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Identification and Synthesis of a Novel Selenium–Sulfur Amino Acid Found in Selenized Yeast: Rapid Indirect Detection NMR Methods for Characterizing Low-Level Organoselenium Compounds in Complex Matrices

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After proteolytic digestion, aqueous extraction, and derivatization with diethyl pyrocarbonate or ethyl chloroformate, HPLC–inductively coupled plasma (ICP)-MS, GC–atomic emission detection (AED), and GC–MS analysis of high-selenium yeast stored at room temperature for more than 10 years showed selenomethionine as the major Se product along with substantial amounts of selenomethionine selenoxide hydrate and the previously unreported selenoamino acid having a Se–S bond, *S*-(methylseleno)cysteine. The identity of the latter compound was confirmed by synthesis. The natural product was shown to be different from a synthetic sample of the isomeric compound *Se*-(methylthio)-selenocysteine. Selenium-specific NMR spectroscopic methods were developed to directly analyze the aqueous extracts of the hydrolyzed selenized yeast without derivatization or separation. Selenomethionine and *S*-(methylseleno)cysteine were identified by ^{77}Se–^{1}H HMQC–TOCSY experiments.

KEYWORDS: Selenized yeast; selenoamino acids; *S*-(methylseleno)cysteine; 1D HMQC–TOCSY

INTRODUCTION

There is considerable interest in Se as a micronutrient because it is an essential element (*1*, *2*). In addition, it has been reported by Clark et al. (*3*) to dramatically decrease the incidence of colon, prostate, and lung cancer in humans when administered as selenized yeast tablets (one tablet containing 200 μg of total Se per day). Follow-up studies on this report are currently under way using selenized yeast tablets (*4*) or pure selenomethionine (*5*). Therefore, the chemical speciation of Se in selenized yeast is of great interest to determine what Se compounds are present and to monitor their stability in tablets over time.

Analytical techniques for chemical speciation of Se compounds have been reviewed (*6*, *7*). Determination of Se compounds in biological samples has been especially challenging owing to the small amounts of Se compounds present, the complexity of the systems, and the potential instability of the Se compounds. In the most successful of the methods used, the samples are hydrolyzed with proteases and the components of the aqueous extracts are analyzed for nonvolatiles such as selenium anions and selenoamino acids by HPLC and further derivatized and separated by GC for volatiles. Selenium-containing compounds are determined quantitatively by element-specific atomic spectroscopy: atomic emission detection (AED) with GC or inductively coupled plasma mass spectrometry (ICP-MS) with HPLC. However, these compounds must generally be identified by comparison with known standards unless sufficient material is available for spectroscopic characterization. For example, following digestion of yeast samples and chromatographic separation, Se-containing glutathione *S*-conjugates were identified by nanoelectrospray MS/MS (*8*), selenoadenosyl-methionine and homocysteine were identified by ICP-MS (*9*–*11*), and the selenopentapeptide SeMet-Asn-Ala-Gly-Arg was identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) (*12*, *13*). By combining the use of chiral HPLC columns with ICP-MS, chiral speciation of D- and L-selenomethionine in selenized yeast can be accomplished (*14*). Capillary zone electrophoresis (CE)–ICP-MS has also been applied to Se speciation of yeast hydrolysates (*15*).

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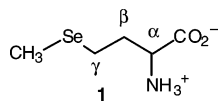[§] University of Massachusetts.
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Figure 1. Selenomethionine (**1**).

The major form of Se in selenized yeast is selenomethionine (**1**; **Figure 1**) (ca. 65%) corresponding to 85−90% of the chromatographically elutable Se species, but at least eight other inorganic or organic Se compounds were shown to be present by HPLC−ICP-MS examination of the hydrolyzed aqueous extracts (*10*). Analysis of archived samples of selenized yeast from the Clark trials showed that two additional Se compounds were sometimes present, in some cases in amounts together approaching or exceeding the level of **1**. One of these unidentified compounds corresponds in retention time and mass spectral fragmentation to the hydrate of selenomethionine *Se*-oxide (**2**, $MeSe(OH)_2CH_2CH_2CH(NH_2)CO_2H$) (*16−19*).

The present study was designed to determine the structure of the additional selenium compound in the archived selenized yeast by established analytical techniques and by the new use of NMR spectroscopic methodology (*20*). In addition, the potential advantages and disadvantages of NMR spectroscopic methodology over the previously established methods were evaluated.

MATERIALS AND METHODS

ICP-MS, GC−AED, and GC−MS Instrumentation. An Elan 5000 inductively coupled plasma mass spectrometer (Perkin-Elmer Sciex, Norwalk, CT) was used for total Se determination and HPLC−ICP-MS. Samples were introduced using a cross-flow nebulizer and double-pass spray chamber. For total Se determination ^{82}Se and ^{77}Se were monitored with ^{74}Ge as an internal standard. The chromatographic system consisted of a SP8810 liquid chromatographic pump (Spectra-Physics, San Jose, CA). The column used was (i) a 15 cm × 3.9 mm i.d., 5 μm Symmetry Shield reversed-phase C8 column or (ii) a 15 cm × 3.9 mm i.d., 5 μm XTerra RP-C8 column, a hybrid particle that has a polar modifier group between the C8 group and the silica base (both columns were from Waters Corp., Milford, MA). The column was connected to the nebulizer with PEEK tubing (30 cm × 0.25 mm i.d.). The mobile-phase compositions were as follows: 99/1 (v/v) water/methanol was used in each case with 0.1% trifluoroacetic acid (TFA) or 0.1% heptafluorobutanoic acid (HFBA).

An Agilent/Hewlett-Packard HP 5921A atomic emission detector interfaced with an HP 5890II gas chromatograph was used. The injection port (splitless) was maintained at 250 °C: the GC oven was programmed from 100 °C (initial temperature for 5 min) to 200 °C at 5 °C/min, holding at 200 °C for 5 min. A 25 m × 0.32 mm, 0.17 μm (film thickness) HP-1 column was used. The helium plasma gas flow was kept at 180 mL/min. Hydrogen was used as a reagent gas, with detection at 181 nm (S) and 196 nm (Se). An HP mass-selective detector (Agilent/Hewlett-Packard Co.) interfaced with an HP 6890 gas chromatograph was used for GC−MS analysis, using a 30 m × 0.25 mm, 0.25 μm HP-5 (5% phenyl, 95% poly(dimethylsiloxane)) column. The oven was run at an isothermal temperature of 160 °C, and helium was used as a carrier gas at a flow rate of 2 mL/min. The split ratio was 10:1, and the column head pressure was 13.28 psi.

NMR Instrumentation and Methods. NMR data were acquired on a Bruker Avance DRX-500 spectrometer operating at a ^1H frequency of 499.93 MHz and a ^{77}Se frequency of 95.35 MHz, using a 5 mm Nalorac inverse broad-band three-axis gradient probe at a temperature of 25 °C. All 1D ^1H spectra were acquired with a spectral width of 3.50 ppm, and with oversampling (96×) and digital filtering to give 2048 complex data points. Simple 1D ^1H spectra were acquired with presaturation of the residual H_2O signal (4.71 ppm) for 1.5 s, moving the carrier to 3.25 ppm for the excitation pulse and acquisition of the FID, which was zero-filled to 4096 complex points and transformed after applying a skewed, 45° shifted sine-bell window function.

Direct ^{77}Se experiments were carried out with ^1H waltz-16 decoupling during acquisition of the FID with a decoupling field strength of 1.2 kHz. This was determined to be the lowest decoupling power which gives good decoupling of a 60% dimethyl selenide in $CDCl_3$ sample with the ^1H decoupler 5 ppm off-resonance. At higher power levels, sample heating was observed in D_2O samples. The ^{77}Se 90° pulse was measured at 15 μs using ^1H detection of the ^{77}Se satellites of the CH_3 group of selenomethionine. The ^{77}Se T_1 of selenomethionine in D_2O was measured as 2.3 s in an inversion−recovery experiment.

