CHAPTER 5.

BIOPHYSICAL STUDIES AND APPLICATIONS OF BACKBONE MODIFIED DNA CONTAINING CHIRAL ACYCLIC ANALOGS
5.1 INTRODUCTION

Earlier studies on acyclic oligonucleotides revealed that the use of L-serinyl acyclic analogs\(^1\) incorporated oligonucleotides disfavored duplex formation. Recently, Benhida et al.\(^2\) incorporated similar analogs into oligonucleotides and found the similar deleterious effect on duplex and triplex formation. This again emphasized the need for restricted flexibility of this type of backbone for stable duplex and triplex formation. The synthesis of the monomer units (Chapter 4) now permitted the investigation of the consequences of restricted backbone rotation on the hybridization properties and nuclease stability of oligonucleotides.

5.2 PRESENT OBJECTIVES

The objectives of this chapter are (1) the incorporation of chiral acyclic 2(R/S)- (N-thymin-1-ylacetyl)-amino-1(R/S)-aryl-1,3-propanediol unit (I) having different stereochemistries and substitutions into designed oligonucleotides at predetermined positions, (2) the study of the hybridization properties of these analogs with the complementary unmodified oligonucleotides, (3) examining the effect of exonuclease on these oligonucleotides, (4) to demonstrate the application of a fluorescent analog derived from the chiral acyclic unit for probing DNA complementation employing energy transfer between the two strands of the DNA duplex.
5.3 RESULTS

5.3.1 Synthesis and Characterization of ODNs Containing Chiral Acyclic Backbone

The phosphoramidites of \(2(R/S)\)- (N-thymin-1-ylacetyl)-amino-1\((R/S)\)-aryl-1,3-propanediol were synthesized as described in the previous chapter and incorporated at various desired sites in oligonucleotides (ODN) using solid phase (CPG resin) synthesis on an automated DNA synthesizer (see experimental, Chapter 2). The syntheses of unmodified ODNs were done using standard \(\beta\)-cyanoethyl phosphoramidite chemistry. The modified ODNs were synthesized by a slight modification in the standard procedure with the coupling time of the modified monomers increased to 15 min (instead of the normal 1 min), to ensure completion of the reaction. The coupling efficiencies of the modified amidites were similar (> 99%) to that of the normal amidites. After the completion of synthesis, ODNs were cleaved from the resin and all protecting groups were removed simultaneously by aqueous ammonia treatment to yield fully deprotected oligonucleotides. The modified ODNs were purified by polyacrylamide gel electrophoresis using 20% acrylamide. The purity of ODNs was ascertained by reverse phase HPLC (Appendix 5.10, p-186) and the retention of modification supported by MALDI-TOF mass spectral results (Appendix 5.10, p-187).

5.3.2 UV-Melting Studies

5.3.2a Duplex Melting Studies

The various oligonucleotide duplexes were constituted from the unmodified oligonucleotide 4 and the modified 5-8, using the common complementary unmodified 18-mer 3. The duplexes were annealed by mixing equimolar amount (1 \(\mu\)M) of the two appropriate strands, heating at 80 °C for 3 min, followed by slow cooling in sodium
cacodylate buffer. The stabilities of duplexes were measured by UV melting experiments, following the UV absorbance change at 260 nm in the temperature range 5-80 °C. Monophasic sigmoidal transitions are indicative of duplex formation. The $T_m$ values were confirmed by distinct peaks in the first derivative plots.

**Oligonucleotide Sequences:**

1  3'- A G G T T C T T C T T C T T C T T C T T T T A T A -5'
2  5'- T C C A A G A A G A A G A A A A G A A A A T A T -3'
3  3' - A A G A A G A A G A A A A A A A A - 5'
4  5' - T T C T T C T C T T C T C T T C T T T T - 3'
5  5' - T T C T T C T C T T C T C T C T C T C T C T T T T - 3'
6  5' - T T C T T C T C T T C T C T T C T C T C T C T C T T T T - 3'
7  5' - T T C T T C T C T T C T C T T C T C T T C T C T C T T T T - 3'
8  5' - T T C T T C T C T T C T C T C T C T C T C T C T T T T - 3'

$t$ = modified monomer (l) with different stereochemistry and substitutions.

- $1S, 2S, 1R, 2R, 1S, 2S (Ar = Ph)$
- $1R, 2R; 1S, 2R (Ar = p-NO_2-Ph)$
- $1R, 2R (Ar = p-NH_2-Ph)$

**Trivial Names** used in the Figures are as follows:

<table>
<thead>
<tr>
<th>Names</th>
<th>Corresponding Modified Sequence in Duplex or Triplex</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-3' or RR-3'</td>
<td>Sequence 5 with 1S, 2S or 1R, 2R modification (Ar= Ph) at 3'-end</td>
</tr>
<tr>
<td>SS-M</td>
<td>Sequence 6 with 1S, 2S modification (Ar= Ph) in the middle.</td>
</tr>
<tr>
<td>SS-5' or RR-5'</td>
<td>Sequence 7 with 1S, 2S or 1R, 2R modification (Ar= Ph) at 5'-end</td>
</tr>
<tr>
<td>SS-3t or RR-3t</td>
<td>Sequence 8 with three 1S, 2S or 1R, 2R modifications (Ar= Ph) at 3'-end</td>
</tr>
<tr>
<td>RR(NO_2)-3' or</td>
<td>Sequence 5 with 1R, 2R or 1S, 2R modification (Ar= p-NO_2-Ph) at 3'-end</td>
</tr>
<tr>
<td>SR(NO_2)-3'</td>
<td>respectively</td>
</tr>
<tr>
<td>RR(NO_2)-3t or</td>
<td>Sequence 8 with three 1R, 2R or 1S, 2R modifications (Ar= p-NO_2-Ph) at</td>
</tr>
<tr>
<td>SR(NO_2)-3t</td>
<td>3'-end respectively</td>
</tr>
<tr>
<td>RS-3'</td>
<td>Sequence 5 with 1R, 2S modification (Ar= Ph) at 3'-end</td>
</tr>
<tr>
<td>RR(NH_2)-3'</td>
<td>Sequence 5 with 1R, 2R modification (Ar= p-NH_2-Ph) at 3'-end</td>
</tr>
<tr>
<td>Entry</td>
<td>UV-T&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------</td>
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<td>10</td>
<td>3.4</td>
</tr>
<tr>
<td>11</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 1. UV-T<sub>m</sub> (°C) data for duplexes.
Table 1 summarizes the $T_m$ data obtained from UV-melting experiments of control (unmodified) and modified duplexes. The inclusion of a chiral acyclic moiety into the ODN backbone does not hinder the formation of duplexes. It is seen that incorporation of $1S, 2S$ isomer in single sites at 3'-end (Table 1, entry 2, Figure 1) or 5'-end (Table 1, entry 4, Figure 2) has no effect on duplex $T_m$ as compared to the control (Table 1, entry 1). Increasing the number of modifications to three at the 3'-end (Table 1, entry 5) slightly destabilized the duplex ($\Delta T_m = -3 ^\circ C$). In contrast, even a single modification at the center of the duplex (Table 1, entry 3) effected a large destabilization ($\Delta T_m = -9 ^\circ C$) (Figure 2). In case of ODNs containing $1R, 2R$ isomer, the duplex $T_m$ showed a similar trend i.e., the 5'-end modification was as good as control (Table 1, entry 7) (Figure 1). Three modifications at the 3'-end (Table 1, entry 6) caused slight destabilization ($\Delta T_m = -4 ^\circ C$), with an effective destabilization of about 1 °C per substitution.
Effect of substitution on phenyl ring: The effect of substitution on phenyl ring on the duplex stability was studied with ODNs containing p-nitro substitution on phenyl ring in 1R, 2R analog. A single modification at the 3'-end (Table 1, entry 8) showed $T_m$ negligibly differing from that of the control duplex and also three modifications at the 3'-end (Table 1, entry 9) effected a slight destabilization ($\Delta T_m = -5^\circ C$ or about 1.5 °C per substitution).

