Amino Acids and Peptides. XXXIV. Synthesis of Mouse Metallothionein I. (1). Synthesis of Dotriacontapeptide Corresponding to C-Terminal Sequence 30—61 (α-Fragment) of Mouse Metallothionein I and Related Peptides and Examination of Their Heavy Metal-Binding Properties[1,2]

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The dotriacontapeptide corresponding to the C-terminal sequence of mouse metallothionein (MT) I and related peptides which contain Cys–X–Cys–Cys (X: amino acid residue except for Cys) sequence were synthesized by the conventional solution method employing the HF deprotection method and their heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding properties were examined.

Keywords mouse metallothionein I; C-terminal dotriacontapeptide; α-fragment; related peptide; chemical synthesis; heavy metal binding property

Metallothioneins (MTs) are heavy metals-containing, cysteine-rich proteins of low molecular weight. Due to their metal (Cd, Zn, Hg, Cu, *etc.*) binding ability, they act as heavy metal (Cd, Hg) detoxifying agents[3,4] and participate in heavy metal (Zn, Cu) metabolism, such as storage function[5,6] and metal transfer to apometalloproteins.[7–9] It has also been reported that MTs can trap radicals and alkylating agents, providing a protective function against the effects of irradiation and carcinogenesis.[10,11] However, their precise role or roles remain to be defined.

As shown in Fig. 1, mammalian MTs consist of 61 amino acid residues, and show extensive homologies and invariance of the positions of 20 cysteines.

Concerning the metal cysteinate structure of MT, Otvos and Armitage[12] demonstrated the existence of two separate Cd clusters, one containing four Cd^{2+} ions (cluster A, C-terminal portion) and the other containing three Cd^{2+} ions (cluster B, N-terminal portion), based on NMR spectral data for rabbit liver ^{113}Cd-MT as shown in Fig. 2. Later Winge and Miklossy[13] isolated a C-terminal fragment which binds four Cd^{2+} ions after subtilisin digestion of rat liver MT. They identified it as a C-terminal dotriacontapeptide (positions 30—61) and designated it as the α-domain. Nielson and Winge[14] isolated a Cu-binding N-terminal peptide after subtilisin digestion; it consisted of the N-terminal untriacontapeptide (positions 1—31), and they designated it as the β-domain. It was also reported that the two clusters exhibited significant differences in their affinity for different metal ions and functioned independently.[15] For example, it was found that the large majority of the Cd^{2+} binds to the four-metal cluster where Cys–Cys sequences exist, while the Zn^{2+} and Cu^{+} ions bind preferentially to the three-metal cluster, when Cd is present.[14,16]

As can be seen in Fig. 1, *Neurospora crassa*[17] and *Agaricus bisporus*[18] MTs consist of 25 amino acid residues with a characteristically high cysteine content and bind 6 mol of Cu^{+} per molecule. Interestingly, the positions of the seven cysteines of these MTs are arranged in the form of Cys–X–Cys clusters and the amino acid sequence is strikingly similar to that of the amino terminal region of mammalian MTs. Although Zn^{2+} and Cd^{2+} are the major natural metallic constituents of mammalian MTs, only Cu is contained in *N. crassa* and *A. bisporus* MTs. The Cu-metallothioneins are assumed to play an important role in both metal storage and detoxification. *In vitro* experiments suggest that these Cu-MTs act as metal donors to the active sites of Cu-containing enzymes.[19]

It is believed that studies on the metal binding properties of the two clusters may provide a clue to clarifying the intrinsic biological roles of MTs.

Under these circumstances, our studies were directed to the systematic synthesis of the α and β-fragments of mouse MT I[20] and related peptides and examination of their heavy

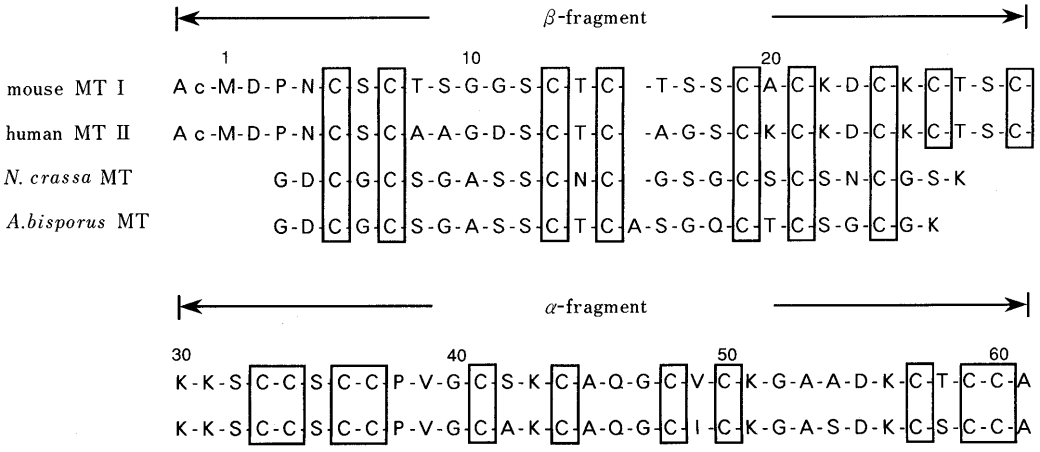

Fig. 1. Primary Structure of Metallothioneins

metal-binding properties with the objectives of studying the structure-heavy metal-binding properties relationship and confirming the differences in their affinities for different metal ions.

Neurospora crassa[21] and *Agaricus bisporus*[22] MTs and related peptides have been synthesized and their heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+}) binding properties examined. It was revealed that the Cu^{2+}- and Cu^{+}-binding activities of various peptides were not greatly dependent on the peptide structure, so far as examined, whereas the Cd^{2+}-binding activities of these peptides were fairly structure-dependent.

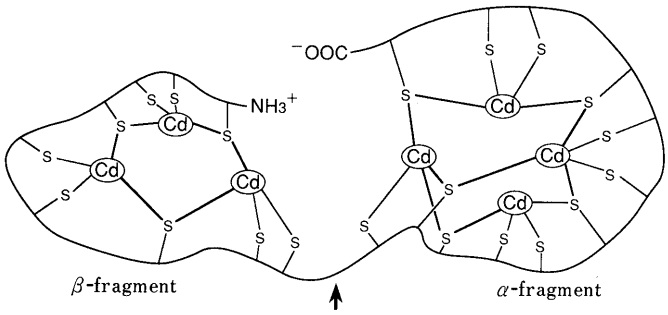

Fig. 2. Schematic Representation of the Structure of Cd_7-MT[12]

This paper deals with the systematic synthesis of α-fragment of mouse MT I and related peptides, which contain Cys–Cys sequences, and examination of their heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+}) binding properties.

As illustrated in Fig. 3, a protected dotriacontapeptide was prepared by the fragment condensation method starting with the C-terminal heptapeptide ester (**1**). Amino acid derivatives bearing protecting groups removable by treatment with HF at 0 °C for 60 min[23] or methanesulfonic acid (MSA) at 20 °C for 60 min,[24] *i.e.* Asp(O-2-Ada),[25] Lys(Z) and Cys(MeBzl),[26] were employed in combination with the TFA-labile Boc-group as the N^{α}-protecting group.

