Synthesis and Configurational Analysis of a Dinucleoside Phosphate Isotopically Chiral at Phosphorus. Stereochemical Course of *Penicillium citrum* Nuclease P1 Reaction†

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ABSTRACT: (R_P)- and (S_P)-5′-*O*-thymidyl 3′-*O*-thymidyl [^{18}O]phosphates have been synthesized by reaction of the respective (S_P)- and (R_P)-phosphorothioate precursors with *N*-bromosuccinimide in dioxane and $H_2{}^{18}O$. Stereochemical analysis of the product derived from the (R_P)-phosphorothioate by digestion with snake venom phosphodiesterase in $H_2{}^{17}O$ and examination of the isotopic chirality of the resulting thymidine 5′-[^{16}O,^{17}O,^{18}O]phosphate demonstrate that the replacement reaction has proceeded with inversion of configuration at phosphorus. Inspection of the ^{31}P NMR spectrum of the methyl esters prepared from (S_P)-5′-*O*-thymidyl 3′-*O*-thymidyl [^{18}O]phosphate confirms that the replacement reaction has proceeded with very little if any racemization. This spectrum also allows the assignment of the absolute configuration of these methyl triesters. (R_P)-5′-*O*-Thymidyl 3′-*O*-thymidyl [^{18}O]phosphate has been used to demonstrate that the stereochemical course of the hydrolytic reaction catalyzed by nuclease P1 from *Penicillium citrum* proceeds with inversion of configuration at phosphorus and therefore probably does not involve the participation of a covalent enzyme intermediate.

Phosphorothioate analogues of nucleotides and nucleotides specifically labeled with isotopes of oxygen have proved to be indispensable in the elucidation of the stereochemical courses of enzyme-catalyzed phosphoryl and nucleotidyl transfer reactions (Knowles, 1980; Eckstein et al., 1982; Frey, 1982). Recently methods have been reported describing the stereospecific replacement of sulfur in nucleoside phosphorothioates by the isotopes of oxygen, making nucleotides stereospecifically ^{17}O or ^{18}O labeled at phosphorus more easily accessible. Such procedures have so far afforded the syntheses of [α-^{18}O]ADP and [α-^{18}O]ATP, [^{18}O]cAMP, [β-^{18}O]ATP, and [α-^{17}O]ATP (Sammons & Frey, 1982; Connolly et al., 1982; Lowe et al., 1982), and [α-^{18}O]GDP and [α-^{18}O]GTP (P. D. Senter, F. Eckstein, A. Mülsch, and E. Böhme, unpublished results).

Since phosphorothioate analogues of nucleotides are sometimes poor substrates for enzymes, the availability of di- or oligonucleotides stereospecifically labeled by the isotopes of oxygen at phosphorus would increase the number of enzymes, in particular nucleases, for which the stereochemical course of the reaction can be investigated. In addition, stereospecific introduction of the NMR[1] active oxygen-17 nucleus should provide a useful environmental probe for conformational and binding studies as well as facilitating investigations of metal–nucleotide interactions by EPR spectroscopy (Reed & Leyh, 1980; Eccleston et al., 1981).

As a first step in this direction we report here the synthesis and configurational analysis of (R_P)- and (S_P)-5′-*O*-thymidyl 3′-*O*-thymidyl [^{18}O]phosphate ([^{18}O]TpT) from (S_P)- and (R_P)-5′-*O*-thymidyl 3′-*O*-thymidyl phosphorothioate [Tp(S)T]. We also demonstrate the use of such substrates by the determination of the stereochemical course of the reaction catalyzed by nuclease P1 from *Penicillium citrum*.

Materials and Methods

Crotalus durissus terrificus snake venom phosphodiesterase was obtained from Boehringer Mannheim GmbH as a suspension in 50% glycerol, pH 6.0, and had a specific activity of 52 units/mg with UDP-glucose as substrate. Nuclease P1 from *Penicillium citrum* was obtained from Boehringer Mannheim GmbH as a lyophilized powder with a specific activity of 430 units/mg of protein. Triisopropylbenzenesulfonyl chloride was obtained from Aldrich Europe (Nettetal, West Germany) and was recrystallized from pentane in the presence of thionyl chloride (Seth & Jay, 1980) before use. 3′-*O*-Acetylthymidine was purchased from Serva (Heidelberg, West Germany). 5′-*O*-Monomethoxytritylthymidine was prepared as previously described (Schaller et al., 1963). TpT was obtained form Sigma (München, West Germany).

^{18}O-enriched water (99 atom %) was obtained from Ventron (Karlsruhe, West Germany). ^{17}O-enriched water (12.5% ^{16}O, 52.4% ^{17}O, 35.1% ^{18}O) was obtained from the Monsanto Research Corp., U.S. Department of Energy. High-pressure liquid chromatographic analyses were performed on Waters Associates chromatographs by using either 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 or a reverse-phase octadecyl column (ODS-Hypersil, Shandon Southern Products Ltd., Runcorn, Great Britain) with 20 mM KH_2PO_4, pH 6, containing 15% CH_3OH. Nucleotides eluted from the column were detected by a Model 440 absorbance detector operating at 254 nm.

^{31}P NMR spectra were recorded on a Bruker WP200SY spectrometer operating at 81.01 MHz with ^{1}H broad band decoupling. Samples were contained in 5-mm precision tubes with a concentric capilliary containing 85% H_3PO_4 as reference. Aqueous samples were recorded in 100 mM EDTA adjusted to pH 8 with triethylamine (25% D_2O), and methyl esters in a 1:1 mixture of Me_2SO-d_6–Me_2SO containing 8-

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[1] Abbreviations: (R_P)- and (S_P)-Tp(S)T, the R_P and S_P diastereoisomers of 5′-*O*-thymidyl 3′-*O*-thymidyl phosphorothioate; (R_P)- and (S_P)-[^{18}O]TpT, the R_P and S_P isotopomers of 5′-*O*-thymidyl 3′-*O*-thymidyl [^{18}O]phosphate; d[Tp(S)A], 5′-*O*-(2-deoxyadenosyl) 3′-*O*-thymidyl phosphorothioate; cAMPS, adenosine cyclic 3′,5′-phosphorothioate; ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); GDPαS, guanosine 5′-*O*-(1-thiodiphosphate); DMF, dimethylformamide; Me_2SO (DMSO in Scheme I), dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TEAB, triethylammonium bicarbonate; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance spectroscopy.

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hydroxyquinoline. Chemical shifts are given in ppm and are positive when downfield from H_3PO_4.

Synthesis of (R_PS_P)-Tp(S)T and TpT. These compounds were prepared by the method used to synthesize (R_P)- and (S_P)-d[Tp(S)A] and d[TpA] (Romaniuk & Eckstein, 1982). The starting nucleosides were 5′-*O*-monomethyoxytrityl-thymidine (1 mmol) and 3′-*O*-acetylthymidine (1.2 mmol). The protocol given was followed with the following modifications. The fully protected dinucleoside 3′,5′-phosphorothioate was not purified by silica gel chromatography, but the 5′-*O*-monomethoxytrityl protecting group was immediately removed by dissolving the product in 40 mL of ice-cold glacial acetic acid containing 10 mL of water. After 4 h on ice the reaction appeared complete as judged by silica gel TLC using $CHCl_3$–CH_3OH (9:1 v/v) as eluant (R_f of monomethoxytrityl positive material changed from 0.5 to 1.0). The mixture was dried down and further evaporated from pyridine (2 × 25 mL) and chloroform (2 × 25 mL) to yield a yellow oil. The product was partially purified by chromatography over a 25 × 2.5 cm column packed with silica gel (0.040–0.063-mm particle size). The column was developed with 250 mL of $CHCl_3$–CH_3OH (99:1 v/v) followed by 250 mL of $CHCl_3$–CH_3OH (97:3 v/v) under a positive nitrogen pressure of 0.5 atm. The product eluted between 50 and 100 mL of the second solvent which was evaporated to give a white foam. Silica gel TLC using $CHCl_3$–CH_3OH (9:1 v/v) showed the product to be about 80% pure (R_f = 0.3) with 3′-*O*-acetylthymidine as the main contaminant (R_f = 0.4).

