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PRACTICAL SYNTHESIS OF A CYCLIC PENTAPEPTIDE IN SOLUTION: LARGE-SCALE PREPARATION OF A REPRESENTATIVE ET_A-SELECTIVE ANTAGONIST, BQ-123Na

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Abstract : A 100-g scale preparation of a representative endothelin-A receptor (ET_A) selective antagonist, BQ-123Na (a sodium salt of BQ-123), is described. Application of a methyl ester as the side chain carboxy-protecting group for the D-Asp residue made it possible to use a benzyl ester as a carboxy-protecting group for the C-terminal Leu throughout the synthesis.

The term endothelin (ET) describes three isopeptides, ET-1, ET-2 and ET-3, each of which is a potent vasoconstrictor consisting of 21 amino acid residues and having sustained and potent pressor activity. ET is known to exert various biological effects on vascular and nonvascular tissues through at least two distinct receptor subtypes (ET_A and ET_B); however, the physiological and/or pathophysiological roles of ET and its receptor subtypes are still unclear.[1] Specific ET antagonists may be used as tools for elucidating the biological roles of ET and possibly as therapy in putative ET-related disorders. In the course of our anti-endothelin program, we discovered a series of potent, water soluble and highly selective ET_A antagonistic cyclic pentapeptides represented by BQ-123 (IC_{max50} for ET_A: 22 nM; IC_{50} for ET_B: 18 μM; solubility of Na salt: >1 g/ml saline).[2] To supply a representative compound for pharmacological studies, a 100-g scale preparation was attempted. In this communication, we describe a short-step and practical synthesis of BQ-123Na in solution.

cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu-)	BQ-123	**(1)**
cyclo (-D-Trp-D-Glu-Ala-D-Val-Leu-)	BE-18257A	**(2)**

For the chemical modification of the natural lead BE-18257A (**2**), we have established a highly efficient, rapid and less laborious method for the preparation of cyclic pentapeptide analogues using solid-phase peptide synthesis.[2,3] This method is suitable for 10- to 500-mg scale preparation of screening samples but is not applicable to large-scale preparation of a specific compound due to the excessive use of agents and solvents. To confirm the chemical structure of **2**, we have also synthesized this compound in solution using a benzyloxycarbonyl (Z) group as the protecting group for the indole NH moiety in the D-Trp residue.[4] Although synthetic **2** was identical to the authentic sample of natural origin with regard to physico-chemical and spectral properties, the low solubility of some synthetic intermediates in conventional solvents caused a reduction in conversion and/or chemical yield in the cyclization and final deprotection steps. The low solubility of some intermediates was mainly attributed to protection of the indole NH moiety.

Based on the above-mentioned results, we devised a synthetic strategy for **1** as follows: a linear pentapeptide is prepared in solution according to the Boc strategy using 1-(3-dimethylaminopropyl)-3-ethylcarbo-

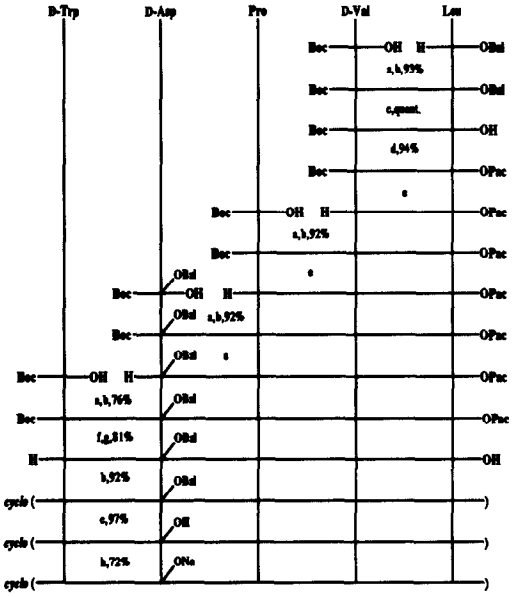
diimide hydrochloride (EDCI) and 1-hydroxybenzotriazole monohydrate (HOBT) as coupling agents. Leu was selected as the C-terminal amino acid residue of the precursor linear peptide and stepwise elongation of the peptide chain toward the N-terminus was conducted. We expected that there would be two principal advantages to selecting the linear pentapeptide D-Trp-D-Asp-Pro-D-Val-Leu as the precursor of **1**, one of which would be that the D-Trp residue was introduced into the peptide chain last.[5] Appropriate selection of deprotection conditions for the protected linear pentapeptide would probably make it possible to use D-Trp without any protection for the indole NH moiety. The other advantage would be that cyclization between the primary amino group of D-Trp and the carboxyl group of Leu could be less affected by steric hindrance between the side-chains than cyclization between Pro and D-Val, or D-Val and Leu. In addition, conformational analysis of the cyclic pentapeptide ET antagonists with a DDLDL sequence using NMR techniques revealed the presence of two intramolecular hydrogen bonds: namely, in the case of **1**, a γ'-turn in the D-Asp-Pro-D-Val region and a type II β-turn in the D-Val-Leu-D-Trp-D-Asp region.[6,7] Therefore, if these intramolecular hydrogen bonds were partially formed during cyclization, the hydrogen bonds would bring both the N-terminal amino group and the activated C-terminal carboxyl group close to each other and would facilitate cyclization. From this viewpoint, the precursor also seemed to be promising. Cyclization of the linear pentapeptide was performed not by the azide method but by the HOBT-EDCI method[8] under dilution conditions by considering applicability to a large-scale (100 g - 1 Kg) preparation.

