Amino Acids and Peptides. XXVII. Synthesis of Phytochelatin-Related Peptides and Examination of Their Heavy Metal-Binding Properties[1,2]

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Phytochelatin (PC)-related peptides were prepared by a conventional solution method and their heavy metal-binding properties were examined. Different from the Cu^{2+} and Cu^{+}-binding properties of metallothionein (MT)-related peptides, the Cu^{2+} and Cu^{+}-binding properties of PC-related peptides were fairly dependent on structure. It is of interest that γ-Glu–Cys–Gly (glutathione) exhibited quite different Cu^{2+} and Cu^{+}-binding properties from those of other PC-related peptides and its binding abilities were comparable to those of MT-related peptides. The Cd^{2+}-binding properties of glutathione were similar to those of Cys, and the Cd^{2+}-binding abilities of PC-related peptides increased in proportion to the increase of γ-Glu–Cys peptide unit.

Keywords phytochelatin; chemical synthesis; heavy metal-binding; structure–binding activity relationship

Recently, heavy metal-binding peptides different from metallothioneins (MTs) have been found in various plants[3] and fission yeast, *Schizosaccharomyces pombe*[4,5] and designated as phytochelatins (PCs) or cadystin. As shown in Fig. 1, these peptides possess the general structure $(\gamma\text{-Glu–Cys})_n\text{–Gly}$ ($n=2$—11, PC_2—PC_{11}) and are homologs of glutathione. Some plants contain homologs of PC_n in which the C-terminal glycine is replaced by β-alanine.[6] They are presumably formed by ribosome-independent enzyme reactions, involving glutathione synthetase and γ-glutamylcysteine dipeptidyl transpeptidase (PC synthetase).[7]

Quite recently, it was reported that *Candida glabrata* could express both MTs and γ-glutamyl peptides and the biosynthesis of each was regulated in a metal-specific manner, namely, Cu could induce MTs and Cd could induce PCs.[8]

