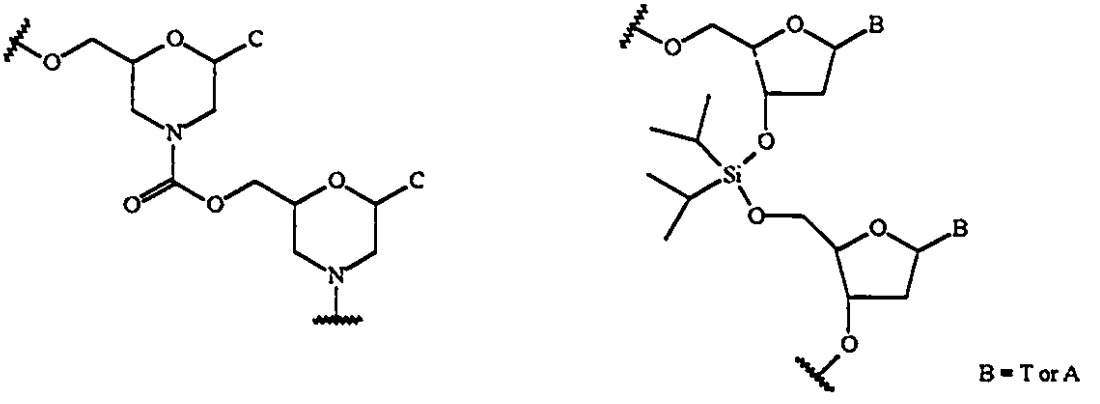


Figure 14. Stirchak's morpholino and Ogilvie's siloxane hexamers.

Similarly, the novel cytidine morpholinocarbamate hexamer, also shown in Figure 14, which was synthesized by Stirchak and Summerton had a solubility of only 4 μM in a pH 7.5 aqueous buffer.[55] Solubility was increased by covalently attaching a poly(ethylene glycol) tail, M.W.1000, to the the hexamer.

Poly(ethylene glycol), usually referred to as PEG, is a biocompatible polymer. It has been used extensively during the last 20 years, among other things, to modify biologically important molecules. PEG is known to enhance certain desirable pharmacological properties, such as water solubility and cell wall penetration.

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[54] Ogilvie, K.K.; Cormier, J.F. *Nucleic Acids Res.* **1988**, *16*, 4583.
[55] Stirchak, E.P.; Summerton, J.E. *Nucleic Acids Res.* **1989**, *17*, 6129.

This was sufficient reason for us to further investigate the possibility of using PEG to increase the solubility of the H-amide linked thymidine homopolymer, described in section 2.10.

2.12 An introduction to poly(ethylene glycol).

PEG is a polyether diol, commercially available in a wide range of molecular weights. Its general formula is $HOCH_2CH_2(OCH_2CH_2)_nOH$. The two hydroxyl end groups provide convenient handles for chemical modification. In many instances, however, it is preferable to have only one reactive site, in which case the monomethyl ether, $MeOCH_2CH_2(OCH_2CH_2)_nOH$ (mPEG) is a better choice. Both, PEG and mPEG are prepared from ethylene oxide by anionic polymerization. The lower molecular weight PEGs (M.W.<1000) are viscous liquids. As their molecular weight increases, the polymers become waxy solids with increasing melting points that start to level out at ~ 67° C.

PEG has many interesting physical properties, which makes it very useful for industrial, as well as biomedical and biotechnical purposes.[56] The most striking is PEG's unique solubility behaviour. It is amphiphilic, meaning that it is soluble in water, as well as many organic solvents, such as toluene and methylene chloride. It is, however, totally insoluble in diethyl ether and hexane.

The higher molecular weight PEGs exist in solution in a helical form, the oxygen atoms pointing inwards, making a cavity, the size of which is related to the length of the molecule. PEG can thus selectively form complexes with cations, similar to crown ethers, and has found application as a transfer catalyst.[57]

PEG in aqueous solution is a highly mobile, heavily hydrated molecule with a large exclusion volume. It can form two-phase systems with aqueous solutions of other polymers, making it an extremely useful tool for the purification of biopolymers. PEG becomes insoluble, however, at higher temperatures. It possesses a lower consolute temperature of ~ 100° C, the exact temperature depending on factors, such as molecular

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[56] Harris, J.M., Ed., *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* **1992**, Plenum Press, New York.
[57] Harris, J.M.; Hundley, N.H.; Shannon, T.G.; Struck, E.C. *J. Org. Chem.* **1982**, *47*, 4789.

weight, concentration and pH. The cloud point of a PEG solution can be lowered by the addition of electrolytes.

PEG's physical properties, especially its behaviour in aqueous solution, have lead to its rapidly growing pharmaceutical use.

It is non-toxic at molecular weights above 400. Lower molecular weight PEG (less than 350 Dalton) were reported, however, to be toxic due to oxidation by alcohol dehydrogenase (ADH), leading to diacid and hydroxyacid metabolites.[58] The authors further suggested that ethanol would appear to be an excellent antidote, because of its potential as a competitive ADH inhibitor.

The larger PEG molecules are not metabolized by living organisms and are readily excreted, unchanged.[59]

PEG has been extensively used for conjugation to a variety of biologically important substrates, ranging from small molecules, such as aspirin and antibiotics, to large peptides and proteins. The PEG conjugation is responsible for altered pharmacokinetics; for instance, the increase in bulk reduces the rate at which a drug is cleared through the kidneys. The following example, which involves the enzyme superoxide dismutase as an effective scavenger of superoxide ions, illustrates this phenomenon. Superoxide ions have been implicated in pathogenic states associated with inflammation, burns, and kidney transplants. The use of superoxide dismutase as a therapeutic agent is, however, severely limited by its rapid clearance from the body. The attachment of 18 molecules of mPEG, molecular weight 5000, per enzyme molecule, resulted in an increase of the enzyme's half-life in the blood of rats from 6 min to 25 h, with a proportional rise in antiinflammatory activity.[60]

PEG is known to impart increased water solubility to its conjugates. For instance,

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[58] Herold, D.A.; Keil, K.; Bruns, D.E. *Biochem. Pharm.* **1989**, *38*, 73.
[59] Yamaoka, T.; Tabata, Y.; Ikada, Y. *J. Pharm. Sci.* **1994**, *83*, 601.
[60] Fuentges, F.; Abuchowski, A. *J. Controlled Release* **1990**, *11*, 139.

taxol, a natural product shown to be effective in the treatment of certain cancers, is poorly soluble in water. In order to solve this problem, mPEGs, molecular weights ranging from 350 to 5000, were covalently attached by urethane or carbonate linkages to the taxol molecule.[61] The solubilities of the conjugates (1.8×10^{-3} *M* for the lower molecular weight to 0.1 M for the higher weight) were found to be greatly increased in comparison to that of unmodified taxol. The PEG conjugation did not affect the cytotoxicity of the taxol.

PEG's well-documented ability to interact with cell membranes may be due to its lipophilic character and is of great importance for facilitating transport of drugs into cells.[62] The process by which this occurs is not well understood at this moment. Direct penetration is not likely in view of the facts that cell membranes have an aliphatic core and PEG is insoluble in alkanes. However, PEG can associate with the phospholipid headgroups and this favourable interaction may be followed by endocytosis.[63]

Nearly twenty years ago Abuchowski *et al.* made the significant discovery that covalent attachment of PEG to a protein renders that protein nonimmunogenic and nonantigenic, but does not result in a significant loss of biological activity.[64] This observation has prompted much research into PEG-modified proteins and peptides. It has since become apparent that other negative drug side effects can also be minimized by PEG conjugation. It was found to lessen drug uptake by the liver and the spleen[65] and to reduce thrombogenicity.[66]

Finally, PEG has been proven to be an effective soluble polymeric support for the

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[61] Greenwald, R.B.; Pendri, A.; Bolikal, D. *J. Org. Chem.* **1995**, *60*, 331.
[62] Beckman, J.S.; Minor, R.L.; White, C.W.; Repine, J.E.; Rosen, G.M.; Freeman, B.A. *J. Biol. Chem.* **1988**, *263*, 6884.
[63] Yamazaki, M.; Ito, T.: *Biochemistry* **1990**, *29*, 1309.
[64] Abuchowski, A.; van Es, T.; Palczuk, N.C.; Davis, F.F. *J. Biol. Chem.* **1977**, *252*, 3578.
[65] Merrill, E.W. See Reference 56, Ch.14, 199.
[66] Woodle, M.C.; Lasic, D.D. *Biochim. Biophys. Acta* **1992**, *1113*, 171.

syntheses of peptides, oligosaccharides and oligonucleotides.

Royer and Ananthramaiah reported the use of the carboxylic acid derivative of mPEG, molecular weight 6000, as a support for synthesis of a tripeptide.[67] The growing chain was elongated from the carboxyl end and the reactions were performed in aqueous solution. The final cleavage of the peptide from the PEG support was mediated by enzymes. Solution chemistry methods, such as extraction, were used throughout the synthesis. Other researchers have successfully prepared PEG-bound octapeptides in organic solution, with the elongation occurring from the amino end.[68] The first amino acid was attached to the mPEG hydroxyl end group via an ester linkage and the finished octapeptide was easily freed from the PEG by hydrolysis. Stepwise monitoring of the reactions was feasible, because spectroscopic methods could be used while the polymer support was still attached to the growing chain. Membrane filtration and recrystallization were used for the purification of the peptides.

In a recent publication Krepinsky and co-workers described the synthesis of an oligosaccharide, using PEG 5000 or 10000 as polymeric support.[69] D-Mannopentose, a cell component of pathogenic yeasts, was prepared in good yield by this method. The growing sugar chain was attached to mPEG via p-dioxyxylyl diether, which allowed a selective, two step, detachment of the finished product from the PEG support. Mild catalytic hydrogenation cleaved off the PEG, leaving a *p*-tolylmethyl group on the D-mannopentose, while more vigorous hydrogenation conditions completely liberated the oligosaccharide from the PEG and the diether linker.

Bonora *et al.* used mPEG 5000 as soluble supporting polymer for the synthesis

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[67] Royer, G.P.; Ananthramaiah, G.M. *J. Am. Chem. Soc.* **1979**, *101*, 3394.
[68] Bayer, E. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 113.
[69] Douglas, S.P.; Whitfield, D.M.; Krepinsky, J.J. *J. Am. Chem. Soc.* **1995**, *117*, 2116.

of short strands of DNA.[70] The first nucleoside unit was attached to the PEG at the 3' position by a succinate linkage, similar to solid phase nucleotide synthesis. An octamer was prepared in 97 % purity, with coupling yields of >90%. This method of nucleotide preparation is more economical than solid phase synthesis, as it requires significantly smaller quantities of reagents. It also makes it feasible to do larger scale reactions. In view of the current interest in antisense for therapeutic purposes, this is an important advantage.

[70] Bonora, G.M.; Biancotto, G.; Maffini, M.; Scremin, C.L. *Nucleic Acids Res.* **1993**, *21*,1213.

2.13 Synthesis of 3' ester linked mPEG-nucleoside conjugates

After reviewing the literature on poly(ethylene glycol), we decided that we could use PEG to try and solubilize amide-linked nucleoside homopolymers. At the same time PEG could serve as a soluble solid support for the synthesis of the strands.

Any misgivings we had about the PEG conjugation causing a loss of binding of the homopolymers to RNA, were dispelled by a publication by Jäschke *et al.*[71] They reported that PEG conjugation to normal DNA had little effect on the hybridization behaviour. Melting temperatures were very close to those involving unmodified duplexes.

Automated solid phase synthesis of biopolymers eliminates the need for manual purification and provides a means to quickly obtain a desired compound. Solution chemistry synthesis requires tedious and time consuming purification at every step. It has, however, certain advantages. It is easy to monitor the reactions by TLC and NMR and it can lend itself to large scale production.

PEG provides a means of combining the advantages of liquid and solid phase chemistry. PEG is soluble in water and in most organic solvents, with the exception of aliphatic hydrocarbons and diethyl ether, and it carries its conjugates with it into solution.

PEG derives its use as solid support from its insolubility in ether. The addition of ether to a reaction mixture, containing a PEG conjugate, will cause the PEG to precipitate out, leaving excess reagents and by-products in solution. Simple filtration is usually sufficient to separate the desired pure compound.[72]

Before we started the PEG-supported synthesis of amide-linked nucleosides, we needed to consider several options, concerning the PEG-nucleoside linkage. The primary amino nucleotide monomers **25** (5' amine, 3' methyl ester) and **26** (5' azide, 3' carboxylic

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[71] Jäschke, A.; Fürste, J.P.; Nordhoff, E.; Hillenkamp, F.; Cech, D.; Erdmann, V.A. *Nucleic Acids Res.* **1994**, *22*, 4810.

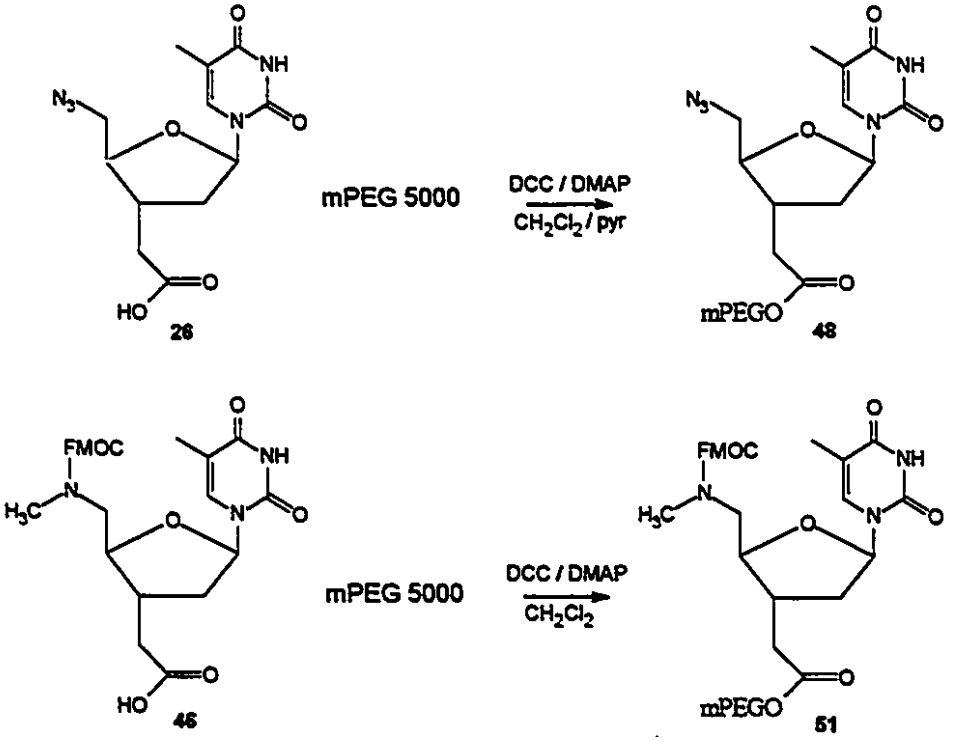
[72] Bonora, G.M.; Scremin, C.L.; Colonna, F.P.; Garbesi, A. *Nucleosides Nucleotides* **1991**, *10*, 269.

acid) or the methylamino monomers **40** (5' methylamine, 3' methylester) and **46** (5' Fmoc methylamine, 3' carboxylic acid), which we had in hand, could be attached to the PEG at the 5' amino or the 3' carboxyl end, respectively. For our initial studies an ester linkage at the 3' position was a practical choice, since we could use low cost, underivatized mPEG 5000 as starting material. A molecular weight of 5000 was chosen because it is the smallest size PEG that is solid and easy to manipulate at room temperature. For our purpose, using higher molecular weight PEGs had no advantage.

After attaching the first nucleoside unit to the support we planned to deprotect the 5' position, add the next unit and repeat these steps.

A slight excess of carboxylic acid **26** (1.2 equivalents), activated by 1,3-dicyclohexylcarbodiimide (DCC) was reacted with mPEG 5000 in the presence of DMAP in methylene chloride, with sufficient pyridine added to bring the carboxylic acid into solution.(Scheme 20)

Scheme 20



The reaction was monitored by TLC (methylene chloride / methanol, 5 / 1). After two hours no more unreacted PEG could be detected. In order to ensure that the reaction went to completion the reaction mixture was stirred for another hour.

The subsequent addition of ether to the mixture produced a voluminous, white precipitate that was easily filtered. No further purification was necessary.

Although the dicyclohexylurea, which was formed as a by-product during the reaction, is not soluble in ether, it did not precipitate out with the PEG conjugate. If the reaction mixture was added to the ether, rather than the reverse, the NMR spectrum indicated that the urea co-precipitated out.

Nucleoside-mPEG ester **48** was characterized by ^{1}H (Appendix 6) and ^{13}C 500 MHz NMR. The molecular weight was verified by matrix-assisted laser desorption / ionization time-of-flight (MALDI TOF) mass spectrometry, using a dithranol (1,8,9-anthracenetriol) matrix.(Appendix 10) The characterization of the mPEG conjugates will be discussed in more detail in Section 2.17.

The mPEG ester **51** of *N*-Fmoc protected methylamine nucleoside carboxylic acid **46** was synthesized the same way as mPEG ester **48**, except that no pyridine was needed to bring the acid **46** into solution and the reaction required less time. The NMR data indicated that mPEG ester **51** exists in solution as two rotamers in a ratio of 2 : 1. The rotamer ratio was the same as for the corresponding methyl ester **44**. Although the NMR data clearly confirmed the structure of the desired compound (Appendix 7), the MALDI mass spectrum, indicated the expected molecular weight minus the Fmoc group. Changing the matrix from dithranol to gentisic acid (2,5-dihydroxybenzoic acid) did not make any difference in the spectrum.

2.14 Reduction of the 5' azide function of the mPEG ester

With the first nucleoside units attached to the PEG support, the next step was the deprotection of the 5' amino functions. Unexpectedly, the clean reduction of the 5' azide function of mPEG ester **48** proved to be difficult. We initially used the same Lindlar-catalyzed hydrogenation method that was used for the azide reduction of the analogous methyl ester **24**. The reaction was considerably slower and the prolonged reaction time favoured the formation of degradation products that could not easily be separated. Changing the catalyst to platinum (IV) oxide did not bring improvement. Since the azide reduction reaction was part of a repeating cycle, we needed a better method.

A wide variety of reagents, such as lithium aluminum hydride, triphenylphosphine, tributyltin hydride, tin chloride etc., have been successfully used to reduce azides.[73] Most of the available reagents were expected to be compatible with nucleoside chemistry. The reduction of azido groups on modified nucleosides by tributyltin hydride / AIBN was reported by Samano and Robins.[74] When we subjected 5' azido mPEG ester **48** to the same reaction conditions, the formation of the free amine was observed by TLC (ninhydrin), but before the reaction went to completion, the amine partially decomposed, releasing mPEG. The same reaction, involving 5' azido methyl ester **24** gave the corresponding 5' amino methyl ester **25** in excellent yield,with no evidence of ester hydrolysis.

A limitation of PEG chemistry is that there exists no efficient and convenient way to separate two different PEG-containing compounds. Although the polarity of the PEG compounds is influenced by their functional groups, it is very difficult, or impossible, to separate the compounds chromatographically. The solubility characteristics of PEG compounds are usually very similar, precluding separation by precipitation or extraction.

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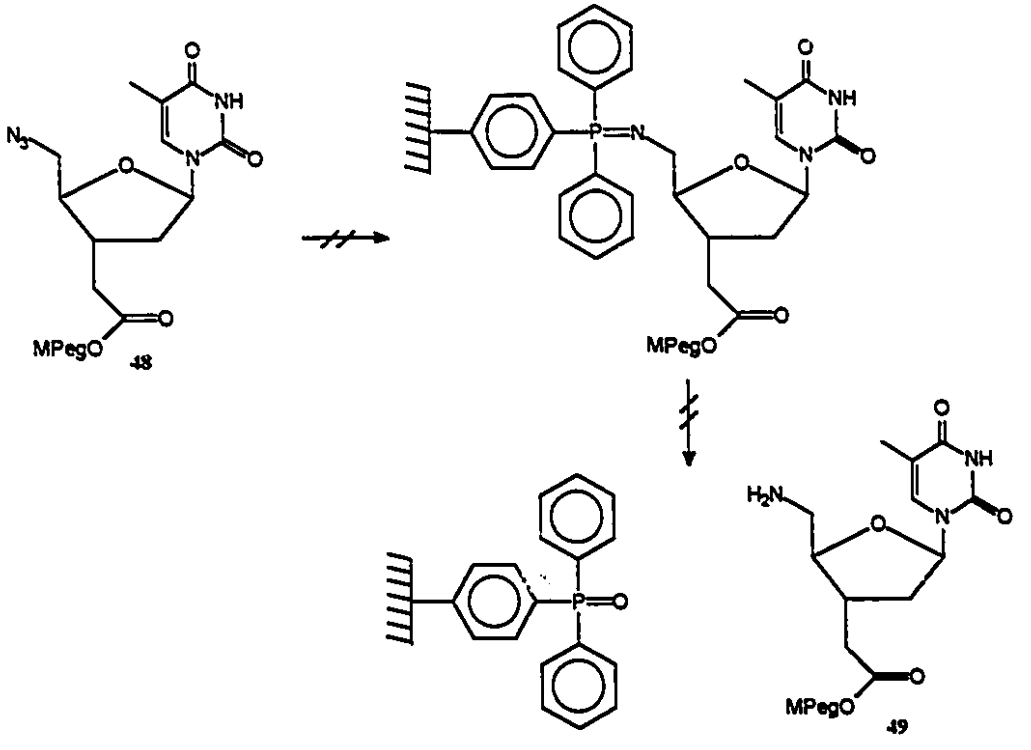
[73] Scriven, E.F.V.; Turnbull, K. *Chem. Rev.* **1988**, *88*, 297.
[74] Samano, M. C.; Robins, M.J. *Tetrahedron Lett.* **1991**, *32*, 6293.

We did, however, perceive of a way to reduce the azide where we could keep the PEG starting material and the PEG product separate, using polymer bound triphenylphosphine.[75] The principle is simple: the PEG azide reacts with the triphenylphosphine to give the polymer bound PEG phosphine imine, as is shown in Scheme 21. Unreacted azide stays in solution and can be washed away. Hydrolysis of the imine releases the pure PEG amine from the polymer. The triphenylphosphine oxide by-product remains on the polymer, as does any unhydrolyzed PEG imine.

