Supplementary Material

Synthesis of CJ-15,208, a novel κ-opioid receptor antagonist

Nicolette C. Ross,[†] Santosh S. Kulkarni,[†] Jay P. McLaughlin[‡] and Jane V. Aldrich*[,†]

[†]*Department of Medicinal Chemistry, The University of Kansas, Lawrence, KS 66045;* [‡]*Department of Psychology, Northeastern University, Boston, MA 02115*

CONTENTS

Abbreviations

DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; PyBOP (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

Materials

2-Chlorotrityl chloride resin (100-200 mesh, 1% divinylbenzene), PyBOP and all N^{α}-Fmoc protected amino acids were purchased from Novabiochem (San Diego, CA). DIEA and HOBt were purchased from Fluka (Milwaukee, WI), and piperidine was obtained from Aldrich (Milwaukee, WI). All solvents for peptide synthesis (HPLC grade DMF and DCM) and HPLC (HPLC grade MeCN and MeOH) and TFA (Pierce Sequanal grade) were purchased from Fisher Scientific (Pittsburgh, PA).

HPLC Analysis and Purification

The peptides were purified by preparative reversed phase HPLC (Shimadzu SCL-10A VP system equipped with a Shimadzu SPD-10A VP detector) on a Vydac C18 column (10 μ, 300 Å, 22 x 250 mm) equipped with a Vydac guard column. The cyclic tetrapeptides were purified using a linear gradient of 30-70% MeOH over 40 min, at a flow rate of 18 mL/min, with monitoring at 220 nm.

The purity of the final peptides was verified by analytical HPLC (Shimadzu SCL-10A VP system equipped with a Shimadzu SPD-10A VP detector) on a Vydac C18 column (5 μ, 300 Å, 4.6 x 50 mm) equipped with a Vydac guard column. The solvent systems used to evaluate purity are listed in Table S1.

Table S1. HPLC conditions used for analysis of the peptides. All methods were performed with a 1 min delay before starting the gradient.

	Method 1	Method 2	Method 3	Method 4	Method 5
Solvent A	Aqueous 0.1% TFA	H_2O	Aqueous 0.1% TFA	Aqueous 0.1% TFA	H_2O
Solvent B	MeCN containing 0.1% TFA	MeOH	MeOH containing 0.1% TFA	MeCN containing 0.1% TFA	MeOH
Gradient	5-50% Solvent B over 45 min	30-70% Solvent B over 40 min	15-55% Solvent B over 40 min	30-70% Solvent B over 40 min	50-90% Solvent B over 40 min
Flow rate	1 mL/min	0.8 mL/min	0.8 mL/min	1 mL/min	0.8 mL/min
Wavelength	214 nm	220 nm	220 nm	214 nm	220 nm

Mass Spectrometry

ESI spectra were acquired on a LCT Premier (Waters Corp., Milford MA) time of flight mass spectrometer. Mass correction for exact mass determinations were made automatically with the lock mass feature in the MassLynx data system. A reference compound ($(M+H)^+$ of leucine enkephalin) in an auxiliary sprayer was sampled every third cycle by toggling a "shutter" between the analysis and reference needles, and the reference mass used for linear mass correction of the analytical cycles. Samples in acetonitrile were injected using an autoinjector (LC PAL, CTC Analytics AG, Zwingen, Switzerland).

Optical Rotation

Optical rotations of the cyclic tetrapeptides were measured in DMSO at 24 °C on an AUTOPOL® Automatic Polarimeter (Rudolph Research, Hackettstown, NJ).

Solid Phase Synthesis of Linear Peptides

Linear peptides were synthesized by a standard Fmoc solid phase synthesis protocol[1] using a custom made manual peptide synthesizer (CHOIR)[2] constructed in house and equipped with 15 mL polypropylene syringes fitted with polytetrafluoroethylene (PTFE) frits. Swelling of the resin, washing, deprotection of the Fmoc group, and capping steps were all performed using 5 mL solvent or solution per 0.5 g resin, and the resin agitated during the reactions and washes using N_2 gas.

Loading of the C-terminal amino acid on to the resin: 2-Chlorotrityl resin (0.50 g, initial loading 1.4 mmol/g) was swollen in DCM (2 x 10 min) and the solvent drained. DIEA (0.61 mL, 3.5 mmol) was added to a solution of Fmoc-Phe or Fmoc-Trp (1.4 mmol) in DCM/DMF (4:1, 5 mL), and the resulting solution added to the resin. The

mixture was gently agitated with N_2 gas for 6 h. DCM was added to the solution every 30 min to maintain the solvent volume, and additional DIEA (0.61 mL, 3.5 mmol) was added to the reaction every 2 h. Following draining the reaction mixture, the resin was washed with DCM/DMF (1/1, 5x). Quantitative Fmoc analysis[1] was used to determine the loading efficiency. A capping step was then performed using 15% MeOH and 5% DIEA in DCM for 2 x 10 min, and the resin washed with DCM/DMF (1:1, 5x).

