Chiral [^{16}O,^{17}O,^{18}O]Phosphate Monoesters. Asymmetric Synthesis and Stereochemical Analysis of [1(*R*)-^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol

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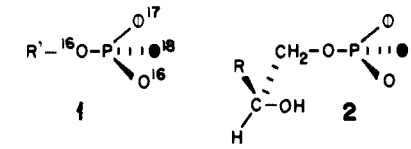
Abstract: To discover the stereochemical consequences of phosphoryl transfer reactions involving phosphate monoesters, a general method for establishing whether retention or inversion occurs at phosphorus is presented. We report here the synthesis of [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol, and an independent evaluation of the absolute configuration of the phosphoryl group in this compound. [^{17}O]-$POCl_3$ reacts with (−)-ephedrine to give predominantly one cyclic chloro adduct, which produces a cyclic phosphoramidate diester on reaction with 2-benzyl-(*S*)-propane-1,2-diol. Ring opening in $H_2{}^{18}O$ followed by catalytic debenzylation gives the title compound. The configuration at phosphorus in this molecule was determined by ring closure to the cyclic diester, followed by methylation with diazomethane and separation of the resulting "syn" and "anti" isomers. Reaction of the separated isomers with methanol followed by linked-scan metastable ion mass spectrometry then allows the absolute configuration at phosphorus to be defined. This analysis shows that our synthetic material is indeed *R* at phosphorus, of 91 ± 8% enantiomer excess.

Introduction

Despite an impressive body of kinetic and mechanistic study on the reactions of phosphate esters both in chemical and in biochemical systems,[2] many of the details of these reactions are still clouded by uncertainty. Thus, although the kinetic effects of changes in substrate structure, temperature, solvent, nucleophile, pH, and ionic strength have all been quite thoroughly investigated, such fundamental questions as the involvement of free monomeric metaphosphate in reactions of phosphate monoesters[3] or the intermediacy of phosphoryl enzymes in reactions catalyzed by phosphokinases[4] cannot be answered unambiguously.

In carbon chemistry, the impact of stereochemistry on mechanistic thinking has been immense, and our views of likely reaction pathways (for instance, of nucleophilic reactions at saturated carbon) would be woolly and tentative without the clarifying and uncompromising results of stereochemical studies. Yet compared with carbon, phosphorus has a much richer stereochemistry, the study of which has already illuminated a number of mechanistic problems.[5] One major area that has remained untouched, however, is that of phosphate monoester chemistry. The ubiquity of phosphorylated substrates in intermediary metabolism and the central importance of phosphorylation–dephosphorylation processes in the transfer and control of energy in biological systems make the mechanistic details of phosphoryl group transfer reactions of special interest. The aim of the present work is to discover the stereochemical consequences *at phosphorus* of reactions in which a phosphoryl group is transferred inter- or intramolecularly.

In order to follow the stereochemistry of reactions of phosphate monoesters the phosphoryl group must be made chiral, which requires that the three formally equivalent oxygen atoms be made distinguishably different. One approach to this problem that has already been taken involves the use of ^{16}O, ^{18}O, and sulfur as the three peripheral atoms. It has been found that many kinases will tolerate phosphorothioates as substrates[6] (even though they react more slowly than the corresponding phosphates) and considerable success has attended the use of these materials as probes of the stereochemical course of the phosphokinases.[7] Phosphorothioates must be used circumspectly, however, since Cohn has demonstrated[8] that the apparent stereochemical course of some kinase reactions can be inverted by changing the essential divalent cation. Moreover, not all phosphate-transferring enzymes (notably some phosphatases[9] and phosphomutases[10]) will handle the sulfur-containing materials. For these and other reasons, therefore, we adopted a second approach, which is to use the three stable oxygen isotopes ^{16}O, ^{17}O, and ^{18}O to create the desired chirality at phosphorus (**1**). This is a challenging objective both in terms of the synthesis and of the analysis of the chiral phosphoryl group, but the isotopic substitution does not, of course, perturb either the chemistry or the enzymology of the labeled species.



We report here the synthesis of a [^{16}O,^{17}O,^{18}O]phosphate monoester of defined configuration at phosphorus, and (since in the absence of a stereoanalytical method the chirality of the synthesized ester would remain cryptic) an independent analysis of its absolute configuration. A preliminary report of part of this work has been published.[11]

Results and Discussion

The aims of the work reported here were (a) to synthesize a phosphate monoester of one configuration, the ester being chiral at phosphorus by virtue only of the three stable isotopes of oxygen, ^{16}O, ^{17}O, and ^{18}O; and (b) to devise an independent method for the determination of the absolute configuration of the monoester and the quantitative assessment of the enantiomeric excess at phosphorus. In order that the stereochemical consequences of chemical and enzymic reactions of phosphate esters could subsequently be studied, it was important that both the synthetic and the stereoanalytical methods be general ones, applicable to a range of phosphate monoesters. The first priority was to design a viable stereoanalytical method, and this is discussed first.

Basis of the Stereoanalytical Method. We chose at the outset a method that would provide the absolute configuration and a quantitative estimate of the enantiomeric excess at phosphorus.

In the first step of the analysis, one of the peripheral (unsubstituted) oxygen atoms of the phosphate monoester is removed stereospecifically. A ring-closure reaction is a convenient way of effecting this removal, which is readily achieved if R′ (in **1**) contains a second hydroxyl group (e.g., **2**). Removal

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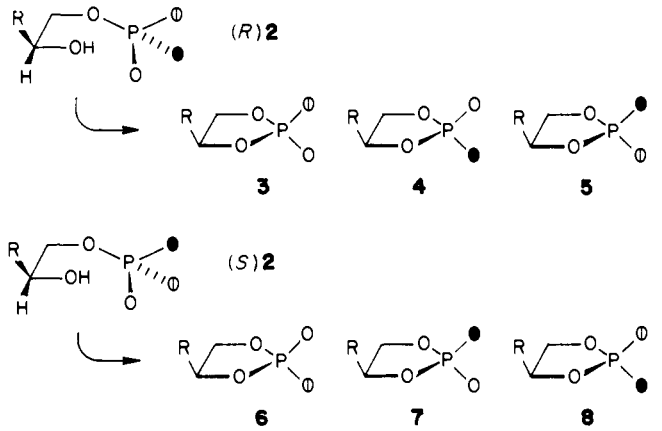


Figure 1. Sets of 1,2-cyclic diesters derived by "in-line" ring closure of 1(*R*)-phospho compound (giving **3–5**) and of 1(*S*)-phospho compound (giving **6–8**).

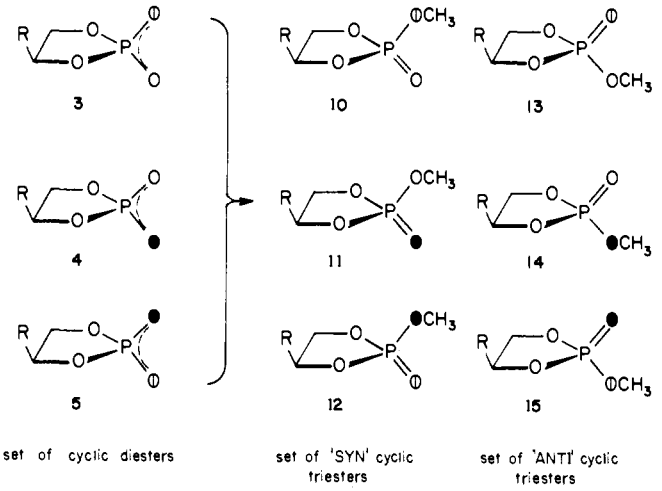
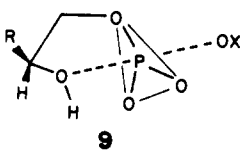


Figure 2. Sets of "syn" and "anti" cyclic triesters derived from methylation of **3–5**.

of ^{16}O, ^{17}O, or ^{18}O (by a route whose stereochemical course is known) then results in a set of three cyclic phosphate diesters (2-hydroxy-2-oxo-1,3,2-dioxaphospholanes). In the absence of kinetic isotope effects (which will in any case be very small), the three cyclic esters from **2** will be formed in equal amounts. The two remaining peripheral oxygens in the cyclic diester are distinguishable if the 1,2-diol itself has a chiral center. In this case, the peripheral oxygens in the cyclic ester are diastereotopic; one is "syn" and the other is "anti" to the R substituent. These relationships are shown in Figure 1, where the diastereoisomeric monoester (*R*)-**2** gives rise to the three cyclic diesters **3, 4,** and **5** if the ring-closure reaction is an "in-line" process (in which the entering oxygen of the 1,2-diol and the leaving peripheral oxygen are on opposite sides of the phosphorus; see **9**). Analogously, "in-line" ring closure of (*S*)-**2** gives rise to **6, 7,** and **8** (see Figure 1).