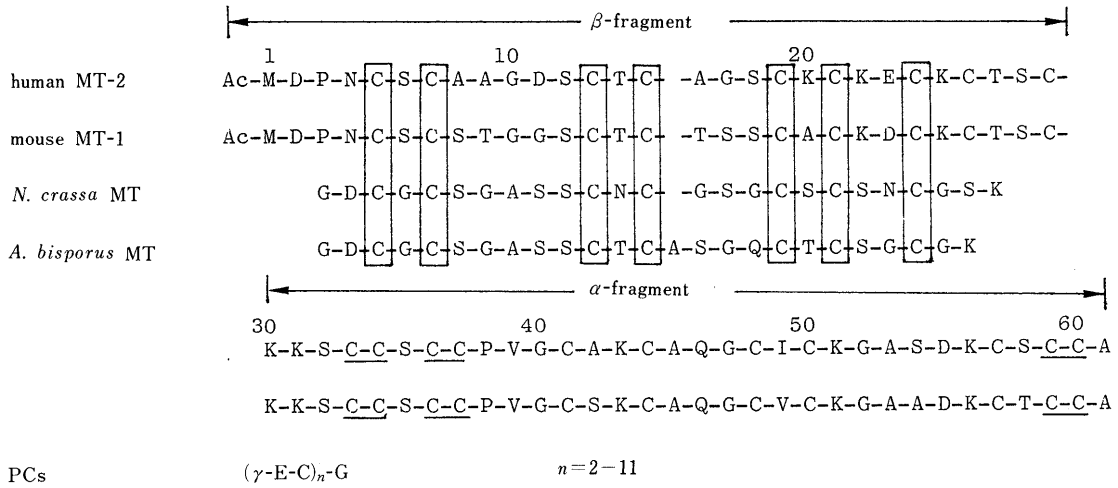

Fig. 1. Primary Structures of MTs and PCs

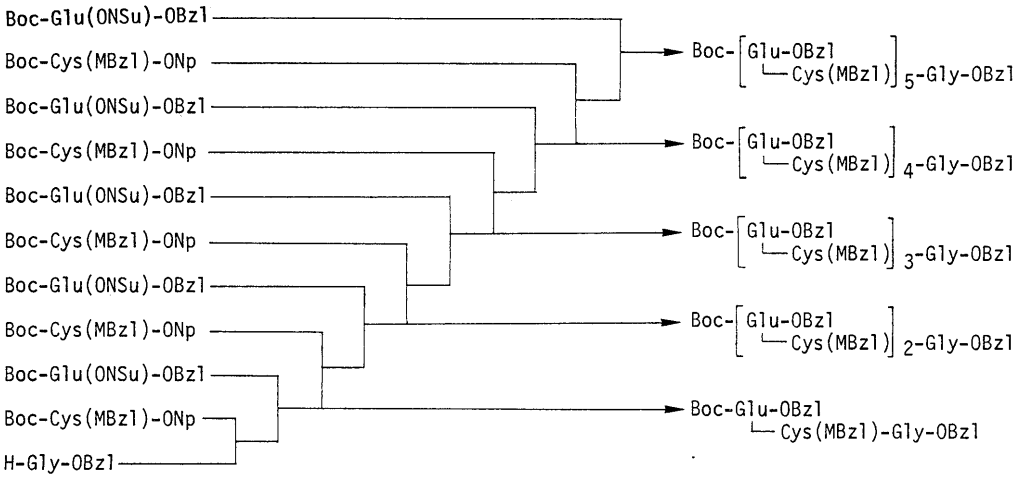

Fig. 2. Synthetic Scheme for Protected PCs

TABLE I. Yields, $[\alpha]_D$ Values, *Rf* Values and Amino Acid Ratios of Deblocked Peptides

Compound	Yield (%)	$[\alpha]_D$ (°) (H_2O, $c=0.2$)	*Rf*[2]	Amino acid ratios of hydrolysate[a] (110 °C, 20 h)	
				Gly	Glu
H–γ-Glu–Cys–Gly–OH (glutathione)	62.9	−21.6	0.89	1.00	1.07
H–(γ-Glu–Cys)$_2$–Gly–OH (PC_2)	69.3	−37.0	0.84	1.00	2.07
H–(γ-Glu–Cys)$_3$–Gly–OH (PC_3)	82.8	−24.9	0.92	1.00	2.97
H–(γ-Glu–Cys)$_4$–Gly–OH (PC_4)	75.7	−37.6	0.85	1.00	3.97
H–(γ-Glu–Cys)$_5$–Gly–OH (PC_5)	70.8	−36.4	0.78	1.00	5.07

a) Cys was not determined.

It has been suggested that PCs are involved in detoxification and metabolism of heavy metals and thus serve functions analogous to those of MTs in animals and fungi, although little is known about the mechanisms by which MTs and PCs might operate. Kondo *et al.*[4,9] synthesized cadystin A and cadystin B (PCs) to confirm their structures. However, the relationship between their structure and heavy metal-binding activity remains to be clarified.

Under these circumstances, our studies were directed to systematic synthesis of PC-related peptides and examination of their heavy metal-binding properties in order to obtain a clue to clarify the functions of PCs.

As shown in Fig. 2, protected PC-related peptides were prepared systematically in a stepwise manner. Amino acid derivatives bearing protecting groups removable by treatment with HF at 0 °C for 60 min,[10] *i.e.*, Cys(MBzl), Glu–OBzl and Gly–OBzl, were employed in combination with the TFA-labile Boc-group as an N^{α}-protecting group.

Starting with H–Gly–OBzl, Boc–Cys(MBzl)–ONp and Boc–Glu(ONSu)–OBzl were coupled successively to give protected PC_1—PC_5. Each protected peptide was purified before deprotection and the homogeneity of the peptide intermediates was ascertained by thin-layer chromatography (TLC), amino acid analysis and elemental analysis. Deprotection of the protected PC-related peptides was performed by the HF method. During the course of the deprotection reaction, oxygen-free water was used and slightly acidic solvent was employed as an eluant for column chromatography on Sephadex G-15 in order to prevent disulfide bond formation.[11]

The homogeneity of the peptides obtained above was ascertained by TLC, high-performance liquid chromatography (HPLC) and amino acid analysis. The yield, *Rf* values, $[\alpha]_D$ value and the results of amino acid analysis are summarized in Table I.

