Stereochemical Course of a Phosphokinase Using a Chiral [^{18}O]Phosphorothioate. Comparison with the Transfer of a Chiral [^{16}O,^{17}O,^{18}O]Phosphoryl Group†

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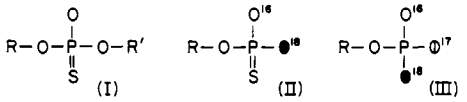
ABSTRACT: Synthetic adenosine 5′-*O*-[3-^{18}O,3-thio]triphosphate having the *R* configuration at the γ-phosphorus has been used as a substrate in the reaction catalyzed by glycerol kinase. The product *sn*-glycerol 3-[^{18}O]phosphorothioate has been isolated, and the configuration at phosphorus has been determined by ring closure to the two diastereoisomeric cyclic 2,3-phosphorothioates of *sn*-glycerol and analysis of the ^{18}O content of each diastereoisomer. The structural identity of these diastereoisomers has been determined by correlation with one of the corresponding diastereoisomers of the cyclic 2,3-phosphorothioate of D-glycerate, whose crystal structure is reported here. From these experiments it is evident that glycerol kinase catalyzes the transfer of a thiophosphoryl group with *inversion* of the configuration at phosphorus, in gratifying agreement with the result from the transfer of a chiral [^{16}O,^{17}O,^{18}O]phosphoryl group [Blättler, W. A., & Knowles, J. R. (1979) *J. Am. Chem. Soc. 101*, 510].

During the past year or so, the use of chiral phosphorothioates in mechanistic investigations of the stereochemical course of enzyme-catalyzed reactions at phosphorus has burgeoned, as work in a number of laboratories has elucidated the stereochemistry of reactions of phosphorothioate diesters (I) [see, e.g., Eckstein (1979)] and of [^{18}O]phosphorothioate



(I) (II) (III)

monoesters (II) (Orr et al., 1978; Richard et al., 1978, 1979). In the case of the phosphorothioate *di*esters (I), the substitution of a phosphate oxygen by sulfur creates chirality at phosphorus, and, if either R or R′ is asymmetric, the difference in physical characteristics of the resulting diastereoisomers often allows their separation chromatographically [for instance, the epimers at phosphorus of ATPαS (Burgers & Eckstein, 1978)]. This elemental substitution usually leads, however, to a marked decrease in the rate of enzyme-catalyzed reactions involving the thioate analogues of both monoesters and diesters. These sluggish reaction rates have caused some anxiety about the validity of the stereochemical results that derive from studies with phosphorothioates. Is it possible that the substitution of oxygen by sulfur actually diverts the stereochemical course of an enzyme from its normal path?

Concurrently with the developments on the phosphorothioate front, the synthesis, analysis, and use of isotopically labeled chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters (III) (Abbott et al., 1978, 1979) have allowed phosphate esters themselves to be employed to probe the stereochemical changes in phosphoryl group transfer reactions (Jones et al., 1978; Blättler & Knowles, 1979a,b). These chiral esters do not, of course, suffer from any measurable rate reduction (when compared with the unlabeled natural substrates) nor can there be any diversion from the enzyme's normal stereochemical course. In order, therefore, to validate the use of the thioate analogues in at least one case, we report here the stereochemical course followed by glycerol kinase in the transfer of an [^{18}*O*]*phosphorothioate* group from ATPγS,γ^{18}O to glycerol and compare this with our earlier determination of the stereochemical course of glycerol kinase using the ^{16}O,^{17}O,^{18}O methodology (Blättler & Knowles, 1979a).

