Production of Polyselenodipenicillamines, Unique Selenium Compounds

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Selenite (H_2SeO_3) reacts with thiol compounds (RSH) under acidic conditions to form selenotrisulfides (RSSeSR, *i.e.* monoselenodithiols). The stoichiometry of the reaction is proposed as $4RSH+H_2SeO_3 \rightarrow RSSeSR+RSSR+3H_2O$. Surprisingly, we found novel polynuclear selenium-containing compounds, *i.e.* polyselenodipenicillamines ($PenSSe_{2-4}SPen$), in the reaction of D-penicillamine (PenSH) with H_2SeO_3. The selenium-centered features of $PenSSe_{2-4}SPen$ were determined by ^{1}H-NMR and LC-MS/MS analyses, showing that the selenium isotope abundance patterns of the compounds were in good agreement with the theoretically-calculated ones. In order to better understand the mechanisms for $PenSSe_{2-4}SPen$ production, various molar ratio of H_2SeO_3 (1/8 to 4 times of PenSH) was reacted with PenSH, and the concentration of the products was calculated from integral values of dimethyl proton signals for $PenSSe_{1-2}SPen$ as compared with methyl proton signals for acetic acid (an internal standard). Total $PenSSe_{1-2}SPen$ concentration was increased with increasing of H_2SeO_3, in which concomitant decrease of PenSSPen (disulfide form of PenSH) was observed. Based on these results, we proposed the $PenSSe_{2-4}SPen$ production mechanisms being involved in penicillamine selenopersulfides ($PenSSe_{1-2}H$).

Key words selenium; polyselenodithiol; selenotrisulfide; penicillamine; cancer chemoprevention

Selenium is an essential micronutrient that is known to play an important role in many physiological functions of the human body. Recent studies have shown that selenium supplements in the diet can reduce the risk of cancer and other diseases.[1,2] To produce unique selenium compounds leading to the therapeutic application, the reaction chemistry of selenium should be studied in greater detail. Selenite (H_2SeO_3) reacts with thiol compounds (RSH) under acidic conditions to form selenotrisulfides (RSSeSR), which was first described by Painter using cysteine.[3] Reactions of glutathione, cysteine, and 2-mercaptoethanol with H_2SeO_3 in a molar ratio of 4 : 1 under acidic conditions lead to form selenodiglutathione, selenodicysteine, and selenodi-2-mercaptoethanol, respectively.[4] Painter and Ganther proposed the following reaction, $4RSH+H_2SeO_3 \rightarrow RSSeSR+RSSR+3H_2O$.

In biological systems, RSSeSR derived from glutathione and cysteine have been reported to play an important role in selenium metabolism, leading to selenium-mediated cancer prevention.[5] The formation of selenodiglutathione, selenocysteineglutathione, and selenodicysteine was demonstrated based on the models of selenium metabolism in cell-free systems and in rats by Gabel-Jensen *et al.*[6] and by Braga *et al.*,[7] who proposed the formation of selenide species—selenopersulfide (RSSeH), hydrogen selenide (H_2Se), and hydrogen selenide anion (HSe^-)—through the reduction of RSSeSR. Recently, selenodipenicillamine (PenSSeSPen), a chemically stable RSSeSR, was isolated and its bioavailability in mice was investigated by Nakayama *et al.*[8,9] In our study aiming at discovering novel selenium compounds, the reaction of D-penicillamine (PenSH) with H_2SeO_3 was found to yield polyselenodipenicillamines ($PenSSe_{2-4}SPen$), which had been overlooked previously (Chart 1). We describe herein the synthesis and characterization of $PenSSe_{2-4}SPen$.