Scheme 21



Azide methyl ester **24** could be cleanly reduced by this method, but, unfortunately, azide PEG ester **48** did not react. Steric hindrance likely prevented this reaction.

In the mean time a publication by Bergbreiter *et al.*, regarding the reactivity of reducible functional groups attached to PEG towards catalytic hydrogenations, came to

[75] Holletz, T.; Cech, D. *Synthesis* **1994**, 789.

our attention.[76] It was found that PEG did not significantly affect the reactivity of a substrate, as long as a sterically accessible catalyst and the right solvent were used. These researchers compared the reaction rates of nitro group reductions in water / ethanol solvent systems, varying the water percentage from 0 % to 100 %. While there was little effect on the rates involving lower weight PEGs, the PEG 5000 compounds showed a large increase in hydrogenation rate when 100 % water, rather than ethanol or ethanol / water mixtures were used.

We felt it would be worthwhile to test if the catalytic hydrogenation of the PEG nucleoside azide **48** would be similarly affected by a change of solvent. We had used ethanol or ethanol / water mixtures for this reaction with unsatisfactory results, as was mentioned before. When the hydrogenation was repeated in water with Lindlar catalyst, there was no increase in reaction rate, but a change of catalyst to platinum (IV) oxide brought the reaction to completion in 2 hours. This was the same reaction time as for the hydrogenation of 5' azide methyl ester **24**. The outcome of these two reactions, however was not the same. While there was no evidence of hydrolysis of the methyl ester, the PEG ester showed partial cleavage of the PEG. These reactions were performed on a small scale, which made it difficult to analyze the by-product, which appeared on TLC on the baseline and tested positive to ninhydrin.

The unexpected hydrolysis of the 5' amino PEG ester in solution can perhaps partially be explained by the electron withdrawing effect of cation impurities, which can be trapped in the PEG, making the PEG a better leaving group. The fact that the hydrolysis was more pronounced for small scale than for larger scale reactions, supports this premise. The addition of a drop of acetic acid to the aqueous hydrogenation mixture lessened, but did not eliminate the PEG hydrolysis. This could indicate that the hydrolysis was assisted to some extent by the free amine, but considering the fact that 5' amino methyl ester **25**

[76] Bergbreiter, D.E.; Kimmel, T.; Caraway, J.W. *Tetrahedron Lett.* **1995**, *36*, 4757.

could be left overnight in solution without showing any signs of hydrolysis, this is not likely. Rather, the lower pH may affect the ability of the PEG to coordinate with the cations.

2.15 mPEG-supported 5' to 3' homologation of the homopolymers.

We had hoped that we could deprotect the 5' methylamino function of PEG ester **51** without having the same hydrolysis problems that we encountered with 5' amino PEG ester **49**.

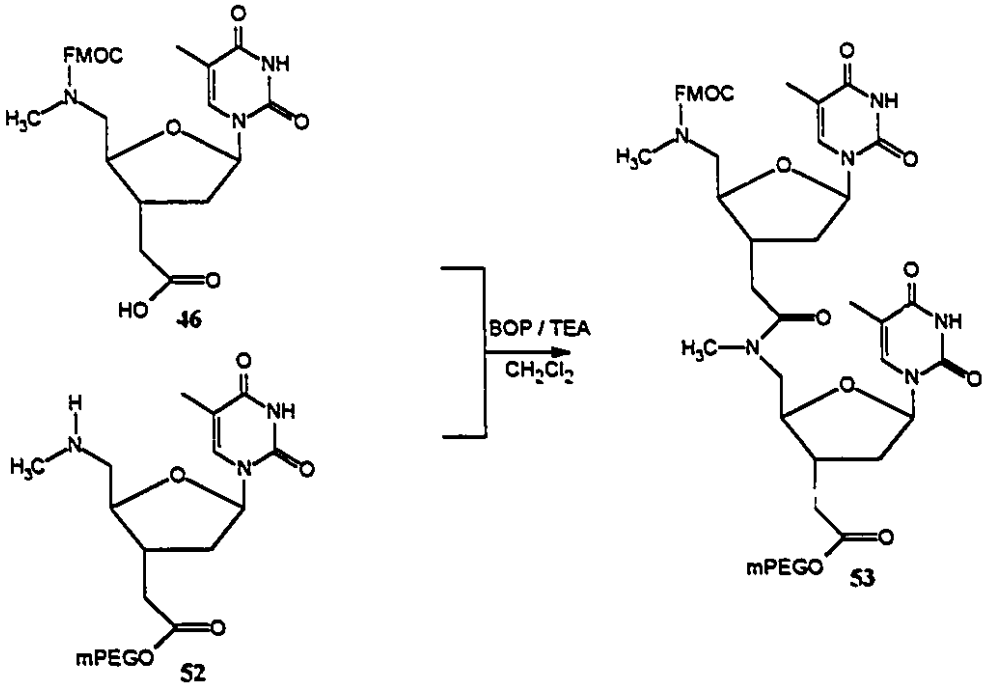
The Fmoc group of ester **51** (shown in Scheme 20) was quickly removed by stirring with piperidine in acetonitrile solution and the resulting free methylamine **52** (shown in Scheme 22) was immediately precipitated from the solution by the addition of ether. No hydrolysis of the PEG ester was observed. If, however, the ester was left in contact with the piperidine solution for longer periods of time, some hydrolysis occurred.

We initially performed the deprotection reaction with piperidine in methylene chloride as solvent. When this reaction was allowed to run for an hour or more, an unexpected white crystalline precipitate formed. This was characterized by NMR as a piperidine salt, probably formed by interaction between methylene chloride and piperidine. Changing the solvent to acetonitrile prevented the formation of this unwanted side product.

With the first deprotected nucleoside in place on the PEG support, we were now ready to attach the next unit as depicted in Scheme 22. Fmoc protected carboxylic acid **46** was coupled to PEG supported amine **52** in a BOP-mediated reaction to yield dimer **53**. The progress of the reaction could be monitored by TLC. The coupling time was considerably longer than for the formation of the analogous methyl ester of methylamide-linked dimer **47**.

Because of the presence of the tertiary amide and carbamate bonds in dimer **53**, four rotamers were expected to be present in solution, resulting in a highly complex NMR spectrum. The integration and the chemical shifts of the poorly resolved signals were compared to those of the methylamide dimer methyl ester **47** and were found to be in agreement with the expected values.

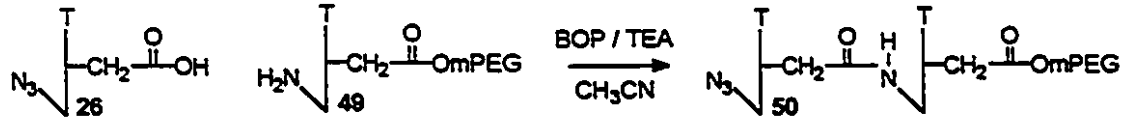
Scheme 22



The described method of synthesizing methylamide thymidine homopolymers on an ester linked PEG support, should be applicable to H-amide homopolymers as well. To completely verify this it would have been necessary to make the primary amine analogue of carboxylic acid **46** as starting material and follow the same deprotection and coupling procedures as for the preparation of dimer **53**. Instead, we used the 5' amino PEG ester **49**, obtained by azide reduction, and coupled it to 5' azido carboxylic acid **26**. Scheme 23 represents the H-amide coupling reaction.

The PEG ester of the H-amide linked dimer **50** was characterized by NMR and the molecular weight confirmed by MALDI mass spectroscopy (dithranol matrix).

Scheme 23



2.16 mPEG-supported 3' to 5' nucleoside chain extension.

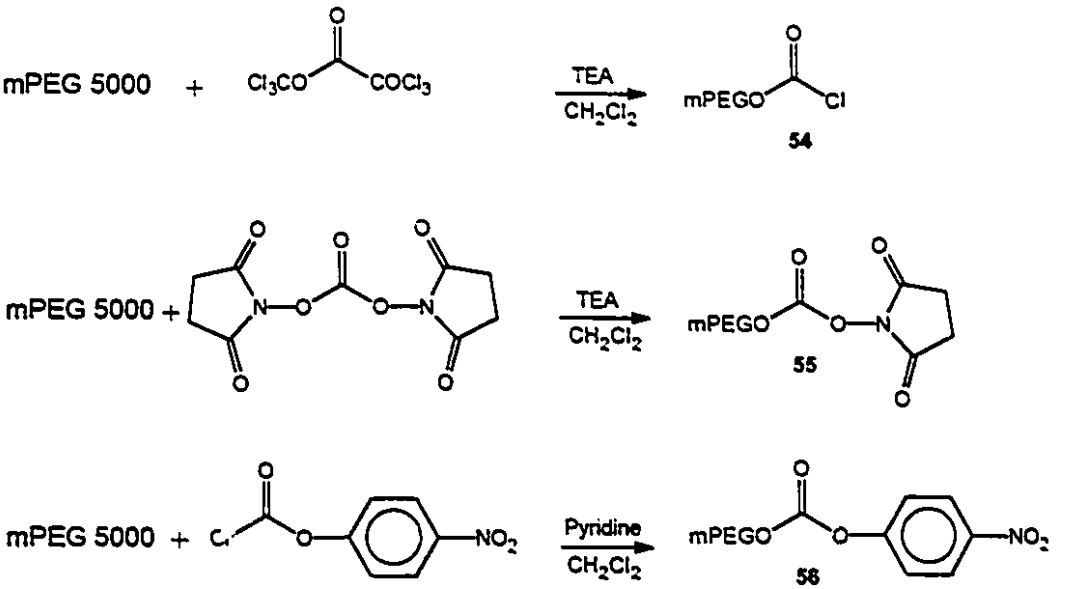
After successfully attaching two nucleoside units to PEG, starting at the 3' carboxylic acid position, we were interested in testing the possibility of linking the nucleoside to PEG at the 5' amino end and making the homopolymer grow in the opposite direction.

We chose a carbamate linker because it is stable under physiological conditions, but can still be hydrolyzed. Although mPEG succinimidyl carbonate 55 or mPEG nitrophenyl carbonate 56, both suitable precursors, were commercially available, they were very expensive. Deterred by the high cost of carbonates 55 and 56, we had first tried to introduce a carbonyl group between mPEG and the 5' amino or methylamino functions of nucleosides 25 and 40, in a one pot reaction with triphosgene. It was not possible to get a clean coupling reaction on a small scale, because of unavoidable inaccuracy in the use of very small quantities of triphosgene. The reaction should work well on a larger scale, however. The intermediate mPEG chloroformate 54 could be isolated, but was very moisture sensitive.

It was found to be possible, however, to synthesize precursor carbonates 55 and 56 quickly and conveniently at a fraction of the commercial cost. (Scheme 24)

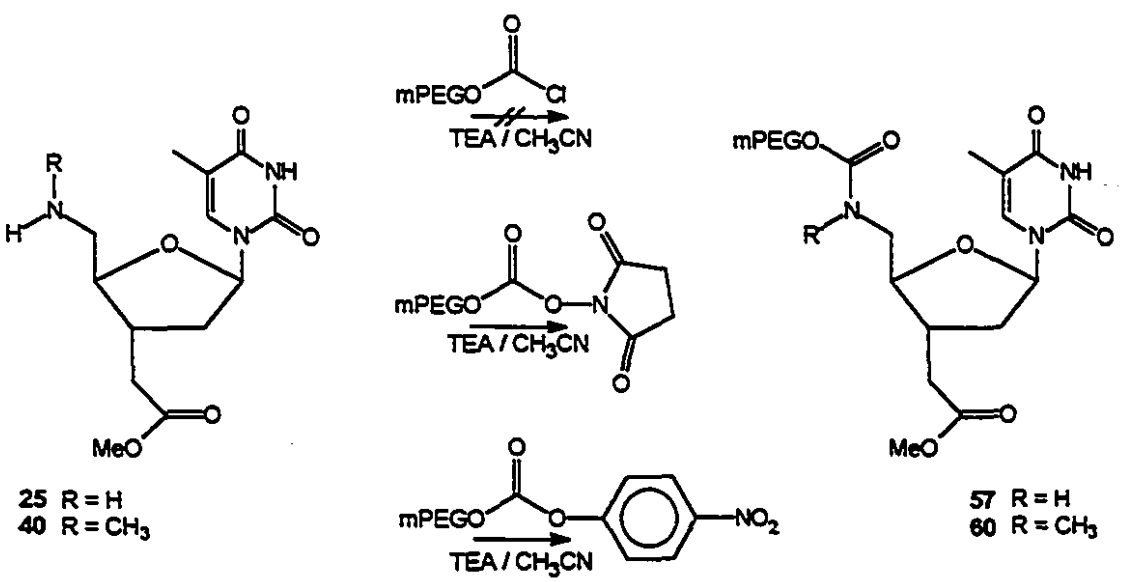
Gram quantities of pure mPEG carbonates could be prepared by stirring mPEG with the pertinent reagents for 3 hours and precipitating out the products with ether. Both reactions were run with triethylamine or pyridine as added base. Triethylamine was the superior reagent for the formation of mPEG succinimidyl carbonate 55, but resulted in a very slow reaction in the case of mPEG nitrophenyl carbonate 56, where pyridine proved to be a better choice. The addition of pyridine to the nitrophenyl chloroformate solution gave an instantaneous white precipitate, which disappeared gradually after adding mPEG.

Scheme 24



The carbonates were synthetically equally satisfactory for coupling to 5' amine **25** and 5' methylamine **40**, as shown in Scheme 25. The mPEG nitrophenyl carbonate has the disadvantage, however, that the nitrophenol, which is released during the coupling reaction, is toxic.

Scheme 25

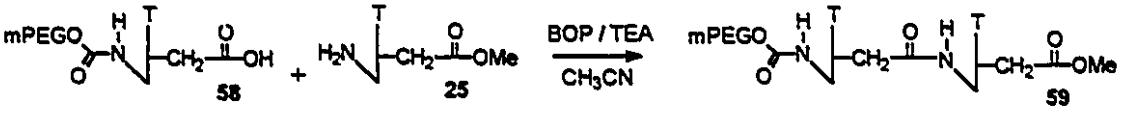


The structure of PEG carbamates **57** and **60** was confirmed by NMR. The molecular weight of PEG carbamate **57** was verified by MALDI mass spectroscopy, using a dithranol matrix.

The next step was the hydrolysis of the 3' ester function of PEG nucleoside carbamates **57** and **60**. This was effected under the mildest possible conditions, by stirring with 0.125 M aqueous KOH, followed by acidification with 0.3 M HCl. Carboxylic acid **58** could be extracted from the aqueous solution with methylene chloride, using PEG's solubility properties to good advantage.

Carboxylic acid **58** was subsequently coupled to the next 5' amino nucleoside unit **25**, depicted in Scheme 26.

Scheme 26



By TLC we had noticed that carboxylic acid **58** was contaminated with a small amount of unreacted mPEG . Careful analysis of the NMR spectra of acid **58** and dimer **59** revealed the presence of 5 to 10 % of unreacted mPEG. How we reached this conclusion will be discussed in a separate chapter, dealing with the characterization of the PEG conjugates in general.

Although the carbamate hydrolysis was not extensive, it was unacceptable in view of the fact that the reaction was part of a repeating cycle. Thus we decided to discontinue the synthesis of the homopolymers attached to mPEG by a carbamate linkage.

We were still interested, however, in assessing the feasibility of producing a longer PEG-supported homopolymer, extending from 3' to 5'.

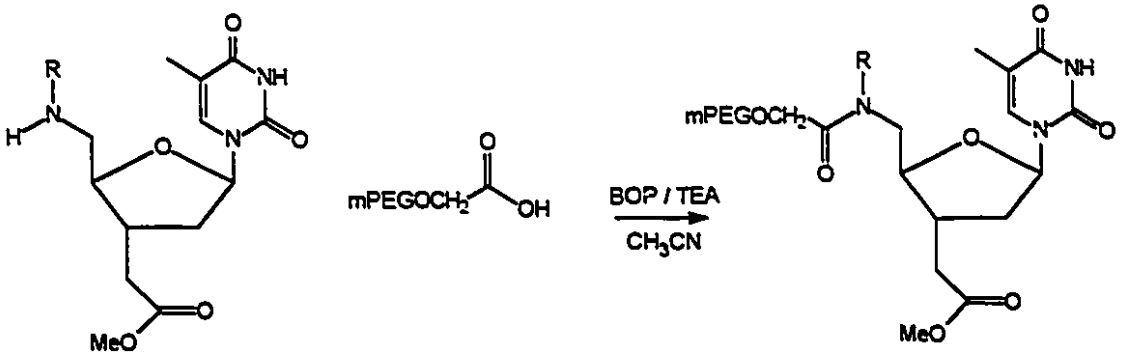
A linker was needed between the mPEG and the 5' nucleoside position which was more than borderline resistant to ester hydrolysis conditions. There were several easily

accessible options open to us. A variety of commercially available, electrophilically activated PEGs, such as PEG tresylate, PEG epoxide or PEG aldehyde, can undergo displacement reactions with amino groups and form an amino linkage. Alternatively, PEG carboxylic acid, also commercially available, could be used to give an amide link. Both linkages are stable. Since the conditions for amide bond formation between the nucleosides were already well established, it seemed reasonable to chose an amide linkage.

mPEG carboxylic acid can be synthesized according to literature procedure by reacting mPEG with ethyl bromoacetate, followed by alkaline hydrolysis.[67] To save time and effort we used the commercial product.

Shown in Scheme 27 is the coupling of the mPEG carboxylic acid to 5' amino nucleoside **25** and to 5' methylamino nucleoside **40** , yielding conjugates **61** and **62**. The reaction conditions were the same as for the formation of PEG-supported dimer **59**.

Scheme 27



25 R = H
40 R = CH_3

61 R = H
62 R = CH_3

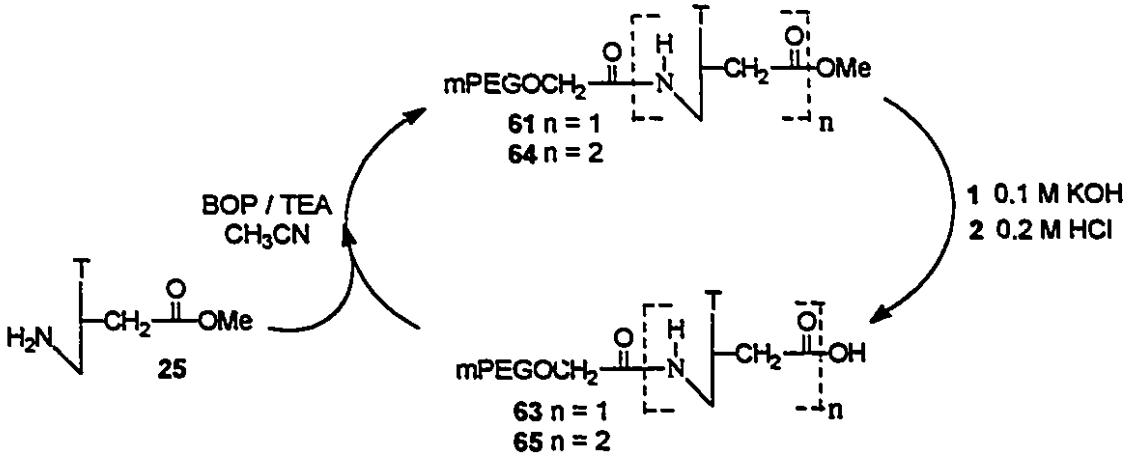
There was, however, a large difference in reaction time. The PEG carboxylic acid was very slow to react. It required three days for the reaction to go to completion, versus 18 hours for dimer **59** , or one hour for non-PEG amide dimers **27** and **47**. Changing the solvent from acetonitrile to DMF made no difference to the reaction rate.

It has been commonly assumed that soluble polymers do not appreciably influence

the reactivity of their terminal groups towards small molecule substrates.[77] We have observed that the reaction rates for PEG-bound nucleosides are generally much slower than those involving non-PEG analogues. This is not in accordance with generally accepted principles.[78]

The alkaline hydrolysis of ester **61** resulted in carboxylic acid **63**, which was coupled to 5' amine **25**, giving dimer **64**. The hydrolysis step was then repeated. These reactions were straightforward and yielded clean products, meeting the criteria for repeating reaction cycles.(Scheme 28) The proton NMR spectra for monomer **61** and dimer **64** are shown in Appendix 8 and 9, respectively.

Scheme 28

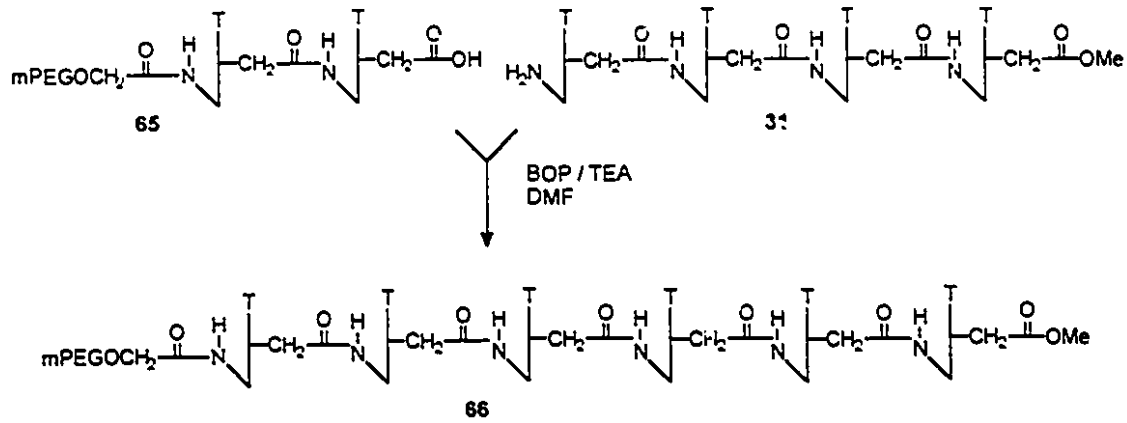


The PEG ester-supported 5' to 3' extension of the homopolymers, as well as the PEG amide-supported 3' to 5' homologation, required only a 20 % excess of nucleoside monomers to make the reactions go to compietion. This compares very favourably to the 20-fold excess commonly used in solid phase nucleotide synthesis.

We still had on hand the previously synthesized tetramer amine **31**. This tetramer was

[77] Bayer, E. *Angew. Chem.* **1991**, *30*, 113.
[78] Billmeyer, F.W. *Textbook of Polymer Science*, **1984**, Wiley-Interscience, New York.