The 2D $^1H-^{77}Se$ HMQC experiment was a phase-sensitive, gradient-selected HMQC (*21*) without refocusing of $J_{Se−H}$ and without ^{77}Se decoupling during acquisition. The defocusing delay (1/2J) was 25.2 ms, and the gradient amplitudes were 17, 52.43, and 62.43 (% of maximum), respectively, using 1 ms sine-shaped gradients on the z-axis. Data were acquired in t_2 with oversampling (128×) and digital filtering with a spectral width of 3.00 ppm, collecting 1024 real points, and in t_1 with 750 FIDs in TPPI mode with a spectral width of 50.0 ppm. The relaxation delay was 1.5 s, and 56 scans were summed to give each FID. Data were processed using Felix (Accelrys, San Diego, CA). A cosine-bell window function was applied in each dimension, with zero-filling to a final real matrix of 2048 (F_2) × 1024 (F_1) points.

The 1D $^1H-^{77}Se$ HMQC−TOCSY experiment used a simple gradient-enhanced HMQC sequence (*22*) with the $t_1/2$ delay fixed at the minimum value (1.2 ms), and a TOCSY mixing sequence inserted just before the FID acquisition. TOCSY mixing was achieved by 30 cycles of MLEV-17 at an rf field strength of 8.33 kHz between two 1 ms trim pulses at full power for a total mixing time of 60.2 ms. Gradient selection was implemented using 1 ms sine-shaped z-axis gradients of amplitude 50, 30, and 35.258 (percent of the maximum).

Selenized Yeast Samples. Selenium-enriched yeast (SelenoExcel, also designated SelenoPrecise) was obtained from Cypress Inc., Fresno, CA. Archived selenized yeast and tablets fabricated from it, as employed in the "Clark" human intervention trials (*3*, *4*) were obtained from The University of Arizona, McKesson Corp., and Cornell University. The materials had been stored at ambient temperatures. The original selenium content of the selenized yeast was 1200 μg/g, and the tablets each contained 200 μg of Se, there being no evidence of a change upon storage. Stock solutions of selenoamino acids were prepared in 0.2 M HCl. A stock solution of sodium selenate was prepared in 2% (v/v) HNO_3, while the plasma Se standard was used as a stock solution of sodium selenite. Working solutions were diluted with mobile phase and stored in the dark between 0 and 4 °C. The standards used in the measurements thus were 0.02−0.002 M in HCl or 0.0002% in HNO_3 after dilution. These acid concentrations have no effect on chromatographic separation and speciation considering the 10 μL volumes injected. Selenized yeast extracts were prepared as follows. A tablet of selenized yeast was ground with a mortar and pestle. The powder was placed in a 50 mL centrifuge tube with purified water (10 mL) and type XIV bacterial protease. The mixture was stirred at room temperature for 24 h and then centrifuged at 5000 rpm for 1 h. The clear supernatant liquid was separated and lyophilized. For NMR experiments the lyophilized powder was dissolved in D_2O (0.5 mL).

Syntheses. *S-(Methylseleno)cysteine (3)*. Potassium hydroxide (0.673 g, 12 mmol) was added to a suspension of L-cysteine (0.485 g, 4 mmol) in anhydrous MeOH (15 mL) under argon. The mixture was stirred until it became homogeneous and was then treated with freshly prepared 1 M methaneselenenyl bromide (MeSeBr; 1.74 g, 10 mmol) in CH_2Cl_2, whereupon a yellow precipitate immediately formed. The mixture was stirred for 15 min, and the precipitate was separated by filtration, washed with MeOH (3 × 250 mL) and dried, giving a 2:1 mixture of **3** (0.61 g, 71% yield) and cystine as a light yellow powder: mp 189 °C dec; ^1H NMR (300 MHz, D_2O) δ 3.95−3.99 (dd, J = 8.5, 3.9 Hz, 1H), 3.30 and 3.10 (AB part of the ABX spectrum, eight lines, J_{AX} = 3.9 Hz, J_{BX} = 8.5 Hz, J_{AB} = 14.9 Hz, 2H), 2.43 (s, 3H) ppm. The ESI mass spectrum of **3** is shown in **Figure 2**. The Se isotope pattern (ions noted for ^{80}Se) can be seen at m/z = 215.9, 198.8, and 126.8. The molecular ion M + H$^+$ is m/z = 215.9, and the major fragments are $M^+ − CH_2CH(NH_2)COOH$ ($M^+ − 89$ at m/z = 126.8) and $M^+ − NH_3$ ($M^+ − 17$ at m/z = 198.8). Also seen are the M + Na$^+$ and M + K$^+$ peaks at m/z 237.8 and 253.8, respectively.
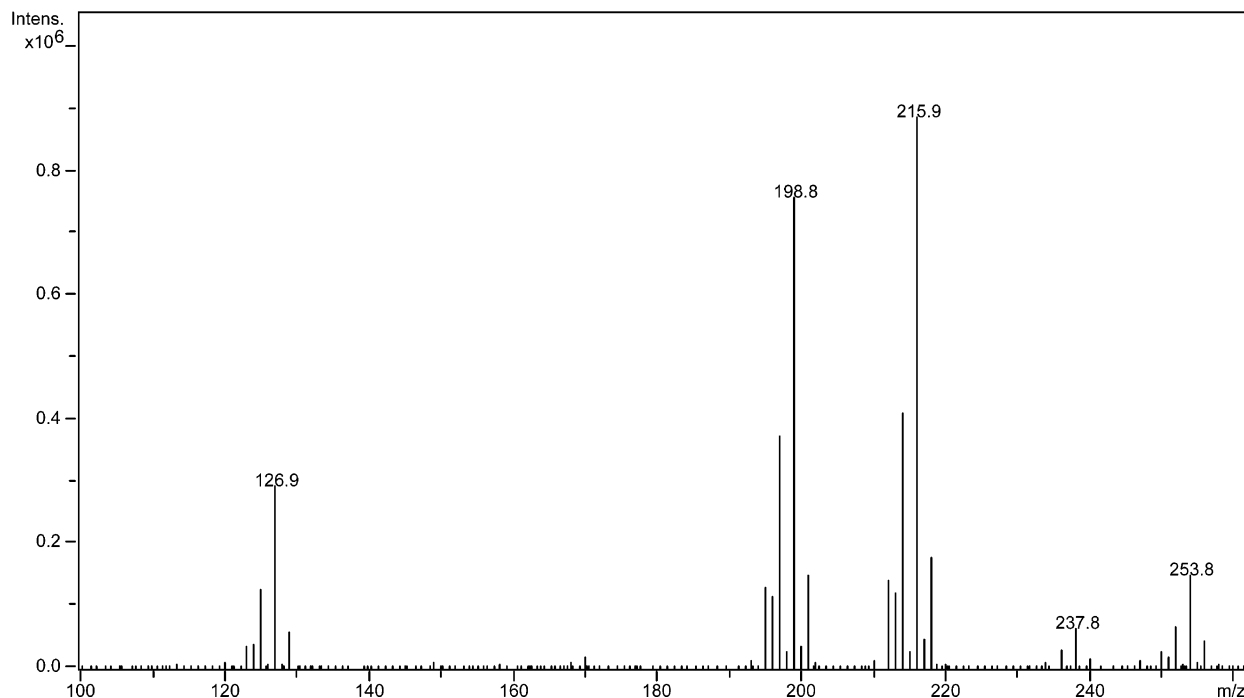
Figure 2. Electrospray ionization mass spectrum of synthetic *S*-(methylseleno)cysteine (**3**).

