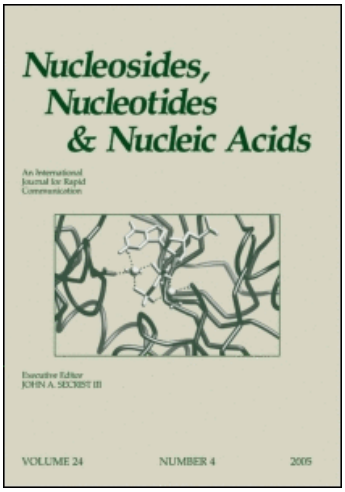

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Synthesis and Biophysical Studies of Modified Oligonucleotides Containing Acyclic Amino Alcohol Nucleoside Analogs

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SYNTHESIS AND BIOPHYSICAL STUDIES OF MODIFIED OLIGONUCLEOTIDES CONTAINING ACYCLIC AMINO ALCOHOL NUCLEOSIDE ANALOGS

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Abstract: Novel serine derivative of thymine was prepared and incorporated into oligonucleotides. These modified oligonucleotides were studied for their binding affinity with complementary DNA/RNA.

Modified oligonucleotides are becoming increasingly important because they specifically recognize messenger RNA[1] and present unique opportunities for the treatment of viral diseases, cancer, and for the study of genetic disorders. The most extensively studied oligonucleotide analogues are those containing modified phosphate linkages such as phosphorothioates[2] and methylphosphonates.[3] Several other modified oligonucleotides have been reported[4-10] in which the phosphodiester linkage has been replaced with other linkages and studied for their biological activities. Peptide nucleic acids[11] and amino acid based nucleic acids[12] have also been prepared in an effort to overcome the problems of passive membrane transport and nuclease susceptibility. Each type of modification has characteristic properties: The peptide-based oligonucleotide derivatives[13] are poorly water soluble, while acyclic oligonucleotides are stable against enzymatic degradation but do not form stable duplexes with complementary oligonucleotides[14,15] presumably because of the highly flexible structures. Moreover, various groups have described the synthesis and the biophysical properties of oligonucleotides containing 2',5' linkages[16-19]

as well as oligonucleotides with alternating 3',3' and 5',5' internucleotide phosphodiester linkages.[20] Thus, the interest to develop new and novel modified oligonucleotides, based on sequence specific interactions between complementary nucleic acids is rapidly growing.

We sought to synthesize a novel class of oligonucleotide reflecting the structural rigidity of DNA coupled with the idea of studying the properties of oligonucleotides containing mixed (2',5' and 3',5') internucleotide linkages has led us to envision amino alcohol nucleic acids (AANA), i.e., molecules where the individual nucleobases are linked to an amino alcohol having an amide bond and phosphodiester backbone. The oligonucleotide analogue derived from serinol is shown in Figure 1, (**2**). Interestingly, this novel acyclic molecule **2** has two modifications. One is the 2',5'-like phophodiester linkage and the other is the acyclic amino alcohol based nucleoside monomer with amide functionality. These modifications have been introduced for the purpose of constructing rigid acyclic oligonucleotides.

The structure of amino alcohol nucleic acid is attractive for several reasons. First, serinol nucleosides[21] are simpler to prepare than ribonucleosides. Second, the AANA has the possibility to form an intramolecular hydrogen bonding (see Figure 1) between the amide "NH" and the phosphate "O" under physiological conditions. We believe that such an intramolecular hydrogen bonding would force the AANA molecule to adopt a rigid conformation. Such a constrained conformation is appropriate for binding of modified oligonucleotides to a target nucleic acid in sequence specific manner. In addition, the presence of amide groups in **2** might reduce the flexibility and also the freedom of rotation of the molecule. Thus, we hoped that the combined properties could have favorable effect on the thermal stability of duplexes formed by **2** with natural oligonulceotide targets.

In this communication, we report the synthesis of a novel oligonucleotide building block **12**, constructed for the first time from an optically active L-serine and thymine, incorporation of **12** into oligonucleotides and measurement of hybridization of the duplex formed between AANA and natural DNA/RNA.

The optically active amino acid nucleoside analogue bearing thymine was prepared from *O*-benzyl-L-serine by the route shown in Scheme 1. Reaction of *O*-

