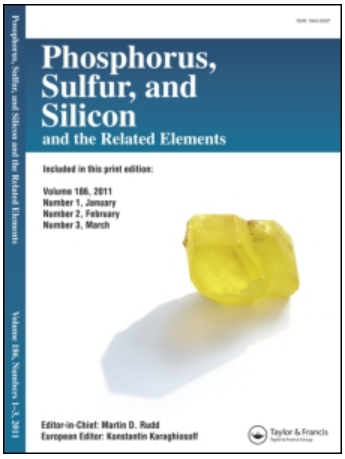

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ASPECTS OF ORGANOSELENIUM CHEMISTRY

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Monoselenophosphate, $SePO_3H_{3-n}^{n-}$, has been chemically synthesized and characterized. It has been shown to be identical with the biological selenium donor SePX. Hydrolysis of selenophosphate is pH dependent and maximal at about pH 7. The dianion is the species which hydrolyzes fastest. The hydrolysis occurs via a dissociative monomeric metaphosphate-like transition state. Alcohols and amines are phosphorylated by monoselenophosphate. The sensitivity of detection of both ^{77}Se and ^{125}Te by NMR spectroscopy has been greatly increased by inverse proton detection using multiple-quantum ^{1}H-{^{77}Se} and ^{1}H-{^{125}Te} correlation spectroscopy. One- and two-dimensional HMQC spectra have been obtained for a variety of organoselenium and tellurium compounds. The signal enhancement obtained by such methods are comparable to the theoretical values.

Key words: monoselenophosphate, indirect ^{77}Se, $^{123,125}Te$ NMR

INTRODUCTION

The labile selenium donor compound SePX is required for the synthesis of selenium-dependent enzymes and seleno-tRNAs. It is formed from ATP and HSe^- by the *sel*D enzyme.[1-3] The structure of SePX has unequivocally been demonstrated to be monoselenophosphate,

$SePO_3H_{3-n}^{n-}$, by comparison with the chemically synthesized and characterized material.[4] A number of enzymes have selenocysteine, Sec, residues and in some cases at their active sites.[5,6] The specific incorporation of Sec in the ribosome is directed by the UGA codon. The selenocysteyl-$tRNA^{Sec}$ which responds to this codon is biosynthesized by selenium donation from $SePO_3H_{3-n}^{n-}$ to the 2,3-aminoacryl-$tRNA^{Sec}$ formed by dehydration of seryl-$tRNA^{Sec}$.[7,8]

Hydrolysis of phosphate monoesters has been extensively studied[9-13] and has been described in terms of a monomeric metaphosphate-like transition state. However, all of the kinetic data fits a preassociation mechanism.[13-15] Hydrolysis of μ-monothiopyrophosphate also occurs by a dissociative transition state in which there is no nucleophilic participation to generate monomeric metaphosphate[16,17] which cannot escape its solvation sphere. Hydrolysis of $SePO_3H_{3-n}^{n-}$ appears to be mechanistically related to these previously studied systems.[18]

^{77}Se NMR spectroscopy provides a powerful method for structural studies of organoselenium compounds and selenoproteins. Selenium-77 has spin I = ½ and a chemical shift range of over 3000 ppm[19-21] rendering it an advantageous structural probe. Nevertheless the low magnetogyric ratio of ^{77}Se and its relatively low natural isotopic abundance of 7.58% render its measurement in proteins difficult. Direct NMR detection of ^{77}Se in the catalytically active form of isotopically enriched glutathione peroxidase was not possible because of its limited solubility.[22] Consequently, the use of inverse detection of ^{77}Se was developed.[23] Inverse detection has been applied to detecting a number

