Oligodeoxyribonucleotide N3′→P5′ Phosphoramidates: Synthesis and Hybridization Properties

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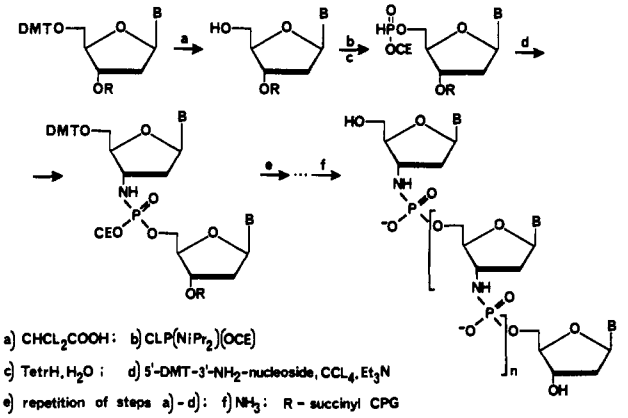
Oligonucleotides have been proposed as potent diagnostic compounds and as new rationally designed therapeutic agents.[1] The mechanism of action of these compounds is based on their specific interaction with RNA or DNA regions of interest. Several modifications of the natural phosphodiester internucleoside bond (phosphomono-[2] or dithioate,[3] methylphosphonate,[4] phosphodiester amidate[5]) have been introduced to improve the stability of the oligomers in biological media, as well as their hybridization properties. Unfortunately, the vast majority of these analogs exhibit somewhat reduced binding with target RNA or DNA strands *via* duplex or triplex formation.[6] Moreover, the presence of the stereoisomers at phosphorus also complicates the binding pattern with complimentary nucleic acids.[7]

Here we describe the synthesis and hybridization properties of oligonucleotides containing achiral internucleoside 3′-NHP(O)(O⁻)O-5′ phosphoramidate linkages.[8] These uniformly modified oligonucleotides were synthesized on a solid support using the step-by-step elongation procedure outlined in Scheme 1.

The synthetic cycle for addition of a single amino nucleoside consists of the following key operations:[9] detritylation; phosphitylation of the 5′-hydroxyl group to generate a polymer-supported 5′-H-phosphonate diester; Atherton–Todd type[10] coupling of a 5′-DMT-3′-aminonucleoside with the 5′-H-phosphonate in the presence of carbon tetrachloride. This cycle can be repeated several times, resulting in a phosphoramidate oligonucleotide after deprotection with ammonia. Average coupling yields were 94–96% per step as judged by DMT-cation assay. Oligonucleotides

Scheme 1



were isolated by IE HPLC, and purity was proven by capillary electrophoresis and slab gel electrophoresis analysis. The presence of the phosphoramidate linkages was also confirmed by ^{31}P-NMR[11] and by selective acid-catalyzed hydrolysis of phosphoramidate linkages.[10b]

Compounds synthesized in this study are presented in Table 1.

The stability of the oligonucleotide phosphoramidates toward hydrolysis by snake venom phosphodiesterase was evaluated in comparison with natural phosphodiester compounds. Phosphodiester decamer **1** (Table 1), treated with snake venom phosphodiesterase (0.2 OD/260 nm of oligonucleotide, 0.02 unit of phosphodiesterase I and 0.8 unit of alkaline phosphatase, both from Sigma in 0.2 mL of 20 mM Tris HCl buffer, pH 8.9), was completely hydrolyzed after 10 min, as judged by RP HPLC. Phosphoramidate analog **3** was practically intact even after 50 min. After 4.5 h, approximately 50% of **3** was converted to the presumed 9-mer $(TnpT)_4T_{NH2}$ with a terminal 3′-amino group, the presence of which retarded further digestion of the oligomer. After 22 h of hydrolysis, the starting 10-mer **3** was completely transformed to the 3′-amino-terminal 9-mer, and only ~20% of further digestion of the latter compound was observed.

