Supplementary Information to:

Oxa-noribogaine reduces alcohol drinking through aversion learning and by altering glutamatergic activity in the mPFC

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1. Materials and Methods.

General Considerations. Reagents and solvents were obtained from commercial sources (Fisher Scientific or Sigma Aldrich) and were used without further purification unless otherwise stated. Reactions were monitored by TLC using solvent mixtures appropriate to each reaction. Column chromatography was performed on silica gel (40 – 63 µm). For preparative TLC, glass plates coated with a 1 mm silica layer were used. Nuclear magnetic resonance spectra were recorded on Bruker 500 MHz instrument. Chemical shifts are reported as δ values in ppm referenced to $CDCl_3$ (^{1}H NMR = 7.26 and ^{13}C NMR = 77.16), methanol-d_4 (^{1}H NMR = 3.31 and ^{13}C NMR = 49.00), water-d_2 (^{1}H NMR = 4.79, ^{13}C NMR was calibrated to MeOH standard 49.50). and DMSO-d_6 (^{1}H NMR = 2.50) Multiplicity is indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); p (pentet); dd (doublet of doublets); td (triplet of doublets); dt (doublet of triplets); dq (doublet of quartets); ddd (doublet of doublet of doublets); ddt (doublet of doublet of triplets); m (multiplet); br (broad). Low-resolution mass spectra were recorded on an Advion CMS quadrupole instrument (ionization mode: APCI+ or ESI+). High-resolution mass spectra (HRMS) were acquired on a high-resolution Waters XEVO G2-XS QToF mass spectrometer equipped with a UPC2 SFC inlet, on-board fluidics and an ESI probe.

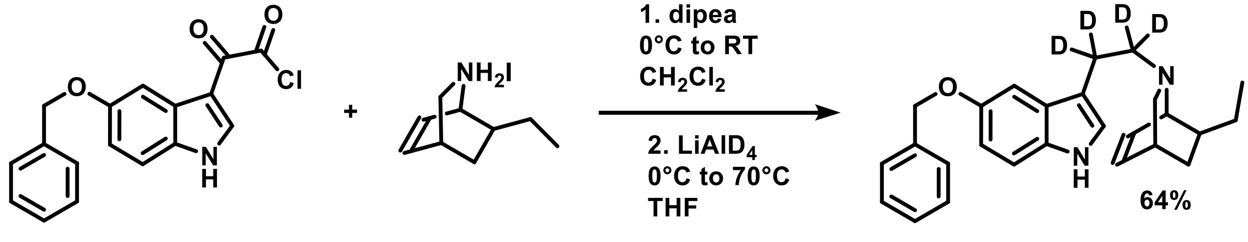
2. Ibogaine hydrochloride

Ibogaine hydrochloride was purchased from Coryn Pharmaceuticals LLC. The material originated from Ghana and was isolated from *Tabernanthe iboga* root bark. The purity and identity of ibogaine hydrochloride was confirmed by an analytical laboratory in Germany to be ≥98%. Minor constituent of the material was identified as ibogamine hydrochloride <1%.

3. Racemic oxa-noribogaine hydrochloride

Oxa-noribogaine hydrochloride was prepared according to the published procedure[1] and was used in the salt form for the biological experiments.

4. Deuterium labeled racemic noribogaine



(Rac)-2-(2-(5-(benzyloxy)-1*H*-indol-3-yl)ethyl-1,1,2,2-d_4)-7-ethyl-2-azabicyclo[2.2.2]oct-5-ene

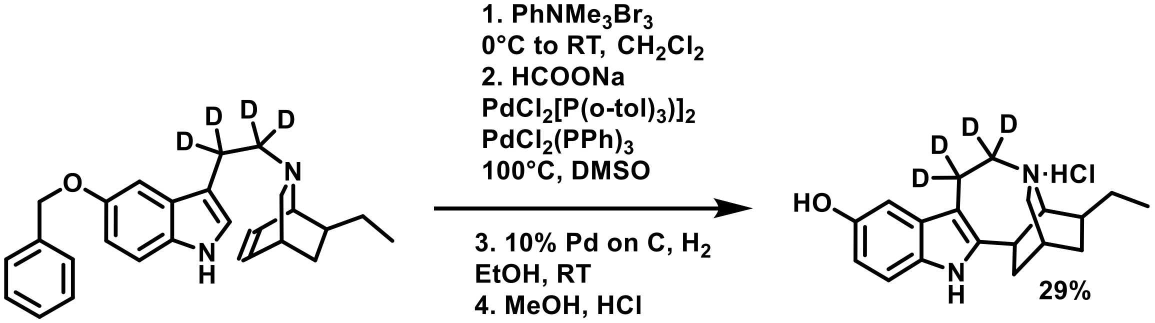
2-(5-(benzyloxy)-1H-indol-3-yl)-2-oxoacetyl chloride (471 mg, 1.50 mmol) was prepared according to published procedure[2] and was combined with a racemic exo-ethyl isoquinuclidine hydroiodide (398 mg, 1.50 mmol) in a reaction vial. Anhydrous CH_2Cl_2 (0.5 M, 3.0 mL) was added, and the suspension was cooled in ice/water bath (~0°C). Dipea (0.78 mL, 4.50 mmol) was added, cooling bath was removed and mixture was further stirred at RT. All solids fully dissolved after the addition of base and reaction was completed within 2 hours. Mixture was then diluted with CH_2Cl_2 (15 mL), washed with aq. HCl (1 M, 10 mL) and sat. aq. $NaHCO_3$ (10 mL). CH_2Cl_2 solution was dried over Na_2SO_4, filtered and concentrated. Residue was purified by column chromatography using a gradient of AcOEt in Hexanes (30, 50 to 100%). Product was isolated as a beige amorphous solid (537 mg).

Isolated glyoxyamide (537 mg, 1.30 mmol) was dissolved in anhydrous THF (5.2 mL, 0.25 M) and the solution was cooled in ice/water bath. Solid lithium aluminum deuteride ($LiAlD_4$, 109 mg, 2.60 mmol) was added in portions to the cold solution (small portions initially, once exothermic reaction subsided all remaining solid was added). Reaction vial was closed with a solid Teflon-lined screw cap and mixture was stirred at 70°C for 3 hours. Mixture was further cooled to RT and then in ice/water bath (~0°C), diluted with diethyl ether (10 mL) and carefully quenched using sequential additions of H_2O, 15% aq. NaOH and H_2O (ratio 1:1:3 mL per g of $LiAlD_4$ used). Mixture was stirred at RT until all salts were discolored and loose, solid was removed by filtration and washed with diethyl ether (50 mL in portions). Collected combined filtrates were concentrated and obtained brown oil was purified by column chromatography using 10% AcOEt in Hexanes + 2% Et_3N. Product was isolated as a pale brown oil (374 mg, 64% over two steps).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (s, 1H), 7.52 – 7.44 (m, 2H), 7.43 – 7.36 (m, 2H), 7.36 – 7.30 (m, 1H), 7.23 (d, *J* = 8.7 Hz, 1H), 7.13 (d, *J* = 2.5 Hz, 1H), 7.01 (d, *J* = 2.4 Hz, 1H), 6.93 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.36 (t, *J* = 6.7 Hz, 1H), 6.30 (ddd, *J* = 8.0, 5.4, 1.6 Hz, 1H), 5.12 (s, 2H), 3.36 – 3.20 (m, 1H), 3.12 (s, 1H), 2.40 (s, 1H), 1.97 (dt, *J* = 9.1, 2.6 Hz, 1H), 1.68 – 1.53 (m, 2H), 1.53 – 1.43 (m, 1H), 1.38 – 1.27 (m, 1H), 0.99 – 0.87 (m, 4H).