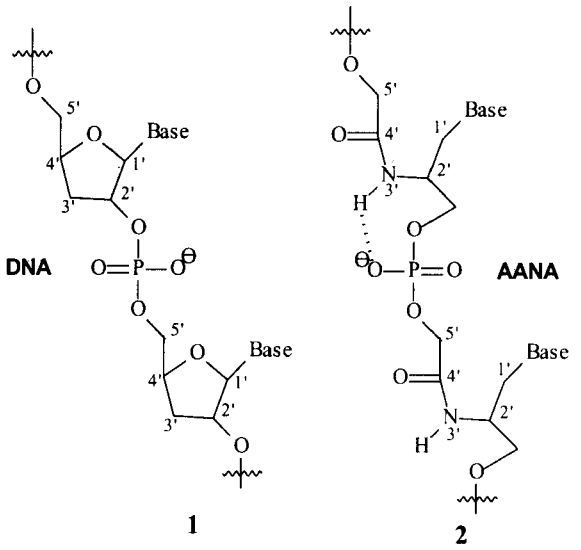
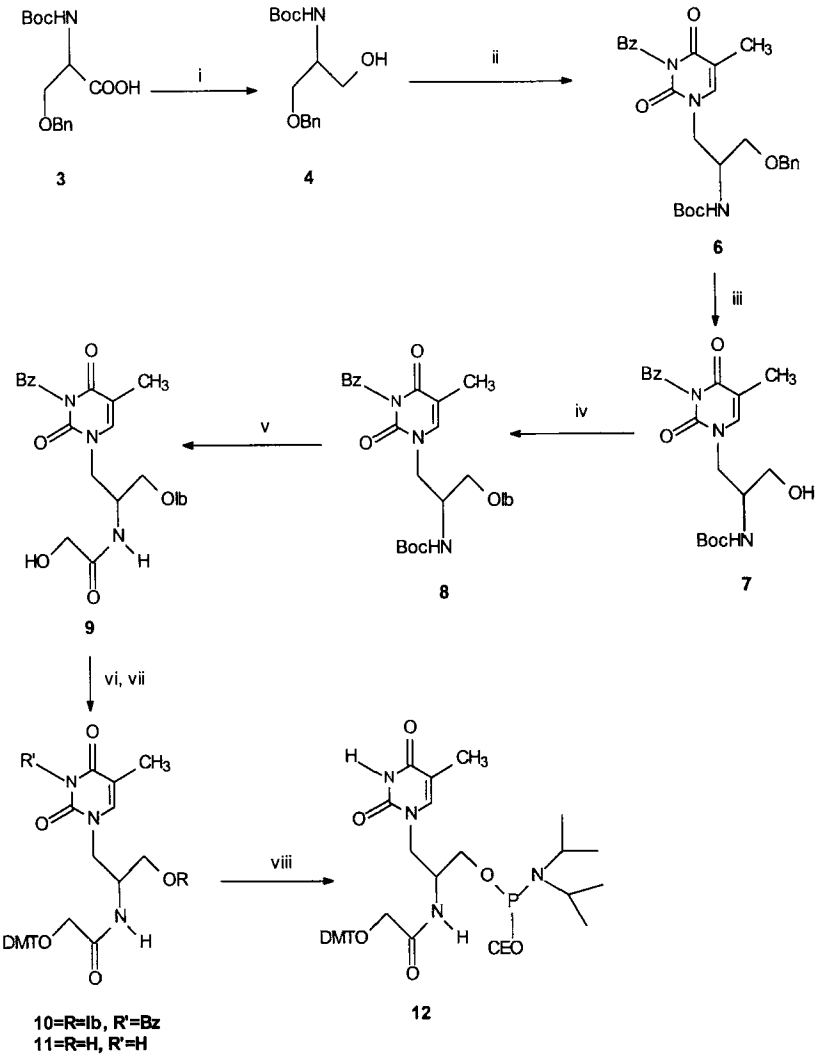


Figure 1

benzyl-L-serine with di-*tert*-butyl dicarbonate provided N^{α}-Boc-*O*-benzyl-L-serine (**3**) in quantitative yield. Treatment of **3** with isobutyl chloroformate followed by N-methylmorpholine at -15°C under argon atmosphere for 30 minutes produced the corresponding active ester, which without isolation was reduced with sodium borohydride in water/tetrahydrofuran mixture to furnish[22] an alcohol (**4**). Mitsunobu alkylation[23] of N_3-Benzoylthymine (**5**)[24] with **4** in the presence of triphenylphosphine and diethyl azodicarboxylate produced serinol nucleoside (**6**)[21] in 86% yield. Hydrogenation of **6** with 10% Pd/C in methanol gave 2-(*tert*-butyloxycarbonyl)amino-3-(N_3-benzoylthymin-1-yl)-L-propan-1-ol (**7**). Protection of the hydroxyl group of **7** with isobutyric anhydride in pyridine provided the isobutyryl derivative (**8**) in quantitative yield. Removal of the *t*-Boc group in **8** with trifluoro acetic acid followed by coupling of the corresponding TFA salt with glycolic acid containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-methylmorpholine afforded an alcohol (**9**) in 92% yield. Dimethoxytritylation[25] of **9** with dimethoxytrityl chloride and triethylamine in pyridine gave the corresponding DMT derivative **10,** which on treatment with 1N NaOH solution in MeOH/THF for 30 minutes at room temperature furnished the key intermediate (**11**) in



Scheme 1[a]

[a]i) IBCF/NMM/$NaBH_4$/THF (86%). ii) DEAD/Ph_3P/N_3-benzoylthymine/THF (86%). iii) 10%Pd/C/MeOH (90%). iv) $(IbO)_2O$/Py (85%). v) (a) TFA/CH_2Cl_2; (b) glycolic acid/EDC/NMM/DMF (92%). vi) DMTCl/TEA/Py (83%). vii) 1N NaOH/MeOH/THF (99%). viii) $EtN(iPr)_2$/P(Cl)$(iPr)_2OCH_2CH_2CN/CH_2Cl_2$ (85%).
t = *tert*-butyloxycarbonyl; CE = Cyanoethyl; Bz = Benzoyl; Ib = Isobutyryl.

