Notes

[Chem. Pharm. Bull. 36(9)3614—3618(1988)]

Amino Acids and Peptides. XXI. Synthesis of N-Terminal Heptapeptide of Mammalian Metallothionein (MT) and Evaluation of Its Immunoreactivity[1,2]

YOSHIO OKADA,*,[a] SHIN IGUCHI,[a] SHIGERU NAKAYAMA,[a] YUTAKA KIKUCHI,[b] MASACHIKA IRIE,[b] JUN-ICHI SAWADA,[c] HIDEHARU IKEBUCHI[c] and TADAO TERAO[c]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Nishi-ku, Kobe 673, Japan, Department of Microbiology, Hoshi College of Pharmacy,[b] Ebara, Shinagawa-ku, Tokyo 142, Japan and Division of Radiochemistry, National Institute of Hygienic Sciences,[c] Kamiyoga, Setagaya-ku, Tokyo 158, Japan

(Received January 26, 1988)

The common N-terminal heptapeptide, Ac–Met–Asp–Pro–Asn–Cys–Ser–Cys–OH, Ac-(MT II 1—7)-OH, of mammalian metallothioneins (MTs) was synthesised by a conventional solution method using newly developed β-2-adamantylaspartate. This peptide was as reactive as native MT with a monoclonal antibody produced against rat Zn-MT II.

Keywords——N-terminal heptapeptide; common sequence; mammalian metallothionein; chemical synthesis; β-2-adamantylaspartate; monoclonal antibody; rat zinc-metallothionein II; crossreactivity

Metallothioneins (MTs) are a class of low-molecular-weight, cysteine-rich metal-binding proteins. Due to their metal (Cd, Zn, Cu, Hg, *etc.*) binding ability, they act as heavy metal (Cd, Hg) detoxifying agents and participate in heavy metal (Zn, Cu) metabolism, maintaining homeostasis, although their precise roles remain enigmatic. Immunological methods are very important for determining low levels of MT[3] and for subcellular localization studies by immunofluorescence.[4] This paper deals with the synthesis of the common N-terminal heptapeptide sequence of mammalian MTs, using the newly developed 2-adamantyl ester protecting group for the β-carboxyl function of aspartic acid,[5] and the evaluation of its immunological properties by the use of monoclonal antibody against rat Zn-MT II.[6]

Quite recently, Kikuchi *et al.*[6] found that a monoclonal antibody against rat Zn-MT II (MT-189-14-7 antibody) well recognized the synthetic N-terminal β-domain of human MT II, abbreviated as Ac–(hMT II 1—29)–OH,[7,8] but did not bind with C-terminal α-domain peptides, H–(hMT II 30—61)–OH[9] and H–(hMT II 29—35)–OH.[10] It was also found [6] that H–(hMT II 4—8)–OH, H–(hMT II 13—19)–OH and H–(hMT 20—28)–OH,[9] which are partial structures in the β-domain of human MT II, could not be recognized by the antibody (MT-189-14-7). Thus, it was expected that an N-terminal oligopeptide sequence containing Ac–Met–Asp–Pro might be an antigenic determinant. In order to clarify this point, we prepared the peptide, Ac–Met–Asp–Pro–Asn–Cys–Ser–Cys–OH (I), which is a common sequence of mammalian MTs, according to the scheme shown in Fig. 1. Boc–Cys(MBzl)–Ser–$NHNH_2$[9] was coupled with H–Cys(MBzl)–OBzl to give Boc–Cys(MBzl)–Ser–Cys(MBzl)–OBzl, which was treated with TFA to give the corresponding amine. Starting with this amine, Boc–Asn–ONp,[11] Boc–Pro–ONp[12] and Boc–Asp(O–2-Ada)–OSu[5] were coupled suc-

