Allium Chemistry: Synthesis, Natural Occurrence, Biological Activity, and Chemistry of *Se*-Alk(en)ylselenocysteines and Their γ-Glutamyl Derivatives and Oxidation Products

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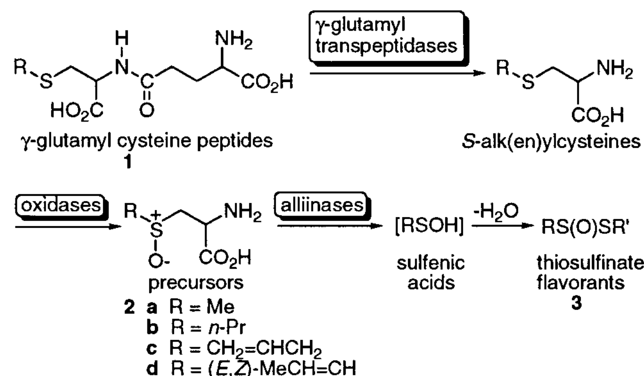
Syntheses are reported for γ-glutamyl *Se*-methylselenocysteine (**8a**), selenolanthionine (**16**), *Se*-1-propenylselenocysteine (**6d**), *Se*-2-methyl-2-propenyl-L-selenocysteine (**6e**), and *Se*-2-propynyl-L-selenocysteine (**6f**). Oxidation of **8a** and *Se*-methylselenocysteine (**6a**) gives methaneseleninic acid (**24**), characterized by X-ray crystallography, and dimethyl diselenide (**25**). Oxidation of *Se*-2-propenyl-L-selenocysteine (**6c**) gives allyl alcohol and 3-seleninoalanine (**22**). Compound **22** is also formed on oxidation of **16** and selenocystine (**4**). Oxidation of **6d** gives 2-[(*E*,*Z*)-1-propenylseleno]-propanal (**36**). These oxidations occur by way of selenoxides, detected by chromatographic and spectroscopic methods. The natural occurrence of many of the *Se*-alk(en)ylselenocysteines and their γ-glutamyl derivatives and oxidation products is discussed. Three homologues of the potent cancer chemoprevention agents **6a** and **6c**, namely **6d**−**f**, were evaluated for effects on cell growth, induction of apoptosis, and DNA-damaging activity using two murine mammary epithelial cell lines. Although each compound displays a unique profile of activity, none of these compounds (**6d**−**f**) is likely to exceed the chemopreventive efficacy of selenocysteine Se-conjugates **6a** and **6c**.

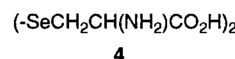
Keywords: *γ-Glutamyl Se-methylselenocysteine; selenoxides; Allium species; selenocysteines*

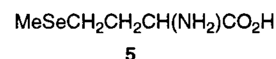
INTRODUCTION

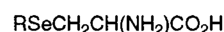
Genus *Allium* plants contain *S*-alk(en)ylcysteine *S*-oxides [**2a**−**d**, $RS(O)CH_2CH(NH_2)COOH$; R = Me, *n*-Pr, $CH_2{=}CHCH_2$, and MeCH=CH], which originate from γ-glutamyl *S*-alk(en)ylcysteine storage compounds (**1**) via *S*-alk(en)ylcysteines (Scheme 1). Crushing these plants releases sulfur-containing flavorants **3**, which are formed through the action of alliinase (β-lyase) enzymes on **2** (*1*, *2*). In 1964 Spåre and Virtanen (*3*) first suggested that analogous types of selenium compounds may also be present in these plants. Thus, from analysis of the extracts of ^{75}Se-treated onions (*Allium cepa*), evidence was obtained for the possible presence of selenocystine (**4**), selenomethionine (**5**), *Se*-β-carboxy-propylselenocysteine (**6g**), *Se*-methylselenocysteine *Se*-oxide (**7a**), *Se*-1-propenylselenocysteine *Se*-oxide (**7d**), and γ-glutamyl *Se*-1-propenylselenocysteine (**8b**). The presence of **4** and **5** was confirmed using authentic compounds (*3*). In an effort to confirm the identity of the other species, Spåre and Virtanen synthesized *Se*-methyl-, *Se*-propyl-, and *Se*-2-propenylselenocysteine (**6a**−**c**, respectively) but were unsuccessful in their attempts to oxidize **6a**−**c** to the corresponding *Se*-oxides **7a**−**c**.

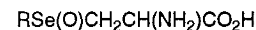
Scheme 1

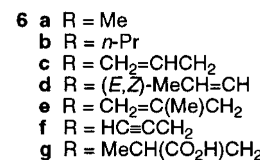


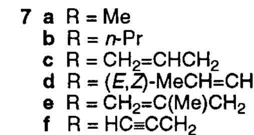


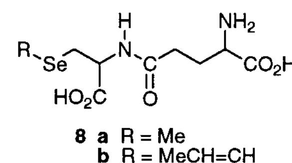


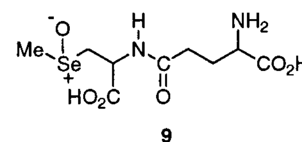












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Since the seminal studies by Spåre and Virtanen (*3*), much progress has been made in *Allium* organoselenium chemistry and, more broadly, in understanding the natural products chemistry of selenium and its importance to human health (*4*). Interest in identifying *Allium* organoselenium compounds has been heightened by the discovery that Se-enriched garlic (*Allium sativum*) is very effective in mammary cancer chemoprevention in a well-characterized experimental model system (*5*−*10*) and that this capacity appears to be generalizable in that Se-enriched broccoli (*Brassica oleracea*) is similarly effective (*11*). Of particular interest is the evidence that Se-enriched *Allium* plants are much more active in cancer prevention than their unenriched *Allium* counterparts and that synthetic *Se*-2-propenylselenocysteine (**6c**) shows stronger chemopreventive effects than *Se*-methylselenocysteine (**6a**). However, in vitro **6c** (but not **6a**) damaged DNA; neither compound damaged DNA in vivo. Against this background several questions emerged in the selenium cancer chemoprevention field: (1) to what extent does the spectrum of *Alllium* organselenium compounds parallel that of sulfur-containing compounds in this genus; (2) does oxidation of *Se*-2-propenylselenocysteine (**6c**) create transitory chemopreventive metabolites responsible for the uniquely stronger chemopreventive effects of **6c** compared to those of *Se*-methylselenocysteine (**6a**); and (3) are there compounds related to **6c** which can be prepared that retain the superior chemopreventive effects without damaging DNA?

The work reported in this paper is a logical extension of our previous investigations in this area wherein the organoselenium compounds in *Allium* extracts (and related plants including broccoli and Se-enriched yeast) were identified using high-performance liquid chromatography with inductively coupled plasma-mass spectrometric (HPLC-ICP-MS) and electrospray mass spectrometric (HPLC-ESI-MS) detection (*12*−*17*). In this previous work the major selenium compound in both Se-enriched and unenriched garlic was identified as γ-glutamyl *Se*-methylselenocysteine (**8a**) along with lesser amounts of *Se*-methylselenocysteine (**6a**), selenocystine (**4**), and selenomethionine (**5**), among other compounds (*16*). In the first part of this paper, details of chemical syntheses of organoselenium compounds necessary for the further development of the field are provided, including **8a**, *Se*-(*E,Z*)-1-propenylselenocysteine (**6d**), derivatives of which are claimed to be present in *Allium* species (*3*), and *Se*-2-methyl-2-propenylselenocysteine (**6e**) and *Se*-2-propynylselenocysteine (**6f**), which were synthesized to compare their biological activity with that of *Se*-2-propenylselenocysteine (**6c**). On the basis of what is known about the occurrence of *Allium* sulfur-containing compounds, the second part of the paper addresses a strategic issue, namely, whether efforts to exploit *Allium* organoselenium chemistry for cancer prevention should be focused on already identified *Se*-alk(en)ylcysteine storage compounds or on their Se-oxides. The formation and novel chemistry of Se-oxides of the *Se*-alk(en)yl storage compounds will be described. Evidence is presented that natural levels of *Se*-alk(en)ylcysteines *Se*-oxides in *Allium* species must be very low. The third part of the paper provides an initial assessment of the biological activities of compounds **6d**−**f**, which are compared with the known activities of **6a**−**c**.

EXPERIMENTAL PROCEDURES

NMR spectra were obtained with Bruker AC200 or AMX 400 NMR spectrometers (200 or 400 MHz) or a Varian Gemini spectrometer (300 MHz), with TMS or sodium 3-(trimethylsilyl)propanesulfonate as internal standard in $CDCl_3$ or aqueous solutions, respectively, and MeSeMe (Strem Chemicals, Newburyport, MA) or MeSeSeMe (Aldrich, Milwaukee, WI) in glass capillary tubes as ^{77}Se standards. Hewlett-Packard HP 5988 or 5898 mass spectrometers were used for GC-MS and LC-MS, a Bruker-Hewlett-Packard Esquire-LC mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) was used for ESI-MS (by M. Kotrebai), and a Finnigan TSQ 7000 (Finnigan MAT, San Jose, CA) equipped with an APCI interface was used for APCI-MS (by Dr. Elizabeth Calvey). Mass spectra are given for the most abundant ^{80}Se isotope only. The following were purchased from commercial sources: Dulbecco's modified Eagle's medium and F-12 medium, Triton X-100, glutaraldehyde, crystal violet (all from Sigma, St. Louis, MO); adult bovine serum (Gemini Bioproducts, Calabasas, CA); insulin and epidermal growth factor (Intergen, Purchase, NY); gentamicin reagent solution and agarose (Gibco BRL, Grand Island, NY); oligreen (Molecular Probes, Eugene, OR); and L-selenocystine, L-selenomethionine, and β-chloro-L-alanine (Aldrich, Milwaukee, WI). Compounds **6a**−**c** were prepared as described elsewhere (*10, 16 18*). Most reactions were carried out under dry N_2 or argon. Diethyl ether ("ether") and THF were distilled under N_2 from sodium-benzophenone ketyl, CH_2Cl_2 was distilled from calcium hydride, MeOH was distilled from Mg, and ethyl acetate was distilled before use. Analytical TLC was performed on precoated silica gel plates (Art. No. 5715, Merck) with a 254 nm fluorescent indicator. HPLC was performed on Rainin HPX and HPXL systems on 5 μm C-18 or normal phase silica columns with UV detection at 254 nm. With the C-18 column, a 7:3 H_2O/CH_3CN mixture was typically employed. A model 7942T Chromatotron (Harrison Research, Palo Alto, CA) was also used with silica gel from Merck (Art. No. 7749).