of insensitive nuclei.[24] In this method applied to selenium the detection of ^{77}Se is enhanced by taking advantage of the high magnetogyric ratio and high natural isotopic abundance of ^{1}H. The method requires that the ^{1}H and ^{77}Se nuclei be scalar coupled. The theoretical enhancement for such inverse detection is given by $N(\gamma_{^1H}/\gamma_{^{77}Se})^{3/2}$ where N is the number of ^{1}H nuclei, and γ is the magnetogyric ratio for the subscripted nucleus.[25] The technique used for inverse detection of ^{77}Se was multiple-quantum ^{1}H-{^{77}Se} correlation spectroscopy. This method has also been applied for the detection of tellurium.[26] Tellurium has two magnetic nuclei: Te-123 and Te-125, both with spin I = ½. Since the natural isotopic abundance of ^{125}Te is greater than that for ^{123}Te, 7.14% versus 0.9%, most work focused on ^{125}Te. Although the magnetogyric ratio of ^{125}Te is greater than that for ^{77}Se, substantial enhancement of ^{125}Te is still expected by inverse ^{1}H detection. An attractive feature of ^{125}Te NMR spectroscopy is its especially large chemical shift range of 7000ppm.[19]

RESULTS AND DISCUSSION

Chemical Synthesis of $SePO_3H_{3-n}^{n-}$ and Identity with SePX

Monoselenophosphate can be synthesized conveniently as shown in Eq.(1)-(4). O,O,O-Trimethylselenophosphate **1** was prepared as previously reported.[27] Treatment of this compound with trimethylsilyl iodide gave **2** in quantitative yield. This material was identical with that reported previously.[28] An aqueous solution of $SePO_3H_{3-n}^{n-}$ can be prepared by hydrolysis of **2** but this is inconvenient because of the water-insolubility of this material. Consequently a more convenient procedure

$$(MeO)_3P + Se \longrightarrow \underset{\mathbf{1}}{(MeO)_3PSe} \quad (1)$$

$$MeO)_3PSe + TMSI \longrightarrow \underset{\mathbf{2}}{(TMSO)_3PSe} \quad (2)$$

$$(TMSO)_3PSe + i\text{-}PrOH + i\text{-}Pr_2NEt \longrightarrow \underset{\mathbf{3}}{(i\text{-}Pr_2NHEt)^+[(TMSO)_2POSe]^-} \quad (3)$$

$$\mathbf{3} + H_2O \longrightarrow SePO_3H_{3-n}^{n-} \quad (4)$$

was developed.[4,29] Selective cleavage of one trimethylsilyl group was achieved by adding a controlled amount of *i*-PrOH and *i*-Pr_2NEt to **2** in CH_2Cl_2. On cooling the solution and adding hexanes, **3** precipitated as a colorless solid. This material can be recrystallized to provide analytically pure salt which can be conveniently stored. Its structure was established by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopic analysis and elemental analysis. Furthermore, selective alkylation at selenium with MeOTf provided the known[28] $MeSeP(O)(OTMS)_2$ identical with the previously reported material. The water-soluble salt **3** can be conveniently hydrolyzed. Owing to the facile oxidation of $SePO_3H_{3-n}^{n-}$, it is essential that its preparation is carried out under rigorously anaerobic conditions. Aqueous solutions of $SePO_3H_{3-n}^{n-}$ were characterized by ^{31}P NMR spectroscopy and the insoluble $Ba_3(SePO_3)_2$ salt obtained by addition of barium chloride to an aqueous alkaline solution. The ^{31}P NMR spectrum of $SePO_3H_{3-n}^{n-}$ showed a strong dependence of the ^{31}P chemical shift on pH. The chemical shift varies by almost 20 ppm over the pH range of 1-12. Similar dependence of the ^{31}P chemical shift on pH has been previously reported for H_3PO_4,[30,31] condensed phosphates,[31] adenine nucleotides,[32,33] and thiamine diphosphate.[34] This sensitivity of chemical shift to ionization

state of $SePO_3H_{3-n}^{n-}$ permits facile determination of pK_2 and pK_3 for H_3PO_3Se. From the inflection points in the plot of ^{31}P chemical shift versus pH, the pK_as of $SePO_3H_{3-n}^{n-}$ acid may be estimated. The pK′ for ADP and ATP were determined in a similar way using NMR spectroscopic data.[35] Shown in Table I are pK_2 and pK_3 corresponding

TABLE I pK_as of H_3PO_3X

Substituent X	pK_2	pK_3
O	7.2[a]	12.3[a]
S	5.6,[b] 5.40,[c] 5.4[d]	10.3,[b] 10.14,[c] 10.2[d]
Se	4.6	8.8

[a]Ref. [36] [b]Ref. [37] [c]Ref. [38] [d]Ref[39]

to Eq. (5) and (6), respectively, determined in this way for H_3PO_3Se and

$$H_2PO_3X^- \rightleftharpoons H^+ + HPO_3X^{2-} \quad (5)$$

$$HPO_3X^{2-} \rightleftharpoons H^+ + PO_3X^{3-} \quad (6)$$

the corresponding pK_as reported for H_3PO_4 and H_3PO_3S. As expected the relative acidities of the corresponding acids follows the order Se>S>O.

