Synthesis and Hybridization Properties of Sugar-Modified Oligonucleotides

by **Ashwani K. Sharma**, **Pradeep Kumar**, and **Kailash C. Gupta***

Nucleic Acids Research Laboratory, Centre for Biochemical Technology, Mall Road, Delhi University Campus, Delhi-110007, India (E-mail: kcgupta15@hotmail.com; fax.: +91 11 7667471; tel.: +91 11 7666156)

L-Threoninol-derived acyclic nucleotide monomers were prepared and incorporated into oligonucleotides at preselected positions *via* phosphoramidite chemistry. Hybridization properties of these modified oligonucleotides with the corresponding natural oligomers were studied, and their *vis-à-vis* comparison with serinol-modified oligonucleotides was made. Stability of the modified oligomers against nuclease in human serum and snake venom phosphodiesterase (SVPD) was examined.

Introduction. – The ability of oligonucleotides to form a double helix by binding to single-stranded nucleic acids when there is a complementary nucleotide sequence makes them useful as diagnostic probes and as tools in molecular genetics [1–4]. Therefore, biological processes involving these nucleic acids can be affected by addition of the respective oligonucleotides. This particular property of modified oligonucleotides makes them useful in biotechnology and, more recently, in medicinal chemistry to an extent that their use as antisense oligonucleotides have been shown to modulate (usually inhibit) the expression either at the transcriptional or translational stage. Consequently, these molecules are being considered as new-generation pharmaceuticals. However, natural oligonucleotides suffer from some limitations, *viz.*, the instability against nucleases, low stability of the duplex formed with medium-chain-length oligonucleotides, and lack of penetration through lipophilic cell membranes. To overcome these obstacles, modifications are generally introduced into synthetic oligonucleotides to make them effective as antisense oligonucleotides. An oligonucleotide consists of bases, sugar residues, and the internucleotide backbone, and all of these are amenable to modification. Therefore, one or more than one type of modification can be introduced into synthetic oligonucleotides. A number of successful modifications or replacements of phosphoramidates [5], methylphosphonate [6], and peptide nucleic acids (PNA) [7] have been shown to produce oligonucleotides that complement DNA and RNA with similar or high stability while maintaining the sequence specificity. Recently, an alternative approach, involving the replacement of sugar-containing nucleotides with acyclic nucleotide analogs derived from serinol [8–10] and phenylserinol [11], was developed; it allows the formation of an intramolecular H-bond between the amide NH of the acetamide tether and the phosphate O-atom to adopt a constrained conformation. These monomers, when incorporated into oligonucleotides, may have an influence stronger than a single H-bond and favor the formation of competent nucleic acid. Furthermore, the H-bond could neutralize the negative charge of the phosphodiester backbone, and this neutralization is expected to enhance the uptake of the oligonucleotide, rendering it potentially more useful as a therapeutic

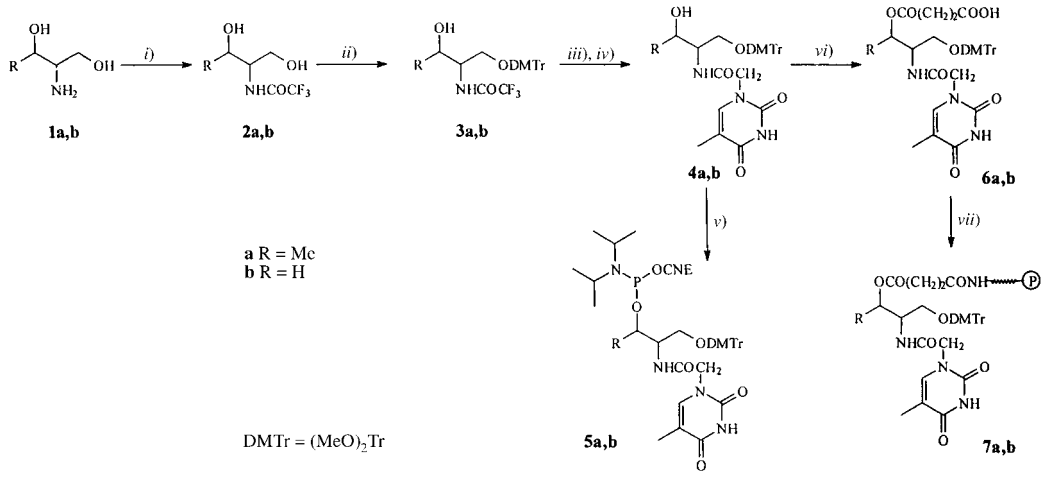
agent. Incorporation of an acyclic L-serinol-modified analog at the 3′ terminus of an oligonucleotide remarkably increased the resistance to 3′-exonucleases but disfavored duplex formation with a natural component. But, in the case of acyclic phenylserinol-modified analogs, the DNA duplex and triplex formations were not hampered. This implies that the side chain at the β-position of the amino alcohols plays a significant role in stabilizing the duplex.