The sodium salt of **1** was initially synthesized according to the route described in Scheme 1. Since commercially available Boc-D-Asp(OBzl)-OH was selected as the protected D-Asp derivative, the carboxyl group of the C-terminal Leu residue was protected with a phenacyl (Pac) group. Boc-Leu-OPac was prepared and deprotection of the Boc group was carried out with TFA. The desired reaction occurred and the reaction mixture produced a single spot on TLC. However, the deprotected compound gradually changed to 3-isobutyl-5-phenyl-2, 3-dihydro-4H-1,3-oxazine-2-one during the removal of TFA under reduced pressure.[9] This made the isolation / purification of Leu-OPac · TFA troublesome; in addition, the yield was not reproducible. To overcome this problem, Leu-OBzl · TsOH was allowed to react with Boc-D-Val-OH and debenzylation of the resulting dipeptide followed by reaction with Pac-Br afforded Boc-D-Val-Leu-OPac in high overall yield. The N- and C-terminal protecting groups of the fully protected linear pentapeptide were deprotected by successive treatments with formic acid and Zn / AcOH. Cyclization of the side-chain protected linear pentapeptide was effectively carried out under dilution (not high dilution !) conditions (the final concentration of the substrate was 20 - 30 mM). After deprotection of the side-chain protecting group, the free acid **1** was neutralized with sodium hydrogen carbonate to afford the sodium salt of **1**. The overall yield of the salt was 29% in 10 steps.

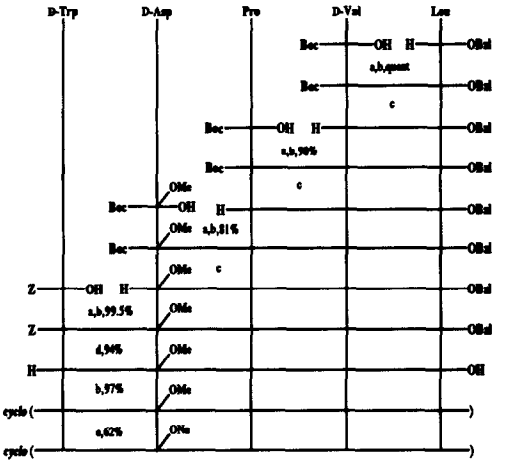
The sodium salt of **1** thus prepared was found to be easily purified by recrystallization from methanol. On the other hand, the methyl ester of **1**, prepared by treatment of **1** with diazomethane, was revealed to be hydrolyzable with 2N NaOH in methanol to regenerate the sodium salt of **1** without any cleavage of the peptide bonds, β-rearrangement at the D-Asp residue or any racemization of amino acid residues. The stability of this cyclic pentapeptide seemed to be mainly dependent on the D-Asp-Pro sequence and the presence of three D-amino acids and two intramolecular hydrogen bonds. Therefore, application of a methyl ester as the side-chain carboxy-protecting group of the D-Asp residue may enable the use of a benzyl ester as the carboxy-protecting group of the C-terminal Leu throughout the synthesis and reduce the number of reaction steps

(hydrolysis of the methyl ester under alkaline conditions followed by purification of the resulting crude product by recrystallization from methanol may directly afford pure **1** as a sodium salt).