Experimental Section

Materials

Adenosine 5′-*O*-[3-^{18}O,3-thio]triphosphate having the *R* configuration at the γ-phosphorus was synthesized as described by Richard et al. (1978). Glycerol kinase (as a freeze-dried powder, from *Escherichia coli*) was obtained from Sigma Chemical Co. Pyridine was dried by reflux over solid KOH under N_2, followed by two distillations from BaO. Benzene and acetonitrile were distilled twice from CaH_2, dioxane was distilled twice from Na, and CH_2Cl_2 was passed through neutral activated alumina. All solvents were stored over 4-Å molecular sieves under N_2. Tri-*n*-octylamine was passed down a column of neutral activated alumina. Diethyl phosphorochloridate was freshly distilled each time it was used and stored under N_2.

rac-Glycerol 1-Phosphorothioate. To a solution of redistilled $PSCl_3$ (16.4 mmol) in dry tetrahydrofuran (10 mL), a solution of (*RS*)-4-(hydroxymethyl)-1,3-dioxalan-2-one (Gigg & Gigg, 1967) (8.8 mmol) in dry pyridine (5 mL) was added over 10 min at room temperature. After being stirred at room temperature for 1 h, the mixture was evaporated to dryness under reduced pressure. Water (40 mL) and solid NaOH (sufficient to raise the pH to >10) were added, and the mixture was left for 1 h at room temperature. After neutralization with 1 N HCl, the product was isolated from a column (500 mL) of DEAE-cellulose (DE-52, from Whatman) equilibrated with 30 mM triethylammonium bicarbonate, pH 7.5, and eluted with a linear gradient (2 L plus 2 L) of triethylammonium bicarbonate (30–200 mM). This column did not cleanly separate the 1-phosphorothioate from the 2-phosphorothioate, but since the purpose of the preparation was to evaluate conditions for the ring-closure reaction, the mixture of 1-phospho and 2-phospho compounds (in 85:15 ratio) was

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used without further purification.

4-Carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholanes were prepared by modification of the general procedure of Zwierzak (1967, 1975). DL-Glycerate (Sigma) was converted into its methyl ester by reaction with diazomethane, and the ester was purified by distillation (bp 114 °C at 4 mmHg). To methyl DL-glycerate (40 mmol) in ethanol (40 mmol) and benzene (40 mL) at 0 °C, a solution of PCl_3 (40 mmol) in benzene (10 mL) was added over 10 min. After stirring at room temperature for 2 h, we added elemental sulfur (sublimed, 40 mmol), and dry N_2 was gently bubbled through the mixture for 30 min. A solution of triethylamine (6 mL) in benzene (5 mL) was then added dropwise over 10 min, and the mixture was stirred at room temperature for 16 h. After being refluxed for 15 min, the solution was cooled to room temperature, and a mixture of triethylamine (5 mL) and water (50 mL) was added. After being stirred at room temperature for 2 h and then overnight at 4 °C, the aqueous phase was separated, washed with benzene (2 × 10 mL), and concentrated under reduced pressure to a viscous yellow oil. The products (the diastereoisomeric 4-carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholanes) were purified by chromatography on silica gel (500 g), eluting with a linear gradient (2 L plus 2 L) of triethylamine–methanol–2-propanol (3:47:50 v/v) to triethylamine–methanol (4:96 v/v). The combined yield of the two phospholanes was 47% (based on methyl glycerate). The ratio of the two isomers was 65:35, as estimated by gas chromatography of the trimethylsilyl derivatives on a 9-ft column of 3% OV1 at 155 °C with a flow rate in N_2 of 80 mL/min (major isomer retention time, 6.8 min; minor isomer retention time, 8.4 min). The ^{31}P NMR spectrum had singlets at −72.150 (major isomer) and −71.502 (minor isomer) ppm.

The syn (2*R*,4*R* and 2*S*,4*S*; i.e., the isomer having sulfur and the carboxyl group on the same side of the five-membered ring) and anti (2*R*,4*S* and 2*S*,4*R*) diastereoisomers were separated by ion-exchange chromatography of the mixture (9.6 mmol) on a column (1200 mL) of DEAE-cellulose (DE-52) equilibrated with 75 mM triethylammonium bicarbonate, pH 7.5, and eluted with a linear gradient (4 L plus 4 L) of triethylammonium bicarbonate (75–250 mM). The triethylammonium salt of the major isomer (purity > 98% as estimated by gas chromatography and ^{31}P NMR) was converted to the bis(cyclohexylammonium salt) by passage down a column of Dowex 50 (cyclohexylammonium form). The bis(cyclohexylammonium salt) was crystallized from aqueous acetone for crystal structure determination (see Appendix) and had mp 175–176.5 °C.

Methods

Glycerol phosphorothioate was assayed by the method described by Bergmeyer (1974) for *sn*-glycerol 3-phosphate.

