**α-Oligodeoxyribonucleotide N3′→P5′ phosphoramidates: synthesis and duplex formation**

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Received August 20, 1997; Revised and Accepted December 14, 1997

ABSTRACT

The synthesis and hybridization properties of novel nucleic acid analogs, α-anomeric oligodeoxyribonucleotide N3′→P5′ phosphoramidates, are described. The α-3′-aminonucleoside building blocks used for oligonucleotide synthesis were synthesized from 3′-azido-3′-deoxynucleosides via acid catalyzed anomerization or transglycosylation reactions. The base-protected α-5′-O-DMT-3′-aminonucleosides were assembled into dimers and oligonucleotides on a solid support using the oxidative phosphorylation method. 1H NMR analysis of the α-N3′→P5′ phosphoramidate dimer structures indicates significant differences in the sugar puckering of these compounds relative to the β-N3′→P5′ phosphoramidates and to the α-phosphodiester counterparts. Additionally, the ability of the α-oligodeoxynucleotide N3′→P5′ phosphoramidates to form duplexes was studied using thermal denaturation experiments. Thus the N3′→P5′ phosphoramidate decamer containing only α-thymidine residues did not bind to poly(A) and exhibited lower duplex thermal stability with poly(dA) than that for the corresponding β-anomeric phosphoramidate counterpart. A mixed base decamer α-CTTCCTCTTT formed duplexes with the RNA and DNA complementary strands only in a parallel orientation. Melting temperatures of these complexes were significantly lower, by 34–47 or 15–25 °C, than for the duplexes formed by the isosequential β-phosphoramidates in antiparallel and parallel orientations respectively. In contrast, the α-decaadenylic N3′→P5′ phosphoramidate formed duplexes with both RNA and DNA complementary strands with a stability similar to that of the corresponding β-anomeric phosphoramidate. Moreover, the self-complementary oligonucleotide α-ATATATATAT did not form an α:α homoduplex. These results demonstrate the effects of 3′-aminonucleoside anomeric configuration on sugar puckering and consequently on stability of the duplexes.

INTRODUCTION

Oligodeoxynucleotides have been proposed as rationally designed therapeutic agents with a mechanism of action based upon specific base pairing with an mRNA region of interest or with certain sequences of double-stranded (ds)DNA targets (1,2). Additionally, properly spatially preorganized oligonucleotides can be used as highly selective and specific protein binding agents; so called aptamers or decoys (3,4). Due to the fact that the oligonucleotides with natural phosphodiester linkages are prone to hydrolysis by nucleases, compounds with structural modifications have been introduced (5,6). Furthermore, the requirement for improved target affinity, cellular uptake and biodistribution has inspired the design and synthesis of a large number of modified oligodeoxynucleotides for possible therapeutic applications. Modifications have been introduced for the heterocyclic bases of nucleosides, ribose residues and the phosphate backbone. Extensive reviews are now available on various physicochemical and in vitro and in vivo properties of oligodeoxynucleotide analogs (5–7). Several antisense oligonucleotide phosphorothioates have been studied in Phase I/II clinical trials (8–10). While the potential for therapeutic efficacy of oligonucleotide phosphorothioates has been demonstrated, several problems remain, mainly: low binding affinity to single-stranded (ss)RNA targets, limited triplex stability and non-specific binding to cellular proteins (11,12). Thus the search for better oligonucleotide analogs continues.

Uniformly modified oligonucleotide N3′→P5′ phosphoramidates where the 3′-hydroxyl was substituted by a 3′-amino group on the sugar residue have recently been introduced (13). These compounds are resistant to nucleases, form stable duplexes with complementary ssRNA strands as well as stable triplexes with dsDNA targets (14–16). Additionally, the oligonucleotide phosphoramidates have demonstrated promising sequence-dependent antisense and antigene activity in various in vitro and in vivo systems (17–19). In addition, α-anomeric phosphodiester oligodeoxynucleotides have been described (20). Studies with uniformly modified α-anomeric oligodeoxynucleotides have demonstrated that these compounds form stable parallel duplexes with the complementary natural β-anomeric ssRNA targets, as well as with ssDNA strands (21–23). These compounds are resistant to nucleases (20,21) and it has been shown that their duplexes with RNA are not substrates for RNase H (22). Also, α-anomeric oligodeoxynucleotides have been shown to inhibit reverse transcription, either as uniformly modified sequences or as chimeras with β-ancomers (24–26). Triple helix formation by the α-oligodeoxynucleotides has also been investigated (27). A recent study has demonstrated parallel binding of α-oligoborinucleotides as a third strand and triplex formation with polypurine-polypyrimidine tracks of DNA duplexes (28).

The aim of the present study was to combine these two types of modified oligonucleotides into one and to synthesize and evaluate

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physicochemical properties of α-anomeric oligonucleotide N3’→P5’ phosphoramidates (Fig. 1).