The SH content of the synthetic PC-related peptides was determined by Ellman's method[12] and the results are summarized in Table II. The SH content values are in good agreement with theoretical values.

The heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding properties of synthetic PC-related peptides were examined. As shown in Fig. 3, addition of Cd^{2+} or Cu^{2+} and Cu^{+} to the apo-PC_4 resulted in ultraviolet (UV) absorptions having a shoulder at 250 or 265 nm, respectively, due to mercaptide formation.[13,14] The intensity of UV absorbance at 250 or 265 nm increases in proportion to the increase of concentration of metals added. It was reported that the increment of the intensity of UV absorption caused by mercaptide formation was proportional to the increment of the amount of metal bound to thiol groups.[13,15]

TABLE II. SH Contents of Synthetic Peptides

Compound	SH content[a] Theoretical	Found
H–γ-Glu–Cys–Gly–OH	1	1.17
H–(γ-Glu–Cys)$_2$–Gly–OH	2	2.02
H–(γ-Glu–Cys)$_3$–Gly–OH	3	2.98
H–(γ-Glu–Cys)$_4$–Gly–OH	4	3.60
H–(γ-Glu–Cys)$_5$–Gly–OH	5	4.73

a) SH content is represented as SH/mol peptide, determined by Ellman's method. Peptide concentration was determined by amino acid analysis of an acid hydrolysate.

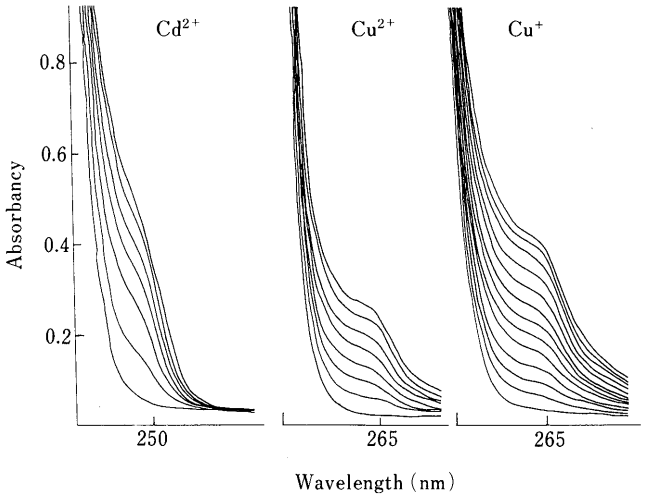

Fig. 3. Binding Study of PC (PC_4) with Heavy Metals

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). The intensity in UV absorbance at 250 nm (Cd^{2+}) or 265 nm (Cu^{2+} and Cu^{+}) increased in proportion to the increase of the concentration of metal added. The concentration of heavy metal was increased as follows (mM): 0, 0.0083, 0.0167, 0.025, 0.0333, 0.0417, 0.050, 0.0583, 0.0667, 0.075, 0.083, 0.0913, 0.0996. In the case of Cd^{2+}, from 0 to 0.050 mM; in the case of Cu^{2+}, from 0 to 0.0583 mM; in the case of Cu^{+}, from 0 to 0.0996 mM.

Therefore, the metal-binding abilities of various peptides were assessed by measuring the increase in absorbance due to mercaptide at 250 or 265 nm as a function of the concentration of Cd^{2+} ($CdCl_2$) or Cu^{2+} ($CuCl_2$) and Cu^{+} [$Cu(CH_3CN)_4ClO_4$],[16] respectively, and the results are illustrated in Fig. 4a, b and c.

The Cu^{+}- and Cu^{2+}-binding properties of PC-related peptides were fairly dependent on their structures. This characteristic is quite different from the metal-binding properties of MTs.[1,17] It is of interest that the Cu^{2+}- and Cu^{+}-binding properties of γ-Glu–Cys–Gly (glutathione) are

