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Amino Acids and Peptides. VIII.[1,2] Synthesis of a Hexacosapeptide corresponding to the C-Terminal Sequence 36—61 of Human Metallothionein II (hMT II) and Determination of Its Heavy Metal Binding Activity

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A C-terminal hexacosapeptide corresponding to residues 36—61 of human liver metallothionein (hMT II) was synthesized by the fragment condensation method using the azide procedure. Protecting groups were removed by the methanesulfonic acid (MSA) method or hydrogen fluoride (HF) method to give the desired peptide. The binding ability of this peptide with Cd and Zn was examined by measuring the absorbance in the ultraviolet region (200—260 nm) as a function of heavy metal concentration and by the gel-filtration method. The heavy metal-binding behavior of this peptide is quite similar to that of thionein.

Keywords——human liver metallothionein; C-terminal hexacosapeptide; chemical synthesis; MSA deprotection; HF deprotection; heavy metal binding

Metallothionein, a cadmium and zinc-containing, cysteine-rich protein of low molecular weight, was recognized in horse renal cortex by Margoshes and Vallee in 1957.[3,4] Similar proteins were found to occur not only in vertebrates but also in invertebrates. The complete amino acid sequences of these proteins from human beings,[5,6] horses,[7] mice,[8] *Neurospora crassa*,[9] and *Scylla serrata* (cram)[10] have been reported, and are shown in Fig. 1. In all mammalian metallothioneins, the polypeptide chain contains 60—61 amino acid residues, among which 20 are cysteines, and it is characteristic that seven Cys–X–Cys (X=amino acid

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        1                   5                  10                  15                  20
I   Ac-Met-Asp-Pro-Asn-Cys-Ser-Cys-Ala-Ala-Gly-Asp-Ser-Cys-Thr-Cys-Ala-Gly-Ser-Cys-Lys-
II  Ac-Met-Asp-Pro-Asn-Cys-Ser-Cys-Val-Ala-Gly-Glu-Ser-Cys-Thr-Cys-Ala-Gly-Ser-Cys-Lys-
III Ac-Met-Asp-Pro-Asn-Cys-Ser-Cys-Ser-Thr-Gly-Gly-Ser-Cys-Thr-Cys-Thr-Ser-Ser-Cys-Ala-
IV      H-Pro-Gly-Pro-Cys-   -Cys-       -Asn-Asp-Lys-Cys-Var-Cys-Lys-Glu-Gly-   -Gly-
V           H-Gly-Asp-Cys-Gly-Cys-Ser-Gly-Ala-Ser-Ser-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-

        21                  25                  30                  35                  40
I   Cys-Lys-Glu-Cys-Lys-Cys-Thr-Ser-Cys-Lys-Lys-Ser-Cys-Cys-Ser-Cys-Cys-Pro-Val-Gly-
II  Cys-Lys-Gln-Cys-Arg-Cys-Ala-Ser-Cys-Lys-Lys-Ser-Cys-Cys-Ser-Cys-Cys-Pro-Val-Gly-
III Cys-Lys-Asp-Cys-Lys-Cys-Thr-Ser-Cys-Lys-Lys-Ser-Cys-Cys-Ser-Cys-Cys-Pro-Val-Gly-
IV  Cys-Lys-Glu* Cys-Gln-Cys-Thr-Ser-Cys-Arg-Cys-Ser-Pro-Cys-Glu-Lys-Cys-Ser-Ser-Gly-
V   Cys-Ser-Asn-Cys-Gly-Ser-Lys-OH

        41                  45                  50                  55                  60
I   Cys-Ala-Lys-Cys-Ala-Gln-Gly-Cys-Ile-Cys-Lys-Gly-Ala-Ser-Asp-Lys-Cys-Ser-Cys-Cys-Ala-OH
II  Cys-Ala-Lys-Cys-Ala-Gln-Gly-Cys-Val-Cys-Lys-Gly-Ala-Ser-Asp-Lys-Cys-Cys-Ser-Cys-Ala-OH
III Cys-Ser-Lys-Cys-Ala-Gln-Gly-Cys-Val-Cys-Lys-Gly-Ala-Ala-Asp-Lys-Cys-Thr-Cys-Cys-Ala-OH
IV  Cys-   -Lys-Cys-Ala-Asn-Lys-Glu-Glu-Cys-Ser-Lys-Thr-Cys-Ser-Lys#Cys-Ser-Cys-Cys-Pro-Thr-OH
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I, human hepatic MT-II; II, horse renal MT-1B; III, mouse hepatic MT-I; IV, *Scylla serrata* MT, * Gly, # Ala, V, *Neurospora crassa* MT

Fig. 1. Primary Structures of Metallothioneins (MT)

residue other than Cys) sequences are found. Although the normal physiological role of thionein is not yet well understood, one important role is to protect against heavy metal (Cd and Hg) poisoning and another is to store essential element such as Zn and Cd, thus maintaining metal homeostasis.[11] In order to identify the physiological roles of the thionein, it is important to study the relationship between the structure of the peptide and metal binding ability. A program was initiated in our laboratory directed to total synthesis of human hepatic metallothionein II (hMT II)[5,6] and to determining the relationship between the structure of Cys-containing peptide fragments and metal-binding activity with the object of clarifying the physiological roles of hMT II in metal metabolism and obtaining Cys-containing peptides for therapeutic use in case of heavy metal poisoning. In this paper, we wish to report the synthesis of the C-terminal hexacosapeptide (residues 36—61) of human metallothionein II (hMT II) with a revised amino acid sequence.[6]

The C-terminal hexacosapeptide was synthesized *via* the route shown in Fig. 2. In order to construct the peptide, starting with the C-terminal heptapeptide ester, H–(hMT 55—61)–OBzl, four peptide fragments were coupled successively by means of the azide procedure[12] in order to minimize racemization. Amino acid derivatives bearing protecting groups removable by treatment with HF at 0°C for 60 min,[13] or methanesulfonic acid (MSA) at 20°C for 30 min,[14] *i.e.* Asp(OBzl), Lys(Z) and Cys(MBzl), were employed in combination with the TFA-labile Boc-group as the N^{α}-protecting group.

Peptide fragment hydrazides except for [2] and [4] were prepared through the corresponding benzyl ester, which could be removed by HF or MSA, in order to obtain Cys-containing peptide fragments for studies on heavy metal-binding ability.

The synthetic scheme for the C-terminal-protected heptapeptide ester, Boc–(hMT 55—61)–OBzl, is illustrated in Fig. 3. Starting with H–Cys(MBzl)–Cys(MBzl)–Ala–OBzl, H–(hMT 59—61)–OBzl, prepared by the azide coupling of H–Ala–OBzl with Boc–Cys(MBzl)–Cys(MBzl)–$NHNH_2$ followed by selective deblocking with TFA, Boc–Cys(MBzl)–Ser–$NHNH_2$, Boc–Lys(Z)–ONp and Boc–Asp(OBzl)–ONp were coupled successively to afford the peptide, Boc–(hMT