The synthetic scheme for the C-terminal heptapeptide ester, Boc–(MT 55—61)–OBzl, is illustrated in Fig. 4. H–Ala–OBzl was coupled with Boc–Cys(MeBzl)–Cys-(MeBzl)–$NHNH_2$, prepared by the coupling of H–Cys-(MeBzl)–OBzl with Boc–Cys(MeBzl)–OH by the DCC method, followed by treatment with hydrazine hydrate, to give Boc–(MT 59—61)–OBzl. After removal of the Boc group of Boc–(MT 59—61)–OBzl, the resultant amine was coupled with Boc–Cys(MeBzl)–Thr–$NHNH_2$ by the azide procedure to give Boc–(MT 57—61)–OBzl. After removal of the Boc group, the resultant amine was coupled with

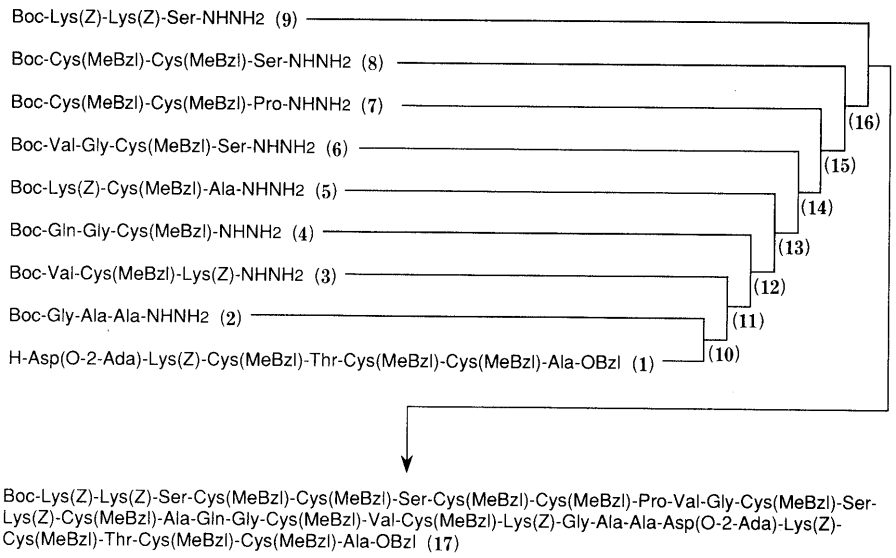

Fig. 3. Synthetic Scheme for α-Fragment of Mouse MT I and Related Peptides

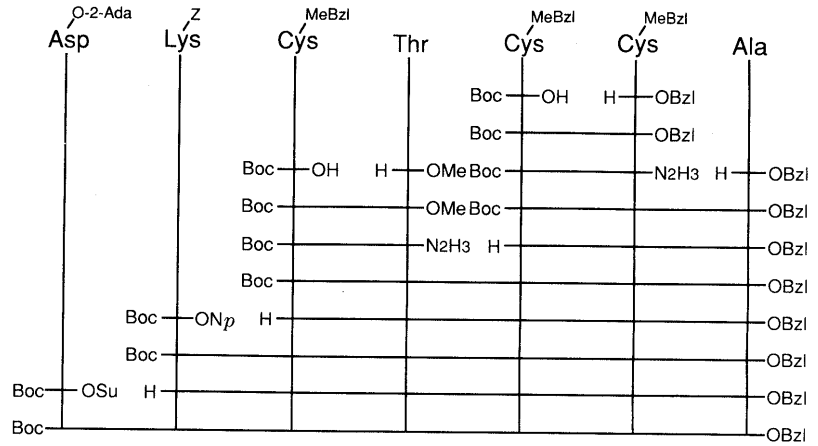

Fig. 4. Synthetic Route to Boc–(MT 55—61)–OBzl

Boc–Lys(Z)–ONp and Boc–Asp(O-2-Ada)–OSu successively to give Boc–(MT 55—61)–OBzl, which was treated with TFA to give the peptide (**1**).

Boc–Gly–Ala–Ala–$NHNH_2$, Boc–(MT 52—54)–$NHNH_2$ (**2**), was prepared by coupling of Boc–Gly–ONp with H–Ala–Ala–OMe, followed by hydrazine hydrate treatment. Boc–Val–Cys(MeBzl)–Lys(Z)–$NHNH_2$, Boc–(MT 49—51)–$NHNH_2$ (**3**), was prepared as follows: Boc–Cys(MeBzl)–ONp was coupled with H–Lys(Z)–OBzl to give Boc–Cys(MeBzl)–Lys(Z)–OBzl, which was treated with TFA. The resultant amine was coupled with Boc–Val–ONp, followed by hydrazine hydrate treatment, to give the desired fragment (**3**).

Boc–Gln–Gly–Cys(MeBzl)–$NHNH_2$, Boc–(MT 46—48)–$NHNH_2$ (**4**), was prepared from H–Cys(MeBzl)–OBzl by successive couplings of Boc–Gly–ONp and Boc–Gln–ONp, followed by hydrazine hydrate treatment. Boc–Lys(Z)–Cys(MeBzl)–Ala–$NHNH_2$, Boc–(MT 43—45)–$NHNH_2$ (**5**) and Boc–Val–Gly–Cys(MeBzl)–Ser–$NHNH_2$, Boc–(MT 39—42)–$NHNH_2$ (**6**) were prepared starting with H–Ala–OBzl and H–Ser–OMe, respectively, in the same manner as used for the synthesis of **4**. Boc–Cys(MeBzl)–Cys(MeBzl)–Pro–$NHNH_2$, Boc–(MT 36—38)–$NHNH_2$ (**7**) was prepared by coupling of Boc–Cys(MeBzl)–Cys(MeBzl)–$NHNH_2$ with H–Pro–OBzl by the azide method, followed by hydrazine hydrate treatment. Boc–Cys(MeBzl)–Cys(MeBzl)–Ser–$NHNH_2$, Boc–(MT 33—35)–$NHNH_2$ (**8**) was prepared by coupling of Boc–Cys(MeBzl)–Cys(MeBzl)–$NHNH_2$ with H–Ser–OMe by the azide method, followed by hydrazine hydrate treatment. Boc–Lys(Z)–Lys(Z)–Ser–$NHNH_2$, Boc–(MT 30—32)–$NHNH_2$ (**9**) was prepared as follows: Boc–Lys(Z)–ONp and H–Lys(Z)–OBzl were coupled to give Boc–Lys(Z)–Lys(Z)–OBzl, which was treated with hydrazine hydrate to give the corresponding hydrazide. This hydrazide was coupled with H–Ser–OMe by the azide method, followed by hydrazine hydrate treatment, to give the peptide (**9**). The homogeneity of the peptide fragments obtained above was ascertained by thin-layer chromatography (TLC), amino acid analysis and elemental analysis.

