Amino Acids and Peptides. XXXV. Synthesis of Mouse Metallothionein I. (2). Synthesis of a Nonacosapeptide Corresponding to N-Terminal Sequence 1—29 (β-Fragment) of Mouse Metallothionein I and Related Peptides and Examination of Their Heavy Metal-Binding Properties[1,2]

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A nonacosapeptide corresponding to the N-terminal sequence 1—29 (β-fragment) of mouse metallothionein I and related peptides were synthesized by the conventional solution method and their heavy metals (Cu^{2+}, Cu^{+} and Cd^{2+})-binding properties were examined. The Cu^{2+}- or Cu^{+}-binding activities of various peptides were not greatly dependent on the peptide structure, so far as examined, as in the cases of *N. crassa* MT and *A. bisporus* MTs. On the contrary, the Cd^{2+}-binding activities of these peptides were fairly structure-dependent.

Keywords mouse metallothionein I; nonacosapeptide; β-fragment; related peptide; chemical synthesis; solution method; heavy metal-binding property; structure–activity relationship

Synthesis of a dotriacontapeptide corresponding to C-terminal sequence 30—61 (α-fragment) of mouse metallothionein (MT) I[3] and related peptides and examination of their heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding properties were reported in the preceding paper.[1]

This paper deals with the synthesis of β-fragment of mouse MT I[3] and related peptides and their heavy metal-binding properties with the objectives of clarifying differences in their affinity for different metal ions and of comparing their heavy metal-binding properties with those of *N. crassa*[4] and *A. bisporus*[5] MTs.

As illustrated in Fig. 1, a protected nonacosapeptide (β-fragment) was prepared by the fragment condensation method, starting with the C-terminal octapeptide ester (**1**). Amino acid derivatives bearing protecting groups removable by treatment with HF at 0 °C for 60 min[6] or methanesulfonic acid (MSA) at 20 °C for 60 min,[7] *i.e.* Asp(O-2-Ada),[8] Lys(Z) and Cys(MeBzl),[9] were employed in combination with the TFA labile Boc-group as the N^{α}-protecting group.

The synthetic scheme for the C-terminal octapeptide ester, H–(MT 22—29)–OBzl, (**1**) is illustrated in Fig. 2. Boc–Thr–Ser–$NHNH_2$, which was prepared by DCC coupling of Boc–Thr–OH with H–Ser–OMe, followed by hydrazine hydrate treatment, was coupled with H–Cys(MeBzl)–OBzl to afford Boc–Thr–Ser–Cys(MeBzl)–OBzl. After treatment of the tripeptide with TFA, the resultant peptide amine was coupled with Boc–Lys(Z)–Cys(MeBzl)–$NHNH_2$, which was prepared by coupling of Boc–Lys(Z)–ON*p* with H–Cys(MeBzl)–OBzl, followed by hydrazine hydrate treatment, by the azide method to give Boc–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl. After treatment of the pentapeptide with TFA, the resultant amine was coupled with Boc–Asp(O-2-Ada)–OSu and Boc–Lys(Z)–ON*p* to give Boc–(MT 22—29)–OBzl, which was treated with TFA to afford the peptide (**1**).

Boc–Cys(MeBzl)–Ala–Cys(MeBzl)–$NHNH_2$, Boc–(MT 19—21)–$NHNH_2$ (**2**), was prepared as follows: Boc–Cys(MeBzl)–ON*p* was coupled with H–Ala–OBzl to afford Boc–Cys(MeBzl)–Ala–OBzl, which was treated with hydrazine hydrate to give the corresponding hydrazide. This hydrazide was coupled with H–Cys(MeBzl)–OBzl by the azide method, followed by hydrazine hydrate treatment to give the peptide (**2**). Boc–Thr–Ser–Ser–$NHNH_2$, Boc–(MT 16—18)–$NHNH_2$ (**3**), was prepared by the coupling of Boc–Thr–Ser–$NHNH_2$ with H–Ser–OMe, followed by hydrazine hydrate treatment. Boc–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–$NHNH_2$, Boc–(MT 12—15)–$NHNH_2$ (**4**), was prepared as follows: Boc–Cys(MeBzl)–Thr–$NHNH_2$ was coupled with H–Cys(MeBzl)–OBzl by the azide procedure to give Boc–Cys(MeBzl)–Thr–Cys(MeBzl)–OBzl, which was treated with TFA to afford the tripeptide ester. This ester was coupled with Boc–Ser–$NHNH_2$ by the azide procedure, followed by hydrazine hydrate treatment to give