The cyanoethyl and 3′-acetyl protecting groups were removed sequentially as described (Romaniuk & Eckstein, 1982). The solution of fully deblocked product was evaporated to dryness, the residue dissolved in 50 mL of 10 mM TEAB, pH 8, and the resulting solution clarified by filtration through coarse filter paper. The solution was applied to a column of DEAE-Sephadex A-25 (25 × 2.5 cm) which was developed with a linear gradient of 1 L each of 10 and 150 mM TEAB, pH 8. TpT (120 μmol, 12%) was eluted at ca. 60 mM buffer and a mixture of the diastereomers of Tp(S)T (500 μmol, 50%) at ca. 120 mM buffer. The TpT was ≥95% pure by reverse-phase HPLC and additionally coeluted with a commercial sample of TpT. Both the prepared and the commercial samples of TpT had identical UV spectra (λ_{max} 267 nm, λ_{min} 233 nm) and ^{31}P NMR spectra (δ 1.2). The diastereomers of (R_PS_P)-Tp(S)T appeared ≥95% pure in the reverse-phase HPLC system with retention times of 5.5 and 7.5 min. The isomer with the shorter retention time is the R_P isomer (^{31}P NMR, δ 55.87), the other the S_P isomer (^{31}P NMR, δ 55.56) (Barlett & Eckstein, 1982). The UV spectrum of the isomeric mixture of Tp(S)T was identical with that of commercial TpT. Both HPLC and ^{31}P NMR showed that the two diastereomers were present in an approximately equal ratio.

Separation of the Diastereomers of Tp(S)T. (1) DEAE-Sephadex A-25 Chromatography. A 1:1 mixture (250 μmol) of the R_P and S_P isomers of Tp(S)T was applied to a column (95 × 5.5 cm) of DEAE-Sephadex A-25 equilibrated with 100 mM TEAB, pH 8. The column was developed with a linear gradient composed of 3 L each of 100 and 300 mM TEAB. Fractions of 15 mL were collected, and the two diastereomers were eluted as a broad peak centered around fraction 300. Individual fractions were analyzed by reverse-phase HPLC. On the basis of this analysis, fractions 277–297, 298–302, and 303–327 were separately pooled and evaporated to dryness in vacuo. Analysis of the three pools by reverse-phase HPLC showed that the first consisted of 101 μmol of a 80:20 mixture of the R_P and S_P diastereomers, respectively, the second 34 μmol of a 50:50 mixture of both diastereomers, and the third 105 μmol of a 20:80 mixture of the R_P and S_P diastereomers.

(2) Semipreparative HPLC. Tp(S)T (50 μmol) (80% R_P diastereomer, 20% S_P) was applied to a Waters 1-in. semipreparative column (2.5 × 30 cm) packed with Waters preparative C_{18} resin. The column was equilibrated with 100 mM triethylammonium acetate, pH 7, containing 7.5% acetonitrile, and products were eluted isocratically with this buffer at a flow rate of 2 mL min^{-1}. Fractions of 10 mL were collected, and two UV-absorbing peaks were eluted. The first between fractions 42 and 52 consisted of 39 μmol of (R_P)-Tp(S)T (isomeric purity ≥95%), and the second between fractions 55 and 67 contained 9 μmol of (S_P)-Tp(S)T (isomeric purity ≥95%) as judged by reverse-phase HPLC (Figure 1). Tp(S)T consisting of 80% of the S_P diastereomer and 20% of the R_P diastereomer could be also resolved similarly into two pools of approximately 95% isomeric enrichment. After elution from this column the appropriate fractions were pooled and evaporated to dryness in vacuo, and most of the triethylammonium acetate was removed by alternate multiple additions and evaporations of methanol and toluene. Final traces of acetic acid were removed by chromatography on DEAE-Sephadex A-25 (10 × 2 cm column) and elution with a gradient consisting of 500 mL each of 10 and 150 mM TEAB.

Synthesis of (R_P)-[^{18}O]TpT and (S_P)-[^{18}O]TPT. A solution of 100 μmol of (R_P)-Tp(S)T (diastereomeric purity 95%) was evaporated to dryness in vacuo and the residue dried by addition and evaporation of anhydrous ethanol (2 × 1 mL). Two volumes of 0.5 mL of dioxane containing 0.1 mL of a 25:75 mixture of H_2O and $H_2{}^{18}O$ were then added and evaporated. The residue obtained was dissolved in 2 mL of dioxane containing 0.4 mL of water of the above composition, and *N*-bromosuccinimide (71 mg, 400 μmol) was added. After 2 min at room temperature, 50 μL of 2-mercaptoethanol was added, followed 1 min later by 15 mL of 10 mM TEAB, pH 8. The pH of the solution was adjusted to pH 8 with triethylamine and the product purified by DEAE-Sephadex A-25 chromatography (20 × 2.5 cm column) with a linear gradient of 1 L each of 10 and 100 mM TEAB. The fractions containing product, which eluted at around 60 mM buffer, were pooled and evaporated to dryness in vacuo. The yield of product in such a reaction varied between 60 and 70%. Reverse-phase HPLC showed that the product was greater than 85% pure and also coeluted with a commercial sample of TpT. ^{31}P NMR spectroscopy (Figure 2A) showed two peaks at 1.23 and 1.20 ppm assigned to TpT and [^{18}O]TpT, respectively. As is discussed later the [^{18}O]TpT prepared from (R_P)-Tp(S)T has the S_P configuration. (R_P)-[^{18}O]TpT was synthesized in an identical fashion from (S_P)-Tp(S)T with 99% enriched $H_2{}^{18}O$. The ^{31}P NMR spectrum (Figure 2B) shows that the [^{18}O]TpT (δ 1.20) contains <5% TpT. The ^{31}P NMR spectra of both the (S_P)- and (R_P)-[^{18}O]TpT additionally showed other resonances, accounting for approximately 15% of the total intensity, between 0 and 1 ppm.

Configurational Analysis of (S_P)-[^{18}O]TpT. [^{18}O]TpT (ca. 40 μmol) prepared from (R_P)-Tp(S)T was thoroughly dried by the evaporation of dry methanol, the residue dissolved in $H_2{}^{17}O$ (100 μL), and the solution evaporated to dryness. This procedure was repeated, and the residue was dissolved in $H_2{}^{17}O$ (400 μL). Tris buffer (1 M), pH 9.2, was added (28 μL), followed by 1 M $MgCl_2$ (3 μL) and snake venom phosphodiesterase (100 μL, ca. 5 units). This mixture was incubated at 37 °C and the hydrolysis to thymidine and [^{16}O,^{17}O,^{18}O]-TMP monitored by anion-exhange HPLC. After 9 h the reaction was approximately 80% complete, and the mixture

was left overnight at room temperature. The mixture was then diluted with 25 mM TEAB and applied to a 20 × 1.5 cm column of DEAE-Sephadex A-25, and the products were eluted by a linear gradient of 25–200 mM TEAB, pH 8.0 (750 mL of each). Fractions of 9 mL were collected, fractions 6–11 yielding thymidine (24 μmol), fractions 33–39 unreacted (S_P)-[^{18}O]TpT (4 μmol), and fractions 58–64 [^{16}O,^{17}O,^{18}O]-TMP (22 μmol).