Effect of stereochemistry: The importance of the nature of the stereogenic center on duplex stability was studied using ODNs containing 1S, 2R isomer of the chiral acyclic analog. Surprisingly, change in stereochemistry seems to have no effect on the stability of duplex. Incorporation of one unit of 1S, 2R isomer at the 3'-end showed $T_m$ as good as that of control (Table 1, entry 10), whereas three modifications with this analog (Table 1, entry 11) showed slight destabilization ($\Delta T_m = 1.5 ^\circ C$ per substitution), similar to that observed with duplexes containing other analogs.
5.3.2b UV-Melting Studies for Triplexes

For studying the effect of chiral acyclic backbone in the third strand of triplex, sequences were designed to generate different combinations of triads involving modified monomer units at different positions in triplexes. Care was taken to avoid self-complementation and a few Cs were introduced into the third strand to break the continuous stretches of T and to prevent its slippage on the duplex. This was further ensured by addition of three base locks at both ends of the duplex. This system provided convenient $T_m$ ranges for studying all combinations of triads at different pHs. UV- $T_m$s were measured at two different pHs viz. pH 5.8 and pH 7.1, since triplex stabilities are sensitive to pH. Triplexes were individually constituted from the unmodified 24 mer duplex 2:1 and individual ODNs 4-8 as third strand. All the three strands were taken in equimolar amounts (1 μM each) in 100 mM sodium cacodylate buffer containing 20 mM MgCl$_2$ and 1 M NaCl at desired pH, either 5.8 or 7.1, heated at 80 °C for 3 min followed by slow cooling. The stability of triplexes was measured by UV melting experiments by following the UV absorbance change at 260 nm in the temperature range of 5-80 °C (Chapter 1, Section B). All the triplexes showed characteristic biphasic sigmoidal transitions. The transition in the lower temperature range corresponds to dissociation of the third strand and that in the higher temperature range arises from duplex denaturation. Accurate $T_m$ values were determined from first derivative curves.

Table 2 summarizes the $T_m$ data obtained for UV melting experiments of various chiral acyclic analogues designed to form triplexes.
Table 2. UV-\(T_m\) (°C) data of triplexes from common duplex 2:1.

<table>
<thead>
<tr>
<th>Entry</th>
<th>3rd strand</th>
<th>pH 5.8</th>
<th>pH 7.1</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2 3 4 5</td>
<td>6 7 8 9</td>
<td>10 11</td>
</tr>
<tr>
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<td>5 8 5</td>
</tr>
<tr>
<td>57</td>
<td>57 40 54 44</td>
<td>54 36 53 40</td>
<td>52.5 41</td>
</tr>
<tr>
<td>39</td>
<td>35 17 31 22</td>
<td>34 17 31 22</td>
<td>33 23 35 22</td>
</tr>
</tbody>
</table>

Parentheses indicate the substitution
At pH 5.8, comparing the results of triplexes containing 1R, 2R and 1S, 2S analogs (where Ar = Ph), it can be inferred that although both, 1R, 2R and 1S, 2S isomers showed triplex formation, 1R, 2R modifications were slightly less stable than 1S, 2S modifications (Figure 3). The stability of triplexes with modifications at different positions in a sequence showed an interesting trend. For either 1R, 2R isomer or 1S, 2S isomer, the most stable triplexes were observed when incorporation of the modified unit was at the 3'-end (Table 2, entries 2,6), followed by slightly lower stability for the 5'-end modification. (Table 2, entries 4,8). Increasing the number of modifications to three at the 3'-end also increased the destabilization (Table 2, entries 5,9) with an effective destabilization of almost 3-4 °C per substitution. The most detrimental effect was observed from modification in the center of the sequence (Table 2, entries 3,7). In general, the stabilization followed the order: control > 3' > 5' > 3'X > M (Figure 4).
The overall melting pattern was similar at pH 7.1. In case of the control as well as the modified third strand, the $T_m$s at pH 5.8 were higher by 18-23 °C compared to those at pH 7.1, as expected for C containing sequences. The differences in $T_m$s among 1S, 2S and 1R, 2R isomers are more at lower pH, 5.8 than that at neutral pH 7.1. The UV-melting profiles for triplexes with 1R, 2R and 1S, 2S isomers at various sites i.e., 3'-end, 5'-end or the center of the sequence are shown in Figures 5 and 6.

**Effect of substitution on the phenyl ring:** In order to study the effect of substitution on the phenyl ring, the p-nitro-substituted monomers were synthesized as mentioned in the previous chapter (chapter 4). The ODNs containing these were studied for their triplex formation. As seen from the results of Table 2, a single modification at the 3'-end (Table 2, entry 8) slightly destabilized the triplex ($\Delta T_m = -4.5$ °C), whereas three modifications at the 3'-end caused a destabilization of almost 5 °C per modification (Table 2, entry 9) (Figure 7).
Figure 5: (A) UV melting profiles for triplexes (at pH 7.1) with third strand containing 1R, 2R (Ar = Ph) isomer and (B) their respective first derivatives. RR-3' is 3'-end modification (5*2:1); RR-5' is 5'-end modification (7*2:1) and RR-M is middle modification (6*2:1).

