

Supporting Information for *New journal of chemistry*

Synthesis of novel Phakellistatin 2 analogs with isoindole moiety and the conformation features affecting their antitumor activities

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Experimental

General

Boc-L-proline(15761-39-4), Boc-D-proline(37784-17-1), L-isoleucine(73-32-5), Boc-L-phenylalanine(13734-34-4), Di-tert-butyl dicarbonate(24424-99-5), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline(EEDQ, 16357-59-8), *N*-[(trimethylsilyl)methyl]benzylamine(53215-95-5), phthalylglycyl chloride(6780-38-7) and trifluoroacetic acid (TFA, 76-05-1) were purchased from Energy Chemical. Dichloromethane, methanol, ethyl acetate, petroleum ether, 1,4-dioxane were analytical reagent. Dulbecco's modied eagle medium (DMEM), penicillin, fetal bovine serum (FBS), and streptomycin were purchased from Beijing Dingguo Biotechnology Co. Phosphatebuffered saline (PBS) purchased from Invitrogen (10010) was used as a balanced salt solution in cell culture. All the solvents were distilled and purified by standard procedures. All the above chemicals reagents were used without further purification. ¹H and ¹³C-NMR spectra were recorded at 400 and 100 MHz, respectively, on an AMX400 spectrometer (Bruker, Bremen, Germany) with tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JEOL JMS-700 spectrometer using the fast atom bombardment (FAB) or electron impact (EI) mode. A 450 W Hanovia medium-pressure mercury lamp surrounded by a Pyrex glass filter ($\lambda > 290$ nm) was used for electronic excitation.

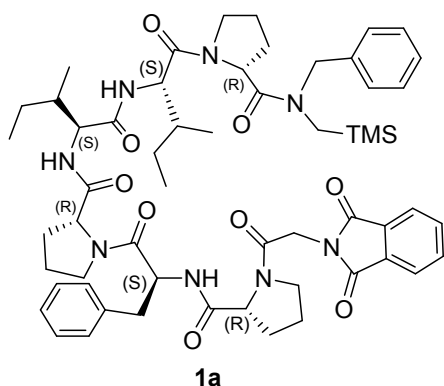
Preparation of trimethylsilylbenzylamido dipeptides

The Boc-proline(2.15 g, 10 mmol) and *N*-[(Trimethylsilyl) methyl]benzylamine (1.93 g, 10 mmol) was dissolved in 50 mL of anhydrous dichloromethane, EEDQ (3.78 g, 15 mmol in 10 mL of THF) was added dropwise with stirring at room temperature. After the reaction, the reaction solution was washed twice with 16 mL of water, the organic layer was dried over anhydrous sodium sulfate and concentrated, the residue was purified by silica gel column chromatography (mobile phase $V_{EA}/V_{PE} = 1:4$) to obtain pure *N*-Boc-Pro-Si(CH₃)₃ (a white solid). The *N*-Boc-Pro-Si(CH₃)₃ was dissolved in 10 mL of anhydrous dichloromethane and added dropwise 10 mL of trifluoroacetic acid, then stirred for 3 h. After removal of trifluoroacetic acid and dichloromethane was concentrated, the residue was dispersed in 20 mL of dichloromethane, washed twice with 10 mL of water, dried over anhydrous sodium sulfate and concentrated to give a chemically pure Pro-Si(CH₃)₃ 2.75 g (yellow oil). The Pro-Si(CH₃)₃ (2.03 g, 7 mmol) and Boc-Isoleucine (1.62 g, 7 mmol) was dissolved in 20 mL of anhydrous dichloromethane. EEDQ (2.59 g, 10.5 mmol in 10 ml of THF) was added dropwise with stirring at room temperature for 72h. After completion of the reaction, the reaction solution was washed twice with 10 mL of water, the organic layer was dried over anhydrous sodium sulfate and concentrated, and the residue was dissolved in 10 mL of anhydrous dichloromethane, added dropwise 3 mL of trifluoroacetic acid and stirred for 3h. After removal of trifluoroacetic acid dichloromethane, the residue was dispersed in 10 mL of dichloromethane, washed twice with 10 mL of water, dried over anhydrous sodium sulfate and concentrated to give Ile-Pro-Si(CH₃)₃ 2.35 g (white solid). The same method was used for synthesis of Ile-Ile-Pro-Si(CH₃)₃, Pro-Ile-Ile-Pro-Si(CH₃)₃, Phe-Pro-Ile-Ile-Pro-Si(CH₃)₃, Pro-Phe-Pro-Ile-Ile-Pro-Si(CH₃)₃.

Preparation of *N*-Phthalimido-Gly-Pro(D)-Phe-Pro(D)-Ile-Ile-Pro(D)-Si(CH₃)₃ and *N*-Phthalimido-Gly-Pro(L)-Phe-Pro(L)-Ile-Ile-Pro(L)-Si(CH₃)₃

Pro-Phe-Pro-Ile-Ile-Pro-Si(CH₃)₃ (0.75 g, 0.01 mol) and triethylamine (3 mL) were dissolved in anhydrous dichloromethane, then phthalimide acetyl chloride (0.25 g, 0.01 mol, in 3 mL of 1,4-dioxane) was added dropwise. After stirring at room temperature for 30 min, the reaction solution washed twice with 20 mL of water. The organic layer was dried over anhydrous sodium sulphate, then concentrated and purified by silica gel column

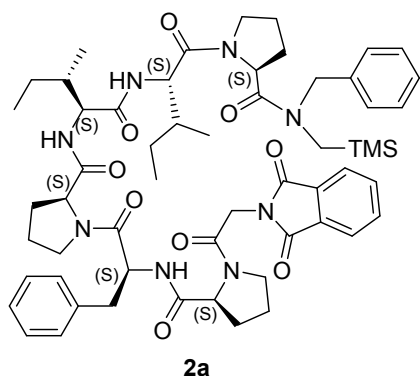
chromatography (mobile phase $V_{EA}/V_{PE} = 1:1$) to obtain a white solid. The similar method was used for synthesis of *N*-Phthalimido-Gly-Pro(D)-Phe-Pro(D)-Ile-Ile-Pro(D)-Si(CH₃)₃ (**1a**) and *N*-Phthalimido-Gly-Pro(L)-Phe-Pro(L)-Ile-Ile-Pro(L)-Si(CH₃)₃ (**2a**).



***N*-Phthalimido-Gly-Pro-Leu-Ile-Pro-Si(CH₃)₃ (**1a**)** white solid (yield 75%).

¹HNMR(CDCl₃, 400 MHz) δ : -0.07~0.01(m, 9H, SiMe₃), 0.67~0.90(m, 12H, CH₃), 0.97~1.17(m, 2H, CH₂CH₃), 1.21~1.33(m, 2H, CH₂CH₃), 1.35~1.48(m, 3H, CHCH₃ and NCH₂CH₂CH₂CH), 1.49~1.79(m, 4H, NCH₂CH₂CH₂CH and NCH₂CH₂CH₂CH), 1.85~2.03(m, 4H, NCH₂CH₂CH₂CH and NCH₂CH₂CH₂CH), 2.05~2.28(m, 2H, NCH₂CH₂CH₂CH), 2.29~2.38(m, 1H, CHCH₃), 2.40~2.43(d, 1H, CHHSiMe₃, $J = 15.2$ Hz), 2.85~2.93(m, 2H, CH₂Ph), 2.95~3.220(m, 2H, CH₂Ph), 3.29~3.33(d, 1H, CHHSiMe₃, $J = 15.2$ Hz), 3.45~3.78(m, 4H, NCH₂CH₂CH₂CH and NCH₂CH₂CH₂CH),

3.80~3.95(m, 1H CHCH₂Ph), 3.98~4.03(m, 1H, NCH₂CH₂CH₂CH), 4.13~4.32(m, 2H, NCH₂CH₂CH₂CH), 4.35~4.48(m, 2H, NCH₂CH₂CH₂CH), 4.50~4.83(m, 2H, NHCH₂CO), 4.85~4.98(m, 1H, HNCHCO), 5.01~5.23(m, 1H, HNCHCO), 7.15~7.79(m, 14H, ArH); ¹³CNMR(CDCl₃, 400 MHz) δ : -0.02, 12.1, 13.3, 16.6, 17.0, 23.3, 23.7, 25.7, 26.3, 30.4, 30.8, 33.8, 36.7, 41.1, 42.7, 47.6, 47.9, 48.4, 48.9, 52.9, 53.7, 57.0, 57.4, 59.4, 60.1, 61.2, 61.8, 62.1, 124.2, 124.5, 124.7, 127.8, 128.6, 129.3, 129.5, 129.7, 129.9, 130.6, 130.9, 133.3, 133.7, 134.6, 135.0, 135.2, 137.5, 137.8, 166.4, 169.0, 170.8, 171.9, 172.0, 172.3, 172.6, 172.9. HRMS (ESI) m/z calcd for C₅₇H₇₇N₈O₉Si⁺ (M+H)⁺ 1045.55773, found 1045.55750.