Mass spectra were run on an AEI MS9 instrument. Gas chromatography–mass spectrometry was done on a Hitachi RMU-6L mass spectrometer interfaced to a Perkin-Elmer 990 gas chromatograph.

^{31}P NMR spectra (proton decoupled) were taken on a Varian XL-100 instrument.

Gas–liquid chromatography was done on a Varian 1400 instrument, using a glass column (6 or 9 ft) of 3% OV1 at 145 °C. Samples (0.1–1 μmol) of phosphorothioates were trimethylsilylated by the addition of pyridine (10 μL) and bis(trimethylsilyl)trifluoroacetamide (15 μL) and heating for 5 min at 80 °C.

Synthesis of sn-Glycerol 3-[^{18}O]Phosphorothioate from (R)-ATPγS,γ^{18}O. The triethylammonium salt of adenosine 5′-*O*-3(*R*)-[3-^{18}O,3-thio]triphosphate (6.04 μmol) was dissolved in 40 mM triethanolamine hydrochloride buffer, pH 8.2, (6.5 mL) containing $MgCl_2$ (30 μmol), glycerol (3 mmol), and 2-mercaptoethanol (150 μmol). Glycerol kinase (208 units) was added, and the mixture was left at room temperature for 1 h. At this time, no remaining ATPγS could be detected by thin-layer chromatography on poly(ethylenimine)–cellulose plates (eluting with 1.5 M aqueous LiCl), and the yield of *sn*-glycerol 3-phosphorothioate was 5.8 μmol (96%). The product was isolated by chromatography on a column (16 mL) of DEAE-cellulose (DE-52) equilibrated with 10 mM triethylammonium bicarbonate, pH 7.7, and eluted with a linear gradient (100 mL plus 100 mL) of triethylammonium bicarbonate (10–200 mM). The fractions containing *sn*-glycerol 3-phosphorothioate were freeze-dried to give 4.2 μmol (73% isolated yield). This material was shown to contain 0.86 g-atom of ^{18}O by mass spectrometric analysis of its trimethylsilyl derivative.

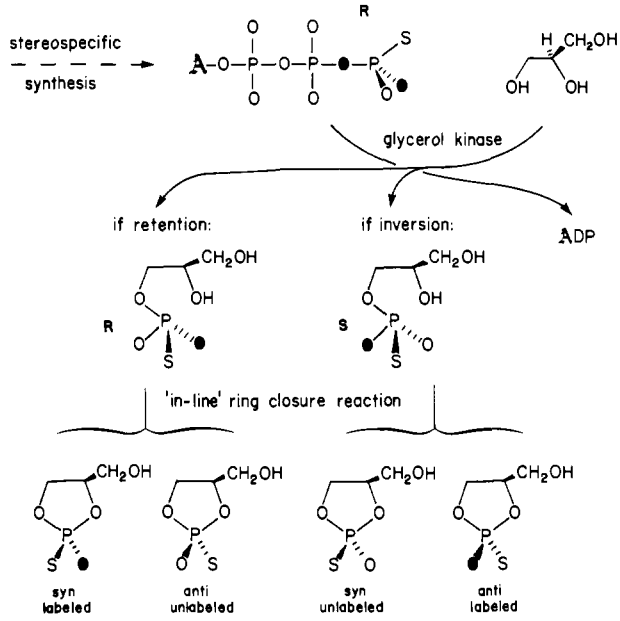
Ring Closure of sn-Glycerol 3-[^{18}O]Phosphorothioate. The triethylammonium salt of the sample *sn*-glycerol 3-[^{18}O]-phosphorothioate (4.2 μmol) was converted into the pyridinium salt by passage down a small column (0.5 mL) of Dowex 50 (pyridinium form). After the column was washed with water (5 mL), the eluate was evaporated to dryness under reduced pressure. After the addition of tri-*n*-octylamine (10.6 μmol) and dry dioxane (1 mL), the mixture was stirred at room temperature for 30 min. The material was then evaporated to dryness under reduced pressure, and a fresh portion of dry dioxane was added. The cycle of addition and removal of dry dioxane was repeated (3 times) until the residue completely dissolved upon the addition of dry dioxane (1 mL). The solvent was removed for the final time, and the sample vial was transferred to a glove bag. Two molecular sieves (4 Å) were added, followed by pyridine (50 μL), acetonitrile (30 μL), and tri-*n*-octylamine (5 μL). After leaving for 4 h, the ring closure was effected by the addition of a fresh solution of diethyl phosphorochloridate (8.2 μmol, 1 μL) in CH_2Cl_2 (10 μL) that had been stored over 4-Å molecular sieves for 4 h. The progress of the reaction was monitored by withdrawal of a portion (3 μL) and analysis of the trimethylsilyl derivatives by gas chromatography. After 150 min at room temperature, water (1 mL) was added, and the mixture was freeze-dried. The residue was dissolved in 100 mM triethylammonium bicarbonate buffer, pH 7.6 (2 mL), and this solution was washed twice with CH_2Cl_2 (1 mL). The washed solution was then freeze-dried in portions, ready for analysis. Under these conditions (i.e., 2 molar equiv of phosphorochloridate), the overall yields of cyclic glycerol phosphorothioate were between 40 and 85%. This variability appears to arise from the small scale on which the closure reaction is performed.

