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Amino Acids and Peptides. XXIV. Synthesis of *Neurospora crassa* Metallothionein and Related Cysteine-Containing 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 *Neurospora crassa* metallothionein and several related cysteine-containing peptides were synthesized by the conventional solution method and their heavy metal-binding properties were examined. The Cu^{2+}- or Cu^{+}-binding properties of the various peptides were similar to each other, whereas the Cd^{2+}-binding properties of these peptides were fairly structure-dependent.

Keywords *Neurospora crassa* 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^{2+}, Cu^{+}, Cd^{2+} and Hg^{+}. These proteins have been obtained from a large variety of organisms.[3)] Their wide occurrence in nature suggests that they have important biological functions. Due to their metal-binding ability, they act as heavy metal (Cd, Hg)-detoxifying agents[4,5)] and participate in heavy metal (Zn, Cu) metabolism, such as the storage function[6,7)] and metal transfer to apometalloproteins.[8—11)] It was also reported that MTs could trap radicals, providing a protective function against the effects of irradiation and against carcinogenesis.[12,13)] However, their precise role is not yet 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 other properties in order to clarify their functions.

This report deals with the synthesis of *Neurospora crassa* MT and related peptides and examination of their metal-binding properties. *N. crassa* MT consists of 25 amino acid residues and contains only the Cu atom.[14)] Interestingly, the amino acid sequence is strikingly similar to that of the amino-terminal region of the Cd/Zn MTs from mammals, as shown in Fig. 1.

As illustrated in Fig. 2, a protected pentacosapeptide was prepared by the fragment condensation method. A key intermediate, the protected C-terminal pentadecapeptide, Boc–(MT 11—25)–OBzl, was synthesized *via* two different routes as shown in Fig. 2. One involves azide couplings[15)] of the C-terminal pentapeptide with two kinds of pentapeptides. The other involves successive azide couplings of the C-terminal pentapeptide with relatively small peptides. These relatively small acylating agents, used in excess, could be readily removed by washing with EtOH or MeOH. Amino acid derivatives bearing protecting groups removable by treatment with HF at 0 °C for 60 min,[16)] or methanesulfonic acid (MSA) at 20 °C for 30 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 pentapeptide ester, Boc–(MT 21—25)–OBzl (**1**), is illustrated in Fig. 3. H–Lys(Z)–OBzl was coupled with Boc–Cys(MBzl)–Gly–Ser–N_2H_3, prepared by the coupling of H–Gly–Ser–OMe[19)] with Boc–Cys(MBzl)–ONp, followed by treatment with hydrazine hydrate, to give Boc–(MT 22—25)–OBzl. After removal of the Boc group by TFA, the resulting amine was coupled with Boc–Asn–ONp to afford the desired pentapeptide. The fragment, Boc–(MT 16—20)–N_2H_3 (**2**), was synthesized by the route shown in Fig. 4. The azide coupling of Boc–Cys(MBzl)–Ser–N_2H_3[20)] with H–Cys(MBzl)–Ser–OBzl[20)] gave Boc–(MT 17—20)–OBzl, which was converted to the corresponding amine by treat-

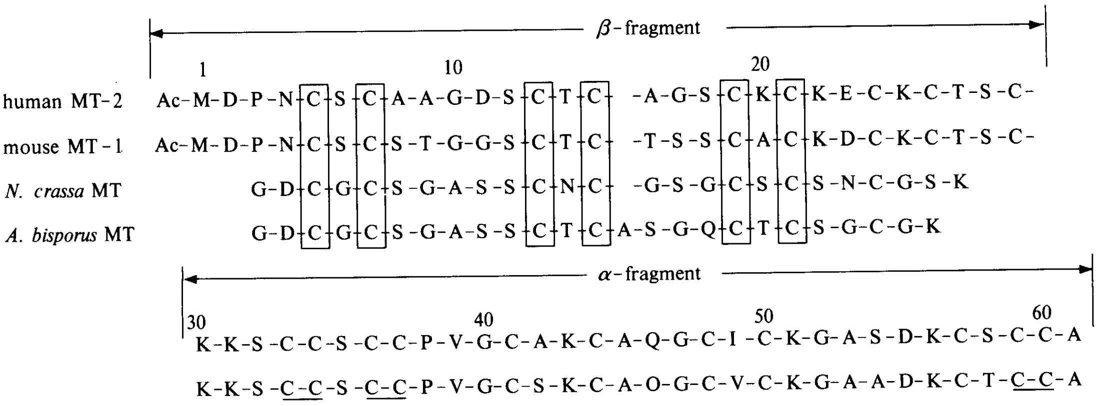

Fig. 1. Primary Structures of MTs

This paper is dedicated to Professor Haruaki Yajima on the occasion of his retirement from Kyoto University in March, 1989.

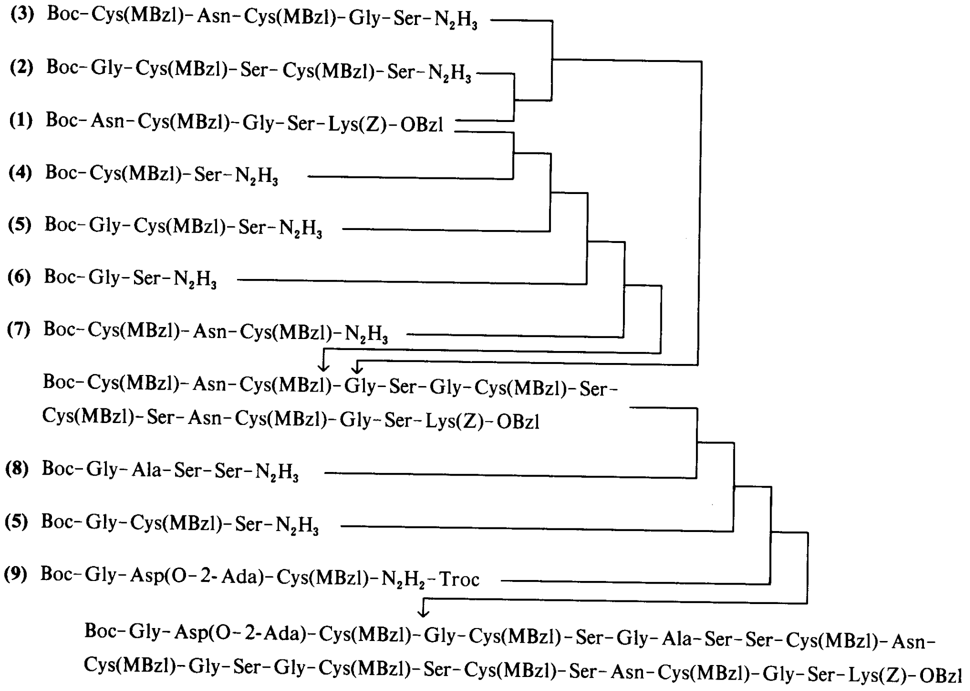

Fig. 2. Synthetic Scheme for Protected Pentacosapeptide Corresponding to the Entire Amino Acid Sequence of *N. crassa* MT

