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Design and synthesis of novel type somatostatin analogs with antiproliferative activities on A431 tumor cells

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Abstract—It was reported that the somatostatin analog TT-232, D-Phe-*c*(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2, exhibited a highly potent antitumor activity in vitro and in vivo. Using pyrazinone analogs and aliphatic amino acids instead of the disulfide bond, we prepared novel type somatostatin analogs including the sequence essential for antitumor activities, Tyr-D-Trp-Lys. These analogs exhibited antiproliferative effect on A431 tumor cells.

Somatostatin is a cyclic tetradecapeptide, which was isolated from bovine hypothalamus and known as a suppressor of growth hormone (GH) secretion from the anterior pituitary gland.[1] Furthermore it also suppresses the release of various hormones including glucagons, insulin, gastrin, secretin, etc.[2,3] Although many somatostatin analogs have been prepared and studied in regard to their biological activities, it is difficult to use them for clinical purpose because of their enzymatic degradation in vivo. Among them, however, sandostatin, D-Phe-*c*(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol, acts longer than somatostatin in vivo and is widely used as the inhibitor of hormone release and for the treatment for endocrine tumors on the clinical level.[4,5] On the other hand, TT-232, D-Phe-*c*(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2, has no GH release inhibiting activity but has only highly potent antitumor activity in vitro and in vivo, indicating that the sequence essential for the antitumor activity might be Tyr-D-Trp-Lys.[6] For clinical purposes, the enzymic stability and the passage through the physical barriers including epithelial membrane and blood–brain barrier of drugs are required. In this paper, we designed and prepared peptidomimetic somatostatin analogs with expectation of their high bioavailability in oral administration.

We designed novel somatostatin analogs that include the Tyr-D-Trp-Lys sequence and tested their antiproliferative activities on tumor cells. We prepared four somatostatin analogs (**I**–**IV**) (Fig. 1). Two of them, **I** and **II** have pyrazinone rings[7] instead of disulfide bond. Previously we established a simple synthetic method for pyrazinone ring formation from dipeptidyl chloromethyl ketone and the procedure has following advantages: the selective introduction of amino and/or carboxyl groups at position 3 and 6 on the pyrazinone ring.[7] It was expected that peptidomimetic containing pyrazinone ring would exhibit a longer period of activity than somatostatin and its peptidic analogs owing to a stability of the ring structures.[7] The most attractive feature is that peptidomimetics containing the pyrazinone rings can pass the epithelial membrane of gastrointestinal tracts and blood–brain barrier.[8,9] These results provided us with an idea to design effective drugs for oral administration. Thus we have prepared numerous pyrazinone derivatives. On the other hand, **III** and **IV** have ω-aliphatic amino acids with different chain lengths in order to compare them with the pyrazinone ring-containing compounds (**I** and **II**). Concerning the number of atoms comprising the backbone in the analogs, the $(CH_2)_6$ analog (**IV**) is almost same as that of TT-232.

