[Chem. Pharm. Bull. 34(3) 986—998 (1986)]

Amino Acids and Peptides. XIII.[1,2] Synthesis of a Nonacosapeptide Corresponding to the N-Terminal Sequence 1—29 (β-Fragment) of Human Liver Metallothionein II (hMT II) and Its Heavy Metal-Binding Properties

YOSHIO OKADA,*,[a] NORIYA OHTA,[a] SHIN IGUCHI,[a] YUKO TSUDA,[a] HIDEAKI SASAKI,[a] TOKUJIRO KITAGAWA,[a] MASAMI YAGYU,[a] KYON-SON MIN,[b] SATOMI ONOSAKA,[b] and KEIICHI TANAKA[b]

Faculty of Pharmaceutical Sciences[a] and Faculty of Nutrition,[b] Kobe-Gakuin University, Ikawadani, Nishi-ku, Kobe 673, Japan

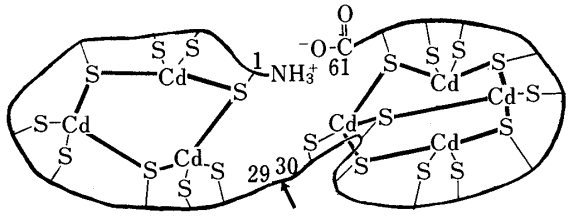
(Received July 26, 1985)

A nonacosapeptide corresponding to the N-terminal sequence 1—29 (β-fragment) of human liver metallothionein II (hMT II) was synthesized by the azide coupling of five peptide fragments [1, 2, 4, 6 and 8], followed by HF deprotection and its heavy metal-binding properties were examined. It was revealed that the Cd-binding ability of the synthetic β-fragment as well as synthetic α-fragment corresponding to the C-terminal sequence 30—61 of hMT II was stronger than that of native rat thionein. Moreover, it was found that both the α- and β-fragments bound preferentially to Cu ions rather than Cd ions.

Keywords——human liver metallothionein II; nonacosapeptide; N-terminal 1—29; β-fragment; chemical synthesis; Asp-containing peptide hydrazide; Asp-Pro-containing peptide hydrazide; fragment condensation; HF deprotection; heavy metal-binding property

Metallothioneins (MT) are a class of low molecular weight cysteine-rich metal-binding proteins. Due to their metal (Cd, Zn, Hg, Cu, *etc.*)-binding ability, they act as heavy metal (Cd, Hg) detoxifying agents and participate in heavy metal (Zn, Cu) metabolism, maintaining homeostasis, although their precise role is not yet well understood. Complete amino acid sequences of these proteins from humans,[3,4] horses,[5] mice,[6] *Neurospora crassa*,[7] and *Scylla serrata*[8] have been reported.

Otvos and Armitage[9] reported that rabbit metallothionein included two separate metal clusters, one containing four Cd^{2+} ions (cluster A, C-terminal portion) and the other containing three (cluster B, N-terminal portion) as shown in Fig. 1. Winge and Miklossy[10] isolated cluster A after proteolytic (subtilisin) digestion of rat MT, identified it as the C-terminal dotriacontapeptide (positions 30—60) and designated it as α-fragment. However, they failed to isolate the N-terminal polypeptide segment (cluster B, β-fragment) due to its



Cd_3-cluster cluster B (β-fragment)

Cd_4-cluster cluster A (α-fragment)

Fig. 1. Schematic Representation of the Structure of Cd_7-MT[9]

degradation into small peptides by subtilisin. It was also shown that the two clusters exhibited significant differences in their affinity for different metal ions and functioned independently. For example, the great majority of Cd^{2+} binds to the four-metal cluster (cluster A, α-fragment), while most of Zn^{2+} binds to the three-metal cluster (cluster B, β-fragment).[10] Thus, we have synthesized the nonacosapeptide corresponding to the N-terminal sequence 1—29 (β-fragment) of human liver metallothionein II (hMT II) in order to examine its heavy metal-binding properties. In this paper, we wish to describe the synthesis of the nonacosapeptide (β-fragment) of hMT II and its heavy metal-binding properties.

The synthetic route to the nonacosapeptide is shown in Fig. 2. The synthetic strategy adopted here is essentially the same as that employed for our previous synthesis of α-fragment of hMT II.[11] Starting with the C-terminal undecapeptide ester [1], four fragments [2, 4, 6 and 8] were coupled successively by the azide procedure[12] in order to minimize racemization. The α-amino function of intermediates was protected with the TFA-labile Boc group. Amino acid derivatives bearing groups removable by hydrogen fluoride[13] or trifluoromethanesulfonic acid (TFMSA)[14] were employed, *i.e.*, Lys(Z), Glu(OBzl), Asp(OBzl) and Cys(MBzl).

The C-terminal protected undecapeptide, Boc–(hMT II 19—29)–OBzl [1] was prepared as shown in Fig. 3. Boc–Thr–Ser–$NHNH_2$ derived from the corresponding benzyl ester[15] was coupled with H–Cys(MBzl)–OBzl by the azide method[12] to give Boc–Thr–Ser–Cys(MBzl)–OBzl, which was treated with TFA. The resultant tripeptide amine was combined with Boc–Cys(MBzl)–Lys(Z)–Cys(MBzl)–$NHNH_2$[15] to afford Boc–(hMT II 24—29)–OBzl. After removal of the Boc group of Boc–(hMT II 24—29)–OBzl with TFA, Boc–Glu(OBzl)–ONp was coupled with the hexapeptide amine to give Boc–(hMT II 23—29)–OBzl. The Boc group of the peptide was removed by TFA treatment and the resultant amine was coupled with Boc–Cys(MBzl)–Lys(Z)–$NHNH_2$[15] by the azide procedure twice to give the desired protected undecapeptide, Boc–(hMT II 19—29)–OBzl [1]. The hexapeptide hydrazide, Boc–(hMT II 13—18)–$NHNH_2$ [2], was prepared by the azide coupling of Boc–Cys(MBzl)–Thr–$NHNH_2$[15] with H–Cys(MBzl)–Ala–Gly–Ser–OMe[15] followed by exposure of the resultant hexapeptide to hydrazine hydrate.

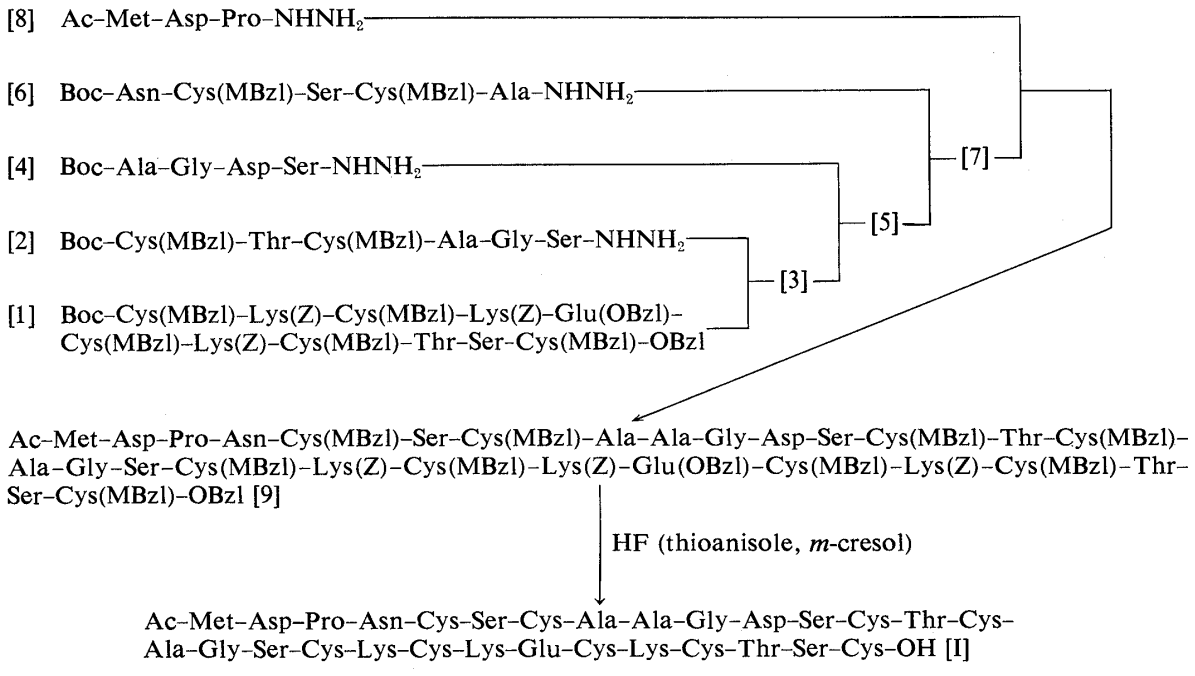


Fig. 2. Synthetic Route to a Nonacosapeptide (hMT II 1—29, β-Fragment) [I]

