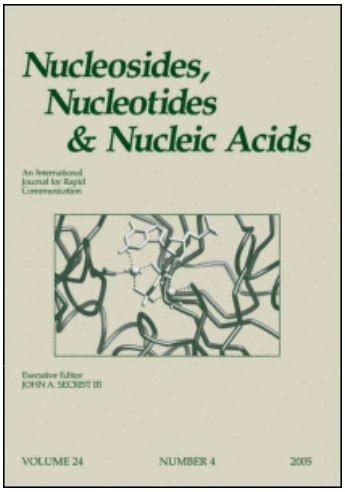

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis and Properties of the Oligonucleotide N3' →P5' Phosphoramidates

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To cite this Article Gryaznov, Sergei M.(1997) 'Synthesis and Properties of the Oligonucleotide N3' →P5' Phosphoramidates', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 899 — 905
To link to this Article: DOI: 10.1080/07328319708006106
URL: http://dx.doi.org/10.1080/07328319708006106

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 899-905 (1997)

SYNTHESIS AND PROPERTIES OF THE OLIGONUCLEOTIDE N3'→P5' PHOSPHORAMIDATES

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ABSTRACT. Uniformly modified oligonucleotide N3'→P5' phosphoramidates were synthesized. The prepared N3'→P5' phosphoramidates form extremely stable duplexes and triplexes with complementary nucleic acids. Moreover, these compounds are highly resistant to enzymatic hydrolysis by snake venom phosphodiesterase and cellular nucleases and they show high antisense activity *in vitro* and *in vivo*.

Synthetic oligonucleotides have become powerful tools in modern molecular biology and nucleic acid-based diagnostics. They may become a new generation of rationally designed therapeutic agents based upon specific interference with gene expression *via* antisense or antigene modes of action (1,2). Additionally, use of natural and modified oligonucleotides as aptamers could offer an interesting approach to specific inactivation of proteins based on their high affinity binding to the defined nucleic acids elements (3,4). Several modifications have been introduced to improve binding properties of oligonucleotides and their resistance to enzymatic hydrolysis (5). Recently, we described a new class of oligonucleotide analogs, N3'→P5' phosphoramidates, where the 3'-oxygen of each 2'-deoxyfuranose was substituted with nitrogen (6-8). These compounds form unusually stable duplexes with complementary DNA and especially RNA oligomers, as well as stable triplexes with polypurine-polypyrimidine double-stranded DNA targets. Thermal stabilization of the duplexes formed by phosphoramidates with single-stranded RNA was enhanced by up to 2.7 °C per modification and with single-stranded DNA by up to 0.7 °C per modification. Additionally, these compounds form very stable homoduplexes, whose melting temperatures, T_m, that were 2.1-2.3 °C per modification higher relative to their phosphodiester counterparts. The nature of the unusual stability of N3'→P5' phosphoramidate hybrids is not yet completely clear. One of the factors

contributing to the enhanced stability of the complex is a preference for N-sugar puckering of the 2'-deoxyfuranose of the phosphoramidates over the favored S-sugar puckering of phosphodiesters (7,9). Structure of the homoduplexes-duplexes formed by the phosphoramidates is similar to that of the isosequential RNAs, but not DNA. The phosphoramidate duplexes exist in an A-form, which is determined by the N-sugar puckering of the 3'-amino nucleosides (9). Interestingly, RRE and TAR elements of the HIV-I being composed by the chimeric N3'→P5' phosphoramidate/RNA duplexes or by homo-phosphoramidate duplexes is recognized by the RNA-binding Rev and Tat proteins with affinities similar to those for unmodified parent RNA complexes (10). Another contributing factor may be increased hydration and rigidity of the phosphoramidates relative to the parent phosphodiesters (8). Thus, the N3'→P5' phosphoramidates are differ from the phosphodiester oligomers in their chromatographic behavior and electroforetic mobility in either single-stranded or homo- or heteroduplexes forms, (FIGURE. 1).

Oligonucleotide phosphoramidates are resistant to enzymatic hydrolysis with phosphodiesterases and cellular nucleases, (FIGURE.2).

