Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting information

Synthesis and pharmacological evaluation of enantiomerically pure *endo*-configured KOR agonists with 2-azabicyclo[3.2.1]octane scaffold

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Content page

1. Purity data of test compounds determined by HPLC

Equipment: Pump: LPG-3600, degasser: DG-1210, autosampler: WPS 3000 PL, UV-detector: VWD-3400RS, interface: DIONEX UltiMate 3000, data acquisition: Chromeleon 7 (Thermo Fisher Scientific); column: LiChrospher® 60 RP-select B (5 µm), LiChroCART® 250-4 mm cartridge; flow rate: 1.0 mL/min; injection volume: 5.0 µL; detection at λ = 210 nm; solvents: A: demineralized water with 0.05 % (V/V) trifluoroacetic acid, B: acetonitrile with 0.05 % (V/V) trifluoroacetic acid; gradient elution (% A): 0 - 4 min: 90 %; 4 - 29 min: gradient from 90 % to 0 %; 29 - 31 min: 0 %; 31 - 31.5 min: gradient from 0 % to 90 %; 31.5 - 40 min: 90 %. Unless otherwise noted, the purity of all test compounds is higher than 95 %.

Purity data of test compounds

compound	*ee*	purity
(1*S*,5*S*,7*R*)-**10a**	53.6 %	99.1 %
(1*S*,5*S*,7*R*)-**10a**	99.9 %	95.1 %
(1*R*,5*R*,7*S*)-**10a**	52.8 %	97.2 %
(1*R*,5*R*,7*S*)-**10a**	96.3 %	95.7 %
(1*S*,5*S*,7*R*)-**10b**	54.3 %	99.2 %
(1*S*,5*S*,7*R*)-**10b**	99.9 %	98.0 %
(1*R*,5*R*,7*S*)-**10b**	49.8 %	98.6 %
(1*R*,5*R*,7*S*)-**10b**	98.8 %	95.1 %

2. NOESY experiments

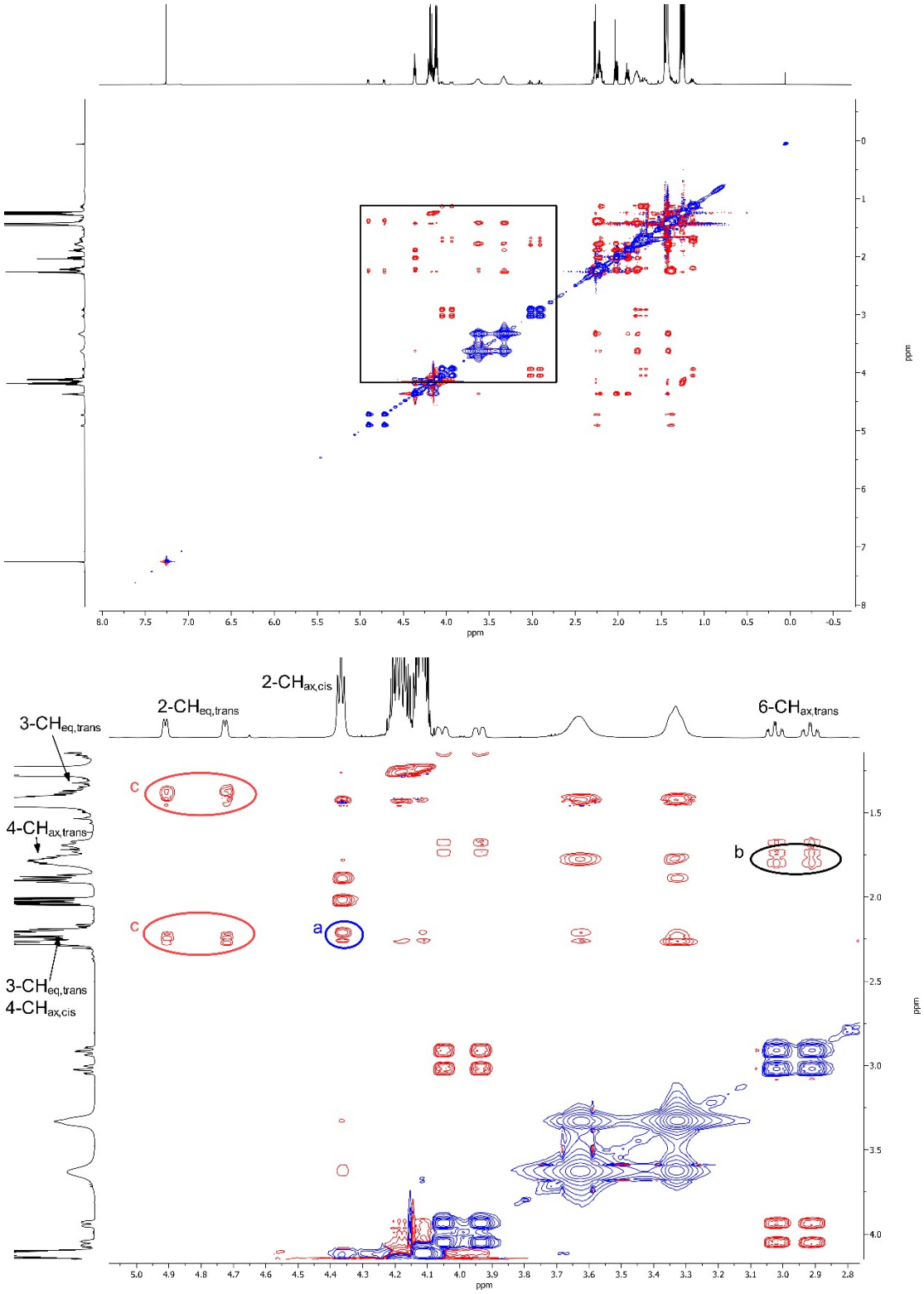
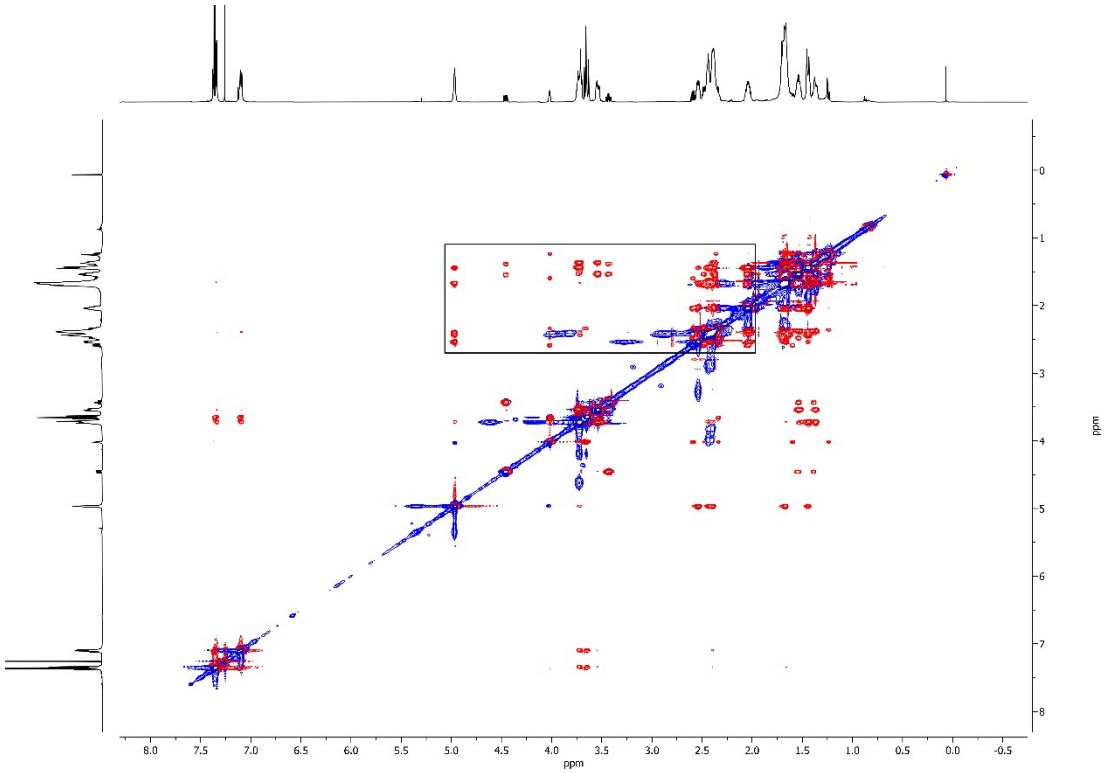