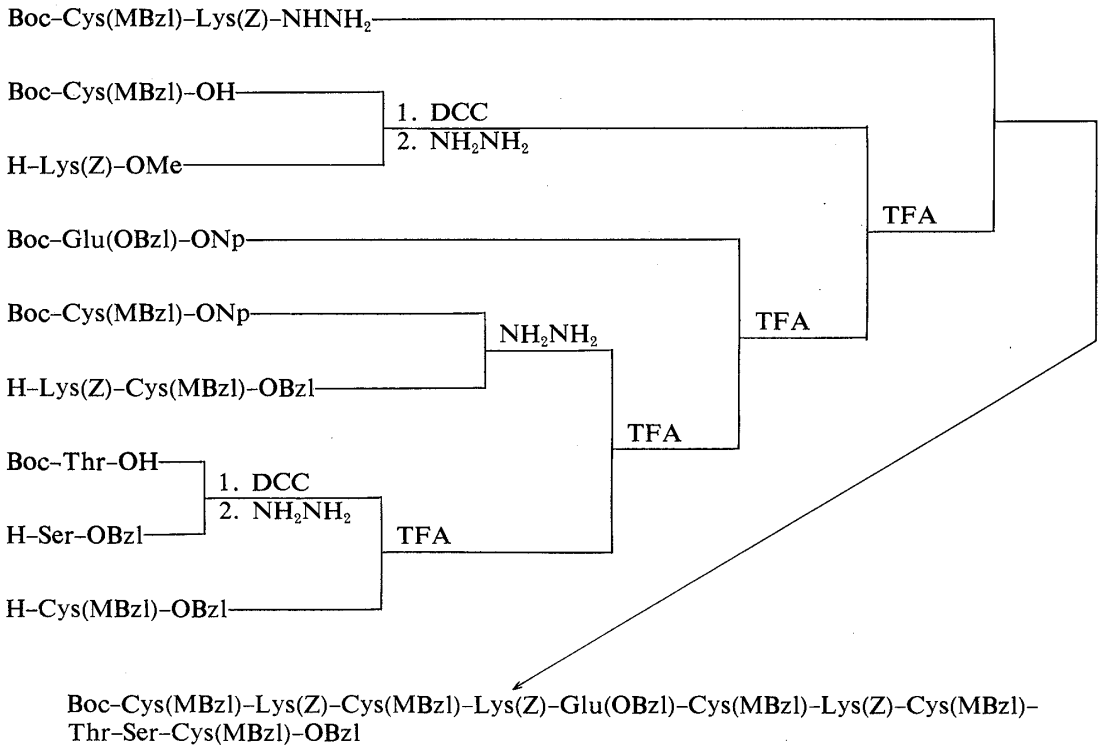


Fig. 3. Synthetic Route to a Protected Undecapeptide, Boc-(hMT II 19—29)-OBzl [1]

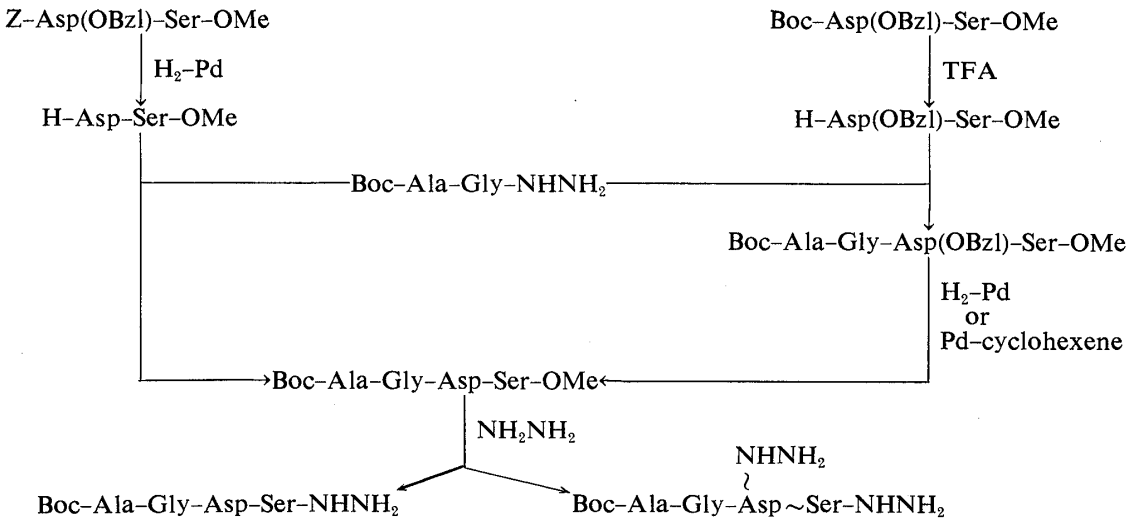


Fig. 4. Synthetic Route to Boc-Ala-Gly-Asp-Ser-$NHNH_2$, Boc-(hMT II 9—12)-$NHNH_2$ [4]

The synthesis of Boc-Ala-Gly-Asp-Ser-$NHNH_2$, Boc-(hMT II 9—12)-$NHNH_2$ [4] was carried out by the route shown in Fig. 4. First of all, we attempted to synthesize Z-Ala-Gly-Asp-Ser-$NHNH_2$ to examine the feasibility of obtaining the desired peptide [4]. Z-Asp(OBzl)-Ser-OMe[16] was hydrogenated over a Pd catalyst to give H-Asp-Ser-OMe. This amine was combined with Z-Ala-Gly-$NHNH_2$[17] to afford Z-Ala-Gly-Asp-Ser-OMe in 13% yield, which was converted to the corresponding hydrazide in a pure form, demonstrating that this synthetic route was suitable for the preparation of [4], except for the yield of the fragment condensation. In fact, the yield of Boc-Ala-Gly-Asp-Ser-OMe from the combination of Boc-Ala-Gly-$NHNH_2$ with H-Asp-Ser-OMe was 3.8%. This low yield was due

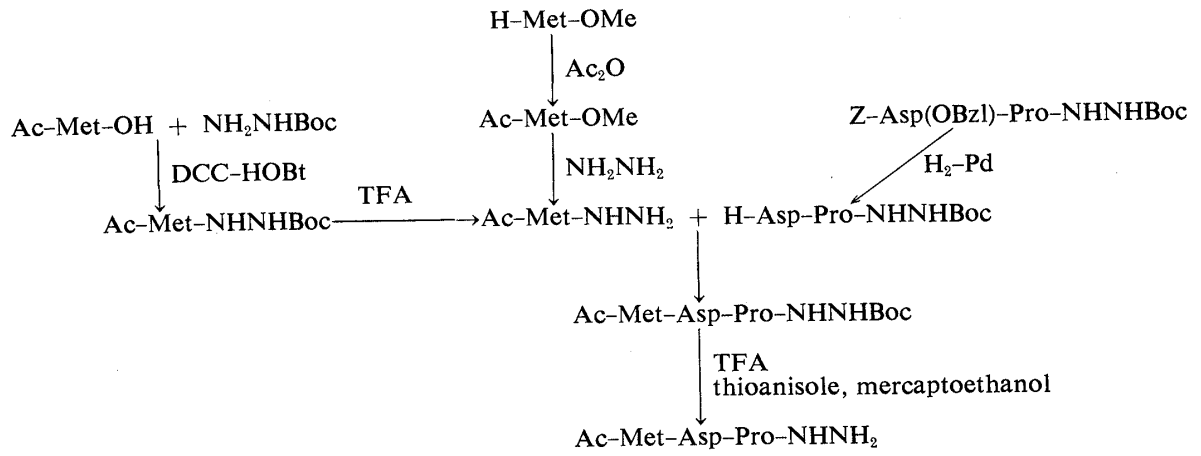


Fig. 5. Synthetic Route to Ac–Met–Asp–Pro–$NHNH_2$, Ac–(hMT II 1—3)–$NHNH_2$ [8]

to the high solubility of the tetrapeptide in water. Thus, Boc–Ala–Gly–$NHNH_2$ was coupled with H–Asp(OBzl)–Ser–OMe derived from Boc–Asp(OBzl)–Ser–OMe by TFA treatment. The Bzl group of the Asp residue in the tetrapeptide was quantitatively removed by catalytic transfer hydrogenation[18] or by usual hydrogenation. The rate of hydrogenation by the former method was much faster than by the latter method. Exposure of Boc–Ala–Gly–Asp–Ser–OMe to hydrazine hydrate gave the desired hydrazide [4] (34.8%) with the dihydrazide Boc–Ala–Gly–Asp($NHNH_2$)–Ser–$NHNH_2$ as a side reaction product, although it is not clear whether Asp residue is at the α or β position. It is likely that this side reaction occurred through succinimide formation. The desired hydrazide was isolated in a pure form by gel-filtration on Sephadex G-25 or by the combination of silica gel column chromatography and gel-filtration.