TABLE 1: Tm values of Oligonucleotides Containing L-Amino Alcohol Nucleic Acids[a]

Sequence	Tm(°C) DNA	Tm(°C) RNA	ΔTm /mod (°C) DNA	ΔTm /mod (°C) RNA
5' GAA AGG AAG CGG AGA GAT 3'				
5' ATC TCT CCG CTT CCT TTC 3'	59.2	66.8		
5' ATC TCT CCG CTT CCT TtC 3'	58.4	66.0	-0.8	-0.8
5' ATC TCT CCG CTT CCt ttC 3'	55.4		-1.3	
5' AtC TCT CCG CTT CCT TTC 3'	58.6	65.8	-0.6	-1.0
5' ATC TCT CCG CtT CCT TTC 3'	50.5	61.0	-8.7	-5.8
5' AtC tCt CCG CTT CCT TTC 3'	45.2	54.8	-4.7	-4.0

[a]Tm is the temperature at the midpoint of the melting curve; The concentrations are as follows: Oligomer strands, 2 μM each. Melting temperatures (Tm) were determined[30] by measuring change in absorbance at 260 nm (cuvette, 1-mm path length) as a function of temperature in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA. All the values are averaged from at least three experiments. The letter "t" denotes modified thymidine. ΔTm /mod represents the change in Tm per modification. The negative (-) values indicates the decrease in Tm value per single incorporation of "t".

excellent (99%) yield. Phosphitylation[26] of **11** with β-cyanoethyl-N,N-diisopropylchlorophosphoramidite in the presence of N,N-diisopropylethylamine in anhydrous CH_2Cl_2 for 2 h provided the novel amino alcohol nucleic acid amidite synthon **12** in 82% yield.[27]

Incorporation of the building block **12** into oligonucleotide sequences was accomplished using ABI 394 DNA synthesizer and protocol,[28] and the coupling efficiency was found to be higher than 99%. Enzymatic degradation and subsequent HPLC analysis of the modified oligonucleotides indicated the expected ratios of the nucleoside components.[29]

Binding properties of the amino alcohol nucleic acid analogs with complementary DNA or RNA strands were evaluated,[30] and the results are summarized in Table 1. Compared to unmodified DNA, substitution of **12** at 3'-end of an oligonucleotide has very little effect on duplex stability. For a single substitution the Tm decreased by 0.8°C (Table 1, line 3). This trend continued up to three incorporations of **12** at the 3'-end (Table 1, line 4). A similar binding behavior is observed when **12** is incorporated at the 5'-end also (Table 1, line 5). On the contrary, substitution of **12** in the middle of an

TABLE 2: Tm values of Oligonucleotides Containing D-Amino Alcohol Nucleic Acids[a]

Sequence	Tm(°C) DNA	Tm(°C) RNA	ΔTm /mod (°C) DNA	ΔTm /mod (°C) RNA
5' GAA AGG AAG CGG AGA GAT 3'				
5' ATC TCT CCG CTT CCT TTC 3'	59.2	66.8		
5' ATC TCT CCG CTT CCT TtC 3'	58.5	66.4	-0.7	-0.4
5' ATC TCT CCG CTT CCt ttC 3'	55.5		-1.2	
5' AtC TCT CCG CTT CCT TTC 3'	58.4	66.2	-0.8	-0.6
5' ATC TCT CCG CtT CCT TTC 3'	52.5	63.0	-6.7	-3.8
5' AtC tCt CCG CTT CCT TTC 3'	47.2	60.0	-4.0	-2.3

[a]Tm is the temperature at the midpoint of the melting curve; The concentrations are as follows: Oligomer strands, 2 μM each. Melting temperatures (Tm) were determined[30] by measuring change in absorbance at 260 nm (cuvette, 1-mm path length) as a function of temperature in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA. All the values are averaged from at least three experiments. The letter "t" denotes modified thymidine. ΔTm/mod represents the change in Tm per modification. The negative (-) values indicates the decrease in Tm value per single incorporation of "t".

oligonucleotide sequence affected the hybridization properties severely and the melting temperature of the duplex is lowered by 8.7°C for one incorporation (Table 1, line 6). Duplex formed by AANA with RNA target is also affected in the same manner as it does to DNA target.

