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# SYNTHESIS, SEPARATION, AND STEREOCHEMISTRY OF DIASTEREOMERIC OLIGODEOXYRIBONUCLEOTIDES HAVING A 5'-TERMINAL INTERNUCLEOTIDE PHOSPHOROTHIOATE LINKAGE

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Abstract: An automated, solid-phase, phosphoramidite-coupling method provided the title compounds, up to a hexadecamer, for HPLC isolation of diastereomerically pure samples, and comparative digestions with snake venom phosphodiesterase and nuclease P1.

Phosphorothioate analogues of oligodeoxyribonucleotides (I) have proven to be powerful tools for studies of the mechanism of action of phosphorolytic enzymes.[2] Stereospecific methods for PS to PO conversion have also been established,[3] and compounds I therefore additionally serve as valuable intermediates for the synthesis of isotopomeric oligonucleotides, wherein chirality at phosphorus results from the presence of different stable isotopes of oxygen.[4] More recently, it has been shown that the chemical synthesis of I can be achieved either by the preparation of dinucleoside phosphorothioates (II) for incorporation of diastereomerically pure II into an oligonucleotide chain,[5,6] or by the automated phosphoramidite method of synthesis, wherein oxidation ($P^{3+}$ to $P^{5+}$) of an internucleotide O-methyl phosphite is achieved by reaction with a solution of elemental sulfur in lutidine during any desired cycle.[7] Successful separation of the latter synthesized diastereomers of I by means of reverse-phase HPLC depends, however, upon the chainlength of I, its base composition and, interestingly, the position of the phosphorothioate linkage within the chain: for example, among the 7 possible monophosphorothioate analogues of the octamer d(GGAATTCC), 5 pairs of diastereomers were separable by HPLC and 2 pairs were not, viz., (Rp)- and (Sp)-d(GGA$_{PS}$ATTCC), and (Rp)- and (Sp)-d(GGAA$_{PS}$TTCC).[7]

In this communication, we wish to report observations regarding the relatively easy HPLC-separation of diastereomers of I when there is a 5'-terminal phosphorothioate linkage and a 5'-dimethoxytrityl (DMT) group, representative examples of which are given in Table I and feature a hexadecamer, IIIi. Compounds IIIa-i were synthesized by means of an Applied Biosystems Model 380A DNA Synthesizer using commercially available phosphoramidite reagents, 5'-DMT-N-P(OMe)N'(iPr)$_2$, and 5'-DMT-N-CPG (CPG="long chain alkyl amine" controlled pore glass,[8a] N = dA$^{Bz}$, dC$^{Bz}$, dG$^{iBu}$, or dT). The last cycle[8] in the 3' to 5' synthesis of each compound was

Table I. 5'-Terminal Phosphorothioate Analogues of Oligonucleotides and Analytical Data