^{13}C NMR (126 MHz, $CDCl_3$ δ 153.14, 137.95, 132.95, 132.91, 131.69, 128.64, 128.23, 127.88, 127.76, 122.56, 112.72, 111.77, 102.85, 71.24, 56.31, 56.01, 41.28, 31.74, 29.90, 27.34, 12.69.

LRMS (APCI+) calcd. for $C_{26}H_{27}D_4N_2O^+$ $[M+H]^+$ 391.3, found 391.3.

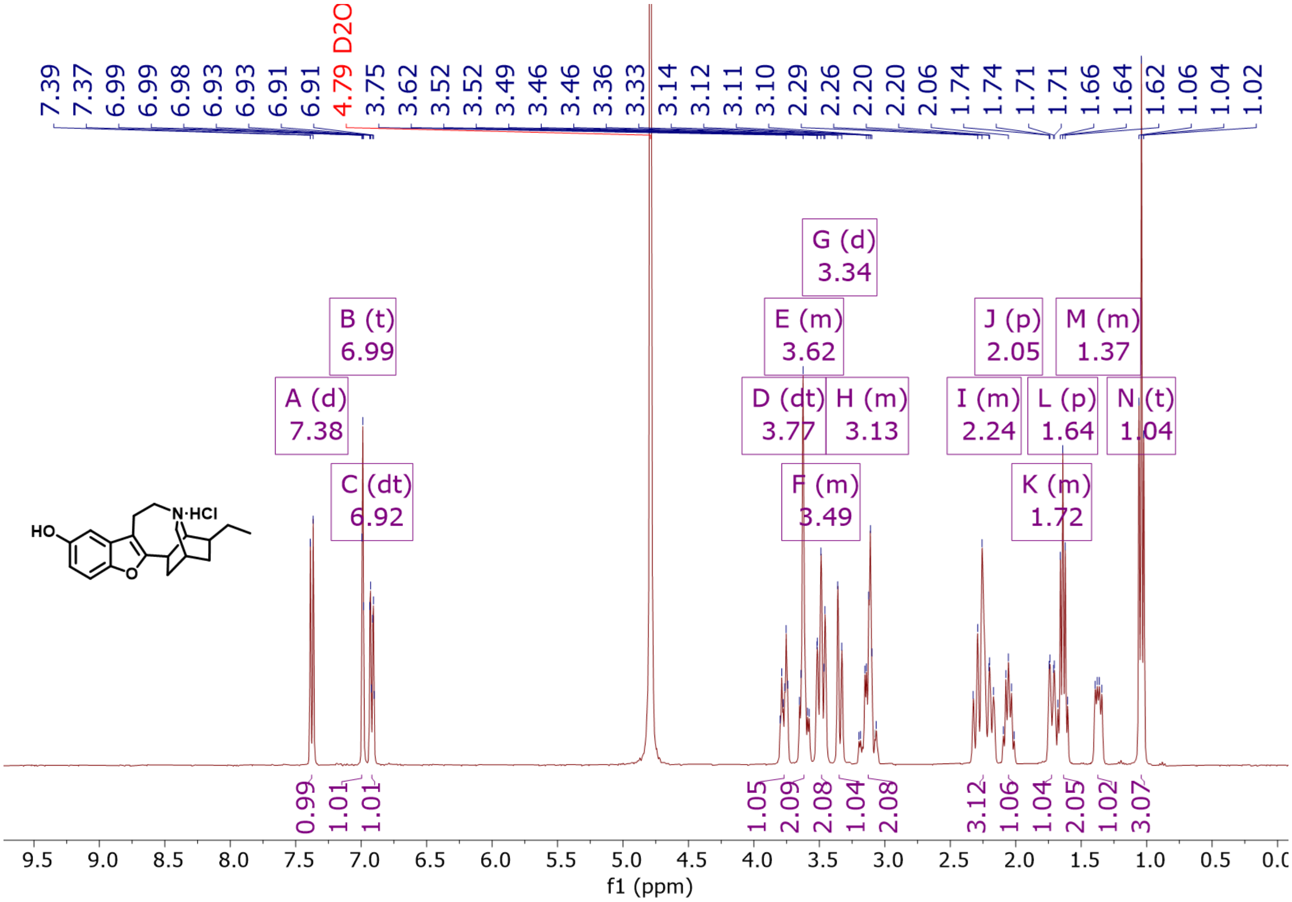


***(Rac)*-noribogaine-d_4 hydrochloride** 7-ethyl-6,6a,7,8,9,10,12,13-octahydro-5*H*-6,9-methanopyrido[1',2':1,2]azepino[4,5-*b*]indol-12,12,13,13-d_4-2-ol hydrochloride
Starting material (357 mg, 0.91 mmol) was dissolved in CH_2Cl_2 (9.1 mL, 0.1 M) and was cooled in ice/water bath. Solution of phenyltrimethylammonium tribromide (378 mg, 1.01 mmol) in CH_2Cl_2 (5.1 mL, 0.2 M) was added dropwise (over 10 min) to the cold solution. Cooling bath was removed, and mixture was further stirred at RT for 20 min. Mixture was then diluted with H_2O (18 mL) and basified with NH_4OH (1 mL). Phases were separated and CH_2Cl_2 phase was washed with H_2O (2 × 10 mL), dried over Na_2SO_4, filtered and concentrated. Residue was further dried together with freshly powdered HCOONa for 2 hours at 50°C under high vacuum. Pd catalyst $PdCl_2[P(o\text{-tolyl})_3]_2$ (36 mg, 0.046 mmol, 5 mol%) and anhydrous DMSO (3.7 mL, 0.25M) were added and mixture was heated for 1 hour at 100 °C. Only a partial conversion was observed at this time, additional Pd cat. was added in the form of $PdCl_2(PPh_3)_2$ (32 mg, 0.046 mmol) and heating continued for additional 30 min. After cooling to RT, mixture was diluted with H_2O (20 mL) and extracted with diethyl ether (4 × 10 mL), dried over Na_2SO_4, filtered and concentrated onto celite. Product was purified by column chromatography (10% AcOEt in Hexanes + 2% Et_3N), followed by preparative TLC (5% AcOEt in Hexanes + 2% Et_3N, plate was developed twice). Slightly impure material was dissolved in EtOH (3.0 mL) 10% Pd on C (18 mg) was carefully added and mixture was stirred under hydrogen atmosphere (40 PSI). Partial reaction detected after 3 hours, hydrogen pressure increased (50 PSI) and reaction continued overnight (18 hours). Mixture was filtered through celite (washed with 10% MeOH in CH_2Cl_2) and combined filtrates were concentrated and dried. Solid residue was dissolved in MeOH and treated with excess of methanolic HCl solution. Solution was concentrated and residue was suspended in MeOH and concentrated again (repeated two times) to remove excess HCl. After drying solid was suspended in MeOH (1.0 mL), sedimented by centrifugation and liquid decanted. Washing was repeated one more time using MeOH (0.5 mL) to yield the pure product as an off-white (grey/beige) solid (84 mg, 29% over 4 steps).
^{1}H NMR (500 MHz, D_2O) δ 7.29 (dd, *J* = 8.7, 0.8 Hz, 1H), 6.96 (dd, *J* = 2.4, 0.7 Hz, 1H), 6.83 – 6.77 (m, 1H), 3.49 (t, *J* = 1.7 Hz, 1H), 3.37 (dt, *J* = 12.3, 2.8 Hz, 1H), 3.31 (ddd, *J* = 12.0, 4.9, 1.9 Hz, 1H), 3.21 (dt, *J* = 12.1, 2.2 Hz, 1H), 2.30 – 2.21 (m, 1H), 2.16 – 2.05 (m, 2H), 2.03 – 1.93 (m, 1H), 1.59 – 1.49 (m, 3H), 1.32 (ddt, *J* = 13.6, 5.4, 2.6 Hz, 1H), 0.96 (t, *J* = 7.3 Hz, 3H).
^{13}C NMR (126 MHz, D_2O) δ 149.58, 141.10, 130.54, 129.02, 112.70, 111.96, 106.37, 102.84, 60.15, 55.82, 51.52, 38.84, 35.05, 31.25, 29.10, 26.58, 24.08, 17.50, 11.42.
HRMS (ESI+) calcd. for $C_{19}H_{20}D_4N_2O^+$ $[M+H]^+$ 301.2214, found 301.2210.