The [^{16}O,^{17}O,^{18}O]TMP so produced was converted to the tri-*n*-octylammonium salt and cyclized with diphenyl phosphorochloridate and potassium *tert*-butoxide (Jarvest et al., 1981). Purification by DEAE-Sephadex A-25 chromatography gave 5.3 μmol (25%) of pure [^{16}O,^{17}O,^{18}O]cTMP. This product was then methylated with methyl iodide in Me_2SO exactly as described for the methylation of cAMP (Jarvest et al., 1981) and TpT below. The ^{31}P NMR spectrum obtained is shown in Figure 3. A ^{31}P NMR spectrum of unlabeled cTMP methyl ester is shown in Figure 4 [δ −3.38 (s) equatorial diastereomer; δ −4.62 (s) axial diastereomer].

Synthesis of the Methyl Esters of TpT and (S_P)-[^{18}O]TpT. TpT (triethylammonium salt) (5 μmol) in water (10 mL) was treated with Dowex 50X-W (1 mL, potassium form) for 15 min. The mixture was filtered from the resin, and the filtrate and washings were evaporated to a small volume. 18-Crown-6 was added (10 mg, 38 μmol) and the solution evaporated to dryness. The residue was dissolved in dry DMF (5 mL) which was evaporated under anhydrous conditions. This was repeated 3 times. The residue was finally dissolved in a 1:1 mixture of Me_2SO-d_6–Me_2SO (400 μL), and methyl iodide (100 μL) was added. After the mixture had been stirred for 12 h, the methyl iodide was removed in vacuo, to give (R_PS_P)-5′-*O*-thymidyl 3′-*O*-thymidyl phosphate methyl ester as a solution in Me_2SO. To the solution were added a few crystals of 8-hydroxyquinoline, the solution was filtered, and the ^{31}P NMR spectrum was recorded [Me_2SO-d_6–Me_2SO, 50:50 v/v; δ 0.60 (s) and δ 0.69 (s)] (Figure 5). The S_P isomer of [^{18}O]TpT (5 μmol) was methylated in an identical fashion. Essentially all the TpT was esterified by this procedure.

K_m and V_{max} Determination for TpT with Nuclease P1. To 10 mM Mes–KOH,pH 6 (total volume 200 μL), containing TpT (concentration varied between 0.3 and 2 mM) was added 3.5 μg of nuclease P1. The mixture was incubated at 37 °C, and 25-μL aliquots were withdrawn after 3, 6, and 9 min. These aliquots were quenched into 25 μL of ice-cold 0.2 M HCl (C_2H_5OH–H_2O, 2:8 v/v). Aliquots were assayed for residual substrate and the products thymidine and TMP by anion-exchange HPLC. The degree of hydrolysis was obtained by integration of the substrate and product peaks. Kinetic parameters were determined by plotting substrate concentration/velocity vs. substrate concentration.

Hydrolysis of (R_P)-[^{18}O]TpT by Nuclease P1 in $H_2{}^{17}O$. A solution of 40 μmol of (R_P)-[^{18}O]TpT was evaporated to dryness in vacuo and the resulting gum dried down twice from 1 mL of anhydrous ethanol and dissolved in 0.4 mL of $H_2{}^{17}O$. Nuclease P1 (140 μg dissolved in 10 μL of H_2O) was added to the substrate solution. The pH of the mixture was 6. Anion-exchange HPLC indicated complete conversion to [^{16}O,^{17}O,^{18}O]TMP and thymidine after 20 min at 37 °C. The mixture was immediately applied to a DEAE-Sephadex A-25 (20 × 2.5 cm) column which was developed with a gradient of 800 mL each of 10 and 20 mM TEAB. Fractions containing [^{16}O,^{17}O,^{18}O]TMP, which eluted around 130 mM buffer, were pooled and evaporated to dryness in vacuo; 30 μmol of [^{16}O,^{17}O,^{18}O]TMP was obtained, and the product was ≥98% pure in the anion-exchange HPLC system and additionally coeluted with standard TMP.

Configurational Analysis of [^{16}O,^{17}O,^{18}O]TMP from the Nuclease P1 Reaction. This was performed by cyclization and methylation of the [^{16}O,^{17}O,^{18}O]TMP as described above. The ^{31}P NMR spectrum obtained is given in Figure 6.

Results

(R_PS_P)-Tp(S)T was synthesized by a similar procedure to that described for d[Tp(S)A] (Romaniuk & Eckstein, 1982) because the previously published procedure for Tp(S)T gives rather poor yields (Bartlett & Eckstein, 1982). We have found it unnecessary to purify the fully protected dinucleoside phosphorothioate but immediately removed the 5′-monomethoxytrityl protecting group with acetic acid. The use of benzenesulfonic acid (Romaniuk & Eckstein, 1982) for deblocking was unsuccessful here due to the unexpected high solubility of the 5′-deblocked dinucleotide in water. Thus, deblocking with benzenesulfonic acid in chloroform followed by washing of the chloroform layer with aqueous bicarbonate led to a considerable loss of product into the aqueous phase. This problem was most simply overcome by deblocking with acetic acid and removing the acetic acid by evaporation in vacuo, thereby eliminating the need for an aqueous extraction. The 5′-deblocked dinucleoside phosphorothioate was partially purified by flash silica gel chromatography at this stage (Still et al., 1978). No separation of the diastereoisomers was observed as has been previously reported for this compound (Bartlett & Eckstein, 1982). Although the two diastereoisomers were still contaminated with 3′-*O*-acetylthymidine and other unidentified impurities these were most easily removed at a later stage, and no further purification was attempted here. Removal of the β-cyanoethyl group with triethylamine was accompanied by some desulfurization yielding after final deblocking TpT in addition to Tp(S)T in accordance with previous observations (Burgers & Eckstein, 1979; Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). The TpT and Tp(S)T are easily separated by DEAE-Sephadex A-25 chromatography which also removes all the other impurities carried through the synthesis.

The separation of diastereoisomers of Tp(S)T proved to be difficult. It has previously been reported that the diastereoisomers of both Tp(S)T and d[Tp(S)A] can be separated by reverse-phase HPLC (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). However, this method was used to prepare pure diastereoisomers in amounts of ca. 1–2 μmol and was clearly unsuitable for our purpose as we required quantities of about 100 μmol. Bartlett & Eckstein (1982) reported that the diastereoisomers of Tp(S)T could not be separated by chromatography on DEAE-Sephadex A-25, and Romaniuk & Eckstein (1982) were also unsuccessful with d[Tp(S)A]. However, some resolution is possible when a large column of DEAE-Sephadex A-25 and a shallow gradient of TEAB are used. When 250 μmol of racemic Tp(S)T was applied to this column, the early fractions in the symmetrical peak which was eluted consisted of 101 μmol of an 80% diastereoisomerically pure R_P isomer, whereas the later fractions contained 105 μmol of an 80% diastereoisomerically pure S_P isomer. The middle fractions consisted of 34 μmol of racemic material. Further purification is possible by recycling of the enriched fractions. However, this method suffers not only from poor resolution but also from long operation times, each column taking 3–4 days to run. A much more convenient method of separation was semipreparative reverse-phase HPLC. Thus 50 μmol of 80% diastereoisomerically pure Tp(S)T (enriched in either the R_P or S_P isomer) could be further refined to about 95% isomeric purity by a single passage over the reverse-phase

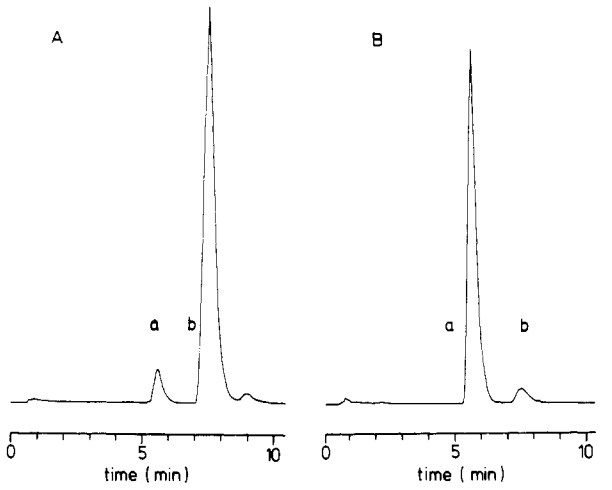


FIGURE 1: HPLC analysis of the diastereoisomers of Tp(S)T. The diastereoisomers were purified by chromatography as under Materials and Methods. (A) (S_P)-Tp(S)T peak b, contaminated with (R_P)-Tp(S)T peak a. (B) (R_P)-Tp(S)T peak a, contaminated with (S_P)-Tp(S)T peak b.