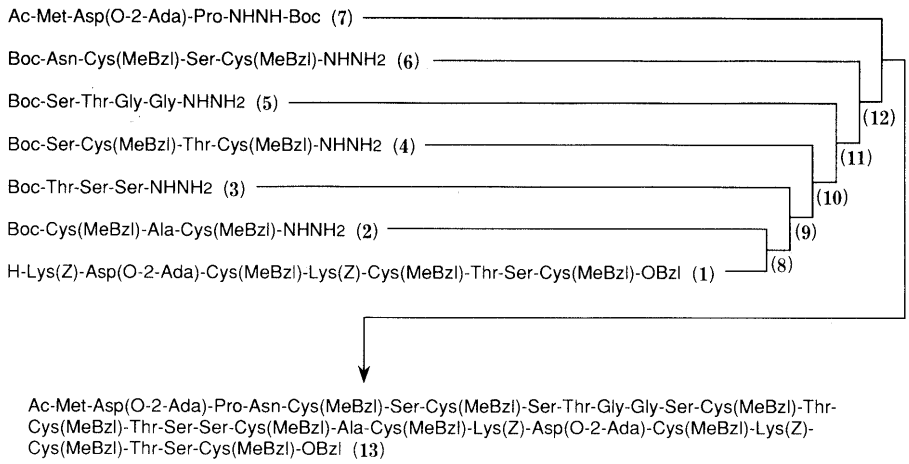

Fig. 1. Synthetic Scheme for β-Fragment of Mouse MT I and Related Peptides

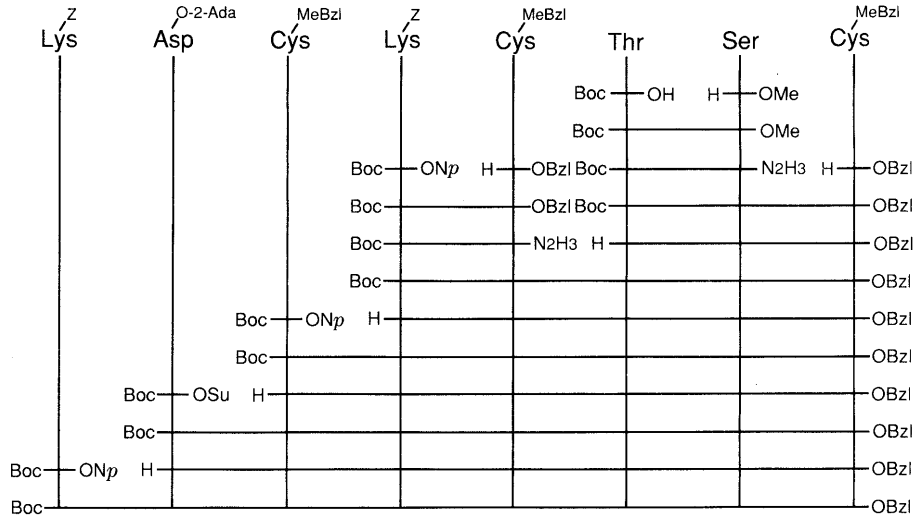

Fig. 2. Synthetic Route to Boc-(MT 22—29)-OBzl

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

Compound	Yield (%)	$[\alpha]_D^{25}$ ($c=0.2$, 3% AcOH)	*Rf*[a]	Amino acid ratios in acid hydrolysate[b]									SH content
				Asp	Thr	Ser	Gly	Ala	Cys	Met	Lys	Pro	
H-[MT 24—29]-OH	73.1	−27.7°	0.71		0.89	0.87			3.15		1.00		2.75
H-[MT 22—29]-OH	41.5	−34.5°	0.75	1.00	0.91	0.85			3.17		2.09		2.79
H-[MT 19—29]-OH	64.0	−48.3°	0.64	1.00	0.87	0.90		1.02	4.72		2.11		4.49
H-[MT 16—29]-OH	65.1	−59.5°	0.50	1.00	1.98	2.47		1.10	4.91		2.14		4.77
H-[MT 12—29]-OH	74.9	−43.3°	0.53	1.00	2.81	3.28		1.01	7.15		2.18		6.57
H-[MT 8—29]-OH	48.3	−43.5°	0.60	1.00	3.51	4.12	1.86	0.97	7.29		2.02		6.65
H-[MT 4—29]-OH	69.4	−37.4°	0.53	2.00	3.57	4.79	1.98	0.93	8.79		2.02		8.19
H-[MT 1—29]-OH (β-fragment)	34.2	−47.8°	0.41	3.00	3.61	4.71	2.10	1.09	8.61	0.65	2.23	1.12	8.38

a) *n*-BuOH : pyridine : AcOH : $H_2O = 1:1:1:1$. *b*) Cys was detected as $CySO_3H$.

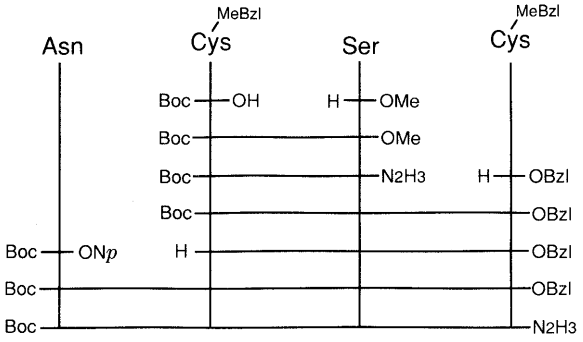

Fig. 3. Synthetic Route to Boc-(MT 4—7)-$NHNH_2$ (**6**)

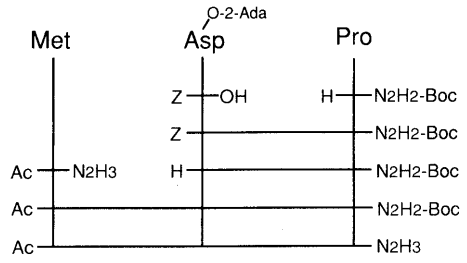

Fig. 4. Synthetic Route to Ac-(MT 1—3)-NHNH-Boc (**7**)

the peptide (**4**).

Boc-Ser-Thr-Gly-Gly-$NHNH_2$, Boc-(MT 8—11)-$NHNH_2$ (**5**), was prepared as follows: Boc-Thr-OH and H-Gly-Gly-OBzl were coupled by DPPA treatment to give Boc-Thr-Gly-Gly-OBzl, which was treated with TFA. The resultant amine was coupled with Boc-Ser-$NHNH_2$, followed by hydrazine hydrate treatment to afford the peptide (**5**).

