SUPPORTING INFORMATION

New synthesis and tritium labeling of a selective ligand for studying high-affinity γ-hydroxybutyrate (GHB) binding sites.

Stine B. Vogensen,[§‡*] Aleš Marek,[†‡] Tina Bay,[§] Petrine Wellendorph,[§] Jan Kehler,[#] Christoffer Bundgaard,[#] Bente Frølund,[§] Martin H.F. Pedersen[†*] and Rasmus P. Clausen[§*]

[§]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark.

[†]The Hevesy Laboratory, DTU Nutech, Technical University of Denmark, 4000 Roskilde, Denmark.

[‡]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 16610 Prague 6, Czech Republic.

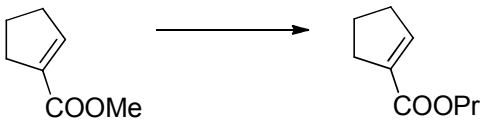
[#]DiscoveryChemistry and DMPK, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark

Chemistry

General experimental section

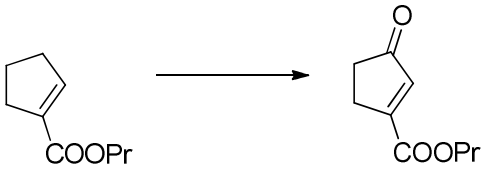
All starting materials were obtained from commercial sources and used without further purification if not stated otherwise. Thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F254 aluminium sheets. The compounds were detected using UV light (254 nm) and anisaldehyde spraying reagent. Flash column chromatography (CC) was performed using Merck Geduran ® Si 60 (0.040–0.063 mm). ^{1}H-NMR and ^{13}C NMR spectra were recorded on a 300 MHz Varian Mercury 300BB or a 400MHz Bruker instrument at room temperature. ^{3}H-NMR spectra were recorded at 320MHz using a Bruker Avance II 300 instrument set at 25 °C. Chemical shifts (δ) are quoted in parts per million (ppm) relative to residual solvent peak or TMS. Coupling constants (*J*) are given in Hertz (Hz). Tritiation reaction was performed on a custom-designed tritium manifold system manufactured by RC Tritec AG, Switzerland. Tritium gas stored as uranium tritide on a uranium bed and was released by heating to approximately 500 °C. All deuterium experiments were carried out on a deuterium manifold system (RC Tritec AG) equipped with pressure bottle contains gaseous deuterium (purity ≥99.9%, enrichment ≥99.8%; Linde AG). HPLC analysis and separation of deuterium samples were performed using a Knauer smartline instrument, pump 1000, UV detector 2500. HPLC analysis and separation of tritium samples were performed using a Waters 1524, binary pump; Waters 2487, dual absorbance detector, equipped with Bioscan Hidex liquid scintillation counter. HPLC grade water obtained by purification using an Aurium 611 UV system from Sartorius, Germany was used. Safety handling with tritium gas was monitored by Canberra T100DSi Tritium air monitor. Liquid scintillation counting was performed by RackBeta 1214 or Hidex 300 SL scintillation counters. Mass spectra of labeled compounds was obtained by a Bruker Daltonics esquire 4000 electrospray ion-trap system by either direct injection or by a Merck Hitachi HPLC for LC-MS analysis. The HR-MS spectra were obtained in the ESI mode either on a Q-Tof micro from Waters or on an LTQ Orbitrap XL from Thermo Fisher Scientific. LC-MS (System B) was performed using a Agilent 1100 HPLC systems with a XBridge 3.5 µm C-18 column (100 x 4.60 mm) using gradient elution from buffer A (H_2O:CH_3CN:HCOOH, 95:5:0.1) to buffer B (H_2O:CH_3CN: HCOOH, 5:95:0.05) over 8 min flow rate: 0.5 mL/min, coupled to an Hewlett Packard 1100 series mass spectrometer with an electrospray ionization source. Melting point was determined on a Stanford Research Systems OptiMelt instrument in an open capillary and is uncorrected. Elemental analysis was performed at the Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria and are within ± 0.4% of the theoretical values.

Propyl cyclopent-1-enecarboxylate (3c)



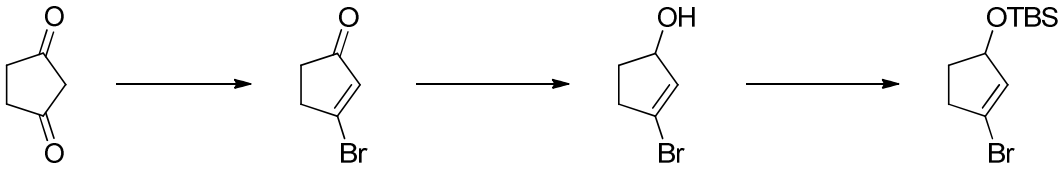
Methyl-1-cyclopentene-1-carboxylate (**3b**) (10.0 g, 79.3 mmol) was dissolved in 1-propanol (50 mL) and conc. H_2SO_4 (0.5 mL) was added. The solution was refluxed for 1h. The solvent was removed in vacuo. Ethyl acetate (200 mL) was added and the organic phase was washed with water (3 x 10 mL) and brine (20 mL). The solvent was removed in vacuo to give the product as yellow oil (8.59 g, 70%). ^{1}H NMR ($CDCl_3$): δ 0.96 (t, *J* = 7.3 Hz, 3H), 1.69 (sextet, *J* = 7.3 Hz, 2H), 1.92–2.00 (pentet, *J* = 7.3 Hz, 2H), 2.46–2.60 (m, 4H), 4.10 (t, J = 7.3 Hz, 2H), 6.75–6.79 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 10.66, 22.25, 23.29, 31.51, 33.49, 65.77, 136.71, 143.45, 165.45.