| Compd no. | Formula, $N_{PS}R$ | Elution time[a] of 5'-DMT-$N_{PS}R$ | Elution time[a] of 5'-HO-$N_{PS}R$ | Product of enzymatic digestion[b] (ret. time)[a] | Absolute config. at PS |
|---|---|---|---|---|---|
| | N=G | 15.77[c] | 15.29[d] | (Sp)-$G_{PS}G$ (15.18)[d] | Sp |
| | R=GG | 16.44 | 13.82 | (Rp)-$G_{PS}G$ (13.29) | Rp |
| | | | 15.79[d] | (Sp)-$G_{PS}G$,(Rp)-$C_{PS}C$(10.06)[d] | Sp,Rp |
| | | 15.33[c] | | | |
| | N=G | | 18.95 | (Sp)-$G_{PS}G$,(Sp)-$C_{PS}C$ (12.38) | Sp,Sp |
| IIIb | | | 17.05 | (Rp)-$G_{PS}G$,(Rp)-$C_{PS}C$ | Rp,Rp |
| | R=$GC_{PS}C$ | 15.88 | | | |
| | | | 18.36 | | |
| | | | | (Rp)-$G_{PS}G$,(Sp)-$C_{PS}C$ | Rp,Sp |
| | N=G | 22.94[e] | 19.65[d] | (Sp)-$G_{PS}A$ (17.20)[d] | Sp |
| | R=AATTCC | 24.64 | 19.32 | (Rp)-$G_{PS}A$ (15.66) | Rp |
| | N=A | 15.30[f] | 23.47[d] | (Sp)-$A_{PS}A$ (20.09)d | Sp |
| | R=ACCTGG | 16.37 | 22.82 | (Rp)-$A_{PS}A$ (18.90) | Rp |
| | N=G | 19.24[g] | 20.11[d,h] | (Sp)-$G_{PS}G$ (15.20)[d] | Sp |
| | R=GAATTCC | 21.12 | 18.63 | (Rp)-$G_{PS}G$ (13.30) | Rp |
| | N=G | 23.53[i] | 17.67[j] | (Rp)-$G_{PS}G$ | Rp |
| IIIf | | | | | |
| | R=GGAATTCCC | 24.60 | 18.10 | (Sp)-$G_{PS}G$ | Sp |
| | N=T | 15.43[f] | 20.62[d] | (Sp)-$T_{PS}G$ (15.77)[d] | Sp |
| IIIg | | | | | |
| | R=GCATATCGAC | 16.28 | 20.68 | (Rp)-$T_{PS}G$ (14.44) | Rp |
| | N=T | 11.20[k] | 23.23[d] | (Sp)-$T_{PS}A$ (14.37)[d] | Sp |
| IIIh | | | | | |
| | R=ATATCGATATA | 12.24 | 22.42 | (Rp)-$T_{PS}A$ (13.47) | Rp |
| | N=T | 10.08[k] | 23.29[d] | (Sp)-$T_{PS}A$[d] | Sp |
| IIIi | | | | | |
| | R=ATATATCGATATATA | 11.14 | 22.85 | (Rp)-$T_{PS}A$ | Rp |

[a] Minutes; cf. text and compds for details. [b] A dry sample of 5'-HO-$N_{PS}R$ (~0.2 $OD_{260}$ unit) was dissolved in 100 µl of 0.1M tris-acetate buffer containing 0.02M $MgCl_2$ (pH 8.8) and a buffered solution (10 µl) of snake venom phosphodiesterase (Sigma, Type II, 1.5 units of protein in 1.5 ml buffer) was added at 37°C. After 6 h at 37°C, the mixtures (except for compd IIIb) were heated (3 min, 100°C), centrifuged, and aliquots were analyzed by HPLC; the incubation mixture from IIIb was treated with alkaline phosphatase (Sigma, Type III R) for 4 h, 37°C, and then heat-denatured before HPLC analysis. To 5'-HO-$N_{PS}R$ (~0.2 $OD_{260}$ unit) in 100 µl of 0.025M tris-HCl buffer (pH 7.0) was added a buffered solution (3 µl) of Nuclease P1 from Penicillium citrinum (Sigma, 370 units of protein in 2 ml of buffer) at 37°C. After 2 h at 37°C the mixtures were processed as described above. [c] Flow rate 4 mL/min, initial conditions 20% $CH_3CN^{(A)}$ -80% $TEAA^{(B)}$, 1%/min gradient. [d] Flow rate 4 mL/min, initial conditions 5% A -95% B, 0.5%/min gradient. [e] Flow rate 4 mL/min, initial conditions 20% A -80% B, after 10 min 25:75, then isocratic. [f] Flow rate 4 mL/min, initial conditions 20% A -80% B, after 10 min 25:75, then isocratic. [g] Flow rate 5 mL/min, initial conditions 20% A -80% B, after 10 min 25:75, then isocratic. [h] $^{31}P$ NMR of unseparated mixture of 5'-OH compd in 0.1M tris containing 1 mg/mL of EDTA, pH 7.6, $\delta_{PS}$ 54.75 and 53.64; $\delta_{PO}$-1.16, -1.44, -1.52, -1.64, -1.00. [i] Flow rate 4 mL/min, initial conditions 20% A -80% B, 0.33%/min gradient. [j] Flow rate 5 mL/min, other conditions as specified in ftnt d. [k] Flow rate 4 mL/min, initial conditions 20% A -80% B, after 5 min 30:70, then isocratic.