Figure S1**:** NOESY spectrum of diesters *cis*-**12** and *trans*-**12** in the ratio 3:1. Due to rotational isomerism along the Boc group, two sets of signals are seen for *trans*-**12**. Top:

Complete NOESY spectrum. Bottom: Magnification of the region of interest. A Cross peak between 4-H (2.16 – 2.18 ppm) and 2-H (4.37 ppm, mark a) for *cis*-**12**, and two cross peaks between 4-H (1.76 – 1.84 ppm) and 6-H (2.92 ppm and 3.02 ppm, mark b) and 2-H (4.72 ppm and 4.91 ppm) and 3-H_{ax} (1.34 – 1.43 ppm) as well as 3-H_{eq} (2.21 – 2.29 ppm, mark c) for *trans*-**12** can be detected.



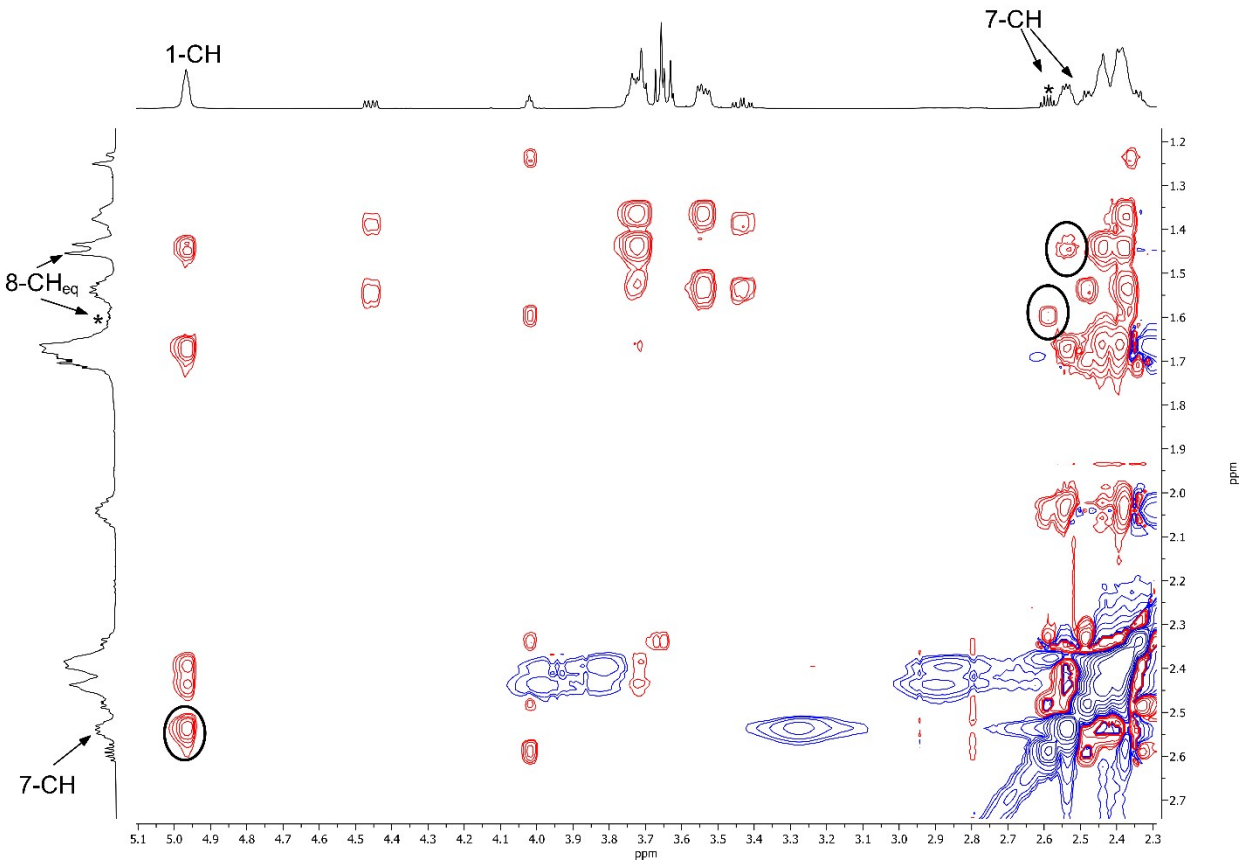


Figure S2: NOESY spectrum of bicyclic amine **10a**. Top: Complete NOESY spectrum. Bottom: Magnification of the region of interest. Cross peaks between 7-H (2.51 – 2.57 ppm) and 8-H_{eq} (1.41 – 1.46 ppm) and 7-H and 1-CH (4.97 – 5.02 ppm) for the major rotational isomer, and a cross peak between 7-H* (2.58 – 2.62 ppm) and 8-H_{eq}* (1.58 – 1.62 ppm) for the minor rotational isomer can be detected. The corresponding cross peaks are marked in black.