Binding properties of the phosphoramidate analogs with complementary DNA or RNA strands were evaluated, and the data is summarized in Table 1. Substitution of the internucleoside phosphodiester for the N3′→P5′ phosphoramidate linkages dramatically changed the oligonucleotides' hybridization properties. Melting temperatures (T_m's) of duplexes formed by the entirely modified 10-mer **3** with poly(dA) and poly(A) were 36.0 and 51.5 °C, respectively, which are 6.3 and 24.5 °C higher than for the phosphodiester counterpart **1** (compare experiments 5 and 6 with 1 and 2, respectively). The same tendency remains for the mixed-base undecanucleotide **6**, where T_m's of duplexes with complementary DNA and RNA strands were 49.2 and 72.4 °C, respectively (experiments 13 and 14), which are 11.7 and 22.9 °C higher than for the parent phosphodiester compound **4** (experiments 8 and 9 and Figure 1). Also, the duplex with the same RNA target formed by phosphoramidate 11-mer **6** is more stable (by 18.0 °C) than one formed by the homologous RNA oligomer **5** (experiment 11). The oligonucleotide with alternating phosphodiester–phosphoramidate linkages also binds more tightly with the RNA strand, T_m 33.7 °C (experiment 4), than the phosphodiester compound, but less strongly with the DNA template, T_m 25.8 °C (experiment 3). Hybridization of the phosphoramidate oligonucleotides with complementary nucleic acids is sequence specific and determined by the proper Watson–Crick base pairing. The duplex formed by phosphoramidate **6** with single mismatched RNA target (experiment 15) is sub-

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(8) Oligonucleotides containing a single N3′→P5′ phosphoramidate linkage have been prepared through chemical ligation in aqueous media (Shabarova, Z. A. *Biochemie* **1988**, *70*, 1323–1334) or on a solid support *via* coupling of the preformed phosphoramidate dimer blocks (ref 10b and the following: Mag, M.; Rainer, S.; Engels, J. W. *Tetrahedron Lett.* **1992**, *33*, 7319–7322). Random size ribooligonucleotide N3′→P5′ phosphoramidates were obtained *via* self-polymerization of dimer blocks: Zielinski, W. S.; Orgel, L. E. *Nucleic Acids Res.* **1987**, *15*, 1699–1715.
(9) Synthesis of the phosphoramidate analogs was carried out either manually in a syringe or automatically on an ABI 384 synthesizer. For a given cycle the chemical steps, reagents, and reaction times were as follows: (a) detritylation, 3% dichloroacetic acid in dichloromethane, 1.5 min; (b) phosphitylation, 0.2 M (2-cyanoethoxy)-(*N*,*N*-diisopropylamino)chlorophosphine and 0.2 M *N*,*N*-diisopropylethylamine in dichloromethane, 10 min; (c) hydrolysis, 0.4 M tetrazole in acetonitrile/water, $^9/_1$ v/v, 5 min; (d) coupling, 0.2 M 5′-DMT-3′-aminonucleoside and 0.2 M triethylamine in carbon tetrachloride/acetonitrile, $^1/_1$ v/v, 20 min.
(10) (a) Atherton, F. R.; Openshaw, H. T.; Todd, A. R. *J. Chem. Soc.* **1945**, 660–663. (b) Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.* **1992**, *20*, 3403–3409. (c) 5′-DMT-3′-aminothymidine and 5′-DMT-3′-aminocytidine were obtained from 5′-DMT-2,3′-anhydronucleosides according to the following: Glinski, R. P.; Khan, S. M.; Kalamas, R. L.; Stevens, C. L. *J. Chem. Soc. D* **1970**, 915–916.
(11) The ^{31}P-NMR spectrum of the 10-mer **3**, Table 1, showed a single resonance peak at 7.12 ppm.