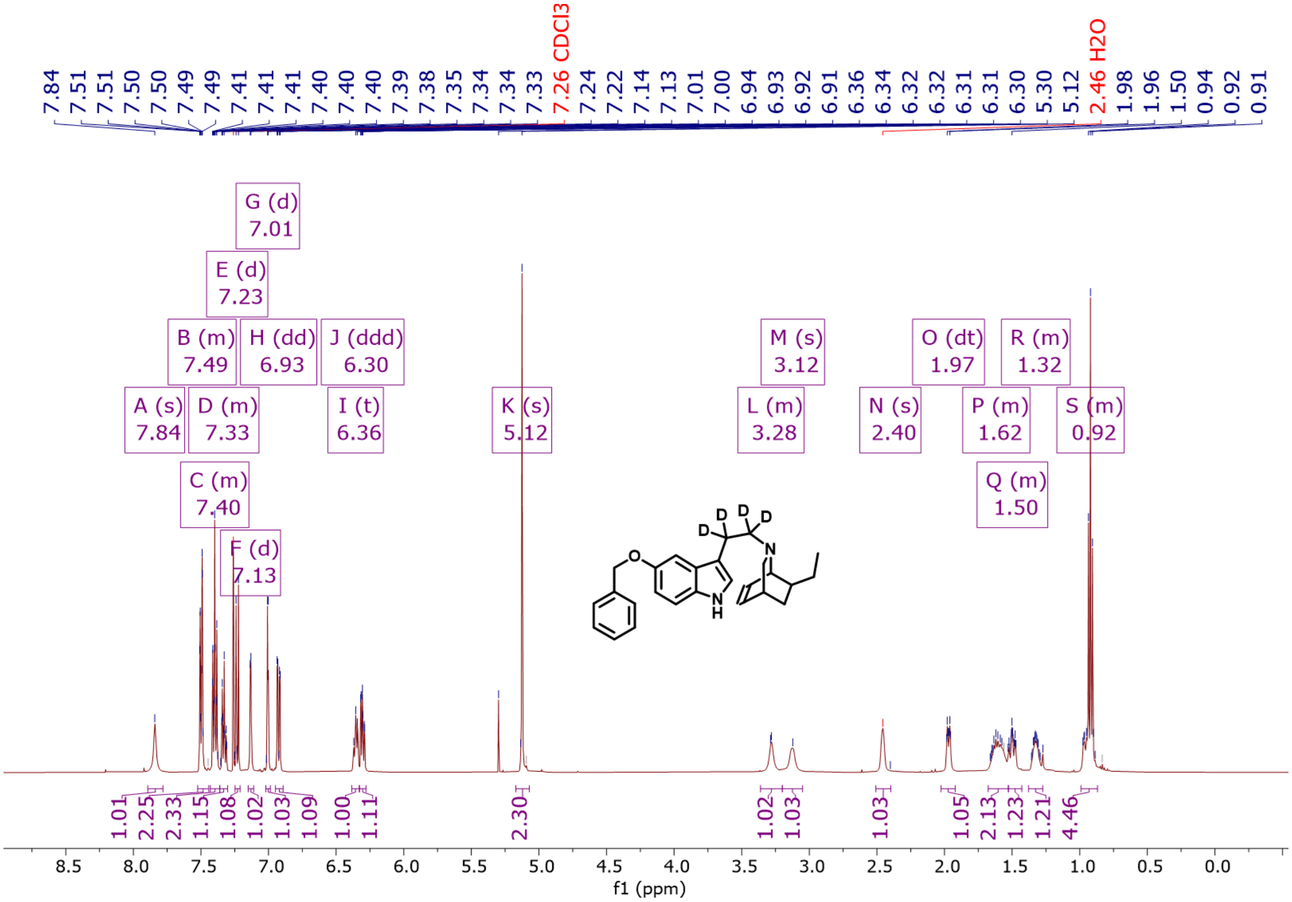
(1) Havel, V.; Kruegel, A. C.; Bechand, B.; McIntosh, S.; Stallings, L.; Hodges, A.; Wulf, M. G.; Nelson, M.; Hunkele, A.; Ansonoff, M.; Pintar, J. E.; Hwu, C.; Ople, R. S.; Abi-Gerges, N.; Zaidi, S. A.; Katritch, V.; Yang, M.; Javitch, J. A.; Majumdar, S.; Hemby, S. E.; Sames, D. Oxa-Iboga Alkaloids Lack Cardiac Risk and Disrupt Opioid Use in

Animal Models. *Nat Commun* **2024**, *15* (1), 8118. https://doi.org/10.1038/s41467-024-51856-y.
(2) Bergman, J.; Janosik, T.; Johnsson, A.-L. Synthesis of Hyrtiosin B, a Bis-Indole Alkaloid from the Okinawan Marine Sponge Hyrtios Erecta. *Synthesis* **1999**, *1999* (04), 580–582. https://doi.org/10.1055/s-1999-3439.

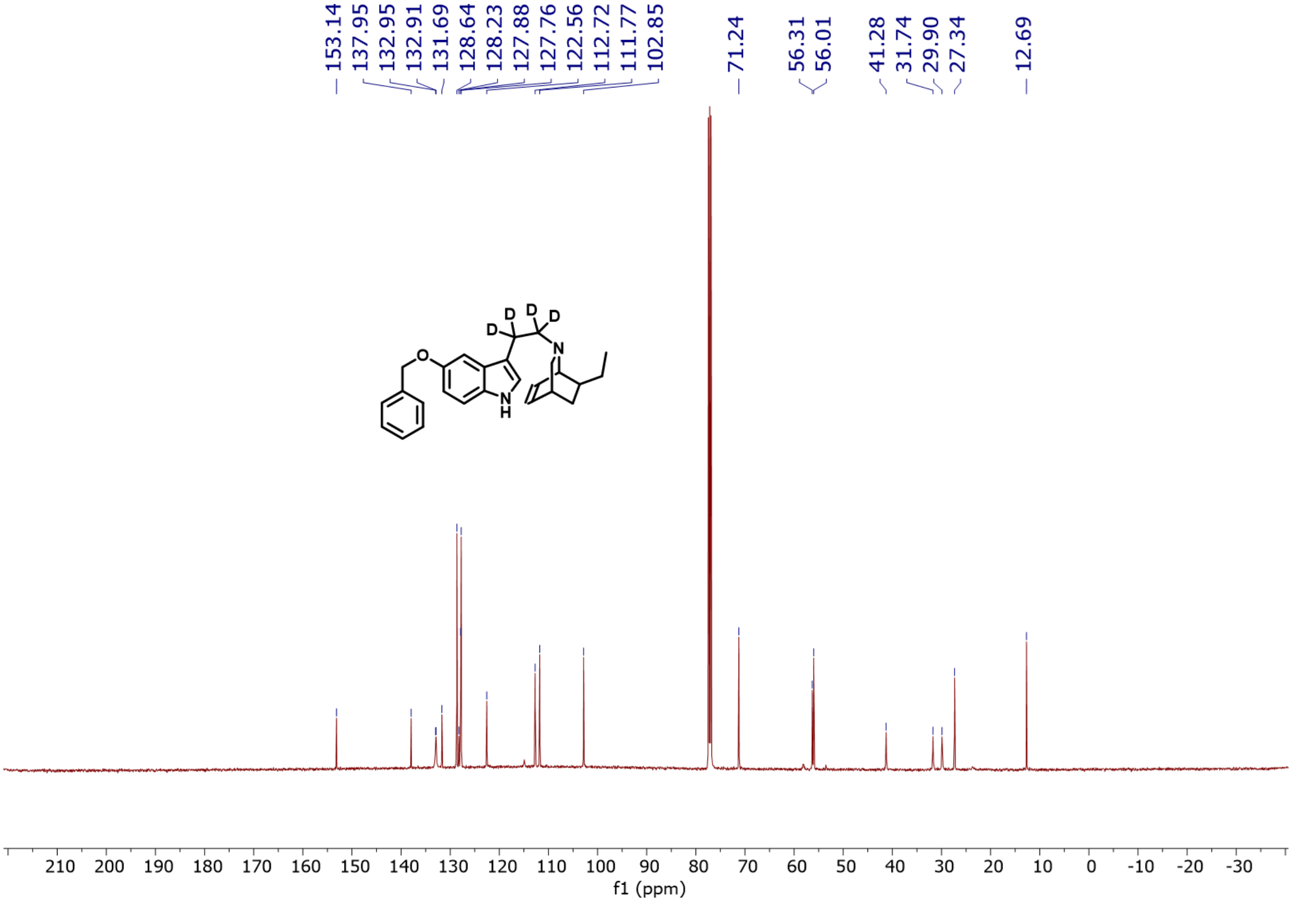
5. NMR and HRMS spectra.



