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Oligonucleotide N3′→P5′ thiophosphoramidates: synthesis and properties

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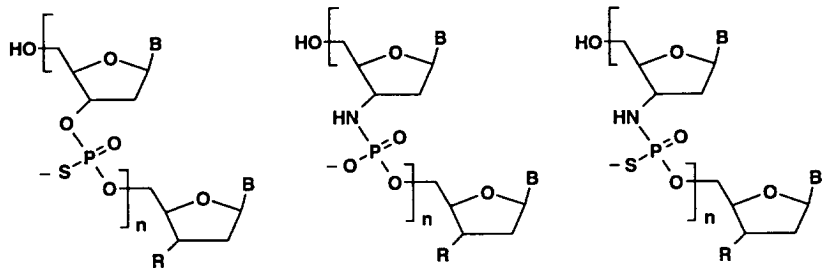
Abstract

Uniformly modified oligonucleotides with a novel sugar–phosphate backbone containing internucleoside 3′-NHP(O)(S⁻)O-5′ linkages were synthesized. These oligonucleotides were found to retain the high RNA binding affinity of the parent oligonucleotide N3′→P5′ phosphoramidates and to exhibit a much higher acid stability.

Synthetic oligonucleotide phosphorothioates (**I**) (Fig. 1), have recently become a new generation of rationally designed therapeutic agents with antisense or anti-mRNA mechanism of action.[1] Meanwhile, active research efforts continue in the area of novel oligonucleotide analogues, which may become the second and third generations of oligonucleotide-based therapeutic and diagnostic agents.[2] Among these compounds are the oligonucleotide N3′→P5′ phosphoramidates (**II**). The synthesis and properties of these oligomers have been recently described. The compounds contain a 3′-amino group at each of the 2′-deoxyfuranose nucleoside residues replacing a 3′-oxygen atom.[3]

The oligonucleotide N3′→P5′ phosphoramidates form unusually stable duplexes with complementary DNA and especially RNA strands, as well as stable triplexes with DNA duplexes, and they are also



(I) (II) (III)

Figure 1.

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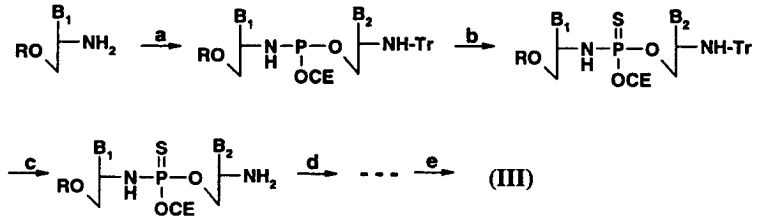
resistant to nucleases.[4,5] Moreover, oligonucleotide N3′→P5′ phosphoramidates were found to be more potent antisense agents than phosphorothioate derivatives both in vitro and in vivo.[6] At the same time the phosphoramidates apparently have a low affinity to the intra- and extracellular proteins and increased acid lability relative to the natural phosphodiester counterparts.[7] These two features of the oligonucleotide phosphoramidates may potentially adversely effect their pharmacological properties for some applications.

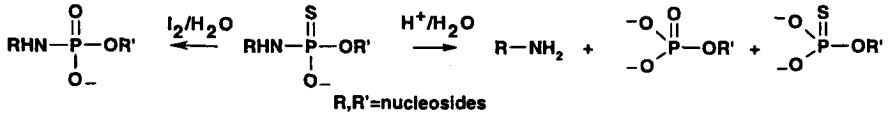
In order to create a new class of compounds which may embody the best characteristics from both oligonucleotide phosphoramidates and phosphorothioates we have synthesized oligonucleotide N3′→P5′ thiophosphoramidates (**III**), (Fig. 1). Oligonucleotide N3′→P5′ thiophosphoramidates were assembled on a solid support using phosphoramidite transfer methodology[8] as described in Scheme 1.



Scheme 1.

The synthetic strategy employs 3′-NH-trityl-protected 3′-aminonucleoside 5′-*O*-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidites.[8] Every synthetic cycle consisted of the following chemical steps: (1) detritylation; (2) coupling; (3) capping; and (4) sulfurization. For a step-wise sulfurization of the internucleoside phosphoramidite group formed after the coupling step, the iodine/water based oxidizing agent was replaced by the sulfurizing agents — either by elemental sulfur S_8 or by the commonly used Beaucage reagent — 3*H*-1,2-benzodithiol-3-one 1,1 dioxide.[9,10] Model thymidine dinucleoside *TnpsTn* with a 3′-NHP(O)(S^-)O-5′ internucleoside group was prepared using both types of sulfurizing agents. The reaction mixtures were analyzed and structure of the compound was confirmed by IE, RP HPLC, and ^{31}P NMR. The analysis revealed that sulfurization of the internucleoside phosphoramidite group with Beaucage reagent resulted in the formation of ~10–15% of the oxidized dinucleoside with 3′-NHP(O)(O^-)O-5′ phosphoramidate linkage (^{31}P NMR δ, ppm 7.0 in D_2O). Alternatively, sulfurization with molecular sulfur S_8 produced the desired dinucleotide containing 3′-NHP(O)(S^-)O-5′ internucleoside group with practically quantitative yield, as was judged by ^{31}P NMR and IE HPLC analysis (^{31}P NMR δ, ppm 56.4, 59.6 in D_2O, *Rp*, *Sp* isomers). Similar results with regards to the sulfurization efficiency were obtained for the synthesis of model oligonucleotide 11-mer GTTAGGGTTAG, where sulfurization with Beaucage reagent resulted in the full length product containing ~15% phosphoramidate linkages, as was judged by ^{31}P NMR analysis of the reaction mixture. Chemical shifts for the main peaks were ~57 and 60 ppm (broad doublets) and 7 ppm (broad singlet) corresponding to the thiophosphoramidate and phosphoramidate groups, respectively. In contrast, sulfurization with S_8 produced only ~2% phosphoramidate linkages in the 11-mer product according to the ^{31}P NMR analysis. The IE HPLC analysis of the oligomer was in good agreement with the ^{31}P NMR spectrum. The structure and purity of the final oligonucleotide products was confirmed by MALDI MS, by ^{31}P NMR, and by PAGE electrophoretic analysis.[11]

