# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600

# SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING α-L- AND β-D-2'-DEOXYNUCLEOSIDES AND ALTERNATING 3', 3'- AND 5', 5'- LINKAGES

by

Margherita Scartozzi

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

Department of Chemistry
McGill University
Montréal, Québec, Canada

# ABSTRACT

Novel chimeric oligonucleotides containing L-α-dC monomeric units linked in the 3'3'→ 5'5' orientation have been synthesized and binding studies were performed. Thermal denaturation studies done on 7-mer sequences containing either an internal 3'3'-L-α-dC-5'5' insert or a 3'3'-D-α-dC-5'5' insert in the same position, with their complementary sequence, showed a comparable destabilization of $\Delta Tm = 6$ °C and 7 °C, respectively in physiological buffer.

A 19-mer sequence, complementary to a sequence near the beginning of the 5'LTR region of HIV-1 genomic mRNA, containing six alternating 3'3'-L-α-dC-5'5' inserts was synthesized. Binding studies showed that this novel oligonucleotide formed stable duplexes with both its DNA and RNA targets, $\Delta T_m = 4$ °C and 8 °C respectively.

Similarly, an 18-mer , also complementary to a sequence near the beginning of the 5'LTR region of HIV-1 genomic mRNA containing a terminal 3'3'-L-β-dC-5'5' unit was synthesized. Binding studies demonstrated that the duplex formed showed minimal destabilization. Inhibition studies done in the presence of this modified oligonucleotide showed that the amount of (-) strong stop DNA synthesized was decreased in comparison to when the inhibtion studies where done in the presence of an unmodified AON. However, due to the presence of two 5'- hydroxyl groups, this modified oligonucleotide did not serve as a substrate for the priming reaction.

The novel synthesis, as well as the characterization, of $N^3$-functionalized 2-β-deoxycytidine phosphoramidite are also described.

**RESUME**

De nouveaux oligonucléotides chimériques contenant des unités de monomère L-α-dC liées selon l'orientation 3'3'→5'5', ont été synthétisés et ultilisés pour réaliser des études de liaison. Des études de dénaturation thermique en solution physiologique ont été effectuées avec des séquences de 7-mères ayant soit une insertion 3'3'-L-α-dC-5'5'interne, soit une insertion 3'3'-D-α-dC-5'5' interne à la même position, incluant également leur sequence complémentaire, ont montré une destabilisation correspondant respectivement à des ΔTm de 6°C et 7°C.

Une séquence de 19-mères, complémentaire à une séquence proche du début de la région 5'LTR de l'ARNm génomique du VIH-1 et ayant six insertions alternantes 3'3'-L-α-dC-5'5' a été synthétisée. Les études de liaison ont montré que ce nouveau nucléotide forme des duplex stables avec ses propres cibles d'ADN et d'ARN; les ΔTm étant respectivement de 4°C et 6°C.

De façon similaire, un 18-mère, également complémentaire à une séquence proche du début de la région 5'LTR de l'ARNm génomique du VIH-1 et contenant une unité terminale 3'3'-L-β-dC-5'5', a été synthétisé. Les études de liaison ont démontré que le duplex formé avait une destabilisation minimale. Les études d'inhibition effectuées en présence de cet oligonucléotide modifié ont montré que la quantité d'ADN synthétisée est moindre par rapport à celle obtenue en présence de l'oligonucléotide "antisense" non modifié. Cependant, à cause de la présence des deux groupes hydroxyle en 5', cet oligonucléotide modifié n'a pas été utilisé pour la réaction initiale de la réplication.

De plus, la synthèse et la caractérisation du $N^3$-2-β-phosphoramidite déoxycytidine fonctionalisé sont également présentées.

# ACKNOWLEDGMENTS

I would like to express my thanks and appreciation to Prof. Masad Damha for his guidance and support during my project.

I wish to thank my family for all their love and encouragement; and for always believing in me. They have been my strength and courage throughout my studies.

A special thanks to Louis Cuccia, for his assistance in the NMR experiments, the figures in the thesis; and above all for his friendship which did not allow me to give up.

I would also like to thank:Emmanuelle Boubour for the translation of the abstract

My colleagues, Sebastian Robidoux, Anne Noronha, Mike Roman, Andre Uddin and Christopher Wilds, in Lab 207 for their helpful discussions and their friendships.

Dr. Anne Marie Lebuis for performing the X-ray diffraction study

Dr. Kristina Kutterer, for her constant encouragement and valued friendship.

And everyone in Lab 200 for their friendships and use of their computers.

I also wish to express my appreciation to the Natural Sciences and Engineering Research Council of Canada for financial support in the form of a postgraduate scholarship.

# GLOSSARY OF ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| Å | angstrom |
| Ac | acetyl |
| AON | antisense oligonucleotide |
| B | base |
| C | cytosine |
| calcd | calculated |
| COSY | correlation spectroscopy |
| d | deoxy |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethylsulfoxide |
| DMTr | dimethoxytrityl |
| DNA | 2'-deoxyribonucleic acid |
| Et | ethyl |
| eq | equivalent(s) |
| FAB | fast atom bombardment |
| g | gram(s) |
| G | Guanine |
| Δ | heat |
| h | hour(s) |
| H | hypochromicity |
| HIV | human immunodeficiency virus |
| Hz | Hertz |
| im | imidazole |
| L | liter(s) |
| LRMS | low-resolution mass spectrometry (spectrum) |
| M | mega |
| M | molar(ity) |
| m | meter(s) |
| m | milli |
| μ | micro |
| Me | methyl |
| min | minute(s) |
| mol | mole(s) |

| | |
|---|---|
| mRNA | messenger RNA |
| n | nano |
| NBA | nitrobenzylalcohol |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| OPC | oligonucleotide purification cartridge |
| Ph | phenyl |
| ppm | parts per million |
| py | pyridine |
| $R_f$ | distance travelled by compound, divided by that travelled by solvent front |
| RNA | ribonucleic acid |
| T | thymine |
| TBAF | tetrabutylammonium fluoride |
| TBDMSi | *tert*-butyldimethylsilyl |
| TEA | triethylamine |
| Tf | trifyl or trifluoromethanesulfonyl |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |
| tlc | thin layer chromatography |
| $T_m$ | melting temperature |
| UV | ultraviolet |
| v | volume |
| W | watt |

# TABLE OF CONTENTS

## FIGURES

**TABLES**

Alla mia carissima Famiglia,

Anita, Guido and Francesco Scartozzi

# 1.INTRODUCTION

## 1.1 Gene Expression

Deoxyribonucleic acid (DNA) is the molecule that stores and transmits genetic information in living systems, as well as directs and controls the synthesis of proteins. DNA molecules are linear polymers consisting of units, composed from four different nucleotide building blocks (A, C, G or T), joined by 3'→ 5' phosphodiester bonds (**Figure 1.1a**). The DNA duplex is made up of two complementary strands that are held together by Watson-Crick base pairing between the purine (adenine and guanine) and pyrimidine bases (cytosine and thymine), to form a right handed double helix (**Figure 1.1b**).

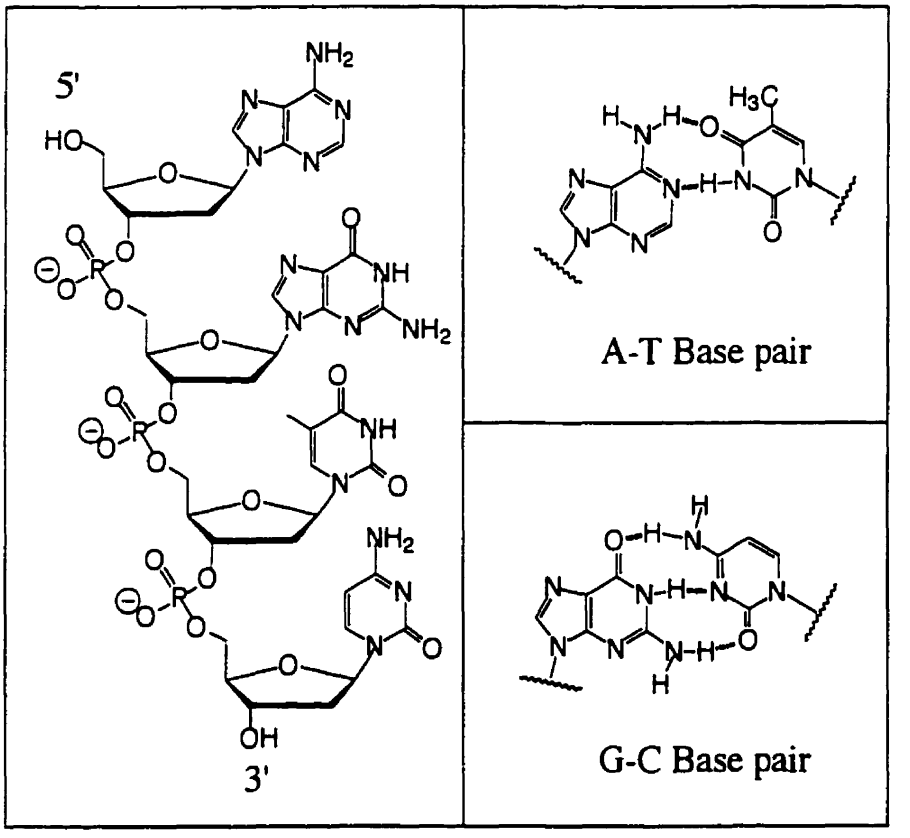



**Figure 1.1**: (**a**) Schematic representation of single stranded DNA. (**b**) Watson-Crick base pairing between complementary base pairs

The genetic information coded in DNA does not serve as a direct template for protein synthesis, however it is used as a template to transcribe messenger RNA (mRNA). In turn it is the mRNA that serves as a direct template for protein synthesis. The mechanism of eukaryotic gene expression is a multi-step process, which is outlined in **Figure 1.2**. In the nucleus of the cell, an entire coding region of the double helical DNA is transcribed into nuclear RNA (pre-mRNA). RNA polymerase recognizes a promoter site on the DNA template, and catalyzes the initiation and elongation of pre-mRNA. The pre-mRNA transcribed is complementary to one of the strands of the DNA template (antisense strand). Inhibition of gene expression by antisense oligonucleotides (AONs) relies on the ability of an AON to bind to a complementary messenger RNA. The primary RNA transcript contains both, coding (exons) and non-coding (introns) regions, which must be excised to give messenger RNA. After this splicing reaction, the mRNA is subsequently transported to the cytoplasm. It is in the cytoplasm that the mRNA becomes transiently associated with ribosomes, the sites of protein synthesis, and the information coded in the linear nucleic acid sequence is translated into a linear amino acid sequence that makes up a specific protein.

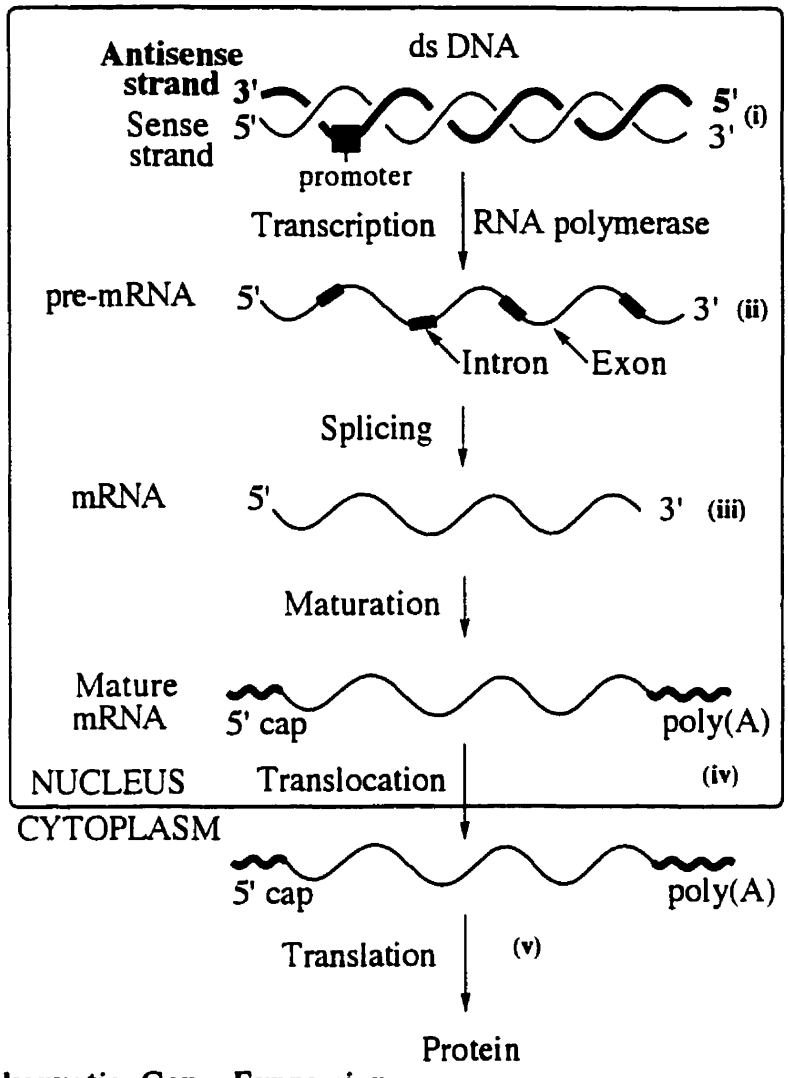



**Figure 1.2: Eukaryotic Gene Expression.**
(i) Transcription of the double helical DNA to the primary RNA transcript occurs in the nucleus and is initiated by the binding of RNA polymerase to a promoter region near the 3' terminal of the antisense strand of the DNA duplex. (ii) DNA serves as a template for the transcription of RNA in the RNA polymerase catalyzed reaction. The growing RNA strand extends in the 5'→3' direction, transiently forming an unstable RNA-DNA duplex. (iii) The primary RNA transcript formed contains noncoding regions (introns), which must be excised from the transcript in order to form messenger RNA (mRNA). Splicing of the RNA occurs in the nucleus. (iv) The mRNA is then transported to the cytoplasm. (v) Once in the cytoplasm,the mRNA forms a complex with ribosomes. Then the mRNA is translated into a linear amino acid sequence (protein).

Many different types of animal viruses contain double-stranded DNA or single-stranded RNA as their genetic material. Viruses containing small genomes encode very few proteins and in turn rely on the host-cell functions for their replication. On the other hand, viruses with larger genomes encode for many enzymes and are able to provide their own replication functions. Viruses, in particular the HIV virus, are the targets of the modified antisense oligonucleotides discussed in the ensuing sections. Reverse transcriptase (RT) is a multi-functional enzyme which catalyzes all the reactions that are outlined in **Figure 1.3**. In retroviruses, such as HIV, reverse transcriptase transcribes the retrovirus' single-stranded RNA genome to a double-stranded DNA. The DNA is then integrated into the host cell chromosome.

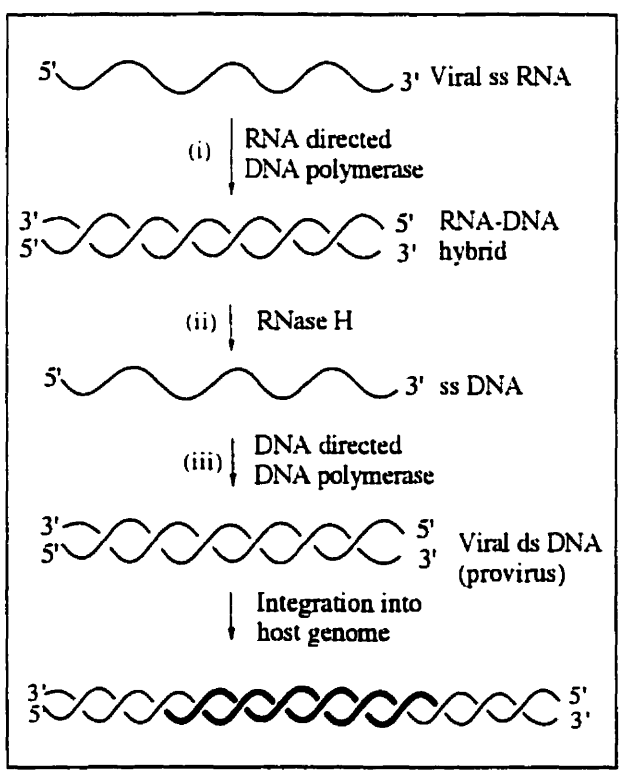



**Figure 1.3: The mechanism of the reactions catalyzed by reverse transcriptase. (RT)**
**(i)** The retroviral RNA acts as a template for the synthesis of its complementary DNA, resulting in an RNA-DNA hybrid by the RNA dependent DNA polymerase function of RT. **(ii)** The RNA strand is then enzymatically degraded by RNase H. **(iii)** The DNA acts as a template for the synthesis of its complementary DNA, yielding double stranded DNA (dsDNA) by the DNA dependent DNA polymerase function of RT. **(iv)** This dsDNA is then integrated into the host genome.

## 1.2. Antisense Oligonucleotides as Potential Inhibitors of Gene Expression

The basic principle upon which the antisense strategy relies on is the complementary Watson-Crick base pairing between the antisense DNA and the sense RNA, to form a stable duplex[1]. Zamecnik and Stephenson[2] were the first to demonstrate the use of short, synthetic oligonucleotides as potential drugs to inhibit viral gene expression. There are various ways in which antisense oligonucleotides (AONs) may inhibit gene expression[3] (**Figure 1.4**).

The specificity that an AON has in recognizing the complementary mRNA, is a major advantage, which allows for the design of an AON to target any gene of a known sequence. As a result, numerous studies focused on the potential antiviral and anticancer activity of AONs.[4] Viruses are attractive targets since their genetic sequence is unique with respect to the host cell. However, in order for antisense oligonucleotides to be viable, they must overcome several obstacles. Primarily, the oligonucleotide must have the ability to permeate the cellular or nuclear membrane, (depending on whether the target is cytoplasmic or nuclear mRNA), and it must be resistant to degradation by endo- and exonucleases. Once the oligonucleotide has penetrated the cell membrane, it must have the capability to bind to the RNA target specifically and with high affinity, in order to inhibit expression of the targeted gene.

---

[1] (a)Belikova, A.M.; Zarytoya, V.F.; Grineva, N.I.; *Tetrahedron Lett.* **1967**, *37*, 3557 - 3560. (b) Paterson, B.M.; Roberts, B.E.; Kuff, E.L.; *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4370 - 4374.

[2] Zamecnik, P.C; Stephenson, M. L.; *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280 - 284.

[3] Hélène, C.; Toulmé, J.-J. *Biochim. Biophys. Acta* **1990**, *1049*, 99 - 125. (and references therein)

[4] (a) Crooke, S.T.; *Annu. Rev. Pharmacol. Toxicol.* **1992**, *32*, 329-376. [4](b) Cook, P. D.; *Anti-cancer Drug Des.* **1991**, *6*, 585 - 607. (c) Milligan, J. F.; Matteucci, M. D.; Martin, J. C.; *J. Med. Chem.* **1993**, Vol. 36, No.14, 1923 - 1937.

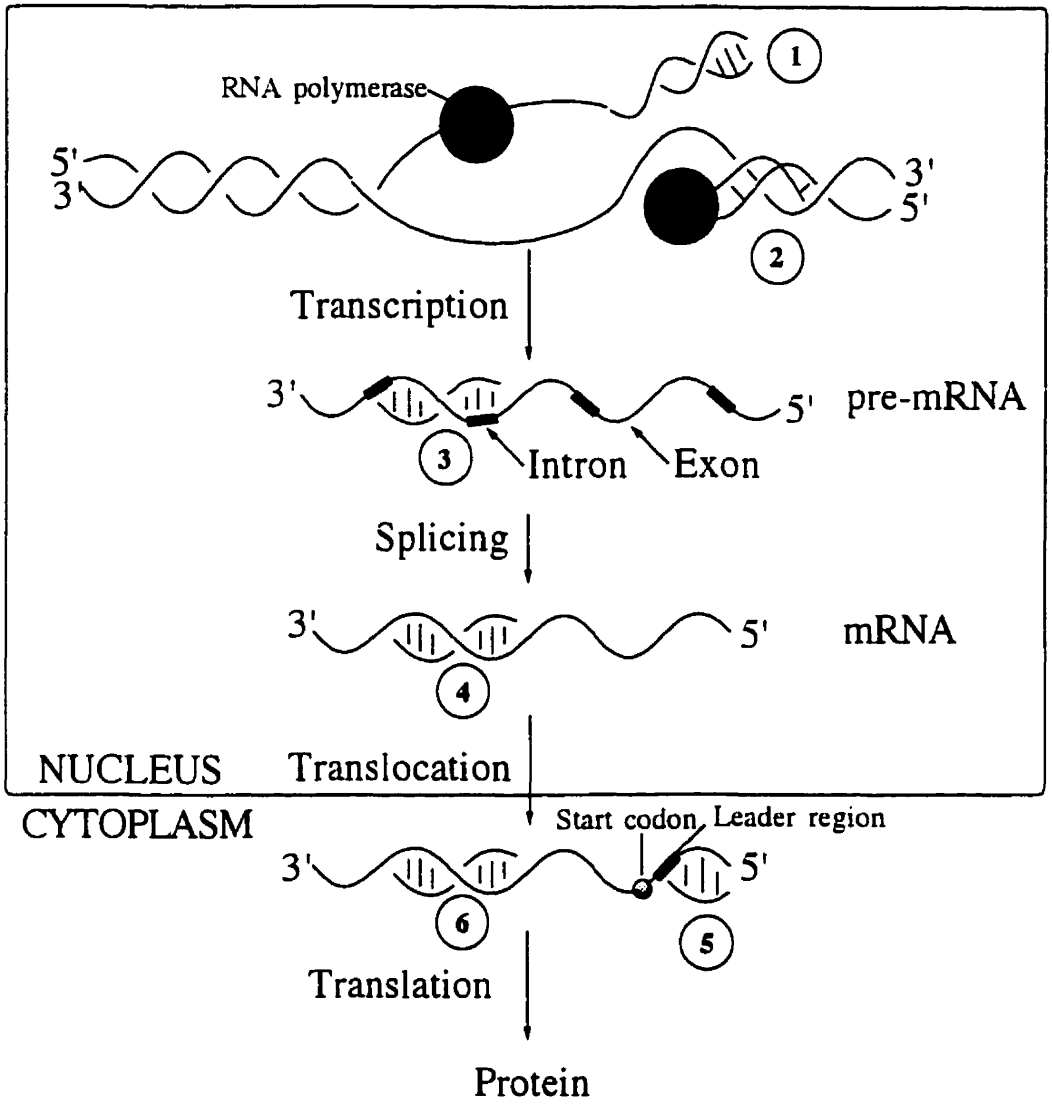



**Figure 1.4: Possible sites of inhibition of gene expression by antisense oligonucleotides** (1) Binding of the AON to the locally unwound loop created by RNA polyermase inhibits transcription by impeding the movement of RNA polymerase along the DNA strand. (2) Hybridization of the AON to the pre-mRNA in an area removed from splice sites, obstructs mRNA formation by either preventing assembly of the spliceosome or by disprupting the pre-mRNA structure. (3) Hybridization of the AON to an intron-exon junction impedes the excision of the intron and/or the ligation of the exons. (4) Hybridization with nuclear mRNA may interfere with the transport of the mRNA to the cytoplasm. Translation of mRNA into proteins may be blocked by an AON in three different ways. By inhibiting the binding of initiation factors and/or the assembly of the ribosomal subunits (5) or by impeding the progress of the ribosome along the mRNA strand.

Single stranded oligodeoxynucleotides (ssDNA) do not meet these criteria, even though they have a high RNA binding affinity, since the phosphodiester linkages are susceptible to cleavage by nucleases; and their highly negative charge impede it's passage into the cell membrane. To overcome the limitations of normal oligonucleotides, many structural variations have been proposed.[5]

One of the most exciting developments in nucleic acid chemistry has been the discovery of remarkably efficient routes to the synthesis of DNA[6], and RNA[7] sequences. The different methods for synthesizing oligonucleotides are primarily differentiated by the method used for the formation of the phosphodiester bond. Hence, according to the nature of the phosphorus component one can distinguish between the triester-, the phosporamidite and the H-phosphonate approach (**Figure 1.5**). In all these processes, synthesis is possible in two directions , by 3'-5' and 5'-3' phosphate coupling. However, due to the higher reactivity of the primary hydroxyl group, the 3'-5' direction is almost exclusively used in chemical synthesis of oligonucleotides.

---

[5] Uhlmann, E.; Peyman, A.; *Chem Rev.***1990**, Vol .*90*, No.*4*, 543. (and references therein)
[6] Letsinger, R.L.; Lunsford, W.B.; *J. Am. Chem. Soc.* **1976**, *98*, 3655 - 3661.
[7] Ogilvie, K.K.; Usman, N.; Nicoghosian, J.; Cedergren, R. J.; *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5764 - 5768.

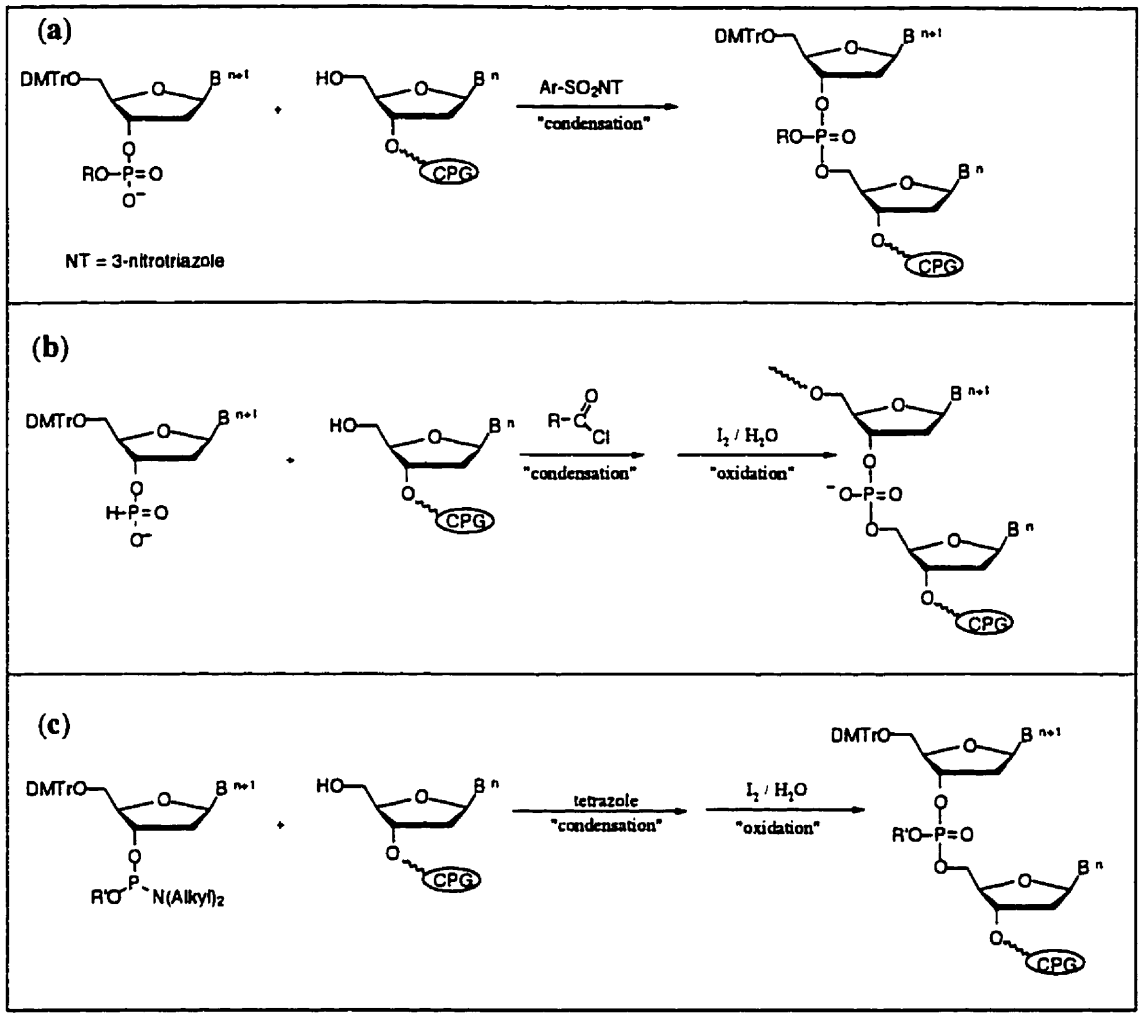



**Figure 1.5:** Methods for oligonucleotide synthesis: (a) Triester, (b) H-phosphonate, and (c) phosphoramidite.

The synthesis via phosphoramidites according to Caruthers[8] is currently the most efficient method for preparing oligodeoxynucleotides (**Figure 1.6**). Assembly of an oligonucleotide starts with the protection of the sugar as well as the heterocyclic base moiety of the nucleoside unit. In conventional chemical oligonucleotide synthesis, the chain grows in the 3'-5' direction, therefore the first nucleoside is linked to the spacer of the solid support (controlled pore glass material, CPG), by its 3'-hydroxyl function. After the removal of the 5'-hydroxyl protecting group of the immobilized nucleoside, the chain grows by reacting this 5'-hydroxyl group with a nucleoside 3'-phosphoramidite function

[8] Beaucage, S.L.; Caruthers, M.H.; *Tetrahedron Lett.* **1981**, *22*, 1859 - 1862.

with catalysis by 1*H*-tetrazole. The unreacted 5'-hydroxyls are capped and then the phosphite triester is oxidized with $I_2$ and it re-enters the cycle.

