

[Chem. Pharm. Bull.]
31(9)3094—3103(1983)

Amino Acids and Peptides. IX.^{1,2)} Synthesis of Cysteine-Containing Peptide Fragments Related to Human Hepatic Metallothionein II (hMT II) and Determination of Their Heavy Metal-Binding Properties

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(Received January 27, 1983)

Eight kinds of cysteine-containing peptides related to human hepatic metallothionein were synthesized by conventional methods. The metal-binding properties of these peptides with Zn^{2+} and Cd^{2+} were indicated by an increase of ultraviolet absorbance resulting from mercaptide formation. The Cd-binding activities of various peptides obtained here were assessed by measuring the increase in absorbance of mercaptide at 250 nm as a function of the concentration of Cd. The binding abilities of the peptides with Cd differed substantially.

Keywords—human hepatic metallothionein; cysteine-containing peptide; chemical synthesis; metal-binding; Zn^{2+} ; Cd^{2+}

Metallothioneins are sulfhydryl-rich, low molecular-weight proteins. Although their exact role is not understood yet, it has been suggested that these proteins act as heavy metal detoxifying agents by sequestering cadmium, mercury and other harmful metal ions and that metallothioneins participate in zinc or copper metabolism or homeostasis, serving in a storage or transport capacity.³⁾ These possible physiological actions are due to their binding ability with heavy metals. Figure 1 shows the amino acid sequence of human hepatic metallothionein II (hMT II), which was determined by Kissling and Kägi⁴⁾ and later revised by Kimura to be Ser-Cys at positions 58 and 59 instead of Cys-Ser.⁵⁾ The polypeptide chain contains 61 amino acid residues, among which 20 are cysteines. It is clear that the spatial distribution of Cys residues in thionein is very important for binding with heavy metals (Cd, Zn, Hg, Ag and Cu). Yoshida *et al.*⁶⁾ reported that some synthetic Cys-containing peptides related to mouse liver thionein I⁷⁾ had higher affinity for Cd^{2+} than Zn^{2+} , while others might bind with Zn^{2+} more strongly than Cd^{2+} . Otovos and Armitage⁸⁾ proposed that metallothionein contains two separate metal clusters, a C-terminal cluster containing four Cd^{2+} ions and an N-terminal

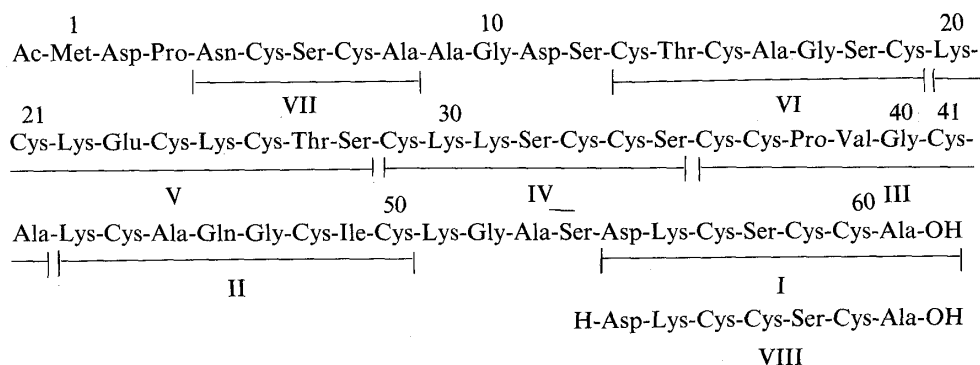


Fig. 1. Amino Acid Sequences of Human Hepatic Metallothionein II (hMT II) and Synthetic Cys-Containing Peptides (I–VIII)

cluster containing three Cd^{2+} ions from the results of ^{113}Cd NMR spectroscopy. They also pointed out that Cd^{2+} ion bound with the C-terminal portion of thionein preferentially. Quite recently, two domains of rat liver metallothionein were separated by Winge and Miklossky.⁹⁾ In that metallothionein, the N-terminal domain contained two Zn^{2+} ions and one Cd^{2+} ion and the C-terminal domain contained four Cd^{2+} ions, suggesting the existence of structure-related differences of metal-binding ability of thionein. Thus, it seems important and interesting to study the relationship between the structure of thionein and binding ability in order to cast light on the physiological roles of thionein. In our previous report,¹⁾ we showed that synthetic C-terminal hexacosapeptide of human liver metallothionein II exhibited heavy metal-binding properties similar to those of native thionein. This report deals with the synthesis of small Cys-containing peptide fragments related to hMT II and with their heavy metal-binding properties in comparison with those of the C-terminal hexacosapeptide.¹⁾

As shown in Fig. 1, 8 kinds of peptide fragments I—VIII, which contain two or three Cys residues were synthesized. The desired peptides I—VIII were derived from the corresponding protected peptides by treatment with hydrogen fluoride (HF)¹⁰⁾ or methanesulfonic acid (MSA)¹¹⁾ containing thioanisole and *m*-cresol as scavengers.^{12,13)}

The peptides [I], H-(hMT 55—61)-OH and [III], H-(hMT 36—42)-OH were derived from Boc-(hMT 55—61)-OBzl¹⁾ and Boc-(hMT 36—42)-OBzl¹⁾ respectively. The peptide [II], H-(hMT 43—50)-OH was prepared from Boc-(hMT 43—50)-OBzl, which was synthesized by azide coupling¹⁴⁾ of Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-NHNH₂¹⁾ and H-Cys(MBzl)-Ile-Cys(MBzl)-OBzl.¹⁾

H-(hMT 29—35)-OH [IV], H-(hMT 20—28)-OH [V] and H-(hMT 13—19)-OH [VI] were synthesized as illustrated in Figs. 2, 3 and 4, respectively.

H-(hMT 4—8)-OH [VII] was derived from Boc-(hMT 4—8)-OBzl, which was synthesized by the reaction of Boc-Asn-ONp and H-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl which was itself derived from Boc-(hMT 5—8)-OBzl by TFA treatment. Boc-(hMT 5—8)-OBzl was prepared by coupling of Boc-Cys(MBzl)-Ser-NHNH₂¹⁾ and H-Cys(MBzl)-Ala-OBzl.¹⁾

The peptide H-Asp-Lys-Cys-Cys-Ser-Cys-Ala-OH [VIII], corresponding to the sequence 55—61 of hMT II, whose amino acid sequence was determined by Kissling and Kägi,⁴⁾ is abbreviated as H-(old hMT 55—61)-OH. VIII was synthesized as follows: Boc-Lys(Z)-Cys(MBzl)-NHNH₂ which was prepared from Boc-Lys(Z)-Cys(MBzl)-OBzl by treatment with hydrazine hydrate was coupled with H-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl to give Boc-Lys(Z)-Cys(MBzl)-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl. After removal of Boc group, the resulting hexapeptide amine was coupled with Boc-Asp(OBzl)-ONp to give Boc-(old hMT