Scheme 29



coupled to dimer acid **65** (Scheme 29), in order to evaluate the solubility of the resulting PEG-conjugated hexamer.

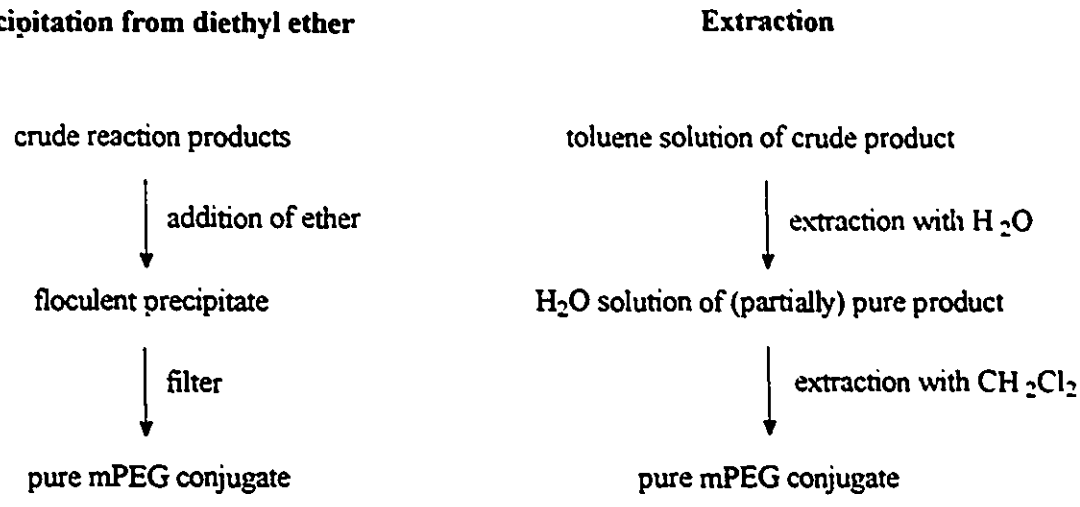
Only 40 mg (0.006 mmol) of the hexamer was available, which made it difficult to prepare a saturated solution. The 40 mg readily dissolved in 0.1 ml of water, giving a solubility of at least 6×10^{-2} M. The concentration of a saturated aqueous solution of non-PEG tetramer amine **31**, calculated from its UV absorbance, was 5.8×10^{-5} M. Given the previously discussed total insolubility of the amide-linked octamer, one can extrapolate that an amide-linked hexamer will be less soluble than the tetramer. Comparing the solubilities of tetramer **31** and the PEG-linked hexamer **66**, it can be concluded that the PEG conjugation increased the solubility of amide-linked thymidine hexamers by at least three orders of magnitude.

Having met our objective to develop methods for the synthesis of certain modified nucleotide chains on PEG support, we terminated our work at this stage.

A following chapter is included to describe the methods that were used for the purification and the characterization of the PEG-adducts in general.

2.17 Purification and characterization of mPEG-conjugated nucleosides.

All PEG adducts were purified by one of the two methods that are shown here in a schematic representation.



The purification by precipitation from ether was generally found to be convenient and efficient. In cases where the crude reaction mixture contained a lot of salt, purification by extraction was more effective. It was possible to extract PEG adducts preferentially from a toluene solution with water and from a water solution with methylene chloride. The final product could also be recrystallized from ethanol.

An important part of PEG methodology is the proper characterization of the PEG conjugates. Since PEG is a very soluble molecule conventional methods are often satisfactory.

The progress of PEG reactions can be monitored by TLC.. We found that an eluent system of methylene chloride / methanol, 5 / 1, resulted in R_f values ranging from 0.1 to 0.5 for the various nucleoside-PEG adducts. Mixtures of PEG and PEG-conjugates tended to run close together, regardless of their individual R_f values. Changing the polarity of the eluent did not give better separation.

We found, however, a way to distinguish between the unreacted PEG and the PEG-nucleoside conjugates. Newly formed nucleoside-PEG conjugates were fluorescent under UV light. A PEG spot by itself is not UV-positive, but acquires a brown colour when the TLC plate is exposed to iodine vapour. Although it was often not possible to get a clear chromatographic separation of the two spots, we could compare the size of the UV-positive spot with the size of the area coloured brown after exposure to iodine vapour. The unreacted PEG usually formed a half ring around the top or the bottom of the nucleoside-PEG conjugate and was thus easily identified. When there was no longer a discernible difference between the size of the fluorescent spot and the brown spot, the reaction was finished. Since this is an imprecise method of monitoring, we always extended the reaction time to ensure completion of the reaction.

^{1}H and ^{13}C NMR can be used for the structure characterization of PEG adducts. The NMR spectra of PEG by itself are very simple. The proton NMR spectrum shows a peak at ~ 3.5 ppm, while the ^{13}C NMR signal appears at 70.0 ppm. The three protons belonging to the methoxy end group on mPEG give rise to a singlet at 3.38 ppm, slightly upfield of the large polyether backbone peak. We found it convenient to use this group as a standard for the quantitative measurement of the degree of substitution of mPEG conjugates. In the case of mPEG nucleosides the methoxy peak was well separated from several distinctive nucleoside signals, for instance, the easily recognizable anomeric protons at ~ 6 ppm.(Appendix 6) This made it possible to compare the integrations of the signals. A well-resolved spectrum was a prerequisite, as the methoxy peak is close to the major PEG signal. It required 500 MHz NMR to give satisfactory results.

^{13}C chemical shifts of PEG linker groups are well-documented and are useful for qualitative analysis of PEG conjugates.[79]

Establishing the molecular weight of the large PEG nucleosides required special

[79] Zalypski, S. *Bioconjugate Chem.* **1995**, *6*, 150.

consideration. Traditional mass spectroscopic methods for determination of exact molecular weights are not satisfactory for biopolymers, such as proteins, carbohydrates and nucleotides. These large, polar molecules must be converted into separate ionized species and the energy required to accomplish this, is also capable of destroying the molecules.

Matrix-assisted laser desorption / ionization (MALDI), time-of-flight spectroscopy mass spectrometry is a method that has been successfully used for biopolymer mass determination.[80] Direct laser irradiation can supply the resonant excitation energy that is needed for ionization, but can also cause photodissociation of bonds. The use of a matrix circumvents this problem. The function of the matrix is to absorb the laser energy and transfer it to the analyte in a controllable and efficient way. It reduces the hardness of the ionization, but the process by which this occurs is not well understood at this time.

MALDI is a mild and sensitive analytical method. A small amount of analyte is embedded in a crystalline matrix, typically a small organic species with a high extinction coefficient, such as dithranol (1,8,9-anthracenetriol), gentisic acid (2,5-dihydroxybenzoic) acid or 2,4,6-trihydroxyacetophenone. The sample must be dilute to prevent complexation of the analyte. A laser is focused on a sample crystal, which is then ionized by short pulses of the laser energy. After ionization the sample is accelerated to a fixed kinetic energy by an electric potential. The ions then pass through a field-free region to a detector which records the time of flight. The mass of the ions is proportional to their velocity and thus to their time of flight.[81]

It was possible to confirm the molecular weight of PEG nucleotides by MALDI. The mass of PEG-adducts is not a single value, but a distribution of weights, 44 mass units apart. MALDI spectra of PEG nucleosides, taken in dithranol or gentisic acid matrices, showed well resolved Gaussian distribution curves, confirming the exact molecular

[80] Pieles, U.; Zürcher, W.; Schär, M.; Moser, H.E. *Nucleic Acids Res.* **1993**, *21*, 3191.
[81] Hillenkamp, F.; Karas, M. *Anal. Chem.* **1991**, *63*,1193.

masses. An example of the MALDI spectrum of PEG nucleside **48** can be found in Appendix 10.

3. CONTRIBUTIONS TO KNOWLEDGE

1) Amide- and methylamide-linked thymidine dimers were efficiently prepared from thymidine.

2) It was established by thermal denaturation studies that the substitution of the DNA phosphate group by an amide or *N*-methylamide moiety does not have an adverse effect on the strength and specificity of DNA-RNA hybridization.

3) An amide-linked thymidine tetramer was successfully prepared by block synthesis. A method was devised for one-by-one homologation of *N*-methylamide-linked homopolymers.

4) Methods were developed to increase the solubility of amide-backbone nucleosides by covalent attachment to poly(ethylene glycol), which at the same time served as a solid support for the homologation reactions.

4. EXPERIMENTAL

4.1 General methods.

Melting points were determined using a Gallenkamp block and are uncorrected. Low-resolution chemical ionization mass spectra were obtained on a DuPont 21-492B mass spectrometer in the direct probe mode. Low-resolution FAB mass spectra were obtained on a DuPont 21-492B mass spectrometer in the direct probe mode and on a VG ZAB-2F-HS sector mass spectrometer in the direct-inlet mode. A VG ZAB-2F-HS sector mass spectrometer in the direct-inlet mode was also used for high-resolution mass spectra. A Kratos Kompact MALDI III instrument with a 337 nm Class 3b invisible laser was used for the MALDI TOF mass spectra. All compounds were shown to be homogeneous by TLC and high-field NMR. In some cases purity was verified by HPLC, using a Waters Millipore chromatograph, equipped with a Model 450 variable wave length detector, a Waters 501 HPLC pump and a M 45 pressure unit. ^{1}H NMR spectra were obtained on either a Varian XL-200, Varian XL-300 or a Varian Unity 500 spectometer at 200 MHz, 300 MHz and 500 MHz, respectively. The peak assignments are based on homonuclear decoupling and / or COSY and / or NOESY experiments. Chemical shifts are reported as parts per million (ppm). The residual proton signals of chloroform, methanol and DMSO (assigned values of δ 7.24, 3.30 and 2.49, respectively) were used as references for spectra recorded in these solvents. The multiplicities are reported using the following abbreviations: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; l m, large multiplet; br, broad. ^{13}C NMR spectra were obtained on a Varian XL-300 or a Varian Unity 500 spectrometer at 75.4 MHz and 125.7 MHz, respectively. The peak assignments were based, in some cases, on APT or HMQC experiments. The ^{13}C signals of $CDCl_3$, CD_3OD and DMSO-d_6 (assigned values of δ 77.00, 49.00 and 39.50, respectively) were used as references. Selected 1- and 2-D experiments are shown in

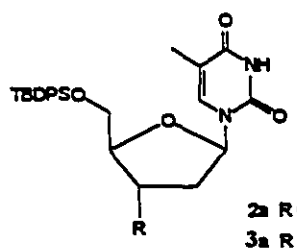
Appendix I. ^{31}P NMR spectra were obtained on a Varian XL-200 or a Varian XL-300 spectrometer at 81.0 MHz and 121.4 MHz. A standard phosphoric acid solution (85 wt. % in water) was used as an external reference.

Methanol was distilled from magnesium. Methylene chloride was distilled from P_2O_5. Pyridine was distilled from BaO. Acetonitrile and triethylamine were distilled from CaH_2. Tetrahydrofuran was distilled from sodium bezophenone ketyl. *N,N*-Dimethylformamide, 99.8 % anhydrous, was obtained from Aldrich. Thin layer chromatography was performed, using Kieselgel 60 F_{254} aluminum-backed plates (0.2 mm thickness). The plates were visualized by UV and / or exposure to iodine vapour, or dipping in a ninhydrin solution (1 g ninhydrin in 80 ml water), or a solution of 2.5 g of ammonium molybdate and 1 g ceric sulfate in 10 % (vol / vol) sulphuric acid. The dipping was followed by heating. Kieselgel 60 (Merck 230-400 mesh) was used for column chromatography.

4.2 Experimental for section 2.2 and 2.3.

5'-*O*-*t*-Butyldiphenylsilyl-3'phenoxythionothymidine (2a).

To a solution of 5'-*O*-*t*-butyldiphenylsilylthymidine (2.80 g, 5.82 mmol) in methylene chloride (60 ml) and pyridine (2.3 ml), under nitrogen atmosphere, was added DMAP (700 mg, 5.82 mmol). The solution was stirred for 5 min in an ice-water bath, before the addition of phenyl thionochloroformate (2.50 ml, 17.5 mmol) over 10 min. The cooling bath was removed and stirring continued for 2.5 h. The reaction mixture was washed with water (3 X 50 ml) and dried over sodium sulfate. After filtration the solvent was evaporated under reduced pressure to give the crude product as a sticky, light brown solid. This solid was triturated with ether (2 X 100 ml) to yield a pale, beige powder (2.87 g, 80 %). The yield could be increased to 89 % by collecting the trituration ether, evaporating it and repeating the triturating step, using 50 ml ether. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.10 (s, 9 H, *t*-butyl), 1.62 (s, 3 H, Me at C5), 2.44 (m, 1 H, H2'a,), 2.74 (m, 1 H, H2'b), 4.09 (br s, 2 H, H5'), 4.36 (m, 1 H, H4'), 5.86 (m, 1 H, H3'), 6.50 (dd, 1 H, H1'), 7.06 - 7.70 (m, 11 H, aromatic + H6), 8.18 (br s, 1 H, H at N3). $J_{H1'-H2'a}$ = 4.9 Hz, $J_{H1'-H2'b}$ = 9.3 Hz.



2a R = OC(S)OPh
3a R = CH_2CHCH_2
4a R = $CH_2C(O)H$
5a R = $CH_2C(O)OMe$
6a R = $CH_2C(O)OH$

5'-*O*-*t*-Butyldiphenylsilyl-3'-allyl-3'-deoxythymidine (3a).

Nucleoside **2a** (3.20 g, 5.19 mmol) was dissolved in toluene (18 ml). A stream of nitrogen was bubbled through the solution for 20 min. To the solution were then added allyltributyl tin (4.6 ml, 15.6 mmol) and AIBN (137 mg, 1.01 mmol) with careful exclusion

of air. The reaction was heated to 80° C under reflux, under nitogen atmosphere, for 22 h The solvent was evaporated and the crude product chromatographed over silicagel (hexanes / ethyl acetate, 7 / 3, vol / vol). The product was isolated as a white foam (1.83 g) in 70 % yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.09 (s, 9 H, *t*-butyl), 1.63 (s, 3 H, Me at C5), 2.00 - 2.56 (m, 5 H, H2', H3', H3"), 3.78 (m, 2 H, H4', H5'b), 4.07 (A of ABX, 1 H, H5'a), 5.07 (m, 2 H, H3""), 5.72 (m, 1 H, H3"'), 6.14 (t, 1 H, H1'), 7.32 - 7.73 (m, 11 H, aromatic + H6), S.32 (br s, 1 H, H at N3). $J_{H1'-H2'a,b}$ = 5.4 Hz. LRMS (FAB, NBA): m/e 505 ($M+H^+$, 16.6 %), 379 ($M+H^+$ - thymine, 52.6 %), 239 ($TBDPS^+$, 18.9 %).

Thymidine 3' aldehyde analogue (4a).

5'-*O*-*t*-Butyldiphenylsilyl-3'-allyl-3'-deoxythimidine **3a** (1.65 g, 3.26 mmol) was dissolved in ether (10 ml). Water (10 ml) and osmium tetroxide (13 mg, 0.051 mmol) were added. Sodium periodate (1.62 g, 7.6 mmol) was added over a period of 30 min, while the mixture was stirred vigorously. Stirring was continued for another 2.5 h, after which the reaction was diluted with methylene chloride (200 ml). The mixture was washed with saturated sodium bicarbonate (2 X 50 ml) and saturated brine (50 ml), before being dried over sodium sulfate. It was filtered and the solvent removed *in vacuo*. The crude product was purified chromatographically (silicagel column, ethyl acetate / hexanes, 3 / 2, vol / vol) to yield a white foam (994 mg, 60 %). ^{1}H NMR (200 MHz, $CDCl_3$):δ 1.09 (s, 9 H, *t*-butyl), 1.66 (d, 3 H, Me at C5), 2.35 - 3.94 (m, 5 H, H2', H3', H3"), 3.71 - 3.86 (overlapping m, 2 H, H4', H5'a), 4.07 (B of ABX, 1 h, H5'b), 6.18 (dd, 1 H, H1'), 7.35 - 7.74 (m, 11 H, aromatic + H6), 8.40 (br s, 1 H, H at N3), 9.73 (s, 1 H, $-CH_2C(O)\mathbf{H}$). $J_{Me\ at\ C5\text{-}H6}$ = 1.1 Hz, $J_{H1'-H2'a}$ = 4.9 Hz, $J_{H1'-H2'b}$ = 6.9 Hz. LRMS (FAB, NBA): m/e 507 ($M+H^+$, 8.0 %), 381 ($M+H^+$ - thymine, 21 %), 239 ($TBDPS^+$, 17.3 %).

5'-*O*-*t*-Butyldiphenylsilyl-3'-carboxymethyl-3'-deoxythymidine (6a).

To aldehyde **4a** (2.22g, 4.38 mmol) were added dry DMF (15 ml) and pyridinium dichromate (3.29 g, 8.76 mmol). The solution was stirred under nitrogen atmosphere for 5h. The reaction mixture was poured into water (150 ml) and the resulting precipitate was filtered off. It was washed with water until the filtrate ran colourless and dried under vacuum. The product (1.83 g, 80%) was used without further purification for the next reaction. LRMS (CI-NH_3): m/e 533 ($M+H^+$, 1.1 %), 397 ($M+H^+$- thymine, 20.9 %), 127 (thymine+H^+, 100 %). The compound was further characterized as its methyl ester **5a**.

5'-*O*-*t*-Butyldiphenylsilyl-3'-carbomethoxymethyl-3'-deoxythymidine (5a). (Method 1)

To acid **6a** (26 mg, 0.050 mmol) were added dry methanol (1 ml) and trimethylsilyl chloride (14µl, 0.11 mmol) under nitrogen atmosphere. The solution was stirred for 3h. A drop of TEA was then added and the solvent was evaporated under vacuum. The crude product was purified by flash chromatography (hexanes / ethyl acetate, 3 / 2, vol / vol) to yield the title compound (14 mg, 50 %) as a pale yellow foam. ^{1}H NMR (200 MHz, $CDCL_3$): δ1.09 (s, 9 H, *t*-butyl), 1.63 (s, 3 H, Me at C5), 2.18 (m, 1 H, H2'a), 2.32 (m, 2 H, H2'b, H3"a), 2.45 (B of ABX, 1 H, H3"b), 2.81 (m, 1 H, H3'), 3.67 (s, 3 H, -OCH_3), 3.78 (m, 2 H, H4', H5'a), 4.03 (B of ABX, 1 H, H5'b), 6.14 (dd, 1 H, H1'), 7.33-7.68 (m, 11 H, aromatic, H6), 8.32 (br.s., H at N3). $J_{H1'-H2'a}$ = 5.0 Hz, $J_{H1'-H2'b}$ = 6.6 Hz. ^{13}C NMR (74.5 MHz, $CDCl_3$): δ 12.22 (Me at C5), 19.38, 27.01 (*t*-butyl), 34.61 (C3'), 36.61 (C3"), 38.68 (C2'), 51.84 (OMe), 63.84 (C5'), 85.58, 85.06 (C1', C4'), 110.87 (C5), 127.91-135.59 (6 lines, aromatic, C6), 150.44 (C2), 163.86 (C4), 171.91 (C3'"). LRMS (FAB, NBA) : m/e 537 ($M+H^+$, 13.0 %), 411 ($M+H^+$ - thymine, 100 %), 353 ($M+H^+$ - thymine - *t*-butyl, 70 %). HRMS (FAB, glycerol): m/e calculated for $C_{29}H_{36}N_2O_6 + H^+$, 537.2372; found 537.2423.

5'-*O*-*t*-Butyldiphenylsilyl-3'-carbomethoxymethyl-3'-deoxythymidine (5a). (Method 2)

To aldehyde **4a** (1.40 g, 3.66 mmol) were added dry DMF (50 ml) and dry methanol (890µl, 22.0 mmol) under nitrogen atmosphere. The solution was stirred for 0.5 h, before the addition of pyridinium dichromate (8.90 g, 22.0 mmol). Stirring was continued for 1h, during which time the colour changed from dark orange to dark green. The reaction mixture was then poured into 100 ml ethyl acetate and the resulting slurry was filtered through 1.5 cm of silica gel, previously wetted with ethyl acetate. It was rised with 20 ml ethyl acetate. The combined solvents were evaporated under vacuum to yield the title compound (1.43 g, 95%) as a pale yellow foam. The spectral data were the same as those obtained from method 1.

5'-*N*-Methylamino-5'-deoxythymidine (12).

A 50 ml round-bottomed flask, stoppered with a rubber septum, vented by a hypodermic needle, was filled with 40 % aqueous methylamine (12 ml, 160 mmol) and 5'-tosylthymidine (2.80 g, 7.07 mmol). The solution was stirred at 55° C for 2 h and allowed to cool to room temperature. The solvent and excess methylamine were evaporated under reduced pressure and the crude residue was purified by flash chromatography (methylene chloride / methanol / TEA, 80 / 20 / 1, vol / vol / vol), to yield 1.50 g (83 %) of a white powder. This powder crystallized upon standing in methylene chloride. m.p 143-145° C. ^{1}H NMR (200 MHz,CD_3OD): δ 1.88 (d, 3H, Me at C5), 2.22 (m, 2H, H2'), 2.42 (s, 3H, N-Me), 2.78 (A of ABX, 1H, H5'a), 2.82 (B of ABX, 1H, H5'b), 3.91 (m, 1H, H4'), 4.22 (m, 1H, H3'), 6.20 (t, 1H, H1'), 7.48 (d, 1H, H6), $J_{\text{Me at C5 - H6}}$ = 1.1 Hz, $J_{\text{H5'a-H5'b}}$ = -12.5 Hz, $J_{\text{H5'a-H4'}}$ = 8.4 Hz, $J_{\text{H5'b-H4'}}$ = 3.73 Hz, $J_{\text{H1'-H2'a,b}}$ = 6.8 Hz. ^{13}C NMR (75.4 MHz, CD_3OD): δ 12.4 (Me at C5), 36.3 (N-Me), 40.1 (C2'), 54.7 (C5'), 73.4 (C3'), 86.2 (C4'), 111.8 (C5), 138.1 (C6), 152.4 (C2), 166.5 (C4); LRMS (CI-NH_3): m/e 256 ($M+H^+$, 100 %), 130 ($M+H^+$-thymine, 44 %), 127

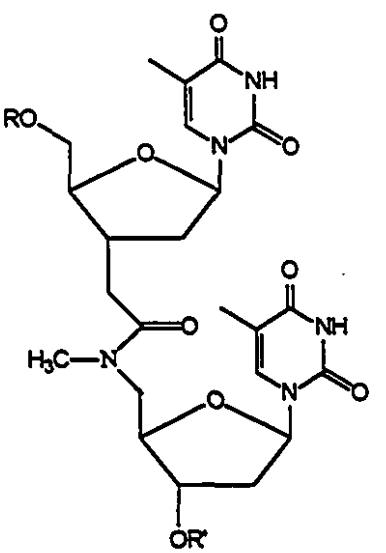
(thymine+H^+, 37 %). HRMS (CI , NH_3): m/e calculated for $C_{11}H_{17}N_3O_4 + H^+$, 256.1297; found 256.1297.