According to the scheme shown in Fig. 3, starting with H–(MT 55—61)–OBzl (**1**), the relatively small peptide hydrazides (**2**—**9**) were coupled successively by the azide method in order to minimize racemization and to avoid the need for protection of side-chain functional groups of the amino acid residues as much as possible during the synthesis, to afford the protected peptides (**10**—**16**) and finally the protected α-fragment (**17**). In each fragment condensation reaction, three equivalents of the azide were employed and the reaction was carried out in DMF or in HMPA without using DMSO, in order to avoid oxidation of Cys residues. All azide components employed were soluble in MeOH. Hence, at each step, the desired peptide could be isolated by evaporation of the reaction mixture, followed by addition of MeOH, and then filtration. Reprecipitation of the desired peptide from DMF and MeOH gave an analytically pure peptide at each step.

The protected α-fragment (**17**) and related peptides, Boc–(MT 57—61)–OBzl, peptides (**10**, **11**, **12**, **13**, **15**), were deprotected by the HF method. During the course of this deprotecting reaction, oxygen-free water was used and a slightly acidic solvent was employed as the eluant for column chromatography on Sephadex G-15 or G-25 in order to prevent disulfide bond formation.[27]

The homogeneity of the peptides obtained was ascertained by TLC and amino acid analysis. The yield, *Rf* value, $[\alpha]_D$ value and the results of amino acid analysis are summarized in Table I. The free SH content of the synthetic peptides was also determined by the Ellman method[28] and the results are summarized in Table I.

The heavy metals (Cd^{2+}, Cu^{2+} and Cu^{+})-binding properties of synthetic α-fragment and related peptides were examined by measuring the increase in absorbance of mercaptide at 250 or at 265 nm as a function of the concentration of Cd^{2+} or Cu^{2+} and Cu^{+}, respectively according to the previously described method.[21] The results are shown in Fig. 5a, b and c. Regarding the Cd^{2+}-binding abilities of the peptides (Fig. 5a), α-fragment exhibited the most potent binding activity, although the other peptides also showed rather similar binding activities. Only the shortest peptide, H–(MT 57—61)–OH exhibited lower Cd^{2+}-binding activity compared with those of the other peptides. In our previous report, the heptapeptide corresponding to the C-terminal sequence (55—61) of human liver MT II exhibited similar Cd^{2+}-binding activity to that of the α-fragment of human liver MT II.[29] From these results, it can be deduced that in order to manifest strong Cd^{2+}-binding activity, the Asp–Lys sequence (positions 55—56) is required in addition to Cys–X–Cys–Cys. The Cys–X–Cys–Cys sequence is quite favorable for Cd^{2+}-binding and the Cd^{2+}-binding activities of various peptides are not greatly dependent on the peptide chain length, so far as examined. It can be deduced that Cys residue or residues which are newly introduced in peptides

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	Glu	Gly	Ala	Cys	Val	Lys	Pro	
H–[MT 57—61]–OH	62.4	−20.9°	0.61		0.90				1.00	3.13				2.67
H–[MT 52—61]–OH	67.9	−64.4°	0.73	1.10	0.88			1.00	3.13	3.02		1.02		2.51
H–[MT 49—61]–OH	74.1	−74.0°	0.89	1.04	0.84	-		1.00	3.13	3.91	0.97	1.84		3.73
H–[MT 46—61]–OH	51.6	−54.4°	0.80	1.12	0.84		1.09	2.00	3.31	4.72	0.99	1.90		4.25
H–[MT 43—61]–OH	49.3	−48.6°	0.81	1.08	0.81		1.13	2.00	3.99	5.62	1.02	2.83		5.48
H–[MT 36—61]–OH	68.0	−39.9°	0.77	1.20	0.90	0.78	1.04	3.00	4.21	8.31	2.07	2.07	0.93	8.09
H–[MT 30—61]–OH (α-fragment)	40.2	−49.0°	0.69	1.23	0.88	2.35	1.04	3.00	4.28	9.96	1.93	4.57	0.95	9.91

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

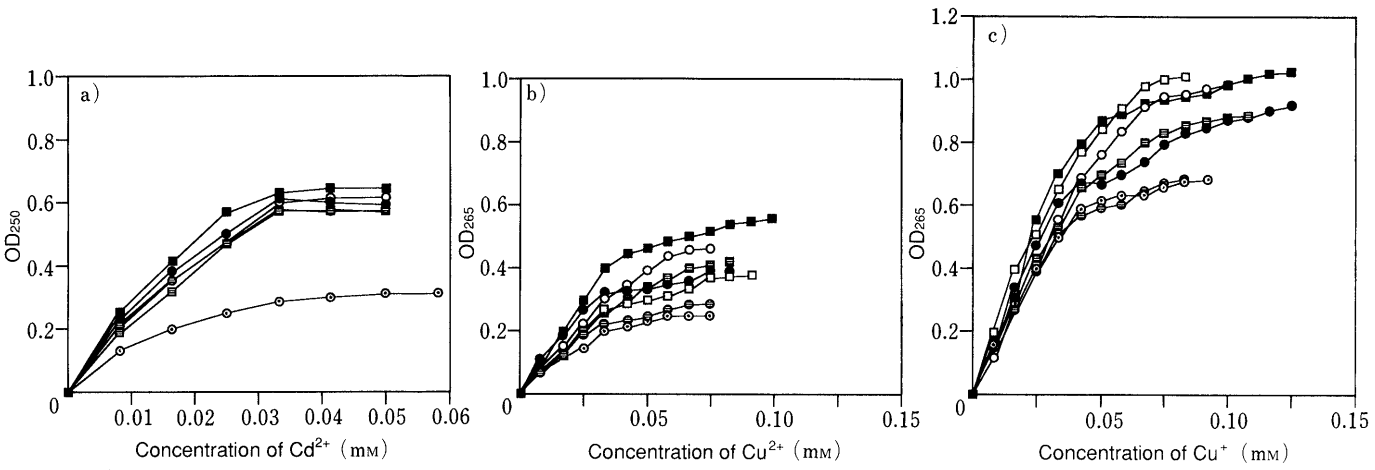

Fig. 5. Binding Properties of Peptides with Heavy Metals, a) with Cd^{2+}, b) with Cu^{2+}, c) with Cu^{+}

Peptide, 0.15 mM as SH in 3 ml of Tris–HCl (10 mM, pH 7.0). ⊙, mouse MT I (57—61); ▤, (52—61); ●, (49—61); ○, (46—61); ●, (43—61); □, (36—61); ■, (30—61).

related to the α-fragment, are suitable to participate in forming Cd^{2+}-mercaptide. In contrast, the Cu^{2+}-binding abilities (Fig. 5b) as well as Cu^{+}-binding abilities (Fig. 5c) were fairly structure-dependent. These phenomena are opposite to those in the cases of *N. crassa* and *A. bisporus* MTs and related peptides.

