"NUCLEOBASE – POLYAMINE CONJUGATES OF OLIGONUCLEOTIDES AND PNA-DNA CHIMERA: SYNTHESIS AND BIOPHYSICAL STUDIES"

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By

PRADEEP S. PALLAN

DIVISION OF ORGANIC CHEMSTRY: (SYNTHESIS)

NATIONAL CHEMICAL LABORATORY

PUNE 411008

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To my Parents...

Dr. K. N. Ganesh
Head

National Chemical Laboratory

Division of Organic Chemistry(Synthesis)
Pune - 411 008, INDIA

TeleFax: +91 20 589 3153
Residence: +91 20 589 8320
E-mail: kng@ems.ncl.res.in

CERTIFICATE

This is to certify that the work presented in the Thesis entitled **"NUCLEOBASE – POLYAMINE CONJUGATES OF OLIGONUCLEOTIDES AND PNA-DNA CHIMERA: SYNTHESIS AND BIOPHYSICAL STUDIES"** submitted by Pradeep S. Pallan was carried out by the candidate at the National Chemical Laboratory Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the Thesis.

(K. N. Ganesh) October 2001

CANDIDATE'S DECLARATION

I hereby declare that the Thesis entitled **"NUCLEOBASE – POLYAMINE CONJUGATES OF OLIGONUCLEOTIDES AND PNA-DNA CHIMERA: SYNTHESIS AND BIOPHYSICAL STUDIES"** submitted for the degree of Doctor of Philosophy in Chemistry to the University of Poona has not been submitted by me to any other university or institution. This work was carried out at the National Chemical Laboratory, Pune, India.

National Chemical Laboratory **(Pradeep S. Pallan)**

Pune 411 008

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Section B Chemical Synthesis and Biophysical Studies of 5-Me-dC-N^4-Alkylguanidino Substituted Oligonucleotides

Publications:

1) Direct Conversion of Alcohols into the Corresponding Iodides.
Joseph, R., **Pallan, P. S.**, Sudalai, A., Ravindranathan, T. *Tetrahedron Lett.* **1995**, *36*, 609-612.

2) DNA Triple Helix Stabilization by Bisguanidinyl Analogues of Biogenic Polyamines.
Pallan P. S and Ganesh K. N. *Biochem. Biophys. Res. Commun.,* **1996**, *222*, 416-420.

3) Modulation of DNA Triplex Stability through Nucleobase Modifications.
Ganesh K. N., Rajeev K.G., **Pallan P. S.**, Rana V. S., Barawkar D. A., and Kumar V. A., *Nucleosides Nucleotides*, **1997,** *16,* 1271-1278.

4) Stabilization of DNA Structures through Nucleobase Modifications and Bisguanidinium Polyamines.
Ganesh K. N., Kumar V.A., Barawkar D.A., Prakash T. P., Rajeev K.G., **Pallan P.S.**, Rana V. S. *Ind. J. Chem.* **1997**, *36*, A & B, 519-524.

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Kumar V. A., **Pallan P. S.**, Meena and Ganesh K. N. *Org.Lett.* **2001**, *3*, 1269-1272.

ABSTRACT

NUCLEOBASE-POLYAMINE CONJUGATES OF OLIGONUCLEOTIDES AND PNA-DNA CHIMERA: SYNTHESIS AND BIOPHYSICAL STUDIES

CHAPTER 1: INTRODUCTION

Hydrogen-bonding interactions are important in the stability of nucleic acid complexes, often composed of simple but fundamental structures of duplexes and hairpin loops. Base-modified nucleoside analogues have provided means to probe the nature of the hydrogen-bonding interactions in these nucleic acid structures. In higher order structures, such as DNA triplexes, a third strand of DNA can bind to the major groove of duplex target DNA in a sequence specific manner. Since binding occurs in the major groove, triplex forming oligonucleotides can interfere with regulatory proteins which bind to the same site, hence controlling gene expression. This perspective on

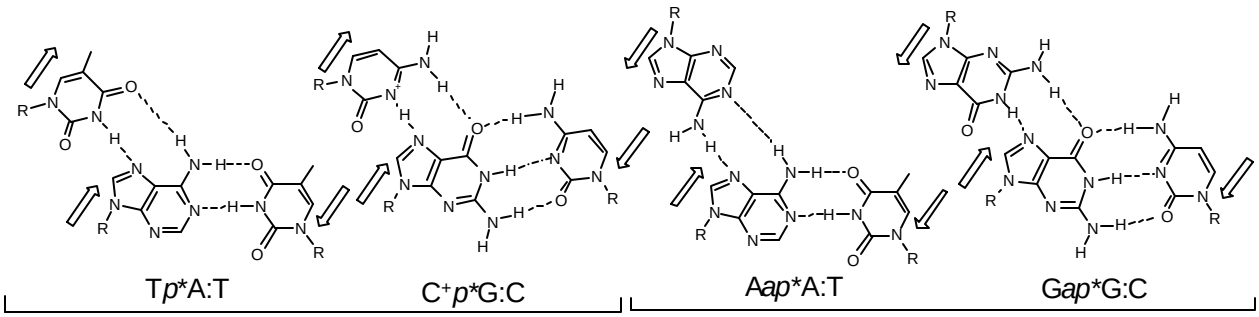


Figure **1**

developing a new strategy for drug discovery has stimulated extensive work on triple helical complexes during the past decade.

There are essentially two families of DNA triple helices (i) the pyrimidine motif, where the polypyrimidine third strand binds parallel to the purine strand of the duplex, forming T*A:T or C+*G:C triplets and (ii) the purine motif, where the purine rich third strand binds in an antiparallel fashion to the purine of the target duplex, thus forming A*A:T or G*G:C triplets as shown in Figure **1**.

Much of the focus has been towards increasing the stability of triplexes under physiological conditions. Although triplex forming oligonucleotides bind with high specificity, their binding is usually weaker than that of the target DNA duplex. In part, this is due to the charge repulsion encountered in bringing together the three poly-anionic DNA strands. Studies have shown that the introduction of cationic groups in the

bases, sugar or backbone can partly compensate such electrostatic repulsions and produce stable triplexes.

This Chapter gives an overview of the various chemical modifications so far known in literature to increase the triplex stability.

The main theme of this thesis is to conjugate cationic ligands such as polyamines and guanidinium functions to generate cat-anionic oligonucleotides and study their biophysical properties. The work is presented in four chapters (1) the synthesis of bisguanidinium derivatives of spermine, spermidine and their effect on stability of DNA duplexes and triplexes (2) covalent conjugation of alkyl guanidiniums to the N-4 position of 5-Me-2'-dC (3) synthesis of oligonucleotides bearing N^6-sperminyl-2-aminoadenine and their DNA binding studies (4) the ability of 2, 6-diaminopurine to form unnatural triplex triads and (5) the synthesis of pyrrolidine containing PNA-DNA dimer and the binding studies of the oligomers comprising this unit with complementary oligonucleotides.

CHAPTER 2 : EFFECT OF GUANIDINIUM DERIVATIVES ON OLIGONUCLEOTIDE COMPLEXES

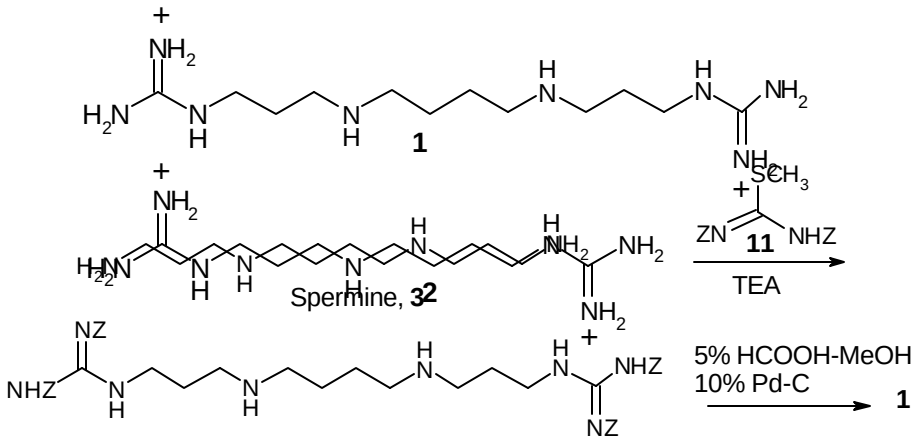
In this chapter the effect of guanidinum groups on DNA duplex and triplex structures is analyzed. The chapter is divided into two sections. Part-I deals with the DNA stabilization by synthetic bisguanidinyl analogues of the polyamines, spermine and spermidine and part-II describes the synthesis and properties of covalently conjugated guanidinium oligonucleotides.

Part–I: DNA Duplex and Triplex Stabilization by Bisguanidinyl Analogues of Spermine and Spermidine

The polycationic nature of biogenic polyamines such as spermine and spermidine plays an important role in selective binding to polyanionic nucleic acids. Amidinium and guanidinium groups do play a significant role in DNA minor groove binding of drugs such as netropsin, distamycin, pentamidine and propamidine. The specific patterns of hydrogen bonding and the high basicity of guanidinium groups (pKa~13.5) allow it to play several key roles in molecular recognition. Spermine is known to stabilize DNA duplexes and triplexes by its specific recognition of the nucleobases and electrostatic interaction with the anionic phosphates. The

transformation of the primary amino groups in these molecules into the corresponding guanidinium functions (e.g. as in **1** and **2**) is expected to amplify the electrostatic component resulting in improved binding.

This section describes the synthesis of the bisguanidinium analogues **1** and **2** by a simple route (Scheme **1**), without involving any extensive protection / deprotection



Scheme 1

strategy. Among the several guanylating reagents N,N'-(bisbenzyloxycarbonyl)-S-methylisothiourea, **11** was found to be the most convenient since it reacted almost exclusively with the primary amino groups. Spermine, **3** and spermidine, **4** as free bases were individually treated with the above reagent in dichloromethane at reflux in presence of triethylamine to yield the corresponding benzyloxycarbonyl protected N,N'-bisguanidinium derivatives. The cleavage of the benzyloxycarbonyl group was achieved by catalytic transfer hydrogenation using HCOOH-Pd/C (10%) to yield the bisguanidines **1** and **2** as the corresponding formate salts, the structures of which were confirmed by ^{1}H, ^{13}C NMR and FAB MS.

The effect of the guanidinium derivatives on duplexes: The effect of bisguanidiums on DNA duplex and triplex stabilities were studied by temperature dependent UV-absorbance. In the UV-melting experiments, it was seen that both spermine and spermidine stabilized DNA duplexes as expected by 3-4°C over the control samples. In comparison to this, the bisguanidinium derivatives **1** and **2** enhanced the T_m of dodecamer **5** by 8.5-9°C and that of the 24 base pair, **6:7** duplex by 7-8°C over control, amounting to ~4°C stabilization over that of parent polyamines (Table **1**).

5 5' CGC GAA TTC GCG

6 5' GCC AAG AAA AAA CAA AAA AGA CGC

7 5' GCG TCT TTT TTG TTT TTT CTT GGC

8 5' TTC TTT TTT CTT TTT TCT

9 5' GCC AAG AAA AAA GAA AAA AGA CGC

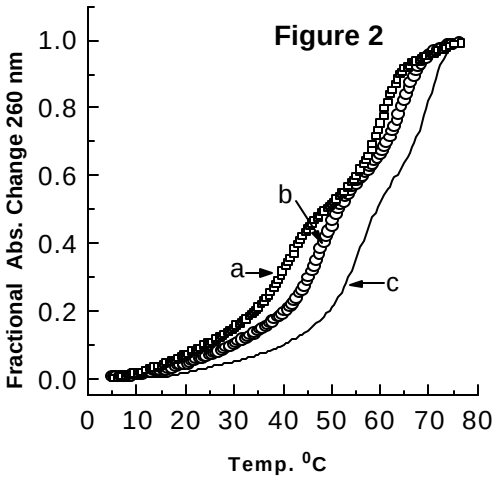
10 5' GCG TCT TTT TTC TTT TTT CTT GGC

Table 1 T_m (°C) of Duplexes / Triplexes

No	Complex	1	2	3	4	Control
1	6:7	68.0 (8)	67.0 (7)	64.0 (4)	63.0 (3)	60.0
2	5	59.0 (9)	58.5 (8.5)	55.0 (5)	54.5 (4.5)	50.0
3	8*9:10	48.5	31.5	36.0	26.0	nd
4	8*9:10	51.5(17.0)	45.0(10.5)	46.0(11.5)	33.5 (-1)	34.5

Values in parentheses = ΔT_m, -sign indicates destabilization.

The effect of the guanidinium derivatives on triplexes: This was examined by carrying out pH-dependent melting experiments on the triplex constituted from the 24-mer **9:10** duplex and 18-mer third strand polypyrimidine **8**. Characteristic biphasic transitions in the melting curves (Figure **2**) unambiguously indicated the formation of triplexes, with the first transition corresponding to the dissociation of the third strand from duplex and the second transition corresponding to duplex melting.



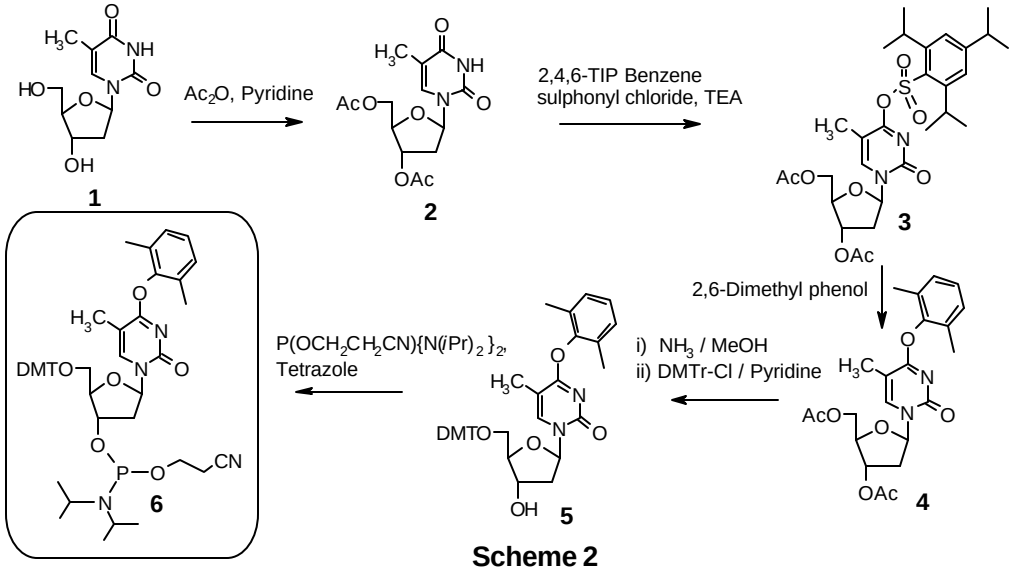
(a) 8*9:10, control (b) 8*9:10+**3**,1mM
(c) 8*9:10+**1**,1mM

In the absence of spermine or spermidine, no triplex formation was seen at pH 7.0 while in their presence, triplexes were observed with the following stability order: **4** ~ **2** < **3** < **1**. The transformation of terminal amino groups of spermine to guanidino functions, resulted in a significant stabilization of the corresponding triplex by 12.5°C. In view of these interesting results, it was considered worthwhile to study the conjugation of alkyl guanidiniums to oligonucleotides.

Part-II: Chemical Synthesis and Biophysical Studies of 5-Me-dC-N^4-alkylguanidino Substituted Oligonucleotides.

Several modified oligonucleotides (ODNs) have been employed as enzymatic and non-enzymatic nucleases, antisense / antigene agents, detection probes and cross-linking agents. These oligonucleotides are synthesized by direct incorporation of the suitably protected unnatural nucleoside into the sequence. If the oligonucleotide bearing an unnatural base having a reacting functionality is incorporated, it can be further treated with an appropriate reagent to get the modified oligonucleotide. For the synthesis of modified oligonucleotides the convertible nucleoside approach was adopted in which the modified nucleoside contained a leaving group, such as 2,6-dimethylphenol, that was completely retained during the ODN synthesis and subsequent deprotection steps. O^4-2,6-Dimethylphenyl-5-Me-T-3'-O-phosphoramidite, *6* ***was synthesized from thymidine as shown in Scheme*** *2.*

The modified nucleoside, O^4-2,6-dimethylphenyl-thymidine ("convertible nucleoside") was incorporated site specifically into ODNs **7-9,** that were later functionalized into guanidinopropyl or guanidinobutyl derivatives by post-synthetic reactions. The "convertible ODNs" containing O^4-dimethylphenyl residues were synthesized, which were functionalized into guanidinopropyl or guanidinobutyl



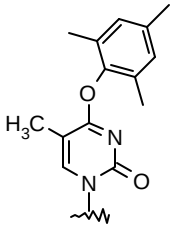
Scheme 2

derivatives by post-synthetic reactions to yield the N^4-propyl/butyl-guanidino-5-Me-

7 5' TTC TTC TTX TTT TCT TTT

8 5' TTX TTC TTC TTT TCT TTT

X =



9 5' TTX TTC TTX TTT TCT TTT

10 5' TTC TTC TTC TTT TCT TTT

11 5' AAA AGA AAA GAA GAA GAA

12 5' TCC AAG AAG AAG AAA AGA AAA TAT

13 5' ATA TTT TCT TTT CTT CTT CTT GGA

Table 2: UV-T_m of DNA duplexes and triplexes

DUPLEXES				TRIPLEXES			
Entry	Complex	$T_m(^0C)$	$\Delta T_m(^0C)$	Entry	Complex	$T_m(^0C)$	$\Delta T_m(^0C)$
1	7a:11	30	-17	8	7a*12:13	nd	---
2	7b:11	33.5	-13.5	9	7b*12:13	12	-13
3	8a:11	38.5	-8.5	10	8a*12:13	16.5	-8.5
4	8b:11	39.5	-7.5	11	8b*12:13	18	-7
5	9a:11	23.5	-23.5	12	9a*12:13	11	-14
6	9b:11	24	-23	13	9b*12:13	14.5	-10.5
7	10:11	47	---	14	10*12:13	25	

Buffer: 25 mM TRIS, 100mM NaCl, pH 7.0. All Tms are accurate to $\pm$ 0.5°C

-sign indicates destabilization.

dC bearing oligonucleotides forming 'zwitterionic DNAs'. The functionalized ODNs were characterized by MALDI-TOF mass spectroscopy and HPLC techniques, and used for biophysical studies. Both duplexes and triplexes formed by the ODNs bearing the guanidino modifications and the respective complementary ODNs were of lower stability, compared to the unmodified ODNs. The reduced stability of the ODN complexes may be because of the *syn* isomer (major) in the case of N^4-alkylated 5-methyl cytidines.

The UV-T_m results obtained for duplexes and triplexes containing the modified nucleosides are shown in Table-**2**. The results show that (i) all oligonucleotides (except **7a**) form triplexes of lower stability than the control (entries 8-14) (ii) the ODNs with modification in the centre (**7a**, **7b**, **9a** & **9b**) considerably destabilize the duplexes and (iii) the ODNs with end modification (**8a**, **8b**) destabilize the duplexes by 8^0 C.

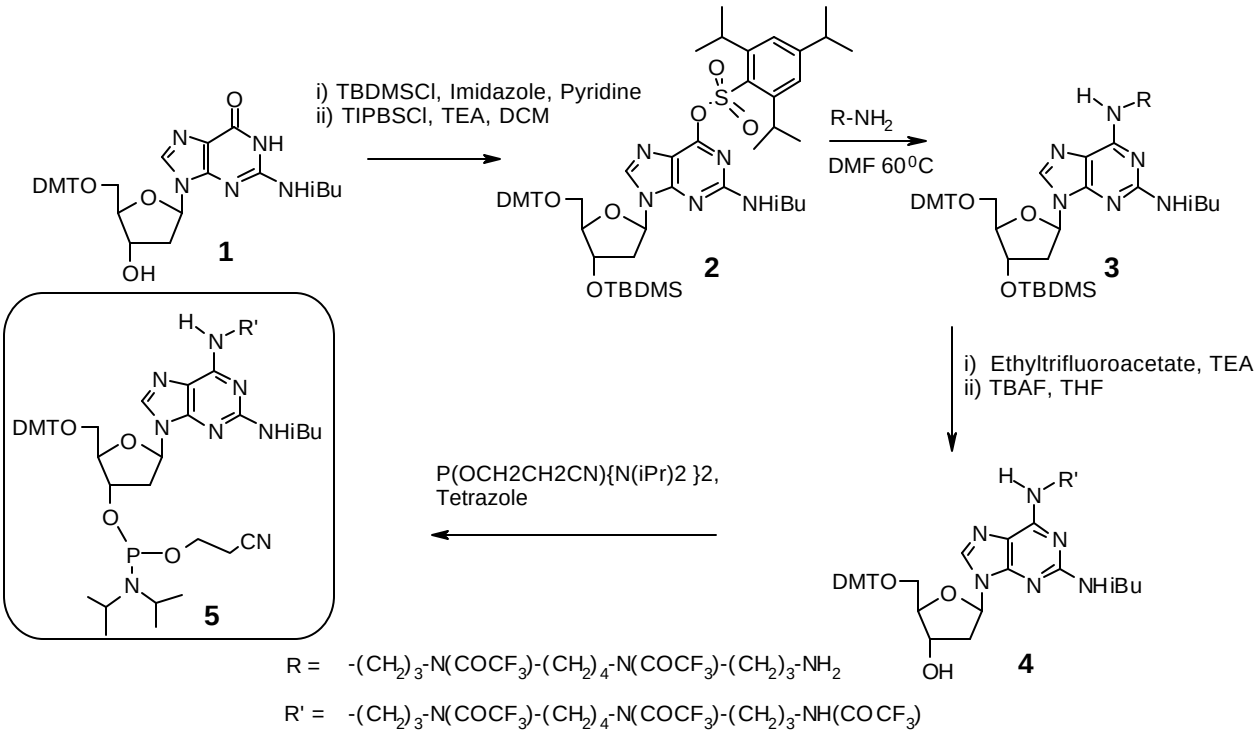
CHAPTER 3: SYNTHESIS AND TRIPLEX FORMATION OF OLIGONUCLEOTIDES CONTAINING N^6-SPERMINYL-2-AMINOADENINE AND 2,6-DIAMINOPURINE

This chapter is concerned with the conjugation of spermine to the N^6-amino group of adenine. Part-I describes the synthesis and biophysical studies of 6-sperminyl 2'-dA while part-II deals with triplex forming abilities of ODNs containing 2,6-diaminopurine.

Part-I : Synthesis of Oligonucleotides bearing N^6-Sperminyl-2-aminoadenine

Spermine has been linked to the 5' end, phosphate or nucleobases of oligonucleotides. It was shown that N^4-sperminyl-dC stabilized DNA triple helices significantly compared to the unmodified oligonucleotides. C-branched spermine tethered at the 5'-end, centre or 2'-end had a stabilizing effect on triplexes. In the purine series, 2'dA and 2'dG carrying protected spermine at N^2 and N^6 respectively have been synthesized. The positive attributes of polyamines in stabilizing DNA duplexes / triplexes and their non-toxic nature encouraged the design of a new purine-sperminyl conjugate such as 6-sperminyl 2'-dA.

The synthesis of the monomer, *5* ***was carried out as shown in Scheme*** *4.* ***The 3'-OH of 5'-O-DMTr-N^2-isobutyryl deoxyguanosine,*** *1* ***was silyl protected using TBDMSCl and imidazole in dry pyridine followed by treatment with triisopropyl benzenesulfonyl chloride in presence of triethyl amine to get the O^6-(2,4,6-triisopropylbenzenesulfonyl) derivative,*** *2.* ***This on treatment with 5,10-bis[N, N'-trifluoroacetyl]-1,5,10,14-tetraazatetradecane gave the sperminyl derivative,*** *3* ***the primary amino group of which was protected as its trifluoroacetyl derivative using ethyl trifluoroacetate in dry ethanol to give the tris-trifluoroacetyl derivative. Deprotection of O-silyl group using TBAF in THF afforded the 3' -OH derivative,*** *4* ***which on phosphitylation gave the required monomer,*** *5.* ***The following ODN sequences were synthesized and their biophysical studies carried out.***



Scheme 4

5 5' TTC TTC TTC T***A***T TCT TTT

6 5' TTC T***A***C TTC T***A***T TCT TTT

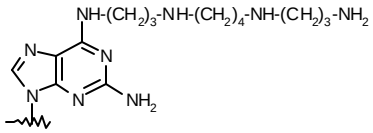
7 5' AAA AGA A***A***A GAA GAA GAA

8 5' TCC AAG AAG A***A***G AAA AGA AAA ATT AT

9 5' TCC AAG AAG AAG AAA AGA AA***A*** ATT AT

10 5' TCC AAG AAG A***A***G AAA AGA AA***A*** ATT AT

A =



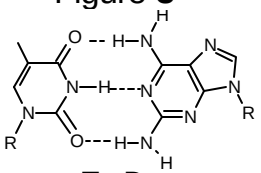
An appropriate control for the modified nucleoside N^6-sperminyl-2-aminoadenine would be 2,6-diaminopurine and the control sequences were synthesized using the commercially available N^2,N^6-bis(N,N-diisobutylformamidine)-2,6-diaminopurine phosphoramidite.

Part-II : Oligonucleotides Containing 2,6-Diaminopurine: Synthesis and Evaluation of Their Effect on Triplex Formation.

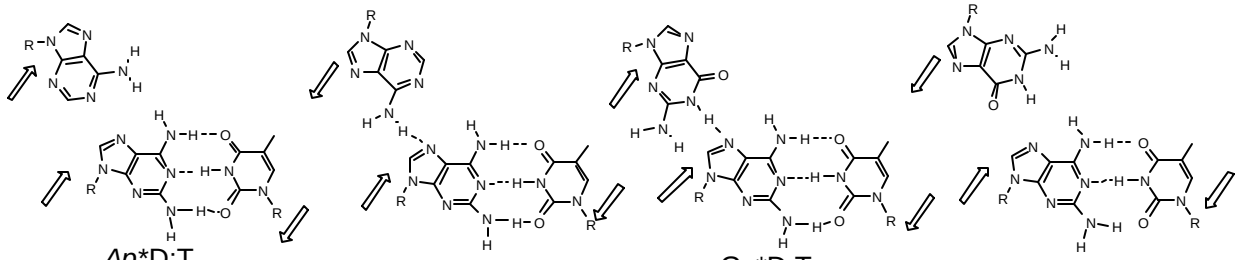
2,6-diaminopurine is an adenine analogue which is found in S-2L cyanophage DNA. The replacement of adenine by 2,6-diaminopurine (D) creates a minor groove modification by introduction of a C2 aminogroup. This increases the stability of the Watson-Crick base pair with thymine by an additional hydrogen bond (Figure **3**). Here, we explored the possibility of 2,6-diaminopurine as the central base of the triplex triad

(X*D:T, X= A, G) both in parallel and antiparallel motifs as shown in the figure **4**. The compatibility of D as the third base and 5-amino dU ($U^{\#}$) in the central postion in the triad was also studied.

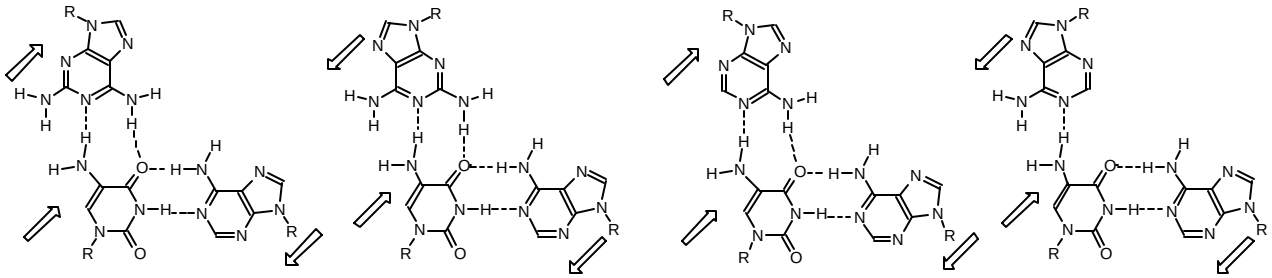
Figure **3**



T : D



*Ap**D:T *Aap**D:T G*p**D:T G*ap**D:T



D*p** $U^{\#}$:A D*ap** $U^{\#}$:A A*p** $U^{\#}$:A D*ap** $U^{\#}$:A

Figure 4

The UV-T_m studies showed that:

(i) A_p*D:T and A_p*A:T triplexes were formed in the parallel motif (*p*) with the same stability; while no triplex formation was observed in the antiparallel mode (*ap*) (ii) G_p*D:T and G_p*A:T triplexes exhibited similar stabilities (iii) D_p*$U^{\#}$:A triplexes were as stable as the control A_p*$U^{\#}$:A (Figure 4**) and (iv) D formed triplexes with higher stabilities than 6-sperminyl 2'-dA.**

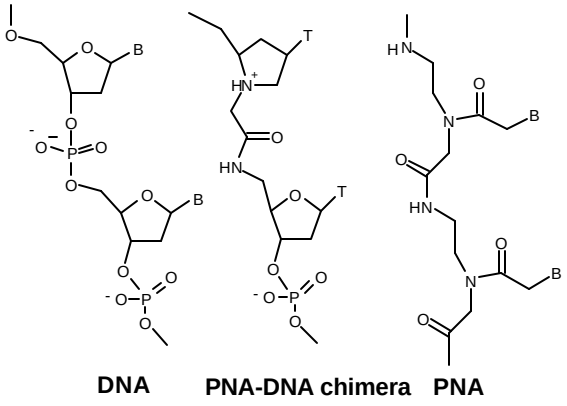
CHAPTER 4: SYNTHESIS OF PYRROLIDINE-CONTAINING PNA-DNA DIMER AND DNA COMPLEMENTATION STUDIES

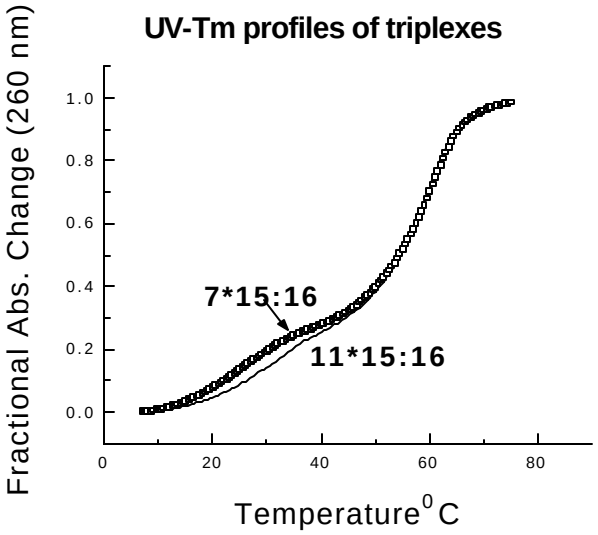
Among the variety of oligonucleotide modifications, the non-ionic oligonucleotide analogues are of particular interest. Besides their increased stability towards nucleases, they have higher binding affinity to their target nucleic acid because of the lack of charge-repulsion effects. The most remarkable non-ionic ODN analogue is the

polyamide nucleic acid (PNA), which binds to complementary DNA and RNA with a higher affinity than the natural oligonucleotides.

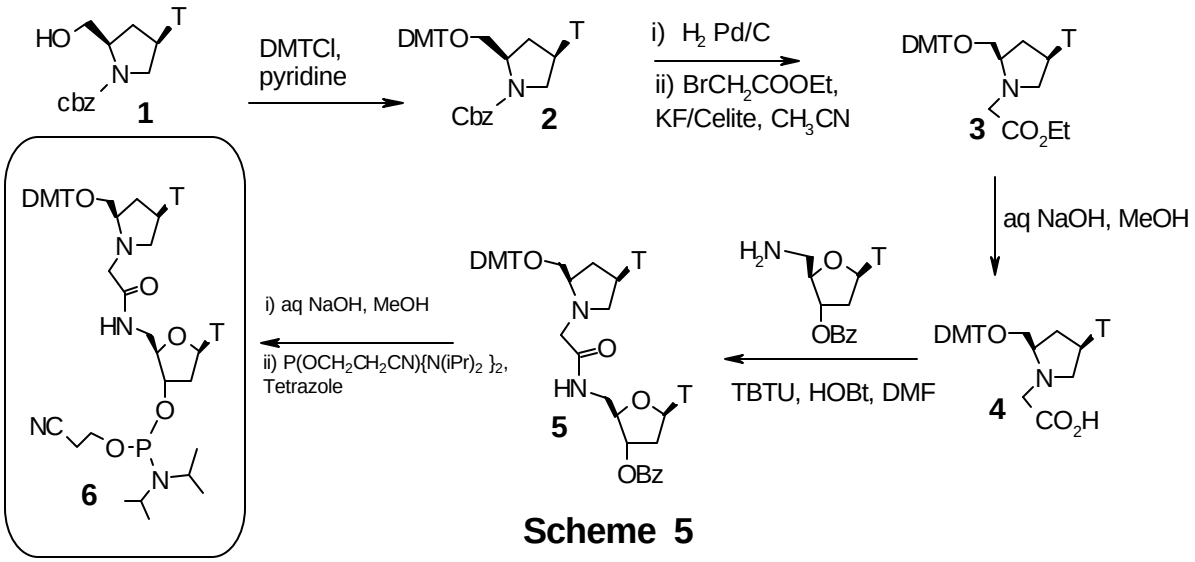
The drawbacks of PNAs are their poor solubility in aqueous systems and their ability to form both parallel and antiparallel duplexes with very similar stabilities. It is found that PNA-DNA

Figure 5





Buffer: 10mM sodium phosphate, 200mM NaCl pH 7.0.



Scheme 5

conjugates (chimerae) bind to their complementary DNA and RNA exclusively in the antiparallel orientation. The anionic nature of the DNA part of the chimerae reduces

the tendency to self-aggregate, thereby improving their solubility. The binding affinities of the PNA-DNA chimerae are usually lower than the corresponding PNAs. Introduction of a positively charged pyrrolidine ring carrying HN^{+}-CH_2-CO-NH-5' linkage for the replacement of the sugar-phosphate backbone in DNA (PNA-DNA chimera in Figure **5**) may therefore be expected to have cumulative advantages, leading to a very attractive antisense construct. The pyrrolidine derivative **1** (*2R,4R*) with stereochemistry equivalent to that of natural nucleosides was used to synthesize the dimer block, **6** suitable for solid phase DNA synthesis as shown in scheme **5**.

7 5' TTC TTC TTC TTT TCT **t*T**T
8 5' CTT GTA CT**t*** **T**T C CGG **t*T**T
9 5' CTT GTA C**t*T t*T**C CGG TTT
10 5' CTT GTA CT**t*** **T**T C CGG TTT
11 5' TTC TTC TTC TTT TCT TTT
12 5' CTT GTA CTT TTC CGG TTT
13 5' AAA AGA AAA GAA GAA GAA
14 5' AAA CCG GAA AAG TAC AAG
15 5' TCC AAG AAG AAG AAA AGA AAA TA
16 5' ATA TTT TCT TTT CTT CTT CTT GG

t*T = dimer block

UV-T_m of DNA duplexes and triplexes

Entry	Complex	T_m (^{0}C)	ΔT_m (^{0}C)
1	**7:13**	45	
2	**11:13**	42	-3
3	**10:14**	51	-6
4	**9:14**	45	-12
5	**8:14**	50	-7
6	**12:14**	57	
7	**7*15:16**	29	
8	**11*15:16**	29	

Buffers: (a) duplexes, 10mM sodium phosphate, pH 7.0, 100mM NaCl, 0.1mM EDTA. (b) triplexes: 10mM sodium phosphate, 200mM NaCl pH 7.0. All T_ms are accurate to $\pm$ 0.5^{0}C and measured in four melting experiments

The duplex with polypyrimidine ODN **7** carrying the amide-linked pyrrolidine-sugar dimer at the 3' terminus, exhibited a 3^{0}C destabilization compared to the control duplex. The duplex with a single modification at the centre in a mixed pyrimidine-purine sequence also showed a destabilization of 6^{0}C compared to the relevant control duplex. The detrimental effect on the stability was more pronounced in the duplex containing two adjacent modified units in the centre (ΔT_m -12^{0}C). The dimer **6** though configurationally equivalent to natural DNA, perhaps induced structural deviations in the duplexes of oligomers **7-10** resulting in their destabilization as compared to the control, unmodified duplexes. The polypyrimidine oligomer **7** also formed the third (Hoogsteen) strand of a triplex with the duplex **15:16**. It was seen that the stability of the triplex **7*15:16** from chimeric oligomer containing the pyrrolidine at the 3'-end was similar to the corresponding unmodified triplex **11*15:16.**

In this Chapter, the synthesis of the above pyrrolidine-PNA-DNA chimerae, their binding studies with DNA and nuclease resistance are discussed.

Abbreviations and Symbols:

A	**Adenine/absorbance**
aeg	Aminoethyl glycine
ala	Alanine
anhy.	Anhydrous
ap	Antiparallel
Ac_2O	Acetic anhydride
β-ala	β-alanine
cm	Centimeter
CH_3CN	Acetonitrile
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
C	Cytosine
m^5C	5-Methylcytosine
CPG	Controlled pore glass
CBz	Benzyloxycarbonyl
CD	Circular Dichroism
DABCO	1,4-Diazabicyclo[2.2.2]octane
DCC	Dicyclohexyl carbodiimide
DCM	Dichloromethane
DCU	Dicyclohexyl urea
DIAD	Diisopropyl azodicarboxylate
DEAD	Diethyl azodicarboxylate
d	Deoxy
dG	2'-deoxyguanosine
EDC	Dichloroethane
dA	2'-Deoxyadenosine
DNG	Deoxynucleoguanidine
dC	2'-Deoxycytidine
dT	2'-deoxythymidine
DMAP	4-N,N'-Dimethylaminopyridine
DMT	Dimethoxytrityl
DIPEA	Diisopropylethylamine
DMF	N,N'-dimethylformamide

DNA	2'-Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESMS	Electrospray mass spectroscopy
FAB	Fast Atom Bombardment
FPLC	Fast Protein purification Liquid Chromatography
g	Gram
G	Guanine
gly	Glycine
h	Hour
HOBt	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IR	Infra-Red
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
mg	Milligram
min	Minute(s)
MHz	Megahertz
µM	Micromolar
mL	Millilter
mM	Millimolar
MS	Mass spectrometry/Mass Spectrum
mmol	Millimole
M	Molar
MeOH	Methanol
N	Normal
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NAP	Nucleic acid purification
ODN	Oligodeoxyribonucleotide
PCR	Polymerase Chain Reaction
PPh_3	Triphenylphosphine
PNA	Peptide Nucleic Acid
Pro	Proline
p	Parallel
psi	Pounds per square inch

R	Rectus
RNA	Ribonucleic Acid
RNAse	Ribonuclease
r.t.	Room temperature
RP	Reverse phase
S	Sinister
sp	Spermine
T	Thymine
tert-Boc	Tert-Butoxycarbonyl
TBDMS	Tert-Butyldimethylsilyl
TBAF	Tetrabutyl ammonium fluoride
TIPBSCl	2,4,6-Triisopropylbenzenesulfonyl chloride
TBTU	O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TEAA	Triethylammoniumacetate
TLC	Thin layer chromatography
Tm	Melting temperature
UV	Ultraviolet
Z	Benzyloxycarbonyl

Deoxyribonucleic acid (DNA) is the basic material in the chromosomes of the cell nucleus. DNA encodes and carries genetic information and is the fundamental element of heredity. It serves as a replicable template for copying of cells, as a scaffold for the organization of proteins, and as a polyfunctional switch for the control of gene expression.

1.1. DNA STRUCTURE

1.1.1. Basis for the Structure of DNA

The elucidation of the structure of DNA by Watson and Crick[1] was the culmination of several experimental results reported by a large number of scientists. Base equivalences in DNA was established by Chargaff and colleagues.[2] Todd and co-workers[3] established that the four deoxynucleotides, containing the two purine bases - adenine, A and guanine, G and the two pyrimidine bases - cytosine, C and thymine, T are linked by bonds joining the 5'-phosphate group of one nucleotide to the 3'-hydroxyl group on the sugar of the adjacent nucleotide to form 3',5'-phosphodiester linkages. In a typical DNA double helix, the base-pairs are located in a plane perpendicular to the axis of the helix, while sugar-phosphate backbones are wound in an antiparallel orientation along the periphery. The aromatic purine and pyrimidine bases are flat and stack above one another. The base stacking is stabilized mainly by London dispersion forces.[4] The two chains of the double helix are held together by specific hydrogen bonds between the nucleobases.

The glycoside bonds that connect the base pair to its sugar rings are not directly opposite to each other and therefore, the two sugar-phosphate backbones of the double helix are not equally spaced along the helical axis. As a result, the grooves that are formed between the backbones are not of equal size; the larger groove is called the major groove and the smaller one, the minor groove (Figure 1).

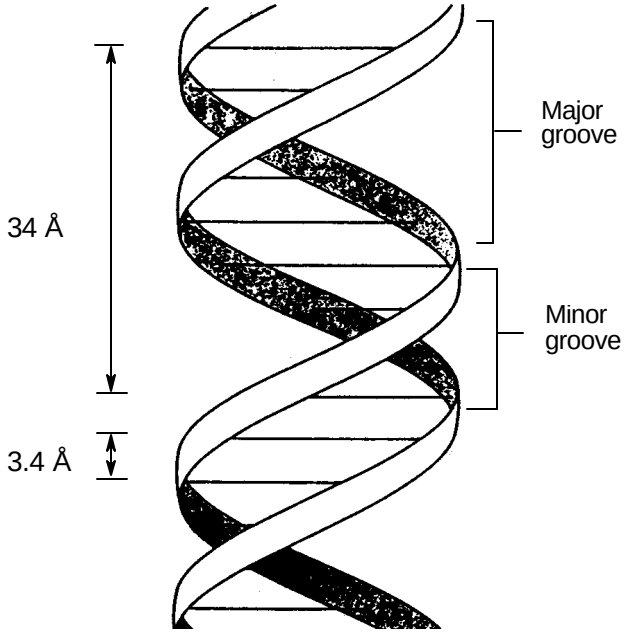


Figure 1. The ribbon model of B DNA. The two ribbons represent the sugar phosphate backbone and the horizontal lines the base pairs, the rise per base-pair 3.4 Å is also shown.

There are several structural families[5,6,7,8] of double stranded DNA of which the B-form, a right-handed helix with 10.5 base pairs per turn and a rise of 3.4 Å per base pair, is believed to be the predominent conformation in living organisms. The major groove of DNA is quite wide, exposing an array of functional groups on the edges of the bases. The floor of the major groove is filled with base pair nitrogen and oxygen atoms, which project inward from their sugar-phosphate backbones toward the center of the DNA. The minor groove is narrower and exposes a limited array of functional groups. Although the B-form structure provides a rough picture of the DNA double helix, X-ray diffraction[9,10] and NMR studies[11,12] have revealed substantial local deviations from this structure for many DNA sequences. The DNA structure is sequence dependent; for example, rungs of adenosines lead to a very narrow minor groove with a high propeller twist of base pairs. Mixed sequences have a more normal groove width but varying local parameters.[8]

1.2. REVERSIBLE INTERACTIONS OF DNA

The structure of the double-stranded DNA as described above, allows it to have a number of reversible interactions through electrostatic, groove-binding or intercalation mechanisms (Figure 2) with other molecules such as proteins, drugs, metal ions etc. Such reversibility of interactions is important in regulation of biological functions.

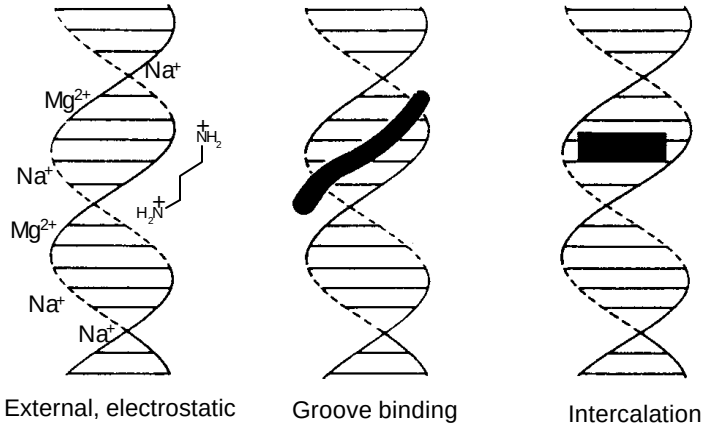


Figure 2. The three primary binding modes seen in DNA.

1.2.1. Intercalators

Generally polycyclic, aromatic or heteroaromatic flat molecules, insert themselves in between the base-pairs (intercalation) and stack within the adjacent bases.[13,14] The driving forces for intercalation are base-stacking and charge-transfer interactions. Electrostatic binding also plays an important role as most structures are equipped with positively charged side chains. Intercalation causes the base pairs to separate vertically, thereby distorting the sugar-phosphate backbone and increasing the pitch of the helix.

1.2.2. Groove-Binders

The general concept of groove binding is shown in Figure 2. Netropsin is a typical molecule that interacts with nucleic acids by this mechanism.[15] The major and minor grooves differ significantly in the electrostatic potential, hydrogen-bonding characteristics, steric effects and hydration. Many proteins and oligonucleotides exhibit binding specificity primarily through major groove interactions while small molecules prefer the minor groove. Typically, minor groove binding ligands have small aromatic rings e.g., pyrrole, furan or benzene, connected by bonds with considerable degree of torsional freedom.[15]

1.2.2.a. Minor groove binders

Dickerson and co-workers[16] have solved the crystal structure of netropsin bound to the DNA duplex d(CGCGAATTCGCG). This structure has thrown much light on the molecular details of the complex formation in the minor groove. Most small molecules bind in the minor groove as it is possible to have a "snug" fit, with good contacts to the wall of the groove.[17] Many minor groove binding ligands interact with AT sequences on account of their narrower minor groove, and because there would be steric clash with the 2-amino group of guanine. For example, netropsin binds to the AATT region of the duplex displacing the spine of hydration in the specified region. Other examples of groove binders include distamycin, berenil etc.

1.2.2.b. Major groove binders

A majority of sequence specific proteins and enzymes bind in the major groove. It is wider and more accessible and its potential for specific hydrogen bond recognition is much greater. In addition the dimensions of the major groove are very similar to that of an alpha helix and a good fit is eaily possible.

1.3. DNA TRIPLE HELIX

Alexander Rich, David R. Davies and Gary Felsenfeld first discovered[18] the triple stranded form of DNA and RNA in 1957 which seemed to be an interesting anomaly of double helical DNA, without much physiological or practical importance. The triplexes formed with synthetic polynucleotides, remained an obscure part of DNA chemistry until 1987 when it was realized that they offered a means for designing DNA sequence specific agents.[19,20]

In order for a ligand to recognize a single sequence in a nucleic acid it should interact with a number of nucleotides or base pairs; this number depends on the size of the targeted nucleic acid. In the case of the human genome, statistical calculations[21] suggest a stretch of minimum 17 base sequence as a target for specific binding at a unique site. The only nucleic acid binding ligands that can achieve such a high degree of selectivity and specificity are the nucleic acids themselves. A few years after the discovery of the DNA double helix structure[1] triple helix formation was first demonstrated with polynucleotides.[22] It was shown that short oligonucleotides bind to duplex DNA to form a triple helix.[19,20] The basis for molecular recognition of double-stranded DNA by oligonucleotides was provided by polynucleotide studies. Thymine and adenine can form two hydrogen bonds with adenine already engaged in a Watson-Crick (WC) A:T base pair. Cytosine and guanine can also form two hydrogen bonds with guanine involved in a WC G:C base pair. However, cytosine N3 must be protonated, otherwise it results in only a single hydrogen bond. Figures 3b & 3c represent the triplets, and it is seen that the third base can form hydrogen-bonds referred to as 'Hoogsteen' and 'reverse Hoogsteen' in accordance with the original hydrogen-bonding scheme observed by Hoogsteen.[23]

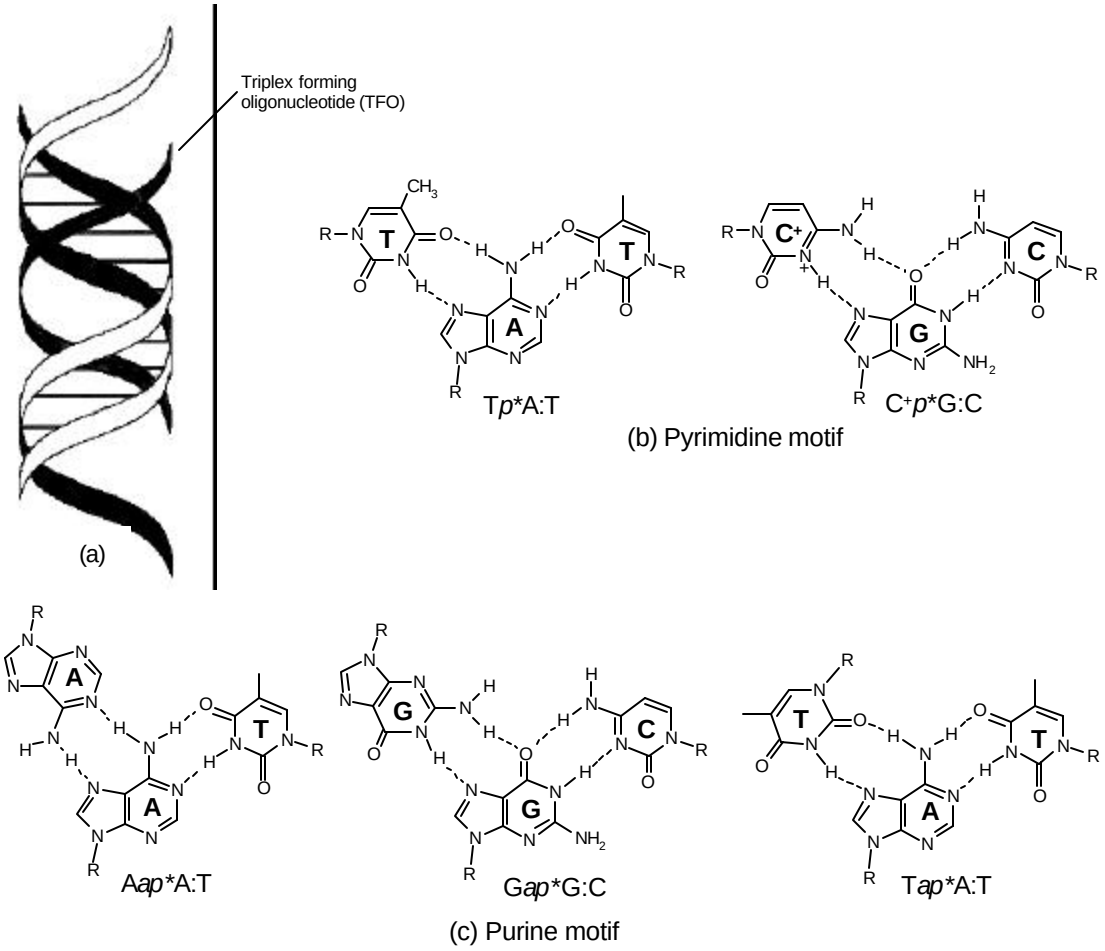


Figure 3. (a) The ribbon model demonstrating the relative position of a triplex forming oligonucleotide (TFO) in the major groove of DNA.
(b) The pyrimidine binding motif- the binding of a TFO in a parallel orientation (*p*) to the polypurine strand of DNA. The two canonical base triplets of this motif (a) involving protonated cytosine in the C^{+}*G:C triplet and T*A:T.
(c) The purine motif- the binding of TFO in antiparallel orientation (*ap*). The three canonical base triplets in this motif: A*p**A:T, G*G:C and T*p**A:T.
' * ' represents Hoogsteen hydrogen-bonds and ' **:** ' denotes Watson-Crick (WC) hydrogen bonds.

DNA triple helix formation results from the major groove binding of a third strand ODN that is either pyrimidine (**Y**)- or purine (**R**)-rich, in parallel (*p*) or antiparallel (*ap*) orientation respectively to the central purine strand as shown in Figure 4.[24,25] A purine-rich third strand binds in antiparallel orientation to the central strand, while a pyrimidine-rich third strand does so in a parallel orientation. The specificity in triplex formation is

derived from Hoogsteen (HG) hydrogen bonding. Thus, T recognizes A of A:T Watson-Crick base pair to form a T_p*A:T triad and protonated C binds to G of G:C base pair to give C_p^+*G:C in the pyrimidine motif (Figure 3b). Similarly in the purine motif, the third strand A binds to A of A:T, leading to a A_{ap}*A:T triad, while G binds to G of G:C base pair forming a G_{ap}*G:C triad in the reverse Hoogsteen mode (Figure 3c).

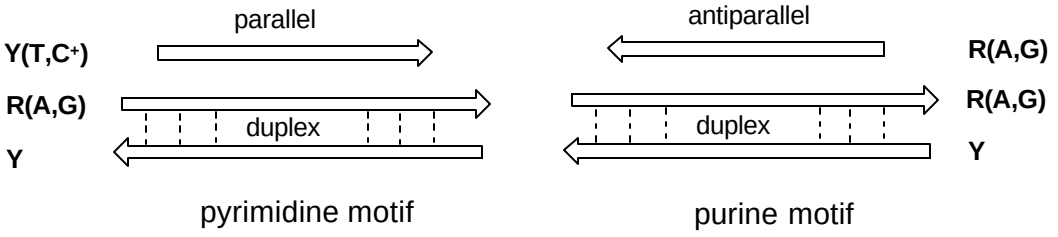


Figure 4. Pyrimidine and purine motifs of triple helix formation.

The widespread occurrence of polypurine/polypyrimidine tracts in eukaryotic DNA suggests that these sequences may have a biological function and consequently, therapeutic significance. The analysis of eukaryotic sequence databases reveals thousands of polypurine/polypyrimidine tracts, with potential for triple helix formation. Triplex formation obeys precise rules imposed by several structural constraints. For a given sequence of DNA it is possible to design therapeutic oligonucleotides (ODNs) that will specifically bind to it and thereby inhibit the gene expression. Different aspects of triple-stranded structures have been discussed in several reviews.[26,27,28,29]

The formation and stabilization of triplexes are achieved through several types of interactions. Usually, the changes in triplex stability are discussed in terms of electrostatic interactions between phosphate backbones and the number of hydrogen bonds between the duplex and the third strand. Other interactions like stacking, hydrophobic effects that also have varying contribution to the overall triplex stability are discussed briefly.

1.3.1. Electrostatic Forces

Electostatic interactions leading to the stabilization of triplex structures probably involve either the Debye-Huckel screening of the negative phosphate charges by positive counterions or by site speciifc neutralization of the phosphate charges by bound cations.[24,30,31]

The cations that neutralize anionic phosphate charges are effective in significantly different concentrations. Triple helices derived from natural polymers [(e.g. poly(A), poly(U), poly(T)] in presence of monovalent cation concentrations between 0.1-1M have been reported.[22,32] The concentrations necessary for divalent cations (e.g. Mg^{2+}, putrescine^{2+}) are in the range 1-10 mM.[22,33] The polyamines spermine and spermidine (bearing 4 and 3 charges respectively) are effective at concentrations of 0.5 μM to 5 μM.[18,33,34,35,36]

Sodium ions alone increase the melting temperature of duplex DNA.[37] However, in the presence of multivalent cations, increasing Na^+ concentration decreases the melting temperature down to a minimum value at a critical Na^+ concentration (10 and 100 mM Na^+ in the presence of Mg^{2+} and spermine respectively). Above the critical Na^+ concentration, the DNA melting proceeds as in cation-free solutions which was explained on the basis of counterion condensation theory.[38,39] This implies that mono and multivalent cations result in differential limiting neutralization of phosphates and when two different cations compete, their occupancies of the binding sites are proportional to their concentrations. The stabilizing efficiency of cations increases in the series $K^+ < Mg^{2+} <$ Spermine^{4+}, the effect of Spermine^{4+} being remarkable that its micromolar concentrations promote the formation of DNA and RNA triplexes.

Even though the problem of polyamine localization on double-stranded and triple-stranded DNA has not been solved, it is clear that polyamines can

electrostatically interact with phosphates and make hydrogen-bonds with the accessible groups of nucleobases. In addition to stabilization caused by phosphate neutralization, polyamines can tie the polynucleotide structure together when the opposite ends of the polyamines interact with different strands.

1.3.2. Stacking and Hydrophobicity Contributions

Experimental studies of mismatches and bulges in triplex structures show the general importance of stacking interactions.[40,41,42] The free energy penalty for introducing a single mismatch in triplexes ranges from 2.5-6 kcal/mole, which is close to the corresponding values for duplex DNA and RNA.[43] The dependence of the triplex stability on stacking interactions is also confirmed by the finding that a single abasic site, which eliminates two stacking interactions, result in a decrease in affinity similar to that observed for imperfectly matched natural triads.[44] Enhanced base stacking interactions of 5-methylcytosine (m^5C) with its neighbours is a likely source of triplex stabilizing influence of C-5 methyl.[45,46] The propyne modification in 5-(1-propynyl)-2'-deoxyuridine/cytidine is planar to the heterocycle and allows for increased stacking of the bases. Additionally, the propyne being more hydrophobic than a methyl group, it may allow for a further increase in the entropy of binding, there by stabilizing the triplexes. [47] Other hydrophobic groups and their influence are discussed in detail, later in this Chapter.

1.3.3. Hoogsteen Hydrogen-bonds

As discussed earlier, triplex formation occurs when a DNA or RNA oligonucleotide binds to a homopurine region of a DNA. The triplex forming oligonucleotides (TFOs) bind specifically in the major groove of DNA, forming

Hoogsteen or reverse Hoogsteen hydrogen-bonds. Several examples of the above binding modes and their effects are discussed in a later section.

1.3.4. Hydration Forces

Some insight into triplex stabilization principles can be gained by considering the hydration states of DNA or oligonucleotide strands. Spines of water were found in double-stranded DNA and were implicated in the stabilization of specific conformations and transitions between them.[48] Experimentally, the role of dehydration was illustrated[49] by the dehydration of poly(dG).poly(dC) in the presence of N-α-acetyl-L-arginine ethylamide leading to triplex formation.

In the case of triple-stranded nucleic acids, organized water molecules may play a significant role in differentiating the stabilities of G*G:C triad containing triplexes and those containing A*A:T and T*A:T triads.[50] Experimental NMR studies of hydration sites in Y*R:Y and R*R:Y type triplexes show the presence of water molecules in all the grooves of the triple helix.[51] The hydration states of various sequences in DNA and its affinity for multivalent cations may be relevant to the overall triplex-forming ability of R:Y tracts. The hydration effects are influenced by the nucleotide sequence, however, the available data do not bring out a specific hypothesis.

Thus, the formation and stability of triplexes is governed by several types of interactions. Triple helical order is due to a combination of electrostatic and stacking interactions, Hoogsteen/reverse Hoogsteen hydrogen-bond formations, hydration forces and hydrophobic interactions. The relative contributions are difficult to evaluate in a system. Sometimes, they can be cumulative and the lack of one contribution can be compensated by another.

1.4. OLIGONUCLEOTIDE MODIFICATIONS FOR ENHANCING TRIPLEX STABILITY

The DNA in the nucleus does not exist in a naked state and is always associated with proteins. In order for the triplex strategy to be effective, it requires oligonucleotides that effectively compete with ligands bound to DNA. Hence one of the major research goals is to achieve better triplex forming abilities with modified oligonucleotides. Triple helix stability can be enhanced by the use of modified nucleobases or by suitable backbone modifications or altering the sugar fragment (Figure 5).

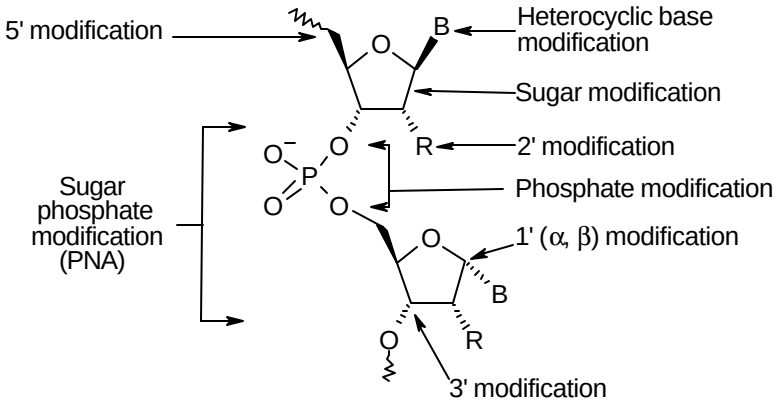


Figure 5. Structurally possible DNA modification sites.

1.4.1. Modified Nucleobases

Modification of the heterocyclic bases may enhance binding affinity with the complementary RNA fragment *via* WC bonding or with the duplex DNA *via* Hoogsteen bonding and may impart nuclease resistance. These modifications may have an impact on stacking interaction, hydrogen-bonding, donor/acceptor properties, pK_a, steric or electrostatic effects.

1.4.1.a. Stabilizing T*A:T and C^+*G:C triads

C-5 Substituted pyrimidines

Several C5 substituted pyrimidines (Figure 6) have been incorporated into ODNs and their triplex formation studied. The C5 or C6 positions lie in the major groove and hence should not affect the hybridization properties due to steric factors. C5 substitutions can change the pK_a of the N3 of cytosine and alter the hydrophobic driving force, base stacking and electronic complementarity of the base pairing for triple helix formations.[8]

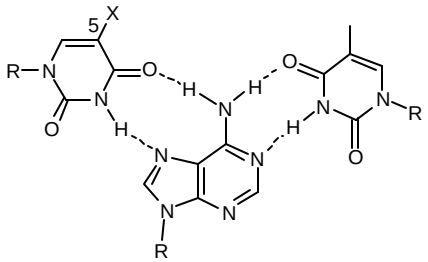


Figure 6. Y*A:T triad with various substitutions in the third strand pyrimidine. X = Br, F, I, $C{\equiv}C{-}CH_3$

5-Methylcytosine:

An electron-releasing substituent such as methyl would help the protonation of N3, which is thus favorable for triplex formation. The duplex stabilizing features of 5-methyl-C (m^5C) are well known.[8] Its effect in triplex formation was first observed in the complex of poly(dGdA) poly[d(Tm^5C)], which formed the complex even at pH 8 in contrast to its unmethylated analogue.[52,53]

Among the C5 substituted derivatives, m^5C has become an important structural element since it allows triplex formation over a wide pH range with enhanced stablity. Thermodynamic analysis has indicated that the additional stability imparted by m^5C is entropic in origin.[54,55] The methyl group also increases the molecular polarizability and

thereby the free energy of stacking, and may stabilize the complex through increased stacking interactions.

5-Halogenouracils:

C5 substitutions in uracil have two opposing electronic effects which influence the hydrogen-bonding ability of X^{5}U*A:T (Figure 6, X = halogen). Electron withdrawing substituents at C5 (F, Br, I) increase the acidity of the N3 amino (making it a better hydrogen-bond donor) but decrease the electron density on the carbonyl oxygen (making it a poorer hydrogen-bond acceptor). The utility of 5-halogenated uracils in triplex formation in parallel as well as antiparallel modes have been evaluated. With the ODNs having 5-Br-U, the relative stabilities of the base triplets were found to be in the following order: 5-BrU*A:T > T*A:T > U*A:T.[55] It was observed that in the antiparallel motif, 5-F-dU has slightly higher affinity for A:T base-pairs than T, and significantly better than 5-Br-dU and 5-I-dU.[56]

5-Propynyl-uridine/cytidine:

The logic of the favourable entropic and stacking interactions provided by m^{5}C has been extended to investigate the effect of 1-propynyl substituent at C5 (Figure 7a).[57] The propynyl group remains coplanar to the pyrimidine allowing an increased stacking of bases enhancing the enthalpic contributions. The functionality being more hydrophobic than the methyl, adds to the entropy component. Experimental studies

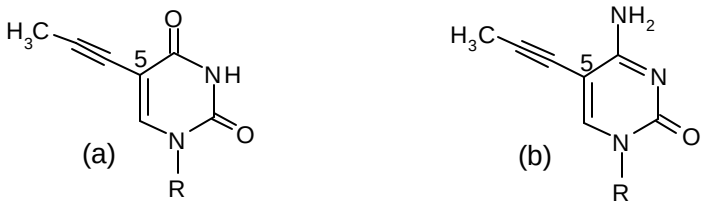


Figure 7. (a) 5-(1-propynyl)-deoxyuridine and (b) 5-(1-propynyl)-deoxycytidine.

with C5-propyne-2'-dU indicated a stabilizing effect relative to thymidine. However, C5-propyne-2'-dC (Figure 7b) destabilized the triple helix, probably due to a decrease in N3-pKa which adversely affected the protonation at physiological pH.

8-Amino-adenine/guanine:

Introduction of an amino group at the C8 position of A (*am*A) has shown remarkable stabilization of Hoogsteen-type base-pairing (Figure 8a) in triplex DNA.[58,59] Third strand binding to a 16-mer purine strand bearing 4 residues of *am*A showed an increased stability of 25°C compared to the unmodified ODN.

Theoretical and experimental studies of the effect of 8-aminoguanine (*am*G) on the stablility of triplexes was presented.[60] The substitution of G by *am*G in a triplex resulted in a high stabilization of the triplex ranging from 6-15°C per substitution. It was suggested that the observed stability is related to (i) the extra hydrogen-bond formed between 8-NH_2 of *am*G & C2=O of C (Figure 8b) and (ii) the favorable electrostatic interactions between the 8-NH_2 group and the anionic major groove. A comparative study of the triplex binding properties of ODNs carrying 8-aminoguanine and 8-aminoadenine was carried out.[61] With either of the 8-aminopurines the stability was greater than the unmodified ODNs, with a higher stabilization being achieved with the dG derivatives.

5-Methyl-2'-O-methyl-uridine/cytidine:

5-Methyl-2'-O-methyluridine (Figure 9a) RNA oligomer was shown to form a triplex which is thermally more stable than the corresponding DNA triplex.[62] The TFOs are chemically more stable and relatively nuclease resistant compared to the unmodified RNAs. Unexpectedly, the cytidine analogue, 5-methyl-2'-O-methylcytidine (Figure 9b) destabilized the triplex. This effect is similar to that observed with 5-propynylpyrimidines.

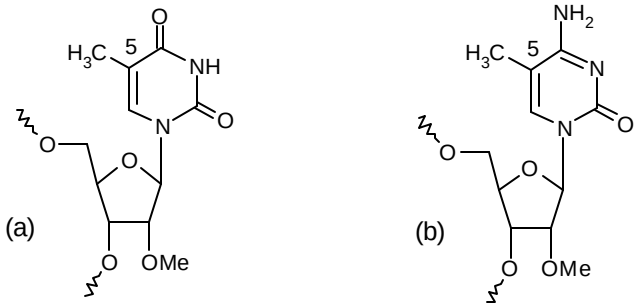


Figure 9. (a) 5-Me-2'-OMe-uridine and (b) 5-Me-2'-OMe-cytidine.

1.4.1.b. Mimics of protonated cytidine

Several base analogues have been synthesized in an attempt to overcome the necessity of cytosine N3 protonation. This requires the presence of two hydrogen-bond donors on the third strand base. The effects of replacement of protonated cytidine (C^+) by analogues having inbuilt hydrogen bond donors mimicking C^+ have been encouraging.

Pseudoisocytidine:

The design and synthesis of ODNs containing 2'-O-methylpseudoisocytidine (Ψ*i*C) which may form Hoogsteen type hydrogen-bonding with guanine in neutral and slightly basic conditions are reported.[63] As depicted in the Figure 10, Ψ*i*C bears a hydrogen atom for bonding with N-7 of guanine which enables the Ψ*i*C*G:C triad formation at neutral pH.

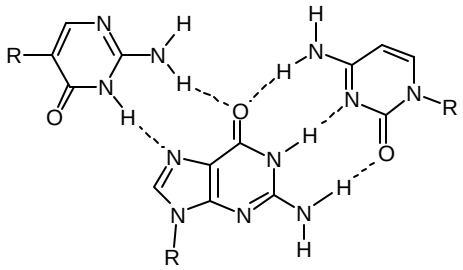


Figure 10. Recognition of Ψ*i*C by G:C in Y*R:Y motif.

8-Oxo-adenine:

Oligonucleotides with 8-oxoadenine were found to be a useful alternative to C or m^5C for use in triple helix formation at neutral and basic pH. Thus 8-oxoadenine in the *syn*-conformation could be a logical mimic of C^+ in a C^+*G:C base triplet where N7H and N^6H_2 of 8-oxo-dA would act like the protonated N3 and 4-amino of C^+ respectively (Figure 11).[64, 65] N^6-Methyl-8-oxo-2'-dA when present with thymidines in the ODN formed a triple helix in the Y*R:Y motif which is not only pH-independent but also superior to m^5C in conferring increased affinity under physiological salt conditions.[66] Formation of the triplex was supported by CD spectral studies, DNA footprinting etc. The oligopyrimidine containing this substitution prevented *in vitro* transcription.

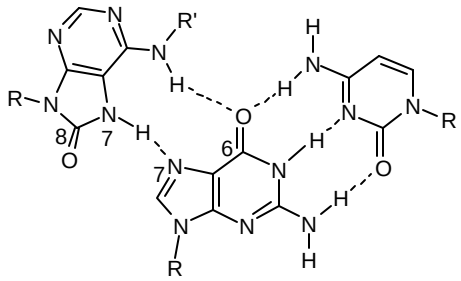


Figure 11. 8-oxo-dA recognizing an G:C duplet, R' = H or CH_3 in the Y*R:Y motif.

N7-guanine:

7-(2-Deoxy-β-D-erythro-pentofuranosyl)guanine (N7-G) upon incorporation into a pyrimidine ODN, binds to GC duplexes (Figure 12a) with remarkable specificity.[67] N7-dG is specific for G:C base pairs and the stability order of triplets containing this modified base is N7-G*G:C > N7-G*C:G > N7-G*A:T ~ N7-G*T:A. The glycosylation of the guanine at N7 position permits it to adopt a conformation such that the Hoogsteen face of the base mimics the arrangement of hydrogen bond donors similar to that seen in protonated cytosine.[68] Another N7-guanine analogue that lacks the rigid

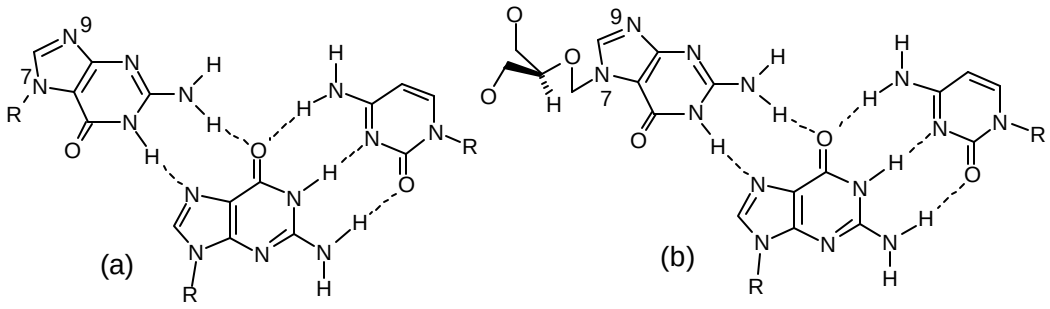


Figure 12. The recognition of G:C by (a) N7-G and (b) *a*N7-G in the Y*R:Y motif.

conformational features of deoxyribose sugar, 7-{[2-hydroxy-1-(hydroxymethyl)ethoxy}methyl}guanine (abbreviated as *a*N7-G) was incorporated into ODNs for studies with DNA triplexes.[69] By comparison, a sequence with five *a*N7-G residues was much more effective than that containing m^5C residues in targeting the sequence containing five adjacent G:C base-pairs (Figure 12b).

Hypoxanthine:

The nucleoside analogues 7-(2'-deoxy-α-D-ribofuranosyl)hypoxanthine (α^7H), 7-(2'-deoxy-β-D-ribofuranosyl)hypoxanthine (β^7H) and 7-(2'-methoxy-β-D-ribofuranosyl) hypoxanthine (β^7H_{OMe}) were prepared (Figure 13) and incorporated into TFOs, designed to bind to DNA in the parallel motif.[70] Among these, β^7H (Figure 14a) was shown to recognize a G:C base pair with high selectivity.[70] Although triplex binding of third strands with α^7H was inferior in stability, β configured nucleosides (β^7H & β^7H_{OMe})

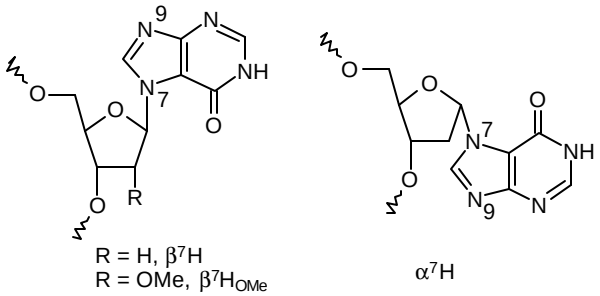


Figure 13. The three N7-hypoxanthine ODNs.

had almost the same stability as that of the control ODNs. The 2'-OMe showed a slight destabilization and the studies demonstrated that recognition of the target duplex by a base residue with one hydrogen-bond can be achieved without losing essential binding energy relative to known, stable models that rely on bidentate base-pair recognition schemes.[70]

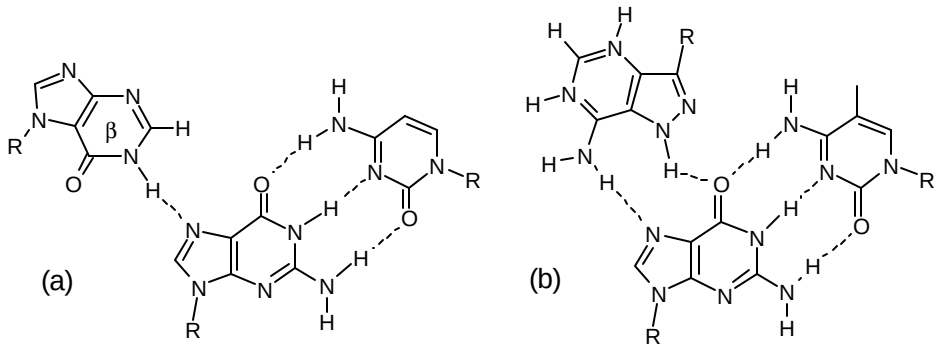


Figure 14. (a) 7-(2'-deoxy-β-D-ribofuanosyl) hypoxanthine recognizing G:C in Y*R:Y motif
(b) Recognition of G:C by 2'-deoxyformycin A in R*R:Y motif.

2'-Deoxyformycin:

Formycin A is a naturally occurring nucleoside antibiotic with structural similarity to adenine. The C-glycosidic bond makes it capable of donating two hydrogen bonds (Figure 14b). Introduction of 2'-deoxyformycin A in a 36-mer ODN resulted in an increase in binding affinity as compared to the unmodified ODN.[71]

3-Methyl-5-amino-1H-pyrazolo-[4,3-d]pyrimidine-7-one (P):

Another nucleoside which mimics protonated cytosine is 3-methyl-5-amino-1H-pyrazolo-[4,3-*d*]pyrimidine-7-one (P) as shown in Figure 15.[72] The results of DNase footprint experiments demonstrated a dramatic effect of sequence composition on triple helix formation by P-containing ODNs. The isomeric nucleoside P was specific for G:C duplets. The decreasing order of duplex recognition was P*G:C > P*C:G > P*A:T ~ P*T:A.

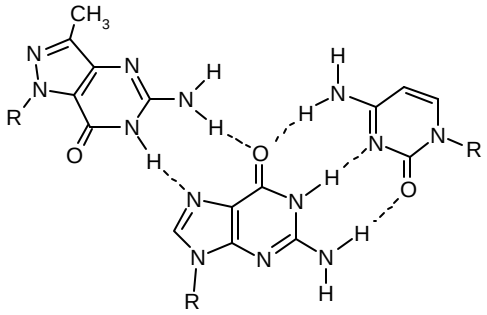


Figure 15. Recognition of G:C by P in the Y*R:Y motif.

6-Oxo-5-methylcytidine:

Another pyrimidine modification in the Y*R:Y motif consists of the 6-keto derivative of 5-methyl-2'-deoxycytidine (Figure 16a) which recognizes G:C base pairs in a wide pH range of 6.5-8.5.[73] This can be considered as an analog of a N3 protonated cytosine, which bears two hydrogen bond donors through its N3H and N^{4}H (Figure 17). The Tm difference between 6-oxo-m^{5}C and m^{5}C is only 2^{o}C in contrast to a difference of 20-25^{o}C between C and m^{5}C.

At pH 8.0-8.5, the derivative is more stabilizing than C and m^{5}C. Similar hydrogen-bonding patterns can be observed in 6-oxo-C$_{OMe}$ and 6-oxo-m^{5}C$_{OMe}$ (Figure 16b). Both 6-oxo-C$_{OMe}$ and 6-oxo-m^{5}C$_{OMe}$ when employed as the third base result in pH independent triple helix formation.[74]

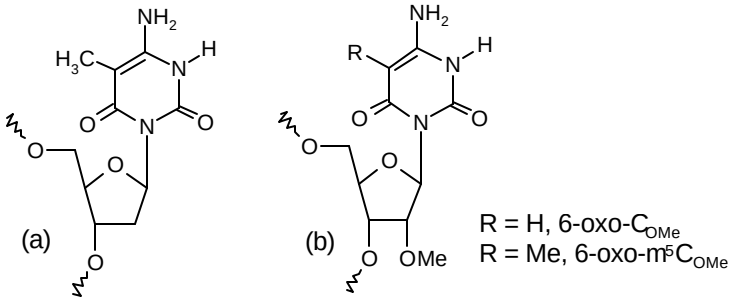


Figure 16. (a) 6-keto-5-methyl-2'-deoxycytidine and
(b) 2'-OMe derivatives of 6-keto-5-methyl-deoxycytidine.

4-Guanidinocytidine:

The 4-guanidino cytidine was designed[75] with an aim that in this particular geometry the targeting residue is shifted away relative to the known Y*R:Y and R*Y:R triple helix families to become equidistant from the strands of the target duplex. As a consequence, molecular recognition of G:C base-pairs is achieved through the α-isomer (Figure 17b).

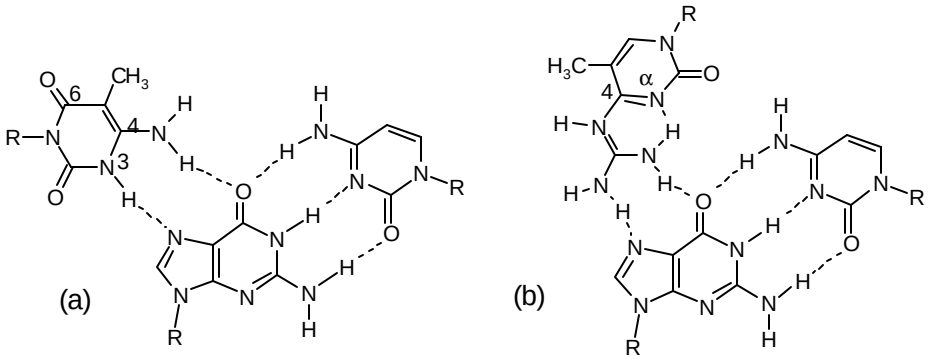


Figure 17. (a) Recognition of G:C by 6-Oxo-m^{5}C in the Y*R:Y motif and (b) recognition of G:C by α-isomer of 4-guanidinocytidine.

Pyrazine:

The pyrazine riboside presents similar hydrogen-bonding patterns as protonated cytosine (Figure 18). The UV-Tm results supported triple helix formation independent of pH in the range 6.3 to 8.0, the pyrazine triplet showing an enhanced stabilization over cytosine ($\Delta T_m = 10^oC$).[76]

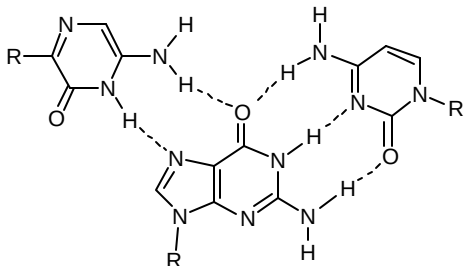


Figure 18. Pyrazine recognizing G:C in the Y*R:Y motif.

2-Aminopyridinylnucleosides:

Substituted pyridine analogs have been incorporated with an aim to mimic protonated cytidines. Examples include ODN containing 2-aminopyridine-5-yl and 2-amino-3-methyl pyridine-5-yl (Figure 19) which have considerable higher binding affinities for the target duplexes from the reference ODNs with C and m^5C,[77,78] clearly illustrating DNA duplex recognition at physiological pH through the Y*R:Y motif. However, 2-amino-2'-O-methyl pyridine-5-yl derived ODNs show distinctly less stable triple helices.[79]

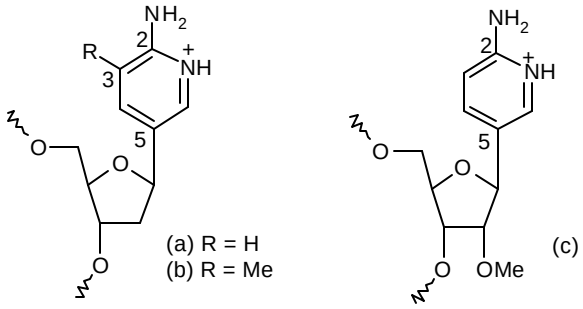


Figure 19. (a) 2-aminopyridine-5-yl (b) 2-amino-3-methyl pyridine-5-yl and (c) 2-amino-2'-O-methyl-pyridine-5-yl containing ODNs.

1.4.1.c. T mimics for stable T*A:T triplets (A:T recognition)

8-Oxo-guanine:

The incorporation of 8-oxo-dG into the third strand exhibited both high thermal stability and cleavage yields like those of the triplex containing G*A:T triplet.[80] This stability may be arising from two hydrogen bonds (i) one involving O^6 of 8-oxo-dG and the 6-amino of dA and (ii) the hydrogen bond between N7-H of 8-oxo-dG and N7 of dA (Figure 20).

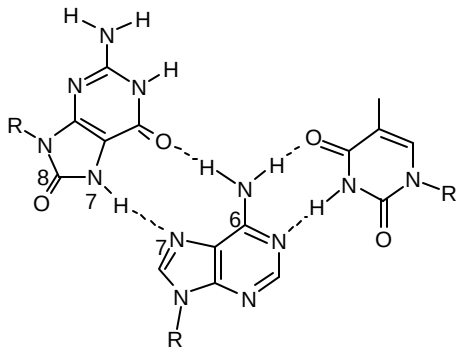


Figure 20. 8-oxo-dG recognizing an A:T duplet in the Y*R:Y motif.

N7-Deazaxanthine:

Xanthine (X) may be considered a true universal base, having an acceptor-donor-acceptor hydrogen bonding configuration. The nonadecanucleotides synthesized were found to have a low Tm and the effect surmised to be due to the negative charge present on X. Replacement of the N7 atom of xanthine with a carbon creates 7-deazaxanthine which increases the pK_a to 7.3 (Xanthine pK_a = 5.3).[81] It was found that ODNs containing 7-deazaxanthine formed triple helices in an antiparallel orientation with respect to the poly purine 15-base strand of the target DNA under physiological K^+ and Mg^{2+} concentrations at pH 7.2[81] and failed to form triplex above 50 mM K^+ concentration.[82] The fact that substitution of 7-deazaxanthine for T significantly increases the anti-parallel triple helix formation (Figure 21), under physiological conditions, may prove useful for the *in vivo* inhibition of gene expression by triple helix formation.

Guanine-rich ODNs have the potential to self-associate and form G-tetrads, which coordinate to K^+ ions. This is a serious drawback of G-rich sequences employed for intermolecular triplex formation. Using modified nucleosides such as 7-deaza-2'-deoxyxanthosine and 6-thioguanine, triplex formation should be facilitated due to

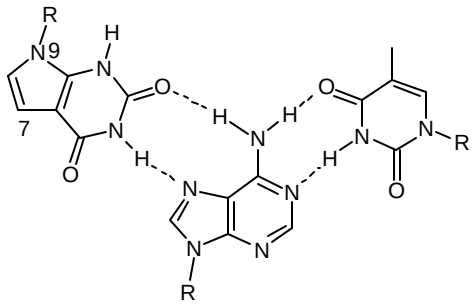


Figure 21. Recognition of A:T by N7-deazaxanthine in the R*R:Y motif.

resistance against guanine-tetraplex formation. In the absence of potassium, successful formation of triplexes were observed with ODNs containing 7-deaza or 6-thioguanine bases.[82]

2,7-Dioxopyrido[5,6-d]pyrimidine:

As shown in Figure 22, both the analogs of 2,7-dioxopyrido[5,6-d]pyrimidine nucleoside (M) recognize A:T base pairs resulting in pH-independent triple helix formation[83] (pH range 6.0-8.0). The M*A:T triplet is slightly less stable than the T*A:T triplet which is evident when the number of the T residues replaced with M are increased. The tautomeric forms possible in M allows it to bind in parallel and antiparallel motifs.[84]

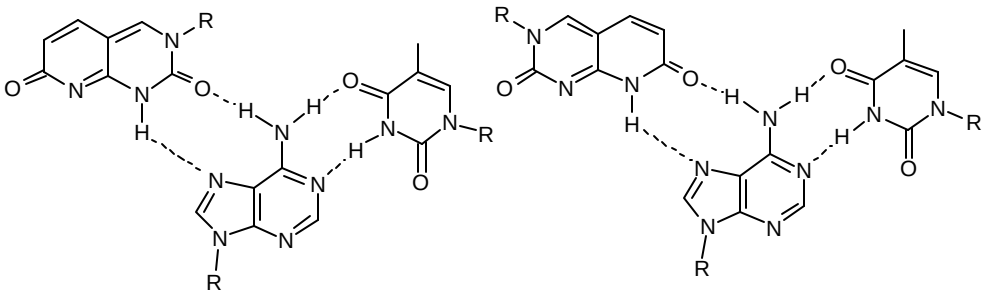


Figure 22. 2,7-dioxopyrido[5,6-d]pyrimidine nucleoside (M) recognizing A:T (a) in the Y*R:Y and (b) R*R:Y motifs.

1.4.1.d. Recognition of pyrimidine in the central strand

N^4-Alkylated cytidines:

The NH_2 of cytosine can be derivatized by a transamination reaction in presence of bisulfite. This reaction is selective with cytosine and does not happen with 5-methyl cytosine. The transaminated oligomers were found to be capable of forming triplexes with the target duplex, but were considerably more destabilized than those formed with an unmodified third strand oligomer. Thus, replacing 2'-dC with the N^4-substituted analogues, decreases the triplex stability considerably. The N^4-(3-aminopropyl)-2'-dC and N^4-(3-acetamido-propyl)-2'-dC nucleosides allow either of the 3-amino group hydrogens or the amide hydrogen to form hydrogen bond to the O^6-carbonyl group of the guanine (Figure 23). N^4-(butyl)-2'-dC and N^4-(carboxypropyl)-2'-dC failed to show triplexes. The lower triplex stability is attributed to the steric interactions between the sidechains and major groove substitutents and/or the different protonation degree of N3 at pH 7.0. [85]

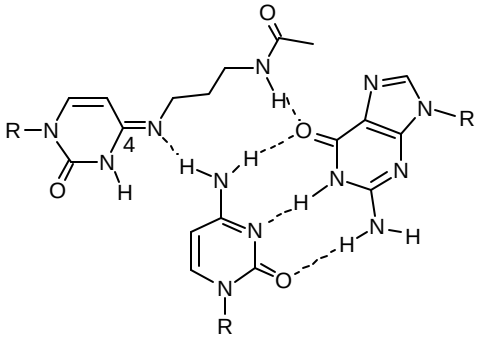


Figure 23. N^4-(3-acetamidopropyl)deoxycytidine recognizing C:G in Y*R:Y motif.

N^4-Pyridinyl cytidines:

The observation with the flexible side chain as discussed in the N^4-alkylated cytidines opened the possibility that further analogs with more rigid side chains might form better triplets. An analog with such a rigid side chain is N^4-(6-amino-2-pyridyl)C.

Triplexes of different stabilities were observed with the base-pair combinations. The base-pairs A:T and C:G in the complementary duplex (Figure 24) showed Tm values higher by 12-14°C than T:A and G:C. Surprisingly, the unusual imino tautomer (at C^4-N) provided the hydrogen-bond acceptor site which was evident from ^{1}H NMR and UV spectroscopic studies.

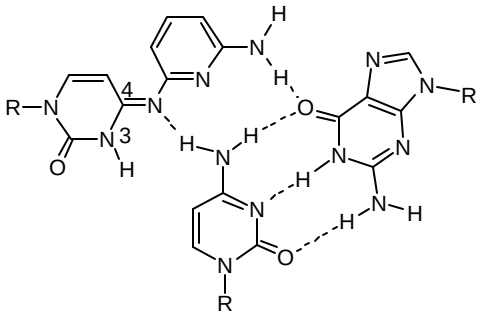


Figure 24. N^4-(6-amino-2-pyridyl)C recognizing C:G.

4-Phenylimidazole and 4-(3-benzamidophenyl) imidazole analogues:

The ODNs bearing base analogs 4-phenyl imidazole D2 and 4(3-benzamidophenylimidazole) D3 were synthesized[86] with the above logic. The single bond connecting the two aromatic rings allows rotational degrees of freedom for the non-natural base to adopt a favorable geometry for interaction with R:Y base-pairs. While D2 may recognize C:G with a single hydrogen bond, D3 spans both strands of the WC C:G base pair (Figure 25).

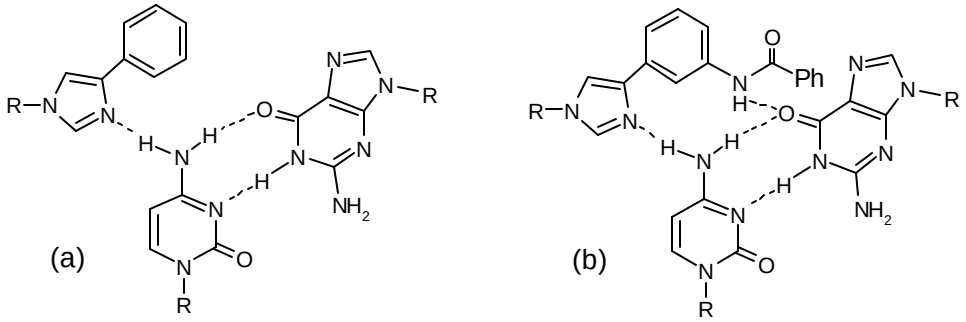


Figure 25. Recognition of C:G by (a) D2 or (b) D3 in the Y*R:Y motif.

D3 recognized both Y:R base-pairs (T:A and CG) in preference to R:Y pairs (A:T and G:C) in the Y*R:Y motif, giving rise to an expanded recognition code for D3*T:A and D3*C:G. The stabilizing order was D3*T:A ~ D3*C:G >> D3*A:T > D3*G:C. The differences in binding between D2 and D3 suggested that the benzamido group is responsible for the site-specific interaction. When this is replaced by acetamido, cyclohexane carboxamide or napthamide, diminished affinity and lower specificity are observed, suggesting a probable shape selectivity.

C6-Substituted purines:

A series of C6-substituted purines were analyzed[87] (Figure 26a) and it was found that the stability of the triplex decreased in the order X=O > X=N > X=CH_2. The CG inversion was stabilized more by the derived oligonucleotides where X=O.

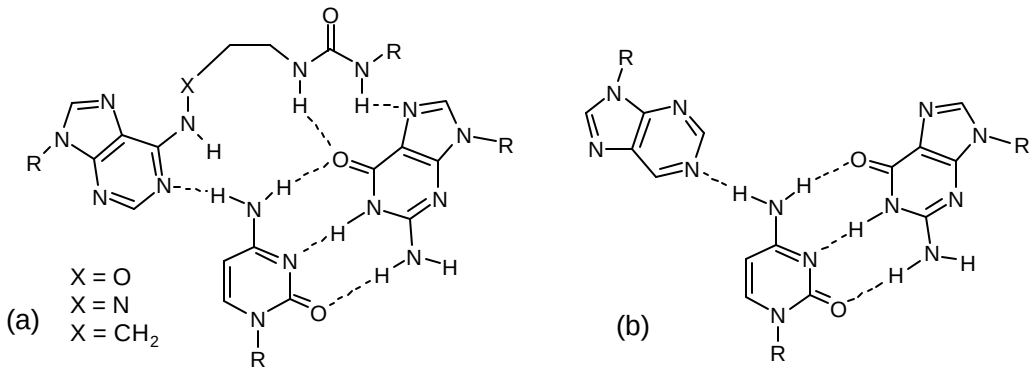


Figure 26. Recognition of C:G by C-6 substituted purines in the Y*R:Y motif and (b) recognition of C:G by N in R*R:Y motif.