Figure 6: (A) UV melting profiles for triplexes with third strand (at pH 7.1) containing 1S, 2S (Ar = Ph) isomer and (B) their respective first derivatives. SS-5' is 5'-end modification (7*2:1) and SS-M is modification in the middle (7*2:1).
Triplexes with p-aminophenyl substituted analogs showed similar $T_m$s (Table 2, entry 14) (Figure 8) as their p-nitrophenyl analogs. Thus, acyclic ODNs containing chloramphenicol backbone showed a remarkable tolerance for different substitutions. The amino substitution on the phenyl ring also renders them suitable for the attachment of various ligands at this position.

![Figure 7](image1.png)

Figure 7: (A) UV melting profiles for triplexes (at pH 5.8) containing 1R, 2R (Ar = Ph-NO$_2$) isomer and (B) their respective first derivatives. RR (NO$_2$)-3' is 3'-end modification (5*2:1) and RR (NO$_2$)-3t is three modifications at 3'-end (8*2:1).

![Figure 8](image2.png)

Figure 8: (A) UV melting profiles for triplexes 5*2:1 (at pH 5.8) and (B) their respective first derivatives. RS-3' is 3'-end modification with 1R, 2S (Ar = Ph) isomer and RR(NH$_2$)-3' is modification at 3'-end with 1R, 2R (Ar = Ph-NO$_2$) isomer.
Effect of stereochemistry: In order to study the sensitivity of \( T_m \) to stereochemistry for the importance of correct geometry in the backbone of the third strand, triplexes were constituted incorporating 1S, 2R and 1R, 2S isomer analogs. Surprisingly, the \( T_m \)s observed were almost indistinguishable from those of their parent counterparts.

Figure 9: UV melting profiles for triplexes 5'2:1 (at pH 5.8) containing 1S, 2R (Ar = Ph-NO$_2$) isomer. SR(NO$_2$)-3' is 3'-end modification and SR(NO$_2$)-3t is three modifications at 3'-end.

In case of 1S, 2R isomer (Ar = p-NO$_2$-Ph) a single modification at the 3'-end gives melting temperature identical to that at pH 5.8 as that of 1R, 2R isomer (Table 2, entry 12). Three substitutions with the same isomer at the 3'-end also show no change in the melting profile (Figure 9). The trend remains the same even at pH 7.1. (Table 2, entry 13). The other isomer 1R, 2S obtained by the inversion of at C1 stereocenter of 1S, 2S isomer for Ar = Ph also indicated no major changes in the binding properties in the triplex (Figure 8) at either pH 5.8 or 7.1 (Table 2, entry 15).

Effect of salt: The triplex stability is highly dependent on ionic strength. The salt concentration was varied over a range from 200 mM to 1 M and the \( T_m \)s were
Figure 10: Bar diagram shows salt [NaCl] dependence of triplex $T_m$ at different pHs.

measured at both pH 5.8 and pH 7.1. Figure 10 graphically represents the data obtained from the UV-$T_m$ of triplexes at various salt concentrations for both pH 5.8 and 7.1. The graph indicates a linear dependence of stability of triplexes on salt concentration from 200 mM to 1 M NaCl.

5.3.3 CD Spectroscopy

Monomers: Figure 11 shows CD spectra of monomers (4a, 4c, 4d, 4e, Chapter 4) taken in CHCl$_3$. The monomers with 1S, 2S stereochemistry (Figure 11a) showed a broad positive band at 270 nm, whereas the isomers with 1R, 2R (Figure 11d) stereochemistry showed a broad negative band at the same wavelength indicating an opposite conformation to that of 1S, 2S isomer. The inversion of 1S, 2S isomer at C1 center generating the 1R, 2S isomer also retained the conformation as that of the parent isomer, i.e. showing the broad positive band at 265 nm (Figure 11b). In a similar way, the spectral pattern of 1S, 2R showed a negative band at 285 nm (Figure 11a), retaining the conformation as that of 1R, 2R isomer.
Figures 11: CD spectra of monomers. (a) 1S, 2S (Ar = Ph); (b) 1R, 2S (Ar = Ph);  
(c) 1R, 2R (Ar = p-NO$_2$-Ph); (d) 1S, 2R (Ar = p-NO$_2$-Ph)

**Single strand oligonucleotides:** Figure 12 shows CD spectra of single stranded DNA containing different chiral acyclic analogs for the comparison with normal ODN. It can be seen from the figure that the basic spectral profiles of all the oligonucleotides are similar, irrespective of the position of modification (at 3'-end or in the middle), as well as the number of modifications (single or three modifications in an oligonucleotide).

**Triplexes:** Figure 13 shows the CD spectra of triplexes constituted from normal duplex (2:1) and modified third strands containing chiral acyclic backbone at different positions (3'-end or middle). These triplexes also showed an identical behavior (CD profiles). This includes a characteristic negative band at around 210 nm, along with a positive band at 275 nm and negative band around 250 nm.
Figure 12: CD spectra of oligonucleotides (a) Control, 4; (b) 3'-end modification 5; (c) Middle modification 6; (d) three modifications at 3'-end 8.

Figure 13: CD spectra of Triplexes. (a) Control (4*2:1); (b) 3'-end modification (5*2:1); (c) Middle modification (8*2:1)
5.3.4 Enzymatic Stability of the Oligomers

The stability against destructive nucleases is an important requirement for antisense ODNs. The stability of ODNs containing the acyclic nucleoside at different positions towards snake venom phosphodiesterase (SVPDE) was studied. The increase in absorbance at 260 nm (hyperchromicity) after addition of SVPDE was followed. The hyperchromicity arises as a consequence of the base stacking of oligonucleotides and consequent π-π orbital overlap, which is destroyed as the oligonucleotide is converted to its deoxynucleotide constituents during the enzymatic digestion. Figure 14 shows the time dependent hydrolysis of modified oligonucleotides 5 and 6 in comparison with that of the unmodified oligomer 4 (Oligonucleotide Sequences, Section 5.2). Since the hyperchromicity upon digestion of a polypyrimidine sequence is less compared to that of polypurines which have a better stacking, it was decided to study the enzymatic degradation of a mixed base sequence. For this purpose the 1R, 2R (Ar = p-NO2-Ph) isomer was incorporated into oligonucleotide 9.