These results support the idea that Cys–Cys sequences and other Cys residues which are contained in the α-fragment, are favorable for Cd^{2+}-binding and the α-domain of mammalian MTs is responsible for Cd^{2+}-binding. A recent study[30] suggested that Cys residues in the α-domain are more critical than those of the β-domain for the Cd-detoxification function. Nearly half (three of seven) of the Cys residues in the β-domain of Chinese hamster MT studied could be replaced with Ser without affecting Cd-detoxification function, while only one out of five Cys residues in the α-domain was found to be dispensable for Cd-detoxification function. Our results obtained here are compatible with that report.

Experimental

The melting points are uncorrected. Optical rotations were measured with automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an acid hydrolysate (110 °C, 6 N HCl, 20 h) 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–Cys(MeBzl)–Cys(MeBzl)–OBzl Boc–Cys(MeBzl)–OH (11.3 g) and H–Cys(MeBzl)–OBzl·TosOH (16.3 g) were dissolved in DMF (100 ml) containing Et_3N (4.8 ml). DCC (8.6 g) was added to the above solution under cooling with ice-salt 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% 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 and recrystallized from MeOH, yield 14.7 g (68.7%), mp 102—104 °C, $[\alpha]_D^{25}$ −52.0° (c = 1.0, DMF), Rf^1 0.82. *Anal.* Calcd for $C_{34}H_{42}N_2O_5S_2 \cdot 0.5H_2O$: C, 64.6; H, 6.86; N, 4.43. Found: C, 64.6; H, 6.83; N, 4.55.

Boc–Cys(MeBzl)–Cys(MeBzl)–$NHNH_2$ Hydrazine hydrate (90%, 2.7 ml) was added to a solution of Boc–Cys(MeBzl)–Cys(MeBzl)–OBzl (11.0 g) in DMF (50 ml) and the reaction mixture was stored 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 8.2 g (84.7%), mp 137—142 °C, $[\alpha]_D^{25}$ −29.8° (c = 1.0, DMF), Rf^1 0.66. *Anal.* Calcd for $C_{27}H_{38}N_4O_4S_2 \cdot 0.5H_2O$: C, 58.4; H, 7.07; N, 10.1. Found: C, 58.3; H, 6.95; N, 10.4.

Boc–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl Boc–Cys(MeBzl)–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (5.0 g) and isopentyl nitrite (1.3 ml) as usual] in DMF (30 ml) was added to a solution of H–Ala–OBzl·TosOH (3.9 g) in DMF (30 ml) containing Et_3N (1.5 ml) under cooling with ice-salt 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. Petroleum ether was added to the residue to afford crystals, which were collected by filtration. The crude product in $CHCl_3$ (10 ml) was applied to a silica gel column (3.5 × 43 cm), equilibrated and eluted with $CHCl_3$. The solvent of the effluent (1200—2000 ml) was removed by evaporation. Petroleum ether was added to the residue to afford crystals, yield 4.1 g (65.1%), mp 115—118 °C, $[\alpha]_D^{25}$ −32.3° (c = 1.0, DMF), Rf^1 0.83. *Anal.* Calcd for $C_{37}H_{47}N_3O_6S_2 \cdot 0.5H_2O$: C, 64.0; H, 6.83; N, 6.06. Found: C, 64.0; H, 6.98; N, 6.22.

Boc–Cys(MeBzl)–Thr–$NHNH_2$ Hydrazine hydrate (90%, 3.4 ml) was added to a solution of Boc–Cys(MeBzl)–Thr–OMe, which was prepared by DCC coupling of Boc–Cys(MeBzl)–OH with H–Thr–OMe, (10.2 g) in MeOH (50 ml), and the reaction mixture was stored at room temperature overnight. The precipitate was collected by filtration, yield 7.2 g (72.0%), mp 125—128 °C, $[\alpha]_D^{25}$ −17.8° (c = 1.0, DMF), Rf^2 0.64. *Anal.* Calcd for $C_{20}H_{32}N_4O_5S$: C, 54.5; H, 7.32; N, 12.7. Found: C, 54.3; H, 7.39; N, 12.7.

Boc–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl Boc–Cys(MeBzl)–Thr–N_3 [prepared from the corresponding hydrazide (3.8 g) and isopentyl nitrite (1.2 ml) as usual] in DMF (50 ml) was added to a solution of H–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl·TFA [prepared from Boc–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl (5.0 g), TFA (8.2 ml) and anisole (2.34 ml) as usual] in DMF (50 ml) containing Et_3N (1.0 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 evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 5.8 g (79.6%), mp 148—153 °C, $[\alpha]_D^{25}$ −45.4° (c = 1.0, DMF), Rf^1 0.76. *Anal.* Calcd for $C_{25}H_{67}N_5O_9S_3 \cdot H_2O$: C, 61.2; H, 6.81; N, 6.86. Found: C, 61.0; H, 6.70; N, 6.87.

Boc–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl Boc–Lys(Z)–ON*p* (1.49 g) and H–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl·TFA [prepared from Boc–(MT 57—61)–OBzl (2.48 g), TFA (2.82 ml) and anisole (0.8 ml) as usual] were dissolved in DMF (50 ml) containing Et_3N (0.4 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 2.64 g (84.5%), mp 143—145 °C, $[\alpha]_D^{25}$ −35.1° (c = 1.0, DMF), Rf^1 0.67. *Anal.* Calcd for $C_{66}H_{85}N_7O_{12}S_3 \cdot 1.5H_2O$: C, 61.4; H, 6.86; N, 7.59. Found: C, 61.1; H, 6.73; N, 7.65.

Boc–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl Boc–Asp(O-2-Ada)–OSu[25] (2.13 g) and H–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl·TFA [prepared

from Boc–(MT 56—61)–OBzl (4.82 g), TFA (4.40 ml) and anisole (1.24 ml) as usual] were dissolved in DMF (50 ml) containing Et_3N (0.64 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 and washed with MeOH, yield 4.73 g (81.9%), mp 226—230 °C, $[\alpha]_D^{25}$ −39.2° (c=1.0, DMF), Rf^1 0.75. *Anal.* Calcd for $C_{80}H_{104}N_8O_{15}S_3$: C, 63.5; H, 6.92; N, 7.40. Found: C, 63.2; H, 6.93; N, 7.56.

Boc–Gly–Ala–Ala–OMe Boc–Gly–ON*p* (5.0 g) and H–Ala–Ala–OMe [prepared from Z–Ala–Ala–OMe[31] (5.2 g) by catalytic hydrogenolysis over Pd] were dissolved in DMF (100 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, yield 3.8 g (68.0 g), mp 108—109 °C, $[\alpha]_D^{25}$ −66.2° (c=1.0, DMF), Rf^2 0.78. *Anal.* Calcd for $C_{14}H_{25}N_3O_6$: C, 50.7; H, 7.60; N, 12.7. Found: C, 50.5; H, 7.70; N, 12.6.