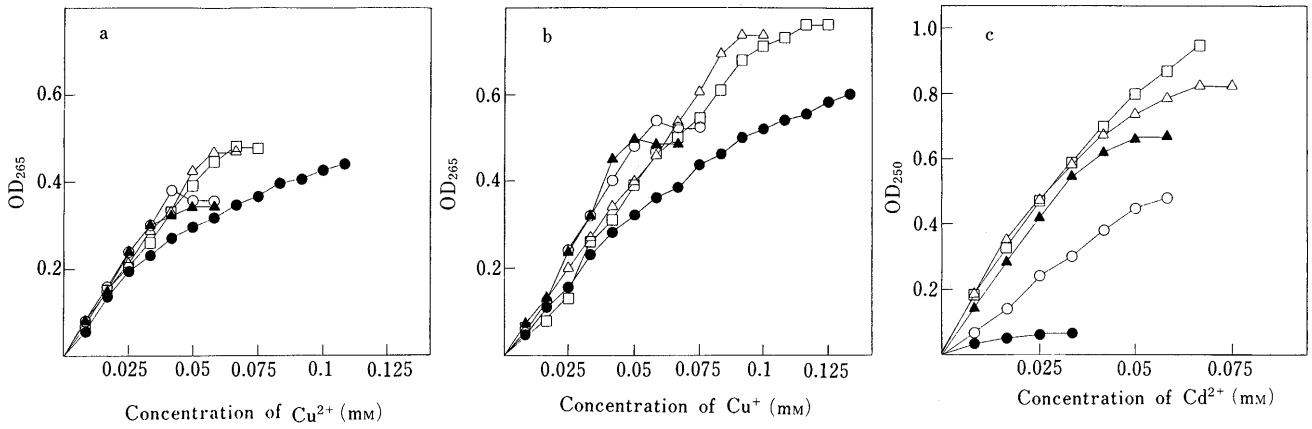

Fig. 4. Binding Properties of Peptides with Heavy Metals, a) with Cu^{2+}, b) with Cu^{+}, c) with Cd^{2+}

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). ●; glutathione, ○; PC_2, ▲: PC_3, △; PC_4, □; PC_5.

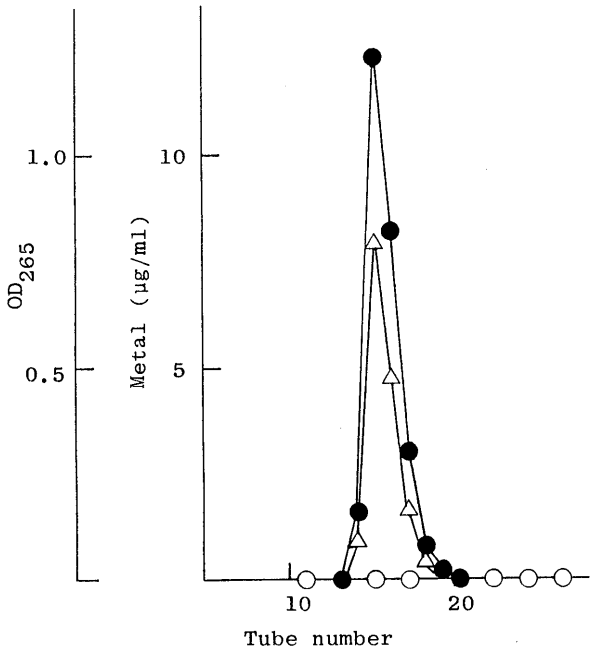

Fig. 5. Gel-Filtration of the Reaction Mixture of PC_5 and Cu^{+} and Cd^{2+} on a Sephadex G-10 Column

Aliquots of the elution fractions were examined for their absorbance at 265 nm (△) and for Cu (●) and Cd (○) concentrations.

different from those of PC_2—PC_5 so far as examined, and the Cu^{2+}- and Cu^{+}-binding abilities of γ-Glu–Cys–Gly (glutathione) are comparable to those of MT-related peptides.

The Cd^{2+}-binding abilities of PC-related peptides increase in proportion to the increase of chain length, as in the cases of fungal MTs.[1,17] The binding abilities of PC_4 and PC_5 were comparable to or greater than those of fungal MTs in spite of their simple structures. The Cd^{2+}-binding ability of glutathione was similar to that of Cys. From these results, it can be deduced that glutathione might play a role in copper metabolism and detoxification of copper.[18] However, for detoxification of Cd, γ-Glu–Cys units are required in addition to glutathione.

