Supporting information for

Pentacyclic spermidine alkaloids with biological activities from *Orychophragmus violaceus*

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Contents of Supporting Information

EXPERIMENTAL SECTION

Plant material

The seeds of *O. violaceus* were purchased from Shuyang county, Jiangsu province of China in June 2014 and identified by Prof. Bin Li, Beijing Institute of Radiation Medicine. A voucher specimen (No.2014-0601) has been deposited in the Herbarium of Beijing Institute of Radiation Medicine.

General experimental procedures

HR-ESI-MS data were taken on an Agilent 6230 Accurate Mass Q-TOFmass spectrometer (Agilent, Santa Clara, USA). UV spectra were recorded on the UV-2500PC spectromter (Shimadzu, Japan). 1D and 2D NMR spectra were obtained using Bruker ECA-400MHz spectrometers with TMS as an internal standard. Optical rotations were acquired on a Autopol V polarimeter (Rudolph, USA). IR spectra were recorded on a Nicolet 6700 spectrometer (Thermo, USA). CD spectra were measured on a JASCO J-815 CD spectrometer (JASCO, Japan). HPLC separation was performed on an LCQ advantage HPLC-MS instrument (Thermo, USA) with a C18 column (IX952505-2, 4.6 mm, 250 mm, 5 μm, Jin Chen Technology Co., Ltd, Beijing, China). The chiral HPLC was performed on Waters e2695 instrument with a chiral OD-H column (4.6 mm 250 mm, 5 μm, Daicel Chiral Technologies Co. Ltd., Tokyo, Japan).

Column chromatography (CC) was carried out on macroporous resin AB-8 (Nan Kai College Chemical Inc., Tianjin, China), silica gel (100-200, 200-300 mesh, Qingdao Marine Chemical Co. China), Reversed phase silica gel Rp-C_{18} (100-200 mesh, YMC, Japan) and Sephadex LH-20 (Pharmacia, Sweden). TLC was carried out on normal phase silica gel 60 (400-600 mesh, Qingdao Marine Chemical Co. China) and GF254 plates pre-coated with silica gel 60 (10-40 μm, Qingdao Marine Chemical Co. China).

Extraction and isolation

The seeds of *O. violaceus* (40 kg) were extracted with 70% EtOH (70 L×3) under reflux for 2 h each time. After evaporation of the solvent, the concentrated residue (4.0 kg) was suspended in water and partitioned with petroleum ether, EtOAc and n-BuOH successively. The n-BuOH extract (800 g) was subjected to macroporous resin column chromatography eluted with EtOH in H_2O (0% ,25 %, 50 %, 75 % and 95%) to yield five fractions (A1-A5).

A2 (250 g) was subjected to silica gel column chromatography (CC) eluted with $CHCl_3$-MeOH gradient (50:1, 20:1, 10:1, 5:1, 2:1 and 1:1) to yield 12 fractions (B1-B12). B10 (30 g) was subjected to silica gel CC with $CHCl_3$-MeOH gradient (30:1, 20:1, 10:1,5:1 and 2:1) to give 10 fractions (C1-C10). C8 (3.3 g) was chromatographed by Sephadex LH-20 with $CHCl_3$-MeOH(0:1) to afford three fractions (D1-D3). D1 (952 mg) was separated by preparative TLC eluted with $CHCl_3$: MeOH : H_2O (40:10:1) to afford four fractions (E1-E4). E2 (39.8 mg) was chromatographed by Sephadex LH-20 eluted with MeOH repeatedly to afford compound (±)-**1** (16.6 mg). E1 (8.1 mg) was chromatographed by Sephadex LH-20 eluted with MeOH repeatedly to afford compound (±)-**2** (5.9 mg).

(±)-**1** and (±)-**2** was loaded on to HPLC over a chiral OD-H column and eluted by n-Hexane : isopropanol : diethylamine (70:30:0.1). The flow rate was 1.0 ml/min at 35℃ and the detection was carried out at 280 nm. In turn, (+)-**1** (t_R = 14.789 min, 4.6 mg), (-)-**1** (t_R = 20.268 min, 4.9 mg), (+)-**2** (t_R = 7.057 min, 1.7 mg) and (-)-**2** (t_R = 9.993 min, 1.9 mg) were obtained, respectively.

Spectroscopic Data of Isolated Compounds

(±)-Orychovioline A ((±)-**1**): white needle crystal; $[\alpha]_D^{19}$ = 0 (c = 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 235 (3.68), 290 (3.32); IR (KBr) ν_{max} 3363, 3254, 3063, 2945, 2761, 1719, 1630, 1486, 1362, 1265, 808 cm^{-1}; ^1H and ^{13}C NMR data (see Table 1); The HR-ESI-MS gave a molecular formula of $C_{25}H_{33}O_4N_3$ at *m/z* 440.2542 $[M+H]^+$ (calcd for $C_{25}H_{34}O_4N_3$, 440.2549). (+)-**1**: $[\alpha]_D^{19}$ +95.2 (0.1, MeOH); (-)-**1**: $[\alpha]_D^{19}$ -95.2 (0.1, MeOH).

(±)-Orychovioline B ((±)-**2**): white needle crystal; $[\alpha]_D^{20}$ = 0 (c = 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 235 (3.45), 290 (3.32); IR (KBr) ν_{max} 3422, 3052, 2944, 2709, 1636, 1484, 1362, 1264, 814 cm^{-1}; ^1H and ^{13}C NMR data (see Table 1); The HR-ESI-MS gave a molecular formula of $C_{27}H_{39}O_5N_3$ at *m/z* 486.2960 $[M+H]^+$ (calcd for $C_{27}H_{40}O_5N_3$, 486.2968). (+)-**2**: $[\alpha]_D^{20}$ +61.6 (0.1, MeOH); (-)-**2**: $[\alpha]_D^{20}$ -61.6 (0.1, MeOH).

Radiation protectionin vitro

HUVEC cell line (Human umbilical vein endothelial cells) was used for in vitro studies. Cells were obtained from Beijing Institute of Radiation Medicine andcultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco, USA) supplemented with 10 % (v/v) fetal calf serum (Gibco, USA), 100 U/mL penicillin and 100 U/mL streptomycin in an atmosphere containing 5 % CO_2 at 37 ℃, according to the manufacturer's protocol.

Cells were seeded in 96-well plates with a density of 5 × 10^2 cells/well and treated with different concentrations of Ex-RAD (positive control) and test compounds (50 μM, 25 μM, 12.5 μM, 6.25 μM and 3.13 μM) for 24 h before irradiation. Cells were irradiated with dose of 8.0 Gy at a dose rate of 0.8 Gy/min. Then 10 μL CCK-8 solution were added to all the wells after continuing cultivation for 24 h. After 4 h incubation, plates were shaken to mix thoroughly. The absorbance at 450 nm of each well was measured in a multiscan photometer. The experiment was repeated three times.

Single-cell electrophoresis (comet assay)

The comet assay was a common technique for measurement of DNA damage in individual cells. Under an electrophoretic field, damaged cellular DNA (containing fragments and strand breaks) migrated further from the intact DNA, yielding a classic "comet tail" shape under the microscope. The extent of DNA damage was usually visually estimated by comet tail measurement. The degree of DNA damage was measured by calculating the tail lengths. The assay was performed according to instructions provided by the manufacturer (OxiSelect™ Comet Assay Kit, Cell Biolabs).

Cells were seeded in 12-well plates with a density of 1 × 10^5 cells/welland treated with Ex-RAD (positive control) and test compounds (25 μM) for 24 h before irradiation. After irradiation with dose of 6.0 Gy at a dose rate of 0.8 Gy/min, cells were mixed with comet agarose at a ratio of 1 : 10 (v/v) and immediately 75 μL per well was onto a 3-well cometslide. The slide was maintained at 4 ℃ for 15 min in the dark for gel solidification. Then the slide was submerged horizontally in the precooled provided lysis buffer in the dark at 4 ℃ for 30 min. Finally, the slide was submerged in an alkaline buffer (200 mM NaOH and 1 mM EDTA, pH > 13) at 4 ℃ for 30 min. Electrophoresis was conducted in a fresh chilled alkaline electrophoresis buffer (200 mM NaOH and 1mM EDTA, pH > 13). The slide was placed in a horizontal electrophoresis apparatus at 20.0 V for 20 min. After electrophoresis, the slide was dried in 70 % ethanol for 5 min, air-dried (15 min), andstained for 5 min with vista green DNA dye (1/10000 dilution of stock supplied by Cell Biolabs). The slide was observed at 100× magnification under an epifluorescence microscope equipped with an excitation filter of 495 nm.

A total of 50 randomly captured comets from each slide were examined. The tail length was measured in micrometers, then calculated and analyzed with Comet Assay Software Project Lab (CASP1.2.3 beta 2). The experiment was repeated three times.

Anti-inflammatory activity assay in vitro

Anti-inflammatory activity and cell viability were tested using Griess reaction and CCK-8 assays, respectively. RAW 264.7 mouse macrophage cell line was used for in vitro studies. Cells were obtained from Beijing Institute of Radiation Medicine and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10 %(v/v) fetal calf serum (Gibco, USA), 100 U/mL penicillin and 100 U/mL streptomycin in an atmosphere containing 5 % CO_2 at 37 ℃.

RAW 264.7 cells (5 × 10^4 cells/well) were seeded in 96-well plates and incubated with different concentrations of Dexamethasone (positive control) and test compounds (50 μM, 25 μM, 12.5 μM, 6.25 μM and 3.13 μM) in presence or absence of LPS (1 μg/mL, Sigma, USA). After 24 h, the cell culture supernatants were collected. NO concentration in the medium was measured with the Griess reagent kit (E1030, Applygen Technologies Inc. China). Samples (50 μl) of culture media were mixed with 50 μl Griess R1 and 50 μl Griess R2 solution at room temperature for 5 min in 96-well microplate. Absorbance at 540 nm

was read using a microplate reader. The experiment was repeated three times.