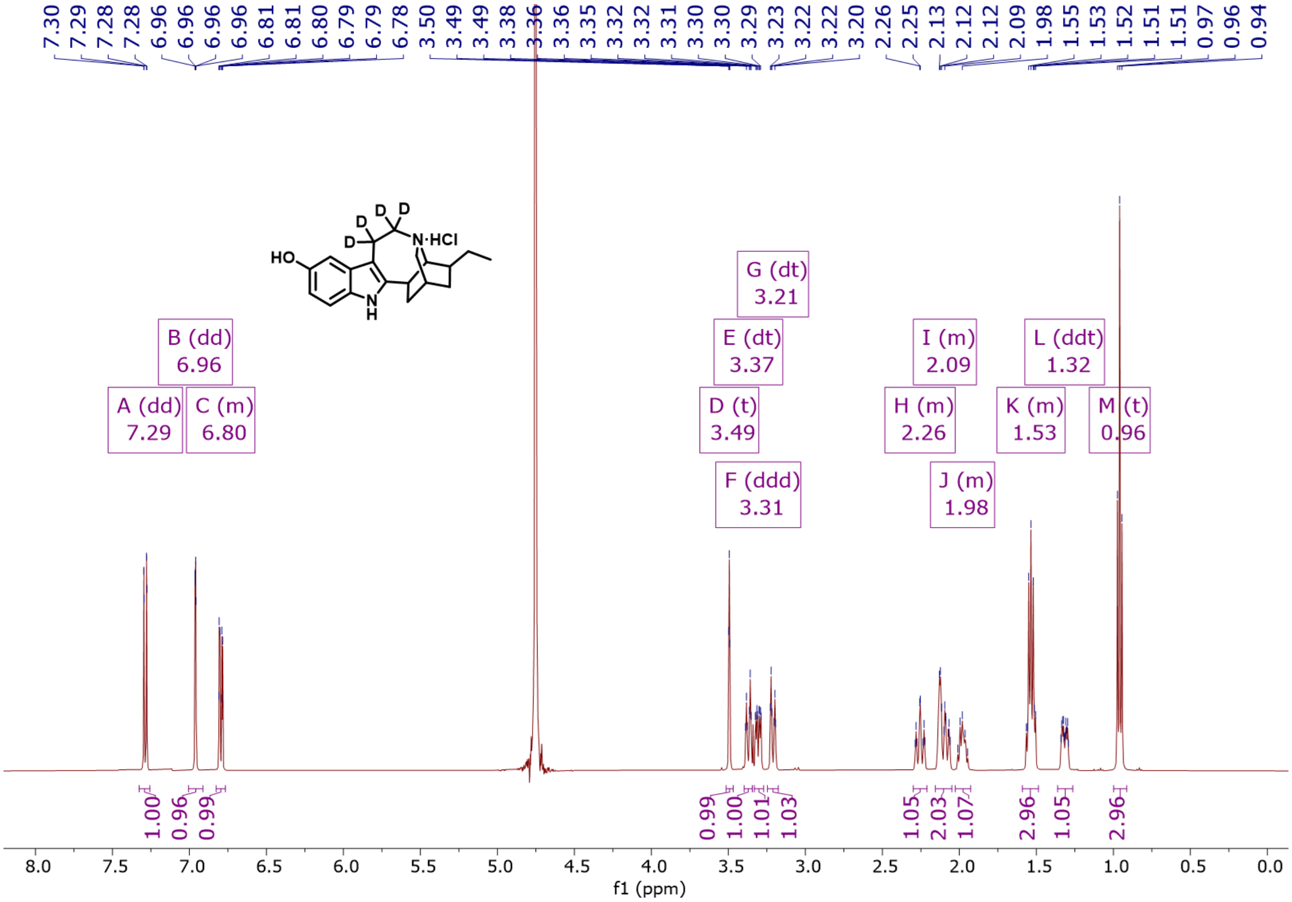
^{1}H NMR spectrum (500 MHz, D_2O) of (*rac*)-oxa-noribogaine hydrochloride (representative example of material used in the study).



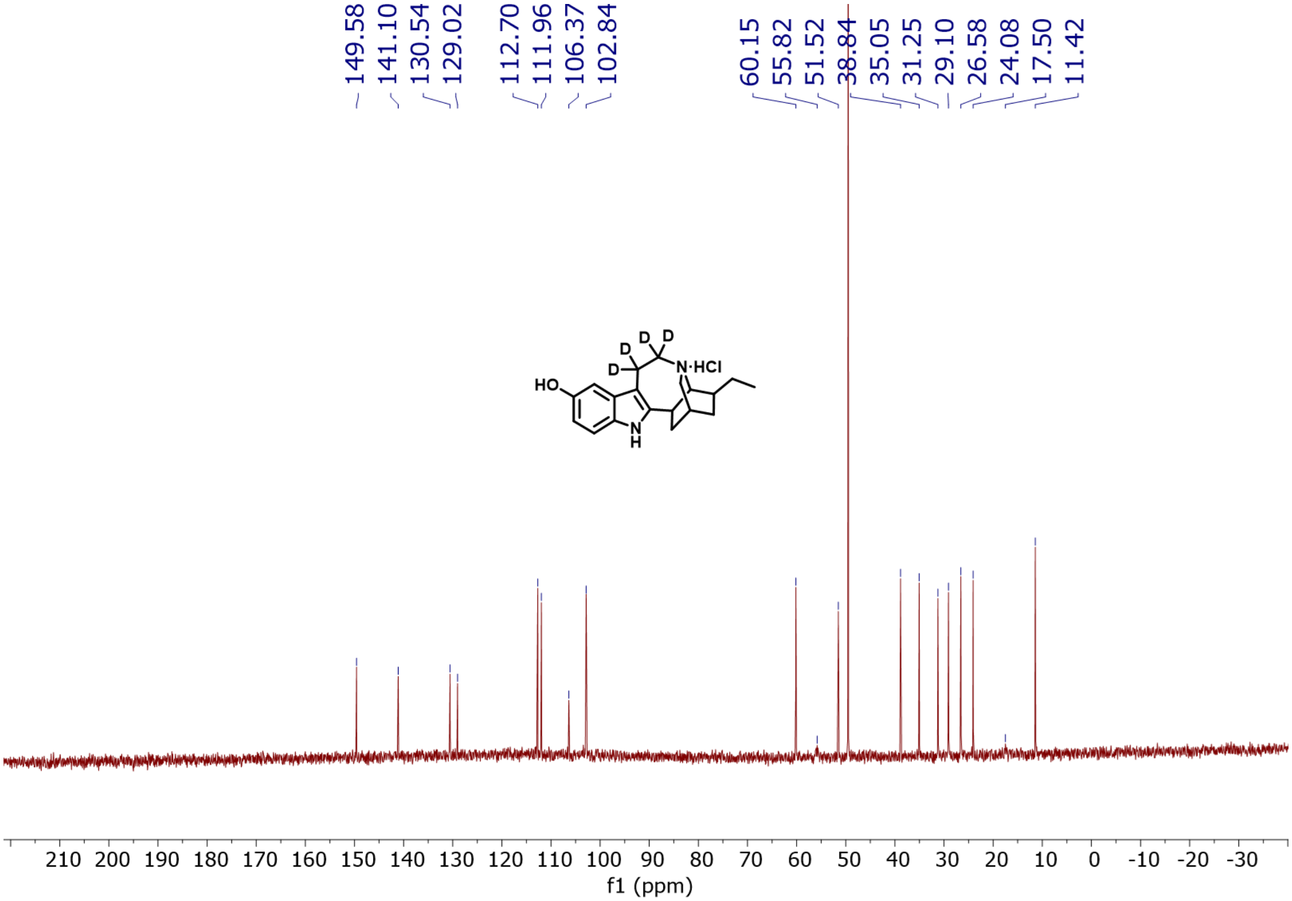
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of (*rac)*-2-(2-(5-(benzyloxy)-1*H*-indol-3-yl)ethyl-1,1,2,2-*d*$_4$)-7-ethyl-2-azabicyclo[2.2.2]oct-5-ene.



