# Amino Acids and Peptides. XXXIII. Synthesis of N-Terminal Epitope Peptides of Mammalian Metallothioneins (MTs)[1,2]

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**In order to determine the fine structure of the mammalian metallothionein (MT) epitope to a monoclonal anti-rat Zn–MT-II antibody (MT 189-14-7), N-terminal peptides of various lengths of mammalian metallothioneins (MTs) were synthesized by a conventional solution method using the newly developed β-2-adamantylaspartate, and their immunological properties were examined. It was found that the N-terminal acetyl group was indispensable for the reaction with the monoclonal antibody and the N-terminally acetylated pentapeptide, Ac–Met–Asp–Pro–Asn–Cys–OH, was the smallest peptide which exhibited a significant reactivity with the antibody.**

**Keywords** N-terminal peptide; mammalian metallothionein; chemical synthesis; β-2-adamantylaspartate; immunological property; crossreactivity; monoclonal antibody; rat zinc metallothionein II

Metallothioneins (MTs) are a class of low-molecular-weight, metal-binding proteins. The amino acid sequences of mammalian MTs are highly conserved, implying their physiological importance.[3] Several functions have been proposed, including metal detoxification or metabolism, control of the intracellular redox potential, activated oxygen detoxification, and sulfur metabolism.[4]

Immunoassay of MT is highly sensitive and useful for determining low levels of MT. Winge and Garvey[5] suggested that the antigenic determinants (epitopes) of MT to rabbit polyclonal antibodies resided in the N-terminal region. Previously, we have shown that an epitope of MT to a murine monoclonal antibody (MT 189-14-7) is located within the N-terminally acetylated heptapeptide common to various animal MTs.[6,7] This paper deals with the synthesis of various kinds of N-terminal peptide of mammalian MTs and examination of their immunoreactivity with the monoclonal antibody.

In order to determine the fine structure of the MT epitope to the MT 189-14-7 antibody (and also to rabbit polyclonal antibodies), N-terminal peptides of various lengths (Fig. 1) were synthesized by using the newly developed β-2-adamantylaspartate.[8] As an example, the synthetic scheme for the N-terminal hexapeptide is illustrated in Fig. 2. Boc–Cys(MBzl)–OH and H–Ser–OBzl were coupled by the DCC method to give Boc–Cys(MBzl)–Ser–OBzl. Treatment of the dipeptide with TFA gave the corresponding amine, which was successively condensed with Boc–Asn–ONp, Boc–Pro–ONp, Boc–Asp(O-2-Ada)–OSu[8] and finally Ac–Met–$N_3$ to give the fully protected hexapeptide. The

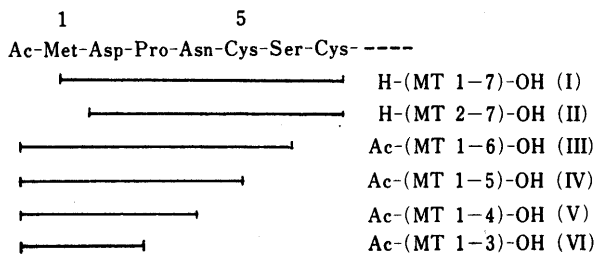


Fig. 1. Structures of Synthesized N-Terminal Peptides of Mammalian MTs

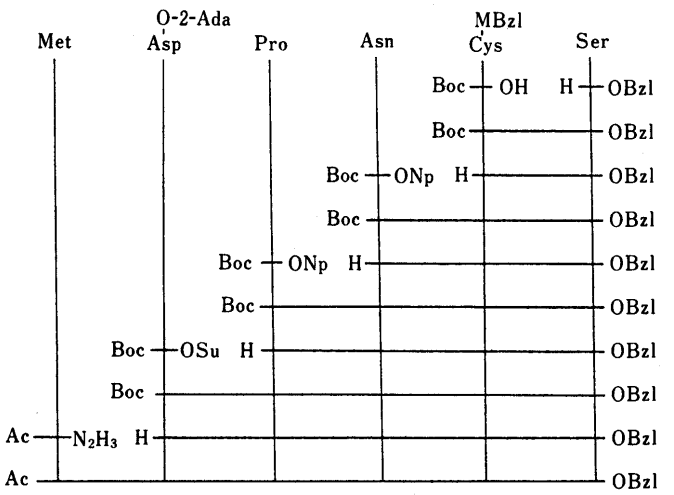


Fig. 2. Synthetic Scheme for the N-Terminal Hexapeptide of Mammalian MTs (MT 1—6)