For product characterization, a sample of *rac*-glycerol 3-phosphorothioate was cyclized, and the mixture of diastereoisomeric cyclic phosphorothioates (5 μmol) was purified by chromatography on a column (10 mL) of DEAE-cellulose (DE-52) equilibrated with 20 mM triethylammonium bicarbonate, pH 7.2, and eluted with a linear gradient (50 mL plus 50 mL) of triethylammonium bicarbonate (20–200 mM). The ratio of the two isomers was 60:40, as estimated by gas chromatography of the trimethylsilyl derivatives on a 9-ft column of 3% OV1 at 150 °C with a flow rate in N_2 of 60 mL/min (major isomer retention time, 16 min; minor isomer retention time, 14 min). The mixture had ^{31}P NMR singlets at −71.967 (major isomer) and −71.808 (minor isomer) ppm.

Results and Discussion

To determine the stereochemical consequence of the transfer of the [^{18}O]thiophosphoryl group catalyzed by glycerol kinase

Scheme I: The Reaction of ATPγS,γ^{16}O in the Glycerol Kinase Reaction and the Nature of the Analysis of the Configuration at Phosphorus in the Product *sn*-Glycerol 3-[^{18}O]Phosphorothioate (O = ^{16}O; ● = ^{18}O)



(see Scheme I), we need to know the configuration at phosphorus in both the starting material (ATPγS,γ^{18}O) and the product (*sn*-glycerol 3-[^{18}O]phosphorothioate). The configuration of the γ-phosphoryl group of the donor molecule, ATPγS,γ^{18}O, was known from its synthetic origin [for a full description, see Richard et al. (1978)] and was *R*. To define the configuration in the product, we adopt the strategy illustrated in Scheme I. If the product *sn*-glycerol 3-phosphorothioate is subjected to ring closure (with oxygen loss) by a reaction of known stereochemical course, two sets of diastereoisomeric cyclic phosphorothioates will be produced. If the ring-closure reaction proceeds with in-line geometry, from the (*R*)-[^{18}O]phosphorothioate, we shall obtain the ^{18}*O-labeled* syn (2*S*,4*R*) isomer and the *unlabeled* anti (2*R*,4*R*) isomer (see Scheme I). This distribution of isotopic label will be reversed if the original phosphoryl group is *S* (see Scheme I). We therefore have three requirements: first, a ring-closure reaction of known stereochemical course; second, a method to separate and identify the syn and anti cyclic phosphorothioates; third, the knowledge of which of the cyclic phosphorothioates is labeled with ^{18}O.

The first requirement is easily met, since it is known that diethyl phosphorochloridate effects an in-line ring closure in systems such as *sn*-glycerol 3-phosphorothioate (Usher et al., 1972; Orr et al., 1978). Although this ring-closure reaction appears not to be completely stereospecific (Orr et al., 1978), we have already shown it to give more than 75% of the product from in-line reaction (Orr et al., 1978). That this imperfect behavior presents no problem will become obvious below.

