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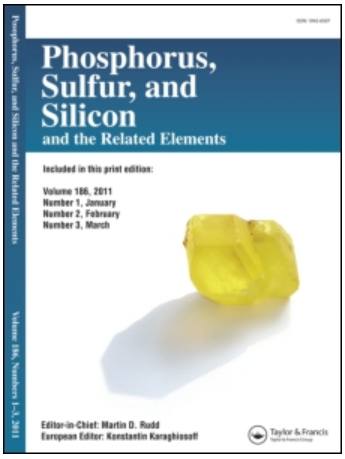

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
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THIONO COMPOUNDS. 10. STRUCTURES AND REACTIONS OF INTERMEDIATES FROM THE OXIDATION OF PHOSPHOROTHIOATES[1,2]

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To cite this Article Swinson, Joel , Field, Lamar , Heimer, Norman E. , Stone, Michael P. and Van Wazer, John R.(1989) 'THIONO COMPOUNDS. 10. STRUCTURES AND REACTIONS OF INTERMEDIATES FROM THE OXIDATION OF PHOSPHOROTHIOATES[1,2]', Phosphorus, Sulfur, and Silicon and the Related Elements, 44: 1, 39 — 52
To link to this Article: DOI: 10.1080/10426508908043707
URL: http://dx.doi.org/10.1080/10426508908043707

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Phosphorus, Sulfur, and Silicon, 1989, Vol. 44, pp. 39–52
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THIONO COMPOUNDS. 10. STRUCTURES AND REACTIONS OF INTERMEDIATES FROM THE OXIDATION OF PHOSPHOROTHIOATES[1,2]

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(Received 23 June, 1988; in final form 3 September, 1988)

Intermediates from the oxidation of phosphorothioates, $(RO)_3PS$, were studied previously at low temperature using ^{31}P NMR, UV and Raman spectra. Now reported is further information about the structure of intermediates and about their reactions, both of which afford significant clues as to how phosphorothioates may produce adverse biological reactions after they have been oxidized biologically. Mass spectra identified intermediates corresponding to $(RO)_3PS_n$ with n up to 7 (although presence of some equivalent masses with two oxygens in place of a sulfur atom is possible). HPLC separated unstable intermediates for which UV and MS evidence again was consistent with the structure $(RO)_3PS_n$. That intermediates can react as nucleophiles is illustrated by reactions with Ellman's Reagent, which produced a maximum of thiolate ion at about the time ^{31}P-NMR and UV indicated a maximum of intermediates. A second illustration of nucleophilicity was reaction with N-ethylmaleimide (and other Michael acceptors), which led to thiiranes and thiirane 1-oxides. That the intermediates can react also as electrophiles is illustrated by reactions (followed by UV and ^{31}P NMR) with trimethyl phosphite, hydroxyl ion, and water (perhaps to some extent); use of $H_2{}^{18}O$ did not introduce ^{18}O into phosphate products, but exchange reactions with $H_2{}^{18}O$ did indicate presence of oxygenated species among the intermediates.

Key words: Ellman's Reagent; N-ethylmaleimide; ^{31}P NMR spectra; Phosphorothioates; Thiiranes; UV spectra.

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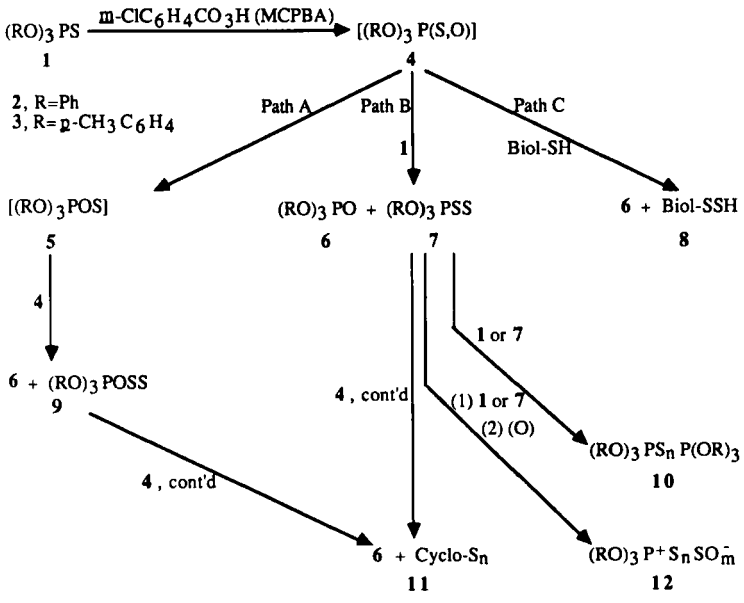
INTRODUCTION

Phosphorothioates (**1**) are widely used, for example as pesticides, despite the fact that they can cause adverse biological effects.[3a] These effects include centrilobular hepatic necrosis and inhibition of monoxygenases containing cytochrome P-450.[3a] Intermediates produced by oxidation evidently are responsible for these adverse effects.[3a] We have investigated model reactions of phosphorothioates with peroxy compounds (chiefly *m*-chloroperoxybenzoic acid, MCPBA) in efforts to clarify the chemistry of these intermediates and thus provide insight as to reasons for the adverse biological effects.[3b] Our previous conclusions and the basis for them can be summarized as follows, in their relevance to the present paper, with key aspects being illustrated in Scheme 1: (1) When a thioate (**1**) is oxidized at ca. 25°C to the phosphate (**6**) by MCPBA, ^{31}P-NMR shows no indication of intermediates; reaction is extremely rapid and elemental sulfur (**11**) precipitates.[4] (2) At lower temperatures, intermediates were discerned by ^{31}P NMR spectra that disappeared with standing or warming, and negligible amounts



SCHEME 1

of sulfur precipitated.[1,4] (3) The lifetimes of the intermediates varied from a few minutes to many hours; they were enhanced by aryl groups more than by alkyl groups and more by electron-donating aryl substituents than by electron-withdrawing ones.[1] (4) At ca. 25°C, the consumption of MCPBA and the mass balance of elemental sulfur plus sulfate ion are consistent with Equation (1).[1] At lower temperatures, with 1:1 molar proportions of

$$\mathbf{1} + 4\,m\text{-}ClC_6H_4CO_3H \rightarrow \mathbf{6} + SO_3(+H_2O \rightarrow H_2SO_4) + 4\,m\text{-}ClC_6H_4CO_2H \quad (1)$$