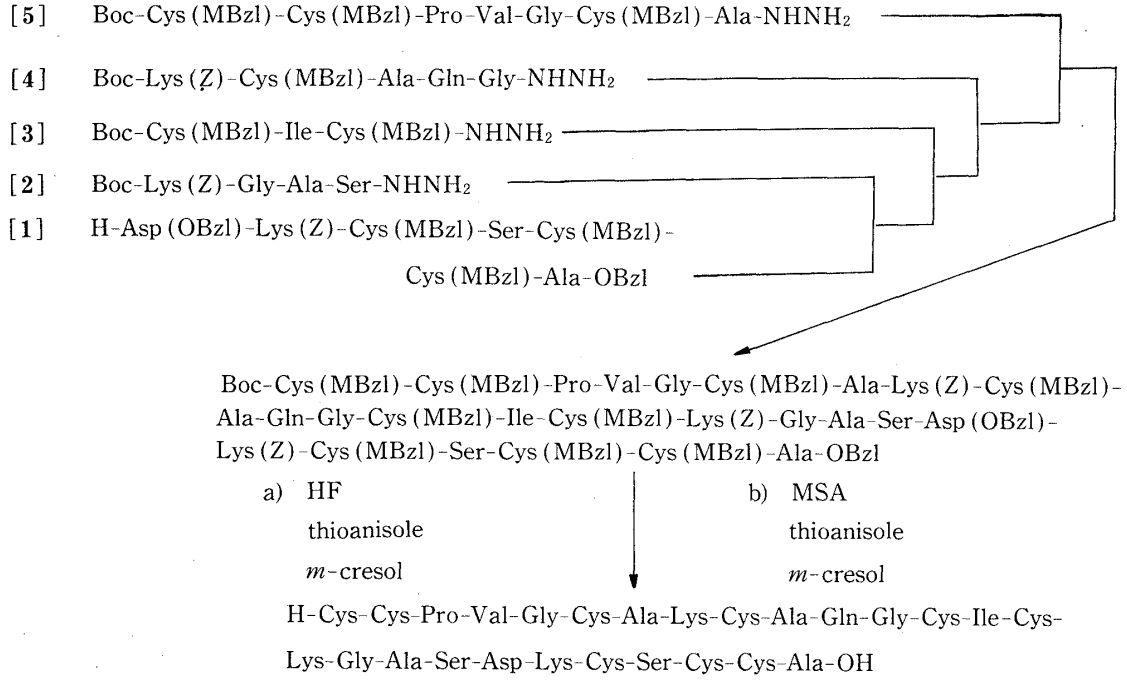

Fig. 2. Synthetic Route to the C-Terminal Hexacosapeptide of Human Metallothionein II, H-(hMT 36—61)-OH

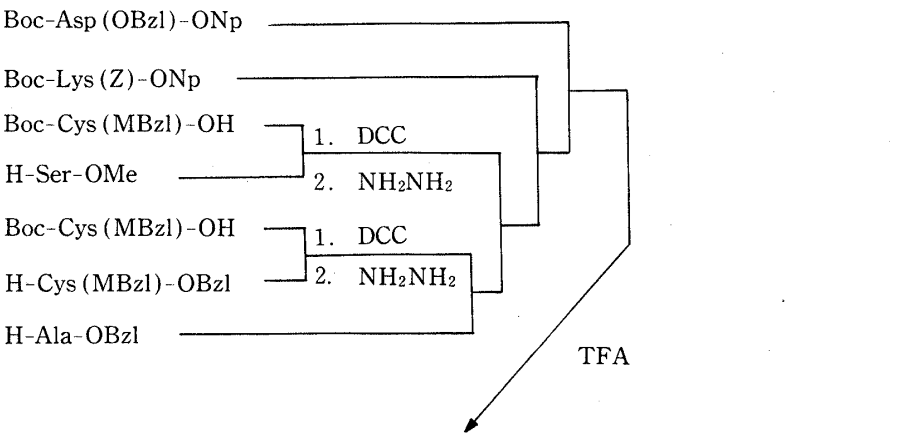

Fig. 3. Synthetic Scheme for the Heptapeptide Ester, H-(hMT 55—61)-OBzl [1]

55—61)–OBzl, which was converted to the corresponding amine [**1**] by treatment with TFA. The fragment Boc–(hMT 51—54)–$NHNH_2$ [**2**] was synthesized as follows. Z–Ala–OH, Z–Gly–OH were coupled successively with H–Ser–OMe by the DCC method and Boc–Lys(Z)–ONp was introduced into the peptide. The resulting ester was converted to the corresponding hydrazide [**2**] in the usual manner. The fragment Boc–(hMT 48—50)–$NHNH_2$ [**3**] was prepared as follows. Boc–Ile–OH and H–Cys(MBzl)–OBzl were coupled by the use of DCC–HOBt to give Boc–Ile–Cys(MBzl)–OBzl. After removal of the Boc group by TFA, the resulting dipeptide ester was coupled with Boc–Cys(MBzl)–ONp to afford Boc–Cys(MBzl)–Ile–Cys–(MBzl)–OBzl, which was smoothly converted to the corresponding hydrazide by hydrazine hydrate treatment. The fragment Boc–(hMT 43—47)–$NHNH_2$ [**4**] was prepared as shown in Fig. 4. C-Terminal peptide, Z–Gln–Gly–OMe, was prepared by the coupling of Z–Gln–OH with H–Gly–OMe by the DCC method. No CN bond vibration[15] was observed in the IR spectrum of this starting material. After removal of the Z group by catalytic hydrogenation, Boc–Lys(Z)–Cys(MBzl)–Ala–$NHNH_2$ was coupled with the corresponding amine component to give the protected pentapeptide ester, which was converted to the hydrazide [**4**] by hydrazine hydrate treatment. Boc–(hMT 36—42)–$NHNH_2$ [**5**] was synthesized in two alternative ways as shown in Fig. 5. One was coupling of the C-terminal pentapeptide, H–Pro–Val–Gly–Cys-

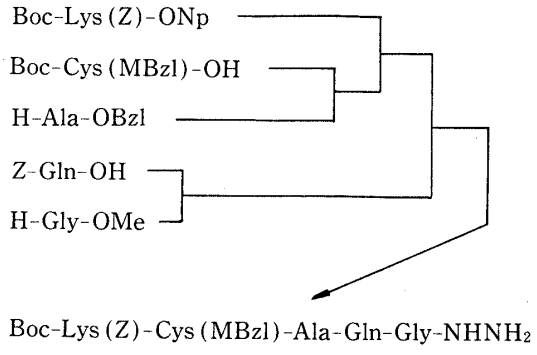

Fig. 4. Synthetic Scheme for the Protected Pentapeptide Hydrazide, Boc-(hMT 43—47)-$NHNH_2$ [4]

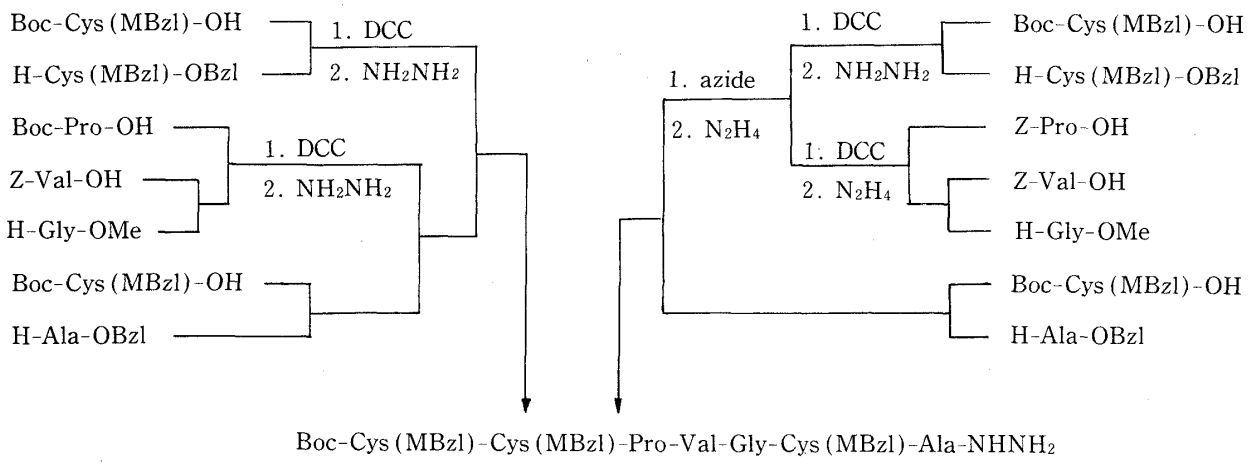

Fig. 5. Synthetic Routes to the Protected Heptapeptide Hydrazide, Boc-(hMT 36—42)-$NHNH_2$ [5]