4.3 Experimental for Sections 2.4 and 2.7.

5'-*O*-*t*-Butyldiphenylsilyl TmaT (17).

(TmaT = methyl amide-linked thymidine dimer)

To dry DMF (5 ml) were added 5'-*O*-*t*-butyldiphenyl-3'-carboxymethyl-5',3'-dideoxythymidine **6a** (484 mg, 0.927 mmol), 5'-methylamino-5'-deoxythymidine **12** (236 mg, 0.927 mmol) and TEA (250 µl, 1.85 mmol). This solution was stirred under nitrogen for 0.5 h, when BOP coupling reagent (408 mg, 0.927 mmol) was added. Stirring was continued for 0.5 h, after which the solvent was removed *in vacuo*. Purification of the crude product (flash chromatography, eluent: methylene chloride / methanol, 23 / 2, vol / vol), gave a white foam (563 mg, 80 %). The proton NMR spectrum indicated the presence of two rotamers in a ratio of ~ 3 : 1. ^{1}H NMR (300 MHz, $CDCl_3$), selected data: δ 1.09 (s, 9 H, *t*-butyl),1.61 (s, 3 H, Me at C5), 1.85 (s, 3 H, Me at C5), 2.99 (s, 3 H, -NMe), 6.10 - 6.38 (unresolved m, 2 H, H1'), 7.02 - 7.64 (12 H, aromatic, 2 X H6), 9.45 (major), 9.68 (minor) (s, s, 1 H, H at N3), 9.80 (s, 1 H, H at N3). LRMS (FAB, glycerol): m/e 760 ($M+H^+$, 21.6 %), 634 ($M+H^+$ - thymine,100 %), 508 ($M+H^+$ - two thymine, 20.2 %). HRMS (FAB, glycerol): m/e calculated for $C_{39}H_{49}N_5O_9Si + H^+$, 760.3378; found 760.3377.



17 R = TBDPS R' = H
18 R = H R' = H
19 R = DMTr R' = H
20 R = DMTr R' = $P(OCH_2CH_2CN)N(i\text{-}Pr)_2$

TmaT diol (18).

To a stirred solution of TBDPS-TmaT 17 (563 mg, 0.724 mmol) in THF (10 ml) under an atmosphere of nitrogen was added tetrabutylammonium fluoride in THF (2.2 ml, 1M) over a period of 5 min. After 45 min, the solvent was removed *in vacuo* to yield a white foam. Purification by flash chromatography (methylene chloride / methanol, 17 / 3, vol / vol) afforded the title compound as a white powder (550 mg, 89 %). The proton NMR spectrum indicated the existence of a mixture of *cis* and *trans* rotamers, in a ratio of 1:2). ^{1}H NMR (500 MHz, CD_3OD): δ 1.87 (overlapping d, 6H, Me at ^{5}C5 and ^{3}C5), 2.03 - 2.15 (m, ^{5}H2'a,b major, minor), 2.20 - 2.32 (m, ^{3}H2'a,b major, ^{3}H2'a minor), 2.47-2.56 (overlapping m, A of ABX, H3", ^{3}H2'a,b minor), 2.54 (B of ABX, H3"), 2.70 (m, ^{5}H3' major, minor), 2.98 (s, -NMe, minor), 3.11 (s, -NMe, major), 3.50-3.92 (m, ^{5}H4', ^{3}H4' minor, ^{5}H5', ^{3}H5'), 4.06 (m, ^{3}H4' major), 4.23 (m, ^{3}H3' major), 4.34 (m, ^{3}H3 minor), 6.00 (dd, ^{5}H1' minor), 6.04 (dd, ^{5}H1' major), 6.08 (dd, ^{3}H1' minor), 6.24 (t, ^{3}H1' major), 7.41 (d, ^{3}H6 minor), 7.59 (d, ^{3}H6 major), 7.99 (d, ^{5}H6 minor), 8.02 (d, ^{5}H6 major), $J^{5}_{H6\text{-}^{5}Me}$ = 1.0 Hz, $J^{3}_{H6\text{-}^{3}Me}$ = 1.4 Hz, $J^{3}_{H1'\text{-}^{3}H2'a,b}$ = 7.1 Hz (major), $J^{3}_{H1'\text{-}^{3}H2'a}$ = 7.8 Hz (minor), $J^{3}_{H1'\text{-}^{3}H2'b}$ = 5.4 Hz (minor), $J^{5}_{H1'\text{-}^{5}H2'a}$ = 7.3 Hz (major), $J^{5}_{H1'\text{-}^{5}H2'b}$ = 3.2 Hz (major), $J^{5}_{H1'\text{-}^{5}H2'a}$ = 6.8 Hz (minor), $J^{5}_{H1'\text{-}^{5}H2'b}$ = 3.4 Hz (minor). ^{13}C NMR (125.7 MHz, CD_3OD): δ 12 .25, 12.37, 12.39 (Me at C5), 34.64, 4.84, 34.85, 34.96 (^{5}C3', N-Me), 36.15, 36.31 (C3"), 39.72, 39.83, 40.10 (C2'), 50.68 (^{3}C5' major), 52.75 (^{3}C5' minor), 62.00, 62.14 (^{5}C5'), 72.54 (^{3}C3' minor), 73.36 (^{3}C3' major), 84.83 (^{3}C4'), 86.17 (^{5}C1'), 86.51 (^{3}C1' major), 87.40 (^{3}C1' minor), 87.45 (^{5}C4'), 110.67, 111.73, 111.88 (C5), 138.03, 138.24, 138.29, 139.06 (C6), 152.00, 152.15, 152.19, 152.26 (C2), 166.16, 16.40, 166.46 (C4), 173.99, 174.02 (C3'"). LRMS (FAB, NBA): m/e 522 ($M+H^+$, 1.1 %), 396 ($M+H^+$ - thymine, 5.5 %).

5'-*O*-DimethoxytritylTmaT (19).

Pyridine (3ml) was co-evaporated from TmaT diol **18** (123 mg, 0.236 mmol), and the residue redissolved in dry pyridine (3ml). To this solution were added, under nitrogen atmosphere, DMAP (20 mg, 0.164 mmol) and dimethoxytrityl chloride (225 mg, 0.664 mmol). After stirring for 3 h, the solvent was evaporated *in vacuo* and the product purified by flash chromatography (methylene chloride / methanol / triethyl amine, 100 / 5 /1, vol / vol / vol). A white foam (175 mg, 90 %) was obtained, which exists as two rotamers in solution, in a ratio of 4 : 1. ^{1}H NMR (200 MHz,$CDCl_3$ + drop of pyridineD_4), selected data: δ 1.42,1.52 (minor, major, s, s, 3 H, Me at C5), 1.86,1.91 (minor, major, s, s, 3 H, Me at C5), 2.94, 2.99 (minor, major, s, s, 3 H, -NMe), 3.78 (s, 6 H, 2 X -OMe), 6.02 - 6.18 (m, 2 H, H1'), 6.78-7.48 (m, 14 H, aromatic plus H6), 7.61 (s, H6). LRMS (FAB, NBA): m/e 824 ($M+H^+$, 0.2 %), 303 (DMT^+, 52.0 %).

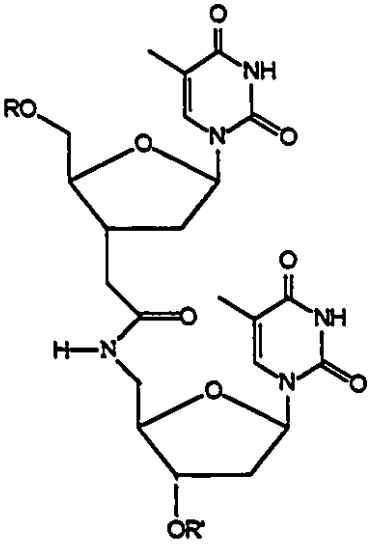
5'-*O*-DimethoxytritylTmaT-3'-phosphoramidite (20).

((DMTrTmaT **19** (68mg, 0.083 mmol) was co-evaporated with dry pyridine (3 ml). The residue was redissolved in dry methylene chloride (4.5 ml), under nitrogen. To this solution was added, while stirring, TEA (60 µl, 0.45 mmol), followed by dropwise addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (41 µl, 0.18 mmol). After 1 h the reaction mixture was diluted with ethyl acetate (10 ml), washed with saturated NaCl (2 ml) and dried over anhydrous sodium sulfate. Purification by flash chromatography (methylene chloride / methanol / TEA, 100 / 5 / 1, vol / vol / vol) yielded the desired product (80 mg, 94%), a cream-coloured foam, which exists in solution as a mixture of rotamers of the two phosphoramidite isomers. ^{31}P NMR (121.4 MHz, $CDCl_3$ + drop of pyridineD_4): δ 144.8 (s, major), 145.0 (s, minor), 145.3 (s, major), 145.6 (s, minor). LRMS (FAB, NBA): m/e ($M + (C_2H_5)_3NH^+$, 33.7%).

5'-*O*-*t*-ButyldiphenylsilylTaT (14).

(TaT = amide-linked thymidine dimer)

In a round-bottomed flask were placed 5'-*O*-*t*-butyldiphenyl-3'-carboxymethyl-5',3'-dideoxythymidine **6a** (522 mg, 1.00 mmol), 5'-amino-5'-deoxythymidine and dry DMF (5 ml) under nitrogen atmosphere. To this solution were added TEA (270 µl, 2.00 mmol) and BOP coupling reagent (442 mg, 1.00 mmol). The reaction mixture was stirred for 1h, after which the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (methylene chloride /methanol, 25 /2, vol / vol), to yield the title compound (599 mg, 80%) as a light yellow foam. The NMR spectrum was not sufficiently well resolved to allow a detailed analysis. ^{1}H NMR (200 MHz, $CDCl_3$) selected data: δ 1.09 (s, 9 H, *t*-butyl), 1.56 (s, 3 H, Me at C5), 1.83 (s, 3 H, Me at C5), 5.91 (br t, 1 H, H1'), 6.05 (br t, 1 H, H1'), 7.11-7.65 (m, 12 H, aromatic, 2 X H6), 8.79 (br s, 1 H, -C(O)NH). MS (FAB, NBA): m/e 746 ($M+H^+$, 5.1 %), 620 ($M+H^+$ -thymine, 100 %), 494 ($M+H^+$ -two thymine, 22.5 %). HRMS (FAB, glycerol): m/e calculated for $C_{38}H_{46}N_5O_9Si + H^+$ 746.3221; found 746.3222.



14 R = TBDPS R' = H
16 R = H R' = H
21 R = DMTr R' = H
22 R = DMTr R' = $P(OCH_2CH_2CN)N(i\text{-}Pr)_2$

TaT diol (16).

Dimer **14** (490 mg, 0.65 mmol) was deprotected by stirring in THF (12 ml) with tetrabutylammonium fluoride (2.5 ml,1M in THF) for 3/4 h. After removal of the solvent under vacuum and flash chromatogaphy (methylene chloride /methanol, 17 /3, vol / vol), the pure product (200 mg, 65%) was isolated as a white powder. ^{1}H NMR (500 MHz, CD_3OD): δ 1.86 (d, 3 H, Me at C5), 1.87 (d, 3H , Me at C5), 2.13-2.28 (m, 5H, H2', H3"a), 2.44 (B of ABX, 1H, H3"b), 2.67 (m, 1H, ^{5}H3'), 3.41 (A of ABX, 1 H, ^{5}H5'a), 3.52 (B of ABX, 1 H, ^{5}H5'b), 3.72 (m, 2 H, ^{5}H4', ^{5}H5'a), 3.90 (m, 2 H, ^{3}H4', ^{5}H5'b), 4.23 (m, 1 H, ^{3}H3'), 6.03 (dd, 1 H, ^{5}H1'), 6.16 (t, 1 H, ^{3}H1'), 7.48 (d, 1H, H6), 7.99 (d, 1H, H6). $J_{^3H1'-^3H2'a,b}$ = 6.8 Hz, $J_{^5H1'-^5H2'a,b}$ = 2.9, 6.8 Hz, 6.83 Hz, $J_{H3"a-H3"b}$ = -14.2 Hz, $J_{Me\ at\ C5\ -H6}$ = 0.9 Hz, $J_{^3H5'a-^3H5'b}$ = -13.9 Hz, $J_{^3H4'-^3H5'a}$ = 7.87 Hz, $J_{^3H4'-^3H5'b}$ = 4.34 Hz. ^{13}C NMR (125.7 MHz, CD_3OD): δ 12.42, 12.45 (Me at C5), 35.36 (^{5}C3'), 38.66 (C3"), 39.76, 39.93 (C2'), 42.39 (^{3}C5'), 61.86 (^{5}C5'), 72.98 (^{3}C3'), 86.10 (^{3}C4'), 86.15 (^{5}C1'), 87.15 (^{3}C1'), 87.35 (^{5}C4'), 110.72 (C5), 111.69 (C5), 138.11 (C6), 138.16 (C6), 152.63 (C2), 152.90 (C2), 167.16 (C4), 167.34 (C4), 174.15 (C3"). LRMS (FAB, NBA): m/e 508 ($M+H^+$, 2.0 %), 382 ($M+H^+$ - thymine, 8.2 %), 256 ($M+H^+$ - 2 thymine, 6.0 %), 242 (5'-amino-5'-deoxythymidine+H^+, 100 %).

5'-*O*-DimethoxytritylTaT (21).

After co-evaporating a solution of TaT **16** (200 mg, 0.394 mmol) in dry pyridine (3 ml), the compound was redissolved in dry pyridine (3 ml) and stirred with TEA (137 µl, 1.00 mmol) and dimethoxytritylchloride (338 mg, 1.00 mmol) for 1 h under nitrogen. The solvent was evaporated under vacuum and the product purified by flash chromatography (methylene chloride / methanol / TEA, 100 / 5 / 1, vol / vol / vol). The title compound (295 mg, 92%) was obtained as a white powder. ^{1}H-NMR (300 MHz, $CDCl_3$ + drop of pyridineD_4), selected data: δ 1.43 (s, 3 H, Me at C5), 1.89 (s, 3 H, Me at C5), 3.73 (s, 6 H, 2 X -OCH_3), 5.70 (t, 1 H, H1'), 6.18 (dd, 1 H, H1'), 6.78-7.48 (m, 14 H, aromatic,

H6), 7.63 (s, 1 H, H6). LRMS (FAB, NBA): m/e 810 ($M+H^+$, 0.4 %), 303 (DMT^+, 49.1 %).

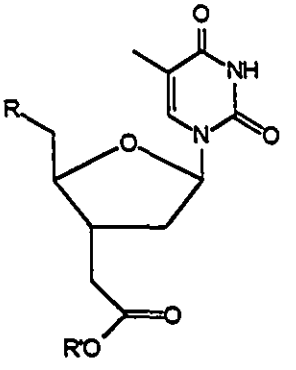
5'-*O*-DimethoxytritylTaT-3'-phosphoramidite (22).

To DMTrTaT **21** was added 5 ml pyridine, which was immediately evaporated under reduced pressure. The residue was redissolved in dry methylene chloride (20 ml) and TEA (264 µl, 2 mmol) was added under nitrogen. Subsequently, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (180 µl, 0.81 mmol) was added, dropwise, while stirring. Stirring was continued for 1 h, after which the reaction mixture was diluted with 50 ml ethyl acetate, followed by washing with saturated brine (10 ml). The solution was dried over anhydrous sodium sulfate, filtered and the solvent evaporated *in vacuo*. Final purification was effected by flash chromatography (methylene chloride / methanol / triethyl amine, 100 / 5 / 1, vol / vol / vol), to yield the title compound as a pale yellow foam (316 mg, 86%). ^{31}P NMR (121.4 MHz, $CDCl_3$ + drop of pyridineD_4): δ 150.63 (s), 151.04 (s). MS (FAB, NBA): m/e 1111 ($M + Et_3NH^+$, 80.6%).

4.3 Experimental for section 2.10.

3'-Carbomethoxymethyl-3'-deoxythymidine (8).

The 5'-silyl protecting group of compound 5 (950 mg, 1.8 mmol) was deprotected by stirring the compound in THF (12 ml) with tetrabutylammonium fluoride (1.8 ml, 1 M solution in THF) for 3/4 h. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (silica gel height, 8 cm), using ethyl acetate as eluent. A white foam was obtained in quantitative (530 mg) yield. The NMR data were in agreement with those obtained by J.-F. Lavallée for the title compound synthesized by a different method.[22]



8 R = OH R' = CH_3
23 R = OMs R' = CH_3
24 R = N_3 R' = CH_3
25 R = NH_2 R' = CH_3
26 R = N_3 R' = H

5'-*O*-Mesyl-3'-carbomethoxymethyl-5',3'-dideoxythymidine (23).[82]

3'-Carbomethoxymethyl-3'-deoxythymidine **8** (829 mg, 2.8 mmol) was dissolved in methylene chloride (30 ml), under nitrogen atmosphere. After adding triethylamine (0.8 ml, 5.6 mmol), the solution was cooled to -10° C in an ice-salt bath and stirred for 15 min. Mesyl chloride (0.25 ml, 3.3 mmol) was then added over a 15 min period. Stirring was continued for 1 h. The reaction mixture was then diluted with methylene chloride (100 ml) and washed with a saturated sodium bicarbonare solution (2 X15 ml) and water (15 ml). The solution was dried over sodium sulfate and filtered. After evaporation, under reduced pressure, of the solvent the title compound was isolated as a cream-coloured foam in

[82] Compounds **23**, **24** and **25** were prepared according to J.-F. Lavallée's methods.

quantitative yield (1.04 g). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.94 (d, 3 H, Me at C5), 2.17- 2.76 (m, 5 H, H2', H3', H3"), 3.07 (s, 3 H, Me on mesyl), 3.70 (s, 3 H, -OMe), 4.00 (m, 1 H, H4'), 4.40 (A of ABX, 1H, H5'a), 4.54 (B of ABX, 1 H, H5'b), 6.14 (dd, 1 H, H1'), 7.42 (d, 1H, H6). $J_{Me\ at\ C5\ -\ H6}$ = 1.2 Hz, $J_{H5'a\ -\ H5'b}$ = -11.3 Hz, $J_{H5'a\ -\ H4'}$ = 3.63 Hz, $J_{H5'b-\ H4'}$ = 2.32 Hz, $J_{H1'\ -\ H2'a}$ = 4.8 Hz, $J_{H1'\ -\ H2'b}$ = 6.4 Hz. ^{13}C NMR (74.5 MHz, $CDCl_3$): δ 12,42 (Me at C5), 34.57 (C3'), 36.43 (C2'), 37.69 (Me on mesyl), 38.03 (C3"), 52.09 (OMe), 69.05 (C5"), 82,21 (C1'), 84.82 (C1'), 111.35 (C5), 135.20 (C6), 150.30 (C2), 159.17 (C4), 171.69 (C3'"). LRMS (CI - NH_3): m/e 377 (M + H^+, 11 %), 251 (M + H^+ - thymine), 127 (thymine + H^+, 53 %).

5'-Azido-3'-carbomethoxymethyl-5',3'-dideoxythymidine (24).

To a stirred solution of mesylate **23** (1.02 g, 2.71 mmol) in DMF, under nitogen atmosphere, was added lithium azide (623 mg, 12.7 mmol). The solution was heated in a 95° C oil bath for 1 h. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography. The eluents were ethyl acetate / hexanes, 2 / 1, vol / vol. After evaporation of the solvents *in vacuo*, the title compound was isolated as a white foam (684 mg, 78 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.92 (s, 3 H, Me at C5), 2.16 (m, 1 H, H2'a), 2.31 (m, 1 H, H2'b), 2.42 (A of ABX, 1 H, H3"a), 2.50 (B of ABX, 1 H, H3"b), 3.53 (A of ABX, 1 H, H5'a), 3.69 (s, 3 H, -OMe), 3.76 (B of ABX, 1 H, H5'b), 6.14 (dd, 1 H, H1'), 6.46 (s, 1 H, H6). $J_{H3'\ -\ H3"a}$ = 7.3 Hz, $J_{H3'\ -\ H3"b}$ = 6.8 Hz, $J_{H3"a\ -\ H3"b}$ = -16.1 Hz, $J_{H4'\ -\ H5'a}$ = 3.7 Hz, $J_{H4'\ -\ H5'b}$ = 2.9 Hz, $J_{H1'-\ H2'a,b}$ = 4.7, 7.0 Hz. ^{13}C NMR (74.5 MHz, $CDCl_3$): δ 12.9 (Me at C5), 35.5 (C3'), 36.6 (C2'), 38.5 (C3"), 52.4 (-OMe), 52.7 (C5'), 83.4 (C4'), 84.8 (C1'),111.6 (C5), 135.8 (C6), 150.9 (C2), 164.3 (C4), 172.4 (C3'"). HRMS (FAB, NBA): m/e calculated for $C_{13}H_{17}N_5O_6$ + H^+, 324.1308; found 324.1307.

5'-Amino-3'-carbomethoxymethyl-5'-3'-dideoxythymidine (25).