To find out the conformational effect of amino alcohol nucleic acid on duplex stability, simultaneously, we prepared[31] the corresponding D-isomer of **12** and incorporated it into oligonucleotide sequences, and studied the binding properties. Hybridization of oligonucleotides containing the D-isomer of **12** was studied by thermodynamic melting experiments and the results are summarized in Table 2. Incorporation of D-isomer of **12** in oligonucleotide sequences has favorable affinity towards RNA, but not to DNA. The presence of D-isomer of **12** in the middle of oligo sequence (Table 2, line 6) decreases the melting temperature significantly (ΔTm = -6.78°C) over DNA target while the duplex stability against RNA was affected less significantly (ΔTm = -3.8°C). Nevertheless, a single substitution at 3' or 5' end of

oligonucleotide does not destabilize hybridization severely. These results suggest that oligonucleotides containing D-amino alcohol nucleoside monomers show selective binding to RNA rather than oligonucleotides having the corresponding L-amino alcohol nucleoside monomers; although it is too early to draw any conclusion.

However, oligonucleotides containing **12** as well as D-isomer of **12** at 3'-end showed an increase in exonuclease stability by many folds than unmodified DNA. The stability of oilgonucleotides to snake venom phosphodiesterase was studied[32] by measuring the increase in absorbance (hyperchromicity) at 260 nm during the digestion and the absorbance versus time was recorded. The half-life times ($t_{1\backslash2}$) are defined as the time when 50% hyperchromicity is reached and the results are shown in Figure 2-3. At these conditions, the unmodified oligonucleotide was completely digested within 20 minutes and had a half-life time of 4.68 minutes. The oligonucleotide containing a D-amino alcohol nucleoside monomer at the 3'-end (Table 2, sequence 3; Fig. 3, sequence 7) had a half-life time of 72 minutes while the oligonucleotide containing a L-amino alcohol monomer (Table1, sequence 3; Fig. 2, sequence 1) had half-life time of 60 minutes. Substitution of more than one amino alcohol modified nucleoside at 3'-end makes the ODNs very stable to nuclease ($t_{1\backslash2}$ > 180 minutes; Fig. 3, sequence 2 & Fig. 2, sequence 3). On the contrary, incorporation of modified amino alcohol nucleosides at 5'-end do not have any effect on the half-life times (Fig. 3, sequence 6 & Fig. 2, sequence 2; $t_{1\backslash2}$=4 minutes) of the corresponding oligonucleotides.

Overall, this study suggests that substitution of either L or D amino alcohol nucleic acid monomer **12** in the middle of the oligonucleotide chain decreases its ability to form stable duplexes with complementary nucleic acids. The weaker binding of AANA is an indicative that neither of the amino alcohol nucleic acid monomer **12** is not adopting a rigid conformation similar to that of furanose sugar puckering. Also, the presence of 2',5'-like linkages should account some responsibility for the observed Tm values. Oligonucleotides having 2',5'-linkages exhibit lower thermal melt rather than 3',5'-linked oligonucleotides and this has been documented.[33]

In summary, oligonucleotides containing novel amino alcohol nucleic acid building block with mixed (2',5' and 3',5') internucleotide linkages have been synthesized for the first time and studied for their ability to form stable duplex.

