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Ene-nucleic acids: A different paradigm to DNA chemistry

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The acyclic prochiral nucleic acids such as FNA, UNA GNA and the cyclic chiral TNA to are all considered as precursors of DNA and RNA in the chemical etiology of nucleic acids. The chemical reasoning would suggest that the unsaturated precursors with constrained flexibility and selectivity based on *cis/trans* isomers could be the missing link between the prochiral-acyclic and chiral-cyclic structures mentioned above. We find that the ene-nucleic acids derived from isoprenoid skeleton possess requisite flexibility and rigidity while forming stable duplex structures with complementary DNA and RNA

Introduction

The path-breaking work of Eschenmoser indicated that Watson-Crick base pairing in nucleic acids could be achieved from the other alternatives of ribose-based natural nucleic acids.[1,2] It is suggested that simple acyclic nucleic acids might be preliminary nucleic acids[3] which ultimately have evolved as present day carriers of genetic information.

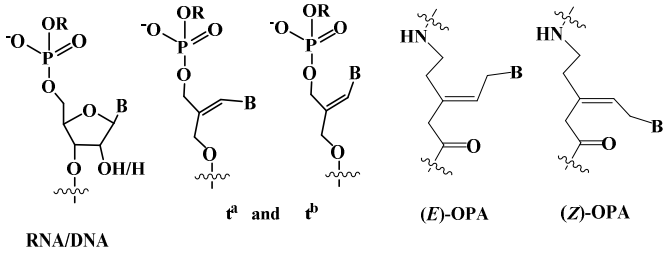

Fig. 1 Chemical structures of DNA/RNA and nucleotide mimics with an ethylene linker to nucleobase

The acyclic nucleic acids such as Flexible nucleic acids FNA,[4] unlocked RNA[5] and the glycerol-based nucleic acids[5] destabilized the duplexes with cDNA, probably due to flexibility in the backbone and large entropic loss while duplex formation. GNA would be less flexible than FNA or UNA due to less number of flexible bonds in the monomer unit. To counter the entropic loss, an attempt was made by introducing a double bond in the acyclic structure. Incorporation of these thymidine nucleosides mimics (Fig. 1, t^{α} and t^{β}) in oligomers was also found to be detrimental to the duplex stability[6] similar to the other acyclic derivatives. We presume that the attachment of nucleobase directly to the double bond in this case may have conferred considerable unnecessary rigidity, leading to reduced ability of the nucleobase to take part in specific W-C

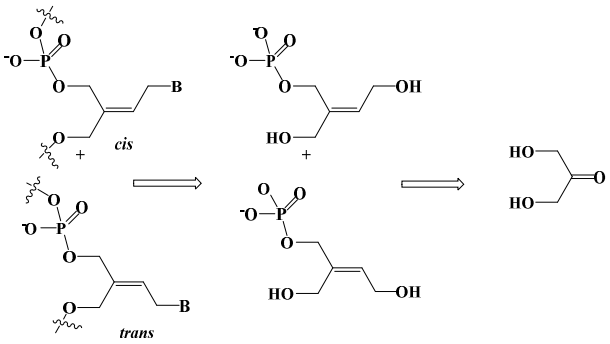

Fig. 2 Proposed *cis*- and *trans*- Ene-nucleic acids

hydrogen bonding. Later, a homooligomeric GNA was synthesized by Meggers [7] The optically pure (*S*)-GNA could also cross-pair with RNA though with much reduced stability. This means that the reduced flexibility in GNA as compared to FNA could lead to stable duplex structures when the nucleobase attachment is kept flexible through a methylene group. The *iso*GNA later studied by Krishnamurthy *et al.* also destabilized duplexes, probably as the nucleobase attachment was directly to the backbone.[8] In an earlier study, the *cis*/*trans* olefinic peptide nucleic acids (Fig. 1, *E/Z* OPAs) were synthesized to delineate the ambiguity regarding rotameric conformations and to elucidate the structural and electronic role of the tertiary amide group in PNA.[9] This design prompted us to visualize an acyclic ene-nucleic acid (Fig. 2, Ene-NA) in which the nucleobase attachment is to a planar double bonded structure through a methylene group, having same number of bonds like natural ribose sugar and a constraint of double bond unsaturation instead of sugar ring. The *cis* or *trans* geometry of the proposed ene-nucleotides would be interesting to study with respect to th

