A stereospecifically ^{18}O-labelled deoxydinucleoside phosphate block for incorporation into an oligonucleotide

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ABSTRACT

Fully protected diastereoisomers of deoxyguanylyl(3' → 5')deoxyadenosine stereospecifically labelled on phosphorus with oxygen-18 have been synthesized by oxidation of phosphite triester intermediates in the presence of ^{18}O-labelled water. The diastereoisomers have been chromatographically separated and their absolute configuration at phosphorus determined. (R_p)-[^{18}O]deoxyguanylyl(3' → 5')deoxyadenosine has been prepared by complete deprotection of the parent diastereoisomer of the S_p configuration. Methylation of the former compound permits assignment of the absolute configurations of the methyl esters of N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine.

INTRODUCTION

The exploitation of nucleotides stereospecifically substituted with sulphur[1] and more recently those labelled with some or all of the stable isotopes of oxygen[2-6] has led to considerable stereochemical understanding and classification of the mechanisms followed by more than 50 phosphoryl transfer reactions to date, catalysed by phosphatases, kinases, mutases, adenyltransferases, diesterases and nucleases. The DNA restriction endonucleases[7] however, a group of more than 350 sequence specific nucleases, remain as yet unexplored with respect to their stereochemical mechanisms. This is primarily due to the lack of a straightforward synthetic method whereby a chemically synthesised oligonucleotide substrate containing the recognition sequence for a restriction endonuclease can be stereospecifically labelled with an oxygen isotope or sulphur at the appropriate specific cleavage point.

We wish to report here a method for the synthesis of stereospecifically labelled fully protected diastereoisomeric deoxydinucleoside phosphate blocks of [^{18}O]-deoxyguanylyl(3' → 5')deoxyadenosine, possessing suitable 5' and 3' blocking groups which can be selectively removed for chain extension in either direction, and are therefore suitable for incorporation into oligonucleotides.

MATERIALS AND METHODS

Nuclease P1 from Penicillium Citrum was obtained from Sigma, Munich as a lyophilized powder. 2'-Deoxyguanosine and 2'-deoxyadenosine were obtained from Pharma Waldhof (Düsseldorf, West Germany). Dimethoxytrityl chloride was obtained from EGA Chemie. N^6-Benzoyldeoxyadenosine and N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine were prepared by standard procedures[11]. Blocked nucleosides were dried for 3 days over P_2O_5 in vacuo before use. Methoxydichlorophosphine was prepared as described[12], and samples (ca. 200 mg) were sealed into ampoules in vacuo. Collidine was dried with calcium hydride and distilled before use. Chloroform was dried before use by stirring over P_2O_5. THF was refluxed and distilled from calcium hydride, then from sodium hydride and stored over $LiAlH_4$. DMF and dioxan were dried over Linde 4A molecular sieves.

^{18}O-enriched water (99 atom %) was obtained from Ventron Ltd. (Karlsruhe, West Germany) and ^{17}O-enriched water (^{16}O, 9.4 %; ^{17}O, 52.8 %; ^{18}O, 37.8 %) from the Monsanto Research Corp., Miamisburg, Ohio, USA.

NMR spectra were recorded on a Bruker WP200SY spectrometer operating at 81.01 MHz with ^1H broad band decoupling. Samples were contained in 5 mm precision tubes with a concentric capillary containing unless stated otherwise trimethyl phosphate as reference. Aqueous samples were measured in 100 mM EDTA adjusted to pH 8 with triethylamine (25 % D_2O), and methyl esters in d_6-DMSO containing 8-hydroxyquinoline. Chemical shifts are given in ppm and are positive when downfield from the appropriate reference.

Tlc was performed on Silica gel 60F plates with solvents as indicated, and preparative layer chromatography on 20 x 20 cm Silica gel 60F plates (Merck, Darmstadt) with solvents as indicated. Column chromatography was performed on Silica gel 60 (230-400 mesh). Ion-exchange chromatography was performed on DEAE-Sephadex A25 (Pharmacia, Sweden) and columns run with gradients of triethylamine bicarbonate pH 8.0. The columns were connected to a Uvicord UV detector and an Ultrorac fraction collector (LKB instruments, Sweden). HPLC was performed on a Waters Associates chromatograph using an anion exchange column (Nucleosil 10SB from Machery and Nagel, Düren, West Germany) with a pH 4.5 buffer, 200 mM in KH_2PO_4 and 300 mM in CH_3COOK as eluant. Nucleotides eluted from the column were detected by a Model 440 absorbance detector operating at 254 nm.

EXPERIMENTAL

Methoxyacetic anhydride

Methoxyacetic acid (90 g, 1 mol) was dissolved in ethylacetate (200 ml) and dicyclohexylcarbodiimide (103 g, 0.5 mol) added. After stirring for 1 h at room temperature the reaction mixture was filtered. The filtrate was distilled first at atmospheric pressure to remove the solvent, then at reduced pressure. Yield 38 g; bp_{12} 112° (Lit.[13] 111-113°C).