Boc-Asn-Cys(MeBzl)-Ser-Cys(MeBzl)-$NHNH_2$, Boc-(MT 4—7)-$NHNH_2$ (**6**) was prepared by the route shown in Fig. 3.

Ac-Met-Asp(O-2-Ada)-Pro-NHNH-Boc (**7**) was prepared as shown in Fig. 4.

According to the scheme shown in Fig. 2, starting with H-(MT 22—29)-OBzl (**1**), Boc-(MT 19—21)-$NHNH_2$ (**2**), Boc-(MT 16—18)-$NHNH_2$ (**3**), Boc-(MT 12—15)-$NHNH_2$ (**4**), Boc-(MT 8—11)-$NHNH_2$ (**5**), Boc-(MT 4—7)-$NHNH_2$ (**6**) and Ac-(MT 1—3)-NHNH-Boc (**7**) were coupled successively to afford peptides (**8**—**12**) and finally the protected β-fragment, Boc-(MT 1—29)-OBzl (**13**).

Next, the protected nonacosapeptide (**13**) and related intermediates, Boc-(MT 24—29)-OBzl, Boc-(MT 22—29)-OBzl and peptides (**8**—**12**), were deprotected by the HF method. During the course of this deprotection reaction, oxygen-free water was used and a slightly acidic solvent was employed as the eluant for column chromatography on Sephadex G-15 or Sephadex G-25 in order to prevent

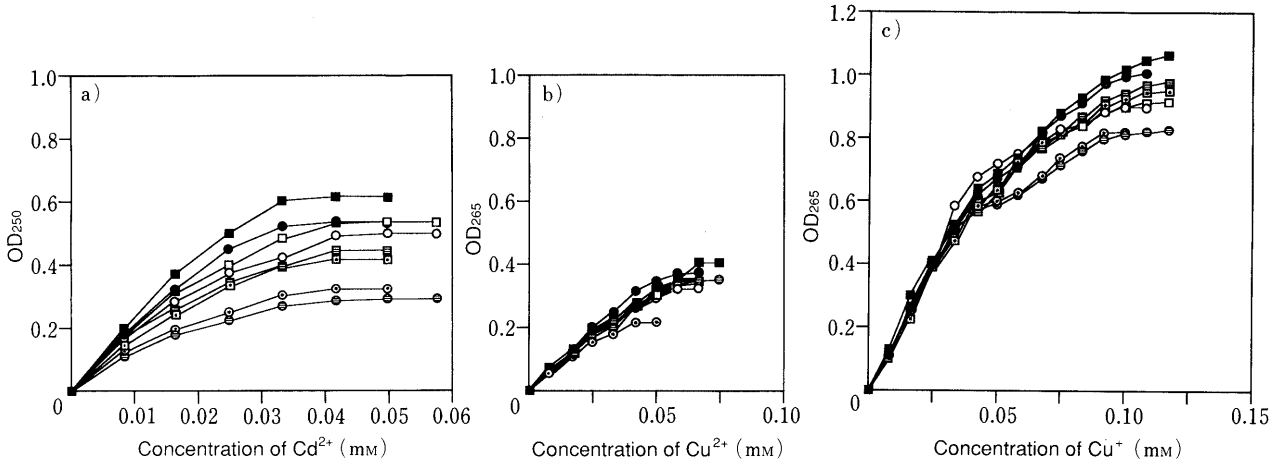

Fig. 5. Binding Properties of Peptides with Heavy Metals, a) with Cd^{2+}, b) with Cu^{2+}, c) with Cu^{+}

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). ⊙, mouse MT I (24—29); ⊡, (22—29); ⊜, (19—29); ▤, (16—29); ○, (12—29); ●, (8—29); □, (4—29); ■, (1—29).

disulfide formation.

The homogeneity of the peptides obtained was ascertained by TLC and amino acid analysis. The free SH content was also determined by the Ellman method.[10] Yield, *Rf* and $[\alpha]_D$ values, the results of amino acid analysis and the value of the SH content are summarized in Table I.

The metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding abilities of various peptides obtained above were assessed by the same procedure as described previously and the results are shown in Fig. 5a, b and c. As shown in Fig. 5, the heavy metals-binding properties of β-fragment and related peptides are quite different from those of α-fragment and related peptides,[1] which contain Cys–X–Cys–Cys sequence in addition to Cys–X–Cys sequence, and similar to those of *N. crassa*[4] and *A. bisporus*[5] MTs and related peptides, which contain only Cys–X–Cys sequences. Namely, Cd^{2+}-binding activity (Fig. 5a) increases in proportion to the increment of peptide chain length from the C-terminus. The whole structure of β-fragment is most suitable for Cd^{2+} binding in comparison with other shorter peptides. On the contrary, the Cu^{2+}-binding abilities (Fig. 5b) as well as Cu^{+}-binding abilities (Fig. 5c) of the various peptides are similar to each other, although there is a difference in the intensity of absorbance between Cu^{2+}- and Cu^{+}-peptides. The positions of Cys residues in β-fragment and related peptides might be suitable for Cu-mercaptide formation. These factors may explain why Cu ions can preferentially bind with β-fragment in mammalian MTs when Cd^{2+} is present and *N. crassa* and *A. bisporus* MTs, which are much shorter peptides than mammalian MTs,[11,12] can participate in Cu-binding.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of acid hydrolysates (110 °C, 6 N HCl, 20 h; Cys was determined as cysteic acid) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co.). Absorption spectra were recorded with a Hitachi 323 recording spectrometer. On TLC (Kieselgel G, Merck), Rf^1, Rf^2 and Rf^3 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (8:3:1, lower phase) and *n*-BuOH, pyridine, AcOH and H_2O (1:1:1:1), respectively.