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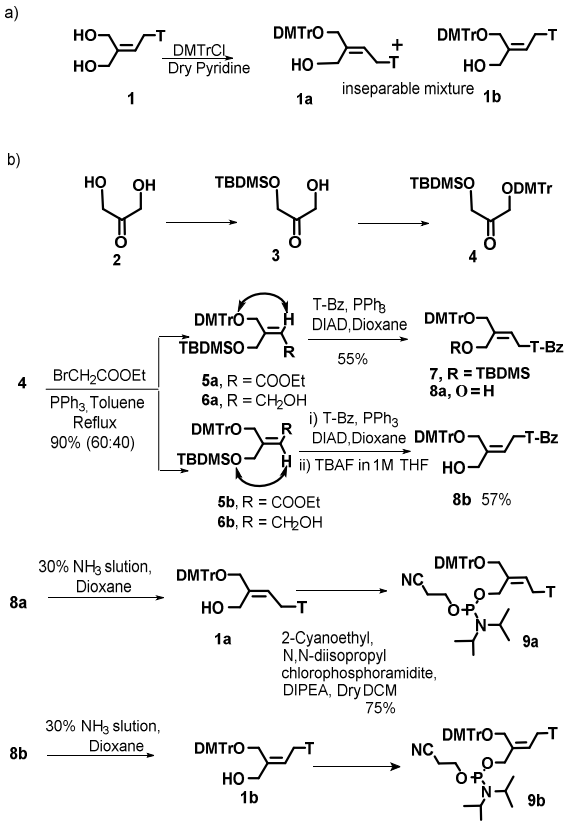
thermal stability of the nucleic acid complexes as well as the stability of the modified oligomers against enzymatic degradation. In this paper we describe the synthesis of the *cis* and *trans* thymine containing monomers, synthesis of oligomers comprising these monomers and show that the mixed Pu/Py duplexes with cDNA and cRNA are quite stable. The pyrimidine sequences with multiple units were destabilized, and the replacement of thymidines in the loop region of quadruplex DNA was found less acceptable. Interestingly, the cis isomer was found to impart stability towards enzymatic digestion compared to the trans isomer.

Results and discussion

Synthesis of monomers

The nucleoside derivative **1** was synthesized by a known procedure in the literarture.[10] Conversion of **1** to mono-DMTr derivatives could be accomplished but the *trans* **1a** and *cis* **1b** compounds could not be separated (Scheme 1a) by repeated flash column chromatography as there was no difference in the Rf on silica gel.



Scheme 1 Synthesis of *cis*- and *trans*- ene-nucleoside phosphoramidites.

We therefore started the synthesis all over again from dihydroxy acetone **2.** Compound **2** was monoprotected[11] with TBDMS to get **3** and subsequently with DMTr to get **4** (Scheme 1, b). Wittig reaction[12] with ethylbromo acetate and triphenyl phosphine yielded the mixture of *trans* **5a** and *cis* **5b** α, β-unsaturated esters in more than 90% yield in 6:4 ratio. At this stage the two compounds *trans* **5a** and *cis* **5b** could be separated with very careful column chromatography and were identified by nOe experiment. (Supporting information). The DMTr*O*- group is considered to be corresponding to 5'-position and compound with nucleobase on the side of 5'-position is considered as *cis* isomer. DIBAL-H reduction of each ester gave allylic alcolhols **6a** and **6b** respectively. Compound **6a** and **6b** were then converted to nucleoside derivatives using N3-Bz-thymine under Mitsunobu conditions.[13] The *trans* compound **7** was isolated by column chromatography but the *cis* isomer was contaminated with triphenylphosphine oxide. Further deprotection of silyl group using TBAF in THF gave pure compounds **8a** and **8b** in 55-57 % overall yield. The silyl-deprotection followed by ammonia treatment in dioxane:water gave DMTr-protected *trans* **1a** and *cis* **1b** ene- nucleosides. The compounds **1a** and **1b** were then subjected to phosphitylation[14] to get corresponding amidites **9a** and **9b** respectively. All new compounds in Scheme 1b were characterized by ^{1}H, ^{13}C, HRMS analysis. The phosphoramidite derivatives **9a** and **9b** were characterized by ^{31}P NMR spectroscopy (Supporting information).