N^6-Benzoyl-3'-methoxyacetyldeoxyadenosine (2)

N^6-Benzoyldeoxyadenosine (4.14 g, 11.7 mmol) was prepared from deoxyadenosine (5 g, 20 mmol) in 59 % yield, and converted into N^6-benzoyl-5'-O-dimethoxytrityldeoxyadenosine (5.68 g, 87 %)[11]. To a solution of N^6-benzoyl-5'-O-dimethoxytrityldeoxyadenosine (5 g, 7.6 mmol) dissolved in dry pyridine (30 ml) was added methoxyacetic anhydride (2 ml, 11.3 mmol). After 1 hour with stirring at room temperature tlc ($CHCl_3$:MeOH, 10:1 v/v) showed that reaction was complete. The solution was evaporated to dryness in vacuo. The resulting gum was dissolved in 80 % acetic acid (50 ml) and the reaction mixture kept at room temperature for 3 h. The deblocking reaction was monitored by tlc ($CHCl_3$:MeOH, 10:1 v/v). The pale orange solution was evaporated to dryness and the residue dissolved in chloroform (150 ml) and washed with a 5 % sodium bicarbonate-crushed ice mixture. The organic layer was dried with Na_2SO_4, filtered and evaporated to dryness. The crude product was purified on a column of silica gel by eluting first with $CHCl_3$:MeOH (100:1 v/v, 200 ml), and then 20:1 v/v (300 ml). Pure N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (2.64 g) was isolated in 81 % yield.

(R_p,S_p)-N^2-Isobutyryldeoxyguanylyl(3' → 5')-3'-methoxyacetyl-N^6-benzoyldeoxyguanosine methyl ester (4a/b)

Coupling of N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine (1) and N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (2) was accomplished using a high vacuum line apparatus.

Solutions of N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine (1.07 g, 1.64 mmol) in dry THF (3 ml) and of N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (0.69 g, 1.63 mmol) in dry chloroform (3 ml) were prepared on the vacuum line by distilling the appropriate solvent from a reservoir into a two-necked pear shaped flask equipped with a septum cap.

Dry THF (ca. 30 ml) was transferred from a reservoir attached to the vacuum line to a three-necked flask equipped with a stirrer bar, septum cap, a tap connected to the vacuum line, and an adaptor to which was connected by a short piece of PVC tubing a sealed ampoule of methoxydichlorophosphine

(218 mg, 1.64 mmol). The neck of the ampoule was located inside the tubing. The flask was isolated from the vacuum line, cooled in a CO_2-isopropanol bath and the neck of the ampoule broken to allow the methoxydichlorophosphine to distil into the cold THF. Dry nitrogen was introduced into the flask through the septum cap and dry collidine (450 µl, 3.1 mmol) injected into the stirred solution.

Dry nitrogen was introduced into the flask containing the solution of N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine in THF and this solution removed by syringe and slowly injected over 10 min into the reaction vessel containing the vigorously stirred solution of methoxydichlorophosphine and collidine in THF at -70°C. Subsequently, the solution of N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine was transferred in a similar fashion to the reaction mixture. The reaction mixture was then stirred at 0°C (ice bath) for 2 h, and collidine was added (0.5 ml). A solution of iodine (462 mg, 1.82 mmol) in water (3 ml) and THF (2 ml) was then added at 0°C with stirring, and the mixture stirred at 0°C for a further 30 min. The volume was reduced to ca. 10 ml in vacuo and the dark brown solution mixed with chloroform (100 ml) and extracted with a crushed ice-water mixture. A 5 % solution of sodium bisulphite was then added until the brown color in the chloroform layer disappeared, and this organic layer was then washed with 5 % sodium bicarbonate, dried with Na_2SO_4 and evaporated to give crude product. This material was dissolved in a small volume of chloroform and applied to a 80 g column of silica gel 60 (200-400 mesh) packed in benzene, and pure product was eluted with a $CHCl_3$:benzene:MeOH solvent system (20:7:1, v/v, 800 ml). Fractions containing product were collected, pooled and evaporated to dryness in vacuo to give the title compound as a diastereoisomeric mixture (1.30 g, 58 %). ^{31}P-NMR ($CDCl_3$) s, -3.62, R_p; s, -3.71, S_p; (d_6-DMSO) s, -3.52, S_p; s, -3.74, R_p.

Separation of the diastereoisomers of N^2-isobutyryl-3'-O-dimethoxytrityl (3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (4a/b)

The diastereoisomers of the title compound were dissolved in chloroform and the solution applied to 10 preparative tlc plates with a concentrating zone, which were then developed in the following solvent system: $CHCl_3$:MeOH: ether:H_2O (180:20:60:1, v/v, an ultrasonic bath was used for the mixing of solvents). After four consecutive developments, three fractions were extracted from the plates with $CHCl_3$/MeOH. The "fast moving" zone yielded pure "fast" diastereoisomer (150 mg, 8 %) after short column filtration through silica gel 60 using $CHCl_3$:MeOH (10:1, v/v) as eluant. ^{31}P-NMR ($CDCl_3$) s,

-3.62, R_p diastereomer.

The "slow moving" zone after extraction and chromatography as above gave pure "slow" diastereoisomer (190 mg, 10 %). ^{31}P-NMR ($CDCl_3$) s, -3.71, S_p diastereoisomer.

The middle zone after extraction and chromatography as above gave a mixture of diastereomers (140 mg, 7 %).

(R_p,S_p)-[^{18}O]-N^2-Isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (5a/b)

The diastereoisomers of the title compound were prepared essentially as described for the unlabelled material using the following components: For the coupling, methoxydichlorophosphine (242 mg, 1.8 mmol), N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine (1.19 g, 1.8 mmol), N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (0.75 g, 1.77 mmol), collidine (499 µl); for the oxidation, collidine (550 µl), iodine (512 mg, 2 mmol), $H_2{}^{18}O$ (2 ml, 100 mmol) and THF (5 ml).

After work-up the crude product was purified on a 70 g column of silica gel 60H packed in CCl_4, and the title compound eluted by a mixture of $CHCl_3$:MeOH:ether (9:1:2 v/v) in the partially purified zones "fast" (800 mg) and "slow" (470 mg). ^{31}P-NMR ($CDCl_3$) s, -3.66, R_p diastereomer; s, -3.75, S_p diastereomer.