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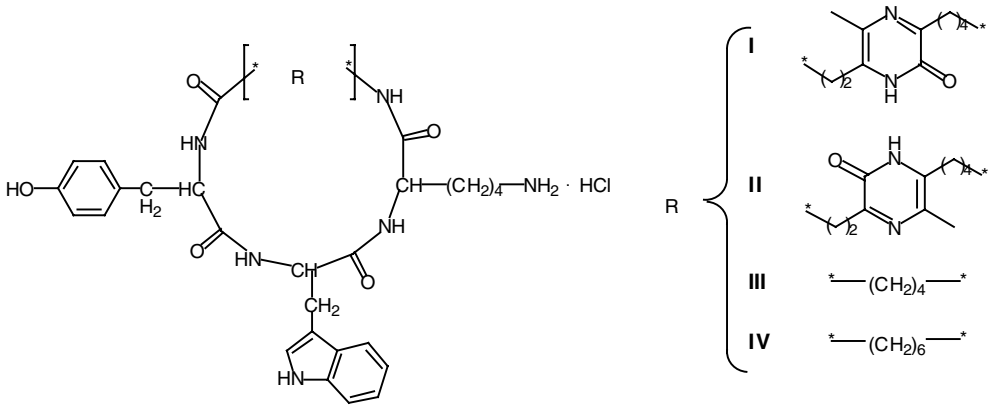
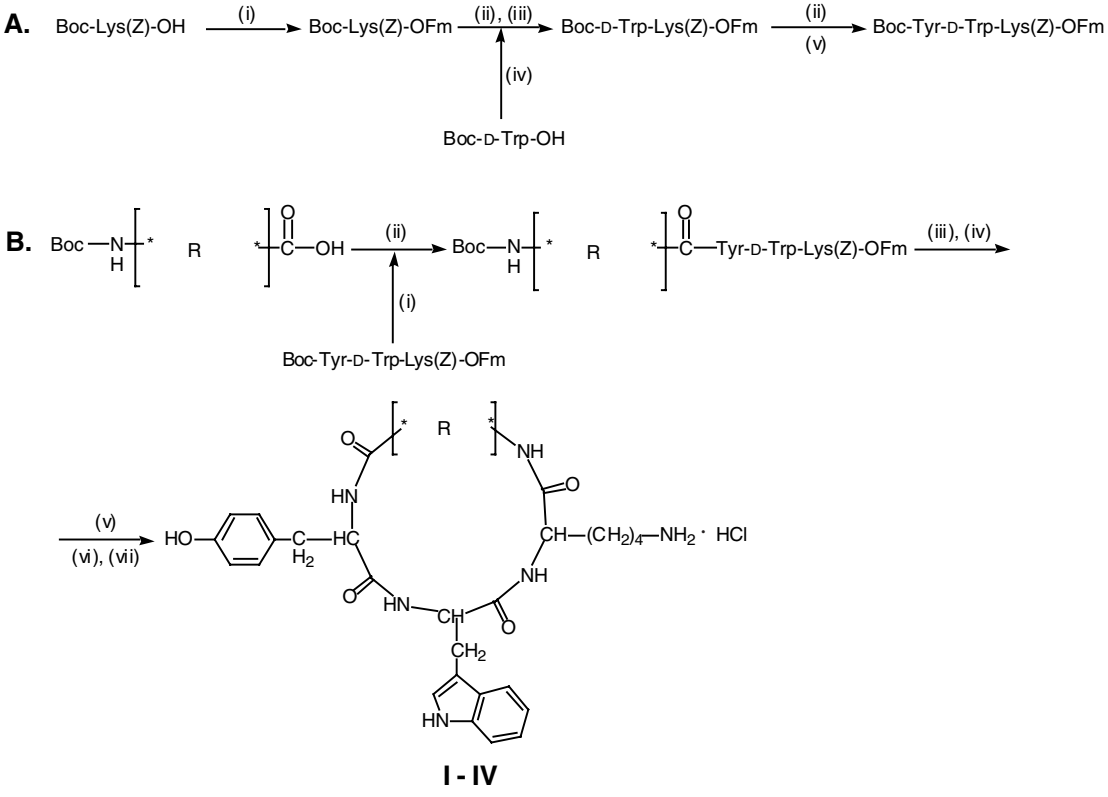


Figure 1. Somatostatin analogs. R: **I**, a pyrazinone ring prepared from Boc-Lys(Z)-Glu(OBzl)-CH_2Cl; **II**, a pyrazinone ring prepared from Boc-Glu(OBzl)-Lys(Z)-CH_2Cl; **III**, an aliphatic amino acid ($n=4$); **IV,** an aliphatic amino acid ($n=6$).

The antiproliferative activities of these compounds were examined on tumor cells by methylene blue test.[10] Furthermore to clarify their structure–activity relationships, CD and NMR spectroscopy measurements were performed.