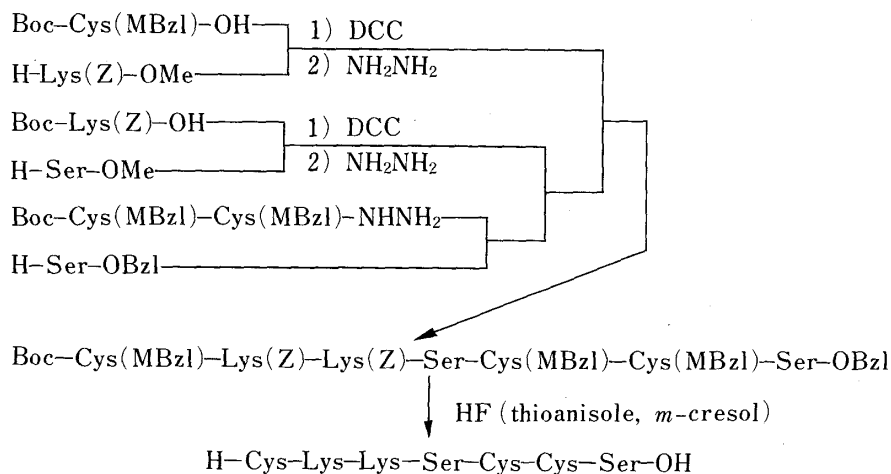


Fig. 2. Synthetic Scheme for H-(hMT 29—35)-OH, [IV]

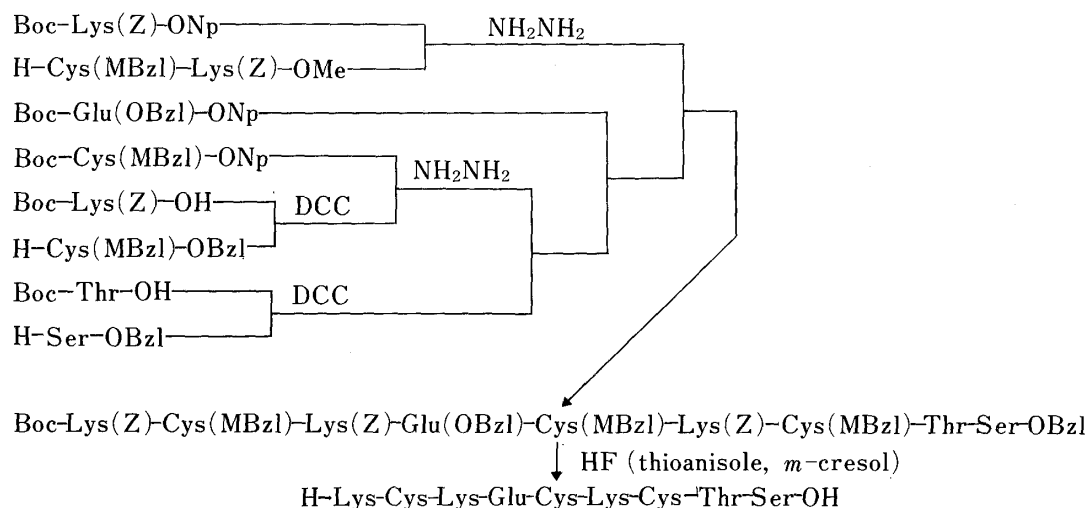


Fig. 3. Synthetic Scheme for H-(hMT 20—28)-OH, [V]

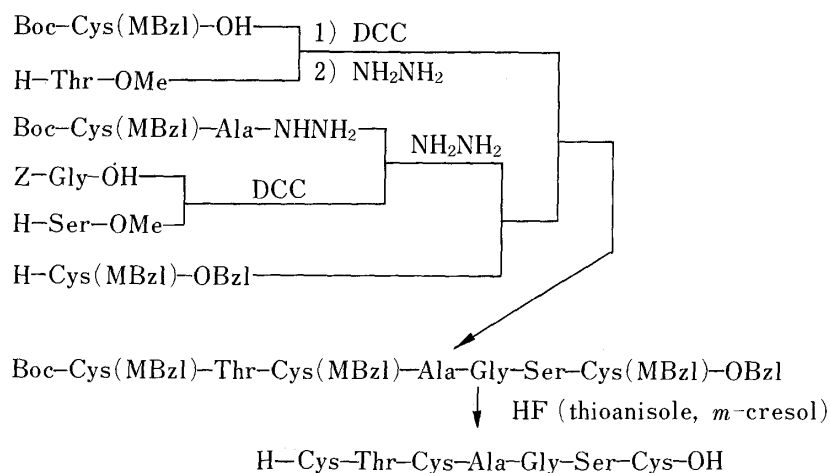


Fig. 4. Synthetic Scheme for H-(hMT 13—19)-OH, [VI]

55—61)-OBzl, which was converted to the desired peptide, [VIII]. These peptides, I—VIII, obtained above were purified by gel-filtration on Sephadex G-15 using 0.5% AcOH as an eluent. The yields, $[\alpha]_D$, R_f values and analytical data are summarized in Table I. As can be seen in Table I, the homogeneity of the peptides was ascertained by thin-layer chromatography (TLC) (ninhydrin, sulfur test and nitroprusside test) and amino acid analysis after acid hydrolysis. Ultraviolet (UV) absorptions of those peptides are quite similar to that of metal-free metallothionein¹⁷⁾ and metal-binding of those peptides with Cd^{2+} and Zn^{2+} was indicated by the increase of UV absorbance caused by mercaptide formation, as reported by Kägi and Vallee¹⁸⁾ and Vasak *et al.*¹⁹⁾ Figure 5 shows the UV absorptions of peptide I and peptide I-metal complexes as examples. The difference spectra of Zn-peptide I and Cd-peptide I shown in Fig. 6 are similar to those of Zn-hexacosapeptide and Cd-hexacosapeptide¹⁾ and those of Zn-thionein and Cd-thionein.¹⁸⁾

Cd-peptides and Zn-peptides were purified by gel-filtration on Sephadex G-10. The eluted material was detected by measuring the UV absorbance due to the metal cluster,^{18,19)} the metal content by atomic absorption spectrometry and the SH content by the Ellman method²⁰⁾ as described previously.¹⁾ In each purification, a single symmetrical peak was detected by all three methods, suggesting that metals (Cd and Zn) bind with the small peptides obtained above and that the resulting complexes are stable.