1 and MCPBA, much MCPBA must be consumed in oxidizing intermediates (and to some extent elemental sulfur) since much of the thioate remains unconsumed and since sulfur does not precipitate. Hence oxygenated intermediates such as **12** are probable.[1,4] Dioxide intermediates, $(RO)_3PSO_2$, are unlikely to be a major source of the phosphate product (**6**), since 1:1 proportions of **1** and MCPBA can produce yields of **6** exceeding 50%.[4] (5) The major ^{31}P-NMR peaks for intermediates appeared about midway between those for the thioate (**1**) and the phosphate (**6**).[1,4] Presence of these peaks in a common region points to the similarity of the corresponding intermediates, and the position of this common region (13–33 ppm downfield from 85% H_3PO_4) points to tetracovalency and consistency with structures of the type $(RO)_3PS_x$ or $(RO)_3POS_x$, with $x \geq 1$; these structures are produced by the growing chains of **7** or/and **9** in Scheme 1.[1] UV spectra support intermediates of types **7** and/or **9**, since UV absorbance increases during the reaction and then decreases.[1] En route to **6**, the intermediate **4** in Scheme 1 has been proposed to be a phosphoxathiirane, $(RO)_3P\langle^{S}_{O}$; we find

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phosphoxathiiranes to be quite useful in explaining reactions of **4**, but **4** is intended to be noncommittal as to their reality (for further discussion of this point, see References 1–3a). (6) Atomic sulfur has been proposed as the culprit in biological damage,[3a] but trapping experiments reveal no such indication, either of singlet or triplet sulfur.[4] (7) EPR experiments show that free radicals do not appear to play any significant role.[1] (8) Enhancement of survival time of intermediates at low concentrations implies that loss occurs by reaction orders greater than unity.[1] (9) UV, Raman, and ^{31}P NMR spectra indicate that longer lived species such as **10** also are involved.[1]

RESULTS AND DISCUSSION

Further Evidence for the Structure of Intermediates

Since the intermediates proposed in Scheme 1 involve growing chains of sulfur atoms in polysulfide species, such as **7** and/or **9**,[1,4] mass spectrometry (MS) was an attractive tool for studying them. When equimolar amounts of the triphenyl ester **2** and MCPBA were allowed to react at −5°C for 6–7 h, aliquots showed MS peaks corresponding to the organic materials of Equation (1) at a probe temperature of ca. 25°C. When the probe temperature was increased to 50–70°C, peaks for intermediates were observed. Confirmations that these peaks arose from intermediates and were not artifacts are that in a repetition of the experiment at ca. 25°C no MS peaks were seen above that of M^+ for **2** (even when the probe was heated), and that after a reaction mixture had been allowed to stand at −5°C for three days, only one very weak peak was found, even at a probe temperature of 110°C. Furthermore, no peaks exceeding M^+ ever were seen only with **2** or the corresponding phosphate (**6**, R = Ph). Table I.A shows the results with **2**.

Compounds with P=S linkages characteristically lose ·SH from the molecular ion (although phosphoramidothioates and phosphorochloridothioates apparently do so more readily than phosphorothioates).[5] Accordingly, the peak from **2** at m/z 501 reasonably can be attributed to $(PhO)_3PS_7 - (SH)$. Similarly, other peaks from **2** in Table I.A can be attributed as shown to loss of SH from counterparts where $n = 2$–6, as well as to loss of sulfur atoms in succession from a peak thus generated by loss of SH. Of course, some of the peaks could have been comprised of structures having two oxygen atoms instead of one of the sulfur atoms.

The peak at m/z 514 produced by oxidizing **2** can be accounted for by loss of SH from a polysulfide built up through addition of three sulfur atoms to the thioate **2**, followed by monoxidation and acquisition of PhO ($m = 93$) (Table I.A). Loss of PhO from the peak at m/z 514 then would account for the peak at m/z 421. Comparable peaks with one sulfur atom less occurred at m/z 482 and 389. Addition of PhO by rearrangement and cleavage of species such as **10** (Scheme 1) is consistent with the involvement of species like **10**, as mentioned under (9) above. Equation (2) illustrates hypothetically how species of the proposed kind might be formed and then lead to a MS peak at m/z 514 (the position of oxygen on the sulfane chains is arbitrary).

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TABLE I

Important fragments in the mass spectra of sulfur-containing products obtained by oxidizing triphenyl (**2**) and tri(*p*-tolyl) phosphorothioate (**3**) at −5°C

A. M/z observed from oxidized $(PhO)_3PS$ (**2**)	341	342	358	373	389	405	421	437	469	482	501	514
Calc'd. for $(PhO)_3PS_n$ − SH, n =	2			3		4		5	6		7	
Other assignments		$M^{\ddagger}$ for **2**	**2** + O		m/z 482 − PhO or 373 + O		m/z 514 − PhO or 405 + O			[**2** + 2S + PhO + O] − SH		[**2** + 3S + PhO + O] − SH
Rel. intensity, %[a]	1	3	b	1	52	4	b	54	9	9	1	b
B. M/z observed, from oxidized (p-CH_3 $C_6H_4O)_3PS$ (**3**)	383	384	400	415	431	447	463			538		570
Calc'd. for $(p\text{-}CH_3C_6H_4O)_3PS_n$ − SH, n =	2			3		4						
Other assignments		$M^{\ddagger}$ for **3**	**3** + O		m/z 538 − MePhO or 415 + O		m/z 570 − MePhO or 447 + O			[**3** + 2S + MePhO + O] − SH		[**3** + 3S + MePhO + O] − SH
Rel. intensity, %[a]	100	b	6	19	54	20	40			22		7

[a] Since intensity varied during the expts. as intermediates disappeared, the % of the base peak (m/z 266 for **2**, 383 for **3**) merely is a rough guide to relative magnitudes.

[b] Peaks where intensities are omitted were observed in other spectra than the one from which the values shown were taken.

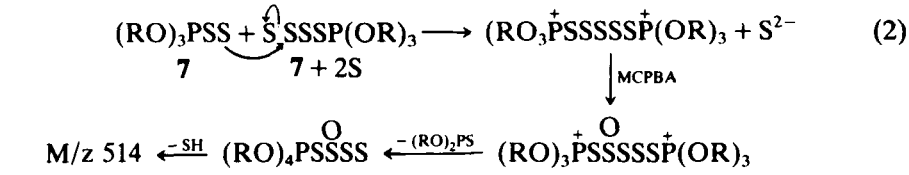

Table I.B shows similar increases in mass for the *p*-tolyl ester (**3**). Shown under peaks from **2** in Table I.A are peaks from **3** that appear to arise from the same causes; the only comparable peaks missing are those for $n = 5$–7. Additionally, a strong new peak at m/z 507 may correspond to **3** + MePhS (not tabulated; relative intensity 63%), again pointing to rearrangement of an aryl species in a polysulfide such as **10** of Scheme 1. In any event, irrespective of the precise assignment of peaks to sulfur and/or oxygen in Tables I.A and I.B, the large number of peaks with masses exceeding those of the molecular ions of **2** and **3** clearly points to polysulfides as major intermediates in the oxidation of phosphorothioates.

