**Introduction**

Nucleic acids play a fundamental role in life. These polyanionic polymers, as well as numerous synthetic analogs, are also used in biochemistry, molecular biology, and other fields. Enantiomerically pure, protected acyclic nucleosides of the GNA type (glycol nucleic acids) (GNA), obtained from (R)-(+) and (S)-(−)-glycidols and the four canonical DNA nucleobases (Ad, Cyt, Gu, and Thy), were transformed into 3′-O-DMT-protected 2-thio-4,4-pentamethylene-1,3,2-oxathiaiphospholane derivatives (OTP-GN) containing a second stereogenic center at the phosphorus atom. These monomers were chromatographically separated into P-diastereoisomers, which were further used for the synthesis of P-stereodefined “dinucleoside” phosphorothioates \( ^{\text{GS}} \text{NPS} \) \( (\text{P} = \text{C}, \text{G}, \text{N}, \text{T}) \). The absolute configuration at the phosphorus atom for all eight \( ^{\text{GS}} \text{NPS} \) was established enzymatically and verified chemically, and correlated with chromatographic mobility of the OTP-GN monomers on silica gel. The GSNPS units (derived from (R)-(+) glycidol) were introduced into self-complementary PS-(DNA/GNA) octamers of preselected, uniform absolute configuration at P-atoms. Thermal dissociation experiments showed that the thermodynamic stability of the duplexes depends on the stereochemistry of the phosphorus centers and relative arrangement of the GS units in the oligonucleotide strands. These results correlate with the changes of conformation assessed from circular dichroism spectra.

A single O → S replacement creates a new stereogenic center, thus a PS-DNA decamer synthesized using a non-stereocontrolled method consists of 2\(^9\) = 512 isomers (usually of comparable abundance), which may have considerably different properties. Over several years P-stereodefined oligonucleotides (up to 15–18 nt in length) of PS-DNA and PS-LNA series, were obtained using an oxathiaphospholane method. In several instances these oligomers showed P-stereodependent biological properties.

Recent advances in nucleic acids chemistry enable synthesis of analogs of DNA or RNA with improved chemical and biological stability. These polymers (often denoted XNA) usually carry standard nucleobases but have profoundly altered sugar-phosphate backbones. One of the most fundamental changes is the replacement of the cyclic ribose (deoxyribose) moiety with an acyclic structure. Because many of those analogs are poor substrates of nucleases, they are explored in modulation of the properties of nucleic acid and in synthetic biology. The inherent flexibility of acyclic “nucleosides”, combined with specific “contact points” offered by a given scaffold, make them suitable for building of molecular devices. Glycol nucleic acid (GNAs; Fig. 1B) attracted our attention because of interesting physico-chemical properties, which make them potentially suitable in biotechnology or nanotechnology.

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**P-Stereodefined phosphorothioate analogs of glycol nucleic acids—synthesis and structural properties†‡**

Agnieszka Tomaszewska-Antczak, * Katarzyna Jastrzębska, Anna Maciaszek, Barbara Mikotajczyk and Piotr Guga

Enantiomerically pure, protected acyclic nucleosides of the GNA type (glycol nucleic acids) (GNA), obtained from (R)-(+) and (S)-(−)-glycidols and the four canonical DNA nucleobases (Ad, Cyt, Gu and Thy), were transformed into 3′-O-DMT-protected 2-thio-4,4-pentamethylene-1,3,2-oxathiaphospholane derivatives (OTP-GN) containing a second stereogenic center at the phosphorus atom. These monomers were chromatographically separated into P-diastereoisomers, which were further used for the synthesis of P-stereodefined “dinucleoside” phosphorothioates \( ^{\text{GS}} \text{NPS} \) \( (\text{P} = \text{C}, \text{G}, \text{N}, \text{T}) \). The absolute configuration at the phosphorus atom for all eight \( ^{\text{GS}} \text{NPS} \) was established enzymatically and verified chemically, and correlated with chromatographic mobility of the OTP-GN monomers on silica gel. The GSNPS units (derived from (R)-(+) glycidol) were introduced into self-complementary PS-(DNA/GNA) octamers of preselected, uniform absolute configuration at P-atoms. Thermal dissociation experiments showed that the thermodynamic stability of the duplexes depends on the stereochemistry of the phosphorus centers and relative arrangement of the GS units in the oligonucleotide strands. These results correlate with the changes of conformation assessed from circular dichroism spectra.
The glycol \textsuperscript{4}Gnucleoside units (\textsuperscript{4}N) can exist in two enantiomeric forms, \textit{i.e.} \((R)\textsuperscript{4}N\) and \((S)\textsuperscript{4}N\) \((1a\ and\ 1b\), respectively, \(B = \text{Ade, Cyt, Gua, Thy})\).

![Fig. 1 Structures of (A) P-stereodefined PS-DNA, (B) C-stereodefined GNA, and (C) P,C-stereodefined PS-(DNA/GNA) oligomers.](image)

The GNA oligomers hybridize according to the Watson–Crick’s scheme and form highly stable duplexes with a complementary GNA strand.\(^\text{28}\) Hybridization with natural nucleic acids is only observed for \((S)\textsuperscript{4}N\)-GNA (and not \((R)\textsuperscript{4}N\)-GNA) with complementary ssRNA templates whereas duplexes with ssDNA are much less stable. The reasons of this difference were thoroughly discussed based on X-ray analysis results.\(^\text{21}\)

Because interesting \(P\)-stereodependent effects were observed in PS-DNA (\textit{e.g.}, different thermodynamic stability of antiparallel duplexes (PS-DNA : DNA)\(^\text{22}\)) or different propensity for the B \(\rightarrow\, Z\) conversion\(^\text{23}\), as well as in conformationally more restricted “locked” PS-LNA,\(^\text{12}\) we have turned our efforts toward synthesis of \(P\)-stereodefined phosphorothioate analogs of conformationally flexible, glycol-based GNA (PS-GNA). Taking into account poor hybridization properties of GNA and the fact that the phosphorothioate modification usually decreases thermal stability of the modified duplexes\(^\text{16}\) we assumed that PS-GNA oligomers would not be able to act as antisense or antigen probes specifically interacting with mRNA or genomic DNA strands. Nonetheless, certain acyclic, thermally destabilizing modifications (including GNA) enhance RNAi-mediated gene silencing, because the Ago2 protein favors loading of the 5’-end of the strand from the less stable end of an siRNA duplex.\(^\text{21,24,25}\)

Thus, one cannot reject PS-GNA from future application in the RNAi methodology.

\(*\) There is only one known exception, where homopurine \((R)\textsuperscript{4}P\text-DNA\) oligomers form thermally highly stable parallel triplexes (RNA : PS-DNA : RNA)\(^\text{46}\) and parallel duplexes (PS-DNA : RNA)\(^\text{46}\). They are supposed to be stabilized not only by the Hoogsteen interactions but also by water bridges, which span the O-2 atom in pyrimidines and the sulfur atom of the phosphorothioate moiety.\(^\text{41}\)

Theoretical studies on the influence of the phosphorothioate modification on the electronic properties of model single (ss) and double-stranded (ds) dinucleotides revealed that the electron migration process through an ss-PS-DNA molecule may be slowed down or, in extreme cases, quenched.\(^\text{26}\) Also, interesting differences in the electron transfer potentials of \(P\)-stereodefined chimeric DNA decamers d(CGCGCGCGCG), as well as their duplexes formed with complementary strands d(TCGGCps-GGCCG), were found by cyclic voltammetry assay.\(^\text{27}\) The cited above electronic properties of PS-DNA depend on hybridization status and/or stereochemistry of the phosphorothioate linkages.

