DESIGNER OLIGONUCLEOTIDES WITH NUCLEOBASE MODIFICATION: SYNTHESIS AND APPLICATIONS FOR STUDYING MOLECULAR RECOGNITION OF NUCLEIC ACIDS

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CERTIFICATE

Certified that the work incorporated in the thesis entitled ***"Designer Oligonucleotides With Nucleobase Modification: Synthesis and Applications for Studying Molecular Recognition of Nucleic Acids"*** submitted by **Mr. Dinesh A. Barawkar** was carried out by the candidate under my supervision. Such material as obtained from other sources has been duly acknowledged in the thesis.

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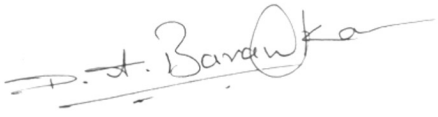
CANDIDATES DECLARATION

I hereby declare that the thesis entitled ***"Designer Oligonucleotides With Nucleobase Modification: Synthesis and Applications for Studying Molecular Recognition of Nucleic Acids"*** submitted for Ph. D. Degree to the University of Poona has not been submitted by me for a degree to any other university.



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CHAPTER 4 OLIGONUCLEOIDE-POLYAMINE CONJUGATES.

LIST OF PUBLICATIONS

1. Effect of C5-amino Substituent on 2'-Deoxyuridine Base Pairing with 2'-deoxyadenosine: Investigation by ^{1}H And ^{13}C NMR Spectroscopy **Dinesh A. Barawkar**, R.Krishna Kumar and K.N.Ganesh. *Tetrahedron*. **1992**, *48*, 8505-8514.

2. Solid Phase Synthesis of DNA Containing 5-NH_2-2'-deoxyuridine. **Dinesh A. Barawkar** and Krishna N. Ganesh. *BioMed. Chem. Lett.* **1993**, *3*, 347-352.

3. Exploring DNA Minor Groove Interactions Through a Probe Conjugate in Major Groove: Fluorescence Studies on Netropsin Complexation with dU-5-aminodansyl-DNA. **Dinesh A. Barawkar** and Krishna N. Ganesh. *Biochem. Biophys. Res. Comm.* **1994**, *203*, 53-58.

4. Fluorescent d(CGCGAATTCGCG): characterization of major groove polarity and study of minor groove interactions through a major groove semantophore conjugate. **Dinesh A. Barawkar** and Krishna N. Ganesh. *Nucleic Acids Research* **1995**, *23*, 159-164.

5. Synthesis of Site-Specific Oligonucleotide-Polyamine Conjugates. T. P. Prakash, **Dinesh A. Barawkar**, Vaijayanti Kumar and K. N. Ganesh. *BioMed. Chem. Lett.* **1994**, *4*, 1733-1738.

6. Triplex Formation at Physioligical pH by Oligonucleotides Incorporating 5-Me-Dc-(N^4-Spermine). **Dinesh A. Barawkar**, Vaijayanti A. Kumar and Krishna N. Ganesh. *Biochem. Biophys. Res. Comm.* **1994**, *205*, 1665-1670.

7. Site-specific oligonucleotide-spermine conjugates: Triplex formation at physiological pH by 5-Me-dC-(N4-Spermine) tethered oligonucleotide **Dinesh A. Barawkar**, Vaijayanti A. Kumar and Krishna N. Ganesh. (Manuscript under preparation).

ABSTRACT

CHAPTER 1: Introduction

Oligonucleotides bind specifically to single stranded nucleic acids to form a double helix through Watson-Crick hydrogen bonding. This process is fundamental to the event that define life: the storage, transmission and translation of genetic information. Even shortest oligonucleotides retain this ability for specific hybridization like their larger relatives. Thus they can be viewed as informational molecules and are valuable models to investigate the physical and biological properties of DNA and RNA. The effect of these oligonucleotides and the fields of their potential application can be broadened by the introduction of chemically modified nucleotides (*designer oligonucleotides*).

An understanding of the molecular basis of DNA recognition by proteins, drugs and various ligands is crucial to discern the chemistry underlying the basic cellular processes, their regulation and rational design of drugs. The major and minor grooves in duplex DNA act as conduits of molecular information required for DNA association with other molecules since hydrogen bonding centers in bases are pointed into these grooves. Large molecules such as proteins binding to nucleic acids, recognize DNA via specific interactions in the major groove, smaller DNA binders such as antibiotics interact with DNA either by intercalating the base pairs or by association in the minor groove or both.

The chemical modifications can be effected at bases, sugars, ends of the chain or at the phosphate group of backbone. These modifications which provide additional hydrogen bonding sites on bases or remove the existing hydrogen bonding sites offer a systematic method to study molecular recognition of nucleic acids. Many conjugates groups have been used with oligonucleotides such as fluorescent dyes, proteins, chemiluminescent groups etc. As a result, the range of application of oligonucleotides has extended from conventional once in molecular biology such as linkers, probes and primers in sequencing etc. to novel applications such as fluorescent oligonucleotides for biophysical studies, affinity labelling for separation chromatography, non-radioactive labelling in PCR diagnostics. Cross linking agents like psoralen or intercalator such as acridine improve duplex or triplex hybridization stability, while internucleotide phosphate modification (e.g. phosphorothioate) confer nuclease resistance and the non-ionic methylphosphonates render lipophillicity. All these lead to the possible usage of modified

oligonucleotides as therapeutic agents in antisense or antigene approach. Chain cleaving agents like EDTA/Fe^{II} covalently linked to DNA allow sequence specific cleavage of DNA (artificial nuclease). This chapter gives an overview of the importance of chemically modified nucleic acids and their emerging applications.

CHAPTER 2: Effect of C5-Amino Substituent on 2'-deoxyuridine Base Pairing With 2'-deoxyadenosine and Incorporation of 5-Amino-2'-deoxyuridine into Oligonucleotides.

Substituents at C5-position of pyrimidine base are known to affect its base pairing properties with complementary purines, either by altering the imino N3-H acidity or by modifying the acceptor strength of C2 and C4 carbonyls. This chapter describes the comparative base pairing property of 5-Methyl-2'-deoxyuridine (dT) and 5-amino-2'deoxyuridine ($dUNH_2$) with 2'-deoxyadenosine as their 3',5'-di-t-butyldimethylsilyl derivatives (Figure 1) in chloroform-d. Using ^1H and ^{13}C NMR, it is demonstrated that the 5-amino substituent in 2'-deoxyuridine results in (i) decreased association (lower K_a) with 2'-dA compared to dA:dT complexation, (ii) increased receptor strength of C2 carbonyl compared to C4 carbonyl and (iii) lower temperature for separation of 6-NH_2 protons of dA due to its complexation with $dUNH_2$ compared to that with dT.

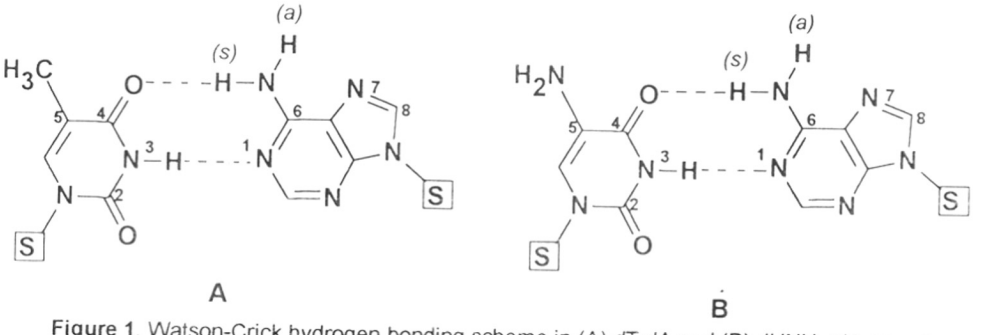


Figure 1. Watson-Crick hydrogen bonding scheme in (A) dT:dA and (B) $dUNH_2$:dA complexes. *s* and *a* refers to *syn* (hydrogen bonded) *anti* (non hydrogen bonded) protons on 6-NH_2 function of dA. S refers to 2'-deoxysugar of nucleosides.

The modified nucleobase has been incorporated into the well studied Dickerson's dodecamer d-(CGCGAATTCGCG) in place of dT. When 5-NH_2-pyrimidine is incorporated into an oligonucleotide, the additional hydrogen bonding sites present at C5 are directed into the major groove and may influence the biophysical properties and interaction of DNA with groove binding ligands. The oligonucleotide containing 5-amino-2'-dU shows slightly lower Tm as compared to that of unmodified, and it was found to be stable to digestion by *EcoR*1 restriction enzyme confirming the modification within the recognition site (GAATTC).

CHAPTER 3: Fluorescent Oligonucleotide probes

The additional amino group at C5 of pyrimidine is suitable chemical center for covalent anchoring of extraneous ligands such as fluorophores. Direct conjugation of ligands projecting it into the major groove with short and rigid linker arm have useful consequence in control of fluorescence properties and precision in footprinting experiments. This chapter describes the synthesis of different monomers for enzymatic and chemical incorporation of 5-amino-2'-dU conjugated with fluorescent groups such as dansyl, indole acetyl and fluorescein and their applications.

The major and minor grooves in duplex DNA are sites of specific molecular recognition by DNA-binding agents such as proteins, drugs and metal complexes and have functional significance. In view of this, understanding of the inherent differences in their environment and the allosteric information exchange/transfer between them induced by DNA-binding agents assumes importance. Site-specific incorporation of 5-aminodansyl-2'-dU (**U**$^{\wedge}$) in oligonucleotides d-(CGCGAA**U**$^{\wedge}$TCGCG) and d-(CGCGAAT**U**$^{\wedge}$CGCG) leads to fluorogenic nucleic acids (Figure 2), in which the reporter group resides in the major groove. The fluorescence observable from such a probe are used to estimate the dielectric constant of the major groove to be ~55D, in comparison to reported nonpolar environment of the minor groove (~20D) in poly d[AT]-poly d[AT]. An exclusive minor groove event such DNA-netropsin association can be quantitatively monitored by fluorescence of the dansyl moiety located in the major groove. This suggests existence of an information network among the grooves.

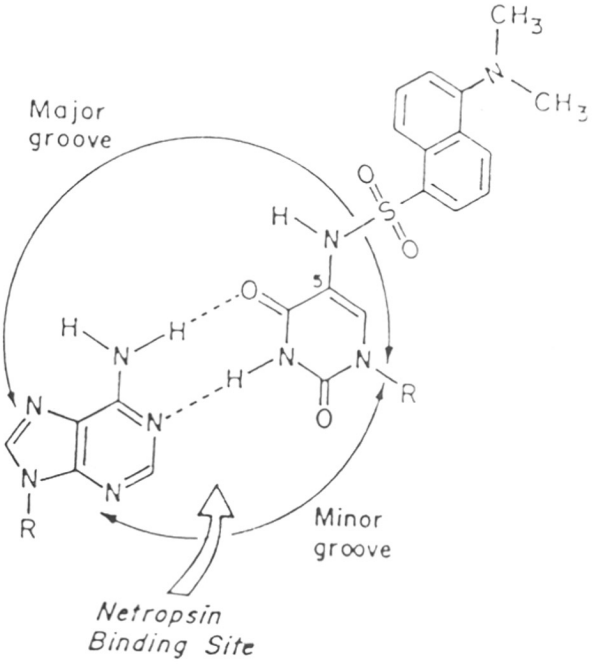


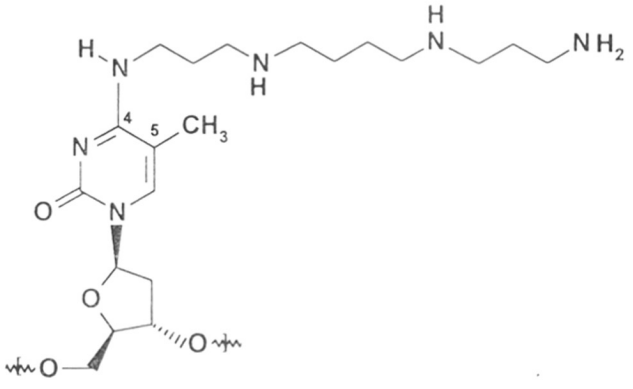
Figure 2. dA:5-aminodansyl-dU base pairing pattern indicating fluoroprobe in the major groove and netropsin binding site in the minor groove.

The reactivity of 5-amino group of 5-NH_2-2'-dU is different from exocyclic amino groups and it undergoes condensation with pentafluorophenol active esters allowing the synthesis of a wide variety of oligonucleotide conjugates. Oligonucleotide containing indole acetyl group linked to C5-amino of 5-amino-2'-dU were also synthesized and their duplexing property with complementary DNS-DNA have been studied by fluorescence energy transfer among the above two spectroscopically compatible donor-acceptor pair. Fluorescein conjugated oligonucleotides were synthesized as primers for PCR (Polymerase Chain Reaction) and these primers are shown to undergo successful amplification reaction to give fluorescent amplified product which can be detected easily.

These fluorescent DNA probes may have potential applications in study of structural polymorphisms in DNA, DNA-ligand interactions and triple helix structure. The 5'-triphosphate of 5-aminodansyl-2'-dU is also synthesized for its enzymatic incorporation into the DNA.

CHAPTER 4: Oligonucleotide-Polyamine Conjugates.

Oligonucleotide-directed triple helix formation has recently emerged as a powerful chemical approach to block transcription of specific gene (antigene strategy) and hence has a great therapeutic potential. Sequence specific binding of polypyrimidine oligonucleotides in parallel orientation to a polypurine strand in a major groove of DNA duplex depends on Hoogsteen hydrogen bonds between thymine and A:T base pairs (T*A:T triplet) and between protonated cytosine and G:C base pairs (CH+*G:C triplet). Due to the molecular necessity of protonated C (CH+) in the third strand, triplex formation in natural nucleic acids is pH dependent with an optimum stability at non-physiological pH 5.6-6.0. This limited specificity and pH stability of triad base pairing in triple helix formation have led to design of chemical modifications that could increase the affinity of third strand at intracellular pH 7.1-7.6. Most approaches have employed either modified base/sugar derivatives to raise the critical pKa of the N3 of cytosine or protonated CH+ mimetics including non-natural heterocycles.



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Substitution of 5-Me-dC for dC in third strand has extended the compatible pH range for triplex formation under non-physiological salt conditions. Oligonucleotides containing dC-(N^4-amidoalkyl) residues are reported to form triplexes, by selective interaction with a mismatched C:G base pair on target duplex with a higher affinity than with normal G:C base pair of duplex.

The biogenic polyamine, spermine, which is predominantly protonated at physiological pH, is well known to favor triple helix formation and it is evident from the literature, that conjugation of spermine to 5'-end of oligonucleotide leads to improved triple helix stability at pH 6.5. In view of the positive attributes of both 5-Me-dC and spermine in promoting triple helix formation, it was envisioned that oligonucleotide containing 5-Me-2'-dC-(N^4-spermine) conjugation (Figure 3) may have a constitutive effect on triplex formation at physiological pH. This chapter describes the synthesis of spermine conjugated oligonucleotides via N^4, and its effect on duplex and triplex stability. Spermine oligonucleotides formed duplexes with lower Tm than the unmodified control duplex, upon incorporation as a third strand, lead to triplex formation at physiological pH, with enhanced stability compared to unmodified triplex, even in the absence of divalent cations like Mg^{2+}. Some of the possible structural factors in *sp*-ODNs responsible for destabilization of duplex and stabilization of triplex are discussed.

ABBREVIATIONS

Ac_2O	-Acetic anhydried
Aem	-Acceptor emission
Aex	-Acceptor excitation
aq	-aqueous
Arg	-Arginine
CAP	-Catabolite activator protein
CH_3CN	-Acetonitrile
$CHCl_3$	-Chloroform
Cro	-Cro repressor protein
Cys	-Cysteine
dA	-deoxyadenosine
DABCO	-1,4-diazabicyclo-[2,2,2]-octane
dC	-deoxycytidine
DCC	-Dicyclohexylcarbodiimide
DCE	-Dichloroethane
DCM	-Dichloromethane
Dem	-Donor emission
Dex	-Donor excitation
dG	-deoxyguanosine
DMF	-Dimethylformamide
DMT-Cl	-Dimethoxytrityl chloride
DNA	-deoxyribonucleic acid
DNS	-Dansyl
DNS-DNA	-Dansyl conjugated oligonucleotide
dU	-deoxyuridine
EDTA	-Ethylenediaminetetraacetic acid
EtOH	-Ethanol
FET	-Fluorescence Energy Transfer
FPLC	-Fast Protein Liquid Chromatograph
gm	-grams
µgm	-microgram
hr	-hour

His	-Histidine
HOBT	-Hydroxybenzotriazole
HPLC	-High Pressure Liquid Chromatograph
µl	-microlitre
µM	-micromolar
M	-Molar
MeOH	-Methanol
Met	-Methionine
MHz	-Megahertz
min	-minute (s)
mM	-millimolar
mmol	-millimole
MsCl	-Mesitylene sulfonyl chloride
nm	-nanometers
NMR	-Nuclear Magnetic Resonance
ODN	-Oligodeoxynucleotide
PAGE	-Polyacrylamide Gel Electrophoresis
PFP	-Pentafluorophenol
PNA	-Peptide Nucleic Acid
RNA	-ribonucleic acid
RPC	-Reverse Phase Chromatography
sp-ODN	-Spermine conjugated oligodeoxynucleotide
T	-Thymine
TBDMS-Cl	-t-Butyldimethylsilyl chloride
TEA	-Triethylamine
TEAA	-Triethyl ammonium acetate
TEAB	-Triethyl ammonium bicarbonate
TFA	-Trifluoroacetate
TLC	-Thin Layer Chromatography
Tris	-Tris(hydroxymethyl)methylamine(2-amino-2-(hydroxymethyl)-1,3-propanediol
Trp	-Tryptophan repressor
UV	-Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

Nucleic acids (DNA, RNA) are perhaps the most important of all biomolecules. DNA is primarily responsible for storage of genetic information and its expression. The genetic expression occurs through the intermediacy of RNA. In certain viruses (retro-viruses), RNA is the genetic material and information propagates through initial transcription to DNA. Nucleic acids perform their vital functions in cell replication and regulation through interaction with a variety of small and large molecules via specific molecular recognition. Accurate DNA replication is most necessary for cells survival and at a molecular level, this is achieved through Watson-Crick hydrogen bonding between complementary base pairs A:T and C:G (Watson & Crick, 1953) (Figure 1). This mutual recognition of bases by use of hydrogen bonds primarily ensures the fidelity of DNA transcription and translation. There are two hydrogen bonds in an A:T base pair and three in C:G base pair involved in Watson-Crick pairing. This results in an isomorphous geometry whereby the four base combinations A:T, T:A, C:G and G:C can all be built into same geometrical framework. Further it leads to an antiparallel double helical structure for DNA in which the two strands held by hydrogen bonds and are twisted around each other. The topological consequence of this is the formation of helical grooves major and minor grooves in B form of DNA (Figure 2). Most specific interactions of DNA with other molecules such as proteins, drugs, water and metal ions take place in these grooves.

Among other patterns of hydrogen bonding, Hoogsteen (Hoogsteen, 1963) and Wobble base pairs (Crick, 1966; Soll et al., 1967) are the most significant (Figure 1). Hoogsteen base pairing is not isomorphous with Watson-Crick base pairs and has importance in triple helix formation (see section 1.5). In Wobble base pairing, a single purine is able to recognize a non-complementary pyrimidine (eg. G:U). Wobble base pairs have importance in m-RNA, t-RNA, and codon:anticodon interactions. Several

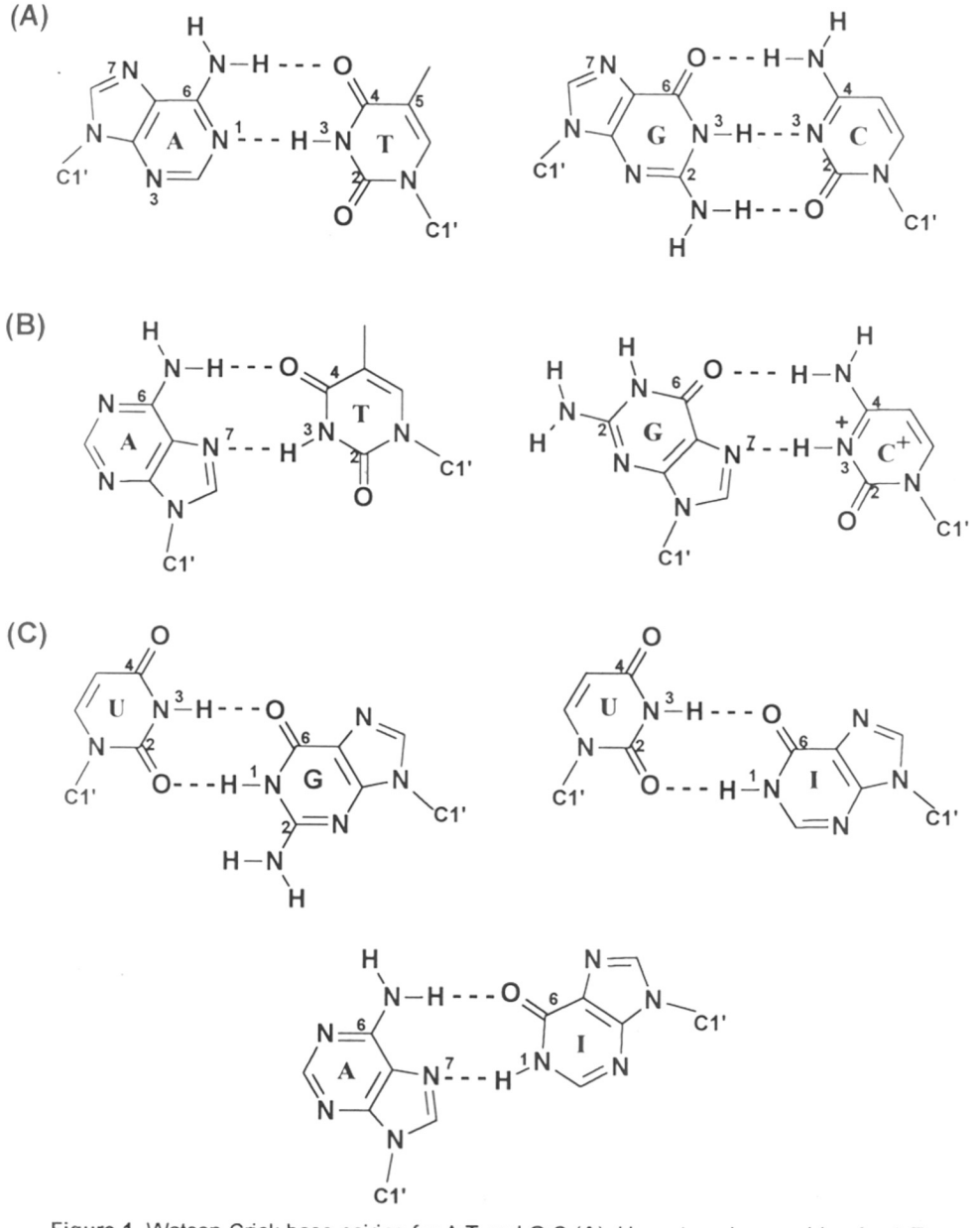


Figure 1. Watson-Crick base pairing for A:T and G:C (**A**). Hoogsteen base pairing for A:T and G:C (**B**). `Wobble base pairing for G:U, I:U and I:A (**C**).

mismatched base pairs and anomalous hydrogen bonding patterns have been seen in X-ray studies of synthetic oligodeoxynucleotides (Dickerson, 1988).

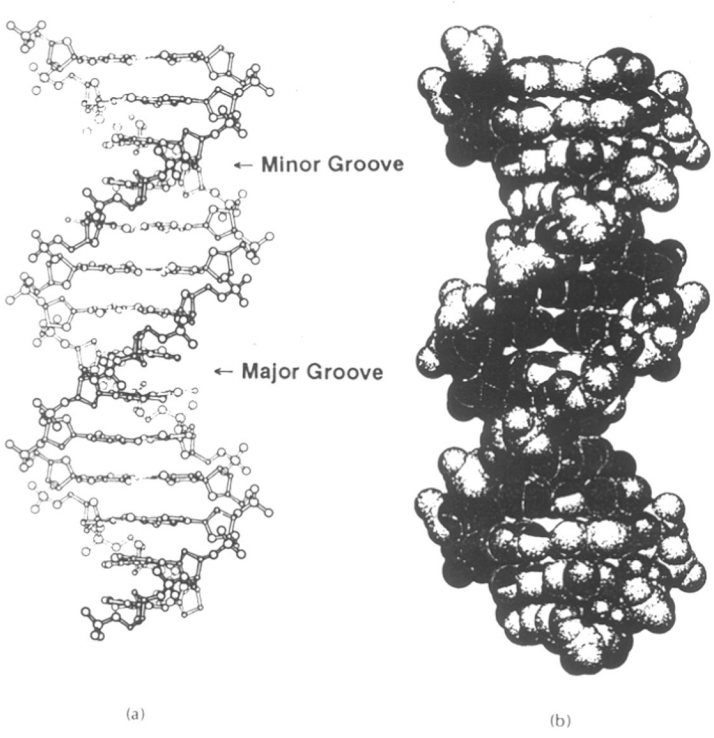


Figure 2. Structure of B-DNA.

Depending on the base sequences and environment, DNA duplex can exist in 3 major conformations: A, B and Z-DNA (Saenger, 1984). A-DNA and B-DNA are major regular DNA secondary structures with right handed double helices and Watson-Crick base pairs. Some of the structural characteristics of these DNA polymorphs are shown in Table 1. B-DNA has a wide major groove and a narrow minor groove, both of which are solvated by water moleclules. Watson-Crick base pairing is maintained by an *anti*

glycosidic conformation and C2' endo sugar pucker features. Two particular hallmarks of B-DNA in contrast to A and Z forms are its flexibility and capacity to modulate local helix structure in response to base sequences.

Table 1: Average helix parameters for the major DNA conformations.

Structure Type	Residues per turn	Sugar pucker	Glycoside torsion	Groove Width minor	major
A-DNA	11	C-3'-*endo*	*anti*	11.0	2.7
B-DNA	10	C-2'-*endo*	*anti*	5.7	11.7
Z-DNA	12	C-3'-*endo*	*syn*	2.0	8.8

The detailed X-ray structure analysis of the dodecamer d(CGCGAATTCGCG) (Wing et al., 1980; Drew et al., 1981) and related sequences have given us much insight about the versatility of B-DNA. The minor groove in AT regions is narrower than GC regions. Steric clashes between adjacent purine rings on complementary strands are compensated by changes in propeller twist. This along with other adjustments in base stacks causes changes in sequence dependent groove size, helical twist, curvature etc. Base stacking plays an important role in DNA structure with 5'-pu:py-3' structures being different from 5'-py:pu-3' for same base combinations and the energetic requirements are accommodated by changes in backbone conformation. Mismatched base pairs also cause local structural irregularities. A-DNA has a more rigid structure and shows little sequence dependent variation in structure. The major groove is deep and narrow with the minor groove broad and shallow. Z-DNA is a left handed double helical structure stabilized by high concentrations of $MgCl_2$, NaCl and EtOH and is favoured for alternating G:C sequences. In Z-DNA, the minor groove is narrow and deep while the major groove is broad and shallow. Z-DNA has characteristic zig-zag phosphate backbone and Watson-Crick base pairing is achieved by purines adopting *syn* glycosidic conformation with C3' endo sugar pucker.

1.2. MOLECULAR RECOGNITION OF NUCLEIC ACID

1.2.1. *Interaction with other molecules: General Aspects*

Nucleic acids interact reversibly with a broad range of chemical species that include water, metal ions and their complexes, small organic molecules and proteins. There are three primary ways in which these interactions take place (Figure 3).

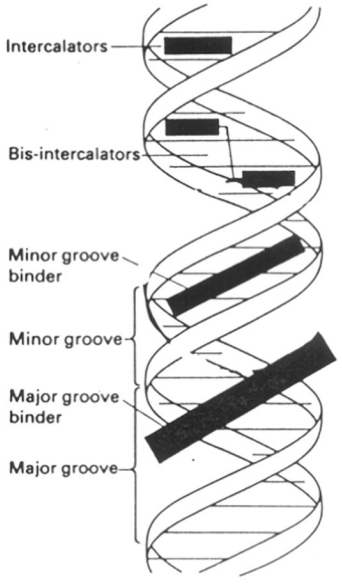


Figure 3. Modes of DNA-Ligand interaction.

(a) **Electrostatic interactions**: The negative charges on DNA sugar-phosphate backbone on the exterior of the helix interact with positive charges on substrates. These are non-specific with respect to DNA sequence.

(b) **Groove binding**: These are the direct interactions of the bound molecules with the edges of the base pairs in either the major or the minor groove of nucleic acids and are mediated by non-covalent forces such as hydrogen bonding and hydrophobic forces.

(c) **Intercalation**: This involves insertion of planer aromatic systems between the base pair stacks.

The first two modes of binding may induce slight changes in DNA conformations while intercalation introduces major distortions in sugar phosphate geometry including unwinding of helix.

The major and the minor grooves of DNA differ significantly in electrostatic potential, hydrogen bonding characteristics, steric effects and hydration. Figure 1a shows the hydrogen bond donors and acceptors that are accessible in B-DNA for binding with other molecules in major and minor grooves. Hydrogen bonds can be accepted by A:T/T:A base pairs from the bound molecule in major groove by C4 carbonyl of T and N7 of A, while in minor groove, this is via C2 carbonyl of T and N3 of A. The only hydrogen bond donor in the major groove for A:T base pair is N^6-H of A while none exists in the minor groove. The picture is different for C:G/G:C base pairs. The hydrogen bond acceptors in major groove are N7 and O^6 for G while in the minor groove, these are O2 of C and N3 of G. The hydrogen bond donor in major groove is N^4-H of C while in the minor groove, it is N^2-H of G.

The salient and significant outcome of this distribution of hydrogen bond donors and acceptors in the naturally occurring base pairs is that binding molecules can discriminate the A:T base pair from C:G base pair efficiently from the major groove side, while it is not so efficient in the minor groove. Two further features of molecular discrimination are noteworthy. In A:T/T:A base pairs, C5 methyl group of T offers substantial hydrophobic recognition in the major groove which is absent in C:G/G:C base pairs. However, in the latter, the N^2-amino group of G presents a steric block to hydrogen-bond formation at N3 of G and C2 carbonyl of C in the minor groove. It is thus possible to distinguish A:T from T:A and C:G from G:C in the major groove since the ordered array of hydrogen bonding sites and hydrophobic centers differ among the four

pairs (Seeman et al., 1976). The negative electrostatic potential due to phosphate charges is greater in A:T minor groove than in G:C rich regions and this provides an additional important source for A:T specific minor groove recognition.

Specificity in binding of nucleic acids results from molecular contacts between the bound ligand and the edges of base pairs on the floor of the groove. Many proteins exhibit binding specificity with nucleic acids primarily via major groove interactions while smaller molecules in general bind in the minor groove.

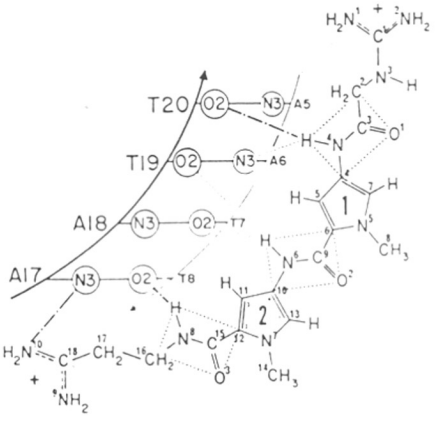


Figure 4. Schematic diagram of the interaction between netropsin and adenine N-3 or thymine O-2 atoms on the floor of minor groove.

1.2.2. *DNA-Drug Minor Groove Interactions*

Much of the intimate structural details of minor groove binding has come as a consequence of crystal structure studies of DNA-netropsin complex (Kopka et al., 1985). Netropsin is an antitumour, antibiotic that binds at the AATT center of the DNA d(CGCGAATTCGCG) by displacing the spine of hydration present in free DNA. The three amide groups of netropsin (Figure 4) point inwards and form bifurcated H-bonds with N3 of A and O2 of T. The two cationic ends of netropsin are centered in the minor groove in AATT region and associate with N3 of outer A and central AT base pairs. The

pyrrole ring C-Hs of netropsin point to the minor groove and make close contact with A:T base pairs. Netropsin binding to DNA causes a slight widening of the minor groove in AATT region and bending of the helix axis. The N^2 amino group of G sterically prevents the drug from sliding in the minor groove and the binding of netropsin to G:C is weaker compared to AT sequences.

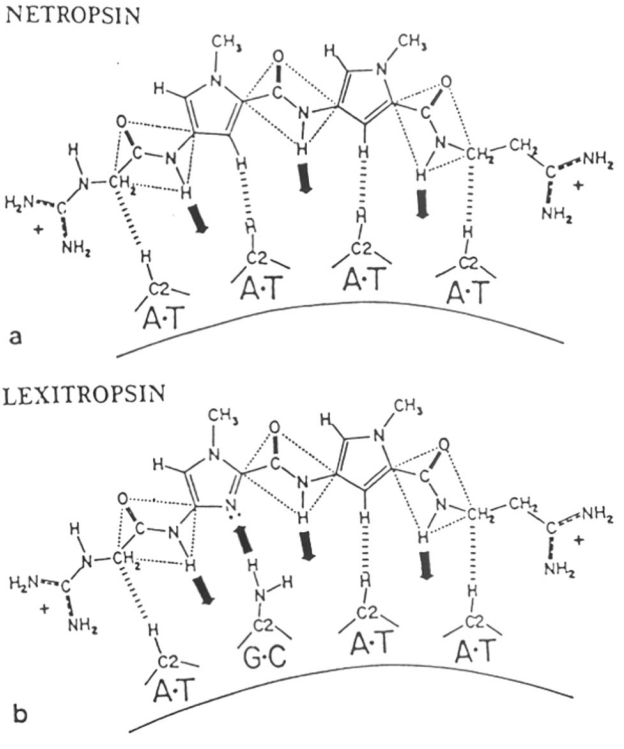


Figure 5 Design of lexitropsins based on netropsin. (a) netropsin; (b) lexitropsin. Solid arrow shows hydrogen bond; broken line shows van der Waals interaction.

Understanding the molecular basis of specific recognition of netropsin by DNA has led to the design of interesting new sequence-specific binding agents known as "lexitropsins" (Lown et al., 1986). In contrast to netropsin, these recognize both A:T and G:C pairs. In lexitropsins, one or more of the pyrrole rings is replaced by H-bonding acceptor

heterocycles such as imidazole (Figure 5). This alleviates steric clash with N^2H of G and confers selectivity for lexitropsins. Such "molecular engineering" of DNA binding agents have primary consequences in rational drug design and will emerge as an important area as our understanding of DNA-drug complexes grow.

1.2.3. *Intercalation*

This mode of binding is prevalent among aromatic molecules that bind to DNA whereby the ring is inserted into the base stacks of a helix (Waring & Wakelin, 1990). The substituents on the ring may protrude into one of the DNA grooves and interact specifically with the base pairs. Intercalation leads to theoretical lengthening of DNA by ~3.4 A/site and in practice, the net lengthening observed is much less due to compensating helical unwinding caused by intercalation. This changes the optical properties of both DNA and the intercalator and affects the base stacking (Porumb, 1978). The intercalaton induced changes in DNA structure have been well established by several crystallographic studies of DNA-drug complexes (Niedle et al., 1987) and involve distortion of phosphate backbone and a rise in base pair separation. Solution studies have indicated that at saturation of intercalating species, binding occurs at most between base pairs at alternate sites on DNA. This observation termed 'neighbor exclusion principle' means that upon binding of an intercalator at one site, it is almost impossible to bind another at adjacent site (Waring & Wakelin, 1990). Specificity in intercalation binding generally favours C:G base pairs. Groove binding molecules display greater binding selectivity than intercalator since they contact more base pairs in the groove.

The binding specificity of intercalators have been elegantly enhanced by linking them covalently to oligonucleotides (Figure 6) at specific sequences (Helene et al., 1985,1994; Vlassov et al., 1986,1988). These adducts bind to complementary single-stranded regions with high precision. Intercalators have also been linked to groove binding molecules (Nielsen, 1991; Bailly & Henichart, 1991) and EDTA derivatives to

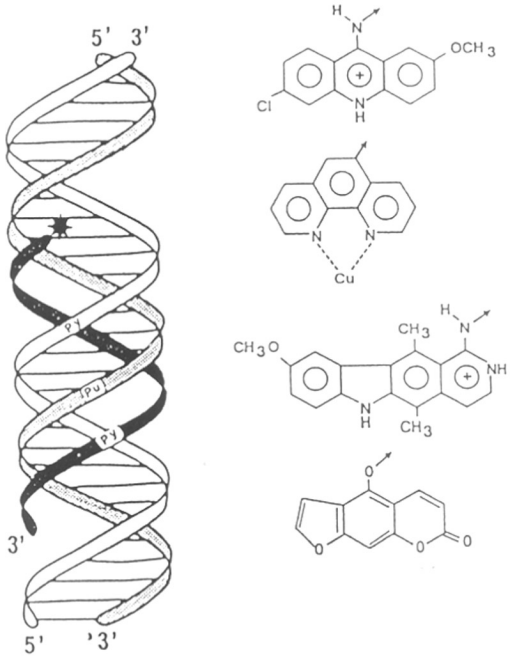


Figure 6. Triple helix forming oligonucleotide conjugate.

obtain selective recognition and DNA cleavage in presence of Fe (II) and oxygen (Moser & Dervan, 1987). This has developed into a powerful strategy to achieve targeted DNA cleavage via DNA triple helix (Dervan, 1992). Several bis intercalators (Atwell et al, 1985) have also been synthesized and studied to understand and mimic the naturally occurring antibiotics such as triostin A and echinomycin.

The "non-classical" intercalators constitute aromatic planer systems with bulky substituents such as tetraphenylporphyrin (Pasternack, 1989). Here the substituents not only provide good stacking of the intercalated system with DNA base pairs but also perturb the kinetics of interaction. The natural analogues of these are the antibiotics nogalamycin, anticancer drugs mitoxantrone and bisantrene (Niedle et al, 1987). Some

molecules containing non-fused, twisted, aromatic ring systems interact with DNA in a fashion paradigm for a groove binding molecule and the examples are HOECHST 33258 (Pjura et al., 1987) and DAPI (Wilson et al., 1989).

Since DNA and RNA are prime targets as receptors for development of antitumour, anticancer and antiviral drugs, understanding of nucleic acid-ligand interactions as described above is important to design molecular recognition based medicinal agents (Hurley, 1989). This approach has already led to several promising drug candidates.

1.2.4. *DNA-Protein Interactions*

Nucleic acids are rarely found alone in the cells and occur as complexes with proteins. These proteins/enzymes mediate all nucleic acid transformations such as replication, gene regulation, splicing or degradation. Protein-nucleic acid interactions are far more complex than protein-ligand or nucleic acid-ligand interactions (Saenger & Heinemann, 1989). The complexity of the problem is reflected in the fact that innumerable methods ranging from X ray crystallography, NMR and Fluorescence spectroscopy to biochemical/molecular biological techniques such as footprinting etc. have been used to study these interactions (Jost and Saluz, 1991). A full description and analysis of these is beyond the scope of present thesis and only those aspects concerned with specific molecular recognition are dealt here.

The main electrostatic component of DNA-protein binding comes from interaction of negatively charged phosphate backbone with cationic side chains of lycine and arginine. These are however, incapable of providing sequence specificity on their own and are predominant in non-specific nuclease-DNA binding. Much of the specificity in DNA-protein interactions is derived from hydrogen bonding of protein side chains with specific base pairs in the major/minor grooves of DNA. Some of the normally observed pairwise hydrogen bonding interactions are shown in the Figure 7. The topological interaction of proteins with DNA occurs via insertion of an α-helix into major groove of

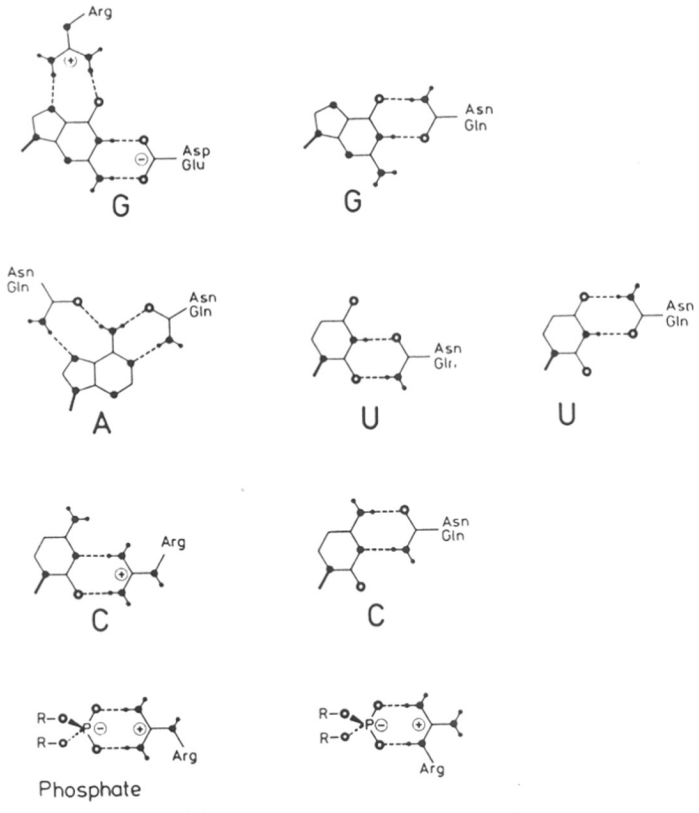


Figure 7. Some possible interactions of nucleic acid bases and phosphate groups with amino acid side chains.

DNA duplex and a β-sheet interaction with DNA in the minor groove. In such complexes the side chains of amino acids recognize the edges of base pairs to form a specific recognition system (Helene & Lancelot, 1982). In case of single stranded nucleic acids, aromatic side chains may stack with nucleic acid bases. Much of the present knowledge on sequence-specific interactions has grown from extensive studies of interaction of regulatory proteins with operator DNA, DNA-polymerase complexes, DNA restriction enzymes and recent work on eukaryotic transcription factors.

The proteins Cro, λ, Trp repressors, CAP and homeodomain all have quite different overall structures, but each bind tightly to specific DNA sequences. All these share a common structural feature in their DNA-binding region: they contain a pair of α-helices termed helix-turn-helix motif (Figure 8a). One of these pairs is the recognition helix which resides in the major groove of DNA, making specific contacts. Any mutation of the base pair on DNA or the amino acids of proteins involved in these specific contacts alters the stability and specificity of complexes in a major way. There are two other structural motifs which have recently been identified as major determinants of DNA-protein interactions. In the zinc-finger motif (Figure 8b), which is most commonly found in several eukaryotic transcription factors, the recognition element is held by zinc in place through interaction of β-sheet/α-helix substructures via Cys/His side chains, forming Zn-S and Zn-N coordinating bonds. Several variations have been noticed within this motif such as all Cys complexes and binuclear Zn complexes. In leucine zipper proteins (Figure 8c), the interacting recognition elements are constructed via two protein chains containing leucine at every seventh residue. This forces a stable zipper like hydrophobic locking of leucine side chains, thus holding the recognition units together in space, for a dimeric complexation with DNA. Some prokaryotic repressors, Met and Arc recognize DNA by way of two antiparallel β-strands positioned in the DNA major groove (Rafferty et al., 1989, Breg et al., 1990).

Restriction enzymes present a class of DNA-binding proteins which show extremely high specificity for binding DNA sequences and cleave them hydrolytically with great precision. These have, therefore, evolved as major tools in genetic engineering and recombinant DNA technology. The enzyme *EcoR*1 recognizes the symmetric hexameric sequence GAATTC and cleaves it specifically between G and A to produce sticky ends (Figure 9). Structural analysis of its complex with synthetic DNA has revealed several interesting facets. The DNA binding element itself shares no

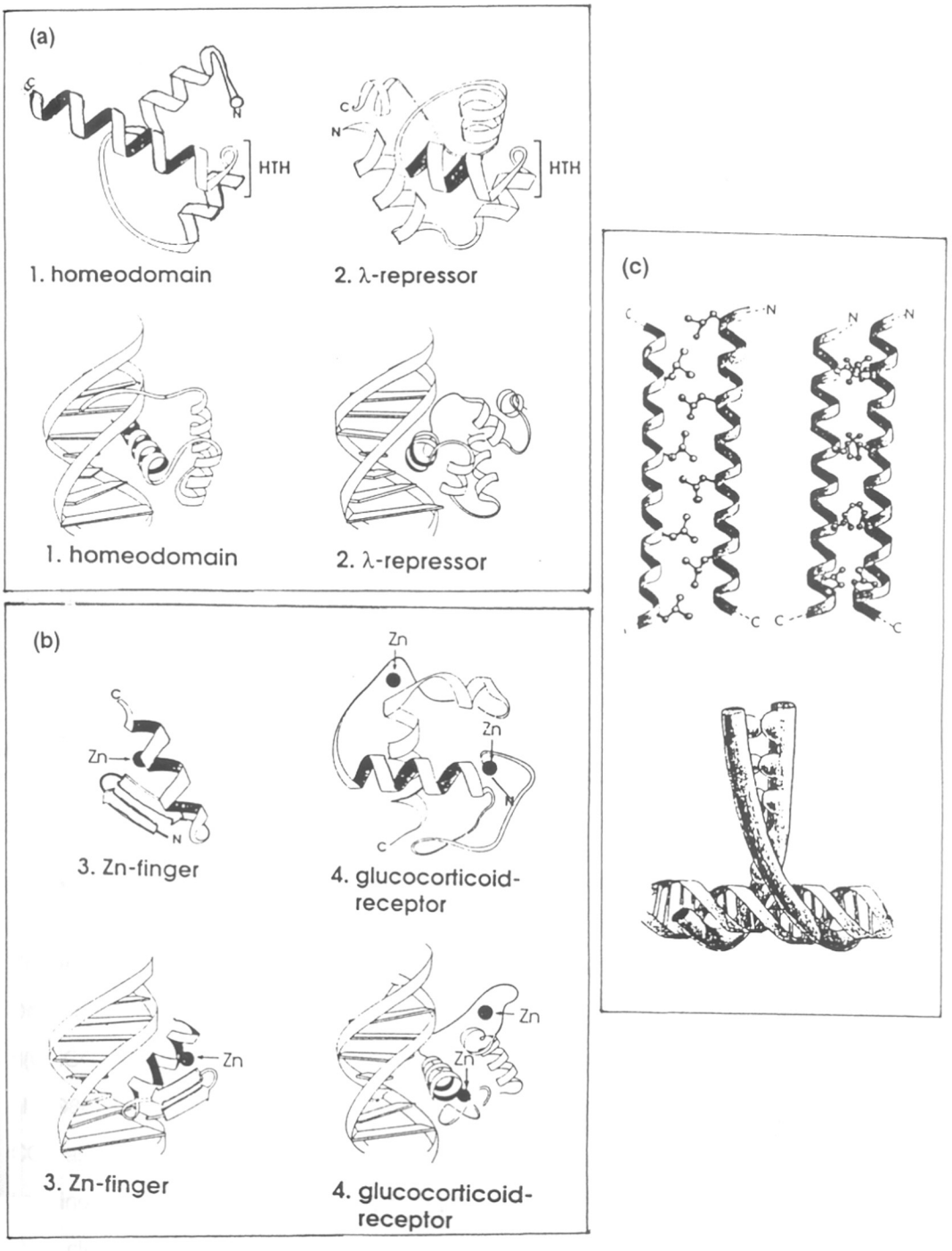


Figure 8. Different structural motifs, helix-turn-helix (a); Zinc finger (b) and Leucine zipper motif (c).

homology to other established motifs such as helix-turn-helix, zinc finger, etc. It employs a four α-helix bundle with 2 extended side chains that project into the major groove of DNA to contact the substrate purines and pyrimidines.

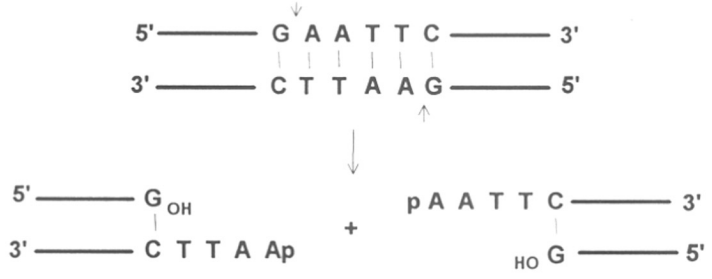


Figure 9. Recognition sequence of *EcoR*1.

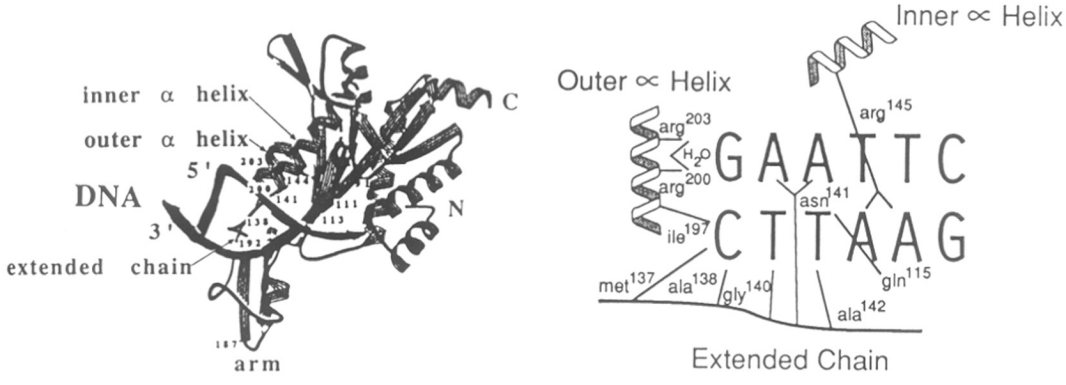


Figure 10. Crystal stucture of an *EcoR*1-DNA complex (A). *EcoR*1 substrate recognition model (B), one subunit contacts are shown.

The enzyme binds (Figure 10) as a dimer symmetrically with each monomer enwrapping DNA and anchoring the scissile bonds at active sites. In its complex with d(CGCGAATTCGCG) studies by X-ray studies (Dickerson & Drew, 1981; McClarine et al., 1986), have shown that the central sequence GAATTC is in B-form while the flanking CGC and GCG are more like in A-form. The binding induces unwinding of DNA by 25°, bending of axis by 50° and widening of major groove by 3.5 A°, leading to a kink at the DNA cleavage site. Such structural changes are lacking in *Eco R*1 interaction with

non-specific DNA. The discrimination in specificity is a result of specific recognition mechanism involving hydrogen bonding with purines, hydrogen bonding and van der Waals interaction with pyrimidines and sequence dependent phosphate interactions which lead to structure distortion.

1.2.5. *DNA-DNA Recognition*

A decisive interaction occurring in DNA major groove is the recognition of single-stranded DNA by duplex DNA to form a triple helix, which is discussed in a separate section 1.5. Other forms of molecular recognition of DNA is, by itself or its structural analogues are briefly mentioned below.

PNA-DNA Interaction: The interaction of double stranded DNA with small as well as with large molecules are often divided into contributions from sugar phosphate backbone and from nucleobases. The main electrostatic component of the binding energy of cationic ligands is attributed to negative phosphates of DNA backbone, while nucleobases contribute a hydrophobic and dispersive components. The relative importance of these two components in recognition and binding is far from clear even for rather simple ligands. A DNA analog called PNA (peptide nucleic acid) contains uncharged

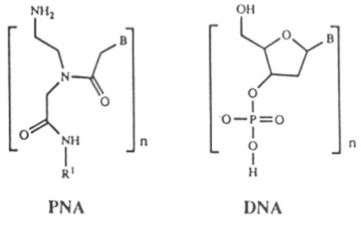


Figure 11. Chemical structures of DNA and PNA.

pseudo-peptide backbone composed of N-(2-aminoethyl)glycine units carrying nucleobases (Figure 11). These are excellent DNA mimics, in terms of ability to form Watson-Crick base paired helical duplexes, with complementary oligonucleotides (Nielsen et al., 1994; Wittung et al., 1994). PNA-DNA stable duplexes have also been

shown to form inspite of the fact that their backbone is uncharged. Structural homologues of PNA's show dramatic effects on their binding affinity. They also displace a DNA strand from duplex in competition experiment, inhibit cleavage of DNA by restriction enzymes, shows antisense/antigene activity in *in vitro* translation experiments (Nielsen et al., 1994). These interesting properties have provided a conceptual leap in our present knowledge on storage of genetic information and much work is required to assess the real practical implication of PNA's.

***DNA Backbone Modifications: Phospho and Dephosphono Analogues*:** The main phosphoro analogues of nucleic acids, which have attracted lot of attention and have emerged as potential antisense compounds from nuclease resistance point of view are, phosphorodithioates, phosphorothiotes and methyl phosphonates (Crooke and Lebleu, 1993). The first one is achiral at phosphorus while the latter two are chiral and hence any synthesis of ODNs is accompanied by problem of mixtures. ODNs containing these units have been extensively studied for their hybridization properties, stability to nucleases, membrane permeability and pharmacokinetics and these are fast emerging as first generation ODN therapeutic agents. The promising results of these studies have also led to investigations of several dephosphono analogues containing non-phosphate backbones (Sanghvi, 1994). However, in these cases, synthesis becomes more complex and still can not be routinely achieved as fast as the phosphoro analogues. The field is still in infancy but growing rapidly.

G-tetraplexes: Self recognition of guanine-rich DNA sequences leads to G-tetraplexes (Sen & Gilbert, 1988). Repeats of $(G)_n$ sequences have been detected at the ends of eukaryotic chromosomes and are known as telomeres. These structures are implied in replication and maintenance of chromosomal termini during mitosis and meiosis (Blackburn, 1991). A variety of telomeric repeat DNA sequences have been identified at 3'-end of chromosomes of different organisms. Of these, the sequences from

Tetrahymena $d(T_2G_4)_n$, oxytricha $d(T_4G_4)_n$ and that of human chromosome $d(T_2AG_3)_n$ have been structurally well characterized (Brahmachari and Balagurumoorthy, 1994). In presence of specific monovalent and divalent cations, guanines adopt planer G tetrads stacking on each other (Figure 12), leading to tetraplexes (Sen & Gilbert, 1988, 1990; Sundquist and Klug, 1989).

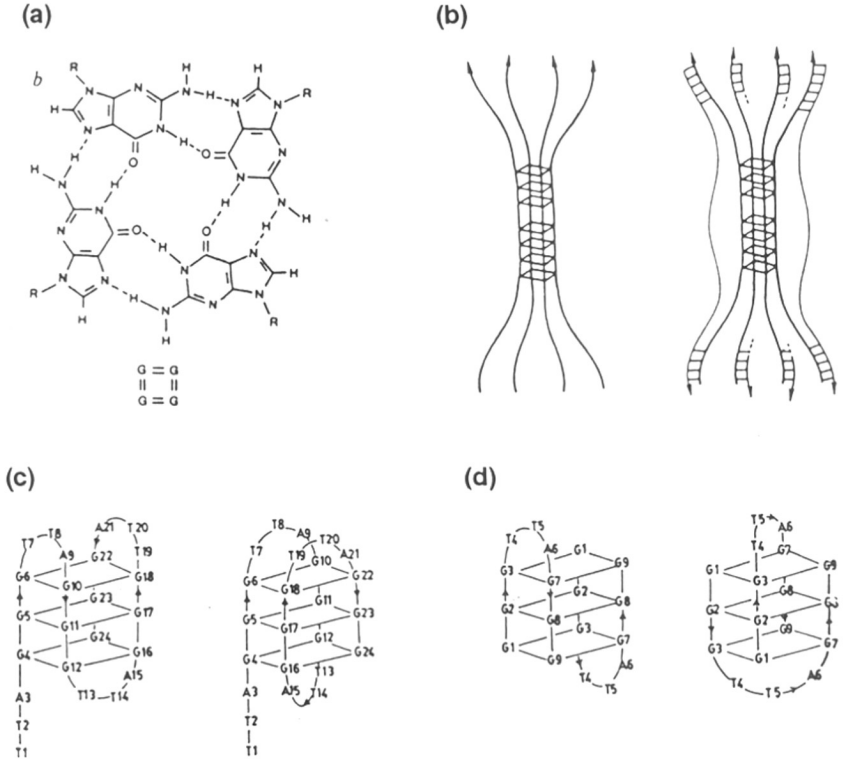


Figure 12. Structure of guanine tetrad (a), schematic diagrams (b-d) showing various possible quadruplex strctures.