Efforts to isolate intermediates by high performance liquid chromatography (HPLC) were made. These were largely defeated by decomposition when the fractions separated were concentrated, but nevertheless they were structurally quite informative. Oxidation of **2** for the usual time of maximal intermediate formation, followed by HPLC (Expt. 1), led to an initial peak which had a UV spectrum that decreased almost linearly from high transmittance at 250 nm to low transmittance at 400 nm, as one would expect for a polysulfide (cf. Reference 6, as well as Reference 1). The fact that this peak appeared significantly before any others (1.45 min) from the nonpolar reversed-phase column used points to a polar species consistent with a formulation of $(RO)_3P^+S_nS^-$, accreted from species such as **7**. After ca. 3 h at −78°C in another analysis, the intensity of the peak dropped to about half. Later HPLC fractions included S_6 and S_8 (cf. Experimental).

In Expt. 2 with **2**, several fractions were collected at 1.0–1.6 min by separate injections and were combined. Evaporation to half volume caused precipitation of gray solid. The solution then was too dilute for good mass spectra, but a UV spectrum like that mentioned above was obtained again. The transmittance of this solution above 300 nm was markedly reduced upon desulfurization with trimethyl phosphite (*vide infra*), again pointing to a polysulfide. Further concentration produced more gray solid, but the solution nevertheless then did show masses above that of the molecular ion of **2** (389, 405, 482, all of which appear in Table I.A).

Additional evidence that the intermediates are indeed as represented in Scheme 1 will be apparent in the reactions of the intermediates as nucleophiles and electrophiles, which will be discussed next. These reactions indicate how intermediates from the oxidation of thionophosphorus compounds may exert adverse biological effects.

Reaction of Intermediates as Nucleophiles

Since a thiolate ion, RS^- (Equation 3), reacts with Ellman's Reagent (**13**) to engender an arenethiolate ion (**14**), which is readily determinable spectrophotometrically,[7]

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TABLE II

Reaction of oxidation products from **2** with Ellman's Reagent (**13**)

Time of oxidation (h)	Temp (°C)	Absorbance	% of total phos. as thiolate[a]	Time of oxidation (h)	Temp (°C)	Absorbance	% of total phos. as thiolate[a]
0.02	−9	0.03	0.7	7	−8	0.18	4.2
1	−7	0.04	0.9	8	−6	0.30	7.1
2	−6	0.14	3.3	9	−6	0.16	3.7
3	−6	0.15	3.5	10	−6	0.18	4.1
4	−6	0.18	4.2	12	−5	0.07	1.7
5	−6	0.20	4.7	23	−5	0.01	0.0
6	−5	0.28	6.6				

[a] (0.4 Absorbance/maximum theoretical absorbance)(100); 0.4 is a dilution factor reflecting the fact that only 20 μmol of **13** was used with the products (owing to low concentration) from 50 μmol of **2**; cf. Experimental.

intermediates such as **7**, which can be formulated as $(RO)_3P^+SS^-$, should respond similarly, if they are indeed nucleophilic sulfanethiolates. To pursue this approach, the triphenyl ester **2** and MCPBA were allowed to react in the usual

$$RS^- + [3\text{-}HO_2C\text{-}4\text{-}O_2NC_6H_3S]_2 \rightarrow 3\text{-}HO_2C\text{-}4\text{-}O_2NC_6H_3S^-$$
$$\mathbf{13} \qquad\qquad \mathbf{14}(\varepsilon_{412}13{,}600)^7$$
$$+ RSSC_6H_3\text{-}3\text{-}CO_2H\text{-}4NO_2 \qquad (3)$$

way at ca. −10°C to −5°C. An equimolar amount of Reagent **13** was used separately with phenylmethanethiol to establish the absorbance for the maximum reaction, so that comparison of this maximum absorbance with that of aliquots from the oxidation mixture could reflect the proportion of **2** converted to a thiolate species. Table II shows the results. After six hours, the absorbance showed ca. 7% conversion to thiolate-type intermediates. After seven hours, the value dropped to ca. 4% but after eight hours it rose again to ca. 7% before slowly decreasing to 0% after 23 hours. It seems significant that a similar second maximum was seen in UV spectra at eight hours (cf. Table II of Reference 1) and that a discontinuity was seen in NMR spectra at nine hours (cf. Curve A of Figure I, Reference 1). All three techniques thus seem to point to formation of an intermediate, then its disappearance, and finally its reappearance, in a manner reminiscent of an oscillating chemical reaction. In any event, the fact that the intermediates did indeed lead to the response expected of a thiolate (Equation 3) indicates that biological effects of the intermediates may result from their reactions as thiolate-type nucleophiles.

As further evidence for the nucleophilic character of species formed from **7** (formulated as **15** in Scheme 2), reactions with NEM (**16**) were studied. Since NEM is an effective trap for thiols,[8] our thought was that reaction of NEM with the sulfanethiolate **15** could trap **15** to give **17**, which by protonation might give the addition product of **15** and NEM. Instead, however, the carbanion **17** displaced a sulfanethiolate with one less sulfur atom to form the thiirane **19** (Scheme 2). LaLonde *et al.* invoked a similar displacement in explaining the reaction of α,β-unsaturated carbonyl compounds with polysulfide anions to give

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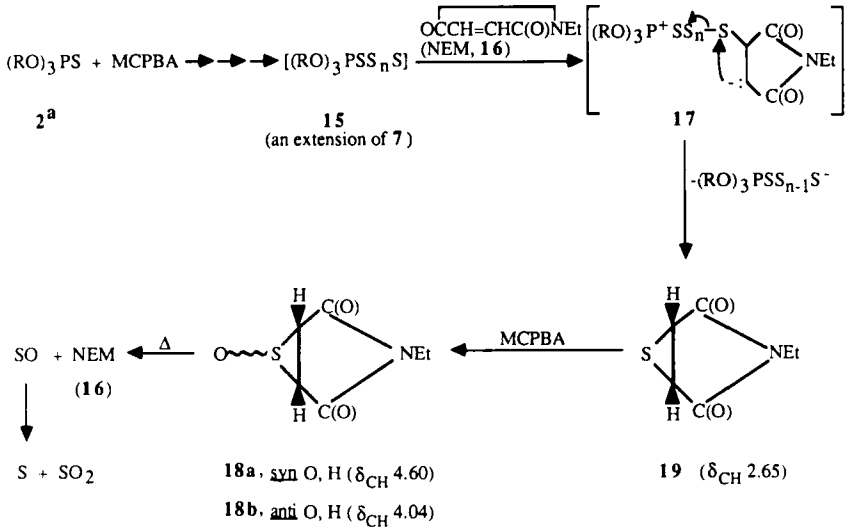