Chemically, these compounds are stable under neutral and alkaline conditions, and somewhat labile under acidic conditions. Acid-catalyzed hydrolysis of the phophoramidate presumably proceeds *via* protonation of 3'-nitrogen, followed by nucleophilic attack at phosphorus, or by metaphosphate formation. This leads to cleavage of the internucleoside N-P bond and formation of nucleotide fragments with terminal 3'-amino or 5'-phosphate groups (11). One of the possible approaches to increase acid stability of oligonucleotide phosphoramidates is to reduce the basicity of the 3'-nitrogen atom by placing a strong electron-withdrawing group nearby. An optimal candidate for this role could be fluorine, which is both strongly electron-withdrawing and sterecally undemanding. Phosphodiester and phosphorothioate oligonucleotides containing 2'-fluoro-2'-deoxynucleosides have been synthesized for antisense (12,13) and ribozyme (14) applications and they appear to adopt A-form duplexes determined by 3'-endo, or N-sugar, puckering (15,16).

Thus we synthesized the oligo-2'-fluoro-N3'→P5'phosphoramidates. Two different approaches to these compounds were developed: one utilizing Atherton-Todd type oxidative phosphorylation coupling similar to the procedure used for assembly of the 2'-deoxy-N3'→P5' phosphoramidates, and another, new and more efficient method utilizing a phosphoramidite transfer reaction (17). Several oligo-2'-fluoronucleotide phosphoramidates were synthesized using these procedures and their sequences and some physico-chemical characteristics are given in TABLE 1. A representative IE HPLC profile of a crude oligomer synthesis is shown in FIGURE 3.

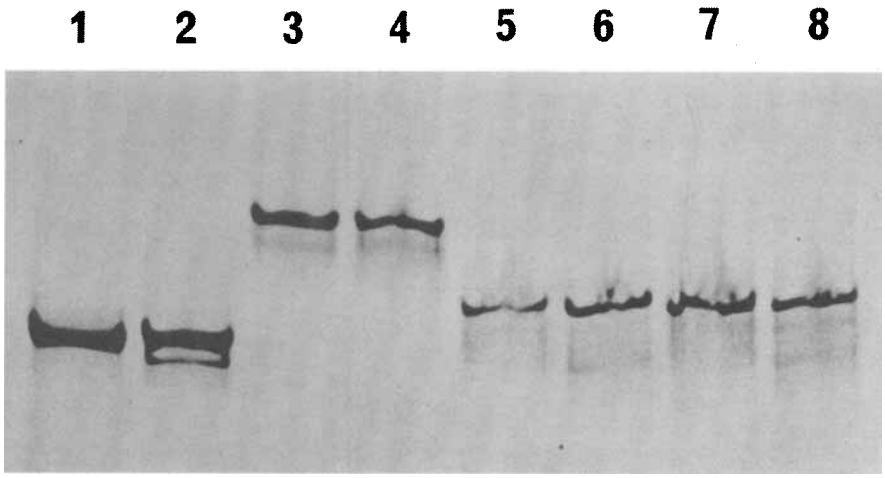

FIGURE 1. Slab gel electrophoretic analysis under native conditions of the duplexes formed by two different pairs of the isosequential phosphodiester and N3'→P5' phosphoramidate hexadecanucleotides. Lanes: 1, 2 - DNA homoduplexes; 3,4 - N3'→P5' phosphoramidate homoduplexes; 5 - 8 - DNA/N3'→P5' phosphoramidate heteroduplexes.

As follows from TABLE 1, the oligo-2'-fluorophosphoramidates form exceptionally stable duplexes with complementary DNA and RNA strands, even more stable than the 2'-deoxyphosphoramidates do. Thus, Tm values for the oligopyrimidines duplexes were increased by 16-25 °C (compare expt. 2 and 5, TABLE 1). Addition of magnesium cation to the melting buffer stabilized duplexes formed by phosphoramidates with DNA to a greater extent than those formed with RNA, suggesting a probable different secondary structure and solvation pattern of the complexes being formed.

