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

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Enantiomerically pure, protected acyclic nucleosides of the GNA type (glycol nucleic acids) (GNAs; Fig. 1B) attracted our attention because of interesting 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.

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. There is also growing interest in their application in nanotechnology. Certain applications require nucleic acids with specific modifications, e.g., at the nucleobases, sugars (ribose or deoxyribose), or inter-nucleotide phosphate linkages. In phosphorothioate analogs of DNA (PS-DNA) one of the non-bridging phosphate oxygen atoms is replaced with a sulfur atom (Fig. 1A). PS-DNA oligomers are remarkably resistant towards nucleolytic enzymes. Although phosphorothioate and phosphate diesters are formally isoelectronic, in PS-DNA there is a greater density of negative charge on the sulfur atom. A single O → S replacement creates a new stereogenic center, thus a PS-DNA decamer synthesized using a non-stereocntrolled method consists of 2⁹ = 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.

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The glycol "nucleoside" units (N) can exist in two enantiomeric forms, i.e. \( (R)_n \)N and \( (S)_n \)N (1a and 1b, respectively, \( B = \text{Ade, Cyt, Gua, Thy} \)).

The GNA oligomers hybridize according to the Watson–Crick’s scheme and form highly stable duplexes with a complementary GNA strand.\(^{20}\) Hybridization with natural nucleic acids is only observed for \( (S)_n \)GNA (and not \( (R)_n \)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.\(^{21}\)

Because interesting P-stereodependent effects were observed in PS-DNA (e.g., different thermodinamic stability of antiparallel duplexes (PS-DNA : DNA)\(^{22}\)) or different propensity for the B → Z conversion\(^{23}\), as well as in conformationally more restricted "locked" PS-LNA,\(^{18}\) 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\(^\ddagger\), 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.\(^{21,24,25}\) Thus, one cannot reject PS-GNA from future application in the RNAi methodology.

\(^\ddagger\) There is only one known exception, where homopurine \( (R)_n \)PS-DNA oligomers form thermally highly stable parallel triplexes (RNA : PS-DNA : RNA)\(^{26}\) and parallel duplexes (PS-DNA : RNA)\(^{28}\). 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.\(^{41}\)

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.\(^{26}\) Also, interesting differences in the electron transfer potentials of P-stereodefined chimeric DNA decamers \( d(CG_{PS}GCCGCGCA) \), as well as their duplexes formed with complementary strands \( d(TCGCGG_{PS}GCG) \), were found by cyclic voltammetry assay.\(^{27}\) 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 \( (R)_n \) and \( (S)_n \)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-oxathiaplophane derivatives of the 3’-O-DMT protected glycidol-derived "nucleosides" (OTP-N), and enzymatic and chemical determination of the absolute configuration of the P-atoms in the assembled \( GN_{PS} \) "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 "nucleosides" (DMT-N)\(^{\ddagger}\)**

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

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quantitatively the corresponding dimethoxytritylated glycidols 3. The regioselective and stereospecific epoxide ring opening reaction with a nucleobase (thymine, adenine or N-benzoylcytosine) in the presence of NaH afforded DMT-GN’s 4a (B = Thy), 4b* (B = Ade) or 4c (B = Cyt)*. DMT-GA (4b*) was next protected at the exocyclic amino group with benzoyl chloride to yield DMT-GA-Re (4b). The guanine derivative 4d was obtained via a different method (Scheme 2), since the literature protocol didn’t work in our hands. In this case we decided to work only with (R)-(+)-glycidol because the related (S)-GNA has more useful properties than (R)-GNA. The epoxide 2 was opened with 2-amino-6-chloropurine to give compound 6, which was further treated with sodium thiosulfate. The resultant 6-thioguanosine derivative 7 was oxidized to 8 using hydrogen peroxide. The amino group in 8 was acylated with iso-butyryl chloride to yield diol 9, which was further protected with DMT-Cl to furnish DMT-GBu (4d).