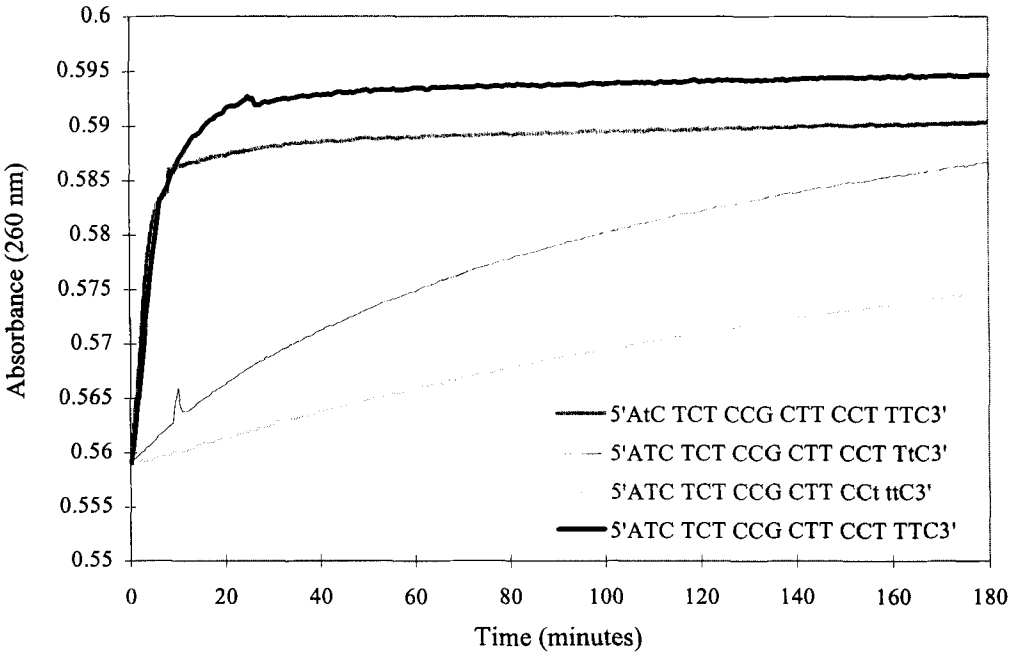


Figure 2. Enzyme digestion time course of ODNs having L-Amino Alcohol Monomers

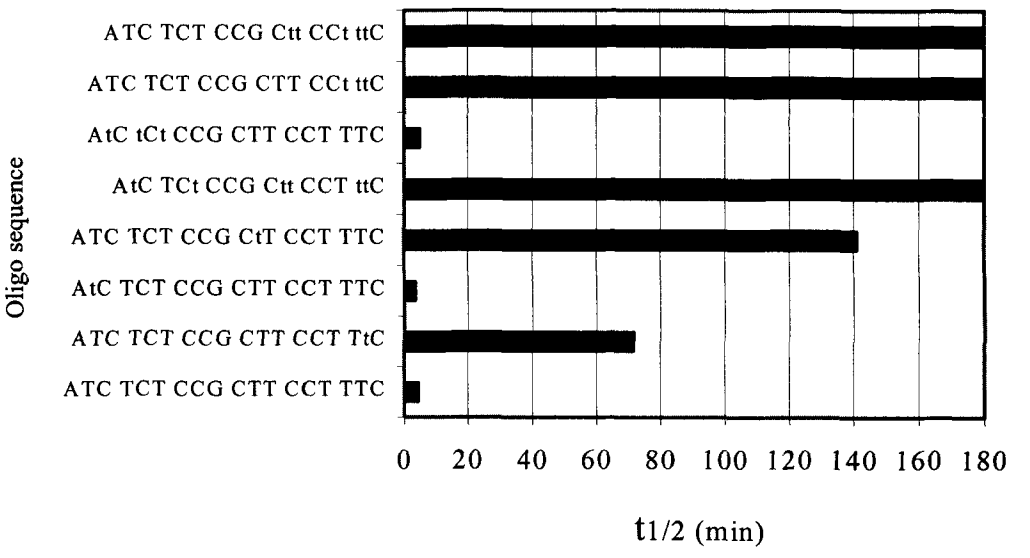


Figure 3. Half-life times of oligonucleotides having D-Amino Alcohol Monomers

Interestingly, AANA modified oligonucleotides showed remarkable resistance to 3'-exonucleases but destabilize the formation of duplex with DNA and RNA.

EXPERIMENTAL

Melting points were taken on a Haake Buchler capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Varian Mercury 300 MHz spectrometer. The chemical shifts are expressed in δ values (ppm) relative to tetramethylsilane as internal standard. Elemental analyses were performed by Quantitative Technologies Inc., WhiteHouse, NJ. Thin layer chromatography (tlc) was performed on plates of silica gel $60F_{254}$ coated on aluminium sheets (5x10 cm; EM Science) using different solvens prepared freshly. ICN silica gel 18-32 (60 A) was used for flash column chromatography. All solvents used were reagent grade. Most of the dry solvents were purchased from Fluka and used as such without further purification. β-Cyanoethyl-N,N-diisopropylchlorophosphoramidite was obtained from Aldrich chemical company. Triethylamine was stored over solid KOH pellets. Most of the reactions were conducted under argon atmosphere. Evaporations were carried out under reduced pressure with the bath temperature below 35°C.

N-(*tert*-Butyloxycarbonyl)-*O*-Benzyl-L-Serine (3): *O*-Benzyl-L-Serine (10 g, 51.28 mmol) was suspended in THF/H_2O (8:2, 100 ml) mixture at room temperature. To this stirred mixture was added triethylamine (6.06 g, 60 mmol) followed by di-*tert*-butyl dicarbonate (13.08 g, 60 mmol), and the stirring continued at room temperature overnight. The homogenous solution was evaporated to dryness and the residue dissolved in ethyl acetate (300 ml). The organic extract was washed with 0.5N solution of potassium hydrogen sulfate (100 ml), water (2x100 ml) and brine (50 ml). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness to give 14 g (93%) of an oily residue. Anal. Calcd. for $C_{15}H_{21}NO_5$(295.33): C, 60.99; H, 7.17; N, 4.74. Found: C, 61.02; H, 7.32; N, 4.91.

N-(*tert*-Butyloxycarbonyl)-*O*-Benzyl-L-Serinol (4): Compound **3** (6.0 g, 20.34 mmol) was dissolved in dry THF and cooled to -20°C under argon atmosphere. To this cold stirred solution was added TEA (2.32 g, 23 mmol) and isobutyl chloroformate (3.13 g, 23 mmol). The stirring was continued for 30 min at -20°C under argon atmosphere.