modified by replacing the $I_2$-$H_2O$-lutidine oxidizing reagent with 0.4M $S_8$ in lutidine (30 min, 60°C). Sequential O-demethylation, cleavage from the support, and base-deprotection[8c] gave 5'-DMT-III with the general formula $Np_SR$, which were eluted from a $C_{18}$ μBondapak® column (7.8mm X 30cm, Waters Assoc.) using a gradient of $CH_3CN$-triethylammonium acetate buffer (0.1M, pH 7) at a flow rate of 4 mL/min, starting with 20:80 $CH_3CN$:buffer (see Table I for details). Inspection of Table I indicates that while the shorter oligonucleotides were also separable as diastereomeric 5'-HO species, the longer products tended to give better resolution of diastereomers as their 5'-DMT derivatives, which suggested that chromatographic stereodifferentiation between diastereomeric pairs was enhanced by juxtaposition of the DMT and phosphorothioate moieties. Assignment of absolute configuration at the chiral phosphorus position(s) was achieved by enzymatic digestion with snake venom phosphodiesterase[9] (SVPDE) and, independently, with nuclease P1,[10] followed by HPLC comparisons of the digests with authentic samples of stereochemically defined II. The latter compounds having the Rp absolute configuration were found in the nuclease P1 digest of a given diastereomer, while the corresponding digest obtained with SVPDE was devoid of detectable II. Converse results were obtained with the second diastereomer: SVPDE led to (Sp)-II and nuclease P1 gave a product mixture devoid of detectable II. Qualitatively, it also appeared that while the relative rates of SVPDE-catalyzed hydrolysis of diastereomers of 5'-HO-$Np_SR$ differed dramatically for N = dA, dC or dT, this stereodifferentiation was somewhat less for N = dG, and much less with a $Gp_SG$ fragment. Control studies proved that (Rp)-$Gp_SG$ undergoes hydrolysis in the presence of SVPDE, but the rate is much slower than that observed with, e.g., (Rp)-$Tp_ST$. An important conclusion from these observations is that the assignment of absolute configuration at phosphorus in phosphorothioate analogues of oligonucleotides having a $Gp_SG$ fragment requires a more quantitative analysis of the SVPDE digests, the use of genuine (Rp)- and (Sp)-$Gp_SG$, and chromatographic conditions which are known to separate these dimers. In a few cases, we also observed that digestion of diastereomerically pure (Rp)-5'-HO-$Np_SR$ with SVPDE led to small amounts of residual (Rp)-$Gp_SG$ as well as some (Sp)-$Gp_SG$. This may indicate that SVPDE causes epimerization of (Rp)-$Gp_SG$ into (Sp)-$Gp_SG$; however, further studies are needed to eliminate the possibility of trace cross-contamination among the diastereomeric starting materials. In any event, nuclease P1 was completely selective toward (Sp)-5'-HO-$Np_SR$ and led to (Rp)-II without detectable amounts of the corresponding Sp dimer. These results imply that independent digestions of separated diastereomers with SVPDE and with nuclease P1 are required for reliable assignment of absolute configuration in I. If the diastereomers of I are unseparable, then their treatment with both enzymes, followed by indentification of (Rp)-and (Sp)-$Np_SN'$ in the digest, should be used for the stereochemical identification of I. It is worthwhile to note here that in those cases where the phosphorothioate group is not at the 5' end, digestion with SVPDE and nuclease P1 was followed by by treatment with alkaline phosphatase, which converts $pNp_SN'$ into more readily available $Np_SN'$.

In agreement with earlier reports [2,7,11,12] from several laboratories, 5'-HO-I with the Rp configuration displays, when separation is achievable, shorter elution times upon $C_{18}$ HPLC, relative to its Sp counterpart. On the other hand, the present data indicate that the compounds (Sp)-5'-DMT-$N_{PS}$R exhibit shorter elution times relative to their Rp counterparts, with only one exception found to date. These empirically derived relative chromatographic mobilities may thus be used as additional evidence for absolute configurational assignments in I. The automated solid-phase synthesis of diastereomers of $N_{PS}$R, their separation by HPLC, and assignment of absolute configuration, as presented in this communication, provide a basis for the stereospecific synthesis of oligonucleotides and hence, DNA's that contain a phosphorothioate moiety at any desired position, given that the individual diastereomers of $N_{PS}$R can be joined either to each other or to DNA fragments by means of biochemical techniques.

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