2'-Deoxynebularine (N):

Nebularine, a naturally occurring nucleoside in certain mushrooms, was found to be a base with suitable acceptor-donor system. It was shown to recognize cytosine or adenine[88] of C:G or A:T WC duplexes through one hydrogen bond in the purine motif (Figure 26b).

The stability of deoxynebularine (N) containing triplexes at pH 7.4 decrease in the order N*C:G ~ N*A:T >> N*G:C ~ N*T:A. The stability of N*C:G is comparable in strength with that of T*C:G. The stability order was N*C:G > N*A:T > N*G:C > N*T:A. T is the only natural base that binds to some extent to C:G base pairs in the R*R:Y motif. However, it also interacts well with the A:T base pair. In contrast, N can bind with C:G as strongly as T, but shows a weak binding with A:T unlike T, making N more specific for C:G than T.

Azole derivatives:

Simple azole-2'deoxyribonucleosides have been proposed as non-specific base analogs for binding to pyrimidine interruptions at C:G or T:A in antiparallel triplexes.[89,90] The azole bases (Figure 27) were incorporated in anticipation to avoid steric clash with the inverted base pair. The triazole analog showed weaker binding to C:G while pyrazole showed no interaction with C:G, binding only to T:A and G:C.[91] The imidazole and tetrazole containing oligomers bind better to C:G base-pairs than triazole oligomers.

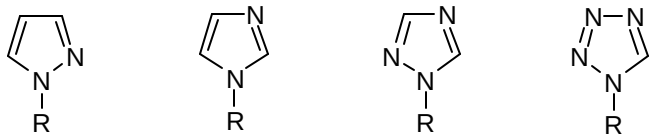


Figure 27. (a) Pyrazole (b) Imidazole (c) Triazole (d) Tetrazole-nucleosides.

4-Guanidinocytidine:

An alternative strategy for recognizing C:G inversions is to design base analogs which reach across the major groove, forming hydrogen bonds with the guanine. The natural bases cannot achieve this hydrogen bonding because of the large distortion required (3.0-5.0 Å). In this regard 2'-deoxyformycin A has been shown to stabilize antiparallel triplexes at C:G inversions by 10-fold compared with natural base

oligonucleotides.[92] Triplex formation using β-isomer of 4-guanidinocytidine capable of binding C:G was suggested[75] (Figure 28). This untested analog is proposed to have its phosphodiester backbone positioned in the center of the major groove, in contrast to most triplexes in which it is closer to the purine strand.

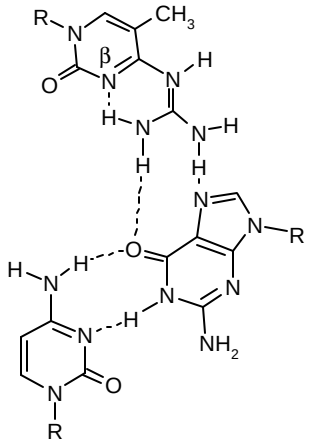


Figure 28. Recognition of G:C by β-isomers of 4-guanidinocytidine.

Benzimidazole-glycyl and 2-methyl-8 (N'-n-butyluriedo) naphth [1,2-d]-imidazole):

All natural bases and most of the synthetic base analogues used in triplex formation make use of specific hydrogen bonding contacts with one base (usually

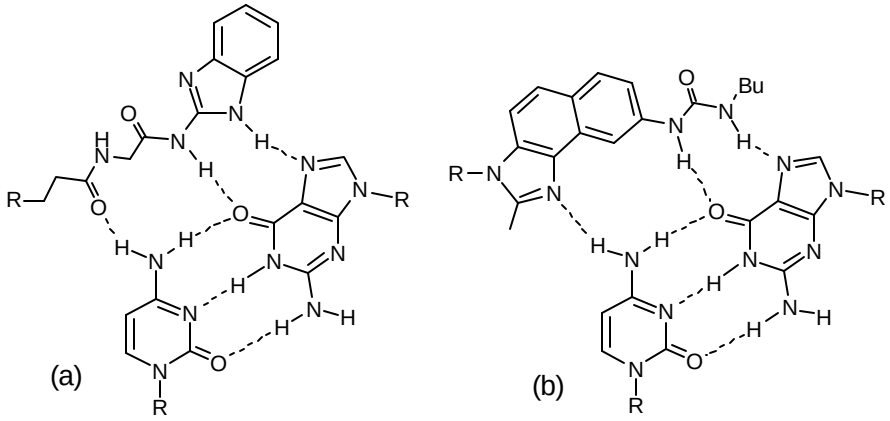


Figure 29. Recognition of C:G by (a) benzimidazole-glycyl- and 2-methyl-8 (N'-n-butyluriedo) naphth [1,2-d]-imidazole)-nucleosides.

purine) of the base-pair. As a result the recognition and the binding strength is weaker. Another strategy to improve recognition is the design of base analogues which form hydrogen bond contacts with substituents on both bases of the Watson-Crick base pair. Examples in this category include recognition of C:G base by benzimidazole-glycyl[93] and 2-methyl-8 (N'-n-butyluriedo) naphth [1,2-d]-imidazole) as shown in Figure 29.[94]

[(1H-Pyrrol-2-yl)carbonyl]-2-aminothiazole-5-carboxamide (L1) and 2-benzamidothiazole-5-carboxamide (L2):

Novel base analogues L1 and L2 have also been designed for C:G recognition (Figure 30).[95] These analogues is expected to form two and one hydrogen bonds respectively with C:G base-pairs. These nucleoside analogs likely share a common binding mode of sequence-specific intercalation.

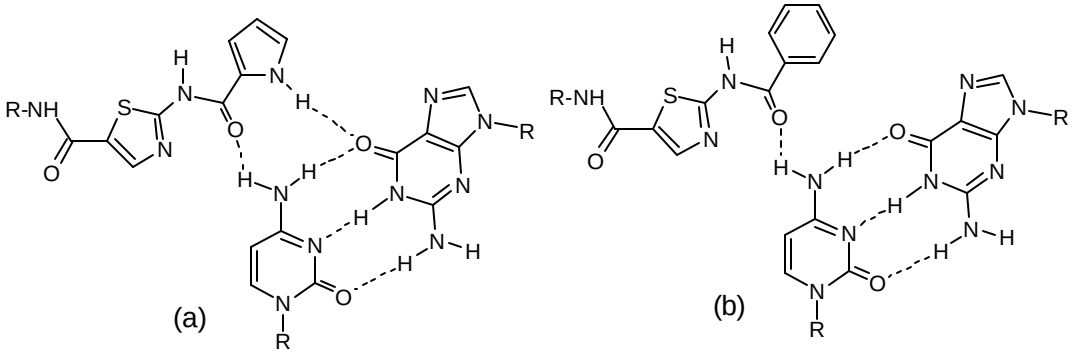


Figure 30. Recognition of C:G base-pairs by base analogues (a) L1 and (b) L2

5-Aminouridine

The effect of 5-aminouridine in the central strand of a DNA triple helix in parallel and antiparallel motifs has been studied.[96,97] The availability of one donor and acceptor site in U-NH_2 provides orientation specificity for recognition of the U-NH_2:A duplet by a third strand base in the major groove. The third strand base A is accepted by U-NH_2:A duplet in the parallel motif (Figure 31) while G is accepted as the third base in the

antiparallel motif. Triplex formation with 5-aminouridine is also a subject of current work discussed in a later part of this Thesis (Chapter 3, Section B).

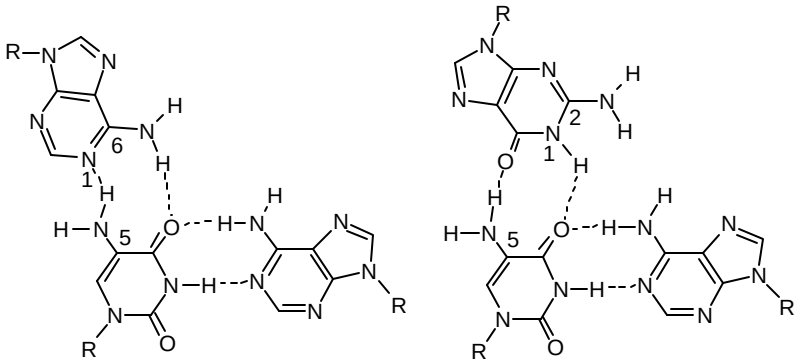


Figure 31. Recognition of U-NH_2:A by (a) A in Y*R:Y motif and (b) G in R*R:Y motif.

1.4.1.e. Enhancement of triplex stability by cationic appendages

C5-Norspermidine:

ODNs bearing 5-{4[N,N-bis(3-aminopropyl)amino]butyl}-dU at C-5 (Figure 32) were evaluated for their duplex and triplex hybridization studies.[98] The triamino group stabilized duplex and triplex of the heptadecadeoxynucleotides with a complementary strand and a target duplex, respectively. Further more, the ODNs were found to be more resistant to nuclease P1 and snake venom phosphodiesterase than the unmodified ODNs.

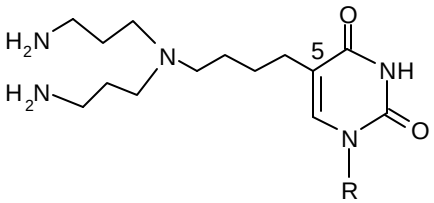


Figure 32. The C -5-polyamino deoxyuridine

2'-Aminoethoxy thymidine:

The properties of 2'-aminoethoxy modified ODN (Figure 33) with regard to triplex formation were also examined.[99] The ODNs offered high affinity, fast rate of association and high nuclease resistance. Extension of the spacer between the protonated amino group and the 2'-oxygen atom by one additional methylene group, 2'-aminopropoxy substituent, resulted in a significant decrease in triplex stablilty. This indicated that the protonated aminoethoxy side chain is ideally positioned to interact specifically with the nearby phosphate group, and that the enhanced triplex stability is not the result of a mere nonspecific electrostatic effect.

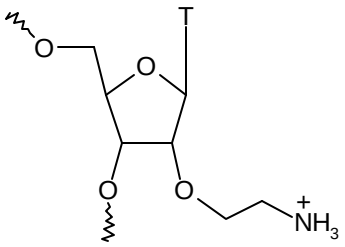


Figure 33. The 2'-aminoethoxy ODN

5-Propargylaminouridine

The effect of 5-(1-propargylamino)-2'-deoxyuridine (U^P, Figure 34a) on triplex formation was examined. It was demonstrated that the ODNs containing propargylamino-dU generate much more stable triplexes than those containing thymine and the difference was greatest at lower pHs.[100] Although the stacking effect may be important for the increased stability of U^P containing triplets, it cannot be the only factor since the propynyl-dU containing ODNs melt ~10°C lower than those with U^P.

A combination of the modification which stabilize triplexes individually, if employed in a single nucleoside should produce stable triplexes with more potential. An example includes the 5-aminopropargyl and 2'-amino-ethoxy modifications in a

thymidine analogue (U^{P}_{AE}, Figure 34b).[101] An ODN bearing two substitutions of U^{P}_{AE} showed an increased stability of 23°C relative to the U^{P} which contains two propargyl modifications. In this case the stabilization is due to the combined effect of the positive charge and the stacking of the propargyl function.

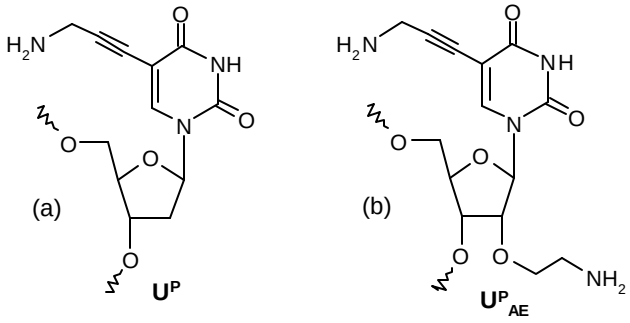


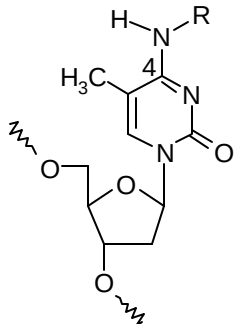
Figure 34. (a) 5-(1-propargylamino)-2'-deoxyuridine (U^{P}) and (b) 5-aminopropargyl-2'-amino-ethoxy-2'-deoxyuridine (U^{P}_{AE}).

N^4-Polyamino cytidines:

When N^4 of m^5C carried a polycationic species (Figure 35) such as spermine in the third strand, stable triplex formation was observed even at neutral pH.[102] Under identical conditions, ODNs carrying m^5C or C without any spermine conjugation did not form triple helices. Although the triplex Tm is slightly decreased with the increasing number of modifications, triplex formation was still observable with the trisubstituted ODN at physiological pH. The molecular origin of this stability could be due to the polycationic side chain, which decreases the net negative charge on the triplex by intramolecular charge neutralization and internal molecular recognition of the duplex by the spermine chain through hydrogen bonding.

Tetraethyleneoxyamine at the N^4 of m^5C[103] showed similar behavior as spermine conjugated at N^4 of m^5C. However, in this case with increasing number of modifications, the destabilization of the triplexes is to a lesser extent compared to the

spermine conjugated ODN. This is possibly because of the favoured N3 protonation of the N^4-tetraethyleneoxyamino analog which is unfavorable in the case of dC-sperminyl ODNs mainly due to the protonated sperminyl chain. In addition, the polyethylene linkage might provide a favourable microenvironment by hydrophobic desolvation, thus leading to a better triple helix formation.



R = $(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2
R = $(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$-NH_2

Figure 35. N^4 (sperminyl/tetraethyleneglycol amine)-5-Me-dC ODNs

5'-Polyamine conjugation:

The 5'-spermine conjugation[104] on a 21-mer homopyrimidine sequence was found to form stable triple helices under which conditions the reference ODN complex failed to form a triplex. The thermal stabilities of the spermine (3,4,3 Figure 36) conjugate was better than the related polyamine conjugates 3, 2, 3 or 3, 3, 3.

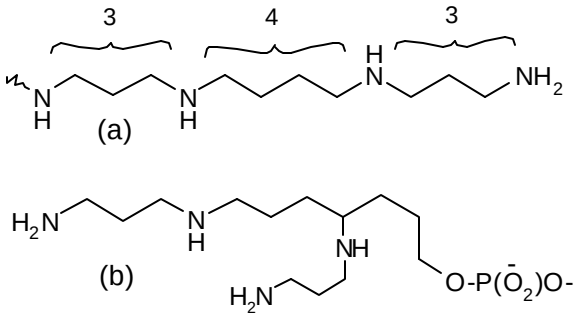


Figure 36. (a) spermine and (b) C-branched spermine.

C-Branched spermine:

The effect of C-branched spermine (Figure 36) on the triplex formation has been the subject of investigation.[105] Stable triplexes were observed in the case of these analogues at the terminals, while triplexes were not observed in the case of the middle derivatized analogue.

1.5. BACKBONE MODIFICATIONS

Early experimentation with unmodified oligonucleotides confirmed that the use of natural DNA as an antisense or antigene molecule had significant limitations. As an example, natural DNA or RNA oligonucleotides are rapidly degraded under physiological conditions by a variety of cellular nucleases that primarily hydrolyze the phosphodiester linkage. As a consequence, a significant number of the phosphate backbone modifications have been made.

Another obvious reason for the growth of backbone modifications is because it does not directly interfere with the key element of recognition i. e., Watson-Crick base-pairing. The backbone modifications can be broadly classified into two: (1) Phosphorus-containing linkages and modifications thereof and (2) non-phosphate or dephosphono linkages containing other heteroatoms.

1.5.1. Phosphate-Modified Linkages

Anionic linkages most closely resemble the native phosphate (PO, Figure 37a) in terms of structure and charge density, often referred to as first generation backbone modifications. The most widely studied and effective analogue in this group is the phosphorothioate (PS) derivative (Figure 37b).[106] Although first introduced into DNA enzymatically by Eckstein and co-workers[107] the phosphoramidite method has greatly facilitated the synthesis of these ODNs. The automated synthesis is nonstereospecific, giving a pair of diastereomers so as to generate 2^n diastereomers for n linkages. Stec

and Wilk[108] introduced the stereocontrolled synthesis of this analogue with a predetermined sense of chirality at each PS function. PS-containing ODNs show considerable retardation in the rate of hydrolysis by a variety of nucleases.

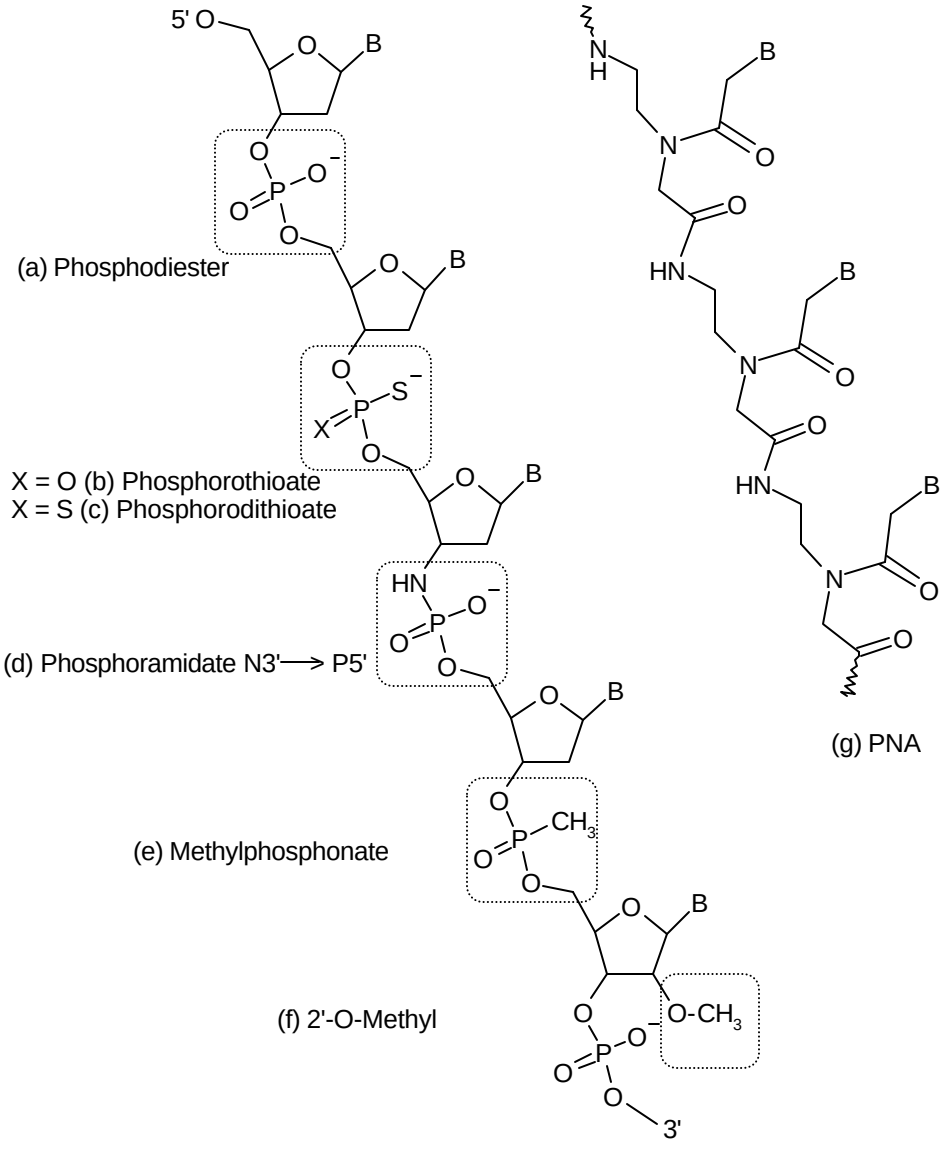


Figure 37. Different backbone modifications conferring stability towards intracellular nucleases and used in antisense/antigene strategies.

The substitution of both non-bridging oxygen atoms with sulfur gives rise to a phosphorodithioate linkage (Figure 37c), which like natural DNA is achiral at phosphorus. Phosphorodithioate (PS) ODNs are completely stable toward nuclease degradation, form duplexes with complementary DNA and RNA, and direct RNase-mediated degradation of RNA. Another analogue (Figure 37d), which has gained attention is the ODN with N3'-P5' phosphoramidate linkages. 3'-NH-P(O)(O-)-O5'-internucleoside linkages reported by Gryaznov and Chen.[109,110] The oligomers containing this modification form remarkably stable duplexes with complementary RNA and triplexes with duplex DNA.[111]

A common problem for all anionic analogs is the ineffective permeation of cellular membranes. Anionic ODNs are taken up in endosomes, but are unable to cross the endosomal membrane in the absence of cationic lipids. Based on this observation, several neutral isosteres of the phosphodiester linkage have been employed of which methylphosphonate oligomers are extensively studied. This derivative (Figure 37e), like the PS analogues, render the linkage chiral. Different diastereomers have shown affinity for RNA.[112]

Apart from methylphosphonates, there are a number of neutral ODN analogs and the most promising ones are discussed in this section. Sulfones,[113] sulfonamides[114] and sulfamates[115] have been prepared as DNA analogs. Replacement of the phosphate backbone by amide groups resulted in neutral and achiral linkages which showed good binding affinity to the RNA targets and high nuclease stability.[116,117]

Interestingly, several analogs having cationic internucleotide linkages have been synthesized. Cationic oligonucleotides having phosphoramidate linkages have shown interesting hybridization properties.[118] These cationic analogues, under appropriate conditions were found to bind more effectively than their natural counterparts. Oligonucleotide in which the backbone consisted of isomerically pure,

alternating (2-aminoethyl)-phosphonate and phosphodiester linkages have been prepared of which the *R*p isomer formed a more stable duplex with DNA or RNA than its corresponding natural oligonucleotide.[119] (Both modified ODNs formed duplexes with DNA and RNA with enhanced melting temperature.[120]) The synthesis of pentameric thymidyl deoxyribonucleic guanidine (DNG) in which the phosphodiester linkages of DNA are replaced by guanidinium linkages (-NHC(=NH_2^+)NH-) showed interestingly high stability of the DNG:DNA complex.[121]

1.5.1.a. Peptide nucleic acids

Peptide Nucleic Acids (PNA) constitute one of the most important backbone modifications of DNA where the entire sugar-phosphate backbone is replaced by a homomorphous, achiral, and uncharged peptide backbone based on N-(2-aminoethyl)glycine (*aeg*-PNA, Figure 37g).[122,123,124] Owing to the higher binding affinity of PNAs to the complementary DNAs and RNAs, these are considered as one of the most remarkable of the non-ionic oligonucleotide analogs. Oligopyrimidine PNAs were shown to bind to single- or double-stranded DNA, forming a 2:1 complex with the purine target sequence. In the case of duplex target, the complementary pyrimidine sequence is displaced, thus resulting in the formation of a P-loop structure (Figure 38). Strand displacement was also reported with an oligopurine PNA forming a very stable 1:1 complex with the oligopyrimidine DNA sequence.[125]

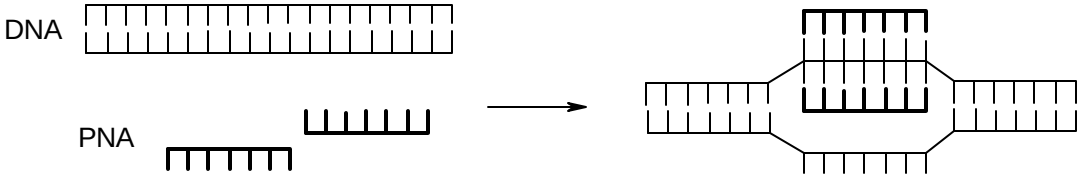


Figure 38. Duplex invasion by PNA forming PNA:DNA (2:1) complex.

The drawbacks of PNAs are their poor solubility in aqueous systems and they form both parallel and antiparallel duplexes with very similar stabilities. In addition, they are not suitable for some biological applications (e.g. PNA is not accepted as substrate for DNA polymerases, DNA kinases and so on). These drawbacks were overcome to a great extent by modifying the *aeg*-PNA or by employing a combination of PNA and DNA in one molecule resulting in PNA-DNA chimeras. These PNA-DNA chimeric (PDC) oligomers were prepared that contained thymine and cytosine PNA and blocks of normal nucleotides.[126,127] PNA-DNA chimeras show improved solubility in aqueous solution as compared to pure PNA.[128] The DNA part can induce a directionality for the chimera at the same time making the analysis and purification simple. Interestingly, PNA-DNA chimeras can also assume biological functions e.g. they can serve as primers for DNA polymerases and can mediate RNase H cleavage upon binding to RNA. Some interesting work on pyrrolidine PNA-DNA chimera is presented in this Thesis (Chapter 4).

1.6. SPECTROSCOPIC METHODS FOR STUDYING DNA DUPLEXES, TRIPLEXES AND DNA-PNA CHIMERIC COMPLEXES

A number of physicochemical techniques have been used to study the properties of duplexes and triplexes of nucleic acids. In the spectroscopic methods, UV absorbance and circular dichroism are very sensitive to the interactions of nearby bases which are stacked in the strands. The principles are outlined in the following sections.

1.6.1. UV Spectroscopy

UV-spectroscopy has been extensively used in studying the thermal stability of nucleic acids[129] by monitoring the absorption at 260 nm as a function of temperature. During this process, as the temperature is increased, structural transitions are

observed resulting from the disruption of hydrogen bonds between the base pairs which also get disturbed. Duplexes exhibit a single transition into single strands (melting) accompanied by an increase in UV absorption termed as *hyperchromicity*. According to the 'all or none model',[129] the UV absorbance value at any given temperature is an average of the absorbance of duplex and single strands.

Duplex melting: A plot of absorbance against temperature gives a sigmoidal curve in case of duplexes and the midpoint of the sigmoidal curve (Figure 39a) called as the 'melting temperature' (T_m) is the temperature at which the duplex and the single strands exist in equal proportions.

Triplex melting: In the case of triplexes, the first dissociation leads to the duplex (WC duplex) and third strand (Hoogsteen strand), followed by the duplex dissociation to two single strands.[28] The DNA triplex melting shows characteristic double sigmoid transition (Figure 39b) and the UV melting temperature for each transition is obtained from the

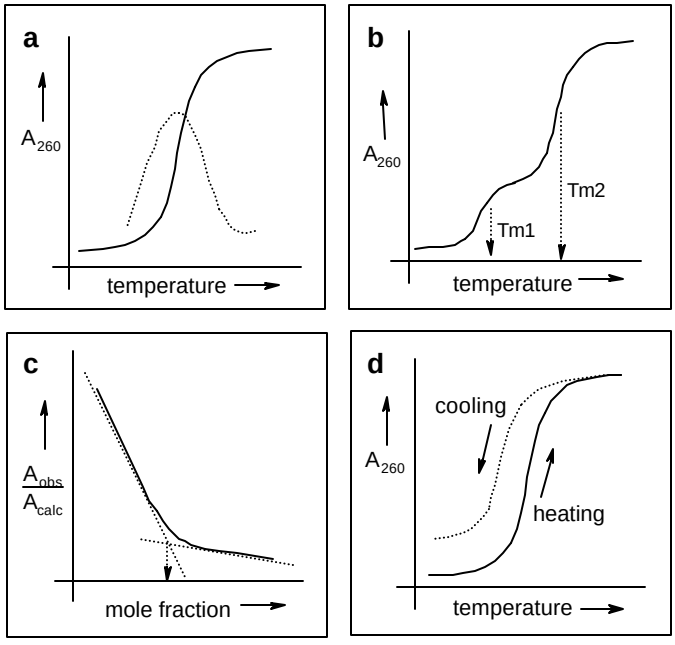


Figure 39. Schematic representation of **a** Duplex UV-melting (thermal stability), **b** Triplex UV-melting (thermal stability), **c** UV-mixing UV-titration (stoichiometry) and **d** Hysteresis (rate of hybridization).

first derivative plots. The lower melting temperature (T_{m1}) corresponds to triplex-to-duplex transition while the second transition gives the T_m of the duplex-to-single strand transition (T_{m2}). In the presence of DNA binding ligands, the T_m shifts to a higher or a lower temperature depending on the stabilization or destabilization induced by the ligand. The DNA binding ability of the polyamines and their corresponding guanylated derivatives (Chapter 1, Section A) have been investigated by such temperature-dependent absorbance experiments. A detailed analysis of the melting curves also enables evaluation of thermodynamic parameters such as enthalpy, entropy and free energy for the melting transitions.[130] The melting transitions of duplexes and triplexes constituted from the modified ODNs or the appropriate references were determined by the UV-T_m experiments throughout this Thesis.

Stoichiometry: The stoichiometry of complexation can be determined by UV-titration (Figure 39c). In this method, one of the strands involved in complexation is sequentially added in aliquots to a fixed amount of the complementary component and the UV-absorbance is recorded at each addition. Upon successive additions of the complementary strand, complex formation results in hypochromicity which leads to a progressive decrease in the ratio of the observed to the calculated absorbance. After the first strand present in the buffer is exhausted, the absorbance reaches a plateau in the plot of the ratio of the observed to the calculated absorbance against the nucleic acid mole fraction. The point at which the plateau is reached indicates the stoichiometry of complexation.

Hysteresis: DNA strands bearing charged groups can be tested for hysteresis by thermal dissociation versus re-association plots. The experiment consists of recording the UV absorbance by first heating the duplexes/triplexes (UV-melting) followed by cooling the sample while recording the absorbance (re-association, cooling curve). In general, for DNA duplexes or triple helical complexes, the cooling curve does not follow

the melting curve and exhibits a hysteresis (Figure 39d). This is due to the fact that the re-association of duplexes or triplexes is much slower than their melting due to the interstrand repulsion on account of the negative phosphate groups. When one of the strands bears cationic charges, the net repulsion between the two strands is reduced, leading to a lower hysteresis in the heating-cooling plots.

1.6.2. Circular Dichroism (CD) Spectroscopy

Circular dichroism[131,132,133] is a technique to study chiral molecules that have chromophores. In the case of nucleic acids, the sugar units of the backbone provide chirality and the bases attached to the sugars are the chromophores. In the CD spectrum of a polynucleotide with stacked bases, the magnitude of CD signals is larger in the 260-280 nm region and significantly higher at 200 nm than that of individual bases. The base stacking in a chiral fashion induces the coupling of CD transitions leading to characteristic patterns. Single stranded ODNs are structurally less well-defined than duplex ODNs and their CD signal is smaller. The CD pattern of the nucleic acid reflects the polymorphic forms of DNA such as A-, B-, and Z- forms. The CD signature of B-form DNA as seen from longer to shorter wavelength is a positive band centered at 275 nm, a negative band at 240 nm, with a crossover at 258 nm. A-DNA is characterized by a positive CD band at 260 nm that is larger than the corresponding B-DNA band, a fairly intense negative band at 210 nm. Naturally occurring RNAs and RNA-DNA hybrids adopt this polymorphic form. The left handed Z-form DNA shows a negative CD band at 290 nm and a positive band at 260 nm.

1.7. PRESENT WORK

The literature cited in this Chapter clearly highlights the importance of triplex forming oligonucleotides (TFOs), their selectivity and binding abilities. This area has

gained importance because triple helix formation provides a powerful tool for manipulating DNA.

There are many difficulties in using triplex DNA as a tool in molecular genetics. For example, it is often difficult to find homopurine stretches in the genome to provide stable triplex binding in pyrimidine motif. A single pyrimidine in a purine binding site (inversion) may lead to potentially destabilizing mismatches during triplex formation. Other challenges include the need for cytosine protonation in the pyrimidine motif, the susceptibility of G-rich sequences to form intramolecular complexes, and problems inherent to all oligonucleotide-based therapies such as reagent delivery, cellular uptake, target specificity and nuclease stability. Because of these issues a library of modifications have been carried out which includes nucleobases, backbone and sugar residues.

In order to get an in-depth understanding of triplex binding, this Thesis includes the study of polyamine analogues in triplex formation, the modifications on the nucleobases aiming at ODNs with better binding capabilities, and chimeric ODNs with the sugar replaced by a pyrrolidine ring.

Throughout this Thesis, we focus on DNA derivatives, and therefore the term "oligonucleotide" (ODN) is used to refer oligodeoxyribonucleotides, and not oligoribonucleotides.

Chapter 2

Model compounds of spermine and spermidine with their α,ω-primary amino groups transformed into the corresponding guanidinium functions (Figure 40) were synthesized from the parent polyamines spermine and spermidine. The effect of these bisguanidiniums on DNA duplexes and triplexes were evaluated using UV-Tm measurements and the results are discussed.

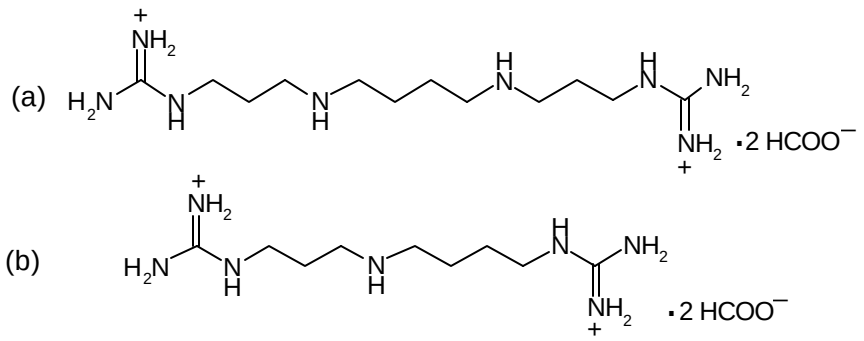


Figure 40. Bisguanidines of (a) spermine and (b) spermidine

Further, the synthesis of covalently conjugated guanidinium (at the N^4 of dC) containing oligonucleotides (ODNs) were targeted. The "convertible ODNs" containing O^4-dimethylphenyl residues were synthesized, which were functionalized into guanidinopropyl or guanidinobutyl derivatives by post-synthetic reactions to yield the N^4-propyl/butyl-guanidino-5-Me-dC bearing oligonucleotides (Figure 41) forming 'zwitterionic DNAs'. The functionalized ODNs were characterized, and used for biophysical studies.

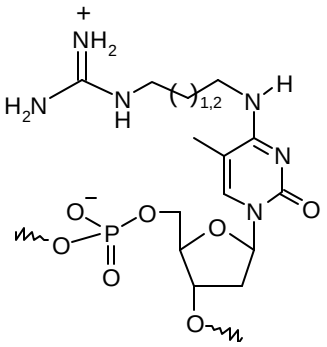


Figure 41. 5-Me-dC-N^4-alkyl guanidino ODNs.

Chapter 3

This Chapter describes the design and synthesis of a novel purine analogue 2-amino-6-sperminylpurine having a spermine moiety at the C6 of purine, suitably protected and incorporated into ODNs (**D**^{sp}, Figure 42a). The sperminyl ODNs obtained were characterized and analyzed for their triplex forming abilities at different pHs.

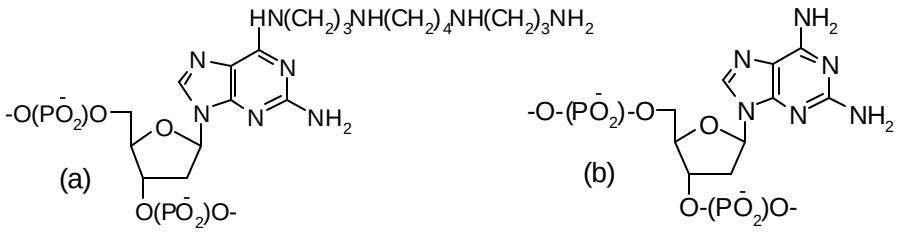
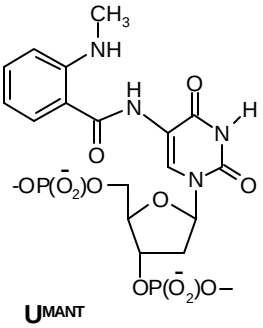


Figure 41. (a) 2-Amino-6-sperminyl-purine-2'-deoxy-ribonucleoside, $\mathbf{D}^{sp}$ and (b) 2-Amino-deoxyadenosine, **D.**

The ODNs bearing 2,6-diaminopurine (**D**, having an additional NH_2 at C-2 when compared to A) were used as the reference ODNs for the $\mathbf{D}^{sp}$ ODNs. In addition to this, studies using ODNs containing **D** as the central base or as the third base were carried out. All the possible triplex triads **X*****Y**:**Z** were generated by suitably designed ODN sequences. The results from UV-Tm are discussed in detail.

Synthesis of ODNs bearing N-methylanthraniloyl at the C5 of 2'-deoxyuridine ($\mathbf{U}^{MANT}$, Figure 43) was also carried out to generate fluorescent ODNs. The fluorescent properties of the derived ODNs are discussed.



$\mathbf{U}^{MANT}$

Figure 43. N-methylanthraniloyl-5-NH_2-dU, $\mathbf{U}^{MANT}$

Chapter 4

This Chapter discusses the design and synthesis of chimeric ODNs containing a pyrrolidine ring for replacement of the sugar phosphate backbone. For this, a suitable

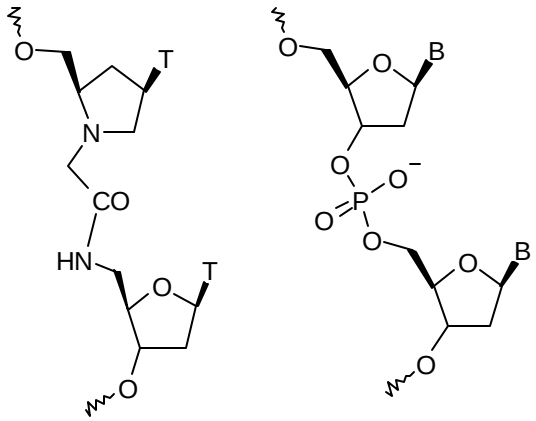


Figure 44. The structures of (a) chimera and (b) DNA.

dimeric unit (Figure 44) was chosen to have configurational equivalence to natural DNA. This corresponds to a D-*cis* configuration for the pyrrolidine unit. The chimeric ODNs synthesized using phosphoramidite chemistry were used for the DNA complementation studies. The hybridization results and nuclease resistance of these chimeric ODNs are outlined.

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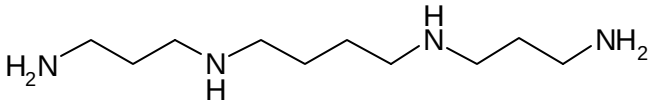
Chapter 2

Section A:

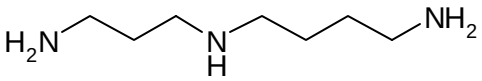
DNA Duplex and Triplex Stabilization by Bisguanidinyl Analogues of Spermine and Spermidine

2.1. INTRODUCTION

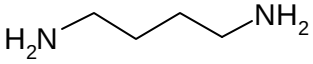
The polyamines spermine (1,12-diamino-4,9-diazadodecane, **1**), spermidine (1,8-diamino-4-azaoctane, **2**) as well as the precursor molecule putrescine (1,4-diaminobutane, **3**) (Figure 1) are polycationic compounds which are abundant in prokaryotic and eukaryotic cells.[1,2] Inspite of intense research in elucidating their precise role, no comprehensive understanding of polyamine action has emerged. The use of polyamine analogues as chemotherapeutic agents has gained importance in the last two decades, and a number of polyamine analogues have emerged as promising anti-cancer agents.[2] The ubiquitous biogenic polyamines such as spermine, **1** and spermidine, **2** have a multitude of functions *in vivo*, important in regulation of cell growth, proliferation, differentiation and stabilization of chromatin structure.[1] The polycationic nature of these polyamines is important for their cellular uptake, transport and for *in vivo* interaction with macromolecules such as enzymes and the polyanionic nucleic acids. Spermine is well known to stabilize DNA duplexes although the exact



Spermine, **1**



Spermidine, **2**



Putrescine, **3**

Figure 1. Structures of the natural polyamines: spermine (**1**), spermidine (**2**) and putrescine (**3**).

molecular details are not yet clear. The terminal ammonium cations may bind to anionic phosphates while the internal amino moieties may be involved in specific hydrogen bonding with nucleobases particularly with G and C in the major groove or A and T in the minor groove.[2,3,4] Spermine is well known to stabilize DNA duplex[5,3] and triplex[4,6] structures *via* specific recognition using both hydrogen bonding and electrostatic interactions. Since spermine and spermidine can carry 4 and 3 positive charges respectively, they bind strongly to negatively charged DNA by electrostatic interaction or to the nucleobases by hydrogen bonding and may influence the secondary structure.[7] The favorable effect of polyamines in DNA triplex formation at neutral pH has also been studied.[8]

Structural modifications of polyamines by variation in the number and distance of carbons between the nitrogens and/or terminal N-substitutions have been carried out. Novel analogues of polyamines have been synthesized for evaluation of biological activity involving chain modifications by insertion of aromatic groups[9] or additional methylenes[10] between the amino groups and modulation of charge distribution through introduction of pyridine/piperidine,[11] or anthracene-9-carbonyl terminals.[12]

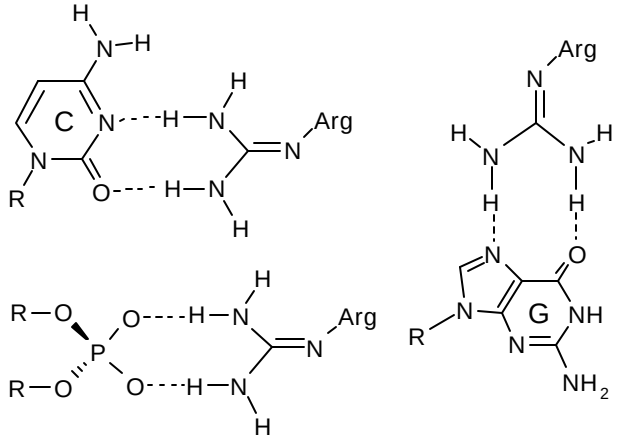


Figure 2. Examples of two point hydrogen bonding interactions between guanidinium group and nucleic acid backbone/nucleobases.[13]

The guanidinium group is most commonly utilized by proteins and enzymes to recognize and bind anions through ion-pairing and nucleobase hydrogen bonding (Figure 2).[13] The guanidinium group of arginine is the most polar of all the common amino acid side chains found in proteins.[14] The side-chain guanidine has a $pK_a \geq 13$ and in interacting with other macromolecules, the positively charged planar guanidinium group can be an efficient hydrogen donor in the formation of upto 5 hydrogen bonds (Figure 3a). Arginine plays a major role in the binding of negatively charged substrates, cofactors, and effectors to protein active sites.[15] In addition, it is often involved in the formation of singly or doubly hydrogen bonded salt bridges, usually with aspartate or glutamate side chains. The salt bridges stabilize the tertiary and quaternary structures of numerous proteins[13] and are an important mode of interaction between different proteins.[16] It has been reported that a specific type of binding to a guanidinium of an arginine residue allows recognition of nucleic acid loops and bulges. A motif termed as "The Arginine Fork"[17] is one where a guanidinium group recognizes phosphodiester linkages of two different phosphate groups that are close enough in space (Figure 3b).

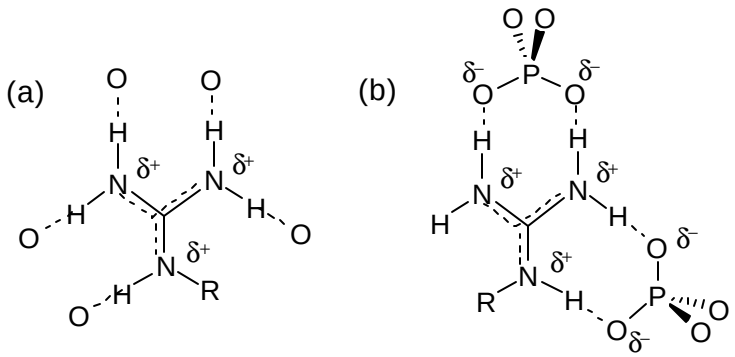


Figure 3. (a) Diagram of the planar protonated guanidinium group showing 5 hydrogen bonds to 5 different oxygen atoms (b) An example where the guanidinium is complementary to two phosphates forming "The Arginine Fork".

Amidinium and guanidinium groups also play a significant role in DNA minor groove binding of drugs such as netropsin, distamycin, pentamidine and propamidine (Figure 4). The specific patterns of hydrogen bonding and the high basicity of guanidinium groups (pK_a ~13.5) allow it to play several key roles in molecular recognition.

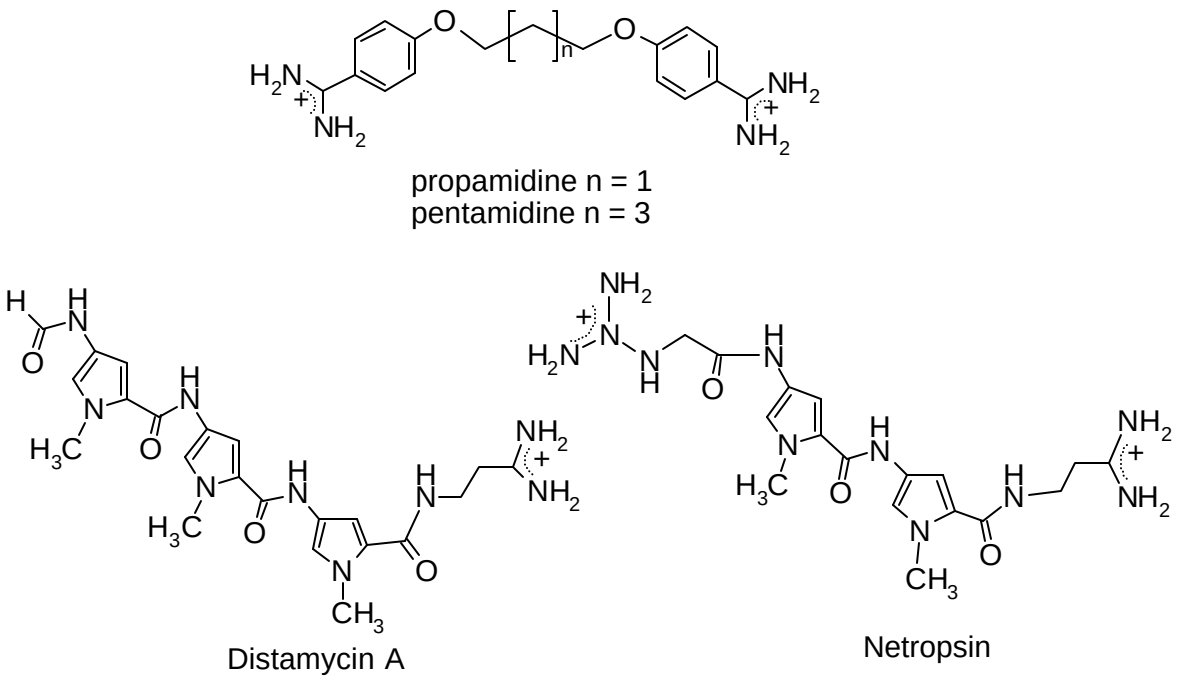


Figure 4. Examples of minor groove binding drugs.

2.2. OBJECTIVES

This Section aims at the synthesis of novel analogues of spermine and spermidine that possess highly basic guanidinium groups. The transformation of the primary amino groups in these molecules into the corresponding guanidinium functions [e.g. as in **6** (SPMG) and **12** (SPDG), Figure 5] was expected to amplify the electrostatic component resulting in improved binding to polyanionic DNA. Spermine and its guanidinyl derivative SPMG **6** differ from spermidine and its respective

guanidinyl derivative SPDG **12** in the number of methylene groups bridging the positively charged terminal amino/guanidino and secondary amino groups. The polyamines spermine, spermidine and their respective guanidinyl derivatives, however, bear the same ionic charge (+4 for spermine and **6**, and +3 for spermidine and **12**).

The objectives of this chapter are:

- Synthesis and characterization of the bisguanidines **6** and **12** (Figure 5).
- The evaluation of DNA duplex formation and triplex formation in presence of the synthesized compounds **6** and **12**.

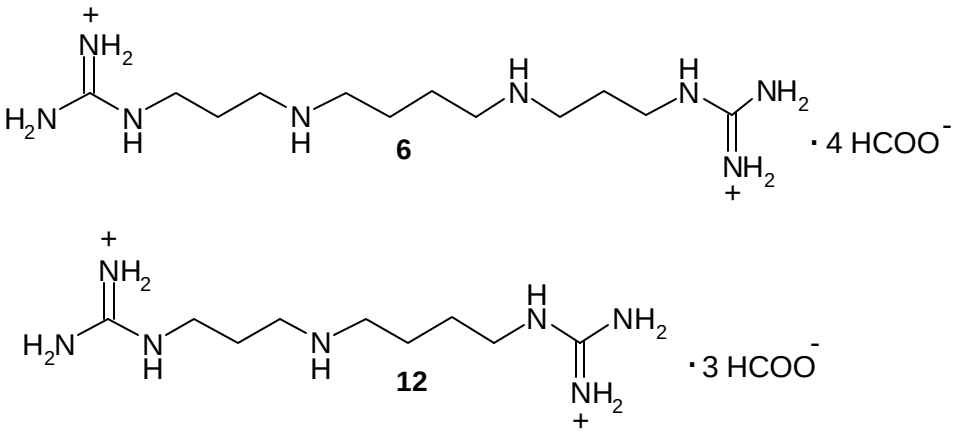


Figure 5. Bisguanidinyl derivatives of spermine (**6**) and spermidine (**12**)

2.3. PRESENT WORK

2.3.1. Synthetic Methods for Guanylation

Guanidinium compounds can be prepared starting from the corresponding primary amino derivatives by reaction with guanylating reagents. Variations of three general methods are typically employed for the synthesis of guanidines from amines (Figure 6). The simplest is the reaction of an amine with commercially available *S*-ethylthiouronium bromide or chloride,[18,19] or *O*-methylisouronium sulfate[20] which

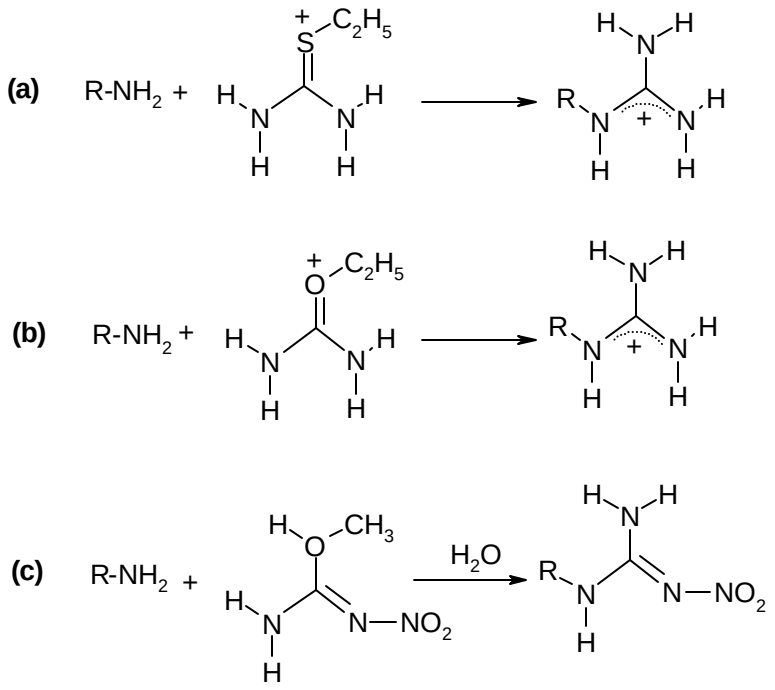


Figure 6. General methods for guanylation of amines.[18-21]

proceed with poor yields. These methods are ideal for the synthesis of monoguanidinium compounds, but when more than one guanidinium is desired in close proximity, these methods are not generally satisfactory. The problem perhaps arises from electrostatic repulsion caused by the formation of two positive charges at close quarters. The method of choice for forming guanidiniums in close proximity is to use 2-methyl-1-nitroisourea[21] (Figure 6c), which gives the nitroguanidines that lack charge. Nitro groups are versatile protecting groups for the guanidine function and can be removed by hydrogenolysis. Other guanylation methods involve the use of cyanamide,[22,23] aminoiminosulfonic acids,[24] 3,5-dimethylpyrazole-1-carboxamidine nitrate[25] or pyrazole-1-carboxamidine hydrochloride.[26,27]

The use of protecting groups other than nitro as part of guanylating reagents led to new reagents for the synthesis of protected guanidines. The use of carbamate protecting groups increases the electrophilicity of the amidino carbon relative to the

unprotected reagents. The examples (Figure 7) include N,N'-bis(*tert*-butyloxycarbonyl)- and N,N'-bis(benzyloxycarbonyl) derivatives of *S*-methylisothiourea,[28,29] N,N'-bis(tert-butyloxycarbonyl)- and N,N'-bis(benzyloxycarbonyl) derivatives of pyrazole-1-carboxamidine[27] or protected thioureas.[30]

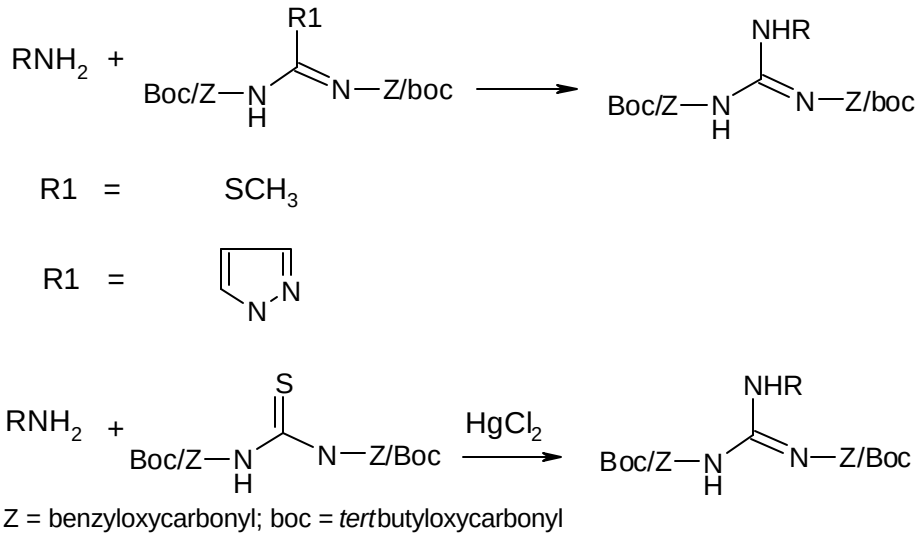


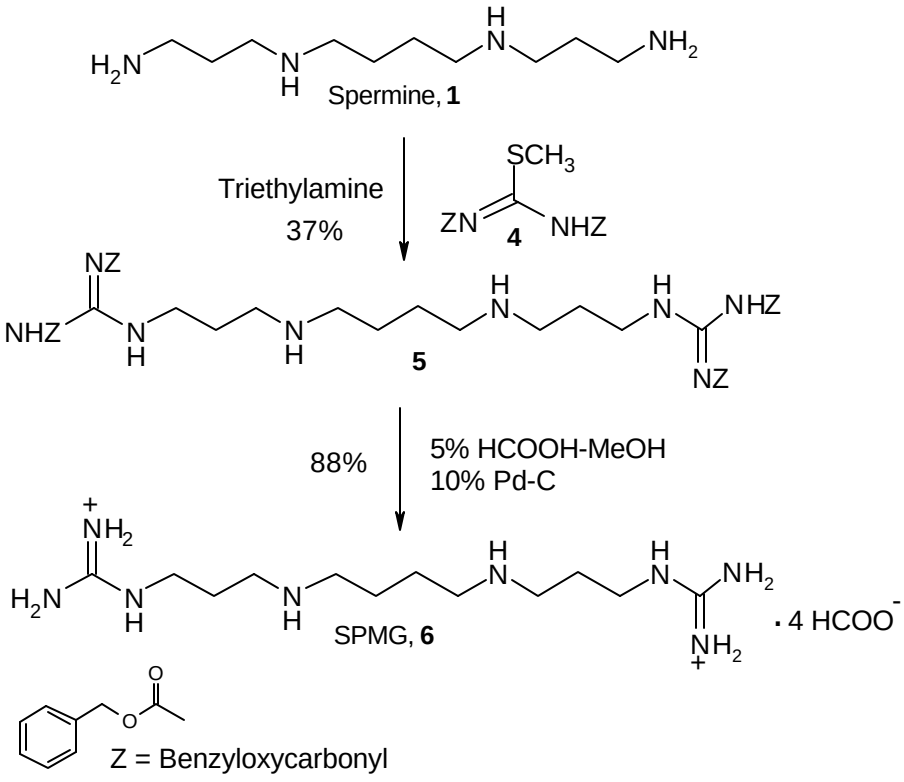
Figure 7. Methods for synthesizing protected guanidines.[27-30]

In the present syntheses, the common procedures mentioned in Figure 6a (or using pyrazole-1-carboxamidine reagent) by treating the free polyamines spermine or spermidine to yield guanidinium derivatives were not found suitable, because of the poor solubility of the reagents and/or products in addition to the isolation problems, and hence methods that yield protected guanidines such as N,N'-bis(benzyloxycarbonyl)-*S*-methylisothiourea[28,29] was chosen as the guanylating reagent.

2.3.2. Chemical Synthesis and Characterization of Bisguanidine 6

Spermine and spermidine have both primary and secondary amino groups and attempts to guanylate the terminal primary amino groups may also lead to reaction at secondary amine sites. The bisguanidine **6** was synthesized by a direct route (Scheme 1) without involving any protection of the polyamines. The guanylation reaction of

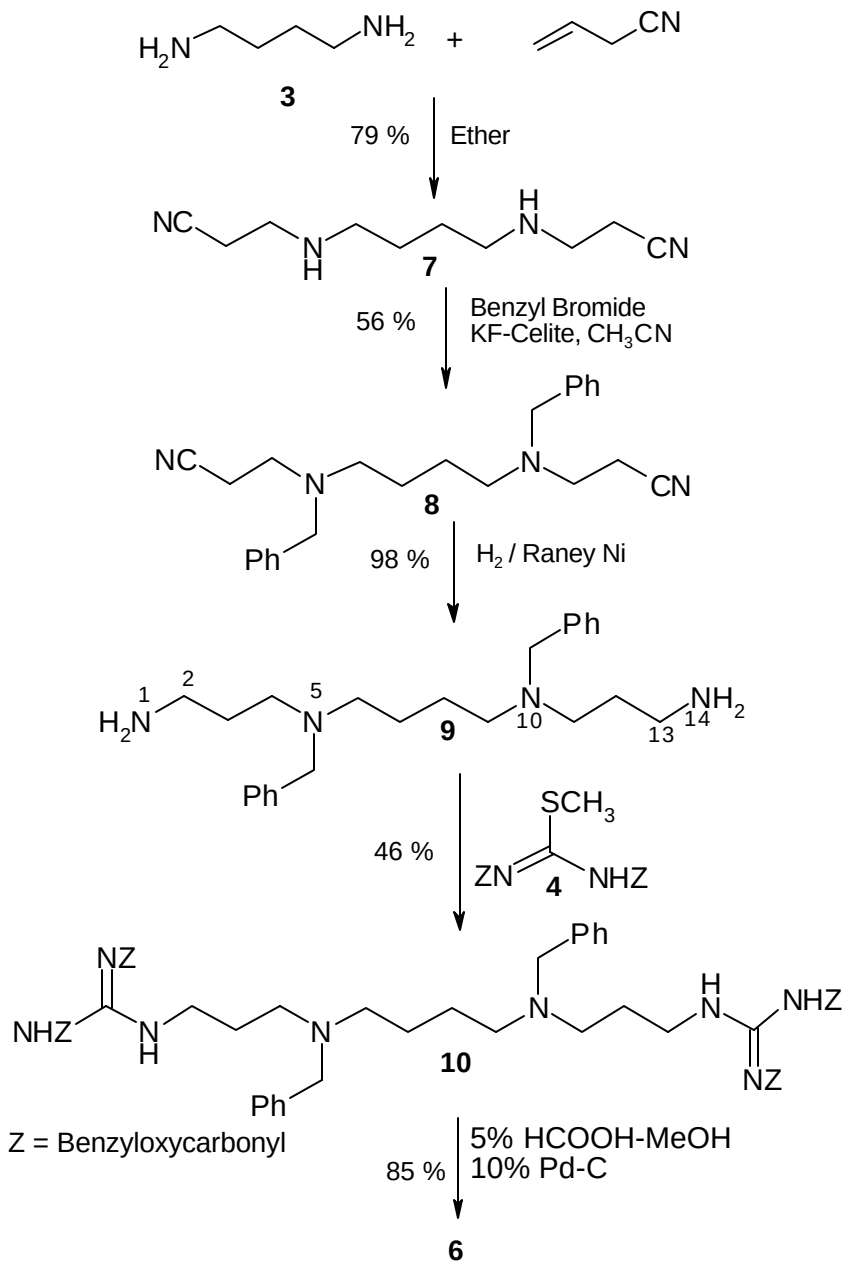
spermine was successfully carried out using *S*-methylisouronium sulfate, but the guanidinium product could not be precipitated out from the reaction mixture. Similar was the case when pyrazole-1-carboxamidine hydrochloride was used. However, N,N'-(bisbenzyloxycarbonyl)-*S*-methyl isothiourea,[28] **4** was found to be the most convenient reagent since it reacted almost exclusively with the primary amino groups of the polyamines and furnished the desired product in moderate yields. Further, there was no reaction when the reagent was treated with a secondary amine such as piperidine, showing the selectivity of the reagent to the primary amino function in the proposed synthesis. Spermine (SPM, **1**) was treated with the reagent **4** in dichloromethane at reflux in presence of triethylamine to yield the corresponding benzyloxycarbonyl protected N1,N14-bisguanidinium derivative **5** (Scheme 1). The product was characterized by ^{1}H NMR in which the aromatic protons of benzyloxycarbonyl (Z) were



Scheme 1. Synthesis of bisguanidine **6**.

seen at δ 7.60-7.20 and the benzylic protons as two singlets at δ 5.20 & 5.10; the remaining sets of methylenes appeared as multiplets at δ 3.45, 2.75, 2.60, 1.90 & 1.55. The removal of the benzyloxycarbonyl in **5** was achieved by catalytic transfer hydrogenation[31] using HCOOH-Pd/C (10%) to yield the bisguanidine **6** as the corresponding formate salt. The ^{1}H NMR of **6** exhibited four sets of mutiplets, the set at δ 3.25 characteristic of methylene α to the guanidino function [$NH_2(C{=}NH)NH\text{-}C\underline{H}_2$-]. The ^{13}C NMR spectrum of compound **6** exhibited two signals for the guanidinum carbons at δ 158.0 and 156.5. The mass spectrum gave the expected molecular ion at 284 (FAB-MS) confirming the structure of the product.

The compound **6** being symmetric, was expected to give only one signal for the guanidinium carbon in the ^{13}C NMR. However, two signals were observed for this functionality. In order to investigate this anomaly, the compound **6** was synthesized by another unambiguous route involving selective blocking of the secondary amino functions to direct the guanylation to the primary amino sites as shown in Scheme 2. N,N'-bis(2-cyanoethyl)butanediamine **7** was prepared by the treatment of acrylonitrile (2 eq.) with 1,4-diaminobutane **3**.[32] In the IR spectrum of **7**, an absorption band at 2251 cm^{-1} indicated the presence of cyano function. In the ^{1}H NMR spectrum, the compound **7** exhibited four sets of methylenes at δ 3.50, 3.25, 3.10 and 1.90. The ^{13}C NMR spectrum indicated all the carbon signals, the signal at δ 118.6 corresponding to the cyano carbon. The formation of ω,ω'-bisaminoalkylated product (and not tetra-alkylated product) was further supported by the mass spectrum which exhibited a peak at 194 (M^{+}). The free amino groups (secondary amines) of **7** were protected with benzyl function using benzyl bromide and KF-celite to get **8**, the ^{1}H NMR of which exhibited the aromatic protons at δ 7.40-7.20 and the benzylic singlet at δ 3.60. Compound **8** was subjected to hydrogenation using Ra-Ni/H_2 to get the tetramine **9**. In the IR spectrum of **9**, the characteristic absorption band of the cyano group was absent, indicating

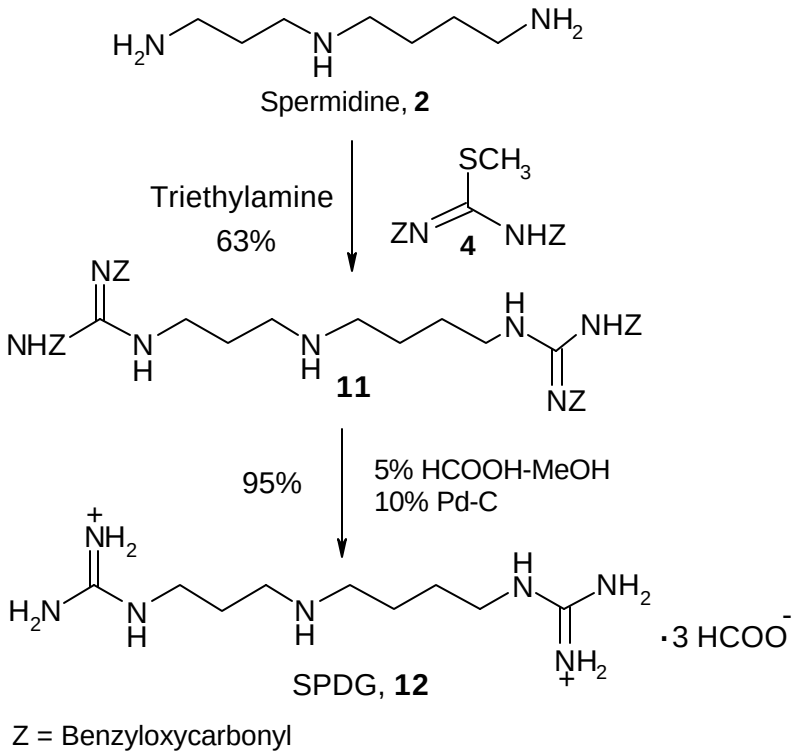


Scheme 2. Synthesis of bisguanidine **6** by a different route.

complete reduction of the cyano group to amine. The presence of the primary amino groups was indicated by a broad band at 3480 cm^{-1}. Compound **9** was subjected to guanylation reaction using N,N'-(bisbenzyloxycarbonyl)-*S*-methyl isothiourea **4** in dichloromethane in presence of triethylamine at reflux to afford the corresponding N5,N10-(dibenzyl) benzyloxycarbonyl protected bisguanidinum derivative **10**. The

structure of **10** was confirmed by ^{1}H and ^{13}C NMR. The ^{1}H NMR spectrum exhibited four sets of multiplets at δ 3.50-3.35, 2.60-2.30, 1.90-1.60 & 1.60-1.40 corresponding to the four different methylenes of the sperminyl chain along with the aromatic and benzylic protons from the benzyloxycarbonyl and benzyl protecting groups. The removal of the N-benzyl as well as Nbenzyloxycarbonyl were achieved in one step using transfer hydrogenation[31] using Pd-C/HCOOH in methanol. The product obtained had identical spectral features as compound **6** obtained by direct guanylation of spermine (Scheme 1) which confirmed the structure of the compound **6**. The two ^{13}C signals may arise due to two different conformations experienced by the guanidino moieties.

2.3.3 Synthesis and Characterization of Bisguanidine 12



Scheme 3. Synthesis of bisguanidine **12**

In a similar manner as described for spermine, spermidine **2** on reaction with the reagent **4** gave the benzyloxy-protected derivative **11** that on catalytic transfer hydrogenation gave the bisguanidine **12** (Scheme 3). The structure of the compound **12** was confirmed by ^{1}H, ^{13}C NMR and FAB-MS techniques. In the ^{13}C NMR spectrum only one guanidinium carbon signal at δ 157.9 could be seen and in the mass spectrum, the molecular ion was seen at 230, confirming the product to be a N1,N10-bisguanidinyl spermidine **12**.

2.3.4. Synthesis of Oligonucleotide Sequences

The effect of the bisguanidines on the stability of oligonucleotide duplexes and triplexes of the oligonucleotide sequences **13-18** was studied. The sequence **13** corresponds to the Dickerson's dodecamer, a self-complementary sequence for which crystal structure is well known. The purine-rich ODN **14** and the complementary pyrimidine-rich **15** form the duplex **14:15** required for the Tm studies. Likewise, the purine-rich ODN **17** and pyrimidine-rich **18** form the duplex **17:18** for triplex studies. The ODN sequence **16** is the third strand which can bind to the target duplex **17:18** forming the triplex. The duplex **17:18** has an overhang of 3 base-pairs each on either side which serves to effectively differentiate the third strand dissociation from the duplex melting.

The oligonucleotides **13-18** were synthesized by standard phosphoramidite methodology on an automated DNA-synthesizer using the commercial β-cyanoethylphosphoramidites. The crude oligonucleotides obtained after deprotection with NH_4OH were desalted and the purity of ODNs was checked by RP-HPLC which showed >95% purity. Therefore, they were used without further purification for the biophysical studies.

13 5'- CGC GAA TTC GCG
14 5'- GCC AAG AAA AAA CAA AAA AGA CGC
15 5'- GCG TCT TTT TTG TTT TTT CTT GGC
16 5'- TTC TTT TTT CTT TTT TCT
17 5'- GCC AAG AAA AAA GAA AAA AGA CGC
18 5'- GCG TCT TTT TTC TTT TTT CTT GGC

2.3.5. Duplex and Triplex Formation of Oligonucleotides

The biophysical effect of guanidinium groups in SPMG **6** and SPDG **12** upon DNA duplex binding, was studied by UV-Tm studies on the self-complementary dodecamer d(CGCGAATTCGCG) and a 24-mer duplex DNA **14:15**. The duplexes were constituted by taking equimolar quantities of ODNs **14** & **15** in TRIS buffer, annealed (by heating the sample in a water bath followed by slow cooling) in the presence of the desired polyamines/guanidines at a concentration of 1mM. The ODN **13** which is self-complementary was also subjected to similar treatment to form a duplex. In both the cases, ODN duplexes constituted in the absence of polyamines/guanidines were taken as the references.

The polypyrimidine ODN **16** binds as the third strand to the target duplex **17:18**. The thermal stabilities of the triplexes in the presence of polyamines/guanidines were assessed by UV-Tm experiments in 25 mM TRIS buffer containing 100 mM NaCl, 0.1 mM EDTA at pH 7.0.

Since the triplex formation in the polypyrimidine motif is favoured at acidic pH,[33,34] due to the required protonation of N3 of cytidine for the Hoogsteen bond formation, UV-Tm experiments were also carried out at pH 5.5 in sodium acetate buffer (50 mM) containing 100 mM NaCl in a similar manner as described above.

2.4. RESULTS AND DISCUSSION

2.4.1. Duplex Stabilization by SPMG 6 and SPDG 12

The UV-melting profiles of the duplexes are shown in Figure 8 and the data shown in Table 1. Under the experimental conditions it is seen that both spermine (SPM) and spermidine (SPD) stabilized both DNA duplexes (**13:13** and **14:15**) by enhancing their T_ms by 3-5°C (Table 1, entry 2 & 3) over the control samples lacking these polyamines (entry 1). In comparison to this, the bisguanidinium derivatives SPMG **6** and SPDG **12** enhanced the T_m of dodecamer **13:13** by 8-9°C and that of the 24 base pair duplex **14:15** by 7-8°C over the control (entry 4 & 5). This amounts to ~4°C stabilization of duplexes by guanidinium compounds over that of parent polyamines. The percentage hyperchromicities upon DNA melting was around 17% for the dodecamer **13** and 27% for the 24-mer duplex (**14:15**) irrespective of the nature of the polyamines present. Thus, DNA duplexes are more stabilized by bisguanidinium

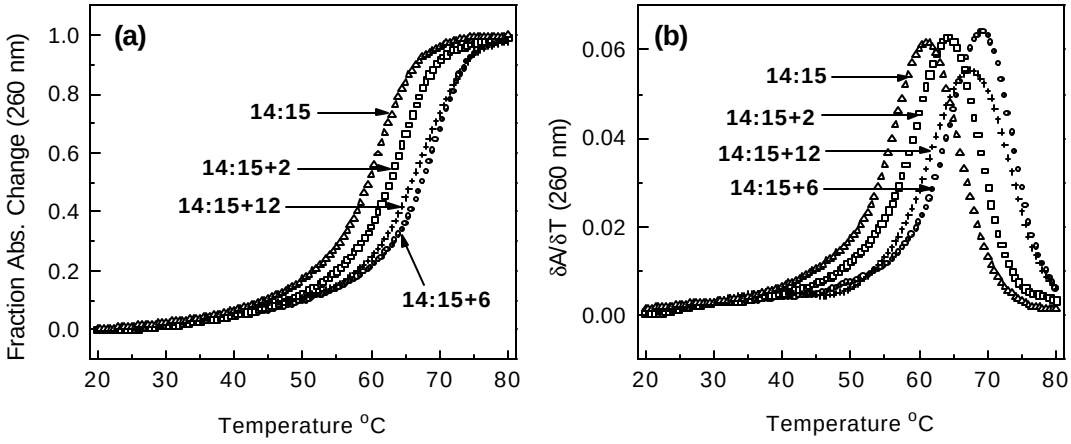


Figure 8. (a) UV-melting profiles of the duplex **14:15** in the presence or absence of polyamines /guanidines and (b) their first derivative curves. **14:15** is without added amines/guanidines; **14:15+2** is duplex in presence of **2**; **14:15+6** is duplex in presence of **6** and **14:15+12** is duplex in presence of **12**. Buffer: 25mM TRIS, 100 mM NaCl, 0.1 mM EDTA at pH 7.0.

analogues **6** and **12** as compared to the parent polyamines spermine **1** and spermidine **2**. However, no discrimination in binding among the two polyamines **1** and **2** or their guanidinium analogues **6** and **12** was seen at pH 7.0 (Table 1, entries 2 & 3 and 4 & 5 respectively).

Table 1. Melting Tms (°C) of duplexes[#]

Entry	Complex →	14:15		13:13	
	Compound ↓	Tm (°C)	ΔTm(°C)	Tm (°C)	ΔTm(°C)
1	None	60.0	---	50.0	---
2	Spermine, **1**	64.0	+4.0	55.0	+5.0
3	Spermidine, **2**	63.0	+3.0	54.5	+4.5
4	SPMG, **6**	68.0	+8.0	59.0	+9.0
5	SPDG, **12**	67.0	+7.0	58.5	+8.5

[#]Buffer: 25mM TRIS, 100 mM NaCl, 0.1 mM EDTA at pH 7.0. All Tms are accurate to ± 0.5°C and measured in three melting experiments. ΔTm is the difference between the Tms in presence of compound and absence of compounds.

13 5'- CGC GAA TTC GCG
14 5'- GCC AAG AAA AAA CAA AAA AGA CGC
15 5'- GCG TCT TTT TTG TTT TTT CTT GGC

2.4.2. Triplex Stabilization by SPMG 6 and SPDG 12

The effect of bisguanidines SPMG **6** and SPDG **12** on DNA triplex formation was examined by carrying out pH-dependent melting experiments of the triplex **16*17:18** constituted from the 24-mer duplex **17:18** and the 18-mer polypyrimidine third strand, **16**. The melting profiles shown in Figure 9 exhibit characteristic biphasic transitions which unambiguously indicate the formation of triplexes. The first transition (lower Tm) corresponds to the dissociation of the third strand **16** from the duplex **17:18** and the second transition (higher Tm) corresponds to the duplex melting.

In the absence of spermine or spermidine at pH 7.0, no triplex formation was seen (Table 2, entry 1), while in the presence of the polyamines, triplexes were observed with the following stability order: SPMG > SPM > SPDG ~ SPD. The transformation of the terminal amino groups of spermine to guanidino functions, resulted in a significant stabilization of the triplex by 12.5°C (ΔT_{m1}, entry 4) over spermine at pH 7.0, while the transformation of the primary amino functions of spermidine enhanced the T_m of the triplex by 5.5°C (ΔT_{m1}, entry 5) over spermidine.

Table 2. Melting Tms (°C) of triplexes at pH 7.0[#]

Entry	Compound	Complex (16*17:18)		
		T_{m1} (°C)	T_{m2} (°C)	ΔT_{m2}(°C)
1	None	nd	60.0	---
2	Spermine, **1**	36.0	64.0	+4.0
3	Spermidine, **2**	26.0	63.0	+3.0
4	SPMG, **6**	48.5 (+12.5)	69.0	+9.0
5	SPDG, **12**	31.5 (+ 5.5)	67.5	+7.5

[#]Buffer: 25mM TRIS, 100 mM NaCl, 0.1 mM EDTA at pH 7.0. All Tms are accurate to ± 0.5°C and measured in three melting experiments. T_{m1} indicates the triplex-to-duplex transition temperature and T_{m2} the duplex-to-single strands. ΔT_{m2} is the difference in duplex Tms in comparison to the reference in absence of compound. Values in brackets indicate the difference in Tms between the samples containing the guanidines in comparison to the parent polyamines.

16 5'- TTC TTT TTT CTT TTT TCT
17 5'- GCC AAG AAA AAA GAA AAA AGA CGC
18 3'- CGG TTC T TT T T TCTT T T T TCT GCG

At acidic pH 5.5, triplex formation was observed (Figure 9) even in the absence of any polyamines (Table 3, entry 1). In presence of polyamines, the triplex was stabilized in all the cases and the stabilizing order was SPMG > SPM > SPDG > SPD,

with the bisguanidinium **6** showing maximum triplex stability (ΔT_{m1}, +17.5°C, entry 4) at pH 5.5. The stabilization effect of acidic pH over neutral pH is relatively more for spermine, **1** (ΔT_m, +10°C) as compared to the corresponding bisguanidine, **6** (ΔT_m, +3°C).

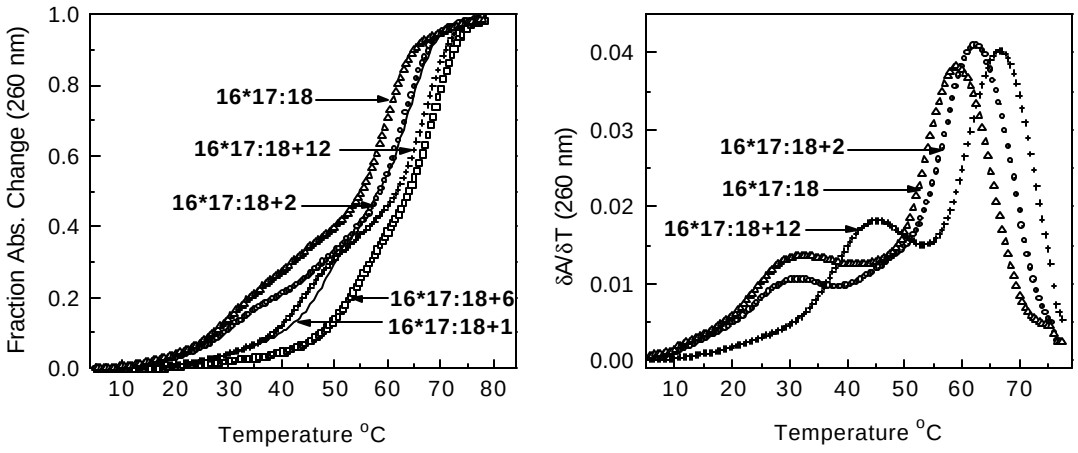


Figure 9. (a) UV-melting profiles of the complex **16*17:18** in the absence or presence of polyamines or guanidines and (b) representative first derivatives. Buffer: 50mM NaOAc, 100 mM NaCl, 0.1 mM EDTA at pH 5.5. All Tms are accurate to ± 0.5°C and measured in three melting experiments.

The lower triplex stablization effect of guanidines (**6** & **12**) compared to the polyamines spermine and spermidine (**1** & **2** respectively) is due to the differential protonation effects and relative pK_a differences. The guanidinium groups having higher pK_a, are predominantly protonated at pH 7.0 and lowering the pH to 5.5 does not significantly enhance their protonation. However, for spermine and spermidine with pK_as lower than guanidinium, the significant enhancement in protonation is seen from pH 7.0-5.5, leading to a lower pH-induced triplex stability effects.

Table 3. Melting Tms (°C) of triplexes at pH 5.5[#]

Entry	Compound	Complex (16*17:18)			
		Tm1 (°C)	DTm1(°C)	Tm2 (°C)	DTm2 (°C)
1	None	34.5	---	60.0	---
2	Spermine, **1**	46.0	+11.5	64.5	+4.5
3	Spermidine, **2**	33.5	-1.0	62.5	+2.5
4	SPMG, **6**	51.5	+17.5	69.0	+9.0
5	SPDG, **12**	45.0	+10.5	68.0	+8.0

[#]Buffer: 50 mM NaOAc, 100 mM NaCl, pH 5.5. All Tms are accurate to ± 0.5°C and measured in three melting experiments. Tm1 indicates the triplex-to-duplex transition temperature and Tm2 the duplex-to-single strands. ΔTm1 is the difference between the Tm1 in presence and absence of compound. Similarly, ΔTm2 is the difference in duplex Tms.

16 5'- TTC TTT TTT CTT TTT TCT

19 5'- GCC AAG AAA AAA GAA AAA AGA CGC

20 3'- CGG TTC T TT T T TCTT T T T TCT GCG

2.5. CONCLUSIONS

In this section, the synthesis and characterization of novel guanidinium analogues of spermine and spermidine are reported. The DNA duplex and triplex formation in the presence of these compounds demonstrate that the bisguanidines **6** and **12** offer a higher stability to duplexes and triplexes over that seen with the parent polyamines spermine **1** and spermidine **2**. This is due to the dominance of electrostatic interactions between the cationic amines and anionic phosphates in DNA-polyamine interactions. The pH-induced stabilization is much larger in case of the parent polyamines as compared to their bisguanidine derivatives, since the higher pK_a of guanidinium makes the fully protonated species more predominant at pH 7.0 compared to that in the polyamines. In comparison, the polyamines are incompletely protonated at pH 7.0 and the protonation is substantially enhanced at pH 5.5. The present results

suggest the use of guanylated polyamine analogues for stable DNA triplex formation at physiological conditions which is important for therapeutic considerations.

Amidinium and guanidinium groups do play significant roles in DNA minor groove binding of drugs such as netropsin,[35] distamycin,[36] pentamidine and propamidine.[37,38] Cationic guanidinium linkages have been recently introduced into DNA in place of anionic phosphates for enhanced binding to duplex DNA.[39] From this view-point, results presented here indicate that electrostatic stabilization can be enhanced by the conversion of amino groups into guanidino functions. This will have importance in designing better analogues of polyamines and related DNA binding agents[49,40] for effective interaction with DNA duplexes and triplexes.

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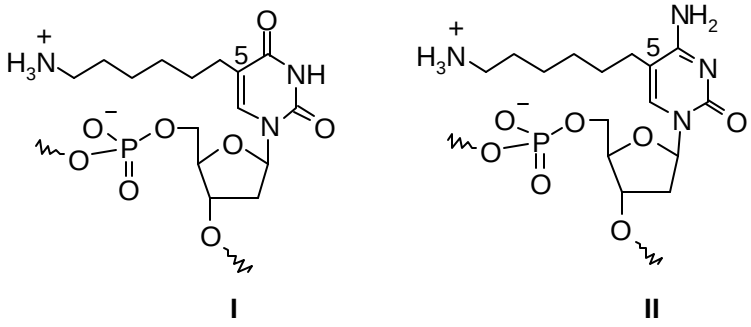
Chapter 2

Section B:

Chemical Synthesis and Biophysical Studies of 5-Me-dC-N^4-Alkylguanidino Substituted Oligonucleotides

2.7. INTRODUCTION

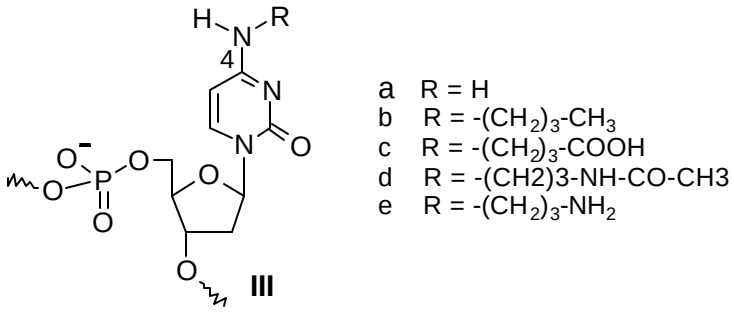
Unnatural nucleosides with altered or additional hydrogen bond donor and acceptor groups and appended cations when incorporated into oligonucleotides can impart different recognition specificities towards proteins and nucleic acids. ODNs bearing pyrimidine 5-ω-aminohexyl substituents which are 'zwitterionic ODNs' (Figure 1) bind to natural DNA (having complementary sequence) at low ionic strength equally or even better than natural DNA itself. [1]



I **II**

Figure 1. Oligonucleotides with 5-ω-aminohexyluridine (**I**) and 5-ω-aminohexylcytidine (**II**).

Among the ODNs containing N^4(butyl)dC **IIIb**, N^4-(3-carboxypropyl)-dC **IIIc**, N^4-(3-acetamidopropyl)dC **IIId**, or N^4-(3-aminopropyl)dC **IIIe** (Figure 2), complementation with the target duplex DNA suggested favoured triplex formation in the case of N^4-(3-aminopropyl)dC **IIIe**, although with a lower stability.[2]



III

a R = H
b R = $-(CH_2)_3-CH_3$
c R = $-(CH_2)_3-COOH$
d R = -(CH2)3-NH-CO-CH3
e R = $-(CH_2)_3-NH_2$

Figure 2. Triplex forming ODNs with different N^4-substituents on dC.

Tetra-cationic peptides attached to ODNs were shown to favour triplex formation.[3] The family of ODN-conjugates with a constant peptide component (arginine) but different nucleic acid chain lengths -15, 18 or 21-mers showed a near constancy of the triplex Tm emphasizing the importance of cationic charge in influencing the triplex formation. The synthesis of pentameric thymidyl deoxyribonucleic guanidine (DNG, Figure 3) in which the phosphodiester linkages of DNA are replaced by guanidinium linkages (-NHC(=NH_2^+)NH-) demonstrated unprecedented stability of the DNG:DNA complex.[4] In the previous section of this Chapter, the non-covalent effect of guanidinum groups on the stabilization of DNA complexes was demonstrated. The interesting results encouraged us to suitably conjugate guanidinium group to nucleobases of ODNs and study their triplex formation.

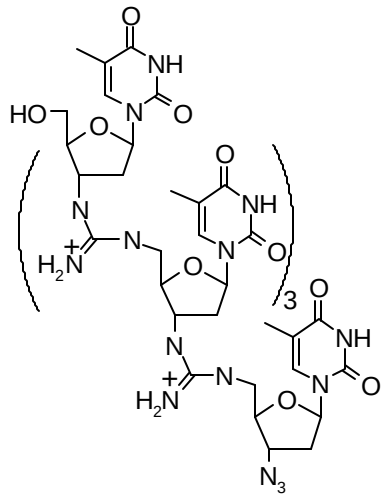


Figure 3. DNG-Deoxyribonucleic guanidine.[4]

2.8. OBJECTIVES

The present Section is aimed at the synthesis and complementation studies of N^4-propyl/butyl-guanidino-5-Me-dC containing oligonucleotides (Figure 4). The guanidine conjugation through a methylene spacer was chosen to be on a pyrimidine at

the 4-position which is relatively easy to synthesize by the convertible nucleoside approach. The guanidine group which is highly basic (pK_a > 13) would remain protonated at neutral pH, thereby imparting a zwitterionic nature to the ODNs which may lead to better hybridization.

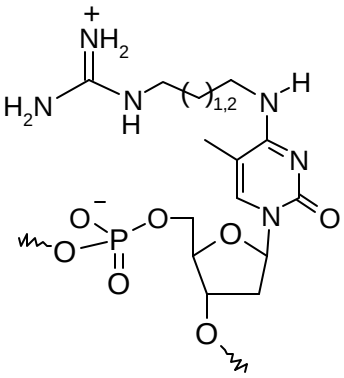
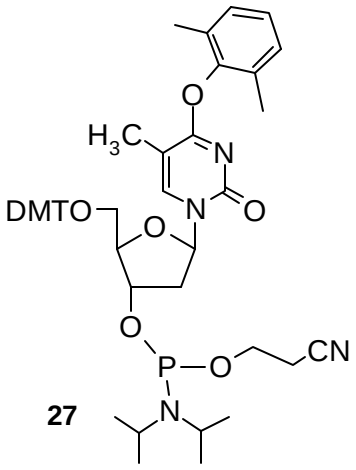


Figure 4. 5-Me-dC-N^4-alkyl guanidino ODNs.

The objectives of this section are:

- Synthesis of "convertible nucleoside"- O^4-2,6-dimethylphenyl thymidine-3'-phosphoramidite **27** and its incorporation into ODNs by solid phase synthesis.
- Functionalization of the "convertible ODNs" into the corresponding 5-Me-N^4-guanidino propyl/butyl derivatives and their characterization.
- Biophysical studies of the N^4-guanidino-propyl/butyl dC containing ODNs.



27

2.9. PRESENT WORK

2.9.1. Strategies for Functionalized ODN Synthesis

Two strategies are normally employed for the synthesis of oligonucleotides bearing tethered functionality (functionally tethered oligonucleotides, FTOs): (i) the dedicated monomer strategy and (ii) the convertible nucleoside strategy.

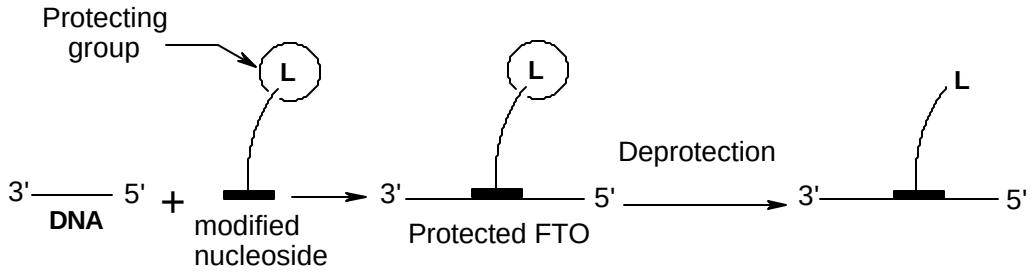


Figure 5. Dedicated monomer strategy.

In the first method (Figure 5) a suitable nucleoside monomer carrying the desired modification with the appropriate protecting group compatible with the conventional oligonucleotide synthesis, is incorporated into the ODNs by automated DNA synthesis. This has limitations in terms of complex protection-deprotection strategies. The second method, the "convertible nucleoside strategy" (Figure 6)

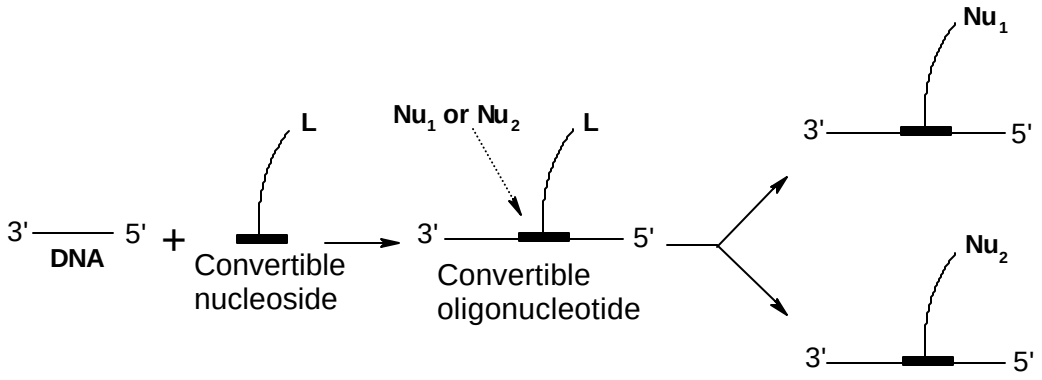


Figure 6. Convertible nucleoside strategy.

employs a nucleoside containing a leaving group, such as 2,6-dimethyl phenol, that is completely retained during the ODN synthesis and subsequent deprotection steps. After the oligonucleotide assembly, the leaving group **L** can be displaced by different nucleophiles, leading to various modified ODNs. This strategy was used for the synthesis of dC-N^4-guanidinopropyl or guanidinobutyl substituted ODNs.