S-(Methylseleno)cysteine Ethyl Ester N-Ethylcarbamate (3a). The derivatization procedure of Husek was followed (*23*). Diethyl pyrocarbonate (700 μL, 4.76 mmol) was added to a suspension of **3** (110 mg, 0.51 mmol) in H$_2$O/Py/EtOH (60:8:32 v/v/v; 1.5 mL). Foaming occurred due to CO$_2$ evolution. Derivatization was completed within a few seconds after mixing and shaking. The reaction mixture was extracted with CHCl$_3$ (3 × 5 mL), and the organic layer was dried (Na$_2$SO$_4$), concentrated in vacuo, and chromatographed (hexane/ethyl acetate, 3:1) to give **3a** as a light yellow oil, which became semisolid on cooling (77 mg, 47% yield): R_f = 0.51; IR (film) ν_{max} 3344, 2980, 2928, 1720, 1523, 1372, 1338, 1262, 1209, 1057 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 5.44 (br d, J = 6 Hz, 1H) 4.59 (dd, J = 4.8, 5.4 Hz, 1H) 4.19 (q, J = 7.2 Hz, 2H) 4.10 (q, J = 7.0 Hz, 2H) 3.16−3.31 (AB part of the ABX spectrum, eight lines, J_{AX} = 5.4 Hz, J_{BX} = 4.8 Hz, J_{AB} = 13 Hz, 2H) 2.43 (s, 3H), 1.25 (t, J = 7.2 Hz, 3 H), 1.20 ppm (t, J = 7.0 Hz, 3 H) ppm; ^{13}C NMR (90 MHz) δ 170.6, 155.9, 61.8, 61.3, 54.1, 40.0, 14.6, 14.2, 13.2 ppm; ^{77}Se NMR (95.3 MHz, CH$_2$Cl$_2$) δ 327 ppm; EI-GC−MS *m/z* (rel intens) 315 (M$^+$, ^{80}Se, 10), 242 (M$^+$ − CO$_2$Et, 21), 220 (M$^+$ − MeSe, 100), 188 (M$^+$ − MeSeS, 47), 174 (M$^+$ − MeSeSCH$_2$, 100), 160 (34), 146 (57); HRMS *m/z* 316.0129 (calcd for C$_9$H$_{18}$NO$_4$SSe [MH$^+$] 316.0122).

Se-(Methylthio)selenocysteine (4). To a suspension of L-selenocystine (100 mg, 0.299 mmol) in anhydrous EtOH (5 mL) was added NaBH$_4$ (102 mg, 2.69 mmol), and the mixture was stirred under reflux until the yellow solution became colorless. While the mixture was cooled to room temperature, freshly prepared methanesulfenyl chloride (61 mg, 0.748 mmol) was added, and a yellow precipitate immediately formed. The reaction was then stirred for 20 min at room temperature, and the precipitate was decanted, rinsed several times with EtOH, and dried in vacuo to give a 1:1 mixture of **4** (77 mg, 60%) together with selenocystine as a light yellow powder: ^{1}H NMR (300 MHz, D$_2$O) δ 3.74 (dd, 1H) 3.37−3.20 (AB part of the ABX spectrum, eight lines, 2H), 2.51 (s, 3H).

Selenocystine Ethyl Ester N-Ethylcarbamate (5). The derivatization procedure of Husek was followed (*23*). A solution of selenocystine (40 mg, 0.12 mmol) in aqueous HCl (0.1 M; 4 mL) was diluted with H$_2$O/Py/EtOH (60:8:32 v/v/v; 40 mL) and then treated with ethyl pyrocarbonate (2 mL, 13.5 mmol), whereupon foaming and CO$_2$ evolution occurred. The derivatization was completed within a few seconds after mixing and shaking. The reaction mixture was extracted (CHCl$_3$), and the organic layer was separated, dried (Na$_2$SO$_4$), concentrated in vacuo, and chromatographed (silica gel, 1:1 hexane/ethyl acetate), giving **5** as a yellow solid (33 mg, 63% yield): R_f =

0.62; 33 mg; mp 74 °C dec; IR (film) ν_{max} 3427, 2929, 1718, 1700, 1507, 1473, 1374, 1339, 1059 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 5.57 (br d, J = 6.5 Hz, 1H), 4.61 (dd, J = 5.0, 5.8 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 4.10 (q, J = 7.1 Hz, 2H), 3.44−3.30 (AB part of the ABX spectrum, eight lines, J_{AX} = 5.8 Hz, J_{BX} = 5.0 Hz, J_{AB} = 13 Hz, 2H), 1.26 (t, J = 7.1 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (90 MHz) δ 170.6, 156.0, 62.0, 61.4, 54.3, 32.5, 14.5, 14.1 ppm; ^{77}Se NMR (95.3 MHz, CH$_2$Cl$_2$) δ 302 ppm.

Se-(Methylthio)selenocysteine Ethyl Ester N-Ethylcarbamate (4a). Compound **5** (40 mg, 0.10 mmol) in EtOH (3 mL) under argon was treated with NaBH$_4$ (39 mg, 0.34 mmol). After the mixture was stirred for 15 min, the yellow color disappeared. Freshly prepared methanesulfenyl chloride (MeSCl; 102 mg, 1.24 mmol) was then added all at once, and the mixture was stirred at room temperature for 1 h, diluted with CHCl$_3$ (3 mL), and extracted with water and brine (2 × 10 mL). The organic layer was separated, dried (Na$_2$SO$_4$), concentrated in vacuo, and chromatographed (silica gel, 1:3 ethyl acetate/hexane), giving **4a** (19 mg, 26% yield, 42% based on unrecovered **5**) as a light yellow oil together with 17 mg of **5**. Data for **4a**: R_f 0.47; IR (film) ν_{max} 3427, 2978, 1733, 1717, 1506, 1375, 1338, 1065, 1026 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 5.51 (br d, J = 5.4 Hz, 1H), 4.67 (dd, J = 5.0, 5.9 Hz, 1H), 4.19 (q, J = 7.2 Hz, 2H), 4.10 (q, J = 7.1 Hz, 2H), 3.16−3.31 (AB part of the ABX spectrum, eight lines, J_{AX} = 5.9 Hz, J_{BX} = 5.0 Hz, J_{AB} = 13 Hz, 2H), 2.43 (s, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (90 MHz) δ 170.7, 155.9, 61.9, 61.4, 53.7, 32.9, 22.6, 14.6, 14.1 ppm; ^{77}Se NMR (95.3 MHz, CH$_2$Cl$_2$) δ 401 ppm; HRMS *m/z* 316.0121 (calcd for C$_9$H$_{18}$NO$_4$SSe [MH$^+$] 316.0122).

Compound **4a** could also be prepared from **4** in low yield by reaction with ethyl pyrocarbonate as described above for the preparation of **3a** and **5**.

3-(Methyldiseleno)-L-alanine (6). To a suspension of L-selenocystine (167 mg, 0.500 mmol) in anhydrous EtOH (5 mL) was added NaBH$_4$ (170 mg, 4.50 mmol), and the yellow solution was stirred under reflux until it became colorless. After the mixture was cooled to room temperature, freshly prepared methaneselenenyl bromide (217 mg, 1.25 mmol) was added, whereupon a yellow precipitate immediately formed. The reaction was then stirred for 20 min at room temperature, and the precipitate was decanted, rinsed with ethanol several times, and dried in vacuo to give a 1:1 mixture of unstable **6** (162 mg, 62%) together with selenocystine as a light yellow powder: ^{1}H NMR (300 MHz, D$_2$O) δ 3.66 (dd, 1H) 3.40−3.20 (AB part of the ABX spectrum, eight lines, 2H), 2.44 (s, 3H) ppm.
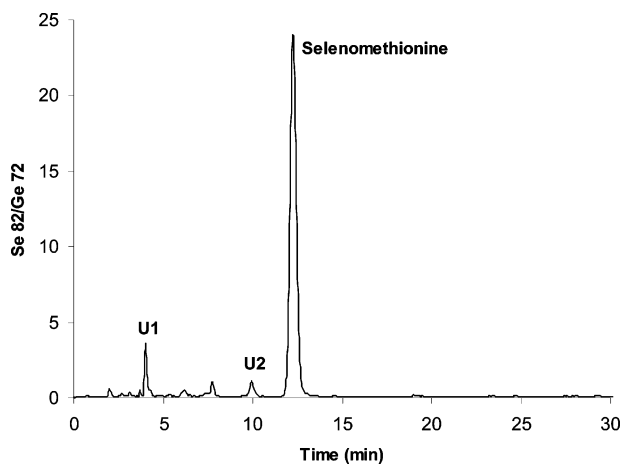
Figure 3. Se-specific HPLC–ICP-MS chromatogram of enzymatic hydrolysis of 1250 ppm Se yeast (reference) using 0.1% HFBA ion-pairing agent, Symmetry Shield column.