<table>
<thead>
<tr>
<th></th>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>5' - T T C T T C T T C T T T T T T T C T T T T T T T T T - 3'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5' - T T C T T C T T C T T T T T T T C T T T C T T T - 3'</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5' - T T C T T C T T C T T T T T T T C T T T T T T T T - 3'</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5' - T T C T T C T T C T T T T T T T C T T T T T T T T T T T T T - 3'</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5' - G A A G G G G C T T T T G A A C T C T C T C T C T C T C T C T C - 3'</td>
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</tr>
<tr>
<td>10</td>
<td>5' - G A A G G G C T T T T G A A C T C T C T C T C T C T C T C T C T C - 3'</td>
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</tr>
</tbody>
</table>

The hyperchromicity change for the digestion of this oligonucleotide along with its control 10 was observed. The results of the enzymatic stability studies are tabulated in Table 3. From table 1, it can be deduced that the oligonucleotides with one acyclic analog substitution (1S, 2S or 1R, 2R), towards the 3'-end are stable to enzymatic
Table 3. Enzymatic Stability of Modified Oligonucleotides towards Snake Venom Phosphodiesterase (SVPDE)

<table>
<thead>
<tr>
<th>Oligonucleotide Sequence</th>
<th>Half-life (min)</th>
<th>% H*</th>
</tr>
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<tbody>
<tr>
<td>4</td>
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<td>11</td>
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<tr>
<td>6</td>
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<td>8*</td>
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<td>6</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>&gt;120</td>
<td>0</td>
</tr>
</tbody>
</table>

*Modified oligonucleotides containing isomers 1S, 2S; 1R, 2R; 1S, 2R (Ar = Ph); 1R, 2R, 2S (Ar = Ph); 1S, 2R, 2S (Ar = p-NO₂-Ph).

# %H = \frac{(A_{260 \text{ after degradation}}) - (A_{260 \text{ before addition of SVPDE}})}{A_{260 \text{ before addition of SVPDE}}} \times 100

Figure 14: Time course of Snake Venom Phosphodiesterase digestion of oligonucleotides. (a) Control 4; (b) Middle modification 6 (1S, 2S; Ar = Ph); (c) 3'-end modification 5 (1S, 2S; Ar = Ph); (d) Mixed sequence control 10; (e) Mixed sequence 9 with 1R, 2R (Ar = p-NO₂-Ph) modification at 3'-end.
degradation for more than two hours, whereas an unmodified oligonucleotide is completely digested in 8 minutes. An oligomer with an acyclic nucleoside in the middle shows a hyperchromicity of about 50% of that observed for the unmodified oligonucleotide. This observation most probably results from the sequential degradation of a 19mer oligonucleotide to the stage of a 10mer having 3'-terminal acyclic substitution, which stabilizes its 3'-end. These results can be represented graphically as shown in Figure 14. Thus, all different isomers of chiral acyclic nucleosides protected oligonucleosides against enzymatic degradation.

5.4 DISCUSSION

The UV- $T_m$ data obtained from the melting experiments suggest that the DNA duplex formation is not hampered by the insertion of chiral acyclic 2-amino-1-phenyl-1, 3-propanediol into the regular 2-deoxyribose phosphate backbone of DNA. Many of the acyclic oligonucleotides reported so far strongly decreased the stability of the complex formed between the modified oligonucleotides and natural DNA or RNA. The reason attributed to this destabilization is that the conformational freedom upon duplex formation from rigid oligonucleotides favorably overrules the entropy loss resulting from hybridization of ODN bearing flexible acyclic analogues with complementary unmodified oligonucleotides. This emphasized the need for introducing restricted conformational flexibility to a range that is near optimal for base-base interactions.

The incorporation of acyclic 2-amino-1-phenyl-1, 3-propanediol unit seems to serve the same purpose with the phenyl ring enforcing conformational rigidity on the otherwise, conformationally mobile acyclic backbone. Water plays an important role in the formation of hydrogen bonding network in the major and minor grooves of DNA duplex. Any hydrophobic substitution in this groove or the addition of salt displaces some of the water molecules to the bulk. This phenomenon of displacement of water molecule from the ordered hydration network to the disordered surroundings gives an
entropic advantage and thereby stabilizes the complex. The phenyl substitution in the described acyclic backbone may cause similar hydrophobic desolvation locally in the major groove.\textsuperscript{10}

The stability of complexes containing modified oligonucleotides showed stereochemistry independence for 1S, 2S and 1R, 2R incorporated units with similar positional dependence. The end modifications were much more stable than the modification in the center of the sequence. The deleterious effect by the central modification was much greater than multiple modifications at the end. The different substitutions on the phenyl ring were explored to engineer a better set of analogs. p-Nitro or amino substitution on phenyl ring caused negligible change in the stability of the complexes and hence, showed the acceptance of different substitutions in the backbone part of the duplex or triplex. This implies that the electronic effect caused by different substitutions in the backbone part is negligible whereas the steric effect caused by bulky phenyl substitution is major. This may be of potential use, as amino substitution can be used as a handle for attachment of useful linkers or fluorophores.

The study of sensitivity of $T_m$ to stereochemistry for the importance of correct geometry in the backbone of the third strand has an interesting outcome. The different stereoisomers of this chiral acyclic backbone were prepared to derive the stereochemistry most resembling that of the native 2'-deoxy nucleotides. Incorporation of 1R, 2S isomer (Ar = Ph) and 1S, 2R isomer (Ar = p-NO\textsubscript{2}-Ph) into oligonucleotides showed $T_m$s almost indistinguishable from those of their parent counterparts. This implies that the nature of the stereogenic center is relatively unimportant in the stability of these complexes provided the basic conformation is optimum for base-base interaction. More definitive studies are possible only by molecular modeling. The overall results suggest that the incorporation of different isomers does not change the basic conformation of ODNs. This was further confirmed by CD spectral studies. The
CD spectra of monomers and oligomers indicated that though the monomer spectral profiles of 1R, 2R and 1S, 2S are opposite to each other, when incorporated into oligonucleotides, the basic spectral structure is similar to that of the normal ODN. This result proves that the intramolecular base stacking of thymine in the acyclic backbone is the same as that in natural ODNs. The CD spectra of triplexes containing modified ODNs showed a pattern similar to that of the unmodified triplex, including a characteristic negative band at 210 nm. This further confirmed the overall similarity in spectral structure of both, modified and unmodified complexes.

The important requirement to be met by oligonucleotides as potential therapeutic agents in the antisense or antigene strategy, is their stability against degradation by nucleases present within the cell and in serum. The main nuclease activity present in fetal calf serum is 3'-exonuclease. Oligomers containing chiral acyclic nucleosides I, with different isomers at their 3'-end, show extremely high stability towards snake venom phosphodiesterase (SVPDE). From the described enzymatic digestion experiments, it could be concluded that SVPDE is not able to cleave the phosphodiester moiety between a natural nucleoside and acyclic nucleoside I. As a result, ODNs with the modification at the 3'-end did not show any hyperchromicity, whereas, an unmodified oligonucleotide is completely broken down within 8 min. An oligomer with an acyclic nucleoside in the middle showed a hyperchromicity of about 50 % of that observed for the unmodified oligonucleotide. This observation, most probably, results from the degradation of a 19 mer oligonucleotide to a 10 mer, which is stabilized by the presence of acyclic nucleoside at its 3'-end.