Encouraged by these results, we decided to use threoninol in place of serinol or phenylserinol. On comparing the structure of serinol with phenylserinol, we find that an H-atom in serinol is replaced by a Ph group, which imparts much stability to the duplex. The present study was undertaken to reveal the effect of other substituents on serinol. L-Threoninol was selected for two reasons: *i*) its preparation is straightforward, and *ii*) it resembles serinol in structure as just one H-atom is replaced by a Me group. Moreover, we assume that the extra Me group in the side chain of the threoninal moiety will favor the stabilization of the duplex at least as well as the corresponding serinol-derived nucleotide. Thus, we report here on L-threoninol-derived phosphoramidite monomers and their incorporation in synthetic oligonucleotides at preselected sites (3′- or 5′-terminus, or in the middle of the chains). The modified oligomers were then used to study various properties, such as hybridization with natural oligomers and stability against snake-venom phosphodiesterase (SVPD) and in human serum *etc.* Results were also compared with the corresponding serinol-derived oligonucleotides.

Results and Discussion. – Syntheses of phosphoramidite monomers (synthons) of L-threoninol and serinol were carried out as depicted in the *Scheme*. L-Threoninol (**1a**) was prepared from L-threonine by a published procedure [12], and serinol (**1b**) was commercially available. L-Threoninol-derived phosphoramidite **5a** and serinol-derived phosphoramidite **5b** were obtained in almost 35% yields starting from the corresponding diols **1a** and **1b**, respectively. The synthesis strategy involved the following steps: selective protection of the primary-amino group in the amino-diol **1** by a CF_3CO group ($\rightarrow$**2**) and subsequent protection of one of the OH functions by the 4,4′-dimethoxytrityl ($(MeO)_2Tr$) group yielded **3** in 65–68% yield. Then the CF_3CO group was removed selectively by treatment with aqueous NH_4OH solution, and the crude product was submitted to the reaction with 1-(carboxymethyl)thymine. After silica-gel chromatography, the thymine derivatives **4** were obtained in 64–65% yield and characterized by MALDI-TOF (*Fig. 1, a* and *Fig. 2, a*) and NMR. Phosphitylation of **4** with 2-cyanoethyl diisopropylphosphoramidochloridite resulted in the desired synthons **5** in 85–88% yield.

Oligonucleotide synthesis was performed by means of phosphoramidite chemistry. The L-threoninol- and serinol-modified reagents **5** were introduced at the 5′-terminus and other preselected sites of the oligonucleotides. These reagents were found to be as efficient as the normal nucleoside phosphoramidites during the coupling reaction. Unlike the 5′-terminus, the 3′-terminus of the oligonucleotide is not available for any manipulation during solid-phase synthesis, as it is anchored on the polymer support. Therefore, the prederivatized supports **7a** and **7b** were employed to introduce the modification at the 3′-terminus. These supports were functionalized *via* **6a**,**b**, essentially according to a procedure [13] reported from this laboratory, with a loading in the range 35–40 μmol/g support.

Scheme. *Preparation of Acyclic Phosphoramidites.*



i) CF_2COOEt, dioxane. *ii*) $(MeO)_2TrCl$, pyridine. *iii*) Aq. NH_4OH soln. *iv*) T-CH_2COOH, EDC, *N*-hydroxysuccinimide. *v*) Phosphitylation. *vi*) Succinic anhydride, *N,N*-dimethylpyridin-4-amine (DMAP), Et_3N. *vii*) LCAA-CPG, Ph_3P, DMAP, $BrCCl_3$.