3-(Methyldiseleno)-L-*alanine Ethyl Ester N-Ethylcarbamate (**6a**).* The derivatization procedure of Husek was followed (*23*). Diethyl pyrocarbonate (700 μL, 4.76 mmol) was added to a suspension of **6** (130 mg, 0.50 mmol) in ethanol/water/pyridine (32:60:8 v/v/v; 1.5 mL). Foaming occurred due to CO_2 evolution. Derivatization was completed within a few seconds after mixing and brief shaking. The reaction mixture was extracted with $CHCl_3$ (3 × 2 mL), and the organic layer was dried (Na_2SO_4), concentrated in vacuo, and repeatedly chromatographed (silica gel, 3:1 hexane/ethyl acetate) to give **6a** as an unstable light yellow oil (5 mg, 3%) which rapidly turns red-brown on standing: $R_f = 0.46$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.43 (br d, 1H), 4.63 (dd, $J = 4.6$, 5.8 Hz, 1H) 4.20 (q, $J = 7.2$ Hz, 2H) 4.12 (q, $J = 7.1$ Hz, 2H) 3.45−3.31 (AB part of the ABX spectrum, eight lines, $J_{AX} = 5.8$ Hz, $J_{BX} = 4.6$ Hz, $J_{AB} = 13$ Hz, 2H) 2.5 (s, 3H) 1.28 (t, $J = 7.2$ 3H), 1.23 (t, $J = 7.1$) ppm.

RESULTS AND DISCUSSION

There is considerable doubt concerning the nature and quantity of Se compounds in apparently similar Se-enriched yeast products, due to the absence of reference standards, as well as the fact that different production methods are used by different producers. We report an extensive characterization study of Se speciation in a number of yeast samples held in lengthy storage following use in the human intervention trial of Clark et al. (*3*).

Figure 3 depicts a typical Se-specific HPLC−ICP-MS chromatogram of enzymatic (protease XIV) hydrolyzate products from a currently produced 1250 ppm reference yeast (Seleno-Excell). Selenomethionine is the major Se species eluted, comprising 84% of the eluted Se. The other positively identified Se compounds present were selenite (0.1%), γ-glutamyl-*Se*-methylselenocysteine (0.5%), and *Se*-adenosylselenohomo-cysteine (0.5%), as authenticated by retention of standards and HPLC−MS (*10*, *16*). In these chromatograms an "unknown" Se peak that does not correspond to any known standards, but comprises ca. 5% of the eluted Se, elutes at ca. 4 min (U1). Another small unidentified peak (ca. 2% eluted Se) appears at ca. 10 min (U2). Peak U1 proves to be related to oxidation of selenomethionine, as is shown subsequently; peak U2 is a previously unidentified organoselenium species.

Representative tablets prepared for the Clark study, 1986−1993 (designated as "N" series N1−N13 from the duration of that study), were crushed, after titanium dioxide coatings were first manually removed if present, and treated enzymatically. Tablets were chosen from various time periods of the trial. A more comprehensive survey of the overall data from this study
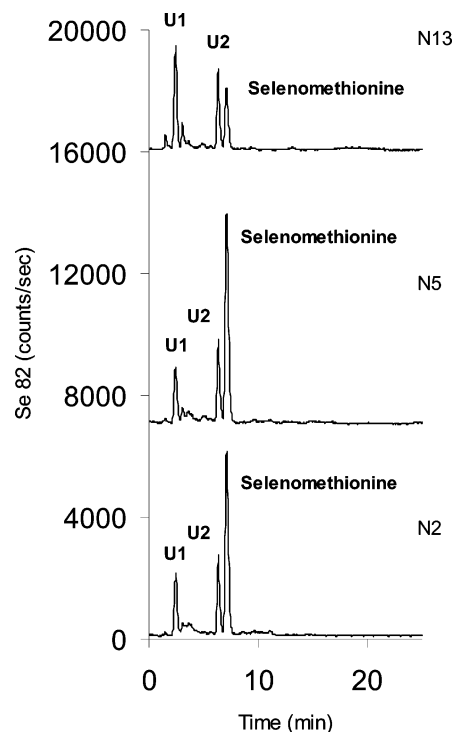

Figure 4. HPLC–ICP-MS chromatograms of the enzymatic hydrolysis of N2, N5, and N13 Clark yeast tablets using 0.1% HFBA as ion-pairing agent, XTerra column.

will be published elsewhere. A representative HPLC−ICP-MS chromatogram is shown in **Figure 4** for samples N2, N5, and N13. A striking difference is evident from the selenized yeasts analyzed earlier. Selenomethionine is still present in a lower proportion, and unknowns U1 and U2 now appear as predominant components. In all archived tablets examined these three peaks occur in various proportions with greater levels of U1 and U2 than are seen in contemporary selenized yeast samples. It may be that these Se species were originally present in the manufactured selenized yeast, but such data are not available. Our current studies on freshly synthesized selenized yeast, prior to any heat treatment, indicate the absence of U2. A likelier conclusion is that changes in speciation have occurred, from lengthy storage perhaps by oxidation, by interaction with tablet excipients, or by linked processes. It was noted that the proportion of U2 remained little changed from sample to sample, while the relative proportions of selenomethionine and U1 varied. A selenized yeast powder obtained from The University of Arizona archives which was used in the preparation of the N series samples gave a profile very similar to that of N5 (*16*).

Since "U1" elutes in the early part of the chromatogram, it is considered to be hydrated selenomethionine selenoxide. This is minimally retained due to the enhanced polarity imparted by the addition of oxygen and the elements of water to the Se atom, producing greater affinity for the acidic aqueous mobile phase. The formation of the hydrate under such acid conditions has been reported elsewhere (*18*, *19*). The mass spectrum of oxidized selenomethionine was confirmed to indicate the presence of hydrated selenomethionine selenoxide with a Se ion cluster (^{80}Se molecular ion at *m/z* 232).

Capillary gas chromatography provides sensitive, high-resolution analysis for analytes of sufficient volatility, or following chemical derivatization to convert nonvolatile species to volatile ones. When GC is coupled with AED, Se in target molecules is directly monitored by spectroscopic detection of atomic emission radiation at a wavelength characteristic of the
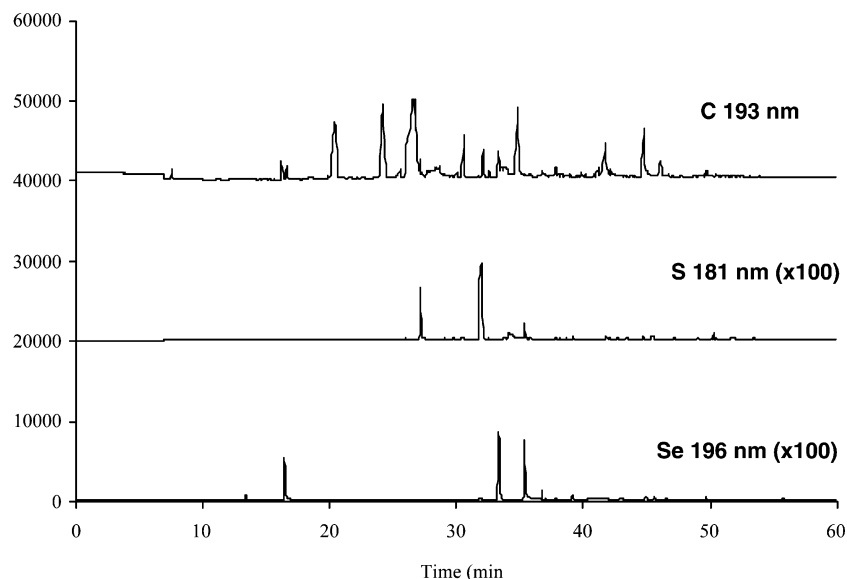
Figure 5. GC—AED chromatogram of derivatized Clark yeast using enzymatic extraction and treatment with ethyl chloroformate, simultaneous detection at 193 nm (C), 181 nm (S), and 196 nm (Se).

element, providing both qualitative elemental speciation and quantitation (*24, 25*). Derivatization with alkyl chloroformates (or alkyl pyrocarbonates) provides an effective route to volatile species for selenoamino acid GC, *Se*-methylselenocysteine and selenomethionine having been determined in normal and Se-enriched plants using GC—AED following ethyl chloroformate derivatization (*26*).