(MBzl)–Ala–OBzl [prepared from Boc–Pro–Val–Gly–$NHNH_2$ and H–Cys(MBzl)–Ala–OBzl followed by selective deblocking of the resulting peptide by TFA treatment] with the N-terminal dipeptide, Boc–Cys(MBzl)–Cys(MBzl)–$NHNH_2$. The other was azide coupling of the C-terminal dipeptide, H–Cys (MBzl)–Ala–OBzl, with the N-terminal pentapeptide hydrazide, Boc-Cys(MBzl)-Cys(MBzl)–Pro–Val–Gly–$NHNH_2$ [constructed by azide coupling of the C-terminal tripeptide methyl ester, H–Pro–Val–Gly–OMe, with Boc–Cys(MBzl)–Cys(MBzl)–$NHNH_2$ followed by treatment with hydrazine hydrate]. The protected heptapeptide esters, Boc–(hMT 36—42)–OBzl, obtained by the two routes were identical (melting points, *Rf* values on thin-layer chromatography (TLC) and optical rotation). However, the latter method gave a better overall yield. Boc–(hMT 36—42)–OBzl was converted to the corresponding hydrazide by hydrazine hydrate treatment in the usual manner.

In each condensation reaction in Fig. 2, 3 equivalents of the azide were employed and the reaction was carried out in DMF. At each step, the desired peptide was isolated by concentration of the reaction mixture followed by the addition of MeOH, then filtration. Reprecipitation of the desired peptide from DMF and MeOH gave analytically pure peptide at each step. The purity of peptide intermediates was ascertained by TLC, elemental analysis and amino acid analysis of acid hydrolysates.

Next,the deprotection of the protected hexacosapeptide by HF or MSA was performed. Inboth cases, thioanisole and *m*-cresol were employed as scavengers.[16] Fujii *et al.* reported that thioanisole had the ability to accelerate the acidolytic cleavage by trapping alkyl cations as the *S*-sulfonium compound.[17] During the course of this deprotection reaction, oxygen-free water was used and a slightly acidic solvent was employed as an eluant for column chromatography on Sephadex G-15 in order to prevent disulfide bond formation. In gel-filtration, the effluent corresponding to that of cysteic acid derivative of the hexacosapeptide was collected and lyophilized to give a fluffy powder, indicating that this peptide had the desired peptide chain length without interchain disulfide bond formation. The homogeneity of the peptides obtained by the two different methods was ascertained by TLC (ninhydrin, sulfur test and nitroprusside test), amino acid analysis after acid hydrolysis, and aminopeptidase-M(AP-M) digestion of the corresponding cysteic acid derivative.[18] At the step of the deprotection, the MSA method gave a better yield than the HF method. Ultraviolet (UV) absorptions of the products were indistinguishable and were quite similar to that of metal-free human metallothionein,[19] as shown in Fig. 6. Metal-binding of this

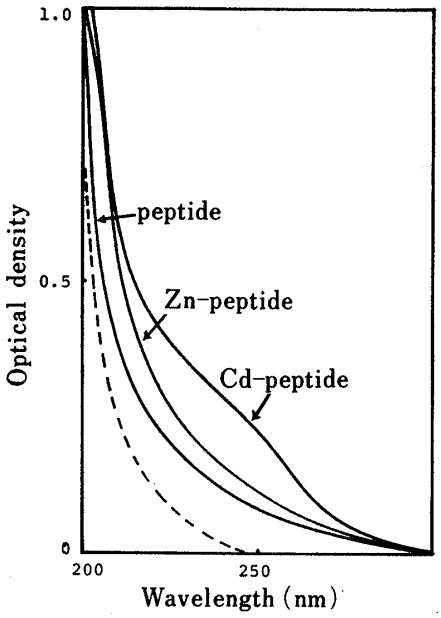

Fig. 6. Absorption Spectra of Human Thionein and Metallopeptides

-----, Absorbance of human thionein[22] (0.05 mg/ml); ——, absorbances of peptide, H-(hMT 36—61)-OH, and metallopeptides; 0.15 mM peptide as SH in 0.9 ml of Tris-HCl (10 mM, pH 7.0), with or without 20 mM Cd^{2+} or Zn^{2+} in 10 μl of the same buffer.

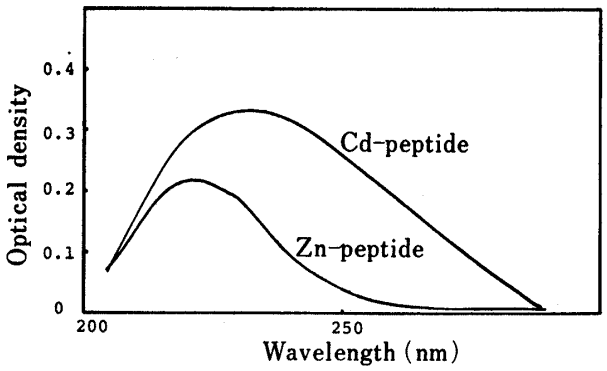

Fig. 7. Difference Spectra of Cd- and Zn-peptide

Peptide, H-(hMT 36—61)-OH: 0.15 mM as SH in 0.9 ml of Tris-HCl (10 mM, pH 7.0)
Cd^{2+} or Zn^{2+}: 20 mM in 10 μl of the same buffer.

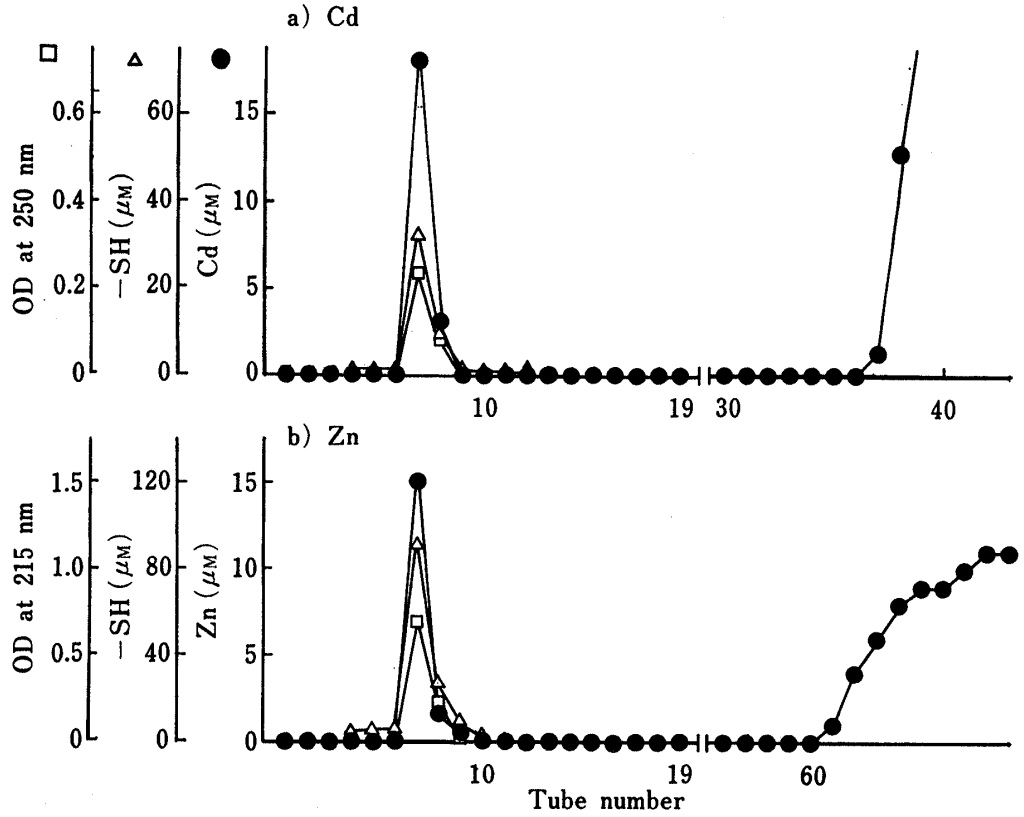

Fig. 8. Gel-filtration of Reaction Mixture of Peptide and Cadmium or Zinc on a Sephadex G-10 Column

a) 1.87 ml of 0.15 mM (as -SH) peptide and 0.22 ml of 20 mM Cd^{2+} or b) 2.1 ml of 0.17 mM (as -SH) peptide and 0.25 ml of 20 mM Zn^{2+} was charged on a Sephadex G-10 column (1.5×42.5 cm) previously equilibrated with 50 mM Tris/HCl, pH 8.0. The column was eluted with the same buffer and fractions of 4.0 ml were collected.