The four G's are held together by Hoogsteen bonds in a cyclical fashion. The metal ion is located between the stacks and bind to O^6 of G's on adjacent stacks analogus to crown ethers (Sundquist and Klug, 1989). Single dGn segments can form four parallel stranded tetraplexes in presence of Na^+ and all G's are *anti* within each strand (Sen & Gilbert, 1988). $d(G_nT_mG_n)$ and $d(G_nT_mG_nT_mG_nT_m)$ segments have potential to form

intramolecular foldback structures. $d(G_4T_4G_4)$ forms hairpin which dimerizes depending on conditions, to align either parallel or antiparallel to each other, in effect leading to G4 tetraplexes (Sundquist and Klug, 1989). In the folded sequences, G's adopt glycosidic torsion angles with *syn/anti* alterations of guanines around G tetrad coupled with antiparallel alignment of adjacent strands.

The polymorphic nature of G tetraplexes is apparent from noticeable structural differences seen among the solid state and solution NMR studies. X-ray structure $d(G_4T_4G_4)$ shows antiparallel strands with laterally connected loops (Kang et al., 1992) while for the same sequence NMR (Smith et al., 1993) studies revealed both parallel and antiparallel strands with cross diagonal loops. In the human telomeric repeat $d[AG_3(T_2AG_3)_3]$, both diagonal and lateral loops were observed (Wang and Patel, 1993). A DNA aptamer which binds to and inhibits thrombin exhibits a new motif related to the telomeric structures (Wang et al., 1993). The structural diversity of G tetraplexes is dictated by both environmental and context effects and these along with the recently discovered interdigitated $C:C^+$ tetraplex (Gehring et al., 1993) bear evidence to the versatility of natural nucleobases to form highly interesting self recognition systems.

The above description illustrates the major elements of DNA molecular recognition, namely the hydrogen bonding interactions of base pairs in the major and minor grooves of DNA. Each of the allowed base pairs in DNA have distinct horizontal array of hydrogen bonding elements whose vertical combinations as present in DNA duplex lead to strategies for sequence specific recognition. It should be possible to fine tune this by employing chemically modified bases possessing additional, suitable donor/acceptor groups as modulators of recognition elements. The next section presents a review of the role and scope of such modifications in altering DNA structure.

1.3. OLIGONUCLEOTIDES WITH MODIFIED BASES

The role of nucleobases in DNA is not just restricted to defining the sequence and maintain base pairing fidelity. They also influence the profile of DNA such as helix diameter, length of repeating unit, tilt and twist of nucleobase pair, etc. Inosine can be inserted opposite to all four regular nucleobases without disrupting the double helix and hence useful for design of nucleic acid probes (Martin et al., 1985). It has an advantage in hybridization probes when uncertainty exists with respect to a base. Two other useful modified bases are those with altered aromatic nucleus including C-nucleosides and those containing new substitutions on the ring (Figures 13 a,b). 2'-Deoxynebularine and 2'-deoxyxanthosine are purines (Eritja et al., 1986) devoid of exocyclic substituents and form wobble base pairs. Double helices containing these nucleobases are generally less stable than those with normal bases. In contrast, helix stability is increased by inserting 2-amino adenine (Gaffney et al., 1984) as well as a fluorescent pyridopyrimidine (Inoue & Ohtsuka, 1985), which can pair with both G and A. The aromatic nucleus of bases have also been modified to yield several deaza and aza purines (Seela & Driller, 1985; Seela et al., 1989; Seela & Kaiser, 1988; Crosstick et al., 1990) and altered glycosidic link at N7 instead of N9 (Seela & Winter, 1993). Exocyclic substituents include N^6 alkylation of A or N^2 of G and 8-substitution on purine ring. Similarly, a number of pyrimidine nucleoside analogues are known: O^4-alkyl T, 2'-deoxyuridine, 5-methyl C, 5-putrescinyl and other 5-substituted thymines and azapyrimidines (Sanghvi, 1993).

A series of double helical nucleic acid analogues based on pairing of isopurines with isopyrimidines have been described (Bain, et al., 1992; Switzer et al., 1989). Normally base modifications lead to destabilization of duplexes although they provide hydrogen bonding complementarity. This is due to the fact that the base stacking with neighbouring bases, which are an important source of stability to nucleic acids (Saenger, 1984) may be severely affected. Recently this fact has been intelligently exploited in

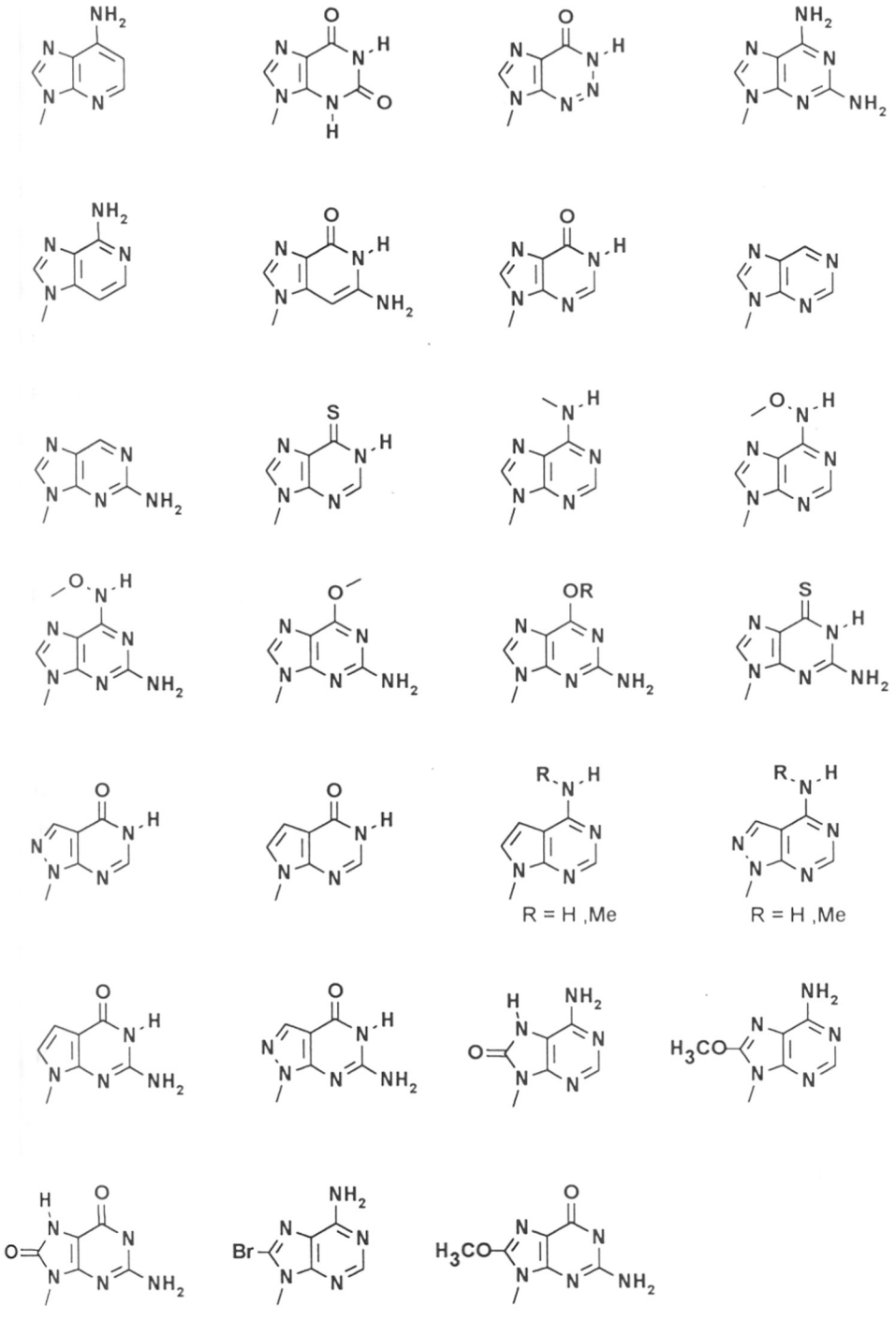


Figure 13a Purine modifications.

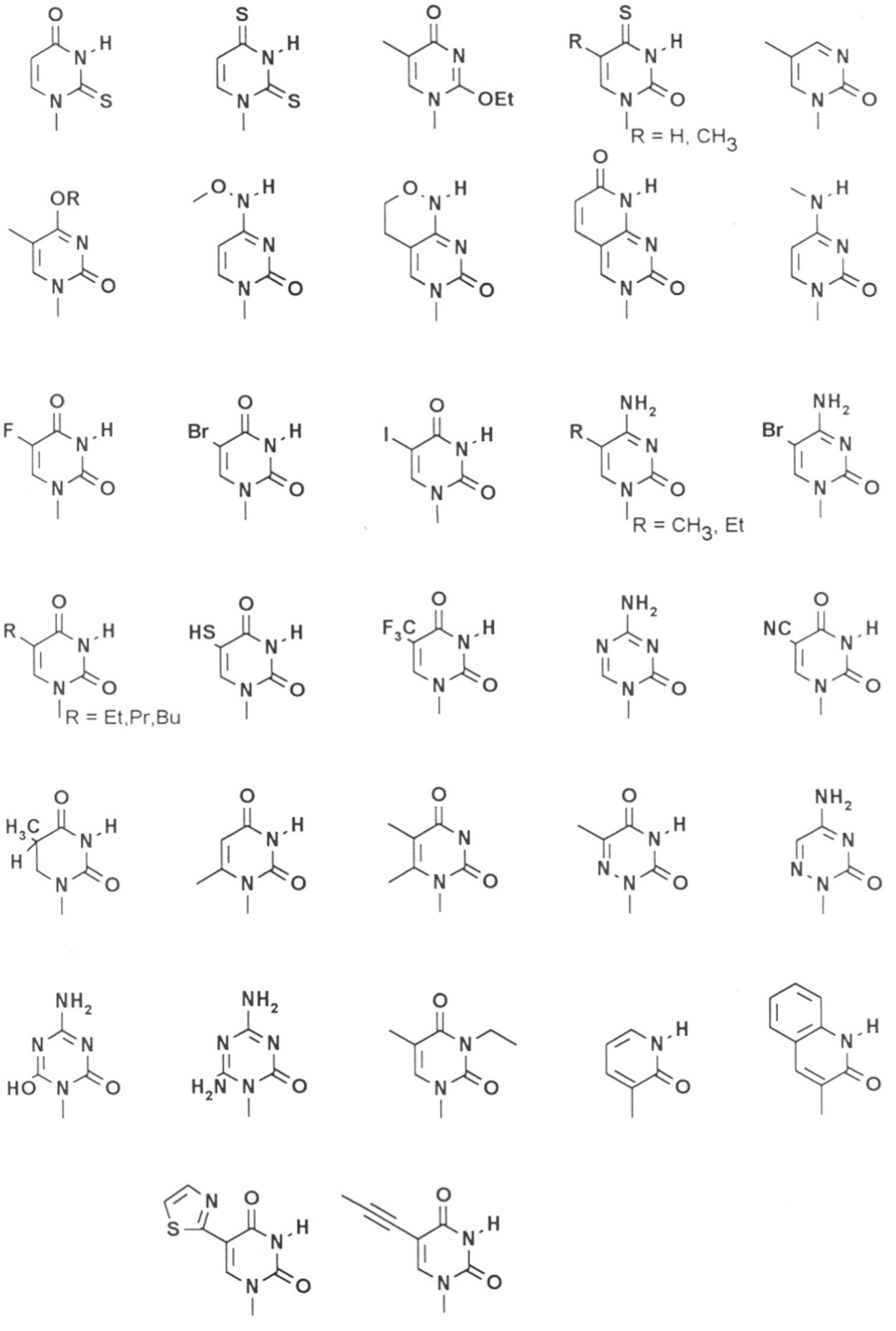


Figure 13b Pyrimidine modifications.

synthesis of ODN's containing 3-nitro pyrrole as a heterocyclic base (Nicholas et al., 1994; 1995). This although cannot form complementary hydrogen bonding with any of the standard bases, stabilizes adjacent base stacking due to favorable interaction of dipole moments/dispersion forces in 3-nitropyrrole ring. Modified DNA with this base has been utilized successfully in priming DNA sequencing and PCR applications. 5-Heteroaryl (Gutierrez et al., 1994) and 5-propenyl 2'-deoxyuridine (Froehler et al., 1992) are shown to enhance the duplex stability since these modifications do not interfere in standard Watson-Crick hydrogen bonding and provide additional stability from better stacking interaction. Several excellent reviews are available on base modifications achieved till now and their effects on DNA duplex structure (Sanghvi 1993; Englisch & Gauss, 1991; Beaucage & Iyer 1993).

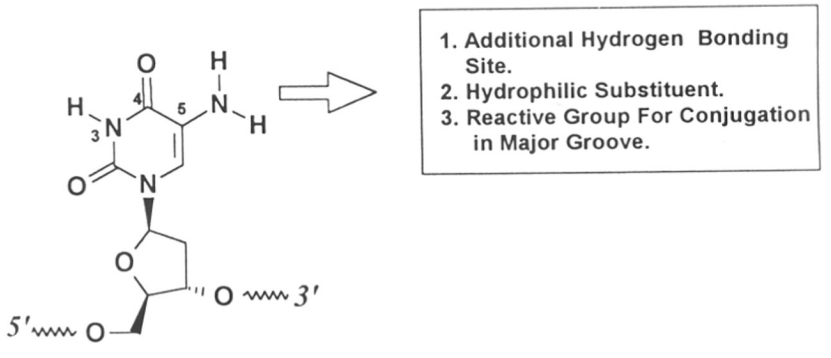


Figure 14. 5-Amino-2'-deoxyuridine.

1.3.1. *Present Work*

In this context, the utility and applications of a new pyrimidine substituent, 5-amino-deoxyuridine, has been described in Chapter 2 of this thesis. The introduction of an amino group at 5-position of a pyrimidine has several interesting consequences from the point of DNA molecular recognition (Figure 14). This position is not involved in standard Watson-Crick base pairing and hence modification will not perturb this

complementation. It introduces a new hydrogen bonding center in the major groove of DNA thus providing an additional repertoire for molecular recognition. C5 of a pyrimidine is normally a hydrophobic center with either a methyl group in T or a hydrogen in C. Amino substituent being hydrophilic, when present at C5, modifies the polarity characteristics of the major groove. 5-NH_2 group may also be useful to explore possibilities of a pyrimidine in the central strand of a triple helix since hydrogen bonding is possible from both directions of this pyrimidine. Finally, an amino group provides a reactive center to derivatize a pyrimidine with useful ligands. Chapter 2 present studies on introduction of 5-amino-deoxyuridine in oligonucleotides to investigate some of the above possibilities.

1.4. OLIGONUCLEOTIDE CONJUGATES

Oligonucleotides carrying a reactive group (chemical/physical) are emerging as new performance molecules since they can be used to carry out several intelligent operations on target DNA depending on the conjugated group. They can covalently modify the binding partner, stabilize DNA structures or achieve targeted DNA cleavage. The reactive functions are conjugated to DNA at either 5' or 3'-terminus or at internal sites by linking to heterocyclic bases, sugars or phosphates (Goodchild, 1990). The groups include psoralen (photocrosslinker) (Pieles & Englisch, 1989; Piles et al., 1989), metal complexes derived from EDTA (Dreyer & Dervan, 1985), phenanthroline (Chen & Sigman, 1986), porphyrins (Doan et al., 1986), bleomycin (Hecht, 1986), desferal (Joshi et al., 1994), cis-platin (Naser et al., 1988) and even enzymes and proteins such as nucleases (Corey et al., 1989). These have utility as targeted cleavage agents. Oligonucleotides covalently linked to a reporter molecule such as a fluorescent dye (fluorescein, rhodamine, acridine, etc.) or affinity ligand (biotin) are useful as hybridization probes. As mentioned earlier, conjugation of DNA with intercalators would improve the DNA duplex or triplex stability. Several important biochemical applications

are emerging by use of these molecules, the most significant ones being detection and localization of m-RNA or its gene. Many medical applications require detection of bacterial or viral sequences in infected hosts. There is also a great interest in inhibition of translation of m-RNA and transcription of DNA via "antisense" oligonucleotides and these involve ODNs conjugated with reactive ligands (Crooke & Lebleu, 1993).

The ligands may be coupled to oligonucleotides either through reactive sites already present in nucleic acids or via linker groups introduced specifically for this purpose. The inherent groups on nucleic acids useful for conjugation are, the exocyclic amino groups on bases, the terminal 5'/3' hydroxyl groups on sugars (DNA) and the 2'-OH of RNA, or the internal and terminal phosphates. The coupling may be achieved via the primary amine, thiol, aldehyde or COOH groups on linkers. Such moieties can be introduced into ODN at one of the three stages of DNA synthesis. (i) In the dedicated monomer strategy, the group is linked to nucleotide before incorporation. The modified monomer can be used either for chemical or enzymatic synthesis of oligonucleotide. The positions amenable for this type of modification are heterocyclic bases and 2'-OH of riboses. (ii) The modification can be effected after synthesis and deprotection of oligonucleotides, as in convertible nucleoside approach. (iii) Some linkers may be introduced during or at the end of oligonucleotide assembly eg. a glycerol backbone (Misiura et al., 1990) or a 5'-amino linker. After deprotection, the reactive group is coupled to this linker-ODN.

1.4.1. *Incorporation of Conjugates and Linker Groups during Chemical Synthesis of Oligonucleotides.*

From a synthetic point of view, incorporation of conjugates and linkers during the assembly of an oligonucleotide rather than afterwards is the most rigorous approach. It gives greatest control over the number and location of the modifications; side reactions are minimized by the protecting groups on the nucleotides, and advantage is taken of

the benefits of solid-support synthesis for workup and purification. This approach is appealing for chemical synthesis as the nucleotide building block carrying the desired modifier can be introduced precisely at any internal or terminal position in the oligonucleotide with assurance that modification at that position is complete. As a result, concerns over the uniformity of the product and its identity should be less than those with some other methods. However, it is necessary to first prepare and purify the nucleotide, and the modifications must be able to withstand the coupling reactions and the rigors of acidic and basic deblocking conditions. In some cases nonstandard protecting groups may be necessary. Though substituents may be attached to nucleotides at the base, sugar or phosphate residues, ideally, such changes should not interfere with hybridization. Two sites on bases which are easy to manipulate chemically without preventing base pairing are C5 of 2'-deoxyuridine and N^4 of 2'-deoxycytidine, several nucleotide phosphoramidite synthons have been prepared with protected linkers at these positions (Table 1).

Table 2: Linkers incorporated into oligonucleotides

Position	Substituent	Refs
N^4 of dC	$(CH_2)_6OH$	Horn & Urdea, 1989
	$(CH_2)_4NH_2$	MacMillan & Verdine, 1990
	$(CH_2)_2OH$	Ferentz, et al., 1993
	CH_2CO_2H	
	$(CH_2)_2S\text{-}S(CH_2)_2NH_2$	
	$(CH_2)NHCO(CH_2)_5NH_2$	Urdea et al., 1988
C5 of dU	$(CH_2)_3NH_2$	Gibson & Benkovic, 1987; Meyer et al., 1989; Allen et al, 1989
	$HC{=}CH(CH_2)_2NH_2$	Haralambidis et al, 1987
	$HC{=}CHCONH(CH_2)_6NH_2$	Jablonski et al, 1986

A number of nucleotides already bearing conjugate groups at above positions have been used. Examples are EDTA (Dreyer & Dervan, 1985), biotinyl, dinitrophenyl, pyrenyl and dansyl groups (Roget et al, 1989), 5-heteroaryl-2'-dU analogs to enhance

thermal stability with complementary RNA (Gutierrez, et al., 1994), and covalently linked DNA-peptide conjugates to alter transport and reactivity properties of DNA (Bashkin, et al., 1991). In case of purines, C8 of adenine was used as an attachment site for the photoactivable cross-linking reagent psoralen (Pieles et al., 1989) and for dansyl conjugation (Singh et al., 1990).

The strategy used for incorporating modified nucleotides chemically can be applied to enzymatic synthesis of oligonucleotides. A variety of DNA and RNA polymerases can be used for this purpose, but most commonly employed are *Escherichia coli* DNA polymerase I and terminal deoxynucleotidyl transferase. Several modified nucleotide triphosphates have been prepared and incorporated enzymatically (Table 3).

Table 3: Linkers and conjugate groups incorported enzymatically into oligonucleotide.

Position	Substituent	Refs
C5 of dUTP	naphthalene derivative	Allen et al., 1989
	biotin	Langer et al., 1981; Shimkus et al., 1985
	$(CH_2)_{12}NH_2$	Kumar et al., 1988
	$HC{=}CHCH_2NH_2$	Kumar et al., 1988
	N_3	Evan et al., 1986
	fluorescein	Trainer & Jenson, 1988
	SCH_3	Iveron & Dervan, 1987
N^4 of dCTP	$(CH_2)_6NH_2$	Gillam & Tener, 1986
C8 of ATP	$NH(CH_2)_6NH_2$	Kumar, et al., 1988
	2,4-dinitrobenzene	Vincent et al., 1982
N^6 of ATP	$CH_2CONH(CH_2)_6NH_2$	Kumar et al., 1988

1.4.2. *Incorporation of Nonnucleotides*

A number of non-nucleotidic phosphoramidite reagents have been developed for incorporation during oligonucleotide synthesis (Figure 15). They are used most

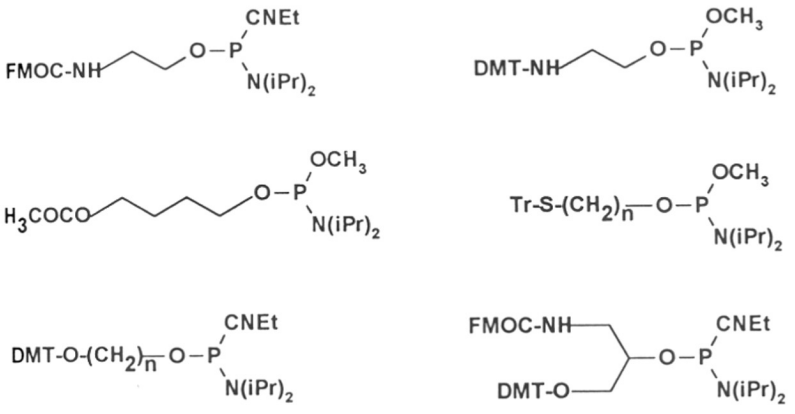


Figure 15. Non-nucleotidic nucleophilic linkers for preparation of oligonucleotide conjugates.

commonly to link a group at the 5'-end of an oligonucleotide while it is still fixed to the solid support. Most groups introduced in this way have been used to give, after deblocking, a nucleophilic linker for preparation of conjugates.

Another site amenable for ready modification during oligonucleotide synthesis is the internucleoside phosphate. This approach involves generation of phosphoramidates during *H*-phosphonate synthesis (Jager et al., 1988; Letsinger et al., 1988). This has been used to introduce a protected amino linker for post-synthetic derivatization. Another easily accessible phosphate derivative is the phosphorothiote, the sulfur atom reacts readily with alkylating reagents (Cosstick et al., 1984; Fidanza & McLaughlin, 1992; Agrawal & Zamecnik, 1990).

1.4.3. *Post synthetic modifications of oligonucleotides*

Conjugate groups are also introduced after the synthesis and deblocking of the oligonucleotide. As oligonucleotides are polyionic, postsynthetic reactions are usually performed in water or in an aqueous solvent in which the reagents must be sufficiently soluble and stable. This, in itself, is restricting as very few synthetic organic reactions

meet these conditions. Post-synthetic reactions may also lead to oligonucleotides bearing different numbers of conjugate groups per oligonucleotide chain depending upon the extent of reaction, and as a result, purification and characterization of products prove difficult.

The length of the spacer arm used to link the active group to ODN has an important role in the final performance of ODN (Englisch & Gauss, 1990; Teare & Wollenzien, 1989). For intercalating groups, small changes in spacer length may decisively influence its effect. A short spacer of two carbons is required for crosslinking psoralen to the opposite strand. A longer spacer of eleven to twelve atoms was preferable to link biotin and fluorescent tags in order to minimize a steric inhibition of hybridization.

1.4.4. *Non-radioactive probes: Fluorescent Nucleic Acids*

Radiolabelling of nucleic acids (^{32}P, ^{35}S) although highly sensitive for detecting hybridization efficiency, has major disadvantages such as short half life and pose problems of health hazard, storage and disposal. This places limitations on their use as nucleic acid hybridization probes, particularly for routine clinical diagnosis of diseases. Hence, efforts to develop alternative, non-radioactive labels (Kricka, 1992) are assuming importance. The detection of hybridization using nucleic acid probes with target DNA may be accomplished directly or indirectly by use of appropriate labels. The detection sensitivity may be enhanced by amplification of either the direct/indirect signal or target DNA. The main non-radio labels employed are biotin (Misiura et al., 1990; Pieles et al., 1990), and fluorophores (Chehab & Kan, 1989). Biotin is detected by its high affinity binding to avidin or streptavidin. The binding signal can be amplified by coupling this assay to enzyme reactions which produce colour or light e.g. peroxidase, etc. ELISA type of detecting systems are also used to obtain enhanced signals (Voss et al., 1991). A direct method of detecting hybridization is the use of fluorescent probes. DNA can be labelled with single or multi fluorophores to increase the sensitivity. Any of

the detecting systems would have problems of background signal, non-specific binding, etc. and requires suitable design of experimental protocols to eliminate or minimize these artifacts.

Alternative method of target amplification to increase the amount of sample is possible by the revolutionary technique of PCR (Erlich, 1989). PCR employs 2 primers for target DNA duplex and by repeated cycles, each comprising of denaturation, annealing and polymerization, the target DNA can be amplified several million folds. The PCR experiment is amenable to several variations to improve its utility. When coupled with the use of non-radioactive labelled primers, PCR offers a unique way of enhancing both the signal and the target. Fluorescent primers lead to amplified DNA which is fluorescent, thus making the detection easy with least manipulations (Chehab & Kan, 1989). Alternatively, fluorescent triphosphates can also be used in PCR as substrate leading to a high degree of labelling of target DNA.

Fluorescent nucleic acids have also found utility in biophysical studies of DNA interactions with other molecules. Some notable examples are interaction with Klenow DNA polymerase I (Guest, et al., 1991), interaction with minor groove binding drug netropsin (Patel et al., 1992), helicase catalyzed DNA unwinding (Bjorson et al., 1994), determination of 4-way DNA junction (Clegg et al., 1992) and DNA-DNA interaction at duplex and triplex level (Cardullo et al., 1988; Mergny et al., 1994).

1.4.5. *Present Work*

Chapter 3 describes synthesis of fluorescent nucleosides 5-aminodansyl, 5-amino indoleacetyl and 5-amino carboxyfluorescein derivatives of 2'-deoxyuridine and their incorporation into DNA. The fluorescent 5-amino dansyl ODN was employed to investigate DNA-netropsin interactions and the polarity of major groove. The indoleacetyl

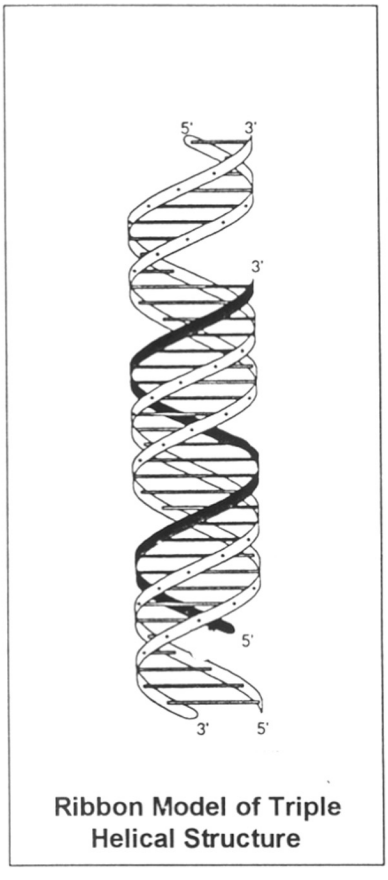
ODN derivative was used along with 5-amino dansyl to examine DNA-DNA duplex interaction by FET. The fluorescein labelled primers when employed in PCR reaction has been demonstrated to give good amplification.

1.5. TRIPLE HELIX AND DNA MOLECULAR RECOGNITION

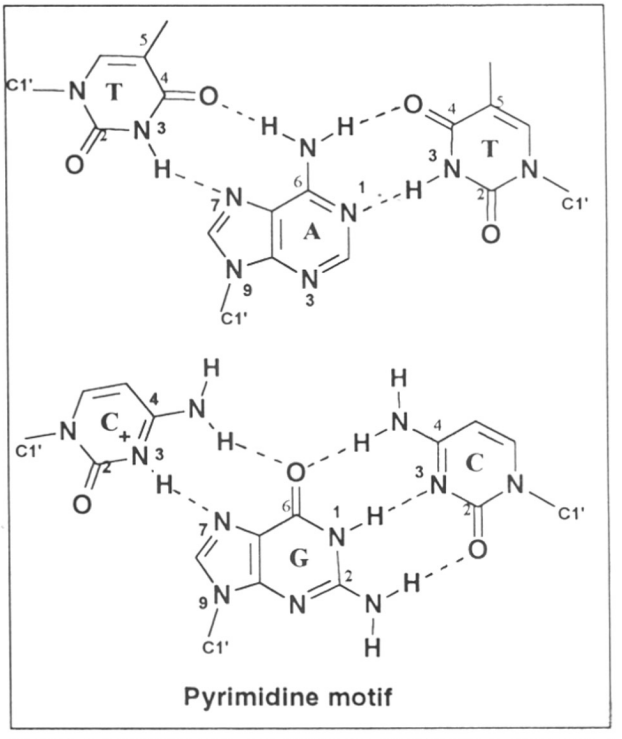
1.5.1. *Triple Helix Moitifs*

One of the most recent exciting developments in DNA molecular recognition with a high potential for practical utility is the discovery of oligonucleotide-directed triple helix formation (Moser & Dervan 1987; Uhlmann & Peyman, 1990; Thuong & Helene, 1993; Radhakishnan & Patel, 1994a). This is a versatile structural motif for sequence specific recognition of double helical DNA. It is possible to form base triads T*A:T and C^+*G:C (Figure 16) in which Hoogsteen base pairing recognition of Watson-Crick base pairs A:T and G:C from the major groove side by pyrimidine bases leads to formation of triple helix. Two important features of this motif are that (i) while A:T base pairs are recognized by neutral T, G:C Watson-Crick pair requires protonated C^+ for triad formation with full complementation and (ii) a purine must occupy the central position of the triad. These limitations have led to exploration of base pair recognition diversity in nucleic acids to accommodate other Watson-Crick base pairs.

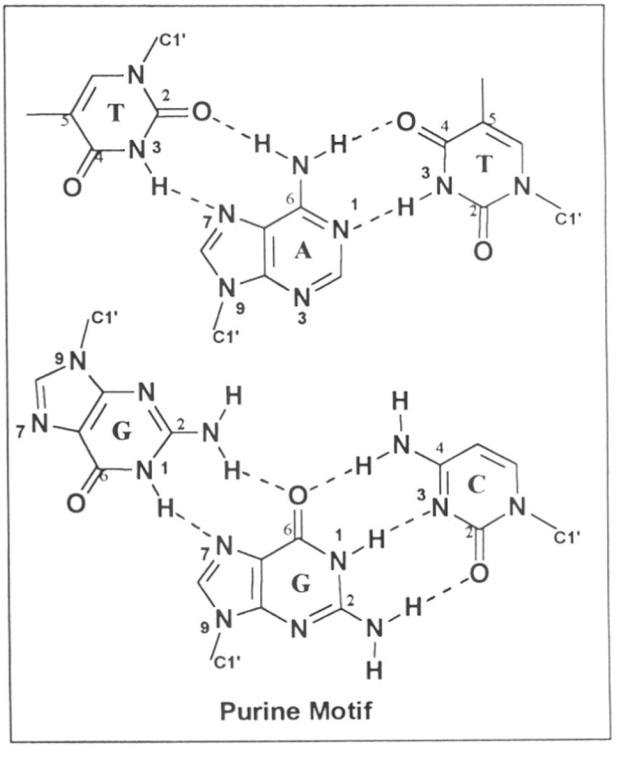
In DNA triple helix, an additional strand of DNA binds to the major groove of a Watson-Crick duplex. The formation of such structures in general, is restricted to homopurine-homopyrimidine DNA sequences, where purine bases participate in Hoogsteen interactions with bases in third strand. In this motif called the pyrimidine motif, the third strand (H strand) is parallel to the purine strand (Moser & Dervan, 1987). Alternatively, in the purine motif, (Cooney et al., 1988; Beal & Dervan, 1991) the third strand consisting of guanines, adenines and thymidines recognize specific bases giving rise to G*G:C and A*A:T and T*A:T triples. Here the third strand is antiparallel to the middle purine strand. Triple helices composed of combinations of these two motifs can



Ribbon Model of Triple Helical Structure



Pyrimidine motif



Purine Motif

Figure 16.

also be formed with some sequence contexts. Triple helix formation is sensitive to several structural and environmental factors such as the length of the third strand (Singleton & Dervan, 1992b), single base mismatches (Moser & Dervan, 1987; Rougee et al., 1992; Best & Dervan, 1995), pH (Povsic & Dervan, 1989; Xodo et al., 1991), cation concentration and valence (Sigleton & Dervan, 1993), temperature and backbone composition of the three strands (Roberts & Crothers, 1992; Han & Dervan, 1994; Escude et al., 1993).

An important factor in the stability of triple helical complexes is the protonation at N3 of C in third strand which is favoured at acidic pH (~5.8-6.0) (Povsic & Dervan 1989). The potential applications of oligonucleotide-directed triple helix formation for repressing gene expression (Cooney et al., 1988; Maher et al., 1989) *in vivo* has stimulated efforts to stabilize protonated C$^+$*G:C by oligonucleotide modification and to design nucleosides capable of GC base pair recognition without protonation. Replacing C in third strand with 5-Me-C increases the apparent stability and the pH limits of triple helix complexes. ODNs containing 5-Me-C are efficient inhibitors of site-specific DNA binding by proteins near pH 7.0. Several other nucleobase modifications have been attempted (Table 4) with varying degrees of success in triplex stabilization.

Table 4a: C modifications in pyrimidine motif (C$^+$*G:C) at extended pH.*

No	Base (H)	C:W	pH	Stability	Reference
1	dC	G:C	5.8	-	Povsic & Dervan, 1989.
2	5-Me-dC	"	6.8	+	Singleton & Dervan, 1992b; Xodo et al., 1991; Lee et al., 1984.
3	5-propyne-dC	"	6.6	-	Froehler et al., 1992.
4	2'-O-methyl-5-Me-C	"	5.0	-	Shimizu, et al., 1994a.
5	Carbocyclic 5-Me-dC	"	7.2	+	Froehler & Ricca, 1992.
6	N^4-(3-carboxypropyl)-dC	"	7.0	-	Miller & Cushman., 1992.
7	N^4-(4-aminobutyl)-dC	"	7.0	-	"
8	N^4-(butyl)-dC	"	7.0	-	"
9	6-amino-2'-O-methyl-C	"	7.2	-	Pudlo et al., 1994.
10	N^4,N^4-ethano-5-Me-dC	"	8.3	+	Shaw et al., 1991.

Table 4b: Base analogs to mimic pyrimidine motif (C⁺*G:C) at physiological pH.

No	Base (H)	C:W	pH	Stability	Reference
1	2'-O-Methyl-pseudoisocytidine	G:C	7.2	+	Ono et al., 1992.
2	Pyrazole P1	"	7.4	+	Koh & Dervan., 1992. Radhakrishnan et al., 1993.
3	Pyrazole P2	"	7.4	-	Koh & Dervan, 1992.
4	N^6-methyl-8-oxo-A	"	7.6	+	Krawczyk et al., 1992.
5	8-oxo-A	"	8.0	+	Miller et al., 1992; Jetter & Hobbs 1993.
6	M^{5ox}-dC	"	8.0	+	Xiang et al., 1994.
7	deoxyinosine	"	5.0	-	Shimizu et al., 1994b.
8	deoxyxanthosine	"	5.0	-	"
9	dG	"	5.0	-	"

Table 4c: Modified bases in T*A:T triplets in pyrimidine motif.

No	Base (H)	C:W	pH	Stability	Reference
1	5-Br-dU	A:T	7.0	+	Povsic & Dervan, 1989.
2	5-propyne-dU	A:T	6.4	+	Froehler et al., 1992; Colocci & Dervan, 1994.
3	Carbocyclic T	A:T	6.6	-	Froehler & Ricca, 1992.
4	8-oxo-dA	A:T	7.0	-	Miller et al., 1992.
5	8-oxo-dG	A:T	5.0	+	Shimizu et al., 1994b.
6	N^4-(6-aminopyridinyl)-dC	A:T	7.0	+	Huang & Miller, 1993.

Table 4d: Base analogs to recognize pyrimidine in central strand (pyrimidine motif).

No	Base (H)	C:W	pH	Stability	Reference
1	4-phenylimidazole D2	C:G	7.4	-	Griffin et al., 1992.
		T:A	"		
2	4-(3-benzamidopropyl)-imidazole)	C:G	7.4	+	"
	D3	T:A	"	+	
3	N^4-(3-acetamidopropyl)-dC	C:G	7.0	+	Huang et al., 1993.
4	N^4-(3-acetamidopyidinyl)-dC	C:G	7.0	+	Huang & Miller., 1993.
5	dG	T:A	7.0	+	Griffin & Dervan, 1989; Wang et al., 1992.
6	T	C:G	7.0	+	Mergny et al., 1991; Radhakrishnan & Patel, 1994.
7	dC	U:A	7.0	+	Miller & Cushman, 1993.
8	T	T:A	8.0	-	Goodwin et al., 1994.

Table 4e: Base modifications in purine motif.

No	Base (H)	C:W	pH	Stability	Reference
1	2'-deoxynebularine	C:G	7.4	+	Stilz & Dervan, 1993.
		A:T	"	+	
2	T	C:G	6.5	+	"
3	5-F-dU	C:G	6.5	+	Durland et al., 1994.
		A:T	6.5	+	
4	pyridine-2-one	C:G	6.5	-	"
5	pyridine-4-one	C:G	6.5	+	"
6	2'-deoxyformycin	C:G	6.5	+	Rao et al., 1994.
7	7-deaza-2'-deoxyxanthosine	A:T	7.2	+	Milligan et al., 1993.
8	2'-deoxy-9-deazaguanosine	G:C	7.6	+	Rao et al., 1993.
9	Imidazole	T:A	8.0	+	Gee et al., 1995.
10	2'-deoxy-6-thioguanosine	G:C	7.6	+	Rao et al., 1995.

* **H**, Hoogsteen (third strand); **C**, Crick (central strand); **W**, Watson (second strand). + indicates stable triplex formation, while - indicates less stable triplex formation.

Several experimental techniques have been employed to follow triple helix formation and stability. These include affinity cleavage (Beal & Dervan, 1992a; Schimizu et al., 1994b), optical melting (Mergny et al., 1991; Miller & Cushman, 1993) and gel retardation (Yoon et al., 1992). The specific interactions implied in triple helix formation have been well characterized by NMR spectroscopy (Live et al., 1991; Radhakrishnan & Patel, 1994a). Quantitative affinity cleavage experiments have recently given data on energetics of formation of triple helical complexes in which a single position within a triad was variable and found that T*A:T > T*G:C, T*C:G or T*T:A and C^{+}*G:C > ^{Me}C*A:T, ^{Me}C*C:G or ^{Me}C*T:A, and G*T:A > G*A:T, G*C:G or G*G:C (Best & Dervan, 1995).

The recognition of mixed DNA sequences in duplex by triple helix formation would find several important applications in not only DNA structure-function analysis, but also in development of therapeutic agents based on selective gene inactivation. The search for such a general solution to triple helix mediated recognition would involve sequence context effects as well and recent studies have extended the pyrimidine motif through

incorporation of both purine and non-natural bases (Best & Dervan 1995). The best characterized of these are G-TA, D_3-TA and P_1-GC triplets (Figure 17).

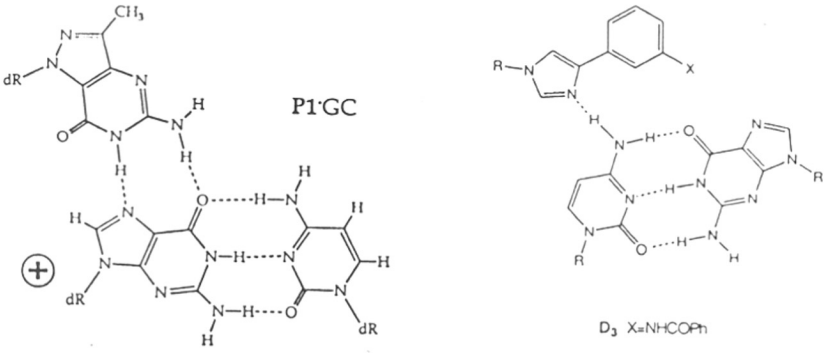


Figure 17. Design rationale for recognition of Watson-Crick C:G base pair by non-natural bases D_3 and P_1 within pyrimidine-purine-pyrimidine triple helix motif.

1.5.2. *Stability of Triple Helices*

A very important parameter for stabilization of triple helices is the control of electrostatics by pH and ionic strength of the medium (Volker & Klump, 1994). Formation of triple helix is accompanied by an increase in charge density resulting from three negatively charged phosphate backbones (sequence independence effect). In addition, the interaction of adjacent protonated cytosines with each other (sequence dependent local effect) and that of protonated cytosines with the backbone (global effect) modulates the stability of triplexes. Thus, depending on the number of C^+ in third strand and their relative positions, the binding affinity of Hoogsteen strand can either enhance or decrease i.e. the local effects can reverse the effect of global composition. Although several groups have attempted at thermodynamic study of triple helix formation, there are discrepancies in the literature between calorimetrically dependent enthalpy values and those derived from UV-melting curves (Volker & Klump, 1994).

The increased negative charge density can be overall compensated by inclusion of cations in the buffer system (Wilson et al., 1994). Thus, divalent cations such as Mg^{++} achieve better stabilization of triple helices compared to monocations. A very interesting and useful case is that offered by polycation spermine which has 4 positive charges at neutral pH. Spermine binds to B-DNA and produces a conformational change from B to Z at physiological pH, induces helical bending and DNA condensation at high concentrations. X-ray structure of A-DNA octamer ($d(GTGTACAC)_2$ shows spermine bound deep in the major groove of the helix (Jain et al., 1989), while that of a Z-DNA $d(CGCGCG)_2$ shows 2 modes of binding interaction with phosphate backbone as well as with base pairs (Bancroft et al., 1994). The structural simplicity of polyamine is deceptive and it is possible that both specific and non-specific interactions may be involved. Much of the solution studies point to spermine binding in major groove of B-DNA. However, considerable ambiguity exists on the nature of binding and sequence specificity of binding. Although there is no general agreement in literature on these points, much of the experimental and theoretical data has been recently rationalized with a model in which spermine may bind in the major groove across the base pair or down the groove (Haworth et al., 1991) (see chapter 4). Spermine is known to favour triple helix formation and in principle, the major groove of duplex can accommodate both the spermine as well as third strand of DNA. However, it is not clear at present how this stabilization is achieved at molecular level.

1.5.3. *Present Work*

In view of the importance of this, **Chapter 4** of the present thesis describes covalent linking of a spermine specifically at N^4 of 5-Me-dC followed by its incorporation into oligonucleotide. Studies on duplex and triple-helix forming potential of these spermine conjugated oligonucleotides are reported and triple helix stabilization is discussed.

This chapter describes the modes of molecular recognition of nucleic acids by drugs, proteins, metal ions etc. The importance of nucleobase modification on structure and molecular recognition have been discussed. Attention is also drawn at the end of each section, to the relevence of these to the work presented in the rest of the thesis. No attempt is made to exhaustively cover all the literature on this topic. However, most leading references have been cited along with typical examples to illustrate the principle in the context of the thesis work.

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CHAPTER 2

EFFECT OF C5-AMINO SUBSTITUENT ON 2'-DEOXYURIDINE BASE PAIRING WITH 2'-DEOXYADENOSINE AND INCORPORATION OF 5-AMINO-2'-DEOXYURIDINE INTO OLIGONUCLEOTIDES.

2.1. INTRODUCTION

The specific association of purine and pyrimidine bases is fundamental to the function of nucleic acids which are involved in stable storage, transmission and expression of genetic information. Forces that contribute to stability of double stranded nucleic acids can be conceptually decomposed into base-base hydrogen bonding interactions (horizontal) and stacking interactions (vertical). The hydrogen bonding between A and T and between C and G bases proposed by Watson-Crick in 1953, provides an explanation for the specificity of pairing. However, a complete understanding of the physical basis for this observed complementarity, has proved difficult. Of late, this has rekindled interest due to the importance of structurally modified bases in controlling DNA association and in generation of novel higher order DNA structures such as triplexes and quadruplexes. These have also attracted attention from a therapeutic angle since DNA is now emerging as a novel medicinal agent in antisense approach.

There have been several approaches to model the nucleic acid interactions. The properties of nucleic acid monomers have provided considerable insight into those of polymeric nucleic acids. The hydrogen bonding interactions in the low dielectric environment of the interior of polymeric nucleic acids can be modelled by soluble nucleic acid monomers in non aqueous solvent such as chloroform and DMSO. Spectroscopic technique such as NMR (^{1}H and ^{13}C) and IR are particularly useful in identifying hydrogen bonds in these complementary base pairs and characterization of important structural motifs such as Watson-Crick and Hoogsteen base pairing (Williams et al., 1989; Kyogoku et al., 1969). While WC base pair dimers are observed between monomeric G and C in low dielectric medium, higher order structures such as $C:G_2$ trimers and $(C:G)_2$ tetramers have also been seen to co-exist (Williams et al., 1989).

The effect of substituents on purine and pyrimidine bases, in particular, on their ability to form complementary hydrogen bonding pairs is not only important but also has

practical relevance in designing new antisense agents. The substituents may directly affect the delicate balance of the electronic and geometric complementarity existing in the natural base pairing. It has been demonstrated from IR studies (Kyogoku et al., 1967) of modelled substituted bases (1-cyclohexyl-uracil and 9-methyl-adenine) that, base substitution also determines the strength of association and the extent of self association relative to the complementary base pairing.

Substitution at 5-position of a pyrimidine base is known to affect its base pairing properties with complementary purines, either by altering the imino N3-H acidity or by modifying the acceptor strength of C2 and C4 carbonyls involved in hydrogen bonding (Kyogoku & Iwahashi, 1977; Saenger, 1984). 5-(Propynyl)-2'-deoxyuridine and 5-(Propynyl)-2'-deoxycyitdine significantly enhance double helix formation with single stranded RNA (Froehler et al., 1992; Sagi et al., 1993) The propyne modification is planer with respect to the heterocycle and provides additional stability through increased stacking with bases. The pyridopyrimidine base pairs with guanine more strongly than does natural cytosine and thus stabilizes the duplex. Substituents at 2 and 8 positions of a purine base may change their complementation strength, either electronically by affecting the hydrogen bond donor/acceptor potential or geometrically by shifting *syn-/anti* equilibrium. 2-amino-2'-deoxyadenosine forms additional hydrogen bond in Watson-Crick base pair with 2 keto of thymidine resulting in increased selectivity and hybridization strength (Chollet & Kawashima, 1988).

Recent interest in modified nucleosides and nucleotides is not only for their antiviral properties, but also for their applications in oligonucleotide therapeutics (Ulhmann & Peyman, 1990; Tamm & Strazewski, 1990; Goodchild, 1990; Englisch & Gauss, 1991). 5-Aminouridine and its acyl derivatives are known to posses a wide range of biological effects (Visser & Roberts, 1951; Smith & Visser, 1966; Ivanovics et al., 1971),

including antibacterial and antiviral activities and interference in purine biosynthesis. 5-Aminouridine partially replaces thymidine as a growth factor requirement in a thymine requiring strain of *Escherichia Coli* (Visser et al., 1963).

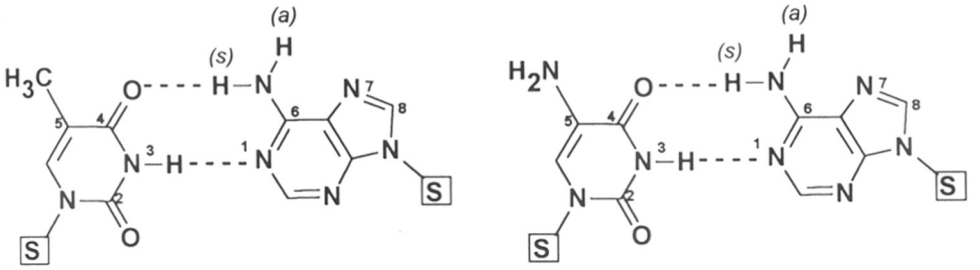


Figure 1. (**A**) Watson-Crick hydrogen bonding scheme in dT:dA and (**B**) $dUNH_2$:dA complexes. *s* and *a* refers to *syn* (hydrogen bonded) *anti* (non hydrogen bonded) protons on 6-NH_2 function of dA. **S** refers to 2'-deoxysugar of nucleosides.

In view of this, it was thought worthwhile to investigate the role of 5-NH_2 group on base pairing properties and its effect on DNA duplex stability. The normal strategy for understanding hydrogen bonding and DNA recognition involves study of effect of a substituent by incorporation of modified bases into DNA sequences such that a potential interacting site is removed from the DNA. 5-amino group on 2'-deoxyuridine is able to maintain the usual Watson-Crick hydrogen bonding scheme (Figure 1). It also exchanges the hydrophobic methyl group of dT with hydrophilic amino group of the same size in major groove and hence has potential to modify the major groove environment. 5-Amino-2'-deoxyuridine with additional hydrogen bonding sites, on incorporation into an oligonucleotide may have potential to study the effect of specific major groove interactions of DNA with other molecules.

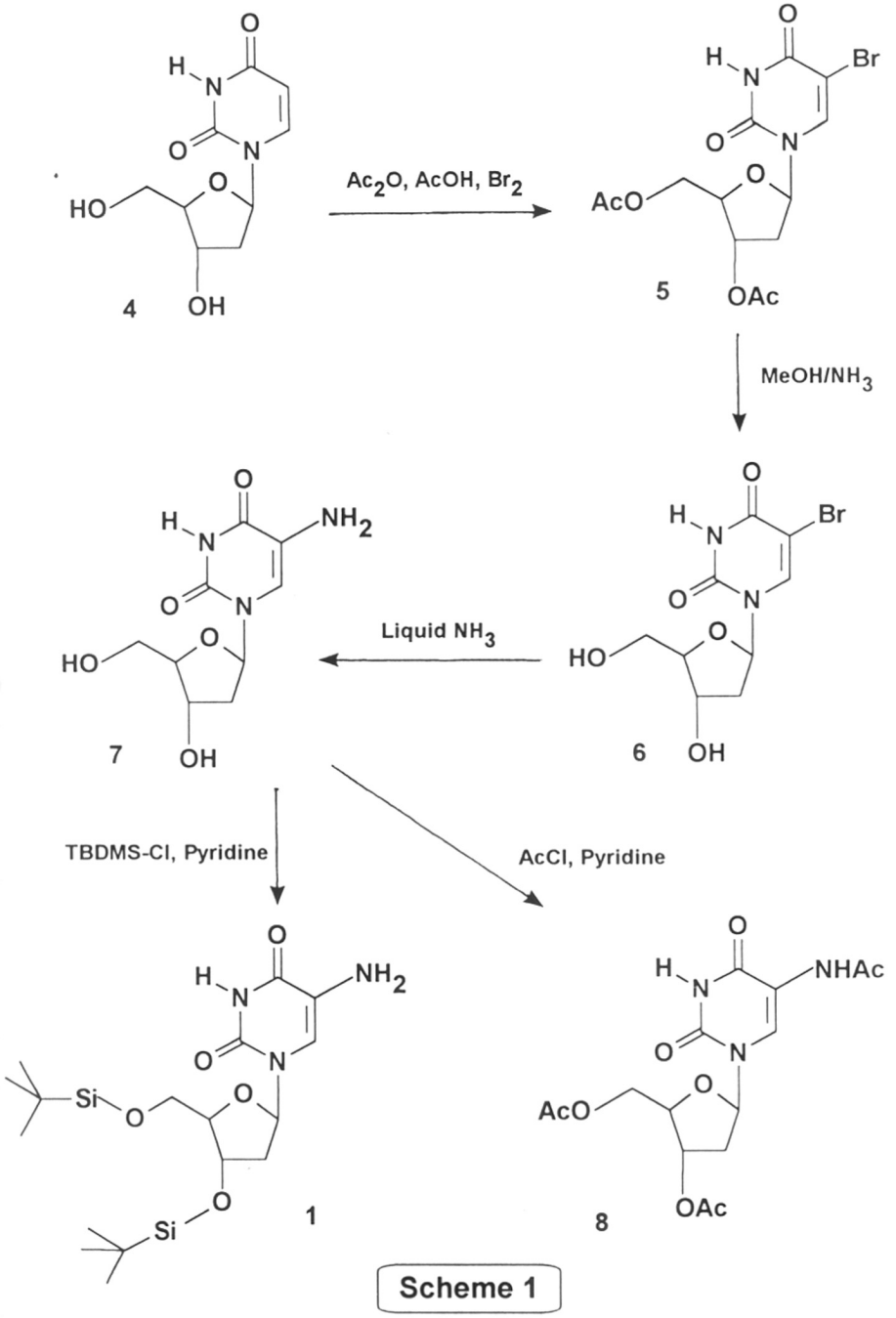
2.2. PRESENT OBJECTIVES

The objectives of this chapter are (i) chemical synthesis of 5-NH_2-2'-dU and study of the effect of 5-NH_2 group on hydrogen bonding ability of 2'-dU with complementary 2'-dA at monomer level (Figure 1) in lipophilic solvent such as chloroform and (ii) chemical incorporation of 5-NH_2-2'-dU into oligonucleotides using solid phase phosphoramidite chemistry (iii) the effect of 5-NH_2-2'dU on Tm of oligonucleotides and recognition/cleavage by restriction endonuclease *EcoR*1. The association at monomeric level is studied by ^1H and ^{13}C NMR by titrimetry and variable temperature techniques, while stability of 5-NH_2-2'-dU containing oligonucleotides are studied by UV spectroscopy.