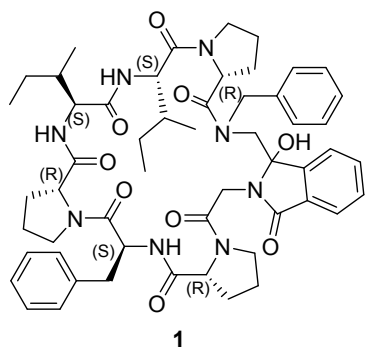
***N*-Phthalimido-Gly-Pro-Phe-Pro-Ile-Ile-Pro-Si(CH₃)₃ (**2a**)** white solid (yield 80%).

¹HNMR(CDCl₃, 400 MHz) δ : 0.03~0.13(m, 9H, SiMe₃), 0.84~1.11(m, 12H, CH₃), 1.12~1.25(m, 2H, (CHCH₃)₂), 1.80~1.98(m, 6H, (NCH₂CH₂CH₂CH)₃), 2.03~2.12(m, 6H, (NCH₂CH₂CH₂CH)₃), 2.19~2.23(m, 4H, (CH₂CH₃)₂), 2.66~2.69(d, 1H, CHHSiMe₃, $J = 14.8$ Hz), 2.86~2.97(m, 2H, CH₂Ph), 3.06~3.10(d, 1H, CHHSiMe₃, $J = 14.8$ Hz), 3.20~3.95(m, 6H, (NCH₂CH₂CH₂CH)₃), 4.20~4.30(m, 2H, CH₂Ph), 4.43~4.60(m, 5H, (NCH₂CH₂CH₂CH)₃ and (HNCHCO)₂), 4.76~4.89(m, 3H, NHCH₂CO and CHCH₂Ph), 7.19~7.89(m, 14H, ArH); ¹³CNMR(CDCl₃, 400 MHz) δ : -1.2, 11.0,

11.4, 15.4, 15.7, 24.4, 24.6, 24.8, 25.0, 25.1, 25.2, 27.1, 27.2, 27.3, 27.4, 29.6, 36.7, 37.5, 38.5, 39.3, 39.7, 46.4, 47.4, 47.7, 50.4, 52.2, 53.3, 54.9, 56.4, 58.3, 60.1, 60.2, 123.6, 126.7, 126.8, 127.5, 128.3, 128.5, 128.8, 129.5, 132.2, 134.1, 136.4, 137.1, 165.8, 167.8, 170.0, 170.2, 170.8, 171.0, 171.1, 171.2, 171.5. HRMS (ESI) m/z calcd for C₅₇H₇₇N₈O₉Si⁺ (M+H)⁺ 1045.55773, found 1045.55701.

Irradiation of compound 1a and compound 2a to obtain compound 1 and compound 2

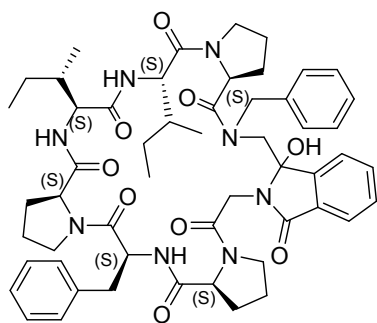
Nitrogen purged solutions of the substrates in the indicated solvents were irradiated by using Pyrex glass filtered light in a water cooled immersion reactor for time periods required. Concentration of the photoproducts were followed by column chromatography to yield the pure products listed below. In brief, 0.5 g of compound **1a** or compound **2a** in 200 mL of anhydrous methanol were placed in a reactor, then ventilated nitrogen flow for 30 min. Upon maintaining the ventilation of nitrogen, the solutions were irradiated by ultraviolet light (Pyrex tube filtered-light $\lambda > 290$ nm).



1

3-Hydroxy-isoindolinone-cyclo-Gly-Pro(D)-Phe-Pro(D)-Ile-Ile-Pro(D) (1): white solid (yield 45%). $^1\text{H NMR}(\text{CDCl}_3, 400 \text{ MHz}) \delta$: 0.80~0.92(m, 12H, CH_3), 0.93~1.02(m, 4H, CH_2CH_3 and CH_2CH_3), 1.28~1.37(m, 2H, CHCH_3 and CHCH_3), 1.70~1.78(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.80~1.98(m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.00~2.41(m, 6H, $(\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH})_3$), 2.48~2.59(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.60~2.95(m, 1H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.98~3.20(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.33~3.49(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.50~3.79(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.80~3.90(m, 1H, HNCHCO), 3.95~4.28(m,

2H, CH_2Ph), 4.30~4.38(m, 1H, HNCHCO), 4.40~4.49(m, 2H, CH_2Ph), 4.50~4.89(m, 4H, NCH_2CO and $\text{NCH}_2\text{C(OH)}$), 4.92~5.13(m, 1H, CHCH_2Ph), 7.09~8.03(m, 14H, ArH); $^{13}\text{C NMR}(\text{CDCl}_3, 400 \text{ MHz}) \delta$: 10.3, 12.2, 15.2, 16.1, 24.0, 24.7, 25.3, 28.9, 29.3, 29.6, 29.7, 34.9, 35.0, 38.3, 43.5, 47.4, 47.9, 50.1, 52.8, 53.5, 55.5, 57.8, 58.1, 60.0, 61.4, 62.1, 88.8, 121.6, 124.0, 125.9, 127.4, 127.8, 127.9, 128.4, 128.6, 128.9, 129.4, 129.6, 129.8, 132.8, 137.2, 139.0, 169.7, 169.9, 170.2, 170.8, 171.2, 171.4, 171.8, 174.5. HRMS (ESI) m/z calcd for $\text{C}_{54}\text{H}_{69}\text{N}_8\text{O}_9^+$ ($\text{M}+\text{H}$) $^+$ 973.51820, found 973.51721.



2

3-Hydroxy-isoindolinone-cyclo-Gly-Pro(L)-Phe-Pro(L)-Ile-Ile-Pro(L) (2): white solid (yield 40%). $^1\text{H NMR}(\text{CDCl}_3, 400 \text{ MHz}) \delta$: 0.70~0.89(m, 12H, CH_3), 0.90~0.98(m, 2H, CH_2CH_3), 1.03~1.08(m, 2H, CH_2CH_3), 1.09~1.15(s, 1H, CHCH_3), 1.22~1.38(s, 1H, CHCH_3), 1.40~1.48(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.52~1.62(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.64~1.70(m, 1H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.79~1.80(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.89~2.11(m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.13~2.21(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.25~2.32(m, 1H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.35~2.41(m, 1H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.73~2.98(m, 2H, CH_2Ph), 3.01~3.31(m, 2H,

CH_2Ph), 3.32~3.70(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.72~3.89(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.92~4.26(m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}$), 4.28~4.38(m, 1H, HNCHCO), 4.44~4.68(m, 1H, HNCHCO), 4.70~4.78(m, 2H, NCH_2CO), 4.79~4.93(m, 2H, $\text{NCH}_2\text{C(OH)}$), 4.95~5.08(m, 1H, CHCH_2Ph), 7.27~7.91(m, 14H, ArH); $^{13}\text{C NMR}(\text{CDCl}_3, 400 \text{ MHz}) \delta$: 10.1, 11.0, 14.0, 15.4, 22.1, 22.6, 24.7, 24.9, 25.1, 29.3, 29.6, 36.9, 37.5, 39.3, 39.7, 46.5, 47.5, 47.7, 47.9, 52.3, 53.2, 54.9, 56.6, 58.4, 60.1, 64.9, 85.0, 121.9, 122.5, 123.6, 123.9, 126.6, 126.9, 127.6, 128.3, 128.5, 128.8, 129.5, 129.7, 130.0, 131.1, 132.2, 132.4, 134.1, 164.9, 167.7, 169.0, 170.1, 170.9, 171.0, 171.6, 173.6. HRMS (ESI) m/z calcd for $\text{C}_{54}\text{H}_{69}\text{N}_8\text{O}_9^+$ ($\text{M}+\text{Na}$) $^+$ 995.50015, found 995.49713.

Cell culture

HepG-2 cells and L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 g/mL). The HepG-2 cell lines and L929 cell lines were received from Harbin engineering University. Cells were cultured at 37 °C in a humidified incubator with 5 % CO_2 . Cells were grown on plates and were subcultured after 0.25% trypsin treatment. The experiments were performed when the ratio of cell fusion reached 80%.

MTT assay for cell viability

The activity test were grouped into two groups: cyclic peptide groups and blank group without peptides. HepG-2 cells were seeded in a 96-well plate at an initial density of 4×10^3 cells per well in DMEM complete medium and incubated at 37 °C in 5% CO₂ for 24 h. Then, they were treated with various concentrations (150, 100, 75, 50, 37.5, 25, 18.75, 12.25 and 9.325 μg/mL) of samples. Each dosage was replicated in six wells. After 48h incubation, MTT dyes (100 μL, 0.5 mg/mL) were added to the wells, and incubated for 4 h. The MTT solutions were then removed and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals generated. Then microplate reader was used to detect the absorbance of each well at 490 nm. Cell viability (%) was calculated by the following formula:

$$\text{Cell viability (\%)} = A_{490 \text{ (sample)}}/A_{490 \text{ (control)}} \times 100\%$$

where $A_{490 \text{ (sample)}}$ represents A values of the wells treated with various concentrations of samples, and $A_{490 \text{ (control)}}$ represents those of the wells treated with DMEM+10% FBS, without any samples. Statistical analyses were performed using the SPSS statistical software version (SPSS Inc., Chicago, IL, USA).