The nucleoside carrying a functionalized protecting group required for the dedicated monomer strategy or that containing a convertible function can be prepared in different ways as follows. One method is by introducing a leaving group [e.g. 4-(3-nitro-1,2,4-triazolyl)[5] or 4-(1,2,4-triazolyl)-[6,7]] at C4 in an appropriately protected thymidine (Figure 7) followed by nucleophilic displacement. In another procedure N^4-cytidine modified oligonucleotides were synthesized by the reaction of the oligonucleotide containing cytidine at appropriate positions with the selected amine in the presence of sodium bisulfite.[2] This reaction does not work with 5-methylcytosines which does not form an adduct with bisulfite.[8]

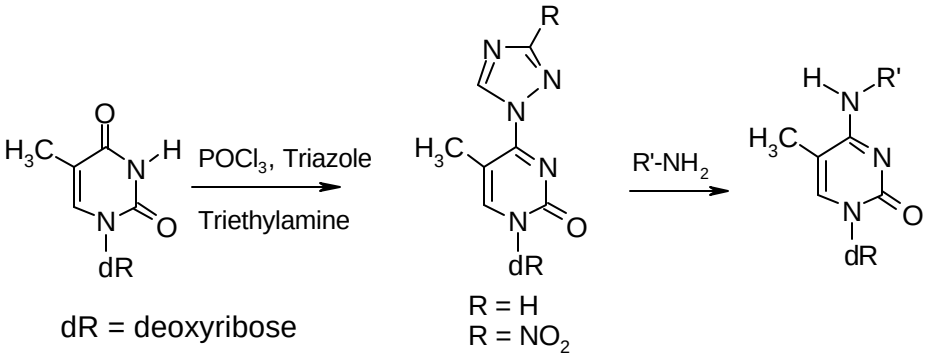
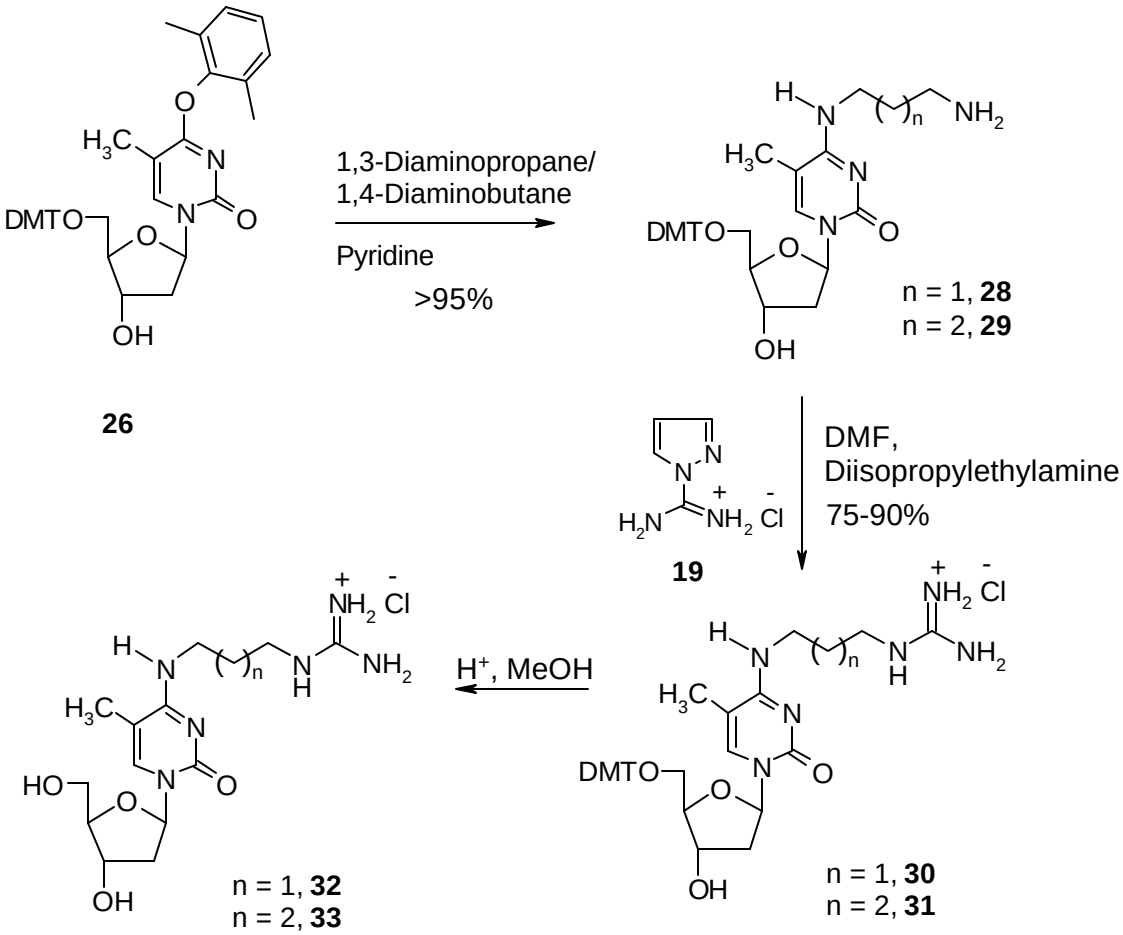


Figure 7. Literature methods[5-7] for N^4-derivatization of 5-Me-dC.

In the present work O^4-2,6-dimethylphenyl-thymidine-3'-O-phosphoramidite (**27**) was incorporated into ODNs, followed by transformation to the desired guanidinium-ODN conjugates in a post-synthetic reaction.

2.9.2. Synthetic Compatibility of the Nucleoside



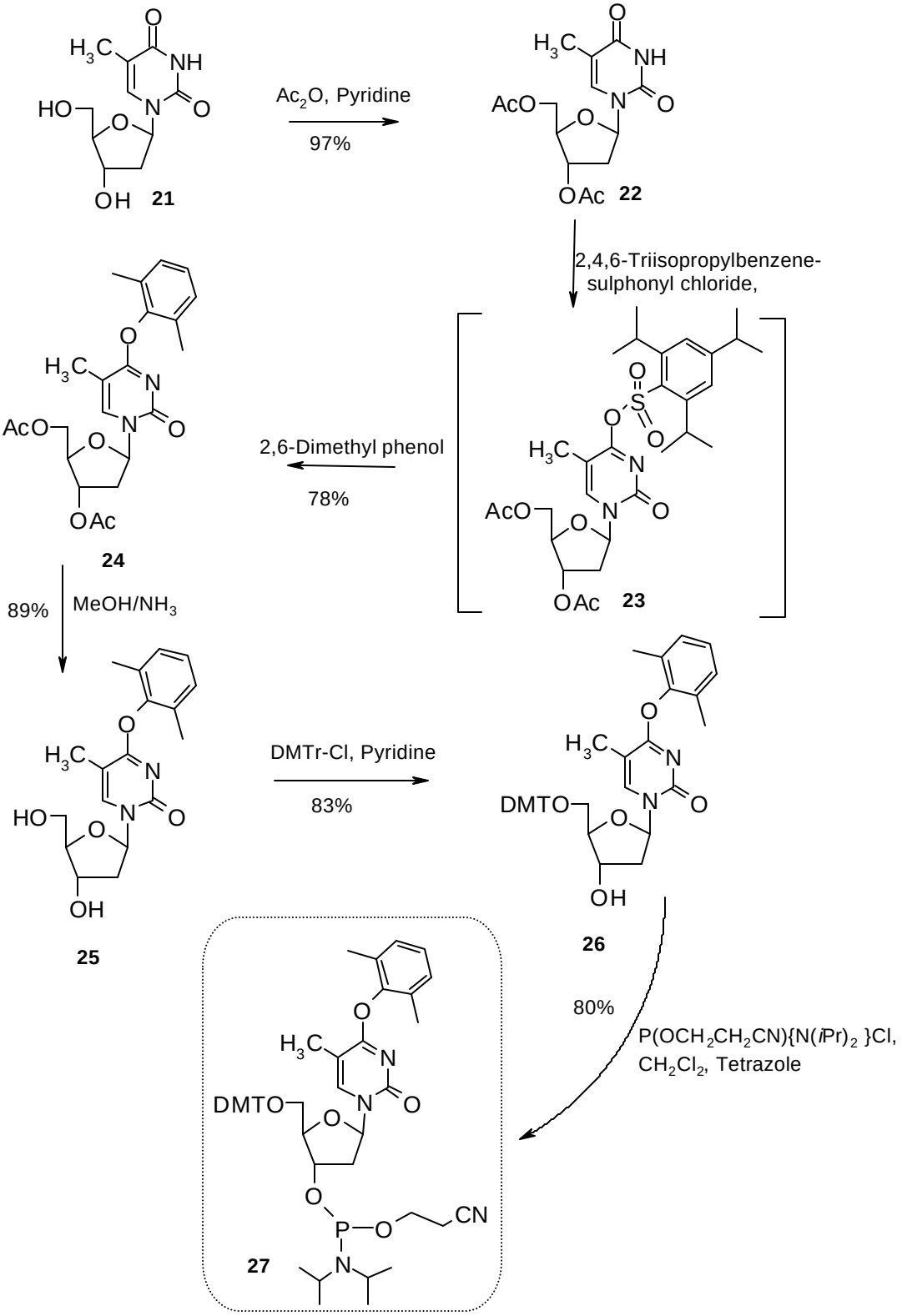
Scheme 3. Functionalization of the convertible nucleoside.

In order to evaluate the synthetic utility of the convertible function in 5'-O-DMT-*O*^{4}-(2,6-dimethylphenyl)-2'-deoxythymidine **26**, (synthesized in Section 2.9.3.) was treated individually with 1,3-diaminopropane or 1,4-diaminobutane (Scheme 3), in pyridine to get the corresponding N^4-3-aminopropyl- (**28**) or N^4-4-aminobutyl-5-Me-dC (**29**) respectively. The success of the reaction was monitored and the products characterized by ^{1}H NMR spectrum. The adducts **28** and **29** were separately treated with the guanylating reagent pyrazole carboxamidine hydrochloride[9] **19** to get the

respective guanidinium derivatives **30** and **31**. These compounds were characterized by ^{1}H NMR and FAB-MS techniques. The ^{1}H NMR spectra of **30** and **31** exhibited the N-C$\underline{H}_2$ protons of the propyl/butyl groups at δ 3.50-3.10 integrating to 4 protons and ascribed to N^4-C$\underline{H}_2$ & C$\underline{H}_2$-NH(C=NH)-N$\underline{H}_2$ methylene protons of the propyl/butyl chains. The molecular ion peaks seen at 643 (M^+) in the mass spectra of **30** and at 657 (M^+) in the case of **31** confirmed the N-alkylguanidino structures. These results suggested that (i) the reactivity of dimethylphenyl derivative for nucleophilic reaction with primary amine and (ii) the reactivity of amino group for converting to guanidino function are appropriate for post-synthetic ODN syntheses. DMT deprotection in **30** and **31** was achieved by treating them separately with methanolic HCl to afford the respective unprotected nucleosides **32** and **33**.

2.9.3. Synthesis of O^4-2,6-Dimethylphenyl-thymidine-3'-O-Phosphoramidite 27

The *O^4*-2,6-dimethylphenyl thymidine-3'-*O*-phosphoramidite was synthesized by the reported method[10,11] starting from thymidine as shown in Scheme 4. The 3' and 5'-hydroxyls of thymidine **21** were first protected as *O*-acetates to get the 3',5'-*O*-diacetate **22**. This was converted *in situ* to 3',5'-diacetyl-4-*O*-(2,4,6-triisopropylbenzene sulfonyl)-2'-dT **23**, by treatment with 2,4,6-triisopropyl benzenesulfonyl chloride and triethylamine and transformed to its 4-*O*-(2,6-dimethylphenyl) derivative **24**, upon addition of 2,6-dimethylphenol, triethylamine and DABCO. In the ^{1}H NMR spectrum of **24**, the aromatic protons and the Ar-C$\underline{H}_3$ of 2,6-dimethylphenyl component could be seen at δ 7.10-6.95 & 2.10 respectively[11] indicating the formation of **24**. The appearance of the C4 signal at δ 168.8 in ^{13}C NMR is another indication of the *O^4*-dimethylphenyl derivatization. The deprotection of 3',5'-*O*-diacetates using methanolic ammonia gave **25** having the hydroxyl groups free. The primary 5'-hydroxyl group in **25** was protected as DMT derivative **26** by treatment with DMTr-Cl in anhydrous pyridine. The ^{1}H NMR of the dimethoxytrityl ether showed a multiplet for aromatic



Scheme 4. Synthesis of O^4-2,6-dimethylphenyl thymidine monomer.

protons at δ 7.50-6.80 (13 H) and a characteristic singlet at δ 3.80 corresponding to 6H of the methoxy groups. The 5'-O-DMT compound **26** was converted to the phosphoramidite derivative **27** through phosphitylation reaction employing chloro-N,N-diisopropyl-2-cyanoethoxyphosphine in dry CH_2Cl_2 in presence of diisopropylethylamine.[12] In the ^{31}P NMR spectrum, signals at δ 150.31 & 149.85 confirmed the formation of the product **27** as a diastereomeric mixture.

2.9.4. Design and Synthesis of Oligonucleotide Sequences

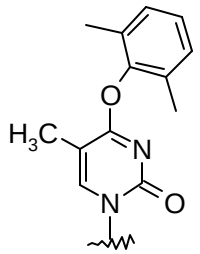
For the study of duplex and triplex stability effects of alkyl guanidinum ODNs, the sequences **34-40** were designed. The target duplex sequence for the triplex studies was the 24-mer **39:40**. The sequences for the third strand were 18-mer polypyrimidines containing one modified site each in **34** and **35**, and two in sequence **36**. The third strand upon binding to the double-stranded target forms a triplex with an overhang of 3 base pairs each on either side. This difference in lengths of the duplex and third strand (and hence triplex) would help to differentiate their melting temperatures. The sequence design also avoided self-complementation and a few cytosines were introduced into the polypyrimidine strands to prevent its slippage on the duplex.

The oligonucleotides **34-40** (given in the next page) were synthesized by standard phosphoramidite methodology[13] on an automated DNA synthesizer (Pharmacia GA Plus) using the phosphoramidite **27** along with other standard PAC-protected β-cyanoethylphosphoramidites. The coupling yields of **27**, as quantified by the released DMT cation, were approximately 95% compared to the average yield with unmodified amidites ~99%. The concomitant use of deoxyribonucleoside phosphoramidites having the phenoxyacetyl group[14] for protection of the exocyclic amino function of adenine and guanine, and the isobutyryl group for that of cytosine,

was necessary to ensure rapid ODN deprotection with conc. NH_4OH without affecting the O^4-arylated thymidine residues.[11]

34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT
37 5' TTC TTC TTC TTT TCT TTT
38 5' AAA AGA AAA GAA GAA GAA
39 5' TCC AAG AAG AAG AAA AGA AAA TAT
40 5' ATA TTT TCT TTT CTT CTT CTT GGA

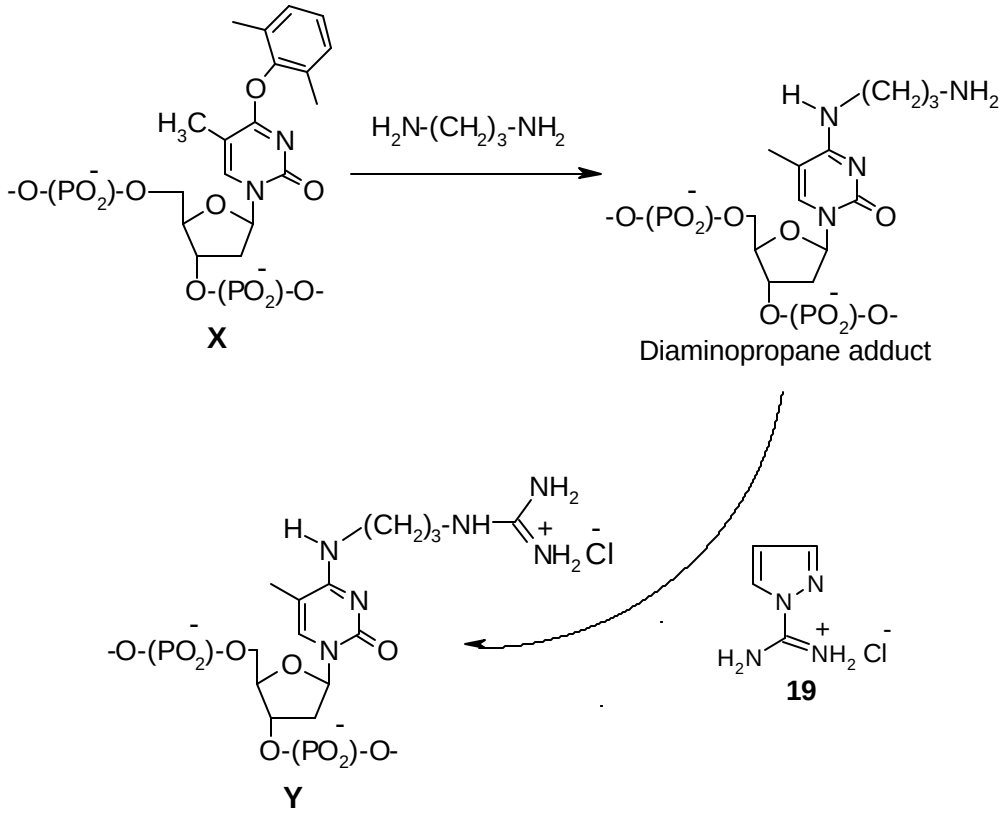
X =



Upon completion of the synthesis, the resin was treated with conc. NH_4OH at room temperature for 8h. Under these conditions, the cleavage from the resin and base deprotection are complete, while the 2,6-dimethylphenyl moiety is completely retained.[11] The analytical HPLC of the crude convertible ODNs indicated that they were >95% pure and were used for functionalization into the guanidino derivatives.

2.9.5. Functionalization of the Convertible ODNs into the Corresponding Guanidino-propyl/butyl Derivatives

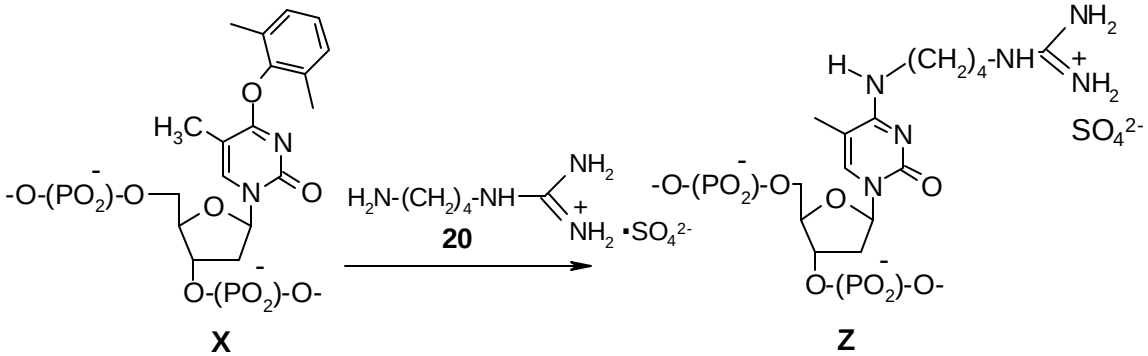
Aliquots of synthesized ODNs bearing 2,6-dimethylphenyl groups (**34-36**) were treated with aqueous solutions of diaminopropane to get the diaminopropyl substituted ODNs (Scheme 5). The treatment of the diaminopropane adduct obtained with pyrazole carboxamidine reagent **19**, gave the corresponding 3-guanidinopropyl derivatized ODNs. The reactions were monitored by analytical HPLC (Figure 8), the reaction mixtures desalted and were found to be of high purity (>95%). The ODNs were characterized by MALDI-TOF mass spectroscopy. The ODNs possessed the expected molecular weight (**41** $M_{calc.}$ 5468, $M_{obs.}$5471; **44** $M_{calc.}$ 5482, $M_{obs.}$ 5481; **45** $M_{calc.}$ 5482, $M_{obs.}$ 5481; **46** $M_{calc.}$5610, $M_{obs.}$ 5609) and were used as such for biophysical studies.



34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT

41 5' TTC TTC TT**Y** TTT TCT TTT
42 5' TT**Y** TTC TTC TTT TCT TTT
43 5' TT**Y** TTC TT**Y** TTT TCT TTT

Scheme 5. Synthesis of guanidinopropyl ODNs.



34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT

44 5' TTC TTC TT**Z** TTT TCT TTT
45 5' TT**Z** TTC TTC TTT TCT TTT
46 5' TT**Z** TTC TT**Z** TTT TCT TTT

Scheme 6. Synthesis of guanidinobutyl ODNs.

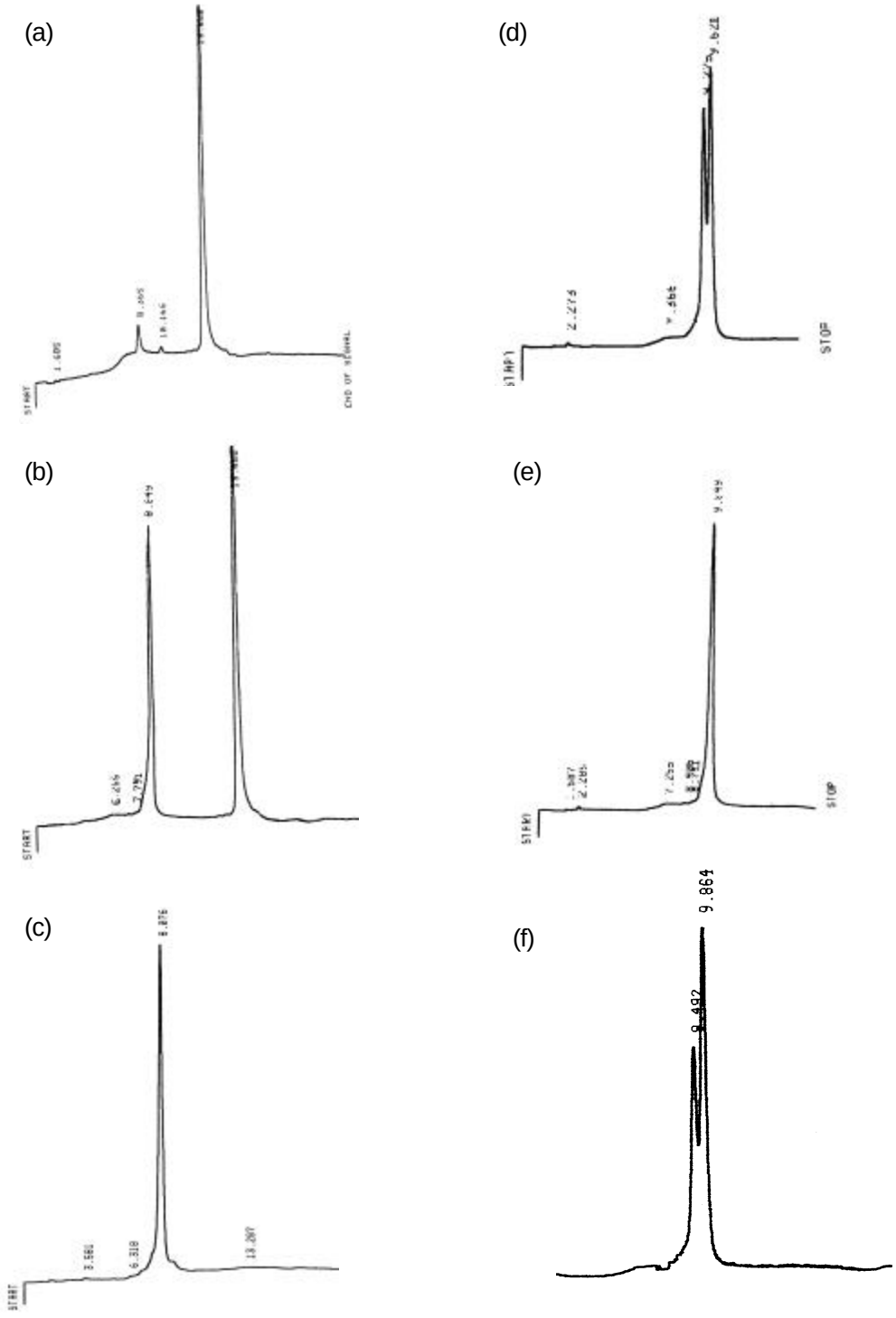


Figure 8. HPLC profiles of (a) **34** upon treatment with 1,3-diaminopropane (b) after 8h (c) after 16h. Diaminopropane adduct on treatment with 1*H*-pyrazole carboxamidine.HCl, **19** (d) after 10h (e) after 18h, the peak corresponds to **41** (f) co-injection of diaminopropane adduct and **40**.

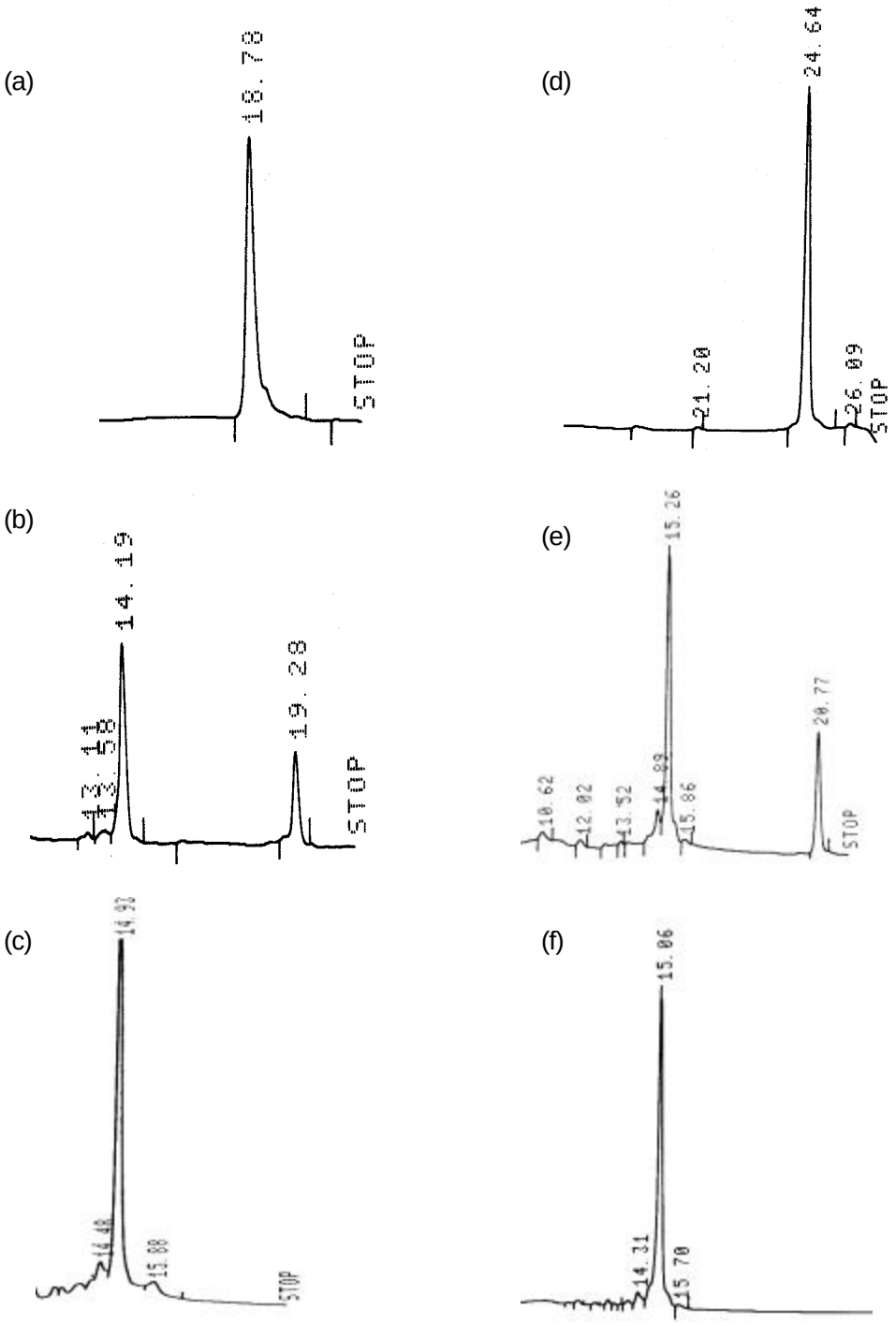


Figure 9. HPLC profiles of (a) **34** upon treatment with agmatine sulphate **20** (b) after 16h (c) after 36h, the peak corresponds to **44**; (d) **36** upon treatment with **20** (e) after 36h (f) after 72h, the peak corresponds to **46**.

The 4-guanidinobutyl sequences were obtained by individually treating the appropriate convertible ODNs with agmatine sulphate **20**, Scheme 6. The reactions were monitored by analytical HPLC. HPLC chromatograms after different intervals of time is given in Figure-9. In the case of ODN **36**, the time required for the completion of reaction was 76h as compared to 36h for the ODNs **34** and **35**. The ODNs after desalting were found to be of high purity (>96%).

2.9.6. UV-Tm Experiments

The hybridization properties of the modified ODNs (**41-46**) were evaluated by determining the melting temperatures (T_m) of the duplexes formed with the complementary DNA strand **38**. The triplex stabilites of ODNs (**41-46**) were assessed by measuring the T_m values of the complexes formed by hybridizing the respective ODNs with the duplex **39:40** in TRIS buffer (pH 7.0).

2.10. RESULTS AND DISCUSSION

2.10.1. Duplex Formation by N^4-Propyl/butyl-guanidino ODNs

The duplexes **41:38** and **44:38** having a single propyl and butyl guanidinium respectively at the centre showed a destabilization of 17°C and 13.5°C respectively

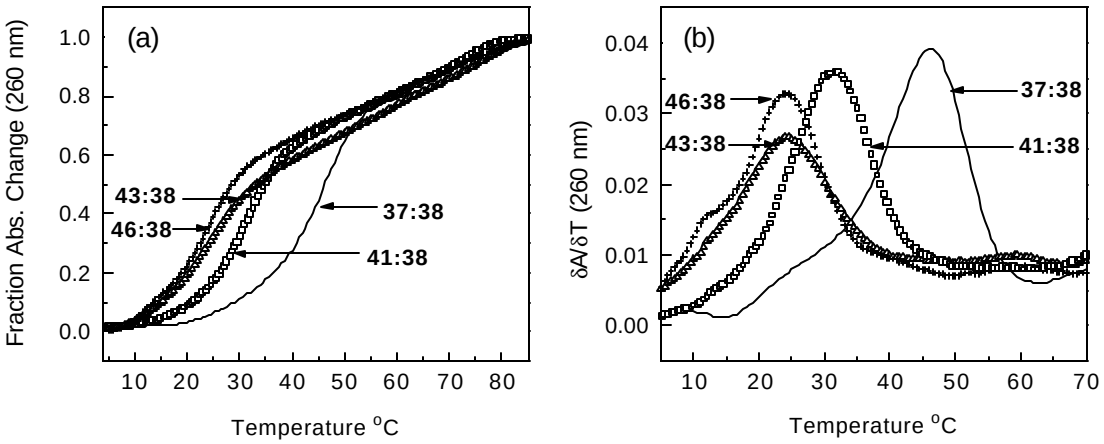


Figure 10. (a) UV-melting profiles of the duplexes and (b) their derivative curves. Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0.

(Table 1, entry 2) compared to the reference **37:38** (entry 1). The duplexes constituted from **42:38** and **45:38**, having 5'-modifications, showed a lesser destabilization (ΔT_m = -8.5°C and -7.5°C respectively, Table-1, entry 3) compared to the reference. The sequences **43** and **46** carrying two modified units formed duplexes with a higher magnitude of destabilization (ΔT_m ~ -23°C, entry 4).

Table 1. UV-Tms of the duplexes[#]

No	Unmodified	Complex			Tm (^{0}C)		
1	5'TT C TTC TT C TTT TCT TTT 3'AA G AAG AA G AAA AGA AAA	**37:38**			47.0		
	Duplexes with modification	**C*** Propylguanidino			**C*** Butylguanidino		
		Complex	Tm ($^\circ$C)	ΔTm ($^\circ$C)	Complex	Tm ($^\circ$C)	ΔTm ($^\circ$C)
2	5' TTC TTC TT**C*** TTT TCT TTT 3' AAG AAG AAG AAA AGA AAA	**41:38**	30.0	-17.0	**44:38**	33.5	-13.5
3	5'TT **C*** TTC TTC TTT TCT TTT 3'AA G AAG AAG AAA AGA AAA	**42:38**	38.5	-8.5	**45:38**	39.5	-7.5
4	5' TTX TTC TT**C*** TTT TCT TTT 3' AAGAAG AA G AAA AGA AAA	**43:38**	23.5	-23.5	**46:38**	24.0	-23.0

[#]Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0. All Tms are accurate to ± 0.5^{o}C and measured in three melting experiments. ΔT_m is the difference between the test and the reference Tms.

In order to rule out the formation of any secondary structures involving the sequences **41-46**, control thermal melting experiments were carried out in the absence of complementary strands. The resulting uneventful thermal denaturation curves indicated the non-existence of any ordered structure in ODNs **41-46** alone.

The introduction of guanidinum at the terminals was less destabilizing than the cases with modifications in the centre. The introduction of two modifications (one at the centre and the other at the 5'-terminus) as in **43** & **46** caused a significant reduction in binding (ΔT_m = ~ -23 °C).

The free rotation around C4-N^4 bond leads to the existence of the *syn* and *anti* conformations. Of these the predominant *syn* isomer[15] (Figure 11) is not capable of

hydrogen bonding *via* the N^4-H, as shown in Figure 11a. The destabilization is perhaps a consequence of this unfavourable conformational effect and the steric effects (Figure 11b). The steric factor imposed by propyl/butyl chain may overweigh the favourable electrostatic contribution of the guanidinium group to complex formation. A similar observation has been made with dC-N^4-sperminyl oligonucleotides[16] which decreased the complementary binding due to similar reasons.

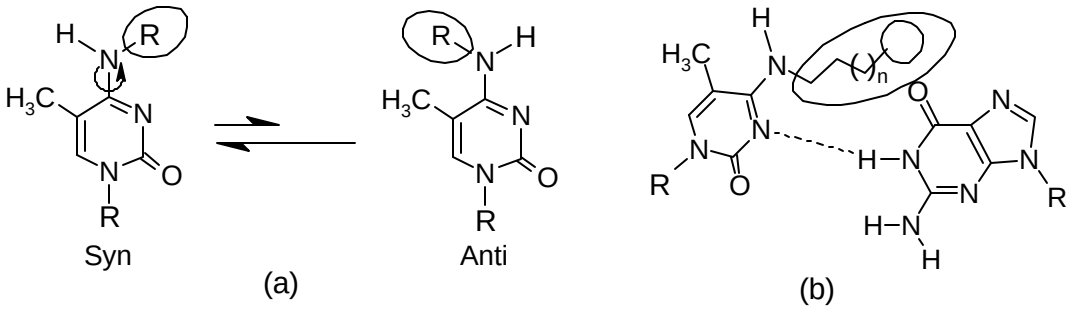


Figure 11. (a) *Syn-anti* isomers of N^4-alkyl derivatives of 5-Me-dC and (b) the weak Watson-Crick hydrogen bond due to the bulky N^4 substituent on 5-Me-dC.

2.10.2. Triplex Formation by N^4-Propyl/butyl-Guanidino ODNs

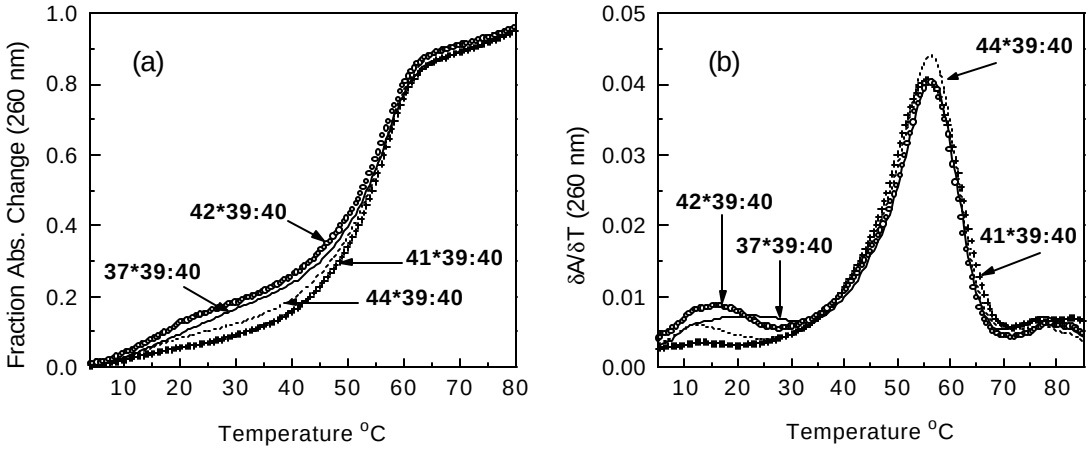


Figure 12. (a) Triplex UV-melting profiles of the complexes **36*38:39, 40*38:39, 41*38:39, 43*38:39** and (b) their derivative curves. Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0

Among the third strand polypyrimidine ODNs **41-46**, **41** having a modification in the middle of the sequence did not form a triplex. Other modified ODNs formed triplexes with the duplex **39:40** with varying magnitudes of destabilization (Table 2, entries 2-7) in comparison to the unmodified triplex **37*39:40** which exhibited a Tm of 25°C (entry 1). The representative melting curves of triplexes are depicted in Figure 12 and show biphasic transitions characteristic of triplex-to-duplex-to-single strand transitions. Triplex Tms were measured both in the absence and presence of $MgCl_2$ as salt is expected to show stabilization triplexes.[17,18] The data in Table 2 also indicates that salt indeed stabilized the triplexes with the stabilization in Tm ranging from 10 to 28°C.

Table 2. UV-Tms of the triplexes[#]

Entry	Sequences	Complex	No Mg^{2+}	Mg^{2+}	Salt induced
			Tm (°C)	Tm (°C)	ΔTm (°C)
1	5' TTC TTC TT C TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAATAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**37*39:40**	25.0	53.0	28.0
2	5' TTC TTC TT **Y** TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAA TAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**41*39:40**	nd	21.0 (-32.0)	
3	5' TTC TTCTT **Z** TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAA TAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**44*39:40**	12.0 (-13.0)	22.0 (-31.0)	10.0
4	5' TT **Y** TTC TTC TTT TCT TTT 5'TCC AA G AAGAAG AAA AGA AAA TAT 3' AGG TT C TTC TTC TTT TCT TTT ATA	**42*39:40**	16.5 (-8.5)	33.0 (-20.0)	16.5
5	5' TT **Z** TTC TTC TTT TCT TTT 5'TCC AA G AAG AAG AAA AGA AAA TAT 3' AGGTT C TTC TTC TTT TCT TTT ATA	**45*39:40**	18.0 (-7.0)	30.0 (-23.0)	12.0
6	5' TT **Y** TTC TT **Y** TTT TCT TTT 5'TCCAA G AAG AA G AAA AGA AAA TAT 3'AGGTT C TTC TT C TTT TCT TTT ATA	**43*39:40**	11.0 (-14.0)	22.0 (-31.0)	11.0
7	5' TT **Z** TTC TT **Z** TTT TCT TTT 5'TCCAA G AAG AA G AAA AGA AAA TAT 3'AGGTT C TTC TT C TTT TCT TTT ATA	**46*39:40**	14.5 (-10.5)	29.0 (-21.0)	14.5

[#]Buffer: 25 mM TRIS, 100mM NaCl, pH 7.0 in the presence or absence Mg^{2+}(20 mM). ΔTm is the difference between the test Tm and the reference Tm. All Tms are accurate to ± 0.5°C and measured in three melting experiments. **Y** indicates N^4-3-propylguanidino-dC and **Z** indicates N^4-4-butylguanidino-dC. Values in brackets indicates the difference between the test and reference Tms.

As discussed in case of duplexes, the incapability of *syn* isomer in the N^4-substituted 5-methyl-dC (m^5-dC) derivatives to form Hoogsteen hydrogen bonds is possibly the main reason for this decreased stability (Figure 13 a).

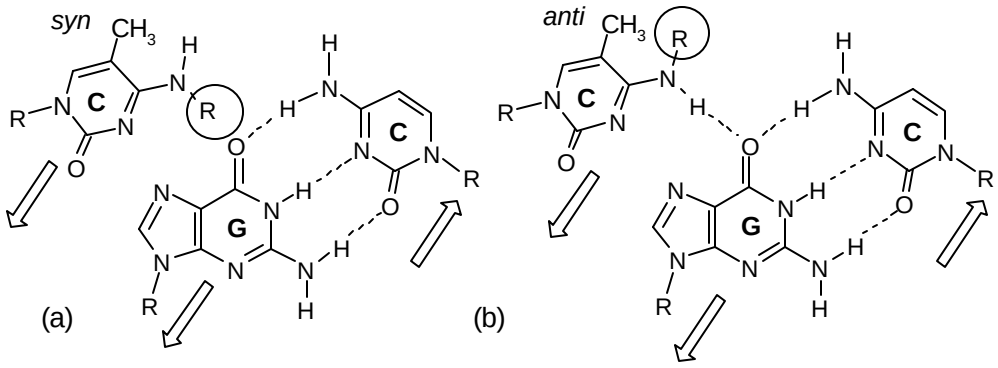


Figure 13. The possible hydrogen bonding patterns with the N^4-alkyl guanidinum derivatives of m^5-dC(a) No Hoogsteen hydrogen bonding (b) One Hoogsteen hydrogen bonding.

2.10.3. Hysteresis in Triplex Transitions

The reassociation or hybridization event of DNA can be monitored by the decrease in absorbance (hypochromicity) with lowering of temperature.[19] Hysteresis is observed between the heating and cooling curves because the rate of association of DNA strands to form triplexes or duplexes is, slower than the rate of dissociation (at lower concentrations). To understand the effect of tethered guanidiniums on the association event, relative heating and cooling profiles were examined for the butyl/propyl or the unmodified complexes under identical conditions. Figure 14 shows the results of such hysteresis experiments.

Factors that increase the rate of association should help in enhancing the association rate and hence decrease the hysteresis effect. As seen from Figure 14, no considerable difference in the hysteresis patterns of the guanidinum tethered ODNs

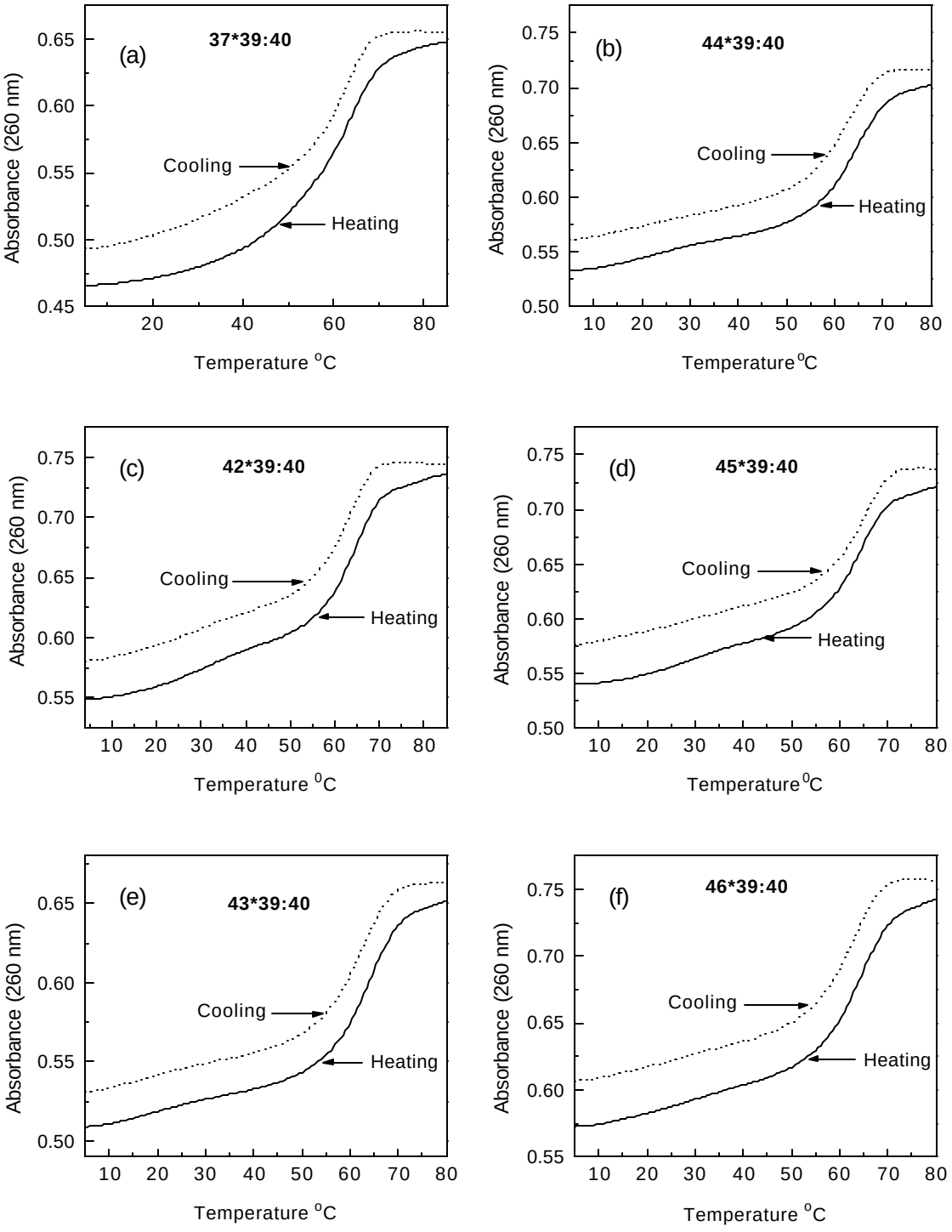


Figure 14. Hysteresis of the denaturation and re-naturation phenomena of the triplexes (a) Unmodified complex **37*39:40,** (b) butyl guanidinium ODN complex **44*39:40** (c) propyl guanidinium ODN complex **42*39:40** (d) butyl guanidinium ODN complex **45*39:40** (e) propyl guanidinium ODN complex **43*39:40** (f) propyl guanidinium ODN complex **46*39:40**.

and the unmodified ODNs were observed. This suggests that the positive charges present in the propyl/butyl guanidinium group do not significantly contribute to accelerate the reassociation by electrostatic interactions. This is unlike dC-(N^4-sperminyl) ODNs which enhance the triplex stability as well as accelerate the reassociation by electrostatic effects.[16]

Additionally, it is possible that the N3-pK_a may differ in the parent and modified cytidines. In order to check this possibility the N3-pK_a of N^4-propyl guanidinium was measured (see experimental) and found to be 3.8 which is much lower than in unmodified cytidine (4.4). *This suggests that protonation of dC in presence of pre-exisiting charge in guanidinium is unfavourable.* In this case, protonation of the modified cytidine would be less likely to occur at pH 7.0 and this results in the formation of only a single Hoogsteen hydrogen-bond to the C^6=O of G in the G:C base-pair of the target (Figure 15). This may perhaps be a cause for destabilization of the triplex.

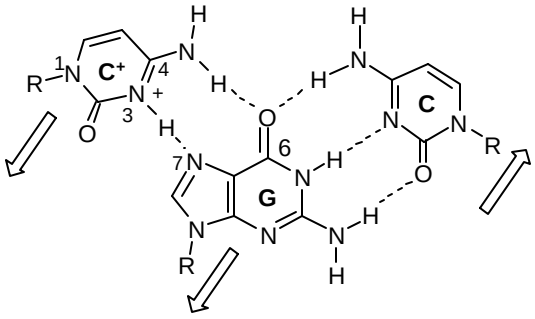


Figure 15. C^+*G:C triad which shows the N3-Cytidine protonation for Hoogsteen bond formation.

2.11. CONCLUSIONS

This section describes the synthesis of "convertible nucleoside" O^4-2,6-dimethylphenyl thymidine-3'-O-phosphoramidite starting from thymidine. The "convertible nucleoside" was successfully incorporated into ODNs and functionalized

into guanidinopropyl or guanidinobutyl derivatives by post-synthetic reactions. The functionalized ODNs were unambiguously characterized by ES-MS/MALDI-TOF mass spectroscopic techniques and used for biophysical studies.

The hybridization studies of the functionalized ODNs were carried out with their complementary strands and indicated that both duplexes and triplexes formed were of lower stability. The reduced stability of the ODN complexes may be because of the existence as the *syn* isomer (which is the major one) in the case of N^4-alkylated cytidines. In addition to the *syn* conformation, the N3 pK_a of N^4-guanidino substituted cytidine being lower than that of cytidine may contribute to the decreased stability of the triplex complexes.

Covalent hybrid oligonucleotides bearing effectors that range from small hydrocarbons to peptides have been used as enzymatic[20,21] and non-enzymatic[22,23] restriction endonucleases, detection probes[24,25] and photoaffinity agents.[26] In view of these, the findings on covalent and non-covalent interactions of guanidinium groups in ODNs may have utility in biochemical studies.

During the course of this work, reports on other C4 substituted pyrimidines with different donor/acceptor groups have appeared.[27] Very recently, the synthesis of modified oligonucleotides containing 4-guanidino-2-pyrimidinone nucleobases[28] has been reported to have potential as protonated cytidine analogues. The findings on covalent and non-covalent interaction of guanidinium groups in ODNs may have utility in biochemical studies.

2.12. REFERENCES

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General Remarks

All reactions requiring anhydrous conditions were performed under a positive pressure of argon using oven-dried glassware (120°C) which was cooled in a desiccator. Commercial reagents for synthesis were obtained from Aldrich Chemical Company (U.S.A.), Lancaster (U.K) or S. D. Fine Chemicals (India) and were generally of the highest purity available. The solvents used for extraction and chromatography were of technical grade and distilled before use. Solvents used for reactions were of the highest quality available and dried, wherever necessary according to the literature procedures.[i] TLC was performed on E. Merck pre-coated silica gel plates 60 HF254 using the appropriate solvent systems and were visualized under UV light, iodine and/or perchloric acid spray (60% ethanol) followed by charring.

^{1}H NMR spectra were recorded on a Bruker AC-200 spectrophotometer at 200 MHz, using TMS as an internal standard. ^{31}P NMR spectra were recorded on the same instrument at 81 MHz with 85% H_3PO_4 as external reference. ^{13}C NMR spectra were recorded at 50 MHz on the same instrument or on 500 MHz (Bruker DRX 500) at 125 MHz. The NMR values are quoted in δ (ppm) scale.

FABMS were recorded on VG Autospec (M Series). Mass spectrometry analyses of the oligonucleotides have been performed using a Voyager-Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction. Optical rotations were measured on JASCO DIP-181 polarimeter. All melting points are uncorrected in degrees Celsius and were recorded on an electrothermal melting point apparatus. Flash chromatography was performed using silica gel (230-400 mesh) from SRL India.

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i. Armarego, W. L. F., Perrin, D. D. (Eds.) *Purification of Laboratory Chemicals*, Butterworth-Heineman **1996**, The Bath Press, Bath, Great Britain.

2.13. EXPERIMENTAL (SECTION A)

2.13.1. Synthesis of Compounds 4-12

N,N'-Bis-(benzyloxycarbonyl)-S-methylisothiourea ***4***[ii]

To a cooled solution of *S*-Me-isothiourea sulphate (1.11 g, 4 mmol) in 1N NaOH (8 mL), were added $NaHCO_3$ (0.75 g, 7.0 mmol), CH_2Cl_2 (12 mL) and benzyl chloroformate (1.72 mL, 12 mmol). The reaction mixture was stirred for 3h and the organic layer was separated out. The aqueous layer was back extracted with CH_2Cl_2 (2 x 20 mL) and the extracts pooled with the organic layer which on concentration gave the crude product. Column chromatography (silica gel, light petroleum/EtOAc) gave the pure product (1.4 g, 97%) as a wax.

TLC: (light petroleum:EtOAc 1:4) R_f 0.60.

^{1}H NMR, $CDCl_3$ δ: 11.90 (bs, 1H, N<u>H</u>), 7.50-7.30 (m, 10H, Ar<u>H</u>), 5.25 (s, 4H, -O-C<u>H</u>$_2$-Ph), 2.45 (s, 3H, S-C<u>H</u>$_3$).

Tetra-benzyloxycarbonyl-N1,N14-bisguanidino-spermine ***5***

To spermine (0.4 g, 2 mmol, free base) were added N,N'-bis(benzyloxycarbonyl)-*S*-methylisothiourea (1.6 g, 4.5 mmol) and triethylamine (1.42 mL, 10.0 mmol). The reaction mixture was refluxed for 4h when TLC indicated the completion of reaction. The reaction was concentrated, dissolved in CH_2Cl_2 (50 mL) and washed with $NaHCO_3$ solution. The organic layer was dried over anhy. Na_2SO_4 to get the crude product. This was purified by column chromatography (silica gel, CH_2Cl_2/MeOH) to get pure product **5** (0.62 g, 37%) as a wax.

TLC: (CH_2Cl_2 MeOH 9:1) R_f 0.45.

ii. Bergeron, R. J., Mcmanis, J. S. *J. Org. Chem.* **1987**, *52*, 1700-1703.

^{1}H NMR, $CDCl_3$ δ: 8.50 (bm, NH), 7.60-7.15 (m, 20H, ArH), 5.15 (2s, 8H, -O-CH_2-Ph), 3.50 (t, J = 6.6 Hz, 4H), 2.75 (t, 6.6 Hz, 4H), 2.55 (m, 4H), 1.90 (m, 4H), 1.55 (m, 4H).

^{13}C NMR, $CDCl_3$ δ: 163.7, 163.1, 158.7, 157.30, 153.8, 138.0, 136.5, 134.7, 129.1, 128.9, 128.7, 128.6, 128.3, 127.7, 68.8, 66.6, 47.8, 45.5, 45.1, 39.1, 38.0, 26.5, 24.8, 22.8, 21.2.

*N1,N14-Bisguanidino-spermine **6***

To tetra-benzyloxycarbonyl-N1,N14-bisguanidino-spermine **5** in 5% HCOOH-MeOH (10 mL) was added Pd-C (10%, 0.11 g) and stirred at r.t. for 3h when TLC indicated the disappearance of starting material, the reaction mixture was filtered through a celite-pad, washed with MeOH and concentrated to get the pure product **6** (0.10 g, 88%) as the formate salt.

^{1}H NMR, D_2O δ: 8.40 (s, 4H, HCOO$^-$), 3.25 (t, J = 6.9 Hz, 4H), 3.15-2.95 (m, 8H), 1.95 (m, 4H), 1.70 (m, 4H).

^{13}C NMR, D_2O δ: 171.9 (HCOO$^-$), 158.0 & 156.5 [NH_2(C=NH)NH-], 56.3, 54.2, 47.8, 45.7, 40.6, 39.1, 25.9, 24.2, 23.7, 22.0.

*N,N'-Bis(2-cyanoethyl) butanediamine **7***[iii]

To a solution of 1,4-diaminobutane (5.80 g, 66 mmol) in ether (5 mL), acrylonitrile (7.0 g 132 mmol) was added with stirring in about 8h. The resulting solution was stirred at r.t. for 15 h, then heated on a steam-bath for 1h, and stirring continued at r.t. (room temperature) for 1h. The reaction mixture was cooled and a little excess ethanolic-HCl (20 mL) was added to get the hydrochloride salt. Recrystallization from ethanol:water (3:1) afforded the pure product **7** (14.1g, 79%).

iii. Schultz, H. P. *J. Am. Chem. Soc*. **1948**, *70*, 2666-2667.

MS: 194 (M^+)

m.p. 220°C.

^{1}H, NMR, D_2O δ: 3.53 (t, J = 6.8 Hz, 4H), 3.25 (m, 4H), 3.10 (t, J = 6.7 Hz, 4H), 1.80 (m, 4H).

^{13}C NMR, D_2O δ: 118.6 (CN), 47.8, 43.5, 23.3, 15.7.

*N,N'-Bis(2-cyanoethyl)-N,N'-di(benzyl)-butanediamine **8***

To N,N'-bis(2-cyanoethyl) butanediamine **7** (3.5 g, 18 mmol) in anhy. CH_3CN (150 mL), were added anhy. K_2CO_3 (12.4 g, 90 mmol) and benzyl bromide (6.4 mL, 54 mmol) and stirred overnight at r.t.. The reaction mixture was filtered through a sintered funnel to remove the residue of potassium salts and washed with CH_3CN. The filtrates were pooled and concentrated to dryness to get the crude product. Column chromatography (silica gel, light petroleum/EtOAc) afforded the pure product **8** (3.70 g, 56%) as a gum.

TLC: (light petroleum:EtOAc 1:1) R_f 0.65

^{1}H NMR, $CDCl_3$ δ: 7.50-7.20 (m, 10 H, ArH), 3.60 (s, 4H, $-CH_2$-Ph), 2.75 (t, J = 7.2 Hz, 4H, $-CH_2$-CN), 2.55-2.30 (m, 8H, N-CH_2-CH_2), 1.60-1.45 (m, 4H).

*N5,N10-dibenzyl-spermine **9***

Ra-Ni (0.35 g) was added to a solution of **8** (1.0 g, 2.69 mmol) in ethanol (10 mL) followed by sodium hydroxide solution (0.1 g of NaOH in 10 mL of 95 % EtOH). The mixture was hydrogenated under 65 psi of H_2 for 18h. The catalyst was then filtered through a celite pad (taking care that it did not become dry), washed with ethanol and concentrated to get the diamine **9** (1.0 g, 98%) as a thick oil.

TLC: (CH_2Cl_2 MeOH 9:1) R_f 0.20.

^{1}H NMR, $CDCl_3$ δ: 7.40-7.20 (m, 10H, ArH), 3.50 (2s, 4H, -N-CH_2), 2.80-2.60 (m, 4H), 2.55-2.30 (m, 8H), 1.70-1.40 (m, 8H).

N5,N10-Dibenzyl-tetra-benzyloxycarbonyl-N1,N14-bisguanidino-spermine ***10***

To the diamine **9** (0.57 g, 1.5 mmol) in CH_2Cl_2 (30 mL), N,N'-bis(benzyloxycarbonyl)-S-methylisothiourea (1.08 g, 3.0 mmol) and triethylamine (1.5 mL 10.6 mmol) were added. The reaction mixture was refluxed for 7h when TLC indicated the completion of reaction. The reaction mixture upon concentration followed by the usual work-up gave the crude product which was purified by column chromatography (silica gel, CH_2Cl_2/MeOH) to get the pure product **10** (0.69 g, 46%) as a wax.

TLC: (CH_2Cl_2 MeOH 9.5:0.5) R_f 0.35.

^{1}H NMR, $CDCl_3$ δ: 11.90-11.60 (bs, 2H, NH), 8.40 (bm, 2H, NH), 7.60-7.10 (m, 30H, ArH), 5.20 (2s, 8H, -O-CH_2-Ph), 3.70-3.50 (bs, 4H, N-CH_2-Ph), 3.50-3.35 (m, 4H), 2.65-2.30 (m, 8H), 1.90-1.60 (m, 4H), 1.60-1.40 (m, 4H).

^{13}C NMR, $CDCl_3$ δ: 163.9, 156.0, 153.7, 137.0, 134.9, 129.3, 128.8, 128.5, 128.4, 128.3, 128.0, 127.3, 68.1, 67.3, 58.6, 53.6, 50.9, 39.6, 26.2, 24.3.

Tetra-benzyloxycarbonyl-N1,N10-bisguanidino-spermidine ***11***

N,N'-Bis(benzyloxycarbonyl)-*S*-methylisothiourea **4** (0.95 g, 2.66 mmol) and triethylamine (1.1 mL, 8 mmol) were added to spermidine (0.2 g, 1.2 mmol, free base) in DCM (30 mL) and refluxed for 7h when the TLC indicated the completion of reaction. The mixture was concentrated and the work-up gave the crude compound. It was purified by column chromatography (silica gel, CH_2Cl_2/MeOH) to get pure **11** (0.62 g, 63%) as a gum.

TLC: (CH_2Cl_2 MeOH 9.5:0.5) R_f 0.45.

^{1}H NMR, $CDCl_3$ δ: 8.60 (m, 1H, NH), 8.35 (m, 1H, NH), 7.50-7.20 (m, 20 H, ArH), 5.15 (3 x s, 8H, N-CH_2-Ph), 3.55-3.40 (m, 4H), 2.7 (m, 4H), 1.85 (m, 2H), 1.60 (m, 4H).

*N1,N10-bisguanidino-spermidine **12***

To 0.51 g (0.66 mmol) of tetra-benzyloxycarbonyl-spermidine bisguanidinum **11** in 5% HCOOH-MeOH (25 mL), Pd-C (10%, 0.25 g) was added and stirred at r.t. TLC showed the completion of reaction after 3h. The reaction mixture was filtered through a celite pad and the filtrate was concentrated to dryness to get the pure product **12** (0.23 g, 95%) as its formate salt.

^{1}H NMR, D_2O δ: 8.45 (s, 3H, $HCOO^-$), 3.45-2.95 (m, 8H), 2.00 (m, 2H) , 1.75 (m, 4H).

^{13}C NMR, D_2O δ: 171.7 ($HCOO^-$), 166.7, 157.9, 48.0, 45.6, 41.4, 39.2, 26.0, 25.9, 23.7.

2.13.2. Oligonucleotide synthesis

The synthesis of ODNs was carried out on a Pharmacia GA Plus automated synthesizer. CPG resins and the protected nucleoside β-cyanoethylphosphoramidite derivatives of 2'-dA, 2'-dC, 2'-dG and 2'-dT were obtained from Amersham Pharmacia.

Dry solvents were used for DNA synthesis. Acetonitrile was distilled twice over P_2O_5 and finally over CaH_2 immediately before use. Dichloroethane was dried by distilling twice over P_2O_5. The commercially available amidites were dissolved in dry acetonitrile to obtain 0.1 M solutions and 4Å molecular sieves were added to them to remove traces of moisture. Tetrazole was employed as the activator for the coupling reactions. For oxidation, after each coupling, 0.01 M iodine in collidine, water and acetonitrile, while for capping 20% acetic anhydride in acetonitrile was used.

The oligonucleotides **13-18** were synthesized at 0.2 μ mole scale with 'trityl off'. The crude oligonucleotides obtained after deprotection with NH_4OH were desalted and the purity of ODNs was checked by RP-HPLC (Refer Section 3.20.2 for HPLC conditions) which showed >95% purity.

2.13.3. Sample Preparation for Duplex and Triplex Melting Studies

The duplexes were constituted by taking equimolar amounts (1.0 μM) of the appropriate single strands based on the UV absorbance at 260 nm calculated using the molar extinction coefficients of A = 15.4, C = 7.3, G = 11.7 and T = 8.8 $cm^2/\mu mol$ in 2 mL of TRIS buffer (25 mM TRIS buffer, 100 mM NaCl, 0.1 mM EDTA, pH 7.0). To the sample the desired polyamines/guanidines were added to attain a final concentration of 1mM. The samples were annealed by heating at 80°C for 2 minutes, followed by slow cooling to room temperature and refrigeration for at least 4h prior to commencing the experiments. The ODN duplexes constituted in the absence of polyamines/guanidines served as the references.

In a similar manner the samples for triplex studies were prepared in TRIS buffer (25 mM TRIS, 100 mM NaCl, 0.1 mM EDTA, pH 7.0) and sodium acetate buffer (sodium acetate, 50 mM, 100 mM NaCl, pH 5.8).

2.13.4. UV-Tm Studies

The thermal stability of duplexes and triplexes was measured by following UV absorbance changes at 260 nm in the temperature range 5-80°C with a heating rate of 0.5°C/min, using a Perkin Elmer Lambda 15 UV/VIS spectrophotometer, equipped with a water jacketed cell holder and a Julabo temperature programmer. Below 15°C, the cell compartment was flushed with nitrogen gas to prevent condensation of water on the UV cells. *For all the UV-Tm experiments, the Tms [the duplex dissociation*

temperature (Tm) or the triplex dissociation temperatures (Tm1)] were determined from the midpoint of the transition/s in the plots of normalized absorbance versus temperature and further confirmed by the maxima in differential (dA/dT Vs Temperature) curves. The experiments were repeated at least three times and are accurate to ±0.5°C.

2.14. EXPERIMENTAL (SECTION B)

2.14.1. Synthesis of Compounds 19-33

1H-Pyrazole-1-carboxamidine hydrochloride ***19***[iv]

Pyrazole (3.4 g, 50 mmol) and cyanamide (2.1 g, 50 mmol) were dissolved in dioxane (50 mL) to which dioxane containing HCl was added (4N, 13 mL). The mixture was refluxed with stirring for 2h under nitrogen. It was cooled to r.t., dry ether (15 mL) was added and the mixture was allowed to stand for 30 min. The white crystalline product was filtered out, washed with anhy. ether and dried to get pure **19** (7.0 g, 96%).
m.p. 176-178°C.

^{1}H NMR, D_2O δ: 8.35 (d, 1H), 8.00 (d, 1H), 6.75 (d, 1H).

^{13}C NMR, D_2O δ: 153.1, 147.6, 131.4, 113.2.

3',5'-O-Diacetyl thymidine ***22***

Thymidine, **21** (2.5 g, 10.3 mmol) was suspended in dry pyridine (10 mL) to which acetic anhydride (3.1 mL, 31 mmol) was added and the mixture was stirred at r.t. overnight. The reaction mixture was concentrated to dryness, dissolved in CH_2Cl_2 (40 mL) and washed with 10% aqueous $NaHCO_3$ solution (20 mL). The organic layer was

iv. Bernatowicz, M. S., Wu, Y. Matsueda, G. R. *J. Org. Chem.* **1992**, *57*, 2497-2502.

dried over anhy. Na_2SO_4 and concentrated to get pure diacetyl thymidine, **22** (3.26 g, 97%).

m.p. 131°C. TLC: (CH_2Cl_2 MeOH 9:1) R_f 0.60.

^{1}H NMR, $CDCl_3$ δ: 9.10 (s, 1H, NH), 7.30 (s, 1H, H6-Thy), 6.35 (m, 1H, H1'), 5.25 (m, 1H, H3'), 4.40 (m, 2H, H5', H5''), 4.25 (m, 1H, H4'), 2.50 (m, 1H, H2'), 2.20 (m, 1H, H2''), 2.15 (2s, 6H, CO CH_3), 1.95 (s, 3H, Thy-CH_3)

*3',5'-O-Diacetyl-O^4-(2,6-dimethylphenyl)-thymidine **24***

Compound **22** (2.94 g, 9.0 mmol), freshly crystallised TPS-Cl (7.8 g, 25.7 mmol), triethylamine (5 mL, 36 mmol) and N,N'-dimethyl-4-aminopyridine (DMAP, 0.22 g, 1.8 mmol) were taken in dry CH_2Cl_2 (40 mL) and the mixture was stirred at room temperature for 8h when TLC indicated the formation of the sulfonate intermediate, **23**. 2,6-Dimethylphenol (5.5 g, 45.0 mmol), triethylamine (6 mL, 43.0 mmol) and a catalytic amount of DABCO (0.12 g, 1.1 mmol) were added into the reaction mixture and stirring was continued for another 16h. The reaction mixture was worked up as usual, to get the crude product. Purification by column chromatography (silica gel, CH_2Cl_2) furnished the desired compound **24** (3.02 g, 78%) as a white foam.

m.p. 60°C; TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.50.

^{1}H NMR, $CDCl_3$ δ: 7.70 (s, 1H, H6-Thy), 7.05 (s, 3H, ArH), 6.30 (m, 1H, H1'), 4.40 (m, 2H, H5', H5''), 4.30 (m, 1H, H4'), 2.70 (m, 1H, H2'), 2.25-2.05 (m, 16H, H2'', Ph-CH_3, 2 x -(CO)CH_3, Thy-CH_3).

^{13}C NMR, $CDCl_3$ δ: 169.5 ($COCH_3$), 168.8 (C4), 154.7 (C2), 148.8 (C6), 139.9, 129.3, 128.0,125.1 (Ar C), 103.4 (C5), 86.0 (C4'), 82.0 (C1'), 73.7 (C3'), 63.2 (C5'), 37.7 (C2'), 20.1 (CO CH_3), 5.8 (Ar-CH_3), 11.9 (Thy-CH_3).

O^4-(2,6-Dimethylphenyl)-2'-deoxythymidine ***25***

Compound **24** (2.23 g, 5.18 mmol) was stirred in saturated methanolic ammonia (10 mL) for 3h at ambient temperature to effect the hydrolysis of the *O*-acetyl groups. The reaction was monitored by TLC. After completion of the reaction, methanol and ammonia were completely removed and column chromatography (silica gel, CH_2Cl_2/MeOH) gave the pure product **25** (1.6 g, 89%).

m.p. >220°C (dec). TLC: (CH_2Cl_2:MeOH 9:1) R_f 0.30.

^{1}H NMR, $CDCl_3$:$DMSOd_6$ (6:4) d: 8.20 (s, 1H, H6), 7.00 (s, 3H, ArH), 6.15 (t, J = 6.2 Hz, 1H, H1'), 4.50 (brs, 2H, 5'- & 3'-OH), 4.30 (m, 1H, H3'), 3.85 (m, 1H, $H_{4'}$), 3.70 (m, 2H, H5', H5''), 2.35 (m, 1H, H2'), 2.15 (m, 10H, H2'', 2 x Ar-CH_3 & Thy-CH_3).

5'-O-DMT-O^4-(2,6-Dimethylphenyl)-2'-deoxythymidine ***26***

Compound **25** (0.94g, 2.59 mmol) was co-evaporated with dry pyridine and the residue dissolved in dry pyridine (10mL). DMTr-Cl (1.05g, 3.11 mmol) was added to the above solution and stirred at r.t. for 7h. The reaction mixture was concentrated, dissolved in DCM (25mL) and subjected to the usual work-up to get the crude product. The compound **26** (1.44 g, 83%) was obtained as a yellowish foam after column chromatography (silica gel, CH_2Cl_2/MeOH, 0.2% pyridine).

m.p. 115-117°C. TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.50.

^{1}H NMR, $CDCl_3$ 8.05 (s, 1H, H6-Thy), 7.50-7.25 (m, 9H, ArH), 7.05 (s, 3H, ArH), 6.85 (d, 4H, ArH), 6.35 (t, 1H, H1'), 4.55 (m, 1H, H3'), 4.15 (m, 1H, H4'), 3.8 (s, 6H, -OCH_3), 3.45 (m, 2H, H5', H5''), 2.4-2.0 (m, 8H, H2', H2'', Ar-$(CH_3)_2$, 1.75 (s, 3H, Thy-CH_3).

3'-O-(2-Cyanoethyl-N,N-diisoprpylphosphoramido)-5'-O-(4',4'-dimethoxytrityl)-O^4-(2,6-dimethylphenyl)-2'-deoxythymidine ***27***

Compound **26** (0.2 g, 0.3 mmol) was dried by co-evaporation with dry acetonitrile. The residue was dissolved in dry CH_2Cl_2 (1 mL), dry diisopropylethylamine (80 µL, 0.23 mmol) and chloro-N,N-diisopropyl-2-cyanoethoxy phoshphine (50 µL, 0.23 mmol) were added under Argon atmosphere. After 1h MeOH was added (0.1 mL) and the usual work-up gave the crude product which was purified by column chromatography (silica gel, CH_2Cl_2/EtOAc/triethylamine).

TLC: (CH_2Cl_2:EtOAc 1:1) R_f 0.80.

^{31}P NMR, $CDCl_3$ d: 150.31, 149.85.

*5'-O-DMT-5-Methyl-N^4-(4-guanidinopropyl)-2'-deoxycytidine hydrochloride **30***

Compound **26** (0.42 g, 0.64 mmol) was dissolved in dry pyridine (1 mL) and treated with 1,3-diaminopropane (0.5 mL, 6 mmol) at 65°C for 36h. The reaction mixture was concentrated to dryness and the residue was dissolved in CH_2Cl_2 (20 mL), washed with water (5 mL) and the organic layer was concentrated to get **28** as a pasty mass. To the compound **28** (0.25 g, 0.4 mmol) in DMF (0.5 mL), 1-H-pyrazole carboxamidine hydrochloride (0.06 g, 0.4 mmol), diisopropylethylamine (0.07 mL, 0.4 mmol) were added and stirred under argon atmosphere for 16h. The reaction mixture was concentrated and column chromatography (silica gel, CH_2Cl_2/MeOH containing 0.2% pyridine) gave the pure product **30** (0.25 g, 90%).

m.p. 155-160°C. TLC: (CH_2Cl_2:MeOH:NH_4OH 4:1:0.2) R_f 0.55.

MS (FAB): 643 (M^+).

^{1}H NMR, $CDCl_3$ 8.55 (s, 1H, H6), 7.50-6.90 (m, 9H, ArH), 6.80 (d, 4H, ArH), 6.30 (m, 1H, H1'), 4.60 (m, 1H, H3'), 4.10 (m, 1H, H4'), 3.70 (s, 6H, -OCH_3), 3.55-3.05 (m, 6H, H5', H5'' & 2 x CH_2), 2.40-2.05 (m, 2H, H2', H2''), 2.05-1.80 (m, 2H, CH_2), 1.90 1.55 (s, 3H, Thy-CH_3).

5'-O-DMT-5-Methyl-N^4-(4-guanidinobutyl)-2'-deoxycytidine hydrochloride ***31***

The same procedure for the synthesis of **30** was adopted here using compound **26** (0.52 g, 0.8 mmol) in pyridine (1 mL), 1,4-diaminobutane (0.8 mL, 8 mmol) to get **29** which on treatment with 1-H-pyrazole carboxamidine hydrochloride (0.12 g, 0.8 mmol), diisopropylethylamine (0.14 mL, 0.8 mmol) gave the product **31** (0.42 g, 75%).

TLC: (CH_2Cl_2:MeOH:NH_4OH 4:1:0.2) R_f 0.50.

MS (FAB): 657 (M^+)

^{1}H NMR, $CDCl_3$ 7.50-6.90 (m, 10H, ArH, H6), 6.75 (d, 4H, ArH), 6.30 (m, 1H, H1'), 4.50 (m, 1H, H3'), 4.05 (m, 1H, H4'), 3.65 (s, 6H, -OCH_3), 3.50-3.00 (m, 6H, H5', H5'' & 2 x CH_2), 2.60-2.35 (m, 1H, H2'), 2.20-2.00 (m, 1H, H2''), 1.80-1.25 (m, 7H, Thy CH_3 & CH_2-CH_2).

N^4-(4-Guanidinopropyl)-5-methyl-2'-deoxycytidine ***32***

Compound **30** (0.1 g, 0.15 mmol) was stirred with methanolic HCl at room temperature for 5 min. The reaction mixture was evaporated to dryness and the residue was taken in water (2 mL) and washed with diethylether (5 mL x 3) to remove the 4,4'-dimethoxytritanol. The aqueous layer on lyophilization gave the pure product **32** (0.051 g, 90%).

^{1}H NMR, MeOH-d_5 8.13 (s, 1H, H6), 6.14 (t, 1H, H1'), 4.32 (m, 1H, H3'), 3.76 (m, 1H, H4'), 3.50 (m, 6H, H5', H5'' & 2 x CH_2), 2.40-2.05 (m, 2H, H2', H2''), 2.05-1.80 (m, 2H, CH_2), 1.55 (s, 3H, Thy-CH_3).

4-N-(4-Guanidinobutyl)-5-methyl-2'-deoxycytidine hydrochloride ***33***

Compound **31** (0.1 g, 0.14mmol) on detritylation, as described for compond **34**, gave compound **33** (0.047 g, 86%).

2.14.2. Determination of N3 pK_a of Modified dC Nucleosides 32 and 33

The N3 pK_a of **32**, and **33** 5-methyl-N^4-(4-aminopropyl/butyl)-2'-deoxycytidine were determined by titration of their individual aqueous solution (2 mg/mL) with aqueous NaOH (5 mM) by incremental addition of aliquots of 10 μL, at 25°C under constant stirring. After each addition the solution was allowed to equilibrate for 2 min followed by pH measurements using standard pH electrode. pK_a were determined from the plots of volume of NaOH used against corresponding pHs of the solution. pK_as of **32** and **33** were found to be 3.8 and 3.9 respectively.

2.14.3. Oligonucleotide Synthesis

The synthesis was carried out on a Pharmacia GA Plus automated synthesizer. CPG resins and the natural protected nucleoside derivatives 2'-dA-PAC-CED, 2'-dC-*i*Bu-CED, 2'-dG-PAC-CED and 2'-T-CED were obtained from Amersham Pharmacia. The phenoxyacetyl and isobutyryl protecting groups enable nucleoside deprotection in a shorter time. The oligonucleotides **34-40** were synthesized using the phosphoramidite **27** along with other standard PAC-protected β-cyanoethylphosphoramidites. The coupling yields of **27**, as quantified by the released DMT cation, were approximately 95% compared to the average yield with unmodified amidites ~99%.

The oligonucleotides were deprotected and cleaved from the resin by incubation of the resin for 8h at r.t. in NH_4OH in a Wheaton vial. After the cleavage reaction, the excess of NH_3 was removed by bubbling argon through the solution for about 1h. It was then lyophilized, desalted and checked for purity by HPLC.

A typical synthesis report of the solid phase synthesis of ODN **36** is given in page No. 109. The Scheme outlines the various steps involved in solid phase ODN synthesis using phosphoramidite strategy.

Gene Assembler Plus (10/11/1998)

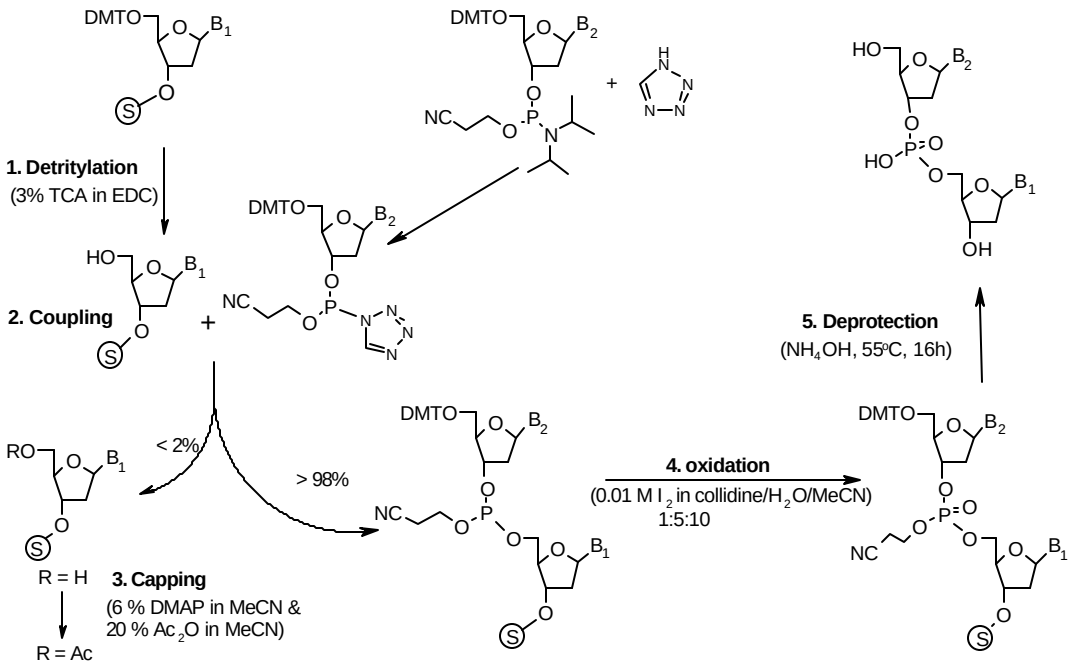
Sequence:	PSPJ-16	Scale:	0.2 micromole
Sequence Length:	18	Column:	1
Final Detritylation:	Yes	Coupling Efficiency Threshold:	40%

Position	Base	Retention min	Duration min	Peak ht %FS	Acc Area % min	Last eff %	Ave eff %
18	T	0.65	0.81	352	58.23	-	-
17	T	0.54	0.82	417	68.23	-	-
16	T	0.54	0.90	396	68.11	99.8	99.8
15	T	0.54	0.79	400	66.58	97.8	98.8
14	C	0.52	0.79	433	64.73	-	98.8
13	T	0.54	0.90	397	65.47	99.2	98.9
12	T	0.55	0.90	384	64.62	98.7	98.9
11	T	0.54	0.82	378	62.35	96.5	98.4
10	T	0.56	0.87	370	62.44	100.1	98.7
9	X	0.57	0.90	344	59.26	-	-
8	T	0.55	0.85	351	58.90	97.1	98.5
7	T	0.55	0.84	349	57.96	98.4	98.5
6	C	0.54	0.81	373	56.34	98.3	98.4
5	T	0.55	0.80	352	54.82	97.3	98.3
4	T	0.56	0.84	332	55.73	101.7	98.6
3	X	0.58	0.88	300	52.63	-	-
2	T	0.56	0.83	309	52.01	96.6	98.4
1	T	0.56	0.86	302	51.55	99.1	98.5

Total synthesis yield from start = 77.4%

Solid Phase synthesis report of ODN **36** on Pharmacia GA Plus DNA synthesizer;

X = O^4-(2,6-dimethylphenyl)-2'-deoxythymidine.



Scheme showing a single cycle involved in the Solid phase synthesis of ODNs

2.14.4. Oligonucleotides Having N^4-3-Guanidinopropyl 5-Me-2'-dC Adducts

Nucleotide solutions **34**, **35** and **36** (~3 OD each) were incubated with 1,3-diaminopropane in water (0.1 M 400 µL) at 65°C for 15h. Analytical HPLC showed the reactions to be complete. ODNs were passed through Sephadex G-15 column (2 cm x 10 cm) and eluted with water. Fractions of 0.5 mL were collected and those having UV absorbance at 260 nm were pooled and concentrated. The ODNs eluted approximately in 10^{th} to 15^{th} fractions.

Oligonucleotide solutions obtained above were treated with a solution of 0.2 M pyrazole carboxamidine hydrochloride (200 µL, pH 10.5 - adjusted using 0.4 M K_2CO_3), and heated at 65°C for 36h. The reaction was monitored by analytical HPLC, each time taking an aliquot of the reaction mixture passing it through a small bed of G-15 Sephadex, and analyzing by HPLC. The ODNs were characterized by MALDI-TOF mass spectroscopy (negative-ion mode).

2.14.5. Oligonucleotides Having N^4-4-Guanidinobutyl-5-Me-2'-dC Adducts

Oligonucleotide solutions (~3 OD each) were treated with a solution of agmatine sulphate (400 µL, pH 11.0 adjusted using 1N NaOH) and heated at 65°C for 36h. In the case of ODN **36** the time required was 72h as indicated by HPLC analysis. N^4-4-guanidinobutyl-5-Me-2'-dC ODNs were isolated by following the procedures adopted for N^4-guanidinopropyl derivatives.

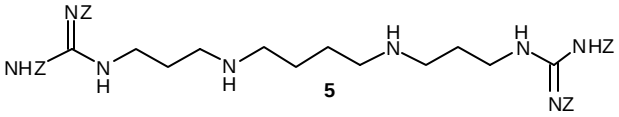
2.14.6. UV-Tm Experiments

The concentration of the DNA oligomers was calculated based on their absorption at 260nm and using the molar extinction co-efficients 7.3 $cm^2/\mu mol$ for N^4-guanidino-propyl/butyl-5-Me-2'-dC. Oligonucleotide complexes were constituted by mixing together stoichiometric quantities (0.5 µM) of the corresponding complementary

strands in 25 mM TRIS buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0 or 50 mM NaOAc, 100 mM NaCl and 0.1 mM EDTA at pH 5.5 or 25mM TRIS, 100 mM NaCl at pH 7.0 or 25mM TRIS, 100 mM NaCl, 20 mM Mg^{2+} at pH 7.0.

The samples were annealed as described in Section 2.13.3. and UV-Tm experiments carried out in a similar fashion (Section 2.13.4). Heating was carried out at a rate of 0.5°C/min and the absorbance at 260 nm was recorded every minute. In order to study the relative rates of melting and re-association, the complexes were held at 75°C for 5min upon completion of the heating process and then cooled to 5°C at a rate of 0.5°C/min. As for the heating process, the absorbance values were recorded every minute and plotted as a function of the temperature along with the heating profile. The Tm of the duplexes or triplexes were determined from the peaks in first derivative plots of the absorbance or percent hyperchromicity versus temperature graphs.