RESULTS AND DISCUSSION

Synthesis of the α-3′-aminonucleosides

The synthetic scheme for preparation of the α-N3’→P5’ phosphoramidate oligonucleotide building blocks is outlined in Schemes 1 and 2. Thus 5′-O-anisoyl-3′-azidothymidine 1t was obtained from thymidine using Mitsunobu reaction conditions to form 5′-O-anisoyl-2,3′-anhydrothymidine, followed by treatment with lithium azide (29). Anomerization of 1t into a mixture of α and β isomers was in general according to the literature procedure with a catalyst containing concentrated sulfuric acid and acetic anhydride in acetonitrile (30). This reaction for the 3′-azidonucleoside required higher concentrations of the catalyst to be used as compared with a described process for 3′,5′-O-diacetylthymidine (30). Thus the α anomer 2t was obtained with an α to β isomer ratio of ~3:2. Also, a significant amount of by-products, likely associated with nucleoside sugar ring opening, was observed. The α,β-anomeric mixture of 3′-azidothymidine was not separable in the 5′-O-protected form, but after removal of the anisoyl group pure 3t was isolated by silica gel column chromatography. Protection of the 5′-hydroxyl group with dimethoxytrityl chloride (4t) and subsequent reduction of the 3′-azido moiety with hydrogen sulfide produced 5 in a total yield of 17.4% based on 1t. The starting material 1u for preparation of α-cytidine-containing monomer 6 was obtained in a manner analogous to 1t (Scheme 1). Anomerization of 1u to 2u proceeded as for 2t, but with slightly higher loss of the nucleoside sugar ring. After dimethoxytritylation of 3u the pure α-anomer 4u was isolated by silica gel chromatography. Reaction of 4u with phosphorus oxychloride and triazole according to a literature procedure (14,36) resulted in a 4-triazolo derivative which, upon replacement with ammonia, gave the base-unprotected cytosine nucleoside. Then N-benzoylation of the N-4 amino group followed by reduction of the 3′-azido moiety with hydrogen sulfide resulted in monomer 6 in 16.4% yield based on 1u.

The 3′-amino-α-adenosine monomer 9 was also synthesized from the same starting compound 5′-O-anisoyl-3′-azidothymidine 1t, as
HPLC. The dimers were characterized using \(^1\)H and \(^31\)P NMR and phosphoramidates (14). First, the model dimers to that used for preparation of tetrachloride-driven oxidative phosphorylation reaction, analogous to those for the Coupling yields for the were analyzed and purified by reversed phase (RP) HPLC. and \(5'\)-terminal (\(\alpha\)-Tnp) and \(5'\)-terminal (\(\beta\)-Tnp) nucleosides respectively.

Figure 2. The H1' region of the proton NMR spectra of the (A) \(\alpha\)- and (B) \(\beta\)-N3'→P5' phosphoramidate dithymidylates. Peaks I and II assigned to H1' of 3'-terminal (\(\alpha\)-pT and \(\beta\)-pT) and 5'-terminal (\(\alpha\)-Tnp and \(\beta\)-Tnp) nucleosides respectively.

shown in Scheme 2. We used a transglycosylation reaction catalyzed by TMS-triflate, which has been described before for the preparation of \(\alpha\)-purine nucleosides (32–34). The \(\alpha\) and \(\beta\) anomers could be separated only after deprotection of the 5'-hydroxyl group of the nucleosides to produce compound 7. The subsequent 5'-O-dimethoxytritylation of 7 and reduction of the 3'-azido group with hydrogen sulfide resulted in \(\alpha\)-3'-amino-nucleoside 9 in 8.1% overall yield based on It.

Synthesis of the \(\alpha\)-oligonucleotide N3'→P5' phosphoramidites

The title compounds were assembled on a solid support from the 5'-O-DMT-3'-aminonucleosides 5, 6 and 9 using a carbon tetrachloride-driven oxidative phosphorylation reaction, analogous to that used for preparation of \(\beta\)-oligonucleotide N3'→P5' phosphoramidates (14). First, the model dimers \(\alpha\)-TnpT, \(\alpha\)-CnpT and \(\alpha\)-AnpT' were synthesized using this method. These compounds were analyzed and purified by reversed phase (RP) HPLC. Coupling yields for the \(\alpha\)-3'-aminonucleosides were similar to those for the \(\beta\) counterparts and were 95–96% as judged by RP HPLC. The dimers were characterized using \(^1\)H and \(^31\)P NMR and acid hydrolysis. \(^1\)H NMR of the dimers confirmed the presence of the \(\alpha\)-aminonucleoside and \(\alpha\)-thymidine in a 1:1 ratio. Coupling constants \(^3J\) for H1'-H2'/H2' were 6.8, 3.7 and 7.0 Hz (\(\alpha\)-TnpT), 6.4, 3.3 and 7.1 Hz (\(\alpha\)-CnpT) and 6.4, 4.4 and 6.4 Hz (\(\alpha\)-AnpT'). The first number for each dimer corresponds to the equal H1'-H2'/H2' coupling constants for the \(\alpha\)-3'-amino-nucleoside and the next two numbers to the different coupling constants for the \(\alpha\)-hydroxy-nucleoside (Fig. 2). This indicates differences in sugar puckering of the \(\alpha\)-3'-amino and \(\beta\)-3'-amino or the \(\alpha\)-hydroxy-nucleosides. Coupling constants \(^3J\) H1'-H2' in the 6.0–7.0 Hz range are characteristic of an S-type sugar puckering for the \(\beta\)-nucleosides, whereas 3'-amino-\(\beta\)-nucleosides typically have smaller \(^3J\) H1'-H2', within the range 2.4–3.0 Hz, which is typical for a N-type sugar conformation (37,38). Thus sugar puckering of \(\alpha\)-aminonucleosides is apparently similar to that for the \(\beta\)-3'-hydroxynucleosides, rather than to the \(\beta\)-3'-amino counterparts (see Fig. 2). The \(^31\)P NMR spectra of the model dimers also confirmed their purity and the presence of an internucleoside phosphoramidate monooester linking group with characteristic resonances at 6–8 p.p.m. Acid hydrolysis of the dimer \(\alpha\)-TnpT' with 40% acetic acid produced the expected 3'-amino-\(\alpha\)-thymidine and 5'-\(\alpha\)-thymidylid acid in a 1:1 ratio, as judged by RP HPLC analysis of the hydrolysate (see Materials and Methods). Then the same oligonucleotide chain assembly protocol was extended to synthesis of the \(\alpha\)-oligonucleotide N3'→P5' phosphoramidates \(\alpha\)-TnpT's, \(\alpha\)-(AT)\(_3\) and \(\alpha\)-A\(_{10}\)T. Stepwise coupling yields for the \(\alpha\)-oligonucleotide phosphoramidates were similar to those for \(\beta\) counterparts and were within the range 93–97%, as judged by stepwise release of the DMT cation, except for \(\alpha\)-oligoadenylate, where coupling yields were 88–94% per step. The synthesized \(\alpha\)-oligonucleotide phosphoramidates had practically the same retention times on an ion exchange (IE) HPLC column as the isosequential \(\beta\) counterparts (see Materials and Methods for the HPLC conditions used). Additionally, \(\alpha\)-decathymidylic phosphoramidate was characterized by electrospray ionization mass spectrometry in the negative ion mode (mol. wt measured 2968.8, mol. wt calculated 2969.6).