Boc–Thr–Ser–$NHNH_2$ Boc–Thr–OH (6.0 g), H–Ser–OMe·HCl (3.6 g), Et_3N (3.5 ml) and HOBt (3.5 g) were dissolved in DMF (100 ml). DCC (5.2 g) was added to the above solution under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the dicyclohexylurea 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. Hydrazine hydrate (90%, 1.64 ml) was added to a solution of the above residual oil in MeOH (100 ml) and the reaction mixture was stored at room temperature overnight. The resultant precipitate was collected by filtration and washed with MeOH, yield 3.0 g (41.0%), mp 140—143 °C, $[\alpha]_D^{25}$ +7.27° (c=1.0, DMF), Rf^2 0.54. *Anal.* Calcd for $C_{12}H_{24}N_4O_6$: C, 45.0; H, 7.55; N, 17.5. Found: C, 44.7; H, 7.49; N, 17.8.

Boc–Thr–Ser–Cys(MeBzl)–OBzl Boc–Thr–Ser–N_3 [prepared from the corresponding hydrazide (5.0 g) and isopentyl nitrite (2.2 ml) as usual] in DMF (100 ml) was added to a solution of H–Cys(MeBzl)–OBzl·TosOH (11.0 g) in DMF (200 ml) containing Et_3N (3.3 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The residual oil in $CHCl_3$ (10 ml) was applied to a silica gel column (3.5×45 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (2000 ml), 1% MeOH in $CHCl_3$ (2000 ml) and 2% MeOH in $CHCl_3$ (2000 ml), successively. The solvent of the effluent (4400—5600 ml) was removed by evaporation to give an amorphous powder, yield 6.0 g (63.7%), mp 44—45 °C, $[\alpha]_D^{25}$ −41.5° (c=1.0, MeOH), Rf^1 0.68. *Anal.* Calcd for $C_{30}H_{41}N_3O_8S$: C, 59.7; H, 6.85; N, 6.96. Found: C, 59.7; H, 7.00; N, 6.86.

Boc–Lys(Z)–Cys(MeBzl)–OBzl Boc–Lys(Z)–ON*p* (10.0 g) and H–Cys(MeBzl)–OBzl·TosOH (11.3 g) were dissolved in DMF (200 ml) containing Et_3N (3.4 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 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 8.8 g (64.6%), mp 113—117 °C, $[\alpha]_D^{25}$ −32.0° (c=1.0, MeOH), Rf^1 0.80. *Anal.* Calcd for $C_{37}H_{47}N_3O_7S$: C, 65.6; H, 6.99; N, 6.20. Found: C, 65.4; H, 7.07; N, 6.30.

Boc–Lys(Z)–Cys(MeBzl)–$NHNH_2$ Hydrazine hydrate (90%, 3.5 ml) was added to a solution of Boc–Lys(Z)–Cys(MeBzl)–OBzl (7.8 g) in DMF (50 ml). The reaction mixture was stored at room temperature overnight. MeOH was added to the solution to afford a precipitate, which was collected by filtration and washed with MeOH, yield 6.2 g (89.5%), mp 135—140 °C, $[\alpha]_D^{25}$ −18.3° (c=1.0, MeOH), Rf^1 0.55. *Anal.* Calcd for $C_{30}H_{43}N_5O_6S$: C, 59.9; H, 7.20; N, 11.6. Found: C, 59.8; H, 7.24; N, 11.7.

Bos–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl Boc–Lys(Z)–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (3.0 g) and isopentyl nitrite (0.70 ml) as usual] in DMF (50 ml) was added to a solution of H–Thr–Ser–Cys(MeBzl)–OBzl·TFA [prepared from Boc–Thr–Ser–Cys(MeBzl)–OBzl (3.0 g), TFA (5.7 ml) and anisole (1.56 ml) as usual] in DMF (50 ml) containing Et_3N (0.70 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 4.0 g (75.0%), mp 102—109 °C, $[\alpha]_D^{25}$ −20.1° (c=1.0, DMF), Rf^1 0.78. *Anal.* Calcd for $C_{55}H_{72}N_6O_{12}S_2 \cdot H_2O$: C, 59.6; H, 6.91; N, 7.58. Found: C, 59.7; H, 7.09;

N, 7.79.

Boc–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl Boc–Cys(MeBzl)–ON*p* (2.4 g) and H–Lys(Z)–Cys(MeBzl)–The–Ser–Cys-(MeBzl)–OBzl·TFA [prepared from Boc–(MT 25—29)–OBzl (3.9 g), TFA (4.1 ml) and anisole (1.20 ml) as usual] were dissolved in DMF (100 ml) containing Et_3N (1.2 ml). The reaction mixture was stirred at room temperature for 2 d. After removal of the solvent, MeOH was added to the residue to afford crystals which were collected by filtration, yield 3.8 g (81.7%), mp 131—135 °C, $[\alpha]_D^{25}$ −21.3° ($c=1.0$, DMF), Rf^1 0.62. *Anal.* Calcd for $C_{66}H_{85}N_7O_{13}S_3 \cdot H_2O$: C, 61.0; H, 6.75; N, 7.55. Found: C, 61.0; H, 6.70; N, 7.74.