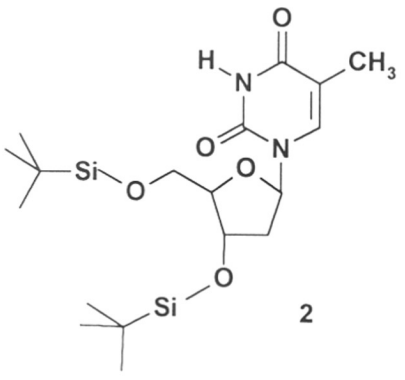
2.3. RESULTS AND DISCUSSION

2.3.1. *Synthesis and Characterization of bis(t-butyldimethylsilyl) (TBDMS) Derivatives of dUNH$_2$ 1, dT 2, and dA 3*

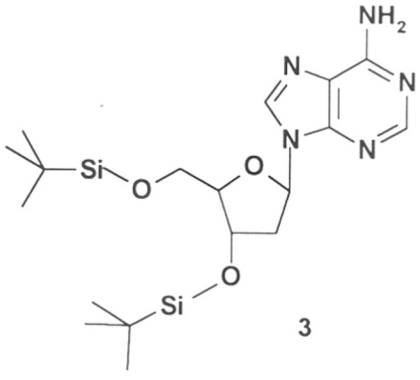
Williams et al., 1987 and Williams et al., 1989 have demonstrated that lipophilic [3',5'-bis(triisopropyl)] derivatives of 2'-deoxycytidine (dC) and 2'-deoxyguanosine (dG) can be usefully employed for ^1H NMR study of base pair formation through hydrogen bonding in non-aqueous solvents. These solvents inhibit the vertical stacking interactions of purines and pyrimidines and as the lipophilic derivatives are uncharged, interionic forces are also absent. In addition, the interactions are not modified by the stereochemical constraints which are present when nucleotides are arranged in a polynucleotide chain, and this allow one to isolate the horizontal hydrogen bonding interactions from other factors which are present at polynucleotide level. In view of this, similar lipophilic derivatives of the corresponding to dUNH$_2$ **1**, dT **2**, and dA **3** were used in the present study (Scheme 1). dUNH$_2$ **7** was synthesized from dU by literature procedure (Visser, 1968) as follows. 2'-dU **4** was first treated with acetic anhydride to yield the corresponding 3',5'-di-O-acetyl derivative, to which bromine in glacial acetic acid


Ac2O, AcOH, Br2
MeOH/NH3
Liquid NH3
TBDMS-Cl, Pyridine
AcCl, Pyridine
4
5
6
7
1
8

Scheme 1



2



3

was added in cold to give 3'-5'-di-O-acetyl-5-bromo-2'-dU **5**. The diester **5** was hydrolyzed with anhydrous methanolic ammonia to obtain 5-bromo-2'-dU **6**. The formation of 5-bromo derivative is shown by the disappearance of signal due to H5 and a downfield shift of H6 in ^{1}H NMR. Compound **6** on treatment with liquid ammonia in a steel bomb afforded 5-amino-2'-dU **7**. The displacement of 5-bromine by NH_2 group was indicated by an upfield shift of H6 in ^{1}H NMR (Figure 2a) accompanied by a downfield shift for C5 and an upfield shift for C6 in ^{13}C NMR (Figure 2b) as compared to 5-bromo derivative **6**. The amino compound **7** was also characterized by conversion into the corresponding 3'-5'-O-N-5-triacetyl-5-amino-2-deoxyuridine derivative **8**, that could be purified by silica gel chromatography. ^{1}H and ^{13}C NMR of **8** indicated presence of two O-acetates and one acetamido group. The nucleoside 5-amino-2'-dU **7**, thymidine (dT) and 2'-deoxyadenosine (dA) were converted into their 3',5'-bis(TBDMS) derivatives **1**, **2** and **3** respectively, using t-butyldimethylsilylchloride in DMF or pyridine following the literature procedure (Oglivie, 1973). The disilyl derivatives were purified by silica gel chromatography to spectroscopic purity (Figure 3).

2.3.2. *Binding Stoichiometry of dA:dUNH$_2$ complexation at ambient temperature*

The base pair formation between the soluble derivatives of 5-amino-dU and dA was investigated by following induced shifts of the exchangeable protons N3-H of dU

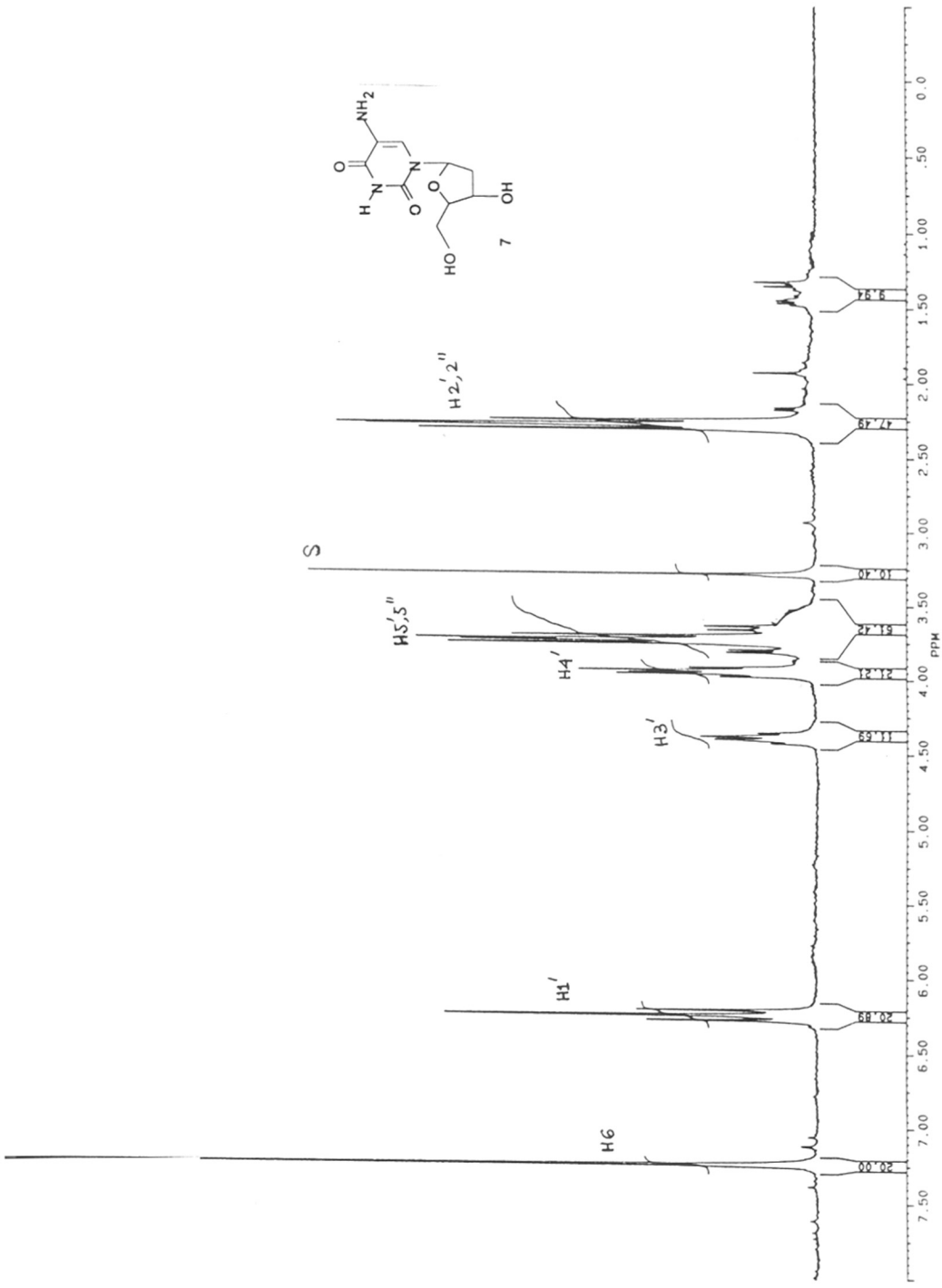


Figure 2a. ^{1}H NMR of 7.

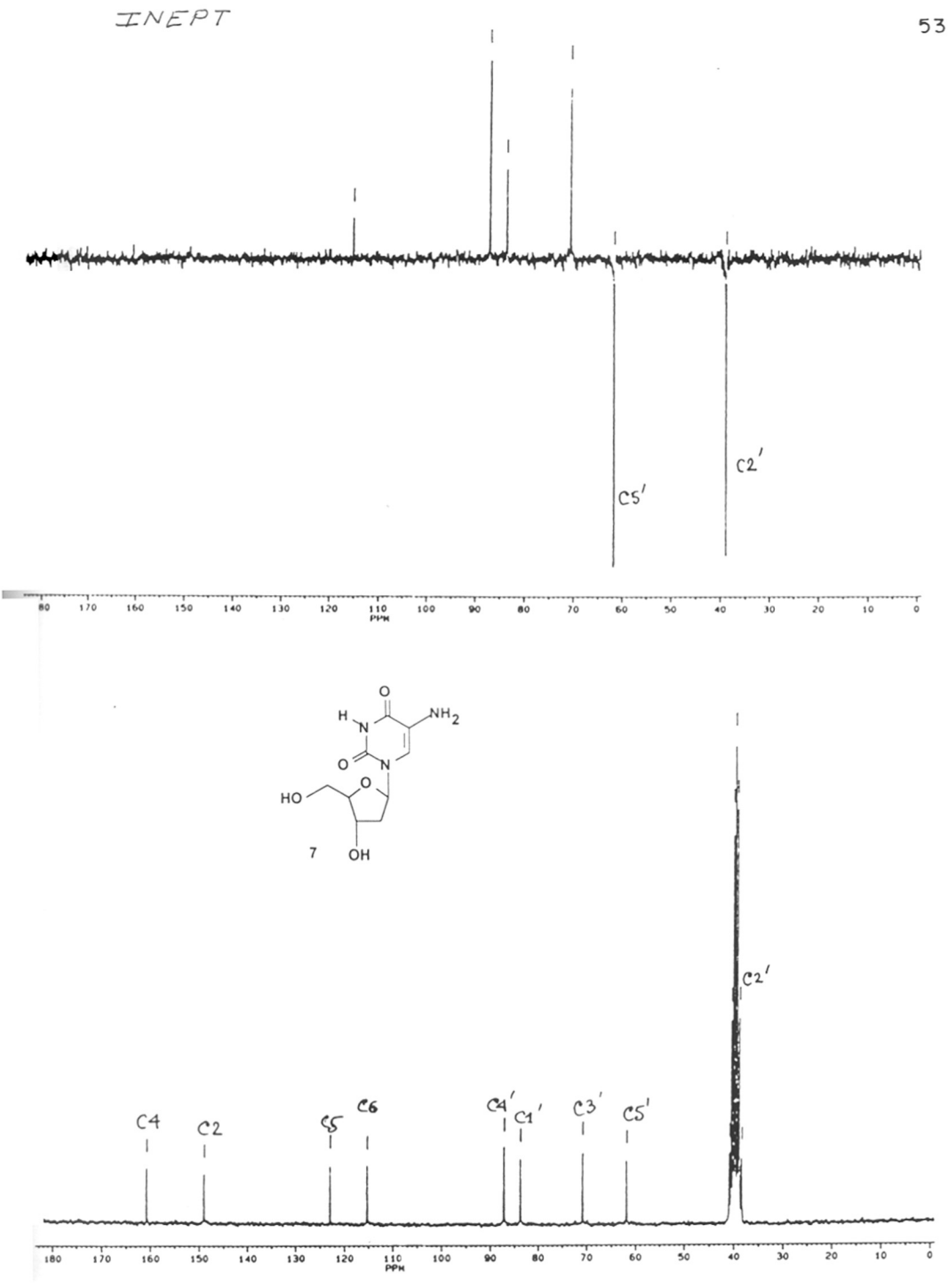


Figure 2b. ^{13}C NMR of **7**.

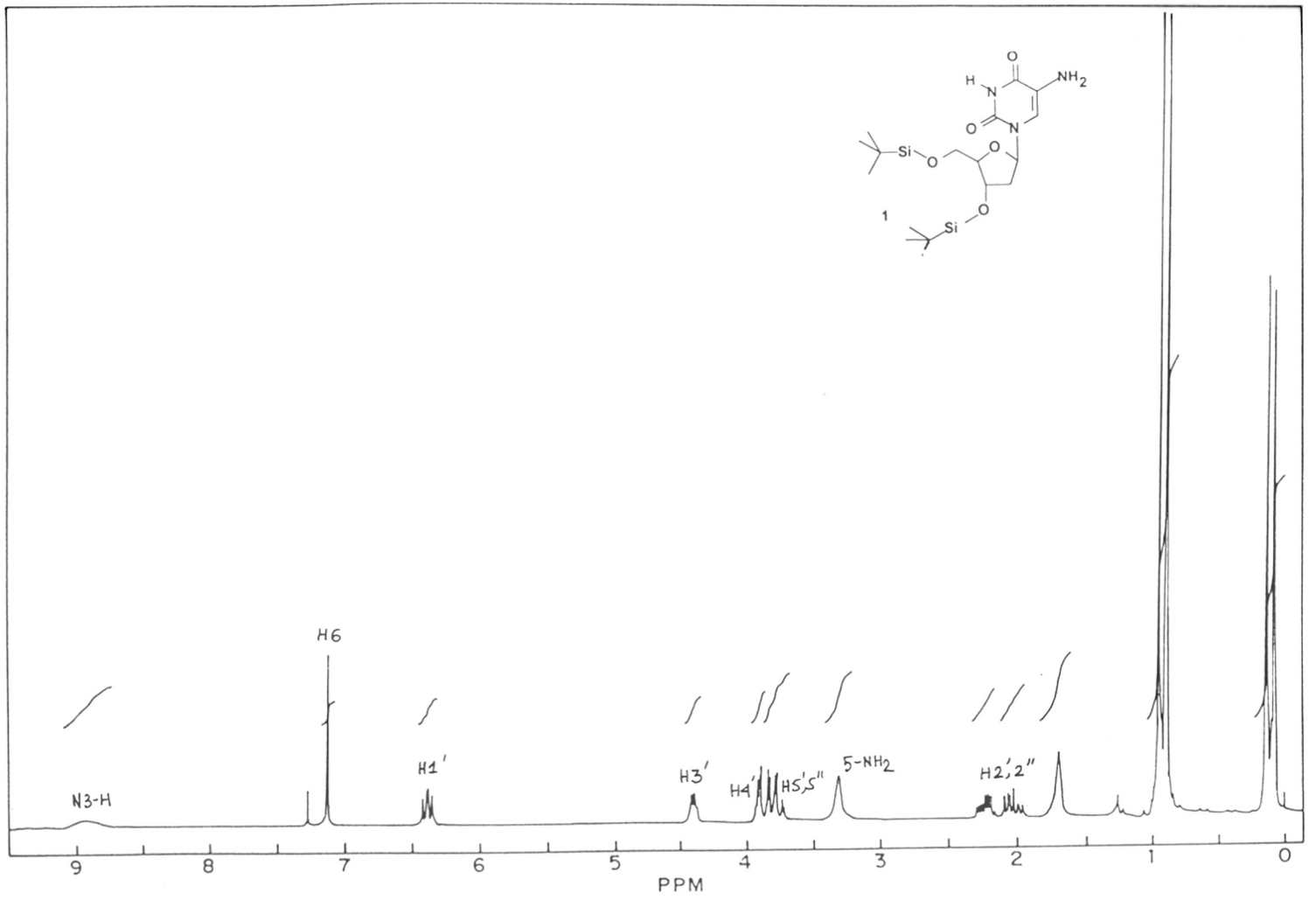


Figure 3a. ^{1}H NMR of **1**.

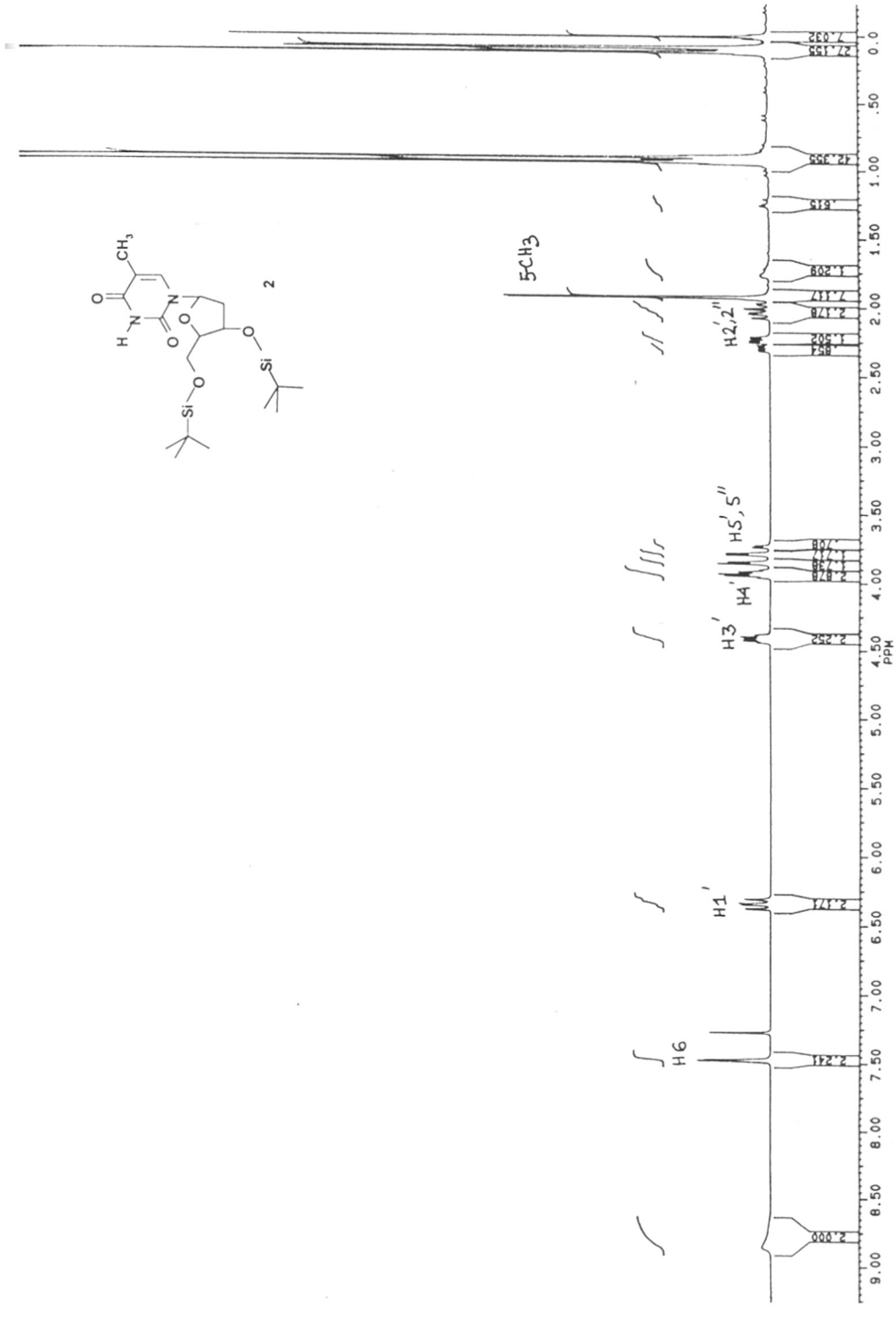


Figure 3b. ^{1}H NMR of 2.

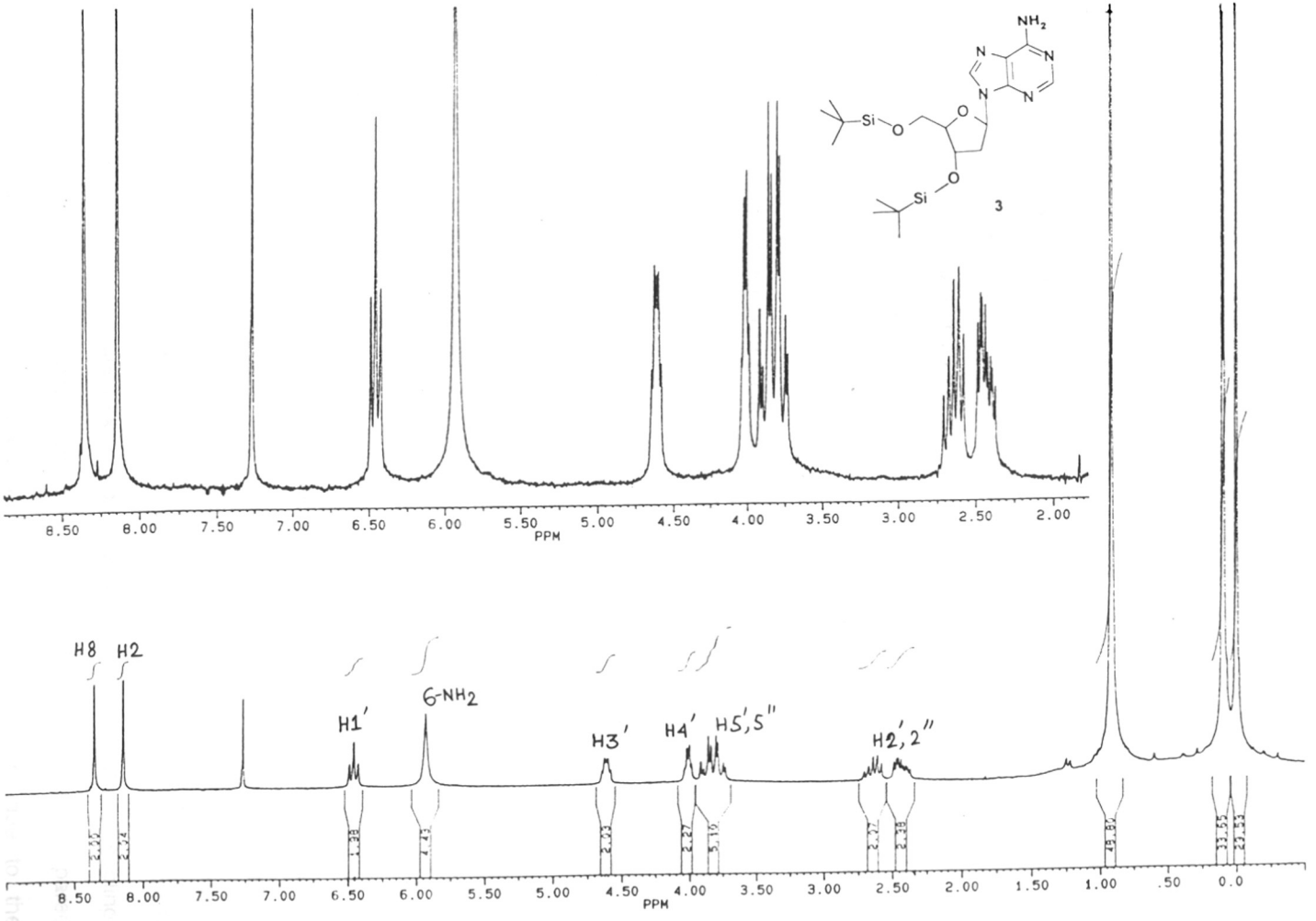


Figure 3c. ^{1}H NMR of **3**.

and 6-NH_2 of dA. Such induced shifts are characteristic of hydrogen bond formation between complementary base pairs. Before computation of actual association constants, it is imperative to determine the binding stoichiometry. This was done by using the well known continuous variation method or Job's plot (Connors, 1987). The experimental procedure for this consists of preparing a series of solutions of the two binding components for example **3** and **1**, by varying concentration of each in such a way that the sum of total concentration of both components remains constant. Such samples of the base pairs dA:dT (**3**:**2**) and dA:$dUNH_2$ (**3**:**1**) at a total concentration of 30 mM were studied by ^{1}H NMR in deuterated chloroform solutions at ambient temperature (21°C). The plot of chemical shift of 6-NH_2 of dA and N3-H of dT/5-NH_2-dU in each binding pair (Job's plot) gave a symmetric pattern characteristic of a 1:1 binding stoichiometry (Figure 4) for both base pairs at the concentration range (30 mM) and temperature (21°C) studied. It is demonstrated in the next section that this corresponds to Watson-Crick mode of base pairing.

2.3.3. *Complexation titrimetry for evaluation of binding constants*

The association constants between the complementary pairs dA:dT and dA:$dUNH_2$ were determined by titrimetry involving incremental, stoichiometric additions of the purine (dA) to the individual pyrimidine component (dT/$dUNH_2$) in chloroform-d. The titration was monitored by ^{1}H NMR spectral changes after each addition. In both pairs, the imino N3 proton (N3-H) of pyrimidine exhibited significant downfield shifts whose magnitude varied as a function of purine concentration (Figure 5). In contrast to N3-H, other protons on nucleobase and sugar moieties showed negligible shifts during titration. The selective downfield shift of N3-H is indicative of its involvement in hydrogen bonding (Figure 1). This is further confirmed by low temperature ^{1}H NMR experiments (see later) where it exhibited enhanced downfield shifts upon cooling. The protons of the exocyclic 6-NH_2 group of dA also shifted downfield compared to free dA but as the titration proceeded, an upfield shift was noticed due to the

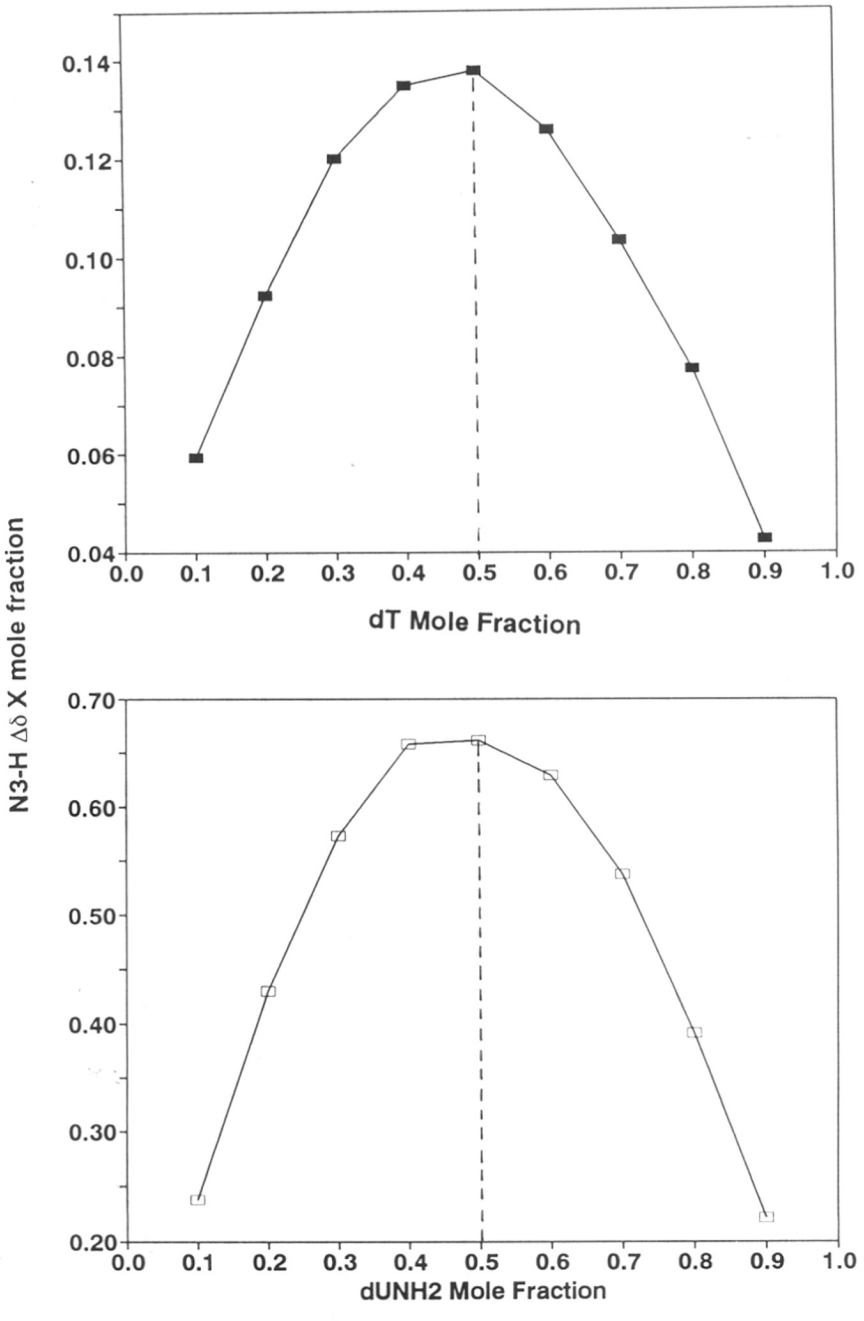


Figure 4. Job's plots for the complexation of dA (**3**) with dT (**2**) (■) and dA (**3**) with $dUNH_2$ (**1**) (□) at 25°C.

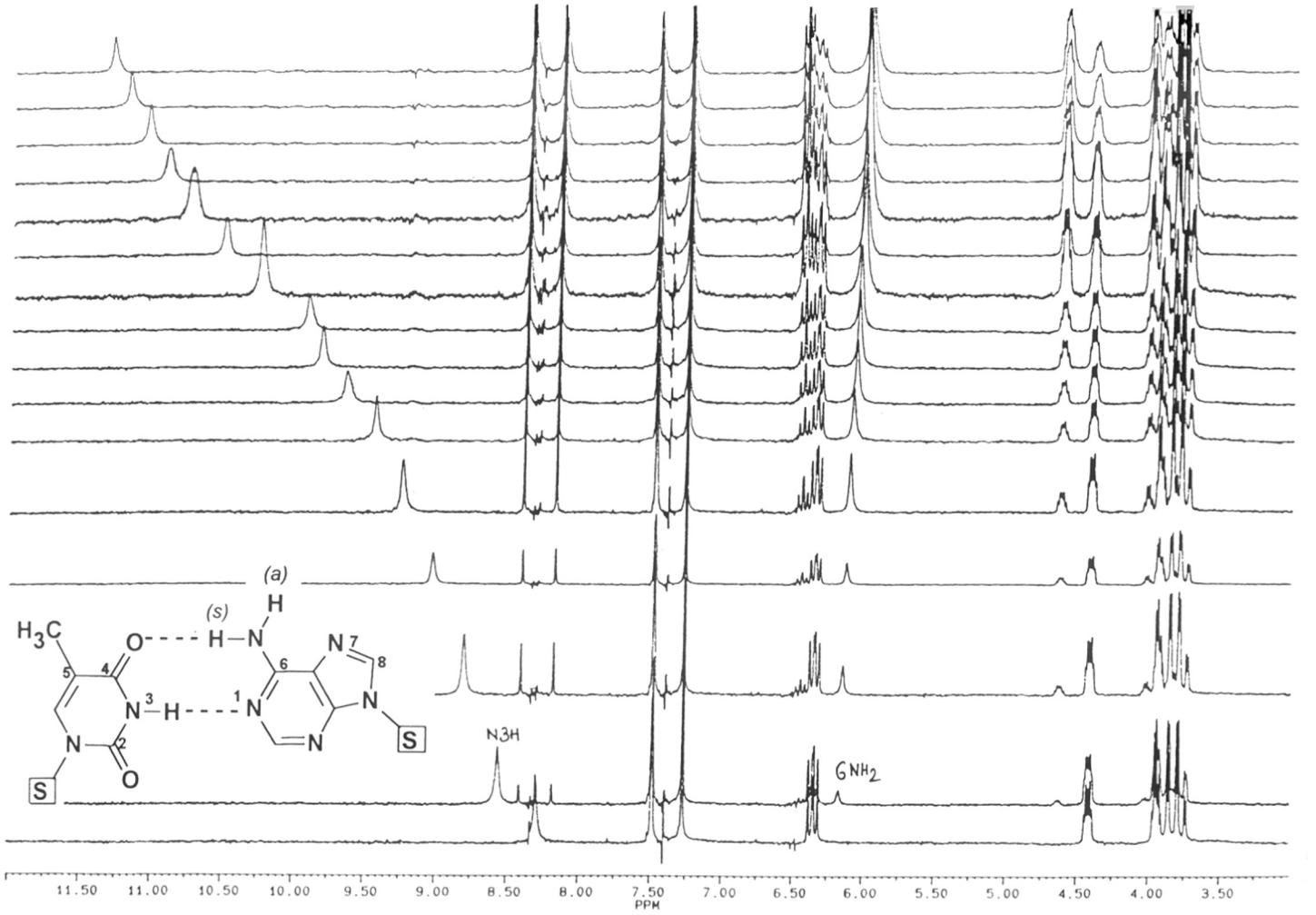


Figure 5. Stacked plot of **2** on incremental stoichiometric addition of the purine (dA) to the pyrimidine (dT).

gradual increase in the fractional concentration of free purine (beyond 1:1 stoichiometry). Figure 6 shows the mole ratio plot for N3-H shifts in pairwise complexation of dA:dT and dA:dUNH2. The binding isotherms point to a complexation stoichiometry of 1:1, beyond which the induced shifts reach a plateau to a limiting value. This is in agreement with the data obtained from Job's plot. The binding constants were computed for 1:1 complexation using HOSTEST II programme (Wilcox, 1990), incorporating a linear regression algorithm and were found to be 330 M^{-1} for dA:dT and 245 M^{-1} for dA:$dUNH_2$. Thus the $dUNH_2$ complexation with dA appears to be 30% weaker than dA:dT complexation.

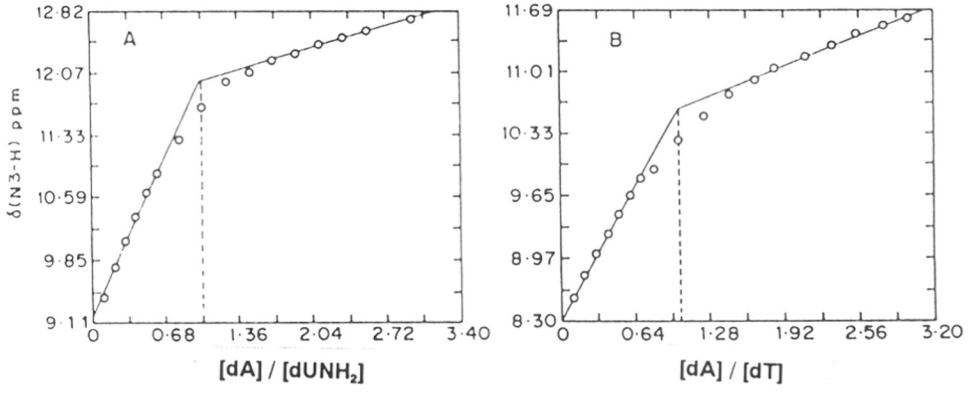


Figure 6. Plots of shifts in ^1H resonance of N3-H as a function of added dA (**3**). Vertical dotted line at break point corresponds to 1:1 stoichiometry.

2.3.4. *Temperature dependant ^1H NMR spectral studies*

In order to check whether the additional hydrogen bonding function (5-NH_2) promotes any non Watson-Crick patterns, temperature dependant ^1H NMR of a 1:1 stoichiometric mixture of individual pairs dA:dT and dA:$dUNH_2$ were studied. Upon mixing equimolar amounts of purine and pyrimidine components in chloroform-d, the N3-H of dT and 6-NH_2 of dA moved downfield as expected for association through hydrogen bonding. Figure 7 shows the ^1H NMR of 1:1 mixture of **3** and **1** at room temperature.

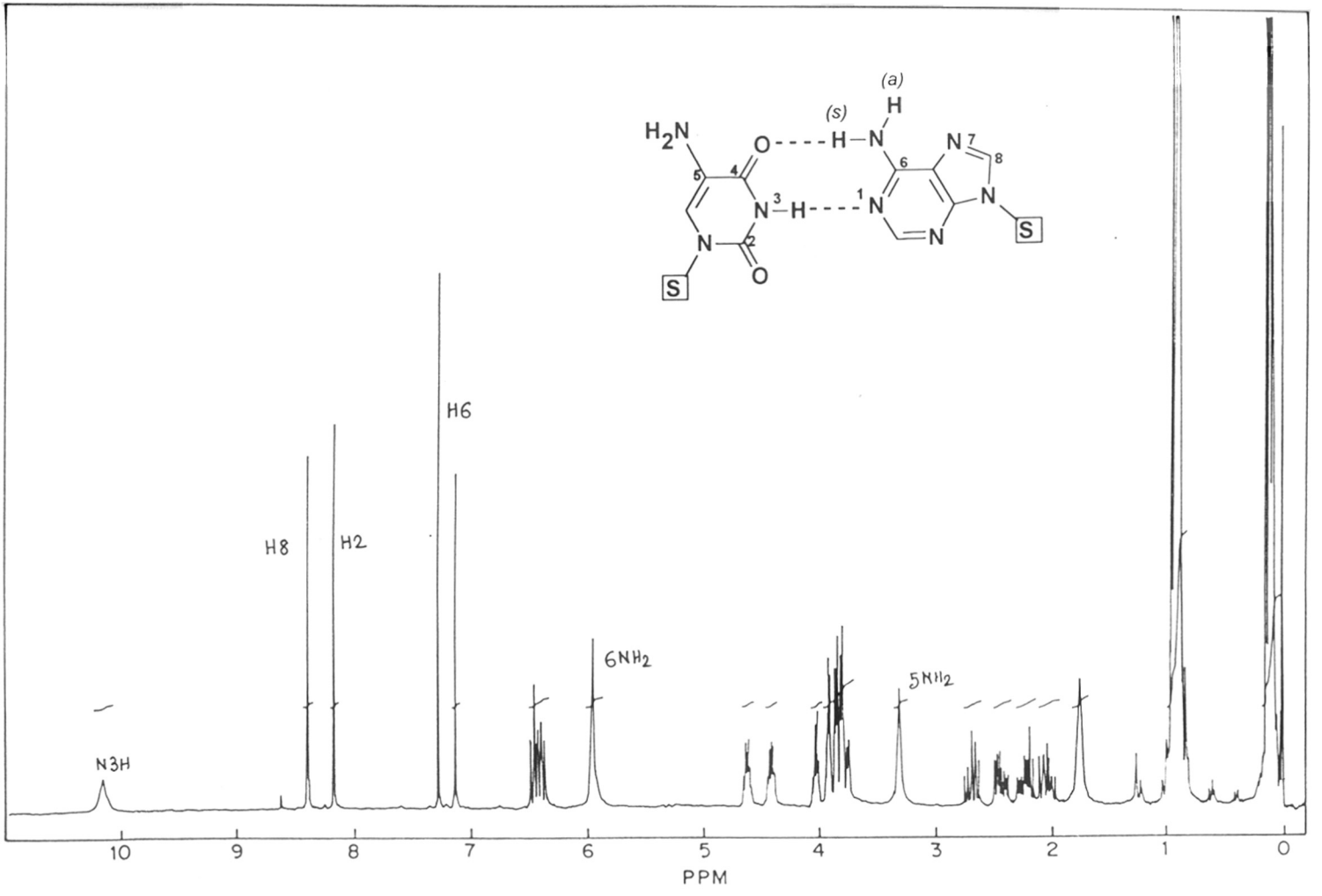


Figure 7. ^{1}H NMR of 1:1 stoichiometric mixture of dA (**3**):$dUNH_2$ (**1**) at 25°C.

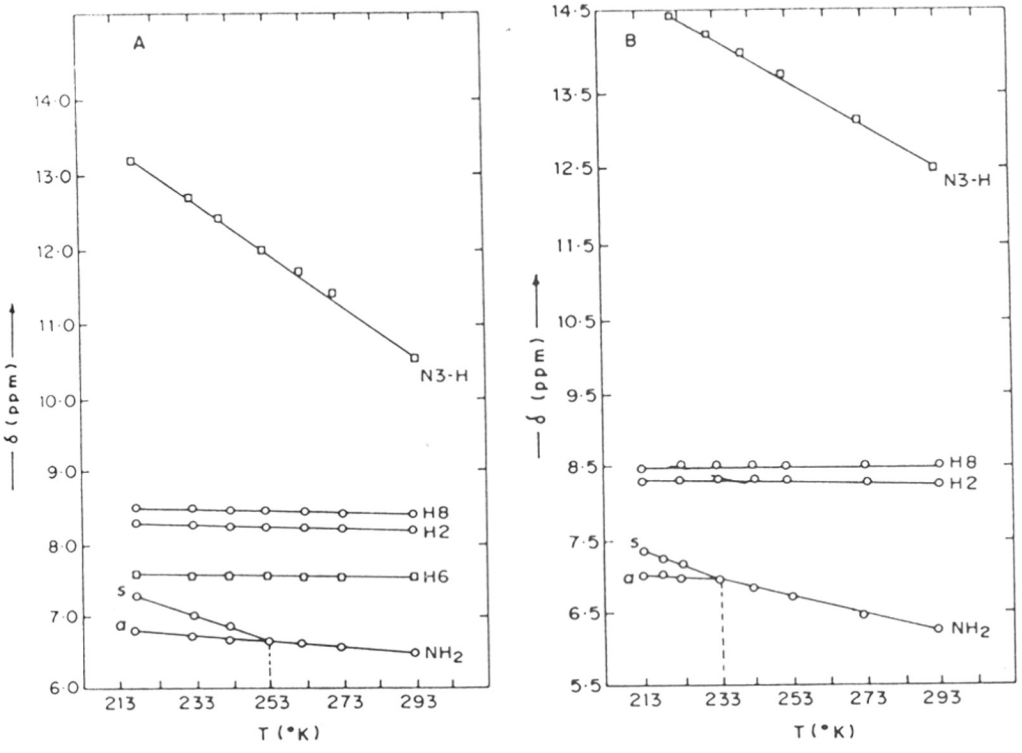


Figure 8. Plot of shift of various ^1H resonances in (A) dA:dT and (B) dA:$dUNH_2$ complexes at 1:1 stoichiometry as a function of temperature. 'o' indicates protons in dA and □ refers to protons in dT (A) and $dUNH_2$ (B). The corresponding proton assignments are shown on right side. Splitting temperature of 6-NH_2 protons in each is indicated by vertical dotted line.

The 5-NH_2 protons did not show any such shift indicating their non-involvment in base pairing. As the temperature of the mixture was lowered, the protons involved in hydrogen bonding (N3-H of dT and 6-NH_2 of dA) suffered further downfield shifts (Figure 8). In contrast to these exchangeable protons (N^6-amino and N3-imino), H2 and H8 of purine and H6 of pyrimidine did not shift significantly. This confirms that selective downfield shift observed for 6-NH_2 (purines) and N3-H (pyrimidines) protons are a consequence

of hydrogen bonding rather than the possible stacking or aggregation at lower temperature. In both pairs, the observed shifts had a linear dependence on temperature and the magnitude of shifts observed for N3-H protons was approximately twice that seen for 6-NH_2 protons. This is characteristic of complexation by Watson-Crick hydrogen bonding mode (Katz & Penman, 1966; Newmark & Cantor, 1968).

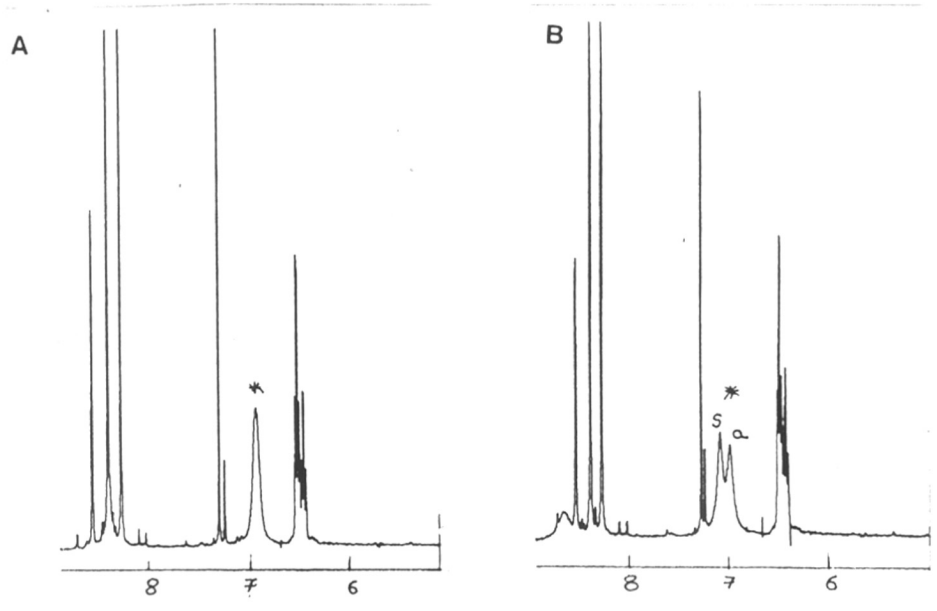


Figure 9. Partial ^1H NMR spectra of dA:dUNH_2 (1:1) complex at (A) 233°K and (B) 223°K. The 6-NH_2 resonances are indicated by *. In (B) *s* and *a* refers to *syn* (hydrogen bonded) and *anti* (non hydrogen bonded) protons of 6-NH_2 of dA.

Significant differences were noticed for 6-NH_2 protons of dA upon cooling the 1:1 complexes. At low temperatures, the 6-NH_2 protons exhibited separated signals (Figure 9) in both complexes and this is due to hindered rotation about N6-C6 bond which has partial double bond character. The hindered rotation is perhaps assisted by hydrogen bonding of 6-NH_2 group with C4 carbonyl of dT. Such an effect has been earlier noticed for 4-NH_2 protons of cytidine (Becker et al., 1965; Raszka & Kaplan, 1972), 2-NH_2 protons

of guanosine (Williams et al., 1987, Williams et al., 1989) and 6-NH_2 protons of adenosine (Kyogoku et al., 1982) upon base pair complexation. Interestingly, the 6-NH_2 protons of dA exhibited separated signals below -2O°C in dA:dT complex, whereas in dA:$dUNH_2$ complex, similar splitting occurred only at much lower temperature (-4O°C) (Figure 8). This change in 6-NH_2 proton splitting temperature may be directly attributed to a difference in the 6-NH_2--O=C hydrogen bond strength among the two complexes; a stronger hydrogen bond leads to a slower rotation of 6-NH_2 group and hence a higher coalescence temperature. The observed results imply that in dA:$dUNH_2$ complex, the 6-NH_2--O=C hydrogen bond is weaker compared to analogous bonding in dA:dT complex. Thus 5-NH_2 substituent in pyrimidine lowers the strength of the amino-carbonyl hydrogen bond in Watson-Crick base pairing mode. This fact is in agreement with a lower association constant obtained for dA:$dUNH_2$ pairing compared to dA:dT complex.

Table 1: Temperature coefficients ($\Delta\delta/\Delta T$, ppm/deg)*

Complex	N3-H	6-NH_2	
		1*	2#
dA:dT	0.03	0.01	0.018(*s*),0.006(*a*)
dA:$dUNH_2$	0.029	0.012	0.02(*s*),0.006(*a*)

* Signs are ignored since in all cases a downfield shift is observed.

* Column 1: temperature coefficient upto break point in Fig. 3.

\# Column 2: *s, syn; a, anti.*

Table 1 shows the temperature coefficients ($\Delta\delta/\Delta T$) for the exchangeable protons N3-H and 6-NH_2 in dA:dT and dA:$dUNH_2$ complexes, as computed from experimental results. The imino proton (N3-H) in both complexes have almost identical values and are higher than 6-amino protons. Among the separated 6-NH_2 protons (column 2), the downfield proton (syn) which is hydrogen bonded has a higher value than the non hydrogen bonded (anti) proton. Although no significant differences were noticed in the

pattern of temperature coefficients observed for analogous protons in the two complexes, the variations seen among the imino and amino protons are quite diagnostic of their relative hydrogen bonding strengths.

2.3.5 *^{13}C NMR spectral studies of 1:1 complexation at room temperature*

^{13}C chemical shifts directly reflect the electron density at carbon and are therefore useful as analytical probes for study of hydrogen bond formation involving amide carbonyls in nucleosides (Jones et al., 1970 a,b, Kyogoku & Iwahashi, 1977). Hydrogen bonding promotes polarization of the carbonyl group which drains electron density from the carbon, leading to its downfield shift. The electron density at C4 may be influenced by a C5 substituent in pyrimidines and this inturn may perturb the acceptor capacity of C4 carbonyl group. A comparison of the base pair induced shifts of C4 and C2 carbonyls in pyrimidines may enable delineation of association through Watson-Crick (C4) or reversed Watson-Crick (C2) base pairing modes.

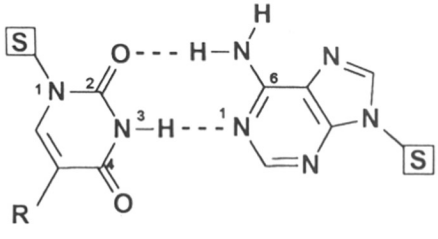


Figure 10. Reverse Watson-Crick hydrogen bonding.

In dA:dT complex, the results were interpreted as due to simultaneous existence of both Watson-Crick and reverse Watson-Crick bonding patterns with a greater population of molecules using C4 carbonyl as acceptor site, compared to C2 carbonyl. In dA:dUBr complex, the reverse situation was seen (Figure 10). Such ^{13}C shift derived results also have support from IR data (Kyogoku et al., 1967).

Table 2 gives the magnitude of induced ^{13}C NMR shifts for selected nucleobase carbons in dA:dT and dA:$dUNH_2$ complexes. It is observed that C2 and C4 signals of pyrimidine component shift downfield by larger amounts than other signals, clearly attributable to their direct participation in base pairing. In dA:dT complex, C4 exhibits a downfield shift (0.8ppm) higher than C2 (0.53ppm), while in dA:$dUNH_2$, the situation is reversed (C4, 0.68; C2, 0.76 ppm). The results for dA:dT complex compares well with that reported earlier (Jones et al., 1970 a,b; Kyogoku & Iwahashi, 1977) while the pattern obtained for dA:$dUNH_2$ complex resembles that seen in case of C5-Br nucleosides (Jones et al 1970 a, b, Kyogoku & Iwahashi, 1977). Although the differences seen in ^{13}C induced shifts among C4 and C2 carbonyls in dA:$dUNH_2$ complex is not as pronounced as corresponding values in dA:dUBr case, the observed qualitative identity suggests a predominance of reverse Watson-Crick base pairing in dA:$dUNH_2$ complex (Figure 10). The induced changes seen for dA (C5, C6 and C8) in both complexes are similar indicating identical electron density changes in dA upon complexation with dT and $dUNH_2$.

Table 2: Complexation induced shifts in ^{13}C NMR*

Complex	dT/$dUNH_2$				dA		
	C2	C4	C5	C6	C5	C6	C8
dA:dT	-0.53	-0.79	-0.04	+0.005	+0.009	-0.016	-0.07
dA:$dUNH_2$	-0.76	-0.68	+0.16	-0.11	+0.36	-0.16	-0.1

* Signs of induced shifts; - indicates downfield shift, + indicates upfield.

2.3.6. *Effect of 5-NH_2 substitution on dA:dU complexation*

It is known from earlier literature that electronic effect of C5 substituents on pyrimidine ring markedly influence the base pairing patterns(Saenger, 1984; Kyogoku et al., 1967). C5-CH_3 substituent (in dT) which is a weak electron releasing group increases the electron density at C4 thereby enhancing the polarization of C4 carbonyl. This causes C4 carbonyl to become a better hydrogen acceptor for hydrogen bonding

compared to C2 carbonyl. On the other hand, Br being more electronegative, C5 substitution with Br drains electron density from the ring, leading to a decreased polarizability of C4 carbonyl and a weaker hydrogen bonding capacity compared to C2 carbonyl. However, this is also accompanied by an increase in N3-H acidity, which enhances its hydrogen bond donor property, and hence resulting in a higher association constant. Amino group, although not as much electronegative as Br, is a better electron releasing function (by resonance) and it is therefore interesting to know its effect on base pairing. The following interesting facts emerge from present study on effects of C5-NH_2-2'-dU substituent on dA:dU base pair association even at monomer level.

1. Compared to dT, $dUNH_2$ has a weaker association constant with dA. This can be attributed to a decrease in polarizability of C4 carbonyl, thereby leading to a weaker 6-NH_2(dA)--O=C4($dUNH_2$) hydrogen bonding. This is evidenced by a lower splitting temperature for 6-NH_2 protons. The 5-NH_2 function has a rapid rotation around C5-N5 bond and no separation of 5-NH_2 protons is observed even at -50°C. No direct evidence could be obtained for its participation in intramolecular hydrogen bonding with C4 carbonyl. If such intramolecular hydrogen bonding does exist, it will certainly contribute to alter the acceptor strength of C4 carbonyl and may assume significance even at DNA level.

2. The electron releasing mesomeric effect of C5-NH_2 group does not contribute to enhance the C4 carbonyl polarization. Existence of such an effect is borne by a marked upfield shift of C6 in $dUNH_2$ compared to dU or dT.

3. C5-NH_2 substitution causes an increase in population of molecules with $6NH_2$(dA)--O=C2(dU) hydrogen bonding (reverse Watson-Crick) compared to $6NH_2$(dA)--O=C4(dU) bonding mode (Watson-Crick). As this is sterically more hindered, the overall association by base pair complementation is a weaker process.

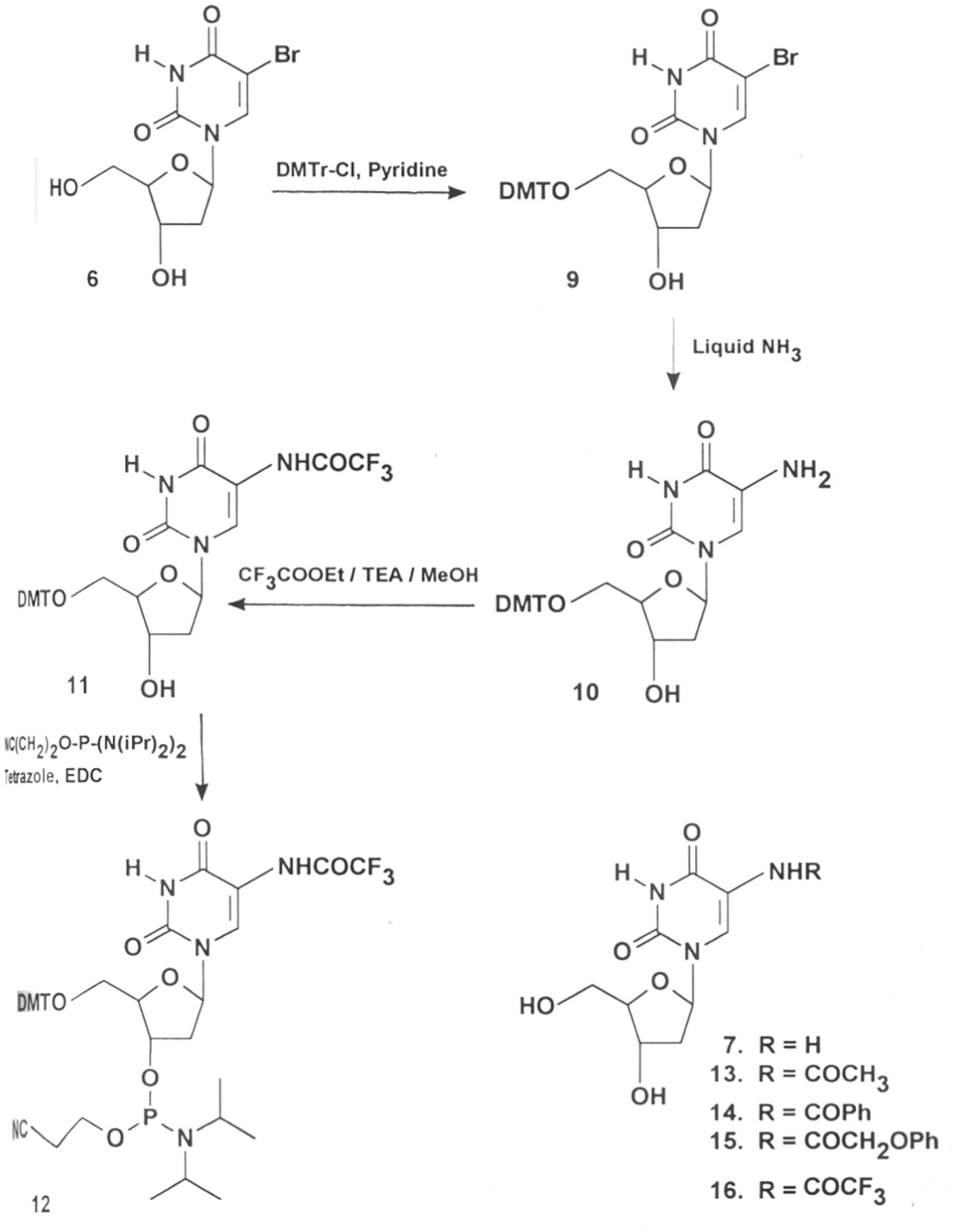
Thus C5-NH_2 substituent can cause subtle changes of base pairing properties of pyrimidine nucleobase. In addition to the above, the 5-NH_2 function can be a suitable center to covalently anchor extraneous ligands for modulation of DNA properties. The next section describes incorporation of dUNH_2 into synthetic oligonucleotides and preliminary study of their biophysical properties, such as Tm and recognition by restriction endonuclease, followed by chemical derivatization in chapter 3.