Benzyl (*Z*)-1-Propenyl Selenide [(*Z*)-10]. Magnesium turnings (0.67 g, 28 mmol) were placed in a 100 mL three-neck round-bottom flask equipped with reflux condenser, addition funnel, and spin bar. A small amount of (*Z*)-1-bromopropene (3.4 g, 28 mmol) in dry THF (25 mL) was added with stirring to the magnesium to initiate the vigorous reaction. Subsequent addition was at a rate sufficient to maintain gentle reflux. The yellow solution was stirred for 0.5 h and cooled to 0 °C, and dibenzyl diselenide (4.9 g, 14 mmol) in THF (20 mL) was added dropwise. The mixture was warmed to 20 °C and poured into 10% HCl solution, and the organic layer was washed with 5% NaOH, saturated $NaHCO_3$, and brine, dried (Na_2SO_4), and purified using a Chromatotron to give (*Z*)-**10** (2.55 g, 43%) as a yellow liquid: ^{1}H NMR ($CDCl_3$) δ 7.22 (m, 5 H), 6.14 (dq, $J = 9.0$, 1.4 Hz, 1 H), 5.86 (m, 1 H), 3.82 (s, 2 H), 1.59 (dd, $J = 6.6$, 1.4 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 139.1, 128.8, 128.5, 128.3, 126.8, 29.4, 16.7; ^{77}Se NMR ($CDCl_3$, MeSeSeMe) δ 282; EI-GC-MS, *m*/*z* 212 (M^+, 11%), 91 (100%).

Benzyl (*E*)-1-Propenyl Selenide [(*E*)-10]. In a similar manner using (*E*)-1-bromopropene, a 3:1 mixture of (*E*)-/(*Z*)-**10** was prepared in 43% yield. Compound (*E,Z*)-**10** showed the following: ^{1}H NMR ($CDCl_3$) δ 7.27 (m, 5 H), 6.20 (dq, $J = 15.4$, 1.4 Hz, 1 H), 5.91 (m, 1 H), 3.87 (s, 2 H), 1.74 (dd, $J = 6.5$, 1.5 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 132.9, 128.8, 128.4, 126.7, 116.6, 29.8, 16.8; ^{77}Se NMR ($CDCl_3$, MeSeSeMe) δ 326; EI-GC-MS, *m*/*z* 212 (M^+, 11%), 91 (100%). Distillation of another batch containing 1:2.3 (*E*)-/(*Z*)-**10** gave a yellow oil, bp 139 °C (16 mbar). Anal. Calcd for $C_{10}H_{12}Se$: C, 56.87; H, 5.72. Found: C, 56.99; H, 5.71.

Bis[(*Z*)-1-propenyl] Diselenide [(*Z,Z*)-13]. Lithium (0.14 g, 20 mmol) was added to liquid ammonia (200 mL) at −78 °C under argon. After the lithium had dissolved, (*Z*)-**10** (1.67 g, 7.9 mmol) in THF (40 mL) was added dropwise with stirring. The reaction mixture was stirred at −65 °C for 1.5 h, warmed to room temperature, and concentrated in vacuo. At 0 °C, water (100 mL) and ether (150 mL) were added followed by an

aqueous solution (40 mL) of KI_3 [from I_2 (1.2 g, 7.5 mmol) and KI (8 g, 48 mmol)]. The ether layer was separated, the aqueous layer was extracted with ether (2 × 200 mL), and the combined organic layers were washed with sodium thiosulfate solution (3 × 100 mL) and NH_4Cl solution, dried ($MgSO_4$), filtered, and concentrated in vacuo. Chromatography (Chromatotron) gave (*Z,Z*)-**13** (0.69 g, 72%) as a yellow liquid: ^1H NMR ($CDCl_3$) δ 6.66 (dq, *J* = 8.9, 1.4 Hz, 2 H), 5.92 (m, 2 H), 1.79 (dd, *J* = 6.9, 1.4 Hz, 6 H); ^{13}C NMR ($CDCl_3$) δ 130.8, 123.3, 16.3; ^{77}Se NMR ($CDCl_3$, MeSeSeMe) δ 356; EI-GC-MS, *m/z* 242 (M^+, 81%), 41 (100%).

Bis(2-methyl-2-propyl) Diselenide (14). 2-Bromo-2-methylpropane (27.4 g, 0.2 mol) and Et_2O (100 mL) were added to magnesium turnings (4.86 g, 0.2 mol). After completion of the reaction, selenium (14.2 g, 0.18 mol) was added gradually over a period of 30 min at such a rate as to maintain gentle refluxing without heating. Stirring was continued for an additional 30 min. At 0 °C, NH_4Cl solution (100 mL) was added. An aqueous solution (40 mL) of KI_3 (prepared as described for **13**) was added slowly. The organic layer was separated, and the aqueous solution was extracted with ether (2 × 200 mL). The combined organic layers were washed [10% sodium thiosulfate (3 × 10 mL) and NH_4Cl solution], dried ($MgSO_4$), filtered, and concentrated. Distillation gave **14**, a yellow liquid (11.0 g, 45% yield), bp 100 °C (11 Torr) (Lit. 53−56 °C, 0.15 Torr; *19*).

2-Methyl-2-propyl (*E,Z*)-1-Propenyl Selenide (15). In a similar manner the Grignard reagent prepared from (*Z*)-1-bromopropene (5.6 g, 46 mmol) was reacted with **14** (6.0 g, 22 mmol). After workup and concentration, the residue was distilled under vacuum using a Vigreux column to give (*E,Z*)-**15** (3.1 g, 40%), bp 53−55 °C (11 Torr): ^1H NMR ($CDCl_3$) δ 5.8−6.5 (m, 2 H), 1.68 (d, *J* = 5.4 Hz), 1.66 (d, *J* = 3.3 Hz; 3 H total), 1.44, 1.43 (s, 9 H total); ^{13}C NMR ($CDCl_3$) δ 129.4, 127.6, 120.7, 118.8 (**C**H=), 41.4, 41.3 (**C**Me_3), 32.3, 32.2 (**C**H_3-CH=), 16.9, 16.6 [(**C**$H_3)_3$C]; EI-GC-MS, *m/z* 178 (M^+, 48%), 121 (62%), 93 (38%), 57 (100%).

***Se*-(*E,Z*)-1-Propenyl-L-selenocysteine (6d).** Lithium (7.5 mg, 1.07 mmol) was added to liquid ammonia (20 mL) at −78 °C in a 50 mL three-neck round-bottom flask. After stirring for 10 min, (*E,Z*)-**10** (200 mg, 0.94 mmol) in THF (2 mL) was added dropwise. The reaction mixture was stirred at −70 °C for 1 h, and then β-chloro-L-alanine (**12**, 0.137 g, 1 mmol) was added as a solid all at once. After 20 min, the ammonia was evaporated and water was added. Compound **6d** (138 mg, 65%, 1:1.8 *E*:*Z*) was obtained as a mixture of isomers after RP-HPLC. For (*Z*)-**6d**: ^1H NMR (D_2O; ref. H_2O δ 4.63) δ 6.12 (dq, *J* = 15.3, 1.2 Hz, 1 H), 5.88−5.97 (m, 1 H), 3.82 (dd, *J* = 7.5, 4.2 Hz, 1 H), 2.86−3.13 (m, 2 H), 1.60 (dd, *J* = 6.3, 1.5 Hz, 3 H); ^{13}C NMR (D_2O, acetone-d_6 internal ref) δ 172.7, 136.4, 114.4, 54.5, 26.0, 19.2; ^{77}Se NMR (D_2O, MeSeSeMe) δ 154; EI-MS, *m/z* 209 (M^+, 12%), 43 (100%). For (*E*)-**6d**: ^1H NMR (D_2O) δ 6.14 (dq, *J* = 8.8, 1.4 Hz, 1 H), 5.91−6.04 (m, 1 H), 3.84 (dd, *J* = 6.6, 4.4 Hz, 1 H), 2.86−3.13 (m, 2 H), 1.54 (dd, *J* = 6.9, 1.5 Hz, 3 H); ^{13}C NMR (D_2O, acetone-d_6) δ 172.7, 131.0, 118.8, 54.7, 26.0, 16.2; ^{77}Se NMR (D_2O, MeSeSeMe) δ 199; EI-MS same as (*Z*)-**6d**. A 1:2.3 *E*:*Z* mixture of crystalline light yellow **6d** had mp 164 °C (dec). Anal. Calcd for $C_6H_{11}NO_2Se$: C, 34.63; H, 5.33. Found: C, 34.58; H, 5.39.

***Se*-2-Methyl-2-propenyl-L-selenocysteine (6e).** Selenocystine (125 mg, 0.375 mmol) was dissolved in 1 M HCl (2 mL). The yellow solution was flushed with argon, and $NaBH_4$ was added until the solution turned colorless. A solution of NaOH was added to give pH 7 as monitored by a glass electrode. 3-Chloro-2-methylpropene (0.15 mL, 1.52 mmol) was added, and the mixture was stirred at room temperature overnight. The mixture was then concentrated in vacuo to ∼1 mL, and the residue was subjected to reverse phase (RP) HPLC (7:3 H_2O/CH_3CN) to give **6e** (64 mg, 48%) as a colorless solid: ^1H NMR (D_2O) δ 4.77 (m, 2 H), 3.79 (dd, *J* = 6.7, 4.7 Hz, 1 H), 3.15 (d, *J* = 3 Hz, 1 H), 2.88 (dd, *J* = 13.8, 5.1 Hz, 1 H), 2.82 (dd, *J* = 13.8, 6.9 Hz, 1 H), 1.69 (s, 3 H); ^{13}C NMR (D_2O, acetone-d_6) δ 173.2, 142.5, 114.0, 54.2, 31.6, 23.3, 21.3; ^{77}Se NMR (D_2O, MeSeSeMe) δ 133; EI-MS, *m/z* 223 (M^+, 0.4%), 135 (15%), 109 (13%), 67 (14%), 55 (89%), 44 (100%).