Thermal stability of the phosphoramidate duplexes

We have studied the ability of the synthesized \(\alpha\)-oligonucleotide N3'→P5' phosphoramidates to form duplexes with complementary DNA and RNA strands using thermal dissociation experiments. Melting temperatures for the complexes were determined from the recorded melting curves and data from this study are summarized in Table 1. The results show that the pyrimidine-containing \(\alpha\)-N3'→P5' phosphoramidates form much less stable duplexes with both DNA and RNA complements, relative to the \(\beta\)-N3'→P5' phosphoramidate counterparts. Thus duplex melting temperatures \(T_m\) for the \(\alpha\) and \(\beta\)-decathymidylates with complementary poly(dA) and poly(A) were 29.3 and \(\leq\)0°C and 51.2 and 58.2°C respectively (experiments 3 and 4 and 1 and 2, Table 1). Analogous results were observed with the C,T-containing \(\alpha\) and \(\beta\)-decamers having sequence 5'-TTCTTTCTTT-3', where \(T_m\) values for the parallel duplexes formed by the \(\alpha\)-oligonucleotide were 23.3 and 34.6°C and for the antiparallel duplexes for the \(\beta\) counterpart 57.8 and 82.0°C with DNA and RNA complements respectively (experiments 9 and 10 and 5 and 6, Table 1). The self-complementary \(\alpha\)-oligonucleotide phosphoramidate \(\alpha\)-(AT)\(_3\) apparently did not form an \(\alpha\)-\(\alpha\) phosphoramidate homoduplex, as judged by the absence of both sigmoidal melting curves and hypochromic changes during thermal dissociation experiments. It is important to emphasize that the \(\alpha\)-oligonucleotide N3'→P5' phosphoramidates form only parallel duplexes with either DNA or RNA complementary strands. Formation of the antiparallel...
duplexes by α-phosphoramidates was not observed (experiments 11 and 12, Table 1). In contrast, β-oligonucleotide N3→P5' phosphoramidates formed stable antiparallel as well as parallel duplexes with both DNA and RNA complements (experiments 7 and 8, Table 1). In general we observed thermal stability of the parallel duplexes to be 17–19°C lower than that for the antiparallel counterparts. Melting curves for the parallel duplexes formed by the β-phosphoramidates have a typical sigmoidal transition and also good hypochromicity of 20–25% for the melting transitions. It is interesting that the thermal stability of the parallel β-N3→P5' phosphoramidate duplexes with RNA was significantly higher than that for the antiparallel duplexes formed by the isosequential phosphodiester (compare experiments 8 and 18, Table 1).

Duplex formation in a parallel orientation by the β-T,C-containing phosphoramidate oligomer may be due to formation of either reversed Watson–Crick or Hoogsteen type C:G base pairs. In the latter case cytosine should be protonated. It was demonstrated previously that for the natural phosphodiester oligonucleotides reversed Watson–Crick C:G base pairs are very unstable and their incorporation into a homo(T):homot(A)-containing parallel duplex results in significant destabilization of the complexes (39). Thus one can suggest that the increased stability of the parallel C:G base pairs for the N3→P5' phosphoramidates might be due to an ability of the 3'-amino-2'-deoxycytidines to adopt an imino resonance form, resulting in formation of two hydrogen bonds between guanine and iminocytosine heterocycles (Fig. 3A).