A key advantage of GC—AED is the facility to display simultaneously element-specific chromatograms for different elements, e.g., carbon, sulfur, and selenium. **Figure 5** shows comparative C, S, and Se chromatograms for derivatized Clark yeast. The Se and S profiles show the presence of both S and Se in the peak eluting at ca. 35 min, the region for derivatized selenoamino acids. From independent calibrations to estimate relative responses of S and Se under plasma conditions, it was found that the presumed selenoamino acid parent species contained one Se and one S atom. Sulfur emission lines at 181, 182, and 183 nm were also seen in addition to the Se 196 nm emission line. To determine the relationship between the U2 peak in the HPLC—ICP-MS Se-specific chromatogram and the derivatized GC—AED peak, the U2 peak was carefully trapped from a number of injections and concentrated by freeze-drying. After ethyl chloroformate derivatization, this fraction showed the identical 35 min peak in S and Se channels by GC—AED.

To gain further information on the identity of the postulated selenoamino acid U2, the ethyl chloroformate derivative was examined by GC—MS. The peak in the total ion chromatogram corresponding to the 35 min GC—AED peak showed a molecular ion at m/z 315 (^{80}Se) and had the Se isotope pattern. A Se ion cluster at m/z 242 (^{80}Se) indicated the loss of COOCH$_2$CH$_3$ (m/z 73) from the molecular ion. A prominent ion at m/z 220 (no Se isotope pattern) indicated the loss of SeCH$_3$ (m/z 95 (^{80}Se)). An ion at m/z 188 is interpreted as loss of SSeCH$_3$ (m/z 127 (^{80}Se)), and that at m/z 174 as the loss of CH$_2$SSeCH$_3$. A GC—MS spectrum of derivatized *S*-(methylseleno)cysteine (**3**) (see below) produced the same fragmentation pattern (*16*).

Because by HPLC—ICP-MS, GC—AED, and GC—MS, the second unknown (U2) in the Clark samples had both Se and S in a 1:1 ratio and an M$^+$ suggestive of either **3** or *Se*-(methylthio)selenocysteine (**4**); both of these unknown compounds were synthesized via the procedures shown in **Figure**
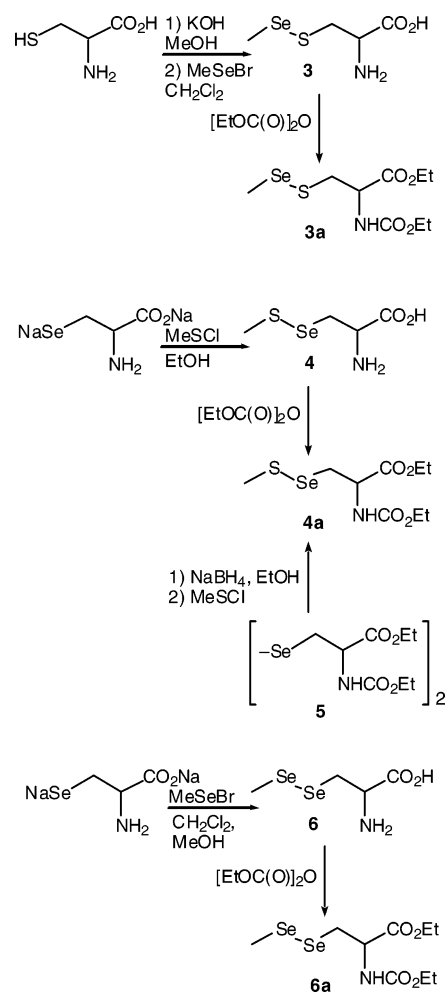

Figure 6. Syntheses of compounds **3**, **3a**, **4**, **4a**, **6**, and **6a**.

6. Synthetic **3** was contaminated with cystine, while **4** was contaminated with selenocystine; both **3** and **4** were unstable to storage in aqueous solution, limiting the possibilities for characterization. The mixtures of **3** and **4** with their respective contaminants were used as standards in conjunction with HPLC—ICP-MS studies. Compounds **3** and **4** were derivatized
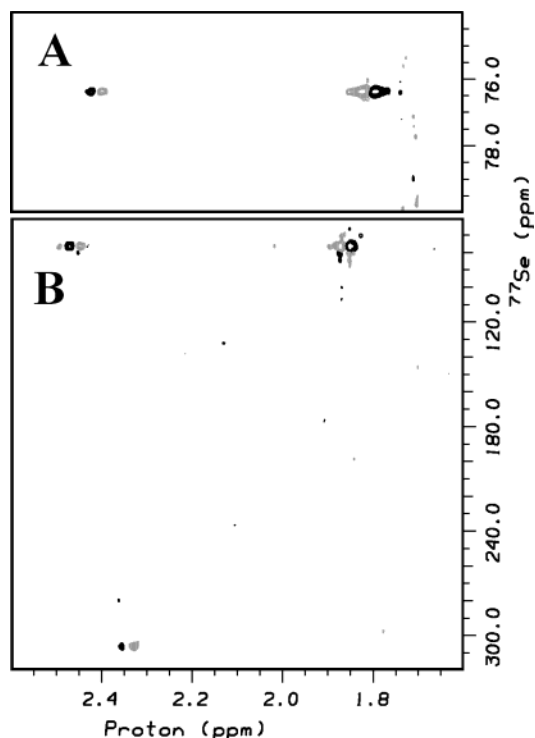
Figure 7. Portions of the 2D ^1H–^{77}Se HMQC spectra of aqueous extracts of selenized yeast after protease XIV digestion, in D_2O. Positive contours are shown in black, negative contours in gray. (**A**) Selenized yeast from the Celecoxib/selenium study. The ^{77}Se carrier was set at 64.9 ppm. The total experiment time was 12.7 h. (**B**) Selenized yeast from the Clark trial. The ^{77}Se carrier was set at 137.8 ppm. The total experiment time was 9.5 h.

with ethyl pyrocarbonate (23), giving *N*-ethylcarbamate *S*-(methylseleno)cysteine ethyl ester (**3a**) and *N*-ethylcarbamate *Se*-(methylthio)selenocysteine ethyl ester (**4a**), respectively, which could be rigorously purified and fully characterized by spectroscopic methods. These two compounds were used as standards for GC−AED and NMR studies. While **3/3a** and **4/4a** are new compounds, derivatives of both *S*-(benzylseleno)-L-cysteine and *Se*-(benzylthio)-L-selenocysteine have been previously synthesized (27, 28). These latter studies noted the instability of compounds with S−Se bonds. For comparison purposes we also prepared previously unknown 3-(methyldiseleno)-L-alanine (**6**, $MeSeSeCH_2CH(NH_2)CO_2H$) and, with ethyl pyrocarbonate, the corresponding *N*-ethylcarbamate 3-(methyldiseleno)-L-alanine ethyl ester (**6a**).