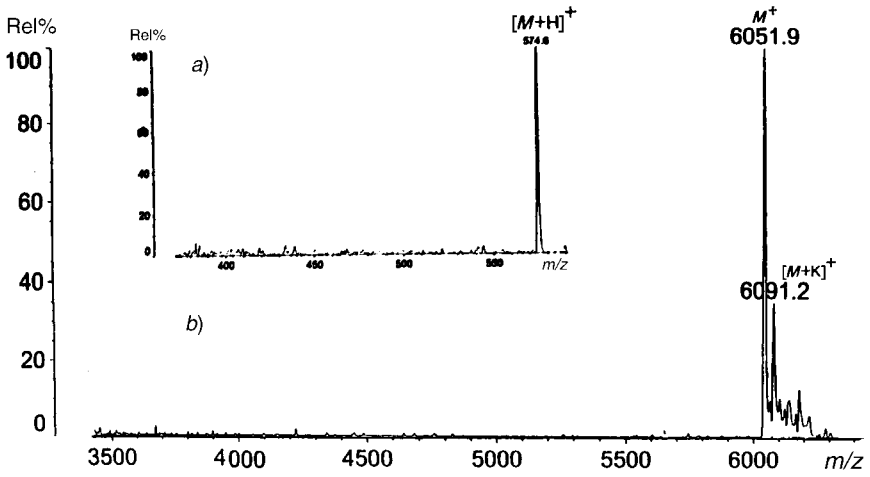


Fig. 1. *MALDI-TOF Mass spectra of* a) *compound* **4a** *and* b) *oligomer d(xTT TTT TTT TTT TTT TTT TT)* (**I**)

The three 3′-terminal modified oligomers **II**, **V**, and **IX**, a number of other modified oligonucleotides, **I**, **III**, **IV**, **VI** – **VIII**, and **X** – **XII**, along with the standard d(TTT TTT TTT TTT TTT TTT TT) (**XIII**), d(AAA AAA AAA AAA AAA AAA AA) (**XIV**), d(AAG AAG AAA AAG A) (**XV**), and d(TTC TTC TTT TTC T) (**XVI**) were synthesized (see *Table1*). The modified as well as normal oligonucleotides were purified by fast protein liquid chromatography (FPLC; *Mono-Q* anion exchange column) and their purity ascertained analytically by FPLC and MALDI-TOF MS (*Fig. 1, b* and *Fig. 2, b*).

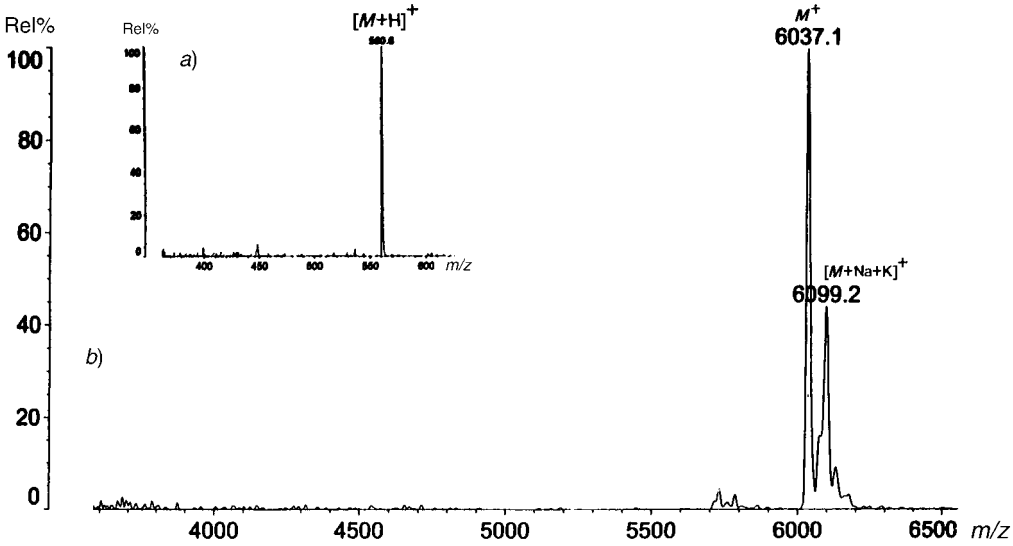


Fig. 2. *MALDI-TOF Mass spectra of* a) *compound* **4b** *and* b) *oligomer d(yTT TTT TTT TTT TTT TTT TT)* (**VIII**)