***Se*-2-Propynyl-L-selenocysteine (6f).** As in the synthesis of **6e**, using 3-chloropropyne (0.15 mL, 2.07 mmol), **6f** (19 mg, 30%) was prepared as a colorless solid: ^1H NMR (300 MHz, D_2O) δ 4.14 (dd, *J* = 7.0, 4.6 Hz, 1 H, $C_\alpha H$), 3.42 (d, *J* = 2.8 Hz, 2 H, ≡CCH_2), 3.40 (dd, *J* = 12.7, 4.6 Hz, 1 H, $C_\beta H_2$), 3.31 (dd, *J* = 14.1, 7.1 Hz, 2 H, $C_\beta H_2$), 2.80 (t, *J* = 2.6 Hz, 1 H, HC≡); ^{13}C NMR (D_2O, acetone-d_6) δ 172.8, 81.5, 72.9, 54.2, 24.3, 7.8; ^{77}Se NMR (D_2O, MeSeSeMe) δ 210.

***Se*-Methyl-L-methylselenocysteinate Hydrochloride (17).** Compound **6a** (1.41 g, 7.74 mmol) was suspended in dry methanol (100 mL) in a 250 mL three-neck flask, fitted with gas inlet and reflux condenser. Dry HCl gas was bubbled into the suspension until the exothermic reaction subsided. The solution was evaporated in vacuo. The oily residue was extracted with CH_2Cl_2, and the extracts were dried (Na_2SO_4). After evaporation, **17** crystallized (1.80 g, 100%): ^1H NMR ($CDCl_3$) δ 8.8 (br s, 3 H), 4.53 (br s, 1 H), 3.86 (s, 3 H), 3.27 (br s, 2 H), 2.12 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 168.7, 53.5, 53.2, 24.1, 5.9.

L-γ-Glutamyl-*Se*-methyl-L-selenocysteine (8a). Compounds **18** (6.33 g, 12.9 mmol) (*20*) and **17** (3.00 g, 12.9 mmol) were dissolved in CH_2Cl_2 (180 mL) and cooled to −10 °C. A solution of dicyclohexylcarbodiimide (3.20 g, 15.5 mmol) in CH_2Cl_2 (20 mL) was added, and the mixture was stirred for 20 h, filtered, and then concentrated in vacuo. The residue was dissolved in EtOAc (200 mL) and filtered again. The filtrate was washed with 1 N HCl (3 × 10 mL) and brine (2 × 20 mL) and dried (Na_2SO_4). The mixture was concentrated to give an oil, which was chromatographed (silica gel; $CHCl_3$:MeOH 20:1). The eluate was evaporated to an oil (**19**; Scheme 6), which was directly used for the next step. The oil was suspended in H_2O (20 mL), heated to reflux and then treated with acetic acid (20 mL). Refluxing was continued for 5 min, the solution was cooled to room temperature, and H_2O (40 mL) was added, whereupon triphenylcarbinol precipitated and was removed by filtration. Concentration of the residue gave a solid, which was dissolved in a mixture of water and MeOH (1:1) and cooled to 15 °C. The pH was continuously adjusted to 12 with aqueous 1 N LiOH over a period of 3 h. The solution was then acidified with 2 N HCl to pH 6 and evaporated below 40 °C to a volume of 15 mL. The solution was applied to an Amberlite IR45 column (OH^- form, 1.5 × 30 cm). After the column had been sequentially washed with water, 0.25 N NaOH, and water, the product was eluted with water. The eluate was evaporated to 2 mL, and dry ethanol was added to precipitate the peptide. The white solid **8a** was collected and dried under reduced pressure (476 mg, 12%; two-step overall yield), mp 159−161 °C: ^1H NMR (300 MHz, D_2O) δ 4.22 (dd, *J* = 4.5, 8.4 Hz, 1 H), 3.59 (t, *J* = 6.2, 1 H), 2.85 (dd, *J* = 4.5, 13.0 Hz, 1 H), 2.69 (dd, *J* = 8.5, 17 Hz, 1 H), 2.33 (t, *J* = 7.6, 2 H), 1.99 (m, 2 H), 1.85 (s, 3 H); ^{13}C NMR (75.5 MHz, D_2O) δ 182.3, 179.9, 177.7 (C=O), 60.l, 57.4, 34.9, 32.1, 29.7, 6.89 (Me); ^{77}Se NMR (D_2O, MeSeSeMe) δ 53; HPLC-ESI-MS, *m/z* 313 (MH^+); APCI-MS, *m/z* 313 (MH^+, 100%), 130, (23%); MS-MS of *m/z* = 313 gave *m/z* 295 (26%), 224 (4%), 184 (58%), 167 (100%).

Bis(α-aminopropionic acid)selenide (16, Selenolanthionine). Selenocystine (**4**), 200 mg, 0.6 mmol) was suspended in EtOH (5 mL) under argon atmosphere. Solid $NaBH_4$ (100 mg, 3 mmol) was added, and the solution was refluxed for 5 min until the solution was clear and colorless. β-Chloro-L-alanine (150 mg, 1.2 mmol) was added, and after 3 min, a precipitate was formed. The solution was refluxed for an additional 10 min and cooled to room temperature. The precipitate was filtered and dried. A solution of the precipitate in water (3 mL) was applied to an Amberlite column (OH^- form) and eluted as described for **8a**. After concentration in vacuo, **16** was precipitated with ethanol as a colorless solid (190 mg, 68%), mp 225−226 °C (dec): ^1H NMR (300 MHz, D_2O) δ 2.90 (d, ^3J = 5.5 Hz, 2 H, CH_2), 3.50 (t, ^3J = 5.5 Hz, 1 H, CH); ^{13}C NMR (75 MHz, D_2O) δ 22.6 (C_β), 48.6 (C_α), 173.3 (COO); ^{77}Se NMR (D_2O, MeSeMe) δ 82; HPLC-ESI-MS, *m/z* 257 (MH^+), 279 ($M + Na^+$), 168 [$HO_2C(NH_2)CHCH_2Se^+$]. Anal. Calcd for $C_6H_{12}N_2O_4Se$: C, 28.25; H, 4.74. Found: C, 27.91; H, 4.40.

2-[(*E,Z*)-1-Propenylseleno]propanal (36) from Oxidation of 6d. A stirred solution of **6d** (80 mg, 0.39 mmol) in

sodium phosphate buffer (pH 8, 1 mL) was cooled to 10 °C, and H_2O_2 (30%, 0.04 mL, 44 mg, 0.39 mmol) was added dropwise via syringe, leading to a vigorous reaction. The mixture was stirred for 10 min, whereupon a yellow oil separated. The organic layer was extracted with ether (3 × 2 mL). The combined extracts were washed with brine, dried (Na_2SO_4), and purified by chromatography to give **36** (48 mg, 69%) as a yellow liquid, which was an inseparable *E*/*Z* mixture: ^1H NMR ($CDCl_3$) δ 9.35, 9.36 (two d, 1 H, C**H**=O), 6.2−6.0 (m, 2H, C**H**=C), 3.57, 3.50 [two q, *J* = 7 Hz, 1 H, MeC**H**(SeR)CHO], 1.79, 1.75 (two d, *J* = 6.4, 3H), 1.53 (t, *J* = 7 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 192.8, 192.7 (H**C**=O), 139.0, 132.3, 115.8, 112.2 (**C**H=**C**′H), 44.4, 44.0 [CH_3**C**H(SeR)CHO], 19.9, 16.9 (**C**H_3CH=), 13.5, 13.3 [**C**H_3CH(SeR)CHO]; ^{77}Se NMR ($CDCl_3$, MeSeMe) δ 240; EI-GC-MS, *m*/*z* 178 (M^+, 14%), 149 (M^+ − CHO, 16%), 121 [M^+ − MeCH(CHO), 14%], 107 (CH_2-CHSe, 27%), 93 (HCSe, 64), 67 (100%); IR (ν_{max}) 2859, 1702 cm^{-1}. Oxidation of **13** and **15** was done in a similar manner. The compound (1 mmol) was dissolved in THF (2 mL) and cooled, and an equimolar amount of H_2O_2 (30%) was added via syringe. After 10 min, the solvent was evaporated and the residue was dissolved in ether, washed, dried, and purified by chromatography as described above. In both cases **36** was the major product, isolated in >50% yield.

General Oxidation Conditions (Oxidation in an NMR Tube). The selenoamino acid or γ-glutamylpeptide (50−80 mg) was dissolved in deuterated sodium phosphate buffer (pD 8, 1 mL) and cooled to 10 °C, and a crystal of internal standard [sodium 3-(trimethylsilyl)propanesulfonate] was added. Equimolar H_2O_2 (30%) was added dropwise via syringe. NMR spectra were taken and, in case of gas evolution, the mixture was shaken. When an organic layer separated, it was extracted by $CDCl_3$ and NMR spectra were taken. Some reactions were monitored by HPLC (15 cm C8 or C18 RP column, 0.1% TFA + 2% MeOH).

Oxidation of *Se*-Methyl-L-selenocysteine (6a). With H_2O_2 at pD 8 the ^1H NMR spectrum of selenoxide **7a** shows a peak at δ 2.80, which at high resolution is resolved into two singlets at δ 2.797 and 2.803, shifted from the δ 2.05 peak for **6a**. Two signals also appear in the ^{77}Se NMR spectrum at δ 853 and 854 ppm, shifted from the signal for **6a** at δ 38. In the ^1H NMR spectrum a singlet at δ 2.45 appeared after 17 min, which could be a hydrate. The oxidation was complete in ~24 h. After 5 days, the final products were methaneseleninic acid (**24**; δ 2.79, s) and dimethyl diselenide (**25**, δ 2.61, s) in a 2:1 ratio and ammonium pyruvate (δ 2.19, s, 3H; 7.15, t, 4 H). Identities of products were confirmed by spiking with authentic materials and, in some cases by ESI-MS [e.g., *m*/*z* 129 for $MeSe(OH)_2^+$]. With H_2O_2 at pD 1 a fast exothermic reaction occurs (in the first 2 min the δ 2.13 ^1H NMR singlet was replaced by one at δ 3.28), which was finished after 1 h. By HPLC, the retention time of the major peak changes from 3.4 min for **6a** to 2.0 min for **7a**; after 1 min, no signal of the starting material was found. The final products are **24**, **25** (2:1 ratio), and ammonium pyruvate.