TABLE I. Yields, $[\alpha]_D$ Values, *Rf* Values and the Results of Amino Acid Analysis of Deprotected Peptides

| Compound | Yield (%) | $[\alpha]_D^{25}$ ($c$=0.4, 3% AcOH) | *Rf*[a] | Amino acid ratios[b] in acid hydrolysate | | | | | Average recovery (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Met | Asp | Pro | Cys | Ser | |
| Ac–Met–Asp–Pro–Asn–Cys–Ser–OH | 67.0 | −34.8° | 0.87 | 0.60 | 2.00 | 1.14 | — | 0.99 | 83 |
| Ac–Met–Asp–Pro–Asn–Cys–OH | 65.3 | −83.2° | 0.86 | 0.86 | 2.00 | 1.05 | — | | 81 |
| Ac–Met–Asp–Pro–Asn–OH | 68.4 | −92.3° | 0.88 | 0.95 | 2.00 | 1.08 | | | 83 |
| Ac–Met–Asp–Pro–OH | 70.6 | −93.0° | 0.65 | 0.75 | 1.00 | 1.01 | | | 86 |

*a*) Solvent: *n*-BuOH, pyridine, AcOH and $H_2O$ (4:1:1:1). *b*) Cys was not determined.

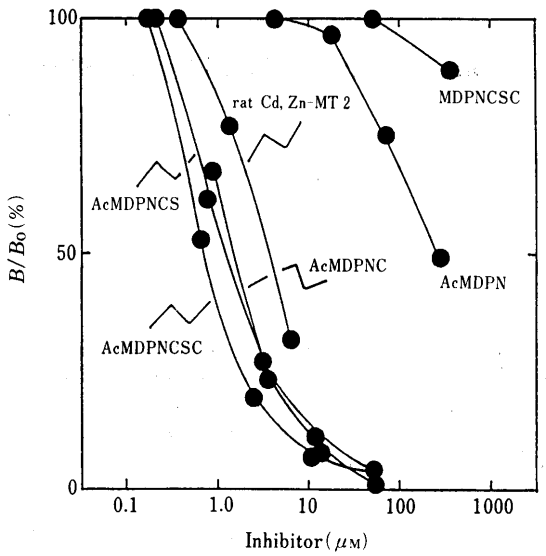


Fig. 3. Inhibition by Chemically Synthesized N-Terminal Peptides of Mammalian MTs

homogeneity of the protected final peptide and intermediates was ascertained by thin-layer chromatography (TLC) on silica gel and by elemental analysis. All protecting groups were removed by employing anhydrous HF[9] in the presence of thioanisole and *m*-cresol.[10] The crude product was extracted with 3% AcOH. The extract was washed with AcOEt and the water layer was lyophilized to give a white fluffy powder. The deblocked peptide, Ac-(MT 1—6)-OH, was homogeneous upon TLC on silicagel and reverse phase high performance liquid chromatography (RP-HPLC).[11] The amino acid ratios in an acid hydrolysate were in agreement with the theoretically expected values. The $[\alpha]_D$, *Rf* values and amino acid ratios of I—V are summarized in Table I.

The crossreactivity of the peptides (I—V) obtained above with the monoclonal antibody MT 189-14-7 was determined by the competitive radioimmunoassay (RIA) method.[12] The extents of crossreactivity of H-(MT 1—7)-OH (I)[6] and H-(MT 2—7)-OH (II)[6] with the monoclonal antibody are very low ($IC_{50}$ values: $1.9 \times 10^{-4}$ M, $2.3 \times 10^{-4}$ M, respectively) compared with that of Ac-(MT 1—7)-OH ($IC_{50}$ value: $7.5 \times 10^{-7}$ M) or native MT ($IC_{50}$ value: $3.0 \times 10^{-6}$ M), indicating that the N-terminal acetyl group and methionine residue are very important for crossreactivity with the monoclonal antibody. The crossreactivities of these peptide (I—V) are summarized in Fig. 3 in comparison with those of Ac-(MT 1—7)-OH and native rat Cd, Zn-MT. Ac-(MT 1—5)-OH was the smallest peptide which exhibited similar reactivity ($IC_{50}$ value: $3.2 \times 10^{-6}$ M) to that of native rat Cd, Zn-MT ($IC_{50}$ value: $3.0 \times 10^{-6}$ M).

