Synthesis of a β-Tetrapeptide Analog as a Mother Compound for the Development of Matrix Metalloproteinase-2-Imaging Agents

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Matrix metalloproteinase-2 (MMP-2) is an attractive target for the diagnosis of cancer and atherosclerosis in nuclear imaging. A cyclic decapeptide, cCTTHWGFTLC (cCTT), has been used as the mother compound for the development of MMP-2-imaging agents with high potency and selectivity. Most of radiolabeled derivatives of cCTT currently developed for *in vivo* studies of MMP-2, however, suffer from low accumulation in the target tissues, such as tumors. For enhanced *in vivo* stability and tissue penetration, we designed a linear β-tetrapeptide analog, H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (1), to mimic cCTT. The component β-amino acids were prepared by reduction of N-protected α-amino acid methyl esters to the alcohols, followed by conversion into the cyanides, and subsequent hydrolysis. Compound 1 was obtained from these β-amino acids by the conventional solution method. In MMP-2 inhibition assay, compound 1 displayed desirably significant inhibition, which was comparable to cCTT. These findings suggest that compound 1 may serve as a mother compound in the design and development of *in vivo* MMP-2-imaging agents.

Key words matrix metalloproteinase-2; β-tetrapeptide; inhibitor; radiopharmaceutical

Matrix metalloproteinases (MMPs) comprise a family of enzymes that degrade the basement membrane and extracellular matrix, thus contributing to tissue remodeling and cell migration.[1,2] Numerous studies have demonstrated the various roles of MMPs in pathological processes associated with cancer and atherosclerosis, making MMPs potential targets for diagnosis using noninvasive imaging.[3,4] Among MMPs, MMP-2, which degrades a major component of the basement membrane, has been studied extensively and shown to be involved in tumor invasion and metastasis,[5,6] and atherosclerotic plaque rupture.[7,8] For *in vivo* imaging of MMP-2 activity, we and others used a cyclic decapeptide, cCTTHWGFTLC (cCTT), as the mother compound because of the clinical and radiopharmaceutical utility of peptide radiopharmaceuticals.[9—11] The cyclic decapeptide cCTT was screened as a selective gelatinase (especially MMP-2) inhibitor using phage display technology. Koivunen *et al.* showed that cyclic decapeptides containing the amino acid sequence His-Trp-Gly-Phe (HWGF) exhibited selective inhibition toward MMP-2 and MMP-9 activities while a liner peptide containing the HWGF sequence had little inhibitory activity.[12] Kuhnast *et al.* first reported ^{125}I-labeled derivative of cCTT as a potential agent for *in vivo* imaging.[9] Sprague *et al.* recently developed ^{64}Cu-labeled cCTT as a positron emission tomography (PET) imaging agent.[10] In tumor-bearing mice, however, both radiolabeled cCTTs exhibited low accumulation in MMP-2-positive tumors. They considered the poor stabilities of these peptides *in vivo* as one of the causes of low tumor accumulations. Our previously designed compound, ^{111}In-labeled diethylenetriaminepentaacetic acid (DTPA)-conjugated cCTT, showed a good correlation between its tumor accumulation and MMP-2 activity in the tumor, but the accumulations in MMP-2-expressing tumors were relatively low.[11]

Oligomers of β-amino acids, β-peptides, have emerged as a promissing class of peptidomimetics.[13—15] It is recognized that one of their important characteristics is to form stable secondary structures with as few as four β-amino acid residues. Furthermore, they are entirely stable against proteolytic degradation *in vivo*. Gademann *et al.* synthesized a linear β-tetrapeptide analog, Ac-β^3-HThr-β^2-HLys-β^3-HTrp-β^3-HPhe-NH_2, to mimic a somatostatin (cyclic 14-mer) in its binding to the human somatostatin receptors, which is known to rest upon a turn containing the amino acid sequence Phe-Trp-Lys-Thr.[16] The turn of the linear β-tetrapeptide analog which has the retro-sequence, is comparable to that of the cyclic α-peptide both in size and orientation of the side chains, although the amide bonds are reversed. These findings led to the design of a linear β-tetrapeptide analog, H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (**1**), as a downsized derivative to mimic cCTT. In the present study, β-tetrapeptide (**1**) was prepared by solution-phase synthesis techniques and its inhibitory effect on MMP-2 activity was compared with that of cCTT. To investigate the validity of the drug design, the linear α-tetrapeptides, H-His-Trp-Gly-Phe-OH (**2**) and H-Phe-Gly-Trp-His-OH (**3**), were also prepared for comparison (Fig. 1).

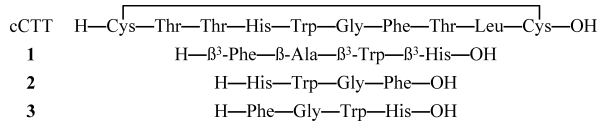

Fig. 1. Structures of cCTT Analogs
A disulfide linkage is shown by a solid line.