2.4. INCORPORATION OF 5-NH_2-2'-DEOXYURIDINE INTO OLIGONUCLEOTIDE AND ITS BIOPHYSICAL STUDIES

5-NH_2-2'-deoxyuridine upon incorporation into an oligonucleotide provides an additional hydrogen bonding site at C5 which is directed into the major groove and thereby may influence the biophysical properties and interactions of DNA with groove binding ligands. As pointed out earlier, 5-NH_2 group can also be a suitable active center for covalent anchoring of extraneous ligands such as fluorophores or DNA cleaving moieties. The incorporation of 5-amino-2'-deoxyuridine into oligonucleotide needed development of a selective protector for 5-NH_2 group which is stable to chemistry of oligonucleotide synthesis. The chemical reactivity of 5-NH_2 group of pyrimidines is different from that of exocyclic 4-NH_2 group in dC. To the best of our knowledge, no previous report exists on the synthesis of either an appropriately protected precursor monomer such as **12** required for phosphoramidite approach or 5-NH_2 pyrimidine consisting DNA.

2.4.1. *Synthesis of oligonucleotides containing 5-amino-2'-deoxyuridine*

The target compound **12** was synthesized according to Scheme 2. 5-Br-dU **6**, was converted to its corresponding 5'-O-DMT derivative **9**. This was treated with liquid NH_3 in a steel bomb and the removal of excess NH_3 gave compound **10** in quantitative yield (95%). In order to standardize the protecting group for modified nucleobase, the corresponding acetyl **13**, benzoyl **14** and phenoxyacetyl **15** derivatives were synthesized


DMTr-Cl, Pyridine
Liquid NH3
CF3COOEt / TEA / MeOH
NC(CH2)2O-P-(N(iPr)2)2
Tetrazole, EDC
7. R = H
13. R = COCH3
14. R = COPh
15. R = COCH2OPh
16. R = COCF3

Scheme 2

and studied for efficient deprotection under usual oligonucleotide deprotection conditions using different ammoniacal reagents. Compounds **13** and **14** were prepared using transient protection method with acetyl and benzoyl chloride respectively. Compound **15** was prepared by peracylation with phenoxyacetyl chloride to give triphenoxyacetyl derivative which on treatment with methanolic ammonia at room temperature gives 5-aminophenoxyacetyl-2'-dU **15**. N^5-acetyl and N^5-benzoyl groups of **13** and **14** were found to be stable to final deprotecting condition of oligonucleotides i.e, aqueous ammonia treatment at 60°C for 24 h, while N^5-phenoxyacetyl group was found to get hydrolyzed to 25-30% only, under the same conditions. Thus, none of these protecting groups are suitable for effective protection/deprotection of 5-amino group of modified nucleoside and hence trifluoroacetyl group (TFA) was tried as a 5-NH_2 protector. So far, this group has not been used as a protecting group for nucleobase exocyclic amino functions in DNA synthesis due to its high lability but it has been used for aliphatic amino protection (Sonveaux, 1986; Beaucage & Iyer, 1992). We found that $COCF_3$ as a protector of 5-NH_2 group had right stability and ammoniacal lability (aq NH_3, 1 hr, 60°C) for use in oligonucleotide synthesis. 5-N-trifluoroacetyl-5'-O-DMT-5-amino-2'-deoxyuridine **11** was made by reacting **10** with ethyltrifluoroacetate (Takeda et al., 1987) in MeOH/TEA in 75% yield. This was characterized by ^1H and ^{13}C NMR (Figure 11).

Alternatively, **11** was also synthesized by first converting **6** into 5-NH_2 derivative **7** (Visser & Roberts, 1951; Smith & Visser; 1966, Ivanovics et al., 1971) followed by N^5-trifluoroacetylation to give **16**. The latter crystallizes out from methanol, and subsequent dimethoxytritylation of **16** gives **11**. The earlier route as shown in Scheme 2, had two advantages: (i) it gave a better overall yield (52%) than the alternative method (26%) and (ii) practical isolation of **10** from liquid ammonia reaction of **9** was easier than that of **7** from **6**. The desired β-cyanoethyl phosphoramidite **12** was obtained from **11** and 2-cyanoethyl N,N,N',N'-tetraisopropylphosphoroamidite activated with tetrazole.

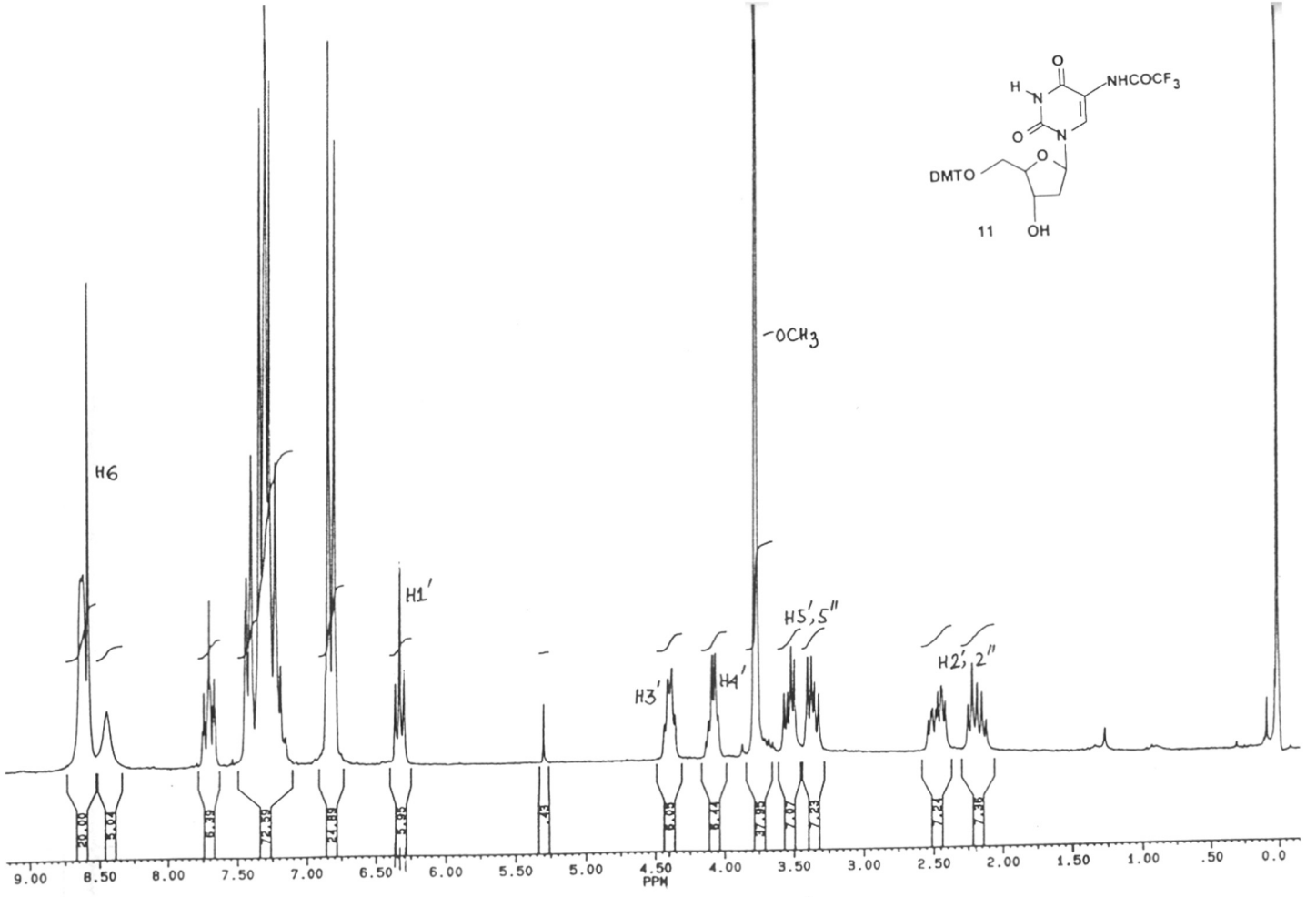


Figure 11a ^{1}H NMR of **11**.

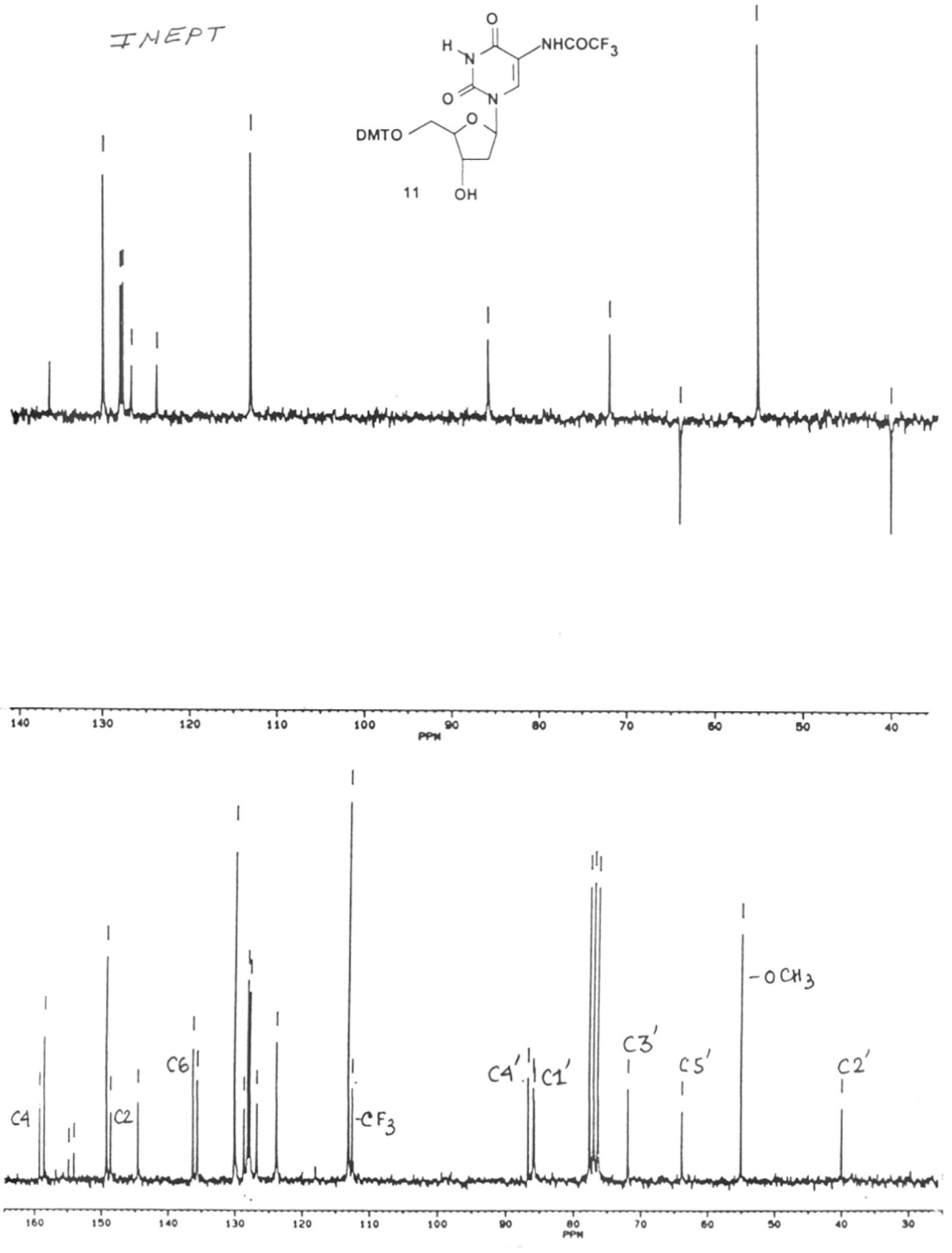


Figure 11b ^{13}C NMR of **11**.

This reagent allows *in situ* preparation of **12** by using standard procedure (Sinha et al., 1984). The monomer **12** gave ^{31}P NMR (s, 149.5 and 149.4 ppm, ref, 85% H3PO4) characteristic of 3'O-phosphoramidite (Figure 12) and no N5-phosphonylation was observed.

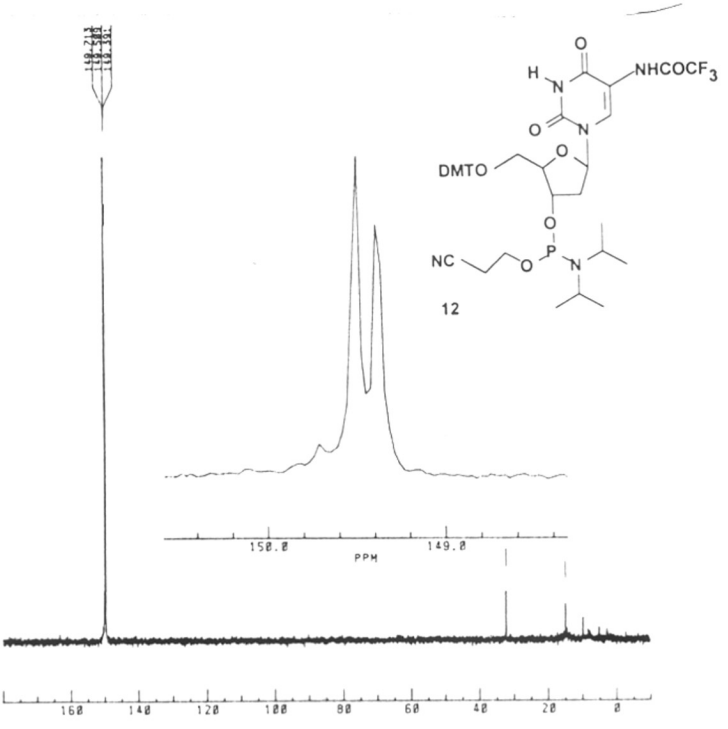


Figure 12. ^{31}P NMR of **12**.

17	5'-	C	G	C	G	A	A	T	T	C	G	C	G
18	5'-	C	G	C	G	A	A	**U#**	T	C	G	C	G
19	5'-	C	G	C	G	A	A	T	**U#**	C	G	C	G
20	5'-	C	G	C	G	A	A	**U#**	**U#**	C	G	C	G

U# is 5-Amino-2'-dU.

The activated amidite monomer of 5-amino-2'-dU **12** was incorporated into well studied Dickerson's dodecamer to prepare sequences **18-20** which differ only in the position of modification. This was achieved by usual protocol on an automated DNA synthesizer (Pharmacia GA plus). The coupling efficiencies of modified amidites were similar to the commercial phosphoramidites of normal nucleosides. After the completion of synthesis, final on-column detritylation was followed by NH_3 treatment to yield fully deprotected oligonucleotides. These were purified by FPLC (Pharmacia) on RPC column and rechecked by HPLC and denaturing PAGE after ^{32}P 5'-end labelling (Figure 13).

2.4.2. *Biochemical and biophysical studies of modified oligonucleotides*

The transition between an ordered duplex structure and a single stranded random coil in a nucleic acid can be conveniently monitored using ultraviolet (UV) absorbance (Puglisi et al., 1989). As the ordered stacks of base pairs are disrupted, the UV absorbance increases due to hypochromicity; the amount of hypochromicity is a measure of base stacking as a result of base pairing. The absorbance in the disordered state approaches the sum of absorbances of constituent nucleotides. The easiest way to denature a nucleic acid is by heating. The resulting profile of absorbance versus temperature is called a melting curve which is normally sigmoidal in form corresponding to two state transitions. The mid point of this transition, defined as the melting temperature "Tm", is characteristic of base composition, sequence and the structure of DNA. The dependence of Tm on DNA concentration yields information on the molecularity and quantitative thermodynamic data for the melting transition (Longfellow et al., 1990).

The thermal stability of various duplexes constituted from the synthesized self-complementary oligonucleotides **17-20** were determined by UV-melting experiments. Since all the sequences are self complementary, any single substitution, makes two

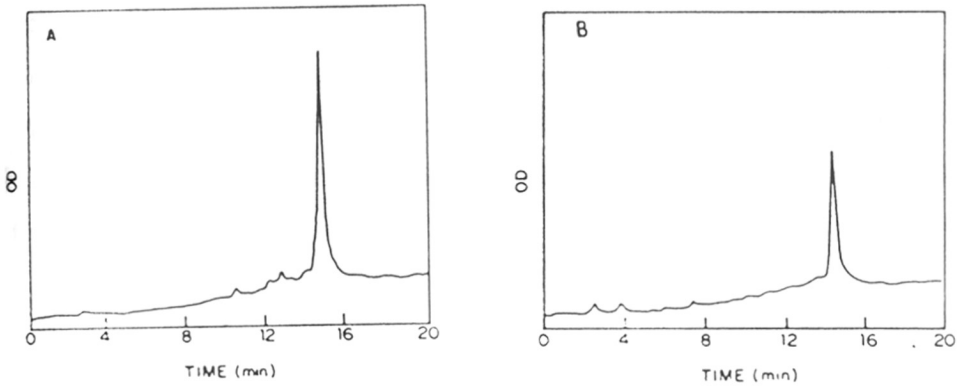


Figure 13a Reverse phase HPLC of **19** (A) and **20** (B).

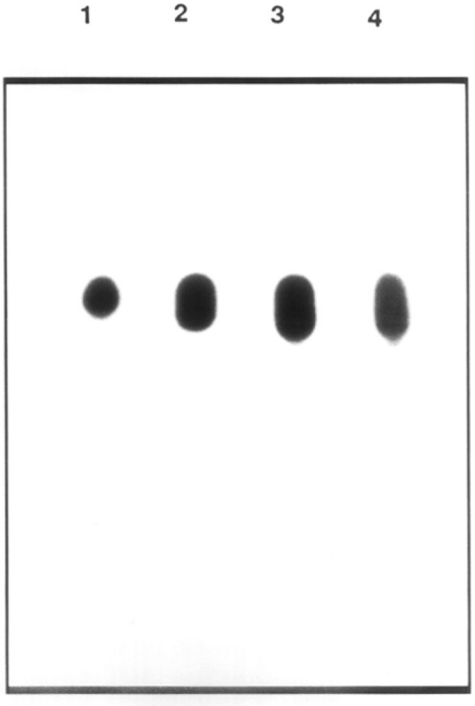


Figure 13b Autoradiogram indicating purity of oligonucleotides **17-20** in lanes 1-4 respectively.

symmetrically located alterations in the antiparallel duplex and hence the observed biochemical/biophysical properties are a consequence of two alterations. UV-melting experiments were carried out on all samples using identical buffers, premelting annealing conditions and heating rates. These experiments were optimized with respect to above conditions to obtain best melting curves. The Tm's were derived from a plot of fraction absorbance change against temperature and rechecked by differential curves.

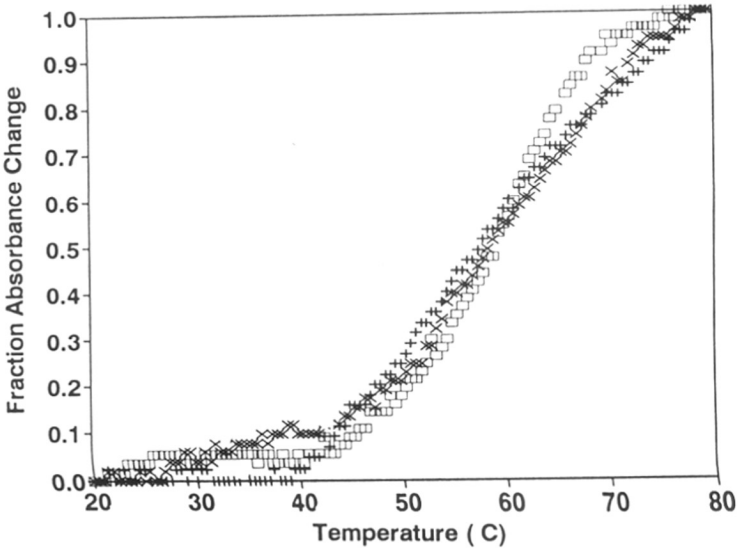


Figure 14. Melting curves for duplexes **17** (X, Tm 60°C), **18** (□ , Tm 59°C) and **20** (+, Tm 58°C) in 10 mM Tris, 80 mM NaCl and 20 mM $MgCl_2$, pH 7.6.

The oligonucleotides **18-20** containing 5-NH_2-dU residues gave slightly lower Tm (Figure 14) (**18**, 57°C; **20**, 55°C in 10 mM Tris.HCl, pH 7.6, 80 mM NaCl and 20 mM $MgCl_2$) as compared to that of unmodified dodecamer **17** (Tm, 60°C). The oligonucleotide substitutions are conservative in that they do not disturb the hydrogen bonding patterns with their complements and hence not expected to greatly affect the thermal stabilities of the oligonucleotides. The Tm for helix-to-coil transition of **17-20** which were in the

range 55-60°C, is a favorable to study them as a substrates for enzyme hydrolysis, since under the experimental conditions of enzymatic hydrolysis, major fraction of oligonucleotides will be present in duplex form.

	1	2	3	4	5	6	7	8	9	10	11	12	
5'-	C	G	C	**G**	**A**	**A**	**T**	**U#**	**C**	G	C	G	-3'
3'-	G	C	G	**C**	**U#**	**T**	**A**	**A**	**G**	C	G	C	-5'
	12	11	10	9	8	7	6	5	4	3	2	1	

The restriction enzyme digestion of the 5'-^{32}P end labelled DNA control **17** and the modified oligonucleotides **18-20** were carried out under identical conditions. While the control DNA gave a pattern characteristic of expected cleavages, oligonucleotides **18-20** were resistant to cleavage. Thus the presence of 5-amino substituent at C5 may play a significant role in inhibition of restriction enzyme cleavage. The Dickerson's dodecamers **17-20** contain six base pair recognition sequence for restriction endonuclease *EcoR*I. X-ray crystal structures of dodecamer (Drew et al., 1981), its derivatives containing base mismatches (Kennard, 1985) or 5-bromocytosine substitution (Fratini et al., 1982) and that of *EcoR*I endonuclease-DNA **17** co-crystal (McClarine et al., 1986) are known. The substrate sequences for the endonuclease is self complementary and are only active as double strand helices (Alves et al., 1984).

It is reported in literature (Brennan et al., 1986) that substitution of uracil for thymine at position 8 which removes 5-methyl group from the major groove and replaces it with hydrogen atom, completely inactivated the substrate. In contrast 5-bromo substitution at either positions 7 or 8 restores the activity of substrate. Bromine atom, though more electronegative has a van der Waal radius similar to that of methyl group. It may therefore compensate for the loss of 5-methyl group by forming a direct contact with the protein or by strengthening a nearby interaction. The delineation of contributions from the steric and hydrophobic parameters (Hansch et al., 1973) for 5-substituents of uracil derivatives are shown in Table 3. The small size and low hydrophobic parameter for hydrogen atom

explains the observed inhibition of hydrolysis of dU substituted ODN's by *EcoR*I. Conversely, the increased size and positive hydrophobic parameter of the bromine atom like that of methyl group restores the substrate activity.

Table 3: Comparison of selected substituent effects.*

Substituent	Steric parameter	Hydrophobic parameter	Enzyme Activity
- CH_3	5.63	0.56	Yes
- H	1.03	0	No
- Br	8.83	0.86	Yes
- NH_2	5.42	- 1.23	No'

* Taken from Hansch et al., 1973.

' Present result

When compared with the above substituents, the amino group has steric parameter similar to that of methyl group, but has a more negative hydrophobicity i.e more hydrophilic than other substituents. The small difference in steric parameters of the methyl group and amino group may not directly affect the rate of hydrolysis by *EcoR*I, since bromine atom was tolerated for hydrolysis under the conditions. *EcoR*I inhibition of the oligonucletide containing 5-amino-2'-deoxyuridine may be attributed to the enzyme sensitivity to hydrophobic environment in the binding pocket which is reversed by amino group substitution. The hydrophobic requirement as of 5-methyl group in thymine has been implicated in the specific binding of the *lac* repressor (Caruthers, 1980), and of Mnt repressor (Knight & Sauer, 1989; Rajendrakumar et al., 1990) to their corresponding DNA, and in the cleavage of octamers (Dwyer-Hallquist et al., 1982) and DNA (Berkner & Folk, 1979), by the *Hpa*I restriction endonuclease.

2.5. CONCLUSIONS

The effect of C5 amino substituent on 2'-dU base pairing with 2'-dA was investigated using ^1H and ^{13}C NMR spectroscopy in low dielectric solvent ($CDCl_3$). It was found that 5-amino substituent in 2'-dU results in (i) decreased association constant (lower

Ka) with 2'-dA compared to dA:dT complexation (ii) lower temperature for separation of 6-NH_2 protons of dA due to its complexation with $dUNH_2$ compared to that of dT and (iii) increased receptor strength of C2 carbonyl compared to C4 carbonyl. This 5-amino-2'-dU was incorporated into oligonucleotide using solid phase phosphoramidite chemistry. Trifluoroacetyl was found to be a suitable protecting group for 5-amino functionality of 5-NH_2-2'-dU. 5-NH_2-2'-dU was found to destabilize the duplex DNA (2.5-2.0°C/substitution) and was found to inhibit the restriction endonuclease activity of *EcoR*1.

2.6. EXPERIMENTAL

All chemicals used were of reagent or better grades. Base protected nucleoside phosphoramidites and nucleoside derivatized controlled pore glass supports were purchased from Cruachem, UK. For DNA synthesizer dry solvents were used. The commercial and modified amidites were dissolved in dry acetonitrile to give 0.1 M solution and 4A molecular sieves were added to it to remove traces of moisture. Acetonitrile was distilled twice over P_2O_5 and finally over CaH_2 immediate before use. Dichloroethane was dried by distilling twice over P_2O_5. For oxidation after each coupling 0.01 M iodine in collidine, water and acetonitrile, while for capping 20% acetic anhydride in acetonitrile were used. TLC was carried out on using E. Merck precoated silica gel 60 F254 plates (Cat No 5554) and using the following solvent systems: A, CH_2Cl_2:MeOH (85:15); B, CH_2Cl_2:MeOH (90:10) and C, CH_2Cl_2:MeOH (95:5). Compounds were visualized on TLC plate either under UV light and/or as dark spots after spray with perchloric acid in ethanol (60%) followed by charring. Column chromatographic separations were done using silica gel (100-200 mesh, Loba-Chemie). ^1H (200 MHz) and ^{13}C (80 MHz) NMR spectra were recorded on Bruker ACF200 spectrometer, fitted with an Aspect 3000 computer. Low temperature ^1H NMR were recorded on Bruker MSL300 NMR spectrometer using BVT1000 temperature controller. For ^1H and ^{13}C NMR, TMS and solvent chloroform-d respectively were used as internal standards and chemical shifts expressed in δ scale (ppm). ^{31}P NMR spectra are recorded at 81 MHz with 85% H_3PO_4 as external reference. The ^1H NMR titration data were analyzed after volume corrections, using HOSTEST II software. The confidence level in the analysis were more than 0.95.

3',5'-Di-O-acetyl-5-bromo-2'-deoxyuridine 5

A suspension of 2'-deoxyuridine **4** (5 gm, 21.9 mmoles) in 30 ml of acetic anhydride was heated until dissolution occurs. A solution of bromine (3.8 gm, 23.8 mmoles) in 3 ml glacial acetic acid was added, with cooling, to maintain a temperature of 25°C. After

keeping overnight in cold (4°C), the solution was evaporated to a thick syrup under reduced pressure. On addition of about 30 ml of ethanol, the diacetate **5** crystallizes slowly (7.2 gm, yield 84%), m.p. 153°C. Rf (system B) = 0.8.

^{1}H NMR ($CDCl_3$) δ 7.8 (s, 1H, H6), 6.3 (t, 1H, H1'), 5.2 (m, 1H, H3'), 4.4-4.15 (m, 3H, H4', H5' and H5"), 2.55 (m, 2H, H2' and H2"), 2.15 and 2.0 (s, 6H, 2xOCO$\underline{C}H_3$). **^{13}C NMR** ($CDCl_3$) δ 170 and 169.8 (O$\underline{C}$OCH_3), 158.5 (C4), 149.4 (C2), 138.4 (C6), 97.2 (C5), 85.6 (C4'), 82.5 (C1'), 73.7 (C3'), 63.5 (C5'), 38 (C2'), 20.56 (OCO$\underline{C}H_3$).

5-Bromo-2'-deoxyuridine 6

Compound **5** (3.91 gm, 10 mmol) was dissolved in 85 ml of anhydrous methanolic ammonia (10%), and the solution was kept at room temperature for 24 h. The solvent was evaporated and the residue was dissolved in a minimum volume of hot, anhydrous ethanol. On cooling crystals of **6** were obtained (5.3 gm, yield 85%), m.p. 193°C. Rf (system B) = 0.4.

^{1}H NMR (DMSO-d_6) δ 8.4 (s, 1H, H6), 6.1 (t, 1H, H1'), 5.4-5.1 (brd, 2H, 3'-OH and 5'-OH), 4.25 (m, 1H, H3'), 3.8 (m, 1H, H4'), 3.6 (m, 2H, H5' and H5"), 2.1 (m, 2H, H2' and H2"). **^{13}C NMR** (DMSO-d_6) δ 159.2 (C4), 149.8 (C2), 140.4 (C6), 95.8 (C5), 87.7 (C4'), 85 (C1'), 70.1 (C3'), 60.9 (C5'), 40.2 (C2').

5-Amino-2'-deoxyuridine 7

Compound **6** (3.10 gm, 10 mmol) was treated with liquid ammonia (25 ml) in a stainless steel bomb and heated at 55°C for 24 hr. After removal of excess ammonia, the resulting product was treated with pyridine (25 ml) when a solid separated out. This was filtered and the filtrate was concentrated to a gum, which was chromatographed on silica gel (50 gm). The column was eluted with CH_2Cl_2 containing increasing proportions of MeOH, when the required compound **7** eluted at 20% MeOH in CH_2Cl_2 (1.8 gm, yield 74%). Rf (System A)= 0.2, Ninhydrin positive.

^{1}H NMR (D_2O) δ 7.25 (s, 1H, H6), 6.25 (t, 1H, H1', J = 7 Hz), 4.4 (m, 1H, H3'), 3.95

(m, 1H, H4'), 3.7 (m, 2H, H5'and H5''), 2.25 (m, 2H, H2' and H2''). **^{13}C NMR** (DMSO-d_6) δ 160.6 (C4), 148.9 (C2), 122.8 (C5), 115.1 (C6), 87 (C4), 83.6 (C1'), 70.6 (C3'), 61.7 (C5'), 38.7 (C2').

3',5'-O-5-N-triacetyl-5-amino-2'-deoxyuridine 8

Compound **7** (0.24 gm, 1mmol) was taken in dry pyridine (2 ml) to which acetic anhydride (1.4 ml, 15mmol) was added and kept stirred for 2 hr. It was then concentrated to a gum, which was chromatographed on silica gel (6 gm) and on elution with 8% MeOH in CH_2Cl_2 gave the desired triacetate **8** as a white solid, (0.26 gm, yield 70%), m.p., 241-242°C, Rf (System A) = 0.83, Ninhydrin negative.

^{1}H NMR ($CDCl_3$-$DMSOd_6$) δ 10.95 (bs, 1H, N3-H), 8.65 (s, 1H, H6), 7.95 (s, 1H, 5-NHCO), 6.4 (t, 1H, H1', J = 6.9 Hz), 5.3 (m, 1H, H3'), 4.35 (m, 2H, H5'and 5''), 4.2 (m, 1H, H4'), 2.4 (m, 2H, H2'and 2''), 2.2 to 1.0 (3s, 9H, 3 2xOCOCH3). **^{13}C NMR** ($CDCl_3$-$DMSOd_6$) δ 170.1 and 169.5 (2x$\underline{C}OCH_3$), 168 (NH$\underline{C}OCH_3$), 159.1 (C4), 148.2 (C2), 124.5 (C5), 114.8 (C6), 84.2 (C4'), 81.4 (C1'), 73.7 (C3'), 63.1 (C5'), 36.2 (C2'), 20.1, 20.2 and 23.1 (3xOCO$\underline{C}H_3$).

5-Amino-3',5'-di-O-(t-butyldimethylsilyl)-2'-deoxyuridine 1

5-Amino-2'-deoxyuridine (1.20 gm, 5 mmol), was taken in dry pyridine (2 ml) to which TBDMSC (3 gm, 20 mmol), was added and kept stirred at room temperature for 24 hr. The reaction was monitored by TLC and after completion it was concentrated to an oil and chromatographed on silica gel (20gm). Elution with n-Hexane:CH_2Cl_2 (1:4) followed by increasing amounts of MeOH in CH_2Cl_2 gave the required disilyl derivative 1, (1.40 gm, yield 60%), Rf (System C) = 0.53, Ninhydrin positive.

^{1}H NMR ($CDCl_3$) δ 9.81 (s, 1H, N3-H), 7.15 (s, 1H, H6), 6.4 (t, 1H, H1', J = 6.5 Hz), 4.45 (m, 1H, H3'), 3.9 (m, 1H, H4'), 3.73 and 3.83 (m, 2H, H5' and 5''), 3.3 (s, 2H, 5-NH_2), 2.05 and 2.3 (2xddd, 1H each, H2' and 2'', J = 3.2, 6.5 and 13.5 Hz), 0.90 and 0.93 (2xs, 18H, 2x$C(CH_3)_3$), 0.08 and 0.10 (2xs, 12H, 2x$Si(CH_3)_2$). **^{13}C NMR**

($CDCl_3$) δ 159.5 (C4), 148.2 (C2), 126.4 (C5), 114.7 (C6), 87.9 (C4'), 85.7 (C1'), 72.3 (C3'), 63.2 (C5'), 40.1 (C2'). Analysis, Calc. $C_{21}H_{41}O_5N_3Si_2$: C, 53.47; H, 8.75; N, 8.90. Found: C, 53.60; H, 9.20; N, 8.45.

3',5'-Di-O-(t-butyldimethylsilyl)-2'-deoxythymidine, 2 and 3',5'-Di-O-(t-butyldimethylsilyl)-2'-deoxyadenosine, 3

These were synthesized according to above procedure using DMF as a solvent instead of pyridine.

3',5'-Di-O-(t-butyldimethylsilyl)-2'-deoxythymidine, 2

^{1}H NMR ($CDCl_3$) δ 8.85 (s, 1H, N3-H), 7.4 (s, 1H, H6), 6.33 (dd, 1H, H1', J = 5.9, 7.8 Hz), 4.41 (m, 1H, H3', J = 2.7 Hz), 3.92 (m, 1H, H4'), 3.8 and 3.7 (ddd, 2H, H5' and 5"), 2.0 and 2.2 (ddd, 2H, H2' and 2", J = 2.7 and 5.9 Hz), 1.9 (s, 3H, 5-CH_3), 0.9 and 0.92 (2xs, 18H, 2x$C(CH_3)_3$), 0.06 and 0.13 (2xs, 12H, 2x$Si(CH_3)_2$). **^{13}C NMR** ($CDCl_3$) δ 163.5(C4), 150.1 (C2), 135.4 (C6), 110.7 (C5), 87.8 (C4'), 84.8 (C1'), 72.2 (C3'), 62.9 (C5'), 41.3 (C2'). Analysis, Calc. $C_{22}H_{42}O_5N_2Si_2$: C, 56.13; H, 8.98; N, 5.95. Found: C, 55.94; H, 9.5; N, 5.75.

3',5'-Di-O-(t-butyldimethylsilyl)-2'-deoxyadenosine, 3.

^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H, H8), 8.15 (s, 1H, H2), 6.45 (t, 1H, H1', J = 6.4 Hz) 5.9 (s, 2H, $6NH_2$) , 4.61 (m, 1H, H3'),4.01 (m, 1H, H4'), 3.7 and 3.8 (ddd, 2H, H5' and 5"), 2.4 and 2.5 (ddd, 2H, H2' and 2", J = 6.4, 4.1 and 13.0 Hz), 0.93 (s, 18H, 2x$C(CH_3)_3$), 0.15 (s, 12H, 2x$Si(CH_3)_2$). **^{13}C NMR** ($CDCl_3$) δ 155.3 (C6), 152.9 (C2), 149.6 (C4), 139.1 (C8), 120.1 (C5), 87.9 (C4'), 84.3 (C1'), 71.9 (C3'), 62.8 (C5'), 41.2 (C2'). Analysis, Calc. $C_{22}H_{41}O_3N_5Si_2$: C, 55.08; H, 8.60; N, 14.59. Found: C, 55; H, 8.50; N, 14.36.

5-Bromo-5'-O-(4,4'-dimetoxytrityl)-2'-deoxyuridine 9

Compound **6** (3.07 gm, 10 mmol) was dried by co-evaporation with dry pyridine and redissolved in dry pyridine (150 ml). To this 4,4'-dimethoxytrityl chloride (3.7 gm, 1.1

eq) was added and reaction mixture was kept stirring at room temperature for 3h. The progress of the reaction as monitored by TLC was complete in 3 hr. It was quenched by adding MeOH (1 ml), concentrated to dryness, dissolved in CH_2Cl_2 and washed with water, purified by column chromatography. (3.65 gm, yield 65%). Rf (system C) = 0.5. **^{1}H NMR** ($CDCl_3$) δ s 8.15 (s, 1H, H6), 7.25 to 7.75 (m, 9H, ArH DMT), 6.85 (m, 4H, Ar H DMT), 6.35 (q, 1H, H1'), 4.6 (m, 1H, H3'), 4.15 (m, 1H, H4'), 3.8 (s, 6H, 2xOCH_3 DMT), 3.4 (m, 2H, H5'and H5"), 2.6 and 2.35 (m, 2H, H2'and H2"). **^{13}C NMR** ($CDCl_3$) δ 159.4 (C4), 149.1 (C2), 139.3 (C6), 97.1 (C5), 86.6 (C4'), 85.7 (C1'), 72.1 (C3'), 63.4 (C5'), 55.1 (OCH3), 41.2 (C2').

5-Amino-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine 10

Compound **9** (3 gm, 5 mmol) was treated with liquid ammonia (20ml) in a stainless steel bomb at 60 °C for 24 h. After removal of ammonia, the residue was taken in CH_2Cl_2 and washed with water to remove NH_4Br formed during the reaction. The product gave a single spot on TLC and was used without any further purification (yield 2.63 gm, 98%). Rf (system C) = 0.25, ninhydrin positive.

^{1}H NMR ($CDCl_3$) δ 7.7 to 7.2 (m, 10H, H6 and ArH DMT), 6.85 (m, 4H, ArH DMT), 6.5 (t, J=6.5Hz, 1H, H1'), 4.65 (m, 1H, H3'), 4.1 (m, 1H, H4'), 3.75 (S, 6H, 2xOCH_3 DMT), 3.45 (m, 2H, H5' and H5"), 2.4 (m, 2H, H2' and H2"). **^{13}C NMR** ($CDCl_3$) δ 160.7 (C4), 150 (C2), 127 (C6), 116.6 (C5), 86.1 (C4'), 84.6 (C1'), 72 (C3'), 63.6 (C5'), 55.1 (OCH_3), 40.4 (C2').

5'-O-(4,4'-dimethoxytrityl)-5-N-trifuoroacetyl-5-amino-2'-deoxyuridine 11

Compound **10** (0.5 g, 1mmol) was taken in dry MeOH (12 ml) to which dry TEA (0.69 ml, 10mmol) was added and the mixture stirred for 2 min. To this, CF_3COOEt (1.2 ml, 10mmol) was added and the reaction mixture was kept stirring for 18 hr. Reaction was monitored by TLC where the product showed a faster moving ninhydrin negative spot. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2

and organic was washed with water and product was isolated by column chromatography. (yield 0.5 gm, 78%), Rf (system C) = 0.3 ninhydrin negative spot.

^{1}H NMR ($CDCl_3$) δ 8.6 (s, 1H, H6), 7.75 to 7.2 (m, 9H, ArH DMT), 6.8 (m, 4H, ArH DMT), 6.35 (t, J=6Hz, 1H, H1'), 4.4 (m, 1H, H3'), 4.1 (m, 1H, H4'), 3.8 (s, 6H, 2xOCH_3 DMT), 4.45 to 3.35 (m, 2H, H5'and H5"), 2.45 to 2.2 (m, 2H, H2' and H2"). **^{13}C NMR** ($CDCl_3$) δ 159.2 (C4), 148.6 (C2), 144.5 ($\underline{C}OCF_3$), 136.4 (C6), 129 (C5), 112.3 (CF_3), 86 (C4'), 85.7 (C1'), 71.6 (C3'), 63.(C5'),55.2 (OCH_3), 39.9 (C2').

5-N-acetyl-5-amino-2'-deoxyuridine 13 and **5-N-benzoyl-5-amino-2'-deoxyuridine 14**.

Compound **7** (0.240 gm, 1 mmol) was co evaporated with dry pyridine and redissolved same solvent (10 ml). It was cooled in ice bath, and trimethylchlorosilane (0.61 ml, 5 eq) was added dropwise under anhydrous conditions. After 30 min either acetyl chloride (0.35 ml, 5 eq) or benzoyl chloride (0.58 ml, 5 eq) was added, reaction mixture was brought to room temperature and stirred for another 2 h. It was then cooled, cold water (2 ml) was added and after 15 min concentrated aq ammonia (2ml) was added dropwise. After 30 min, the reaction mixture was concentrated to dryness and purified over silica gel (3 gm) column.

Compound 13 ^{1}H NMR (DMSO-d_6) δ 11.7 (brs, 1H, 5-NH), 9.2 (brs, 1H, N3H), 8.35 (s, 1H, H6), 6.2 (t, 1H, H1'), 5.25 (d, 1H, 3'-OH), 4.9 (t, 1H, 5'-OH), 4.2 (m, 1H, H3'), 3.75 (m, 1H, H4'), 3.5 (m, 2H, H5' and H5"), 2.1 (m, 2H, H2' and H2"), 2.0 (s, 3H, $COCH_3$).

Compound 14 ^{1}H NMR (DMSO-d_6) δ 11.7 (brs, 1H, 5-NH), 8.9 (s, 1H, H6), 8.6 (brs, 1H, N3H), 7.9-7.4 (m , 5H, ArH), 6.4 (t, 1H, H1'), 5.1 (d, 1H, 3'-OH), 4.5 (m, 2H, 5'-OH and H3'), 4.0 (m, 1H, H4'), 3.8 (m, 2H, H5' and H5"), 2.3-2.2 (m, 2H, H2' and H2"), **^{13}C NMR** (DMSO-d_6) δ 164.1 ($\underline{C}OBz$), 158.7 (C4), 147.6 (C2), 130.6 (C6), 112.6 (C5), 86 (C4'), 83.6 (C1'), 69.4 (C3'), 60.5 (C5'), 38.3 (C2').

5-N-Phenoxyacetyl-5-amino-2'-deoxyuridine 15

Compound **7** (0.24 gm, 1 mmol) was coevaporated with dry pyridine, redissolved in dry pyridine (10 ml) and cooled in an ice bath. To this phenoxyacetyl chloride (1.35 ml, 10 eq) was added dropwise. The reaction mixture was kept stirring at room temperature for 3h after which it was concentrated to an oil. This was dissolved in CH_2Cl_2, washed with saturated aq $NaHCO_3$ and the organic layer was concentrated to obtain 3',5'-di-O-5-N-triphenoxyacetyl-5-amino-2'-deoxyuridine. This was dissolved in methanolic ammonia (10 ml) and kept at room temperature for 3 hr. It was concentrated and the product **15** was purified by silica gel column to give **15**.

^{1}H NMR (DMSO-d_6) δ 8.6 (s, 1H, H6), 7.5-6.8 (m, 5H, ArH), 6.3 (t, 1H, H1'), 5.3 (s, 2H, $C\underline{H}_2$COPh), 4.2 (m, 1H, H3'), 3.9 (m, 1H, H4"), 3.5 (m, 2H, H5' and H5"), 2.5 (m, 2H, H2' and H2").

5-N-Trifluoroacetyl-5-amino-2'-deoxyuridine 16

Compound **7** (0.9 gm, 3.7 mmol) was taken in dry MeOH (45 ml) containing TEA (2.5 ml, 18.5 mmol) and to this, CF_3COOEt (3.25 ml, 7.5 eq) was added. The reaction was kept stirring at room temperature for 20 hr and was monitored by TLC, when the product appeared as a ninhydrin negative spot. After work up the product **16** was crystalized from MeOH. (0.65 gm, yield 51%). m.p 96-98°C, Rf (system A) = 0.6, ninhydrin negative. **^{1}H NMR** (DMSO-d_6) δ 8.25 (s, 1H, H6), 6.15 (t, J=8 Hz, 1H, H1'), 4.2 (m, 1H, H3'), 3.8 (m, 1H, H4'), 3.55 (m, 2H, H5'and H5"), 2.2-2.0 (m, 2H, H2'and H2"). **^{13}C NMR** (DMSO-d_6) δ 159.2 (C4),149.1 (C2), 144.1 ($\underline{C}OCF_3$), 142.8 (C6), 130.5 (C5), 112 (CF_3), 87.7 (C4'), 85.7 (C1'), 71.1 (C3'), 62.1 (C5'), 40.5 (C2').

5'-O-(4,4'-dimethoxytrityl)-5N-trifluoroacetyl-5-amino-2'deoxyuridine 3'-O-(N,N-diisopropylamino)(β-cyanoethoxy)phosphine 12

Compound **11** (0.32gm, 0.5 mmol) and tetrazole (35 mg, 0.5 mmol) were dried by coevaporation and suspension in dichloroethane (4 ml). To this 2-cyanoethyl

N,N,N',N'-tetraisopropylphosphorodiamidite (0.15 ml, 0.5 mmol) was added with stirring at 20°C. After stirring for 3 hr the precipitate of diisopropylammonium tetrazolide was removed by filtration and the solution was diluted with dry CH_2Cl_2 and washed with 5% $NaHCO_3$, concentrated to dryness and purified by precipitation from DCM by cold hexane. Rf (EtOAc:CH_2Cl_2, 1:1 and 0.5% TEA) = 0.62, two close moving spots for two distereoisomers. **^{1}H NMR** ($CDCl_3$) δ 8.6 (s, 1H, H6), 7.15 to 7.55 (m, 9H, ArH DMT), 6.8 (m, 4H, ArH DMT), 6.35 (t, 1H, H1'), 4.5 (m, 1H, H3'), 4.2 (m, 1H, H4'), 3.7 (s, 6H, 2xOCH_3, DMT), 3.2 to 3.6 (m, 6H, OCH2, 2xNCH, H5' and H5"), 2.4 to 2.8 (m, 4H, CH_2CN, H2' and H2"), 1.25 (d, 12H, 2x$NC(CH_3)_2$).

Oligonucleotide Synthesis, purification and labelling

All oligonucleotides were synthesized on 1.3 μmol scale on a Pharmacia GA plus DNA synthesizer using controlled pore glass support and base protected 5'-O-(4,4'-dimethoxytrityl)deoxyribonucleoside-3'-O-[(diisopropylamino)-β-cyanoethylphopsphoramidite] monomers which was followed by deprotection with aqueous NH_3. For synthesis of oligonucleotides **18-20** containing 5-amino-2'-deoxyuridine, appropriate amidite monomer **12** was employed in place of normal amidite for coupling at desired positions. All oligonucleotides were purified by reversed phase HPLC on Novapak C18 column using the buffer systems A: 5% CH_3CN in 0.1 M triethylammoniumacetate (TEAA) and B: 30% CH_3CN in 0.1 M TEAA using a gradient A to B of 1.5%/min at a flow rate of 2 ml/min. The purified oligonucleotides were labeled at the 5'-end with T4 polynucleotide kinase and 5'-[γ-^{32}P]ATP according to standard procedures. The radiolabeled oligonucleotide samples were run on a 20% polyacrylamide gel containing 7M urea and with Tris-Borate-EDTA (pH 8.3) as buffer. Samples were loaded in formamide, heated to 70°C and cooled on ice bath before loading on the gel. Autoradiograms were developed within 1 h with an intensifying screen.

Gene Assembler Plus

Date : 17.06.1992
Sequence : DIN-22
Synthesis : DIN-22
Scale : 1.3 micromole
Sequence Length : 12
Column : 1
Final Detritylation : Yes
Coupling Efficiency Threshold : 90 %

Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %	Ave eff %
G	0.39	1.00	1072	197.87	-	-
C	0.42	1.00	3001	476.22	-	-
G	0.39	1.00	3001	384.40	-	-
C	0.43	0.99	3001	463.86	98.7	98.7
Y	0.43	0.99	3001	448.51	-	-
T	0.44	0.99	2882	475.23	-	98.7
A	0.44	0.99	3001	410.58	-	98.7
A	0.44	1.00	3001	408.58	99.5	99.1
G	0.42	1.00	3001	369.90	99.4	99.2
C	0.44	1.00	2957	447.52	99.4	99.2
G	0.42	1.00	3001	364.55	99.3	99.2
C	0.45	1.68	2858	473.59	102.9	99.9

synthesis yield from start = 98.4 %

Typical synthesis report of solid phase oligonucleotide synthesis, where Y is 5-aminodansyl-2'-dU.

Melting experiments

Melting experiments were carried out in buffer 10 mM Tris-HCl, 80 mM NaCl, 20 mM $MgCl_2$, pH 7.5. heated to 70°C for 3 min, cooled to room temperature and then stored at 4°C overnight. The A_{260} at various temperatures were recorded using Perkin Elmer Lambda 15 UV/VIS spectrophotometer, fitted with a temperature programmer and heating at a rate of 0.5°C/min over the range 20-75°C, duplex Tm, was determined from the midpoint in the plots of fraction absorbance change versus temperature and were further confirmed by differential (*dA/dT vs T*) curves. The Tm values are accurate to $\pm$ 1°C over the reported values.

***EcoR*I Endonuclease Hydrolysis**

The oligonucleotide **17-20** were tested as substrate for the *EcoR*I endonuclease by 5'-^{32}P labeled and unlabeled dodecamer in the final reaction volume of 10-20 μl. It was dried under vaccum, redissolved in 5 μl of reaction buffer (20 mM Tris-HCl, pH 7.3, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) and annealed to form duplex. The reaction was started by adding enzyme mixture that contained reaction buffer, bovine serum albumin (50 μg/ml) and endonuclease (2 units), incubated at 30°C for 1 hr. The reaction was quenched by addition of 0.5 M EDTA, part of the reaction mixture was dried, added to the loading dye containing formamide. This sample was heated in boiling water for 3 min and cooled on ice and loaded on 14% polyacrylamide, 7 M urea gel. Electrphoresis was done till the bromophenol dye moved 3/4 the distance on 20 cm long (0.4 mm thick) gel, followed by development of electrophotogramm using intensifying screen.

2.7. REFERENCES

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CHAPTER 3

FLUORESCENT OLIGONUCLEOTIDE PROBES

3.1. INTRODUCTION

As described in earlier chapter the amino functionality at C5 of 5-Amino-2'-deoxyuridine is directed into the major groove of the helix and does not interfere in usual Watson-Crick hydrogen bonding. The chemical reactivity of this C5 amino group is different from the exocyclic amino groups of nucleobases as evident from the requirement of a different protecting group (Barawkar & Ganesh, 1993). It also offers possibilities of direct conjugation of ligands to C5 via a amide linkage with or without spacer chain.

The length of the spacer arm used to link the conjugate group to the oligonucleotide plays an important role on the effect of the modification (Englisch & Gauss, 1990). For the intercalating reagents that have to interact with the helix, a very small change in spacer length influences their interaction. For psoralen to cross-link with the opposite strand, a short spacer of two carbons was required (Teare & Wollenzien, 1989). For reporter groups, biotin and fluorescent tags that do not interact with the helix, a longer spacer of eleven to twelve atoms was preferable to minimize steric inhibition to hybridization. Direct conjugation of the ligands with short spacer chain have useful consequences in control of the fluorescence properties (Singh et al., 1990) and the precision in footprinting experiments (Guest et al., 1991).

The methodologies for substitution at C5 of dU derivatives are centered around Pd(II) mediated coupling of either 5-HgCl derivative with a,β-unsaturated esters (Dervan & Dreyer, 1985) and allylamines (Cook et al., 1988) or Pd(0) coupling of 5-iodo nucleosides with alkynylamines in presence of added CuI (Hobbs Jr, 1989). The method presented here by amino conjugation route is not only comparable to the reported methods in terms of ease of synthesis and overall yields, but also offers possibilities of direct conjugation of ligands to C5 via amide linkage and control over the spacer chain.

3.2. PRESENT OBJECTIVES

The main objectives of this chapter are (i) synthesis of suitable fluorescent oligonucleotide conjugates for studying DNA-drug interaction, (ii) characterization of major groove polarity, (iii) hybridization of nucleic acids in solution and (iv) application of primers in PCR-based diagnostics. Accordingly, this chapter is divided into three sections.

SECTION A

3.3. STUDY OF MINOR GROOVE INTERACTION AND CHARACTERIZATION OF MAJOR GROOVE POLARITY THROUGH A MAJOR GROOVE SEMANTOPHORE CONJUGATE.

An understanding of the molecular basis of DNA recognition by proteins, drugs and various ligands is crucial to discern the chemistry underlying the basic cellular processes, their regulation and rational design of drugs. The major and minor grooves in duplex DNA act as conduits of molecular information required for DNA association with other molecules since hydrogen bonding centers in bases are pointed into these grooves (Saenger, 1984). Large molecules such as proteins binding to nucleic acids, recognize DNA via specific interactions in the major groove (Schleif, 1988; Steitz, 1990); smaller DNA binders such as antibiotics interact with DNA either by intercalating the base pairs or by association in the minor groove or both (Zimmer & Wahnert, 1986; Niedle, et al., 1987). Extensive X-ray crystallographic studies have indicated specific structural changes induced in DNA upon complexation with other molecules (Saenger & Heinemann, 1989; Travers, 1989) and are well supported in many cases by spectroscopic studies in solution (Zimmer & Wahnert, 1986; Niedle, et al., 1987; Saenger & Heinemann, 1989; Travers, 1989; Patel, 1982). The expression of the molecular forces that dictate and control the affinities/specificities of DNA binding agents (proteins/drugs)

are modulated by the local micro-environment. Hence characterization of the environment in the grooves of DNA complexes, assumes importance in delineating the relative contributions of various molecular interactions in stabilizing DNA complexes.

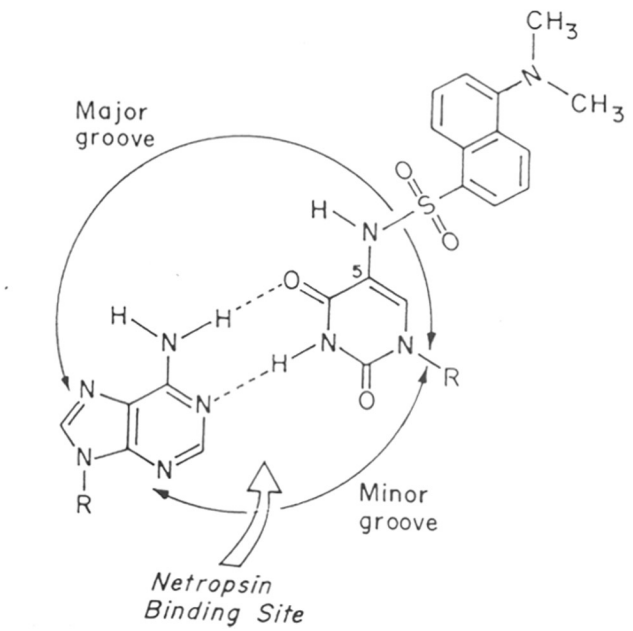


Figure 1. 5-aminodansyl-dU:dA base pairing with the fluoroprobe in the major groove and netropsin binding site in the minor groove.