peptide with Zn^{2+} and Cd^{2+} was indicated by the increase of UV absorbance caused by mercaptide formation[20] (Fig. 6). The difference spectra of Zn-peptide and Cd-peptide shown in Fig. 7 are quite similar to those of Zn-thionein and Cd-thionein respectively. Fig. 8 shows the gel-filtration pattern of peptide-metal complexes. The eluted material was detected by measuring the UV absorbance due to metal clusturs,[20] the metal content by atomic absorption spectrometry and the SH content by the Ellman method.[21] A single symmetrical peak was detected by all three method, suggesting that metals (Cd and Zn) bind with the peptide and that the complexes are fairly stable. Finally, the metal-binding activity of the hexacosapeptide was measured. A fixed amount of the peptide solution was mixed with various concentrations of Cd^{2+}, resulting mercaptide formation,[20] which was measured by following the increase in absorbance of mercaptide at 250 nm. As shown in Fig.9, the binding abilities of the peptides obtained by the HF deprotection method and MSA deprotection method are indistinguishable, and are much stronger than that of cysteine. The metal-binding properties of the C-terminal hexacosapeptide described here are similar to those of the C-terminal dotriacontapeptide of rat liver metallothionein isolated and designated as fragment α by Winge and Miklossky.[22] An examination of the relationship between the structure and metal-binding activities of various peptides related to thionein is under way in our laboratory, and the results will be described elsewhere.

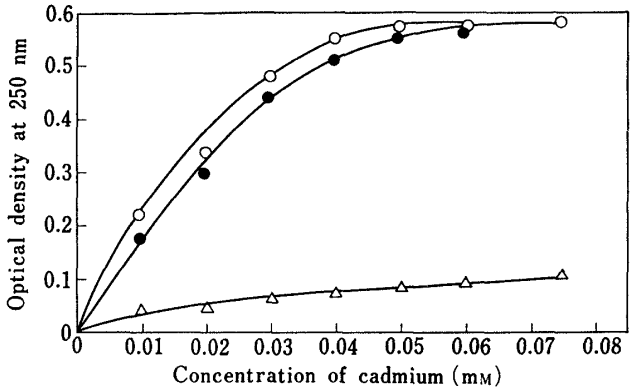

Fig. 9. Binding of Peptides with Cadmium

Peptide: 0.15 mM as -SH in 3 ml of Tris-HCl (10 mM, pH 7.0). ○, H-(hMT 36—61)-OH (HF); ●, H-(hMT 36—61) -OH (MSA); △, Cysteine.

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 and enzymatic hydrolysates were determined with a JEOL JLC-6AH amino acid analyzer. 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, Rf^6, Rf^7 values refer to the systems of $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), $CHCl_3$, MeOH and AcOH (90 : 8 : 2), benzene and AcOEt (1 : 1), *n*-BuOH, pyridine, AcOH and H_2O (4 : 1 : 1 : 2), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase), *n*-BuOH, pyridine, AcOH and H_2O (1 : 1 : 1 : 1) and *n*-BuOH, pyridine, AcOH and H_2O (4 : 1 : 1 : 1), respectively.

Boc-Cys(MBzl)-Cys(MBzl)-OBzl——Boc–Cys(MBzl)–OH (12.97 g) and H–Cys(MBzl)–OBzl·TosOH (20 g) were dissolved in a mixture of DMF and dioxane (40 ml+40 ml) containing triethylamine (Et_3N, 5.3 ml) and the solution was cooled with ice-salt. DCC (5.9 g) was added to the above cold solution, and the mixture was stirred in a cold room (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 concentrated to a small volume. Petroleum ether was added to the residue to afford a crystalline material, which was collected by filtration and recrystallized from AcOEt and petroleum ether, yield 18.0 g (71%), mp 90—92°C, $[\alpha]_D^{31}$ −39.5° (c=1.0, DMF), Rf^1 0.85. *Anal.* Calcd for $C_{34}H_{42}N_2O_7S_2$: C, 62.4; H, 6.46; N, 4.3. Found: C, 62.5; H, 6. 71; N, 4.4.

Boc-Cys(MBzl)-Cys(MBzl)-NHNH$_2$——Hydrazine hydrate (80%, 3.9 ml) was added to a solution of Boc–Cys(MBzl)–Cys(MBzl)–OBzl (18.0 g) in MeOH (50 ml). The reaction mixture was kept at room temperature overnight. A crystalline precipitate was collected by filtration and recrystallized from EtOH, yield 12.4 g (80.0%), mp 151—152°C, $[\alpha]_D^{31}$ −28.6° (c=1.0, DMF). *Anal.* Calcd for $C_{27}H_{38}N_4O_6S_2$: C, 56.0; H, 6.62; N, 9.7. Found: C, 56.0; H, 6.88; N, 9.6.

Boc-Cys(MBzl)-Cys(MBzl)-Ala-OBzl——Boc–Cys(MBzl)–Cys(MBzl)–N_3 (prepared from 12.4 g of the corresponding hydrazide with 4.9 ml of isopentyl nitrite in the usual manner) was added to a solution of

H–Ala–OBzl·TosOH (7.7 g) in DMF (30 ml) containing Et_3N (3.1 ml) under cooling in an ice-bath. This reaction mixture was stirred at 4°C for 2 d. After removal of the solvent, the residue was dissolved in AcOEt. The organic phase was washed with 10% citric acid and water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a crystalline material, which was collected by filtration and recrystallized from ether and petroleum ether, yield 12.5 g (81%), mp 110—112°C, $[\alpha]_D^{31}$ −34.0° (c=1.0, DMF), Rf^1 0.87. *Anal.* Calcd for $C_{37}H_{47}N_3O_8S_2$: C, 61.2; H, 6.53; N, 5.8. Found: C, 61.0; H, 6.47; N, 5.8.

Boc-Cys(MBzl)-Ser-NHNH$_2$——Boc–Cys(MBzl)–OH (13.0 g) and H–Ser–OMe·HCl (5.9 g) were dissolved in DMF and dioxane (50 ml+50 ml) containing Et_3N (5.3 ml) and the solution was cooled with ice-salt. DCC (10.0 g) was added to the above cold 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 H_2O, dried over Na_2SO_4 and evaporated down to give Boc–Cys(MBzl)–Ser–OMe as an oily material (Rf^2 0.86). This oily material was dissolved in MeOH (20 ml). Hydrazine hydrate (80%, 5.5 ml) was added to the above solution and this reaction mixture was stored at room temperature overnight. The precipitate was collected by filtration and recrystallized from EtOH, yield 12.5 g (60.5%), mp 143—145°C, $[\alpha]_D^{31}$ −15.1° (c=1.0, DMF), Rf^2 0.27. *Anal.* Calcd for $C_{19}H_{20}N_4O_6S$: C, 64.1; H, 6.83; N, 6.1. Found: C, 64.3; H, 6.68; N, 6.3.