Oligonucleotide duplexes were constituted from unmodified oligomer d(AAA AAA AAA AAA AAA AAA AA) (**XIV**) and modified **I**–**XI**, oligomer **XIII** and **XIV**, modified hetero oligomer **XII** and oligomer **XV**, and oligomer **XVI** and **XV** (see *Table 1*). The stability of the duplex was measured by UV melting experiments, by detection of the change in UV absorbance at 260 nm in the temperature range 15–60°. T_m Values were confirmed by distinct peaks of first-derivative plots, and the data

Table 1. *Sequences of Modified and Normal Oligonucleotides*

	Sequence[a])
I	d(xTT TTT TTT TTT TTT TTT TT)
II	d(TTT TTT TTT TTT TTT TTT Tx)
III	d(TTT TTT TTT TTT TTT TTx xT)
IV	d(TTT TTT TTT xTT TTT TTT TT)
V	d(xTT TTT TTT TTT TTT TTT Tx)
VI	d(TTT TTT TTT TTT TTT TTT xT)
VII	d(TxT TTT TTT TTT TTT TTT TT)
VIII	d(yTT TTT TTT TTT TTT TTT TT)
IX	d(TTT TTT TTT TTT TTT TTT Ty)
X	d(TTT TTT TTT TTT TTT TTy yT)
XI	d(TTT TTT TTT yTT TTT TTT TT)
XII	d(TTC TTC TxT TTC T)
XIII	d(TTT TTT TTT TTT TTT TTT TT)
XIV	d(AAA AAA AAA AAA AAA AAA AA)
XV	d(AAG AAG AAA AAG A)
XVI	d(TTC TTC TTT TTC T)

[a]) x = L-Threoninol-modified residue; y = serinol-modified residue.

obtained for control (unmodified) and modified duplexes are shown in *Table 2*. These data establish that L-threoninol modifications introduced in oligonucleotides do not hamper the duplex formation. A single L-threoninol or serinol modification at the 5′-terminus decreased the duplex stability by a ΔT_m of -2.7 and $-3.7°$, respectively, per modification and a corresponding single modification at the 3′-terminus resulted in a ΔT_m of -3.2 and $-5.4°$, respectively, per modification. Two L-threoninol modifications at the $n-1$ and $n-2$ position from the 3′-terminus (see **III**) decreased the duplex stability only by a ΔT_m of $-1.2°$ per modification, while the same serinol modifications (see **X**) resulted in a ΔT_m of $-1.6°$ per modification. On the contrary, the insertion of a L-threoninol residue in the middle of the oligomer (see **IV**) resulted in a ΔT_m of $-5.5°$ per modification, while a ΔT_m of $-9.7°$ was noticed in the case of the corresponding serinol-modified oligomer (see **XI**). This clearly indicates that the L-threoninol-modified oligonucleotides show a better hybridization behavior than the corresponding serinol-modified oligonucleotides. On comparing the thermal stability of the duplexes formed between the hetero oligomers **XII** and **XV** and between the unmodified hetero oligomers **XVI** and **XV**, one can see that introduction of a L-threoninol residue in the middle of the oligomers destabilizes the duplex by ΔT_m $-11.5°$.

Table 2. *UV* T_m *Data for Duplexes*

S.No.	Duplex	T_m [°]	ΔT_m/mod. [°]
1.	**I**·**XIV**	40.0	– 2.7
2.	**II**·**XIV**	39.5	– 3.2
3.	**III**·**XIV**	40.2	– 1.2
4.	**IV**·**XIV**	37.2	– 5.5
5.	**V**·**XIV**	40.9	– 1.8
6.	**VI**·**XIV**	40.0	– 2.7
7.	**VII**·**XIV**	40.6	– 2.1
8.	**VIII**·**XIV**	39.0	– 3.7
9.	**IX**·**XIV**	37.2	– 5.4
10.	**X**·**XIV**	39.4	– 1.6
11.	**XI**·**XIV**	33.0	– 9.7
12.	**XII**·**XV**	29.5	– 11.5
13.	**XIII**·**XIV**	42.7	–
14.	**XVI**·**XV**	41.0	–

As expected, the modification at 5′- or near the 5′-terminus did not improve the stability of the modified oligonucleotides against exonucleases (human serum) and snake-venom phospodiesterase (SVPD). However, the 3′- or near- 3′-terminus-modified oligonucleotides showed very good stability against exonucleases (human serum) and against SVPD. Interestingly, the oligomer modified in the middle was digested quickly up to the modified position and, from then on, was quite stable against exonucleases and SVPD.