Synthesis of oligomers and UV-melting studies

These ene-thyminyl amidites were used to synthesize modified DNA sequences by substituting the thymidine residues at predetermined positions in the sequences using solid phase DNA synthesis.[15] We used the deprotection and cleavage conditions for obtaining the modified oligomers as described earlier to avoid cleavage at the site of modification.[6] All the oligomers were purified by HPLC and purity was checked by gel-electrophoretic mobility studies (Supporting information). The unmodified 18 mer DNA sequence (DNA1) used in this study and the modified sequences are listed in Table 1. It is seen that the sequences modified with T-*cis* as well as T-*trans* are able to form stable sequences 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 (Supporting information). The results obtained are indeed in accordance with our design and as the base is separated by a methylelne group away from the central C = C the deviation caused is minimum for each individual case of *cis*/*trans* isomers as well. This is much more satisfactory than observed earlier *i.e* about 10-15 °C in the case of FNA[4] and UNA[5] and about 8 °C in the case of GNA[5] 5-6 °C in the case of t^{α}/ t^{β} [6] when single modified unit was present in the center of the sequence. In the case of 10 mer PNA, containing *cis*- and *trans*- olefinic PNA modification in the center of the sequence, the complexes with cDNA were destabilized by 14 °C and 6.5 °C, respectively.[9]

Table 1. 18 mer DNA sequence and DNA sequences in which the T^{cis} and T^{trans} units are present at the defined position, MALDI-TOF mass analysis and their UV-melting studies with complementary DNA and RNA sequences.

Name	Sequence[a] 5'→ 3'	MALDI TOF mass Cal /obs	UV T_m °C[b] DNA	RNA
DNA1	caccattgtcacactcca	5363/5367	63.5	62.7
DNA1-15T^{trans}	caccattgtcacac**T**^{trans}cca	5347/5342	60.2	59.7
DNA1-9T^{trans}	caccattg**T**^{trans}cacactcca	5347/5347	59.6	61
DNA1-15T^{cis}	caccattgtcacac**T**^{cis}cca	5347/5343	59.3	59.1
DNA1-9T^{cis}	caccattg**T**^{cis}cacactcca	5347/5344	60.8	59.6

a. lower case denote DNA backbone, upper case denote modified site in the sequence. b. The T_m values correspond to the mean values of minimum three experiments where the strand concentration is 1 µM each. All values are an average of at least 3 experiments and accurate to within ± 0.5 °C.

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 alternately in the sequence. The destabilization was about 8 °C/mod when the thymine units were continuously substituted and ~ 5 °C/mod when they were alternately substituted. The results are documented in

Table 2 12 mer DNA sequence and DNA sequences in which the T^{cis} units are present at the defined position, MALDI-TOF mass analysis and their UV-melting studies with complementary DNA sequences.

Name	Sequence[a] 5'→ 3'	MALDI TOF mass Cal /obs	UV T_m °C[b] cDNA
DNA2	gcg ttt ttt gct	3633/3635	51
DNA3	gcg ttT^{cis} T^{cis} T^{cis}tgct	3585/3585	26
DNA4	gcg T^{cis} tT^{cis} tT^{cis} tgct	3585/3582	34

a. lower case letters denote DNA backbone, upper case letters denote modified site in the sequence. b. The T_m values correspond to the mean values of minimum three experiments where the strand concentration is 1 μM each. All values are an average of at least 3 experiments and accurate to within ± 0.5 °C.