Alternatively, Hoogsteen type C:G along with Hoogsteen type T:A base pairs (Fig. 3B) between the phosphoramidate and DNA or RNA strands can also result in formation of stable parallel duplexes. This type of strand binding may be possible because the C,G-containing oligonucleotide N3→P5' phosphoramidates form very stable triplexes with polypurine regions of nucleic acids, where the N3→P5' phosphoramidate strand hybridizes via Hoogsteen type hydrogen bonding (15,16). A further study needs to be conducted to elucidate the exact nature of the hydrogen bonds in the amide parallel duplexes. Additionally, duplex formation by the β-oligonucleotide N3→P5' phosphoramidates with complementary RNA strands was studied by gel mobility shift assays using capillary electrophoresis (CE) gels. This analytical method also demonstrated formation of stable parallel duplexes by the β-oligonucleotide N3→P5' phosphoramidates (data not shown).

### Table 1. Oligonucleotides and Tm values of their duplexes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Oligonucleotidea</th>
<th>Target</th>
<th>Duplex typeb</th>
<th>Tm (°C)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-TnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>poly(dA)</td>
<td>ap</td>
<td>34.0, 51.2</td>
</tr>
<tr>
<td>2</td>
<td>β-TnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>poly(A)</td>
<td>ap</td>
<td>54.7, 58.2</td>
</tr>
<tr>
<td>3</td>
<td>α-TnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>poly(dA)</td>
<td>p</td>
<td>&lt;10, 29.3</td>
</tr>
<tr>
<td>4</td>
<td>α-TnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>poly(A)</td>
<td>p</td>
<td>no, no</td>
</tr>
<tr>
<td>5</td>
<td>β-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>d-TAAAGAAGAAAGC</td>
<td>ap</td>
<td>52.8, 57.8</td>
</tr>
<tr>
<td>6</td>
<td>β-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>r-UAAGAGAAAGAGA</td>
<td>ap</td>
<td>70.8, 82.0</td>
</tr>
<tr>
<td>7</td>
<td>β-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>d-AGAGAAGAAGA</td>
<td>p</td>
<td>34.3, 38.3</td>
</tr>
<tr>
<td>8</td>
<td>β-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>r-GAAGAGAAAGA</td>
<td>p</td>
<td>57.7, 64.9</td>
</tr>
<tr>
<td>9</td>
<td>α-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>d-GAAGAAGAAGA</td>
<td>p</td>
<td>17.5, 23.3</td>
</tr>
<tr>
<td>10</td>
<td>α-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>r-GAAGAAGAAAGA</td>
<td>p</td>
<td>32.8, 34.6</td>
</tr>
<tr>
<td>11</td>
<td>α-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>d-AGAGAAGAAGA</td>
<td>ap</td>
<td>no, no</td>
</tr>
<tr>
<td>12</td>
<td>α-CnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpTnpT</td>
<td>r-UAAGAGAAAGAGA</td>
<td>ap</td>
<td>no, no</td>
</tr>
<tr>
<td>13</td>
<td>β-AnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpA</td>
<td>poly(dT)</td>
<td>ap</td>
<td>20.0, nd</td>
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<td>14</td>
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<td>poly(U)</td>
<td>p</td>
<td>50.8, nd</td>
</tr>
<tr>
<td>15</td>
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<td>poly(dT)</td>
<td>p</td>
<td>36.5, 51.0</td>
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<tr>
<td>16</td>
<td>α-AnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpAnpT</td>
<td>poly(U)</td>
<td>p</td>
<td>41.9, 58.6</td>
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<tr>
<td>17</td>
<td>CpTpTpCpTpTpCpTpTpT</td>
<td>d-TAAAGAAGAAAGC</td>
<td>ap</td>
<td>35.5, nd</td>
</tr>
<tr>
<td>18</td>
<td>CpTpTpCpTpTpCpTpTpT</td>
<td>r-UAAGAGAAAGA</td>
<td>ap</td>
<td>47.5, nd</td>
</tr>
</tbody>
</table>

aAll compounds are 2-deoxy. p and np correspond to the internucleoside phosphodiester or N3→P5' phosphoramidate groups respectively.
bap and p correspond to the presumed antiparallel and parallel oligomer strand orientations respectively.
cTm was determined with ~3 μM oligonucleotides. First value in a buffer containing 10 mM sodium phosphate, 150 mM NaCl, pH 7.0; second value in the same buffer containing an additional 10 mM MgCl2. no, duplex formation was not observed; nd, not determined.
evaluated using 1H NMR spectroscopy. The study demonstrated puckering of the synthesized model dimer compounds was amidates, were prepared and characterized. Nucleoside sugar ring conformations, while the α275–280 nm and a negative CD band at 247–252 nm. The positive signal, suggesting an overall A-type conformation (40). A exact structure of the duplexes with both DNA and RNA complements with thermal stabilities close to those for the β-phosphoramidate and β-phosphodiester counterparts (compare experiments 13 and 14 and 16, Table 1). This difference in the binding properties of the α-oligopyrimidine and α-oligopyrimidyl phosphatephosphorimidyl phosphorimidates may be due to the different sugar puckering of the α-purine and α-pyrimidine 3′-aminonucleosides, as seen from the model dimer 1H NMR study. Better base stacking interactions for the α-oligoadenylate than for the α-oligothymidylyl phosphatephosphorate may also contribute to better binding of the former compound. The overall structure of the α- and β-N3′→P5′ phosphorimidate duplexes was evaluated by CD spectroscopy. The uniformly modified α-T10 oligodeoxyribonucleotide N3′→P5′ phosphoramidate forms a duplex with poly(dA) (experiment 3, Table 1) and exhibits positive CD bands at 220 and 280 nm and a negative CD band at 250 nm. These bands are characteristic of a general B-type helix. Also, the CD spectra of the parallel duplexes formed with DNA or RNA complements by the mixed base oligonucleotide 5′-α-CTTCCTCCTT-3′ N3′→P5′ phosphorimidates were compared with those formed by the isosequential β anomer (Fig. 4.). The CD spectra of all duplexes contain a positive CD band at 275–280 nm and a negative CD band at 247–252 nm. The α-phosphorimidate CD spectra indicate more of a B-type helical conformation, while the β counterpart shows a more intensive positive signal, suggesting an overall A-type conformation (40). A detailed NMR or X-ray analysis will be needed to determine the exact structure of the α-phosphorimidate duplexes.