Treatment of a pulverized tablet of selenized yeast used in the current Celecoxib/selenium study in water with protease XIV in a manner similar to that reported before (9) provided an aqueous extract which was analyzed by ^{77}Se NMR spectroscopy. Remarkably the 2D $^{77}Se−^1H$ HMQC spectrum shown in **Figure 7A** (**Figure 7B** is discussed below), which is similar to that observed before (20) for an authentic selenomethionine (**1**) sample, was obtained after a relatively short period of data collection (no other selenium signal was detected even after prolonged data collection). Note that less than 200 nmol of ^{77}Se is detected (200 mg of Se in the tablet, and the natural isotopic abundance of ^{77}Se is 7.58%). In the ^{77}Se dimension of the 2D spectrum a signal at δ 76.4 ppm is observed which correlates with signals at δ 1.9 and 2.5 ppm in the ^1H dimension. The signal at δ 1.9 ppm is due to the methyl hydrogens on selenomethionine which have a two-bond $^{77}Se−^1H$ coupling constant of about 10 Hz. The signal at δ 2.5 ppm is due to the γ-hydrogens, which are scalar coupled to ^{77}Se with a slightly
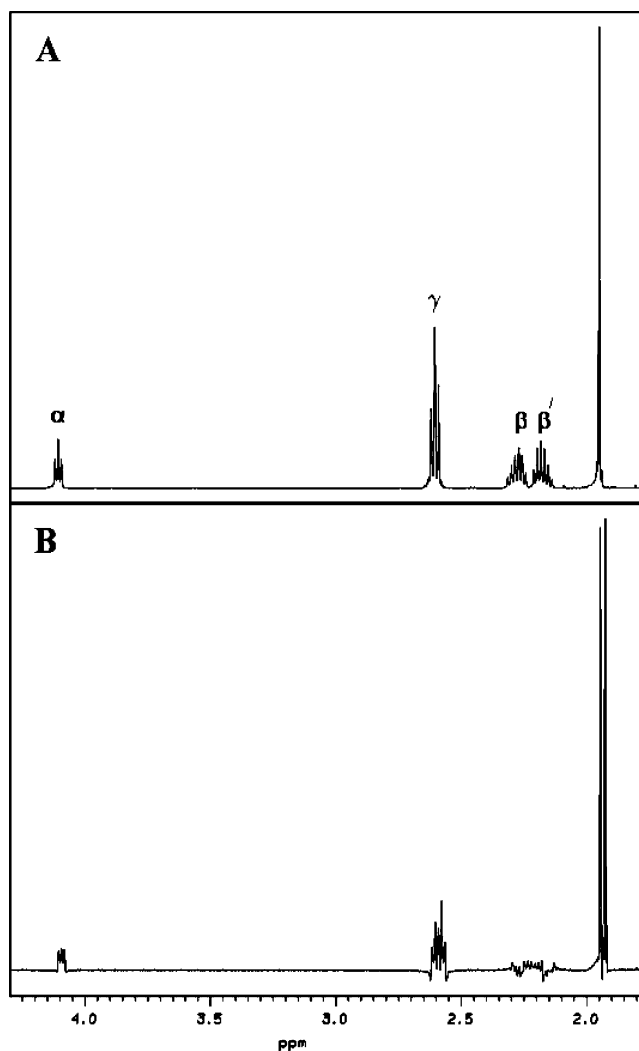

Figure 8. (**A**) ^1H NMR spectrum (4 scans) and (**B**) 1D ^1H–^{77}Se HMQC–TOCSY spectrum (16 scans) of selenomethionine, pH $\approx$ 1 in D_2O. The ^{77}Se carrier was set at 64.9 ppm.

smaller coupling constant than that of the methyl hydrogens. No correlation is seen with the α- or β-hydrogens because their coupling to ^{77}Se is too small.

It would be more useful for structural assignments if the signals due to the hydrogens not coupled to ^{77}Se could be measured in the Se-containing compounds in this complex mixture. Consequently, a standard TOCSY pulse sequence was added following the $^{77}Se−^1H$ HMQC pulse sequence (29, 30). In principle this methodology would allow the observation of all of the spin-coupled protons in the Se compound being measured. That is, in **1** (**Figure 1**) the β-hydrogens would be observed because they are spin-coupled to the γ-hydrogens; the α-hydrogen, which is spin-coupled to the β-hydrogens, would also be measured. This method was validated with an authentic sample of **1** in a 1D $^{77}Se−^1H$ HMQC−TOCSY experiment. **Figure 8** shows the resulting spectrum compared with the ^1H NMR spectrum measured with this sample. The signals due to the methyl hydrogens and α-, β-, and γ-hydrogens are all clearly observed. This methodology was applied to the aqueous extract of the selenized yeast sample (Celecoxib/selenium study) to give the spectrum shown in **Figure 9**, which is compared with the ^1H spectrum of this sample to illustrate the selectivity of the method for selenium-containing compounds. Comparison of the 1D $^1H−^{77}Se$ HMQC−TOCSY spectrum with that of authentic **1** shows that the two spectra are similar but not identical. There
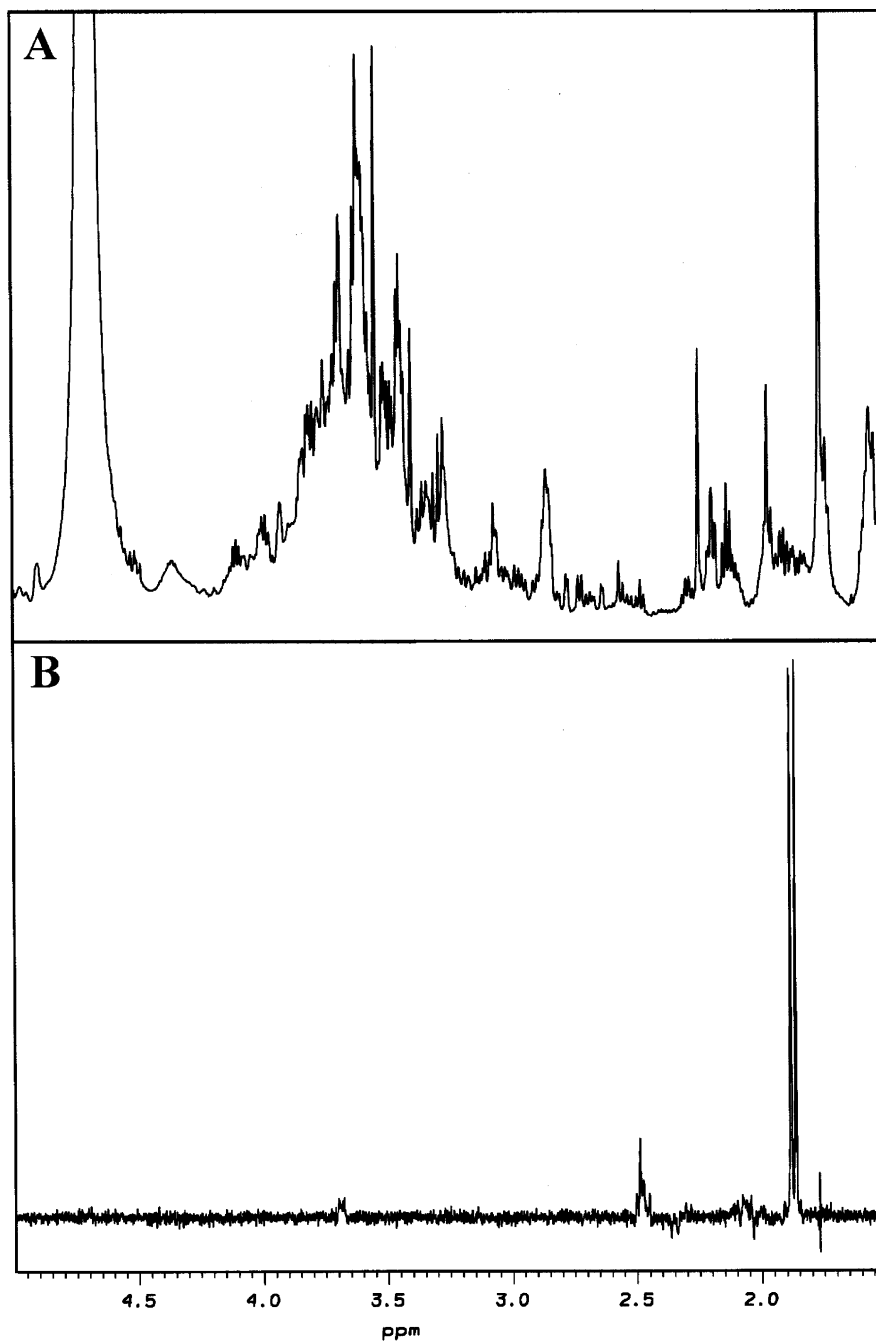
Figure 9. (**A**) ^{1}H NMR spectrum and (**B**) 1D ^{1}H–^{77}Se HMQC–TOCSY spectrum of aqueous extracts of hydrolyzed yeast from the Celecoxib/selenium study. The ^{77}Se carrier was set at 64.9 ppm. The total experiment time for the HMQC–TOCSY was 15.1 h.

are chemical shift differences between them. However, the pH values of the solutions are different (pH ca. 1 for the authentic **1** sample and pH ca. 7 for the yeast sample). Consequently, authentic **1** was added in an approximately equimolar amount to the yeast sample. The resulting spectrum was identical to that of the original spectrum for the yeast sample except that there was a corresponding increase in signal intensity.

