Amino Acids and Peptides. XXVI. Synthesis of *Agaricus bisporus* Metallothionein and Related Peptides and Examination of Their Heavy Metal-Binding Properties[1,2]

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A pentacosapeptide corresponding to the entire amino acid sequence of *Agaricus bisporus* metallothionein (MT) and related cysteine-containing peptides were prepared by the conventional solution method and their heavy metal-binding properties were examined. The Cu^{2+}- or Cu^{+}-binding activities of various peptides were not greatly dependent on the peptide structure, so far as examined, although the pentacosapeptide, *A. bisporus* MT, exhibited slightly higher binding activity than the other peptides. On the contrary, the Cd^{2+}-binding activities of these peptides were fairly structure-dependent.

Keywords *Agaricus bisporus* metallothionein; related peptide; chemical synthesis; heavy metal-binding property; structure–activity relationship

Metallothioneins (MTs) are a class of low-molecular-weight, cysteine-rich proteins binding with various kinds of heavy metal ions, such as Zn^{2+}, Cu^{+}, Cu^{2+}, Cd^{2+} and Hg^{+}. Their wide occurrence in nature suggests that they have important biological functions. Due to their heavy metal-binding ability, they act as heavy metal (Cd, Hg or Cu)-detoxifying agents[3,4] and participate in heavy metal (Zn or Cu) metabolism, such as the storage function[5,6] and metal transfer to apometalloproteins.[7-10] It was also reported that MTs could trap radicals and alkylating agents,[11,12] providing a protective function against the effects of irradiation and carcinogenesis.[13,14] However, their precise role is not well understood.

Under these circumstances, our studies have been directed to the synthesis of various kinds of MTs and related peptides and examination of their heavy metal-binding and their biological properties in order to obtain a clue to clarify their intrinsic functions.

This report deals with the synthesis of *Agaricus bisporus* MT and related cysteine-containing peptides and examination of their heavy metal-binding properties. *A. bisporus* MT,[15] like *N. crassa* MT,[16] consists of 25 amino acid residues and contains only the Cu atom. Interestingly, the positions of their seven cysteines are homologous to those of the amino terminal region of mammalian MTs, as shown in Fig. 1.

As illustrated in Fig. 2, a protected pentacosapeptide was prepared by the fragment condensation method starting with the C-terminal hexapeptide ester (I). Amino acid derivatives bearing protecting groups removable by treatment with HF at 0 °C for 60 min,[17] *i.e.* Asp(O-2-Ada),[18] Lys(Z) and Cys(MBzl), were employed in combination with the TFA-labile Boc-group as the N^{α}-protecting group.

The synthetic scheme for the C-terminal hexapeptide ester, H–(MT 20—25)–OBzl (I), is illustrated in Fig. 3. Starting with H–Lys(Z)–OBzl, stepwise coupling of Boc–Gly–OH, Boc–Cys(MBzl)–ONp, Boc–Gly–ONp, Boc–Ser–OH and Boc–Cys(MBzl)–ONp was performed to give Boc–(MT 20—25)–OBzl, which was treated with TFA to give peptide (I).

Boc–Gly–Gln–Cys(MBzl)–Thr–$NHNH_2$ (II) was prepared as follows: Boc–Cys(MBzl)–OH and H–Thr–OMe were coupled by the DCC–HOBt method[19] to give Boc–Cys(MBzl)–Thr–OMe, which was treated with TFA to afford H–Cys(MBzl)–Thr–OMe. H–Cys(MBzl)–Thr–OMe was coupled with Boc–Gln–ONp and Boc–Gly–ONp, followed by treatment with hydrazine hydrate to afford peptide (II).

Boc–Ala–ONp and H–Ser–OMe were coupled and the resultant dipeptide was treated with hydrazine hydrate to give Boc–Ala–Ser–$NHNH_2$ (III).

Boc–Cys(MBzl)–Thr–$NHNH_2$, which was prepared from Boc–Cys–(MBzl)–Thr–OMe by hydrazine hydrate treatment, was coupled with H–Cys(MBzl)–OBzl by the azide method to give Boc–Cys(MBzl)–Thr–Cys(MBzl)–OBzl. The