5.5 CONCLUSION

In conclusion, the present study showed that the incorporation of chiral acyclic nucleosides within the normal DNA backbone could still lead to formation of stable DNA duplexes and triplexes unlike many ODNs with other acyclic analogs. Different
substitutions on the phenyl ring did not hamper the stability of the complexes, thereby proving the versatility of this backbone. The insensitivity of \( T_m \) to stereochemistry at C1 indicates that the nature of this stereogenic center is relatively unimportant in the stability of these complexes. This observation suggests that it is the flexibility of these chiral acyclic analogs that permits more effective duplex and triplex formation, and not a specific conformation dictated by the presence of a single stereogenic site in the backbone of the acyclic analog. The most important fact is that the incorporation of this chiral acyclic nucleoside protected the oligonucleotide against enzymatic degradation.

The presently observed superior hybridization properties along with the exhibited enzymatic stability may be of potential use to design chimeric backbone based second generation antisense/antigene therapeutic agents. A novel class of nucleoside analogs (\( \beta \)-lactam nucleoside chimeras) have been recently reported as examples of potential dual action drugs. The presently used modification is a close substructure of active pharmacophore chloramphenicol and the hybrid molecules (monomers and oligomers) as designed here could be further examples of this class of molecules.

This is probably the first example of a chiral acyclic nucleoside analog (in third strand) stabilizing triplexes.
5.6 APPLICATION OF ACRYCLIC BACKBONE AS FLUORESCENT PROBE

Fluorescent labeling of nucleic acids has attracted wide attention for probing nucleic acid hybridization and DNA sequence identification, along with their use in DNA sequencing, nucleic acid-protein interactions and for applications in DNA diagnostics. The lower sensitivity of this technique compared to conventional labels can be overcome either by multiple labelling or signal amplification through PCR techniques.

The reported fluorescent labeling methods involve conjugation of fluorescent dye to the 5'3' terminus of oligonucleotides, phosphate backbone, sugar or nucleoside bases using spacer arms.

Solution-based fluorescence methods usually require a strategy for engineering a change in spectral properties when the DNA target of interest is detected, relative to other sequences, which may be present in the sample. For example, useful changes can result from either quenching or change in emission wavelength of fluorescence, which is triggered by hybridization. It is often advantageous in solution based sequence probing to utilize a change in fluorescence emission wavelength as a consequence of hybridization, rather than intensity alone. The appearance of a new emission band upon hybridization is a definitive signature for the presence of a specific sequence in the target DNA. The ratios of intensities at two wavelengths can also be used for this purpose and interference from background fluorescence of unbound probes and other fluorescent species in a given sample can be avoided. Use of two probes for the detection of target provides an added advantage since a single mismatch can alter their binding properties and thereby increase the specificity for target.
Pyrene is one such well-characterized excimer forming fluorophore, which has been incorporated into synthetic oligonucleotides by several research groups. A few of them have exploited the excimer formation by adjacent pyrenes as a color-changing reporter of the presence of specific sequences in solution. Pyrene has the longest fluorescence lifetime among the common fluorophores, making it useful for fluorescence depolarization studies.

Recent studies have made use of excimer formation by adjacent pyrenes as a color-changing reporter of the presence of specific sequences in solution. Pyrene labeled oligonucleotides have been used by Rippe et al to establish the existence of parallel stranded duplexes, revealed by means of the pyrene excimer. When two pyrene molecules are in close proximity, the fluorescence spectrum exhibits a characteristic excimer band, red-shifted compared to the pyrene monomer emission. Mohammedi et al extended this concept to study triple helix formation and homologous strand exchange in pyrene labeled oligonucleotides. Paris et al reported a new chemical strategy for the incorporation of single or multiple pyrene fluorophores into DNA oligonucleotides, where a pyrene derivative (II) replaces a standard nucleoside phosphoramidite. When this new fluorescent nucleoside analog was employed in dual oligonucleotide probes with optimal spacing of the hybridization probes, adjacent binding resulted in the appearance of efficient excimer emission, coupled with a large drop in monomer emission intensity.

5.6.1 Synthesis and Incorporation of Fluorescent Monomer

In our quest for generating fluorescent probes, we thought of extending the utility of the acyclic backbone for this purpose by incorporating a chiral acyclic fluorescent unit (III) in the oligonucleotide. It has been shown in the previous
section that the achiral chloramphenicol backbone unit is ideally suited as it has little or no effect on the duplex/triplex structures when incorporated either at 3' or 5' terminus of the oligonucleotides. The fluorescent monomer amidite containing chiral acyclic backbone was synthesized as described in Scheme 1. The pyrene acetyl group was attached to 2-amino group of 2(S)-amino-1(S)-phenyl-1,3-propanediol unit 11 through amide linkage. The reaction was carried out using standard condition of peptide bond formation between amine 11 and pyrene acetic acid using diisopropylcarbodiimide (DIPCDI) as a coupling agent, and hydroxybenzotriazole (HOBt) as an activator to get the diol 12. The primary hydroxyl was then protected with DMTr group to give 13 and
the secondary hydroxyl in 13 was converted to the β-cyanoethylphosphoramidite 14, that gave the expected \(^{31}\)P NMR signals (150.0 & 149.7 ppm) characteristic of O-amidites. The amide linkage in pyrenylacetyl was found quite stable to final deprotection conditions of oligonucleotides. The unit 14 is suitable for automated incorporation into an oligonucleotide sequence at any position during DNA synthesis. Because of the placement of the fluorophore in a position analogous to a DNA base, it is well situated to form excimer with other adjacent pyrene residues.

5.6.2 Sequence Design and Synthesis of 2(S)-N-(pyrene-1ylacetyl)amino-1(S)-phenyl-1,3-propanediol Incorporated Oligonucleotides

Since pyrene excimer formation depends strongly on a well-defined geometry of fluorophore interaction,\(^{35}\) experiments were aimed at investigating single and double probe hybridization. A pair of singly labeled pyrene containing sequences P1 and P2 were synthesized along with the complementary sequence P3 that contains two consecutive pyrene labels. The sequences P1 and P2, when used as a dual probe,

**Oligonucleotide Sequences.**

| T   | 3' - C T T C C G A A A A C T T G A G A - 5' |
| P1  | 5' - G A A G G G C T T P G - 3' |
| P2  | 5' - P T T G A A C T C T - 3' |
| P3  | 5' - G A A G G G C T P P T G A A C T C T - 3' |

P is pyrene monomer

\[\text{(a)}\] 
\[\text{(b)}\] 
\[\text{(c)}\] 
\[\text{(d)}\]

were expected to show an excimer band upon hybridization with the target sequence T. The oligonucleotides T, P1, P2 and P3 were synthesized on a Pharmacia GA plus DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry (Experimental, Section A), but with extended (15 min) coupling times for the pyrene residue 14. A pyrene phosphoramidite concentration of 0.15 M in acetonitrile was used, and shown not to compromise with the normal coupling efficiency.