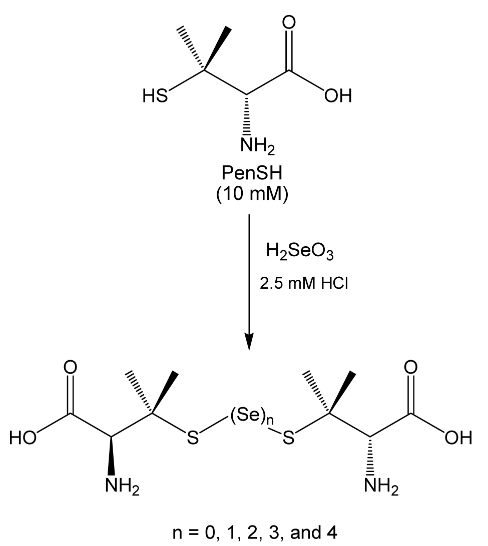

Chart 1

Experimental

Preparation of $PenSSe_{2-4}SPen$ $PenSSe_{2-4}SPen$ were prepared by mixing with 10 mM PenSH (Tokyo Kasei Kogyo, Tokyo, Japan) and 40 mM H_2SeO_3 (Wako Pure Chemical, Osaka, Japan) in 400 μl of 2.5 mM HCl. $PenSSe_{2-4}SPen$ production was confirmed using LC-MS/MS and ^{1}H-NMR. Products were purified using preparative HPLC equipped with reversed-phase column (Develosil C-18, 250×20 mm i.d., 5 μm-pore size, Nomura Chemical, Aichi, Japan) and were analyzed by LC-MS/MS and ^{1}H-NMR.

LC-MS/MS Analysis of $PenSSe_{2-4}SPen$ LC-MS/MS experiments were conducted using an Agilent 1100 Series HPLC system (Agilent Technologies Japan Ltd., Tokyo, Japan) coupled to an LCQ DECA XP ion trap mass spectrometer (Thermo Fisher Scientific, Kanagawa, Japan). LC was carried out using a reversed-phase column (Develosil C-18, 50×4.6 mm i.d., 3 μm-pore size, Nomura Chemical). Mobile phase A consisted of 0.05% formic acid and mobile phase B consisted of methanol. The separation of $PenSSe_{0-4}SPen$ was performed in 15 min using a linear gradient elution from A to B at a flow rate of 0.8 ml/min.

^{1}H-NMR Analysis ^{1}H-NMR spectra were obtained using an ECP500 spectrometer (Jeol Ltd., Tokyo, Japan). All NMR spectra were measured in D_2O (Wako) unless otherwise indicated and the signal positions are expressed in parts per million (ppm) based on the D_2O signal as a reference at 4.80 ppm.

Results and Discussion

As shown in Fig. 1, through LC-MS analysis, we found

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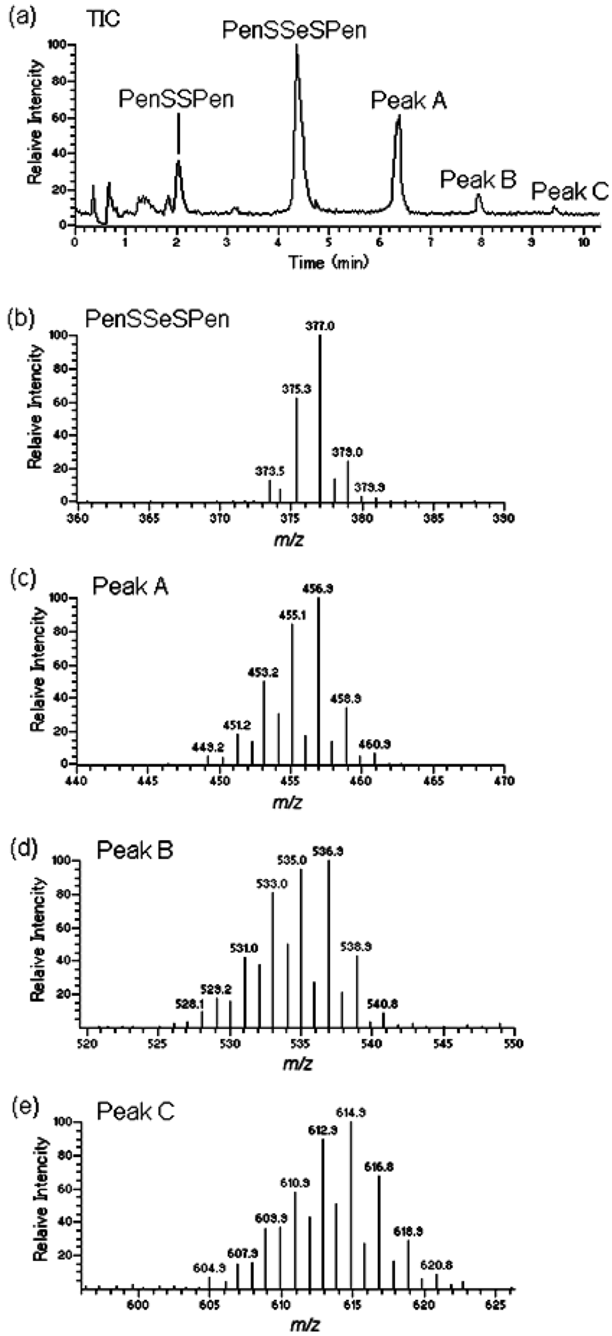