Boc-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 57—61)-OBzl]——A solution of Boc–Cys(MBzl)–Cys(MBzl)–Ala–OBzl (8.7 g) in TFA (8.9 ml) containing anisole (2.6 ml) was kept at room temperature for 45 min and at 0°C for 25 min. 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*. This tripeptide amine was dissolved in DMF (40 ml) containing Et_3N (1.7 ml). Boc–Cys(MBzl)–Ser–N_3 (prepared from 6.6 g of the corresponding hydrazide with 2.3 ml of isopentyl nitrite in the usual manner) was added to the above cold solution. The reaction mixture was stirred at 4°C for 2 d. After removal of the solvent, AcOEt and H_2O were added to the residue to afford a crystalline material, which was collected by filtration and recrystallized from AcOEt and ether, yield 5.5 g (43%), mp 123—124°C, $[\alpha]_D^{31}$ −41.6° (c=1.0, DMF), *Anal.* Calcd for $C_{51}H_{65}N_5O_{12}S_3\cdot 2H_2O$: C, 57.1; H, 6.49; N, 6.5. Found: C, 57.3; H, 6.49; N, 7.0.

Boc-Lys(Z)-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 56—61)-OBzl]——H–(hMT 56—61)–OBzl·TFA (prepared from 4.0 g of Boc–(hMT 56—61)–OBzl, 3.0 ml of TFA and 0.87 ml of anisole in the usual manner) and Boc–Lys(Z)–ONp (2.5 g) were dissolved in DMF (30 ml) containing Et_3N (0.54 ml) and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford a crystalline material, yield 3.5 g (70%), mp 193—195°C, $[\alpha]_D^{31}$ −36.9° (c=1.0, DMF). *Anal.* Calcd for $C_{65}H_{82}N_6O_{15}S_3$: C, 60.2; H, 6.37; N, 7.6. Found: C, 59.8; H, 6.48; N, 7.7.

Boc-Asp(OBzl)-Lys(Z)-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 55—61)-OBzl]——H–(hMT 56—61)–OBzl·TFA (prepared from 3.0 g of Boc–(hMT 56—61)–OBzl, 3.0 ml of TFA and 0.5 ml of anisole) and Boc–Asp(OBzl)–ONp (1.5 g) in DMF (40 ml) containing Et_3N (0.32 ml) were stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford a crystalline material, which was collected by filtration, washed with MeOH and dried, yield 2.8 g (82.6%), mp 224—226°C, $[\alpha]_D^{31}$ −39.5° (c=1.0, DMF), Rf^1 0.56. *Anal.* Calcd for $C_{76}H_{94}N_8O_{17}S_3\cdot 2H_2O$: C, 59.9; H, 6.49; N, 7.4. Found: C, 59.7; H, 6.31; N, 7.6. Amino acid ratios in an acid hydrolysate: Asp, 0.89; Ser, 0.84; Ala, 1.00; Lys, 0.93 (average recovrery 92%). Cys was not determined.

Z-Gly-Ala-Ser-OMe——Z–Gly–OH (10.9 g), H–Ala–Ser–OMe (prepared from 17.0 g of Z–Ala–Ser–OMe[23] by catalytic hydrogenation) and HOBt (7.1 g) were dissolved in DMF (80 ml) and the solution was cooled with ice-salt. DCC (2.8 g) was added to the cold solution and the reaction mixture was stirred at 4°C overnight. After removal of the dicyclohexyl urea and the solvent, AcOEt was added to the residue to form crystals, which were collected by filtration and recrystallized from MeOH, yield 16.0 g (75.9%), mp 179—181°C, $[\alpha]_D^{32}$ −6.5° (c=1.0, DMF). *Anal.* Calcd for $C_{17}H_{23}N_3O_7$: C, 53.5; H, 6.08; N, 11.0. Found: C, 53.6; H, 6.14; N, 11.3.

Boc-Lys(Z)-Gly-Ala-Ser-OMe——Boc–Lys(Z)–ONp (7.9 g) and H–Gly–Ala–Ser–OMe (prepared from 6.0 g of Z–Gly–Ala–Ser–OMe by catalytic hydrogenation) were dissolved in DMF (40 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt. The organic layer was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford a crystalline material, which was collected by filtration and recrystallized from AcOEt, yield 5.5 g (57.6%), mp 138—139°C, $[\alpha]_D^{32}$ −20.0° (c=0.5, DMF), Rf^1 0.70, Rf^2 0.30. *Anal.* Calcd for $C_{28}H_{43}N_5O_{10}$: C, 55.2; H, 7.11; N, 11.5. Found: C, 55.3; H, 7.20; N, 11.3.

Boc-Lys(Z)-Gly-Ala-Ser-NHNH$_2$ [Boc-(hMT 51—54)-NHNH$_2$, (2)]——Hydrazine hydrate (80%, 0.61 ml) was added to a solution of Boc–(hMT 51—54)–OMe (3.76 g) in MeOH (20 ml) and the solution was kept at room temperature overnight. The crystalline precipitate was collected by filtration and recrystallized from EtOH, yield 3.49 g (91.9%), mp 165—166°C, $[\alpha]_D^{32}$ −11.6° (c=1.0, DMF), Rf^1 0.18. *Anal.* Calcd for $C_{27}H_{43}N_7O_9$: C, 53.2; H, 7.11; N, 16.1. Found: C, 53.0; H, 7.14; N, 16.0. Amino acid ratios in an acid hydrolysate: Ser, 1.00; Ala, 1.00; Gly, 0.98; Lys, 1.05 (average recovery 95.6%).

Boc-Ile-Cys(MBzl)-OBzl——Boc–Ile–OH (4.79 g) and H–Cys(MBzl)–OBzl·Tos–OH (10.69 g) were dissolved in DMF (40 ml) containing Et_3N (2.9 ml) and the solution was cooled with ice-salt. DCC (6.08 g)

and HOBt (3.32 g) were added to the cold solution. The reaction mixture was stirred at 4°C overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% 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 a crystalline material, yield 8.8 g (77%), mp 90—91°C, $[\alpha]_D^{28}$ −49.3° (c=1.0, MeOH), Rf^3 0.83. *Anal.* Calcd for $C_{29}H_{40}N_2O_6S$: C, 64.0; H, 7.23; N, 5.2. Found: C, 64.4; H, 7.62; N, 5.6.

Boc-Cys(MBzl)-Ile-Cys(MBzl)-OBzl——A solution of Boc–Ile–Cys(MBzl)–OBzl (6.8 g) in TFA (11.85 ml) containing anisole (1.5 ml) was kept at room temperature for 30 min and at 0°C for 40 min. Petroleum ether and ether (1 : 1) were added to the solution to afford a precipitate, which was collected by decantation and washed with petroleum ether and ether (1 : 1), and dried over KOH pellets *in vacuo*. This TFA salt was dissolved in DMF (50 ml) together with Boc–Cys(MBzl)–ONp (7.24 g) and Et_3N (1.7 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt. The organic phase was washed with 5% Na_2CO_3 and water, then dried over Na_2SO_4 and the solvent was evaporated off. Petroleum ether was added to the residue to give a crystalline material, which was collected by filtration and recrystallized from AcOEt and petroleum ether, yield 6.83 g (74.2%), mp 89—92°C, $[\alpha]_D^{28}$ −29.0° (c=1.0, DMF), Rf^3 0.92. *Anal.* Calcd for $C_{40}H_{53}N_3O_8S_2$: C, 62.3; H, 6.80; N, 5.5. Found: C, 62.6; H, 6.96; N, 5.5.