The compounds (**I**–**IV**) were synthesized by solution-phase method using fragment condensation (Scheme 1). The Boc–pyrazinones were prepared by a method described previously.[7] The tripeptide was prepared starting from Boc-Lys(Z)-OH. Boc-Lys(Z)-OH was reacted with 9-fluorenylmethanol, WSCI·HCl and 4-dimethylaminopyridine to give Boc-Lys(Z)-OFm. After removal of Boc group by HCl/dioxane, the resulting HCl salt was coupled with Boc-D-Trp-OH by a mixed anhydride method to give Boc-D-Trp-Lys(Z)-OFm. After removal of Boc group from the dipeptide by HCl/dioxane, the resulting amine was coupled with Boc-Tyr-OH to give crude tripeptide. The crude crystal was purified by silica gel column chromatography (3% methanol/chloroform) to give Boc-Tyr-D-Trp-Lys(Z)-OFm (Scheme 1, **A**). The Boc group was removed from the tripeptide by HCl/



Scheme 1. Reagents and conditions. **A**: (i) 9-fluorenylmethanol, WSCI·HCl, 4-dimethylaminopyridine, DMF; (ii) HCl/dioxane; (iii) triethylamine, DMF; (iv) triethylamine, isobutyl chloroformate, THF, −15 °C; (v) Boc-Tyr-OH, PyBop, *N*,*N*-diisopropylethylamine, in DMF. **B**: (i) HCl/dioxane; (ii) PyBop, *N*,*N*-diisopropylethylamine, in DMF; (iii) 20% piperidine/DMF; (iv) trifluoroacetic acid, 1,2-ethanedithiol; (v) triethylamine, DPPA, in 0.5 mM DMF; (vi) 25% HBr/acetic acid, thioanisole, 1,2-ethanedithiol; (vii) 1 M HCl. (R: see Fig. 1).

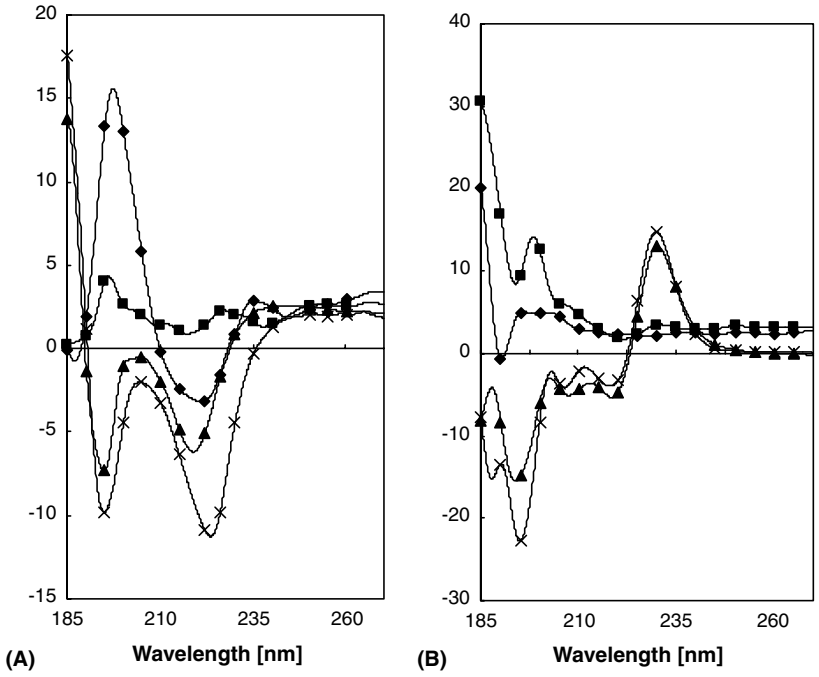


Figure 2. The CD spectra pattern of **I–IV** in **A** (trifluoroethanol) and **B** (methanol). I: ■, II: ◆, III: ▲, IV: ×.

dioxane and the resulting amine was coupled with Boc–pyrazinones or Boc–aliphatic amino acids by PyBop reagent as described above. The Fm and Boc groups were removed using 20% piperidine/DMF and trifluoroacetic acid containing 1,2-ethanedithiol, respectively. The compounds purified by reversed-phase HPLC, were cyclized by diphenyl phosphoryl azide (DPPA) and triethylamine in 0.5 mM DMF solution at room temperature, monitoring the reaction by reversed-phase HPLC. The crude crystal was recrystallized. Finally, the Z group was removed by HBr/acetic acid containing thioanisole and 1,2-ethanedithiol. The crude compounds were purified by reversed-phase HPLC [column: COSMOSIL C18 (4.6×250 nm); solvents: from 0.05% trifluoroacetic acid in water (90), 0.05% trifluoroacetic acid in CH_3CN (10) to 0.05% trifluoroacetic acid in water (50), 0.05% trifluoroacetic acid in CH_3CN (50) for 40 min at a flow rate of 1 mL/min with detection at 220 nm], (Scheme 1, **B**) and identified by NMR, MALDI TOF-MS, and elemental analysis.[11–14]