Based on these considerations, improvement of the synthetic route was investigated (shown in Scheme 2). In the second route, the Boc group was deprotected by HCl / dioxane, which simplified the work-up and isolation procedures of N-terminal deprotected linear intermediates (all of the hydrochloric acid salts of linear intermediates were solids, not oils). Since D-Trp was introduced into the peptide chain as Z-D-Trp, both the N- and C-terminal protecting groups were simultaneously deprotected under hydrogenation conditions. The resulting side-chain protected linear pentapeptide was cyclized in the same manner as described in Scheme 1, followed by hydrolysis of the methyl ester to afford the sodium salt of **1**. The overall yield was 45% in 7 steps (not optimized) without any purification of the intermediates and the final product by column chromatography.[10]



Scheme 1. a: NMM, rt; b: HOBT, EDCI, 0°C-rt; c: H_2, 10% Pd-C, MeOH; d: i) Cs_2CO_3, H_2O-MeOH, ii) PacBr, DMF; e: TFA; f: Zn, 90% AcOH, 0°C; g: HCOOH, rt; h: $NaHCO_3$, MeOH.



Scheme 2. a: NMM, rt; b: HOBT, EDCI, 0°C-rt; c: HCl, dioxane, 0°C; d: H_2, 10% Pd-C, MeOH; e: 2N NaOH, MeOH, 0°C-rt

In this route, the condensation reaction of Boc-D-Asp(OMe)-OH[11] and the linear tripeptide ester proceeded in relatively low yield because the methanol contained in dichloromethane as a stabilizer competed with the less reactive N-terminal imino group of the Pro residue. Using additional amount of Boc-D-Asp(OMe)-OH would surely overcome the low yield, however, the use of methanol-free dichloromethane will avoid this side reaction (consuming an activated ester of Boc-D-Asp(OMe)) to improve the coupling yield.[12] Compared with the first route described in Scheme 1, the second route was more suitable for a Kg scale preparation of the sodium salt of **1** in one batch.

In conclusion, we established a short-step and practical way to prepare a representative ET_A-selective antagonist, BQ-123Na, by considering its structural and physicochemical properties. Using thus obtained BQ-123Na, the *in vivo* activity of an ET_A-selective antagonist is currently under evaluation.

Boc-Pro-D-Val-Leu-OBzl: Boc-D-Val-Leu-OBzl (231.5 g, 0.551 mol), prepared from Leu-OBzl · TsOH and Boc-D-Val-OH in the presence of N-methylmorpholine (NMM), HOBT and EDCI using dichloromethane (DCM) as a solvent, was suspended in 4N HCl-dioxane (1000 ml) and DCM (100 ml). The suspension was stirred at rt for 1 hr and concentrated under reduced pressure. The residual dioxane was removed *in vacuo* for 16 hr to afford H-D-Val-Leu-OBzl · HCl (196.5 g, quant.). To the suspension of the HCl salt (196.5 g, 0.551 mol) in DCM (1000 ml) were added NMM (67 ml, 0.606 mol), Boc-Pro-OH (120.4 g, 0.560 mol), and HOBT (101.2 g, 0.606 mol) at 0 - 5 °C. To the resulting solution were added NMM (10 ml) and EDCI (116.6 g, 0.606 mol) at 0 - 5 °C using 150 ml of DCM. The mixture was stirred at rt for 16 hr, then washed with sat. $NaHCO_3$ (500 ml x 4), 5% $KHSO_4$ (500 ml x 4) and brine (500 ml x 2), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was dried *in vacuo* for 4 hr to give Boc-Pro-D-Val-Leu-OBzl (279.2 g, 98%).