**Experimental**

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6N HCl, 110°C, 18h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). On TLC (Kieselgel G, Merck), $Rf^1$, $Rf^2$, $Rf^3$, $Rf^4$ and $Rf^5$ 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), *n*-BuOH, pyridine, AcOH and $H_2O$ (1:1:1:1), *n*-BuOH, pyridine, AcOH and $H_2O$ (4:1:1:2) and $CHCl_3$, MeOH and AcOH (17:2:1), respectively. HPLC was conducted with a Waters M 600 instrument.

**Boc-Cys(MBzl)-Ser-OBzl** Boc-Cys(MBzl)-OH (3.41g, 10.0mmol) and H-Ser-OBzl·$C_6H_5SO_3H$[13] (3.53g, 10.0mmol) were dissolved in DMF containing $Et_3N$ (1.4ml, 10.0mmol) and the solution was cooled with ice-salt. DCC (2.48g, 12.0mmol) was added to the above cold solution and the reaction mixture was stirred at 40°C overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$, dried over $Na_2SO_4$ and concentrated to a small volume. Petroleum ether was added to the residue to give a crystalline material, which was collected by filtration and recrystallized from $CH_2Cl_2$-hexane, yield (4.04g, 77.9%), mp 83—84°C, $[\alpha]_D^{27}$ +13.7 ($c$=1.0, $CHCl_3$), $Rf^2$ 0.92, $Rf^5$ 0.55. *Anal.* Calcd for $C_{26}H_{34}N_2O_2S$: C, 60.2; H, 6.61; N, 5.40. Found: C, 59.9; H, 6.66; N, 5.22.

**A Representative Procedure for Deprotection with TFA** A solution of Boc-Cys(MBzl)-Ser-OBzl (3.0g, 5.87mmol) in TFA (6.6ml, 86.8mmol) containing anisole (1.25ml, 11.6mmol) was stirred at room temperature for 40min and at 0°C for 30min. Ether was added to the solution to give a white precipitate, which was collected by decantation, washed with ether, and dried over KOH pellets *in vacuo*.

**Boc-Asn-Cys(MBzl)-Ser-OBzl** Boc-Asn-ONp (2.24g, 6.36mmol) and H-Cys(MBzl)-Ser-OBzl·TFA [prepared from Boc-Cys(MBzl)-Ser-OBzl (3.0g, 5.87mmol) and TFA (6.6ml, 57.8mmol) containing anisole (1.25ml)] were dissolved in DMF (50ml) containing $Et_3N$ (6.6ml). 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% $NaHCO_3$, 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. Petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 2.08g (57.0%), mp 160—163°C, $[\alpha]_D^{27}$ −27.0° ($c$=1.0, DMF), $Rf^1$ 0.80. *Anal.* Calcd for $C_{30}H_{40}N_4O_9S \cdot 4.5H_2O$: C, 50.5; H, 6.92; N, 7.85. Found: C, 50.5; H, 6.63; N, 7.80.

**Boc-Pro-Asn-Cys(MBzl)-Ser-OBzl** Boc-Pro-ONp (637mg, 1.89mmol) and H-Asn-Cys(MBzl)-Ser-OBzl·TFA [prepared from Boc-Asn-Cys(MBzl)-Ser-OBzl (1.0g, 1.58mmol) and TFA (1.8ml, 15.8mmol) containing anisole (0.51ml)] were dissolved in DMF (30ml) containing $Et_3N$ (0.30ml). The reaction mixture was stirred at room temperature overnight and the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 5% 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 660mg (57.2%), mp 180—182°C, $[\alpha]_D^{27}$ −68.2° ($c$=1.0, MeOH), $Rf^1$ 0.50. *Anal.* Calcd for $C_{35}H_{47}N_5O_{10}S \cdot 3H_2O$: C, 53.6; H, 6.82; N, 8.93. Found: C, 53.5; H, 6.81; N, 8.69.