2.15. APPENDIX



5

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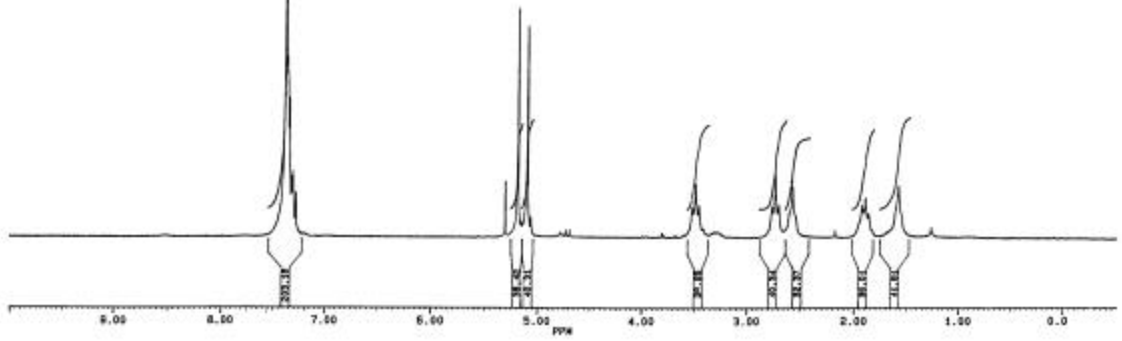

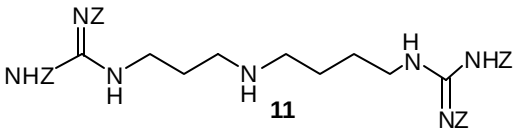

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(Z = Benzyloxycarbonyl)

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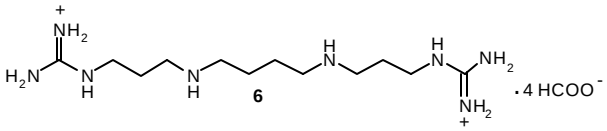

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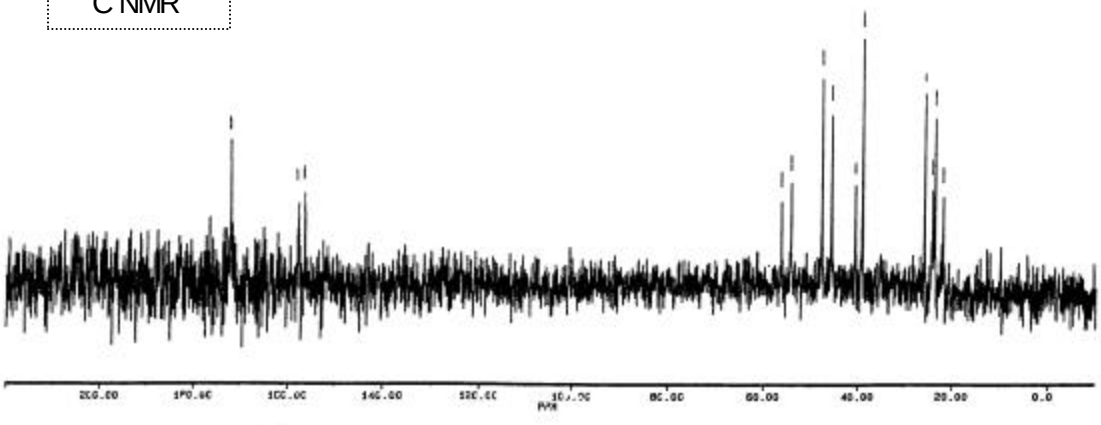
^{13}C NMR



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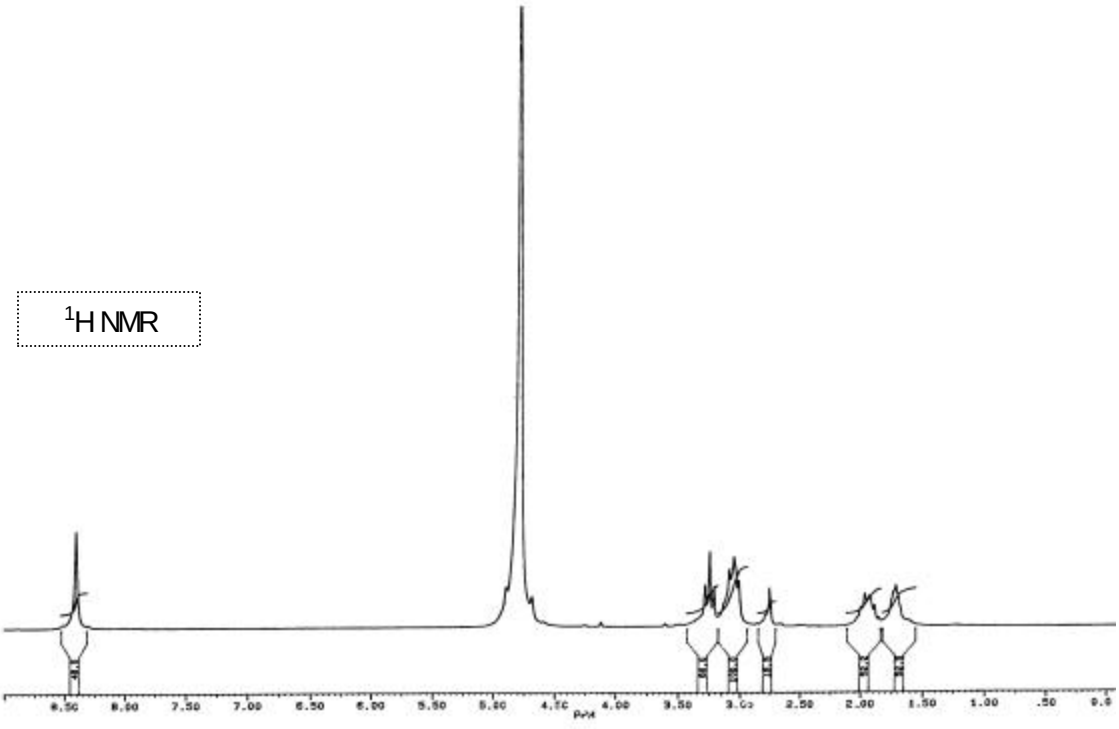

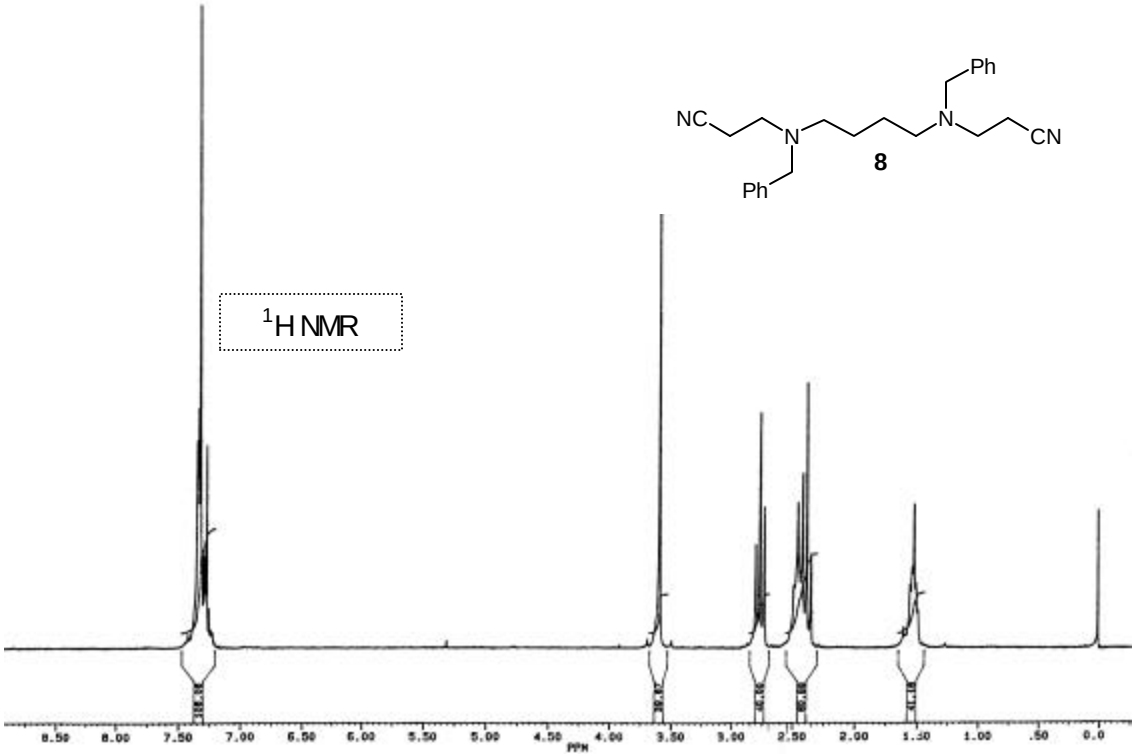
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N
CN
Ph
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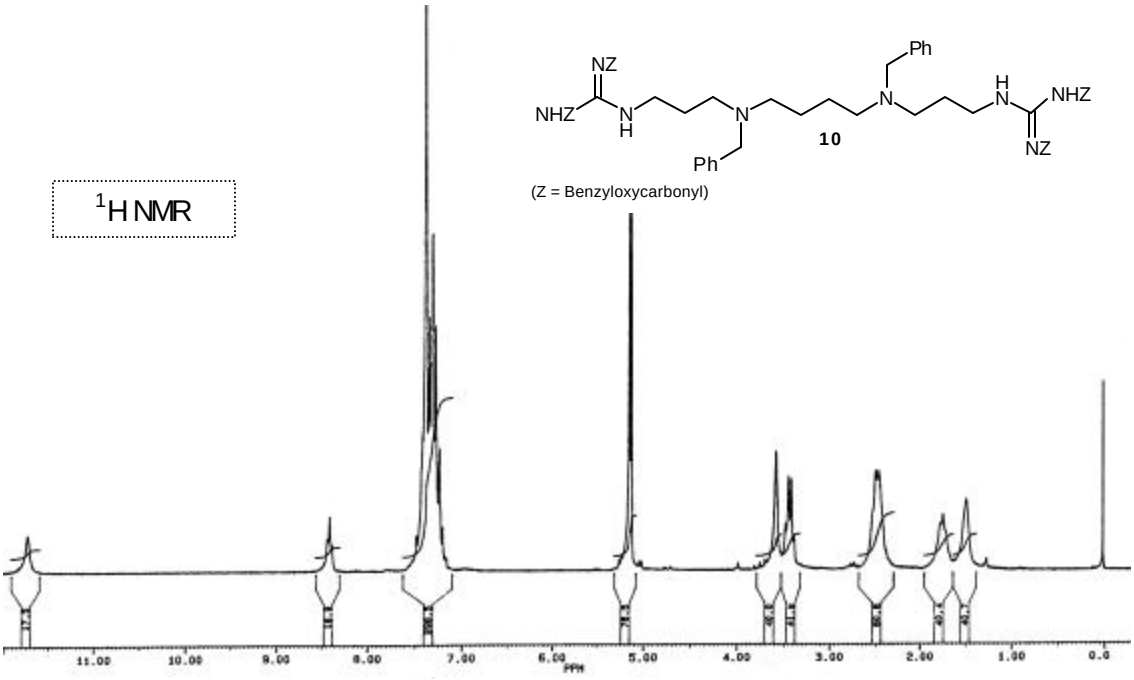
^{1}H NMR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Ph
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NZ
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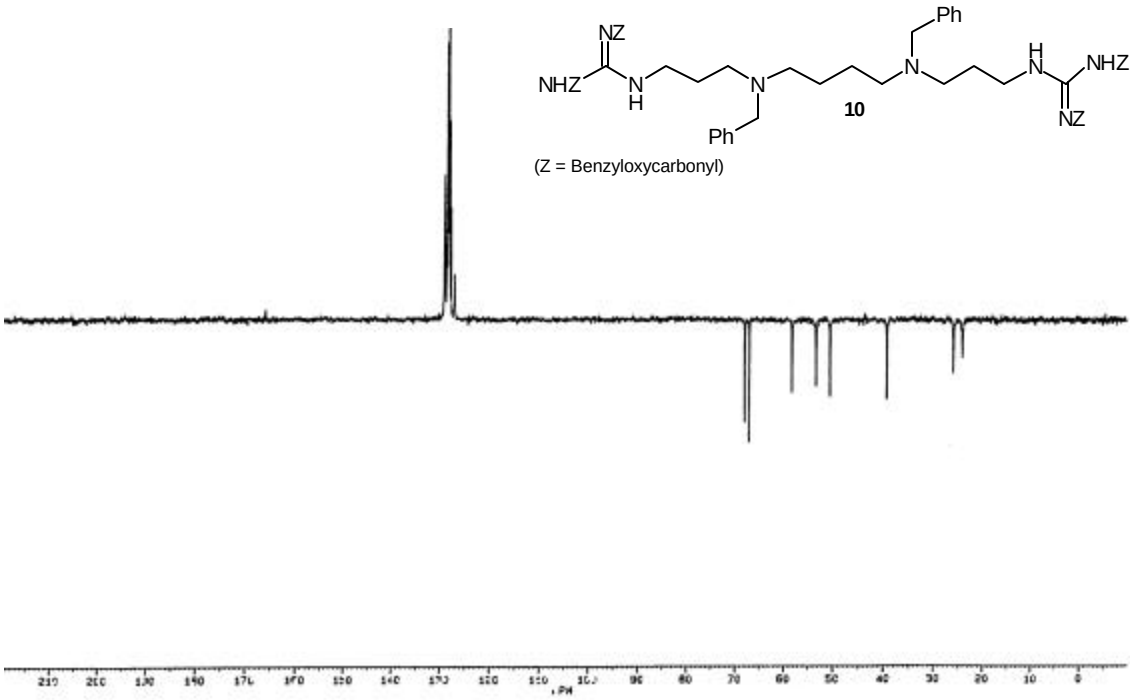
(Z = Benzyloxycarbonyl)
^{1}H NMR



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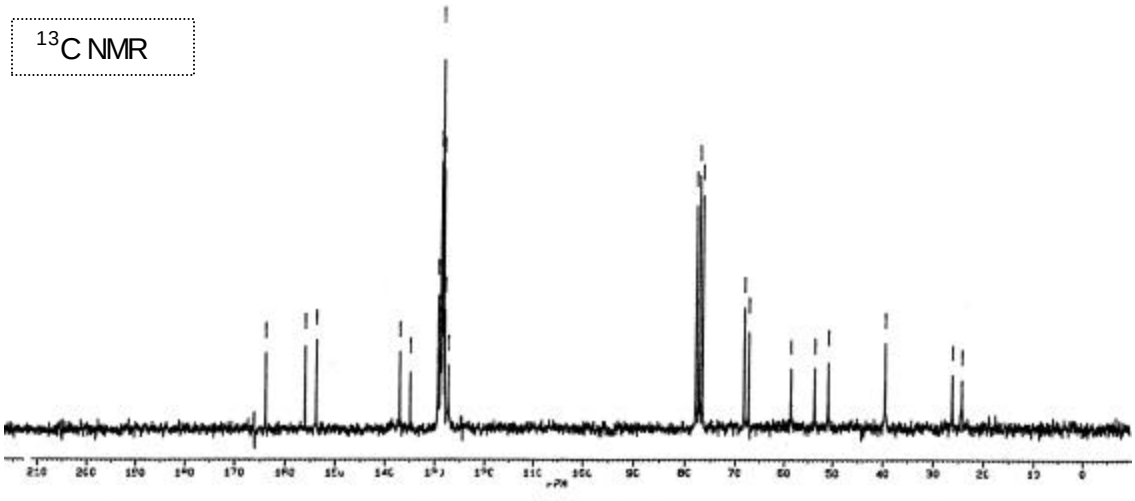
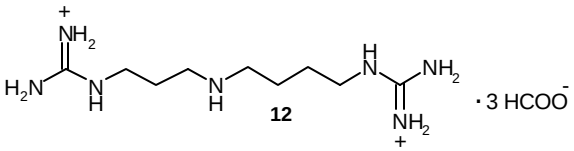
(Z = Benzyloxycarbonyl)

^{13}C NMR





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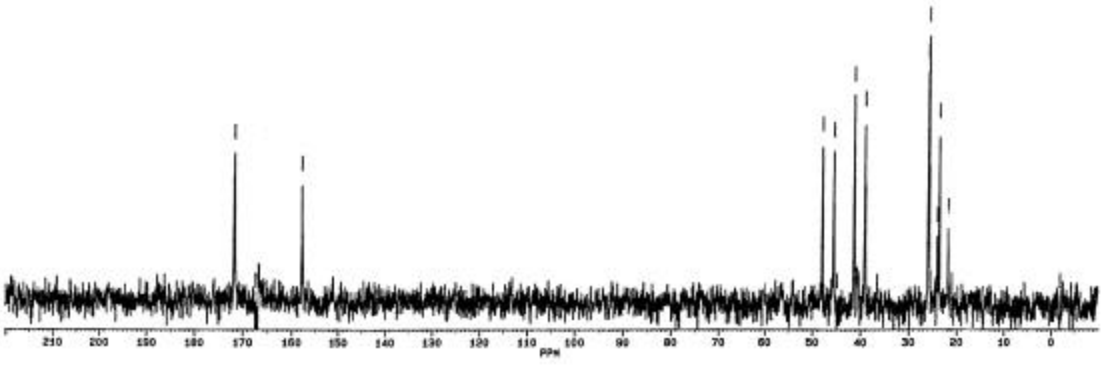
^{13}C NMR



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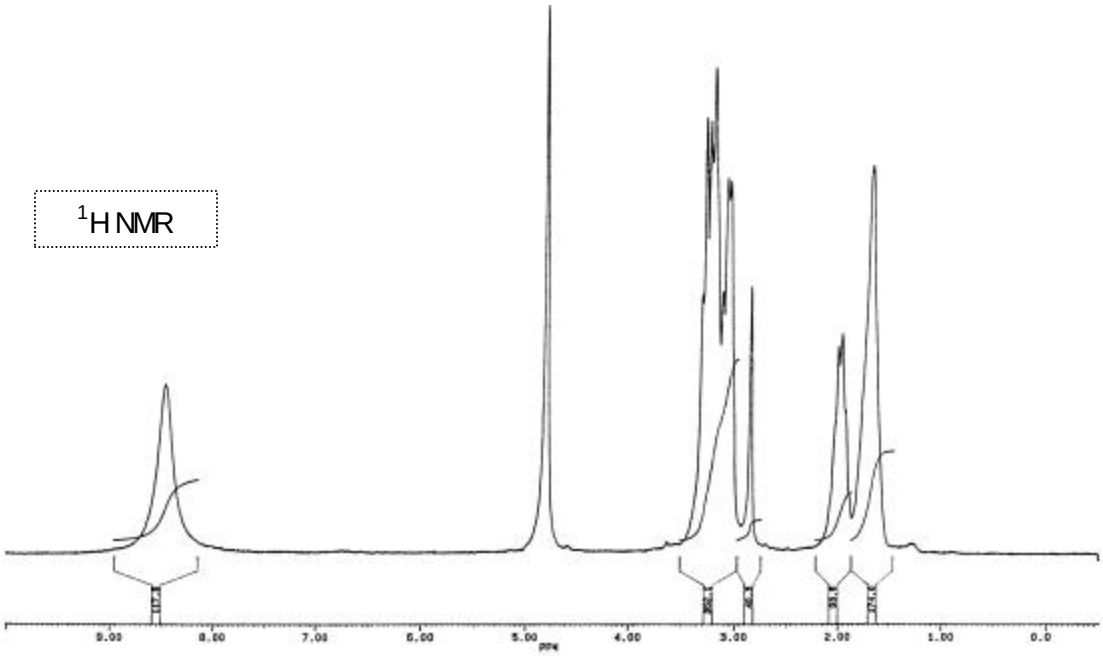

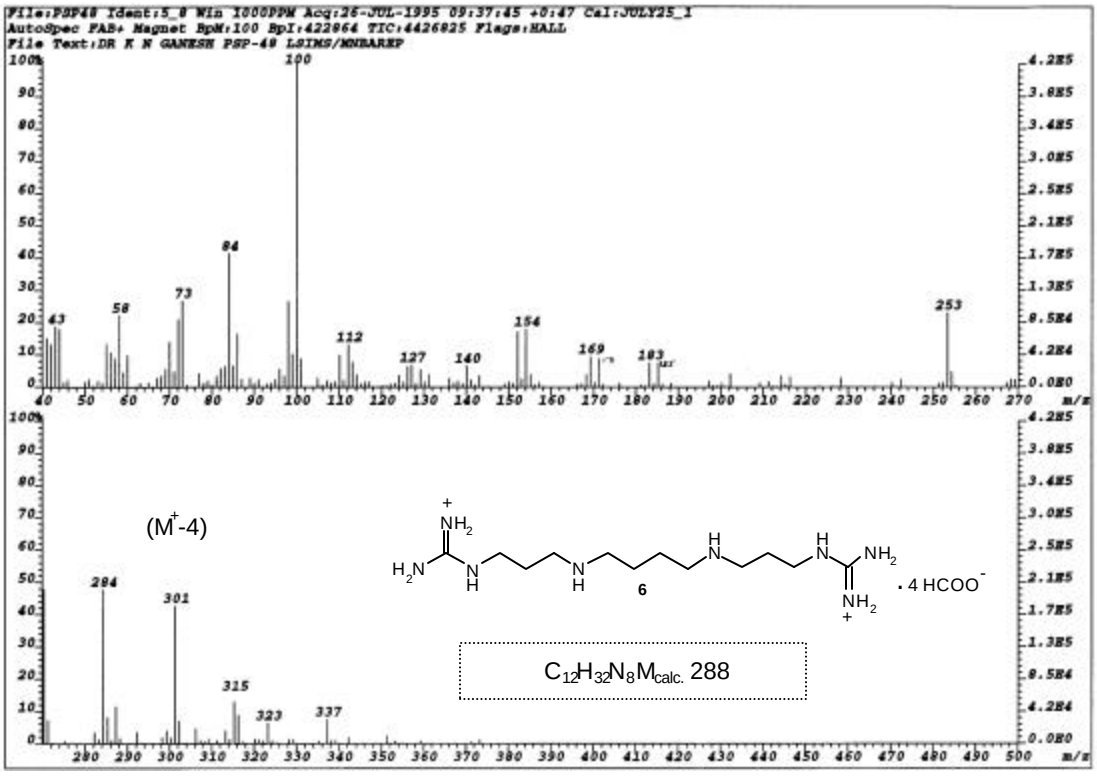
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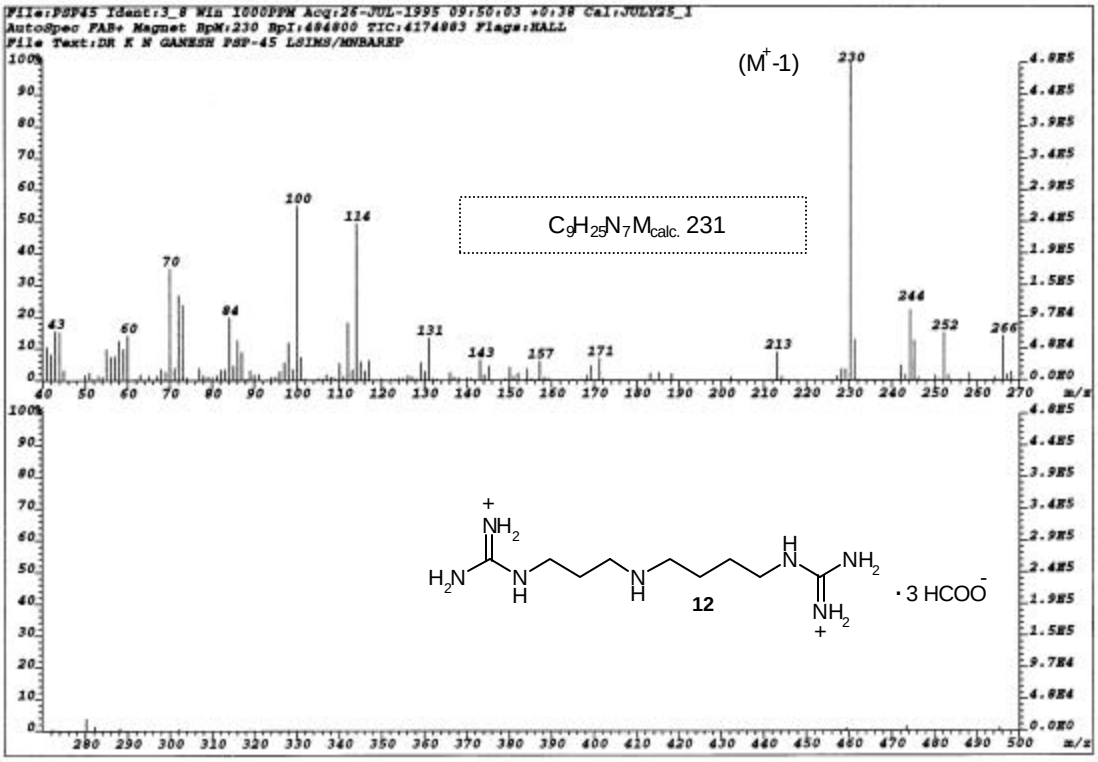
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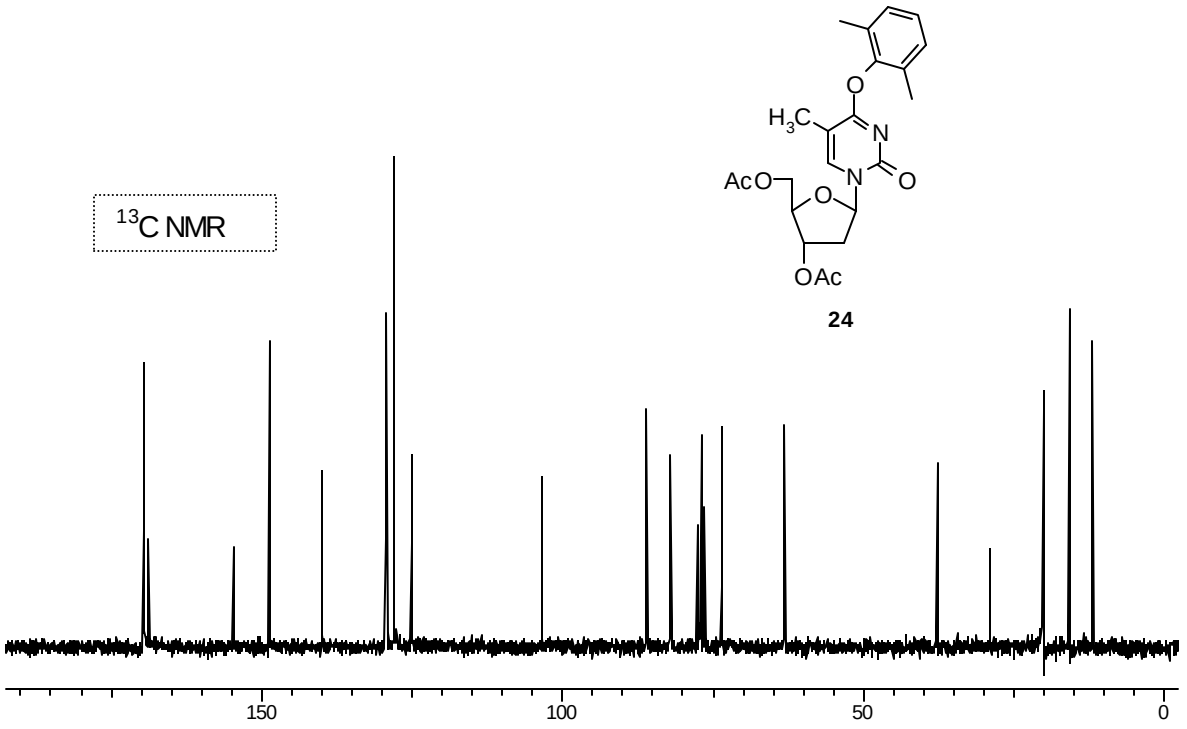
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50
0

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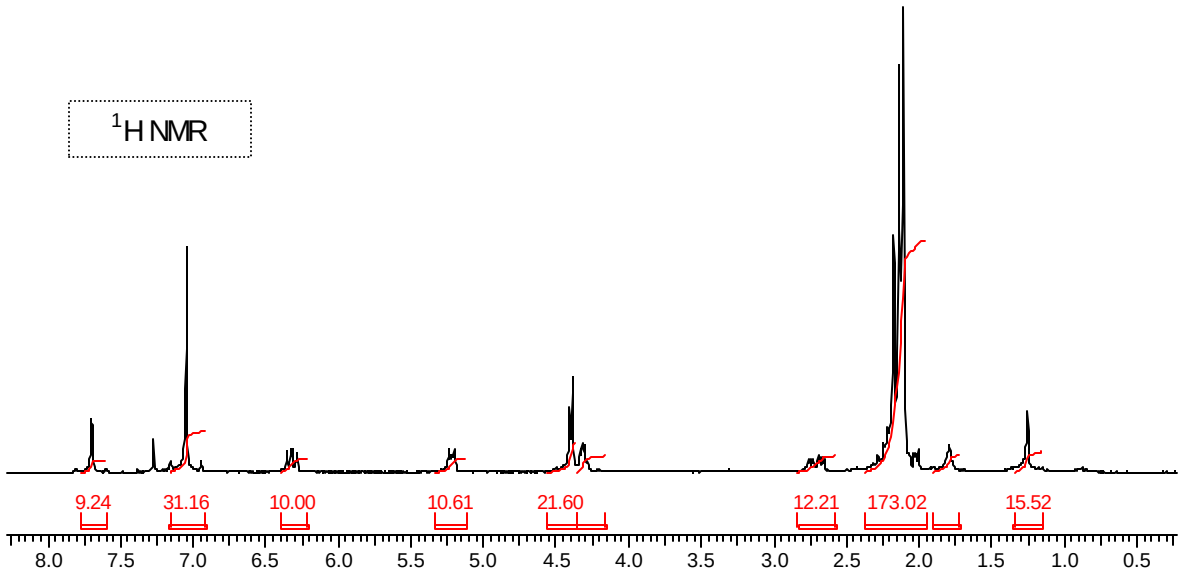
N
AcO
N
O
O
OAc

24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6.5
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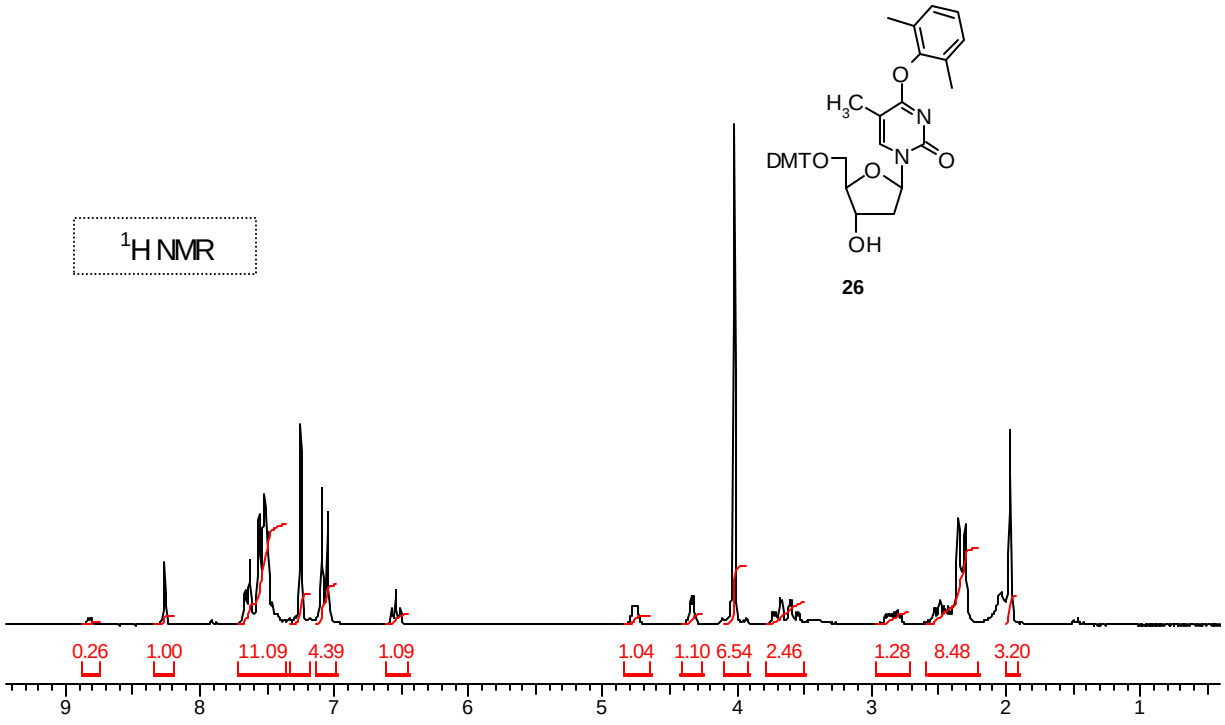
0.5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OH
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1.00
11.09
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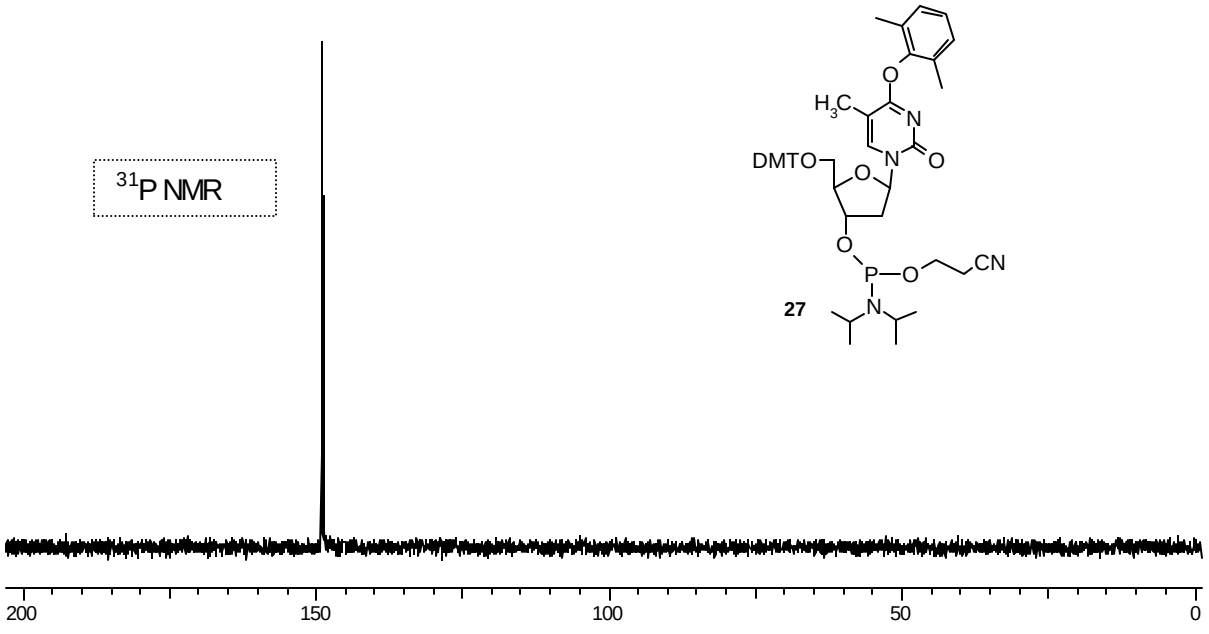
7
6
5
4
3
2
1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50
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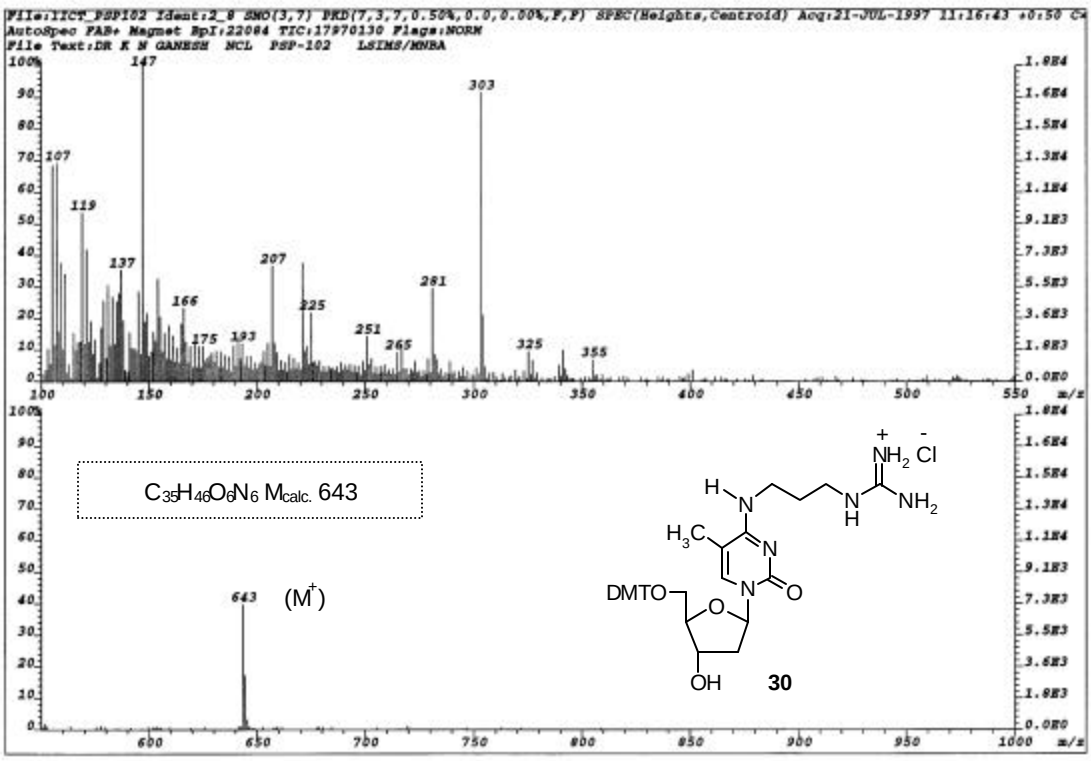
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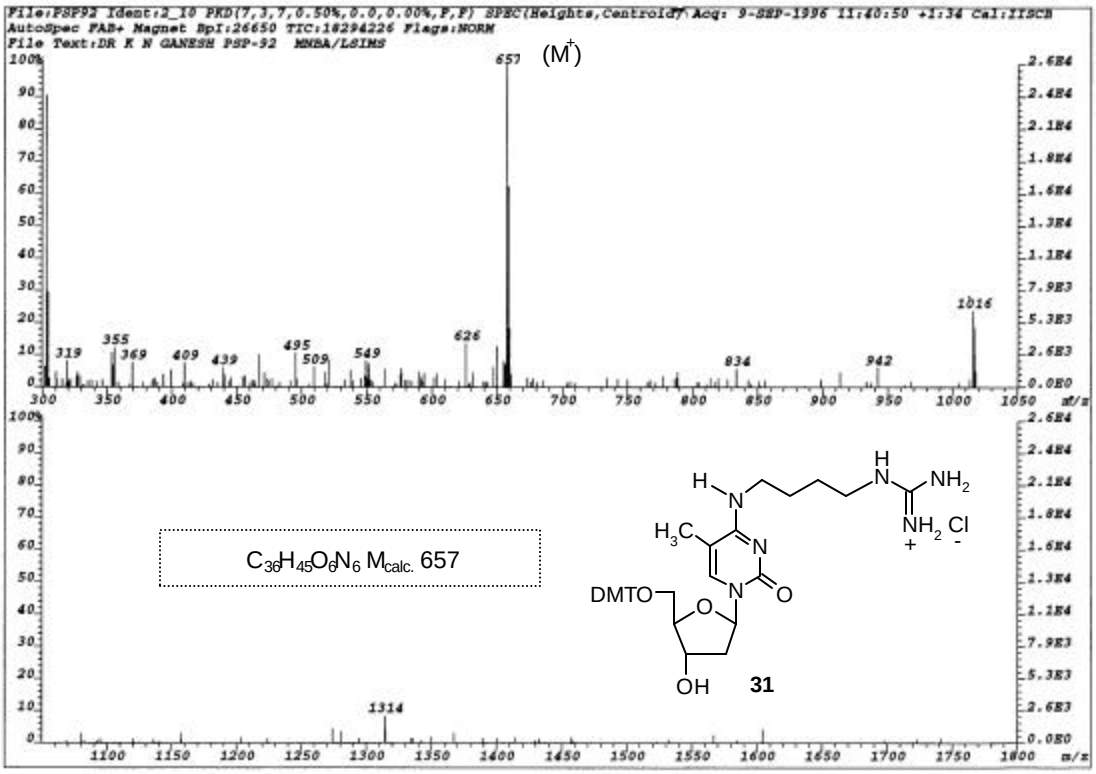
(M+)
DMTO
OH
30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(M+)
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OH
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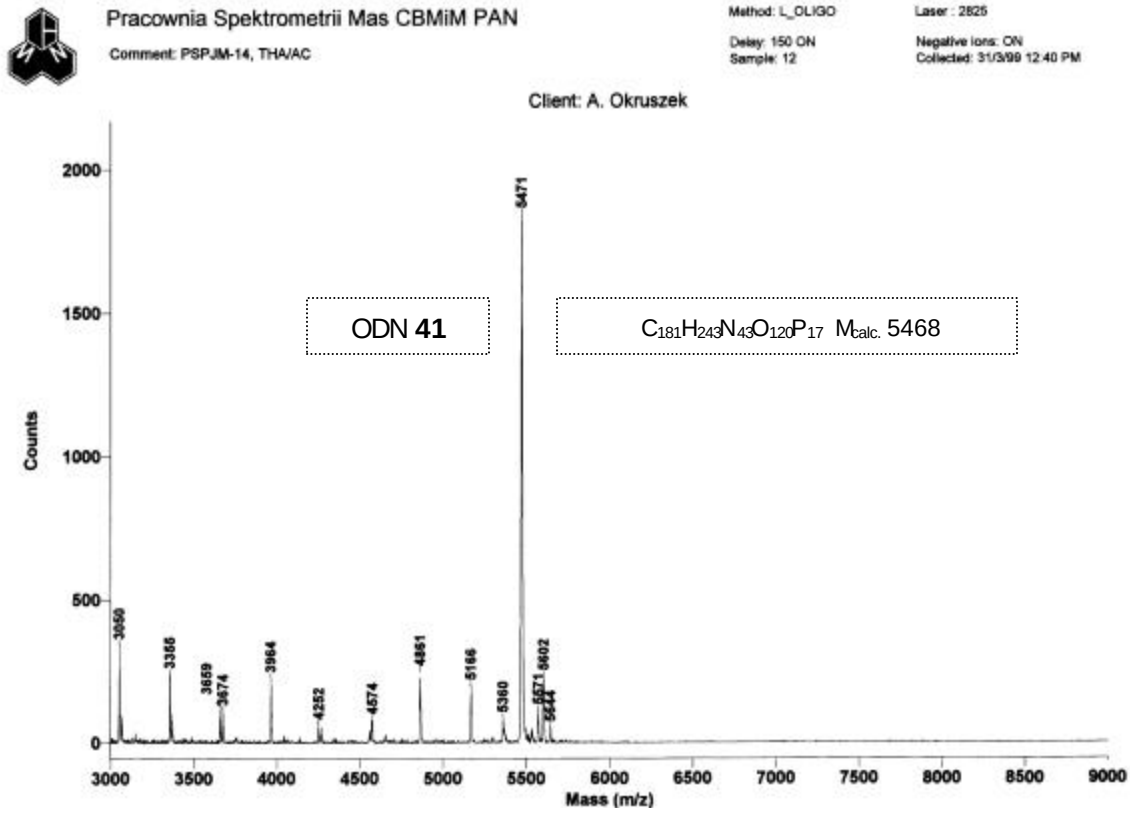
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Sample: 12
Laser : 2825
Negative Ions: ON
Collected: 31/3/99 12:40 PM
Client: A. Okruszek
ODN **41**
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Counts
Mass (m/z)
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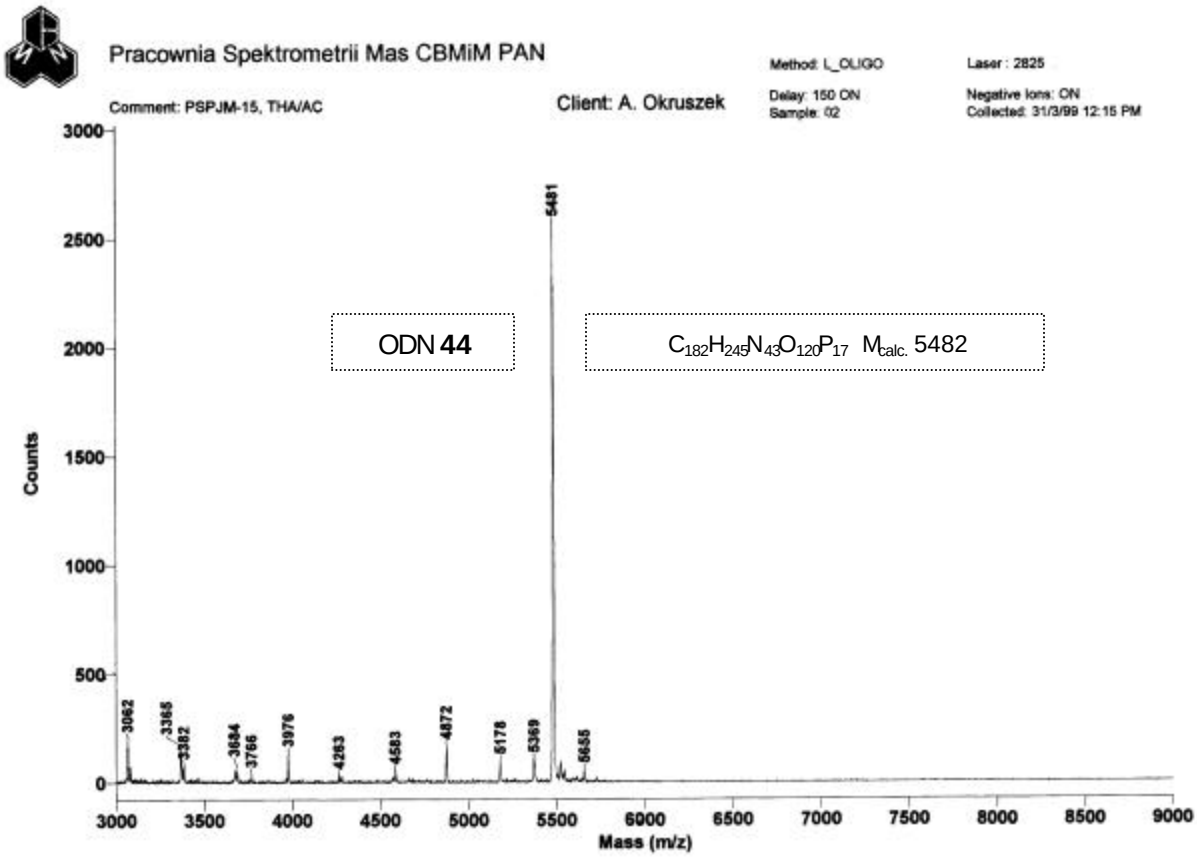
5671
5602
5444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Laser : 2825
Negative Ions: ON
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ODN **44**
$C_{182}H_{245}N_{43}O_{120}P_{17}$ $M_{calc.}$ 5482
Counts
Mass (m/z)
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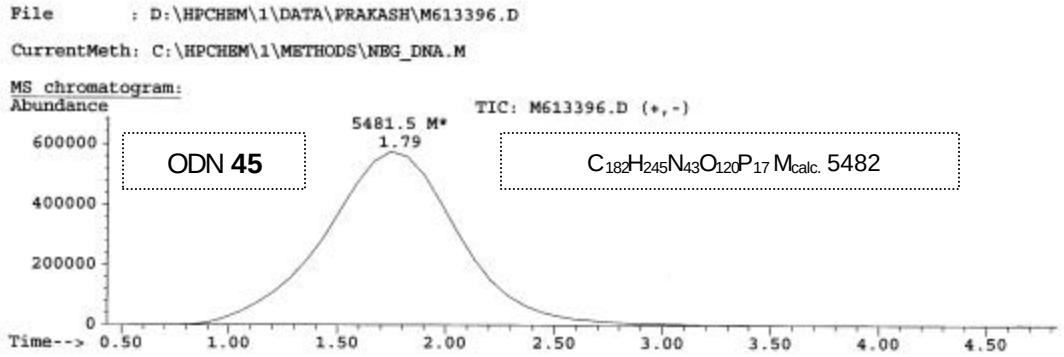
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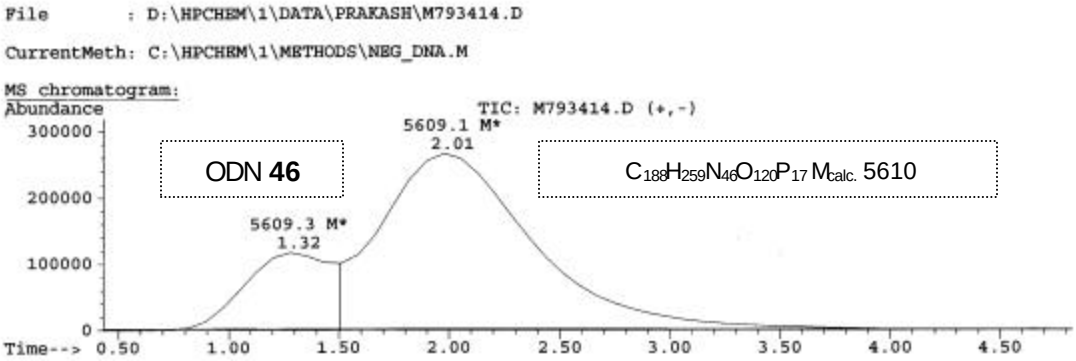
1.79
ODN 45
C182H245N43O120P17 Mcalc. 5482
600000
400000
200000
0
Time--> 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50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Abundance
TIC: M793414.D (+,-)
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2.01
5609.3 M*
1.32
ODN 46
C188H259N46O120P17 Mcalc. 5610
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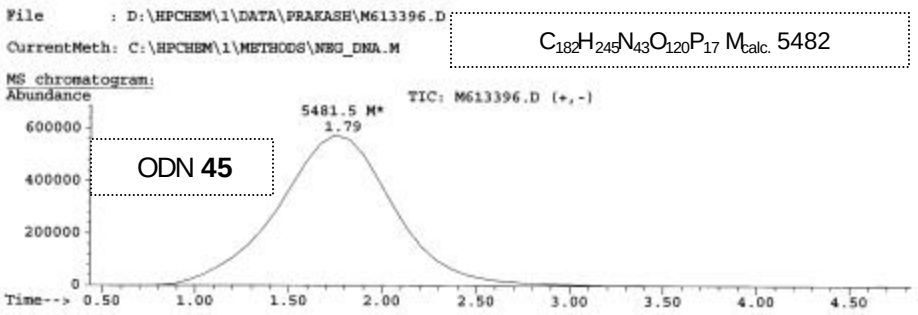
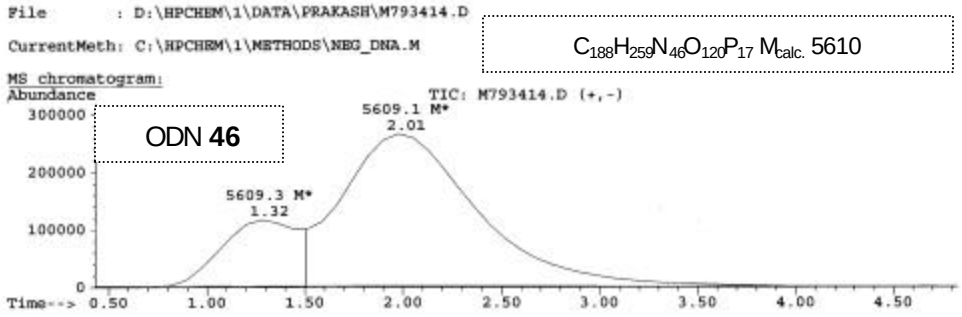
200000
100000
0
Time--> 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50



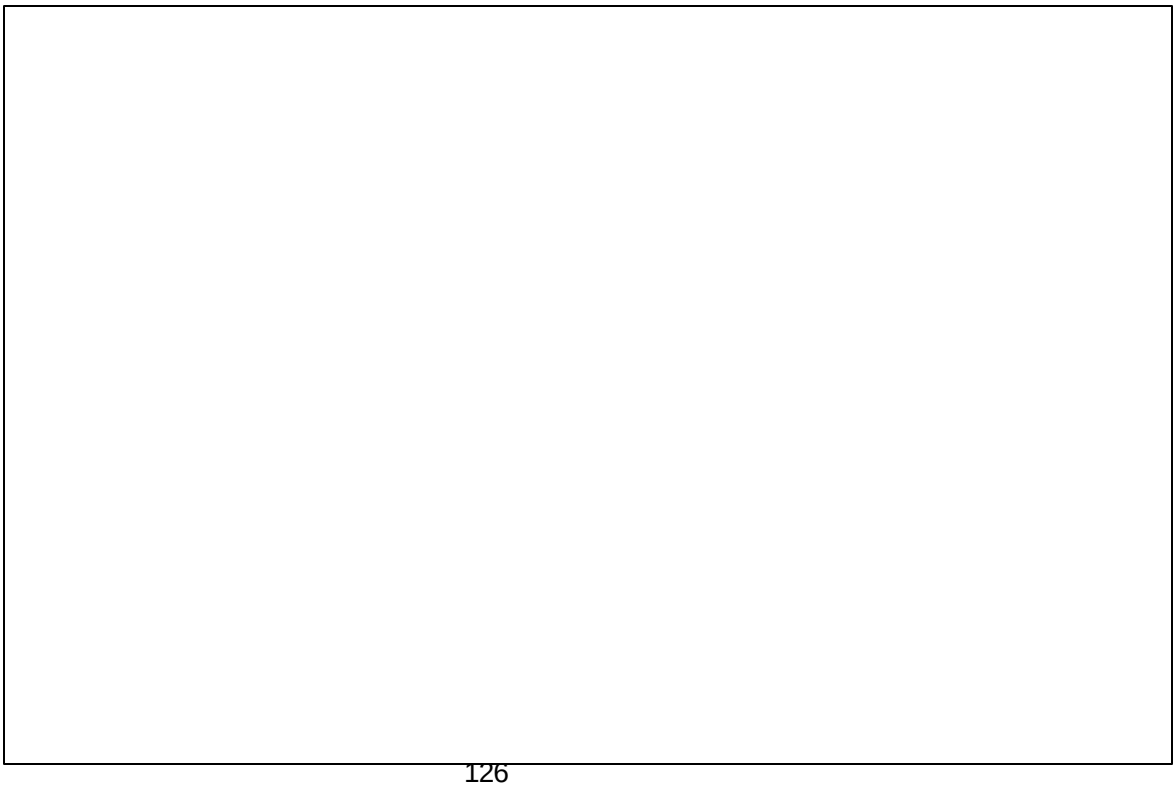
$C_{182}H_{245}N_{43}O_{120}P_{17}$ $M_{calc.}$ 5482

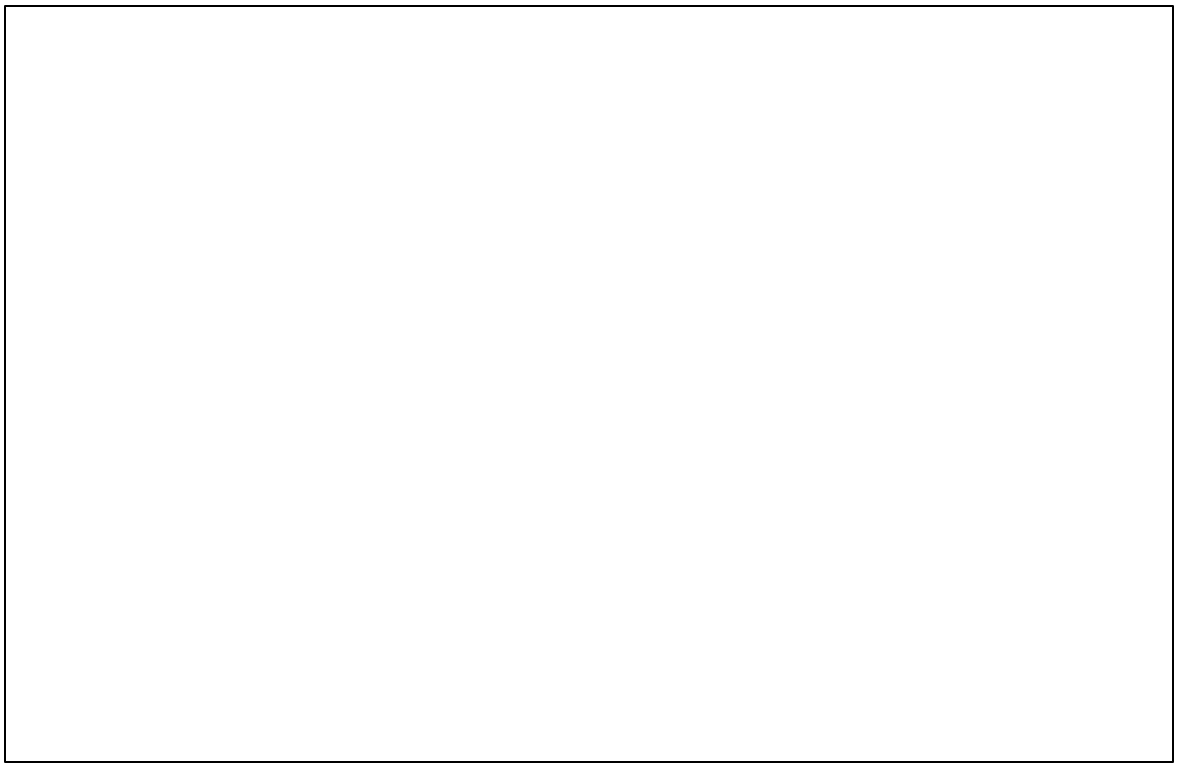
ODN **45**

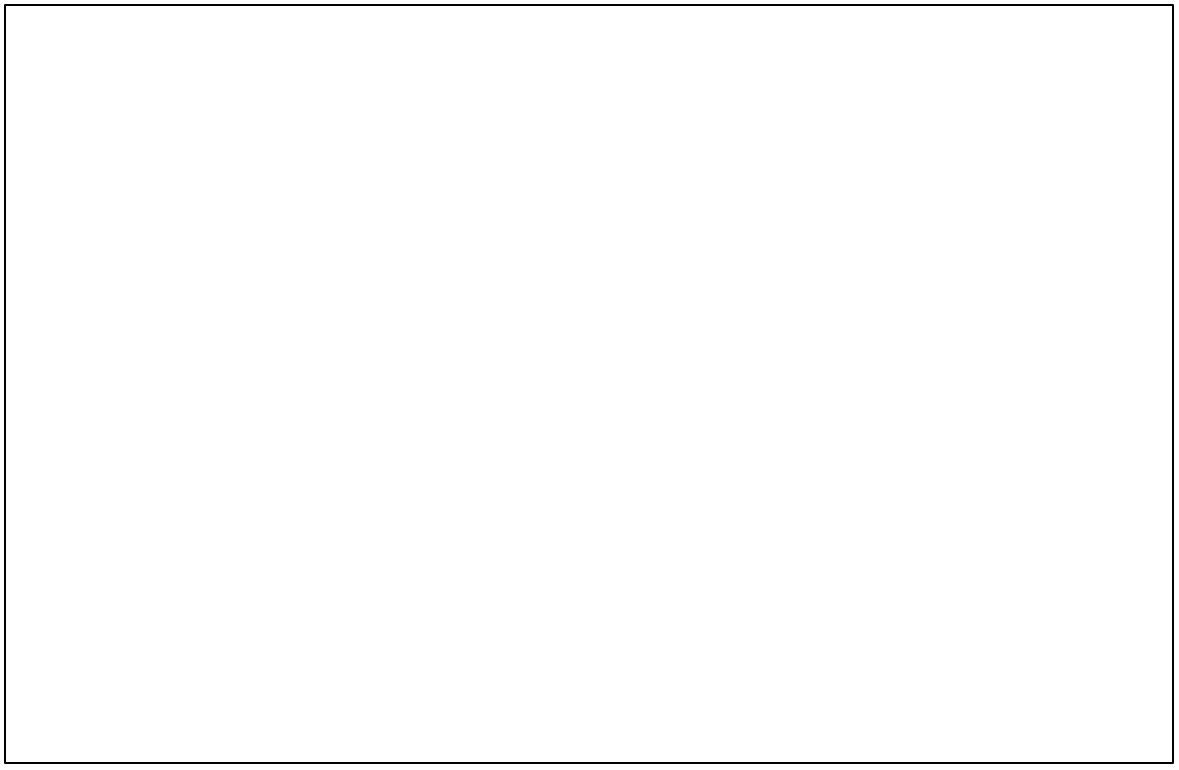


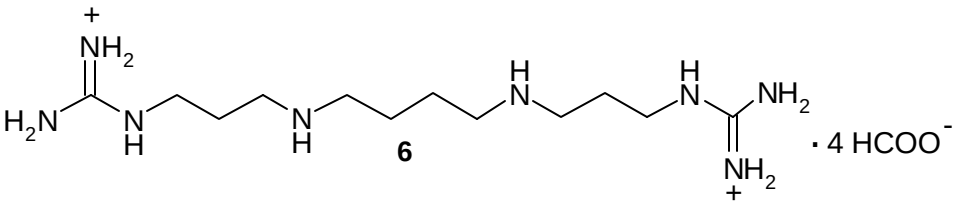
$C_{188}H_{259}N_{46}O_{120}P_{17}$ $M_{calc.}$ 5610

ODN **46**

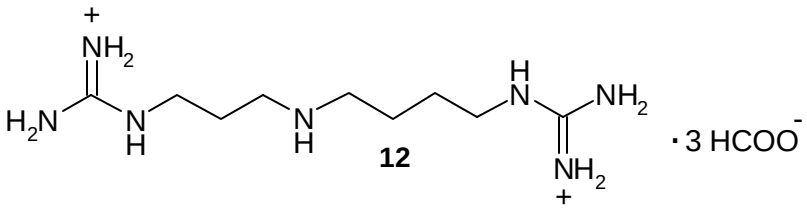




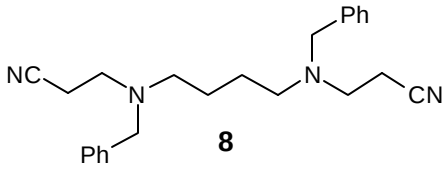




6 · 4 HCOO⁻



12 · 3 HCOO⁻



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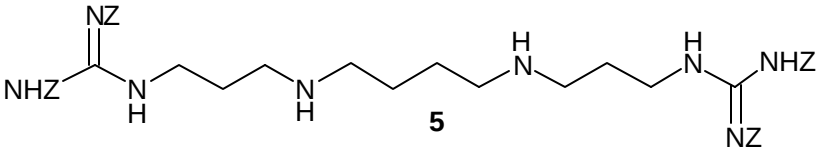

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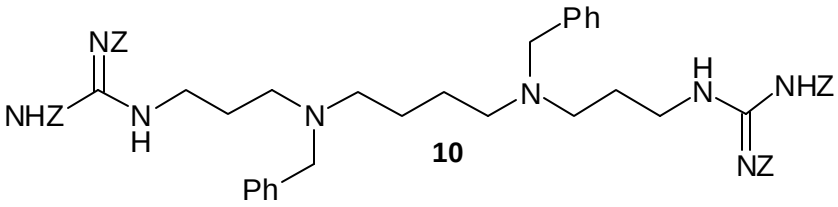

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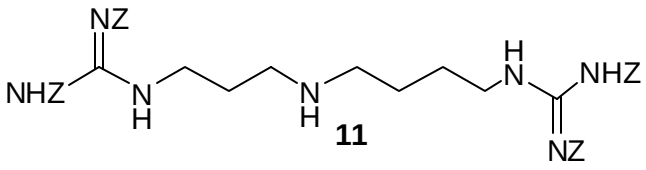

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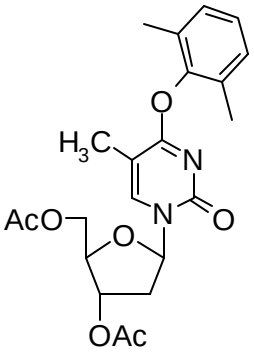
(Z = Benzyloxycarbonyl)



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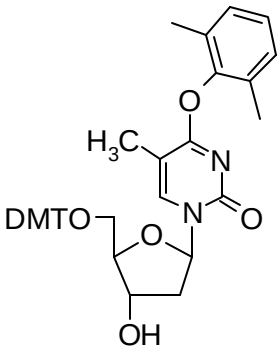

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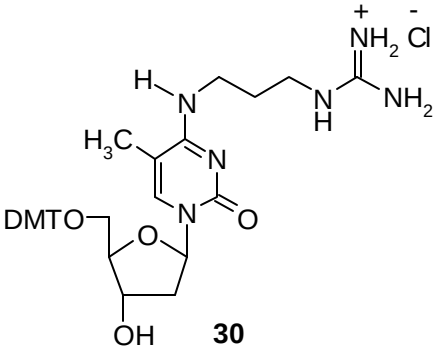

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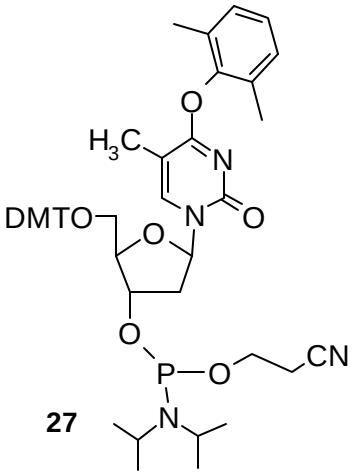

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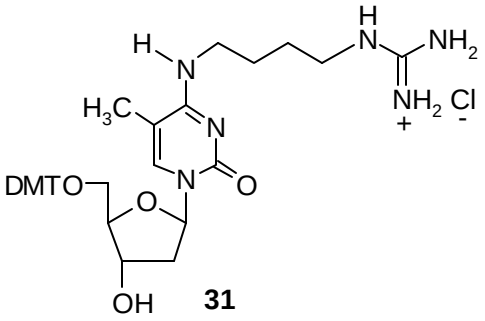

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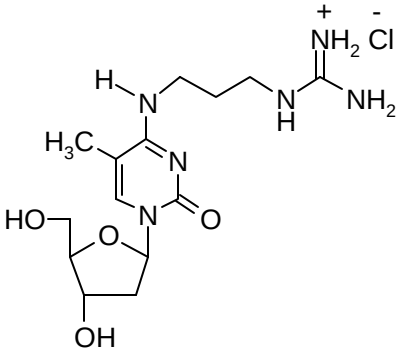


32

ODN **45**

ODN **46**

^{13}C NMR

^{31}P NMR

$C_{36}H_{45}O_6N_6$ $M_{calc.}$ 657

$C_{35}H_{46}O_6N_6$ $M_{calc.}$ 643

^{1}H NMR

$C_{181}H_{243}N_{43}O_{120}P_{17}$ $M_{calc.}$ 5468

$C_9H_{25}N_7$ $M_{calc.}$ 230

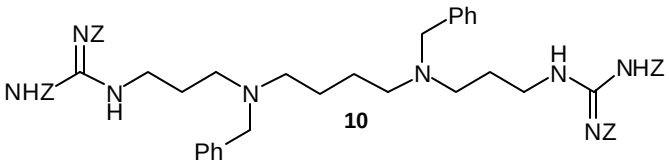
$(M^{+}+1)$

^{1}H NMR

^{13}C NMR

^{1}H NMR

^{13}C NMR



10

(Z = Benzyloxycarbonyl)

Chapter 3

Section A:

Synthesis and DNA Hybridization Studies of Oligonucleotides Bearing 2-Amino-6-Sperminylpurine

3.1. INTRODUCTION

As described previously, triplex forming oligonucleotides bind to the major groove of double-stranded DNA through Hoogsteen or reverse Hoogsteen hydrogen-bonding and are capable of inhibiting transcription of genes.[1,2] Though the association of the third strand with the duplex is sequence-specific, the strength of its binding is much weaker than that of the target duplex. This is due to the phosphate charge repulsion encountered in bringing together the three poly-anionic DNA strands. This can be potentially overcome by charge neutralization *via* conjugation of cationic ligands such as polyamines at different positions on nucleotides. Conjugating a ligand increases its concentration in the local microenvironment, enhancing its binding effect. Among the several polyamines, naturally occurring spermine is an attractive candidate for this purpose because of its high duplex/triplex DNA stabilizing features[3,4,5,6] and non-toxicity. Spermine remains protonated at neutral pH since the pK_as of all the amino functions are above 7 (10.97, 10.27, 9.04, 8.03).[7] A number of reports on spermine conjugated oligonucleotides have emerged in recent years and several strategies have been employed to conjugate spermine to DNA (Figure 1) at various positions. These include tethering of spermine to 5' phosphate,[8] C4 of 5-methyl-2'-deoxycytidine,[9] at the 5' terminus,[10] C2 of inosine[11] and to C2' of the ribose sugar.[12]

3.1.1. Effect of Conjugated Spermine on Duplexes

The C4 sperminyl 5-Me-dC (Figure 1a) was shown to destabilize the duplexes by 3-7°C per modified site over the reference ODNs lacking the modification.[13] Schmid and Behr[11] employed C2-sperminyl deoxyinosine (*sp*-dI, Figure 1c) to place it in the minor groove. The study showed that the spermine containing duplex (having two *sp*-dI

residues) is more stable by 25°C than the natural one. Sund et al.[12] studied oligonucleotides containing C-branched spermine (Figure 1d) on the phosphodiester

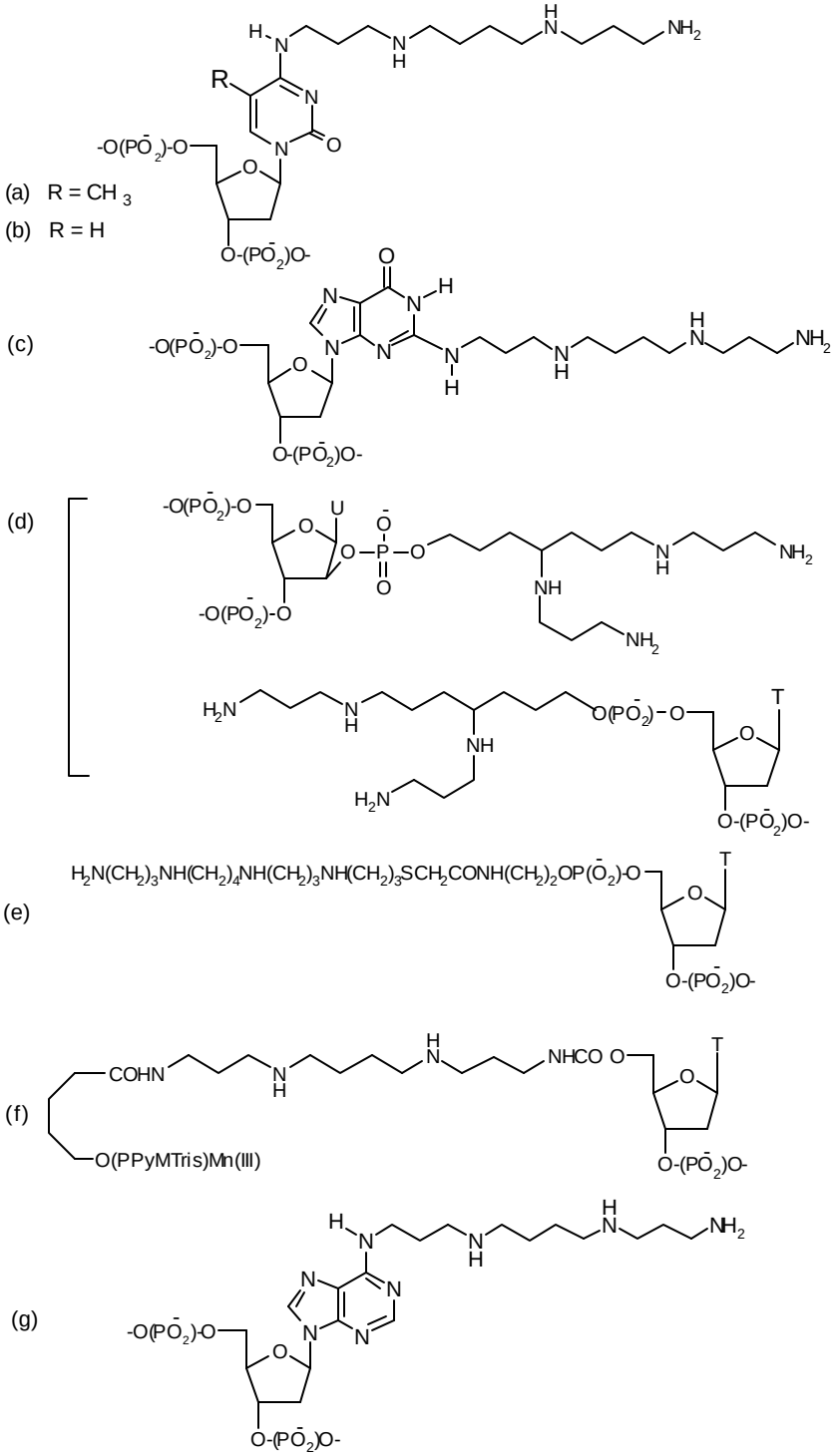


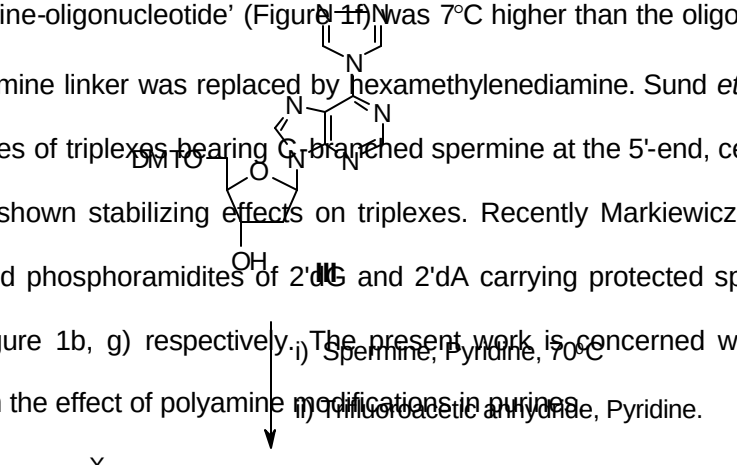
Figure 1.[8-12,14,16,17] Spermine conjugated oligonucleotides (a) C4-5-methyl-2'-deoxycytidine (b) C4-2'-deoxycytidine (c) C2-deoxyinosine (d) C-branched spermine (e) 5'-end conjugation (f) 'manganese porphyrin-spermine-oligonucleotide' and (g) C6-purine.

backbone tethered at the 5'-end, center or 3'-end. The 5'-modification induced a small duplex stabilization of +2°C over the unmodified one, the 3'-conjugation did not alter the stability while the middle modification gave a weakly associated complex. Markiewicz *et al.*[14] have studied N^4-sperminyl-2'-dC oligonucleotide (Figure 1b) combinatorial library by hybridization with the complementary fluorescein labeled ODN probe. The results indicated that introduction of spermine in 2'-dC resulted in strong improvement of duplex stability.

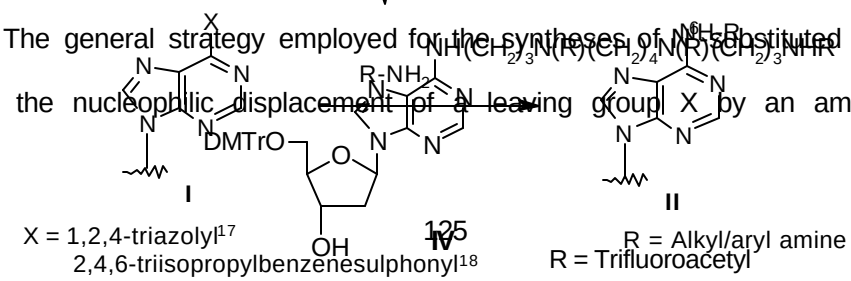
3.1.2. Effect of Conjugated Spermine on Triplexes

The 5'-spermine conjugation[8] (Figure 1e) to a 21-mer homopyrimidine sequence was found to lead to stable triple helices under identical conditions where the reference ODN complex failed to form a triplex. The C4 conjugation of spermine on 5-Me-dC,[13,15] the first of the kind carrying a sperminyl chain in a nucleobase, places the sperminyl moiety in the major groove of DNA. The studies carried out by Barawkar *et al*.[13,15] established that covalent conjugation at C4 of 5-Me-dC led to triplex stabilization by spermine bearing oligonucleotides when used as the third strand at physiological pH.

It was shown by Bigey *et al*.[10] that the triplex stability of the 'manganese porphyrin-spermine-oligonucleotide' (Figure 1f) was 7°C higher than the oligonucleotide where the polyamine linker was replaced by hexamethylenediamine. Sund *et al.*[12] have carried out studies of triplexes bearing C-branched spermine at the 5'-end, center or 3'-end which has shown stabilizing effects on triplexes. Recently Markiewicz *et al*.[16,17] have synthesized phosphoramidites of 2'dC and 2'dA carrying protected spermine at C2 and C6 (Figure 1b, g) respectively. The present work is concerned with further investigations on the effect of polyamine modifications in purines.



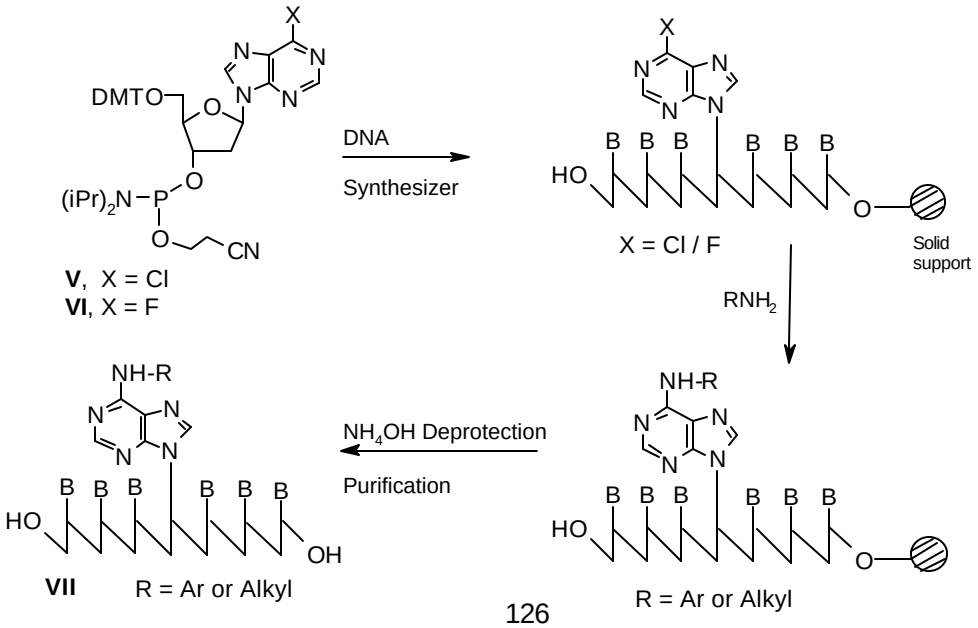
The general strategy employed for the syntheses of N^6-substituted A analogs involve the nucleophilic displacement of a leaving group[I] X by an amine in an



intermediate substrate **I** (Scheme 1). X at C6 is a good leaving group such as 1,2,4-triazolyl[17] or 2,4,6-triisopropylbenzene sulphonyl[18] and the reaction leads to the N^6-derived product **II**.

For the synthesis of polyaminooligonucleotides that bear the polyamine at C6 of purines, 6-(1,2,4-triazol-4-yl)purine-2'-deoxyribonucleoside was used[17] by Markiewicz *et al.* The 5'-O-DMT-derivative **III,** on treatment with spermine, followed by trifluoroacetylation gave compound **IV** (Scheme 2) which was converted to its 3'-O-phosphoramidite for incorporation into oligonucleotides. Likewise, the O^6-(2,4,6-triisopropylbenzenesulfonyl) purine nucleoside on treatment with amines is reported[18] to give the N^6-amino substituted derivatives.

Post synthetic halogenopurinenucleoside strategy was also employed for the synthesis of oligonucleosides with C6 adducts of purine. The 6-chloropurinenucleoside- or 6-fluoropurinenucleoside phosphoramidite, **V** or **VI** was used[19,20] in the automated solid-phase synthesis of DNA. After oligomer assembly, the product on the solid support was treated with different amines to get the corresponding adenine N^6-derivatives (Scheme 3). The excess amine was washed off with methanol followed by the standard NH_4OH cleavage to yield the N^6-derivatives, **VII**.



Scheme 3. Post synthetic halonucleotide strategy for the synthesis of N^6-substituted adenines.[19,20]

3.2. OBJECTIVES

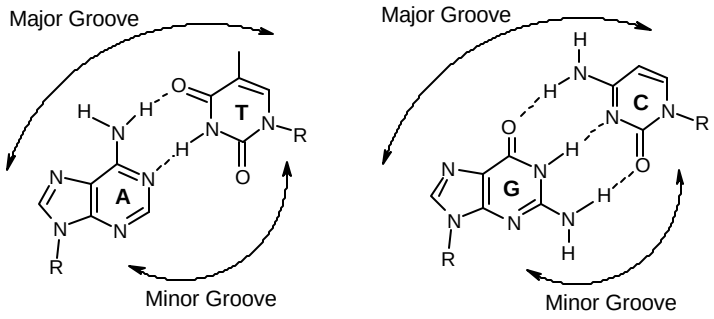


Figure 2. Schematic representation of the 'major groove' and 'minor groove' of Watson-Crick hydrogen-bonded duplets.

The effect of a sperminyl chain linked to a purine, in the major groove (Figure 2), was studied by synthesis and incorporation of 2-amino-6-sperminylpurinyl-2'-deoxyribonucleoside (**D**^{sp}, Figure 3), in a suitably protected form and investigation of the biophysical properties of the derivatized oligonucleotides. This sperminyl analogue **D**^{sp} is different from the C6-sperminyl purine reported in the literature[17] in having an additional C2 amino group which is known to stabilize DNA duplexes.[21,22] In the newly designed **D**^{sp} an additional hydrogen-bond arises in the Watson-Crick pair **D**^{sp}:T. It was envisioned that this additional hydrogen bond in **D**^{sp} together with the sperminyl chain (which has its amino functions protonated at physiological pH) in the major groove would lead to improved stability of the derived duplexes and triplexes.

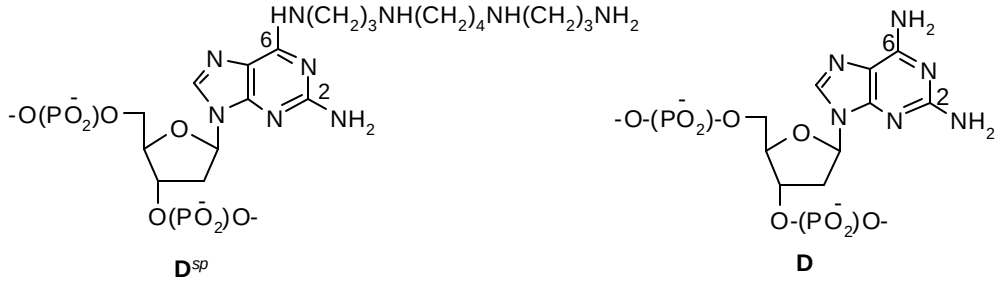
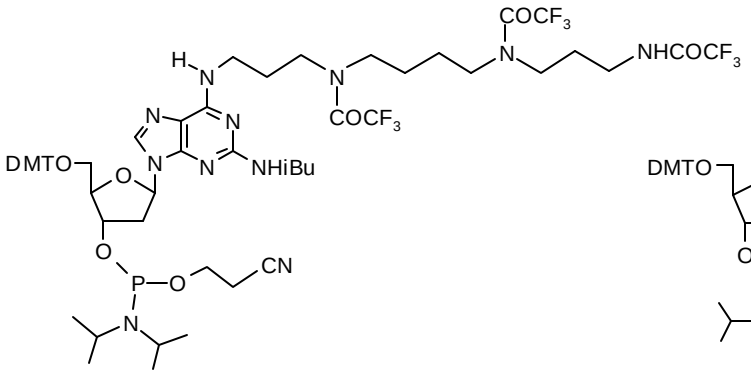


Figure 3. Designed **D**^{sp} and **D** in ODNs.

2-Amino-dA (2,6-diaminopurine, **D**) having amino groups at C2 and C6 positions (Figure 4), was chosen as the reference for 2-amino-6-sperminylpurine for comparison of stability effects.

The objectives of this section are:

- Synthesis of the fully protected monomer, 2-amino-6-sperminylpurine-3'-O-phosphoramidite **21**.
- Synthesis of oligonucleotides having 2-amino-6-sperminylpurine (**D**^{sp}) and 2-amino-dA (**D**) at the desired sites using the phosphoramidites **21** and **22** respectively.
- Study of the effect of spermine conjugation in **D**^{sp} on the formation and stability of



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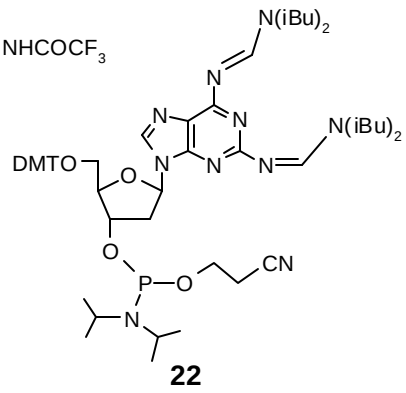
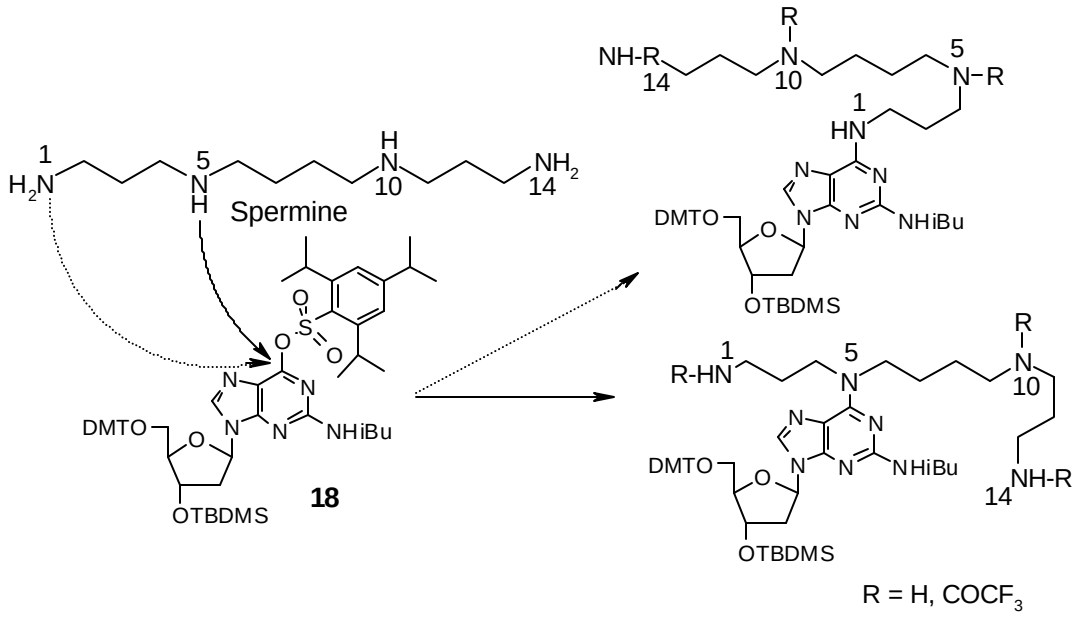


22

oligonucleotide duplexes and triplexes.

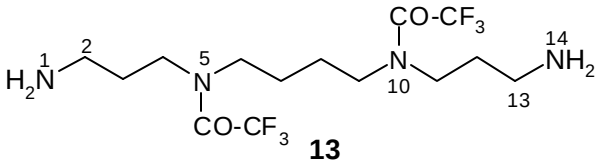
3.3. PRESENT WORK

In the light of the above literature reports, the method employed here for the synthesis of C6 substituted adenines involved the reaction of O^6-(2,4,6-triisopropylbenzenesulfonyl) derivative, **18** with spermine (Scheme 4). The compound **18** was prepared from deoxyguanosine as per literature,[23] and on treatment with spermine (free base) followed by protection of the amino groups as trifluoroacetamides



Scheme 4. Attempted synthesis of 2-amino-6-sperminyl purine.

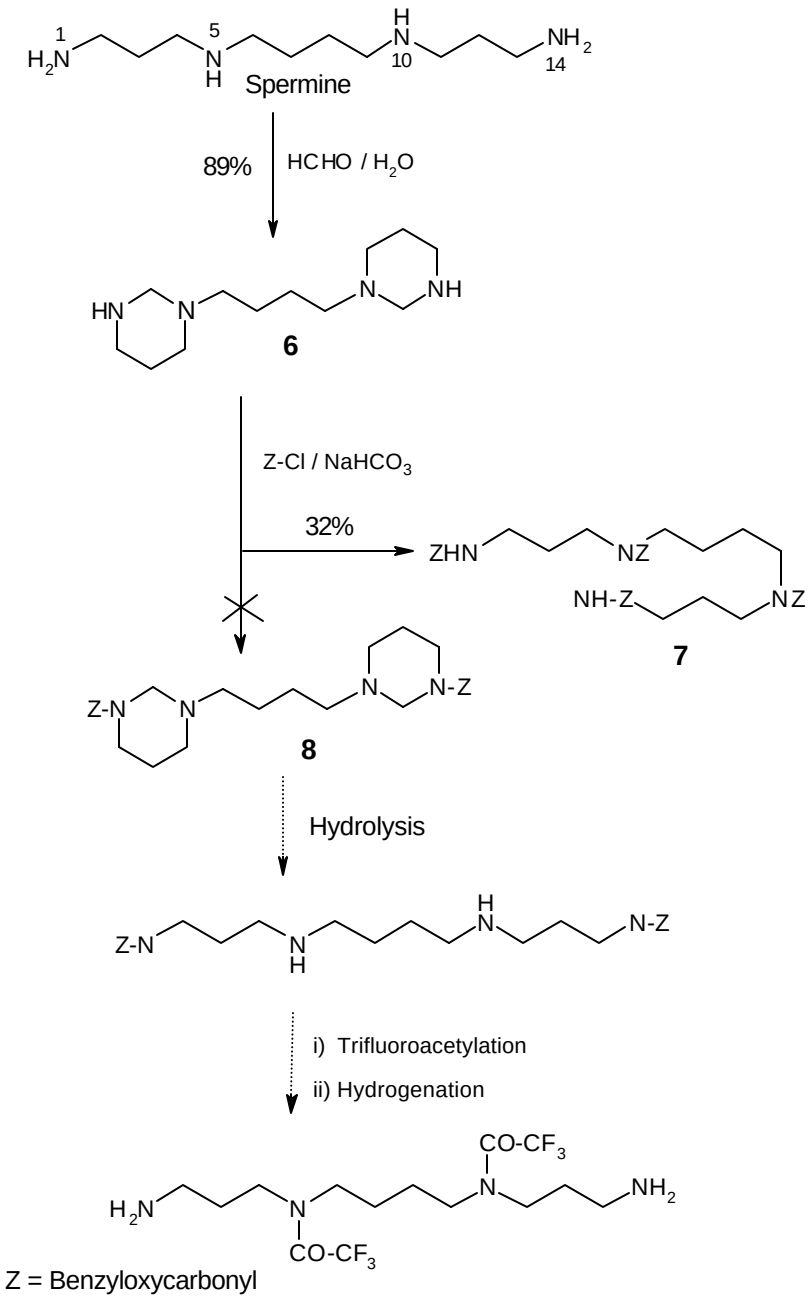
gave a mixture of products, which were not separable by silica gel column chromatography. The products possibly arise by the reaction of both N1 amino (primary amino group) and N5 amino (secondary amino group) of free spermine (Scheme 4). This necessitated the protection of the N5 and N10 amino groups of spermine as in derivative **13**, for the regiospecific reaction of its N1 aminogroups. The trifluoroacetyl group was the protecting group of choice since it has been shown to be suitable for



13

protecting aliphatic amino groups in oligonucleotide synthesis by phosphoramidite chemistry.[24,25]

3.3.1. Synthesis of N5,N10-Bistrifluoroacetyl Spermine 13



Scheme 5. Planned route for the synthesis of N5,N10-protected spermine.

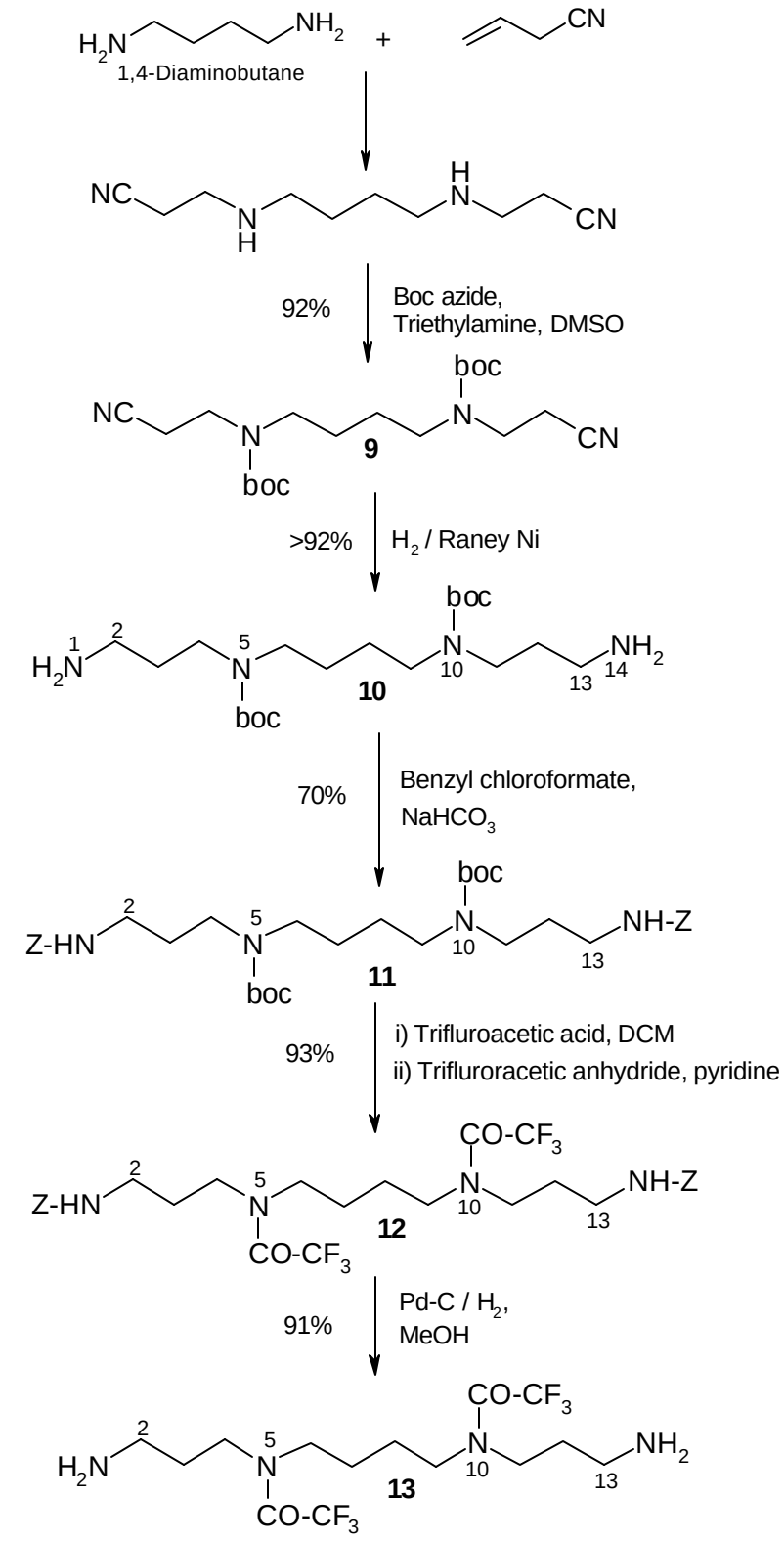
Spermine on treatment with 2 equivalents of formalin in water yielded the bis-hexahydropyrimidine derivative **6** (Scheme 5),[26] characterized by ^{1}H and ^{13}C NMR spectroscopy. In the ^{1}H NMR of **6**, the –NC$\underline{H}_2$N- appeared at δ 3.40-3.25 and the three sets of –NC$\underline{H}_2$C-methylenes at δ 2.85-.2.70, 2.60-2.45 and 2.25-2.10. The ^{13}C NMR

spectrum showed the characteristic signal of –N<u>C</u>H_2N- at δ 69.2. Compound **6** on treatment with benzyl chloroformate in presence of $NaHCO_3$ gave the tetrabenzyloxycarbonyl compound **7** instead of the expected bis-benzyloxycarbonyl product, **8**. The expected product **8** was not obtained even when lower equivalents of benzyl chloroformate were used. The reactions shown in dotted lines in Scheme 5 corresponds to the planned route in case the compound **8** was obtained.

Since regiospecific protection of primary or secondary amino groups in spermine was not directly possible to access the target secondary amino-protected spermine, the synthesis of the target compound was carried out starting from 1,4-diaminobutane, as outlined in Scheme 6. The free amino groups (secondary amines) of N,N'-bis(2-cyanoethyl) butanediamine[27] (compound **7**, Chapter 2) were protected as the *tert*-butylcarbamate derivative using ^{t}boc-azide in DMSO to get **9**, which gave characteristic *tert*-butyl singlet peak at δ 1.45 in the ^{1}H NMR spectrum. Compound **9** was subjected to hydrogenation using Ra-Ni/H_2 to get the tetra-amine **10**, the IR spectrum of which lacked the characteristic absorption band of the cyano group at 2247 cm^{-1}, indicating complete conversion of the cyano group to amine. The presence of the primary amino groups was indicated by a broad band at 3480 cm^{-1}. In the ^{1}H NMR spectrum, the emergence of a multiplet corresponding to the C^2, C^{13} methylenes at δ 2.80-2.65 supported the conversion.

The bis-benzyloxycarbonyl protection of the primary amino groups (N1 & N14) was carried out using benzylchloroformate in presence of $NaHCO_3$ to give compound **11** the structure of which was confirmed by NMR spectroscopy. The ^{1}H NMR spectrum showed aromatic peaks at δ 7.40-7.30, the benzylic protons (–NCO-O-C<u>H_2</u>Ph) at 5.10 and a downfield shift of the terminal -NC<u>H_2</u>- methylenes from δ 2.80-2.65 to 3.40-3.00.

The removal of the *tert*-butyloxycarbonyl groups in **11** was carried out using trifluoroacetic acid. The liberated free secondary amines were then transformed into N5,N10-(bis)-trifluoroacetamide derivative **12** by treatment with trifluoroacetic anhydride in pyridine. In the ^{13}C NMR spectrum, all the methylene carbons showed



Z = Benzyloxycarbonyl

Scheme 6. Synthesis of N5,N10-bistrifluoroacetyl spermine.