TABLE I. Yields, $[\alpha]_D$, R_f Values and Amino Acid Ratios of Synthetic Peptides (I—VIII)

Peptide	Yield (%)	$[\alpha]_D^{28}$ (0.5% AcOH)	R_f^4	Amino acid analysis of acid hydrolysate (Average recovery) ^{a)}
H-(hMT 55—61)-OH, I	62.5	−44.0°	0.80	Asp 1.00; Ser 0.65; Ala 1.00; Lys 1.20 (85.0%)
H-(hMT 43—50)-OH, II	36.4 (HF) 56.6 (MSA)	−40.0°	0.78	Glu 1.00; Gly 1.10; Ala 1.00; Ile 0.80; Lys 0.90 (96.0%)
H-(hMT 36—42)-OH, III	61.0	−81.0°	0.70	Pro 1.00; Gly 0.97; Ala 1.00; Val 1.16 (91.0%)
H-(hMT 29—35)-OH, IV	80.1	−33.0°	0.74	Ser 1.41; Lys 2.00 (72.5%)
H-(hMT 20—28)-OH, V	82.9	−28.0°	0.80	Thr 1.03; Ser 0.81; Glu 1.03; Lys 3.00 (82.0%)
H-(hMT 13—19)-OH, VI	56.0	−23.0°	0.75	Thr 0.78; Ser 0.80; Gly 1.04; Ala 1.00 (84.5%)
H-(hMT 4—8)-OH, VII	74.1	−43.0°	0.71	Asp 1.07; Ser 0.70; Ala 1.00 (73.7%)
H-(old hMT 55—61)-OH, VIII	80.5	−39.0°	0.80	Asp 0.87; Ser 0.70; Ala 1.00; Lys 1.11 (80.0%)

a) Cys was not determined.

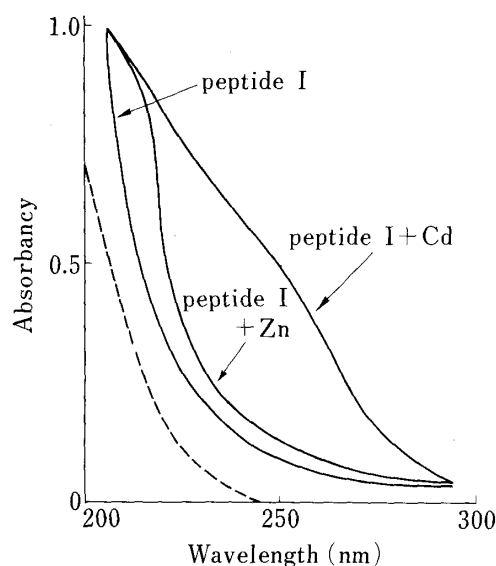


Fig. 5. Absorption Spectra of Human Thionein and Metallopeptide

-----, absorbance of human thionein¹⁷⁾ (0.05 mg/ml); —, absorbances of peptide and metallopeptides. Peptide: H-(hMT 55—61)-OH [I], 0.15 mM as SH, 0.9 ml; Cd²⁺ or Zn²⁺, 20 mM in 10 μ l of Tris/HCl (10 mM, pH 7.0).

Finally, the Cd-binding activities of various peptides obtained above were assessed by measuring the increase in absorbance of mercaptide at 250 nm as a function of the concentration of Cd. The results are illustrated in Fig. 7. It can be seen that the peptides belonging to the C-terminal domain of hMT II bind with Cd ion more strongly than those of the N-terminal domain. The Cd-binding activities of I, III and VIII are comparable to that of the C-terminal hexacosapeptide.¹⁾ Peptides I and VIII have the sequences Cys-Ser-Cys-Cys and Cys-Cys-Ser-Cys, respectively. These sequences might be favorable for binding with Cd ion. In peptide III, there is the sequence Pro-Val-Gly between Cys-Cys and Cys, but the distance between Cys-Cys and Cys might be similar to that of I or VIII because of the presence of the Pro residue. With regard to heavy metal-binding properties, even small peptides which contain Cys residues at suitable positions exhibit behavior similar to that of thionein. In view of these results, it would be surprising if thioneins which contain 61 amino acid residues are produced in mammals solely in order to bind with heavy metals. Further

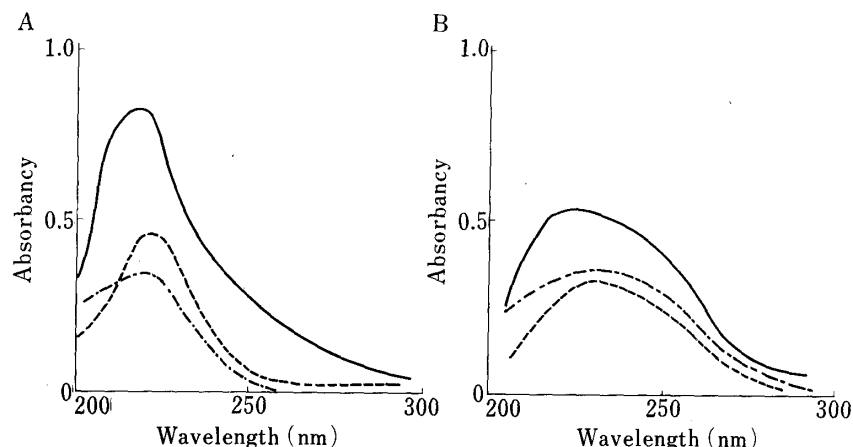


Fig. 6. Difference Spectra of Metallothionein and Metallopeptide

—, metallothionein;¹⁸⁾ ----, metal-hexacosapeptide;¹⁾ — · —, metal-peptide. Peptide: H-(hMT 55—61)-OH [I], 0.15 mM as SH, 0.9 ml; (A), with Zn^{2+} , 20 mM in 10 μ l of Tris/HCl (10 mM, pH 7.0); (B), with Cd^{2+} , 20 mM in 10 μ l of Tris/HCl (10 mM, pH 7.0).

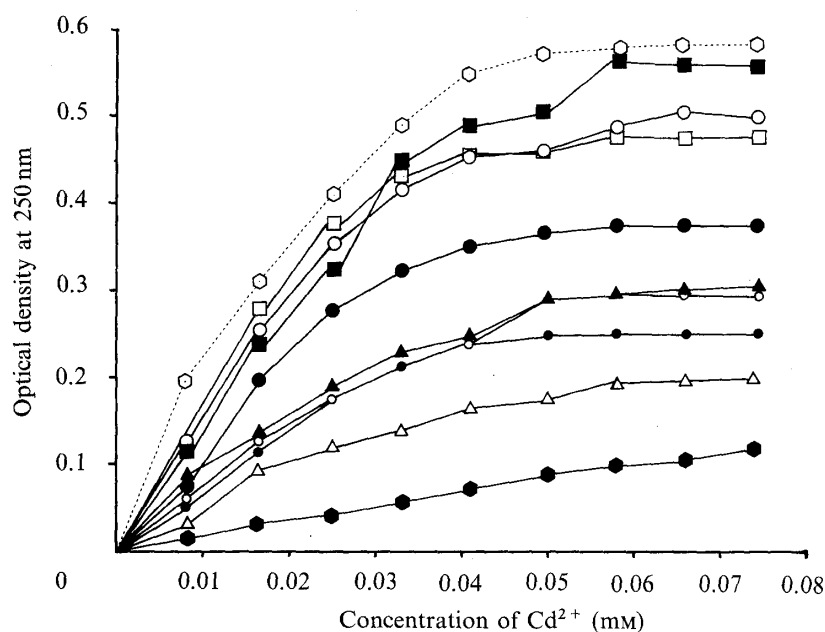


Fig. 7. Binding Ability of Peptides (I—VIII) and Hexacosapeptide¹¹⁾ with Cd^{2+}

Peptide: 0.15 mM as SH in Tris/HCl (3 ml, 10 mM, pH 7.0). ■, peptide I; ●, peptide II; ○, peptide III; ▲, peptide IV; ●, peptide V; △, peptide VI; ○, peptide VII; □, peptide VIII; ●, cysteine ○, C-terminal hexacosapeptide.¹¹⁾