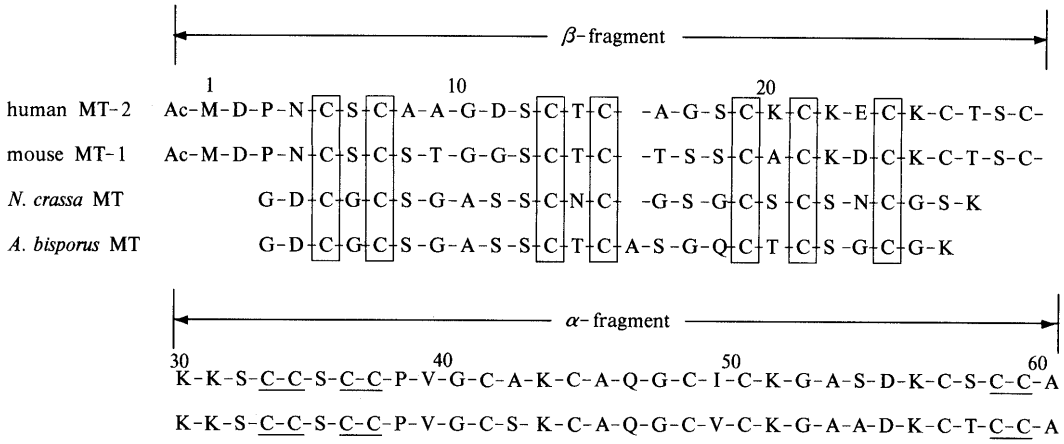

Fig. 1. Primary Structures of Metallothioneins

tripeptide ester was treated with hydrazine hydrate to give the desired hydrazide (IV). The homogeneity of the peptide fragments obtained above was ascertained by thin-layer chromatography (TLC), amino acid analysis and elemental analysis.

According to the scheme shown in Fig. 2, starting with H–(MT 20—25)–OBzl (I), Boc–(MT 16—19)–$NHNH_2$ (II), Boc–(MT 14—15)–$NHNH_2$ (III), Boc–(MT 11—13)–$NHNH_2$ (IV), Boc–(MT 7—10)–$NHNH_2$ (V),[20] Boc–(MT 4—6)–$NHNH_2$ (VI)[20] and Boc–(MT 1—3)–$NHNH_2$[20] were coupled successively by the azide method to afford a protected pentacosapeptide, Boc–(MT 1—25)–OBzl.

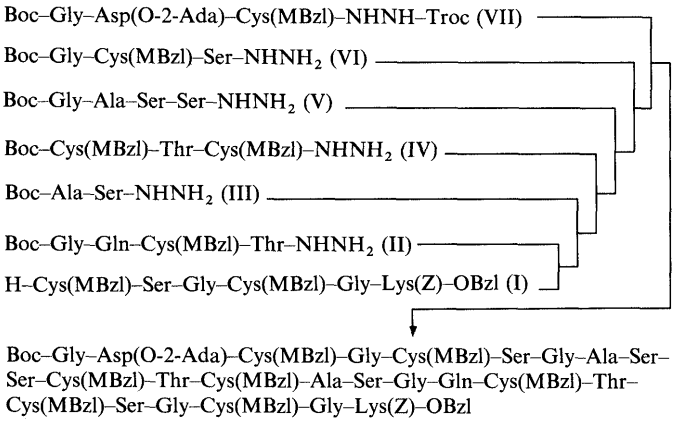

Fig. 2. Synthetic Scheme for *A. bisporus* Metallothionein

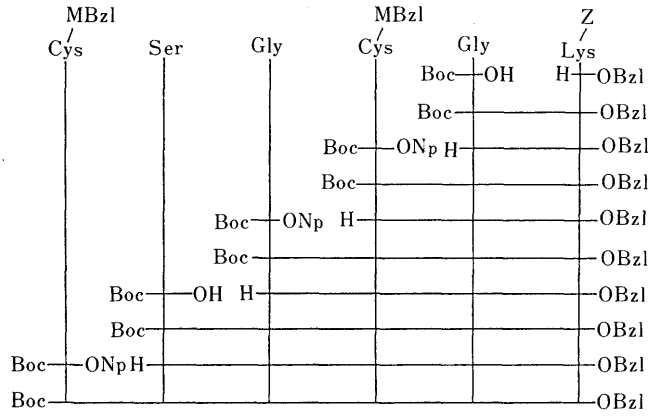

Fig. 3. Synthetic Route to Protected C-Terminal Hexapeptide

The homogeneity of the peptides obtained was ascertained by TLC and amino acid analysis. The yield, *Rf* value, $[\alpha]_D$ value and the results of amino acid analysis are summarized in Table I. The synthetic pentacosapeptide was treated with $HCOOH–H_2O_2$[22] and then hydrolyzed with 6 N HCl and leucine amino peptidase (LAP). The amino acid analyses of those hydrolysates were also peformed. The free SH content of the synthetic *A. bisporus* MT was 6.3/peptide as calculated from the value of the SH content determined by the Ellman method.[23]

The heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding properties of synthetic *A. bisporus* MT and related peptides were examined. $Cu(CH_3CN)_4ClO_4$[24] was employed for Cu^{+}-binding studies because of the stability of the reagent, rather than CuCl as reported previously.[20] As shown in Fig. 4, addition of Cd^{2+} or Cu^{2+} and Cu^{+} to the synthetic