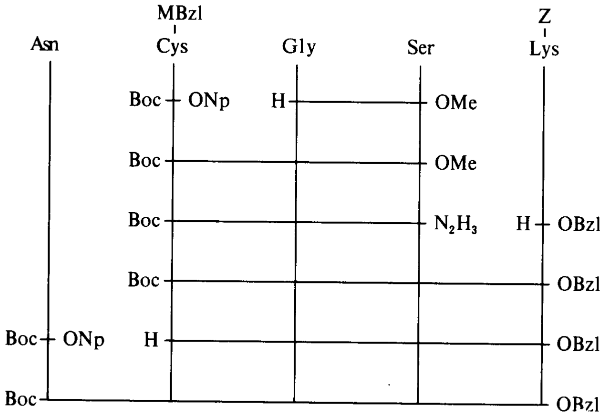

Fig. 3. Synthetic Scheme for the C-Terminal Pentapeptide, Boc-(MT 21—25)-OBzl

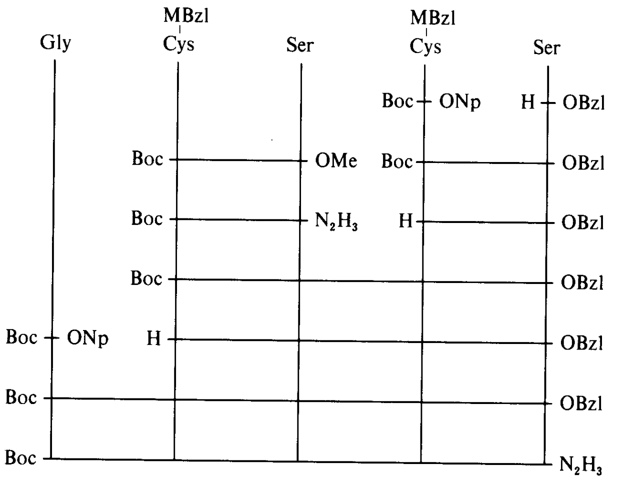

Fig. 4. Synthetic Scheme for the Protected Pentapeptide Hydrazide, Boc-(MT 16—20)-N$_2$H$_3$

ment with TFA and coupled with Boc-Gly-ONp. The resulting pentapeptide ester was treated with hydrazine hydrate to give Boc-(MT 16—20)-N$_2$H$_3$.

The fragment, Boc-(MT 11—15)-N$_2$H$_3$ (**3**), was prepared by the route shown in Fig. 5. Starting with H-Ser-OBzl, Boc-Gly-ONp, Boc-Cys(MBzl)-ONp, Boc-Asn-ONp and Boc-Cys(MBzl)-ONp were coupled successively. The resulting pentapeptide ester was treated with hydrazine hydrate to afford Boc-(MT 11—15)-N$_2$H$_3$. The fragment Boc-Gly-Cys(MBzl)-Ser-N$_2$H$_3$ (**5**) was prepared by the coupling of Boc-Gly-ONp with H-Cys(MBzl)-Ser-OBzl,[20] followed by treatment with hydrazine hydrate. Boc-Gly-Ser-N$_2$H$_3$ (**6**) was synthesized by the coupling of Boc-Gly-OH and H-Ser-OBzl by the DCC method, followed by treatment with hydrazine hydrate. Boc-Cys(MBzl)-Asn-Cys(MBzl)-N$_2$H$_3$ (**7**) was prepared by the successive coupling of Boc-Asn-ONp and Boc-Cys(MBzl)-ONp with H-Cys(MBzl)-OBzl, followed by treatment with hydrazine hydrate. 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 21—25)-OBzl, Boc-(MT 16—20)-N$_2$H$_3$ (**2**) and Boc-(MT 11—15)-N$_2$H$_3$ (**3**) were coupled successively to give Boc-(MT 11—25)-OBzl, while in the alternative synthesis, Boc-(MT 19—20)-N$_2$H$_3$[20] (**4**), Boc-(MT 16—18)-N$_2$H$_3$ (**5**), Boc-(MT 14—15)-N$_2$H$_3$ (**6**) and Boc-(MT 11—13)-N$_2$H$_3$ (**7**) were coupled successively to give Boc-(MT 11—25)-OBzl. The latter method gave a better result because excess amounts of azide component, which was relatively easy to remove, could be employed at each azide coupling step. After removal of the Boc group of Boc-(MT 11—25)-OBzl, the resulting amine was coupled with Boc-Gly-Ala-Ser-Ser-N$_2$H$_3$ (**8**), Boc-Gly-Cys(MBzl)-Ser-N$_2$H$_3$ (**5**) and Boc-Gly-Asp(O-2-Ada)-Cys(MBzl)-N$_2$H$_3$ (**9**) successively to afford the protected pentacosapeptide, Boc-(MT 1—25)-OBzl.

Next, the deprotection of the protected pentacosapeptide and related peptides by HF treatment was performed. During the course of this deprotection reaction, oxygen-free water was used and a slightly acidic solvent was

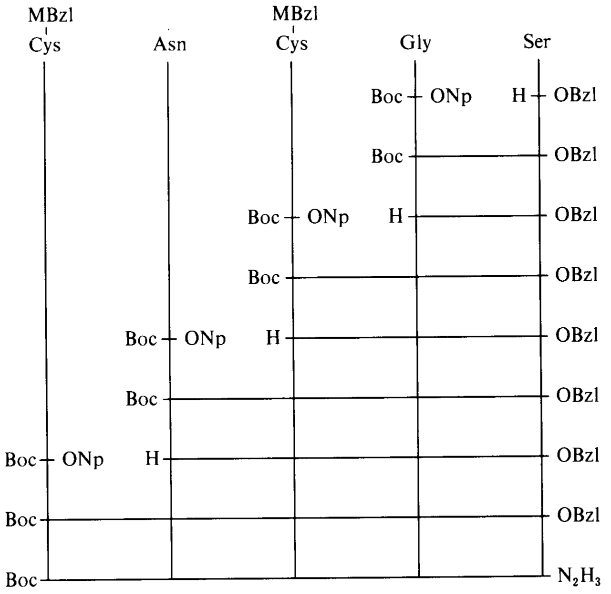

Fig. 5. Synthetic Scheme for the Protected Pentapeptide Hydrazide, Boc-(MT 11—15)-N_2H_3

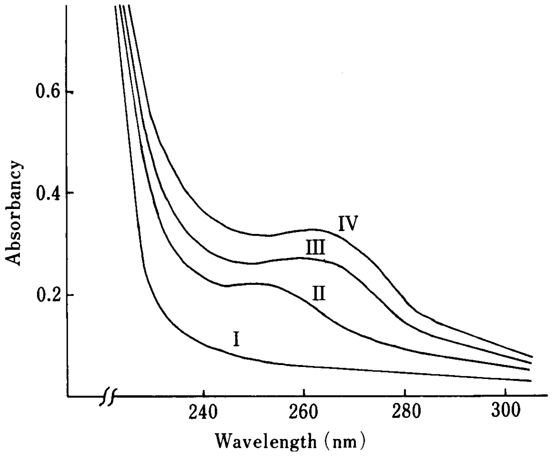

Fig. 7. Binding Study of Peptide with Heavy Metals

Peptide, 0.15 mM as SH in 3 ml of Tris-HCl (10 mM, pH 7.0). I, synthetic *N. crassa* apo-MT; II, apo-MT+Cd^{2+}; III, apo-MT+Cu^{2+}; IV, apo-MT+Cu^{+}.