Fig. 1. LC-MS Data of the Reaction Mixture of 10 mM PenSH and 40 mM H_2SeO_3

(a) Total ion chromatogram (TIC). Mass spectra of PenSSeSPen (b), peak A (c), peak B (d), and peak C (e) indicate the isotope patterns of Se, 2Se, 3Se, and 4Se, respectively.

that the reaction of PenSH with H_2SeO_3 under acidic conditions yielded PenSSeSPen (*m*/*z* 377) as well as unique selenium-containing compounds (indicated by peaks A—C). The mass spectra of peaks A—C gave $[M+H]^+$ ions at *m*/*z* 457, 537, and 617, respectively, corresponding to the addition of 80 (Se), 160 (2Se), and 240 (3Se) atomic mass units of PenSSeSPen. The observed isotope patterns were in good agreement with the theoretically-calculated ones assuming two, three, and four selenium atoms. LC-MS/MS analyses showed typical fragment ion patterns of PenSSeSPen and peaks A—C as follows: PenSSeSPen, *m*/*z* 150 $[PenSH+H]^+$, *m*/*z* 228 $[PenSSe]^+$; peak A, *m*/*z* 150 $[PenSH+H]^+$, *m*/*z* 228 $[PenSSe]^+$, *m*/*z* 341 $[PenSSe_2SH+H]^+$; peak B, *m*/*z* 150 $[PenSH+H]^+$, *m*/*z* 228 $[PenSSe]^+$, *m*/*z* 388 $[PenSSe_3]^+$; peak C, *m*/*z* 468 $[PenSSe_4]^+$, *m*/*z* 501 $[PenSSe_4SH+H]^+$. PenSSPen (a disulfide form of PenSH) gave typical fragment ion $[PenSS]^+$ (*m*/*z* 180), whereas $[PenSS]^+$ was not found in LC-MS/MS spectra of peaks A—C. These results suggest that PenSSeSPen and peaks A—C would share common basic structures. In [1]H-NMR analyses, the dimethyl group of PenSH [R–C$(\underline{CH_3})_2$–SH, R: CH(NH_2)COOH] were observed as a twin (nonequivalent) signal at δ 1.53/δ 1.60 ppm (Fig. 2). PenSSPen [R-C$(\underline{CH_3})_2$–SS–C$(\underline{CH_3})_2$–R] and PenSSeSPen [R–C$(\underline{CH_3})_2$–SSeS–C$(\underline{CH_3})_2$–R], both of which have a symmetrical center, showed a twin signal at δ 1.51/δ 1.58 ppm and δ 1.55/δ 1.65 ppm, respectively. Also, two dimethyl groups of peak A were observed as a twin signal at δ 1.57/δ 1.67 ppm. These results indicate that peak A has a symmetrical structure about linearly-arranged 2Se atoms, $PenSSe_2SPen$ [R–C$(\underline{CH_3})_2$–SSe_2S–C$(\underline{CH_3})_2$–R]. The proton signals of peaks B and C were lower than detection limit due to their rather small yields. However, in combination with the [1]H-NMR data of peak A and the fragment ion patterns of PenSSeSPen and peaks A—C, it can be deduced that peaks B and C have a symmetrical structure like $PenSSe_3SPen$ and $PenSSe_4SPen$, respectively. The product having the nonequivalent methyl signals at δ 1.14/δ 1.23 ppm was deduced to be dipenicillamine ether (PenSOSPen, bridging two sulfides *via* oxygen atom) from LC-MS/MS analysis.