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[It may be noted that the first step in the analysis is conceptually related to that used in the stereochemical analysis of a chiral [^1H,^2H,^3H]methyl group.[12] One of the hydrogens on the chiral carbon atom is removed stereospecifically to give three products, that have lost either ^1H, ^2H, or ^3H. In this case,

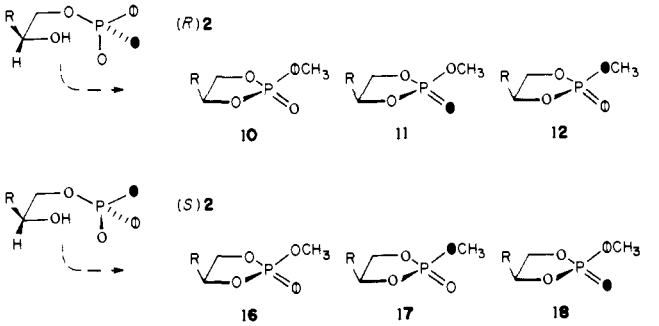


Figure 3. Sets of "syn" cyclic triesters derived by (a) "in-line" ring closure, (b) methylation, and (c) separation of "syn" from "anti" isomers.

Table I. Masses of Parent Ions and Daughter Ions (after Loss of Formaldehyde from the Exocyclic Methoxyl Group) of the Cyclic Triesters Shown in Figure 3

	mass of parent ion[a]	mass of daughter ion[b]
From the (*R*)-Phosphate Ester		
10	$m+1$	m'
11	$m+2$	$m'+2$
12	$m+3$	$m'+1$
From the (*S*)-Phosphate Ester		
16	$m+1$	$m'+1$
17	$m+2$	m'
18	$m+3$	$m'+2$

[a] m is the mass of the parent triester if all the oxygen isotopes were ^{16}O. [b] m' is the mass of the daughter ion (after loss of the $-{}^{i}OCH_3$ group) if all the oxygen isotopes were ^{16}O.

however, the primary kinetic isotope effects involved in the hydrogen removal step result in the formation of the three products in *unequal* amounts, which allows the products to be analyzed directly. In the case of a [^{16}O,^{17}O,^{18}O]phosphate ester, the absence of a useful primary isotope effect in the ring-closure reaction means that a more circuitous path must be taken to solve the analytical problem (see below).]

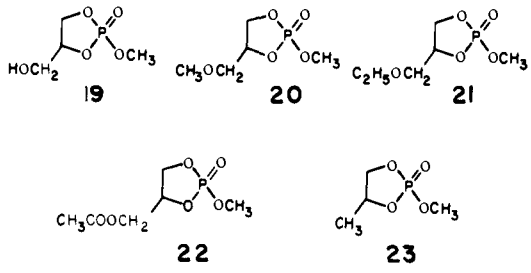
The second step in the analysis involves the accentuation of the difference between the "syn" and "anti" oxygens in the sets of cyclic esters (**3–5** or **6–8**). To make these oxygens *chemically* different rather than merely topologically different, the cyclic diesters are methylated with diazomethane. In general, either of the peripheral oxygens can be methylated, and from **3–5** we obtain **10–15** (Figure 2). Triesters **10, 11,** and **12** derive from methylation of the "syn" oxygen of **3, 4,** and **5** (respectively), and **13, 14,** and **15** come from the alkylation of the "anti" oxygen. Since the "syn" triesters (**10–12**) are diastereoisomeric with respect to the "anti" triesters (**13–15**), the two sets of isomers can be separated from each other chromatographically. This is the third step of the analysis. In Figure 3 we illustrate the "syn" set of triesters (**10–12**) from **3–5** (which in turn derived from (*R*)-**2**), and the "syn" set of triesters (**16–18**) from **6–8** (which in turn derived from (*S*)-**2**). It is evident from a comparison of Figure 3 with Figure 1 that the methylation–separation sequence puts a chemical "handle" (the methyl group) on the "syn" and the "anti" oxygens of the compounds in Figure 1.

In the fourth step of the analysis, we have to differentiate between the two "syn" sets of cyclic methyl triesters illustrated in Figure 3. (We illustrate only the "syn" sets in Figure 3, but the problem is analogous for the "anti" sets.) The two "syn" sets differ only in the disposition of the isotopic labels, and the mass spectra of each set would be identical. That this is true is evident from Table I: the mass spectrum of a 1:1:1 mixture of **10, 11,** and **12** will contain parent ions of the same *m/z* as

a 1:1:1 mixture of **16, 17,** and **18**. Moreover, if we look at the three putative daughter ions deriving from loss of the $-^{i}OCH_3$ group from the parent ions, the same pattern of daughter ions will be produced by each mixture. Simple mass spectrometry cannot therefore distinguish between the two sets of "syn" isomers shown in Figure 3. The two sets of "syn" isomers *do* differ, however, *in the relationships between individual daughter ions and their parents*. Thus **12,** which has a parental mass of $m + 3$, produces a daughter ion of mass $m' + 1$, whereas the corresponding $m + 3$ parent ion in the other set, **18**, produces a daughter ion of mass $m' + 2$ (Table I and Figure 3). Analogously, the parental ions of **11** and **17** ($m + 2$) and of **10** and **16** ($m + 1$) also give daughter ions of different masses. If, therefore, individual parent-daughter relationships can be established, an absolute and quantitative discrimination between **10 + 11 + 12** and **16 + 17 + 18** can be made. Metastable-ion[13,14] (MI) or collisional activation[15] (CA) mass spectrometry can establish the relationship between parent and daughter ions. These methods require only that the appropriate fragmentation process can be measured with sufficient sensitivity and with insignificant interference from other fragmentation or isotopic scrambling processes. We therefore need a fragmentation of the cyclic triesters shown in Figure 3 in which one of the two exocyclic oxygens is lost: this can in principle be effected by cleavage of the P-OCH_3 bond.

The above describes the basis of the stereoanalytical method, the practice of which is now outlined.

Realization of the Stereoanalysis. Since one of our main goals was to determine the stereochemical course of enzyme-catalyzed phosphoryl transfer reactions, the ideal R group (in **2**) would be structurally simple, and derive from an accessible intermediary metabolite into which a range of phosphate esters could be readily transformed. The compound must also, however, have suitable chemical and mass spectrometric properties. Although the hydroxymethyl group is an obvious candidate for R (the key metabolite would then be *sn*-glycerol 3-phosphate), glycerol phosphate cannot be used. Ring closure



followed by methylation (the first two steps of the analysis) gives the cyclic methyl triester **19,** which, like all such compounds, is extremely sensitive to nucleophiles. The triester **19** decomposed in all but very dilute solutions (presumably the hydroxyl group of one molecule attacks the triester group of another), and this precluded the separation of the "syn" and "anti" diastereoisomers (the third step of the analysis) of this material. In the expectation that for compounds **20–23** there would be no serious problems with the first three steps of the analysis (cyclization, alkylation, and separation of diastereoisomers), their mass spectrometric fragmentation behavior was investigated.

As discussed above, to relate the parent ions to their daughters, the fourth step of the analysis requires an MI or CA fragmentation in which the exocyclic $-OCH_3$ group is lost (since we have "labeled" the "syn" oxygen of the cyclized materials **3–8** by methylation). Such a fragmentation would involve loss of CH_2O or CH_3O. In order to define the structures of the fragment ions from compounds **20–23**, especially of those ions that have lost the exocyclic $-OCH_3$ group, the exocyclic trideuteriomethoxyl analogues of **20–23** were also

Table II. Ions Formed in the Fragmentation of **20–23**[a]

compd	M^+	fragment ions
20	182	183 (186) 10%; 167 (170) 8%; 152 (155) 100%; 139 (142) 34%; 137 (140) 33%; 113 (116) 60%; 109 (112) 60%; 96 (99) 31%; 95 (98) 39%; 79 (82) 91%
21	196	197 (200) 8%; 167 (170) 6%; 166 (169) 10%; 152 (155) 85%; 139 (142) 24%; 138 (141) 25%; 137 (140) 24%; 113 (116) 87%; 95 (98) 100%; 79 (82) 58%
22	210	180 (183) 18%; 168 (171) 16%; 150 (153) 33%; 138 (141) 100%; 137 (140) 90%; 113 (116) 30%; 95 (98) 28%; 79 (82) 65%; 43 (43) 95%
23	152	152 (155) 15%; 137 (140) 28%; 123 (126) 3%; 122 (123) 5%; 113 (116) 58%; 96 (99) 54%; 79 (82) 100%

[a] The numbers listed for the fragment ions are: mass (mass of corresponding ion derived from the exocyclic trideuteriomethyl compound), percentage intensity of base peak.

synthesized. The major fragments from these materials are listed in Table II.[16] Unfortunately, it is clear that the 1-*O*-methyl-, 1-*O*-ethyl-, and 1-*O*-acetylglycerol 3-phosphates yield cyclized triesters (**20, 21,** and **22**) whose M^+ ions do *not* exhibit suitable fragmentations. Although each of the parent ions produce daughter ions of mass $(M - 30)^+$ or $(M - 31)^+$, these cannot involve the loss of CH_2O or CH_3O from the exocyclic methoxyl group, as the trideuterio (exocyclic $-OCD_3$) analogues do not show a corresponding loss of CD_2O or CD_3O.[16]

In the case of the propane-1,2-diol derivative, **23,** however, there is an $(M - 30)^+$ daughter ion at m/z 122 that clearly derives from the loss of the exocyclic methoxyl group, as this peak only shifts to m/z 123 in the exocyclic $-OCD_3$ derivative (see Table II). Unfortunately, there is in the MI spectrum a more abundant m/z 152 → 123 transition that does *not* involve the exocyclic methoxyl group. This interferes with measurement of the MI transitions corresponding to the m/z 152 → 122 fragmentation for the set of ions (e.g., **10 + 11 + 12**) deriving from a chiral [^{16}O,^{17}O,^{18}O]ester, using a normal double-focusing mass spectrometer. (While such interference should be much less serious using a "reversed-geometry" instrument,[16] normal double-focusing mass spectrometers are much more widely available, so that a method using such equipment would be more generally useful.)