**Boc-Asp(O-2-Ada)-Pro-Asn-Cys(MBzl)-Ser-OBzl** Boc-Asp(O-2-Ada)-OSu (613mg, 1.32mmol) and H-Pro-Asn-Cys(MBzl)-Ser-OBzl·TFA [prepared from 800mg (1.10mmol) of Boc-Pro-Asn-Cys(MBzl)-Ser-OBzl and 1.3ml (11.0mmol) of TFA containing 0.36ml of anisole] were dissolved in DMF (30ml) containing $Et_3N$ (0.16ml). The reaction mixture was stirred at room temperature overnight and the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 5% citric acid and water, dried over $Na_2SO_4$ and evaporated. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 660mg (57.2%), mp 142—151°C, $[\alpha]_D^{27}$ −49.9° ($c$=1.0, DMF), $Rf^1$ 0.77. *Anal.* Calcd for $C_{49}H_{66}N_6O_{13}S \cdot 2H_2O$: C, 58.0; H, 6.55; N, 8.27. Found: C, 57.7; H, 6.52; N, 8.51.

**Ac-Met-Asp(O-2-Ada)-Pro-Asn-Cys(MBzl)-Ser-OBzl** Ac-Met-$N_3$ [prepared from Ac-Met-$NHNH_2$ 83.8mg (0.41mmol), 6.4N HCl/dioxane (0.17ml) and isopentyl nitrite (0.56ml, 0.41mmol) in the usual manner] in DMF (10ml) cooled to −10°C was combined with H-Asp(O-2-Ada)-Pro-Asn-Cys(MBzl)-Ser-OBzl·TFA [prepared from 200mg (0.20mmol) of Boc-Asp(O-2-Ada)-Pro-Asn-Cys(MBzl)-Ser-OBzl and 0.22ml (2.0mmol) of TFA containing anisole (0.05ml)] in DMF (10ml) containing $Et_3N$ (0.22ml). The reaction mixture was stirred at 4°C overnight. After removal of the solvent, AcOEt and water were added to the residue to afford a solid mass, which was collected by filtration, yield 140mg (65.3%), mp 135—150°C, $[\alpha]_D^{27}$ −47.7° ($c$=0.3, DMF), $Rf^1$ 0.48, $Rf^2$ 0.68. Amino acid ratios in an acid hydrolysate: $Asp_{2.00(2)}$, $Ser_{1.00(1)}$, $Met_{0.77(1)}$, $Pro_{1.12(1)}$ (average recovery 94%). Cys was not determined. *Anal.* Calcd for $C_{51}H_{61}N_7O_{13}S_2 \cdot 1.5H_2O$: C, 56.8; H, 6.72; N, 9.09. Found: C, 56.5; H, 6.68; N, 9.27.

**Boc-Asn-Cys(MBzl)-OBzl** Boc-Asn-ONp (9.11g, 25.9mmol) and H-Cys(MBzl)-OBzl·TosOH (13.0g, 25.9mmol) were dissolved in DMF (100ml) containing $Et_3N$ (7.3ml). 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% $NaHCO_3$, 5% 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 8.90 g (63.0%), mp 145—148 °C, $[\alpha]_D^{27}$ −33.0° ($c$=1.0, DMF), $Rf^1$ 0.79. *Anal.* Calcd for $C_{27}H_{35}N_3O_7S$: C, 59.4; H, 6.47; N, 7.70. Found: C, 59.7; H, 6.51; N, 7.83.

