Synthetic Study on Selenocystine-Containing Peptides[1)]

Takaki KOIDE,[a] Hiromi ITOH,[a] Akira OTAKA,[a] Hiroyuki YASUI,[a] Masataka KURODA,[a] Nobuyoshi ESAKI,[b] Kenji SODA,[b] and Nobutaka FUJII*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto 606, Japan and Institute for Chemical Research, Kyoto University,[b] Uji, Kyoto 611, Japan. Received September 22, 1992

***N*-9-Fluorenylmethoxycarbonyl-*Se*-4-methoxybenzylselenocysteine [Fmoc–Sec(MBzl)–OH] was synthesized from selenocystine and successfully applied to Fmoc-based solid-phase peptide synthesis. The stability and the deprotection conditions of the *Se*-MBzl group were examined. The diselenide bond of a peptide was directly and effectively established between Sec(MBzl) residues by treatment with iodine or the dimethyl sulfoxide–trifluoroacetic acid system. Reduction kinetics of diselenide and disulfide in model peptides by reduced glutathione were also studied comparatively.**

Keywords selenocystine; selenocysteine; Fmoc-based solid-phase peptide synthesis; *N*-9-fluorenylmethoxycarbonyl-*Se*-methoxybenzylselenocysteine; diselenide bond formation; disulfide bond formation

Selenocysteine is a naturally occurring amino acid that constitutes the catalytic centers of some redox enzymes such as glycine reductase, formate dehydrogenases, and glutathione peroxidase.[2)] Selenocysteine and cysteine have similar structures and resemble each other in chemical behavior under physiological conditions, that is, both amino acids are oxidized to the corresponding dimers (selenocystine and cystine). Selenolates and thiolates can be good nucleophiles and leaving groups. However, the acidity of the selenol group (pK_a 5.73) differs from that of the thiol group (pK_a 8.53).[3)] In addition, the redox potentials of selenols are much lower than those of corresponding thiols.[4)] Therefore, selenocysteine or selenocystine replacement for cysteine or cystine residue(s) in peptides might provide interesting chemical properties and/or biological activities.

There is limited information available on the synthesis of selenocysteine- or selenocystine-containing peptides. For example, the benzyl group, which was cleaved by sodium in liquid ammonia (Na/NH_3 liq.), was introduced for the protection of the selenol group in the synthesis of selenium analogues of glutathione,[5)] oxytocin,[6)] and somatostatin.[7)] However, this method is unsatisfactory for chemical manipulations because the cleavage reaction with Na/NH_3 liq. is accompanied with side reactions. Recently, Soda and his colleagues synthesized metalloselenonein, a selenocysteine analogue of metallothionein, by the Boc-based solid-phase method using *Se*-4-methylbenzylselenocysteine, which is cleaved by HF, as a selenocysteine precursor.[8)] However, detailed synthetic studies on selenocysteine- or selenocystine-containing peptides have not been reported. Development of an efficient synthetic method for such peptides, which is generally applicable in automatic solid-phase peptide synthesis, would be valuable.

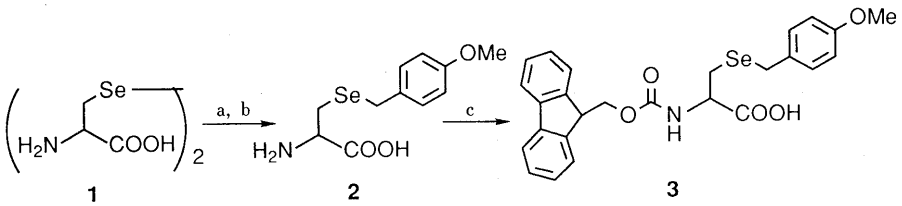
We have synthesized *N*-9-fluorenylmethoxycarbonyl-*Se*-4-methoxybenzylselenocysteine [Fmoc–Sec(MBzl)–OH] and investigated the stability and the deprotecting conditions of the side chain protecting group. Fmoc–Sec(MBzl)–OH was successfully applied to the standard Fmoc-based solid-phase peptide synthesis. Diselenide bond-forming reactions between Sec(MBzl) residues were studied using a model pentapeptide. To obtain information about the properties of selenocystine-containing peptides, reduction kinetics of diselenide and disulfide in model peptides were also studied comparatively.