The reaction mixture was filtered immediately under a blanket of argon; the precipitate was washed with dry THF (50 ml). The combined filtrate was added slowly into a cold (0°C) solution of $NaBH_4$ (7.4 g, 200 mmol) in THF/water (80:20, 200 ml) during 10 min period. After the addition, the reaction mixture was stirred for 2 h at 0°C and the pH adjusted to 7 with acetic acid. The solution was evaporated to dryness, partitioned between ethyl acetate/water (300:150 ml) and extracted in ethyl acetate. The organic extract was washed with brine (100 ml), dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by flash column chromatography over silica gel using CH_2Cl_2 → EtOAc as the eluent. The pure product was pooled together and evaporated to dryness to give 4.7 g (82%) of the pure product as an oil. ^{1}HNMR ($CDCl_3$): δ 1.41 (s, 9H, Boc), 3.60 - 3.70 (m, 4H), 3.82 (d, 2H), 4.53 (s, 2H, OCH_2Ph), 5.20 (bs, 1H, NH) and 7.30 - 7.40 (m, 5H, Ph). Anal. Calcd. for $C_{15}H_{23}NO_4$(281.34): C, 64.03; H, 8.24; N, 4.98. Found: C, 64.27; H, 8.21; N, 4.96.

1-*O*-Benzyl-2-[(*tert*-butyloxycarbonyl)amino]-3-[N_3-benzoyl(thymin-1-yl)]-L-propanol (6): To a stirred solution of N_3-benzoylthymine (**5**)[24] (5.75 g, 25 mmol) in dry THF (200 ml) under argon was added triphenyl phosphine (10.48 g, 40 mmol) and **4** (5.3 g, 18.86 mmol) at room temperature. After 15 min, diethylazodicarboxylate (6.96 g, 40 mmol) was added slowly during 30 min period. The reaction mixture was covered with aluminum foil and allowed to stir at room temperature under argon for 24 h. The solvent was evaporated to dryness and the residue dissolved in EtOAc (300 ml). The organic extract was washed with 5% $NaHCO_3$ solution (100 ml), water (100 ml) and brine (100 ml), and dried over anhydrous Na_2SO_4. The dried EtOAc extract was evaporated to dryness to give an orange oil. The crude product was purified by flash chromatography over silica gel using hexane → EtOAc as the eluent. The fractions having the required product were pooled and evaporated to give a pale pink oil. Yield: 8.0 g (86%). ^{1}HNMR ($CDCl_3$): δ 1.41 (s, 9H, Boc), 1.72 (s, 3H, CH_3), 3.56 (m, 2H), 4.20 (m, 2H), 4.32 (m, 1H), 4.52 (d, 2H, OCH_2Ph), 5.20 (d, 1H, NH), 7.06 (s, 1H, C_6H) and 7.20 - 7.60 (m, 10H, Ph). Anal. Calcd. for $C_{27}H_{31}N_3O_6$(493.54): C, 65.70; H, 6.33; N, 8.51. Found: C, 65.92; H, 6.51; N, 8.72.

2-[(*tert*-Butyloxycarbonyl)amino]-3-[N_3-benzoyl(thymin-1-yl)]-L-propan-1-ol (7): Compound **6** (4.93 g, 10 mmol) was dissolved in MeOH (100 ml) and treated with

Pd/C (10%, 1 g). The reaction mixture was hydrogenated at 50 psi of hydrogen for 12 h. The catalyst was filtered, washed with MeOH (50 ml) and the filtrate was evaporated to dryness. The residue was crystallized from acetone/hexane to give 3.70 g (92%) of pure product. Mp: 156-159°C. ^{1}HNMR ($CDCl_3$): δ 1.42 (s, 9H, Boc), 1.94 (s, 3H, CH_3), 3.64 (m, 4H), 3.84 (m, 1H), 4.14 (m, 1H), 5.22 (d, 1H, NH), 7.18 (s, 1H, C_6H), 7.48 (t, 2H, Ph), 7.62 (t, 1H, Ph) and 7.98 (d, 2H, Ph). Anal. Calcd. for $C_{20}H_{25}N_3O_6.1/2H_2O$(412.41): C, 58.24; H, 6.35; N, 10.19. Found: C, 58.60; H, 6.38; N, 10.05.

1-*O*-Isobutyryl-2-[(*tert*-butyloxycarbonyl)amino]-3-[N_3-benzoyl(thymin-1-yl)]-L-propanol (8): Compound **7** (1.60 g, 3.97 mmol) was dissolved in dry pyridine (30 ml) and allowed to stir at room temperature under argon. To this stirred solution was added TEA (0.51 g, 5 mmol) and isobutyric anhydride (0.79 g, 5 mmol). The reaction mixture was stirred at room temperature for 12 h and evaporated to dryness. The residue was dissolved in EtOAc (150 ml) and washed with 5% $NaHCO_3$ solution (100 ml), water (100 ml) and brine (50 ml). The organic extract was dried and evaporated to dryness. The residue was purified by flash column chromatography over silica gel using CH_2Cl_2 → EtOAc as the eluent. The pure fractions were collected together and evaporated to give 1.6 g (85%) of foam. The pure product was crystallized from acetone/hexane. Mp: 165-167°C. ^{1}HNMR ($CDCl_3$): δ 1.16 (d, 6H, $IbCH_3$), 1.42 (s, 9H, Boc), 1.94 (s, 3H, CH_3), 2.52 (m, 1H), 3.64 (m, 4H), 3.84 (m, 1H), 4.14 (m, 1H), 5.22 (d, 1H, NH), 7.18 (s, 1H, C_6H), 7.48 (t, 2H, Ph), 7.62 (t, 1H, Ph) and 7.98 (d, 2H, Ph). Anal. Calcd. for $C_{24}H_{31}N_3O_7$(473.51): C, 60.87; H, 6.59; N, 8.87. Found: C, 61.00; H, 6.72; N, 8.78.