Cell viability was measured by a Cell Counting Kit-8 (CCK-8) assay. Cells were seeded in 96-well plates with a density of 2×10^4 cells/well and treated with different concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, and 6.25 μM) of test compounds for 24 h. Then 10 μL CCK-8 solution were added to each well. After 4 h incubation, plates were shaken to mix thoroughly. The absorbance at 450 nm of each well was measured in a multiscan photometer. The experiment was repeated three times.

Statistical analysis

Data were represented as mean ± SD of three independent experiments. The statistical analyses were performed using the one-wayanalysis of ANOVA in GraphPad Prism 5 in comparison with the control. Differences of 5 % were considered significant (*$p < 0.05$).

Conformational Analysis and Calculations of the ECD and UV Spectra of (+)-1 and (+)-2

Conformational analysis and quantum computations were performed using the MOE and Gaussian 16 program package. Conductor-like polarizable continuum model (CPCM) was adopted to consider solvent effects using the dielectric constant of MeCN (ε = 35.69).

Conformational searches of (+)-**1** and (+)-**2** showed 1 and 22 lowest energy conformers with relative energy within 3 kcal/mol, respectively. After re-optimized using DFT at B3LYP/6-31G (d, p) level, the conformers with Boltzmann distributions larger than 1% and without imaginary frequency (Tables S1 and S2 and Figure S1 and S2) were calculated using the TDDFT methodology at the B3LYP/6-31G (d, p) level for their energies, oscillator strengths, rotational strengths. The ECD and UV spectra were simulated by the Gaussian function (σ = 0.28 eV). The final spectra were accomplished by averaging of relative conformational Gibbs free energy (G) combined with UV correction, respectively.

Table S1. The Boltzmann distribution and energies of conformers (within 3 kcal/mol) for (+)-**1**.

conf.	rel.E (kcal/mol)	Boltzmann distribution
1a C1	0.000	1.000

Table S2. The Boltzmann distribution and energies of conformers (within 3 kcal/mol) for (+)-**2**.

conf.	rel.E	Boltzmann distribution
2aC1	1.604	0.016
2aC2	1.422	0.022
2aC3	0.001	0.245
2aC4	0.000	0.246
2aC5	2.501	0.004
2aC6	2.964	0.002
2aC7	2.435	0.004
2aC8	3.056	0.001
2aC9	0.377	0.130
2aC10	2.773	0.002
2aC11	1.247	0.030
2aC12	2.773	0.002
2aC13	0.380	0.130
2aC14	4.157	0.000
2aC15	4.356	0.000
2aC16	2.394	0.004
2aC17	1.527	0.019
2aC18	0.826	0.061
2aC19	1.536	0.018
2aC20	3.107	0.001
2aC21	0.828	0.061
2aC22	4.200	0.000

Table S3. X-ray crystallographic data for (±)-**1**

Identification code	20200627li_znz8277_0m_a
Empirical formula	C26 H38 Cl N3 O5
Formula weight	508.04
Temperature/K	189.99 K
Wavelength	1.34139
Crystal system	Monoclinic
Space group	C 1 2/c 1
Volume	5175.3(9)
Z	8
Density (calculated)	1.304 mg/mm^3
Absorption coefficient	1.069 mm^{-1}
F(000)	2176
Crystal size	0.12 x 0.11 x 0.08 mm^3
Theta range for data collection	2.976 to 54.370°
Index ranges	-20<=h<=21, -13<=k<=13, -28<=l<=31
Reflections collected	33846
Independent reflections	4750 [R(int) = 0.0606]
Completeness to theta = 53.594	99.9 %
Absorption correction	None
Max. and min. transmission	0.3012 and 0.1954
Refinement method	Full-matrix least-squares on F2
Data / restraints / parameters	4750 / 0 / 318
Goodness-of-fit on F^2	1.029
Final R indices [I>2sigma(I)]	R_1 = 0.0360, wR_2 = 0.0879
R indices (all data)	R_1 = 0.0451, wR_2 = 0.0951
Extinction coefficient	n/a
Largest diff. peak and hole	0.280 and -0.286 e^{-3}

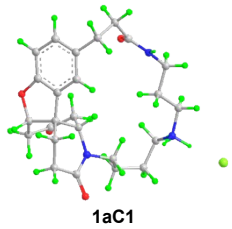


Figure S1. The lowest energy conformers of (+)-**1** re-optimized at B3LYP/6-31+G (d, p).

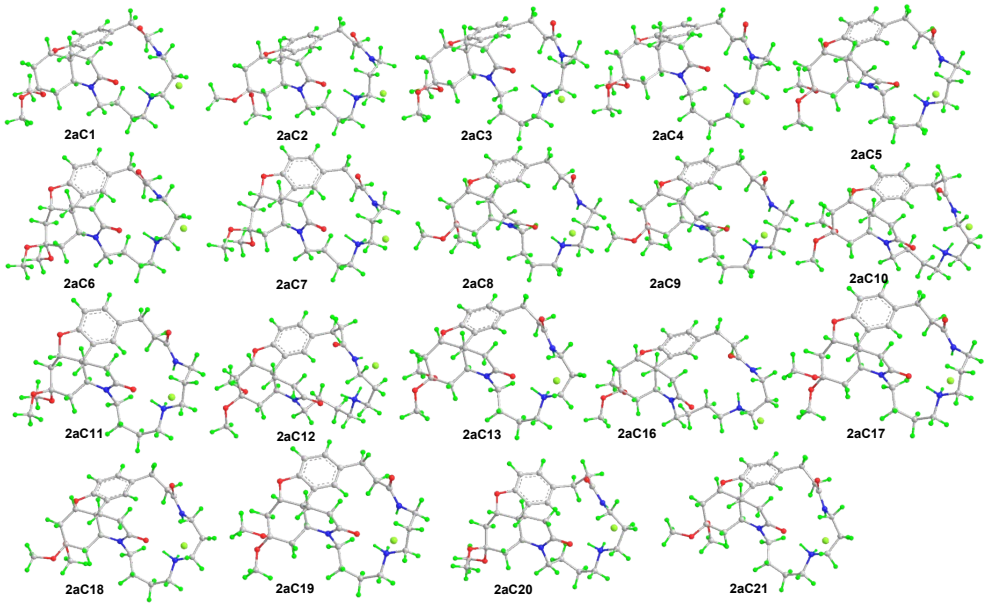


Figure S2. The lowest energy conformers of (+)-**2** re-optimized at B3LYP/6-31+G (d, p).

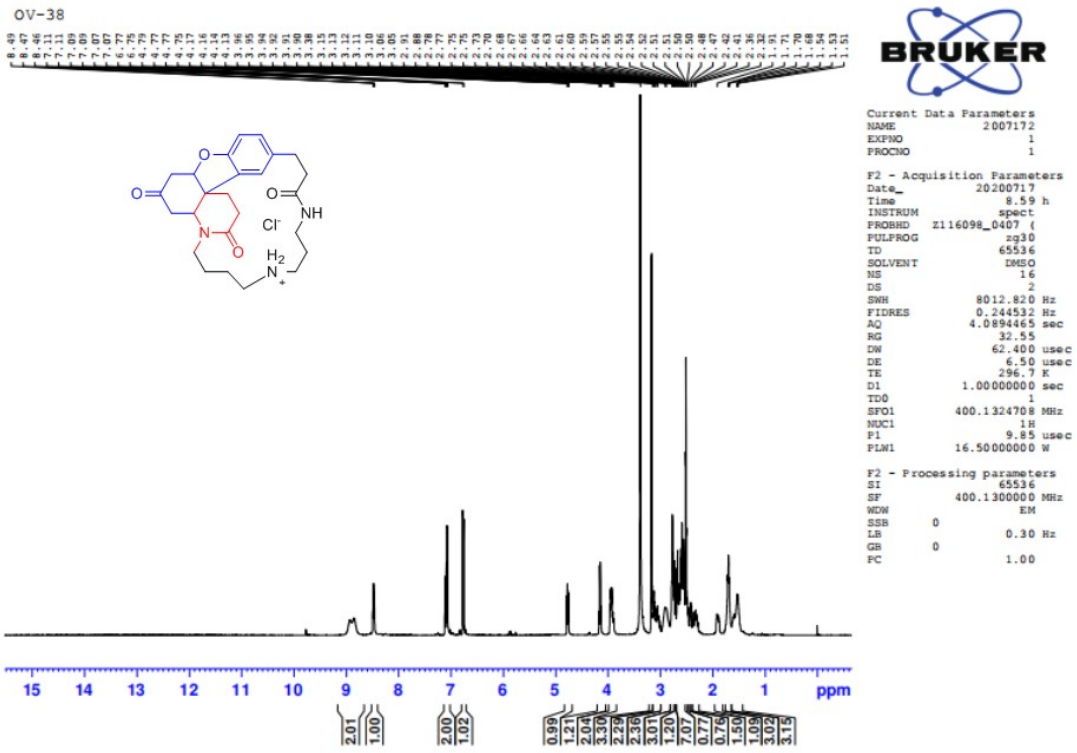


Figure S3. ^{1}H-NMR (400 MHz) spectrum of (±)-**1** in DMSO-d_6.