Starting from (R)- and (S)-glycidol (2), the corresponding DMT-GN’s (4) of Sc and Rc absolute configuration, respectively, were obtained (see inset in Scheme 1; note that the methylene group in the oxirane ring (marked as CH2) has priority over the hydroxymethyl group, as shown by the numbers in parentheses).

Preparation of P-diastereomercerically pure oxathiaphospholane derivatives of glycol “nucleosides” (OTP-GN)†

The enantiomerically pure “nucleoside” substrates (DMT-GN, 4a-d) were phosphorylated 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-5N) were obtained in good yield (78%, 74%, 71%, 79%, respectively) and characterized by 31P NMR, 1H NMR, 13C NMR (for representative spectra see Fig. 1S–3S, ESI†) and HRMS (for the spectra of 5a–d see Fig. 4S, ESI†).

A molecule of OTP-5N (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-5N 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† and LNA‡ series, we tried to remove the DMT group in 5a–d to obtain 3’-OH OTP-5N derivatives 5a–d (shown in square brackets in Scheme 1) using earlier successful p-toluenesulfonyl acid monohydrate, yet with no success. Another proic acid (dichloroacetic acid), Lewis’ acids (ZnCl2, SnCl4), tetra-n-butylammonium peroxydisulfate and NaHSO4/SiO2 (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 31P NMR and MS (Fig. 6S, ESI†) as the corresponding thymidine cyclic 2’,3’-O,3’-phosphorothioate (c5TMPS) bearing a 5-membered ring (Scheme 3, B = Thy). The reasons of this unexpected elimination are unclear. It should be noted that the conversion of the detritylated OTP derivative of dBu into the corresponding cyclic 3’,5’-O,3’-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.† The possible mechanism of forming this five membered byproduct is shown in Scheme 3.

Assignment of the absolute configuration of the P-atom in “dinucleotides” 5NPS obtained using OTP-5N†

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 DMT-GN’s 5-PSAc (10a–d, Scheme 4, Fig. 7S, ESI†), which after routine two-step deprotection furnished the phosphorothioates 5NPS (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 5NPS (11a–d), the reactions with snake venom phosphodiesterase (sPDE, an Rp-specific nuclease) and nuclease P1 (nP1, an Sp-specific nuclease) were
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) showed that compounds 11a–d obtained from slow-eluting (Rc)-OTP-GN (synthesized from S(−)-glycidol) or fast-eluting (Sc)-OTP-GN (obtained from R(+)-glycidol) were hydrolyzed to yield \(^{\text{c}}\)N (1, Scheme 4) and thymidine 5′-O-phosphorothioate (TMPs), identified by means of RP HPLC (Fig. 11S, ESI). Thus the svPDE-digestable diastereomers were tentatively assigned an \(R_\text{p}\) configuration. Those obtained from fast-eluting (Rc)-OTP-\(^{\text{c}}\)N or slow-eluting (Sc)-OTP-\(^{\text{c}}\)N remained intact after 24 h incubation, so were assigned an \(S_\text{p}\) 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 \(^{\text{c}}\)Nps\(_\text{T}\) (11a–d; the synthesis of 11b is shown in Scheme 5). We used fast- and slow-eluting \(P\)-diastereoisomers of the 5′-O-oxathiaphospholane derivative of 3′-O-camphanoylated thymidine (5′-OTP-T\(_\text{Camph}\), 12), which are known to be the precursors of \((S_\text{p})\)-Nps\(_\text{T}\) and \((R_\text{p})\)-Nps\(_\text{T}\), respectively.\(^{35}\) In the presented example fast-12 was reacted with \((S_\text{c} + R_\text{c})\)-DMT-G\(_{\text{A Bz}}\) (4b, prepared from racemic glycidol) to yield a pair of protected \(\text{DMT-G}_{\text{A Bz}}\) ps\(_\text{T Camph}\) (13b). By a routine two-step deprotection both isomers of 13b were converted into a pair of \(S_\text{c}R_\text{c}\) and \(R_\text{c}S_\text{c}\), but this time the absolute configuration at P-atoms (i.e. \(S_\text{p}\)) was known.\(^{1}\) The second pair of 11b consisting of \(S_\text{p}R_\text{p}\) and \(R_\text{p}S_\text{p}\) isomers was obtained using slow-eluting 12. The \(R_\text{p}\) and \(S_\text{p}\) pairs of 11b 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).