5.6.3 Fluorescence Studies of Oligonucleotides and Duplexes

Figure 15 shows the fluorescence spectra of oligonucleotides P1, P2 and P3. As seen from the spectra, the monolabeled oligonucleotides P1 and P2 showed two major bands at 385 and 405 nm. No other structureless bands in the fluorescence spectrum were observed. The oligonucleotide P3 containing double pyrenyl residues, in addition to the 385 and 405 nm emission bands, similar to that seen with oligomers P1 and P2, exhibited a significant, predominantly broad band at a longer wavelength around 480 nm. The fluorescence excitation spectra of P3 corresponding to bands at

Figure 15: Fluorescence emission spectra of single strand oligonucleotides P1, P2 and P3 (1μM each) upon excitation at 348 nm.
385 and 405 nm were identical, thus indicating that these bands are ascribable to the pyrene monomer fluorescence and the 480 nm band for intramolecular excimer emission respectively. The absence of excimer band in P1 and P2 suggests no aggregation of single stranded forms in solution due to the presence of hydrophobic pyrene units.

![Figure 16: Fluorescence emission spectra of (A) duplexes containing single pyrene labeled probes P1, P2 and dual probe P1+ P2 with target T and (B) doubly labeled probe P3 before and after hybridization with target T.](image)

Upon hybridization to the complementary target strand T, a significant increase in the pyrene monomer fluorescence of P1 and P2 was observed (Figure 16A) without appearance of any other new bands. The oligonucleotide containing two pyrene residues P3, upon complexing with T, also showed a similar effect of enhancement in its monomer emission bands at 385 and 405 nm, and the intensity of the excimer band at 480 nm was decreased (Figure 16b). However, hybridization of target strand, T, with both probes together P1 and P2, not only resulted in an enormous increase in the
monomer emission bands (385 nm and 405 nm), but also showed the appearance of a new broad band at 480 nm, ascribable to intermolecular excimer emission.

The intensity of the excimer band at 480 nm in dual probe hybridization (P1+P2+T) is ~5 times more compared to the intensity at 480 nm in P1 and P2 in the absence of the target strand. This strongly supports the detection of target sequence due to specific simultaneous binding of P1 and P2 with the target strand T. The pyrene probes in P1 and P2 are now somewhat favorably oriented in the complex P1+P2+T, leading to a weak, but detectable excimer band at 480 nm, which is practically absent in the ODN mixtures P1+T, P2+T or P1+P2. Conversely, the doubly labeled single strand probe, P3 alone, shows a strong excimer band at 480 nm, the intensity of which is reduced by 70%, in the presence of the target strand (P3+T). This could be a consequence of either a disturbance in the duplex of favored geometry of pyrenes for excimer formation seen in single strand P3 or an inherent quenching of fluorescence upon duplex formation. The use of dual probe P1+P2, should therefore be more effective in the detection of even a single mismatch in target, as compared to the doubly labeled probe P3, in which the two bases in the target facing adjacent pyrenes are as such mismatches.

5.7 ENERGY TRANSFER IN FLUORESCENT DNA DUPLEX

The present section describes an approach by which two complementary fluorescent oligonucleotides involved in the process of non-radiative fluorescence resonance energy transfer (FRET) can be used to study nucleic acid hybridization in solution. FRET is a consequence of hybridization of two nucleic acid strands carrying appropriate donor and acceptor fluorophores and effects a distinct signal in response to the cause. The implications of this for analytical and diagnostic methods for detecting hybridization events in vivo are obvious.¹⁴a
Principle: Fluorescence Resonance Energy Transfer (FRET) with overlapping emission and excitation spectra is a dipole-dipole resonance interaction between two geometrically close fluorescent chromophores, where one of the chromophores called the "donor" transfers its excitation energy to the other chromophore, called the "acceptor." 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. As FRET is dependent on distance and orientation of chromophores, rigid locations of the donor and acceptor groups on oligonucleotides are essential to provide meaningful structural information (for DNA, RNA and protein-nucleic acid complexes).

We have previously employed the fluorescent analog, 2-aminopurine for the molecular recognition of 5-amino dU using its hydrogen bonding capacities. The fluorescence observables of 2-aminopurine (λ_{ex} 314 nm, λ_{em} 358 nm) seems to satisfy the FRET requirement for resonance pairing with pyrene (λ_{ex} 348 nm, λ_{em} 385, 405 nm) (Figure 17, Table 4). The unknown application of the fluorophores 2-aminopurine (donor) and pyrene (acceptor) as a suitable donor and acceptor pair in FRET was therefore explored. Complementary oligonucleotides containing this pair may form a duplex and thereby generate a FRET signal. The synthesis of 2-aminopurine (AP) oligonucleotides has been described in Chapter 2, while the pyrene containing oligonucleotide was synthesized as described in the previous part of this section.

15 (D) 5' - T T C T T T X T T C T T C T - 3'
16 (A) 3' - A A G A A A A T A A P P A G A - 5'
X is 2-aminopurine unit and P is pyrene unit

The pyrenylacetyl phosphoramidite (14) was incorporated into oligonucleotide 16, which is a complementary sequence for oligonucleotide containing AP 15. The
oligonucleotides were designed in such a way that constitution of duplex from 15:16 permitted generation of double strands in which the donor/acceptor fluorophores were separated by 3-4 base pairs.

5.7.1 Spectral Properties of Donor and Acceptor

![Figure 17: Spectral overlap in fluorescence spectra of 2-aminopurene and pyrene; (a) emission spectrum of 2-aminopurine (donor), (b) excitation spectrum of pyrene (acceptor) and (c) emission spectrum of pyrene (acceptor).](image)

**Table 4: Spectral Properties of AP (15) and pyrene (16) oligonucleotides**

<table>
<thead>
<tr>
<th>Label</th>
<th>$\lambda_{ex}$ (nm)</th>
<th>$\lambda_{em}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP (15)</td>
<td>314</td>
<td>358</td>
</tr>
<tr>
<td>Pyrene (16)</td>
<td>348</td>
<td>385, 405 (monomer emission)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>470 (excimer emission)</td>
</tr>
</tbody>
</table>
Figure 17 and Table 4 show the fluorescence excitation and emission spectra of the individual single strands labeled with either AP (donor) or pyrene (acceptor). As seen from these, spectral characteristics and frequency overlap of AP and pyrene pair are highly favorable for FRET studies. The maximum excitation wavelength for AP is 314 nm, whereas its maximum emission wavelength is 358 nm. The maximum excitation wavelength for pyrene is 348 nm, while its maximum emission wavelength is 385 and 405 nm for monomer emission and 470 nm for excimer emission. Significantly, the emission spectra of AP ODNs (donor) has considerable overlap with the excitation spectra of pyrene ODNs (acceptor) (Shaded area, Figure 17). Thus, if the AP and pyrene fluorophores are in proximity, excitation of AP may lead to a non-radiative transfer of energy from AP to pyrene, resulting in emission by pyrene. The characteristic excimer peak (470 nm) shown by the acceptor allows effective monitoring of energy transfer.