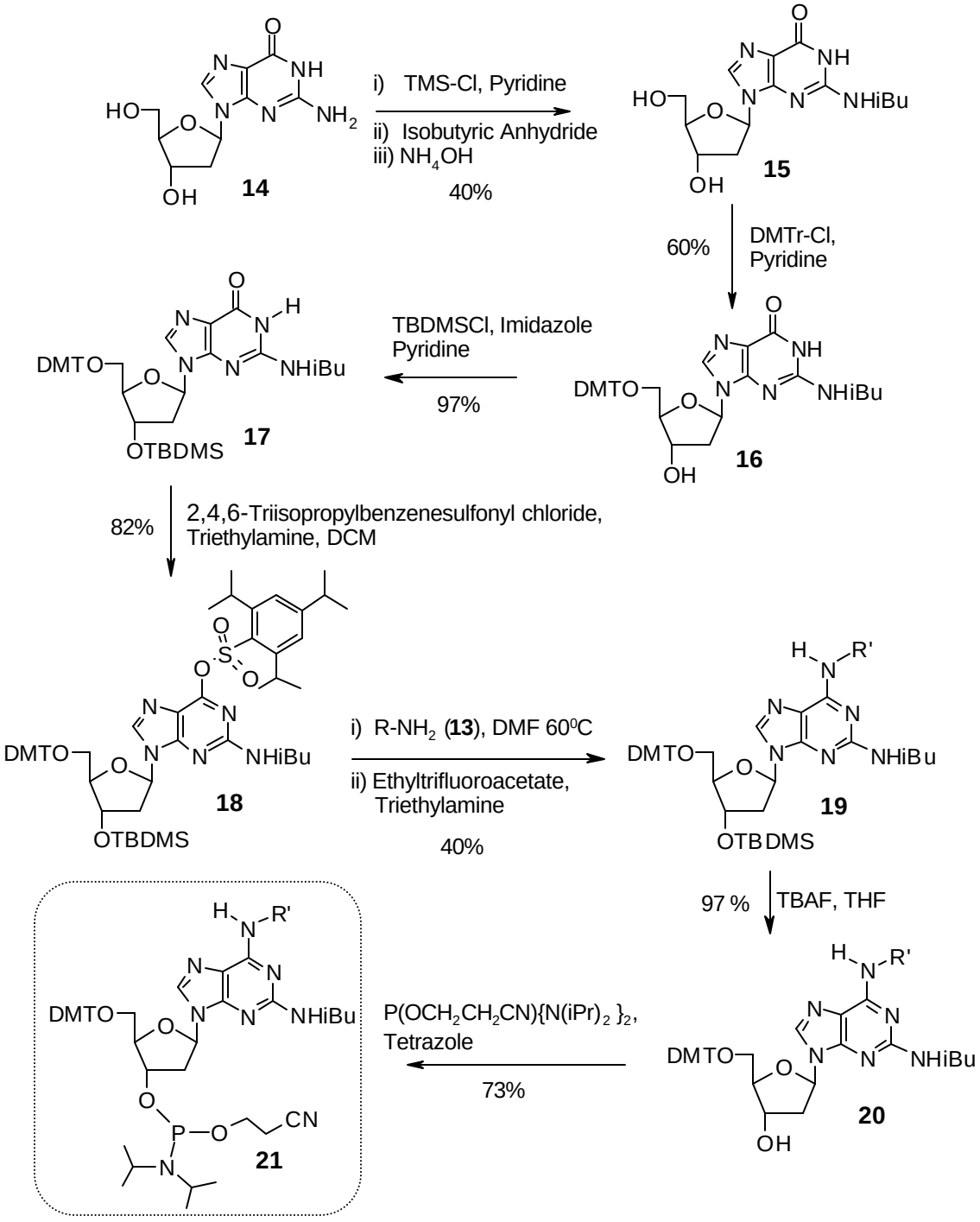
separate signals as expected in the region δ 46.0-23.0. In the signals corresponding to $COCF_3$, the fluorine coupling with carbon was characteristic giving rise to a quartet, with $^1J_{C\text{-}F}$ = 287.8 Hz ($CO\underline{C}F_3$) and $^2J_{C\text{-}F}$ = 37.2 Hz ($\underline{C}OCF_3$). The mass spectrum of the product showed a peak at 663 (M^+), corresponding to **12** as the structure of the compound.

The benzyloxycarbonyl protecting groups in **12** were removed by hydrogenation over Pd-C to get the spermine analogue **13**, in which the primary amines are free while both the secondary amines are blocked as trifluoroacetyl protecting groups. The ^1H NMR spectrum of **13** exhibited a multiplet at δ 3.40 for the terminal methylenes ($H_2N\text{-}C\underline{H}_2$) along with the other four sets of methylenes. The ^{13}C spectrum of the compound showed only five methylene carbon signals as per expectation of the symmetry of the compound. The carbon signals of $\text{-}\underline{C}O\underline{C}F_3$ were seen as quartets with the appropriate C-F coupling [$^1J_{C\text{-}F}$ = 286.6 Hz ($CO\underline{C}F_3$) and $^2J_{C\text{-}F}$ = 37.0 Hz ($\underline{C}OCF_3$)]. The stability of compound **13** which is susceptible for transamidation (from secondary amino to primary) was studied by keeping the sample at 50°C over a period of 18h and scanning ^1H and ^{13}C NMR at intervals of 6h. No transamidation was detected indicating that compound **13** maintained its integrity during the period of study.

3.3.2. Synthesis of 2-Amino-6-sperminylpurine Phosphoramidite 21

The phosphoramidite **21** was synthesized starting from dG as shown in Scheme 7. The transient protection of the 3' and 5' hydroxyl groups of deoxyguanosine using trimethylchlorosilane in dry pyridine followed by treatment with isobutyric anhydride and the removal of O-trimethylsilyl groups using NH_4OH gave N^2-isobutyryl deoxyguanosine **15**. This on reaction with DMTr-Cl in pyridine gave the 5'-O-

dimethoxytrityl ether **16**.[23] The 3'-hydroxyl group of **16** was protected as silyl ether in **17** using TBDMS-Cl and imidazole in dry pyridine. The ^{1}H NMR spectrum showed the



Scheme 7. Synthesis of the phosphoramidite **21**.

presence of the *tert*-butyldimethyl silyl group and an upfield shift of H3' from δ 4.15 to 3.95. This was converted to O^6-(2,4,6-triisopropylbenzenesulfonyl) derivative **18** by treatment with 2,4,6-triisopropyl benzenesulfonyl chloride in presence of triethylamine. The structures of all the compounds were confirmed by ^{1}H NMR and mass spectroscopic techniques and confirmed by comparison with literature values.

The conversion of the sulfonate derivative **18** into the N^6-alkyl derivative **19** was carried out as follows: Compound **18** on treatment with 5,10-bis(trifluoroacetyl) spermine **13** (R-NH_2) gave the sperminyl derivative with the ω-amino group free. The free amino function was protected as the trifluoroacetate using ethyl trifluoroacetate in dry ethanol to get **19**. In the ^{1}H NMR spectrum, in addition to the expected signals from the nucleoside structure, methylene protons appeared at δ 3.50-3.00 indicating the formation of the spermine adduct. The ^{13}C NMR spectrum revealed all the required carbon signals of the product including the quartet due to C-F couplings in the $\underline{C}F_3$ signals with a coupling of $^1J_{C\text{-}F}$ = 287.8 Hz (CF_3). The mass spectrum of the product showed a molecular ion peak at 1249 (M^++Na). The desilylation of **19** using tetrabutylammonium fluoride (TBAF) in THF afforded the 3'-OH derivative **20** with a free 3' hydroxyl group. The ^{1}H NMR of this derivative showed the disappearance of *tert*-butyldimethyl silyl group (TBDMS) and a downfield shift of H3' from δ 4.50 to 4.65. Further support was obtained from mass spectral analysis of the compound **20** which exhibited the molecular ion at 1134 (M^++Na). Compound **20** on phosphitylation gave the required amidite monomer **21**. In the ^{31}P NMR spectrum, signals at δ 149.73, 149.33 characteristic of the phosphoramidites were observed.

3.3.3. Design and Synthesis of Oligonucleotide Sequences

In order to evaluate the effect of conjugated spermine on the duplex and triplex formation, the oligonucleotide sequences **23-29** were designed (Section 3.3.3.a). The

sequences include $\mathbf{D}^{sp}$-ODNs with one $\mathbf{D}^{sp}$ unit (**23, 24, 26**, **27** & **28**) as well as with two $\mathbf{D}^{sp}$ units at internal sites (**25** & **29**). From the hydrogen bonding schemes with $\mathbf{D}^{sp}$ in the third strand (Figure 4), it is seen that $\mathbf{D}^{sp}$ can be accommodated in the triads

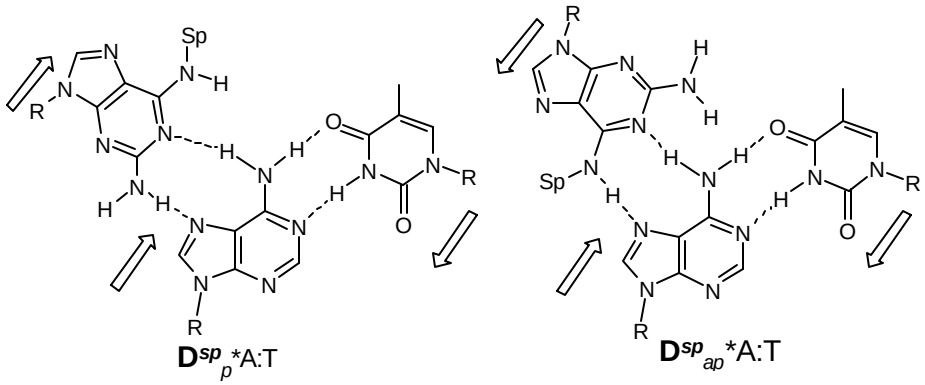


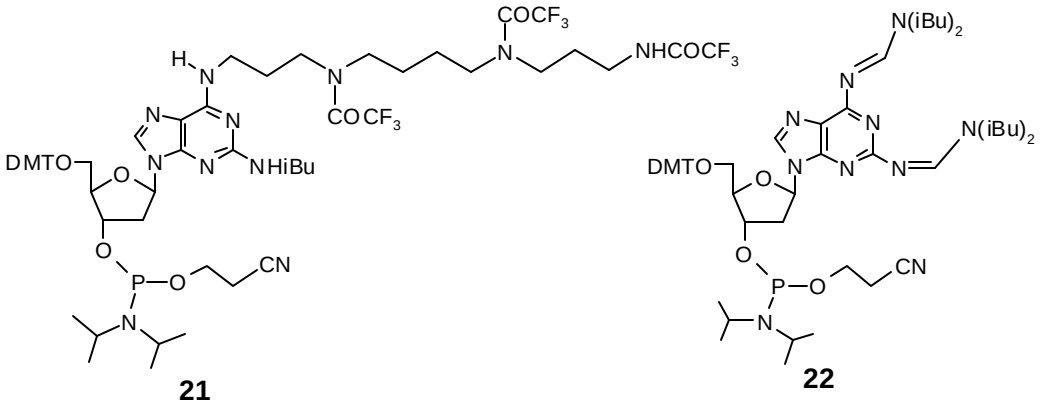
Figure 4. The hydrogen bonding patterns of $\mathbf{D}^{sp}$. The notations * and : represent Hoogsten and Watson-Crick while '*p*' and '*ap*', the parallel and antiparallel orientations respectively; Sp indicates sperminyl chain.

$\mathbf{D}^{sp}$*A:T in both parallel and antiparallel orientations.

The modifications can be introduced in the pyrimidine-rich third strand (parallel motif), the purine-rich third strand (antiparallel motif) or the purine-rich Watson-Crick central strand. In accordance, the oligonucleotides **23-25** (homopyrimidine strand), **26** (homopurine strand) and **27-29** (homopurine central strand) were designed.

As a reference for the $\mathbf{D}^{sp}$-ODNs, oligonucleotides with 2,6-diaminopurine **D** (2-aminoadenine) at the corresponding positions in **30-36** were also designed (Section 3.3.3.b). The normal sequences carrying A instead of $\mathbf{D}^{sp}$ or **D** were also constituted (**37-41**, Section 3.3.3.c.) for comparison and the sequences **42-46** served as the complementary oligonucleotides.

Oligonucleotide synthesis was carried out following the standard protocols.[28] The phosphoramidite **21** was employed in the automated synthesis of sequences **23**-**29** using a Pharmacia GA Plus synthesizer. The coupling yield of **21**, as quantified by the



21 **22**

released DMT cation, was comparable to the average yield with unmodified amidites.

3.3.3.a. Spermine-bearing oligonucleotides (D^{sp}-ODNs)

23 5'-T T C T T C T T C T **D^{sp}** T T C T T T T

24 5'-T T C T **D^{sp}** C T T C T T T T C T T T T

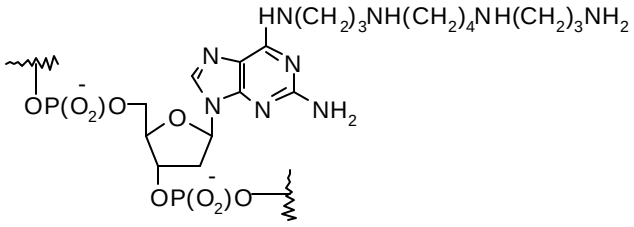
25 5'-T T C T **D^{sp}** C T T C T **D^{sp}** T T C T T T T

26 5'-A A A A G A A **D^{sp}** A G A A G A A G A A

27 5'-T C C A A G A A G A **D^{sp}** G A A A A G A A A A A T A T

28 5'-T C C A A G A A G A A G A A A A G A **D^{sp}** A A A T A T

29 5'-T C C A A G A A G A **D^{sp}** G A A A A G A **D^{sp}** A A A T A T



2-Amino-6-sperminyl-purinyl fragment, **D^{sp}**

The reference oligonucleotide sequences having 2,6-diaminopurine, **D** (2-amino-dA) substitution for **D*sp*** were synthesized by using commercially available 5'-dimethoxytrityl-N^2,N^6-bis(diisobutylaminomethylidene)-2,6-diamino-2'-deoxypurine riboside-3'-[(2-cyanoethyl) -(N,N-diisopropyl)]- phosphoramidite **22**.

3.3.3.b. Oligonucleotides bearing 2-amino-dA (D)

30 5'- T T C T T C T T C T **D** T T C T T T T

31 5'- T T C T **D** C T T C T T T T C T T T T

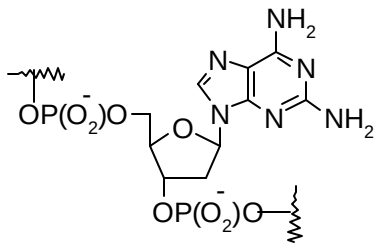
32 5'- T T C T **D** C T T C T **D** T T C T T T T

33 5'- A A A A G A A **D** A G A A G A A G A A

34 5'- T C C A A G A A G A **D** G A A A A G A A A A A T A T

35 5'- T C C A A G A A G A A G A A A G A **D** A A A T A T

36 5'- T C C A A G A A G A **D** G A A A A G A **D** A A A T A T



2-Amino-deoxyadenosine, **D**

3.3.3.c. Unmodified oligonucleotides

37 5'- T T C T T C T T C T A T T C T T T T (control for **23 & 30**)

38 5'- T T C T A C T T C T T T T C T T T T (control for **24 & 31**)

39 5'- T T C T A C T T C T A T T C T T T T (control for **25 & 32**)

40 5'- A A A A G A A A A G A A G A A G A A (control for **26**)

41 5'- T C C A A G A A G A A G A A A A A G A A A A T A T (control for **27-29**)

42 5'- A T A T T T T T C T T T T C T T C T T C T T G G A

43 5'- A A A A A G A A T A G A A G A A G A A A (compl. to **23, 30, 37**)

44 5'- A A A A A G A A A A A G A A G T A G A A (compl. to **24, 31, 38**)

45 5'- A A A A A G A A T A G A A G T A G A A (compl. to **25, 32, 39**)

46 5'- T T C T T C T T C T T T T C T T T T (compl. to **26, 33, 40**)

It is reported in the literature that the normal deacylation conditions (concentrated NH_4OH at 55°C, 16h) which also release the oligonucleotide from the

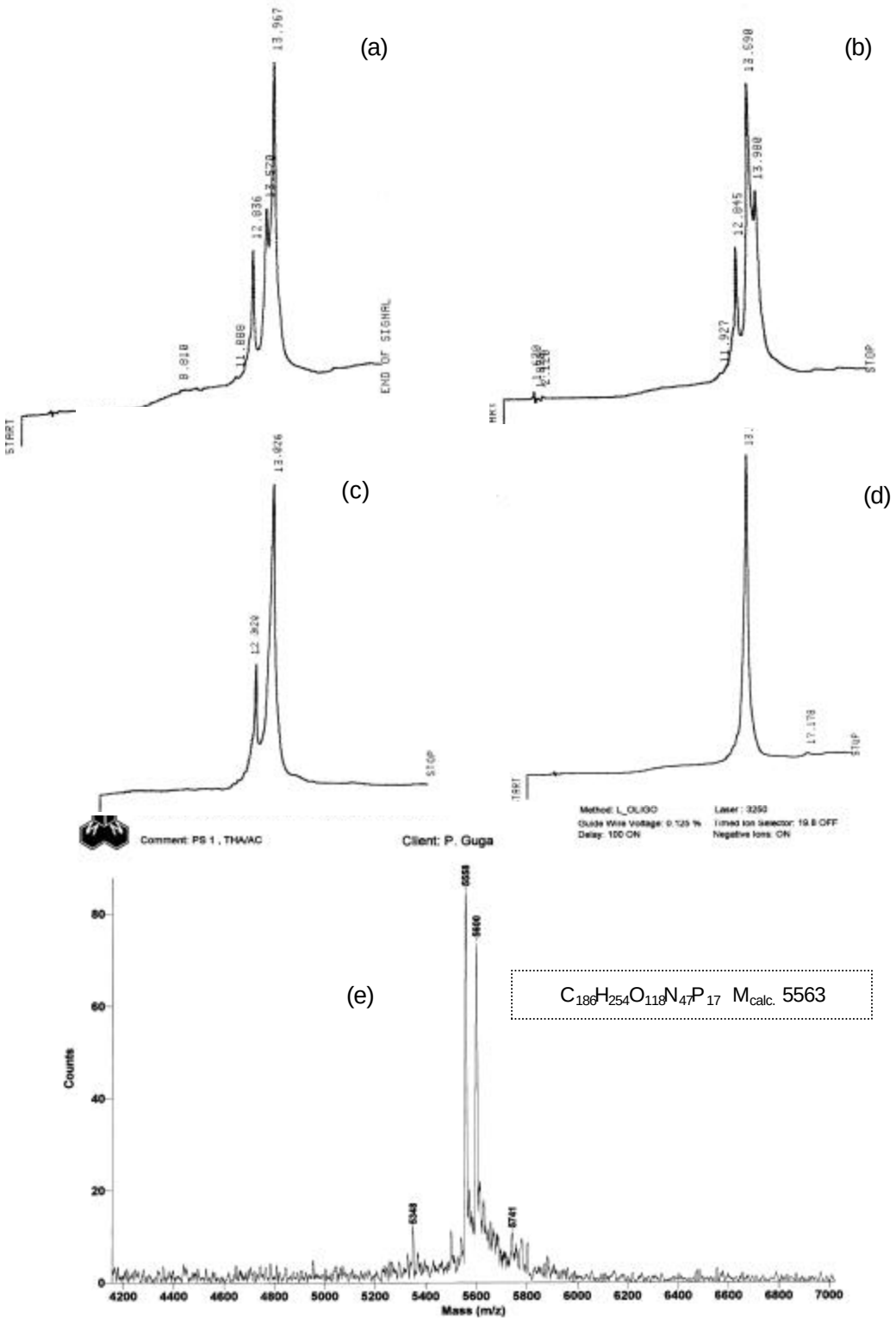


Figure 5. Analytical RP-HPLC profiles of ODN **23** after NH_4OH treatment at 65°C for (a) 18h (b) 36h (c) 72h (d) purified ODN **23** and (e) MALDI-TOF (negative-ion mode) mass spectrum of **23**.

solid support, do not cleave the N^2-isobutyryl group from N^2-isobutyryl-O^6-alkylguanine moiety in the oligonucleotide.[29] The isobutyryl group from such modified bases can be completely removed from a blocked oligonucleotide using stronger conditions such as concentrated NH_4OH at 65°C, 72h.[30] Hence the oligonucleotides having $\mathbf{D^{sp}}$, where the C2-amino protecting group is isobutyryl, was subjected to deprotecting conditions of NH_4OH at 65°C for 72h and the deprotection was monitored by analytical RP-HPLC carried out at intervals of 18h (Figure 2a-c). Initially three peaks at 12.83, 13.52 and 13.96 min were seen (18h). The peak eluting at 13.5 min increased in intensity at 36h and then became the major peak after 72h, thus demonstrating the course of deprotection. The deprotections were complete by about 70h. After the deprotection, the oligonucleotide solutions were processed (lyophilized, desalted) and their purity checked by HPLC. A representative MALDI-TOF mass spectrum (negative-ion mode) is shown in Figure 2e. The crude oligonucleotides were purified by RP-HPLC on a semipreparative RP-column (see experimental section for details).

3.4. RESULTS

3.4.1. Duplex Formation of $\mathbf{D^{sp}}$*-Oligonucleotides*

The duplex formation for the modified oligonucleotides was examined by carrying out thermal denaturation experiments in sodium phosphate buffer (10 mM) containing 100 mM NaCl, pH 7.0. The duplexes were constituted by taking equimolar ratios of the appropriate single strands in phosphate buffer, followed by annealing the samples. The UV-T_m data as derived from thermal denaturation profiles (Figure 6) are shown in Table 1. The duplex **23:43** derived from $\mathbf{D^{sp}}$-ODN **23** having a modification at the center exhibited a T_m of 32°C which was lower by 12°C compared to the control (Table 1, entries 3 & 2). When the sperminyl modification was towards the 5'-terminus, a destabilization of 13°C was noticed (entry 6). The sequence **25** carrying two

modifications exhibited a Tm of 25.5°C (entry 9) which was lower by 19°C compared to the reference **D**-ODN duplex (**32:45**) or unmodified duplex **39:45** (entries 8 & 7). This suggests a destabilization of $\mathbf{D}^{sp}$-ODN duplex by ~10-13°C per unit when present in a pyrimidine-rich strand.

The 18-mer purine rich $\mathbf{D}^{sp}$-ODN **26** formed a duplex (**26:46**) with a Tm of 33.5°C which was lower than the reference **D**-ODN **33** (ΔTm = -11.0°C, entry 12). In the longer duplexes **27:42** and **28:42**, the $\mathbf{D}^{sp}$ modification is in a purine environment and a lower destabilization of 5.5°C was seen (entries 15 & 17) compared to the corresponding reference duplexes **34:42** and **35:42** respectively (entries 14 & 16). Changing the position of $\mathbf{D}^{sp}$ in the sequence does not seem to affect the stability. However, upon increasing the number of modifications, as in **29** carrying two $\mathbf{D}^{sp}$ units, the binding was further reduced by a considerable amount (ΔTm = -13°C, entry 19).

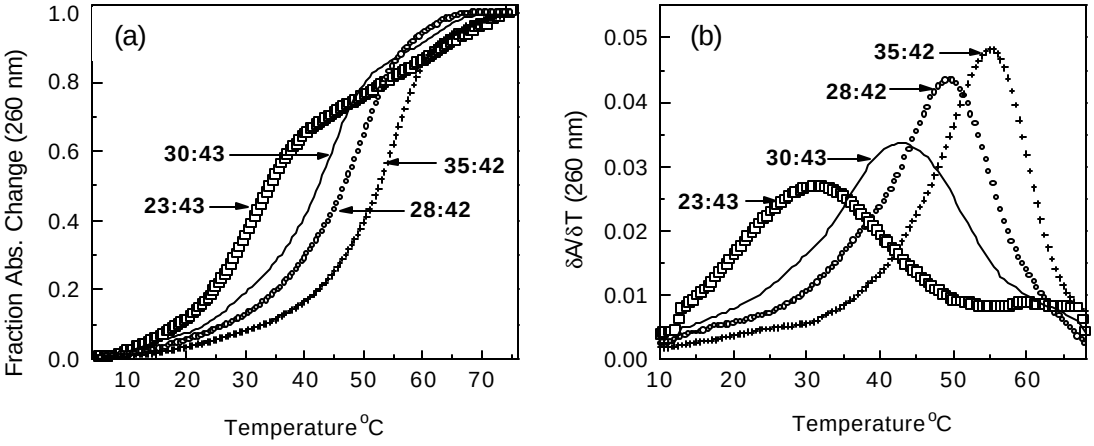


Figure 6. (a) Selected UV-melting profiles of duplexes and (b) their first derivative curves. Buffer: 10 mM sodium phosphate, 100 mM NaCl, pH 7.0.

Table 1. Duplex melting data*

Entry	Complex	Duplets	Tm (°C)	ΔTm (°C)
1	5'-T TCTT CTTCT A TTC TTTT 3'-AAGAAGAAGA T AAGAAAA	**37:43** (A:T)	44	
2	5'-TT CTT CTTCT **D** TTCT TTT 3'-AAGAAGAAGA T AAGAAAA	**30:43** (**D**:T)	44	
3	5'-TT CTT CTTCT **D**^{sp} T TC TTTT 3'-AAGAA GAAGA T AAGAAAA	**23:43** (**D**^{sp}:T)	32	-12.0
4	5'-TT CT A CTTCT TTTC TTTT 3'-AAGA T GAAGAAAAGAAAA	**38:44** (A:T)	46.0	
5	5'-TTCT **D** CTTCT TTTCT TTT 3'-AAGA T GAAGAAAAGAAAA	**31:44** (**D**:T)	46.0	
6	5'-TT CT **D**^{sp} CT TCTTTT CT TTT 3'-AAGA A GAAGAAAAGAAAA	**24:44** (**D**^{sp}:T)	33.0	-13.0
7	5'-TT CT A CTTCT ATTCT TTT 3'-AAGA T GAAGATAAGAAAA	**39:45** (A:T)	44.5	
8	5'-TT CT **D** CTTC T **D** TT CT TTT 3'-AAGA T GAAGA T AAGAAAA	**32:45** (**D**:T)	44.5	
9	5'-TT CT **D**^{sp} CTT CT **D**^{sp} TT CTT TT 3'-AAGA T GAAGA T AAGAAAA	**25:45** (**D**^{sp}:T)	25.5	-19.0
10	5'-AAAAGAA A AGAAGAAGAA 3'-TT TTCT T T TCTT CTTCT T	**40:46** (A:T)	44.0	
11	5'-AAAAGAA **D** AGAAGAAGA A 3'-TT TTCT T T TCTT CT TCT T	**33:46** (**D**:T)	44.5	
12	5'-AAAAGAA **D**^{sp} AGAAGAAGA A 3'-TT TTCTT T T CTTCTT CT T	**26:46** (**D**^{sp}:T)	33.5	-11.0
13	5'-TCCAAGAAGA A GAAAAGAAAAATAT 3'-AGGTTCTTC T T CTT TTCTT TT TATA	**41:42** (A:T)	56	
14	5'-TCCAAGAAGA **D** GAAAAGAAAAATAT 3'-AGGTTCTTC T T CT TTTCT TTT TATA	**34:42** (**D**:T)	55.5	
15	5'-TCCAAGAAGA **D**^{sp} GAAAAGAAAAATAT 3'-AGGTTCTTC T T CT TT TCT TTTTATA	**27:42** (**D**^{sp}:T)	50	-5.5
16	5'-TCC AAGAAGAAGAAAAGA **D** AAATAT 3'-AGGTT CTT CTTCTT TTCT T T TTATA	**35:42** (**D**:T)	55.5	
17	5'-TCCAAGAAGAAGAAAAGA **D**^{sp} AAATAT 3'-AGGTTCTTCT TCTT TTCT T T TTATA	**28:42** (**D**^{sp}:T)	49.5	-5.5
18	5'-TCCAAGAAGA **D** GA AAAGA **D** AAATAT 3'-AGGTTCTT CT T CT TTT CT T TT TATA	**36:42** (**D**:T)	55.5	
19	5'-TCCAAGAAG **D**^{sp} GAAAAGA **D**^{sp} AAA TAT 3'-AGGTTCTTC T T CT TTTC T TTTTATA	**29:42** (**D**^{sp}:T)	42.5	-13.0

*Buffer: 10 mM sodium phosphate, 100 mM NaCl, pH 7.0. All Tm values are accurate to ± 0.5°C and are measured in at least three melting experiments. ΔTm is the difference between the test and reference Tms.

The UV-melting temperatures of ODNs with A substitution for **D**/**D**^{sp} were also measured. The duplex Tms of **37:43** (Table 1, entry 1) and **30:43** (entry 2) were the same, 44°C. The duplexes **38:44** showed a similar behaviour when compared to the duplex **31:44** (entries 4 & 5). Similar was the case with the duplex Tms of the polypyrimidine rich ODN strand **39** with two A and **32** with two **D** residues (entries 7 & 8). Likewise, the thermal stability of the unmodified polypurine sequences **40** and **41** (entries 10 & 13) were comparable with that of the **D**-ODNs (entries 11, 14). *In general, the Tms of duplexes with **D** were comparable with that of unmodified ODNs. This again suggests that **D**^{sp}-ODNs cause destabilization compared to the reference ODNs with **D** or A.*

3.4.2. Triplex Formation of **D**^{sp}*-Oligonucleotides*

The UV-Tm of triplexes derived from the **D**^{sp}-ODNs and their complementary duplexes are summarized in Table 2. The experiments were carried out in sodium cacodylate buffer (10 mM) containing NaCl (100 mM) in the presence or absence of $MgCl_2$ (10 mM).

3.4.2.a. Triplex formation of oligonucleotides with modification in the third strand

Pyrimidine motif: The polypyrimidine **D**^{sp} sequence **23** was analyzed for its triplex formation (pyrimidne motif) with the complementary duplex **41:42** in sodium cacodylate buffer at pH 7.0. From the UV-Tm profiles (Figure 7) it is seen that the ODN **23** formed a triplex with a Tm of 30°C (Table 2, entry 3). Interestingly, the ODN **30** (with D) and the ODN **37** (with A substitution) failed to form triplex with the duplex **41:42** under identical conditions (entries 2 & 1). Thus the presence of purine bases A or **D** in a pyrimidine rich

third strand does not encourage triplex formation either in the presence or absence of

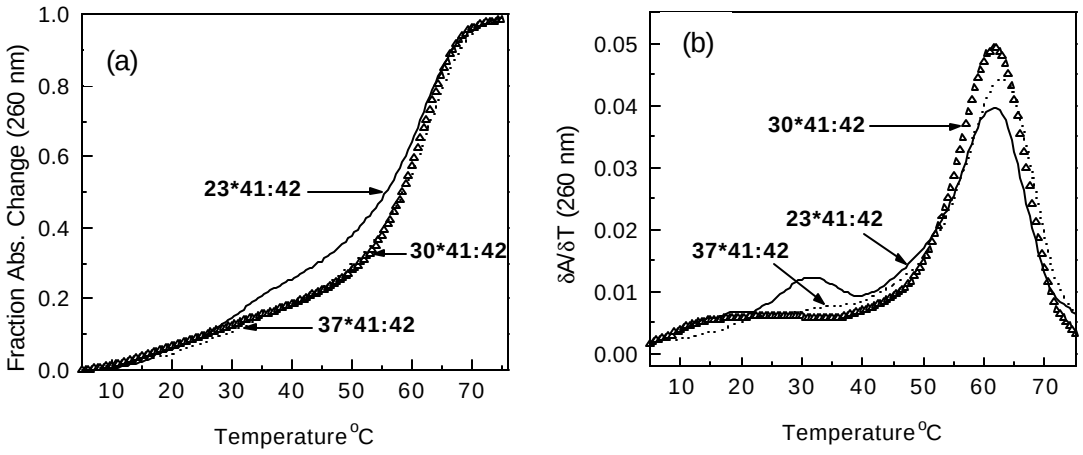


Figure 7. (a) Selected UV-melting profiles of triplexes and (b) their first derivative curves. Buffer: 10 mM Sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.0

Mg^{2+}, while the presence of spermine analog $\mathbf{D}^{sp}$ favors triplex formation.

When the sperminyl modification $\mathbf{D}^{sp}$ was present at the terminus as in **24**, a triplex T_m of 31°C was observed (Table 2, entry 6) which was higher by 4°C than the relevant reference **D**-ODN **31** (entry 5). No triplex formation was seen with the corresponding A-ODN (entry 4) in the presence or absence of Mg^{2+}.The $\mathbf{D}^{sp}$-ODN **25** with two sperminyl residues in the pyrimidine motif formed a stable triplex compared to the **D**-ODN (ΔT_{m1} = +5°C, entries 9 & 8). Once again no triplex formation was seen with A-ODNs in the presence or absence of Mg^{2+} (entry 7).

Purine motif: The $\mathbf{D}^{sp}$-ODN **26** in a purine sequence formed an antiparallel triplex with a T_m of 34°C (entry 12) while the reference **D**-ODN **33** exhibited a T_m of 31°C (entry 11) with stability equal to that of A-ODN (entry 10). This is an interesting observation since

the modification being a purine base, remains in a poly-purine context. *In general, the* ***D^{sp}****-ODNs acting as the third strand supported triplex formation at neutral pH.*

Table 2. Triplex Melting Tms[#]

No.	Sequences	Complex	10 mM Mg^{2+}		---	
			Tm1	Tm2	Tm1	Tm2
	Pyrimidine motif					
1	5'-TTCTT CTT CT A TT CTTTT 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTTCTT CT T TT CTTTTTATA	**37*41:42** (A*A:T)	nd	62.0	nd	55.5
2	5'-TT CTT CTT CT **D** TT CTTTT 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTT CTT CT T TTCTT TTTATA	**30*41:42** (**D***A:T)	nd	62.0	nd	56.0
3	5'-TT CTT CTTCT $\underline{\mathbf{D}}^{sp}$ T T CTTTT 5'-TCCAAGAAGAAGA A AA GAAAAATAT 3'-AGGTTCTT CTT CT T T T CTTTTTATA	**23*41:42** ($\mathbf{D}^{sp}$*A:T)	30.0	62.0	nd	55.5
4	5'-TTCT A CTT CT T TT CTTTT 5'-TCCAAGA A GAAGAAAA GAAAAATAT 3'-AGGTTCT T CTT CTTTT CTT TTTATA	**38*41:42** (A*A:T)	nd	62.0	nd	55.5
5	5'-TTCT **D** CTTCT T TTCTTTT 5'-TCCAAGA A GAA GAAAA GAAAAATAT 3'-AGGTTCT T CTT CTT T T CTTTTTATA	**31*41:42** (**D***A:T)	27.0	62.5	nd	55.5
6	5'-TTCT $\mathbf{D}^{sp}$ CTT CT T TT CTTTT 5'-TCCAAGA A GAA GAAAA GAAAAATAT 3'-AGGTTCT T CTT CTTTT CTTTTTATA	**24*41:42** ($\mathbf{D}^{sp}$*A:T)	31.0	62.5	nd	55.5
7	5'-TT CT A CTTCTT A TC TTTT 5'-TCCAAGA A GAAGAA A AGAAAAATAT 3'-AGGTTCT T CTT CT T T TCTT TTTATA	**39*41:42** (A*A:T)	nd	63.0	nd	55.5
8	5'-TTCT **D** CTTCT T **D** TCT TTT 5'-TCCAAGA A GAAGAA A AGAAAAATAT 3'-AGGTTCT T CTT CTT T TCT TTTTATA	**32*41:42** (**D***A:T)	29.0	63.0	nd	55.0
9	5'-TT CT $\mathbf{D}^{sp}$ CTT CTT $\mathbf{D}^{sp}$ TCTTTT 5'-TCCAAGA A GAAGAA A AGAAAA ATAT 3'-AGGTTCT T CTT CTT T TCT TTT TATA	**25*41:42** ($\mathbf{D}^{sp}$*A:T)	34.0	63.0	nd	55.0
	Purine motif					
10	3'-AAGAAGAAGA A AA GAAAA 5'-TCCAAGAAGAAGA A AA GAAAAA TAT 3'-AGGTTCTT CTTCT T TT C TT TTT ATA	**40*41:42** (A*A:T)	30.5	63.0	nd	55.5
11	3'-AAGAAGAAGA **D** AAGAAAA 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTTCTTCT T TTCTT TTTATA	**33*41:42** (**D***A:T)	31.0	63.0	nd	56.0
12	3'-AAGAAGAAGA $\underline{\mathbf{D}}^{sp}$ AAGAAAA 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTT CTT CT T TTCTTTT TATA	**26*41:42** ($\mathbf{D}^{sp}$*A:T)	34.0	63.0	nd	55.5

[#]Buffer: 10 mM sodium cacodylate, 100 mM NaCl, pH 7.0 in the presence or absence of 10 mM $MgCl_2$. All Tm values are accurate to $\pm$ 0.5 °C and are measured in at least three melting experiments. Tm1 indicates the triplex-to-duplex transition temperature and Tm2 the duplex-to-single strands. 'nd' indicates the transition not detected.

3.4.2.b. Modification in the middle strand

The triplex formation of ODNs with 2-amino-6-sperminylpurine in the target duplex to give the triad T*$\mathbf{D}^{sp}$:T was also studied and the results tabulated in Table 3. The unmodified third strand ODN **46** was hybridized with the $\mathbf{D}^{sp}$ duplex **28:42** or **D** duplex **35:42** to form the corresponding triplexes **46*28:42** (T*$\mathbf{D}^{sp}$:T) or **46*35:42** (T***D**:T) respectively with the modified unit towards the 3'-end of the central strand. The triplex stabilities in these cases were comparable (Table 3, entries 3 & 2). Under similar conditions, the reference complex **46*41:42** (T*A:T) also showed a triplex transition (Figure 8) with similar Tm (entry 1). These experiments indicated that purine modifications are tolerated in the central strand for triplex formation which is observed even in the absence of Mg^{2+}. On the other hand, when the modification was present in the middle as in **46*27:42** or the complex with multiple modification (**46*29:42**), the triplex Tms were lower by 11°C (Table 3, entries 5 & 7) than their respective reference complexes **46*34:42** and **46*36:42** (entries 4 & 6) in the presence of Mg^{2+}. However, in the absence of Mg^{2+} these complexes formed triplexes of comparable stabilities

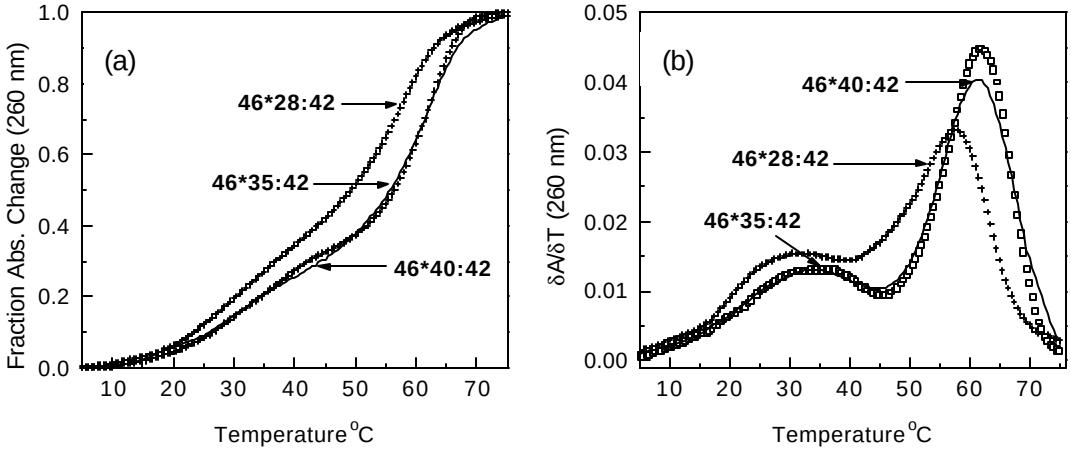


Figure 8. (a) Selected UV-melting profiles of triplexes and (b) their first derivative curves. Buffer: 10 mM Sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$ pH 7.0.

(entries 4-7).

The triplex formation was not observed in the absence of Mg^{2+} in the cases where the third strand carried the sperminyl modification and the respective reference ODNs (Table 2, entries 1-12). When the modifications were in the target duplexes, the triplexes were observed with nearly identical stabilities of ~21°C (Table 3, entries 1-7).

Table 3. Triplex T_ms of middle strand modified ODNs[#]

No.		Complex	10 mM Mg^{2+}		---	
			Tm1	Tm2	Tm1	Tm2
1	5'-TTC TTC TTCTT TTCT T TT 5'-TCCAAGAAGAAGAAAAGA A AAATAT 3'-AGGTTCTT CTTCTTTTCTT T TTATA	**46*41:42** (T*A:T)	34.0	62.0	21.0	54.0
2	5'-TTCTT C TTCTT TTCT T TT 5'-TCCAAGAAGAAGAAAAGA **D** AAATAT 3'-AGGTTCTT CTTCTTTT CT T TTATA	**46*35:42** (T***D**:T)	34.0	61.5	22.0	54.5
3	5'-TTCTT CTTCTT TTCT T T T 5'-TCCAAGAAGAAGAAAAGA **D**^{sp} AAATAT 3'-AGGTTCTT CTT CTT TTCT T T T TATA	**46*28:42** (T***D**^{sp}:T)	33.0	56.0	22.0	49.0
4	5'-TT CTT CT T C TT TT C T T T T 5'-TCCAAGAAGA **D** GAAAA G A A A A ATAT 3'-AGGTTCTT CT T CTT TT CT T T T TATA	**46*34:42** (T***D**:T)	34.0	62.5	21.0	55.5
5	5'-TTCTTCT T CTT TTC T T TT 5'-TCCAAGAAGA **D**^{sp} GAAAAGA A AAATAT 3'-AGGTTCTTCT T CTTTT CT T T TTATA	**46*27:42** (T***D**^{sp}:T)	23.0	57.0	21.0	50.0
6	5'-TTCTTCT T CTT TTCT T TT 5'-TCCAAGAAGA **D** GAAAAGA **D** AA ATAT 3'-AGGTTCTTCT T CTTTT CT T TT TA TA	**46*36:42** (T***D**:T)	36.0	63.0	22.0	55.5
7	5'-TT CTT CT T CTT TTCT T T T 5'-TCCAAGAAGA **D**^{sp} GAAAAGA **D**^{sp} AA ATAT 3'-AGGTT CTTCT T CTTTT CT T T TTATA	**46*29:42** (T***D**^{sp}:T)	25.0	49.0	20.5	42.0

[#]Buffer: 10 mM sodium cacodylate, 100 mM NaCl, pH 7.0 in the presence or absence of 10 mM $MgCl_2$. All Tm values are accurate to ± 0.5 °C and are measured in at least three melting experiments. Tm1 indicates the triplex-to-duplex transition temperature and Tm2 the duplex-to-single strands. 'nd' indicates the transition not detected.

The formation of triplexes was supported by UV-mixing curves (Figure 9), generated by addition of third strand to the preformed duplex by continuous variation method.[31,32] Increasing the mole fraction of the third strand to the duplex led to a decrease in the value of $A_{obs./calc.}$, and the inflection point at ~1 mole fraction of the third

strand indicated the formation of triplexes with a stoichiometry of 1:1 for the third-strand:duplex hybrids.

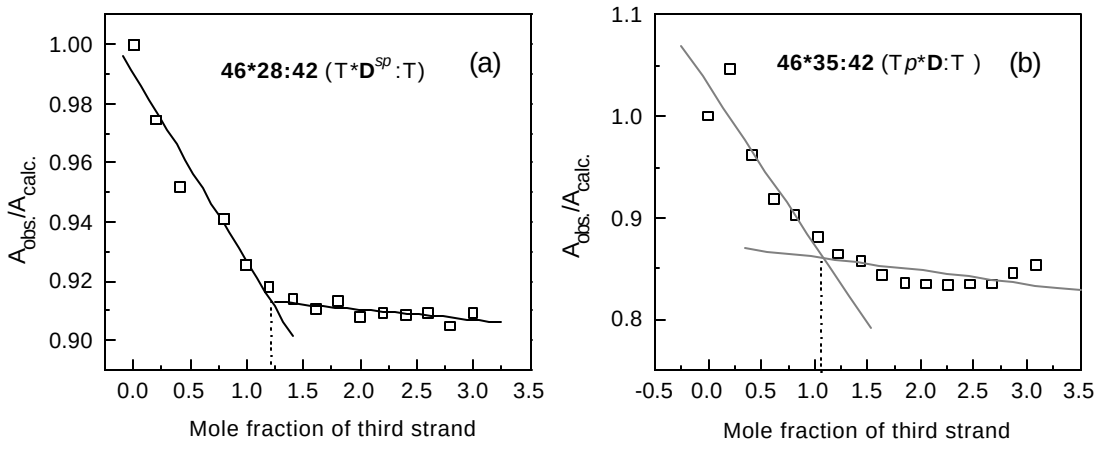


Figure 9. UV-mixing curves for the formation of parallel triplex complexes obtained by mixing third strand T (**46**) with the preformed duplex (a) **D**^{sp}:T and (b) **D**:T

3.4.2.c. Effect of D^{sp} unit (T* D^{sp}:T) on the duplex stability in triplex complexes

It was interesting to note the effect of substitution in the middle strand on the underlying duplex transitions (T_{m2}) in triplex-forming complexes. In all the cases with **D**^{sp}-ODNs acting as the middle strands in the triplex triads (T* **D**^{sp}:T), a destabilization of ~5-13°C was observed compared to the reference **D**/A-ODNs (Table 3, T_{m2}). This corresponds to the observed duplex destabilizations in the case of duplexes alone (Table 1). The observed differences in the duplex T_{ms} in the triplexes were in the same range in the presence and absence of Mg^{2+}. A large duplex destabilization seen for the **D**^{sp}-ODN complex **46*29:42** (Table 3, entry 7) arises from **D**^{sp}-substitution in the middle of the sequence, which also considerably reduced the stability of the derived triplexes.

3.4.2.d. pH Dependence of Triplex Formation

Since the triplex formation of a polypyrimidine third strand sequence with C is known to be pH-dependent,[33,34,35,36] UV-Tm experiments were also carried out at pH 5.8. The results are indicated in Table 4. At pH 7.0 in cacodylate buffer, **D**^{sp}-ODN **23** showed a triplex Tm of 30°C (Table 4, entry 3, pH 7.0) while at a lower pH of 5.8 no considerable change in Tm was observed (entry 3, pH 5.8). The complexes having **D** or A that did form triplexes at pH 7.0, did so at lower pH 5.8 with melting transitions of 34°C and 35°C respectively (entries 1 & 2).

Table 4. pH Dependence of Triplex Tms[#]

Entry	Complex	Triad	pH 7.0		pH 5.8	
			Mg^{2+}	---	Mg^{2+}	---
1	5'-TT CTT CTT CT A TT CTTTT 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTT CTT CTT CT T TTCTTTT TATA	**37*41:42** (A*A:T)	nd	nd	35.0	22.0
2	5'-TTCTT CTT CT **D** TTCTTTT 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTT CTT CT T TTCTTTTTATA	**30*41:42** (**D***A:T)	nd	nd	34.0	22.0
3	5'-TT CTT CTTCT **D**^{sp} TT CTTTT 5'-TCCAAGAAGAAGA A AAGAAAAATAT 3'-AGGTTCTT CTTC T T TT CTTTTTATA	**23*41:42** (**D**^{sp}*A:T)	30.0	nd	31.0	22.0
4	5'-TT CT A CTTCT TTTCTTTT 5'-TCCAAGA A GAAGAAAAGAAAAATAT 3'-AGGTTCT T CTT CTTTT CTTTTTATA	**38*41:42** (A*A:T)	nd	nd	36.0	22.5
5	5'-TTCT **D** CTTCT TTTCTTTT 5'-TCCAAGA A GAAGAAAAGAAAAATAT 3'-AGGTTCT T CTT CTTTT CTTTTTATA	**31*41:42** (**D***A:T)	28.0	nd	36.0	24.0
6	5'-TTCT **D**^{sp} CTTCT T TTCTTTT 5'-TCCAAGA A GAAGAAAAGAAAAATAT 3'-AGGTTCT T CTT CTT TTCTTTTTATA	**24*41:42** (**D**^{sp}*A:T)	31.0	nd	34.0	24.0
7	5'-T TCT A CTTCT T A TCT TTT 5'-TCCAAGA A GAAGAA A AGAAAAATAT 3'-AGGTTCT T CT TCT T T T CTTTTTATA	**39*41:42** (A*A:T)	nd	nd	36.5	24.5
8	5'-TT CT **D** CTTCT T **D** TCT TTT 5'-TCCAAGA A GAAGAA A AGAAAA ATAT 3'-AGGTTCT T CTT CT T T TCTTTTTATA	**32*41:42** (**D***A:T)	nd	nd	32.0	25.0
9	5'-TTCT **D**^{sp} CTTCT **D**^{sp} TTCTTTT 5'-TCCAAGA A GAAGA A AAGAAAAATAT 3'-AGGTTCT T CTTCT T TTCTTTTTATA	**25*41:42** (**D**^{sp}*A:T)	29.0	nd	28.0	28.0

[#]Buffer: 10 mM sodium cacodylate, 100 mM NaCl, (0/10 mM) Mg^{2+} at pHs 6.5 and 7.0; 50 mM NaOAc, 100 mM NaCl, 10 mM Mg^{2+}, pH 5.8. All Tm values are accurate to $\pm$ 0.5 °C and measured in three melting experiments. 'nd' indicates the triplex formation not detected.

The **D**^{sp}-ODN **25** with two modified units also exhibited a similar trend with Tms of 29°C and 28°C at pHs 7.0 and 5.8 respectively. The corresponding reference ODN complexes with double A (**39*41:42**) and **D** substitutions (**32*41:42**) formed triplexes only at acidic pH (entry 7 & 8, pH 5.8). It was seen that increasing the degree of substitution of **D** or **D**^{sp} altered the stability of the triplexes (entries 8 & 9) and the unmodified A-ODNs were able to form triplexes only at a lower pH of 5.8 (entry 7). The **D**^{sp}-ODN **24** with spermine conjugation towards the 5'-end showed a different behaviour. The melting temperature at pH 5.8 was higher by 3°C (entry 6) than that at pH 7.0.

pH dependent triplex formation in the absence of divalent cation (Mg^{2+}) was also evaluated. None of the ODNs formed triplexes at pH 7.0 in the absence of Mg^{2+} and triplexes could be observed only at acidic pH 5.8. No difference between the Tms of ODNs **23**, **30**, **37** could be observed at pH 5.8 (Table 3, entries 1, 2 & 3, pH 5.8).

3.5. DISCUSSION

3.5.1. Duplex Formation by D^{sp}-ODNs

It is observed from Table 1 that **D**^{sp}-ODN sequences **23-29** have a detrimental effect on duplex formation which ranged from -5.5°C to -13.0°C per modification. The stability seems to depend significantly on the sequence context. With one modification in the 25-mer polypurine strands, the destabilization was almost -5.5°C (Table 1, entries 15 & 17) which increased to -13.0°C for the **D**^{sp}-ODN **29** bearing two sperminyl modifications (entry 19). The destabilization seen for a single modification in a polypyrimidine strand (**D**^{sp}-ODNs **23 & 24**, entries 3 & 6) is slightly higher than the **D**^{sp} substitution in the polypurine sequence (entry 12). Such destabilization effects are similar to those observed earlier with sperminyl-pyrimidine modification.[15] These results

suggest that the destabilization of duplexes results perhaps from a conformational rotation about C6-N bond, placing the spermine chain towards the complementary base, leading to the loss of one hydrogen bond (Figure 8). In this conformation, the spermine chain may span across the DNA duplex and interact with adjacent bases leading to sequence dependent effects.

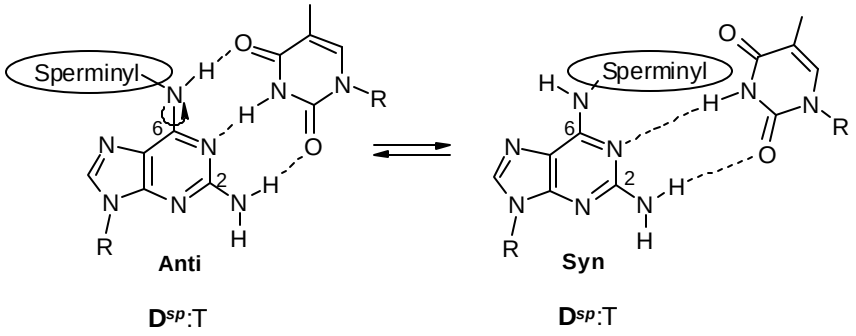


Figure 8. *Syn-anti* conformations expected in **D**^{sp} derivatives.

3.5.2. Triplex Formation by D^{sp}-ODNs

The triplex Tm data (Table 2) indicates that the polypyrimidine **D**^{sp}-ODN **23** (entry 3) forms a stable triplex at pH 7.0 while the ODN **30** (entry 2) bearing 2-aminoadenine, **D** (the appropriate reference for 2-amino-6-sperminyl-dA, **D**^{sp}) as well as the unmodified ODN **37** (entry 1) having A, do not show any triplex transition. Triplex stabilization is a well known feature of polyamines which arises from the combined

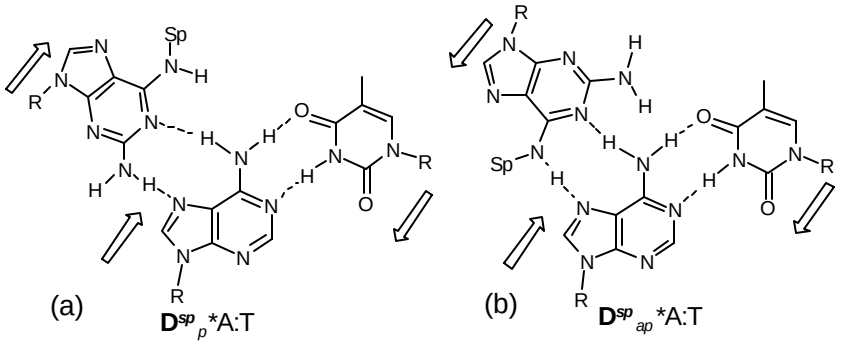
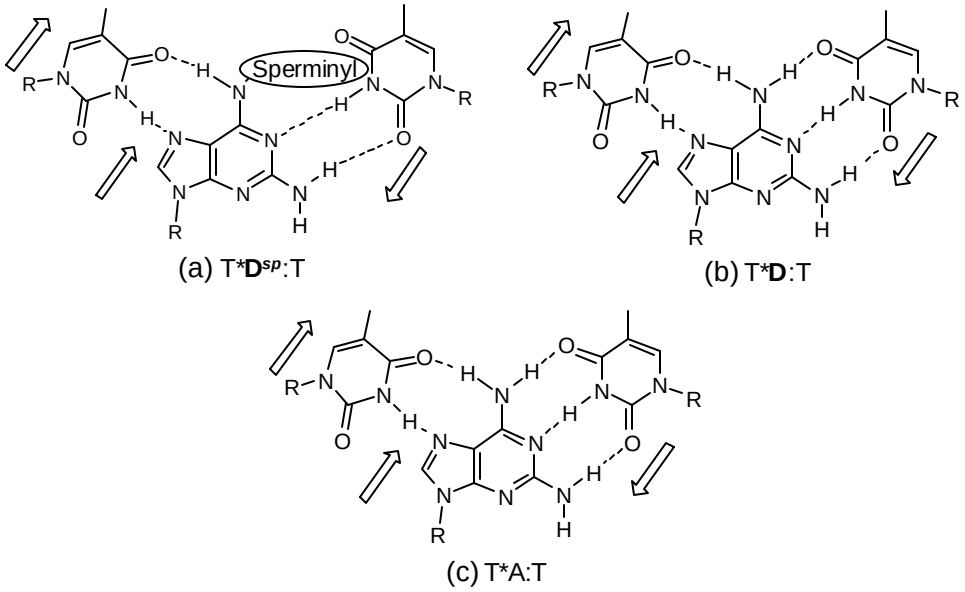


Figure 9. The hydrogen bonding patterns of **D**^{sp} in (a) parallel and (b) antiparallel orientations. 'Sp' indicates sperminyl chain.

influence of their ionic charge as well as chemical structure. In the present case, **D**^{sp} forms Hoogsteen hydrogen-bonds (Figure 9a) and the cationic sperminyl chain may assist in shielding the anionic charge density of the oligonucleotide thereby facilitating triplex formation. The observation is similar to the reported[15] dC-N^4-spermine ODNs which also stabilize triplex formation when present in the third strand at pH 7.0. A similar trend was observed with third strand **D**^{sp}-ODNs **24-26**, giving rise to stable triple helices at neutral pH.

The incorporation of **D**^{sp} in a polypurine third strand gave similar stability compared to its presence in a polypyrimidine strand. *In the present case a better stacking of the purine* ***D***^{sp} *is possible in a third strand comprising purines than in a pyrimidine motif.*

Under identical conditions, **D**^{sp} present in the middle strand in **46*28:42** (T***D**^{sp}:T) showed a nominal destabilization of 1°C for triplex formation when compared either with **46*35:42** (T***D**:T) or the unmodified complex **46*41:42** (T*A:T) (Table 3, entries 3, 2 & 1). Since **D**^{sp} destabilized the duplexes in the case of **D**^{sp}:T as discussed



(a) T***D**^{sp}:T (b) T***D**:T

(c) T*A:T

Figure 10. The recognition of (a) **D**^{sp}:T (b) **D**:T and (c) A:T by third strand T in pyrimidine motif.

earlier (Section 3.4.1.), the underlying duplex to single strand transition was also lowered by 5.5°C. This fact suggests that the **D**^{sp} in the duplex of a triplex is similar to that in the duplex alone, with the sperminyl chain pointing towards the Watson-Crick complementary base pair (Figure 10a). This results in Hoogsteen base pairing in T*$\mathbf{D}^{sp}$:T similar to that in T***D**:T and T*A:T (Figure 10b & 10c respectively) and hence these have similar triplex Tms. The identical stability of triplexes **46*28:42** (T*$\mathbf{D}^{sp}$:T), **46*35:42** (T***D**:T) and **46*41:42** (T*A:T) with lowering of duplex stability only in **46*28:42** (T*$\mathbf{D}^{sp}$:T) reinforces that the origin of destabilization in the duplex alone or that in the triplex arises from the conformational incompatibility (due to the C6-N bond rotation) in duplexes.

3.5.3. Effect of pH

In the pyrimidine motif, the protonation of N3 of cytidine is required to establish two Hoogsteen hydrogen bonds with guanosine. The pK_a of N3 in cytidine nucleoside is 4.3. However its apparent pKa increases when incorporated into ODNs due to the polyanionic environment, leaving an optimum pH of around 5.5 for triplex formation.[33,34,35] The pK_a values shift towards physiological pH in duplexes and triplexes as a result of microenvironment effects favouring protonation. The triplex stability of ODN forming complexes were measured at different pHs. It is seen that at pH 5.8, in the absence of Mg^{2+}, $\mathbf{D}^{sp}$-ODN **23** as well as the **D**-ODN **30** and the unmodified ODN **37** formed triplexes with the same thermal stability (Table 4, entries 1-3, pH 5.8). In the presence of Mg^{2+}, all exhibited higher Tms, the complex with **D** (**30*41:42**) having a ΔT_m of +3.0°C compared to the $\mathbf{D}^{sp}$-ODN complex **23*41:42** (entries 2 & 3). The complexes having the modifications near the 5'-terminus (**24*41:42** & **31*41:42**) or the complexes having multiple modifications (**25*41:42** & **32*41:42**) behaved in a similar manner.

The above observations showed that triplex stability is strongly dependent on pH within the range 5.8 and 7.0 and may be explained as follows. The third strand cytosine would be protonated at 5.8 pH and may generate two Hoogsteen hydrogen-bonds (Figure 11) to stablilize the triple helix structure. The cytosine N3 would be less protonated at neutral pH, decreasing the extent of hydrogen bonding thereby decreasing the stability of triple helical complexes. *With the C6-sperminyl moiety, however, the third strand can still form a triple helix at neutral pH due to the extra binding energy resulting from interaction of sperminyl moiety with the nucleobases or the anionic phosphates. The sperminyl chain which is protonated and positively charged even at physiological pH, may help to reduce the electrostatic repulsion between the charged phosphates - the determining factor in triplex formation.*

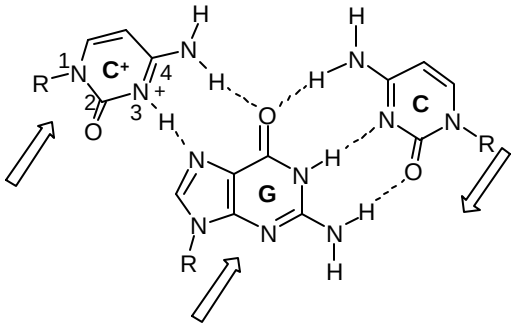


Figure 11. Hoogsteen hydrogen bonding of C^+*G:C.

The association constant K_a for triplex formation is ~1000 times lower than that for the duplex and the rate of third strand association with the duplex is ~100 times slower than association of duplex from single strands.[37] As a consequence, hysteresis is observed for triple helix dissociation-reassociation which is dependent on both, pH and ionic strength.[38] To examine the role of appended spermine in triplex stabilization through strand association and dissociation events, the relative heating and cooling curves were examined for both **D**^{sp}-ODNs and corresponding **D**-ODNs. A

representative hysteresis curve is shown in the Figure 12. The **D**^{sp}-ODNs exhibited a small hysteresis which shows a kinetically faster association while the reference ODNs showed an exceedingly slow association. This implies that appended spermine partly favours the association process in triplex formation by increasing the affinity to the target duplex.

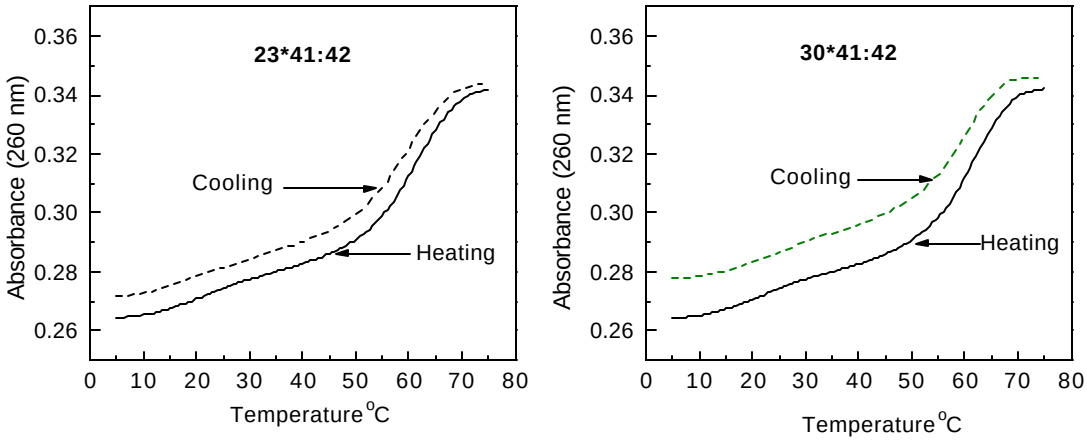


Figure 12. Hysterisis of the denaturation and re-naturation phenomena of the triplexes **23*41:42** (**D**^{sp}*A:T) and **30*41:42** (**D***A:T). Buffer: 10 mM sodium cacodylate, 100 mM NaCl, 10 mM Mg^{2+} at pH 5.8.

3.6. CONCLUSIONS

This section describes the synthesis of 2-amino-C6-sperminylpurine suitable for incorporation into ODN sequences by solid phase synthesis. The **D**^{sp}-ODNs thus synthesized were purifed by RP-HPLC and characterized by MALDI-TOF mass spectroscopy. The results presented here show the effect of spermine conjugated to the purine (**D**^{sp}) acting as the third strand in forming triple helical complexes. At neutral pH 7.0, where the reference ODN **30** failed to form a triplex, **D**^{sp}-ODN **23** acting as the third strand formed a stable triplex. The polypyrimidine sequences **24** and **25** also formed stable triplexes at neutral pH.

The site-specific spermine incorporation through the purinyl analogue allows the introduction of cationic environment at the desired internal sites of oligonucleotides thereby giving rise to zwitterionic DNA. The triplex formation by **D**^{sp}-ODNs at physiological pH embedded in a polypyrimdine sequence with multiple cytosines demonstrates the ability of sperminyl oligonucleotides to overcome the N3 protonation barrier of cytosine for Hoogsteen hydrogen-bond formation. This remarkable ability of **D**^{sp}-ODNs may be attributed to the interaction of the cationic sperminyl chain with the nucleobases in addition to the charge neutralization. Studies involving **D**^{sp} in the middle strand of triple helical complexes T*$\mathbf{D}^{sp}$:T showed destabilization of the duplexes to a large extent. Interestingly, the **D**^{sp}-ODNs were better in the re-association process which is clear from the hysteresis data indicating a kinetically faster association phenomenon.

For the purpose of a therapeutic agent, *in vivo* stability and cellular uptake are important concerns. End-blocked ODNs have been shown to be more nuclease resistant.[39] The synthesis of **D**^{sp}-ODNs carrying the modification at the 3' or 5' ends can improve the nuclease resistance, in addition to the triplex stabilizing effect. A lower net negative charge arising from **D**^{sp}-ODNs may assist the cellular uptake of ODNs.[40] Furthermore, the use of polyamine transport system for drug delivery has been reported[41] which may be applicable to these polyamine tethered ODNs.

3.7. REFERENCES

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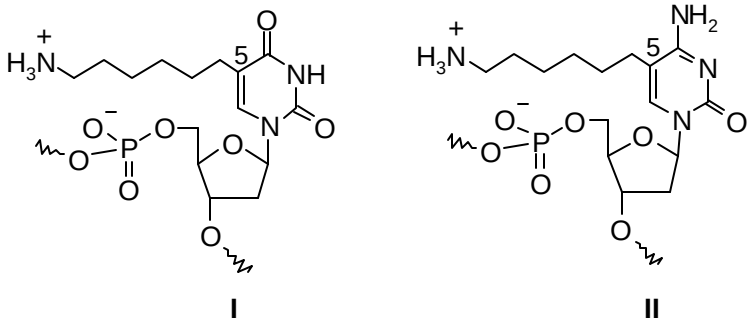
Chapter 2

Section B:

Chemical Synthesis and Biophysical Studies of 5-Me-dC-N^4-Alkylguanidino Substituted Oligonucleotides

2.7. INTRODUCTION

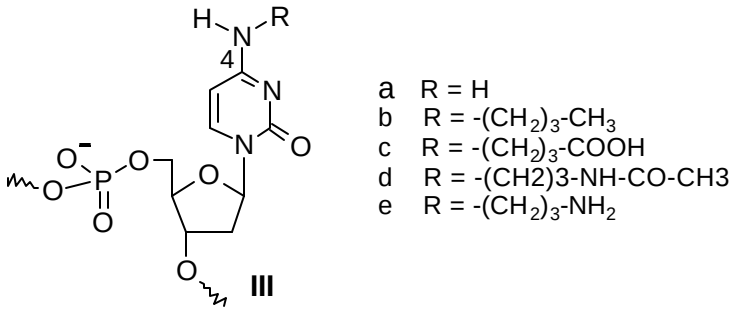
Unnatural nucleosides with altered or additional hydrogen bond donor and acceptor groups and appended cations when incorporated into oligonucleotides can impart different recognition specificities towards proteins and nucleic acids. ODNs bearing pyrimidine 5-ω-aminohexyl substituents which are 'zwitterionic ODNs' (Figure 1) bind to natural DNA (having complementary sequence) at low ionic strength equally or even better than natural DNA itself. [1]



I **II**

Figure 1. Oligonucleotides with 5-ω-aminohexyluridine (**I**) and 5-ω-aminohexylcytidine (**II**).

Among the ODNs containing N^4(butyl)dC **IIIb**, N^4-(3-carboxypropyl)-dC **IIIc**, N^4-(3-acetamidopropyl)dC **IIId**, or N^4-(3-aminopropyl)dC **IIIe** (Figure 2), complementation with the target duplex DNA suggested favoured triplex formation in the case of N^4-(3-aminopropyl)dC **IIIe**, although with a lower stability.[2]



III

a R = H
b R = $-(CH_2)_3-CH_3$
c R = $-(CH_2)_3-COOH$
d R = -(CH2)3-NH-CO-CH3
e R = $-(CH_2)_3-NH_2$

Figure 2. Triplex forming ODNs with different N^4-substituents on dC.

Tetra-cationic peptides attached to ODNs were shown to favour triplex formation.[3] The family of ODN-conjugates with a constant peptide component (arginine) but different nucleic acid chain lengths -15, 18 or 21-mers showed a near constancy of the triplex Tm emphasizing the importance of cationic charge in influencing the triplex formation. The synthesis of pentameric thymidyl deoxyribonucleic guanidine (DNG, Figure 3) in which the phosphodiester linkages of DNA are replaced by guanidinium linkages (-NHC(=NH_2^+)NH-) demonstrated unprecedented stability of the DNG:DNA complex.[4] In the previous section of this Chapter, the non-covalent effect of guanidinum groups on the stabilization of DNA complexes was demonstrated. The interesting results encouraged us to suitably conjugate guanidinium group to nucleobases of ODNs and study their triplex formation.

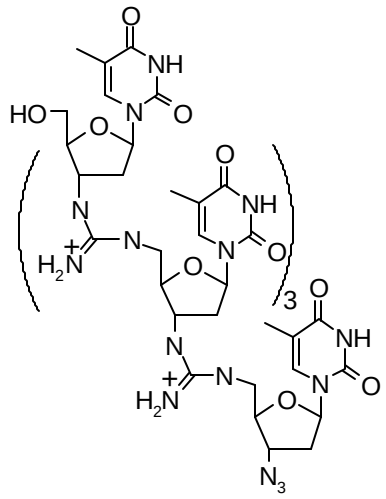


Figure 3. DNG-Deoxyribonucleic guanidine.[4]

2.8. OBJECTIVES

The present Section is aimed at the synthesis and complementation studies of N^4-propyl/butyl-guanidino-5-Me-dC containing oligonucleotides (Figure 4). The guanidine conjugation through a methylene spacer was chosen to be on a pyrimidine at

the 4-position which is relatively easy to synthesize by the convertible nucleoside approach. The guanidine group which is highly basic (pK_a > 13) would remain protonated at neutral pH, thereby imparting a zwitterionic nature to the ODNs which may lead to better hybridization.

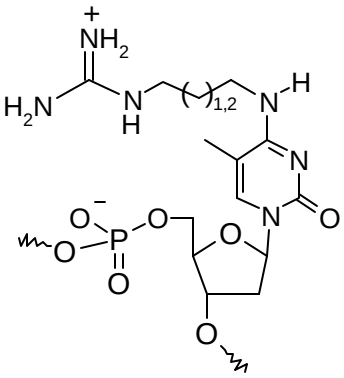
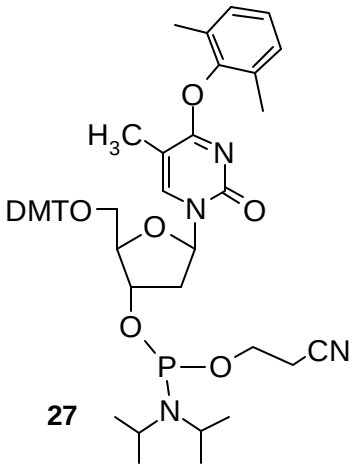


Figure 4. 5-Me-dC-N^4-alkyl guanidino ODNs.

The objectives of this section are:

- Synthesis of "convertible nucleoside"- O^4-2,6-dimethylphenyl thymidine-3'-phosphoramidite **27** and its incorporation into ODNs by solid phase synthesis.
- Functionalization of the "convertible ODNs" into the corresponding 5-Me-N^4-guanidino propyl/butyl derivatives and their characterization.
- Biophysical studies of the N^4-guanidino-propyl/butyl dC containing ODNs.



27

2.9. PRESENT WORK

2.9.1. Strategies for Functionalized ODN Synthesis

Two strategies are normally employed for the synthesis of oligonucleotides bearing tethered functionality (functionally tethered oligonucleotides, FTOs): (i) the dedicated monomer strategy and (ii) the convertible nucleoside strategy.

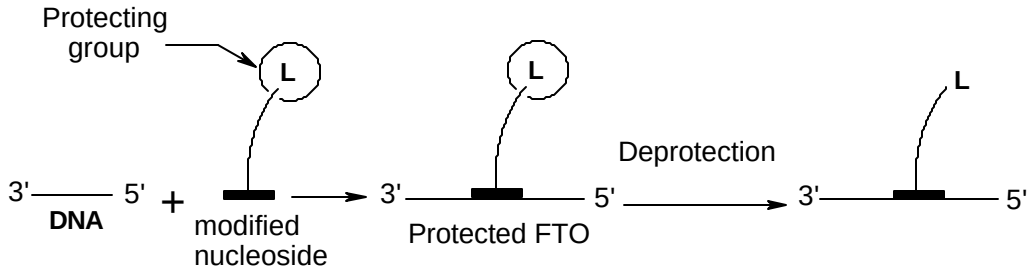


Figure 5. Dedicated monomer strategy.

In the first method (Figure 5) a suitable nucleoside monomer carrying the desired modification with the appropriate protecting group compatible with the conventional oligonucleotide synthesis, is incorporated into the ODNs by automated DNA synthesis. This has limitations in terms of complex protection-deprotection strategies. The second method, the "convertible nucleoside strategy" (Figure 6)

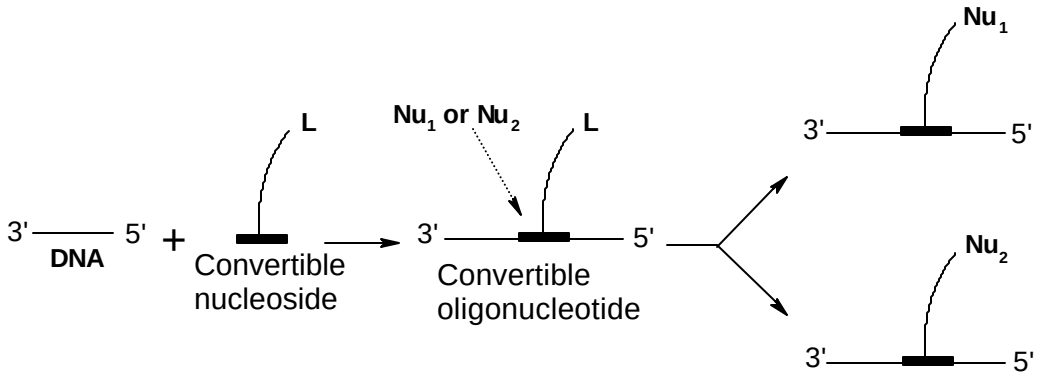


Figure 6. Convertible nucleoside strategy.

employs a nucleoside containing a leaving group, such as 2,6-dimethyl phenol, that is completely retained during the ODN synthesis and subsequent deprotection steps. After the oligonucleotide assembly, the leaving group **L** can be displaced by different nucleophiles, leading to various modified ODNs. This strategy was used for the synthesis of dC-N^4-guanidinopropyl or guanidinobutyl substituted ODNs.

The nucleoside carrying a functionalized protecting group required for the dedicated monomer strategy or that containing a convertible function can be prepared in different ways as follows. One method is by introducing a leaving group [e.g. 4-(3-nitro-1,2,4-triazolyl)[5] or 4-(1,2,4-triazolyl)-[6,7]] at C4 in an appropriately protected thymidine (Figure 7) followed by nucleophilic displacement. In another procedure N^4-cytidine modified oligonucleotides were synthesized by the reaction of the oligonucleotide containing cytidine at appropriate positions with the selected amine in the presence of sodium bisulfite.[2] This reaction does not work with 5-methylcytosines which does not form an adduct with bisulfite.[8]

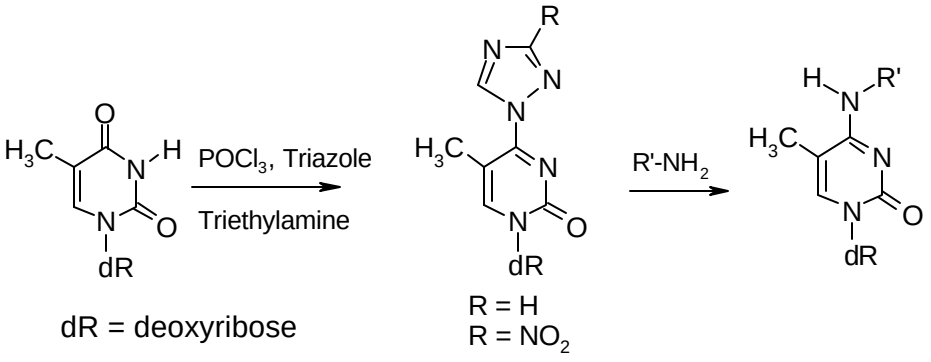
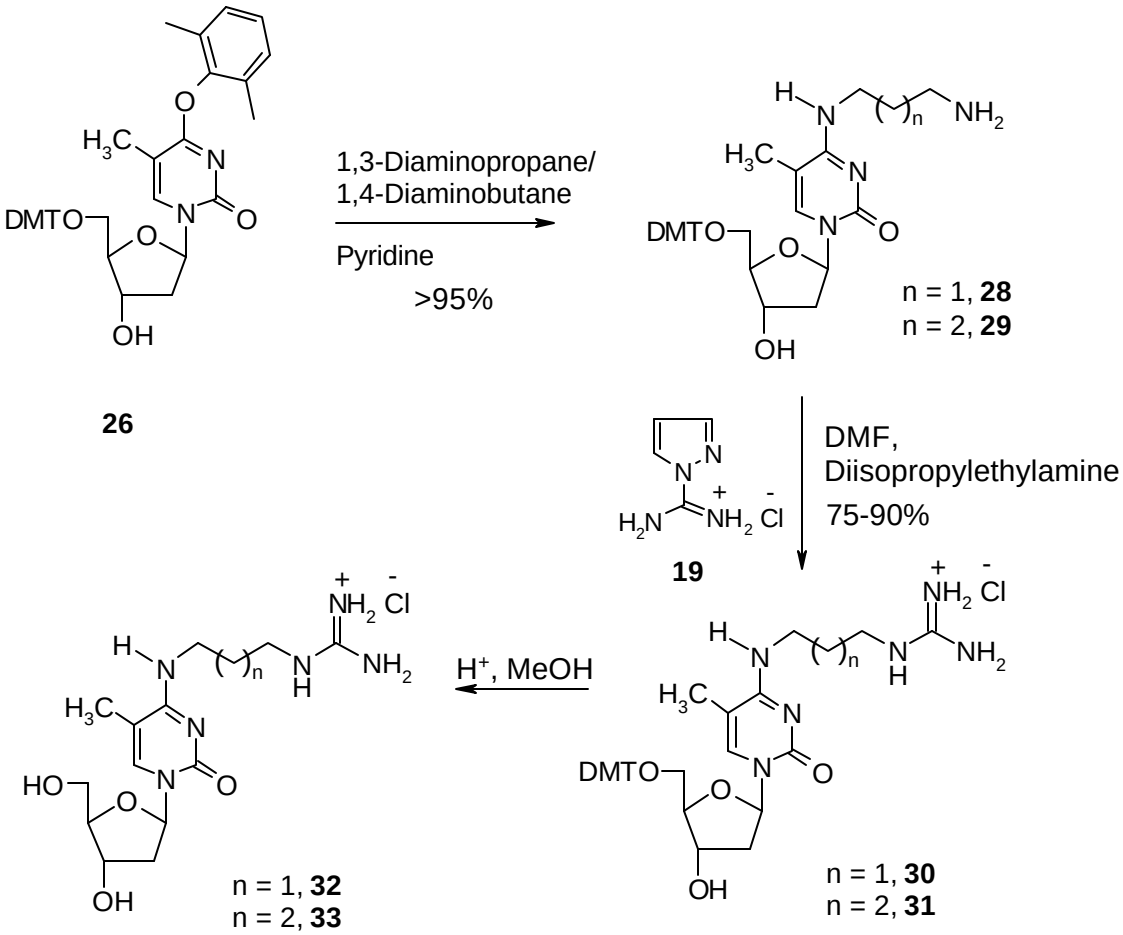


Figure 7. Literature methods[5-7] for N^4-derivatization of 5-Me-dC.

In the present work O^4-2,6-dimethylphenyl-thymidine-3'-O-phosphoramidite (**27**) was incorporated into ODNs, followed by transformation to the desired guanidinium-ODN conjugates in a post-synthetic reaction.

2.9.2. Synthetic Compatibility of the Nucleoside



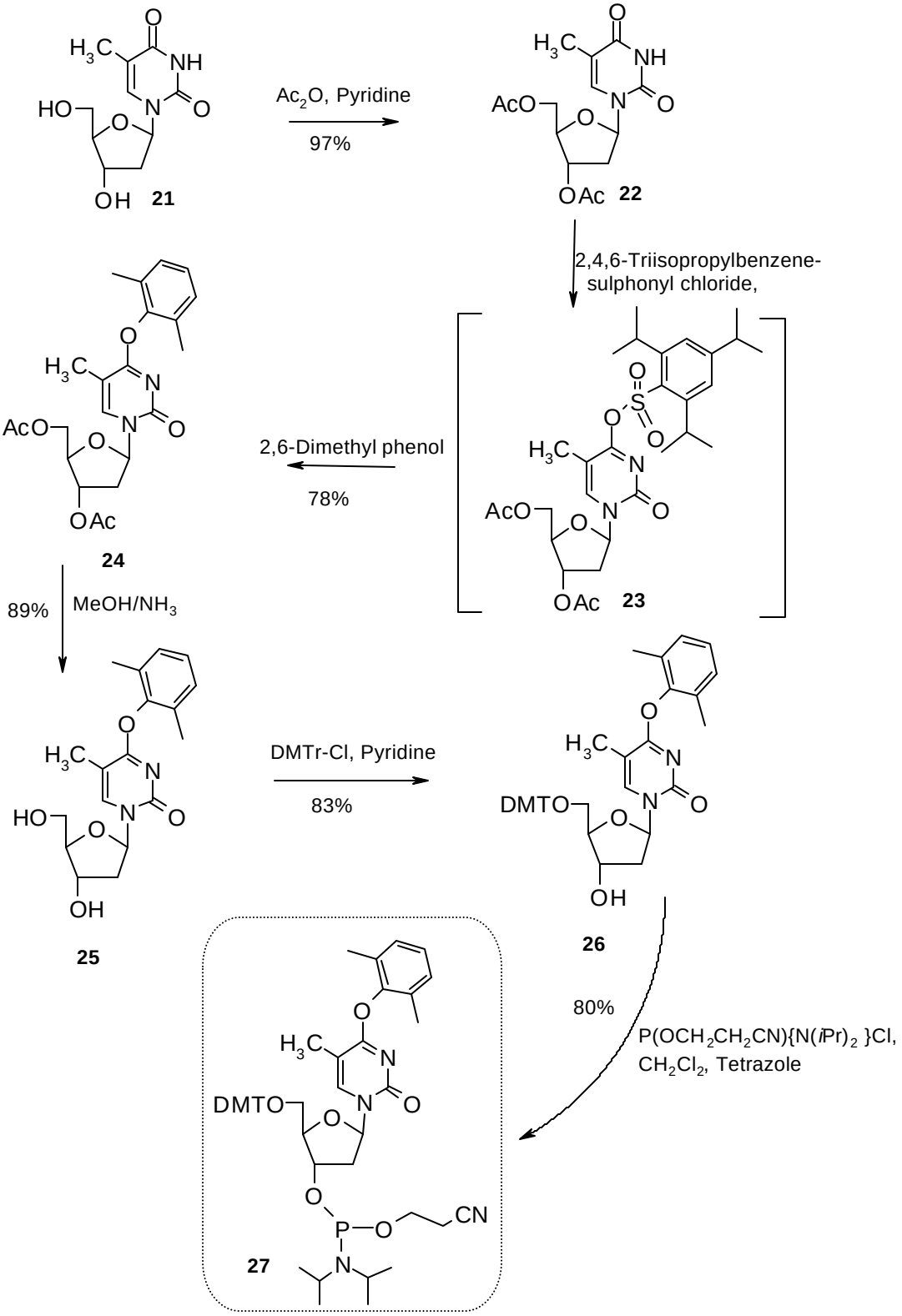
Scheme 3. Functionalization of the convertible nucleoside.

In order to evaluate the synthetic utility of the convertible function in 5'-O-DMT-*O*^{4}-(2,6-dimethylphenyl)-2'-deoxythymidine **26**, (synthesized in Section 2.9.3.) was treated individually with 1,3-diaminopropane or 1,4-diaminobutane (Scheme 3), in pyridine to get the corresponding N^4-3-aminopropyl- (**28**) or N^4-4-aminobutyl-5-Me-dC (**29**) respectively. The success of the reaction was monitored and the products characterized by ^{1}H NMR spectrum. The adducts **28** and **29** were separately treated with the guanylating reagent pyrazole carboxamidine hydrochloride[9] **19** to get the

respective guanidinium derivatives **30** and **31**. These compounds were characterized by ^{1}H NMR and FAB-MS techniques. The ^{1}H NMR spectra of **30** and **31** exhibited the N-C$\underline{H}_2$ protons of the propyl/butyl groups at δ 3.50-3.10 integrating to 4 protons and ascribed to N^4-C$\underline{H}_2$ & C$\underline{H}_2$-NH(C=NH)-N$\underline{H}_2$ methylene protons of the propyl/butyl chains. The molecular ion peaks seen at 643 (M^+) in the mass spectra of **30** and at 657 (M^+) in the case of **31** confirmed the N-alkylguanidino structures. These results suggested that (i) the reactivity of dimethylphenyl derivative for nucleophilic reaction with primary amine and (ii) the reactivity of amino group for converting to guanidino function are appropriate for post-synthetic ODN syntheses. DMT deprotection in **30** and **31** was achieved by treating them separately with methanolic HCl to afford the respective unprotected nucleosides **32** and **33**.

2.9.3. Synthesis of O^4-2,6-Dimethylphenyl-thymidine-3'-O-Phosphoramidite 27

The *O^4*-2,6-dimethylphenyl thymidine-3'-*O*-phosphoramidite was synthesized by the reported method[10,11] starting from thymidine as shown in Scheme 4. The 3' and 5'-hydroxyls of thymidine **21** were first protected as *O*-acetates to get the 3',5'-*O*-diacetate **22**. This was converted *in situ* to 3',5'-diacetyl-4-*O*-(2,4,6-triisopropylbenzene sulfonyl)-2'-dT **23**, by treatment with 2,4,6-triisopropyl benzenesulfonyl chloride and triethylamine and transformed to its 4-*O*-(2,6-dimethylphenyl) derivative **24**, upon addition of 2,6-dimethylphenol, triethylamine and DABCO. In the ^{1}H NMR spectrum of **24**, the aromatic protons and the Ar-C$\underline{H}_3$ of 2,6-dimethylphenyl component could be seen at δ 7.10-6.95 & 2.10 respectively[11] indicating the formation of **24**. The appearance of the C4 signal at δ 168.8 in ^{13}C NMR is another indication of the *O^4*-dimethylphenyl derivatization. The deprotection of 3',5'-*O*-diacetates using methanolic ammonia gave **25** having the hydroxyl groups free. The primary 5'-hydroxyl group in **25** was protected as DMT derivative **26** by treatment with DMTr-Cl in anhydrous pyridine. The ^{1}H NMR of the dimethoxytrityl ether showed a multiplet for aromatic



Scheme 4. Synthesis of O^4-2,6-dimethylphenyl thymidine monomer.

protons at δ 7.50-6.80 (13 H) and a characteristic singlet at δ 3.80 corresponding to 6H of the methoxy groups. The 5'-O-DMT compound **26** was converted to the phosphoramidite derivative **27** through phosphitylation reaction employing chloro-N,N-diisopropyl-2-cyanoethoxyphosphine in dry CH_2Cl_2 in presence of diisopropylethylamine.[12] In the ^{31}P NMR spectrum, signals at δ 150.31 & 149.85 confirmed the formation of the product **27** as a diastereomeric mixture.

2.9.4. Design and Synthesis of Oligonucleotide Sequences

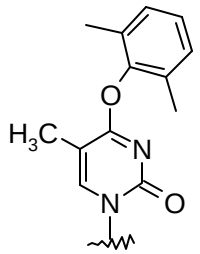
For the study of duplex and triplex stability effects of alkyl guanidinum ODNs, the sequences **34-40** were designed. The target duplex sequence for the triplex studies was the 24-mer **39:40**. The sequences for the third strand were 18-mer polypyrimidines containing one modified site each in **34** and **35**, and two in sequence **36**. The third strand upon binding to the double-stranded target forms a triplex with an overhang of 3 base pairs each on either side. This difference in lengths of the duplex and third strand (and hence triplex) would help to differentiate their melting temperatures. The sequence design also avoided self-complementation and a few cytosines were introduced into the polypyrimidine strands to prevent its slippage on the duplex.

The oligonucleotides **34-40** (given in the next page) were synthesized by standard phosphoramidite methodology[13] on an automated DNA synthesizer (Pharmacia GA Plus) using the phosphoramidite **27** along with other standard PAC-protected β-cyanoethylphosphoramidites. The coupling yields of **27**, as quantified by the released DMT cation, were approximately 95% compared to the average yield with unmodified amidites ~99%. The concomitant use of deoxyribonucleoside phosphoramidites having the phenoxyacetyl group[14] for protection of the exocyclic amino function of adenine and guanine, and the isobutyryl group for that of cytosine,

was necessary to ensure rapid ODN deprotection with conc. NH_4OH without affecting the O^4-arylated thymidine residues.[11]

34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT
37 5' TTC TTC TTC TTT TCT TTT
38 5' AAA AGA AAA GAA GAA GAA
39 5' TCC AAG AAG AAG AAA AGA AAA TAT
40 5' ATA TTT TCT TTT CTT CTT CTT GGA

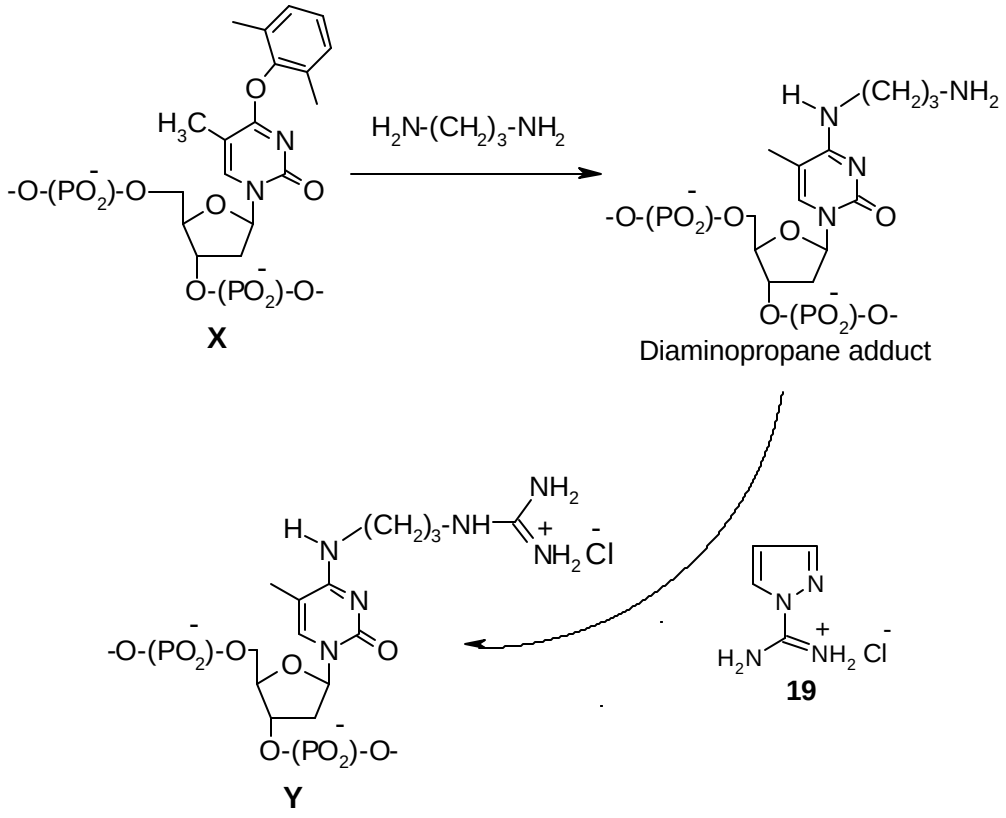
X =



Upon completion of the synthesis, the resin was treated with conc. NH_4OH at room temperature for 8h. Under these conditions, the cleavage from the resin and base deprotection are complete, while the 2,6-dimethylphenyl moiety is completely retained.[11] The analytical HPLC of the crude convertible ODNs indicated that they were >95% pure and were used for functionalization into the guanidino derivatives.

2.9.5. Functionalization of the Convertible ODNs into the Corresponding Guanidino-propyl/butyl Derivatives

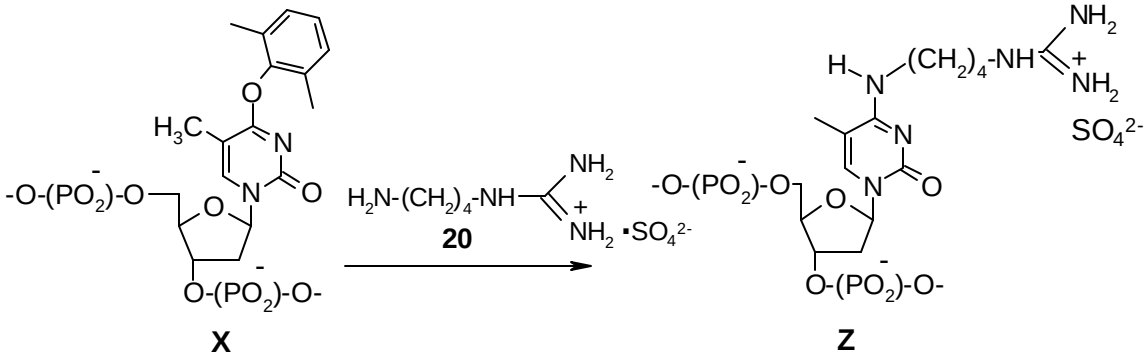
Aliquots of synthesized ODNs bearing 2,6-dimethylphenyl groups (**34-36**) were treated with aqueous solutions of diaminopropane to get the diaminopropyl substituted ODNs (Scheme 5). The treatment of the diaminopropane adduct obtained with pyrazole carboxamidine reagent **19**, gave the corresponding 3-guanidinopropyl derivatized ODNs. The reactions were monitored by analytical HPLC (Figure 8), the reaction mixtures desalted and were found to be of high purity (>95%). The ODNs were characterized by MALDI-TOF mass spectroscopy. The ODNs possessed the expected molecular weight (**41** $M_{calc.}$ 5468, $M_{obs.}$5471; **44** $M_{calc.}$ 5482, $M_{obs.}$ 5481; **45** $M_{calc.}$ 5482, $M_{obs.}$ 5481; **46** $M_{calc.}$5610, $M_{obs.}$ 5609) and were used as such for biophysical studies.