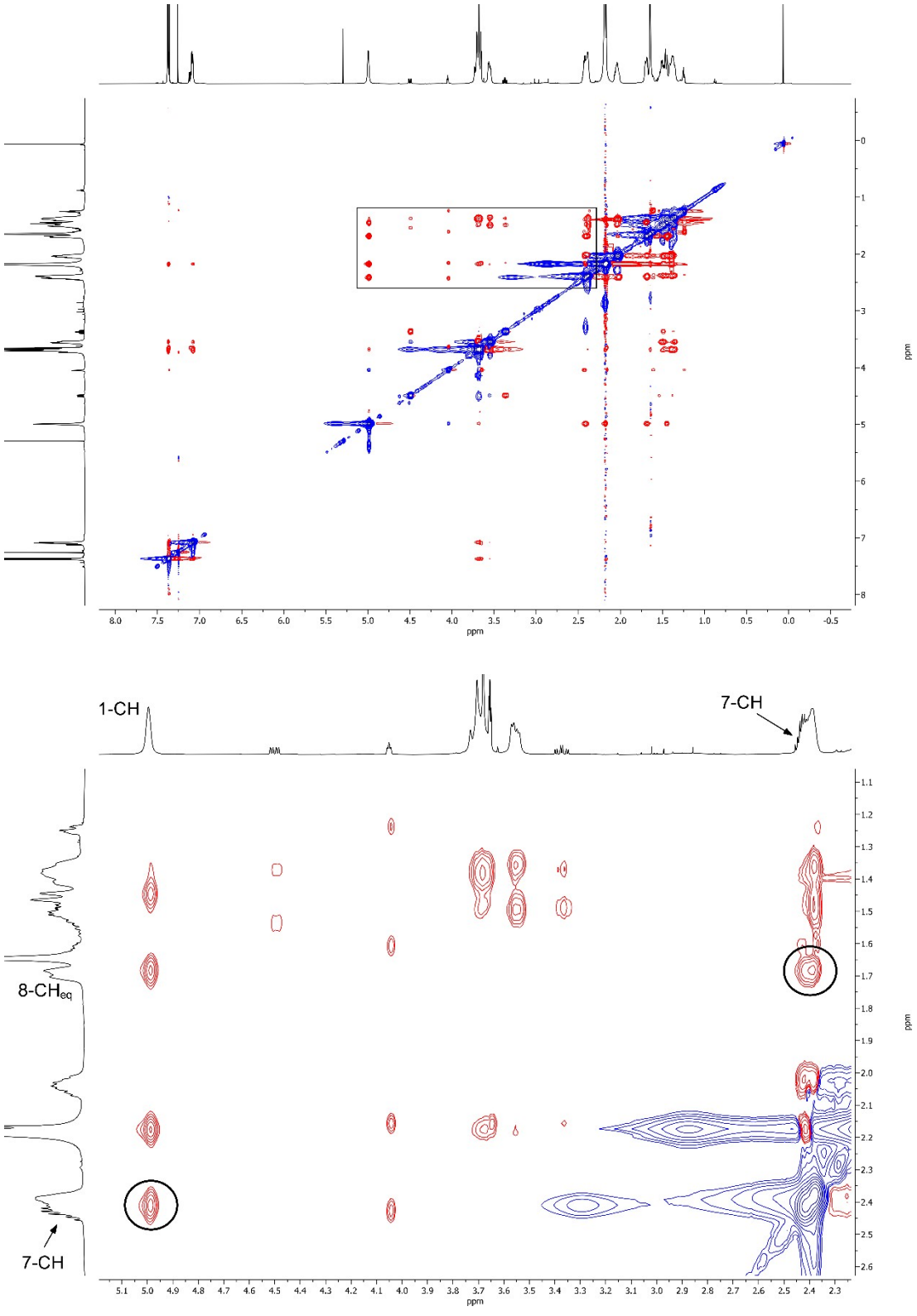


Figure S3**:** NOESY spectrum of bicyclic amine **10b**. Top: Complete NOESY spectrum. Bottom: Magnification of the region of interest. A Cross peak between 7-H (2.36 – 2.46

ppm) and 8-H_{eq} (1.66 – 1.72 ppm) and a cross peak between 7-H (2.36 – 2.46 ppm) and 1-H (4.97 – 5.02 ppm) for the minor rotational isomer can be detected. The corresponding cross peaks are marked in black.

3. Chiral HPLC

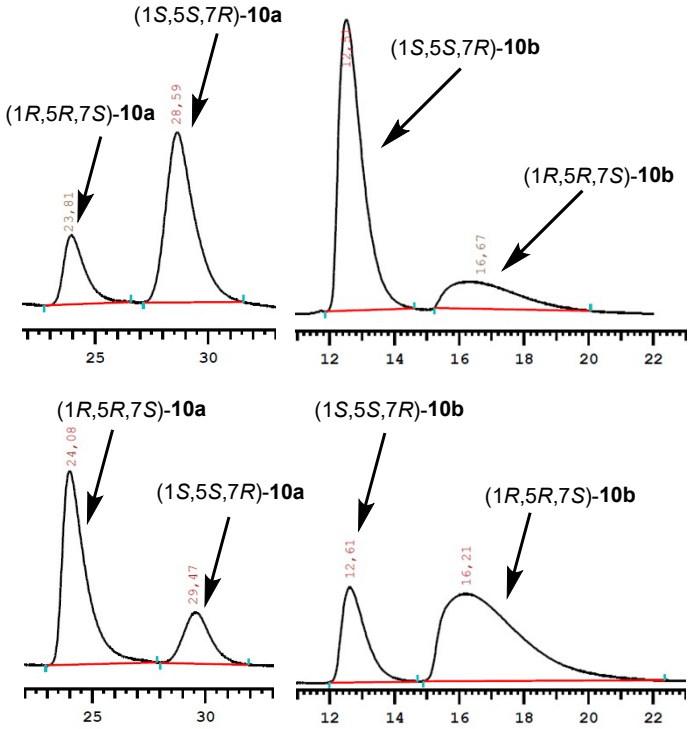


Figure S4: HPLC chromatogram before the separation of enantiomers **10a,b**. Column: Daicel Chiralpak IA; eluent: *iso*-hexane/ EtOH = 9:1 + 0.1 % diethylamine + 0.1 % formic acid (**10a**), *iso*-hexane/ *iso*-propanol = 95:5 + 0.1 % diethylamine (**10b**), isocratic elution; flow rate: 1.0 mL/min; detection l = 275 nm. The addition of formic acid led to an inversion of the signals.

4. Receptor binding studies

4.1. Materials

Guinea pig brains, rat brains and rat livers were commercially available (Harlan-Winkelmann, Borchen, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep® 150 (MSE, London, UK). Centrifuges: Cooling centrifuge model Eppendorf 5427R (Eppendorf, Hamburg, Germany) and High-speed cooling centrifuge model Sorvall® RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96 well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta® FilterMate 96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex® (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta® Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

4.2. Preparation of membrane homogenates from guinea pig brain[1-3]

5 guinea pig brains were homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

4.3. Preparation of membrane homogenates from rat brain[4-6]

5 rat brains (species: Sprague Dawley rats) were homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

4.4. Preparation of membrane homogenates from rat liver[1-3]

2 rat livers were cut into small pieces and homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at rt for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x g for 20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80 °C in 1.5 mL portions containing about 2 mg protein/mL.

4.5. Protein determination

The protein concentration was determined by the method of Bradford,[7] modified by Stoscheck.[8] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95 %, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85 %, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg /mL). In a 96 well standard multiplate, 10 µL of the calibration solution or 10 µL of the membrane receptor preparation were mixed with 190 µL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye complex at λ = 595 nm was measured with a plate reader (Tecan Genios®, Tecan, Crailsheim, Germany).

4.6. General procedures for the binding assays

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5 % aqueous polyethylenimine solution for 2 h at rt before use. All binding experiments were carried out in duplicates in the 96 well multiplates. The concentrations given are the final

concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL of test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 µL of the corresponding radioligand solution and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500-600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration, each well was washed five times with 300 µL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at rt, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20 %. The IC_{50} values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC_{50} values were transformed into K_i values using the equation of Cheng and Prusoff.[9] The K_i values are given as mean value ± SEM from three independent experiments.

4.7. Performance of the KOR assay[4-6]

The assay was performed with the radioligand [^{3}H]U-69,593 (55 Ci/mmol, BIOTREND). The thawed guinea pig brain membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 1 nM [^{3}H]U-69,593, and TRIS-$MgCl_2$-buffer (50 mM TRIS, 8 mM $MgCl_2$, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled U-69,593. The K_d value of U-69,593 is 0.69 nM.

4.8. Performance of the MOR assay[4-6]

The assay was performed with the radioligand [^{3}H]DAMGO (51 Ci/mmol, Perkin Elmer). The thawed guinea pig brain membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]DAMGO, and TRIS-$MgCl_2$-buffer (50 mM TRIS, 8 mM $MgCl_2$, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled naloxone. The K_d value of DAMGO is 0.57 nM.

4.9. Performance of the DOR assay[4-6]

The assay was performed with the radioligand [^{3}H]DPDPE (69 Ci/mmol, BIOTREND). The thawed rat brain membrane preparation (about 75 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]DPDPE, and TRIS-$MgCl_2$-buffer (50 mM TRIS, 8 mM $MgCl_2$, pH 7.4) supplemented with SIGMAFAST® protease inhibitor mix (Sigma Aldrich Biochemicals, Hamburg, Germany; 1 tablet dissolved in 100 mL of buffer) at 37 °C. The non-specific binding was determined with 10 µM unlabeled morphine. The K_d value of DPDPE is 0.65 nM.