The financial assistance from the Department of Science and Technology, New Delhi, is gratefully acknowledged.

Experimental Part

1. *General.* All solvents and reagents employed in the present investigation were purified prior to their use. LCAA-CPG, *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide (EDC) and thymine were from *Sigma Chemical Co.*, USA. CF_3COOEt, *N*-hydroxysuccinimide (*N*-HOSu), and diisopropylethylamine were from *Aldrich Chemical Co.*, USA. Other chemicals and reagents were obtained from local suppliers and purified before use. CC = Column chromatography. Reversed-phase HPLC: *Shimadzu-LC-4A* system, UV detection at 254 nm, recording with a *Shimadzu-C-R7A* chromatopac. Anion-exchange fast protein liquid chromatography (FPLC): *Pharmacia FPLC* system, two *P-500* pumps, single-path UV monitor (*UV-1*) operating at 254 nm; *Mono-Q-HR-5/5* (*10/10*) column; elution with 1M NaCl buffer (pH 12). ^{1}H-NMR Spectra: *Bruker Avance-DPX-300* at 300 MHz; δ in ppm, *J* in Hz. MALDI-TOF-MS: *Kompact SEQ* (*Kratos*, UK).

2. N-*{1-[[(4,4'-Dimethoxytrityl)oxy]methyl}-2-hydroxypropyl}-2,2,2-trifluoroacetamide* (**3a**). To a suspension of 2-aminobutan-1,3-diol (**1a**; 55 mmol) in dry 1,4-dioxane, CF_3COOEt (100 mmol, 10 ml) was added. The mixture was stirred at r.t. for 48 h and then evaporated. The product **2a** was dried by co-evaporation with anh. pyridine (100 ml). $(MeO)_2TrCl$ (60 mmol) was added and the mixture stirred overnight. After evaporation and removal of traces of pyridine by several co-evaporations with toluene, the syrupy material thus obtained was taken up in AcOEt (250 ml), and the soln. was sequentially washed with sat. aq. $NaHCO_3$ soln. (2 × 100 ml), 5% aq. citric acid soln. (2 × 50 ml), and sat. NaCl soln. (1 × 100 ml), dried (Na_2SO_4), and evaporated: **3a** (65%).

N-*{2-[(4,4'-Dimethoxytrityl)oxy]-1-(hydroxymethyl)ethyl}-2,2,2-trifluoroacetamide* (**3b**). Similarly, **3b** was prepared starting from 2-aminopropan-1,3-diol (**1b**) in 68% yield.

3. *2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)*-N-*{1-{[(4,4'-dimethoxytrityl)oxy]methyl}-2-hydroxypropyl}acetamide* (**4a**). A soln. of **3a** (5 mmol) in MeOH (20 ml) was treated with aq. NH_4OH soln. at 5° for 2 h and then evaporated. The residue was dissolved in AcOEt, the soln. washed with sat. $NaHCO_3$ soln. (2 × 25 ml) and sat. NaCl soln. (1 × 25 ml), dried (Na_2SO_4), and evaporated, and the oily mass (almost quant.) dried by co-evaporation with DMF and finally dissolved in DMF (50 ml). After addition of *N*-hydroxysuccinamide (5 mmol), 1-(carboxymethyl)thymine (=2-(1,3-dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)acetic acid; 5 mmol), and EDC (6 mmol), the mixture was stirred overnight. The soln. was evaporated, the residue redissolved in AcOEt (75 ml), the soln. subsequently washed with aq. sat. Na_2CO_3 soln. (2 × 25 ml), 5% aq. citric acid soln. (2 × 25 ml), and sat. NaCl soln. (1 × 25 ml), dried (Na_2SO_4), and evaporated, and the crude product purified by CC (gradient MeOH/CH_2Cl_2 containing 1% Et_3N): **4a** (65%). ^{1}H-NMR ($CDCl_3$): 1.1 (*d*, 1 Me); 1.89 (*s*, 1 Me); 3.22–3.4 (br. *m*, CHOH, $CH_2O[(MeO)_2Tr]$); 3.78 (*s*, 2 MeO); 3.96–4.38 (*m*, CHN, CH_2CO); 6.81–7.37 (*m*, 14 H, NH, $(MeO)_2Tr$). MALDI-TOF-MS: 574.6.

