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Oligonucleotides with Alternating Anionic and Cationic Phosphoramidate Linkages: Synthesis and Hybridization of Stereo-uniform Isomers

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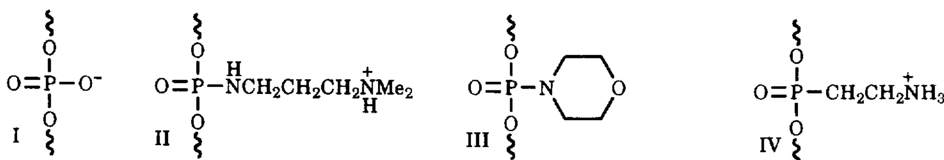
Abstract. An oligonucleotide containing alternating anionic and stereo-uniform cationic dimethylaminopropyl-phosphoramidate linkages, $d(T+T-)_7T$, is shown to bind with unusually high affinity to DNA [poly(dA) and $d(C_2A_{15}C_2)$] and RNA [poly(rA)] targets in solutions of low ionic strength. The isomeric oligomer with the opposite configuration at the phosphoramidate links binds much less efficiently.

Oligonucleotides containing pendant amino group acquire positive charges in neutral aqueous solutions that markedly influence their hybridization properties.[1-4] Thus, in the first report describing duplexes of cationic oligonucleotides it was shown that oligomers containing alternating phosphodiester (I) and basic aminoalkylphosphoramidate linkages behave as electrically neutral species at pH 7; they do not migrate on polyacrylamide gel electrophoresis, and the thermal dissociation curves for complexes formed with natural, anionic oligonucleotide targets are essentially independent of salt concentration.[1] Of special interest with respect to potential diagnostic applications was the observation that an anionic-cationic phosphoramidate derivative binds with higher affinity than the corresponding natural phosphodiester probe to a complementary oligonucleotide target in solutions of low ionic strength. This and a related study[2] were carried out with phosphoramidate derivatives generated without control of stereochemistry at the P-N bonds. As a consequence, the oligomers comprised a large number of stereoisomers.[5] Other groups have reported that the hybridization properties of oligonucleotides containing phosphodiester linkages alternating with methylphosphonate,[6] uncharged phosphoramidate[7] (III) or protonated aminoethylphosphonate linkages[4] (IV) depend on chirality at the phosphorus atoms bearing the modifications.



Phosphodiester Cationic Phosphoramidate Uncharged Phosphoramidate Cationic aminoethyl phosphonate

In hope of developing oligonucleotide probes with improved binding affinity, we have investigated a family of homothymidine oligomers $d[(T+T-)_7T]$ containing alternating anionic (I) and stereo-uniform cationic phosphoramidate (II) linkages. Stereochemical homogeneity was achieved by stepwise synthesis of the oligomers using dimer building blocks, DMT-d(T+T)-3'-$P(OCH_2CH_2CN)N(iPr)_2$, where + denotes a chirally selected phosphoramidate internucleoside linkage (II minus the proton). The synthesis and hybridization characteristics of these oligonucleotides are described in the present communication.

The dimer blocks were obtained by coupling 5'-DMT-dT-3'-P(OMe)N(iPr)$_2$ and dT-3'-TBDMS in the presence of tetrazole, followed by oxidation of the intermediate methyl phosphite triester with 3-dimethyl-aminopropylamine/I_2[8] and separation of the mixture of diastereoisomers by chromatography on silica gel. The dimer eluting first is designated here as **1a**, the one eluting later, as **2a**.[9] It proved important to separate the stereoisomers prior to removal of the 3'-O-*t*-butyldimethylsilyl group. After cleavage of the TBDMS group by treatment with tetrabutylammonium fluoride, each dimer unit was converted to the corresponding phosphoramidite derivative[10] using a one-fold excess of chloro-(diisopropylamino)-2-cyanoethoxyphosphine. These blocks were then employed individually to synthesize the pentadecathymidylate derivatives using standard protocols for coupling phosphoramidite reagents on a controlled pore glass support. Oligomers **1** (termed the "fast" isomer), **2** (termed the "slow" isomer), and **3** (the "mixed" isomer) were obtained using the phosphoramidate derivatives prepared from **1a**, **2a**, and an equimolar mixture of **1a** and **2a**, respectively. All syntheses proceeded in high yield as indicated by the DMT cation determination. The oligomers were purified by RP-HPLC in the DMT-form; then they were detritylated and lyophilized, and the purity of each was checked by IE-HPLC[11] and ^{31}P NMR.[12]