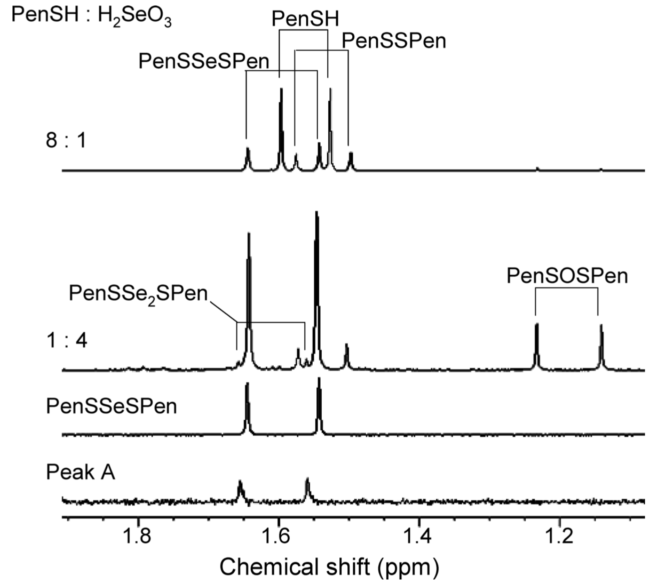

Fig. 2. [1]H-NMR Spectra of the Reaction Mixtures ([PenSH : H_2SeO_3] = 8 : 1 or 1 : 4)

Dimethyl group of PenSH (δ 1.53/δ 1.60 ppm), two dimethyl groups of PenSSPen (δ 1.51/δ 1.58 ppm), PenSOSPen (δ 1.14/δ 1.23 ppm), PenSSeSPen (δ 1.55/δ 1.65 ppm), and peak A ($PenSSe_2SPen$, δ 1.57/δ 1.67 ppm) were observed as a twin signal.

In order to elucidate the mechanism for $PenSSe_2SPen$ production, reaction mixtures consisting of 10 mM PenSH and H_2SeO_3 at various molar ratios (1 : 0, 8 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 4) in 2.5 mM HCl and 10 mM acetic acid (an internal standard for the calculation of the molar concentration) were prepared in D_2O and directly used for [1]H-NMR analysis. The molar concentrations of PenSH, PenSSPen, PenSSeSPen, $PenSSe_2SPen$, and PenSOSPen were calculated from their integral values based on the methyl proton value of 10 mM acetic acid (δ 2.10), as shown in Fig. 3. In the molar ratio range from 2 : 1 to 1 : 4 (PenSH to H_2SeO_3), PenSH (originally 10 mM) was converted to the detected symmetric dipenicillamines (PenSSPen, PenSSeSPen, $PenSSe_2SPen$, and PenSOSPen: 5 mM in total with a theoretical stoichiometry, see Fig. 3). Kice *et al.* proposed the reaction of RSH compounds with H_2SeO_3 using 1-butanethiol (*n*-BuSH) and its isomer with a sterically shielded sulfur atom, 2-methyl-2-propanethiol (*t*-BuSH), as summarized in Chart 2.[10] In the chart, the reaction of RSH compounds with a selenium intermediate, RSSe(O)SR, proceeds *via* two possible pathways (attacks on S and on O) depending on the steric effect of the R-group. The reaction of *n*-BuSH (no steric hindrance) with RSSe(O)SR proceeds *via* an attack on S (Painter/Ganther reaction), and the amount of disulfide (*n*-BuSSBu-*n*) is equal to that of RSSeSR (*n*-BuSSeSBu-*n*). However, the reaction of *t*-BuSH proceeds *via* two attacks on S and on O (Kice reaction in addition to Painter/Ganther reaction) due to steric hindrance by the *tert*-butyl group in the nucleophilic attack on S, leading to the formation of RSSeSR (*t*-BuSSeSBu-*t*) and sulfenic acid (*t*-BuSOH). The reaction of *t*-BuSOH with *t*-BuSH to form disulfide (*t*-BuSSBu-*t*) is

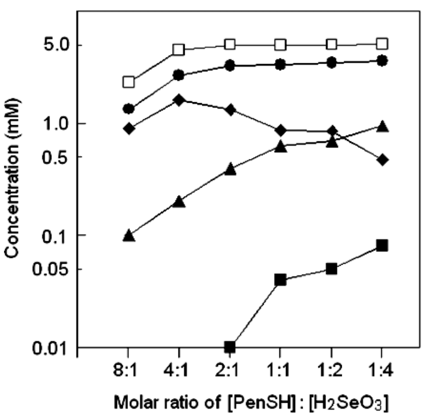

Fig. 3. Yields of PenSH, PenSSPen, PenSSeSPen, $PenSSe_2SPen$, and PenSOSPen in the Reaction between 10 mM PenSH and H_2SeO_3 at Their Various Molar Ratios

□, Sum of PenSSPen, PenSOSPen, PenSSeSPen, and $PenSSe_2SPen$; ●, PenSSeSPen; ▲, PenSOSPen; ◆, PenSSPen; ■, $PenSSe_2SPen$.