2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)-N-*{2-[(4,4'-dimethoxytrityl)oxy]-1-(hydroxymethyl)ethyl}acetamide* (**4b**). Similarly, **4b** was prepared starting from **3b** in *ca.* 64% yield. ^{1}H-NMR ($CDCl_3$): 1.87 (*s*, Me); 3.06–3.31 (br. *m*, CH_2OH, $CH_2O[(MeO)_2Tr]$); 3.78 (*s*, 6 H, 2 MeO); 4.28–4.4 (*m*, CHN, CH_2CO); 6.81–7.36 (*m*, 14 H, NH, $(MeO)_2Tr$). MALDI-TOF-MS: 560.6.

4. *2-Cyanoethyl 2-{[2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)-1-oxoethyl]amino}-3-[(4,4'-dimethoxytrityl)oxy]-1-methylpropyl Diisopropylphosphoramidite* (**5a**). To **4a** (2 mmol) in dry dichloroethane (15 ml), $^iPr_2EtN$ (8 mmol) was added. The mixture was cooled in an ice bath and 2-cyanoethyl diisopropylphosphoramidochloridite (3 mmol) was added dropwise through a glass syringe within 5 min with vigorous stirring. The reaction was allowed to proceed at r.t. for 1 h (TLC monitoring). Dry MeOH (0.5 ml) was added, the mixture was diluted with dichloroethane (50 ml), the soln. washed with 10% Na_2CO_3 soln. (2 × 30 ml) and sat. NaCl soln. (2 × 30 ml), dried (Na_2SO_4), and evaporated, and the oily mass purified by CC (silica gel, dichloroethane/AcOEt/Et_3N 45 : 45 : 10): **5a** (85%).

2-{[2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)-1-oxoethyl]amino}-3-[(4,4'-dimethoxytrityl)oxy]-propyl 2-Cyanoethyl Diisopropylphosphoramidite (**5b**). In the same manner, derivative **5b** was prepared from **4b** in 88% yield.

5. *4-{2-{[2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)-1-oxoethyl]amino}-3-[(4,4'-dimethoxytrityl)oxy]-1-methylpropoxy}-4-oxobutanoic Acid* (**6a**). A mixture of **4a** (1.0 mmol), DMAP (=*N,N*-dimethylpyridine-4-amine; 0.5 mmol), succinic anhydride (1.5 mmol), and Et_3N (1 mmol) in 1,2-dichloroethene (5 ml) was stirred at r.t. for 30 min and then monitored by TLC. After completion of the reaction, the mixture was further diluted with 1,2-dichloroethene (50 ml) and the soln. washed with 5% aq. citric acid soln. (2 × 25 ml) and sat. NaCl soln. (2 × 25 ml), dried (Na_2SO_4), and evaporated: **6a** (quant.).

4-{2-{[2-(1,3-Dihydro-5-methyl-2,4-dioxopyrimidin-1-yl)-1-oxoethyl]amino}-3-[(4,4'-dimethoxytrityl)oxy]-propoxy}-4-oxobutanoic Acid (**6b**). Similarly, **6b** was prepared from **4b**.

6. Derivatized Polymer Supports **7a**,**b**. A soln. of **6a** or **6b** (0.2 mmol), DMAP (0.4 mmol) and bromotrichloromethane (0.2 mmol) in DMF (1 ml) was mixed with triphenylphosphine (0.2 mmol) dissolved in DMF (0.5 ml), prior to the addition to long chain alkylamine controlled pore glass (500 mg). The suspension was agitated for 30 min at r.t. Then the support was filtered through a sintered disc glass funnel. The support was washed sequentially with DMF, MeOH, and Et_2O (10 ml of each). The loading on the supports was in the range of 35–40 μmol/g of support based on the trityl assay.