Boc–(hMT II 4—8)–$NHNH_2$ [6] was prepared by treating Boc–Asn–Cys(MBzl)–Ser–Cys(MBzl)–Ala–OBzl[15] with hydrazine hydrate.

Ac–Met–Asp–Pro–$NHNH_2$, Ac–(hMT II 1—3)–$NHNH_2$ [8], contains an acid labile Asp–Pro bond,[19] an Ac group and a Met residue, which trends to undergo sulfoxide formation and S-alkylation during deprotection. Consequently, the synthetic route to the desired peptide [8] was planned as shown in Fig. 5. Z–Asp(OBzl)–Pro–NHNHBoc[20] was hydrogenated over Pd catalyst to give H–Asp–Pro–NHNHBoc, which was condensed with Ac–Met–$NHNH_2$ to afford Ac–Met–Asp–Pro–NHNHBoc. The optimum conditions for removal of the Boc group of Ac–Met–Asp–Pro–NHNHBoc were examined. As can be seen in Table I, TFA treatment was more suitable than HCl treatment to avoid cleavage of the acid-labile peptide bond.[19] The HF–thioanisole or HF–*m*-cresol system, which would be employed for deprotection at the final stage, was also examined. The peptide hydrazide thus obtained exhibited a single spot on silica gel thin-layer chromatography (TLC) (positive to the methionine test and hydrazine test) with the same *Rf* value as the peptide hydrazide [8] obtained by TFA treatment, as shown in Table I, suggesting that HF–thioanisole–*m*-cresol treatment of the protected nonacosapeptide at the final stage would not result in any side reaction at the N-terminal peptide segment. The stability of the Ac group and the methionine residue was confirmed by the nuclear magnetic resonance (NMR) data of the peptide [8]. As can be seen in Fig. 6, CH_3 groups of CH_3CO and CH_3–S– exhibited sharp singlet peaks at δ 1.98 and 2.08, respectively. Thus we obtained the five peptide fragments required to construct the β-fragment of hMT II and established the optimum conditions for the deprotection procedure at the final stage.

In each fragment condensation reaction in Fig. 2, three or more equivalents of the azide

TABLE I. Removal of Boc Group of Ac–Met–Asp–Pro–NHNHBoc

Conditions				TLC	
Acid	Additive	Temperature (°C)	Time (min)	Rf^1	Rf^3
TFA	Thioanisole Mercaptoethanol	25	60	0	0.48
	Anisole Mercaptoethanol	25	60	0	0.50
3 N HCl–dioxane	Thioanisole Mercaptoethanol	25	60	0 (main) 0.67 0.82	0.50 (main) 0.75
	Anisole Mercaptoethanol	25	60	0 (main) 0.63 0.88	0.50 (main) 0.75
HF	Thioanisole	0	60	0	0.50
	m-Cresol	0	60	0	0.50

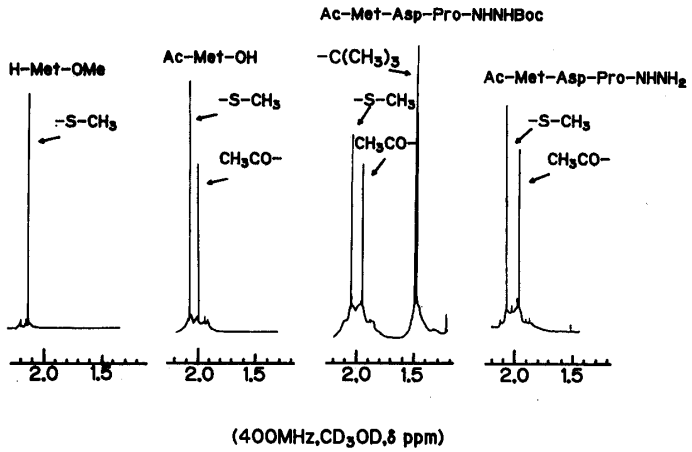


Fig. 6. NMR Spectra of C-Terminal Tripeptide Derivatives

component [2, 4, 6 or 8] were employed and the reaction was carried out in dimethylformamide (DMF) without using dimethyl sulfoxide (DMSO) in order to avoid oxidation of the cysteine or methionine residue. All azide components employed were soluble in MeOH. Hence, at each step, the desired peptide could be isolated by condensation of the reaction mixture followed by addition of MeOH and then filtration. The reprecipitation of the desired peptide from DMF and MeOH gave an analytically pure peptide at each step. This method is very convenient for isolation of the peptide, especially in the case where the desired peptide is fairly insoluble in organic solvents, as described previously.[21]

In order to remove all protecting groups employed, the peptide [9] was exposed to hydrogen fluoride[13] in an ice bath for 60 min. Thioanisole and *m*-cresol were employed as scavengers[22,23] to avoid alkylation.[24] The resultant deblocked peptide was converted to the corresponding acetate with Amberlite IRA-45 (acetate form) and reduced with dithiothreitol and mercaptoethanol. During the course of these reactions, oxygen-free water was used and a slightly acidic solvent (3% AcOH)[11] was employed as an eluant for column chromatography on Sephadex G-25 in order to prevent disulfide bond formation. This purified nonacosapeptide exhibited a single spot upon TLC on silica gel (ninhydrin test, H_2PtCl_6–KI test and nitroprusside test positive). The free SH content of the nonacosapeptide [I] was 8.7/peptide as calculated from the value for SH content determined by the Ellman method[25] and

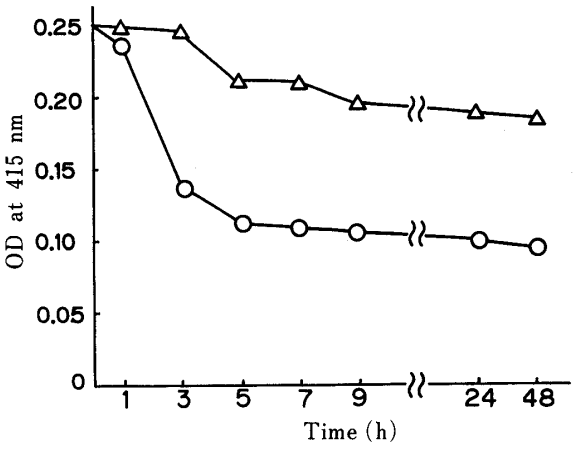


Fig. 7. Stability of SH Groups of β-Fragment [I]

The free SH groups of synthetic β-fragment [I] in oxygen-free water (○—○) and 3% AcOH (△—△) were measured at 0 °C by the Ellman method as a function of time.

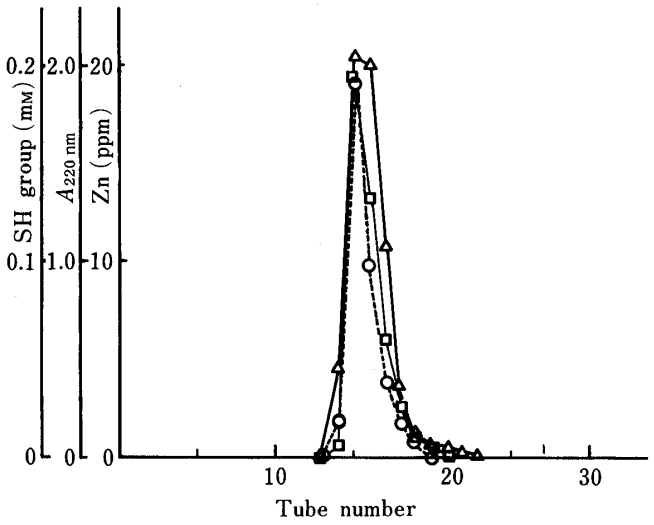


Fig. 8. Gel-Filtration of Zn–β-Fragment [I] Complex on Sephadex G-10

Aliquots of the eluate fractions were examined for UV absorption at 220 nm (—△—), Zn content by atomic absorption spectrometry (---○---) and SH content by the Ellman method (—□—).