Boc–Gly–Ala–Ala–NHNH$_2$ (2) Hydrazine hydrate (90%, 1.58 ml) was added to a solution of Boc–Gly–Ala–Ala–OMe (3.5 g) in MeOH (30 ml). The reaction mixture was stored at room temperature overnight. The resultant precipitate was collected by filtration, yield 3.2 g (91.4%), mp 199—200 °C, $[\alpha]_D^{25}$ −4.75° (c=1.0, DMF), Rf^2 0.50. *Anal.* Calcd for $C_{13}H_{25}N_5O_5 \cdot 0.5H_2O$: C, 45.9; H, 7.69; N, 20.6. Found: C, 46.0; H, 7.45; N, 20.7.

Boc–Cys(MeBzl)–Lys(Z)–OBzl Boc–Cys(MeBzl)–ON*p* (5.4 g) and H–Lys(Z)–OBzl·TosOH (4.5 g) were dissolved in DMF (100 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 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 and recrystallized from EtOH, yield 3.9 g (80.8%), mp 109—110 °C, $[\alpha]_D^{25}$ −26.0° (c=1.0, DMF), Rf^1 0.90. *Anal.* Calcd for $C_{37}H_{47}N_3O_7S$: C, 65.6; H, 6.99; N, 6.20. Found: C, 65.3; H, 7.01; N, 6.17.

Boc–Val–Cys(MeBzl)–Lys(Z)–OBzl Boc–Val–ON*p* (4.7 g) and H–Cys(MeBzl)–Lys(Z)–OBzl·TFA [prepared from Boc–Cys(MeBzl)–Lys(Z)–OBzl (7.8 g), TFA (13.1 ml) and anisole (3.7 ml) as usual] were dissolved in DMF (100 ml) containing Et_3N (1.9 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 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 and recrystallized from EtOH, yield 5.5 g (61.6%), mp 148—149 °C, $[\alpha]_D^{25}$ −30.6° (c=1.0, DMF), Rf^1 0.81. *Anal.* Calcd for $C_{42}H_{56}N_4O_8S$: C, 64.9; H, 7.26; N, 7.21. Found: C, 64.7; H, 7.36; N, 7.21.

Boc–Val–Cys(MeBzl)–Lys(Z)–NHNH$_2$, Boc–(MT 49—51)–NHNH$_2$ (3) Hydrazine hydrate (90%, 1.7 ml) was added to a solution of Boc–(MT 49—51)–OBzl (4.4 g) in DMF (20 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 MeOH, yield 2.9 g (73.1%), mp 203—204 °C, $[\alpha]_D^{25}$ −17.6° (c=1.0, DMF), Rf^1 0.51. *Anal.* Calcd for $C_{35}H_{52}N_6O_8S \cdot 0.5H_2O$: C, 59.2; H, 7.53; N, 11.8. Found: C, 59.5; H, 7.54; N, 12.1.

Boc–Gly–Cys(MeBzl)–OBzl Boc–Gly–OH (1.8 g), H–Cys(MeBzl)–OBzl·TosOH (4.7 g) and DCC (2.2 g) were dissolved in DMF (50 ml) containing Et_3N (4.8 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of 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. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 3.9 g (82.5%), mp 43—45 °C, $[\alpha]_D^{25}$ −25.7° (c=1.0, DMF), Rf^1 0.75. *Anal.* Calcd for $C_{25}H_{32}N_2O_5S$: C, 63.5; H, 6.83; N, 5.93. Found: C, 63.6; H, 6.90; N, 6.18.

Boc–Gln–Gly–Cys(MeBzl)–OBzl Boc–Gln–ON*p* (3.7 g) and H–Gly–Cys(MeBzl)–OBzl·TFA [prepared from Boc–Gly–Cys(MeBzl)–OBzl (3.0 g), TFA (7.24 ml) and anisole (2.0 ml) as usual] were dissolved in DMF (50 ml) containing Et_3N (0.9 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and washed with AcOEt, yield 2.6 g (68.2%), mp 159—160 °C, $[\alpha]_D^{25}$ −15.7° (c=1.0, DMF), Rf^1 0.60. *Anal.* Calcd for $C_{30}H_{40}N_4O_7S$: C, 60.0; H, 6.71; N, 9.33. Found: C, 60.2; H, 6.94; N, 9.42.

Boc–Gln–Gly–Cys(MeBzl)–NHNH$_2$, Boc–(MT 46—48)–NHNH$_2$ (4) Hydrazine hydrate (90%, 0.65 ml) was added to a solution of Boc–(MT 46—48)–OBzl (2.6 g) in DMF (20 ml). The reaction mixture was stored at room temperature overnight. The resultant precipitate was collected by filtration and washed with MeOH, yield 2.1 g (93.1%), mp 186—188 °C, $[\alpha]_D^{25}$ −18.1° (c=0.5, DMF), Rf^1 0.50. *Anal.* Calcd for $C_{23}H_{36}N_6O_6S \cdot 0.5H_2O$: C, 52.7; H, 6.92; N, 16.0. Found: C, 53.0; H, 7.09; N, 15.8.

Boc–Cys(MeBzl)–Ala–OBzl Boc–Cys(MeBzl)–ON*p* (13.4 g) and H–Ala–OBzl·TosOH (12.6 g) were dissolved in DMF (200 ml) containing Et_3N (5.0 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and 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 EtOH, yield 10.8 g (74.0%), mp 76—78 °C, $[\alpha]_D^{25}$ −28.7° (c=1.0, DMF), Rf^1 0.67. *Anal.* Calcd for $C_{20}H_{30}N_2O_5S$: C, 58.5; H, 7.36; N, 6.83. Found: C, 58.3; H, 7.21; N, 6.90.

Boc–Lys(Z)–Cys(MeBzl)–Ala–OBzl Boc–Lys(Z)–ON*p* (9.0 g) and H–Cys(MeBzl)–Ala–OBzl·TFA [prepared from Boc–Cys(MeBzl)–Ala–OBzl (7.3 g), TFA (17.1 ml) and anisole (4.9 ml) as usual] were dissolved in DMF (150 ml) containing Et_3N (2.5 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 9.1 g (81.0%), mp 101—104 °C, $[\alpha]_D^{25}$ −35.4° (c=1.0, DMF), Rf^1 0.75. *Anal.* Calcd for $C_{34}H_{48}N_4O_8S$: C, 60.7; H, 7.19; N, 8.33. Found: C, 60.7; H, 7.21; N, 8.32.

Boc–Lys(Z)–Cys(MeBzl)–Ala–NHNH$_2$, Boc–(MT 43—45)–NHNH$_2$ (5) Hydrazine hydrate (90%, 0.81 ml) was added to a solution of Boc–(MT 43—45)–OBzl (4.0 g) in DMF (30 ml). The reaction mixture was stored at room temperature overnight. MeOH was added to the reaction mixture to afford a precipitate, which was collected by filtration and washed with MeOH, yield 2.8 g (77.9%), mp 173—176 °C, $[\alpha]_D^{25}$ −17.0° (c=1.0, DMF), Rf^1 0.64. *Anal.* Calcd for $C_{33}H_{48}N_6O_7S$: C, 58.9; H, 7.19; N, 12.5. Found: C, 58.7; H, 7.13; N, 12.6.