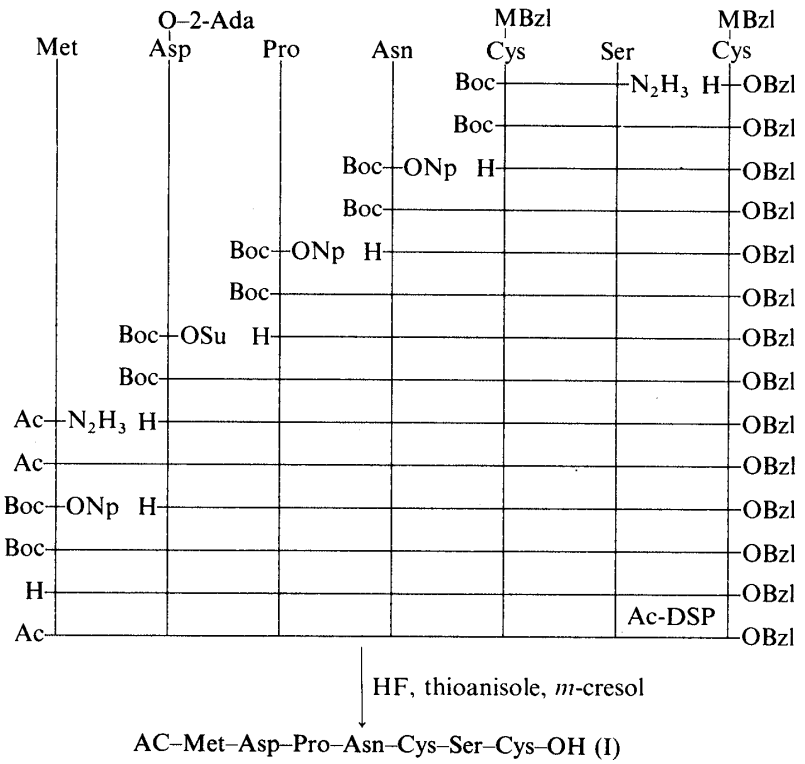

Fig. 1. Synthetic Scheme for the N-Terminal Heptapeptide Ac–(MT II 1—7)–OH of Mammalian MTs

cessively to afford Boc–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl in good yield. Introduction of Boc–Asp(O–2-Ada)–OH into the peptide increased the solubility of the peptide in organic solvent. After removal of the Boc group of the hexapeptide, the resultant amine was coupled with Ac–Met–$NHNH_2$[7] or coupled with Boc–Met–ONp,[13] followed by acetylation with Ac-DSP[14] to give Ac–Met–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl. All protecting groups were removed by employing anhydrous HF[15] in the presence of thioanisole and *m*-cresole. After reduction with dithiothreitol, the acetylated peptide was purified by gel-filtration on Sephadex G 25 using 3% AcOH as a solvent in order to suppress disulfide formation.[16] The purified peptide, [Ac–(MT II 1—7)–OH, I] was homogeneous upon thin layer chromatography (TLC) on silica gel and the amino acid ratios in an acid hydrolysate were in good agreement with the theoretically expected values.

The crossreactivity of the peptide (I) with a monoclonal antibody, MT 189-14-7 was determined by two kinds of competitive radioimmunoassay (RIA) methods, the conventional double antibody method and the polyethylene glycol method. The peptide (I) exhibited similar reactivity (IC_{50} value, 7.5×10^{-7} M) to that of native rat Cd, Zn-MT II (IC_{50} value, 1.1×10^{-6} M) in the former RIA method and also in the latter RIA method (IC_{50} values, 1.4×10^{-7} M and 3.5×10^{-7} M, respectively). The reason for the discrepancy of IC_{50} values between the conventional double antibody method (incubation time, 40 h) and the polyethylene glycol method (incubation time, 50 min) remains to be solved. However, it seems clear that the intra- or intermolecular disulfide bond formation of the peptide does not affect the crossreactivity with the antibody.

The displacement curve of peptide (I) in comparison with that of native rat Cd, Zn-MT II is shown in Fig. 2, from which it can be seen that both curves are parallel.