Boc–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys-(MeBzl)–OBzl Boc–Asp(O-2-Ada)–OSu (1.74 g) and H–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl·TFA [prepared from Boc–(MT 24—29)–OBzl (3.2 g), TFA (2.9 ml) and anisole (0.81 ml) as usual] were dissolved in DMF (100 ml) containing Et_3N (0.35 ml). The reaction mixture was stirred at room temperature for 2 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 3.1 g (81.1%), mp 205—208 °C, $[\alpha]_D^{25}$ −15.3° ($c=0.5$, DMF), Rf^1 0.56. *Anal.* Calcd for $C_{80}H_{104}N_8O_{16}S_3 \cdot H_2O$: C, 62.1; H, 6.90; N, 7.24. Found: C, 61.9; H, 6.83; N, 7.35.

Boc–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 22—29)–OBzl Boc–Lys(Z)–ON*p* (1.7 g) and H–(MT 23—29)–OBzl·TFA [prepared from Boc–(MT 23—29)–OBzl (4.4 g), TFA (6.6 ml) and anisole (0.93 ml) as usual] were dissolved in DMF (150 ml) containing Et_3N (0.40 ml). The reaction mixture was stirred at room temperature for 2 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 4.5 g (87.3%), mp 208—211 °C, $[\alpha]_D^{25}$ −37.5° ($c=0.5$, DMF), Rf^1 0.50. *Anal.* Calcd for $C_{94}H_{122}N_{10}O_{19}S_3 \cdot 1.25H_2O$: C, 61.6; H, 6.85; N, 7.64. Found: C, 61.8; H, 6.71; N, 7.83.

Bos–Cys(MeBzl)–Ala–Cys(MeBzl)–OBzl Boc–Cys(MeBzl)–Ala–N_3 [prepared from the corresponding hydrazide (4.7 g) and isopentyl nitrite (1.59 ml) as usual] in DMF (100 ml) was added to a solution of H–Cys(MeBzl)–OBzl·TosOH (6.5 g) in DMF (100 ml) containing Et_3N (1.92 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collectec by filtration and recrystallized from MeOH, yield 4.5 g (56.6%), mp 125—132 °C, $[\alpha]_D^{25}$ −31.1° ($c=1.0$, DMF), Rf^1 0.88. *Anal.* Calcd for $C_{37}H_{47}N_3O_6S_2$: C, 64.0; H, 6.83; N, 6.05. Found: C, 63.8; H, 6.77; N, 6.07.

Boc–Cys(MeBzl)–Ala–Cys(MeBzl)–$NHNH_2$, Boc–(MT 19—21)–$NHNH_2$ (2) Hydrazine hydrate (90%, 0.84 ml) was added to a solution of Boc–Cys(MeBzl)–Ala–Cys(MeBzl)–OBzl (3.9 g) in DMF (30 ml). The reaction mixture was stored at room temperature overnight. MeOH was added to the solution to afford a precipitate, which was collected by filtration and washed with MeOH, yield 3.1 g (89.2%), mp 183—187 °C, $[\alpha]_D^{25}$ −25.0° ($c=1.0$, DMF), Rf^1 0.55. *Anal.* Calcd for $C_{30}H_{43}N_5O_5S_2$: C, 58.3; H, 7.02; N, 11.3. Found: C, 58.3; H, 7.12; N, 11.3.

Boc–Thr–Ser–Ser–OMe Boc–Thr–Ser–N_3 [prepared from the corresponding hydrazide (4.0 g) and isopentyl nitrite (1.74 ml) as usual] in DMF (80 ml) was added to a solution of H–Ser–OMe·HCl (2.9 g) in DMF (50 ml) containing Et_3N (2.62 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with *n*-BuOH. The extract was washed with 3% AcOH and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration, yield 4.0 g (78.6%), mp 157—160 °C, $[\alpha]_D^{25}$ −16.1° ($c=1.0$, MeOH), Rf^2 0.61. *Anal.* Calcd for $C_{16}H_{29}N_3O_9$: C, 47.2; H, 7.17; N, 10.3. Found: C, 47.2; H, 7.36; N, 10.3.

Boc–Thr–Ser–Ser–$NHNH_2$, Boc–(MT 16—18)–$NHNH_2$ (3) Hydrazine hydrate (90%, 1.29 ml) was added to a solution of Boc–Thr–Ser–Ser–OMe (3.5 g) in MeOH (30 ml). The reaction mixture was stored at room temperature overnight. A small amount of ether was added to the solution to afford a precipitate, which was collected by filtration and washed with EtOH, yield 1.8 g (51.4%), mp 186—190 °C, $[\alpha]_D^{25}$ −11.3° ($c=0.5$, MeOH), Rf^2 0.37. *Anal.* Calcd for $C_{15}H_{29}N_5O_8$: C, 44.2; H, 7.17; N, 17.2. Found: C, 44.0; H, 7.24; N, 17.1.

Boc–Cys(MeBzl)–Thr–Cys(MeBzl)–OBzl Boc–Cys(MeBzl)–Thr–N_3 [prepared from the corresponding hydrazide (2.6 g) and isopentyl nitrite (0.82 ml) as usual] in DMF (50 ml) was added to a solution of H–Cys(MeBzl)–OBzl·TosOH (3.6 g) in DMF (50 ml) containing Et_3N (1.07 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 2.0 g (46.8%), mp 74—80 °C, $[\alpha]_D^{25}$ −9.8° ($c=1.0$, DMF), Rf^1 0.79. *Anal.* Calcd for $C_{38}H_{49}N_3O_7S_2 \cdot H_2O$: C, 61.5; H, 6.93; N, 5.66. Found: C, 61.5; H, 6.92; N, 5.72.