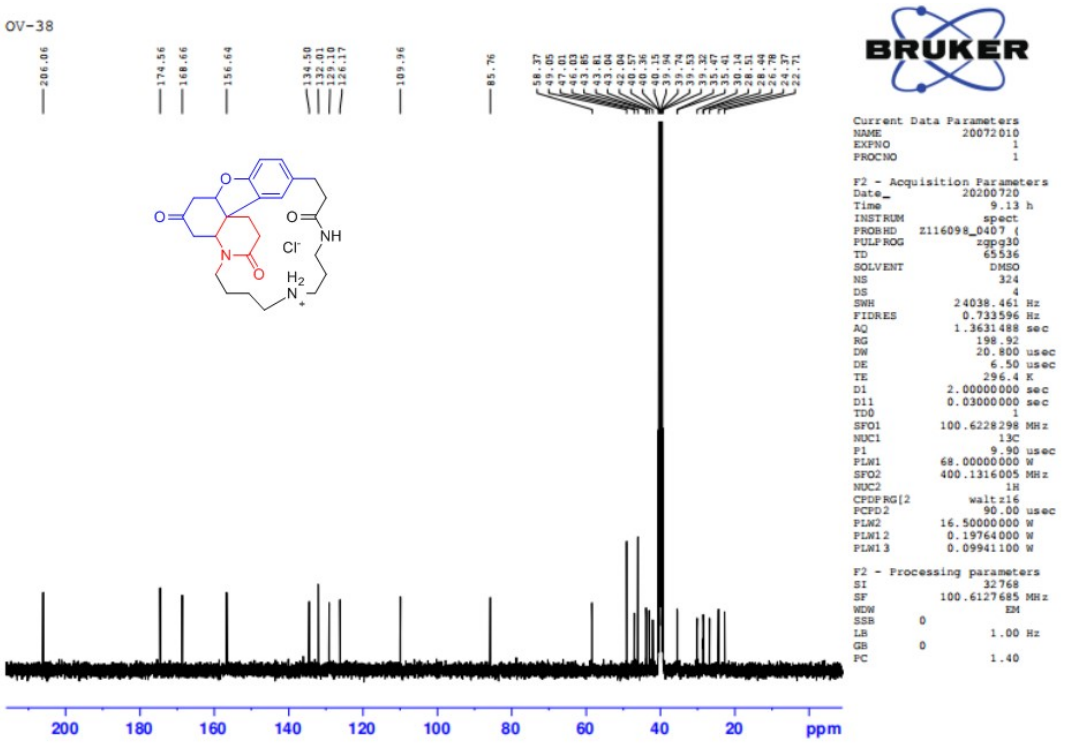


Figure S4. ^{13}C-NMR (100 MHz) spectra of (±)-**1** in DMSO-d_6.

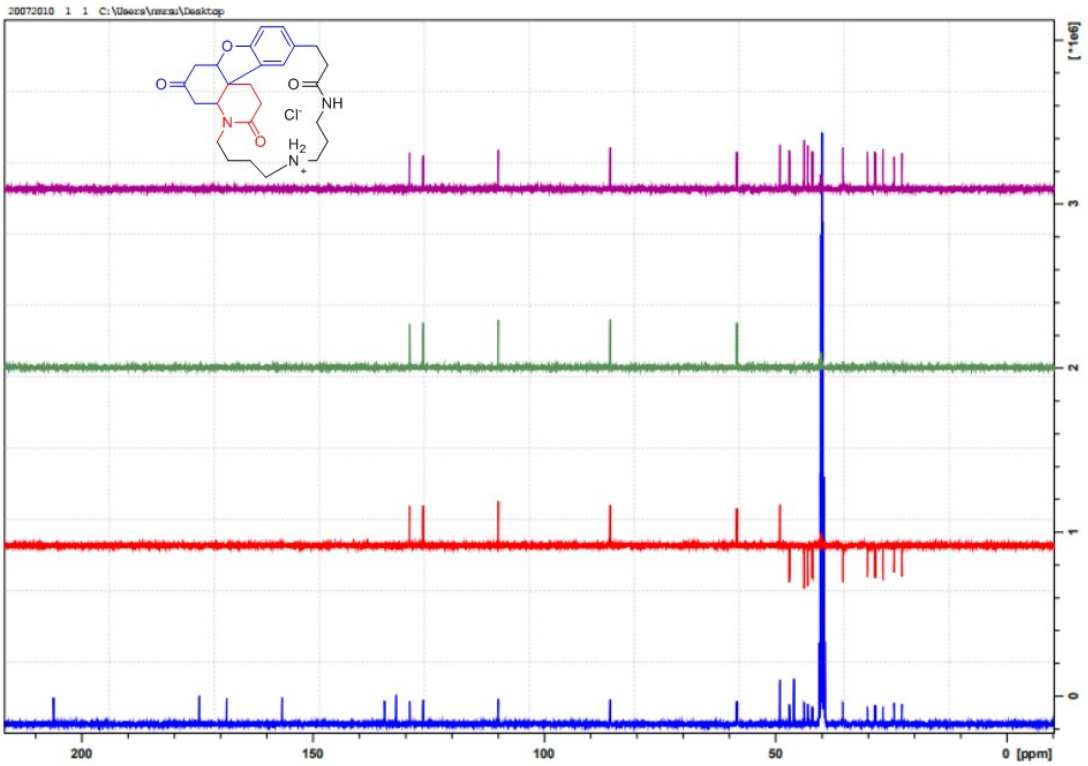


Figure S5. DEPT (100 MHz) spectrum of (±)-**1** in DMSO-d_6.

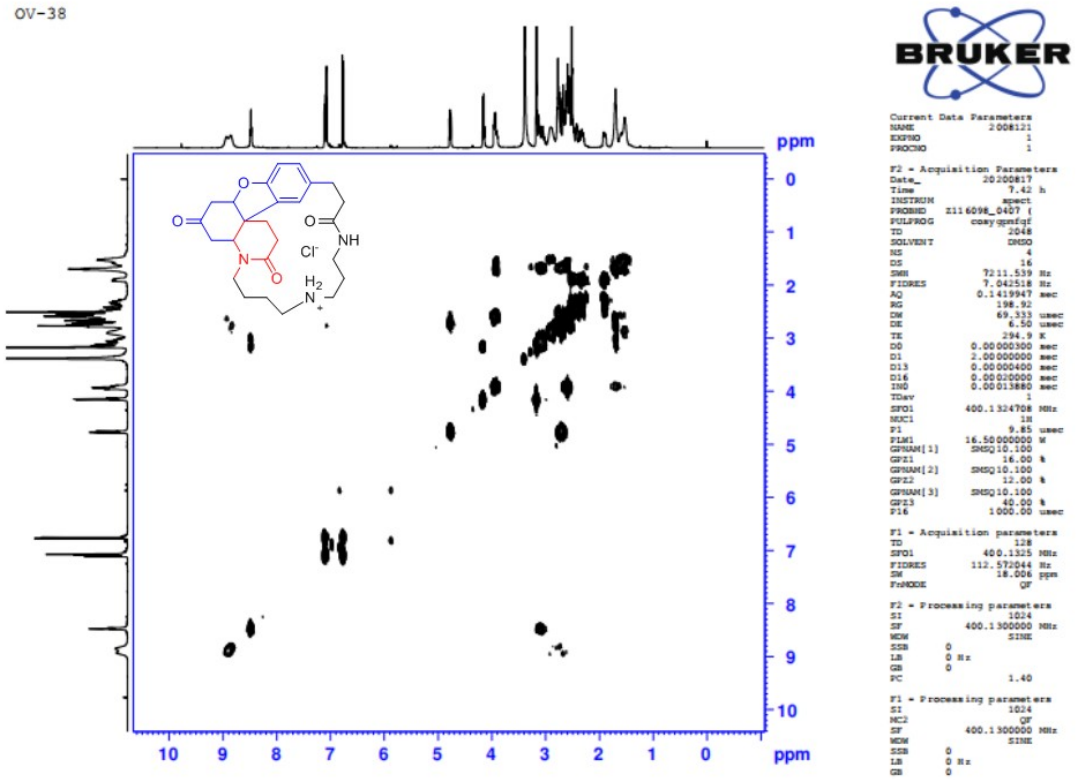


Figure S6. ^{1}H-^{1}H COSY (400 MHz) spectrum of (±)-**1** in DMSO-d_6.

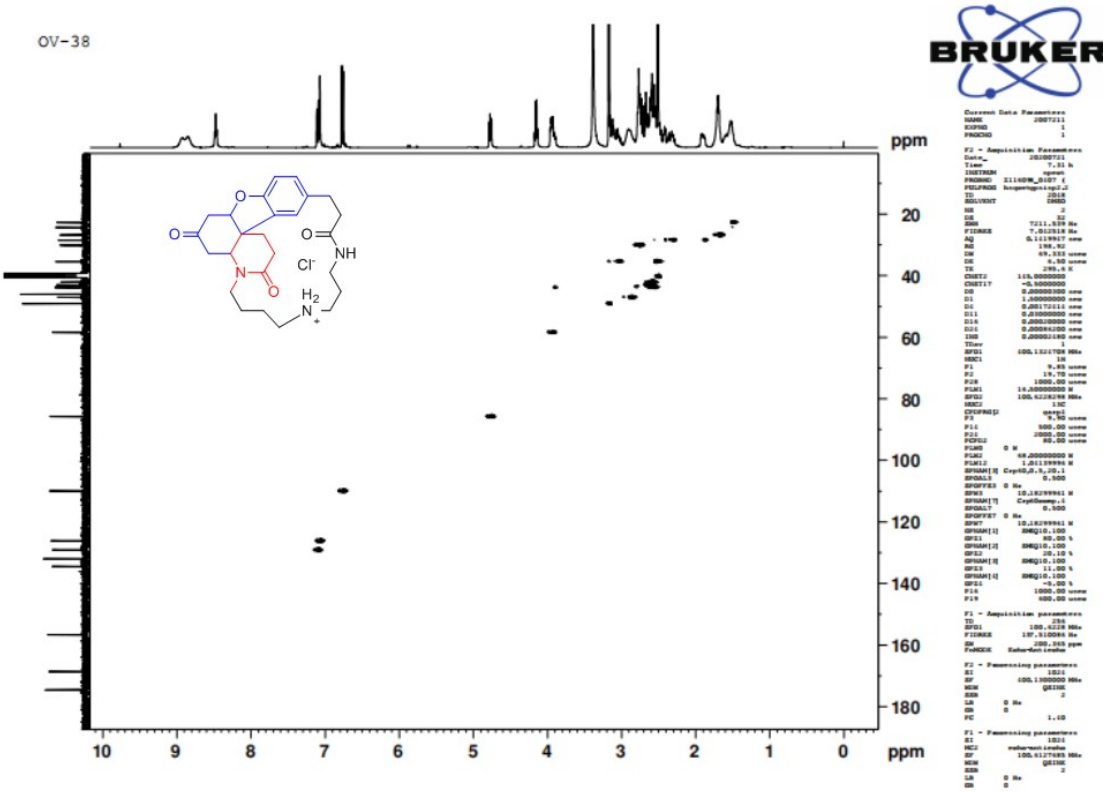


Figure S7. HSQC (400 MHz) spectrum of (±)-**1** in DMSO-d_6.