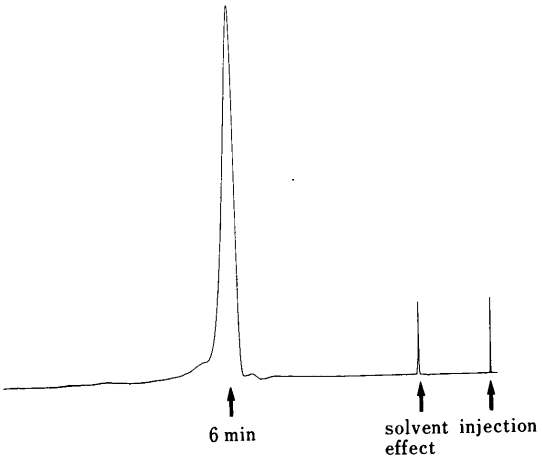

Fig. 6. HPLC Profile of the Synthetic Pentacosapeptide

Column, YMC-PAC A-302 ODS (4.6 × 250 mm), solvent, H_2O-CH_3CN (93 : 7) containing 0.1% TFA; absorbance, 214 nm; flow rate, 1 ml/min.

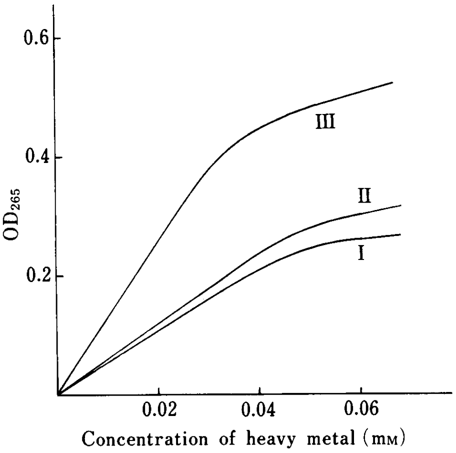

Fig. 8. Binding Study of Peptide with Cu

Peptide (pentacosapeptide): 0.15 mM as SH in 3 ml of Tris-HCl (10 mM, pH 7.0). I, peptide+$CuCl_2$; II, peptide+CuCl; III, peptide+$Cu(CH_3CN)_4ClO_4$.

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

N. crassa MT (positions)	Yield (%)	Rf^6	$[\alpha]_D$ (H_2O, $c=0.2$)	Amino acid ratios of hydrolysate[a] (110 °C, 18 h) Asp	Ser	Gly	Lys	Ala
H-Asn-Cys-Gly-Ser-Lys-OH (21—25)	66.4	0.76	−31.9°	1.00	0.84	1.02	0.88	
H-Gly-Cys-Ser-Cys-Ser-OH (16—20)	81.6	0.82	−50.2°		1.34	1.00		
H-Cys-Asn-Cys-Gly-Ser-OH (11—15)	52.9	0.86	−33.2°	1.00	0.74	1.06		
H-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH (16—25)	90.7	0.80	−53.3°	1.00	2.38	2.01	0.84	
H-Gly-Ser-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH (14—25)	90.2	0.77	−47.3°	1.00	3.06	2.94	0.93	
H-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH (11—25)	72.8	0.84	−51.7°	1.88	3.55	3.07	1.00	
H-Gly-Ala-Ser-Ser-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH (7—25)	82.7	0.79	−57.0°	1.82	5.21	4.33	1.00	1.25
H-Gly-Asp-Cys-Gly-Cys-Ser-Gly-Ala-Ser-Ser-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH (1—25)	88.8	0.90	−41.3°	2.76	6.52	5.94	1.00	1.33
H-Gly-Asp-Cys-Gly-Cys-Ser-Gly-Ala-Ser-Ser-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH · Cu complex (1—25 · Cu)	—	—	—	2.74	7.07	5.63	1.00	1.20

a) Cys was not determined.

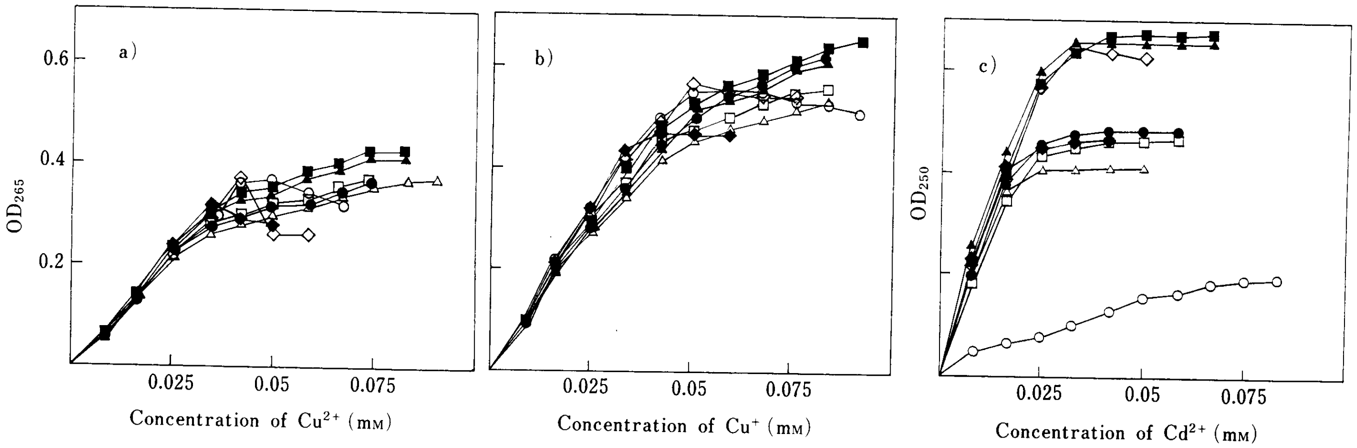

Fig. 9. Binding Properties of Peptides with Heavy Metals, a) with Cu^{2+}, b) with Cu^{+}, c) with Cd^{2+}

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). (○) *N. crassa* MT (21—25), (△) (16—25), (□) (14—25), (●) (11—25), (▲) (7—25), (■) (1—25), (◇) (16—20), (◆) (11—25).

employed as an eluant for column chromatography on Sephadex G-15 in order to prevent disulfide bond formation. The homogeneity of the peptides obtained was ascertained by TLC, high perfomance 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 synthetic pentacosapeptide was treated with $HCOOH$–H_2O_2[21] and then hydrolyzed with 6 N HCl and leucine aminopeptidase (LAP). The amino acid analysis of those hydrolysates were also performed.

The HPLC profile of the synthetic pentacosapeptide is shown in Fig. 6. The free SH content of the synthetic *N. crassa* MT was 6.5/peptide as calculated from the value of the SH content determined by the Ellman method.[22]

Heavy metals (Cd^{2+}, Cu^{2+}, and Cu^{+})-binding properties of synthetic *N. crassa* MT and related peptides were next examined. As shown in Fig. 7, synthetic *N. crassa* apo-MT exhibits ultraviolet (UV) absorption (I). Addition of Cd^{2+}, Cu^{2+} and Cu^{+} to the apo-MT solution resulted in UV absorptions (II, III and IV, respectively), having a shoulder at 250 or 265 nm due to mercaptide formation.[23,24] Therefore, the metal-binding properties of various peptides obtained above 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.

First of all, Cu-binding activities of synthetic *N. crassa* apo-MT were examined using $CuCl_2$, CuCl and $Cu(CH_3CN)_4ClO_4$[25] and the results are summarized in Fig. 8.

The binding property of apo-MT with Cu^{+} of CuCl is quite similar to that with Cu^{2+}, though the binding property of Cu^{+} of $Cu(CH_3CN)_4ClO_4$ is different from that of Cu^{2+}. This difference might be explained by the fact that Cu^{+} of CuCl is unstable and easily oxidized to Cu^{2+}, and Cu^{+} in $Cu(CH_3CN)_4ClO_4$ is quite stable. In fact, $Cu(CH_3CN)_4ClO_4$ is especially stable in a CH_3CN solution. Thus, $Cu(CH_3CN)_4ClO_4$ was employed for the examination of Cu^{+}-binding activity with the peptides.