Propyl 3-oxocyclopent-1-enecarboxylate (4c)



To a stirred ice-cooled solution of Ac_2O (18 mL) and AcOH (36 mL) was added CrO_3 (15.31 g, 153.2 mmol). When all of the CrO_3 was dissolved, the solution was added drop wise to a solution of propyl cyclopent-1-enecarboxylate (**3c**) (8.59 g, 55.7 mmol) in DCM (100 mL). The solution was stirred at rt for 1h. The solution was cooled on ice and aq. KOH (12.5M, 90 mL) was added to neutral pH. Water (200 mL) was added and extracted with diethyl ether (3 x 400 mL). The combined organic phase was washed with sat. $NaHCO_3$ (2 x 50 mL) and brine (2 x 50 mL) and dried ($MgSO_4$) and the solvent was removed in vacuo. CC (4:1 heptane/ethyl acetate) gave the product (4.03 g, 43%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.01 (t, *J* = 6.8 Hz, 3H), 1.77 (sextet, *J* = 6.8 Hz, 2H), 2.55–2.57 (m, 2H), 2.87–2.90 (m, 2H), 4.25 (t, *J* = 6.8 Hz, 2H) 6.78 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 10.20, 21.70, 27.30, 35.33, 66.81, 137.50, 163.87, 163.98, 208.55.

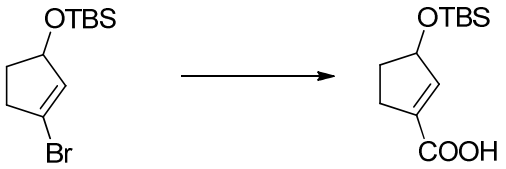
((3-bromocyclopent-2-en-1-yl)oxy)(*tert*-butyl)dimethylsilane (6)



Dibromo-triphenylphosphine (18.93 g, 44.85 mmol) was suspended in dry benzene (65 mL) and cyclopentane-1,3-dione (4.0g, 40.77 mmol) and triethylamine (6.25 mL, 44.85 mmol, freshly distilled from $LiAlH_4$) were added. The suspension was stirred at rt for 18h. The solvent was removed in vacuo (temp. < 30°C). CC (diethyl ether) gave 3-bromocyclopent-2-enone as colorless oil. ^{1}H ($CDCl_3$): δ 2.51–2.56 (m, 2H), 2.95–3.00 (m, 2H), 6.40–6.43 (m, 1H). ^{13}C ($CDCl_3$): δ 36.98, 37.19, 135.59, 161.16, 204.67. According to ^{1}H NMR 0.5 equiv of

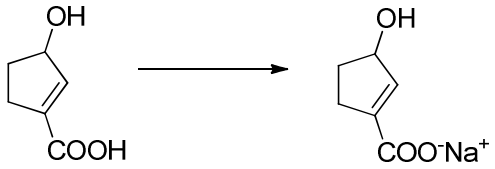
benzene and 0.1 eq of diethyl ether were present. The oil was dissolved in MeOH (175 mL) and $CeCl_3 \cdot 7H_2O$ was added (1.34 g, 3.60 mmol). The colorless solution was cooled to 0 °C and $NaBH_4$ (1.05 g, 27.8 mmol) was added in portions. After 1h the ice bath was removed and the reaction was stirred at room temperature for 1.5h. The solvent was removed in vacuo (temp. < 30°C) and water (60 mL) was added. pH was adjusted to 3 with aq. HCl (1M) and the aqueous phase was extracted with diethyl ether (4 x 100 mL). The combined organic phase was washed with brine (2 x 20 mL), dried ($MgSO_4$) and the solvent was removed in vacuo (temp. < 30°C) to give colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.67 (bs, 1H), 1.75 (dddd, J = 13.6, 10.3, 4.5 and 3.5 Hz, 1H), 2.34 (dddd, J = 16.6, 9.0, 7.5 and 4.8 Hz, 1H), 2.44–2.53 (m, 2H), 2.68–2.77 (m, 1H), 4.70–4.74 (m, 1H), 5.91 (q, J = 2.3 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 34.21, 38.37, 76.56, 127.58, 133.60. According to ^{1}H NMR 0.1 equiv of diethyl ether was present. The oil was dissolved in dry DCM (235 mL) and imidazole (6.14 g, 90.2 mmol), 4-(dimethylamino)pyridine (1.32 g, 10.82 mmol) and *tert*-butyl(chloro)dimethylsilane (6.53 g, 43.3 mmol) was added. The solution turned milky right away. The reaction was stirred for 16h at room temperature. Water was added and the aqueous phase was extracted with DCM (3 x 100 mL). The combined organic phase was washed with brine (2 x 50 mL), dried ($MgSO_4$) and the solvent removed in vacuo. CC (P-ether 40–70 °C) gave the product as colorless oil (9.7 g, 34.9 mmol, 86% over three steps). ^{1}H NMR ($CDCl_3$): δ 0.82 (s, 6H), 1.47 (s, 9H), 1.73 (dddd, *J* = 15.1, 9.5, 5.8 and 4.2 Hz, 1H), 2.25 (dddd, *J* = 16.6, 9.0, 7.5 and 4.0, 1H), 2.39–2.47 (m, 1H), 2.63–2.72 (m, 1H), 4.72–4.77 (m, 1H), 5.78 (q, J = 2.0, 1H). ^{13}C NMR ($CDCl_3$): δ –4.65, –4.62, 18.22, 25.89, 34.53, 38.38, 76.89, 125.82, 134.11. The intermediates are volatile and the yield is obtained if the intermediates are used right away.

3-((*tert*-butyldimethylsilyl)oxy)cyclopent-1-enecarboxylic acid (7)



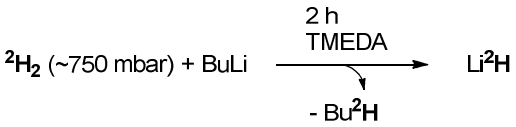
((3-bromocyclopent-2-en-1-yl)oxy)(*tert*-butyl)dimethylsilane (**6**) (4.202 g, 15.15 mmol) was dissolved in dry THF (150 mL) and the solution was cooled to –78 °C. t-BuLi (18.7 mL, 1.7M in pentane, 31.8 mmol) was added drop wise resulting in a persisting yellow solution. The solution was warmed to –50 °C over 2h. The solution was cooled to –78 °C and CO_2 (g) was bobbled through the solution for 10 min. The now colorless solution was stirred for another 15 min and sat. NH_4Cl (80 mL) and diethyl ether 100 (mL) was slowly added and the reaction was warmed to room temperature. The aqueous phase was extracted with diethyl ether (3 x 100 mL) and the combined diethyl ether phase was dried ($MgSO_4$) and the solvent removed in vacuo. CC (6:1 heptane/ethyl acetate + 2% acetic acid) gave the product (3.21 g, 87%) as white crystals. Mp: 79–82°C. ^{1}H NMR ($CDCl_3$): δ 0.00 (s, 6H), 0.80 (s, 9H), 1.67–1.75 (m, 1H), 2.20–2.39 (m, 2H), 2.58–2.66 (m, 1H), 4.88–4.93 (m, 1H), 6.65 (q, *J* = 1.8 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ –4.70, –4.68, 18.21, 25.86, 29.50, 34.08, 77.58, 136.75, 146.74, 170.24.