Cell morphological changes of HepG-2 Cells after treatment of sample

After being cultured with compound **1** for 0h, 6h, 36h, 48h, respectively, the cell phenotype in bright field were analyzed by Leica DM IL LED Fluorescence inverted microscope (FIM). HepG-2 cell lines were incubated on 6 well plates and incubated 24 h. The compound **1** (1 mL, 15 μg/mL) was added to each well and then incubated for additional 6 h. Then nucleus's morphological variation was immediately observed under FIM.

Lactate Dehydrogenase (LDH) Assay.

In order to monitor membrane leakage, an LDH assay was performed according to the manufacturer's instructions. Briefly, HepG-2 cells were plated into a 96-well plate in quadruplicate as described above and allowed to incubate for 24 h. The media was subsequently removed, and cells were incubated with various concentrations of peptide at 200 μL/well for 24 h. Serum-free RPMI-1640 growth media was used as a control for background LDH release, and 2% triton X-100 in serum-free media was used as a control for maximal LDH release. To measure LDH release, 100 μL/well of assay media was transferred from the cell culture plate into a new 96-well plate, followed by the addition of 100 μL of freshly prepared LDH assay solution into each well. The plates were incubated for 30 min at room temperature. Then, the absorbance was measured at 490 nm using a microplate reader (Biotek, Winooski, VT). Percent LDH release was calculated using the following equation:

$$\left(\frac{\text{Absorbance}_{\text{peptide-treated cells}} - \text{Absorbance}_{\text{untreated cells}}}{\text{Absorbance}_{\text{triton-X100 treated cells}} - \text{Absorbance}_{\text{untreated cells}}} \right) \times 100.$$

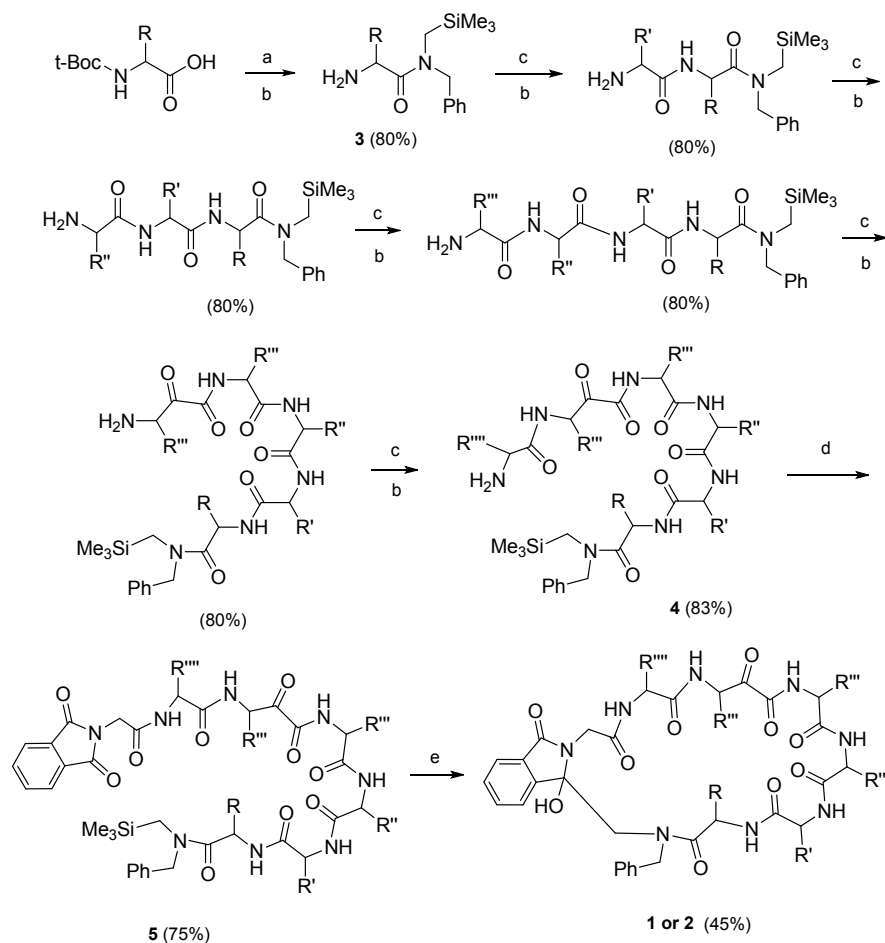
Dose response curves shown are an average of three independent experiments.

Computational details:

The conformational analysis was performed by arbitrarily fixing the absolute configuration of C-3 for compound **1** and compound **2**, using the Spartan 08 package¹ with the MMFF94 molecular mechanics force field and Monte Carlo searching. The obtained conformers were geometrically optimized at the DFT/B3LYP/6-31G** level of theory in the program package Gaussian 09.² TDDFT/3LYP/6311++(2d,2p) was employed to calculate excitation energy (denoted by wavelength in nm) and rotatory strength R. ECD curves were calculated based on rotatory strengths using half bandwidth of 0.3 eV with conformers by Specdis 1.61.³

References:

1. Spartan'08, Wavefunction, Inc. Irvine, CA
2. Gaussian 09, Revision C.01; Gaussian, Inc.: Wallingford, CT, 2010.
3. T. Bruhn, A. Schaumloffel, Y. Hemberger, G. Bringmann, *Chirality*, 2013, **25**, 243.



Scheme 1. The synthesis of target cyclopeptides. (a) BnTMSA, EEDQ, CH₂Cl₂, separation by column chromatography; (b) TFA, CH₂Cl₂, rt; (c) N-Boc-amino acid, EEDQ, CH₂Cl₂, rt; (d) N-phthalimido-glycine acid chloride, benzene, rt; (e) Irradiated by medium pressure mercury lamp, the concentration of **5** is 3.4×10⁻³ mol/L in MeOH.

1. HPLC of compound 1 and compound 2.

Shiseido Capcell PAK C18 (150×4.0 mm, 5 μm) was used as the column at 30 °C , and the mobile phase flow rate was 1 mL/min. During the analytical run, the elution was carried out using mobile phases A (Ultrapure water) and B (acetonitrile), the percentage of mobile phases B was 50%, while the detection wavelength was 192 nm. From HPLC analysis, the retention time of compound 1 is 11.2 min, and the purity of compound 1 is approximately 98.5%. The retention time of compound 2 is 10.8 min, and the purity of compound 2 is approximately 98.2%.

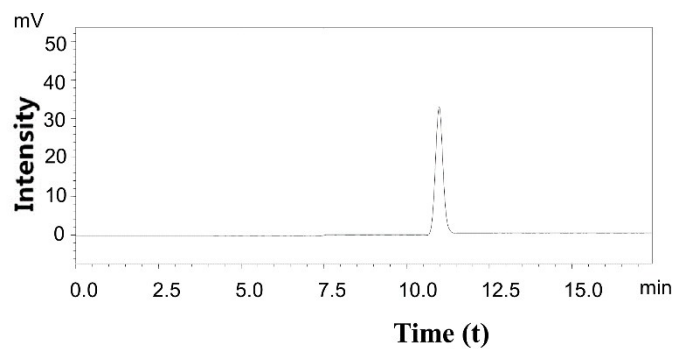


Figure S1. HPLC spectrum of compound 1.

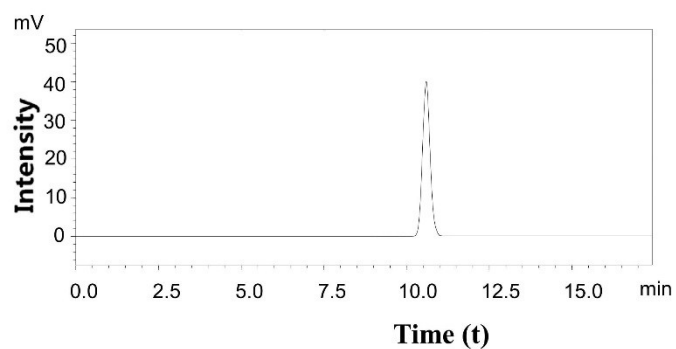


Figure S2. HPLC spectrum of compound 2.

2. UV of compound 1 and compound 2.

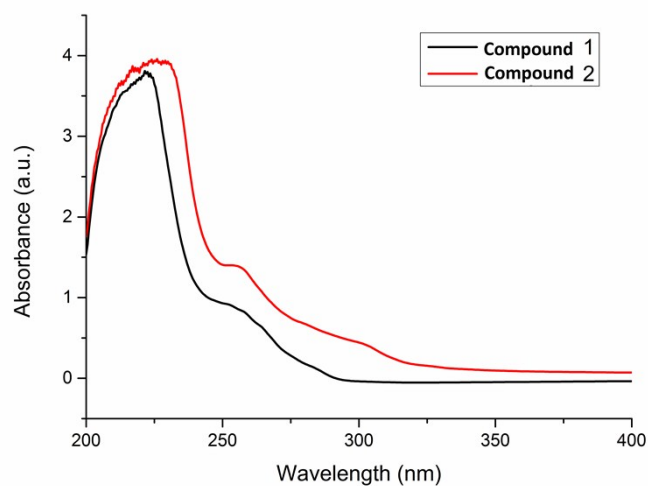


Figure S3. UV spectrum of compound 1 and compound 2.