SCHEME 2[a]

[a] R = C_6H_5; MCPBA = M-$ClC_6H_4CO_3H$; NEM(**16**) = N-ethylmaleimide

intermediary thiiranes.[9] Earlier results rule out the possibility that **19** was formed by reaction of singlet [S(^{1}D)] or triplet [S(^{3}P)] forms of atomic sulfur with NEM.[4] Oxidation of **19** gave the monoxides **18a** and **18b**, which when warmed lost SO and regenerated NEM, a chaacteristic type of reaction for thiirane monoxides.[10] Rapid disproportionation of SO to S and SO_2 is well known (cf. Reference 11).

In typical experiments, one mmol of **2** was allowed to react with 0.05–2 mmol of NEM and 0–4 mmol of MCPBA in Me_2CO-d_6 (so that the reaction could be followed by NMR). Reactions were done at ca. 25°C (since several hours were required at lower temperatures). With the loss of NEM, new singlets appeared at δ 2.65 for **19** and at δ 4.60 and 4.04 for the forms of **18** in which the S—O bond was *syn* (**18a**) and *anti* (**18b**), respectively, to the methinyl hydrogens; the signals for the N-ethyl group became a multiplet.

The loss of NEM increased when the amount of MCPBA was increased, up to three equivalents. Four equivalents of MCPBA led to a decrease, however, perhaps either because of oxidation of **15** to nonnucleophilic species such as $(RO)_3PSS_nSO_m$, or of oxidation of **18** to the sulfone which would be expected to lose SO_2 readily and regenerate NEM.[12] With an excess of NEM and MCPBA, 1 mmol of the thioate **2** led to loss of 0.6–0.8 mmol of NEM. That sulfur did not precipitate in this reaction at ca. 25°C confirms that it was trapped as shown in Scheme 2 rather than liberated as in Scheme 1. No loss of NEM was detected in control experiments in which either the thioate **2** or the MCPBA was omitted.

Several forms of evidence support the structures of **18a** and **18b**: (1) Mass spectra showed the molecular ion for **18a,b** (although fragmentation studies were infeasible because of interfering fragments from MCPBA and diphenyl hydrogen phosphate). (2) Heating up to 60°C resulted in progressive conversion of **18a,b** to NEM (Scheme 2), as expected of a thiirane S-oxide.[10] (3) Of the two forms of **18**, that with δ 4.60 (**18a**) is considered to have the S—O bond *syn* to the

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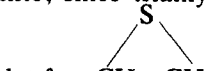
methinyl hydrogen atom, since hydrogens *syn* to S—O bonds appear downfield in ^{1}H NMR spectra from hydrogens that are *anti*;[13] **18b**, then, is the *anti* isomer. Several pieces of evidence also support the structure of **19**: (1) A molecular ion in mass spectra. (2) Material isolated from a typical trapping experiment was divided into two parts. One was treated with trimethyl phosphite, since trialkyl phosphites are known to abstract sulfur (vide infra).[14a] The singlet for  virtually disappeared, and the doublet of $(MeO)_3PS$ appeared at δ 3.73. (3) The second portion, when treated with MCPBA, lost most of the singlet at δ 2.65, indicating oxidation of **19** to **18a,b**/or the unstable S,S-dioxide.

Efforts to isolate pure **18a,b** were unpromising. Preparative TLC seemed to lead to decomposition on silica gel, and reversed phase TLC on C_{18}-capped silica gel did not remove *m*-chlorobenzoic acid (consistent with reports that separation of acids and thiirane S-oxides is difficult).[13] The most promising approach was dilution with Et_2O and extraction into H_2O; *m*-chlorobenzoic acid and NEM remained in the Et_2O, but separation then could not be achieved of **18, 19** and diphenyl hydrogen phosphate in the H_2O after evaporation of the H_2O (however, mass spectra were consistent).

Use of diethyl and dimethyl maleate as Michael acceptors in place of NEM gave similar results (see the Experimental Section), and acrylonitrile led to the large number of peaks expected from incorporation of a chiral center into the thiiranes produced. The amount of these acceptors that reacted was consistent with conversion to ca. 5–25% of thiirane. Efforts to isolate the products, however, indicated that they were even less stable than that from NEM.

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Reactions of Intermediates as Electrophiles

It seemed likely that sulfanethiolate intermediates such as **7** or **9** in Scheme 1 might exert adverse biological effects not only by *attacking* biological macromolecules as *nucleophiles* but also by *being attacked* as *electrophiles* by macromolecular biological nucleophiles. As electrophiles, the intermediates could be subject to nucleophilic displacements either on the sulfur atoms of the sulfane chain or on the phosphorus atom.

An attractive nucleophile for examining attack on the sulfane chain was trimethyl phosphite (**20**), as illustrated by Equation (4). Trivalent phosphorus is well known to attack a variety of sulfur species as a nucleophile and abstract sulfur

$$(MeO)_3P + \mathbf{7, 9, 10,} \text{ etc.} \rightarrow (MeO)_3PS \qquad (4)$$

20(^{31}P NMR, 139.50 ppm) **21**(^{31}P NMR, 72.62 ppm)

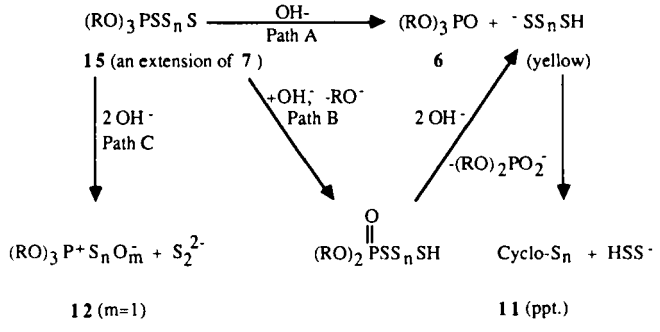
with formation of products such as **21**; substrates include S_8, thiols, disulfides, episulfides and sulfenyl halides.[14a]

UV spectra were used first to follow reactions of **20**. We reported earlier that when the triphenyl ester **2** reacted with MCPBA UV absorption typical of a polysulfide developed and then disappeared, except for slight residual absorption attributed to species like **10**.[1] Essentially as before,[1] reaction of equimolar

amounts of **2** and MCPBA at −10°C for 4–5 hours led to an absorbance at 325 nm of 0.55–0.85. The fact that addition of **20** then led within 5 minutes at −25°C to zero absorbance at 325 nm shows that the sulfane chain can indeed react as an electrophile toward an appropriate nucleophile. It might be added that this result also further supports the sulfane structure, as well as the probability that the residual UV absorption mentioned is indeed attributable to species like **10**, which normally are relatively stable but can lose sulfur to **20**.