Sodium 3-hydroxycyclopent-1-enecarboxylate (1 as sodium salt)



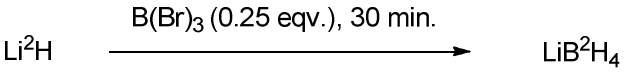
3-hydroxycyclopent-1-enecarboxylic acid (**1**) (1.234 g, 9.63 mmol) was dissolved in ethanol (10 mL) and NaOH (aq) (19.26 mL, 9.63 mmol, 0.5M Tritisol) was added. The solvent was removed in vacuo to give the product (1.428 g, 99%) as white solid. Mp. > 300 °C. ^{1}H NMR (D_2O): δ 1.69–1.77 (m, 1H), 2.29–2.45 (m, 2H), 2.57–2.66 (m, 1H), 4.88–4.94 (m, 1H), 6.29 (q, *J* = 2.0 Hz, 1H). ^{13}C NMR (D_2O): δ 30.65, 32.44, 76.67, 136.86, 145.31, 174.55. Calculated for $C_6H_7O_3Na \cdot 0.1\ H_2O$: C, 47.44; H, 4.78. Found: C, 47.34; H, 4.59. LC-MS (system B): $(M-H_2O)H^+$: 111.1.

Lithium deuteride[1]



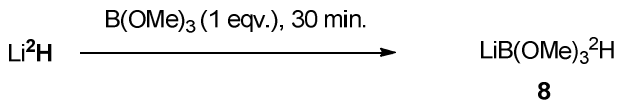
A 2-necked round-bottom flask (1 mL) equipped with a magnetic stir bar was mounted onto deuterium manifold system and flame-dried and applying vacuum-argon sequence and terminally filled with D_2 (800 mbar). N,N,N‘,N‘-Tetramethylethylenediamine (TMEDA) (50 µL, 330 µmol) and a solution of *n*-BuLi (100 µL, 160 µmol, 1.6 M in heptane) was drop wise added. The reaction mixture was stirred for 2 h at room temperature forming a white solid of Li^2H precipitate. Generated Li^2H was used directly in synthesis of borodeuterides without further isolation. One attempt of synthesis of [^{2}H]-**1** using Li^2H was performed in the screening of reaction conditions (table S1 entry 10).

Lithium borodeuteride



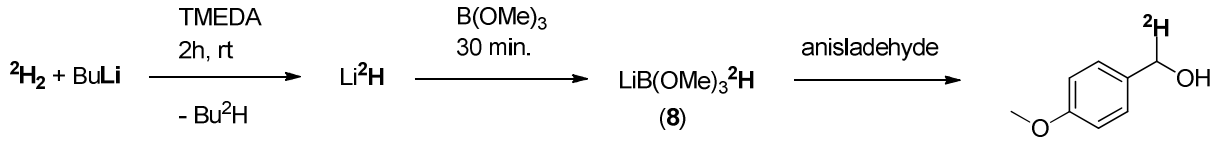
To freshly prepared lithium deuteride (theoretical 160 µmol based on the amount of *n*-BuLi) was drop wise added boron tribromide (40 µL, 1M in hexane) and the reaction was stirred for 30 min at room temperature. Precipitated lithium borodeuteride was used directly in the optimization reactions (table S1 entry 11) without further isolation.

Lithium trimethoxyborodeuteride (8)



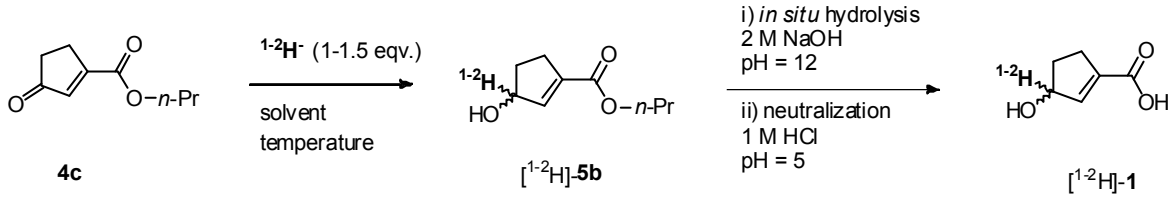
To freshly prepared lithium deuteride (theoretical 160 µmol based on the amount of *n*-BuLi) was drop wise added trimethyl borate (18 µL, 160 µmol) and the reaction was stirred for 30 min at room temperature. Lithium trimethoxyborodeuteride (**8**) slowly precipitated as a white solid and such prepared suspension of **8** was used directly without further isolation in the optimization reactions (table S1 entry 2-9). The yield of in situ generated **8** was indirectly determined in the range 40-47% (see the experiment below).

Determination of the yield of lithium trimethoxyborodeuteride (8)



In a series of up scaled reactions the yield of **8** was determined. A freshly prepared solution of LiB(OMe)$_3$^{2}H (theoretical 160 µmol based on the amount of *n*-BuLi) was added a solution of 4-methoxybenzaldehyde (65 µL, 0.53 mmol; 129 µL, 1.06 mmol; and 258 µL, 2.12 mmol respectively). The reaction was stirred for 2h, then quenched with H_2O (500 µL), extracted into $CHCl_3$ (3 × 1 mL), dried ($MgSO_4$) and the combined organic layer was evaporated. ^{1}H NMR and HPLC established an overall yield of 40–47%. Yield of generated borodeuteride was determined by the assumption of quantitative reduction of *p*-anisaldehyde. HPLC analysis was performed with a Princerton Spher C30 column (250 × 10 mm, 5 µm) with a flow rate of 3 mL/min and UV detection at 230 nm. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–2 min, 0% B; 2–20 min, 0–100% B; 20–21 min, 100% B; 21-25 min, 0-100% A. The retention time of 1-(*p*-methoxyphenyl)-1-methanol was 15.2 min and 18.2 min for *p*-anisaldehyde.