Boc-Cys(MBzl)-Ile-Cys(MBzl)-NHNH$_2$ [Boc-(hMT 48—50)-NHNH$_2$, (3)]——A solution of Boc–Cys(MBzl)–Ile–Cys(MBzl)–OBzl (3.38 g) in MeOH (80 ml) was treated with hydrazine hydrate (100%, 0.87 ml). After 12 h at room temperature, crystals were collected by filtration and recrystallized from EtOH, yield 2.75 g (90.3%), mp 151—152°C, $[\alpha]_D^{30}$ −28.8° (c=0.5, DMF), Rf^1 0.40. *Anal.* Calcd for $C_{33}H_{49}N_5O_7S_2$: C, 57.3; H, 7.14; N, 10.1. Found: C, 57.4; H, 7.23; N, 10.1.

Boc-Cys(MBzl)-Ala-OBzl——H–Ala–OBzl·Tos–OH (24.24 g) and Boc–Cys(MBzl)–OH (23.48 g) were dissolved in DMF and dioxane (30 ml+30 ml) containing Et_3N (9.8 ml) and the solution was cooled with ice-salt. DCC (17.17 g) was added to the cold solution. 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% $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to afford crystals, yield 26.35 g (75.4%), mp 60°C, $[\alpha]_D^{28}$ −17.2° (c=1.0, MeOH), Rf^3 0.90. *Anal.* Calcd for $C_{26}H_{34}N_2O_6S$: C, 62.1; H, 6.83; N, 5.6. Found: C, 62.2; H, 7.02; N, 6.5.

Boc-Lys(Z)-Cys(MBzl)-Ala-OBzl——H–Cys(MBzl)–Ala–OBzl·TFA (prepared from 5.17 g of Boc–Cys(MBzl)–Ala–OBzl, 7.4 ml of TFA and 2.0 ml of anisole in the usual manner) and Boc–Lys(Z)–ONp (5.01 g) were dissolved in DMF (60 ml) containing Et_3N (1.4 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Ether and petroleum ether (1 : 1) were added to the residue to give crystals, yield 5.0 g (65.4%), mp 58—62°C, $[\alpha]_D^{25}$ −29.0° (c=1.0, DMF), Rf^3 0.62. *Anal.* Calcd for $C_{40}H_{52}N_4O_9S$: C, 62.8; H, 6.85; N, 7.3. Found: C, 62.4; H, 6.78; N, 7.5.

Boc-Lys(Z)-Cys(MBzl)-Ala-NHNH$_2$——Hydrazine hydrate (80%, 0.73 ml) was added to a solution of Boc–Lys(Z)–Cys(MBzl)–Ala–OBzl (4.0 g) in MeOH (40 ml). The reaction mixture was allowed to stand at room temperature overnight. Ether was added to the solution to give a precipitate, which was collected by filtration and recrystallized from EtOH, yield 2.1 g (60%), mp 159—161°C, $[\alpha]_D^{24}$ −17.3° (c=1.0, DMF), Rf^1 0.55. *Anal.* Calcd for $C_{33}H_{48}N_6O_8S$: C, 57.5; H, 7.02; N, 12.2. Found: C, 57.4; H, 7.12; N, 12.1.

Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-OMe——H–Gln–Gly–OMe (prepared from 0.77 g of Z–Gln–Gly–OMe[24] by catalytic hydrogenation) was dissolved in DMF (20 ml) and the solution was cooled with ice. Boc–Lys(Z)–Cys(MBzl)–Ala–N_3 (prepared from 1.6 g of Boc–Lys(Z)–Cys(MBzl)–Ala–$NHNH_2$, 0.16 ml of 7.6 N HCl/dioxane and 0.33 ml of isopentyl nitrite in the usual manner) was added to the above cold solution, and the reaction mixture was stirred at 4°C for 2 d. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and washed with AcOEt, yield 1.60 g (84.4%), mp 174—178°C, $[\alpha]_D^{30}$ −19.1° (c=1.0, DMF), Rf^1 0.69. *Anal.* Calcd for $C_{41}H_{59}N_7O_{12}S\cdot 1/2H_2O$: C, 55.8; H, 6.80; N, 11.1. Found: C, 55.6; H, 7.01; N, 10.8.

Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-NHNH$_2$ [Boc-(hMT 43—47)-NHNH$_2$, (4)]——Hydrazine hydrate (80%, 0.28 ml) was added to a solution of Boc–Lys(Z)–Cys(MBzl)–Ala–Gln–Gly–OMe (1.6 g) in DMF (15 ml) and MeOH (15 ml). The reaction mixture was kept at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford crystals, yield 1.21 g (73.7%), mp 193—194°C, $[\alpha]_D^{26}$ −14.5° (c=1.0, DMF), Rf^1 0.50. *Anal.* Calcd for $C_{40}H_{59}N_9O_{11}S\cdot 3H_2O$: C, 51.7; H, 7.06; N, 13.7. Found: C, 51.7; H, 6.90; N, 14.0. Amino acid ratios in an acid hydrolysate: Glu, 0.95; Gly, 0.98; Ala, 1.00; Lys, 1.00 (average recovery 98.3%). Cys was not determined.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-OMe——Boc–Cys(MBzl)–Cys(MBzl)–N_3 (prepared from 6.25 g of the corresponding hydrazide by addition of 2.9 ml of 7.5 N HCl in dioxane followed by 2.9 ml of isopentyl nitrite as usual) was added to the solution of H–Pro–Val–Gly–OMe (prepared from 3.0 g of Z–Pro–Val–Gly–OMe[25] by catalytic hydrogenation) in DMF (30 ml). The pH was adjusted to 8 with Et_3N, and 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. Ether and petroleum ether were added to the residue to give crystals, yield 5.66 g (94.4%), mp 68—69°C, $[\alpha]_D^{23}$ −45.4° (c=0.5, DMF), Rf^1 0.80. *Anal.* Calcd for $C_{40}H_{57}N_5O_{10}S$: C, 57.7; H, 6.91; N, 8.4. Found: C, 58.3; H, 6.97; N, 7.8.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-NHNH$_2$——Hydrazine hydrate (80%, 1.5 ml) was added to a solution of Boc–Cys(MBzl)–Cys(MBzl)–Pro–Val–Gly–OMe (4.4 g) in MeOH (20 ml). This solution was allowed to stand at room temperature overnight, then concentrated to half the original volume. H_2O was added to the residue to give a precipitate, which was collected by filtration and recrystallized from EtOH, yield 2.44 g (48.3%), mp 102°C, $[\alpha]_D^{23}$ −51.5° (c=0.9, DMF), Rf^1 0.78. *Anal.* Calcd for $C_{39}H_{57}N_7O_9S_2 \cdot H_2O$: C, 55.1; H, 6.99; N, 11.5. Found: C, 55.5; H, 6.86; N, 11.0.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-Cys(MBzl)-Ala-OBzl [Boc-(hMT 36—42)-OBzl]——Boc–Cys(MBzl)–Cys(MBzl)–Pro–Val–Gly–N_3 (prepared from 1.0 g of the corresponding hydrazide, 0.38 ml of 6.4 N HCl/dioxane and 1.9 ml of isopentyl nitrite as usual) in DMF (20 ml) was combined with a solution of H–Cys(MBzl)–Ala–OBzl·TFA (prepared from 1.21 g of Boc–Cys(MBzl)–Ala–OBzl and 1.8 ml of TFA containing 0.52 ml of anisole as usual). The reaction mixture was stirred at 4°C for 2 d. During the reaction, the pH of the solution was kept at 8 by addition of Et_3N. 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 concentrated to a small volume. Ether and petroleum ether were added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH and ether, yield 0.83 g (68%), mp 120°C, $[\alpha]_D^{25}$ −50.1° (c=1.0, DMF), Rf^1 0.75. *Anal.* Calcd for $C_{60}H_{79}N_7O_{13}S_3 \cdot 3H_2O$: C, 57.4; H, 6.82; N, 7.8. Found: C, 57.6; H, 6.72; N, 7.9. Amino acid ratios in an acid hydrolysate: Pro, 0.95; Gly, 1.00; Ala, 1.15; Val, 0.91 (average recovery 95%). Cys was not determined.