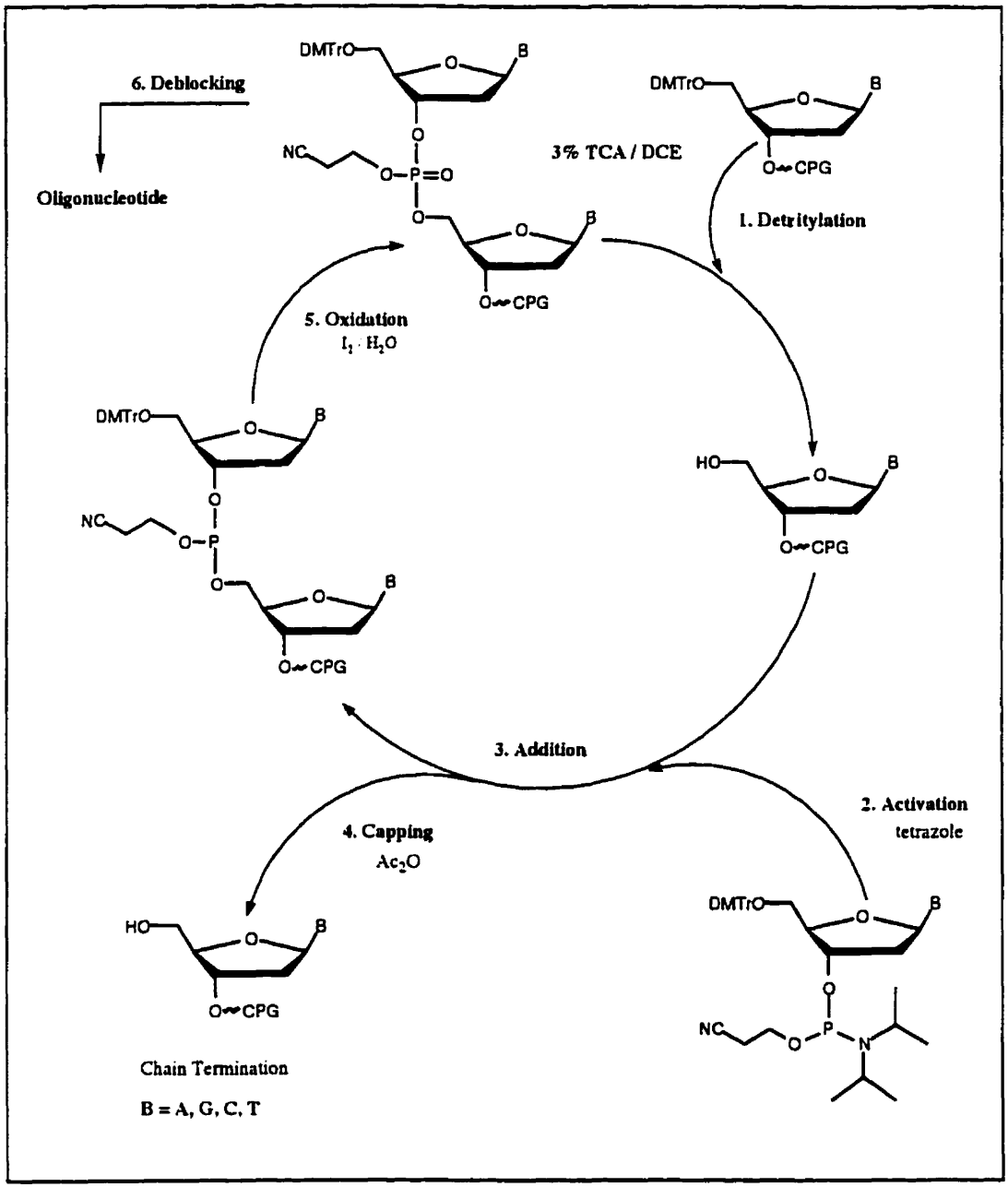



**Figure 1.6**: The phosphoramidite cycle

These advancements in oligonucleotide synthesis, allow for the production of any desired natural sequence as well as sequences having any modification in the basic nucleotide structure. Alterations in nucleotide structure may be categorized as: (i)

phosphodiester backbone modifications , (ii) sugar modifications, (iii) base modifications or (iv) 5' or 3' conjugates (**Figure 1.7**). It has already been demonstrated that modified oligonucleotides containing a phosphate or sugar modification, such as, methlyphosphonates[9], phosphorothioates[10] , D-α sugars, and L-β sugars, have increased resistance to degradation by nucleases and increased cellular uptake.[5] However, some drawbacks of these modified oligonucleotides, are that they usually bind with a lower affinity to the RNA target. One of the modes of action of these antisense molecules is to elicit RNase H activity, which leads to the degradation of the mRNA bound to the AON. However, RNase H is very sensitive to any structural modification, hence most of the modified AONs' do not exhibit any RNase H activity, excluding this mode of action.

[9] (a) Miller, P.S.; Agris, C.H.; Blake, K.R.; Murakami, A.; Reddy, M.P.; Spitz, ,S.A.; Ts'O, P.O.P.; In *Nucleic Acids: The Vectors of Life*; **1983** Pullman, B.; Jortner, J.; Eds.; D. Reidel Publishing Co.: Dordrecht. (b) Miller, P.S.; Agris, C.H.; Aurelian, L.; Blake, K.R.; Murakami, A.; Reddy, M.P.; Spitz, ,S.A.; Ts'O, P.O.P.; *Biochimie*, **1985**, *67*, 769.

[10] (a) Eckstein, F.; *Agnew. Chem.* **1983**, *6*, 431. (b) Eckstein, F.; *Annu. Rev. Biochem.* **1985**, *228*. 541.

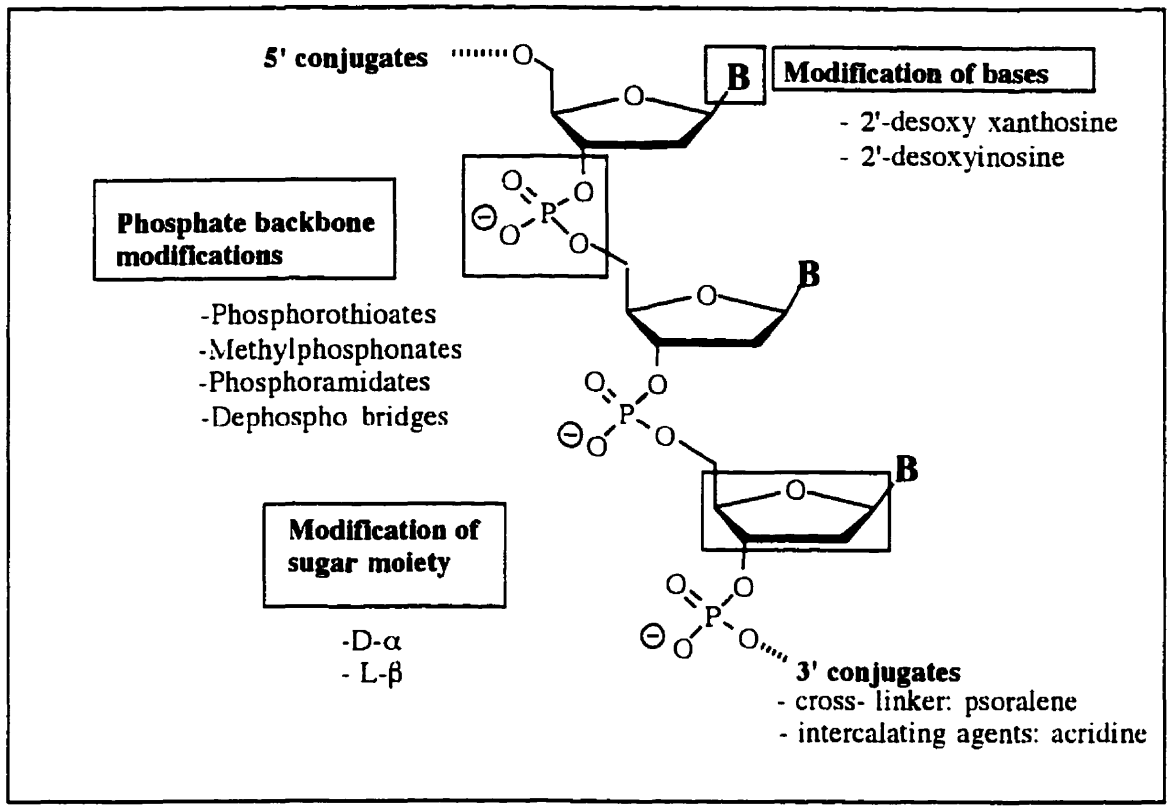



**Figure 1.7**: Possibilities for modifying oligonucleotides.

Our laboratory has focused on modified oligonucleotides and oligoribonucleotides containing, 2',5'-phosphodiester linkages,[11] arabinonucleosides,[12] covalent attachment of intercalators for stabilization of triple helix formation, $N^3$-functionalized deoxythymidines,[13] and L-β nucleosides.[14] The focus of this thesis deals with novel modifications of the sugar moiety, which is reviewed in the next section.

[11] Giannaris, P.A.; Damha M.J., *Nucleic Acids Research*, **1993**, Vol.*21*, No.*20*, 4742-4749.
[12] Giannaris, P.A.; Damha M.J., *Canadian Journal of Chemistry*, **1993**
[13] Uddin, A.H.; Roman M.A.; Anderson J.R.; Damha M.J.; *Chem Commun.*, **1996**, 171.
[14] Damha, M. J.; Giannaris, P.A.; Marfey P.; Reid L. S.; *Tetrahedron Lett.* **1991**, Vol. 32, No. 23, 2573 - 2576.

## 1.3 Literature review of chimeric DNA containing D-α, L-β and L-α nucleosides.

Many structural, binding, and biological studies have been reported on oligonucleotides containing a modification at the anomeric carbon $C^1$ (especially D-α-anomers). Even though much of the focus has been on D-α- modification, L-β sugars (enantiomer of natural D-β) and L-α- sugars (enantiomer of D-α) have also been studied. The structure of these modified nucleoside units are shown in **Figure 1.8.**

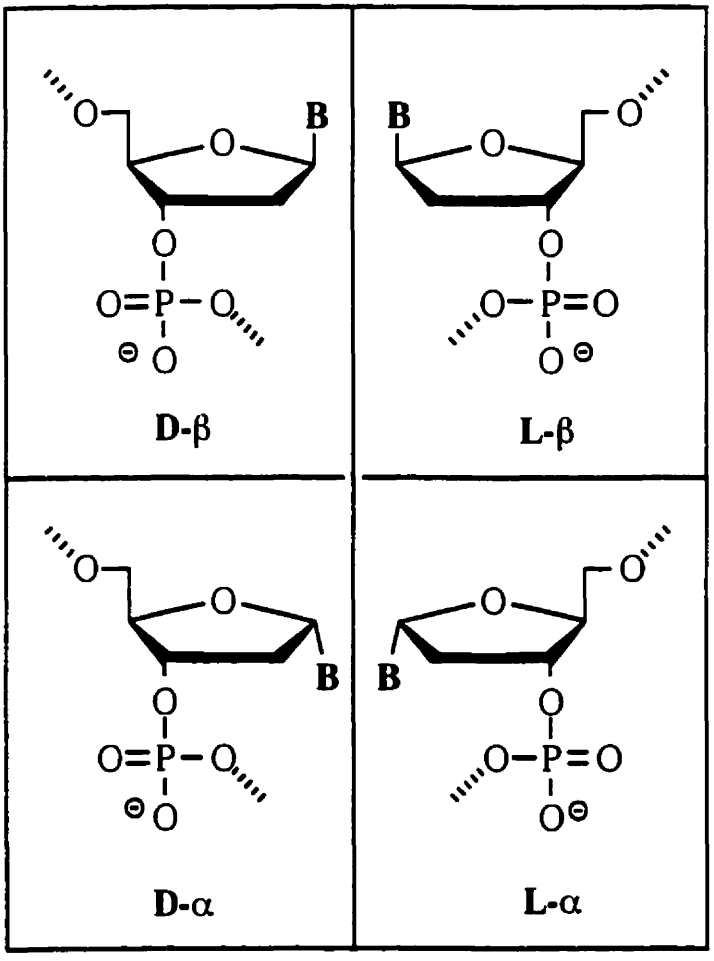



**Figure 1.8**: Structure of oligonucleotides constructed from D-β, L-β, D-α and L-α nucleotide units.

### 1.3.a D-α-DNA

Studies on the synthesis, characterization and base pairing properties of unnatural α- oligodeoxyribonucleotides have been carried out extensively by Imbach and co-workers[15]. The D-α-hexamer, synthesized by Imbach *et al.*, consisting only of pyrimidine monomeric units exhibited increased resistance to nuclease degradation, and a secondary structure similar to that of the natural nucleic acids.[15b] These initial studies spawned further research into oligonucleotides consisting of D-α nucleoside units [16-20].

The unnatural α-oligomers, which differ from the natural oligomers only by exhibiting the α-configuration at the anomeric carbon atom ($C^1$) (**Figure 1.8**), have been shown to have the following features: (i) the ability to form duplexes with complementary α- or β- strands, and these duplexes show anti-parallel and parallel polarity respectively[16,17,18] (**Figure 1.9**), (ii) Watson-Crick base pairing specificity is retained;[19,20] (iii) heteroduplexes obtained from hybridization between complementary α- and β-strands belong to the B-DNA family[4] and (iv) these unnatural α-oligomers are more stable than their β-counterparts against hydrolysis by nucleases.[21,22] Furthermore, intercalating agents linked to α-oligonucleotides have to shown to form an even stronger complex than α-

---

[15] (a) Morvan, F.; Rayner, B.; Imbach, J.L.; Chang, D.K., Lown, J.W. *Nucl. Acid Res.* **1986**, *14*, 5016 (b) Morvan, F.; Rayner, B.; Imbach, J.L.; Chang, D.K., Lown, J.W. *Nucleosides & Nucleotides*. **1987**, *6*, 471-472.
[16] Morvan, F.; Rayner, B.; Imbach, J.L.; Chang, D.K., Lown, J.W. *Nucl. Acid Res.* **1987**, *15*, 4241-4255.
[17] Morvan, F.; Rayner, B.; Imbach, J.L.; Lee, M.; Hartley, J. A.; Chang, D.K., Lown, J.W. *Nucl. Acid Res.* **1987**, *15*, 7027-7043.
[18] Praseuth, D.; Chassignol, M.; Takasugi, M.; Le Doan, T.; Thuong, N.T.; Helene, C. *J. Mol. Biol.* **1987**, *196*, 939-942.
[19] Lancelot, G.; Guesnet, J.L.; Roig, V.; Thuong, N.T. *Nucl. Acid Res.* **1987**, *15*, 7531-7547.
[20] Gmeiner, W.H. ; Rao, K.E.; Rayner, B. Vasseur, J.J.; Morvan F.; Imbach, J.L.; Lown, J.W. *Biochemisty*, **1990**, *29*, 10329-10341.
[21] Morvan, F.; Rayner, B.; Imbach, J.L.; Thenet, S.; Bertrand, J.R.; Paoletti, J.; Malvy, C.; Paoletti C. *Nucl. Acid Res.* **1987**, *15*, 3421-3437.
[22] Cazenave, C.; Chevrier, M.; Nguyen, T.T.; Helene, C. *Nucl. Acid Res.* **1987**, *15*, 9909-9919.

oligomers that do not have these conjugates.[23, 24, 25] These features have made D-α -oligonucleotides very attractive agents for the antisense strategy for the control of gene expression. However, biological studies have demonstrated the inability of RNase H to degrade RNA in α-DNA:RNA duplexes.[26] Although these latter duplexes do not trigger RNase H-mediated translation inhibition, α-oligomers were shown to efficiently inhibit translation initiation in cell-free systems[27] and *Xenopus* oocytes.[28] This prompted further investigation into the synthesis of α-anomeric oligoribonucledotides, since it had been found that RNA:RNA duplexes are more stable than the DNA:RNA heteroduplexes. The results of the study indicated that α-RNA oligomers also showed a marked resistance to hydrolysis by nucleases, as well as the ability to inhibit reverse transcriptase synthesis in HIV-1 Infected MT4 cells.[29]

---

[23] Gauthier, C.; Morvan, F.; Rayner, B.; Tam, H-D.; Imbach, J.L.; Paoletti, J.; Paoletti C. *Nucl. Acid Res.* **1987**, *15*, 6625-6641.
[24] Thuong, N.T.; Asseline, U.; Roig, V.; Takasugi, M.; Helene, C. *Biochemistry*, **1987**, *84*, 5129-5133.
[25] Durand, M.; Maurizot, J.C.; Asseline, U.; Thuong, N.T.; Helene, C. *Bioconjugate Chem.* **1993**, *4*, 206-211.
[26] Gagnor, C.; Bertrand, J.R.; Thenet, S.; Lemaitre, M. Morvan, F.; Rayner, B. Malvy, C.; Lebleu, B.; Imbach, J.L.; Paoletti, C. *Nucl.Acid Res.* **1987**, *15*, 10419-10436.
[27] Bertrand, J.R.; Imbach, J.L.; Paoletti, C.; Malvy, C. *Biochem. Biophys. Res. Commun.***1989**, *164*, 311-318.
[28] Boizeau, C.K.; Cazenave, C.; Roig, V.; Thuong, N.T.; Toulme, J.J *Nucl. Acid. Res.* **1991**, *19*, 1113-1119.
[29] Debart, F.; Rayner, B.; Degols, G.; Imbach, J.L. *Nucl. Acid. Res.* **1992, *20***, 1193-1200.

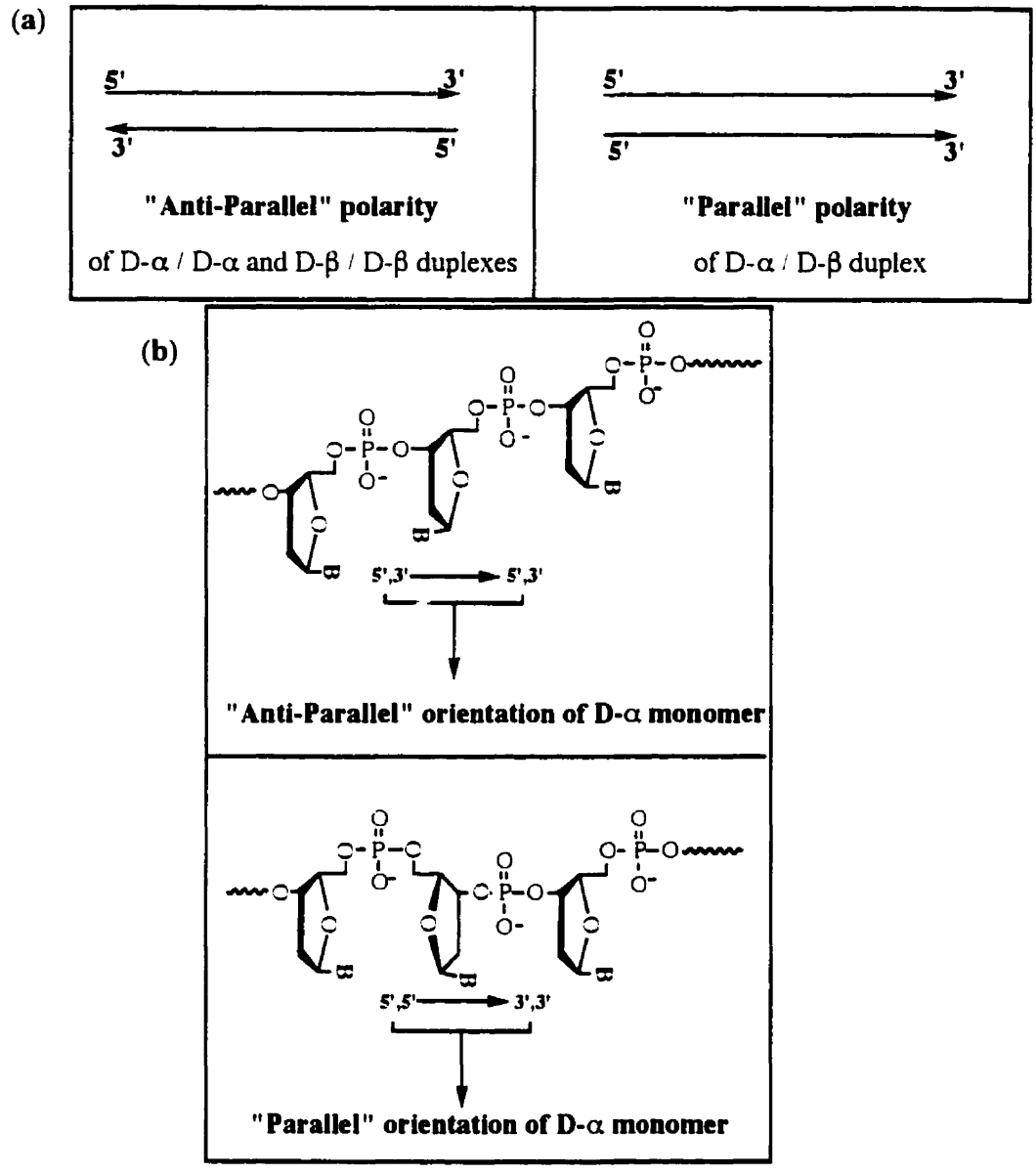



**Figure 1.9**: Parallel and Anti-parallel orientation of (a) oligonucleotide and (b) monomers within an oligonucleotide.

Koga *et al.*[30] originally hypothesized that the alternate substitution of a β-monodeoxyribose nucleotide for an α-deoxyribonucleotide, through a (3'→3') and (5'→5') internucleotidic phosphate linkage motif, (**Figure 1.10 C**) should impart base-pairing that would be similar to those of natural oligonucleotides. This hypothesis was based on the previous studies done on α-DNA, (**Figure 1.10 B**) which indicated the preferred parallel orientation of α-DNA to its β- counterpart. Also, it was believed that

[30] Koga, M.; Moore, M. F.; Beaucage, S.L. *J. Org. Chem.* **1991**, *56*, 3757-3759.

insertion of these D-α monomeric units within a natural oligomer sequence might retain the ability of such oligonucleotides to elicit RNase H activity, which is a property of natural β-oligonucleotides. Concomitantly, it was hypothesized that the insertion of unnatural 3'→3' and 5'→5' phosphodiester linkages might also impart nuclease resistance, since these unnatural linkages might not be recognized by nucleases. Therefore these phosphodiester linkages would not be hydrolyzed as rapidly as the 3'→5' phosphodiester functions of unmodified oligonucleotides. Binding studies as well as NMR studies have confirmed that the orientation previously found for α- oligomers for duplex formation with either natural DNA or RNA complementary strands were also valid for chimeric oligonucleotides consisting of α- and β- stretches. Even though the NMR studies have shown the capability of these α- momomeric inserts with the reverse polarity are able to form Watson-Crick base pairs with its complementary sequence[31], weaker hybrids were obtained with $T_m$ values decreasing with an increasing number of unnatural 3'→3' or 5'→5' phosphodiester linkages.[32] The decrease in thermal stability was greater with complementary RNA than for complementary DNA.[33] Promising results have been obtained for these chimeric α,β-oligonucleotides, where the β- sequence of eight nucleotides was flanked at the 3'- and/or 5' end by α- strands. This specific design of chimeric DNA has shown the ability to form stable complexes with both DNA and RNA as well as serving as substrate for RNase H activity, even though it is decreased relative to natural DNA. However, when the chimeric α,β-oligonucleotides consists of an α-fragment bound by its 3'-end to the 3'-end of a β-octanucleotide, it directed more specific RNA cleavage by RNase H than its ββ counterpart [34]. In both cases it is the central β-8-mer by itself that supports RNase H- mediated

[31] Aramini, J. M.; Kalisch, B. W.; Pon, R. T.; van de Sande, J. H.; Germann, M. W. *Biochemistry*, **1996**, *35*, 9355 - 9365.
[32] Debart, F.; Tosquellas, G.; Rayner, B.; Imbach, J.L. *Bioorganic & Medicinal Chemistry Letters*, **1994** Vol.4, No.8, 1041 - 1046.
[33] Koga, M., Wilk, A., Moore, M.F.; Scremin, C.L.; Zhou L.; Beaucage S.I. *J.Org.Chem.* **1995**, *60*, 1520 - 1530.
[34] Gottikh, M.; Bertrand, J.-R.; Baud-Demattei, M.V.; Lescot, E.; Giorgi-Renault, S.; Shabarova Z.; Malvy, C. *Antisense Research and Development* **1994**, *4*, 251 - 258.

cleavage of mRNA. This supports other results obtained that D-α oligonucleotides do not induce RNase H activity.

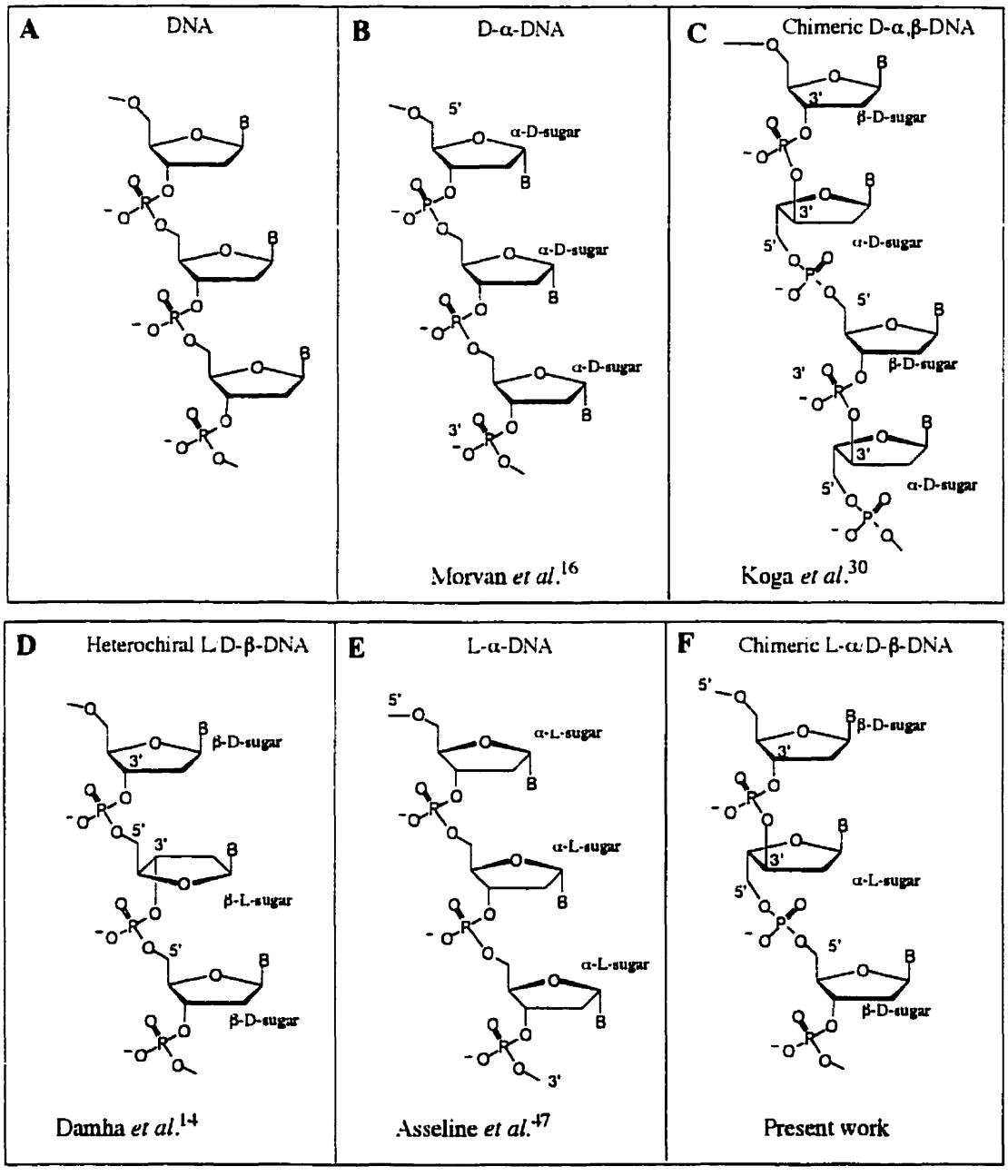



**Figure 1.10:** Schematic representation of **A**: Natural D-β-DNA, **B**: D-α-DNA, **C**: Chimeric D-α,β-DNA, **D**: Heterochiral L/D-β-DNA, **E**: L-α-DNA, and **F**: Chimeric L-α/D-β-DNA.

### 1.3b. L-β and L-α oligonucleotides

The synthesis of L-α and L-β nucleosides has been described by Robins[35] and Holy[36],[37] respectively. Subsequently L-α and L-β oligonucleotides have been synthesized and their resistance towards hydrolysis by nucleases was confirmed[38]. The work of Ts'o[39] demonstrated that dinucleotides of adenosine (ApA) containing both D- and L- ribose possessed similar binding properties with poly U. This spawned further research into "enantio-DNA". In an early study done by Fujimoro *et al.*[40], it was demonstrated that even though the duplex formed between L-$dA_6$ and poly(U) is less stable than the $dA_6$/poly(U) complex, , L-$dA_6$ possesses the striking ability to bind selectively to RNA over DNA, which might be very advantageous for an antisense oligonucleotide. However, a strategy based on completely enantiomeric (L)-DNA, appears unlikely to be successful due to the relatively poor hybridization with natural RNA.[29,32,41] Furthermore, L-ODNs containing all four natural base residues did not interact with complementary D-DNA or RNA sequences.[42] As a result, our laboratory[43] and others have independently focused upon heterochiral ODNs having both D- and L-deoxynucleosides ("mixed" L/D- or

---

[35] Robins, M.J.; Khwaja, T.A.; Robins, R.K.; *J .Org. Chem.* **1970**,. *35*, 636 - 639.
[36] (a) Holy, A.; Sorm, F.; *Collection Czechoslov. Chem. Commun.* **1971**, Vol. *36*, 3282 - 3299. (b) Holy, A.; *Collection Czechoslov. Chem. Commun.* **1972**, Vol. *37*, 4072 - 4087.
[37] Anderson, D.J.; Reischer, R.J.; Taylor, A.J. Wechter, W. J. *Nucleosides & Nucleotides*. **1984**, *3(5)*, 499-512.
[38] Holy, A. *Chemica Scripta*, **1985**, *26*, 6-2.
[39] Tazawa, I.; Tazawa, S.; Stempel, M.; Ts'o, P.O. P.; *Biochemistry*, **1970**, Vol. *9*, No. *18*, 3499 -3514.
[40] Fujimoro, S.; Shudo, K.; *J. Am. Chem.* **1990**, *112* 7436-7438.
[41] Morvan, F.; Genu, C.; Rayner, B.; Gosselin, G.; Imbach, J.L. *Biochem. Biophys. Chem. Res.***1990**, *172*, 537-543.
[42] Garbesi, A.; Capobianco, M. L.; Colonna, F.P.; Tondelli, L.; Arcamone, F.; Manzini, G.; Hilbers C. W.; Awlwn, J. M.E.; Blommers M.J.J. *Nucl. Acid Res.* **1993**, *21*, 4159-4165.
[43] Damha, M.J.; Giannaris, P.A.; Marfey P.; *Biochemistry*, **1994**, *33*, 7877 - 7884.

"heterochiral"-DNA, **Figure 1.10 D**).[44,45,46] These studies have shown that heterochiral ODNs are significantly resistant toward exonuclease digestion. Also, stable Watson-Crick base pairing between, D- and L- nucleotide residues in these the L-DNA/DNA duplexes was observed.[43] The stable base pairing observed between the duplex formed from L/D heterochiral DNA and its complementary sequence is expected since, only a little distortion in the base stacking should occur with the insertion of one or more L-β- nucleoside units. Such base-pairing between D- and L- nucleotide units is also supported in an NMR study done by Blommers *et al.*[45]

Much research has been devoted to the study of oligonucleotides containing D-α or L-β monomeric units. However, very little is known about oligonucleotides consisting of L-α nucleotidic units (**Figure 1.10 E**). In a recent study done by Morvan *et. al.*[41], L-α-octathymidylates were found to be resistant toward enzymatic degradation; however they did not form stable complexes with complementary RNA or DNA strands. Similarly, Asseline *et al*[47] performed similar studies on L-α tetramers. However, in order to promote duplex formation, an acridine derivative was covalently linked to the 3'-end of L-α oligomers. This modification resulted in binding of the L-α DNA to its complementary DNA and RNA sequences. This prompted a computer modeling study of DNA double helices composed of a L-α-oligodeoxynucleotide and a complementary D-β- strand. The results of this study showed that the L-α-oligomer preferably forms a duplex with the natural D-β-strand in the parallel fashion.[48]

---

[44] Urata, H.; Ueda, Y.; Suhara, H.; Nishioka, E.; Akagi, M. *J. Am. Chem. Soc.* **1993**, *115*, 9852-9853.
[45] Blommers, M.J.J.; Tondelli, L.; Garbesi, A.; *Biochemistry*, **1994**, 33, 786-7896.
[46] Hashimoto, K.; Iwanami, N.; Fujimori, S.; Shudo, K.. *J. Am. Chem. Soc.* **1993**, *115*, 9883-9887.
[47] Asseline, U.; Hau, J-F.; Czernecki, S.; Le Diguarher, T.; Perlat, M-C.; Valery, J-M.; Thuong, T. *Nucl. Acid Res*, **1991**, *19*, 4067 - 4074.
[48] Kienginger, M.; Suhai, S. *Anti-Cancer drug Design*, **1995**, *10*, 189 - 201.

### 1.3d Specific Goals

Based on the literature review, few studies have been done on L-α-oligomers. To the best of our knowledge, chimeric L-α/D-β-oligonucleotides have not yet been synthesized, and their binding properties with complementary D-β DNA or RNA are still unknown. Therefore the objective of this work was to synthesize L-α-2'-deoxycytidine monomer and incorporate it into an oligomer sequence in the 3'3'→5'5' orientation (**Figure 1.10 F**).

