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SYNTHESIS AND ABSOLUTE CONFIGURATION OF P-CHIRAL O-ISOPROPYL OLIGONUCLEOTIDE TRIESTERS

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Abstract: New O-isopropylphosphomorpholidite reagents provided the title compounds as mixtures of P-chiral diastereomers, which were separated by HPLC for enzymatic digestion studies and assignment of configuration at phosphorus by chemical correlation with known phosphorothioates.

Among the synthetic analogues of oligodeoxyribonucleotides, those having alkylated internucleotide phosphate moieties (1, DNA triesters) are especially interesting. In addition to their relevance as products of DNA alkylation, synthetic 1 are resistant to nucleases and are taken up by cells, which makes them candidates for antiviral drugs and drug-carriers.[2] Consequently, our investigations of backbone-modified DNA analogues, such as phosphorothioates[3-5] and alkanephosphonates,[6] have led to the presently described synthesis of isopropoxy 1 (1-OiPr) and the design of a potentially general chemical method for assigning absolute stereochemistry at stereogenic phosphorus centers in 1.

Our synthetic route to 1-OiPr utilizes base-protected 5'-dimethoxytrityl (5'-DMT) nucleosides having an O-iPr phosphomorpholidite function attached to the 3'-oxygen atom (2), which are variants of Caruthers' O-Me phosphoramidites.[7] Distilled chloro-morpholino-i-propoxyphosphine (bp 54-56°C/0.02 mm, 1.1 equiv, obtained from i-propyl phosphodichloridite and N-$SiMe_3$ morpholine) was added dropwise to a solution of either 5'-DMT-Ade^{Bz}, -Gua^{iBu}, -Cyt^{Bz}, or -Thy (1g) and $EtN(iPr)_2$ (1.3 equiv) in dry CH_2Cl_2 (30 mL), and after 15 min the reaction mixture was poured into ice-cooled aq. $NaHCO_3$ (50 mL) and extracted with $CHCl_3$ (3 x 30 mL). The organic layer was dried ($MgSO_4$), volatiles were removed in vacuo, and the residue was dissolved in toluene (4 mL) for addition to n-pentane (300 mL). Precipitated 2 was collected by filtration, dried in vacuo, and used without further purification [70-80% yields; ^{31}P NMR ($CDCl_3$): 1:1 ratio of diastereomers, ca. 142 ppm, rel. to H_3PO_4 in D_2O; FAB-MS: $(M+H)^+$ ion].

Manual coupling (3 min) of O-iPr G-type morpholidite to support-bound 5'-HO-Gua^{iBu} (3 μmol) in the presence of 1H-tetrazole (10 equiv) gave, after standard[7] oxidation, ammoniolytic release from the support, and base-deprotection with conc. NH_4OH (10 h, 60°C), a 30% isolated yield of diguanosyl(3'→5')O-iPr phosphate (G_*G, 3) as a 1:1 mixture of diastereomers, which were separated by reverse-phase HPLC (Table I). ^{31}P NMR spectra of 3 indicated isochronous signals at -3.82 ppm. FAB-MS analysis of each diastereomer gave an $(M+H)^+$ ion, m/z 639, as well as $(M+Na)^+$ and $(M+K)^+$ ions at m/z 661 and 677, respectively. Among the dimers synthesized in this manner (e.g., 3-11, Table I), some of the diastereomers (e.g., 9 and G_*C) were unseparable by C_{18} HPLC. The 30-90% isolated yields of the dimers indicated that the relatively bulky O-iPr group in 2 did not impede coupling, and that O-iPr phosphates were not extensively hydrolyzed during base-deprotection with conc. NH_4OH. Nevertheless, the possibility of the loss of

some triester, especially in more complex products (vide infra), led to the use of ethylenediamine - EtOH[8] (1:1 v/v, 7 h, 25°C) in place of conc. NH_4OH.