Boc-D-Asp(OMe)-Pro-D-Val-Leu-OBzl: To Boc-Pro-D-Val-Leu-OBzl (273.7 g, 0.529 mol) was added 4N HCl-dioxane in four portions (860 ml) at 0 - 5 °C over a period of 2 hr. The mixture set to gel. The gel was concentrated under reduced pressure. The resulting material was dried *in vacuo* for 17 hr to give H-Pro-D-Val-Leu-OBzl · HCl (240.1 g, quant.). To the HCl salt (240.1 g, 0.529 mol) were added DCM (800 ml), NMM (64 ml, 0.582 mol), HOBT (97.2 g, 0.635 mol), and Boc-D-Asp(OMe)-OH (137.3 g, 0.556 mol) at rt. To the resulting solution was added EDCI (112 g, 0.582 mol) at 0 - 5 °C. The mixture was stirred at rt for 11 hr. Since the pH value of the reaction mixture (initially ca. 8) dropped to ca. 4, 60 ml of NMM was added. One hour later, to the mixture were added Boc-D-Asp(OMe)-OH (3.0 g, 12 mmol), HOBT (2.05 g, 13.4 mmol), and EDCI (2.57 g, 13.4 mmol). The reaction mixture was stirred at rt for additional 4 hr, then washed with 5% $NaHCO_3$ (500 ml x 4), 5% $KHSO_4$ (500 ml x 6) and brine (500 ml x 2), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an orange foam, which was recrystallized from CH_2Cl_2-/hexane (300 ml/750 ml, 300 ml/1500 ml) to afford the first and the second crops of Boc-D-Asp(OMe)-Pro-D-Val-Leu-OBzl (137.7 g, 7.8 g). The residual yellow foam (160.9 g) was triturated with EtOAc (250 ml) to afford Pro-D-Val-Leu-OBzl (11.2 g). The mother liquid was concentrated under reduced pressure and the residue was recrystallized from EtOAc (200 ml) to give the third crop of the tetrapeptide (37.7 g). The residue of the mother liquid was recrystallized from EtOAc/hexane (150 ml/400 ml) (at 4 °C, 16 hr) to give a fourth crop of the tetrapeptide (29.4 g). The residual oil (89.5 g) was triturated with EtOAc/hexane to afford the fifth crop of the tetrapeptide (43.7 g). The mother liquid was concentrated and the residue was triturated with hexane to give the second crop of the tripeptide (4.7 g). Overall, 256.3 g (75%, 81% estimated yield based on the consumed tripeptide) of the tetrapeptide was obtained and 15.9 g (7.2%) of the tripeptide was recovered.

Z-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OBzl: To 4N HCl-dioxane (500 ml) were added Boc-D-Asp(OMe)-Pro-D-Val-Leu-OBzl (an aliquot of the first four crops, 191.4 g, 296 mmol) at 0 - 5 °C. The mixture was stirred at rt for 1.5 hr and then concentrated under reduced pressure. The residue was dried *in vacuo* for 24 hr to give the Boc-deprotected tetrapeptide hydrochloride (206.5 g, 120%; dioxane was not completely removed) as a slightly viscous solid. The HCl salt was dissolved in DCM (500 ml) and NMM (40 ml) at 0 - 5 °C. To the solution were added HOBT (52.1 g, 341 mmol) and Z-D-Trp-OH (105.2 g, 311 mmol) using 200 ml of DCM. EDCI (65.4 g, 341 mmol) was then added to the reaction mixture at 0 - 5 °C using 100 ml of DCM. The resulting reaction mixture was stirred at rt. Three hours later, it was found that the pH of the mixture had dropped to ca. 4. 24 ml of NMM and 50 ml of DCM were added to adjust the pH of the solution

to 7 - 8, and the mixture was stirred in a cold room (4 °C) for 60 hr. The reaction mixture was washed with 5% $NaHCO_3$ (500 ml x 4), 1N HCl (600 ml x 4) and brine (500 ml x 4), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was dried *in vacuo* for 24 hr to give Z-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OBzl (255.1 g, 99.5%).

H-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OH: Z-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OBzl (250.1 g, 288.8 mmol) was dissolved in methanol (750 ml). Under nitrogen atmosphere, 10% Pd-C (17.5 g) was added to the solution, and then nitrogen in the reaction vessel was replaced by hydrogen. The deprotection of the N-terminal Z and C-terminal benzyl groups was effected by hydrogenation under an atmospheric pressure of hydrogen at rt for two days. The reaction mixture was then filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was dried *in vacuo* and reconstituted in 95% MeOH-water (800 ml). 10% Pd-C (21 g) was added and the mixture was stirred under an atmospheric pressure of hydrogen for one day. The reaction mixture was filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was dried *in vacuo* for 16 hr and the resulting solid was pulverized. The powder was further dried *in vacuo* to give H-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OH (174.3 g, 94%).