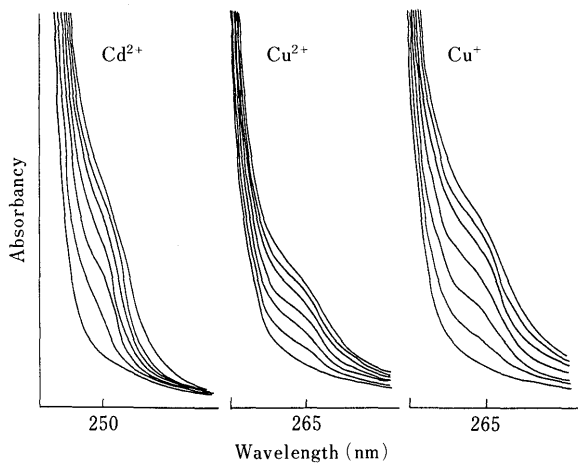

Fig. 4. Binding Study of *A. bisporus* MT with Heavy Metals

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.

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

A. bisporus MT (positions)	Yield (%)	*Rf*[a]	$[\alpha]_D$ (H_2O, $c=0.2$)	Amino acid ratios of hydrolysate[a] (110 °C, 18 h)						
				Gly	Ser	Thr	Glu	Lys	Ala	Asp
H–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (16—25)	79.5	0.61	−41.0°	2.86	0.98	0.96	1.14	1.00		
H–Ala–Ser–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (14—25)	94.9	0.64	−36.3°	2.99	2.07	0.73	1.30	1.23	1.00	
H–Cys–Thr–Cys–Ala–Ser–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (11—25)	95.6	0.80	−41.7°	3.00	1.96	1.38	0.98	0.83	1.06	
H–Gly–Ala–Ser–Ser–Cys–Thr–Cys–Ala–Ser–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (7—25)	85.0	0.84	−45.1°	4.00	3.85	1.37	1.38	1.01	1.06	
H–Gly–Cys–Ser–Gly–Ala–Ser–Ser–Cys–Thr–Cys–Ala–Ser–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (4—25)	88.8	0.89	−48.9°	4.64	4.42	1.42	1.08	1.00	2.22	
H–Gly–Asp–Cys–Gly–Cys–Ser–Gly–Ala–Ser–Ser–Cys–Thr–Cys–Ala–Ser–Gly–Gln–Cys–Thr–Cys–Ser–Gly–Cys–Gly–Lys–OH (1—25)	87.2	0.92	−48.5°[b]	6.11	4.80	1.65	1.20	1.00	2.06	0.84

a) Cys was not determined. b) 3% AcOH, $c=0.2$.

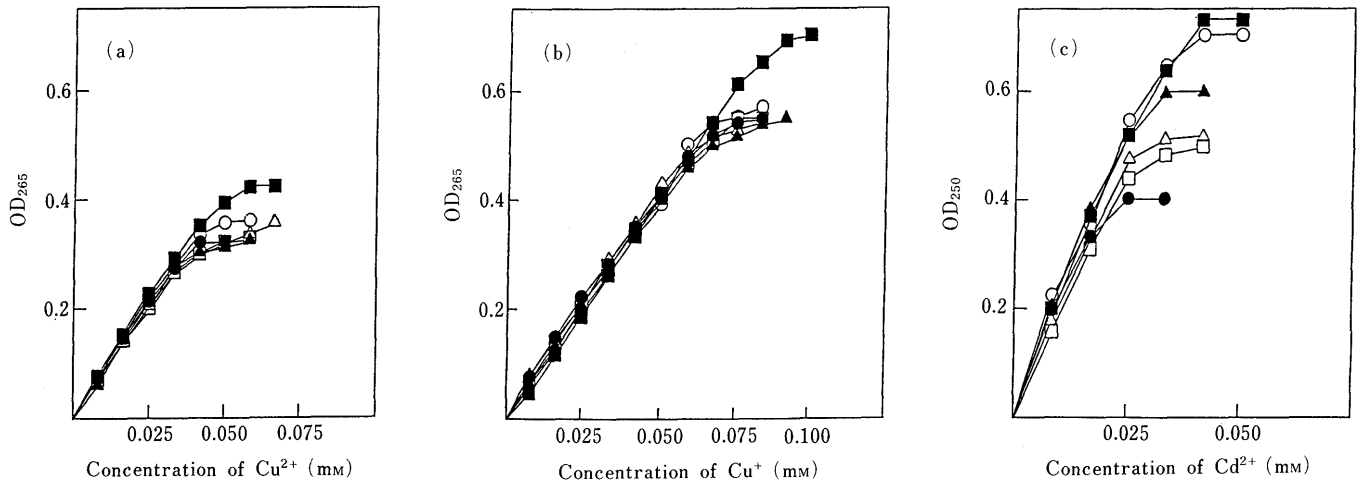

Fig. 5. Binding Properties of Peptides with Heavy Metals, a) with Cu^{2+}, b) with Cu^{+}, c) with Cd^{2+}

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). △, *A. bisporus* MT (16—25); □, (14—25); ●, (11—25); ▲, (7—25); ○, (4—25); ■, (1—25).