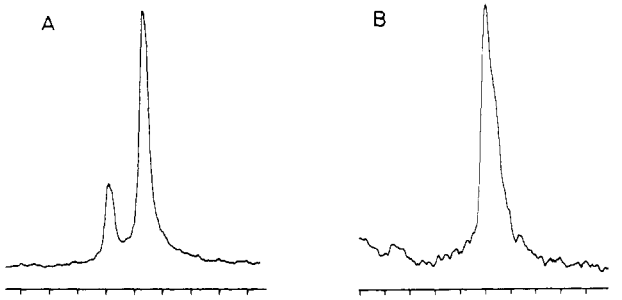


FIGURE 2: ^{31}P NMR spectra of (R_P)- and (S_P)-[^{18}O]TpT. (A) Spectrum of (S_P)-[^{18}O]TpT containing approximately 25% TpT derived from (R_P)-Tp(S)T; δ 1.23 and 1.20. (B) Spectrum of (R_P)-[^{18}O]TpT derived from (S_P)-Tp(S)T; δ 1.20. Spectra were taken in 100 mM EDTA, pH 8.0, and 25% D_2O. Parameters were the following: sweep width 1000 Hz; pulse width 18 μs; acquisition time 8.19 s; data collection in 16K; 1026 transients; line broadening 0.12 Hz; scale 2 Hz/division.

column (Figure 1). This process takes about 7–8 h and can also be used to purify racemic Tp(S)T. Clearly this is the method of choice.

When the R_P diastereomer of Tp(S)T was reacted with *N*-bromosuccinimide in dioxane and a mixture of H_2O and $H_2{}^{18}O$ (25% ^{16}O, 75% ^{18}O), it gave [^{18}O]TpT which after chromatography on DEAE-Sephadex had a chemical purity of 85% as determined by both reverse-phase HPLC and ^{31}P NMR spectroscopy. The impurities did not interfere with the chemical and enzymatic steps used to determine the stereochemical course of this reaction. Additionally, neither were they hydrolyzed by nuclease P1 nor did they significantly inhibit this enzyme. Thus, these impurities were of no significance in this study, and no further attempts were made to remove them. Should further purification be required, semipreparative reverse-phase HPLC will probably suffice. This is based on our observation that TpT (retention time 4 min) and the impurities (retention time 2–3 min) are easily separated by analytical reverse-phase HPLC. A ^{31}P NMR spectrum indicated that the material consisted of a mixture of approximately 26% [^{16}O]TpT and 74% [^{18}O]TpT (Figure 2A). The isotope shift on phosphorus at 81.01 MHz was 2.44 Hz. The configuration of the labeled compound was expected by precedent (Connolly et al., 1982) to be S_P at phosphorus. This has been verified by digestion of this material with snake venom phosphodiesterase in ^{17}O-enriched water. The resulting

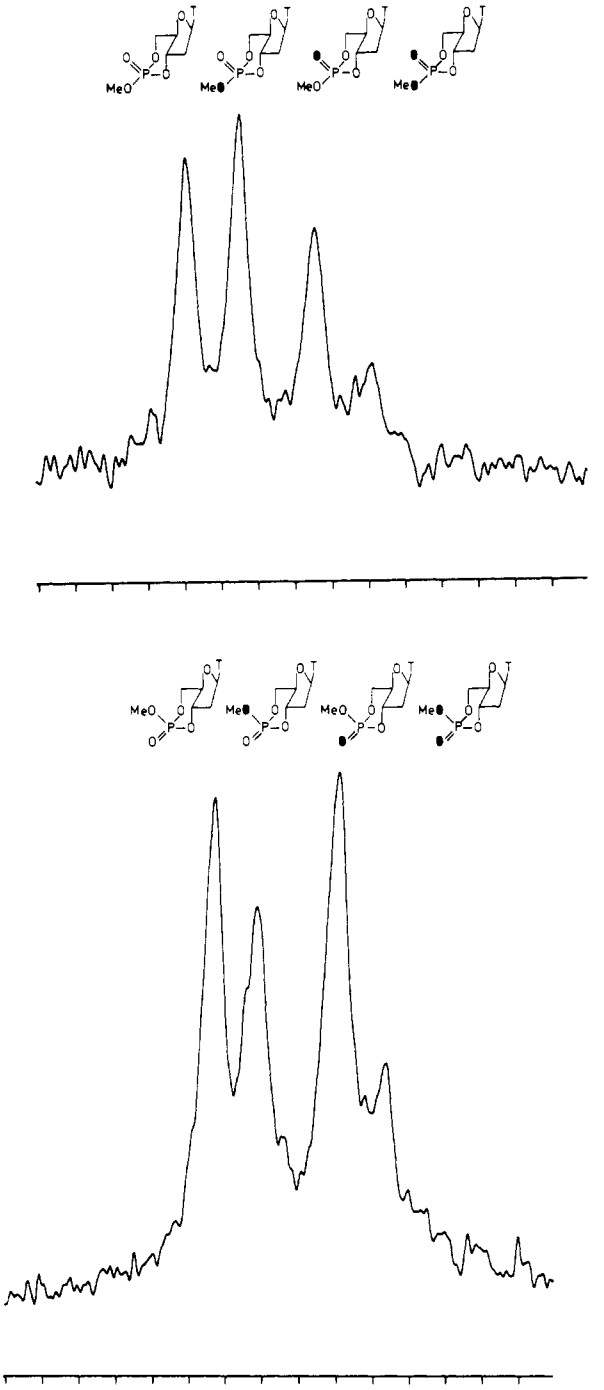


FIGURE 3: ^{31}P NMR spectra of isotopomers of cTMP methyl ester derived from [^{16}O,^{17}O,^{18}O]TMP obtained from snake venom phosphodiesterase digestion of (S_P)-[^{18}O]TpT. (Upper spectrum) Equatorial diastereomers; (lower spectrum) axial diastereomers. Spectra were obtained in Me_2SO-d_6–Me_2SO (50:50 v/v) containing 8-hydroxyquinoline. Parameters were the following: sweep width 600 Hz; pulse width 10 μs; acquisition time 6.82 s; data collection in 8K and Fourier transform in 32K; 9300 transients; line broadening 0.5 Hz; scale 0.2 ppm/division.