We next sought a daughter ion of **23** that still contained both the exocyclic methoxyl group and the exocyclic P=O group, which would fragment to a granddaughter ion by loss of $-OCH_3$. Such a secondary transition of a daughter ion could be suitable, provided that the structural identity of the two exocyclic oxygen atoms of that daughter have not been compromised in its formation. A thorough investigation of all the progeny ions from **23** was therefore undertaken. (Many of the dominant daughter ions of **23** are the same as those from **20–22**,[16] so a search of the second-generation fragmentations of **23** would render separate investigation of **20–22** unnecessary.) Using both the high-voltage (HV) scan[14] and the "linked-scan" mode[17] with the normal double-focusing instrument, the major MI and CA fragmentation pathways of **23** were determined (see Table III). Of the transitions that involve loss of the exocyclic methoxyl group, most are compromised by the existence of an interfering neighboring transition, which rules out the use of m/z 152 → 123, 152 → 122, 123 → 93, 122 → 92, 112-109 → 82-79, 96 → 66, and 95 → 65 (see Table III). Additionally, the fact that the "best" obtainable $H_2{}^{17}O$ contains about 25% ^{18}O and 25% ^{16}O (this leads, in a chiral ^{16}O,^{17}O,^{18}O molecule, to the existence of some $m + 4$ ions from species that should contain ^{17}O and ^{18}O but

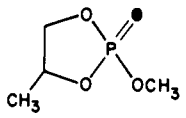
Table III. M1 Fragmentations from Ions Produced by **23**

parent ion	daughter ion (intensity)[a]
152	137 (w), 123 (w), 122 (vw), 113 (s), 112 (w), 111 (w), 110 (w)
137	109 (s), 107 (w)
123	93 (vw)
122	92 (vw)
113	95 (m), 82 (vw), 81 (vw), 82 (s, CA), 81 (s, CA)
112	82 (s, CA)
111	81 (s, CA)
110	80 (s, CA)
109	79 (s, CA)
96	66 (w)
95	65 (w)
79	49 (w), 49 (s, CA)

[a] w, weak; m, medium; s, strong; CA, collisional activation.

actually contain two ^{18}O) also makes the *m/z* 113 → 82 and 113 → 81 transitions undesirable for normal double-focusing instruments. This leaves two possibilities: *m/z* 137 → 107 and 79 → 49.

If these daughter to granddaughter fragmentations are to be useful, the methyl group must not migrate from one exocyclic oxygen to the other during the generation or decomposition of the daughter ion, else all the stereochemical information would be lost. To investigate this point, the specifically ^{18}O-labeled compounds **24** and **25** were prepared. If the methyl



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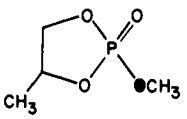

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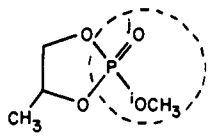
group does not migrate between the exocyclic oxygens in the mass spectrometer, then the *m/z* 137 → 107 transition for the unlabeled compound **23** will become exclusively *m/z* 139 → 109 (for **24**) and 139 → 107 (for **25**). Analogous differences will be seen for the *m/z* 79 → 49 transition. However, the measured transitions, both M1 and CA, from the *m/z* 139 daughter ion of *either* **24** or **25** produced granddaughter ions at *m/z* 107 and 109 of *equal intensity*. The *m/z* 79 → 49 transition behaved analogously. We must conclude, therefore, that the methyl "label" on the exocyclic oxygen in **24** and **25** is equilibrated between the two exocyclic oxygens during the mass spectrometric analysis. None of the fragmentations investigated, either parent to daughter or daughter to granddaughter, can therefore be used to discriminate between the two sets of cyclic triesters in Figure 3, **10–12** and **16–18**.

All is not lost, however. Consider again the strategy that we are using to distinguish between the two exocyclic oxygens. It is clear from Figure 2 that, *once the separation of the "syn" and "anti" sets of methylated cyclic esters has been achieved, the three-carbon propanediol unit serves no further purpose.* After the separation of "syn" and "anti" isomers (of Figure 2) all the stereochemical information is contained in the encircled fragment shown in **26**. From any such fragment, we only need to know the fragment mass and which oxygen isotope (^{l}O or ^{J}O) in it is methylated. The feasibility of this course was investigated by studying the mass spectrum of **27**, which would derive from methanolysis of the cyclic triester (e.g., **26**). It was



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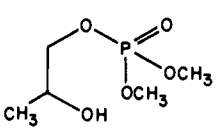

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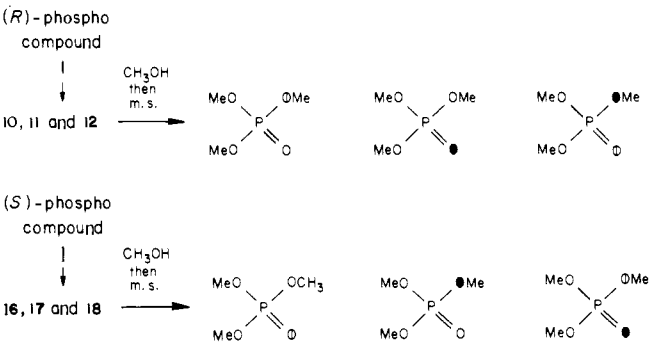
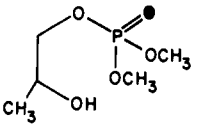


Figure 4. Labeling patterns in the trimethylphosphate ions that derive from the two sets of "syn" cyclic triesters after methanolysis.

found that a daughter ion of *m/z* 140, which appears to be the trimethyl phosphate ion (see below), fragmented with loss of formaldehyde to a granddaughter ion at *m/z* 110. Moreover, the *m/z* 140 → 110 fragmentation is attended by a strong metastable transition in the absence of any collisional activation. This transition is uncomplicated by fragmentations from ions of *m/z* 136 to 139 and 141 to 144. When the labeled triester **28** was studied, the appropriate transition (*m/z* 142 →



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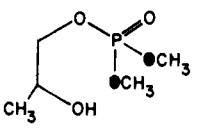

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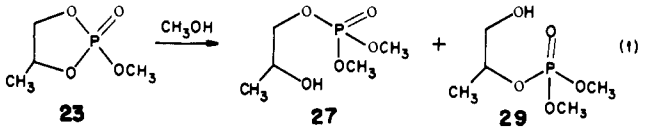
112) was observed. Less than 0.1% of the *m/z* 142 → 110 transition could be detected, showing that the methyl group was not migrating to the ^{18}O label. Finally, since in any real analysis involving methanolytic ring opening of the cyclic triester (**23**), we expect to generate both the 1 and 2 isomers of phosphopropanediol (**27** and **29**; see eq 1), authentic **29** was



23 **27** **29** (1)

synthesized and its mass spectral behavior compared to that of **27**. The two spectra were very similar; the *m/z* 140 ion is also formed from **29** and fragments in a manner identical with the *m/z* 140 ion generated from **27**. This eliminated any need to consider the separation of **27** and **29** after methanolysis.

Two further checks were required before the *m/z* 140 → 110 transition could be accepted. First, it was shown that the third methoxyl group of the trimethylphosphate ion does not arise from *inter*molecular transfer of methyl or of methoxyl groups. (Such processes would vitiate the analysis by scrambling methyl groups among phosphoryl oxygens or methoxyl groups between trimethylphosphate species.) This was shown from the mass spectrum of the products from ring opening of the cyclic triester **23** in CD_3OD (cf. eq 1), which gave a peak at *m/z* 143 corresponding to $O{=}P(OCH_3)_2OCD_3^+$ and none at *m/z* 146 from $O{=}P(OCD_3)_2OCH_3^+$. So, in the trimethylphosphate ions studied (Figure 4), one methyl group is the one that "labeled" the "syn" exocyclic oxygens in the cyclic esters of Figure 3, the second methyl group comes from the methanol used to open the cyclic esters (eq 1 and Figure 4), and the third methyl group derives from the *intra*molecular fragmentation of the propanediol skeleton. Secondly, it was shown that the three methoxyl groups of *m/z* 140 are equivalent, and that oxygen isotope effects in the fragmentation of labeled trimethylphosphate ions are negligible. The CA mass spectrum of the *m/z* 140 ion derived from **27** was identical with that from ionized trimethylphosphate, showing that the ions from both

Table IV. Predicted Fragmentation Patterns from the Two Sets of Trimethylphosphate Ions Shown in Figure 4

set of ions derived from	parental mass[a]	daughter ion mass (rel intensity)[b]
SYN set from (*R*)-phospho	141	111 (2) + 110 (1)
or	142	112 (3)
ANTI set from (*S*)-phospho	143	113 (2) + 111 (1)
SYN set from (*S*)-phospho	141	111 (3)
or	142	112 (2) + 110 (1)
ANTI set from (*R*)-phospho	143	113 (2) + 112 (1)

[a] The parental mass of the all-^{16}O ion is 140. [b] On the basis that each isotope enrichment is 100%.

sources have the same structure.[15] Further, the products from the methanolysis of the ^{18}O-labeled ester **25** in CH_3OH gave a *m/z* 142 ion whose MI spectrum has peaks at *m/z* 112 (minus $CH_2{}^{16}O$) and 110 (minus $CH_2{}^{18}O$) in the ratio 2:1, which is expected only if the three methoxyl groups are completely equivalent and are lost with equal ease. This result was confirmed using a sample containing the doubly labeled species **30,** where the *m/z* 144 → 114 and 144 → 112 transitions occurred in a ratio of 1:2.