The second requirement is that the syn and anti diastereoisomers be separated and identified. It was not possible to effect the preparative separation of the two diastereoisomers of cyclized glycerol phosphorothioate by ion-exchange chromatography, although gas chromatography of their trimethylsilyl derivatives provides an analytical separation. Since a crystal structure is the only entirely unambiguous way of identifying which diastereoisomer is syn and which is anti, we chose to relate the cyclic phosphorothioates of *glycerol* to the cyclic phosphorothioates of *glycerate*. The latter compounds can be separated preparatively, and the major diastereoisomer

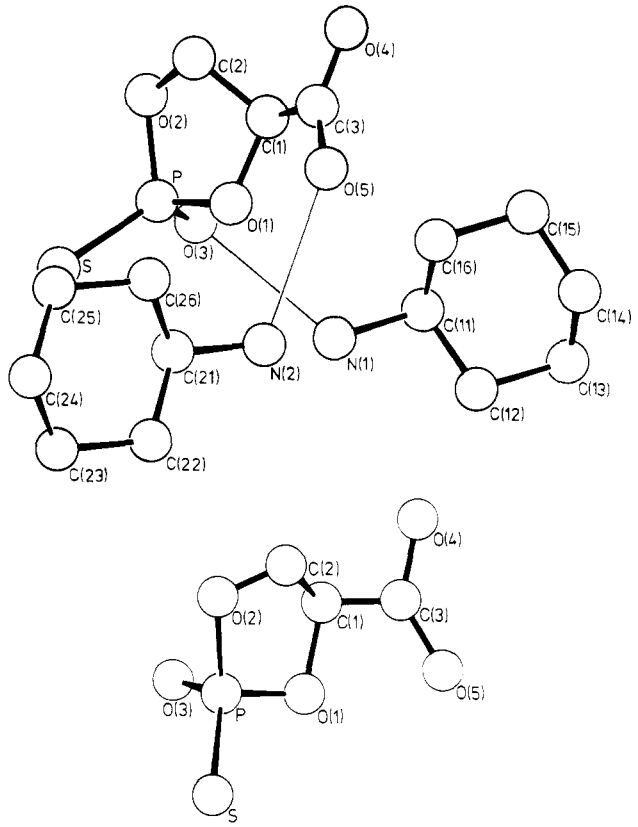
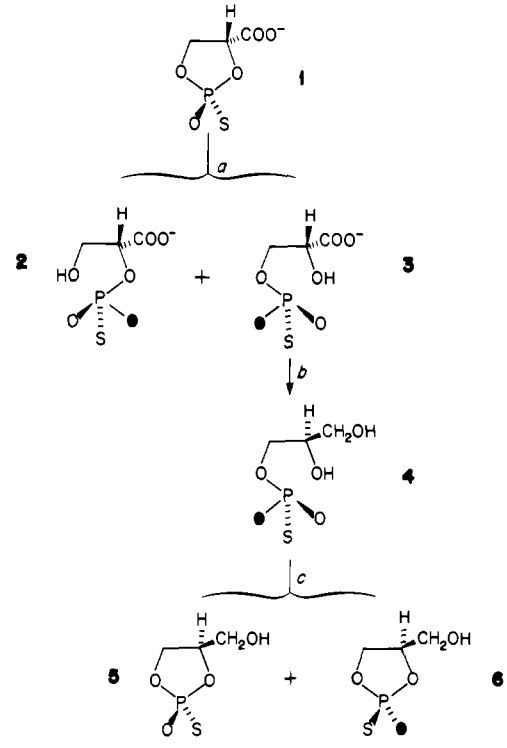


FIGURE 1: Structure of the major diastereoisomer of the cyclic phosphorothioate of glycerate.