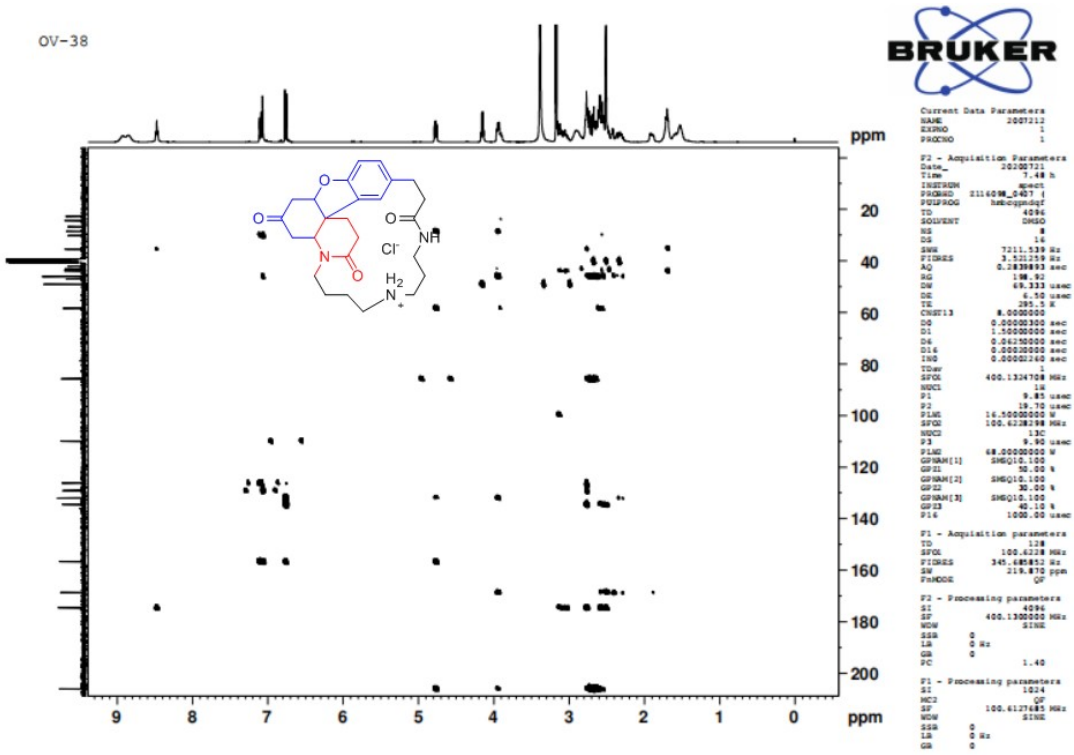


Figure S8. HMBC (400 MHz) spectrum of (±)-**1** in DMSO-d_6.

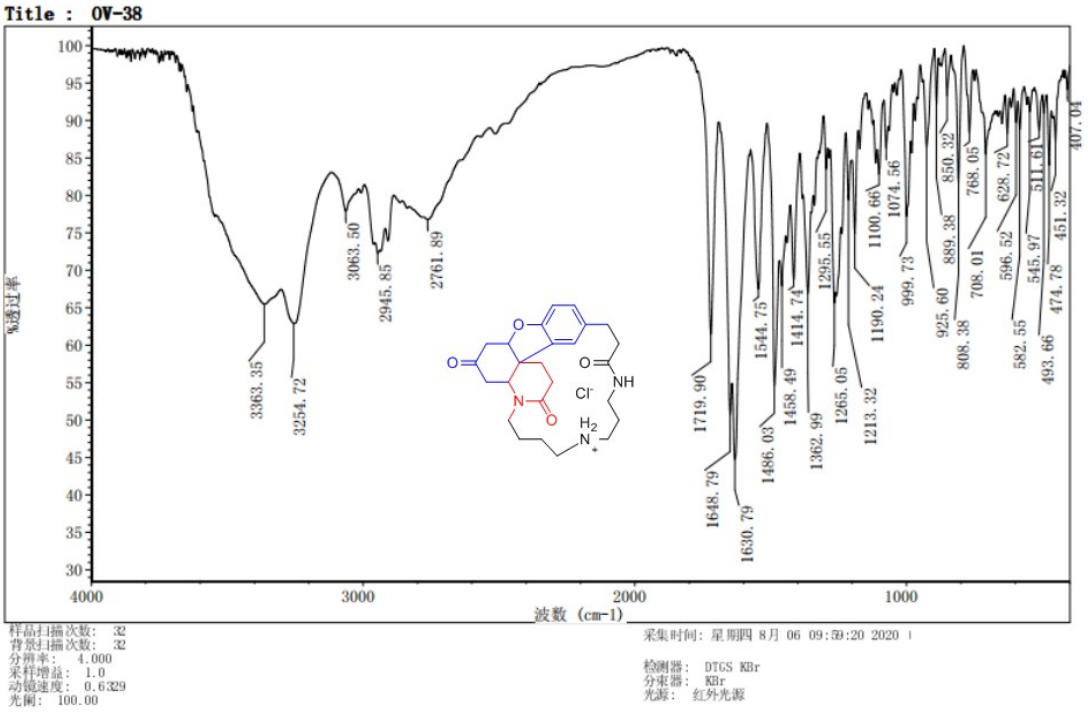


Figure S9. IR spectrum of (±)-**1**.