[^{16}O,^{17}O,^{18}O]TMP was shown to have the S_P configuration by a stereochemical analysis consisting of cyclization to the isotopomers of cTMP followed by methylation (Jarvest et al., 1981) (Figures 3 and 4; Table I). Since snake venom phosphodiesterase is known to hydrolyze internucleotidic linkages with retention of configuration at phosphorus (Burgers et al., 1979; Bryant & Benkovic, 1979; Jarvest & Lowe, 1981; Mehdi & Gerlt, 1981), the original [^{18}O]TpT may be stereochemically characterized as (S_P)-[^{18}O]TpT, and so the oxygen for sulfur replacement reaction had proceeded with

Table I: Configurational Analysis of (S_P)-[^{18}O]TpT[a]

	axial diastereoisomer			equatorial diastereoisomer		
		calcd			calcd	
	obsd	(R_P)-[^{18}O]TpT	(S_P)-[^{18}O]TpT	obsd	(R_P)-[^{18}O]TpT	(S_P)-[^{18}O]TpT
MeO–P=O	0.91	0.75	0.75	0.86	0.75	0.75
Me●–P=O	0.74	1.00	0.64	1.00	0.64	1.00
MeO–P=●	1.00	0.64	1.00	0.71	1.00	0.64
Me●–P=●	0.44	0.27	0.27	0.47	0.27	0.27

[a] The observed relative peak intensities of the ^{31}P NMR resonances (from Figure 3) of the diastereomeric triesters derived from the cyclization and methylation of [^{16}O, ^{17}O, ^{18}O]TMP derived from the snake venom phosphodiesterase catalyzed hydrolysis of [^{18}O]TpT from (R_P)-Tp(S)T are compared with the calculated values for the hydrolysis of either isotopomer of [^{18}O]TpT with retention of configuration at phosphorus. These values were calculated on the basis of the following assumptions: (R_P)-Tp(S)T starting material contained 5% of the S_P diastereoisomer; [^{18}O]TpT contained 26% [^{16}O]TpT; the hydrolysis reaction was performed in water of the isotopic composition 27.2% ^{16}O, 43.6% ^{17}O, and 29.2% ^{18}O.

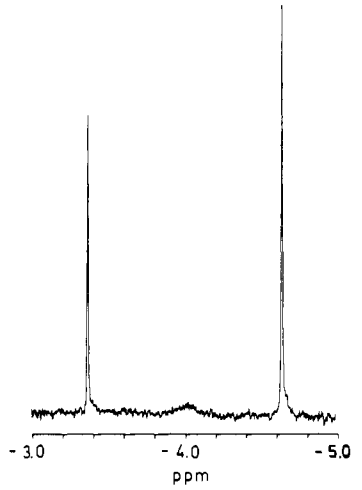


FIGURE 4: ^{31}P NMR spectrum of diastereoisomeric mixture of cTMP methyl ester. Spectrum was recorded in Me_2SO-d_6–Me_2SO (50:50 v/v) containing 8-hydroxyquinoline. Parameters were as for Figure 3 with line broadening 0.15 Hz and 1808 transients. δ −3.38 and −4.62.

inversion of configuration as anticipated.

As an independent check of the stereospecificity of the replacement reactions, the methyl esters of TpT were prepared. Methylation of the potassium-18-crown-6 salt of TpT by methyl iodide gave the diastereoisomeric TpT methyl esters. The ^{31}P NMR spectrum (Figure 5) of this compound exhibited two resonances at 0.60 and 0.69 ppm corresponding to the two diastereoisomers. We have not determined whether methylation of the thymidine base also occurs under these conditions although in view of the inertness of thymidine to methyl iodide and similar alkylating agents (Kochetkov & Budovskii, 1972) we consider this unlikely.

Methylation in the same fashion of [^{18}O]TpT derived from (R_P)-Tp(S)T gave the ^{31}P NMR spectrum shown in Figure 5 (lower spectrum). It consists of six resonances assignable to the two unlabeled diastereoisomers of TpT methyl ester and the two additional isotopomers belonging to each diastereoisomer as is shown on the spectrum and discussed in greater detail later. This spectrum allows for the first time the determination of the absolute configuration of a dinucleoside phosphate triester. The downfield set of resonances can be assigned to the S_P-isotopomeric triesters and the upfield to the R_P triesters (see Discussion for further details). The assignments are shown in Figure 5 (lower spectrum). Furthermore, integration of the NMR signals of the two pairs of ^{18}O-containing triesters showed that the minor one in each pair was present in quantities corresponding to approximately 10% of the total ^{18}O-containing material. When the 5% contamination of the R_P-Tp(S)T precursor by the S_P diastereomer is taken into account, this shows that the chemical replacement of oxygen for sulfur proceeded with at least 95% inversion of configuration at phosphorus.

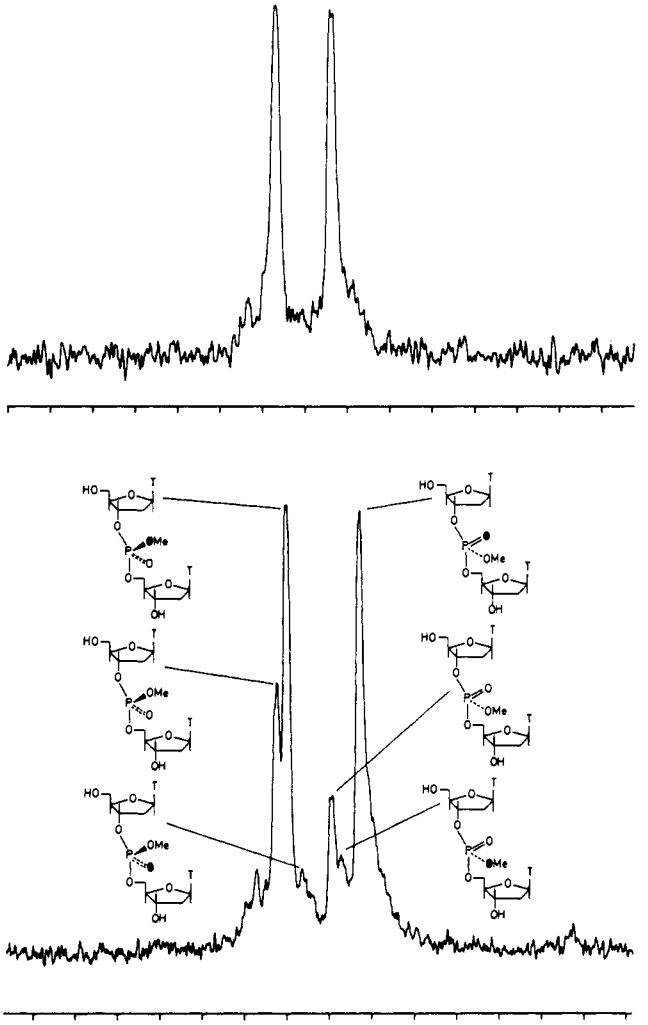


FIGURE 5: ^{31}P NMR spectra of TpT methyl ester and [^{18}O]TpT methyl ester. The upper spectrum is of the mixture of diastereomers of TpT methyl ester (δ 0.60 and 0.69); the lower spectrum is of the mixture of diastereomers of [^{18}O]TpT methyl esters containing approximately 25% of TpT methyl ester. Spectra were taken in Me_2SO-d_6–Me_2SO (50:50 v/v) containing 8-hydroxyquinoline. Parameters were the following: sweep width 500 Hz; pluse width 20 μs; acquisition time 8 s; data collecton in 8K and Fourier transform in 32K; 1200 transients; line broadening 0.1 Hz; scale 5 Hz/division.