4.10. Performance of the σ_1 receptor assay[1-3]

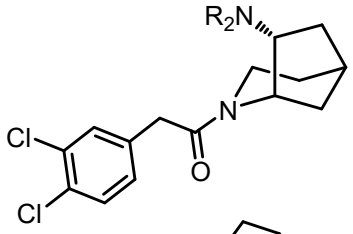
The assay was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d value of (+)-pentazocine is 2.9 nM.[10]

4.11. Performance of the σ_2 receptor assay[1-3]

The assays were performed with the radioligand [^{3}H]di-*o*-tolylguanidine (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed rat liver membrane preparation (about 100 µg protein) was incubated with various concentrations of the test compound, 3 nM [^{3}H]di-*o*-tolylguanidine and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in TRIS buffer (50 mM TRIS, pH 8.0)) at rt. The non-specific binding was determined with 10 µM non-labeled di-*o*-tolylguanidine. The K_d value of di-*o*-tolylguanidine is 17.9 nM.[11]

5. Receptor affinity of non-racemic amines (ee ~*50 %)*

Table SI1: Affinities of non-racemic 2-azabicyclo[3.2.1]octanes **10aa** and **10b** (ee ~50 %).



10a: NR_2 = –N

10b: $NR_2 = N(CH_3)_2$

compd.	NR_2	*ee* [%]	K_i ± SEM [nM] (n = 3)[a)]				
			KOR	MOR	DOR	σ_1	σ_2
			[^{3}H] U-69,593	[^{3}H] DAMGO	[^{3}H] DPDPE	(+)-[^{3}H]pentazocine	[^{3}H] DTG
10a	–N	53.6	12 ± 2	8%	3200	7 ± 4	310
ent-**10a**		52.8	53 ± 5	0%	1800	20 ± 4	243
10b	$N(CH_3)_2$	54.3	18 ± 2	10%	0%	76 ± 6	17%
ent-**10b**		49.8	49 ± 18	5%	565	32 ± 10	366

[a)] A value in % reflects the inhibition of the radioligand binding at a test compound concentration of 1 µM. K_i values without SEM values represent the mean of two experiments (n = 2), K_i values with SEM values represent the mean of three experiments (n = 3).

6. References

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4. Wittig, C.; Schepmann, D.; Soeberdt, M.; Daniliuc, C. G.; Wünsch, B. Stereoselective Synthesis of Conformationally Restricted KOR Agonists Based on the 2,5-diazabicyclo[2.2.2]octane Scaffold. *Org. Biomol. Chem.* **2017**, *15* (31), 6520–6540.
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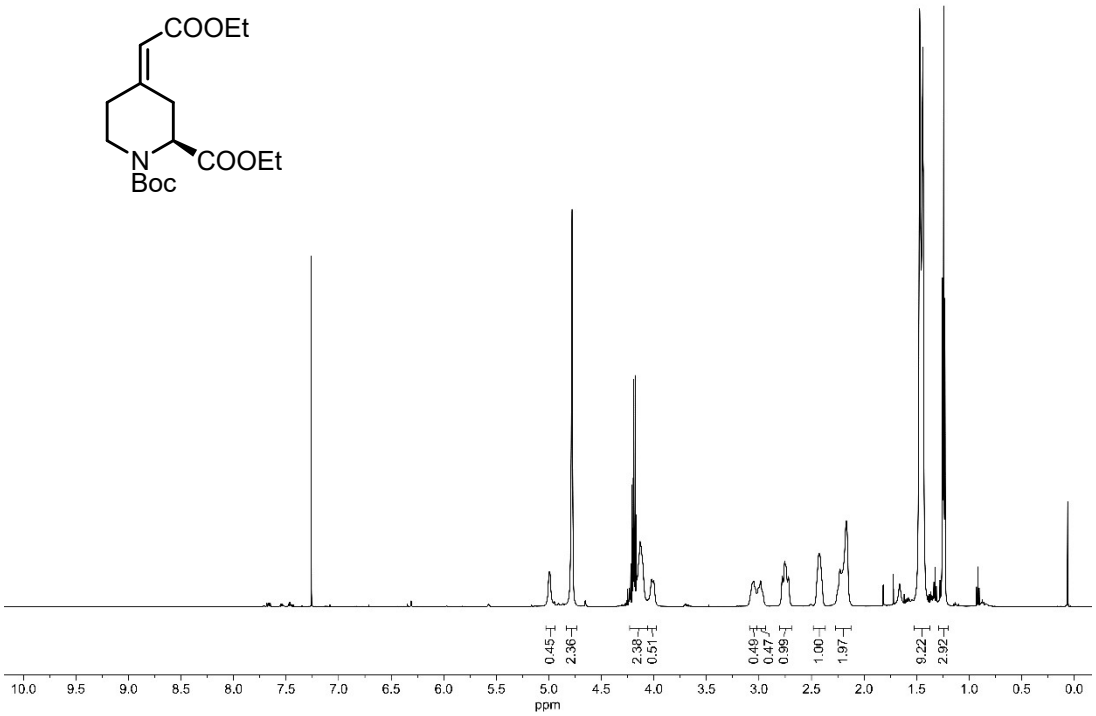
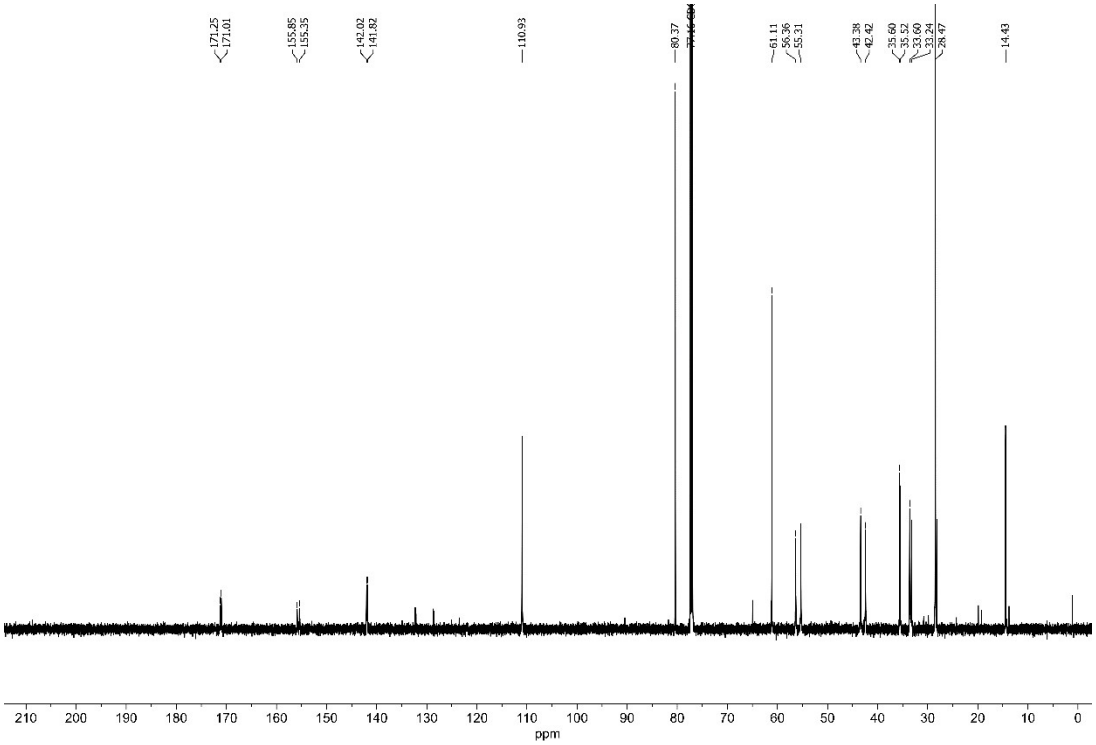
11. Mach, R. H.; Smith, C. R.; Childers, S. R. Ibogaine Possesses a Selective Affinity for Sigma 2 Receptors. *Life Sci.* **1995**, *57* (4), 57-62.

7. NMR spectra



^{1}H NMR spectrum ($CDCl_3$) of **14**.