The reason that we chose to synthesize L-α-dC rather than the other nucleosides (A, G or T) was based on a study done by Imbach *et al.*[49], where it was shown that the D-α,β duplex stability was dependent on the base composition. It was deduced that the pyrimidine α-nucleosides gave more stability than purine α-nucleosides. The newly synthesized L-α-dC monomer would hence be inserted into an oligonucleotide sequence in the "parallel" 3'3'→5'5' orientation since it has already been demonstrated that this is the preferred orientation of L-α-oligomers with respect to their complementary D-β-oligonucleotides[48]. Also, the introduction of such a modification within an oligonucleotide has already been shown to impart nuclease resistance towards hydrolysis,[47,49] therefore increasing its potential as a candidate for antisense inhibition.

The sequences that were chosen in which to insert the L-α-dC monomer were: (i) a G-C rich heptanucleotide (7-mer) and (ii) a 18-mer that was complementary to an 18 nt sequence present near the beginning of the 5'LTR region of HIV-1 genomic RNA. In the case of the 7-mer, the L-α-dC unit would be inserted within the sequence in the reversed orientation. In order to determine the binding properties of this modified 7-mer, thermal denaturation studies would be performed and compared to those obtained for the same 7-mer containing a D-α-dC also oriented in the 3'3'→5'5' orientation. This would allow us to determine whether the destabilization caused by the insertion of a L-α-dC monomer is similar to that imparted by its D-α counterpart. Similarly we wanted to investigate the

[49] Imbach, J-L.; Rayner, B.; Morvan, F. *Nucleosides & Nucleotides*, **1989**, *8*, 627 - 648.

binding properties of a longer oligonucleotide containing six, somewhat alternating, L-α-dC units.

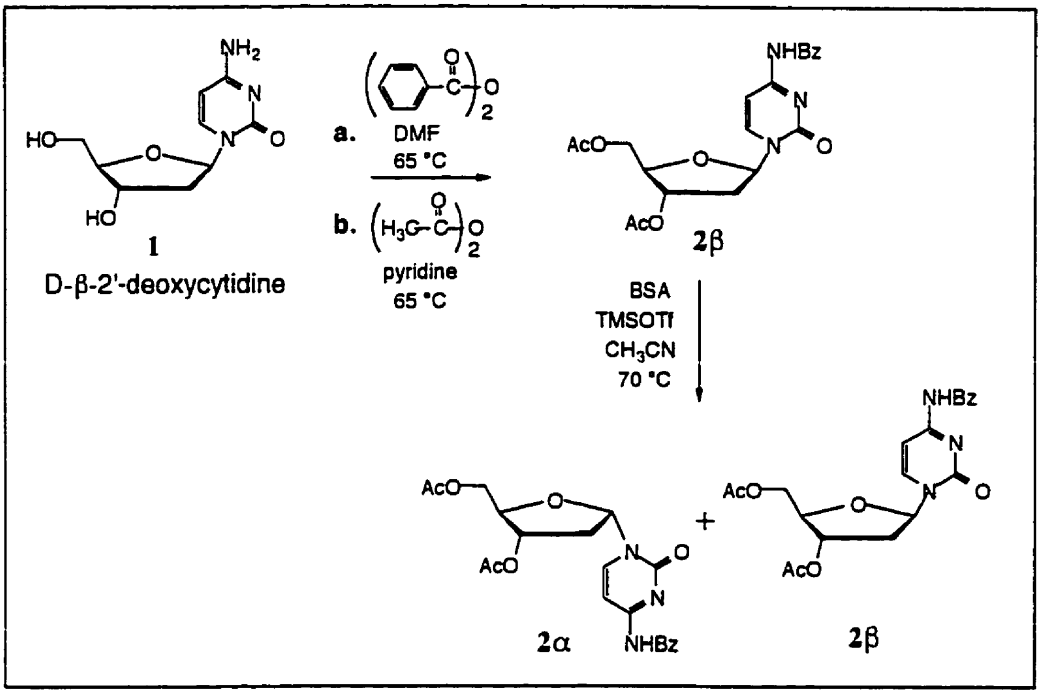
It was also our goal to investigate the binding properties of the 18-mer containing a L-β-dC unit at the 3'-end in the parallel orientation. Furthermore we wished to probe this modified oligonucleotides' ability to inhibit HIV-1 reverse transcription in vitro, as well as to determine whether or not this modified 18-mer may serve as a primer for reverse transcriptase.

# 2. RESULTS AND DISCUSSION

## 2.1 Attempted synthesis of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidine-$N^4$-benzoyl-D-α -2'-deoxycytidine

A substantial amount of research has been devoted to oligonucleotides containing D-α nucleosides. Initially, the main focus was to synthesize unnatural oligonucleotides consisting exclusively of D-α-nucleotide units, to study their structure and conformation, their substrate activities towards nucleases, as well as their ability to form duplexes with complementary D-β- and D-α-strands. Recently however, research has also focused on chimeric DNA, consisting of D-α- and D-β- nucleotide units, where the D-α-units are oriented in the 3'3'→5'5' orientation (**Figure 1.10 C**). These studies have indicated that oligonucleotides containing D-α-nucleoside units oriented in the 3'3'→5'5' direction are able to form stable duplexes with both complementary DNA and RNA, as well as having increased nuclease resistance[30,31,32,34] compared to the natural D-β oligomers. Since the L-α- and D-α-monomers are enantiomers, and binding studies have already been performed on the D-α-monomer inserts in oligonucleotides, we thought that in addition to the natural D-β oligonucleotides, the D-α-monomer inserts may also serve as a control for the L-α modified oligonucleotides in our investigation (See Section 2.3.2, for further elaboration). This would enable us to determine if the L-α inserts have similar binding properties as their D-α counterparts. Our plan was to synthesize the L-α-cytidine monomer in order to determine the effect the insertion of this modified nucleoside in an oligomer sequence would have on binding to both complementary DNA and RNA. Therefore, we felt it would be desirable to also synthesize the D-α-cytidine nucleoside for comparison purposes.

**Scheme 1**

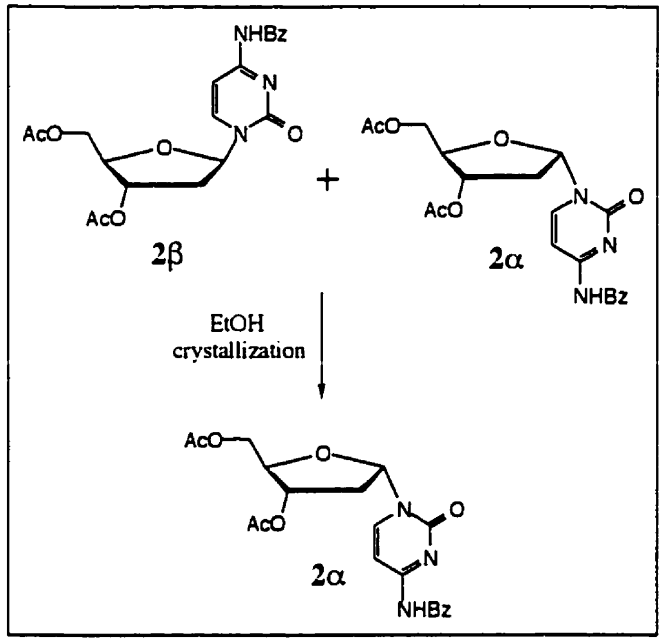



In the past few years, a number of procedures for the synthesis of α-nucleoside derivatives have been developed.[50] Due to its apparent simplicity, we decided to use the method reported by Yamaguchi and Saneyoshi[51] to synthesize D-α-2'-deoxycytidine. Since enantiomers have the same physical properties, this methodology would subsequently be applied to the synthesis of L-α-2'-deoxycytidine. It was advantageous to attempt the synthesis of the D-α-2'-deoxycytidine from the starting material, D-β-2'-deoxycytidine, due to its availability and low cost (**Scheme 1**). Specifically, the synthetic approach consisted of benzoylation of the $N^4$ position followed by the acetylation of the 5' and 3' hydroxyls of D-β-2'-deoxycytidine, as illustrated in **Scheme 1**. This resulted in the fully protected deoxyribonucleoside **2β**, which upon treatment with *N,O*-bis-(trimetylsilyl)acetamide (BSA) and trimethylsilyl triflate (TMSOTf) produced a mixture of

[50] Morvan, F.; Rayner, B.; Imbach, J.L. From: *Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs* Ch. 13, 261 - 283. Edited by: S. Agrawal Copyright 1993 Humana press Inc., Totowa, N.J.
[51] Yamaguchi, T.; Saneyoshi, M. *Chem. Pharm. Bull.* **1984**, *32* 1441 - 1450.

α- and β-5',3'-di-*O*-acetyl-($N^4$-benzoyl)-2'-deoxycytidine anomers (**2α** and **2β**; $R_f$ = 0.33 and 0.37 respectively in 5% MeOH in $CH_2Cl_2$). However, after our attempted synthesis, Koga *et al*[33] described a more straightforward approach for the synthesis of **2α**, by reducing the number of synthetic steps described above in **Scheme 1**. The acetylation step was deemed unnecessary because the free hydroxyl groups would be protected by silylation with BSA prior to the anomerization reaction. This approach may be more advantageous when starting from the benzoylated cytidine since only one step is required to achieve the desired product, potentially cutting down on possible side products. The α-anomer was crystallized from ethanol after silica gel purification of the mixture and obtained in 40 % yield (**Scheme 2**). By thin layer chromatography it appeared that the desired product was obtained, namely **2α** nucleoside, since the $R_f$'s corresponded to those cited in the literature ($R_f$ = 0.31($CH_2Cl_2$ : MeOH, 19 : 1 v/v) or $R_f$ = 0.67 ($CHCl_3$ : ethyl acetate, 1 :1 v/v).[49,50] However, the $^1$H NMR spectrum of the synthesized compound revealed that, the desired compound was only present in small amounts with respect to a contaminant that was also present (1:8 respectively). Since the $R_f$'s of the desired product and the contaminant were very close and a small quantity of **2α** had been synthesized, we decided not to proceed with the synthesis of nucleoside **2α**. Our decision was also influenced by two other factors, namely that previous thermal denaturation studies had been performed on oligomers containing D-α-insert in the 3'3→5'5' orientation, which could be used to compare with our L-α- modified nucleoside (Section 2.3.2), and also that Dr. Richard Pon (University of Calgary, Alberta) had generously supplied us with an oligonucleotide containing a D-α-insert in the 3'3→5'5' orientation.

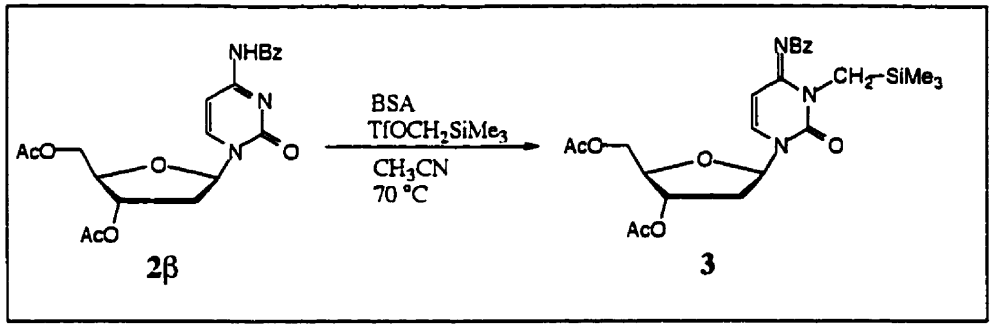
**Scheme 2**




### 2.1.1. Synthesis and characterization of a novel base-modified nucleoside

During the synthesis of D-α-cytidine (**2α**), the reagent trimethylsilyl-triflate was serendipitously substituted with trimethylsilylmethyl-triflate resulting in **3**, 3',5'-di-*O*-acetyl-$N^3$-methyl,trimethylsilyl-$N^4$-benzoyl-2'-deoxycytidine (**Scheme 3**).

**Scheme 3**



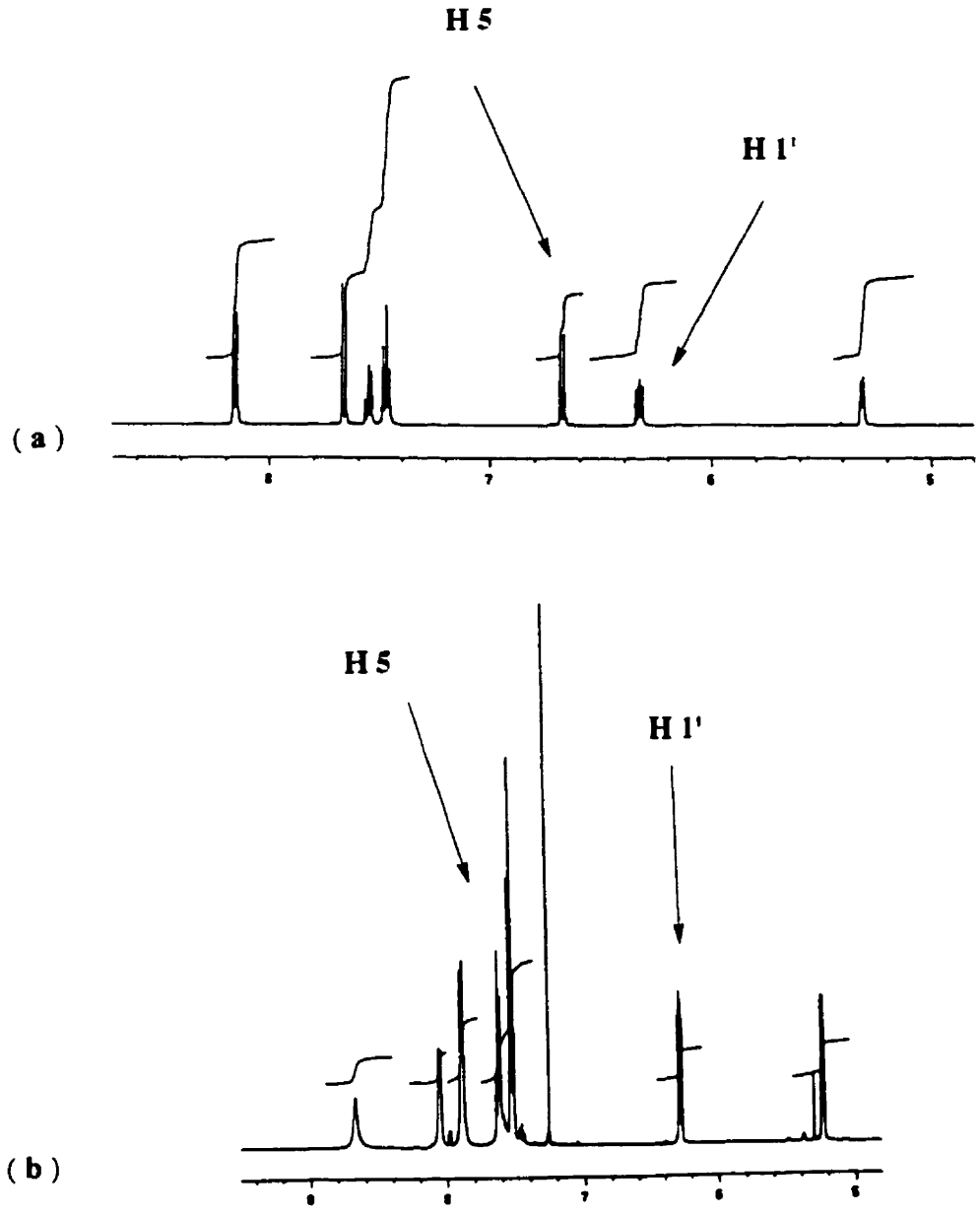



**Figure 2.1**: (**a**) $^{1}$H-NMR (in Acetone-$d_6$) of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'-deoxycytidine (**3**), (**b**) $^{1}$H-NMR (in Acetone-$d_6$) of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl- 2'-β-deoxycytidine (**2β**).

The $^{1}$H NMR (**Figure 2.1 a**) of this interesting new compound showed a large peak at a chemical shift of 0.1 ppm characteristic of methyl groups adjacent to a silyl moiety. The peak usually observed at 9 ppm for the exocyclic amino group at the 4 position (**Figure 2.1 b**) of the base was absent, suggesting deprotonation at the $N^4$ position. Also, from the NMR analysis, it was evident that a modification had occurred since the chemical shift of the H5 proton on the base and the H 1' proton of the sugar moiety of 5',3'-Di-*O*-Acetyl-

$N^4$-benzoyl- 2'-β-deoxycytidine (**2β**)., had drastically changed in the case of nucleoside **3** (**Figure 2.1**). In order to confirm our NMR analysis, an X-ray crystallographic structure of this modified nucleoside **3** was obtained (**Figure 2.2**). The X-ray structure clearly indicates that alkylation of the base at the $N^3$ position had occurred.

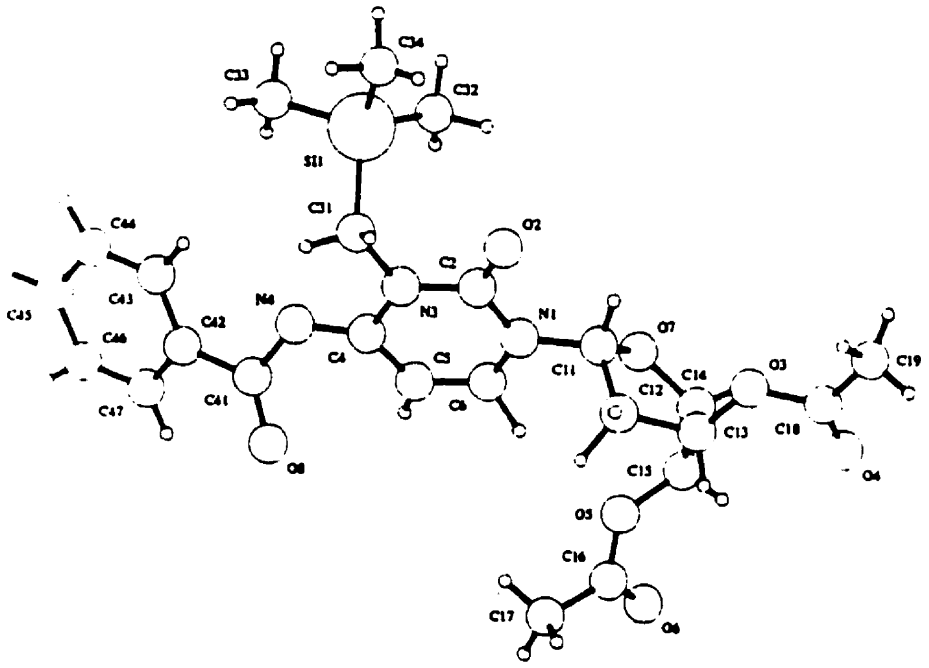


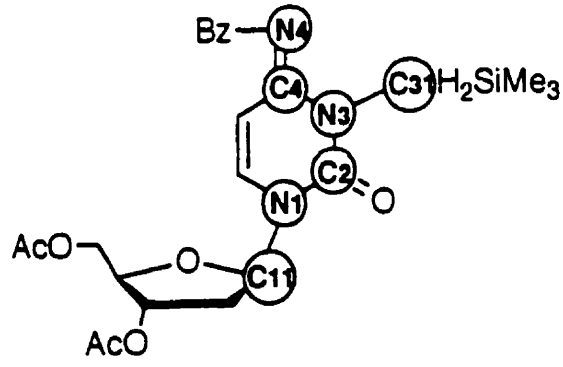

**Figure 2.2**: X-ray crystallographic structure of modified nucleoside **3**

The decrease in the $C^4$-$N^4$ carbon-nitrogen bond length, obtained from the X-ray crystallographic parameters, clearly indicates that the $N^4$ position is $sp^2$ hybridized in comparison to the longer $sp^3$ hybridized C-N bonds (**Table 2.1**). The C-N bond lengths in the vicinity of 1.49 Å, are representative of $sp^3$ hybridization, whereas those in the vicinity of 1.39 Å are shorter due to the possible resonance in the amide moiety. This $sp^2$ hybridization, in addition to the $sp^3$ character of the $N^3$-$C^4$ bond, indicates the loss of

aromaticity of the base. Details of the crystallographic study of nucleoside **3** are shown in Appendix 1.

**Table 2.1:** Carbon - Nitrogen Bond Lengths in the Base Moiety of **3**.

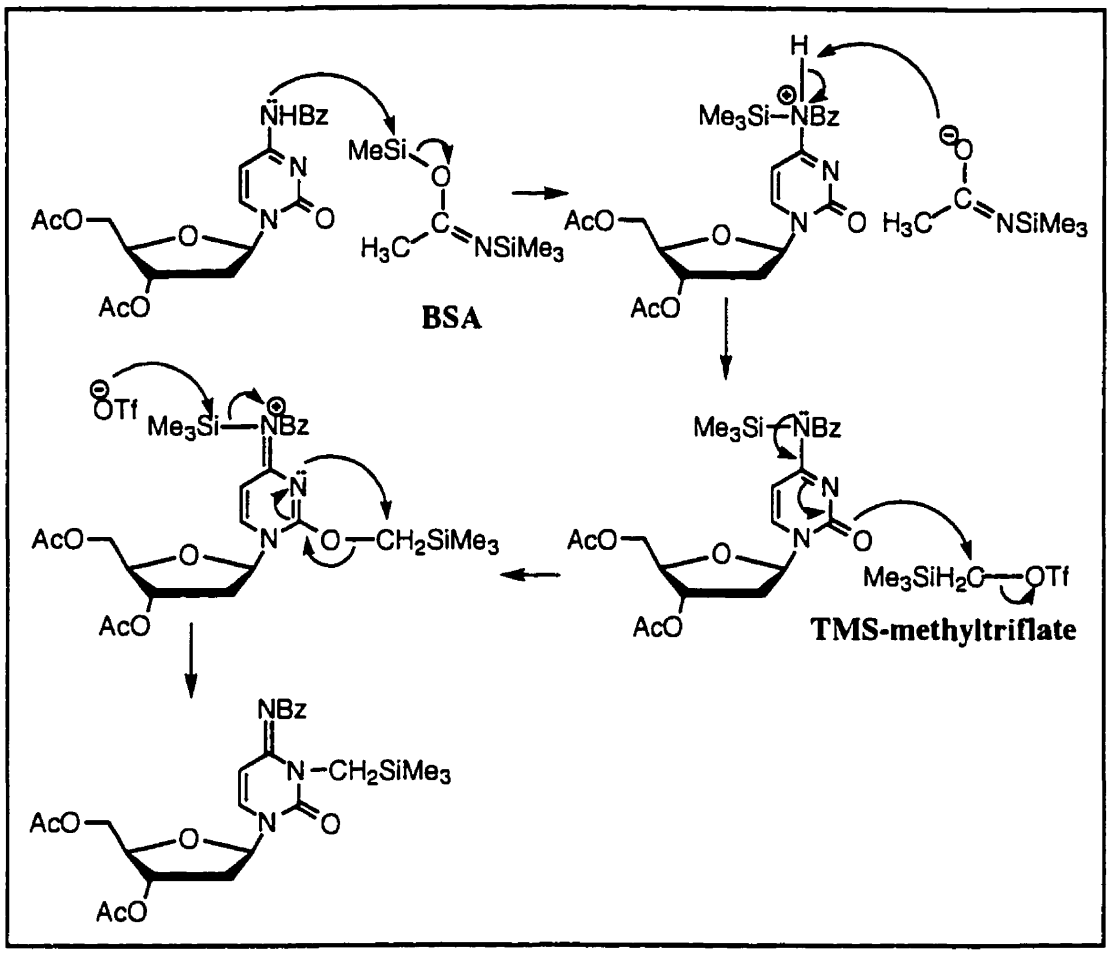
| Bond | Bond Length (Å) |
|---|---|
| C11 - N1 | 1.476 |
| C2 - N1 | 1.384 |
| C2 - N3 | 1.394 |
| C31 - N3 | 1.482 |
| C4 - N3 | 1.406 |
| C4 - N4 | 1.298 |




We proposed a mechanism for the formation of base modified nucleoside **3** (**Scheme 4**) based on its elucidated structure. This mechanism is an adaptation of that proposed by Vorbruggen[52] for the Hilbert-Johnson reaction of persilylated heterocyclic bases with peracylated sugars in the presence of a Friedel-Crafts catalyst (**Scheme 5**).

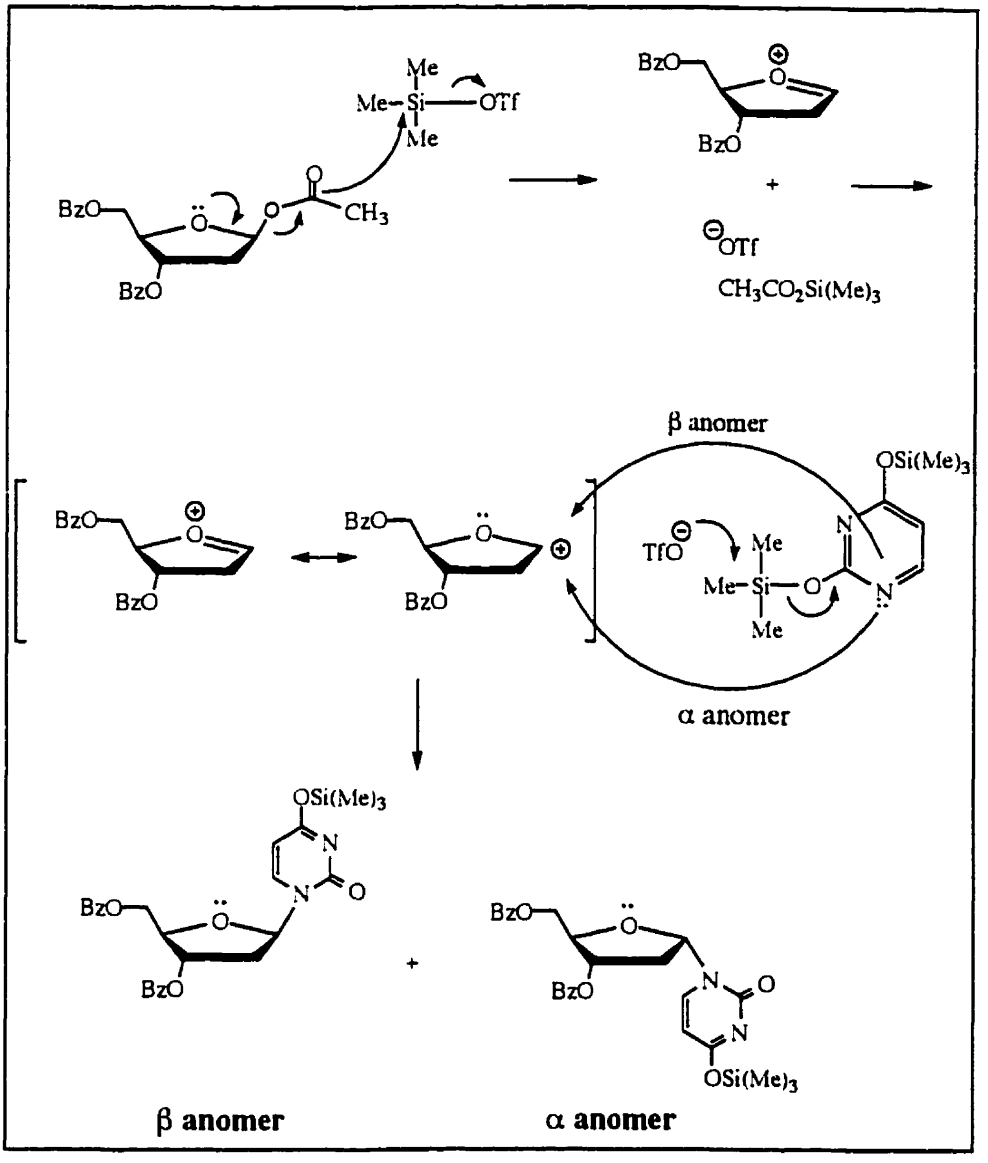
[52] Vörbruggen, H.; Hölfe, G. *Chem. Ber.* **1981**, *114*, 1256 - 1268.

**Scheme 4**




In contrast to Vorbruggen's nucleoside synthesis, the base moiety is already attached to the di-acetylated sugar. The first step in the reaction that we performed was the treatment of the 5',3'-diacetylated nucleoside (**2β**) with BSA, a silylating agent. We propose that the $N^4$ position becomes silylated by BSA in the first step, as opposed to the $O^2$ position, even though the O-silyl form would be favored due to the greater thermal stability of the Si-O bond in comparison with the Si-N bond.

**Scheme 5**




However, in this case, the N-silyl form would be favored due to the possibility of amide resonance and also because $N^1$ is more nucleophilic than $O^2$. Previously it has been shown that both N- and O-TMS derivatives could be prepared, but of the tautomeric forms of an amide, RCONHSi dominates over RC(OSi)=NH. [53] In the second step the $O^2$ position becomes methylated by TMS-methyltriflate. Then we propose that a 1-3 rearrangement takes place in order to give the observed product.

[53] Pierce, A.E. *Silylation of Organic Compounds*. **1979**.

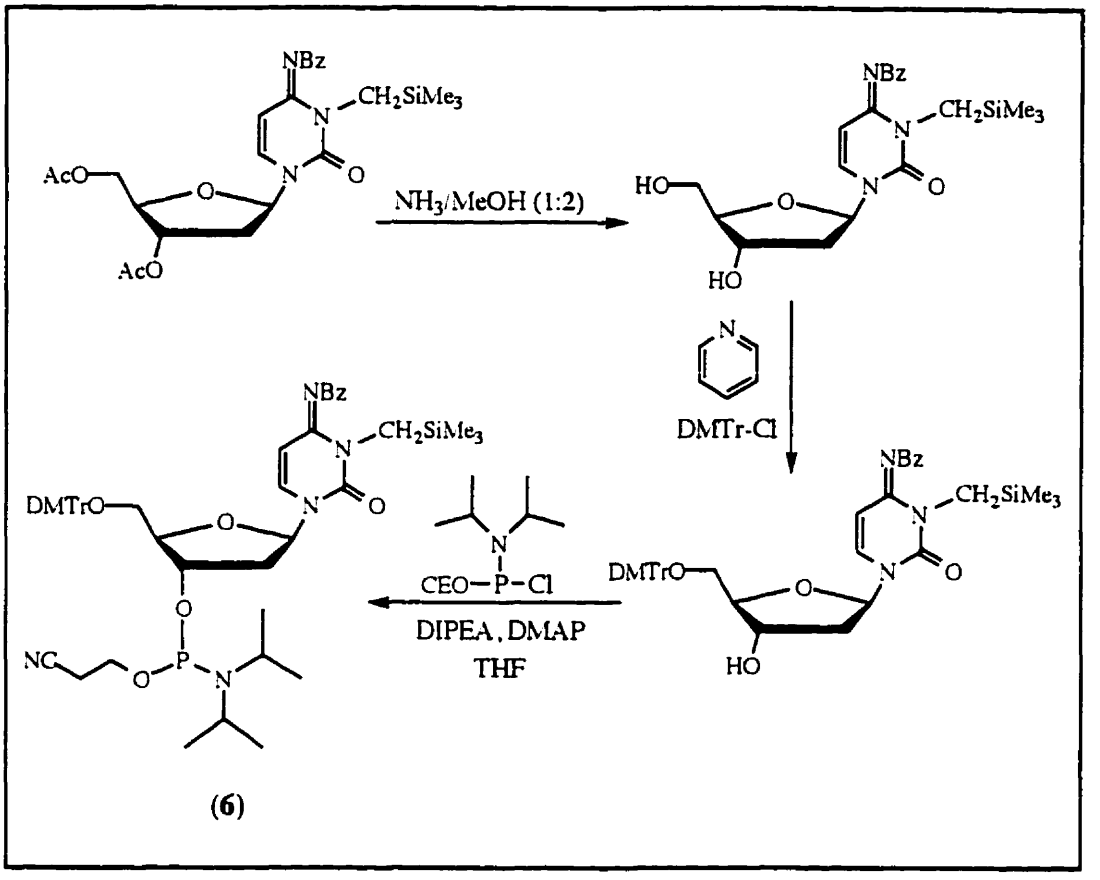
### 2.1.2 Synthesis and possible applications of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidine-$N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'-deoxycytidine(6)

Since we had regioselectively modified cytidine at the $N^3$-position, we decided to synthesize its phosphoramidite derivative. Such a derivative may find use in being implemented into an oligonucleotide sequence for further binding studies.