Oxidation of Selenomethionine (5). Oxidation was carried out in D_2O with an equimolar amount of H_2O_2. By HPLC (0.1% HFBA), the retention time of the major peak changes from 12.95 min for **5** to 2.61 min for selenomethionine *Se*-oxide (**20**). The oxidized product gives two ^1H NMR signals (δ 3.01 and 3.05, shifted from δ 2.02 in **5**), which correspond to the methyl protons of the two diastereomers **20**. The ^{77}Se-NMR spectrum of **20** showed signals at δ 838 and 845 ppm, whereas HPLC-ESI-MS showed the presence of the selenoxide hydrate at *m*/*z* 232 [$MeSe(OH)_2CH_2CH_2CH(NH_3)CO_2H^+$].

Oxidation of L-γ-Glutamyl-*Se*-methyl-L-selenocysteine (8a). Oxidation with H_2O_2 at pH 7 occurs rapidly, and two ^1H NMR signals for the methyl protons were found at δ 2.74 and 2.76. After 2 h, a new signal, presumed to be a hydrate, could be seen rising under the γ-protons at δ 2.4 and was the major signal after 3 days but was gone after 6 days. After 3 days, elimination was complete [the ^1H NMR spectrum showed olefinic protons at δ 5.69 and 5.89 and two singlets for methaneseleninic acid (**24**) and dimethyl diselenide (**25**), as described for **6a**]. Compounds **24** and **25** were the final products after 6 days.

Oxidation of *Se*-2-Propenyl-L-selenocysteine (6c). Oxidation with H_2O_2 at pH 8 led to a reaction that was 95% complete after 1.5 h. The final products were allyl alcohol [δ 6.0 (m, 1 H), 5.29 (dd, 1H), 5.19 (dd, 1H), 4.12 (ddt, 2 H); ^1H NMR spectrum and GC-MS identical to those of authentic material], selenate (by ICP-MS), and a third compound, identified as 3-seleninoalanine (**22**), showing ^1H NMR δ 4.32 (dd, *J* = 10.7, 6.2 Hz; 1 H; CH), δ 3.03 (dd, *J* = 14.3, 6.2 Hz), and 2.88 (dd, *J* = 14.3, 10.7 Hz; CH_2, 2 H); ^{13}C NMR δ 53.1 (CH_2; ^{77}Se-satellites with *J* = 75 Hz), 54.9 (CH), 180.1 ppm (COOH). On oxidation at pD 5 the same rapid exothermic reaction could be observed. Besides allyl alcohol, a second product had the same NMR patterns as seen at pH 8, but slightly shifted chemical shifts [^1H NMR δ 4.32 for CH (dd, *J* = 10.7, 6.2 Hz), 3.48 (dd, *J* = 14.3, 10.7 Hz), and 3.28 (dd, *J* = 14.3, 6.2 Hz) for CH_2; ^{13}C NMR δ 52.5 (Cβ), 55.9(Cα) and 175.2 (COOH)]. No elimination products (ammonium pyruvate) were observed. (The above NMR data were obtained in Siegen at 200 MHz for ^1H and 50 MHz for ^{13}C.) After 2 h, HPLC-ESI-MS of selenium products showed one Se-containing peak with *m*/*z* 155 (100%), 137, and 109, possibly $AllSe(OH)_2^+$ from **7c**, and a second Se-containing peak with *m*/*z* 226, 208, and 168. On oxidation the HPLC retention time changes from 12 min (**6c**) to 3.85 min (**7c**).

Oxidation of Selenolanthionine (16). Oxidation was achieved using excess H_2O_2 (30%) under neutral conditions. The ^{77}Se NMR spectrum (D_2O, MeSeMe) changes from a single peak at 82 ppm to double peaks at 858 and 865 ppm to a product with a single peak at 1195 ppm. The ^1H NMR spectrum (300 MHz) showed δ 4.33 for CH (dd, *J* = 10.0, 6.1 Hz), 3.38 (dd, *J* = 12.5, 9.1 Hz), and 3.18 (dd, *J* = 12.5, 7.1 Hz) for CH_2; ^{13}C NMR spectrum (75 MHz) δ 50 (Cβ), 53 (Cα), and 172 (COOH). After 1.5 h, ammonium pyruvate could be detected. The final product is identical to 3-seleninoalanine (**22**) from **6c**. By ICP-MS SeO_4^{2-} was also found.

Oxidation of Selenocystine (4). Oxidation was achieved using excess H_2O_2 (30%) in D_2O/DCl (1 M). The ^1H NMR spectrum (300 MHz) showed δ 4.32 for CH (dd, *J* = 10.6, 6.1 Hz), 3.48 (dd, *J* = 12.2, 10.7 Hz), and 3.26 (dd, *J* = 12.2, 6.2 Hz) for CH_2; ^{13}C NMR spectrum (75 MHz) δ 50 (Cβ), 54 (Cα), and 172 (COOH). Oxidation at pH 7 gave a product with ^{77}Se 1208 ppm (in D_2O), shifted from the position of **4** at 290 ppm (DCl/D_2O). This final product is identical to 3-seleninoalanine (**22**) from **6c** [note that selenoamino acid ^{77}Se NMR shifts are known to vary with pH (*21*)].

Oxidation of 6b, (*E*)- and (*Z*)-6d, 6e, and 6f. On oxidation, HPLC retention times changed from 21 min for **6b** to 4.8 min for **7b**, from 15 and 17 min for (*E*)- and (*Z*)-**6d** to 3.8−4 min for (*E*,*Z*)-**7d**, from 38 min for **6e** to 2 min for **7e**, and from 5 min for **6f** to 1.9 min for **7f**. Treatment of **7b**,**d**−**f** with sodium thiosulfate shortly after oxidation regenerated **6b**,**d**−**f**. By LC-ESI-MS, oxidation of **6b** gave a product showing *m*/*z* 157 (*n*-$PrSeO_2H_2^+$).

Cell Culture. The mouse mammary hyperplastic epithelial cell lines TM2H and TM12 were obtained from the laboratory of Daniel Medina (*22*). Cells were grown at 37 °C in a humidified incubator containing 5% CO_2 in Dulbecco's modified Eagle's medium and F-12 medium (1:1 DMEM/F-12) containing 2% adult bovine serum, 10 μg/mL insulin, 5 ng/mL epidermal growth factor, and 5 μg/mL gentamicin. TM12 cells have an intact functional (wild-type) p53 gene. This gene is part of the genetic circuitry that participates in cellular responses to genotoxicity. Thus, it was anticipated that this cell line would be very responsive to genotoxic effects of the compounds investigated. The TM2H cell line is biologically similar to the TM12 cell line in terms of its origin: both are derived from a mouse mammary epithelial hyperplasias, but TM2H cells lack p53 activity; functionally, this eliminates a component of the cellular response to genotoxic insult. By using both cell lines, the opportunity to detect a wider spectrum of biological responses to a particular compound is enhanced.

Analysis of Cell Growth Inhibition. The overall effect of **6d**−**f** on cell number homeostasis was determined by evaluating the number of adherent cells accumulating over different periods of time. This approach provides an estimate of the net effect of a compound on cell proliferation and cell death. The assay used crystal violet staining of adherent cells as an endpoint (*23*). Briefly, cells (1000 cells/well) were seeded in flat-bottom 96-well plates in 100 μL of culture medium. Twenty-four hours after initial seeding, cells were allowed to continue growing in either the same medium (no Se supplement) or that medium supplemented with **6d**−**f** at various concentrations of Se. After 24 h of incubation, the medium was removed, and the cells were fixed for 15 min with 100 μL of 1% glutaraldehyde in phosphate-buffered saline (PBS) per well. The fixative was removed and replaced with 150 μL of PBS per well, and the plates were stored under PBS at 4 °C. At the end of the experiment, all plates were stained simultaneously with 0.02% aqueous crystal violet solution (100 μL/well) for 30 min. Excess dye was removed by rinsing the plates with distilled water, and excess water was blotted out. The stain bound by the cells was redissolved in 70% ethanol (180 μL/well) while the microplates were shaken for 2 h on a Titertek shaker (Titertek Instruments Inc., Huntsville, AL). Absorbance was measured at 590 nm using a Thermo$_{max}$ Microplate Reader (Molecular Devices, Sunnyvale, CA).

Comet Assay. We adapted without modification the previously reported single-cell gel electrophoresis assay as a method for assessing DNA damage (*24*). The assay, more commonly known as the comet assay, is a rapid and sensitive method for measuring DNA strand breaks at the level of individual cells. Quantification of DNA damage was performed as recommended (*25*).

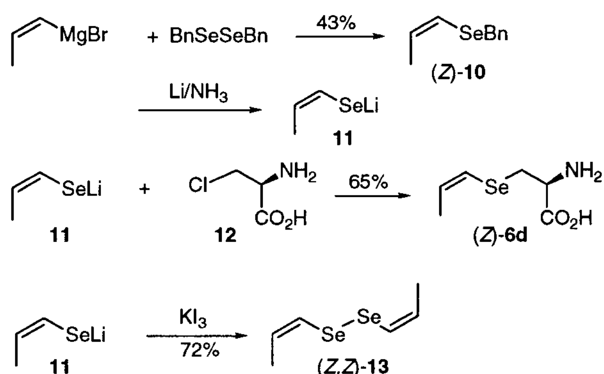
Apoptosis Counting. Effects of selenium compounds on apoptosis of cultured cells were determined morphologically by fluorescent microscopy after labeling with acridine orange and ethidium bromide as reported (*26*).

Statistical Analyses. Differences in cellular responses to selenium compounds were evaluated by factorial analysis of variance (ANOVA). The factors investigated were dose of compound and treatment duration. Post hoc comparisons among treatment conditions were made using the Bonferroni multiple-range test (*27*).