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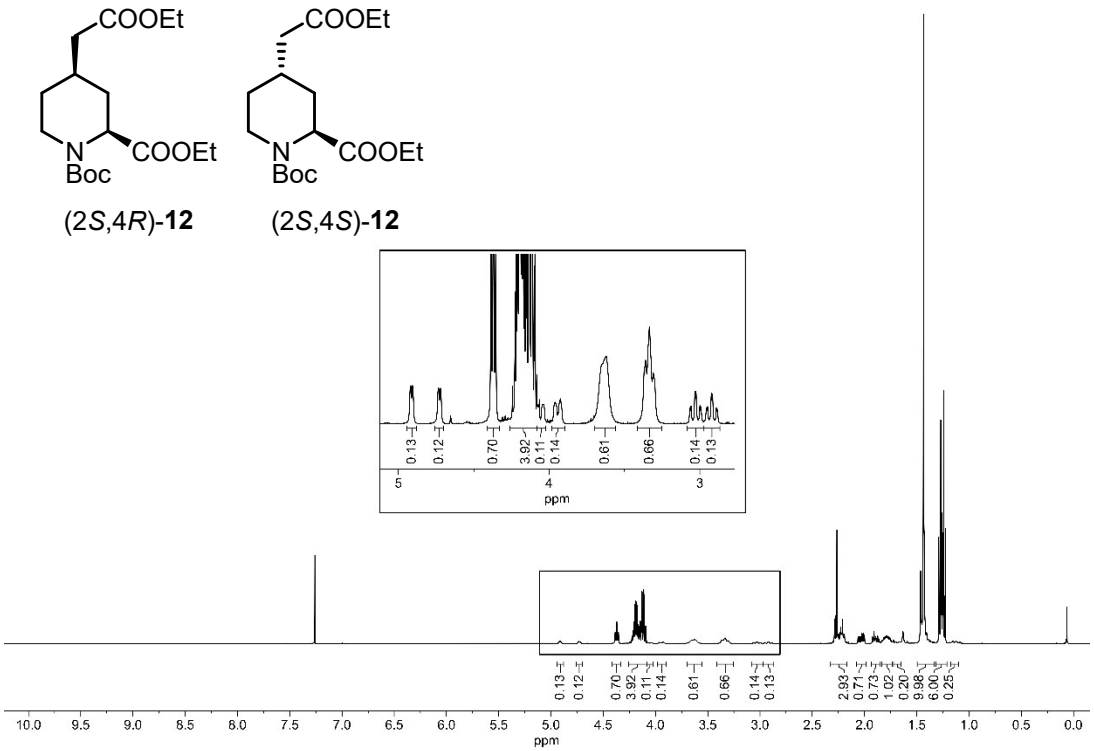
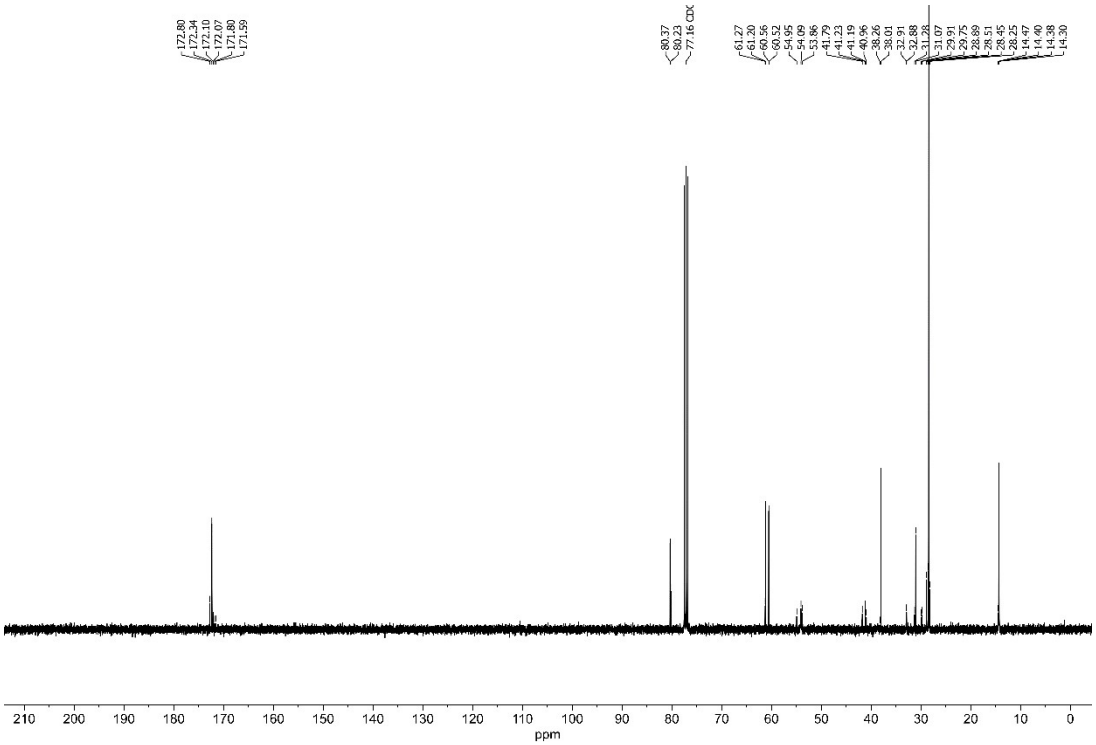
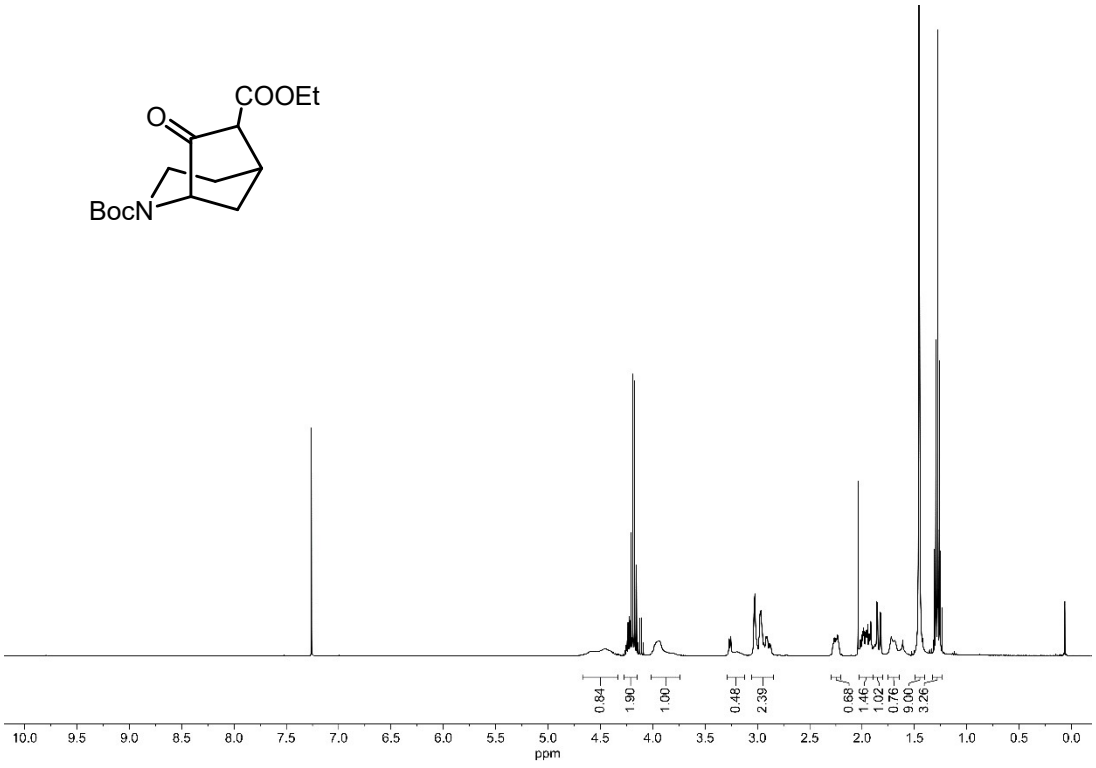


^{1}H NMR spectrum ($CDCl_3$) of (2*S*,4*R*)-**12** (mayor) and (2*S*,4*S*)-**12** (minor). The diastereomers were obtained in a 3:1 ratio. Due to rotational isomerism along the Boc group, the minor diastereomer shows two sets of signals.