(R_p)-[^{18}O]-N^2-Isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (5a)

Material from the "fast" zone was further purified on a 70 g medium pressure (2.4 atm) column of silica gel packed in CCl_4, by elution with a mixture of $CHCl_3$:MeOH:ether (8:1:4 v/v) to give pure "fast" diastereoisomer (330 mg, 19 %). ^{31}P-NMR ($CDCl_3$) s, -3.66, R_p diastereoisomer. Addition of corresponding [^{16}O]d[GpA] gave rise to an isotope shift of 3.15 Hz.

(S_p)-[^{18}O]-N^2-Isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (5b)

The material from the "slow" zone was further purified as above to give pure "slow" diastereoisomer (250 mg, 14 %). ^{31}P-NMR ($CDCl_3$) s, -3.75, S_p diastereomer. Addition of corresponding [^{16}O]d[GpA] gave rise to an isotope shift of 3.25 Hz.

Deoxyguanylyl(3' → 5')deoxyadenosine (6)

(R_p,S_p)-N^2-Isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (4a/b) (70 mg, 65 µmol) was suspended in a mixture of dioxan (1 ml), triethylamine (0.5 ml) and thiophenol (0.5 ml). A few drops more of dioxan were added until the mixture

became clear, and the solution was left stirring at room temperature. Tlc $CHCl_3$:MeOH, 9:1 v/v) showed complete reaction. The nucleotide product was precipitated with heptane and the residue triturated twice with heptane and dissolved in 25 % ammonia solution (10 ml) and incubated at 50°C overnight to remove base protecting groups.

After thorough evaporation of solvent in vacuo the residue was dissolved in 80 % acetic acid (10 ml) and the solution stirred for 1/2 h at room temperature. After extraction with ether the solution was evaporated to dryness and the residual d[GpA] purified on a column of DEAE-Sephadex A25 run in a linear gradient of 50-200 mM triethylammonium bicarbonate. Fractions containing product were pooled and evaporated to dryness to yield d[GpA] (25 µmol, 38.8 %) as a glassy triethylammonium salt. This material was >95 % pure by HPLC, coeluted with an authentic sample of d[GpA] and was completely digested by nuclease P1 to deoxyguanosine and dAMP. ^{31}P-NMR (D_2O) s, -4.07.

(R_p,S_p)-N^1-Methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine methyl ester (7a/b)

Deoxyguanylyl(3' → 5')deoxyadenosine (6) (10 µmol) was methylated as previously described for TpT[10] to give N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine methyl ester as a solution in DMSO. ^{31}P-NMR (d_6-DMSO) 2s, -3.67, S_p; -3.68, R_p.

(R_p)-[^{18}O]-Deoxyguanylyl(3' → 5')deoxyadenosine (8a)

(S_p)-[^{18}O]-N^2-Isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (5b) (200 mg, 230 µmol) was deblocked and purified essentially as described for the unlabelled compound to yield the triethylammonium salt of (R_p)-[^{18}O]d[GpA]. This material was >99 % pure by HPLC, coeluted with an authentic sample of d[GpA], and was >95 % ^{18}O-labelled by ^{31}P-NMR. ^{31}P-NMR (D_2O) s, -4.10. Addition of [^{16}O]d[GpA] gave a value for the isotope shift of 2.40 Hz.

Configurational analysis of (R_p)-[^{18}O]guanylyl(3' → 5')adenosine (8a)

a) Hydrolysis of (R_p)-[^{18}O]d[GpA] by nuclease P1 in $H_2{}^{17}O$. [^{18}O]d[GpA] (8a) (60 µmol) derived by complete deprotection of (S_p)-[^{18}O]-N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanylyl(3' → 5')-N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine methyl ester (5b) was dissolved in $H_2{}^{17}O$ (100 µl) and left at room temperature for 30 min. The $H_2{}^{17}O$ was removed in vacuo and the solid residue left on a vacuum line overnight. The residue was then dissolved in $H_2{}^{17}O$ (350 µl) and nuclease P1 (350 µg, ca. 140 units) dissolved in $H_2{}^{17}O$ (50 µl) was then added. The solution in a well sealed flask was incubated at 37°C and the cleavage reaction monitored by HPLC. The reaction

was seen to be essentially complete after 75 min and the solution was then applied to a column of DEAE-Sephadex A25 (20 x 2.5 cm) and the products eluted with a gradient of 750 ml of each of 25-200 mM TEAB. Deoxyguanosine (55.7 µmol) was eluted in fractions 9 to 17 and [^{16}O,^{17}O,^{18}O]dAMP (50.3 µmol) in fraction 43 to 55. The latter fractions were pooled and evaporated to dryness in vacuo, excess triethylammonium bicarbonate being removed by the evaporation of several volumes at methanol. A colourless glass of [^{16}O,^{17}O,^{18}O]dAMP was obtained (83 %).

b) Configurational analysis of (S_p)-[^{16}O,^{17}O,^{18}O]dAMP (9). [^{16}O,^{17}O,^{18}O] dAMP triethylammonium salt (50.3 µmol) was converted to the tri-n-octyl-ammonium salt via the pyridinium salt and cyclised with diphenylphosphoro-chloridate and t-butoxide to the isotopomers of [^{16}O,^{17}O,^{18}O]cdAMP by the method of Jarvest et al.[9]. Purification by DEAE-Sephadex A25 chromatography as described above gave 12.2 µmol (24.2 %) of [^{16}O,^{17}O,^{18}O]cdAMP. After evaporation of solvent in vacuo and conversion to the potassium-18-crown 6 salt this product was methylated using methyl iodide in DMSO according to the method of Jarvest et al.[9].