7. *Oligonucleotide Synthesis and Deprotection.* Oligonucleotide synthesis was carried out on a 0.2-μmol scale with a *Pharmacia-LKB Gene Assembler Plus* according to the standard protocol [14]. A large number of oligonucleotides, **I**–**XVI**, unmodified or modified at preselected sites, were prepared (see *Table 1*) from standard nucleoside phosphoramidites, from supports **7a**,**b** for 3′-terminal modifications, and from **5a**,**b** for modifications at preselected sites other than the 3′-terminus. The cleavage of oligomers from the support and their deprotection were achieved in a single step by treatment with 30% aq. NH_4OH soln. at 55° for 16 h. The fully deprotected oligomers were purified by ion-exchange FPLC.

8. *MALDI-TOF-MS.* The mass spectra of the purified oligomers were recorded in the negative linear mode. The matrix used for monomer units was norharmane (10 mg/ml in 50% MeCN/H_2O) and 3-hydroxypicolinic acid (10mg/ml in 20% MeCN/H_2O) for oligonucleotides along with diammonium citrate.

9. *Melting Experiments.* The melting experiments were carried out in a buffer system consisting of 10 mM sodium phosphate, 100 mM NaCl, and 0.1 mM EDTA (pH 7.1), and containing equal concentrations of the two complementary strands. The increase in absorbance at 260 nm as a function of time was recorded while the temp. was raised from 15 to 60° in 0.5° increments.

10. *Enzymatic Studies of the Modified Oligonucleotides.* The stability of the oligodeoxyribonucleotides containing the modified nucleosides at different positions towards snake-venom phosphodiesterase (SVPD) and human serum (HS) was studied by polyacrylamide-gel electrophoresis after labeling the oligomers with ^{32}P [15]. The cleavage of the oligonucleotides was studied over the time period 0–90 min. Aliquots after intervals of 15 min were withdrawn and loaded onto the gel. After running, the gel was exposed onto the X-ray film and the spots visualized after developing the autoradiogram.

REFERENCES

[1] G. Zon, *Pharm. Res.* **1998**, *5*, 539.
[2] B. J. Donich, *Cancer Invest.* **1991**, *9*, 185.
[3] S. T. Crooke, *Annu. Rev. Pharmacol. Toxicol.* **1992**, *32*, 329.
[4] C. Cazenave, C. Helene, in 'Antisense Nucleic Acids and Proteins: Fundamentals and Applications', Eds. J. N. M. Mol and A. R. V. Krol, Marcel Dekker Press, New York 1991, p. 47.
[5] S. Gryaznov, K. J. Chen, *J. Am. Chem. Soc.* **1994**, *116*, 3143.
[6] T. Loschner, J. W. Engels, *Nucleosides Nucleotides* **1988**, *7*, 729.
[7] M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, *J. Am. Chem. Soc.* **1992**, *114*, 1895.
[8] K. S. Ramasamy, W. Seifert, *BioMed. Chem. Lett.* **1996**, *6*, 1799.
[9] R. Benhida, M. Devys, J. L. Fourrey, F. Lecubin, J.S. Sun, *Tetrahedron Lett.* **1998**, *39*, 6167.
[10] V. A. Efimov, M. V. Choob, A. A. Buryakova, O. G. Chakhmakhcheva, *Nucleosides Nucleotides* **1999**, *18*, 1425.
[11] V. S. Rana, V. A. Kumar , K. N. Ganesh, *BioMed. Chem. Lett.* **1997**, *7*, 2837.
[12] D. Shapiro, H. M. Flowers, E. Hecht, *J. Org. Chem.* **1957**, *22*, 461.
[13] P. Kumar, A. K. Sharma, P. Sharma, B. S. Garg, K. C. Gupta, *Nucleosides Nucleotides* **1996**, *15*, 879.
[14] 'Pharmacia-LKB Gene Assembler Plus Manual', Upsala, Sweden.
[15] R. Frank, H. Blocker, in 'Chemical and Enzymatic Synthesis of Gene Fragments', Eds. H. G. Gassen, A. Lang, Verlag Chemie Press, Weinheim 1982, p. 225.

Received June 2, 2001