The aqueous extracts of the hydrolyzed sample of selenized yeast from the Clark trial were next examined. Using 2D ^{77}Se–^{1}H HMQC spectroscopy, two Se signals were observed which correlated with proton signals (**Figure 7B**). The ^{77}Se chemical shifts for these compounds are 76.4 and 306 ppm. The compound giving rise to the Se signal at 76.4 ppm was identified as **1**. This assignment is based on this chemical shift and that of its correlated hydrogens as well as its 1D ^{77}Se–^{1}H HMQC– TOCSY spectrum. To determine the structure of the other Se

compound, **3a** and **4a** were studied by NMR spectroscopic methods. The directly determined ^{77}Se chemical shifts of **3a** and **4a** in CH_2Cl_2 were 327 and 401 ppm, respectively (the similarly measured ^{77}Se chemical shift for **5** in CH_2Cl_2 was 302 ppm). The reported typical ranges for ^{77}Se chemical shifts for selenenyl sulfides and dialkyl selenides are 500−700 and 178− 584 ppm, respectively (*31*). However, for selenenyl sulfides derived from glutathione, penicillamine, and cysteine with selenocysteine, the ^{77}Se chemical shift range in water is 250− 340 ppm, which overlaps with the ^{77}Se chemical shift range for comparable diselenides in water of 230−360 ppm (*32*). Nevertheless, in cases where the groups attached to the SeS moiety are the same, the Se is deshielded in the selenenyl sulfide relative to the diselenide as expected on the basis of the greater electronegativity of sulfur than selenium. Comparison of the chemical shifts for comparably substituted selenenyl sulfide **4a**
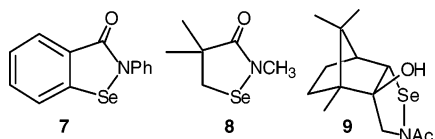
Figure 10. Glutathione peroxidase analogues 7–9.

and diselenide 5 reflects this trend: 401 and 302 ppm, respectively.

Selenenyl sulfides have attracted interest because of their proposed intermediacy in the catalytic cycle of glutathione peroxidase (33, 34). It is proposed that glutathione reacts with the selenenic acid formed by peroxide oxidation of the seleno-cysteine residue of the enzyme to form ESeSG. Ebselen (7; **Figure 10**) has glutathione peroxidase activity and reacts with thiols to give selenenyl sulfides which have been well-characterized (35, 36). Other arylselenenyl sulfides have also

been prepared (37, 38) as well as such species in which the sulfur is part of a cysteinyl residue of a denatured protein (39, 40). Other selenamide analogues of glutathione peroxi-dase, **8** (41) and **9** (42), have been reported and converted to selenenyl sulfides by reactions with thiols. As already mentioned, mixed selenenyl sulfides from glutathione, pen-icillamine, and selenocysteine and related compounds have been made in solution (32). Selenenyl sulfides are easily reduced by selenols or thiols, and some are very susceptible to dis-proportionation (43). The selenenyl sulfides reported in this paper are susceptible to disproportionation in solution. Conse-quently, some of their NMR spectra contain signals due to disproportionation products. Nevertheless, the spectroscopic assignments for the selenenyl sulfide signals are secure because the spectra of the pure corresponding disproportionation products were measured.
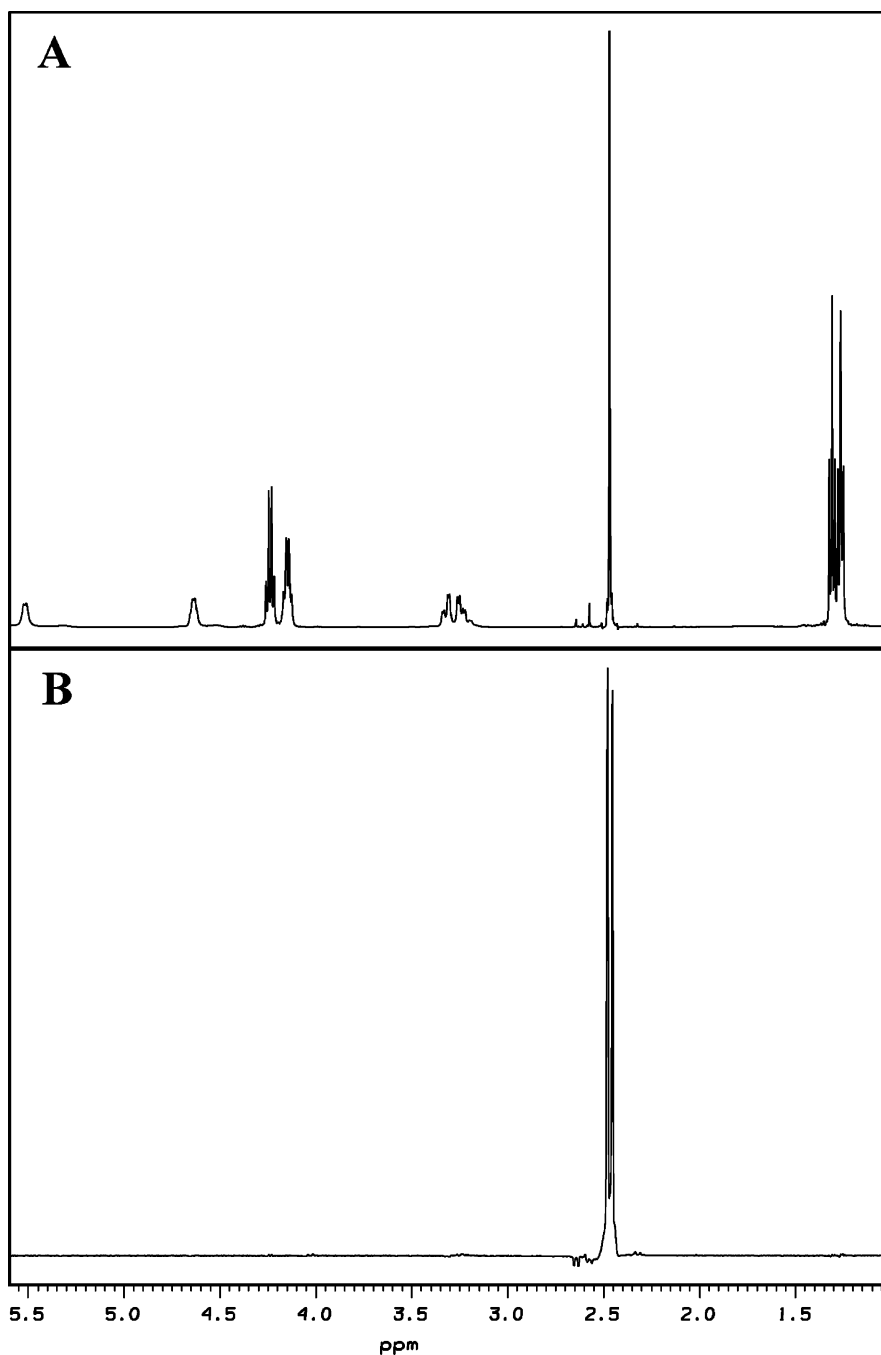