Results and Discussion

In general, the preparation of β-amino acids can be achieved by two different procedures. One procedure involves homologation of the appropriate α-amino acid by using a Wolff rearrangement of the diazo ketone (Arndt–Eistert approach).[17,18] The other procedure is by reduction to an N(α)-protected amino alcohol, followed by conversion to the corresponding cyanide, then giving the desired carboxylic acid after hydrolysis.[19,20] In this study, the latter procedure

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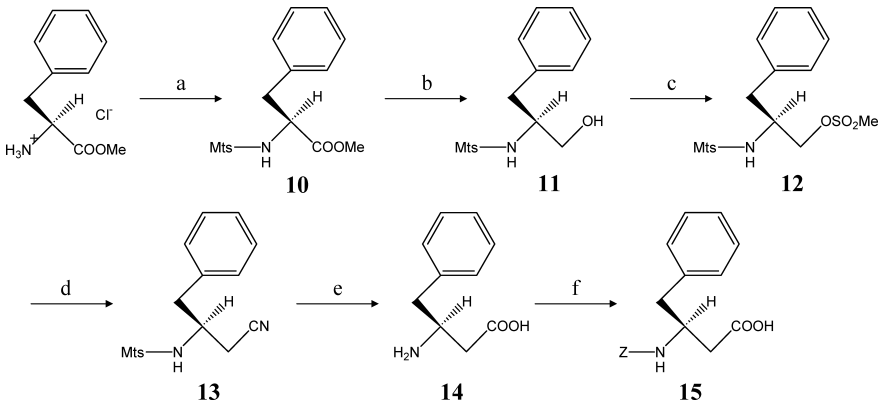
was adopted because the former suffers from handling difficulties—for example, α-diazomethyl ketones are hazardous and there are skin irritant intermediates.[21)]

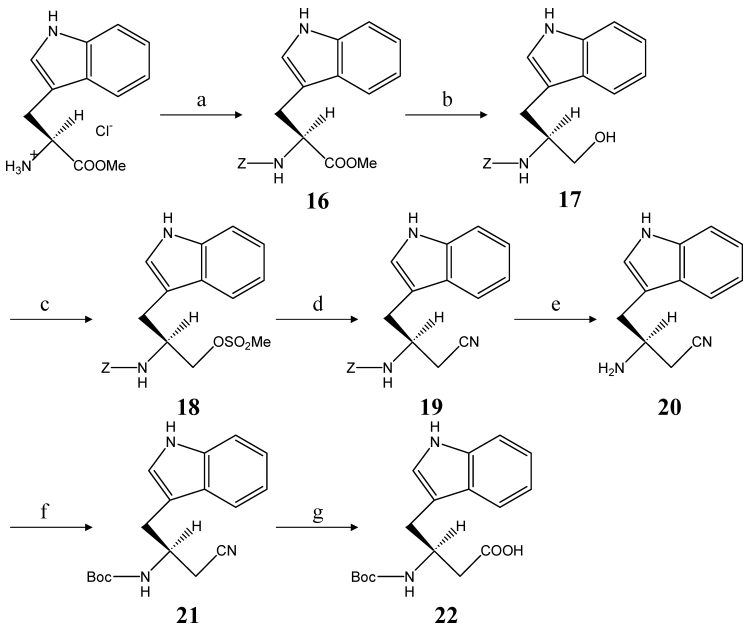
Initially, three protected β-amino acids were prepared for the required peptide synthesis. H-β^3-His-OH (**8**), which was prepared from L-histidine methyl ester according to the procedure described previously,[22)] was protected as benzyl ester **9** by heating with benzyl alcohol and *p*-toluenesulfonic acid in toluene (31%). Similarly, for the preparation of H-β^3-Phe-OH (**14**), the synthesis was carried out from L-phenylalanine methyl ester, as outlined in Chart 1. The amino group of L-phenylalanine methyl ester was protected as its sulfonamide (**10**) by treatment with 2,4,6-trimethylbenzenesulfonyl chloride (MtsCl) (86%). Reduction of the methyl ester **10** to alcohol **11** was achieved using $NaBH_4$ in methanol in 91% yield. Compound **11** was then activated as the mesylate analog **12** (78%), followed by treatment with NaCN in *N*,*N*-dimethylformamide (DMF) to give the desired nitrile **13** (96%). Treatment of **13** with 48% aqueous HBr in phenol resulted in the hydrolysis of the nitrile group with concomitant cleavage of the Mts group to the β-amino acid **14** (61%). The amino group of **14** was again protected as its benzyl carbamate to give the desired protected β-amino acid **15** (61%).

For the preparation of β-amino acid **22**, the amino group of L-tryptophan methyl ester was protected with a Z group to give **16** quantitatively. The corresponding nitrile **19** was accessible by a similar reaction sequence described for the phenylalanine analog (Chart 2). Alkali treatment of **19** with sodium hydroxide, unexpectedly, resulted in the deprotection of Z group as well as the hydrolysis of the nitrile group, to give H-β^3-Trp-OH, although the yield was extremely low and its isolation from the reaction mixture was awkward. Though the detailed mechanism of release of the Z group of compound **19** by alkaline hydrolysis remains unclear, it might be affected by the electron-withdrawing ability of the nitrile group. Thus, the Z group of **19** was replaced with a Boc group, *via* the free amine intermediate **20**, to afford **21** (55%). Hydrolysis of **21** with potassium hydroxide gave Boc-