3. Cell viability of compound 1 and compound 2.

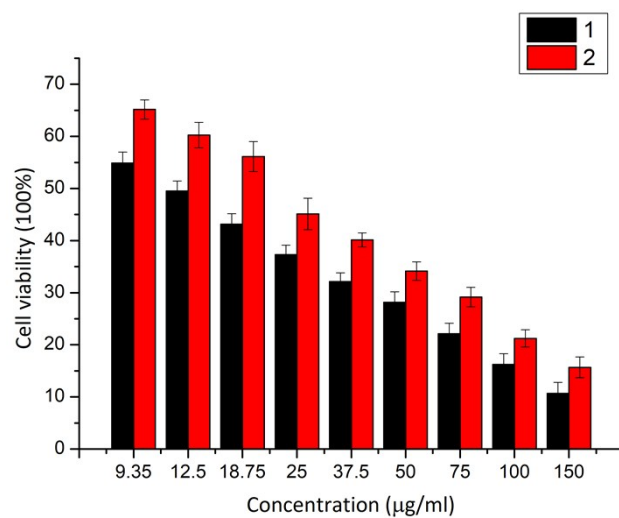
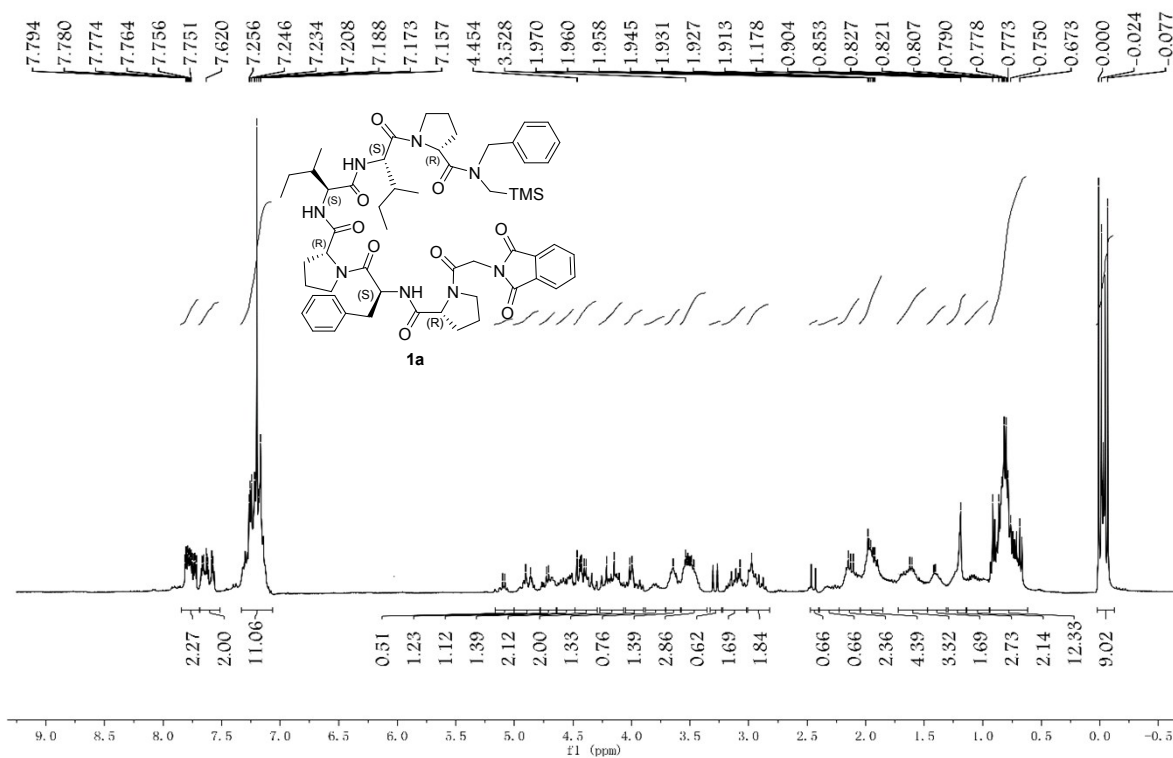
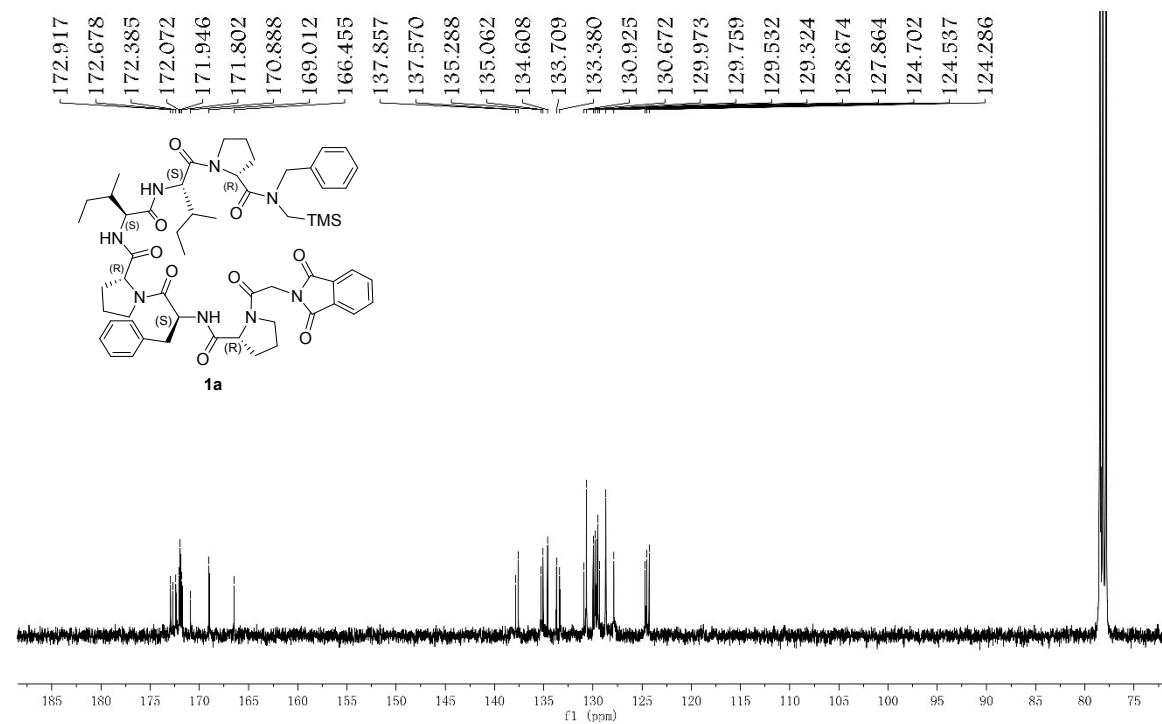
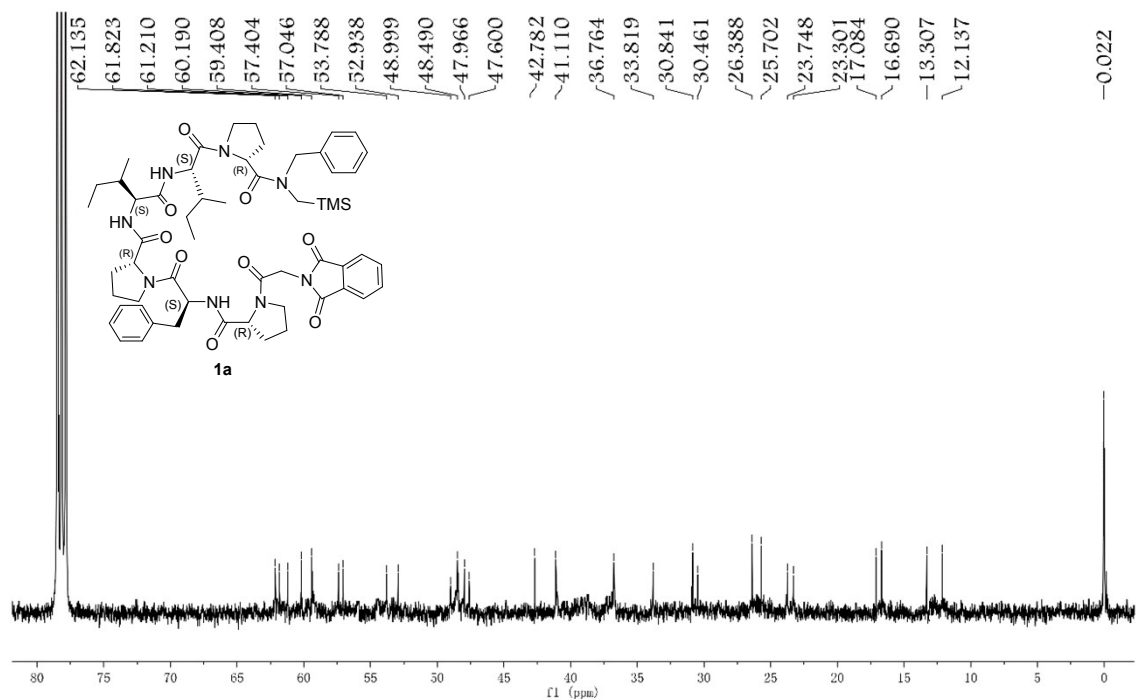