Results and Discussion

Preparation of Fmoc–Sec(MBzl)–OH and Properties of the *Se*-MBzl Group For protection of the selenol group we chose the 4-methoxybenzyl group which was expected to be cleavable by acid, by analogy with the *S*-4-methoxybenzyl group.[9)]

L-Selenocystine (**1**) was prepared from β-L-chloroalanine and disodium diselenide as described in the literature.[10)] Selenocystine (**1**) was reduced to selenocysteine with sodium borohydride in an aqueous solution of NaOH. *Se*-4-Methoxybenzylation was successively performed without isolation of selenocysteine because free selenols are highly oxidizable under aerobic conditions. Fmoc–Sec(MBzl)–OH (**3**) was easily prepared from the amino acid **2** by using Fmoc–OSu.

On amino acid analysis, selenocystine (**1**) and H–Sec(MBzl)–OH (**2**) were eluted at the same retention times



a, $NaBH_4$; b, MBzl-Cl, 74%; c, Fmoc-OSu, 89%

Chart 1. Preparation of Fmoc–Sec(MBzl)–OH

© 1993 Pharmaceutical Society of Japan

NII-Electronic Library Service

TABLE I. Stability of the *Se*-MBzl Group

Reagents	Temperature[a]	Time (h)	Stability
TFA–water (9 : 1)	r.t.	2	Stable
TFA–thioanisole–*m*-cresol–EDT–water (80 : 5 : 5 : 5 : 5)	r.t.	2	Stable
20% piperidine–DMF	r.t.	4	Stable
1 M TMSBr–thioanisole–*m*-cresol–TFA	4 °C	1	Cleavable
1 M TMSOTf–thioanisole–*m*-cresol–TFA	4 °C	1	Cleavable

a) r.t., room temperature.

as those of valine (44.9 min) and arginine (92.6 min), respectively.

The stability and deprotecting conditions of the *Se*-MBzl group under various conditions, which are generally used in peptide synthesis, were examined by amino acid analysis. Here we noted that most of the selenocysteine generated by deprotection of the *Se*-MBzl group was oxidized to selenocystine during amino acid analysis. As shown in Table I, the *Se*-MBzl group was stable toward 90% (v/v) TFA aq., TFA–thioanisole–*m*-cresol–EDT–water (80 : 5 : 5 : 5 : 5, v/v), and 20% (v/v) piperidine–DMF, but cleavable with the TMSBr–*m*-cresol–thioanisole–TFA[11] or the TMSOTf–*m*-cresol–thioanisole–TFA[12] system.

Solid-Phase Synthesis of Model Pentapeptides To investigate the applicability of Fmoc–Sec(MBzl)–OH to solid-phase peptide synthesis, the model pentapeptide H–Leu–Lys–Gly–Sec(MBzl)–Ala–OH : P–Se(MBzl) was synthesized. For a comparative study, a peptide in which the Sec(MBzl) residue is replaced by Cys(MBzl): P–S(MBzl), was also synthesized.

Protected peptidyl–resins for both model peptides were constructed manually using the Fmoc-based solid-phase method[13] on Wang-type polystyrene supports[14] using diisopropylcarbodiimide–HOBt coupling. In all cases, the ninhydrin tests were negative after a single coupling. Cleavage of the peptides from the resin and simultaneous deprotection of the Boc group of the Lys residue were performed with TFA–*m*-cresol–thioanisole–EDT–water (80 : 5 : 5 : 5 : 5, v/v). The crude products of both P–Se(MBzl) and P–S(MBzl) were relatively pure on HPLC analysis (>90%). These peptides were purified by HPLC and characterized by FAB-mass spectrometry. These results revealed that no significant side reactions involving the selenium atom occurred during the coupling step.

Diselenide Bond Formation between Sec(MBzl) Groups Next, to develop efficient synthetic methods for obtaining diselenide-containing peptides as analogues of disulfide-containing peptides, various reagent systems currently used in disulfide bond formation, such as iodine,[15] $Tl(TFA)_3$,[16] DMSO–TFA,[17] and DMSO–TMSCl–TFA[18] were tested for diselenide bond formation of the model peptide. Here we aimed to form the diselenide bond directly between the protected selenocysteine residues, because free selenol is generally difficult to handle due to its susceptibility of air oxidation.