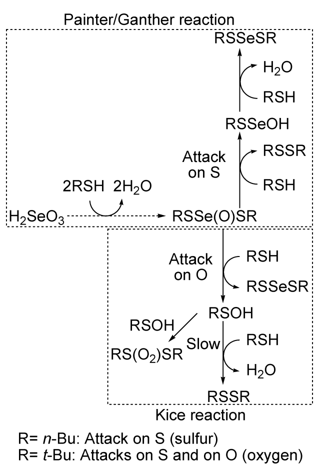

Chart 2

quite slow, and it allows time for much of *t*-BuSOH to undergo another reaction yielding thiosulfonate (*t*-$BuSO_2SBu$-*t*). Therefore, the amount of disulfide (*t*-BuSSBu-*t*) is lower than that of RSSeSR (*t*-BuSSeSBu-*t*).

PenSH has a 1-methylethanethiol group in a manner similar to a *tert*-butyl system such as *t*-BuSH. Therefore, it is believed that the reaction of PenSH with H_2SeO_3 proceeds *via* two attacks on S and on O, and the stoichiometry is similar to the reaction of *t*-BuSH with H_2SeO_3. We now consider how $PenSSe_{2-4}SPen$ are formed in Chart 3. Figure 3 indicates the decreasing yields of PenSSPen and increasing ones of $PenSSe_{1-2}SPen$ with decreasing molar ratios of [PenSH]/[H_2SeO_3]. The yield of PenSSPen at a molar ratio of 4 : 1 (Painter/Ganther reaction) decreased during the changes of molar ratios from 4 : 1 to 1 : 4, whereas that of $PenSSe_{1-2}SPen$ increased during the same changes. Inorganic selenium generally exists as several chemical species such as H_2Se, HSe^-, Se^0, H_2SeO_3, $HSeO_3^-$, SeO_3^{2-}, $HSeO_4^-$, and SeO_4^{2-}, corresponding to different redox potential levels, of which three, H_2Se, H_2SeO_3, and Se^0, are the main components in acidic conditions.[11] H_2Se may allow the following two reactions: i) reduction of PenSSPen into 2 PenSH, which then reacts with H_2SeO_3 again; this reaction results in a decrease in PenSSPen and increase in PenSSeSPen, ii) reduction of sulfenic acid (RSOH) and thioselenic acid (RSSeOH) by the Painter/Ganther reaction and Kice reaction, respectively; this reaction would lead to the formation of selenopersulfides ($PenSSe_{1-2}H$), which are key intermediates in the formation of $PenSSe_{2-4}SPen$. $PenSSe_{1-2}H$ could react with PenSSe(O)SPen, PenSOH, and $PenSSe_{1-2}OH$ in competition with PenSH and produce $PenSSe_{1-4}SPen$, as shown in Chart 3.

We have discovered unique polynuclear selenium-containing compounds, $PenSSe_{2-4}SPen$, in the reaction of PenSH with H_2SeO_3. We will be reporting their beneficial effects such as protection from oxidative DNA damage and inhibition of cancer cell proliferation. Moreover, a series of non-symmetric polyselenodisulfides ($RSSe_{2-4}SR'$) are also being synthesized. Organic selenium compounds such as Se-methylselenocysteine have been reported to be more effective and safer than inorganic selenium in animal models of cancer chemoprevention.[12] Therefore, our efforts are currently focused on chemical/biological studies of a series of diverse polyselenodisulfides aiming at achieving significantly effective chemoprevention and reducing the toxic side effects as well as providing a useful tool for new research on the metabolism of selenium.

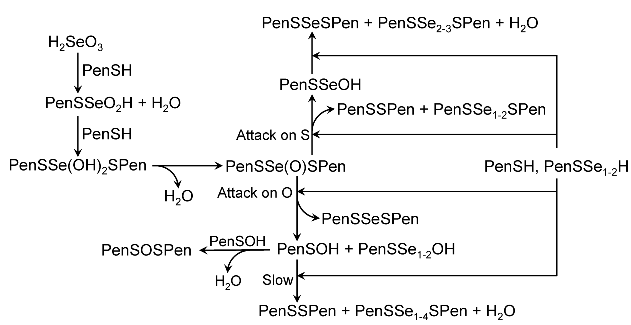

Chart 3

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