CHAPTER 3 / SECTION-A

Design, synthesis and biophysical evaluation of Open chain analogues of cyclohexenyl nucleic acids
Section 3A: Design, synthesis and biophysical evaluation of Open chain analogues of cyclohexenyl nucleic acids

3A.1 Introduction

Nucleic acids store the hereditary information passed from one generation to the next. Chemically modified nucleic acid analogues are required to perform functions in DNA therapeutics. Artificial nucleotide analogues have been widely explored to modulate the properties of nucleic acids, making them promising therapeutic agents and important building blocks for constructing molecular devices. Researchers have investigated various artificial nucleic acid systems containing non-ribose sugars as well as acyclic scaffolds. The acyclic nucleic acids grabbed the attention of chemists and biologists alike for nearly three decades because of their structural simplicity. Early work in the field of nucleoside mimetics led to the discovery that certain acyclic nucleosides such as acyclovir to possess significant therapeutic activity. The acyclic nucleic acids such as FNA, UNA, GNA and isoGNA (Figure 1a) have also been examined as progenitor candidates to RNA in the early development of nucleic acids. FNA is an open chain analogue of DNA in which the 2'-carbon of ribose ring system was absent. Wengel designed unlocked RNA (Figure 1a, UNA) system by removing 2'-3' C-C bond in RNA. GNA and FNA are the very interesting acyclic nucleoside candidates, as these molecules are structurally much simpler than natural DNA or RNA. Indeed, both acyclic nucleosides can be obtained under primitive reaction conditions using prebiotically plausible molecules. Wengel also studied the glycerol-based nucleic acids (Figure 1a, GNA) which would be less flexible than FNA or UNA due to less number of flexible bonds in the monomer unit.

Figure 1a Natural DNA/RNA and acyclic analogues
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All these acyclic structures were considered to be the plausible precursors of DNA/RNA, largely destabilized DNA /RNA duplexes. It was inferred that these acyclic structures would incur large entropic loss while duplex formation which was responsible for the incapability of acyclic nucleic acids to form stable cross-paired duplex structures with cDNA.\(^5,6\)

The incorporation of prochiral acyclic nucleosides into oligonucleotides causes the central C-atom of the monomer to become stereogenic, with each monomer adopting either a d-like or l-like orientation in the chain. However, the sugar backbone might, in the case of FNA or related acyclonucleotides, be sufficiently flexible to allow racemic mixtures of d- and l-like monomers to adopt similar conformations such that replication could take place without being affected by the absolute configuration of the stereogenic center. Later, homooligomeric chiral R-GNA and S-GNA were synthesized by Meggers which could form highly stable antiparallel helical duplex structures.\(^7\) The optically pure (S)-GNA could also cross-pair with RNA though with much reduced stability and was proposed to be a potential precursor of RNA as genetic material. This means that the reduced flexibility in GNA as compared to FNA could lead to stable duplex structures when nucleobase attachment is kept flexible through methylene group. As a class of promising RNA progenitors, acyclic nucleic acid systems have been extensively investigated, and experimental evidence has strengthen the hypothesis that structurally simplified analogs might have played a transitional role, which eventually led to the emergence of catalytic RNA molecules.

3A.2 Design of Open chain analogues of Cyclohexenyl nucleic acids and rationale

The evolutionary chemistry with respect to nucleic acids suggested that simple acyclic nucleic acids might be preliminary nucleic acids\(^10\) which ultimately have evolved as present day carriers of genetic information. To counter the entropic loss in acyclic nucleic acids, an attempt was made by introducing a double bond in the acyclic structure. Incorporation of these thymidine nucleosides mimics (Figure 1b, \(t^9\) and \(t^9\)) in oligomers was also found to be detrimental to the duplex stability similar to the other acyclic derivatives.\(^11\) We presume that the attachment of nucleobase directly to the double bond in this case may have conferred considerable rigidity, leading to
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reduced ability of the nucleobase to take part in specific W-C hydrogen bonding. The isoGNA (Figure 1b, isoGNA) later studied by Krishnamurthy et al also destabilized duplexes, probably as the nucleobase attachment was directly to the backbone.⁸

![isoGNA](image)

**Figure 1b** GNA and acyclic analogues

These modifications prompted us to visualize an open chain ene-nucleic acids (Figure 2a) in which the nucleobase attachment is to a planar double bonded structure through a methylene group, having same number of atoms in the backbone like natural sugar. This would also have a constraint of double bond unsaturation and act as an acyclic version of cyclohexene nucleic acid.

![CeNA](image)

**Figure 2a** CeNA and Proposed cis- and trans- open chain analogues of CeNA

The proposed isoprenoid 5-carbon unit could be envisaged to be a part of evolutionary pre-biotic soup containing dihydroxy acetone and acetaldehyde (Figure 2b) similar to acrolein from formaldehyde and acetaldehyde.¹² At this stage, although the specific stereochemistry of prochiral glycerol or other D-sugars may not be explained chemically in the prebiotic world, the possibility of favored cis or trans geometry of the proposed ene-nucleotides and directionality thus imparted may not be ruled out.
3A.3 Synthesis of cis and trans thymine monomers

The synthesis started with the mono TBS protection of commercially available dihydroxyacetone\(^3\) to furnish compound 2. Compound 2 subjected to DMTrCl in dry pyridine to result 3 and further reacted with ethylbromoacetate, PPh\(_3\) under wittig reaction conditions yielded the geometrical isomeric products mixture of trans (4a) and cis (4b) \(\alpha,\beta\)-unsaturated esters in more than 90% yield in 6:4 ratio. At this stage the two compounds trans (4a) and cis (4b) could be separated with very careful column chromatography and identified by nOe experiment. The DMTrO- group is considered to be corresponding to 5'-position and compound with nucleobase on the side of 5'-position is considered as cis isomer (scheme 1).

Scheme 1 Synthesis of key intermediates 7a, 7b
Reagents and conditions (i) TBDMSCl, imidazole, dry DMF (ii) DMTrCl, dry pyridine, overnight (iii) ethylbromoacetate, PPh₃, Toluene, reflux, 5h (iv) DIBAL-H, dry DCM, -78 °C (v) T-Bz, PPh₃, DIAD, dry dioxane, overnight (vi) TBAF in 1M THF, THF, rt, 2h.

Compounds 4a, 4b were treated with DIBAL-H at -78 °C in dry DCM individually to obtain the corresponding allylic alcohols 5a, 5b. Compound 5a was subjected for Mitsunobu reaction condition, hydroxy group was substituted with benzoyl protected thymine (T°C) nucleobase 6 which was further treated with TBAF yielded 7a. In case of 5b Mitsunobu product was contaminated with triphenylphosphineoxide. Due to the contamination without purification reaction mixture subjected for silyl group deprotection using TBAF in THF yielded 7b in 57 %. To obtain the trans and cis thymine monomers, compounds 7a, 7b subjected for ammonia treatment to remove the N³-benzoyl group of thymine nucleobase followed by phosphitylation of free hydroxyl group in 8a, 8b delivered the required amidite monomers 9a, 9b respectively, which could be incorporated into modified oligonucleotides using a DNA synthesizer (scheme 2).

Scheme 2 Synthesis of trans and cis phosphoramidite monomers

Reagents and conditions (i) 30% aq ammonia solution, dioxane, rt, 4h (ii) 2-cyanoethyl-N,N-diisopropylchlorophosphine, DIPEA, dry DCM.