\[\text{Note, that priority of substituents around the stereogenic phosphorus atom in} \quad \text{A}_\text{ps}\text{T and A}_\text{ps}\text{T (generally in psN and psN) is the same.}\]

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

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

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

The diastereomerically pure OTP-GN' 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 then with double coupling used at each condensation step (earlier effectively applied in synthesis of PS-LNA), 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 amount (0.75 = 0.133, 0.75² = 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 DNA and LNA series. To acquire an insight into a possible mechanism contributing to the problem, a PS-GNA oligonucleotide ([4⁺UP₃]₄) dA has been synthesized using a modified phosphoramidite/sulfurization methodology and the sarcosine linker. A 2-cyanoethyl-N,N-diisopropylphosphoramidite derivative of DMT-tetrazole 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 pipedine/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 dephosphorylated 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 6-uridine cyclic 2',3'-O-phosphorothioate (c6UMPS, Scheme 3, B = Ura), which is an analog of c6TMPs, 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-stereodefined 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 $^14$N units, using appropriate OTP-GN' and OTP-N' monomers.** The syntheses were performed manually at a 1
μmol scale. Selfcomplementary oligomers of a general sequence PS-d(AGCGCAT) were synthesized in two DNA/GNA variants (each chimera oligomer in two P-stereoregular forms, Table 1), bearing either purine 4Ns (PS-4ATGGCGCAT; Rp, 14; Sp, 15) or pyrimidine 6Ns (PS-4ATGGCGCAT; Rp, 16; Sp, 17).†† Only Sp- OTP-6N were used in the syntheses. Decay of the trityl cation absorption after consecutive coupling steps was measured photometrically (Fig. 1S, 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(AGCGCAT)-3′ (18) and (Sp)-PS-5′-d(AGCGCAT)-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 4N 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 Θ254 = −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 conformation 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 Θ254 = −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.
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 3NnpT. The absolute configuration of the P-atom in 3NnpT was established by enzymatic and chemical methods. Then, four self-complementary P-stereodefined 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 stereochernistry at phosphorus and relative arrangement of the N-units in the oligonucleotide strands. Most interestingly, a duplex formed by 17 (S)p-PS-(A†G-G-C-G-C) was thermally more stable than that formed by the corresponding PS-DNA congener 19 (ΔT_m = 8 °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-stereodefined phosphorothioate glycol nucleic acid are underway.

Experimental section

1H NMR and 31P NMR spectra were recorded using a Bruker instrument (AV-200, 200 MHz for 1H). Chemical shift values (δ) are given in ppm, relative to internal tetramethylsilane (TMS) or residual solvent protons for 1H NMR, and external 85% H3PO4 for 31P 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).

Dimethoxytritylation of glycidol – synthesis of enantiomeric compounds 3

To a solution of (R)-(+) or (S)-(−)-glycidol 2 (1.0 mL, 15.1 mmol) and Et3N (5.4 mL, 40.7 mmol) in CH2Cl2 (50 mL) solid DMT-Cl (6.45 g, 19 mmol) was added. After 12 h, the reaction mixture was treated with saturated aqueous NaHCO3 (50 mL). The organic layer was separated, dried with MgSO4 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–Et₃N (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 dimethylthionylated glycidol 3 (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–Et₃N (1 : 2 : 0.01, v/v/v), then with EtOAc–MeOH 9 : 1, v/v; FAB MS calc. for C₃₆H₃₃N₃O₆: 591.67, found: 592.1 (+VE), 590.3 (–VE).