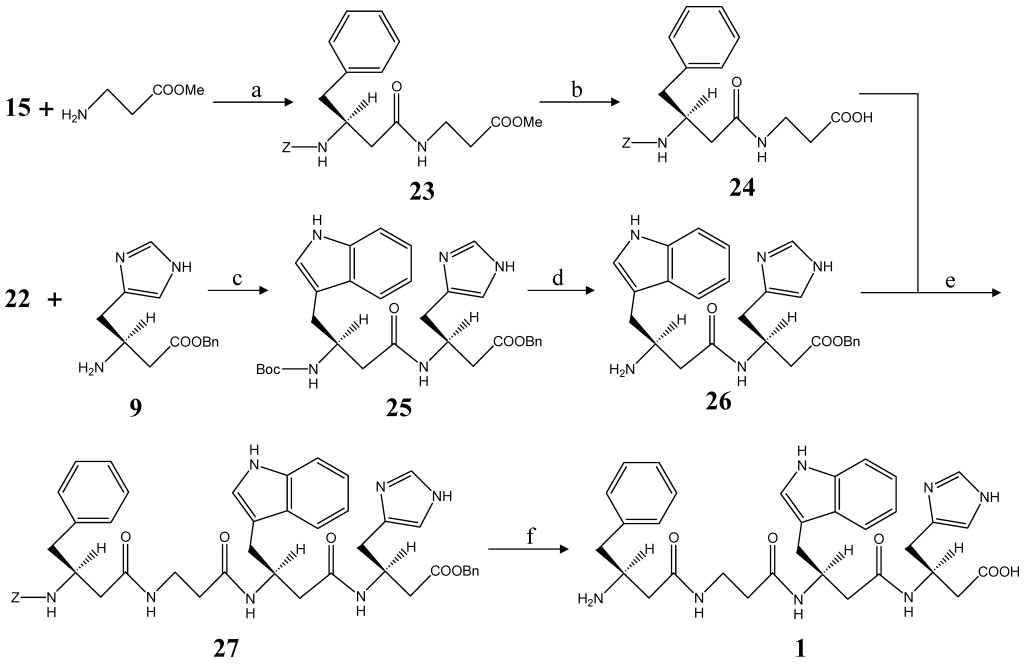
Reagents and conditions: (a) MtsCl, THF/H_2O, Na_2CO_3 (85.6%); (b) $NaBH_4$, MeOH (90.8%); (c) $MeSO_2Cl$, Et_3N, CH_2Cl_2 (78.2%); (d) NaCN, DMF (96.1%); (e) HBr, H_2O, phenol (61.4%); (f) *N*-(benzyloxycabonyloxy)succinimide, Et_3N, dioxane/H_2O (60.9%).

Chart 1



Reagents and conditions: (a) *N*-(Benzyloxycabonyloxy)succinimide, Et_3N, dioxane/H_2O (99.6%); (b) $NaBH_4$, MeOH (85.8%); (c) $MeSO_2Cl$, Et_3N, CH_2Cl_2 (84.4%); (d) NaCN, DMF (53.2%); (e) MeOH, Pd/C, H_2; (f) $(Boc)_2O$, $NaHCO_3$, THF/H_2O (55.4%); (g) EtOH/H_2O, KOH (29.3%).

Chart 2



Reagents and conditions: (a) EDC, HOBt, Et_3N, DMF/CH_2Cl_2 (55.1%); (b) 1 M NaOH, MeOH; (c) EDC, HOBt, Et_3N, DMF/CH_2Cl_2 (57.6%); (d) TFA; (e) EDC, HOBt, Et_3N, DMF/CH_2Cl_2 (32.3%); (f) Pd/C, H_2, DMF (62.3%).

Chart 3

β^3-Trp-OH (**22**) in 29% yield.

The required peptides were prepared by the conventional solution method (Chart 3). The acid **15** was coupled with β-alanine methyl ester by using 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) in dichloromethane to afford the dipeptide **23** in 55% yield. The coupling of the acid **22** with the amine **9** was performed in a similar manner to give the dipeptide **25** in 58% yield. After removal of the appropriate protecting groups, the tetrapeptide **27** was obtained in 32% yield by coupling of the acid **24** and the amine **26**. Treatment of **27** with hydrogen gas in the presence of catalyst and subsequent HPLC purification afforded the required **1** in 62% yield. The ^{1}H-NMR and MS spectra were consistent with the assigned structures.

To elucidate the validity of the design of the β-tetrapeptide, the inhibitory activity of compound **1** toward MMP-2 was examined. As shown in Fig. 2, compound **1** inhibited the gelatinolytic activity of MMP-2 in a concentration-dependent fashion. The IC_{50} value obtained from the concentration–response curve was 225 μM, which was comparable to that of cCTT (212 μM). On the other hand, linear α-tetrapeptides, H-His-Trp-Gly-Phe-OH (**2**) and H-Phe-Gly-Trp-His-OH (**3**), exhibited no significant inhibition of the gelatinolytic activity of MMP-2 (>1 mM). These results indicate that the linear β-tetrapeptide, H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (**1**), has a similar affinity for MMP-2 to the cyclic decapeptide, cCTT. Not only the generally known high *in vivo* stability of β-peptides but also downsizing from decapeptide to tetrapepide would be advantageous for enhanced accumulation in the target tissues. Therefore, compound **1** may be superior to cCTT itself as a mother compound in the design and development of optimal MMP-2-imaging agents.