Although Kikuchi *et al.*,[6] pointed out that the antigenicity of metal-protein would not be associated with the metal-dependent structures since the apo-form of the β-domain of human

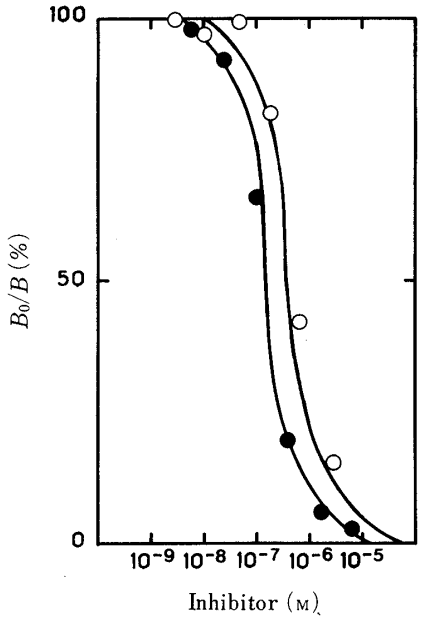

Fig. 2. Competitive RIA for Ac–(hMT II 1—7)–OH (I) and Native Rat Cd, Zn-MT II

Inhibitors used were Ac–(hMT II 1—7)–OH (I) (●) and rat Cd, Zn-MT II (○). The MT 189-14-7 antibody (1.4 μg) was incubated with various concentrations of inhibitors and ^{125}I-rat Cd, Zn-MT II and the bound ^{125}I-rat Cd, Zn-MT II was precipitated as described in the experimental section (polyethylene glycol method). The ratio of the radioactivity of the bound ^{125}I-labeled MT in the presence of inhibitors to that in the absence of inhibitors (B/B_0) was plotted against the concentration of inhibitor added.

MT II exhibited the reactivity with the antibody with a similar IC_{50} value to that of native rat Cd, Zn-MT II, it is still very important to compare the reactivities of metal-free Ac–(hMT II 1—7)–OH (I) and metal-bound I with the antibody. Examination of the metal-binding properties of I and the isolation of Cd-bound I are under way in our laboratory and the results will be reported in the future.

In conclusion, since the N-terminal heptapeptide (I) is common to mammalian MTs, the MT 189-14-7 antibody could react with various kinds of mammalian MTs and might be a useful tool for further studies on MTs.

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 (110 °C, 18 h, 6 N HCl) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). 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 water (8 : 3 : 1, lower phase) and *n*-BuOH, AcOH and water (4 : 1 : 5, upper phase), respectively.

Boc–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——Boc–Cys(MBzl)–Ser–N_3 [prepared from 1.56 g (0.35 mmol) of Boc–Cys(MBzl)–Ser–$NHNH_2$, 0.92 ml (0.71 mmol) of 7.6 N HCl/dioxane and 0.49 ml (0.35 mmol) of isopentyl nitrite in the usual manner] in DMF (10 ml) cooled to −10 °C was combined with H–Cys(MBzl)–OBzl [prepared from 2.5 g (0.50 mmol) of H–Cys(MBzl)–OBzl·Tos–OH and 0.7 ml (0.50 mmol) of Et_3N] in DMF (10 ml). The reaction mixture was stirred at 4 °C overnight and the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_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 material (2.22 g, 84.9%). This crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (2 × 45 cm), equilibrated and eluted with $CHCl_3$. After removal of the solvent of the effluent (1000—1500 ml), petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 1.34 g (51.1%), mp 90 °C with sintering at 70 °C, $[\alpha]_D^{25}$ −26.7° (*c* = 1.0, DMF), Rf^1 0.76, Rf^2 0.92. *Anal.* Calcd for $C_{37}H_{47}N_3O_9S_2$: C, 59.9; H, 6.39; N, 5.66. Found: C, 59.8; H, 6.55; N, 5.76.

Boc–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——Boc–Asn–ONp (477 mg, 1.35 mmol) and H–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 1.0 g (1.35 mmol) of Boc–Cys(MBzl)–Ser–Cys(MBzl)–OBzl and 1.5 ml (19.7 mmol) of TFA containing 0.1 ml of anisole] were dissolved in DMF (10 ml) containing Et_3N (0.189 ml, 0.135 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford a gelatinous material, which was collected by filtration and washed with AcOEt, yield 700 mg (60.6%), mp 168—170 °C, $[\alpha]_D^{25}$ −26.7° (*c* = 1.0, DMF), Rf^1 0.52. *Anal.* Calcd for $C_{41}H_{53}N_5O_{11}S \cdot H_2O$: C, 56.3; H, 6.34; N, 8.01. Found: C, 56.3; H, 6.26; N, 8.01.