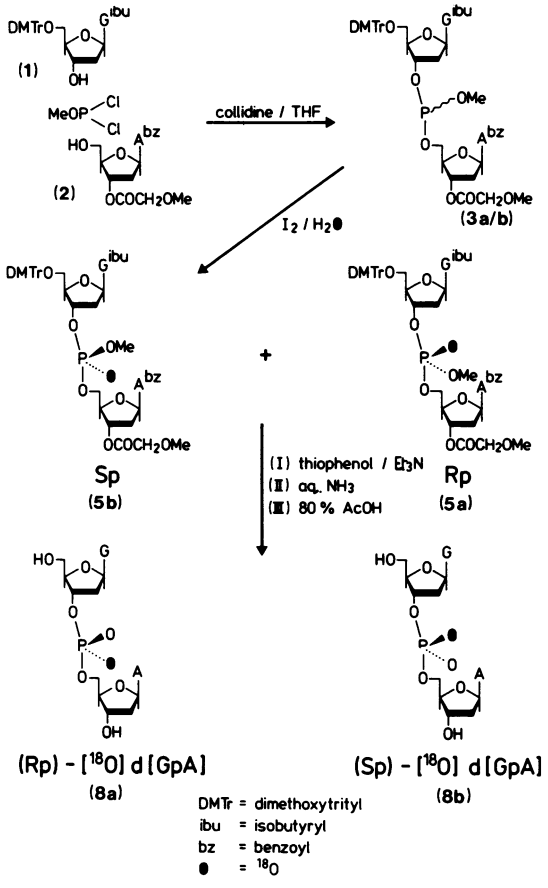
(R_p,S_p)-[^{18}O]-N^1-Methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine methyl ester (10a/b).

(R_p)-[^{18}O]-Deoxyguanylyl(3' → 5')deoxyadenosine (5.2 µmol) was methylated as described for the unlabelled compound. ^{31}P-NMR (d_6-DMSO) s, -3.69, R_p; s, -3.71, S_p.

After mixing with an approximately equal amount of unlabelled material the following isotope shifts were recorded. P-^{18}OMe, 1.35 Hz ± 0.08 Hz; P=^{18}O, 3.25 Hz ± 0.08 Hz.

RESULTS

Coupling of N^2-isobutyryl-5'-O-dimethoxytrityl-2'-deoxyguanosine (1) and N^6-benzoyl-3'-methoxyacetyl-2'-deoxyadenosine (2) by the phosphite method of oligonucleotide synthesis using methoxydichlorophosphine[8] yielded a phosphite triester intermediate as a pair of diastereoisomers (3a/b). Oxidation of this intermediate by iodine but using ^{18}O-labelled water instead of ^{16}O water yielded a mixture of two diastereoisomeric fully protected phosphate triesters (5a/b) containing the ^{18}O label in the P=O bond of each diastereoisomer. Separation of these diastereoisomers by flash chromatography produced two zones, from which pure diastereoisomers were isolated and denoted as "fast" or "slow" according to their chromatographic mobility (Fig. 1).



Removal of the methyl protecting group from the phosphate oxygen by thiophenolate[14,15] with retention of configuration at phosphorus for the "slow" diastereoisomer (5b), followed by removal of the base labile blocking group with ammonia and subsequent deblocking of the 5'-dimethoxytrityl group gave after purification, [^{18}O]-deoxyguanylyl(3' → 5')deoxyadenosine ([^{18}O] d[GpA]) (8a), stereospecifically labelled on one of the phosphate oxygens. The isotopic purity was judged to be >95 % by ^{31}P-NMR (Fig. 2).

Cleavage of this [^{18}O]d[GpA] in ^{17}O-labelled water by nuclease P1 yielded deoxyguanosine and [^{16}O,^{17}O,^{18}O]dAMP (9) of unknown absolute isotopic configuration at phosphorus. Stereochemical analysis of the [^{16}O,^{17}O,^{18}O]dAMP[9] showed it to be of the S_p configuration (Fig. 3, Table 1). Knowing the nuclease P1 cleavage to proceed with inversion of configuration