Peptide chain assembly: The Fmoc group was removed using a solution of 20% piperidine in DMF for 2 x 20 min, and the resin washed with DCM/DMF (1:1, 5x) and DCM (5x). Fmoc-protected amino acids (2 equiv) were coupled using PyBOP (2 equiv), HOBt (2 equiv) and DIEA (5 equiv) in DCM/DMF (1:1, 5 mL) for 2-4 h. The resin was washed after the coupling reactions with DCM/DMF (1:1, 5x) and DCM (5x). Coupling reactions were monitored to determine completion using the Kaiser test for primary amines or the chloranil test for the secondary amine of Pro. The deprotection/coupling cycle was repeated to assemble the peptide chain. Finally, the resin was washed with DCM/DMF (1:1, 5x), DCM (10x), *i*PrOH (2x), hexane (2x), DCM (2x), MeOH (2x), and finally DCM (2x).

Cleavage: The peptides were cleaved from the resin using a solution of 1% TFA in DCM (85 mL total per 0.5 g of resin). The TFA solution was bubbled through the resin (5 mL x 2 min) and the cleavage solution was drained into a round bottom flask. This procedure was repeated until all 85 mL of the cleavage solution was collected in the round bottom flask. Following the cleavage, the resin was washed with DCM (2x) and MeOH (2x). The combined solutions were evaporated to give the crude peptides.

H-Phe-D-Pro-Phe-Trp-OH

Fmoc-Trp-OH (0.60 g, 1.4 mmol, 2 equiv) was loaded onto the resin and the tetrapeptide assembled using the procedures described above. Following the final Fmoc deprotection and cleavage from the resin, the crude peptide was obtained as a tan colored solid (221 mg): HPLC t_R = 18.6 min (method 1) and t_R = 23.4 min (method 3); ESI-MS *m/z* 596.28 observed, 596.29 calculated $(M+H)^+$. The crude peptide was ≥98% pure by analytical HPLC and was used in the cyclization without purification.

H-D-Trp-Phe-D-Pro-Phe-OH

Fmoc-Phe-OH (0.55 g, 1.4 mmol, 2 equiv) was loaded onto the resin and the tetrapeptide assembled using the procedures described above. Following the final Fmoc deprotection and cleavage from the resin, the crude peptide was obtained as a tan colored solid (310 mg): HPLC t_R = 19.8 min (method 1) and t_R = 26.6 min (method 3); ESI-MS *m/z* 596.28 observed, 596.29 calculated $(M+H)^+$. The crude peptide was ≥98% by analytical HLPC and was used in the cyclization without purification.

Cyclization of the Peptides:

L-Trp Isomer of CJ-15,208

Cyclization of H-Phe-D-Pro-Phe-Trp-OH (50 mg, 0.072 mmol) as described above gave the L-Trp isomer of CJ-15,208 as a tan colored solid (95% cyclic tetrapeptide by HPLC, method 2). Purification by preparative HPLC gave the cyclic tetrapeptide (29 mg, 71% yield) as a white solid. HPLC and ESI-MS spectra are shown in Figures S1-S2; purity was >99% in both analytical HPLC systems. The cyclic tetrapeptide could be readily distinguished from the dimeric cyclic octapeptide[3] based on HPLC (see Figures S1 and S5, respectively) and mass spectra (Figures S2 and S6, respectively).

D-Trp Isomer of CJ-15,208

Cyclization of H-D-Trp-Phe-D-Pro-Phe-OH (50 mg, 0.072 mmol) in two portions as described above gave the D-Trp isomer of CJ-15,208 as a tan colored solid (95% cyclic tetrapeptide by HPLC, method 2). Purification by preparative HPLC gave the cyclic tetrapeptide (13 mg, 32% yield) as a white solid. HPLC and ESI-MS spectra are shown in Figures S3-S4; purity was >99% in both analytical HPLC systems. The cyclic tetrapeptide could be readily distinguished from the dimeric cyclic octapeptide[3] based on HPLC (Figures S3 and S7, respectively) and mass spectra (Figures S4 and S8, respectively).

Kappa Opioid Receptor Ligand Binding to Murine Brain Membranes.

Whole brains were dissected from C57Bl/6J mice; the cerebellum was removed and membranes were prepared as described previously.[4] A dounce homogenizer was used to homogenize tissue, and prepared membranes were used immediately in experiments to avoid changes resulting from freezing and thawing. The protein concentration of the membranes after harvest was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

The affinity of the L-Trp and D-Trp cyclic tetrapeptides for the kappa opioid receptor (KOR) was determined by incubating 0.5 mg of membrane protein with peptide ligand (0.1-1,000 nM) with 1 nM of the KOR-selective radioligand [^{3}H]U69,593 for 60 min at 25°C in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.5. Nonspecific binding was measured by inclusion of 10 μM naloxone. Binding was terminated by filtering the samples through Schleicher & Schuell No. 32 glass fiber filters (Keene, NH) using a Brandel 24-well cell harvester (Brandel, Gaithersburg, MD). Filters were first soaked for at least 60 min in 0.25% polyethylenimine prior to filtration. After filtration, filters were washed three times with 3 mL of cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 mL of Ecoscint A scintillation fluid.