studies using synthetic Cys-containing peptides with various amino acid sequences related to that of thionein are required in order to clarify the relationship between the structure of peptides and their metal-binding propensities.

Experimental

General experimental methods employed here were essentially the same as those described in the previous paper¹⁾ of this series. Thin layer chromatography was performed on silica gel (Kieselgel G, Merck). R_f^1 , R_f^2 , R_f^3 and R_f^4 values refer to the systems of benzene and AcOH (1:1), $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (8:3:1, lower phase) and n -butanol, pyridine, AcOH and H_2O (1:1:1:1), respectively.

Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-Cys(MBzl)-Ile-Cys(MBzl)-OBzl, **Boc-(hMT 43—50)-OBzl**—**Boc-Lys(Z)-Cys(MBzl)-Ala-Gln-Gly-N₃** (prepared from 1.0 g of the corresponding hydrazide¹⁾ with 0.16 ml of isopentyl

nitrite and 0.32 ml of 7.4 N HCl in dioxane in a usual manner) in DMF (20 ml) was combined with H-Cys(MBzl)-Ile-Cys(MBzl)-OBzl (prepared from 1.06 g of Boc-(hMT 48—50)-OBzl¹⁾ with 1.0 ml of TFA containing 0.28 ml of anisole) in DMF (15 ml) containing Et₃N (0.19 ml). This reaction mixture was stirred in a cold room (4 °C) for 2 d. After removal of the solvent, AcOEt and H₂O were added to the residue to give a solid, which was collected by filtration and reprecipitated from DMF and MeOH, yield 0.90 g (85%), mp 230 °C, $[\alpha]_D^{25} - 25.0^\circ$ ($c = 1.0$, DMF), R_f^3 0.63. *Anal.* Calcd for C₇₅H₁₀₀N₁₀O₁₇S₃: C, 59.7; H, 6.68; N, 9.2. Found: C, 59.8; H, 6.77; N, 9.2. Amino acid ratios in an acid hydrolysate: Glu 0.95; Gly 0.97; Ala 1.00; Ile 0.90; Lys 1.00 (average recovery 96.4%). Cys was not determined.

Boc-Cys(MBzl)-Cys(MBzl)-Ser-OBzl—Boc-Cys(MBzl)-Cys(MBzl)-N₃ (prepared from 5.79 g of the corresponding hydrazide,¹⁾ 3.13 ml of 6.4 N HCl in dioxane and 1.47 ml of isopentyl nitrite as usual) in DMF (20 ml) was added to a solution of H-Ser-OBzl·C₆H₅SO₃H (5.3 g) in DMF (20 ml) containing Et₃N (2.1 ml). This 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 H₂O, dried over Na₂SO₄ and concentrated. Petroleum ether was added to the residue to give a solid, which was recrystallized from AcOEt and ether, yield 6.72 g (90.6%), mp 70—71 °C, $[\alpha]_D^{21} - 6.8^\circ$ ($c = 1.0$, MeOH), R_f^1 0.50. *Anal.* Calcd for C₃₇H₄₇N₃O₉S₂: C, 59.9; H, 6.39; N, 5.7. Found: C, 59.4; H, 6.35; N, 5.6.

Boc-Lys(Z)-Ser-OMe—Boc-Lys(Z)-OH (9.13 g) and H-Ser-OMe·HCl (3.73 g) were dissolved in DMF (20 ml) and dioxane (20 ml) containing Et₃N (3.35 ml) and the solution was cooled to -15 °C. DCC (5.98 g) was added to the cold solution and the reaction mixture was stirred at 4 °C overnight. After removal of the *N,N'*-dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na₂CO₃ and water, dried over Na₂SO₄ and concentrated. Petroleum ether was added to the residue to give a crystalline material, yield 8.79 g (79%), mp 53 °C, $[\alpha]_D^{20} - 6.2^\circ$ ($c = 1.0$, MeOH), R_f^3 0.70. *Anal.* Calcd for C₂₂H₃₃N₃O₈: C, 56.5; H, 7.12; N, 9.00. Found: C, 57.0; H, 7.04; N, 8.9.

Boc-Lys(Z)-Ser-NHNH₂—Hydrazine hydrate (80%, 2.7 ml) was added to a solution of Boc-Lys-Ser-OMe (5.0 g) in MeOH (20 ml). The reaction mixture was kept at room temperature overnight. Crystals formed were collected by filtration and recrystallized from EtOH, yield 4.45 g (86.4%), mp 142—144 °C, $[\alpha]_D^{20} - 7.0^\circ$ ($c = 1.0$, DMF), R_f^3 0.61. *Anal.* Calcd for C₂₂H₃₃N₅O₇: C, 55.1; H, 6.94; N, 14.6. Found: C, 55.0; H, 7.45; N, 14.8.

Boc-Lys(Z)-Ser-Cys(MBzl)-Cys(MBzl)-Ser-OBzl—A solution of Boc-Cys(MBzl)-Cys(MBzl)-Ser-OMe (4.07 g) in TFA (5.18 ml) containing anisole (1.5 ml) was stored at room temperature for 45 min and then at 0 °C for 25 min. Ether and petroleum ether were added to the solution to give a precipitate, which was washed with ether and petroleum ether by decantation and dried over KOH pellets *in vacuo*. Boc-Lys(Z)-Ser-NHNH₂ (3.27 g) in DMF (20 ml) was converted to the corresponding azide with 5.4 N HCl in dioxane (2.59 ml) and isopentyl nitrite (1.07 ml) as usual and combined with the solution of H-Cys(MBzl)-Cys(MBzl)-Ser-OMe·TFA in DMF (20 ml) containing Et₃N (0.98 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was dissolved in AcOEt. The organic layer was washed with 10% citric acid and H₂O, dried over Na₂SO₄ and concentrated. Petroleum ether and ether were added to the residue to afford a solid mass, which was recrystallized from AcOEt and ether, yield 4.0 g (52.4%), mp 103—105 °C, $[\alpha]_D^{20} - 3.4^\circ$ ($c = 1.0$, DMF), R_f^1 0.50. *Anal.* Calcd for C₅₄H₇₀N₆O₁₄S₂·H₂O: C, 58.5; H, 6.54; N, 8.0. Found: C, 58.5; H, 6.60; N, 8.0.