Next, the Cd^{2+}, Cu^{2+} and Cu^{+}-binding properties of various peptides were examined and the results are shown in Fig. 9a, b and c. It can be seen that the Cu^{2+}-binding properties (Fig. 9a) as well as Cu^{+}-binding properties (Fig. 9b) of various kinds of peptides are similar to each other.

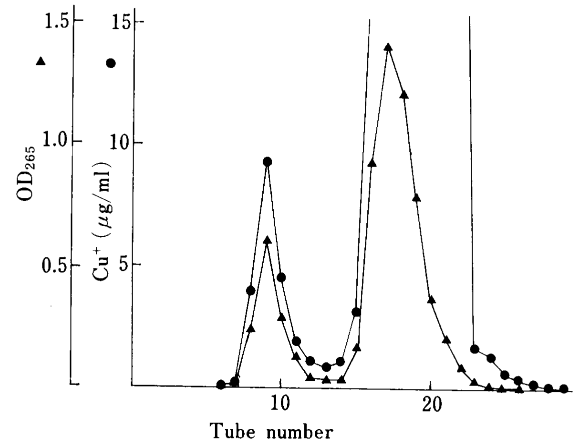

Fig. 10. Gel-Filtration of Reaction Mixture of *N. crassa* apo-MT and Cu^{+} on a Sephadex G-10 column

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

However, there was a difference in the intensity of absorbancy between Cu^{2+} and Cu^{+}-peptides. This discrepancy might be attributed to the difference in the structure of Cu-mercaptide or the difference in the binding ability between Cu^{2+} and Cu^{+}. On the contrary, the Cd^{2+}-binding activities of peptides were fairly structure-dependent. The Cd^{2+}-binding property of *N. crassa* MT (21—25), which contains only one Cys residue, is similar to that of cysteine. The structures of *N. crassa* MT (7—25) and (1—25) are more favorable for Cd^{2+}-binding than those of *N. crassa* MT (16—25), (14—25) and (11—25). It is interesting that the small peptide, *N. crassa* MT (16—20), H–Gly–Cys–Ser–Cys–Ser–OH, exhibited a similar Cd^{2+}-binding activity to that of *N. crassa* apo-MT. These results are compatible with our previous reports that the structure containing Cys–X–Cys–Cys (X: amino acid), which exists in α-fragment of mammalian MTs, is favorable for Cd^{2+}-binding.[26,27]

In order to determine the Cu^{+} content in synthetic *N. crassa* MT, the Cu^{+}-peptide complex was purified over Sephadex G-10. The desired complex exhibited a symmetrical peak, as shown in Fig. 10. Amino acid analysis of the compound present in this peak after acid hydrolysis gave molar ratios in good agreement with the theoretically expected values from *N. crassa* MT. The values for Cu content determined by the atomic absorption method and the average recovery of amino acids indicated that the ratio of Cu to peptide was 4.0 : 1. The discrepancy between this value and the expected value of 6[14] may reflect incomplete saturation of the metal binding site or inherent errors in the quantitative method employed.

In a similar manner, Cu^{2+} content in the Cu^{2+}-pentacosapeptide complex was also determined and it was revealed that the ratio of Cu to peptide was 2.7 : 1.

During this investigation, it was revealed that Cd-binding activity was fairly structure-dependent in peptides related to *N. crassa* MT, whereas the Cu-binding activity was not so much dependent on peptide structure, so far as examined. These results are consistent with our previous reports[26,27] 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 an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an acid hydrolysate (110°C, 6 N HCl, 20 h) and enzymatic digest (leucine amino peptidase, 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 spectrophotometer. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 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), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase), $CHCl_3$ and ether (4 : 1), $CHCl_3$, MeOH and H_2O (89 : 10 : 1) and *n*-BuOH, pyridine, AcOH and H_2O (1 : 1 : 1 : 1), respectively.

Boc–Cys(MBzl)–Gly–Ser–OMe H–Gly–Ser–OMe · HCl(prepared from 8.5 g of Z–Gly–Ser–OMe by catalytic hydrogenation) and Boc–Cys(MBzl)–ONp (12.7 g) were dissolved in DMF (150 ml) containing Et_3N (4.6 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 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a column of silica gel (6 × 45 cm), which was eluted with $CHCl_3$ (3000 ml) and then with 2% MeOH in $CHCl_3$ (2000 ml). The solvent of the latter eluate was removed by evaporation to afford a white powder, yield 9.1 g (64.7%), $[\alpha]_D^{25}$ +7.3° (*c* = 1.0, $CHCl_3$), Rf^1 0.54. *Anal.* Calcd for $C_{22}H_{33}N_3O_8S$: C, 52.9; H, 6.66; N, 8.41. Found: C, 52.7; H, 6.84; N, 8.16.

Boc–Cys(MBzl)–Gly–Ser–N_2H_3 Hydrazine hydrate (90%, 2.2 ml) was added to a solution of Boc–Cys(MBzl)–Gly–Ser–OMe (6.4 g) in MeOH (50 ml) and the solution was kept at room temperature overnight. The solution was concentrated to a small volume and ether was added to the residue. The precipitate was collected by filtration and recrystallized from EtOH, yield 4.8 g (75.0%), mp 151—152 °C, $[\alpha]_D^{25}$ −21.7° (*c* = 1.0, DMF), Rf^2 0.63. *Anal.* Calcd for $C_{21}H_{33}N_5O_7S$: C, 50.5; H, 6.66; N, 14.0. Found: C, 50.2; H, 6.76; N, 13.8.

Boc–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl Boc–Cys(MBzl)–Gly–Ser–N_3 (prepared from 3.5 g of the corresponding hydrazide with 1.0 ml of isopentyl nitrite in the usual manner) in DMF (30 ml) was added to a solution of H–Lys(Z)–OBzl · TosOH (3.0 g) in DMF (30 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at 4°C overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 4.2 g (71.8%), mp 110—115 °C, $[\alpha]_D^{25}$ −21.2° (*c* = 1.0, DMF), Rf^2 0.73, Rf^3 0.88. *Anal.* Calcd for $C_{42}H_{55}N_5O_{11}S$: C, 60.1; H, 6.73; N, 8.35. Found: C, 59.9; H, 6.75; N, 8.38.

Boc–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT 21—25)–OBzl] (1) Boc–Asn–ONp (1.8 g) and H–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl · TFA [prepared from 3.5 g of Boc–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl and 5 ml of TFA containing 1.1 ml of anisole at room temperature for 2 h] were dissolved in DMF (50 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and washed with AcOEt yield 3.2 g (80.8%), mp 188—193 °C, $[\alpha]_D^{25}$ −19.2° (*c* = 1.0, DMF), Rf^1 0.39, Rf^2 0.78. *Anal.* Calcd for $C_{48}H_{61}N_7O_{13}S \cdot H_2O$: C, 56.9; H, 6.62; N, 10.3. Found: C, 56.7; H, 6.44; N, 10.6.

Boc–Cys(MBzl)–Ser–Cys(MBzl)–Ser–OBzl Boc–Cys(MBzl)–Ser–N_3 (prepared from 1.6 g of the corresponding hydrazide and 0.41 ml of isopentyl nitrite in the usual manner) in DMF (20 ml) was added to a solution of H–Cys(MBzl)–Ser–OBzl · HCl [prepared from 2.0 g of Boc–Cys(MBzl)–Ser–OBzl and 5.3 ml of 7.3 N HCl/dioxane in the usual manner] in DMF (20 ml) containing Et_3N (0.49 ml). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 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 1.3 g (44.8%), mp 160—163 °C, $[\alpha]_D^{25}$ −31.9° (*c* = 1.0, MeOH), Rf^1 0.42, Rf^5 0.50. *Anal.* Calcd for $C_{40}H_{52}N_4O_{11}S_2 \cdot 1.5H_2O$: C, 56.1; H, 6.48; N, 6.55. Found: C, 56.3; H, 6.66; N, 6.78.