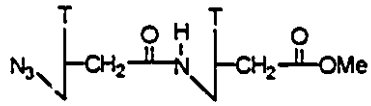
A solution of 5' azidothymidine analogue **24** (450 mg, 1.4 mmol) in methanol (40 ml) was stirred in the presence of Lindlar catalyst (150 mg) under hydrogen at atmospheric pressure for 5 h. The reaction mixture was then filtered through 1 cm celite in a sintered glass filter. The filtrate was evaporated to dryness *in vacuo* to give amine **25** in quantitative yield (412 mg). ^{1}H NMR (200 MHz, CD_3OD): δ 1.88 (s, 3 H, Me at C5), 2.10 - 2.60 (m, 5 H, H2', H3', H3"), 2.85 (A of ABX, 1 H, H5'a), 2.96 (B of ABX, 1 H, H5'b), 3.67 (s, 3 H, -OMe), 3.65 - 3.71 (m, 1 H ,H4'), 6.07 (dd, 1 H, H1'), 7.56 (s, 1 H, H6). $J_{H1'-H2'a}$ = 4.2 Hz, $J_{H1'-H2'b}$ = 7.4 Hz, $J_{H5'a,b}$ = -13.5 Hz, $J_{H4'-H5'a}$ = 7.3 Hz, $J_{H4'-H5'b}$ = 2.0 Hz. ^{13}C NMR (74.5 MHz, CD_3OD): δ 12.3 (Me at C5), 36.9 (C3'), 37.2 (C2'), 38.8 (C3"), 44.8 (C4'), 52.3 (-OMe), 54.7 (C5'), 86.3, 86.9 (C1', C4'), 111.8 (C5), 138.4 (C6), 152.7 (C2), 166.8 (C4), 174.5 (C3'"). HRMS (FAB, glycerol): m/e calculated for $C_{13}H_{19}N_3O_5 + H^+$, 298.1403; found 298.1403.

5'-Azido-3'-carboxymethyl-5',3'-dideoxythymidine (26).

Ester **24** (150 mg, 0.47 mmol) was dissolved in THF (5 ml) and 10% aqueous KOH (0.8 ml) was added. The mixture was stirred for 40 min. It was subsequently acidified with 1M HCl to pH ~ 2 and concentrated under reduced pressure to 1 ml. This was extracted 5 times, each with 20 ml CH_2Cl_2. The organic phases were combined and dried over Na_2SO_4. Evaporation of the solvent yielded the desired product as a white foam (129 mg, 90%). ^{1}H NMR (300 MHz, CD_3OD): δ 1.91 (d, 3H, Me at C5), 2.23 (m, 1H, H2'a), 2.33 - 2.67 (m, 4H, H2'b, H3', H3"), 3.90 (m, 1H, H4'), 6.13 (q, 1H, H1'), 7.64 (d, 1H, H6). $J_{Me\ at\ C5-H6}$ = 1.2Hz, $J_{H4'-H5'a}$ = 2.2 Hz, $J_{H4'-H5'b}$ = 3.5 Hz, $J_{H5'a-H5'b}$ = -13.5Hz, $J_{H1'-H2'a}$ = 7.4Hz, $J_{H1'-H2'b}$ = 4.0 Hz. ^{13}C NMR (75.4 MHz, CD_3OD): δ 12.50 (Me at C5), 36.77 (C3'), 36.97 (C2'), 38.79 (C3"), 53.39 (C5'), 84.78 (C4'), 86.02 (C1'), 111.56 (C5), 137.74 (C6), 152,35 (C2), 166.40 (C4), 175.36 (C3'"). LRMS (CI-NH_3): m/e 310 (10%, M+H$^+$), 184 (10% , M+H$^+$), 127 (100%, Thymine +

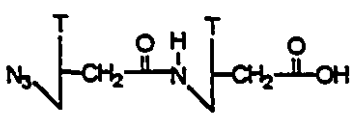
H^+). HRMS (FAB, glycerol): m/e calculated for $C_{13}H_{15}N_5O_5 + H^+$, 310.1152; found 310.1152.

Amide-linked ^{5}5'-azido-^{3}3'-carbomethoxymethyl thymidine dimer (27).



Carboxylic acid **26** (110 mg, 0.36 mmol) and amine **25** (106 mg, 0.36 mmol), DMF (5 ml) and triethylamine (80 µl, 0.58 mmol) were combined in a flask, under nitrogen, and stirred for 10 min. BOP coupling reagent (159 mg, 0.36 mmol) was added and stirring was continued for 1 h. The solvent was then removed *in vacuo*. The brown, oily residue was subjected to silicagel column chromatographic purification (CH_2Cl_2 / methanol, 92 / 8, vol / vol). Dimer **27** was isolated as a colourless sticky foam (182 mg, 87 %). ^{1}H NMR (500 MHz, CD_3OD): δ 1.88 (d, 3H, CH_3 at C5), 1.91 (d, 3H, CH_3 at C5), 2.40 - 2.67 (m, 10 H, H2', H3', H3"), 3.49 -3.72 (m, 4H, H5'), 3.67 (s, 3H, -OMe), 3.79 (m, 1H, H4'), 3.89 (m, 1H, H4'), 6.00 (dd, 1H, ^{5}H1'), 6.11 (dd, 1H, ^{3}H1'), 7.52 (d, 1H, H6), 7.62 (d, 1H, H6). $J_{H6\text{ - Me at C5}}$ = 1.1 Hz, 1.2Hz, $J_{^3H1'\text{-}^3H2'a,b}$ = 3.9 , 7.3 Hz, $J_{^5H1'\text{-}^5H2'a,b}$= 4.2, 7.8 Hz. ^{13}C NMR (125.7 MHz, CD_3OD): δ 12.2 (Me at C6), 36.6 (C3"), 38.5 (C2'), 36.9 (C3'), 38.5 (C2'), 42.0 (C5'), 52.0 (OMe), 53.2 (C5'), 84.5 (C4'), 84.9 (C4'), 85.8 (C1'), 87.8 (C1'), 113.2 (C5), 137.8 (C6), 138.4 (C6), 153.8 (C2), 154.0 (C2), 164.5 (C4), 167.1 (C4), 169.0 (^{5}C3'"), 175.6 (^{3}C'"). LRMS (FAB, NBA): m/e 589 (13%, M+H$^+$), 337 (100%, M+H$^+$-2 thymine). HRMS (FAB, glycerol): m/e calculated for $C_{25}H_{22}N_8O_9 + H^+$, 589.2370; found 589.2370.

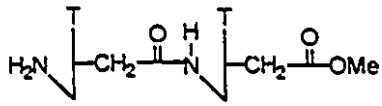
Dimer carboxylic acid (28).



To a solution of dimer **27** (90 mg, 0.15 mmol) in THF (2 ml) was added aqueous KOH (0.3 ml, 1M). After stirring 1.5 h, the solution was acidified with 0.1 M HCl to

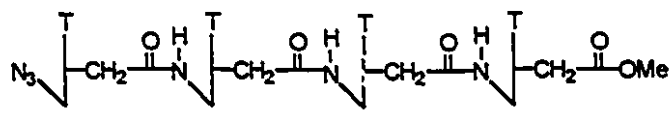
pH ~ 6. The reaction mixture was evaporated to dryness under reduced pressure. The white, powdery residue was used without further purification for the synthesis of tetramer **30**.

Amino dimer (29) .



A solution of dimer **27** (75 mg, 0.13 mmol) in methanol (10 ml) was placed in a hydrogenation bottle and shaken for 5 h under hydrogen pressure (5 lbs) in the presence of Lindlar catalyst (30 mg). The reaction was followed by TLC. When all the starting material had been replaced by a new, more polar, spot which tested positive to ninhydrin, the hydrogenation was stopped. Methanol (10 ml) was added prior to filtration through a sintered glass filter, containing 1 cm celite. After filtration the celite was rinsed with methanol (15 ml). Evaporation of the solvent under reduced pressure yielded a white powder (60 mg, 84%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.89 (d, 3H, Me at C6), 1.91 (d, 3H, Me at C6), 3.68 (s, 3H, OMe), 5.98 (dd, 1H, H1'), 6.04 (dd, 1H, H1'), 7.99 (d. 1H, H6), 7.53 (d, 1H. H6). $J_{\text{Me at C5 - H6}}$ = 1.0 Hz and 1.0 Hz, $J_{\text{H1' - H2'a,b}}$ = 4.4, 7.8 Hz and 3.9, 7.3 Hz.

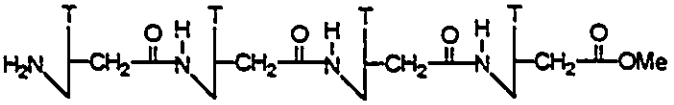
Tetramer (30).



The reaction conditions for the synthesis of tetramer **30** were the same as those for the formation of dimer **27**. Equimolar amounts of acid **28** (43 mg, 0.075 mmol) and amine **29** (42 mg, 0.075 mmol) were dissolved in DMF (5 ml). Triethylamine (20 µl, 0.15 mmol) and BOP coupling reagent (35 mg, 0.80 mmol) were added. The eluent that was used for the silcagel chromatography of the tetramer was methylene chloride / methanol, 100 / 12,

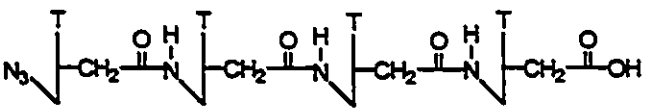
vol / vol. The title compound was isolated as a white, amorphous, solid (68 mg, 80%). ^{1}H NMR (500 MHz, $CDCl_3$ / CD_3OD, 5 / 1): δ 1.73 (s, 12 H, 4 X Me at C5), 3.52 (s, 3 H, -OMe), 5.75 (m, 3 H, 3 X H1'), 5.91 (dd, 1H, H1'), 7.12 (s, 1H, H6), 7.14 (s, 1H, H6), 7.18 (s, 1H, H6), 7.24 (s, 1H, H6). LRMS (FAB, NBA): 1123 (18.6%, $M+H^+ + 4H$), 997 (21.0%, $M+H^+ + 4H$- thymine), 995 (19.5%, $M+H^+ + 2H$ - thymine), 869 (22.3%, $M+H^+ + 2H$ - 2 thymine), 743 (20.2%, $M+H^+ + 2H$ - 3 thymine), 617 ($M+H^+ + 2H$ - 4 thymine).

Tetramer amine (31).



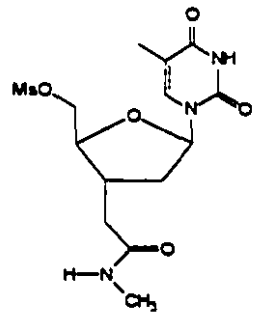
5'-Azido tetramer **30** (26 mg, 0.023 mmol) was dissolved in a mixture of water (5 ml) and methanol (5ml). The solution was transferred to a hydrogenation bottle and platinum (IV) oxide (5 mg) was added. The mixture was shaken under hydrogen at 30 lbs pressure for 0.5 h, after which the pressure was increased to 40 lbs and shaking continued for 1.5 h. The reaction was monitored by TLC, the same way as was the hydrogenation of azido dimer **27**. When the reaction was finished, a white precipitate had formed which was dissolved by the addition of 1M HCl (23 µl). The catalyst was filtered off through 1 cm celite in a sintered glass filter and rinsed with 5 ml water. After the solvent was evaporated, a white powder (22 mg) was obtained. ^{1}H NMR (500 MHz, CD_3OD). selected data: δ 1.91 (s, 3 H, Me at C5), 1.93 (s, 9 H, 3 X Me at C5), 3.34 (s, 3 H, -OMe). 5.98 (m, 3 H, 3 X H1'), 6.03 (m, 1 H, H1'), 7.45 (s, 1 H, H6), 7.50 (s, 2 H, 2 X H6), 7.52 (s, 1 H, H6).

Tetramer acid (32).



The hydrolysis of tetramer **30** was effected in the following manner. The tetramer (26 mg, 0.023 mmol) was dissolved in a mixture of methanol (0.7 ml) and 0.5 M KOH (0.3 ml). The reaction was stirred for 5 h. It was then acidified to pH ~ 6 with 0.1 M HCl. The solution was evaporated to dryness under reduced pressure and used without further purification for coupling.

5'-*O*-Mesyl-3'-methylamide thymidine analogue (33).



5'-*O*-Mesyl-3'-carbomethoxymethyl-3'-deoxythymidine **23** (80 mg, 0.21 mmol) was dissolved in 40 % aqueous methylamine (1 ml) in an open round-bottomed flask. It was stirred and the reaction was monitored by TLC. After 0.5 h the starting material spot had disappeared in favour of a more polar spot. The solvent was subsequently removed *in vacuo*. The residue was a white foam (80 mg, quantitative yield). ^{1}H NMR (200 MHz, CD_3OD): δ 1.91 (s, 3 H, Me at C5), 2.13 - 2.48 (m, 5 H, H2', H3', H3"), 2.72 (m, 5 H, H5', -NMe), 3.14 (s, 3 H, Me on mesyl), 4.03 (m, 1 H, H4'), 4.42 (A of ABX, 1 H, H5'a), 4.56 (B of ABX, 1 H, H5'b), 6.12 (dd, 1 H, H1'), 7.64 (s, 1 H, H6). $J_{H1'-H2'a}$ = 4.5 Hz, $J_{H1'-H2'b}$ = 6.9 Hz, $J_{H5'a-H5'b}$ = -11.7 Hz, $J_{H4'-H5'a}$ = 2.52 Hz, $J_{H4'-H5'b}$ = 3.71 Hz.

5'-*O*-Mesyl-3'-allyl-3'-deoxythymidine (34).

3'-Allyl-3'-deoxythymidine (3.53 g, 13.3 mmol) was dissolved in methylene chloride under nitrogen atmosphere. After addition of triethylamine (2.4 ml, 18.0 mmol)), the solution was cooled to -10° C in an ice-salt bath. Mesyl chloride (1.24 ml, 16.0 mmol) was added gradually over 10 min. The reaction mixture was stirred at -10° for 1h. The ice-salt bath was then removed and the reaction quenched with an aqueous saturated ammonium chloride solution (25 ml). The mixture was diluted with 500 ml methylene chloride and washed twice with saturated sodium bicarbonate (25 ml, each) and once with water (25 ml). It was then dried over sodium sulfate and filtered. The solvent was subsequently removed under reduced pressure to yield the title compound as a cream-coloured foam (4.00 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94 (s, 3 H, Me at C5),

2.16 - 2.36 (m, 5 H, H2', H3', H3"), 3.03 (s, 3 H, Me on mesyl), 3.91 (m, 1H, H4'), 4.36 (A of ABX, 1H, H5'a), 4.48 (B of ABX, 1H, H5'b), 5.10 (m, 2H, H3""), 5.71 (m, 1H, H3""), 6.12 (dd, 1H, H1'), 7.39 (s, 1H, H6), 9.18 (br s, 1H, H on N3). $J_{H5'a - H5'b}$ = -11.3 Hz, $J_{H5'a - H4'}$ = 3.6 Hz, $J_{H5'b - H4'}$ = 2.3 Hz, $J_{H1' - H2'a}$ = 5.0 Hz, $J_{H1' - H2'b}$ = 6.0 Hz. ^{13}C NMR (74.5 MHz, $CDCl_3$): δ 12.35 (Me at C5), 36.24 (C2'), 37.45 (Me on mesyl), 37.73 (C3'), 38.12 (C3"), 68.99 (C5'), 82.49 (C4'), 85.09 (C1'), 111.12 (C5), 119.23 (C3""), 137.83 (C6), 149.87 (C2), 149.89 (C3""), 169.80 (C4). LRMS (FAB, NBA): m/e 345 (M + H^+, 13.9 %), 219 (M + H^+ - thymine, 27.8 %), 127 (thymine + H^+, 100 %)

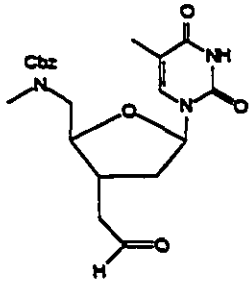
5'-*N*-Methylamino-3'-allyl-5',3'-dideoxythymidine (35).

Mesylate **34** (2.0 g, 5.8 mmol) was dissolved in 40 % aqueous methylamine solution (10 ml). The reaction conditions were the same as those for the synthesis of 5'-*N*-methylamino-5'-deoxythymidine **12**, except for the reaction time which was 6 h in this case. The crude product was purified by flash chromatography (methylene chloride / methanol, 100 / 4, vol / vol, then methylene chloride / methanol / triethylamine, 100 / 10 / 1, vol / vol / vol). After removal of the solvents a cream-coloured foam (1.15 g, 71 %) was obtained. ^1H NMR (200 MHz, CD_3OD): δ 1.90 (d, 3H, Me at C5), 2.12 - 2.40 (m, 5 H, H2', H3', H3"), 2.45 (s, -NMe), 2.81 (A of ABX, 1 H, H5'a), 2.85 (B of ABX, 1 H, H5'b), 3.80 (m, 1 H, H4'), 5.09 (m, 2 H, H3""), 5.83 (m, 1 H, H3""), 6.06 (dd, 1 H, H1'), 7.52 (d, 1 H, H6). $J_{H5'a - H5'b}$ = -12.6 Hz, $J_{H5'a - H4'}$ = 8.2 Hz, $J_{H5'b - H4'}$ = 3.7 Hz, $J_{H1' - H2'a}$ = 4.0 Hz, $J_{H1' - H2'b}$ = 6.6 Hz, $J_{H6 - Me\ at\ C5}$ = 1.1 Hz. ^{13}C NMR (74.5 MHz, CD_3OD): δ 12.05 (Me at C5), 33.05 (N-Me), 35.34, 35.78 (C2', C3'), 38.60 (C3"), 50.79 (C5'), 79.19 (C4'), 84.22 (C1'), 109.83 (C5), 116.88 (C3""), 135.87 (C6), 136.56 (C3""), 150.37 (C2), 163.68 (C4). LRMS (FAB, NBA): m/e 280 (M + H^+, 42.6 %), 154 (M+H^+ - thymine, 100 %), 127 (thymine + H^+, 98.8 %).

5'-*N*-Benzyloxycarbonyl-*N*-methylamino-3'-allyl-5',3'-dideoxythymidine (36).

5'-*N*-Methylamino-3'-allyl-5',3'-dideoxythymidine (1.40 g, 5.00 mmol) was dissolved in aqueous potassium bicarbonate (25 ml, 1 M) and left to stir in an ice bath for 10 min. A solution of benzoyl chloroformate (930 µl, 6.52 mmol) in ethyl acetate (25 ml) was added, dropwise, over 20 min. The reaction was stirred vigorously at 0° C for 2 h. Ethyl acetate (100 ml) was then added to the mixture and the aqueous and organic phases were separated. The organic phase was washed with water (2 X 10 ml), dried over sodium sulfate and filtered. The crude oil was purified by flash chromatography (ethyl acetate / hexanes, 1 / 1, vol / vol). After evaporation under reduced pressure of the solvent, a cream-coloured foam was obtained (1. 76 g, 85 %). The NMR spectrum indicated the presence of two rotamers in a 3 : 1 ratio. ^{1} H NMR (200 MHz, $CDCl_3$): δ 1.89,1.91 (minor, major, s, s, 3 H, Me at C 5), 1.95 - 2.40 (m, 5 H, H2', H3', H3"), 3.00 (s, 3 H, -NMe), 3.43 - 3.93 (m, 3 H, H4', H5'), 4.92 - 5.23 (m, 4 H, benzylic -CH_2, H3""), 5.49 (m, 1 H, H3'"), 6.07 (m, 1H, H1'), 7.10, 7.42 (minor, major, s, s, 1 H, H6), 7.32 (s, 5 H, benzyl aromatic), 8.39 (br s, 1 H, H at N3).

Thymidine 3' aldehyde analogue (37).



To a solution of 3'-allyl nucleoside **36** (1.75 g, 4.24 mmol) in THF (40 ml) were added water (20 ml) and osmium tetroxide (26 mg, 0.10 mmol). The mixture was stirred vigorously, while sodium periodate (1.82 g, 8.50 mmol) was added in six portions over 20 min. Stirring was continued for 0.5 h. The volume of the solvents was reduced by half, *in vacuo*, before ethyl actate (150 ml) was added. The mixture was washed with saturated bicarbonate (20 ml) and water (2 X 15 ml). The crude ethyl acetate solution was dried

over sodium sulfate and filtered. After evaporation of the solvent the brown, oily, residue was purified by column chromatography, with ethyl acetate as eluent. The title compound was isolated as a white foam (1.32 g) in 75 % yield. The NMR spectrum indicated the presence of two rotamers in a ratio of 3 : 1. ^{1}H NMR (200 MHz, $CDCl_3$) : δ 1.91 (s, 3 H, Me at C5), 2.22 -2.61 (m, 5 H, H2', H3', H3"), 2.99 (s, 3 H, -NMe), 3.45 - 3.83 (m, 3 H, H4', H5'), 5.12 (s, 2 H, benzylic -CH_2), 6.13 (m, 1H, H1'), 7.08, 7.31 (minor, major, s, s, 1 H, H6), 7.35 (s, 5 H, benzyl aromatic), 8.20 (br s, 1 H, H at N3), 9.40, 9.78 (minor, major, s, s, 1 H, aldehyde). LRMS (FAB, NBA): m/e 416 ($M+H^+$, 7.6 %), 290 ($M+H^+$-thymine, 19.5 %).

5'-*N*-Benzyloxycarbonyl-5'-*N*-methylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (38).

A solution of aldehyde 37 (1.00 g, 2.41 mmol) and dry methanol (580 µl, 14.4 mmol) in dry DMF (22 ml) was allowed to stir under nitrogen atmosphere for 3/4 h, before the addition of pyridinium dichromate (5.43 g, 14.4 mmol). After stirring the reaction for 2 h, the colour had changed from deep orange to dark green. The mixture was poured into ethyl acetate (100 ml), filtered through silicagel (1.5 cm) and rinsed through with ethyl acetate (30 ml). The solvent was evaporated, under reduced pressure, from the colourless solution, to yield a white foam (929 mg, 87 %). The proton NMR spectrum showed the presence of two rotamers in a 1 : 3 ratio. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.89 (s, 3 H, Me at C5), 2.09 - 2.72 (m, 5 H, H2', H3', H3"), 2.99 (s, 3 H, -NMe), 3.42 - 3.92 (m, 6 H, H4', H5', -OMe), 5.13 (unresolved ABX, 2 H, benzylic -CH_2), 6.12 (m, 1 H, H1'), 7.09, 7.38 (minor, major, s, s, 1 H, H6), 7.32 (s, 5 H, benzyl aromatic), 9.34 (br s, 1 H, H at N3). LRMS (CI, NH_3): m/e 446 ($M+H^+$, 9.9 %), 320 ($M+H^+$ - thymine, 100 %), 127 (thymine + H^+, 25.9 %).

5'-*N*-Benzyloxycarbonyl-5'-*N*-methylamino-3'-carboxymethyl-5',3'-dideoxythymidine (39).