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

To a solution of (6-chloropurine)G nucleoside (241 mg, 1 mmol) in NH₄OH (5 mL) was allowed to stand at room temperature for 5 h. The reaction mixture was filtered and the residue was dissolved in EtOAc and loaded on a silica gel column. The column was eluted with a mixture of CHCl₃–MeOH 9 : 1, v/v. The mixture was cooled to 0 °C and was then stirred for 2–3 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-GC₄T. (553 mg, 78%); Rₜ = 0.71 (CHCl₃–MeOH 9 : 1, v/v); HRMS for C₄₃H₄₀N₁₀O₅PS₂: calc. 707.2015, found 707.2015 (Fig. 4SA, ESI†); 3¹P NMR: δ 105.8 ppm, δ 105.5 ppm (no deuterated solvent).

5b OTP-GA₅′. 607 mg (74%); Rₜ = 0.75 (CHCl₃–MeOH 9 : 1, v/v); HRMS for C₄₅H₴₂N₁₀O₅PS₂: calc. 820.2287, found 820.2280 (Fig. 4SB, ESI†); 3¹P NMR: δ 105.9 ppm, δ 105.2 ppm (no deuterated solvent).

5c OTP-GC₅Bu. 566 mg (71%); Rₜ = 0.69 (CHCl₃–MeOH 9 : 1, v/v); HRMS for C₄₇H₴₄N₁₀O₈PS₂: calc. 976.2280, found 976.2287 (Fig. 4SC, ESI†); 3¹P NMR: δ 106.5 ppm, δ 105.1 ppm (no deuterated solvent).

5d OTP-GC₆Bu. 634 mg (79%); Rₜ = 0.70 (CHCl₃–MeOH 9 : 1, v/v); HRMS for C₄₉H₴₆N₁₀O₈PS₂: calc. 1124.2548, found 1124.2514 (Fig. 4SD, ESI†); 3¹P NMR: δ 106.1 ppm, δ 105.6 ppm (no deuterated solvent).
the corresponding nucleoside \( \text{G} \) as white solid material (155 mg, 69%). \( R_t = 0.42 \) (CHCl\(_3\)-MeOH 9:1, v/v); FAB MS: calc. for C\(_{24}\)H\(_{32}\)N\(_2\)O\(_4\)S\(_2\) 225.2, found: 226.2 (+VE), 224.1 (−VE).

**Iso-butylation of \( \text{G} \) – synthesis of compound 9**

To a solution of \( \text{G} \) (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-butylin 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\(_2\)O (2 mL) at 0°C. After 10 min, conc. NH\(_2\)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 \( \text{G}_{\text{Bun}} \). \( R_t = 0.10 \) (CHCl\(_3\)-MeOH 9:1, v/v); FAB MS: calc. for C\(_{23}\)H\(_{31}\)N\(_2\)O\(_4\)S\(_2\) 295.3, found: 296.1 (+VE), 294.2 (−VE).

**Dimethoxymethylolation of \( \text{G} \) – synthesis of \( \text{DMT-G} \) (compound 4d)**

To a solution of \( \text{G} \) (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\(_2\)N (1:2:0.01, v/v/v), then with EtOAc-Et\(_2\)N (100:1, v/v), and finally with EtOAc-MeOH-Et\(_2\)N (25:1:0.01, v/v/v). The fraction containing \( \text{DMT-G}_{\text{Bun}} \) (\( R_t = 0.46 \) (CHCl\(_3\)-MeOH 9:1, v/v)) was concentrated in vacuo to yield 430 mg (colorless foam, 72%); FAB MS: calc. for C\(_{33}\)H\(_{31}\)N\(_2\)O\(_4\)S\(_2\) 597.7 found: 296.1 [M-DMT] (+); +VE.

**HPLC separation of P-diastereomers of GNA oxathiaiphospholane 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\(^{-1}\) 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\(^{-1}\) 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 \(^{31}\)P NMR analysis (CDCl\(_3\), an estimated limit of detection of a minor component 0.2%). They gave satisfactory \(^1\)H NMR, \(^{31}\)P NMR, \(^{13}\)C NMR and MALDI-TOF mass spectra.