Boc–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——Boc–Pro–ONp (202 mg, 0.60 mmol) and H–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 450 mg (0.53 mmol) of Boc–Asn–Cys(MBzl)–Ser–

Cys(MBzl)–OBzl and 0.40 ml (5.3 mmol) of TFA containing 0.1 ml of anisole] were dissolved in DMF (10 ml) containing Et_3N (0.074 ml, 0.53 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to give a gelatinous material, which was collected by filtration and washed with AcOEt, yield 460 mg (91.2%), mp 161—164 °C, $[\alpha]_D^{25} -51.7°$ ($c=0.9$, DMF), Rf^1 0.55, Rf^3 0.78. *Anal.* Calcd for $C_{46}H_{60}N_6O_{12}S_2 \cdot H_2O$: C, 56.9; H, 6.43; N, 8.65. Found: C, 56.9; H, 6.38; N, 8.72.

Boc–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——Boc–Asp(O–2-Ada)–OSu[5] (158 mg, 0.34 mmol) and H–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 270 mg (0.28 mmol) of Boc–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl and 0.21 ml (2.84 mmol) of TFA containing 0.05 ml of anisole] were dissolved in DMF (10 ml) containing Et_3N (0.04 ml, 0.84 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford a gelatinous material, which was collected by filtration and washed with AcOEt and water, yield 250 mg (73.2%), mp 130—131 °C, $[\alpha]_D^{25} -47.4°$ ($c=0.8$, DMF), Rf^1 0.58. *Anal.* Calcd for $C_{60}H_{79}N_7O_{15}S_2 \cdot H_2O$: C, 59.0; H, 6.69; N, 8.03. Found: C, 58.7; H, 6.51; N, 8.28.

Boc–Met–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——H–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 295 mg, 0.246 mmol of Boc–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl and 1.5 ml (19.7 mmol) of TFA containing 0.3 ml of anisole in usual manner] and Boc–Met–ONp (109 mg, 0.295 mmol) were dissolved in DMF (2 ml) containing Et_3N (0.069 ml, 0.49 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford a solid mass, which was collected by filtration and washed with ether: yield 185 mg (56.5%); mp 150 °C with sintering at 120 °C; $[\alpha]_D^{25} -52.6°$ ($c=0.4$, DMF), Rf^1 0.50, Rf^2 0.92. *Anal.* Calcd for $C_{65}H_{88}N_8O_{16}S_3$: C, 58.5; H, 6.65; N, 8.40. Found: C, 58.7; H, 6.56; N, 8.20. Amino acid analysis after acid hydrolysis gave the following composition, expressed in molar ratios: $Asp_{2.00(2)}Ser_{0.96(1)}Met_{0.92(1)}Pro_{0.90(1)}$ (average amino acid recovery 96%); Cys was not determined.

Ac–Met–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl——1) Ac–Met–N_3 [prepared from 61.5 mg (0.30 mmol) of Ac–Met–$NHNH_2$, 0.079 ml (0.6 mmol) of 7.6 N HCl/dioxane and 0.42 ml (0.30 mmol) of isopentyl nitrite] in DMF (3 ml) cooled to −10 °C was combined with a solution of H–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 121 mg (0.10 mmol) of Boc–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl and 0.50 ml (6.6 mmol) of TFA containing 0.1 ml of anisole] in DMF (5 ml) containing Et_3N (0.014 ml, 0.1 mmol). 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 water and concentrated to a small volume. Ether was added to the residue to give a gelatinous material, which was collected by filtration, yield 110 mg (86.2%), mp 151—153 °C with sintering at 130 °C, $[\alpha]_D^{25} -42.9°$ ($c=0.6$, DMF), Rf^1 0.27. *Anal.* Calcd for $C_{62}H_{79}N_8O_{15}S_3 \cdot 3H_2O$: C, 56.0; H, 6.67; N, 8.43. Found: C, 55.7; H, 6.37; N, 8.61. Amino acid analysis of an acid hydrolysate gave the following molar ratios: $Asp_{1.90(2)}Ser_{0.74(1)}Met_{0.83(1)}Pro_{1.00(1)}$ (average amino acid recovery 86%), Cys was not determined.