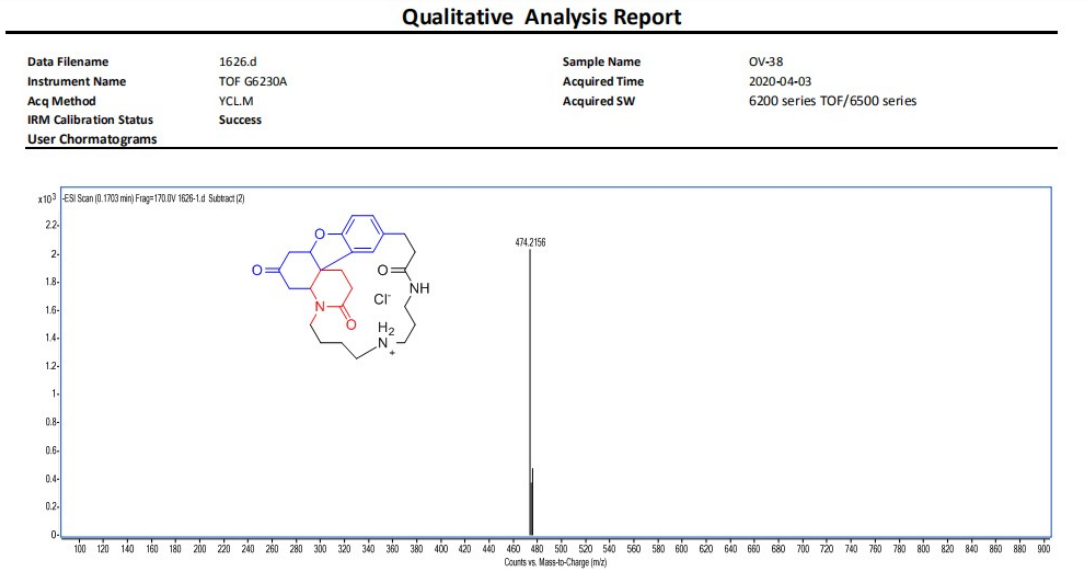
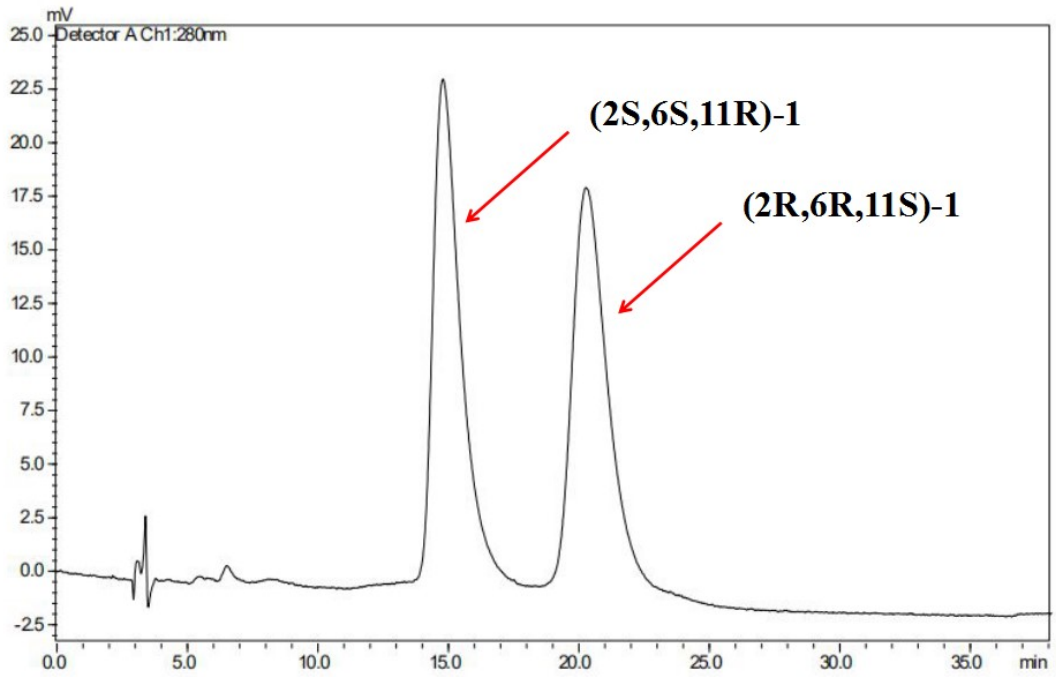
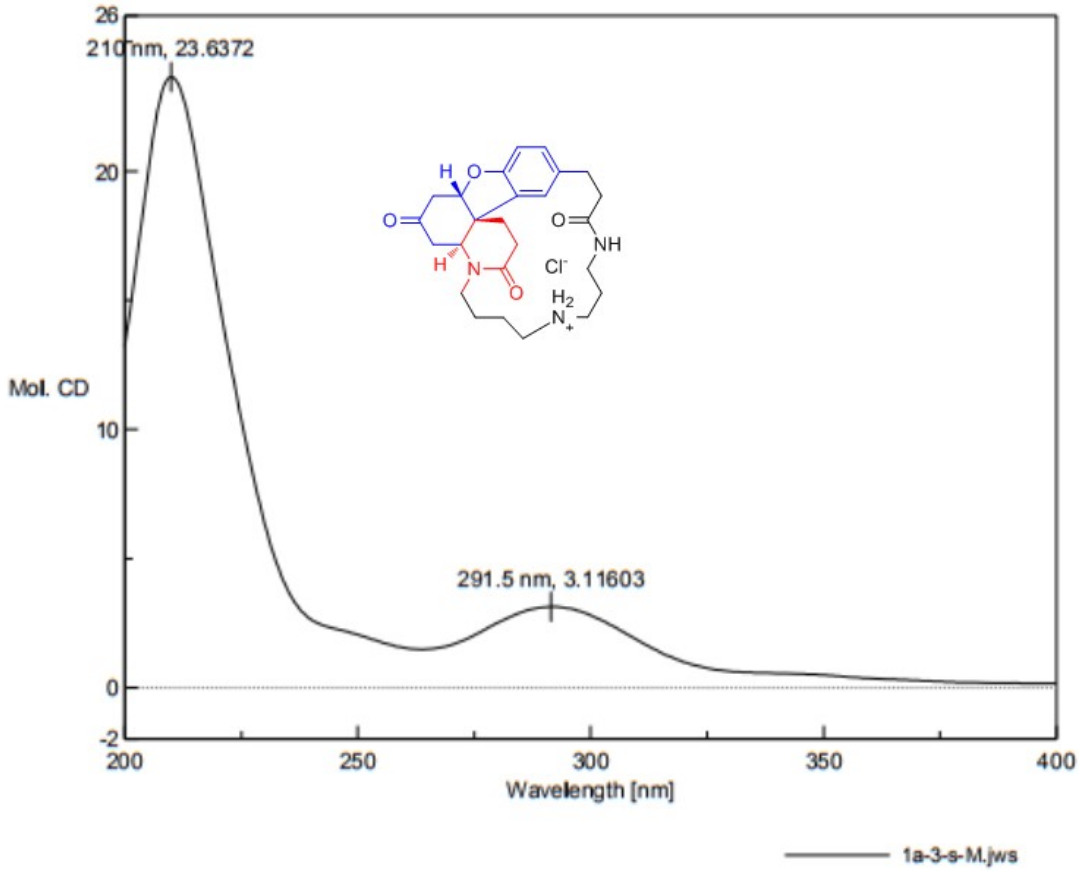


Figure S10. HR-ESI-MS data of (±)-**1**.



Peak No.	Time	Area	Area %	Plate number	Tailing	Resolution
1	14.789	1590588	49.7062	1061.951	1.687	--
2	20.268	1609392	50.2938	1162.410	1.428	2.614

Figure S11. The chiral HPLC separation chromatogram of (±)-**1**.



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Accessory S/N	A024461168
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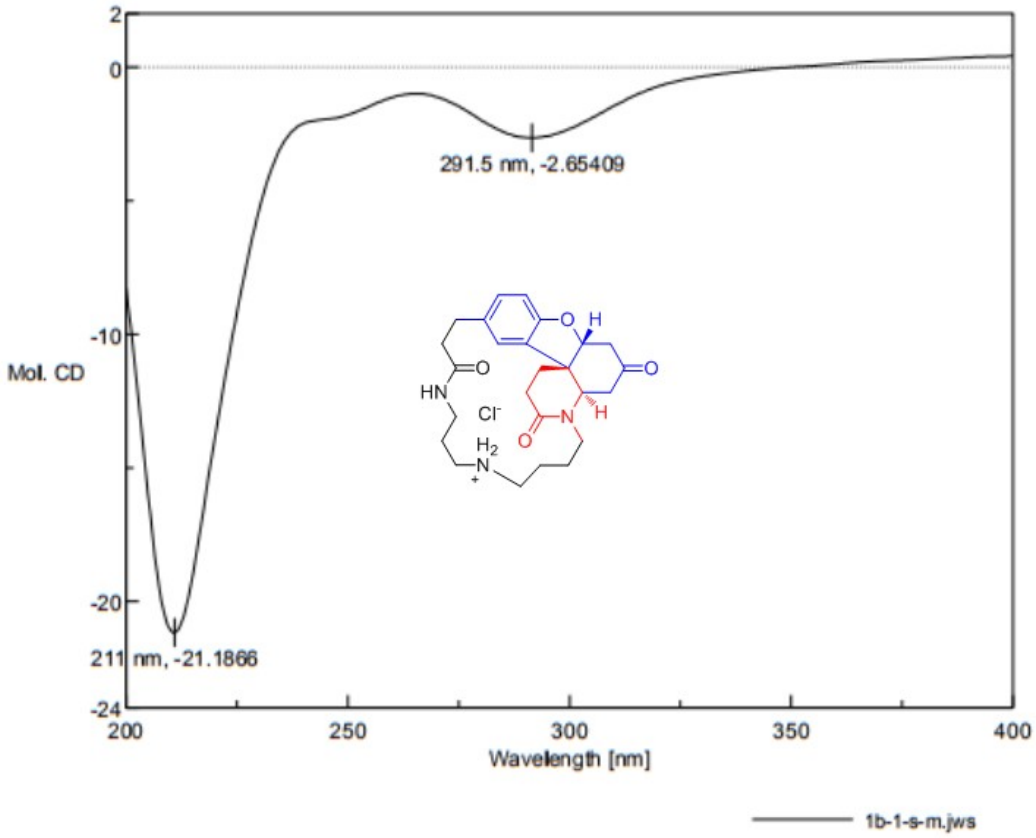
Photometric Mode	CD, HT, Abs
Measure Range	400 - 200 nm
Data pitch	0.5 nm
Sensitivity	Standard
D.I.T.	1 sec
Bandwidth	1.00 nm
Start Mode	Immediately
Scanning Speed	100 nm/min
Baseline Correction	Baseline
Shutter Control	Auto
CD Detector	PMT
PMT Voltage	Auto
Accumulations	2
Solvent	MEOH
Concentration	0.2 (w/v)%

Figure S12. CD spectrum of (+)-**1**.



[Measurement Information]	
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Model Name	J-815
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Accessory	Standard
Accessory S/N	A024461168
Cell Length	1 mm
Measurement date	2020/11/9 10:57
Photometric Mode	CD, HT, Abs
Measure Range	400 - 200 nm
Data pitch	0.5 nm
Sensitivity	Standard
D.I.T.	1 sec
Bandwidth	1.00 nm
Start Mode	Immediately
Scanning Speed	100 nm/min
Baseline Correction	Baseline
Shutter Control	Auto
CD Detector	PMT
PMT Voltage	Auto
Accumulations	2
Solvent	MEOH
Concentration	0.2 (w/v)%

Figure S13. CD spectrum of (-)-**1**.

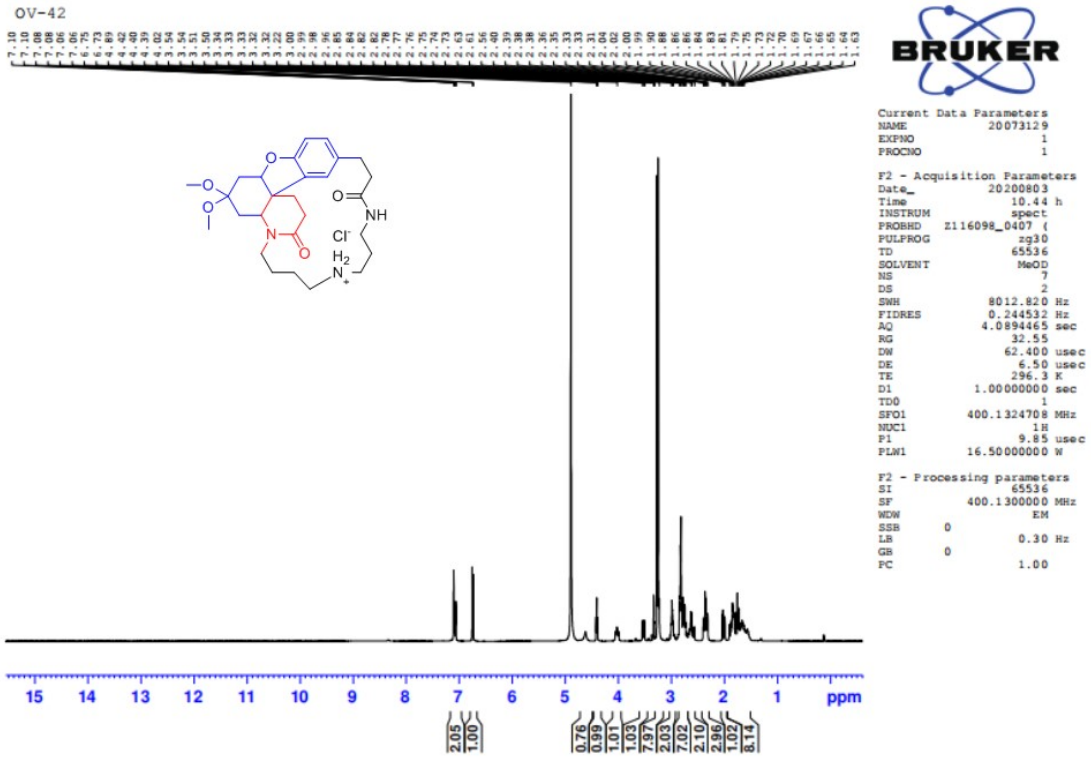


Figure S14. ^{1}H-NMR (400 MHz) spectrum of (±)-**2** in CD_3OD-d_4.