3A.4 Solid phase synthesis of oligonucleotide using phosphoramidite chemistry

Oligonucleotide synthesis can be carried out with two types of solid supports. One is the standard support with preloaded nucleosidic unit, which becomes the 3’-terminal residue, as the synthesis is done in 3’—5’ direction. To use standard supports one requires four different supports for DNA synthesis carrying the individual canonical nucleobases. The other type is the universal solid support, which...
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carries an abasic sugar unit rather than the 3’-nucleoside unit. The advantage of using
the universal support the possibility to synthesize 3’-end modified nucleic acids
which is not possible using conventional standard supports. The natural DNA was
synthesized in the 3’ to 5’ direction, on a polystyrene solid support with the required
end nucleoside (A, T, C, G) attached to it via a linker. The oligonucleotides were
cleaved from solid support by ammonia treatment, their purity ascertained by RP-
HPLC on a C18 column, which further used for biophysical studies (scheme 3).

Scheme 3 The sequence of chemical reactions involved in the solid phase synthesis of
oligonucleotide

Reagents and conditions: (i) 3% TCA in DCM (ii) 0.25 M 5-(S-ethyhetrazole) in ACN (iii)
Ac$_2$O/Py (iv) 10% N-methylimidazole in THF 0.1 M (v) I$_2$/Py/H$_2$O/THF (vi) aq. NH$_3$, 55 °C.

3A.5 Synthesis of modified oligonucleotides, characterization and UV-$T_m$ studies

The 18mer DNA sequence chosen for the current study of biological
relevance, DNA1 is used for miRNA down-regulation.$^{14}$ Unmodified oligomers were
synthesized using a Bioautomation MM4 DNA synthesizer by using commercially
available phosphoramidite building blocks. Modified oligonucleotides were
synthesized on Bioautomation MM4 DNA synthesizer, using phenoxyacetyl (Pac) protected cyanoethyl phosphoramidites and modified amidite building blocks 9a, 9b. The modified phosphoramidites 0.1M in CH$_3$CN were manually coupled for 6min, followed by washing step with 10% H$_2$O, 0.2% Ac$_2$O, 0.2% Lutidine v/v/v in THF done to avoid the unwanted phosphitylation at bases of highly reactive acyclic olefinic monomers. After washing step, capping followed by oxidation with 0.5M tert-butyl hydroperoxide in CH$_2$Cl$_2$-acetone (1:1) used instead of iodine/water, because it is known that iodine/water cleaved the allylic C-O bond. This is known to occur for other phosphites with allylic or tertiary substituents$^{12}$. Deprotection and cleavage were performed by shaking the support bound oligonucleotide with neat dry diisopropylamine, washing with diethylether followed by shaking with conc aqueous ammonia for 2h at rt$^{12}$. The crude oligomer was purified by RP-HPLC, purity confirmed by gel-electrophoretic mobility studies (Figure 3a) and characterized by MALDI-TOF mass spectrometry (Table 1).

Table 1 Modified DNA sequences, their MALDI-TOF mass analyses and biophysical evaluation by UV-$T_m$ measurements$^a$

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence$^b$ 5'→ 3'</th>
<th>mass cal/obs</th>
<th>UV $T_m$ °C</th>
<th>DNA$^c$</th>
<th>RNA$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA1</td>
<td>caccattgtcacactcra</td>
<td>5363/5367</td>
<td>63.5</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
<td>DNA1-15T$^{trans}$</td>
<td>caccattgtcacT$^{trans}$cra</td>
<td>5347/5342</td>
<td>60.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DNA1-9T$^{trans}$</td>
<td>caccattgcacT$^{trans}$cra</td>
<td>5347/5347</td>
<td>59.6</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>DNA1-15T$^{cis}$</td>
<td>caccattgcacT$^{cis}$cra</td>
<td>5347/5344</td>
<td>59.3</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td>DNA1-9T$^{cis}$</td>
<td>caccattgcacT$^{cis}$cra</td>
<td>5347/5344</td>
<td>60.8</td>
<td>59.6</td>
<td></td>
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</table>

$^a$UV-$T_m$ values were measured by using 1μM sequences with 1μM cDNA/cRNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is ±0.5 °C). $^b$The lower case letters indicate unmodified DNA and upper case indicate modified site. $^c$5'tggagtgtgacaatggtg was the complementary DNA sequence. $^a$5' uggagugugacaauggug was the complementary RNA sequence.
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Figure 3 (a) Gel picture of purified sequences and UV melting profiles of modified DNA sequences with, (b) cDNA, (c) cRNA

It is seen that the sequences modified with T-cis as well as T-trans are able to form stable duplexes with both DNA as well as RNA independent of the site of modification i.e towards 3'-end or in the middle of the sequence. The destabilization observed is 2-4°C in each case (Table 1).

We further studied multiple modifications in the sequence containing continuous stretch of cis-thymine units so that the modified units could be inserted continuously or alternatingly in the sequence. In homothyminyl sequences the acyclic units were seldom tolerated and the duplexes formed were destabilized (Table 2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence[5'→3']</th>
<th>mass cal/obs</th>
<th>UV T_m °C DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA2</td>
<td>gcgttttttgcct</td>
<td>3633/3635</td>
<td>51</td>
</tr>
<tr>
<td>DNA3</td>
<td>gcgtT^{cis}T^{cis}T^{cis}gcct</td>
<td>3585/3586</td>
<td>26</td>
</tr>
<tr>
<td>DNA4</td>
<td>gcgtT^{cis}T^{cis}T^{cis}gcct</td>
<td>3585/3582</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 2 Modified DNA sequences, their MALDI-TOF mass analysis and biophysical evaluation by UV-T_m measurements

UV-\(T_m\) values were measured by using 1\(\mu\)M sequences with 1\(\mu\)M cDNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is \(\pm 0.5\) °C). The lower case letters indicate unmodified DNA and upper case indicate modified site. \(\text{S}^\prime\text{agcaaaaaacgc}\) was the complementary DNA sequence.

**Figure 4** (a) Gel picture of purified sequences, (b) UV melting profiles of modified DNA sequences with cDNA

### 3A.6 Stability of oligonucleotides to SVPD

The antisense technology AS-ONs must have specific characteristic properties such as sequence specificity, high target-binding affinity, and nuclease resistance. Among all of these, nuclease resistance is key factor, which determines the effectiveness of AS-ONs in vivo. The exo- and endo- nucleases cleave the phosphodiester bonds between the nucleotide subunits of AS-ONs in vivo. Exonucleases are enzymes which cleave nucleotides from the ends (exo) (either the 3'‐ or the 5'‐end) of a polynucleotide and one at a time, where as endonucleases, cleave phosphodiester bonds in the middle (endo) of a polynucleotide chain. 3'‐exonuclease catalyzes the degradation of nucleic acid in 3'→5' direction with the removal of a nucleoside-5'‐phosphate from the 3'-end of DNA (Figure 5).

Natural DNA/RNA oligonucleotides are not good antisense candidates mainly because of lack of nuclease resistance. This has encouraged enormous chemical efforts to produce therapeutically significant modified ONs to provide improved stability, with maintaining high target affinity and specificity. First generation AS-ONs (phosphorothioate) have shown improved nuclease resistance but reduced affinity towards a complementary RNA sequence. Second generation of AS-ONs involves various 2'-modifications of the ribose moiety, results considerable improvement of binding affinity to the target RNA.
Figure 5 Exonucleolytic cleavage in 3’- to 5’-direction to yield nucleoside 5’-phosphates

In this case, size, electronegativity, and configuration of the 2’-substituents are very important factors for the target affinity and nuclease resistance. The new third generation of AS-ONs, containing conformationally constrained LNA (also called BNA) shows unusual high affinity towards complementary RNA strand (3-8°C per modification). Unfortunately, though LNA containing AS-ONs are more nucleolytically stable than the native PO AS-ONs, they do not have nuclease resistance as good as that of the PS AS-ONs.