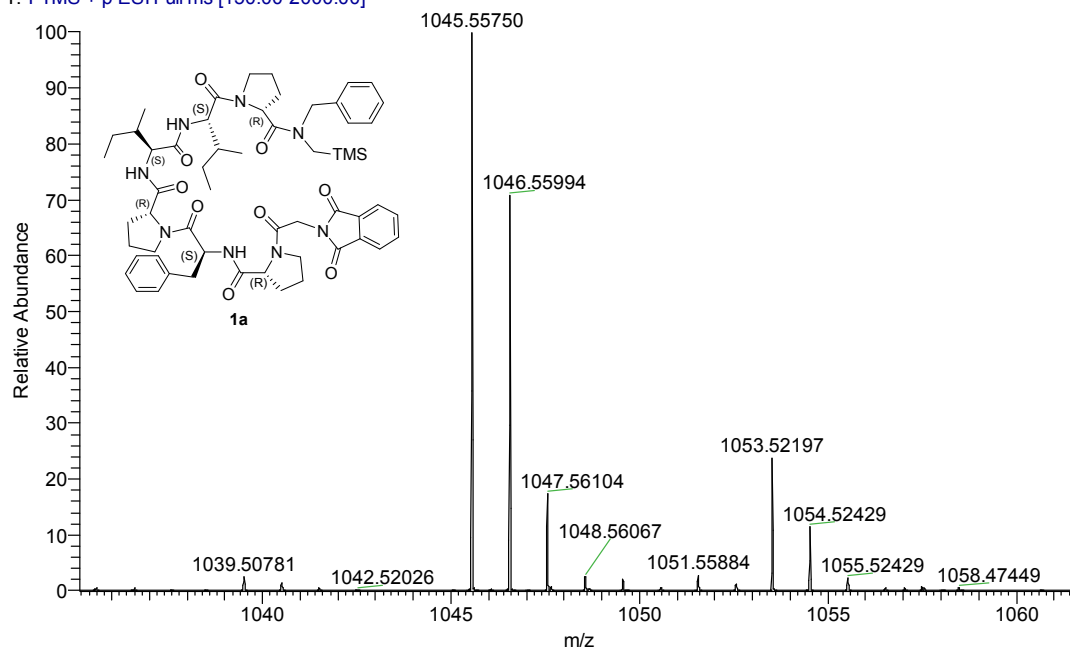
Figure S4. Cell viability of two groups: compound 1 experiment groups (black columns), compound 2 experimental groups (red columns).

4. ¹H, ¹³C-NMR and HRMS of compound 1a.

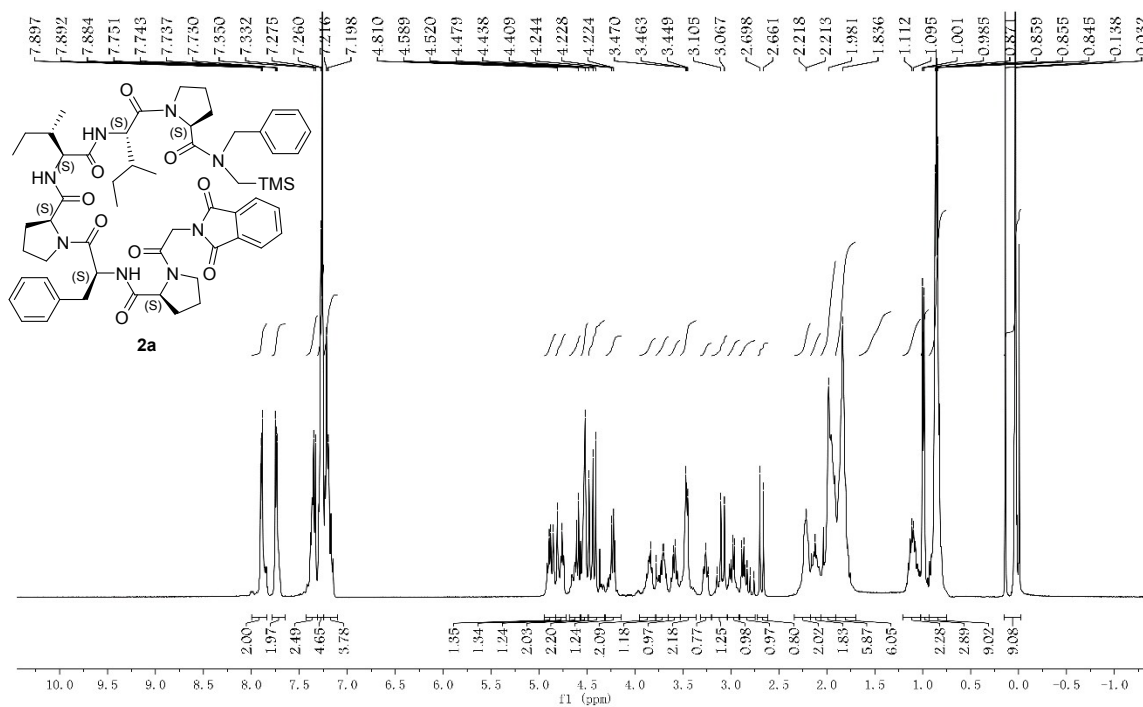


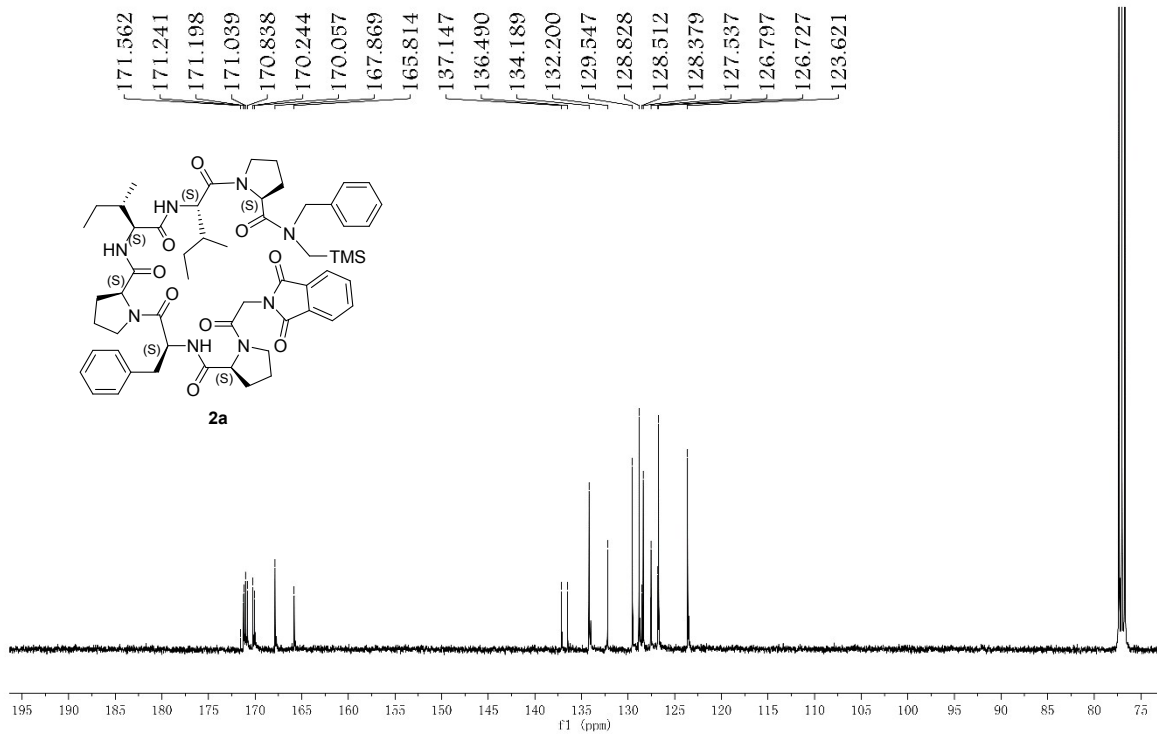
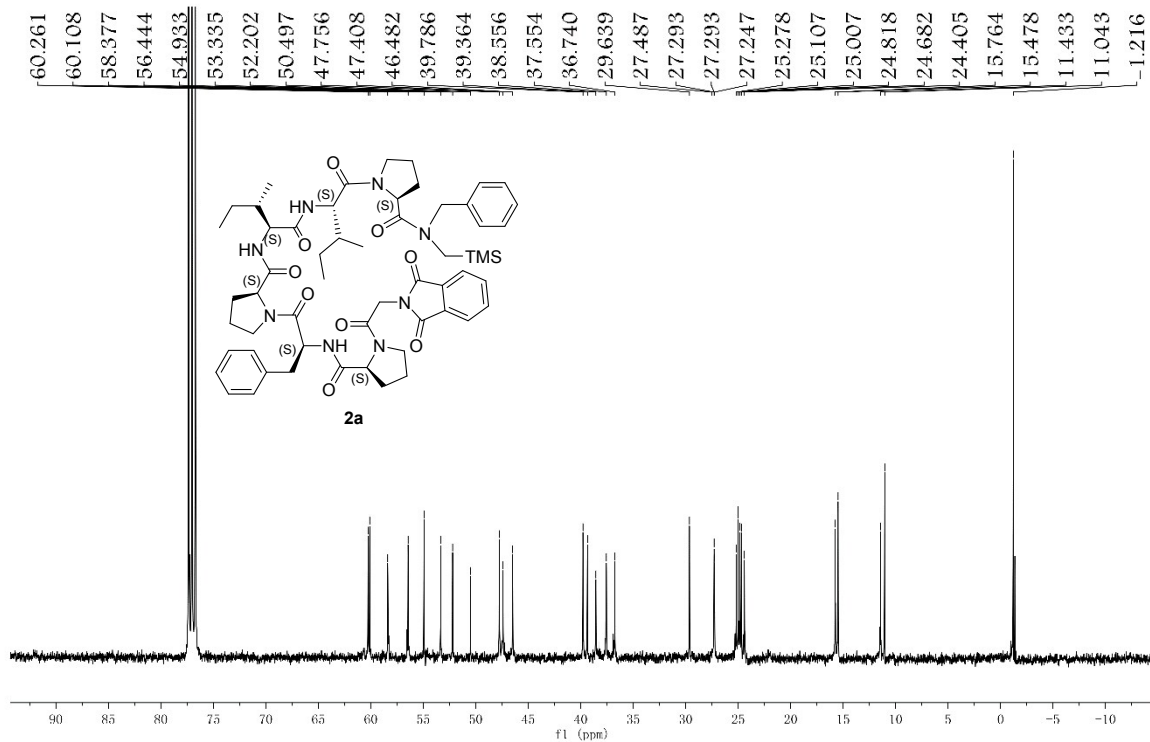