The efficiency of diselenide bond formation was evaluated by quantitation of the dimerization yield of the model peptide P–SeSe–P. Parallel experiments using Cys(MBzl) peptide P–S(MBzl) were also done for comparison (Chart 2). Yields of the diselenide and disulfide bond-forming reaction were determined by using analytical HPLC, by comparing the peak areas of the dimeric product in the reaction mixture to that of the standard dimer of known concentration.

H–Leu–Lys–Gly–X–Ala–OH P–Se(MBzl): X = Sec(MBzl)
P–S(MBzl): X = Cys(MBzl)
H–Leu–Lys–Gly–X–Ala–OH P–SeSe–P: X = Sec
H–Leu–Lys–Gly–X–Ala–OH P–SS–P: X = Cys

Chart 2. Structures of Model Peptides; P–Se(MBzl), P–S(MBzl), P–SeSe–P, and P–SS–P

TABLE II. Diselenide and Disulfide Bond Formation by Various Reagents

Reagents	Temperature[a]	Time (min)	Yield of dimer (%)	
			P–SeSe–P	P–SS–P
I_2 (10 eq, in 80% AcOH)	r.t.	15	98	0
I_2 (10 eq, in 50% MeOH)	r.t.	15	101	0
$Tl(TFA)_3$ (2 eq, in TFA)	4 °C	2	50	69
10% (v/v) DMSO–TFA	r.t.	15	94	102
0.2 M DMSO–1 M TMSCl–TFA	4 °C	2	82	88

a) r.t., room temperature.

As shown in Table II, the diselenide bond was quantitatively established between the Sec(MBzl) residues by iodine. It is interesting that iodine, which does not affect the *S*-MBzl group at all, is the most useful reagent for diselenide bond formation. This selectivity could be due to the high nucleophilicity of the selenium atom compared to that of the sulfur atom. This result suggests the possibility of selective formation of diselenide and disulfide bridges in peptides containing Sec(MBzl) and Cys(MBzl).

The diselenide bond as well as disulfide bond was effectively established by treatment with the DMSO–TFA system. On the other hand, when using $Tl(TFA)_3$, a by-product which elutes faster than the desired dimeric product was observed on analytical HPLC. Unfortunately, the instability of this by-product prevented us from identifying it. However, we assume that this is an over-oxidized product, because prolonged treatment with the 0.2 M DMSO–1 M TMSCl–TFA system afforded the same by-product.

Study of Reduction Kinetics of Diselenide and Disulfide by GSH Thiol–disulfide exchanges involving glutathione, coenzyme A, and other cysteine-containing proteins are important reactions in regulating intracellular redox systems.[19] The catalytic activities of selenocysteine-containing enzymes can be expressed by the selenol group of the selonocystine residue, which is thought to act as a nucleophile and a leaving group.[20] Concerning these physiological redox reactions, basic kinetic studies of the thiol–disulfide and selenol–diselenide exchange reactions using low-molecular-weight compounds were reported.[21]

To obtain basic information on the chemical behavior of selenocystine-containing peptides in aqueous solvents, we compared the reactivity of diselenide and disulfide to GSH using the model peptides.

The model peptide of dimer form (P–SeSe–P, or P–SS–P,

NII-Electronic Library Service

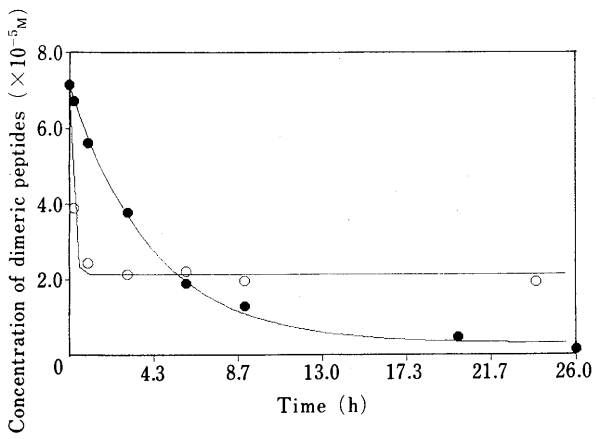


Fig. 1. Reduction Time Course of P–SeSe–P (○) and P–SS–P (●) with GSH

7.14×10^{-5} M each) was incubated in a buffer of pH 7.5 with GSH (1.00×10^{-1} M) at 37 °C under anaerobic conditions. The concentrations of the dimeric peptides remaining in the reaction mixtures were periodically determined by measuring the peak areas on analytical HPLC. During the reduction reaction only peaks of fully reduced peptide (P–SeH or P–SH) and dimeric peptide were detected, and no peaks of mixed selenenylsulfide or disulfide, that would be generated as an intermediate in the reducing reaction, were detected. Therefore this reaction can be simply described as in Eq. 1.