Monoselenophosphate, prepared and characterized chemically, was compared with the prokaryotic biological selenium donor SePX.[4] The ^{31}P chemical shift and ^{31}P-^{77}Se coupling constant for each of the two species were identical under the same conditions. In addition, ^{75}Se-radiolabeled SePX was prepared from $Na^{75}SeH$ and ATP using purified selenophosphate synthetase. This material and chemically prepared $SePO_3H_{3-n}^{n-}$ coeluted on ion-pairing HPLC. Furthermore, addition of

chemically prepared unlabeled $SePO_3H_{3-n}^{n-}$ to ^{75}Se labeled SePX generated *in situ* decreased the amount of ^{75}Se incorporated into seleno t-RNAs by a partially purified enzyme in a dose dependent manner. Consequently, the biological intermediate SePX and chemically prepared and characterized $SePO_3H_{3-n}^{n-}$ are concluded to be the same species.

Hydrolysis of $SePO_3H_{3-n}^{n-}$

Since $SePO_3H_{3-n}^{n-}$ is a new chemical species, its chemical behavior, particularly that which may be relevant to its biological activity, is being explored. The first reaction investigated was its hydrolysis.[18] Since the ^{31}P NMR signal for $SePO_3H_{3-n}^{n-}$ is well-removed from that of $H_{3-n}PO_4^{n-}$, the hydrolysis of $SePO_3H_{3-n}^{n-}$ can be conveniently monitored by ^{31}P NMR spectroscopy. The rates of hydrolysis at various pHs were determined by measuring the decrease in the ^{31}P signal for $SePO_3H_{3-n}^{n-}$ and the corresponding increase in the signal due to $H_{3-n}PO_4^{n-}$. These rates could be conveniently monitored at 53.7° in NMR tubes containing aqueous buffer solutions of $SePO_3H_{3-n}^{n-}$ sealed under inert gas. The rates of the reaction depended on the pH of the solution and obeyed first order kinetics. The rates were determined in the pH range of 2-12 and a plot of the dependence of the rate on pH is shown in Fig. 1. A variety of different buffers was used and, where there was overlap between two different buffers, at the same pH, the rates of hydrolysis were the same within experimental error. The pH-rate profile could be fitted analytically and a best least-squares fit of the data gave the following rate constants for each of the ionic states of $SePO_3H_{3-n}^{n-}$: $k_0<10^{-6}s^{-1}$ (free acid), $k_1=0.3\times10^{-5}s^{-1}$ (monoanion), $k_2=45.1\times10^{-5}s^{-1}$ (dianion) and $k_3<10^{-6}s^{-1}$ (trianion).

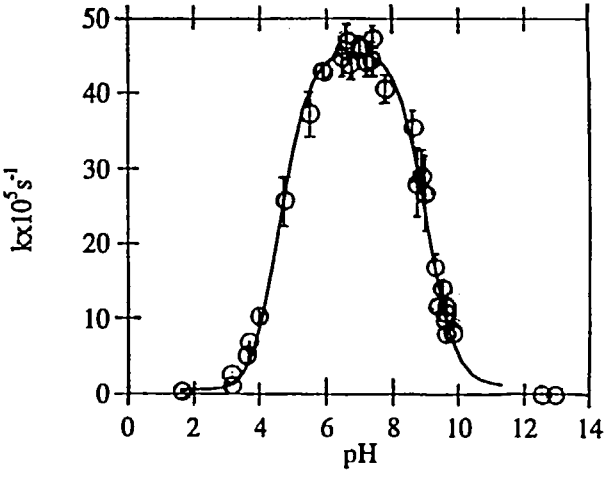


FIGURE 1. Plot of the rate constant versus pH for the hydrolysis of $SePO_3H_{3-n}^{n-}$