The model phosphoramidate nucleoside *TnpsTn* was quantitatively converted into the phosphoramidate counterpart *TnpTn*, by treatment with 0.1 M iodine solution in pyridine/THF/H_2O 1/4/0.1, v/v, 55°C, 15 min, as judged by IE HPLC and ^{31}P NMR (^{31}P NMR δ, ppm 7.0) (Scheme 2). Treatment of the *TnpsTn* dinucleotide with 10% acetic acid, at 55°C for 48 h unexpectedly resulted in only partial hydrolysis (~10%) of internucleoside phosphoramidate linkage. For comparison under these conditions the parent phosphoramidate dimer *TnpTn* was completely hydrolyzed. Cleavage of the N–P bond in the dinucleotide thiophosphoramidate was accompanied by concomitant de-sulfurization process (~15%), followed by a rapid hydrolysis of the resultant phosphoramidate -NHP(O)(O^-)O- group as revealed by IE HPLC and ^{31}P NMR (Scheme 2).



Scheme 2.

Oligonucleotide thiophosphoramidates also demonstrated an increased acid stability. The half-lives of thiophosphoramidate $TAG_3T_2AGACA_2$ and its phosphoramidate counterpart in 40% aqueous acetic acid at room temperature were approximately 6 h and 0.5 h, respectively, according to IE HPLC analysis. Moreover, the composition of the hydrolysis products was different. The acid hydrolysis of the thiophosphoramidate appears to initially result in de-sulfurization, rather than cleavage of internucleoside N–P groups, as it occurs for the phosphoramidates. These results indicated a much higher resistance to acidic conditions of the thiophosphoramidates than that of the phosphoramidate oligonucleotides.

Duplex formation properties of oligonucleotide phosphoramidates with complementary RNA strand were evaluated using thermal dissociation experiments. The results are summarized in Table 1. The presented data show that the oligonucleotide thiophosphoramidates formed significantly more stable complexes than the isosequential natural phosphodiester oligomers do—ΔTm was ~25–27°C per oligomer. Also, the increase in the termal stability of duplexes is similar to that observed for the phosphoramidate oligomers. This indicates, that the substitution of non-bridging oxygen by sulfur atom in internucleoside phosphoramidate group does not alter the RNA binding properties of these compounds significantly, which is determined by *N*-type sugar puckering of the 3′-aminonucleosides and by increased sugar–phosphate backbone hydration.[5]

In summary, a new class of oligonucleotide analogs was synthesized. These compounds form stable duplexes and are resistant to acid catalyzed hydrolysis.

Table 1

Expt	Oligomer	Type[a]	Tm, °C[b]	ΔTm, °C[c]
1.	GTTAGGGTTAG	po	44.2	-
2.	TAGGGTTAGACAA	po	45.2	-
3.	Same as expt 1	np	72.1	27.9
4.	Same as expt 2	np	71.7	26.5
5.	Same as expt 1	nps	71.5	27.3
6.	Same as expt 2	nps	70.0	24.8

[a]po, np, nps correspond to phosphodiester, N3'→P5' phosphoramidate and thiophosphoramidate groups respectively; [b]melting temperature, Tm (±0.5°C) of the duplexes formed with a complementary natural RNA oligomer in 150 mM NaCl, 10 mM sodium phosphate buffer pH 7.4;

[c]increase of Tm relative to the natural phosphodiester counterpart.

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10. Oligonucleotide syntheses were performed with a 1% solution of Beaucage reagent in anhydrous acetonitrile or 15% S_8 in CS_2/Et_3N, 99/1, v/v as the sulfurizing agent on 1 μmole scale.
11. Molecular mass for thiophosphoramidate oligomers $GTAG_3T_2AG$ and $TAG_3T_2AGACA_2$ calculated 3 577.11 and 4 202.69, found by MALDI MS 3577 and 4203, respectively; mobility in 15% PAGE relative to isosequential phosphoramidates was 0.95 and 0.97, respectively.