Boc–Cys(MeBzl)–Ser–OMe Boc–Cys(MeBzl)–ON*p* (17.9 g) and H–Ser–OMe·HCl (7.0 g) were dissolved in DMF (100 ml) containing Et_3N (6.3 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 15.8 g (92.6%), mp 53—56 °C, $[\alpha]_D^{25}$ −31.1° (c=1.0, MeOH), Rf^1 0.63. *Anal.* Calcd for $C_{20}H_{28}N_2O_6S$: C, 56.6; H, 6.65; N, 6.60. Found: C, 56.6; H, 6.88; N, 6.59.

Boc–Gly–Cys(MeBzl)–Ser–OMe Boc–Gly–ON*p* (5.0 g) and H–Cys(MeBzl)–Ser–OMe·TFA [prepared from Boc–Cys(MeBzl)–Ser–OMe (8.0 g), TFA (21.4 ml) and anisole (6.1 ml) as usual] were dissolved in DMF (100 ml) containing Et_3N (2.6 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 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 and recrystallized from EtOH, yield 5.3 g (58.3%), mp 118—121 °C, $[\alpha]_D^{25}$ −19.9° (c=1.0, MeOH), Rf^1 0.53. *Anal.* Calcd for $C_{22}H_{31}N_3O_7S$: C, 54.9; H, 6.49; N, 8.73. Found: C, 54.6; H, 6.63; N, 8.71.

Boc–Val–Gly–Cys(MeBzl)–Ser–OMe Boc–Val–ON*p* (3.7 g) and H–Gly–Cys(MeBzl)–Ser–OMe·TFA [prepared from Boc–Gly–Cys(MeBzl)–Ser–OMe (5.3 g), TFA (12.5 ml) and anisole (3.6 ml) as usual] were dissolved in DMF (100 ml) containing Et_3N (1.5 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 AcOEt, yield 4.6 g (70.9%), mp 145—148 °C, $[\alpha]_D^{25}$ −32.5° (c=1.0, DMF), Rf^1 0.53. *Anal.* Calcd for $C_{27}H_{40}N_4O_8 \cdot H_2O$: C, 54.2; H, 7.18; N, 9.36. Found: C, 54.0; H, 7.06; N, 9.43.

Boc–Val–Gly–Cys(MeBzl)–Ser–NHNH$_2$, Boc–(MT 39—42)–NHNH$_2$ (6) Hydrazine hydrate (90%, 1.14 ml) was added to a solution of Boc–(MT 39—42)–OBzl (4.5 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 3.9 g (86.7%), mp 207—208 °C, $[\alpha]_D^{25}$ −20.1° (c=1.0, DMF), Rf^2 0.71. *Anal.* Calcd for $C_{26}H_{40}N_6O_7S \cdot 0.5H_2O$: C, 52.9; H, 7.01; N, 14.3. Found: C, 52.6; H, 7.22; N, 14.5.

Boc–Cys(MeBzl)–Cys(MeBzl)–Pro–NHNH$_2$, Boc–(MT 36—38)–NHNH$_2$ (7) Boc–Cys(MeBzl)–Cys(MeBzl)–N_3 [prepared from the

corresponding hydrazide (4.0 g) and isopentyl nitrite (1.02 ml) as usual] in DMF (50 ml) was added to the solution of H–Pro–OBzl·HCl (4.0 g) in DMF (50 ml) containing Et_3N (2.1 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The residual oil was dissolved in MeOH (50 ml). Hydrazine hydrate (90%, 0.67 ml) was added to the above solution, and the reaction mixture was stored at room temperature overnight. A small amount of ether was added to the solution and the resultant precipitate was collected by filtration, yield 2.7 g (57.3%), mp 115—117 °C, $[\alpha]_D^{25}$ −65.8° (c = 1.0, MeOH), Rf^1 0.53. *Anal.* Calcd for $C_{32}H_{45}N_5O_5S_2$: C, 59.7; H, 7.04; N, 10.9. Found: C, 59.6; H, 7.02; N, 10.8.

Boc–Cys(MeBzl)–Cys(MeBzl)–Ser–OMe Boc–Cys(MeBzl)–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (4.0 g) and isopentyl nitrite (1.02 ml) as usual] in DMF (50 ml) was added to a solution of H–Ser–Me·HCl (1.7 g) in DMF (50 ml) containing Et_3N (1.03 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 evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 3.7 g (80.8%), mp 158—159 °C, $[\alpha]_D^{25}$ −34.2° (c = 0.8, DMF), Rf^1 0.71. *Anal.* Calcd for $C_{31}H_{43}N_3O_7S_2$: C, 58.7; H, 6.84; N, 6.63. Found: C, 58.5; H, 6.74; N, 6.49.

Boc–Cys(MeBzl)–Cys(MeBzl)–Ser–$NHNH_2$, Boc–(MT 33—35)–$NHNH_2$ (8) Hydrazine hydrate (90%, 0.61 ml) was added to a solution of Boc–(MT 33—35)–OMe (1.5 g) in DMF (20 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 1.4 g (93.5%), mp 191—194 °C, $[\alpha]_D^{25}$ −15.8° (c = 0.5, DMF), Rf^1 0.41. *Anal.* Calcd for $C_{30}H_{43}N_5O_6S_2 \cdot 0.5H_2O$: C, 56.1; H, 6.89; N, 10.9. Found: C, 56.3; H, 6.80; N, 11.0.

Boc–Lys(Z)–Lys(Z)–OBzl Boc–Lys(Z)–ON*p* (7.0 g) and H–Lys(Z)–OBzl·TosOH (9.4 g) were dissolved in DMF (100 ml) containing Et_3N (2.3 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. Ether and petroleum ether were added to the residue to afford crystals, which were collected by filtration, yield 6.9 g (68.0%), mp 64—68 °C, $[\alpha]_D^{25}$ −17.2° (c = 1.0, MeOH), Rf^2 0.69. *Anal.* Calcd for $C_{40}H_{52}N_4O_9$: C, 65.6; H, 7.38; N, 7.66. Found: C, 65.6; H, 7.15; N, 7.65.

Boc–Lys(Z)–Lys(Z)–$NHNH_2$ Hydrazine hydrate (90%, 1.02 ml) was added to a solution of Boc–Lys(Z)–Lys(Z)–OBzl (5.0 g) in MeOH (50 ml). The reaction mixture was stored at room temperature overnight. A small amount of ether was added to the solution and the resultant precipitate was collected by filtration, yield 3.5 g (78.1%), mp 122—126 °C, $[\alpha]_D^{25}$ −30.1° (c = 1.0, DMF), Rf^2 0.77. *Anal.* Calcd for $C_{33}H_{48}N_6O_8 \cdot H_2O$: C, 58.7; H, 7.47; N, 12.5. Found: C, 58.9; H, 7.39; N, 12.6.