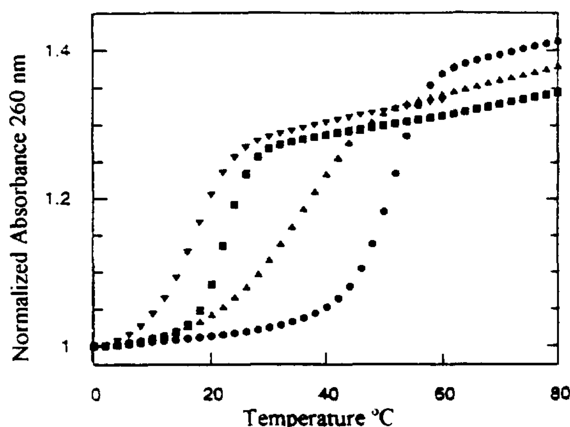


Figure 1. Melting curves for complexes of d($C_2A_{15}C_2$) with "slow oligomer" **2** (▼); dT_{15} (■); "mixed oligomer" **3** "(▲); and "fast oligomer" **1** (●) in 10 mM phosphate buffer, pH 7.0, no NaCl.

The hybridization properties of these oligomers were determined using two DNA targets [poly(dA) and d($C_2A_{15}C_2$)] and an RNA target [poly(rA)]. Representative thermal denaturation curves are shown in Figure 1, and Tm values are summarized in Table 1.[13] In agreement with related studies,[4,6,7] these data show that the stability of the complexes depends strongly on the configuration at the phosphorus atoms bearing the substituents. For both the DNA and the RNA targets, oligomer **1** forms duplexes of much higher stability than oligomer **2**; the binding affinity of oligomer **3** falls in between values for the pure isomers. Also, the melting curves for **1** and **2** are considerably sharper than for **3**, as expected for comparison of transitions involving stereo-uniform probes relative to the transition involving a probe consisting of many stereoisomers.

A striking result is the high affinity exhibited by oligomer **1** in solutions of low ionic strength. The enhancement in Tm relative to that for T_{15} is >30°C for the deoxyribonucleotide targets in 10 mM phosphate (no NaCl), i.e. about +4.3°C/modification. This increase is about twice that observed with anionic-cationic probe **3**, which has a mixture of stereoisomeric phosphorus atoms. The relative enhancement decreases with increasing salt concentration since, in contrast to the case for dT_{15}, the Tm values are independent of the salt concentration. However, even in 1 M NaCl the binding affinity of **1** is comparable to that for the all-phosphodiester probe.

Table 1. Tm values (°C) for complexes of $d(T+T-)_7T$ [13]

	poly(dA)			$d(C_2A_{15}C_2)$			poly(rA)		
Oligomer	No salt	0.1M	1.0M	No salt	0.1M	1.0M	No salt	0.1M	1.0M
dT_{15}	22	40	58	21.5	40	53	21	39	52
$d(T+T-)_7T$ (**1**) fast	58	58	58	52	52	52	37	39	41
$d(T+T-)_7T$ (**2**) slow	20	21	18*	19	19	26	18	19	20
$d(T+T-)_7T$ (**3**) mixed	40	40	44	38.5	39	40	27	27	29

* A second, higher value (39°C) represents another transition indicative of a novel structure, which is under study and will be described at a later time.

The effect of the stereo-uniform cationic substituents was also found to be significant, though smaller, for complexes formed with poly(rA). Under optimum conditions (oligomer **1**, no NaCl) the enhancement in Tm relative to dT_{15} is 16°C, or +2.3°/modification, for binding to poly(rA). This value may be compared to +0.9°/modification for the oligomer containing mixed isomers at phosphorus.

In summary, we have demonstrated that a family of oligodeoxyribonucleotides containing alternating anionic phosphodiester and cationic stereo-uniform phosphoramidate linkages can form unusually stable hybrids with DNA and RNA strands under conditions of low ionic strength. Hybridization is highly dependent on chirality at the phosphoramidate centers and is not sensitive to salt concentrations. The compounds offer especially attractive possibilities as probes for deoxyribo- and ribonucleotide segments that form secondary structures at high salt concentrations. Since the aminoalkylphosphoramidate group (II) is compatible with syntheses involving all four common nucleosides,[2] the chemistry should be useful in constructing high affinity probes directed toward any desired oligonucleotide sequence. Work concerning other nucleosides, including 2'-O-methyl derivatives, and the absolute configuration at the phosphoramidate linkage is in progress.