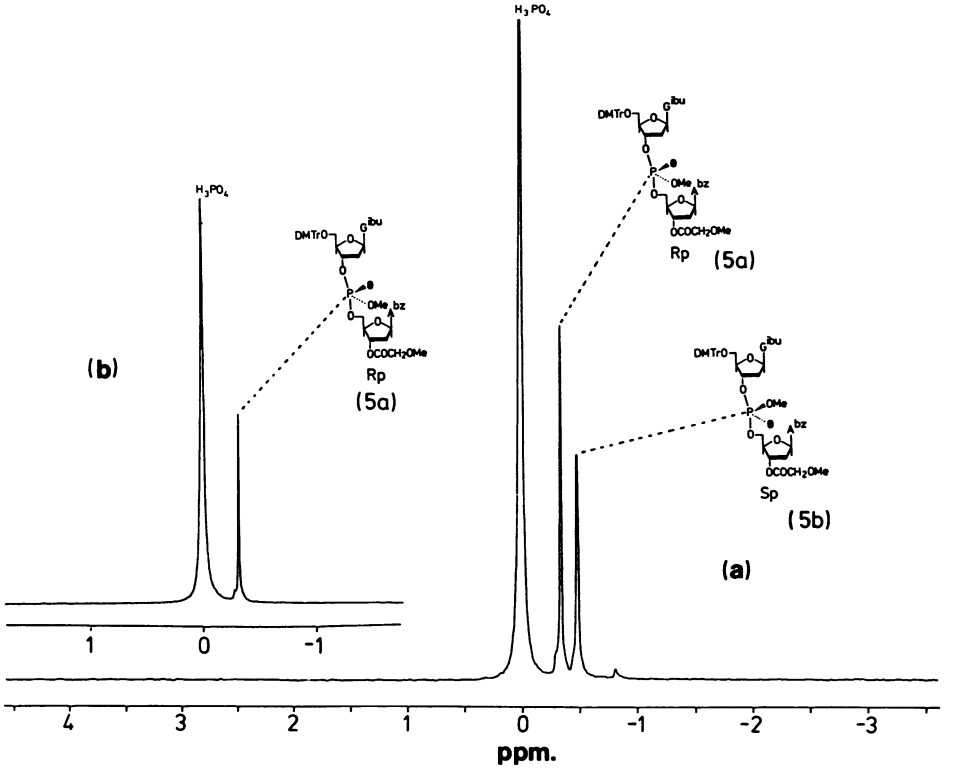


FIGURE 1: ^{31}P-NMR spectra in $CDCl_3$ of (a) the diastereoisomeric mixture of (R_p,S_p)-[^{18}O]-N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanyly(3' → 5')3'-methoxyacetyl-N^6-benzoyldeoxyadenosine (5a/b) obtained from the oxidation with $I_2/H_2{}^{18}O$. (b) the separated R_p-diastereoisomer (5a) from (5a/b). ^{31}P-NMR parameters were: Offset, 1000 Hz; sweep width, 1805 Hz; pulse width, 14 µs; 16K; acquisition time, 4.54s; line broadening, 0.4 Hz. Chemical shifts relative to H_3PO_4, R_p-isomer: -0.340 ppm; S_p-isomer: -0.489 ppm.

at phosphorus[10] the absolute configuration of the chosen isotopomer of [^{18}O]d[GpA] was assigned as R_p. The fully protected "slow" diastereoisomeric precursor was thus assigned the S_p configuration. The correlations of absolute configurations, chromatographic mobilities and ^{31}P-NMR chemical shifts for these compounds are thus as in Table 2. ^{31}P-NMR configurational assignments are reversed by changes of solvent.

Methylation of [^{16}O]d[GpA] (6) produces two signals in the ^{31}P-NMR spectrum corresponding to the two diastereoisomeric methyl esters of N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine (7a/b) (Fig. 4a). The absolute configurations of these diastereoisomers were assigned by

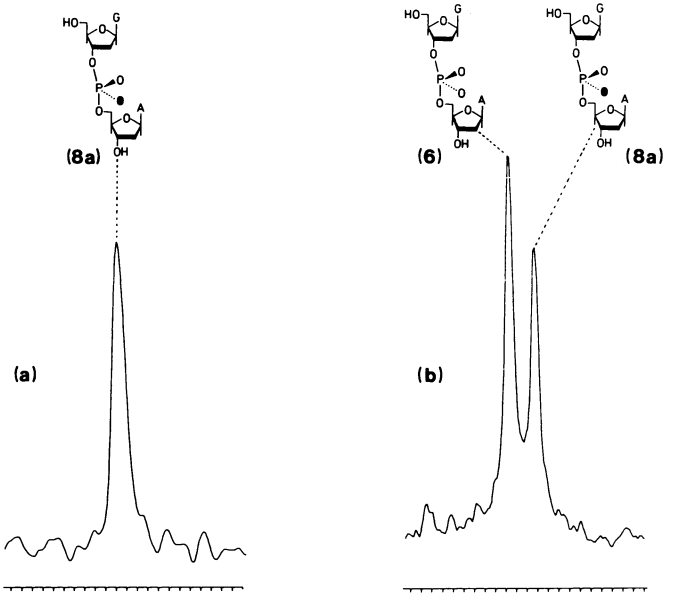
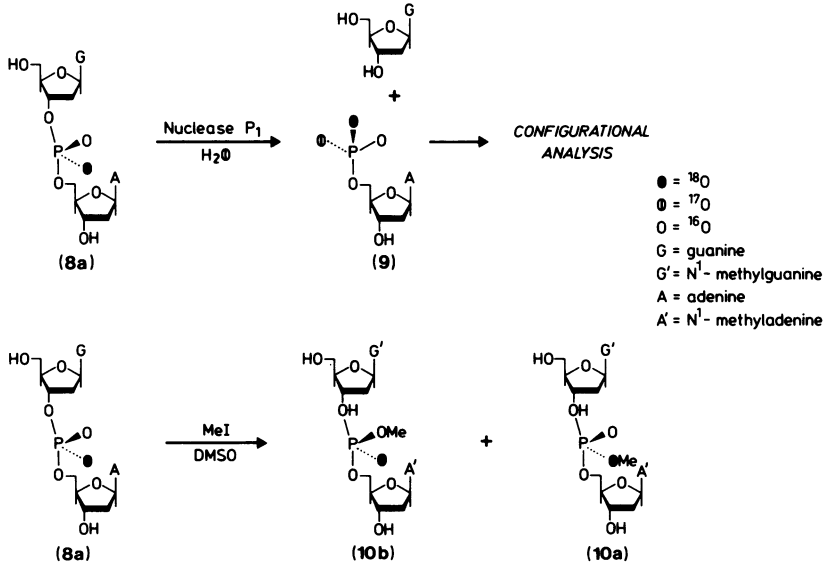


FIGURE 2: ^{31}P-NMR spectra in D_2O/EDTA of (a) 5 mM (R_p)-[^{18}O]d[GpA] (δ, -4.10) (8a) and (b) after addition of [^{16}O]d GpA (δ, -4.07). Isotope shift is 2.40 Hz. ^{31}P-NMR parameters were: Offset, 1000 Hz; sweep width, 1500 Hz; pulse width, 6 μs; 16K; acquisition time, 5.46s; line broadening, 0.4 Hz; no. of transients, (a) 877, (b) 1495; 1 division = 1 Hz.