The pK_as determined kinetically were 4.61 and 9.1 for pK_2 and pK_3, respectively. Although these pK_as refer to a different temperature than the pK_as determined by ^{31}P NMR spectroscopy as outlined above, they are in reasonable agreement. The activation parameters for the hydrolysis were determined at various pHs. In the alkaline pH range, the values for E_a were found to be 27.5 ± 1.8 and 31.1 ± 1.3 kcal/mol, $\Delta H_{298}^{\neq}$ 26.9± 1.8 and 30.5 ± 1.3 kcal/mol, and $\Delta S_{298}^{\neq}$ 7.5± 5.6 and 16.2 ± 4.2 cal/mol deg.

The fortuitous monitoring of the hydrolysis of $SePO_3H_{3-n}^{n-}$ by ^{31}P NMR spectroscopy provided an unexpected result. During the hydrolysis of $SePO_3H_{3-n}^{n-}$ in Tris, $H_2NC(CH_2OH)_3$, buffer a small signal appeared in the ^{31}P NMR spectrum in addition to those due to the starting material and $H_{3-n}PO_4^{n-}$. Increasing the concentration of buffer did not significantly change the rate of reaction but resulted in an increase in the unassigned

signal. It was surmised that this signal was due to phosphorylated buffer. Indeed it was proved that Tris was O-phosphorylated in the following way. A triplet was observed for the unassigned ^{31}P signal when there was no ^{1}H decoupling and the chemical shift was identical with authentic O-phosphorylated Tris[16] prepared by the reaction of Tris with ammonium hydrogen phosphoramidate.[40] Once it had been established that Tris could be O-phosphorylated other alcohols were added to determine if this phosphorylation were general. Indeed ethylene glycol and glycerol were phosphorylated by $H_{3-n}PO_4^{n-}$ in aqueous Hepes, N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid, or Mops, (3-N-morpholino) propane sulfonic acid, buffers.[41] The structures of the phosphorylated products were established by comparison with authentic compounds. In addition to comparison of the ^{31}P chemical shifts, the products from phosphorylation of ethylene glycol and glycerol were silylated and analyzed by GC-MS.[42,43] The materials obtained in this way from ethylene glycol and glycerol and $SePO_3H_{3-n}^{n-}$ gave identical GC retention times and MS as those of authentic materials. The results with glycerol are particularly interesting. As shown in Eq. (7) two phosphorylation

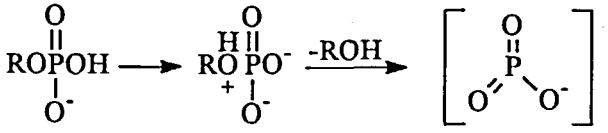
$$\begin{array}{l} CH_2OH \\ | \\ CHOH + SePO_3H_{3-n}^{n-} \\ | \\ CH_2OH \end{array} \longrightarrow \begin{array}{l} CH_2OPO_3H_{2-n}^{n-} \\ | \\ CHOH \\ | \\ CH_2OH \end{array} + \begin{array}{l} CH_2OH \\ | \\ CHOPO_3H_{2-n}^{n-} \\ | \\ CH_2OH \end{array} \qquad (7)$$

products were obtained. These two products were shown to be identical with authentic α- and β-glycerol phosphate as outlined above. In addition, these products were formed in approximately 2:1 ratio of α:β isomers. Since there are two equivalent primary alcohol moieties in

glycerol which on phosphorylation lead to the α-product but only one secondary alcohol group which leads to the β-product, the observed ratio of products is statistical. That is, the rate of phosphorylation of either the primary or secondary alcohol is about the same. This lack of selectivity in the phosphorylation step was generally observed. Thus, the ratios of added alcohol to water are within a factor of 15 or less of the ratio of O-phosphate to $H_{3-n}PO_4^{n-}$. Amines could be phosphorylated by $SePO_3H_{3-n}^{n-}$ as well. With 2-aminoethanol both the product of O-phosphorylation and N-phosphorylation were obtained. The amino group in 1,2-diaminoethane and morpholine were also phosphorylated. The products of these phosphorylations were confirmed by comparison with authentic compounds. Again the phosphorylation of amines, like that of alcohols, was relatively unselective.