***cyclo* (-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-):**[13] H-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OH thus obtained (172.0 g, 267.9 mmol) was separated into three portions (61 g, 61 g and 50 g) and each portion was submitted to cyclization as follows: To a solution of HOBT (21.8 g, 142.5 mmol) and EDCI (27.4 g, 142.5 mmol) in DMF (1500 ml) and DCM (1500 ml) was dropwise added a solution of H-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-OH (61.0 g, 95.0 mmol) in DMF (500 ml) and DCM (500 ml) at rt over a period of 28 hr, and the resulting solution was stirred 14 hr. The reaction mixture was concentrated under reduced pressure (15 mmHg) and then *in vacuo* (ca. 0.2 mmHg). The residue was partitioned between DCM (800 ml) and 5% $NaHCO_3$ (500 ml). The organic layer was washed with 5% $NaHCO_3$ (500 ml x 2), 1N HCl (500 ml x 4) and brine (500 ml x 3), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was triturated with EtOAc/hexane and the resulting solid was dried *in vacuo* to give *cyclo* (-D-Trp-D-Asp-(OMe)-Pro-D-Val-Leu-) (57.5 g, 97%) as a pale yellow solid. Overall, 161.4 g (97%) of the cyclic pentapeptide methyl ester was obtained.

A sodium salt of BQ-123:[14] *cyclo* (-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-) (156.7 g, 251.1 mmol) was dissolved in methanol (930 ml). To the solution was added dropwise 2N NaOH (146 ml) at 0 - 5 °C over a period of 1 hr. The resulting solution was stirred at rt for 22 hr. Additional 2N NaOH (24 ml) was then added and the mixture was stirred at rt for 1.5 hr. The reaction mixture was concentrated under reduced pressure and dried *in vacuo*. The residue (153.8 g) was recrystallized from methanol (960 ml). The crystals were collected by suction filtration and washed with a minimum amount of cold methanol to give *cyclo*(-D-Trp-D-Asp(ONa)-Pro-D-Val-Leu-) (104.9 g) as a colorless solid. The filtrate and washings were concentrated under reduced pressure and the residual solid was recrystallized from methanol (300 ml) to give the second crop (20.0 g). The purities of the first and second crops were found to be 99.6% and 98.4% (area %), respectively, by HPLC analysis. The first crop was pulverized and dried *in vacuo* at 100 °C to give BQ-123Na (98.42 g, 62%) as a colorless hygroscopic crystalline powder (The residual methanol was estimated 0.07% by GC analysis).