1-*O*-Isobutyryl-2-[(β-hydroxyacetyl)amino]-3-[N_3-benzoyl(thymin-1-yl)]-L-propanol (9): Compound **8** (1.6 g, 3.38 mmol) was allowed to stir in a mixture of TFA (5 ml) and CH_2Cl_2 (10 ml) at room temperature for 30 min and evaporated to dryness. The residue was dissolved in dry MeOH (10 ml) and evaporated again. The residue that obtained was dried over solid NaOH under vacuum overnight. The dried material was used as such for the next reaction.

To a stirred solution of glycolic acid (0.53 g, 7 mmol) in dry DMF (50 ml) was added 1-hydroxybenzotriazole (0.67 g, 5 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (1.91 g, 10 mmol). After stirring for 15 min, TEA (1.01 g, 10 mmol) and the above TFA salt in DMF (20 ml) were added at room

temperature. The reaction mixture was stirred for 12 h and evaporated to dryness. The residue was partitioned between CH_2Cl_2 (150 ml) and water (100 ml), and extracted in CH_2Cl_2. The organic extract was washed with brine (50 ml), dried and evaporated to dryness. The residue was purified by flash chromatography over silica gel using CH_2Cl_2 → acetone as the eluent. The fractions having the required product were collected and evaporated to give 1.35 g (92%) of foam. ^{1}HNMR ($CDCl_3$): δ 1.16 (d, 6H, IbCH$_3$), 1.94 (s, 3H, CH$_3$), 2.52 (m, 1H), 3.20 (bs, 1H), 3.80 - 4.30 (m, 6H), 4.56 (m, 1H), 7.14 (d, 2H, C$_6$H and NH), 7.50 (t, 2H, Ph), 7.64 (t, 1H, Ph) and 7.94 (d, 2H, Ph). Anal. Calcd. for $C_{21}H_{25}N_3O_7$(431.43): C, 58.45; H, 5.84; N, 9.74. Found: C, 58.09; H, 5.78; N, 9.49.

1-*O*-Isobutyryl-2-[[β-(4, 4'-dimethoxytrityl)-*O*-acetyl]amino]-3-[N_3-benzoyl-(thymin-1-yl)]-L-propanol (10): Compound **9** (1.2 g, 2.78 mmol) was dissolved in dry pyridine (50 ml) and allowed to stir at room temperature under argon atmosphere. To this stirred solution was added TEA (0.35 g, 3.5 mmol) and 4,4'-dimethoxytrityl chloride (1.18 g, 3.5 mmol). The reaction mixture was stirred at room temperature for 12 h, quenched with MeOH (10 ml) and evaporated to dryness. The residue was dissolved in EtOAc (150 ml), washed with 5% $NaHCO_3$ solution (100 ml), water (100 ml) and brine (50 ml). The organic extract was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography over silica gel using CH_2Cl_2 → EtOAc as the eluent. The pure fractions were pooled and evaporated to give 1.7 g (83%) of pure product. ^{1}HNMR ($CDCl_3$): δ 1.16 (d, 6H, IbCH$_3$), 1.94 (s, 3H, CH$_3$), 2.52 (m, 1H), 3.74 (s, 6H, 2.OCH$_3$), 3.80 - 4.30 (m, 6H), 4.56 (m, 1H), 6.82 (d, 4H, Ph), 7.14 (d, 2H, C$_6$H and NH) and 7.26 - 8.00 (m, 14H, Ph). Anal. Calcd. for $C_{42}H_{43}N_3O_9$(733.78): C, 68.74; H, 5.91; N, 5.73. Found: C, 68.51; H, 5.99; N, 5.70.