50 #12 RT: 0.15 AV: 1 NL: 1.79E8
T: FTMS + p ESI Full ms [150.00-2000.00]

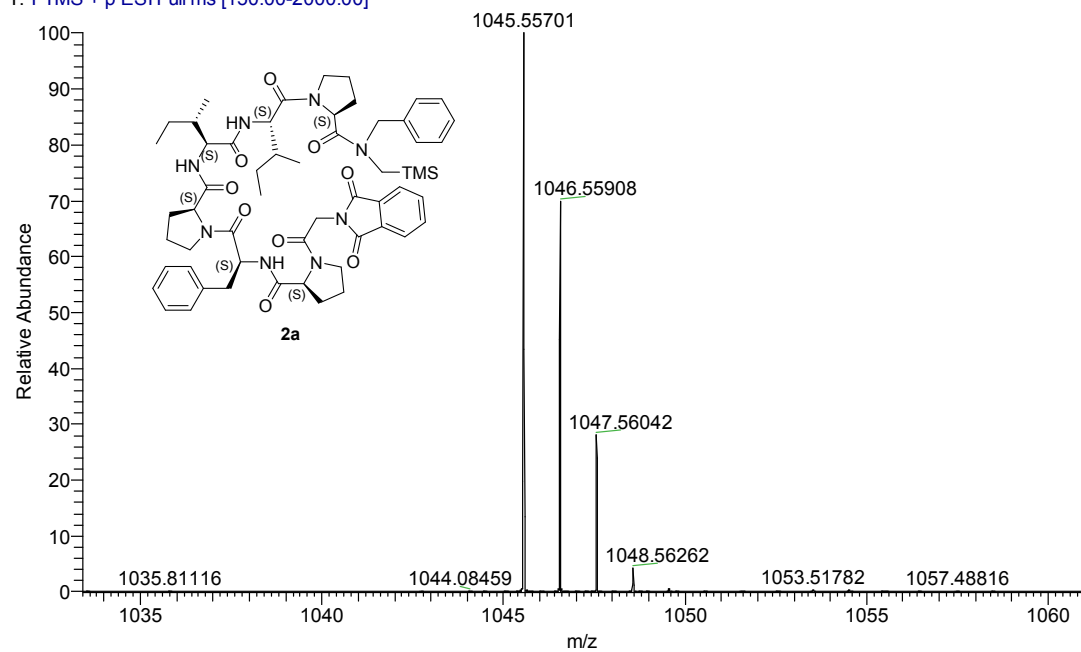


5. ^1H , ^{13}C -NMR and HRMS of compound **2a**.