Ester **38** (22 mg, 0.05 mmol) was hydrolyzed in the following manner. It was first dissolved in THF (1.5 ml). Aqueous potassium hydroxyde (0.2 ml, 1 M) was subsequently added and the reaction was stirred for 0.5 h. The mixture was acidified by stirring for 10 min with 50 mg weakly acidic resin (Rexyn* RG 51 (H), medium porosity, exchange capacity 10.3 m eq /g). It was filtered and the solvents evaporated under reduced pressure, to leave a white foam (22 mg, 100 %).

The reaction was monitored by TLC (methylene chloride / methanol, 10 / 1, vol / vol). The R_f of the starting material was 0.8, while that of the product was 0.3. The reaction was stopped when all the starting material had disappeared. No UV-positive impurities were observed. The proton NMR was too poorly resolved to be analyzed.

5'-*N*-Methylamino- 3'-carbomethoxymethyl-5',3'-dideoxythymidine (40). (Method I)

A solution of Cbz-protected methylamine **38** (150 mg, 0.33 mmol) in absolute ethanol (20 ml) and Pd/C (10 %, 60 mg) were placed in a hydrogenation vessel. The reaction mixture was shaken under 10 lb hydrogen atmosphere for 5 h, before being filtered through a sintered glass filter, containing 1 cm celite. The filter was subsequently rinsed with 5 ml ethanol. The solvent was removed under vacuum to yield a cream-coloured foam, which was purified by flash chromatography (methylene chloride / methanol / TEA, 100 / 20 / 1, vol / vol / vol). Compound **40** (60 mg) was isolated in 58 % yield. ^{1}H NMR (300 MHz, CD_3OD): 1.90 (d, 3H, Me at C5), 2.21 (m, 1 H, H2'a), 2.53 (m, 1 H, H2'b), 2,54 - 2.72 (m, 3 H, H3', H3"), 2.75 (s, 3 H, -NMe), 3.29 - 3.39 (ABX, obscured by solvent lines, 2H, H5'), 3.98 (m, 1H, H4'), 6.08 (dd, 1 H, H1'), 7.49 (d, 1H, H6). $J_{Me\ at\ C5\ -\ H6}$ =1.1 Hz, $J_{H1'-H2'a}$ = 4.7 Hz, $J_{H1'-H2'b}$ = 7.7 Hz. ^{13}C NMR (74.9 MHz, CD_3OD): δ 12.29 (Me at C5), 35.53 (-NMe), 36.83 ((C2'), 37.77 (C3"), 38.39 (C3'), 52.31 (-OMe), 53.27 (C5'), 81.78 (C1'), 87.83 (C4'), 118.78 (C5), 138.92 (C6), 152.25

(C2), 168.75 (C4), 173.90 (C3'''). LRMS (FAB, NBA): m/e 312 ($M + H^+$, 96.6 %), 186 ($M + H^+$ - thymine, 100 %), 127 (thymine + H^+, 82.0 %).

_N_-9-Fluorenylmethoxycarbonylglycine ethyl ester (41).

The ethyl ester of glycine.HCl (70 mg, 0.50 mmol) was dissolved in aqueous KOH (2.5 ml, 1 M) and cooled in an ice bath to 0°. To this solution was added, over 10 min, fmoc chloride (143mg, 0.55 mmol), previously dissolved in ethyl acetate (2.5 ml). The reaction mixture was stirred vigorously for 0.5 h. The ice bath was then removed and stirring continued for 0.5 h. Ethyl acetate (10 ml) was added to the mixture and the aqueous and organic phases were separated. The organic phase was washed with water (2 X 2 ml), dried over sodium sulfate and filtered. After evaporation of the solvent, the product was isolated as a white powder. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.30 (t, 3 H, CH_2CH_3), 3.82 (d, 2 H,-$NCH_2C(O)$-), 4.23 (m, 3 H, -OCH_2CH_3 + fluorenyl non-aromatic H), 4.42 (d, 2H, -$CHCH_2O$-), 5.31 (br s, 1 H, -NH-), 7.29 -7.43 (m, 4 H, fluorenyl aromatic Hb, Hc), 7.58 (d, 2 H, fluorenyl aromatic Ha), 7.75 (d, 2 H, fluorenyl aromatic Hd). $J_{CH2-CH3}$ = 6.7 Hz, $J_{CH(fmoc)-CH2(fmoc)}$ = 6.6 Hz, $J_{Ha(fmoc)-Hb(fmoc)}$ = 7.2 Hz, $J_{Hc(fmoc)-Hd(fmoc)}$ = 7.7 Hz.

Hydrolysis of ester 41.

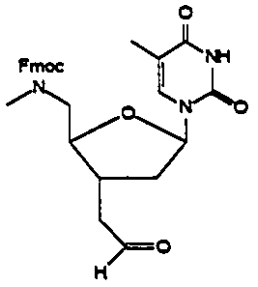
To a solution of the ethyl ester of fmoc-glycine (33 mg, 0.10 mmol) in toluene (1 ml) under nitrogen atmosphere, was added bis(*n*-tributyltin) oxide (100 µl, 0.20 mmol). The reaction mixture was heated under reflux, while stirring, for 6 h in a 75° C oil bath and left at room temperature overnight. After the addition of 0.5 M HCl (0.5 ml) a white precipitate formed, which was dissoved by adding ethyl acetate (2 ml). The organic and aqueous layers were separated. The organic phase was extracted with 5 % aqueous potassium carbonate (3 X 5 ml) and the aqueous layer acidified with 1 M HCl to pH~3. The carboxylic acid was extracted from the aqueous layer with ethyl acetate (3 X 5 ml).

This solution was dried over sodium sulfate and filtered. The solvent was evaporated under reduced pressure, leaving the product (23 mg, 70 %) as a white powder. ^{1}H NMR (200 MHz, CD_3OD): δ 3.82 (d, 2 H, -$NCH_2C(O)OH$), 4.22 - 4.39 (3 H, m, -$CHCH_2O$-), 7.25 -7.85 (m, 8 H, fluorenyl aromatic.

5'-*N*-9-Fluorenylmethoxycarbonyl-*N*-methylamino-3'-allyl-5',3'-dideoxythymidine (42).

A solution of 5'-*N*-methylamino-3'-allyl-5',3'-dideoxythymidine 35 (40 mg, 0.14 mmol) in aqueous potassium bicarbonate (0.75 ml, 1M) was cooled to 0° in an ice-water bath under nitrogen atmosphere. Fmoc chloride (43 mg, 0.16 mmol), dissolved in ethyl acetate (0.75 ml) was added dropwise over 5 min, after which the reaction mixture was stirred for 10 min. The ice bath was then removed and stirring was continued for 1.5 h. The reaction mixture was subsequently diluted with ethyl acetate (30 ml) and washed two times with water (5 ml, each). The organic phase was dried over anhydrous sodium sulfate and filtered. The solvent was removed under reduced pressure, leaving a lightly coloured syrup. After column chromatography (hexanes / ethyl acetate, 1 / 1, vol / vol), the title compound was isolated as a white foam (57 mg, 80 %). The NMR proton spectrum indicated the presence of two rotamers in a ratio of one to two. Due to the complexity of the spectrum no detailed analysis was performed. ^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.88, 1.92 (minor, major, s, s, 3 H, Me at C5), 2.92, 2.99 (minor, major, s, s, 3 H, -NMe), 5.94, 6.06 (minor, major, dd, t, 1 H, H1'), 6.99, 7.38 (minor, major, s, s, 1H, H6), 7.28 - 7.78 (m. 8 H, fluorenyl aromatic), 8.75, 8.82 (major, minor, s, s, 1 H, H at N3). LRMS (FAB, NBA): m/e 502 ($M + H^+$,11.1 %), 376 ($M + H^+$ - thymine, 5.5 %). HRMS (FAB, glycerol): m/e calculated for $C_{29}H_{31}N_3O_5 + H^+$, 502.2340; found 502.23443.

Thymidine 3' aldehyde analogue (43).



To a solution of 3'-allyl compound 42 (1.20 g., 2.4 mmol) in THF (15 ml), were added water (15 ml) and osmium tetroxide (28 mg, 0.10 mmol). Sodium periodate (1.17 g, 5.4 mmol) was added in four equal portions over a period of 10 min, while the mixture was stirred vigorously. Stirring was continued for a total of 45 min, after which methylene chloride (500 ml) was added. This mixture was washed with saturated sodium bicarbonate (2 times, 50 ml each) and water (50 ml), before being dried over anhydrous sodium sulfate. The solution was filtered and the solvent removed under vacuum. The crude product was purified by column chromatography (hexanes / ethyl acetate, 1 / 1, vol / vol, then ethyl acetate). After evaporation of the solvents under reduced pressure, a white foam (965 mg) was obtained in 80 % yield. The NMR proton spectrum showed the presence of two rotamers in a one to two ratio. ^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.95 (s, 3 H, Me at C5), 2.92, 2.99 (minor, major, s, s, 3 H, -NMe), 5 94, 6.13 (minor, major, dd, t, 1 H, H1'), 6.95, 7.32 (minor, major, s, s, 1 H, H6), 7.26 - 7.78 (m, 8 H, fluorenyl aromatic), 8.76, 8.80 (major, minor, s, s, 1H, H at N3), 9.50, 9.78 (minor, major, s, s, 1H, aldehyde). ^{13}C (MHz, $CDCl_3$), selected data: δ 12.5 (Me at C5), 199.70, 199.76 (C3'''). LRMS (FAB, NBA): m/e 504 (M + H^+, 19.4 %), 378 (M + H^+ - thymine, 31.7 %). HRMS (FAB, glycerol): m/e calculated for $C_{28}H_{29}N_3O_6$ + H^+, 504.21346; found 504.21363.

5'-*N*-9-Fluorenylmethoxycarbonyl-*N*-methylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (44).

A solution of aldehyde **43** (960 mg,1.9 mmol) in dry DMF (20 ml) was stirred with dry methanol (460 µl, 11.4 mmol) under nitrogen atmosphere for 45 min, before the addition of pyridinium dichromate (4.28 g, 11.4 mmol). Stirring was continued for 1.5 h, while the colour of the mixture changed from dark orange to dark green. When the reaction was complete, the mixture was poured into ethyl acetate (150 ml). The resulting slurry was filtered through 1.5 cm silicagel, previously wetted with ethyl acetate, and rinsed with 50 ml ethyl acetate. The colourless filtrate was collected and the solvent removed under reduced pressure to yield the the title compound as a pale yellow foam (990 mg, 98 %). The NMR proton spectrum indicated the presence of two rotamers (one to two ratio). ^{1}H NMR (300 MHz, $CDCl_3$), selected data: δ 1.90 (s, 3 H, Me at C5), 2.90, 2.99 (minor, major, s, s, 3 H, -NMe), 3.67 (s, 3 H, -OMe), 4.23 (t, 1 H, fluorenyl non-aromatic -C**H**-), 4.42 , 4.50 - 4.85 (major d, minor ABX, 2H, fluorenyl non-aromatic -CH_2-), 5.95, 6.11 (minor, major, br s, t, 1 H, H1'), 6.96, 7.35 (minor, major, s, s, 1 H, H6), 7.34 (m, 4 H, fluorenyl aromatic b, c), 7.57 (d, 2 H, fluorenyl aromatic a), 7.78 (d, 2 H, fluorenyl aromatic d), 8.69 (br s,1H, H on N3). LRMS (FAB, NBA): m/e 534 (1.6 %, M + H^+), 408 (M + H^+ -thymine, 28.6 %).

5'-*N*-Fluorenylmethoxycarbonylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (45).

A solution of 5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine **25** (30 mg, 0.10 mmol) in dry pyridine (0.3 ml), under nitrogen atmosphere, was cooled in an ice-water bath. Fmoc chloride (39 mg, 0.15 mmol) was added and the mixture was stirred for 5 min. The cooling bath was then removed and stirring continued for 5 h.The solvent was evaporated and the crude product was purified by flash chromatography, using ethyl acetate / hexanes, 3 / 2, vol / vol as eluent. The title compound (36 mg, 69 %) was

obtained as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.24 (s, 3 H, Me at C5), 2.05 - 2.68 (overlapping m, 5 H, H2', H3', H3"), 3.31 -3.80 (m, 3 H, H4', H5'), 3.69 (s, 3 H, -OMe), 4.22 (t, 1 H, fluorenyl non-aromatic -CH-), 4.42 (d, 2 H, fluorenyl non-atomatic -CH_2-), 5.40 (br t, 1 H, carbamate -NH-), 6.06 (dd, 1 H, H1'), 7.35 (s, 1 H, H6), 7.21 -7.42 (m, 4 H, fluorenyl aromatic Hb, Hc), 7.58 (d, 2 H, fluorenyl aromatic Ha), 7.74 (d, 2 H, fluorenyl aromatic Hd), 9.04 (s, 1 H, H at N3). $J_{CH(fmoc)-CH2(fmoc)}$ = 6.6 Hz, $J_{H1'-H2a'}$ = 6.7 Hz, $J_{H1'-H2b'}$ = 4.4 Hz, $J_{Ha(fmoc)-Hb(fmoc)}$ = 7.3 Hz, $J_{Hc(fmoc)-Hd(fmoc)}$ = 7.6 Hz.

5'-Methylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (40). (Method II)

The amino group of compound **44** (350 mg, 0.66 mmol) was deprotected by dissolving the compound in methylene chloride (1.4 ml) and stirring it in the presence of piperidine (0.35 ml) for 10 min.The solvent and the excess piperidine were evaporated under reduced pressure. The crude product was chromatographed over silica gel, with methylene chloride / methanol / triethylamine, 90 / 10 / 1, vol / vol / vol, as eluent. After removal of the solvents *in vacuo*, the title compound was obtained as a hard, white foam (164 mg, 80 %). The NMR data were identical to those of methylamine **40**, obtained by method I. HRMS (FAB, glycerol): m/e calculated for $C_{14}H_{21}N_3O_5 + H^+$, 312.15594; found 312.15582.

5'-*N*-9-Fluorenylmethoxycarbonyl-*N*-methylamino-3'-carboxymethyl-5',3'-dideoxythymidine (46). (Method I)

Ester **44** (41 mg, 0.075 mmol) was placed in a round-bottomed flask, equipped with a reflux condenser, and dissolved under nitrogen atmosphere in dry toluene (0.75 ml). Bis(*n*-tributyltin)oxide (75 µl, 0.15 mmol) was added and the reaction mixture was stirred and heated in a 90° C oil bath for 10 h. It was then allowed to cool to room temperature before 0.5 M HCl (0.5 ml) was added. The mixture was diluted with ethyl

acetate (30 ml) and the aqueous and the organic phases were separated. The organic layer was extracted with a cold 5 % aqueous potassium carbonate solution (3 X 4 ml). The aqeous extracts were combined and acidified to pH~3 with 0.5 M HCl. The resulting precipitate was filtered off and dried under vacuum to give the pure title compound (22 mg) in 50 % yield. The poorly resolved proton NMR spectrum showed two rotamers in a one to four ratio. ^{1}H NMR (300 MHz, CD_3OD), selected data: δ 1.80 (s, 3 H, Me at C5), 2.85 , 2.90 (s, s, 3 H, -NMe), 5.91- 6.12 (unresolved m, 1 H, H1'), 7.22-7.85 (m, 9 H, fluorenyl aromatic + H6). ^{13}C NMR (74.9 MHz, CD_3OD): δ 12.2, 15.1 (Me at C5), 34.9 (C2'), 35.8 (C3"), 36.4 (C3'), 37.9 (-NMe), 51,2 (C5'), 65.7 (fmoc CH_2), 67.5 (fmoc CH), 82.3 82.5 (C1'), 84.4, 85.0 (C4'), 110.8 (C5), 119.9,124.2, 124.4,126.7, 127.6 ,141.3,143.9 (fmoc aromatic),135.3,135.8 (C6),150.2 (C2),156.8 (carbamate carbonyl),164.4 (C4), 175.0 (carboxyl). LRMS (FAB, NBA): m/e 520 (5.1 %, $M+H^+$), 394 (8.8 %, $M+H^+$ - thymine).

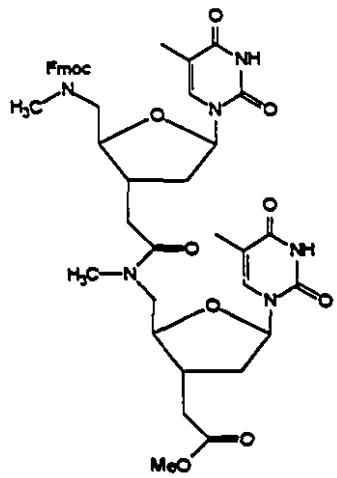
Alternatively, after the acidification of the aqueous extracts, the product was extracted with ethyl acetate (3 X 10 ml). This increased the yield to 68 %, but the NMR spectrum of the compound, obtained by this method, indicated the presence of some impurities.

5'-*N*-9-Fluorenylmethoxycarbonyl-*N*-methylamino-3'-carboxymethyl-5',3'-dideoxythymidine (46). (Method II)

A solution of aldehyde **43** (250 mg, 0.52 mmol) in dry DMF (1.6 ml) was stirred with pyridinium dichromate (780 mg, 3.08 mmol) under nitrogen atmosphere for 2.5 h. The resulting dark green solution was poured into water (100 ml). A precipitate formed which was filtered off and rinsed with water until the water ran clear. The precipitate was redissolved in a mixture of ethyl acetate (40 ml) and methylene chloride (10 ml). This solution was dried over sodium sulfate and filtered. The solvents were removed under reduced pressure, leaving the title compound as a cream-coloured, hard foam (210 mg, 82 %). NMR data were the same as those for this compound obtained by the first method.

HRMS (FAB, glycerol): m/e calculated for $C_{28}H_{29}N_3O_7 + H^+$, 520.20837; found 520.20820.

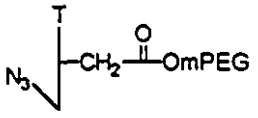
Methylamide homo dimer (47).



Carboxylic acid **46** (11mg, 0.02 mmol) and methylamine **40** (7 mg, 0.02 mmol) were combined in a flask under nitrogen atmosphere. Dry DMF (0.5 ml) and TEA (6 µl, 0.04 mmol) were added and the solution was stirred for 0.5 h, before the addition of BOP coupling reagent (8 mg, 0.02 mmol). Stirring was continued for 2 h.The solvent was then evaporated under vacuum, taking care that no residual DMF remained in the crude product. The resulting brown oil was purified by silicagel chromatography (methylene chloride / methanol, 20 / 1, vol / vol). After removal of the solvents the title compound was isolated as a white foam (14 mg) in 80 % yield. The ^1H NMR spectrum was highly complex and gave multiple overlapping signals between 5.78 ppm and 6.21 ppm for the 2 anomeric protons, indicating the presence of the expected 4 rotamers. LRMS (FAB, NBA): m/e 687 ($M + H^+$ - thymine, 1.7 %), 561 ($M + H^+$ - 2 thymine, 0.9 %). HRMS (FAB, glycerol): m/e calculated for $C_{42}H_{48}N_6O_{11} + H^+$, 813.3459; found 813.3459.

4.4 Experimental for section 2.13, 2.14 and 2.15.

mPEG ester of 5'-azido-5'-deoxy-3'-carboxymethyl- 3'-deoxythymidine (48).



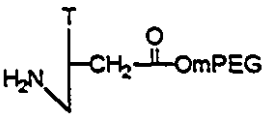
Carboxylic acid **26** (30 mg, 0.10 mol) and methylene chloride (3 ml) were combined under nitrogen atmosphere in a flask. Sufficient pyridine (60 µl) was added to bring the carboxylic acid into solution. DCC coupling reagent (30 mg, 0.15 mmol) was added and the solution stirred for 10 min.

During this time a fine white precipitate had formed. mPEG 5000 (400 mg, 0.08 mmol) and DMAP (10 mg, 0.08 mmol) were then added and stirring was continued for 3h. The reaction was monitored by TLC (methylene chloride / methanol, 5 / 1, vol / vol) for disappearance of mPEG. This was done by comparing the size of the UV-positive spot with the size of the area, coloured brown after development in an iodine chamber. When there was no longer a difference, the reaction mixture was transferred to a beaker and the flask was rinsed with 1 ml methylene chloride which was also poured into the beaker. To this mixture was added diethyl ether (200 ml). A voluminous, floculent, white precipitate formed, which was allowed to stand for 30 min before being filtered through a paper filter and washed with 30 ml ether. After drying under reduced pressure, the title compound (410 mg, 95 %) was isolated as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.88 (s, 3 H, Me at C5), 2.12 (m, 1 H, H2'a), 2.25 (m, 1 H, H2'b), 2.40 (A of ABX, 1 H, H3"a), 2.48 (B of ABX, 1 H, H3"b), 2.63 (m, 1 H, H3'), 3.31 (s, 3 H, Me end group of mPEG), 3.28 - 3.80 (large m, PEG, H4', H5'), 4.19 (m, 2 H, -CH_2O(CO)-), 6.07 (dd, 1H, H1'), 7.38 (s, 1 H, H6), 8.68 (s, 1 H, H at N3). $J_{H3"a-H3"b}$ = -16.6 Hz, $J_{H3'-H3"a}$ = 7.3 Hz, $J_{H3'-H3"b}$ = 6.8 Hz, $J_{H1'-H2'a}$ = 4.9 Hz, $J_{H1'-H2'b}$ = 6.8 Hz.

^{13}C NMR (125.7 MHz, $CDCl_3$): δ 12.6 (Me at C5), 35.1 (C3"), 36.5 (C3'), 38.1 (C2'), 52.3 (C5', -OCH_3), 58.9 (-$CH_2CH_2OCH_3$), 63.9 (-$CH_2CH_2OC(O)$-), 68.8

(-$CH_2CH_2OC(O)$-), 70.5 (PEG), 71.8 (-$CH_2CH_2OCH_3$), 82.9 (C4'), 84.3 (C1'), 112.6 (C5), 135.1 (C6), 158.9 (C2), 164.5 (C4), 171.1 (-C(O)O-). MALDI (dithranol): m/e 5296; peak distribution 44 mass units apart. Calculated mass: 5295 for n = 113.

mPEG ester of 5'-amino-5'-deoxy-3'-carboxymethyl-3'-deoxythymidine (49). Method I.