From the experiments described above, it appeared that metastable ion mass spectrometry of the transitions from the daughter ions based on *m/z* 140 would satisfy all the requirements for the quantitative analysis of the methanolysis products from **10** + **11** + **12** and from **16** + **17** + **18**. Assuming for the moment that each of the isotopic labels is enriched to 100%, the trimethylphosphate ions deriving from ring opening of **10–12** and **16–18** in $CH_3{}^{16}OH$ are as shown in Figure 4. The set of ions deriving from the "syn" cyclic esters **10–12** (from an (*R*)-phospho compound) are, of course, complementary to the set from the "syn" cyclic esters **16–18** (from an (*S*)-phospho compound). But it will also be noticed that the "anti" cyclic esters from an (*R*)-phospho compound (**13–15**; see Figure 2) have the *same* isotopic distribution as the "syn" set from an (*S*)-phospho compound (**16–18**) in terms of an analysis of the derived trimethylphosphate ions. This means that a comparative analysis of "syn" and of "anti" isomers that derive from a chiral phosphate monoester of one configuration provides precisely the same analytical information as does a comparison of the two "syn" sets from phosphate monoesters of opposite configuration. Any experimental analysis of "syn" and "anti" sets from a phosphate ester of one configuration thus provides an internal check on the integrity of the analysis. The anticipated daughter to granddaughter mass relationships for the various sets of trimethylphosphate ions are shown in Table IV. This establishes the basis for the analysis.

Stereoselective Synthesis of [^{16}O,^{17}O,^{18}O]Phosphate Monoesters. The synthesis of [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol is outlined in Figure 5. Two principles governed the design of this scheme: first, the synthetic method should be a general one that would readily allow the synthesis of a range of phosphorylated intermediary metabolites such as γ[^{16}O,^{17}O,^{18}O]-ATP; secondly, the oxygen isotopes should be introduced from $H_2{}^{i}O$ with minimal dilution of label. The method is based upon the chemistry developed by Inch and his group.[18]

[^{17}O]-$POCl_3$ was prepared in 85% yield from water enriched in ^{17}O and allowed to react with (−)-ephedrine to yield the two chloro adducts **31** and **32** (Figure 5). These materials could be separated chromatographically or the major isomer (**31**) could be fractionally crystallized, but more conveniently the mixture was allowed to react with 2-benzyl-(*S*)-propane-1,2-diol to give **33** and **34,** which were separated chromatographically to obtain yields of 65 and 7% (based on $POCl_3$), respectively. The ^1H NMR spectra of **31, 33,** and **34** are consistent with the

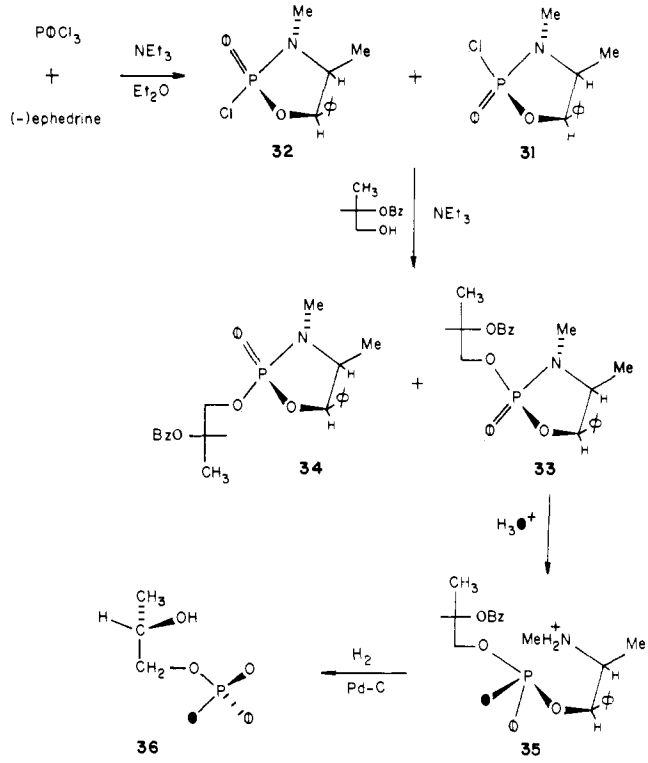


Figure 5. Stereoselective synthesis of [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol: ^{16}O ≡ O; ^{17}O ≡ Φ; ^{18}O ≡ ●

structures shown (see the analysis of Inch and co-workers[18,19]). As has been frequently observed, the alcoholysis proceeds with retention of configuration at phosphorus.[18] [When racemic 2-benzyl-(*RS*)-propane-1,2-diol was allowed to react with **31**, the product shows two sets of peaks from NCH_3 in the ^1H NMR and two equally intense signals in the ^{31}P NMR. Using **31** as reactant, the NMR spectra allowed an estimate of the enantiomeric excess of the starting 2-benzylpropane-1,2-diol, which was 78%.] Acid-catalyzed hydrolytic ring opening of purified **33** in $H_2{}^{18}O$ yields **35,** which (by analogy with the acid-catalyzed alcoholysis reaction[18]) is presumed to proceed by "in-line" displacement. To prevent any dilution of the ^{18}O label, the aqueous acid solution was prepared from 0.5 equiv of trifluoroacetic anhydride in 30 molar equiv of $H_2{}^{18}O$. Hydrogenolysis of **35** in two steps yielded, after purification by ion-exchange chromatography, [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**36**) in 72% yield from **33**. (Test syntheses incorporating one isotopic label either from [^{18}O]-$POCl_3$ or from $H_2{}^{18}O$ in the acid-catalyzed ring-opening step showed that no label was lost at any stage of the synthesis.) The isotopic composition of the three peripheral oxygens of the product **36** was found to be ^{16}O, 42.9; ^{17}O, 15.8; ^{18}O, 41.3%. That predicted on the basis of the percentage enrichment of the labeled water samples used was ^{16}O, 42.8; ^{17}O, 15.8; ^{18}O, 41.4%.

It will be evident that the synthetic approach is a general one. Phosphate monoesters of either chirality at phosphorus can be synthesized, the chirality being determined simply by the order in which the ^{17}O and the ^{18}O are introduced. Further, the method should allow a range of phosphate esters to be synthesized. Indeed, phenyl [^{16}O,^{17}O,^{18}O]phosphate[20] and γ-[^{16}O,^{17}O,^{18}O]-ATP[21] have recently been synthesized in our laboratory.

Stereoanalysis. Chemical Aspects. The first step of the analytical method requires ring closure by a pathway of *defined and known* stereochemistry. It is self-evident that no loss or exchange of the isotopic labels on phosphorus is acceptable. On the basis of the known "in-line" ring closure of 2-hy-

Table V. Ring Closure of 1-[$^{18}O_1$]Phosphopropane-1,2-diol

reagent	conditions	^{18}O atom % excess: in starting material	^{18}O atom % excess: predicted in product (if no wash-out)	^{18}O atom % excess: obsd in product	% loss of ^{18}O label
$(EtO)_2POCl$	*a, b, c,* pyridine, 2 h	96	64	45	30
$(EtO)_2POCl$	*a, d, e,* CH_2Cl_2, 24 h	80	53	45	15
$(PhO)_2POCl$	*a, c, d, f,* CH_2Cl_2, 24 h, high dilution	80	53	50	6
Ph_3P, dipyridyl disulfide	*c, d, g,* dioxane	80	53	52	2
diphenylphosphorylimidazole	*a, c, d, f, h,* CH_2Cl_2, 16 h	80	53	52	2

[a] 1 equiv of reagent. [b] Cyclohexylammonium salt. [c] 25 °C. [d] Tri-*n*-octylammonium salt. [e] −78 °C. [f] Plus diisopropylethylamine (1.7 equiv). [g] 5 equiv of reagent(s). [h] In the presence of 4 Å molecular sieves.