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Table 1. Oligonucleotides and T_m Values of the Duplexes and Triplexes[a]

expt	oligonucleotide	target	T_m (°C)[b]
1	TTTTTTTTTT, **1**	poly(dA)	29.7
2	same as expt 1	poly(A)	27.0
3	TnpTTnpTTnpTTnpTTnpT, **2**	poly(dA)	25.8
4	same as expt 3	poly(A)	33.7
5	TnpTnpTnpTnpTnpTnpTnpTnpTnpT, **3**	poly(dA)	36.0
6	same as expt 5	poly(A)	51.5
7	same as expt 5	d-AAAAAAAAAAC_4 TTTTTTTTTT	32.0; 47.2[c]
8	d-CTTCTTCCTTA, **4**	d-ATAAGGAAGAAGC	37.5
9	same as expt 8	r-AUAAGGAAGAAGC	49.5
10	same as expt 8	r-AUAAGGUAGAAGC[d]	35.1
11	r-CTTCTTCCTTA, **5**	r-AUAAGGAAGAAGC	54.4
12	same as expt 11	r-AUAAGGUAGAAGC	42.0
13	d-CnpTnpTnpCnpTnpTnpCnpCnpTnpTnpA, **6**	d-ATAAGGAAGAAGC	49.2
14	same as expt 13	r-AUAAGGAAGAAGC	72.4
15	same as expt 13	r-AUAAGGUAGAAGC	60.2

[a] T_m is the temperature at the midpoint of the melting curve; np is the abbreviation for the 3′-NHP(O)(O^-)O-5′ phosphoramidate link. The concentrations are as follows: oligomer strands, 5 μM; buffer A, 10 mM Tris HCl, 150 mM NaCl, pH 7.02; buffer B, 10 mM Tris HCl, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.02. [b] The T_m's of complexes in the buffer A. [c] T_m in the buffer B; the T_m of the hairpin duplex itself was 55.7 and 61.5 °C in buffers A and B, respectively. [d] Mismatched nucleotide is underlined.

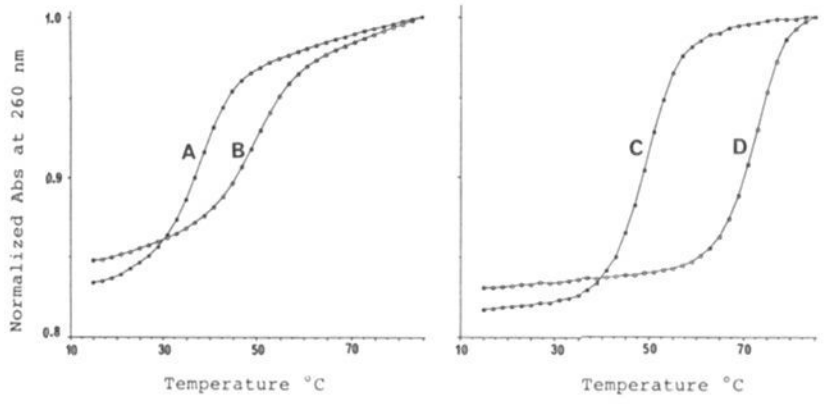

Figure 1. Melting curves for the duplexes formed by phosphodiester and phosphoramidate oligomers: A, C and B, D correspond to experiments 8 and 9 (phosphodiester **4**) and 13 and 14 (phosphoramidate **6**) in Table 1, respectively.