apo-MT solution resulted in ultraviolet (UV) absorptions having a shoulder at 250 or 265 nm, respectively, due to mercaptide formation.[25,26] It was also reported that the increment of the intensity of the absorbance due to mercaptide was proportional to the increment of mercaptide formation.[25,27]

Therefore, the metal-binding abilities of various peptides were assessed by measuring the increase in absorbance of mercaptide at 250 or at 265 nm as a function of the concentration of Cd^{2+} or Cu^{2+} and Cu^{+}, respectively, and the results are shown in Fig. 5a, b and c. The Cu^{2+}-binding abilities (Fig. 5a) as well as Cu^{+}-binding abilities (Fig. 5b) of the various peptides are similar to each other, although the pentacosapeptide, corresponding to the entire amino acid sequence of *A. bisporus* MT exhibits a slightly higher binding ability in both cases. It can be seen that there is a difference in the intensity of absorbance between Cu^{2+}- and Cu^{+}-peptides. This discrepancy might be attributed to a difference in the structure of Cu-mercaptide or a difference in binding ability between Cu^{2+} and Cu^{+}. On the contrary, the Cd^{2+}-binding abilities of the peptides (Fig. 5c) are fairly structure-dependent. The Cd^{2+}-binding activity increases in proportion to the increment of peptide chain length from the C-terminus. *A. bisporus* MT (1—25) and (4—25) exhibited higher binding activity than other *A. bisporus* MT-related peptides. These results support our previous findings on the structure-metal-binding activities relationship of *N. crassa* MT and related peptides.[20] The structure-dependency of Cd^{2+}-binding is also compatible with our previous finding that the structure containing Cys–X–Cys–Cys (X: amino acid other than Cys), which exists in the α-fragment of mammalian MTs, is favorable for Cd^{2+}-binding.[28,29]

In order to determine the Cu^{+} content in synthetic *A. bisporus* MT, the Cu^{+}-peptide complex was purified over Sephadex G-10. The desired complex exhibited a symmetrical peak, as shown in Fig. 6. Amino acid analysis of the compound present in this peak after acid hydrolysis gave molar ratios in good agreement with the theoretically expected values for *A. bisporus* MT. The values for Cu content determined by the atomic absorption method and the average recovery of amino acids indicate that the ratio

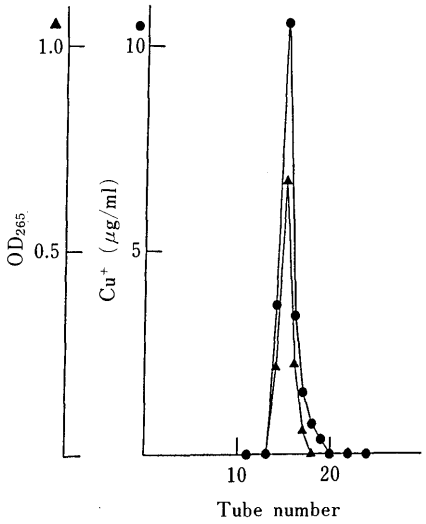

Fig. 6. Gel-Filtration of Reaction Mixture of *A. bisporus* Apo-MT and Cu^{+} on a Sephadex G-10 Column

Aliquots of the eluate fractions were examined for their absorbance at 265 nm and Cu concentration.

of Cu to peptide was 4.4 : 1. The discrepancy between this value and the expected value of 6[15] may be attributed to incomplete saturation of the metal-binding site due to the disulfide formation. The ratio of Cu to peptide in the Cu^{2+}-pentacosapeptide was 2.8 : 1.

In conclusion, Cd-binding activity was fairly structure-dependent in peptides related to *A. bisporus* MT, whereas the Cu-binding activity was not greatly dependent on peptide structure, so far as examined. These results are consistent with our previous reports[20,21,28] and provide us with a possible answer to our question as to why further evolved MTs have an additional C-terminal peptide part (α-fragment).

Experimental

The melting points are uncorrected. Optical rotations were measured with automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an acid hydrolysate (110 °C, 6 N HCl, 20 h; Thr residue was partially destroyed during acid hydrolysis) and enzymatic digest (leucine aminopeptidase, Sigma, lot No. 106F-8050) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co.).

Absorption spectra were recorded with a Hitachi 323 recording spectrometer. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3 and Rf^4 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), $CHCl_3$, MeOH and H_2O (89 : 10 : 1) and *n*-BuOH, pyridine, AcOH and H_2O (1 : 1 : 1 : 1), respectively.