In view of the functional importance of the major and the minor grooves in DNA recognition, it would be appropriate to study the inherent differences in their environments and the information exchange/transfer that is possible among them upon DNA binding with other molecules. Herein, it is demonstrated that an exclusive minor groove event such as DNA-netropsin association can be quantitatively monitored by changes in dansyl fluorescence as observed from the major groove. The major groove polarity of DNA in

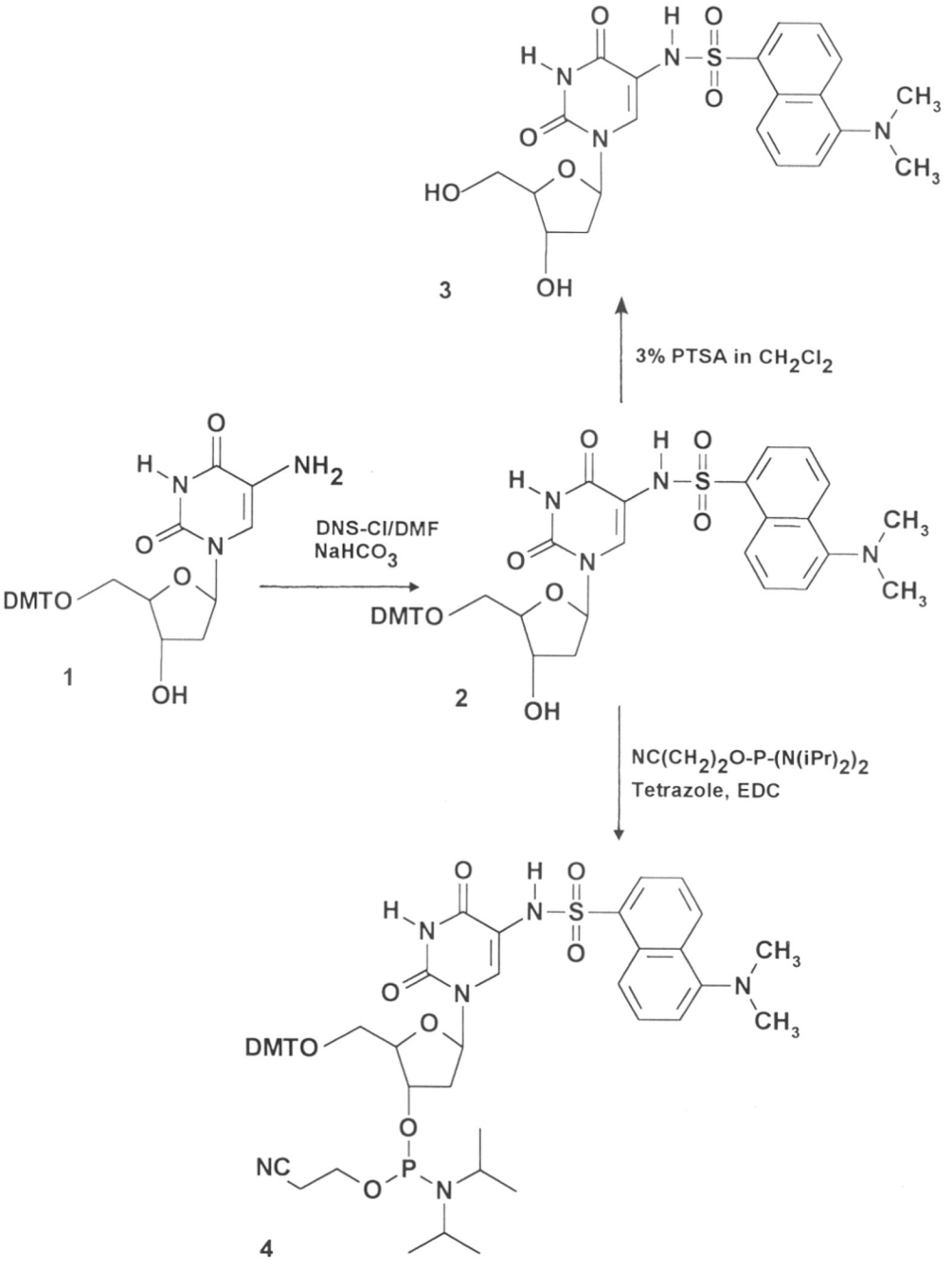
oligonucleotides **6-9** has been characterized using an environment sensitive fluoroprobe, the dansyl group, rigidly linked to C-5 of dU and directed in the major groove (Figure 1) as a "semantophore".

3.3.1. *Results and Discussion*

Dansyl was chosen as a fluoroprobe because it has large Stoke's shift and further, it responds to perturbations in local environments such as changes in solvation, ligand binding, etc by undergoing spectral shifts (Chen, 1967; Bramhall, 1986). Such alterations in the spectral properties of dansyl fluorophore have been previously used to detect substrate binding to protein (Onedra et al., 1976; Skorka et al., 1981) and to study interaction of DNA with the Klenow fragment of DNA polymerase I (Guest et al., 1991).

3.3.2. *Synthesis, Characterization and Stability of 5-N-dansyl-5-amino-2'-dU-Oligonucleotide.*

Synthesis: In chapter II, successful synthesis of oligonucleotide containing 5-amino-dU using trifluoroacetyl as a NH_2 protector was reported. This reactive 5-amino group was used to link dansyl fluoroprobe to nucleobase (Scheme 1). 5-amino-5'-O-DMT-2'-deoxyuridine **1** was reacted with dansyl chloride in DMF containing saturated $NaHCO_3$ to yield 5-N-dansyl-5'-O-DMT-5-amino-2'-deoxyuridine **2**. Under these conditions dansyl chloride reacted selectively with 5-amino group and did not require protection of 3'-hydroxyl. The presence of the dansyl group in compound **2** was clearly shown in ^1H NMR (Figure 2a) by characteristic signals for $N\text{-}(CH_3)_2$ at 2.8 ppm and the aromatic protons at 8.4-7.2 overlapping with those of DMT. ^{13}C NMR shows signals for $N\text{-}(CH_3)_2$ at 44.8 ppm (Figure 2b). The compound **2** on treatment with *p*-toluenesulfonic acid, underwent detritylation to give 5-N-dansyl-5-amino-2'-dU **3** in which, the presence of dansyl group could be unambiguously assigned by ^1H NMR (Figure 2c) since there is no interference from DMT in aromatic region. Compound **2** was converted into the desired β-cyanoethyl phosphoramidite **4** by standard procedure. This amidite was used


DMTO
NH2
1
OH
DNS-Cl/DMF
NaHCO3
2
3% PTSA in CH2Cl2
3
HO
NC(CH2)2O-P-(N(iPr)2)2
Tetrazole, EDC
4
NC
CH3
N
S

Scheme - 1

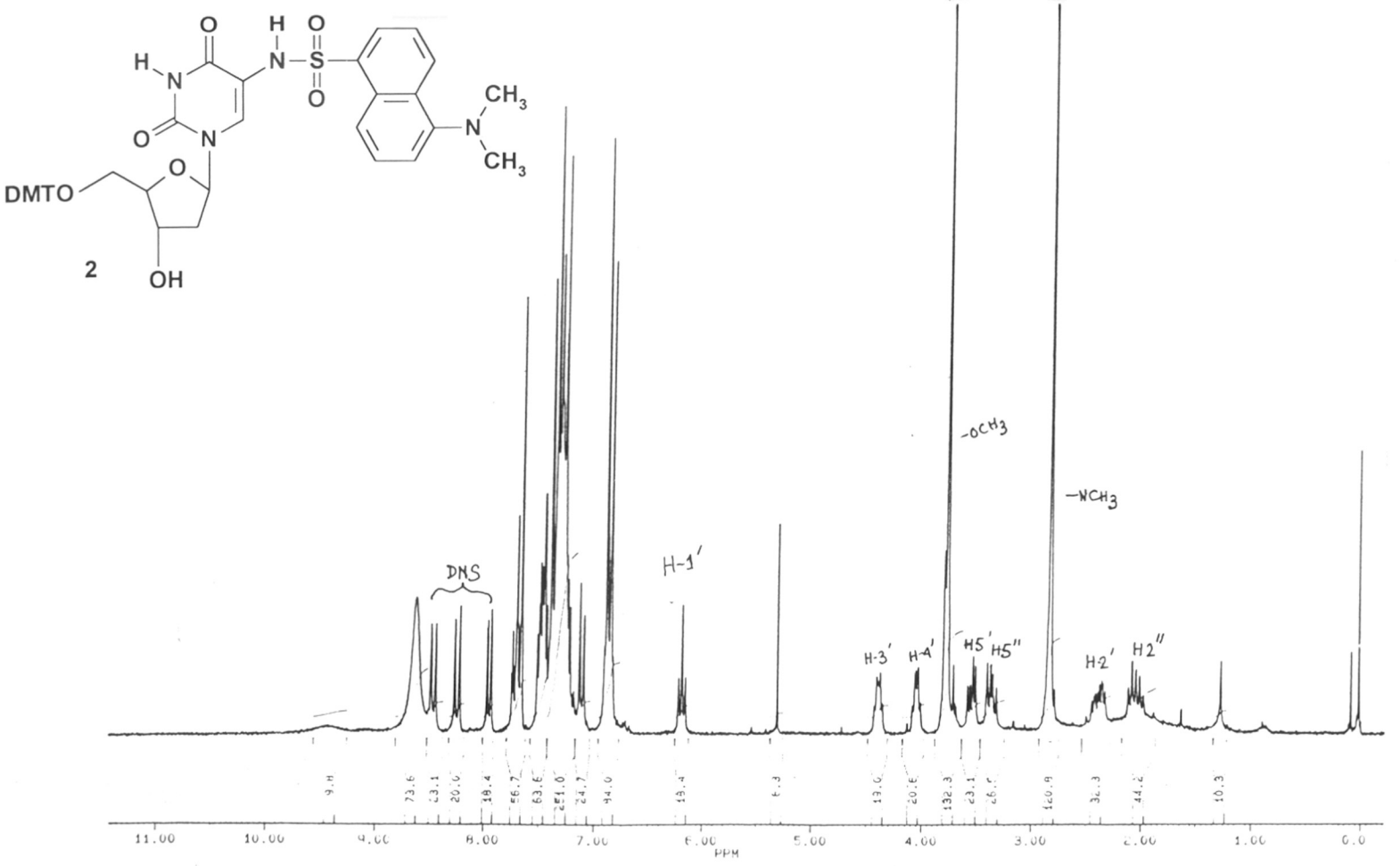


Figure 2a. ^{1}H NMR of **2**.

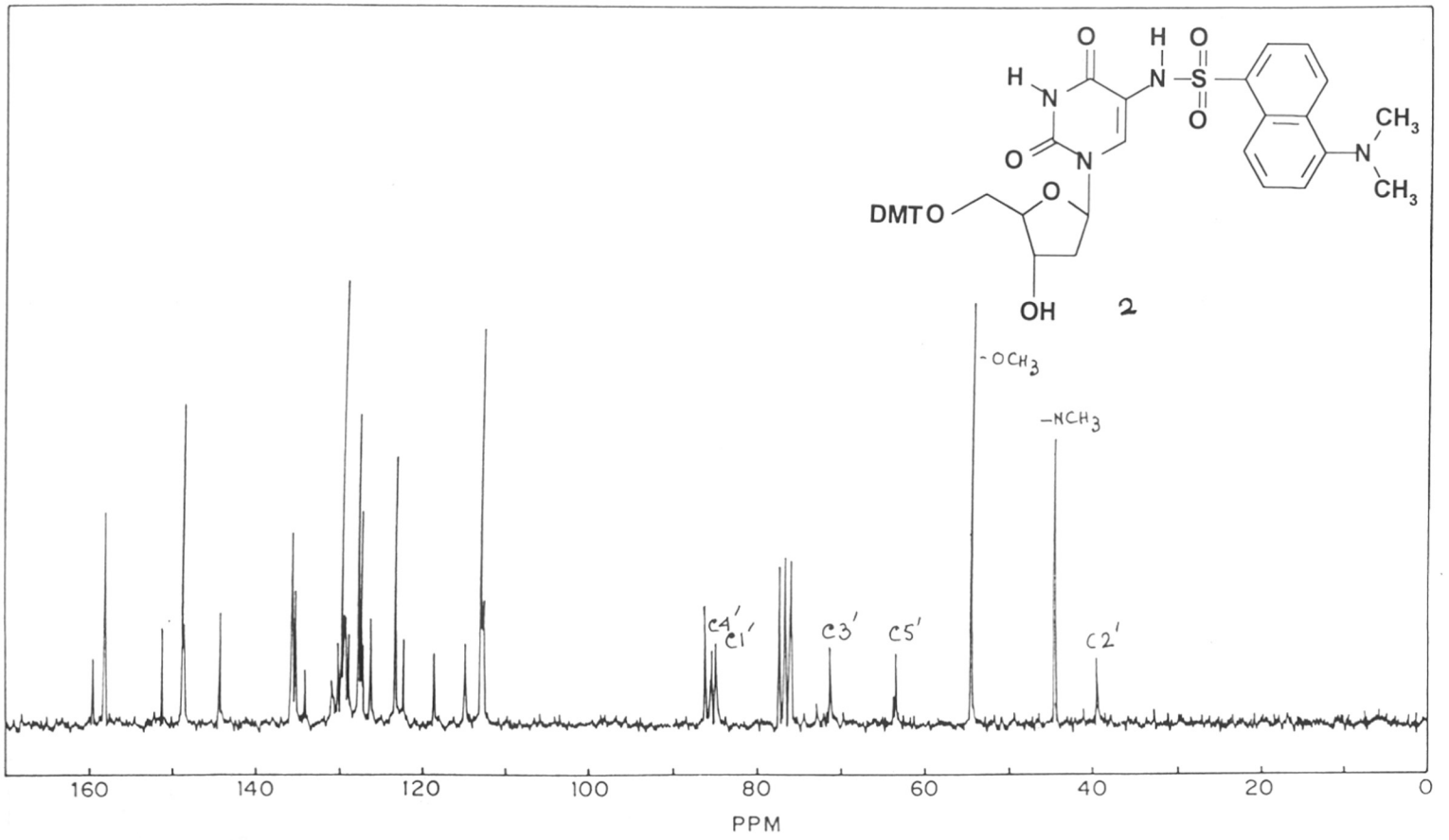


Figure 2b. ^{13}C NMR of **2**.

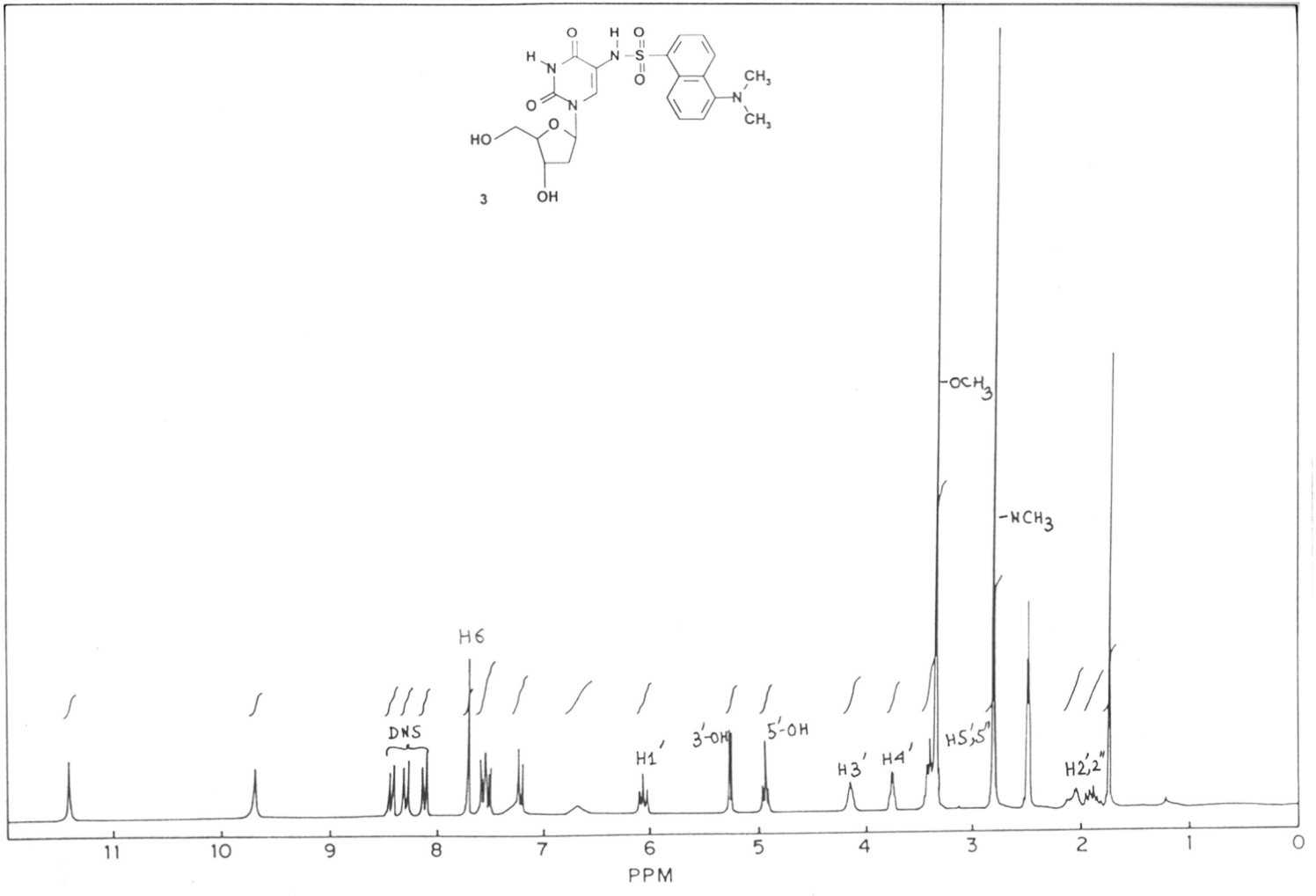


Figure 2c. ^{1}H NMR of **3**.

for incorporation at desired positions in oligonucleotides **6-9** (Table 1) using an automated DNA synthesizer. The coupling efficiency of dansyl amidite **4** was similar to that of commercial amidites of normal nucleosides. The sulphonamide linkage of the dansyl group was stable to oligonucleotide synthesis and deprotection conditions and retention of the dansyl group in the final products was indicated by the detection of dansyl fluorescence at oligonucleotide levels. The oligonucleotides were purified by FPLC and a rechecking by HPLC indicated high purity desirable for biophysical studies (Figure 3).

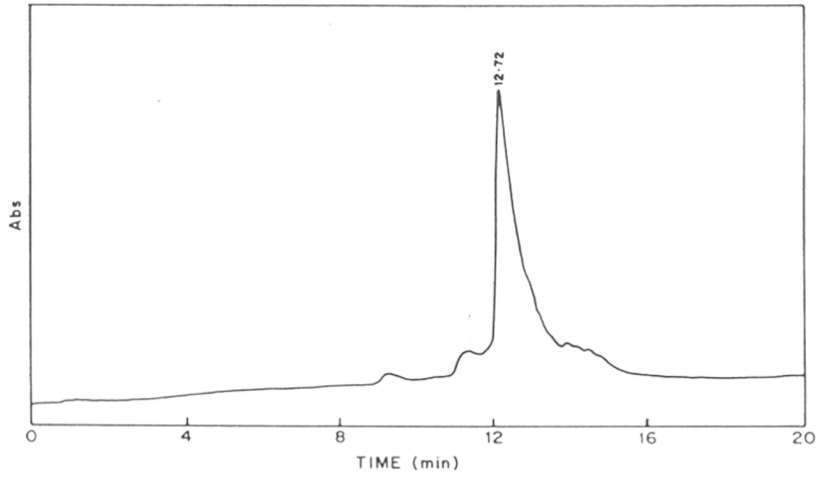


Figure 3. Reverse phase HPLC of **6**.

Characterization: The successful incorporation and retention of 5-aminodansyl-2'-dU (**U***) in the final oligonucleotides was also confirmed by the following experiments.

(i) *Resistance to Eco*R1: The modification is effected within the recognition site of the restriction enzyme *Eco*R1. Hence the modified sequences were resistant to cleavage by *Eco*R1 restriction enzyme. Under same reaction conditions, the unmodified sequences exhibited susceptibility to *Eco*R1.

(ii) *Base Composition Analysis*: The non-specific nucleases such as snake venom

phosphodiesterase degrade nucleic acids from 3'-end to monomeric units. This generates the four nucleoside 5'-monophosphates which can be enzymatically dephosphorylated to deoxynucleosides dA, dC, dG & T for identification and quantification by HPLC. The presence of modified nucleoside should generate additional peaks in HPLC of SVP-alkaline phosphatase reaction of oligonucleotides. As expected, the modified oligonucleotides after the above sequence of reactions exhibited a peak identifiable to 5-N-dansyl-5-amino-2'-dU.

(iii) *Optical Spectroscopy:* Dansyl group absorbs in UV at 335 nm, outside the spectral range of DNA absorbance. The presence of the dansyl group in the finally purified oligonucleotide could be confirmed by this. Further, excitation at this wavelength led to fluorescence emission at 500 nm.

Thermal Stability: Dickerson's dodecamer d(CGCGAATTCGCG) (Kopka, et al., 1985a) is one of the most well studied oligonucleotides, both in free form and as a complex with a variety of ligands. The two T residues in the above sequence were replaced one at a time by 5-aminodansyl-dU (U*), to yield the oligonucleotides **6** and **7**. Modifications at base residues may in principle alter the hydrogen bonding mode as well as base stacking in DNA. Substitution at C-5 of a pyrimidine residue is not expected

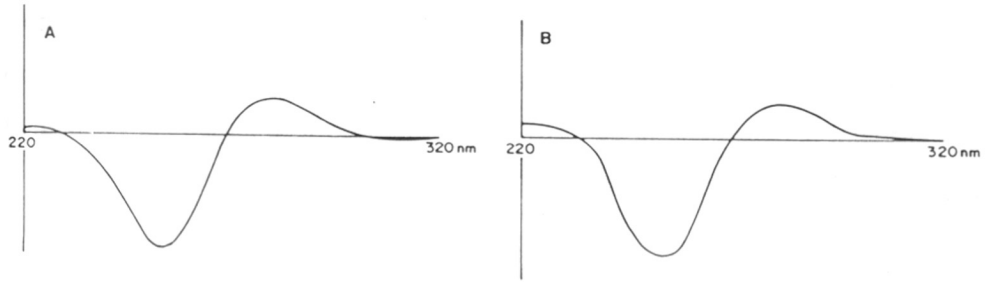


Figure 4. CD spectra of Dickerson's dodecamer **5** (A) and modified oligonucleotide **6** (B).

to significantly affect the standard Watson-Crick mode of hydrogen bonding in dA-dT base pair (Barawkar, et al., 1992; Kopka, et al., 1985b) since this substituent projects into the major groove of DNA. The CD spectra of modified oligonucleotides **6** (Figure 4) was similar to that of Dickerson's dodecamer d(CGCGAATTCGCG) **5** in the region 220-320 nm (negative Cotton effect at 253 nm, positive Cotton effect at 280 nm with cross over at 270 nm) indicating no major alterations of base stacking in **6**. The CD profile overall corresponds to B-form of DNA. However, as followed by temperature dependent UV changes (Figure 5), the dansylated oligonucleotides **6** and **7** gave a lower Tm (48°C) compared to the unmodified dodecamer **5** (60°C) suggesting a slight destabilizing effect on duplex, locally induced by aminodansyl group in dU.

Fluorescence Tm: The oligonucleotides **6-9** are self complementary and hence their duplexes contain two dansyl groups, one in each strand symmetrically located in the major groove. The observed fluorescence properties are therefore a sum of contribution from both dansyl groups of duplex. In duplexes derived from **6** and **7**, the two dansyls are within the binding site (AATT) for the antibiotic netropsin, while in duplexes from **8** and **9** they are outside the binding site, separated by 10 base pairs in case of **8** and 6 base pairs in **9**. The λ_{em} of **8** and **9** were blue shifted by 8-10 nm compared to that of **6** and **7**. The Tm of **6** and **7** were also determined from temperature dependent fluorescence studies. The melting studies showed an enhancement in fluorescence intensity with increasing temperature, the intensity attaining a maximum (Figure 6) just around the melting temperature of oligonucleotide. The rise in fluorescence intensity as a function of temperature is invariant with the oligonucleotide concentration in the range 0.1 μM to 10 μM. From this fact and that of an earlier report (Patel et al., 1992; Lycksell et al., 1987), the observed temperature of maximum fluorescence intensity was considered as an approximate measure of melting of duplex. This Tm value thus derived was in close agreement with that obtained from UV melting studies. The fact that

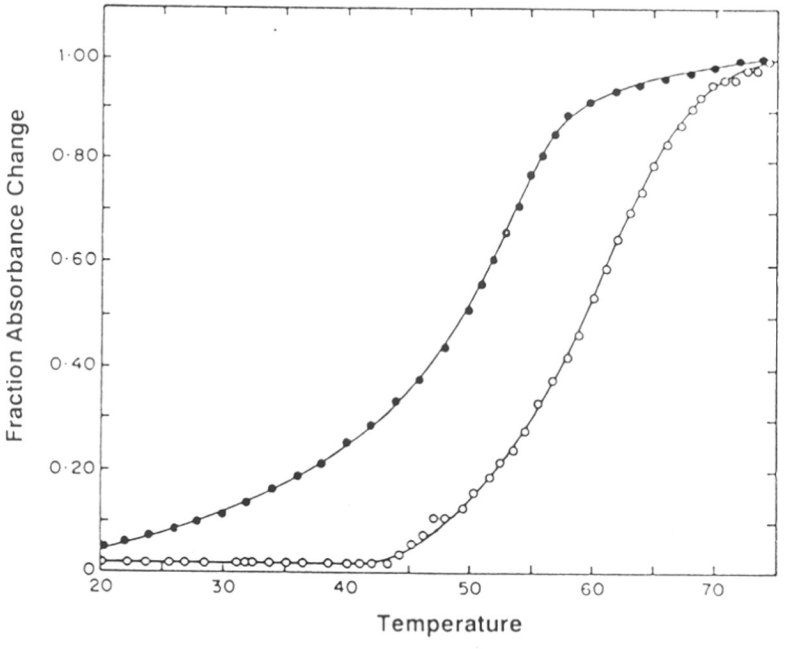


Figure 5. UV melting curves for Dickerson's dodecamer (o) and DNS-DNA **6** (●).

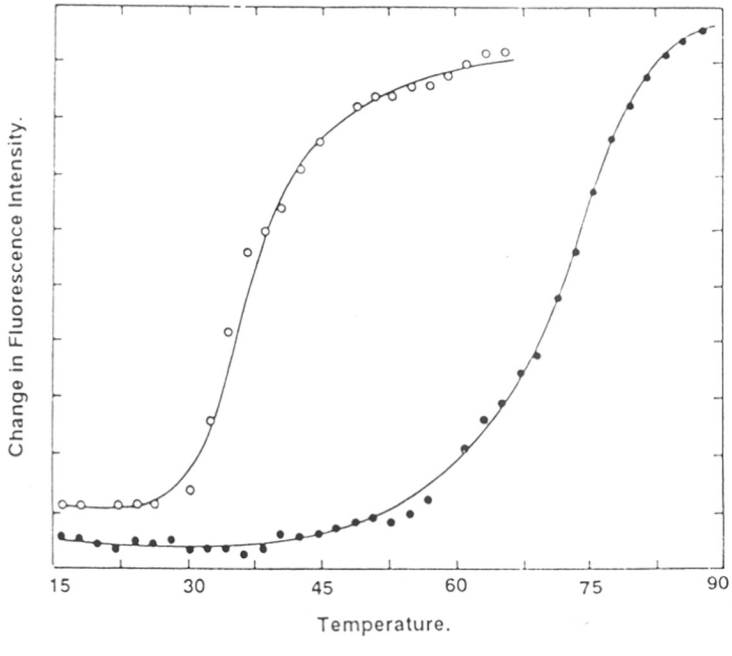


Figure 6. Fluorescence melting curves for DNS-DNA **6** (1μM) in absence (o) and in presence of equimolar netropsin (●).

DNS-DNA exists in B-form at room temperature (as seen by CD profile and sigmoidal UV/fluorescence Tm curves) substantially supports the use of DNS-DNA **6** and **7** as suitable models for studying DNA interactions.

3.3.3. *Interaction of Netropsin with DNS-DNA*

As stated earlier, 5-aminodansyl group protrudes into the major groove of DNA and hence should be an ideal reporter to study molecular interactions of DNA. The observed resistance of sequences **6** and **7** to *Eco*R1 enzyme indicated interference of major groove interactions by the dansyl group. Netropsin is an antiviral minor groove binding drug (Zimmer & Wahnert, 1986; Breslauer & Marky, 1987) and its complexation with B-DNA has been well studied by using a combination of spectroscopic and calorimetric techniques. The molecular details of its interaction in minor groove was established by NMR spectroscopy (Patel, 1982) and X-ray crystallographic studies (Kopka et al., 1985a). In the present work, it was sought to examine the effect of netropsin binding to DNA in the minor groove using the fluorescent dansyl probe located in the major groove.

Upon stoichiometric addition of netropsin into DNS-DNA **6**, an increase in intensity was observed in the dansyl fluorescence emission at 500 nm as a function of ligand concentration. The fluorescence enhancement reached near saturation at 1:1 stoichiometry (Figure 7). The association constants were calculated (Table 1) from the plot of 1/a vs 1/[L] at 25°C (Figure 8) (Pesce et al., 1971) and were found to be in the range 10^7-10^8 M^{-1}. This corresponds to a binding free energy ΔG of -10.8 to -12.1 kcal M^{-1} which is in close agreement with the literature reported value (Breslauer & Marky, 1987) for netropsin binding to a decamer d(GCGAATTCGC). The stoichiometry of binding as computed from a plot of 1/a vs 1/[L] was also close to 1. The modified oligonucleotide **7** behaved in a similar way and in both cases, no appreciable shifts in λ_{ex} and λ_{em} were seen upon netropsin addition. The presence of dansyl group at C5 therefore does not

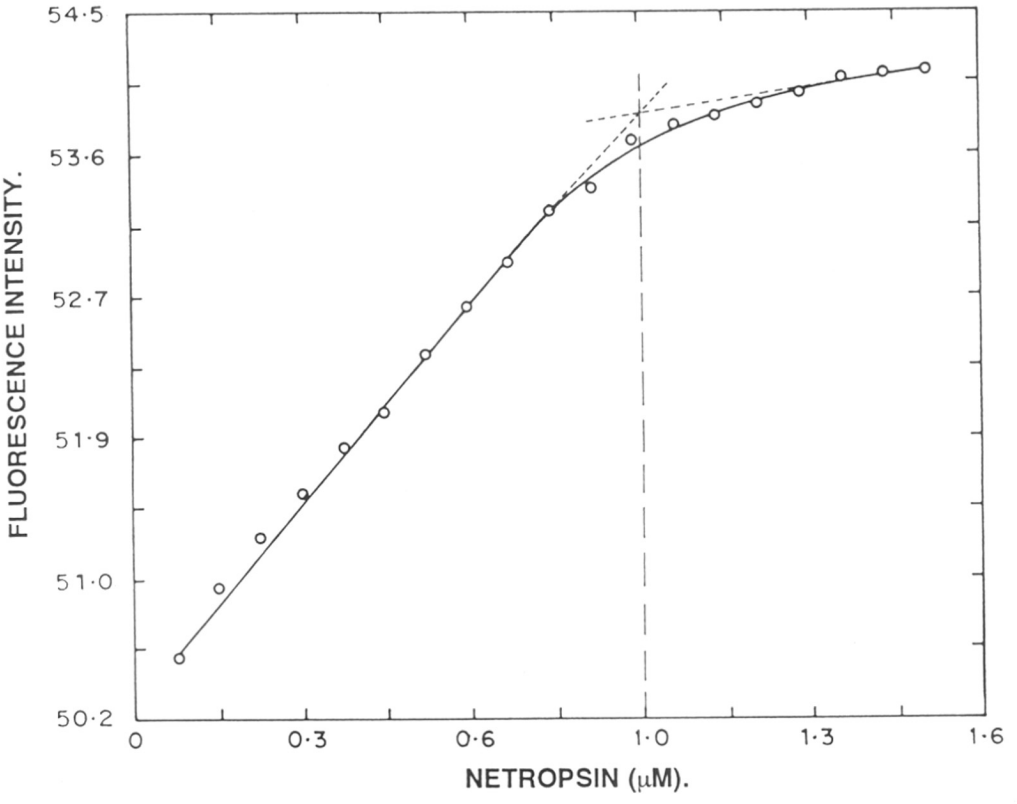


Figure 7. Plot of fluorescence enhancement of DNS-DNA **6** as a function of added netropsin indicating 1:1 stoichiometry.

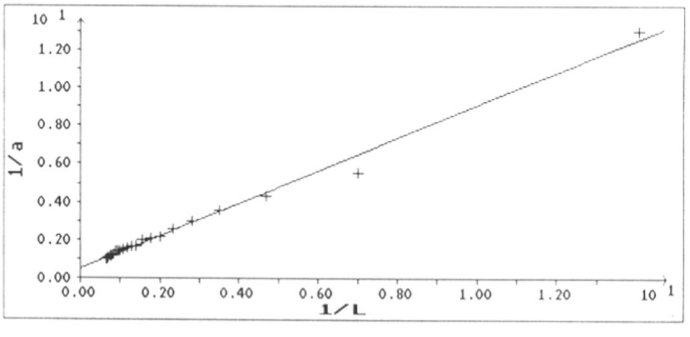


Figure 8. Double reciprocal plot of DNS-DNA **6** used for calculating binding constant.

affect the association strength of netropsin to DNA. Thus although the dansyl fluorophore is present in the major groove of DNA, it efficiently monitors binding of netropsin occurring in the minor groove. Netropsin binding is known to stabilize DNA duplex and hence the binding of netropsin with the oligonucleotides **6** and **7** was also monitored by UV and fluorescence (Figure 5 and 6) melting experiments. Compared to DNS-DNA duplex alone, in 1:1 complex of DNS-DNA:netropsin, the Tm as measured by both UV and fluorescence, was significantly enhanced by similar extents ($\Delta Tm \sim 31^0C$) confirming the thermal stabilization of DNS-DNA by netropsin binding.

Table 1. Association constants and free energy changes for netropsin binding with fluorescent DNA.[®]

Compound No	Oligonucleotide.	Ka (M^{-1})	ΔG (kcal M^{-1})
	GCGAATTCGC[#]	2.8×10^8	- 11.5
6	CGCGAAU[•]TCGCG	5.9×10^7	- 10.8
7	CGCGAATU[•]CGCG	7.5×10^7	- 10.9
8	GCU[•]GTGAATTCACAGC	1.5×10^8	- 11.1
9	GCTGU[•]GAATTCACAGC	7.8×10^8	- 12.1

® All binding experiments were perfoírmed at 25°C.

Taken from (Breslauer & Marky, 1987).

U[•] is 5-N-dansyl-5-amino-2'-dU

The oligonucleotide **7** has the fluorophore located on an adjacent base to that in **6** and the fluorescence titration of **7** with netropsin also gave similar results. In both **6** and **7**, dansyl is linked from the major groove side to a base pair that is directly involved in hydrogen bonding with netropsin in the minor groove. It is possible that the observed changes in fluorescence properties is a direct consequence of the electronic changes induced in base pairs by netropsin binding. To examine this, the oligonucleotides **8** and **9** which have the fluorophore located outside the region of netropsin binding (AATT) were synthesized. Upon netropsin titration, both **8** and **9** gave a higher percent enhancement (~30%) in intensity compared to **6** and **7** (~10%) and there was no shift

in λ_{em} due to complexation (Figure 9). The computed binding constants for oligonucleotides **6-9** are shown in Table 1.

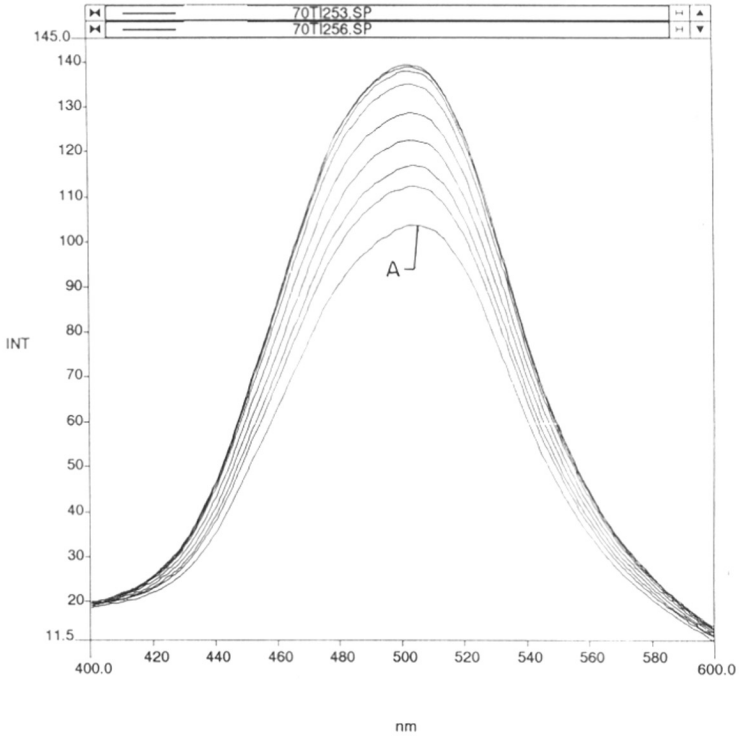


Figure 9. Overlay of fluorescence spectra of DNS-DNA **8**, without netropsin (A) and increasing netropsin concentration indicating no shift in λ_{em} due to complexation.

The strong association of netropsin to DNA is believed to be caused by the displacement of the spine of hydration in the DNA minor groove corresponding to AATT stretch (Kopka et al., 1985a; Breslauer & Marky, 1987) with a major entropic contribution. Further, the binding also widens the minor groove by 0.5 - 2.5 A, accompanied by a bending of helix axis by 8°, but without unwinding or elongation of the double helix

(Kopka et al., 1985b). The observed changes in fluorescence intensity of DNS located in the major groove of DNS-DNA may be a resultant of local structural changes induced in DNA upon minor groove binding by netropsin.

By employing the fluorescent base analogue 2-amino purine, the interaction of netropsin with the fluorescent oligomer has been studied (Patel et al., 1992; Lycksell et al.; 1987) and the drug binding found to be weak due to partial blockage of tight fit of netropsin into the preferred minor groove by the 2-NH_2 group of the host. Such was not the case with the present fluorescent oligonucleotides since the fluoroprobe is located in the major groove through a short and rigid spacer arm.

The above results can only be accounted by the fact that the DNS fluorophore, though located in the major groove, away from the binding site (as in **6-9**), still senses the binding event in the minor groove. This may occur through a pathway involving correlated structural changes in DNA, which mediate an information transfer among the two grooves leading to major groove/minor groove "cross talk". Such an intergroove conversation has recently been observed in netropsin complexation to DNA triple helix (Park & Breslauer, 1992; Durand, et al., 1992). This lead to a decrease in the co-operativity of triplex to duplex transition, in contrast to the stabilization of duplex to single strand transition. The occupancy of the minor groove of DNA by a ligand like netropsin can therefore exert a profound impact on the properties of a resident guest in major groove, for example, an oligonucleotide in a triplex or a fluorophore as in the present case. A study of the conformational perturbations associated with the cross talk among the two main receptor sites of duplex DNA (major and minor groove) may provide an approach for modulating the affinity and specificity of DNA binding agents.

3.3.4. *Characterization of Major Groove Polarity*

Principle: The fluorescence emission spectra of many fluorophores are sensitive to the polarity of their surrounding environment (Lakowicz, 1983). This polarity dependence

arises from (i) interaction of dipole moment of the fluorophore in the excited state with the electrical field induced by the surrounding solvent dipoles and (ii) specific chemical interactions such as hydrogen bonding, charge-transfer interactions etc between the fluorophore and the solvent molecules. The former is a general solvent effect governed by the orientation polarity (*f*) which is derived from the refractive index (n) and dielectric constant (ε)

$$f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

of the medium. The orientation polarity of the solvent reflects the local polarity changes due to reorientation of solvent molecules around the excited fluorophore. In the absence of solvent-fluorophore specific interactions, orientation polarity effects provide a major contribution to the emission spectral shifts. The physico-chemical interactions between fluorophore and the solvent molecules causing the general solvent effects is described by Lippert equation (Lakowicz, 1983).

$$\Delta\nu = \frac{2\Delta\mu^2}{hca^3} \times f$$

where $\Delta\nu$ is the Stoke's shift (the difference between excitation and emission wavelength, expressed as energy difference in cm^{-1}), $\Delta\mu$ is the difference in dipole moment between the excited and the ground states of the fluorophore, h is Planck's constant, c is the speed of light and *a* is the radius of the cavity in which the fluorophore resides. Since for a given fluorophore, *a* and $\Delta\mu$ remain constant, the above equation implies that the Stoke's shift is directly related to solvent polarity (*f*) changes. A calibration curve (Lippert plot) may be generated for variation of Stoke's shift ($\Delta\nu$) with respect to orientational polarity (*f*) and this exhibits a linear behavior if general solvent effects

predominante over specific solvent effects. When the fluorophore is in an unknown environment, from the measured Stoke's shift the orientation polarity (f) can be obtained which is interpolated to yield the dielectric constant (ε) of the medium.

Application to DNA Groove Polarity: It has been reported that HOECHST 33258 (Figure 10) which is a highly fluorescent environment-sensitive drug selectively binds to AT regions in the minor groove and induces sequence specific structural changes in the resulting DNA complex (Breslauer & Jin, 1988). A comparison of the fluorescent observables (λex and λem as a function ν) of this complex with the corresponding properties of free ligand in different solvent systems of variable polarity (neat organic and mixed organic/aqueous solvents) has enabled the determination of the polarity of the minor groove which is around 20D.

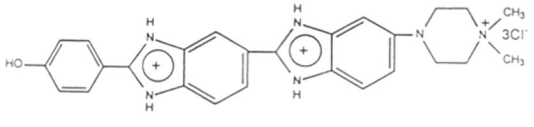


Figure 10. Structure of fluorescent environment-sensitive drug HOECHST 33258.

In order to estimate the dielectric constant of the major groove in DNS-DNA, the fluorescence parameters (λ_{ex} and λ_{em}) of the monomer 5-aminodansyl-dU **3**, were first measured in media of different dielectric constants generated by varying ratios of dioxane-water (Table 2). The Stoke's shift $\Delta\nu$ calculated from the above parameters, exhibited a linear correlation with the orientation polarity (Figure 11). This suggests the dominant influence of general solvent effect in the observed fluorescence properties of **3**. Assuming that such a correlation of orientation polarity and dielectric constant for 5-aminodansyl-dU **3** is also valid on its incorporation into the major groove of Dickerson's dodecamer, the observed Stoke's shift of DNS-DNA **6-9** can be used to estimate the

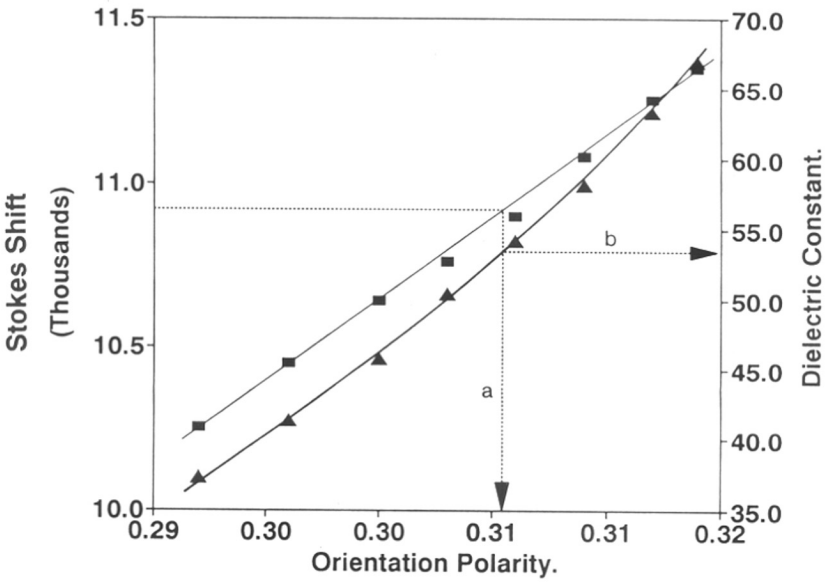


Figure 11. A combined plot of Stoke's shift ($\Delta\nu$) versus orientation polarity (f) (■) and dielectric constant (ε) versus orientation polarity (▲). Arrow a correlates $\Delta\nu$ with f for **3** and arrow b interpolates this f with ε for DNS-DNA **6-9**.

polarity reported by conjugated dansyl in the major groove. The Stoke's shift among differentially labeled oligonucleotides **6-9** was found to be in the range of 10959-10842 cm^{-1}, indicating only a small variation of $\Delta\nu$ with position of dansyl in oligonucleotide sequences. An average value of 10900 cm^{-1} was thus used for interpolation to deduce the orientation polarity, (Figure 11, arrow a) which was ~0.307. This corresponds to a dielectric constant of ~55D (Figure 11, arrow b) which implies that the major groove in **6-9** is non-polar compared to the bulk water (80D), but considerably more polar than the minor groove (~20D). Slight variation seen in Stoke's shift among **6-9**, ($\delta\Delta\nu$ ~ 100 cm^{-1}) is much less to effect significant changes in f and hence the value of the interpolated ε. It may be added that, upon netropsin complexation, no changes were seen in λ_{ex} and λ_{em} of DNS-DNA **6-9** and extension of the above principle suggests that there is no appreciable alteration of the polarity of the major groove upon netropsin complexation in the minor groove. The fluoroprobe is regiospecifically and rigidly conjugated to DNA

by a sulphonamide bond, without much freedom for flexible averaging over different environments. Hence the present method of polarity estimation using dansyl moiety in **6-9** is appropriate.

It may be pointed out that the minor groove polarity estimated for DNA complexes with a non-conjugated fluorophore may have significant contribution from the free, uncomplexed ligand and so may lead to substantial errors in polarity estimation. The presently described method employing site-specific, covalent conjugation is devoid of such a shortcoming. The molecular rigidity due to covalent conjugation exhibits negligible effect on the Stoke's shift of a fluorophore since it has been shown that there is

Table 2. Stoke's shifts of 5-aminodansyl-2'-dU **3** in media of different dielectric constants generated from varying ratio of dioxane:water.⊕

% 1,4-Dioxane	ε	*n*	*f*	λex nm	λem nm	Δν cm^{-1}
0	78.5	1.333	0.320	330	523	11180
5	72.5	1.338	0.317	328	523	11367
10	67.0	1.343	0.314	330	531	11470
15	63.3	1.349	0.312	332	530	11253
20	58.2	1.354	0.309	334	530	11080
25	**54.2**	**1.359**	**0.306**	**336**	**530**	**10900**
30	50.4	1.364	0.303	338	529	10680
35	45.8	1.369	0.300	338	528	10641
40	41.3	1.374	0.296	340	528	10481
45	37.3	1.379	0.292	342	527	10250
50	32.7	1.383	0.288	342	526	10220
55	28.2	1.389	0.283	343	524	10060
60	24.0	1.392	0.277	343	523	10030
65	20.0	1.397	0.270	343	522	10000
DNS-DNA 6			**0.307**	**323**	**500**	**10959**
DNS-DNA 7			**0.307**	**323**	**499**	**10919**
DNS-DNA 8			**0.307**	**320**	**492**	**10925**
DNS-DNA 9			**0.307**	**320**	**490**	**10842**

⊕ Values for ε, *n*, *f* corresponding to various Dioxane:H_2O compositions are taken from (Breslauer & Jin, 1988)

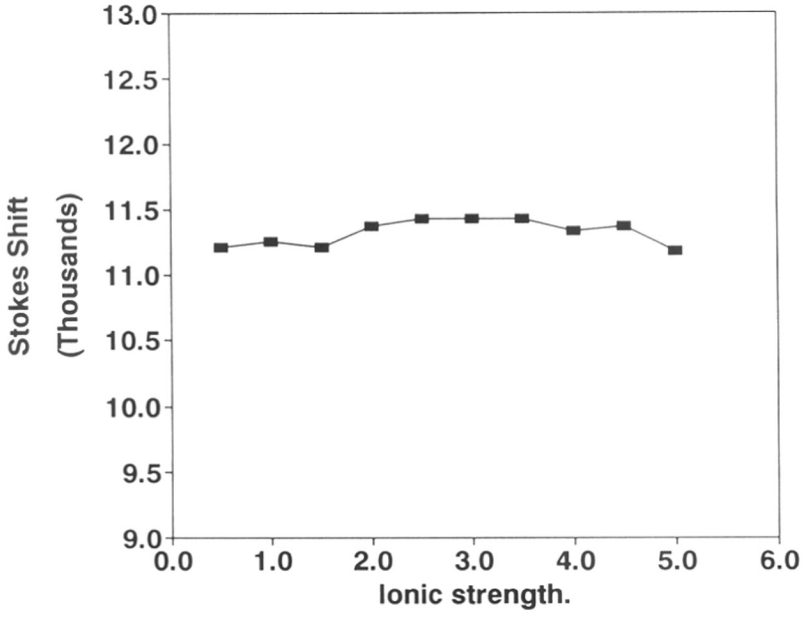


Figure 12a A plot of Stoke's shift, $\Delta\nu$, of **3** at pH 7.0 in 10 mM Tris buffer versus the concentration of added NaCl.

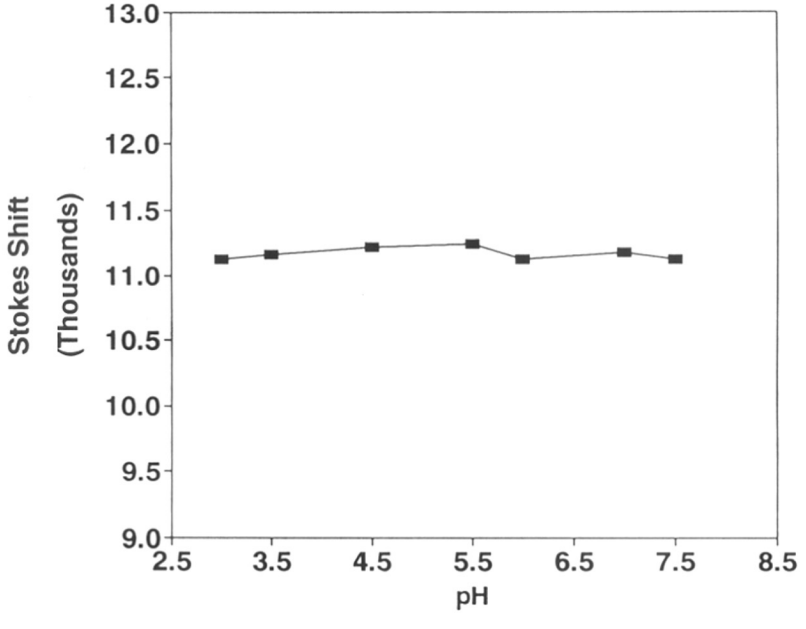


Figure 12b A plot of Stoke's shift, $\Delta\nu$, of **3** in 10 mM Tris buffer versus the buffer pH.

little, if any effect on the optical properties of a free fluorescent ligand upon conjugation to a synthetic polymer (Breslauer & Jin, 1988).

It is well known that fluorescence properties are also influenced by ionic strength and pH of the medium and the exact values of the latter are difficult to obtain for organic/aqueous mixed solvents. In order to examine such effects on dansyl fluorescence, the dependence of Stoke's shift of dansyl in **3** was measured over a range of ionic strength (0.1 M to 5M) and pH (3 to 7.5). It was observed that the Stoke's shift was invariant under these conditions (Figure 12) and hence the induced differences in fluorescence properties in various solvent systems is due to bulk solvent properties.

SECTION B

3.4. ENERGY TRANSFER IN FLUORESCENT DNA DUPLEX

Physical studies of nucleic acid hybridization in solution depend on methods which can separately monitor the paired and unpaired nucleic acids. This may be achieved by several techniques which include absorption spectroscopy (hypochromicity and circular dichroism) calorimetry and nuclear magnetic resonance. All these techniques require large amounts of nucleic acids and most often nucleic acids of interest are not available in such quantities. Radioactivity measurments allow the detection of minute amounts (pmoles) of radioisotope-labeled DNA; however, true solution phase measurements are not possible since physical separation of hybridized and unhybridized nucleic acids is required. Any separation technique perturbs the hybridization equilibria under study and rules out the possibility of monitoring hybridization continuously in real time.

Fluorescence spectroscopy (Lakowicz, 1983) offers a more sensitive method for measurement of nucleic acid concentration than the conventional absorption spectroscopy techniques, such as UV and CD. In addition, the sensitivity of fluorophore

characteristics to the environment offers a means to distinguish the hybridized form from the unhybridized complement without resorting to separation techniques (Cardullo et al., 1988; Morisson et al., 1989).

3.4.1. *Fluorescence Energy Transfer*

The present section describes an approach by which two complementary fluorescent oligonucleotides involved in the process of nonradiative fluorescence energy transfer (FET) can be used to study nucleic acid hybridization in solution. This FET is a consequence of hybridization of nucleic acid strands and creates a distinct signal to reflect this. The implication of this for analytical and diagnostic methods for detecting hybridization events *in vivo* are obvious (Kricka, 1992).

Principle: Fluorescence energy transfer (FET) is a dipole-dipole resonance interaction between two close fluorescent chromophores, where one of the chromophore called "donor", transfers its excitation energy to the other chromophore called "acceptor" (Forster, 1948). The consequences of this energy transfer are i) decrease in donor lifetime, ii) quenching of donor fluorescence by acceptor and iii) an enhancement of acceptor fluorescence intensity. The efficiency of energy transfer E decreases with distance between donor and acceptor molecules (r) and is expressed as

$$E = \frac{R^6o}{R^6o + r^6} \qquad (1)$$

where Ro is the distance at which 50% energy transfer occurs i.e the rate of energy transfer is equal to the rate of the de-excitation in the absence of energy transfer. To observe significant amount of energy transfer the distance separating the donor and acceptor chromophores should be of the order of Ro or smaller.

$$Ro^6 = (8.79 \times 10^5)\ K^2\ n^{-4}\ \Phi_D\ J \quad \text{(in A)} \qquad \textbf{(2)}$$

where Φ_D, is the quantum yield of donor in the absence of acceptor

K, is the orientation factor between donor and acceptor dipole moments

n, is refractive index of solution

J, is the overlap integral between the emission spectrum of the donor and the excitation spectrum of the acceptor

$$J = \frac{f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{f_D(\lambda)d(\lambda)} \qquad \textbf{(3)}$$

$f_D(\lambda)$, is the fluorescence intensity (in arbitrary units) of the energy donor at wavelength λ (in cm).

$\varepsilon_A(\lambda)$, is the extinction coefficient of the acceptor ($M^{-1}\ cm^{-1}$)

The efficiency of energy transfer can be calculated from the steady state fluorescence emission intensities of donor alone (I_D) and its decrease in the presence of acceptor.

$$E = \frac{I_D - I_{DA}}{I_D} \qquad \textbf{(4)}$$

The experimentally measured values of E from eq 4 and R_o from eq 2 can be used to calculate the distance r by eq 1.