5.7.2 Duplex Formation Monitored by Fluorescence Energy Transfer between Donor and Acceptor Chromophores on Complementary DNA Strands

To detect the fluorescence energy transfer between AP and pyrene chromophores attached to separate strands of oligonucleotides, the emission spectra of acceptor upon excitation of donor (AP at 314 nm) were monitored as a function of increasing acceptor concentration at constant concentration of donor.

Figure 18 shows the emission spectra obtained upon incremental stoichiometric addition of pyrene conjugated oligonucleotide (16) to a constant amount of AP oligonucleotide (15). Spectrum 1 is the emission spectrum of AP (donor, 1 μM) oligonucleotide in the absence of acceptor, whereas, spectra 2-5 are the emission spectra upon incremental addition (0.3 μM per addition) of pyrene (acceptor) labeled oligonucleotide (excitation at 314 nm). The comparison of spectra 1-5 shows a
Figure 18: Modulation of fluorescence intensity upon duplex 15:16 formation at a constant amount of AP oligonucleotide 15 (1 μM, Spectrum 1), with increasing concentration of pyrene oligonucleotide 16 (0.3 μM/addition, Spectra 2-5)

continuous decrease in the emission intensity of AP (358 nm), accompanied by a corresponding increase in the sensitized pyrene monomer emission (385 & 405 nm) as well as the excimer emission band (470 nm). This enhancement of acceptor emission at the expense of donor’s emission is characteristic of FRET. It is noticed from the Figure 18 that all the spectra cross through an “isoblistic” point (analogous to isobestic point in UV titration) at 365 nm, which arises due to simultaneous decrease in emission of AP and a rise in pyrene emission. This synchronous effect is clearly a consequence of hybridization and demonstrates that AP and pyrene are a successful donor/acceptor pair for FRET experiments. More quantitative studies are necessary to get accurate structural details of optimum distance requirement on efficient energy transfer by this
method. Since 2-aminopurene is an intrinsically fluorescent base, rigidly locked in base-pairing with complementary base, the energy transfer experiments may be more efficient compared to other pairs in nucleic acid hybridization which comprise donor/acceptor both covalently linked through flexible spacers (non-rigid) or non-covalent intercalators as donor/acceptor components.38

In conclusion, the overall results presented in this section successfully demonstrate the useful applications of fluorescent probe containing chiral acyclic backbone. Future potential applications of such fluorescent DNA probes include studying structural polymorphism in DNA, DNA-peptide interactions and investigation of triple helix formation by using fluorescent DNA as the Hoogsteen strand.
5.8 EXPERIMENTAL

5.8.1 Oligonucleotide Synthesis

N-protected standard nucleoside phosphoramidites (A, T, G and C) and nucleoside derivatized controlled pore glass supports were purchased from Cruachem, UK. The DNA synthesis was carried out on Pharmacia LKB-Gene Assembler Plus. Dry solvents were used for DNA synthesis. The commercially available amidites (0.1 M) were dissolved in dry acetonitrile, while 0.15 M solutions were prepared for modified amidites and 4A molecular sieves were added to it to remove traces of moisture. Acetonitrile was distilled twice over P₂O₅ and finally over CaH₂ immediately before use. Dichloroethane was dried by distilling twice over P₂O₅. For oxidation, after each coupling, 0.01 M iodine in collidine, water and acetonitrile, while for capping 20% acetic anhydride in acetonitrile was used. The solid phase synthesis protocol is summarized in chapter 2. The modified amidites were incorporated at desired sites of ODN by using automated Pharmacia GA Plus DNA synthesizer with extended coupling time (15 min). Modified phosphoramidite concentration of 0.15 M in acetonitrile was used, and shown not to compromise coupling efficiency.

5.8.2 Oligonucleotide Purification

The crude oligonucleotides were cleaved from support, deprotected using aqueous ammonia at 55 °C for 16 h and desalted using NAP-10 gel filtration columns. The desalted oligonucleotides were purified by gel electrophoresis under denaturing conditions. The oligonucleotides (10 O.D.) were dissolved in 200 µl of 95 % formamide, heated at 80 °C for 5 min and loaded on 20% polyacrylamide gel containing 7 M urea. The gels were run at constant voltage (200 V) till the dye reached the bottom of gel. Modified and unmodified oligonucleotides were detected by UV shadowing. The gel slices containing full-length oligonucleotides were cut and crushed in a dry tube. To
this, 5 ml of sterile water was added and the tubes were kept at 50 °C overnight. The solution was filtered through Whatman paper to remove polyacrylamide particles. The filtrate was concentrated, dissolved in minimum amount of water (~500 μl) and purified twice over NAP-10 gel filtration column (Pharmacia) to remove dissolved acrylamide and urea. The purity of oligonucleotide was rechecked by reverse phase HPLC using the buffer systems A: 5 % CH$_3$CN in 0.1 M triethylammoniumacetate pH 7.0 (TEAA) and B: 30 % CH$_3$CN in 0.1 M TEAA using a gradient A to B of 1.5 %/min at a flow rate of 1.5 ml/min. The absorption spectrum of each peak was scanned in the range of 200-600 nm using diode array detector.

5.8.3 UV Melting Experiments

Duplex and triplex melting experiments were carried out in the buffer 100 mM sodium cacodylate containing 20 mM MgCl$_2$ and 1 M NaCl at pH mentioned in each case. Appropriate oligonucleotides, each at a strand concentration of 1 μM based on UV absorbance at 260 nm calculated using molar extinction coefficients of dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 cm$^2$/μmol were mixed and heated at 80 °C for 3 min, cooled to room temperature followed by overnight storage at 4 °C. The $A_{260}$ at various temperatures were recorded using Perkin Elmer lambda 15 UV/VIS spectrophotometer, fitted with a water jacketed 5-cell holder and a Julabo temperature programmer with a heating rate of 0.5 °C/min over a range of 5-80 °C. Dry nitrogen gas was flushed in the spectrophotometer chamber to prevent moisture condensation at temperatures below 15 °C. The melting temperature $T_m$ were determined from the midpoint of the transition in the plots of fraction absorbance change versus temperature and were further confirmed by differential ($dA/dT$ vs T) curves. The $T_m$ values are accurate to ±0.5 °C over the reported values and are the average of three sets of experiments.
5.8.4 Circular Dichroic Spectral Studies

Circular Dichroism spectra were recorded on JASCO J-715 spectropolarimeter attached to Julabo water circulator for maintaining temperatures. The samples were scanned in the range of 320-200 nm at scan speed of 200 nm, band width 1.0 nm, sensitivity 10 mdeg, resolution 0.1 nm and response factor 2 sec. Each spectrum was taken as an average of 5 scans using 10 mm cell. The samples were made in the similar manner as that for UV melting experiments by taking 1 μM of each appropriate strands. The cell was thermostated using Julabo water circulator at 10 °C for all measurements.