3A.7 Enzymatic stability studies of modified DNA sequences

Considering nuclease resistance as an important factor, we examined the 3’-exonuclease sensitivity of unmodified homooligomer t₁₀ as well as cis and trans modified sequences t₅T₆cis and t₅T₆trans by using phosphodiesterase I from Crotalus adamanteus venom [snake venom phosphodiesterase (SVPD)] (Table 3). Enzymatic hydrolysis of the ONs (7.5 μM) was carried out at 37 °C in buffer (100 μl) containing 100 mM Tris-HCl (pH 8.5), 15 mM MgCl₂, 100 mM NaCl and SVPD (10μg/mL). Aliquots were removed at several time-points; a portion of each reaction mixture was removed and heated to 90 °C for 2 min to inactivate the nuclease. The amount of intact ONs was analyzed at several time points by RP-HPLC (Figure 6). The
percentage of intact ON was then plotted against the exposure time to obtain the ON degradation curve with time.

**Table 3** Oligomers used for 3'-exonuclease degradation study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'→3'</th>
<th>mass cal/obs</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;10&lt;/sub&gt;</td>
<td>tttttttttt</td>
<td>2980/2977</td>
</tr>
<tr>
<td>t&lt;sub&gt;8&lt;/sub&gt;T&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>tttttttT&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>2964/2961</td>
</tr>
<tr>
<td>t&lt;sub&gt;8&lt;/sub&gt;T&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>tttttttT&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>2964/2961</td>
</tr>
</tbody>
</table>

The unmodified single strand DNA sequence t<sub>10</sub> did not show any 3'-exonuclease resistance and was completely degraded within 4min. The synthesis of t<sub>8</sub>T<sup>trans</sup> was carried out by using standard support with preloaded thymine nucleosidic unit. We incorporated our trans modified monomer at 9th position from 5'- end of 10mer sequence. 3'- terminal thymidine residue was started cleaving within 0.5min and t<sub>8</sub>T<sup>trans</sup> fragment was observed simultaneously. The sequence t<sub>8</sub>T<sup>trans</sup> was stable up to 10min, which is comparatively stable than unmodified t<sub>10</sub>. In case of t<sub>8</sub>T<sup>cis</sup>, 3'-terminal thymidine residue was completely cleaved within 4min and t<sub>8</sub>T<sup>cis</sup> was observed to be stable up to 5h, which was clearly indicated the high stability of cis modified sequence.

**t<sub>10</sub>: RP-HPLC at several time-points for SVPD study**

(Black- No SVPD, Blue- 0.5min, Green-2min,cyan-4min, Magenta-6min)
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$t_{8T^{\text{trans}}}$: RP-HPLC at several time-points for SVPD study

(Black- No SVPD, Blue- 0.5min, Green- 4min, Cyan-8min, Magenta-20min)

$t_{8T^{\text{cis}}}$: RP-HPLC at several time-points for SVPD study

(Black- No SVPD, Blue- 0.5min, Green- 6min, Cyan-1h, Magenta-3h, Brown-5h)
Figure 6: RP-HPLC at several time-points for SVPD study for $t_{10}$, $t_8^{\text{trans}}$, $t_8^{\text{cis}}$

3A.8 Conclusions:

- Successfully cis and trans open chain analogues of cyclohexenyl nucleosides were synthesized from a prebiotic precursor, dihydroxy acetone.

- These cis and trans modified nucleosides were incorporated for the first time in mixed Pu/Py DNA sequences and purity was confirmed by gel-electrophoretic mobility studies.

- Duplexes with cDNA and cRNA are quite stable compared to other known acyclic modifications.

- Alternative modifications are stabilizing the duplex structure with cDNA than consecutive positions.

- Nuclease resistance of cis and trans modified sequences were stable to SVPD enzyme than unmodified $t_{10}$ and the stability order was $t_8^{\text{cis}} > t_8^{\text{trans}} > t_{10}$. 
3A.9 Experimental section

General information:

All the non-aqueous reactions were carried out under the inert atmosphere of Nitrogen/Argon and the chemicals used were of laboratory or analytical grade. All solvents used were dried and distilled according to standard protocols. TLCs were carried out on precoated silica gel 60 F254 (Merck). Column chromatographic separations were performed using silica gel 60-120 mesh (Merck) or 200-400 mesh (Merck) and using the solvent systems EtOAc/Petroleum ether and MeOH/DCM. 1H and 13C NMR spectra were obtained using Bruker AC-200, AC-400 and AC-500 NMR spectrometers. The chemical shifts are reported in delta (δ) values and referred to internal standard TMS for 1H. High resolution mass spectra were recorded on a Thermo Fisher Scientific Q Exactive mass spectrometer.

1-((tert-butyldimethylsilyl)oxy)-3-hydroxypropan-2-one (2)

\[
\text{HO} \quad \text{TBDMSO} \\
\text{O} \\
\text{O}
\]

Compound 1 (9.4 g, 104.4 mmol) was dissolved in dry DMF (100 mL), then TBS-Cl (5.0 g, 33.5 mmol) and imidazole (2.95 g, 43.4 mmol) was added under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 10h then quenched with water (100 mL). Compound was extracted with ethyl acetate from crude reaction mixture and organic layer washed with brine solution, dried over Na2SO4 and concentrated on rotavapor in vacuo. Crude compound purified through column chromatography (pet ether:EtOAc, 90:10) to result 2 (6.2 g, 55%) as a colour less thick liquid.

\[^1\text{H NMR}\ (200\text{ MHz},\ \text{CDCl}_3)\ \delta\ 0.10\ (s,\ 6\text{ H}),\ 0.93\ (s,\ 9\text{ H}),\ 3.01\ (t,\ J=4.99\text{ Hz},\ 1\text{ H}),\ 4.32\ (s,\ 2\text{ H}),\ 4.51\ (d,\ J=4.93\text{ Hz},\ 2\text{ H})\ \text{ppm};\ ^{13}\text{C NMR}\ (50\text{ MHz},\ \text{CDCl}_3)\ \delta\ -5.7,\ 18.1,\ 25.7,\ 66.6,\ 67.7,\ 211.1\ \text{ppm};\ HRMS\ (EI):\ Mass\ calculated\ for\ C_9H_{20}O_3NaSi\ (M^+Na^+),\ 227.1074,\ found\ 227.1069.\]

1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-((tert-butyldimethylsilyl)oxy)propan-2-one (3)

To a solution of 2 (5 g, 24.5 mmol) in pyridine (15 mL) DMTr chloride (10g, 29.5 mmol) and catalytic amount of DMAP were added, stirred at rt for 6h. Pyridine was
removed in vacuo and the residue was diluted with EtOAc. Water wash and brinewash were given to the organic layer, dried over Na$_2$SO$_4$, concentrated in vacuo. The residue was subjected to silica gel column chromatography (pet ether: EtOAc, 95:5) to afford 3 (9.3 g) in 75% yield.