CONCLUSIONS

In contrast to the α-oligothymidylic or α-cytidine and α-thymidine containing oligonucleotide N3′→P5′ phosphorimidates, the α-decaadenylic N3′→P5′ phosphoramidate formed duplexes with both DNA and RNA complements with thermal stabilities close to those for the β-phosphoramidate and β-phosphodiester counterparts (compare experiments 13 and 14 and 16, Table 1). This difference in the binding properties of the α-oligopyrimidine and α-oligopyrimidyl phosphorimidates may be due to the different sugar puckering of the α-purine and α-pyrimidine 3′-aminonucleosides, as seen from the model dimer 1H NMR study. Better base stacking interactions for the α-oligoadenylate than for the α-oligothymidylyl phosphatephosphate may also contribute to better binding of the former compound. The overall structure of the α- and β-N3′→P5′ phosphorimidate duplexes was evaluated by CD spectroscopy. The uniformly modified α-T10 oligodeoxyribonucleotide N3′→P5′ phosphoramidate forms a duplex with poly(dA) (experiment 3, Table 1) and exhibits positive CD bands at 220 and 280 nm and a negative CD band at 250 nm. These bands are characteristic of a general B-type helix. Also, the CD spectra of the parallel duplexes formed with DNA or RNA complements by the mixed base oligonucleotide 5′-α-CTTCCTCCTT-3′ N3′→P5′ phosphorimidates were compared with those formed by the isosequential β anomer (Fig. 4.). The CD spectra of all duplexes contain a positive CD band at 275–280 nm and a negative CD band at 247–252 nm. The α-phosphorimidate CD spectra indicate more of a B-type helical conformation, while the β counterpart shows a more intensive positive signal, suggesting an overall A-type conformation (40). A detailed NMR or X-ray analysis will be needed to determine the exact structure of the α-phosphorimidate duplexes.

CONCLUSIONS

Novel nucleic acid analogs, α-oligonucleotide N3′→P5′ phosphorimidates, were prepared and characterized. Nucleoside sugar ring puckering of the synthesized model dimer compounds was evaluated using 1H NMR spectroscopy. The study demonstrated different sugar conformations of pyrimidine-containing β-phosphorimidates and their α counterparts, being C3′-endo and presumably C2′-endo respectively. In contrast, 3′-aminodeoxyfuranose conformations of α- and β-adenosine-containing phosphorimidates are more similar to each other and likely correspond to C3′-endo puckering. This emphasizes the importance and influence of the anomeric positioning of bases on the 3′-amino nucleoside sugar ring conformation and, consequently, its influence on the oligonucleotide duplex formation properties. Thus pyrimidine-containing α-oligonucleotide phosphorimidates form much less stable parallel duplexes with DNA and RNA strands then their β counterparts in either the parallel or antiparallel orientations, while the thermal stabilities of α- and β-oligoadenylic phosphorimidate duplexes are similar. The pyrimidine-rich β-oligonucleotide N3′→P5′ phosphorimidate apparently formed parallel duplexes with complementary DNA and RNA strands, although their thermal stability was much lower than that for the antiparallel duplexes.

MATERIALS AND METHODS

General methods

α-Oligonucleotide N3′→P5′ phosphorimidates were synthesized on the 1 μmol scale using an ABI 394 synthesizer and 5′-O-(4,4′-dimethoxytrityl)-N-acyl-3′-amino-2′,3′-dideoxy-α-nucleosides as building blocks according to the described procedure (14). Oligonucleotides were purified by IE HPLC on a Pharmacia Mono Q 10/10 column at pH 12 (10 mM NaOH) with a 1% per min gradient of 1.5 M NaCl in 10 mM NaOH and a flow rate of 2 ml/min. Oligonucleotides were desalted on Pharmacia NAP-5 gel filtration columns and then lyophilized in vacuo. RP HPLC was performed on a Waters 510 system using a Hypersil ODS 5 μm 4.6 × 250 mm column. The compounds were eluted with a linear gradient of 1% acetonitrile/min in 50 mM triethylammonium acetate buffer, pH 7.0, at a flow rate of 1 ml/min. Silica gel flash column chromatography was performed with 230–400 mesh 60 Å silica from Aldrich. TLC analysis was performed on 0.2 mm thick precoated silica gel plates from Merck containing a fluorescent indicator.