Recently, the concept of multivalent peptides has been considered as a promising approach to obtain peptide-based

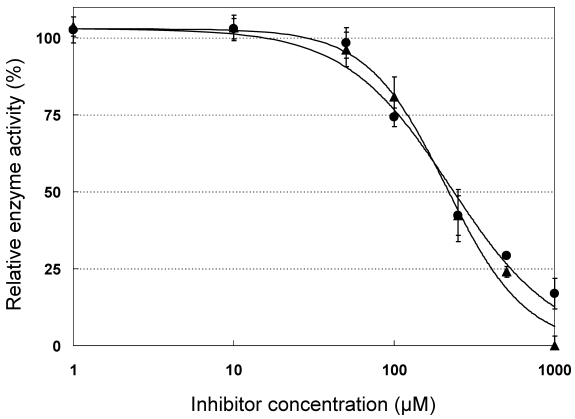


Fig. 2. Inhibition of the Gelatinolytic Activity of MMP-2 by H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (**1**) (Circles) and cCTT (Triangles)

Each value represents the mean±S.D. of three experiments.

radiopharmaceuticals.[23,24] For example, Arg-Gly-Asp sequence-containing peptides, RGD peptides, were synthesized in the form of multivalent ligand systems and were labeled with several radionuclides such as ^{18}F, ^{64}Cu and ^{99m}Tc. The dimeric and tetrameric RGD derivatives showed a higher binding affinity toward the corresponding receptor than the monomeric peptides. Moreover, the tetrameric peptide exhibited high tumor uptake and prolonged tumor retention *in vivo*.[25] Although a weak interaction between MMP-2 and cCTT, as indicated by its IC_{50} value, was suggested as a cause of low tumor accumulation of cCTT,[10] the multivalent approach using downsized analogs of cCTT may hold potential promise to enhance the affinity of cCTT derivatives for MMP-2. Thus, the downsized analog **1**, which has a significant affinity for MMP-2, would be a useful tool for the development of MMP-2-imaging agents.

In summary, the present study describes the synthesis of a linear β-tetrapeptide analog of cCTT which is a cyclic decapeptide inhibitor of MMP-2. The β-peptide, H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (**1**), displayed desirably significant inhibition of the gelatinolytic activity of MMP-2, which was comparable to cCTT. Although additional studies are needed, the present findings provide useful information about the chemical design of peptide-based radiopharmaceuticals. Studies using radiolabeled analogs are proceeding to assess the suitability for *in vivo* imaging of MMP-2.

Experimental

Chemical reagents and solvents were of commercial quality and were used without further purification unless otherwise noted. cCTT was prepared according to the procedure described previously.[11] All melting points were determined on a Yanaco melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO DIP-370 digital polarimeter. ^{1}H-NMR spectra were obtained on a Varian Unity 400 (400 MHz), and the chemical shifts are reported in parts per million downfield from tetramethylsilane. Infrared (IR) spectra were recorded with a Shimadzu FTIR-8400 spectrometer. Mass spectra were obtained with a JEOL JMS DX-610 (FAB-MS), or an Applied Biosystems Mariner System 5299 spectrometer (ESI-MS). Column chromatography was performed on Silica gel 60N (63—210 μm, Kanto Chemical), and the progress of the reaction was monitored by TLC on Silica gel 60F 254 plates (Merck). In the synthetic procedures, organic extracts were routinely dried over anhydrous Na_2SO_4 and evaporated with a rotary evaporator under reduced pressure. All reactions involving air- or moisture-sensitive compounds were performed under an argon atmosphere. HPLC was done using a Hitachi L-7000 system fitted with an Inertsil WP300C_{18} column (20×250 mm, GL Sciences) with monitoring of UV absorption at 220 nm.

Synthesis. H-β^3-His-OBn (9) H-β^3-His-OH (**8**) was prepared in a five-step process from L-histidine methyl ester according to the procedure described in previous literature.[22] To a solution of **8** (100 mg, 0.59 mmol) in dichloromethane (1 ml) was added benzyl alcohol (0.45 ml, 4.43 mmol) and *p*-toluenesulfonic acid (202 mg, 1.06 mmol). The reaction mixture was refluxed for 8 h with a Dean–Stark trap to remove water. After cooling to room temperature, the solvent was evaporated. The residue was purified by silica gel chromatography (chloroform : methanol : aqueous ammonia = 5 : 1 : 0.1) to afford **9** (46.8 mg, 30.6%) as a black oil. ^{1}H-NMR (CD_3OD) δ (ppm): 2.36—2.68 (4H, m), 3.42—3.51 (1H, m), 5.12 (2H, s), 6.85 (1H, s), 7.29—7.35 (5H, m), 7.60 (1H, s); IR (KBr) cm^{-1}: 3400—2600, 1728; ESI-MS (*m*/*z*): 260.1 $(M+H)^+$; $[\alpha]_D^{27}$ −17.1° (*c* = 1.87, $CHCl_3$).

***N*-(2,4,6-Trimethylbenzenesulfonyl)-L-phenylalanine Methyl Ester (10)** To a solution of L-phenylalanine methyl ester (4.0 g, 18.5 mmol) in THF/H_2O = 1 : 1 (186 ml) was added 2,4,6-trimethylbenzenesulfonyl chloride (5.3 g, 23.9 mmol) and sodium carbonate (4.92 g, 46.3 mmol). The mixture was stirred at room temperature for 5.5 h, and extracted with ethyl acetate. After the organic layer was dried and concentrated *in vacuo*, the residue was recrystallized from methanol to obtain **10** (5.73 g, 85.6%) as a white solid, mp 87—89 °C. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.26 (3H, s), 2.53 (6H, s), 2.94—3.04 (2H, 2), 3.50 (3H, s), 4.06—4.12 (1H, m), 5.09 (2H, d, *J* = 8.8 Hz), 6.87 (2H, s), 7.00—7.02 (2H, m), 7.18—7.20 (3H, m); IR (KBr) cm^{-1}: 3296, 2950, 1743, 1337, 1157; FAB-MS (*m*/*z*): 362.1 $(M+H)^+$.