In this work, we wanted to answer a question whether the stereochemistry of the phosphorus atoms would affect the hybridization and/or structural properties of PS-GNA oligomers. Anticipated differences in hybridization properties between \(R\textsuperscript{4}P\)- and \(S\textsuperscript{4}P\)-PS-(DNA/GNA) oligomers and possible \(P\)-stereodependent electronic properties may be advantageous for the applications of \(P\)-stereodefined PS-GNA units in nanotechnology. Toward this goal, we present synthesis and separation of \(P\)-diastereomers of 2-thio-4,4-pentamethylene-1,3,2-
oraxathiphospholane derivatives of the 3’-O-DMT protected glycidol-derivided \textsuperscript{4}Gnucleosides \((\text{OTP-}^{4}\text{N})\), and enzymatic and chemical determination of the absolute configuration of the \(P\)-atoms in the assembled \(\text{G}^{\text{N}}\text{psT}\) “dinucleotides”. Because of unexpected destructive side-reactions encountered during oligonucleotide assembly, we synthesized chimeric self-complementary PS-(DNA/GNA) oligomers (Fig. 1C), which were subjected to melting experiments and circular dichroism analysis.

### Results

#### Preparation of DMT-protected glycidol \textsuperscript{4}Gnucleosides (\textit{DMT-}\textsuperscript{4}G)

The 3’-O-DMT-protected glycidol \textsuperscript{4}Gnucleosides \((\text{DMT-}^{4}\text{N}, \, 4a-c\), Scheme 1) were obtained by a literature method.\(^\text{28}\) In brief, the commercially available enantiomerically pure \((R)\)- and \((S)\)-\textsuperscript{4}G-glycidols \(2\) were protected with DMT-Cl to yield almost

\[\text{Scheme 1 Synthesis of DMT-}^{4}\text{G}^{\text{N}}\text{(}4a-c\text{) and their conversion into the oxathiaphospholane monomers 5a-d. In the inset, the numbers in parentheses indicate the priority of substituents (in terms of the Cahn–Ingold–Prelog’s rules) around the stereogenic centers. The synthesis of DMT-}^{4}\text{G}^{\text{Bu}}\text{(4d) is shown in Scheme 2.}\]
quantitatively the corresponding dimethoxytrytilylated glycolids 3. The regioselective and stereospecific epoxide ring opening reaction with a nucleobase (thymine, adenine, or N-benzoylcytosine) in the presence of NaH afforded \( ^{\text{DMT-GN\text{-N}}} \) 4a \((B = \text{Thy})\), \( ^{\text{4b}\,*} \) \((B = \text{Ade})\) or 4c \((B = \text{Cyt}_{\text{Br}})\). \( ^{\text{28}} \) \( ^{\text{DMT-G\text{-A}}\text{B}} \) was next protected at the exocyclic amino group with benzoyl chloride to yield \( ^{\text{28}} \) \( ^{\text{DMT-G\text{-A}}\text{B}} \) (4b). The guanine derivative 4d was obtained via a different method (Scheme 2), since the literature protocol\(^{28} \) didn’t work in our hands. In this case we decided to work only with \((\Delta R)^{(+)}\)-glycidol because the related \((S\sub{R})\)-GNA has more useful properties than \((R\sub{C})\)-GNA.\(^{29} \) The epoxide 2 was opened with 2-amino-6-chloropurine to yield compound 6, which was further treated with sodium thiosulfate. The resultant 6-thio-guanosine derivative 7 was oxidized to \( ^\text{6G} \) (8) using hydrogen peroxide.\(^{29} \) The amino group in 8 was acylated with iso-butyryl chloride to yield diol 9, which was further protected with DMT-Cl to furnish \( ^{\text{DMT-G\text{-Bu}}} \) (4d).

Starting from \((\Delta R)^{(+)}\) and \((S\sub{R})\)-(–)-glycidol (2), the corresponding \( ^{\text{DMT-GN\text{-N}}} \)‘s (4) of \( S \sub{C} \) and \( R \sub{C} \) absolute configuration, respectively, were obtained (see inset in Scheme 1; note that the methylene group in the oxirane ring (marked as \( \text{CH}_2 \)) has priority over the hydroxymethyl group, as shown by the numbers in parentheses).

**Preparation of \( P \)-diastereomerically pure oxathiaphospholane derivatives of glycol \( ^{\text{4G-N}} \) (OTP-GN)**

The enantiomerically pure \( ^{\text{4G}} \) (nucleoside) substrates \( ^{\text{DMT-GN\text{-N}}} \), 4a–d were phosphitylated with 2-chloro-4,4-pentamethylene-1,3,2-oxathiaphospholane (prepared according to ref. 11) in the presence of DIPEA and elemental sulfur (see Scheme 1). After purification on a silica gel column the derivatives 5a–d (OTP-GN\text{-N}) were obtained in good yield (78%, 74%, 71%, 79%, respectively) and characterized by \( ^{31} \) P NMR, \( ^{1} \) H NMR, \( ^{13} \) C NMR (for representative spectra see Fig. 1S–3S, ESI†) and HRMS (for the spectra of 5a–d see Fig. 4G, ESI†).

A molecule of OTP-GN\text{-N} (5) has two stereogenic centers (at the carbon atom C-2 and the phosphorus atom, both marked with asterisks in Scheme 1), therefore, each of 5a–d may exist in four diastereomeric forms. If the absolute configuration at the carbon center is preselected by the use of a particular enantiomer of glycidol, the number of possible forms is reduced to two \( P \)-diastereomers. For each compound of 5a–d we were able to separate these \( P \)-diastereomers by semi-preparative HPLC on a silica gel column. MALDI-TOF MS spectra for fast- and slow-eluting 5a are shown in Fig. 5S (ESI),‡ and further characterization of fast and slow-eluting isomers is given in Table 1S (ESI).‡

**Attempts to prepare crystals of OTP-GN\text{-N} for X-ray analysis**

Our efforts to obtain crystals of 5a–d for X-ray analysis were unsuccessful. Following the previous studies on crystallization of the oxathiaphospholane derivatives in DNA\(^{11} \) and LNA\(^{32} \) series, we tried to remove the DMT group in 5a–d to obtain 3'-OH OTP-GN\text{-N} derivatives 5a–d (shown in square brackets in Scheme 1) using earlier successful \( p \)-toluenesulfonylic acid monohydrate, yet with no success. Another protic acid (dichloroacetic acid), Lewis’ acids (ZnCl\(_2\), SnCl\(_4\)), tetra-\( n \)-butylammonium peroxysulfate\(^{30} \) and NaHSO\(_4\)/SiO\(_2\) (ref. 31) did not work either. In each case we observed the formation of a very polar compound, and in the case of 5a that polar compound was isolated and identified by \( ^{31} \) P NMR and MS (Fig. 6S, ESI†) as the corresponding \( ^{14} \) thymidine cyclic 2',3'-O,O- phosphorothioate \( (c\text{-TMPS}) \) bearing a 5-membered ring (Scheme 3, \( B = \text{Thy} \)). The reasons of this unexpected elimination are unclear. It should be noted that the conversion of the detritylated OTP derivative of d\( ^{\text{cB}} \) into the corresponding cyclic 3',5'-O,O-phosphorothioate, which has an entropically favored 6-membered ring, occurred only after DBU (1,4-diazabicyclo[5.4.0]undec-7-ene, an activator of high basicity) was added.\(^{32} \) The possible mechanism of forming this five membered by-product is shown in Scheme 3.