Boc-Cys(MBzl)-Gly-Lys(Z)-OBzl H-Gly-Lys(Z)-OBzl·TFA [prepared from Boc-Gly-Lys(Z)-OBzl[29] (22.6 g), TFA (49.0 ml) and anisole (14.0 ml) as usual] and Et_3N (6.0 ml) were dissolved in DMF (150 ml). Boc-Cys(MBzl)-ONp (19.9 g) was added to the above solution under cooling with ice. The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and H_2O were added and the AcOEt layer was concentrated to a small volume to give crystals, which were collected by filtration and recrystallized from EtOH and AcOEt, yield 29.5 g (91.4%), mp 123—126 °C, $[\alpha]_D^{25}$ −17.3° (c = 1.0, DMF), Rf^3 0.88. *Anal.* Calcd for $C_{39}H_{50}N_4O_9S$: C, 62.4; H, 6.71; N, 7.46. Found: C, 62.3; H, 6.68; N, 7.36.

Boc-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl H-Cys(MBzl)-Gly-Lys(Z)-OBzl·TFA [prepared from Boc-Cys(MBzl)-Gly-Lys(Z)-OBzl (13.0 g), TFA (19.4 ml) and anisole (5.5 ml) as usual] and Et_3N (2.4 ml) were dissolved in DMF (100 ml). Boc-Gly-ONp (6.0 g) was added to the solution under cooling with ice and 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 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 11.2 g (81.7%), mp 111—115 °C, $[\alpha]_D^{25}$ −23.6° (c = 1.0, DMF), Rf^1 0.57, Rf^2 0.84. *Anal.* Calcd for $C_{41}H_{53}N_5O_{10}S$: C, 61.0; H, 6.61; N, 8.67. Found: C, 60.9; H, 6.66; N, 8.71.

Boc-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl H-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl·TFA [prepared from Boc-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl (8.1 g), TFA (11.4 ml) and anisole (3.3 ml) as usual], Boc-Ser-OH (3.1 g) and Et_3N (1.4 ml) were dissolved in DMF (50 ml). DCC (3.1 g) was added to the above solution under cooling with ice, and the reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 5% 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 5.58 g (62.3%), mp 138—140 °C, $[\alpha]_D^{25}$ −21.2° (c = 1.0, DMF), Rf^1 0.31, Rf^2 0.89. *Anal.* Calcd for $C_{44}H_{58}N_6O_{12}S \cdot H_2O$: C, 57.9; H, 6.62; N, 9.20. Found: C, 58.0; H, 6.27; N, 9.06.

Boc-Cys(MBzl)-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 20—25)-OBzl] H-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl·TFA [prepared from Boc-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl (5.0 g), TFA (5.7 ml) and anisole (1.8 ml) as usual], Boc-Cys(MBzl)-ONp (3.1 g) and Et_3N (0.8 ml) were dissolved in DMF (70 ml). 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 and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield, 4.93 g (78.8%), mp 112—116 °C, $[\alpha]_D^{25}$ −18.9° (c = 1.0, DMF), Rf^1 0.39, Rf^2 0.57. *Anal.* Calcd for $C_{55}H_{71}N_7O_{14}S_2 \cdot H_2O$: C, 58.1; H, 6.48; N, 8.63. Found: C, 58.2; H, 6.31; N, 8.65. Amino acid ratios in an acid hydrolysate: $Ser_{0.98}$-$Gly_{2.14}Lys_{1.00}$ (average recovery 86.1%). Cys was not determined.

Boc-Cys(MBzl)-Thr-OMe Boc-Cys(MBzl)-OH (25.0 g), H-Thr-OMe·HCl (12.4 g), HOBt (9.9 g) and Et_3N (10.3 ml) were dissolved in DMF (100 ml). DCC (16.5 g) was added to the above solution under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. The residual oil in $CHCl_3$ (15 ml) was applied to a silica gel column (3.8 × 50 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (1500 ml) and then 2% MeOH in $CHCl_3$ (1000 ml). The solvent of the fractions (1000—2500 ml) was removed by evaporation. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 21.1 g (63.3%), mp 70—72 °C, $[\alpha]_D^{25}$ −33.5° (c = 1.0, DMF), Rf^1 0.47. *Anal.* Calcd for $C_{21}H_{32}N_2O_7S$: C, 55.3; H, 7.07; N, 6.14. Found: C, 55.2; H, 7.07; N, 6.00.

Boc-Gln-Cys(MBzl)-Thr-OMe Boc-Gln-ONp (2.7 g), H-Cys(MBzl)-Thr-OMe·TFA [prepared from Boc-Cys(MBzl)-Thr-OMe (3.0 g), TFA (7.5 ml) and anisole (2.1 ml) as usual] and Et_3N (1.0 ml) were dissolved in DMF (50 ml). 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, 5% 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, 3.0 g (78.3%), mp 143—147 °C, $[\alpha]_D^{25}$ −32.6° (c = 1.0, DMF), Rf^2 0.60. *Anal.* Calcd for $C_{26}H_{40}N_4O_9S$: C, 53.4; H, 6.90; N, 9.58. Found: C, 53.2; H, 6.89; N, 9.48.