The same trend in duplex thermal stability was observed for mixed-base 11-mer **8**, TABLE 1, which formed more stable hybrids with complementary DNA and RNA than did the analogous oligo-2'-deoxynucleoside phosphoramidate **7** (compare expt. 7 and 8, TABLE 1).

It is noteworthy that Tm values of the duplexes formed by 2'-fluoroamidates were 38-44 °C higher than those of isosequential phosphodiester compounds, with 4-5 °C per modification increase in melting temperatures (compare expt. 1, 5, 6, TABLE 1).

The stability of the oligo-2'-fluoro- in comparison with the oligo-2'-deoxynucleotide N3'→P5' phosphoramidates and with parent phosphodiesters toward enzymatic hydrolysis was evaluated next. Thus, phosphoramidates **2** and **5**, TABLE 1,

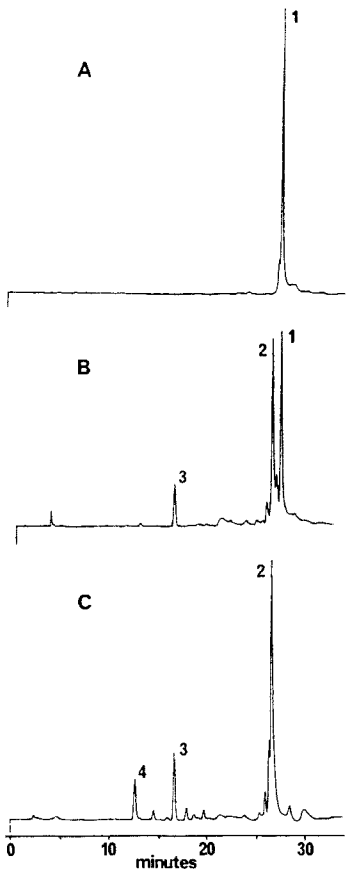

FIGURE 2. Reversed phase HPLC profiles of the reaction mixtures after incubation of the phosphoramidate decathymidilate with snake venom phosphodiesterase and alkaline phosphatase for 10 minutes, 4.5 and 22 hoirs A, B and C respectively; peaks 1, 2, 3 and 4 correspond to the starting compound, 9-mer with terminal 3'-amino group, thymidine and 3'-amino thymidine respectively.

were treated with snake venom phosphodiesterase and alkaline phosphatase and analyzed at successive time points by IE HPLC. Under the conditions used, oligo-2'-deoxyphosphoramidate **2** and oligo-2'-fluorophosphoramidate **5** were hydrolyzed progressively and at similar rates, with calculated half-lives of the full-length strands equal to 4.9 and 5.4 h, respectively. In comparison, decathymidylic acid with natural phosphodiester linkages was completely digested to thymidine within 10 min under the same conditions.

TABLE 1.

Oligonucleotides and T_m values of their duplexes.

Expt	Oligonucleotide[a]	T_m, °C[b]	
		DNA[c]	RNA[c]
1	UUUUUUUUUT, **1**	16.7; 24.6	17.9; 20.3
2	$U_{np}U_{np}U_{np}U_{np}U_{np}U_{np}U_{np}U_{np}U_{np}T$, **2**	18.5; 38.2	38.1; 47.2
3	$U_{np}U_{np}U_{np}U_{np}U^{f}_{np}U_{np}U_{np}U_{np}U_{np}T$, **3**	20.0; 41.0	40.1; 49.3
4	$U_{np}U_{np}U_{np}U^{f}_{np}U^{f}_{np}U_{np}U_{np}U_{np}U_{np}T$, **4**	23.4; 44.6	44.5; 52.7
5	$U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}T$, **5**	37.4; 56.3	55.6; 61.9
6	$U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{n}$, **6**	39.4; 58.0	55.6; 61.7
7	$_{p}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}T$, **7**	34.6; 63.0	55.2; 64.6
8	$_{p}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{np}U^{f}_{n}$, **8**	39.5; 63.2	56.4; 64.0
9	$C_{np}U_{np}U_{np}C_{np}U_{np}U_{np}C_{np}C_{np}U_{np}U_{np}A$, **9**	44.2; –	66.0; –
10	$C^{f}_{np}U^{f}_{np}U^{f}_{np}C^{f}_{np}U^{f}_{np}U^{f}_{np}C^{f}_{np}C^{f}_{np}U^{f}_{np}U^{f}_{np}A$, **10**	56.9; –	81.6; –