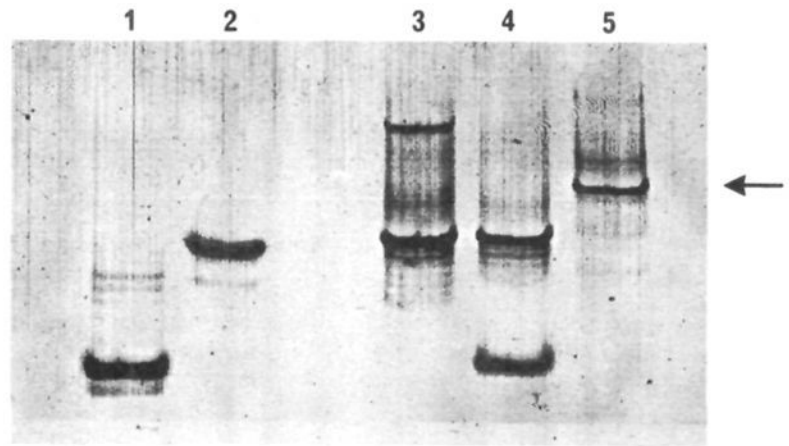

Figure 2. Gel-electrophoresis analysis of the oligonucleotide triplex formation under native conditions; 20% acrylamide, 5% bis-acrylamide in 10 mM $MgCl_2$, 80 mM Tris-borate buffer, pH 8.2, 10 °C. Lane 1: 10-mer phosphodiester **1**. Lane 2: 10-mer phosphoramidate **3**. Lane 3: 24-mer hairpin target d($A_{10}C_4T_{10}$); slow moving minor band is likely to correspond to the bimolecular duplex of d($A_{10}C_4T_{10}$). Lane 4: hairpin target and **1**. Lane 5: hairpin target and **3**; mobility of the triplex is denoted by arrow. Gel was stained with Stains-all (Kodak) and imaged on a Molecular Dynamics densitometer; note that efficiency of the phosphoramidate **3** staining is different from that for phosphodiester compounds.

stantially less stable (ΔT_m −12.2 °C) than one with the fully complementary RNA oligomer (experiment 13). About the same mismatch discrimination was observed for the phosphodiester deoxyribo- and ribooligonucleotides, where ΔT_m was −14.4 and −12.4 °C, respectively (experiments 10 and 12).

In addition, we evaluated the ability of the phosphoramidate analogs to form triplexes with double-stranded DNA, using thermal dissociation experiments and gel-shift analysis. Obtained melting curves show that decathymidilic phosphoramidate **6** forms a relatively stable triplex with the dA_{10}·dT_{10} duplex region of the hairpin DNA target d($A_{10}C_4T_{10}$), T_m 32 °C, under close to physiological conditions. A more stable triplex (T_m 42.2 °C) was observed in magnesium-containing buffer (experiment 7). The same T_m value was obtained for triplexes formed by phosphoramidate **3** with poly(dA)·poly(dT) duplex. Thermal dissociation of the triplexes was monitored by change of absorbance at 260 nm, as well as at 284 nm, which is characteristic for T·AT triplexes.[12] Results of the gel-shift experiments under native conditions also demonstrate formation of the stable triplex by phosphoramidate decamer **3** and dsDNA target (Figure 2). It is important to mention that under the same hybridization conditions phosphodiester decathymidilic acid **1** did not form triplexes with the same double-stranded DNA targets, as judged by the melting curves and by the gel-shift experiments (Figure 2).

Why do oligonucleotide phosphoramidates form more stable complexes than diesters with ssDNA and particularly with ssRNA and dsDNA targets? We think that substitution of the 3′-oxygen by nitrogen changes the 2′-deoxyribose conformation,[13] favoring the hydrogen binding between bases in presumably A-form of a double helix.[14] This spatial arrangement of the nucleotides is stabilized by the relatively rigid phosphoramidate backbone.

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Supplementary Material Available: Experimental details for synthesis, purification, and analysis of oligonucleotide N3′→P5′ phosphoramidates including IE HPLC and CE profiles and ^{31}P NMR spectra (Figure 3) (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(13) ^1H-NMR spectra of 3′-aminothymidine and the dTnpA dimer show differences in sugar puckering compared with dT and the dTA dimer; coupling constants J^3(H3′–H4′) for the former compounds are ~2 Hz larger than those for the latter ones, indicating increasing of the H3′–C3′–C4′–H4′ dihedral angle, which is characteristic of a C3′-endo sugar ring conformation.

(14) An additional interstrand hydrogen binding (mediated by a water molecule) between 3′-amino and 2′-hydroxyl or phosphate groups through the narrow major groove of the A-form phosphoramidate·RNA duplex is also possible.