$^1$H NMR (200 MHz, CDCl$_3$) δ 0.02 (s, 6 H), 0.85 (s, 9 H), 3.80 (s, 7 H), 3.96 (s, 2 H), 4.38 (s, 2 H), 6.79 - 6.89 (m, 5 H), 7.26 - 7.39 (m, 9 H), 7.41 - 7.49 (m, 2 H) ppm; $^{13}$C NMR (50 MHz, CDCl$_3$) δ -5.6, 18.2, 25.7, 55.2, 68.2, 68.4, 86.9, 113.3, 127.0, 128.0, 130.0, 135.4, 144.3, 158.7 ppm; HRMS (EI): Mass calculated for C$_{30}$H$_{38}$O$_5$NaSi (M+ Na$^+$) 529.2381, found 529.2369.

ethyl(Z)-4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl)but-2-enoate (4a & 4b)

Solution of 3 (10 g, 19.7 mmol) and two carbon wittig ylide (9.5 g, 29.6 mmol) in 100 mL toluene was refluxed for 4 h. Solvent was removed in vacuo, residue diluted with the EtOAc and water wash, saturated aqueous NaHCO$_3$ wash and finally brine wash were given. The organic layer was dried over Na$_2$SO$_4$, concentrated in vacuo followed by column chromatography (pet ether: EtOAc, 98:2) to give a 4a and 4b (90%) in 60:40 ratio.

$^1$H NMR(4a) (200 MHz, CDCl$_3$) δ -0.08 (s, 6 H), 0.72 (s, 9 H), 1.34 (t, $J$=7.14 Hz, 4 H), 3.80 (s, 6 H), 3.90 (s, 2 H), 4.21 (q, $J$=7.07 Hz, 2 H), 4.81 (s, 2 H), 6.38 (t, $J$=1.77 Hz, 1 H), 6.84 (d, $J$=8.72 Hz, 4 H), 7.23 7.39 (m, 8 H), 7.41 - 7.49 (m, 2 H) ppm; $^{13}$C NMR (125 MHz, CDCl$_3$) δ -5.7, 14.4, 18.0, 25.7, 55.2, 59.9, 61.9, 64.0, 86.6, 112.2, 113.2, 126.8, 127.9, 128.0, 129.9, 136.1, 144.8, 158.5, 160.1, 166.7 ppm; HRMS (EI): Mass calculated for C$_{34}$H$_{44}$O$_6$NaSi (M+ Na$^+$) 599.2799, found 599.2789.

$^1$H NMR(4b) (200 MHz, CDCl$_3$) δ 0.10 (s, 6 H), 0.94 (s, 9 H), 1.19 (t, $J$=7.14 Hz, 4 H), 3.77 - 3.81 (m, 8 H), 4.05 (q, $J$=7.07 Hz, 2 H), 4.39 (s, 2 H), 4.52 (s, 2 H), 5.94 - 5.99 (m, 1 H), 6.82 (d, $J$=8.84 Hz, 5 H), 7.25 - 7.43 (m, 13 H) ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) δ -5.4, 14.3, 18.4, 26.0, 55.2,
(Z)-4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl) but-2-en-l-ol (5a)

DIBAL-H was added to a solution of ester 4a (1g, 2.6 mmol) in DCM at -78 °C. After 45 min at same temperature aq. sodium potassium tartarate and diethyl ether added. The resultant cloudy reaction mixture was then vigorously stirred for 1h at which organic layer appears like clear solution. Organic layer was separated and washed with brine solution and extracted with DCM, dried over Na$_2$SO$_4$.Compound was purified through column chromatography (pet ether:EtOAc, 70:30) to obtain 5a (0.71g) in 79% yield.

$^1$H NMR (200 MHz, CDCl$_3$) δ -0.01 (s, 6 H), 0.82 (s, 9 H), 3.62 (s, 2 H), 3.80 (s, 8 H), 4.19 (s, 2 H), 4.22 - 4.31 (m, 2 H), 6.02 (t, $J=6.57$ Hz, 1 H), 6.83 (d, $J=8.84$ Hz, 6 H), 7.25 (d, $J=2.65$ Hz, 2 H), 7.28 - 7.52 (m, 11 H) ppm; $^{13}$C NMR (50 MHz, CDCl$_3$) δ -5.5, 18.2, 25.8, 55.2, 58.7, 59.9, 65.3, 86.2, 113.1, 126.2, 126.7, 127.8, 128.1, 130.0, 136.3, 139.5, 145.0, 158.4 ppm; HRMS (EI): Mass calculated for C$_{32}$H$_{42}$O$_5$NaSi (M+ Na$^+$) 557.2694, found 557.2677.

(Z)-3-benzoyl-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)dione (6)

To a solution of 5a (0.5g, 0.93 mmol) in dry dioxane (4ml) was added triphenyl phosphine(0.37g, 1.4mmol) and N$_2$-benzoyl protected thymine (0.32g, 1.4mmol) stirred for 15 min. DIAD (0.36mL, 1.86mmol) was dissolved in 1mL dry dioxane and added to the reaction mixture, continued the stirring for overnight at room temperature. Dioxane was removed in vacuo and the residue was diluted with EtOAc. Water wash and brine wash were given to the organic layer, dried over Na$_2$SO$_4$, concentrated in vacuo. Crude residue was subjected
to silica gel column chromatography (pet ether:EtOAc, 70:30) to obtain 6 (0.38 g) in 55\% yield.

$^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 0.01 (s, 6 H), 0.83 (s, 9 H), 1.97 (s, 3 H), 3.67 (s, 2 H), 3.80 (s, 6 H), 4.23 (s, 2 H), 4.57 (d, $J$=7.58 Hz, 2 H), 5.79 (t, $J$=7.71 Hz, 1 H), 6.84 (d, $J$=8.84 Hz, 5 H), 7.23 (br. s., 2 H), 7.29 - 7.54 (m, 13 H), 7.59 - 7.69 (m, 1 H), 7.91 - 7.98 (m, 2 H) ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ -5.3, 12.6, 18.3, 25.9, 25.9, 44.3, 55.3, 59.7, 65.4, 86.6, 110.9, 113.2, 120.0, 126.9, 128.0, 128.1, 129.2, 130.0, 130.6, 131.7, 135.0, 136.1, 139.6, 142.9, 144.9, 150.1, 158.6, 163.3, 169.3 ppm.

LCMS (EI): Mass calculated for C$_{44}$H$_{50}$N$_2$O$_7$NaSi (M+ Na$^+$) 769.3387, found 769.17.

(Z)-3-benzoyl-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(hydroxymethyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (7a)

Compound 6 (1g, 1.28mmol) was dissolved in 15mL THF and TBAF (0.394 g, 1.5 mmol) was added. The reaction mixture was stirred for 2h at room temperature. The solvent was removed in vacuo. The residue was dissolved into 50 mL of ethyl acetate, washed with water (3 x 25 mL) and then with brine. The organic layer dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The resulting residue was purified on silica gel column chromatography (pet ether:EtOAc, 60:40) to yield 7a (0.7g) in 83\%.

$^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.89 (s, 3 H), 3.68 (s, 3 H), 3.71 (s, 8 H), 4.10 (s, 2 H), 4.41 (d, $J$=7.58 Hz, 2 H), 5.62 (t, $J$=7.64 Hz, 1 H), 6.76 (d, $J$=8.84 Hz, 5 H), 7.12 - 7.24 (m, 9 H), 7.29 - 7.43 (m, 6 H), 7.48 - 7.61 (m, 2 H), 7.79 - 7.88 (m, 3 H) ppm; $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 12.4, 25.8, 44.2, 55.2, 59.6, 65.3, 86.5, 110.8, 113.1, 119.9, 126.8, 127.9, 128.0, 129.1, 129.9, 130, 131.6, 134.9, 136.0, 139.5, 140.0, 142.8, 144.7, 150.0, 158.5, 163, 169.2 ppm; HRMS (EI): Mass calculated for C$_{38}$H$_{36}$O$_7$N$_2$Na (M+ Na$^+$) 655.2415, found 655.2398.