[a]All 2'-deoxy compounds; np, f, p, and n represent 3'-NHP(O)(O^-)O-5' internucleoside link, 2'-fluorine, 5'-phosphate, and 3'-amine, respectively; [b]T_m was determined with 3 μM of oligonucleotides; first values were determined in 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.04; second values were determined in same buffer containing an additional 10 mM magnesium chloride; dashes are for not determined T_ms. [c]complementary target; poly(dA) or poly(rA) for experiments 1-6, d(ATAAGGAAGAAGC) or r(AUAAGGAAGAAGC) for experiments 9 and10.

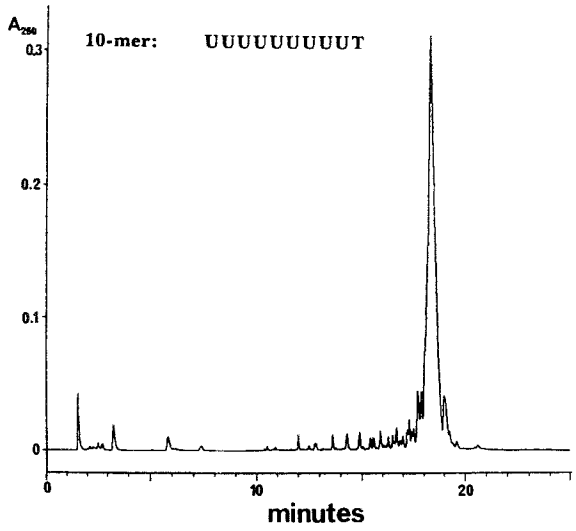

FIGURE 3. Ion exchange HPLC profile of the crude reaction mixture from synthesis of oligonucleotide **5**, TABLE 1. Pharmacia MonoQ 5/5 column and gradient 1.5% per minute of 1.5 M of NaCl in 10 mM NaOH were used on a Dionex DX 500 HPLC system.

Additionally, the stability of the phosphoramidates toward acid-catalyzed hydrolysis was studied. Oligonucleotides **2** and **5** were incubated at room temperature in 10% acetic acid, pH 2.2 or in 20 mM sodium acetate buffers, pH 4.7 and 5.3. The hydrolysis reactions were monitored by IE HPLC. The observed half-lives of full-length oligonucleotide **2** at pH 2.2, 4.7, and 5.3 were 21.5 min, 12.3 h, and 68 h, respectively. The oligo-2'-fluorophosphoramidate **5** was noticeably more stable under these conditions, with respective half-lives of this full-length oligomer of 61 min, 66 h, and 309 h. These results demonstrate a markedly reduced 3'-nitrogen basicity due to electron-withdrawing 2'-fluorine, with consequently greater acid stability of oligo-2'-fluorophosphoramidates relative to the parent oligo-2'-deoxyphosphoramidates.

In conclusion, the 2'-deoxy and 2'-fluoro oligonucleotide N3'→P5' phosphoramidates were synthesized using two different approaches, with the phosphoramidite transfer reaction shown as an efficient method for assembly of uniformly modified oligomers. The phosphoramidates form extremely stable duplexes with complementary DNA and RNA under close to physiological conditions, where T_m values were increased up to 4-5 °C per modification relative to natural phosphodiesters. In addition, 2'-fluoro amidates were more stable in acidic media than 2'-deoxynucleoside

phosphoramidates and are comparably resistant to enzymatic digestion by snake venom phosphodiesterase. The described properties of the oligonucleotide N3'→P5' phosphoramidates indicate that they have good potential as diagnostic and possible antisense agents.

ACKNOWLEDGEMENTS. I wish to thank Ronald Schultz, Jer-Kang Chen, David Lloyd, Larry DeDionisio, and Maria Koziolkiewicz for their contributions to this work.

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