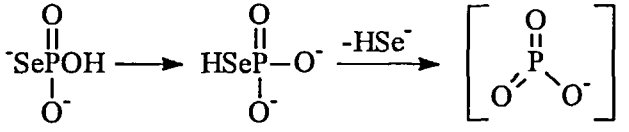
The proposed mechanism for the hydrolysis of $SePO_3H_{3-n}^{n-}$ was based on the following factors: first order dependence, the positive $\Delta S^{\neq}$ the lack of significant rate changes on the addition of alcohols and amines which, nevertheless, were phosphorylated, and the relatively indiscriminant phosphorylation of nucleophiles. From these data it was concluded that this hydrolysis is dissociative in nature involving a monomeric metaphosphate-like transition state. There is no evidence for free monomeric metaphosphate as a reaction intermediate with a finite lifetime but rather a $D_N{}^*A_N$ mechanism.[44] A further interesting issue is the structure of the species undergoing decomposition. In the hydrolysis of monophosphate esters of phenols in which the pK_a of the phenol is greater than 5.5, the monoanion is the most reactive species.[9] The monoanion decomposes by first protic isomerization to generate a better

leaving group and better electrofugal moiety as shown in Eq. (8).



(8)

Similarly, decomposition of the $SePO_3H^{2-}$, whose structure is presumed to be **4**, occurs fastest of the various ionic species. In analogy with phosphate monoesters, it is proposed to decompose as shown in Eq. (9).



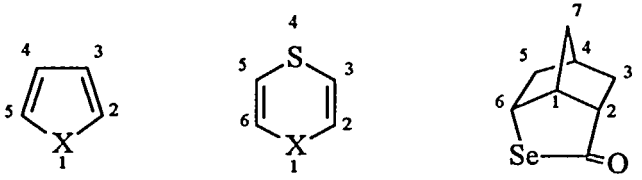
(9)

4

Indirect Detection of Se-77 in NMR Spectroscopy

As pointed out in the introduction $SePO_3H_{3-n}^{n-}$ serves as the biological donor of selenium in the formation of selenocysteyl t-RNA. This Sec is then incorporated cotranslationally into selenoproteins. Since Sec is often at the active site of such proteins it is of interest to develop ^{77}Se NMR spectroscopy which can provide structural information about Sec and its environment.

As pointed out in the Introduction ^1H-{^{77}Se} HMQC spectroscopy was used to increase the sensitivity of NMR detection of selenium. Selenophene **5a**, Me_2Se, Me_2Se_2, and selenomethionine, whose direct ^{77}Se NMR spectra have previously been reported were used to validate the method. Satisfactory one- and two-dimensional ^1H-{^{77}Se} heteronuclear multiple quantum coherence (HMQC)[45,46] spectra were obtained for all of these compounds. Illustrated in Fig. 2 is the spectrum obtained for **5a** in the region where the H(2,5) absorption appears. Note



5a, X=Se
b, X=Te

6a, X=Se
b, X=Te

7

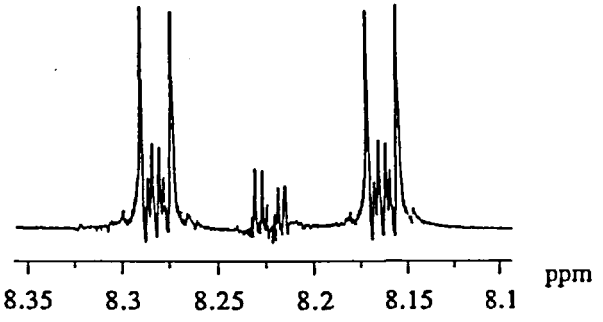


FIGURE 2. 1-D ^{1}H-{^{77}Se}HMQC spectrum of selenophene, **5a**

that the absorption at 8.22 ppm, where the main absorption peak would appear in an ordinary ^{1}H NMR spectrum of this compound is suppressed. This results from the fact that this signal is due to ^{1}H nuclei that are not coupled to ^{77}Se because they are in molecules containing other, non-magnetic selenium isotopes. However, the absorptions at 8.16 and 8.28 ppm, which appear as satellites in an ordinary ^{1}H NMR spectrum of **5a**, are enhanced because they are due to ^{1}H in molecules of 5a containing the Se-77 isotope and are coupled to ^{77}Se. To estimate the experimental enhancement in such NMR experiments, Me_2Se was investigated. The absorption obtained in the inverse ^{1}H-{^{77}Se} HMQC spectrum was compared with that obtained by direct detection. After correcting for the