Scheme II: Structural Correlation between the Diastereoisomers of the Cyclic Phosphorothioate of D-Glycerate (One of Which Is Shown in 1) and the Diastereoisomers of the Cyclic 2,3-Phosphorothioate of *sn*-Glycerol (5 and 6) (O = ^{16}O; ● = ^{18}O)



crystallizes nicely. The structure of this material [the bis(cyclohexylammonium salt) of 4-carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholane] was solved by X-ray diffraction (see Appendix), and the structure is shown in Figure 1. This syn

Table I: ^{18}O Distribution[a] (%) between the Two Diastereoisomers of the Cyclic Phosphorothioates of *sn*-Glycerol 3-[^{18}O] Phosphorothioate

diastereoisomer[b]	configuration[c]	sample of *sn*-glycerol 3-[^{18}O] phosphorothioate	
		A[d]	B[e]
I	syn (2*S*,4*R*)	76	15.6
II	anti (2*R*,4*R*)	24	84.4

[a] The ^{18}O content was determined from gas chromatography–mass spectrometry of the trimethylsilyl derivatives. The peak at $M^+ - 15$ was used in all determinations. The percentages quoted refer to the distribution of the ^{18}O present between the two diastereoisomers (taking into account the relative amount of each diastereoisomer formed in the ring-closure reaction). At least 10 mass spectra were averaged for each determination. [b] Designated on the basis of the order of elution on gas chromatographic separation on OV1. [c] Based on the correlation outlined in Scheme II and the known crystal structure shown in Figure 1 (for details, see the text). [d] Control sample derived from the known diastereoisomer 1 (Scheme II) via the path shown in Scheme II [data from Orr et al. (1978)]. [e] Sample derived from the glycerol kinase reaction (this work).

diastereoisomer (having sulfur and the carboxyl group on the same side of the five-membered ring; Scheme II, **1**) is the major isomer formed synthetically by sulfuration of the cyclic phosphite, has the shorter retention time on gas chromatography, and has the downfield ^{31}P NMR signal. Now, when this compound is treated with [^{18}O]hydroxide ion (Orr et al., 1978), the ring is opened to give both the 2- and 3-[^{18}O]-phosphorothioate of D-glycerate (Scheme II, **2** and **3**, respectively). The 3 isomer (**3**) has the *R* configuration at phosphorus, since the hydroxide ion attack is known to be an in-line process (Usher et al., 1972). This glycerate derivative **3** can now be converted to the corresponding *glycerol* derivative **4**, by using the four glycolytic enzymes phosphoglycerate kinase/ATP, glyceraldehyde-3-phosphate dehydrogenase/NADH, triosephosphate isomerase, and glycerol phosphate dehydrogenase/NADH as we have described previously (Orr et al., 1978). These reactions invert the configuration at C(2) but leave the configuration at phosphorus untouched (Scheme II). Cyclization of the product **4** [*sn*-glycerol 3-(*R*)-[^{18}O]-phosphorothioate] by the known in-line path using diethyl phosphorochloridate yields the ^{18}O-labeled syn and the unlabeled anti diastereoisomers of the cyclic phosphorothioate of glycerol (**5** and **6**). From the work of Orr et al. (1978), we know that the isomer having the shorter retention time on gas chromatography [referred to as "diastereoisomer I" in Orr et al. (1978)] contains the ^{18}O label, so we can assign the syn stereochemistry (2*S*,4*R*) to the first isomer eluted on gas chromatographic separation and anti stereochemistry (2*R*,4*R*) to the isomer of cyclized *sn*-glycerol 3-phosphorothioate that elutes second.

The third requirement of the stereochemical analysis is to discover which of the cyclic diastereoisomers (syn or anti) derived from the product of the glycerol kinase reaction (Scheme I) contains ^{18}O. When the mixture of cyclic diastereoisomers was separated and analyzed by gas chromatography–mass spectrometry, it was found that most of the ^{18}O label was associated with the *anti* (2*R*,4*R*) diastereoisomer (see Table I). As illustrated in Scheme I, only an *S*-phosphoryl group in the glycerol derivative will produce unlabeled syn and labeled anti. The product of the glycerol kinase reaction therefore has the *S* configuration at phosphorus, and glycerol kinase has thus mediated the thiophosphoryl group transfer with *inversion*.

The finding that glycerol kinase catalyzes the transfer of a chiral [^{18}O]thiophosphoryl group from ATP to glycerol with inversion of the configuration at phosphorus is in gratifying agreement with our earlier demonstration of inversion using a phosphoryl group chiral by virtue only of the three stable isotopes of oxygen. This is the first demonstration that the stereochemical behavior of phosphorothioates and the stereochemical behavior of phosphates are in concord and should remove at least some of the lingering fears that the often much slower rates of reaction of the phosphorothioates could be the consequence of a change in reaction mechanism.