Boc-Cys(MBzl)-Lys(Z)-OMe—Boc-Cys(MBzl)-OH (6.83 g) and H-Lys(Z)-OMe·HCl (6.62 g) were dissolved in DMF (25 ml) containing Et₃N (2.8 ml) and the solution was cooled to -10 °C. DCC (4.95 g) was added to the above solution and the reaction mixture was stirred at 4 °C overnight. After removal of the dicyclohexylurea and the solvent, the residue was dissolved in AcOEt. The organic layer was washed with 10% citric acid, 5% Na₂CO₃ and H₂O, dried over Na₂SO₄ and evaporated down. Petroleum ether was added to the residue to afford a solid mass, yield 7.63 g (62%), mp 70—74 °C, $[\alpha]_D^{20} - 2.0^\circ$ ($c = 1.0$, MeOH), R_f^1 0.75. *Anal.* Calcd for C₃₀H₄₃N₃O₈S: C, 60.3; H, 7.15; N, 6.8. Found: C, 60.7; H, 7.02; N, 7.3.

Boc-Cys(MBzl)-Lys(Z)-NHNH₂—Hydrazine hydrate (80%, 2.43 ml) was added to a solution of Boc-Cys(MBzl)-Lys(Z)-OMe (7.63 g) in MeOH (20 ml). The reaction mixture was kept at room temperature overnight. Crystalline material was collected by filtration and recrystallized from EtOH, yield 6.51 g (89%), mp 132—134 °C, $[\alpha]_D^{20} + 3.7^\circ$ ($c = 1.0$, DMF), R_f^1 0.58. *Anal.* Calcd for C₃₀H₄₃N₅O₇S: C, 58.3; H, 7.02; N, 11.3. Found: C, 58.6; H, 7.02; N, 11.6.

Boc-Cys(MBzl)-Lys(Z)-Lys(Z)-Ser-Cys(MBzl)-Cys(MBzl)-Ser-OBzl, Boc-(hMT 29—35)-OBzl—A solution of Boc-Lys(Z)-Ser-Cys(MBzl)-Cys(MBzl)-Ser-OBzl (3.6 g) in TFA (2.4 ml) containing anisole (0.27 ml) was stored at room temperature for 45 min and then at 0 °C for 25 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration, washed with ether and dried over NaOH pellets *in vacuo*. The resulting pentapeptide ester trifluoroacetate was dissolved in DMF (30 ml) containing Et₃N (0.46 ml) and cooled in an ice bath. Boc-Cys(MBzl)-Lys(Z)-N₃ (prepared from 3.1 g of the corresponding hydrazide with 1.85 ml of 5.4 N HCl in dioxane and 0.8 ml of isopentyl nitrite as usual) in DMF (25 ml) was combined with the cold DMF solution of the amino component obtained above. 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 solid mass, which was collected by filtration and washed with MeOH, yield 3.93 g (75.4%), mp 196—201 °C, $[\alpha]_D^{20} - 10.0^\circ$ ($c = 1.0$, DMF), R_f^1 0.64. *Anal.* Calcd for C₇₉H₁₀₁N₉O₁₉S₃·2H₂O:

C, 58.8; H, 6.56; N, 7.8. Found: C, 59.0; H, 6.44; N, 8.1. Amino acid ratios in an acid hydrolysate: Lys 2.00; Ser 1.43 (average recovery 86%). Cys was not determined.

Boc-Thr-Ser-OBzl—Boc-Thr-OH (5.5 g) and H-Ser-OBzl·C₆H₅SO₃H (8.84 g) were dissolved in CH₃CN (40 ml) containing Et₃N (3.49 ml) and the solution was cooled to −10 °C. DCC (6.19 g) was added to the solution and the reaction mixture was stirred at 4 °C overnight. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na₂CO₃ and H₂O, dried over Na₂SO₄ and evaporated down. Ether and petroleum ether were added to the residue to afford a solid mass, yield 5.2 g (52.0%), mp 56–57 °C, $[\alpha]_D^{22} + 0.2^\circ$ ($c = 1.0$, DMF), R_f^1 0.40. *Anal.* Calcd for C₁₉H₂₈N₂O₇: C, 57.6; H, 7.12; N, 7.1. Found: C, 57.3; H, 7.10; N, 7.3.

Boc-Lys(Z)-Cys(MBzl)-OBzl—Boc-Lys(Z)-OH (3.1 g) and H-Cys(MBzl)-OBzl·Tos-OH (4.22 g) were dissolved in DMF (20 ml) and dioxane (20 ml) containing Et₃N (1.13 ml) and the solution was cooled to −10 °C. DCC (2.06 g) was added to the cold solution and the reaction mixture was stirred at 4 °C overnight. After removal of the *N,N'*-dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na₂CO₃ and water, dried over Na₂SO₄ and evaporated down. Petroleum ether was added to the residue to afford a crystalline material, yield 5.0 g (88.9%), mp 69–71 °C, $[\alpha]_D^{26} - 14.2^\circ$ ($c = 1.0$, MeOH), R_f^1 0.70. *Anal.* Calcd for C₃₇H₄₃N₃O₈S: C, 64.1; H, 6.83; N, 6.1. Found: C, 64.3; H, 6.68; N, 6.3.

Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-OBzl—H-Lys(Z)-Cys(MBzl)-OBzl·TFA (prepared from 6.94 g of Boc-Lys(Z)-Cys(MBzl)-OBzl and 7.4 ml of TFA containing 2.17 ml of anisole as usual) and Boc-Cys(MBzl)-ONp (4.63 g) were dissolved in DMF (40 ml) containing Et₃N (1.39 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% NaHCO₃ and H₂O, dried over Na₂SO₄ and evaporated down. Petroleum ether was added to the residue to give a crystalline material, yield 7.82 g (85.5%), mp 91–92 °C, $[\alpha]_D^{22} - 34.0^\circ$ ($c = 1.0$, DMF), R_f^2 0.72. *Anal.* Calcd for C₄₈H₅₈N₄O₁₀S₂: C, 62.3; H, 6.39; N, 6.1. Found: C, 62.6; H, 6.38; N, 6.0.

Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-NHNH₂—Hydrazine hydrate (80%, 1.84 ml) was added to a solution of Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-OBzl (7.0 g) in MeOH (30 ml). The reaction mixture was stored at room temperature overnight. Crystals that appeared were collected by filtration and recrystallized from EtOH, yield 4.99 g (78.3%), mp 140–142 °C, $[\alpha]_D^{22} - 4.0^\circ$ ($c = 1.0$, DMF), R_f^2 0.45. *Anal.* Calcd for C₄₁H₅₄N₆O₉S₂: C, 58.7; H, 6.49; N, 10.0. Found: C, 58.8; H, 6.57; N, 9.9.

Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl—H-Thr-Ser-OBzl·TFA (prepared from 2.0 g of Boc-Thr-Ser-OBzl and 3.7 ml of TFA containing 1.1 ml of anisole as usual) was dissolved in DMF (20 ml) containing Et₃N (0.7 ml) and the solution was cooled to 0 °C. To this solution, Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-N₃ (prepared from 4.19 g of Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-NHNH₂, 0.8 ml of isopentyl nitrite and 1.56 ml of 6.4 N HCl in dioxane as usual) in DMF (25 ml) was added. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and H₂O were added to the residue to give crystals, which were collected by filtration and washed with AcOEt, yield 4.44 g (80.0%), mp 152–154 °C, $[\alpha]_D^{23} - 11.8^\circ$ ($c = 1.0$, DMF), R_f^1 0.51. *Anal.* Calcd for C₅₅H₇₂N₆O₁₄S₂: C, 57.9; H, 6.71; N, 7.4. Found: C, 57.8; H, 6.57; N, 7.6.

Boc-Glu(OBzl)-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl—H-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl·TFA (prepared from 3.0 g of Boc-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl and 2.0 ml of TFA containing 0.59 ml of anisole) and Boc-Glu(OBzl)-ONp (1.38 g) were dissolved in DMF (40 ml) containing Et₃N (0.38 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration and washed with MeOH, yield 2.4 g (66.7%), mp 165–167 °C, $[\alpha]_D^{24} - 10.9^\circ$ ($c = 1.0$, DMF), R_f^2 0.82. *Anal.* Calcd for C₆₇H₈₅N₇O₁₇S₂·H₂O: C, 59.9; H, 6.53; N, 7.3. Found: C, 59.7; H, 6.37; N, 7.4.

Boc-Lys(Z)-Cys(MBzl)-Lys(Z)-OMe—H-Cys(MBzl)-Lys(Z)-OMe·TFA (prepared from 3.1 g of Boc-Cys(MBzl)-Lys(Z)-OMe and 3.7 ml of TFA containing 1.1 ml of anisole as usual) and Boc-Lys(Z)-ONp (3.0 g) were dissolved in DMF (25 ml) containing Et₃N (0.7 ml). The reaction mixture was stirred at room temperature for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% NaHCO₃ and H₂O, dried over Na₂SO₄ and concentrated. Ether and petroleum ether were added to the residue to afford crystals, yield 3.2 g (76.0%), mp 66–68 °C, $[\alpha]_D^{25} - 14.5^\circ$ ($c = 1.0$, DMF), R_f^1 0.49. *Anal.* Calcd for C₄₃H₆₁N₅O₁₁S: C, 60.3; H, 7.18; N, 8.2. Found: C, 60.0; H, 6.88; N, 8.2.

Boc-Lys(Z)-Cys(MBzl)-Lys(Z)-NHNH₂—Hydrazine hydrate (80%, 0.53 ml) was added to a solution of Boc-Lys(Z)-Cys(MBzl)-Lys(Z)-OMe (3.2 g) in MeOH (20 ml). The reaction mixture was stored at room temperature overnight and concentrated to half the initial volume. Ether was added to the residue to give crystals, which were recrystallized from EtOH, yield 1.54 g (48.2%), mp 130–141 °C, $[\alpha]_D^{25} + 5.0^\circ$ ($c = 1.0$, DMF), R_f^3 0.75. *Anal.* Calcd for C₄₂H₆₁N₇O₁₀S: C, 58.9; H, 7.18; N, 11.5. Found: C, 58.7; H, 6.93; N, 11.3.

Boc-Lys(Z)-Cys(MBzl)-Lys(Z)-Glu(OBzl)-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl, Boc-(hMT 20–28)-OBzl—A solution of Boc-Glu(OBzl)-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Lys(Z)-Cys(MBzl)-Thr-Ser-OBzl (1.0 g) in TFA (0.56 ml) containing anisole (0.16 ml) was stored at room temperature for 45 min and then at 0 °C for 25 min. Ether was added to the solution to afford a precipitate, which was washed with ether by decantation and dried over NaOH pellets *in vacuo*. Boc-Lys(Z)-Cys(MBzl)-Lys(Z)-N₃ (prepared from 0.7 g of the corresponding hydrazide,

0.17 ml of isopentyl nitrite and 0.41 ml of 5.4 N HCl in dioxane as usual) in DMF (10 ml) was mixed with the hexapeptide amine TFA salt in DMF (20 ml) containing Et₃N (0.1 ml). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration and washed with MeOH, yield 0.86 g (54.7%), mp 242–244 °C, $[\alpha]_D^{25} -2.5^\circ$ ($c=1.0$, DMF), R_f^3 0.86. *Anal.* Calcd for C₁₀₆H₁₃₄N₁₂O₂₅S₃·2H₂O: C, 60.4; H, 6.60; N, 8.0. Found: C, 60.3; H, 6.56; N, 8.1. Amino acid ratios in an acid hydrolysate: Thr 0.84; Ser 0.70; Glu 0.90; Lys 2.90 (average recovery 86%). Cys was not determined.

Boc-Cys(MBzl)-Ala-NHNH₂—Hydrazine hydrate (80%, 3.9 ml) was added to a solution of Boc-Cys(MBzl)-Ala-OBzl¹¹ (7.00 g) in MeOH (20 ml). The reaction mixture was stored at room temperature overnight. Crystals were collected by filtration and recrystallized from EtOH, yield 5.92 g (85.7%), mp 135–138 °C, $[\alpha]_D^{25} -1.4^\circ$ ($c=1.0$, DMF), R_f^1 0.66. *Anal.* Calcd for C₁₉H₃₀N₄O₅S: C, 53.5; H, 7.09; N, 13.1. Found: C, 53.6; H, 7.00; N, 13.1.

Boc-Cys(MBzl)-Ala-Gly-Ser-OMe—Boc-Cys(MBzl)-Ala-N₃ (prepared from 4.3 g of Boc-Cys(MBzl)-Ala-NHNH₂, 0.14 ml of isopentyl nitrite and 0.4 ml of 5.4 N HCl in dioxane) in DMF (30 ml) was added to a cold solution of H-Gly-Ser-OMe (prepared from 3.1 g of Z-Gly-Ser-OMe¹⁶) by catalytic hydrogenation) in DMF (30 ml) containing Et₃N (1.39 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 H₂O, dried over Na₂SO₄ and concentrated. Ether and petroleum ether were added to the residue to afford crystals, yield 5.16 g (90.4%), mp 90–94 °C, $[\alpha]_D^{25} -32.0^\circ$ ($c=1.0$, MeOH), R_f^1 0.62. *Anal.* Calcd for C₂₅H₃₈N₄O₉S: C, 52.6; H, 6.71; N, 9.8. Found: C, 52.4; H, 6.52; N, 9.5.