As shown in Fig. 1, at the beginning of the reaction, P–SeSe–P was reduced to the corresponding selenol, P–SeH, at a high rate, and the reaction reached equilibrium within one hour, whereas the concentration of the disulfide-peptide, P–SS–P, decreased gradually over the reaction period.

The observed rate constants of the reactions, k_1(obsd.) and k_{-1}(obsd.), were estimated from curve fitting based on the third-order rate equation (Eq. 2) using the nonlinear least-squares regression program MULTI (RUNGE)[22] with the Runge–Kutta–Gill method. The values of the rate constants thus determined were as follows; when X is Se, the values of $k_1(\text{obsd.})^{Se}$ and $k_{-1}(\text{obsd.})^{Se}$ are 8.8×10^{-2} ($\text{M}^{-2}\,\text{s}^{-1}$) and 3.8×10^{4} ($\text{M}^{-2}\,\text{s}^{-1}$), and when X is S, those of $k_1(\text{obsd.})^{S}$ and $k_{-1}(\text{obsd.})^{S}$ are 6.1×10^{-3} ($\text{M}^{-2}\,\text{s}^{-1}$) and 1.5×10^{2} ($\text{M}^{-2}\,\text{s}^{-1}$), respectively.

The equilibrium constants of Eq. 3 were also calculated from the ratio of k_1(obsd.) to k_{-1}(obsd.). The values of K_{eq}^{Se} and K_{eq}^{S} are 2.3×10^{-6} and 4.1×10^{-5}, respectively.

$$\text{P–XX–P} + 2\text{GSH} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2\text{P–XH} + \text{GSSG} \qquad (\text{X} = \text{Se, S}) \tag{1}$$

$$\frac{d[\text{P–XX–P}]}{dt} = -k_1[\text{P–XX–P}][\text{GSH}]^2 + k_{-1}[\text{P–XH}]^2[\text{GSSG}]$$
$$= -k_1[\text{P–XX–P}]\{0.1 - 2(7.14 \times 10^{-5} - [\text{PXXP}])\}^2$$
$$+ 4k_{-1}(7.14 \times 10^{-5} - [\text{P–XX–P}])^3 \tag{2}$$

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{[\text{P–XH}]^2[\text{GSSG}]}{[\text{P–XX–P}][\text{GSH}]^2} \tag{3}$$

These results indicated that the kinetic behavior of the selenocystine-containing peptide is quite different from that of the cystine-containing peptide in this system. The rate constant for P–SeSe–P is much larger than that of P–SS–P; $k_1(\text{obsd.})^{Se}/k_1(\text{obsd.})^{S} = 14.4$ and $k_{-1}(\text{obsd.})^{Se}/k_{-1}(\text{obsd.})^{S} = 253$. The higher reactivity of diselenide might be accounted for by the following factors; first, the energy of the Se–Se bond (46 kcal/mol) is smaller than that of the S–S bond (64 kcal/mol),[23] and second, selenolate is a good leaving group compared to the corresponding thiolate.[24] The much larger value of $k_{-1}(\text{obsd.})^{Se}$ would result from the lower dissociation constant of the selenol group; at pH 7.5, most selenol exists in the form of the selenolate anion, which possesses high nucleophilicity.

Conclusion

In this study, we have developed a general method for the Fmoc-based solid-phase synthesis of a selenocysteine-or a selenocystine-containing peptide using Fmoc–Sec(MBzl)–OH, a new precursor of selenocysteine. The character of the *Se*-MBzl group was found to be very similar to that of the *S*-MBzl group; both are cleaved by either the 1 M TMSBr–thioanisole–*m*-cresol–TFA or the 1 M TMSOTf–thioanisole–*m*-cresol–TFA system. The diselenide and disulfide bonds are both efficiently established directly from *Se*-MBzl and *S*-MBzl, by the DMSO–TFA system. In contrast, a clear selectivity in diselenide and disulfide bond formation was found when iodine was used as an oxidant.