Screening of reaction conditions for synthesis of [^{2}H]-1:



A freshly prepared solution of appropriate deuteride (theoretical 160 µmol based on the amount of *n*-BuLi) was cooled using an ice bath (entry 5), dry ice (entry 6) or left at room temperature and a solution of **4c** (14 mg, 83 µmol or 21 mg, 125 µmol) in dry solvent (0.5 mL) (Table S1) was drop wise added. To follow Luche reaction conditions a solution of $CeCl_3$ (20.5 mg, 83 µmol) in MeOH (100 µL) was added (entry 1-3 and 7). The reaction mixture was stirred for 2h, and all volatile components were lyophilized. Solid residue was quenched with water (1 mL) and $NaOH_{(aq)}$ (2M, 100 µL) was added and the reaction was stirred for 1h at room temperature.

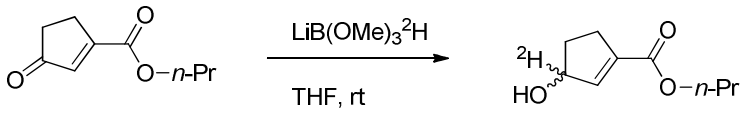
The reaction mixture was neutralized to pH 5 by addition of 1M HCl. The yield of [$^{1\text{-}2}$H]-**1**[a] was determined by HPLC analysis using a Princerton Spher C30 column (250 × 10 mm, 5 µm) with a flow rate of 3 mL/min and UV detection at 230 nm. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–5 min, 0% B; 5–20 min, 0–100% B; 25–30 min, 100% B; 30-33 min, 0–100% A. The reaction conditions shown in entry 9 were used in the subsequent radiolabeling reaction .

Table S1. Screening of reaction conditions for reduction of **4c** to give [$^{1\text{-}2}$H]-**1**[a]

Entry	Hydride	Eq. of hydride to substrate	Solvent	Temp.	$CeCl_3$	4c (%)	Hydrolized 4c (%)	1 (%)
1	$NaBH_4$	1.0	MeOH	rt	+	–	–	98
2	$LiB(OMe)_3{}^2H$	1.0	EtOH	rt	+	94	4	2
3	$LiB(OMe)_3{}^2H$	1.5	EtOH	rt	+	–	34	66
4	$LiB(OMe)_3{}^2H$	1.5	EtOH	rt	–	–	9	91
5	$LiB(OMe)_3{}^2H$	1.5	EtOH	0 °C	–	–	39	61
6	$LiB(OMe)_3{}^2H$	1.5	EtOH	–78 °C	–	–	16	84
7	$LiB(OMe)_3{}^2H$	1.0	DMF	rt	+	–	28	72
8	$LiB(OMe)_3{}^2H$	1.0	DMF	rt	–	–	10	90
9	$LiB(OMe)_3{}^2H$	1.0	THF	rt	–	–	12	88
10	$Li_2{}^2H$	1.0	THF	rt	–	–	56[b]	–
11	LiB^2H_4	1.0	THF	rt	–	–	44[b]	36

[a]The yield were determined by HPLC at 230 nm. [b]Mixture of unidentified products.

Synthesis of propyl (*R*, *S*)-3-[^{2}H]-3-hydroxy-cyclopent-1-en carboxylate ([^{2}H]-5b)

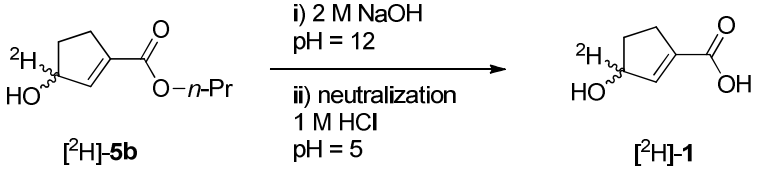


4c [^{2}H]-**5b**

To a freshly prepared solution of $LiB(OCH)_3{}^2H$ (theoretical 160 µmol based on the amount of *n*-BuLi) was drop wise added a solution of **4c** (14 mg, 83 µmol) in dry THF (0.5 mL). The reaction mixture was stirred for 2h, and all volatile components were lyophilized. Solid residue was quenched with 1 mL water and pH adjusted with 1M HCl to 5. The HPLC analysis showed [^{2}H]-labeled ester [^{2}H]-**5b** (88%, 230 nm). The pure product was isolated as colorless oil by preparative HPLC. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.87 (3H, t, *J* = 7.5 Hz, C*H*$_3$); 1.56–1.61 (2H, m, C*H*$_2$CH$_3$); 2.26–2.3 and 2.60–2.68 (2 × 1H, 2 × m, 5-C*H*$_2$), 2.34–2.41 and 3.27–3.30 (2 × 1H, 2 × m, 4-C*H*$_2$), 4.01

(2H, t, *J* = 6.5 Hz, OCH_2); 6.59 (1H, s, 2-C*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 10.32 (*C*H_3), 21.99 (*C*H_2CH$_3$), 29.87 (*C*H_2), 33.04 (*C*H_2), 66.30 (O*C*H_2), 138.57 (1-*C*), 143.42 (2-*C*H), 165.90 (*C*O), (3-*C*D) missing. MS (ESI): 193.2 (100, M-1 + Na). HPLC purification and analysis of [^{2}H]-**5b** was performed with a Princerton Spher C30 column (250 × 10 mm, 5 µm) with a flow rate of 3 mL/min and UV detection at 230 nm. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–5 min, 0% B; 5–20 min, 0–100% B; 25–30 min, 100% B; 30-33 min, 0–100% A. The retention time of [^{2}H]-**5b** was detected at 16.0 min, **4c** was not visible (standard at 17.5 min).