IC_{50} values were calculated by a least squares fit to a logarithm-probit analysis. The K_i values of unlabeled compounds were calculated from the Cheng and Prusoff equation $K_i=IC_{50}/(1+[L]/K_D)$, where [L] = the concentration of radioligand;[5] the K_D for [^{3}H]U69,593 was 1.0 nM.

Acknowledgments

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References and Footnotes

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3. The linear octapeptides H-Phe-D-Pro-Phe-Trp-Phe-D-Pro-Phe-Trp-OH and H-D-Trp-Phe-D-Pro-Phe-D-Trp-Phe-D-Pro-Phe-OH were assembled using standard Fmoc solid phase synthesis protocol as described above. The crude peptides were ≥98% by analytical HLPC and were used in the cyclization without purification. The cyclic octopeptides were obtained as follows: A solution of the linear octapeptide (100 mg, 0.072 mmol) in DCM (20 mL) was added dropwise along with DIEA (0.54 mL, 0.31 mmol) to a solution of HATU (46 mg, 0.12 mmol, 1.7 equiv) in acetonitrile (25 mL) and DCM (56 mL) over a few minutes, and the reaction stirred at room temperature for 24 h. A second portion of HATU (46 mg, 0.12 mmol, 1.7 equiv) was added to the reaction in one portion along with DIEA (0.54 mL, 0.31 mmol), and the reaction stirred for an additional 24 h. Following removal of the solvent under reduced pressure the residue was dissolved in EtOAc or DCM (20 mL), and the solution washed with 2N citric acid (3x), saturated bicarbonate (3x) and brine (3x). The organic layer was separated, dried ($MgSO_4$), and the solvent was removed under reduced pressure to give the crude cyclic peptide.
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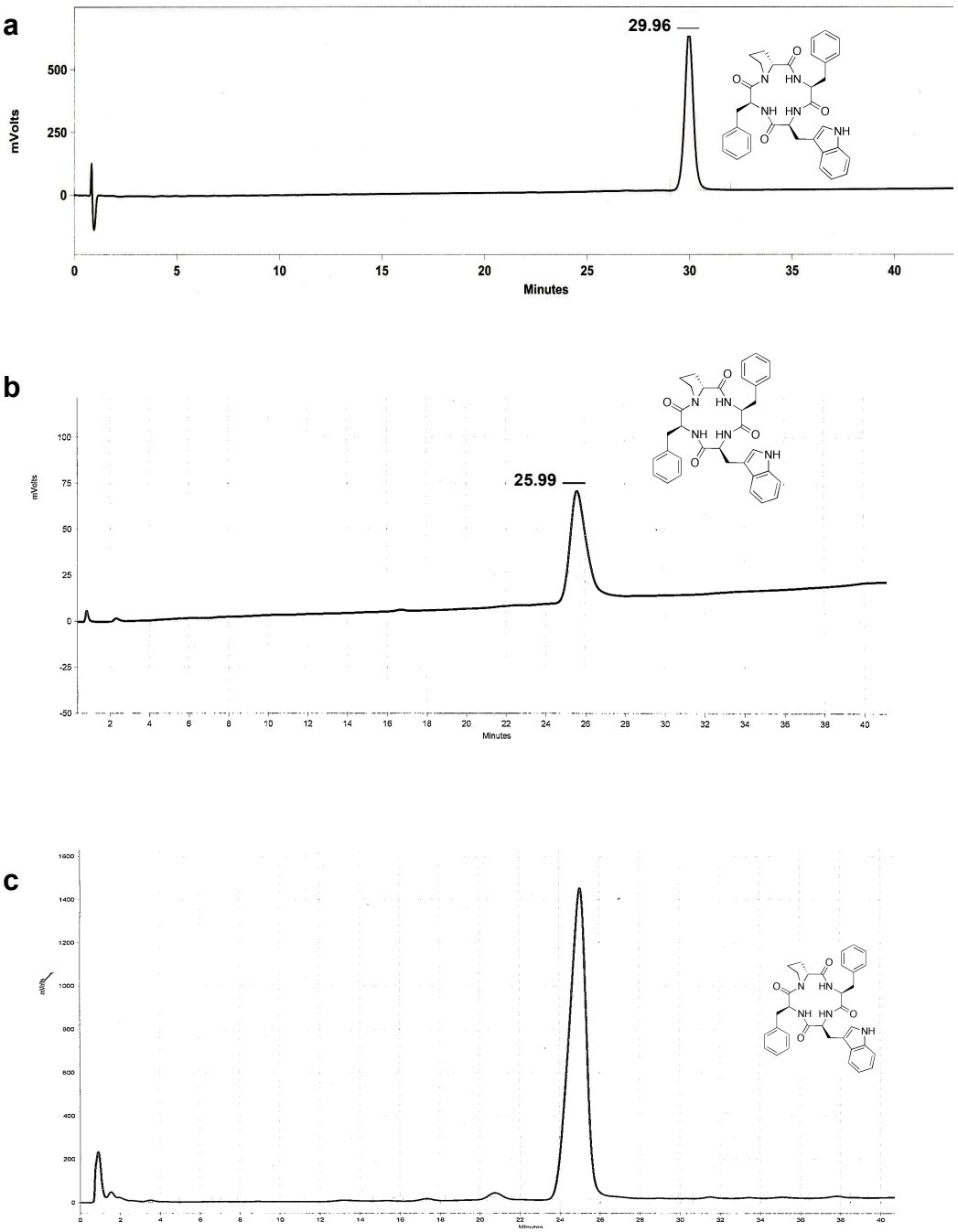