The data obtained from the above exchange experiments with GSH indicate that the diselenide bond in a peptide is more reactive to thiols than disulfide, though generated selenol has a stronger reducing ability than thiol and is itself apt to be reoxidized to diselenide. Consequently, under equilibrium conditions, the proportion of diselenide to selenol is large compared with that of disulfide. In some biologically active peptides, such as insulin,[25] insulin-like growth factor,[26] and endothelin,[27] thiol–disulfide interchanges between the cystine residue of ligands and the thiol group of their receptors are assumed to trigger the expressions of their bioactions. If this assumption is valid, replacement of the reactive cystine residue by selenocystine might provide the peptides with altered biological activity. In this context, application of the present new method to the synthesis of biologically active peptides containing selenocystine is under investigation.

Experimental

General Melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. TLC was performed on silica gel (Kieselgel 60G, Merck) and the *Rf* value refers to the following solvent system: $CHCl_3$–MeOH–water (8 : 3 : 1). Optical rotations were measured with the JASCO DIP-360 digital polarimeter. The ^{1}H-NMR spectra were obtained on a Brucker AC-300 spectrometer. FAB-mass spectra were recorded on a VG analytical ZAB-SE instrument. Amino acid analysis was performed with a Hitachi 835 amino acid analyzer. HPLC was performed on the Waters single pump system equipped with the 600E system controller, the 484 tunable absorbance detector and the Hitachi D-2500 chromato-integrator. The solvents for HPLC were water and acetonitrile, both containing 0.1% (v/v) TFA, and both were degassed before use. For analytical HPLC, Cosmosil C18-AR (5 μm particle size, 0.46 × 15 cm) was used at a flow rate of 1 ml/min. Preparative HPLC was performed on Cosmosil C18-AR (5 μm particle size, 2.0 × 25 cm) or YMC Pack D-ODS-5 (5 μm particle size, 2.0 × 25 cm).

H–Sec(MBzl)–OH (2) Selenocystine (**1**) was prepared from β-L-chloroalanine and disodium diselenide according to the literature.[10] $NaBH_4$ (1.80 g, 44.9 mmol) was added portionwise to an ice-cooled solution of **1** (1.90 g, 5.69 mmol) in 0.5 N NaOH (5 ml), and the mixture was stirred at room temperature until the yellow color disappeared. The mixture was cooled in an ice bath and 2 N NaOH (15 ml) was added, followed by

NII-Electronic Library Service

dropwise addition of MBzl–Cl (4.06 ml, 29.9 mmol). After vigorous stirring (4 °C, 4 h), the mixture was acidified with concentrated HCl to form a precipitate of crude **2**. The product was collected by filtration, washed with ether, and recrystallized from hot water; yield 2.44 g (74%), mp 173—176 °C (dec.), $[\alpha]_D^{26}$ +23.5° (c=0.22, 1 N HCl). TLC: *Rf* 0.26. ^{1}H-NMR (0.1 N DCl–D_2O) δ: 2.99—3.15 (m, 2H), 3.86 (s, 3H), 3.94 (s, 2H), 4.16—4.20 (q, 1H), 7.00—7.04 (m, 2H), 7.36—7.40 (m, 2H). *Anal.* Calcd for $C_{11}H_{15}NO_3Se$: C, 45.84; H, 5.25; N, 4.86. Found: C, 45.98; H, 5.40; N, 4.82.