TABLE II. Amino Acid Ratios in Acid Hydrolysates of β-Fragment [I] and Its Derivatives

Compound	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Met	Lys
Ac–(hMT II 1—29)–OH [I]	3.09	1.71	3.47	0.89	0.80	2.42	3.28	—[a]	0.80	3.00
Oxidized [I] by performic acid	3.33	1.87	3.43	1.00	1.10	2.40	3.03	8.50[d]	—[b]	3.00
Zn–β-fragment complex	3.27	1.71	3.85	0.80	0.90	2.10	3.02	—[a]	0.52[c]	3.00

a) Cys was not determined. *b*) Met was oxidized with performic acid. *c*) Met was partially oxidized due to the presence of Zn^{2+}. *d*) Cys was determined as cysteic acid.

the average recovery of amino acids. However, the SH content in oxygen-free water solution decreased rapidly even at 0 °C as shown in Fig. 7. It was noted that in 3% AcOH solution, disulfide formation was prevented significantly. During storage of the synthetic β-fragment [I] in a lyophilized powdered form at 0 °C for 1 month, no disulfide bond formation was observed. However, longer storage resulted in a decrease of SH content, indicating that disulfide bond formation occurred even in the powdered form. We thought that it might be possible to protect the SH groups by the formation of the Zn^{2+} complex, which might be used to capture Cd^{2+} and Hg^{2+} based on the differences in binding ability with various heavy metals.[26] The β-fragment [I] obtained above was combined with Zn^{2+} and the resultant complex was purified by gel-filtration on Sephadex G-10 using 10 mM Tris–HCl buffer (pH 8.0) as an eluant. The eluted material was detected by measuring the absorbancy at 220 nm due to Zn–thiolate formation, the Zn content by atomic absorption spectrometry and the SH content by the Ellman method.[25] As can be seen in the chromatographic patterns shown in Fig. 8, a single peak was detected by these three methods. The amino acid ratios in an acid hydrolysate of the Zn–β-fragment complex are summarized in Table II. Met residue was partially converted to Met sulfoxide during acid hydrolysis due to the presence of Zn^{2+} ions.

From the value of Zn content and the average recovery of amino acids, it was found that the Zn content of this complex was 2.7 atoms/peptide. This value is in good agreement with

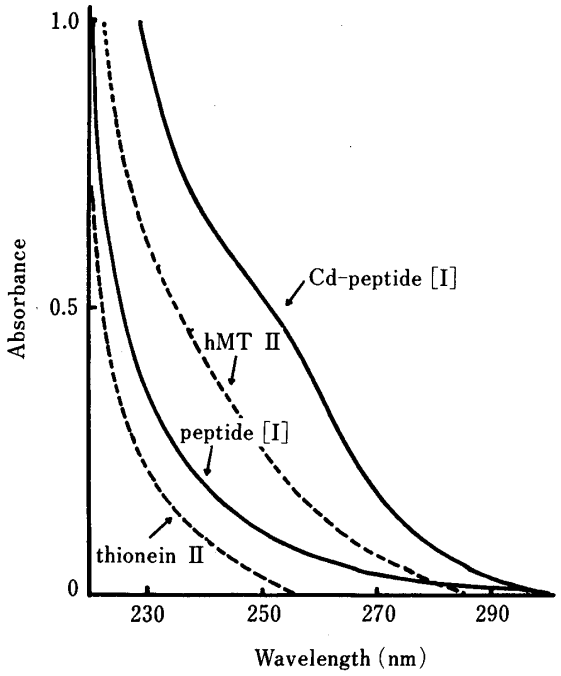


Fig. 9. Absorption Spectra of β-Fragment [I] and Cd–β-Fragment Complex

β-Fragment [I], 0.15 mM as SH content; Cd, 20 mM in 3 ml of Tris–HCl buffer (10 mM, pH 7.0). ——, β-fragment; – – – –, human thionein.[28]

the previously reported value.[27]

The ultraviolet (UV) absorptions of the β-fragment [I] and Cd–β-fragment [I] complex were similar to those of metal-free human MT and human MT, respectively[28] as shown in Fig. 9. A broad absorption shoulder at 250 nm of the Cd–β-fragment is due to Cd–thiolate formation.[28] The extinction at 250 nm is proportional to the Cd content.[28,29] By measuring the absorbance at 250 nm due to Cd–thiolate formation, the Cd^{2+}-binding abilities of the β-fragment [I] and native rat thionein were examined. The binding abilities of the β-fragment [I] and synthetic α-fragment[11] so far as examined by the method described above were superior to that of native thionein, as reported previously.[30] In the above experiment, it was observed that the β-fragment [I] was saturated with 2.7—3.0 Cd^{2+} atoms/peptide. As previously reported,[11] the synthetic α-fragment was saturated with 3.6—3.9 Cd^{2+} atoms/peptide. These values are in good agreement with the theoretically expected values.[9]

As stated above, the great majority of Cd^{2+} binds to the four-metal cluster (α-fragment), while most of Zn^{2+} binds in the three-metal cluster (β-fragment).[10] It was also reported that Cu ions bound preferentially to the β-domain (cluster B) and this order of cluster formation by Cu ions was opposite to that observed with Cd ions.[31] Synthetic α-fragment or β-fragment [I] was mixed with an excess amount of both Cu^{2+} and Cd^{2+} at equal molarities, and the resultant metal complex was separated by gel-filtration on Sephadex G-10. In the above experiment, only Cu–α-fragment complex or Cu–β-fragment complex was obtained and Cd–peptide complex was not formed. These results indicated that Cu ions bound preferentially to both fragments, demonstrating that the α-fragment and β-fragment could not discriminate between Cu^{2+} and Cd^{2+} when sufficient amounts of Cu and Cd were present at the same time. Since the affinity of Cu ions for thiol group is stronger than that of Cd ions, both fragments bind to Cu ions.

The heavy metal-binding properties of synthetic β-fragment [I] and α-fragment[11] studied here and the fact that the MT of *Neurospora crassa*[7] consist of 25 amino acid residues and is homologous with the β-fragment of mammalian MTs raise the question of why further-evolved MTs have two metal clusters. One possibility is that these two clusters might participate in heavy metal metabolism cooperatively. Further studies on systematic synthesis of Cys-containing peptides and their heavy metal-binding properties along the lines of those

reported previously[15,30,32] should provide an answer to the above question, and clarify the intrinsic role of MT.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). TLC was performed on silica gel plates (Kieselgel G, Merck) using the following solvent systems: Rf^1, $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase); Rf^2 $CHCl_3$–MeOH–AcOH (90 : 8 : 2); Rf^3, *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase); Rf^4, *n*-BuOH–pyridine–AcOH–H_2O (4 : 1 : 1 : 2); Rf^5, *n*-BuOH–pyridine–AcOH–H_2O (1 : 1 : 1 : 1). Absorption spectra were recorded with a Hitachi 323 recording spectrophotometer.

Boc–Thr–Ser–$NHNH_2$——Hydrazine hydrate (80%, 4.5 ml) was added to a solution of Boc–Thr–Ser–OBzl[15] (12.1 g) in MeOH (50 ml) and the solution was kept at room temperature overnight. The crystalline precipitate was collected by filtration and recrystallized from EtOH and ether, yield 6.31 g (64.6%), mp 147—152 °C, $[\alpha]_D^{26}$ +5.8° (c = 1.0, MeOH), Rf^1, 0.12; Rf^2, 0.36; Rf^3, 0.57. *Anal.* Calcd for $C_{12}H_{24}N_4O_6$: C, 44.9; H, 7.84; N, 17.4. Found: C, 44.6; H, 7.64; N, 17.6.

Boc–Thr–Ser–Cys(MBzl)–OBzl——Boc–Thr–Ser–N_3 (prepared from 8.5 g of the corresponding hydrazide with 4.3 ml of isopentyl nitrite in the usual manner) in DMF (80 ml) was added to a solution of 13.8 g of H–Cys(MBzl)–OBzl·TosOH in DMF (40 ml) containing Et_3N (3.7 ml) under cooling with ice. 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 10% citric acid 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 (3.3 × 40 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (100 ml each) were collected and the solvent of the desired eluate (tube Nos. 27—32) was removed by evaporation. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield, 6.8 g (41.7%), mp 42—45 °C, $[\alpha]_D^{26}$ −48.0° (c = 1.0, MeOH), Rf^1 0.78, Rf^2 0.44. *Anal.* Calcd for $C_{30}H_{41}N_3O_9S$: C, 58.1; H, 6.67; N, 6.8. Found: C, 58.1; H, 6.82; N, 6.7.

Boc–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl——Boc–Cys(MBzl)–Lys(Z)–Cys(MBzl)–N_3 (prepared from 6.8 g of the corresponding hydrazide and 1.3 ml of isopentyl nitrite in the usual manner) in DMF (40 ml) was added to a cold solution of H–Thr–Ser–Cys(MBzl)–OBzl·TFA (prepared from 5.0 g of Boc–Thr–Ser–Cys(MBzl)–OBzl and 5.9 ml of TFA containing 1.7 ml of anisole at room temperature for 2 h) in DMF (30 ml) containing Et_3N (1.4 ml). The reaction mixture was stirred at 4 °C for 3 d. 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 and ether, yield 7.9 g (73.7%), mp 130—140 °C, $[\alpha]_D^{25}$ −25.2° (c=0.5, DMF), Rf^1 0.84. *Anal.* Calcd for $C_{66}H_{85}N_7O_{16}S_3 \cdot 2H_2O$: C, 58.1; H, 6.57: N, 7.2. Found: C, 58.2; H, 6.57; N, 7.4.