***N*-(2,4,6-Trimethylbenzenesulfonyl)-L-phenylalaninol (11)** Under argon, a solution of **10** (400 mg, 1.11 mmol) in dry methanol (10.7 ml) was cooled to 0 °C and sodium borohydride (950 mg, 0.025 mol) was added to the mixture in small portions during 15 min in an ice bath. The mixture was kept at room temperature for 30 min with stirring, was then diluted by the addition of water in an ice bath, and extracted with chloroform. The organic layer was dried, concentrated *in vacuo*, and purified by silica gel chromatography (chloroform : methanol = 20 : 1) to afford **11** (334.6 mg, 90.8%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.14 (1H, s), 2.29 (3H, s), 2.52 (6H, s), 2.71—2.83 (2H, m), 3.42—3.43 (1H, m), 3.50—3.66 (2H, m), 4.76 (1H, d, *J* = 1.6 Hz), 6.89 (2H, s), 6.98—6.99 (2H, m), 7.18—7.20 (3H, m); IR (neat) cm^{-1}: 3340, 1319, 1151; FAB-MS (*m*/*z*): 334.4 $(M+H)^+$.

***N*-[(1*S*)-1-Methanesulfonylmethyl-2-phenylethyl]-2,4,6-trimethyl-benzenesulfonamide (12)** Under argon, a solution of **11** (334.6 mg, 1.0 mmol) in dry dichloromethane (1.5 ml) was cooled to 0 °C and treated with triethylamine (0.33 ml, 2.35 mmol) followed by methanesulfonyl chloride (0.15 ml, 1.94 mmol). The mixture was kept at room temperature for 2 h with stirring, then diluted by the addition of water, and extracted with chloroform. The organic layer was dried, concentrated *in vacuo*, and purified by silica gel chromatography (chloroform : ethyl acetate = 20 : 1) to afford **12** (322.7 mg, 78.2%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.30 (3H, s), 2.50 (6H, s), 2.75—2.92 (2H, m), 3.01 (3H, s), 3.61—3.67 (1H, m), 4.18—4.23 (2H, m), 4.75 (1H, d, *J* = 8.4 Hz), 6.89 (2H, s), 6.97—6.99 (2H, m), 7.19—7.21 (3H, m); IR (neat) cm^{-1}: 2939, 1334, 1155; FAB-MS (*m*/*z*): 412.3 $(M+H)^+$; $[\alpha]_D^{27}$ −38.9° (*c* = 0.91, CH_2Cl_2).

***N*-[(1*S*)-1-Cyanomethyl-2-phenylethyl]-2,4,6-trimethylbenzenesulfonamide (13)** Under argon, to a solution of **12** (322.7 mg, 0.78 mmol) in dry DMF (4.8 ml) was added sodium cyanide (95.1 mg, 1.97 mmol). The mixture was stirred at room temperature for 24 h, then diluted with ethyl acetate and washed with brine. The organic layer was dried, and evaporated to leave a residue which was purified by silica gel chromatography (chloroform : ethyl acetate = 25 : 1) to afford **13** (258.1 mg, 96.1%) as a yellow oil. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.31 (3H, s), 2.49 (6H, s), 2.53—2.64 (2H, m), 2.81—2.97 (2H, m), 3.62—3.66 (1H, m), 4.69 (1H, d, *J* = 7.2 Hz), 6.90 (2H, s), 7.00—7.02 (2H, m), 7.21—7.24 (3H, m); IR (neat) cm^{-1}: 1326, 1155; ESI-MS (*m*/*z*): 343.2 $(M+H)^+$; $[\alpha]_D^{27}$ −37.9° (*c* = 0.99, CH_2Cl_2).

H-β^3-Phe-OH (14) A mixture of compound **13** (3.3 g, 9.6 mmol), 48% aqueous HBr (25 ml) and phenol (9.0 g, 96 mmol) were heated at 110 °C for 121 h. The mixture was then diluted with distilled water and washed with chloroform. After the aqueous layer was concentrated *in vacuo*, the residue was recrystallized from distilled water to obtain **14** (1.06 g, 61.4%) as a white solid, mp 173—176 °C. ^{1}H-NMR (CD_3OD) δ (ppm): 2.34—2.53 (2H, m), 2.87—3.00 (2H, m), 3.64 (1H, br s), 7.25—7.30 (3H, m), 7.33—7.37 (2H, m); IR (KBr) cm^{-1}: 3300—2900, 1652; FAB-MS (*m*/*z*): 180.1 $(M+H)^+$; $[\alpha]_D^{27}$ −176.2° (*c* = 0.99, CH_2Cl_2).