Figure S1. HPLC chromatograms of the purified L-Trp cyclic tetrapeptide in (a) method 1 and (b) method 2. (c) Chromatogram of the crude L-Trp isomer (method 2).

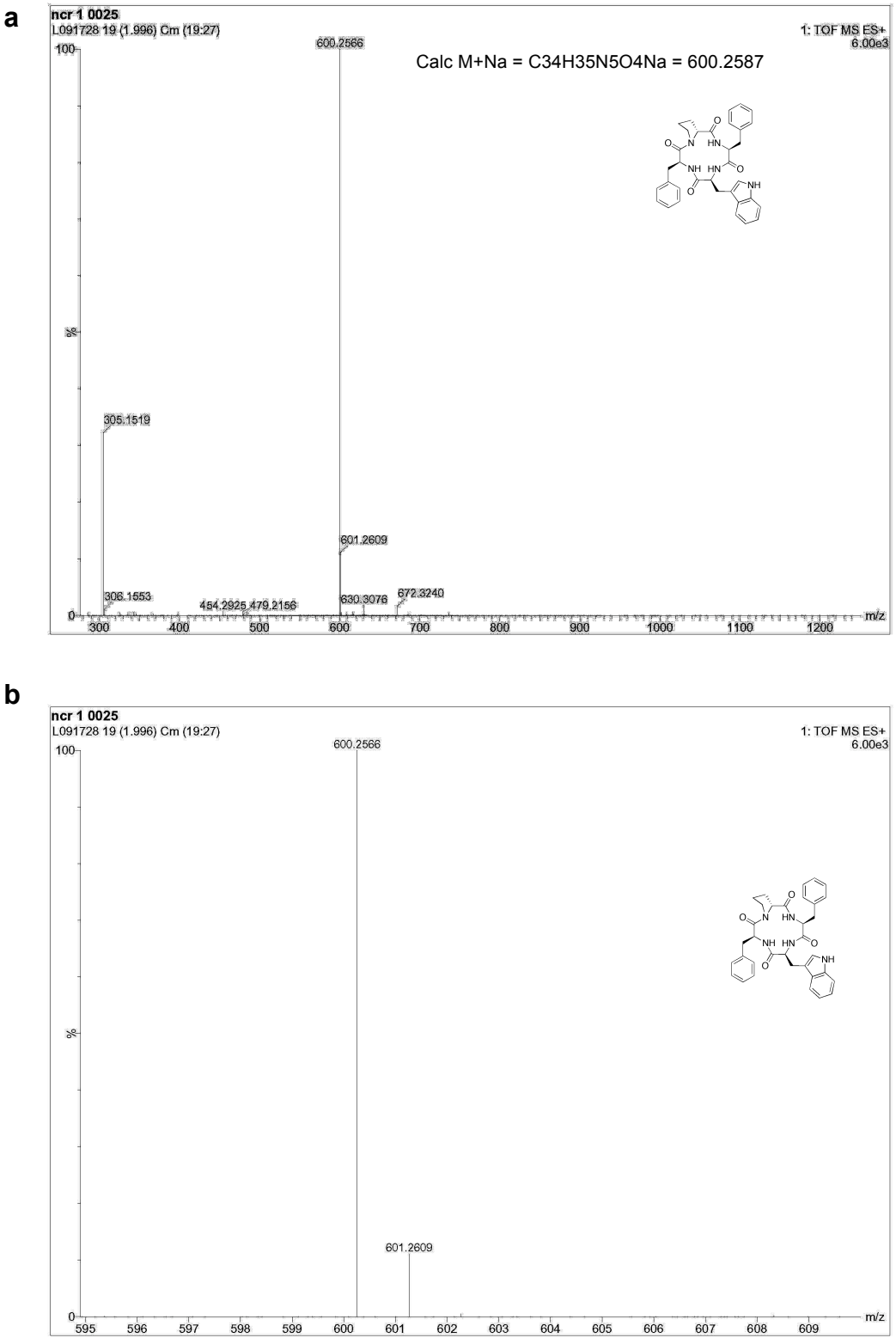


Figure S2. ESI-MS of the L-Trp cyclic tetrapeptide: (a) full spectrum, and (b) expansion of the region from 595-610 amu.