2-[[β-(4, 4'-Dimethoxytrityl)-*O*-acetyl]amino]-3-(thymin-1-yl)-L-propanol (11): Compound **10** (1.55 g, 2.05 mmol) was dissolved in MeOH (20 ml) and cooled to 0°C in an ice bath. To this cold stirred solution was added 2N NaOH (5 ml, 10 mmol) and the stirring continued for 30 min at 0°C. The pH of the solution was adjusted to 7 with acetic acid and evaporated to dryness. The residue was partitioned between water (50 ml) and CH_2Cl_2 (150 ml) and extracted in CH_2Cl_2. The aqueous layer was extracted again with CH_2Cl_2 (50 ml). The combined organic extract was washed with brine (50 ml), dried and evaporated to dryness. The residue was purified by flash column chromatography

over silica gel using CH_2Cl_2 → acetone as the eluent. Yield: 1.0 g (99%). ^{1}HNMR ($CDCl_3$): δ 1.94 (s, 3H, CH_3), 3.74 (s, 6H, 2.OCH_3), 3.80 - 4.30 (m, 6H), 4.56 (m, 1H), 6.82 (d, 4H, Ph), 7.14 (d, 2H, C_6H and NH) and 7.26 - 8.00 (m, 14H, Ph). Anal. Calcd. for $C_{31}H_{33}N_3O_7$(559.59): C, 66.59; H, 5.94; N, 7.50. Found: C, 66.38; H, 5.95; N, 7.01.

2-[[β-(4, 4'-Dimethoxytrityl)-*O*-acetyl]amino]-3-(thymin-1-yl)-L-propan-1-*O*-(N,N-diisopropyl)-β-cyanoethylphosphoramidite (12): Compound **11** (1.00 g, 2.09 mmol) was dried over solid NaOH under vacuum overnight and dissolved in dry CH_2Cl_2 (50 ml). The solution was cooled to 0°C under argon atmosphere. To this cold stirred solution was added N,N-diisopropylethylamine (0.54 g, 4.2 mmol) followed by β-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.73 g, 3.1 mmol). The reaction mixture was stirred at 0°C for 1 h and at room temperature for 2 h. The reaction was diluted with CH_2Cl_2 (100 ml) and the organic layer was washed with 5% $NaHCO_3$ solution (100 ml), water (100 ml) and brine (50 ml). The CH_2Cl_2 extract was dried and evaporated to dryness to give an oily residue. The residue was purified by flash chromatography over silica gel using CH_2Cl_2 → EtOAc containing 0.1% TEA as the eluent. The pure fractions were pooled together and evaporated to give a foam. The foam was dried over solid NaOH under vacuum overnight. The dried foam was dissolved in dry CH_2Cl_2 (10 ml) and dropped into a stirred solution of dry hexane (800 ml) under argon during 30 min period. After the addition, the precipitate formed was stirred for additional 30 min and filtered, washed with dry hexane (100 ml) and the solid was dried over solid NaOH under vacuum for 4 h. Yield: 1.3 g (82%).

Oligonucleotide synthesis:

Oligonucleotides were synthesized on an automated ABI 394 DNA/RNA synthesizer with automatic dimethoxytrityl cation detection, which monitors the coupling yields. Software and the hardware of the synthesizer were modified to accommodate the synthesis of modified oligonucleotides. The oligos were deprotected at 55°C for 8 h using ammonium hydroxide solution. The crude oligos were purified on Waters HPLC system (Waters 600 pump with 490 E variable wavelength detector) using reverse phase C18 column (Beckman, ODS ultrasphere, dp 5μ, 10 x 250mm, Part NO:235328) and gradient buffer (Buffer A: 5% acetonitrile in 0.1 TEAA, pH 7.50; Buffer B; 100% acetonitrile)

with the flow rate of 3ml/min. Appropriate fractions were collected and evaporated using rotary evaporator. The residue obtained was treated with 80% acetic acid for 1 h at room temperature, and then 1.0M sodium acetate was added and 2-propanol. The mixture was kept at -20°C for 1 h. Precipitated product was removed by centrifugation. The colorless product was dissolved in HPLC-grade water, filtered through 0.45 μm pore size filter and lyophilized to give white powder.

Hybridization studies:

The thermodynamic melting experiments were conducted on a Varian UV spectrometer equipped with an electronic temperature controller and Cary hybridization software. The complementary DNA (5' GAA AGG AAG CGG AGA GAT 3') was synthesized as described above and the complementary RNA (5' GAA AGG AAG CGG AGA GAU 3') was purchased from Genset, San Diego. The samples for Tm measurements containing 2 μM of modified oligonucleotides and 2.0 μM of either the complementary DNA or RNA were mixed in a buffer (10 mM sodium phosphate, 0.1 mM EDTA and 0.1 M sodium chloride, pH=7.0). The absorbance versus temperature curves of duplexes was measured from 20°C to 90°C. These data points were used to fit in a two-state model with linear sloping baselines. From these the Tm values in Table 1 were calculated with Cary software based on hyperchromicity.

Enzyme stability studies:

Oligonucleotides (0.75 OD) were incubated with snake venom phosphodiesterase (1.2 units) in 1.5 mL of buffer (0.1 M Tris.HCl, pH 7.5; 0.1 M NaCl; 14 mM $MgCl_2$) in a cuvette on a Varian UV spectrometer at 25°C, and the increase of absorbency at 260 nm during the digestion time was monitored every 30 seconds. From these absorbency value curves, the half-life times of oligonucleotides were calculated.

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