As stated above,[8] in *Candida grabrata*, Cu salts could induce MT-like peptides and Cd salts could induce γ-glutamyl peptides, and only Cd–γ-glutamyl peptide and Cu–MT-like peptide were obtained. Therefore, PC (PC_5) was mixed with excess amount of Cu^{+} and Cd^{2+} at the same time and the resultant metal complex was isolated by gel-filtration on Sephadex G-10. As shown in Fig. 5, the eluted material was examined by measuring the Cu and Cd contents by atomic absorption spectrometry and by measuring the absorbance at 265 nm. Only Cu–peptide complex was obtained, and the ratio of Cu^{+} to PC_5 was 4 : 1, suggesting that PC_5 bound with Cu^{+} preferentially and not with Cd^{2+} due to the difference in affinity of Cu^{+} and Cd^{2+} to the peptide.

The results obtained here provide a possible answer to our question as to why different heavy metal-binding proteins, MTs and PCs, exist.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an acid hydrolysate (110 °C, 6 N HCl, 20 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co). Absorption spectra were recorded with a Hitachi 323 recording spectrophotometer. On TLC (Kieselgel G, Merck), Rf^1 and Rf^2 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2) and *n*-BuOH, pyridine, AcOH and H_2O (1 : 1 : 1 : 1), respectively.

Boc-Cys(MBzl)-Gly-OBzl Boc–Cys(MBzl)–ONp (18.4 g, 0.04 mol) and H–Gly–OBzl·Tos–OH (13.4 g, 0.04 mol) were dissolved in DMF (200 ml) containing Et_3N (5.6 ml, 0.04 mol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (15 ml) was applied to a silica gel column (4.0 × 50 cm), equilibrated and eluted with $CHCl_3$. After removal of the solvent of the effluent (1000—2300 ml), petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 17.3 g (88.7%), mp 58—59 °C, $[\alpha]_D^{25}$ −25.9° (c = 1.0, MeOH), Rf^1 0.90. *Anal.* Calcd for $C_{25}H_{32}N_2O_6S$: C, 61.4; H, 6.61; N, 5.73. Found: C, 61.2; H, 6.60; N, 5.70.

Boc-γ-Glu(OBzl)-Cys(MBzl)-Gly-OBzl [Boc-Glutathione-OBzl] Boc–Glu(ONSu)–OBzl (4.4 g, 0.01 mol) and H–Cys(MBzl)–Gly–OBzl·TFA [prepared from Boc–Cys(MBzl)–Gly–OBzl (5.0 g, 0.01 mol), TFA (11.7 ml, 0.15 mol) and anisole (3.3 ml, 0.03 mol) as usual] were dissolved in DMF (150 ml) containing Et_3N (1.4 ml, 0.01 mol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 6.4 g (88.3%), mp 125—127 °C, $[\alpha]_D^{25}$ −30.3° (c = 1.0, DMF), Rf^1 0.73. *Anal.* Calcd for $C_{37}H_{45}N_3O_9S$: C, 62.8; H, 6.41; N, 5.93. Found: C, 62.9; H, 6.65; N, 6.21.

Boc-Cys(MBzl)-γ-Glu(OBzl)-Cys(MBzl)-Gly-OBzl Boc–Cys(MBzl)–

ONp (3.3 g, 7.1 mmol) and H–γ-Glu(OBzl)–Cys(MBzl)–Gly–OBzl·TFA [prepared from Boc–glutathione–OBzl (5.0 g, 7.1 mmol), TFA (8 ml, 100 mmol) and anisole (2.3 ml, 21.3 mmol) as usual] were dissolved in DMF (150 ml) containing Et_3N (1.0 ml, 7.1 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 6.1 g (92.4%), mp 97—99 °C, $[\alpha]_D^{25}$ −33.0° (c=1.0, DMF), Rf^1 0.82. *Anal.* Calcd for $C_{48}H_{58}N_4O_{11}S_2$: C, 61.9; H, 6.28; N, 6.01. Found: C, 62.1; H, 6.47; N, 6.26.