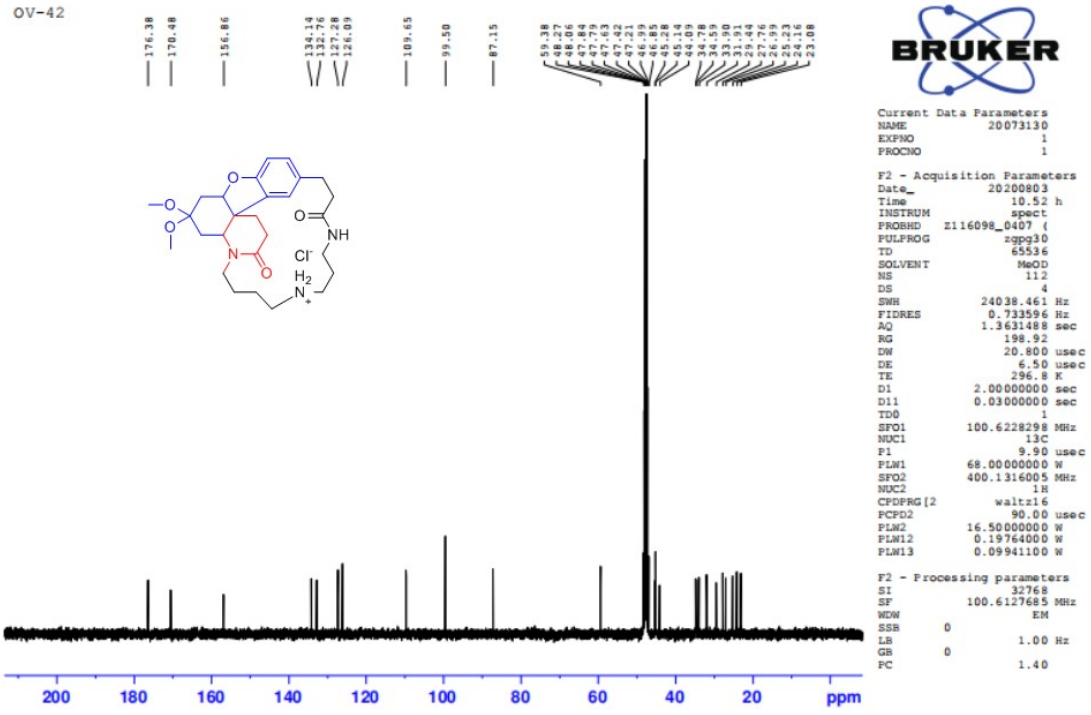


Figure S15. ^{13}C-NMR (100 MHz) spectra of (±)-**2** in CD_3OD-d_4.

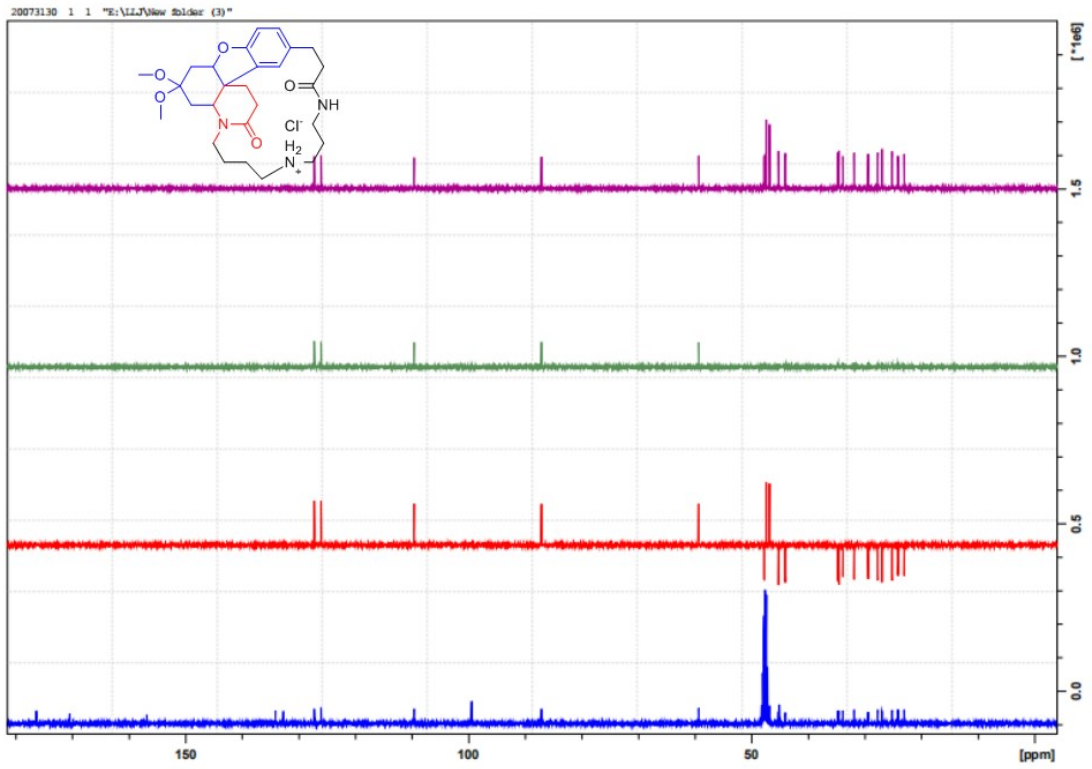


Figure S16. DEPT (100 MHz) spectrum of (±)-**2** in CD_3OD-d_4.

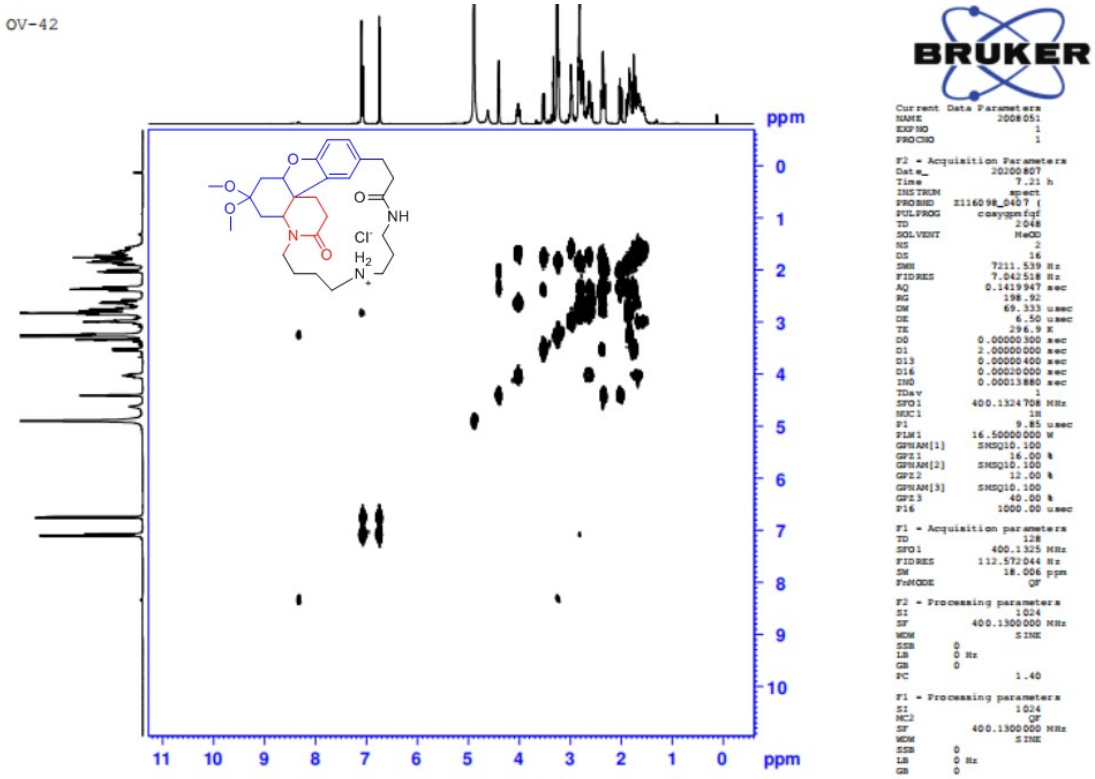


Figure S17. ^{1}H-^{1}H COSY (400 MHz) spectrum of (±)-**2** in CD_3OD-d_4.