Table I. Triester- and Thiono Triester-Containing Oligodeoxyribonucleotides and Analytical Data

compd	formula[a]	^{31}P, δ[b]	elution time, min 5'-DMT[c]	elution time, min 5'-HO[d]
3	G_*G[e]	(-3.82)	-	16.1,16.6
4	G_*A	(-3.26)	-	15.7,16.4
5	$G_{P(S)OiPr}A$	(-64.5)	-	24.7,25.1
6	T_*T[e,f]		-	36.0,38.5
7	$T_{P(S)OiPr}T$[g]		-	21.3,24.3
8	A_*A[e]	(-3.89,-3.95)	-	11.4,11.8[h]
9	A_*T[e]	(-2.36,-2.48)	-	13.0[h]
10	A_*G[e]		-	17.9,18.4
11	A_*C[e]		-	18.6,19.0
12	A_*TA_*T		23.1[i] 23.5	13.7,14.1 13.6,14.0
13	$G_*G_*A_*A_*T$	(-3.34[j])	16.6-18.1[i]	11.5-13.2[k]

compd	formula[a]	elution time, min 5'-DMT[c]	elution time, min 5'-HO[d]
14	$G_*G_*A_*A_*TTCC$[l]	23.7-24.5	19.8-21.5[m]
15	$G_*GAATTCC$	16.7 22.2	16.0 16.6
16	$GG_*AATTCC$	12.7 13.4	14.3 13.9
17	GGA_*ATTCC	8.4[n]	13.1 13.7
18	$GGAA_*TTCC$	9.3[n] 10.4	15.3 14.4
19	$GG_{P(S)OiPr}AATTCC$	14.0 14.4	15.8 15.5
		^{31}P: 64.2, -1.1 to -1.5 63.0, -1.3 to -1.6	

[a]An asterisk and P(S)OiPr refer to O-iPr triester and thiono triester groups, respectively. [b]Chemical shifts, rel. to ext. 25% H_3PO_4 in D_2O, measured for 5'-HO compd at 20°C in 1:1 v/v EtOH-0.1M Tris, pH 7.6 with 1 mg/mL EDTA, except for 13. [c]HPLC of 5'-DMT compd using a Waters C_{18} μBondapak column with a 1%/min linear gradient (G) of CH_3CN (A) vs. 0.1M triethylammonium acetate (B), pH 7 for 10 min at 4 mL/min, then isocratic; initially 20% A. [d]HPLC of 5'-HO compd as described in ftnt c unless specified otherwise. [e]Negative and positive FAB-MS gave the molecular ion. [f]Isocratic using $CH_3CN:H_2O$=13:87, 1.5 mL/min. [g]Isocratic using $CH_3CN:H_2O$=22:78. [h]Initially 5% A, G=3%/min for 5 min then G=0.25%/min. [i]Initially 5% A, G=2%/min. [j]Measured for 5'-DMT compd in ethylenediamine - EtOH (1:1 v/v) containg 30% v/v $CDCl_3$. [k]Initially 30% A, G=2%/min. [l]Initially 30% A, G=1.7%/min. [m]Initially 5% A, G=1.5%/min for 10 min then G=1%/min. [n]Initially 25% A, G=0.25%/min.

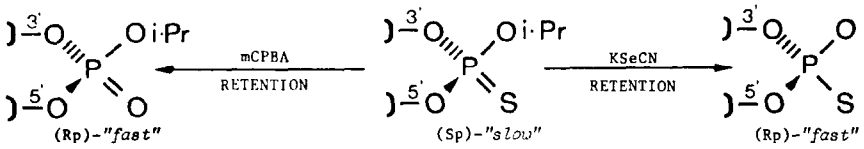
To examine the suitability of 2 and O-Me $N(iPr)_2$ phosphoramidites for automated synthesis of "mixed" triester/diester oligonucleotides, which requires chemoselective O-demethylation with, e.g., PhSH-Et_3N,[7] the two types of amidites were used in the standard synthetic cycle[6,7] for a 3-column DNA synthesizer (Applied Biosystems). Three parallel syntheses of G_*A using 30 min, 3 h, and 8 h times for O-demethylation gave the same yield (HPLC) of this dimer, and thus indicated that the 30 min normally alloted for O-demethylation did not lead to loss of the iPr group. The selection of "mixed" triester/diester synthetic targets was related to our previous studies[3] of the octamer $G_1G_2A_3A_4T_5T_6C_7C$, which contains the recognition sequence (GAATTC) for EcoRI endonuclease: O-iPr at position 2 for "protection" of the scissile bond, and O-iPr at positions 1, 3, and 4 for modification of purported backbone-contacts.[9] Analytical data for these and other products (12-19) are given in Table I. Our reported[3,4] 2-stage "tandem" HPLC purification procedure was employed, and led to the collection of a "cluster" of product peaks for

5'-DMT 13 and 14 (16 possible diastereomers). All 4 diastereomers of 12 were isolated by pairwise separation of 5'-DMT and 5'-HO species, as was previously found[3] for cognate phosphorothioates, $N_{PS}N'N_{PS}N'$. The diastereomers of 15, 16, 18, and 19 were separated as their respec-5'-DMT derivatives, whereas 17 required detritylation before separation was possible.