Z-β^3-Phe-OH (15) To a solution of **14** (100 mg, 0.56 mmol) in dioxane/H_2O = 1 : 1 (2 ml) was added triethylamine (94 μl, 0.67 mmol) and a solution of *N*-(benzyloxycarbonyloxy)succinimide (168 mg, 0.67 mmol) in dioxane (1 ml). The mixture was kept at room temperature for 1 h and concentrated *in vacuo*. The residue was partitioned between ethyl acetate and water. The organic layer was dried, concentrated *in vacuo*, and purified by silica gel chromatography (chloroform : methanol = 20 : 1) to afford **15** (106.9 mg, 60.9%) as a white solid, mp 100—103 °C. ^{1}H-NMR (CD_3OD) δ (ppm): 2.42—2.52 (2H, m), 2.76—2.87 (2H, m), 4.16—4.19 (1H, m), 4.95—5.04 (2H, m), 7.18—7.36 (10H, m); IR (KBr) cm^{-1}: 3400—2900, 1697, 1662; FAB-MS (*m*/*z*): 314.2 $(M+H)^+$; $[\alpha]_D^{27}$ −16.4° (*c* = 1.07, $CHCl_3$).

***N*-(Benzyloxycarbony)-L-tryptophan Methyl Ester (16)** Compound **16** was obtained as a colorless oil in 99.6% yield from L-tryptophan methyl ester according to the procedure described for **15**. ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.32 (2H, d, *J* = 5.2 Hz), 3.68 (3H, s), 4.70—4.74 (1H, m), 5.06—5.14 (2H, m), 5.32 (1H, s), 6.97 (1H, s), 7.09 (1H, t, *J* = 7.6 Hz), 7.19 (1H, t, *J* = 7.6 Hz), 7.34—7.40 (5H, m), 7.52 (1H, d, *J* = 7.6 Hz), 8.04 (1H, s); IR (neat) cm^{-1}: 3358, 1713; FAB-MS (*m*/*z*): 352.1 $(M+H)^+$.

***N*-(Benzyloxycarbony)-L-tryptophanol (17)** Compound **17** was obtained as a white solid in 85.8% yield, mp 103—105 °C, from **16** according to the procedure described for **11**. ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.02 (2H, d, *J* = 6.8 Hz), 3.64—3.69 (2H, m), 4.50 (1H, m), 5.09 (2H, s), 7.01 (1H, s), 7.11 (1H, t, *J* = 7.6 Hz), 7.20 (1H, t, *J* = 7.4 Hz), 7.29—7.36 (6H, m), 7.65 (1H, s), 8.03 (1H, s); IR (KBr) cm^{-1}: 3400, 1690; FAB-MS (*m*/*z*): 325.2 $(M+H)^+$; $[\alpha]_D^{27}$ −23.6° (*c* = 0.89, CH_2Cl_2).

(2*S*)-2-[(Benzyloxycarbonyl)amino]-3-(1*H*-indol-3-yl)propyl Methanesulfonate (18) Compound **18** was obtained as a yellow oil in 84.4% yield from **17** according to the procedure described for **12**. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.94 (3H, s), 3.00—3.23 (2H, m), 4.18—4.27 (3H, m), 5.03 (1H, br s), 5.11 (2H, s), 7.08 (1H, s), 7.14 (1H, t, *J* = 7.6 Hz), 7.21 (1H, t, *J* = 7.2 Hz), 7.32—7.41 (6H, m), 7.63 (1H, s), 8.06 (1H, s); IR (neat) cm^{-1}: 3389, 1703, 1352; ESI-MS (*m*/*z*): 403.07 $(M+H)^+$.

Benzyl [(1*S*)-2-Cyano-1-(1*H*-indol-3-ylmethyl)ethyl]carbamate (19) Compound **19** was obtained as a yellow oil in 53.2% yield from **18** according to the procedure described for **13**. ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.48—2.74 (2H, m), 3.04—3.21 (2H, m), 4.28—4.29 (1H, m), 5.02 (1H, br s), 5.11 (2H, s), 7.09 (1H, s), 7.14 (1H, t, *J* = 7.0 Hz), 7.23 (1H, t, *J* = 7.0 Hz), 7.31—7.39 (6H, m), 7.62 (1H, d, *J* = 8.0 Hz), 8.10 (1H, s); IR (neat) cm^{-1}: 3339, 1699; FAB-MS (*m*/*z*): 333.3 $(M+H)^+$; $[\alpha]_D^{27}$ −17.1° (*c* = 1.04, CH_2Cl_2).

(3*S*)-3-Amino-4-(1*H*-indol-3-yl)butanenitrile (20) A mixture of **19** (476.8 mg, 1.43 mmol) and Pd–C (10%, *ca.* 100 mg) in dry methanol (5.0 ml) was stirred at room temperature for 19 h under atmospheric pressure of H_2. The catalysts were filtered through Celite, and the filtrate was evaporated to give **20** (740 mg, quant.) as a yellow oil. ^{1}H-NMR (CD_3OD) δ (ppm): 2.43—2.57 (2H, m), 2.88—2.99 (2H, m), 3.35—3.42 (1H, m), 6.99—7.12 (3H, m), 7.35 (1H, d, *J* = 8.0 Hz), 7.57 (1H, d, *J* = 8.4 Hz); IR

(neat) cm^{-1}: 3400—2800; ESI-MS (*m/z*): 200.13 $(M+H)^+$.