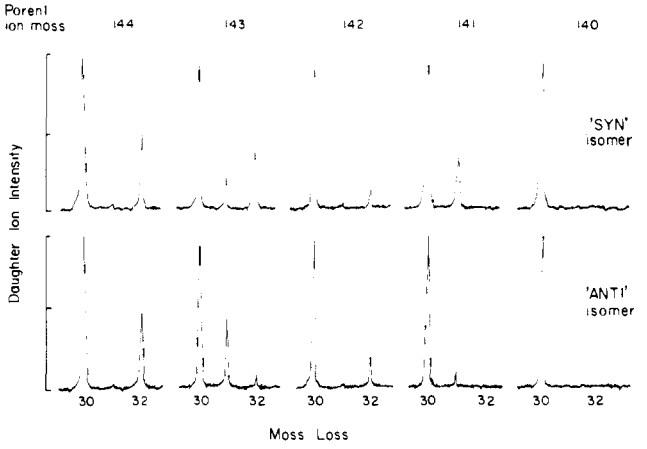


Figure 6. "Linked-scan" metastable ion mass spectra of the trimethylphosphate ions (the isotopically unlabeled ion has *m/z* of 140) that derive from the "syn" and "anti" cyclic triesters (**10–12; 13–15**) from the (*R*)-phospho compound **36** (see Figure 5).

droxy-1-phosphorothioates evaluated by Usher and Eckstein,[22] diethyl phosphochloridate–pyridine was investigated first. It was found, however, that when 1-[^{18}O]phosphopropane-1,2-diol was subjected to this procedure, the loss of the single ^{18}O label in the cyclization was 15% more than that predicted. The recovered starting material showed no label loss. Since the loss of 15% of one label could mean 15% loss of each of two labels, up to 30% of the stereochemical information could be lost in such a process. This was considered unsatisfactory. Diphenyl phosphochloridate–diisopropylethylamine was also tried under various conditions, with only somewhat better results. Accordingly, other chemical methods were investigated the stereochemistry of which was predicted to be by "in-line" pathways. Dipyridyl disulfide–triphenylphosphine[23] gave cyclized diester with essentially no loss of isotopic label (Table V), and the diester triethylammonium salt was methylated to give the "syn" and "anti" cyclic methyl triesters. Separation and stereoanalysis as outlined above suggested that there was indeed an excess of the *R* configuration in the phosphopropanediol starting material, though the estimated enantiomer excess was only 40%. Since it seemed possible that the ring-closure reaction was proceeding by the desired "in-line" pathway but that subsequent reaction of the product (the cyclic diester monoanion) with the ring-closure reagent was leading to the scrambling of the exocyclic oxygens of the cyclic diester, a more selective reagent was sought.

Diphenylphosphorylimidazole seemed likely to react more selectively (with the acyclic phosphate dianion rather than the cyclic phosphate monoanion), and, under conditions where cyclization by diphenyl phosphochloridate was complete in minutes, diphenylphosphorylimidazole gave >75% yields of cyclized product only after 12 h. Moreover, only 1.8% of isotopic label was lost (over that predicted) during the closure reaction using this reagent. Both on the basis of the preference rules[24] and by analogy with the diethyl phosphochloridate reaction with 2-hydroxy-1-phosphorothioates,[22] it is expected that diphenylphosphorylimidazole closure proceeds with "in-line" stereochemistry. That this is true experimentally is clear from what follows.

After ring closure, the cyclic diesters were methylated using diazomethane, and the "syn" and "anti" sets of isomers were separated by high-pressure liquid chromatography. The cyclic triesters are extremely sensitive to nucleophiles, and were treated with methanol immediately after separation. It is necessary, of course, that this reaction occurs with no loss of isotopic label (i.e., no exchange of methoxyl groups), and it was found possible to achieve this by treatment of the cyclic triesters with methanol–triethylamine at −78 °C. That this reaction proceeds as desired was confirmed by using CD_3OD for the methanolysis.

The synthetic [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**2**) was subjected to the sequence of ring closure, methylation, and separation as described above. The resulting "syn" and "anti" isomers (**10** + **11** + **12** and **13** + **14** + **15**) were then subjected to methanolysis (eq 1) and analyzed mass spectrometrically. The results of "linked-scan" metastable ion mass spectrometry on the fragmentation of the trimethylphosphate ions based on *m/z* 140 are shown in Figure 6. The quantitative analysis of these data is discussed below.

Stereoanalysis. Quantitative Aspects. The apparent enantiomeric excess at phosphorus as deduced from the intensities of the peaks in Figure 6 is affected by a number of factors, which require brief discussion.

A. Enantiomeric Excess of the Propane-1,2-diol. Since our analytical method relies upon the separation of diastereoisomers (the "syn" and "anti" sets of Figure 2), any inadequacy in the optical purity of the propanediol will be reflected directly in an *apparent* fall in the enantiomer excess at phosphorus as

deduced from Figure 6. The enantiomeric purity of the propanediol can, however, be quantified independently, from the ^{31}P NMR of **33**, from the specific rotation of the starting 2-benzylpropane-1,2-diol, or from the specific rotation of the 1-phosphopropane-1,2-diol. In the present experiments, the enantiomeric excess of the propane-1,2-diol was 78 ± 1%.

B. Imperfect Separation of "Syn" and "Anti" Diastereoisomers. Any cross contamination of the "syn" and "anti" sets of diastereoisomeric cyclic triesters shown in Figure 2 will also reduce the *apparent* enantiomeric excess at phosphorus, since "syn" from *S* (at phosphorus) analyzes equivalently to "anti" from *R* (at phosphorus). The cross contamination of "syn" and "anti" sets was higher in these first experiments owing to fears (since shown to be unjustified) of shortage of material for mass spectrometry. The contamination was, however, quantitated by GLC analysis of the samples of diastereoisomers resulting from the preparative high-pressure liquid chromatography. The "anti" isomer fraction contained 5 ± 2% of "syn" and the "syn" isomer fraction contained 15 ± 3% of "anti".

C. Isotopic Enrichment. While $H_2{}^{18}O$ is commercially available at 97% atom excess or better, the most highly enriched available $H_2{}^{17}O$ contains no more than 50% or so of ^{17}O. Exact knowledge of these levels is, of course, essential for the quantitative analysis of the results. In the present experiments, the ^{16}O:^{17}O:^{18}O fractional ratios in the $H_2{}^{17}O$ were 0.26:0.47:0.28, and in the $H_2{}^{18}O$ were 0.02:0.01:0.97. Each trimethylphosphate ion in the two sets of such ions (Figure 5) contains two out of the three original isotopic labels. In practice, there will be ions at $m + 4$ (m is 140) which contain two ^{18}O (one bona fide ^{18}O and one from the ^{17}O used) and give no stereochemical information. Ions at $m + 3$ *must* contain one ^{18}O and one ^{17}O, and the chance of these labels being the "correct" way round rather than the "incorrect" way round is 1.0:0.006. "Incorrect" ions at $m + 2$ can arise in a number of ways, and the ratio of "correct" to "incorrect" is 1.0:0.55. For $m + 1$ (ions containing only one ^{17}O), the ratio is 1.0:0.047. Ions at m are unlabeled and stereochemically uninformative. It is evident, then, that the $m + 3$ and $m + 1$ ions are the least contaminated by ions from undesired sources.

A second issue arises, however, with respect to label loss during synthesis or analysis, where ^{17}O or ^{18}O may be replaced by ^{16}O. Clearly, spurious $m + 1$ and $m + 2$ peaks may arise from label washout from $m + 3$ and $m + 4$, and this reduces the usefulness of the $m + 1$ and $m + 2$ ions. Even though there is no detectable label loss in the synthetic steps, the loss during the ring-closure step of the analysis is perceptible and it is desirable to study an ion whose stereochemical information content cannot be reduced by isotope washout. In summary, the ion at $m + 3$ provides the more precise information; $m + 1$ yields good information but is susceptible to (and has to be corrected for) label washout; $m + 2$ is the least precise of those ions that contain stereochemical information.

D. Isotopes of Elements Other Than Oxygen. The contribution of natural-abundance levels of ^{13}C and ^{2}H to the trimethylphosphate ions based on *m/z* 140 is easily evaluated. (In practice, only the consequences of an $m + 2$ ion contribution to $m + 3$ needs consideration, and the correction is but a small one.)