34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT

41 5' TTC TTC TT**Y** TTT TCT TTT
42 5' TT**Y** TTC TTC TTT TCT TTT
43 5' TT**Y** TTC TT**Y** TTT TCT TTT

Scheme 5. Synthesis of guanidinopropyl ODNs.



34 5' TTC TTC TT**X** TTT TCT TTT
35 5' TT**X** TTC TTC TTT TCT TTT
36 5' TT**X** TTC TT**X** TTT TCT TTT

44 5' TTC TTC TT**Z** TTT TCT TTT
45 5' TT**Z** TTC TTC TTT TCT TTT
46 5' TT**Z** TTC TT**Z** TTT TCT TTT

Scheme 6. Synthesis of guanidinobutyl ODNs.

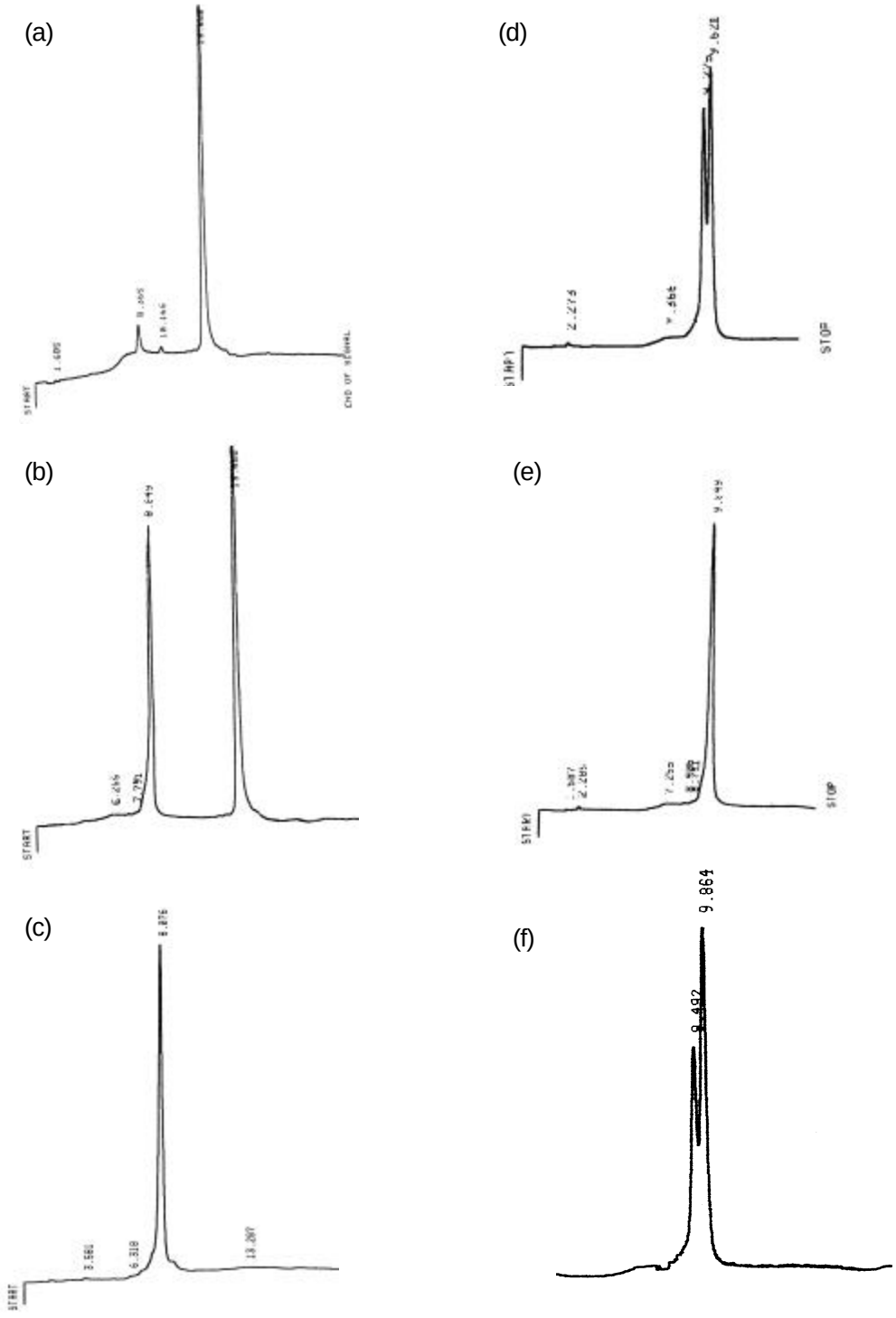


Figure 8. HPLC profiles of (a) **34** upon treatment with 1,3-diaminopropane (b) after 8h (c) after 16h. Diaminopropane adduct on treatment with 1*H*-pyrazole carboxamidine.HCl, **19** (d) after 10h (e) after 18h, the peak corresponds to **41** (f) co-injection of diaminopropane adduct and **40**.

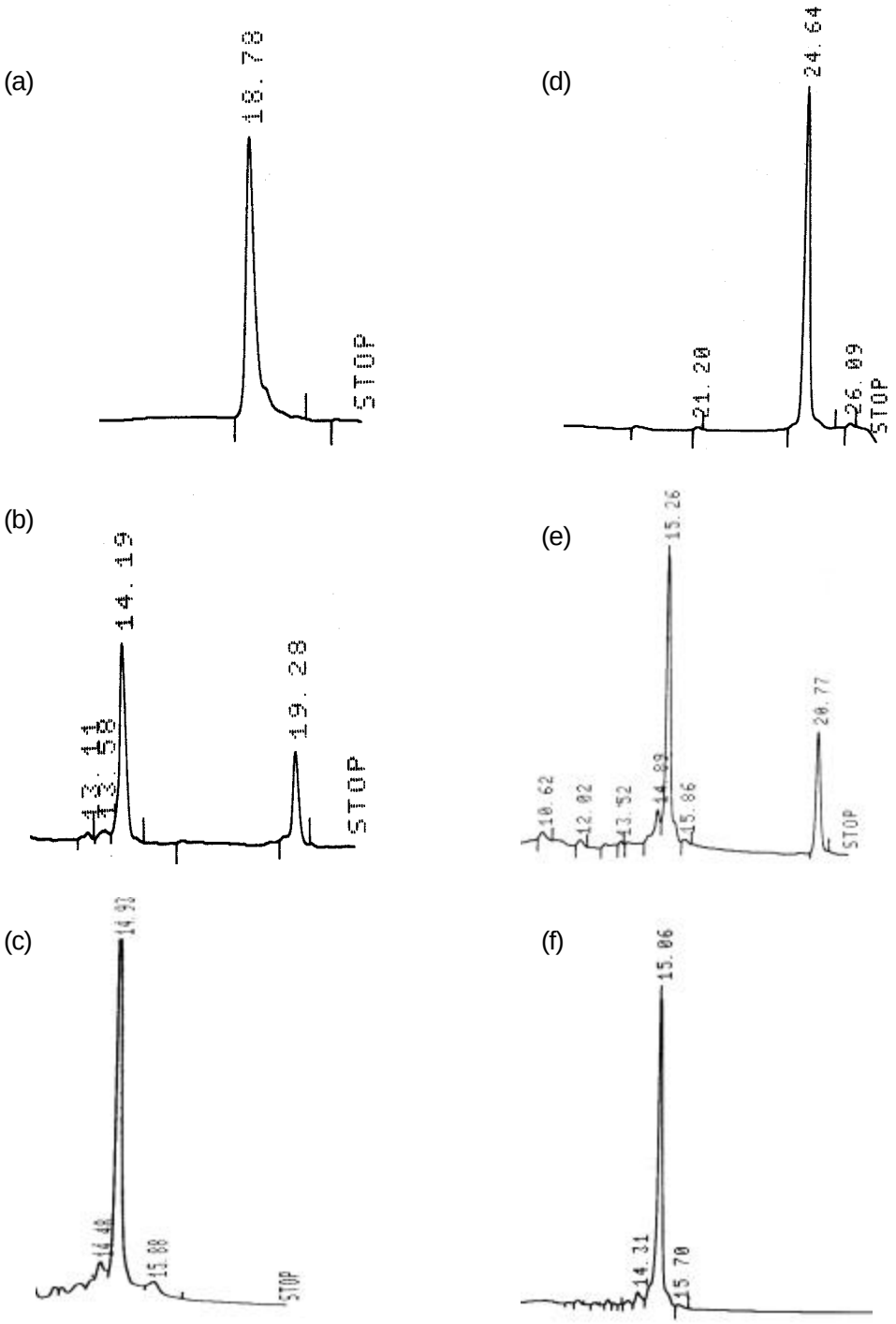


Figure 9. HPLC profiles of (a) **34** upon treatment with agmatine sulphate **20** (b) after 16h (c) after 36h, the peak corresponds to **44**; (d) **36** upon treatment with **20** (e) after 36h (f) after 72h, the peak corresponds to **46**.

The 4-guanidinobutyl sequences were obtained by individually treating the appropriate convertible ODNs with agmatine sulphate **20**, Scheme 6. The reactions were monitored by analytical HPLC. HPLC chromatograms after different intervals of time is given in Figure-9. In the case of ODN **36**, the time required for the completion of reaction was 76h as compared to 36h for the ODNs **34** and **35**. The ODNs after desalting were found to be of high purity (>96%).

2.9.6. UV-Tm Experiments

The hybridization properties of the modified ODNs (**41-46**) were evaluated by determining the melting temperatures (T_m) of the duplexes formed with the complementary DNA strand **38**. The triplex stabilites of ODNs (**41-46**) were assessed by measuring the T_m values of the complexes formed by hybridizing the respective ODNs with the duplex **39:40** in TRIS buffer (pH 7.0).

2.10. RESULTS AND DISCUSSION

2.10.1. Duplex Formation by N^4-Propyl/butyl-guanidino ODNs

The duplexes **41:38** and **44:38** having a single propyl and butyl guanidinium respectively at the centre showed a destabilization of 17°C and 13.5°C respectively

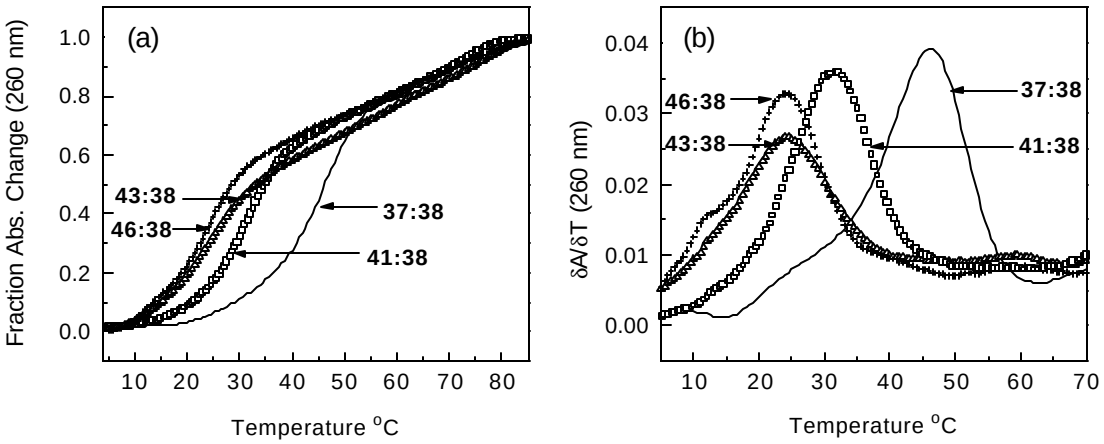


Figure 10. (a) UV-melting profiles of the duplexes and (b) their derivative curves. Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0.

(Table 1, entry 2) compared to the reference **37:38** (entry 1). The duplexes constituted from **42:38** and **45:38**, having 5'-modifications, showed a lesser destabilization (ΔT_m = -8.5°C and -7.5°C respectively, Table-1, entry 3) compared to the reference. The sequences **43** and **46** carrying two modified units formed duplexes with a higher magnitude of destabilization (ΔT_m ~ -23°C, entry 4).

Table 1. UV-Tms of the duplexes[#]

No	Unmodified	Complex			Tm (^{0}C)		
1	5'TT C TTC TT C TTT TCT TTT 3'AA G AAG AA G AAA AGA AAA	**37:38**			47.0		
	Duplexes with modification	**C*** Propylguanidino			**C*** Butylguanidino		
		Complex	Tm ($^\circ$C)	ΔTm ($^\circ$C)	Complex	Tm ($^\circ$C)	ΔTm ($^\circ$C)
2	5' TTC TTC TT**C*** TTT TCT TTT 3' AAG AAG AAG AAA AGA AAA	**41:38**	30.0	-17.0	**44:38**	33.5	-13.5
3	5'TT **C*** TTC TTC TTT TCT TTT 3'AA G AAG AAG AAA AGA AAA	**42:38**	38.5	-8.5	**45:38**	39.5	-7.5
4	5' TTX TTC TT**C*** TTT TCT TTT 3' AAGAAG AA G AAA AGA AAA	**43:38**	23.5	-23.5	**46:38**	24.0	-23.0

[#]Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0. All Tms are accurate to ± 0.5^{o}C and measured in three melting experiments. ΔT_m is the difference between the test and the reference Tms.

In order to rule out the formation of any secondary structures involving the sequences **41-46**, control thermal melting experiments were carried out in the absence of complementary strands. The resulting uneventful thermal denaturation curves indicated the non-existence of any ordered structure in ODNs **41-46** alone.

The introduction of guanidinum at the terminals was less destabilizing than the cases with modifications in the centre. The introduction of two modifications (one at the centre and the other at the 5'-terminus) as in **43** & **46** caused a significant reduction in binding (ΔT_m = ~ -23 °C).

The free rotation around C4-N^4 bond leads to the existence of the *syn* and *anti* conformations. Of these the predominant *syn* isomer[15] (Figure 11) is not capable of

hydrogen bonding *via* the N^4-H, as shown in Figure 11a. The destabilization is perhaps a consequence of this unfavourable conformational effect and the steric effects (Figure 11b). The steric factor imposed by propyl/butyl chain may overweigh the favourable electrostatic contribution of the guanidinium group to complex formation. A similar observation has been made with dC-N^4-sperminyl oligonucleotides[16] which decreased the complementary binding due to similar reasons.

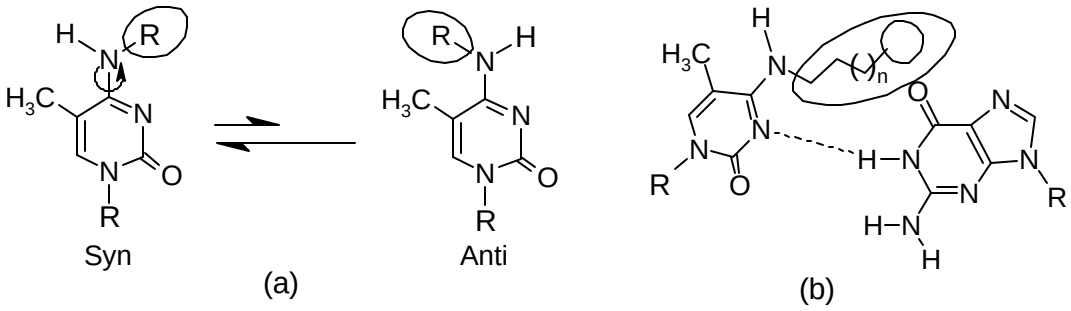


Figure 11. (a) *Syn-anti* isomers of N^4-alkyl derivatives of 5-Me-dC and (b) the weak Watson-Crick hydrogen bond due to the bulky N^4 substituent on 5-Me-dC.

2.10.2. Triplex Formation by N^4-Propyl/butyl-Guanidino ODNs

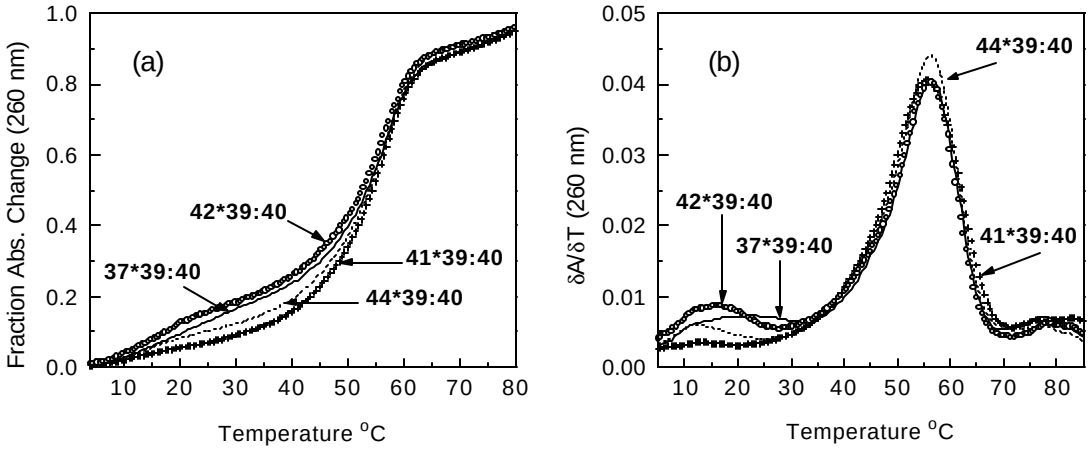


Figure 12. (a) Triplex UV-melting profiles of the complexes **36*38:39, 40*38:39, 41*38:39, 43*38:39** and (b) their derivative curves. Buffer: 25mM TRIS, 100 mM NaCl at pH 7.0

Among the third strand polypyrimidine ODNs **41-46**, **41** having a modification in the middle of the sequence did not form a triplex. Other modified ODNs formed triplexes with the duplex **39:40** with varying magnitudes of destabilization (Table 2, entries 2-7) in comparison to the unmodified triplex **37*39:40** which exhibited a Tm of 25°C (entry 1). The representative melting curves of triplexes are depicted in Figure 12 and show biphasic transitions characteristic of triplex-to-duplex-to-single strand transitions. Triplex Tms were measured both in the absence and presence of $MgCl_2$ as salt is expected to show stabilization triplexes.[17,18] The data in Table 2 also indicates that salt indeed stabilized the triplexes with the stabilization in Tm ranging from 10 to 28°C.

Table 2. UV-Tms of the triplexes[#]

Entry	Sequences	Complex	No Mg^{2+}	Mg^{2+}	Salt induced
			Tm (°C)	Tm (°C)	ΔTm (°C)
1	5' TTC TTC TT C TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAATAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**37*39:40**	25.0	53.0	28.0
2	5' TTC TTC TT **Y** TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAA TAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**41*39:40**	nd	21.0 (-32.0)	
3	5' TTC TTCTT **Z** TTT TCT TTT 5' TCC AAG AAG AA G AAA AGA AAA TAT 3' AGG TTC TTC TT C TTT TCT TTT ATA	**44*39:40**	12.0 (-13.0)	22.0 (-31.0)	10.0
4	5' TT **Y** TTC TTC TTT TCT TTT 5'TCC AA G AAGAAG AAA AGA AAA TAT 3' AGG TT C TTC TTC TTT TCT TTT ATA	**42*39:40**	16.5 (-8.5)	33.0 (-20.0)	16.5
5	5' TT **Z** TTC TTC TTT TCT TTT 5'TCC AA G AAG AAG AAA AGA AAA TAT 3' AGGTT C TTC TTC TTT TCT TTT ATA	**45*39:40**	18.0 (-7.0)	30.0 (-23.0)	12.0
6	5' TT **Y** TTC TT **Y** TTT TCT TTT 5'TCCAA G AAG AA G AAA AGA AAA TAT 3'AGGTT C TTC TT C TTT TCT TTT ATA	**43*39:40**	11.0 (-14.0)	22.0 (-31.0)	11.0
7	5' TT **Z** TTC TT **Z** TTT TCT TTT 5'TCCAA G AAG AA G AAA AGA AAA TAT 3'AGGTT C TTC TT C TTT TCT TTT ATA	**46*39:40**	14.5 (-10.5)	29.0 (-21.0)	14.5

[#]Buffer: 25 mM TRIS, 100mM NaCl, pH 7.0 in the presence or absence Mg^{2+}(20 mM). ΔTm is the difference between the test Tm and the reference Tm. All Tms are accurate to ± 0.5°C and measured in three melting experiments. **Y** indicates N^4-3-propylguanidino-dC and **Z** indicates N^4-4-butylguanidino-dC. Values in brackets indicates the difference between the test and reference Tms.

As discussed in case of duplexes, the incapability of *syn* isomer in the N^4-substituted 5-methyl-dC (m^5-dC) derivatives to form Hoogsteen hydrogen bonds is possibly the main reason for this decreased stability (Figure 13 a).

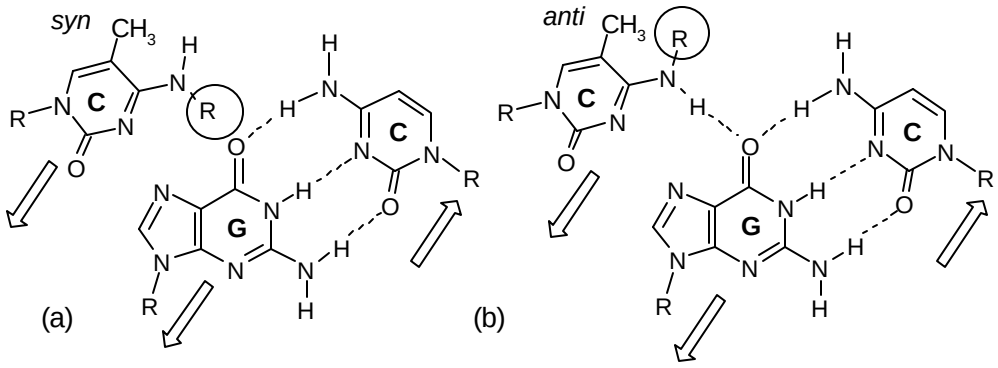


Figure 13. The possible hydrogen bonding patterns with the N^4-alkyl guanidinum derivatives of m^5-dC(a) No Hoogsteen hydrogen bonding (b) One Hoogsteen hydrogen bonding.

2.10.3. Hysteresis in Triplex Transitions

The reassociation or hybridization event of DNA can be monitored by the decrease in absorbance (hypochromicity) with lowering of temperature.[19] Hysteresis is observed between the heating and cooling curves because the rate of association of DNA strands to form triplexes or duplexes is, slower than the rate of dissociation (at lower concentrations). To understand the effect of tethered guanidiniums on the association event, relative heating and cooling profiles were examined for the butyl/propyl or the unmodified complexes under identical conditions. Figure 14 shows the results of such hysteresis experiments.

Factors that increase the rate of association should help in enhancing the association rate and hence decrease the hysteresis effect. As seen from Figure 14, no considerable difference in the hysteresis patterns of the guanidinum tethered ODNs

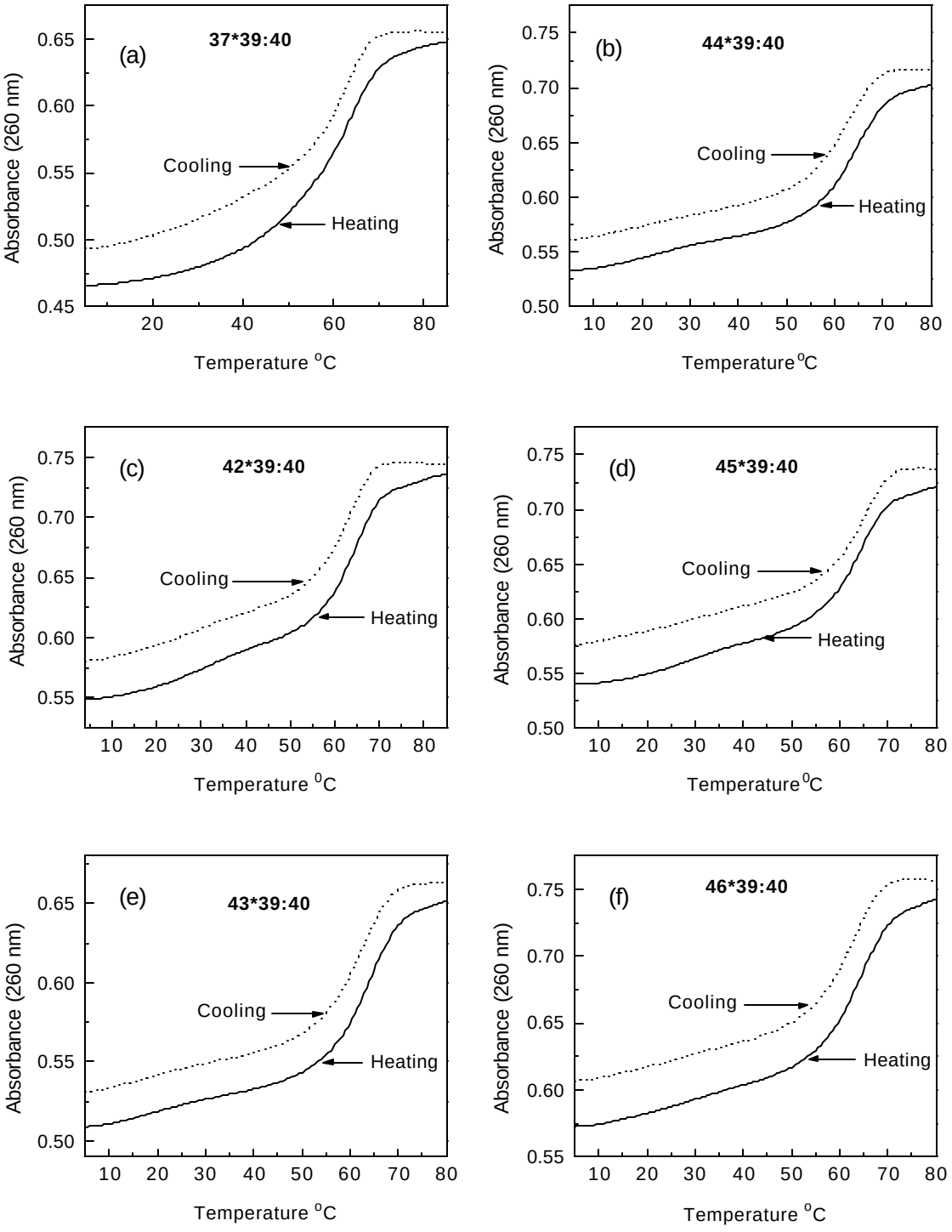


Figure 14. Hysteresis of the denaturation and re-naturation phenomena of the triplexes (a) Unmodified complex **37*39:40,** (b) butyl guanidinium ODN complex **44*39:40** (c) propyl guanidinium ODN complex **42*39:40** (d) butyl guanidinium ODN complex **45*39:40** (e) propyl guanidinium ODN complex **43*39:40** (f) propyl guanidinium ODN complex **46*39:40**.

and the unmodified ODNs were observed. This suggests that the positive charges present in the propyl/butyl guanidinium group do not significantly contribute to accelerate the reassociation by electrostatic interactions. This is unlike dC-(N^4-sperminyl) ODNs which enhance the triplex stability as well as accelerate the reassociation by electrostatic effects.[16]

Additionally, it is possible that the N3-pK_a may differ in the parent and modified cytidines. In order to check this possibility the N3-pK_a of N^4-propyl guanidinium was measured (see experimental) and found to be 3.8 which is much lower than in unmodified cytidine (4.4). *This suggests that protonation of dC in presence of pre-exisiting charge in guanidinium is unfavourable.* In this case, protonation of the modified cytidine would be less likely to occur at pH 7.0 and this results in the formation of only a single Hoogsteen hydrogen-bond to the C^6=O of G in the G:C base-pair of the target (Figure 15). This may perhaps be a cause for destabilization of the triplex.

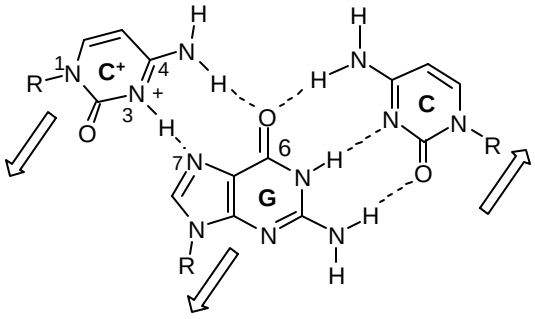


Figure 15. C^+*G:C triad which shows the N3-Cytidine protonation for Hoogsteen bond formation.

2.11. CONCLUSIONS

This section describes the synthesis of "convertible nucleoside" O^4-2,6-dimethylphenyl thymidine-3'-O-phosphoramidite starting from thymidine. The "convertible nucleoside" was successfully incorporated into ODNs and functionalized

into guanidinopropyl or guanidinobutyl derivatives by post-synthetic reactions. The functionalized ODNs were unambiguously characterized by ES-MS/MALDI-TOF mass spectroscopic techniques and used for biophysical studies.

The hybridization studies of the functionalized ODNs were carried out with their complementary strands and indicated that both duplexes and triplexes formed were of lower stability. The reduced stability of the ODN complexes may be because of the existence as the *syn* isomer (which is the major one) in the case of N^4-alkylated cytidines. In addition to the *syn* conformation, the N3 pK_a of N^4-guanidino substituted cytidine being lower than that of cytidine may contribute to the decreased stability of the triplex complexes.

Covalent hybrid oligonucleotides bearing effectors that range from small hydrocarbons to peptides have been used as enzymatic[20,21] and non-enzymatic[22,23] restriction endonucleases, detection probes[24,25] and photoaffinity agents.[26] In view of these, the findings on covalent and non-covalent interactions of guanidinium groups in ODNs may have utility in biochemical studies.

During the course of this work, reports on other C4 substituted pyrimidines with different donor/acceptor groups have appeared.[27] Very recently, the synthesis of modified oligonucleotides containing 4-guanidino-2-pyrimidinone nucleobases[28] has been reported to have potential as protonated cytidine analogues. The findings on covalent and non-covalent interaction of guanidinium groups in ODNs may have utility in biochemical studies.

2.12. REFERENCES

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Chapter 3

Section C:

Synthesis of 5-(N-Methylanthraniloyl) -2'-Deoxyuridine Labeled Oligonucleotides

3.14. INTRODUCTION

Recent years have seen considerable interest in the synthesis of various fluorescent labeled oligonucleotides as DNA probes, for use in polymerase chain reaction (PCR) or in experiments involving fluorescence resonance energy transfer (FRET).[1,2,3,4] FRET, a phenomenon first observed by Foster[5] occurs when the emission spectrum of one fluorophore overlaps with the excitation spectrum of the second fluorophore (Figure 1a). FRET is a non-radiative energy transfer involving a dipole-dipole resonance interaction between two spatially close molecules, where one molecule, called the "donor", transfers its emission energy to the other, called the

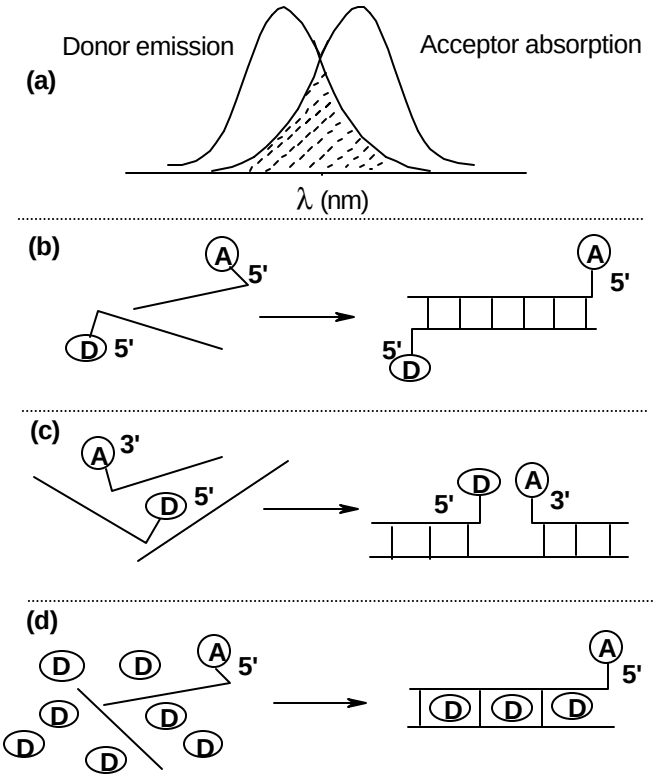


Figure 1. (a) Schematic representation of spectral overlap of the emission of donar and excitation of acceptor which results in resonance energy transfer.
The different strategies for determining nucleic acid hybridization by FRET
(b) Fluorescent probes are covalently attached to the 5'-ends of the complementary ODNs, allowing energy transfer to occur between a donor **D** and an acceptor **A**, (c) fluorescent molecules attached to two ODNs, one at the 3'-end and the other at the 5'-end; the two labeled ODNs are complementary to the unlabeled ODN (d) an intercalating dye functioning as a donor for an acceptor fluorophore covalently attached at the 5'-end.

"acceptor". FRET has given valuable information on the structure of nucleic acids and nucleic acid-drug interactions because of its dependence on distance and orientation.[6,7] Hybridization of two fluorescent oligonucleotides to adjacent single-stranded sequences has been shown to lead to fluorescence energy transfer from a donor molecule to an acceptor, thus allowing detection of adjacent single stranded sequences,[8,9,10] which has been a valuable tool to monitor nucleic acid hybridization (Figure 1b-d).

Table 1.

Donor	Acceptor
Fluorescein	X-Rhodamine
Acridine	Rhodamine
Acridine orange	Rhodamine
Indole acetyl	Dansyl

Fluoroscence measurements offer a considerably more sensitive method for nucleic acid detection compared to the conventional solution-phase techniques like UV. Proper selection of the fluorophores lead to detectable energy transfer between the "donor" and "acceptor" as represented in Figure 1. Some of the common fluorescence resonance energy pairs used in ODNs are given in Table 1.

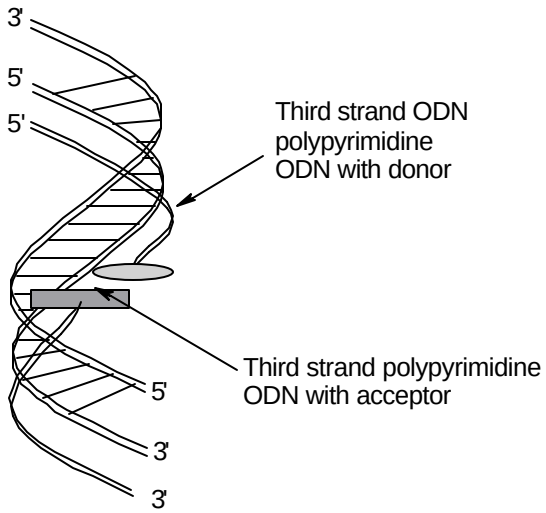


Figure 2. Schematic representation of donor and acceptor brought close to each other by formation of two triple helices on neighboring sites giving rise to FRET.[12]

FRET between two triple helix forming oligonucleotides bound to a duplex DNA was demonstrated by Mergny *et al.* (Figure 2).[11] As was shown in the previous Section (Section 3.11.3.b.) from the UV-T_m measurements of the antiparallel motif, the distinction of the triplex melting from duplex melting was found to be ambiguous because of the merging of triplex-to-duplex and duplex-to-single strand transitions. In this context, it was envisaged to recruit the phenomenon of FRET by using fluorophores with suitable donor/acceptor properties, linked to the third strand and middle stand of ODNs forming the triplex in order to confirm the existance of the triplex

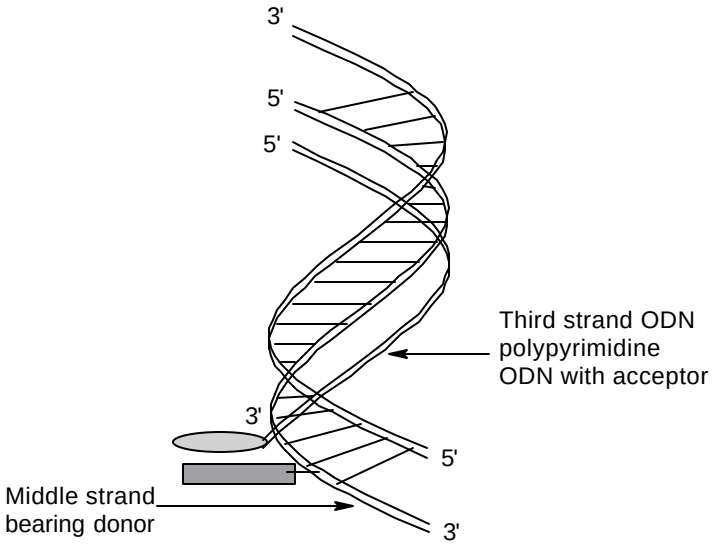


Figure 3. Schematic representation of the designed FRET pair with donor in the middle strand and acceptor in the third strand of a triple helix.

as shown in Figure 3.

Fluorescent derivatives of nucleotides are appealing because of their sensitivity to small changes in the environment as well as low concentrations required to carry out biophysical studies. The desirable attributes of a fluorophoric nucleoside are high quantum yield, and absorption/emission maxima distinct from those of proteins and nucleic acids. It has been shown[12] that anthraniloyl and N-methylanthraniloyl groups are excellent fluorophores. In addition, their small size produces only minor

perturbations upon introduction into a system. Hiratsuka[13] has synthesized the 3'-O-anthraniloyl/3'-O-N-methylanthraniloyl derivatives of nucleosides and nucleotides. In addition to being a good fluorophore and smaller in size, N-methylanthraniloyl (MANT) derivatives have a greater quantum yield (0.9 in DMF and 0.3 in H_2O)[13]. Hence, it was thought to prepare a nucleoside derivative with N-methylanthraniloyl attached to the nucleobase. Further, it could form a FRET pair with 2-aminopurine (λ_{ex} 305 nm and λ_{em} 369 nm),[14] which can serve as a donor while N-methylanthraniloyl derivative forms the

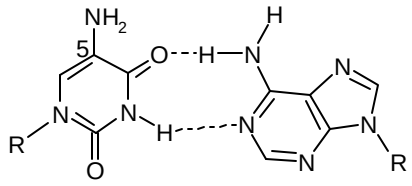


Figure 4. Watson-Crick hydrogen bonding in dU-NH_2:dA complexes.

acceptor (λ_{ex} 350 nm and λ_{em} 425-445 nm).[13]

The amino functionality at C5 of 5-amino-2'-dU is directed into the major groove of the DNA double-helix and does not interfere in the Watson-Crick base-pairing (Figure 4). It was proposed to link the N-methylanthraniloyl group to the C5-NH_2 of 5-amino-dU to get the required fluorescent nucleoside. Earlier, 5/6-carboxyfluorescein has been linked to 5-amino-dU and the derived ODNs were used as primers in fluorescent PCR.[15]

3.15. OBJECTIVES

The main objectives of this part remain:

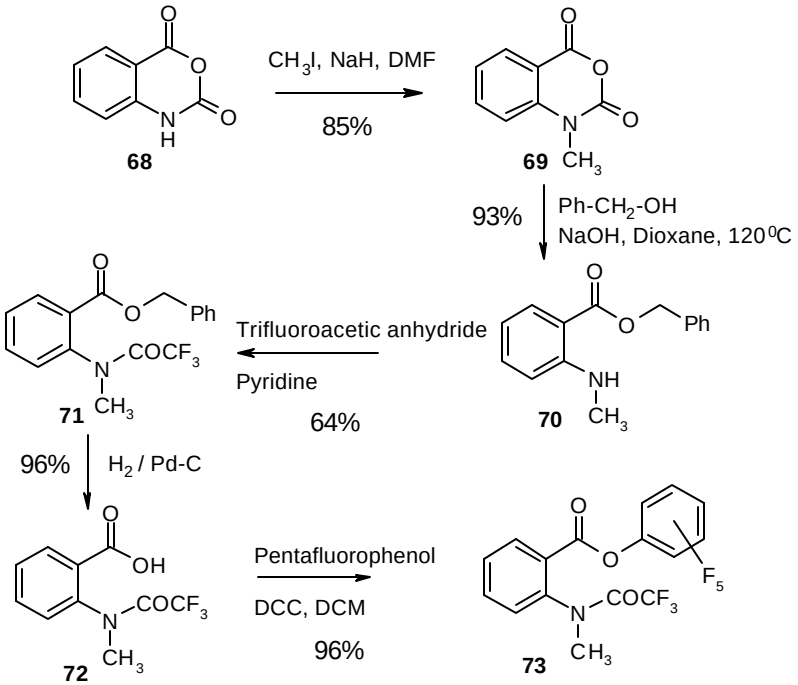
- Synthesis of N-methylanthraniloyl-5-NH_2-dU phosphoramidite.
- Incorporation of the fluorescent residue into ODN sequences.

3.16. PRESENT WORK

3.16.1. Synthesis and Characterization of Fluorescent Monomer

3.16.1.a. Synthesis of N-trifluoracetyl-N-Me-anthranilic acid pentafluorophenyl ester 73

The synthesis of N-trifluoroacetyl-N-Me-anthranilic acid pentafluorphenyl ester was achieved starting from isatoic anhydride as shown in Scheme 1. N-Methylisatoic anhydride was obtained by methylation of isatoic anhydride **68**, with methyl iodide in presence of NaH in DMF. The formation of N-methylated product **69** was indicated in the ^{1}H NMR spectrum, where the methyl protons appeared at δ 3.60 as a singlet. The anhydride was opened up using benzyl alcohol and a catalytic amount of NaOH to get the benzyl-N-Me-anthranilate **70** which was characterized by ^{1}H, ^{13}C NMR and mass spectral analysis. In the ^{1}H NMR spectrum, the Ar<u>H</u> was seen at δ 7.50-7.20, Ph-C<u>H</u>$_2$-O at δ 5.30 along with the other signals. The benzyl ester was treated with trifluoroacetic anhydride in pyridine to yield the N-trifluoroacetyl derivative **71**. The conversion was



Scheme 1. Synthesis of pentafluorophenyl ester **73**.

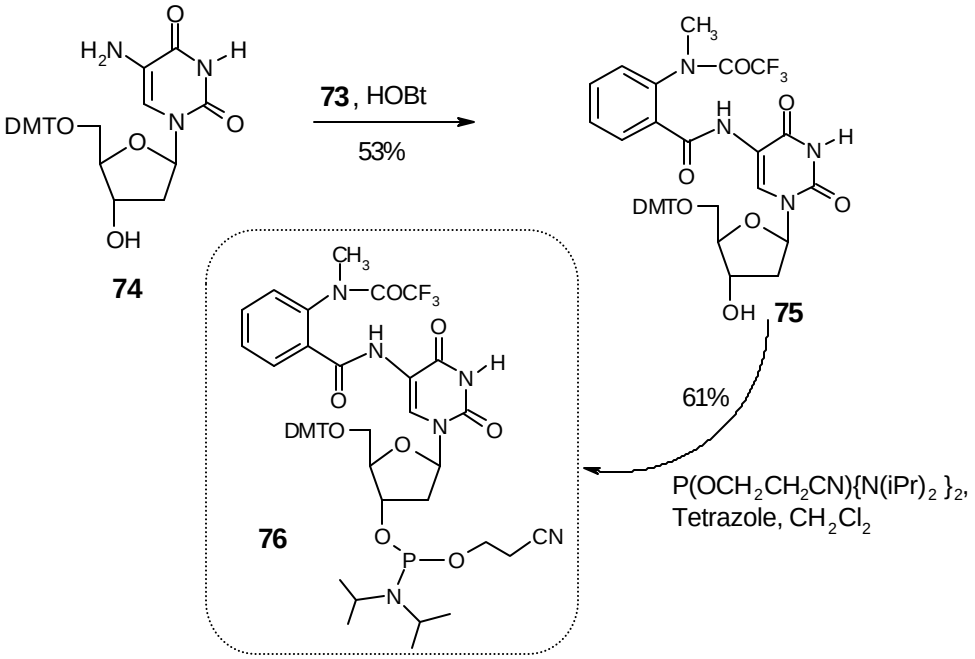
evident from the disappearance of the IR NH band at 3383 cm^{-1}. In the ^1H NMR spectrum, the downfield shift of -N-C$\underline{H}_3$ from δ 2.95 to 3.25 supported the transformation. In addition, the carbon signals of -$\underline{C}$O$\underline{C}F_3$ were seen as quartets with the appropriate C-F coupling [$^1J_{C\text{-}F}$ = 288.6 Hz (CF_3) and $^2J_{C\text{-}F}$ = 35.1 Hz (C=O)]. The mass spectral analysis showed a molecular ion at 337, confirming the structure of **71**.

Hydrogenation of **71** over Pd-C gave the required acid **72**, the ^1H NMR spectrum of which was supportive of the right structure of the compound. The mass spectrum showed the molecular ion peak at 247 (M^+). The pentafluorophenyl ester of the acid **73** was obtained by esterification with pentafluorophenol in presence of dicyclohexylcarbodiimide (DCC) as the coupling agent. The structure of **73** was confirmed by ^1H, ^{13}C NMR and mass spectra. The ^{13}C NMR spectrum exhibited all the required carbon signals, and the characteristic splitting of the carbon signals due to fluorine coupling could be seen. The mass spectral analysis gave the expected M^++1 peak at 414 in support of the structure.

3.16.1.b. Synthesis of 5-N-(N-methylanthraniloyl-N-trifluoroacetyl)-5-amino-2'-deoxyuridine U^{MANT} monomer 76

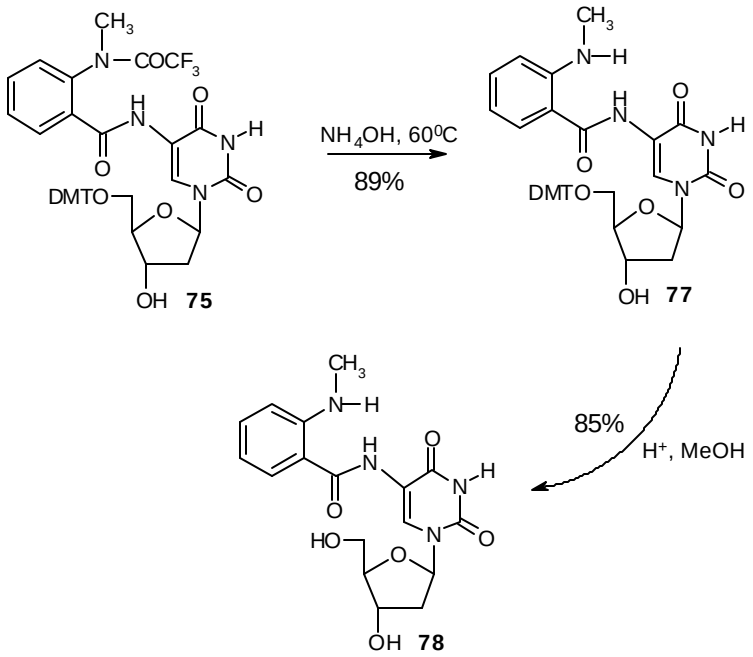
The pentafluorophenyl ester **73** was treated with 5-NH_2-dU-5'-O-DMT **74**[16] in presence of 1-hydroxybenzotriazole (HOBt) as catalyst at room temperature to get the coupled product **75**. In the ^1H NMR spectrum, the nucleoside protons were seen along with the aromatic protons of the anthraniloyl fragment appearing at δ 7.70-7.20 overlapping with those of DMT protons. In the ^{13}C NMR spectrum most of the carbon signals were split, the amide CO appearing at δ 163.4, N-CH3 at δ 39.4, C1' at 86.1, 85.9, C4' at 85.6, 85.1, C3' at 71.1, 70.9. Due to C-F coupling, the $\underline{C}F_3$ signals were split into quartets, $^1J_{C\text{-}F}$ = 287.9 Hz (CF_3). In addition, the mass spectrum gave the expected molecular ion peak at 797 (M^++Na). Compound **75** on phosphitylation gave

the required phosphoramidite monomer **76**. In the ^{31}P NMR spectrum, the signals at δ 149.42, 149.14 were characteristic of the phosphoramidites.



Scheme 2. Synthesis of monomer **76**.

In order to determine the stability of **75** to the reagents and conditions used in automated DNA synthesis, the dU derivative **75** was exposed to the I_2/pyridine/H_2O/CH_3CN (oxidation reagent) and the reaction was monitored by TLC and ^1H NMR. The compound remained unaffected even after extended exposure, significantly longer than the actual DNA synthesis durations. The treatment of **75** with NH_4OH at 55°C for 8h, gave the expected trifluoroacetyl hydrolyzed product **77** indicating the ease of trifluoroacetyl removal using the standard protocols. The compound **77** was characterized by ^1H NMR and mass spectroscopic techniques. The expected molecular ion was observed at 701 (M^++Na) confirming the structure of the product. Detritylation of **77** using methanolic HCl afforded the free nucleoside **78**.



Scheme 3. Preparation of nucleoside **78**.

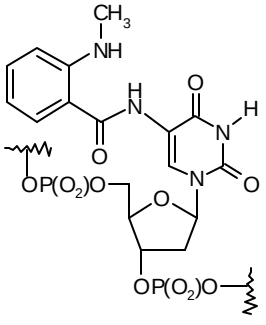
3.16.2. Synthesis of ODNs Bearing the Fluorophore

The N-methylanthraniloyl nucleoside phosphoramidite **76** was incorporated into ODNs **79** and **80** at the desired sites as indicated, for the preliminary studies. The synthesized oligomers were cleaved and deprotected by standard protocols and were characterized by MALDI-TOF mass spectroscopy (Figure 5).

79 5'- C C **U**^{MANT} G G C C A G G

80 5'- T T C T T C **U**^{MANT} T C T **D**^{sp} T T C T T T T

(**D**^{sp} C6-sperminyl-2-aminopurine)



U^{MANT}

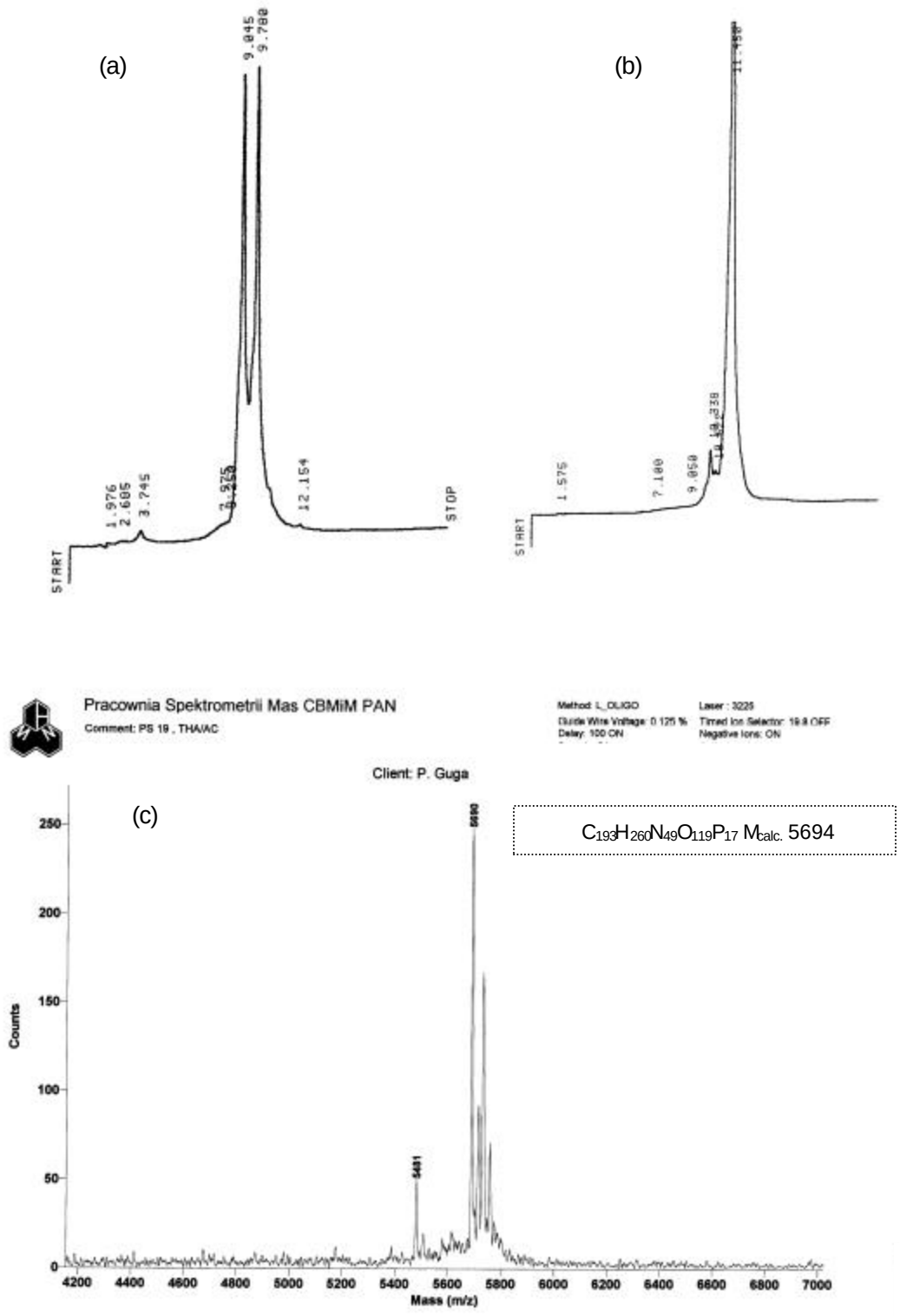


Figure 5. Analytical RP-HPLC profiles of (a) ODN **79**, self-complementary sequence (b) **80** and (c) MALDI-TOF mass spectrum (negative ion-mode) of **80**.

3.17. RESULTS AND DISCUSSION

3.17.1. Fluorescence Properties of the N-Methylanthraniloyl-2'-deoxyuridine Nucleoside 78 (U^{MANT})

The N-methylanthraniloyl nucleoside **78** emits strongly in the range 430-450 nm with λ_{ex}^{max} at 440 nm (Figure 6b) upon excitation at 350-360 nm region (Figure 6a). The excitation spectrum thus overlaps with the emission spectrum of 2-aminopurine (λ_{em} = 369 nm)[14] indicating that these two would form a FRET pair.

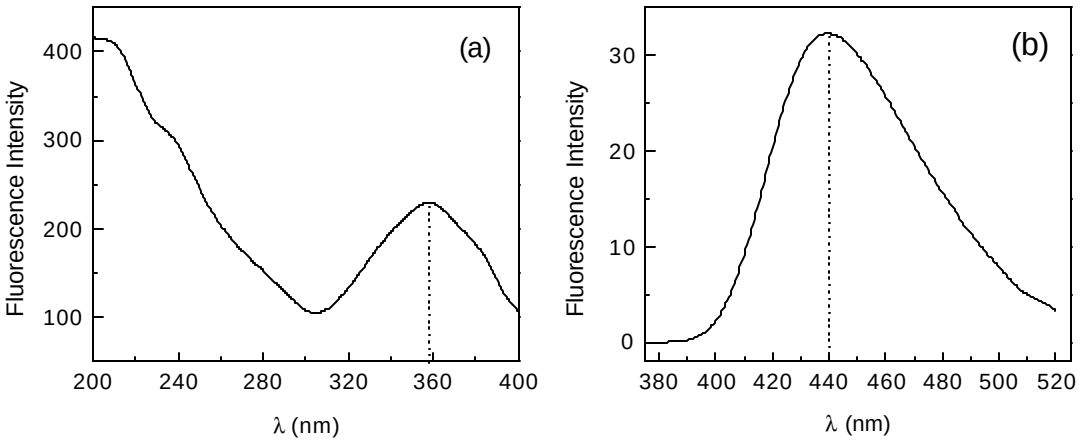


Figure 6. (a) Fluorescence excitation spectra and (b) emission spectra of **78** in phosphate buffer on excitation at 350 .nm.

3.17.2. Synthesis and Properties of ODNs Bearing the Fluorophore

Surprisingly, it is seen that the excitation maximum of the fluorescent ODNs were different from that observed for the monomer **78**. The N-methylanthraniloyl nucleoside **78** showed a fluorescence excitation maximum (λ_{ex}^{max}) at 358 nm and emission maximum (λ_{em}^{max}) at 440 nm. Upon incorporation into ODNs **79** and **80**, the λ_{ex}^{max} and λ_{em}^{max} experienced a blue shift to 210 nm and 380 nm respectively. The

fluorescence excitation and emission spectra of ODN **79** is represented in Figure 7. Similar behaviour was observed for the ODN **80** bearing $\mathbf{U}^{MANT}$ and $\mathbf{D}^{sp}$ (2-amino-6-sperminylpurine).

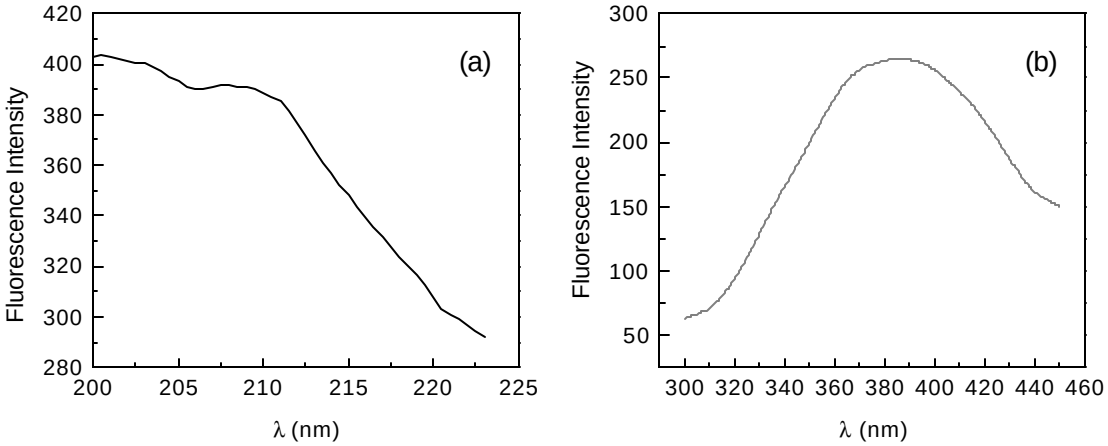


Figure 7. (a) Fluorescence excitation spectra (λ_{em} 386nm) and (b) Fluorescence emission spectra of ODN **79** ($\lambda_{exc.}$ 210nm), in phosphate buffer (100 mM), pH 7.4.

The fluorescence λ_{ex} and λ_{em} values of 2'-O-(N-Methylanthraniloyl) derivatives of cyclic nucleoside phosphates reported[17] were 350 nm and 425-445 nm respectively. The observations concerning the λ_{ex} and λ_{em} of the ODNs clearly suggests a change in the environment of the fluorophore.

In order to investigate the effect of polarity on the fluorophore, the free nucleoside was studied in water-dioxane mixtures, which expand the range of solvent polarities. It was seen that in dioxane:water, 1:3, the excitation maximum for the free nucleoside was 210-215 nm which suggests the polarity difference experienced by the MANT group when incorporated into ODNs. Further studies on the fluorescence properties of the free nucleoside and the derived ODNs are in progress.

3.18. CONCLUSIONS

The synthesis of N-methylanthraniloyl conjugated at the C5 of 2'-deoxyuridine was achieved. The fluorescent nucleoside monomer was incorporated into ODNs and the derived ODNs were characterized by MALDI-TOF mass spectroscopy. Fluorescence properties of the ODNs were carried out which indicated a large blue shift in the fluorecence excitation and emission maxima of the ODNs when compared to the nucleoside. The results suggest that the fluorophore experiences a different polarity in the major groove of the duplex DNA than the bulk solvent.

3.19. REFERENCES

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3.20. EXPERIMENTAL (SECTION A)

General experimental techniques have been described in the experimental section of Chapter 2.

3.20.1. Synthesis of Compounds 6-21

*Bis-hexahydropyrimidine **6***

To spermine (free base, 4.0 g, 19.8 mmol) was added formalin (38% solution, 3 mL, 40 mmol) at 0°C and stirred for 1h when the crystalline product separated out. The crystals were filtered out and washed with ether (10 mL) to get the pure compound **6** (4.0 g, 89%).

m.p. 60-70°C.

^{1}H NMR, $CDCl_3$ δ: 3.40-3.25 (s, 4H, -NC$\underline{H_2}$N-), 2.85-2.70 (t, 4H), 2.60-2.45 (m, 4H), 2.25-2.10 (m, 4H), 1.65-1.50 (m, 4H), 1.50-1.35 (m, 4H).

^{13}C NMR, $CDCl_3$ δ: 69.2, 54.6, 52.3, 44.5, 26.4, 24.2.

*N,N',N'',N'''-Tetrabenzyloxycarbonyl spermine **7***

The compound **6** (1.7 g, 7.5 mmol) was dissolved in CH_2Cl_2 (5 mL) to which water (5 mL), $NaHCO_3$ (3.36 g, 40 mmol) and benzylchloroformate (6.8 mL of 50% solution in toluene, 20 mmol) were added. The reaction mixture was stirred for 3h, diluted with CH_2Cl_2 (50 mL) and washed with $NaHCO_3$ solution. The organic layer was dried over anhy. Na_2SO_4 to get the crude material which was purified by column chromatography (silica gel, light petroleum/EtOAc) to get the pure product **7** (1.84 g, 32%) as a gum.

TLC: (light petroleum:EtOAc 1:1) R_f 0.50.

^{1}H NMR, $CDCl_3$ δ: 7.40-7.10 (m, 20H, Ar<u>H</u>), 5.20-5.00 (m, 8H, -O-C<u>H</u>$_2$-Ph), 3.40-3.00 (m, 12H, N-C<u>H</u>$_2$-C), 2.00-1.50 (m, 4H), 1.50-1.30 (m, 4H).

N,N'-Bis(t-Boc)-N,N'-bis(2-cyanoethyl)-1,4-butanediamine ***9***

To N,N'-bis(2-cyanoethyl) butanediamine,[i] (6 g, 22.5 mmol) in DMSO (40 mL) were added triethylamine (14 mL, 100 mmol) and ^{t}Boc azide (8.8 g, 61.5 mmol) and stirred under nitrogen atmosphere at 60°C for 12h. The reaction mixture was poured into water (200 mL) and extracted with CH_2Cl_2 (60 mL x 4). The combined organic extracts were washed with 5% $NaHCO_3$ solution (50 mL) dried over anhy. Na_2SO_4 and concentrated. Crystallization from CH_2Cl_2 afforded the pure product **9** (8.16 g, 92%).

m.p. 95-96°C. TLC: (CH_2Cl_2:MeOH 9.75:0.25) R_f 0.60.

^{1}H NMR, $CDCl_3$ δ: 3.55-3.40 (t, 4H), 3.35-3.20 (m, 4H), 2.75-2.50 (m, 4H), 1.60-1.40 (m, 22H).

1,14-Bis(benzyloxycarbonyl)-5,10-bis(tert-butoxycarbonyl)-1,5,10,14-tetraazatetradecane ***11***

Ra-Ni (0.7 g) was added to a solution of **9** (2.6 g, 5.77 mmol) in ethanol (10 mL) followed by sodium hydroxide solution (0.1 g of NaOH in 10 mL of 95% EtOH). The mixture was hydrogenated under 65 psi of H_2 for 18h. The catalyst was then filtered (taking care that it did not become dry) through a celite pad, washed with ethanol and concentrated to get the diamine **10**. It was dissolved in 1:1 water dioxane (8 mL), and $NaHCO_3$ (2.4 g, 28 mmol) and benzyl chloroformate (5 mL of a 50% solution in toluene, 14 mmol) were added and stirred overnight. When TLC showed the disappearance of starting material, the mixture was concentrated to remove dioxane. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with 5% $NaHCO_3$ solution (25 mL). The

organic layer was concentrated and purified by column chromatography (silica gel, light petroleum/EtOAc) to get **11** (2.7 g, 70%) as a viscous oil.

TLC: (light petroleum:EtOAc 1:1) R_f 0.50.

^{1}H NMR, $CDCl_3$ δ: 7.35 (m, 10 H, ArH), 5.75 (bs, 2H, NH), 5.10 (s, 4H, -O-CH_2-Ph), 3.20 (12H, m), 1.65 (4H, m), 1.45 (22H, m).

^{13}C NMR, $CDCl_3$ δ: 155.9, 155.0, 136.3, 127.6, 127.1, 78.6, 65.5, 46.0, 43.5, 37.6, 27.7, 25.1.

1,14-Bis(benzyloxycarbonyl)-5,10-bis-(trifluoroacetyl)-1,5,10,14-tetraazatetra-decane ***12***

A solution of trifluoroacetic acid in CH_2Cl_2 (1:1, 5 mL) was added to compound **11** (2.1 g, 2.89 mmol) in CH_2Cl_2 (2 mL) and the solution was left as such for 30 min. The reaction mixture was concentrated to dryness, diisopropylethylamine solution (5% in CH_2Cl_2, 5 mL x 3) was added and then concentrated. The residue was dissolved in CH_2Cl_2 (5 mL), pyridine (3 mL) was added followed by trifluoroacetic anhydride (3.6 mL, 25 mmol). The progress of the reaction was monitored by TLC. After 1h the reaction mixture was concentrated to dryness, dissolved in CH_2Cl_2 (150 mL), washed with 5% $NaHCO_3$ solution (25 mL). The organic layer was concentrated and purified by column chromatography (silica gel, light petroleum/EtOAc) to get **12** (1.78 g, 93%).

m.p. 79-80°C. TLC: (light petroleum:EtOAc 1:1) R_f 0.25.

MS (FAB): 663 (M^+).

^{1}H NMR, $CDCl_3$ δ: 7.40 (s, 10H), 5.10 (s, 4H), 3.40 (m, 8H), 3.20 (m, 4H), 1.80 (m, 4H), 1.60 (m, 4H).

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^{1}H NMR, $CDCl_3$ δ: 157.3, 157.1, 156.5, 136.5, 136.3, 136.2, 128.4, 127.9, 124.9, 119.2, 113.5, 107.8, 66.7, 66.5, 46.1, 45.1, 44.0, 38.1, 37.9, 29.3, 27.2, 25.6, 23.8.

5,10-Bis-(Trifluoroacetyl)-1,5,10,14-tetraazatetradecane ***13***

To a solution of **12** (1.5 g, 2.26 mmol) in MeOH (15 mL) was added 10% Pd-C (0.5 g) and hydrogenated under 50 psi of H_2 for 3h. The mixture was filtered through a celite pad and concentrated to get the pure product **13** (0.78 g, 91%) as a gum.

^{1}H NMR, MeOH-d_4 δ: 3.40-3.30 (m, 4H), 2.80-2.60 (m, 8H), 1.85-1.70 (m, 4H), 1.65-1.50 (m, 4H).

^{13}C NMR, MeOH-d_4 δ: 160.2, 159.5, 158.7, 158.0 (q, J_{C-F} = 37.0 Hz ($\underline{C}OCF_3$), 126.1, 120.4, 114.7, 109.0 (q, J_{C-F} = 286.6 Hz, $CO\underline{C}F_3$) 50.2, 47.6, 38.8, 29.2, 27.9.

5'-O-DMT-N^2-Isobutyryl deoxyguanosine ***16***[ii]

To 2'-deoxyguanosine **14** (2.68 g, 20 mmol) dried by co-evaporation with pyridine and suspended in dry pyridine (75 mL) was added trimethylchlorosilane (20 mL, 160 mmol) at ice-bath temperature. After stirring for 15 min, isobutyric anhydride (16.5 mL, 100 mmol) was added and stirring was continued at room temperature for 10h. The reaction was then cooled in an ice bath and water (10 mL) was added. After 5 min conc. NH_4OH was added (20 mL) and the reaction mixture stirred for 15 min. The solution was evaporated to near dryness and the residue was dissolved in water (100 mL) and washed once with ethyl acetate:ether (1:1, 100 mL). The organic layer was extracted with water (50 mL) and the combined aqueous layers were concentrated to about 50 mL. The N^2-isobutyryl-2'-deoxyguanosine **15** crystallized out as a white solid (2.7g, 40%). The compound obtained was filtered out, dried in a desiccator, co-evaporated with dry pyridine and suspended in dry pyridine (50 mL). To this solution,

4,4'-dimethoxytrityl chloride (4.06 g, 12 mmol), triethylamine (Et_3N,1.67 mL, 12 mmol) and N,N'-dimethylaminopyridine (DMAP, 30 mg, 0.25 mmol) were added. After 4h TLC showed the completion of reaction. The reaction mixture was cooled in an ice bath and 5% $NaHCO_3$ (50 mL) was added. The mixture was extracted with ethyl acetate (3 x 100 mL). The combined organic layers were concentrated and the residue purified by flash chromatography (silica gel, CH_2Cl_2/MeOH/Et_3N, 9.5:0.5:0.1) to get the pure compound **16** (3.1 g, 60%).

158-161°C. TLC: (CH_2Cl_2:MeOH 9:1) R_f 0.60.

^{1}H NMR, $CDCl_3$ d: 12.20 (bs, 1H, NH), 9.15 (bs, 1H, NH), 7.80 (s, 1H, H8), 7.25 (m, 9H, ArH), 6.70 (d, 4H, ArH), 6.20 (t, J = 7.1 Hz, 1H, H1'), 4.70 (bs, 1H, H4'), 4.15 (m, 1H, H3'), 3.75 (s, 6H, OCH_3), 3.40 (m, 2H, H5', H5''), 2.80 (m, 1H, H2'), 2.40 (m, 1H, H2''), 1.10 (dd, $J_{1,2}$ = 6.8 Hz, $J_{1,3}$ = 12.2 Hz, 6H).

5'-O-DMT-3'-O- (tert-Butyldimethylsilyl)-N^2-isobutyryl-2'-deoxyguanosine ***17***

5'-O-DMT-N^2-isobutyryl-2'-deoxy-guanosine, **16** (2.24 g, 3.5 mmol) was dissolved in anhydrous pyridine (20 mL). Imidazole (0.6 g, 8.8 mmol) and TBDMS-Cl (1.06 g, 7 mmol) were added to the above solution and stirred for 10h at room temperature. The progress of the reaction was monitored by TLC. After removal of pyridine under vacuum the residue was taken in dichloromethane (40 mL) and washed with 5% $NaHCO_3$ solution. The compound **17** (2.56 g, 97%) was obtained as a foam after flash column chromatography (silica gel, CH_2Cl_2/MeOH/Et_3N, 100:0.5:0.2) of the crude product.

m.p. 78-81°C. TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.40.

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ii. Ti, G. S., Gaffney, B. L., Jones, R. A., *J. Am. Chem. Soc.* **1982**, *104*, 1316-1319.

^{1}H NMR, $CDCl_3$ δ: 11.95 (s, 1H, NH), 7.70 (s, 1H, H8), 7.40-7.10 (m, 9H, ArH), 6.70 (d, 4H, ArH), 6.10 (m, 1H, H1'), 4.45 (m, 1H, H3'), 3.95 (m, 1H, H4'), 3.70 (s, 6H, OCH_3), 3.40-3.00 (m, 2H, H5', H5''), 2.70-2.50 (m, 1H, H2'), 2.30-1.95 (m, 2H, H2'', $CH(CH_3)_2$), 1.00-0.80 (dd, $J_{1,2}$ = 6.9, $J_{1,3}$ = 8.1 Hz, 6H, *i*Bu CH_3), 0.70 (s, 9H, *t*Bu), -0.1 (d, J = 7.3 Hz, 2 x CH_3).

O^6-(2,4,6-Triisopropylbenzenesulfonyl)-5'-O-dimethoxytrityl-3'-O-(tert-butyldimethylsilyl)-N^2-isobutyryl-2'-deoxyguanosine ***18***

Compound **17** (1.5 g, 2 mmol) was dissolved in dry dichloromethane (45 mL) followed by the addition of triethylamine (1.4 mL, 10 mmol) and DMAP (30 mg, 0.25 mmol) with constant stirring at ice bath temperature. Freshly recrystallized 2,4,6-triisopropylbenzenesulfonyl chloride (1.5 g, 5.0 mmol) was added to the above solution and the stirring was continued for 4h maintaining the temperature between 5-10°C. The reaction mixture was diluted with CH_2Cl_2 (25 mL), washed with 5% $NaHCO_3$ (30 mL), the organic layer dried over anhy. Na_2SO_4 and concentrated. The residue was purified by column chromatography (silica gel CH_2Cl_2/Et_3N, 99:1) to get the sulfonate, **18** (1.66 g, 82%) as a white foam.

m.p. 83-92°C. TLC: (CH_2Cl_2:MeOH 9.8:0.2) R_f 0.40.

^{1}H NMR, $CDCl_3$ δ: 8.05 (s, 1H, H8), 7.65 (s, 1H, NH), 7.40-7.00 (m, 11H, ArH), 6.70 (d, 4H, ArH), 6.35 (t, J = 6.4 Hz, 1H, H1'), 4.50 (m, 1H, H4'), 4.20 (q, 2H, 2 x *o* -CH-Me_2 of SO_2Ar), 4.00 (m, 1H, H3'), 3.70 (s, 6H, -OCH_3), 3.25 (m, 2H, H5', H5''), 3.05-2.70 (m, 2H, CH of N^2-isobutyryl & *p* -CH -Me_2 of SO_2Ar), 2.70-2.30 (m, 2H, H2', H2''), 1.40-1.00 (m, 24H, 3 x $(CH_3)_2$ of *i*Pr & *i*Bu CH_3), 0.80 (s, 9H, *t*Bu), -.05 (d, J = 7.8 Hz, 6H, $Si(CH_3)_2$).

5'-O-(4,4'-Dimethoxy)trityl-3'-O-(tert-butyldimethyl)silyl-N^2-isobutyryl-(C6-[N',N'',N'''tristrifluoroacetamido]spermino)purinyl-2'-deoxyribonucleoside ***19***

The sulfonate **18** (0.5 g, 0.55 mmol) was dissolved in dry DMF (2 mL) and spermine derivative 5,10-bis(-trifluoroacetyl)-1,5,10,14-tetraazatetradecane **13** (0.68 g, 1.02 mmol) was added to the above solution and stirred at 55°C for 20h when the TLC indicated completion of reaction. The reaction mixture was then concentrated to a gum and dissolved in dichloromethane (50 mL), washed with 5% $NaHCO_3$ solution (20 mL) and the organic layer dried over anhydrous sodium sulfate. The residue was dried by co-evaporation with dry ethanol, redissolved in dry ethanol (1 mL), treated with ethyltrifluoroacetate (2 mL) and triethylamine (1.0 mL, 7.2 mmol) and stirred at room temperature for 36h. The progress of the reaction was monitored by TLC. The residue obtained after removal of solvents and excess ethyltrifluoroacetate was taken in CH_2Cl_2 (50 mL) and subjected to usual work-up. Flash column chromatography of the residue (silica gel, CH_2Cl_2/MeOH/Et_3N, 9.5:0.5:0.1) yielded the desired compound **19** (0.24 g, 40%) as a pale yellow foam.

m.p. 66°C. TLC: (CH_2Cl_2:MeOH, 9.5:0.5) R_f 0.50.

MS (FAB): 1248 (M^++Na).

^{1}H NMR, $CDCl_3$ δ: 9.75 (bs 1H, NH), 7.80 (s, 1H, H8), 7.45-7.1 (m, 9H, ArH), 6.75 (d, 4H, ArH), 6.30 (m, 1H, H1'), 4.50 (bs, 1H, H3'), 4.00 (bs, 1H, H4'), 3.75 (s, 6H, OCH_3), 3.60-3.00 (m, 14H, spermine-CH_2, H5', H5''), 2.70-2.50 (m, 2H, H2' and $CH(CH_3)_2$), 2.50-2.25 (m, 1H, H2''), 2.10-1.60 (m, 8H spermine-CH2), 1.25-1.00 (m, 6H, *i*Bu 2 x CH_3), 0.85 (s, 9H, *t*Bu), 0.00 (d, J = 6.7 Hz, 6H, $Si(CH_3)_2$).

^{13}C NMR, $CDCl_3$ + Pyridine d_5 δ: 175.4 (CO of *i*Bu), 158.1, 157.6, 157.3, ($COCF_3$), 153.8, 151.1, 144.2, 136.1, 129.5, 127.6, 127.2, 126.3, 119.1, 117.2, 116.8, 114.9,

114.5, 112.6, 86.3 (C1'), 85.9, 83.6 (C4'), 72.1 (C3'), 62.9, 54.5 (OCH_3), 47.0, 46.0, 45.7, 44.7, 44.5, 44.0, 43.8, 40.1, 36.6, 36.5, 27.9, 26.4, 26.0, 25.2 [$C(\underline{C}H_3)_3$], 23.4, 18.7, 17.4, -5.4 [$Si(\underline{C}H_3)_2$].

5'-O-(4,4'-Dimethoxy)trityl-3'-hydroxy-N^2-isobutyryl-(C6-[N',N'',N'''-tris-trifluoroacetamido]spermino)-purinyl-2'-deoxyribonucleoside ***20***

Tetrabutylammonium fluoride in THF (1M, 0.35 mL) was added dropwise to compound **19** (0.29 g, 0.24 mmol) in dry THF (10 mL) and the reaction mixture was stirred at room temperature when the TLC indicated completion of reaction after 1h. The reaction mixture was concentrated to dryness, the resulting mass was dissolved in CH_2Cl_2 (20 mL) and washed with 5% $NaHCO_3$ solution. The organic layer was co-evaporated with toluene to get pure compound **20** (0.26 g, 97%).

m.p. 66-68°C. TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.35.

MS (FAB): 1134 (M^++Na).

^{1}H NMR, $CDCl_3$ d: 7.85 (s, 1H, H8), 7.50- 7.10 (m, 9H, ArH), 6.90-6.70 (d, 4H, ArH), 6.40 (m, 1H, H1'), 4.70 (m, 1H, H3'), 4.15 (m, 1H, H4'), 3.75 (s, 6H, OCH_3), 3.60-3.10 (m, 14H, H5', H5'', spermine-CH_2), 2.80-2.30 (m, 3H, H2', H2''), 2.10-1.50 (m, 8H, spermine-CH_2), 1.40-1.20 (m, 6H, $CH(CH_3)_2$).

3'-O-(2-Cyanoethyl-N,N-diisopropylphosphoramido)-5'-O-(4,4'-dimethoxy)trityl-N^2-isobutyryl-(N^6-[N',N'',N'''-tristrifluoroacetamido]spermino)purinyl-2'-deoxyribonucleoside ***21***

Compound **20** (0.24 g, 0.22 mmol) was dissolved in dry CH_2Cl_2 (2.5 mL) followed by the addition of tetrazole (6 mg, 0.09 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphorodiamidite (0.10 g, 0.33 mmol) and the reaction mixture was

stirred at room temperature for 3h when the TLC indicated ~90% completion of reaction. The reaction mixture was then diluted with CH_2Cl_2 and washed with 10% $NaHCO_3$ solution. The organic phase was dried over anhy. Na_2SO_4 and concentrated to a foam. The residue was then purified using flash column chromatography (silica gel, CH_2Cl_2/ EtOAc/Et_3N, 1:1:0.05) gave the phosphoramidite **21** (0.21 g, 73%).

TLC: (CH_2Cl_2:EtOAc 4:1) R_f 0.35.

^{31}P NMR, $CDCl_3$ d: 149.73, 149.33.

3.20.2. Oligonucleotide Synthesis and Purification

All the oligonucleotides were synthesized on Pharmacia Gene Assembler Plus using conventional β-cyanoethyl phosphoramidite chemistry. 5'-Dimethoxytrityl-N^2,N^6-bis(diisobutylaminomethylidene)-2,6-diamino-2'-deoxypurine riboside-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite **22** was procured from Glen Research.

The oligonucleotide sequences were synthesized 'trityl off' (0.2 μmol scale) using the commercial amidites and $\mathbf{D}^{sp}$ amidite **21** or **D** amidite **22** wherever necessary. The synthesized oligonucleotides were cleaved from support, deprotected using NH_4OH at 55°C for 16h and desalted using NAP-10 gel filtration columns. The purity of the oligonucleotides were checked by reverse phase HPLC using the buffer systems A: 5% CH_3CN in 0.1 M triethylammmoniumacetate pH 7.0 (TEAA) and B: 30% CH_3CN in 0.1 M TEAA using a gradient from A to B (10% B to 30% B) at a flow rate of 1.5 mL/min. The impure oligonucleotides were purified by RP-HPLC on a Waters C-18 semipreparative column using the above buffer systems.

The purity and composition of the ODNs were confirmed by MALDI-TOF mass spectroscopy (negative ion mode). Mass spectrometry analyses have been performed using a Voyager-Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction.

3.20.3. Sample Preparation for Duplex Studies

The duplexes were constituted by taking equimolar amounts of the appropriate single strands based on the UV absorbance at 260 nm calculated using the molar extinction coefficients of $\mathbf{D}^{sp}$/**D** = 7.6, A = 15.4, C = 7.3, G = 11.7 and T = 8.8 $cm^2/\mu mol$ in 2 mL of sodium phosphate buffer (10 mM sodium phosphate, 100 mM NaCl, pH 7.0). The samples were annealed by heating at 80°C for 2 minutes, followed by slow cooling

to room temperature and refrigeration for at least 4h prior to commencing the experiments.

3.20.4. Sample Preparation for Triplex Studies

The various triplexes were individually constituted by taking 0.5 μM each of the appropriate single strands based on the UV absorbance at 260 nm. The three constituents were taken in 10 mM sodium cacodylate buffer containing 0/10 mM $MgCl_2$ and 100 mM NaCl at pH 7.0 and were annealed as described above. For the UV thermal measurements at acidic pH 5.8, sodium cacodylate buffer was used (10 mM sodium cacodylate, 0/10 mM $MgCl_2$, 100 mM NaCl, pH 5.8).

3.20.5. UV-Tm Experiments

The thermal stability of duplexes and triplexes was measured by following UV absorbance changes at 260 nm in the temperature range 5-80^{o}C with a heating rate of 0.5^{o}C/min, using a Perkin Elmer Lambda 15 UV/VIS spectrophotometer, equipped with a water jacketed cell holder and a Julabo temperature programmer. Below 15°C, the cell compartment was flushed with nitrogen gas to prevent condensation of water on the UV cells. The triplex dissociation temperatures (T_{m1}) were determined from the midpoint of the first transition in the plots of normalized absorbance versus temperature and further confirmed by the maxima in differential ($\delta A/\delta T$ Vs Temperature) curves. The experiments were repeated at least three times and are accurate to ±0.5^{o}C. The hysteresis experiments were carried out as described in Chapter 2, Section 2.14.6.

3.20.6. UV-Mixing

Stock solutions of the individual strands were prepared, and equal amounts (0.5 μmol) were added to constitute the duplexes. The samples were annealed and stored at

4°C for a minimum of 4h prior to the commencement of the experiments. To construct a triplex mixing curve, aliquots of the third strand were added to a 1 cm pathlength cuvette containing a solution of the target duplex. After each addition, the cuvette was shaken repeatedly to ensure complete mixing followed by equilibration at 10°C for 20-30 min. Upon equilibration, the absorbance at 260 nm was measured and the plot of $A_{obs.}/A_{calc.}$ Vs mole fraction of the third strand gave the mixing curve, which generally had an inflection point near 1 indicating a 1:1 stoichiometry of binding.

3.21. EXPERIMENTAL (SECTION B)

3.21.1. Oligonucleotide Synthesis and Purification

The oligonucleotides were synthesized in a similar manner as described in Section 3.20.2. The phosphoramidites of **D** (**22**) and $\mathbf{U}^{\#}$ (**47**) were dissolved in dry acetonitrile to get 0.15 M solutions and 4Å molecular sieves were added to remove traces of moisture. The ODNs synthesized were analyzed by HPLC and purified as described in Section 3.20.2.

3.21.2. Sample Preparation for Duplex Studies

The duplexes were constituted by taking equimolar amounts of the appropriate single strands based on the UV absorbance at 260 nm calculated using the molar extinction coefficients of **D** = 7.6, A = 15.4, C = 7.3, G = 11.7 and T/$\mathbf{U}^{\#}$ = 8.8 $cm^2/\mu mol$ in 10 mM sodium cacodylate buffer containing 2 mM $MgCl_2$ and 100 mM NaCl at pH 7.0.

3.21.3. Sample Preparation for Triplex Studies

The various triplexes containing the respective triads (as in Table 2-5 *Section B*) were individually constituted by taking 0.5 μM each of the appropriate single strands based on the UV absorbance at 260 nm. The three constituents were taken in 100 mM

sodium cacodylate buffer containing 20 mM $MgCl_2$ and 1M NaCl at pH 5.8 and were annealed. The thermal stability of duplexes and triplexes was measured by following UV-Tm experiments.

3.21.4. UV-Mixing

The UV-mixing experiments were carried out as described in Section 3.20.5. and the derived plots of $A_{obs.}/A_{calc.}$ Vs mole fraction of the third strand gave the mixing curve, indicating a 1:1 stoichiometry of complex formation.

3.22. EXPERIMENTAL (SECTION C)

3.22.1. Synthesis of Compounds 69-78

N-Methyl isatoic anhydride ***69***

To isatoic anhydride **68** (4.0 g, 24.5 mmole) in dry DMF (30 ml) cooled in an ice bath was added NaH (60%, 1.1 g, 27.5 mmol) slowly with stirring. After 1h, MeI (1.8 mL, 29 mmol) was added in about 2 min and the mixture was stirred overnight. The reaction mixture was concentrated to dryness and ice-cold water was added (75 mL) when a beige coloured precipitate was obtained. The precipitate was filtered out, washed with ice-cold water (10 mL) and desiccated to get the pure product **69** (3.72 g, 85%).

m.p. 171°C. TLC: (light petroleum:EtOAc 9:1) R_f 0.60.

^{1}H NMR ($CDCl_3$) δ: 8.15 (d, J = 7.8 Hz, 1H, ArH), 7.80 (t, 7.4 Hz, 1H, ArH), 7.30 (m, 1H, ArH), 7.20 (m, 1H, ArH), 3.60 (s, 3H, N-CH_3).

Benzyl-N-methyl anthranilate ***70***

To N-methylisatoic anhydride **69** (3.54 g, 20 mmole) in dioxane (40 mL) was added benzyl alcohol (2.2 mL, 21.2 mmole), NaOH (0.04 g, 1 mmole) and refluxed for

6h when the TLC indicated the complete consumption of the starting material. The solution was concentrated to dryness, redissolved in ethylacetate (75 mL) and washed with water (15 mL x 2). The organic layer was concentrated to get pure **70** (4.5 g, 93%) as an oil.

TLC: (light petroleum:EtOAc 9.5:0.5) R_f 0.60.

MS: 337 (M^+)

^{1}H NMR ($CDCl_3$) δ: 7.90 (d, J = 8.3 Hz, 1H, Ar<u>H</u>), 7.70-7.50 (bs, 1H, N<u>H</u>), 7.50-7.20 (m, 6H, Ar<u>H</u>), 6.70-6.50 (m, 2H, Ar<u>H</u>), 2.90 (d, J = 4.4 Hz, 3H, N-C<u>H</u>$_3$).

Benzyl-N-trifluoroacetyl-N-methyl anthranilate ***71***

The benzyl ester **70** (2.5 g, 10.4 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and pyridine (3.9 mL, 49 mmol) and trifluoroacetic anhydride (5.0 mL, 35 mmole) was added drop-wise. The reaction mixture was stirred for 2h when the TLC indicated product formation. After removal of the solvent, the residue was taken in CH_2Cl_2 (75 mL), washed with $NaHCO_3$ (10% solution, 15 mL) and concentrated to get the crude product. Column chromatography of the product (silica gel, light petroleum/EtOAc) gave the pure product **71** (2.15 g, 64%) as an oil.

TLC: (light petroleum:EtOAc 9:1) R_f 0.30.

^{1}H NMR ($CDCl_3$) δ: 8.10 (d, J = 5.9 Hz, 1H, Ar<u>H</u>), 7.70-7.20 (m, 8H, Ar<u>H</u>), 5.30 (d, J = 4.9 Hz, 2H, O-C<u>H</u>$_2$Ph), 3.25 (s, 3H, N-C<u>H</u>$_3$).

^{13}C NMR ($CDCl_3$) δ:164.1 (<u>C</u>=O), 157.1, 156.4, 155.7, 155.0 (q, J_{C-F} = 288.6 Hz, <u>C</u>O<u>C</u>F$_3$), 140.1, 135.0, 133.8, 133.4, 131.8, 129.7, 129.2, 128.4, 127.9, 124.7, 119.0, 113.2, 107.5 (q, J_{C-F} = 35.1, CO<u>C</u>F$_3$), 67.1, 66.85 (O-<u>C</u>H$_2$), 38.9, 38.2 (N-<u>C</u>H$_3$).

N-Trifluoroacetyl-N-methyl anthranilic acid ***72***

Pd-C (10%, 0.05 g) was added to a solution of **71** (0.5 g, 1.53 mmol) in MeOH (10 mL). The mixture was hydrogenated under 50 psi of H_2 for 3h. The catalyst was filtered off and the solution concentrated to dryness to get the desired compound **72** (0.36 g, 96%).

m.p. 151-151°C. TLC: (light petroleum:EtOAc 3:1) R_f 0.20.

MS: 247 (M^+)

^{1}H NMR (Acetone-d_6) δ: 8.10 (m, 1H, ArH), 7.80-7.40 (m, 3H, ArH), 3.30 (s, 3H, N-CH_3).

N-Trifluoracetyl-N-Me-anthranilic acid pentafluorophenyl ester ***73***

To the compound **72** (0.22 g, 0.93 mmole) in dry CH_2Cl_2 (3.0 mL) was added pentafluorophenol (0.2 g, 1.08 mmol) followed by dicyclohexylcarbodiimide (DCC, 0.21 g, 1 mmole) in CH_2Cl_2 (1 mL). The reaction mixture was stirred at r.t. for 5h. The white solid dicyclohexyl urea formed was filtered out and washed with CH_2Cl_2. The filtrates were pooled and concentrated to get the crude product. This was purified using column chromatography (silica gel, light petroleum/EtOAc) to get the pure product **73** (0.34 g, 96%).

m.p. 60-61°C. TLC: (light petroleum:EtOAc 9:1) R_f 0.30.

MS (FAB): 414 (M^++1).

^{1}H NMR ($CDCl_3$) δ: 8.30 (d, 7.8 Hz, 1H, ArH), 7.80-7.55 (m, 2H, ArH), 7.40-7.30 (m, 1H, ArH), 3.35 (s, 3H, N-CH_3).

^{13}C NMR ($CDCl_3$) δ: 160.4 (COO), 157.2, 156.4, 155.7, 155.0 ($COCF_3$), 143.9, 143.1, 141.3, 140.3, 137.21, 135.7, 135.4, 132.6, 130.6, 129.8, 128.9, 124.9, 124.8, 119.0, 113.3 & 107.6 ($COCF_3$), 38.7, 38.1 (N-CH_3).

5-N-(N-Methylanthraniloyl-N-trifluoroacetyl)-5'-O-DMT-5-amino-2'-deoxyuridine ***75***

To the pentafluorophenyl ester **73** (0.42 g, 1.0 mmol) in pyridine (3 mL), HOBt (0.14 g, 1.0 mmol) was added followed by 5-NH_2-dU-5'-O-DMT **74** (0.51 g, 0.93 mmol). The reaction mixture was stirred at r.t. for 6h when TLC indicated the completion of the reaction. The reaction mixture was concentrated to dryness, redissolved in CH_2Cl_2 (25 mL), washed with $NaHCO_3$ (5% solution, 10 mL). The organic layer was concentrated and purified by flash column chromatography (silica gel, CH_2Cl_2, MeOH) to get the pure product **75** (0.41 g, 53%).

m.p. 77-78°C. TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.35.

MS (FAB): 797 (M^++Na).

^{1}H NMR ($CDCl_3$) δ: 10.40 (bs,1H, NH), 8.10 (s, 1H, H6), 7.80-7.00 (m, 13H, ArH), 6.75 (d, 2H, ArH), 6.30 (m, 1H, H1'), 4.40 (m, 1H, H3'), 4.05 (m, 1H, H4'), 3.70 (s, 6H, O-CH_3), 3.60-3.30 (m, 2H, H5', H5"), 3.24 (d, J = 3.4 Hz, 3H, N-CH_3), 2.50-2.20 (m, 2H, H2', H2').