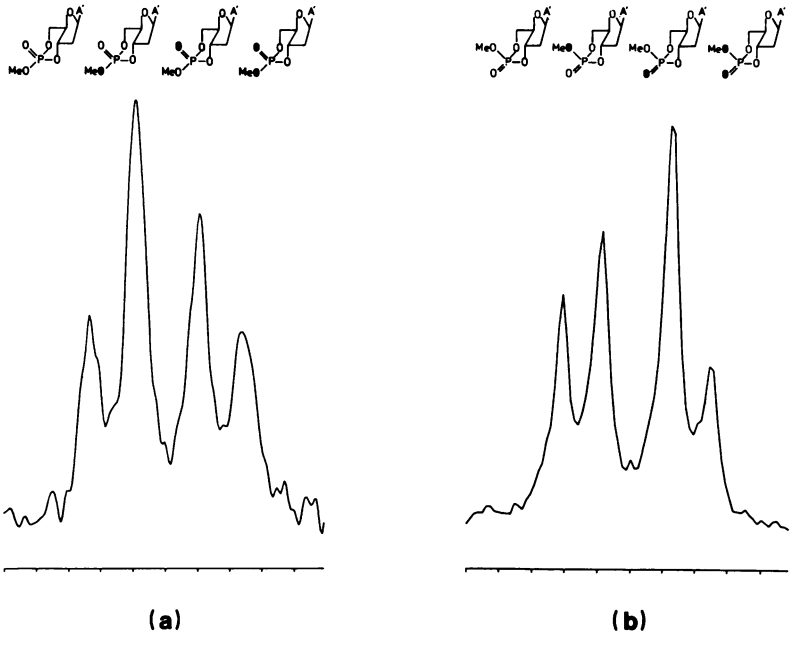


FIGURE 3: ^{31}P-NMR spectra in d_6-DMSO (containing 8-hydroxyquinoline) of (a) the equatorial isotopomers (δ, -3.12, -3.14, -3.16, -3.18) and (b) the axial isotopomers (δ, -4.42, -4.44, -4.47, -4.49) of N^1-methyl cdAMP derived from [^{16}O,^{17}O,^{18}O]dAMP obtained via the nuclease P1 digestion of (R_p)-[^{18}O]d[GpA] (8a). ^{31}P-NMR parameters were: Offset, 1205 Hz; sweep width, 1000 Hz; pulse width, 4 μs; 16K; acquisition time, 8.19s; line broadening, 0.12 Hz; no. of transients, 2309. A' = N^1-methyl cdAMP.

inspection of the ^{31}P-NMR spectrum consequent on addition to the above sample of material resulting from a similar methylation of (R_p)-[^{18}O]d[GpA] (8a) in DMSO (Fig. 4b). The ^{31}P-NMR resonance to low field is assigned to the S_p diastereoisomer (7a), and that to high field to the R_p diastereoisomer (7b) when the spectrum is taken of a DMSO solution.

DISCUSSION

Amongst the enzymes which have been studied with respect to their stereochemical mechanisms are six deoxyribonucleases[1-5]. Four of these catalyse phosphodiester cleavage with inversion of configuration and two with retention of configuration at phosphorus. Further studies within this group are desirable, and moreover, methods whereby the stereochemical approach could be extended to the study of the large number of sequence specific DNA

Table 1. Configurational analysis of (R_p)-[^{18}O]d[GpA] (8a)

	Axial Diastereisomer			Equatorial Diastereoisomer		
	Observed	Calculated for [^{18}O]d[GpA]		Observed	Calculated for [^{18}O]d[GpA]	
		R_p	S_p		R_p	S_p
MeO-P=O	0.52	0.16	0.16	0.48	0.16	0.16
Me^{18}O-P=O	0.71	0.53	1.00	1.00	1.00	0.53
MeO-P=^{18}O	1.00	1.00	0.53	0.72	0.53	1.00
Me^{18}O-P=^{18}O	0.39	0.38	0.38	0.45	0.38	0.38

The observed relative peak intensities of the ^{31}P-NMR resonances (from Fig. 3) of the isotopomers of the diastereoisomeric triesters obtained via the cyclisation and methylation of [^{16}O,^{17}O,^{18}O]dAMP (9) derived from the nuclease P1 catalyzed hydrolysis of (R_p)-[^{18}O]d[GpA] are compared with the calculated values for the hydrolysis of both isotopomers with inversion of configuration at phosphorus. These values were calculated on the basis of the following assumptions: (R_p)-[^{18}O]d[GpA] starting material contained ^{16}O, 5.0 %; ^{18}O, 95.0 % and the hydrolysis reaction with nuclease P1 was performed in water of the following isotopic composition: ^{16}O, 9.4 %; ^{17}O, 52.8 %; ^{18}O, 37.8 %.

restriction endonucleases would be of great interest in opening this field of enzymes to investigation. Despite the fact that restriction endonucleases are able to recognise and cleave small pieces of synthetic DNA containing appropriate sequences[16,17,18], the absence of a suitable method for specific labelling of the cleavage sites with sulphur or stable isotopes of oxygen has precluded the possibility of stereochemical investigations. A method whereby the NMR active ^{17}O nucleus could be specifically incorporated at certain sites in an oligonucleotide would also be extremely useful.