Table 2. In homothyminyl sequences the acyclic units were seldom tolerated and the duplexes formed were destabilized. Similar results were also obtained earlier in the case of GNA and UNA,[5] *iso*GNA[8] and OPA.[9]

Stability of oligonucleotide to SVPD

The phosphodiester linkages of DNA are cleaved by snake venom phoshodiesterase (SVPD) as 3'-exonuclease. We introduced the *cis*- and *trans*- thymine monomers at the 3'-end of thyminyl 10 mer sequence (t_{10}, $t_8\mathbf{T}^{trans}$t and $t_8\mathbf{T}^{cis}$t, Fig. 3). In our experiments with SVPD, we found that the unmodified t_{10} oligomer was completely digested within 10 min as expected. Surprisingly, we found differential tolerence of the *cis*/*trans* isomers to SVPD digestion. After the cleavage of 3'-terminal thymidine, the 9 mer $t_8\mathbf{T}^{trans}$ oligomer, containing *trans* isomer was completely digested by SVPD within 15 min whereas the the 9mer $t_8\mathbf{T}^{cis}$ oligomer containing *cis* isomer was stable with a half life of 1h and was about 10-15% available after 5 h (Fig. 3). The 9 mer sequence was isolated by HPLC and was confirmed by MALDI-TOF mass spectrometry (Supporting information).

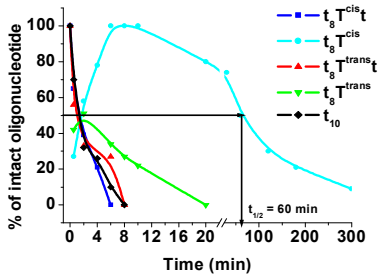


Fig. 3 Stability assay of ONS to degredation of SVPD; Digestion condition: Enzymatic hydrolysis of the ONs (7.5 μM) was carried out at 37 °C in buffer containing 100 mM Tris-HCl (pH 8.5), 15 mM $MgCl_2$, 100 mM NaCl and SVPD (100 μg/mL).

Such kind of discrimination towards hydrolytic enzymes is observed only in the case of enantiomers[16] and probably would be the first example in the literature when *cis-trans* isomers are differentiated by the SVPD enzyme digestion reaction.

Synthesis of G-quadruplex forming TBA sequences

Table 3 15 mer TBA sequence[a] and TBA sequences in which the T^{cis} and T^{trans} units are present at the defined position, MALDI-TOF mass analysis and their CD melting studies

Name	Sequence[a] 5'→ 3'	MALDI TOF mass Cal /obs	CD T_m °C[b]
TBA	ggttggtgtggttgg	4726/4730	49.5
TBA-3T^{cis}	gg$\mathbf{T}^{cis}$tggtgtggttgg	4710/4709	38
TBA-7T^{cis}	ggttgg$\mathbf{T}^{cis}$ gtggttgg	4710/4709	41.4
TBA-3T^{trans}	gg$\mathbf{T}^{trans}$tggtgtggttgg	4710/4714	36.1
TBA-7T^{trans}	ggtgg$\mathbf{T}^{trans}$gtggttgg	4710/4708	43.7

a. lower case letters denote DNA backbone, upper case letters denote modified site in the sequence All values are an average of at least 3 experiments and accurate to within ± 0.5 °C.