**Assignment of the absolute configuration of the P-atom in \( \text{“dinucleotides” \( ^{\text{N}\text{Ps-T}} \) obtained using OTP-GN\text{-N}}**

**Enzymatic approach.** Pure \( P \)-diastereomers of 5a–d were reacted with 3'-O-acetyl-thymidine in the presence of DBU to yield the corresponding protected \( ^{\text{DMT-G\text{-N}}\text{Ps-T}}\text{Ac} \) (10a–d, Scheme 4, Fig. 7S, ESI‡), which after routine two-step deprotection furnished the phosphorothioates \( ^{\text{NPs-T}} \) (11a–d). The products were isolated by RP-HPLC and identified by MALDI-TOF MS (Fig. 8S, ESI‡). In order to assign the absolute configuration at phosphorus atom in diastereomeric \( ^{\text{NPs-T}} \) (11a–d), the reactions with snake venom phosphodiesterase (svPDE, an \( R \sub{P} \)-specific nuclease) and nuclease P1 (nP1, an \( S \sub{P} \)-specific nuclease) were
attempted.\textsuperscript{11,14} It was found that neither diastereomer of 11 was hydrolyzed by nP1 to a measurable extent.

The experiments with svPDE gave more useful results. The HPLC profiles recorded after hydrolysis (Fig. 9S and 10S, ESI\textsuperscript{2}) showed that compounds 11a-d obtained from slow-eluting (R\textsubscript{C})-OTP-\textsuperscript{3}N\textsuperscript{0}N (synthesized from S(-)-glycidol) or fast-eluting (S\textsubscript{C})-OTP-\textsuperscript{3}N\textsuperscript{0}N (obtained from R(+)-glycidol) were hydrolyzed to yield \textsuperscript{3}N (1, Scheme 4) and thymidine 5'-O-phosphorothioate (TMPS), identified by means of RP HPLC (Fig. 11S, ESI\textsuperscript{2}). Thus the svPDE-digestable diastereomers were tentatively assigned an R\textsubscript{p} configuration. Those obtained from fast-eluting (R\textsubscript{C})-OTP-\textsuperscript{3}N\textsuperscript{0}N or slow-eluting (S\textsubscript{C})-OTP-\textsuperscript{3}N\textsuperscript{0}N remained intact after 24 h incubation, so were assigned an S\textsubscript{p} configuration.

Chemical verification of the enzymatic assignment

Because phosphorothioates 11 are much different from svPDE natural substrates, one cannot exclude the possibility that these compounds do not conform the general observation of known stereoselectivity of the enzyme towards PS-DNA. To strengthen our stereochemical assignment, a chemical verification was carried out, based on the “inverted” synthesis of \textsuperscript{3}N\textsubscript{PS,T} (11a-d; the synthesis of 11\textsuperscript{*}b is shown in Scheme 5). We used fast- and slow-eluting P-diastereoisomers of the 5'-O-oxathiaiphospholane derivative of 3'-O-camphanoylated thymidine (5'-OTP-T\textsubscript{Camph}, 12), which are known to be the precursors of (S\textsubscript{p})-N\textsubscript{PS,T} and (R\textsubscript{p})-N\textsubscript{PS,T}, respectively.\textsuperscript{35} In the presented example fast-12 was reacted with (S\textsubscript{C} + R\textsubscript{C})\textsuperscript{DMT-GA}\textsuperscript{Bz} (4b, prepared from racemic glycidol) to yield a pair of protected \textsuperscript{DMT-GA}\textsuperscript{Bz} (13b). By a routine two-step deprotection both isomers of 13b were converted into a pair of S\textsubscript{p}S\textsubscript{p}- and R\textsubscript{p}S\textsubscript{p}-11\textsuperscript{*}b, but this time the absolute configuration at P-atoms (i.e. S\textsubscript{p}) was known.\textsuperscript{11a} The second pair of 11\textsuperscript{*}b consisting of S\textsubscript{p}R\textsubscript{p}- and R\textsubscript{p}R\textsubscript{p}-isomers was obtained using slow-eluting 12. The R\textsubscript{p} and S\textsubscript{p} pairs of 11\textsuperscript{*}b were mixed at ca. 1 : 2 molar ratio and we found RP-HPLC conditions allowing for baseline separation of all four components (Fig. 2, an inset in the panel A).

[Note, that priority of substituents around the stereogenic phosphorus atom in \textsuperscript{3}A\textsubscript{PS,T} and A\textsubscript{PS,T} (generally in \textsuperscript{3}N\textsubscript{PS,N} and N\textsubscript{PS,N}) is the same.]

In next HPLC analyses this mixture of standards was spiked with a given 11b and it was found that 11b derived from fast-R\textsubscript{C}-5b (Fig. 2, panel A) and slow-R\textsubscript{C}-5b (panel B) (both obtained from S(-)-glycidol) were precursors of the internucleotide phosphorothioate linkages of S\textsubscript{p} and R\textsubscript{p} configuration, respectively.

The analogous analysis indicated (data not shown) that the fast- and slow-eluting S\textsubscript{C}-5b (obtained from R(+)-glycidol) were precursors of the internucleotide phosphorothioate linkages of R\textsubscript{p} and S\textsubscript{p} configuration, respectively. In conclusion, the

![Scheme 4](image)

Scheme 4 Synthesis of 'dinucleotides' \textsuperscript{3}N\textsubscript{PS,T} (11a-d) using OTP-\textsuperscript{3}N\textsuperscript{0}N (5a-d) and products of their hydrolysis (if successful) with svPDE.

![Scheme 5](image)

Scheme 5 The "inverted" synthesis of \textsuperscript{3}A\textsubscript{PS,T} (11\textsuperscript{*}b) using \textsuperscript{DMT-GA}\textsuperscript{Bz} and 5'-OTP-T\textsubscript{Camph} (12).

![Fig. 2](image)

Fig. 2 HPLC profiles for the mixture of standards 11\textsuperscript{*}b (an inset in panel A) and co-injections of 11\textsuperscript{*}b and \textsuperscript{3}A\textsubscript{PS,T} (11b) obtained from fast- and slow-eluting (R\textsubscript{C})-OTP-\textsuperscript{3}A\textsubscript{Bz} 5b (panels A and B, respectively). HPLC conditions: a Kinetex column, 250 × 4.6 mm; flow rate 1 mL min\textsuperscript{−1}; buffer A: 0.1 M TEAB, buffer B: 40% CH\textsubscript{3}CN in 0.1 TEAB, gradient: 0–38% of buffer B over 20 min.
the experiment shown in Scheme 5, as well as those carried out with 4a, c, d (data not shown) proved that the absolute configurations at the phosphorus atom in 11a-d assigned using sivPDE were correct.