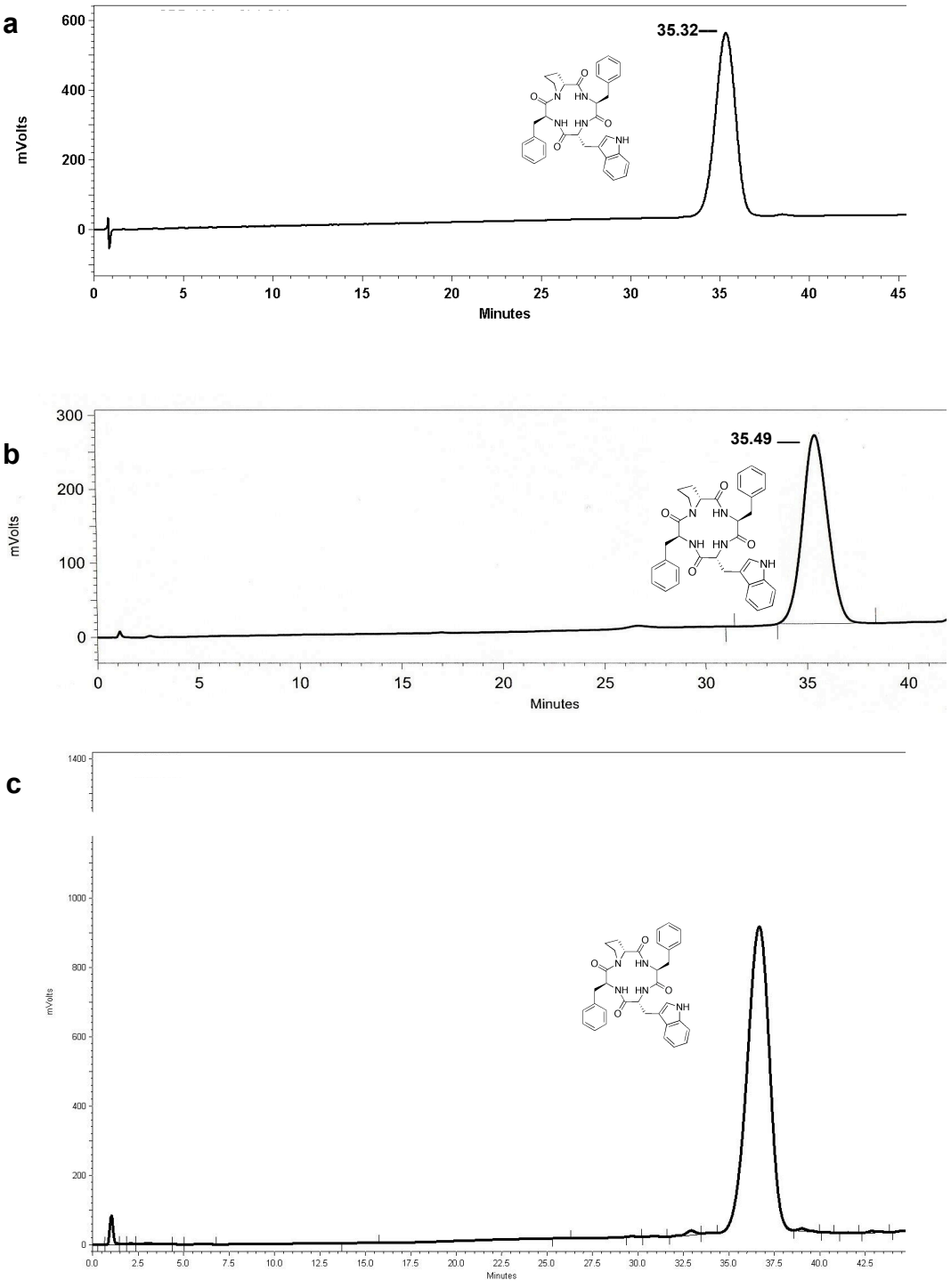
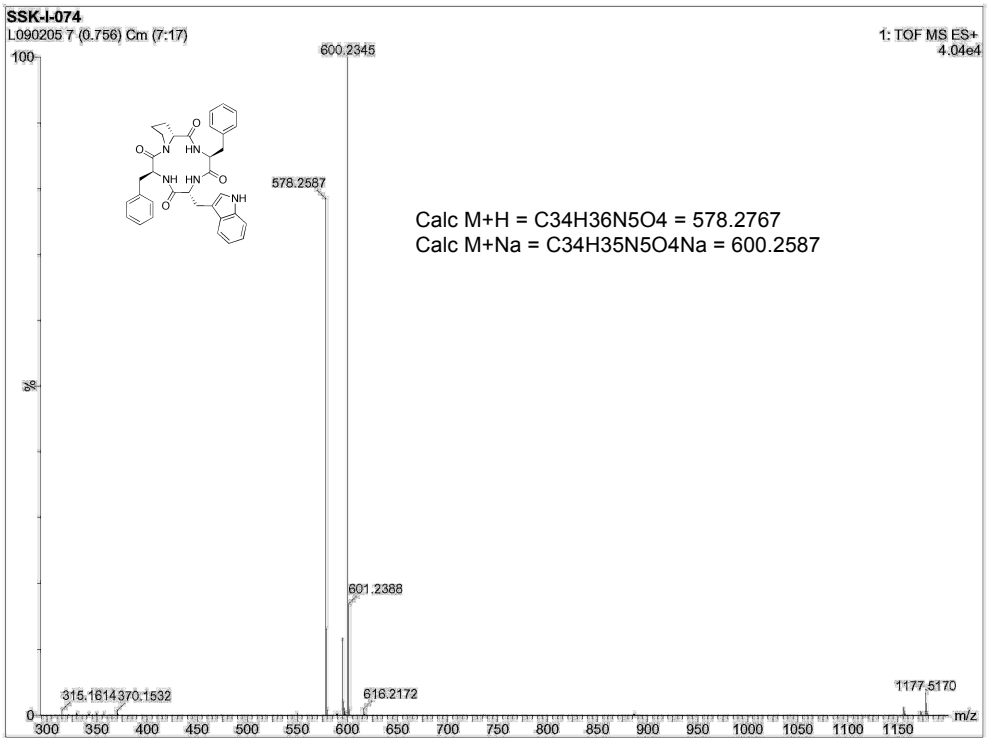