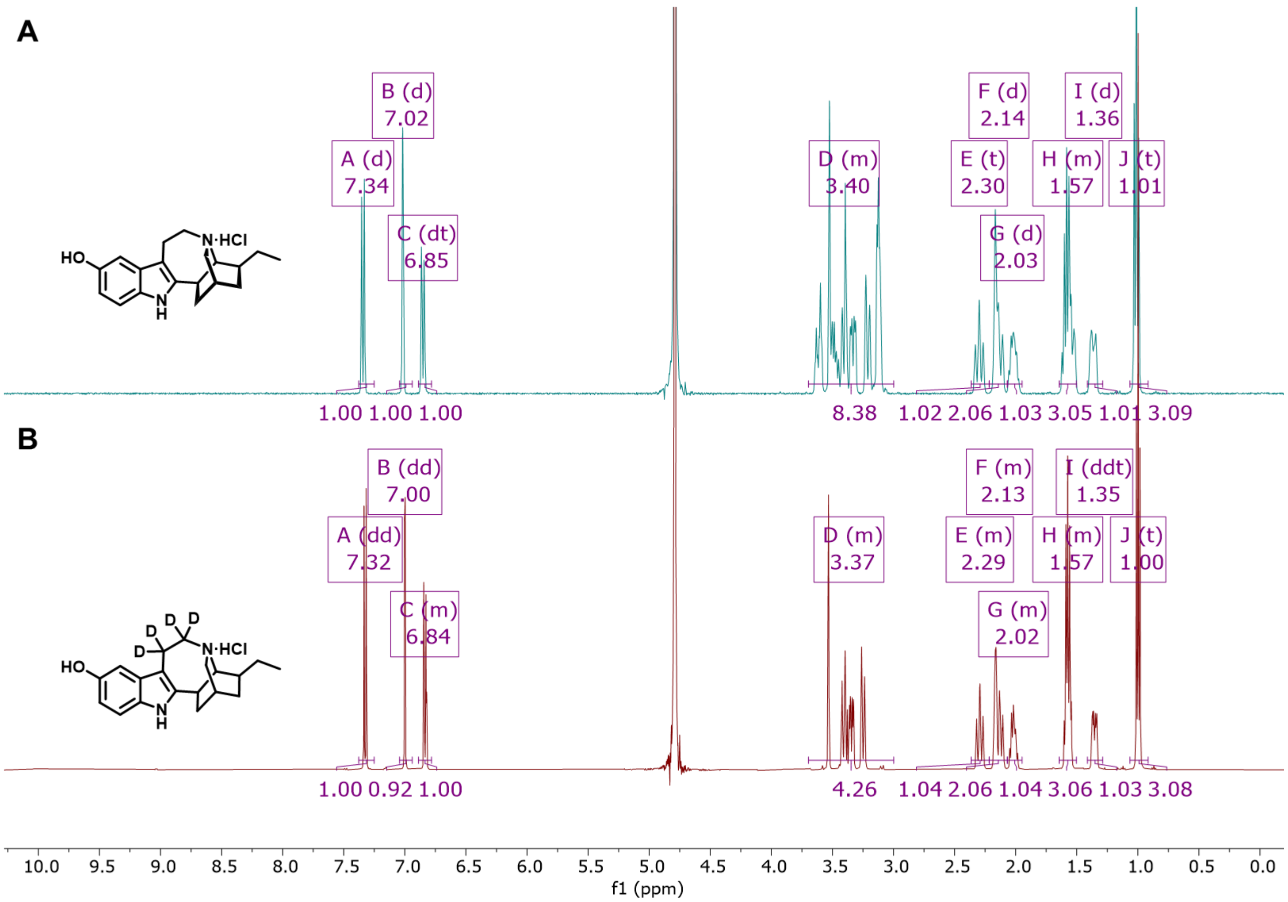
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of (*rac)*-2-(2-(5-(benzyloxy)-1*H*-indol-3-yl)ethyl-1,1,2,2-*d*$_4$)-7-ethyl-2-azabicyclo[2.2.2]oct-5-ene.