NMR spectra provided further evidence that the sulfane could act as an electrophile toward **20**. Appearance and disappearance of ^{31}P NMR signals for intermediates was discussed at length earlier.[1] After development of new signals, produced by reaction of equimolar amounts of **2** and MCPBA for 7 hours at −5°C signals were seen at 53.40 (**2**), 20.00 (intermediates) and −17.73 ppm [$(C_6H_5O)_3PO$; **6**, R = C_6H_5]. Addition of **20** then led within 30 min at −5°C to new ^{31}P signals corresponding precisely to residual **20** (139.50 ppm), to trimethyl phosphorothioate (**21**, 72.61 ppm) and to trimethyl phosphate (**6**, R = Me; 1.85 ppm). The signal for the intermediates disappeared, seemingly in favor of a new peak 0.7 ppm downfield, which however was never more than 40% as intense as originally; this result seems consistent with partial survival of partly desulfurized species of type **10.** Elemental sulfur precipitated during the oxidation of **2** but was not responsible for converting **20** to **21**, since both sulfur and **20** still were present even after several days. As a further control datum, a mixture of the phenyl thioate **2**, *m*-chlorobenzoic acid, and **20** showed no indication after a day at ca. 25°C of any transfer of sulfur from **2** to **20** to give **21**.

The intermediates also acted as electrophiles toward hydroxyl ion as a nucleophile, although it was unclear whether OH^- attacked predominantly at phosphorus, sulfur, or both. The reaction of **2** and MCPBA was carried out as in the UV and NMR experiments with the phosphite **20**. In the use of UV with OH^-, the absorbance at 325 nm after the oxidation was 0.84. When the CH_2Cl_2 solution was shaken with saturated aqueous NaOH for 5 min at −10°C, the absorbance decreased to 0.1, signifying destruction of the intermediates; a yellow aqueous layer (indicative of polysulfide ions) and precipitation of sulfur were consistent with Scheme 3. This attack of OH^- could have occurred on the



SCHEME 3[a]

[a]R = C_6H_5

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phosphorus atom, with displacement of the sulfane chain (Path A) or phenoxide ion (Path B), as well as on the sulfane chain to give oxygenated species like **12** (Path C; subsequent oxidation of course could lead to **12** with $m > 1$). Similar reactions appeared to occur with water alone but much more slowly (over several hours, a slight decrease of absorbance occurred in one instance but not in another).

Use of ^{31}P NMR led to similar conclusions. Hydroxyl ion caused loss of the signal at ca. 20 ppm for intermediates in 5 min, and water led to a 45% decrease in absorbance in 0.5 hour; in both instances phosphate ion appeared to increase, indicating at least partial attack on the phosphorus atom (further conclusions are mentioned in the Experimental). As with **20** therefore, the intermediates could act as electrophiles toward hydroxyl ion and apparently to some extent toward water. After this paper had been written, we learned that the intermediate from the oxidation of $(EtO)_2P(S)OPh$ reportedly phosphorylates MeOH to give $(EtO)_2P(O)OMe$;[14b] hence such intermediates apparently can act also as electrophiles toward MeOH.

When the NMR experiment with water was repeated with $H_2{}^{18}O$, mass spectra showed no incorporation of ^{18}O in the peak for triphenyl phosphate. On the other hand, two major fragments from the intermediates of Table I.A did show entry of ^{18}O, probably because of exchange with terminally oxidized species such as **12** (see the Experimental).

Here may be the best place to introduce an incidental matter on the oxidation *per se,* which also relates to water. When 0.5 mmol of **2** was oxidized in Me_2CO-d_6 with 0.5 mmol of MCPBA in the presence of 0.1–2.5 mmol of $H_2{}^{18}O$ at temperatures of −5°C to reflux, mass spectra showed no incorporation of ^{18}O into the triphenyl phosphate produced (**6**, $R = C_6H_5$); hence byproduct or adventitious water normally plays no role in phosphate formation, and all oxygen of the phosphate (**6**, $R = C_6H_5$) comes from the MCPBA. Interestingly, however, the oxidations were slowed by increased amounts of water, probably because of hydrogen bonding of the MCPBA.

The new information above about the oxidation of phosphorothioates permits expansion of Conclusions (1)–(9) in the following respects: (10) Mass spectrometry shows intermediates of the structure $(RO)_3PS_n$, where n can range from 2 to 7 (although some peaks could result from presence of two oxygen atoms in lieu of one of sulfur). These results thus confirm Conclusion (5), i.e. that polysulfides play a crucial role. (11) HPLC separation of a sulfane fraction, followed by its loss of absorbance, mass spectra, and desulfurization with the phosphite **20**, also confirms Conclusions 5 and 10 as to polysulfide involvement. (12) Bisphosphonium species like **10** appear to be long-lived products, since the residual UV absorption mentioned was obliterated by **20** and since supporting changes were seen in the NMR spectra when **20** was used. (13) Species appear to be involved in which the sulfane chain is terminated with oxygen atoms to give species like **12**, thus supporting Conclusion (4), since $H_2{}^{18}O$ exchange occurred that did not involve the phosphorus. (14) The intermediates can react as nucleophiles, as shown by reaction with Ellman's Reagent (**13**) and N-ethylmaleimide (**16**). (15) The intermediates *also* can react as electrophiles, as shown by the reactions with **20** and hydroxyl ion (and perhaps water) as nucleophiles. (16) The adverse

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biological effects of the intermediates produced by oxidation of phosphorothioates thus may be attributable to their action as nucleophiles and/or electrophiles toward biomacromolecules.