Figure S3. HPLC chromatograms of the purified D-Trp cyclic tetrapeptide in (a) method 1 and (b) method 2. (c) Chromatogram of the crude D-Trp isomer (method 2).

a



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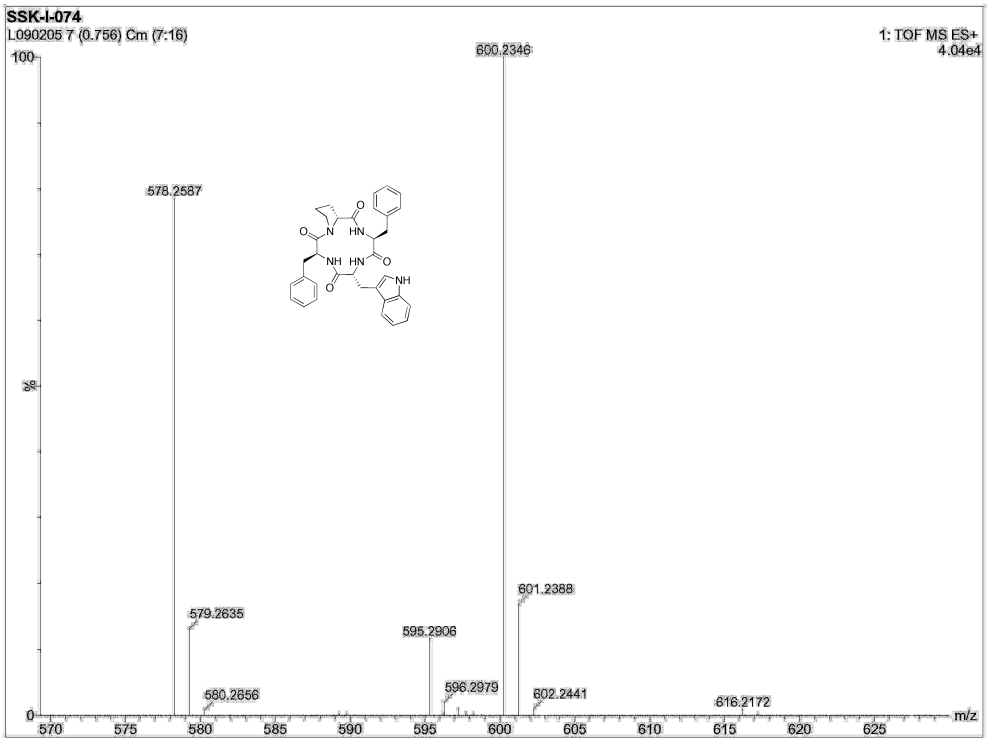

Figure S4. ESI-MS of the D-Trp cyclic tetrapeptide: (a) full spectrum, and (b) expansion of the region from 570-630 amu.