**Attempts at solid phase synthesis of P-stereodefiniend PS-GNA oligomers**

The diastereomerically pure OTP-GiN’ monomers 5a-d were tested in a manually performed, solid-phase synthesis of PS-GNA oligonucleotides. To prevent the DBU-promoted cleavage of the standard lca linker, the first nucleoside was attached to the solid support via a DBU-resistant sarcosine linker. Even with double coupling used at each condensation step (earlier effectively applied in synthesis of PS-LNA13), only ca. 75% repetitive yield was achieved (based on a DMT cation assay), so synthesis of octamers and decamers would provide only ca. 13% and 7%, respectively, of the theoretical amounts (0.75% = 0.133, 0.75% = 0.07), whereas the amount of actually isolated products would be further reduced at least by half. This is unacceptably low efficiency, compared to 92–94% repetitive yield noted for OTP monomers of DNA14 and LNA15 series. To acquire an insight into a possible mechanism contributing to the problem, a PS-GNA oligonucleotide ([5m]PS)m dA has been synthesized using a modified phosphoramidite/sulfurization methodology and the sarcosine linker. A 2-cyanoethyl-N,N-diisopropylphosphoramidite derivative of DMT-4 was used (prepared according to ref. 28) and >99.2% repetitive yield was noted (based on a DMT cation assay, Fig. 12S, ESI†). After the synthesis was complete, the oligomer (still being attached to the support) was treated with piperdine/acetonitrile solution (1 M) to remove the 2-cyanoethyl protecting groups, and the resultant phosphorothioate diester compound was subjected to a DBU/acetonitrile solution (1.5 M) for 1 h. The remaining material was cleaved from the support and isolated by RP-HPLC (a broad peak eluting at 15.65 min, Fig. 13S, ESI†). A MALDI-TOF MS analysis showed three ladders of ions corresponding to the molecular ion (m/z 3154), the depurinated (due to the acidic matrix) derivative (m/z 3020), and the depurinated/hydroxylated oligomer (m/z 3037), all consecutively truncated by up to 5 residues, each of 264 amu (Fig. 14S, ESI†). The relative intensities of the consecutive bands related to the most intense band at m/z 3037 are 1 : 0.69 : 0.30 : 0.13 : 0.09 : 0.06, and the other two sets basically follow this pattern. Thus one can assume that the cleavage took place at the end of the consecutively truncated oligomer and not randomly inside the chain. The 264 Da residue seems to be Guridine cyclic 2’,3’-O-phosphorothioate (c’GUMPS, Scheme 3, B = Ura), which is an analog of c’GMPs, although this time it was formed upon intramolecular attack of the sequentially released terminal hydroxyl groups at the phosphorus atom of the nearest internucleotide linkage, with the concomitant release of next primary hydroxyl group, so the cleavage process could go further. Undoubtedly, to avoid that “walking” truncation some mechanistic studies and optimization of the synthetic protocol are necessary.

**Solid phase synthesis of P-stereodefiniend chimeric PS-(DNA/GNA) and PS-DNA oligomers**

Because of the unsatisfactory coupling efficiency we decided to synthesize “chimeric” PS-(DNA/GNA) oligonucleotide octamers containing 3 or 4 4N units, using appropriate OTP-GiN’ and OTP-N’ monomers.** The syntheses were performed manually at a 1

<table>
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<th>Duplex</th>
<th>P-Chirality</th>
<th>Tm (°C)</th>
<th>∆A254 (%)</th>
<th>θ254 [mdeg]</th>
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<tbody>
<tr>
<td>14 5′-(CA TGG CCG CCA T)-3′</td>
<td>Rv</td>
<td>24</td>
<td>2.4</td>
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<tr>
<td>14 3′-(TC GAC CGG CCG T)-5′</td>
<td>Rv</td>
<td>27</td>
<td>5.9</td>
<td>−2.9</td>
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<tr>
<td>15 5′-(CA TGG CCG CCA T)-3′</td>
<td>Sβ</td>
<td>&lt;12</td>
<td>2.0</td>
<td>−1.1</td>
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<tr>
<td>16 3′-(TC GAC CGG CCG T)-5′</td>
<td>Sβ</td>
<td>22</td>
<td>7.9</td>
<td>−2.4</td>
</tr>
<tr>
<td>17 5′-(CA TGG CCG CCA T)-3′</td>
<td>Sβ</td>
<td>33</td>
<td>&gt;7</td>
<td>−3.7</td>
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<tr>
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</tr>
<tr>
<td>18 3′-d(ATGCCGAT)-3′</td>
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<td>29</td>
<td>7.6</td>
<td>−3.1</td>
</tr>
<tr>
<td>19 3′-d(ATGCCGAT)-3′</td>
<td>Sβ</td>
<td>25</td>
<td>10.7</td>
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Table 1 Melting temperatures, hyperchromicity (∆A254) and ellipticity values at 254 nm (θ254) for duplexes formed by oligomers 14–19 in pH 7.2 buffer containing 10 mM Tris–HCl, 100 mM NaCl, and 10 mM MgCl2.
μmol scale. Selfcomplementary oligomers of a general sequence PS-d(ATGCCGAT) were synthesized in two DNA/GNA variants (each chimeric oligomer in two P-stereoregular forms, Table 1), bearing either purine 6Ns (PS-5′AT6G6GC6GAT; Rp, 14; Sp, 15) or pyrimidine 6Ns (PS-A6T6G6GC6GAT; Rp, 16; Sp, 17).†† Only S-OTP-6N were used in the syntheses. Decay of the trityl cation absorption after consecutive coupling steps was measured photometrically (Fig. 15S, ESI‡). After each synthesis was complete, the DMT tagged oligomer was cleaved from the support and the N-protecting groups were removed using routine treatment with conc. NH4OH. The oligonucleotides were isolated by RP-HPLC, then detritylated with 50% acetic acid, purified by RP-HPLC (Fig. 16S, ESI‡), and finally characterized by MALDI-TOF MS (Fig. 17S, ESI‡). The syntheses furnished ca. 3 OD260 of each oligomer.

The reference oligomers (Rp)-PS-5’-d(ATGCCGAT)-3’ (18) and (Sp)-PS-5’-d(ATGCCGAT)-3’ (19) were obtained in an analogous way (a single coupling was used).

Thermal stability and circular dichroism analysis of complexes formed by P-stereodefined chimeric PS-(DNA/GNA) octamers 14–17

The P-stereoregular, selfcomplementary chimeric PS-(DNA/GNA) oligomers 14–17, where the 6N units in the related duplexes occupy “alternate” positions (see the duplex structures in Table 1), and the reference PS-DNA oligomers 18 and 19 were dissolved in pH 7.2 buffer containing 10 mM Tris–HCl, 100 mM NaCl, and 10 mM MgCl2. The annealing was done from 40 °C to 5 °C with a temperature gradient of 1 °C min⁻¹. Then the melting profiles were recorded over a 5 → 40 °C range (0.5 °C min⁻¹) and melting temperatures (Tm) were calculated using the first order derivative method. For the reference compounds 18 and 19 we found Tm = 29 °C and 25 °C (Table 1), hyperchromicity of 7.6% and 10.7% (Fig. 18S, ESI‡) and Θ295 = −3.1 and −1.7 mdeg (Fig. 3), respectively.