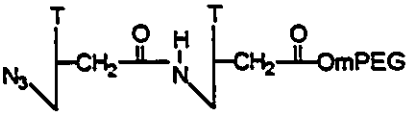


Azido mPEG conjugate **48** (700 mg, 0.13 mmol) was dissolved in a mixture of water (10 ml) and ethanol (10 ml). Lindlar catalyst (20 mg) was added. The reaction mixture was stirred vigorously under hydrogen at atmospheric pressure for 4 h, when a further 10 mg catalyst was added. The reaction was allowed to proceed for another 2h. Hydrogenation was then discontinued and the reaction mixture stored under nitrogen overnight. The next day hydrogenation was resumed under the same conditions and continued for 6 h. At that point the starting material had disappeared, as monitored by TLC (methylene chloride / methanol, 100 / 20, vol / vol); R_f azide = 0.5, R_f amine = 0.4. The newly formed spot turned intense purple when the TLC was developed in ninhydrin dip. When the TLC was developed in an iodine chamber a small spot of UV-negative material was observed above the amine spot. The mixture was twice filtered through two layers of filter paper and 1 cm of celite, each time rinsed through with 5 ml ethanol. The solvent was removed under reduced pressure. The product was obtained as a white powder (600 mg, 86 %). In order to separate the pure product from unreacted mPEG, preparative TLC was performed on 100 mg of the reaction product, using the same eluent mixture as described above. The pure product was extracted from the silica gel with methylene chloride (3 X 10 ml). The mixture was filtered and the solvent evaporated, yielding 70 mg of the title compound.

Method II.

To water (10 ml) was added a drop of acetic acid ; the pH of this solution measured approximately 4. Azido mPEG conjugate **48** (50 mg, 0.01 mmol) was dissolved in the acidified water (3 ml) and platinum (IV) oxide (10 mg) was added. This mixture was shaken under hydrogen pressure (40 lbs). The reaction was monitored by TLC (methylene chloride / methanol, 5 / 1). After 2 h the starting material spot (R_f ~ 0.5) was gone and a new spot, which tested intense purple to ninhydrin, had appeared on the baseline. The mixture was then filtered through a sintered glass filter, containing 1 cm celite. It was rinsed through with 3 ml of the acidified water and the combined solvents were evaporated under reduced pressure. The title amine was isolated as its acetic acid salt. (45 mg, 90 %). ^{1}H NMR (500 MHz, CD_3OD): δ 1.90 (s, 3 H, CH_3COO^-), 1.95 (s, 3 H, Me at C5), 2.20 - 2.74 (m, 5 H, H2', H3', H3"), 3.35 (s, 3 H, Me end group of mPEG), 3.45 - 3.82 (large m, H4', H5', PEG), 4.26 (m, 2 H, -CH_2OC(O)-), 6.08 (br s, 1 H, H1'), 7.50 (s, 1 H, H6).

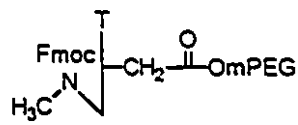
Amide-linked thymidine dimer ^{3}3'-mPEG ester conjugate (50).



Amine **49** (500 mg, 0.10 mmol) and 5'-azido-5'-deoxy-3'-carboxymethyl-3'-deoxythimidine **26** (36 mg, 0.12 mmol) were combined under nitrogen atmosphere. After addition of acetonitrile (5 ml) and TEA (36 µl, 0.24 mmol) the mixture was stirred for 15 min, when BOP coupling reagent was added. Stirring was continued for 5h, at which time the reaction mixture was poured into a beaker. The reaction flask was rinsed with 1 ml acetonitrile, which was also added to the beaker. Diethyl ether (200 ml) was poured into the reaction mixrure. A thick, white precipitate formed immediately.Total precipitation of the reaction product was ensured by letting the mixture stand for 30 min before filtration. The product was washed with 30 ml ether and dried *in vacuo*, to yield 480 mg (90 %) of

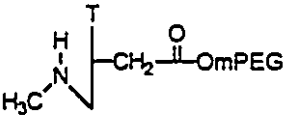
white powder. ^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.90 (s, 6 H, 2 X Me at C5), 3.35 (s, 3 H, Me end group on mPEG), 4.21 (m, 2 H, -C**H**$_2$O(CO)-), 5.78 (dd, 1 H, H1'), 6.13 (dd, 1 H, H1'), 6.87 (t, 1 H, amide -N**H**), 7.16 (s, 1H, H6), 7.42 (s, 1 H, H6),8.72 (s, 1 H, H at N3), 8.97 (s, 1 H, H at N3). MALDI (dithranol): m/e 5426, peak distribution 44 mass units apart. Calculated mass: 5428 for n = 110.

5'-*N*-Fmoc-*N*-methylamino- 3'-mPEG ester conjugate (51).



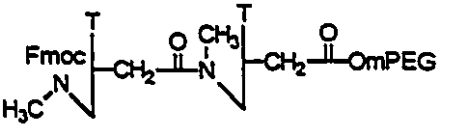
To a solution of 5'-*N*-Fmoc-*N*-methylamino-3'-carboxylic acid **46** (50 mg, 0.10 mmol) in methylene chloride (3 ml) was added DCC coupling reagent (30 mg, 0.15 mmol). This solution was stirred under nitrogen until no more white precipitate was forming (10 min). At that point mPEG 5000 (400 mg, 0.08 mmol) and DMAP (10 mg, 0.08 mmol) were added to the reaction mixture. Stirring was continued for 1.5 h. The work-up was the same as for azido compound **48**. The title compound (412 mg, 95 %) was isolated as a white powder. The NMR spectrum indicated that this compound exists in solution as two rotamers in a ratio of one to two.^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.85, 1.88 (minor, major, s, s, 3 H, Me at C5), 2.88, 2.98 (minor, major, s, s, 3 H, -NC**H**$_3$), 3.38 (s, 3 H, Me end group of mPEG), 5.94, 6.12 (minor, major, br s, br s, 1 H, H1'), 6.96 - 7.78 (m, 9 H, Fmoc aromatic + H6), 8.18 (br s, 1 H, H on N3). MALDI (dithranol), two sets of overlapping signals. Major set: m/e 5209, peak distribution 44 mass units apart. Calculated mass: 5417 for n = 111. Calculated mass for M - Fmoc: 5209 for n = 111. Minor set: m/e 5224, peak distribution 44 mass units apart. Calculated mass for mPEG: 5224 for n = 118.

mPEG ester of 5'-methylamino-3'-carboxymethyl-5',3'-dideoxythymidine (52).



Compound **51** (100 mg,0.018 mmol) and acetonitrile (1 ml) were combined in a flask. To this suspension was added, while stirring, piperidine (50 µl). The solution became clear and was stirred for a total of 15 min. The work-up was the same as for azido compound **51**, except that the flask was rinsed with 0.5 ml acetonitrile and the amount of ether used for the precipitation was 100 ml. The yield of title compound **52** was 90 mg, 94 %. ^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.95 (s, 3 H, Me at C5), 3.19 (s, 3 H, -NMe),3.38 (s, 3 H, Me end group of mPEG), 4.24 (collapsed ABX, 2 H, -CH_2O(CO)-), 6.09 (dd, 1 H, H1'), 7.22 (s, 1H, H6), 8.18 (br s, 1H, H at N3). $J_{H1'-H2'a}$ = 4.3 Hz, $J_{H1'-H2'b}$ = 7.3 Hz. MALDI (dithranol), two sets of overlapping signals. Minor set: m/e 5341, peak distribution 44 mass units apart. Calculated mass: 5341 for n = 114. Major set: m/e 5224, peak distribution, 44 mass units apart. Calculated mass for mPEG: 5224, n = 118.

***N*-Methylamide-linked thymidine dimer ^{3}3'-mPEG ester conjugate (53).**



Methylaminothymidine mPEG ester conjugate **52** (50 mg, 0.094 mmol) and *N*-Fmoc protected thymidine carboxylic acid **46** (8 mg, 0.015 mmol) were coupled in a BOP-mediated (7 mg, 0.015 mmol) reaction, in the same manner as is described for dimer **50**. In this case, however, the solvent used was methylene chloride (0.5 ml). The reaction time was 18 h and the yield was 40 mg (76 %) of white powder. Four rotamers were expected to be present in solution, resulting in a poorly resolved NMR spectrum. ^{1}NMR (500 MHz, $CDCl_3$), selected data:δ 1.90, 1.92 (s, s, 6 H, 2 X Me at C6), 2.82 (d, 6 H,

2 X -NMe), 3.38 (Me end group on mPEG), 5.86 - 6.15 (br s, 2 H, 2 X H1'), 6.92 - 7.78 (m, 10 H, Fmoc aromatic + 2 X H6).

4.5 Experimental for section 2.16.

mPEG chloroformate (54).

A solution of mPEG 5000 (134 mg, 0.027 mmol) and TEA (6 µl, 0.040 mmol) in methylene chloride (1 ml), under nitrogen, was cooled in an ice bath for 15 min, when triphosgene (4 mg, 0.014 mmol) was added. After stirring the reaction mixture for 1 h, it was poured into 50 ml of ether. The voluminous, white precipitate was immediately filtered off, washed with 25 ml ether and dried *in vacuo*. The mPEG chloroformate (130 mg, 97 %) was used immediately for mPEG carbamate coupling.

mPEG succinimidyl carbonate (55).

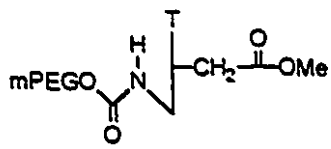
mPEG 5000 (1000 mg, 0.2 mmol) was dissolved in acetonitrile (2 ml) under nitrogen. To this solution were added TEA (112 µl, 0.8 mmol) and disuccinimidyl carbonate (104 mg, 0.4 mmol). The reaction mixture was stirred for 3 h, before being transferred to a beaker. It was then diluted with 8 ml acetonitrile and 200 ml ether was added. A thick, floculent, white precipitate formed immediately. It was allowed to stand for 15 min before being filtered and rinsed with an additional 20 ml of ether. It was dried under reduced pressure and yielded 1000 mg (100 %) of white, flaky powder. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.82 (s, 4 H, succinimidyl), 3.35 (s, 3 H, Me end group on mPEG), 3.44 -3.75 (l m, $(-CH_2CH_2O-)n$, 4.43 (br t, 2 H, $-CH_2OC(O)O-$).

mPEG 4-nitrophenyl carbonate (56).

To a solution of 4-nitrophenyl chloroformate (80 mg, 0.40 mmol) under nitrogen atmosphere, was added pyridine (60 µl, 0.90 mmol). This solution was stirred for 5 min, during which time a white precipitate formed. mPEG 5000 (1000 mg, 0.20 mmol) was added and stirring was continued for 3 h, while the mixture gradually became clear again. The solution was then diluted with 3 ml methylene chloride.The work-up procedure was

the same as described for carbonate 55. The title compound was isolated as a white powder (990 mg, 99 %).1H NMR (500 MHz, $CDCl_3$): δ 3.33 (s, 3 H, Me end group on mPEG), 3.42 - 3.79 (l m, (-CH_2CH_2O-)n, 4.39 (t, 2H, -$CH_2OC(O)O$-), 7.35 (d, 2H, aromatic), 8.23 (d, 2H, aromatic).

5'- mPEG carbamate of 5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (57).



5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine 25 (18 mg, 0.06 mmol), acetonitrile (3 ml) and TEA (32 µl, 0.23 mmol) were combined under nitrogen and stirred for 5 min, before succinimidyl mPEG carbonate (200 mg, 0.04 mmol) was added. The reaction was allowed to run overnight. The next morning acetonitrile (2 ml) was added, prior to precipitation from ether (120 ml). The powdery, white precipitate was filtered, washed with 20 ml ether and dried under reduced pressure (190 mg, 95%). 1H NMR (500 MHz, $CDCl_3$): δ 1.87 (s, 3 H, Me at C5), 2.14 (m, 1 H, H2'a), 2.30 (m, 1 H, H2'b), 2.36 (A of ABX, 1H, H3"a), 2.45 (m, 1H, H3'), 2.56 (B of ABX, 1 H, H3"b), 3.38 (s, 3H, Me end group on mPEG), 3.44- 3.79 (l m, (-CH_2CH_2O-)n), 4.20 (m, 2 H, -$CH_2OC(O)N$-), 5.33 (br t, 1 H, -OC(O)NH-), 6.01 (dd, 1H, H1'),7.23 (s, 1 H, H6), 8.19 (s, 1 H, H on N3). $J_{H3"a-H3"b}$ = - 16.1 Hz, $J_{H3"a-H3'}$ = 7.8 Hz, $J_{H3"b-H3'}$ = 6.0 Hz, $J_{H1'-H2'a}$ = 3.9 Hz, $J_{H1'-H2'b}$ = 7.3 Hz. MALDI (dithranol): m/e 5370, peak distribution 44 mass units apart. Calculated mass: 5371 for n = 114.

Carboxylic acid (58).

The hydrolysis of the methyl ester group of compound 57 was effected in the following manner: ester 57 (60 mg, 0.011 mmol) was stirred with aqueous KOH (1 ml, 0.125 M) for 0.5 h. The solution was acidified to pH ~ 2 with 0.3 M HCl and extracted

with 2 X 20 ml methylene chloride. It was dried over Na_2SO_4,, filtered and the solvent removed *in vacuo*. The product (55 mg, 92 %) was a transparent, waxy solid. ^1H NMR (500 MHz, $CDCl_3$), selected data: δ 1.92 (s, 3H, Me at C5), 2.12 - 2.59 (m, 5 H, H2', H3', H3"), 3.38 (s, 3 H, me end group on mPEG), 4.21 (br d, 2 H, -CH_2OC(O)N-), 5.52 (br t, 1 H, -OC(O)N**H**-),6.01 (br s, 1 H, H1'), 7.27 (s, 1 H, H6), 8.39 (s, 1 H, H at N3).

5'-mPEG carbamate dimer (59).

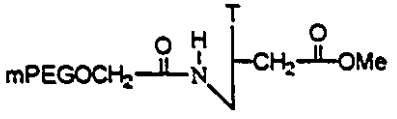
Carboxylic acid **58** (50 mg, 0.0094 mmol), 5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine **25** (4 mg, 0.013 mmol), acetonitrile (0.5 ml) and TEA (5 µl, 0.36 mmol) were combined under nitrogen and stirred for 5 min before BOP coupling reagent (6 mg, 0.013 mmol) was added. Stirring was continued for 18 h. The reaction mixture was diluted with acetonitrile to a total volume of 1 ml and the white, powdery product (50 mg, 97 %) was isolated after the usual work-up, using 50 ml of ether and allowing 1 h for precipitation. ^1H NMR (500 MHz, $CDCl_3$), selected data: δ 1.89, 1.91 (s, s, 6 H, 2 X Me at C5), 3.38 (s, 3 H, Me end group on mPEG), 4.21 (m, 2 H, -CH_2OC(O)N-), 5.54 (br t, 1 H, -OC(O)NH-), 5.80 (dd, 1H, H1'), 6.02 (dd, 1 H, H1'), 6.85 (br t, 1 H, ribose-linking amide NH), 7.15 (s, 1H, H6), 7.35 (s, 1 H, H6).

5'-mPEG carbamate of 5'-*N*-methylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine (60).

The title compound was prepared from 5'-methylamino-3'-carbomethoxymethyl-5',3'-dideoxythymidine **40** (8 mg, 0.025 mmol), mPEG succinimidyl carbonate **55** (80 mg, 0.016 mmol), TEA (14 µl, 0.10 mmol) and acetonitrile (1 ml). The procedure was analogous to that used for the synthesis of the corresponding primary amine carbamate derivative **57**. The NMR data indicated the presence of two rotamers in a ratio of 4 : 1. ^1H NMR (500 MHz, $CDCl_3$), selected data: δ 1.97 (s, 3 H, Me at C5), 2.98 (s, 3 H, -NMe), 3.38 (s, 3 H, Me end group on mPEG), 4.22 (m, 2H, -CH_2O(CO)N(Me)-), 5.54, 6.19

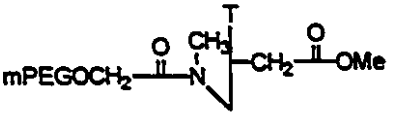
(minor, major, br s, br s, 1H, H1'), 7.12, 7.28 (minor, major, s, s, 1 H, H6), 8.19 (br d, 1 H, H on N3).

5'-mPEG amide of 5'-amino-5'-3'-carbomethoxymethyl-5',3'-dideoxy-thymidine (61).



mPEG carboxylic acid (50 mg, 0.010 mmol), 5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine **25** (5 mg, 0.017 mmol), acetonitrile (0.5 ml) and TEA (7 µl, 0.05 mmol) were stirred under nitrogen for 5 min when BOP coupling reagent (9 mg, 0.020) was added. After 3 days stirring the reaction mixture was worked up in the usual manner, using 0.5 ml acetonitrile for dilution and 40 ml ether for precipitation. The precipitation time was 2 h. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.91 (s, 3 H, Me at C5), 2.15 (m, 1 H, H2'a), 2.28 - 2.45 (m, 4 H, H2'b, H3', H3"a), 2.65 (ABX, 1 H, H3"b), 3.38 (s, 3 H, Me end group of mPEG), 3.43 - 3.79 (l m, (-CH_2CH_2O-)n), 3.98 (AB system, 2 H, -$OCH_2C(O)N$-), 6.05 (dd, 1 H, H1'), 7.26 (s, 1 H, H6), 7.46 (br t , 1H, -$OCH_2C(O)NH$-), 8 59 (s, 1 H, H at N3). $J_{H3"a-H3"b}$ = -16.2 Hz, $J_{H1'-H2'a}$ = 7.32 Hz, $J_{H1'-H2'b}$ = 3.91 Hz, J = -15.8 Hz for signal at 3.98 ppm.

5'-mPEG amide of 5'-*N*-methylamino-3'-carbomethoxymethyl-5,3'-dideoxythymidine (62).



The title compound was prepared the same way as compound 61, substituting 5'-*N*-methylamino nucleoside **40** for 5'-amino nucleoside **25**. The molar ratios, reaction scale and reaction time were also the same. The NMR spectral data indicated the presence of two rotamers in a 4 : 1 ratio. ^{1}H NMR (500 MHz, $CDCl_3$),selected data: δ 1.96, 1.98

(minor, major, s, s, 3 H, Me at C5), 2.97, 3.02 (minor, major, s, s, 3 H, -NC**H**$_3$),3.38 (s, 3 H, Me end group on mPEG), 4.21 (s, 2 H, -OC**H**$_2$C(O)N(CH$_3$)-), 6.06 (m, 1 H, H1'), 6.19, 6.31 (minor, major, s, s, 1 H, H6).

Carboxylic acid (63).

Methyl ester **61** (65 mg, 0.0125 mmol) was stirred with aqueous KOH (0.3 ml, 0.1 M) for 1 h. The solution was then acidified with 0.2 M HCl and extracted with 3 X 5 ml methylene chloride. It was dried over Na_2SO_4 and filtered to yield the carboxylic acid as a transparent, waxy solid (65 mg, 100 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.91 (s, 1 H, Me at C5), 2.17 (m, 1 H, H2'a), 2.30 (m, 1H, H2'b), 2.44 (m, 2 H, H3', H3"a), 2.58 (B of ABX, 1 H, H3"b), 3.38 (s, 3 H, Me end group of mPEG), 3.42 - 3.79 (l m, (-C**H**$_2$C**H**$_2$O-)n), 3.98 (AB system, 2 H, -C**H**$_2$C(O)N-), 6.03 (dd, 1 H, H1'), 7.35 (s, 1 H, H6), 7.52 (br t, 1 H, -OCH$_2$C(O)N**H**-), 8.58 (s, 1 H, H on N3). $J_{H1'-H2'a}$ = 4.2 Hz, $J_{H1'-H2'b}$ = 7.1 Hz. $J_{H3"a-H3"b}$ = - 15.6 Hz.

5'-mPEG amide dimer (64).

The reaction conditions for the coupling of mPEG-conjugated carboxylic acid **63** (50 mg, 0.094 mmol) and 5'-amino-3'-carbomethoxymethyl-5',3'-dideoxythymidine **25** were the same as for compound **61**. The same quantities of reagents were used, but the reaction time was only 18 h. The title dimer (45 mg, 90 %) was isolated as a white, waxy powder.^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.96 (s, 6 H, 2 X Me at C5), 3.38 (s, 3 H, Me end group on mPEG), 3.98 (AB system, 2 H, -OC**H**$_2$C(O)N-), 5.79 (dd, 1 H, H1'), 6.08 (t, 1 H, H1'), 7.16 (s, 1H, H6), 7.19 (br t, 1 H, ribose-linking amide -N**H**),7.22 (s, 1 H, H6), 7.68 (br t, 1 H, -OCH$_2$C(O)N**H**-), 8.72 (s, 1 H, H at N3), 9.42 (s, 1 H, H at N3). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 12.2 (Me at C5), 35.8 - 37.9 (6 lines, C2', C3',C3", C5'), 52.0 (-C(O)OMe), 52.3 (-CH$_2$CH$_2$OMe), (58.9 (-CH$_2$CH$_2$OMe), 70.0 ((-OCH$_2$CH$_2$-)n), 71.9 (-OCH$_2$CH$_2$OMe), 83.0 (C4'), 83.9 (C4'), 84.7 (C1'), 87.9 (C1'),

111.0 (C5), 135.6 (C6),137.8 (C6), 150.6 (C2), 150.9 (C2), 162.0 (C4), 163.4 (C4),169.3 (amide), 169.5 (amide), 169.9 (ester).

5'-mPEG amide dimer carboxylic acid (65).

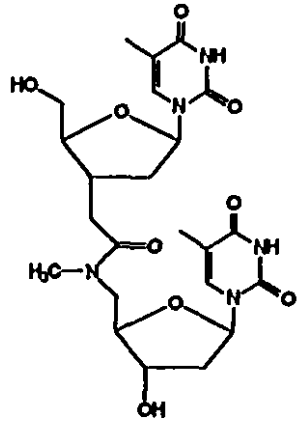
The reaction conditions for the hydrolysis of 5'-mPEG dimer **64** (45 mg, 0.085 mmol) were the same as for the synthesis of carboxylic acid **63**. The reaction time was extended to 1.5 h. The isolated yield of carboxylic acid **65** was 45 mg, 100 %, of white waxy solid. ^{1}H NMR (500 MHz, $CDCl_3$), selected data: δ 1.96 (s, 6 H, 2 × Me at C5), 3.38 (s, 3 H, Me end group of mPEG), 3.43 - 3.79 (l m, poly ether backbone), 3.98 (AB system, 2 H, -$OCH_2C(O)N$-), 5.82 (unresolved, 1 H, H1'), 6.06 (unresolved, 1 H, H1'), 7.31 (s, 1 H, H6), 7.71 (br t, 1 H, -$OCH_2C(O)NH$-), 8.70 (s, 1 H, H at N3), 9.38 (s, 1 H, H at N3).