1H NMR spectra were recorded on a Bruker 400 MHz instrument. Liquid secondary ionization mass spectra (LSIMS) were recorded on a VG 70-SE mass spectrometer (VG Analytical Ltd, Manchester, UK). Oligonucleotides were analyzed by
electrospray ionization mass spectrometry (ESI) using a SCIEX API-300 instrument.

Thermal dissociation experiments were performed on a Cary-1E spectrophotometer equipped with a temperature controller and data processor. Absorbance values at 260 nm were collected at 1 min intervals at a heating rate of 1.0°C/min.

3′-Azido-3′-deoxy-5′-O-(4-methoxybenzoyl)thymidine 1t

This compound was synthesized according to published procedures using thymidine as the starting material (29).

1H NMR (CDCl3/TMS) 1.723 (s, 3H, Me), 2.376, 2.552 (2m, 2H, H-2′), 3.880 (s, 3H, OMe), 4.228 (m, 1H, H-4′), 4.367 (m, 1H, H-3′), 4.544, 4.663 (2m, 2H, H-5′), 6.192 (pseudo-t, 1H, H-1′), J = 6.33 Hz, 6.951, 6.995 (dd, 4H, aromatic protons, J = 8.8 Hz), 7.235 (s, 1H, H-6), 9.281 (br s, 1H, NH) p.p.m.

Anomerization procedure for 3′-azido-nucleosides (1t, 1u)

Nucleosides 1t and 1u were anomerized using a published method for anomerization of 3′′-di-O-acetylthymidine with the exception that a 5-fold excess of the catalyst was used (30). Typically 7.5 mmol 1t or 1u were dissolved in 50 ml dry acetonitrile. To 50 ml dry acetonitrile were added 2.4 ml freshly distilled acetic anhydride and 635 µl 98% sulfuric acid. This mixture was then added to the solution of 1t or 1u and stirred for 40 min at room temperature. The color of the solution changes to orange and later to grayish brown. At the end of the reaction the reaction mixture was neutralized using saturated aqueous NaHCO3 and extracted with ethyl acetate. The organic phase was washed with diethylether and the organic phase was evaporated to dryness to yield a yellow-brown oil which was used without further purification.

1H NMR (CDCl3/TMS) anomic protons for α anomers: 2t 6.279 (dd, 1H, H-1′, JH1′-H2′ = 6.9 Hz, JH1′-H2″ = 3.9 Hz), 2u 6.255 (dd, 1H, H-1′, JH1′-H2′ = 6.8 Hz, JH1′-H2″ = 2.9 Hz) p.p.m.

3′-Azido-2′,3′-dideoxy-α-thymidine 3t

Compound 2t (3.01 g, 7.5 mmol) was dissolved in 100 ml methanol and 15 ml 1 N NaOH solution was added. After 30 min stirring at room temperature the reaction mixture was neutralized with 1 N HCl, and after addition of 100 ml water, evaporated to half the volume. The remaining aqueous solution was extracted with diethyl ether. The aqueous layer was evaporated to dryness and co-evaporated several times with ethanol. Flash chromatography using chloroform/methanol (19:1 v/v) for elution gave 500 mg (25.0%) 3t.

1H NMR (CDCl3/TMS) 1.944 (s, 3H, Me), 2.122, 2.872 (2m, 2H, H-2′), 3.693, 3.826 (2m, 2H, H-5′), 4.325 (m, 2H, H-3′, H-4′), 6.300 (dd, 1H, H-1′, JH1′-H2′ = 6.95 Hz, JH1′-H2″ = 4.3 Hz), 7.344 (s, 1H, H-6) 9.941 (br s 1H, NH) p.p.m.

3′-Azido-2′,3′-dideoxy-α-uridine 3u

This compound was prepared as described for 3t using 2a as the starting material.

1H NMR (CDCl3/TMS) 2.216, 2.877 (2m, 2H, H-2′), 3.718, 3.839 (2m, 2H, H-5′, H-6), 4.330 (m, 2H, H-3′, H-4′), 5.805 (d, 1H, H-5, J = 8.2 Hz), 6.271 (dd, 1H, H-1′, JH1′-H2″ = 7.04 Hz, JH1′-H2″ = 3.6 Hz), 7.548 (d, 1H, H-6 J = 8.2 Hz) p.p.m.

3′-Azido-2′,3′-dideoxy-5′-O-(4,4′-dimethoxytrityl)-α-thymidine 4t

To 500 mg (1.87 mmol) 3t previously co-evaporated with dry pyridine and dissolved in 50 ml pyridine was added 761 mg (2.25 mmol) dimethoxytritylchloride, 10 mg dimethylamino-pyridine and 0.5 ml triethylamine and the reaction mixture stirred at room temperature for 2 h. The reaction was quenched with 5 ml methanol and then 100 ml water were added. The product was extracted with diethyl ether and the organic phase washed with brine. After drying the organic layer over Na2SO4 the solvent was removed in vacuo and the resulting oil precipitated from dichloromethane/hexane to give 950 mg (89.1%) 4t.