TpT was found to be a reasonable substrate for nuclease P1 from *Penicillium citrum* with K_m and V_{max} values of 3.25 mM and 20.5 μmol min^{-1} (mg of enzyme)$^{-1}$. This compares with the manufacturer's specification of 430 μmol of phos-

Table II: Stereochemical Analysis of [^{16}O,^{17}O,^{18}O]TMP from the Nuclease P1 Reaction[a]

	axial diastereoisomer			equatorial diastereoisomer		
		calcd			calcd	
	obsd	inversion	retention	obsd	inversion	retention
MeO–P=O	0.39	0.20	0.20	0.44	0.20	0.20
Me●–P=O	0.65	0.54	1.00	1.00	1.00	0.54
MeO–P=●	1.00	1.00	0.54	0.61	0.54	1.00
Me●–P=●	0.38	0.35	0.35	0.40	0.35	0.35

[a] The observed peak intensities of the ^{31}P NMR resonances (from Figure 6) of the diastereoisomeric triesters derived by cyclization and methylation of [^{16}O,^{17}O,^{18}O]TMP derived from the nuclease P1 catalyzed hydrolysis of (R_P)-[^{18}O]TpT are compared with the calculated values for hydrolysis with inversion of configuration at phosphorus. These values were calculated on the basis of the following assumptions: (S_P)-Tp(S)T starting material contained 6% of the R_P diastereoisomer; (R_P)-[^{18}O]TpT was 95% labeled; the nuclease P1 hydrolysis was performed in water of the isotopic composition 14.6% ^{16}O, 51.2% ^{17}O, and 34.2% ^{18}O.

phodiester equivalents hydrolyzed min^{-1} (mg of enzyme)$^{-1}$ with RNA as substrate. (R_P)-[^{18}O]TpT containing ≥95% oxygen-18 was prepared from (S_P)-Tp(S)T with 99% enriched $H_2{}^{18}O$ (Figure 2B). This was digested to thymidine and [^{16}O,^{17}O,^{18}O]TMP by the action of nuclease P1 in ^{17}O-enriched water. Stereochemical analysis as before showed that the [^{16}O,^{17}O,^{18}O]TMP had the S_P configuration at phosphorus (Figure 6 and Table II). Thus the *Penicillium citrum* nuclease P1 reaction proceeds with inversion of configuration at phosphorus.

Discussion

Although a number of nucleotides have been synthesized with phosphorus stereospecifically labeled by the isotopes of oxygen, no dinucleoside [^{18}O]phosphates have yet been described. However, it is envisaged that such compounds will be of interest not only for the determination of the stereochemical course of nucleases in cases where the corresponding phosphorothioates prove to be unsuitable but also for incorporation into oligo- or polynucleotides, for those of restriction endonucleases. The primary goal of this investigation was therefore to prepare a dinucleoside phosphate chiral due to the presence of oxygen-18 at phosphorus. As the simplest representative [^{18}O]TpT was selected for synthesis. The recently published methods for the stereospecific replacement of sulfur for oxygen in nucleoside phosphorothioates (Sammons & Frey, 1982; Connolly et al., 1982; Lowe et al., 1982) made the diastereomers of Tp(S)T (Bartlett & Eckstein, 1982) an obvious choice as starting material.

The diastereomers of Tp(S)T were prepared by analogy to the synthesis of d[Tp(S)A] (Romaniuk & Eckstein, 1982) and separated by DEAE-Sephadex chromatography and preparative-reverse phase HPLC and their absolute configurations determined as described (Bartlett & Eckstein, 1982). The diastereomeric purity of each isomer was approximately 95% (Figure 1). The reaction of Tp(S)T with *N*-bromosuccinimide in dioxane/water gave rise to TpT in yields of 60–70%. *N*-Bromosuccinimide can be used with all the common bases (Connolly et al., 1982), and so this reaction should be applicable to the isotopic labeling of all dinucleotides, regardless of base composition. It should be noted, however, that side reactions are possible between some of the bases and *N*-bromosuccinimide. In our experience adenosine is inert toward *N*-bromosuccinimide whereas guanosine is rather reactive. The pyrimidines lie between these two extremes. The use of the

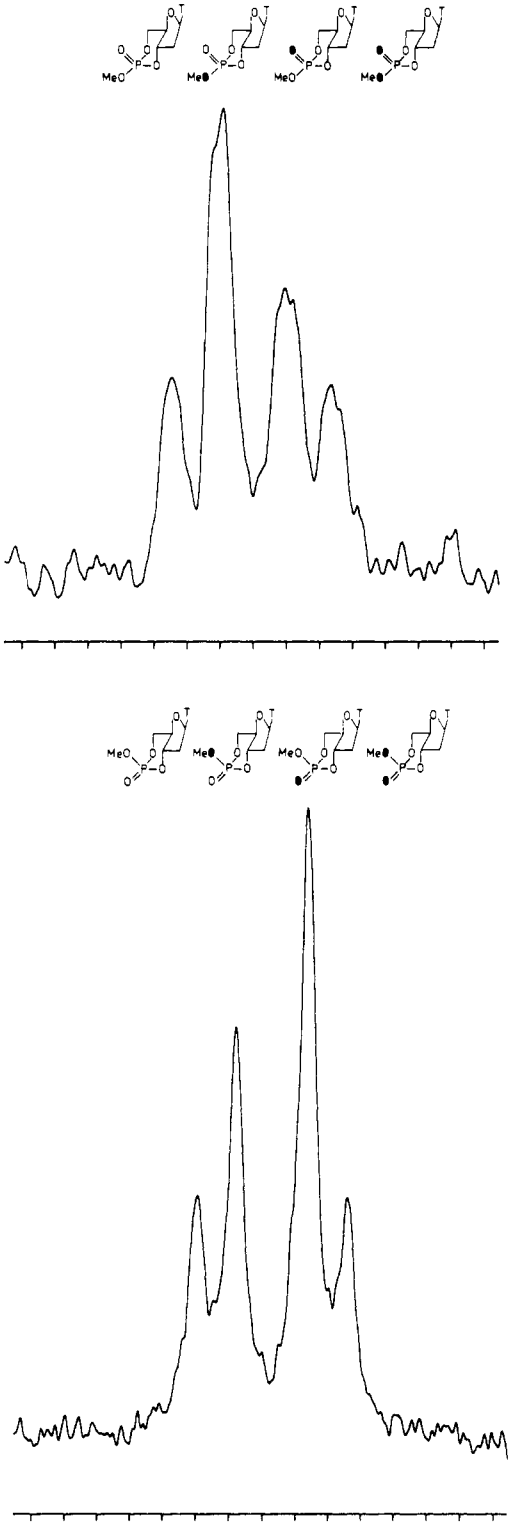
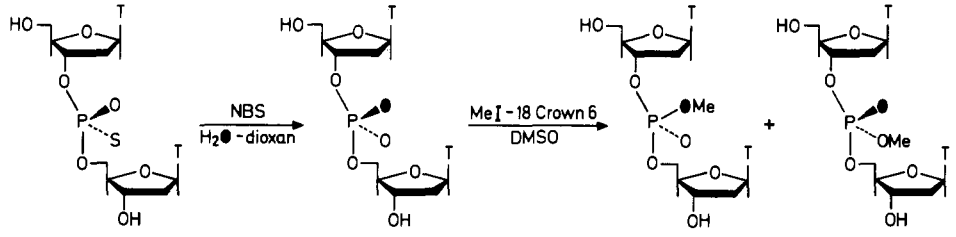


FIGURE 6: ^{31}P NMR spectra of isotopomers of cTMP methyl ester derived from [^{16}O,^{17}O,^{18}O]TMP obtained from nuclease P1 digestion of (R_P)-[^{18}O]TpT. (Upper spectrum) Equatorial diastereomers; (lower spectrum) axial diastereomers. Spectra were obtained in Me_2SO-d_6–Me_2SO (50:50 v/v) containing 8-hydroxyquinoline. Parameters were as for Figure 4 with line broadening for the equatorial diastereomers of 0.18 Hz and 0.88 Hz for the axial diastereomers, number of transients 8280, and scale 1 Hz/division.

conditions given here and earlier (Connolly et al., 1982; P. D. Senter, F. Eckstein, A. Mülsch, and E. Böhme, unpublished results), namely, 3–4-fold excess of *N*-bromosuccinimide and a 2-min reaction time, tends to reduce any side reactions. However, it should be noted that the replacement reaction as

Scheme I: Synthesis and Methylation of (S_P)-[^{18}O]TpT[a]



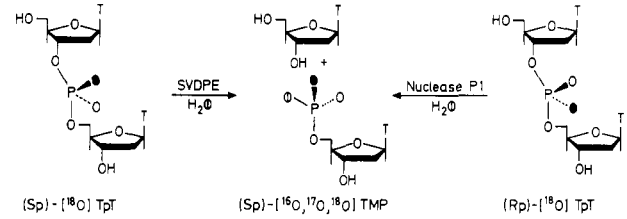
[a] Solid circle = ^{18}O; open circle = ^{16}O.

described here may not be applicable to the stereospecific labeling of diribonucleoside phosphates due to the strong possibility of neighboring group participation by the 2′-hydroxyl group during the activation step.