The acyclic UNA analogues mentioned earlier[5] were used by Wengel and co-workers to moderate the unrequired high stability of LNA:DNA duplexes.[17] In addition to modulating DNA:RNA duplex stability in LNA/UNA mixmers, the acyclic UNA analogue found excellent application in stabilizing loop structure in aptamers due to its ability to alleviate strain in quadruplex loop structure[18] of thrombin binding aptamer (TBA).[19] We studied the constrained flexibility parameter of our ene-NA modification 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 or T7 positions of thymidine by UNA units was found to stabilize the quadruplex structure of TBA. We chose these two positions for replacing the thyminyl units of TBA by $\mathbf{T}^{trans}$ and $\mathbf{T}^{cis}$ monomers to study its effect on the quadruplex stability. The synthesized sequences are listed in Table 3. The stability of the quadruplex formed as studied by temperature dependent CD studies (Table 3, Supporting information).[18,20] Substitution of thymidines by UNA stabilized the quadruplex structures by 1.6 °C and 4 °C at T3 and T7 positions respectively whereas in our studies the structures were destabilized at both T3 and T7 positions by *cis* as well as *trans* modified units. This may indicate that the ene-NA modification is indeed more constrained compared to UNA and is less suitable for quadruplex formation compared to the highly evolved DNA quadruplexes.

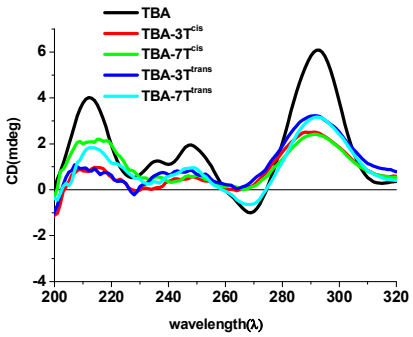


Fig. 4 CD spectra of TBA and modified TBA sequences

The reduced stability of the G-quadruplex structures due to the introduction of modification by $\mathbf{T}^{cis}$ or $\mathbf{T}^{trans}$ units was also evident by the CD signals at 295 nm (+ve band) and 265 nm (– ve band), known to be the signature for antiparallel G-quadruplexes,[21] formed by TBA sequences (Figure 4). The positive CD signal at 295 nm showed reduced intensity in each case where modified $\mathbf{T}^{cis}$ or $\mathbf{T}^{trans}$ units were present. The –ve CD band was absent when the modified units destabilized the structure to a larger extent (ΔTm = 8-12 ^{o}C). Only $\mathbf{T}^{trans}$ unit when present at T7 position retained all the CD signals as in unmodified TBA, when the destabilization was minimum (ΔTm = 6 ^{o}C).

Conclusions

In conclusion, we designed, synthesized and studied the compatibility of novel prebiotically plausible ene-NA anlogue in duplex and quadruplex DNA for the first time. The stability of duplexes formed by ene-NA modified oligomers with cDNA/RNA was found to be better compared to other reported acyclic DNA analogues. The constrained structure however, destabilized quadruplex TBA structure compared to UNA. The *cis*/*trans* ene-NA showed diffrential enzymatic stability towards hydrolytic enzyme, the *trans* isomer being almost as prone to hydrolytic cleavage as natural DNA in comparison with the more stable *cis* isomer. This may suggest that this novel ene-DNA analogue could be a missing link between the other suggested acyclic prochiral nucleic acids and the chiral DNA/RNA.