The base modified cytidine was treated with a solution of ammonium hydroxide and methanol at room temperature in order to selectively remove the acetyl protecting groups on the 5'- and 3'- hydroxyls, and not the $N^4$-benzoyl protecting group (**Scheme 6**). The subsequent steps leading to the synthesis of the base modified phosphoramidite (**6**) are the same as for non-modified nucleosides.[54] The first step being the protection of the 5'-hydroxyl group with dimethoxytrityl chloride, (69% yield) followed by the coupling reaction with *N,N*-diisopropyl (2-cyanoethyl) phosphonamidic chloride. This reaction gave the desired phosphoramidite (**6**) in a yield of 87%, which is analogous to yields obtained for unmodified phosphoramidites. The product was characterized by both $^1$H and $^{31}$P NMR. (Appendix II)

[54] (a) Gait, M. J, Ed. *Oligonucleotide Synthesis: A Practical Approach*, **1984**, IRL Press, Oxford. (b) Damha M. J.; Ogilvie, K. K. in *Protocols for Oligonucleotides and Analogs: Synthesis and Properties* **1993**, (Agrawal, S.,Ed.), 81 - 114, The Humana Press, Inc., Totowa, NJ.

**Scheme 6**




It has already been shown in our laboratory that deoxythymidine can be regioselectively functionalized at the $N^3$-postition with a linker in order to stabilize triple helical structures.[13] In the case of the novel $N^3$-functionalized cytidine, the addition of a linker would entail the cleavage of the C-Si bond, which would be very challenging due to the high stability of this bond. However a possible application for this novel phosphoramidite (**6**) is insertion into an oligonucleotide sequence. It would be interesting to see how the stability of the resulting duplex structure would compare to the natural duplex, as well as a duplex containing a mismatch at the same position where the $N^3$-funtionalized unit would be inserted. The insertion of the $N^3$-funtionalized nucleoside unit (**6**) should cause a greater destabilization in the duplex structure as compared to a mismatch in the same position. This destabilization, as well as distortion of the duplex structure,

would result from the loss of the $N^3$-H donor which partakes in Watson-Crick base pairing, as well as increased steric hinderance imparted by the bulkiness of the methyltrimethysilyl moiety at the $N^3$-position.

### 2.1.3. Synthesis of 5'-*O*-Dimethoxytrityl-3'-*O*-*N*,*N*-diisoproyl-β-cyanoethyl-phosphoramidine-$N^4$-benzoyl-L-α -2'-deoxycytidine (8)

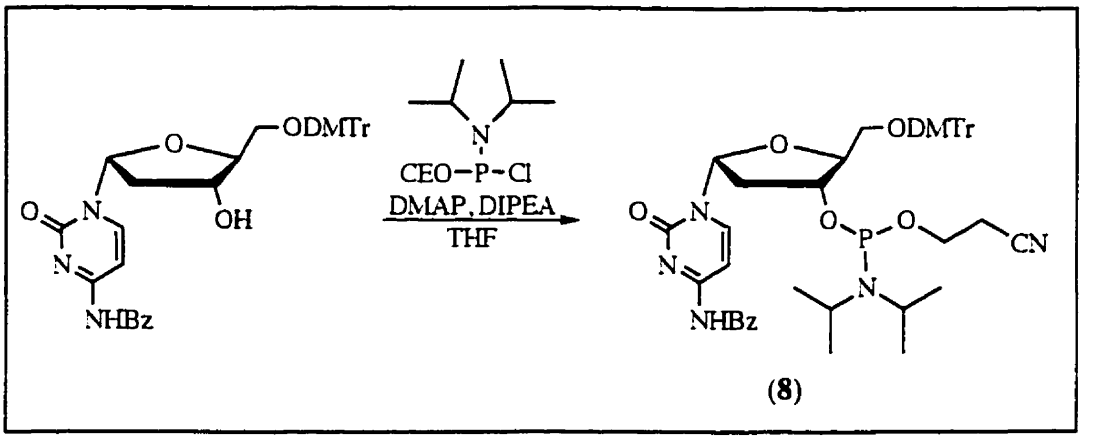
The pyrimidine nucleoside, L-α-2'-deoxycytidine (L-α-dC), was chosen for insertion into an oligonucleotide sequence, since it can be readily prepared from the naturally occurring L-arabinose.[35,36] Also, if desired, the purine nucleosides L-dA and L-dG can be obtained from L-dU by transglycosylation.[15a] Recently, a shorter synthetic approach to L-pyrimidine nucleosides has been reported by Spadari *et al.*[55] This synthetic strategy is also applicable to L-α-pyrimidine nucleosides.

The synthesis of 5'-*O*-Dimethoxytrityl-3'-*O*-*N*,*N*-diisoproyl-β-cyanoethyl-phosphoramidine-$N^4$-benzoyl-L-α -2'-deoxycytidine (**8**), was achieved from a phosphytilation reaction with the precursor, 5'-*O*-Dimethoxytrityl- -$N^4$-benzoyl-L-α -2'-deoxycytidine, which was synthesized and given to us by Dr. A. Garbesi (CNR - Bologna, Italy) (**Scheme 7**). The reaction conditions are the same as those for the synthesis of the aforementioned phosphoramidite (**6**). The product (**8**), was obtained in 87% yield and characterized by UV, $^1$H and $^{31}$P NMR spectroscopy (Appendix II).

---

[55] Spadari, S.; Maga, G.; Focher, F.; Ciarrocchi, G.; Manservigi, R.; Arcamone F.; Capobianco, M.; Carcuro, A.; Colonna, F.; Iotti, S.; Garbesi, A. *J. Med. Chem.* **1992**, *35*, 4212 -4220.

**Scheme 7**




(8)

## 2.2. Preparation and Binding Studies of Sugar Modified Oligonucleotides

### 2.2.1 Synthesis and Purification of Oligomers

Once we obtained the purified 5'-*O*-Dimethoxytrityl-3'-*O*-*N*,*N*-diisoproyl-β-cyanoethylphosphoramidine-$N^4$-benzoyl-L-α-2'-deoxycytidine monomer, it was our goal to incorporate it into an oligonucleotide sequence in the 3',3' →5,'5' direction in order to study binding effects. Two sequences were chosen for the binding studies. One sequence was an 18-mer that was complementary to an 18 nt (nucleotide) sequence present near the beginning of the 5' LTR region of HIV-1 genomic RNA. The other sequence chosen was a G, C rich 7-mer. Two 7-mer sequences were synthesized, one containing a L-α-dC insert (**K**) and the other containing a D-α-dC insert (**J**), in which both inserts where oriented it the 3'3'→5' 5'orientation. This 7-mer sequence chosen is similar to the one that Bloomers *et al.*[45] used in their NMR study of the structural effects of an internal L-β insert in the normal 3'5'-L-β-3' 5' orientation. All the oligonucleotides that were used in the thermal denaturation studies are given in **Table**

**2.2**. The syntheses of oligomers **A - E , I, J** and **K** was performed on an Applied Biosystems 381A DNA Synthesizer using standard protocols.[56]

**Table 2.2**: Oligomer sequences used in thermal denaturation studies

| Oligomer Notation | Oligomer Sequence |
|---|---|
| **A** | 5' GAT CTG AGC CTG GGA GCT 3' |
| **B** | 5' AGC TCC CAG GCT CAG ATC 3' |
| **C** | 5' TAC GCA CGT CAC GTA CCG 3' |
| **D** | 5' AGC TCC C<u>C</u>G GCT CAG ATC 3' |
| **E** | 5' GAU CUG AGC CUG GGA GCU 3' |
| **F** | 5' AGC TCC CAG GCT CAG AT(**L-β-C**) 5' <br> 5' 3'3' 5' |
| **G** | 5' AG(**L-α-C**) T(**L-α-C**) C (**L-α-C**) AG G (**L-α-C**) T (**L-α-C**) AG AT(**L-α-C**) T 3' <br> 5' 3'3' 5'5' 3'3' 5'5' 3'3' 5'5' 3'3' 5'5' 3'3' 5'5' 3'3' 5'5' 3' |
| **H** | 5' GCA CGC G 3' |
| **I** | 5' CGC GTG C 3' |
| **J** | 5' GCA (**D-α-C**)GC G 3' <br> 5' 3'3' 5'5, 3' |
| **K** | 5' GCA (**L-α-C**)GC G 3' <br> 5' 3'3' 5'5, 3' |

Footnote: <u>C</u> refers to a mismatch compared to oligonucleotide **B**.

→ refers to a parallel orientation of the internucleotidic linkage.

← refers to an anti-parallel orientation of the internucleotidic linkage.

All oligonucleotide sequences are composed of deoxyribo- oligonucleosides, except for ribonucleotide **E**.

[56] Sequences B and C were purchased from Dalton Chemical Laboratories, Inc. (Toronto), whereas G, H, I and J were synthesized by Dr. Richard Pon (University Core DNA Services, The University of Calgary, Alberta)

In the case of the 7-mer **K**, the polarity reversal of the L-α insert with a 3'3' and 5'5' phosphate linkage, necessitated automated chemical synthesis in the 5' to 3' direction (**Figure 2.3a**) rather than in the conventional 3' to 5' direction[57] (**Figure 2.3b**), because the L-α-dC, 3'-phosphoramidite had been synthesized. This also required that the other monomer be 5'-phosphoramidites in order to ensure the correct phosphodiester connectivity. In order to obtain the reversal in the phosphate linkage for oligomer **F** a solid support with 3'-dimethoxytrityl-$N^4$-benzoyl- L-β-2'-dC bound through its 5' end was used in the conventional 3' to 5' automated synthesis. This resulted in oligomer **F** having two 5' ends. The coupling efficiencies of the aforementioned oligonucleotides was monitored by the release of the orange colored dimethoxytrityl cation, and was found to be greater than 92% (average) for all the oligomers prepared.

[57] Van de Sande, J. H.; Ramsing, N. B.; Germann, M. W. Elhorst, W.; Kalisch, B. W.; Kitzing E. V.; Pon, R. T.; Clegg, R. C.; Jovin, T. M. *Science* **1988**, *241*, 551 - 557.

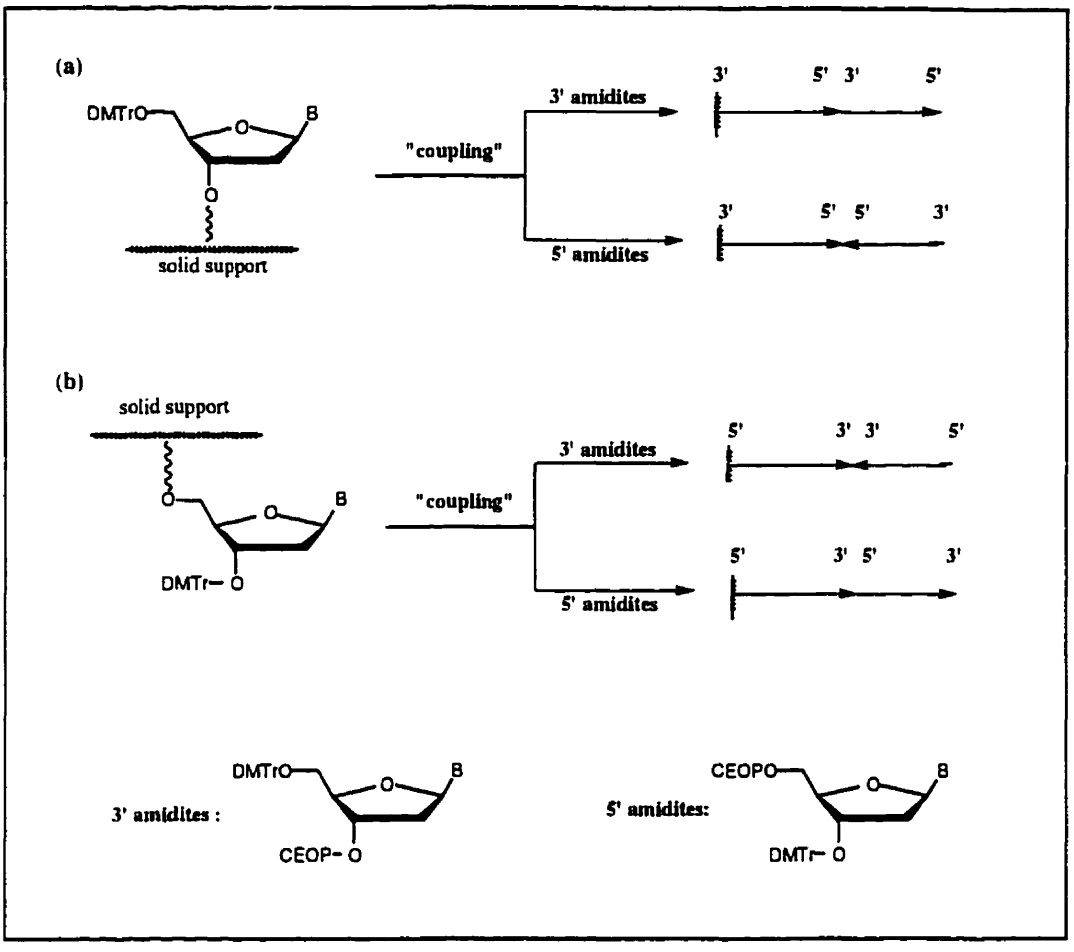



**Figure 2.3**: Automated DNA synthesis in the (**a**) conventional 3'→5' direction, and (**b**) the 5'→3' direction.

Cleavage of the oligomers from the solid support as well as deprotection of the bases was accomplished using concentrated ammonia at 50 °C for 16 hours. Following the removal from the solid support cassette, the oligonucleotides were purified either by using a reversed-phase oligonucleotide purification cartridge (OPC) or by preparatory polyacrylamide gel electrophoresis. The purity of the oligomers was >95% as assessed by polyacrylamide gel electrophoresis. For example, as can be seen in **Figure 2.4**, the heptamers **H** - **K** migrated as single bands .

1 2 3 4

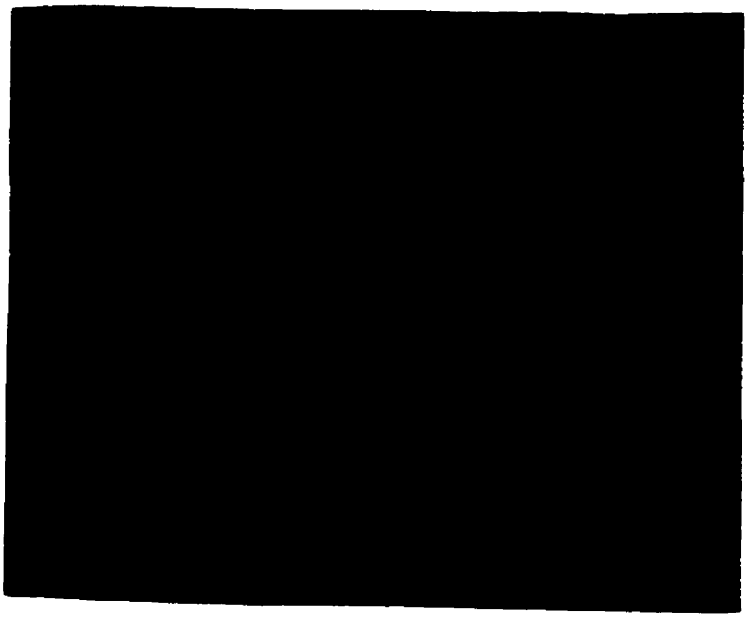


**Figure 2.4**: Polyacrylamide gel electrophoresis 0.35 OD units of purified oligomers **H** to **K**. Lanes: 1) **H**; 2) **I**; 3) **J**; 4) **K**.

## 2.3 Thermal Denaturation Studies

The physicochemical properties are one of the major determinants of the efficiency of antisense oligonucleoties. The affinity of binding between the modified oligonucleotide and its target sequence is characterized by the melting temperature $T_m$ of the duplex formed by association. $T_m$ is the temperature at which 50% of the double strands has dissociated into two single strands. The major factors contributing to duplex stability are the base stacking and hydrogen bonding between complementary base pairs.

The UV spectrum is a convenient way to monitor the formation and breakdown of double helices since they are sensitive to base stacking effects and therefore sensitive to any structural modifications on the natural oligonucleotide. As the ordered regions of stacked

base pairs are disrupted, the UV absorbance increases. The increase in absorbance is called hyperchormicity, thus the absorbance of a native nucleic acid is hypochromic relative to its nucleotides; the amount of hypocromicity is a measure of the base pairing and stacking of the secondary structure.

In a thermal denaturation study, the temperature of a solution containing double-helical DNA or RNA is slowly raised. This results in a sudden increase in the UV absorption at a certain temperature because the ordered double helices dissociate in a cooperative manner. The midpoint of this transitions is the melting point, $T_m$. The $T_m$ value is dependent on the base composition, especially on G-C content; on the concentration of the oligonucleotide, and on the ionic strength of the buffer used.

### 2.3.1 Binding Studies on 18-mer containing a terminal L-β unit

Our laboratory was one of the first to study mixed L/D-oligodeoxynucleotides.[14,58] It was demonstrated that L-β monomer units in the terminal positions of the natural sequence, significantly enhance its stability toward exonuclease digestion without considerably altering binding to the natural target sequence. A subsequent study in our laboratory also showed that the same qualities applied to oligonucleotides containing the L-β-C inserts in the 3'3'→ 5'5' orientation[14], although a significantly greater destabilization was observed. This provided the basis for synthesizing the oligonucleotide sequence containing the "reversed" 3'3'- L-β-dC 5' nucleoside unit at the terminal position, where destabilization of potential duplex formation is minimized. It was also our intention to determine whether this modified nucleoside may act as a primer for the RT of HIV since it is complementary to an 18 nt sequence present near the beginning of the 5' LTR region of HIV-1 genomic RNA (Section 2.4.1).

Thermal denaturation studies were performed on the modified oligonucleotide (**F**) with both the DNA and RNA targets. For comparison, the $T_m$'s for the unmodified

[58] Capobianco, M.L.; Garbesi, A.; Arcamone, F.; Maschera, B.; Palu, G. *Nucleic Acids Symp. Series*, **1991**, *24*, 274.

oligomer (**B**) as well as for the oligonucleotide containing a mismatch (**D**) were measured. The $T_m$ values as well as the percent hyperchromicity (%H) are reported in **Table 2.3**.

**Table 2.3:** Duplex hybridization data for oligomer containing a L-β unit (**F**).

| Oligomer | DNA Target (A) | | RNA Target (E) | |
|---|---|---|---|---|
| | $T_m$ | %H | $T_m$ | %H |
| **B** | 69 | 12 | 71 | 12 |
| **D** | 66 | 15 | 64 | 17 |
| **F** | 68 | 13 | 68 | 12 |

The thermal denaturation data indicate that there is no significant destabilization in the hybrids formed between the modified oligonucleotide (**F**) and the DNA (**A**) and RNA (**E**) target, as compared to the natural sequence (**B**). This is also evident in the melting profiles (**Figure 2.5 a and b**), where it can be seen that the melting curves for the modified oligomer **F** are nearly superimposed with that of the natural oligomer. It is also an indication that the thermodynamics of hybridization (ΔH, ΔS) are very similar to that of the natural oligomer.[21] This is as expected since a modification at the terminal position should not have a great destabilizing effect. However, it has been shown that when an L-β-dC monomer is inserted with a 3'3' to 5'5' linkage within a 13-mer, a greater destabilization is observed.[43] Even though destabilization also occurs when the same monomer is linked with the conventional 3' to 5' phosphate linkage, 3',5'-L-β-dC-3'5' does so to a lesser extent confirming other studies, that the most favorable orientation for an L-β monomer is anti-parallel to it's complementary sequence (See **Figure 1.10D**). Similarly, when a mismatch is incorporated in an internal position of the sequence, the decrease in $T_m$ is greater (See data complexes formed by **D** and target DNA or RNA, **Table 2.3**).

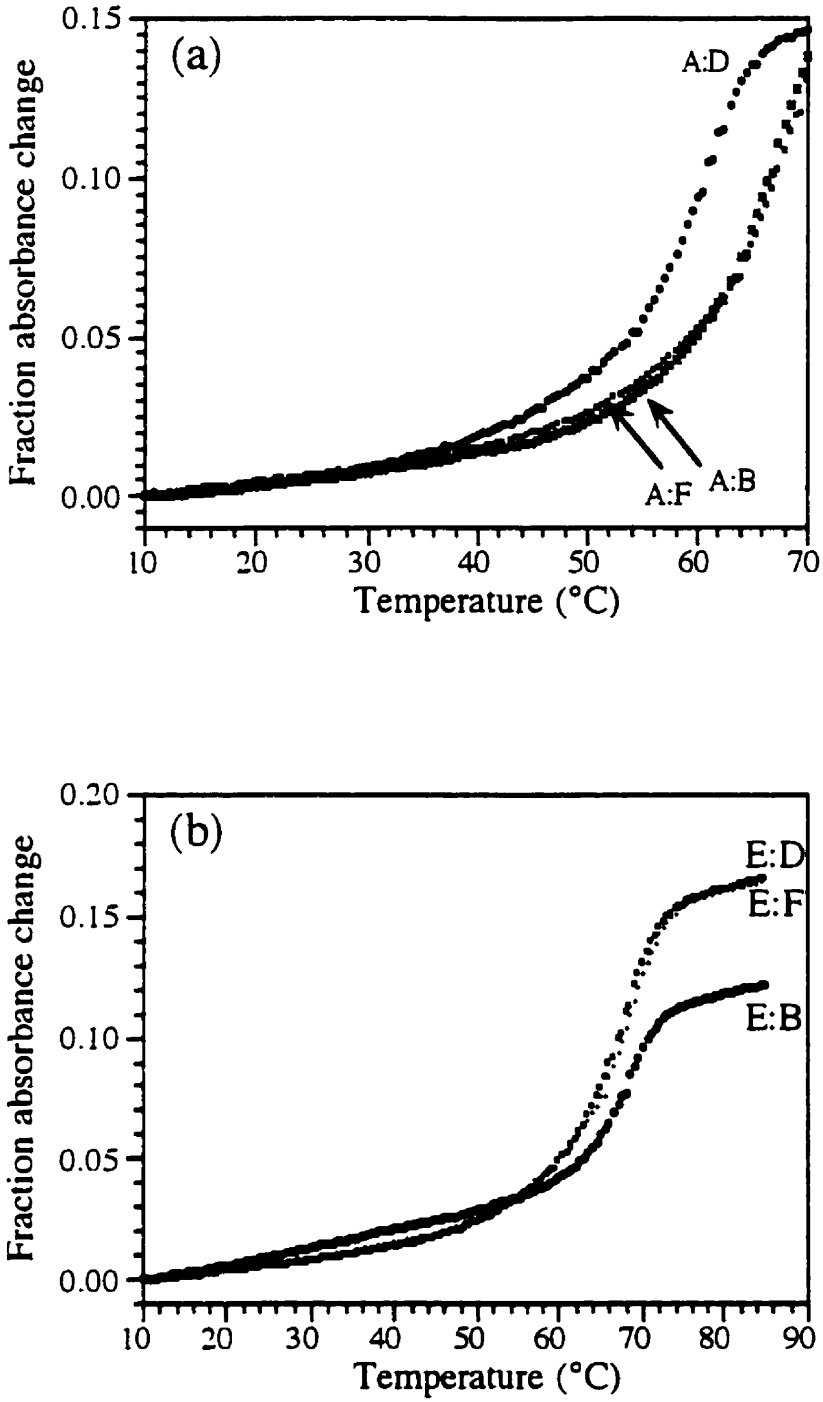



**Figure 2.5** :(**a**) Thermal denaturation curves of duplexes formed by natural oligomer **B**, L-β modified oligomer **F** and mismatch oligomer **D** with their complement DNA (**A**) in physiological buffer (140 mM KCl, 5 mM $Na_2HPO_4$, 1 mM $MgCl_2$, pH = 7.2 .
(**b**) Thermal denaturation curves of aforementioned oligomers with their RNA complement (**E**) in physiological buffer.

The ability of this modified oligonucleotide (**F**) to hybridize to its complementary RNA sequence (**E**) is crucial for its use as an antisense agent. From the binding studies we have shown that this requirement has been met. A previous study in our laboratory has shown that an L-β insert at the terminal positions increases the stability toward exonucleases.[14] These considerations suggest that this modified oligonucleotide would be a good candidate to study the inhibition of DNA synthesis from viral genomic RNA, as well as to probe DNA priming during reverse transcription (Section 2.4).

### 2.3.2 Comparison of thermal stability between D-α and L-α inserts

Prior to studying the effects on thermal stability that one or more L-α inserts would have in an oligomer, we decided to compare the thermal stability between an oligomer containing a L-α-dC insert and the same sequence containing a D-α-dC monomer, in which both monomers are inserted in the 3'3' →5'5' direction. Interaction of the heptamers containing an L-α insert (**K**), the D-α insert (**J**) and the natural heptamer (**H**), with their complementary DNA sequence (**I**) was followed by UV spectroscopy. The binding studies were performed in two different buffers and the data is given in **Table 2.4**.

**Table 2.4**: Thermal denaturation data from binding study on heptamers.

| Oligomer | Physiological Buffer | | 1.0 M NaCl | |
|---|---|---|---|---|
| | $T_m$ (°C) | % H | $T_m$ (°C) | % H |
| **H** | 42 | 12 | 45 | 3 |
| **J** | 36 | 6 | 40 | 8 |
| **K** | 35 | 5 | 33 | 2 |

The thermal denaturation data in physiological buffer shows that there is a destabilization of 6 °C and 7 °C, with heptamers **J** and **K**, respectively, in comparison to the natural sequence **H**.[30] The difference in length of the (3'→3') and (5'→5') -phosphodiester bridges relative to the natural (3'→5')-phosphodiester bridge may account for the lower $T_m$ values observed with the complexes formed with **J** and **K** relative to those formed with the native oligomer **H**. There is also a substantial decrease in the hyperchromicity, suggesting that the nucleobases of the modified oligomers, **J** and **K**, induced less base stacking than the unmodified sequence **H**, presumably due to structural differences.[30] The melting profiles show well defined sigmoidal curves for both modified oligomers, which indicate a cooperative transition (**Figure 2.6 a**).

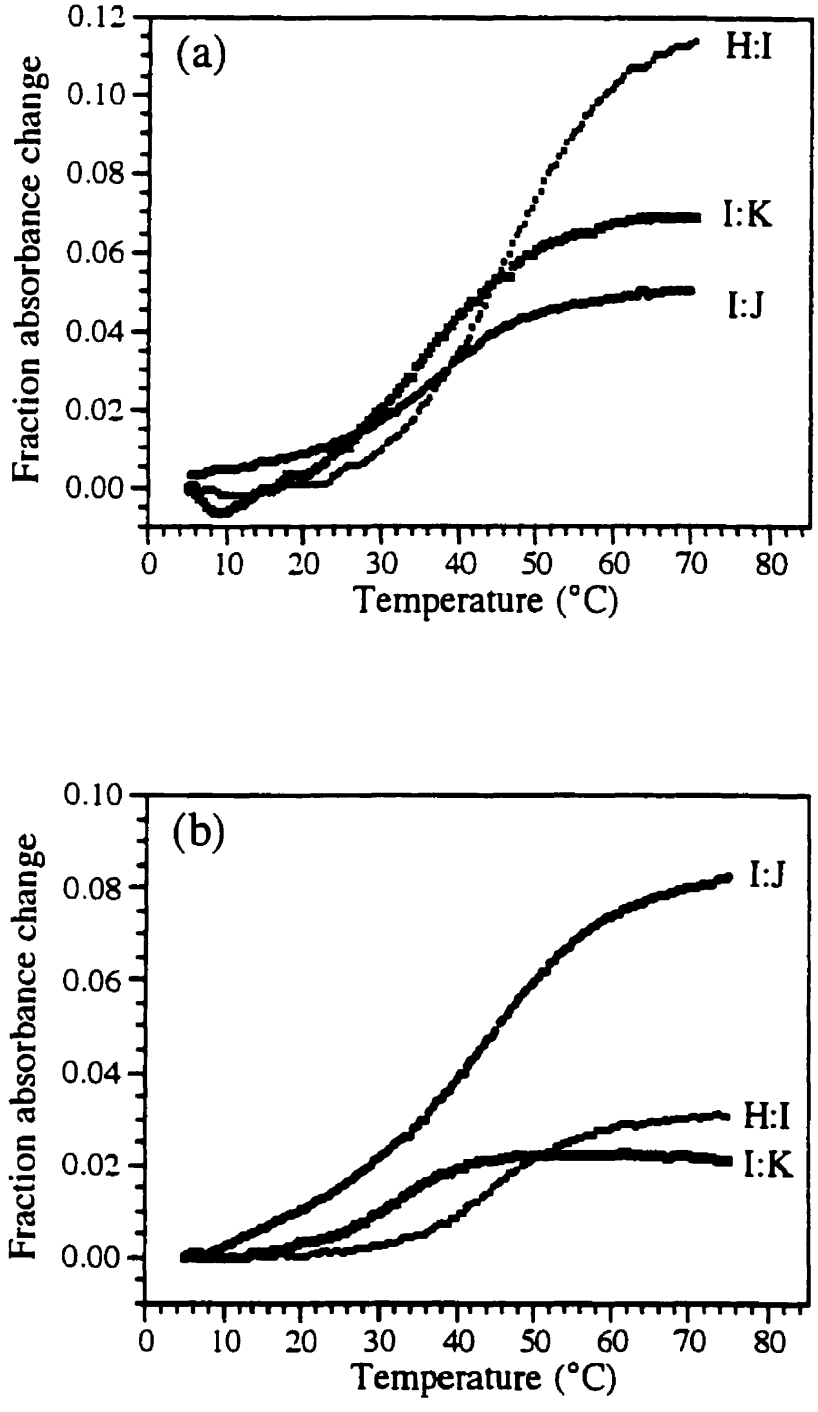



**Figure 2.6** : Melting profiles of the duplexes formed between heptamers **H** (natural sequence), **J** (D-α insert), and **K** (L-α-insert) with their complementary sequence **I** in both (**a**) physiological buffer [140 mM KCl, 5 mM $Na_2HPO_4$, 1 mM $MgCl_2$, pH = 7.2] and, (**b**) 1.0 M NaCl buffer [10 mM $Na_2HPO_4$, 1.0 M NaCl, pH = 7.0].

The results obtained in 1.0 M NaCl buffer shows a greater decrease in $T_m$ for the heptamer containing the L-α insert ($\Delta T_m$ = 12 °C), however a very minimal decrease in hyperchromicity is observed with respect to the unmodified heptamer **H**. The data obtained for the D-α insert in 1.0 M NaCl buffer is also somewhat ambiguous in comparison to that obtained in physiological buffer. In 1.0 M NaCl, only a slight decrease in $T_m$ is observed and a large increase in hyperchromicity. This would suggest that the high salt concentration stabilizes the duplex formed between the modified oligomer **J** and its complementary sequence **I**.