(E)-4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl)but-2-en-1-ol (5b)
DIBAL-H was added to a solution of ester 4b (1g, 2.6 mmol) in DCM at -78 °C. After 45 min at same temperature aq. sodium potassium tartarate and diethyl ether added. The resultant cloudy reaction mixture was then vigorously stirred for 1h at which organic layer appears like clear solution. Organic layer was separated and washed with brine solution and extracted with DCM, dried over Na₂SO₄. Compound was purified through column chromatography (pet ether:EtOAc, 70:30) to obtain 5b (0.67g) in 75% yield.

**¹H NMR** (200 MHz, CDCl₃) δ 0.08 (s, 6 H), 0.92 (s, 9 H), 3.66 (s, 2 H), 3.80 (s, 7 H), 4.07 (d, J=6.82 Hz, 2 H), 4.22 (s, 2 H), 5.88 (t, J=6.82 Hz, 1 H), 6.85 (d, J=8.84 Hz, 5 H), 7.24 - 7.50 (m, 12 H) ppm; **¹³C NMR** (125 MHz, CDCl₃) δ -5.3, 18.4, 26.0, 55.2, 58.8, 59.6, 65.1, 86.6, 113.2, 113.3, 113.3, 126.4, 126.8, 127.9, 128.0, 128.1, 129.9, 130.0, 130.0, 136.1, 139.3, 144.9, 158.5 ppm; **HRMS (EI):** Mass calculated for C₃₂H₄₂O₅NaSi (M⁺ Na⁺) 557.2694, found 557.2684.

(E)-3-benzoyl-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(hydroxymethyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (7b)

Compound 5b was subjected for Mitsunobu reaction and without purification the crude mixture used for TBDMS deprotection to obtain 7b in 57% yield over two steps.

**¹H NMR** (500 MHz, CDCl₃) δ 1.86 (s, 3 H), 3.78 (s, 7 H), 3.80 (s, 2 H), 4.19 - 4.23 (m, 4 H), 5.65 (t, J=7.02 Hz, 1 H), 6.85 (d, J=8.85 Hz, 5 H), 7.27 - 7.37 (m, 8 H), 7.42 - 7.50 (m, 5 H), 7.90 (d, J=7.32 Hz, 2 H) ppm; **¹³C NMR** (125 MHz, CDCl₃) δ 12.3, 44.9, 55.3, 59.6, 65.5, 87.0, 110.9, 113.4, 121.7, 127.1, 128.0, 128.1, 129.1, 130.0, 131.7, 135.0, 135.5, 139.5, 142.2, 144.4, 149.8, 158.8, 163.1, 169.1 ppm; **HRMS (EI):** Mass calculated for C₃₈H₃₆O₇N₂Na (M⁺ Na⁺) 655.2415, found 655.2396.

(Z)-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(hydroxymethyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (8a)
30% aq. ammonia solution (0.5mL) was added to a solution of 7a (0.5g, 0.76mmol) in 10mL dioxane and stirred for 7h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved into 50 mL of ethyl acetate, washed with water (3 x 25 mL) and then with brine. The organic layer dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The resulting residue was purified on silica gel column. The product was eluted with 50% ethyl acetate in petroleum ether to afford 8a (0.4 g, 85 %) as a white solid.

$^1$H NMR (500 MHz, CDCl$_3$) δ 1.83 (s, 3 H), 3.80 (s, 7 H), 3.81 (br. s., 2 H), 4.17 (d, $J$=7.02 Hz, 2 H), 4.23 (br. s., 2 H), 5.62 (t, $J$=7.02 Hz, 1 H), 6.85 (d, $J$=9.16 Hz, 5 H), 7.21 - 7.26 (m, 1 H), 7.28 - 7.36 (m, 7 H), 7.44 (d, $J$=7.02 Hz, 2 H), 8.82 (br. s., 1 H) ppm; $^{13}$C NMR (126 MHz, CDCl$_3$) δ 12.2, 44.7, 55.3, 59.7, 65.5, 87.0, 110.9, 113.3, 122.1, 127.1, 128.0, 128.0, 130.0, 135.5, 139.8, 141.8, 144.4, 150.8, 158.7, 164.1 ppm; HRMS (EI): Mass calculated for C$_{31}$H$_{32}$O$_6$N$_2$Na (M+ Na$^+$) 551.2153, found 551.2147.

(E)-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(hydroxymethyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (8b)

30% aq. ammonia solution (0.5mL) was added to a solution of 7b (0.5g, 0.76mmol) in 10mL dioxane and stirred for 7h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved into 50 mL of ethyl acetate, washed with water (3 x 25 mL) and then with brine. The organic layer dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The resulting residue was purified on silica gel column. The product was eluted with 50% ethyl acetate in petroleum ether to afford 8b (0.33 g, 80 %) as a white solid.

$^1$H NMR (500 MHz, CDCl$_3$) δ 1.92 (s, 3 H), 3.76 (s, 2 H), 3.77 (s, 6 H), 4.19 (s, 2 H), 4.43 (d, $J$=7.63 Hz, 2 H), 5.62 (t, $J$=7.63 Hz, 1 H), 6.81 (d, $J$=8.85 Hz, 4 H), 7.21 (d, $J$=7.32 Hz, 1 H), 7.24 - 7.32 (m, 7 H), 7.40 (d, $J$=7.32 Hz, 2 H), 9.41 (br. s., 1 H) ppm ; $^{13}$C NMR (126 MHz, CDCl$_3$) δ 12.3, 45.4, 55.2, 58.7, 66.3, 86.8, 111.3, 113.2, 120.6, 126.9, 127.9, 128.1, 130.0, 135.9, 140.2, 142.6, 144.7, 151.2, 158.6, 164 ppm;
Chapter 3

HRMS (EI): Mass calculated for C$_{31}$H$_{32}$O$_6$N$_2$Na (M$^+$ Na$^+$) 551.2153, found 551.2145.

**General procedure followed for synthesis of phosphoramidite derivatives 9a, 9b**

To the compound 8a, 8b (100 mg, 0.17 mmol) dissolved in dry DCM (3 mL), DIPEA (0.64 mmol, 0.12 mL) was added. 2-cyanoethyl-N,N-diisopropyl-chloro phosphine (0.35 mmol, 0.08 mL) was added to the reaction mixture at 0 °C and stirring continued at room temperature for 1 hour. The contents were diluted with DCM and washed with 5% NaHCO$_3$ solution. The organic phase was dried over anhydrous sodium sulphate and concentrated to white foam. The residue was re-dissolved in DCM and the compound was precipitated with n-hexane to obtain corresponding phosphoramidite derivatives in 70-75 % yield.

**phosphoramidite derivative 9a**

$^{31}$P NMR (500MHz, CDCl$_3$) $\delta$ 148.82

HRMS (EI): Mass calculated for C$_{40}$H$_{49}$O$_7$N$_4$NaP (M$^+$ Na$^+$) 751.3231, found 751.3212.