Boc–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–OBzl Boc–Ser–N_3 [prepared from Boc–Ser–$NHNH_2$ (0.70 g) and isoamyl nitrite (0.41 ml) as usual] in DMF (50 ml) was added to a solution of H–Cys(MeBzl)–Thr–Cys-(MeBzl)–OBzl·TFA [prepared from Boc–Cys(MeBzl)–Thr–Cys(MeBzl)–OBzl(1.8 g), TFA (2.83 ml) and anisole (0.81 ml) as usual] in DMF (30 ml) containing Et_3N (2.83 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 1.9 g (94.2%), mp 133—139 °C, $[\alpha]_D^{25}$ −18.6° ($c=1.0$, DMF), Rf^1 0.57. *Anal.* Calcd for $C_{41}H_{54}N_4O_9S_2 \cdot 1.5H_2O$: C, 58.8; H, 6.86; N, 6.69. Found: C, 58.9; H, 6.80; N, 6.61.

Boc–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–$NHNH_2$, Boc–(MT 12—15)–$NHNH_2$ (4) Hydrazine hydrate (90%, 0.28 ml) was added to a solution of Boc–(MT 12—15)–OBzl (1.5 g) in DMF (40 ml). The reaction mixture was stored at room temperature overnight. MeOH was added to the solution to afford a precipitate, which was collected by filtration and washed with MeOH, yield 0.9 g (66.2%), mp 196—202 °C, $[\alpha]_D^{25}$ −20.5 ($c=1.0$, DMF), Rf^2 0.63. *Anal.* Calcd for $C_{34}H_{50}N_6O_8S_2 \cdot 0.25H_2O$: C, 55.2; H, 6.88; N, 11.4. Found: C, 55.1; H, 6.88; N, 11.3.

Boc–Thr–Gly–Gly–OBzl Boc–Thr–OH (2.0 g) and H–Gly–Gly–OBzl·HCl [prepared from Boc–Gly–Gly–OBzl[13] (3.0 g) and 3.5 N HCl/dioxane (8.5 ml) as usual] were dissolved in DMF (50 ml) containing Et_3N (2.9 ml). DPPA (3.1 ml) was added to the solution under cooling with ice-salt. 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 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down to give an oily material, yield 3.5 g (88.9%), $[\alpha]_D^{25}$ −8.5° ($c=1.0$, MeOH), Rf^2 0.74. *Anal.* Calcd for $C_{20}H_{29}N_3O_7 \cdot H_2O$: C, 54.4; H, 7.59; N, 9.52. Found: C, 54.2; H, 7.88; N, 9.39.

Boc–Ser–Thr–Gly–Gly–$NHNH_2$, Boc–(MT 8—11)–$NHNH_2$ (5) Boc–Ser–N_3 [prepared from Boc–Ser–$NHNH_2$ (1.4 g) and isopentyl nitrite (0.89 ml) as usual] in DMF (50 ml) was added to a solution of H–Thr–Gly–Gly–OBzl·HCl [prepared from Boc–Thr–Gly–Gly–OBzl (1.8 g) and 3.6 N HCl/dioxane (5.9 ml) as usual] in DMF (30 ml) containing Et_3N (0.60 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Hydrazine hydrate (90%, 1.64 ml) was added to a solution of the residual oil in MeOH (30 ml). The solution was stored at room temperature overnight. Ether was added to the solution to afford a precipitate, which was collected by filtration and washed with EtOH, yield 0.9 g (48.7%), mp 160—162 °C, $[\alpha]_D^{25}$ −15.7° ($c=1.0$, DMF), Rf^2 0.36. *Anal.* Calcd for $C_{16}H_{30}N_6O_8$: C, 44.2; H, 6.99; N, 19.3. Found: C, 44.1; H, 7.03; N, 19.2.

Boc–Cys(MeBzl)–Ser–$NHNH_2$ Hydrazine hydrate (90%, 0.35 ml) was added to a solution of Boc–Cys(MeBzl)–Ser–OMe[1] (3.0 g) in MeOH (100 ml). The reaction mixture was stored at room temperature overnight. After removal of the solvent, EtOH was added to the residue to afford crystals, which were collected by filtration and washed with EtOH, yield 2.53 g (83.9%), mp 136—139 °C, $[\alpha]_D^{25}$ −15.3° ($c=1.0$, DMF), Rf^1 0.26. *Anal.* Calcd for $C_{19}H_{30}N_4O_5S$: C, 53.5; H, 7.09; N, 13.1. Found: C, 53.3; H, 6.95; N, 13.0.

Boc–Cys(MeBzl)–Ser–Cys(MeBzl)–OBzl Boc–Cys(MeBzl)–Ser–N_3 [prepared from the corresponding hydrazide (2.1 g) and isopentyl nitrite (0.68 ml) as usual] in DMF (50 ml) was added to a solution of H–Cys(MeBzl)–OBzl·TosOH (3.5 g) in DMF (50 ml) containing Et_3N (1.03 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH, yield 2.3 g (46.8%), mp 135—139 °C, $[\alpha]_D^{25}$ −30.8° ($c=1.0$, DMF), Rf^1 0.89. *Anal.* Calcd for $C_{37}H_{47}N_3O_7S_2$: C, 62.6; H, 6.67; N, 5.92. Found: C, 62.6; H, 6.61; N, 5.89.