**Boc–Pro–Asn–Cys(MBzl)–OBzl** Boc–Pro–ONp (2.03 g, 6.00 mmol) and H–Asn–Cys(MBzl)–OBzl·TFA [prepared from Boc–Asn–Cys(MBzl)–OBzl (3.0 g, 5.5 mmol) and TFA (6 ml, 55 mmol) containing anisole (0.45 ml)] were dissolved in DMF (30 ml) containing $Et_3N$ (0.8 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% $NaHCO_3$, 5% 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 2.34 g (66.4%), mp 70—72 °C, $[\alpha]_D^{27}$ −49.6° ($c$=1.0, DMF), $Rf^1$ 0.58. *Anal.* Calcd for $C_{32}H_{42}N_4O_8S\cdot 0.5H_2O$: C, 59.4; N, 6.61; N, 8.66. Found: C, 59.2; H, 6.60; N, 8.71.

**Boc–Asp(O-2-Ada)–Pro–Asn–Cys(MBzl)–OBzl** Boc–Asp(O-2-Ada)–OSu (1.08 g, 2.3 mmol) and H–Pro–Asn–Cys(MBzl)–OBzl·TFA [prepared from Boc–Pro–Asn–Cys(MBzl)–OBzl (1.00 g, 1.56 mmol) and TFA (1.77 ml, 15.6 mmol) containing anisole (0.50 ml)] were dissolved in DMF (10 ml) containing $Et_3N$ (0.20 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% $NaHCO_3$, 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. Petroleum ether was added to the residue to afford a crude product. The crude product in $CHCl_3$ (3 ml) was applied to a silica gel column (2.0 × 30 cm), equilibrated and eluted with $CHCl_3$. After evaporation of the effluent (300—1100 ml), petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 1.15 g (82.7%), mp 70—75 °C, $[\alpha]_D^{27}$ −43.0° ($c$=0.8, DMF), $Rf^1$ 0.56. *Anal.* Calcd for $C_{46}H_{61}N_5O_{11}S\cdot H_2O$: C, 60.7; H, 6.89; N, 7.69. Found: C, 60.7; H, 6.90; N, 7.64.

**Ac–Met–Asp(O-2-Ada)–Pro–Asn–Cys(MBzl)–OBzl** Ac–Met–$N_3$ [prepared from Ac–Met–$NHNH_2$ (459 mg, 2.24 mmol), HCl/dioxane (0.89 ml) and isopentyl nitrite (0.30 ml) in the usual manner] in DMF (5 ml) cooled to −10 °C was combined with H–Asp(O-2-Ada)–Pro–Asn–Cys(MBzl)–OBzl·TFA [prepared from Boc–Asp(O-2-Ada)–Pro–Asn–Cys(MBzl)–OBzl (1.0 g, 1.12 mmol) and TFA (1.27 ml, 11.2 mmol) containing anisole (0.36 ml)] in DMF (10 ml) containing $Et_3N$ (1.4 ml). 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 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. The residue in $CHCl_3$ (3 ml) was applied to a silica gel column (1.5 × 20 cm), equilibrated and eluted with $CHCl_3$. After evaporation of the effluent (100—2100 ml), ether was added to the residue to give crystals, which were collected by filtration, yield 339 mg (56.0%), mp 80—85 °C, $[\alpha]_D^{27}$ −41.0° ($c$=1.0, DMF), $Rf^1$ 0.67, $Rf^2$ 0.56. Amino acid ratios in an acid hydrolysate: $Asp_{2.00(2)}$, $Met_{0.67(1)}$, $Pro_{1.09(1)}$ (average recovery 88%). Cys was not determined. *Anal.* Calcd for $C_{48}H_{64}N_6O_{11}S_2\cdot H_2O$: C, 58.6; H, 6.77; N, 8.55. Found: C, 58.7; H, 6.65; N, 8.53.

**Boc–Pro–Asn–OBzl** Boc–Pro–ONp (2.29 g, 6.8 mmol) and H–Asn–OBzl·TFA [prepared from Boc–Asn–OBzl[14] (2.00 g, 6.2 mmol) and TFA (7.0 ml, 62.0 mmol) containing anisole (2.0 ml)] were dissolved in DMF (20 ml) containing $Et_3N$ (1.00 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% $NaHCO_3$, 5% 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 1.55 g (59.4%), mp 140—145 °C, $[\alpha]_D^{27}$ −41.3° ($c$=1.0, DMF), $Rf^1$ 0.48, $Rf^2$ 0.72. *Anal.* Calcd for $C_{21}H_{29}N_3O_6$: C, 60.1; H, 6.99; N, 10.0. Found: C, 59.9; H, 6.92; N, 9.99.