**phosphoramidite derivative 9b**

$^{31}$P NMR (500MHz, CDCl$_3$) $\delta$ 148.27

HRMS (EI): Mass calculated for C$_{40}$H$_{49}$O$_7$N$_4$NaP (M$^+$ Na$^+$) 751.3231, found 751.3212.
### Compounds - Spectral data

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<td>4a- DEPT &amp; HRMS</td>
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<td>HPLC &amp; MALDI-TOF of DNA 4</td>
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NMR spectral data:

**Chemical Shift (ppm):**
- 2.11, 1.96, 1.00
- 9.26, 6.07

**Normalized Intensity:**
- Chloroform-d
- TBDMSO
- HO

**Chemical Shift (ppm):**
- 2.11, 1.96, 1.00
- 9.26, 6.07
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[Diagram showing chemical shift and normalized intensity plots with various peak annotations and values.

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![Diagram of NMR spectra with chemical shifts and normalized intensity plots.]

Chemical Shift (ppm):
- Chloroform-d
- DMTrO
- TBDMSO

Normalized Intensity:
- Values ranging from 0.08 to 1.00

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The image shows two NMR spectra plots. The top plot is labeled with chemical shifts in ppm (parts per million) and normalized intensity on the y-axis. The bottom plot is similar with additional chemical shift values in ppm. The spectra contain labeled peaks denoted by chemical structures such as DMTTO and TBDMSO. The chemical shifts range from approximately -1.97 to -0.01 ppm in the top plot and from -44.30 to 172.10 ppm in the bottom plot.
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[Image of a graph showing chemical shifts and normalized intensity with molecular structures labeled as DMTro and TBDMSO attached to a compound 6.

Chemical Shift (ppm) and m/z values are listed.

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[Chemical Shift graph]

DMTrO

7b

[Chemical Shift graph]
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Normalized Intensity

Chemical Shift (ppm)

Normalized Intensity

Chemical Shift (ppm)
Chapter 3

1.0-

S 0.5-

o

0.0

Normalized Intensity

DMT\text{rO}

\text{HO}

\text{T}

\text{Ba}

Chemical Shift (ppm)

0

50

100

150

200

250

300

350

400

450

500

550

600

650

700

750

800

Normalized Abundance

DMT\text{rO}

\text{HO}

\text{T}

\text{Ba}

517.2626

R=47207

\text{C}_{10} \text{H}_{10} \text{O}_{2} \text{Na} \cdot \text{Na} = 581.2153

-0.9727 ppm

483.2793

R=49207

\text{C}_{10} \text{H}_{10} \text{O}_{2} \text{Na} \cdot \text{Na} = 517.2614

1.9823 ppm

454.6246

R=41207

544.5246

R=43207

573.1965

R=46507

613.1851

R=46407

641.1862

R=43007

657.1794

R=45007

703.1546

R=38007

725.1889

R=37507

771.1409

R=38307

460

500

540

580

620

660

700

740

780

420

460

500

540

580
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HPLC & MALDI-TOF of DNA 1:

DNA 1
Mass calcd. 5363
obsd. 5367
HPLC & MALDI-TOF of DNA1-15^trans.

DNA1-15^trans
Mass calcd. 5347
obsd. 5342
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HPLC & MALDI-TOF of DNA1-9T$_{\text{trans}}$.

![Graph showing HPLC analysis]

![Graph showing MALDI-TOF analysis]

DNA1-9T$_{\text{trans}}$

Mass calcd. 5347
obsd. 5347
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HPLC & MALDI-TOF of DNA1-15T<sup>cis</sup>:

![HPLC & MALDI-TOF graph]

**DNA1-15T<sup>cis</sup>**
Mass calcd. 5347
obsd. 5344
HPLC & MALDI-TOF of DNA1-9\textsuperscript{cis}.
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HPLC & MALDI-TOF of DNA 2:

DNA2
Mass calcd. 3633
obsd. 3635
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HPLC & MALDI-TOF of DNA 3:

[Graph showing HPLC profile]

DNA3
Mass calcd. 3585
obsd. 3586
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HPLC & MALDI-TOF of DNA 4:

[Graph showing chromatogram]

DNA 4
Mass calcd. 3585
obsd. 3582
CHAPTER 3 / SECTION-B

Synthesis of cis and trans modified thrombin binding aptamers and their quadruplex formation study
Section 3B: Synthesis of *cis* and *trans* modified thrombin binding aptamers and their quadruplex formation study

3B.1 Introduction

G-quadruplexes are a unique class of highly ordered nucleic acid structures, which are formed by four G-rich oligonucleotide strands associate through hydrogen-bonding. The stacked tetrads are stabilized by sandwiched monovalent cations coordinated to the O6 oxygen atoms of the guanines (Figure 7). G-quadruplex structures plays important role of regulatory functions in many biological processes\(^5\) such as telomeres, promoters, centromeres, etc. The d(TTAGGG) repeat sequences, called as telomere, they have recently received great attention because of their potential links to cancer, HIV and other diseases. A unique G-rich DNA sequence in the telomeres was found to protect the chromosomes from recombination, end to end fusion, and degradation through forming G-quadruplexes with highly polymorphic structures in the presence of alkali metal cations. This regular occurrence of quadruplex structures in the genome\(^6\) suggests the designing of potential drug molecules based on DNA/RNA G-quartets.\(^7\)

![G-tetrad motif and a G-quadruplex](http://www.al-nasir.com)

3B.2 Aptamers: An emerging class of therapeutics

From past 25 years, huge number of nucleic acid ligands also termed aptamers have been developed, which can inhibit the activity of many pathogenic proteins. Aptamers are using for treatment of a variety of human maladies, cancer, infectious diseases, and cardiovascular disease.\(^8\) Aptamers became an attractive class of...
therapeutic compounds because of their affinity and specificity towards the targets, such as small molecules to peptides, proteins or even whole cells. Their binding affinity and specificity may be equal or even superior to that of antibodies. Natural aptamers also exist in riboswitches. Aptamers can differentiate the small structural differences that may exist as a result of the presence or absence of a small functional group such as methyl or hydroxyl or even as a result of differing chirality. Aptamers were first developed by an *in vitro* selection process termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) independently in the laboratories of Joyce, Szostak and Gold in 1990 (Figure 8). The word ‘aptamer’ was first coined by Ellington and Szostak in 1990.

![SELEX in vitro selection protocol](www.lookfordiagnosis.com)

Till date, hundreds of aptamers have been introduced against a range of different targets. These can be used for basic research, clinical purposes, in diagnostics, as well as in therapeutics and macromolecular drugs.
3B.3 Discovery, structural features of Thrombin-binding aptamer (TBA)

Thrombin is a key regulatory enzyme in the coagulation cascade. It is a serine protease produced from prothrombin by the action of factor Xa. Thrombin will catalyze the conversion of fibrinogen into fibrin, which is the building block of the fibrin matrix of blood clots.\textsuperscript{28}

The **thrombin binding aptamer (TBA)** was discovered in 1992 by *in vitro* selection and found to inhibit fibrin-clot formation with high selectivity and affinity. NMR and X-ray structural study reveals that TBA forms an intramolecular, antiparallel G-quadruplex structure with chair like conformation (Figure 9a). This G-quadruplex consists of two G-quartets connected by three edge wise loops, a central TGT loop and two TT loops. The aptamer interacts with two thrombin molecules, inactivating only one of them. X-ray studies indicated that inhibition of fibrinogen-clotting is a result of specific blocking of the thrombin anion exosite I. The central TGT loop (Figure 9b)\textsuperscript{29} and two TT loops are involved in ionic interactions with the electropositive heparin binding site of a second thrombin molecule to compensate the residual negative charge of the aptamer. In contrast, NMR studies indicated that the two TT loops interact with the thrombin anion exosite I (Figure 9c),\textsuperscript{30} while the TGT loop is in close proximity to the heparin binding site of a neighbouring thrombin molecule.