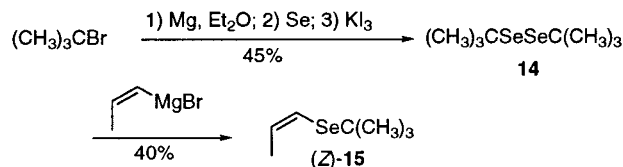
RESULTS AND DISCUSSION

Synthesis. *Se*-Methylselenocysteine (**6a**), *Se*-propyl-L-selenocysteine (**6b**), and *Se*-2-propenyl-L-selenocysteine (**6c**) were synthesized according to known procedures [**6a**,**b** (*10*, *28*)], which were also used in the synthesis of *Se*-(*E*,*Z*)-1-propenylselenocysteine (**6d**), *Se*-2-methyl-2-propenyl-L-selenocysteine (**6e**), and *Se*-2-propynyl-L-selenocysteine (**6f**). Thus, **6d** was prepared by alkylation of β-chloro-L-alanine (**12**) with lithium (*E*,*Z*)-1-propeneselenolate (**11**), from Li−NH_3 reduction of benzyl (*E*,*Z*)-1-propenyl selenide [**10**; Scheme 2; only (*Z*)-isomers shown]. Oxidation of **11** gave bis(1-propenyl) diselenide **13**, used in connection with studies of the oxidation of **6d** described below. These latter studies also required 2-methyl-2-propyl (*Z*)-1-propenyl selenide (**15**), prepared from bis(2-methyl-2-propyl) diselenide (**14**; Scheme 3), synthesized according to an improved procedure. Compounds **6e** and **6f** were prepared by alkylation of the sodium salt of selenocysteine [from borohydride reduction of selenocystine (**4**)] with 3-chloro-2-methylpropene or 3-chloropropyne, respectively (Scheme 4). The ^{1}H and ^{13}C NMR spectra of **6f** were similar to those of *S*-2-propynyl-L-cystine (E. Block and T. Nakahodo, unpublished data; *29*). Reaction of the sodium salt of selenocysteine with β-chloro-L-alanine (**12**) gave selenolanthionine (**16**, Scheme 5), used in connection with studies of the oxidation of **6c** described below. γ-Glutamyl *Se*-methylselenocysteine (**8a**) was prepared by condensing *Se*-methyl-L-methylselenocysteinate hydrochloride (**17**) with triethylammonium-*N*-(trityl)-L-γ-glutamate (**18**) (*20*) in the presence of dicyclohexylcarbodiimide (DCC) and then deprotecting condensation product **19** (Scheme 6). The ^{1}H NMR spectrum of **8a** was very similar to that of L-γ-glutamyl-*S*-methyl-L-cysteine (**1**, R = Me), run under identical conditions, and to the published ^{1}H and ^{13}C NMR spectra of related L-γ-glutamyl-*S*-alk(en)yl-L-cysteines (*30*).

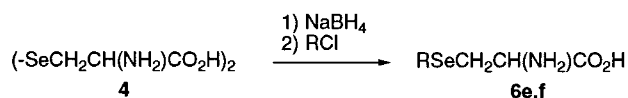
Scheme 2



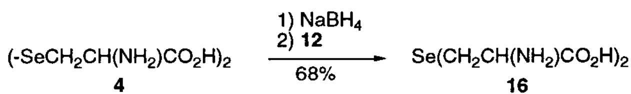
Scheme 3



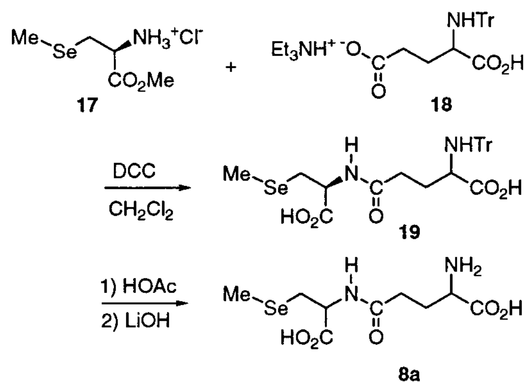
Scheme 4



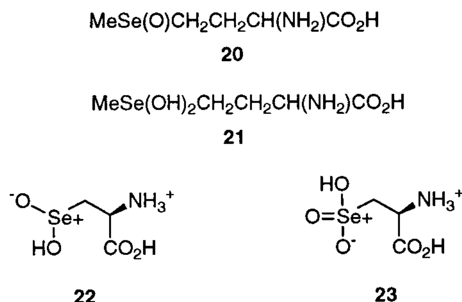
Scheme 5



Scheme 6



Oxidation Studies. *Background.* Selenomethionine (**5**) is said to give a stable selenoxide **20** or its hydrate **21** (*31*, *32*), readily reducible back to **5** with thiosulfate (*33*) and by other means (*34*, *35*). Under the same conditions, selenocysteine derivatives afforded only dehydroalanines, by elimination (*31*). Selenomethionine



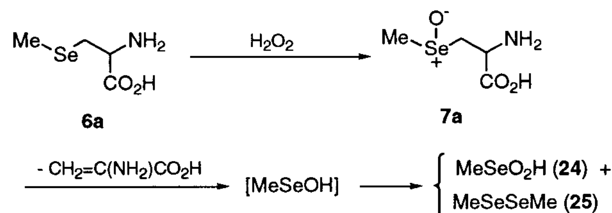
Se-oxide (**20**) and *Se*-methylselenocysteine *Se*-oxide (**7a**) are reported to be present in marine phytoplankton (*36*, *37*), clover (*38*), and cabbage (*39*). Several papers (*37*, *40*–*43*) propose the possible natural occurrence of 3-seleninoalanine [**22**, $HO_2SeCH_2CH(NH_2)COOH$; "selenocysteine seleninic acid"] and/or 3-selenonoalanine [**23**, $HO_3SeCH_2CH(NH_2)COOH$; "selenocysteic acid"] but provide no spectroscopic proof or authentic samples.

In collaboration with Professor Peter Uden and coworkers, oxidation of selenoamino standards **6a**–**f** with excess H_2O_2 was investigated using HPLC-ICP-MS (*17*). For each selenoamino acid oxidized a new selenium-containing peak was produced and the original selenoamino acid peak disappeared. The selenoamino acid oxidation products eluted at much shorter retention times than their precursors on the reverse phase (RP) column, consistent with the increased polarity expected for selenoxides. Oxidation of a mixture of (*E*/*Z*)-*Se*-1-propenyl selenocysteine (**6d**) led to a pair of new peaks, indicating that geometrical isomerism persisted unchanged in the oxidation products. Treatment of solutions of the oxidized selenoamino acids with thiosulfate restored the original selenoamino acids if the thiosulfate treatment was performed shortly after oxidation, before selenoxide decomposition took place.

*Oxidation of Selenomethionine (**5**).* As monitored by RP-HPLC, addition of an equivalent of H_2O_2 to **5** led to an immediate change in retention time from 7.3 to 2.2 min. With H_2O_2 at pD 8 in D_2O, the ^1H NMR spectrum showed two singlets at δ 3.05 and 3.01 replacing the δ 2.02 peak for **5**; similarly, the ^{77}Se NMR spectrum showed two singlets at δ 845 and 838 replacing the δ 75 (D_2O, pD 4) peak for **5**. These observations are consistent with the conversion of **5** to a pair of diastereomeric selenoxides **20**. Analysis of a solution of oxidized **5** by HPLC-ESI-MS showed the presence of the selenoxide hydrate (**21**) at *m*/*z* 232.

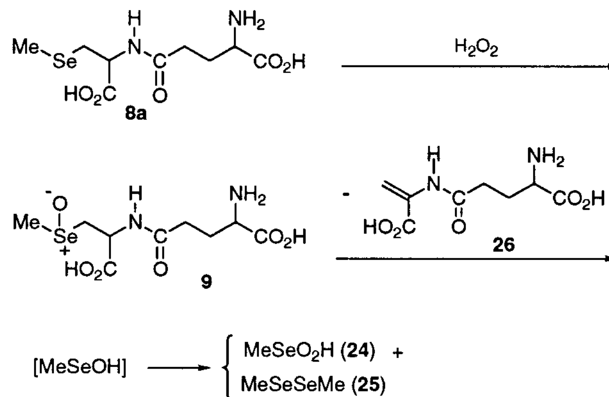
*Oxidation of Se-Methyl-L-selenocysteine (**6a**) and L-γ-Glutamyl-Se-methyl-L-selenocysteine (**8a**).* As monitored by RP-HPLC, addition of an equivalent of H_2O_2 to **6a** led to an immediate change in retention time from 3.4 to 2.0 min. With H_2O_2 at pD 8 in D_2O, the ^1H NMR spectrum of selenoxide **7a** shows a peak at δ 2.80, which at high resolution is resolved into two singlets at δ 2.797 and 2.803 replacing the δ 2.05 peak for **6a**; similarly, the ^{77}Se NMR spectrum showed two singlets at δ 853 and 854 replacing the δ 38 peak for **6a**. These observations are consistent with the conversion of **6a** to a pair of diastereomeric selenoxides **7a**. After 17 min, a ^1H NMR singlet at δ 2.45 appeared, which could be a hydrate. The oxidation was complete in 24 h. The final products were 2:1 methaneseleninic acid (**24**)/dimethyl diselenide (**25**) and ammonium pyruvate, as confirmed by NMR by spiking with authentic samples and by HPLC-ESI-MS (*44*) (Scheme 7). The results were similar

Scheme 7



at pD 1, but the reactions were much faster. In a similar manner, oxidation of **8a** rapidly led to elimination products **24**–**26** via selenoxide **9** (Scheme 8). Colorless

Scheme 8



crystals of **24**, obtained by slow evaporation of a saturated aqueous solution at 0–5 °C, were characterized by X-ray crystallography. The configuration about the selenium atom is pyramidal with Se–C = 1.925(8) Å, Se–O = 1.672(7) Å, Se–OH = 1.756(7) Å, ∠OSeO = 103.0(3)°, ∠HO–Se–C = 93.5(3)°, and ∠OSeC = 101.4(3)°. Hydrogen bonds link the molecules together in spirals along the *c* axis (Figure 1). The structure is isomorphous to that of methanesulfinic acid (*45*). Although structures of aliphatic seleninic acids have not

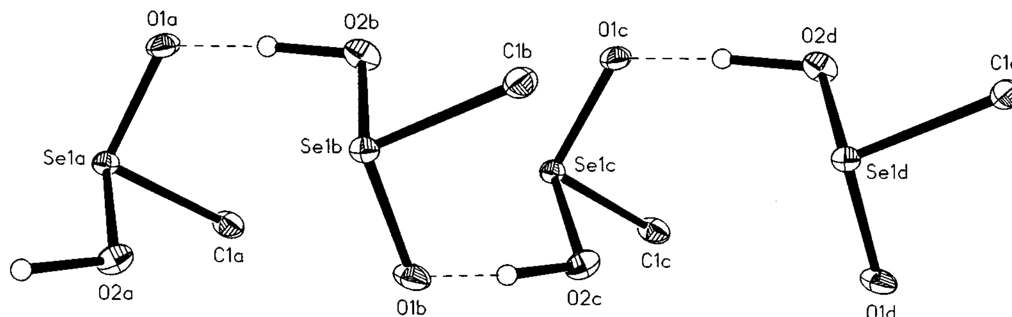


Figure 1. X-ray crystal structure of methaneseleninic acid (**24**) showing intermolecular hydrogen bonding.