**Boc–Asp(O-2-Ada)–Pro–Asn–OBzl** Boc–Asp(O-2-Ada)–OSu (1.33 g, 2.86 mmol) and H–Pro–Asn–OBzl·TFA [prepared from Boc–Pro–Asn–OBzl (1.00 g, 2.38 mmol) and TFA (2.6 ml, 23.8 mmol) containing anisole (0.8 ml)] were dissolved in DMF (10 ml) containing $Et_3N$ (0.40 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% $NaHCO_3$, 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. The residue in $CHCl_3$ was applied to a silica gel column (3.0 × 20 cm), equilibrated and eluted with $CHCl_3$. After evaporation of the effluent (100—730 ml), petroleum ether was added to the residue to give crystals, yield 1.08 g (68.2%), mp 80—88 °C, $[\alpha]_D^{27}$ −41.6° ($c$=1.0, DMF), $Rf^1$ 0.53, $Rf^2$ 0.77. *Anal.* Calcd for $C_{35}H_{48}N_4O_9\cdot 0.5H_2O$: C, 62.0; H, 7.42; N, 8.27. Found: C, 62.2; H, 7.29; N, 8.07.

**Ac–Met–Asp(O-2-Ada)–Pro–Asn–OBzl** Ac–Met–$N_3$ [prepared from Ac–Met–$NHNH_2$ (174 mg, 0.85 mmol), 7.5 N HCl/dioxane (0.22 ml) and isopentyl nitrite (0.11 ml) in the usual manner) in DMF (1 ml) cooled to −10 °C was combined with H–Asp(O-2-Ada)–Pro–Asn–OBzl·TFA [prepared from Boc–Asp(O-2-Ada)–Pro–Asn–OBzl (500 mg, 0.71 mmol) and TFA (0.80 ml, 7.1 mmol) containing anisole (0.23 ml)] in DMF (2 ml) containing $Et_3N$ (0.8 ml). 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 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. Ether was added to the residue to give crystals, which were collected by filtration, yield 305 mg (58.1%), mp 84—89 °C, $[\alpha]_D^{27}$ −27.7° ($c$=0.7, DMF), $Rf^1$ 0.61, $Rf^2$ 0.86. Amino acid ratios in an acid hydrolysate: $Asp_{2.00(2)}$, $Met_{0.72(1)}$, $Pro_{1.07(1)}$ (average recovery 85%). Cys was not determined. *Anal.* Calcd for $C_{37}N_{51}N_5O_9S\cdot 0.5H_2O$: C, 59.2; H, 6.98; N, 9.32. Found: C, 59.4; H, 7.00; N, 9.04.

**Boc–Asp(O-2-Ada)–Pro–OBzl** Boc–Asp(O-2-Ada)–OSu (1.10 g, 2.36 mmol) and H–Pro–OBzl·HCl[15] (0.52 g, 2.15 mmol) were dissolved in DMF (10 ml) containing $Et_3N$ (0.40 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% $NaHCO_3$, 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. Petroleum ether was added to the residue to afford crystals, yield 350 mg (30%), mp 83—87 °C, $[\alpha]_D^{27}$ −35.5° ($c$=1.0, DMF), $Rf^1$ 0.80. *Anal.* Calcd for $C_{31}H_{42}N_2O_7$: C, 67.1; H, 7.63; N, 5.05. Found: C, 66.9; H, 7.65; N, 5.05.