The melting curves for homoduplexes formed by 15–17, showed hyperchromicity (in a range 5.9–7.9%) and cooperativity similar to 18 and 19 (Fig. 18S, ESI‡). However, for 14 only 2.4% hyperchromicity was noted and the cooperativity was very poor (Fig. 19S, ESI‡). This dichotomy was also observed in the corresponding CD spectra, which were recorded after 15 minutes incubation of the samples at 15 °C. The spectra for homoduplexes 15 and 16 were similar to those for 18 and 19, as they have strong negative bands around 254 nm and weak negative signals around 280 nm (Fig. 3). In contrast, for compound 17 a positive band around 294 nm (Θ294 = 0.52 mdeg) had a wide shoulder around 280 nm. Interestingly, the CD spectrum for Rp-14 (on contrary to that recorded for the Sp-15 counterpart) has a weak signal at 254 nm and a positive signal around 280 nm, so one can assume that the conformations got substantially changed compared to Rp-18. Furthermore, the Tm value for Sp-17 was by 6 °C higher than for Rp-16 and by 8 °C higher than for the reference Sp-19. Also, ellipticity Θ295 = −3.7 mdeg found for

†† For simplicity, in the sequences of chimeric DNA/GNA oligonucleotides 14–17 the letters A, C and G are used instead of dA, dC and dG, respectively.

S_P-17 was the highest among all the oligomers studied, including the reference homoduplexes formed by PS-DNA oligomers 18 and 19. These observations indicate that the stereochirality of the phosphorothioate linkages is important for the mode of hybridization of the oligomers and the resultant conformation of the duplexes.

For the heteroduplexes 14/16 and 15/17, where the 6N units occupy the “face-to-face” positions (see Table 1), rather unexpected effects were noted. In both cases very low hyperchromicities (2%) and low Tm values were observed. For 14/16 the Tm value was only roughly assessed at <12 °C, and for 15/17 was 20 °C higher than for 15/17. For those systems one might expect the presence of certain amounts of the duplexes formed by 16 (Tm = 24 °C) or 17 (Tm = 33 °C), with the latter duplex expected to be quite abundant. However, the melting curves did not show the relevant inflection points, as both profiles reached a plateau at much lower temperatures (Fig. 19S, ESI‡). The melting profile for 16 is given as a reference). Also the corresponding CD spectra (Fig. 4) indicated a transition of both heteroduplexes to the conformation already observed for 14, with the absence of bands characteristic for homoduplexes formed by 16 and 17. At that moment we cannot offer any possible reasons for that behavior.

Thermal stability of complexes formed by PS-(DNA/GNA) octamers 14–17 with DNA and RNA templates

The unexpected hybridizing properties of chimeric PS-(DNA/GNA) oligomers prompted us (despite of the most often observed destabilizing effect of the phosphorothioate modification) to investigate the interactions of 14–17 with natural DNA (DNA, (dATGCCGAT), isosequential to 18 and (2′-OMe)-RNA ((m)RNA, (2′-OMe)-AUGGCCAU) templates. However, the results of melting experiments (Table 2S‡) indicate that the Tm value recorded for the reference homoduplex formed by DNA (43 °C) has been only very little reduced (<2 °C) by the presence of equinolar amount of any of the 14–17 oligomer. In the case of (m)RNA (Tm = 62 °C) the reduction was only slightly bigger (up to 4 °C for Sp-17). Because the melting curves also showed very good cooperativity (Fig. 20S, ESI‡), one can conclude that the oligomers 14–17 only weakly interact with DNA or (m)RNA, which preferentially form thermodynamically much more stable
homoduplexes. This was confirmed by CD spectra (recorded at room temperature, Fig. 21S, ESI) which showed that the bands characteristic for the (m)RNA/(m)RNA homoduplex remained virtually unchanged, except for their lowered intensity resulting from the reduced by half concentration of (m)RNA.

Conclusions

P-Diastereomers of OTP-GN’s (obtained from enantiomerically pure (R)-(+) and (S)-(−)-glycidols 2) were chromatographically separated and used in synthesis of “dinucleotide” phosphorothioates 3N_{PS}T. The absolute configuration of the P-atom in 3N_{PS}T was established by enzymatic and chemical methods. Then, four self-complementary P-stereoredefined chimeric oligomers PS-(DNA/GNA) were synthesized, although even double coupling at the condensation steps utilizing OTP-GN’ led to a significant drop in reaction efficiency. Thermal dissociation experiments showed that the thermodynamic stability of the duplexes depends on the stereochemistry at phosphorus and relative arrangement of the N-units in the oligonucleotide strands. Most interestingly, a duplex formed by 17 (S,o)-PS-(A/GTG/CG/CAT) was thermally more stable than that formed by the corresponding PS-DNA congener 19 ($\Delta T_m = 8 \pm 3 \degree C$). The results on thermal stability correlate with the changes of overall conformation assessed from circular dichroism spectra.

Further studies on the adaptation of the oxathiaphospholane approach to synthesis of P-stereoredefined phosphorothioate glycol nucleic acid are underway.

Experimental section

$^1$H NMR and $^{31}$P NMR spectra were recorded using a Bruker instrument (AV-200, 200 MHz for $^1$H). Chemical shift values ($\delta$) are given in ppm, relative to internal tetramethylsilane (TMS) or residual solvent protons for $^1$H NMR, and external 85% H$_3$PO$_4$ for $^{31}$P NMR.

Mass spectra (FAB-MS) were recorded on a Finnigan MAT 95 spectrometer, in the positive and negative ion modes. MALDI-TOF MS analyses of oligonucleotides were performed using a Voyager-Elite instrument (PerSeptive Biosystems Inc., Framingham, MA) operating in the reflector mode with the detection of negative ions, unless otherwise stated. HRMS measurements were performed on a qTOF Waters ESI SYNAPT G2-Si high definition mass spectrometer fitted with an atmospheric pressure ionization electrospray source (Waters Corporation, Milford, MA). The instrument was operated in negative-ion mode, with capillary voltage 2.5 kV, cone 40.0 V, and source offset 50 V. Desolvation temperature 150 °C was applied.

Routine UV spectra were recorded on a CINTRA 10e spectrophotometer (GBC, Dandenong, Australia), using a quartz cuvette of 1 cm path length. UV monitored melting experiments were carried out in 1 cm path length cells, using a spectrophotometer CINTRA 4040 (GBC), equipped with a Peltier thermocell.

CD measurements were done on a Jasco J-815 dichrograph using a cuvette with a 5 mm path-length, thermostated (with a Peltier effect accessory) at 15 °C. The spectra were recorded over a 200–400 nm range with a 1.00 nm bandwidth, at a scanning speed 100 nm min$^{-1}$, and a data pitch of 0.2 nm. After 5 spectra were accumulated, the baseline was subtracted and the resultant spectrum was smoothed with a means-movement algorithm using a 5–25 point filter.

Preparative HPLC separation of the oxathiaphospholane monomers was performed using a Shimadzu Prominence HPLC system consisting of an LC-20AP preparative pump (a 2 mL sample loop in the Rheodyne valve was used) and an SPD-M20A UV detector (set at 260 nm) equipped with a 0.2 mm path length preparative cell. A silica gel column Pursuit XRs (10 µm, 250 x 21.2 mm) was eluted at a flow rate of 25 mL min$^{-1}$.