Experimental

General information

All the reagents were purchased from Sigma-Aldrich and used without further purification. SVPD was purchased from Sigma. DMF, pyridine were dried over KOH and 4 Å molecular sieves. TLCs were run on pre-coated silica gel GF254 sheets (Merck 5554). All reactions were monitored by TLC and usual workup implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulphate and evaporation under vacuum. Column chromatography was performed for purification of compounds on silica gel (60-120 mesh or 100–200 mesh, Merck). TLCs were carried out on precoated silica gel 60 F254 (Merck), and were performed using dichloromethane–methanol or petroleum ether–ethyl acetate solvent systems for most compounds. Compounds were visualized with UV light and/or by spraying with 30% perchloric acid/EtOH solution and heating. ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker ACF 200 spectrometer fitted with an Aspect 3000 computer and ^{31}P NMR spectra were recorded on a 400 MHz Bruker ACF instrument. All the chemical shifts (δ/ppm) are referred to internal TMS for ^{1}H and chloroform-*d* / DMSO-*d*6 for ^{13}C NMR. ^{1}H NMR data are reported in the order of chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet and/or multiple resonance), number of protons. Mass spectra were recorded on an APQSTAR spectrometer, LC-MS on a Finnigan-Matt instrument. High resolution mass spectra were recorded on a Thermo Fisher Scientific Q Exactive mass spectrometer. DNA oligomers were synthesized on CPG solid support using Bioautomation Mer-Made 4 synthesizer. The RNA oligonucleotides were obtained commercially (Sigma-Aldrich). RP-HPLC was carried out on a C18 column using a Waters system (Waters Delta 600e quaternary solvent delivery system and 2998 photodiode array detector and Empower2 chromatography software). MALDI-TOF spectra were recorded on a AB Sciex TOF/TOF™ Series Explorer™ 72085 instrument and the matrix used for analysis was THAP (2', 4', 6'-trihydroxyacetophenone). UV experiments were performed on a Varian Cary 300 UV-VIS spectrophotometer fitted with a Peltier-controlled temperature programmer. CD spectra were recorded on a Jasco J-715 Spectropolarimeter, with a ThermoHaake K20 programmable water circulator for temperature control of the sample.

1-((tert-butyldimethylsilyl)oxy)-3-hydroxypropan-2-one (3) Compound **2** (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 10 h 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 Na_2SO_4 and concentrated on rotavapor *in vacuo*. Crude compound purified through column chromatography (pet ether:EtOAc, 90:10) to result **3** (6.2 g, 55%) as a colour less thick liquid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.10 (s, 6 H), 0.93 (s, 9 H), 3.01 (t, *J*=4.99 Hz, 1 H), 4.32 (s, 2 H), 4.51 (d, *J*=4.93 Hz, 2 H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$) δ -5.7, 18.1, 25.7, 66.6, 67.7, 211.1 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-butyl dimethyl silyl) oxy) propan-2-one (4) To a solution of **3** (5 g, 24.5 mmol) in pyridine (15 mL) DMTr chloride (10 g, 29.5 mmol) and catalytic amount of DMAP were added, stirred at rt for 6 h. Pyridine 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_2SO_4, concentrated in *vacuo*. The residue was subjected to silica gel column chromatography (pet ether:EtOAc, 95:5) to afford **4** (9.3 g) in 75% yield.

^{1}H NMR (200 MHz, $CDCl_3$) 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, 158.7 206.8 ppm; HRMS (EI): Mass calculated for $C_{30}H_{38}O_5NaSi$ (M+Na) 529.2381, found 529.2369.

Ethyl (Z)-4-(bis (4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy) methyl)but-2-enoate (5a & 5b) Solution of **4** (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_2SO_4, concentrated in *vacuo* followed by column chromatography (pet ether: EtOAc, 98:2) to give a **5a** and **5b** (90%) in 60:40 ratio. ^{1}H NMR(5a) (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 H, 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 $C_{34}H_{44}O_6NaSi$ (M+Na) 599.2799, found 599.2789. ^{1}H NMR(5b) (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, 59.8, 62.3, 63.5, 86.5, 113.0, 113.1, 126.8, 127.8, 128.1, 129.1, 129.9, 130.0, 136.0, 144.8, 158.5, 158.8, 166.4 ppm**;** HRMS (EI): Mass calculated for $C_{34}H_{44}O_6NaSi$ (M+Na) 599.2799, found 599.2790.