E. Cross-Talk. If the energy resolution in the mass spectrometer is not fine enough, there will be some contribution to a particular daughter ion intensity from a neighboring metastable peak. Experimentally, with the normal double-focusing mass spectrometer,[14,17] such cross-talk was reduced to less than 1% of the neighboring intensity by reducing the energy-resolving slit width to the minimum consistent with acceptable losses in ion intensity. A second type of cross-talk, not affected by the energy-resolving slit, occurs if there is substantial ion fragmentation in the accelerating region. This so-called "downward" cross-talk can be quantitated, and appropriate

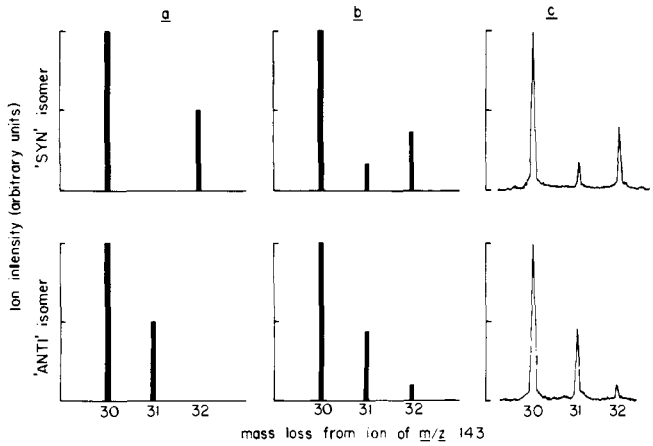


Figure 7. Metastable ion spectra for the ($m + 3$) ions at *m/z* 143 which come from the "syn" and "anti" cyclic triesters derived from **36**: (a) simplified spectra predicted for the fragmentation of the ion at *m/z* 143; (b) predicted spectra as in (a), taking into account the measured isomeric purity of the "syn" and "anti" cyclic triesters, the measured enantiomer excess of the starting diol, and the known contributions from the natural abundance of ^{13}C and ^{2}H; (c) observed spectra.

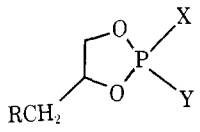
allowance made in the predicted spectra. In the present experiments, some 3% of the neighboring metastable ion intensity appears in the corresponding daughter ion region. (For a clear explanation of cross-talk, see ref 17c.)

On the basis of the five factors discussed above, a quantitative estimate of the metastable-ion spectrum can be made, and this is shown for the fragmentation of the $m + 3$ ion (*m/z* 143) in Figure 7b. The experimental spectra are also shown (Figure 7c) for the "syn" and "anti" isomers deriving from a phosphate ester of one chirality (*R*). The mass spectra were obtained by repeated slow scanning, and the relative peak intensities are precise to better than 3%. Comparison of these data (Figure 7c) with those predicted (Figure 7b) show that (a) the absolute configuration of the synthetic phosphoryl group is indeed *R*, and that (b) the enantiomer excesses are 91 ± 8% (from the analysis of "anti") and 90 ± 20% (from the analysis of the more cross-contaminated "syn"). The analysis is, for the reasons discussed, most accurately performed on the $m + 3$ ion, though the $m + 1$ and $m + 2$ ions provide less precise results in complete agreement with those from the $m + 3$ ion.

Validity of the Configurational Assignments. We are reporting here the stereoselective synthesis of a [^{16}O,^{17}O,^{18}O]-phosphate monoester that is *R* at phosphorus and an independent analysis of its absolute configuration as an *R* center. To an extent, the synthesis and the analysis are mutually supportive, and there would have to be the same number of errors in *both* the synthesis *and* in the analysis to be consistent with the results presented. It is as well, however, to summarize here the structural and chemical features on which both the synthesis and the analysis are based.

Synthesis. In the synthetic scheme (Figure 5), the assignment of configuration depends upon two facts: that the major isomer is **33** (not **34**), and that the ring-opening reaction **33** → **35** goes with "in-line" stereochemistry. That the configuration of **33** is as shown is confirmed by the ^1H NMR spectra of **33** and **34**,[18,19] by the formation of **33** from **31** (i.e., the major isomer is formed from the major isomer: the alcoholysis of five-membered cyclic phosphochloridates proceeds with retention[18,19]), and by the ^1H NMR spectrum of the product of **31** and phenol[25] (en route to phenylphosphate[18,23]). Secondly, the "in-line" stereochemistry of the hydrolytic ring opening of the cyclic phosphoramidate **33** is not only predicted by the preference rules,[24] but is known to proceed in this way in alcoholysis.[18] The final reductive steps of the synthesis (**35** →

Table VI. Boiling Points and Yields of Substituted 1,3,2-Dioxaphospholanes



R	X	Y		bp °C	pressure mmHg	yield, %
OCH_3	Cl			77–78	11	60
OC_2H_5	Cl			85–91	11	71
H	Cl			55–60	20	70
OCH_3	OCH_3			91–92	22	72
OC_2H_5	OCH_3			80–82	7	61
$OCOCH_3$	OCH_3			83–85	1.3	35
H	OCH_3			52–53	19	60
OCH_3	OCH_3	O	**20**	112–113	0.5	71
OC_2H_5	OCH_3	O	**21**	118–119	0.7	72
H	OCH_3	O	**23**	87–93	1.1	60

36) do not involve phosphorus–oxygen bonds (no isotopic labels are lost in these steps), and could only racemize the phosphorus center (which, from the quantitative stereoanalysis, they clearly do not).

Analysis. There are, analogously, two features of the analysis that could affect the determined configuration: that the ring-closure reaction proceeds with "in-line" stereochemistry and that the "syn" isomer (of the cyclic methyl triester) is indeed "syn" (not "anti"). The evidence for the "in-line" course of the ring-closure derives, as discussed, both from the predictions of the preference rules[24] and from experimental findings on the closure of phosphorothioates.[22] Secondly, the identity of the "syn" cyclic triester (e.g., **10–12**) is strongly indicated by the ^1H NMR spectrum, and has been thoroughly discussed by Bentrude et al.[26]

Recently, Cullis and Lowe[27] have reported the synthesis of methyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate, and have demonstrated that this material has a detectable circular dichroic absorption at 208 nm. This chiroptical approach, while not providing the absolute configuration independently, should be an alternative method for the analysis of such esters.

In summary, it appears that both the synthetic approach and the stereoanalytical method are valid in terms of the absolute stereochemistry presented. We have recently applied the methodology described here to evaluate the stereochemical course of the alkaline phosphatase[20] and of glycerol kinase[21] and we look forward to the stereochemical definition of a number of reactions of phosphate monoesters in both chemistry and enzymology.

Experimental Section

^1H NMR spectra were recorded on a Varian CFT20 or a Varian XL100 instrument in $CDCl_3$ or D_2O. Chemical shifts from tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt are reported on the δ scale. ^{31}P NMR spectra were recorded on the XL100 machine; the chemical shifts are relative to external 85% H_3PO_4 (downfield negative). Gas chromatographic separations were done on a Varian Aerograph 1400 instrument using all-glass columns and a flame ionization detector. High-pressure liquid chromatography was done on an instrument from Waters Associates. Mass spectra were recorded on an AEI MS9 double-focusing instrument, modified to allow linked-scan metastable-ion spectra to be obtained.[28] The direct inlet system at 120 °C was used. To provide a steady flux of material into the instrument, the ceramic tip was replaced by a glass capillary of similar size, inside which the sample had been deposited. Ion-exchange chromatography was performed either using diethylaminoethylcellulose (DE-52, from Whatman) or Dowex-1 (200–400 mesh, 8% cross-linked). Dowex-50 was 100–200 mesh, 8% cross-linked. Optical rotations were recorded on a Perkin-Elmer 141 instrument.

All solvents were distilled immediately before use. $CHCl_3$ and CH_2Cl_2 were dried over P_2O_5 or (for small-scale work) by passage through a column of alumina (neutral grade 1). Benzene, ether, and dioxane were dried over sodium, acetonitrile over CaH_2, and methanol over magnesium methoxide prepared in situ. Triethylamine was distilled from solid KOH and redistilled from naphthyl isocyanate. Pyridine was distilled from solid KOH and stored over 4 Å molecular sieves. Ethyl acetate was distilled from 4 Å molecular sieves.

Preparation of 2-Chloro-1,3,2-dioxaphospholanes, 2-Methoxy-1,3,2-dioxaphospholanes, and the 2-Methoxy-2-oxo-1,3,2-dioxaphospholanes, 20–23. The methods of Lucas et al.[29] and Denny et al.[30] were used in the synthesis of **20–23**. The starting glycols were prepared by standard procedures. For **20**, **21**, and **23**, glycol (0.1 mol) in $CHCl_3$ (30 mL) and PCl_3 (0.1 mol) in $CHCl_3$ (30 mL) were each added over 2 h to $CHCl_3$ (50 mL) using a Lucas addition tube.[29] A continuous stream of dry N_2 removed the HCl formed. After the reagents had been added, the solvent was removed at atmospheric pressure and the product 2-chloro-1,3,2-dioxaphospholane was distilled in vacuo. Boiling points and yields are listed in Table VI.

To a stirred solution of the 2-chloro-1,3,2-dioxaphospholane (0.025 mol) in dry benzene (25 mL) at 0 °C were added methanol or trideuteriomethanol (0.025 mol) and pyridine (0.025 mol) in benzene (25 mL), over 2 h, with stirring at 0 °C. The precipitate of pyridinium hydrochloride was removed by filtration, and the solvent was evaporated under reduced pressure. The product 2-methoxy-1,3,2-dioxaphospholane was distilled in vacuo. Boiling points and yields are listed in Table VI.

The 2-methoxy-1,3,2-dioxaphospholane (as a 10% v/v solution in dry CH_2Cl_2) was cooled to 0 °C, and N_2O_4 was bubbled through until a bright blue-green color resulted. The solvent and excess N_2O_4 were removed at atmospheric pressure, and the 2-methoxy-2-oxo-1,3,2-dioxaphospholane was distilled in vacuo. Boiling points and yields are listed in Table VI.