![Figure 9](image)

Figure 9 (a) Quadruplex structure of the thrombin binding aptamer (TBA), interaction with the thrombin anion exosite I according to (b) X-ray and (c) NMR studies. (*Nucleic Acids Research, 2011, Vol. 39, No. 3*)
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3B.4 Modifications in TBA

Although several DNA/RNA aptamers are able to show interesting and promising pharmacological properties, rarely used as therapeutic agents without modifications due to less stability towards hydrolytic enzymes. To overcome these problems lot of attempts has been done via chemical and structural modifications.\(^{31}\)

Unlocked nucleic acids substitution at T-7 position of TGT loop, resulted in enhanced the quadruplex stability, anticoagulation activity as well as enzymatic stability.\(^{32}\) LNA substitutions showed the reduction of anticoagulation activity.\(^{33}\) The effect on quadruplex stability of North-nucleoside in the loops of TBA was reported by Eritja and coworkers.\(^{34}\) The replacement of thymidines in the TGT loop of the TBA quadruplex by uridine (U) and 2'-fluorouridine (FU) induced greater stability to the antiparallel quadruplex structure, determined by UV-\(T_m\) experiments and CD spectroscopy. However the presence of North-methanocarbathymidine (NT) in the same positions destabilized the quadruplex structure. Also, substitution of thymidines in the TT loops by U, FU and NT destabilized the antiparallel quadruplex structure. Thus the changes in sugar conformations of the nucleotides in the loop region of TBA are important in determining the stability of TBA quadruplex structure.

3B.5 Present work

The acyclic UNA analogues were introduced by Wengel and co-workers to study the stability duplexes.\(^{35}\) They also introduced UNA analogues in TBA\(^{36}\), found to be an excellent application in stabilizing the structure due to its ability to alleviate strain in quadruplex loop structure\(^{31}\). We studied the flexibility parameter of our open chain-NA modification (Figure 10) by introducing it in the loop region of TBA quadruplex in comparison with unmodified TBA and with the UNA modification of TBA. The replacement of T3 and T7 positions of thymidine by UNA units were found to stabilize the quadruplex structure of TBA. We chose these two positions for replacing the thymidinyl units of TBA to study its effect on the quadruplex stability.
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3B.6 Chemical synthesis of cis and trans modified thrombin binding aptamers

Using an automated Bioautomation MM-4 DNA synthesizer and commercially available phenoxyacetyl (Pac) protected 5’-O-dimethoxytrityl-2’-deoxy-3’-phosphoramidites four sequences, TBA-3T\textsuperscript{cis}, TBA-7T\textsuperscript{cis}, TBA-3T\textsuperscript{trans} and TBA-7T\textsuperscript{trans} were synthesized by following the reported protocol which was already discussed in (section-A). The 3’-5’-linked TBA was synthesized for control experiments. All modified sequences were purified by HPLC and purity was confirmed by gel-electrophoretic mobility studies (Figure 11) and characterized by MALDI-TOF mass spectrometry (Table 4).

3B.7 G-quadruplex formation in the presence of monovalent cation using CD spectroscopy

The G-quadruplex formation for the synthesized sequences was studied by CD spectroscopy\textsuperscript{37} in the presence of added monovalent cation such as K\textsuperscript{+} and their stability was determined as a function of temperature-dependent change in CD amplitude at 295nm.

All four modified sequences showed maxima at 295nm which is characteristic CD signature for formation of stable antiparallel quadruplex (Figure 12a). The stability of the G-quadruplexes was studied by the change in the amplitude of the CD signal at 295nm with temperature (Figure 12b, Table 4). The CD melting results tells that T7 position modifications are showing the stabilization of antiparallel quadruplex structures as compared to their corresponding T3 position modifications. The modifications of cis and trans units at both T3 and T7 positions showed the destabilization of tetraplex structure compared to unmodified TBA. This may indicate that the open chain-NA modification is indeed more strained and is less suitable for quadruplex formation compared to the highly evolved DNA quadruplexes.
Table 4 Modified TBA sequences, their MALDI-TOF mass analysis and biophysical evaluation by CD-$T_m$ measurements

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<tr>
<th>Name</th>
<th>Sequence$^a$ 5'→ 3'</th>
<th>mass cal/obs</th>
<th>HPLC $t_R$ (min)</th>
<th>CD $T_m$ °C</th>
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<td>9.8</td>
<td>49.5</td>
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$^a$The lower case letters indicate unmodified DNA and upper case indicate modified site. Gel-electrophoretic mobility studies revealed that all the modified sequences were more than 95% pure.

Figure 11 Gel picture of purified sequences

Figure 12 (a) CD spectra of oligomers TBA, TBA-3T$^{cis}$, TBA-7T$^{cis}$, TBA-3T$^{trans}$, TBA-7T$^{trans}$ sequences of 5μM concentration in 10mM potassium phosphate buffer (pH 7.5) containing 100mM KCl at 5°C. (b) Temperature-dependent changes in CD amplitude at 295nm plotted against temperature, first derivative plots at strand concentration 5μM in 10mM potassium phosphate buffer (pH 7.5) containing 100mM KCl.
3B.8 Duplex stability studies of modified TBA oligomers

The binding affinity of 15mer TBA ONs TBA, TBA-3Tcis, TBA-7Tcis, TBA-3Ttrans and TBA-7Ttrans with complementary DNA and RNA was also investigated by measuring the melting temperatures (UV Tm) of the duplexes (Table 5).

Table 5 UV Tm (°C) values of TBA and modified TBA: DNA/RNA duplexes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequenceb 5'→3'</th>
<th>mass cal/obs</th>
<th>CD Tm°C</th>
<th>cDNAc</th>
<th>cRNA d</th>
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<td>53</td>
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UV-Tm values were measured by using 1µM sequences with 1µM cDNA/cRNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is ±0.5 °C). The lower case letters indicate unmodified DNA and upper case indicate modified site. 5'ccacccacacaac was the complementary DNA sequence. 5'ccacccacacaac was the complementary RNA sequence.

Figure 13 (a) UV melting profiles of TBA and modified TBA sequences with cDNA, (b) UV melting profiles of TBA and modified TBA sequences with cRNA.

UV Tm results showed that all modified and unmodified sequences were forming stable duplexes with cDNA as well as cRNA. Independent to the nucleoside
and position of modifications, $T_m$ values were same for all sequences with cDNA, cRNA. These *cis* and *trans* modifications are destabilizing the duplexes compared to unmodified TBA.

3B.9 Conclusions:

- Successfully synthesized the *cis* and *trans* modified TBA sequences for the first time and purity was confirmed by gel-electrophoretic mobility studies.

- *cis* and *trans* modified TBA sequences are forming stable antiparallel quadruplex in the presence of $K^+$ ions.

- CD melting results showed that modifications at T7 (TGT loop) position, forming more stable quadruplex structures than T3 (TT loop) position. The stability of both T7 and T3 modifications was less, compared to unmodified TBA.

- The destabilization results may indicate that the open chain-NA modification is indeed more strained compared to UNA and is less suitable for quadruplex formation compared to the highly evolved DNA quadruplexes.