^{13}C NMR spectrum ($CDCl_3$) of (2*S*,4*R*)-**12** (maior) and (2*S*,4*S*)-**12** (minor).



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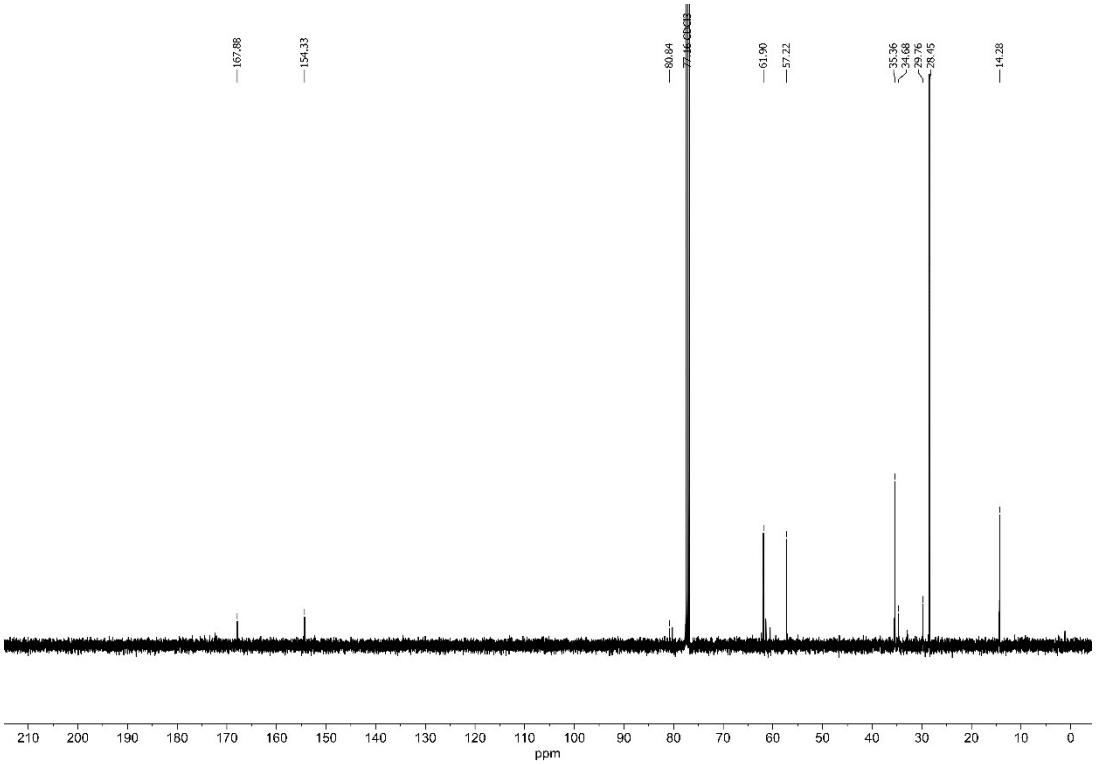

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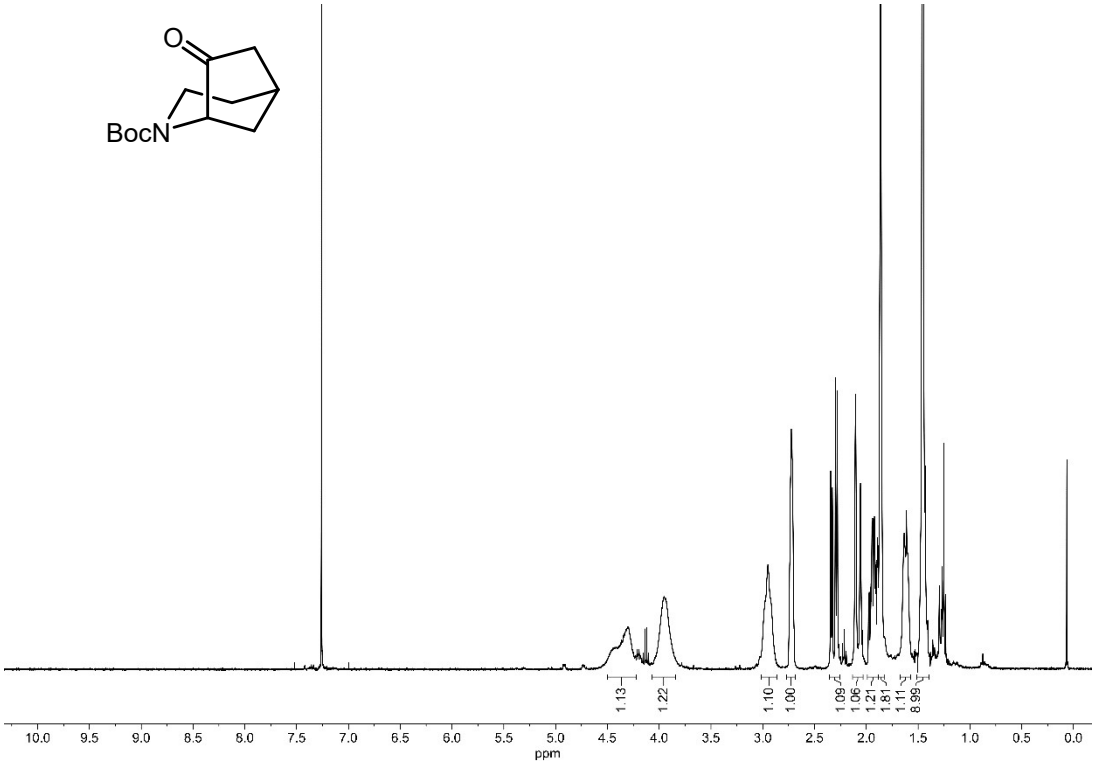
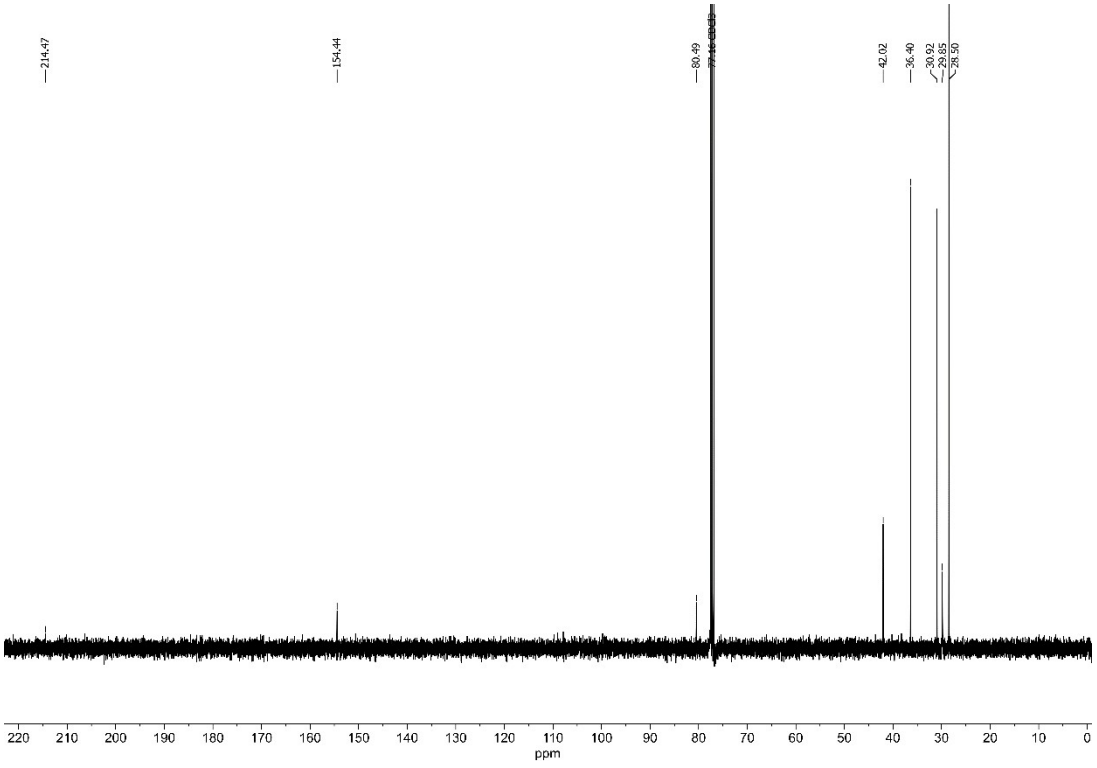


^{1}H NMR spectrum ($CDCl_3$) of **16**.