The acetoxy compound **22** was prepared by reaction of the glycol (0.25 mol) with methoxydichlorophosphine (0.25 mol) in ether (30 mL), in the presence of triethylamine (0.05 mol) at 0 °C. [The methoxydichlorophosphine was prepared from methanol (0.13 mol) and PCl_3 (0.13 mol) in ether (100 mL) in the presence of triethylamine (0.13 mol).] The 2-methoxy-1,3,2-dioxaphospholane intermediate was oxidized on the small scale by N_2O_4 to **22**.

The ^1H NMR and ^{31}P NMR spectra of the 2-methoxy-2-oxo-1,3,2-dioxaphospholanes, **20–23**, were consistent in all cases with mixtures of the diastereoisomers of the desired products.

2-Benzyl-(*S*)-propane-1,2-diol. 2-Benzyl-(*S*)-propane-1,2-diol was prepared from ethyl (*S*)-lactate by a method similar to that of Mislow et al.[31] Freshly prepared silver oxide (26 g) was added over 30 min to a solution of ethyl (*S*)-lactate (Aldrich) (13.2 g) and benzyl bromide (28.6 g) in ether (60 mL), so as to maintain gentle reflux. When the addition was complete, the mixture was refluxed for a further 30 min. The solid material was removed by filtration and triturated with ether. The solvent was removed from the combined ether solution, and the residue was distilled in vacuo. The desired product was contaminated with dibenzyl ether, and the distillate was subjected to chromatography on silica gel (Merck), eluting with light petroleum–ethyl acetate. Ethyl 2-benzyl-(*S*)-lactate was isolated in 93% yield. This ester was reduced with excess $LiAlH_4$ in THF to give 2-benzyl-(*S*)-propane-1,2-diol [bp 95–99 °C (0.9 mmHg); $[\alpha]_D^{27}$ 27.7° (neat liquid)] in 89% yield. ^1H NMR (in Me_2SO-d_6) δ 7.3 (broad s, 5 H), 4.60 (t, 1 H, J = 5.5 Hz), 4.51 (s, 2 H), 3.42 (m, 3 H), 1.08 (d, 3 H, J = 5.8 Hz).

2-Phospho-(*RS*)-propane-1,2-diol. 1-Trityl-(*RS*)-propane-1,2-diol (prepared by standard procedures from (*RS*)-propane-1,2-diol and trityl chloride in pyridine at 100 °C for 1 h) (640 mg, 2 mmol) in pyridine (2 mL) was added slowly to $POCl_3$ (400 μL) in pyridine (2 mL) over 10 min at 0 °C. The mixture was stirred for a further 30 min. The solvent was removed under reduced pressure and the resulting solid triturated with CH_2Cl_2. The CH_2Cl_2 extracts were chromatographed on silica (eluting with 2-propanol–water–concentrated aqueous ammonia, 80:15:5 by volume) to give 1-trityl-2-phospho-(*RS*)-propane-1,2-diol as a glass. This material was detritylated by standing in acetonitrile (5 mL) plus water (5 mL, adjusted to pH 2 with concentrated HCl) overnight. The reaction mixture was neutralized with solid NH_4HCO_3 and filtered, and the product was isolated by chromatography on DEAE-cellulose (equilibrated with 60

mM triethylammonium bicarbonate and eluted with a linear gradient of 60–200 mM triethylammonium bicarbonate). The appropriate fractions were evaporated to dryness, and the product was passed through Dowex-50 (H^+), then immediately neutralized with aqueous cyclohexylamine to pH 8.5. Evaporation produced a white solid that was recrystallized from acetone–water to give the dicyclohexylammonium salt of 2-phospho-(*RS*)-propane-1,2-diol in 48% yield: ^1H NMR (in D_2O) δ 4.30 (broad m, 1 H), 3.64–3.50 (m, 2 H), 3.15 (broad m, 2 H), 2.10–1.10 (broad m, 20 H), 1.21 (d, 3 H, J = 6.5 Hz); ^{31}P NMR (in D_2O) − 3.5 ppm (d, J = 8 Hz).

1- and 2-(dimethylphosphoryl)propane-1,2-diol (27 and 29) were prepared by methylation of the dicyclohexylammonium salts of the monoesters. To 1- or 2-phosphopropane-1,2-diol (25 mg, 300 μmol) in 2-propanol (50 mL) and water (0.5 mL) was added an excess of freshly distilled ethereal diazomethane until the solution remained yellow for 15 min at room temperature. The solvents were removed under reduced pressure. The resulting oil was purified by preparative TLC on silica (eluting with ethyl acetate–EtOH, 80:20 v/v) to give **27** or **29**. ^1H NMR (in $CDCl_3$) for **27**: δ 4.1–3.6 (m, 3 H), 3.77 (d, 6 H, J = 11.1 Hz), 1.18 (d, 3 H, J = 6.2 Hz). ^1H NMR (in $CDCl_3$) for **29**: δ 4.52 (broad m, 1 H), 3.78 (d, 6 H, J = 11.1 Hz), 3.8–3.6 (m, 2 H), 1.6 (broad s, 1 H), 1.31 (d, 3 H, J = 6.4 Hz).

^{18}O-Labeled 1-(Dimethylphosphoryl)propane-1,2-diol (28). To PCl_5 (100 mg, 0.48 mmol) was added $H_2{}^{18}O$ (9 μL, 0.45 mmol) at room temperature, and the resulting solution heated to 100 °C for 10 min. After cooling, anhydrous pyridine (500 μL) was added, followed by the slow addition of 2-benzyl-(*S*)-propane-1,2-diol (0.45 mmol) over 20 min. After 2 h at room temperature, MeOH (1 mL) was added, and the solution was then poured into CH_2Cl_2 and washed successively with aqueous H_2SO_4 (0.1 M), water, and 5% aqueous Na_2CO_3. The organic solution was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The resulting oil was dissolved in EtOH and hydrogenolyzed over 10% Pd/C at 4 atm overnight. The product was purified by preparative TLC on silica, eluting with ethyl acetate–EtOH (80:20 v/v). The mass spectrum of the product showed that the unalkylated phosphoryl oxygen was 88% ^{18}O.

^{18}O-Labeled 1-(Dimethylphosphoryl)propane-1,2-diol (30). This material was prepared by the same method as **28** (described above) except that unlabeled $POCl_3$ and [^{18}O]methanol (see below) were used.

2-Methoxy-2-[^{18}O]oxo-4-methyl-1,3,2-dioxaphospholane (24). ^{18}O-Labeled 1-(dimethylphosphoryl)propane-1,2-diol (**28**) was heated at 250 °C and 70 mmHg and the distillate collected. This material (**24**) was purified by high-pressure liquid chromatography (see later).

2-[^{18}O]Methoxy-2-oxo-4-methyl-1,3,2-dioxaphospholane (25). [^{18}O]Methanol was prepared by a modification of the method of Sawyer.[32] Tributyl orthoformate (3.75 mL) in diglyme (2.5 mL) and $H_2{}^{18}O$ (0.22 mL) were stirred at room temperature. Dry HCl gas (1 mL) was added, and the mixture was heated to 150 °C. The product was distilled through a 15-cm Vigreux column, the material boiling below 110 °C being collected. This distillate was added dropwise to lithium aluminum hydride (0.02 mol) in diglyme (20 mL) and left at room temperature for 1 h. Ethylene glycol (4 mL) was then added carefully, followed by benzene (10 mL). The mixture was then fractionally distilled, and to the crude product molecular sieves (4 Å) were added until no methanol could be detected in the supernatant by GLC. The sieves were removed by filtration, washed with anhydrous benzene, dried at room temperature in a stream of dry N_2, and then dried under reduced pressure (15 mmHg) at room temperature for 1 min. The methanol was then recovered by heating the sieves to 250 °C at 1 mm, collecting the labeled methanol in a liquid N_2 trap: yield 252 mg (67%); single peak on GLC; ^{18}O content, 93%. 2-[^{18}O]Methoxy-2-oxo-4-methyl-1,3,2-dioxaphospholane (**25**) was then prepared from the corresponding 2-chloro-1,3,2-dioxaphospholane by reaction with [^{18}O]methanol followed by N_2O_4 oxidation as described for the unlabeled compound, **23**.

1- and 2-(Methyltrideuteriomethylphosphoryl)propane-1,2-diol. The trideuterio analogue of **27** (labeled in one of the methoxyl groups) was prepared by the reaction of 2-methoxy-2-oxo-4-methyl-1,3,2-dioxaphospholane (**23**) with CD_3OD–triethylamine (1:1 v/v) at −78 °C. The products were purified by preparative TLC as described above.

[1(*R*)-^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol (2, R = CH_3). [^{17}O]-$POCl_3$ was prepared as follows. $H_2{}^{17}O$ (165 μL, 9.1 mmol) was added over 15 min with rapid stirring to powdered PCl_5 at −78 °C. A stream of dry N_2 removed the HCl produced. The mixture was allowed to warm to room temperature, then refluxed for 30 min. Distillation gave [^{17}O]-$POCl_3$ (1.2 g, bp 105 °C, 85% yield). The isotopic composition was found by reaction with excess methanol, followed by mass spectrometric analysis of the resulting trimethylphosphate: ^{16}O, 26%; ^{17}O, 47%; ^{18}O, 28%.