Boc-Gly-Gln-Cys(MBzl)-Thr-OMe Boc-Gly-ONp (1.2 g), H-Gln-Cys(MBzl)-Thr-OMe·TFA [prepared from Boc-Gln-Cys(MBzl)-Thr-OMe (1.5 g), TFA (2.9 ml) and anisole (0.8 ml) as usual] and Et_3N (0.4 ml) were dissolved in DMF (50 ml). 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, 5% 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 1.47 g (89.0%), mp 180—184 °C, $[\alpha]_D^{25}$ −45.5° (c = 1.0, DMF), Rf^2 0.53. *Anal.* Calcd for $C_{28}H_{43}N_5O_{10}S$: C, 52.4; H, 6.75; N, 10.9. Found: C, 52.5; H, 6.78; N, 10.9.

Boc-Gly-Gln-Cys(MBzl)-Thr-NHNH$_2$ [Boc-(MT 16—19)-NHNH$_2$] (II) Hydrazine hydrate (98%, 1.0 ml) was added to a solution of Boc-Gly-Gln-Cys(MBzl)-Thr-OMe (4.6 g) in DMF (50 ml). The reaction mixture was kept at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 4.5 g (97.9%), mp 217—219 °C, $[\alpha]_D^{25}$ −32.0° (c = 1.0, DMF), Rf^1 0.26. *Anal.* Calcd for $C_{27}H_{43}N_7O_9S \cdot H_2O$: C, 49.2; H, 6.88; N, 14.9. Found: C, 49.1; H, 6.82; N, 15.2.

Boc-Ala-Ser-NHNH$_2$ [Boc-(MT 14—15)-NHNH$_2$] (III) Boc-Ala-ONp (2.2 g), H-Ser-OMe·HCl (1.0 g) and Et_3N (0.9 ml) were dissolved in DMF (20 ml). 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, 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. The residual oil in $CHCl_3$ (1 ml) was applied to a silica gel column (3 × 18 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (450 ml) and then 2% MeOH in $CHCl_3$ (500 ml). The solvent of the fractions (450—950 ml) was removed by evaporation to leave an oily residue [yield 1.1 g (59.2%), Rf^1 0.55]. Hydrazine hydrate (98%, 0.6 ml) was added to a solution of Boc-Ala-Ser-OMe (1.1 g) in MeOH (20 ml) and the reaction mixture was kept at room temperature for 2 h. The precipitate formed was collected by filtration and washed with MeOH, yield 0.70 g (37.6%), mp 175—181 °C, $[\alpha]_D^{25}$ −8.8° (c = 1.0, DMF), Rf^2 0.34. *Anal.* Calcd for $C_{11}H_{22}N_4O_5$: C, 45.5; H, 7.64; N, 19.3. Found: C, 45.4; H, 7.92; N, 19.2.

Boc-Cys(MBzl)-Thr-NHNH$_2$ Hydrazine hydrate (0.3 ml) was added to a solution of Boc-Cys(MBzl)-Thr-OMe (1.0 g) in MeOH (20 ml). The reaction mixture was kept at room temperature overnight. After removal of the solvent, water was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from EtOH, yield 985 mg (98.5%), mp 114—116 °C, $[\alpha]_D^{25}$ −20.3° (c = 1.0, DMF), Rf^2 0.41. *Anal.* Calcd for $C_{20}H_{32}N_4O_6S$: C, 52.6; H, 7.07; N, 12.3. Found; C, 52.4; H, 7.13; N, 12.1.

Boc-Cys(MBzl)-Thr-Cys(MBzl)-OBzl A solution of Boc-Cys(MBzl)-Thr-N_3 [prepared from Boc-Cys(MBzl)-Thr-NHNH$_2$ (4.0 g) and isopentyl nitrite (1.3 ml) as usual] in DMF (50 ml) was added to a solution of H-Cys(MBzl)-OBzl [prepared from H-Cys(MBzl)-OBzl·Tos-OH (4.4 g) and 10% Na_2CO_3 as usual] in DMF (50 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 2.7 g (41.1%), mp 114—116 °C, $[\alpha]_D^{25}$ −20.8° (c = 1.0, DMF), Rf^2 0.63. *Anal.* Calcd for $C_{38}H_{49}N_3O_9$: C, 60.4; H, 6.53; N, 5.56. Found: C, 60.4; H, 6.47; N, 5.53.

Boc-Cys(MBzl)-Thr-Cys(MBzl)-NHNH$_2$ [Boc-(MT 11—13)-NHNH$_2$] (IV) Hydrazine hydrate (98%, 0.6 ml) was added to a solution of Boc-Cys(MBzl)-Thr-Cys(MBzl)-OBzl (3.0 g) in DMF (70 ml). The reaction mixture was kept at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 2.0 g (66.7%), mp 174—177 °C, $[\alpha]_D^{25}$ −15.7° (c = 1.0, DMF), Rf^2 0.60. *Anal.* Calcd for $C_{31}H_{45}N_5O_8S_2$: C, 54.8; H, 6.67; N, 10.3. Found: C, 54.8; H, 6.65; N, 10.3.