Acknowledgments

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Appendix

Crystal Structure Determination of the Major Isomer of 4-Carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholane [*Bis-(cyclohexylammonium salt)*]. The crystalline salt of the major isomer of 4-carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholane isolated from aqueous acetone is triclinic, space group $P\bar{1}$ with $z = 2$ formula units per cell. The cell parameters are as follows: $a = 12.003$ (3), $b = 12.888$ (2), and $c = 6.755$ (2) Å; $\alpha = 107.36$ (1), $\beta = 95.09$ (2), and $\gamma = 84.92$ (2)°; $d_c = 1.28$ g cm^{-3}. Intensity data were collected with a Syntex $P2_1$ diffractometer (Cu Kα, $\gamma = 1.54178$ Å) in the θ–2θ mode with range $3.0° \leq 2\theta \leq 135°$ and scan speeds of 2.93–29.3°/min depending on the intensity of the reflection. The crystal was twinned with each twin having equally strong reflections, related by a 180° rotation about the C axis. This results in the superimposition of equivalent reflections at $l = 0$ and the superimposition of nonequivalent reflections at $l = 3$ and 6. Reflections having $l = 3$ or 6 were therefore deleted from the data set and the measured intensities of reflections at $l = 0$ were halved. Lorentz and polarization factors were applied, but no absorption correction was made ($\mu = 23.15$ cm^{-1} for Cu K_α radiation). After data reduction, 1824 independent reflections [$F > 3.0\sigma(F)$] were retained for the refinement of

Table AI: Positional Parameters × 10^4 (Standard Deviations in Parentheses)

	parameters × 10^4		
	x	*y*	*z*
P	6700 (1)	8520 (1)	1595 (3)
S	8084 (1)	7807 (1)	3206 (3)
O(1)	5931 (3)	7693 (2)	772 (5)
O(2)	6991 (3)	8756 (3)	−596 (6)
O(3)	5884 (3)	9531 (2)	2477 (6)
C(1)	5557 (4)	7872 (4)	−1296 (9)
C(2)	6601 (6)	8079 (5)	−2134 (10)
C(3)	5164 (4)	6952 (4)	−2361 (9)
O(4)	4878 (3)	7072 (3)	−4211 (6)
O(5)	5119 (3)	6183 (2)	−1445 (6)
N(1)	3892 (4)	9166 (4)	4166 (9)
C(11)	2724 (4)	9283 (3)	3095 (9)
C(12)	1946 (5)	8920 (5)	4396 (10)
C(13)	765 (5)	8985 (6)	3279 (12)
C(14)	899 (6)	8349 (6)	1280 (12)
C(15)	1660 (6)	8731 (6)	−4 (11)
C(16)	2851 (5)	8663 (5)	1094 (9)
N(2)	5762 (4)	5596 (3)	2567 (8)
C(21)	7061 (4)	5268 (4)	3036 (10)
C(22)	7379 (5)	4555 (5)	4758 (11)
C(23)	8706 (7)	4238 (6)	5260 (14)
C(24)	9360 (6)	3668 (6)	3424 (18)
C(25)	9012 (6)	4369 (7)	1675 (16)
C(26)	7681 (6)	4696 (6)	1190 (12)

Table AII: Bond Distances (in Å) (Standard Deviations in Parentheses)

P-S	1.932 (2)	P-O(1)	1.639 (4)
P-O(2)	1.625 (5)	P-O(3)	1.489 (3)
O(1)-C(1)	1.446 (7)	O(2)-C(2)	1.433 (8)
C(1)-C(2)	1.521 (9)	C(1)-C(3)	1.506 (7)
C(3)-O(4)	1.276 (7)	C(3)-O(5)	1.232 (7)
N(1)-C(11)	1.492 (7)	N(2)-C(21)	1.500 (7)
C(11)-C(12)	1.530 (9)	C(21)-C(22)	1.498 (10)
C(11)-C(16)	1.503 (8)	C(21)-C(26)	1.525 (10)
C(12)-C(13)	1.529 (9)	C(22)-C(23)	1.536 (10)
C(13)-C(14)	1.511 (11)	C(23)-C(24)	1.537 (13)
C(14)-C(15)	1.522 (12)	C(24)-C(25)	1.504 (14)
C(15)-C(16)	1.533 (9)	C(25)-C(26)	1.538 (9)
C-H	0.82 (6)-1.14 (4)	N-H	0.84 (6)-1.14 (6)