Boc–[γ-Glu(OBzl)–Cys(MBzl)]$_2$–Gly–OBzl Boc–Glu(ONSu)–OBzl (2.6 g, 6.0 mmol) and H–Cys(MBzl)–γ-Glu(OBzl)–Cys(MBzl)–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (5.0 g, 5.4 mmol), TFA (6.0 ml, 80 mmol) and anisole (1.7 ml, 16 mmol) as usual] were dissolved in DMF (100 ml) containing Et_3N (0.9 ml, 6.4 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH and H_2O were added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 5.0 g (81.0%), mp 146—148 °C, $[\alpha]_D^{25}$ −29.3° (c=1.0, DMF), Rf^1 0.80. *Anal.* Calcd for $C_{60}H_{71}N_5O_{14}S\cdot H_2O$: C, 61.7; H, 6.30; N, 6.00. Found: C, 61.5; H, 6.31; N, 6.29.

Boc–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_2$–Gly–OBzl Boc–Cys(MBzl)–ONp (3.8 g, 8.2 mmol) and H–[γ-Glu(OBzl)–Cys(MBzl)]$_2$–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (8.0 g, 7.0 mmol), TFA (16 ml, 210 mmol) and anisole (2.3 ml, 21 mmol) as usual] were dissolved in DMF (150 ml) containing Et_3N (1.3 ml, 9.2 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH and H_2O were added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 8.0 g (78.1%), mp 141—144 °C, $[\alpha]_D^{25}$ −32.7° (c=1.0, DMF), Rf^1 0.66. *Anal.* Calcd for $C_{71}H_{84}N_6O_{16}S_3\cdot H_2O$: C, 61.3; H, 6.23; N, 6.04. Found: C, 61.4; H, 6.18; N, 6.26.

Boc–[γ-Glu(OBzl)–Cys(MBzl)]$_3$–Gly–OBzl Boc–Glu(ONSu)–OBzl (2.2 g, 5.2 mmol) and H–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_2$–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (7.0 g, 4.7 mmol), TFA (8.0 ml, 100 mmol) and anisole (1.5 ml, 14 mmol) as usual] were dissolved in DMF (200 ml) containing Et_3N (0.84 ml, 6.0 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with EtOH, yield 6.6 g (82.3%), mp 154—156 °C, $[\alpha]_D^{25}$ −45.7° (c=1.0, DMF), Rf^1 0.70. *Anal.* Calcd for $C_{83}H_{97}N_7O_{19}S_3$: C, 62.5; H, 6.13; N, 6.15. Found: C, 62.3; H, 6.21; N, 6.24.

Boc–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_3$–Gly–OBzl Boc–Cys(MBzl)–ONp (0.86 g, 1.9 mmol) and H–[γ-Glu(OBzl)–Cys(MBzl)]$_3$–Gly–OBzl [prepared from the corresponding N^{α}-Boc-protected peptide (3.0 g), 1.7 mmol), TFA (3.0 ml, 40 mmol) and anisole (0.52 ml, 5.1 mmol) as usual] were dissolved in DMF (100 ml) containing Et_3N (0.3 ml, 2.1 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with EtOH, yield 2.7 g (80.0%), mp 140—143 °C, $[\alpha]_D^{25}$ −31.0° (c=1.0, DMF), Rf^1 0.65. *Anal.* Calcd for $C_{49}H_{110}N_8O_{21}S_4\cdot H_2O$: C, 61.6; H, 6.05; N, 6.11. Found: C, 61.5; H, 6.11; N, 6.23.

Boc–[γ-Glu(OBzl)–Cys(MBzl)]$_4$–Gly–OBzl Boc–Glu(ONSu)–OBzl (1.0 g, 2.3 mmol) and H–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_3$–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (3.9 g, 2.1 mmol), TFA (2.3 ml, 32 mmol) and anisole (0.7 ml, 6.3 mmol) as usual] were dissolved in DMF (100 ml) containing Et_3N (0.4 ml, 2.8 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 3.8 g (98.4%), mp 160—162 °C, $[\alpha]_D^{25}$ −33.1° (c=1.0, DMF), Rf^1 0.70. *Anal.* Calcd for $C_{106}H_{123}N_9O_{24}S_4\cdot H_2O$: C, 62.0; H, 6.14; N, 6.14. Found: C, 62.2; H, 6.20; N, 6.27.