***tert*-Butyl [(1*S*)-2-Cyano-1-(1*H*-indol-3-ylmethyl)ethyl]carbamate (21)** Sodium bicarbonate (282 mg, 3.35 mmol) in water (5.0 ml) and di-*tert*-butyl dicarbonate (491.3 mg, 2.25 mmol) in THF (5.0 ml) were added to compound **20** (335.1 mg, 1.68 mmol) in an ice bath. After stirring at room temperature overnight, the solvent was concentrated and ethyl acetate was added. The mixture was washed with 10% aqueous citric acid, then dried and evaporated *in vacuo*. The residue was purified by silica gel chromatography (chloroform : acetone=20 : 1) to afford **21** (278.8 mg, 55.4%) as a brown solid, mp 48—50 °C. ^{1}H-NMR (CD_3OD) δ (ppm): 1.45 (9H, s), 2.44—2.71 (2H, m), 3.02—3.20 (2H, m), 4.21 (1H, m), 4.77 (1H, bs), 7.13—7.25 (3H, m), 7.39 (1H, d, J=8.4 Hz), 7.65 (1H, d, J=7.6 Hz), 8.10 (1H, s); IR (KBr) cm^{-1}: 3350, 3100—2800, 1693; ESI-MS (*m/z*): 300.19 $(M+H)^+$; $[\alpha]_D^{27}$ −8.9° (c=1.35, CH_2Cl_2).

Boc-β^3-Trp-OH (22) To a solution of **21** (18 mg, 0.06 mmol) in ethanol/H_2O=5 : 1 (1.3 ml) was added potassium hydroxide (33.7 mg, 0.6 mmol). The mixture was heated under reflux for 9 h and concentrated *in vacuo*. The reaction mixture, after addition of water (2 ml) and neutralization with 10% aqueous citric acid, was extracted with ethyl acetate. The organic layer was dried, concentrated *in vacuo*, and purified by silica gel chromatography (chloroform : methanol=20 : 1) to afford **22** (5.6 mg, 29.3%) as a pale brown solid, mp 137—138 °C. ^{1}H-NMR (CD_3OD) δ (ppm): 1.37 (9H, s), 2.39—2.59 (2H, m), 2.95 (2H, d, J=6.4 Hz), 4.19—4.22 (1H, m), 6.97—7.09 (3H, m), 7.13 (1H, d, J=8.0 Hz), 7.60 (1H, d, J=8.0 Hz); IR (neat) cm^{-1}: 3338, 3100—2800, 1683; ESI-MS (*m/z*): 319.2 $(M+H)^+$; $[\alpha]_D^{27}$ −8.9° (c=0.45, CH_2Cl_2).

Z-β^3-Phe-β-Ala-OMe (23) Under argon, to a solution of β-alanine methyl ester (22.3 mg, 0.16 mmol) in dry dichloromethane (0.5 ml) was added triethylamine (0.12 ml, 0.8 mmol), **15** (50 mg, 0.16 mmol) in dry DMF (0.5 ml), HOBt (29.4 mg, 0.19 mmol), and EDC (36.4 mg, 0.19 mmol) in an ice bath. After stirring for 10 h at room temperature, chloroform (10 ml) was added. The mixture was washed with a 1 M hydrogen chloride solution, a saturated sodium hydrogencarbonate solution and brine, and then dried and evaporated *in vacuo*. The residue was purified by chromatography on a silica gel column (chloroform : methanol=30 : 1) to afford **23** (35.1 mg, 55.1%) as a white solid, mp 108—110 °C. ^{1}H-NMR (CD_3OD) δ (ppm): 2.25—2.43 (2H, m), 2.52 (2H, t, J=6.0 Hz), 2.78—3.01 (2H, m), 3.46—3.51 (2H, q, J=6.0 Hz), 3.68 (3H, s), 4.09—4.18 (1H, m), 5.07 (2H, s), 5.75 (1H, br s), 6.13 (1H, br s), 7.15—7.36 (10H, m); IR (KBr) cm^{-1}: 3312, 3032, 1693, 1636; FAB-MS (*m/z*): 399.2 $(M+H)^+$; $[\alpha]_D^{27}$ +10.5° (c=0.21, $CHCl_3$).

Boc-β^3-Trp-β^3-His-OBn (25) Compound **25** was obtained as a brown solid in 57.6% yield, mp 92—94 °C, from **22** and **9** according to the procedure described for **23**. ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.41 (9H, s), 2.22—2.36 (2H, m), 2.43—2.62 (2H, m), 2.78—3.04 (4H, m), 4.21 (1H, m), 4.52—4.57 (1H, m), 5.11 (2H, s), 5.52 (1H, br s), 6.67 (1H, bs), 6.74 (1H, s), 6.97 (1H, d, J=2.0 Hz), 7.09 (1H, t, J=7.4 Hz), 7.17 (1H, t, J=7.6 Hz), 7.26—7.35 (5H, m), 7.51 (1H, s), 7.63 (1H, d, J=8.0 Hz), 8.16 (1H, s); IR (KBr) cm^{-1}: 3361, 2900, 1680; ESI-MS (*m/z*): 560.2 $(M+H)^+$; $[\alpha]_D^{27}$ +47.7° (c=0.13, $CHCl_3$).

Z-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OBn (27) To a solution of **23** (285 mg, 0.72 mmol) in dry methanol (5.0 ml) was added a 1 M sodium hydroxide solution (1.44 ml) in an ice bath. The mixture was stirred at room temperature for 30 min, neutralized by a 1 M hydrogen chloride solution and then concentrated *in vacuo*. The residue was diluted with a saturated sodium hydrogencarbonate solution and washed with ether. After acidification by the addition of a 1 M hydrogen chloride solution, the mixture was extracted by ethyl acetate. The organic layer was dried, and evaporated *in vacuo* to obtain crude dipeptide (Z-β^3-Phe-β-Ala-OH, **24**) (260.7 mg) as a white solid.