Generally, somatostatin, sandostatin, and many other somatostatin analogs form β-turn structures. For example, sandostatin forms an antiparallel β-pleated sheet with a type β-turn spanning residues D-Trp^4 and Lys^5, and is stabilized by the hydrogen bond between Thr^6 ($N^{\alpha}H$) and Phe^3 (C═O). This is supported by the strong NOE between $C^{\alpha}H$ of D-Trp^4 and $N^{\alpha}H$ of Lys^5, the average NOE between $N^{\alpha}H$ of Lys^5, and $N^{\alpha}H$ of Thr^6 and the low temperature coefficient measured for the Thr$^6N^{\alpha}H$ resonance.[15,16] In the above case, the side chain of D-Trp^4 and Lys^5 were in juxtaposition, which was supported by the upfield chemical shift (<0.4 ppm) of γ-methylene protons of Lys^5. [16,17] With **I–IV**, we expected similar results; however, they failed to exhibit the above NOE. The aromatic side chain orientation of D-Trp^4 was not determined due to the overlap of the chemical shifts. The side chains of amino acids were flexible, such that the interactions between them were not observed. CD spectra were recorded in methanol and trifluoroethanol (Fig. 2). In the both cases, all analogs showed random structures and the spectra patterns of analogs containing pyrazinone rings were different from those including aliphatic amino acids. As shown in Figure 2, insertion of this constraint extremely changed the backbone conformation of the cyclic peptide.

Finally, the antiproliferative effects of analogs **I–IV** on A431 cells (human epithelial tumor cells) in which the somatostatin receptor is expressed were measured by methylene blue test (Fig. 3).[10] The concentrations of the analogs were 0.08, 0.4, 2.0, 10, and 50 μmol/L. The cycloheximide inducing apoptosis by inhibiting protein synthesis was used as positive control. After incubation of A431 cells with **I–IV** and cycloheximide for 6 or 48 h, the cells dyed by methylene blue were counted. On the

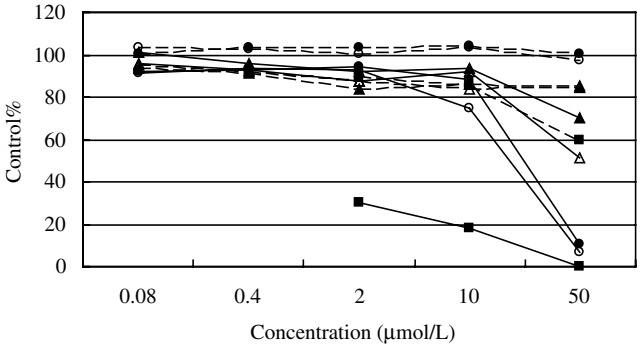


Figure 3. The result of antiproliferative activity of **I–IV** on A431 tumor cells. The dotted line and solid line show the case of incubation for 6 and 48 h, respectively. The ●, ○, ▲, △, and ■ show **I–IV**, and cycloheximide, respectively.

incubation for 48 h, their activities increase depending on concentration and all analogs (**I**–**IV**) have the highest efficient at 50 μmol/L (90%, 90%, 30%, and 50% inhibition, respectively). The analogs containing pyrazinone ring (**I** and **II**) showed highly potent activities at 50 μmol/L although the activity was weaker than that of cycloheximide at low concentration. Interestingly **I** and **II** were more potent than analogs containing aliphatic amino acid (**III** and **IV**). The differences of activity between **I** and **II** were not observed, on the other hand, the $(CH_2)_6$ analog (**IV**), the number of atoms comprising the backbone was same as that of TT-232, was slightly stronger than the $(CH_2)_4$ analog (**III**).

In conclusion, we designed and prepared novel type somatostatin analogs **I**–**IV**. As shown in Figure 3, these analogs exhibited high antiproliferative effects on A431 cells, although their conformations determined by NMR and CD were different from those of typical somatostatin analogs. Compounds **I** and **II** had high degree of antiproliferative activity at 50 μmol/L and we can expect the more effective uptake by oral administration owing to the stability and physicochemical features in vivo[8,9] as well as their potent antiproliferative activity.