In search for the condition suitable for HPLC separation of the P-diastereomers of the oxathiaphospholane monomers a Phenomenex Luna 5u Silica column (100 Å; 250 x 10 mm; flow rate 5 mL min$^{-1}$) and a binary Varian HPLC system (two PrepStar 210 pumps, 25 mL pump heads, a ProStar 320 UV/VIS detector set at 275 nm) were used.

HPLC solvents and reagents of gradient grade (from Sigma-Aldrich, Baker or ChemPur) were used.

All moisture-sensitive operations were carried out under anhydrous argon.

Note: compounds 3, 4a, 4b, 4c and 4b were obtained according to ref. 28.

Unmodified DNA d(ATGCGCAT, DNA), and (2’-OME)-RNA ((2’-OMe)-AUGCGCAU, (m)RNA) oligonucleotides were synthesized at a 0.2 µmol scale, using commercially available LCA-CPG supports (Biosearch Technologies, Inc., Petaluma, CA) and standard DNA and 2’-OME-RNA phosphoramidite monomers (Glen Research, Sterling, VA).

Dimethoxycrylation of glycidol – synthesis of enantiomeric compounds 3

To a solution of (R)-(+) or (S)-(−)-glycidol 2 (1.0 mL, 15.1 mmol) and Et$_3$N (5.4 mL, 40.7 mmol) in CH$_2$Cl$_2$ (50 mL) solid DMT-Cl (6.45 g, 19 mmol) was added. After 12 h, the reaction mixture was treated with saturated aqueous NaHCO$_3$ (50 mL). The organic layer was separated, dried with MgSO$_4$ and...
concentrated. The residue was dissolved in EtOAc and the solution was washed with saturated aq. NaHCO₃. The solvent was evaporated and the product was isolated chromatographically on a silica gel column eluted with hexane-EtOAc-Et₂N (from 95 : 5 : 0.1 to 85 : 15 : 0.1, v/v/v) to give compounds 3 (typically 5.4 g, 95% yield).

Opening of the oxirane ring in 3 with nucleobases – synthesis of compounds 4a, 4b*, and 4c

A mixture of nucleobase (1 mmol, 126 mg of thymine, 135 mg of adenine, or 215 mg of N-benzoylcytosine) and NaH (60% suspension in mineral oil, ~10 mg, 0.2 mmol) in DMF (2 mL) was stirred at room temperature for 2 h. A solution of dimethoxytritylated glycidol (340 mg, 0.9 mmol) in DMF (2–3 mL) was added and the resulting mixture was heated at 110 °C for 20 h. The solvent was evaporated under reduced pressure (an oil pump), and the residue was dissolved in EtOAc and loaded on a silica gel column, initially eluted with hexane-EtOAc-Et₂N (1 : 2 : 0.01, v/v/v), then with EtOAc (1 mL) and later with EtOAc in vacuo (1 mL), then with EtOAc–Et₂N (100 : 1, v/v), with stirring, the mixture was extracted with CH₂Cl₂ (2 mL) at room temperature the mixture was cooled to 0 °C and elemental sulfur (64 mg, 2-fold molar excess) was added. The reaction was complete in 5 h and elemental sulfur (64 mg, 2-fold molar excess) was added. Stirring was continued for 24 h and excess sulfur was filtered off. After evaporation of the solvent, the residue was dissolved in chloroform (3 mL) and loaded on a silica gel column. The column was eluted with a mixture of CHCl₃ and iPrOH (a gradient from 100 : 0 to 95 : 5, v/v) containing 0.1% of pyridine. Appropriate fractions (vide infra) were collected and the solvent was evaporated under reduced pressure.

5a OTP-GT. (553 mg, 78%); Rf = 0.71 (CHCl₃-MeOH 9 : 1, v/v); HRMS for C₂₈H₂₄O₃N₅PS₂: calc. 707.2099, found 707.2100 (Fig. 4SA, ESI†); ⁳¹P NMR: δ 105.8 ppm, δ 105.5 ppm (no deuterated solvent).

5b OTP-GA. 607 mg (74%); Rf = 0.75 (CHCl₃-MeOH 9 : 1, v/v); HRMS for C₃₄H₂₅N₃O₇PS₂: calc. 820.2280, found 820.2287 (Fig. 4SB, ESI†); ⁳¹P NMR: δ 105.9 ppm, δ 105.2 ppm (no deuterated solvent).

5c OTP-GB. 566 mg (71%); Rf = 0.69 (CHCl₃-MeOH 9 : 1, v/v); HRMS for C₃₄H₃₄N₃O₇PS₂: calc. 796.2280, found 796.2287 (Fig. 4SC, ESI†); ⁳¹P NMR: δ 105.6 ppm, δ 105.1 ppm (no deuterated solvent).

5d OTP-GBu. 634 mg (79%); Rf = 0.70 (CHCl₃-MeOH 9 : 1, v/v); HRMS for C₃₄H₃₄N₃O₇PS₂: calc. 802.2498, found 802.2514 (Fig. 4SD, ESI†); ⁳¹P NMR: δ 106.1 ppm, δ 106.5 ppm (no deuterated solvent).

Synthesis of (6-chloropurine)G nucleoside (6)

A mixture of 2-amino-6-chloropurine (169 mg, 1 mmol), glycidol (73 µL, 1.1 mmol) and K₂CO₃ (23.5 mg, 0.17 mmol) in anhydrous methylene chloride (2 mL) was stirred at 100 °C for 18 h. The reaction mixture was concentrated in vacuo, the residue was dissolved (not easily) in pyridine and loaded on a silica gel column. Initially the column was eluted with CHCl₃ and later with CHCl₃–MeOH (1 : 1, v/v) containing 0.1% of pyridine. Fractons containing (6-chloropurine)G nucleoside (6, Rf = 0.41 CHCl₃-MeOH 9 : 1, v/v) were collected and evaporated to yield 90 mg (37%). FAB MS (Cl): calc. for C₂₉H₁₉ClN₅O₂ 423.6, found: 244.1 (+VE).

Conversion of (6-chloropurine)G nucleoside into the 6-thio derivative 7

A solution of 6 (243 mg, 1 mmol) in water (25 mL) Na₂S₂O₃ × 5H₂O (1.24 g, 5 mmol) was added and the mixture was refluxed with stirring (ca. 16 h) until no starting (6-chloropurine)G nucleoside was detected by TLC. The mixture was cooled down to room temperature, and extracted with ethyl acetate (2 × 50 mL). The aqueous phase was concentrated under reduced pressure and the residue was chromatographed on a reverse phase silica gel column using water as eluent to yield 180 mg of 7 (75%); Rf = 0.55 (i-PrOHconc. NH₄OH/H₂O 7 : 1, v/v/v); FAB MS (Cl): calc. for C₂₉H₁₉ClN₅O₃S 243.6, found: 242.0 (+VE).