It has been recently demonstrated that deoxydinucleoside phosphates[10] and ribodinucleoside phosphates[19] can be stereospecifically labelled with either ^{18}O or ^{17}O isotopes on phosphorus, respectively. In the first case this was achieved by the stereospecific replacement of a sulphur atom in the appropriate separated diastereoisomers of the deoxydinucleoside phosphorothioate, and in the second case by the oxidation of a phosphite intermediate in the presence of ^{17}O labelled water. Both methods lead to labelled dinucleoside phosphates which are intrinsically applicable to the investigation of non-specific ribo- and deoxyribonuclease reactions. In the case

Table 2. Comparison of data for deoxy- and ribo-series

Dinucleoside Phosphate	Protected Diastereomer Absolute Configuration	Mobility on Silica gel	Protected Diastereomer ^{31}P-NMR Chemical Shift (in $CDCl_3$)	Deblocked Isotopomers Absolute Configuration
[^{18}O]d[GpA]	R_p	"fast"	-3.62	S_p
	S_p	"slow"	-3.71	R_p
[^{17,18}O]UpA[19]	R_p	"fast"	+0.71*	S_p
	S_p	"slow"	+0.50*	R_p

*relative to 85 % H_3PO_4

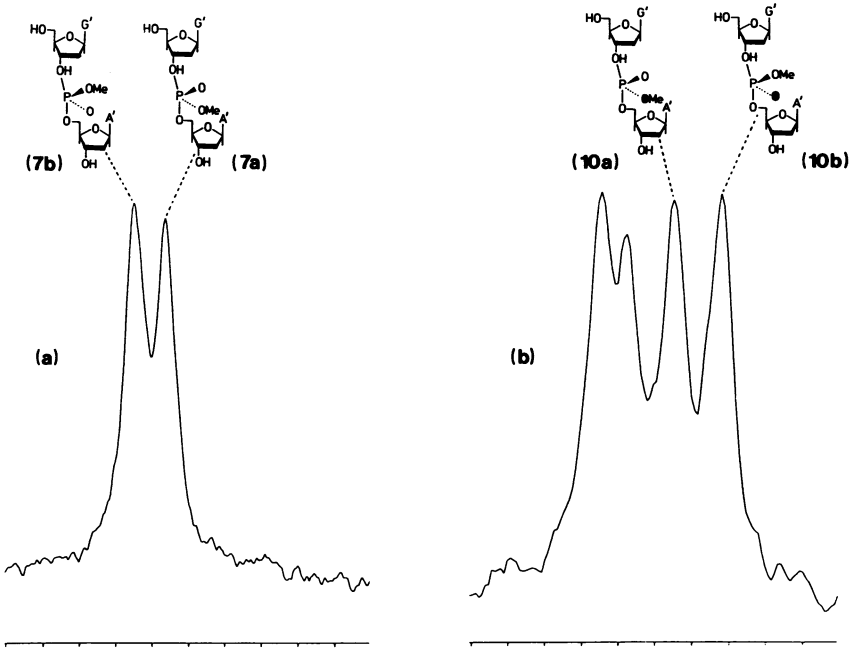


FIGURE 4: ^{31}P-NMR spectra in d_6-DMSO (containing 8-hydroxyquinoline) of (a) 5 mM N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine methyl ester (7a/b; δ, -3.67 and -3.62) and (b) after addition of the methyl esters (10a/b); δ, -3.69 and -3.71) resulting from methylation of (R_P)-[^{18}O]d[GpA] (8a). ^{31}P-NMR parameters were: Offset, 1500 Hz; sweep width, 700 Hz; pulse width, 7μs; 16K; acquisition time, 11.7s; line broadening, 0.09 Hz; no. of transients, (a) 695, (b) 298.

of the sulphur replacement method, however, the labelled product possesses no protecting groups on the base, sugar or phosphate moieties, and the phosphite oxidation method, which relies on the separability of the resulting diastereoisomers, has only so far been demonstrated for the ribodinucleoside phosphate series, and moreover, only with the use of silyl protecting groups at centres which could not be subsequently selectively deblocked. These methods are clearly inapplicable to the more ambitious aims of the stereospecific labelling of oligonucleotides.

We wished to demonstrate that labelling by the phosphite oxidation procedure was also applicable to the deoxydinucleoside phosphate series, that the diastereomers could be separated and that intermediate blocks could be prepared which could be selectively deblocked to permit further chain extension. Since the restriction endonuclease EcoRI has been particularly well characterised with respect to its interaction with small DNA substrates

[16,18] and cleaves between guanosine and adenosine in the recognition sequence, we chose the synthesis of the fully protected stereospecifically ^{18}O-labelled diastereoisomers of d[GpA]. As an acid sensitive 5' blocking group we chose the dimethoxytrityl group, and for a 3' blocking group sensitive to weak base the methoxyacetyl group[20].

Similarly to Seela et al.[19] who oxidised the phosphite precursor of a silylated ribodinucleoside phosphate with ^{17}O-labelled water, we oxidised the phosphite intermediate generated from the coupling of N^2-isobutyryl-5'-O-dimethoxytrityldeoxyguanosine (1) and N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (2) with methoxydichlorophosphine. The ^{31}P-NMR spectrum of the resulting approx. 3:2 mixture of labelled diastereoisomers is shown in Fig. 1. After separation of the diastereoisomers on preparative plates or by flash chromatography, ^{31}P-NMR spectroscopy (Fig. 1) demonstrated that they were >95 % diastereoisomerically pure. The diastereoisomer with the "fast" mobility on silica gel was seen to resonate to lower field than that with the "slow" mobility when the spectrum was recorded in chloroform. The assignments established in this work are shown on the spectrum.