An important question in this study is the stereochemical course of the replacement of sulfur by oxygen in Tp(S)T. We originally reported that cAMPS is stereospecifically converted to [^{18}O]cAMP by *N*-bromosuccinimide in dioxane/$H_2{}^{18}O$ with inversion of configuration. However, we have recently found that this reaction proceeds with 80% inversion and 20% retention of configuration at phosphorus. A similar result has been obtained with thymidine cyclic 3′,5′-phosphorothioate (Lowe et al., 1982). The replacement of sulfur in ADPαS to give [α-^{18}O]ADP also appeared to proceed with complete inversion of configuration (Connolly et al., 1982), although the method used to assess the configurational purity of the [α-^{18}O]ADP may not have detected up to 20% racemization. Probably the best evidence that this reaction is stereospecific comes from a study of the stereochemical course of the reaction catalyzed by guanylate cyclase (P. D. Senter, F. Eckstein, A. Mülsch, and E. Böhme, unpublished results). (S_P)-GDPαS was converted to (R_P)-[α-^{18}O]GDP with *N*-bromosuccinimide, and this material was used to prepare [α-^{18}O]GTP. The [α-^{18}O]GTP was enzymatically cyclized to [^{18}O]cGMP by using guanylate cyclase, and following methylation ^{31}P NMR spectroscopy indicated absolute stereospecificity in the chemical replacement reaction. With these results in mind and particularly those obtained with cAMPS, we have determined the stereochemical course of the Tp(S)T to TpT conversion by two distinct methods.

When (R_P)-Tp(S)T was reacted with *N*-bromosuccinimide in $H_2{}^{18}O$ (25% ^{16}O, 75% ^{18}O) a mixture of TpT and [^{18}O]TpT of unknown but suspected S_P configuration at phosphorus was obtained (Scheme I). This material was digested by snake venom phosphodiesterase in ^{17}O-enriched water to thymidine and [^{16}O,^{17}O,^{18}O]TMP (Scheme II). The absolute configuration of the [^{16}O,^{17}O,^{18}O]TMP so formed can be determined by ^{31}P NMR spectroscopy following chemical cyclization of this compound to the isotopomers of cTMP and methylation to a mixture of the equatorial and axial triesters (Jarvest et al., 1981). When ^{18}O is bonded to phosphorus, it causes an upfield shift in the ^{31}P NMR signal that is greater for doubly as compared to singly bonded oxygen (Lowe et al., 1979; Cohn & Hu, 1980). Additionally when ^{17}O is bonded to phosphorus, the quadrupolar moment of the ^{17}O nucleus causes broadening of the ^{31}P NMR signal to such an extent that this resonance is not observed (Lowe et al., 1979; Tsai & Chang, 1980). These two effects have been combined to facilitate the stereochemical analysis of [^{16}O,^{17}O,^{18}O]phosphate esters by NMR spectroscopy (Buchwald & Knowles, 1980; Jarvest et al., 1981), and more specifically of [^{16}O,^{17}O,^{18}O]AMP (Jarvest et al., 1981). This method is clearly applicable to the analysis

Scheme II: Configurational Analysis of (S_P)-[^{18}O]TpT by Digestion with Snake Venom Phosphodiesterase and Stereochemical Course of Nuclease P1 Reaction[a]



[a] Solid circle = ^{18}O; slashed circle = ^{17}O; open circle = ^{16}O.

of [^{16}O,^{17}O,^{18}O]TMP. Thus, cyclization of [^{16}O,^{17}O,^{18}O]TMP proceeds with inversion of configuration and random loss of one of the three oxygen atoms present to give the isotopomers of cTMP. Subsequent methylation permits the location of the various isotopes in this compound from which the configuration of the original [^{16}O,^{17}O,^{18}O]TMP can be deduced.

Figure 3 shows that in the set of four resonances assigned to the axial triester the largest peak contains a nonbridging oxygen whereas for the equatorial triesters the largest peak contains a bridging ^{18}O. Additional peaks in the spectrum arise because the $H_2{}^{17}O$ was not isotopically pure and the original [^{18}O]TpT also contained TpT. After allowing for this, the pattern shown in Figure 3 establishes that the [^{16}O,^{17}O,^{18}O]TMP has the S_P configuration at phosphorus (Table I). As snake venom phosphodiesterase catalyzes hydrolysis with retention of configuration at phosphorus (Burgers et al., 1979; Bryant & Benkovic, 1979; Mehdi & Gerlt, 1981; Jarvest & Lowe, 1981), the starting [^{18}O]TpT must also have had the S_P configuration, and so, as expected, the oxygen for sulfur replacement reaction proceeds with predominant inversion of configuration at phosphorus.

As a further check on the diastereoisomeric purity of the (S_P)-[^{18}O]TpT, we have prepared the methyl esters of this compound and also of unlabeled TpT (Scheme I). TpT methylester contains a chiral phosphorus and therefore exists as a pair of diastereoisomers. These compounds have previously been synthesized by esterification of protected TpT with methanol and a sulfonyl chloride, but neither were the diastereoisomers separated nor was a ^{31}P NMR spectrum reported (Miller et al., 1971). We esterified the potassium-18-crown-6 salt of TpT with methyl iodide, a procedure developed previously for esterification of cAMP (Jarvest et al., 1981), and which was found to give nearly quantitative yields in this case. In the ^{31}P NMR spectrum (Figure 5, upper spectrum) two signals of equal height were observed with chemical shifts of 0.60 and 0.69 ppm when recorded in Me_2SO. Addition of methanol was found to considerably reduce the separation from 7.5 to 3 Hz at 81.01 MHz. To the best of our knowledge no literature precedent exists that enables the assignment of these

resonances to a particular diastereomer.

When this methylation was performed on (S_P)-[^{18}O]TpT containing 25% unlabeled TpT, the ^{31}P NMR spectrum shown in Figure 5 (lower spectrum) was obtained. This spectrum consists of two groups of signals, each group containing three resonances. The two strongest signals derive from the two diastereomeric [^{18}O]TpT methyl esters. When referenced to the unlabeled TpT methyl ester in each group, the strongest signal in the upfield group shows a larger isotope shift than the strongest signal in the downfield group. This indicates that the former contains a nonbridging oxygen-18 whereas in the latter the oxygen-18 is bridging. Knowing the configuration of [^{18}O]TpT as S_P, this means that the low-field resonances are due to the isotopomers of the S_P diastereomer, and the upfield resonances are due to the isotopomers of the R_P diastereomer of the methyl ester. The smallest signals are from the methyl esters of the contaminating (R_P)-[^{18}O]TpT that must be present in at least 5% quantities due to the presence of the 5% (S_P)-Tp(S)T in the starting (R_P)-Tp(S)T. Integration of the peaks is difficult because of the overlap of small resonances with larger peaks but yields a value of approximately 10% contamination by (R_P)-[^{18}O]TpT in the original (S_P)-[^{18}O]TpT. Since a value of 5% was expected in any case, this value is consistent with the idea that very little, if any, racemization at phosphorus during the replacement reaction had occurred under our conditions. This result is in agreement with the formation of [α-^{18}O]ADP and [α-^{18}O]GDP from the appropriate phosphorothioate precursors. The assignment of the upfield and downfield ^{31}P NMR signals to the R_P and the S_P diastereomers, respectively, in this acyclic phosphate triester contrasts with the much better documented cyclic (six-membered ring) triester case. Here the R_P and S_P isomers resonate at low and high field, respectively (Engels & Schlaeger, 1977; Engels, 1979). Additionally the ^{31}P NMR signals for the acyclic triesters are relatively poorly separated (separation about 0.1 ppm) when compared to the cyclic compounds (separation usually about 2 ppm) when measured in the same solvent.