Fmoc–Sec(MBzl)–OH (3) A solution of Fmoc–OSu (2.24 g, 6.65 mmol) in CH_3CN (7 ml) was added to an ice-cooled suspension of **2** (2.00 g, 6.93 mmol) in a mixture of water (12 ml) and TEA (0.967 ml, 6.93 mmol). After the addition of TEA (0.967 ml, 6.93 mmol), the mixture was stirred at room temperature for 1 h. The solution was acidified with 1 N HCl and extracted with ethyl acetate. The organic phase was washed successively with 1 N HCl and water saturated with NaCl, and then dried over $MgSO_4$. After removal of the solvent by evaporation, *n*-hexane was added to give the title compound **3** as a white powder; yield 3.01 g (89%), mp 138—140 °C (dec.), $[\alpha]_D^{25}$ −33.1° (*c*=0.87, DMF). TLC: *Rf* 0.67. ^{1}H-NMR ($CDCl_3$) δ: 2.95 (m, 2H), 3.75 (s, 3H), 4.2—4.45 (m, 4H), 4.6 (m, 1H), 5.5 (m, 1H), 6.75—6.84 (m, 2H), 7.13—7.22 (m, 2H), 7.28—7.44 (m, 5H), 7.53—7.61 (m, 2H), 7.70—7.78 (m, 2H). *Anal.* Calcd for $C_{26}H_{25}NO_5Se$: C, 61.18; H, 4.94; N, 2.74. Found: C, 61.46; H, 5.10; N, 2.75.

Stability of the *Se*-MBzl Group The amino acid **2** (20 μmol) and glycine (20 μmol), as an internal standard were treated with 1 ml of the following deprotecting reagent systems; i) TFA–water (9 : 1, v/v) at room temperature for 2 h, ii) TFA–*m*-cresol–EDT–thioanisole–water (80 : 5 : 5 : 5 : 5, v/v) at room temperature for 2 h, iii) *m*-cresol–thioanisole–TFA–TMSBr (50 : 120 : 750 : 132, v/v) at 4 °C for 1 h, iv) *m*-cresol–thioanisole–TFA–TMSOTf (50 : 120 : 690 : 194, v/v) at 4 °C for 1 h, v) 20% (v/v) piperidine–DMF at room temperature for 4 h. After treatment, each reaction mixture was diluted with 10 ml of water and then applied to the amino acid analyzer.

Solid-Phase Synthesis of Model Pentapeptide: P–Se(MBzl) and P–S(MBzl) Protected H–Leu–Lys–Gly–Sec(MBzl)–Ala–OH [P–Se(MBzl)] and H–Leu–Lys–Gly–Cys(MBzl)–Ala–OH [P–S(MBzl)] were prepared manually by Fmoc-based solid-phase peptide synthesis[13] on a *p*-benzyloxybenzyl alcohol polystyrene support.[14]

The syntheses were started on a 0.2 mmol scale on the Fmoc–Ala–resin (Kokusan Chemical Co., 0.45 mmol/g). Removal of Fmoc group was performed with 20% (v/v) piperidine–DMF followed by washing with DMF. For protection of N^{ε} of Lys, the Boc group was used. Each coupling reaction was performed at room temperature for 2 h in DMF using the Fmoc amino acid (2.5 eq) with diisopropylcarbodiimide and HOBt (2.5 eq each). In all of the coupling reactions, the ninhydrin tests were negative after a single coupling.

The peptidyl-resin for P–Se(MBzl) (75 mg) or P–S(MBzl) (65 mg) was treated with TFA–*m*-cresol–EDT–thioanisole–water (80 : 5 : 5 : 5 : 5, v/v, 2.5 ml) at 4 °C for 1 h. The resin was removed by filtration and the TFA was evaporated. Then ice-chilled ether was added to precipitate the product. After centrifugation, the precipitate was dissolved in water (1 ml). The crude product was purified by preparative HPLC, and the solvent was removed by lyophilization to produce a powder; yield P–Se(MBzl); 19 mg (86%); P–S(MBzl); 16 mg (86%). FAB-mass *m/z*: P–Se(MBzl): Found 659.3 $(M+H)^+$; Calcd for $C_{28}H_{47}N_6O_7Se$, P–S(MBzl): Found 611.3 $(M+H)^+$; Calcd for $C_{28}H_{47}N_6O_7S$.

Diselenide or Disulfide-Bond Forming Reactions Partially protected peptide, P–Se(MBzl) or P–S(MBzl) (200 μg), was treated with 200 μl of the following reagent systems; i) iodine (10 eq, in 50% MeOH) at room temperature for 15 min, ii) Tl $(TFA)_3$ (2 eq, in TFA) at 4 °C for 2 min, iii) 10% (v/v) DMSO–TFA at room temperature for 15 min, iv) 0.2 M DMSO–1 M TMSCl–TFA at 4 °C for 2 min. Twenty μl of the reaction mixture was diluted with 980 μl of water and the sample was immediately analyzed by HPLC using a 30 min linear gradient from 7% to 27% acetonitrile in 0.1% aqueous TFA. Retention times of P–SeSe–P and P–SS–P were 19.6 and 18.4 min, respectively. The yields of the dimeric peptides (P–SeSe–P and P–SS–P) were determined by comparing the relative peak areas to those of the standard peptide solution.