(−)-Ephedrine monohydrate (10 g) was dried by azeotropic distillation with benzene (50 mL) for 15 min, and the benzene solution was stored overnight over 4 Å molecular sieves. The filtered solution was then evaporated to dryness. To anhydrous ephedrine (1.09 g, 6.6 mmol) in dry ether (35 mL) containing triethylamine (1.33 g, 13.2 mmol) at 0 °C was added a solution of [^{17}O]-$POCl_3$ (1.02 g, 6.6 mmol) in dry ether (5 mL) over 1 h with stirring. The mixture was then allowed to stand at room temperature for 2 h. The precipitate was removed by filtration and washed with ether. The combined ether solution and washings were evaporated to give a white, crystalline solid (1.69 g), which is a mixture of **31** and **32**.

A sample (1.0 g, 4 mmol) of the mixture of **31** and **32** was stirred overnight with excess 2-benzyl-(*S*)-propane-1,2-diol (1.2 mL, ~8 mmol) in ether (1 mL) and triethylamine (0.6 mL) at room temperature. The reaction mixture was then chromatographed on a column (200 g) of silica, eluting with ethyl acetate–triethylamine (99:1 v/v), to give unchanged alcohol, and the two product esters (as oils) **33** (0.99 g, 65% based on $POCl_3$) and **34** (0.11 g, 7%). The major isomer, **33**, had a signal in the ^{31}P NMR spectrum (proton decoupled, in dioxane) at −18.91 ppm and a small peak at −18.77 ppm from the compound with the epimeric carbon center (the propane-1,2-diol has 78% enantiomer excess). When 2-benzyl-(*RS*)-propanediol was used, these peaks were of equal intensity. The ^1H NMR of **33** (in $CDCl_3$) had δ 7.26 (broad s, 10 H), 5.61 (dd, 1 H, J = 2.1 and 5.5 Hz), 4.61 (s, 2 H), 4.24–3.35 (m, 4 H), 2.68 (d, 3 H, J = 10.6 Hz), 1.26 (d, 3 H, J = 6.2 Hz), 0.76 (d, 3 H, J = 6.7 Hz). When the *RS* diol is used, two doublets are observed, at δ 1.26 and 1.24, having equal intensity. The minor isomer, **34**, had ^1H NMR δ 7.28 (broad s, 10 H), 5.45 (dd, 1 H, J = 3.8 and 5.5 Hz), 4.56 (s, 2 H), 4.24–3.35 (m, 4 H), 2.62 (d, 3 H, J = 10.2 Hz), 1.23 (d, 3 H, J = 6 Hz), 0.76 (d, 3 H, J = 6.7 Hz). When the *RS* diol is used, two doublets of equal intensity at δ 2.63 and 2.62, and two singlets of equal intensity at δ 4.58 and 4.56, are seen.

To the purified major adduct **33** (100 mg, 0.27 mmol) in dioxane (500 μL) was added trifluoroacetic acid in $H_2{}^{18}O$ (prepared from trifluoroacetic anhydride (20 μL, 0.15 mmol) in $H_2{}^{18}O$ (20 μL, 10 mmol)) at room temperature. Analysis by TLC showed the absence of **33** after 30 s, and the reaction was quenched with solid NH_4HCO_3 (200 mg), EtOH (5 mL), and water (5 mL). This reaction gives only a single product by decoupled ^{31}P NMR (singlet at 0.5 ppm) and the mixture was hydrogenolyzed directly in ethanol–water (1:1 v/v) (3 atm, 10% Pd/C, 2 h) to give the diammonium salt of 2-benzyl-1[(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol [^{31}P NMR (proton decoupled): singlet at −2 ppm. ^1H NMR: δ 7.46 (broad s, 5 H), 4.66 (s, 2 H), 4.04–3.70 (m, 3 H), 1.22 (d, 3 H, J = 6 Hz)]. The reaction mixture was diluted with water (10 mL) and washed with CH_2Cl_2 until all the deoxyephedrine (the first-formed product, which inhibits further hydrogenolysis) had been removed. The combined CH_2Cl_2 extracts were back-extracted with water and the combined aqueous solution and washings were concentrated under reduced pressure and hydrogenolyzed again (3 atm, 10% Pd/C, overnight). After removal of the catalyst, the mixture was diluted with water (200 mL), the pH was adjusted to 7 by bubbling CO_2 through the solution, and the product was isolated by chromatography on DEAE-cellulose equilibrated with 50 mM aqueous $Et_3NH \cdot HCO_3$ and eluted with a linear gradient (50–200 mM) of aqueous $Et_3NH \cdot HCO_3$. Fractions containing phosphopropanediol were pooled and evaporated to dryness. The material was then converted to the dicyclohexylammonium salt (see above under the preparation of the unlabeled 2-phospho isomer) which was recrystallized from acetone–water to give 70 mg (72% based on **33**, 47% based on $POCl_3$) of [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**36**), mp 160–162 °C (uncor). ^{31}P NMR (in D_2O) gave, decoupled, a singlet at −2.8 ppm; undecoupled, a triplet of J = 11.5 Hz. Unlabeled 1-phospho-(*S*)-propane-1,2-diol was prepared by the same method as that described, and showed identical characteristics. In addition this material gave ^1H NMR (in D_2O) δ 4.1–3.5 (m, 3 H), 3.13 (m, 2 H), 2.10–1.10 (m, 20 H), 1.12 (d, 3 H, J = 6.3 Hz).

Ring Closure of 1-Phosphopropane-1,2-diol. The outlines of five methods for the ring closure of 1-phosphopropane-1,2-diol are given in Table V. Since it was shown that only one of these methods gave

a clean and defined stereochemical course (at least on the small scale that our experiments require), only this method will be described in detail. Diphenylphosphorylimidazole was prepared by a route analogous to that used for the synthesis of the sulfonylimidazolides.[33] Diphenyl phosphochloridate was added to imidazole (1 equiv) and triethylamine (1 equiv) in dry CH_2Cl_2 at 0 °C, and the mixture was stirred at room temperature for 2 h. The imidazolium hydrochloride was removed by filtration, and the solvent was then removed under reduced pressure. The product was recrystallized from ether in 86% yield. The sample of 1-phosphopropane-1,2-diol as its dicyclohexylammonium salt (12.5 mg, 35 μmol) was converted into the di(tri-*n*-octylammonium) salt by passage through a column of Dowex-50 (pyridinium form). The eluate was treated with tri-*n*-octylamine (24.9 mg, 70 μmol) in dioxane (5 mL) and evaporated to dryness. Dioxane (5 mL) was added and the solution again evaporated under reduced pressure. The dioxane addition-evaporation sequence was repeated four more times. The crystalline salt was then dissolved in CH_2Cl_2 (160 μL) containing diisopropylethylamine (10.5 μL) and the mixture stirred with four or five molecular sieves (4 Å) for 4 h. Diphenylphosphorylimidazole (1 equiv, 145 μL of a solution in CH_2Cl_2 containing 71.3 mg/mL, stored over molecular sieves for 4 h) was then added and the mixture stirred for 16 h at room temperature. Aqueous triethylammonium bicarbonate (3 mL, 50 mM, pH 7.0) was then added, and the aqueous layer washed with CH_2Cl_2 (5 × 10 mL) to remove amines and diphenyl phosphate. The aqueous solution (which is near pH 7) was freeze-dried to an oil. Yields of the cyclization reaction were commonly ~75% as estimated by ^{31}P NMR.

Methylation of the Cyclic Phosphate Diester. The oil obtained above was sonicated with acetonitrile (400 μL) and dry ethereal diazomethane was added until the solution remained yellow. The solvents were removed (taking care to exclude atmospheric moisture) and the product (which is an approximately 1:1 mixture of the "syn" and "anti" cyclic methyl triesters **10–15**) was dissolved in dry ether (200 μL).

Separation of "Syn" and "Anti" Cyclic Methyl Triesters. The ethereal mixture was subjected in four portions to chromatography on Corasil II (5 × 24 in. columns, o.d. 0.125 in.) in dry ether at 1.0 mL/min, room temperature. It is crucial that anhydrous conditions be used; sodium-dried ether was refluxed under N_2 over CaH_2 and led from this closed system directly onto the column. The isomers were located by the refractive index detector. The diastereoisomeric purity of the "syn" (**10–12**) and "anti" (**13–15**) sets of triesters was estimated by GLC on 5% XE-60 at 180 °C.

Ring Opening of the Separated "Syn" and "Anti" Triesters. Each of the triesters was allowed to react with methanol (2 mL) and triethylamine (0.5 mL) at −78 °C overnight. After this period, the solvents were removed under high vacuum at −78 °C. Transfer to the glass capillary for mass spectrometric analysis was effected using dry acetonitrile.

Acknowledgments. We are indebted to Dr. George Orr for his valuable help in the early stages of this work, to Dr. G. Dudek for helping to implement the linked-scan system, and to the National Institutes of Health and Merck Sharp & Dohme for support.

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