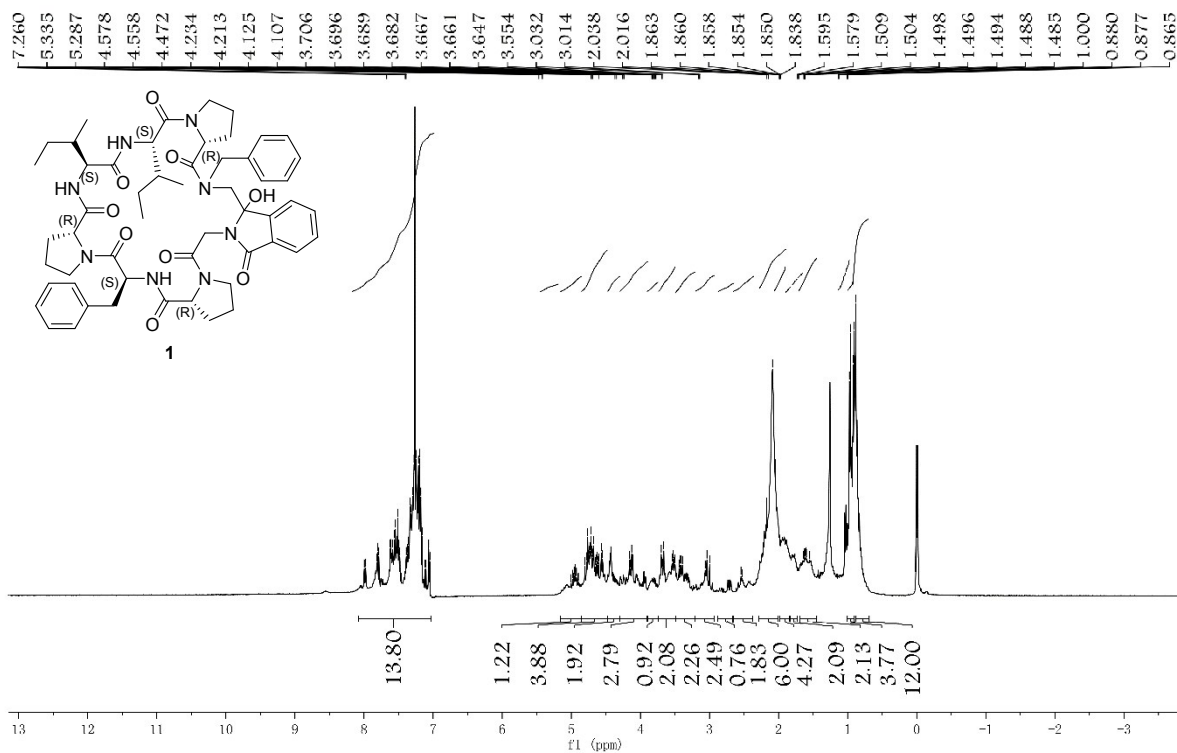


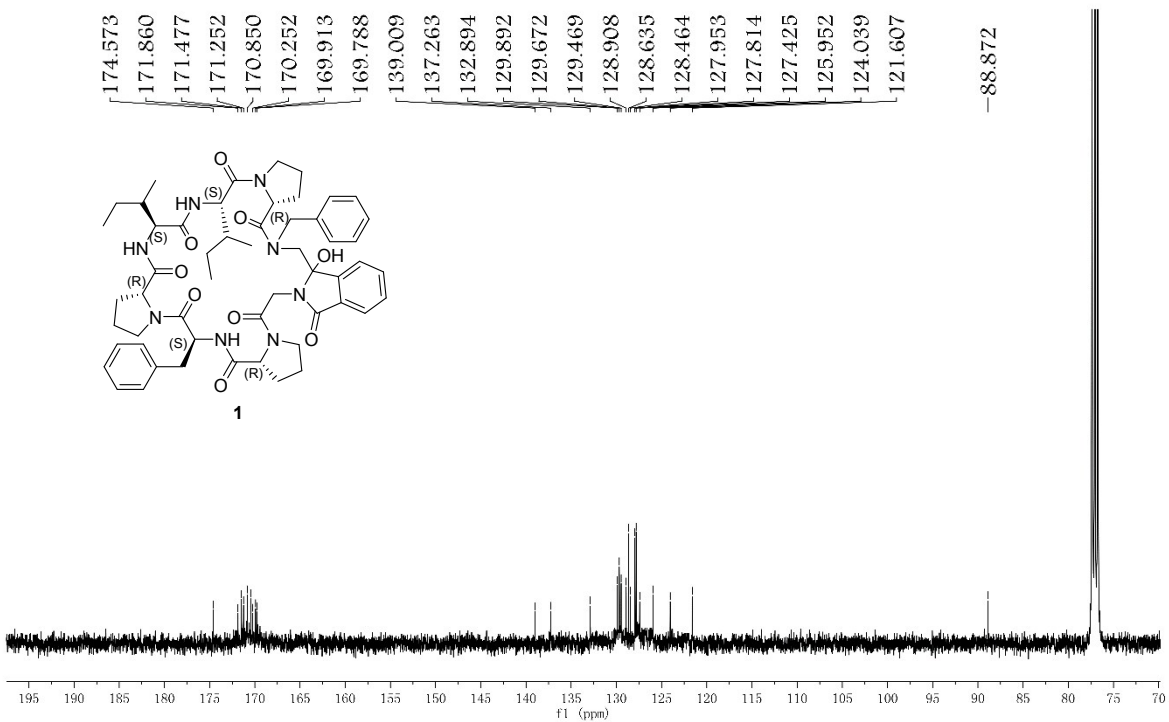
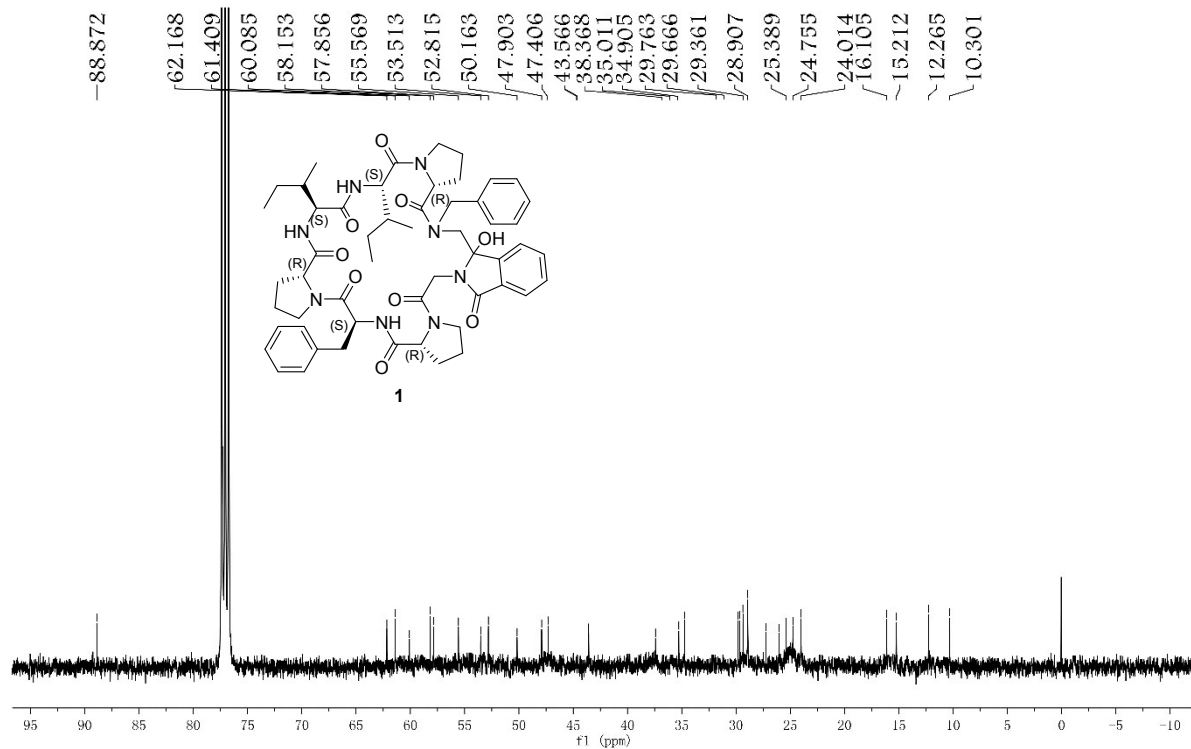


47 #18 RT: 0.26 AV: 1 NL: 1.71E8
 T: FTMS + p ESI Full ms [150.00-2000.00]

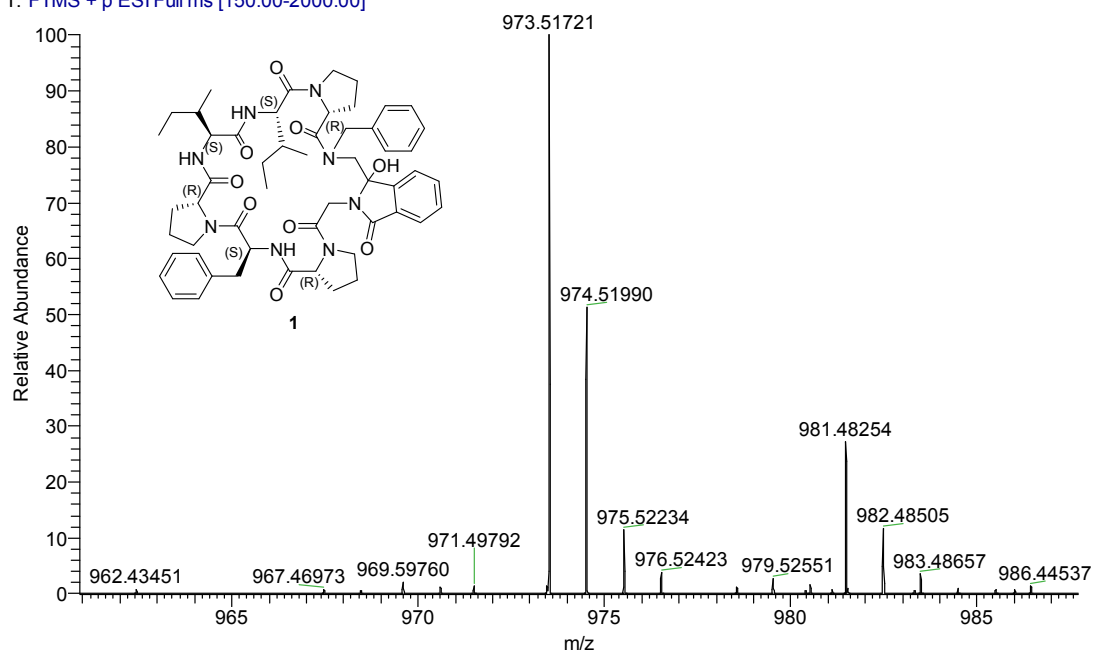


6. ^1H , ^{13}C -NMR and HRMS of compound 1.