differences in number of acquisitions, signal-to-noise ratio, and filling factors the experimental enhancement was found to be approximately 68. This compares very favorably with the theoretical enhancement value of 72. Consequently, combining the use of inverse detection with isotopic enrichment an increase of sensitivity in detecting ^{77}Se of approximately 800 × is obtained.

This method has also been used to measure the ^{77}Se NMR spectroscopic parameters of compounds **6a** and **7**. Use of 2D ^{1}H-{^{77}Se} HMQC and COSY NMR spectroscopic methods were vital in providing a complete assignment of the ^{1}H NMR spectrum of **7**.[47] In particular, H(6) could be unequivocally assigned based on its large two-bond coupling constant with ^{77}Se of 29.8 Hz. Furthermore, there are three-bond couplings between H(5exo), H(5endo) and ^{77}Se of 3.8 and 10.7 Hz, respectively but not between H(1) and ^{77}Se. This lack of a measurable coupling constant between H(1) and ^{77}Se is ascribed to the Se-C(6)-C(1)-H(1) dihedral angle of 84°. Finally this method has also been successfully applied to Protein A, a selenoprotein which is part of the *Clostridium sticklandii* glycine reductase complex.[48]

Indirect Detection of Te in NMR Spectroscopy

Since inverse detection of ^{77}Se proved so advantageous, this method was applied to tellurium.[26] Satisfactory one- and two-dimensional ^{1}H-{^{125}Te} HMQC spectra were secured for Me_2Te, *n*-Bu_2Te, Me_2Te_2, $PhCH_2TeCN$, Me_3TeCl, telluromethionine, **5b**, and **6b**. An illustration of the spectra obtained is given in Fig. 3 for **6b** which shows the region in which H(2,6) resonance occurs. In the ordinary ^{1}H

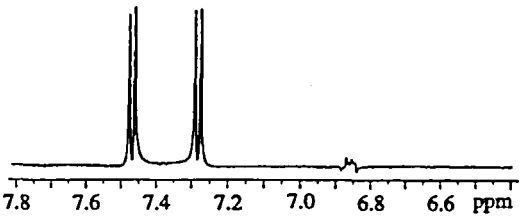


FIGURE 3 1-D ^{1}H-{^{125}Te} HMQC spectrum of 1,4-thiatellurin, **6b**

NMR spectrum of this compound H(2,6) appears as a doublet with a chemical shift of 7.37 ppm. This absorption is suppressed in the indirect spectrum shown because it is due to ^{1}Hs not coupled to ^{125}Te. It is further suppressed by nulling the signal using the BIRD pulse sequence and then averaging away any residual signal by alternating the phase of the receiver. The doublets centered at 7.28 and 7.47 ppm correspond to the satellites in an ordinary ^{1}H NMR spectrum and are due to the absorption of H(2,6) in molecules containing ^{125}Te in which ^{1}H and ^{125}Te are coupled to each other. The experimental enhancement due to inverse detection of ^{125}Te was determined in the same way as that for ^{77}Se using Me_3TeCl. The experimentally observed enhancement was approximately 46 which is close to the theoretical enhancement value of 50.7. To demonstrate that this method is also applicable to the Te-123 nucleus, compound **6b** was studied. A satisfactory one-dimensional ^{1}H-{^{123}Te} HMQC spectrum was obtained.

In conclusion, the biological selenium donor compound SePX was shown to be $SePO_3H_{3-n}^{n-}$; hydrolysis of $SePO_3H_{3-n}^{n-}$ is dissociative involving a monomeric metaphosphate-like transition state; inverse detection of ^{77}Se and ^{125}Te using HMQC spectroscopy is advantageous.

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