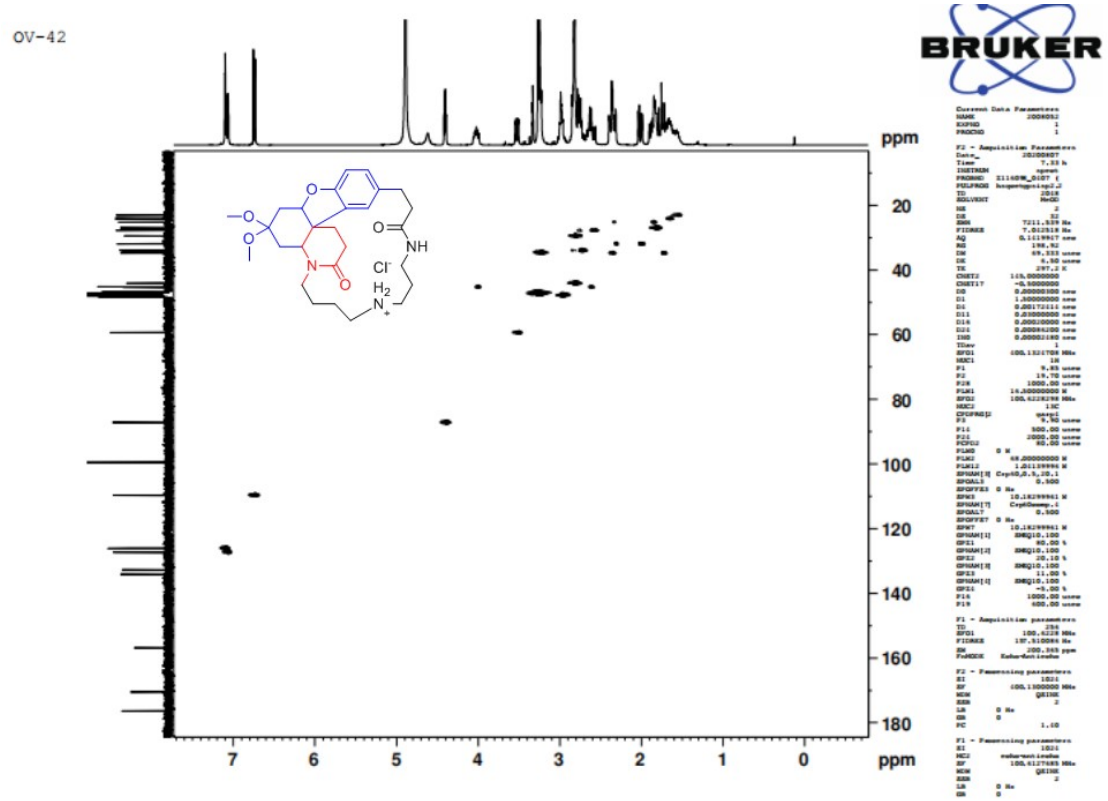


Figure S18. HSQC (400 MHz) spectrum of (±)-**2** in CD_3OD-d_4.

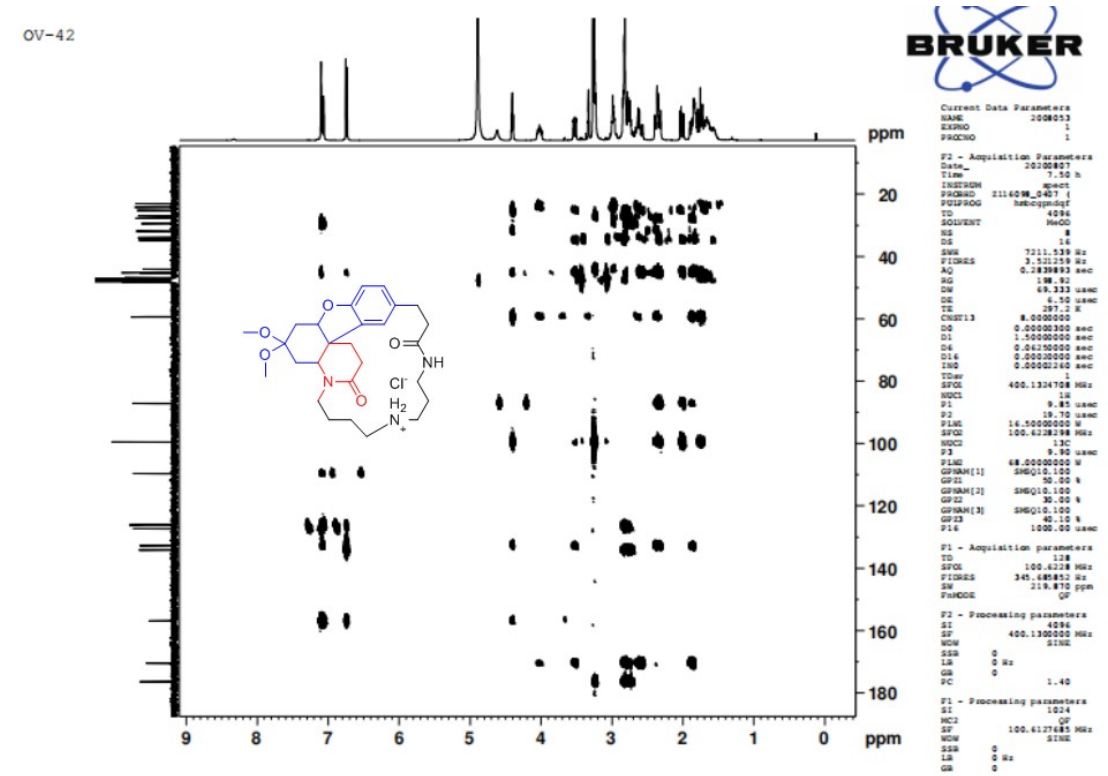


Figure S19. HMBC (400 MHz) spectrum of (±)-**2** in CD_3OD-d_4.

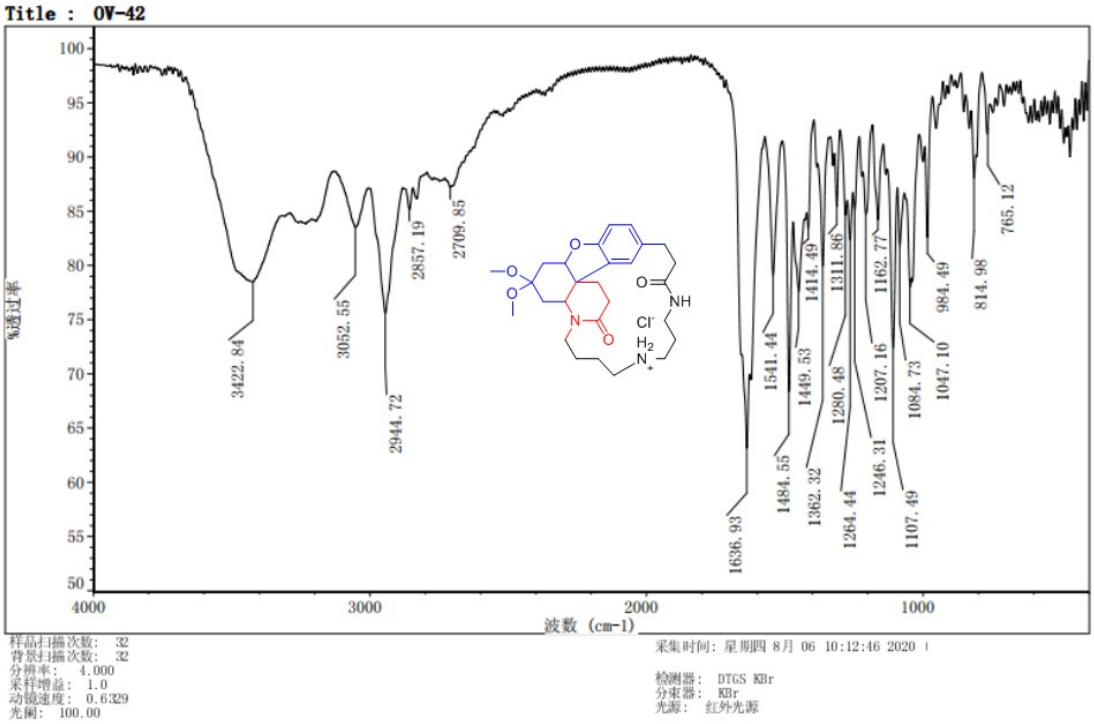


Figure S20. IR spectrum of (±)-**2**.