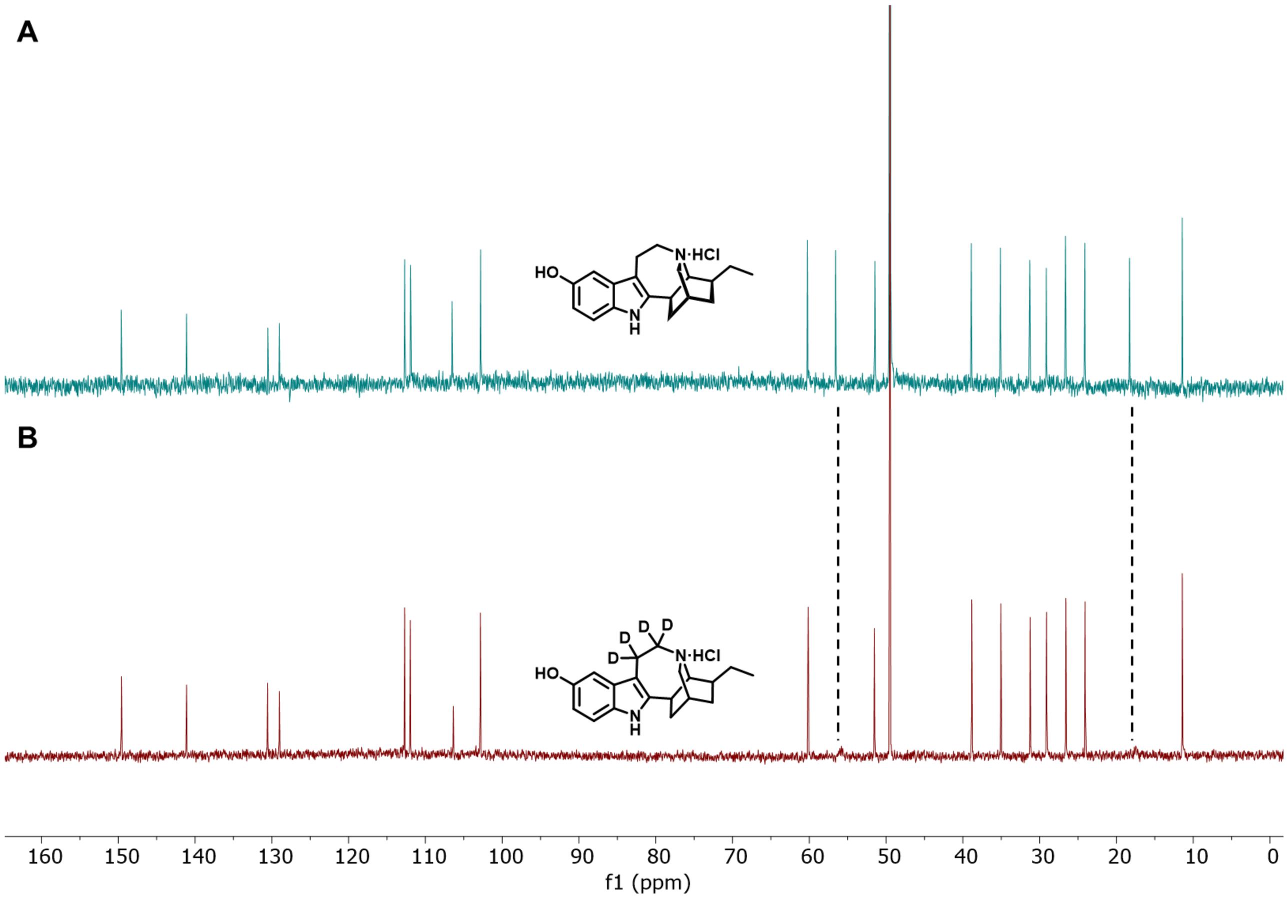
^{1}H NMR spectrum (500 MHz, D_2O) of (*rac)*-noribogaine-*d*$_4$ hydrochloride.



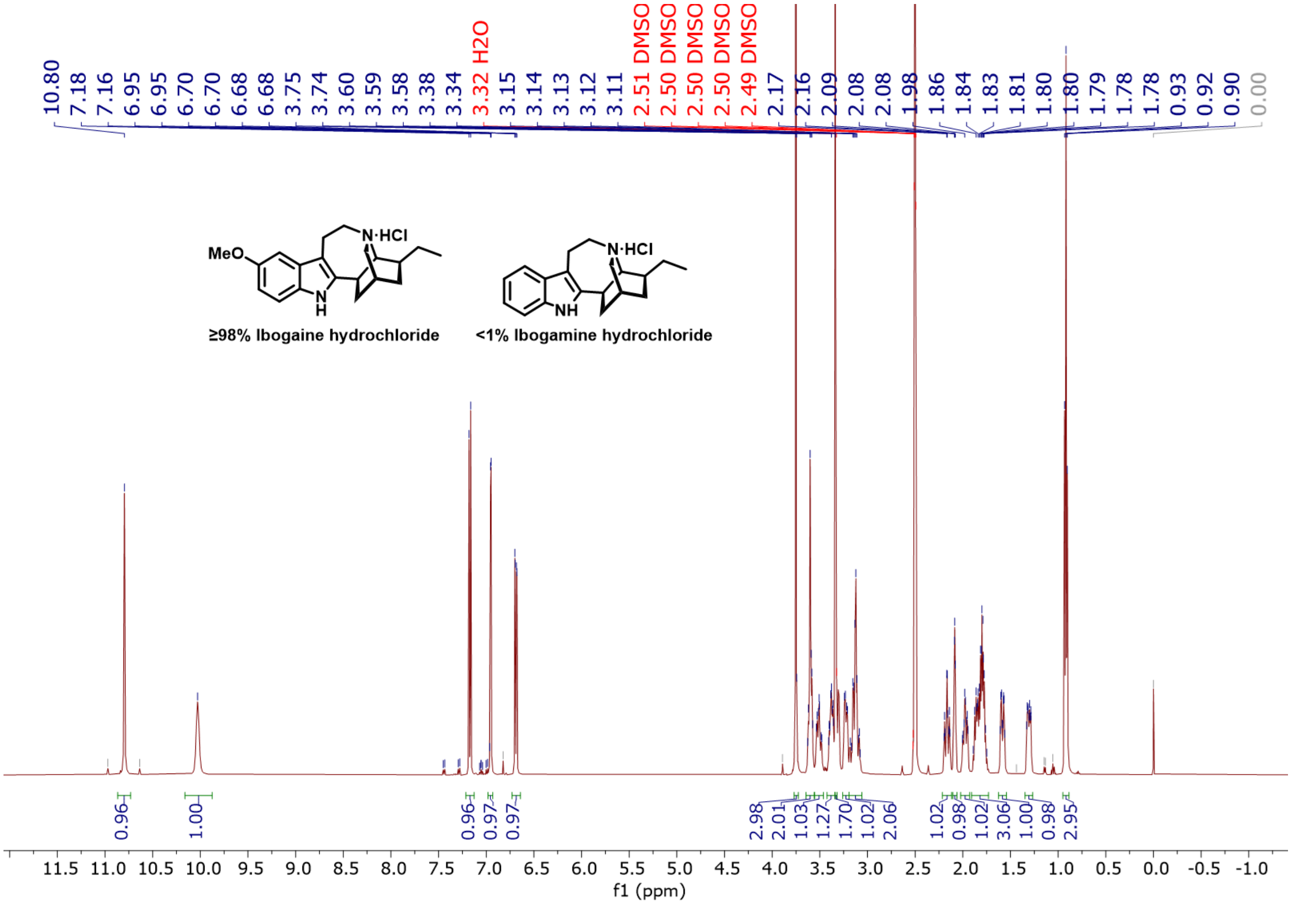
^{13}C NMR spectrum (126 MHz, D_2O) of (*rac)*-noribogaine-*d*$_4$ hydrochloride.