5a OTP-\(^{4}\)Br fast: \( R_t = 0.68 \) (CHCl\(_3\)-MeOH 9:1, v/v); \( R_t = 19.2 \) min (EtOAc–hexane 50:50, v/v); MALDI TOF MS: calc. for C\(_{34}\)H\(_{34}\)N\(_2\)O\(_4\)PS\(_2\) 708.8 found: 747.6 (+); 708.8 + K; \(^{31}\)P NMR \( \delta \) (CDCl\(_3\), ppm) 105.6; \(^1\)H NMR \( \delta \) (CDCl\(_3\), ppm) 8.36 (1H, N3-\( \text{H} \)), 7.43 (1H, C6-\( \text{H} \)), 7.40–6.79 (13H, DMT), 5.13–5.06 (1H, C2'-\( \text{H} \)), 4.16–4.15 (2H, C1'-\( \text{H} \)), 4.08–4.05 (2H, P-O-CH\(_2\)C-\( \text{S} \)), 3.75 (6H, 2 \( \times \) OCH\(_3\)), 3.33–3.25 (2H, C3'-\( \text{H} \)), 1.89–1.20 (3H, C5-CH\(_3\)-10H, \(-\text{CH}_2\text{O}^\text{spiro}^\text{spiro}\)); \(^{13}\)C NMR \( \delta \) (CDCl\(_3\), ppm) 162.6, 157.3, 143.0, 140.5, 134.2, 128.7, 126.7, 126.5, 111.9, 78.0, 76.4, 75.9, 75.1, 62.2, 53.9, 36.0, 35.3, 23.9, 22.7, 22.2, 11.0; 5a OTP-\(^{4}\)I fast: \( R_t = 0.60 \) (CHCl\(_3\)-MeOH 9:1, v/v); \( R_t = 21.5 \) min (EtOAc–hexane 50:50, v/v); MALDI TOF MS: calc. for C\(_{35}\)H\(_{36}\)N\(_2\)O\(_4\)PS\(_2\) 708.8 found: 747.6 (+); 708.8 + K; \(^{31}\)P NMR \( \delta \) (CDCl\(_3\), ppm) 106.1; \(^1\)H NMR \( \delta \) (CDCl\(_3\), ppm) 11.98 (1H, NHCOC), 11.98 (1H, NHCOC), 7.58 (1H, C8-\( \text{H} \)), 7.24–6.76 (13H, DMT), 5.17–5.10 (1H, C2’-\( \text{H} \)), 4.41–4.31 (2H, C1’-\( \text{H} \)), 4.08–3.94 (2H, P-O-CH\(_2\)C-\( \text{S} \)), 3.76 (6H, 2 \( \times \) OCH\(_3\)), 3.17–3.15 (2H, C3’-\( \text{H} \)), 3.17–3.15 (2H, C3’-\( \text{H} \)), 3.17–3.15 (2H, C3’-\( \text{H} \)), 3.17–3.15 (2H, C3’-\( \text{H} \))
General procedure for synthesis of compound 11

A given P-diastereoisomer (fast or slow eluting) of OTP-\(^{15}\)N' 5 (0.04 mmol; 28 mg for 5a, 33 mg for 5b, 32 mg for 5c, or 32 mg for 5d) and 3'-O-acetylthymidine (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 (\(\sim\)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-\(^{15}\)N'\(_{\text{Ac}}\)T._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); \(S_{\text{C}-5}\)-substitutes: \(^{15}\)DMT-\(_{\text{T}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.46 ppm (from fast 5a), \(\delta\) 5.49 ppm (from slow); \(^{15}\)DMT-GAB\(_{\text{T}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.56 ppm (from fast 5b), \(\delta\) 5.59 ppm (from slow); \(^{15}\)DMT-GGP\(_{\text{T}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.62 ppm (from fast 5c), \(\delta\) 5.68 ppm (from slow); \(^{15}\)DMT-GTP\(_{\text{T}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.56 ppm (from fast 5d), \(\delta\) 5.67 ppm (from slow); \(R_{\text{C}-5}\)-substitutes: \(^{15}\)DMT-GAB\(_{\text{R}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.65 ppm (from fast 5a), \(\delta\) 5.63 ppm (from slow); \(^{15}\)DMT-GGP\(_{\text{R}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.58 ppm (from fast 5b), \(\delta\) 5.60 ppm (from slow); \(^{15}\)DMT-GTP\(_{\text{R}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(\delta\) 5.67 ppm (from fast 5c), \(\delta\) 5.63 ppm (from slow).