Conversion of (6-thiopurine)G nucleoside into compound 8

A solution of 7 (241 mg, 1 mmol) in NH₄OH – 30% H₂O₂ (5 : 1, v/v, 8 mL) was allowed to stand at room temperature for 5 h. The solvents were removed under reduced pressure. The obtained crude residue was purified by crystallization from H₂O to give...
the corresponding 4-nucleoside 8 as white solid material (155 mg, 69%). R<sub>t</sub> = 0.42 (CHCl<sub>3</sub>-MeOH 9 : 1, v/v); FAB MS: calc. for C<sub>4</sub>H<sub>11</sub>N<sub>2</sub>O<sub>3</sub> 225.2, found: 226.2 (+VE), 224.1 (−VE).

Iso-butylation of 4G – synthesis of compound 9

To a solution of 8 (225 mg, 1 mmol) in anhydrous pyridine (30 mL) trimethylsilyl chloride (1.01 mL, 8 mmol) was added at 0 °C. The reaction mixture was stirred at 0 °C for 15 min, followed by 40 min at room temperature. The mixture was cooled to 0 °C and iso-butyral anhydride (415 µL, 2.5 mmol) was added dropwise. The mixture was allowed to warm slowly to room temperature and was stirred for 2 h. The reaction was stopped by addition of H<sub>2</sub>O (2 mL) at 0 °C. After 10 min, conc. NH<sub>4</sub>OH (2 mL) was added and the mixture was left for 15 min. The volatile components were removed in vacuo. The residue was dissolved in MeOH, loaded onto a silica gel column and the column was eluted with EtOAc, EtOAc-MeOH (9 : 1, v/v), and finally with EtOAc-MeOH (9 : 2, v/v) to yield 239 mg (81%) of 4G<sup>Br</sup>. R<sub>t</sub> = 0.10 (CHCl<sub>3</sub>-MeOH 9 : 1, v/v); FAB MS: calc. for C<sub>12</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub> 295.3, found: 296.1 (+VE), 294.2 (−VE).

Dimethoxytritylation of 9 – synthesis of DMT-GG (compound 4d)

To a solution of 9 (295 mg, 1 mmol) in anhydrous pyridine (4 mL) solid DMT-Cl (406 mg, 1.2 mmol) was added at room temperature. After 2 h, the reaction mixture was concentrated in vacuo. The residue was purified by chromatography on a silica gel column, first eluted with hexane-EtOAc-Et<sub>3</sub>N (1 : 2 : 0.01, v/v/v), then with EtOAc-EtN (100 : 1, v/v), and finally with EtOAc-MeOH-Et<sub>3</sub>N (25 : 1 : 0.01, v/v/v). The fraction containing DMT-GG (R<sub>t</sub> = 0.46 (CHCl<sub>3</sub>-MeOH 9 : 1, v/v)) was concentrated in vacuo to yield 430 mg (colorless foam, 72%); FAB MS: calc. for C<sub>13</sub>H<sub>23</sub>N<sub>2</sub>O<sub>3</sub> 597.7 found: 598.1 [M+D-MT]<sup>+</sup> + VE).

HPLC separation of P-diastereomers of oxathiaphospholane monomers 5a–d

A preparative HPLC silica gel column (Pursuit XRs 10 µm, 250 × 21.2 mm) was equilibrated with a given eluent mixture (see Table 1) at a flow rate 25 mL min<sup>−1</sup> for 20 minutes. A solution of 5 (100 mg; 300 mg for 5a) in ethyl acetate (1.5 mL, with 1% TEA) was filtered through a membrane filter (0.2 µm pores, organic solvent resistant) and the filtrate was applied onto the column. The column was eluted at the flow rate of 25 mL min<sup>−1</sup> and baseline separation of P-diastereomers was achieved. The collected fractions were concentrated using a rotary evaporator with a “cold finger” condenser filled with isopropyl alcohol/dry ice coolant, with the temperature of a water bath not exceeding 30 °C. Pure P-diastereomers were recovered in ca. 90% and their diastereomeric purity was confirmed by 31P NMR analysis (CDCl<sub>3</sub>, an estimated limit of detection of a minor component 0.2%). They gave satisfactory 1H NMR, 31P NMR, 13C NMR and MALDI-TOF mass spectra.

5a OTP<sup>4-Br</sup> fast: R<sub>t</sub> = 0.68 (CHCl<sub>3</sub>-MeOH 9 : 1, v/v); R<sub>t</sub> = 19.2 min (EtOAc–hexane 50 : 50, v/v); MALDI TOF MS: calc. for C<sub>13</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub>-PS<sub>2</sub> 708.8 found: 747.6 (+); 708.8 + K<sup>+</sup>; 31P NMR δ (CDCl<sub>3</sub>, ppm) 105.6; 31P NMR δ (CDCl<sub>3</sub>, ppm) 8.36 (1H, N3–H), 7.43 (1H, C6–H), 7.40–6.79 (13H, DMT), 5.13–5.06 (1H, C2′–H), 4.16–4.15 (2H, C1′–H), 4.08–4.05 (2H, P–O–CH<sub>2</sub>C–S), 3.75 (6H, 2 × OCH<sub>3</sub>), 3.33–3.25 (2H, C3′–H), 1.89–1.20 (1H, C5–CH<sub>3</sub>), 10H, (CH<sub>3</sub>;<sup>2</sup>p-sphiro); 13C NMR δ (CDCl<sub>3</sub>, ppm) 162.6, 157.3, 143.0, 140.0, 134.2, 128.7, 126.7, 126.5, 125.7, 111.9, 78.0, 76.4, 75.7, 75.1, 62.2, 57.9, 36.0, 35.3, 23.9, 22.7, 22.2, 11.0. 5a OTP<sup>4-</sup>slow: R<sub>t</sub> = 0.60 (CHCl<sub>3</sub>-MeOH 9 : 1, v/v); R<sub>t</sub> = 21.5 min (EtOAc–hexane 50 : 50, v/v); MALDI TOF MS: calc. for C<sub>34</sub>H<sub>41</sub>N<sub>2</sub>O<sub>7</sub>P<sub>2</sub> 708.8 found: 747.6 (+); 708.8 + K<sup>+</sup>).
2.53–2.50 (1H, COCH), 2.02–1.50 (10H, -(CH$_2$)$_5$–“spiro”), 1.22–1.19 (6H, 2 × CH$_3$); $^{13}$C NMR δ (CDCl$_3$, ppm) 177.0, 157.4, 145.8, 143.0, 138.2, 134.1, 128.7, 126.7, 126.6, 125.7, 111.9, 85.3, 76.4, 75.7, 75.1, 67.6, 61.3, 54.0, 35.2, 23.8, 22.4, 17.7. 5d OTP.$^{GHM}$ slow: $R_f = 0.50$ (CHCl$_3$:MeOH 9 : 1, v/v); $R_f = 17$ min (EtOAc–hexane 85 : 15, v/v); MALDI TOF MS calc. 804.9 found 804.6 (+).

Attempt at detritylation of compound 5a

To a solution of 5a (100 mg) in CH$_2$CN (10 mL) a catalytic amount of NaHSO$_4$/SiO$_2$ (prepared according to ref. 38) was added. The suspension was stirred for 30 minutes and TLC analysis showed no substrate remaining. The product did not migrate on a TLC plate when a mixture CHCl$_3$ : MeOH (9 : 1) was applied as an eluent. It was isolated using a CHCl$_3$ : MeOH gradient 6 : 4 → 3 : 7 (v/v). MS analysis revealed the presence of a band at m/z 277. (Fig. 6S, ESI$^+$).