Boc–Lys(Z)–Lys(Z)–Ser–OMe Boc–Lys(Z)–Lys(Z)–N_3 [prepared from the corresponding hydrazide (3.4 g) and isopentylnitrite (0.72 ml) as usual] in DMF (50 ml) was added to a solution of H–Ser–OMe·HCl (1.2 g) in DMF (50 ml) containing Et_3N (1.1 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 evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 2.8 g (72.7%), mp 88—90 °C, $[\alpha]_D^{25}$ −19.4° (c = 1.0, MeOH), Rf^1 0.51. *Anal.* Calcd for $C_{37}H_{53}N_5O_{11}$: C, 59.7; H, 7.18; N, 9.14. Found: C, 59.6; H, 7.40; N, 9.38.

Boc–Lys(Z)-Lys(Z)–Ser–$NHNH_2$, Boc–(MT 30—32)–$NHNH_2$ (9) Hydrazine hydrate (90%, 0.73 ml) was added to a solution of Boc–(MT 30—32)–OMe (1.8 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 1.5 g (85.0%), mp 185—186 °C, $[\alpha]_D^{25}$ −11.3° (c = 1.0, DMF), Rf^2 0.75. *Anal.* Calcd for $C_{36}H_{53}N_7O_{10}$: C, 58.1; H, 7.18; N, 13.2. Found: C, 57.9; H, 7.32; N, 13.2.

Boc–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 52—61)–OBzl (10) Boc–Gly–Ala–Ala–N_3 [prepared from the corresponding hydrazide (1.2 g) and isopentyl nitrite (0.5 ml) as usual] in DMF (30 ml) was added to a solution of H–(MT 55—61)–OBzl·TFA [prepared from Boc–(MT 55—61)–OBzl (2.74 g), TFA (2.0 ml) and anisole (0.60 ml) as usual] in DMF (30 ml) containing Et_3N (0.25 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 2.50 g (88.8%), mp 258—259 °C, $[\alpha]_D^{25}$ −18.7° (c = 1.0, DMF), Rf^1 0.55. *Anal.* Calcd for $C_{88}H_{117}N_{11}O_{18}S_3 \cdot 0.5H_2O$: C, 61.4; H, 6.91; N, 8.95. Found: C, 61.2; H, 6.91; N, 8.97.

Boc–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 49—61)–OBzl (11) Boc–Val–Cys(MeBzl)–Lys(Z)–N_3 [prepared from the corresponding hydrazide (900 mg) and isopentyl nitrite (0.18 ml) as usual] in DMF (30 ml) was added to a solution of H–(MT 52—61)–OBzl·TFA [prepared from Boc–(MT 52—61)–OBzl (1.1 g), TFA (1.46 ml) and anisole (0.20 ml) as usual] in DMF (30 ml) containing Et_3N (0.09 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 1.25 g (85.3%), mp 250 °C (dec.), $[\alpha]_D^{25}$ −23.2° (c = 0.2, DMF), Rf^1 0.71. *Anal.* Calcd for $C_{118}H_{157}N_{15}O_{23}S_4 \cdot 2H_2O$: C, 61.1; H, 7.00; N, 9.06. Found: C, 60.8; H, 6.91; N, 9.15.

Boc–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 46—61)–OBzl (12) Boc–Gln–Gly–Cys(MeBzl)–N_3 [prepared from the corresponding hydrazide (690 mg) and isopentyl nitrite (0.18 ml) as usual] in DMF (30 ml) was added to a solution of H–(MT 49—61)–OBzl·TFA [prepared from Boc–(MT 49—61)–OBzl (1.00 g), TFA (1.50 ml) and anisole (0.28 ml) as usual] in DMF (30 ml) containing Et_3N (0.06 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 1.09 g (93.0%), mp 255 °C (dec.), $[\alpha]_D^{25}$ −28.0° (c = 0.2, DMSO), Rf^1 0.66. *Anal.* Calcd for $C_{136}H_{181}N_{19}O_{27}S_5 \cdot 3.5H_2O$: C, 59.6; H, 6.92; N, 9.72. Found: C, 59.3; H, 6.94; N, 10.1.

Boc–Lys(Z)–Cys(MeBzl)–Ala–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 43—61)–OBzl (13) Boc–Lys(Z)–Cys(MeBzl)–Ala–N_3 [prepared from the corresponding hydrazide (303 mg) and isopentyl nitrite (0.06 ml) as usual] in DMF (20 ml) was added to a solution of H–(MT 46—61)–OBzl·TFA [prepared from Boc–(MT 46—61)–OBzl (400 mg), TFA (1.20 ml) and anisole (0.05 ml) as usual] in DMF (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 470 mg (91.2%), mp 245 °C, $[\alpha]_D^{25}$ −10.0° (c = 0.2, DMSO), Rf^1 0.45, Rf^2 0.65. *Anal.* Calcd for $C_{164}H_{217}N_{23}O_{32}S_6 \cdot 6H_2O$: C, 59.3; H, 6.95; N, 9.70. Found: C, 59.0; H, 6.90; N, 10.1.

Boc–Val–Gly–Cys(MeBzl)–Ser–Lys(Z)–Cys(MeBzl)–Ala–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 39—61)–OBzl (14) Boc–Val–Gly–Cys(MeBzl)–Ser–N_3 [prepared from the corresponding hydrazide (492 mg) and isopentyl nitrite (0.12 ml) as usual] in DMF (20 ml) was added to a solution of H–(MT 43—61)–OBzl·TFA [prepared from Boc–(MT 43—61)–OBzl (670 mg), TFA (1.40 ml) and anisole (0.07 ml) as usual] in DMF (50 ml) and HMPA (50 ml) containing Et_3N (0.03 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 650 mg (85.1%), mp 258 °C (dec.), $[\alpha]_D^{25}$ −15.5° (c = 0.2, DMF), Rf^1 0.50. *Anal.* Calcd for $C_{185}H_{247}N_{27}O_{37}S_7 \cdot 6H_2O$: C, 58.9; H, 6.91; N, 10.0. Found: C, 58.6; H, 6.63; N, 10.1.

Boc–Cys(MeBzl)–Cys(MeBzl)–Pro–Val–Gly–Cys(MeBzl)–Ser–Lys(Z)–Cys(MeBzl)–Ala–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 36—61)–OBzl (15) Boc–Cys(MeBzl)–Cys(MeBzl)–Pro–N_3 [prepared from the corresponding hydrazide (211 mg) and isopentyl nitrite (0.05 ml) as usual] in DMF (20 ml) was added to a solution of H–(MT 39—61)–OBzl·TFA [prepared from Boc–(MT 39—61)–OBzl (300 mg), TFA (0.84 ml) and anisole (0.03 ml) as usual] in DMF (30 ml) and HMPA (30 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 290 mg (85.3%), mp 268—269 °C (dec.), $[\alpha]_D^{25}$ −18.2 °C (c = 0.2, DMSO), Rf^1 0.48. *Anal.* Calcd for $C_{212}H_{280}N_{30}O_{40}S_9 \cdot 7H_2O$: C, 59.2; H, 6.89; N, 9.76. Found: C, 59.1; H, 6.69; N, 9.75.