Boc–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl——Boc–Glu(OBzl)–ONp (2.3 g) and H–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl·TFA [prepared from 7.0 g of Boc–(hMT II 24—29)–OBzl and 7.0 ml of TFA containing 1.2 ml of anisole at room temperature for 2 h] were dissolved in DMF (40 ml) containing Et_3N (0.84 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford precipitate, which was collected by filtration and washed with MeOH, yield 4.9 g (59.6%), mp 179—186 °C, $[\alpha]_D^{28}$ −22.6° (c=0.5, DMF), Rf^1 0.95, Rf^2 0.64. *Anal.* Calcd for $C_{78}H_{98}N_8O_{19}S_3 \cdot 2H_2O$: C, 59.2; H, 6.49; N, 7.1. Found: C, 59.2; H, 6.28; N, 7.1. Amino acid ratios in an acid hydrolysate: Thr 1.08; Ser 0.84; Glu 0.87; Lys 1.00 (average recovery 88.0%), Cys was not determined.

Boc–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl——Boc–Cys–(MBzl)–Lys(Z)–N_3 (prepared from 4.5 g of the corresponding hydrazide and 1.2 ml of isopentyl nitrite in the usual manner) was added to a solution of H–(hMT II 23—29)–OBzl [prepared from 4.5 g of Boc–(hMT II 23—29)–OBzl and 8.0 ml of TFA containing 0.65 ml of anisole in the usual manner] in DMF (40 ml) containing Et_3N (2.0 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 reprecipitated from DMF and MeOH, yield 4.3 g (72.4%), mp 238—247 °C, $[\alpha]_D^{27}$ −24.2° (c=0.5, DMF), Rf^2 0.82. *Anal.* Calcd for $C_{103}H_{129}N_{11}O_{24}S_4 \cdot 4H_2O$: C, 58.8; H, 6.56; N, 7.3. Found: C, 58.8; H, 6.57; N, 7.7. Amino acid ratios in an acid hydrolysate: Thr 1.04; Ser 0.89; Glu 0.99; Lys 2.00 (average recovery 93.7%), Cys was not determined.

Boc–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl, Boc–(hMT II 19—29)–OBzl [1]——Boc–Cys(MBzl)–Lys(Z)–N_3 (prepared from 3.7 g of the corresponding hydrazide and 0.95 ml of isopentyl nitrite in the usual manner) in DMF (40 ml) was added to a solution of H–(hMT II 21—29)–OBzl·TFA [prepared from 4.0 g of Boc–(hMT II 21—29)–OBzl and 8.0 ml of TFA containing 0.43 ml of anisole in the usual manner] in DMF (40 ml) containing Et_3N (0.42 ml). The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to yield a precipitate, which was collected by filtration and reprecipitated from DMF and MeOH, yield 4.22 g (84.8%), mp 263—272 °C, $[\alpha]_D^{27}$ −20.6° (c=1.0, DMF), Rf^2 0.77. *Anal.* Calcd for $C_{128}H_{160}N_{14}O_{29}S_5 \cdot 5H_2O$: C, 58.9; H, 6.57; N, 7.5. Found: C, 58.6; H, 6.67; N, 7.9.

Amino acid ratios in an acid hydrolysate: Thr 1.00; Ser 0.89; Glu 0.98; Lys 3.09 (average recovery 88.0%), Cys was not determined.

Boc–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–OMe——Boc–Cys(MBzl)–Thr–N_3 (prepared from 6.4 g of the corresponding hydrazide and 2.4 ml of isopentyl nitrite in the usual manner) was added to a solution of H–Cys(MBzl)–Ala–Gly–Ser–OMe·TFA (prepared from 4.0 g of Boc–Cys(MBzl)–Ala–Gly–Ser–OMe[15] and 5.2 ml of TFA containing 1.5 ml of anisole in the usual manner) in DMF (40 ml) containing Et_3N (1.3 ml). The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water. Crystals formed were collected by filtration and recrystallized from AcOEt, yield 1.7 g (27.1%), mp 205—209 °C, $[\alpha]_D^{28}$ −23.4° (c=1.0, DMF), Rf^1 0.66. *Anal.* Calcd for $C_{40}H_{58}N_6O_{13}S_2$: C, 53.6; H, 6.53; N, 9.3. Found: C, 53.5; H, 6.70; N, 9.1. Amino acid ratios in an acid hydrolysate: Thr 0.86; Ser 0.87; Gly 1.14; Ala 1.04 (average recovery 73.2%), Cys was not determined.

Boc–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–$NHNH_2$, Boc–(hMT II 13—18)–$NHNH_2$ [2]——Hydrazine hydrate (80%, 0.22 ml) was added to a solution of Boc–(hMT II 13—18)–OMe (1.0 g) in DMF (20 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 and recrystallized from EtOH, yield 0.53 g (53.0%), mp 230—235 °C, $[\alpha]_D^{27}$ −22.4° (c=0.5, DMF), Rf^1 0.45. *Anal.* Calcd for $C_{39}H_{58}N_8O_{12}S_2 \cdot 1/2H_2O$: C, 51.8; H, 6.58; N, 12.3. Found: C, 51.7; H, 6.58; N, 12.2. Amino acid ratios in an acid hydrolysate: Thr 0.88; Ser 0.80; Gly 1.08; Ala 1.00 (average recovery 82.2%), Cys was not determined.

Z–Ala–Gly–Asp–Ser–OMe——Z–Ala–Gly–N_3 (prepared from 2.2 g of Z–Ala–Gly–$NHNH_2$[17] and 0.97 ml of isopentyl nitrite) in DMF (12 ml) was added to a solution of H–Asp–Ser–OMe·HCl (prepared from 3.2 g of Z–Asp(OBzl)–Ser–OMe[16] by catalytic hydrogenation) in DMF (20 ml) containing Et_3N (2.0 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was dissolved in AcOEt; this solution was washed with 1 N HCl and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give crystals, which were collected by filtration, yield 0.45 g (12.9%), mp 96—107 °C, $[\alpha]_D^{24}$ −24.2° (c=1.0, MeOH), Rf^3 0.45. *Anal.* Calcd for $C_{21}H_{28}N_4O_{10}$: C, 50.8; H, 5.68; N, 11.3. Found: C, 51.1; H, 5.81; N, 11.2. Amino acid ratios in an acid hydrolysate: Ala 1.00; Gly 1.05; Asp 0.99; Ser 0.70 (average recovery 86.5%).

Z–Ala–Gly–Asp–Ser–$NHNH_2$, Z–(hMT II 9—12)–$NHNH_2$——Hydrazine hydrate (80%, 0.04 ml) was added to a solution of Z–Ala–Gly–Asp–Ser–OMe (0.20 g) in MeOH (10 ml). The solution was kept at room temperature overnight and concentrated to a small volume. The pH of the solution was adjusted to 4 by adding AcOH. Crystals were collected by filtration and washed with MeOH, yield 0.038 g (18.4%), mp 167—178 °C, $[\alpha]_D^{24}$ −11.0° (c=0.5, MeOH), Rf^3 0.44. *Anal.* Calcd for $C_{20}H_{28}N_6O_9 \cdot 1/2H_2O$: C, 47.5; H, 5.78; N, 16.6. Found: C, 47.9; H, 6.00; N, 16.5. Amino acid ratios in an acid hydrolysate: Ala 1.09; Gly 1.00; Asp 1.04; Ser 0.91 (average recovery 96.0%).

Boc–Ala–Gly–$NHNH_2$——Boc–Ala–OH (7.6 g), H–Gly–OMe (prepared from 5.0 g of H–Gly–OMe·HCl and 5.6 ml of Et_3N) and HOBt (5.4 g) were dissolved in CH_3CN (25 ml) and DMF (25 ml) and cooled with ice-salt. DCC (9.9 g) was added to the cold solution 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, 10% citric acid and water, dried over Na_2SO_4 and evaporated down (Rf^2 0.64, 9.7 g, 93.3%). Hydrazine hydrate (80%, 3.6 ml) was added to a solution of Boc–Ala–Gly–OMe (9.7 g) in MeOH (50 ml) and the solution was kept at room temperature overnight. Crystals formed were collected by filtration and recrystallized from EtOH, yield 7.2 g (74.2%), mp 143—145 °C, $[\alpha]_D^{24}$ −4.9° (c=1.0, DMF), Rf^1 0.48. *Anal.* Calcd for $C_{10}H_{20}N_4O_4$: C, 46.1; H, 7.75; N, 21.5. Found: C, 46.0; H, 7.71; N, 21.3.