Table 1. NMR Data for Selected Selenium-Containing Amino Acids and Related Model Systems[a]

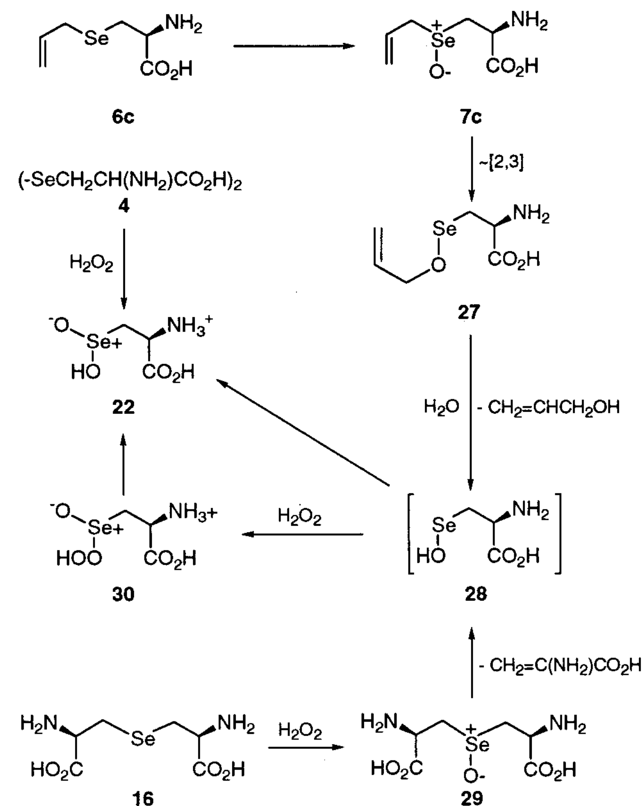
compd	ECH_2	CH	CO_2H	^{77}Se
4[b]	3.34 (dd, *7.7, 14.1*) 3.48 (*4.6, 14.1*) [26.6]	4.34 (dd, *4.6, 7.7*) [53.0]	[170.2]	295
6a[c]	3.20 (t, *7.2*) [24.1] $SeCH_3$: 2.13 [5.7]	4.47 (t, *7.2*) [53.1]	[170.5]	38
16	2.90 (*5.5*) [22.6]	3.50 (*5.5*) [48.6]	[173.3]	82
Me_2Se	2.00 [6.0]			0
Me_2SeO	3.05			812[e]
22[d]	3.28 (*6.2, 14.3*) 3.48 (*10.7, 14.3*) [52.5 (*75*)]	4.32 (*6.2, 10.7*) [55.9]	[175.2]	1195
24	2.79 [39.2]			1216[e]
25	2.61			275[e]
A	2.82 (*9.0, 14.0*) 2.99 (*3.5, 14.0*) [54.2]	4.50 (*3.5, 9.0*) [45.8]		

[a] E = S or Se; ^1H NMR shifts, ppm (*coupling constants in hertz*)[^{13}C NMR shifts, ppm (*coupling constants in hertz*)]; **A** = $HO_2SCH_2CH(NH_3^+)CO_2H$; **4**, selenocystine; **6a**, *Se*-methylselenocysteine; **16**, selenolanthionine; **24**, methaneseleninic acid; **25**, dimethyl diselenide; **22**, 3-seleninoalanine. [b] Stocking et al. (*52*). [c] M. Birringer, Ph.D. Thesis, Universität-Siegen, 1997; solvent is DMF-d_7. [d] From **6c**, at pH (pD) 5. [e] Paulmier, C. *Selenium Reagents and Intermediates in Organic Synthesis*; Pergamon Press: Oxford, U.K., 1986.

been previously reported, the crystal structures of both benzeneseleninic acid and *p*-chlorobenzeneseleninic acid are known (*46*, *47*), and the crystal structure at 2.0-Å resolution of the seleninic acid from selenosubtilisin has been published (*48*).

*Oxidation of Se-2-Propenyl-L-selenocysteine (**6c**) and Selenolanthionine (**16**).* Oxidation of **6c** with an equivalent of H_2O_2 at pD 8 in D_2O led to a reaction that was 95% complete after 1.5 h as judged by NMR, giving allyl alcohol (proven by ^1H NMR and GC-MS) and a selenium-containing compound **22**. The latter compound is also formed on oxidation of selenolanthionine (**16**). On the basis of the spectroscopic data summarized in Table 1 and mechanistic grounds (Scheme 9), **22** is identified

Scheme 9



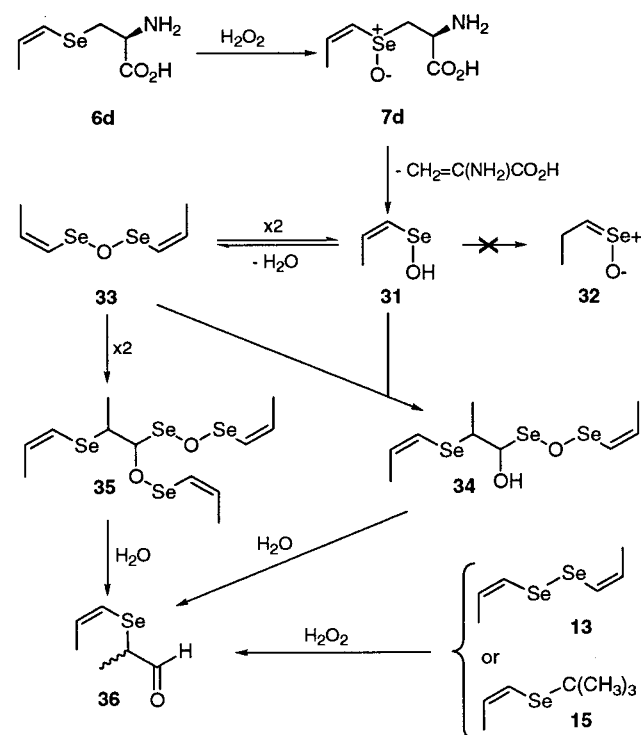
as 3-seleninoalanine (selenocysteine seleninic acid). We postulate that allyl alcohol and **22** are produced by a facile [2,3]-sigmatropic rearrangement of *Se*-2-propenyl-L-selenocysteine *Se*-oxide (**7c**) (*49*) followed by hydrolysis of the resultant selenenic ester **27** to 3-selenenoalanine (**28**). The latter compound then undergoes further reactions. The ^{13}C NMR spectrum of **22** indicated two aliphatic carbons (CH, δ 54.9; and CH_2, 53.1) and a COOH carbon at 180.1 ppm. The CH_2 group, which shows ^{77}Se-satellites, is deshielded compared to model compounds such as selenocystine (**4**) (δ 26.6) but is similar to the chemical shift of the CH_2 group in 3-sulfinoalanine [$HO_2SCH_2CH(NH_3^+)CO_2H$; 54.2]. The ^1H NMR spectrum of **22** is consistent with a compound of type $X{-}SeCH_aH_bCH(NH_3^+)CO_2^-$. The results of oxidation at pD 5 were similar. Using RP-HPLC-ICP-MS and RP-HPLC-ESI-MS within 2 h of addition of H_2O_2, as well as 24 h later, the formation of a short-lived, polar selenium-containing oxidation product, most likely **7c** or **27**, was indicated by a peak of *m/z* 226, and a second, more persistent, polar selenium-containing compound was indicated by a peak with the highest mass at *m/z* 185 (*44*).

In an effort to identify the unknown selenium-containing product, we examined the oxidations of selenolanthionine (**16**) and selenocystine (**4**). We anticipated that the selenoxide **29** of **16** would decompose to pyruvate and **28** (Scheme 9). According to the literature (*37*), oxidation of **4** gives 3-seleninoalanine (**22**) or 3-selenonoalanine (**23**). Oxidation of **16** with H_2O_2 gave ammonium pyruvate after 1.5 h. As monitored by RP-HPLC-ICP-MS, addition of an equivalent of H_2O_2 to **16** led to immediate formation of a polar selenium-containing compound with a retention time similar to that of the more persistent *m/z* 185 product from oxidation of **6c** (*44*). Initially this peak had a (presumed) MH^+ ion at *m/z* 218, corresponding to selenocysteine perseleninic acid (**30**). After 24 h, this peak was replaced with a peak of retention time and a mass spectral pattern (*m/z* 185, 154, 131, and 111) identical to that of the more persistent *m/z* 185 product from oxidation of **6c** (*44*). Although the identical, persistent *m/z* 185 peaks from oxidation of **6c** and **16** might be selenenic acid **28** (perhaps stabilized by intramolecular hydrogen bonding), it is more likely that the *m/z* 185 peak is a fragment ion derived from a heavier parent, for example, loss of $HO^•$ from selenocysteine seleninic acid, because under the ESI-MS conditions used, compounds **6a**, **6c**, and **6d** show no parent ions but only fragment ions. Furthermore, in the mass spectrum of $MeSeO_2H$ the fragment resulting from loss of $HO^•$ is 58% as abundant as the MH^+ ion.

It is known that selenenic acids can be oxidized to seleninic acids when generated in the presence of H_2O_2 (*50*) in addition to undergoing disproportionation to seleninic acids. During the course of the oxidation of **16**, the ^{77}Se NMR spectrum underwent a change from a single peak in the starting material at δ 82 to a pair of peaks at δ 858 and 865 (presumably selenoxide **29**) to a final product showing a peak at δ 1195, which is within the range of chemical shifts reported for seleninic acids (δ 1240−1175) (*21*) but distinct from the chemical shifts for selenenic acids (δ ~1143−1066) (*51*, *53 54*) and selenonic acids (δ 1022) (*55*). In particular, the observed shift of δ 1195 is quite close to the values of δ 1188 and 1190 reported for the 3-seleninoalanine component of oxidized selenosubtilisin. Efforts to observe the selenenic acid corresponding to selenosubtilisin were unsuccessful (*21*). We also find that oxidation of selenocystine (**4**) with H_2O_2 gives **22**.