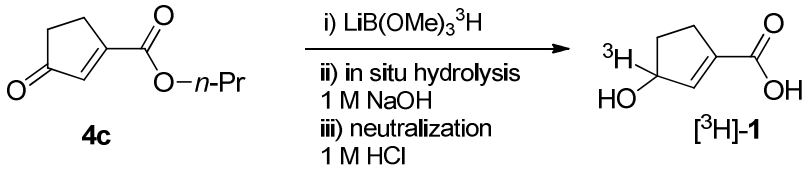
Synthesis of (*R*, *S*)-3-[^{2}H]-3-hydroxy-cyclopent-1-en carboxylate ([^{2}H]-1)



[^{2}H]-**5b** [^{2}H]-**1**

To a solution of [^{2}H]-**5b** (5 mg, 30 µmol) in water-acetonitrile 5:1 (1 mL) $NaOH_{(aq)}$ (2M, 100 µL) was added and the reaction was stirred for 1h at room temperature. HPLC analysis showed no starting material ([^{2}H]-**5b**), and showed predominantly the product [^{2}H]-**1** (95%, 230 nm). The reaction mixture was neutralized to pH 5 by addition of 1M HCl and preparative HPLC gave [^{2}H]-**1** as a white solid. Mp. 133–135°C. ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD 6:1) δ: 1.71–1.75 and 1.90–2.00 (2 × 1H, 2 × m, CH_2), 2.29–2.34 (1H, m, CH_2), 2.39–2.45 (1H, m, CH_2), 2.63-2.67 (1H, m, CH_2), 6.65 (1H, t, ^wJ (H, H) = 2.0 Hz, 2-C*H*). ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD 6:1) δ: 27.79 and 29.96 (*C*H_2), 33.33 and 35.40 (*C*H_2), 138.95 (1-*C*), 143.79 (2-*C*H), 167.61 (*C*O), (3-*C*D) missing. MS (ESI): 128.1 (100, M-1). HRMS: for $C_6H_6{}^2HO_3$: calculated 128.04634, found 128.04591.
HPLC analysis and purification of [^{2}H]-**1** was performed with a Princerton Spher C30 column (250 × 10 mm, 5 µm) with a flow rate of 3 ml/min and UV detection at 230 nm. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–5 min, 0% B; 5–20 min, 0–100% B; 25–30 min, 100% B; 30-33 min, 0–100% A. The retention time of [^{2}H]-**1** was detected at 12.5 min.

Synthesis of (*R*, *S*)-3-[^{3}H]-3-hydroxy-cyclopent-1-en carboxylate ([^{3}H]-1)



4c [^{3}H]-**1**

See main article for a description of the synthesis.

A fraction (1/10) of the total crude reaction mixture was purified by preparative HPLC. The collected fraction was then lyophilized and reconstituted in four different formulations (in a concentration of 6.3 mCi/ml) for specific activity analysis and initial radiochemical stability studies.

Radiochemical stability of [^{3}H]-1.

Stability studies were performed in order to determine the best formulation for storage and use in pharmacological investigations of the radioligand.

The four different formulations were:

1) 50% EtOH : 10^{-3}M NaOH (Figure S1)
2) Saline phosphate buffer (DPBS) (Figure S2)
3) Water stabilized with ascorbic acid (50 mM) (Figure S3)
4) Water stabilized with gentisic acid (50 mM) (Figure S4)

The final formulation for [^{3}H]-**1** was after this study then chosen to be the ligand dissolved in pure EtOH at a radioactive concentration of 1 mCi/ml. This formulation showed a stabile radioligand over a longer period of time (Figure S10).

Preparative HPLC: Column: Princerton Spher C30 (250mm × 10 mm, 5 µm). Flow: 2 ml/min. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0–6 min, 0% B; 6–40 min, 0–50% B; 40–41 min, 50-100% B; 41–43 min, 100%B; 43–44 min, 100–0% B; 44-46 min, 0% B. The retention time of [^{3}H]-(**1**) was detected at 18.4 min, [^{2}H]-**5b** at 37.9 min (refer to fig. 6 and 7 for chromatograms).

Analytical HPLC: Column: Phenomenex Luna C18 (2) (250 mm x 4.6 mm). Flow 1 ml/ml. Eluents: (A) 95% purified water, 5% acetonitrile, 0.1% TFA; (B) 99.9% acetonitrile, 0.1% TFA. Gradient: 0-3 min, 0% B; 3-20 min, 0–60% B; 20–21 min, 60–100% B; 21–23 min, 100% B; 23–24 min, 100–0% B; 24–26 min, 0% B. The retention time of [^{3}H]-**1** was detected at 10.9 min.