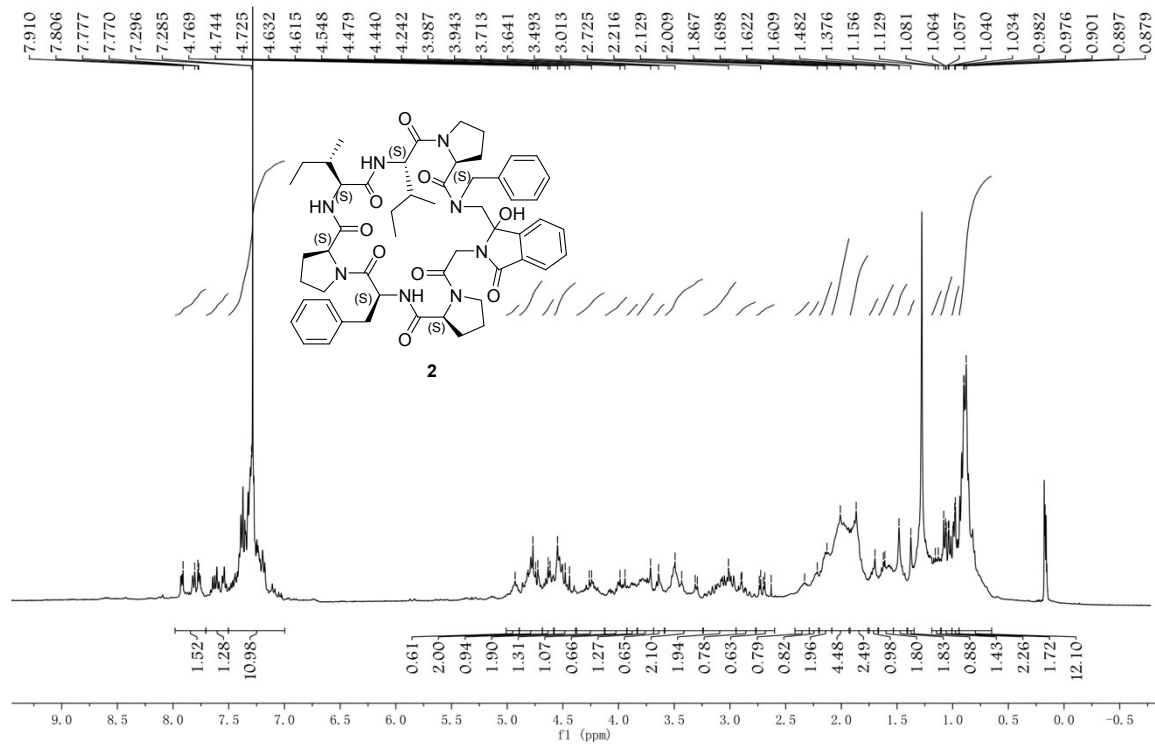


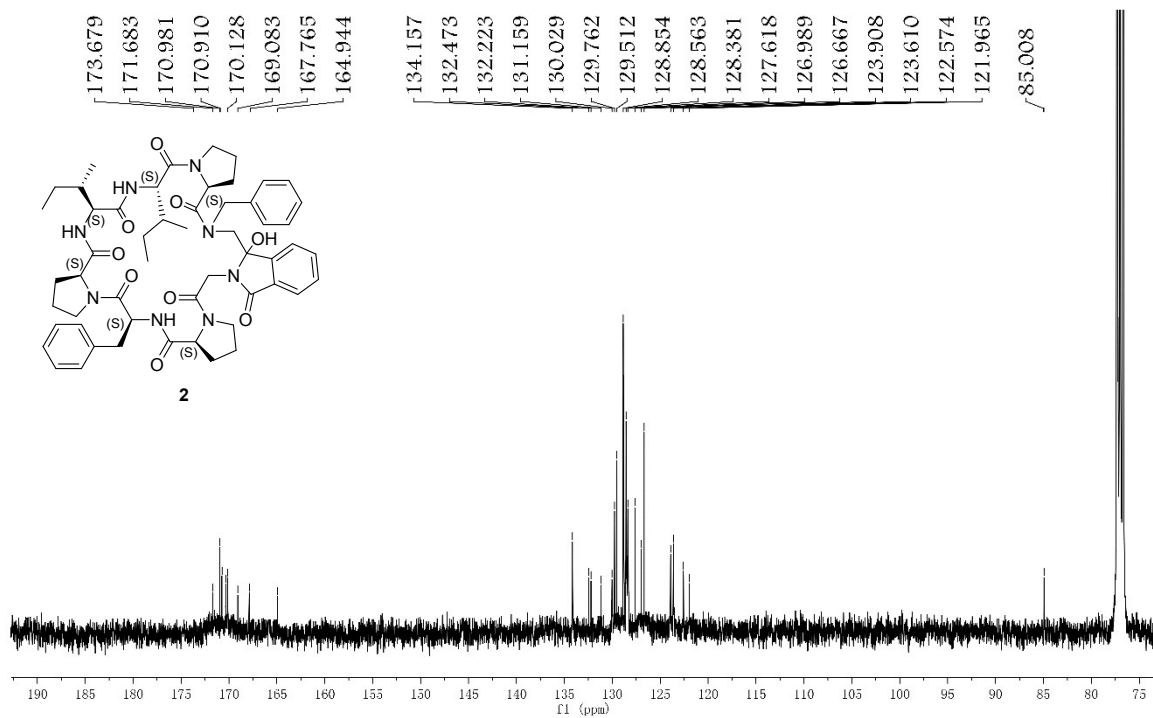
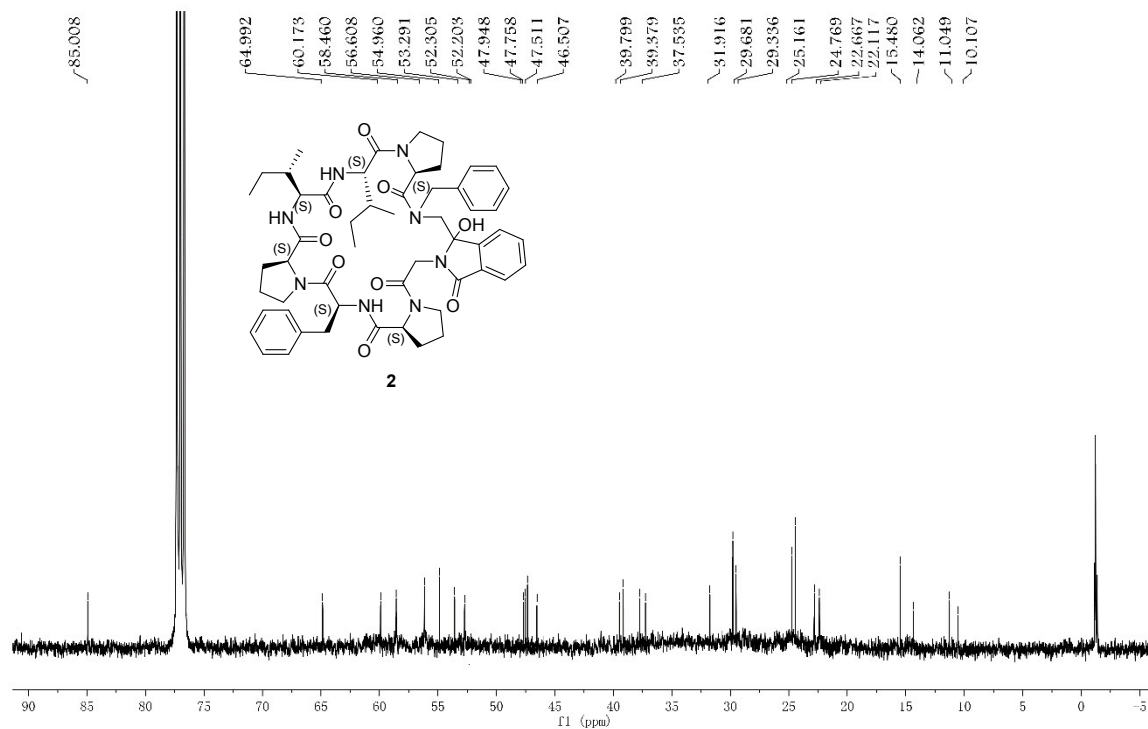


51 #15 RT: 0.20 AV: 1 NL: 1.93E7
T: FTMS + p ESI Full ms [150.00-2000.00]

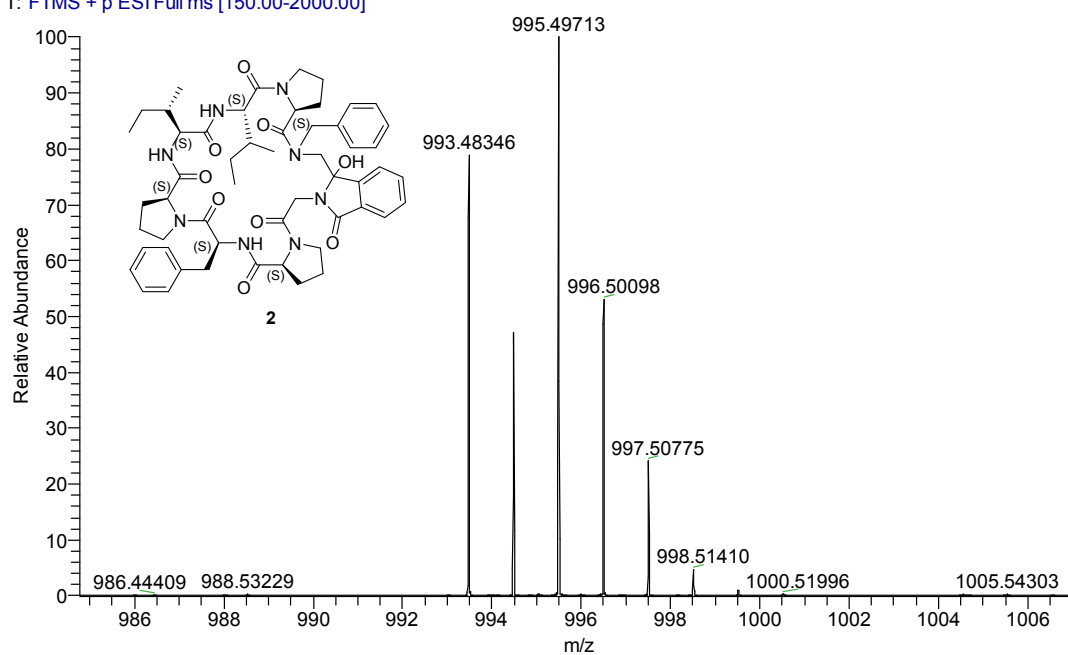


7. ¹H, ¹³C-NMR and HRMS of compound 2.





49 #45 RT: 0.69 AV: 1 NL: 3.46E7
 T: FTMS + p ESI Full ms [150.00-2000.00]



8. Table S1. Relative free energies and dipole moment of the conformers as determined at the level of B3LYP/6-31G(d,p) in MeOH (PCM model).

Table S1. Relative free energies and dipole moment of the conformers as determined at the level of B3LYP/6-31G(d,p) in MeOH (PCM model).

Conf	G(kcal/mol)	ΔG (kcal/mol)	Dipole moment
DS	-	0	12.42
DR	-	1.098	6.97
LR	-	0	13.62
LS	-	26.532	4.76