- Duplex stability studies also done for the TBA modified sequences with cDNA/cRNA, modification are destabilizing the duplex structures.
Chapter 3

3B.10 Experimental Section

MALDI-TOF mass

Mass was obtained by MALDI-TOF mass spectrometry. The MALDI-TOF spectra were recorded on Voyager-De-STR (Applied Biosystems). The matrix used for analysis was THAP (2’, 4’, 6’-trihydroxyacetophenone).

CD spectroscopy

CD spectra were recorded on Jasco J-815 CD Spectrometer equipped with a Jasco PTC-424S/15 peltier system. 2 mm path-length quartz cuvettes were used for a sample volume 500 µl and strand concentration of 5 µM in 10mMol Na/K-phosphate buffer( pH 7.5) containing 100mM NaCl/KCl respectively. Oligomers prepared in buffer were annealed by heating at 95° C for 5 minutes then slowly cooling to room temperature followed by refrigeration for 5 to 6 hours before use. Spectral scans were collected over a wavelength range 200- 320nm at a scanning rate of 100 nm min⁻¹. Three scans were averaged for each sample. CD thermal denaturation of the TBA sequences, Thrombin binding studies were performed.

Buffers used for CD experiments

Phosphate buffer (pH = 7.2, 100 mM NaCl)

Na₂HPO₄ (110mg, 10mM), NaH₂PO₄·H₂O (35.3mg,10mM), NaCl (585mg,100mM) was dissolved in minimum quantity of water and the total volume was made 100 mL. The pH of the solution was adjusted 7.2 with aq. NaOH solution in de-ionised water (DI), and stored at 4°C.

Phosphate buffer (pH = 7.2, 100 mM KCl)

K₂HPO₄ (174mg,10mM), KH₂PO₄ (136mg,10mM), KCl (746mg,100mM) was dissolved in minimum quantity of water the total volume was made 100 mL. The pH of the solution was adjusted 7.2 with aq. KOH solution in DI water, stored at 4°C.

UV-θₘ experiments

Thermal denaturation of the cis and trans modified oligomers was performed using a 10 mm quartz cell in a Cary 300 Bio UV-Visible Spectrophotometer Varian.
mixture of 1µM oligomers and 1µM cDNA/cRNA were annealed in a 10 mMol potassium phosphate buffer pH 7.5, 150mMol NaCl. The concentration was calculated on the basis of absorbance from molar extinction coefficients of the corresponding nucleobases of DNA/RNA. Absorbance versus temperature profiles were obtained by monitoring the absorbance at 254 nm from 10–85°C at a ramp rate of 0.5°C per minute. A stream of dry nitrogen was gently applied through the sample compartment to prevent condensation of water on the cuvette at low temperatures.

**Snake venom phosphodiesterase stability experiments**

Enzymatic hydrolysis of the oligonucleotides (7.5 µM) was carried out at 37 °C in buffer (100 µl) containing 100 mM Tris-HCl (pH 8.5), 15 mM MgCl2, 100 mM NaCl and SVPD (10µg/mL). Aliquots were removed at several time-points; a portion of each reaction mixture was removed and heated to 90 °C for 2 min to inactivate the nuclease. The amount of intact ONs was analyzed at several time points by RP-HPLC. The percentage of intact ON was then plotted against the exposure time to obtain the ON degradation curve with time.

**Polyacrylamide Gel Electrophoresis (PAGE)**

**Preparation of Gel and buffer solutions**

1. **5X TBE buffer (500 ml,pH 8.0)**
   
   Tris (hydroxymethyl)methyl amine (Tris base) 27g, boric acid 13.75g and EDTA 1.462g was dissolved in 500 ml of deionised water (DI water). The pH was adjusted to 8.0 with tris base or HCl.

2. **1X TBE buffer (500 ml)**
   
   100ml of 5X TBE buffer was diluted to 500ml with DI water.

3. **29:1 acrylamide : N,N-methylene bis-acrylamide solution.**
   
   29g of acrylamideand 1g of N,N-methylene bis-acrylamide was dissolved in 100ml of DI water.

4. **Bromophenol Blue dye (marker dye)**
   
   40% of glycerol solution in DI water (V/V) + 0.25% Bromophenol Blue dye solution was prepared.

5. **40% sucrose solution in DI water (w/v).**
This solution was mixed with the Bromophenol Blue dye solution and the sample to be loaded in 1:1 ratio, to make the loading solution viscous and heavy for proper loading into the gel wells.

6. **10% ammonium persulfate solution.**

50mg ammonium persulfate was dissolved in 500 µl DI water. Fresh solution prepared just before preparing the gel for casting.

7. **20% acrylamide gel.**

To prepare 10 ml gel solution, 6.66 ml of acrylamide (29:1) + 1.27 ml DI water + 2 ml of 5X TBE buffer were mixed and degassed for 15-20 minutes under vacuum. Then 70 µl of ammonium persulfate was added followed by 4.6 µl of TEMED (N,N,N',N'-tetraethylmethylenediamine)

8. **Gel casting procedure.**

The above prepared gel solution after swirling for a minute was immediately poured into the previously cleaned and fixed gel plates. The appropriate gel comb was inserted for the formation of wells. Then the cast gel was allowed to stand for 40 minutes or more till it was set to a proper polymerised gel.

9. **Gel experiment**

**Pre-run:** The set gel was rinsed with DI water to remove any excess of unpolymerised gel after the comb was removed. For a pre-run or blank run, the gel plate assembly was placed in the gel run chamber and completely immersed in the 1X TBE buffer. Each well was loaded with 2 µl of the bromophenol blue dye in 40% sucrose solution(1:1). Then 200V voltage was applied and the gel run was carried out at 4°C for 1 hour till the marker dye had travelled down and washed out along with any unpolymerised gel.

**Sample run:**

The DNA oligomer samples 2 µl solution (350 µmol concentration) were mixed with equal volume of the 40% sucrose solution and loaded into the appropriately numbered wells. The marker dye was loaded in the first and last wells to monitor the run. The gel was run with the voltage set at 150V for 120 min till the marker was visible at 3/4 th the gel height.

**Gel visualization:**

The gels after run were washed with DI water and then were visualized by UV-shadowing.
### 3B.11 Appendix

<table>
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<tr>
<th>Compounds - Spectral data</th>
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<td>HPLC &amp; MALDI-TOF of TBA-3T&lt;sup&gt;cis&lt;/sup&gt;</td>
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<tr>
<td>HPLC &amp; MALDI-TOF of TBA-7T&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>160</td>
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</table>
HPLC & MALDI-TOF of TBA-3^{cis}.

![HPLC and MALDI-TOF chromatograms](image)
HPLC & MALDI-TOF of TBA-7T\textsuperscript{cis}:

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hplc_maldi.png}
\caption{HPLC-TOF fingerprint of TBA-7T\textsuperscript{cis} in Negative Ion Mode.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mass_spectra.png}
\caption{Mass spectrum of TBA-7T\textsuperscript{cis}.}
\end{figure}

TBA-7T\textsuperscript{cis}

Mass calcd. 4710
obsd. 4709
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HPLC & MALDI-TOF of TBA-3T$^{\text{trans}}$: 

![HPLC and MALDI-TOF spectra](image)

**TBA-7T$^{\text{trans}}$**

Mass calcd. 4710
obsd. 4714
Chapter 3

HPLC & MALDI-TOF of TBA-7T$^\text{trans}$:

![HPLC & MALDI-TOF Graph]

**TBA-7T$^\text{trans}$**
Mass calcd. 4710
obsd. 4708
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3B.12 References


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