Boc–Asn–Cys(MeBzl)–Ser–Cys(MeBzl)–OBzl Boc–Asn–ON*p* (1.3 g) and H–Cys(MeBzl)–Ser–Cys(MeBzl)–OBzl·TFA [prepared from Boc–Cys(MeBzl)–Ser–Cys(MeBzl)–OBzl (2.3 g), TFA (3.70 ml) and anisole

(1.05 ml) as usual] were dissolved in DMF (80 ml) containing Et_3N (0.45 ml). The reaction mixture was stirred at room temperature for 2 d. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and washed with MeOH, yield 1.7 g (70.2%), mp 186—189 °C, $[\alpha]_D^{25}$ −25.2° (c=1.0, DMF), Rf^1 0.60. *Anal.* Calcd for $C_{41}H_{53}N_5O_9S_2$: C, 59.5; H, 6.48; N, 8.50. Found: C, 59.8; H, 6.48; N, 8.50.

Boc–Asn–Cys(MeBzl)–Ser–Cys(MeBzl)–NHNH$_2$, Boc–(MT 4—7)–NHNH$_2$ (6) Hydrazine hydrate (90%, 0.34 ml) was added to a solution of Boc–(MT 4—7)–OBzl (1.7 g) in DMF (30 ml). The solution was stored at room temperature overnight. MeOH was added to the solution to afford a precipitate, which was collected by filtration and washed with MeOH, yield 1.5 g (88.2%), mp 203—205 °C, $[\alpha]_D^{25}$ −29.1° (c=1.0, DMF), Rf^2 0.59. *Anal.* Calcd for $C_{34}H_{49}N_5O_9S_2$: C, 54.6; H, 6.60; N, 13.1. Found: C, 54.4; H, 6.42; N, 13.2.

Z–Asp(O-2-Ada)–Pro–NHNH–Boc Z–Asp(O-2-Ada)–OH (2.3 g), H–Pro–NHNH–Boc (1.3 g) and HOBt (0.8 g) were dissolved in DMF (70 ml). DCC (1.4 g) was added to the above solution under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of the 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 amorphous powder, yield 2.4 g (68.4%), mp 56—61 °C, $[\alpha]_D^{25}$ −34.0° (c=0.5, MeOH), Rf^2 0.70. *Anal.* Calcd for $C_{32}H_{44}N_4O_8 \cdot 2H_2O$: C, 59.2; H, 8.08; N, 8.64. Found: C, 59.4; H, 7.83; N, 8.97.

Ac–Met–Asp(O-2-Ada)–Pro–NHNH–Boc, Ac–(MT 1—3)–NHNH–Boc (7) Ac–Met–N_3 [prepared from Ac–Met–$NHNH_2$ (0.8 g) and isopentyl nitrite (0.63 ml) as usual] in DMF (20 ml) was added to a solution of H–Asp(O-2-Ada)–Pro–NHNH–Boc [prepared from Z–Asp(O-2-Ada)–Pro–NHNH–Boc (2.3 g) by catalytic hydrogenation over Pd] in DMF (50 ml) under cooling with ice-salt. The reaction mixture was sirred 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 was added to the residue to afford crystals, which were collected by filtration, yield 1.5 g (61.3%), mp 90—94 °C, $[\alpha]_D^{25}$ −21.9° (c=0.5, MeOH), Rf^2 0.55. *Anal.* Calcd for $C_{31}H_{49}N_5O_8S \cdot 2H_2O$: C, 54.1; H, 7.77; N, 10.1. Found: C, 53.9; H, 7.72; N, 10.1.

Boc–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 19—29)–OBzl (8) Boc–Cys(MeBzl)–Ala–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (950 mg) and isopentyl nitrite (0.21 ml) as usual] in DMF (50 ml) was added to a solution of H–(MT 22—29)–OBzl·TFA [prepared from Boc–(MT 22—29)–OBzl (2.3 g), TFA (2.93 ml) and anisole (0.42 ml) as usual] in DMF (80 ml) containing Et_3N (0.18 ml) under cooling with ice-salt. 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, yield 2.2 g (75.3%), mp 248—251 °C, $[\alpha]_D^{25}$ −23.1° (c=0.5, DMSO), Rf^1 0.61. *Anal.* Calcd for $C_{119}H_{153}N_{13}O_{22}S_5 \cdot 2H_2O$: C, 61.8; H, 6.84; N, 7.87. Found: C, 61.7; H, 6.75; N, 7.98.

Boc–Thr–Ser–Ser–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 16—29)–OBzl (9) Boc–Thr–Ser–Ser–N_3 [prepared from the corresponding hydrazide (715 mg) and isopentyl nitrite (0.24 ml) as usual] in DMF (30 ml) was added to a solution of H–(MT 19—29)–OBzl·TFA [prepared from Boc–(MT 19—29)–OBzl (1.00 g), TFA (1.50 ml) and anisole (0.14 ml) as usual] in DMF (100 ml) containing Et_3N (0.06 ml) under cooling with ice-salt. 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, yield 995 mg (88.8%), mp 260—261 °C, $[\alpha]_D^{25}$ −15.1° (c=0.5, DMSO), Rf^1 0.52. *Anal.* Calcd for $C_{129}H_{170}N_{16}O_{28}S_5 \cdot 5H_2O$: C, 58.6; H, 6.86; N, 8.48. Found: C, 58.6; H, 6.64; N, 8.70.

Boc–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–Thr–Ser–Ser–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 12—29)–OBzl (10) Boc–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (1.15 g) and isopentyl nitrite (0.22 ml) as usual] in DMF (50 ml) was added to a solution of H–(MT 16—29)–OBzl·TFA [prepared from Boc–(MT 16—29)–OBzl (1.00 g), TFA (2.23 ml) and anisole (0.13 ml) as usual] in DMF (50 ml) and HMPA (30 ml) containing Et_3N (0.05 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 1.29 g (95.8%), mp 236—237 °C, $[\alpha]_D^{25}$ −17.1° (c=0.1, DMSO), Rf^1 0.48. *Anal.* Calcd for $C_{158}H_{208}N_{20}O_{34}S_7 \cdot 6H_2O$: C, 58.1; H, 6.79; N, 8.58. Found: C, 58.1; H, 6.82; N, 8.78.