Boc–Ala–Gly–Asp(OBzl)–Ser–OMe——Boc–Asp(OBzl)–OH (3.1 g) and H–Ser–OMe (prepared from 6.5 g of H–Ser–OMe·HCl and 2.8 ml of Et_3N) were dissolved in DMF (40 ml) and cooled with ice-salt. DCC (4.1 g) was added to the solution 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 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down to give an oily material, yield 7.0 g (82.7%), Rf^1 0.66. This material was converted to the corresponding amine, H–Asp(OBzl)–Ser–OMe, by treatment with TFA–anisole. Boc–Ala–Gly–N_3 (prepared from 5.2 g of Boc–Ala–Gly–$NHNH_2$ and 3.2 ml of isopentyl nitrite as usual) in DMF (40 ml) was added to a solution of H–Asp(OBzl)–Ser–OMe·TFA in DMF (40 ml) containing Et_3N (2.31 ml) under cooling with ice-salt. 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 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether and ether were added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 3.1 g (33.5%), mp 147—150 °C, $[\alpha]_D^{31}$ −26.7° (c=1.0, MeOH), Rf^1 0.59. *Anal.* Calcd for $C_{25}H_{36}N_4O_{10}$: C, 54.3; H, 6.57; N, 10.1. Found: C, 54.7; H, 6.66; N, 10.1. Amino acid ratios in an acid hydrolysate: Asp 1.12; Ser 0.80; Gly 1.00; Ala 1.00 (average recovery 88.2%).

Boc–Ala–Gly–Asp–Ser–OMe——a) Boc–Ala–Gly–N_3 (prepared from 6.3 g of Boc–Ala–Gly–$NHNH_2$ and 3.9 ml of isopentyl nitrite as usual) in DMF (40 ml) was combined with H–Asp–Ser–OMe·HCl (4.3 g) in DMF (30 ml) containing Et_3N (2.8 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 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether and ether were added to the residue to afford crystals, which were collected by

filtration and recrystallized from AcOEt and ether, yield 0.35 g (3.8%), mp 120—127 °C, $[\alpha]_D^{25}$ −33.8° (c=0.5, MeOH), Rf^3 0.50. *Anal.* Calcd for $C_{18}H_{30}N_4O_{10}$: C, 46.8; H, 6.54; N, 12.1. Found: C, 47.1; H, 6.60; N, 11.8.

b) Boc–Ala–Gly–Asp(OBzl)–Ser–OMe (1.0 g) in MeOH (50 ml) was hydrogenated over a Pd catalyst for 18 h. After removal of Pd and the solvent, petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 0.74 g (88.9%), mp 127—134 °C, $[\alpha]_D^{22}$ −35.8° (c=0.5, MeOH), Rf^3 0.50. *Anal.* Calcd for $C_{18}H_{30}N_4O_{10}$: C, 46.8; H, 6.54; N, 12.1. Found: C, 47.3; H, 6.87; N, 11.8.

c) Boc–Ala–Gly–Asp(OBzl)–Ser–OMe (1.0 g) was dissolved in EtOH (25 ml) and cyclohexene (25 ml) containing Pd (0.8 g). The reaction mixture was stirred at 40 °C for 15 min. After removal of Pd and the solvent, petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 0.8 g (94.4%), mp 126—132 °C, $[\alpha]_D^{22}$ −35.4° (c=0.5, MeOH), Rf^3 0.50. *Anal.* Calcd for $C_{18}H_{30}N_4O_{10}$: C, 46.8; H, 6.54; N, 12.1. Found: C, 47.0; H, 6.60; N, 11.8.

Boc–Ala–Gly–Asp–Ser–NHNH$_2$, Boc–(hMT II 9—12)–NHNH$_2$ [4]——a) Hydrazine hydrate (80%, 0.43 ml) was added to a solution of 1.2 g of Boc–Ala–Gly–Asp–Ser–OMe in MeOH (20 ml). The reaction mixture was allowed to stand at room temperature overnight to give crystals. The crude material in 5% AcOH (5 ml) was applied to a column of Sephadex G-25 (2.3 × 108 cm), equilibrated and eluted with the same solvent. Individual fractions (3 g each) were collected. The solvent of the desired effluent fractions (Nos. 148—152) was removed by evaporation followed by lyophilization to give an amorphous powder, yield 0.40 g (38.4%), $[\alpha]_D^{25}$ −28.3° (c=0.8, AcOH), Rf^4 0.47. *Anal.* Calcd for $C_{17}H_{30}N_6O_9 \cdot CH_3COOH \cdot H_2O$: C, 42.2; H, 6.71; N, 15.5. Found: C, 42.1; H, 6.59; N, 15.3. Amino acid ratios in an acid hydrolysate: Asp 1.00; Ser 0.78; Gly 0.87; Ala 1.00 (average recovery 78.0%). After removal of the solvent of the effluent (Nos. 153—162), AcOEt was added to the residue to give crystals, which were collected by filtration, yield 0.50 g (43.5%). The crystals thus obtained exhibited two spots on TLC (ninhydrin test, hydrazine test positive) Rf^4 0.26, 0.47. The compound having the lower Rf value (0.26) corresponds to the dihydrazide (mixture of α and β positions of the Asp residue). The authentic dihydrazide, Boc–Ala–Gly–Asp(NHNH$_2$)–Ser–NHNH$_2$, was derived from Boc–Ala–Gly–Asp(OBzl)–Ser–OMe by treatment with hydrazine hydrate: yield 37.8%, mp 197—200 °C, $[\alpha]_D^{25}$ −22.4° (c=1.0, AcOH), Rf^4 0.26. *Anal.* Calcd for $C_{17}H_{32}N_8O_8 \cdot 1/2H_2O$: C, 42.1; H, 6.85; N, 23.1. Found: C, 42.3; H, 6.75; N, 23.0. Amino acid ratios in an acid hydrolysate: Asp 1.00; Ser 0.88; Gly 1.06; Ala 1.02 (average recovery 73.0%).

b) Silica Gel Column: Boc–Ala–Gly–Asp–Ser–OMe (1.5 g) was converted to the hydrazide. The crude material obtained was applied to a column of silica gel (2 × 40 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (10 g each) were collected. After removal of the solvent of the desired effluent (fraction Nos. 8—13), the residue in 5% AcOH (5 ml) was applied to a column of Sephadex G-25 (2.3 × 108 cm), equilibrated and eluted with 5% AcOH. Individual fractions (5 g each) were collected and the solvent of the desired effluent (fraction Nos. 46—55) was removed by evaporation, followed by lyophilization to give an amorphous powder, yield 0.70 g (46.6%), $[\alpha]_D^{25}$ −31.0° (c=0.7, AcOH), Rf^4 0.47. *Anal.* Calcd for $C_{17}H_{30}N_6O_9 \cdot 3/2H_2O$: C, 41.7; H, 6.79; N, 17.2. Found: C, 41.9; H, 6.54; N, 17.2. Amino acid ratios in an acid hydrolysate: Asp 1.07; Ser 0.90; Gly 1.00; Ala 1.00 (average recovery 75%).

Boc–Asn–Cys(MBzl)–Ser–Cys(MBzl)–Ala–NHNH$_2$, Boc–(hMT II 4—8)–NHNH$_2$ [6]——Hydrazine hydrate (80%, 0.39 ml) was added to a solution of Boc–(hMT II 4—8)–OBzl[14] (1.78 g) in DMF (20 ml). The reaction mixture was stored at room temperature overnight. After removal of the solvent, MeOH was added to the residue to give crystals, which were collected by filtration and washed with EtOH, yield 1.20 g (71.2%), mp 237—242 °C, $[\alpha]_D^{32}$ −37.0° (c=0.5, DMF), Rf^1 0.50. *Anal.* Calcd for $C_{37}H_{54}N_8O_{11}S_2$: C, 52.2; H, 6.40; N, 13.2. Found: C, 51.8; H, 6.40; N, 13.1. Amino acid ratios in an acid hydrolysate: Asp 1.10; Ser 0.95; Ala 1.00 (average recovery 92.0%), Cys was not determined.

Ac–Met–NHNHBoc——Ac–Met–OH (6.0 g), Boc–NHNH$_2$ (4.2 g) and HOBt (4.2 g) were dissolved in DMF (30 ml) and cooled with ice-salt. DCC (7.7 g) was added to the cold solution and the reaction mixture was stirred at room temperature overnight. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford an amorphous powder. This powder in $CHCl_3$ (5 ml) was applied to a silica gel column (3.8 × 43 cm), equilibrated and eluted with $CHCl_3$, followed by 1% MeOH in $CHCl_3$. After evaporation of the latter effluent (1600—4000 ml), petroleum ether was added to the residue to afford an amorphous powder, yield 4.0 g (41.9%), $[\alpha]_D^{29}$ −35.2° (c=0.8, MeOH), Rf^2 0.45. *Anal.* Calcd for $C_{12}H_{23}N_3O_4S$: C, 47.2; H, 7.59; N, 13.8. Found: C, 47.0; H, 7.64; N, 13.6.