EXPERIMENTAL

Melting points were determined using a Thomas-Hoover stirred-liquid apparatus and are corrected. ^{1}H NMR spectra were recorded on a JEOL-JNM-MH 100 or JEOL FX90Q (also used for ^{13}C spectra) spectrometer in Me_2CO-d_6 or $CDCl_3$ unless otherwise specified, using as internal standards Me_4Si or proton bands present in Me_2CO-d_6 or $CDCl_3$. The ^{31}P NMR spectra were done on a Bruker AM400-NB spectrometer with 85% H_3PO_4 as an external reference. All NMR spectra are reported in parts per million (^{31}P downfield from H_3PO_4). Mass spectra were obtained with use of a LKB9000 instrument and were checked under typical conditions with a Neurmag R10.10 mass spectrometer. UV spectra were obtained with a Cary Model 14 spectrophotometer or, in HPLC work, with a Hewlett-Packard diode-array detector. Moist extracts usually were dried using anhydrous $MgSO_4$, and solvents then were removed with a rotary-flash evaporator under reduced pressure. Analytical TLC was performed on Eastman Chromagram silica gel plates (catalog no. 13181), with visualization by I_2 vapor or UV. Preparative TLC was done on 1000-μM silica gel plates (Whatman PK6F or PLK5F), and reversed-phase TLC was with Whatman KC18F C_{18}-capped silica gel plates (catalog no. 4803-800). HPLC was performed on a IBM LC/9533 or Spectrophysics 8700 LC HPLC system, using a Whatman 4.6 mm × 25 cm Partisil 10 ODS-3 C_{18}-capped silica gel column (catalog no. 4228-001). Elemental analyses were by Galbraith Laboratories, Knoxville, Tenn. Commercially available MCPBA (Aldrich) either was purified to greater than 99% purity as reported,[15] or was titrated with aqueous $KI/Na_2S_2O_3$ solutions to determine purity. The following compounds prepared by us had spectra and properties in agreement with reported values: triphenyl (**2**),[16] tri(*p*-tolyl) (**3**),[1] and trimethyl (**20**)[2] phosphorothioate. All other compounds were commercial ($H_2{}^{18}O$ was from Merck, Sharp and Dohme).

Mass spectrometric studies. One mmol each of **2** or **3** and of MCPBA was dissolved in 2 mL portions of Me_2CO-d_6. For experiments at −5°C, the reactants were cooled to −10°C, mixed, and then were allowed to warm to ca −5°C. Mass spectra then were acquired after 6–10 h of reaction at ca. −5°C, as appropriate for maximum development of intermediates (for times, cf. References 1 and 4). The probe temperature was increased gradually, and spectra were acquired as the ion current detector indicated appearance of charged species. Table I shows the results.

With triphenyl phosphorothioate (**2**) and MCPBA at 25°C, after 30 min no peaks were observed above m/z 342 (M^+ for **2**) at probe temperatures up to ca. 50°C. In expts. with **2** at −5°C, peaks corresponding to MCPBA and *m*-chlorobenzoic acid were observed 1–2 min after an aliquot (6–7 h of oxidation time) was placed on the probe of the spectrometer at ca. 25°C, and soon thereafter peaks appeared for **2** and **6** (R = Ph). As the probe was warmed gradually, after ca. 1–3 min at ca. 50–70°C, several peaks were observed (Table I.A). Essentially the same results were obtained when the expt. was repeated with the Neurmag spectrometer. However, when a reaction mixture had stood for three days at −5°C the only peak seen above m/z 342 up to 110° at the probe was one very weak one at 389.

With tri(*p*-tolyl) phosphorothioate (**3**) and MCPBA at −5°, after 8–10 h MS of an aliquot initially showed MCPBA and *m*-chlorobenzoic acid as before, followed in a few minutes by **3** and the phosphate (**6**, R = *p*-$CH_3C_6H_4$). As the probe was heated slowly to ca. 100°C, the peaks shown in Table I.B were seen. The Neurmag spectrometer gave essentially the same results with a 10-h aliquot.

Studies with high performance liquid chromatography (HPLC). In Expt. 1, oxidation was done at ca. −5°C for 6–8 h as described for the mass spectrometric studies, with 1 mmol each of **2** and MCPBA in a total of 4 mL of Me_2CO. After 6–8 h at ca. −5°C the mixture was chilled to −78°C to quench reaction and then was centrifuged while cold to remove sulfur. It was kept at −75°C during ca. 1 h while 10-μL aliquots were injected into the HPLC system. Elution with MeCN and monitoring at 280 nm led to the first peak at 1.45 min, for which the diode array showed a UV spectrum that decreased almost linearly from near 90% absorbance at 250 nm to near 10% at 400 nm (the diode array system connected to the HPLC system allowed complete UV spectra to be taken, each at any desired time). Other peaks, identified by comparison of retention times or UV spectra with those of authentic samples, were as follows (elution time, min, in parentheses): $(C_6H_5O)_2PO_2H$ (2.20), Me_2CO (2.8–3), MCPBA (3.05), *m*-$ClC_6H_4CO_2H$ (3.15), $(C_6H_5O)_3PS$ (3.35), and $(C_6H_5O)_3PO$ (3.65); for S_6 (5.10), and S_8 (6.0), identification was by comparison of the UV spectra of the relevant peaks with those reported in the literature,[17] and by determining the retention time of an authentic

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sample of S_8 (6.0 min). Expt. 2 was done in the same manner as Expt. 1, except that several aliquots were injected so that fractions emerging at 1–1.6 min could be combined. Evaporation was done by passing a stream of N_2 over the solution. In addition to the peaks stated in the discussion, peaks were seen that corresponded to $(C_6H_2O)_2PO_2H$, **2**, and **6** (R = C_6H_5).

Reaction of Intermediates as Nucleophiles

a. *With Ellman's Reagent [5,5'-dithiobis(2-nitrobenzoic acid), 13].*[7] Solutions of 1.00 mmol each of triphenyl phosphorothioate (**2**) and of MCPBA in 2 mL each of Me_2CO were mixed. At the time specified in Table II, 200-μL aliquots (i.e., from 50 μmol of **2**) of the mixture were added to a solution consisting of 5 mL of deionized H_2O, 5 mL of pH 8 phosphate buffer, and 2 mL of pH 7 phosphate buffer containing 20 μmol of Ellman's Reagent (**13**).[7] The mixtures were shaken for 5 min, washed with an equal volume of Et_2O, and centrifuged for 10 min, after which the absorbance was determined spectrophotometrically at 412 nm; the wash and centrifugation dispelled turbidity up to ca. 10 h, after which turbidity could not be completely dispelled (sulfur ?). For calculation of the % of total phosphorus in the form of thiolate ion, the theoretical maximum absorbance (1.70) was determined by reaction of 50 μmol of phenylmethanethiol (2.5-fold excess) with the usual solution containing 20 μmol of **13**. Since the aliquots from the oxidation contained 50 μmol of phosphorus species rather than 20 for the model thiol (to enhance the amount of thiolate species, owing to the low yield), the % of phosphorus species present as thiolate = (absorbance for the aliquot × 20/50)(100)/1.7. Table II shows the results.