Boc–Ser–Thr–Gly–Gly–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–Thr–Ser–Ser–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 8—29)–OBzl (11) Boc–Ser–Thr–Gly–Gly–N_3 [prepared from the corresponding hydrazide (372 mg) and isopentyl nitrite (0.12 ml) as usual] in DMF (30 ml) was added to a solution of H–(MT 12—29)–OBzl·TFA [prepared from Boc–(MT 12—29)–OBzl (900 mg), TFA (1.30 ml) and anisole (0.09 ml) as usual] in DMF (40 ml) and HMPA (20 ml) containing Et_3N (0.04 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford cystals, which were collected by filtration, yield 720 mg (73.0%), mp 256 °C, $[\alpha]_D^{25}$ −13.9° (c=0.1, DMSO), Rf^1 0.50. *Anal.* Calcd for $C_{169}H_{226}N_{24}O_{40}S_7 \cdot 6H_2O$: C, 56.9; H, 6.72; N, 9.44. Found: C, 56.8; H, 6.52; N, 9.74.

Boc–Asn–Cys(MeBzl)–Ser–Cys(MeBzl)–Ser–Thr–Gly–Gly–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–Thr–Ser–Ser–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 4—29)–OBzl (12) Boc–Asn–Cys(MeBzl)–Ser–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (324 mg) and isopentyl nitrite (0.06 ml) as usual] in DMF (20 ml) was added to a solution of H–(MT 8—29)–OBzl·TFA [prepared from Boc–(MT 8—29)–OBzl (500 mg), TFA (0.82 ml) and anisole (0.05 ml) as usual] in DMF (50 ml) and HMPA (20 ml) containing Et_3N (0.02 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 500 mg (84.9%), mp 252 °C, $[\alpha]_D^{25}$ −19.1° (c=0.1, DMSO), Rf^1 0.40. *Anal.* Calcd for $C_{198}H_{263}N_{29}O_{46}S_9 \cdot 9H_2O$: C, 56.1; H, 6.68; N, 9.59. Found: C, 55.9; H, 6.40; N, 9.68.

Ac–Met–Asp(O-2-Ada)–Pro–Asn–Cys(MeBzl)–Ser–Cys(MeBzl)–Ser–Thr–Gly–Gly–Ser–Cys(MeBzl)–Thr–Cys(MeBzl)–Thr–Ser–Ser–Cys(MeBzl)–Ala–Cys(MeBzl)–Lys(Z)–Asp(O-2-Ada)–Cys(MeBzl)–Lys(Z)–Cys(MeBzl)–Thr–Ser–Cys(MeBzl)–OBzl, Boc–(MT 1—29)–OBzl (13, Protected β-Fragment) Ac–Met–Asp(O-2-Ada)–Pro–N_3 [prepared from the corresponding hydrazide (240 mg) and isopentyl nitrite (0.05 ml) as usual] in DMF (10 ml) was added to a solution of H–(MT 4—29)–OBzl·TFA [prepared from Boc–(MT 4—29)–OBzl (300 mg), TFA (0.84 ml) and anisole (0.02 ml) as usual] in DMF (30 ml) and HMPA (20 ml) containing Et_3N (0.01 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 3 d. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration, yield 280 mg (82.2%), mp 261 °C, $[\alpha]_D^{25}$ −30.3° (c=0.1, DMSO), Rf^1 0.43. *Anal.* Calcd for $C_{219}H_{292}N_{32}O_{50}S_{10} \cdot 12H_2O$: C, 55.0; H, 6.82; N, 9.37. Found: C, 54.9; H, 6.51; N, 9.53.

General Procedure for Deprotection by HF The protected peptide (0.03 mmol) was treated with anhydrous HF (10 ml) containing thioanisole (0.17 ml) and *m*-cresol (0.73 ml) at 0 °C for 90 min. After removal of HF, the residue was extracted with 3% AcOH. The extract was washed with ether and lyophilized to give a fluffy powder. The crude peptide in water (10 ml) was treated with dithiothreitol (140 ml) at room temperature overnight. The solution was applied to a Sephadex G-15 column (3.5 × 48 cm) for H–(MT 24—29)–OH, H–(MT 22—29)–OH and H–(MT 19—29)–OH or a Sephadex G-25 column (3 × 55 cm) for H–(MT 16—29)–OH, H–(MT 12—29)–OH, H–(MT 8—29)–OH, H–(MT 4—29)–OH and H–(MT 1—29)–OH (β-fragment). These column were equilibrated and eluted with 3% AcOH. Individual fractions (3 g each) were collected. The desired fractions were combined and lyophilized to give a fluffy powder. Yield, $[\alpha]_D$, value, amino acid ratios in an acid hydrolysate, *Rf* value and free SH content of each peptide are summarized in Table I.

General Procedure for Examination of Binding Ability of Peptides with Cd^{2+}, Cu^{2+} and Cu^{+} A 5—45 μl aliquot of $CdCl_2$, $CuCl_2$ and $(CH_3CN)_4ClO_4$ solution (5 mM) was added to 5 ml of peptide solution (0.15 mM as SH in 10 mM Tris–HCl, pH 7.00). The UV absorbance at 250 nm (for Cd-mercaptide) or 265 nm (for Cu-mercaptide) of the mixture was measured and the increase was plotted against metal concentration.

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References and Notes

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