The distance dependance of FET was elegantly verified experimentally by Stryer (Stryer & Haugland 1967) for a series of oligo-L-proline molecules of varying length using α-naphthyl group as donor and dansyl as acceptor. Since then, FET has given valuable information on nucleic acid-drug interactions, the structure of nucleic acids (Clegg et al., 1992; Cooper & Hagerman, 1990; Murchie et al., 1989), as means of monitoring the hybridization process of oligodeoxynucleotides (Cardullo et al., 1988;

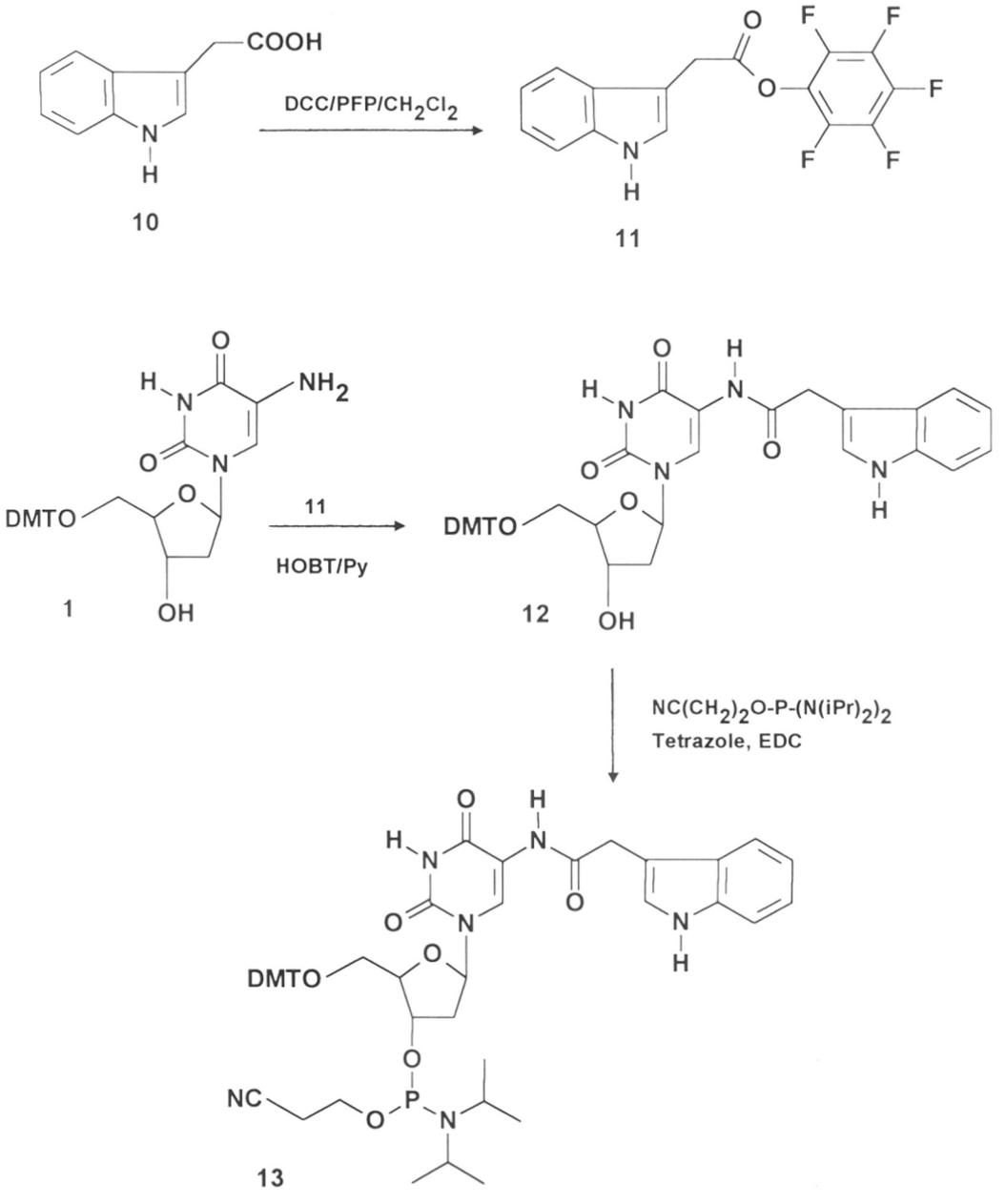
Morisson et al., 1989; Mergny et al., 1994; Yang et al., 1994) and to monitor helicase catalyzed unwinding of duplex DNA continuously in real time (Bjornson et al., 1994; Houston & Kodadek, 1994; Raney et al., 1994).

3.4.2. *Results and Discussion*

In most applications employing nucleic acid-FET, 3' or 5'- terminal positions of oligonucleotides were used for conjugation of donor and acceptor chromophores through long spacer arms. The present work reports the conjugation of donor/acceptor chromophores on nucleobases through dedicated monomer strategy. This approach allows to label the oligonucleotides either at the terminal positions or almost anywhere across the oligonucleotide length through a short and rigid spacer arm. As FET is dependent on distance and orientation of chromophores, rigid locations of the donor and acceptor groups on oligonucleotide, is essential to provide meaningful structural information (for DNA, RNA, and protein-nucleic acid complexes). The location of the reporter groups (donor, acceptor or both) along the DNA helix may be varied relative to each other to generate continuous profile of FET from which structural data can be extrapolated.

3.4.3. *Synthesis of 5-N-(3-indoleacetyl)-5-amino-2'-dU-Oligonucleotide*

A suitable donor and acceptor pair of fluorophores useful for FET are indole acetyl (donor) and dansyl (acceptor). A similar pair tryptophan and dansyl has been reported in FET studies on Zinc finger motif (Eis & Lakowicz, 1993). Complementary oligonucleotides containing this pair would form a suitable duplex system to generate FET. The synthesis of 5-aminodansyl oligonucleotides has already been described in the earlier section of this chapter. Indole acetyl group was also attached to C5 of dU through amide linkage as shown in scheme 2. The carboxyl moiety of indole acetic acid **10** was activated by pentafluorophenol using dicyclohexylcarbodiimide activation to give the ester **11**. The downfield shift of acetate methylene in ^{1}H NMR confirmed the structure. The


COOH
DCC/PFP/CH2Cl2
10
11
NH2
DMTO
OH
1
11
HOBT/Py
12
NC(CH2)2O-P-(N(iPr)2)2
Tetrazole, EDC
13

Scheme - 2

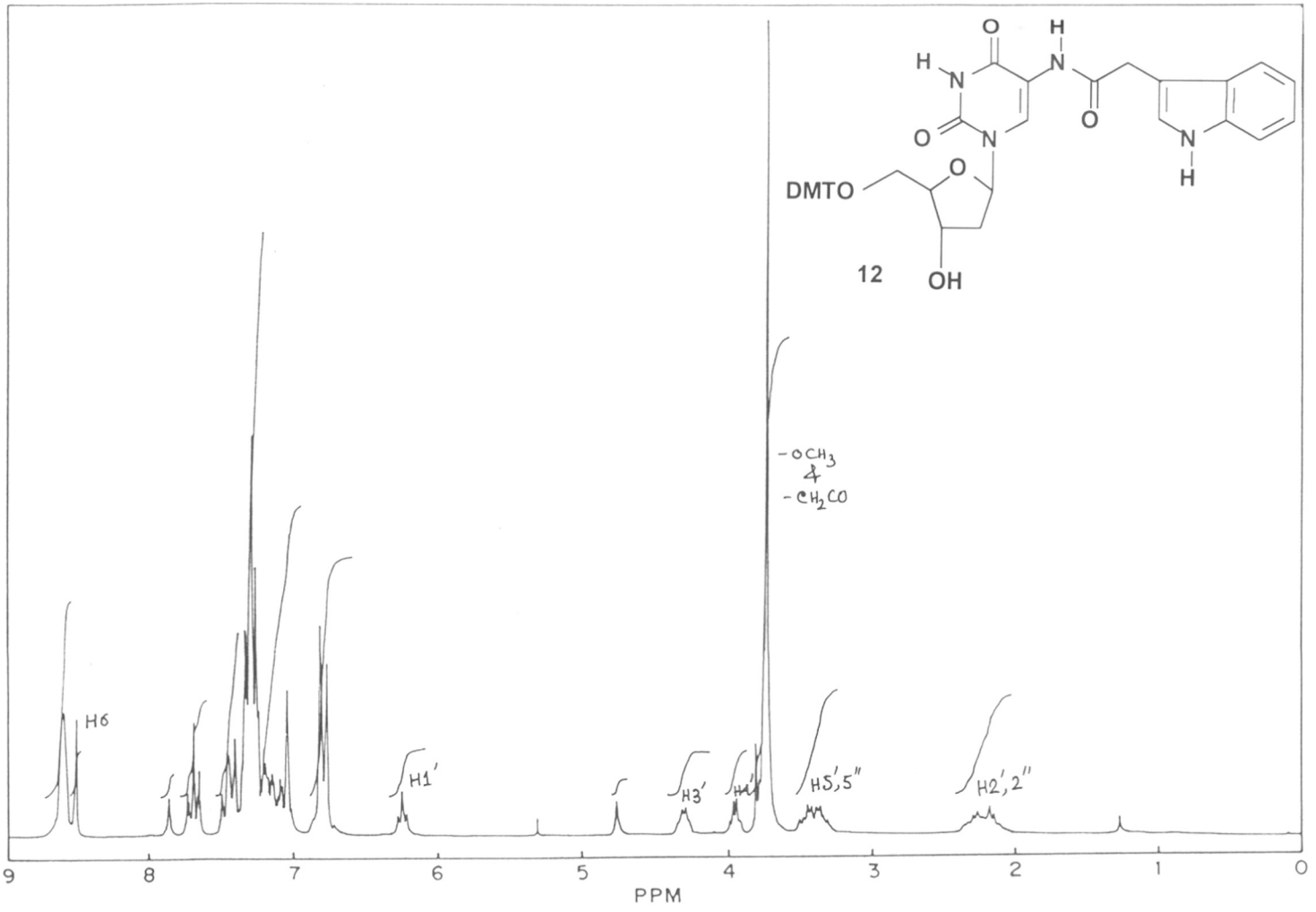


Figure 13a ^{1}H NMR of **12**.

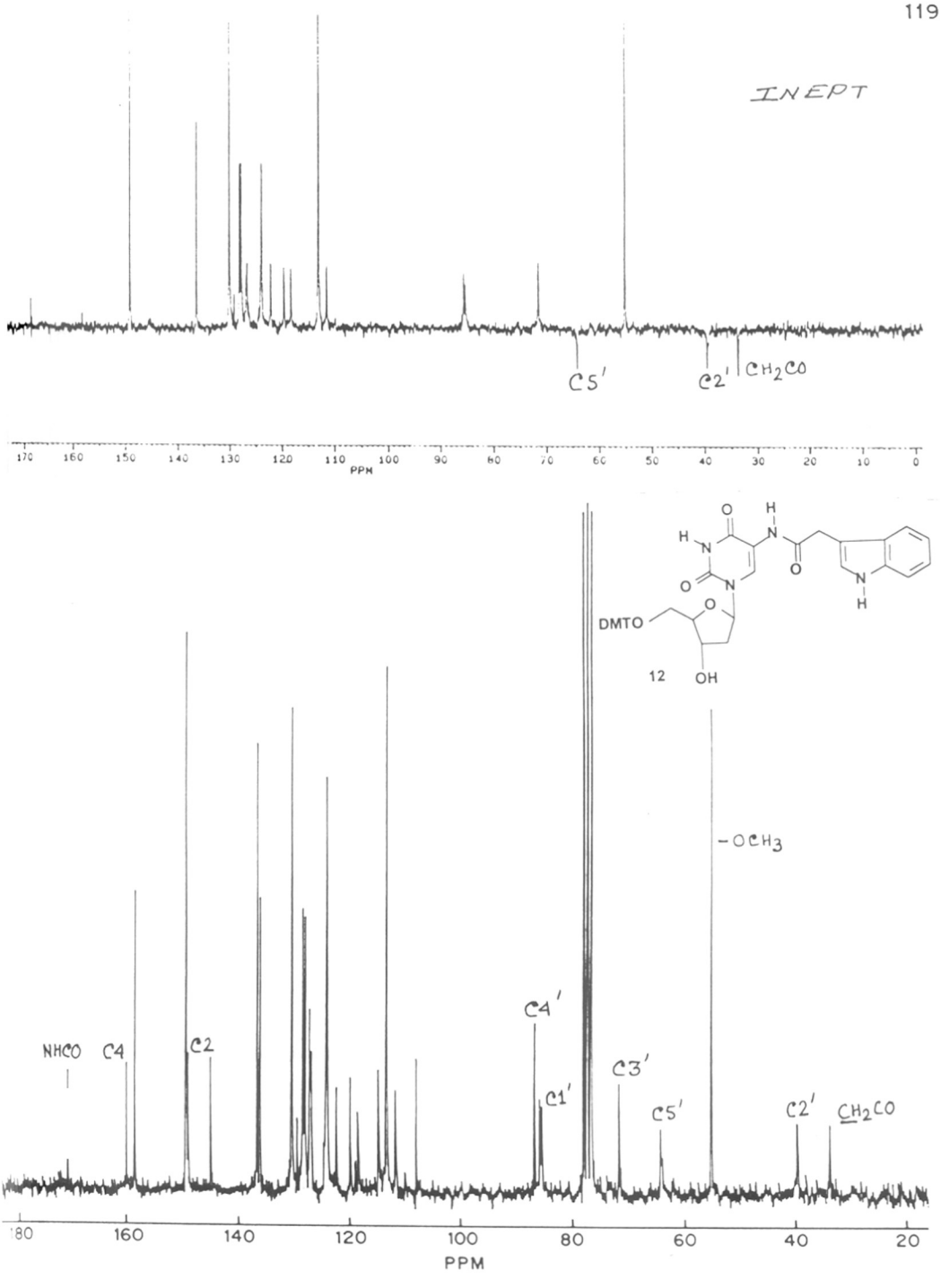


Figure 13b ^{13}C NMR of 12.

pentafluorophenol ester of indole acetic acid **11** was coupled with 5-amino-5'-O-DMT-2'-dU **1** in the presence of HOBT as an activating agent to yield the desired 5-N-(3-indoleacetyl)-5'-O-DMT-5-amino-2'-dU **12**. The ^{1}H NMR of **12** (Figure 13a) showed a downfield shift for H6 (8.5 ppm), with the singlet for two protons of acetyl of indole overlapping with that of $-OCH_3$ of DMT at 3.75 ppm. However ^{13}C NMR of **12** (Figure 13b) showed a distinctive, additional signal for CH_2 (34 ppm) other than that of 5'-CH_2 (64 ppm) and 2'-CH_2 (40 ppm). No indole acetyl ester formation at 3'-OH was observed as seen from no changes in the chemical shift of H3' (4.35 ppm). The fluorophore labeled nucleosides **12** was converted into the corresponding β-cyanoethyl phosphoramidites **13** by normal procedure and the product gave expected ^{31}P signal (150.0 & 149.7 ppm) characteristic of O-amidites (Figure 14). The ring nitrogen of indole acetic acid did not need protection as N-phosphonylation was not observed. The amide linkage of indole acetyl was found to be quite stable to final deprotection condition of oligonucleotide (aq NH_3, 60°C for 24 hr).

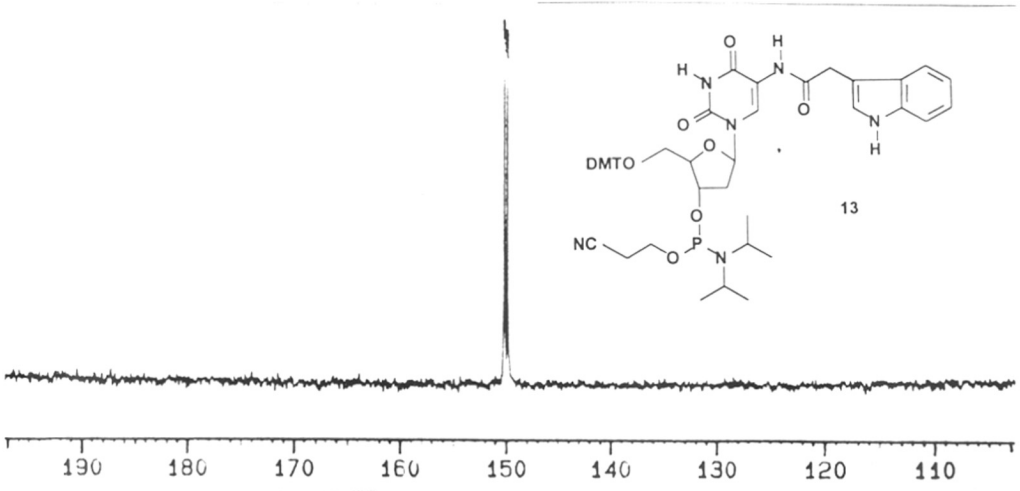


Figure 14. ^{31}P NMR of **13**.

The indole acetyl phosporamidite **13** (U^D) was incorporated into oligonucleotides **15-20**, while dansyl phosphoramidite **4** (U^A) into oligonucleotides **22** and **23** at desired positions as indicated. The oligonucleotides are designed in such a way that constitution of duplexes from **22**:**15-20** and **23**:**15-20** permitted generation of double strands in which the donor and acceptor groups were separated by 1-6 and 6-11 base pairs respectively. As donor and acceptor could be moved either close or away from each other over 11 base pairs, this range would allow to scanning of a complete helical turn of B-DNA. All oligonucleotides were purified by FPLC as described before.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
14	5'	-	G	C	A	C	G	G	A	A	T	T	T	T	T	T	C	C	-	3'
15	5'	-	G	C	A	C	G	G	A	A	U^D	T	T	T	T	T	C	C	-	3'
16	5'	-	G	C	A	C	G	G	A	A	T	U^D	T	T	T	T	C	C	-	3'
17	5'	-	G	C	A	C	G	G	A	A	T	T	U^D	T	T	T	C	C	-	3'
18	5'	-	G	C	A	C	G	G	A	A	T	T	T	U^D	T	T	C	C	-	3'
19	5'	-	G	C	A	C	G	G	A	A	T	T	T	T	U^D	T	C	C	-	3'
20	5'	-	G	C	A	C	G	G	A	A	T	T	T	T	T	U^D	C	C	-	3'
21	3'	-	C	G	T	G	C	C	T	T	A	A	A	A	A	A	G	G	-	5'
22	3'	-	C	G	T	G	C	C	T	U^A	A	A	A	A	A	A	G	G	-	5'
23	3'	-	C	G	U^A	G	C	C	T	T	A	A	A	A	A	A	G	G	-	5'

U^D is donor 5-N-(3-indoleacetyl)-5-amino-2'-dU
U^A is acceptor 5-N-dansyl-5-amino-2'-dU

3.4.4. *UV Melting Studies*

For performing energy transfer experiments, it was important to ensure that duplexes were prevalent under the experimental conditions and this required determination of Tm. Table 3 shows the UV-melting temperature values for different sequences. It was found that (Figure 15) duplexes in which the donor and acceptor groups were present close to each other (separated by one or two base pairs) show decreased

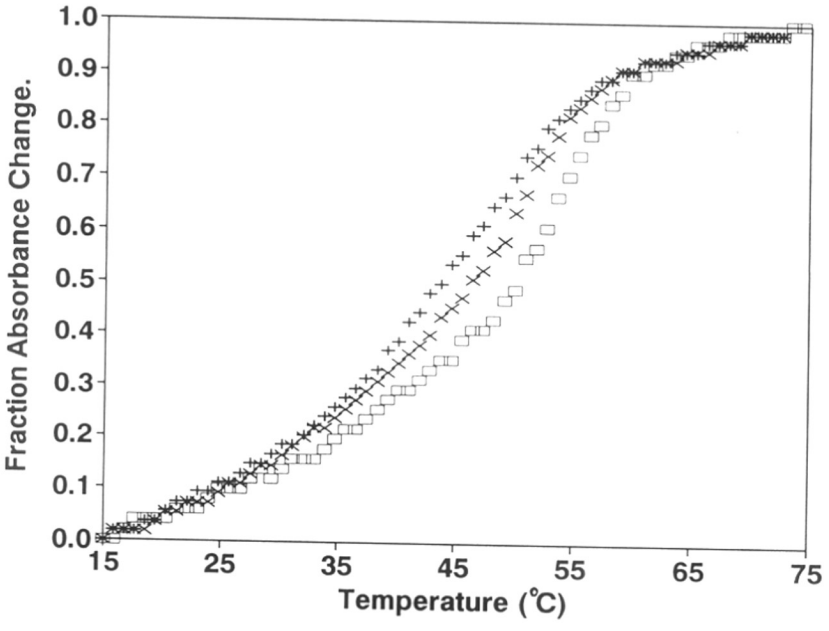


Figure 15. UV melting curves of duplexes **20:22** (+, Tm 44°C), **16:23** (X, Tm 46.5°C) and **20:23** (□, Tm 50°C) in 10 mM Tris, 200mM NaCl, 10 mM $MgCl_2$, pH 7.0.

Table 3:
Melting Temperatures of DNA duplexes used for FET.

Duplex	Base Pair Separation	Tm
14:21		58.0
15:22	0	40.5
16:22	1	40.0
17:22	2	43.0
18:22	3	43.0
19:22	4	42.0
20:22	5	44.0
16:23	6	46.5
17:23	7	43.0
18:23	8	50.0
19:23	9	47.5
20:23	10	50.0

stability. As the chromophores are moved away from each other and towards the end of oligonucleotides, the destabilization is decreased. It was evident from this experiment that antiparallel complementary oligonucleotides carrying donor and acceptor fluorophores (Table 3) do form stable duplexes without any interference of the standard Watson-Crick hydrogen bonding.

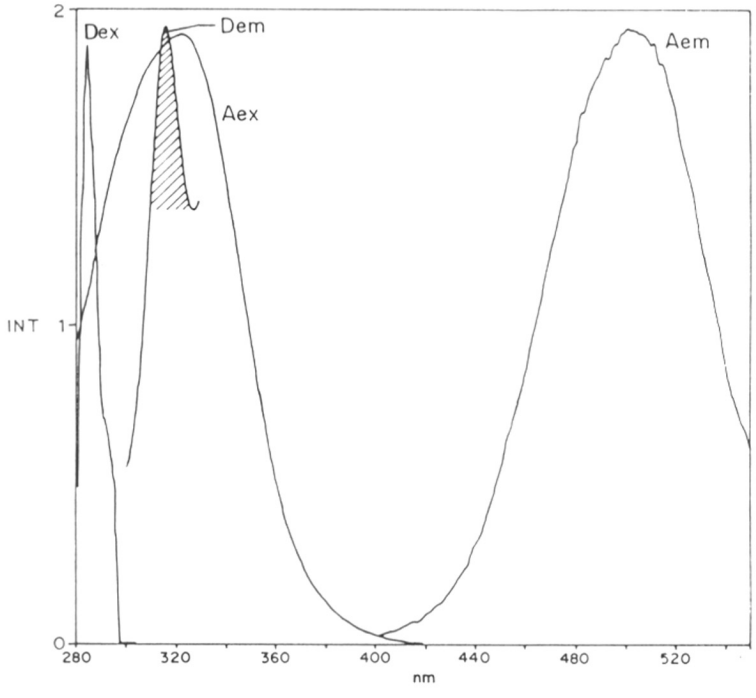


Figure 16. Fluorescence spectral characteristics of indole acetyl (donor) and dansyl (acceptor). The shaded area represent the spectral overlap between the fluorescently labeled DNA donor emission (D_{em}) and the acceptor excitation (A_{ex}) required for fluorescence energy transfer.

3.4.5. *Spectral Properties of Donor and Acceptor*

Figure 16 shows the fluorescence excitation and emission spectra of the individual single strands labeled with either indole acetyl (D) or dansyl (A). As seen from this, the spectral characteristics and spectral overlap of indole acetyl and dansyl pair are highly

favorable for FET studies. The maximum excitation wavelength for D (indole acetyl) is 285 nm, whereas its maximum emission wavelength is 316 nm. The maximum excitation wavelength for A (dansyl) is 322 nm, while its maximum emission wavelength is 500 nm. Significantly, the emission spectrum of D has considerable overlap with the excitation spectrum of A (shaded area). Thus if the D and A fluorophores are in close proximity, upon excitation of D, nonradiative transfer of energy from D to A can occur resulting in emission by A, leading to a decrease of fluorescence emission of D.

3.4.6. *Duplex Formation Monitored by Fluorescence Energy Transfer between Donor and Acceptor Chromphores on Complementary DNA strands*

To determine the maximum efficiency of fluorescence energy transfer between donor and acceptor chromophores attached to oligonucleotides, the emission spectra of donor and acceptor were followed as a function of increasing acceptor concentration at a fixed number of donor molecules. The titration was monitored at a excitation wavelength of donor (indole acetyl at 285 nm). Three separate titration experiments (at 15°C) were performed using three sets of two complementary oligonucleotides **18**:**22**, **20**:**22** and **18**:**23** in which the donor and acceptor are separated from each other by 3, 5, and 8 base pairs respectively.

Figure 17a shows emission spectra obtained upon stoichiometric addition of dansyl conjugated oligonucleotide to a constant amount of indole acetyl conjugated oligonucleotide molecules. Spectrum 1 is the emission spectra of indole acetyl (D) (1 μM) labeled oligonucleotide in the absence of acceptor, whereas spectra 2-5 are emission spectra upon incremental addition (0.3 μM per addition) of dansyl (A) labeled oligonucleotide (excitation at 285 nm). Comparison of spectra 1 and 5 shows a continuous decrease in the emission intensity of indole acetyl (D) (316 nm), accompanied by a corresponding increase in the sensitized dansyl emission (A) (514 nm). This enhancement of acceptor emission at the expense of donor's emission is characteristic of FET.

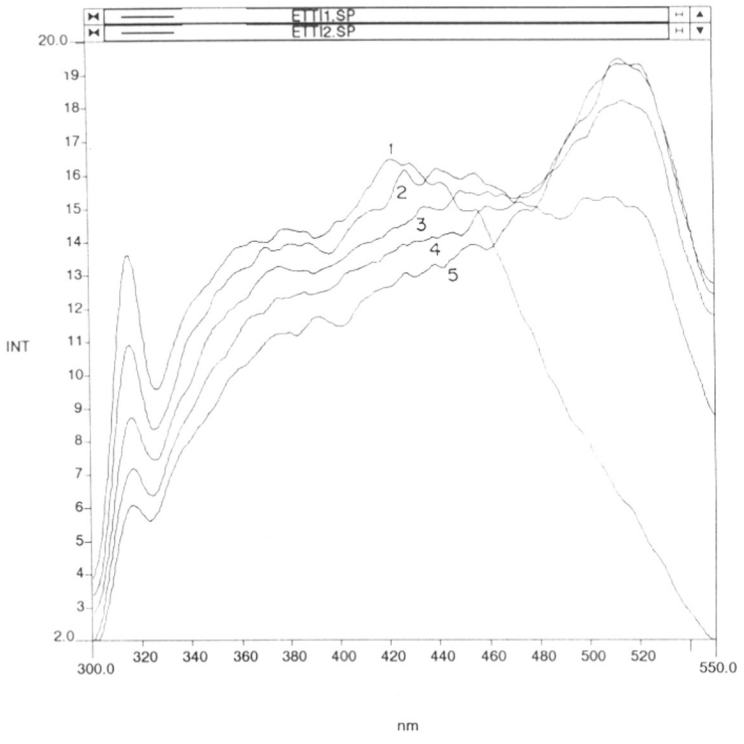


Figure 17a Modulation of fluorescence intensity upon duplex **18**:**22** hybridization at a constant amount of indole acetyl conjugated oligonucleotide molecules (**18**, 1 μM, spectrum 1) and increasing concentration of dansyl conjugated oligonucleotide (**22**, 0.3 μM/addition, spectrum 2-5). Energy transfer occurred between the indole acetyl donor and the dansyl acceptor as evident by a decrease in indole acetyl emission intensity (316 nm, 56% quenching) and an increase in dansyl emission intensity at 514 nm with increasing acceptor concentration.

The emission intensity of the acceptor reaches saturation at a D:A stoichiometry 1:1.2, suggesting that the observed FET is a consequence of the hybridization event of duplex formation. It is noticed from Figure 17a that all the spectra cross through an "isoblistic" point (analogous to isobestic point in UV titration) at 475 nm which arises due to simultaneous decrease in emission of indole acetyl and a rise in dansyl emission. This synchronous effect is a consequence of hybridization.

The magnitude of quenching, of indole acetyl conjugated DNA emission intensity, upon saturation (1:1.2) with DNS-DNA was 56% of its intensity in the absence of acceptor. This decrease may also result from mere hybridization to duplex and may not originate entirely from FET effect. To examine this an identical experiment was performed with indole acetyl oligonucleotide **18** titrated with its unlabeled complement **21**.

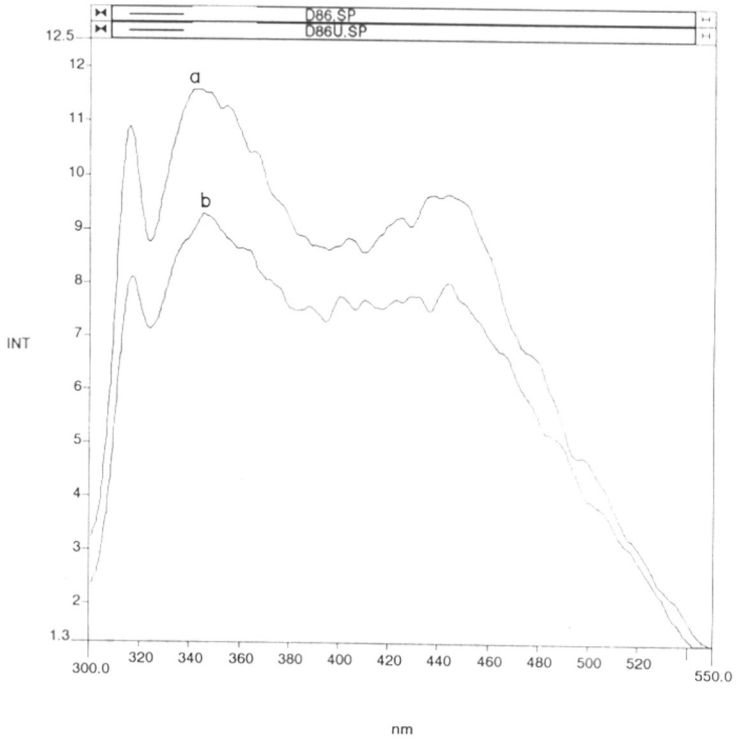


Figure 17b Spectrum (a) is the emission spectrum of single-stranded indole acetyl linked oligonucleotide **18**, while spectrum (b) is after addition of equimolar amount of unlabeled complementary strand **21**. The observed quenching of indole acetyl is only 25% and is result of mere hybridization.

The emission intensity of indole acetyl was now quenched by only 25% (Figure 17b), and as expected no detectable increase in emission intensity at 510 nm. Thus fluorescence was modulated in three components upon hybridization (i) a loss of donor,

indole acetyl emission (25%) due to mere hybridization, (ii) a larger decrease in donor fluorescence emission intensity (56%) upon duplexing with dansyl oligonucleotide and (iii) detection of dansyl emission intensity upon hybridization to indole acetyl conjugated oligonucleotide. The first phenomenon represents a quenching of the fluorophore upon mere hybridization to its unlabeled complement, while the latter two phenomena represent modulation of fluorescence intensity due to energy transfer.

Energy transfer efficiencies (E, equation 4) were measured at 15°C, for duplex pairs in which the donor and acceptor were separated by 3, 5 and 8 base pairs. The efficiencies were found to vary noticeably among these with obtained values of 0.49, 0.46 and 0.32 respectively. Base pair separation along the axis (3.4 A/base) is not linear with chromophore separation at the edge of bases, since the base pairs also undergo a twist due to helix propagation (Clegg, et al., 1993). More quantitative studies are necessary to get accurate structural details by this method. However, the present experiments qualitatively indicate that the efficiency of FET does change as the donor and acceptor groups are moved away from each other on the DNA helix.

SECTION C

3.5. FLUORESCENT PCR: USE OF 5-AMINOFLUORESCEIN-2'-dU LABELED OLIGONUCLEOTIDE PRIMERS

Application of nucleic acid hybridization tests for detection of specific DNA and RNA sequences are extensively being performed in genetic analysis and forensic identity tests using the recently discovered PCR based diagnostic assay (Erlich, 1989). Sensitivity of assay can be improved either by signal amplification or target amplification. PCR is a gene amplification technique which allows many copies of a specific DNA to be generated from only a few target molecules within a period of 1-2 h (Saiki et al, 1985). The amplified DNA is normally detected by electrophoretic analysis using appropriate

staining procedures. Alternatively, if primers or the nucleoside triphosphates having fluorophore conjugates are used then all the amplified product becomes fluorescent making the detection easier (Chehab & Kan, 1989). Consequently this will have applications in nucleic acid based nonradioactive diagnostic systems which are generally limited by low sensitivity. Introduction of procedures that do not require an electrophoretic step, is highly desirable to extend applications in mass screening, as needed in the detection of HIV. We have explored the use of fluorescent primers towards this end as described in the present section.

3.5.1. *Results and Discussion*

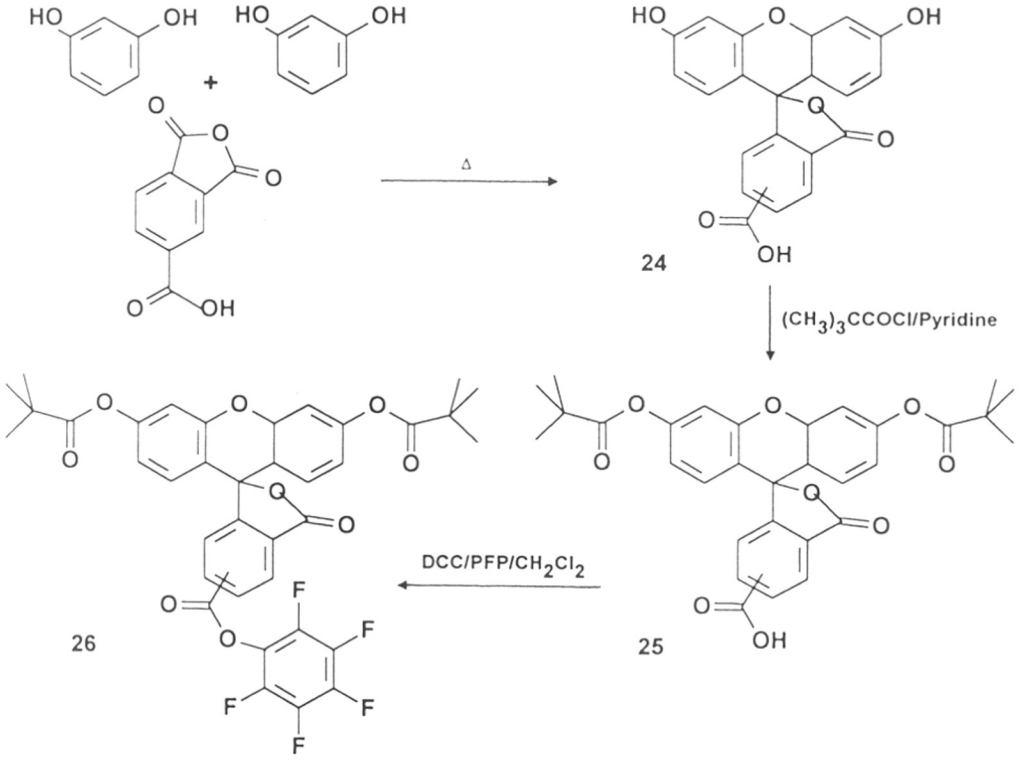
There are several reports for preparation of fluorescent end labeled oligonucleotides, most of which involve coupling reaction between the 5'-terminal amino linked oligonucleotide and active ester of the fluorescent dye (Chu, et al., 1983; Smith, et al., 1987) or coupling of fluorescent dye phosphoramidite at the 5'-end of oligonucleotide (Theisen, et al., 1992a). These approaches allowed the mono labeling of oligonucleotides at 5'- terminus. Multiple labeling of oligonucleotides with 5-carboxyfluorescein and solketal has recently been reported (Theisen, et al., 1992b), using non-nucleosidic solketal backbone. Such incorporation at the internal sites of oligonucleotides may distort the helical backbone and thus its hybridization ability.

Dansyl a is weak fluorophore and is inherently insensitive for use in practical diagnostic assay. To improve the efficiency, fluorescein was employed which has a higher quantum yield and shows emission in visible region. The 5-amino position of 5-amino-2'-deoxyuridine was utilized for covalent linkage of carboxyfluorescein. As it is evident from earlier sections, functionalization at C5 of 2'-dU confines the label into the major groove of nucleic acid and does not perturb hydrogen bonding necessary for hybrid formation. This derivatization of heterocyclic nucleobase 5-amino-2'-dU with carboxyfluorescein also allows multiple labeling of oligonucleotides at internal sites with

marginal effects on its hybridization abilities.

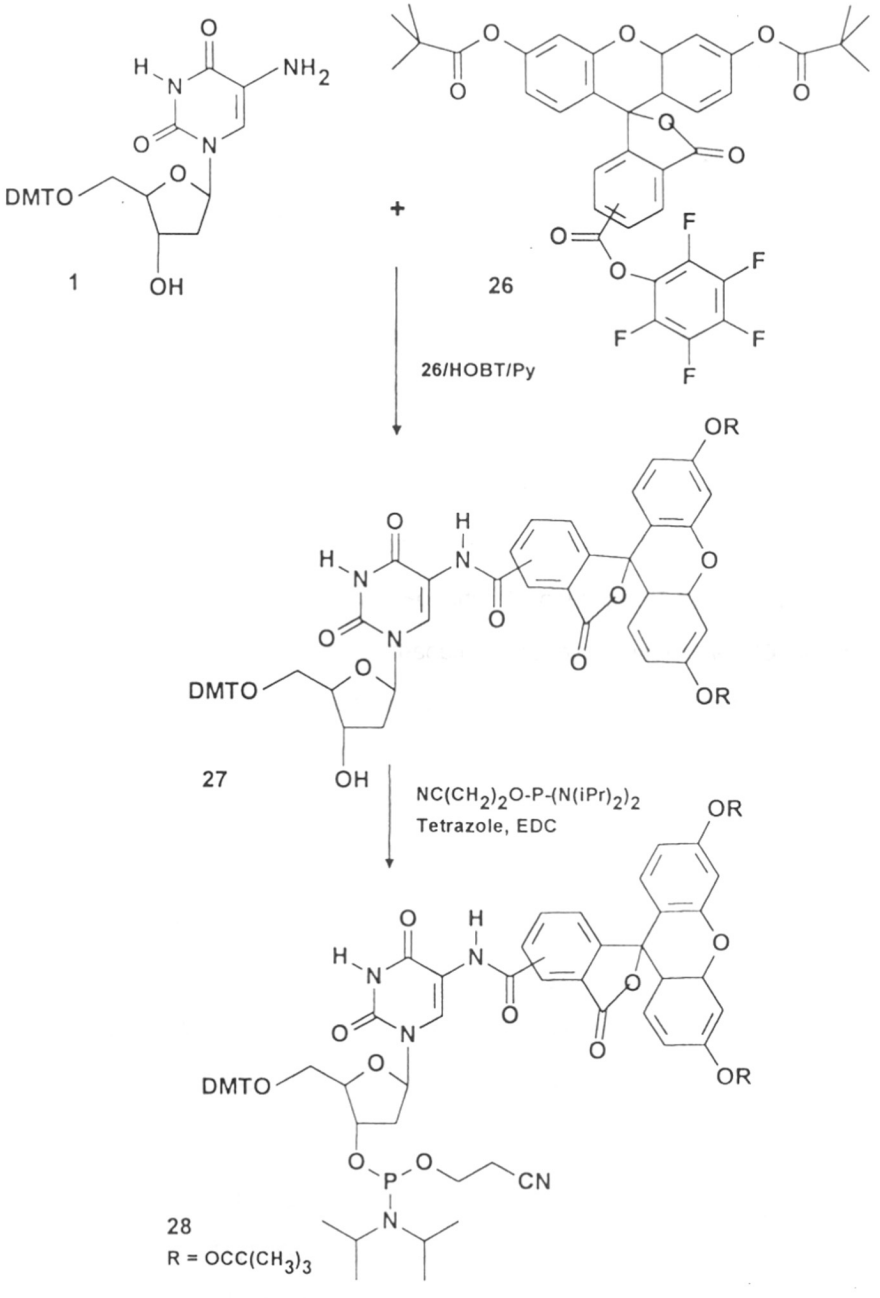
3.5.2. *Synthesis of fluorescein Nucleoside*

The synthesis of modified nucleoside **27** was achieved using 5(and-6)carboxyfluorescein dye **24** (Scheme 3). This was prepared according to literature



Scheme - 3

procedure by heating trimellitic anhydride and resorcinol together in 1:2 ratio, which gave rise to a mixture of two products, isomers 5 and 6-carboxyfluorescein (Haralambidis, et al., 1990). The phenolic hydroxyl groups of **24** were protected with trimethyl acetyl chloride to give the non-fluorescent lactone **25**. The pentafluorophenol (PFP)


DMTO
1
OH
NH2
+
26
F
26/HOBT/Py
OR
27
NC(CH2)2O-P-(N(iPr)2)2
Tetrazole, EDC
CN
28
R = OCC(CH3)3

Scheme - 4

ester **26** was formed with DCC and PFP. The PFP active ester of 5(and-6)carboxyfluorescein **26** (Scheme 4) was condensed with 5-amino-2'-deoxyuridine **1** in presence of activating agent hydroxybenzotriazole (HOBT) in pyridine, to yield the fluorescein conjugated nucleoside **27**, figure 18 shows its ^{1}H NMR. This was converted into the required β-cyanoethyl phosphoramidite **28** by usual procedure (^{31}P 149.5 and 149.2 ppm).

3.5.3. *Design and Synthesis of the Fluorescein Labeled Primers*

The fluorescent amidites were incorporated into oligonucleotides at desired positions by synthesis on a DNA synthesizer (Pharmacia GA plus). After final deprotection with ammonia, oligonucleotides were purified on denaturing PAGE. The sequences correspond to the primer regions which amplify the 285 base pair region of β-globin gene of human genomic DNA. Specific T residues of these primers were replaced by 5-amido-5(and-6)carboxyfluorescein-2'-dU, to obtain mono (**30**, **34**), di (**31**, **35**), and tri (**32**) labeled primers. In multiple labelled primers, two fluorescein molecules were separated from each other by at least 5 or 6 base pairs in order to reduce the loss in emission intensity of fluorescein by self quenching.

Primer A

29 5'- CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG - 3'

30 5'- CTC CT**U*** AAA CCT GTC TTG TAA CCT TGT TAG - 3'

31 5'- CTC CT**U*** AAA CC**U*** GTC TTG TAA CCT TGT TAG - 3'

32 5'- CTC CT**U*** AAA CC**U*** GTC TTG **U***AA CCT TGT TAG - 3'

Primer B

33 5'- ACC TCA CCC TGT GGA GCC AC - 3'

34 5'- ACC **U***CA CCC TGT GGA GCC AC - 3'

35 5'- ACC **U***CA CCC **U***GT GGA GCC AC - 3'

U* is 5-N-carboxyfluorescein-5-amino-2'-dU

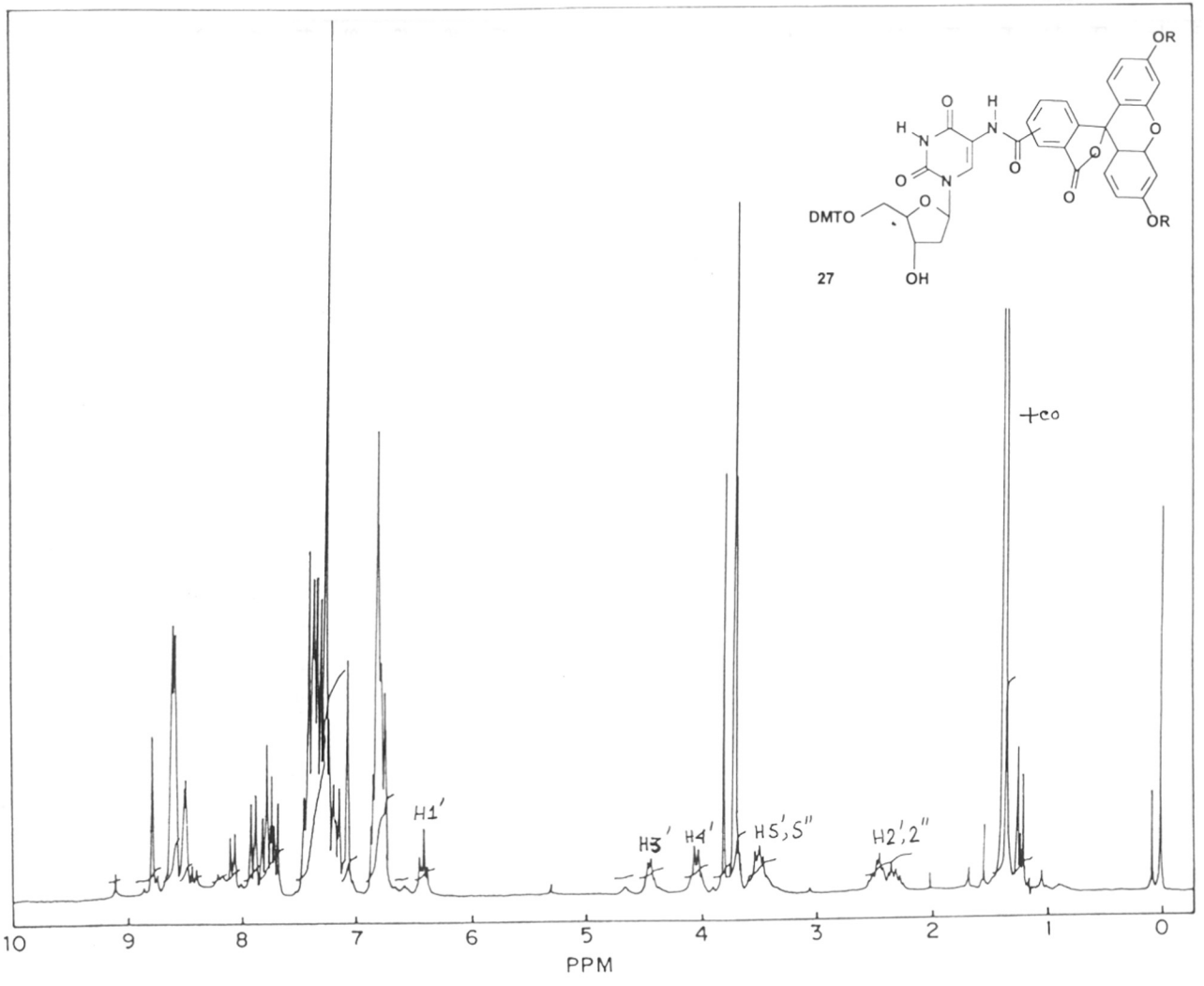


Figure 18. ^{1}H NMR **27**.

A notable feature of these fluorescent primers is that, the fluorophore is directly linked to DNA via the rigid amide linker arm on C5, unlike most other reported examples. This decreases the possibility of interference of fluorescein label in the hybridization process and minimizes any interference with the enzyme DNA polymerase bound at the 3'-terminus of the oligonucleotide in PCR reaction. These nonradioactive primers have also advantages over isotopically-labeled probes in being more stable for extended periods of time and safer to handle.

3.5.4. *Amplification Reaction Using Fluorescein Labeled Primers*

A 285 base pair region of β-globin gene of human genomic DNA was amplified using a pair of fluorescein labeled 30 base pair (primer A) and 20 base pair long (primer B) primers. The control amplification reaction was performed using primers **29** and **33**. The fluorescent primers were used in following pairs **30**:**34**, **31**:**35**, and **32**:**35** so that the amplified product had two, four, and five fluorescein molecules respectively. The amplified products were passed through a NAP column (sephadex G75) to remove excess fluorescent primers. The NAP fractions were monitored directly with a UV illuminator when the amplified products eluted in void volume showed fluorescence. The amplified product were also checked by gel electrophoresis and figure 19 shows the agarose gel photograph before (Figure 19a) and after (Figure 19b) ethidium bromide staining. In figure 19a lanes 1-3 show fluorescent amplified product in which number of fluorescein labels increases in the order from two, four, and five per duplex respectively. As the number of labels increased, fluorescence intensities enhanced for same concentration of labeled DNA. The gel after ethidium bromide staining lights up the marker lane (Figure 19b) and the PCR products from control reaction (lane 1). This result indicates that the fluorescein labeled primers give rise to similar amplified product (lanes 2-4) of 285 base pairs as from the normal primers **29** and **33** (lane 1). The gel electrophoresis step thus can be excluded in detection of fluorescent amplified product for routine tests if unincorporated primers are removed from amplified product. This can

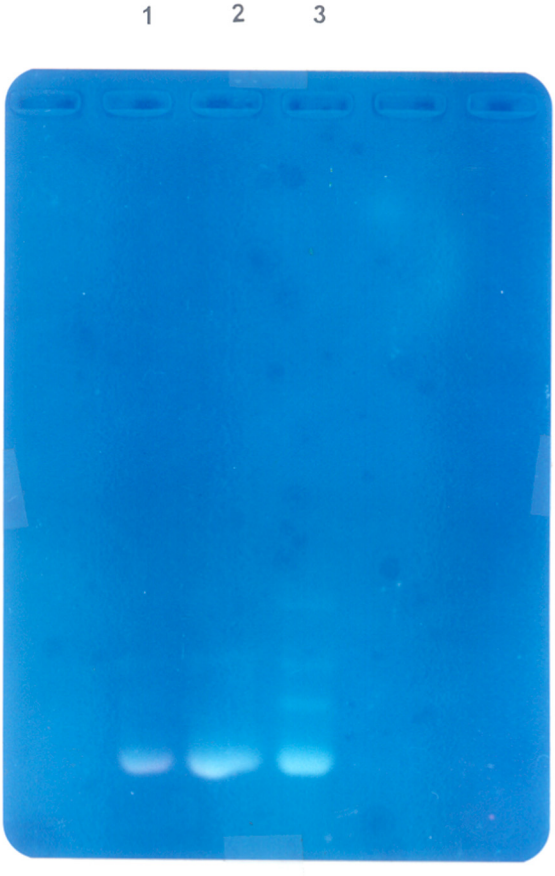


Figure 19a Agarose gel photograph before ethidium bromide staining, lanes 1-3 show fluorescent amplified product with increasing number of fluorescein labels 2, 4 and 5 respectively in amplified product.

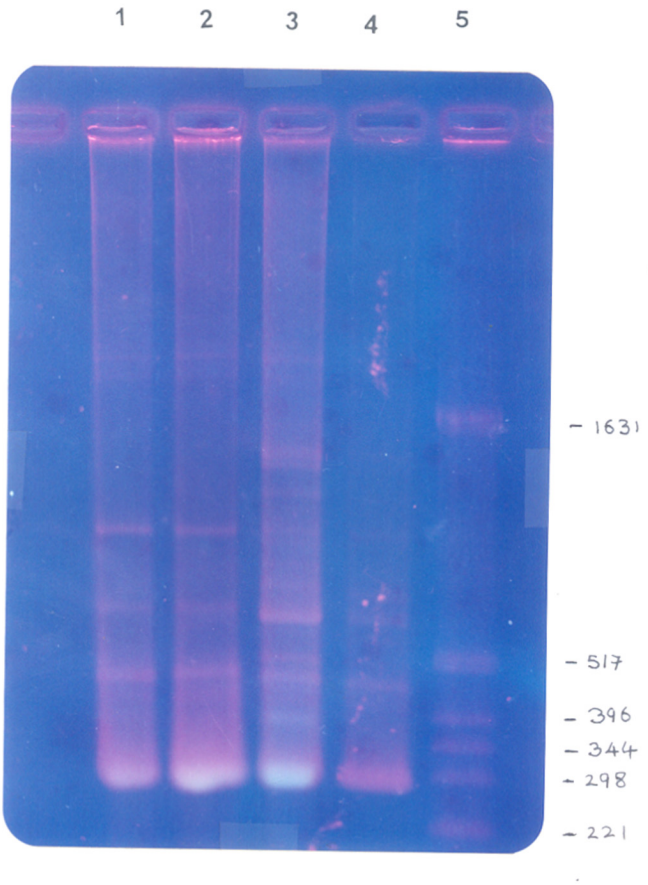


Figure 19b Agarose gel photograph after ethidium bromide staining, lanes 1-3 are similar to Figure 19a. Lane 4 is control PCR reaction using unlabeled primers **29** and **33**, lane 5 is marker lane.

be conveniently achieved by a simple exclusion chromatography, since the amplified product differs from the primers in size and molecular weight. Centrifugation (5000 rpm, 10 min) of the PCR reaction mixture on microfiltration tube (Amicon) gave the amplified product DNA on top of the filter which could be easily visualized under light.

3.5.5. *Synthesis of 5-N-dansyl-5-amino-2'-deoxyuridine-5'-triphosphate*

In an alternative labeling approach, the fluorescent group could be incorporated into the PCR product by using the modified (fluorescein) nucleoside triphosphates (Canard & Sarfati, 1994). 5-N-dansyl-5-amino-2'-deoxyuridine-5'-triphosphate **41** was prepared with starting from **2** according to literature procedure (Ludwig & Eckstein, 1989) (Scheme 5). First the 3'-hydroxyl of **2** was protected as acetate **36** and then 5'-hydroxy detritylation gave **37**. This on treatment with the reagent 2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one **38** which phosphitylates the 5'-hydroxyl group of **37** gave the intermediate **39**, which on subsequent reaction with pyrophosphate produced **40**. Oxidation of **40** with iodine/water, followed by ammonia treatment for deacetylation of 3'-hydroxyl yielded the nucleotide triphosphate **41**. This was purified by chromatography on DEAE-Sephadex and characterized by ^{31}P NMR (Figure 20) spectroscopy.