Boc–Cys(MeBzl)–Cys(MeBzl)–Ser–Cys(MeBzl)–Cys(MeBzl)–Pro–Val–Gly–Cys(MeBzl)–Ser–Lys(Z)–Cys(MeBzl)–Ala–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 33—61)–

OBzl (16) Boc–Cys(MeBzl)–Cys(MeBzl)–Ser–N_3 [prepared from the corresponding hydrazide (159 mg) and isopentyl nitrite (0.03 ml) as usual] in DMF (15 ml) was added to a solution of H–(MT 36—61)–OBzl·TFA [prepared from Boc–(MT 36—61)–OBzl (350 mg), TFA (0.96 ml) and anisole (0.03 ml) as usual] in DMF (30 ml) and HMPA (30 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 300 mg (76.5 mg), mp 261 °C (dec.), $[\alpha]_D^{25}$ −11.2° (c=0.2, DMSO), Rf^1 0.61. *Anal.* Calcd for $C_{237}H_{311}N_{33}O_{44}S_{11}\cdot 8H_2O$: C, 58.8; H, 6.85; N, 9.55. Found: C, 58.6; H, 6.66; N, 9.82.

Boc–Lys(Z)–Lys(Z)–Ser–Cys(MeBzl)–Cys(MeBzl)–Ser–Cys(MeBzl)–Cys(MeBzl)–Pro–Val–Gly–Cys(MeBzl)–Ser–Lys(Z)–Cys(MeBzl)–Ala–Gln–Gly–Cys(MeBzl)–Val–Cys(MeBzl)–Lys(Z)–Gly–Ala–Ala–Asp(O-2-Ada)–Lys(Z)–Cys(MeBzl)–Thr–Cys(MeBzl)–Cys(MeBzl)–Ala–OBzl, Boc–(MT 30—61)–OBzl (17) Boc–Lys(Z)–Lys(Z)–Ser–N_3 [prepared from the corresponding hydrazide (113 mg) and isopentyl nitrite (0.02 ml) as usual] in DMF (15 ml) was added to a solution of H–(MT 33—61)–OBzl·TFA [prepared from Boc–(MT 33—61)–OBzl (230 mg), TFA 0.60 ml) and anisole (0.02 ml) as usual] in DMF (30 ml) and HMPA (30 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 200 mg (74.6%), mp 263 °C (dec.), $[\alpha]_D^{25}$ −20.2° (c=0.1, DMSO), Rf^1 0.55. *Anal.* Calcd for $C_{268}H_{352}N_{38}O_{52}S_{11}\cdot 10H_2O$: C, 58.1; H, 6.88; N, 9.67. Found: C, 58.1; N, 6.62; N, 9.88.

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. The residue in H_2O (10 ml) was reduced with dithiothreitol (140 mg) at room temperature overnight. This reaction mixture was applied to a Sephadex G-15 column (2.2×118 cm) for MT (57—61), (52—61) or a Sephadex G-25 column (2.2×95 cm) for MT (49—61), (46—61), (43—61), (36—61), (30—61, α-fragment). These columns 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, $[\alpha]_D$, amino acid ratios in acid hydrolysate, Rf^3 value and SH content 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 $CdCl_2$, $CuCl_2$ or $(CH_3CN)_4ClO_4$ 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

1) Part XXXIII: Y. Okada, S. Nakayama, S. Iguchi, Y. Kikuchi, M. Irie, J. Sawada, H. Ikebuchi and T. Terao, *Chem. Pharm. Bull.*, **40**, 1029 (1992).
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; MeBzl, 4-methylbenzyl; ON*p*, *p*-nitrophenyl ester; OSu, *N*-hydroxysuccinimide ester; O-2-Ada, 2-adamantyl ester; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide; TFA, trifluoroacetic acid; AcOH, acetic acid; AcOEt, ethyl acetate; *n*-BuOH, *n*-butanol; TosOH, *p*-toluenesulfonic acid.
3) M. Piscator, *Nord. Hyg. Tidskr.*, **45**, 76 (1964).
4) G. F. Nordberg, *Environ. Physiol. Biochem.*, **2**, 7 (1972).
5) I. Bremner and N. T. Davies, *Biochem. J.*, **149**, 733 (1975).
6) L. Ryden and J. F. Deutsch, *J. Biol. Chem.*, **253**, 519 (1978).
7) O. M. Udom and F. O. Brady, *Biochem. J.*, **187**, 329 (1980).
8) M. Beltramini and K. Lerch, *FEBS Lett.*, **142**, 219 (1982).
9) B. L. Gello and D. R. Winge, *Arch. Biochem. Biophys.*, **213**, 109 (1982).
10) A. Bakka and M. Webb, *Biochem. Pharmacol.*, **30**, 721 (1981).
11) B. B. Griffith, R. A. Walters, M. D. Enger, C. E. Hilderband and J. K. Griffith, *Nucleic Acids Res.*, **11**, 901 (1983).
12) J. D. Otvos and I. M. Armitage, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7094 (1980).
13) D. R. Winge and K. A. Miklossy, *J. Biol. Chem.*, **257**, 3471 (1982).
14) K. B. Nielson and D. R. Winge, *J. Biol. Chem.*, **259**, 4941 (1984).
15) Y. Boulanger, I. M. Armitage, K. A. Miklossy and D. R. Winge, *J. Biol. Chem.*, **257**, 13717 (1982).
16) I. M. Armitage, J. D. Otvos, R. W. Briggs and Y. Boulanger, *Fed. Proc.*, **41**, 2941 (1982).
17) K. Lerch, *Nature* (London), **284**, 368 (1980).
18) K. Munger and K. Lerch, *Biochemistry*, **24**, 6751 (1985).
19) K. Lerch, *Met. Ions Biol. Syst.*, **13**, 299 (1981).
20) I-Y. Huang, A. Yoshida, H. Tsunoo and H. Nakajima, *J. Biol. Chem.*, **252**, 8217 (1977).
21) Y. Okada, Y. Matsuno, Y. Nishiyama, Y. Tsuda, S. Iguchi, K. Min, S. Onosaka and K. Tanaka, *Chem. Pharm. Bull.*, **37**, 2322 (1989).
22) Y. Nishiyama, S. Nakayama, Y. Okada, K. Min, S. Onosaka and K. Tanaka, *Chem. Pharm. Bull.*, **38**, 2112 (1990).
23) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
24) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii and H. Irie, *Chem. Pharm. Bull.*, **30**, 1706 (1980).
25) Y. Okada and S. Iguchi, *J. Chem. Soc., Perkin Trans. 1*, **1988**, 2129.
26) B. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).
27) Y. Okada, N. Ohta, M. Yagyu, K. Min, S. Onosaka and K. Tanaka, *J. Protein Chem.*, **3**, 243 (1984).
28) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
29) N. Ohta, Y. Okada and K. Tanaka, *Chem. Pharm. Bull.*, **31**, 3094 (1983).
30) M. L. Cherian and P. C. Huang, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 3024 (1991).
31) Y. Okada, M. Okinaka, M. Yagyu, K. Watabe, K. Sano and Y. Kakiuchi, *Chem. Pharm. Bull.*, **24**, 3081 (1976).