5'-mPEG amide hexamer (66).

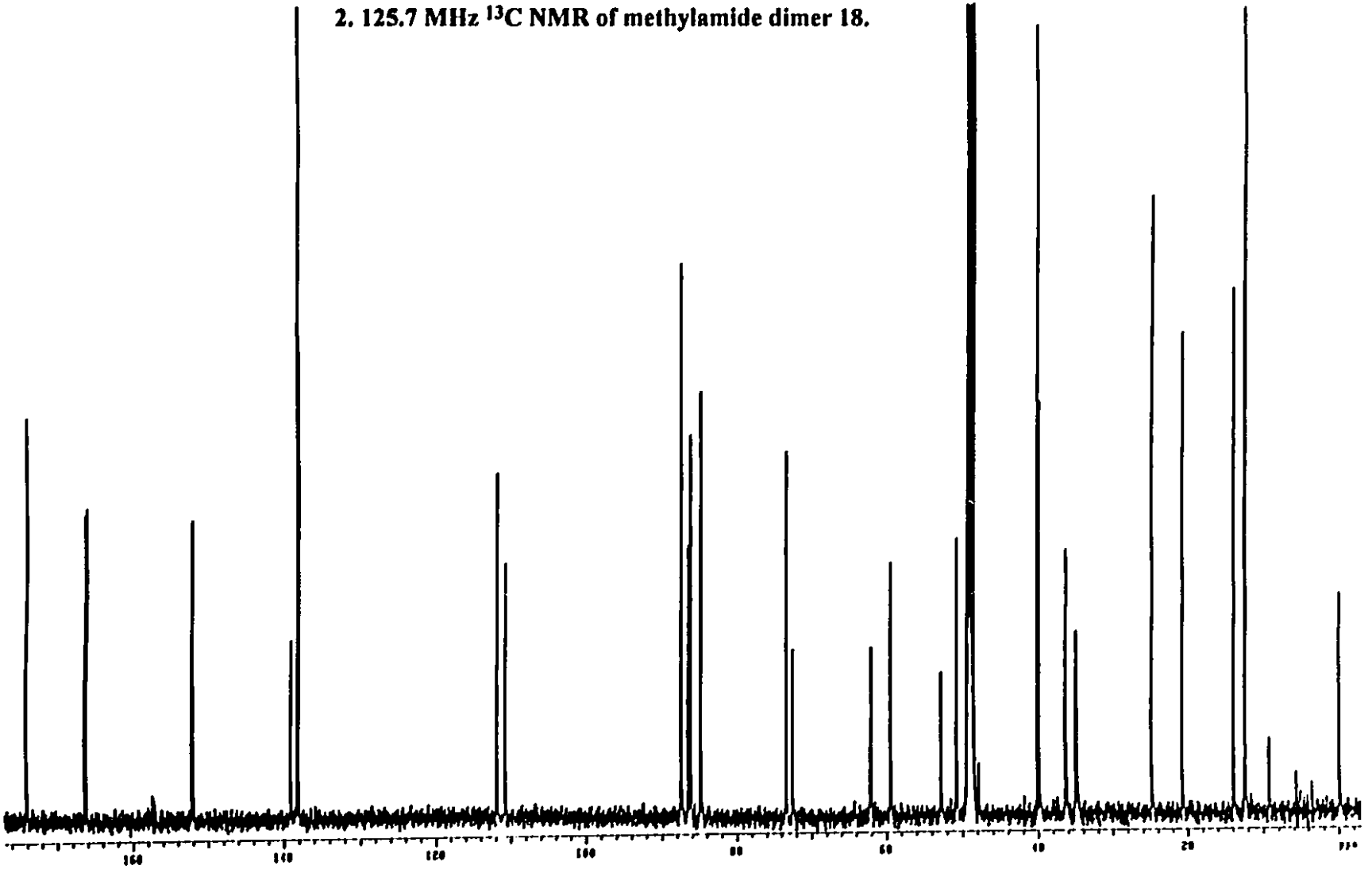
Carboxylic acid **65** (35 mg, 0.006 mmol) and 5'-amino tetramer **31** (8 mg, 0.007 mmol) were combined under nitrogen atmosphere and dissolved in DMF (0.5 ml) . To the stirred solution was then added TEA (5 µl, 0.035 mmol), followed by BOP coupling reagent (5 mg, 0.011 mmol). Stirring was continued for 24 h. The solution was then transferred to a beaker from the flask, which was rinsed with acetonitrile (0.5 ml). To the combined solutions was added ether (50 ml). A hard white precipitate formed, which was subsequently filtered and dried. The product was redissolved in methylene chloride, the solution filtered and the solvent evaporated *in vacuo*, to give hexamer **66** (40 mg) in 93 % yield.

5. APPENDIX

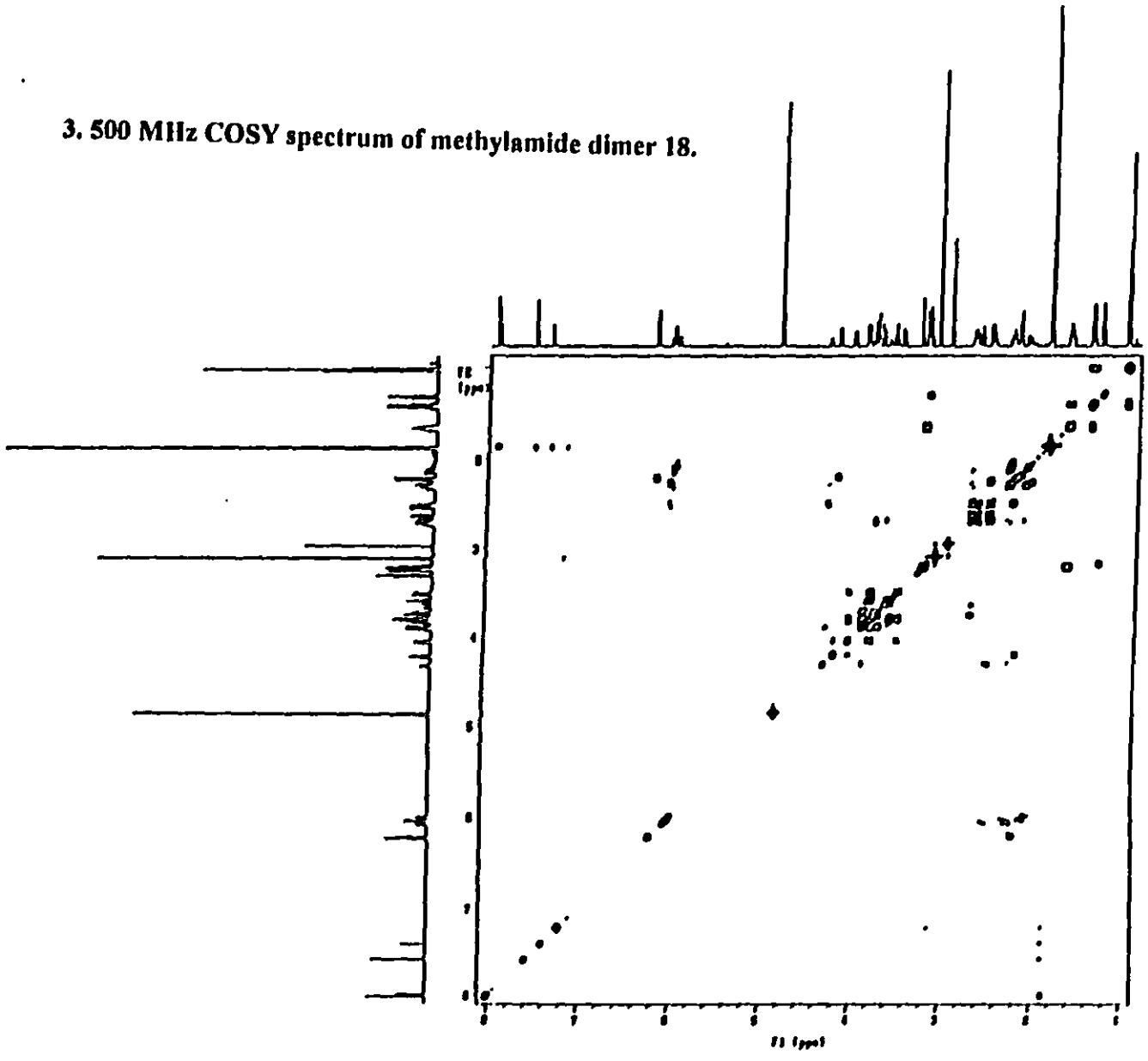
1. 500 MHz ^{1}H NMR spectrum of methylamide dimer 18



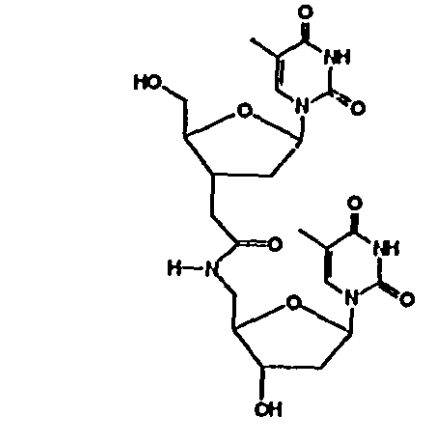
2. 125.7 MHz ^{13}C NMR of methylamide dimer 18.



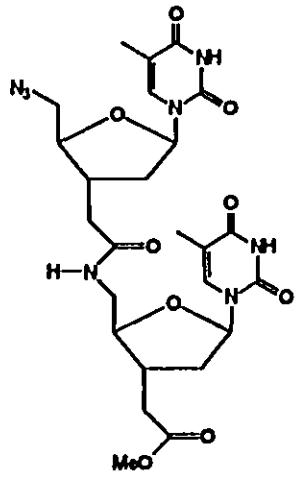
3. 500 MHz COSY spectrum of methylamide dimer 18.



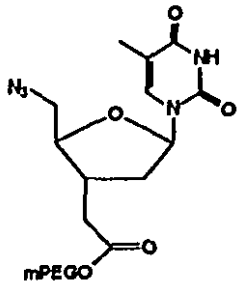
4. 500 MHz ^{1}H NMR spectrum of amide dimer 16.



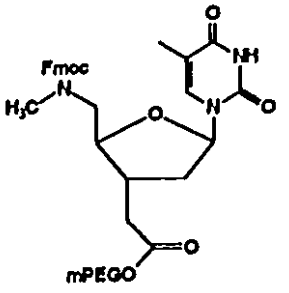
5. 500 MHz ^{1}H NMR spectrum of homodimer 27.



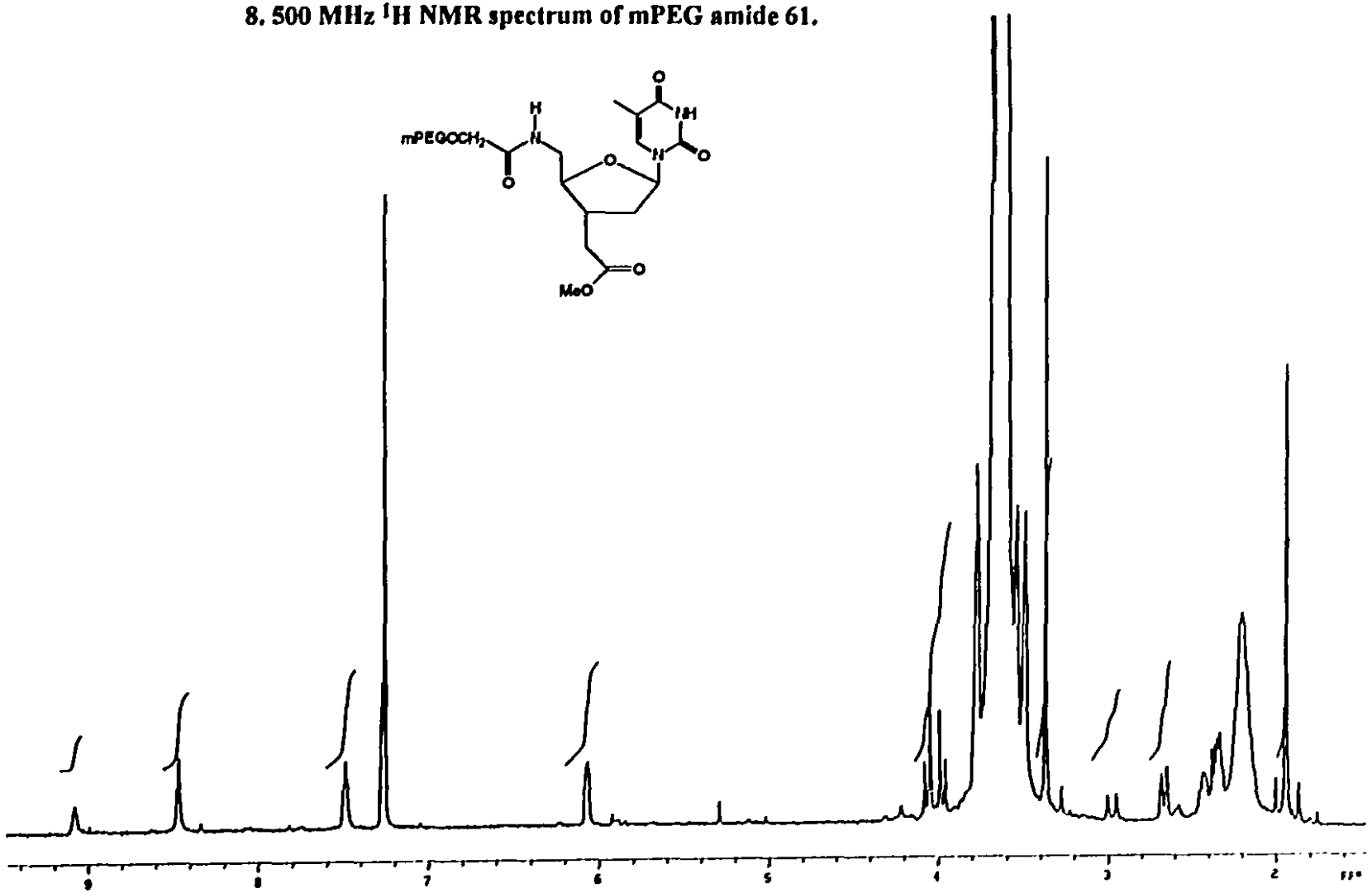
6. 500 MHz ^{1}H NMR partial spectrum of mPEG ester 48.



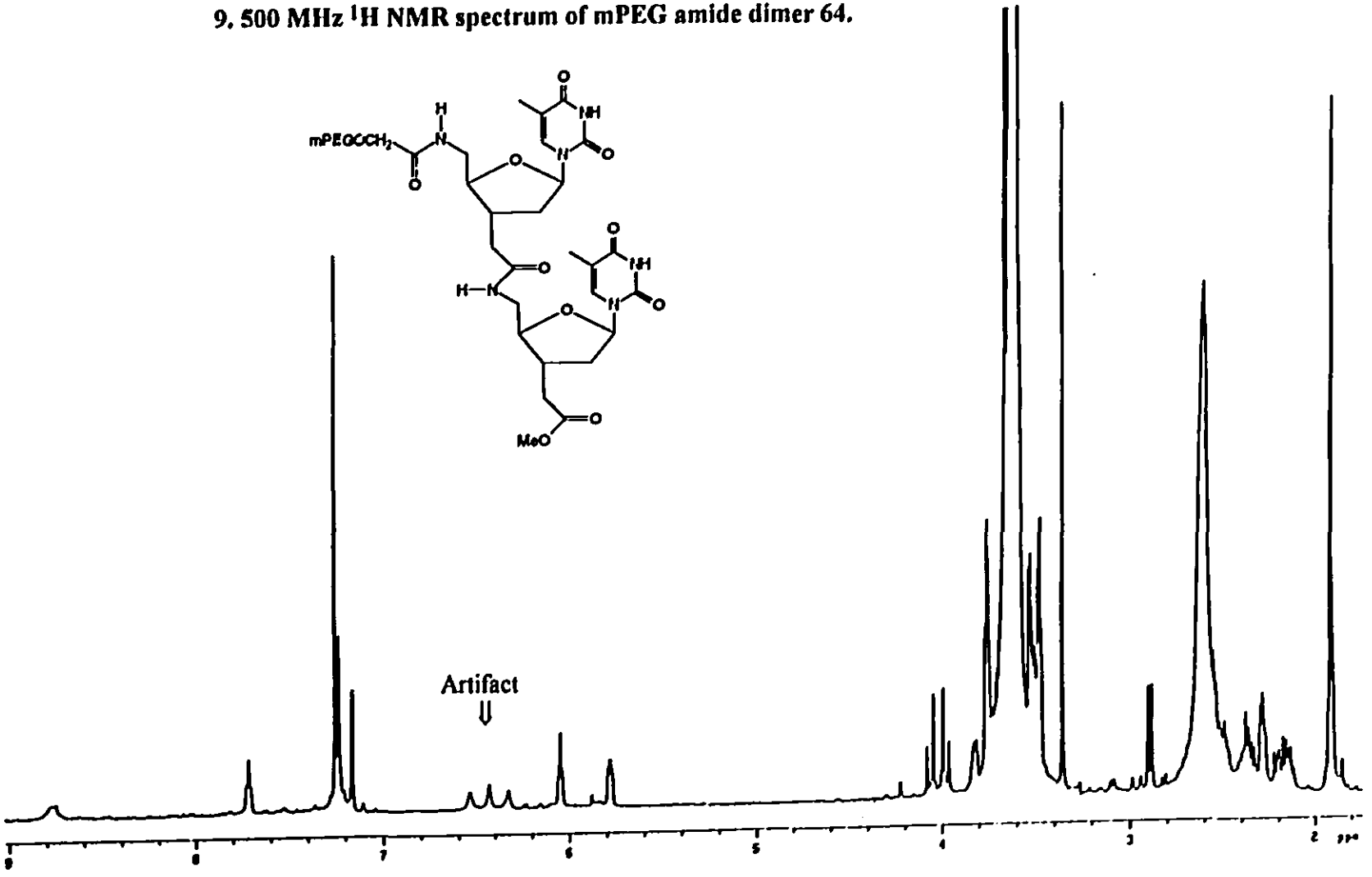
7. 500 MHz ^{1}H NMR spectrum of mPEG ester 51.

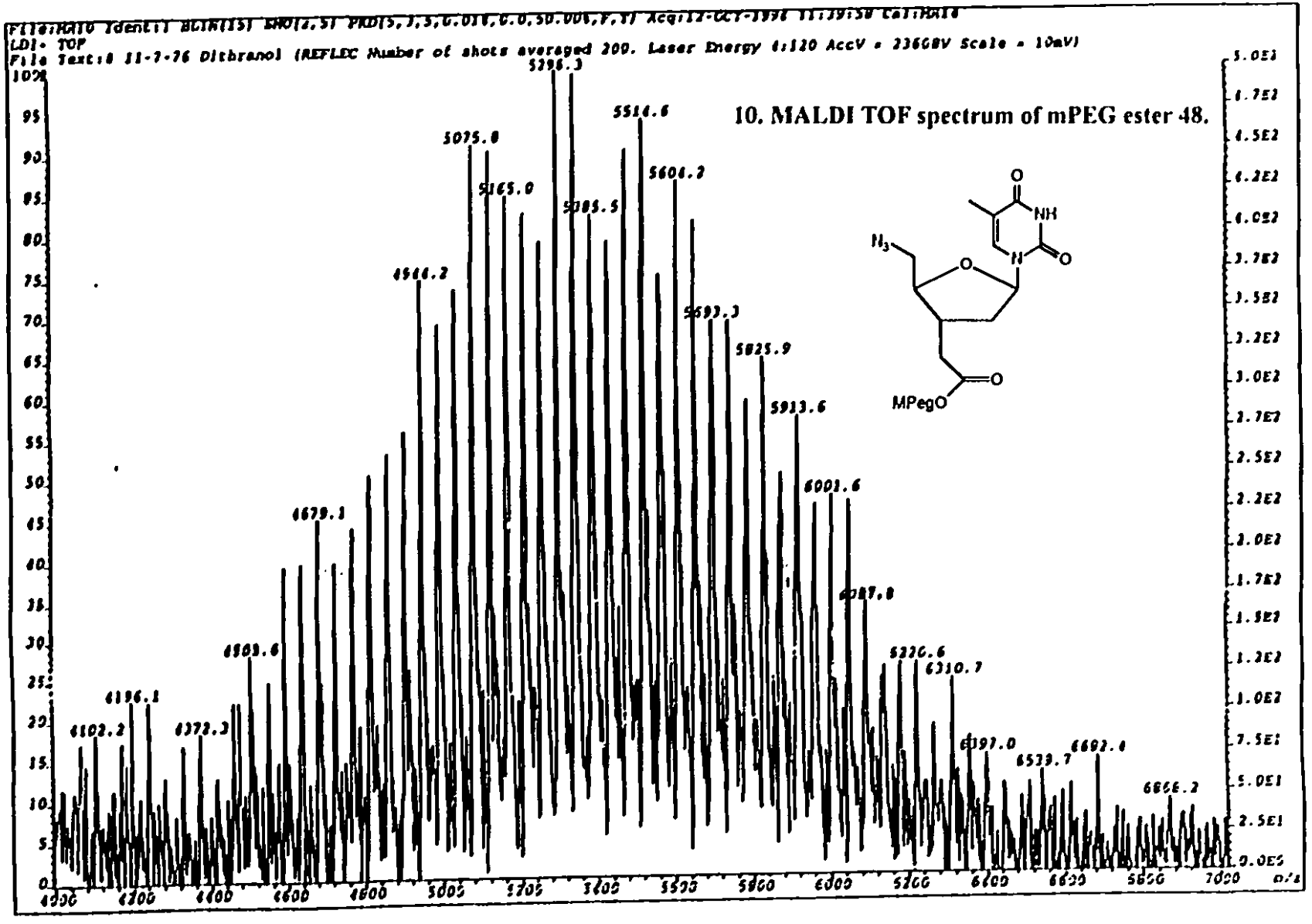


8. 500 MHz ^{1}H NMR spectrum of mPEG amide 61.



9. 500 MHz ^{1}H NMR spectrum of mPEG amide dimer 64.





10. MALDI TOF spectrum of mPEG ester 48.