b. *With N-ethylmaleimide (NEM, 16).* Typically, triphenyl phosphorothioate (**2**, 1.00 mmol) and the amount of NEM specified below were dissolved in 2 mL of Me_2CO-d_6. The specified amount of MCPBA in 2 mL of Me_2CO-d_6 then was added at ca. 25°C. After 0.5 h, an aliquot (ca. 1 mL) of the reaction mixture was removed. ^1H NMR then typically showed singlets at δ 7.00 [$(PhO)_2PO_2H$], 4.60 (*syn* CHS(O)CH, **18a**), 4.04 (*anti* CHS(O)CH, **18b**) and 2.65 (CHSCH, **19**). The loss of NEM was determined by comparing the NMR integral of the olefinic peak with those of the N-ethyl protons. A mass spectrum (EI) showed a molecular ion (quickly lost) at m/z 173 for **18**.

With 1 mmol each of **2** and NEM, use of 1, 2, and 3 mmol of MCPBA led to respective losses of 0.2, 0.3, and 0.4 mmol of NEM, but 4 mmol led to loss only of 0.25–0.3 mmol. The most informative experiments were done with 1 mmol of **2** and 2 mmol each of NEM and MCPBA; unless otherwise noted, the experiments below were done with use of this ratio in the above procedure; here, 0.6–0.8 mmol of NEM was lost (use of 1 mmol of MCPBA led to loss of 0.3 mmol of NEM, and use of 3 mol led to loss of 0.5–0.6 mmol); use of only 0.05 mmol of NEM with 1 each of **2** and MCPBA led to loss of 0.023 mmol of NEM. In control experiments with 1 mmol each of NEM and MCPBA but no **2**, or with 1 mmol each of **2** and NEM but no MCPBA (with *m*-chlorobenzoic acid as a catalyst), there was no loss of NEM, even though observations were continued for 2 and 7 days respectively.

In order to assess the thermal stability of **18a,b**, a reaction mixture from **2**, NEM and MCPBA (1:2:2), which showed the usual signals, was heated at 60° during 6 h; the ^1H signals for **18a,b** decreased regularly and finally disappeared, and the ethyl multiplet became simpler; the only peaks left after 6 h corresponded to phosphate, *m*-chlorobenzoic acid and regenerated NEM. In another experiment, **18a,b** seemed unchanged after 24 h in $CDCl_3$ at ca. 25°C and then 1 h at 40°C.

The best approach to isolation of **18**, although not entirely successful, involved dilution of the product from the usual preferred 1:2:2 mixture with Et_2O (10 mL), followed by extraction into H_2O (10 mL). The aqueous layer was washed twice with CCl_4 and concentrated at 45–50°C. The resulting oil in Me_2CO-d_6 showed a NMR multiplet at δ 7.00 [$(C_6H_5O)_2PO_2H$], along with singlets at δ 4.60 (**18a**), 4.04 (**18b**) and 2.65 (**19**). EI mass spectra indicated two main components (via ion currents); one showed molecular ions at m/z 173 (calcd. for **18a,b**, 173) and m/z 157 (smaller; calcd. for **19**, 157). The second component exhibited a molecular ion at m/z 250 [calcd for $(C_6H_5O)_2PO_2H$, 250]; attempts failed to remove the phosphate by preparative TLC on C_{18}-capped silica gel. It might be added that reversed-phase TLC of another reaction mixture with 7:3 MeOH—H_2O did lead to a spot with R_f 0.90 that may have been **18a,b**, but it was lost on attempted elution.

To confirm the structure of the thiirane **19**, the oil obtained by using the preferred procedure (1 mmol of **2** with 2 each of MCPBA and NEM), followed by the extraction procedure just described for the isolation, was divided into two parts. One part in Me_2CO-d_6 was treated with 0.15 mmol of trimethyl phosphite (**20**) and the other with 0.15 mmol of MCPBA. NMR spectra after 30 min of the phosphite-produced product no longer exhibited the singlet of **19** at δ 2.65; a new doublet at δ 3.73 corresponded to authentic trimethyl phosphorothioate (**21**); these results are consistent with abstraction of a sulfur atom from **19** to give $(MeO)_3PS$ and regenerate NEM. The mixture treated

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with MCPBA showed only a very small singlet at δ 2.65, indicating a significant loss of **19** by oxidation to **18a,b** and/or the S,S-dioxide.

c. *With other Michael-type acceptors.* Use of diethyl maleate instead of NEM in the preferred procedure (1:2:2 mixture of **2**, the maleate and MCPBA) led to a ratio of olefinic to ethyl protons consistent with conversion of ca. 17% of the maleate to thiiranes (evidence for the thiiranes per se, however, was precluded by overlapping ethyl peaks in the expected region of ca. 2.5–4.5 ppm). With dimethyl malcate, three new singlets at δ 4.54, 4.26 and 2.89 were consistent with formation of a thiirane and its S-oxide, and the methyl/olefinic proton ratio indicated disappearance of ca. 5% of the maleate. Acrylonitrile led to a complex of at least 20 small peaks at δ 3.67–2.70 (as one would expect because of the chiral centers in the thiiranes), with indication of ca. 25% conversion to thiiranes. Subjection of each of the mixtures to the isolation procedure that had seemed most promising with the mixture of **18a,b** and **19** (dilution with Et_2O, extraction into H_2O) caused loss of the signals attributed to thiiranes, indicating that the thiirane products were much less stable than those from NEM.

Reactions of Intermediates as Electrophiles

a. *With trimethyl phosphite (**20**).* In the UV experiments, solutions of 1.00 mmol each of the triphenyl ester **2** and MCPBA in CH_2Cl_2 (4 mL total volume) were cooled to $-10°C$, mixed, and allowed to react at $-10°C$ for 4 h. A 20-μL aliquot of the solution, diluted to 2 mL with cold CH_2Cl_2, then had a UV absorbance of 0.55 at 325 nm. As soon as possible after the first aliquot was analyzed (ca. 10 min), a second aliquot was removed and diluted with cold CH_2Cl_2 containing 4.00 mmol of trimethyl phosphate (**20**). After 5 min at $-25°C$, the UV spectrum was perfectly flat between 305 and 400 nm (A = 0.00). When the procedure was repeated one hour later, for confirmation, the first aliquot (30 μL) produced an absorbance at 325 nm of 0.85 and the second aliquot again led to A = 0.00 at 325 nm.