Acknowledgement

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11. *c*[(*Lys-Glu*)-*Tyr*-D-*Trp-Lys*]·*HCl* (**I**): (Lys-Glu) shows 3-(4-aminobutyl)-6-(2-carboxyethyl)-5-methyl-2(1*H*)-pyrazinone. Yield 75.5 mg (34.0%): amorphous; $[\alpha]_D^{25} + 75.4$ (*c* 1.0, H_2O); R_f (*n*-butanol/acetic acid/pyridine/H_2O = 4:1:1:2) = 0.50; ^1H NMR (500 MHz, DMSO-d_6) δ: 10.85 (1H, s, 1-NH of indole), 8.45 (1H, d, J = 7.8 Hz, α-NH of D-Trp), 8.19 (1H, d, J = 8.3 Hz, α-NH of Lys), 7.88 (2H, s, ε-NH_2 of Lys), 7.78 (1H, d, J = 8.5 Hz, α-NH of Tyr), 7.69 (1H, d, J = 7.8 Hz, 4-CH of indole), 7.65 (1H, t, J = 5.0 Hz, 3-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 7.31 (1H, d, J = 8.0 Hz, 7-CH of indole), 7.18 (1H, d, J = 1.8 Hz, 2-CH of indole), 7.04 (1H, t, J = 7.3 Hz, 6-CH of indole), 6.98 (1H, t, J = 7.3 Hz, 5-CH of indole), 6.78 (2H, d, J = 8.3 Hz, 2, 6-OH of Tyr), 6.51 (2H, d, J = 8.3 Hz, 3, 5-CH of Tyr), 4.60–4.52 (2H, m, α-CH of D-Trp and α-CH of Tyr), 4.13 (1H, td, J = 8.5, 5.7 Hz, α-CH of Lys), 3.18 (1H, m, 3-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 3.01 (1H, dd, J = 14.5, 5.8 Hz, β-CH_2 of Tyr), 2.89–2.72 (4H, m, 3-$CH_2CH_2CH_2CH_2NH$ of pyrazinone, 6-CH_2CH_2CO of pyrazinone and β-CH_2 of Tyr), 2.66 (2H, m, ε-CH_2 of Lys), 2.56–2.17 (6H, m, 3-$CH_2CH_2CH_2CH_2NH$ of pyrazinone, 6-CH_2CH_2CO of pyrazinone and β-CH_2 of D-Trp), 2.14 (3H, s, 5-CH_3 of pyrazinone), 1.60–1.23 (8H, m, 3-$CH_2CH_2CH_2CH_2NH$ of pyrazinone, β-CH_2 and δ-CH_2 of Lys),1.05 (2H, m, γ-CH_2 of Lys); MS $(M+H)^+$ Calcd 713.8. Found. 713.8. Anal. Calcd for $C_{38}H_{49}ClN_8O_6$ (+4.5H_2O): C, 54.9; H, 7.04; N, 13.5. Found: C, 54.7; H, 6.95; N, 13.7.
12. *c*[(*Glu-Lys*)-*Tyr*-D-*Trp-Lys*]·*HCl* (**II**): (Glu-Lys) shows 6-(4-aminobutyl)-3-(2-carboxyethyl)-5-methyl-2(1*H*)-pyrazinone. Yield 7.00 mg (14.4%): amorphous; $[\alpha]_D^{25} + 32.1$ (*c* 1.0, H_2O); R_f (*n*-butanol/acetic acid/pyridine/H_2O = 4:1:1:2) = 0.55; ^1H NMR (500 MHz, DMSO-d_6) δ: 10.85 (1H, s, 1-NH of indole), 9.05 (1H, s, 4-OH of Tyr), 8.42 (1H, d, J = 8.3 Hz, α-NH of Lys), 8.19 (1H, d, J = 8.7 Hz, α-NH of D-Trp), 7.96 (2H, s, ε-NH_2 of Lys), 7.81 (1H, d, J = 6.4 Hz, 6-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 7.73 (1H, d, J = 7.8 Hz, 4-CH of indole), 7.50 (1H, d, J = 8.8 Hz, α-NH of Tyr), 7.30 (1H, d, J = 8.0 Hz, 7-CH of indole), 7.18 (1H, d, J = 1.8 Hz, 2-CH of indole), 7.05 (1H, t, J = 7.4 Hz, 6-CH of indole), 6.98 (1H, t, J = 7.4 Hz, 5-CH of indole), 6.70 (2H, d-like, J = 8.3 Hz, 2, 6-OH of Tyr), 6.47 (2H, d-like, J = 8.3 Hz, 3, 5-CH of Tyr), 4.72 (1H, td, J = 8.9, 5.3 Hz, α-CH of Tyr), 4.59 (1H, td, J = 8.6, 4.8 Hz, α-CH of D-Trp), 4.15 (1H, td, J = 8.3, 5.9 Hz, α-CH of Lys), 3.57 (1H, m, 6-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 2.97(1H, dd, J = 14.3, 5.1 Hz, β-CH_2 of D-Trp), 2.91–2.87 (1H, m, 3-CH_2CH_2CO of pyrazinone), 2.84 (1H, dd, J = 14.3, 9.6 Hz, β-CH_2 of D-Trp), 2.69 (2H, br, ε-CH_2 of Lys), 2.63–2.50 (3H, m, 3-CH_2CH_2CO of pyrazinone and 6-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 2.39 (1H, dd, J = 13.5, 4.5 Hz, β-CH_2 of Tyr), 2.31 (1H, dd, J = 13.5, 8.5 Hz, β-CH_2 of Tyr), 2.14 (1H, m, 6-$CH_2CH_2CH_2CH_2NH$ of pyrazinone), 2.04 (1H, d, J = 14.4 Hz, 3-CH_2CH_2CO of pyrazinone), 1.90 (3H, s, 5-CH_3 of pyrazinone), 1.55–1.30 (8H, m, 6-$CH_2CH_2CH_2CH_2NH$ of pyrazinone, β-CH_2 and δ-CH_2 of Lys), 1.22–1.07 (2H, m, γ-CH_2 of Lys); MS $(M+H)^+$ Calcd 713.8. Found. 713.2. Anal. Calcd for $C_{38}H_{49}ClN_8O_6$ (+3H_2O): C, 56.8; H, 6.90; N, 14.0. Found: C, 56.7; H, 6.88; N, 14.2.
13. *c*[$(CH_2)_4$-*Tyr*-D-*Trp-Lys*]·*HCl* (**III**): Yield 14.7 mg (49.2%): amorphous; $[\alpha]_D^{25} - 22.3$ (*c* 1.0, H_2O); R_f (*n*-butanol/acetic acid/pyridine/H_2O = 4:1:1:2) = 0.60; ^1H NMR (500 MHz, DMSO-d_6) δ: 10.93 (1H, s, 1-NH of indole), 9.22 (1H, s, 4-OH of Tyr), 8.66 (1H, d, J = 6.9 Hz, α-NH of D-Trp), 8.32 (1H, d, J = 8.1 Hz, α-NH of Lys), 8.00 (1H, d, J = 8.7 Hz, α-NH of Tyr), 7.51 (1H, d, J = 7.8 Hz, 4-CH of indole), 7.34 (1H, d, J = 8.0 Hz, 7-CH of indole), 7.20 (1H, t, J = 5.5 Hz, NH of Val), 7.17 (1H, d, J = 1.3 Hz, 2-CH of indole), 7.06 (1H, t, J = 7.5 Hz, 6-CH of indole), 7.01 (2H, d, J = 8.4 Hz, 2, 6-OH of Tyr), 6.98 (1H, t, J = 7.4 Hz, 5-CH of indole),