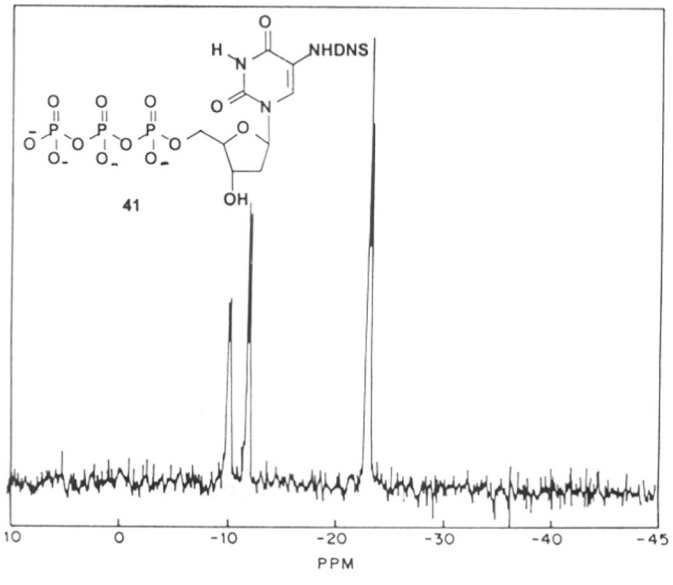
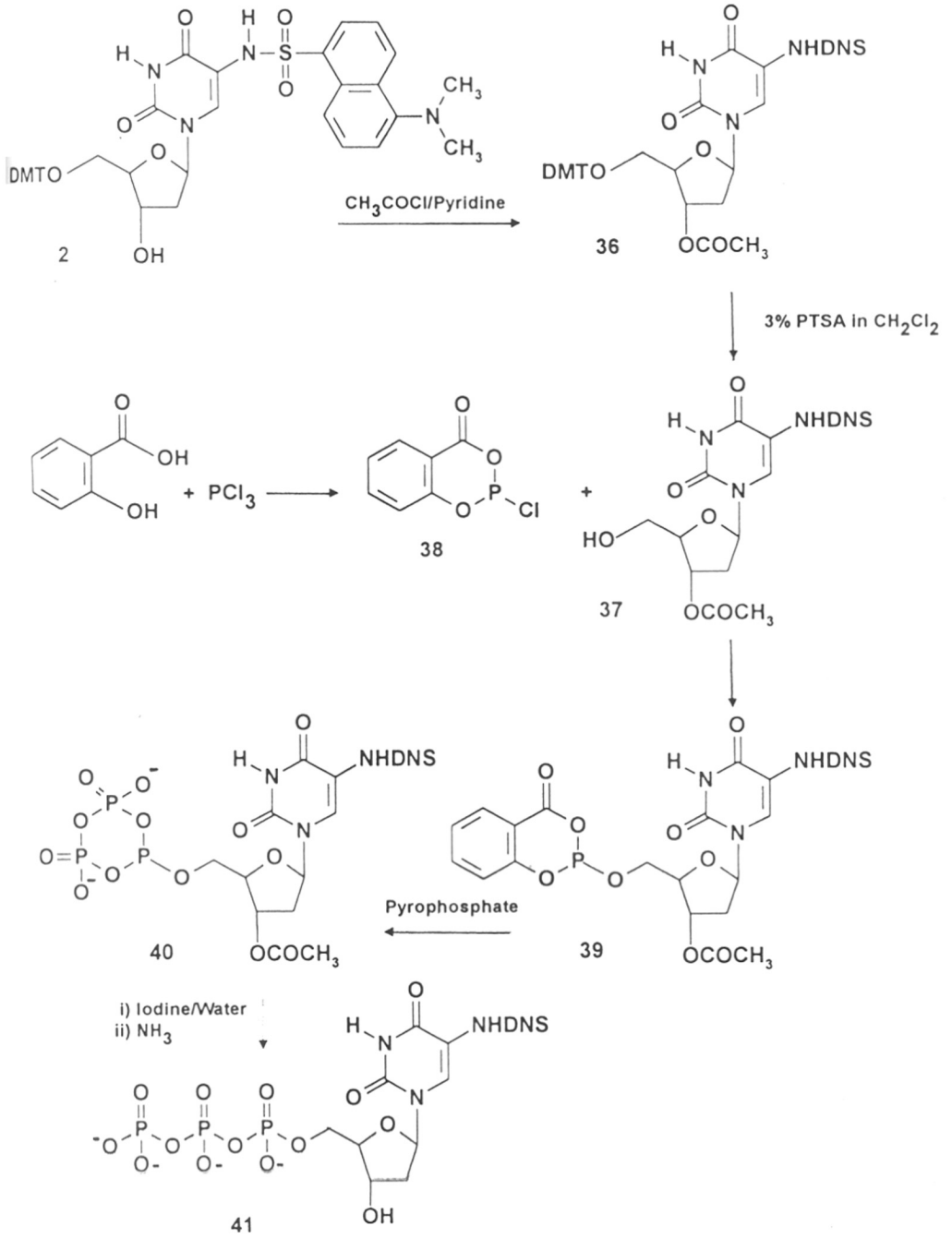


Figure 20. ^{31}P NMR of **41**.


DMTO
2
OH
CH3COCl/Pyridine
NHDNS
36
OCOCH3
3% PTSA in CH2Cl2
+ PCl3
38
37
HO
40
39
Pyrophosphate
i) Iodine/Water
ii) NH3
41

Scheme 5

3.6. CONCLUSIONS

The 5-amino function of 5-amino-2'-deoxyuridine was used as a handle to conjugate oligonucleotides with different fluorophores. Since the conjugation is on heterocyclic base, site-specific and multiple labeling could be achieved. An environment sensitive fluoroprobe, dansyl, covalently linked to dU at C5 was employed to quantitate the major groove dielectric constant which turns out to be ~55D in contrast to the more non-polar minor groove (20D). It was also found that significant "groove cross talk" exists between the major and minor groove as indicated by fluorescence changes in dansyl fluoroprobe in the major groove upon binding of netropsin in the minor groove. The fluorophores having reasonable spectral overlap for fluorescence energy transfer, indole acetyl and dansyl conjugated to complementary oligonucleotides were used to monitor hybridization process. The distance dependence of FET in above oligonucleotide duplexes conceptually was demonstrated, since the donor and acceptor can be located from each other at various distances. The oligonucleotide primers, multiply labelled with fluorescein were found to undergo amplification in polymerase chain reaction using Taq Polymerase. As the resulting amplified product was fluorescent, it has application in non-radioactive labelling and detection of nucleic acids. Further potential applications of such fluorescent DNA probes include study of structural polymorphism in DNA, DNA-peptide interactions and investigation of triple helix formation by using fluorescent DNA as the Hoogsteen strand.

3.7. EXPERIMENTAL

All chemicals used were of the highest purity available. Netropsin was procured from Boehringer Mannheim. Taq polymerase and nucleoside triphosphates from Bangalore Genie.

5-N-dansyl-5'-O-(4,4'-dimethoxytrityl)-5-amino-2'-deoxyuridine 2.

A solution of dansyl chloride (0.3 gm, 1.1 mmole) in 2 ml of DMF was added slowly to **1** (0.54 gm, 1 mmole) in 5 ml DMF containing 1.5 ml of saturated aqueous $NaHCO_3$ solution. After 30 min, 1 ml of 20% ammonia solution, was added and after another 15 min, reaction mixture was evaporated to dryness and precipitated from water. The residue was taken up in CH_2Cl_2 (50 ml) and washed with water (2x20 ml), organic layer was dried over anhydrous Na_2SO_4 and organic concentrated and purified by silica gel column (9 gm). Compound **2** was eluted with gradient of methanol in CH_2Cl_2 containing 0.5% pyridine (Yield 0.57 gm, 74%). Rf (5% MeOH in CH_2Cl_2) = 0.46, ninhydrin negative, fluorescent spot.

^{1}H NMR ($CDCl_3$) δ 8.65-7.9 (3xd, 3H, dansyl ArH), 7.8-7.0 (m, 13H, H6, DMT & dansyl ArH), 6.85 (d, 4H, DMT ArH), 6.16 (t, J=6.3 Hz, 1H, H1'), 4.36 (m, 1H, H3'), 4.1 (m, 1H, H4'), 3.8 (s, 6H, 2xOCH_3 DMT), 3.53-3.35 (m, 2H, H5' & H5"), 2.8 (s, 6H, 2xNCH_3), 2.45-2.0 (m, H2' & H2"). **^{13}C NMR** ($CDCl_3$) δ 159.6 (C4), 148.5 (C2), 85.7 (C4'), 85.1 (C1'), 71.5 (C3'), 63.7 (C5'), 54.8 (OCH_3), 44.8 (NCH_3), 39.7 (C2').

5-N-dansyl-5-amino-2'-deoxyuridine 3.

Compound **2** (0.38 gm, 0.5 mmole) was treated with 15 ml CH_2Cl_2 to which *p*-toluenesufonic acid (0.48 gm, 2.5 mmole, 3% solution). The reaction mixture was kept stirring at room temperature for 1 hr after which it was washed with saturated aqueous $NaHCO_3$, and concentrated to dryness. This was subjected to column purification over silica gel. Rf (5% MeOH in CH_2Cl_2) = 0.28.

^{1}H NMR (DMSO-d_6): δ 11.4 (s, 1H, N<u>H</u>SO_2-), 9.7 (s, 1H, N3<u>H</u>), 8.45, 8.3 and 8.1 (3xd,

3H, dansyl ArH), 7.75 (s, 1H, H6), 7.6 (m, 2H, dansyl Ar-H), 7.25 (d, 1H, dansyl Ar-H), 6.1 (t, 1H, H1'), 5.3 (d, 1H, 3'OH), 4.9 (t, 1H, 5'-OH), 4.2 (m, 1H, H4'), 3.75 (m, 1H, H3'), 3.4 (m, 2H, H5'and H5"), 2.8 (s, 6H, $N(CH_3)_2$ dansyl), 2.1 and 1.9 (m, 2H, H2' and H2").

Pentafluorophenyl indole-3-acetate 11.

Indole-3-acetic acid **10** (1 gm, 5.7 mmole) and pentafluorophenol (1.15 gm, 6.25 mmole) were taken in 10 ml dry CH_2Cl_2. To this DCC (1.29 gm, 6.25 mmole) in 5 ml dry CH_2Cl_2 was added slowly and reaction mixture was kept stirring at room temperature for 24 hr. The precipitate of dicyclohexyl urea was filtered and the filtrate was evaporated to dryness followed by column purification over silica gel (30 gm).

^{1}H NMR ($CDCl_3$) δ 8.2 (brs, 1H, NH), 7.65 (d, 1H, H2), 7.45-7.15 (m, 4H, Ar-H), 4.15 (s, 2H, CH_2).

5-N-(3-indoleacetyl)-5'-O-(4,4'-dimethoxytrityl)-5-amino-2'-deoxyuridine 12.

To compound **1** (0.41 gm, 0.75 mmole) in 5 ml dry pyridine **11** (0.4 gm, 1.1 mmole), HOBT (0.1 gm, 0.75 mmole) was added and stirred overnight at room temperature. The reaction was monitored by TLC, with the product showing a ninhydrin negative test. The reaction mixture was evaporated to dryness, taken in CH_2Cl_2 (20 ml) and washed with aqueous $NaHCO_3$ (2x5 ml), organic layer was dried over anhydrous Na_2SO_4 followed by column purification over silica gel. (Yield 0.39 gm, 59%). Rf (5% MeOH in CH_2Cl_2) = 0.3.

^{1}H NMR ($CDCl_3$) δ 8.5 (s, 1H, H6), 7.6-7.0 (m, 14H, DMT and Ar-H of indole), 6.8 (d, 4H, DMT ArH), 6.25 (t, 1H, H1'), 4.3 (m, 1H, H3'), 3.95 (m, 1H, H4'), 3.75 (s, 8H, 2xOCH_3 DMT and indole-CH_2), 3.5-3.3 (m, 2H, H5' & H5"), 2.4-2.1 (m, 2H, H2' & H2"). **^{13}C NMR** ($CDCl_3$) δ 171 (indole carbonyl), 161 (C4), 150 (C2), 86.5 (C4'), 86 (C1'), 71.5 (C3'), 64 (C5'), 55 (OCH_3), 40 (C2'), 34 $\underline{C}H_2CO$ indole).

Spiro[isobenzofuran-1-(3H)-9'-[9H]xanthene]-5(and-6)carboxylic acid, 3',6'-(2,2-dimethylpropanoyloxy)-3oxo 25.

Trimellitic anhydride (4.8 gm, 25 mmol) and resorcinol (5.5 gm, 55 mmol) were mixed thoroughly and placed in a 190°C oil bath for 1 hr. The temperature was then increased to 210°C and was maintained for 5 hr, by which time the melt gets solidified to a dark red solid. It was then allowed to cool down and dissolved in hot ethanol followed by precipitation from water. The precipitate was filtered out and dried to yield a fluorescent compound **24** (excitation maxima 489 nm and emission maxima 518 nm). This was dissolved in 30 ml of dry pyridine, to which was added trimethylacetyl chloride (9.2 ml, 0.15 mol). This was allowed to stir for 24 hr, was filtered, and the filtrate was concentrated to a thick syrup. The resultant was redissolved in 400 ml of EtOAc, washed with 1M H_2SO_4 (2x300 ml) and H_2O (1x300 ml), dried over anhydrous (Na_2SO_4), and reconcentrated to a syrup and which was chromatographed over silica gel column to give 6.2 gm (yield 48%) of crude non-fluorescent lactone **25**.

Spiro[isobenzofuran-1-(3H)-9'-[9H]xanthene]-5(and-6)carboxylic acid, 3',6'-(2,2-dimethylpropanoyloxy)-3oxo pentafluorophenol 26

To a mixture of **25** (1 gm, 2 mmole) and pentafluorophenol (0.4 gm, 2.2 mmole) taken in 10 ml dry CH_2Cl_2, DCC (0.45 gm, 2.2 mmole) in 5 ml CH_2Cl_2 was added slowly and allowed to stir for 24 hr. The reaction was monitored by TLC and precipitated dicyclohexyl urea was filtred out. The filtrate was evaporated to dryness followed by purification over silica gel column (20 gm). (yield 0.9 gm, 63%). Rf (System B) 0.71 & 0.68, corresponding to 5 and 6 isomers.

^{1}H NMR ($CDCl_3$) δ 8.20 (m, 1H, H_4 & H_5 of 6-isomer), 8.87 (s, 0.5 H, H4 of 5-isomer), 7.96 (s, 0.5H, 0.5 H, H_7 of 6-isomer), 7.38 (d, J=8 Hz, 0.5 H, H_7 of 5-isomer), 7.11 (s, 2H, H4'), 6.83 (s, 4H, H1' and H2'), 1.4 (s, 18H, 2x$C(CH_3)_3$).

5-N-carboxyflurescein-5'-O-(4,4'-dimethoxytrityl)-5-amino-2'-deoxyuridine 27.

To compound **1** (0.27 gm, 0.5 mmole) in 5 ml dry pyridine **26** (0.42 gm, 0.6 mmole), HOBT (0.06 gm, 0.5 mmole) was added and stirred overnight at room temperature. The

reaction was monitored by TLC, evaporated to dryness, and redissolved in CH_2Cl_2 (30 ml) and the solution was washed with aqueous $NaHCO_3$, (2x10 ml) concentrated and followed purified over silica gel. (Yield 0.33 gm, 63%), Rf (5% MeOH in CH_2Cl_2) = 0.59. **^{1}H NMR** ($CDCl_3$) δ 8.9-7.5 (m, 4H, H6 & ArH), 7.5-6.7 (m, 18H, DMT & ArH), 6.4 (t, 1H, H1'), 4.5 (m, 1H, H3'), 4.1 (m, 1H, H4'), 3.7 (s, 6H, -OCH_3), 3.5 (m, 2H, H5' & H5"), 2.55-2.3 (m, 2H, H2' & H2"), 1.4 (s, 18H, 2x$C(CH_3)_3$).

General procedure for phosphitylation of 2, 12, and 27 to yield corresponding 3'-O-(N,N-Diisopropylamino)(β-cyanoethoxy)phosphines 4, 13, and 28.

Compound **2, 12** or **27** (0.5 mmol) and tetrazole (35 mg, 0.5 mmol) were dried by coevaporation with dichloroethane and suspended in dichloroethane (4 ml). To this 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (0.15 ml, 0.5 mmol) was added with stirring at 20°C. After stirring for 3 hr the precipitate of diisopropylammonium tetrazolide was removed by filtration and the solution was diluted with dry CH_2Cl_2 and washed with 5% aqueous $NaHCO_3$, concentrated to dryness and purified by precipitation from CH_2Cl_2 by cold hexane. These phosphoramidites were dried overnight over P_2O_5 and KOH in desiccator before using on DNA synthesizer. Rf (EtOAc:CH_2Cl_2, 1:1 and 0.5% TEA) it shows two close moving spots for two distereoisomers, **4** = 0.65 & 0.48, **13** = 0.65 & 0.59, **28** = 0.68 & 0.62.

^{31}P NMR ($CDCl_3$) **4** 149.3 and 149.1 ppm, **13** 150.1 and 149.7, **28** 149.6 and 149.3.

3'-O-Acetyl-5-N-dansyl-5'-O-(4,4'dimethoxytrityl)-5-amino-2'-deoxyuridine 36.

Compound **2** (0.3 g, 0.36 mmol) was taken in dry pyridine (3 ml) to which acetic anhydride (0.18 ml, 1.8 mmol) was added. The reaction mixture was allowed to stir for 2 hr, concentrated to dryness. The residue was dissolved in CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$, evaporated to dryness, and purified by silica gel column chromatography. (Yield 0.27 g, 85%). Rf (5% MeOH in CH_2Cl_2) = 0.6. **^{1}H NMR** ($CDCl_3$)

δ 8.7-7.9 (3xd, 3H, dansyl), 7.9 -7.0 (m, 13H, H6, DMT, dansyl), 6.8 (d, 4H, DMT), 6.1 (t, 1H, H1'), 5.45 (m, 1H, H3'), 4.0 (m, 1H, H4'), 3.75 (s, 6H, 2xOCH_3), 3.5-3.4 (m, 2H, H5' & H5"), 2.85 (s, 6H, 2xNCH_3), 2.3-2.1 (m, 2H, H2' & H2"), 1.9 (s, 3H, $COCH_3$).

3'-O-Acetyl-5-aminodansyl-2'-deoxyuridine 37.

Compound **36** was detritylated according to procedure for **2** to **3**. Rf (5% MeOH in CH_2Cl_2) = 0.3.

^{1}H NMR (Acetone d6) δ 8.6 (m, 2H, dansyl), 8.3 (m, 1H, dansyl), 7.65 (m, 2H, dansyl), 7.15 (m, 1H, dansyl), 6.35 (m, 1H, H1'), 5.35 (m, 1H, H3'), 4.3 (m, 1H, H4'), 3.75 (m, 2H, H5' & H5"), 2.9 (s, 6H, 2xNCH_3 dansyl), 2.6-2.3 (m, 2H, H2' & H2"), 2.0 (s, 3H, $COCH_3$).

2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one 38.

Salicylic acid (13.8 g, 0.1 mol) was taken in a 100 ml round bottom flask with reflux condenser, whose outlet was connected to a water container to dissolve the HCl formed during reaction. To this flask toluene (15 ml) and PCl_3 (9.52 ml) were added and reaction mixture was refluxed for 3 hr, by which time evolution of HCl ceased. The initial turbid solution became a clear yellow liquid. The reaction mixture was allowed to cool down to room temperature and then excess of PCl_3 and toluene were removed under reduced pressure. This was followed by vacuum distillation, constant boiling fraction at 130°C (~12 mm Hg) was collected, this reagent on cooling forms white solid. **^{31}P NMR** ($CDCl_3$) δ 149.1 ppm.

5-N-dansyl-5-amino-2'-deoxyuridine-5'-O-triphosphate 41.

The 3'-O protected nucleoside **37** (0.16 g, 300 μmol) was dried by coevaporation with anhydrous pyridine followed by desiccation over P_2O_5. This was dissolved in dry pyridine (300 μl) under argon. A freshly prepared 1 M solution of **38** (330 μl, 330 μmol) was then injected into the well stirred solution of the nucleoside. After 10 min, when white solid precipitates out, 0.5 M of bis(tri-n-butylammonium) pyrophosphate in anhydrous DMF

(300 μl) and tri-n-butylamine (100 μl) was injected quickly when precipitate dissolves and the reaction mixture was stirred for another 10 min. A solution of 1% iodine in pyridine/water (98/2 v/v) (2 ml) was then added. After 15 min excess of iodine was destroyed by adding few drops of 5% aqueous solution of $NaHSO_3$ and the reaction solution was evaporated to dryness. The residue was dissolved in water (10 ml) to which concentrated ammonia (20 ml) was added. After 1 hr, the solution was evaporated to dryness, the residue dissolved in water, and the solution applied to a DEAE sephadex column, which was eluted with a linear gradient of 800 ml each of 0.05 M and 1 M TEAB. The product was eluted between 0.4 to 0.5 M buffer, yield 60%. **^{31}P NMR** (D_2O) δ -10.0 d, -11.8 t, -22.8 d.

Oligonucleotide Synthesis and Purification.

All oligonucleotides were synthesized on 1.3 μmol scale on a Pharmacia GA plus DNA synthesizer using controlled pore glass support and base protected 5'-O-(4,4'-di methoxytrityl)deoxyribonucleoside-3'-O-[(diisopropylamino)-β-cyanoethylphopsphora midite] monomers, which was followed by deprotection with aqueous NH_3. For synthesis of oligonucleotides **6-9**, **22** and **23** containing 5-N-dansyl-5-amino-2'-deoxyuridine, appropriate amidite monomer **4** was employed in place of normal T amidite for coupling at desired positions. Similarly for oligonucleotides **15-20** containing 5-N-(3-indoleacetyl)-5-amino-2'-deoxyuridine monomer amidite **13** and for **30-32**, **34** and **35** containing 5-N-carboxyfluorescein-5-amino-2'-deoxyuridine monomer amidite **28** were used. Oligonucleotides **6-9** and **14-23** were applied to a column of sephadex G-25 and eluted with water containing 20% MeOH. The crude oligonucleotides eluted in void volume were lyophilized and purified by FPLC and rechecked by RP HPLC. FPLC (PepRPC HR 5/5, Pharmacia) Buffer A: 5% CH_3CN in 0.1 M TEAA; Buffer B: 30% CH_3CN in 0.1 M TEAA. Gradient 0%B, 3 min; 0-15%B, 5 min; 15-75%B, 35 min; 75-100%B, 5 min. HPLC, Buffers A and B, same as FPLC. Gradient, A to B 20 min. Oligonucleotides **29-35** were purified by preparative denaturing polyacrylamide gel

electrophoresis. The major fluorescent bands (corresponding two isomers of carboxy-fluorescein) were cut and eluted out in a sterile water and passed through sephadex G-25 lyophilized to dryness.

Melting Experiments

Thermal denaturation profiles of self-complementary DNA duplexes **6-9** were performed with or without netropsin in 10mM Tris buffer, pH 7.0, containing 100mM NaCl and 20mM $MgCl_2$ using Perkin Elmer Lambda 15 UV/VIS spectrophotometer, using five sample cell holder, fitted with a temperature programmer and heating at a rate of 1°/min. Duplex Tm, were determined from the midpoint in the plots of fraction absorbance change versus temperature and Tm values are accurate to $\pm$ 1°C over the reported values. Similarly, UV melting experiments on non self-complementary duplexes **22**:**15-20**, **23**:**15-20** and **15**:**16** were performed in 10 mM Tris buffer, pH 7.0, containing 200 mM NaCl and 10 mM $MgCl_2$. Appropriate oligonucleotides, each at a strand concentration of 1 μM based on UV absorbance calculated using molar extinction coefficients at 260 nm, dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 $cm^2/\mu mol$) were mixed, heated to 70°C for 3 min, cooled to room temperature and then stored at 4°C overnight. CD spectra were recorded on a Jobin Yvon instrument at pH 7.0.

Fluorescence Spectroscopy

Fluorescence measurements were done on a Perkin Elmer model LS-50 B spectrometer attached to a Julabo programmable water circulator for variable temperature experiments (Tm). The fluorescent DNA samples **6-9** dissolved in the above buffer were excited at 323nm and the emission monitored at 500nm using a spectral bandwidth of 2.5nm. Association constants were calculated from the 1/a Vs 1/L plots, where *a* is the fraction of oligonucleotide bound to netropsin and L is the effective netropsin concentration (Pesce et al., 1971). The fraction of oligonucleotide bound to netropsin, a, was calculated using the relationship

$$a = \frac{F/Fo - 1}{Fmax/Fo - 1}$$

where Fo and F are the fluorescence emission intensities of the oligonucleotide in the absence and presence of the netropsin, respectively. The value of Fmax is the fluorescence emission intensity of the oligonucleotide measured at a netropsin concentration beyond which no change in fluorescence intensity is observed. For characterization of the major groove polarity, fluorescence spectra of **3** were recorded in mixed organic/aqueous solvent systems prepared by stirring distilled water with appropriate volume percent of 1,4-dioxane (spectroscopic grade). Emission and excitation wavelength maxima were obtained after smoothening each spectrum by using a quadratic polynomial and are accurate to $\pm$ 0.5 nm.

Polymerase Chain Reaction

PCR amplification of a 285 base pair region of β-globin gene of human genomic DNA was performed. For control amplification reaction, primer **29** and **33** were used, while for amplification reactions using fluorescein conjugated primers **30**:**34**, **31**:**35** and **32**:**35** were used. The 50 μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.15 mM $MgCl_2$, 0.001% w/v gelatin, 200 μM each dNTP, 20 pmoles each primer, 0.1 μg genomic DNA and 2.5 units of AmpliTaq DNA polymerase. These were subjected to 35 cycles of PCR using Perkin-Elmer Cetus DNA Thermal Cycler. The reaction temperature cycle was 96°C, 5 min (before Taq Pol addition); 94°C, 1 min.; 50°C, 1 min.; 72°C, 5 min. The PCR products were analyzed on 1.5% agarose gel and visualized on UV transilluminator with and without ethidium bromide.

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CHAPTER 4

OLIGONUCLEOTIDE-POLYAMINE CONJUGATES

4.1. INTRODUCTION

As described in chapter I, in most commonly occurring pyrimidine motif for triple helix, binding specificity arises from Hoogsteen hydrogen bonds possible between thymine and A:T base pairs (T*A:T triplet) and between protonated cytosine and G:C base pairs (C*G:C triplet) in the major groove of duplex DNA (Thuong & Helene, 1993; Moser & Dervan, 1987; Povsic & Dervan, 1990) (Figure 1).

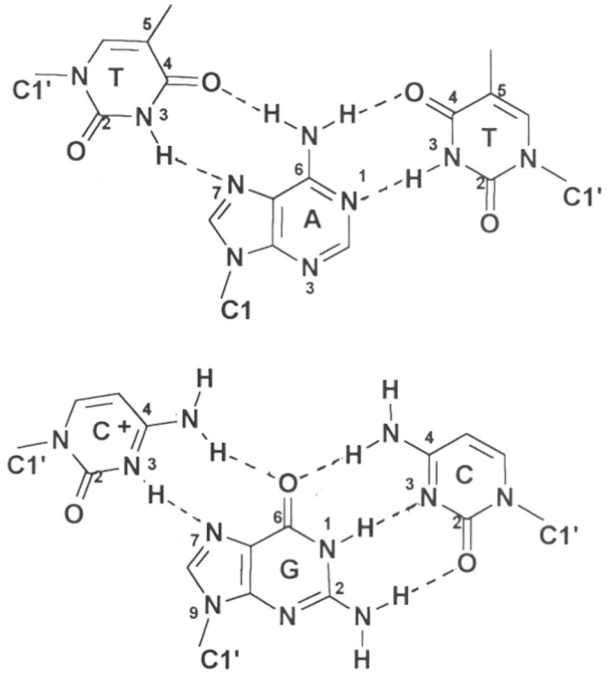


Figure 1. Pyrimidine motif

Due to the molecular necessity of protonated C (CH^+) in the third strand (Francois et al., 1989; Rajagopal & Feigon, 1989; Singleton & Dervan, 1992), triplex formation in natural nucleic acids is pH dependent with an optimum stability at non-physiological pH

5.6-6.0. This specificity and limiting pH dependence of triad base pairing in triple helix formation have led to design of chemical modifications that could increase the affinity of third strand at intracellular pH 7.1-7.6. Most approaches have employed either modified base/sugar derivatives to raise the critical pKa of the N3 cytosine (Froehler & Ricca, 1992; Froehler et al., 1992; Schimizu et al., 1994) or protonated CH^+ mimetics including non-natural heterocycles (Ono et al., 1992; Koh & Dervan, 1992, Krawczyk et al., 1992; Jetter & Hobbs, 1993; Huang & Miller, 1993). Substitution of 5-Me-dC for dC in the third strand has extended the compatible pH range for triplex formation to 6.5 (Povsic & Dervan, 1989; Plum et al., 1990; Lee et al., 1984; Xodo et al., 1991). Oligonucleotides containing dC-(N^4-amidoalkyl) residue are reported to form triplexes, by selective interaction with a mismatched C:G base pair on target duplex with a higher affinity than with normal G:C base pair of duplex (Huang et al., 1993).

4.1.1. *Polyamines-DNA Interaction*

Polyamines and their bio-conjugates have emerged as wide ranging biological effector molecules with bright prospects for medicinal developments (Ganem, 1982; Tabor & Tabor, 1984; Behr, 1993). The naturally occurring polyamines like spermine and spermidine are largely protonated at physiological pH and exhibit net positive charge close to +4 and +3 respectively. Their inherent polycationic nature and the chain conformational mobility encourage electrostatic interaction with the anionic phosphate groups of polynucleotides and specific molecular interaction in duplex DNA. At low concentration, spermine induces B-Z transition in solution (Behe & Felsenfeld, 1981; Basu et al., 1990). The parameters influencing the effect of polyamines in such transitions are the inherent positive charges and the length of alkyl chain separating the positively charged nitrogens (Basu & Marton, 1987).

There is no general agreement in literature about the principal site for polyamine binding to DNA duplex, with experiments supporting a minor groove interaction for

B-DNA (Schimd et al., 1991) and Z-DNA (Bancroft et al., 1994) and major groove interaction for A-DNA (Feurstein et al., 1990, Jain et al., 1989). In the minor groove of B-DNA, each ammonium group is able to establish two interstrand hydrogen bonds with O2 (Pyrimidine) or N3 (Purine) on the floor of minor groove clipping both strands together. The low temperature crystal structure of pure-spermine form of Z-DNA has revealed the presence of both interhelix and intrahelix spermines bound to DNA (Bancroft et al., 1994). The intrahelix spermine binds primarily in the minor groove, displacing the spine of hydration. This also decrease the cross-groove electrostatic repulsion in Z-DNA which is greater than that in B-DNA.

Spermine has not been observed crystallographically in complexes with B-DNA at a higher resolution to understand the structural effects. However, much of experimental data on B-DNA in solution has recently been rationalized (Haworth et al., 1991) with a model (Figure 2) in which spermine may bind in the major groove across the base pair or down the groove. In both cases, the terminal $^{+}NH_3$ interact with phosphate and the internal secondary $^{+}NH_2$ interact with base pairs through hydrogen bonding. The down groove model clearly implicates a sequence dependent interaction since hydrogen bonds are formed with N7 and O6 of G. In view of such ambiguities in the proposed model systems, the investigation of spermine covalently linked to DNA, specifically directed in the major or minor groove, may be able to throw light on their binding modes. N^4 position of cytosine is exposed in the major groove of duplex DNA and attachment of spermine at this position will allow the study of spermine:DNA binding in the major groove of duplex of DNA.

Spermine is also known to favor triple helix formation when present in millimolar concentrations (Hample et al, 1991; Thomas & Thomas, 1993). It was recently shown (Tung et al., 1993) that conjugation of spermine to 5'- end of oligonucleotide led to improved triple helix stability at pH 6.5. Both spermine as well as a third strand may be

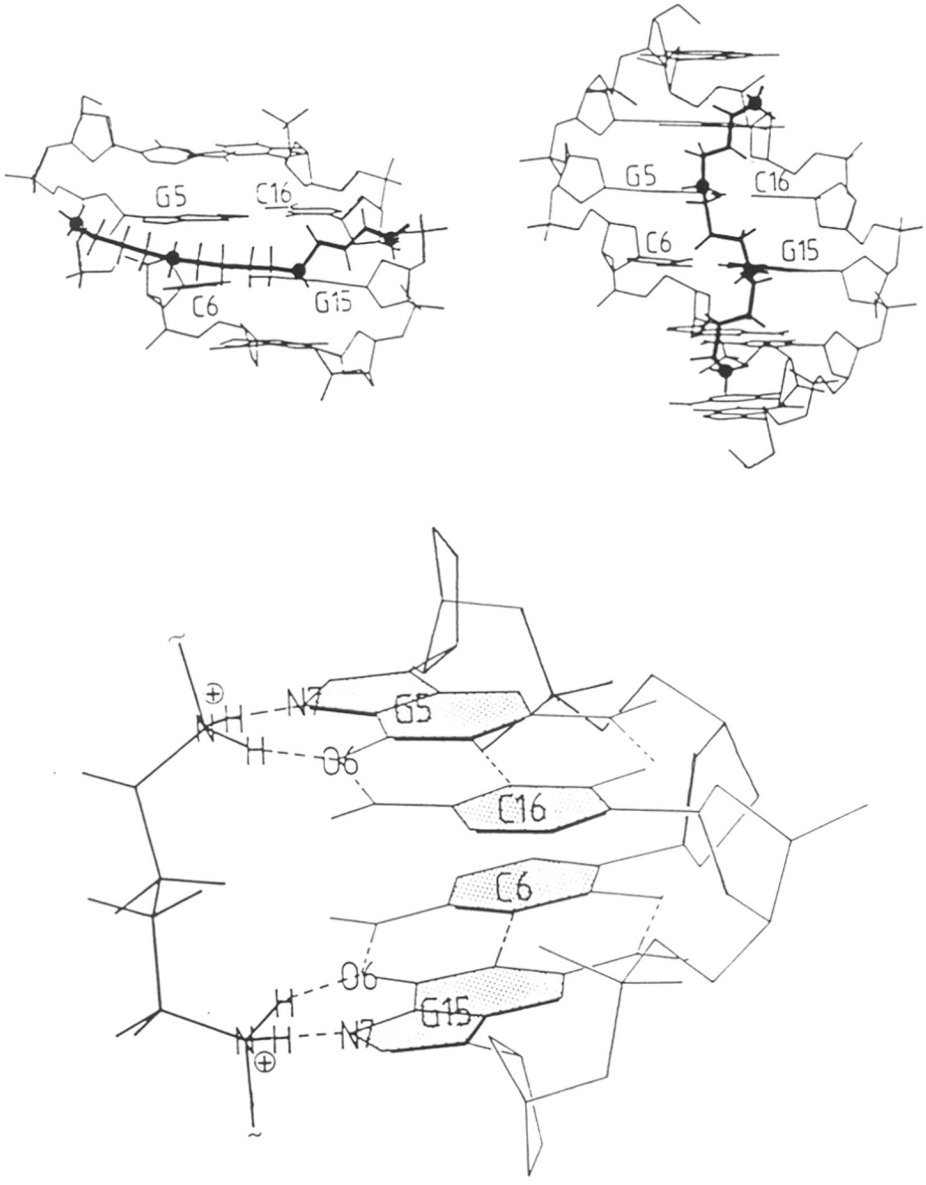
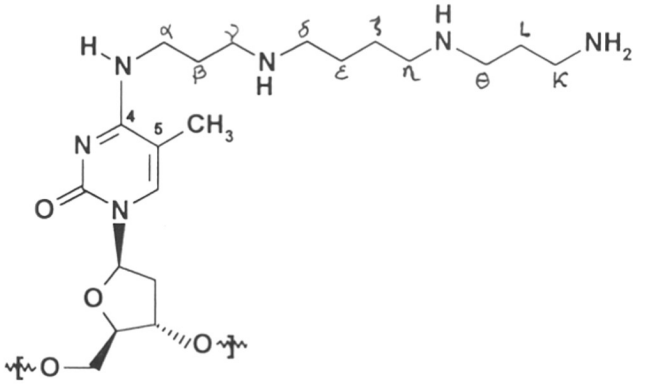


Figure 2. Minimized structures showing the complexation of spermine to a major groove of $d(GC)_5$ in the cross-groove (a) and down-groove (b) binding sites. The proposed model of spermine binding to the poly(dG-dC) major groove (c). (Taken from Haworth et al., 1991).

accommodated in the deep major groove of duplex DNA to yield triplex. In view of the positive attributes of both 5-Me-dC and spermine in promoting triple helix formation it was envisioned (Barawkar et al., 1994) simultaneous modifications, as in 5-Me-dC-N^4-spermine (Figure 3), may have a constitutive effect on triplex formation by oligonucleotide at physiological pH.



X

Figure 3. X = 5-Me-dC-(N^4-spermine)

Further, linking of spermine to a nucleobase also permits multiple incorporation at internal sites leading to an increased local concentration of spermine. Such multiple incorporation of polycationic spermine in the polyanionic oligonucleotide may possibly improve the membrane permeability due to net charge effects and nuclease resistance, which are the essential features of therapeutic antisense/antigene agents. In an elegant study, it has been shown that zwitterionic ODNs carrying a lipophilic cation at each nucleotide base form duplex at physiological pH and low salt conditions (Hashimoto et al., 1993a,b). Here, the base tethered ammonium cations replace metal ions which normally neutralize negative phosphate charges, making ODNs membrane permeable and nuclease resistant as a result of attendant changes in charge and size of monomer

units. This very well supports the premises for the present work. Much after the preliminary publication of this work on N^4-spermine-DNA presented in this chapter (Barawkar et al., 1994), very recently a paper has appeared in which spermine was covalently directed into the minor groove through conjugation at N^2 of G (Schmid & Behr, 1995).

In this chapter, 5-Me-dC tethered to spermine via N^4 is incorporated into the third strands and studies on their triplex forming ability are reported. These form triplexes with foremost stability at physiological pH 7.0-7.5, even in the absence of divalent cations like Mg^{2+}. From a therapeutic perspective, this is an attractive idea since intracellular environment cannot be manipulated to the conditions favorable for triplex existence.

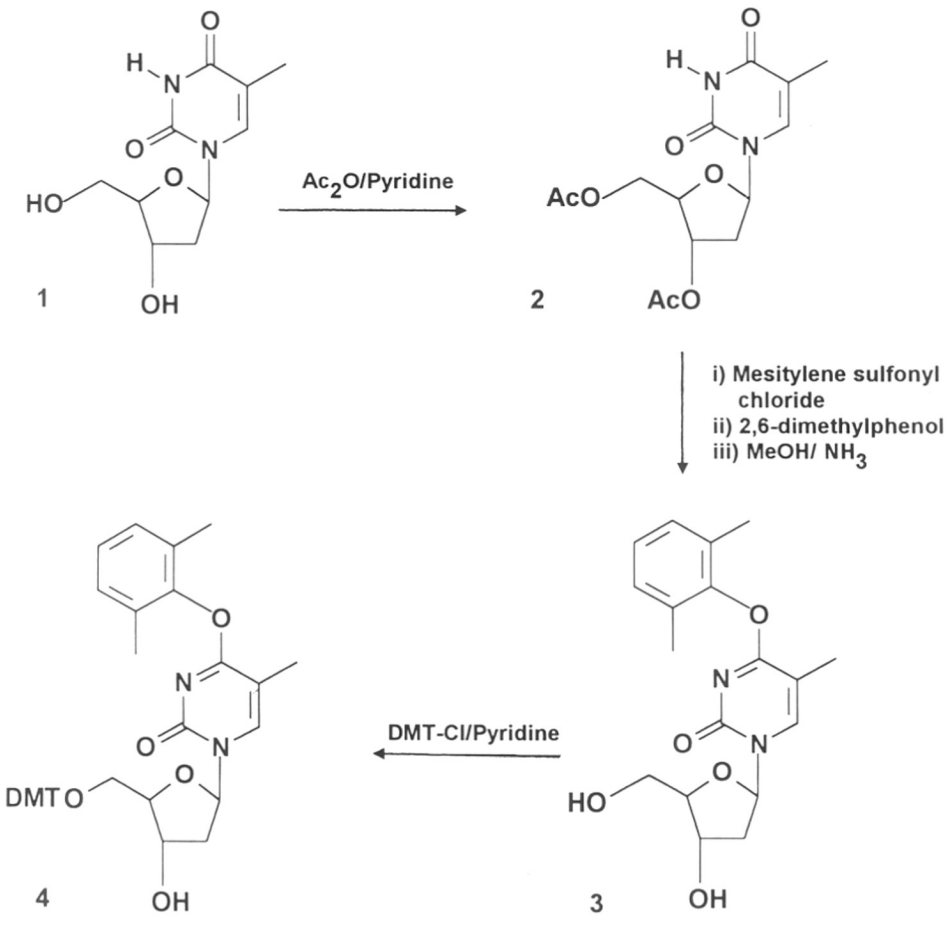
4.2. PRESENT OBJECTIVES

The objectives of this chapter are (i) synthesis of fully protected nucleoside 5'-O -(4,4'-Dimethoxytrityl)-5-methyl-N4-(N',N",N"'-tristrifluoroacetamidospermine)-2'-deox ycytidine **9** (ii) its site-specific and multiple incorporation into oligonucleotides using solid phase phosphoramidite chemistry and (iii) study of the effect of spermine conjugation on oligonucleotide net charge and its duplex and triplex forming ability.

4.3. RESULTS AND DISCUSSION

4.3.1. *Synthesis of protected nucleoside-polyamine conjugates*

For site-specific conjugation of polyamines to oligonucleotides, the monomer amidite **10** (Scheme 2) was synthesized by a variation of convertible nucleoside approach (Zhou & Chattopadhyayaya 1986; MacMillan & Verdine 1991). The convertible nucleoside approach uses the incorporation of monomer carrying a good leaving group such as 4-nitro or 2,6-dimethyl phenol, at O^4 of thymine into the oligonucleotide and displacement of this by nucleophiles after the final deprotection of oligonucleotide is over (post synthetic reaction). In the present case O^4-dimethylphenyl-thymidine was



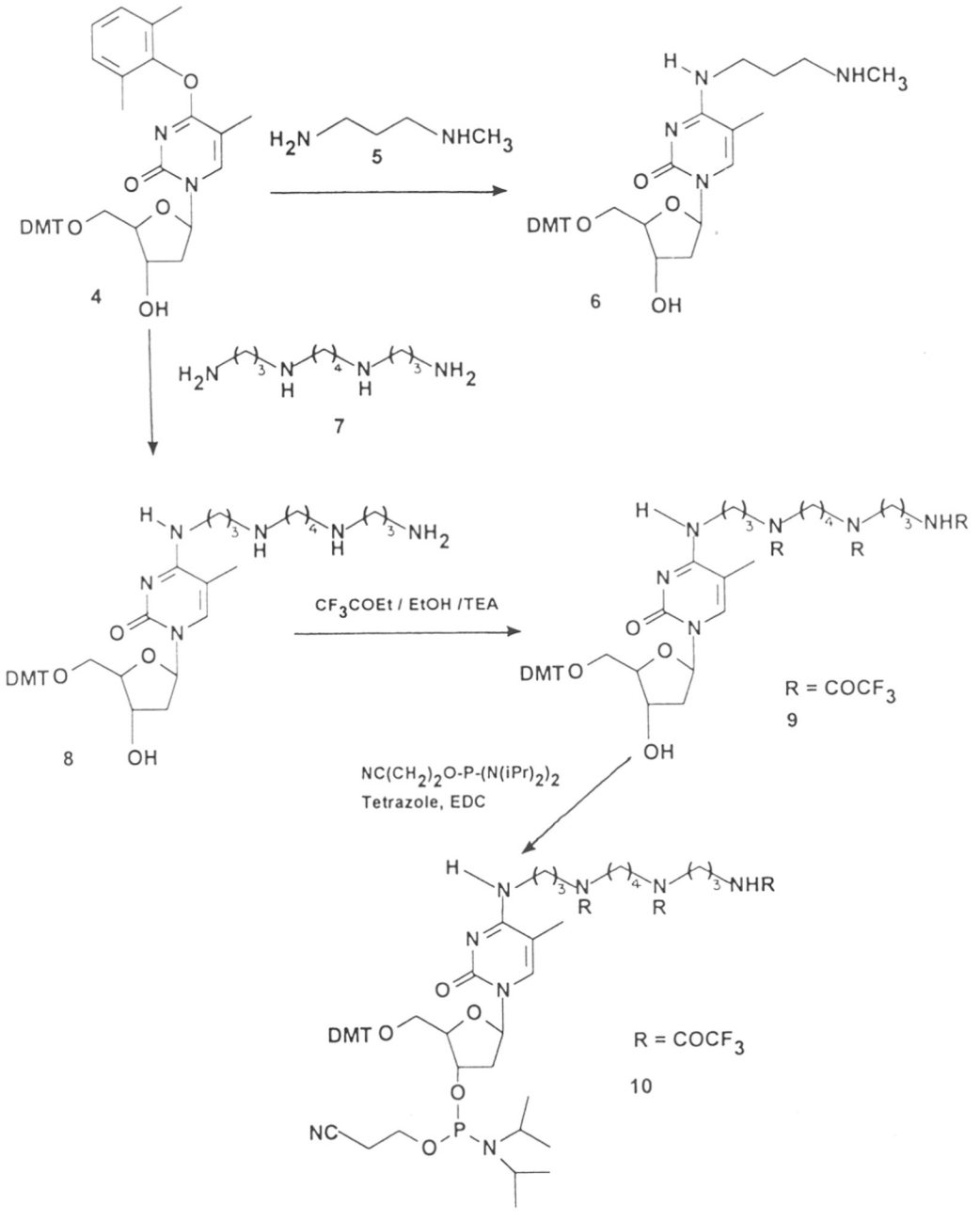
Scheme 1

treated with polyamines at monomeric level before the oligonucleotide assembly (dedicated monomer strategy). This gives an opportunity for unambiguous characterization of the regiospecific presence of the tether in modified nucleobase.

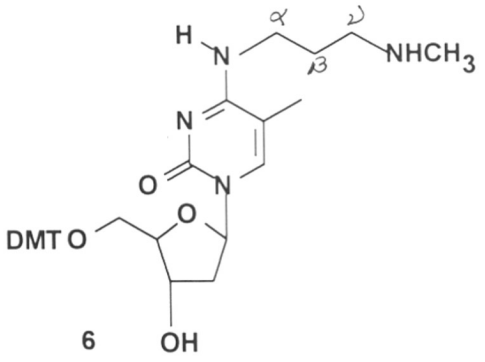
5'-O-4,4'-Dimethoxytrityl-4-O-(2,4-dimethylphenyl)-thymidine **4** was prepared according to Scheme 1. 3' and 5'-Hydroxyl functions of thymidine were first protected

as corresponding O-acetates to yield **2**. This was converted into 4-O-(2,6-dimethylphenyl)-thymidine **3** by treatment with 2,6-dimethylphenol in presence of mesitylenesulfonyl chloride, TEA and DABCO, followed by deprotection of 3',5'-O-acetates using methanolic ammonia to obtain **3**. Selective protection of 5'-hydroxyl was achieved by treatment with DMT-Cl in pyridine. Compound **4** upon treatment with an amine (RNH_2) is known to undergo nucleophilic substitution to yield N^4-alkyl-5-methyl-dC-nucleoside (Zhou & Chattopadhyayaya 1986; MacMillan & Verdine 1991). The reaction proceeds smoothly with primary amines but with polyamines (e.g. $H_2N(CH_2)_nNH(CH_2)_nNH(CH_2)_nNH_2$), the reaction may be hampered due to side reactions. These may arise from the higher nucleophilicity of secondary amino groups of polyamines and would lead to a mixture containing polyamine conjugation either at primary or secondary nitrogens.

To establish the reactivity profile of C4 in **4** towards primary and secondary amino nucleophiles present simultaneously in the same molecule, the amine N-methyl-1,3-diaminopropane **5** was chosen. Compound **4** was reacted in pyridine (16 h, 60°C) with excess of **5** to give a single product **6** in 90% yield and its structure was unambiguously established by ^1H and ^{13}C NMR data. In ^1H NMR (Figure 4a) N-CH_3 gave a peak at 2.65 ppm as against 2.9-3.0 ppm expected for dC-N^4-CH_3. In ^{13}C NMR (Figure 4b) a peak at 33.4 ppm could be assigned for the aliphatic N-CH_3, rather than to NCH_3 directly linked to nucleobase at C4, whose chemical shift is expected around 48 ppm (Pieles et al., 1990). This was also confirmed by INEPT experiment confirming the assigned structure. The relative reactivity of primary and secondary amines towards hindered electrophiles such as **4** is perhaps governed by their nucleophilicity as well as steric effects. The formation of a single product **6** in this case indicates the possible dominance of steric reasons over the higher nucleophilicity of secondary amines.


DMT O
4
OH
H_2N
NHCH$_3$
5
6
7
8
CF_3COEt / EtOH /TEA
R = $COCF_3$
9
$NC(CH_2)_2O$-P-$(N(iPr)_2)_2$
Tetrazole, EDC
10
NC
P
N
O
NHR

Scheme 2



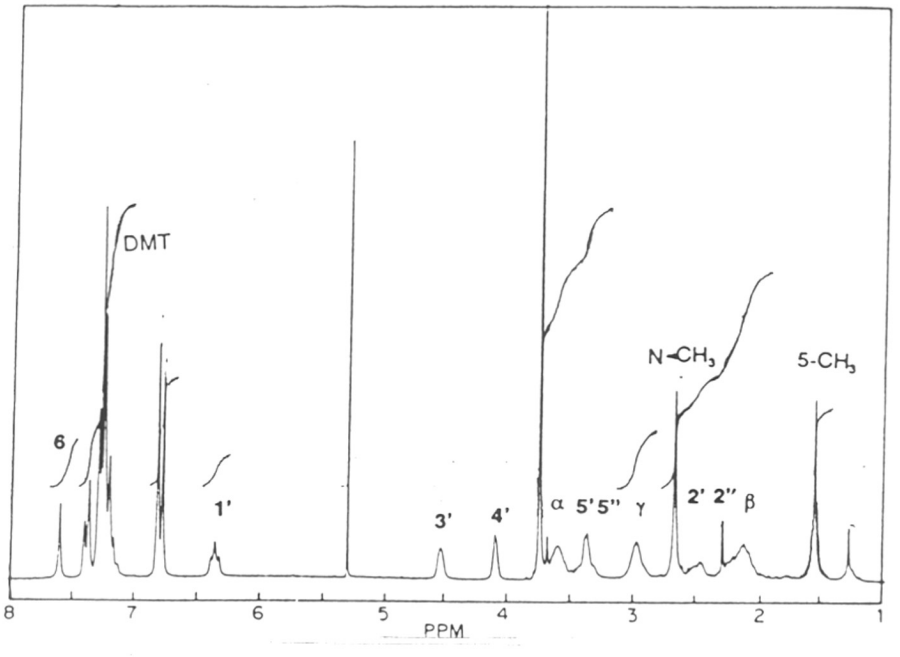


Figure 4a. ^{1}H NMR of **6**.

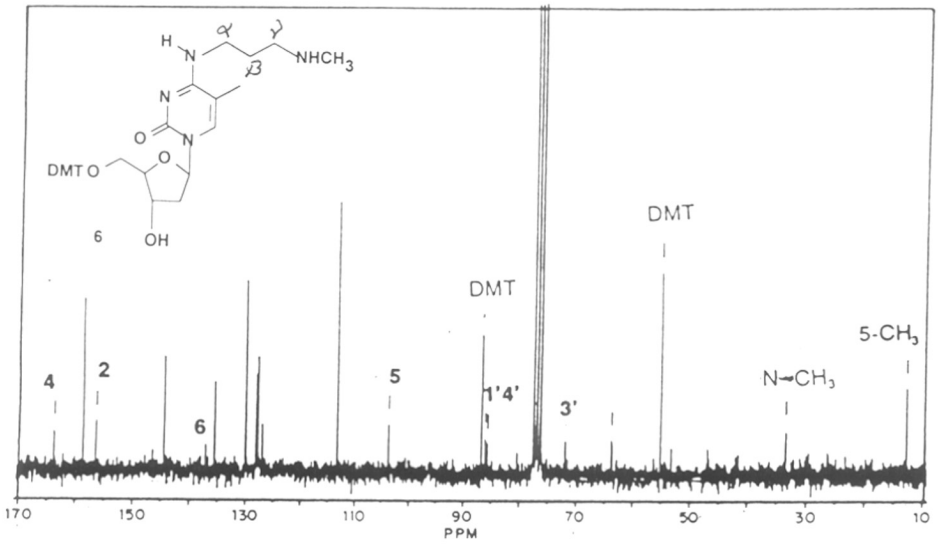


Figure 4b. ^{13}C NMR of 6.

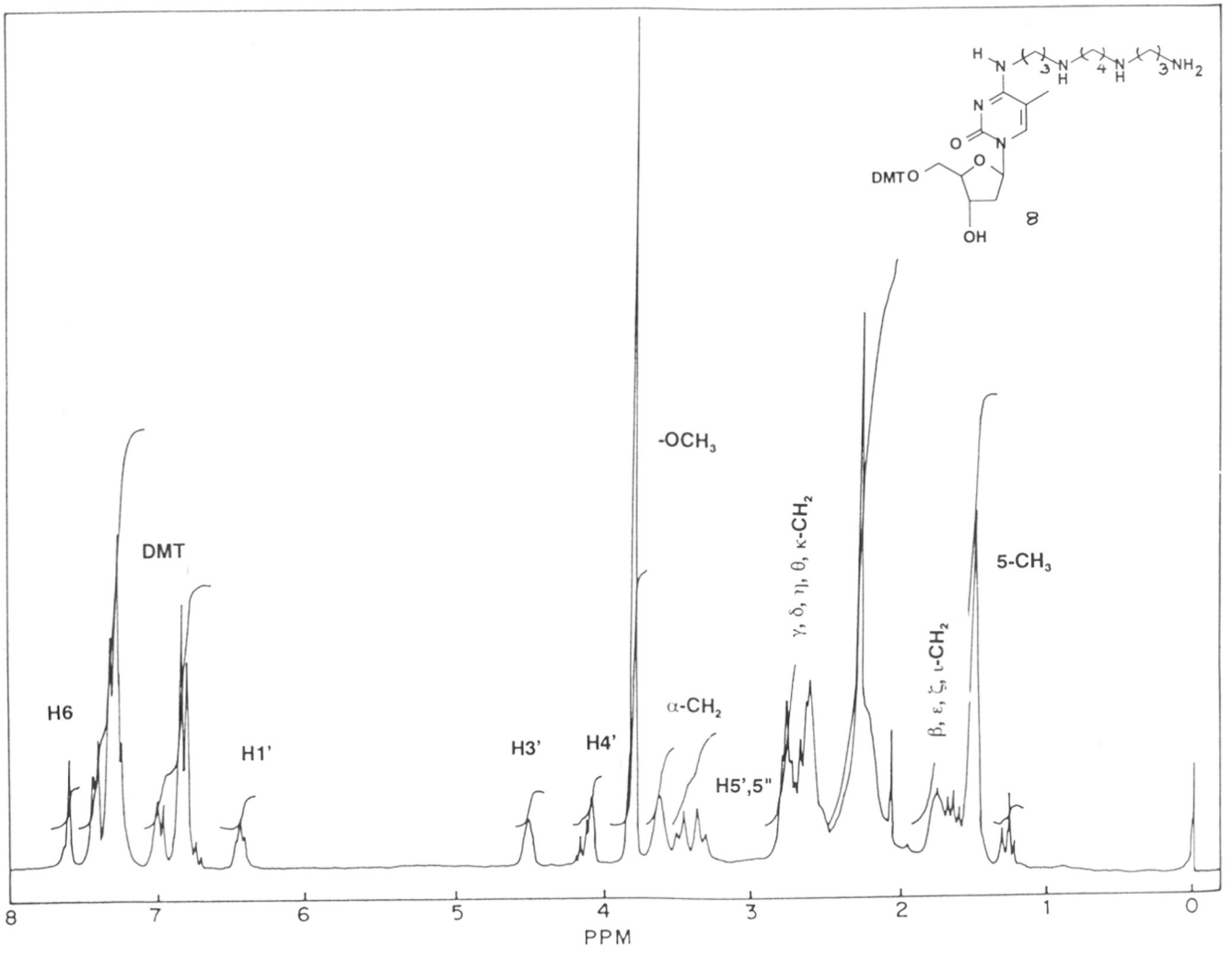


Figure 5a. ^{1}H NMR of 8.