A mixture of **25** (17 mg, 0.03 mmol) and trifluoroacetic acid (0.3 ml) was stirred at room temperature for 30 min, and concentrated *in vacuo*. The residue was dissolved in distilled water, and the solution was lyophilized to give a crude dipeptide (H-β^3-Trp-β^3-His-OBn, **26**) (9.9 mg) as a white solid.

Under argon, to a solution of **26** (15.0 mg, 0.033 mmol) in dry dichloromethane (0.5 ml) was added triethylamine (23 μl, 0.165 mmol), **24** (12.7 mg, 0.033 mmol) in dry DMF (0.5 ml), HOBt (7.7 mg, 0.05 mmol), and EDC (9.6 mg, 0.05 mmol) in an ice bath. After stirring for 10 h at room temperature, chloroform (10 ml) was added. The mixture was washed with water, and then dried and evaporated *in vacuo*. The residue was purified by chromatography on a silica gel column (chloroform : methanol=30 : 1) to afford **27** (8.8 mg, 32.3%) as a white solid. ESI-MS (*m/z*): 826.50 $(M+H)^+$; $[\alpha]_D^{27}$ −8.0° (c=0.20, CH_3OH).

H-β^3-Phe-β-Ala-β^3-Trp-β^3-His-OH (1) A mixture of **27** (13.6 mg, 0.016 mmol) and Pd–C (10%, *ca.* 15 mg) in dry DMF (0.7 ml) was stirred at room temperature for 2.5 h under atmospheric pressure of H_2. After the catalysts were removed by filtration through Celite, dry ether was added to precipitate crude peptide. The peptide was purified by HPLC (Inertsil $WP300C_{18}$ column, 20×250 mm, GL Sciences), eluted with a linear gradient of 15—30% acetonitrile in 0.1% aqueous trifluoroacetic acid in 130 min at a flow rate of 10 ml/min. Fractions containing the peptide were collected, and the solvent was removed by lyophilization to give **1** (6.0 mg, 62.3%) as a brown solid. ^{1}H-NMR (CD_3OD) δ (ppm): 2.20—3.30 (16H, m), 3.75 (1H, m), 4.45 (2H, m), 6.98—7.10 (3H, m), 7.23—7.36 (7H, m), 7.56 (1H, d, J=7.6 Hz), 8.65 (1H, s); ESI-MS (*m/z*): 602.42 $(M+H)^+$.

H-His-Trp-Gly-Phe-OH (2) The protected peptidyl resin was constructed using an Fmoc-based solid phase methodology on a Wang resin. Triethylsilane (60 μl), trifluoroacetic acid (4.9 ml), and distilled water (60 μl) were added to the peptidyl-resin (104 mg), and the mixture was stirred at room temperature for 2 h. After the resins were removed by filtration, dry ether was added to precipitate a crude peptide. The peptide was purified by HPLC (Inertsil $WP300C_{18}$, 20×250 mm), eluted with a linear gradient of 15—22% acetonitrile in 0.1% aqueous trifluoroacetic acid in 40 min at a flow rate of 16 ml/min. Fractions containing the peptide were collected, and the solvent was removed by lyophilization to give **2** (8.8 mg) as a white solid. ^{1}H-NMR (CD_3OD) δ (ppm): 2.95—3.30 (4H, m), 3.62—3.99 (2H, m), 4.09 (1H, m), 4.64—4.67 (2H, m), 6.99—7.34 (10H, m), 7.59 (1H, d, J=8.0 Hz), 8.44 (1H, s); ESI-MS (*m/z*): 546.28 $(M+H)^+$.

H-Phe-Gly-Trp-His-OH (3) Compound **3** was obtained as a white solid according to the procedure described for **2**. ^{1}H-NMR (CD_3OD) δ (ppm): 2.97—3.27 (4H, m), 3.68—3.95 (2H, m), 4.06—4.10 (1H, m), 4.62—4.71 (2H, m), 6.97—7.10 (3H, m), 7.23—7.33 (7H, m), 7.56 (1H, d, J=7.6 Hz), 8.71 (1H, s); ESI-MS (*m/z*): 546.2 $(M+H)^+$.

MMP-2 Inhibition Assay Gelatin (from bovine bone, Wako) was dissolved in phosphate buffer (0.3 M, pH 7.4) to a concentration of 1 mg/ml. To this solution (100 μl) was added [^{125}I]NaI (6.1 MBq, 3 μl, GE Healthcare) and a chloramine T solution (1 mg/ml, 10 μl). After a 20-min reaction, the solution was removed and eluted through a PD-10 column (GE Healthcare) to separate non-reacted [^{125}I]NaI, according to the manufacturer's instructions. The radiochemical purity was determined to be >96% by TLC developed with acetone/water/*n*-butanol/ammonia=13 : 1 : 4 : 2.

Compound **1**, **2**, and **3** and cCTT were dissolved in DMF and diluted in the assay buffer (pH 7.5, 50 mM Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.1% Triton X-100) to the appropriate concentrations (0.001—1 mM final concentration). These compounds at the desired concentrations were added to the enzyme solution (active MMP-2, Calbiochem, 3 nM final concentration) and incubated for 30 min at 37 °C. Finally, ^{125}I-labeled gelatin (30 MBq) was added to each assay and incubated for 1 h at 37 °C. The degradation of ^{125}I-labeled gelatin was determined by counting the radioactivity in the supernatant after precipitation of the undegraded gelatin with 20% trichloroacetic acid.

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