With the establishment of the configuration of the two isotopomers of [^{18}O]TpT, it became possible to use one of them for the elucidation of the stereochemical course of a reaction catalyzed by a nuclease. Nuclease P1 from *Penicillium citrum* (Fujimoto et al., 1974; Fujimoto & Kuninaka, 1974) was chosen for such a study. This enzyme is a member of a family of endonucleases (Lehman, 1981) which have been isolated from diverse fungal sources and exhibit a high degree of specificity for single-stranded polynucleotides. Each of the members of this group hydrolyzes single-stranded RNA or DNA to 5′-phosphorylated products and shows maximal activity at acidic pH values. The possession of a 3′-nucleotidase activity is also common. Because of their ability to hydrolyze single-stranded polynucleotides, these enzymes have become useful tools in the tailoring of DNA for genetic manipulation. Little is known, however, about the mechanisms of action of these enzymes, and the only previous investigation focusing on the stereochemical course of the hydrolysis reaction has been for another member of the group, nuclease S1 (Potter et al., 1983).

Although many similarities exist between nucleases P1 and S1 (both enzymes are glycoproteins and are activated by zinc ions), there are quantitative differences between their catalytic properties. S1 nuclease is much more active on DNA than on RNA or 3′-AMP, whereas the opposite is true for nuclease P1. In addition, the relatively sharp pH optimum for polynucleotide substrates is approximately 1 pH unit lower for nuclease S1. In order to compare these two enzymes at a mechanistic level, we have determined the stereochemical course of the nuclease P1 reaction.

TpT was shown to be a substrate for nuclease P1. The K_m and V_{max} values were found to be 3.25 mM and 20.5 μmol min^{-1} (mg of enzyme)$^{-1}$. These data compare with 1.6 mM and 8.4 μmol min^{-1} (mg of enzyme)$^{-1}$ for nuclease S1 when d[TpA] is used as the substrate (Potter et al., 1983). For determination of the stereochemical course of the reaction, (R_P)-[^{18}O]TpT was digested in ^{17}O-enriched water to thymidine and [^{16}O,^{17}O,^{18}O]TMP by nuclease P1 (Scheme II) and the [^{16}O,^{17}O,^{18}O]TMP stereochemically analyzed as described. As can be seen from the ^{31}P NMR spectra (Figure 6 and Table II), the intensity pattern is similar to that previously discussed for the snake venom phosphodiesterase reaction. It is therefore concluded that the [^{16}O,^{17}O,^{18}O]TMP isolated also has the S_P configuration at phosphorus and that the reaction has proceeded with inversion of configuration at phosphorus. This is most simply interpreted to be the result of a direct displacement reaction not involving a covalent enzyme intermediate. However, this simple interpretation might become more complicated if a carboxyl group is situated at the active site of this enzyme as has been postulated for the related enzyme nuclease S1 (Berg & Witzel, 1982). As has been discussed in the context of the stereochemical course of the staphylococcal nuclease reaction (Mehdi & Gerlt, 1982), an acyl phosphate covalent enzyme intermediate between such a carboxyl group and a nucleoside phosphate can be hydrolyzed in principle by nucleophilic attack of H_2O at either carbon or phosphorus, resulting in overall inversion of configuration in the former and retention in the latter case.

Nuclease S1 also catalyzes hydrolysis with inversion of configuration at phosphorus, using a diastereomer of d[Tp(S)A] as substrate (Potter et al., 1983). A further similarity exists in the stereoselectivity of nuclease P1 and S1 for thiophosphorylated substrates, nuclease S1 accepting the S_P diastereomer of d[Tp(S)A] and nuclease P1 the S_P diastereomer of Tp(S)T (data not shown). It may therefore be concluded that the biochemical similarities between the two enzymes also extend to a mechanistic level.

In summary, this report describes the synthesis and assignment of the configuration of the isotopomers of [^{18}O]TpT as an example of an ^{18}O-labeled dinucleoside phosphate and demonstrates the usefulness of such a compound for the determination of the stereochemical course of nuclease-catalyzed reactions.

Acknowledgments

We thank the Royal Society for a European Science Exchange Fellowship to B.V.L.P., B. Seeger for recording the NMR spectra, and A. Schelper and G. Haag for technical assistance.

Registry No. (R_P)-[^{18}O]TpT, 84332-64-9; (S_P)-[^{18}O]TpT, 84413-98-9; (R_P)-Tp(S)T, 83199-36-4; (S_P)-Tp(S)T, 83199-37-5; Tp(OMe)T, 35002-94-9; (S_P)-[^{18}O]Tp(OMe)T, 84332-65-0; (R_P)-[^{18}O]Tp(OMe)T, 84332-66-1; nuclease P1, 54576-84-0.

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Proton Nuclear Overhauser Effect Study of the Structure of an Actinomycin D Complex with a Self-Complementary Tetranucleoside Triphosphate†

David G. Reid, Stephen A. Salisbury, and Dudley H. Williams*

ABSTRACT: Saturation transfer and nuclear Overhauser effect (NOE) techniques have been used to assign some resonances of nonexchangeable protons in the NMR spectrum of the complex formed between actinomycin D and the self-complementary tetranucleoside triphosphate d(A-G-C-T). Intermolecular NOEs suggest that the drug chromophore intercalates between the two G-C base pairs of the nucleotide double helix, while the pentapeptide lactone rings fill the minor groove. Binding-induced distortions of helix geometry are discussed.

The interaction between actinomycin D (Figure 1) and nucleic acids is probably the most studied of all antitumor compounds (Remers, 1978). Understanding its high selectivity for G-C-rich double-stranded DNA not only has offered the possibility of increasing its therapeutic usefulness but also may have relevance as a model of the contact between proteins and polynucleotides.

The drug binds by intercalation (Muller & Crothers, 1968), but its preference for G-C-rich DNA was accounted for by deductions made from the X-ray crystal structure of its complex with deoxyguanosine (Jain & Sobell, 1972). This relatively simple complex is stabilized by two hydrogen bonds between guanines parallel to the phenoxazone chromophore of the drug and L-threonine in the side chains. These interactions in combination with the overall shape of the actinomycin molecule were used to construct a detailed model of the complex with DNA generally consistent with other experimental data (Sobell & Jain, 1972; Sobell et al., 1977). It has, however, not been possible to examine at high resolution a model system closely resembling the DNA-bound drug; the crystalline complex with d(G-C) (Takusagawa et al., 1982) does not contain a double-helical nucleotide fragment.

NMR spectroscopy has been extensively used in attempts to characterize the binding site and mode of action of actinomycin D. Krugh & Neely (1973a) established that the drug has two binding sites for deoxyguanosine and 5′-dGMP. The same authors (Krugh & Neely, 1973b) studied its interactions with deoxydinucleotides and once again found two binding sites for G-containing species. The G-C sequence was particularly

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