Spectroscopic and NMR studies have already been performed on oligonucleotides containing D-α-anomeric units and 3'3' and 5'5' phosphodiester linkages[31] These studies indicated that the insertion of D-αT-within a 10-mer, in the reversed polarity (3'3' → 5'5') still allowed for Watson-Crick base pairing without significant structural perturbation to the adjacent nucleosides. For this particular modified 10-mer the binding studies showed a $\Delta T_m$ = 5 °C. This corroborates the $\Delta T_m$ (5 °C) obtained for the D-α insert (**J**) in 1.0 M NaCl buffer.

Therefore, a valid continuation of this binding study would be to perform an NMR study on the aforementioned modified heptamers. Such a study would enable us to see the structural modifications of the duplex, imparted by the insertion of L-α versus D-α monomers. This would allow for a better understanding of the binding studies.

### 2.3.3 Thermal denaturation studies of a modified oligonucleotide containing 6 L-α inserts

The $T_m$ data obtained from the binding study of the heptamers showed no significant difference in destabilization between the L-α (**K**) and D-α inserts (**J**) and the natural sequence (**H**). However this may not be the case for longer duplexes, therefore we decided to study the thermal stability of a 19-mer sequence (**G**), which is complementary to the a region of HIV-1 genomic RNA, containing six L-α dC units oriented in the parallel

(3'3' → 5'5') fashion. In physiological buffer, the $T_m$ data obtained (**Table 2.5**) indicates that the duplex formed from the modified oligomer (**G**) and its complementary RNA (oligomer **E**) is more destabilized than the duplex formed with its DNA target (oligomer **A**), $\Delta T_m$= 8 °C and 4 °C / L-α insert respectively. Therefore in physiological buffer the modified oligonucleotide **G** shows selectivity for its DNA target over its RNA target. In order to determine if there was any non-specific binding, we also studied the possible interaction of a random sequence **C** (containing the same base composition as the natural oligomer **B**) and sequence **G**. It is evident from the melting profile (**Figure 2.7 a**), that there is no observable thermal helix to coil transition, between oligomer **C** and **G**, as indicated by the straight line, which is due to a normal change of absorbance as a function of temperature. However, for the duplexes, **A:B**, **B:E**, **C:G**, and **G:E**, a sigmoidal $T_m$ curve is observed.

**Table 2.5:** Thermal Denaturation data of modified oligonucleotide (**G**), with its complementary DNA and RNA targets, in both physiological and 1.0 M NaCl buffers.

| Oligomer | Physio Buffer | | | | 1.0 M NaCl | | | |
|---|---|---|---|---|---|---|---|---|
| | DNA (A) | | RNA (E) | | DNA (A) | | RNA (E) | |
| | Tm | % H | Tm | % H | Tm | % H | Tm | % H |
| **B** | 68 | 20 | 72 | 16 | 76 | 12 | 79 | 11 |
| **G** | 45 | 15 | 25 | 10 | 53 | 10 | 33 | 8 |

We also conducted the binding studies in 1.0 M NaCl buffer, since it has been shown that a buffer of high ionic strength augments the stability of the duplex by balancing the high anionic charge of the phosphate backbone.[59] Indeed, the $T_m$ values are higher than those observed in the physiological buffer and the extent of stabilization is the same for all complexes (**Table 2.5**). In addition, in this buffer, the modified oligonucleotide

[59] Alberts, B. et al. *Molecular Biology of the Cell*, Galland: New York, **1989**, p.301.

(**G**) also shows a greater specificity for the DNA target (**A**) as compared to the RNA target (**E**). (**Figure 2.7 b**). These results coincide with those obtained by Koga (et al)[7] from a thermal denaturation on alternating D-α- and D-β- deoxythymidine units with alternating (3'→3') and (5'→5') linkages[33]. Given that DNA/RNA duplexes adopt an A-type helical geometry[60], it is plausible that the L-α/D- β-oligonucleotide (**G**) does not accommodate the A-type helicity, dictated by **E** during complex formation, as the oligomer **A**. Hence the greater decrease in thermal stability for **G** with its complementary RNA (**E**) may be a result of a conformational incompatibility. However, it has been shown just recently that the insertion of a methylene or ethylene tether between the sugar moiety and the nucleobase of 3'→3'/5'→5'- linked D-α-nucleotidic residues, provides additional selectivity and allows for complementary nucleobases to better align and form more stable-Watson-Crick base pairs.[61] Such modified oligonucleotides, when inserted in the (3'→3') and (5'→5')-internucleotidic polarity, were shown to exhibit a slightly higher affinity for their corresponding unmodified RNA sequence than for the corresponding DNA sequence.

---

[60] Bush, C. A.; Brahms, J. In *Physico-Chemical Properties of Nucleic Acids*: Duchesne, J. Ed: Academic Press: London and New York, **1973**, Vol. 2, 147-186.
[61] Boal, J. H.; Wilk, A.; Scremin, C. L.; Gray, G. N.; Phillips, L.R.; Beaucage S. L. *J.Org.Chem.* **1996**, *61*, 8617-8626.

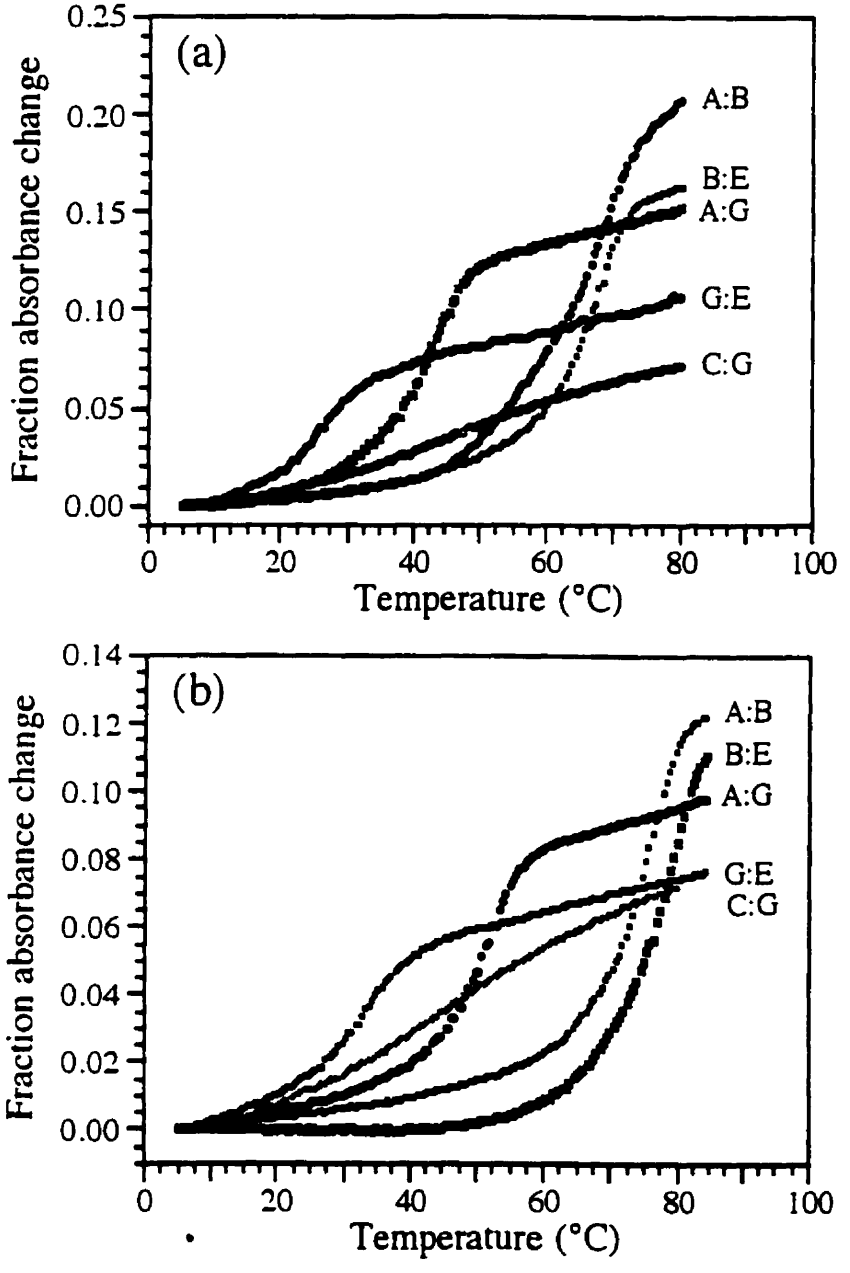



**Figure 2.7:** Melting curves at 260 nm of duplex formation between the native oligomer (**B**), modified oligomer (**G**), and their complementary DNA (**A**) and RNA (**E**) targets. (**a**) Binding studies in physiological buffer, including the melting profile of the random sequence (**C**) with the DNA target (**A**). (**b**) Binding studies in 1.0 M NaCl buffer.

## 2.4 Inhibitory Activity of Antisense Oligonucleotides Against in vitro DNA Polymerization / Inhibition of HIV-1 Infected Cells

### 2.4.1. HIV-1 Reverse Transcription

An essential step in the replication cycle of the human immunodeficiency virus type I (HIV-1), is the transcription of retroviral RNA into double-stranded DNA. As depicted in **Figure 2.8**, this is carried out by a multifunctional viral enzyme, reverse transcriptase (RT) and necessitates a primer (tRNA) annealed to the genomic viral RNA to initiate DNA synthesis. Once the retrovirus has entered the cell, the plus (+) strand viral RNA is transcribed into the minus (-) DNA strand by the RNA-dependent DNA polymerase activity of HIV reverse transcriptase (RT). Subsequently, the RNA that is hybridized to the (-) DNA strand is hydrolyzed by the ribonuclease H activity of RT.

Finally, the (-) DNA strand serves as a template for the synthesis for the second DNA strand, which occurs after the second "strand jump" and is carried out by the DNA-dependent DNA polymerase function of RT. An in-vitro reverse transcription assay has been developed by Parniak and coworkers[62], to study the properties of priming, RNA-dependent DNA polymerization, and template switching by HIV-1 RT, which are the same reactions that occur in infected cells. AONs complementary to the viral mRNA can prevent reverse transcription via different modes of action: competition with the primer, interaction with reverse transcriptase extension of the antisense oligomer via priming of the AON and termination of complementary DNA polymerization. In order to evaluate the potency of antisense oligonucleotides to inhibit HIV replication, we have synthesized 18 nt oligonucleotides with the sequence 5' AGC TCC CAG GCT CAG ATC-3'( Section 2.2.1). This sequence is complementary to a sequence near the 5' end of the viral HIV-1 genomic RNA in the LTR of RNA (R-region). However, antisense compounds composed of natural DNA or RNA sequences, have a limited application in the antisense strategy

[62] Arts, E. J.; Li, X.; Gu, Z.; Kleiman, L.; Parniak, M. A.; Wainberg, M .A. *The Journal of Biological Chemistry*, **1994**, *269*, 14672-14680.

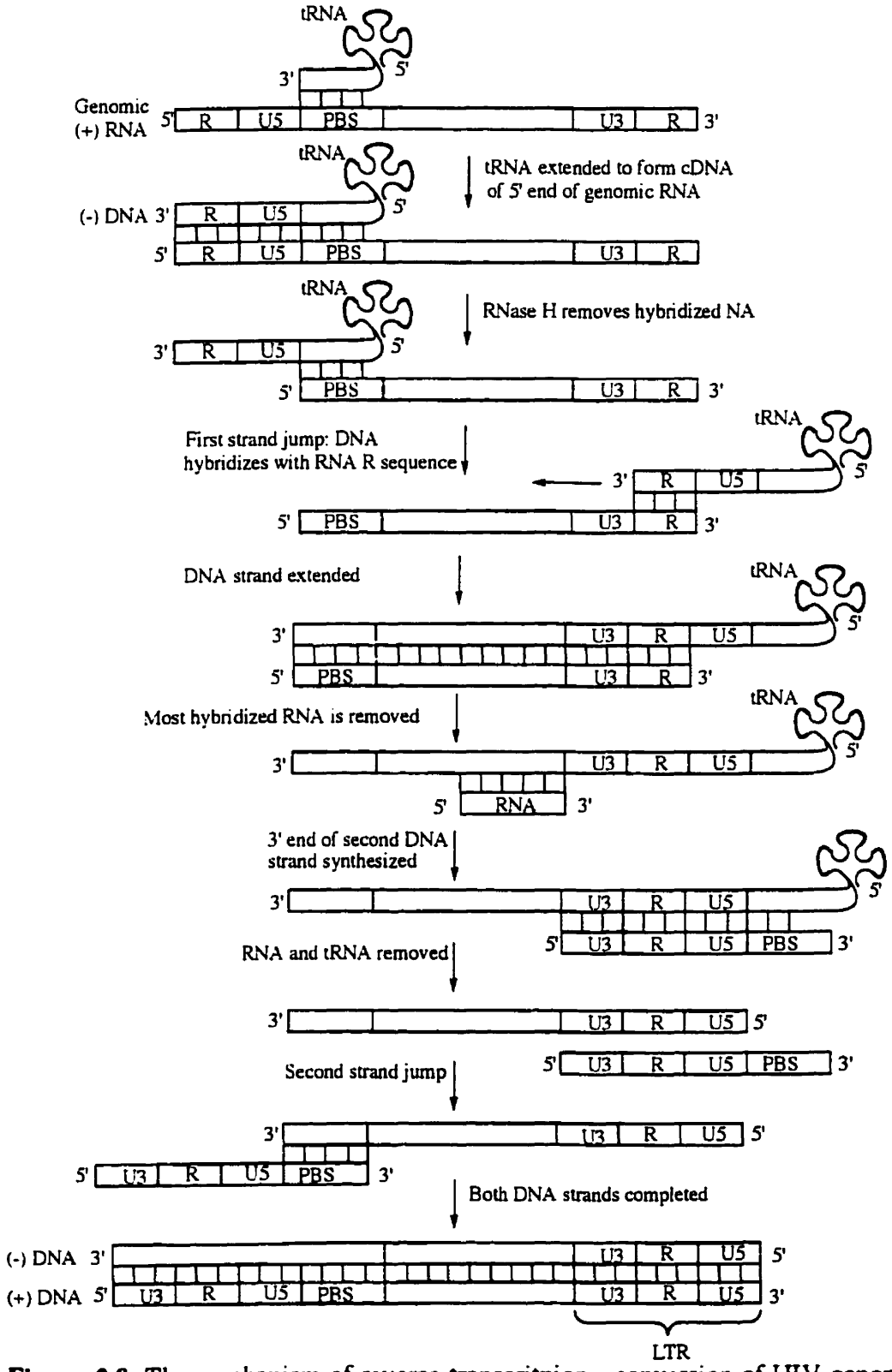



**Figure 2.8**: The mechanism of reverse transcritpion - conversion of HIV genomic viral RNA into double stranded DNA. Adapted from Stryer[63]

[63]Stryer, L. *Biochemistry*, W.H. Freeman and Company, New York, **1988**, p.875.

since they are rapidly degraded by nucleases. Therefore we synthesized the same aforementioned sequence with a modified L-β-dC monomeric unit at the 3' terminal end, linked via a 3'3' to 5'5' phosphodiester linkage. Such a modification has been shown to confer resistance towards nuclease degradation.[43] Thermal denaturation studies have been performed on these AONs as well as on a random sequence containing the same nucleobase composition (Section 2.3.1. and 2.3.3). The $T_m$ values obtained for both the natural AON and the modified L-β AON with their complemtary RNA target are 71 °C and 68 °C respectively. As expected the random sequence was unable to hybridize to the target RNA sequence.

### 2.4.2. Inhibition of (-) strong stop DNA synthesis by AON.

As illustrated in **Figure 2.9**, the (-) strong stop DNA encoded by reverse transcription from the PBS oligonucleotide annealed to the pHIV-PBS RNA template in absence of AON is 192 nt in length. However, shorter DNA polymerization products are also observed (162 nt). This 162 nt polymerization product is not noted in the absence of AON. Hence, the presence of DNA products shorter than 162 nt provides an estimate of the efficacy of AON in inhibiting HIV-1 (-) strong stop DNA synthesis in an *in vitro* system.

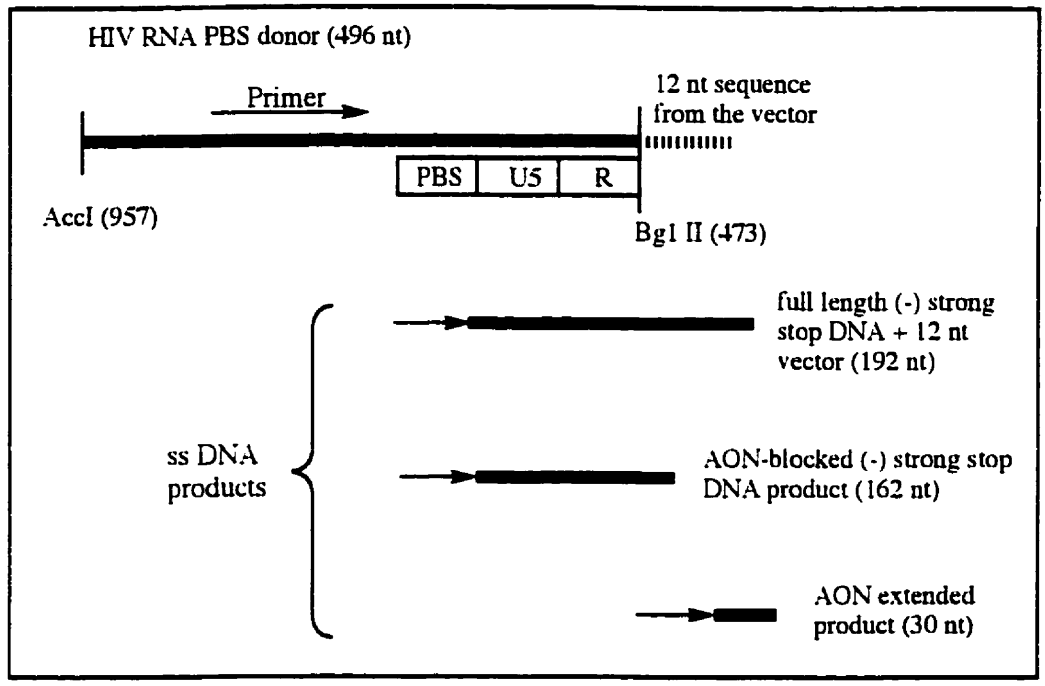



**Figure 2.9**: An illustration of the expected DNA polymerization products in *in vitro* DNA polymerization reaction. Polymerization products anticipated in experiments studying the effect of AONs on (-) strong stop DNA synthesis, using pHIV-PBS RNA containing an additional 12 nt of vector derived sequences.

Both of the AONs studied by Parniak and coworkers (McGill University; Lady Davis Institute), inhibited the synthesis of full length (-) strong stop DNA, as indicated by the appearance of significant amounts of the anticipated 162 nt polymerization product. This product accounted for about 65% of the total polymerization products in both of the DNA- and L- inhibited reactions (data not shown).

Significant amounts of a 30 nt product were noted in the reactions containing the AON DNA, both in absence and in the presence of the PBS primer. This polymerization product is a result of the RT-catalyzed 12 nt extension from the 3' end of the AON. These small DNA products were not seen in L- AON reactions, indicating that the L-β could not serve as polymerization primer for RT. This may be accounted for by the fact that the modified oligonucleotide contains two 5'ends and therefore might not serve as a substrate for the priming reaction, which necessitates a 3' terminal hydroxyl group.

### 2.4.3. AON Inhibition of template switching

In the *in vitro* template switching reaction, in the absence or AON and pHIV-R/U3 acceptor RNA the major DNA polymerization product was a 180 nt full-length (-) strong stop DNA product. The amount of 180 nt (-) strong stop DNA was reduced to about 20% or the control levels in the presence of both DNA and L AONs. Furthermore, the amount of the template switched polymerization product decreased, in the presence of the DNA and L AONs, by 54% and 69% respectively, relative to the control respectively. These results show that even though the AONs studied produce a truncated (-)strong stop DNA product, it is less effective in the first strand transfer process due to the decreased complementarity with the acceptor RNA template. Also, the decreased synthesis of full-length (-)strong stop DNA lowers the concentration of donor DNA, thereby limiting the effective rate of first strand "jump" transfer reaction.

Also, a larger DNA polymerization product (397 nt) when the pHIV-R/U3 acceptor was added in the absence of the AON or in the presence of the random sequence. This larger DNA polymerization product is also present when the AON DNA was used and corresponds to the polymerization product expected from the RT-catalyzed extension of these priming AONs to the pHIV-R/U3 template. This product was not observed in the reactions containing the L-β modified AON, since this oligonucleotide cannot prime RT polymerase activity. It is also important to note that these template switching experiments were carried out by incubating the reaction components at 37 °C only, without an initial heat annealing step to position the AON on the RNA template. The thermal stability data indicate that an AON targeted to a sequence in the R region of the HIV-1 genomic RNA have a high degree of binding and stability at 37 °C.

### 2.4.4 Future Work

Ongoing collaboration with Parniak and coworkers of the McGill Aids Center, will allow us to study the inhibitory potency of the modified 19-mer (**G**) containing six L-α-dC monomeric units attached via 3'3'and 5'5' phosphodiester linkages. This sequence, as the

DNA and L-β AONs,[64] is also complementary to the a region near the 5' end in the LTR region of HIV-1 genomic RNA. Furthermore our laboratory has extended the oligonucleotide by one monomeric unit at the 3' end with natural dT, in order to evaluate the priming ability of this modified oligomer containing a 3'-OH as opposed to the L-β AON.

[64] Borkow, G.; Arion, D.; Noronha, A.; Scartozzi, M.; Damha, M. J.; Parniak, M.A. International Journal of Biochemistry & Cell Biology (in press).

## 2.5 Conclusions

To the best of our knowledge, we have been the first to synthesize the novel oligonucleotides containing L-α-deoxynucleoside units in the 3'3'→5'5' orientation. A 19-mer sequence, complementary to the 5'-LTR region of HIV-mRNA, containing alternating L-α-dC units oriented in the 3'3'→5'5' direction, was synthesized. From the thermal denaturation studies, it may be concluded that this modified oligonucleotide forms a stable duplex with both its DNA and RNA targets (ΔTm = 4 °C and 8 °C respectively). Hence the novel 19-mer showed selectivity for its DNA target. The destabilization imparted by the insertion of the L-α-dC monomeric units in the "parallel" orientation is greater in comparison to that of oligonucleotides containing D-α-dN units in the same orientation (ΔTm/D-α-insert = 1 °C).[33] However, the modified oligonucleotides containing the 3'3'-D-α-dN-5'5' inserts also showed selectivity for its DNA target. Our own comparative study was done on a 7-mer sequence; one containing an internal L-α-dC monomeric unit oriented in the 3'3'→5'5' direction and another containing a D-α-dC monomeric unit in the same position with the same internucleotidic phosphate linkage. The binding study demonstrated that the destabilization imparted by both D-α- and L-α-monomeric insertions is similar in physiological buffer (ΔTm = 6 °C and 7 °C respectively). Since the stability of the duplex formed between the modified 19-mer with both its DNA and RNA targets are comparable to those of oligonucleotides containing D-α-dN inserts, studies that probe the ability of this oligonucleotide to inhibit DNA synthesis as well as to test its priming ability, are currently underway.

Another 18-mer sequence, complementary to the 5'-LTR region of HIV-mRNA, containing a terminal L-β-dC unit in the 3'3'→5'5' orientation was synthesized. Oligonucleotides containing L-β- inserts have alreay been synthesized and studied in by our laboratory. The binding study done on the modified 18-mer oligonucleotide did not show a significant destabilization upon duplex formation with both its DNA and RNA targets. Since the nuclease resistance imparted by the insertion of L-β- monomers has already been

confimed[43], we decided to probe the ability of this modified 18-mer to inhibit the synthesis of DNA, as well as to probe its ability to serve as a primer. The inihibition studies done by Parniak and co-workers showed that the amount of (-) strong stop DNA synthesized decreased by 69% in presence of the L-β- modified 18-mer. It was also shown that the L-β-modified containing two 5'-ends did not serve as a substrate for the priming reaction which requires a 3' terminal hydroxyl group.

We have also described the novel synthesis of $N^3$-functionalized D-β-2'-deoxycytidine phosphoramidite. The structure of this novel oligonucleoside was elucidated by NMR and X-ray crystallography.

# 3. EXPERIMENTAL

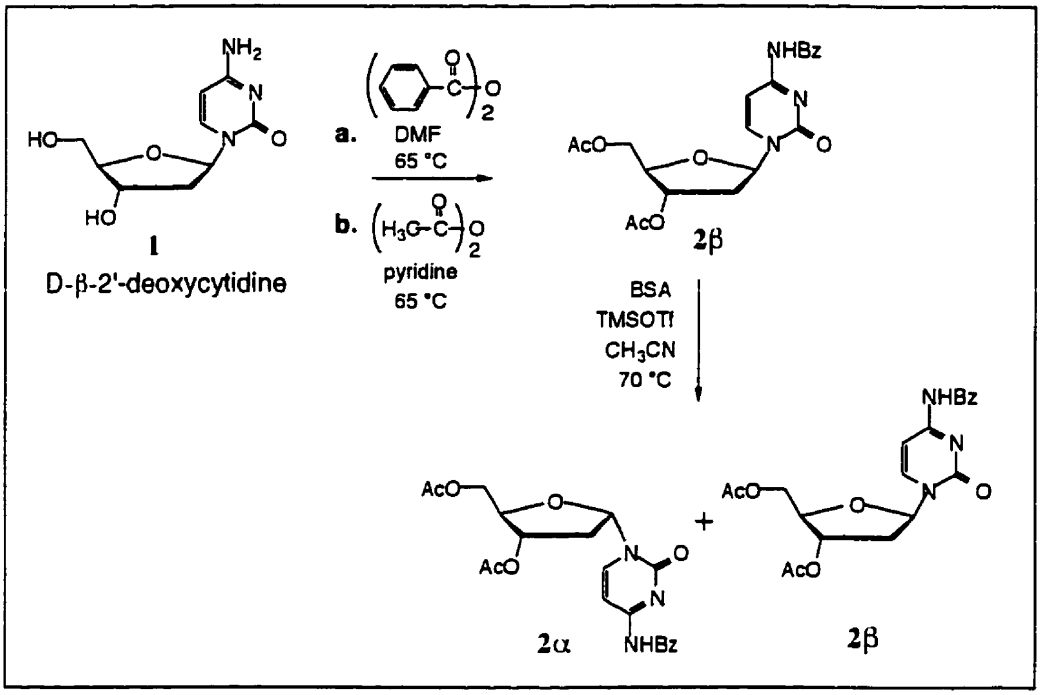
## 3.1 General Methods

X-Ray crystallography was performed using a Rigaku diffractometer using NRCVAX system programs. All details concerning the X-ray structure are shown in Appendix 1. 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. $^{1}$H-NMR spectra were obtained on a Varian Unity 500 spectrometer at a frequency of 499.84 MHz, and the peak assignments were made, in some cases, with the aid of COSY experiments. Chemical shifts are reported with a scale of parts per million (ppm). The residual proton signals of chloroform and acetone (assigned values of δ 7.24 and 2.05 ppm, respectively) were used as reference signals in these solvents. The multiplicities are recorded using the following abbreviations: **s**, singlet; **d**, doublet; **dd**, doublet of doublet; **dt**, doublet of triplets; and **m**, multiplet. Representative 1 & 2-D experiments are shown in Appendix II. $^{31}$P-NMR spectra were obtained on a Varian Unity 500 spectrometer at a frequency of 202.34 MHz. A standard phosphoric acid solution, 85 wt. % solution in water, was used as an external reference for all samples regardless of solvent.

Pyridine, acetonitrile and triethylamine were distilled from calcium hydride. Methylene chloride was distilled from $P_2O_5$. Tetrahydrofuran was distilled from sodium benzophenone ketyl. *N,N*-Dimethylformamide was dried by shaking with KOH followed by distillation from BaO. For the antisense work, all traces of acidity were removed from ethyl acetate by shaking with saturated aqueous $NaHCO_3$, drying over $MgSO_4$ and filtering. Thin-layer chromatography (tlc) was performed on silica gel (Kieselgel 60 $F_{254}$) aluminum-backed plates (0.2 mm thickness) and visualized by UV and/or dipping into vapors of HCl. Kieselgel 60 (Merck 230-400 mesh) silica gel was used for flash chromatography.

## 3.1.1 Experimental for Synthesis of Monomeric Units

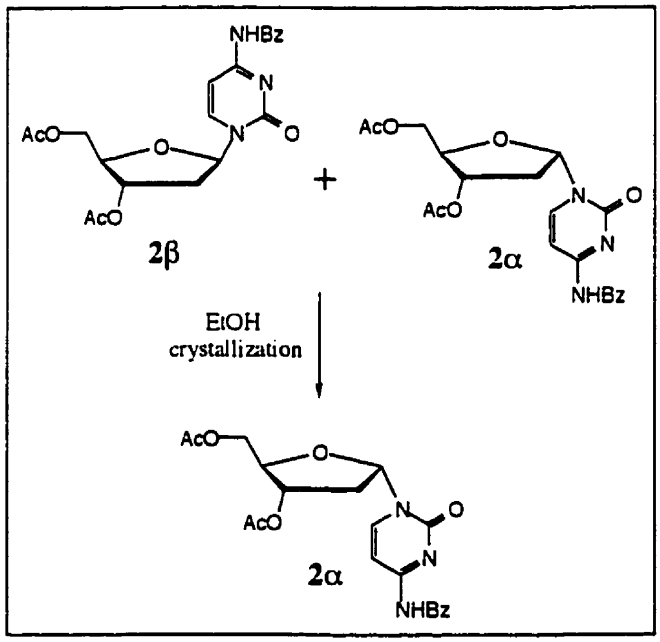
**5',3'-Di-*O*-Acetyl-$N^4$-benzoyl- 2'-β-deoxycytidine (2β).**




2'-β-deoxycytidine (2.84 g, 12.5 mmol), was dissolved in dry DMF. Benzoic anhydride (3.11g, 13.75 mmol), which was stored in a dessicator, was added to the reaction mixture and consequently stirred for 66 hr at room temperature. The solvent (DMF) was removed under reduced pressure, which resulted in a light brown oily residue. The residue was then co-evaporated with ether under reduced pressure, which resulted in a yellow solid ($R_f$ = 0.24; EtOH : $CH_2Cl_2$, 1:9 v/v). The crude product, $N^4$-benzoyl- 2'-β-deoxycytidine (6.7g, 12.5 mmmol) was dissolved in 130 mL of anhydrous pyridine. To this solution, acetic anhydride (8 mL, 109 mmol) was added and the reaction mixture was stirred at 65 °C for 25 hr. The solvent was removed under reduced pressure and the resulting residue was co-evaporated with toluene (3 x 100 mL) which resulted in a light brown residue. The residue was dissolved in hot EtOH, until a clear solution resulted. Activated charcoal was added to the the solution, which was then removed by hot filtration. The filtrate was cooled down to room temperature and then placed in an ice bath. The solution was then filtered and the

resulting white precipitate (4.0g, 77% yield) was dried under vacuum. Tlc analysis (Rf = 0.19; $CHCl_3$ : ethyl acetate, 1:1 v/v), as well as $^1$H-NMR anaysis, showed that only one product was present. UV (EtOH, nm) max, 302, 250; min, 283, 225. $^1$H-NMR (499.84 MHz, $CDCl_3$): δ 8.67 (s, 1H, -N**H**), 8.05 (d, 1H, H6), 7.88 (dd, 2H, $H_{meta}$ of benzoyl), 7.61 - 7.64 (m, 1H, $H_{para}$ of benzoyl), 7.51 - 7.54 (m, 2H, $H_{ortho}$ of benzoyl: d, 1H, H5), 6.28 (dd, 1H, H1'), 5.23 (dt, 1H, H3'), 4.38 - 4.58 (m, 3H, H4' and H5', H5''), 2.8 - 2.87 (m, 2H, H2', H2'), 2.10, 2.11 (2s, 6H, $CH_3$ of acetyl), $J_{5\text{-}6}$ = 7.83 Hz, $J_{1'\text{-}2'a}$ = 7.8 Hz, $J_{1'\text{-}2'b}$ = 5.4 Hz, $J_{Hmeta\ -\ Hortho(benzoyl)}$ = 8.3 Hz, $^4J_{Ha\text{-}Hc(benzoyl)}$ = -1.95 Hz.