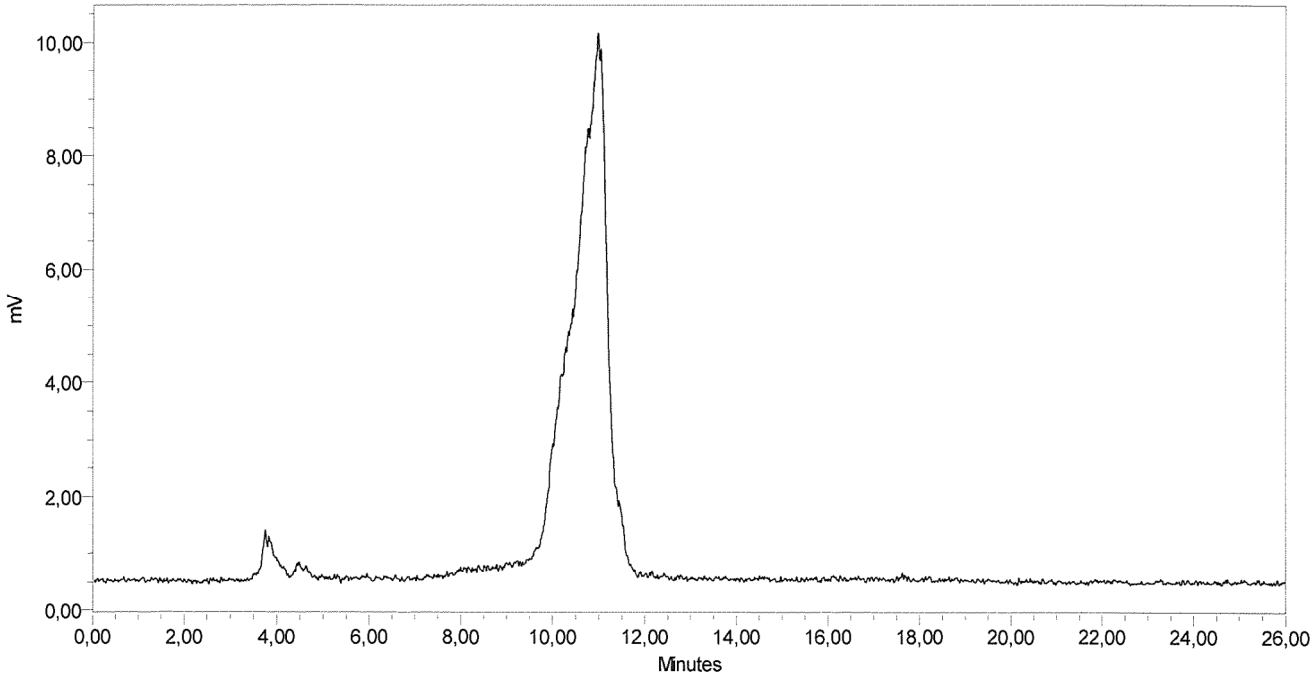


Figure S1. Stability HPLC radiochromatogram of [^{3}H]-**1** in 50% EtOH : 10-3 M NaOH (3 days after formulation / 6.3 mCi/mL).

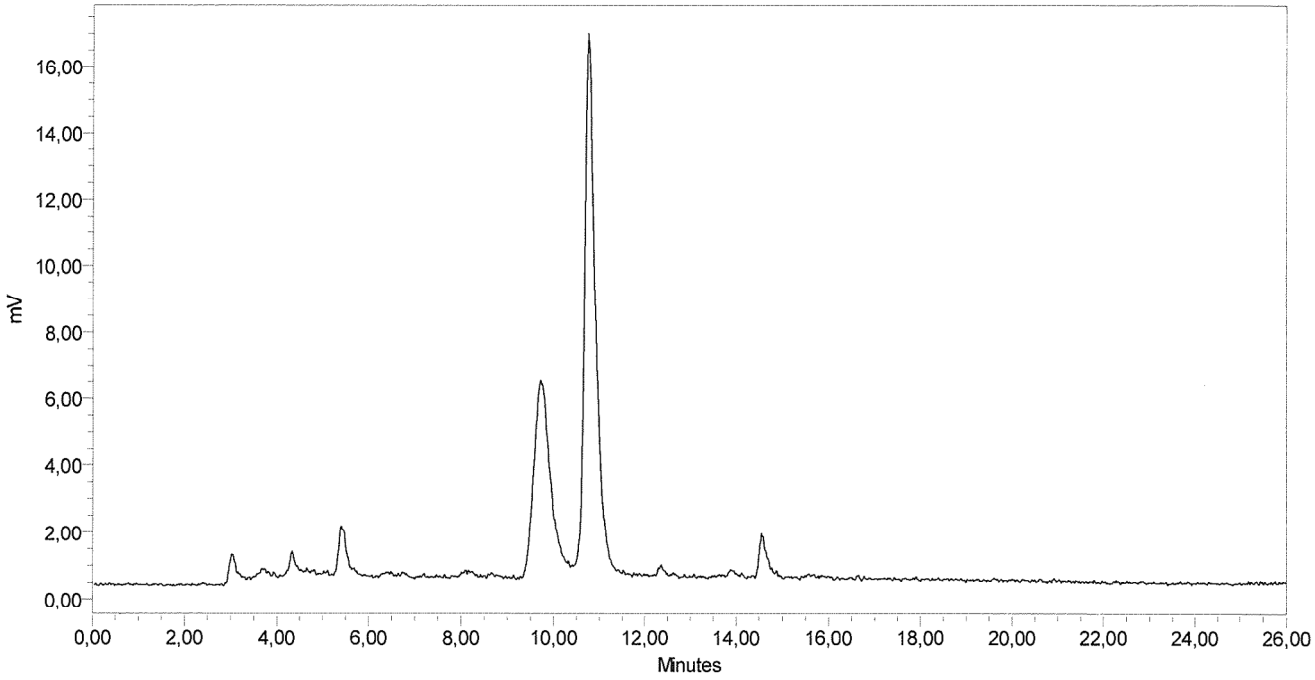


Figure S2. Stability HPLC Radiochromatogram of [^{3}H]-**1** in saline phosphate buffer (DPBS) (3 days after formulation/6.3 mCi/mL).