2) H–Met–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl·TFA [prepared from 90 mg (0.0675 mmol) of Boc–Met–Asp(O–2-Ada)–Pro–Asn–Cys(MBzl)–Ser–Cys(MBzl)–OBzl and 1.0 ml (13.2 mmol) of TFA containing 0.2 ml of anisole in the usual manner] and Ac-DSP[14] (31.2 mg, 0.101 mmol) were dissolved in DMF (2 ml) containing Et_3N (0.025 ml, 0.175 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt (2 ml), water (0.5 ml) and ether (2 ml) were added to the residue to give a solid mass, which was collected by filtration and washed with water and ether: yield 33 mg (38%); mp 150 °C with sintering at 140 °C; $[\alpha]_D^{25} -48.0°$ ($c=0.4$, DMF), Rf^1 0.27, Rf^2 0.66. Amino acid analysis of an acid hydrolysate gave the following molar ratios: $Asp_{2.00(2)}Ser_{0.94(1)}Met_{0.92(1)}Pro_{1.10(1)}$ (average amino acid recovery 82%); Cys was not determined.

Ac–Met–Asp–Pro–Asn–Cys–Ser–Cys–OH [Ac–(MT II 1—7)–OH]——Ac–Met–Asp(O–2-Ada)–Pro–Asn–Cys–(MBzl)–Ser–Cys(MBzl)–OBzl (50 mg, 0.04 mmol) was treated with anhydrous HF (5 ml) containing *m*-cresol (0.20 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. AcOEt was added to the residue to afford a precipitate, which was collected by filtration, washed with AcOEt and dried over KOH pellets *in vacuo*. This was dissolved in water (3 ml) and reduced with dithiothreitol (36 mg, 0.23 mmol) at room temperature overnight. The reaction mixture was applied to a column of Sephadex G-25 (2.2 × 135 cm), equilibrated and eluted with 3% AcOH. Individual fractions (3 g each) were collected. The desired fractions (tube Nos. 53—60) were combined and lyophilized to afford a white fluffy powder: yield 14 mg (43%), Rf^2 0.23, Rf^3 0.05. Amino acid analysis of an acid hydrolysate gave the following molar ratios: $Asp_{1.84(2)}Ser_{0.73(1)}Met_{0.68(1)}Pro_{1.12(1)}$ (average amino acid recovery 85%); Cys was not determined.

Radioimmunoassay (RIA)——1) The reactivity of a monoclonal anti-metallothionein antibody, MT 189-14-7[6] with native and synthetic peptides was determined by competitive RIA. The conditions of double-antibody RIA for MT have been described.[17] Briefly, 1.4 μg of the MT 189-14-7 antibody, normal mouse serum (25 μl) as a carrier, and 14000 cpm of ^{125}I-labeled Cd, Zn-MT II (4.9 μCi/ug) that had been radiolabeled with the Bolton–Hunter reagent (NEN, NEX-120) were incubated in the presence of various concentrations of inhibitor in a total volume of 175 μl in 0.1% gelatin (Difco, 0143)–10 mM phosphate-buffered saline (pH 7.2). After incubation for 16 h at 4 °C, 100 μl of a second antibody (rabbit anti-mouse IgM serum) in the same buffer was added, and the mixture was kept for 24 h at

room temperature. The precipitates were collected by centrifugation, and the radioactivity of the precipitates was measured with a well-type γ-counter (model JDC-751, Aloka, Tokyo). The ratio of the radioactivity of the bound ^{125}I-labeled MT in the presence of inhibitors to that of the ^{125}I-labeled MT in the absence of inhibitors (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.

2) Polyethylene Glycol Method: The conditions of the polyethylene glycol method for competitive RIA have been described.[18] Briefly, 1.4 μg of the MT 189-14-7 antibody and 14000 cpm of ^{125}I-labeled Cd, Zn-MT II (2.3 uCi/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 of the precipitates was measured in the same manner as described above.

Acknowledgement This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan (61303005).

References and Notes

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