Boc–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_4$–Gly–OBzl Boc–Cys(MBzl)–ONp (0.14 g, 0.3 mmol) and H–[γ-Glu(OBzl)–Cys(MBzl)]$_4$–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (0.5 g, 0.25 mmol), TFA (0.5 ml, 6.7 mmol) and anisole (0.1 ml, 0.90 mmol) as usual] were dissolved in DMF (30 ml) containing Et_3N (0.05 ml, 0.35 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 0.5 g (90.9%), mp 163—165 °C, $[\alpha]_D^{25}$ −34.7° (c=1.0, DMF), Rf^1 0.66. *Anal.* Calcd for $C_{117}H_{136}N_{10}O_{26}S_5\cdot H_2O$: C, 61.7; H, 6.11; N, 6.15. Found: C, 61.7; H, 6.12; N, 6.17.

Boc–[γ-Glu(OBzl)–Cys(MBzl)]$_5$–Gly–OBzl Boc–Glu(ONSu)–OBzl (0.25 g, 5.8 mmol) and H–Cys(MBzl)–[γ-Glu(OBzl)–Cys(MBzl)]$_4$–Gly–OBzl·TFA [prepared from the corresponding N^{α}-Boc-protected peptide (1.2 g, 0.53 mmol), TFA (1.2 ml, 16 mmol) and anisole (0.2 ml, 1.8 mmol) as usual] were dissolved in DMF (50 ml) containing Et_3N (0.08 ml, 0.58 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 0.95 g (72.3%), mp 166—168 °C, $[\alpha]_D^{25}$ −35.2° (c=1.0, DMF), Rf^1 0.55. *Anal.* Calcd for $C_{129}H_{149}N_{11}O_{29}S_5$: C, 62.5; H, 6.06; N, 6.22. Found: C, 62.3; H, 6.15; N, 6.28.

General Procedure for Deprotection by HF The protected peptide (0.03 mmol) was treated with anhydrous HF (10 ml) containing thioanisole (0.17 ml) and *m*-cresol (0.73 ml) at 0 °C for 1 h. After removal of HF, the residue was dissolved in oxygen-free water. The solution was washed with AcOEt. The water layer was lyophilized to give a fluffy powder. Dithiothreitol (140 mg) was added to a solution of the above powder in H_2O (1.0 ml) and the reaction mixture was stirred at room temperature overnight. The solution was applied to a column of Sephadex G-15 (1.3 × 50 cm), equilibrated and eluted with 3% AcOH. Individual fractions (1.5 g each) were collected. The desired fractions were combined and lyophilized to give a fluffy powder. The yield, $[\alpha]_D^{25}$ value, Rf value and amino acid ratios in an acid hydrolysate are summarized in Table I.

General Procedure for Examination of Binding Ability of Peptides with Cd^{2+}, Cu^{2+} and Cu^{+} A 5—45 μl of aliquot of $CdCl_2$, $CuCl_2$ or $Cu(CH_3CN)_4ClO_4$ solution (5 mM) was added to 5 ml of peptide solution (0.15 mM as SH in 10 mM Tris/HCl buffer, pH 7.0). The UV absorbance at 250 nm (for Cd^{2+}-mercaptide) or 265 nm (for Cu^{2+} or Cu^{+}-mercaptide) of the mixture was determined and the increase was plotted against metal concentration.

Reaction of PC_5 with Cd^{2+} and Cu^{+} All operations were carried out under an N_2 atmosphere using oxygen-free water. PC_5 (1 μmol as SH) was added to 2 ml of water containing 1 μmol of $Cu(CH_3CN)_4ClO_4$ and 1 μmol of $CdCl_2$. The reaction mixture was stored at room temperature for 5 min. This solution was applied to a column of Sephadex G-10 (1.5 × 28 cm), equilibrated and eluted with Tris–HCl buffer (0.1 mM, pH 7.0). Fractions of 2 ml each were collected. The eluted material was examined by measuring the UV absorbance at 265 nm and Cu and Cd content by atomic absorption spectrometry. The chromatographic pattern is shown in Fig. 5.

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References and Notes

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