For the NMR experiments, solutions of 1.00 mmol each of **2** and MCPBA were cooled to $-10°C$ in a total of 4 mL of Me_2CO-d_6. The solutions were mixed and after 7 h of reaction time at $-5°C$, the mixture was analyzed by ^{31}P NMR. A large signal was seen at 53.40 ppm for **2**, and small signals were seen at 20.00 and -17.73 ppm for the intermediate(s) and triphenyl phosphate (**6**, R = C_6H_5) respectively. Trimethyl phosphite then was added (**20**, 2.00 mmol). After 30 min, new signals corresponded precisely (via known samples) to residual **20** (139.50 ppm), trimethyl phosphate (**6**, R = Me; 1.85 ppm), and trimethyl phosphorothioate (**21**, 72.62 ppm). The signal for intermediates at 20.00 ppm (cf. Reference 1) seemed to disappear as a new peak developed at 20.73 ppm, which however became only 40% as intense as that at 20.00 ppm before the addition of **20** (consistent with attack of **20** on species like **10** to reduce the value of n). Elemental sulfur precipitated; since both it and **20** persisted together for several days at ca. 25°C, the **21** could not have arisen from their reaction.

b. *With hydroxyl ion and water.* For the UV experiments, after reaction of 1.00 mmol each of **2** and MCPBA at $-10°C$ in 4 mL of CH_2Cl_2 for 6 h, a 30-μL aliquot of the reaction mixture was diluted to ca. 2 mL with cold CH_2Cl_2; the absorbance at 325 nm was 0.84. A 200-μL portion of the mixture then was shaken with an equal volume of H_2O saturated with NaOH for 5 min at $-10°C$. The aqueous layer immediately became pale yellow (attributable to H_2S_n or its salts), and a fine precipitate appeared (attributable to sulfur). A UV spectrum of 60 μL of the CH_2Cl_2 solution, diluted to ca. 2 mL with cold CH_2Cl_2, then had an absorbance of only 0.1 at 325 nm, consistent with nucleophilic attack of OH^- on the intermediates. Repetition of the procedure after 7 h of oxidation gave much the same result. The experiment then was repeated with water alone. After 4 h of oxidation, a UV spectrum was acquired of an aliquot of the reaction mixture; the absorbance of 325 nm was 0.45. When H_2O (200 μL) was shaken with the mixture at $-10°C$ for 4 h, the UV absorbance still was 0.45 (in another experiment, the absorbance did decrease from 0.55 to 0.38 in 1 h but was unchanged during 2 h more).

For the NMR experiments, 1.00 mmol each of the phosphorothioate **2** and MCPBA were allowed to react at $-5°C$ in 4 mL of the Me_2CO-d_6. After 7 h, a ^{31}P-NMR spectrum showed signals for **2** (δ 53.38), triphenyl phosphate (δ -17.74) and intermediates (δ 19.89). Water (200 μL) saturated with NaOH then was added with vigorous shaking. After 5–10 min, precipitate was removed. Signals for **2**, triphenyl phosphate and diphenyl hydrogen phosphate (-10.8 ppm) were observed, but that at 19.89 ppm had completely disappeared and the phosphate peak seemed somewhat larger. To determine the effect of H_2O alone, the preceding experiment was repeated, a spectrum was acquired after 8 h, and H_2O (200 μL) then was added. After 30 min, the peak for intermediates at 19.89 ppm had vanished and a peak had appeared at 21.20 ppm but with only about 55% of the initial intensity; the slight shift, downfield, and the partial loss of intensity are consistent with attack of H_2O on species among the intermediates of the form $(RO)_3PS_xSO_m^{n-}$.

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For the $H_2{}^{18}O$ study, after reaction of 0.50 mmol each of **2** and MCPBA in 2 mL of Me_2CO-d_6 at −5°C for ca. 8 h, 100 μL of $H_2{}^{18}O$ was added. After ca. 5 h more, an aliquot was analyzed by mass spectrometry (EI, direct probe injection). The usual fragmentation pattern was observed (cf. Table I.A). No enhancement of the M + 2 peak (m/z 328) of triphenyl phosphate could be detected. On the other hand, enhanced peaks were observed at m/z 391 and 484 (^{18}O peaks corresponding to 389 and 482 of Table I.A), indicating that although ^{18}O was not incorporated into the triphenyl phosphate, it was incorporated into some of the intermediates (e.g. **12**, by exchange).

In the experiments on the oxidation done in the presence of $H_2{}^{18}O$, 0.50 mmol of **2**, $H_2{}^{18}O$ (either 2, 10 or 50 μL), and 0.50 mmol of MCPBA were dissolved in 2 mL of Me_2CO-d_6. Each mixture then was allowed to react completely at −5°C (8.5 h), 25°C (0.5 h), or reflux temperatures (10–20 min). At reflux, with 2 μL of $H_2{}^{18}O$ the mixture became yellow within seconds and sulfur precipitated; with 10 μL of $H_2{}^{18}O$ 2–3 min was required; with 50 μL, only a faint yellow had developed after 5–10 min, and little or no sulfur was seen. It is noteworthy that the greatest amounts of sulfur were produced in the fastest reactions; this observation is consistent with earlier stoichiometric experiments at 25°C,[4] as well as with Scheme 1, and indicates that *slow* generation of species like **7** permits time for oxidation to species like **12** instead of precipitation of sulfur. Similar relationships were obtained at 25°C. The reactions at −5°C led to little precipitate even after several hours. Clearly, therefore, $H_2{}^{18}O$ slowed the oxidation. Aliquots were taken from all of the mixtures and EI mass spectra were determined. Triphenyl phosphate was easily detected (m/z 326). No ^{18}O was incorporated into it in any instance, based on [(intensity of m/z 328)/(intensity of m/z 326)]—[same values without $H_2{}^{18}O$].

ACKNOWLEDGMENTS

This research was supported by PHS Grant No. CA 30321 awarded by the National Cancer Institute, DHSS. We thank the Vanderbilt Center in Molecular Toxicology for support with instruments and secretarial help (PHS Grant No. ES 00267) and Ms. Tracy Wright of the Center for help in the diode-array UV spectroscopy. We are indebted for mass spectra to Prof. B. J. Sweetman and Ms. Betty Fox of the Department of Pharmacology, Vanderbilt University (funds were provided by the N.I.H. Division of Research Resources Grant No. RR 01688).

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