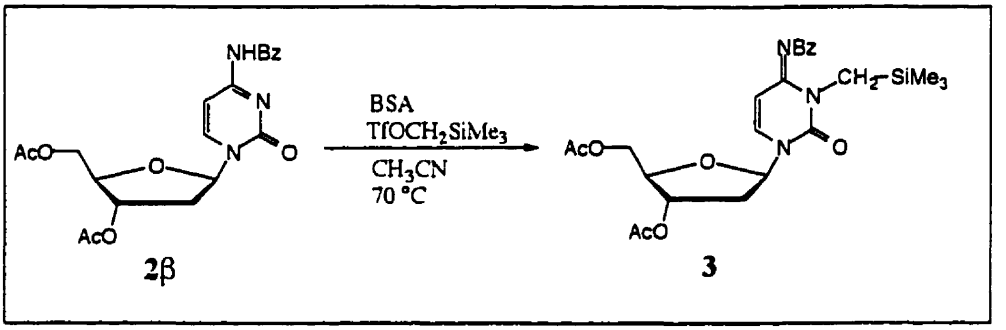
**5',3'-Di-*O*-Acetyl -$N^4$-benzoyl -2'-α-deoxycytidine (2α).**




5',3'-Di-*O*-Acetyl-$N^4$-benzoyl- 2'-β-deoxycytidine (2.0 g, 4.79 mmol) was dissolved in 40 mL of anhydrous pyridine. BSA (1.2 mL, 4.89 mmol) was added and the reaction mixture was refluxed for 10 min at 70°C. Subsequently, TMS-triflate (939 μL, 5.75 mmol) was added and the solution was refluxed for and additional 2 hrs at 70°C. The solution was cooled to room temperature and the solvent was removed under reduced pressure. The resulting crude brown residue, a mixture of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-

2'-β-deoxycytidine and 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl- 2'-α-deoxycytidine, was obtained in an 83 % yield (1.65 g crude). In order to isolate the 2-α- anomer, the brown residue was recrystallized from EtOH. This resulted in white needle-like crystals, (656 mg, 40 % yield) with an $R_f$= 0.33 (solvent system: 5% MeOH / $CH_2Cl_2$). At this point a $^1$H-NMR was taken of the resulting product and even though the desired product was obtained there was a contaminant present ( approximately 80%). Therefore it was decided to discontinue this approach.

**5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-$N^3$-trimethylsilylmethyl-2'-deoxycytidine (3)**

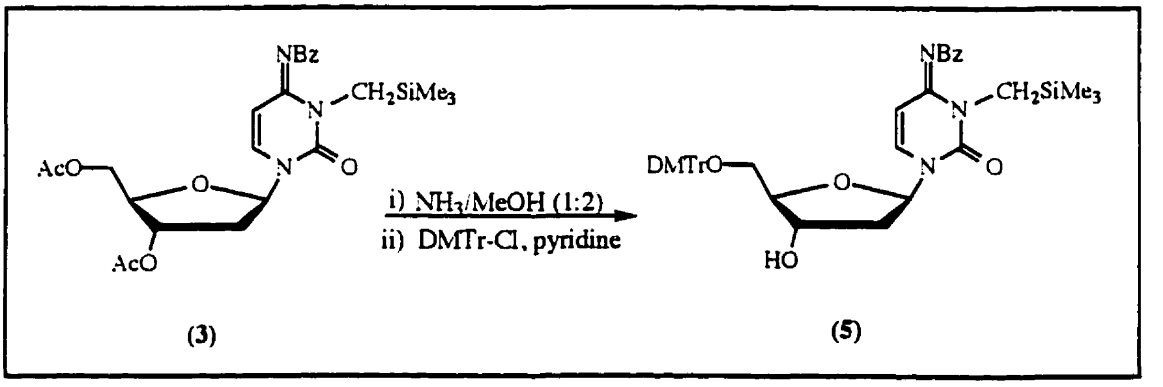



5',3'-Di-O-Acetyl-N4-benzoyl-2'-deoxycytidine (1.92 g, 4.6 mmol) prepared by known methods[65,2] was suspended in 30 ml of anhydrous acetonitrile at room temperature, to which BSA (*N,O*-bis-(trimethylsilyl)acetamide) (1.15 mL, 4.7 mmol) was added. The solution was subsequently heated and stirred at 70 °C for 10 minutes. Trimethylsilylmethyl triflate (1.0 mL, 5.52 mmol) was added and the solution was continuously stirred at 70 °C for 3 hours. The solvent was subsequently removed under reduced pressure and the residue was treated with cold saturated sodium bicarbonate (150 mL) and then filtered. The organic layer was washed with distilled $H_2O$, dried over anhydrous magnesium sulfate and evaporated to dryness. After purification by flash chromatography over silica gel (chloroform / ethyl acetate, 1:1 v/v $R_f$= 0.69), the yellow oily residue was crystallized

[65] Bhat V.; Ugarkar B.G.; Sayeed V.A.; Grimm K.; Kosora N.; Domenico P.A.; Stocker E.; *Nucleosides & Nucleotides*, **1989**, *8*(2), 179 - 183.

using ethanol yielding **3** (605 mg, 28% yield) as white needle-like crystals. LRMS (FAB-nitrobenzyl alcohol): m/e 502([MH+], 78.9%). UV (EtOH, nm) max, 315, 244; min, 266, 230. $^1$H-NMR (499.84 MHz, Acetone-$d_6$): δ 8.16 (dd, 2H, $H_{meta}$ of benzoyl), 7.66 (d, 1H, H6), 7.54 - 7.57 (m, 1H, $H_{para}$ of benzoyl), 7.46 - 7.49 (m, 2H, $H_{ortho}$ of benzoyl), 6.68 (d, 1H, H5), 6.33 (dd, 1H, H1'), 5.31 (dt, 1H, H3'), 4.28 - 4.38 (m, 3H,H4' and H5', H5''), 3.87 (d, 2H, C**H**2-Si(-$CH_3$)$_3$), 2.45 - 2.55 (m, 2H, H2', H2''), 2.07, 2.09 (2s, 6H, $CH_3$ of acetyl), 0.10 (s, 9H, $CH_2$-Si(-C**H**$_3$)$_3$); $J_{5-6}$ = 8.3 Hz, $J_{1'-2'a}$ = 8.3 Hz, $J_{3'-4'}$ = 4.2 Hz, $J_{4'-5'a}$ = 2.9 Hz, $J_{4'-5'b}$ = 2.7 Hz, $J_{5'a-5'b}$ = -10.8 Hz, $J_{H_m-H_o(benzoyl)}$ = 8.3 Hz, $^4J_{H_m-H_p\,(benzoyl)}$ = -1.5 Hz.

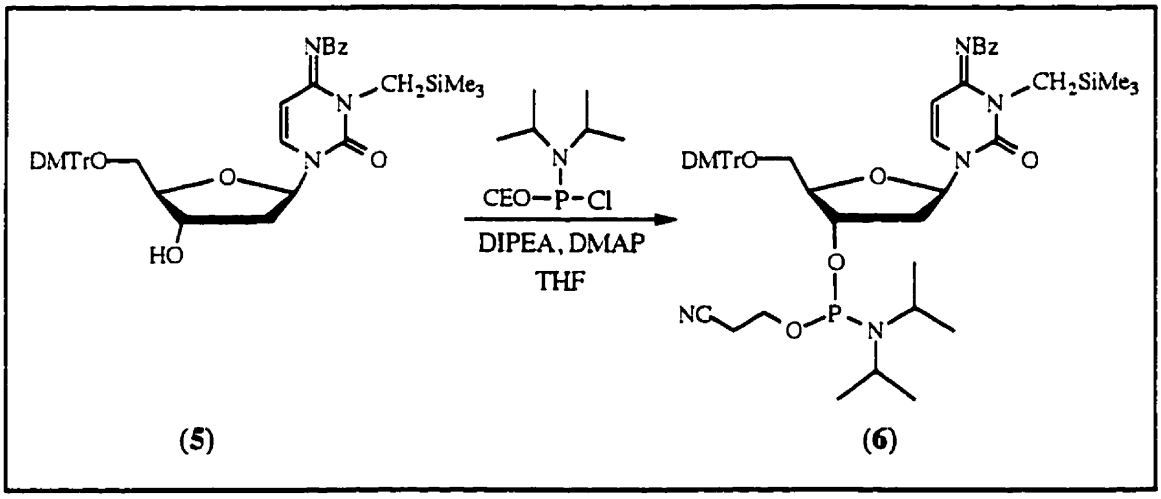
**5'-*O*-Dimethoxytrityl-$N^4$-benzoyl-$N^3$-trimethylsilylmethyl-2'-deoxycytidine (5)**




The deprotection of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-$N^3$-trimethylsilyl (**3**) methyl-2'-deoxycytidine was carried out in a solution of methanol and ammonia (2:1 / v:v) and the reaction mixture was left at room temperature for 50 min. The solvent was removed under reduced pressure and then co-evaporated with 95% ethanol (3 x 10 mL), and ether (3 x 10 mL), yielding the de-acetylated nucleoside ( ethanol / cholorform, 1:10 v/v, $R_f$= 0.40). The crude $N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'deoxycytidine (886 mg, 1.99 mmol), obtained from the deprotection procedure, was dried by co–evaporation with anhydrous pyridine (3×15mL), to ensure that no traces of water were present, and then it was dissolved in 15 mL of anhydrous pyridine. To the reaction mixture, which was stirred and

cooled to 0 °C, DMTrCl (674 mg, 1.99 mmol) dissolved in 5 mL of anhydrous pyridine was added dropwise over a period of 1 hour. The reaction was quenched by adding 30 mL of MeOH. The solvent was subsequently removed under reduced pressure. The resulting residue was dissolved in 50 mL of methylene chloride and washed with 5% $NaHCO_3$ (2 x 30 mL). The organic phase was evaporated and co-evaporated with toluene (3 x 15 mL). The resulting brown viscous residue was purified by flash chromatography (1%-10% EtOH/$CH_2Cl_2$ $R_f$= 0.51) to remove the side product tritanol ($R_f$=0.74), resulting in **5** (500 mg, 69% yield) as a bright yellow foam. LRMS (FAB-nitrobenzyl alcohol): m/e 702([$MH^+$], 5.1%), 303 ([$DMT^+$], 100%). UV (EtOH, nm) max, 316, 229, 283; min, 290, 266, 230.

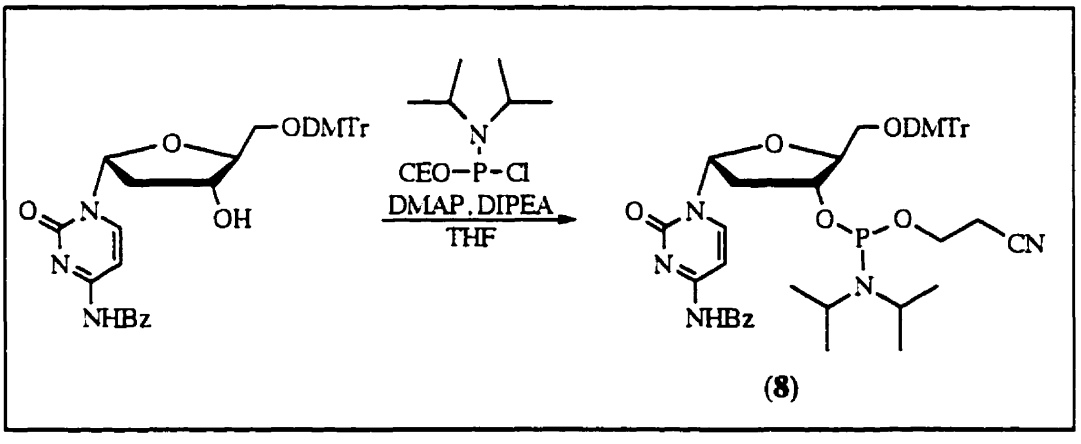
**5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidite-$N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'-deoxycytidine(6)**




To a dry hypovial containing purified 5'*O*- dimethoxytrityl-$N^4$-benzoyl-2'-deoxycytidine (**5**) (500 mg, 0.7 mmol) and DMAP (10.4 mg, 0.085 mmol) in dry THF was added anhydrous diisopropylethlylamine (56 µL, 2.9 mmol). To the stirred solution, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (212 µL, 0.95mmol) was added dropwise over a period of 1 min. After approximately 20 min a white precipitate appeared (formation of the ammonium salt) which indicated the desired reaction had occurred. After

2 hours, the complete consumption of the starting material (**5**), was determined by tlc. The reaction mixture was poured into ethyl acetate (150 mL), washed with brine (3 x 60 mL), dried ($Na_2SO_4$), filtered and the solvents removed *in vacuo*. Purification by flash chromatography over silica gel (methylene chloride / hexanes / triethylamine, 55:44:6, v/v/v) afforded **6** (442 mg, 69% yield) as a light yellow foam after the eluent was co-evaporated with 95% ethanol (2 x 30 mL) and ether (3 x 30 mL) LRMS (FAB-nitrobenzyl alcohol): m/e 920([$MH^+$], 8.5%), 303 ([$DMT^+$], 100%). The $^{31}$P-NMR spectrum of this compound indicated the presence of two isomers with signals at 149.776 and 149.179 ppm as well as a signal at 146.31 ppm, corresponding to excess phosphoramidite reagent. $^1$H-NMR (499.84 MHz, $CDCl_3$), for both diastereomers : δ 8.13 - 8.15 (dd, 2H, $H_{meta}$ of benzoyl), 7.66, 7.58 (d, 1H, H6), aryl region: 7.20 - 7.53 (m, 1H, $H_{para}$ of benzoyl; m, 2H, $H_{ortho}$ of benzoyl), 7.81 - 7.85 (m, 13 H's of DMT), 6.33 - 6.36, 6.28 - 6.32 (m, 1H, H1'), 4.56 - 4.65 (m, 1H. H3'), 4.11 - 4.17 (m, 1H,H4'), 3.79 - 3.88 (m, 2H, -OC**H**$_2$CH$_2$-) 3.78, 3.77 (d, 2H, C**H**2-Si(-$CH_3$)$_3$), 3.33 - 3.50 (m, 2H, H5', H5''), 2.17 - 2.25 (m, 2H, H2', H2''), 0.11 (s, 9H, $CH_2$-Si(-C**H**$_3$)$_3$); $J_{5-6}$ = 8.0 Hz.

**5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidine-*N*$^4$-benzoyl-L-α -2'-deoxycytidine (8)**




**(8)**

Similar to the synthesis of phosphoramidite (**6**), 5'-*O*-Dimethoxytrityl- -$N^4$-benzoyl-L-α -2'-deoxycytidine[66] (500 mg, 0.79 mmol) and DMAP (10 mg, 0.079 mmol) were dissolved in dry THF in a dry hypovial. To the stirred solution at room temperature were added DIPEA (56.4 μL, 3.2 mmol) and CEOP-Cl (212 μL, 0.95 mmol). After 5 h the reaction mixture was quenched, since by tlc it observed that all the starting material (**7**) had reacted. The work-up and purification of (**8**) is the same as described for phosphoramidite (**6**), and yielded 573 mg of product (87% yield) as a white foam. Tlc of the resulting product indicated the presence of two isomers (ethyl acetate:hexanes 2:1 v/v with a drop of triethylamine $R_f$= 0.25, $R_f$= 0.35). The $^{31}$P-NMR spectrum of this compound also indicated the presence of two isomers with peaks at 149.898 and 149.501 ppm. $^1$H-NMR (499.84 MHz, $CDCl_3$), for both diastereomers : δ 8.59 - 8.62 (s, 1H, -N**H**), 8.05, 8.10 (d, 1H, H6), aryl region: 6.83 - 7.63 (m, 5H, of benzoyl; 12H, Hb of DMT), 7.81 - 7.85 (m, 2H, H's of DMT), 6.35, 6.28 (d, 1H, H1'), 4.59 , 4.70 (dt, 1H, H3'), 4.48 - 4.51 (m, 1H,H4'), 4.11 - 4.24 (m, 2H, -OC**H2**$CH_2$) 3.80 (s, 6H, -OC**H**$_3$ of DMT) 3.15 - 3.35 (m, 2H, H5', H5'') 2.40 - 2.55 (m, 2H, H2', H2''), 2.72 - 2.77 (m, 2H, -C**H**$_2$-CN).

## 3.2. Experimental for Synthesis of Oligomers

### 3.2.1. Reagents

For the solid phase synthesis of the oligomers, reagent grade aqueous ammonia, acetic anhydride ($Ac_2O$), trichlororoacetic acid (TCA), 1,2-dicholorethane (DCE), and iodine, where used as received. Anhydrous acetonitrile was predried by distillation from $P_2O_5$ and redistilled from calcium hydride under dry $N_2$. Tetrahydrofuran was predried over KOH pellets, filtered, and distilled immediately prior to use from sodium benzophenone ketyl. Tetrazole was stored in a dessicator over $P_2O_5$. Pyridine, lutidine, and N-methylimidazole, were distilled from calcium hydride (BDH) and stored over

[66] Synthesized and given to us by Dr. Anna Garbesi's laboratory, Istituto ICoCEA-CNR, Bologna, Italy.

molecular sieves. Water was double distilled in glass, treated in diethyl pyrocarbonate, and autoclaved (30 min, 121 °C, 5 atm). Reagents for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad. Sephadex G-25 (Pharmacia) and Acrodisc's were used according to manufacturer's specifications.

### 3.2.2 Synthesis of oligomers.

The oligomer sequences used in the physicochemical studies are outlined in Table 2 of Section 2.2.1. All the modified nucleotides, L-β-C, L-α-C and D-α are incorporated into the sequences in a 3'3'→ 5'5' phosphate linkage.

Solid-phase synthesis of standard deoxyribonucleotide sequences **A**, **D** and **J**, the modified deoxyribonucleoside sequences **F** and **K**, and ribonucleotide **E** (complement RNA), were performed on an Applied Biosystems 381A DNA Synthesizer. The aforementioned oligomers were synthesized in the "trityl on" mode. Both **A** and **F** deoxynucleotides were prepared using the 1.0 μM DNA synthesis cycle; whereas complementary RNA was synthesized utilizing the standard 1 μM RNA cycle. The standard phosphoramidite coupling time of 2 minutes was used. The synthesis of the 7 nt oligomers **F** and **K** used the 0.2 μM DNA cycle, where the phosphoramidite coupling time was 5 min for both oligomers.

The reagents used for the solid-phase synthesis of the oligonucleotides were prepared as described below. Detritylation was performed with 3% tricholoracetic acid in DCE. Activation of the phosphoramidite reagents was achieved with 0.5 M tetrazole in anhydrous acetonitrile. Reagents for acetylation (capping) were prepared as follows: Cap A, 10% acetic anhydride and 10% 2,4-collidine in THF; and Cap B, 16% N-methylimidazole in THF (w/v). Oxidation of the trivalent phosphorus was achieved using iodine, 0.1 M, in THF / pyridine / water (75:20:2, v/v/v). The phosphoramidite reagents for the synthesis of the deoxynucleotides **A**, **D**, **F**, **H** and **K** were:

5'-*O*-DMT-thymidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidite),

$N^6$-Bz-5'-*O*-DMT-adenosine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

$N^4$-Bz-5'-*O*-DMT-cytidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

$N^2$-Ibu-5'-*O*-DMT-guanosine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

5'-*O*-DMT-2'-O-TBDMS-uridine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

$N^6$-Bz-5'-*O*-DMT-2'-O-TBDMS-adenosine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

$N^4$-Bz-5'-*O*-DMT-2'-O-TBDMS-cytidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

$N^2$-Ibu-5'-*O*-DMT-2'-O-TBDMS-guanosine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl-phosphoramidite),

were the phosphoramidite reagents used for the synthesis of the ribonucleotide **E**.

All phosphoramidite reagents were either purchased from Dalton (Toronto) or synthesized in our laboratory according to the methods of Damha and Ogilvie[67]. The modified deoxyoligonucleotides **G** and **K**, containing the L-α-inserts, were synthesized using the phosphoramidite reagent L-α-$N^4$-Bz-5'-*O*-DMT-cytidine-3'-*O*-diisopropyl-phosphoramidite as described in Section 4.2. These phosphoramidite reagents were stored at -20 °C and dessicated under vacuum and over $P_2O_5$ for 24 hr. prior to use. All the phosphoramidite reagents were dissolved in dry, freshly distilled acetonitrile to the concentration of 0.15 M. All solutions were passed through a 0.45-μm-pore Teflon filter prior to placement on the automated synthesizer.

Solid supports used for the synthesis of **A** (bound 3'-terminal thymidine phosphoramidite), **E** (bound 3'-terminal uridine phosphoramidite) and **H** (bound 3'-terminal guanosine phosphoramidite) were prepared according to literature procedure.[68]

---

[67] Damha, M.J.; Ogilvie, K.K.; Ch. 5 , p.81, in Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs, 1993 Humana Press Inc., Totowa, NJ.

[68] Damha, M.J.; Giannaris, P.A.; Zabarylo S.V. *Nucleic Acids Research*, **1990**, *Vol. 28, No. 13*, P.3813-3821.

Whereas for the synthesis of **F**, bound 5'-terminal L-β cytidine phosphoramidite [69]was used as the solid support, and 5'-terminal guanosine phosphoramidite, obtained from Bio/Can Scientific, was used for the synthesis of **K** in order to achieve the desired 3'-3'' 5'5 linkage for the L-α insert.

In order to evaluate the phosphoramidite coupling step during the synthesis of the oligomers, UV spectroscopic quantitation of the trityl cation released during the TCA treatment step (504 nm for DMT) was used. The coupling for the oligonucleotides synthesized were as follows: **A** : 99% , **D**: 99%, **E**: 96%, **F** : 97%, **I** : 99% and **K** : 92%

### 3.2.3. Cleavage and deprotection of oligomers.

Following synthesis, oligomers were cleaved from the support by placing the solid-support cassette into an Eppendorf tube, adding 900 μL of concentrated aqueous ammonia (29% v/v) and 300 μL of 100% EtOH then incubating at 55°C for 16 hr. The cleavage from the solid support occurs rapidly (15-30 min). The prolonged 16 hr incubation period is required for the deprotection of the benzoyl and isobutyryl protecting groups. The Eppendorf tube was then placed in a centrifuge at 2000 rpm for 2 min, in order to decant the solvent from the solid support at the bottom. Following these cleavage and deprotection steps, the oligomers were purified as described below.

In the case of the 7-mers **H** and **K**, prior to the deprotection step one half of the solid support was once again placed on the DNA synthesizer to remove the trityl group. The 7-mers were subjected to cleavage and deprotection as described above.

[69] Synthesized in our laboratory by P.A. Giannaris according to literature procedure.

### 3.2.4. Purification of oligomers.

### 3.2.4a Purification of oligomers: A, B and D

Purification and detritylation of the DNA oligomers **A**, **B** and **D** performed utilizing reverse-phase oligonucleotide purification cartridges (OPC) from Applied Biosystems. The protocol for OPC purification, of both oligomers, is as follows. The cartridge was first flushed with 5 mL of HPLC grade acetonitrile and then with 2.0 M triethylamine acetate (100 mmol TEA; 100 mmol Acetic acid in 50 mL autoclaved water). After centrifuging the ammonia / oligomer solution, the supernatant was carefully removed ( so as not to transfer any solid support which may in turn block the cartridge) and transferred to another Eppendorf tube. This solution (*ca.* 0.6 mL) was diluted to 1.0 mL with autoclaved water and applied to the OPC cartridge for purification. The cartridge consists of a reverse-phase column, which implies that the highly polar molecules exit the column first, while the less polar ones are retained. Hence, by washing the cartridge that has been previously loaded with the oligomer with 10% ammonium hydroxide solution (3 x 5 mL) and autoclaved water (2 x 5 mL), the ammonia salts, obtained from the cleavage and deprotection steps are eluted first leaving the tritylated oligomers (mainly 18-mer) on the column. Detritylation was then performed by using a 2% trifluoroacetic acid (TFA) solution and then the 18-mer was eluted from the OPC cartridge with a 20% acetonitrile solution, leaving the other organic molecules behind on the cartridge. After the purified oligomers **A**, **B** and **D** were lyophilized, the pellets were dissolved in autoclaved water and were quantitated in OD units (**Table 3.1**)

**Table 3.1**: Quantitation of purified oligomers A, **D**, **E** and **F**, reported in OD units at 260 nm for 1/2 of a 1 μM scale synthesis.

| Oligomer | OD Units |
|---|---|
| **A** | 6.2 |
| **D** | 4.8 |
| **E** | 20.4 |
| **F** | 10.6 |

### 3.2.4.b Purification of oligoribonucleotide E

Following the cleavage and deprotection of the oligoribonucleotide **E**, as described above, the sample was redissolved in ethanol and lyophilized once again. The resulting pellet was treated with 50 μL TEA:HF (v:v) and left to spin overnight. Subsequently the sample was lyophilized for 48 hr. and the pellet that resulted was redissolved in double distilled $H_2O$.

Purification of **E** was accomplished with the use of preparatory scale PAGE (**Figure 3.1**), followed by excision of the appropriate band under UV-shadow, extraction of the oligomer from the gel matrix with water followed by filtration using Acrodisc, and desalting using Sephadex G-25F chromatography. The purified sample was then quantitated (**Table 3.1**).

### 3.2.4.c Purification of 7-mers: H, I, J and K

After the synthesis of the 7-mers **I** and **J**, each oligomer was divided in two. One half of each 7-mer was subjected to OPC purification (all details are specified in Sec. 3.2.4a). Two bands are present for both **I** and **J**, (lanes 4 and 6 respectively in **Figure 3.2**), this is seen with the use of a 24% denaturing PAGE. The slower running bands for both 7-mers are suspected to be the tritylated oligomers, implying that the detritylation step was not successful in the OPC purification process. The faster moving bands correspond to the pure oligomers. This was confirmed by comparison with the same oligomers that were put through another detritylation cycle to remove the trityl group, cleaved and deprotected using standard methods (as described in Section 3.2.3) and then subjected to OPC purification. In the latter case oligomers **I** and **J** show only one distinct band in the PAGE gel ( **Fig.3.2**, lanes 5 and 7 respectively).

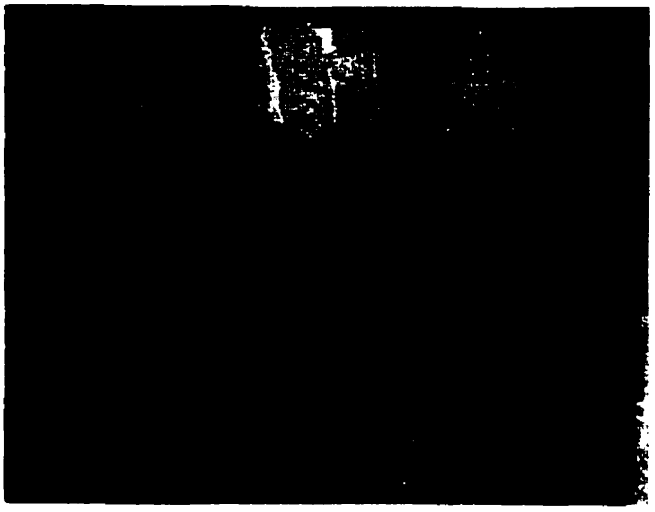


**Figure 3.1:** Preparatory gel electrophoresis of oligoribonucleotide **E**.

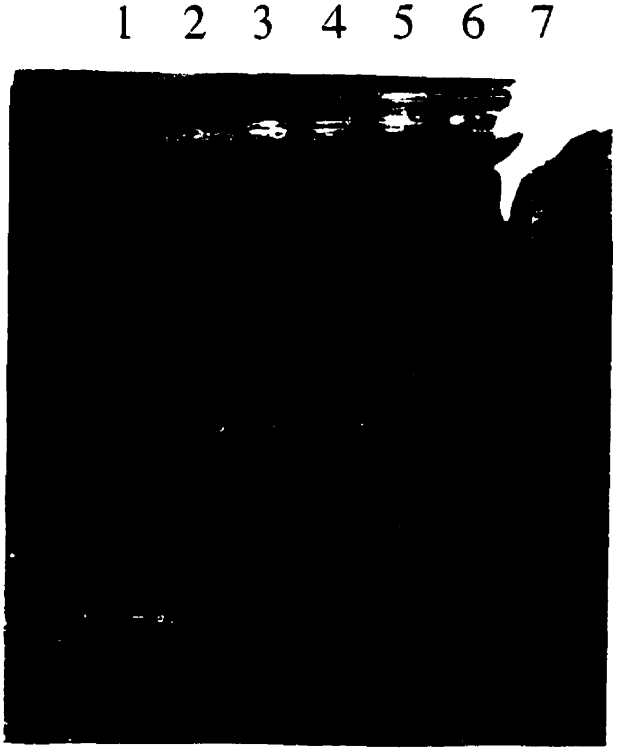



**Figure 3.2:** Polyacrylamide gel electrophoresis of **I** and **J**. Lanes: 1) and 2) Bromophenol Blue dye; 4) **I** (OPC purified); 5) **I** (detritylated and OPC purified); 6) **J** (OPC purified); 7) **J** (detritylated and OPC purified).

The same procedure was applied to the purification of the 7-mers **H** and **K**. Both oligomers were treated with 3% trichloroacetic acid in DCE. The purity of these oligomers was also determined using 24% denaturing PAGE (**Fig 2.4**).All the 7-mers were quantified in OD units (**Table 3.2**).

**Table 3.2**: Quantitation of purified oligomers **H**, **I**, **J** and **K**, reported in OD units at 260nm for 1/2 of a 0.2 μM scale synthesis.

| Oligomer | OD units |
|---|---|
| **H** | 2.6 |
| **I** | 3.5 |
| **J** | 1.5 |
| **K** | 2.5 |

### 3.2.5. Thermal denaturation measurements.

Thermal denaturation studies were carried out using a Varian-Cary 1 UV/VIS spectrophotometer equipped with the Peltier thermal unit accessory. The absorbance vs. temperature curves were recorded at intervals of 0.5°C starting from 5°C up to a maximum of 85°C (depending on the oligomer sequence) at a rate of 1°C/min and a wavelength of 260 nm.

The extinction coefficients were calculated using the nearest-neighbor approximation[70] For sequences **A**, **B**, **C**, **D**, **E**, **G**, **H** and **I**, the extinction coefficients were found to be 177 420, 170 980, 172 440, 165 620, 180 090, 178 700, 66 700 and 62 780 L $mol^{-1}cm^{-1}$. The same extinction coefficients were assumed for the modified 7-mers **J** and **K**, as that of the natural analogue **H**. Similarly the same extinction coefficient was assumed for modified oligomer **F** as that of its natural analogue **B**. Samples for the first thermal denaturation studies were prepared by mixing 0.20 OD units of each of strands **A** and **E** with, 0.193 OD units of either **B** or **F**, and with 0.193 OD units of **D** ( for oligomer

[70] Puglisi,, J. D., Tinoco, I.Jr., *Methods Enzymol.***1989**, *180*, 304 - 324.