Boc–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–OBzl Boc–Gly–ONp (0.21 g) and H–Cys(MBzl)–Ser–Cys(MBzl)–Ser–OBzl · TFA [prepared from 0.80 g of Boc–Cys(MBzl)–Ser–Cys(MBzl)–Ser–OBzl and 1.1 ml of TFA containing 0.35 ml of anisole in the usual manner] were dissolved in DMF (20 ml) containing Et_3N (0.12 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt was added to the residue to afford a precipitate, which was collected by filtration and washed with 5% Na_2CO_3 and EtOH, yield 0.58 g (91.6%), mp 194—195 °C, $[\alpha]_D^{25}$ −70.1° (*c* = 1.0, DMF), Rf^1 0.42. *Anal.* Calcd for $C_{42}H_{55}N_5O_{10}S_2 \cdot 0.5H_2O$: C, 56.4; H, 6.31; N, 7.82. Found: C, 56.3; H, 6.22; N, 7.83.

Boc–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–N_2H_3[Boc–(MT 16—20)–N_2H_3] (2) Hydrazine hydrate (90%, 0.13 ml) was added to a solution of Boc–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–OBzl (0.50 g) in DMF (10 ml). The solution was kept at room temperature overnight. The solution was concentrated to a small volume and EtOH was added to the residue to afford a precipitate, which was collected by filtration and washed with MeOH, yield 1.5 g (77.0%), mp 227—229 °C, $[\alpha]_D^{25}$ −5.9° (*c* = 0.8, DMF), Rf^2 0.84, Rf^3 0.83. *Anal.* Calcd for $C_{35}H_{51}N_7O_{11}S_2 \cdot 0.5H_2O$: C, 51.3; H, 6.40; N, 12.0. Found: C, 51.4; H, 6.40; N, 12.0.

Boc–Asn–Cys(MBzl)–Gly–Ser–OBzl Boc–Asn–ONp (6.0 g) and H–Cys(MBzl)–Gly–Ser–OBzl · TFA [prepared from 9.8 g of Boc–Cys (MBzl)–Gly–Ser–OBzl and 19.0 ml of TFA containing 1.8 ml of anisole in the usual manner] were dissolved in DMF (100 ml) containing Et_3N (2.2 ml). 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 7.2 g (61.0%), mp 180—182 °C, $[\alpha]_D^{25}$ −24.3° (*c* = 0.9, DMF), Rf^1 0.12, Rf^5 0.24. *Anal.* Calcd for $C_{32}H_{43}N_5O_{10}S$: C, 55.7; H, 6.28; N, 10.2. Found: C, 55.7; H, 6.27; N, 9.93.

Boc–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–OBzl Boc–Cys(MBzl)–ONp (4.6 g) and H–Asn–Cys(MBzl)–Gly–Ser–OBzl · TFA [prepared from 6.2 g of Boc–Asn–Cys(MBzl)–Gly–Ser–OBzl and 10 ml of TFA containing 0.97 ml of anisole in the usual manner] were dissolved in DMF (50 ml) containing Et_3N (1.3 ml). 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 recrystallized from MeOH, yield 4.5 g (55.0%), mp 175—178 °C, $[\alpha]_D^{25}$ −28.7° (*c* = 1.0, DMF), Rf^1 0.40, Rf^3 0.83. *Anal.* Calcd for $C_{43}H_{56}N_6O_{12}S_2$: C, 56.6; H, 6.18; N, 9.20. Found: C, 56.3; H, 6.14; N, 9.10.

Boc–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–N_2H_3 [Boc–(MT 11—15)–N_2H_3] (3) Hydrazine hydrate (90%, 0.9 ml) was added to a solution of Boc–Cys (MBzl)–Asn–Cys (MBzl)–Gly–Ser–OBzl (3.0 g) in DMF (20 ml). The solution was kept at room temperature overnight. After removal of the solvent, EtOH was added to the residue to give a precipitate, which was collected by filtration and washed with MeOH, yield 2.5 g (93.0%), mp 192—196 °C, $[\alpha]_D^{25}$ −28.5° (*c* = 1.0, DMF), Rf^1 0.08, Rf^3 0.67. *Anal.* Calcd for $C_{36}H_{52}N_8O_{11}S_2$: C, 51.7; H, 6.26; N, 13.4. Found: C, 51.4; H, 6.37; N, 13.2.

Boc–Gly–Cys(MBzl)–Ser–OBzl Boc–Gly–ONp (3.6 g) and H–Cys(MBzl)–Ser–OBzl · TFA [prepared from 6.0 g of Boc–Cys(MBzl)–Ser–OBzl and 13.7 ml of TFA containing 2.5 ml of anisole in the usual manner] were dissolved in DMF (100 ml) containing Et_3N (1.7 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 10% citric acid, 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, yield 5.1 g (76.1%), mp 100—103 °C, $[\alpha]_D^{25}$ −39.2° (c=1.0, $CHCl_3$), Rf^1 0.56, Rf^5 0.88. *Anal.* Calcd for $C_{28}H_{37}N_3O_8S$: C, 58.4; H, 6.48; N, 7.30. Found: C, 58.4; H, 6.53; N, 7.30.

Boc–Gly–Cys(MBzl)–Ser–N_2H_3 (5) Hydrazine hydrate (90%, 1.3 ml) was added to a solution of Boc–Gly–Cys(MBzl)–Ser–OBzl (5.0 g) in MeOH (50 ml) and the solution was kept at room temperature overnight. The solution was concentrated to a small volume and ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH, yield 3.4 g (77.2%), mp 153—158 °C, $[\alpha]_D^{25}$ −32.8° (c=1.0, DMF), Rf^5 0.13. *Anal.* Calcd for $C_{21}H_{33}N_5O_7S\cdot 0.25H_2O$: C, 50.0; H, 6.70; N, 13.9. Found: C, 50.1; H, 6.63; N, 14.0.

Boc–Gly–Ser–OBzl Boc–Gly–ONp (4.2 g) and H–Ser–OBzl·$C_6H_5SO_3H$ (6.3 g) were dissolved in DMF (100 ml) containing Et_3N (1.2 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 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a column of silica gel (3 × 40 cm), equilibrated and eluted with $CHCl_3$. After removal of the solvent of the effluent (2000—2500 ml), petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 3.2 g (75.7%), mp 71—73 °C, $[\alpha]_D^{25}$ −2.4° (c=1.0, MeOH), Rf^1 0.72, Rf^5 0.40. *Anal.* Calcd for $C_{17}H_{24}N_2O_6$: C, 57.9; H, 6.78; N, 7.95. Found: C, 57.7; H, 6.89; N, 7.95.

Boc–Gly–Ser–N_2H_3 (6) Hydrazine hydrate (90%, 1.0 ml) was added to a solution of Boc–Gly–Ser–OBzl (2.1 g) in EtOH (30 ml). The solution was kept at room temperature overnight. After removal of the solvent, ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from EtOH, yield 1.6 g (96.4%), mp 147—149 °C, $[\alpha]_D^{25}$ +0.5° (c=1.0, DMF), Rf^2 0.65, Rf^5 0.11. *Anal.* Calcd for $C_{10}H_{20}N_4O_5$: C, 43.5; H, 7.30; N, 20.3. Found: C, 43.2; H, 7.35; N, 20.2.