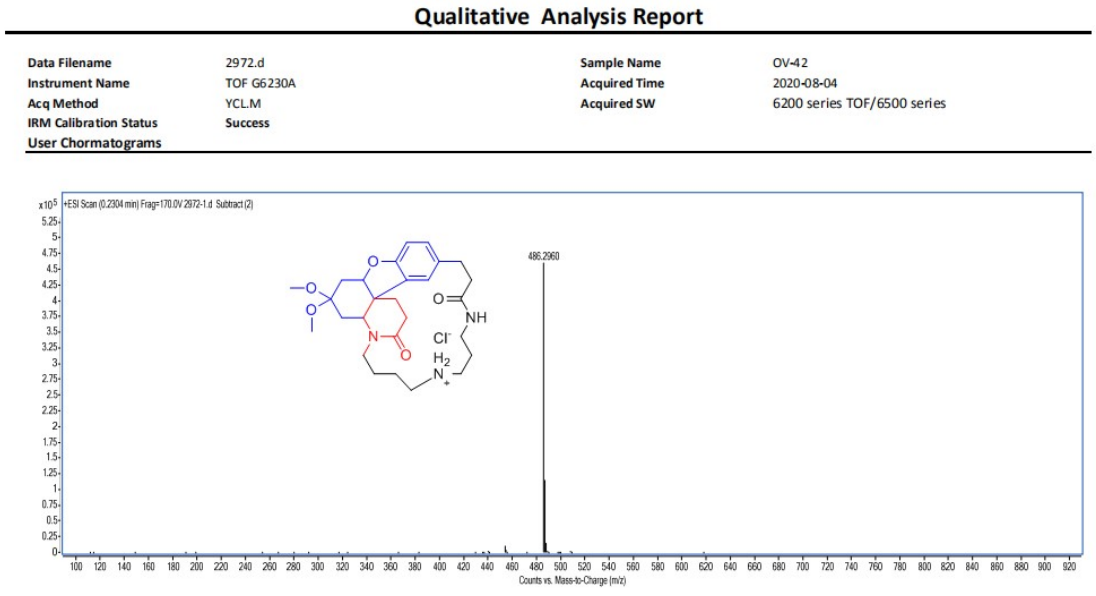
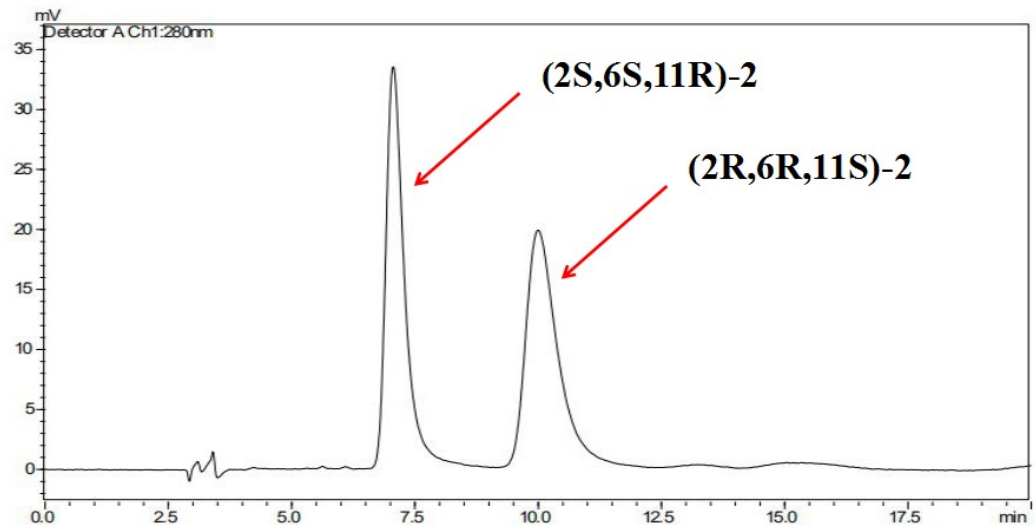
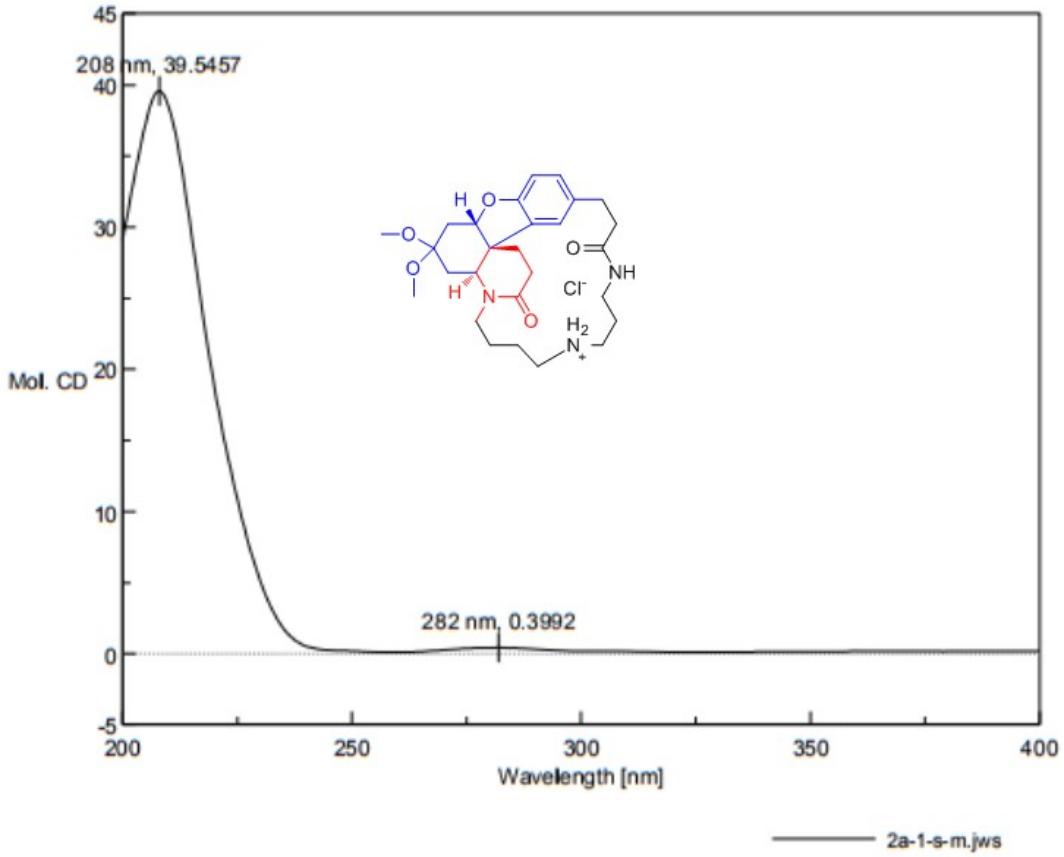


Figure S21. HR-ESI-MS data of (±)-**2**.



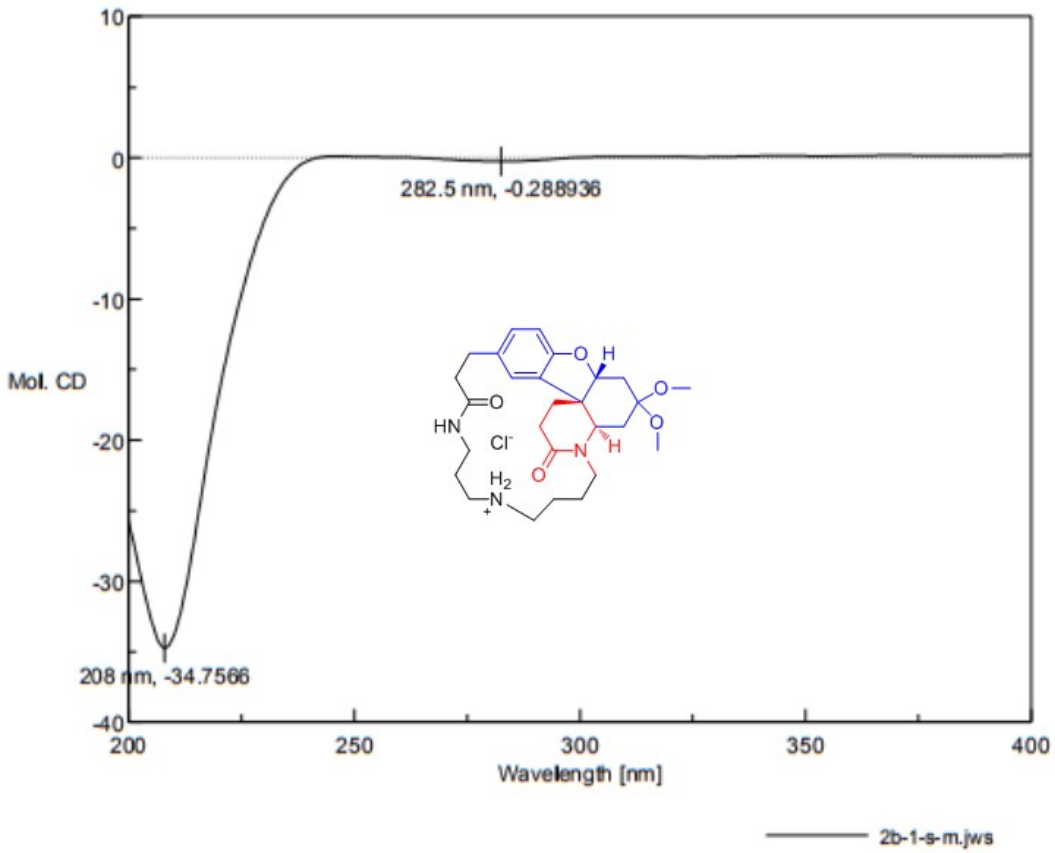
Peak No.	Time	Area	Area %	Plate number	Tailing	Resolution
1	7.057	887554	49.8467	1885.214	1.630	--
2	9.993	893011	50.1533	1245.974	1.640	3.295

Figure S22. The chiral HPLC separation chromatogram of (±)-**2**.



[Measurement Information]	
Instrument Name	J-815
Model Name	J-815
Serial No.	A024461168
Accessory	Standard
Accessory S/N	A024461168
Cell Length	1 mm
Measurement date	2020/11/9 11:05
Photometric Mode	CD, HT, Abs
Measure Range	400 - 200 nm
Data pitch	0.5 nm
Sensitivity	Standard
D.I.T.	1 sec
Bandwidth	1.00 nm
Start Mode	Immediately
Scanning Speed	100 nm/min
Baseline Correction	Baseline
Shutter Control	Auto
CD Detector	PMT
PMT Voltage	Auto
Accumulations	2
Solvent	MEOH
Concentration	0.2 (w/v)%

Figure S23. CD spectrum of (+)-**2**.



[Measurement Information]

Instrument Name	J-815
Model Name	J-815
Serial No.	A024461168
Accessory	Standard
Accessory S/N	A024461168
Cell Length	1 mm
Measurement date	2020/11/9 11:13
Photometric Mode	CD, HT, Abs
Measure Range	400 - 200 nm
Data pitch	0.5 nm
Sensitivity	Standard
D.I.T.	1 sec
Bandwidth	1.00 nm
Start Mode	Immediately
Scanning Speed	100 nm/min
Baseline Correction	Baseline
Shutter Control	Auto
CD Detector	PMT
PMT Voltage	Auto
Accumulations	2
Solvent	MEOH
Concentration	0.2 (w/v)%

Figure S24. CD spectrum of (-)-**2**.