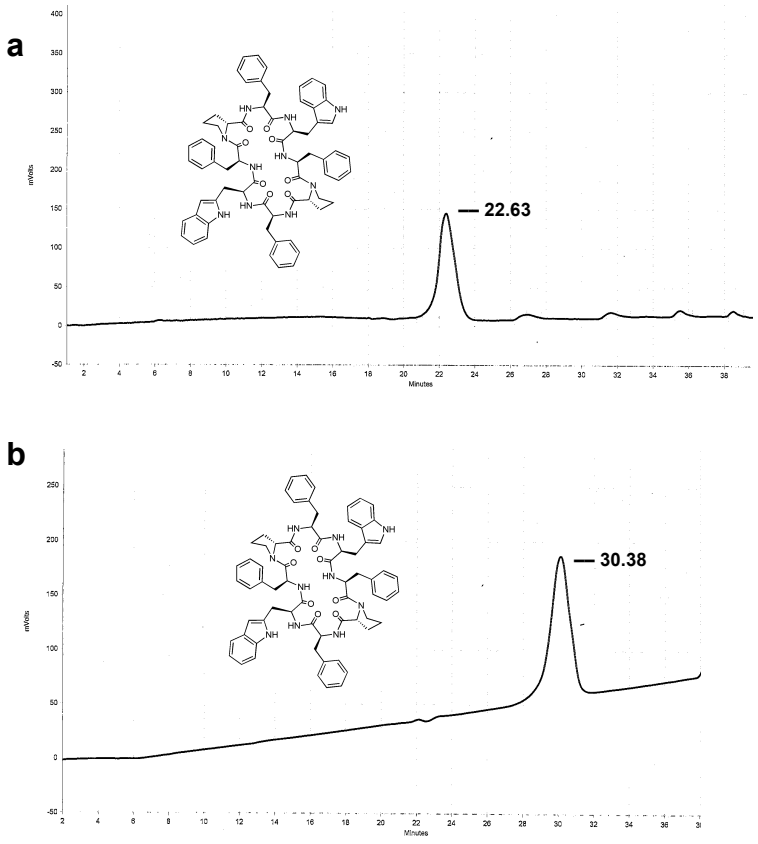

Figure S5. HPLC chromatograms of the purified L-Trp cyclic octapeptide in (a) method 4 and (b) method 5.