Selected products were characterized by $^{31}P/^{1}H$ NMR and FAB-MS, and all products were identified by 1.) hydrolysis with 90% formic acid followed by HPLC analysis of base-ratios,[6] and 2.) enzymatic digestions with nuclease P1 and then alkaline phosphatase followed by product analysis by HPLC.[3,4] Application of the latter method to 12 and 18 gave A_*T as the expected, nuclease P1-resistant fragment, whereas 15-17 gave diastereomers of G_*G, G_*A, and A_*A, respectively. The HPLC profile for 13 was not affected by exposure to nuclease P1, which was consistent with the absence of phosphodiester linkages, whereas digestion of 14 gave material with the same HPLC profile as that of 13. In connection with strategies for the separation of isomers, it is worthwhile to note that the diastereomers of A_*T, which were unseparable by HPLC, were obtained via digestions of the HPLC-separated diastereomers of 18.

It was especially interesting to devise a potentially general method for establishing absolute configurations at chiral phosphorus in 1, independent of the nature of the non-bridging alkoxy group. The method summarized in Scheme I for O-iPr is based upon stereoselective conversion of a thiono triester into both the triester of interest and a thioate product whose absolute configuration is either known or can be readily assigned by enzymatic procedures.[3,4]

Scheme I



Scheme I was tested by conducting 2 parallel syntheses for separate oxidation and sulfurization[3] to afford fast- and slow-eluting diastereomers of T_*T (6) and the $T_{P(S)OiPr}T$ (7) counterparts, respectively. Stereoretentive conversion of 7 to 6 using m-chloroperbenzoic acid (mCPBA)[10] [0.1 mL of 0.1M mCPBA/CH_2Cl_2 was reacted with 2 OD_{260} units of 7 in 1 mL CH_3CN at 0°C/10 min then 20°C/10 min] led to transformation of fast-7 into a 76:24 mixture of slow-:fast-6, and an 87:13 mixture of slow-:fast-7 into an 80:20 mixture of fast-:slow-6.

Potassium selenocyanate[11] was studied as a strongly carbophilic nucleophile for chemoselective dealkylation of 7 to give $T_{PS}T$, the absolute configuration of which is known.[12] Pure fast-7 (2 OD_{260} units) was converted (40%) to pure, slow-eluting, (S_p)-$T_{PS}T$ after 4 h at 120°C in CH_3CN (0.5 mL) containing KSeCN (5 mg), and an 87:13 mixture of slow-:fast-7 gave an 87:13 mixture of (R_p)-:(S_p)-$T_{PS}T$. Since the conversion of 7 to $T_{PS}T$ occurs with retention of configuration,[13] our results indicate that the fast-eluting diastereomers of $T_{PS}T$, T_*T (6), and $T_{P(S)OiPr}T$ (7) all have the R_p configuration.

By application of Scheme I to 3-5 and 8-11, it will be possible to determine whether these results found for 6 and 7 hold for other dimers. That the relative mobilities derived from C_{18} HPLC of diastereomeric triester- and thiono triester-containing oligonucleotides cannot, however, be used as a reliable criterion for assignment of absolute configuration was evident from enzymatic digestions of fast-16 which gave fast-4, whereas similar nuclease P1 and then alkaline

phosphatase treatment of fast-19 gave slow-5. Consequently, further studies may show that configurational correlations[3,6] with HPLC elution times and with ^{31}P NMR chemical shifts will obtain only within a strictly homologous family of compounds of the type described herein.

The octamers 14-18 were incubated with EcoRI endonuclease, in a parallel manner, using GGAATTCC as a positive control to establish enzyme activity. It was found (HPLC) that the substrate GGAATTCC underwent 60% conversion to GG and pAATTCC, whereas none of the triester-containing octamers 14-18 underwent detectable amounts of cleavage. These findings indicated that O-isopropylation either at the scissle position or at neighboring positions may be used to "protect" the recognition sequence, as an alternative to the approach using sulfurization.[3,14]

The presently reported studies have shown that 2 affords diastereomeric mixtures of triester- and thiono triester-containing oligonucleotides, which can be separated by HPLC. Moreover, the latter compounds can be used to chemically correlate the absolute configuration of the triester moiety with the enzymatically determined absolute configuration of a phosphorothioate group. Our future investigations of the molecular dynamics, bioorganic chemistry, and biological properties of these oligonucleotide analogues will be reported elsewhere.

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