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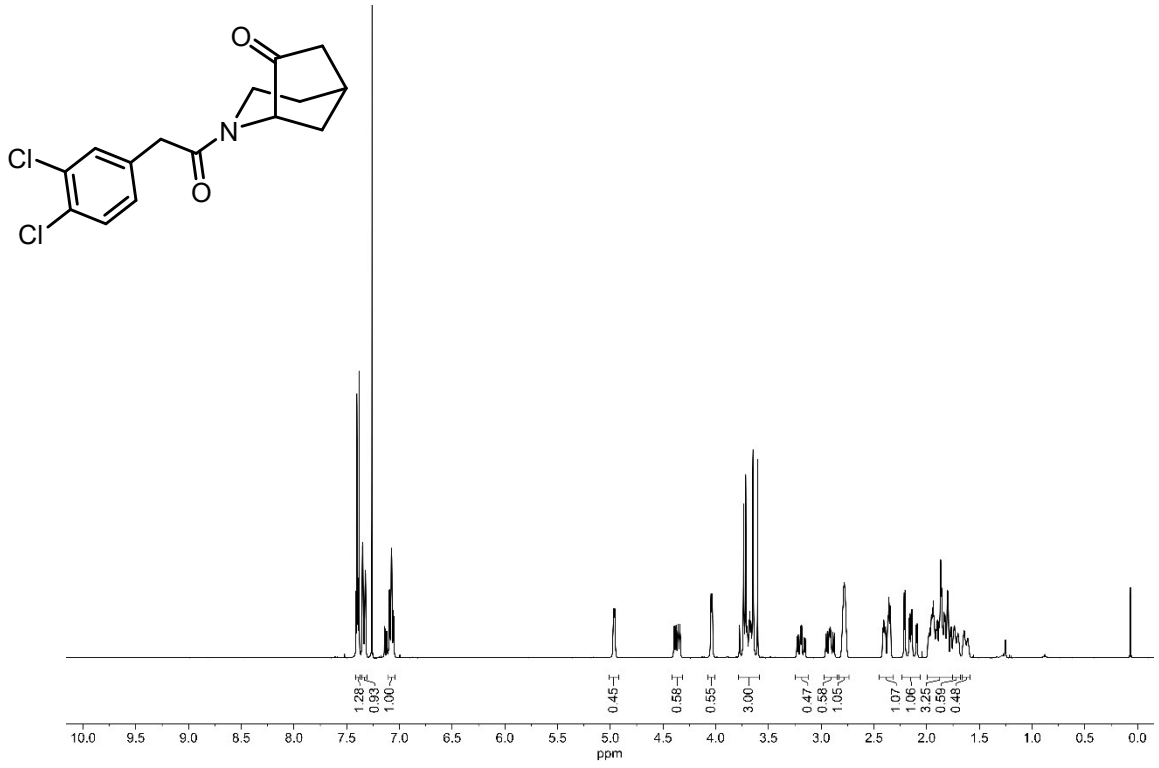

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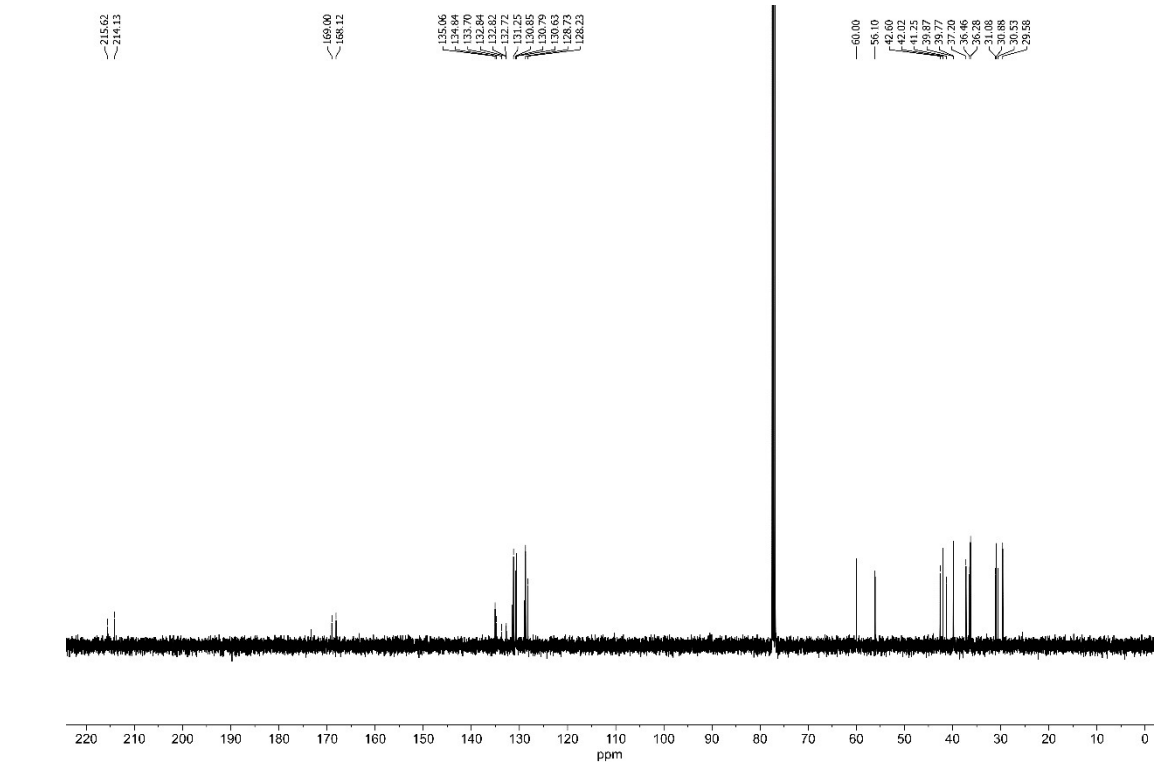