Removal of the methyl group on the phosphate bridge of the "slow" diastereoisomer with retention of configuration by treatment with thiophenolate[14,15], and complete deblocking of other groups gave, after purification, [^{18}O]d[GpA] of unknown absolute isotopic configuration at phosphorus. The ^{31}P-NMR spectrum of this product is shown in Fig. 2. A single peak is observed, and when the same quantity of [^{16}O]d[GpA] (6) is added to the sample a new peak is seen to appear in a downfield position, the two peaks being separated by 2.4 Hz, the value of the ^{18}O isotope shift for this type of labelled molecule[19]. Whereas in the report of Seela et al.[19] considerable isotopic washout was observed in the product after the oxidation step it can be seen from Fig. 2 that in this case the [^{18}O]d[GpA] is >95 % isotopically pure, and thus careful use of a high vacuum line procedure can lead to a product with excellent labelling.

The assignment of absolute isotopic configuration for this type of molecule has been well described for [^{18}O]TpT[10] and [$^{17,18}O$]UpA[19] and will not be discussed further here. [^{18}O]d[GpA] was cleaved by nuclease P1 in ^{17}O-labelled water as shown in Scheme 2 to yield deoxyguanosine and [^{16}O,^{17}O,^{18}O]dAMP (9). Cyclisation and methylation of the latter according to the method of Jarvest et al.[9] and examination of the resulting product by ^{31}P-NMR showed that the [^{16}O,^{17}O,^{18}O]dAMP had the S_p configuration. The spectra are shown in Fig. 3 and are correlated with the calculated data in

Table 1. Since the nuclease P1 cleavage reaction has been shown to involve inversion of configuration at phosphorus[10], we assigned the R_p absolute configuration to this [^{18}O]d[GpA] (8a). Thus the S_p absolute configuration is assigned to the fully protected "slow" diastereoisomeric precursor resonating to high field in the ^{31}P-NMR spectrum. (Although the deprotection reaction proceeds with retention of configuration at phosphorus the R,S sequence rules require that the precursor has the opposite absolute configuration to that of the deblocked product, although no change in stereochemistry at this centre has occurred.) It is to be noted that the correlations set out in Table 2 are all in the same sense as those previously described for the labelled ribodinucleoside phosphates prepared by Seela et al.[19].

Methylation of d[GpA] (6) using methyl iodide gave rise to the two ^{31}P-NMR signals shown in Fig. 4a corresponding to the two diastereoisomers of N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine (7a/b). Assignment of these resonances became possible after addition of an equal amount of labelled material which had been prepared by a similar methylation of (R_p)-[^{18}O]d[GpA] (8a). Two new peaks appeared upfield in the ^{31}P-NMR spectrum corresponding to two isotope shifts (Fig. 4b). Measurement of the separation of the peaks gave two possible sets of values for the isotope shifts, namely 2.03 and 2.64 ± 0.09 Hz or 1.35 and 3.22 ± 0.09 Hz. Only the latter values are reasonable for bridging and double bond shifts respectively[19], and thus the low field resonance in Fig. 4a is associated with a large isotope shift and the signal to high field with a small isotope shift when methylated material derived from (R_p)-[^{18}O]d[GpA] (8a) is present. Thus, the ester resonating at low field has the ^{18}O-label in the doubly bonded position, and the ester at high field has the label in the bridging position when the precursor is labelled in the pro R_p position. The absolute configurations of these N^1-methyldeoxyguanylyl(3' → 5')N^1-methyldeoxyadenosine methyl esters thus follow as S_p for the ester resonating at low field (7b), and R_p for the ester at high field (7a), and are assigned in Fig. 4a. These assignments are in the same sense as those made for TpT methyl ester[10] and for uridylyl(3' → 5')N^1-methyladenosine methyl ester[19].

It is also of interest to note that the configurational assignments made to the ^{31}P-NMR spectra of the methyl esters of the fully protected d[GpA] (4a/b) are solvent dependent, as are presumably those of the methyl esters of the deblocked compounds. When the ^{31}P-NMR spectrum of an unequal mixture of the two diastereoisomers of N^2-isobutyryl-5'-O-dimethoxytrityl (3' → 5')N^6-benzoyl-3'-O-methoxyacetyldeoxyadenosine (4a/b) is recorded in

denteriated chloroform, the R_p diastereoisomer resonates downfield with respect to the S_p diastereoisomer. This was also observed for the fully protected diastereoisomers of UpA[19] but was not further investigated. Exchange of the solvent, however, for d_6-DMSO clearly reverses the order and the S_p diastereoisomer now resonates downfield. Such information was not recorded for the esters of UpA, although it was observed that the NMR configurational assignments of the fully protected methyl esters and those of the re-methylated deblocked material were opposite in different solvents.

Configurational assignments for methyl esters generated by methylation using methyl iodide have been made in the deoxy-series for TpT in d_6-DMSO and for the ribo-series for UpA in DMSO-methanol. In both cases the S_p diastereoisomer was shown to resonate to lower field than the R_p. Now that it is clear from this work that configurational assignments of ^{31}P-NMR resonances can be reversed by solvent changes, the uniform recording of such spectra in DMSO or DMSO-based solvent mixtures may prove to be a valuable criterion of absolute configuration for methyl esters of dinucleoside phosphates.

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