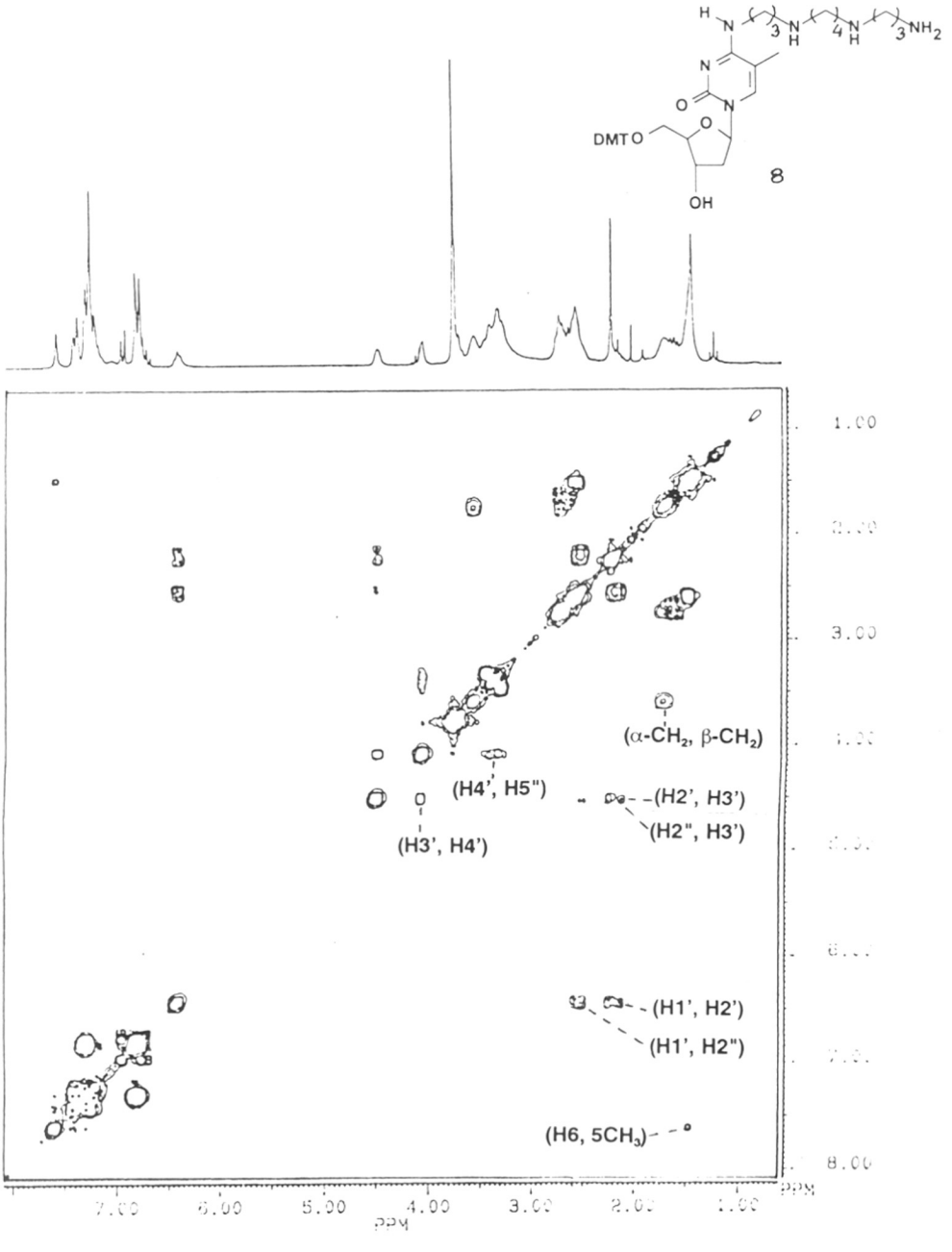


Figure 5b. 2D ^{1}H NMR of **8**.

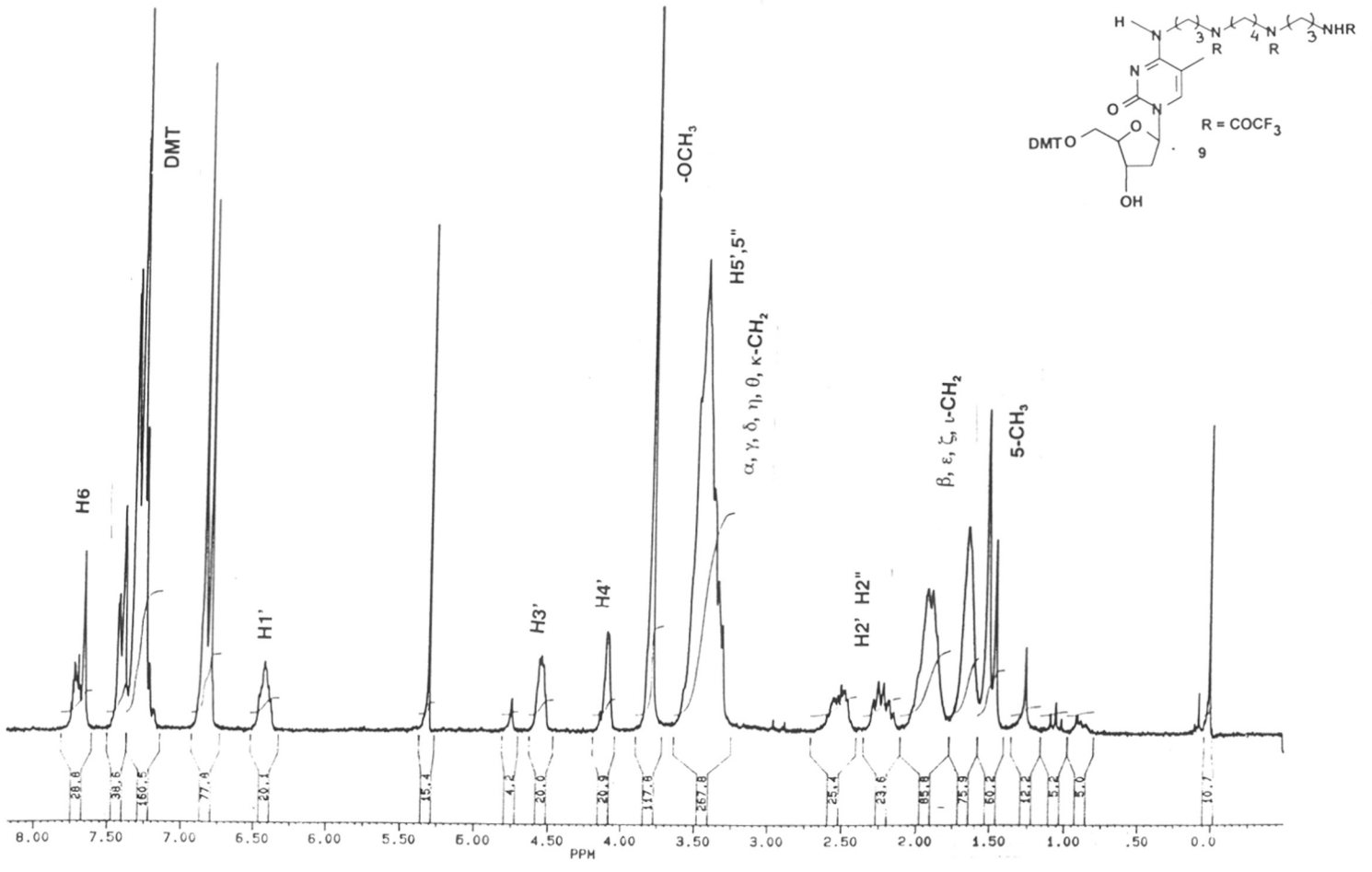


Figure 6a. ^{1}H NMR of **9**.

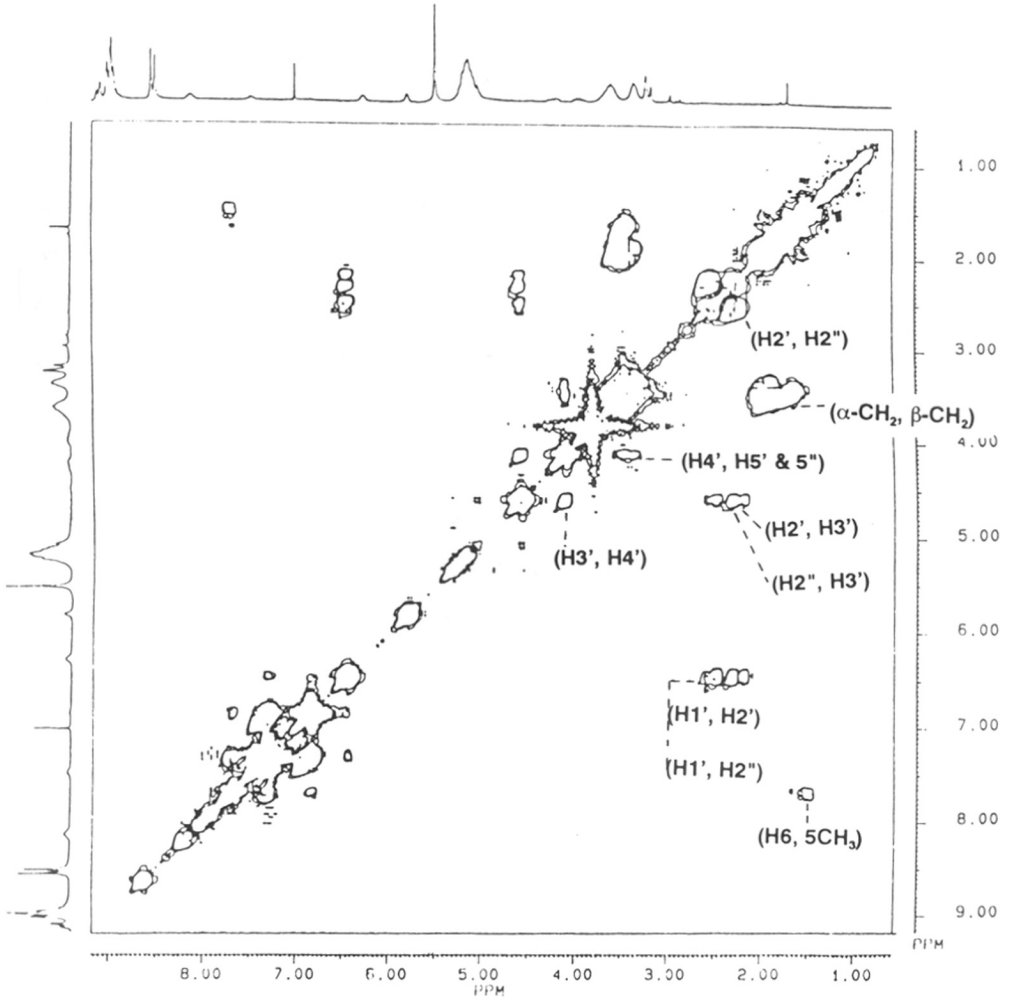
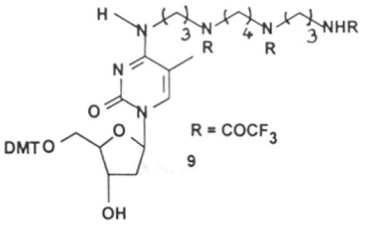


Figure 6b. 2D ^{1}H NMR of **9**.



A similar reaction of free base spermine **7** with **4** also yielded a single product **8** (yield, 85%) in which the polyamine is linked through the primary amino function. This is evident from the ^{1}H NMR of **8** (Figure 5) which showed a characteristic peak at 3.65 ppm integrating to 2 protons and ascribed to methylene in dC-N^{4}H-CH_2-. This is similar to that seen for N^4-C$\underline{H}_2$- in **6**. A reaction at secondary amine would have generated two sets of such protons (dC-N^4-$(CH_2)_2$). The primary and secondary amino functions of **8** were protected by trifluoroacetyl (TFA) group to yield **9** (Figure 6). The TFA group although not satisfactory for exocyclic amino of dA and dC, proved effective for exocyclic 5-amino group as described in chapter 2, and was also found to be suitable for protecting the side chain aliphatic amino groups in oligonucleotide synthesis by phosphoramidite chemistry. The TFA protected polyamine-nucleoside conjugate **9** was converted into the corresponding β-cyanoethyl phosphoramidite **10** (Scheme 2) by using standard methods (Sinha et al., 1984). No N-phosphonylation was observed during this reaction, as seen by ^{31}P NMR, which contained only two signals at 149.87 and 149.48 ppm characteristic of O-amidite (Figure 7).

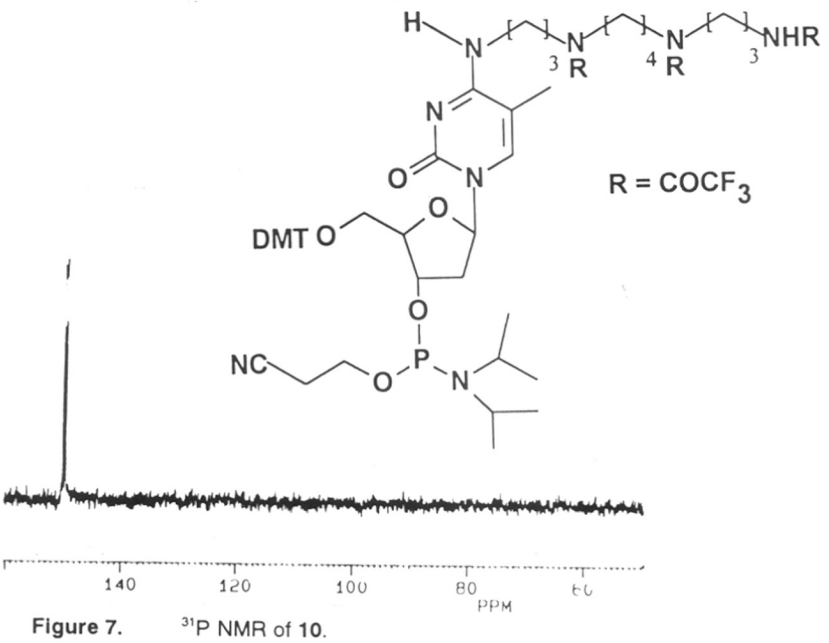


Figure 7. ^{31}P NMR of **10**.

	(positions 3, 10, 17 marked above sequence 11)
11	d - T T X T T T T T T C T T T T T T C T
12	d - T T C T T T T T T C T T T T T T X T
13	d - T T C T T T T T T X T T T T T T C T
14	d - T T X T T T T T T C T T T T T T X T
15	d - T T X T T T T T T X T T T T T T X T
16	d - T T C T T T T T T C T T T T T T C T
17	d - T T C̲ T T T T T T C̲ T T T T T T C̲ T
18	A A G A A A A A A G A A A A A A G A - d
19	d - G C C A A G A A A A A A G A A A A A A G A C G C
20	C G G T T C T T T T T T C T T T T T T C T G C G - d

X 5-Me-dC-N^4-spermine.
C̲ 5-Me-dC.

4.3.2. *Synthesis and characterization of spermine-oligonucleotide (sp-ODN) conjugates*

The amidite monomer **10** was incorporated into various oligonucleotide sequences **11-15** at specific positions on an automated DNA synthesizer (Pharmacia, GA Plus). The coupling efficiency of polyamine tethered amidite **10** was similar to the commercial phosphoramidites of normal nucleosides. After completion of synthesis, final on-column detritylation was followed by aqueous NH_3 treatment (60°C, 18h) to yield the fully deprotected oligonucleotides **11-15**. These were desalted and purified by HPLC and their purity was checked by reversed phase HPLC (Figure 8, inset). The laser desorption mass spectral measurement of the purified oligonucleotide **11** (Figure 8) gave the expected mass 5561.1 (M-H) and 5603.1 (M-2H+K), thus confirming the incorporation and retention of polyamine in final oligonucleotides.

To further ensure that polyamine conjugated nucleobases have survived the synthetic chemistry of oligonucleotide assembly by phosphoramidite approach and the subsequent step of ammoniacal deprotection, enzymatic hydrolysis of **11-15** were done using snake venom phosphodiesterase and alkaline phosphatase (Connoly, 1992).

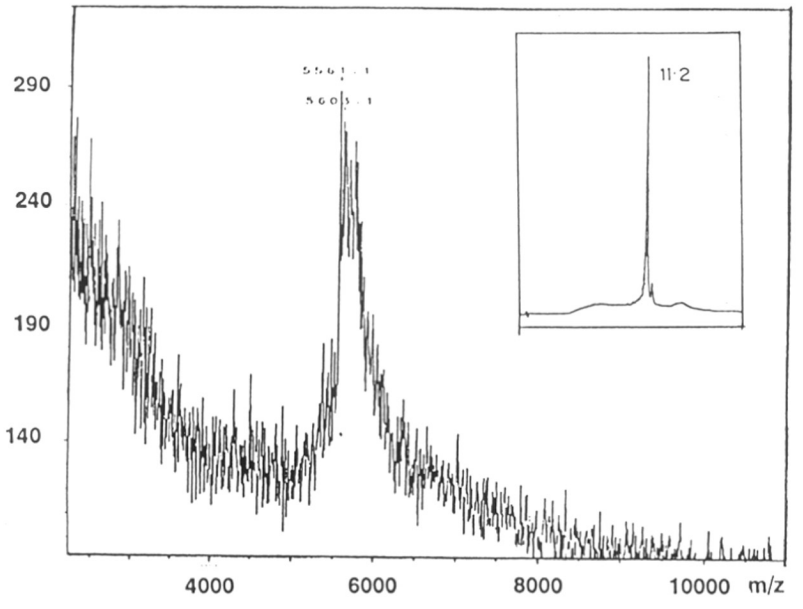


Figure 8. Laser desorption mass spectrum of *sp*-ODN **11**; inset, HPLC purity of **11**.

RP-HPLC analysis of the hydrolysate indicated the presence of the nucleoside base **X** in addition to the normal deoxynucleosides. The modified nucleoside base **X** could also be unambiguously assigned by its different uv spectral shift (280 nm) as compared to standard dC (271 nm). The spermine conjugated oligonucleotides **11-15** used presently consisted of 18-nucleotide oligomers with a single modification towards 5'- side (**11**, **X***3*), 3'-side (**12**, **X***17*), and at the center (**13**, **X***10*) and those with double (**14**, **X***3* & **X***17*) and triple (**15**, **X***3*, **X***10* & **X***17*) insertions of 5-Me-dC-N^4-spermine. ODNs having either dC **16** or 5-Me-dC **17** were also synthesized as above for control melting experiments.

4.3.3. *Electrophoretic mobility of sp-ODN*

The homogeneity of synthesized oligonucleotides were also established by PAGE after radioactive labeling. On denaturing PAGE (Figure 9), *sp*-ODNs exhibited single bands with migratory aptitudes varying with the degree of spermine substitution for the

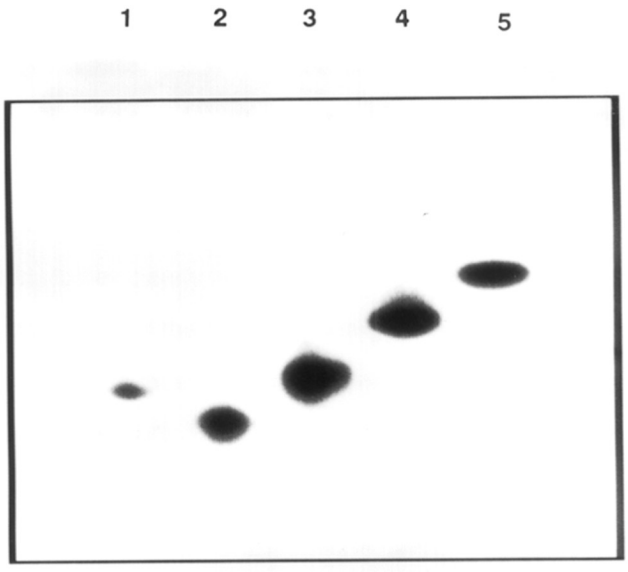


Figure 9. Autoradiogram showing altered electrophoretic mobility of sp-ODNs: lane 1, 24-mer **20**; lane 2, 18-mer **16**; lane 3, **11**; lane 4, **14** and lane 5, **15**.

same length of oligonucleotides (Figure 9). The oligomers **11** (lane 3), **14** (lane 4) and **15** (lane 5) containing one, two and three spermine molecules respectively, per DNA strand, show considerably increased retardation in mobility on the denaturing polyacrylamide gel as compared to unmodified 18-mer **16** (lane 2) and 24-mer **20** (lane 1). This suggests a lowering of net negative charge on *sp*-ODN as a consequence of spermine conjugation. The oligomer **11** with one spermine showed mobility lower than unmodified 18-mer **16** but similar to that of unmodified 24-mer **20** indicating that retardation in gel mobility is due to neutralization of the charge rather than an increase in size. The *sp*-ODN **14** and **15** with higher substitutions show further retardation in their mobility compared to the 24-mer **20** suggesting the additive nature of the neutralization effect on increasing the number of spermine molecules per DNA strand. Thus gel electrophoretic studies clearly indicate the expected zwitterionic character of *sp* ODNs due to covalent linkage of the positively charged spermine with the negatively charged oligonucleotide. On the face of it this behavior appears to be a favorable property for antisense ODN, particularly for transport across the membrane.

4.3.4. *Duplex formation by sp-ODN*

In order to check for ability of *sp*-ODNs to bind to single stranded DNA, the *sp*-ODNs **11-15** were individually hybridized with the complementary strand **18** and T_m of the duplexes were determined, Figure 10, shows the UV-melting curves from which it may be noticed that the polyamine-oligonuclotide conjugates do form duplexes, but with slightly lower T_m compared to the corresponding unmodified duplex **16:18**. The extent of destabilization depended on the degree of modification with an average effect of 5°C lower Tm per substitution. Such a destabilization was reported with N^4-amidoalkyl-dC containing ODNs with an average of 3-7°C destabilization (Cushman & Miller, 1992; Huang et al., 1993; Ono & Ueda., 1987; MacMillan & Verdine 1990). In general, the observed destabilization of duplex with N^4 modification may result from either a steric or electronic perturbation of Watson-Crick base pairing. It may be pointed out that in

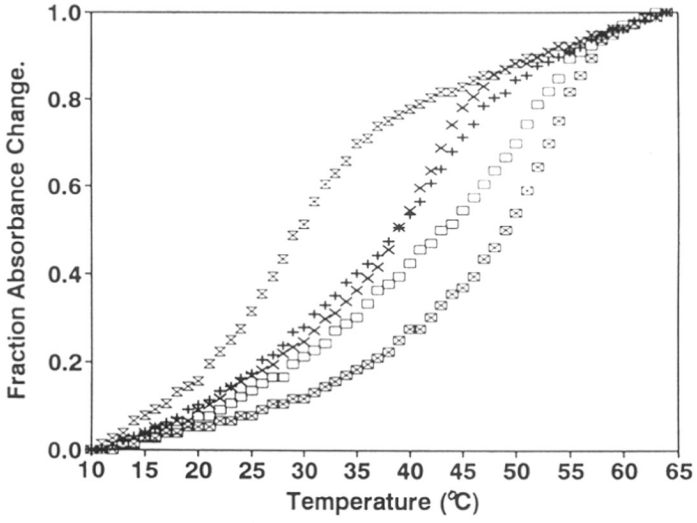


Figure 10. Melting curves of duplexes **16:18** (⊠ , 50°C), **12:18** (□ , 44°C), **13:18** (+ , 40°C), **14:18** (X , 40°C) and **15:18** (⧖ , 31°C), in 25 mM Tris, 100mM NaCl, 20 mM $MgCl_2$, pH 7.0.

N^4-methyl-dC nucleoside, a rotation around C4-N^4 bond leads to rotamers containing the N^4-methyl group in either of the two orientations, *syn* or *anti* with respect to N3 of dC (Figure 11). The preferred *syn* (Engel & Von Hippel, 1974) position leads to methyl group interfering with the Watson-Crick interbase hydrogen bonding. At oligonucleotide level, such an effect, though not same in intensity, nevertheless may lead to significant duplex destabilization and this is actually observed in most cases. In addition to the steric effect, partial contribution to lower stability may arise from the (substituent) electronic effect as well. The N-alkyl group exerts an electron donating inductive effect, thus lowering the hydrogen bonding donor potential of the exocyclic NH group. The combined influence of steric and electronic factors on hydrogen bonding in base pairs is supported by the fact that, electron withdrawing substitution at N^4 when locked in favorable Watson-Crick *anti* conformation (Figure 11) imparts stability to the G:C base pair in duplex DNA (Inoue & Ohtsuka, 1985). The destabilization of duplexes upon

N^4-substitution may also originate partially from the tether chain causing van der waals strain. The chain may also cause a disruption of the hydration network of DNA bases in the major groove. The presence of amino groups in the polyamine tether located in the major groove of the duplex, thus does not seem to provide any extra stability to the duplex, which is in contrast to well known duplex stability offered by externally added spermine.

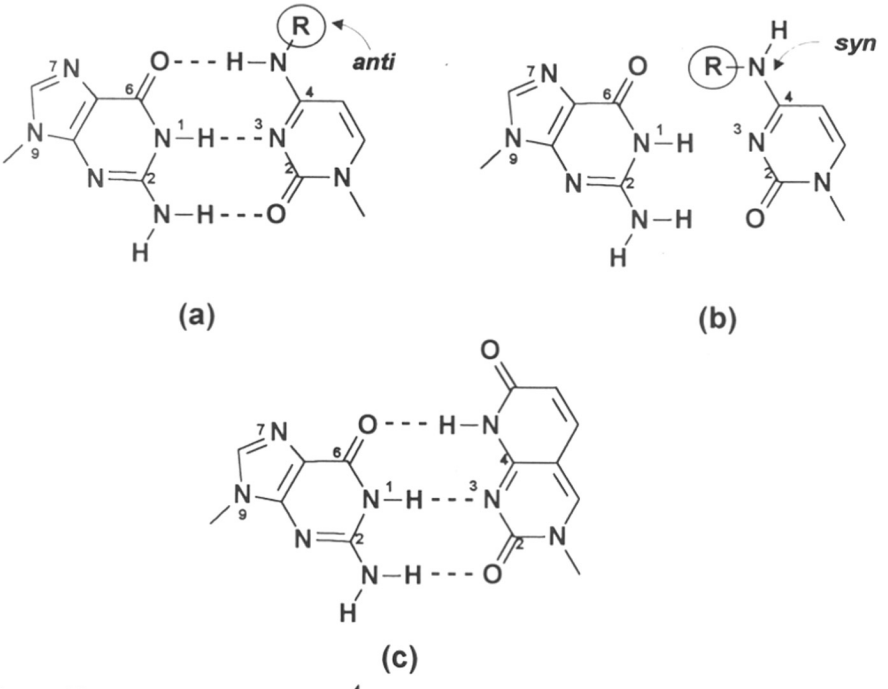


Figure 11. (**a**) The *anti* rotamer of N^4-alkyl-dC required at oligonucleotide level, (**b**) the *syn* rotamer favored at nucleoside level. (**c**) pyridopyrimidine base locked in *anti* rotamer.

Just before the completion of this thesis, an interesting paper appeared (Schmid & Behr, 1995) in which spermine was covalently linked to N^2 of dG in the minor groove of DNA. It formed duplex with its complementary strand, exhibiting 15-25°C higher melting than the unmodified duplex. The melting experiments were done in presence of externally added spermine unlike the work reported here. PAGE showed that this

modified DNA replaced its natural analogue from duplex in a physiological relevant medium. Such displacements are kinetically and thermodynamically favored due to high local concentrations altered by the pendent DNA-binding residue. The polyamine anchored in minor groove at C2 was thought to allow a hydrogen bonding zipper network to extend along the groove. This report is particularly significant for duplex stabilization and has direct relevance to the work reported in this chapter.

4.3.5. *Triplex formation by sp-ODN*

Antisense inhibition is caused by duplex formation of ODNs with single stranded DNA or RNA. Antigene effect operates via blocking of DNA duplex function by a third strand in triplex form. To examine the effect of *sp*-ODNs for this activity, triplexes bearing spermine on a third strand were generated by hybridization of *sp*-ODNs **11-15** with duplex **19**:**20** constituted from unmodified 24-mer oligonucleotides containing polypurine and polypyrimidine stretches. The 5' and 3' ends in duplex **19**:**20** were designed to avoid concatenation and to obtain a triplex Tm well resolved from the duplex melting transition.

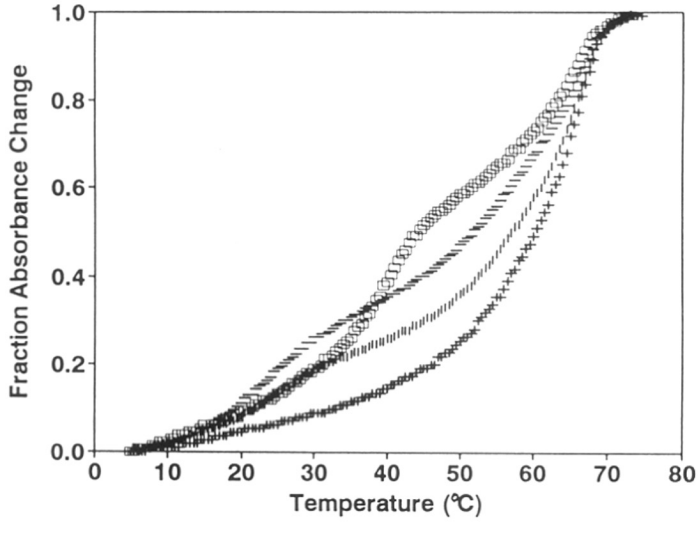


Figure 12. Melting profiles at pH 7.0 of **13*19**:**20** (□), **16*19**:**20** (▮), **19**:**20** (+) in presence of Mg^{2+} and **13*19**:**20** (—) in absence of Mg^{2+}.

The triplex formation was followed by temperature dependent UV absorbance. In contrast to the observed duplex destability, *sp*-ODNs **11-15**, when employed as a third strand in triple helix formation, exhibited very interesting stabilization effects. The thermal denaturation profiles of hybrids constituted from *sp*-ODNs with the control duplex **19:20** in Tris buffer (pH 7.3) containing NaCl (100 mM), showed a biphasic dissociation (Figure 12) in which the transition in the range 25-40°C corresponds to melting of third strand and that at ~60°C to duplex denaturation.

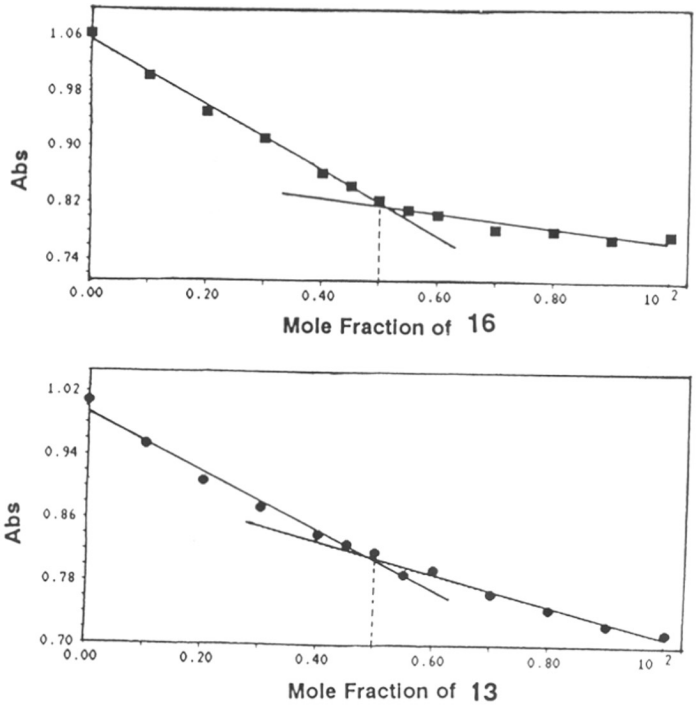


Figure 13. Mixing curve for triplex formation upon titration of third strands (■) **16** and (●) **13** with the duplex **19:20** as followed by UV at A_{260}, 5°C and pH 7.3.

The formation of triplexes was also supported by uv-mixing curves, generated by addition of the third strand to the preformed duplex by continuous variation method

(Plum et al., 1990; Felsenfeld & Rich, 1957). Increasing the mole fraction of the third strand to the duplex, lowered the UV absorbance and inflection point at 0.5 mole fraction of third strand (Figure 13) indicated the formation of expected 1:1 stoichiometry for *sp*-ODN:duplex hybrids. Among the *sp*-ODNs **11-15** which differ in the number and sequence-position of modifications, triplexes derived from third strands containing spermine conjugation towards 5'/3'- ends (**11*19:20** and **13*19:20**) gave better thermal stability (Table 1) compared to modification in the center (**11*19:20**). Although the triplex Tm slightly decreased with the increasing number of modifications, triplex formation was still observable with the trisubstituted *sp*-ODN **15** at physiological pH and temperature. In all cases, no differences were seen for duplex melting (60°C).

Table 1. Melting temperatures (Tm °C) of third strand of triplexes *sp*-ODN (**11-17**)***19:20** in presence and absence of $MgCl_2$.

		-$MgCl_2$			+$MgCl_2$#			
Third Strand	No of Modifications per Strand*	pH 7.3	pH 6.5	pH 6.0	pH 7.3	pH 7.0	pH 6.0	pH 5.5
11	1	40	36	34	47	46	36	35
12	1	40	36	35	46	45	37	35
13	1	33	31	30	41	37	35	35
14	2	33	32	31	40	40	35	34
15	3	25	24	23	31	31	29	28
16	-	nd	nd	28	28	35	48	49
17	3	nd	nd	38	46	48	50	50
17[b]	3	33	-	-	-	-	-	-

\# $MgCl_2$ (20 mM).

* Modification is either N^4-spermine-2'-dC for **11-15** or 5-Me-2'-dC for **17**.

Nd Not detected.

[b] ODN **17** with 1 mM added spermine.

The ODNs **16** and **17** containing dC or 5-Me-dC are devoid of conjugated spermine and showed no triple helix formation under the above conditions. The triplexes from these were obtained at pH 7.3 only in the presence of 20 mM $MgCl_2$ (Table 1). The

stabilities of triplexes from *sp*-ODNs **11-1**5 measured with added $MgCl_2$ showed an enhancement of Tm by 6-7°C. Significantly, triplexes from mono *sp*-ODNs (**11*19**:**20**) and (**12*19**:**20**) were as stable as that from ODN with three 5-Me-dC (**17*19**:**20**) in presence of $MgCl_2$. The formation of the control triplex (**17*19**:**20**) was examined in presence of externally added spermine. A Tm of 33°C obtained in presence of 1 mM extraneous spermine is similar to that from *sp*-ODN **13**, in which the concentration of appended spermine (equivalent to [ODN]) is only ~1μM.

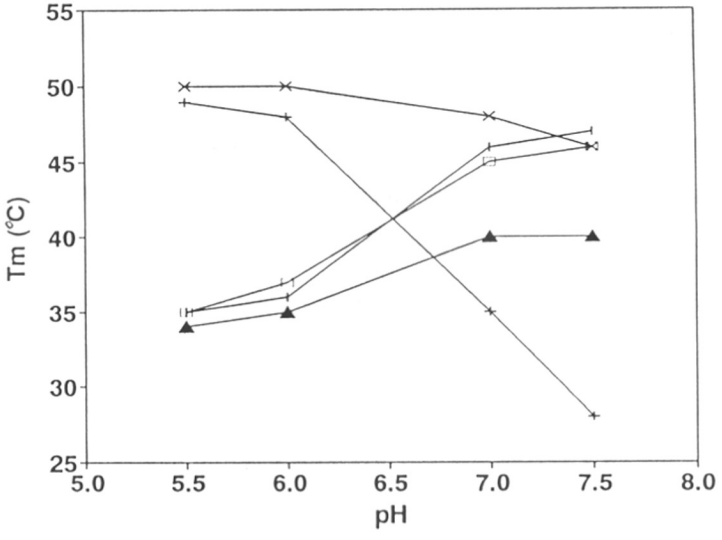


Figure 14. pH dependent Tm of triplexes constituted from the duplex **19**:**20** and **11** (▮), **12** (□), **14** (▲), **16** (+) and **17** (×) as third strand in the presence of Mg2+.

The pH dependence of triplex stability is critical for *in vivo* applications and hence the effect of pH on triplex formation from ODNs **11-15** was examined in presence of Mg^{2+} (Figure 14). As expected, the unmodified triplex (**16*19**:**20**) exhibited a large increase in Tm towards the acidic pH with a plateau around pH 5.6-5.8, while 5-Me-dC containing triplex (**17*19**:**20**) showed only a marginal increase in Tm at lower pH. Interestingly, the pH dependence of Tm of *sp*-ODN triplexes (**11-15*19**:**20**) showed an

opposite pattern; Tm was maximum in the pH range 7.1 to 7.4 and displayed moderate destabilization at acidic pH. This stability pattern was retained (Table 1) in the *sp*-ODN triplexes, even in the absence of Mg^{2+}. From a therapeutic perspective this is an attractive result as intracellular environment can not be manipulated to the condition favorable for triplex existence. The most significant and useful result of these experiments was that *sp*-ODNs had optimum triplex stability at physiological pH.

4.3.6. *Possible Origin of stabilization in sp-ODN triplex*

The rationale for covalent conjugation of spermine to 5-Me-dC present in Hoogsteen strand of a triplex to constitutively enhance the stability is well supported by the UV Tm results. The higher stability of *sp*-ODN containing triplex is perhaps due to the extra binding energy resulting from interaction of duplex with the polyamine appended to the third strand. While triplexes from unmodified analogs (**16*19:20**) are formed only in presence of $MgCl_2$ (20 mM), *sp*-ODN (**11-15**) triplexes are seen even in the absence of $MgCl_2$. Addition of $MgCl_2$ to *sp*-ODN triplexes results in further stabilization and higher Tm for triplex ⇔ duplex transition as expected for the effect of increased ionic strength. In all cases, the duplex Tm (in triplex) was unaffected implicating a direct role of appended spermine in triplex stabilization. The stability of *sp*-ODN triplex (**13*19:20**) containing a single spermine chain in micromolar concentrations (~1 μM) was equivalent to that of the 5-Me-dC containing triplex (**17*19:20**) but in presence of millimolar concentrations of externally added spermine. This points to a thousand fold higher "local" or "effective" concentration effect offered by the conjugated spermine over free spermine (Tung et al., 1993).

The mono *sp*-ODNs **11**, **12** are as effective in triplex formation at physiological pH as ODN containing three 5-Me-dC residues. Either increasing the number of spermine modification per ODN or decreasing the pH to acidic range destabilized the triplex. While the pH effect is opposite to that seen for unmodified or 5-Me-dC containing triplex, even

at the level of three modifications, the *sp*-ODN triplexes had better stability at pH 7.0 compared to the unmodified triplex. In contrast to stabilization seen for triplexes, spermine conjugation significantly destabilized the duplexes where the dC-N^4-spermine unit is involved in Watson-Crick hydrogen bonding mode. This suggests different electronic/steric requirements for Hoogsteen base pairing in triplexes as compared to Watson-Crick base pair of duplex.

The intramolecular phosphate neutralization by polycationic spermine leading to a net diminished charge for *sp*-ODNs **11-15** was clearly apparent from mobility retardation seen for them in gel electrophoresis (Figure 9). Monospermine 18-mer ODN **11** migrates as equal to an unsubstituted 24-mer **20** and the migration was systematically retarded as a function of degree of spermine substitution. Such a correspondence illustrated the zwitterionic character of *sp*-ODNs **11-15** similar to those reported by Hashimoto et al (1993).

An analogous effect of interstrand phosphate charge neutralization is possible in triplex. The third strand of DNA partitions the major groove and the N^4-linked spermine chain may lie either across the major groove accessing the phosphate backbone of Watson-Crick duplex strands or may participate in hydrogen bonding with complementary Watson-Crick base pair or those at the adjacent sites. The latter possibility should lead to considerable sequence dependence of triplex stabilization and may be understood better by a study of base pair mismatch tolerance and nearest neighbor effects of *sp*-ODN triplex.

The factors leading to triplex stability from the appended polyamine may arise as a combined consequence of several effects: (a) intra/inter strand electrostatic phosphate neutralization favoured by polycationic appendage (Hashimato et al., 1993a,b; Tung et al., 1993), (b) entropic changes from counter-ion decondensation during melting as predicted from polyelectrolyte theory (Manning, 1979) and (c) stabilizing hydrogen

bonding interactions of spermine with complementary or adjacent base pair (Feurstein et al., 1990; Jain et al., 1989; Haworth et al., 1991). A recent paper has attempted to investigate the electrostatic effects in triplexes by studying intramolecular triplex formation as a function of pH and ionic strength (Volker & Klump, 1994). It was observed that while electrostatic interactions between protonated C (C^+) of third strand and phosphate backbone offer considerable stabilization in a global, sequence independent manner, repulsion between adjacent C^+ can cause successful negation of the electrostatic advantage, imparting local sequence dependent effects. Application of polyelectrolyte theory to DNA suggests that DNA melting is accompanied by release of a part of counter-ions from DNA bound state to bulk since the condensed charge density is reduced in the process. In zwitterionic DNA such as that from *sp*-ODNs **11-15** described here or reported earlier (Hashimoto et al., 1993b), the higher charge density in duplex/triplex form is partially balanced by the covalently bound, non diffusible cations, offering a unique stabilization effect.

Interestingly, in the present study on *sp*-ODNs **11-15** a decrease of triplex stability was noticed upon lowering the pH, in reverse trend to that seen with unmodified oligonucleotides (Figure 14). This may arise perhaps as a consequence of pH induced change in conformation of appended spermine chain, which at low pH, may assume orientations unfavorable for specific interactions with the backbone or the base pairs. Alternatively, a higher positive charge density on spermine at low pH may discourage efficient protonation at N3 of anchor dC by electrostatic repulsion, leading to a weaker Hoogsteen interaction. The fact that increasing degree of spermine substitution also lowered the triplex stability supports electrostatic repulsion, either among the spermine chains or between spermine and protonated C^+, as a major causant of low pH destabilization of *sp*-ODN triplexes. Further study on the ionic strength and sequence

dependent effects, calorimetric measurements of enthalpy and 'chemical mutation' of spermine chain (replacement by polyaminoether analogs) are necessary to understand the origin of the triplex stability and thereby improve it by this strategy.

4.4. CONCLUSIONS

This chapter reports a strategy for convenient synthesis of oligonucleotides with site-specific conjugation of spermine. Such *sp*-ODNs **11-15** have been shown to form stable triple helices in absence of $MgCl_2$ at physiological pH. Under these conditions ODNs containing natural dC **16** or 5-Me-dC **17** fail to show triplex hybridization. The simple and unambiguous chemistry of conjugation permits introduction of spermine at desired, multiple internal sites in oligonucleotide sequences, thereby imparting significant zwitterionic character to DNA. Although trisubstitution leads to lowering of Tm, it does not completely inhibit the triplex formation. A lower net negative charge arising from multiple substitution, may assist the cellular uptake of ODNs. This conjecture is strengthened by the recent findings that coadministration of ODNs with cationic lipids significantly enhances the membrane permeability (Wagner et al., 1993; Colige et al., 1993). Further evaluation of the effects of sequence and mismatch tolerance on specificity of triplex formation by *sp-ODNs are necessary. Since polyamines can also form* metal complexes, the compounds such as those synthesized here, may have potential utility in site directed cleavage of DNA.

4.5. EXPERIMENTAL

Base-protected nucleoside phopsphoramidites and nucleoside derivatised controlled pore glass supports were purchased from Cruachem. All chemicals used were of reagent or better grade. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker 200 MHz AC-F spectrometer. Laser desorption mass spectrum was recorded on a Finnigan Lasermat mass spectrometer. T4 polynucleotide kinase, snake-venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Mannheim. 5'-[γ-^{32}P]ATP was purchased from Bhabha Atomic Research Center, Bombay.

3',5'-Di-O-acetyl-thymidine 2

Thymidine (4 g, 16.5 mmol) was dissolved in dry pyridine (15 ml) to which acetic anhydride (5 ml, 3 eq) was added slowly and stirred at room temperature for 24 hr. The reaction mixture was evaporated to dryness and redissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$ solution, concentrated to dryness to give **2** (yield 4.26 g, 79%). M.P. = 135°C, Rf ($MeOH:CH_2Cl_2$ 1:9) = 0.6.

1H NMR ($CDCl_3$) δ 9.5 (brs, 1H, N3H), 7.28 (s, 1H, H6), 6.33 (dd, J= 5.4 & 8.1 Hz, 1H, H1'), 5.3 (m, 1H, H3'), 4.35 (m, 2H, H5' & H5"), 4.24 (m, 1H, H4'), 2.5 (m, 1H, H2'), 2.2 (m, 1H, H2"), 2.14 (s, 6H, $2XCOC\underline{H}_3$), 1.85 (s, 3H, $5CH_3$).

4-O-(2,6-Dimethylphenyl)-thymidine 3

5',3'-O-Diacetylthymidine (3.26 g, 10 mmol) was dissolved in dry CH_2Cl_2 (40 ml) to which mesitylenesulphonyl chloride (5.4 g, 2.5 eq) was added. The reaction mixture was cooled in ice bath and to this triethyl amine was added dropwise (3.6 g, 3.5 eq) followed by addition of dimethylaminopyridine (0.24 g, 2 mmol). The reaction was allowed to attain room temperature with continuous stirring for 3 hr after which reaction was monitored by TLC and disappearence of starting material was confirmed. To this reaction mixture 2,6-dimethyl phenol (10.0 g, 8 eq), triethylamine (10.0 g, 10 eq) and DABCO (0.2 g, 1.8 mmol) were added and stirred overnight. The reaction mixture was

concentrated to dryness, redissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$, dried over $NaSO_4$, evaporated to form solid foam. This was further treated with saturated methanolic ammonia for 2 hr, to remove 5' and 3' ester protection. This was concentrated to solid followed by purification on silica gel column, (yield 2.8 gm, 80%), Rf ($MeOH:CH_2Cl_2$ 1:9) = 0.5.

1H NMR ($CDCl_3$:$DMSOd_6$, 7:3), δ 8.2 (s, 1H, H6), 7.0 (s, 3H, ArH), 6.15 (t, J= 6.1 Hz, 1H, H1'), 4.48 (brs, 2H, 5' & 3' OH), 4.3 (m, 1H, H3'), 3.9 (m, 1H, H4'), 3.7 (m, 2H, H5' & H5"), 2.35 (m, 1H, H2'), 2.15 (m, 10H, H2", 2x$ArCH_3$ & 5CH_3).

5'-O-(4,4'-Dimethoxytrityl)-4-O-(2,6-dimethylphenyl)-thymidine 4

Compound **3** (2.4 g, 7.2 mmol) was dried by coevaporation over dry pyridine and dissolved in dry pyridine (35 ml) to which 4,4'-dimethoxytrityl chloride (2.94 g, 8.68 mmol) was added in two batches of 30 min interval and stirred at room temperature for 3 hr. The reaction was monitored by TLC and quenched by adding methanol (2 ml). The reaction mixture was concentrated to oil, dissolved in CH_2Cl_2, washed with saturated aqueous $NaHCO_3$ solution, dried over $NaSO_4$, followed by purification on silica gel column chromatography, eluted with CH_2Cl_2 containing 0.5% pyridine and methanol gradient. (yield 2.86 g, 62%), Rf = ($MeOH:CH_2Cl_2$, 1:9) = 0.57.

1H NMR ($CDCl_3$) δ 8.0 (s, 1H, H6), 7.5-7.2 (m, 9H, DMT) 7.0 (s, 3H, ArH), 6.82 (d, 4H, DMT), 6.3 (t, 1h H1'), 4.5 (m, 1H, H3'), 4.08 (m, 1h, H4'), 3.8 (s, 6H, 2xOCH_3), 3.4 (m, 2H, H5' & H5"), 2.6 (m, 1H, H2'), 2.2 (m, 1H, H2"), 2.1 (s, 3H, Ar-CH_3), 2.02 (s, 3H, Ar-CH_3), 1.7 (s, 3H, 5CH_3).

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-N4(-polyamine)-2'-deoxycytidine.

General procedure: **4** (0.33 g, 0.5 mmol) was dissolved in dry pyridine (1.5 ml) and treated with either N-methyl-1,3-diaminopropane (0.5 ml, 10 eq) or spermine (0.8 gm, 4 eq) at 60 °C for 10-12 h. The reaction mixture was concentrated to dryness and

redissolved into dichloromethane, washed with water, and the organic layer was concentrated to an oil. This was purified by column chromatography over silica gel (10 gm), when the required compound elutes at 5 to 15% of MeOH gradient in dichloromethane containing 0.5% pyridine.

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-N4-(γ-methylaminopropyl)-2'deoxycytidine 6: TLC (silica gel) R_f = 0.4 (15 % MeOH-CH_2Cl_2).

^{1}H NMR ($CDCl_3$) δ 7.6 (s, 1H, H6), 7.5-7.2 (m, 9H, DMT), 6.8 (s, 4H, DMT), 6.35 (t, J = 6.3Hz, 1H, H1'), 4.5 (brs, 1H, H3'), 4.1 (brs, 1H, H4'), 3.75 (s, 6H, 2xOCH_3), 3.6 (m, 2H, α $C\underline{H}_2$), 3.38 (m, 2H, H5' and H5"), 3.0 (m, 2H, γ $C\underline{H}_2$), 2.65 (s, 3H, N-CH_3), 2.45 (m, 1H, H2'), 2.13 (m, 3H, β $C\underline{H}_2$, H2"), 1.53 (s, 3H, 5-CH_3), **^{13}C NMR** ($CDCl_3$), 163.7 (C4), 156.4 (C2), 137 (C6), 103.7 (C5), 86, 85.6 (C1', C4'), 71.9 (C3'), 63.7 (C5'), 53.6 (CH_2 α), 46.7 (CH_2 γ), 41.7 (C2'), 37.6 (CH_2 β), 33.4 (N-$\underline{C}H_3$), 12.8 (5-$\underline{C}H_3$).

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-N4-(spermine)-2'-deoxycytidine 8:

^{1}H NMR $CDCl_3$), δ 7.65 (s, 1H, H6), 7.5-7.0 (m, 9H, DMT), 6.8 (s, 4H, DMT), 6.5 (t, 1H, H1'), 4.5 (brs, 1H, H3'), 4.15 (brs, 1H, H4'), 3.8 (s, 6H, 2xOCH_3), 3.65 (m, 2H, α CH_2), 3.5 (m, 1H, H5'), 3.4 (m, 1H, H5"), 2.8-2.5 (m, 12H, γ, δ, η, θ, κ, H2' and H2"), 1.8-1.6 (m, 8H, β, ε, ζ, ι), 1.5 (s, 3H, 5-CH_3)

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-N4-(N',N",N"'-tristrifluoroacetamidospermine)-2'-deoxycytidine 9: Compound **5** (0.35 gm, 0.5 mmol) was dissolved in anhydrous ethanol (24 ml), to which dry triethylamine (1 ml, 10 eq) and ethyltrifluoroacetate (2.6 gm, 20 eq) were added. The reaction mixture was stirred at room temperature for 24 h, after which ethanol was removed under reduced pressure. The residue was dissolved in dichloromethane (25 ml) and washed with water (3 x 15 ml). The organic phase was dried over anhydrous Na_2SO_4 concentrated to oil and was purified by silica gel column chromatography.

^{1}H NMR ($CDCl_3$) δ 7.65 (s, 1H, H6), 7.5-7.2 (m, 9H, DMT), 6.85 (s, 4H, DMT), 6.5 (t,

1H, H1'), 4.55 (brs, 1H, H3'), 4.1 (brs, 1H, H4'), 3.8 (s, 6H, 2xOCH_3), 3.6-3.25 (m, 14H, α, γ, δ, η, θ, κ, H5' and H5''), 2.6-2.45 (M, 1H, H2'), 2.3-2.15 (m, 1H, H2''), 2.1-1.6 (m, 8H, β, ε, ζ, ι), 1.5 (s, 3H, 5-CH_3). **^{13}C NMR** ($CDCl_3$) δ 163.4 (C4), 156.6 (C2), 158.2, 157.8 and 157.2 (3x$\underline{C}OCF_3$), 136.4 (C6), 113.7 ($\underline{C}F_3$) 102.7 (C5), 86.8 (C1'), 86.3 (C4'), 71.9 (C3'), 63.8 (C5'), 47.3, 46.5, 46.1, 45.4, 44.5 and 41.8 (α, γ, δ, η, θ, and κ CH_2), 37.1 (C2'), 28.3, 26.4, 25.9 and 24 (β, ε, ζ and ιCH_2).

3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramido)-5'-O-(4,4'-Dimethoxytrityl)-5-methyl-4-N-(N',N'',N'''-tristrifluoroacetamidospermine)-2'-deoxycytidine 10: Compound **9** (0.37 gm, .030 mmol) was taken in dry ethylene dichloride. To this, tetrazole (26 mg, 1.25 eq) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidate (0.14 ml, 1.5 eq) was added and stirred at room temperature for 3 h. The completion of reaction was indicated by a faster moving spot on TLC. The reaction mixture was diluted with dichloromethane and washed with 10% aq $NaHCO_3$. Organic layer was dried over anhydrous Na_2SO_4 and was concentrated under reduced pressure to get **7** as a foam. Rf (EtOAc:CH_2Cl_2, 1:1, 0.5% TEA) = 0.32 (2 spots, distereoisomers).

^{31}P NMR ($CDCl_3$), δ 149.87, 149.48.

Oligonucleotide synthesis, purification and labelling

All oligonucleotides were synthesized on 1.3 μmol scale on a Pharmacia GA plus DNA synthesizer using controlled pore glass (CPG) support and base protected 5'-O-(4,4'-dimethoxytrityl)deoxyribonucleoside-3'-O-[(diisopropylamino)-β-cyanoethylphosphoramidite] monomers which was followed by deprotection with aqueous NH_3. For synthesis of oligonucleotides **11-15** containing chemically modified dC units (Figure 2), appropriate amidite monomers were employed in place of normal amidites for coupling at desired positions. All oligonucleotides were purified by reversed phase HPLC on Novapak C18 column using the buffer systems A: 5% CH_3CN in 0.1 M triethylammoniumacetate (TEAA) and B: 30% CH_3CN in 0.1 M TEAA using a gradient A to B of

1.5%/min at a flow rate of 2 ml/min. The purified oligonucleotides were labeled at the 5'-end with T4 polynucleotide kinase and 5'-[γ-^{32}P]ATP according to standard procedures. The radiolabeled oligonucleotide samples were run on a 20% polyacrylamide gel containing 7M urea and with Tris-Borate-EDTA (pH 8.3) as buffer. Samples were loaded in formamide, heated to 70°C and cooled on ice bath before loading on the gel. Autoradiograms were developed within 1 h with an intensifying screen.

Base composition analysis

The base composition of synthesized oligonucleotides were confirmed by enzymatic hydrolysis (29). Oligonucleotides **11-15**, (0.5 OD_{254} Units) were dissolved in 10 mM KH_2PO_4 (100 μl, pH 7), containing $MgCl_2$ (10 mM) and treated with snake venom phosphodiesterase (10 μl, 1 mg/0.5 ml) and alkaline phosphatase (10 μl, 1 unit/μl) at 37°C for 12 hr. This hydrolysate (2 μl) was analyzed on analytical C18 RP-column and eluted with 0.1 M triethyl ammonium acetate, pH 6.5, 1 ml/min and the peaks were detected using photodiode array detector. Standard nucleosides: Rt dC (1.2 min), dG (2.3 min), dT (2.7 min) and dA (4.4 min). Enzymatic hydrolysate: dC (1.2 min, λ_{max} 271 nm), **X** (1.4 min, λ_{max} 280 nm) and dT (2.7 min, λ_{max} 269 nm). The enzymic hydrolysate of **15** showed complete absence of dC and presence of **X** and dT.

Melting experiments

Duplex and triplex melting experiments were carried out in the following buffers, all containing 100 mM NaCl, with or without 20 mM $MgCl_2$. pH 5.5, 50 mM NaOAc; pH 6.0, 10 mM PIPES [piperazine-N-N'-bis(2-ethanesulphonic acid)]; and pH 7.0 - 7.3, 25 mM Tris [2-Amino-2-hydroxymethylpropane-1,3-diol]. Appropriate oligonucleotides, each at a strand concentration of 1μM based on UV absorbance calculated using molar extinction coefficients at 260 nm, dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 $cm^2/\mu mol$) were mixed, heated to 70°C for 3 min, cooled to room temperature and then stored at 4°C overnight. The A_{260} at various temperatures were recorded using Perkin Elmer

Lambda 15 UV/VIS spectrophotometer, fitted with a temperature programmer and heating at a rate of 0.5°C/min over the range 5-75°C and dry nitrogen gas was flushed in the spectrophotometer chamber to prevent moisture condensation at low temperature. The triplex melting temperature, Tm, was determined from the midpoint of the first transition in the plots of fraction absorbance change versus temperature and were further confirmed by differential (*dA/dT vs T*) curves. The Tm values are accurate to $\pm$ 1°C over the reported values.

4.6. REFERENCES

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