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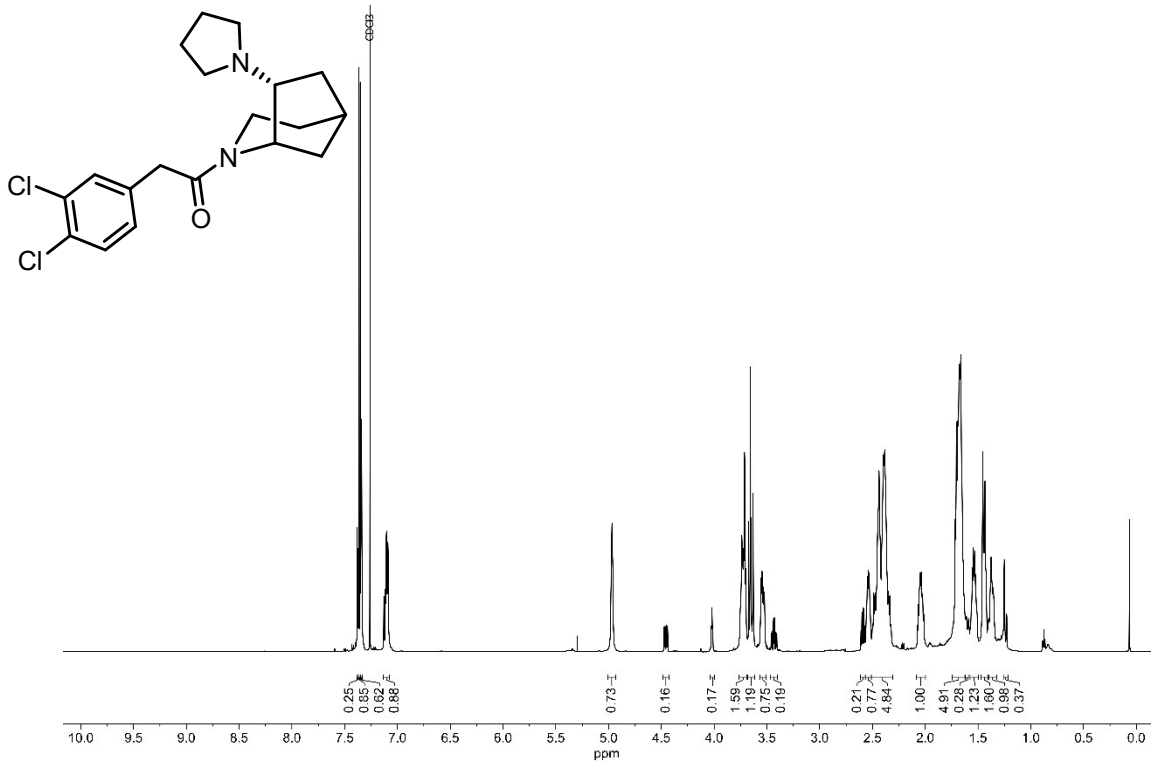

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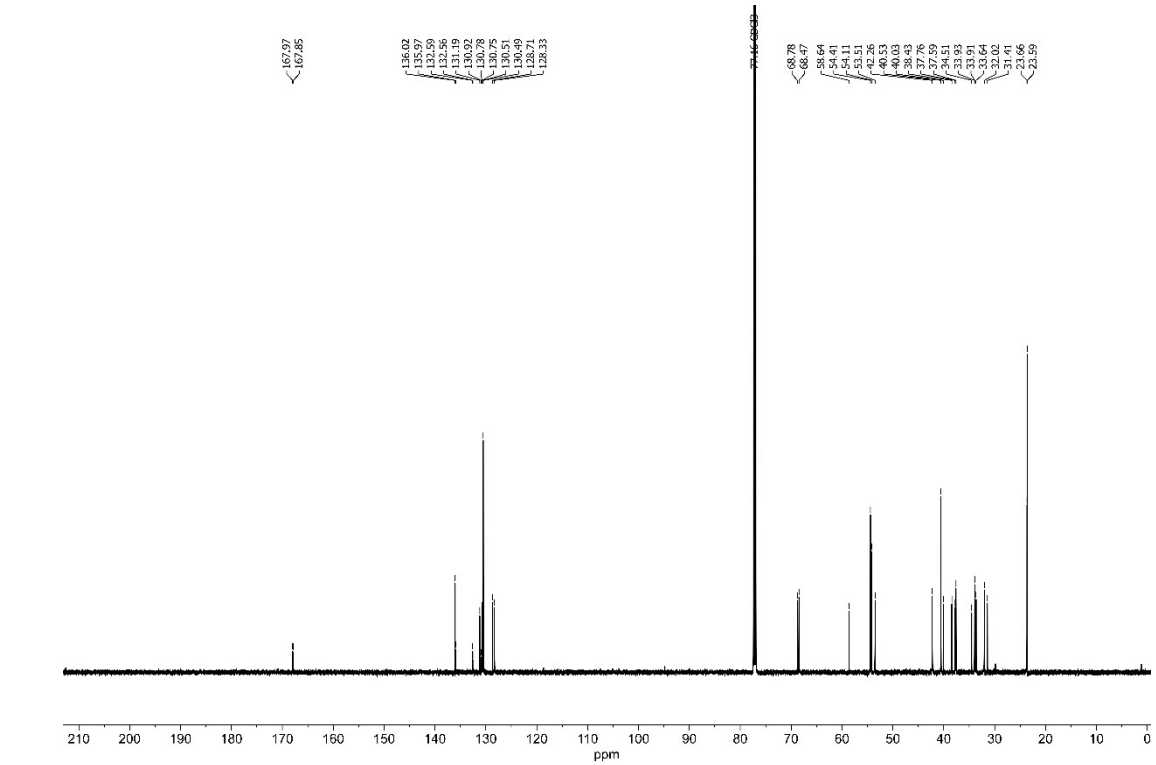
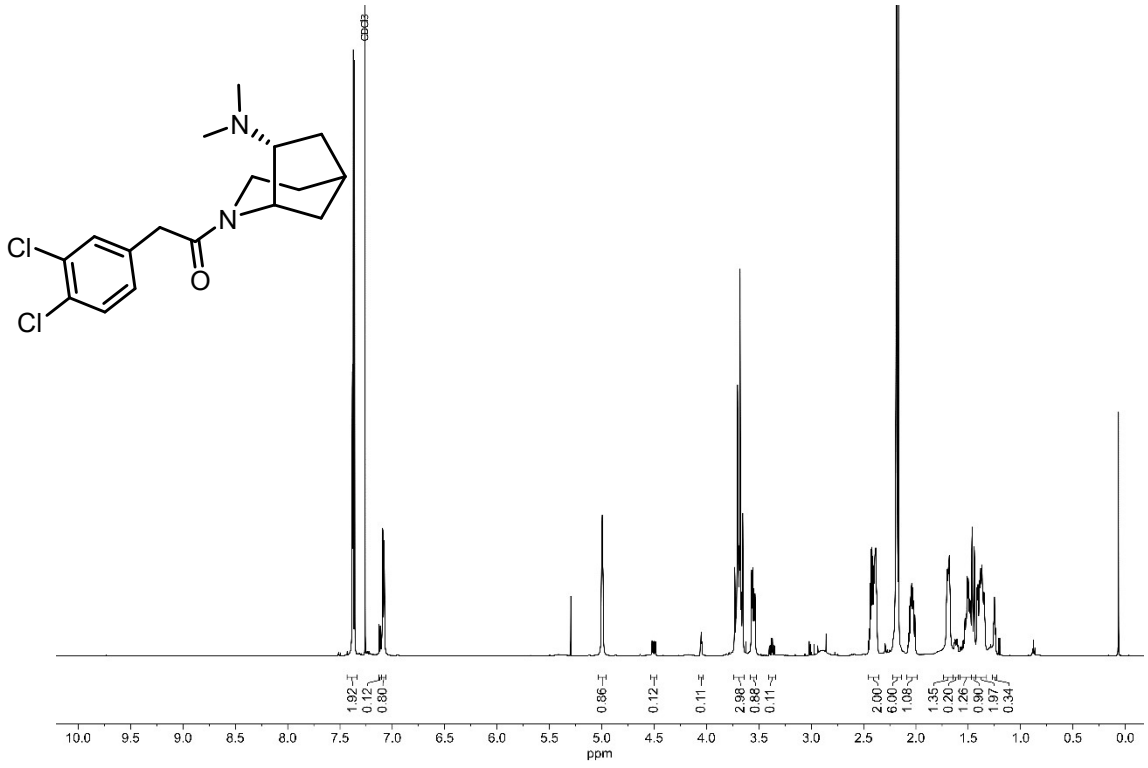


^{13}C NMR spectrum ($CDCl_3$) of **10a**.



^{1}H NMR spectrum ($CDCl_3$) of **10b**.

^{13}C NMR spectrum ($CDCl_3$) of **10b**.