(Z)-4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl) but-2-en-1-ol (6a) DIBAL-H Was added to a solution of ester **5a** (1 g, 2.6 mmol) in DCM at -78 ^{o}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 1 h at which organic layer appears like clear solution. Organic layer was separated and washed with brine solution and extracted with DCM, dried over Na_2SO_4.Compound was purified through column chromatography (pet ether:EtOAc, 70:30) to obtain **6a** (0.71 g) 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 $C_{32}H_{42}O_5NaSi$ (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 (7) To a solution of **6a** (0.5 g, 0.93 mmol) in dry dioxane (4 mL) was added triphenyl phosphine(0.37 g, 1.4 mmol) and N^3-benzoyl protected thymine (0.32 g, 1.4 mmol) stirred for 15 min. DIAD (0.36 mL, 1.86 mmol) was dissolved in 1 mL dry dioxne 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_2SO_4, concentrated in vacuo. Crude residue was subjected to silica gel coloumn chromatography (pet ether:EtOAc, 70:30) to obtain **7** (0.38 g) in 55% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ 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, $CDCl_3$) δ -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.

(Z)-3-benzoyl-1-(4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(hydroxymethyl)but-2-en-1-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (8a) Compound **7** (1 g, 1.28 mmol) was dissolved in 15 mL THF and TBAF (0.394 g, 1.5 mmol) was added. The reaction mixture was stirred for 2 h 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_2SO_4 and concentrated under reduced pressure. The resulting residue was purified on silica gel column chromatography (pet ether:EtOAc, 60:40) to yield **8a** (0.7 g) in 83%. ^{1}H NMR (200 MHz, $CDCl_3$) δ 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, $CDCl_3$) δ 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 $C_{38}H_{36}O_7N_2Na$ (M+Na) 655.2415, found 655.2398.

(E)-4-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-(((tertbutyldimethylsilyl)oxy)methyl) but-2-en-1-ol (6b) DIBAL-H was added to a solution of ester **5b** (1 g, 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 1 h at which organic layer appears like clear solution. Organic layer was separated and washed with brine solution and extracted with DCM, dried over Na_2SO_4.Compound was purified through column chromatography (pet ether:EtOAc, 70:30) to obtain **6b** (0.67 g) in 75% yield. ^{1}H NMR (200 MHz, $CDCl_3$) 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; ^{13}C NMR (125 MHz, $CDCl_3$) 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 $C_{32}H_{42}O_5NaSi$ (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 (8b) Compound **6b** was subjected for mitsunobu reaction and without purification the crude mixture used for TBDMS deprotection to obtain **8b** in 57% yield over two steps. ^{1}H NMR (500 MHz, $CDCl_3$) 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; ^{13}C NMR (125 MHz, $CDCl_3$) 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, 130.5, 131.7, 135.0, 135.5, 139.5, 142.2, 144.4, 149.8, 158,. 163.1, 169.1 ppm; HRMS (EI): Mass calculated for $C_{38}H_{36}O_7N_2Na$ (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 (1a) 30% aq. ammonia solution (0.5 mL) was added to a solution of **8a** (0.5 g, 0.76 mmol) in 10 mL dioxane and stirred for 7 h 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_2SO_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 **1a** (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, 139.3, 141.8, 144.4, 150.8, 158.7, 164.1 ppm; HRMS (EI): Mass calculated for $C_{31}H_{32}O_6N_2Na$ (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 (1b) 30% aq. ammonia solution (0.5 mL) was added to a solution of **8** (0.5 g, 0.76 mmol) in 10 mL dioxane and stirred for 7 h 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_2SO_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 **9** (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; HRMS (EI): Mass calculated for $C_{31}H_{32}O_6N_2Na$ (M+Na) 551.2153, found 551.2145.

General procedure followed for the synthesis of phospharamidite derivatives 9a, 9b To the compound 1a, 1b (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 h. 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. ^{31}P NMR **9a** (500 MHz, $CDCl_3$) δ 148.82 HRMS (EI): Mass calculated for $C_{40}H_{49}O_7N_4NaP$ (M+Na) 751.3231, found 751.3212. ^{31}P NMR **9b** (500MHz, $CDCl_3$) δ 148.27 HRMS (EI): Mass calculated for $C_{40}H_{49}O_7N_4NaP$ (M+Na) 751.3231, found 751.3212.