6.65 (2H, d, J = 8.4 Hz, 3, 5-CH of Tyr), 4.53 (1H, td, J = 9.1, 5.3 Hz, α-CH of Tyr), 4.40 (1H, q-like, J = 7.0 Hz, α-CH of D-Trp), 3.90 (1H, m, α-CH of Lys), 3.11 (1H, dd, J = 14.3, 8.7 Hz, β-CH_2 of D-Trp), 3.04 (1H, dd, J = 13.0, 6.5 Hz, δ-CH_2 of Val), 2.97 (1H, dd, J = 13.0, 5.8 Hz, δ-CH_2 of Val), 2.84 (1H, dd, J = 14.3, 6.5 Hz, β-CH_2 of D-Trp), 2.74 (1H, dd, J = 13.8, 5.2 Hz, β-CH_2 of Tyr), 2.64 (1H, dd, J = 13.8, 9.0 Hz, β-CH_2 of Tyr), 2.63–2.59 (1H, m, ε-CH_2 of Lys), 2.18 (1H, m, α-CH_2 of Val), 1.84 (1H, dt, J = 12.7, 4.6 Hz, α-CH_2 of Val), 1.67–1.52 (2H, m, β-CH_2 of Lys and γ-CH_2 of Val), 1.48–1.30 (5H, m, β-CH_2, δ-CH_2 of Lys and β-CH_2 of Val), 1.28–1.18 (1H, m, γ-CH_2 of Val), 0.92 (2H, q, J = 7.6 Hz, γ-CH_2 of Lys); MS $(M+H)^+$ Calcd 577.7. Found. 577.8. Anal. Calcd for $C_{31}H_{41}ClN_6O_5$ (+3H_2O): C, 60.7; H, 6.74; N, 13.7. Found: C, 61.0; H, 6.75; N, 14.0.