Boc-Pro-Val-Gly-OMe——H–Val–Gly–OMe (prepared from 5.0 g of Z–Val–Gly–OMe[26] by catalytic hydrogenation) and Boc–Pro–OH (6.7 g) were dissolved in DMF (60 ml) and the solution was cooled with ice-salt. DCC (6.4 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% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford crystals, yield 5.65 g (95%), mp 65—70°C, $[\alpha]_D^{25}$ −88.4° (c=1.0, MeOH), Rf^3 0.80. *Anal.* Calcd for $C_{18}H_{31}N_3O_6$: C, 56.1; H, 8.11; N, 10.9. Found: C, 56.3; H, 8.26; N, 10.6.

Boc-Pro-Val-Gly-NHNH$_2$——Hydrazine hydrate (80%, 1.9 ml) was added to a solution of Boc–Pro–Val–Gly–OMe (3.0 g) in MeOH (50 ml). The reaction mixture was stored at room temperature overnight and concentrated to half the original volume. H_2O was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH and H_2O, yield 1.5 g (30%), mp 80—85°C, $[\alpha]_D^{25}$ −29.5° (c=1.0, DMF), Rf^2 0.70. *Anal.* Calcd for $C_{17}H_{31}N_5O_5$: C, 53.0; H, 8.11; N, 18.2. Found: C, 53.2; H, 8.34; N, 18.3.

Boc-Pro-Val-Gly-Cys(MBzl)-Ala-OBzl——Boc–Pro–Val–Gly–N_3 (prepared from 2.4 g of Boc–Pro–Val–Gly–$NHNH_2$, 2.3 ml of 5.4 N HCl/dioxane and 0.91 ml of isopentyl nitrite as usual) in DMF (10 ml) was combined with H–Cys(MBzl)–Ala–OBzl·TFA (prepared from 3.4 g of Boc–Cys(MBzl)–Ala–OBzl, 4.6 ml of TFA containing 1.3 ml of anisole as usual) in DMF (10 ml) containing Et_3N (0.95 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 and water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to afford crystals, which were collected by filtration. These crystals in $CHCl_3$ (4 ml) were applied to a column of silica gel (2×15 cm) and elution with $CHCl_3$ (1000—1500 ml) afforded the title compound, which was recrystallized from ether and petroleum ether, yield 1.4 g (29%), mp 108—109°C, $[\alpha]_D^{21}$ −68.8° (c=1.0, MeOH), Rf^2 0.60. *Anal.* Calcd for $C_{38}H_{53}N_5O_9S$: C, 60.4; H, 7.07; N, 9.3. Found: C, 60.1; H, 7.18; N, 9.0.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-Cys(MBzl)-Ala-OBzl [Boc-(hMT 36—42)-OBzl]——Boc–Cys(MBzl)–Cys(MBzl)–N_3 (prepared from 1.16 g of the corresponding hydrazide, 0.72 ml of 5.4 N HCl/dioxane and 0.29 ml of isopentyl nitrite as usual) in DMF (5 ml) cooled with ice-salt was combined with H–Pro–Val–Gly–Cys(MBzl)–Ala–OBzl·TFA (prepared from 1.0 g of Boc–Pro–Val–Gly–Cys(MBzl)–Ala–OBzl and 0.96 ml of TFA containing 0.28 ml of anisole as usual) in DMF (5 ml) containing Et_3N (0.3 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 H_2O, dried over Na_2SO_4 and concentrated to a small volume. Ether and petroleum ether (1 : 1) were added to the residue to give crystals. This material in $CHCl_3$ (5 ml) was applied to a column of silica gel (2×15 cm), which was eluted first with $CHCl_3$ (500 ml) and then with 1% MeOH in $CHCl_3$ (500 ml). After removal of the solvent of the latter eluate, the residue was crystallized from ether and petroleum ether, yield 0.52 g (44%), mp 118—120°C, $[\alpha]_D^{23}$ −50.1° (c=0.5, MeOH), Rf^1 0.57. *Anal.* Calcd for $C_{60}H_{79}N_7O_{13}S_3$: C, 59.9; H, 6.62; N, 8.2. Found: C, 59.6; H, 6.65; N, 7.9.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-Cys(MBzl)-Ala-NHNH$_2$ [Boc-(hMT 36—42)-NHNH$_2$, (5)]——A solution of Boc–Cys(MBzl)–Cys(MBzl)–Pro–Val–Gly–Cys(MBzl)–Ala–OBzl (4.37 g) in DMF (30 ml) was treated with hydrazine hydrate (100%, 0.7 ml) and the mixture was kept for 12 h at room temperature. The solvent was removed by evaporation and MeOH was added to the residue to give crystals, which were collected by filtration and washed with MeOH and ether, yield 3.0 g (83.3%), mp 108—110°C, $[\alpha]_D^{20}$ −25.1° (c=1.0, DMF), Rf^1 0.38. *Anal.* Calcd for $C_{53}H_{75}N_9O_{12}S_3 \cdot H_2O$: C, 55.6; H, 6.78; N, 10.6. Found: C, 55.8;

H, 6.76; N, 11.0. Amino acid ratios in an acid hydrolysate: Pro, 1.06; Gly, 1.00; Ala, 0.97; Val, 1.19 (average recovery 97%). Cys was not determined.

Boc-Lys(Z)-Gly-Ala-Ser-Asp(OBzl)-Lys(Z)-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 51—61)-OBzl]——Boc–Lys(Z)–Gly–Ala–Ser–N_3 (prepared from 2.77 g of Boc–Lys(Z)–Gly–Ala–Ser–$NHNH_2$, 1.43 ml of 8.4 N HCl/dioxane and 0.94 ml of isopentyl nitrite) in DMF (30 ml) was added to a solution of H-(hMT 55—61)–OBzl (prepared from 4.46 g of Boc–(hMT 55—61)–OBzl, 2.96 ml of TFA and 0.5 ml of anisole) in DMF (30 ml) containing Et_3N (0.42 ml) at 0°C. The reaction mixture was stirred in a cold room (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 3.22 g (54.3%), mp 245—247°C, $[\alpha]_D^{31}$ −23.2° ($c=0.5$, DMSO), Rf^1 0.65. *Anal.* Calcd for $C_{98}H_{125}N_{13}O_{25}S_3 \cdot 2H_2O$: C, 58.3; H, 6.45; N, 9.0. Found: C, 58.4; H, 6.39; N, 9.2. Amino acid ratios in an acid hydrolysate: Asp, 0.85; Ser, 1.95; Gly, 1.00; Ala, 2.13; Lys, 2.06 (average recovery 98%). Cys was not determined.

Boc-Cys(MBzl)-Ile-Cys(MBzl)-Lys(Z)-Gly-Ala-Ser-Asp(OBzl)-Lys(Z)-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 48—61)-OBzl]——Boc–Cys(MBzl)–Ile–Cys(MBzl)–N_3 (prepared from 2.26 g of **3**, 0.78 ml of 8.6 N HCl/dioxane and 0.53 ml of isopentyl nitrite as usual) in DMF (20 ml) was added to a solution of H-(hMT 51—61)–OBzl (prepared from 3.22 g of Boc–(hMT 51—61)–OBzl and 2.4 ml of TFA containing 0.35 ml of anisole as usual) in DMF (50 ml) containing Et_3N (0.23 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/MeOH, yield 4.0 g (96%), mp 275—277°C, $[\alpha]_D^{31}$ −44° ($c=0.2$, DMF), Rf^1 0.83. *Anal.* Calcd for $C_{126}H_{162}N_{16}O_{30}S_5 \cdot 2H_2O$: C, 58.7; H, 6.50; N, 8.7. Found: C, 58.8; H, 6.64; N, 9.0. Amino acid ratios in an acid hydrolysate: Asp, 1.04; Ser, 1.69; Gly, 1.00; Ala, 1.81; Ile, 0.86; Lys, 1.95 (average recovery 85.0%). Cys was not determined.

Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-Cys(MBzl)-Ile-Cys(MBzl)-Lys(Z)-Gly-Ala-Ser-Asp(OBzl)-Lys(Z)-Cys-(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 43—61)-OBzl]——Boc–Lys(Z)–Cys(MBzl)–Ala–Gln–Gly–N_3 (prepared from 2.88 g of **4**, 0.79 ml of 8.4 N HCl/dioxane and isopentyl nitrite) in DMF (30 ml) was combined with a solution of H-(hMT 48—61)–OBzl (prepared from 4.0 g of Boc–(hMT 48—61)–OBzl and 4.0 ml of TFA containing 0.5 ml of anisole as usual) in DMF (50 ml) containing Et_3N (0.23 ml). The reaction mixture was stirred at 4°C for 2 d. After removal of the solvent, MeOH was added to yield a white precipitate, which was collected by filtration, washed with MeOH and reprecipitated from DMF and MeOH, yield 2.87 g (54.6%), mp 284—286°C, $[\alpha]_D^{29}$ −24.5° ($c=0.2$, DMSO), Rf^5 0.80. *Anal.* Calcd for $C_{161}H_{209}N_{23}O_{39}S_6 \cdot 6H_2O$: C, 57.0; H, 6.57; N, 9.5. Found: C, 57.0; H, 6.66; N, 9.8. Amino acid ratios in an acid hydrolysate: Asp, 1.04; Ser, 1.69; Glu, 0.85; Gly, 2.00; Ala, 3.19; Ile, 0.85; Lys, 2.90 (average recovery 92.3%). Cys was not determined.

Boc-Cys(MBzl)-Cys(MBzl)-Pro-Val-Gly-Cys(MBzl)-Ala-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-Cys(MBzl)-Ile-Cys-(MBzl)-Lys(Z)-Gly-Ala-Ser-Asp(OBzl)-Lys(Z)-Cys(MBzl)-Ser-Cys(MBzl)-Cys(MBzl)-Ala-OBzl [Boc-(hMT 36—61)-OBzl]——Boc–Cys(MBzl)–Cys(MBzl)–Pro–Val–Gly–Cys(MBzl)–Ala–N_3 (derived from 2.6 g of **5**) was added to a solution of H–(hMT 43—61)–OBzl·TFA (prepared from 2.88 g of Boc–(hMT 43—61)–OBzl and 5.2 ml of TFA containing 1.0 ml of anisole) in DMF (100 ml) containing Et_3N (0.12 ml). The reaction mixture was stirred at 4°C for 2 d and concentrated to half the initial volume. MeOH was added to the residue to afford crystals, which were collected by filtration, washed with MeOH and dried, yield 2.95 g (78.4 %), mp 295—297°C, $[\alpha]_D^{31}$ −22.0° ($c=0.2$, DMSO), Rf^5 0.78. *Anal.* Calcd for $C_{209}H_{272}N_{30}O_{49}S_9 \cdot 2H_2O$: C, 57.7; H, 6.49; N, 9.5. Found: C, 57.7; H, 6.54; N, 9.8. Amino acid ratios in an acid hydrolysate: Asp, 1.01; Ser, 1.40; Glu, 0.95; Pro, 0.86; Gly, 2.84; Ala, 3.74; Val, 0.71; Ile, 0.83; Lys, 3.00 (average recovery 80%). Cys was not determined.

H-Cys-Cys-Pro-Val-Gly-Cys-Ala-Lys-Cys-Ala-Gln-Gly-Cys-Ile-Cys-Lys-Gly-Ala-Ser-Asp-Lys-Cys-Ser-Cys-Cys-Ala-OH, H-(hMT 36—61)-OH——a) HF Method: Boc–(hMT 36—61)–OBzl (100 mg) was treated with anhydrous HF (approximately 10 ml) in the presence of thioanisole (0.17 ml) and *m*-cresol (0.73 ml) in an ice-bath for 60 min, then the HF was removed under reduced pressure. The residue was dried over KOH pellets *in vacuo* overnight and dissolved in oxygen-free water (10 ml). The solution was treated with Amberlite A-45 (acetate form). The resin was removed by filtration and the filtrate was washed with AcOEt and lyophilized. The product was dissolved in 0.5% AcOH (5 ml) and the solution was applied to a column of Sephadex G-15 (2×60 cm), which was eluted with 0.5% AcOH. Individual fractions of 3 g were collected. The desired fractions (tube Nos. 19—27) were collected and lyophilized, yield 19 mg (32.2%), $[\alpha]_D^{30}$ −43.0° ($c=0.1$, 0.5% AcOH), Rf^4 0.25, Rf^6 0.87 (ninhydrin test, H_2PtCl_6–KI test, nitroprusside test positive). This peptide was treated with performic acid and then hydrolyzed with 6 N HCl or aminopeptidase M (AP–M). Amino acid ratios in an acid hydrolysate: $CySO_3H$, 8.80; Asp, 1.13; Ser, 1.88; Glu, 0.93; Pro, 0.73; Gly, 2.85; Ala, 3.81; Val, 0.71; Ile, 0.84; Lys, 3.00 (average recovery 80%), amino acid ratios in an AP–M digest: $CySO_3H$, 9.00; Asp, 1.23; Ser, 1.98; Glu, 1.07; Pro, 0.64; Gly, 2.63; Ala, 4.18; Val, 0.80; Ile, 0.96; Lys, 3.00 (average recovery 65%).

b) Methanesulfonic Acid (MSA) Method: Under an N_2 atmosphere, Boc–(hMT 36—61)–OBzl (100 mg) was treated with MSA (3.9 ml) in the presence of thioanisole (0.17 ml) and *m*-cresol (0.73 ml) in an ice-bath for 10 min, then at room temperature for 60 min. Dry ether was added to the solution to give a precipitate, which was collected by decantation, washed with dry ether and dried over KOH pellets *in vacuo*. The residue

was dissolved in oxygen-free water (10 ml) and the solution was treated with Amberlite A-45 (acetate form, approximately 5 g). After removal of the resin, the filtrate was washed with AcOEt and lyophilized. This product in 0.5% AcOH (5 ml) was applied to a column of Sephadex G-15 (2 × 60 cm), which was eluted with 0.5% AcOH. Individual fractions of 3 g were collected. The desired fractions (tube Nos. 17—24) were combined and lyophilized to give a fluffy powder, yield 33 mg (56.5%), $[\alpha]_D^{30}$ −37.0° (c=0.1, 0.5% AcOH), Rf^4 0.25, Rf^6 0.85 (ninhydrin test, H_2PtCl_6–KI test, nitroprusside test positive). This peptide was treated with performic acid and hydrolyzed with 6 N HCl. Amino acid ratios in an acid hydrolysate: $CySO_3H$, 9.39; Asp, 1.26; Ser, 1.73; Glu, 1.02; Pro, 0.76; Gly, 2.83; Ala, 4.00; Val, 0.74; Ile, 0.97; Lys, 3.26 (average recovery 91.4%).

References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine. Standard abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *idem*, *ibid.*, **6**, 362 (1967); *idem*, *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; ONp, *p*-nitrophenyl ester; DCC, dicyclohexylcarbodiimide; MBzl, *p*-methoxybenzyl; DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOEt, ethyl acetate; AcOH, acetic acid; *n*-BuOH, *n*-butanol; HOBt, 1-hydroxybenzotriazole.
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