5.8.5 Enzymatic Stability of Oligonucleotides

A solution of the oligonucleotides (0.2-0.3 OD) in 2.0 ml of the pH 8.6 buffer (0.1 M Tris. HCl; 0.1 M NaCl; 14 mM MgCl₂) was digested with 1.2 U snake venom phosphodiesterase (Sigma) (34 ml of a solution of the enzyme in the following buffer: 5 mM Tris. HCl; pH 7.5; 50 % glycerol (v/v)) at 25 °C. During digestion the increase in UV absorbance at 260 nm was followed. The absorption versus time curve of the digestion was plotted from which the hyperchromicity and half-life of the oligomer were evaluated.

5.8.6 Synthesis of Pyrene Phosphoramidite 14

2(S)-N-(pyren-1-ylacetyl)-amino-1(S)-phenyl-1,3-propanediol 12

To a solution of 2(S)-amino-1(S)-phenyl-1,3-propanediol 11 (0.5 g, 3 mmol) in dry DMF (5 ml), HOBt (0.4 g, 3 mmol) and DIPCDI (0.57 ml, 3.6 mmol) were added. The reaction mixture was stirred at room temperature for 10 min. Pyrene acetic acid (0.78 g, 3 mmol) was added to the reaction mixture and stirring was continued for 10 h at room temperature. After the completion of the reaction, the solvent was removed.
The residue was dissolved in CH$_2$Cl$_2$ and purified by column chromatography to get the product 12 (1.1 g, yield 89%).

$^1$H NMR (200 MHz, DMSO-$d_6$) δ 7.7-8.3 (m, 9H, pyrene), 7.1 (b, 5H, Ph), 5.05 (d, 1H, J= 4.7 Hz, C1-H), 4.8, 4.5 (2s, 2H, Py-CH$_2$), 4.1 (m, 1H, C2-H), 3.5 (bs, 2H, C3-H).

3-O-4,4'-dimethoxytrityl-2(S)-N-(pyren-1-ylacetyl)-amino-1(S)-phenyl-1,3-propanediol 13

Compound 12 (1 g, 2.44 mmol) was coevaporated with dry pyridine (5 ml x 3) and redissolved in dry pyridine (10 ml). To this, 4, 4'-dimethoxytrityl chloride (1 g, 2.9 mmol) was added. The reaction mixture was stirred for 4 h at room temperature. Upon completion of the reaction, pyridine was removed completely. The pure product 13 was obtained after column chromatography (1.5 g, yield 88%).

$^1$H NMR (200 MHz, CDCl$_3$) δ 7.5-8.3 (m, 9H, pyrene), 6.6-7.2 (m, 18H, DMTr+Ph), 5.3 (s, 1H, C1-H), 4.7 (d, 2H, J = 5.4 Hz, Py-CH$_2$), 4.2 (m, 1H, C2-H), 3.7 (s, 6H, 2 x OCH$_3$), 3.4 (ddd, 2H, C3-H).

HRMS (FAB+) for C$_{46}$H$_{44}$N$_2$O$_5$: (Appendix 5.10, p-189)
  Calc.; 711.29
  Found: (M+H), 711.0; (M+Na), 734.0

3-O-4,4'-dimethoxytrityl-2(S)-N-(pyren-1-ylacetyl)-amino-1(S)-phenyl-1,3-propanediol-1-O-phosphoramidite 14

A mixture of compound 13 (0.3 g, 0.42 mmol) and tetrazole (0.03 g, 0.5 mmol) was coevaporated with dry CH$_3$CN (3 ml x 2) and suspended in dry CH$_2$Cl$_2$ (3 ml). β-cyanoethyl-N,N,N',N'-tetraisopropylphosphine (0.16 ml, 1.2 eq) was added. The reaction mixture was stirred under nitrogen atmosphere for 2 h. After the completion of the reaction, the reaction mixture was diluted with CH$_2$Cl$_2$ and washed with aqueous
NaHCO₃. The organic layer was dried over anhydrous Na₂SO₄ and concentrated. The pure product 14 was obtained by precipitation in cold hexane (0.34 g, yield 88 %).

³¹P (81 MHz, CDCl₃) δ148.9, 148.2

5.8.7 Fluorescence Spectroscopy

Fluorescence measurements were performed on a Perkin-Elmer Model LS-50B spectrofluorimeter. Oligonucleotide concentrations were determined from the absorbance at 260 nm and using molar extinction coefficients for dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 cm²/µmol at 260 nm. A correction factor for the contribution of pyrene at 260 nm was taken to be 0.5 x the absorbance at 348 nm. An excitation wavelength of 348 nm for pyrene oligonucleotides, and 314 nm for AP oligonucleotide was used. All excitation and emission slits were set to 5 nm. Fluorescence measurements were taken in the right angle mode. Unless otherwise mentioned each oligomer was present at a concentration of 1 µM in 10 mM sodium cacodylate buffer containing 10 mM MgCl₂ and 100 mM NaCl at pH 7.0. The temperature of the sample compartment of the spectrofluorimeter was maintained using Julabo water circulator at 15 °C for all experiments.
5.9 REFERENCES


Pracownia Spektrometrii Mas CBM iM PAN

Method: L_Oligo
Mode: Linear
Accelerating Voltage: 20000
Glow Voltage: 92.000 %
Guide Wire Voltage: 0 150 %
Delay: 150 ON
Sample: 10

Laser: 2925
Scans Averaged: 256
Pressure: 6.00e-07
Low Mass Gate: 740.0
Timed Ion Selector: 19.8 OFF
Negative ions: ON
Collected: 21/3/99 10:58 AM

Savitsky-Golay Order = 2 Points = 5

C_{181}H_{217}N_{41}O_{122}P_{18}
M_{calc}: 5445.47
M_{obs}: 5444.0

Counts

3000 3500 4000 4500 5000 5500 6000 6500 7000 7500 8000 8500 9000
Mass (m/z)

Client: A. Okruszek

Comment: VR-SS-2, THA/AC

Original Filename: c:\voyager\data1999_03\uh73_01.ms
This File # 5: C:\VOYAGER\DATA1999_03\SMOOTH.MS