**A**); and with, 0.190 OD units of either **B** or **F**, and with 0.184 OD units of **D** (for oligomer **E**). In a separate spectroscopic study, 0.2 OD units deoxyribo-oligonucleotide **A** or ribo-oligonucloeotide **E**, with0.199, or 0.198 OD units, respectively, of **G**, also ). For the T*m* study of the 7-mers, 0.188 OD units of oligomer **I** was mixed with 0.2 OD units of either 7-mer **H**, **J**, or **K**. The solutions were lyophilized to dryness and dissolved in 1.0 mL of either physiological (140 mM KCl, 5 mM $Na_2HPO_4$, 1 mM $MgCl_2$ adjusted to a pH of 7.2 using concentrated HCl) or 1.0 M NaCl (10 mM $Na_2HPO_4$, 1.0 mM NaCl, pH = 7.0) buffers. The duplex solutions were then heated to 70°C for 15 min and slowly cooled to room temperature

The mixtures of the oligomers in the physiological or the 1.0 M NaCl buffers were added to a 1 cm path length 1.5 mL quartz cuvet and placed in the spectrophotometer which was set on the dual beam optical mode to reduce optical drift. Unless otherwise specified, the cells were equilibrated at 40°C for 15 min and slowly cooled down at 5° intervals over a period of a half hour or longer to the start temperature (5 or 10°C).

$T_m$ values are reported as the first derivatives (calculated by the Cary 13E software Base system) obtained from the slopes of the curves. These values may also be obtained from the midpoint between the upper and lower linear sloping base lines of the absorbance versus temperature curves obtained directly from the Cary 13E thermal application program. Hypochromicities (%H) were calculated utilizing the following equation: $\%H = (A_f - A_i)/A_f$, where $A_f$ = the absorbance at the highest temperature for the curve and $A_i$ = the absorbance at the initial (or lowest) temperature. For visualization purposes the thermal denaturation data was normalized by presenting the graphs as fraction absorbance change versus temperature. Absorbance readings were extrapolated from the absorbance versus temperature curves at 5°C intervals.

# APPENDIX I

## X-Ray Structure Determination of Nucleoside 3

An X-Ray diffraction study was carried out on nucleoside **3** and the data obtained is shown in the tables XR-1 to XR-5.. The diffraction measurement were performed by Dr. Anne Marie Lebuis, using a Rigaku diffractometer.

### Table XR-1

Crystal data and structure determination

| | |
|---|---|
| Compound name | Nucleoside **3** |
| Molecular Formula | $C_{24}\ H_{31}\ O_7\ N_3\ Si$ |
| Formula Weight | 498.44 |
| X-ray specimen size (mm) | 0.35 x 0.37 x 0.04 |
| Crystal system | Monoclinic |
| Space group | P 21 |
| Lattice constants | |
| *a*(Å) | 10.019(3) |
| b(Å) | 7.7707(22) |
| c(Å) | 17.1234(23) |
| β(°) | 97.076(16) |
| V ($Å^3$) | 1314.76(16) |
| No. of formula in cell | 2 |
| F(000) | 534.15 |
| Calculated density (mg $m^{-3}$) | 1.259 |
| mu ($mm^{-1}$) | 1.15 |
| λ(Å) | 1.54056 |
| 2θ max (°) | 140.0 |

| | |
|---|---|
| (h), (k), (l) ranges | (-12 - 12), (0 - 9), (0 - 20) |
| No. of reflections measured | 9574 |
| No. of unique reflections | 4957 |
| No. of reflections with $I_{net} > 2.5\ \sigma(I_{net})$ | 3227 |
| For significant reflections | RF =0 .063, $R_w$ = 0.056, $G_oF$ = 1.82 |
| For all reflections | RF = 0.133, $R_w$ = 0.080 |
| Maximum shift/$\sigma$ ratio | 0.502 |
| Deepest hole in D-map (e/ $Å^3$) | -0.270 |
| Highest peak in D-map (e/ $Å^3$) | 0.360 |
| Secondary extinction coefficient | 0.325893 |
| $\sigma$ | 0.0844911 |
| Method of structure refinement | Refined using NRCVAX system programs |

Cell dimensions were obtained from 15 reflections with the angle 2θ in the range of 60.00° - 70.00°. The intensity data were collected using the θ/2θ scan mode. The structure was solved by direct methods, methyl groups on Si were found to be disordered over two sites at 60% and 40% occupancy. All non-H atoms were refined anisotropically, hydrogens were introduced in calculated positions, H atoms on disordered carbons were introduced in difference map positions and were not optimized. Bivoet analysis confirmed the enantiomer ( the probability of error is 0.43 x 10exp(-13)). The data was measured over the whole sphere and equivalent reflections were averaged ( Rint = 0.044).

$$RF = \Sigma\ (F_o - F_c) / \Sigma\ (F_o)$$

$$R_w = (\Sigma\ [w(F_o - F_c)^2 / \Sigma\ (wFo^2)])^{1/2}$$

$$G_oF = (\Sigma\ [w(F_o - F_c)^2 / \Sigma\ (\#\ \text{of reflections} - \#\ \text{of parameters})])^{1/2}$$

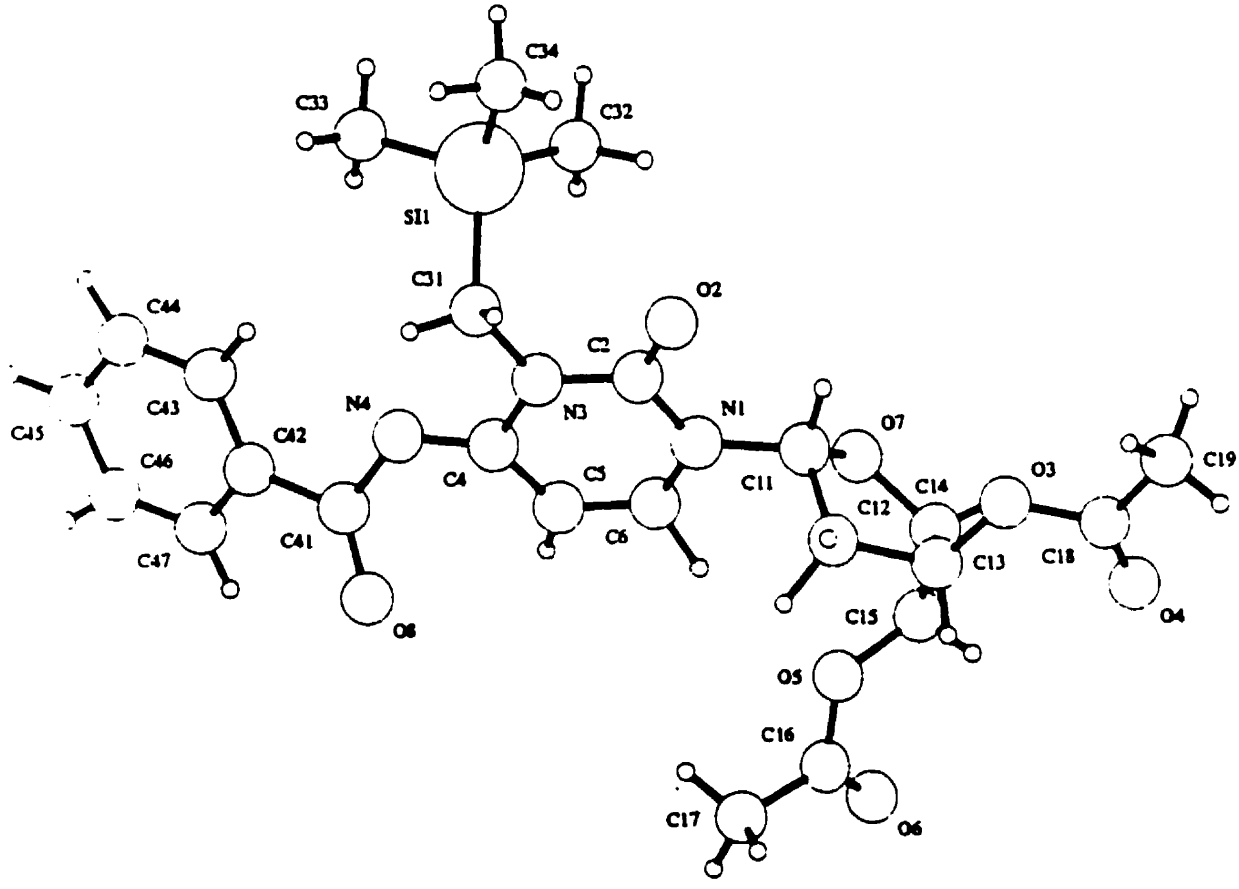



**Figure XR-1.** X-ray crystallographic structure of nucleoside **3.**

**TableXR-2**

Refined Atomic Cooridinates X, Y, Z and $B_{iso}$

| Atom | X | Y | Z | $B_{iso}$ |
|---|---|---|---|---|
| Si1 | 0.2327 | 0.6721 | 0.96384 | 5.92 |
| O2 | 0.3103 | 0.2858 | 0.8892 | 6.1 |
| O3 | 0.5741 | -0.1939 | 0.7807 | 5.1 |
| O4 | 0.7192 | -0.3103 | 0.7070 | 8.5 |
| O5 | 0.4751 | 0.1144 | 0.5805 | 5.8 |
| O6 | 0.5300 | 0.1126 | 0.4631 | 17.1 |
| O7 | 0.5769 | 0.2017 | 0.7429 | 5.7 |
| O8 | 0.0469 | 0.7216 | 0.6029 | 7.4 |
| N1 | 0.3713 | 0.3187 | 0.7664 | 4.5 |
| N3 | 0.2128 | 0.5017 | 0.8130 | 3.9 |
| N4 | 0.1058 | 0.7175 | 0.7390 | 4.4 |
| C2 | 0.2999 | 0.3623 | 0.8278 | 4.8 |
| C3 | 0.1913 | 0.5914 | 0.7413 | 3.8 |
| C5 | 0.2699 | 0.5379 | 0.6829 | 4.5 |
| C6 | 0.3557 | 0.4086 | 0.6970 | 4.9 |
| C11 | 0.4580 | 0.1648 | 0.7763 | 5.0 |
| C12 | 0.3977 | 0.0043 | 0.7371 | 4.4 |
| C13 | 0.5196 | -0.0896 | 0.7149 | 4.5 |
| C14 | 0.6151 | 0.0537 | 0.7008 | 4.9 |
| C15 | 0.6144 | 0.1012 | 0.6143 | 6.0 |
| C16 | 0.4456 | 0.1105 | 0.5045 | 7.4 |
| C17 | 0.2997 | 0.1207 | 0.4814 | 9.5 |
| C18 | 0.6740 | -0.3031 | 0.7677 | 5.8 |
| C19 | 0.7127 | -0.4146 | 0.8388 | 8.3 |
| C31 | 0.1371 | 0.5492 | 0.8789 | 4.9 |
| C32 | 0.4109 | 0.6845 | 0.9514 | 8.6 |
| C33 | 0.1762 | 0.9027 | 0.9577 | 13.0 |
| C34 | 0.2100 | 0.5766 | 1.0603 | 10.5 |
| C35 | 0.390 | 0.757 | 0.944 | 12.6 |
| C36 | 0.103 | 0.818 | 0.989 | 12.3 |
| C37 | 0.257 | 0.501 | 1.045 | 9.5 |
| C41 | 0.0591 | 0.7949 | 0.6671 | 4.9 |
| C42 | 0.0158 | 0.9789 | 0.6721 | 4.2 |
| C43 | 0.0019 | 1.0534 | 0.7438 | 4.6 |
| C44 | -0.0366 | 1.2235 | 0.7472 | 6.2 |
| C45 | -0.0615 | 1.3195 | 0.6788 | 6.9 |
| C46 | -0.0438 | 1.2431 | 0.6072 | 6.9 |
| C47 | -0.0068 | 1.0746 | 0.6044 | 6.0 |

**Table XR-3**

Calculated Hydrogen Atom Parameters

| Atom | X | Y | Z | $B_{iso}$ |
|---|---|---|---|---|
| H5 | 0.280 | 0.588 | 0.633 | 5.1 |
| H6 | 0.384 | 0.339 | 0.648 | 5.5 |
| H11 | 0.478 | 0.142 | 0.831 | 5.9 |
| H12 A | 0.332 | -0.079 | 0.766 | 5.2 |
| H12 B | 0.319 | 0.024 | 0.690 | 5.2 |
| H13 | 0.490 | -0.159 | 0.669 | 5.2 |
| H14 | 0.703 | 0.015 | 0.721 | 5.7 |
| H15 A | 0.631 | -0.012 | 0.600 | 6.7 |
| H15 B | 0.681 | 0.184 | 0.606 | 6.7 |
| H17 A | 0.255 | 0.014 | 0.480 | 9.5 |
| H17 B | 0.280 | 0.166 | 0.430 | 9.5 |
| H17 C | 0.259 | 0.201 | 0.513 | 9.5 |
| H19 A | 0.639 | -0.426 | 0.869 | 9.0 |
| H19 B | 0.787 | -0.369 | 0.872 | 9.0 |
| H19 C | 0.733 | -0.524 | 0.818 | 9.0 |
| H31 A | 0.066 | 0.621 | 0.858 | 5.7 |
| H31 B | 0.101 | 0.447 | 0.899 | 5.7 |
| H32 A | 0.452 | 0.556 | 0.956 | 6.9a |
| H32 B | 0.438 | 0.718 | 0.902 | 6.9a |
| H32 C | 0.467 | 0.739 | 0.993 | 6.9a |
| H33 A | 0.236 | 0.973 | 0.993 | 11.4a |
| H33 B | 0.196 | 0.952 | 0.905 | 11.4a |
| H33 C | 0.090 | 0.920 | 0.963 | 11.4a |
| H34 A | 0.116 | 0.604 | 1.066 | 9.7a |
| H34 B | 0.233 | 0.475 | 1.063 | 9.7a |
| H34 C | 0.262 | 0.657 | 1.098 | 9.7a |
| H35 A | 0.449 | 0.758 | 0.986 | 10.8b |
| H35 B | 0.411 | 0.717 | 0.898 | 10.8b |
| H35 C | 0.366 | 0.889 | 0.932 | 10.8b |
| H36 A | 0.130 | 0.862 | 1.042 | 9.9b |
| H36 B | 0.102 | 0.920 | 0.955 | 9.9b |
| H36 C | 0.021 | 0.769 | 0.985 | 9.9b |
| H37 A | 0.173 | 0.457 | 1.059 | 6.1b |
| H37 B | 0.319 | 0.420 | 1.041 | 6.1b |
| H37 C | 0.292 | 0.566 | 1.099 | 6.1b |
| H43 | 0.023 | 0.989 | 0.791 | 5.3 |
| H44 | -0.059 | 1.283 | 0.793 | 7.0 |
| H45 | -0.095 | 1.434 | 0.684 | 7.5 |
| H46 | -0.071 | 1.331 | 0.571 | 7.9 |
| H47 | 0.011 | 1.030 | 0.555 | 7.0 |

a: occupancy of 0.6
b: occupancy of 0.4

**Table XR-4**

Bond Distances in Angstroms

| Bond | Bond Distance | | Bond | Bond Distance |
|---|---|---|---|---|
| Si(1) - C(32) | 1.83(6) | | N(3) - C(31) | 1.482(12) |
| Si(1) - C(33) | 1.88(4) | | N(4) - C(4) | 1.298(14) |
| Si(1) - C(34) | 1.85(5) | | N(4) - C(41) | 1.398(15) |
| Si(1) - C(35) | 1.78(9) | | C(4) - C(5) | 1.409(14) |
| Si(1) - C(36) | 1.81(6) | | C(5) - C(6) | 1.325(17) |
| Si(1) - C(37) | 1.92(8) | | C(11) - C(12) | 1.507(17) |
| Si(1) - C(31) | 1.898(11) | | C(12) - C(13) | 1.511(14) |
| O(2) - C(2) | 1.201(14) | | C(13) - C(14) | 1.507(16) |
| O(3) - C(13) | 1.439(13) | | C(14) - C(15) | 1.526(17) |
| O(3) - C(18) | 1.351(15) | | C(16) - C(17) | 1.468(19) |
| O(4) - C(18) | 1.185(16) | | C(18) - C(19) | 1.505(19) |
| O(8) - C(41) | 1.231(14) | | C(32) - C(35) | 0.64(12) |
| O(5) - C(15) | 1.448(13) | | C(33) - C(36) | 1.16(8) |
| O(5) - C(16) | 1.298(15) | | C(34) - C(37) | 0.81(7) |
| O(6) - C(16) | 1.169(17) | | C(41) - C(42) | 1.499(18) |
| O(7) - C(11) | 1.413(13) | | C(42) - C(43) | 1.380(16) |
| O(7) - C(14) | 1.434(15) | | C(42) - C(47) | 1.371(17) |
| N(1) - C(2) | 1.384(14) | | C(43) - C(44) | 1.380(18) |
| N(1) - C(6) | 1.370(15) | | C(44) - C(45) | 1.386(22) |
| N(1) - C(11) | 1.476(15) | | C(45) - C(46) | 1.393(25) |
| N(3) - C(2) | 1.394(15) | | C(46) - C(47) | 1.363(21) |
| N(3) - C(4) | 1.406(13) | | | |

**Table XR-5**

Bond Angles in Degrees

| Bond | Bond Angle | | Bond | Bond Angle |
|---|---|---|---|---|
| C(32) - Si(1) - C(33) | 104.6(23) | | C(4) - C(5) - C(6) | 120.1(10) |
| C(32) - Si(1) - C(34) | 110.3(23) | | N(1) - C(6) - C(5) | 123.2(9) |
| C(32) - Si(1) - C(35) | 20(4) | | O(7) - C(11) - N(1) | 107.6(10) |
| C(32) - Si(1) - C(36) | 138(3) | | O(7) - C(11) - C(12) | 107.4(9) |
| C(32) - Si(1) - C(37) | 94(3) | | N(1) - C(11) - C(12) | 114.9(8) |
| C(32) - Si(1) - C(31) | 109.7(19) | | C(11) - C(12) - C(13) | 102.7(8) |
| C(33) - Si(1) - C(34) | 111.3(20) | | O(3) - C(13) - C(12) | 108.4(8) |
| C(33) - Si(1) - C(35) | 84(4) | | O(3) - C(13) - C(14) | 110.7(8) |
| C(33) - Si(1) - C(36) | 36(3) | | C(12) - C(13) - C(14) | 103.4(9) |
| C(33) - Si(1) - C(37) | 135.3(22) | | O(7) - C(14) - C(13) | 107.4(8) |
| C(33) - Si(1) - C(31) | 108.5(14) | | O(7) - C(14) - C(15) | 108.9(10) |
| C(34) - Si(1) - C(35) | 121(3) | | C(13) - C(14) - C(15) | 114.0(9) |
| C(34) - Si(1) - C(36) | 82(3) | | O(5) - C(15) - C(14) | 107.2(8) |
| C(34) - Si(1) - C(37) | 24.8(20) | | O(5) - C(16) - O(6) | 121.0(13) |
| C(34) - Si(1) - C(31) | 112.1(15) | | O(5) - C(16) - C(17) | 111.4(11) |
| C(35) - Si(1) - C(36) | 119(4) | | O(6) - C(16) - C(17) | 127.3(13) |
| C(35) - Si(1) - C(37) | 110(4) | | O(3) - C(18) - O(4) | 123.0(12) |
| C(35) - Si(1) - C(31) | 114(3) | | O(3) - C(18) - C(19) | 110.6(11) |
| C(36) - Si(1) - C(37) | 107(3) | | O(4) - C(18) - C(19) | 126.4(12) |
| C(36) - Si(1) - C(31) | 100.7(20) | | Si(1) - C(32) - C(35) | 75(10) |
| C(37) - Si(1) - C(31) | 102.5(24) | | Si(1) - C(33) - C(36) | 68(4) |
| C(13) - O(3) - C(18) | 116.3(8) | | Si(1) - C(34) - C(37) | 82(6) |
| C(15) - O(5) - C(16) | 119.3(9) | | Si(1) - C(35) - C(32) | 84(10) |
| C(11) - O(7) - C(14) | 109.3(9) | | Si(1) - C(36) - C(33) | 74(4) |
| C(2) - N(1) - C(6) | 121.3(10) | | Si(1) - C(37) - C(34) | 72(7) |
| C(2) - N(1) - C(11) | 117.6(9) | | O(8) - C(41) - N(4) | 124.9(12) |
| C(6) - N(1) - C(11) | 121.1(8) | | O(8) - C(41) - C(42) | 119.6(11) |
| C(2) - N(3) - C(4) | 124.7(8) | | N(4) - C(41) - C(42) | 115.4(10) |
| C(2) - N(3) - C(31) | 115.0(9) | | C(41) - C(42) - C(43) | 120.8(11) |
| C(4) - N(3) - C(31) | 120.3(9) | | C(41) - C(42) - C(47) | 119.3(11) |
| C(4) - N(4) - C(41) | 120.0(9) | | C(43) - C(42) - C(47) | 120.0(12) |
| O(2) - C(2) - N(1) | 123.1(11) | | C(42) - C(43) - C(44) | 119.9(11) |
| O(2) - C(2) - N(3) | 121.9(10) | | C(43) - C(44) - C(45) | 120.1(12) |
| N(1) - C(2) - N(3) | 114.9(10) | | C(44) - C(45) - C(46) | 119.0(12) |
| N(3) - C(4) - N(4) | 115.5(9) | | C(45) - C(46) - C(47) | 120.3(13) |
| N(3) - C(4) - C(5) | 115.7(9) | | C(42) - C(47) - C(46) | 120.6(13) |
| N(4) - C(4) - C(5) | 128.7(10) | | Si(1) - C(31) - N(3) | 116.8(7) |

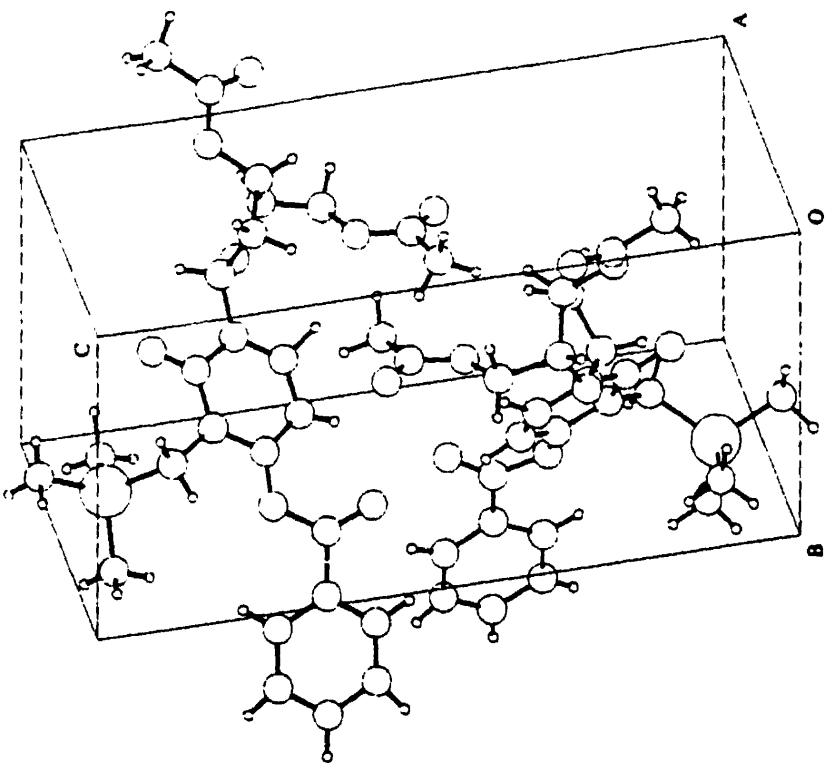



**Figure XR-2.** Unit cell of nucleoside **3**

# APPENDIX II

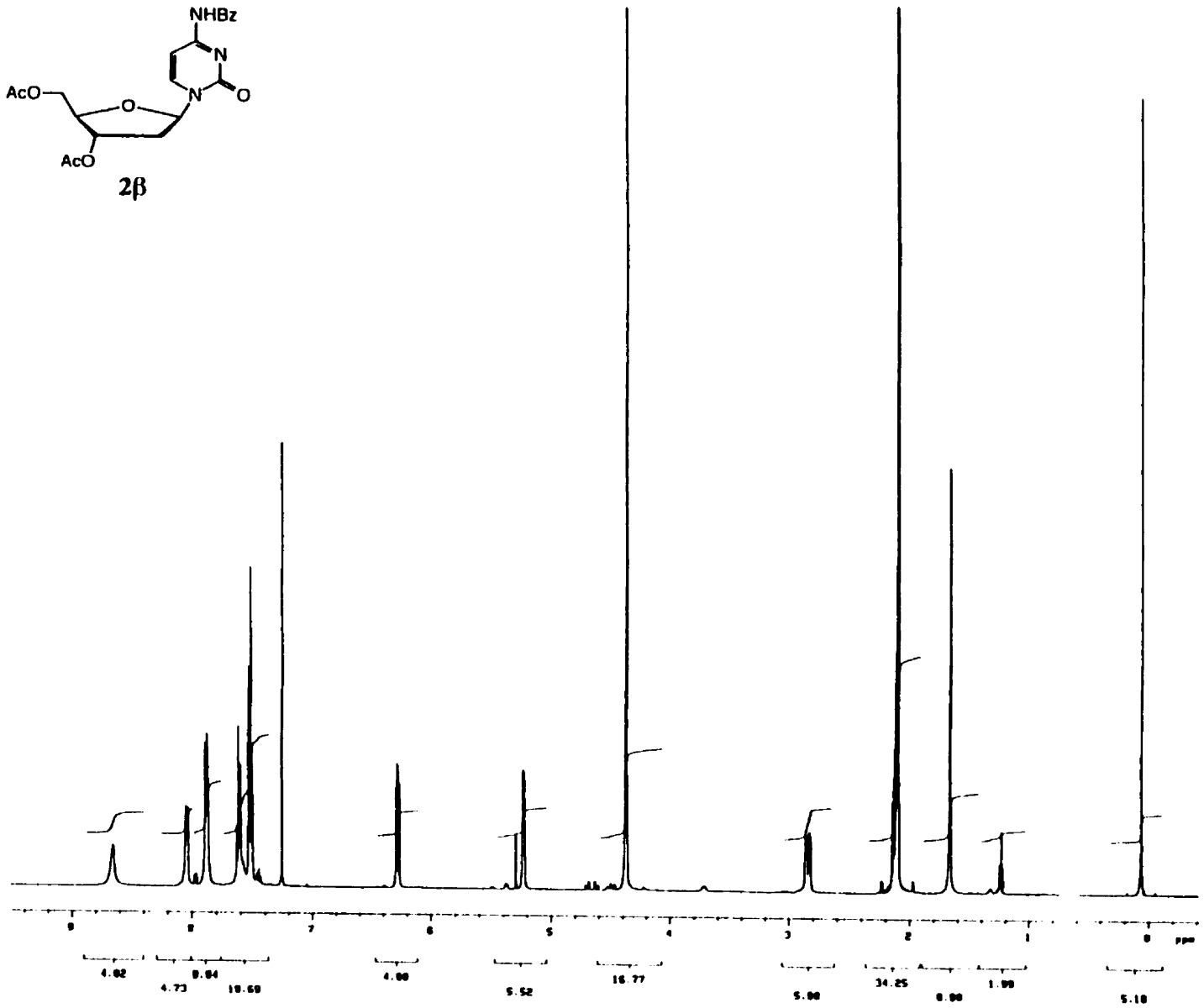



**Figure A.II-1:** $^1$H NMR (499.84 MHz, $CDCl_3$) of 5',3'-Di-*O*-Acetyl-*N*$^4$-benzoyl- 2'-β-deoxycytidine (**2β**).

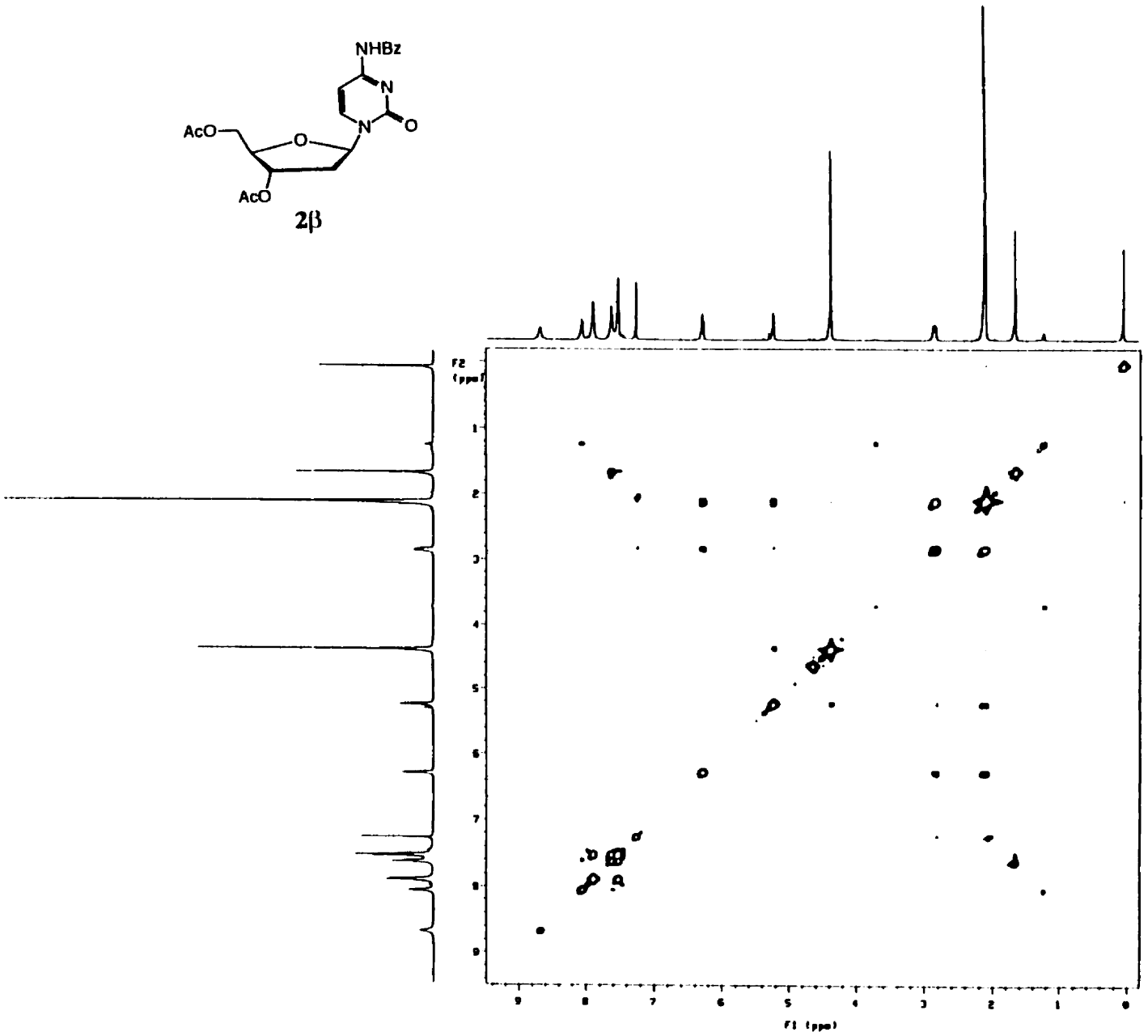



**Figure A.II-2:** 2D-COSY (499.84 MHz, $CDCl_3$) of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl- 2'-β-deoxycytidine (**2β**).