General procedure for synthesis of compound 11

A given P-diastereoisomer (fast or slow eluting) of OTP-5N' 5 (0.04 mmol); 28 mg for 5a, 33 mg for 5b, 32 mg for 5c, or 32 mg for 5d) and 3'-O-acetyl-thymidine (2-fold molar excess; 23 mg, 0.08 mmol), were dried over P$_2$O$_5$ in a vacuum desiccator for 24 h, and dissolved in anhydrous acetonitrile (~1 mL) under dry argon. To the solution 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.1 molar equivalent) was added and after 2 h the reaction was complete to give OTP-5N'Tac 10. The formation of 10 was confirmed by $^{31}$P NMR (in CH$_2$CN, no deuterated solvent – the recorded chemical shifts are not precisely reproducible); S$_2$C-5 substrates: DMT-GTP$_{PS}$T, δ 54.6 ppm (from fast 5a), δ 54.9 ppm (from slow); DMT-GTP$_{AC}$T, δ 55.6 ppm (from fast 5b), δ 55.9 ppm (from slow); DMT-GTP$_{AC}$P, δ 56.2 ppm (from fast 5c), δ 56.8 ppm (from slow); DMT-GTP$_{PS}$A, δ 55.6 ppm (from fast 5d), δ 56.7 ppm (from slow); R$_C$-5 substrates: DMT-GTP$_{PS}$T, δ 56.5 ppm (from fast 5a), δ 56.3 ppm (from slow); DMT-GTP$_{AC}$P, δ 55.8 ppm (from fast 5b), δ 56.0 ppm (from slow); DMT-GTP$_{AC}$P, δ 56.7 ppm (from fast 5c), δ 56.3 ppm (from slow). The mixture was concentrated under reduced pressure and treated (in a tightly closed vessel) with concentrated ammonia solution (2 mL; 10a for 2 h at room temperature, 10b, 10c and 10d for 15 h at 55 °C). After evaporation, the DMT moiety was removed with 50% aqueous acetic acid for 1.5 h at room temperature, followed by evaporation under reduced pressure. The resultant OTP$_{PS}$T 11a–d were isolated using RP-HPLC, a gradient of 0.1 M TEAB, pH 7.3 to 38% CH$_2$CN in 0.1 M TEAB over 20 minutes, elution at 1.0 mL min$^{-1}$; 11a, 11b, 11d: an Alltima C18 column, 250 × 4.6 mm, 5 μm; 11c: a Clarity C18 column, 250 × 4.6 mm, 5 μm. Isolated 11 were characterized by MALDI TOF MS and $R_f$.

From S$_2$C-5a–d substrates: 11a OTP$_{PS}$T; $R_f = 18.5$ min, $R_{r2} = 19.5$ min; MALDI TOF MS: calc. for C$_{18}$H$_{25}$N$_{15}$O$_{10}$PS 519.45, found 519.0 and 519.0. 11b OTP$_{PS}$T: $R_r = 16.5$ min, $R_{r2} = 17.0$ min; MALDI TOF MS: calc. for C$_{18}$H$_{25}$N$_{15}$O$_{10}$PS 530.48, found 528.0 and 528.0. 11c OTP$_{PS}$T: $R_r = 15.5$ min, $R_{r2} = 19.0$ min; MALDI TOF MS: calc. for C$_{18}$H$_{25}$N$_{15}$O$_{10}$PS 544.46, found 544.2 and 544.1. From R$_C$-5a–c substrates: 11a OTP$_{PS}$T: $R_r = 19.9$ min, $R_{r2} = 21.0$ min; MALDI TOF MS: calc. for C$_{18}$H$_{25}$N$_{15}$O$_{10}$PS 519.45, 519.3 and 519.0. 11b OTP$_{PS}$T: $R_r = 17.9$ min, $R_{r2} = 17.6$ min; MALDI TOF MS: calc. for C$_{18}$H$_{25}$N$_{15}$O$_{10}$PS 544.46, found 544.0 and 544.1. MALDI TOF MS: calc. for C$_{17}$H$_{23}$N$_{5}$O$_{10}$PS 504.44, found 504.1 and 506.2 (in a positive ions mode).

Hydrolysis of 11 with $svPDE$

The reaction mixture (20 μL) containing 25 mM Tris−Cl (pH 8.5), 5 mM MgCl$_2$ buffer, 10 μL of the svPDE suspension, and $^{15}$N$_{PS}$T (11, 0.2 OD$_{260}$) was incubated for 24 h at 37 °C. Then, the sample was heat-denatured (for 1 min at 95 °C) and analyzed by HPLC, using the conditions specified above.

Hydrolysis of 11 with nuclease P1

The reaction mixture (20 μL) containing 100 mM Tris−Cl (pH 7.2), 1 mM ZnCl$_2$, 10 μL of nuclease P1 suspension, and $^{15}$N$_{PS}$T (11, 0.2 OD$_{260}$) was incubated for 24 h at 37 °C. Then, the sample was heat-denatured (for 1 min at 95 °C) and analyzed by HPLC, using the conditions specified above.

Chimeric oligonucleotides 14, 15, 16 and 17 – synthesis and purification

The chimeric oligonucleotides 14–17 were synthesized manually at a 1 μmol scale. A published protocol for synthesis utilizing OTP monomers was used, except that the coupling of the OTP-5N' monomers was extended to 20 min. The first nucleoside unit was anchored to the support by a sarcosinyl linker. In the cycles where OTP-5N' monomers 5 were delivered, two consecutive coupling steps were executed (20 mg + 20 mg), separated by washing with acetonitrile and drying with a stream of argon. The coupling efficiency was controlled by measurement of the DMT cation absorption decay at 504 nm. All oligomers were purified by reverse phase HPLC (conditions as above) and analyzed by MALDI-TOF MS. 14: (R) -PS-GATGC-GGC-CA; MALDI TOF MS calc. 2353 found 2352.8. 15: (S)$\text{P}$-GATGC-GGC-CA; calc. 2353 found 2352.9. 16: (R) -PS-AGT-GGC-CA; calc. 2395 found 2394.5. 17: (S)-PS-ACT-GGC-CA; calc. 2395 found 2394.1.

Sample preparation and melting UV profile recording

The concentration of oligomers was determined by UV absorbance at their $\lambda_{max}$ in water, using the extinction coefficients calculated by the standard method. The samples were then lyophilized and re-dissolved in pH 7.2 buffer containing 10 mM Tris−HCl, 100 mM NaCl, and 10 mM MgCl$_2$. For melting profiles, the oligonucleotides were mixed at 2.0 μM concentration each. This concentration is also suitable for CD

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measurements). First, an annealing step was performed from 40 °C to 5 °C with a temperature gradient of 1°C min⁻¹, followed by a melting step, carried out to 40 °C (or to 85 °C for DNA, (m)RNA and for mixtures DNA/(m)RNA, 14/(m)RNA and 16/(m)RNA) with a gradient of 0.5°C min⁻¹. The measurements were done at 260 nm, using a 60 s cycle time and a 2 s integration time. The melting temperatures (see Tables 1 and 2S†) were calculated using the first order derivative method.

**Conflicts of interest**

There are no conflicts to declare.

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**Notes and references**