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10. Purity of BQ-123Na was standardized as follows: Clarity and color of solution (1% in H_2O): clear and colorless; Purity (HPLC, area %): > 99.0%; Residual solvent (MeOH, GC): < 0.1%; Optical Rotation:$[\alpha]_D = +77 \pm 5$ ° (c 1.0, H_2O, 20 °C).
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12. In a mmol scale preliminary experiment, 1.05 - 1.20 equiv of Boc-D-Asp(OMe)-OH was condensed with Pro-D-Val-Leu-OBzl • HCl in 96 - 99% yield by using methanol-free DCM as a solvent.
13. ***cyclo* (-D-Trp-D-Asp(OMe)-Pro-D-Val-Leu-)**: mp: 144 - 150 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) : 0.73 (3H, d, J = 6.4 Hz, Me of Leu), 0.76 (3H, d, J = 6.4 Hz, Me' of Leu), 0.85 (3H, d, J = 6.9 Hz, Me of D-Val), 0.92 (3H, d, J = 6.9 Hz, Me' of D-Val), 1.20 - 2.22 (7H, m, γ-CH and β-CH_2 of Leu, β- CHH' and γ-CH_2 of Pro, and β-CH of D-Val), 2.35 - 2.50 (1H, m, β-CHH' of Pro), 2.37 (1H, dd, J = 3.9 Hz, 16.3 Hz, β-CHH' of D-Asp), 2.86 (1H, dd, J = 9.7 Hz, 16.3 Hz, β-CHH' of D-Asp), 3.27 (1H, dd, J = 5.0 Hz, 14.6 Hz, β-CHH' of D-Trp), 3.33 - 3.55 (2H, m, δ CH_2 of Pro), 3.44 (1H, dd, J = 6.6 Hz, 14.6 Hz, β-CHH' of D-Trp), 3.67 (3H, s, -$COOCH_3$), 3.73 (1H, q, J = 7.6 Hz, α-CH of Leu), 3.91 (1H, t, J = 9.7 Hz, α-CH of D-Val), 4.75 (1H, dt, J = 5.0 Hz, 6.6 Hz, α-CH of D-Trp), 4.79 (1H, d-like, J = 6.8 Hz, α-CH of Pro), 5.14 (1H, dt, J = 3.9 Hz, 9.7 Hz, α-CH of D-Asp), 6.09 (1H, d, J = 7.6 Hz, NH of Leu), 6.57 (1H, d, J = 6.6 Hz, NH of D-Trp), 7.05 (1H, d, J = 2.1 Hz, indole-2), 7.11 (1H, t, J = 7.7 Hz, indole-5), 7.12 (1H, d, J = 9.7 Hz, NH of D-Asp), 7.22 (1H, t, J = 7.7 Hz, indole-6), 7.39 (1H, d, J = 7.7 Hz, indole-7), 7.58 (1H, d, J = 7.7 Hz, indole-4), 7.70 (1H, d, J = 9.7 Hz, NH of D-Val), 8.22 (1H, d, J = 2.1 Hz, indole-NH); IR (KBr, cm^{-1}): 3304, 2962, 1737, 1662, 1536, 1443, 1374, 1344, 1302, 1236, 744; High Resolution FAB-MS (m/e for $(C_{32}H_{44}N_6O_7 + H)^+$): Calcd: 625.3350; Found: 625.3372.
14. **BQ-123Na**: mp: >300 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.61 (3H, d, J = 6.6 Hz, CH_3 of Leu), 0.72 (3H, d, J = 6.4 Hz, CH_3 of Leu), 0.81 (3H, d, J = 7.6 Hz, CH_3 of D-Val), 0.84 (3H, d, J = 7.3 Hz, CH_3 of D-Val), 0.90 - 1.07 (1H, m, γ-CH of Leu), 1.08 - 1.30 (2H, m, β-CH_2 of Leu), 1.46 - 2.08 (5H, m, β- and γ-CH_2 of Pro, β-CH of D-Val and β-CH_2 of D-Asp), 2.18 - 2.31 (1H, m, γ-CH_2 of Pro), 2.61 (1H, dd, J = 11.0 Hz, 14.5 Hz, β-CH_2 of D-Asp), 2.87 (1H, dd, J = 11.9 Hz, 14.5 Hz, β-CH_2 of D-Trp), 3.08 - 3.25 (2H, m, δ-CH_2 of Pro and β-CH_2 of D-Trp), 3.49 - 3.61 (1H, m, δ-CH_2 of Pro), 4.00 - 4.16 (2H, m, α-CH of D-Val and Leu), 4.16 - 4.29 (1H, m, α-CH of D-Trp), 4.66 (1H, d-like, J = 7.3 Hz, α-CH of Pro), 4.82 - 4.95 (1H, m, α-CH of D-Asp), 6.94 (1H, t, J = 7.4 Hz, indole-5), 7.03 (1H, t, J = 7.4 Hz, indole-6), 7.11 (1H, d, J = 2.3 Hz, indole-2), 7.27 (1H, d, J = 7.4 Hz, indole-7), 7.30 (1H, d, J = 7.8 Hz, NH of D-Asp), 7.52 (1H, d, J = 7.4 Hz, indole-4), 7.94 (1H, d, J = 9.5 Hz, NH of D-Val), 8.65 (1H, d, J = 6.1 Hz, NH of Leu), 8.77 (1H, dd, J = 1.3 Hz, 8.0 Hz, NH of D-Trp), 10.79 (1H, d, J = 2.3 Hz, NH of indole); High Resolution FAB-MS (m/e for $(C_{31}H_{42}N_6O_7 + H)^+$): Calcd: 611.3193; Found: 611.3206; EA: Found: C, 56.80; H, 7.09; N, 12.59%; Calcd for $C_{31}H_{41}N_6O_7Na \cdot 1.5H_2O$: C, 56.44; H, 6.72; N, 12.74%.

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