Synthesis of oligonucleotides

The 18 mer DNA sequence chosen for the current study are of biological relevance, **DNA1** is used for miRNA down-regulation.[14] Unmodified oligomers were synthesized using commercially available phosphoramidite building blocks. Modified oligonucleotides were synthesized using phenoxyacetyl (Pac) protected cyanoethyl phosphoramidites and modified amidite building blocks **9a**, **9b**. The modified phosphoramidites 0.1 M in CH_3CN were manually coupled for 6min, followed by washing step with 10% H_2O, 0.2% Ac_2O, 0.2% Lutidine v/v/v in THF done to avoid the unwanted phosphitylation at bases of highly reactive acyclic olefinic monomers. After washing capping followed by oxidation with 0.5 M tert-butyl hydroperoxide in CH_2Cl_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]. For the modified units, double coupling (300 s X 2) was performed. Deprotection and cleavage were performed by shaking the support bound oligonucleotide with neat dry diisopropylamine, washing with diethylether followed by shaking with conc aq. ammonia for 2 h at rt[12]. The crude oligomer was purified by RP-HPLC. Purity of oligomers were confirmed by gel-electrophoretic mobility studies and characterized by MALDI-TOF mass spectrometry.

UV-*Tm* Measurements

The concentration was calculated on the basis of absorbance from molar extinction coefficients of the corresponding nucleobases of DNA/RNA. The experiments were performed at 1 μM concentrations. The complexes were prepared in 10 mM sodium phosphate buffer, pH 7.2 containing NaCl (150 mM) and were annealed by keeping the samples at 90 oC for 2 min followed by slow cooling to room temperature and refrigeration for at least two hours prior to running the expeients. Absorbance versus temperature profiles were obtained by monitoring the absorbance at 260 nm from 10–85 °C at a ramp rate of 0.5 °C per minute. The data were processed using Microcal Origin 6.1 and *T*m (°C) values were derived from the maxima of the first derivative plots.

CD experiments

CD experiments were done for the TBA sequences. The 5 μM concentration of each strand was used for the sample preparation. The complexes were prepared in 10 mM potassium phosphate buffer, pH 7.2 containing KCl (100 mM) and were annealed by keeping the samples at 90 °C for 2 min followed by slow cooling to room temperature and refrigeration for at least four hours prior to running the experiments. CD spectra were recorded in a 2mm pathlength cuvette, using a resolution of 1 nm, bandwidth of 1nm, sensitivity of 20 mdeg, response of 1s and a scan speed of 100 nm/min. Spectral scans were collected at 4 °C over a wavelength range 200- 320 nm at a scanning rate of 100 nm min-1. CD melting was performed for the entire sample by monitoring CD intensity at 295 nm against temperature over a range of 5-90 °C. Three scans were averaged for each sample.

Nuclease resistance study

Enzymatic hydrolysis of the ONs (7.5 μM) was carried out at 37 oC in buffer (100 μl) containing 100 mM Tris-HCl (pH 8.5), 15 mM MgCl2, 100 mM NaCl and SVPD (100 μg/mL). Aliquots were removed at several time-points; a portion of each reaction mixture was removed and heated to 90 oC 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.

Gel Experiments

Purity of synthesized oligomers was assessed by non-denaturing 30% polyacrylamide gel electrophoresis. Pre run was done by loading each well with 2 μl of the bromophenol blue dye in 40% sucrose solution(1:1) and run carried out in 1X TBE buffer applying 200 V voltage at 4 °C for 1 h till the marker dye had travelled down and washed out along with any unpolymerised gel. The DNA oligomer control and samples 2 μl solution (350 μM concentration) mixing with equal volume of the 40% sucrose solution loaded into the appropriately numbered wells. The gel was run with the voltage set at 150 V for 120 min till the marker was visible at 3/4th the gel height. The gels after run were washed with DI water and then were visualized by UV-shadowing. For denaturing gel-experiments 7 M Urea were used for gel casting, while 2 μL of 350 μM sample in DI-water mixed with 2 μL formamide for loading, and gel were run in 1X TBE buffer at 25 °C by applying 150 V voltage.

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