Figure 11. (A) ^{1}H NMR spectrum and (B) 1D ^{1}H–^{77}Se HMQC–TOCSY spectrum of **3a**. The ^{77}Se carrier was set at 326.8 ppm.
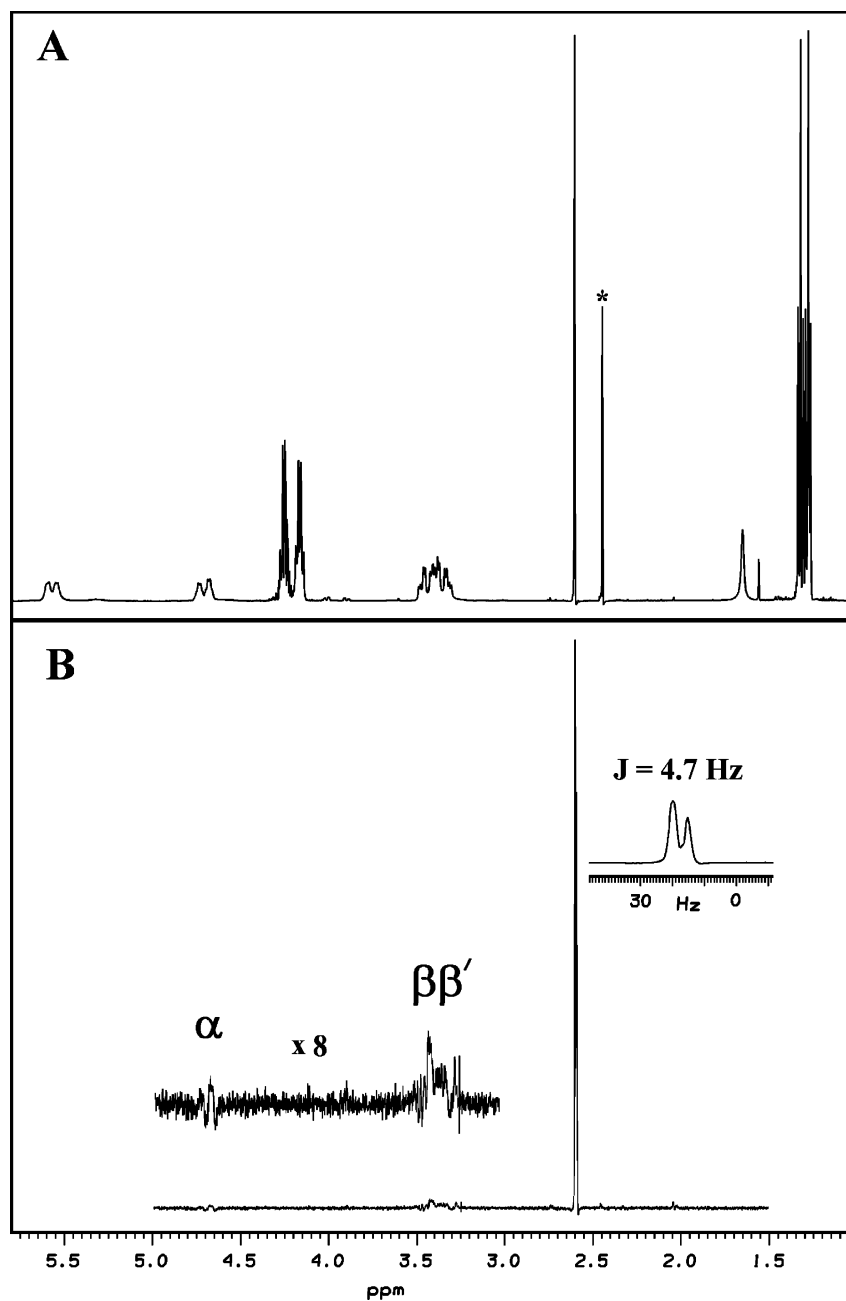
Figure 12. (**A**) ^{1}H NMR spectrum and (**B**) 1D [1]H–[77]Se HMQC–TOCSY spectrum of **4a**. In (**A**) a dimethyl diselenide impurity peak is marked with an asterisk. For (**B**) the [77]Se carrier was set at 203.6 ppm and the total experiment time was 1.5 h. The inset on the left has the vertical scale increased by a factor of 8; the inset on the right is a horizontal expansion of the peak at 2.6 ppm.

The [77]Se−[1]H HMQC−TOCSY spectra for **3a** and **4a** compared with their proton spectra are shown in **Figures 11** and **12**, respectively. As can be seen in **Figure 11**, only the methyl hydrogen signal is observed but not those due to the α- and β-hydrogens. Observation of the β-hydrogen and concomitantly α-hydrogen signals requires three-bond [77]Se−[1]H coupling through the sulfur. Since the spectrum of **4a**, shown in **Figure 12**, reveals the signal due to the methyl group, which also requires three-bond [77]Se−[1]H coupling through sulfur (this coupling constant is about 4.7 Hz), the α- and β-hydrogen signals in **3a** are not detected because their signals are too weak in our experiments. On the basis of these studies with **3a** and **4a**, it is expected that the [77]Se−[1]H HMQC−TOCSY spectrum of **3** will only show methyl absorption whereas, in the corresponding spectrum of **4**, the methyl hydrogen and α- and β-hydrogen signals will all be observed. In the yeast sample

only the methyl hydrogens are observed in the second compound, and its [77]Se chemical shift is in the range expected (on the basis of the chemical shift for **3a**) for **3**. Interestingly, the [77]Se chemical shifts for **5** and selenocystine are also in this region (*31, 32*), but the β-^{1}H resonances for **5** and selenocysteine (*44*) (3.30−3.44 ppm, 3.32 and 3.45 ppm, respectively) are well removed from that observed. Consequently, these studies support the assignment of **3** as the other previously unknown component in the hydrolyzed selenized yeast extracts obtained from samples used in the Clark trials.

The hydrate of selenomethionine *Se*-oxide (**2**) and the new amino acid **3** were shown to be present in archived selenized yeast, in addition to **1**, after proteolytic digestion, aqueous extraction, derivatization, HPLC−ICP-MS, GC−AED, and GC−MS analysis with comparison with authentic samples. Detection of selenomethionine and *S*-(methylseleno)cysteine

could be achieved using ^{77}Se$-^1$H HMQC NMR spectroscopic methods on crude, underivatized aqueous extracts of proteolyzed sample. In addition, ^{77}Se$-^1$H HMQC$-$TOCSY experiments were shown to provide more structural information which confirmed the structure of these two Se compounds. This Se-specific NMR spectroscopic methodology promises to complement and augment the previous methods for determining the structures of trace Se compounds, i.e., Se speciation, in biological materials. The advantages of the new methodology are that no separation or derivatization of the mixture after hydrolysis and aqueous extraction is required, that NMR is more widely available than AED/ICP-MS instrumentation, and that the NMR spectrum provides rich details which may allow structural assignment of the Se compound in the absence of an authentic sample. These advantages may be especially useful in identifying new organoselenium compounds in selenium-enriched foods and selenium metabolites in bodily fluids. While a possible disadvantage of the new methodology is that ^{77}Se is a relatively insensitive NMR nucleus, the indirect detection methods provide an enhancement which, when combined with isotopic enrichment, results in an increase in sensitivity of almost 800 times (20). Indirect detection requires compounds in which hydrogens are spin-coupled to ^{77}Se. Since Se-methylation of selenium compounds is a major metabolic pathway and this method is especially well-suited for detection of SeMe moieties, as illustrated in this paper, the detection and identification of Se-metabolites in this way is a very promising application.

ABBREVIATIONS USED

AED, atomic emission detection; ESI-MS, electrospray ionization mass spectrometry; HMQC, heteronuclear multiple-quantum correlation; ICP-MS, inductively coupled plasma mass spectroscopy; TOCSY, total correlation spectroscopy; TOF-MS, time-of-flight mass spectrometry.

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