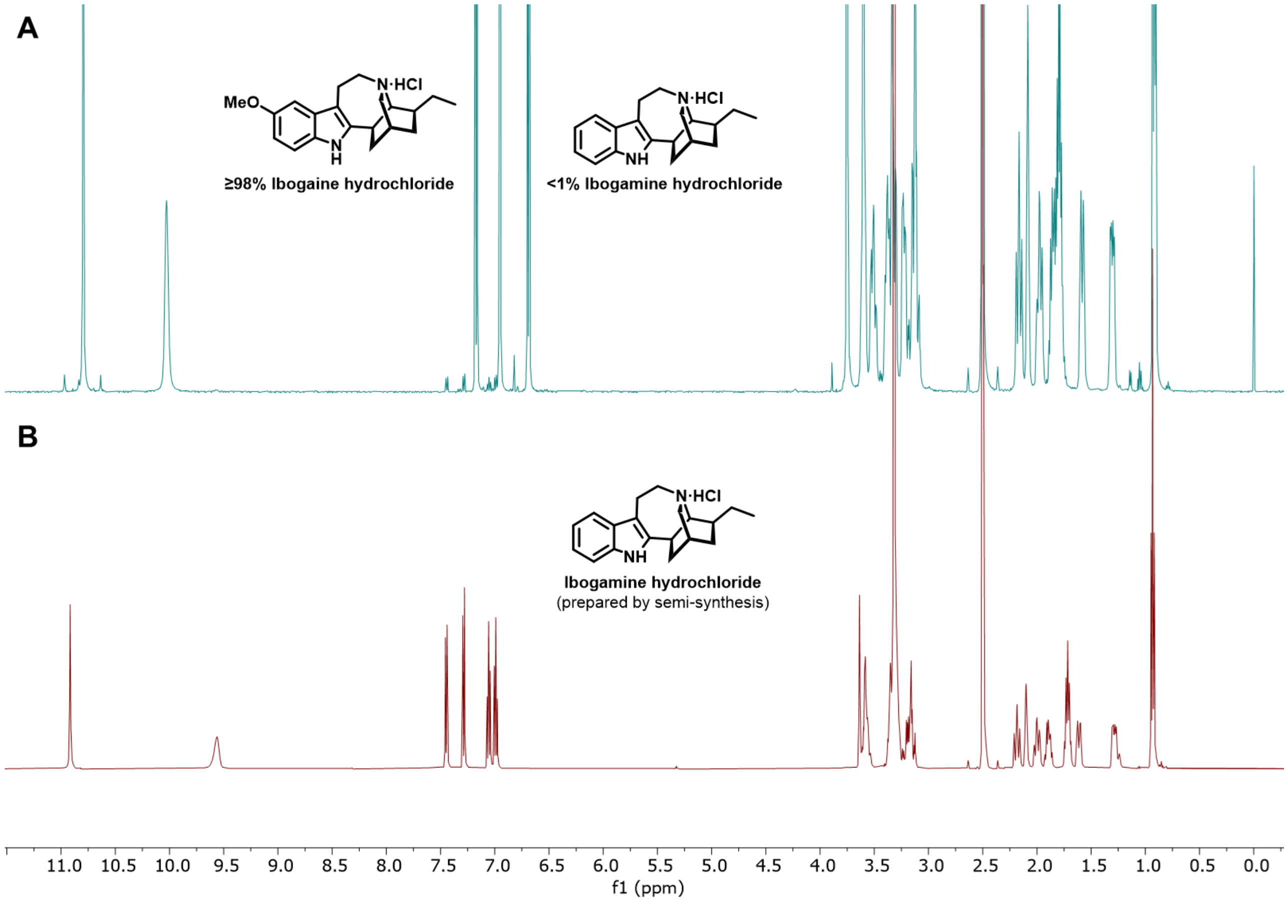
Comparison of ^{1}H NMR spectra (500 MHz, D_2O) of noribogaine hydrochloride (A) and (*rac)*-noribogaine-*d*$_4$ hydrochloride (B).



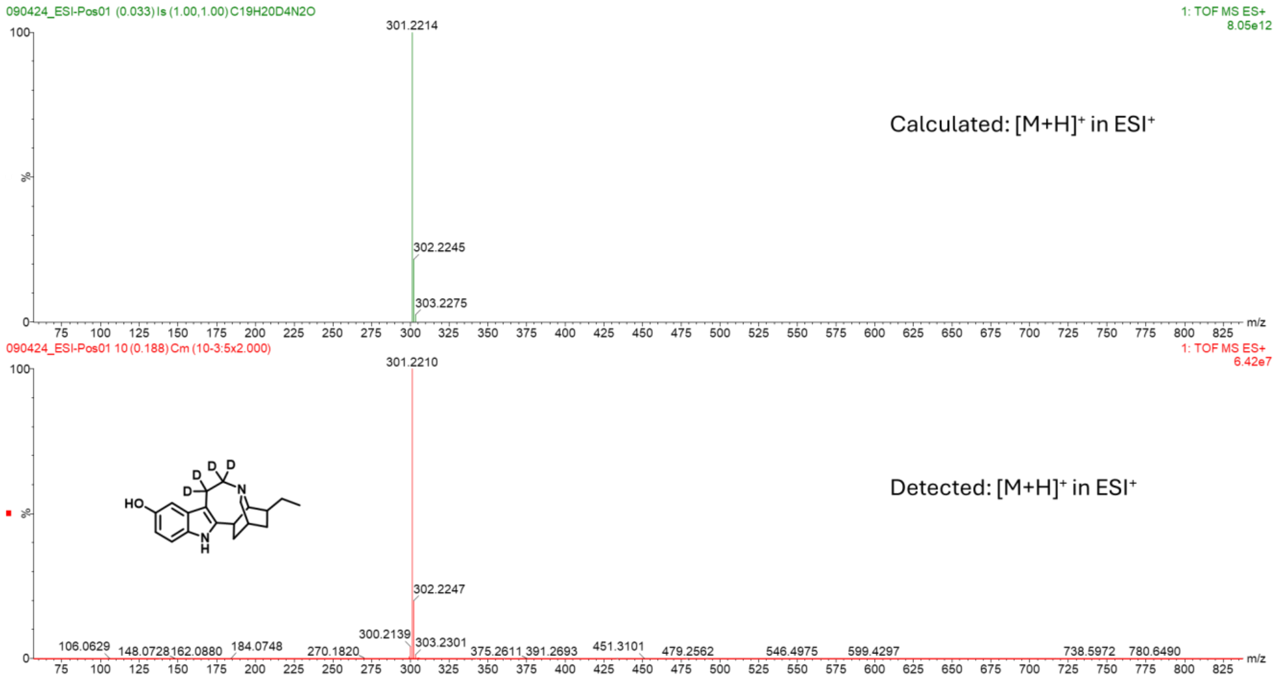
Direct comparison of ^{13}C NMR spectra (126 MHz, D_2O) of noribogaine hydrochloride (A) and (*rac)*-noribogaine-d_4 hydrochloride (B). Intensity of two CD_2 carbon signals of (*rac)*-noribogaine-d_4 hydrochloride are diminished due to the lack of ^1H NOE.



^{1}H NMR spectrum (500 MHz, DMSO) of commercially available ibogaine hydrochloride.



Purchased ibogaine hydrochloride sample (A) contained <1% of ibogamine hydrochloride, identified by comparison with a synthetic ibogamine hydrochloride sample (B), ^{1}H NMR spectra (500 MHz, DMSO).



High resolution mass spectrum of (*rac*)-noribogaine-d_4.

Annotation validation of MALDI MSI

On-tissue MS/MS acquisition. For on-tissue MS/MS validation of glutamate (*m/z* 146.05 $[M–H]^-$) and glutamine (*m/z* 145.06 $[M–H]^-$), spectra were acquired in negative-ion mode from 1,5-DAN-coated brain sections of OXA-treated animals (30 min post-injection time point) using the same timsTOF fleX and source settings as for the MSI acquisition. A precursor isolation window of 1 Da was applied. CID was performed at 15 eV for glutamate and at 15 eV for on-tissue glutamine spectra. Reference compounds L-glutamic acid (Fluka, 49450-1G-KC, Buchs, Switzerland) and L-glutamine (Fluka, 49419-1G-KC, Buchs, Switzerland), each prepared at 1 µM in acetonitrile/water (50/50, v/v) were spotted previously onto the same Slide and measured under identical conditions; reference spectra for glutamine were additionally acquired at 10 eV. Spectra were centroided and exported from DataAnalysis as .mgf files.

Spectral extraction for compound annotation. For MS1-level annotation of OXA, mean spectra were exported from SCiLS Lab software as targeted feature lists comprising the FMP-10-derivatized OXA monoisotopic ion and its isotopologs using Peak Maximum interval processing. Standard spectra were extracted from a 2 µM OXA reference standard spotted on the MALDI target plate, on-tissue spectra were extracted from OXA-treated brain sections (*n* = 3 animals, 30 min post-injection time point). All spectra were normalized to deuterated noribogaine NB-D4 (*m/z* 569.33) as internal standard prior to export and subsequently re-normalized to the OXA-FMP-10 monoisotopic peak intensity for display.