Table AIII: Bond Angle (deg) (Standard Deviations in Parentheses)

O(1)-P-S	112.4 (1)	O(2)-P-S	112.9 (2)
O(1)-P-O(2)	95.3 (2)	O(3)-P-S	117.6 (2)
O(1)-P-O(3)	107.7 (2)	O(2)-P-O(3)	108.6 (2)
P-O(1)-C(1)	108.9 (3)	P-O(2)-C(2)	111.7 (4)
O(1)-C(1)-C(2)	103.6 (4)	O(2)-C(2)-C(1)	103.8 (5)
O(1)-C(1)-C(3)	113.0 (4)	C(2)-C(1)-C(3)	115.0 (5)
O(4)-C(3)-C(1)	114.0 (5)	O(5)-C(3)-C(1)	120.4 (5)
O(4)-C(3)-O(5)	125.6 (5)		
N(1)-C(11)-C(12)	109.6 (5)	N(2)-C(21)-C(22)	110.1 (5)
N(1)-C(11)-C(16)	110.7 (4)	N(2)-C(21)-C(26)	109.5 (5)

endocyclic C-C-C angles 109.3 (6)-112.4 (5)

the structure. The positions of the phosphorus and sulfur atoms were determined by Patterson methods. All other atoms, including hydrogen, were located in difference–Fourier maps. The hydrogen atoms, together with isotropic temperature factors, were included in the refinement. Anisotropic temperature factors were introduced for all other atoms. After several cycles the refinement converged to a final value of $R = 0.064$. In the last cycle of refinement the ratio of the shifts to the standard deviation was smaller than 0.01 for all parameters, and a final difference synthesis displayed no peak above 0.30 e $Å^{-3}$.

The crystal structure determination (Figure 1 and Tables AI–AIII) showed that the major isomer of 4-carboxy-2-hydroxy-2-thio-1,3,2-dioxaphospholane is that having the carboxyl group and the sulfur atom on the same side of the five-membered ring. The geometry of this molecule is similar to analogous five-membered cyclic phosphorothioate esters: 2-hydroxy-4-methyl-2-thio-1,3,2-dioxaphospholane (Mikolajczyk et al., 1976) and uridine cyclic 2′,3′-phosphorothioate (Saenger & Eckstein, 1970). The distances from the least-squares plane (P, −0.127; O(1), 0.137; C(1), −0.230; C(2), 0.229; O(2), −0.139 Å) indicate that the ring has a semichair conformation. As observed for the other cyclic phosphorothioates, there is a significant elongation of the P=S bond to 1.932 Å [cf. 1.89 for 2-chloro-2-thio-1,3,2-dioxaphospholane (Lee et al., 1970)] and a shortening of the P—O single bond to 1.489 Å [cf. 1.54 for P—OMe single bond in 2-methoxy-1,3,2-dioxaphospholane (Chiu & Lipscomb, 1969)]. This has been attributed to some contribution from the mesomeric form of the monothio acid anion having a negative charge on sulfur (Mikolajczyk et al., 1976). Some charge delocalization is also observed at the carboxylate group where the formal double bond is lengthened to 1.232 Å and the single bond is shortened to 1.276 Å [cf. 1.21 Å for C=O and 1.31 Å for C—OH in $HOCH_2COOH$ (Golic & Speakman, 1965)]. The difference between the two carbon–oxygen bond lengths is still significant and, unlike several oxalate salts (Beagley & Small, 1964), the negative charge is not fully delocalized.

The two cyclohexylammonium cations show no significant differences and are in the chair conformation. Both cations use all three ammonium hydrogen atoms in hydrogen bonding to the phosphorothioate and carboxylate oxygens, thereby forming an extended intermolecular hydrogen-bonded network.

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