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References and Notes

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9. A clean separation was confirmed by thin layer chromatography of the products using silica gel plates with 8% methanol and 2% triethylamine in methylene chloride as eluent: Rf for **1a**, 0.55; Rf for **2a**, 0.38.
10. NMR spectra were recorded on a Varian 300 MHz instrument. ^{31}P spectra were run at 121 MHz with 85% aqueous H_3PO_4 as external reference. The ^{31}P NMR spectrum taken in acetonitrile for the "fast" stereoisomer of dT-P(O)($NHCH_2CH_2CH_2NMe_2$)T-P(OCH_2CH_2CN)N(iPr)$_2$ showed peaks at 150.0 and 149.7 ppm (phosphoramidite groups) and a single peak at 10.4 ppm for the internucleoside phosphoramidate group. The ratio of the integrated areas for phosphoramidite/phosphoramidate was 1/1. Similarly, the "slow" stereoisomer exhibited phosphoramidite peaks at 150.0 and 149.7 ppm and a phosphoramidate peak at 10.6 ppm, and the ratio for the areas for phosphoramidite/phosphoramidate was 1:1. A mixture of the two dimer units showed the expected peaks, including well resolved peaks at 10.4 and 10.6 ppm for the two phosphoramidate stereoisomers. The presence of the latter two peaks demonstrates that one of the phosphoramidate stereoisomers can be readily detected in the presence of the other; therefore, appearance of a single peak in the spectrum of each dimer block indicates a high degree of uniformity in chirality at the phosphoramidate centers. All dimer blocks gave satisfactory FABS spectra.
11. RP-HPLC was carried out on a Supelco LC-18 column (25 cm x 4.6 mm) with a 0-50% gradient (2%/min) of acetonitrile in 5% acetonitrile in 0.1M TEAA buffer, pH 7.5; flow rate 1 mL/min; retention time for DMT-d(T+T-)$_7$T prepared from **1a**, 19.8 min; for DMT-d(T+T-)$_7$T prepared from **2a**, 19.3 min. IE-HPLC was carried out with a Dionex DX 500 chromatograph system equipped with a P40 gradient pump, an AD 20 absorbance detector, and a Dionex Nucleopack PA-100 (4X250 mm) column. A flow rate of 1.5 mL/min was used with a 2%/min gradient of Solvent B (10 mM NaOH+ 1M NaCl) in Solvent A (10 mM NaOH). The retention times for the deprotected oligomers were: d-T_{15}, 27.35 min; **1**, 20.15 min; **2**, 20.15 min.
12. In view of the stereochemical stability of a variety of P(V) derivatives, including phosphate triesters, phosphorothioates, and methyl phosphonates, complications due to epimerization at phosphorus in the phosphoramidates are unlikely. Direct evidence that the stereochemical integrity of the phosphoramidate groups indeed remained intact, at least to a preponderant extent, during synthesis of the oligomers was provided by the ^{31}P NMR spectra of the dimer phosphoramidite derivatives (see footnote 10) and oligomers **1** and **2**. The ^{31}P NMR spectra for the oligomers in D_2O showed the following bands, attributable to phosphoramidate groups: **1**, +8.99 ppm; **2**, +9.35 ppm; **1** + **2**, 8.95 + 9.17 ppm. In addition, two bands attributable to phosphodiester groups were found in the region of -2.0 ppm (minor band) and -2.2 ppm (major band). The appearance of a distinctive signal for the phosphoramidate groups in **1** and a different signal for those in **2**, together with resolution of the two bands in the mixture (**1** + **2**), shows that epimerization was minimal if it occurred at all.
13. Thermal melt analyses were performed on a Varian Cary 3E spectrophotometer or a Perkin Elmer Lambda 2 spectrophotometer with 10 mM phosphate buffer, pH 7.0, at the indicated salt concentrations. The oligo(dT) compounds were present at approximately 5 μM concentration, and the dT/dA ratio was 1.0. The change in absorbance was measured at 260 nm with a temperature ramping of 0.5° C/min. Tm specifies the temperature at the midpoint of the region of maximum slope in the plot of A_{260} vs temperature.

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