The standard solutions for HPLC analysis were prepared as follows: P–Se(MBzl) or P–S(MBzl) (2.1 mg) was treated with 10% (v/v) DMSO–TFA (500 μl) at room temperature and after 15 min, ice-chilled ether was added to form a white powder. The main product was purified by HPLC and lyophilized. The product (P–SeSe–P or P–SS–P) was identified by amino acid analysis after LAP digestion and FAB-mass spectrometry; amino acid ratios in LAP digests (values in parentheses are theoretical): P–SeSe–P: Gly 1.03 (1), Ala 1.00 (1), $(Sec)_2$ 0.52 (0.5), Leu 1.03 (1), Lys 0.97 (1), P–SS–P: Gly 1.22 (1), Ala 1.00 (1), $(Cys)_2$ 0.54 (0.5), Leu 1.16 (1), Lys 1.02 (1). FAB-mass *m/z*: P–SeSe–P: Found 1097.24 $(M+Na)^+$; Calcd for $C_{40}H_{74}N_{12}O_{12}Se_2Na$, P–SS–P: Found 979.43 $(M+H)^+$; Calcd for $C_{40}H_{75}N_{12}O_{12}S_2$.

These purified peptides were dissolved in 0.1% TFA and the concentrations were determined accurately by amino acid analysis after 20-h hydrolysis in 6 N HCl containing 0.1% phenol.

Reduction of Dimeric Peptide (P–SeSe–P or P–SS–P) by GSH In this experiment all solvents were degassed and argon-saturated just before use, and all transfers were accomplished using gas-tight syringes. The reaction was carried out in a 1 ml-vial with a rubber septum under an argon atmosphere. A freshly prepared solution of GSH (11.9 mg, 38.8 μmol) in 50 mM Tris–HCl buffer containing 1 mM EDTA (pH 7.5) was added to 200 μl of a solution of dimeric peptide (P–SeSe–P or P–SS–P, 27.7 μmol each) in the same buffer. The final concentrations of the peptide and GSH were 7.14×10^{-5} M and 1.00×10^{-1} M, respectively. The mixture was vortexed and incubated at 37 °C. At intervals, 60 μl aliquots were withdrawn from the mixture and immediately acidified to pH≈2 with 0.1% TFA to quench the reaction. Then each sample was analyzed by HPLC using a 30 min linear gradient from 3% to 27% acetonitrile in 0.1% aqueous TFA. Retention times of P–SeSe–P, P–SS–P, P–SeH, P–SH were 24.2, 23.0, 19.2, and 18.1 min, respectively. The concentration of the remaining dimeric peptide in the reaction mixture was calculated from the peak areas.

Data shown in Fig. 1 are the means of three experiments. Curve fitting was accomplished using the MULTI(RUNGE) program[22] based on differential equation (Eq. 2).

Acknowledgement We are grateful to Drs. Nobuharu Sigematsu and Hirokazu Tanaka of Fujisawa Pharmaceutical Co., Ltd., for measurements of FAB-mass spectra. We thank Dr. Tadao Oikawa of the Faculty of Engineering, Kansai University, for helpful discussions. We also thank Prof. Toshiro Ibuka of the Faculty of Pharmaceutical Sciences, Kyoto University, for his encouragement.