Boc–Asn–Cys(MBzl)–OBzl Boc–Asn–ONp (5.0 g) and H–Cys(MBzl)–OBzl·TosOH were dissolved in DMF (100 ml) containing Et_3N (2.0 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, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH, yield 5.8 g (76.3%), mp 145—148 °C, $[\alpha]_D^{25}$ −33.0° (c=1.0, DMF), Rf^1 0.56. *Anal.* Calcd for $C_{27}H_{35}N_3O_7S\cdot 0.25H_2O$: C, 59.0; H, 6.50; N, 7.64. Found: C, 58.8; H, 6.65; N, 7.61.

Boc–Cys(MBzl)–Asn–Cys(MBzl)–OBzl Boc–Cys(MBzl)–ONp (3.7 g) and H–Asn–Cys(MBzl)–OBzl·TFA [prepared from 4.2 g of Boc–Asn–Cys(MBzl)–OBzl and 9.0 ml of TFA containing 1.7 ml of anisole in the usual manner] were dissolved in DMF (200 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 5.3 g (87.2%), mp 145—150 °C, $[\alpha]_D^{25}$ −17.0° (c=1.0, DMF), Rf^1 0.61. *Anal.* Calcd for $C_{38}H_{48}N_4O_9S_2$: C, 59.4; H, 6.29; N, 7.29. Found: C, 59.6; H, 6.25; N, 7.20.

Boc–Cys(MBzl)–Asn–Cys(MBzl)–N_2H_3 (7) Hydrazine hydrate (90%, 1.0 ml) was added to a solution of Boc–Cys(MBzl)–Asn–Cys(MBzl)–OBzl (5.3 g) in DMF (100 ml) and the solution was kept at room temperature overnight. The solution was concentrated to a small volume and ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from MeOH, yield 3.3 g (68.8%), mp 182—185 °C, $[\alpha]_D^{25}$ −17.0° (c=1.0, DMF), Rf^1 0.27, Rf^2 0.80. *Anal.* Calcd for $C_{31}H_{44}N_6O_8S_2$: C, 53.7; H, 6.40; N, 12.1. Found: C, 54.0; H, 6.42; N, 11.9.

Z–Ala–Ser–Ser–OMe Z–Ala–ONp (5.2 g) and H–Ser–Ser–OMe·HCl (prepared from 5.0 g of Z–Ser–Ser–OMe[28] by catalytic hydrogenation) were dissolved in DMF (200 ml) containing Et_3N (2.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, H_2O was added to the residue to afford a precipitate, which was collected by filtration, washed with AcOEt and recrystallized from EtOH, yield, 4.0 g (65.0%), mp 177—179 °C, $[\alpha]_D^{25}$ −21.9° (c=1.0, MeOH), Rf^1 0.13, Rf^5 0.35. *Anal.* Calcd for $C_{18}H_{25}N_3O_8$: C, 52.6; H, 6.13; N, 10.2. Found: C, 52.4; H, 6.01; N, 10.0.

Boc–Gly–Ala–Ser–Ser–OMe Boc–Gly–ONp (2.7 g) and H–Ala–Ser–Ser–OMe·HCl (prepared from 3.3 g of Z–Ala–Ser–Ser–OMe by catalytic hydrogenation in the presence of 1 N HCl) were dissolved in DMF (50 ml) containing Et_3N (1.3 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with water. The extract was washed with AcOEt and lyophilized to afford a white powder. The crude product in water (3 ml) was applied to a column of Sephadex G-25 (3.5 × 150 cm), equilibrated and eluted with water. Individual fractions (10 g each) were collected. The desired fractions (tube Nos. 61—69) were collected and lyophilized to afford a white fluffy powder, yield 2.2 g (63.4 g), $[\alpha]_D^{25}$ −44.3° (c=0.3, MeOH), Rf^2 0.69. *Anal.* Calcd for $C_{17}H_{30}N_4O_9$: C, 47.0; H, 6.96; N, 12.9. Found: C, 46.7; H, 7.03; N, 12.7.

Boc–Gly–Ala–Ser–Ser–N_2H_3 [Boc–(MT 7—10)–N_2H_3] (8) Hydrazine hydrate (90%, 0.35 ml) was added to a solution of Boc–Gly–Ala–Ser–Ser–OMe (1.0 g) in MeOH (20 ml). The reaction mixture was kept at room temperature overnight. Crystals formed were collected by filtration and recrystallized from MeOH, yield 0.96 g (96.0%), mp 195 °C (dec.), $[\alpha]_D^{25}$ −67.0°, Rf^2 0.37. *Anal.* Calcd for $C_{16}H_{30}N_6O_8\cdot 0.5H_2O$: C, 43.4; H, 6.99; N, 19.0. Found: C, 43.4; H, 6.88; N, 19.0.

Boc–Cys(MBzl)–N_2H_2–Troc Boc–Cys(MBzl)–OH (6.83 g) and NH_2NH–Troc (5.19 g) were dissolved in DMF (100 ml) and cooled with ice-salt. DCC (4.54 g) was added to the solution and the reaction mixture was stirred at room temperature overnight. After removal of the urea derivative and the solvent, ether was added to the residue to afford crystals, which were collected by filtration and washed with ether, yield 7.8 g (73.6%), mp 166—168 °C, $[\alpha]_D^{25}$ +0.5° (c=1.0, DMF), Rf^1 0.61, Rf^2 0.88. *Anal.* Calcd for $C_{19}H_{26}Cl_3N_3O_6S$: C, 43.0; H, 4.94; N, 7.92. Found: C, 43.0; H, 4.97; N, 8.21.

Boc–Asp(O-2-Ada)–Cys(MBzl)–NHNH–Troc Boc–Asp(O-2-Ada)–OSu (2.3 g) and H–Cys(MBzl)–NHNHTroc·TFA [prepared from 1.9 g of Boc–Cys(MBzl)–NHNHTroc and 1.6 ml of TFA containing 0.6 ml of anisole in the usual manner] were dissolved in DMF (20 ml) containing *N*-methylmorpholine (1.0 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 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a precipitate, which was collected by filtration, yield 1.77 g (46.3%), amorphous, $[\alpha]_D^{25}$ −31.5° (c=0.2, MeOH), Rf^1 0.50, Rf^4 0.29. *Anal.* Calcd for $C_{33}H_{46}Cl_3N_4O_9S$: C, 50.7; H, 5.94; N, 7.17. Found: C, 51.2; H, 6.07; N, 6.89.

Boc–Gly–Asp(O-2-Ada)–Cys(MBzl)–NHNH–Troc [Boc–(MT 1—3)–NHNH–Troc] (9) Boc–Gly–ONp (0.4 g) and H–Asp(O-2-Ada)–Cys(MBzl)–NHNHTroc·TFA [prepared from 1.1 g of Boc–Asp(O-2-Ada)–Cys(MBzl)–NHNHTroc and 1.2 ml of TFA containing 0.3 ml of anisole in the usual manner] were dissolved in DMF (20 ml) containing Et_3N (0.2 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 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (3 ml) was applied to a column of silica gel (3.2 × 60 cm), equilibrated and eluted with $CHCl_3$. After removal of the solvent of the effluent (1200—1500 ml), petroleum ether was added to the residue to afford a precipitate, which was collected by filtration, yield 1.0 g (85.5%), amorphous, $[\alpha]_D^{25}$ −39.0° (c=0.2, MeOH), Rf^1 0.62. *Anal.* Calcd for $C_{35}H_{49}Cl_3N_5O_{10}S$: C, 50.2; H, 5.89; N, 8.36. Found: C, 50.4; H, 6.03; N, 8.10.