The mixture was concentrated under reduced pressure and treated (in a tightly closed vessel) with concentrated ammonium 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 \(^{15}\)N\(_{\text{PS}}\)T._11a-d_ were isolated using RP-HPLC, a gradient of 0.1 M TEAB over 20 minutes, elution at 1.0 mL min\(^{-1}\); 11a, b, d: an Alltima C18 column, 250 x 4.6 mm, 5 \(\mu\)m; 11c: a Clarity C18 column, 250 x 4.6 mm, 5 \(\mu\)m. Isolated 11 were characterized by MALDI TOF MS and R$_t$.

From \(S_{\text{C}-5}\)-substitutes: \(^{15}\)DMT-\(_{\text{R}_{\text{Ac}}}\)P\(_{\text{Ac}}\); \(R_{t}\) = 18.5 min, \(R_{t}\) = 19.5 min; MALDI TOF MS: calc. for C$_{18}$H$_{33}$N$_{4}$O$_{10}$PS 519.45, found 519.0 and 519.0. 11b \(^{15}\)APsT: \(R_{t}\) = 16.5 min, \(R_{t}\) = 17.0 min; MALDI TOF MS: calc. for C$_{18}$H$_{33}$N$_{4}$O$_{10}$PS 530.48, found 528.0 and 528.0. 11c \(^{15}\)CpsT: \(R_{t}\) = 15.5 min, \(R_{t}\) = 19.0 min; MALDI TOF MS: calc. for C$_{18}$H$_{32}$N$_{4}$O$_{10}$PS 504.44, found 504.2 and 504.1. 11d \(^{15}\)GpsT: \(R_{t}\) = 18.7 min, \(R_{t}\) = 18.5 min; MALDI TOF MS: calc. for C$_{18}$H$_{32}$N$_{4}$O$_{10}$PS 544.46, found 544.2 and 544.1. From Rp-5a-c-substitutes: \(^{15}\)DMT-PsT: \(R_{t}\) = 19.9 min, \(R_{t}\) = 21.0 min; MALDI TOF MS: calc. for C$_{18}$H$_{32}$N$_{4}$O$_{10}$PS 519.45, found 519.3 and 519.0. 11b \(^{15}\)APsT: \(R_{t}\) = 17.9 min, \(R_{t}\) = 17.6 min; MALDI TOF MS: calc. for C$_{18}$H$_{32}$N$_{4}$O$_{10}$PS 530.48, found 528.0 and 528.0. 11c \(^{15}\)CpsT: \(R_{t}\) = 15.6 min, \(R_{t}\) = 18.2 min; MALDI TOF MS: calc. for C$_{18}$H$_{32}$N$_{4}$O$_{10}$PS 544.44, found 540.1 and 506.2 (in a positive ions mode).

Sample preparation and melting UV profile recording

The concentration of oligomers was determined by UV absorbance at their \(\lambda_{\text{max}}\) 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 \(\mu\)M concentration each. (This concentration is also suitable for CD
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

18 V. Kumar and V. Kesavan, RSC Adv., 2013, 3, 19330.
23 M. Boczkowska, P. Guga, B. Karwowski and A. Maciaszek, Biochemistry, 2000, 39, 11057.