14. *c*[$(CH_2)_6$-*Tyr*-D-*Trp*-*Lys*]·*HCl* (**IV**): Yield 7.10 mg (55.5%): amorphous; $[\alpha]_D^{25}$ − 167 (*c* 1.0, H_2O); R_f (*n*-butanol/acetic acid/pyridine/H_2O = 4:1:1:2) = 0.37; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.94 (1H, s, 1-NH of indole), 9.20 (1H, s, 4-OH of Tyr), 8.46 (1H, d, J = 5.7 Hz, α-NH of D-Trp), 8.32 (1H, d, J = 8.3 Hz, α-NH of Lys), 7.93 (1H, d, J = 8.2 Hz, α-NH of Tyr), 7.54 (1H, d, J = 7.8 Hz, 4-CH of indole), 7.45 (1H, t, J = 5.7 Hz, NH of hep), 7.35 (1H, d, J = 8.0 Hz, 7-CH of indole), 7.20 (1H, d, J = 1.1 Hz, 2-CH of indole), 7.06 (1H, t, J = 7.5 Hz, 6-CH of indole), 6.99 (1H, t, J = 7.5 Hz, 5-CH of indole), 6.97 (2H, d, J = 8.3 Hz, 2, 6-CH of Tyr), 6.61 (2H, J = 8.3 Hz, 3, 5-OH of Tyr), 4.54 (1H, td, J = 9.0, 4.9 Hz, α-CH of Tyr), 4.28 (1H, q, J = 7.1 Hz, α-CH of D-Trp), 3.91 (1H, td, J = 10.2, 3.9 Hz, α-CH of Lys), 3.14–3.09 (1H, m, ω-CH_2 of hep), 3.07 (1H, dd, J = 14.1, 7.8 Hz, β-CH_2 of D-Trp), 2.91 (1H, dd, J = 14.1, 6.8 Hz, β-CH_2 of D-Trp), 2.90–2.87 (1H, m, ω-CH_2 of hep), 2.75–2.70 (1H, m, α-CH_2 of hep), 2.64–2.59(1H, m, α-CH_2 of hep), 1.70–1.62 (1H, m, β-CH_2 of Lys), 1.58–1.34 (5H, m, β-CH_2, ε-CH_2 of hep and δ-CH_2 of Lys), 1.31–1.05 (6H, m, γ-CH_2, δ-CH_2, ε-CH_2 of hep and β-CH_2 of Lys), 0.85 (2H, q, J = 7.2 Hz, γ-CH_2 of Lys); MS $(M+H)^+$ Calcd 605.7. Found. 605.9. Anal. Calcd for $C_{33}H_{45}ClN_6O_5$ (+1.4H_20): C, 59.5; H, 7.23; N, 12.6. Found: C, 59.2; H, 7.34; N, 12.8.

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