Boc–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT 19—25)–OBzl] Boc–Cys(MBzl)–Ser–N_3 (prepared from 1.8 g of the corresponding hydrazide and 0.56 ml of isopentyl nitrite in the usual manner) in DMF (30 ml) was added to a solution of H–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl·TFA [prepared from 1.8 g of Boc–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl and 3 ml of TFA containing 0.2 ml of anisole in the usual manner] in DMF (20 ml) containing Et_3N (0.27 ml). The reaction mixture was stirred at 4 °C for 2 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 1.8 g (75.0%), mp 156—161 °C, $[\alpha]_D^{25}$ −25.2° (c=0.2, DMF), $Rf^1$0.17, Rf^5 0.31. *Anal.* Calcd for $C_{60}H_{79}N_9O_{17}S_2\cdot H_2O$: C, 55.5; H, 6.44; N, 9.71. Found: C, 55.4; H, 6.41; N, 10.0.

Boc–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT 16—25)–OBzl] 1) Boc–Gly–Cys(MBzl)–Ser–N_3 (prepared from 1.25 g of the corresponding hydrazide and 0.35 ml of isopentyl nitrite in the usual manner) in DMF (20 ml) was added to a solution of H–(MT 19—25)–OBzl·TFA [prepared from 1.5 g of Boc–(MT 19—25)–OBzl and 1.5 ml of TFA containing 0.2 ml of anisole in the usual manner] in DMF (20 ml) containing Et_3N (0.2 ml). The reaction mixture was stirred at 4 °C for 2 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 0.92 g (73.0%), mp 198—207 °C, $[\alpha]_D^{25}$ −21.9° (c=0.2, DMF), Rf^2 0.77, Rf^5 0.14. *Anal.* Calcd for $C_{76}H_{100}N_{12}O_{22}S_3\cdot 1.5H_2O$: C, 55.1; H, 6.27; N, 10.3. Found: C, 54.8; H, 6.31; N, 10.6.

2) Boc–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–N_3 (prepared from 0.81 g

of the corresponding hydrazide and 0.14 ml of isopentyl nitrite in the usual manner) in DMF (30 ml) was added to a solution of H–(MT 21—25)–OBzl·TFA [prepared from 1.0 g of Boc–(MT 21—25)–OBzl and 1.2 ml of TFA containing 0.23 ml of anisole in the usual manner] in DMF (20 ml) containing Et_3N (0.15 ml). The reaction mixture was stirred at 4 °C for 2d. After removal of the solvent, EtOH was added to the residue to afford a precipitate, which was collected by filtration. This crude product in DMF (3 ml) was applied to a column of Sephadex LH-20 (4 × 140 cm), equilibrated and eluted with DMF. Individual fractions (10 g each) were collected. After removal of the solvent of the desired fractions (tube Nos. 34—39), EtOH was added to the residue to afford a precipitate, which was collected by filtration, yield 1.09 g (66.9%), mp 199—205 °C, $[\alpha]_D^{25}$ −19.8° (c=0.5, DMF).

Boc–Gly–Ser–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT14—25)–OBzl] Boc–Gly–Ser–N_3 (prepared from 0.3 g of the corresponding hydrazide and 0.16 ml of isopentyl nitrite in the usual manner) in DMF (20 ml) was added to a solution of H–(MT 16—25)–OBzl·TFA [prepared from 0.9 g of Boc–(MT 16—25)–OBzl and 1.0 ml of TFA containing 0.2 ml of anisole in the usual manner] in DMF (20 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at 4 °C for 2 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 0.76 g (78.7%), mp 203—208 °C, $[\alpha]_D^{25}$ −17.7° (c=0.2, DMF), Rf^2 0.78. *Anal.* Calcd for $C_{81}H_{108}N_{14}O_{25}S_3\cdot 4H_2O$: C, 52.7; H, 6.33; N, 10.6. Found: C, 52.5; H, 6.03; N, 10.6.

Boc–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT11—25)–OBzl] 1) Boc–Cys(MBzl)–Asn–Cys(MBzl)–N_3 (prepared from 590 mg of the corresponding hydrazide and 0.12 ml of isopentyl nitrite in the usual manner) in DMF (15 ml) was added to a solution of H–(MT 14—25)–OBzl·TFA [prepared from 750 mg of Boc–(MT 14—25)–OBzl and 1.0 ml of TFA containing 0.2 ml of anisole in the usual manner] in DMF (15 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at 4 °C for 2 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 (67.0%), mp 210—213 °C, $[\alpha]_D^{25}$ −22.1° (c=0.2, DMF), Rf^2 0.76. *Anal.* Calcd for $C_{107}H_{144}N_{18}O_{31}S_5\cdot 2.5H_2O$: C, 53.9; H, 6.30; N, 10.8. Found: C, 53.9; H, 6.33; N, 11.0.

2) Boc–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–N_3 (prepared from 125 mg of the corresponding hydrazide and 0.03 ml of isopentyl nitrite in the usual manner) in DMF (10 ml) was added to a solution of H–(MT 16—25)–OBzl·TFA [prepared from 250 mg of Boc–(MT 16—25)–OBzl and 0.5 ml of TFA containing 0.1 ml of anisole in the usual manner] in DMF (5 ml) containing Et_3N (0.04 ml). The reaction mixture was stirred at 4°C for 2 d. After removal of the solvent, MeOH was added to the residue to afford a precipitate, which was collected by filtration. This crude product in DMF (3 ml) was applied to a column of Sephadex LH-20 (4 × 140 cm), equilibrated and eluted with DMF. Individual fractions (10 g each) were collected. After removal of the solvent of the desired fractions (tube Nos. 45—49), EtOH was added to the residue to afford crystals, which were collected by filtration, yield 230 mg (65.1%), mp 204—212 °C, $[\alpha]_D^{25}$ −19.0° (c=0.2, DMF).

Boc–Gly–Ala–Ser–Ser–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT7—25)–OBzl] Boc–Gly–Ala–Ser–Ser–N_3 (prepared from 500 mg of the corresponding hydrazide and 0.16 ml of isopentyl nitrite in the usual manner) in DMF (10 ml) was added to a solution of H–(MT 11—25)–OBzl·TFA [prepared from 550 mg of Boc–(MT 11—25)–OBzl and 0.7 ml of TFA containing 0.1 ml of anisole in the usual manner] in DMF (15 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, H_2O and MeOH were added to the residue to afford a precipitate, which was collected by filtration and washed with H_2O and MeOH, yield 575 mg (93.7%), mp 140 °C (dec.), $[\alpha]_D^{25}$ −9.0° (c=0.2, DMF), Rf^2 0.66. *Anal.* Calcd for $C_{118}H_{158}N_{22}O_{37}S_5\cdot 8H_2O$: C, 51.0; H, 6.30; N, 11.1. Found: C, 50.6; H, 6.02; N, 11.4.