**Ac–Met–Asp(O-2-Ada)–Pro–OBzl** Ac–Met–$N_3$ [prepared from Ac–Met–$NHNH_2$ (296 mg, 1.44 mmol), 5.0 N HCl/dioxane (0.60 ml) and isopentyl nitrite (0.20 ml) in the usual manner] in DMF (2 ml) cooled to −10 °C was combined with H–Asp(O-2-Ada)–Pro–OBzl·TFA [prepared from BOC–Asp(O-2-Ada)–Pro–OBzl (400 mg, 0.72 mmol) and TFA (0.82 ml, 7.20 mmol) containing anisole (0.24 ml)] in DMF (2 ml) containing $Et_3N$ (0.9 ml). 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 5% citric acid and water, dried over $Na_2SO_4$ and evaporated down. Ether was added to the residue to afford crystals, yield 347 mg (77.1%), mp 146—154 °C, $[\alpha]_D^{27}$ −51.4° ($c$=1.0, DMF), $Rf^1$ 0.55, $Rf^2$ 0.81. Amino acid ratios in an acid hydrolysate: $Asp_{1.00(1)}$, $Met_{0.72(1)}$, $Pro_{1.07(1)}$ (average recovery 85%). Cys was not determined. *Anal.* Calcd for $C_{33}H_{45}N_3O_7S\cdot 2H_2O$: C, 59.7; H, 7.44; N, 6.33. Found: C, 59.8; H, 7.07; N, 6.55.

**General Procedure for Deprotection by HF** The protected peptide (0.04 mmol) was treated with anhydrous HF (5 ml) containing *m*-cresol (0.2 ml) and thioanisole (0.24 ml) at 0 °C for 1 h. After removal of HF, the residue was dried over KOH pellets *in vacuo* overnight. The residue was dissolved in oxygen-free water. The solution was washed with AcOEt. The water layer was lyophilized to give a fluffy powder. The powder in 3% AcOH (1 ml) was applied to a column of Sephadex G-15 (2.2 × 135 cm), equilibrated and eluted with 3% AcOH. Individual fractions (3 g each) were collected. The desired fractions were combined and lyophilized to give a white fluffy powder. Yield, $[\alpha]_D$ value, amino acid ratios in an acid hydrolysate and $Rf$ values are summarized in Table I.

**RIA** The conditions of the polyethylene glycol method for competitive RIA have been described.[16] Briefly, 1.4 μg of the MT 189-14-7 antibody and 14000 cpm of $^{125}$I-labeled Cd, Zn–MT II (2.3 μCi/μg) were incubated in the presence of various concentrations of inhibitor in a total volume of 175 μl of 0.1% bovine serum albumin–10 mM phosphate-buffered saline (pH 7.2). After incubation for 20 min at 4 °C, 100 μl of 1.5% (w/v) bovine-γ-globulin as a carrier in the same buffer and 1 ml of 16% (w/v) polyethylene glycol 6000 (Wako, Tokyo) in 50 mM Tris–HCl buffer (pH 8.2) were added and the mixture was kept for 30 min at 4 °C. The precipitates were collected by centrifugation and the radioactivity was measured with a well-type γ-counter (model JDC-751, Aloka, Tokyo). The ratio of radioactivity of the bound $^{125}$I-labeled MT in the presence of inhibitor to that of the $^{125}$I-labeled MT in the absence of inhibitor ($B/B_0$) was plotted against the concentration of inhibitor added and the $IC_{50}$ value (the concentration of an inhibitor giving 50% inhibition) was determined.

## References and Notes

1) Part XXXII: Y. Okada and S. Tsuboi, *J. Chem. Soc., Perkin Trans. 1*, **1991**, 3321.
2) Amino acids, peptides and their derivatives mentioned in this paper were of the L-configuration. The abbreviations used are those

recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; MBzl, *p*-methoxybenzyl; OBzl, benzyl ester; O-2-Ada, 2-adamantyl ester; ONp, *p*-nitrophenyl ester; OSu, *N*-hydroxysuccinimide ester; Ac, acetyl; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; AcOH, acetic acid; DMF, dimethylformamide; *n*-BuOH, 1-butanol; Tos, *p*-toluenesulfonyl.

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11) The final products thus obtained gave a single peak on RP-HPLC under the following conditions: column, reversed-phase $C_{18}$ column [YMC-pack R-ODS-5 (4.6 × 250 mm)]; solvent, $H_2O$–$CH_3CN$ (90 : 10) containing 0.1% TFA; flow rate, 1 ml/min; absorbance, 220 nm. Retention times (min) of MT-derivatives were determined to be as follows: Ac–(MT 1—6)–OH, 6.0; Ac–(MT 1—5)–OH, 6.8; Ac–(MT 1—4)–OH, 8.0; Ac–(MT 1—3)–OH, 10.0.
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