Boc-Cys(MBzl)-Ala-Gly-Ser-NHNH₂—Hydrazine hydrate (80%, 1.7 ml) was added to a solution of 4.83 g of Boc-Cys(MBzl)-Ala-Gly-Ser-OMe in MeOH (50 ml). The reaction mixture was stored at room temperature overnight. Crystals were collected by filtration and recrystallized from EtOH, yield 2.2 g (45.5%), mp 180–182 °C, $[\alpha]_D^{25} -10.0^\circ$ ($c=1.0$, DMF), R_f^1 0.30. *Anal.* Calcd for C₂₄H₃₈N₆O₈S: C, 50.5; H, 6.71; N, 14.7. Found: C, 50.4; H, 6.73; N, 14.2.

Boc-Cys(MBzl)-Ala-Gly-Ser-Cys(MBzl)-OBzl—Boc-Cys(MBzl)-Ala-Gly-Ser-N₃ (prepared from 1.5 g of the corresponding hydrazide with 0.4 ml of isopentyl nitrite and 0.56 ml of 5.4 N HCl in dioxane as usual) in DMF (20 ml) was combined with a solution of H-Cys(MBzl)-OBzl·Tos-OH (2.0 g) in DMF (10 ml) containing Et₃N (0.56 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, MeOH was added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt and ether, yield 2.18 g (96.2%), mp 89–90 °C, $[\alpha]_D^{25} -11.4^\circ$ ($c=1.0$, DMF), R_f^1 0.70. *Anal.* Calcd for C₄₂H₅₅N₅O₁₁S₂·3H₂O: C, 54.6; H, 6.66; N, 7.6. Found: C, 54.7; H, 6.55; N, 7.1.

Boc-Cys(MBzl)-Thr-NHNH₂—Boc-Cys(MBzl)-OH (6.83 g), H-Thr-OMe·HCl (3.39 g) and Et₃N (2.79 ml) were dissolved in CH₃CN (60 ml) and the solution was cooled with ice-salt. DCC (4.95 g) was added to the cold solution. This reaction mixture was stirred at 4 °C for 2 d. After removal of the *N,N'*-dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% NaHCO₃ and H₂O, dried over Na₂SO₄ and evaporated to dryness to give Boc-Cys(MBzl)-Thr-OMe (R_f^3 0.74) as an oily material. This was dissolved in MeOH (20 ml). Hydrazine hydrate (80%, 3.89 ml) was added to the solution and the reaction mixture was stored at room temperature overnight. Crystalline material that formed was collected by filtration and recrystallized from EtOH, yield 4.16 g (56.9%), mp 117–119 °C, $[\alpha]_D^{25} -11.4^\circ$ ($c=1.0$, DMF), R_f^3 0.55. *Anal.* Calcd for C₂₀H₃₂N₄O₆S: C, 52.6; H, 7.07; N, 12.3. Found: C, 52.7; H, 7.22; N, 12.1.

Boc-Cys(MBzl)-Thr-Cys(MBzl)-Ala-Gly-Ser-Cys(MBzl)-OBzl, Boc-(hMT 13–19)-OBzl—Boc-Cys(MBzl)-Thr-N₃ (prepared from 1.28 g of Boc-Cys(MBzl)-Thr-NHNH₂, 1.04 ml of 5.4 N HCl in dioxane and 0.41 ml of isopentyl nitrite) in DMF (10 ml) was added to a solution of H-Cys(MBzl)-Ala-Gly-Ser-Cys(MBzl)-OBzl (prepared from 2.1 g of Boc-(hMT 15–19)-OBzl with 1.8 ml of TFA containing 0.52 ml of anisole) in DMF (10 ml) containing Et₃N (0.33 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, MeOH was added to the residue to give crystals, which were collected by filtration and washed with MeOH, yield 1.2 g (41.7%), mp 198–202 °C, $[\alpha]_D^{25} -10.5^\circ$ ($c=1.0$, DMF), R_f^2 0.86. *Anal.* Calcd for C₅₇H₇₅N₇O₁₅S₃·2H₂O: C, 55.6; H, 6.47; N, 8.0. Found: C, 55.6; H, 6.30; N, 8.0. Amino acid ratios in an acid hydrolysate: Thr 0.89; Ser 0.83; Gly 1.00; Ala 1.11 (average recovery 95%). Cys was not determined.

Boc-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl—Boc-Cys(MBzl)-Ser-N₃ (prepared from 10.0 g of the corresponding hydrazide,¹¹ 6.05 ml of 7.6 N HCl in dioxane and 4.69 ml of isopentyl nitrite) in DMF (20 ml) was combined with a solution of H-Cys(MBzl)-Ala-OBzl·TFA (prepared from 10.05 g of Boc-Cys(MBzl)-Ala-OBzl with 14.8 ml of TFA containing 4.32 ml of anisole as usual) in DMF (20 ml) containing Et₃N (2.79 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 H₂O, dried over Na₂SO₄ and concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt and ether, yield 10.48 g (65.5%), mp 142–144 °C, $[\alpha]_D^{26} -7.5^\circ$ ($c=1.0$, DMF) R_f^1 0.45. *Anal.* Calcd for C₄₀H₅₂N₄O₁₀S₂: C, 59.1; H, 6.45; N, 6.9. Found: C, 58.9; H, 6.41; N, 6.9.

Boc-Asn-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl, Boc-(hMT 4–8)-OBzl—H-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl·TFA (prepared from 1.2 g of Boc-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl, 1.1 ml of TFA and 0.33 ml of anisole as usual) and Boc-Asn-ONp (0.6 g) were dissolved in DMF (15 ml) containing Et₃N (0.21 ml). The reaction mixture was

stirred 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 MeOH, yield 1.15 g (80.0%), mp 184–187 °C, $[\alpha]_D^{21} -24.0^\circ$ ($c=0.5$, DMF), $R_f^{21} 0.44$. *Anal.* Calcd for $C_{45}H_{60}N_6O_{12}S_2 \cdot 2H_2O$: C, 55.3; H, 6.60; N, 8.6. Found: C, 55.3; H, 6.32; N, 8.8. Amino acid ratios in an acid hydrolysate: Asp 1.02; Ser 0.92; Ala 1.00 (average recovery 80.6%). Cys was not determined.

Boc-Lys(Z)-Cys(MBzl)-NHNH₂—Hydrazine hydrate (80%, 1.46 ml) was added to a solution of Boc-Lys(Z)-Cys(MBzl)-OBzl (5.15 g) in MeOH (25 ml). The reaction mixture was stored at room temperature overnight and concentrated to half the initial volume to give crystals, which were collected by filtration and recrystallized from EtOH and H₂O, yield 4.02 g (87.8%), mp 140–142 °C, $[\alpha]_D^{26} -4.7^\circ$ ($c=0.1$, MeOH), $R_f^{26} 0.60$. *Anal.* Calcd for $C_{30}H_{43}N_5O_7S$: C, 58.3; H, 7.02; N, 11.3. Found: C, 58.2; H, 7.13; N, 11.3.