Boc-Gly-Gln-Cys(MBzl)-Thr-Cys(MBzl)-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 16—25)-OBzl] Boc-Gly-Gln-Cys(MBzl)-Thr-N_3 [prepared from the corresponding hydrazide (5.0 g) and isopentyl nitrite (1.1 ml) as usual] in DMF (50 ml) was added to a solution of H-(MT 20—25)-OBzl·TFA [prepared from Boc-(MT 20—25)-OBzl (4.36 g), TFA (4.4 ml) and anisole (1.3 ml) as usual] in DMF (100 ml) containing Et_3N (0.5 ml) under cooling with ice-salt. The reaction mixture was stirred

at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration and washed with MeOH, yield 3.25 g (51.2%), mp 210—215 °C, $[\alpha]_D^{25}$ −21.0° (c=0.2, DMF), Rf^2 0.51. *Anal.* Calcd for $C_{77}H_{102}N_{12}O_{21}S_3 \cdot 3H_2O$: C, 55.5; H, 6.53; N, 10.1. Found: C, 55.6; H, 6.26; N, 10.3.

Boc-Ala-Ser-Gly-Gln-Cys(MBzl)-Thr-Cys(MBzl)-Ser-Gly-Cys-(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 14—25)-OBzl] Boc-Ala-Ser-N_3 [prepared from the corresponding hydrazide (2.2 g) and isopentyl nitrite as usual] in DMF (50 ml) was added to a solution of H-(MT 16—25)-OBzl·TFA [prepared from Boc-(MT 16—25)-OBzl (2.5 g), TFA (4.0 ml) and anisole (0.5 ml) as usual] in DMF (50 ml) containing Et_3N (0.2 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 2.31 g (83.9%), mp 228—231 °C, $[\alpha]_D^{25}$ −6.7° (c=0.1, DMSO), Rf^2 0.76. *Anal.* Calcd for $C_{83}H_{112}N_{14}O_{24}S_3 \cdot H_2O$: C, 55.3; H, 6.37; N, 10.9. Found: C, 55.1; H, 6.52; N, 10.8.

Boc-Cys(MBzl)-Thr-Cys(MBzl)-Ala-Ser-Gly-Gln-Cys(MBzl)-Thr-Cys(MBzl)-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 11—25)-OBzl] Boc-Cys(MBzl)-Thr-Cys(MBzl)-N_3 [prepared from the corresponding hydrazide (1.1 g) and isopentyl nitrite (0.2 ml) as usual] in DMF (30 ml) was added to a solution of H-(MT 14—25)-OBzl·TFA [prepared from Boc-(MT 14—25)-OBzl (1.0 g), TFA (1.2 ml) and anisole (0.2 ml) as usual] in DMF (50 ml) containing Et_3N (0.1 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 1.14 g (87.2%), mp 215 °C (dec.), $[\alpha]_D^{25}$ −16.8° (c=0.1, DMSO), Rf^2 0.68. *Anal.* Calcd for $C_{109}H_{145}N_{17}O_{30}S_5 \cdot 4H_2O$: C, 54.4; H, 6.41; N, 9.90. Found: C, 54.4; H, 6.17; N, 10.3.

Boc-Gly-Ala-Ser-Ser-Cys(MBzl)-Thr-Cys(MBzl)-Ala-Ser-Gly-Gln-Cys(MBzl)-Thr-Cys(MBzl)-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 7—25)-OBzl] Boc-Gly-Ala-Ser-Ser-N_3 [prepared from the corresponding hydrazide[20] (840 mg) and isopentyl nitrite (0.27 ml) as usual] in DMF (20 ml) was added to a solution of H-(MT 11—25)-OBzl·TFA [prepared from Boc-(MT 11—25)-OBzl (900 mg), TFA (0.9 ml) and anisole (0.1 ml) as usual] in DMF (10 ml) and HMPA (10 ml) containing Et_3N (0.05 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, H_2O and MeOH (1 : 1) were added to the residue to afford a precipitate, which was collected by filtration and washed with H_2O, yield 940 mg (92.4%), mp 230 °C (dec.), $[\alpha]_D^{25}$ −27.8° (c=0.1, DMSO), Rf^2 0.72. *Anal.* Calcd for $C_{120}H_{163}N_{21}O_{36}S_5 \cdot 6H_2O$: C, 52.5; H, 6.43; N, 10.7. Found: C, 52.1; H, 6.08; N, 10.9.