^{13}C NMR ($CDCl_3$) δ: 164.0, 163.3 (NH-CO), 160.06, 160.00, 159.7, 158.1 (C4-dU), 157.1, 156.3 ($COCF_3$), 149.2, 149.0, 148.8, 148.6 (C2-dU), 144.6, 139.9, 138.3, 135.9, 135.7, 135.4, 131.7, 129.9, 129.3, 128.5, 127.9, 127.4, 126.4, 123.5, 123.2, 123.0, 122.8, 117.2, 114.9, 114.6, 114.5, 114.3, 112.8 (q, J_{C-F} = 287.9 Hz, $COCF_3$), 86.1, 85.8 (C1'), 85.7, 85.5, 85.1 (C4'), 71.1, 70.9 (C3'), 64.0, 63.8, 63.8 (C5'), 54.7 (OCH_3), 39.7, 39.6 (C2'), 39.4 (N-CH_3).

3'-O-(2-Cyanoethyl-N,N-diisopropylphosphoramido)-5'-O-(4,4'-dimethoxy)trityl-5-N-(N-methylanthraniloyl-N-trifluoroacetyl)-2'-deoxyuridine ***76***

Compound **75** (0.24 g, 0.22 mmol) was dissolved in dry CH_2Cl_2 (2.0 mL) followed by the addition of tetrazole (6 mg, 0.09 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.12 g, 0.40 mmol) and the reaction mixture was stirred at room temperature for 3h when the TLC indicated ~90% completion of reaction. The reaction mixture was then diluted with dichloromethane and washed with 10% $NaHCO_3$ solution. The organic phase was dried over anhy. Na_2SO_4 and concentrated and the residue after purification using flash column chromatography (silica gel, CH_2Cl_2/EtOAc/Et_3N, 1:1:0.05) gave the phosphoramidite **76** (0.16 g, 61%).

TLC: (CH_2Cl_2:EtOAc, 1:1) R_f 0.60

^{31}P NMR, $CDCl_3$ δ: 149.42, 149.14.

*5-N-(N-methylanthraniloyl)-5'-O-DMT-5-amino-2'-deoxyuridine **77***

To **75** (0.1g, 0.13 mmol) in a Wheaton vial was added 1mL of NH_4OH and heated at 60°C for 12h when TLC indicated the completion of the reaction. The reaction mixture was concentrated to dryness and purified by flash column chromatography (silica gel, CH_2Cl_2/ MeOH) to get the pure product **77** (0.08 g, 89%).

m.p. 74°C. TLC: (CH_2Cl_2:MeOH 9.5:0.5) R_f 0.35.

MS (FAB): 701 (M^++Na).

^{1}H NMR ($CDCl_3$) δ: 9.10 (m,1H, NH), 8.15 (s, 1H, H6), 7.90-7.00 (m, 13H, ArH), 6.80 (d, 2H, ArH), 6.35 (m, 1H, H1'), 4.35 (m, 1H, H3'), 4.00 (m, 1H, H4'), 3.70 (s, 6H, O-CH_3), 3.60-3.30 (m, 2H, H5', H5"), 3.20-3.10 (d, 3H, N-CH_3), 2.50-2.10 (m, 2H, H2', H2').

*5-N-(N-Methylanthraniloyl)-5-amino-2'-deoxyuridine **78***

Compound **77** (0.04 g, 0.52 mmol) was stirred with methanolic HCl at room temperature for 5 min. The reaction mixture was evaporated to dryness and the residue

was taken in water (2 mL) and washed with diethylether (5 mL x 3) to remove the 4,4'-dimethoxytritanol. The aqueous layer on lyophilization gave the pure product **78** (0.017 g, 85%).

m.p. 196-199°C.

^{1}H NMR (D_2O) δ: 8.00-7.80 (s, 2H, H6 & ArH), 7.75-7.50 (m, 1H, ArH), 7.30-6.80 (m, 2H, ArH), 6.30 (m, 1H, H1'), 4.50 (m, 1H, H3'), 4.15 (m, 1H, H4'), 3.70-3.35 (m, 2H, H5', H5"), 3.20-3.10 (s, 3H, N-CH_3), 2.60-2.40 (m, 2H, H2', H2').

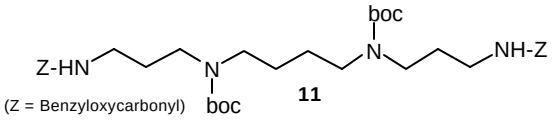
3.22.2. Oligonucleotide synthesis

The oligonucleotides were synthesized by following the protocols as described in Section 3.21.2. The phosphoramidite of $\mathbf{U}^{MANT}$ (**76**) was employed for the synthesis.of self-complementary ODN **79** while the phosphoramidites **76** and **21** ($\mathbf{D}^{sp}$) were utilized for the ODN **80**.

3.22.3. Fluorescence studies

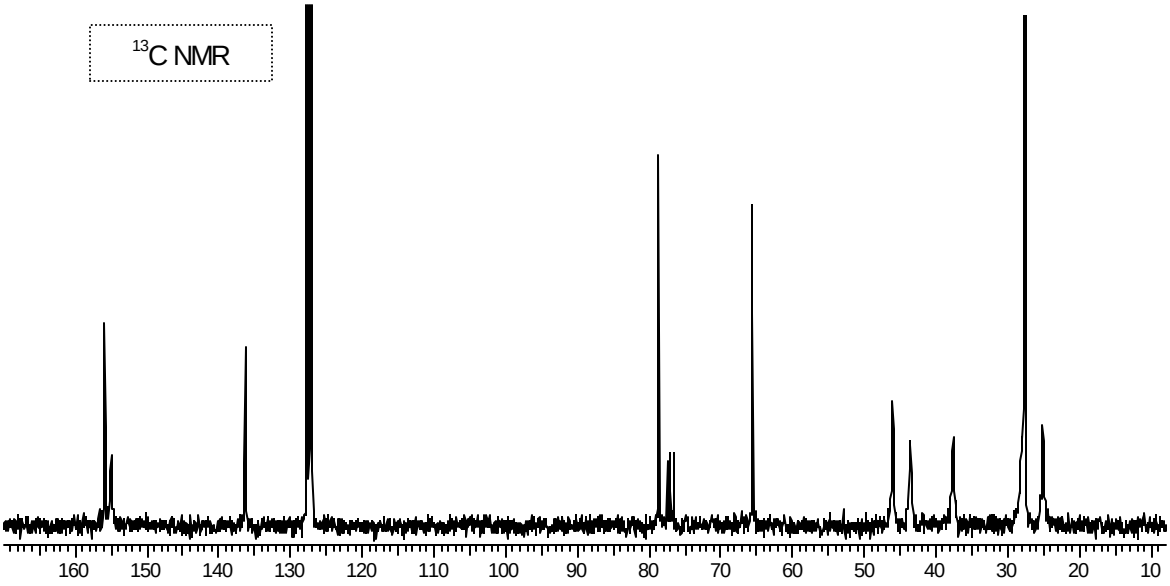
Fluorescence measurements were performed on a Perkin-Elmer LS50 Luminescence spectrometer connected to a Julabo F20 water bath. The fluorescent monomer samples or ODNs were dissolved in phosphate buffer (100 mM) pH 7.4 for the measurements. The monomer sample (0.5 mg/2mL) was excited at 360 nm and the emission was recorded from 375 nm to 520 nm. The ODNs (1 μmole/2mL of buffer) were excited at 210 nm and the emission monitored in the region 300-450 nm. The mixed solvent systems (water/dioxane) were prepared by mixing distilled water with appropriate volumes of dioxane (spectroscopic grade). The slit widths for the excitation and emission measurements were set at 5 nm and the scan speed was 200 nm/min. Fluorescence data were corrected for buffer/solvent and smoothened.

3.23. APPENDIX

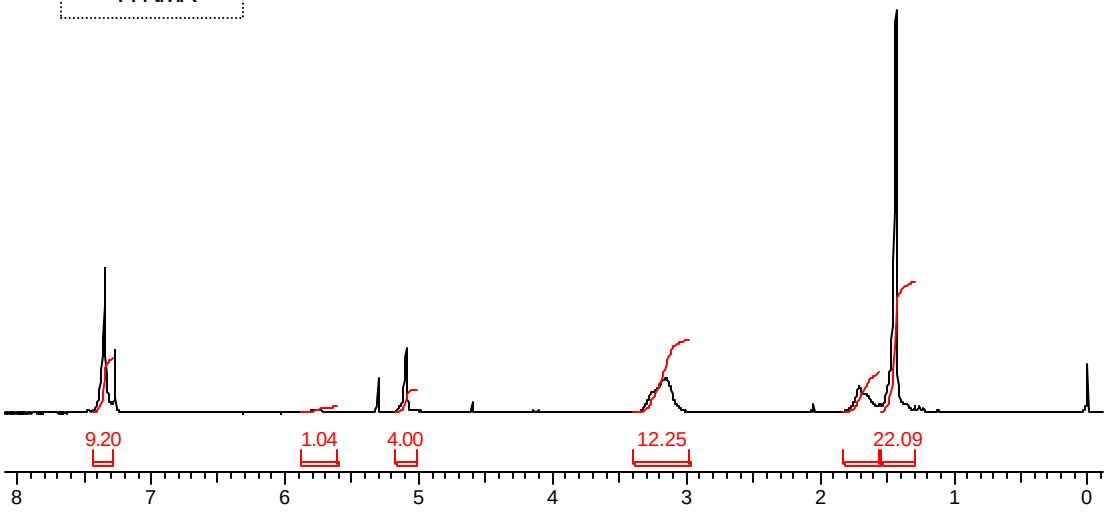


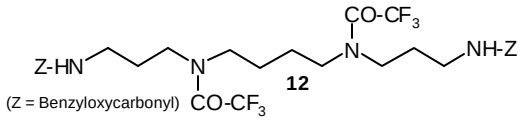
11

^{13}C NMR



^{1}H NMR

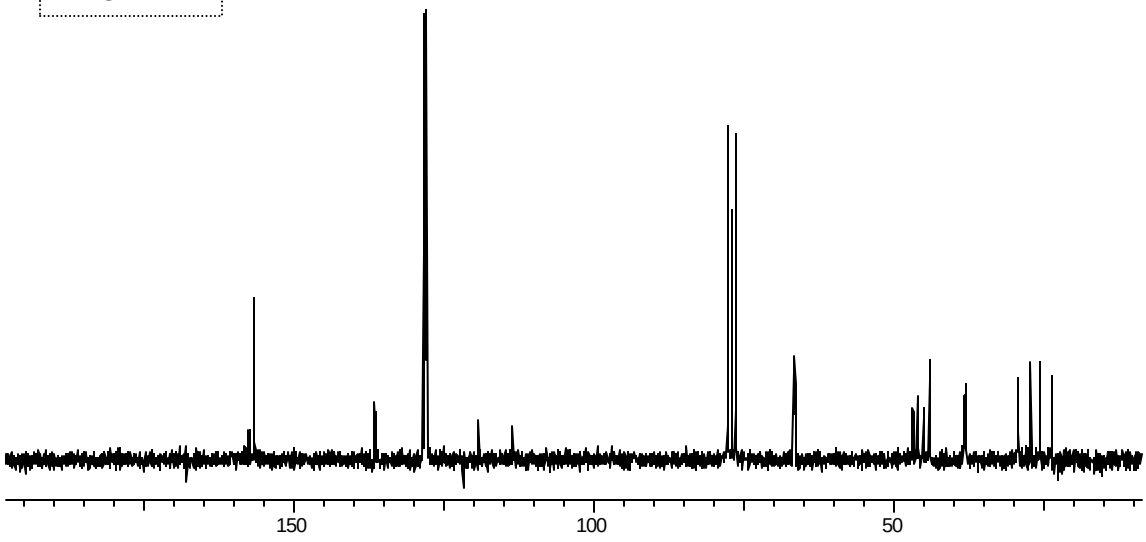




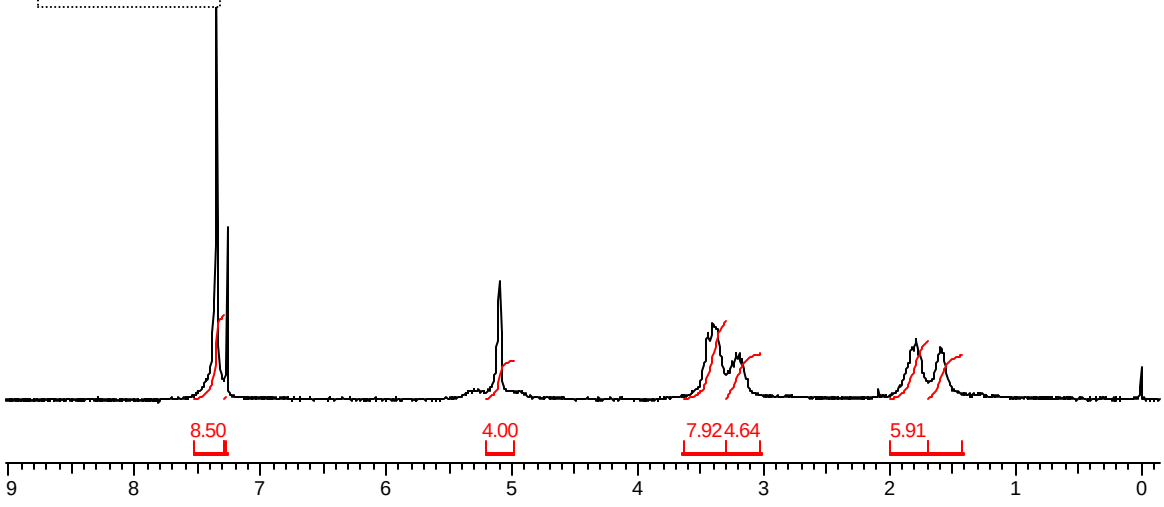
(Z = Benzyloxycarbonyl)

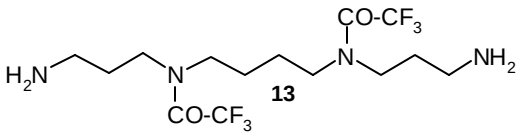
12

^{13}C NMR

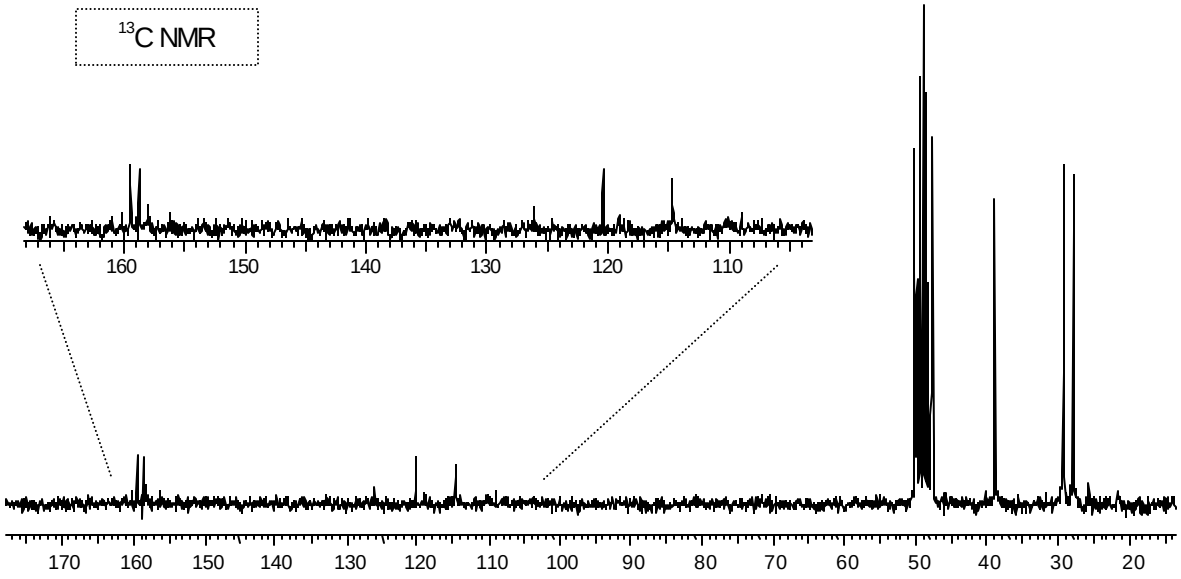


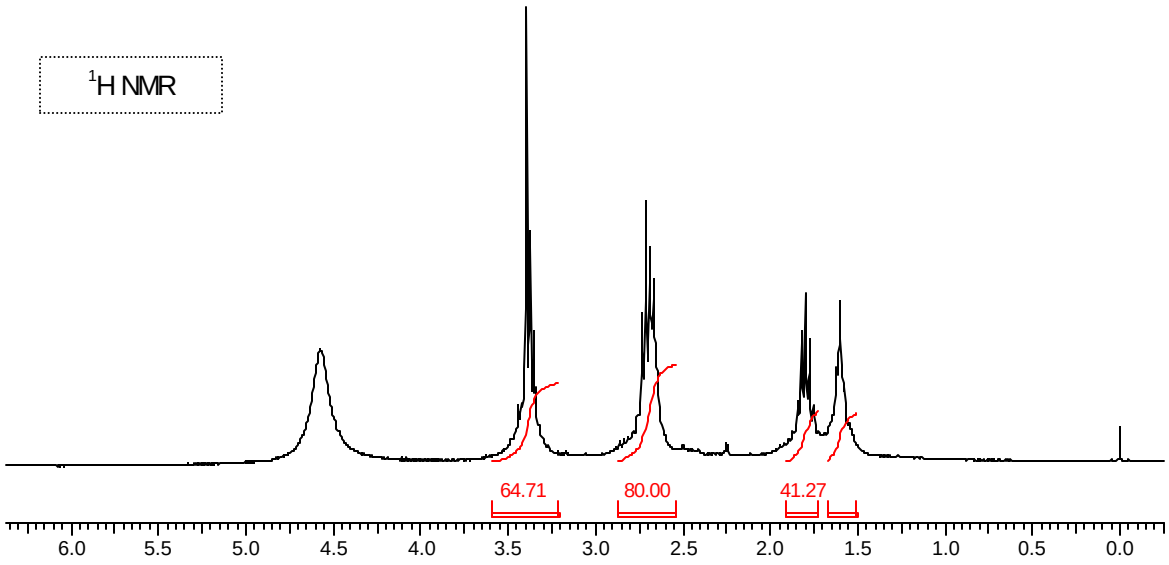
^{1}H NMR

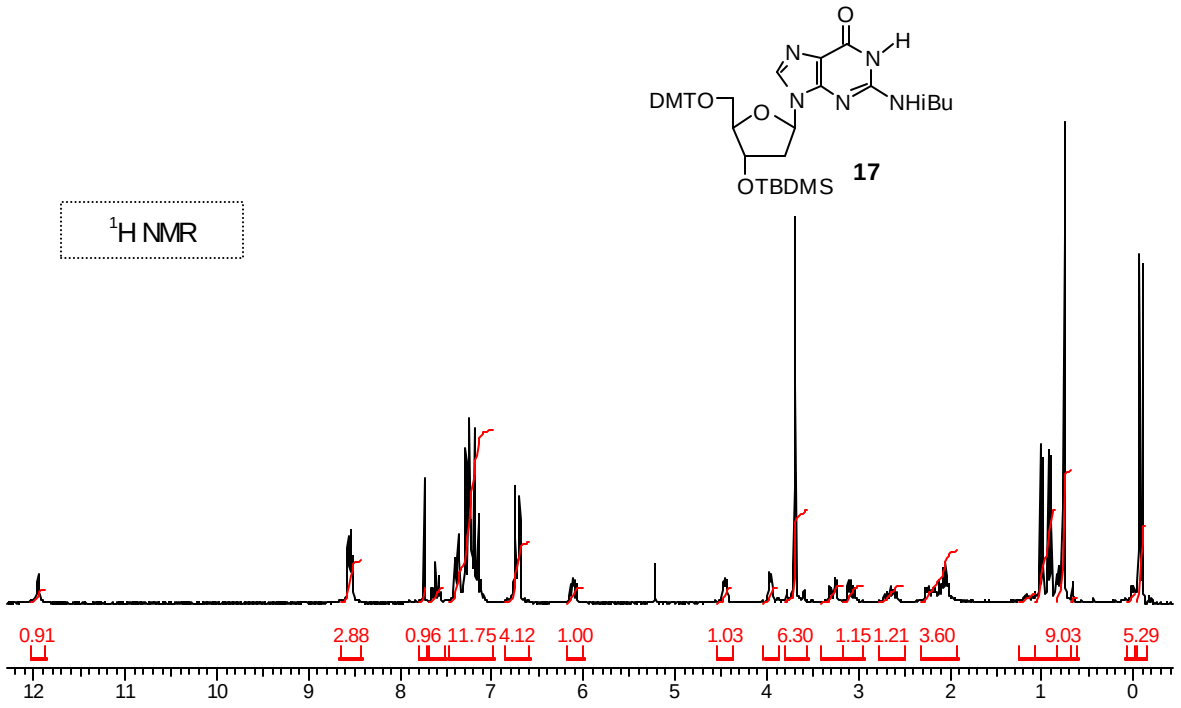


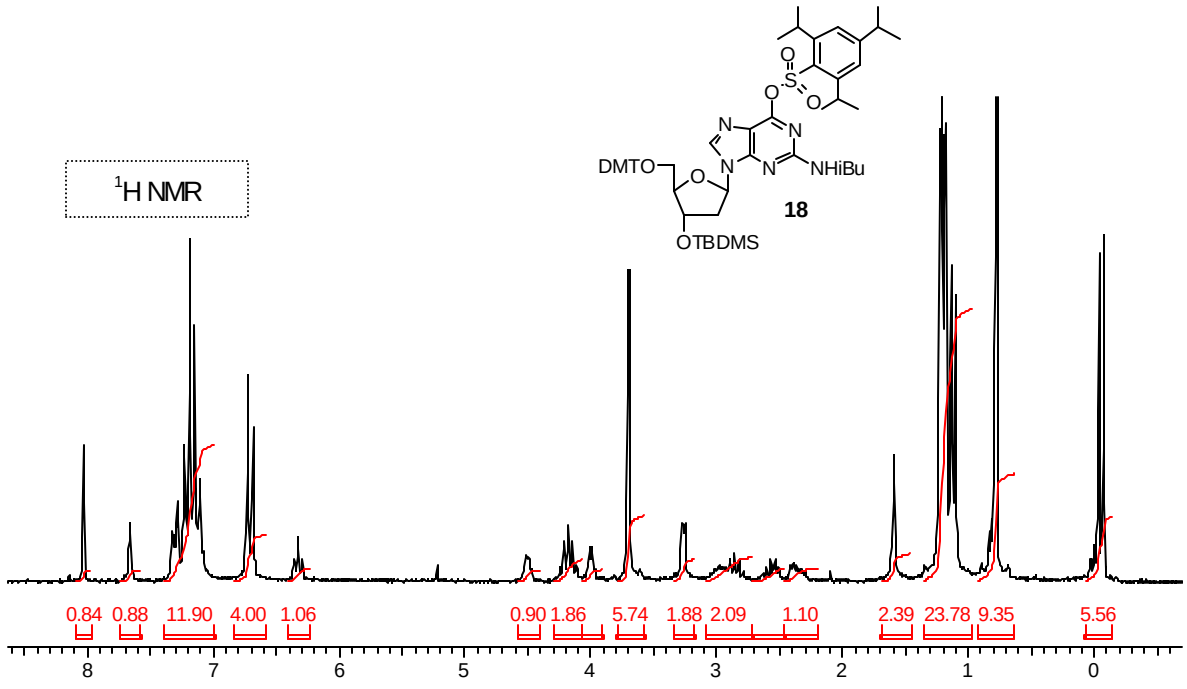

CO-CF3
H2N
N
N
NH2
CO-CF3

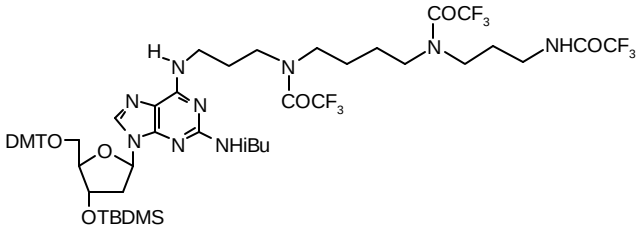
13


13C NMR
160
150
140
130
120
110
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20

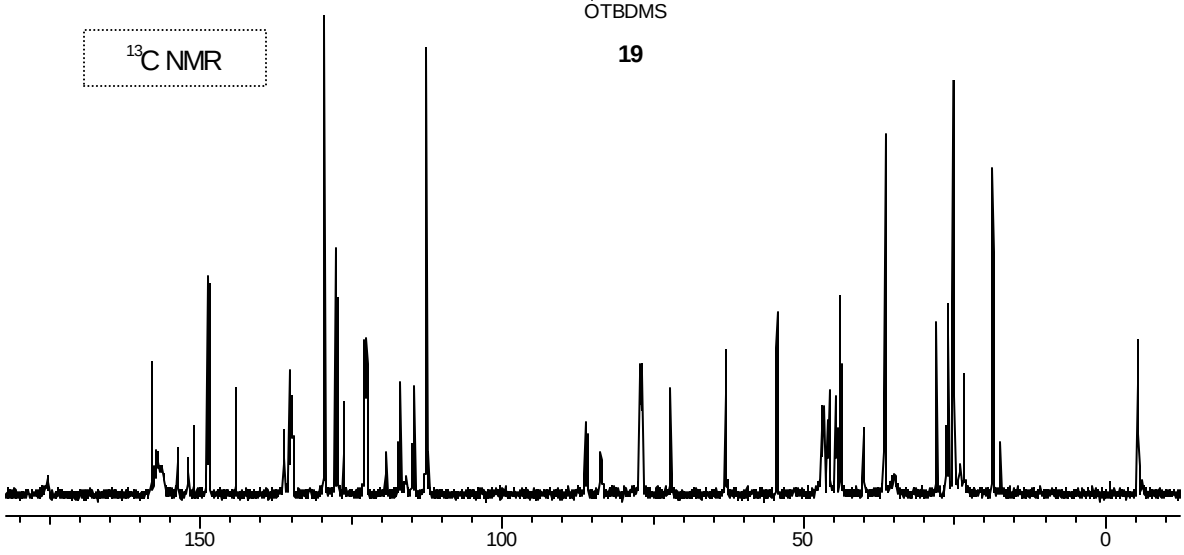

1H NMR
64.71
80.00
41.27
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

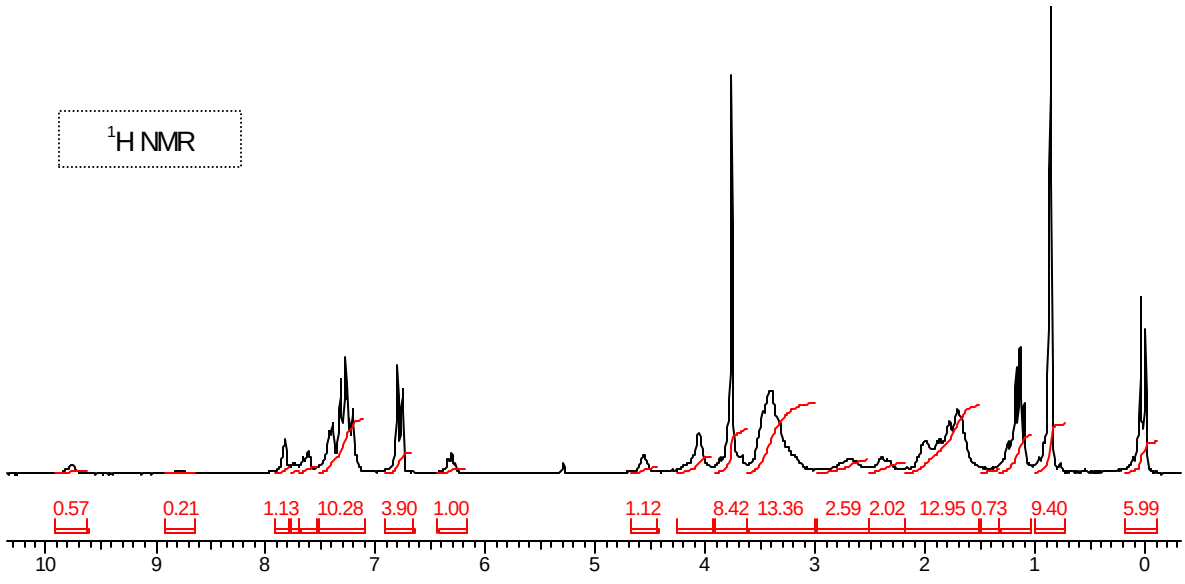

DMTO
O
N
N
H
N
N
NHiBu
OTBDMS
17
^{1}H NMR
0.91
2.88
0.96
11.75
4.12
1.00
1.03
6.30
1.15
1.21
3.60
9.03
5.29
12
11
10
9
8
7
6
5
4
3
2
1
0

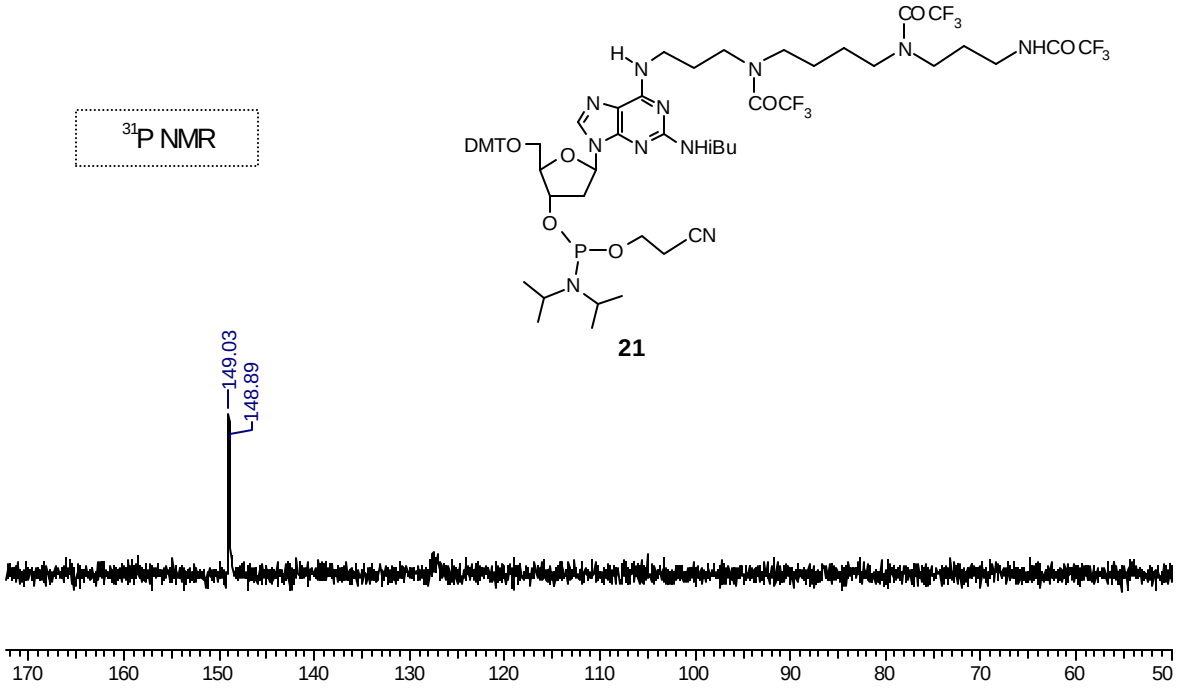

O
S
O
O
N
N
N
N
NHiBu
DMTO
O
OTBDMS
18
^{1}H NMR
0.84
0.88
11.90
4.00
1.06
0.90
1.86
5.74
1.88
2.09
1.10
2.39
23.78
9.35
5.56
8
7
6
5
4
3
2
1
0


COCF3
H
N
N
N
NHCOCF3
COCF3
N
N
N
N
N
NHiBu
DMTO
O
OTBDMS

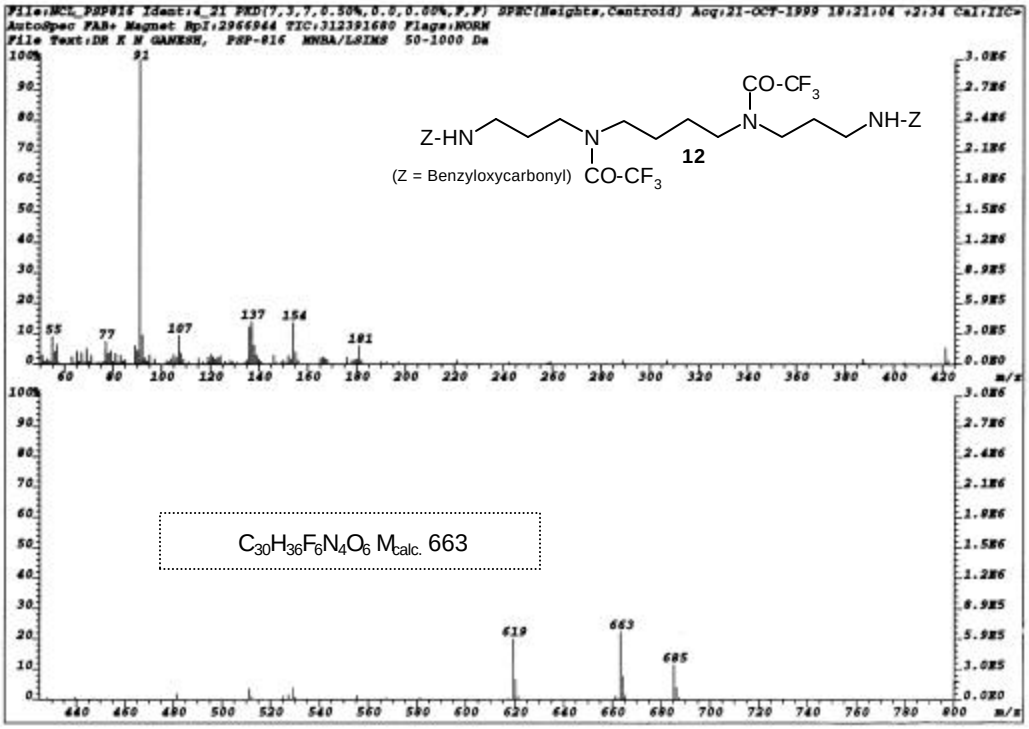
19


13C NMR
150
100
50
0

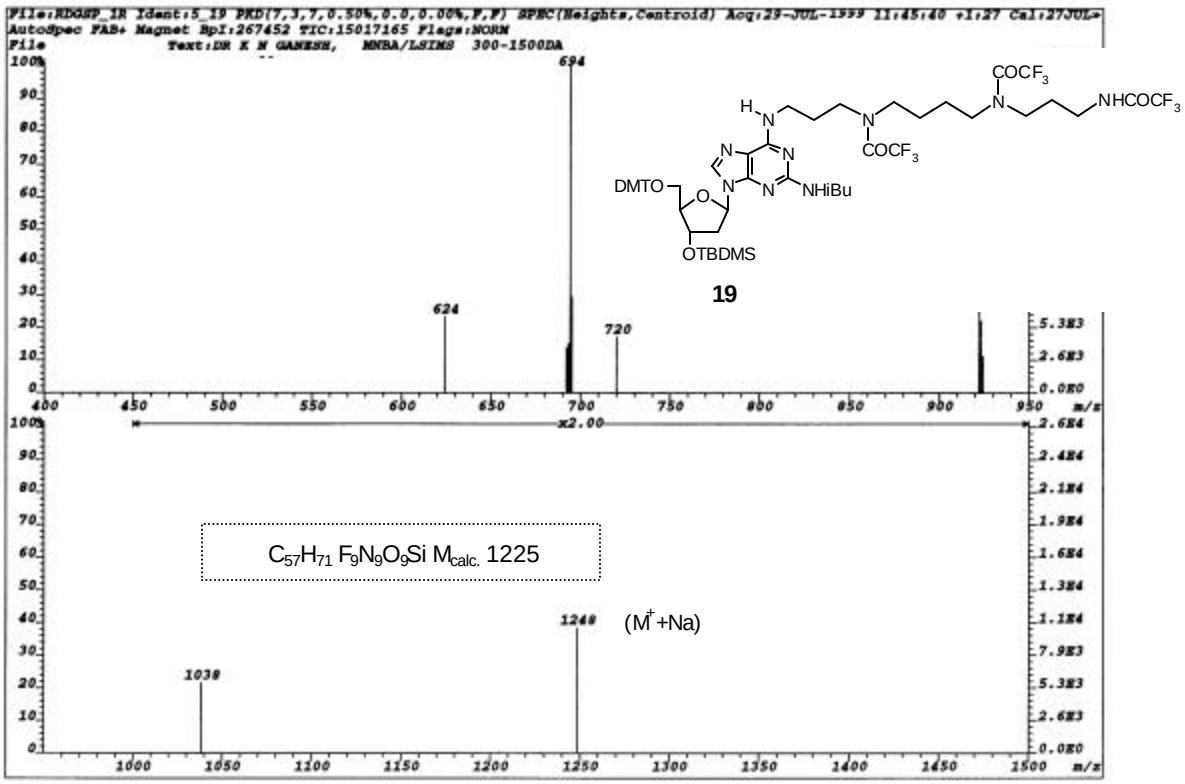

1H NMR
0.57
0.21
1.13
10.28
3.90
1.00
1.12
8.42
13.36
2.59
2.02
12.95
0.73
9.40
5.99
10
9
8
7
6
5
4
3
2
1
0


31P NMR
DMTO
NHiBu
CN
CO CF3
NHCO CF3
COCF3
149.03
148.89
170
160
150
140
130
120
110
100
90
80
70
60
50

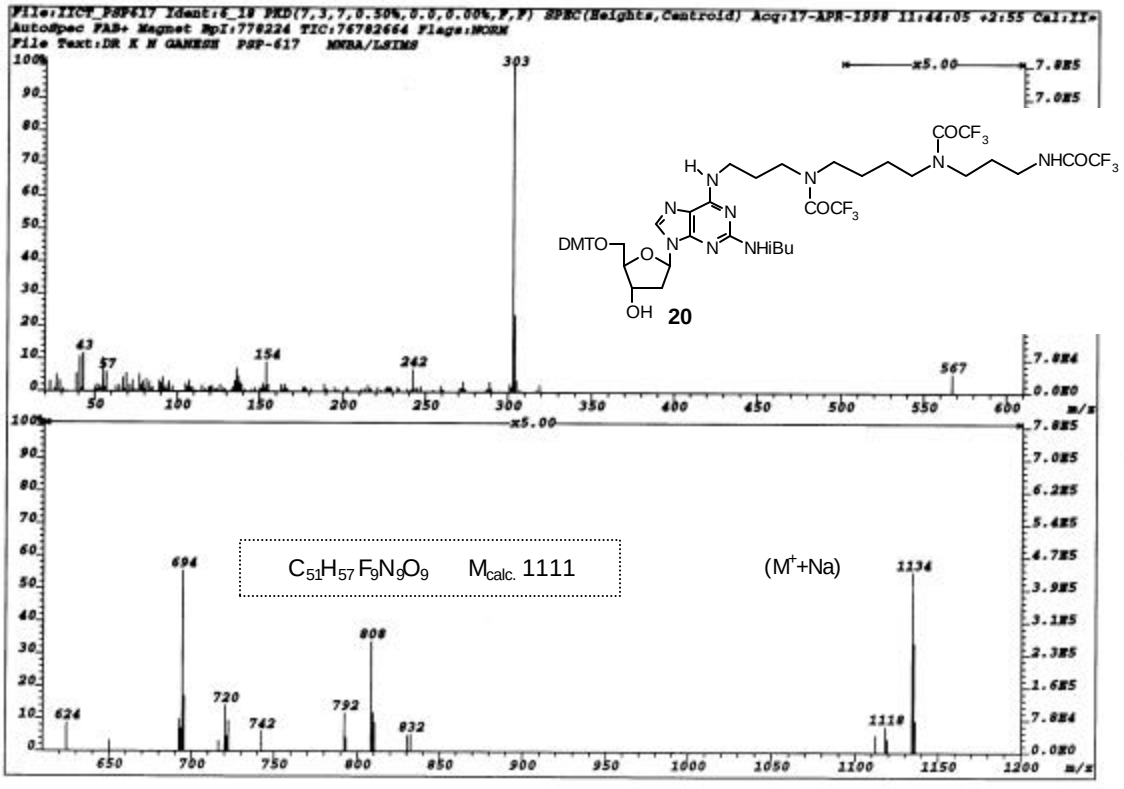
21


Z-HN
NH-Z
CO-CF3
(Z = Benzyloxycarbonyl)
91
55
77
107
137
154
181
619
663
685
C30H36F6N4O6 Mcalc. 663

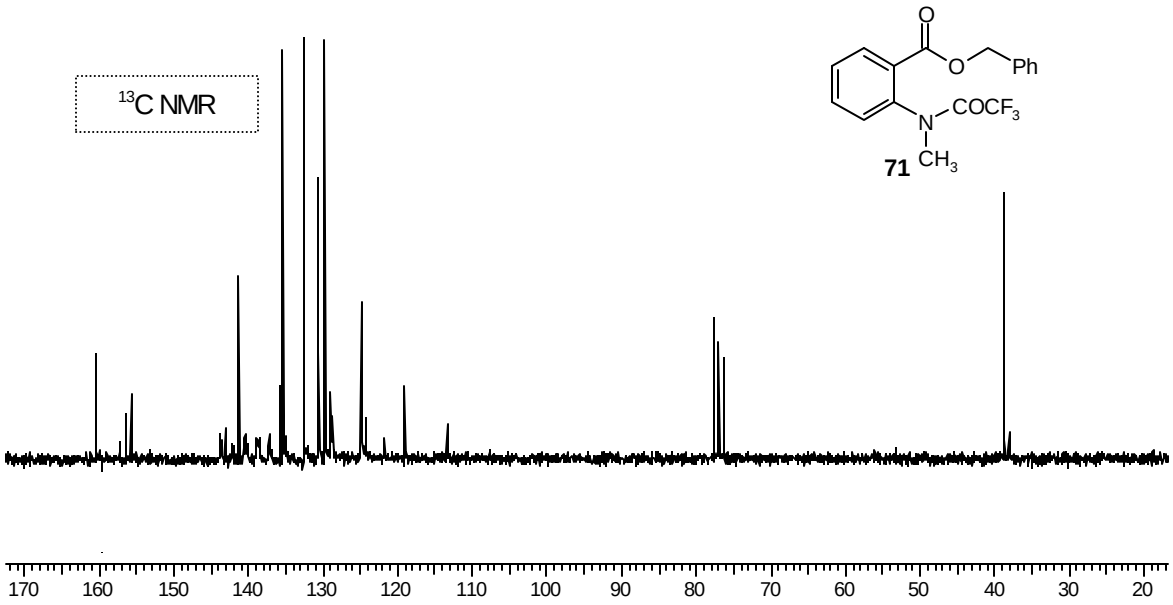
12

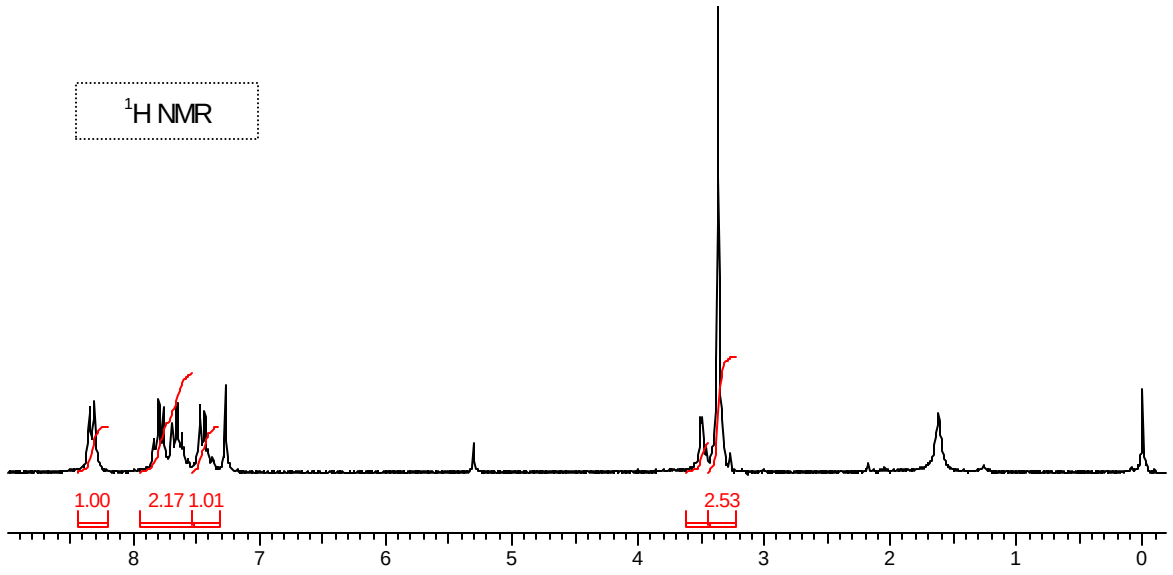

694
624
720
1248
1038
x2.00
DMTO
OTBDMS
NHiBu
COCF3
NHCOCF3
C57H71 F9N9O9Si Mcalc. 1225
(M+Na)

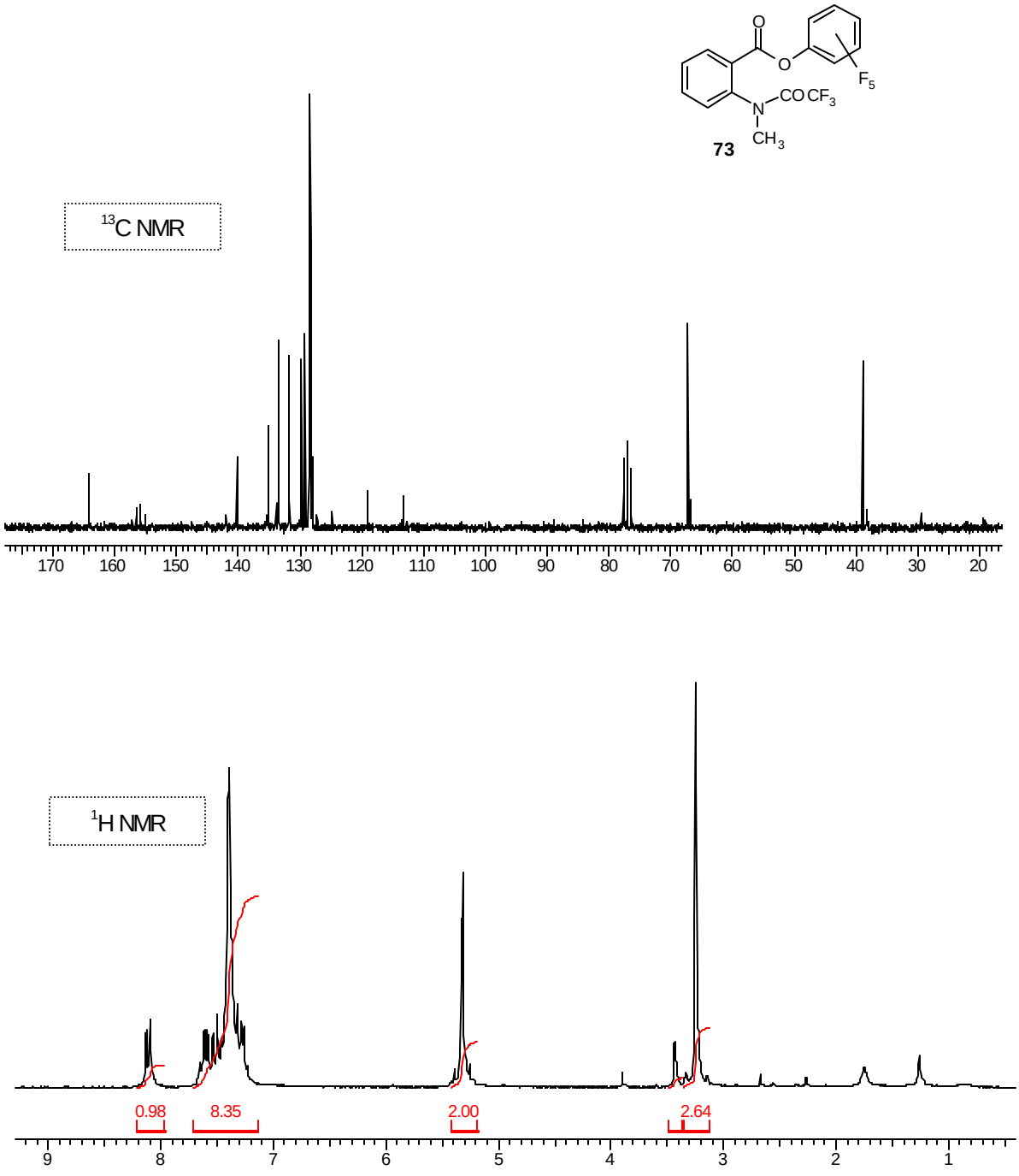
19

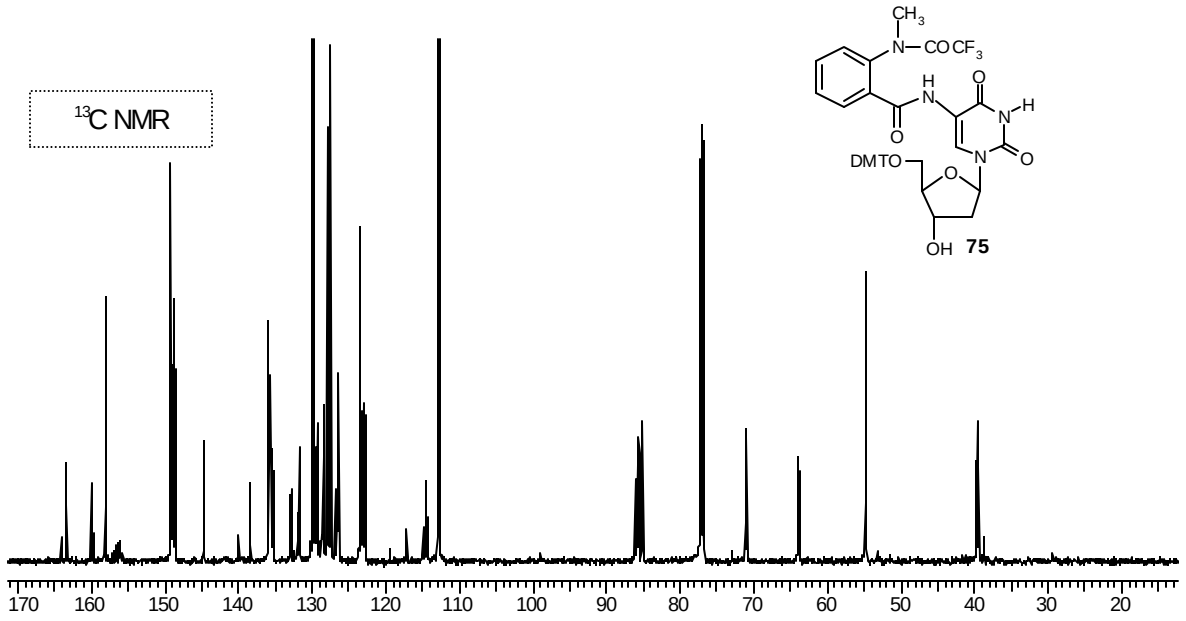

303
x5.00
43
57
154
242
567
694
808
624
720
742
792
832
1134
1118
DMTO
OH
NHiBu
COCF3
NHCOCF3
C51H57 F9N9O9 Mcalc. 1111
(M+Na)

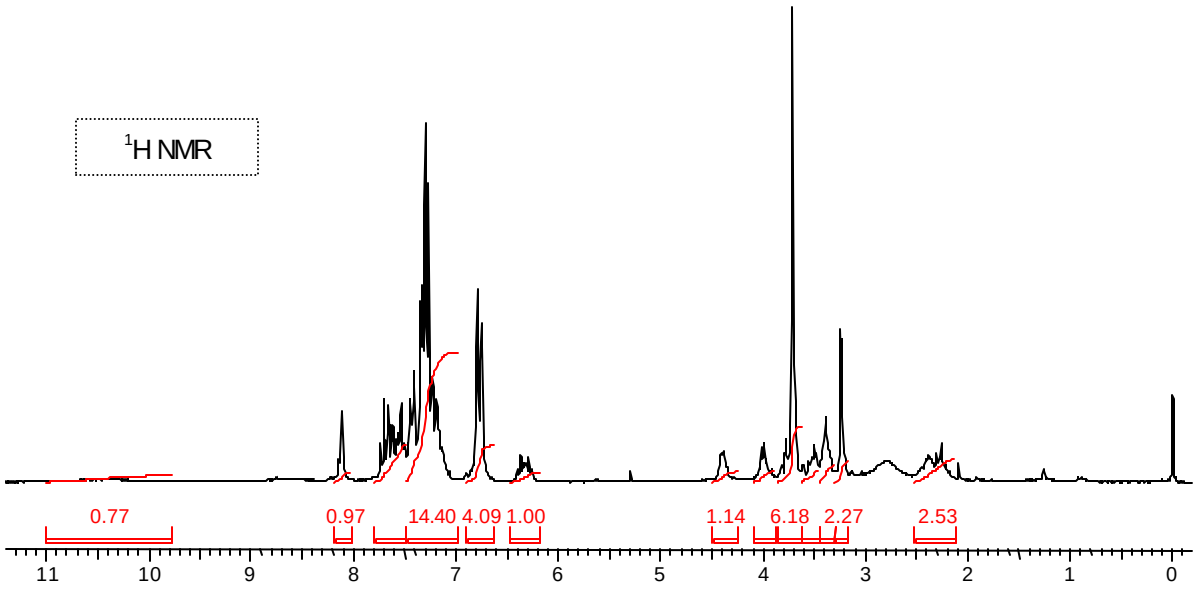
20


^{13}C NMR
O
O
Ph
N
COCF$_3$
CH$_3$
71
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20

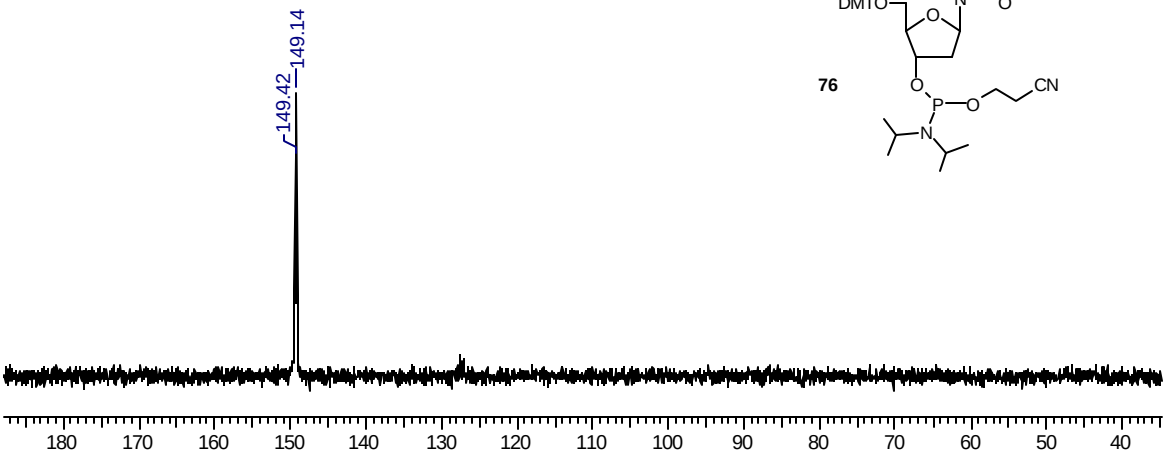

^{1}H NMR
1.00
2.17
1.01
2.53
8
7
6
5
4
3
2
1
0


O
O
F5
CO CF3
N
CH3
73
13C NMR
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20
1H NMR
0.98
8.35
2.00
2.64
9 8 7 6 5 4 3 2 1


^{13}C NMR
CH3
N
CO CF3
H
N
O
O
N
H
N
O
DMTO
O
OH
75
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20

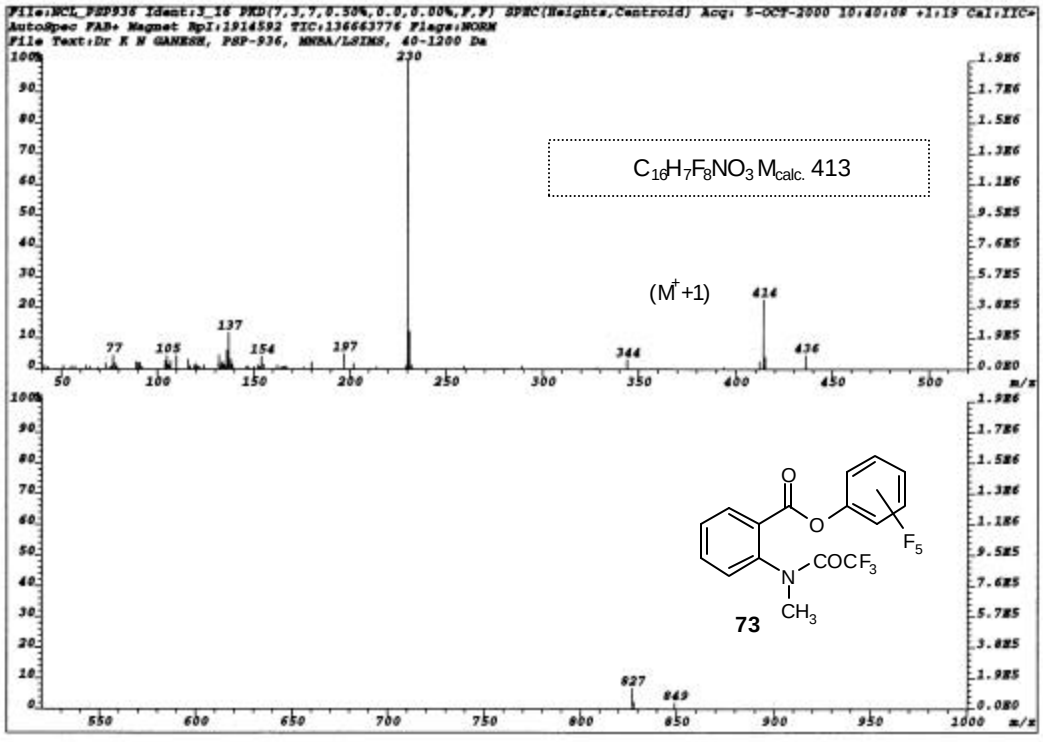

^{1}H NMR
0.77
0.97
14.40
4.09
1.00
1.14
6.18
2.27
2.53
11
10
9
8
7
6
5
4
3
2
1
0

^{31}P NMR


149.42
149.14
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40

CH3
N
COCF3
H
N
O
O
H
N
N
O
DMTO
O
O
P
O
CN
N

76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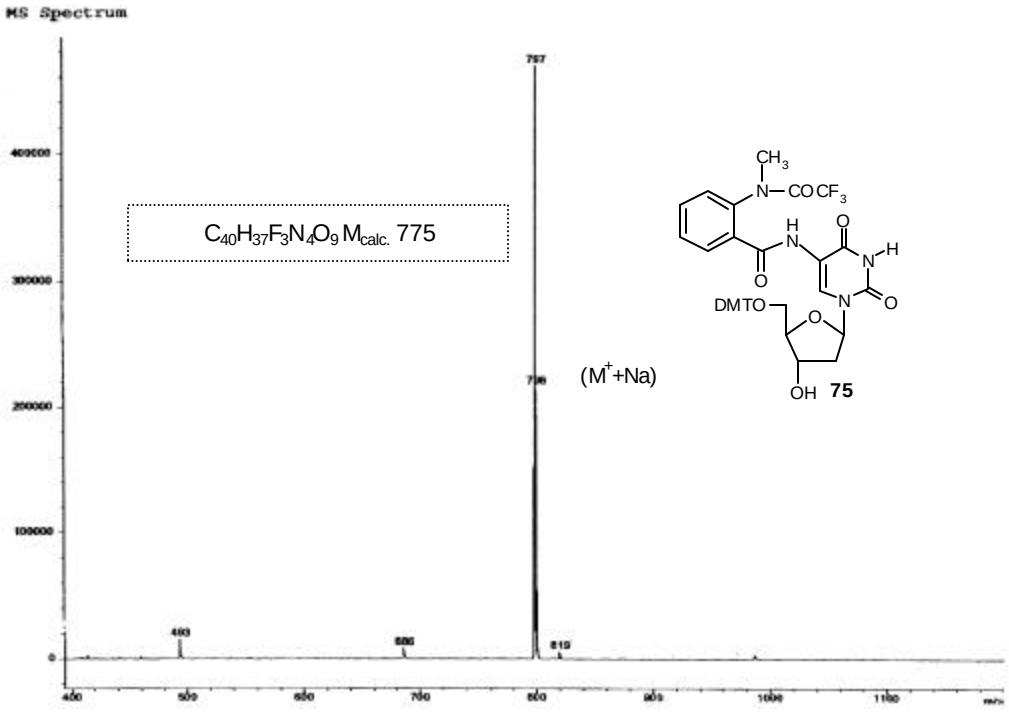
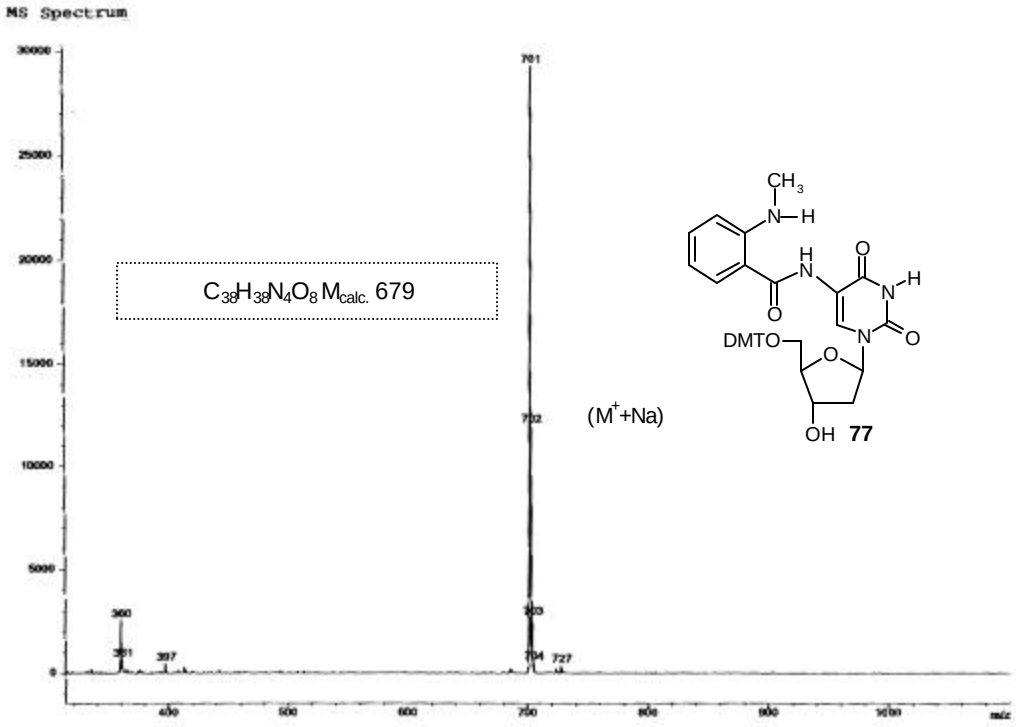
AutoSpec FAB+ Magnet BpI:1914592 TIC:136663776 Flags:NORM
File Text:Dr K N GANESH, PSP-936, MNBA/LSIMS, 40-1200 Da
C16H7F8NO3 Mcalc. 413
(M+ +1)
414
230
137
77
105
154
197
344
436
627
849
F5
COCF3
N
CH3
O
O
m/z

73



$C_{40}H_{37}F_3N_4O_9$ $M_{calc.}$ 775

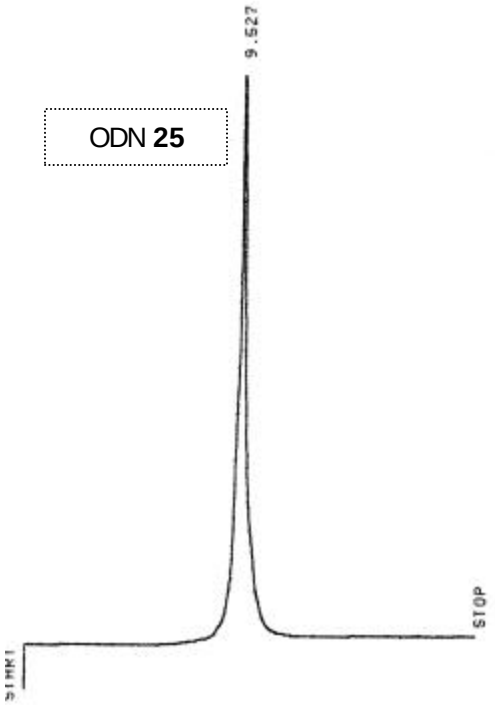
$(M^+ + Na)$ **75**



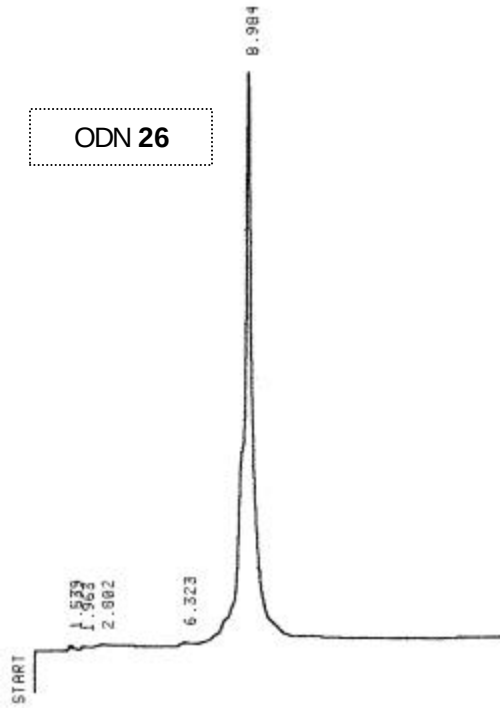
$C_{38}H_{38}N_4O_8$ $M_{calc.}$ 679

$(M^+ + Na)$ **77**

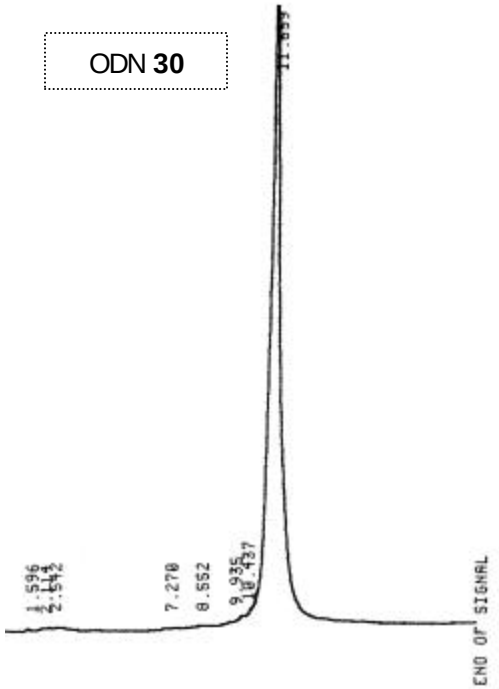
ODN **25**



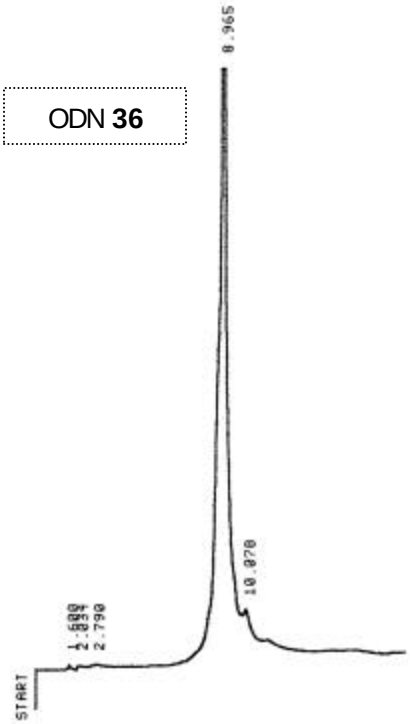
ODN **26**

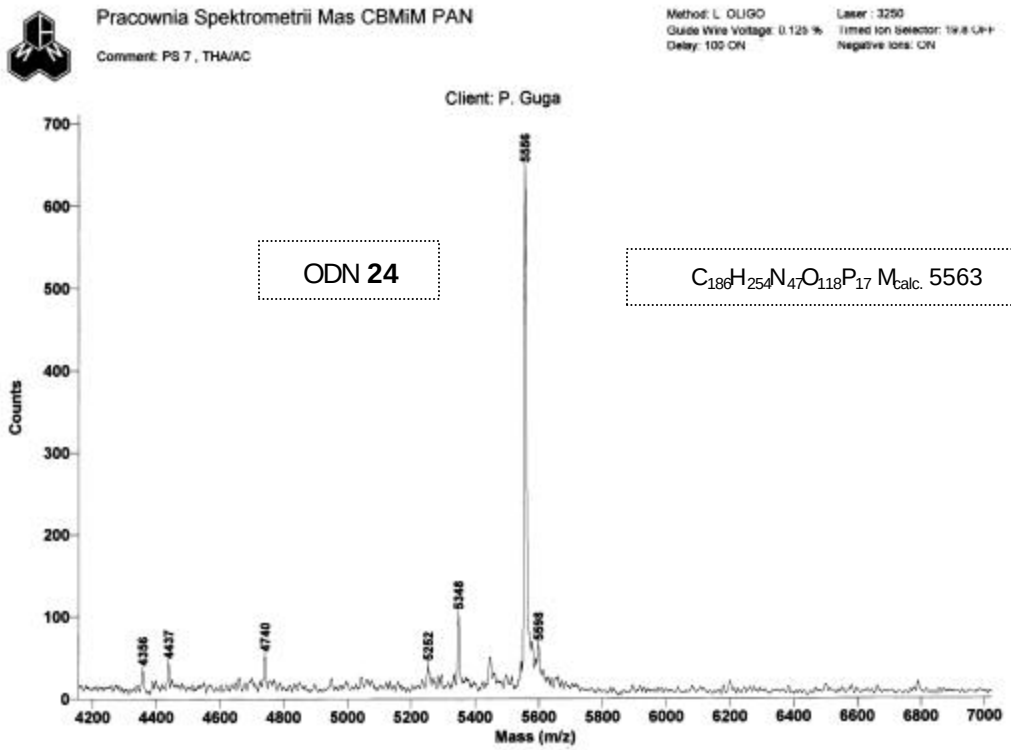


ODN **30**



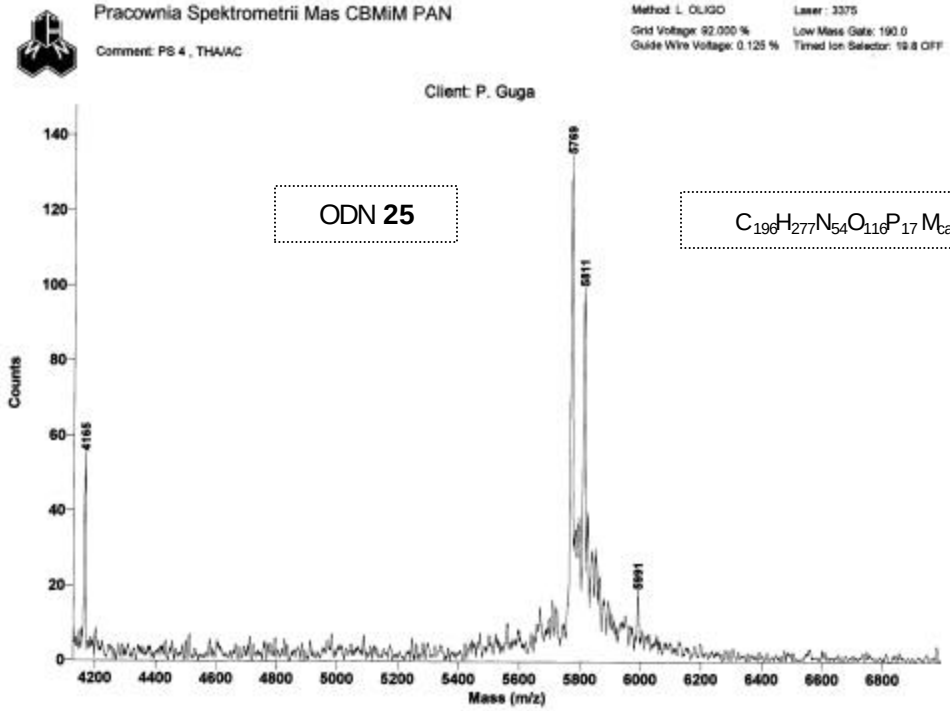
ODN **36**





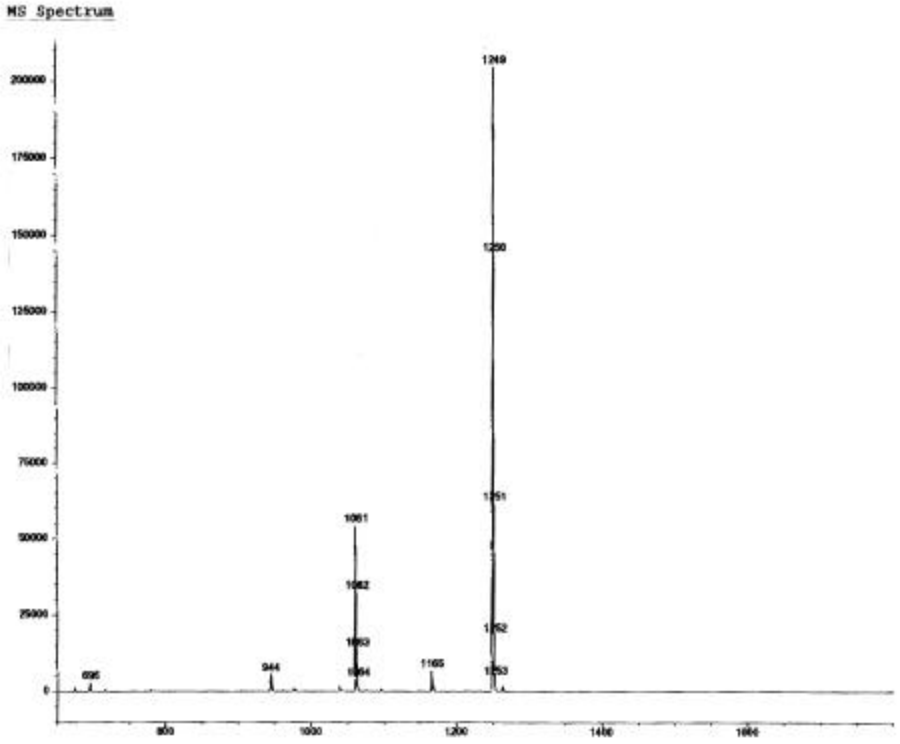
ODN **24**

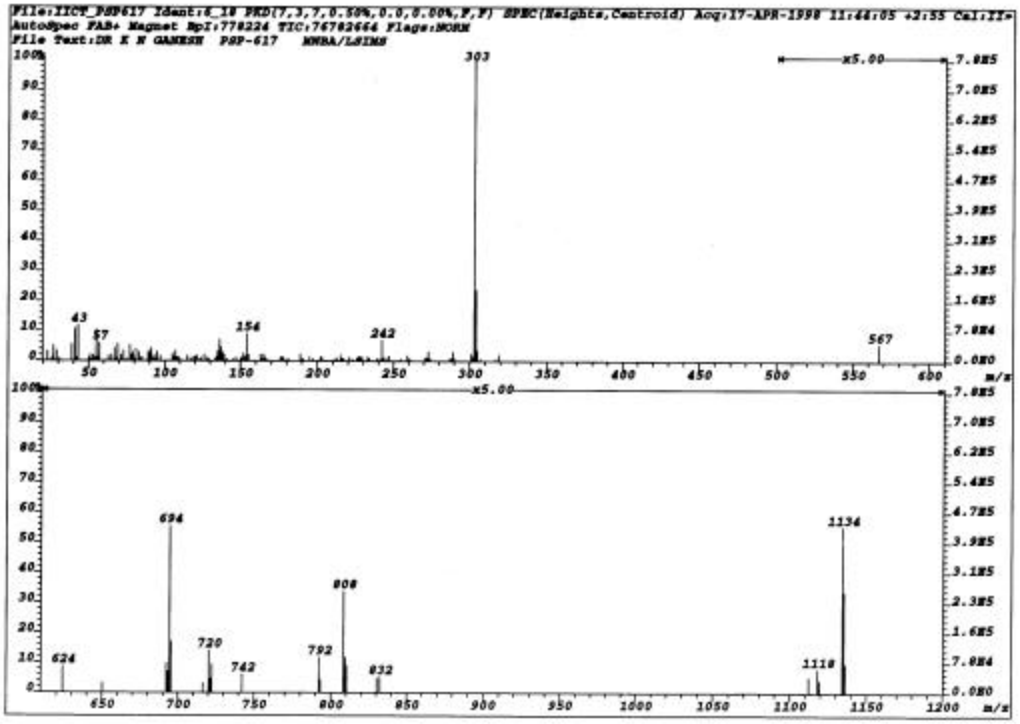
$C_{186}H_{254}N_{47}O_{118}P_{17}$ $M_{calc.}$ 5563

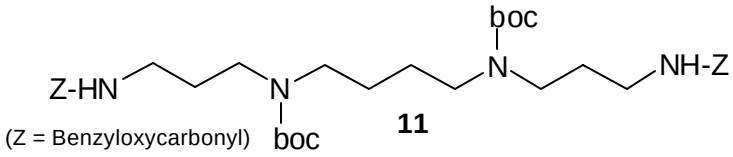


ODN **25**

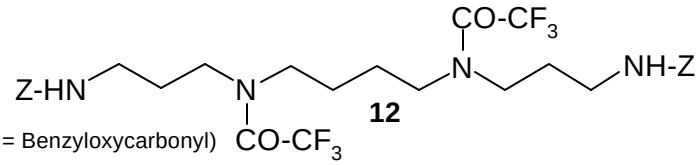
$C_{196}H_{277}N_{54}O_{116}P_{17}$ $M_{calc.}$ 5772


MS Spectrum
1249
1250
1251
1061
1062
1063
1064
1252
1253
1165
944
696
Instrument 1 9/20/2000 3:07:20 PM QP

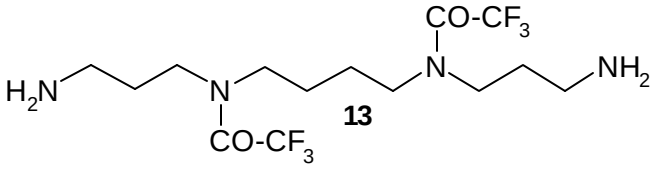

303
x5.00
43
57
154
242
567
694
720
742
792
808
832
624
1134
1118
m/z



(Z = Benzyloxycarbonyl) **11**



(Z = Benzyloxycarbonyl) **12**



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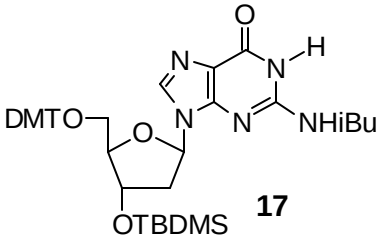

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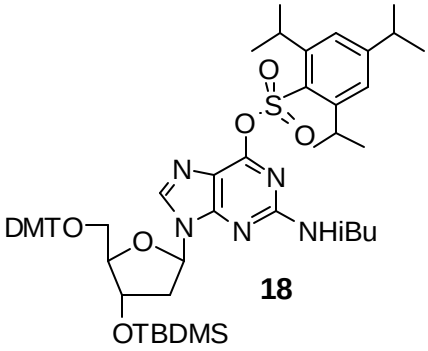


18

$C_{186}H_{254}N_{47}O_{118}P_{17}$ $M_{calc.}$ 5563

$C_{30}H_{36}F_6N_4O_6$ $M_{calc.}$ 663

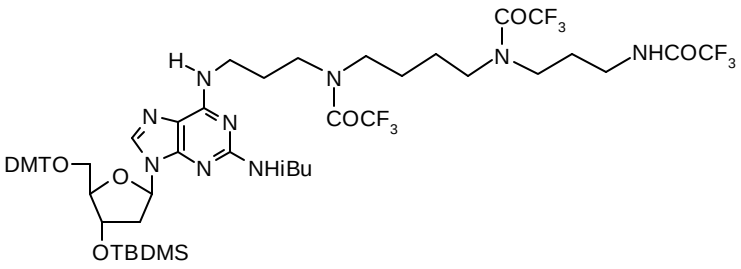
$(M^+ + Na)$

$C_{57}H_{71}F_9N_9O_9Si$ $M_{calc.}$ 1225

$C_{16}H_7F_8NO_3$ $M_{calc.}$ 413

$C_{186}H_{254}N_{47}O_{118}P_{17}$ $M_{calc.}$ 5563

$C_{38}H_{38}N_4O_8$ $M_{calc.}$ 679



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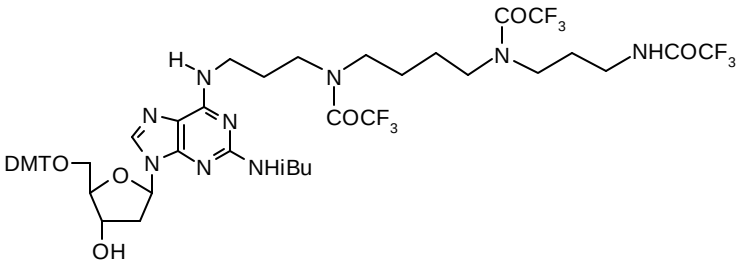
C57H71 F9N9O9Si 1225



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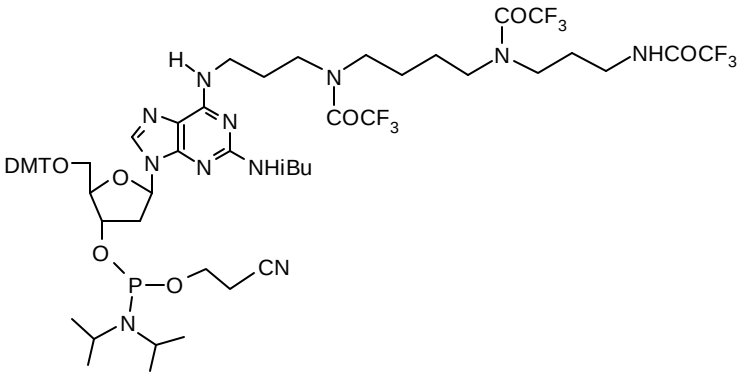


21

ODN **24**

ODN **26**

^{31}P NMR

ODN **25**

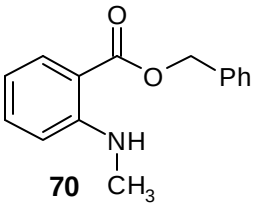
^{1}H NMR

^{13}C NMR

ODN **30**

^{1}H NMR

^{13}C NMR



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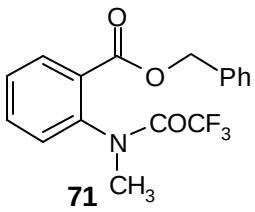

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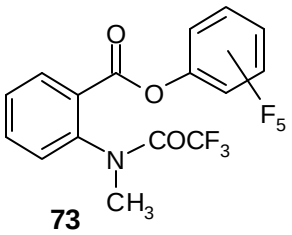

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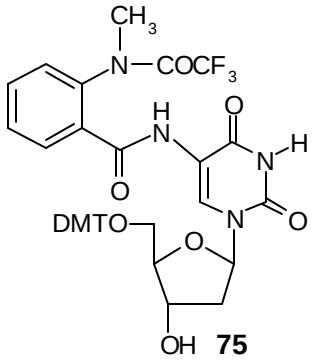

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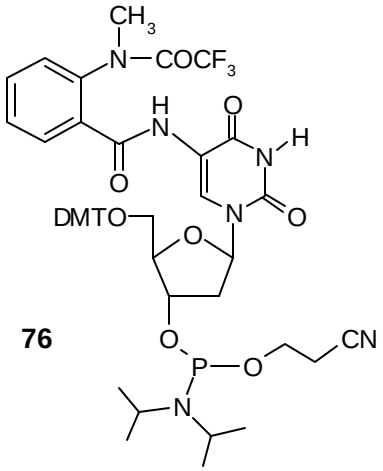

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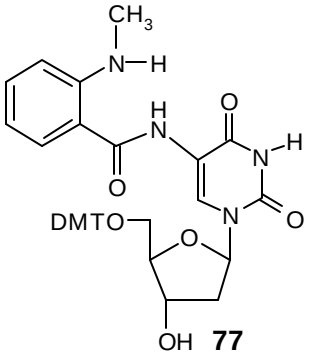

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Chapter 4

Synthesis of Pyrrolidine-Containing PNA-DNA Dimer, Oligomers and their DNA Complementation Studies

4.1. INTRODUCTION

Oligonucleotide analogues are of special interest in molecular biology and medicinal chemistry because of their use as diagnostic agents and their potential application as therapeutic agents.[1,2] To manifest the desired biological activity *in vivo*, oligonucleotides must be stable against the degradative nucleases present in cells. As discussed in Chapter 3, the polyanionic nature of DNA was thought to be a drawback for its efficient uptake by cells. Therefore, non-ionic, neutral oligonucleotides were made in the hope that they would traverse the cell membrane by passive diffusion. Such nonionic oligonucleotides include phosphotriesters, methylphosphonates, phosphoramidates and phosphoramidimates. The most remarkable non-ionic ODN analogue reported till date is the peptide or polyamide nucleic acid[3,4,] (abbreviated as PNA).

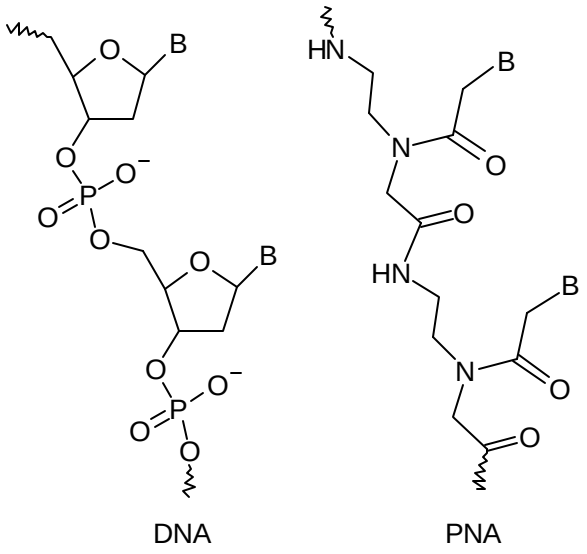


Figure 1. The basic structures of DNA and PNA

PNAs are achiral, homomorphous DNA analogues in which 2-aminoethyl glycine units replace the normal sugar-phosphodiester backbone (Figure 1). The nucleobases are linked to the glycine nitrogens of this backbone by a carboxymethylene linker. PNAs

bind to complementary DNA and RNA with a higher affinity than the natural ODNs.[3,4,5] Because of their enhanced binding properties, PNAs are one of the most interesting oligonucleotide analogues. An added advantage of these analogues is that they are not recognized by either nucleic acid hydrolyzing enzymes[6] or proteases.

PNAs obey the Watson-Crick base pairing rules when binding to complementary DNA/RNA, but the orientation of the strands is ambiguous. Thus, PNAs can form duplexes with DNA in both parallel and antiparallel orientations,[5] the antiparallel mode being slightly preferred over the parallel one.[7] The antiparallel mode refers to the orientation in which the PNA 'N' terminus lies towards the 3'- end and the 'C' terminus, towards the 5'- end of the complementary DNA/RNA oligonucleotide (Figure 2). Likewise, the parallel mode of binding refers to the PNA orientation with the 'N' terminus towards the 5'- end of DNA and the 'C' terminus against the 3'- end of the complementary oligonucleotide.

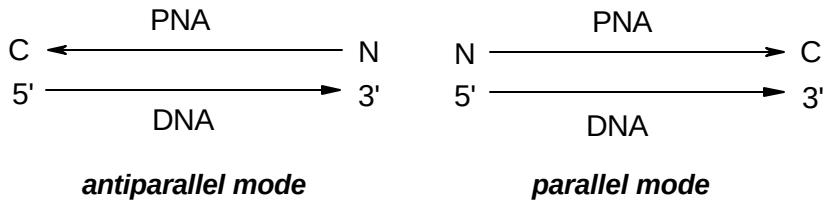


Figure 2. Parallel and antiparallel modes of PNA-DNA binding.

The major obstacles to the use of PNAs in therapeutics are their poor water solubility, tendency to self-aggregate, and their insufficient cellular uptake.[8] Other limitations of PNA with respect to applications include their non-acceptance as substrates for DNA polymerases, DNA kinases or DNA ligases. Further, PNA cannot induce RNase H cleavage of target RNA[9] which is considered a drawback of antisense agents.

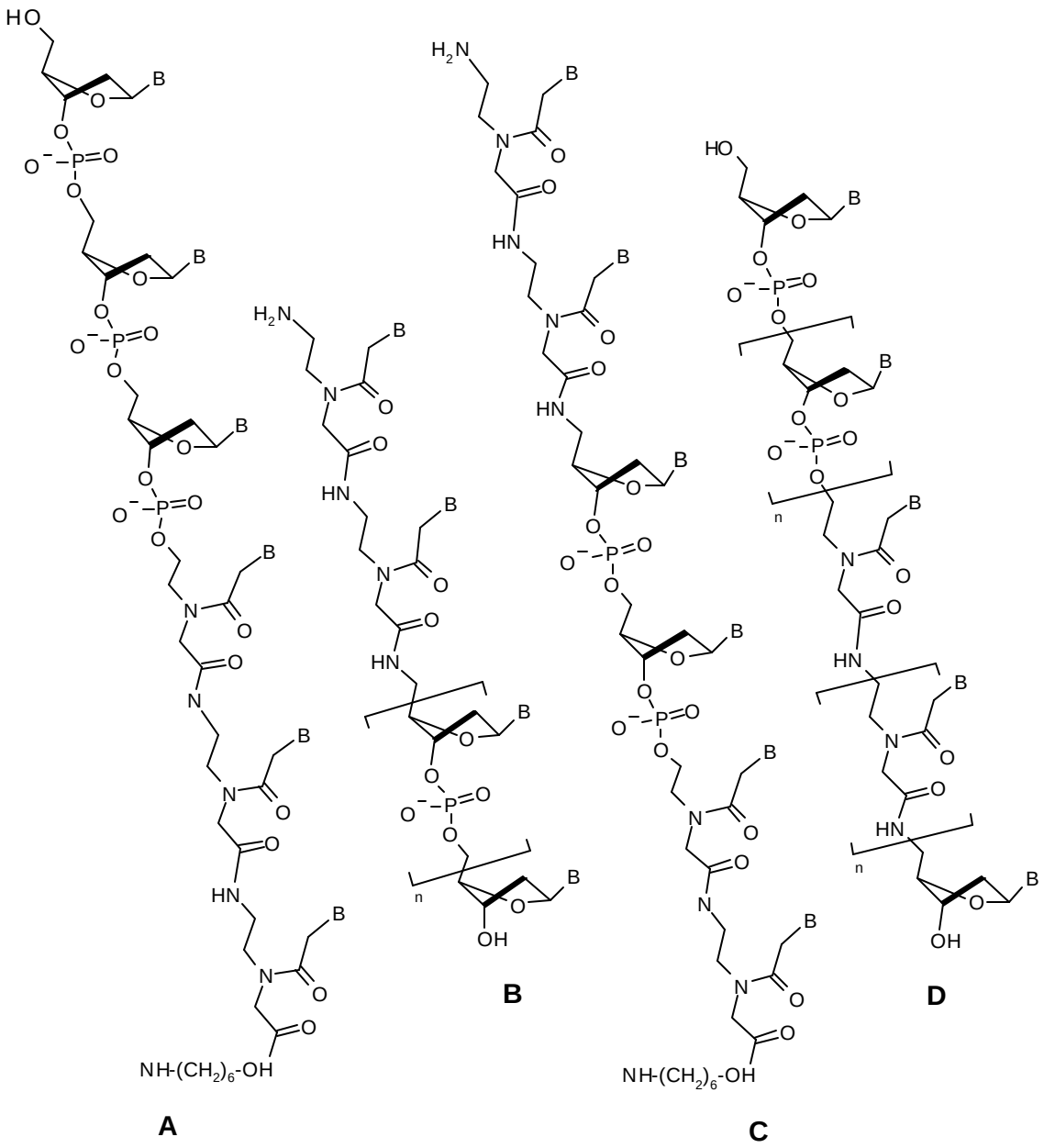


Figure 3. Different types of PNA-DNA chimeras. (A) 5'-DNA-3'-PNA (B) H-PNA-5'-DNA-3' (C) H-PNA-5'-DNA-3'-PNA (D) 5'-DNA-3'-PNA-5'-DNA-3'.