Boc–Gly–Cys(MBzl)–Ser–Gly–Ala–Ser–Ser–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT 4—25)–OBzl] Boc–Gly–Cys(MBzl)–Ser–N_3 (prepared from 470 mg of the corresponding hydrazide and 0.13 ml of isopentyl nitrite in the usual manner) in DMF (10 ml) was added to a solution of H–(MT 7—25)–OBzl·TFA [prepared from 500 mg of Boc–(MT 7—25) –OBzl and 0.7 ml of TFA containing 0.1 ml of anisole in the usual manner] in DMF (15 ml) containing Et_3N (0.1 ml). 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 496 mg (87.5%), mp 165—170 °C, $[\alpha]_D^{25}$ −13.3° (c=0.2, DMF), Rf^2 0.72. *Anal.* Calcd for $C_{134}H_{179}N_{25}O_{42}S_6\cdot 5H_2O$: C, 52.0; H, 6.16; N, 11.3. Found: C, 52.2; H, 6.02; N, 11.3.

Boc–Gly–Asp(O–2-Ada)–Cys(MBzl)–Gly–Cys(MBzl)–Ser–Gly–Ala–Ser–Ser–Cys(MBzl)–Asn–Cys(MBzl)–Gly–Ser–Gly–Cys(MBzl)–Ser–Cys(MBzl)–Ser–Asn–Cys(MBzl)–Gly–Ser–Lys(Z)–OBzl [Boc–(MT1—25)–OBzl] Zinc powder (1.0 g) was added to a solution of Boc–Gly–Asp(O–2-Ada)–Cys(MBzl)–NHNHTroc (400 mg) in AcOH–MeOH (1 : 1). The reaction mixture was stirred at room temperature for 2 d. After removal of zinc and the solvent, water saturated with EDTA was added to the residue to give a precipitate, which was collected by filtration and washed with 5% Na_2CO_3 and H_2O. The precipitate in DMF (15 ml) was converted to Boc–Gly–Asp(O–2-Ada)–Cys(MBzl)–N_3 in the usual manner. This solution was added to a solution of H–(MT 4—25)–OBzl·TFA [prepared from 400 mg of Boc–(MT 4—25)–OBzl and 1.0 ml of TFA containing 0.1 ml of anisole in the usual manner] in DMF (20 ml) containing Et_3N (0.1 ml). 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 373 mg (80.3%), mp 155—162 °C, $[\alpha]_D^{25}$ −25.8°(c=0.2, DMF). *Anal.* Calcd for $C_{161}H_{214}N_{28}O_{48}S_7\cdot 3H_2O$: C, 54.0; H, 6.18; N, 10.9. Found: C, 53.8; H, 6.01; N, 11.2.

General Procedure for Deprotection by HF The protected peptide (0.03 mmol) was treated with anhydrous HF (5 ml) containing *m*-cresol (0.73 ml) and thioanisole (0.17 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. This solution was applied to a column of Sephadex G-15 (2.2 × 135 cm), 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, $[\alpha]_D$ value, amino acid ratios in an acid hydrolysate and *Rf* values are summarized in Table I.

A synthetic pentacosapeptide (3.0 mg) was treated with HCOOH–H_2O_2[21] 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: $CySO_3H_{6.68}Asp_{3.00}Ser_{7.21}Gly_{6.40}Ala_{1.20}Lys_{0.82}$ (average recovery 82.9%). LAP digest: $CySO_3H_{6.50}Ser_{7.10}Gly_{5.89}Ala_{1.30}Lys_{1.00}$ (average recovery 65.6%), Asp was degraded by LAP.[18]

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 determined and the increase was plotted against metal concentration.

Synthesis of Cu–Peptide Complex All operations were carried out under an N_2 atmosphere using oxygen-free water. A pentacosapeptide (0.32 mg, 0.14 μmol) was added to 2 ml of water containing 0.98 mg (3 μmol) of $Cu(CH_3CN)_4ClO_4$. The solution 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 8.0). Fractions of 2 ml each were collected. The eluted material was examined by measuring the UV absorbance at 265 nm and Cu content by atomic absorption spectrometry. The chromatographic pattern is shown in Fig. 10. The Cu content of tube no. 9 was 9.1 μg/ml (69.5 nmol/ml). One-half ml of tube no. 9 was hydrolyzed at 110 °C for 20 h after addition of concentrated HCl, and the acid hydrolysate was analyzed. The amino acid ratios are listed in Table I. The average recovery of amino acid was 17.3 nmol/ml. The Cu content was 4.0 g atom/peptide.

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References and Notes

1) Part XXIII: S. Tsuboi and Y. Okada, *Chem. Pharm. Bull.*, **37**, 46 (1989).

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*-butoxycarbonyl; Bzl, benzyl ester; MBzl, *p*-methoxybenzyl; Troc, 2,2,2-trichloroethyloxycarbonyl; ONp, *p*-nitrophenyl ester; OSu, *N*-hydroxysuccinimide ester; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOH, acetic acid; AcOEt, ethyl acetate; *n*-BuOH, *n*-butanol; TosOH, *p*-toluensulfonic acid; EDTA, ethylenediaminetetraacetic acid disodium salt.

3) M. Nordberg and Y. Kojima, "Metallothioneins," ed. by J. H. R. Kägi and M. Nordberg, Birkhauser, Basel, 1979, p. 41.
4) M. Piscator, *Nord. Hyg. Tidskr.*, **45**, 76 (1964).
5) G. F. Nordberg, *Environ. Physiol. Biochem.*, **2**, 7 (1972).
6) I. Bremner and N. T. Davies, *Biochem. J.*, **149**, 733 (1975).
7) L. Ryden and H. F. Deutsch, *J. Biol. Chem.*, **253**, 519 (1978).
8) O. M. Udom and F. O. Brady, *Biochem. J.*, **187**, 329 (1980).
9) T. Y. Li, A. J. Kraker, C. F. Shaw, III and D. H. Petering, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7094 (1980).
10) M. Beltramini and K. Lerch, *FEBS Lett.*, **142**, 219 (1982).
11) B. L. Geller and D. R. Winge, *Arch. Biochem. Biophys.*, **213**, 109 (1982).
12) A. Bakka and M. Webb, *Biochem. Pharmacol.*, **30**, 721 (1981).
13) B. B. Griffith, R. A. Walters, M. D. Enger, C. E. Hilderband and J. K. Griffith, *Nucleic Acids Res.*, **11**, 901 (1983).
14) K. Lerch, *Nature* (London), **284**, 368 (1980).
15) J. Honzle and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
16) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967)
17) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii and H. Irie, *Chem. Pharm. Bull.*, **30**, 1706 (1980).
18) Y. Okada, S. Iguchi, *J. Chem. Soc., Perkin Trans. 1*, **1988**, 2129.
19) R. G. Hisky, T. Mizoguchi and E. L. Smithwick, *J. Org. Chem.*, **32**, 97 (1967).
20) N. Ohta, Y. Okada and K. Tanaka, *Chem. Pharm. Bull.*, **31**, 1885 (1983).
21) C. H. W. Haris, *Methods Enzymol.*, **11**, 187 (1967).
22) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
23) J. H. R. Kägi and B. L. Vallee, *J. Biol. Chem.*, **236**, 2435 (1961).
24) A. R. Thrower, J. Byrd, E. B. Tarbet, R. K. Mehra, D. H. Hamer and D. R. Winge, *J. Biol. Chem.*, **263**, 7037 (1988).
25) P. Hemmerich and C. Sigwart, *Experientia*, **19**, 488 (1963).
26) N. Ohta, Y. Okada and K. Tanaka, *Chem. Pharm. Bull.*, **31**, 3094 (1983).
27) Y. Okada, N. Ohta, M. Yagyu, K. Min, S. Onosaka and K. Tanaka, *J. Protein Chem.*, **3**, 243 (1984).
28) R. Badiello, G. Vidali and A. Marzotto, *Gazz. Chem. Ital.*, **94**, 322 (1964).