Boc-Gly-Cys(MBzl)-Ser-Gly-Ala-Ser-Ser-Cys(MBzl)-Thr-Cys-(MBzl)-Ala-Ser-Gly-Gln-Cys(MBzl)-Thr-Cys(MBzl)-Ser-Gly-Cys-(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 4—25)-OBzl] Boc-Gly-Cys-(MBzl)-Ser-N_3 [prepared from the corresponding hydrazide[20] (660 mg) and isopentyl nitrite (0.2 ml) as usual] in DMF (10 ml) was added to a solution of H-(MT 7—25)-OBzl·TFA [prepared from Boc-(MT 7—25)-OBzl (700 mg), TFA (0.6 ml) and anisole (0.09 ml) as usual] in DMF (10 ml) and HMPA (10 ml) containing Et_3N (0.04 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 670 mg (84.0%), mp 220 °C (dec.), $[\alpha]_D^{25}$ −20.6° (c=0.1, DMSO), Rf^2 0.69. *Anal.* Calcd for $C_{136}H_{184}N_{24}O_{41}S_6$: C, 54.39; H, 6.18; N, 11.2: Found: C, 54.0; H, 6.54; N, 10.9.

Boc-Gly-Asp(O-2-Ada)-Cys(MBzl)-Gly-Cys(MBzl)-Ser-Gly-Ala-Ser-Ser-Cys(MBzl)-Thr-Cys(MBzl)-Ala-Ser-Gly-Gln-Cys(MBzl)-Thr-Cys-(MBzl)-Ser-Gly-Cys(MBzl)-Gly-Lys(Z)-OBzl [Boc-(MT 1—25)-OBzl] Boc-Gly-Asp(O-2-Ada)-Cys(MBzl)-N_3 [prepared from Boc-Gly-Asp-(O-2-Ada)-Cys(MBzl)-$NHNH_2$[20] (220 mg) and isopentyl nitrite (0.05 ml) as usual] in DMF (20 ml) was added to a solution of H-(MT 4—25)-OBzl·TFA [prepared from Boc-(MT 4—25)-OBzl (200 mg), TFA (0.3 ml) and anisole (0.02 ml) as usual] in DMF (15 ml) and HMPA (15 ml) containing Et_3N (0.01 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 170 mg (72.6%), mp 205 °C (dec.), $[\alpha]_D^{25}$ −22.0° (c=0.1, DMSO), Rf^2 0.65. *Anal.* Calcd for $C_{163}H_{219}N_{27}O_{47}S_7$: C, 55.4; H, 6.25; N, 10.7. Found: C, 55.1; H, 6.55; N, 10.4.

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, ether was added to the residue to afford a precipitate, which was collected by filtration. The precipitate in oxygen-free water (15 ml) was treated with Amberlite IRA-45 (acetate form). After removal of the resin, the filtrate was lyophilized to give a white powder, which was reduced with dithiothreitol (140 mg) in oxygen-free water (10 ml) at room temperature for 2 h. The reaction mixture was applied to a Sephadex G-15 column (2.2 × 118 cm) for MT (16—25) and (14—25) or a Sephadex G-25 column (2.2 × 95 cm) for MT (11—25), (7—25), (4—25) and (1—25). These columns were equilibrated and eluted with 3% AcOH. Individual fractions (3 g each) were collected. The desired fractions were combined and lyophilized to give a fluffy powder. Yield, $[\alpha]_D^{25}$ value, amino acid ratios in an acid hydrolysate and Rf values are summarized in Table I.

The synthetic pentacosapeptide (3 mg) was treated with $HCOOH-H_2O_2$ and the resulting cysteic acid derivative was hydrolyzed by 6 N HCl and digested with LAP. Amino acid ratios in the hydrolysate and the LAP digest were in good agreement with the theoretically expected values. Acid hydrolysate: $CySO_3H_{6.84}Asp_{0.83}Thr_{1.71}Ser_{4.26}Glu_{1.11}Gly_{6.15}Ala_{2.18}$-$Lys_{1.00}$ (average recovery 82%). LAP digest: $CySO_3H_{6.76}Thr+Gln_{2.81}$-$Ser_{4.85}Gly_{6.04}Ala_{2.12}Lys_{1.00}$ (average recovery 75.0%).

General Procedure for Examination of Binding Ability of Peptides with Cd^{2+}, Cu^{2+} and Cu^+ A 5—45 μl 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, pH 7.0). The UV absorbance at 250 nm (for Cd-mercaptide) or 265 nm (for Cu-mercaptide) of the mixture was measured and the increase was plotted against metal concentration.

Synthesis of Cu-Peptide Complex All operations were carried out in the same manner as described in the previous paper.[20]

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References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine. Standard abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *idem, ibid.*, **6**, 362 (1967); *idem, ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; OBzl, benzyl ester; O-2-Ada, 2-adamantyl ester; MBzl, *p*-methoxybenzyl; Troc, 2,2,2-trichloroethoxycarbonyl; ONp, *p*-nitrophenyl ester; HOBt, *N*-hydroxybenzotriazole; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOH, acetic acid; AcOEt, ethyl acetate; *n*-BuOH, *n*-butanol; HMPA, hexamethylphosphoramide; TosOH, *p*-toluenesulfonic acid.
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