Oxidation of Se-(E,Z)-1-Propenyl-L-selenocysteine (***6d***). In view of the ready formation of methaneselenenic acid, MeSeOH, on oxidation of *Se*-methylselenocysteine (**6a**), it was of interest to determine if a similar process would occur with **6d** giving 1-propeneselenenic acid (**31**; Scheme 10), because the latter might then rearrange to give the unknown propaneselenal *Se*-oxide (**32**), the selenium analogue of the onion lachrymatory factor (*56*). Oxidation of **6d** at pH 8 with H_2O_2 afforded in 69% yield a yellow oil identified by spectroscopic means (see Experimental Procedures) as 2-[(*E,Z*)-1-propenylseleno]-propanal (**36**). We propose that *Se-(E,Z)*-1-propenyl-L-selenocysteine *Se*-oxide (**7d**) undergoes elimination, giving 1-propeneselenenic acid (**31**), which is in equilibrium with the corresponding selenenic anhydride (**33**) (*53*, *57*). Compound **33** adds to itself to give intermediate **35**, which on hydrolysis gives **36**. Alternatively, addition of **31** to **33** followed by hydrolysis of adduct **34** could also afford **36**. Similar processes have been reported by others (*58*). In support of this mechanism, oxidation of **13** (which should afford **33**) and **15** (which should yield **31**) both gave **36**, each in >50% yield.

Scheme 10



Natural Occurrence. Apart from the work of Spåre and Virtanen (*3*) on the possible natural occurrence of γ-glutamyl selenoamino acids discussed above, γ-glutamyl *Se*-methylselenocysteine (**8a**) has been detected in *Astralagus bisulcatus* (*59*) and in low levels in *Melilotus indica* L. (an Se-tolerant grassland legume species) (*60*). We have reported on the detection of **8a** and γ-glutamyl selenomethionine in garlic, onion, ramp, selenized yeast, and *Astragulus praleongus* and the detection of selenolanthionine (**16**) in selenized yeast by HPLC-ICP-MS and HPLC-ESI-MS, calibrating our analyses with samples of **8a** and **16** prepared as described in the present paper (*13*−*16*). Because little change was seen following treatment of garlic and onion extracts with thiosulfate, known to readily reduce selenoxides [including those derived from *Se*-alk(en)yl selenocysteines and selenomethionine], we conclude that selenoxides can at most be present in *Allium* species at levels below the detection limits of ICP-MS. These conclusions are also supported by X-ray absorption spectroscopy (XAS) studies of the forms of selenium in normal and Se-enriched garlic (I. Pickering and E. Block, unpublished studies, Stanford Synchrotron). The same can be said about the presence in *Allium* species of *Se*-alk(en)yl selenocysteines **6b**−**d**. The thermal instability found by us for *Se*-alk(en)yl selenocysteine selenoxides also makes their natural occurrence improbable, other than as transient intermediates in redox systems or as artifacts formed by air oxidation during analysis (*61*).

Biological Activity. *Background.* As noted above, Ip and Lisk showed that selenium-enriched garlic had anticancer activity significantly higher than that of unenriched garlic (*6*, *7*). Synthetic *Se*-propylselenocysteine (**6b**) and *Se*-2-propenylselenocysteine (**6c**) were selected for evaluation of their anticancer activity because they are analogues of *Se*-methylselenocysteine (**6a**), which is found in high abundance in Se-enriched garlic. Compound **6c** showed particular promise: at 2 ppm of Se in the diet it caused an 86% inhibition in a chemically induced mammary tumor model in rats (*10*). This magnitude of cancer protection was significantly greater than that observed with equivalent doses of either **6a** (*5*) or **6b**. Differences in the anticancer activity of allyl and propyl organosulfur compounds have been noted; it has been suggested that the presence of a double bond in the allyl moiety may have a unique effect (*62*).

It is well recognized that the biological activity of selenium is an expression of selenium in a variety of chemical forms and not that of the element per se (*63*). We have used defined cell culture models to characterize the responses to different chemical forms of selenium (*64*, *65*). Cell culture models work well for this purpose because the specificity of the assay endpoints can be assessed without the complications of systemic metabolism. In recent work, the effects of **6b** and **6c** have been compared in cell culture (*66*). Compound **6c** inhibited cell growth and induced apoptosis in mammary epithelial cell lines that have either wild-type or mutant p53 activity. Compound **6b** was without effect. However, **6c** also induced a loss of DNA integrity measured as alkaline labile breaks, an activity that could limit its usefulness if this effect were manifest in vivo at doses of **6c** that inhibit the development of cancer (*10*). As noted in Zhu et al. (*66*), and consistent with evidence recently reported by Rooseboom et al. (*67*), selenocysteine Se-conjugates are likely to undergo selenoxidation

Table 2. Effects of 6d−f on TM2H and TM12 Cell Growth[a]

	% untreated control					
	TM2H			TM12		
selenium (μM)	24 h	48 h	72 h	24 h	48 h	72 h
6d						
25	99 ± 5.4^{ab}	92 ± 2.1^{ab}	72 ± 3.8^{b}	95 ± 3.2^{ab}	87 ± 2.0^{b}	78 ± 2.4^{b}
50	91 ± 3.8^{b}	86 ± 2.3^{b}	65 ± 1.0^{b}	87 ± 3.0^{b}	76 ± 0.9^{c}	61 ± 4.0^{c}
100	81 ± 1.8^{b}	67 ± 1.8^{c}	33 ± 0.8^{c}	84 ± 2.4^{b}	66 ± 1.2^{d}	33 ± 0.8^{d}
6e						
25	93 ± 3.4^{ab}	91 ± 3.3^{a}	85 ± 1.8^{b}	98 ± 2.6^{ab}	98 ± 1.8^{ab}	99 ± 1.9^{a}
50	84 ± 4.4^{bc}	80 ± 2.4^{b}	70 ± 3.8^{c}	90 ± 1.4^{ab}	92 ± 1.6^{bc}	97 ± 2.8^{a}
100	78 ± 3.4^{bc}	64 ± 1.3^{c}	54 ± 1.5^{d}	88 ± 3.5^{b}	87 ± 1.1^{c}	95 ± 1.8^{a}
6f						
25	98 ± 3.9^{a}	91 ± 2.3^{ab}	88 ± 1.4^{b}	97 ± 2.1^{a}	91 ± 1.8^{b}	84 ± 3.0^{b}
50	95 ± 2.5^{a}	78 ± 11^{bc}	73 ± 1.3^{c}	85 ± 2.0^{b}	77 ± 3.0^{c}	53 ± 0.8^{c}
100	93 ± 2.6^{a}	66 ± 6.6^{c}	48 ± 1.9^{d}	80 ± 3.3^{b}	59 ± 1.0^{d}	32 ± 0.9^{d}

[a] Effect of treatment with **6d−f** on cell growth inhibition. All experiments were repeated three times. In each experiment, eight replicates of each dose of each compound were analyzed. The results of a representative experiment are presented. Data are expressed as percent of untreated control, mean ± SE ($n = 8$). Cell number was estimated as an absorbance value of crystal violet stained cells, $\lambda = 590$ nm. The absolute absorbance of TM2H cells in the untreated control was 0.081 ± 0.004, 0.170 ± 0.02, and 0.248 ± 0.02 at 24, 48, and 72 h, respectively; the absolute absorbance of TM12 cells in the untreated control was 0.072 ± 0.005, 0.153 ± 0.03, and 0.265 ± 0.02 at 24, 48, and 72 h, respectively. For each compound and treatment duration, values with different superscripts were statistically different, $p < 0.05$.

Table 3. Effects of 24 h of Exposure to 6d−f on Rate of Apoptosis in TM2H and TM12 Cells[a]

	apoptotic cells/100 cells counted					
	6d		**6e**		**6f**	
Se (μM)	TM2H	TM12	TM2H	TM12	TM2H	TM12
0	1.9 ± 0.4^{a}	8.9 ± 1.6^{a}	1.9 ± 0.2^{a}	8.9 ± 1.6^{a}	1.9 ± 0.3^{ab}	8.9 ± 1.6^{a}
12.5	5.5 ± 1.2^{ab}	15.2 ± 2.1^{a}	3.9 ± 0.4^{b}	11.8 ± 1.3^{a}	1.5 ± 0.5^{a}	12.6 ± 2.3^{a}
25	8.0 ± 2.4^{ab}	17.5 ± 2.1^{b}	4.0 ± 0.5^{b}	10.7 ± 2.1^{a}	1.9 ± 0.4^{ab}	13.9 ± 2.3^{a}
50	9.8 ± 2.1^{b}	19.2 ± 2.3^{b}	4.2 ± 0.5^{b}	9.7 ± 1.4^{a}	3.0 ± 0.4^{b}	27.3 ± 2.2^{b}

[a] Effect of treatment with **6d−f** on apoptosis. The data are expressed as a percent of cells counted that were apoptotic, mean ± SE ($n = 6$). Two hundred cells were counted for each apoptosis assay. In an experiment, each treatment was evaluated in duplicate. The effects of each treatment were studied in three independent experiments. The results of all three experiments were combined for the purpose of statistical analysis. For each compound and treatment duration, values with different superscripts were statistically different, $p < 0.05$.

followed by a syn-elimination, a reaction mechanism that proceeds at least to some extent under physiological conditions despite the presence within the cell of glutathione, which is known to be able to rapidly reduce selenoxides (*34*). In this regard, it is interesting to note that **24** ($MeSeO_2H$), the disproportionation product of methaneselenenic acid (MeSeOH) released from **7a** and **9**, shows significant anticancer activity (*68*, *69*). In the case of **6c**, oxidative metabolism could ultimately give rise to allyl alcohol, which could be converted to acrolein, a metabolite that has been reported to have DNA-damaging activity. Compounds **6e** and **6f** would be predicted to form related products, for example, methylallyl and propargyl alcohols, respectively, if they were similarly metabolized. Therefore, we have used previously developed cell culture models and endpoints (*66*) to study the activities of these three synthetic homologues of **6c**, namely, *Se*-(*E*,*Z*)-1-propenylselenocysteine (**6d**), *Se*-2-methyl-2-propenylselenocysteine (**6e**), and *Se*-2-propynylselenocysteine (**6f**). Our goal was to identify a homologue of **6c** that had comparable activities in inhibiting cell growth and inducing apoptosis without affecting DNA integrity.