Boc-Lys(Z)-Cys(MBzl)-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl—Boc-Lys(Z)-Cys(MBzl)-N₃ (prepared from 2.29 g of the corresponding hydrazide, 1.0 ml of 7.6 N HCl in dioxane and 0.6 ml of isopentyl nitrite as usual) was added to a solution of H-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl·TFA (prepared from 3.0 g of Boc-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl, 2.8 ml of TFA and 0.82 ml of anisole as usual) in DMF (20 ml) containing Et₃N (0.53 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and H₂O were added to the residue to give crystals, which were collected by filtration and washed with MeOH, yield 2.7 g (70.1%), mp 178–182 °C, $[\alpha]_D^{26} -10.4^\circ$ ($c=1.0$, DMF), $R_f^{26} 0.72$. *Anal.* Calcd for $C_{65}H_{83}N_7O_{15}S_3 \cdot H_2O$: C, 59.3; H, 6.51; N, 7.5. Found: C, 59.0; H, 6.57; N, 7.6.

Boc-Asp(OBzl)-Lys(Z)-Cys(MBzl)-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl, Boc-(Old hMT 55–61)-OBzl—H-Lys(Z)-Cys(MBzl)-Cys(MBzl)-Ser-Cys(MBzl)-Ala-OBzl·TFA (prepared from 2.0 g of the corresponding protected hexapeptide and 1.5 ml of TFA containing 0.3 ml of anisole as usual) and Boc-Asp(OBzl)-ONp (0.86 g) were dissolved in DMF (30 ml) containing Et₃N (0.25 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford a solid, which was collected by filtration and washed with MeOH, yield 1.24 g (55.6%), mp 208–209 °C, $[\alpha]_D^{25} -22.5^\circ$ ($c=1.0$, DMF), $R_f^{25} 0.45$. *Anal.* Calcd for $C_{76}H_{94}N_8O_{17}S_3 \cdot H_2O$: C, 60.6; H, 6.43; N, 7.4. Found: C, 60.7; H, 6.31; N, 7.5. Amino acid ratios in an acid hydrolysate: Asp 0.89; Ser 0.84; Ala 1.00; Lys 0.93 (average recovery 91.8%). Cys was not determined.

General Procedure for Deblocking in Order to Obtain the Peptides (I–VIII)—a) HF Method: The protected peptide (100 mg each) 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. HF was then 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 (3 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 (3 g each) were collected. The desired fractions (tube Nos. mainly 29–35) were combined and lyophilized. Yield, $[\alpha]_D$, R_f value and analytical data are summarized in Table I.

b) Methanesulfonic Acid (MSA) Method: Boc-(hMT 43–50)-OBzl (100 mg) was treated with MSA (4.0 ml) in the presence of thioanisole (0.17 ml) and *m*-cresol (0.73 ml) in an ice-bath for 10 min and then at room temperature for 60 min. Dry ether was added to the solution to give an oily 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 (3 g each) were collected. The desired fractions (tube Nos. 29–35) were combined and lyophilized to give a fluffy powder. Yield, $[\alpha]_D$, R_f value and analytical data are listed in Table I.

Gel-Filtration of Peptide-Metal Complex—A solution of peptide (0.15 mM as –SH) in 2 ml of 50 mM Tris/HCl, pH 8.0, was mixed with 0.3 ml of CdCl₂ or ZnCl₂ solution (20 mM). The reaction mixture was charged on a Sephadex G-15 column (1.0 × 30.0 cm) previously equilibrated with 50 mM Tris/HCl, pH 8.0. The column was eluted with the same buffer and two-ml fractions were collected. Metal concentration, UV absorbance at 215 nm (for Zn-mercaptide) or 250 nm (for Cd-mercaptide) and –SH concentration of each eluate were determined.¹⁾

Binding Ability of Peptides with Cd²⁺—A 5–45 μl aliquot of CdCl₂ solution (5 mM) was added to 3 ml of peptide solution (0.15 mM as –SH in 10 mM Tris/HCl, pH 7.0). The UV absorbance at 250 nm of the mixture was determined and the increase was plotted against Cd concentration.

References and Notes

- 1) Part VIII: N. Ohta, Y. Okada, and K. Tanaka, *Chem. Pharm. Bull.*, **31**, 1885 (1983).
- 2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine. Standard abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *idem, ibid.*, **6**, 362 (1967); *idem, ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; ONp, *p*-

nitrophenyl ester; DCC, *N,N'*-dicyclohexylcarbodiimide; MBzl, *p*-methoxybenzyl; TFA, trifluoroacetic acid; DMF, dimethylformamide; AcOEt, ethyl acetate; AcOH, acetic acid; *n*-BuOH, *n*-butanol; HOBT, 1-hydroxybenzotriazole.

- 3) Y. Kojima and J. H. R. Kägi, *TIBS*, **1978**, 90.
- 4) M. M. Kissling and J. H. R. Kägi, *FEBS Lett.*, **82**, 247 (1977).
- 5) Personal communication from Dr. M. Kimura
- 6) A. Yoshida, B. E. Kaplan, and M. Kimura, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 486 (1979).
- 7) I-Y. Huang, A. Yoshida, H. Tsunoo, and H. Nakajima, *J. Biol. Chem.*, **252**, 8217 (1977).
- 8) J. D. Otovos and I. M. Armitage, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7094 (1980).
- 9) D. R. Winge and K-A. Miklossky, *J. Biol. Chem.*, **257**, 347 (1982).
- 10) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jnp.*, **40**, 2164 (1967).
- 11) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).
- 12) S. Funakoshi, N. Fujii, H. Yajima, C. Shigeno, I. Yamamoto, R. Morita, and K. Torizuka, *Chem. Pharm. Bull.*, **30**, 1706 (1980).
- 13) N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 650 (1978).
- 14) J. Honzle and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 233 (1966).
- 15) E. Schröder and E. Klieger, *Justus Liebigs Ann. Chem.*, **673**, 208 (1964).
- 16) R. G. Hiskey, T. Mizoguchi, and F. E. L. Smith, Jr., *J. Org. Chem.*, **32**, 97 (1967).
- 17) P. Pulido, J. H. R. Kägi, and B. L. Vallee, *Biochemistry*, **5**, 1768 (1966).
- 18) J. H. R. Kägi and B. L. Vallee, *J. Biol. Chem.*, **236**, 2435 (1961).
- 19) M. Vasak, J. H. R. Kägi, and H. A. O. Hill, *Biochemistry*, **20**, 2852 (1981).
- 20) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).