Spectral similarity scoring. To quantify spectral agreement between reference and on-tissue MS/MS spectra, a normalized cosine similarity score was calculated in R. Both spectra were binned to 0.01 *m/z* resolution and intensities scaled to 0–100%. Cosine similarity was computed as

$$\cos(0) = \frac{\sum_i R_i T_i}{\sqrt{\sum_i R_i^2}\sqrt{\sum_i T_i^2}}$$

Score close to 1 indicate highly similar fragmentation patterns.

OXA detection. Following on-tissue chemical derivatization with FMP-10, OXA-FMP-10 (*m/z* 565.28, $C_{39}H_{37}N_2O_2^+$, theoretical *m/z* 565.2844, $\Delta m/z$ < 1 ppm; Figure SI1) was detected and visualized in brain sections from OXA-treated rats by MALDI-MSI. OXA-FMP-10 signal was absent in control sections from vehicle-treated rats.

Glutamate and glutamine identification. In negative-ion mode MALDI-MSI, glutamate and glutamine were identified by on-tissue MS/MS. Fragment ions at *m/z* 85.03, 102.05 and 128.03 matched the glutamate reference standard spectrum with a cosine similarity of 0.75 (Figure SI2, panel A), and fragment ions at *m/z* 84.04, 109.04 and 127.05 matched the glutamine reference standard with a cosine similarity of 0.98 (Figure SI2, panel B).

OXA was annotated at Level 3 confidence based on sub-ppm accurate mass, close agreement between theoretical and experimental M+1/M+2 isotope abundances in both reference and on-tissue spectra, and reproducible absence of signal in vehicle controls, representing a high-confidence MS1-based assignment in the absence of robust on-tissue MS/MS data. For glutamate and glutamine, cosine similarities of 0.75 and 0.98 support MSI Level 2 identification. Given the 1 Da precursor isolation window in negative-ion mode (m/z 146.05 for glutamate and 145.06 for glutamine), minor co-isolation of isobaric or isotopic species (e.g. the M+1 isotopologue of glutamine during glutamate isolation) cannot be fully excluded; however, the close spectral match to co-measured reference standards indicates that glutamate and glutamine are the predominant contributors to the observed MS/MS spectra.

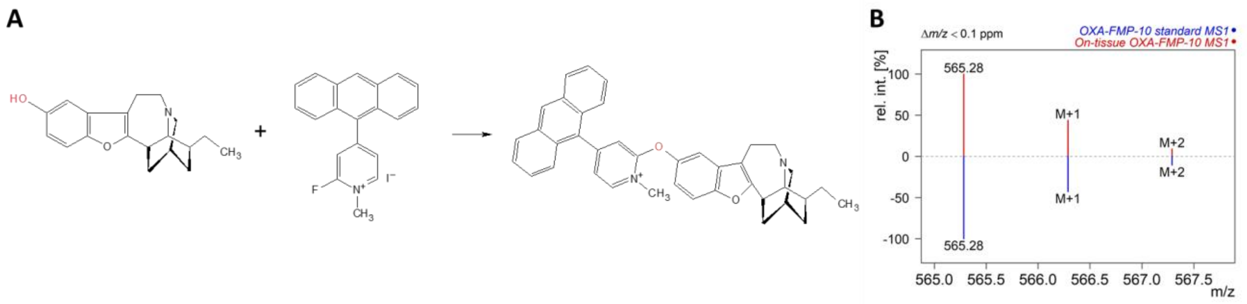


Figure SI1. On-tissue detection and MS1 validation of FMP-10-derivatized oxanoribogaine (OXA-FMP-10) by MALDI-MSI.
(A) On-tissue chemical derivatization (OTCD) of the phenolic hydroxyl group in oxanoribogaine (OXA) with 4-formyl-1-methylpyridinium (FMP-10) enables detection of OXA-FMP-10 by MALDI-MSI in positive-ion mode.
(B) On-tissue MS1 validation of OXA identification. Mirror plot comparing the MS1 spectrum of an OXA-FMP-10 reference standard (blue) with the corresponding on-tissue MS1 spectrum from OXA-treated rat brain (red). The monoisotopic, M+1 and M+2 peaks are consistent in both spectra. Mass accuracy: Δ*m/z* <1 ppm relative to the theoretical monoisotopic mass (*m/z* 565.2844, $C_{39}H_{37}N_2O_2^+$). Spectra were normalized to the OXA-FMP-10 monoisotopic peak intensity. OXA is classified at identification confidence Level 3 (putatively annotated compound) based on sub-ppm accurate mass, matching M+1/M+2 isotopic pattern and specific on-tissue reactivity with the FMP-10 derivatization reagent, confirmed using a co-measured reference standard (Sumner et al., 2007).

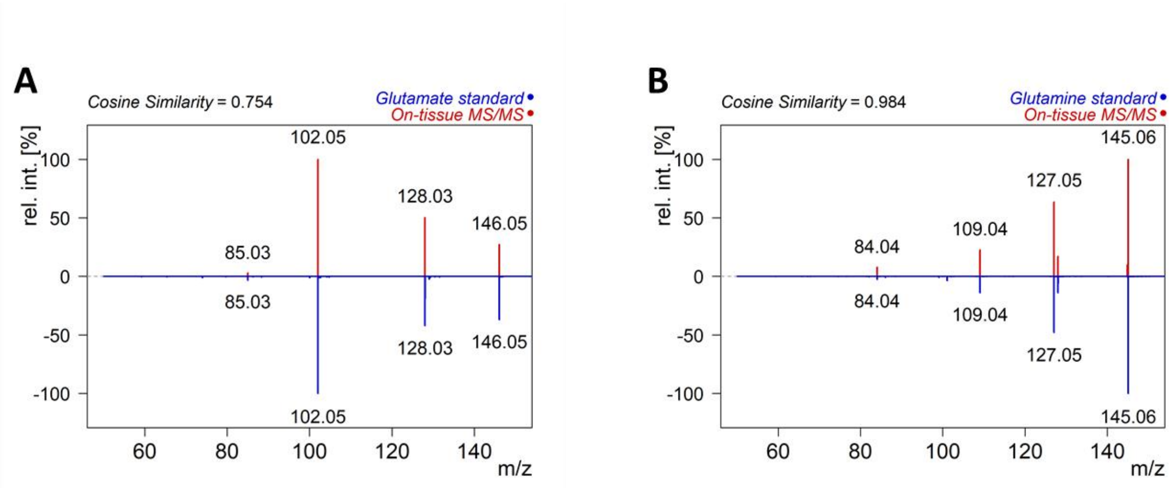


Figure SI2. On-tissue MS/MS validation of glutamate and glutamine identification by MALDI-MSI.
(A) On-tissue MS/MS validation of glutamate identification. Mirror plot comparing the

negative-ion MS/MS spectrum of a glutamate reference standard (blue, 15 eV CID) with the corresponding on-tissue MS/MS spectrum (red, 15 eV CID), acquired using [matrix name] as MALDI matrix. Fragment ions at *m/z* 85.03, 102.05 and 128.03, together with the precursor ion at *m/z* 146.05, show highly similar relative intensities in both spectra. Glutamate is classified at identification confidence Level 2 (putatively annotated compound) based on on-tissue MS/MS matching to an authentic reference standard (cosine similarity = 0.75) (Sumner et al., 2007).

(B) On-tissue MS/MS validation of glutamine identification. Mirror plot comparing the negative-ion MS/MS spectrum of a glutamine reference standard (blue, 10 eV CID) with the corresponding on-tissue MS/MS spectrum (red, 15 eV CID). Fragment ions at *m/z* 84.04, 109.04 and 127.05, together with the precursor ion at *m/z* 145.06, show near-identical relative intensities in both spectra. Glutamine is classified at identification confidence Level 2 based on on-tissue MS/MS with a cosine similarity of 0.98 to the co-measured reference standard (Sumner et al., 2007).