References and Notes

1) All amino acids used here are of the L-configuration. The following abbreviations are used: Sec=selenocysteine, $(Sec)_2$=selenocystine, Fmoc=9-fluorenylmethoxycarbonyl, Boc=*tert*-butoxycarbonyl, MBzl=4-methoxybenzyl, Fmoc–OSu=9-fluorenylmethyl-*N*-succinimidylcarbonate, DMF=*N*,*N*-dimethylformamide, TFA=trifluoroacetic acid, TMSBr=trimethylsilyl bromide, TMSOTf=trimethylsilyl trifluoromethanesulfonate, TMSCl=trimethylsilyl chloride, HOBt=*N*-hydroxybenzotriazole, EDT=1,2-ethanedithiol, Tl$(TFA)_3$=thallic trifluoroacetate, TEA=triethylamine, GSH=glutathione (reduced form), GSSG=glutathione (oxidized form), LAP=leucine aminopeptidase.
2) T. C. Stadtman, *Annu. Rev. Biochem.*, **49**, 93 (1980).
3) B. Nygård, *Arkiv for Kemi*, **27**, 341 (1967).
4) R. J. P. Williams, "New Trends in Bioinorganic Chemistry," ed. by R. J. P. Williams and J. R. R. F. da Silva, Academic Press, New York, 1978, p. 253.
5) D. Theodoroulos, I. L. Shuwartz, and R. Walter, *Biochemistry*, **6**, 3927 (1967).
6) R. Walter and W. Y. Chan, *J. Am. Chem. Soc.*, **89**, 3892 (1967).
7) B. Hartrodt, K. Neubert, B. Bierwolf, W. Blech, and H. D. Jakubke, *Tetrahedron Lett.*, **21**, 2393 (1980).
8) T. Oikawa, N. Esaki, H. Tanaka, and K. Soda, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 3057 (1991).
9) S. Akabori, S. Sakakibara, Y. Shimonishi, and Y. Nobuhara, *Bull. Chem. Soc. Jpn.*, **37**, 433 (1964).
10) P. Chocat, N. Esaki, H. Tanaka, and K. Soda, *Anal. Biochem.*, **148**, 485 (1985).
11) N. Fujii, A. Otaka, N. Sugiyama, M. Hatano, and H. Yajima, *Chem. Pharm. Bull.*, **35**, 3880 (1987).
12) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funsukosi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc. Chem. Commun.*, **1987**, 274.
13) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125; L. Cameron, M. Medal, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, **1987**, 270.
14) S. S. Wang, *J. Am. Chem. Soc.*, **95**, 1328 (1973).
15) B. Kamber, *Helv. Chim. Acta*, **54**, 927 (1971).

NII-Electronic Library Service

16) N. Fujii, A. Otaka, S. Funakoshi, K. Bessho, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 163; N. Fujii, A. Otaka, S. Funakoshi, K. Bessho, T. Watanabe, and H. Yajima, *Chem. Pharm. Bull.*, **35**, 2339 (1987).
17) A. Otaka, T. Koide, and A. Shide, and N. Fujii, *Tetrahedron Lett.*, **32**, 1223 (1991).
18) T. Koide, A. Otaka, H. Suzuki, and N. Fujii, *Synlett*, **1991**, 345.
19) H. F. Gilbert, "Advances in Enzymology," Vol. 63, ed. by A. Meister, Interscience Publishers, Inc., New York, 1990, p. 69.
20) T. C. Stadtman, *J. Biol. Chem.*, **266**, 16257 (1991).
21) G. M. Whitesides, J. E. Liburn, and R. P. Szajewski, *J. Org. Chem.*, **42**, 332 (1977); R. P. Szajewski and G. M. Whitesides, *J. Am. Chem. Soc.*, **102**, 2011 (1980); J. C. Pleasants, W. Guo, and D. L. Rabenstein, *ibid.*, **111**, 6553 (1989); R. Singh and G.M. Whitesides, *ibid.*, **112**, 1190 (1990); *idem, J. Org. Chem.*, **56**, 6931 (1991); D. A. Keire, E. Strauss, W. Guo, B. Noszal, and D. L. Rabenstein, *ibid.*, **57**, 123 (1992).
22) K. Yamaoka and T. Nakagawa, *J. Pharmacobio-Dyn.*, **6**, 595 (1983).
23) D. A. Jhonson, "Sulfur in Organic and Inorganic Chemistry," ed. by A. Senning, Marcel Dekker, New York, 1972, p. 37.
24) C. J. M. Stirling, *Acc. Chem. Res.*, **12**, 198 (1979).
25) B. D. Morrison, M. L. Swanson, L. J. Sweet, and J. E. Pessin, *J. Biol. Chem.*, **263**, 7806 (1988).
26) P. A. Wilden, J. L. Treadway, B. D. Morrison, and J. E. Pessin, *Biochemistry*, **28**, 9734 (1989).
27) M. J. Spinella, A. B. Malik, J. Everitt, and T. T. Andersen, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 7443 (1991).

NII-Electronic Library Service