To overcome such limitations, many PNA analogues and derivatives have been synthesized and their properties evaluated. Ambiguity in binding orientation of PNA with the target DNA sequence may lead to PNA hybridization at undesired sites. The combination of PNA and DNA in a single molecule results in PNA-DNA chimeras with

new properties. Different types of PNA-DNA chimeras are shown in Figure 3. These may have the PNA moiety conjugated (i) to the 3' end of DNA (A), (ii) at the 5'-end of the DNA (B), at the 3' and 5'-ends of DNA (C) or may be within a DNA oligomer (D). PNA-DNA chimeras show improved solubility in aqueous solution as compared to pure PNA due to the anionic nature of the DNA component. The PNA strand now assumes a directionality by virtue of the conjugated DNA and can be manipulated to serve as a substrate for DNA processing enzymes, e.g., as primers for DNA polymerases,[10] or to activate cleavage by RNase H upon binding to RNA.[6]

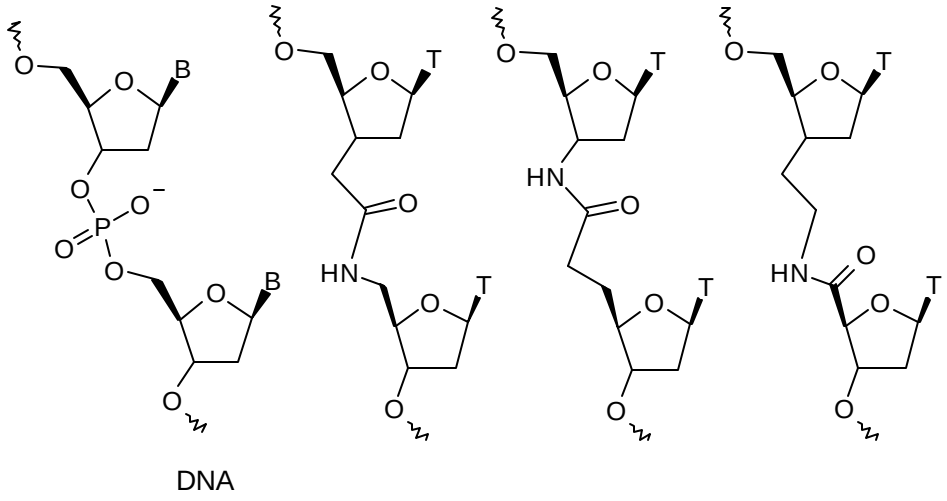


Figure 4. DNA and analogues of DNA having amide linkages.

The replacement of one phosphodiester linkage by an amide unit in oligonucleotides (Figure 4) has been reported and is considered superior in terms of the thermal stability of their complexes with complementary nucleic acids.[11] Further modifications involved changing the conformational flexibility of the backbone[12] as in **b-e**, Figure 5. These were incorporated into ODN sequences and their binding with complementary ODNs studied. The alkane linkage ('**b**') containing hybrids did not lead to duplex formation with its DNA complement. On the other hand, changing the hybridization of one carbon atom from sp^3 to sp^2, and thereby restricting the flexibility of

the linkage as in '**c'** led to an increased thermal stability of the duplexes compared to the very flexible modification '**b'**. The PNA:DNA chimera are also reported to form less stable complexes with the target DNA and the instability is attributed to the flexibility at the junction of these chimeric ODNs.[13]

The replacement of the acyclic PNA junction in the PNA-DNA chimera by a pyrrolidine ring may influence the hybridization properties due to geometric and/or entropic attributes exerted by the rigid cyclic-PNA-DNA junction. Further the protonation of the tertiary amine of the pyrrolidine ring at physiological pH may lead to electrostatic interactions with the anionic phosphate groups of the complementary strand. It may further change the hydration of the groove which alters the thermal stability of the hybrid.

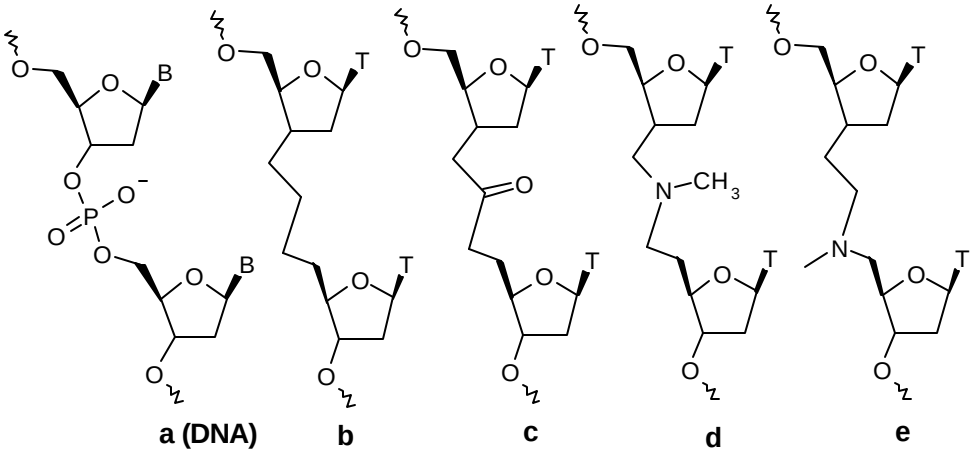


Figure 5. DNA and its modifications involving conformational flexibility.

4.2. OBJECTIVES

This chapter targets the synthesis of chimeric ODNs containing a pyrrolidine ring. The introduction of a positively charged pyrrolidine ring carrying the HN^{+}-CH_2-CO-NH-5' linkage for replacement of the sugar phosphate backbone in DNA (Figure 6) was expected to have advantages over the reported chimeric ODNs. The dimeric unit (Figure

6a) was chosen to have configurational equivalence to natural DNA. This corresponds to a D-*cis* configuration[14] for the pyrrolidine unit. Further, the design of the dimer was such as to enable its introduction into oligonucleotides using the standard 2-cyanoethyl-phosphoramidite methodology.

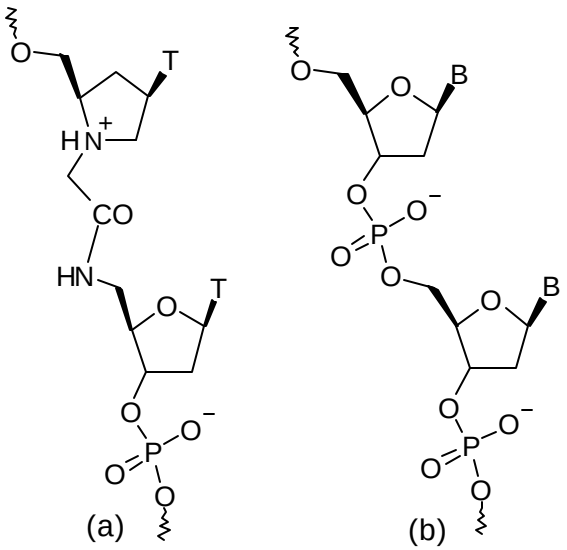
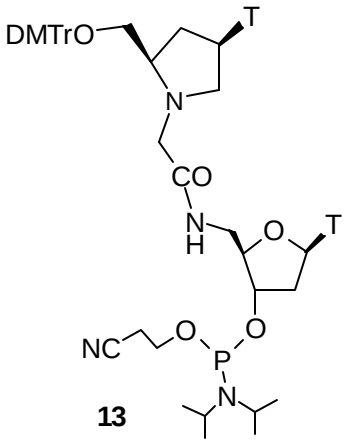


Figure 6. The structures of (a) PNA-DNA chimera and (b) DNA

The main objectives of this chapter are: To synthesize the dimer phosphoramidite **13** (t*T where '*' indicates the amide linkage and 't' indicates pyrrolidine component) and incorporate it into ODN sequences for carrying out the biophysical studies of the chimeric ODNs to examine their complementation properties.



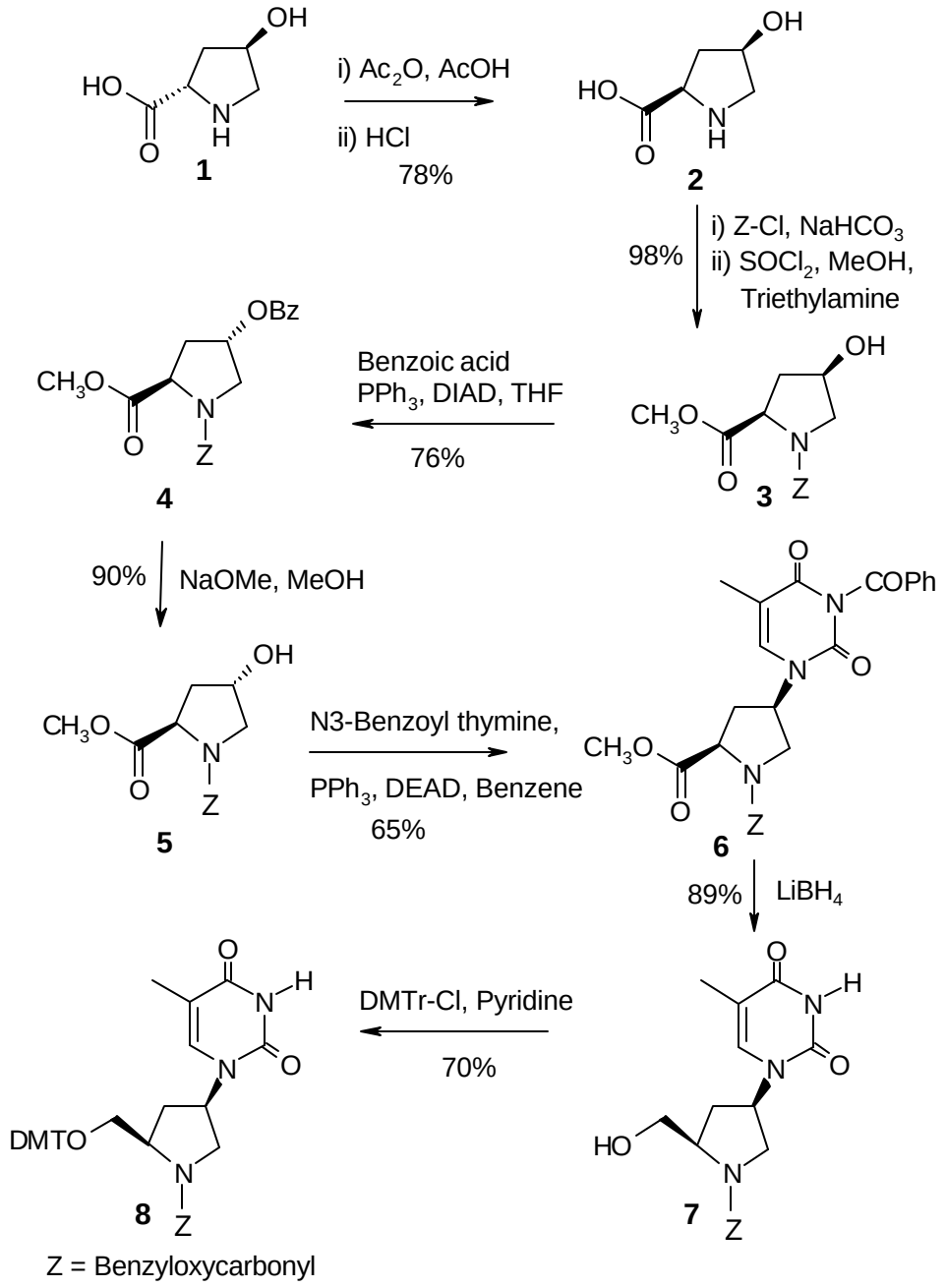
13

4.3. PRESENT WORK

*4.3.1. Synthesis of t*T Dimer Block-3'-O-Phosphoramidite 13*

The dimer phosphoramidite was prepared starting from the naturally occurring amino acid, *trans*-4(*R*)-hydroxy-2(*S*)-proline **1** as shown in Schemes 1 and 2. First the L-isomer (4(*R*)-hydroxy-2(*S*)-proline) was configurationally inverted at C2 to the D-isomer **2**. The inversion was achieved by treatment with acetic acid and acetic anhydride followed by the hydrolysis of the resulting lactone with 2N HCl to give *cis*-4(*R*)-hydroxy-2(*R*)-proline hydrochloride, **2** in good yields.[15] The purity of the D-isomer was evident from the ^{13}C NMR which exhibited single peaks for individual carbon atoms. The ring nitrogen in compound **2** was protected as the N-benzyloxycarbonyl derivative using benzylchloroformate in aqueous $NaHCO_3$. The resulting N-benzyloxycarbonyl-4(*R*)-hydroxy-2(*R*)-proline was esterified by treatment with $SOCl_2$ in methanol to get the methyl ester **3**. The ^1H NMR spectrum of **3** indicated the presence of aromatic and benzylic (C$\underline{H}_2$-O) protons of benzyloxycarbonyl at δ 7.30-7.20 and δ 5.20-5.00 respectively.

The methyl ester **3** was subjected to Mitsunobu reaction conditions to configurationally invert substitution at the C4 center and obtain the 4(*S*)-O-benzoyl derivative **4**. The structure was confirmed by ^1H NMR where the aromatic protons of the benzoyl group appeared at δ 8.05 & 7.70-7.45 accompanied by a downfield shift of H4 from δ 4.40 to δ 5.55, indicating the formation of the O4-benzoate. Solvolysis of the 4(*S*)-O4-benzoate **4** using sodium methoxide/MeOH afforded *trans*-4(*S*)-hydroxy-2(*R*)-proline methyl ester **5**. The removal of the benzoate ester was clear from the ^1H NMR spectrum of **5** which showed the disappearance of aromatic protons and the upfield shift of H4 from δ 5.55 to δ 4.55, while the methyl ester peak at δ 3.75-3.55 was retained. The identity of **5** was further supported by the ^{13}C NMR spectrum which retained the C=O

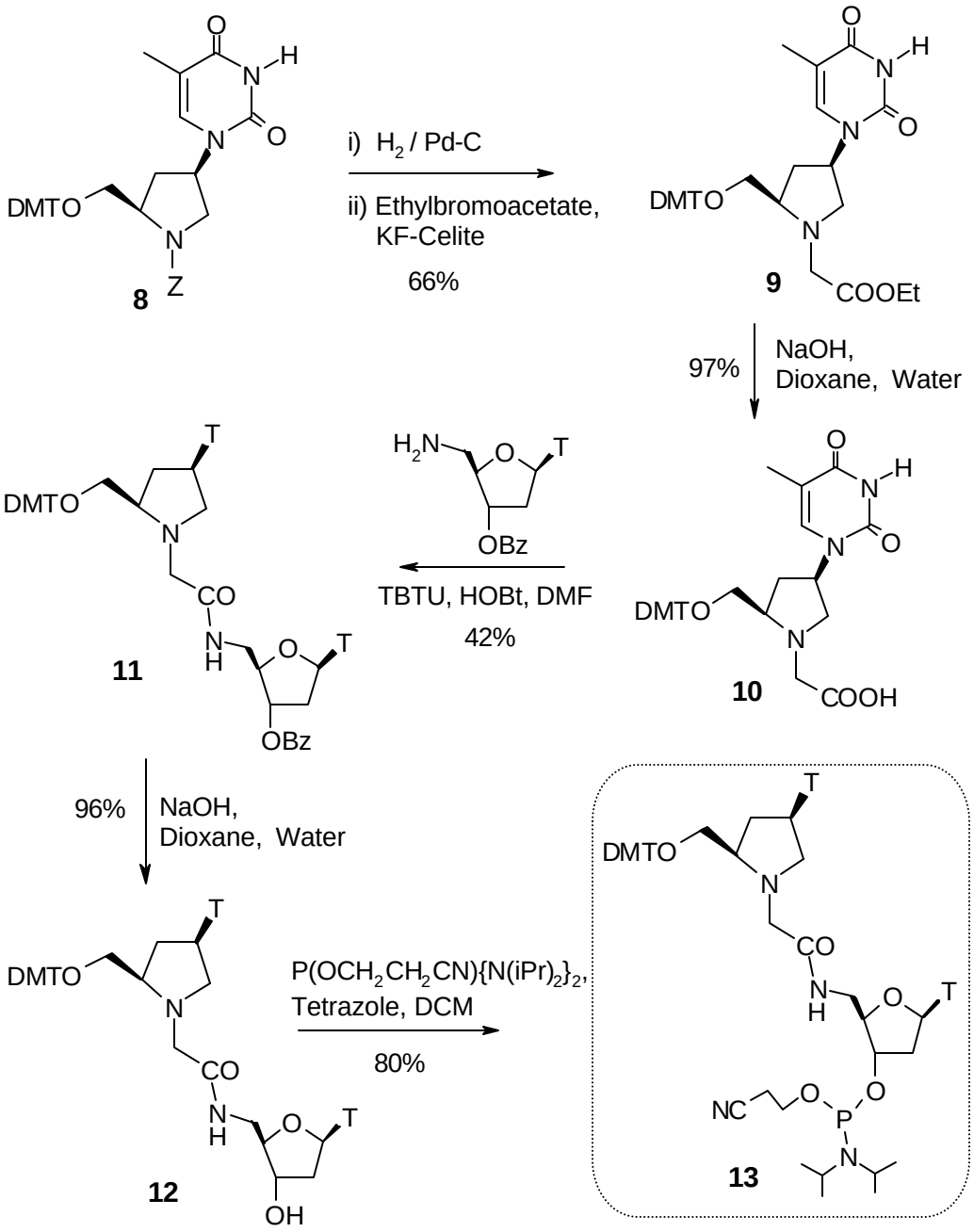


Scheme 1. Synthesis of N-Z-4(*R*)-(thymin-1-yl)-2(*R*)-dimethoxytrityloxymethyl pyrrolidine **8**.

resonance due to the methyl ester at δ 173.0. Compound **5** on treatment with N3-benzoylthymine under Mitsunobu conditions gave pyrrolidyl thymine **6**. The reaction proceeds with inversion of configuration at C4, to yield 4(*R*)-(N3-benzoylthymin-1-yl)-2(*R*)-proline methyl ester **6**. A downfield shift of the H4 multiplet in ^{1}H NMR from δ 4.55

to δ 5.40-5.10 and the appearance of a characteristic peak at δ 1.70 due to CH_3 of thymine confirmed the identity of the Mitsunobu product. The mass spectral analysis gave the expected M^+ ion peak at 491 in support of the structure. The reduction of the ester **6** with excess $LiBH_4$ afforded the alcohol **7** with simultaneous removal of the N3-benzoyl group of thymine. In the ^1H NMR spectrum of **7**, the upfield shift of H2 from δ 4.55 to δ 3.70 and the appearance of HO-C$\underline{H}_2$ at δ 3.80-3.50 indicated the successful reduction of the ester. The IR spectrum exhibited a broad band at 3390 cm^{-1} due to OH, while the disappearance of the ester stretching band at 1740 cm^{-1} was observed. The primary hydroxyl function in **7** was protected by treatment with dimethoxytritylchloride in pyridine to get the dimethoxytrityl ether, **8** the structure of which was confirmed by ^1H and ^{13}C NMR spectroscopy.

Compound **8** (Scheme 2) on hydrogenation over Pd-C for removal of N-benzyloxycarbonyl and subsequent alkylation of the deprotected pyrrolidine ring nitrogen with ethylbromoacetate provided the N-alkylated ester **9**. The ^1H NMR spectrum of **9** exhibited the methylene protons of N-C$\underline{H}_2$ at δ 3.40-3.00 and the ethyl quartet and triplet at δ 4.20 and δ 1.30 respectively. In the ^{13}C NMR, the N-alkylated group appeared as a set of distinct signals at δ 58.5 (N-$\underline{C}H_2$), 60.4 (-COO-$\underline{C}H_2$-), 12.0 (-COO-CH_2-$\underline{C}H_3$) & 170.4 (-$\underline{C}$OOEt). The hydrolysis of the ester group in **9** with aqueous NaOH in dioxane led to the acid **10**. The condensation of **10** with 3'-O-benzoyl-5'-amino-deoxythymidine prepared according to the literature method[16] in presence of *O*-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) yielded the dimer **11**, that was characterized by ^1H NMR and mass spectroscopy. In the ^1H NMR spectrum, characteristic signals of H1', H3' & H4' appeared at δ 6.25, 5.30 & 5.10 respectively. The mass spectral analysis of **11** gave the molecular ion at 936 (M^++Na). The hydrolysis of the 3'-O-benzoyl group of **11** using aqueous NaOH resulted in **12**, the ^1H NMR of which showed all the expected signals, the H1' of thymidine



Scheme 2. Synthesis of dimer phosphoramidite **13**.

appearing at δ 6.00, the C5 methyl groups of the two thymines separated at δ 1.80 and 1.60. The ^{13}C NMR signals were also supportive of the structure of the dimer. The amide C=O appeared at δ 169.7, the C5 methyls of the two thymines at δ 11.6 & 11.4 along with the other carbon signals. The mass spectrum showed the molecular ion at 831

(M^++Na) further confirming the structural authenticity of the product. Compound **12** on phosphitylation with 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite activated with tetrazole gave the protected chimeric dimer phosphoramidite **13** for site-specific incorporation into ODNs by automated solid phase synthesis. The phosphoramidite **13** showed two distinct signals at δ 149.50, 148.42 in the ^{31}P NMR spectrum due to diastereomeric phosphorous atoms.

To check the stability of **12** to oligonucleotide synthesis reagents and conditions, the dimer **12** was exposed to I_2/pyridine/H_2O/CH_3CN, the reagent used for oxidation in automated DNA synthesis, and the reaction monitored by TLC and ^{1}H NMR. The dimer remained unaffected even after exposure times longer than the actual DNA synthesis durations. On the other hand, treatment of **12** with NH_4OH at 55°C, gave products indicating hydrolysis of the amide linkage. The milder cleavage conditions using NH_4OH at room temperature (12h) did not affect the dimer, as indicated by ^{1}H NMR and TLC.

4.3.2. Synthesis of DNA-PNA Chimeric Oligonucleotides

The dimer phosphoramidite **13** was incorporated into ODN sequences at the desired positions by automated DNA synthesis to yield the chimeric ODNs **15-20**. The coupling yields of **13** were >90% as quantified by the released DMT cation. Since the dimer was susceptible to NH_4OH at 55°C, solid phase DNA protocols employed phenoxyacetyl (PAC) phosphoramidites that could be cleaved under milder NH_4OH conditions. The deoxyribonucleoside phosphoramidites having the PAC group[17] for protection of the exocyclic amino function of adenine and guanine, and the isobutyryl group for that of cytosine, were necessary to ensure rapid ODN deprotection with conc. NH_4OH at room temperature without affecting the amide linkage. The synthesized

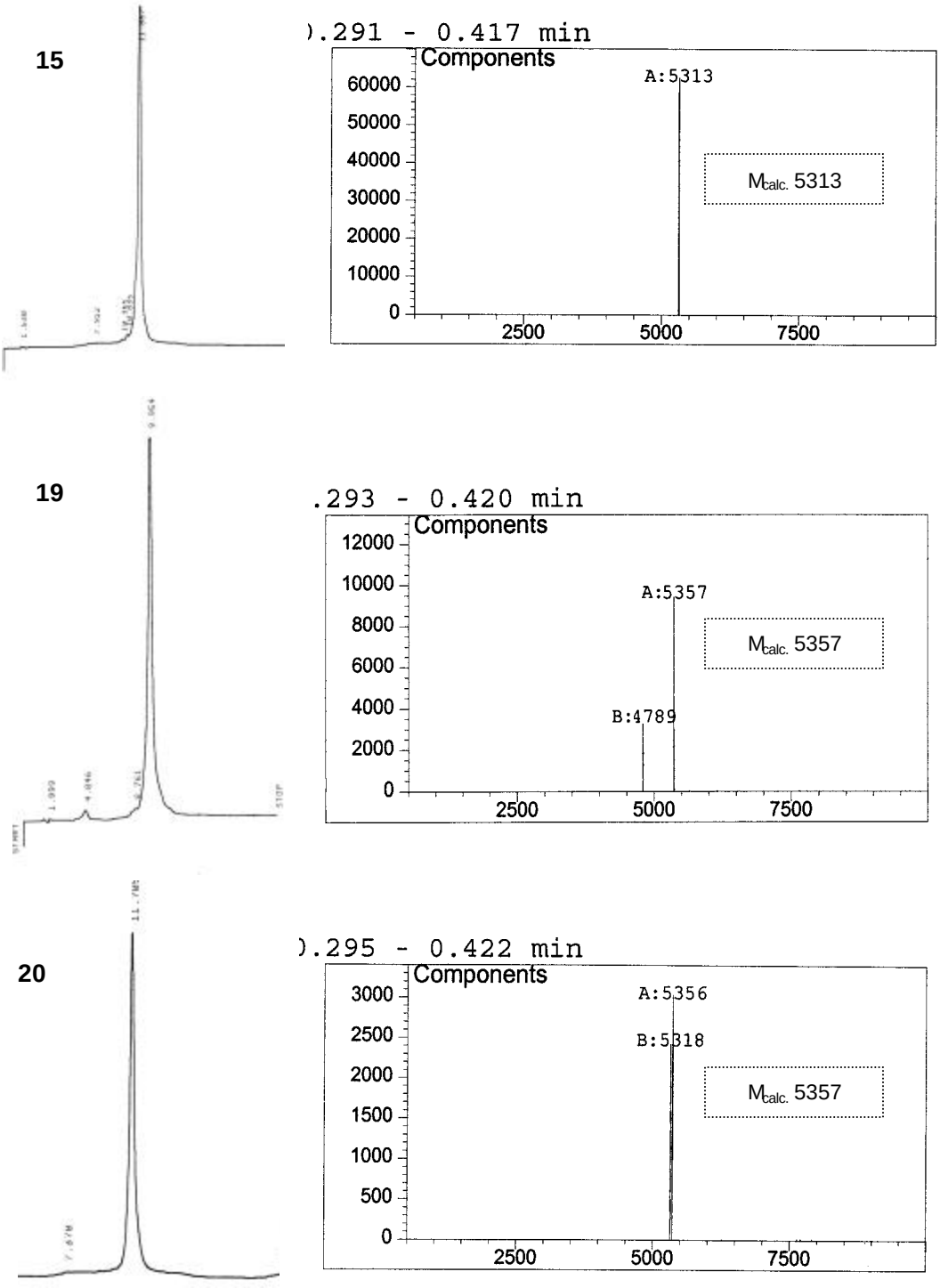
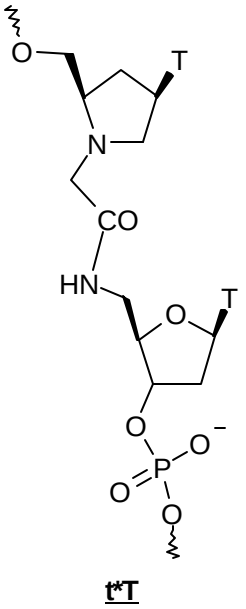


Figure 7. Representative RP-HPLC chromatograms of chimeric ODNs **15**, **19** and **20**. Inset shows the corresponding ES-MS spectra (negative-ion mode).

ODNs after on-column detritylation were cleaved from the solid support with conc. NH_4OH, lyophilized and desalted to get the crude ODNs. These were then purified by RP-HPLC (see experimental section for details) and characterized by negative ion electrospray mass spectrometry (Figure 7).[18]

14 T TC TTC TTC TTT TCT TTT
15 TTC TTC TTC TTT TCT **t*T**T
16 TTC TTC TTC **t*T**T TCT TTT
17 TTC TTC TTC **t*T**T TCT **t*T**T
18 CTT GTA CT**t*T**TC CGG TTT
19 CTT GTA C**t*T t*T**C CGG TTT
20 CTT GTA CT**t*T**TC CGG **t*T**T
21 AAA AGA AAA GAA GAA GAA
22 AAA CCG GAA AAG TAC AAG
23 CTT GTA CTT TTC CGG TTT
24 TCC AAG AAG AAG AAA AGA AAA TAT
25 ATA TTT TCT TTT CTT CTT CTT GGA
t*T = pyrrolidinamide dimer block



t*T

4.4. RESULTS AND DISCUSSION

4.4.1. Duplex Formation by Chimeric Oligonucleotides

The binding affinity of the chimeric oligonucleotides (**15-20**) to their complementary nucleic acid sequences was investigated by measuring the melting temperatures (T_ms) of the duplexes. The T_m experiments of duplexes were carried out in phosphate buffer (pH 7.0) containing 100 mM NaCl. The chimeric ODNs **15**-**17** and **18**-**20** were individually hybridized with the complementary DNA strands **21** and **22** respectively, to obtain duplexes while **14:21** and **23:22** served as the references. The observed sigmoidal transitions (Figure 8) indicated successful formation of duplexes.

The T_m values of various oligomers with the respective complementary DNA sequences are given in Table 1. Comparison of the difference in the T_m values (ΔT_m)

between the duplexes formed by the chimeras with the complementary DNA and the corresponding duplexes formed by the reference ODNs and the complementary strands indicate the degree of stability.

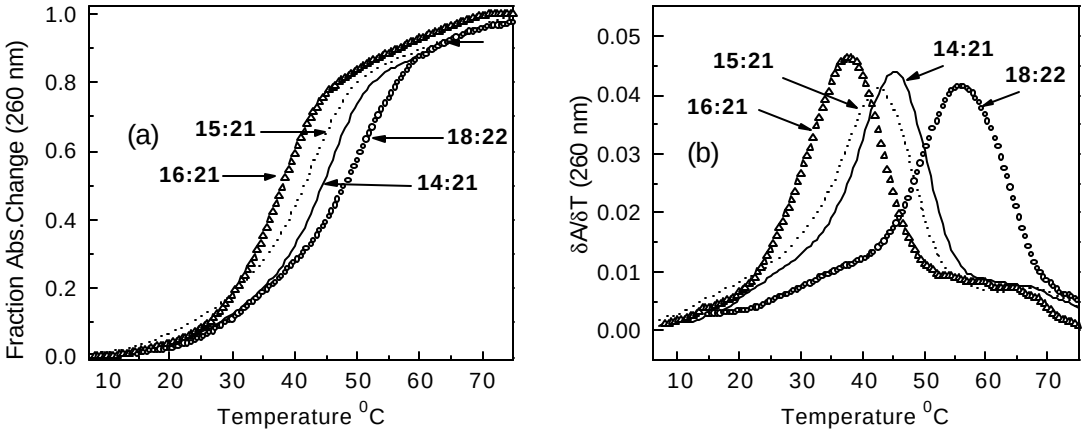


Figure 8. (a) Selected UV-melting profiles of duplexes and (b) their first derivative curves. Buffer: 10mM phosphate, 100mM NaCl, 0.1mM EDTA, pH 7.0. All Tms are accurate to $\pm 0.5^{o}$C and are measured in four melting experiments.

As compared to the control duplex **14:21** (Table 1, entry 1) the duplex with polypyrimidine ODN **15** carrying the amide-linked pyrrolidine-sugar dimer at the 3'-terminus exhibited a 3°C destabilization. When the dimer is present at the center as in **16:21**, a larger destabilization was observed (-7°C, entry 3). The duplex **17:21** having two dimer substitutions showed a Tm of 36°C with more destabilization (ΔT_m = -9°C, entry 4). In the above sequences, the dimer unit is present in a C/T polypyrimidine region and was found to cause destabilization. The results with duplexes of sequences **18-20** represent the consequences of dimer substitution in a mixed purine/pyrimidine sequence (entries 6-8). The duplex **18:22** with a single dimer unit at the center in a mixed pyrimidine-purine sequence also showed a destabilization of 6°C compared to the relevant control **23:22** (Table 1, entry 5). The duplex **19:22** containing two dimer units adjacent to each other in the center (entry 7) was destabilized to a greater extent (ΔT_m =

-12°C), while the introduction of a second dimer unit at the 3' terminus in addition to one central unit as in **20:22** effected a lesser destabilization of 7°C (entry 8).

Table 1. UV-Tm of chimeric duplexes[#]

Entry	Sequences	Complex	Tm °C	ΔTm °C
1	5'-T TC TTC TTC TTT TCT TTT 3'-AAG AAG AAG AAA AGA AAA	**14:21**	45.0	
2	5'-TTC TTC TTC TTT TCT **t*T** T 3'-AAG AAGAAGAAA AGA AAA	**15:21**	42.0	-3.0
3	5'-TTCTTC TTC **t*T** T TCT TTT 3'-AAGAAGAAG AAA AGAAAA	**16:21**	38.0	-7.0
4	5'-TTC TTC TTC **t*T** TT TCT **t*T** T 3'-AAG AAGAAG AAAAGA AAA	**17:21**	36.0	-9.0
5	5'-CTT GTA CTT TTC CGG TTT 3'-GAA CAT GAAAAG GCC AAA	**23:22**	57.0	
6	5'-CTT GTACT **t*T** TTC CGG TTT 3'-GAA CATGA AAAG GCC AAA	**18:22**	51.0	-6.0
7	5'-CTT GTA CT **t*T t*T** C CGG TTT 3'-GAA CAT GA AAAG GCC AAA	**19:22**	45.0	-12.0
8	5'-CTT GTA CT **t*T** TTC CGG **t*T** T 3'-GAA CAT GAAAAG GCC AAA	**20:22**	50.0	-7.0

[#]Buffer: 10mM phosphate, 100mM NaCl, 0.1mM EDTA, pH 7.0. ΔTm is the difference between the test Tm and the reference Tm; '-' sign indicates a destabilization. All Tms are accurate to ± 0.5°C and measured in four melting experiments.

It is seen that the chimeric sequences bind to the complementary DNA sequences with a relatively lower affinity (Table 1, entries 2-4, 6-8) than their control duplexes (entries 1 & 5 respectively). The dimer **13** though configurationally equivalent to natural DNA, perhaps induces structural deviations in the duplexes of oligomers **15-20** resulting in their destabilization as compared to the control, unmodified duplexes. Additionally, the dimer portion in the ODN binds to the complementary DNA part, but with lower binding strength possibly because of the unfavorable spatial orientation of the bases or the rigidity factor of the backbone.

The CD spectrum (Figure 9) of the chimeric duplexes **18:22** and **15:21** were very similar to that of the corresponding reference DNA duplexes **23:22** and **14:21**

respectively. All the duplexes displayed a minimum at 246 nm and a maximum at 220 nm with a cross-over point at ~230 nm. *This indicates that the native and chimeric dimers are adopting similar overall conformations.*

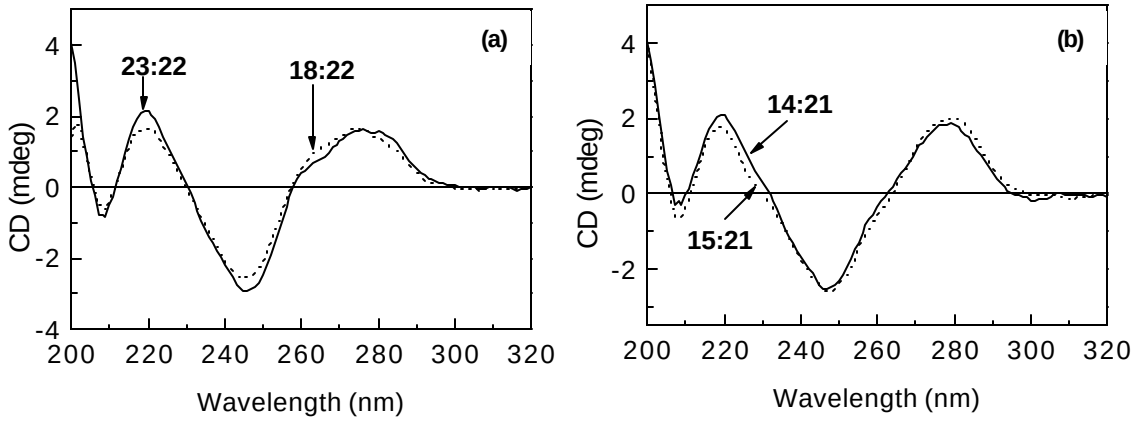


Figure 9. CD profiles of (a) chimeric duplex **18:22** and reference duplex **23:22** and (b) chimeric duplex **15:21** and **14:21**. Buffer: 10mM phosphate, 100mM NaCl, 0.1mM EDTA, pH 7.0.

4.4.2. Triplex Formation by Chimeric Oligonucleotides

The polypyrimidine ODN **14** and the chimeric oligomers **15**, **16** and **17** also form the third strand with the target duplex **24:25**. The thermal stabilities of the triplexes were

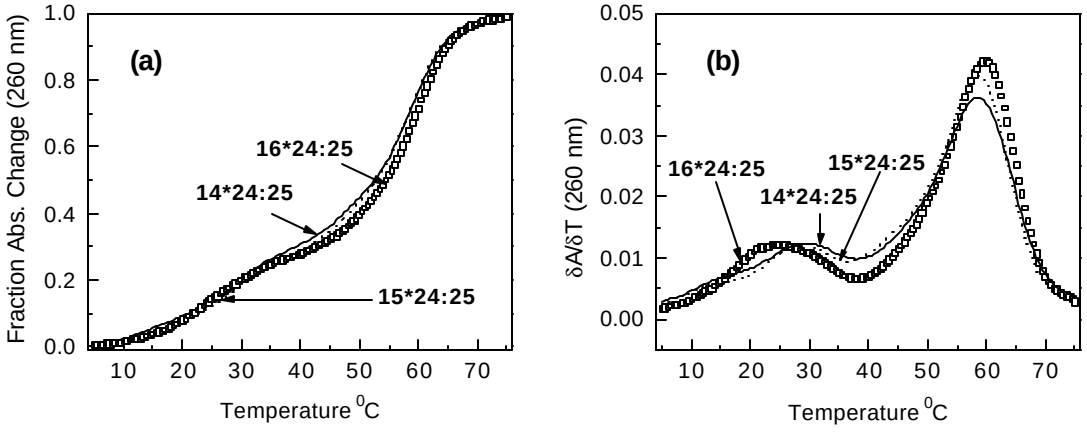


Figure 10. (a) Selected UV-melting profiles of triplexes and (b) their first derivative curves. Buffer: 10mM phosphate, 200mM NaCl, pH 7.0.

assessed by UV-Tm experiments in 10 mM phosphate buffer containing 200 mM NaCl at pH 7.0. Characteristic biphasic transitions in the melting curves were observed (Figure 10) which unambiguously indicated the formation of triplex, with the first transition (lower Tm) corresponding to the dissociation of the third strand **14**, **15**, **16** or **17** from the duplex **24:25** and the second transition (higher Tm) corresponding to the duplex melting.

It was seen that the stability of the triplex **15*24:25** with chimeric oligomer having the pyrrolidine dimer **13** at the 3'-end was similar to the corresponding unmodified triplex **14*24:25**. The triplex with third strand carrying the dimer in the center (**16*24:25**) exhibited a 4°C destabilization (Table 2, entry 3). This indicates that the modification is accommodated better at the 3' terminus compared to the modification at the center of the sequence. Again, the sequence **17** showed a further decreased triplex stability (**17*24:25**) with a ΔTm of -5.5°C, showing the lesser affinity of sequences with multiple modifications. Contrary to the expectation, the effect of protonated pyrrolidine in the dimer did not show any favourable triplex formation.

Table 2. UV-Tm of DNA triplexes[#]

Entry	Sequences	Complex	Tm °C	ΔTm(°C)
1	5'-T TC TTC TTC TTT TCT TTT 5'-TCC AAG AAG AAG AAA AGA AAA TAT 3'-AGG TTC TTC TTC TTT TCT TTT ATA	**14*24:25**	29.0	
2	5'-TTC TTC TTC TTT TCT **t*T** T 5'-TCC AAG AAG AAG AAA AGA AAA TAT 3'-AGG TTC TTC TTC TTT TCT TTT ATA	**15*24:25**	29.0	0
3	5'-TTC TTC TTC **t*T** T TCT TTT 5'-TCC AAG AAG AAG AAA AGA AAA TAT 3'-AGG TTC TTC TTC TT T TCT TTT ATA	**16*24:25**	25.0	-4
4	5'-TTC TTC TTC **t*T** T TCT **t*T** T 5'-TCC AAG AAG AAG AAA AGA AAA TAT 3'-AGG TTC TTC TTC TT T TCT TT T ATA	**17*24:25**	23.5	-5.5

[#]Buffer: 10 mM phosphate, 200 mM NaCl, pH 7.0. ΔTm is the difference between the test and the reference Tms; '-' sign indicates a destabilization. All Tms are accurate to ± 0.5°C and measured in four melting experiments.

4.4.3. Enzymatic Stability of Oligonucleotides

Snake venom phosphodiesterase (SVDPE) is an exonuclease and is known to cleave unmodified oligonucleotides from their 3'-end within 5-30 min at 37°C.[19] The stability towards enzymatic degradation is an important factor for using modified oligonucleotides in different biological systems. For example, modified ODNs containing an acyclic backbone or a replacement of the phosphate linkage, as in phosphorothioates and methylphosphonates were found to be nuclease resistant. The increase in absorbance (hyperchromicity) at 260 nm during the hydrolysis by nuclease can be followed as a function of time and the hyperchromicity half-life ($t_{1/2}$) evaluated.[20] The hypochromicity, a consequence of the base stacking of oligonucleotides and consequent Π-orbital overlap, is destroyed as the oligonucleotide is converted to its nucleotide constituents during the enzyme digestion. This leads to a hyperchromicity that is evident by an increase in the absorbance at 260 nm. The $t_{1/2}$ gives a measure of the resistance of oligonucleotides toward nucleases.

The unmodified oligonucleotides (**14, 23**) exhibited complete degradation when treated with SVDPE. The results are presented in Table 3 and some examples of hyperchromicity versus time plots are depicted in Figure 11. As shown, the 3'-end-modified chimeric oligomer **15** is significantly stabilized compared to the unmodified oligomer **14**. Results for the middle-modified chimeras (**18** & **19**) indicate that the nuclease rapidly digests the oligomers until a modification is reached whereupon further degradation is impeded. These results are in support of the resistance of the chimeric ODNs towards SVDPE and suggest that incorporation of such dimer units at the 3' or 5'-end may enhance their utility without causing destabilization.

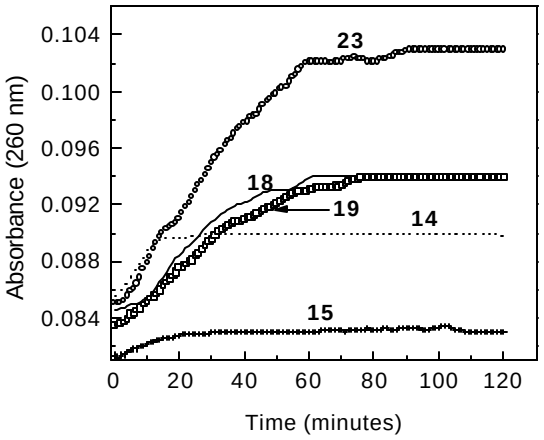


Figure 11. The event of SVDPE digestion of oligonucleotides **14, 15, 18, 19, 23** in TRIS buffer (100mM TRIS, 100mM NaCl, 15 mM Mg2+, pH 8.5)

Table 3. Enzymatic digestion of ODNs.

ODN	$t_{1/2}$(min)
14	<5
15	>12
18	>30
19	>30
23	<25

The table presenting $t_{1/2}$ of ODNs subjected to SVDPE digestion.

4.5. CONCLUSIONS

The automated synthesis of novel DNA-PNA chimeras with standard automated DNA synthesis is demonstrated. The synthesized oligomers were characterized by electrospray mass spectroscopy. The binding studies show that the chimera have a detrimental effect on duplex stability, but are better tolerated in triplex formation. Unlike PNAs, the DNA-PNA chimeras investigated in this section bind only in the antiparallel orientation to their complementary sequences. In addition, the chimeric ODNs have significantly improved enzymatic stability.

The presence of positive charge on pyrrolidine PNA may also favour cell permeation as seen in the case of cationic peptide-PNA conjugates.[21] Further work has to be carried out to delineate the binding preferences of stereochemically divergent dimer units incorporated into the chimera.

The (2*R*,4*R*) stereochemistry used for the pyrrolidine-sugar thymine dimer unit was expected to be compatible with DNA geometry,[14] the nucleobase being *cis* to the

aminomethyl segment of the backbone, as the *cis*-hydroxymethyl in the DNA sugar. However, the pyrrolidine ring nitrogen being positively charged, might adopt a ring pucker that results in a nucleobase orientation detrimental to duplex formation with the complementary DNA. This modification, even though structurally similar to the natural sugar-phosphate backbone, needs further refinement, e.g. having an additional methylene group or a hetero atom between the pyrrolidine N and amide C=O, for attaining better triplex forming abilities. Further studies on the effective stereochemical preferences exerted by these units on the DNA/RNA binding properties are necessary.

4.6. EXPERIMENTAL

4.6.1. Synthesis of Compounds 2-13

(2R,4R)-cis-4-Hydroxy-D-proline ***2***[15]

To *trans*-4-hydroxy-L-proline **1** (10 g, 76.3 mmol) was added acetic anhydride (75 mL) and acetic acid (150 mL) in one portion and the contents were refluxed for 6h. The mixture was cooled and excess of acetic acid and acetic anhydride were removed under vacuum to get a thick oil. The residue was refluxed with 2N HCl (190 mL) for 3h. The reaction mixture was treated with activated charcoal, filtered through celite and the filtrate was concentrated when crystallization began. The crystals were filtered out under suction, washed with ether and dried to get **2** (9.95 g, 78%).

m.p. 150°C. $[\alpha]_D^{25}$ +58.4 (c = 2, H_2O).

^{13}C NMR (D_2O): δ 172.7, 69.8, 59.1, 54.4, 37.6.

N-Benzyloxycarbonyl-4(R)-hydroxy-2(R)-proline methyl ester ***3***

To a solution of 4(*R*)-hydroxy-2(*R*)-proline, **2** (4.5 g, 26.8 mmol and $NaHCO_3$ (8 g, 96.7 mmol) in water (30 mL) was added benzylchloroformate (11 mL of 50% solution in

toluene, 32.2 mmol) dropwise and stirred at room temperature. After completion of the reaction as indicated by TLC (8h), the solvent was removed under vacuum and the residue dissolved in water (30 mL), washed with ether (2 x 10 mL). The aqueous layer was acidified with 4N HCl and extracted with ethyl acetate (3 x 75 mL) to get the pure product (6.67 g, 93%). This was dissolved in methanol, triethylamine was added followed by $SOCl_2$ after cooling the reaction mixture in an ice bath. The solution was stirred overnight, concentrated and dissolved in ethyl acetate (100 mL), washed with water (20 mL). The aqueous layer was back extracted with ethyl acetate (20 mL). The organic layers were pooled, dried over anhy. Na_2SO_4 to get the pure ester, **3** (7.27 g, 98%).

^{1}H NMR $CDCl_3$ δ: 7.50-7.20 (m, 5H, ArH), 5.20-5.00 (m, 2H, -O-CH_2-Ph), 4.50-4.30 (m, 2H, C4H and C2H), 3.80-3.50 (m, 5H, -OCH_3, C5H, C5'H), 2.4-2.2 (m, 1H, C3H), 2.2-2.0 (m, 1H, C3'H).

m.p. 80-82°C. TLC: (light petroleum:EtOAc 1:1) R_f 0.35.

N-Benzyloxycarbonyl-4(S)-O-benzoyl-2(R)-proline methyl ester ***4***

To a stirred solution of N-benzyloxycarbonyl-4(*R*)-hydroxy-2(*R*)-proline methyl ester **3** (4.8 g, 17.2 mmol), benzoic acid (2.61 g, 21.4 mmol) and triphenyl phosphine (5.6 g, 21.4 mmol) in dry THF (50 mL) at 0°C, was added dropwise diisopropylazodicarboxylate (DIAD, 4.2 mL, 21.4 mmol). After completion of the reaction as indicated by TLC (4h), the solvent was removed in vacuum and the residue purified by column chromatography (silica gel, light petroleum/EtOAc) to get the pure benzoyl ester **4** (5 g, 76%).

m.p. 75-76 °C. TLC: (light petroleum:EtOAc 1:1) R_f 0.60.

^{1}H NMR, $CDCl_3$ δ: 8.00 (m, 2H, Bz), 7.60-7.50 (m, 1H, Bz), 7.50-7.40 (m, 2H, Bz), 7.30 (s, 5H, Bn), 5.55 (bs, 1H, H4), 5.25-5.00 (m, 3H, O-CH_2-Ph, C5H), 4.70-4.50 (m, 1H,

C5'H), 3.95-3.85 (m, 1H, H2), 3.80-3.55 (m, 3H, OCH_3), 2.60-2.50 (m, 1H, C3H), 2.40-2.20 (m, 1H, H3').

N-Benzyloxycarbonyl-4(S)-O-hydroxy-2(R)-proline methyl ester ***5***

To compound **4** (3 g, 7.8 mmol) in dry methanol (50 mL) was added NaOMe (2.2 g, 40 mmol) and the reaction mixture was stirred at room temperature for 20 min when TLC indicated completion of reaction, the reaction mixture was acidified with $KHSO_4$ solution. The solution was concentrated and purified using column chromatography (silica gel, light petroleum/ EtOAc) to obtain **5** (2.0 g, 90%).

m.p. 85-86 °C. TLC: (light petroleum:EtOAc 1:1) R_f 0.40.

^{1}H NMR, $CDCl_3$ δ: 7.40-7.20 (m, 5H, ArH), 5.20-4.95 (m, 2H, -O-CH_2-Ph), 4.55-4.40 (m, 2H, H4, H2), 3.75-3.50 (m, 5H, -OCH_3, H5 & H5'), 3.00-2.50 (b, 1H, -OH), 2.40-2.20 (m, 1H, H3), 2.15-2.00 (m, 1H, H3').

^{13}C NMR, $CDCl_3$ δ: 173.0, 172.9 ($COOCH_3$), 154.9, 154.4 (CO-O-CH_2-Ph), 136.0, 135.8, 128.0, 127.4, 69.3 & 68.6 (OCH_3), 66.9 (CH_2Ph), 57.7 & 57.4 (C4), 54.7 (C5), 51.9, 51.7 (C2), 38.7, 37.9 (C3).

N-Benzyloxycarbonyl-4(R)-(N3-benzoyl-thymin-1-yl)-2(R)-proline methyl ester ***6***

To a stirred solution of N-benzyloxycarbonyl-4(*S*)-hydroxy-2(*R*)-proline methyl ester **5** (1.4g, 5mmol), N3-benzoylthymine (2.3 g, 10 mmol) and triphenyl phosphine (2.62 g, 10 mmol) in dry benzene (20 mL) at 0°C temperature, was added dropwise diethylazodicarboxylate (DEAD, 1.6 mL, 10 mmol). After completion of the reaction as indicated by TLC (8h), the solvent was removed in vacuum and the residue purified by column chromatography (silica gel, light petroleum/ EtOAc) to get the pure product **6** (1.6 g, 65%) as a gum.

TLC: (light petroleum:EtOAc 1:1) R_f 0.50.

^{1}H NMR $CDCl_3$ d: 8.00-7.30 (m, 11H, ArH, H6-Thy), 5.40-5.10 (m, 3H, H4 & O-CH_2-Ph), 4.60-4.40 (bs, 1H, H2), 4.15-3.90 (m, 1H, H5), 3.90-3.60 (m, 4H, OCH_3 & H5'), 2.90-2.70 (m, 1H, H3), 2.30-2.10 (m, 1H, H3'), 1.70 (s, 3H, Thy-CH_3).

*N-Benzyloxycarbonyl-4(R)-(thymin-1-yl)-2(R)-hydroxymethyl pyrrolidine **7***

To a solution of **6** (0.69 g, 1.41 mmol) in dry THF (20 mL) at 0°C, $LiBH_4$ (60 mg, 2.82 mmol) was added and stirred at room temperature. The reaction was monitored by TLC (16h), and upon completion, quenched the reaction using saturated NH_4Cl and concentrated to dryness. The residue was extracted with EtOAc/MeOH (80:20), concentrated and purified by column chromatography (silica gel, light petroleum/EtOAc) to get **7** (0.45 g, 89%).

m.p. 70-72 °C. TLC: (EtOAc) R_f 0.45.

^{1}H NMR, $CDCl_3$ + DMSO-d_6 (1:1) d: 10.60 (s, 1H, N3H-Thy) 7.10 (s, 1H, H6-Thy), 7.00 (s, 5H, ArH), 4.80-4.70 (m, 3H, CH_2-Ph & H4), 3.80-3.50 (m, 3H, H5, -CH_2OH), 3.80-3.60 (m, 1H, H2), 3.40-3.25 (m, 1H, H5'), 2.20-1.70 (m, 2H, H3, H3'), 1.52-1.48 (2s, 3H, Thy-CH_3).

*N-Benzyloxycarbonyl-4(R)-(thymin-1-yl)-2(R)-dimethoxytrityloxymethyl pyrrolidine **8***

Compound **7** (0.34 g, 0.94 mmol) was dried by co-evaporation with dry pyridine (2 x 10 mL), re-dissolved in dry pyridine (5 mL). DMTr-Cl (0.4 g, 1.2 mmol) was added to the above solution and stirred at room temperature for 8h. The reaction mixture was concentrated to dryness, dissolved in dichloromethane (30 mL) and washed with $NaHCO_3$ solution. Concentration of the organic fraction gave the crude product which after column chromatography (silica gel, CH_2Cl_2:EtOAc:Et_3N 8:1.5:0.5) afforded the compound **8** (0.44 g, 70 %) as a light yellowish foam.

m.p. 71-72°C. TLC: (CH_2Cl_2:MeOH 0.5:9.5) R_f 0.40. $[\alpha]_D^{25}$ +185.1 (c = 1.5, $CHCl_3$).

^{1}H NMR, $CDCl_3$ δ: 8.60 (d, 1H, H6-Thy) 8.50-8.40 (bs, 1H, N-H), 7.50-7.10 (m, 14 H, Ar-H), 6.90-6.70 (d, 4H, Ar-H), 5.30-5.00 (m, 3H, CH_2-Ph, H4), 4.25-4.00 (m, 2H, O-CH_2), 3.80 (s, 6H, OCH_3), 3.60-3.35 (m, 2H, H5, H5'), 3.20-3.00 (m, 1H, H2), 2.60-2.40 (m, 1H, H3), 2.20-2.00 (m, 1H, H3'), 1.70 (s, 3H, Thy-CH_3).

N-Ethoxycarbonylmethyl-4(R)-(thymin-1-yl)-2(R)-dimethoxytrityloxymethyl pyrrolidine ***9***

To a solution of **8** (0.42 g, 0.8 mmol) in MeOH:EtOAc (1:1,15 mL), was added 10% Pd-C (0.05 g) and hydrogenated under 50 psi of H_2 for 12h. The mixture was filtered through a celite pad and concentrated to get the free amine. The amine was dissolved in DMF:CH_3CN (1:1, 6 mL), KF-celite (50%, 0.32 g) and ethylbromoacetate (0.1 mL, 0.96 mmol) were added and the reaction was stirred at room temperature. After completion of the reaction as indicated by TLC (8h), the reaction mixture was filtered through a celite-pad, the residue was washed with CH_3CN, the filtrate pooled and concentrated to get the product which was purified by column chromatography (silica gel, CH_2Cl_2:MeOH) to get pure **9** (0.32 g, 66%).

TLC: (CH_2Cl_2:MeOH 0.5:9.5) R_f 0.50. $[\alpha]_D^{25}$ -42.2 (c = 0.4, $CHCl_3$).

^{1}H NMR, $CDCl_3$ δ: 9.50-9.20 (bs, 1H, N-H), 8.25 (s, 1H, H6-Thy), 7.50-7.15 (m, 9H, Ar-H), 6.90-6.70 (d. 4H, Ar-H), 5.15-4.95 (m, 1H, H4), 4.30-4.10 (q, 2H, O-CH_2), 3.90-3.80 (m, 1H, H2), 3.75 (s, 6H, OCH_3), 3.50-3.00 (m, 4H, O-CH_2, N-CH_2), 2.90-2.60 (m, 2H, H5, H5'), 2.60-2.40 (m, 2H, H3, H3'), 1.90-1.70 (s, 3H, Thy-CH_3), 1.40-1.20 (t, 3H, CH_3).

^{13}C NMR, CDCl3 δ: 170.4 ($COOCH_3$), 164.0 (C4), 158.4 (C2), 151.3 (C6), 144.6, 138.4, 135.7, 129.8, 127.9, 127.5, 126.5, 112.9, 110.5 (C5), 86.1, 63.8 (C6'), 61.9 (C4'), 60.4 (O-CH_2-CH_3), 58.5 (N-CH_2), 54.9, 53.9 (C5'), 52.3 (C2'), 35.3 (C3'), 13.9 (CH_3-Thy), 12.1 (O-CH_3).

*N-Carboxymethyl-4(R)-(thymin-1-yl)-2(R)-dimethoxytrityloxymethyl pyrrolidine **10***

The ethyl ester **9** (0.28 g, 0.44 mmol) was taken in dioxane (1 mL) and 2N aqueous NaOH (1 mL) was added. Stirring was continued for 30 min, after which, the excess alkali was neutralized with $NaHSO_3$ solution, and the resulting mixture was extracted with chloroform. The organic layer was concentrated under vacuum to get the product **10** in good yield (0.26 g, 97%).

^{1}H NMR, $CDCl_3$ δ: 9.30-9.00 (bs, 1H, NH), 8.25 (s, 1H, H6-Thy), 7.40-7.10 (m, 9H, ArH), 6.90-6.70 (d, 4H, ArH), 5.10-4.90 (m, 1H, H4), 3.90-3.80 (m, 1H, H2), 3.75 (m, 6H, OCH_3), 3.50-3.00 (m, 4H, O-CH_2, N-CH_2), 3.00-2.30 (m, 4H, H5, H5', H3, H3'), 1.80-1.70 (2s, 3H, Thy-CH_3).

*3'-Hydroxy-t*T-Dimer **12***

To compound **10** (0.26 g, 0.42 mmol) in DMF (2 mL) TBTU (0.1 g, 0.31 mmol), triethylamine (0.27 mL, 1.8 mmol) and HOBt (0.112 g, 0.84 mmol) were added and stirred for 1h. To this was added 3'-O-benzoyl-5'-amino-5'-deoxythymidine[16] (0.16 g, 0.44 mmol) and stirred at room temperature for 24h when TLC showed the completion of reaction. The reaction mixture was concentrated to dryness, dissolved in $CHCl_3$ (30 mL) and washed with 5% solution of $NaHCO_3$ (10 mL). The organic layer was dried over anhy. Na_2SO_4 and concentrated to get the crude product. This was purified by column chromatography (silica gel, $CHCl_3$, MeOH) to get the pure product **11**. The 3'-*O*-benzoyl derivative, **11** was dissolved in dioxane (1 mL) and 2N aqueous NaOH (1 mL) was added. Stirring was continued for 30 min, after which, the excess alkali was neutralized with $NaHSO_3$ solution, and the resulting mixture was extracted with chloroform and the organic layer was concentrated to get the crude product. Column chromatography (silica gel, $CHCl_3$, MeOH) of the residue gave the product in moderate yield (0.16 g, 42%).

m.p. 115-120°C. TLC: ($CHCl_3$:MeOH 1:9) R_f 0.45.

MS (FAB): 831 (M^+ + Na).

^{1}H NMR, $CDCl_3$ δ: 8.60 (NHCO), 7.62 (s, 1H, Thy-H6), 7.40-7.20 (m, 11H, ArH, NH-Thy & H6-Thy), 6.75 (d, 4H, ArH), 6.04 (t, 1H, H1'), 5.05 (bs, 1H, C4H-pro.), 4.25 (bs, 1H, H3'), 3.85-3.50 (m, 9H, H5, H5', H4', -OCH_3), 3.50-3.20 (m, 4H, NCH_2, C5'H, C5''H) 3.00-2.60 (m, 2H, CH_2-O), 2.40-2.20 (m, 3H, H2', H2'', C3H-pro), 1.82 (s, 3H, CH_3-Thy), 1.67 (s, 3H, CH_3-Thy).

^{13}C NMR, $CDCl_3$ + Pyridine d_5 δ: 169.7 (CO amide), 163.8, 163.7 (2 x C4-Thy), 157.6 (2 x C2-Thy), 148.7, 148.5 (2 x C6-Thy), 148.3-112.2 (ArC), 109.8 (2 x C5), 85.3 (C1'), 84.5 (C4'), 70.7 (C3'), 62.8 (CH_2CO), 62.1 (C4-pro.), 58.3 (OCH_2), 55.7 (C5), 54.0 (OCH_3), 51.6 (C2-pro.), 40.5 (C5'), 38.9 (C2'), 34.4 (C3-pro.), 11.6 & 11.5 (2 x Thy CH3).

3'-O-(2-Cyanoethyl-N,N-diisopropylphosphoramido)-5'-O-(4,4'-dimethoxy)trityl-dimer ***13***

Compound **12** (0.16 g, 0.2 mmol) was dissolved in dry CH_2Cl_2 (1.5 mL) followed by the addition of tetrazole (4 mg, 0.09 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphorodiamidite (0.09 g, 0.30 mmol) and the reaction mixture was stirred at room temperature for 3h when the TLC indicated ~90% completion of reaction. The reaction mixture was then diluted with dichloromethane and washed with 10% $NaHCO_3$ solution. The organic phase was dried over anhy. Na_2SO_4 and concentrated to a foam. The residue was purified using flash column chromatography (silica gel, CH_2Cl_2/EtOAc/ Et_3N 1:1:0.05) to get the phosphoramidite **13** (0.16 g, 80%).

TLC: (EtOAc) R_f 0.5.

^{31}P NMR, $CDCl_3$ δ: 149.50, 148.42.

4.6.2. Oligonucleotide synthesis

The synthesis was carried out on a Pharmacia GA Plus automated synthesizer as described in Section 2.14.3 employing PAC amidites. The phenoxyacetyl and

isobutyryl protecting groups enable nucleoside deprotection in a shorter time.[17] The oligonucleotides **15-20** were synthesized using the phosphoramidite **13** along with other standard PAC-protected β-cyanoethylphosphoramidites. The oligonucleotides were purified by semi-prepaarative HPLC as described in Section 3.20.2.

4.6.3. UV-Tm Studies

Oligonucleotide duplexes were constituted by mixing together stoichiometric quantities (0.5 μmol) of the corresponding complementary strands in 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0. The samples were annealed and UV-Tm experiments carried out.

The various triplexes containing the respective triads (Table 2, page No. 251) were individually constituted by taking 0.5 μM each of the appropriate single strands based on the UV absorbance at 260 nm. The three constituents were taken in 10 mM sodium phosphate buffer containing 100 mM NaCl at pH 7.0 and were annealed. The thermal stability of triplexes was measured by following UV-Tm experiments.

4.6.4. CD

CD spectra were recorded on a Jasco J-715 spectropolarimeter. The CD spectra of the PNA:DNA complexes and the relevant single strands were recorded in sodium phosphate buffer, pH 7.0 (10 mM sodium phosphate, 100 mM NaCl and 0.1 mM EDTA at pH 7.0). The temperature of the circulating water was kept well below the melting temperature of the chimeric complexes, i. e., at 10°C.

The CD spectra of the duplexes were recorded as an accumulation of 8 scans from 320 to 200 nm using a 1 cm cell, a resolution of 0.1 nm, band-width of 1.0 nm, sensitivity of 2 mdeg, response 2 sec and a scan speed of 50 nm/min.

4.6.4. Enzymatic Stability of Oligonucleotides

To a solution of ODN (0.1-0.2 OD) in 2 mL of buffer (100mM TRIS, 100mM NaCl, 15 mM Mg^{2+}, pH 8.5) at 25°C, was added 0.5U of snake venom phosphodiesterase. During the enzymatic digestion, the increase in UV absorbance was monitored at 260 nm. The absorbance change versus time of the event was plotted from which the half-life of the ODNs were evaluated.

4.7. REFERENCES

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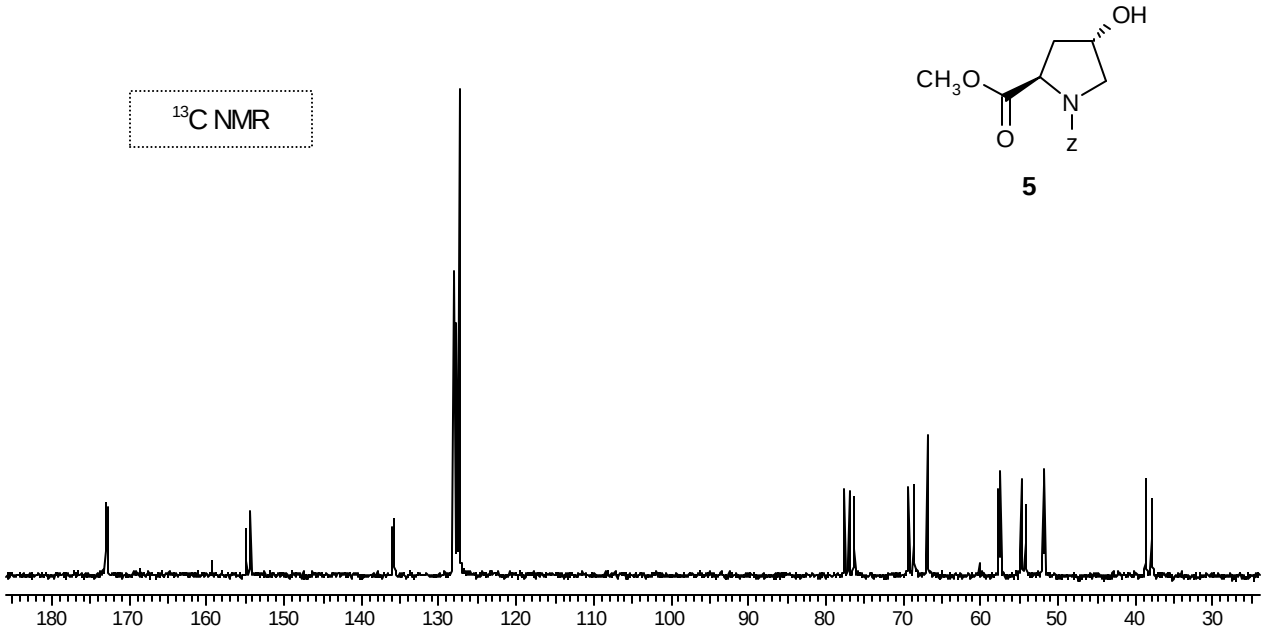
4.8. Appendix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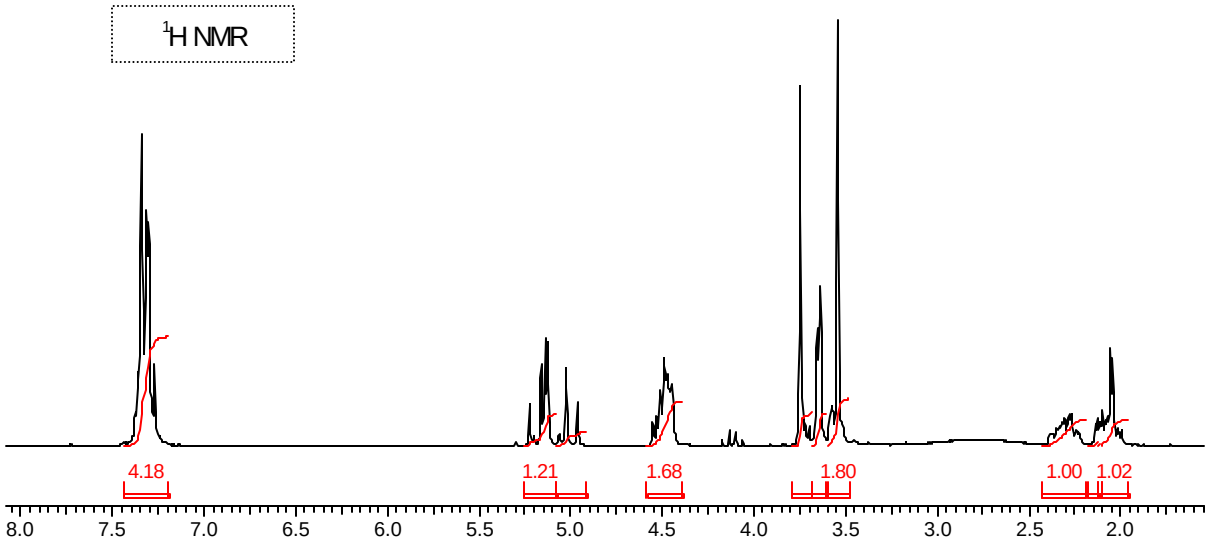
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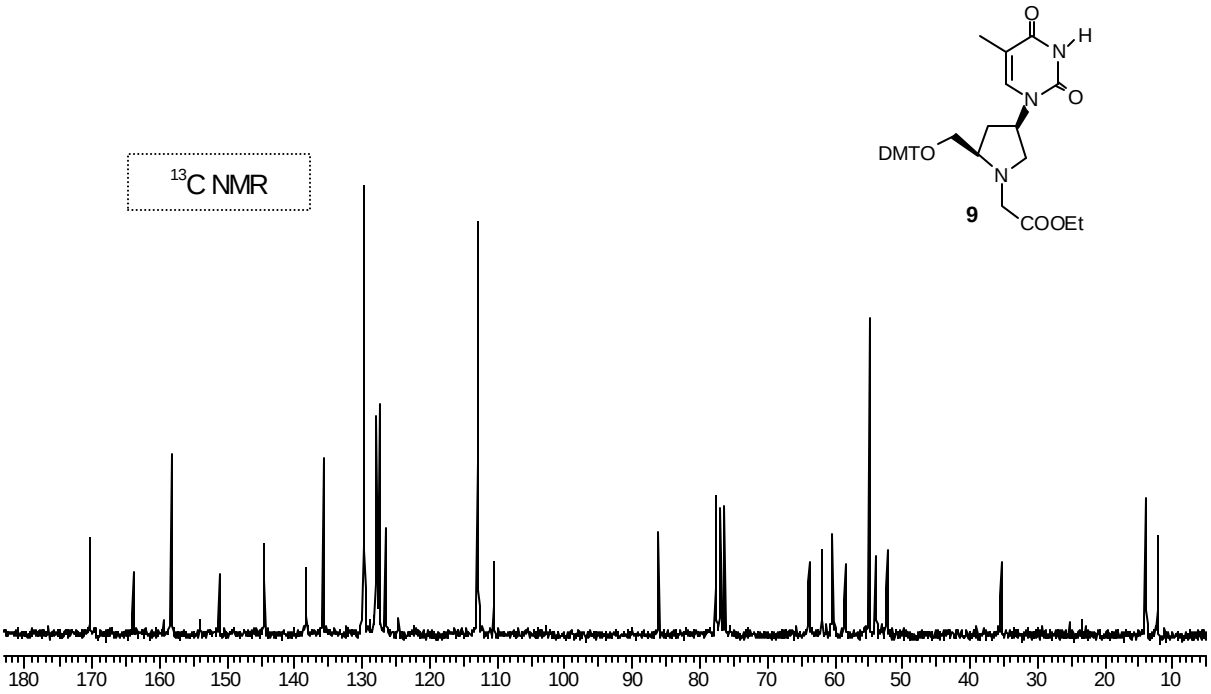
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H
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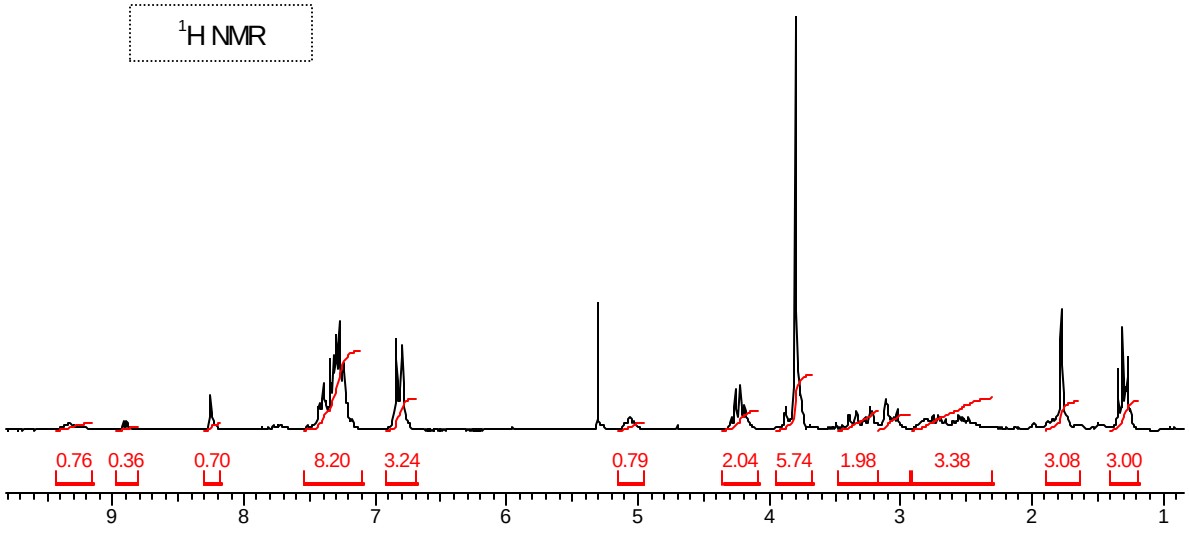
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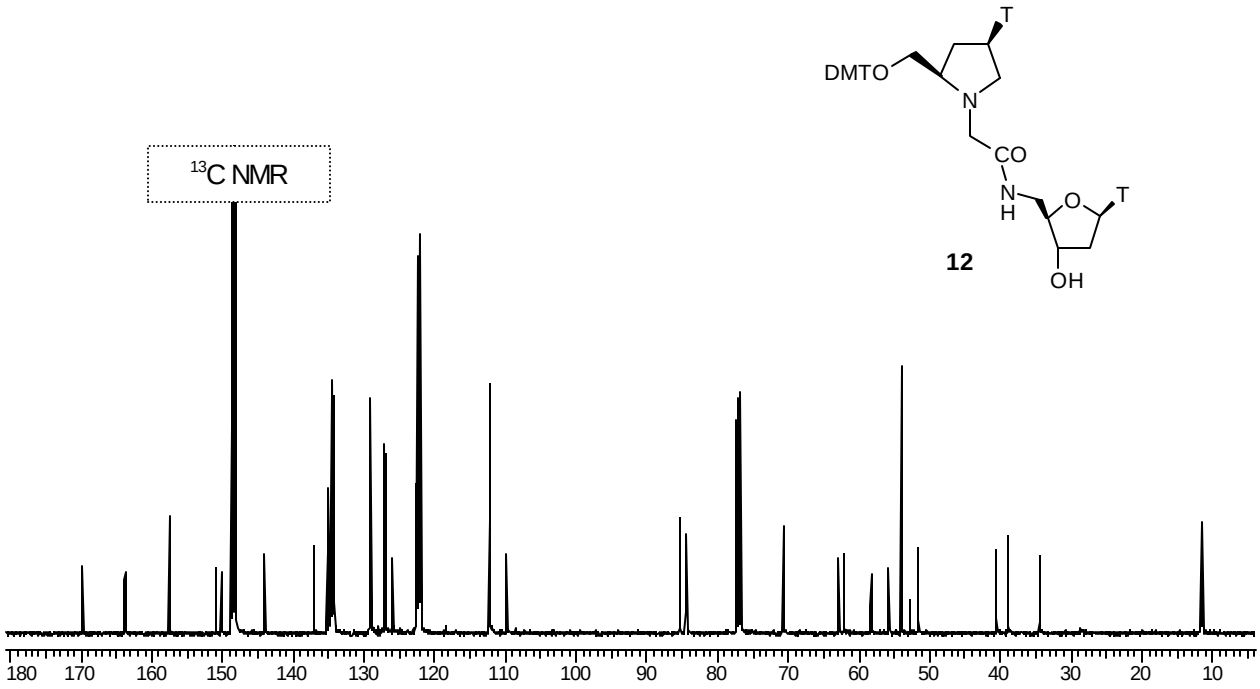
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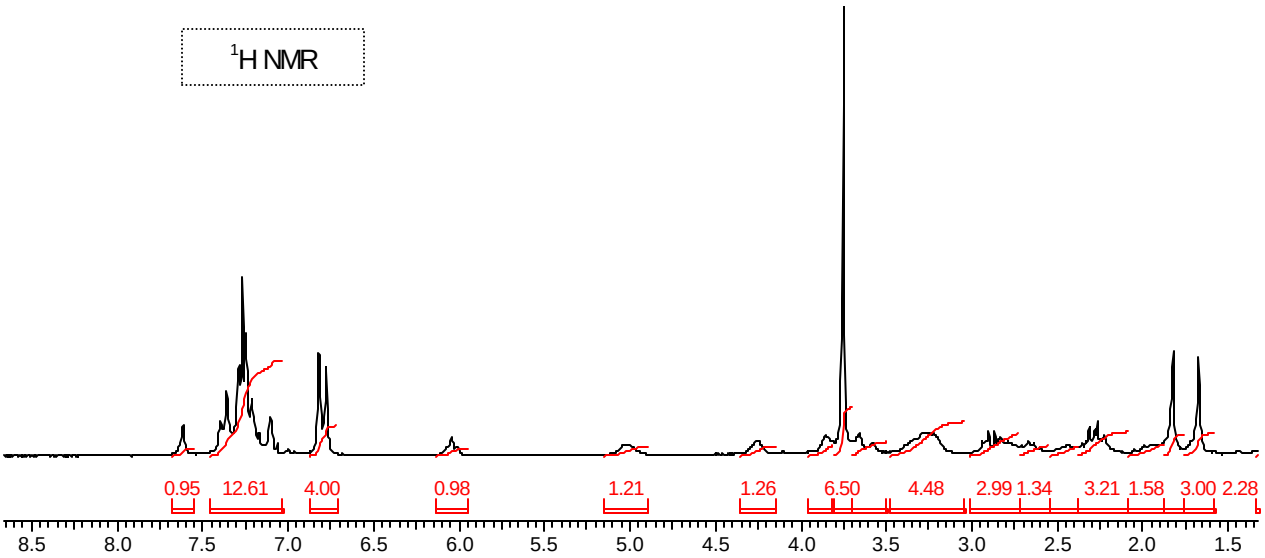
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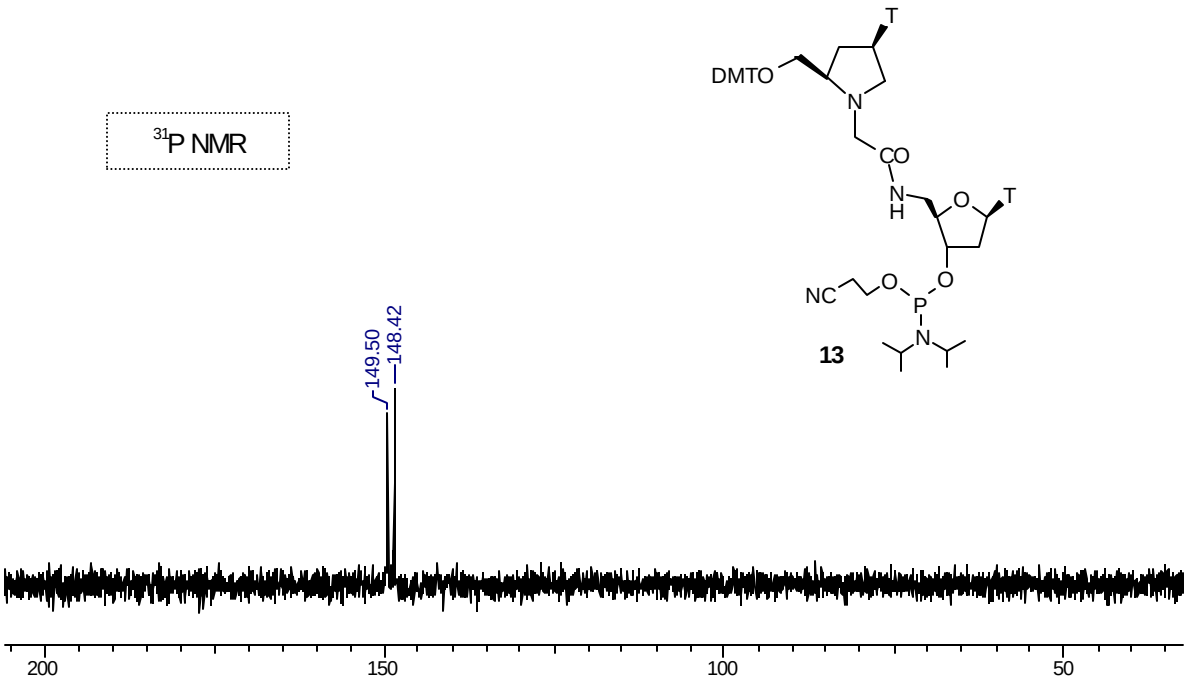
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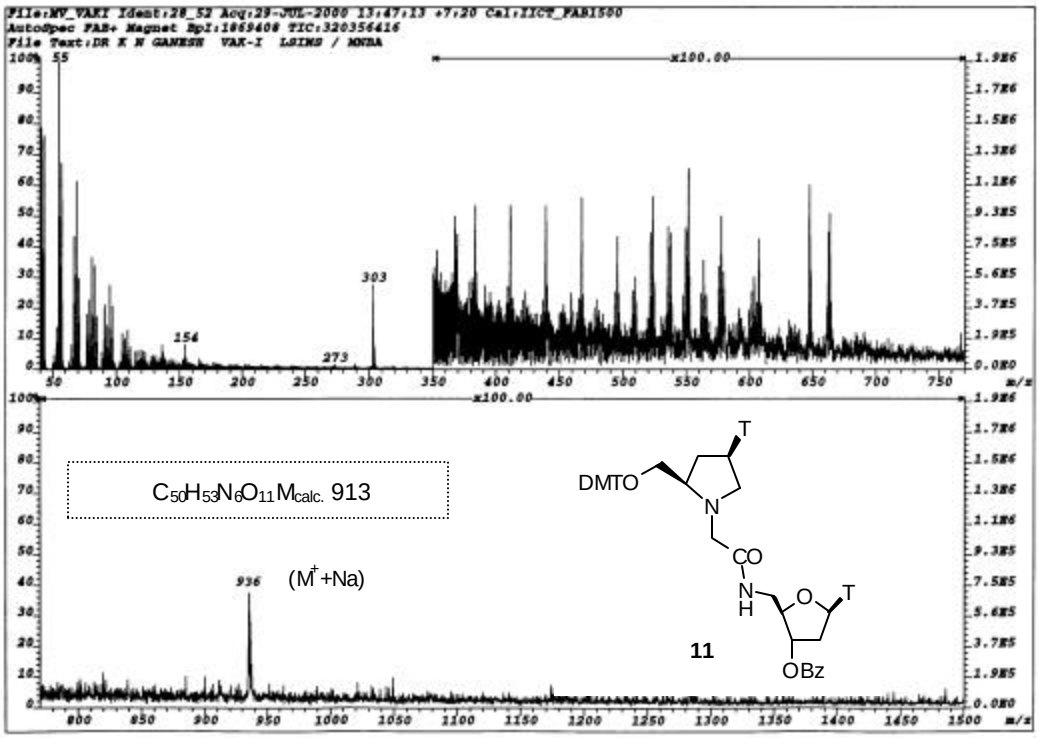
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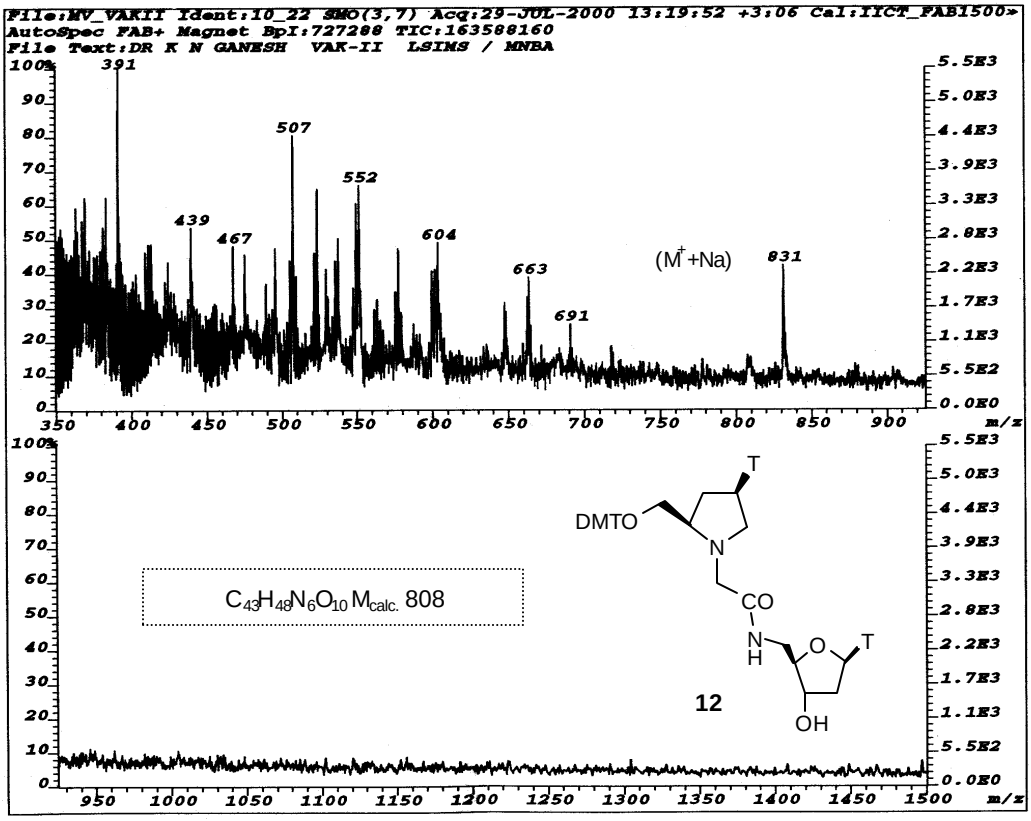
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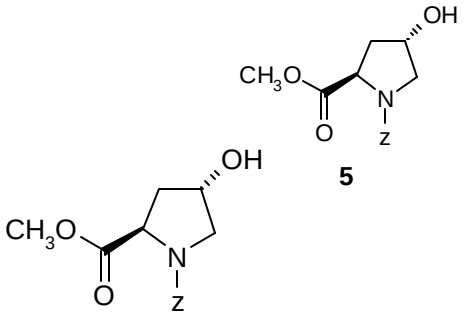

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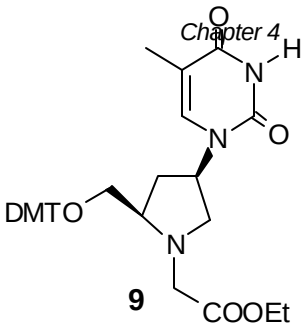

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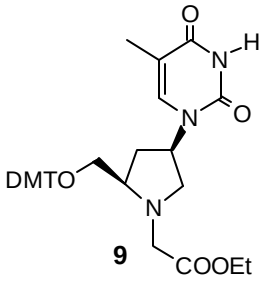

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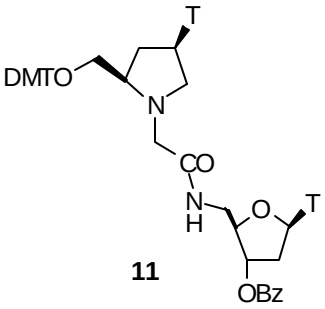

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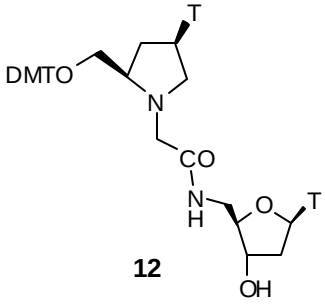

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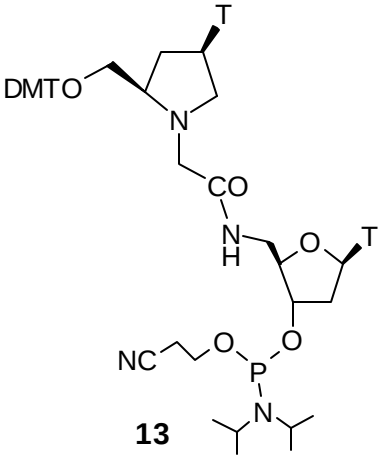

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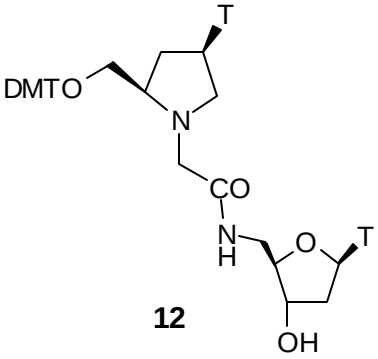

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$(M^+ + Na)$

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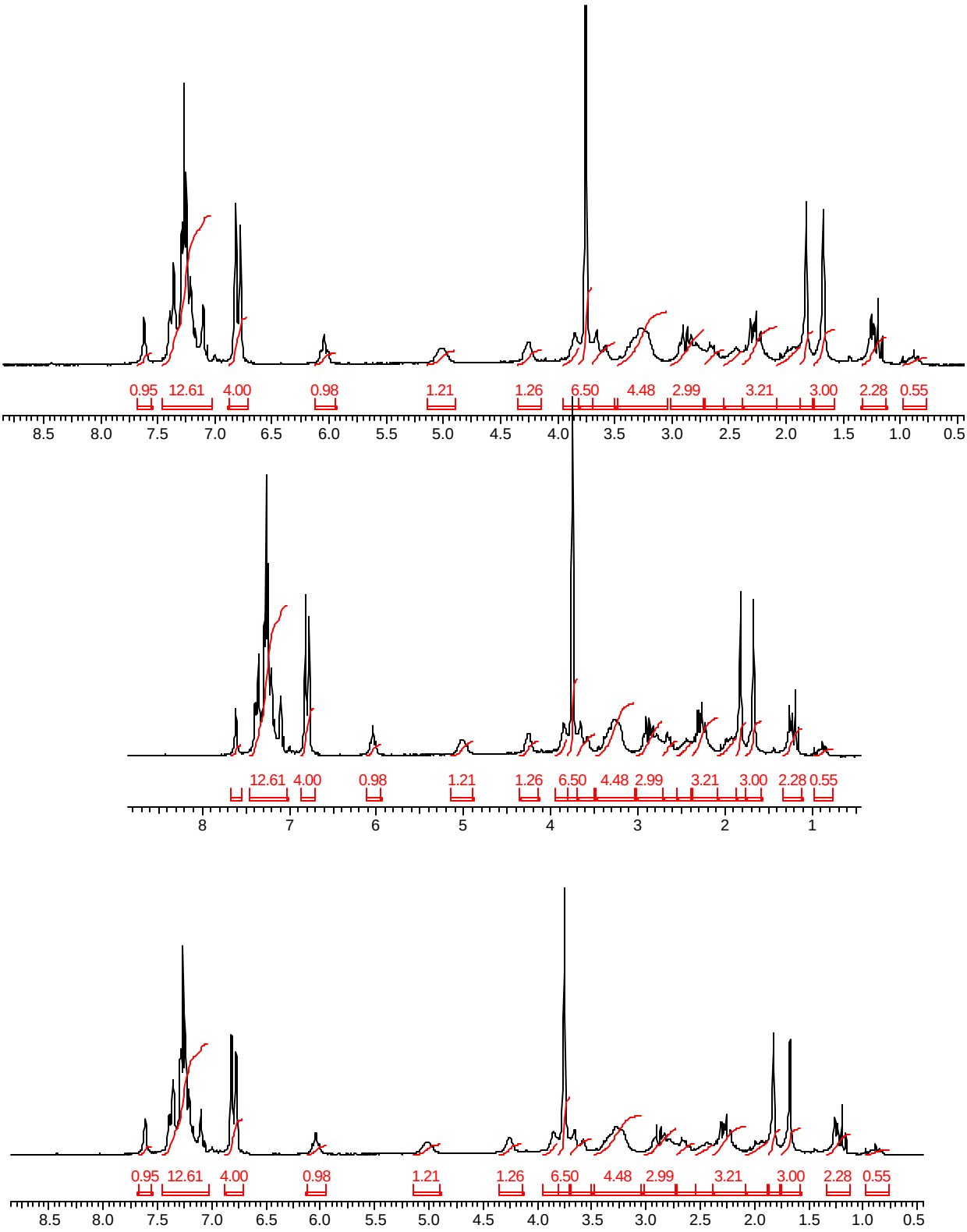
C50H53N6O11 913

(

$(M^+ + Na)$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