Ac–Met–NHNH$_2$——a) H–Met–OMe·HCl (9.5 g) was dissolved in $CHCl_3$ (100 ml) containing Et_3N (7.0 ml) and cooled with ice. Acetic anhydride (4.7 ml) was added to the above solution. The reaction mixture was stirred at 0 °C for 1 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated down to give an oily residue (Rf^2 0.60, I_2 and H_2PtCl_6–KI positive and ninhydrin negative). The residue was dissolved in MeOH (30 ml). Hydrazine hydrate (5.0 ml) was added to the above solution. The reaction mixture was stored at room temperature overnight and concentrated to a small volume. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH and ether twice, yield 2.0 g (10.9%), $[\alpha]_D^{29}$ −21.4° (c=1.0, MeOH), Rf^1 0.47, Rf^2 0.25 (I_2, H_2PtCl_6–KI and hydrazine test positive). *Anal.* Calcd for $C_7H_{15}N_3O_2S$: C, 41.2; H, 7.40; N, 20.6. Found: C, 41.0; H, 7.18; N, 20.3.

b) A solution of Ac–Met–NHNHBoc (3.0 g) in TFA (5.0 ml) containing anisole (2.2 ml) was stored at room temperature for 1.5 h. Addition of ether gave a precipitate, which was collected by filtration, washed with ether and dried over KOH pellets *in vacuo*, yield 1.8 g (86.7%), mp 128—133 °C, Rf^1 0.47, Rf^2 0.25.

H–Asp–Pro–NHNHBoc·HCl——Z–Asp(OBzl)–Pro–NHNHBoc (2.3 g) in EtOH (50 ml) and H_2O (50 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, the residue was dissolved in H_2O (10 ml) containing 1 N HCl (4 ml) and lyophilized, yield 1.5 g (95%), $[\alpha]_D^{29}$ −78.8° (c=0.6, MeOH), Rf^3 0.14, Rf^4 0.51. *Anal.* Calcd for $C_{14}H_{24}N_4O_6 \cdot HCl \cdot H_2O$: C, 42.2; H, 6.82; N, 14.0. Found: C, 42.5; H, 6.84; N, 14.3.

Ac–Met–Asp–Pro–NHNHBoc, Ac–(hMT II 1—3)–NHNHBoc——Ac–Met–N_3 (prepared from 0.82 g of Ac–Met–$NHNH_2$ and 0.56 ml of isopentyl nitrite as usual) in DMF (5 ml) was added to a cold DMF solution (30 ml) of H–Asp–Pro–NHNHBoc·HCl (1.5 g) containing Et_3N (0.87 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was dissolved in H_2O (30 ml), and the pH of the solution was adjusted to 5.0 with AcOH. The water layer was washed with ether (60 ml × 2) and saturated with NaCl. The desired compound was extracted with AcOEt from the water layer. The AcOEt layer was dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a solid material, which was collected by decantation and washed with ether, yield 1.8 g (88.0%), amorphous powder, $[\alpha]_D^{29}$ −110.7° (c=0.9, MeOH), Rf^3 0.46, Rf^4 0.63. *Anal.* Calcd for $C_{21}H_{35}N_5O_8S \cdot 1/2H_2O$: C, 47.9; H, 6.89; N, 13.3. Found: C, 47.9; H, 7.09; N, 12.9. Amino acid ratios in an acid hydrolysate: Asp 1.06; Met 0.81; Pro 1.00 (average recovery 78.0%). NMR data are summarized in Fig. 6.

Ac–Met–Asp–Pro–$NHNH_2$, Ac–(hMT II 1—3)–$NHNH_2$ [8]——a) TFA Method: Ac–Met–Asp–Pro–NHNHBoc (10 mg) was dissolved in TFA (0.1 ml) containing thioanisole (0.02 ml) or anisole (0.02 ml) and mercaptoethanol (0.1 ml). The solution was kept at room temperature for 1 h. Peroxide-free dry ether was added to the solution to give a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*, yield 6.0 mg (57.9%), mp 135—142 °C, $[\alpha]_D^{28}$ −90.0° (c=0.1, MeOH), Rf^1 0.04 (thioanisole or anisole), Rf^3 0.50 (thioanisole or anisole). *Anal.* Calcd for $C_{18}H_{27}N_5O_6 \cdot CF_3COOH \cdot 3H_2O$: C, 38.1; H, 6.01; N, 11.6. Found: C, 38.4; H, 5.86; N, 11.5. Amino acid ratios in an acid hydrolysate: Asp 1.26; Met 0.80; Pro 1.00 (average recovery 82.0%, thioanisole), Asp 0.98; Met 0.92; Pro 1.11 (average recovery 86.0%, anisole). The NMR data are summarized in Fig. 6.

b) HCl–Dioxane Method: Ac–Met–Asp–Pro–NHNHBoc (10 mg) was dissolved in 3 N HCl–dioxane (0.45 ml) containing thioanisole (0.02 ml) or anisole (0.02 ml) and mercaptoethanol (0.1 ml). The solution was kept at room temperature for 1 h. Peroxide-free dry ether was added to the solution to give a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*, yield 6.5 mg (65.8%), mp 125—155 °C, Rf^1 0 (main), 0.67 (minor), 0.82 (minor) (thioanisole); 0 (main), 0.63 (minor), 0.88 (minor) (anisole), Rf^3 0.50 (main), 0.75 (minor) (thioanisole and anisole). Amino acid ratios in an acid hydrolysate: Asp 0.96; Met 0.90; Pro 1.13 (average recovery 75%, thioanisole), Asp 0.94; Met 0.93; Pro 1.10 (average recovery 80%, anisole).

c) HF Method: A solution of Ac–Met–Asp–Pro–NHNHBoc (50 mg) in anhydrous HF (5 ml) containing thioanisole (0.24 ml) or *m*-cresol (0.20 ml) was stirred at 0 °C for 1 h. After removal of HF by evaporation, peroxide-free dry ether was added to the residue to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*. The crude material was dissolved in H_2O (10 ml) containing Amberlite IRA-45 (5 g) and the mixture was stirred at room temperature for 1 h. The resin was collected by filtration. The desired peptide was extracted from the resin with 3% AcOH and the AcOH solution was lyophilized to give Ac–Met–Asp–Pro–$NHNH_2 \cdot CH_3COOH$, yield 35 mg (68%), Rf^3 0.50 (ninhydrin negative, H_2PtCl_6–KI test and hydrazine test positive, thioanisole or *m*-cresol). Amino acid ratios in an acid hydrolysate: Asp 1.20; Met 0.89; Pro 0.91 (average recovery 85.0%, thioanisole); Asp 1.09; Met 0.80; Pro 0.91 (average recovery 76%, *m*-cresol). The NMR data are summarized in Fig. 6.

Boc–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl, Boc–(hMT II 13—29)–OBzl [3]——Boc–(hMT II 19—29)–OBzl (1.5 g) was dissolved in TFA (4 ml) containing anisole (0.25 ml) and the solution was kept at room temperature for 2 h. Ether was added to the solution to form a precipitate, which was collected by decantation, washed with ether and dried over KOH pellets *in vacuo*. H–(hMT II 19—29)–OBzl·TFA obtained was dissolved in DMF (25 ml) containing Et_3N (0.14 ml). Boc–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–N_3 (prepared from 1.2 g of the corresponding hydrazide and 0.19 ml of isopentyl nitrite in the usual manner) in DMF (50 ml) was combined with the above cold solution and 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 reprecipitated from DMF and MeOH, yield 1.2 g (61.7%), mp 300 °C, $[\alpha]_D^{31}$ −23.0° (c=0.5, DMSO), Rf^3 0.81. *Anal.* Calcd for $C_{162}H_{206}N_{20}O_{39}S_7 \cdot 2H_2O$: C, 58.6; H, 6.38; N, 8.4. Found: C, 58.5; H, 6.25; N, 8.6. Amino acid ratios in an acid hydrolysate: Thr 1.87; Ser 1.72; Glu 1.15; Ala 1.09; Lys 3.00 (average recovery 86.0%); Cys was not determined.