1H NMR (CDCl3/TMS) 1.984 (s, 3H, Me), 2.163, 2.877 (2m, 2H, H-2′), 3.198, 3.329 (2m, 2H, H-5′), 3.822 (s, 6H, OMe), 4.227 (m, 1H, H-4′), 4.366 (m, 1H, H-3′), 6.317 (dd, 1H, H-1′, JH1′-H2″ = 7.9 Hz, JH1′-H2″ = 3.1 Hz), 7.184–7.440 (m, 14H, aromatic protons and H-6), 8.565 (br s, 1H, NH) p.p.m.

3′-Azido-2′,3′-dideoxy-5′-O-(4,4′-dimethoxytrityl)-α-uridine 4u

This compound was prepared as above using 3u as the starting material.

1H NMR (CDCl3/TMS) 2.204, 2.894 (2m, 2H, H-2′), 3.195, 3.330 (2m, 2H, H-5′), 3.825 (s, 6H, OMe), 4.230 (m, 1H, H-4′), 4.373 (m, 1H, H-3′), 5.773 (d, 1H, H-5, J = 8.1 Hz), 6.877 (m, 4H, aromatic protons), 7.184–7.430 (m, 9H aromatic protons, 7.544 (d, 1H, H-6, J = 8.1 Hz), 8.202 (br s, 1H, NH) p.p.m.

3′-Amino-2′,3′-dideoxy-5′-O-(4,4′-dimethoxytrityl)-α-thymidine 5

Compound 4t (940 mg, 1.65 mmol) was dissolved in 100 ml pyridine:triethylamine (85:15 v/v) mixture. After cooling the solution to 0°C, dry H2S was bubbled through it for 30 min. The resulting green solution was evaporated to dryness in vacuo, dissolved in methylene chloride and washed with saturated NaHCO3. After drying the organic layer over Na2SO4 and evaporation to dryness the product was purified by flash chromatography using a methylene chloride:methanol (19:1 v/v) solvent system. The yield of 5 was 700 mg (78.0%).

1H NMR (CDCl3/TMS) 1.874, 2.754 (2m, 2H, H-2′), 1.959 (s, 3H, Me), 3.260 (2m, 2H, H-5′), 3.612 (m, 1H, H-4′), 3.788 (s, 6H, OMe), 4.121 (m, 1H, H-3′), 6.248 (pseudo-t, 1H, H-1′, J = 5.66), 6.859 (d, 4H, aromatic protons), 7.221–7.468 (9m, 9H, aromatic protons), 7.662 (s, 1H, H-6) p.p.m. ESI MS (MH+) m/z 544.3 (C31H33N3O6). 543.24.

N4-Benzoyl-3′-amino-2′,3′-dideoxy-5′-O-(4,4′-dimethoxytrityl)-α-cytidine 6

Compound 4u (700 mg, 1.26 mmol) in 50 ml dry acetonitrile was added to an ice-cold mixture of 1.6 g triazole and 0.46 ml distilled phosphorus oxychloride in 50 ml acetonitrile. To this mixture was added 3 ml triethylamine with stirring at 0°C and then the reaction mixture was warmed to room temperature. After 90 min stirring...
triphenylmethylamine (2 ml) and water (1 ml) were added to the red solution and stirring continued for 10 min. The solvents were removed in vacuo and the resulting oil dissolved in ethylacetate and washed with saturated NaHCO₃. After concentration in vacuo the residue was redissolved in 50 ml dioxane and 10 ml concentrated ammonia solution was added and the reaction mixture was stirred overnight at room temperature. The solvents were removed in vacuo and the resulting oil was dissolved in ethylacetate, washed with water and dried over Na₂SO₄. The solution was evaporated to dryness and the resulting oil was dried in a desiccator over P₂O₅.

For the intermediate 5'-O-DMT-3'-azido-α-cytidine: ¹H NMR (CDCl₃/TMS) 2.306, 2.902 (2m, 2H, H-2'), 3.193, 3.291 (2m, 2H, H-5'), 3.821 (s, 6H, OMe), 4.207 (m, 1H, H-4'), 4.376 (m, 1H, H-3'), 5.738 (d, 1H, H-5, J = 7.44 Hz), 6.278 (dd, 1H, H-1', J H1'-H2' = 6.75, J H1'-H2' = 2.05), 6.714, 7.249–7.439 (2m, 13H, aromatic protons), 7.624 (d, 1H, J = 7.44) p.p.m.

The dried residue was taken up in 50 ml methylene chloride:methanol (19:1 v/v) and 159 μl triethylamine, 10 mg dimethylaminopyridine. The product was purified by flash chromatography using methylene chloride:methanol (19:1 v/v) to give 600 mg (69.3%).

Compounds 7 and 8 were dissolved in 100 ml pyridine:triethylamine (85:15 v/v) and H₂S was bubbled through the mixture cooled to 4°C before 1.0 ml benzoyl chloride was added dropwise under stirring. The mixture was warmed to room temperature and then stirred for 2 h. The reaction was quenched with 5 ml water and after 10 min stirring and evaporation it was worked up using ethylacetate and saturated NaHCO₃. For the intermediate 5'-O-DMT-3'-azido-α-cytidine: ¹H NMR (CDCl₃/TMS) 2.422, 2.977 (2m, 2H, H-2'), 3.234, 3.362 (2m, 2H, H-5'), 3.829 (s, 6H, OMe), 4.238 (m, 1H, H-4'), 4.468 (m, 1H, H-3'), 6.301 (dd, 1H, H-1'), 6.888, 7.184–7.999, 8.641 (3m, 20H, aromatic protons, H-5, H-6) p.p.m.