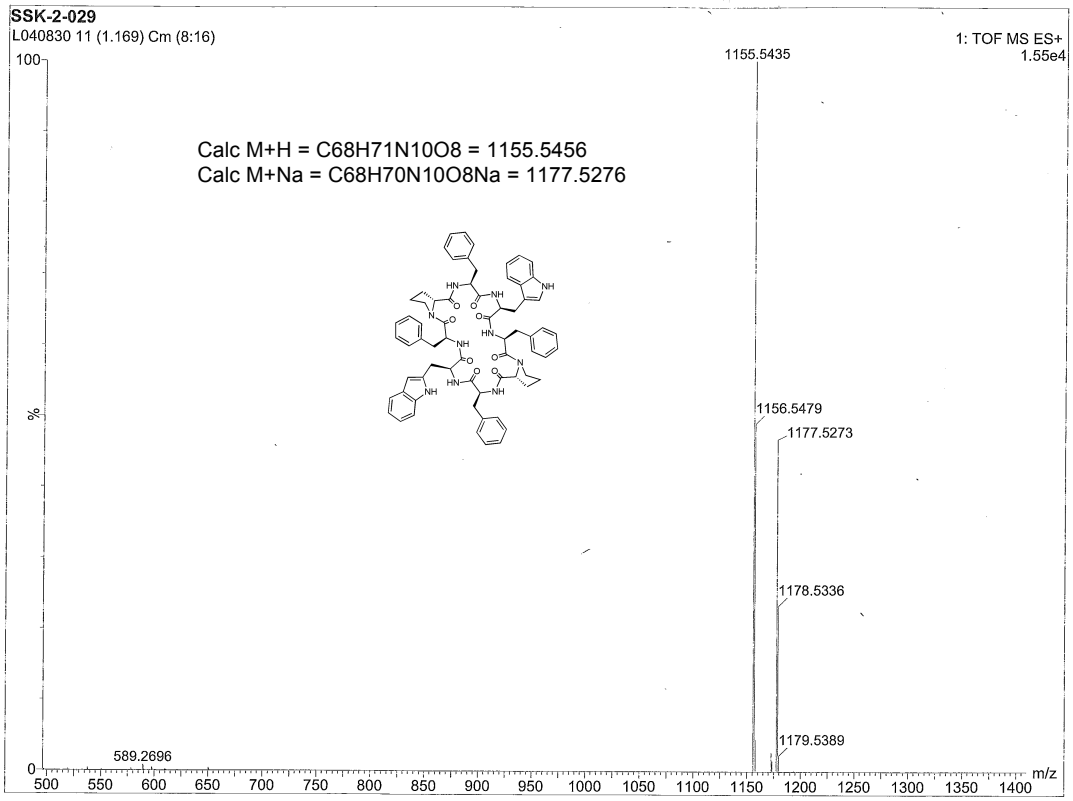

Figure S6. ESI-MS of the L-Trp cyclic octapeptide

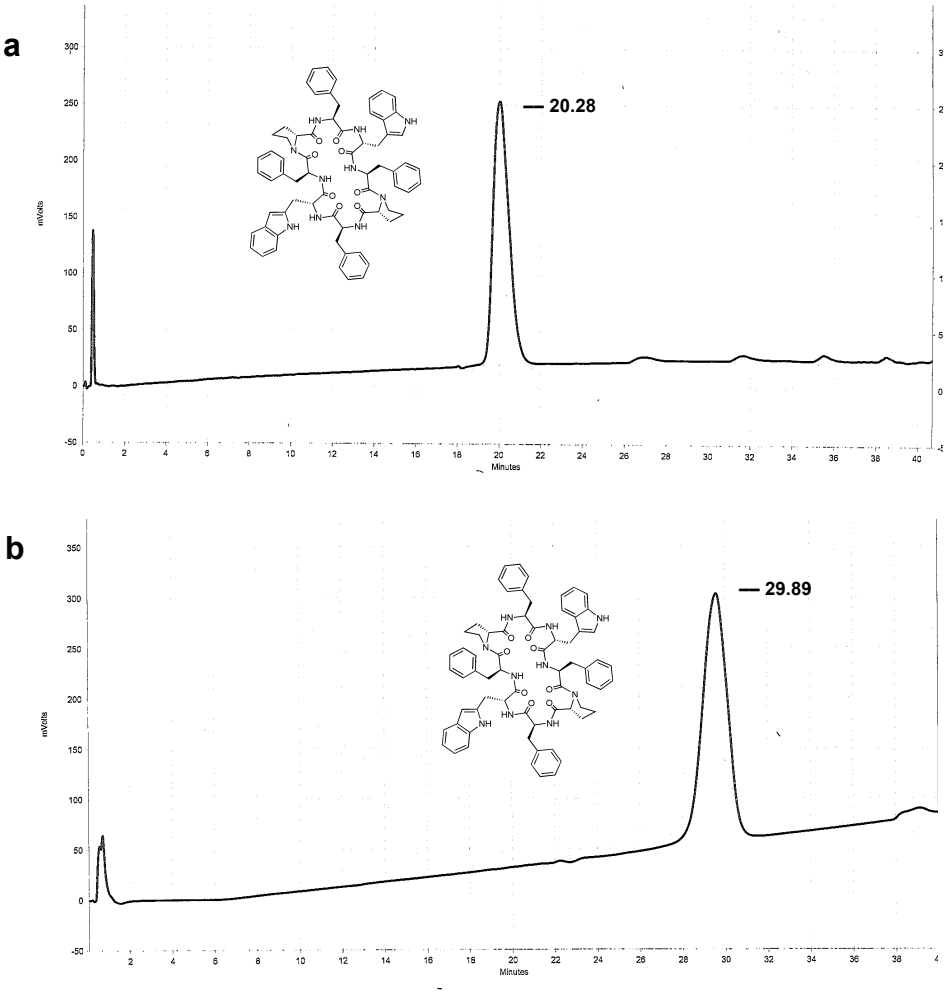

Figure S7. HPLC chromatograms of the purified D-Trp cyclic octapeptide in (a) method 4 and (b) method 5.

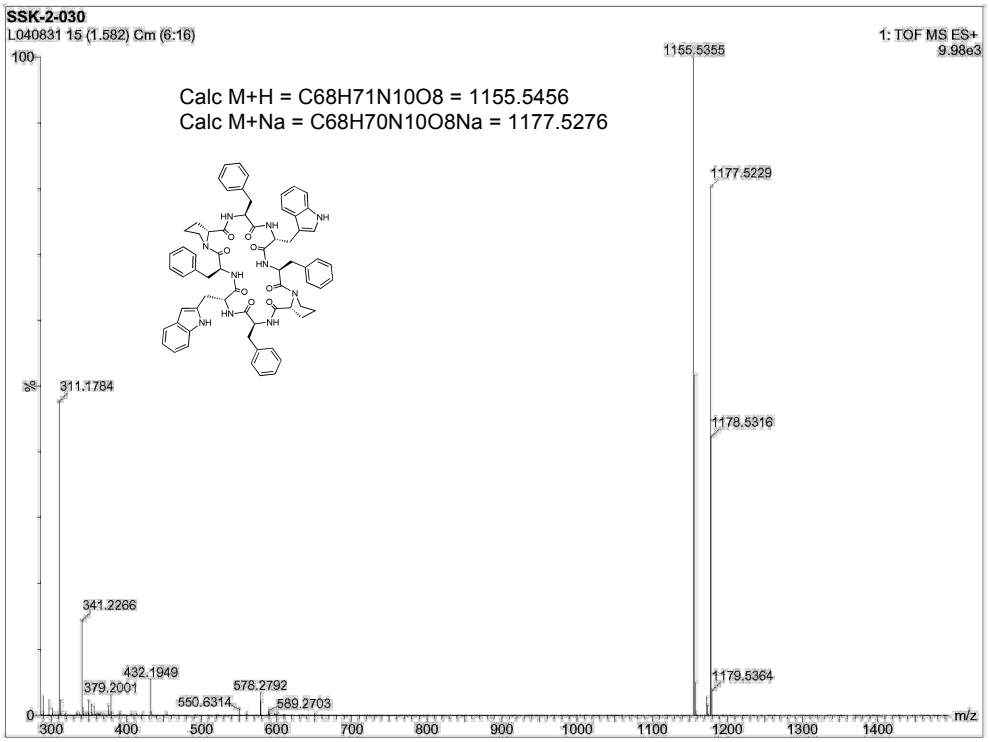

Figure S8. ESI-MS of the D-Trp cyclic octopeptide