Results. Compounds **6d−f** were evaluated for their effects on cell growth, induction of apoptosis, and DNA integrity in two cell lines that differ in their p53 activity: TM12, which has wild-type p53 activity, and TM2H, which lacks p53 activity due to a mutation in that gene (*66*). As noted above, these cell lines differ in the mechanisms by which they respond to genotoxic stimuli and undergo apoptotic cell death. Cell death by apoptosis, which can be induced by several different sequences of cellular events, is recognized as a powerful effector of cancer inhibitory activity and has been reported to be induced by many selenium compounds, some of which induce DNA damage (*63*, *65*). Because some selenium compounds induce DNA damage whereas others do not, we also measured DNA integrity using the comet assay. Selenium compounds that inhibit cell growth and induce apoptosis in the absence of DNA damage in these cell lines have generally been found to be well tolerated and to exert cancer inhibitory activity in vivo (*65*).

In both TM2H and TM12 cells, *Se*-(*E*,*Z*)-1-propenylselenocysteine (**6d**), used as a mixture of isomers, inhibited cell growth in a dose- and time-dependent manner (Table 2) and also induced apoptosis (Table 3). Levels of DNA damage were increased by treatment of either cell line with **6d** (Table 4). Although the addition of a methyl group in the 2-position of the allyl moiety eliminated DNA-damaging activity, the presence of a double bond conjugated to the selenium atom is activating. The levels of DNA damage observed in the TM2H cell line, which cannot eliminate damaged cells by a p53-mediated growth arrest mechanism, were the highest that we have observed with any selenoamino acid tested to date. Consistent with this observation was an increase in levels of apoptosis, which can be a cellular response to genotoxic events. Such effects are likely to account for the progressive increase in the degree of cell growth inhibition by **6d** with increasing treatment duration. Because of the DNA-damaging activity of **6d**,

Table 4. Effects of 6d−f on DNA Integrity Measured by the Comet Assay in TM2H and TM12 Cells for 24 h[a]

	arbitrary units					
	6d		**6e**		**6f**	
Se (μM)	TM2H	TM12	TM2H	TM12	TM2H	TM12
0	35 ± 2.4[a]	45 ± 5.3[a]	35 ± 2.4[a]	45 ± 5.3[a]	35 ± 2.4[a]	45 ± 5.3[a]
12.5	46 ± 2.2[a]	40 ± 11[a]	36 ± 3.1[a]	42 ± 4.5[ac]	34 ± 2.3[a]	33 ± 4.0[ab]
25	154 ± 11[b]	66 ± 11[a]	40 ± 4.7[a]	22 ± 2.3[b]	32 ± 3.0[a]	21 ± 2.4[bc]
50	187 ± 11[c]	125 ± 15[b]	40 ± 4.4[a]	30 ± 1.7[bc]	34 ± 3.6[a]	14 ± 2.5[c]

[a] Effect of treatment with **6d−f** on DNA integrity. The data are expressed as arbitrary units of alkaline labile DNA damage, mean ± SE ($n = 9$). The score ranges from a minimum of 0 to a maximum of 400. Two hundred cells were counted for each comet assay. In an experiment, each treatment was replicated three times. The effects of each treatment were studied in three independent experiments. The results of all three experiments were combined for the purpose of statistical analysis. For each compound and treatment duration, values with different superscripts were statistically different, $p < 0.05$.

Table 5. Comparison of in Vitro Effects of Selenoamino Acids 6a−f in TM2H and TM12 Cells[a]

	% of untreated control					
	cell growth[a]		apoptosis[b]		DNA integrity[b]	
compd	TM2H	TM12	TM2H	TM12	TM2H	TM12
6a[c,d]	28 ± 2.9[c]	47 ± 5.6[c]	225 ± 30[d]	176 ± 15[d]	65 ± 8[d]	95 ± 5[d]
6b[e]	94 ± 1.1	89 ± 1.2	175 ± 25	141 ± 9	65 ± 3	98 ± 2
6c[e]	31 ± 0.7	5 ± 0.4	225 ± 20	318 ± 9	168 ± 10	158 ± 5
6d	65 ± 1.0	61 ± 4.0	516 ± 216	216 ± 26	534 ± 31	278 ± 33
6e	70 ± 3.8	97 ± 2.8	221 ± 26	109 ± 15	114 ± 13	67 ± 4
6f	73 ± 1.3	53 ± 0.8	158 ± 21	307 ± 24	97 ± 10	31 ± 6

[a] Comparison made with 50 μM Se of each compound following 72 h of exposure. Data are represented as percent of control, i.e., cells not treated. [b] Comparison made with 50 μM Se of each compound following 24 h of exposure. Data are represented as percent of control, i.e., cells not treated. [c] Unpublished data. [d] Ip et al. (*68*). [e] Zhu et al. (*66*).

modifications of the propenyl chain in this manner appear to be of limited value relative to chemoprevention because of the potential for genotoxicity.

Se-2-Methyl-2-propenylselenocysteine (**6e**) inhibited cell growth in the TM2H cell line in a dose- and time-dependent manner; however, it had only a small effect in the TM12 cell line (Table 2). Consistent with this observation, **6e** also induced apoptosis in the TM2H cell line (a 2-fold increase), but not in TM12 cells (Table 3). Whereas **6e** was without effect on DNA integrity in TM2H cells, lower levels of DNA damage were observed with increasing concentrations of **6e** in TM12 cells (Table 4). These data indicate that the presence of a methyl group in the 2-position of the allyl moiety of this selenocysteine homologue reduces the level of DNA damage in treated cells in comparison to *Se*-2-propenylselenocysteine (**6c**) (see Table 5). The inhibition of cell growth and induction of apoptosis by **6e** was generally greater than that achieved using *Se*-propylselenocysteine (**6b**) but less than that when **6c** was used (Table 5). These data imply that the addition of a methyl group to the double bond reduces DNA-damaging activity while retaining a modest level of cell growth inhibitory activity and ability to induce apoptosis. These data suggest that effects of **6e** on cell number homeostasis are independent of the DNA-damaging activity that has previously been associated with the allyl moiety of **6c**. The fact that the effects of **6e** were observed primarily in TM2H cells indicates that mechanisms of apoptosis induction were independent of p53. Whether or not the effect on cell growth is mediated by influences on cell cycle regulatory molecules merits consideration because **6c** has recently been shown to dramatically reduce levels of phosphorylated retinoblastoma protein (*70*).

Se-2-Propynylselenocysteine (**6f**) inhibited the growth of both cell lines in a dose- and time-dependent manner (Table 2). Overall, the effects of **6f** on cell growth were relatively small during the first 24 h of exposure but then became marked at 48 and 72 h of exposure. Effects on growth were consistently greater at each dose−time point in TM12 versus TM2H cells. Interestingly, **6f** had a marginal effect on the induction of apoptosis in TM2H cells, whereas a 3-fold increase was observed in TM12 cells. Compound **6f** had no effect on DNA integrity in TM2H, but treatment was associated with lower levels of DNA strand breaks in TM12 cells. These data indicate the substitution of a triple bond for the double bond in the allyl moiety eliminates DNA-damaging activity while retaining the ability to inhibit cell growth and induce apoptosis. Although the effects of **6f** on cell growth were substantial after 72 h of treatment with 50 μM Se (73 and 53% of the cell number in TM2H and TM12 untreated cultures, respectively), **6c** induced greater effects on cell growth under the same treatment conditions (31 and 5% of the cell number in TM2H and TM12 untreated cultures, respectively; see Table 5).

Table 5 summarizes the relative effects of the six selenocysteine compounds tested in our cell culture model system. Although each compound displays a unique profile of activity, the compounds can be rank ordered in terms of their probability of being well tolerated in vivo and having cancer inhibitory activity in animal models. The ranking is **6a** > **6c** ≫ **6f** > **6e** > **6b** > **6d**. This ranking takes into account, in decreasing order of importance, the degree of cell growth inhibition by each compound, the magnitude of DNA-damaging activity, and the consistency of apoptosis induction in both cell lines studied. Thus, on the basis of our assay results, it appears that the compounds evaluated in the biological assays in this study are unlikely to exceed the chemopreventive efficacy of other selenocysteine compounds that have already been evaluated, that is, **6a** and **6c**. This analysis is made with one significant reservation. We have recently observed that the β-lyase activity of cells in this model system may limit the rate of release of the selenol moiety from the selenoamino acid (H. Thompson, unpublished data). Moreover, it is unclear how the K_m and V_{max} of the lyases will vary for

each of the compounds evaluated. The above reservation is significant because one mechanism for bioactivation of selenocysteine Se-conjugates **6** involves β-elimination of selenols from **6** by β-lyases (*18*, *63*, *71*, *72*). Approaches are being developed to minimize this potential limitation.

Finally, the biological activity of γ-glutamyl *Se*-methylselenocysteine (**8a**) has been found to be quite similar to that of *Se*-methylselenocysteine (**6a**); these results will be presented elsewhere (*73*).

NOTE ADDED IN PROOF

Compound **8a** has recently been identified in garlic harvested in naturally seleniferous soil using size exclusion chromatography in conjunction with ICP-MS and ESI-MS; **8a** accounts for 78% of the total selenium present (*74*). It has also been reported that in bulbs from Se-enriched ramps (*Allium tricoccum*), the predominant form of selenium at all concentrations of selenium is **6a**, with lower amounts of selenate, **8a**, and selenocystathionine (*75*).

SAFETY

Caution! Many of the organoselenium compounds used in this work have very unpleasant smells and may be toxic on ingestion or skin contact. All experiments were carried out in a glovebox within a hood.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; MS, mass spectrometry; ESI, electrospray ionization; GC, gas chromatography; RP, reverse phase; PBS, phosphate-buffered saline.

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Supporting Information Available: Details of X-ray structure determination of methaneseleninic acid (**24**). This material is available free of charge via the Internet at http://pubs.acs.org.

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