The agido group of precursor of 6 was reduced to the amino group by H₂S in 50 ml pyridine:triethylamine (85:15 v/v) at 0°C for 30 min. The aminonucleoside was purified as described for 5 using flash chromatography to yield 650 mg (81.6%) as a light yellow foam.

Synthesis of the solid support containing α-thymidine

To 242 mg (11.6 mmol) 11 and 8.3 g (34.8 mmol) N-benzoyl-adenosine dissolved in 100 ml acetonitrile was added 12 ml bis-trimethylsilylacetamide and the mixture was heated to 70°C. After 15 min stirring, 2.6 ml TMS-triflate was added and stirred continuing at 70°C for 4 h. The reaction mixture was cooled and the solvent removed in vacuo. The residue was cooled to 0°C and 100 ml cold saturated methanolic NaHCO₃ were added with vigorous stirring. The suspension was filtered, the salt washed with methanol and the combined methanol solutions evaporated to dryness. The resulting oil was taken up in 50 ml methylene chloride and the white precipitate (unreacted N-benzoyladenosine) removed by filtration. The methylene chloride solution was evaporated and loaded on a flash chromatography column prepared with a methylene chloride:methanol (19:1 v/v) solvent system. The α and β anomers of N⁶-benzoyl-3'-azido-2',3'-dideoxy-5'-O-(4'-methoxybenzoyl)adenosine do not separate well under these conditions and elute with a Rf of 0.7–0.8. Their mixture was collected from the column and evaporated to dryness. Then 100 ml methanol and 9 ml 1 N NaOH were added and the mixture stirred for 30 min. After neutralization with 1 N HCl and evaporation the α anomer was isolated by flash chromatography as a slow migrating compound using a methylene chloride:methanol (19:1 v/v) solvent system. Thus 600 mg 7 were obtained (13.6%).

¹H NMR (CDCl₃/TMS) 2.723, 3.073 (2m, 2H, H-2'), 3.770, 3.884 (2m, 2H, H-5'), 4.398 (m, 2H, H-3', H-4'), 6.512 (dd, 1H, H-1', J H1'-H2' = 7.1, J H1'-H2' = 3.4), 7.479–7.608 (m, 3H, aromatic protons), 8.035 (d, 2H aromatic protons), 8.326 (s, 1H, H-8), 8.747 (s, 1H, H-2), 9.602 (br s, 1H, NH) p.p.m.

N⁶-Benzoyl-3'-azido-2',3'-dideoxy-5'-O-(4',4'-dimethoxytrityl)-α-adenosine 8

Compound 7 was dimethoxytritylated using standard procedures as described for 4a. (600 mg 7, 6.40 mg dimethoxytritylchloride, 1 ml triethylamine, 10 mg dimethymalinopyridine). The product obtained (920 mg, 85.3%) was used without further purification.

¹H NMR (CDCl₃/TMS) 2.731, 3.034 (2m, 2H, H-2'), 3.288, 3.421 (2m, 2H, H-5'), 3.826 (s, 6H, OMe), 4.347 (m, 1H, H-4'), 4.498 (m, 1H, H-3'), 6.628 (dd, 1H, H-1', J H1'-H2' = 7.1 Hz, J H1'-H2' = 2.5 Hz), 6.872, 7.179–7.635, 8.055 (3m, 18H aromatic protons), 8.347 (s, 1H, H-8), 8.842 (s, 1H, H-2), 9.059 (br s, 1H, NH) p.p.m.

N⁶-Benzoyl-3'-amino-2',3'-dideoxy-5'-O-(4',4'-dimethoxytrityl)-α-adenosine 9

Compound 8 (900 mg, 1.32 mmol) was dissolved in 100 ml pyridine:triethylamine (85:15 v/v) and H₂S was bubbled through the cooled solution at 0°C for 30 min. After evaporation and extraction by ethylacetate from saturated aqueous NaHCO₃ the product was purified by flash chromatography using methylene chloride:methanol (19:1 v/v) to give 600 mg 8 (69.3%).

¹H NMR (CDCl₃/TMS) 2.433, 3.002 (2m, 2H, H-2'), 3.172, 3.369 (2m, 2H, H-5'), 3.816 (s, 6H, OMe), 4.277 (m, 1H, H-4'), 4.420 (m, 1H, H-3'), 6.786 (dd, 1H, H-1', J H1'-H2' = 7.2, J H1'-H2' = 2.9), 6.862, 7.303–7.644, 8.051 (3m, 18H, aromatic protons), 8.848 (s, 1H, H-8), 8.950 (s, 1H, H-2), 9.032 (br s, 1H, NH) p.p.m.

LSIMS (MH⁺) m/z: 657.2 (C₁₃H₁₄N₄O₁₂ 656.27)

Nucleic Acids Research, 1994, Vol. 22, No. 1

Nucleic Acids Research, 1998, Vol. 26, No. 4
**Acknowledgements**

We would like to thank W. David Wilson for stimulating discussions and for recording the CD spectra and Gerald Zon for helpful discussions. Mass spectrometry was provided by the UCSF Mass Spectrometry Facility (A.L. Burlingame, Director) supported by NIH NCRR BRTP RR01614.

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