Boc–Ala–Gly–Asp–Ser–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl, Boc–(hMT II 9—29)–OBzl [5]——Boc–(hMT II 13—29)–OBzl (0.90 g) was dissolved in TFA (5.0 ml) containing anisole (0.1 ml), and the reaction mixture was kept at room temperature for 2 h. Addition of ether to the solution gave a precipitate, which was collected by decantation, washed with ether and dried over KOH pellets *in vacuo*. Boc–Ala–Gly–Ser–N_3 (prepared from 0.6 g of **4**

and 0.23 ml of isopentyl nitrite as usual) in DMF (20 ml) was added to a solution of H–(hMT II 13—29)–OBzl·TFA obtained above in DMF (30 ml) containing Et_3N (0.049 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to give a solid mass, which was collected by filtration and reprecipitated from DMF and MeOH. The solid material was further washed with 5% citric acid and MeOH, yield 0.52 g (53.7%), mp 300 °C, $[\alpha]_D^{29}$ −32.0° ($c=0.1$, DMSO), Rf^4 0.78. *Anal.* Calcd for $C_{174}H_{224}N_{24}O_{46}S_7 \cdot 5H_2O$: C, 56.5; H, 6.37; N, 9.1. Found: C, 56.4; H, 6.28; N, 9.4. Amino acid ratios in an acid hydrolysate: Asp 1.17; Thr 1.60; Ser 2.26; Glu 0.86; Gly 2.22; Ala 2.20; Lys 2.82 (average recovery 84.6%), Cys was not determined.

Boc–Asn–Cys(MBzl)–Ser–Cys(MBzl)–Ala–Ala–Gly–Asp–Ser–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl, Boc–(hMT II 4—29)–OBzl [7]——Boc–Asn–Cys(MBzl)–Ser–Cys(MBzl)–Ala–N_3 (prepared from 0.28 g of Boc–(hMT II 4—8)–$NHNH_2$ and 0.05 ml of isopentyl nitrite as usual) in DMF (10 ml) was added to a solution of H–(hMT II 9—29)–OBzl·TFA [prepared from 0.30 g of Boc–(hMT II 9—29)–OBzl and 1.4 ml of TFA containing 0.10 ml of anisole] in DMF (20 ml) containing Et_3N (0.017 ml). The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to give a precipitate, which was collected by filtration and reprecipitated from DMF and MeOH, yield 0.23 g (63.9%), mp 300 °C, $[\alpha]_D^{30}$ −33.0° ($c=0.1$, DMSO), Rf^4 0.56, Rf^5 0.65. *Anal.* Calcd for $C_{206}H_{266}N_{30}O_{55}S_9 \cdot 7H_2O$: C, 55.5; H, 6.33; N, 9.4. Found: C, 55.3; H, 6.05; N, 9.6. Amino acid ratios in an acid hydrolysate: Asp 2.12; Thr 1.69; Ser 3.23; Glu 0.82; Gly 2.28; Ala 3.30; Lys 3.00 (average recovery 79.1%); Cys was not determined.

Ac–Met–Asp–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–Ala–Ala–Gly–Asp–Ser–Cys(MBzl)–Thr–Cys(MBzl)–Ala–Gly–Ser–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Lys(Z)–Glu(OBzl)–Cys(MBzl)–Lys(Z)–Cys(MBzl)–Thr–Ser–Cys(MBzl)–OBzl, Ac–(hMT II 1—29)–OBzl [9]——Ac–Met–Asp–Pro–N_3 (prepared from 0.2 g of Ac–Met–Asp–Pro–$NHNH_2$ and 0.06 ml of isopentyl nitrite as usual) in DMF (6 ml) was added to a DMF (20 ml) solution of H–(hMT II 4—29)–OBzl [prepared from 0.35 g of Boc–(hMT II 4—29)–OBzl, 1.5 ml of TFA and 0.18 ml of anisole as usual]. 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, washed with 10% citric acid, water and MeOH and reprecipitated from DMF and MeOH, yield 0.25 g (66.8%), mp 300 °C (dec.), $[\alpha]_D^{30}$ −52.0° ($c=0.1$, DMSO), Rf^5 0.76. *Anal.* Calcd for $C_{217}H_{281}N_{33}O_{59}S_{10} \cdot 7H_2O$: C, 55.0; H, 6.27; N, 9.8. Found: C, 54.7; H, 6.10; N, 10.1. Amino acid ratios in an acid hydrolysate: Asp 3.25; Thr 1.65; Ser 3.27; Glu 0.96; Ala 3.10; Met 0.95; Lys 3.00; Pro 0.95 (average recovery 77.7%), Cys was not determined.

Ac–Met–Asp–Pro–Asn–Cys–Ser–Cys–Ala–Ala–Gly–Asp–Ser–Cys–Thr–Cys–Ala–Gly–Ser–Cys–Lys–Cys–Lys–Glu–Cys–Lys–Cys–Thr–Ser–Cys–OH, Ac–(hMT II 1—29)–OH, β-Fragment [I]——Ac–(hMT II 1—29)–OBzl (70 mg) was treated with anhydrous HF (10 ml) containing *m*-cresol (0.47 ml) and thioanisole (0.11 ml) at 0 °C for 1 h. After removal of HF, the residue was dried over KOH pellets *in vacuo* overnight. Ether was added to the residue to afford a white precipitate, which was collected by filtration, washed with ether and dried over KOH pellets. This was dissolved in oxygen-free water (10 ml) and treated with Amberlite IRA-45 (acetate form, *ca.* 10 g). After removal of the resin, the filtrate was lyophilized. The white powder in H_2O (5 ml) was reduced with dithiothreitol (0.23 g) and 2-mercaptoethanol (0.32 ml) at room temperature overnight under N_2 gas. The reaction mixture was applied to a column of Sephadex G-15 (2 × 47 cm), equilibrated and eluted with 3% AcOH. Individual fractions (3 g each) were collected. The desired fractions (tube Nos. 17—19) were combined and lyophilized to afford a white fluffy powder, yield 14 mg (31.3%), $[\alpha]_D^{28}$ −84.6° ($c=0.1$, 3% AcOH), Rf^4 0.41, Rf^5 0.80 (ninhydrin, H_2PtCl_6–KI test and nitroprusside test positive). *Anal.* Calcd for $C_{107}H_{178}N_{32}O_{45}S_{10} \cdot 3CH_3COOH \cdot 10H_2O$: C, 41.0; H, 6.39; N, 13.5. Found: C, 40.9; H, 6.09; N, 13.6. SH content: 8.7/peptide. Amino acid ratios in an acid hydrolysate are summarized in Table II.

Experimental Procedure for Formation of Heavy Metal–Peptide Complex——Synthesis of Zn–β-Fragment [I] Complex: All operations were carried out under an N_2 gas atmosphere using oxygen-free water. β-Fragment [I, 3 μmol as SH] was added to 2 ml of water containing $ZnCl_2$ (6 μmol). The pH of the solution was adjusted to 6.0 by adding 0.1 N NaOH and the clear solution was stored at room temperature for 5 min. The solution was then applied to a column of Sephadex G-10 (1.5 × 48 cm), equilibrated and eluted with Tris–HCl buffer (0.1 mM, pH 8.0). Fractions of 2 ml each were collected (see Fig. 3). The eluted material was examined by measuring the UV absorbance at 220 nm, the Zn content by atomic absorption spectrometry, and the SH content by the Ellman method. Tube Nos. 15—17 were collected. One-half of the effluent was hydrolyzed at 110 °C for 20 h after adding 0.5 ml of conc. HCl and the acid hydrolysate was analyzed. The amino acid ratios are summarized in Table II. Zn content was 2.7 atoms/β-fragment [I].

Reaction of Peptide with Cu and Cd Ions: Synthetic α-fragment[11] or β-fragment [I] (5 mg each) was dissolved in H_2O (2 ml). Cu^{2+} and Cd^{2+} (6.68 μmol each) were added to the above solution at the same time. This reaction mixture was stored at room temperature for 5 min, and the clear supernatant was applied to a column of Sephadex G-10 (1.5 × 28 cm), equilibrated and eluted with Tris–HCl buffer (10 mM, pH 7.0). Only Cu–α-fragment complex and Cu–β-fragment [I] complex were obtained. Amino acid ratios in acid hydrolysates: Cu–α-fragment complex: Asp 1.21; Ser 3.76; Glu 1.01; Pro 0.88; Gly 3.17; Ala 4.00; Val 0.82; Ile 0.85; Lys 4.79; Cys was not determined. Cu–β-fragment complex: Asp 3.15; Thr 1.66; Ser 3.67; Glu 0.85; Pro 0.85; Gly 2.00; Ala 3.07; Lys 3.00; Met was oxidized to

Met sulfoxide due to the presence of Cu ions; Cys was not determined. The chromatographic pattern in this experiment was reported previously.[30]

References and Notes

1) Part XII: Y. Okada, N. Teno, J. Miyao, Y. Mori, and M. Irie, *Chem. Pharm. Bull.*, **32**, 4585 (1984).
2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; OMe, methyl ester; OBzl, benzyl ester; MBzl, 4-methoxybenzyl; ONp, *p*-nitrophenyl ester; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Et_3N, triethylamine; TFA, trifluoroacetic acid; AcOH, acetic acid; AcOEt, ethyl acetate; *n*-BuOH, *n*-butanol; hMT, human liver metallothionein.
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