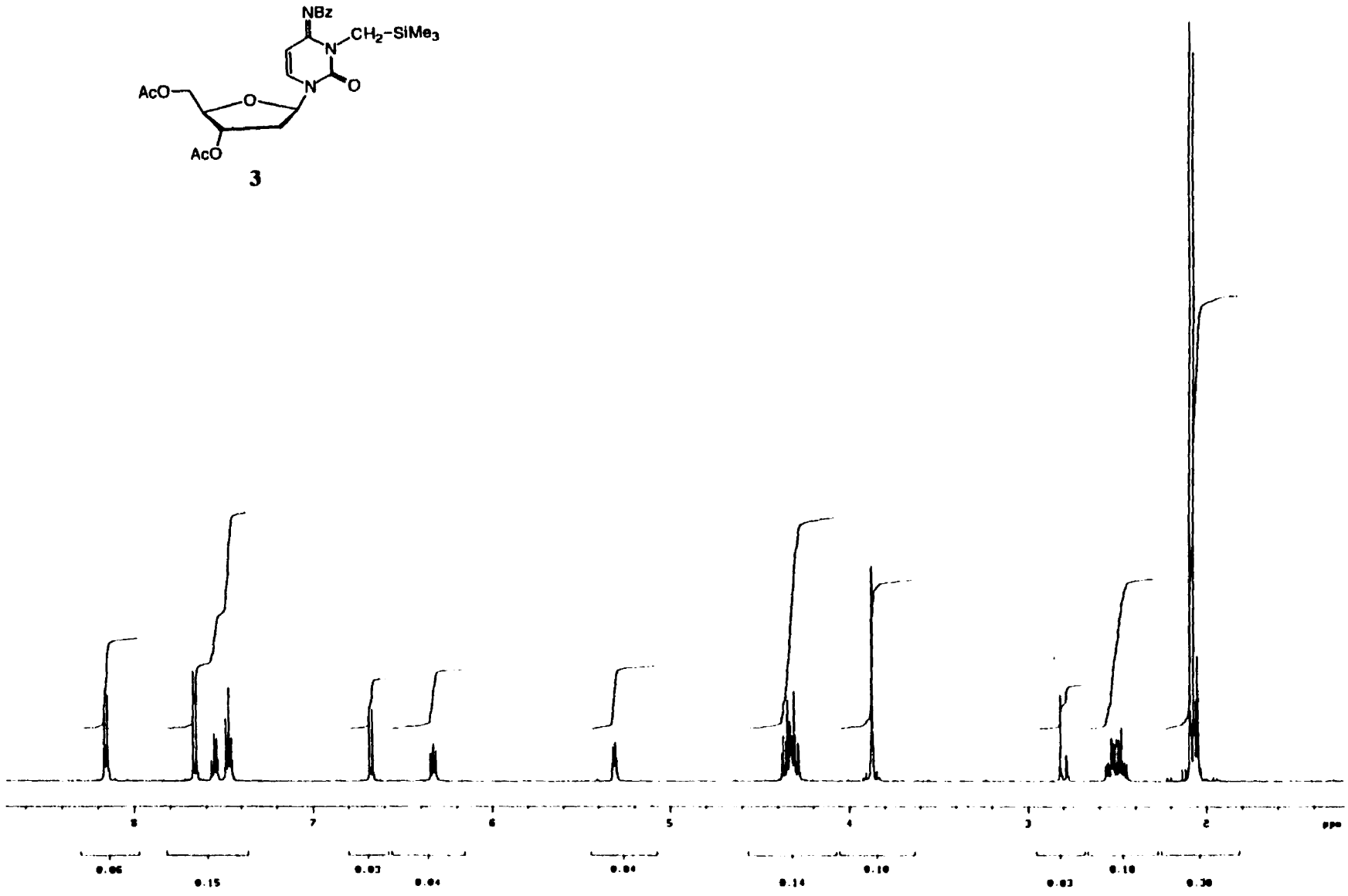



**Figure A.II-3:** $^1$H NMR (499.84 MHz, Acetone-$d_6$) of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'-deoxycytidine (**3**).

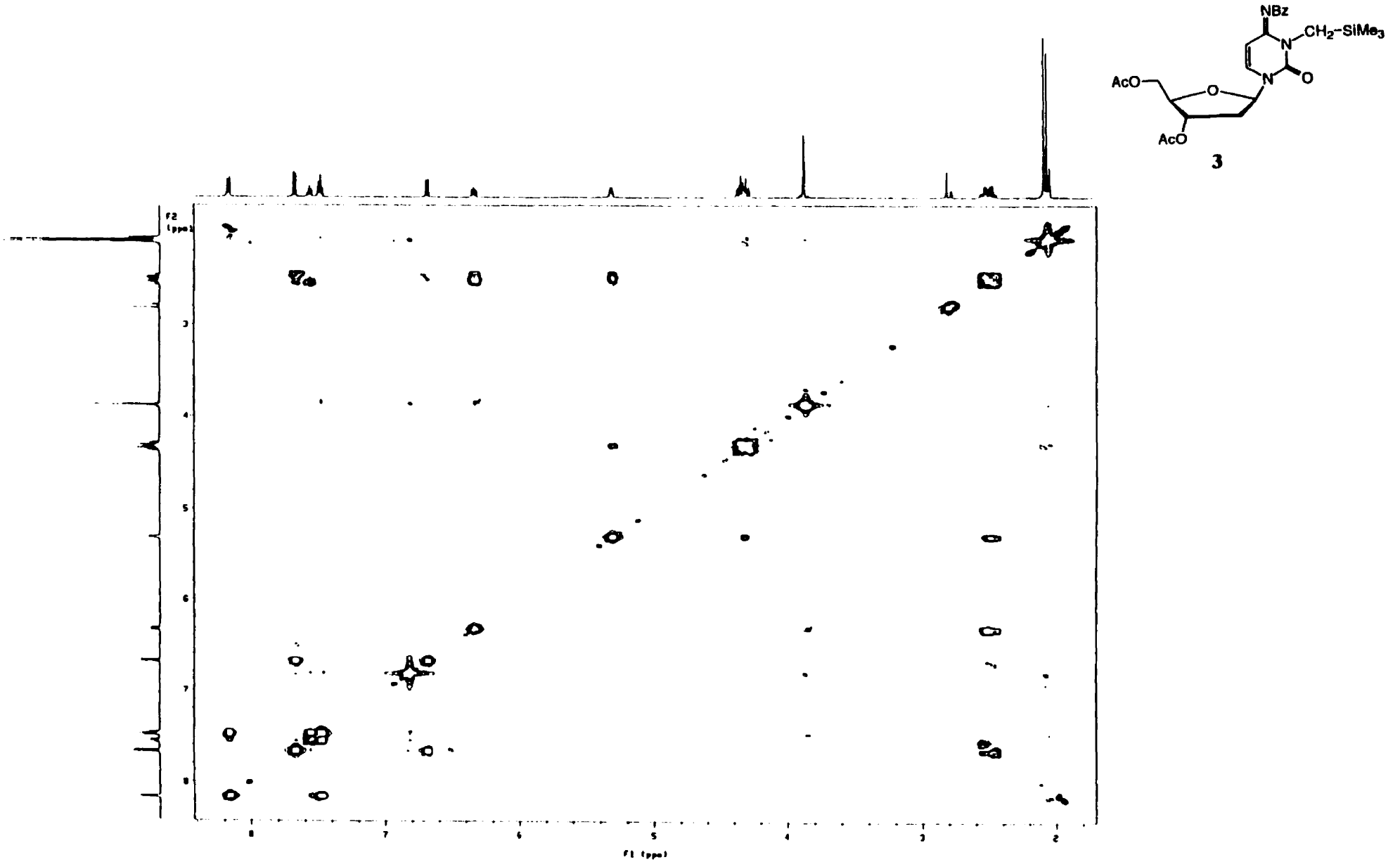



**Figure A.II-4:** 2D-COSY (499.84 MHz, Acetone-$d_6$) of 5',3'-Di-*O*-Acetyl-$N^4$-benzoyl-$N^3$-trimethylsilyl methyl-2'-deoxycytidine (**3**).

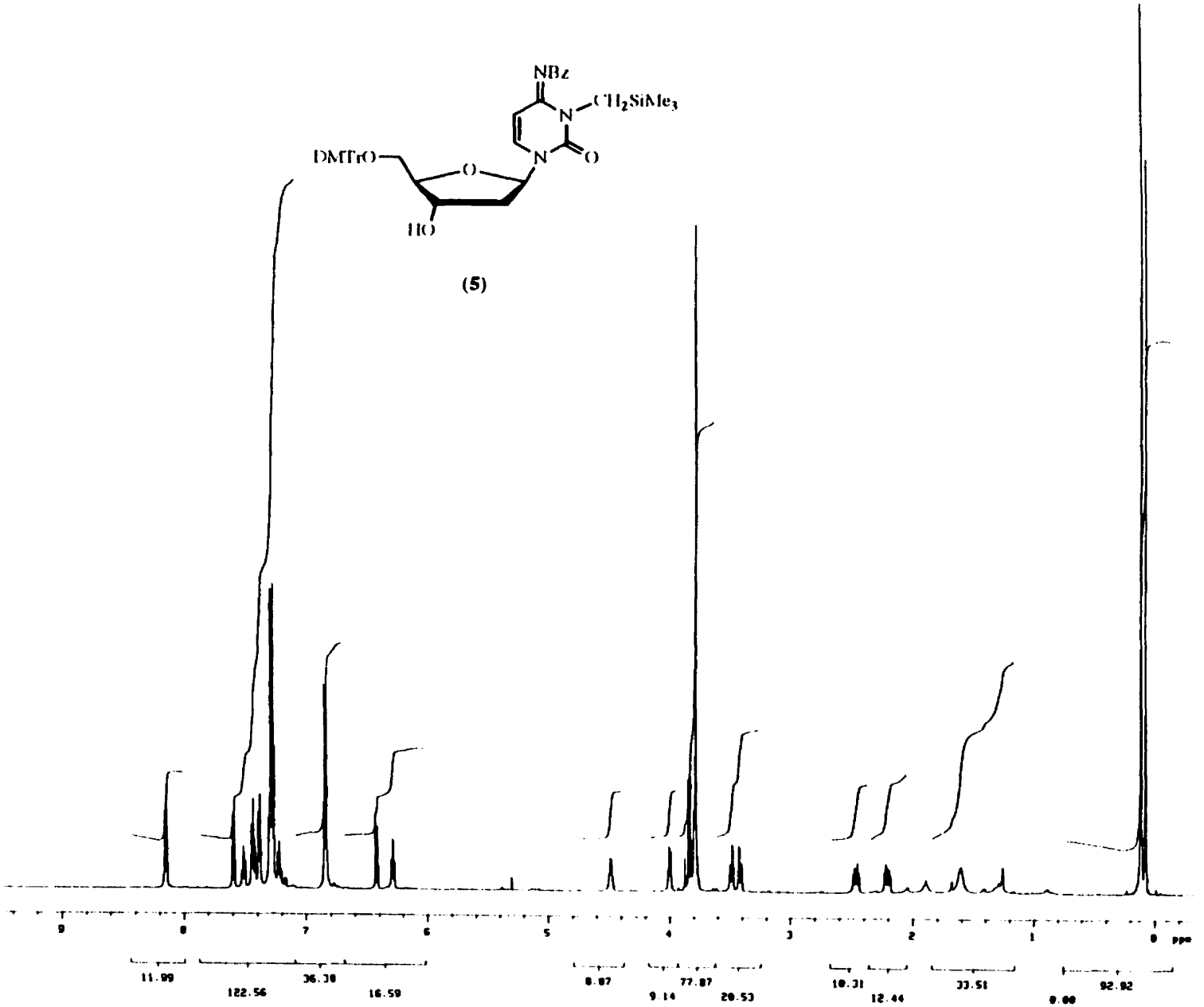



**Figure A.II-5:** $^1$H NMR (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-$N^4$-benzoyl-$N^3$-trimethylsilylmethyl-2'-deoxycytidine (**5**).

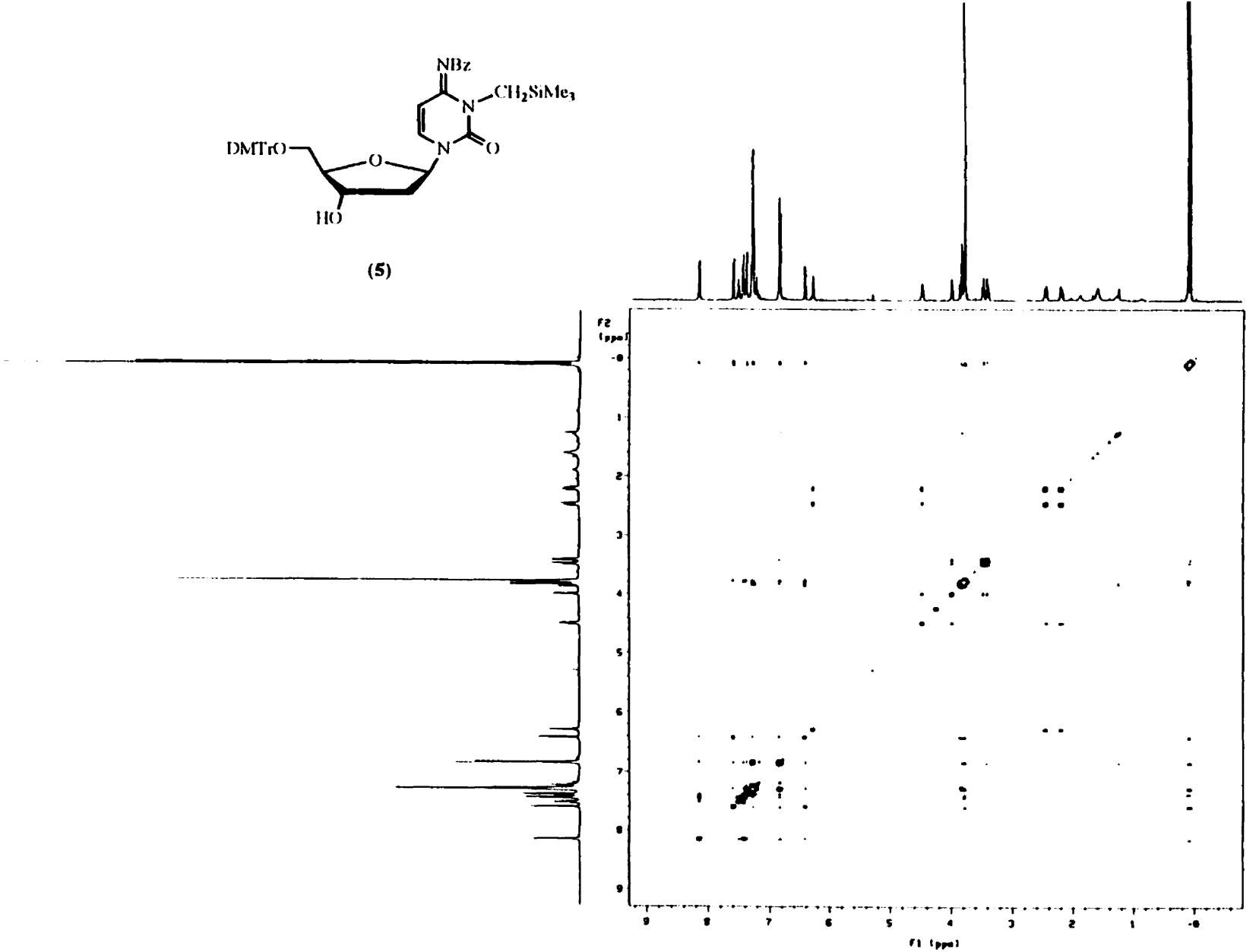



**Figure A.II-6:** 2D-COSY (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-$N^4$-benzoyl-$N^3$-trimethylsilylmethyl-2'-deoxycytidine (**5**).

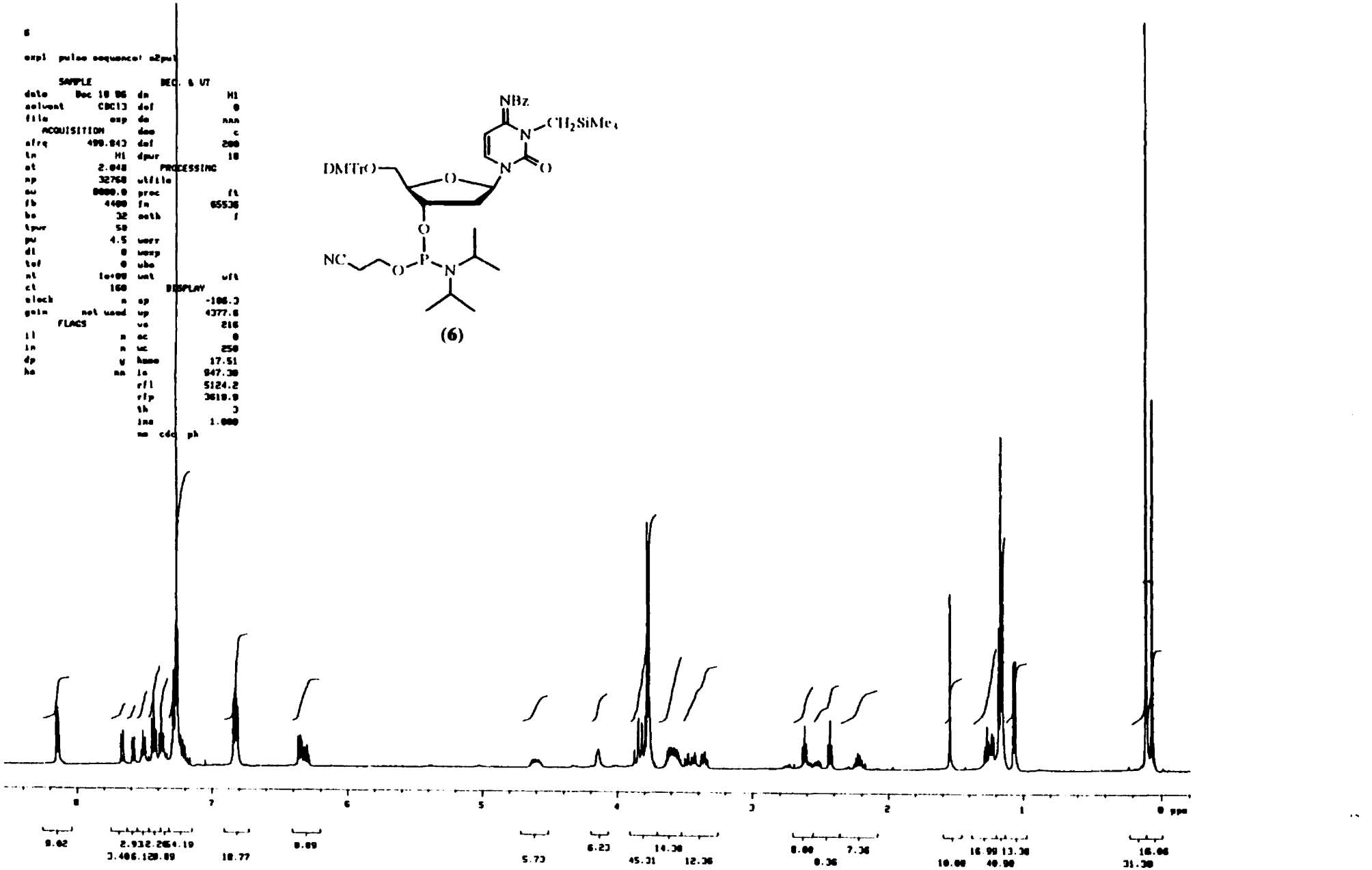
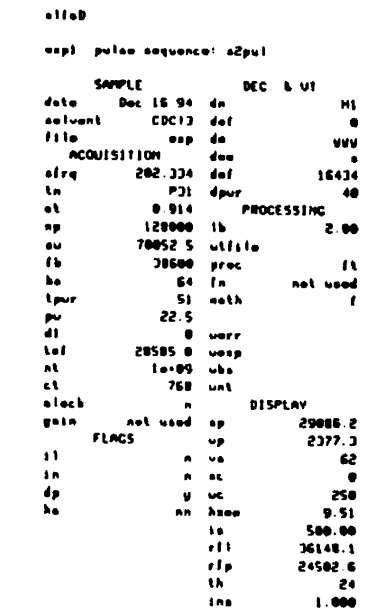
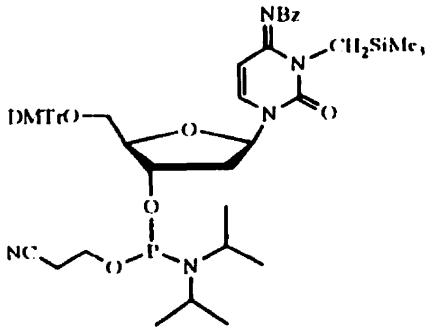



**Figure A.II-7:** $^1$H NMR (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidite-$N^4$-benzoyl-$N^1$-trimethylsilyl methyl-2'-deoxycytidine (**6**).




**(6)**

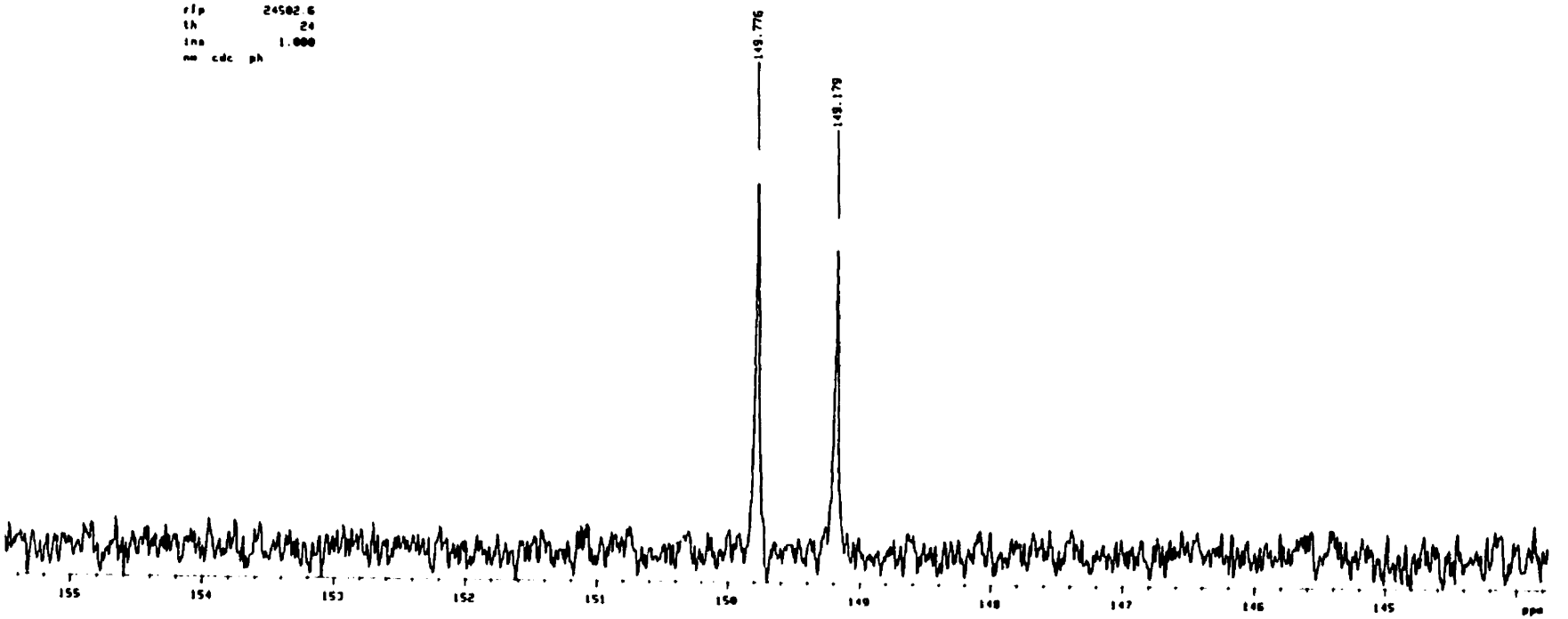



**Figure A.II-8:** $^{31}P$ NMR (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidite-$N^4$-benzoyl-$N^4$-trimethylsilyl methyl-2'-deoxycytidine (**6**).

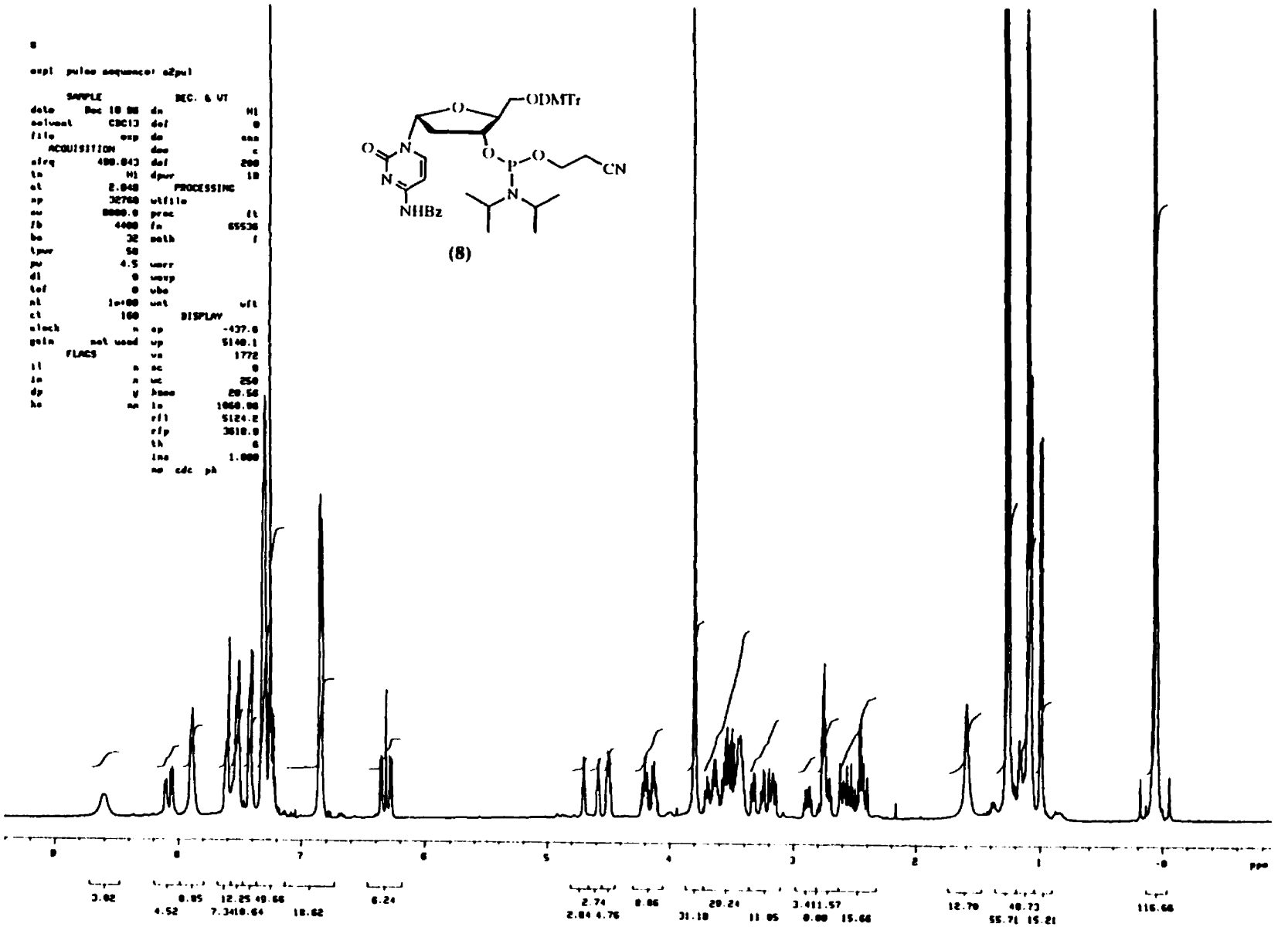
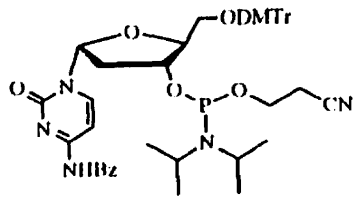



**Figure A.II-9:** $^1$H NMR (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidine-$N^4$-benzoyl-L-α-2'-deoxycytidine (**8**).




(8)

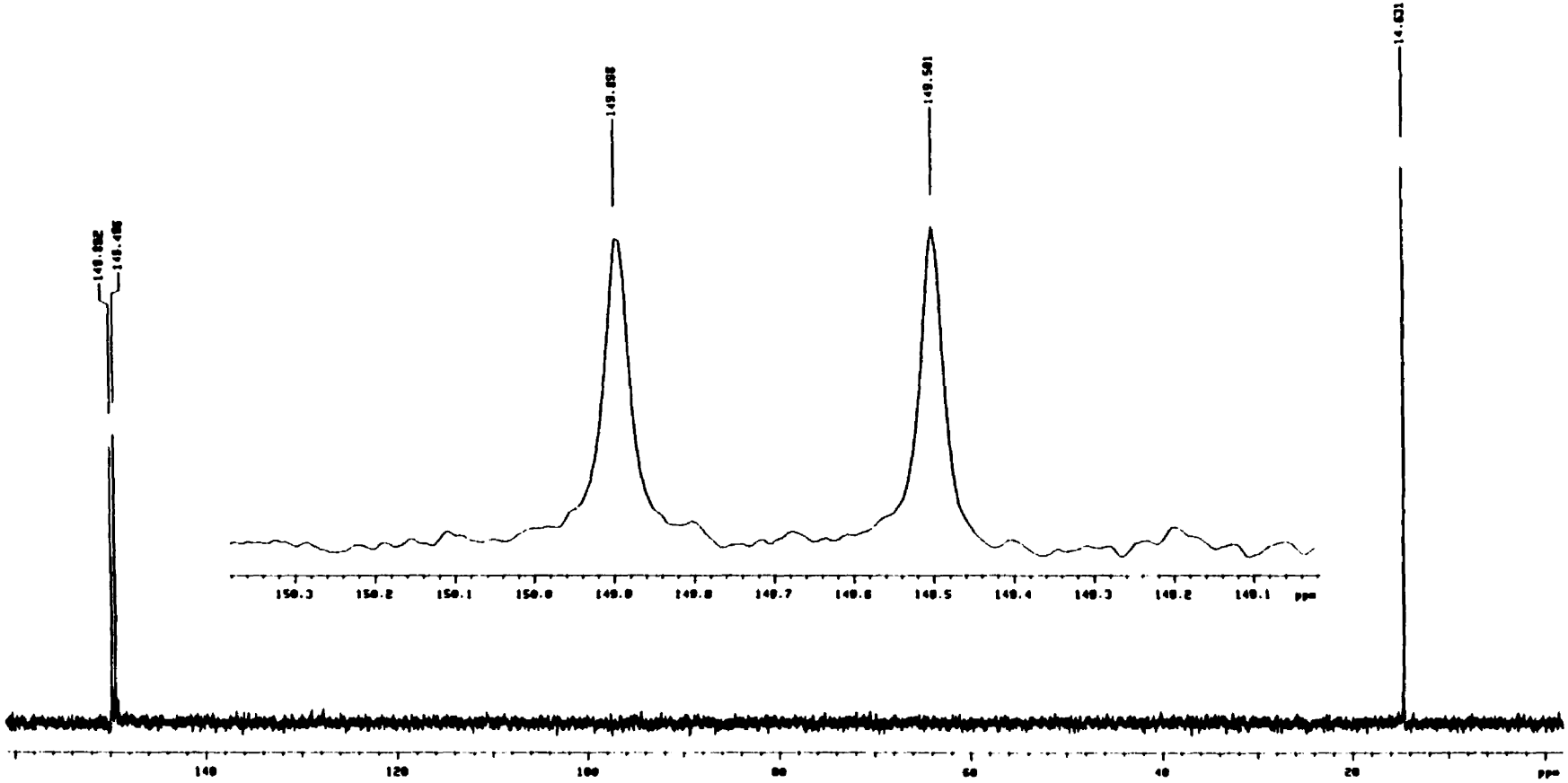



**Figure A.II-10:** $^{31}P$ NMR (499.84 MHz, $CDCl_3$) of 5'-*O*-Dimethoxytrityl-3'-*O*-N,N-diisoproyl-β-cyanoethylphosphoramidine-$N^4$-benzoyl-L-α-2'-deoxycytidine (**8**).