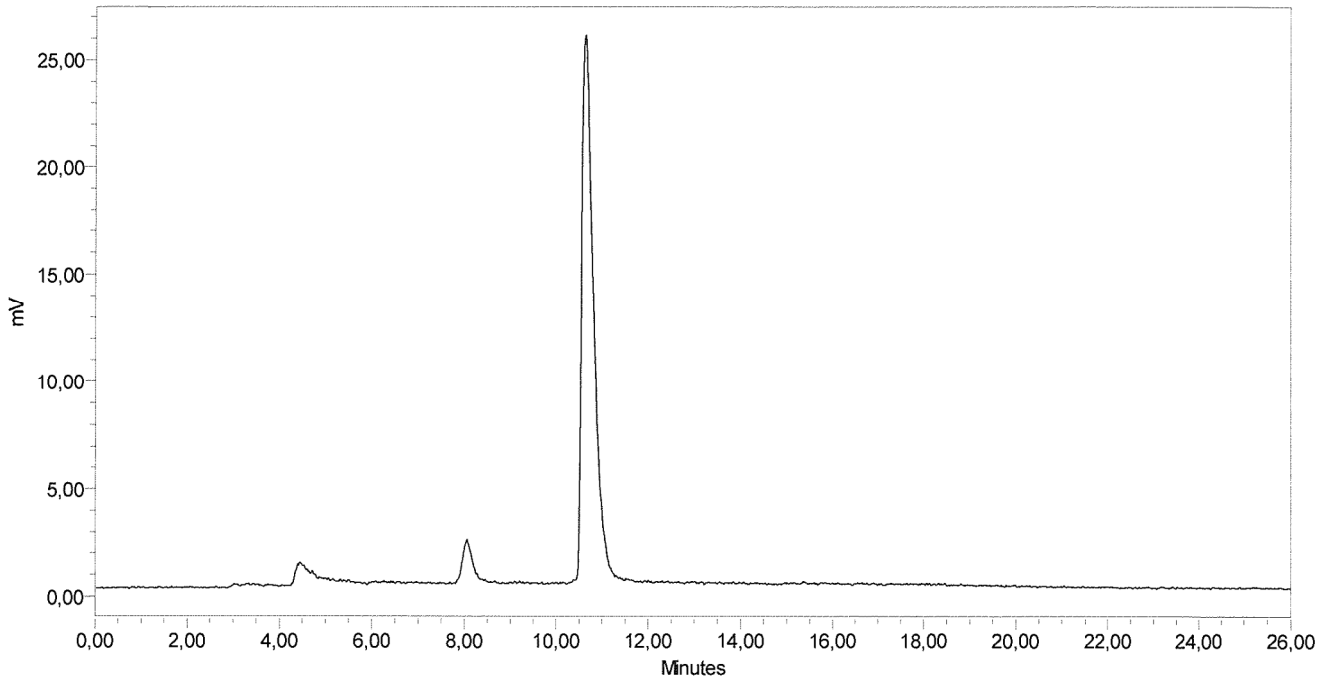


Figure S3. Stability HPLC Radiochromatogram of [^{3}H]-**1** in ascorbic acid (50 mM) (3 days after formulation / 6.3 mCi/mL)

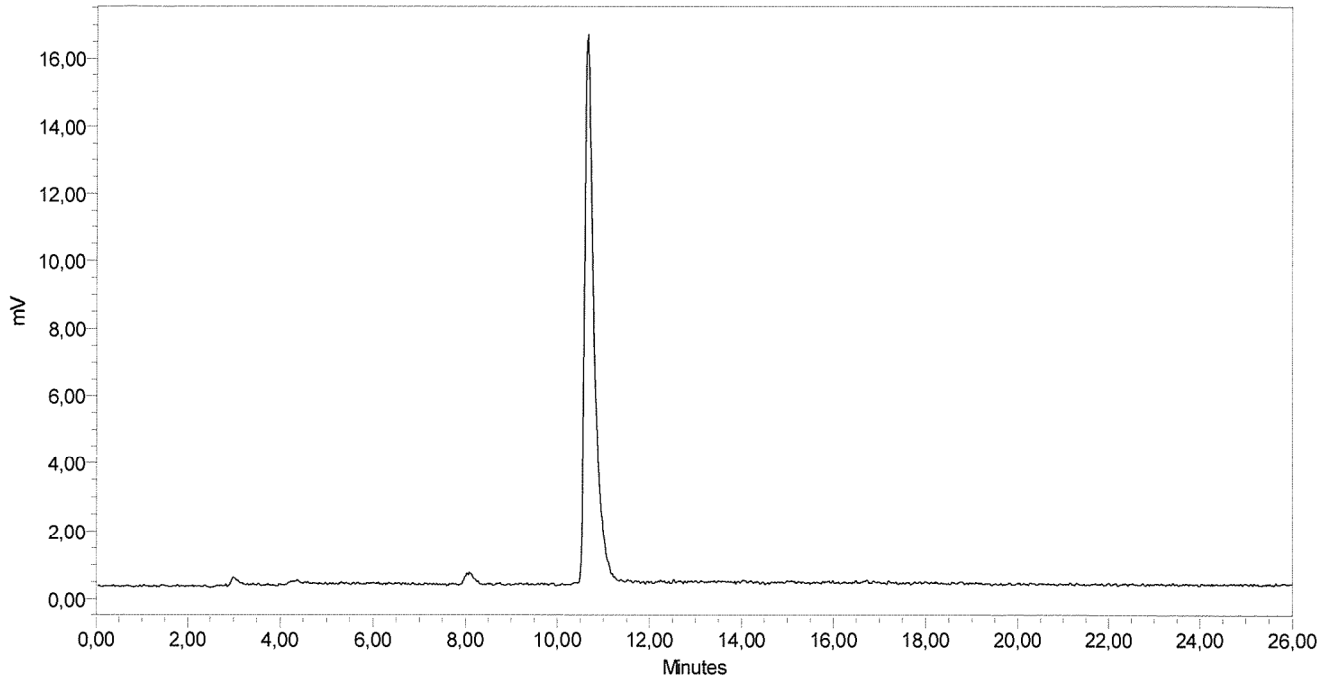


Figure S4. Stability HPLC Radiochromatogram of [^{3}H]-**1** in gentisic acid (50 mM) (3 days after formulation / 6.3 mCi/mL)

Representative NMR spectra and HPLC chromatograms:

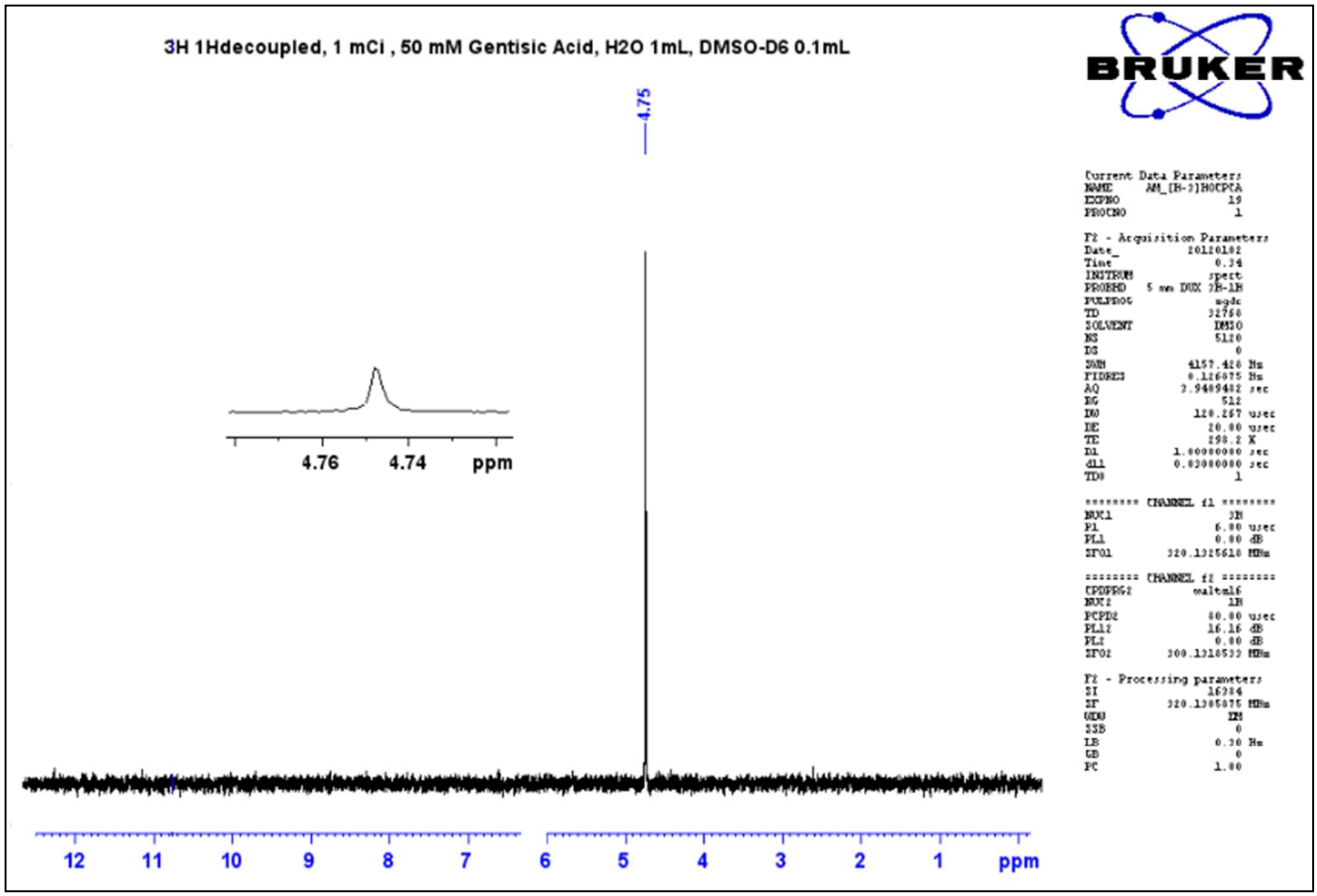


Figure S5. $^{3}H\{^{1}H\}$ NMR of [^{3}H]-**1**, 1 mCi, with gentisic acid 50 mM, in DMSO-D_6 (0.1 mL)/H_2O 1mL, (320 MHz)

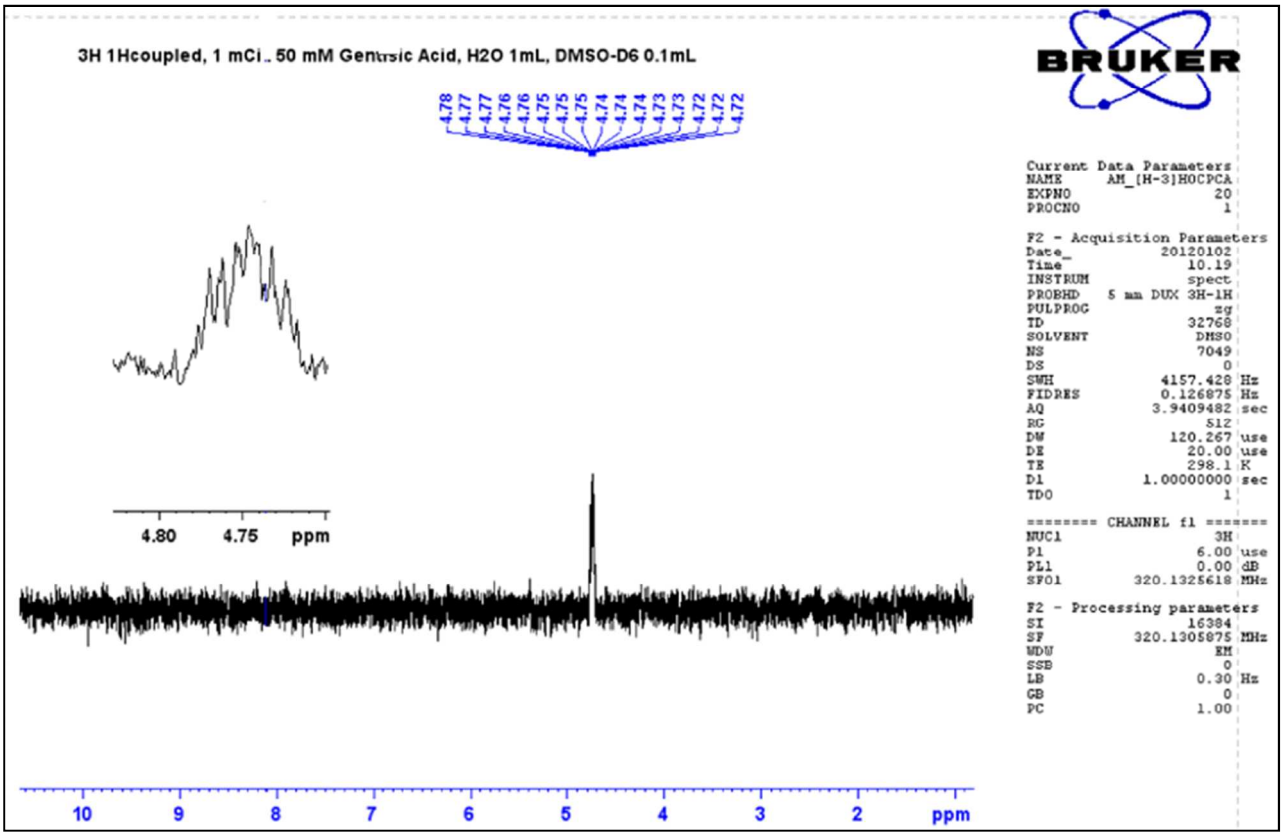


Figure S6. $^{3}H(^{1}H$ coupled) NMR of [^{3}H]-**1**, 1 mCi, with Gentisic acid 50 mM, in DMSO-D_6 (0.1 mL)/H_2O 1mL, (320 MHz)

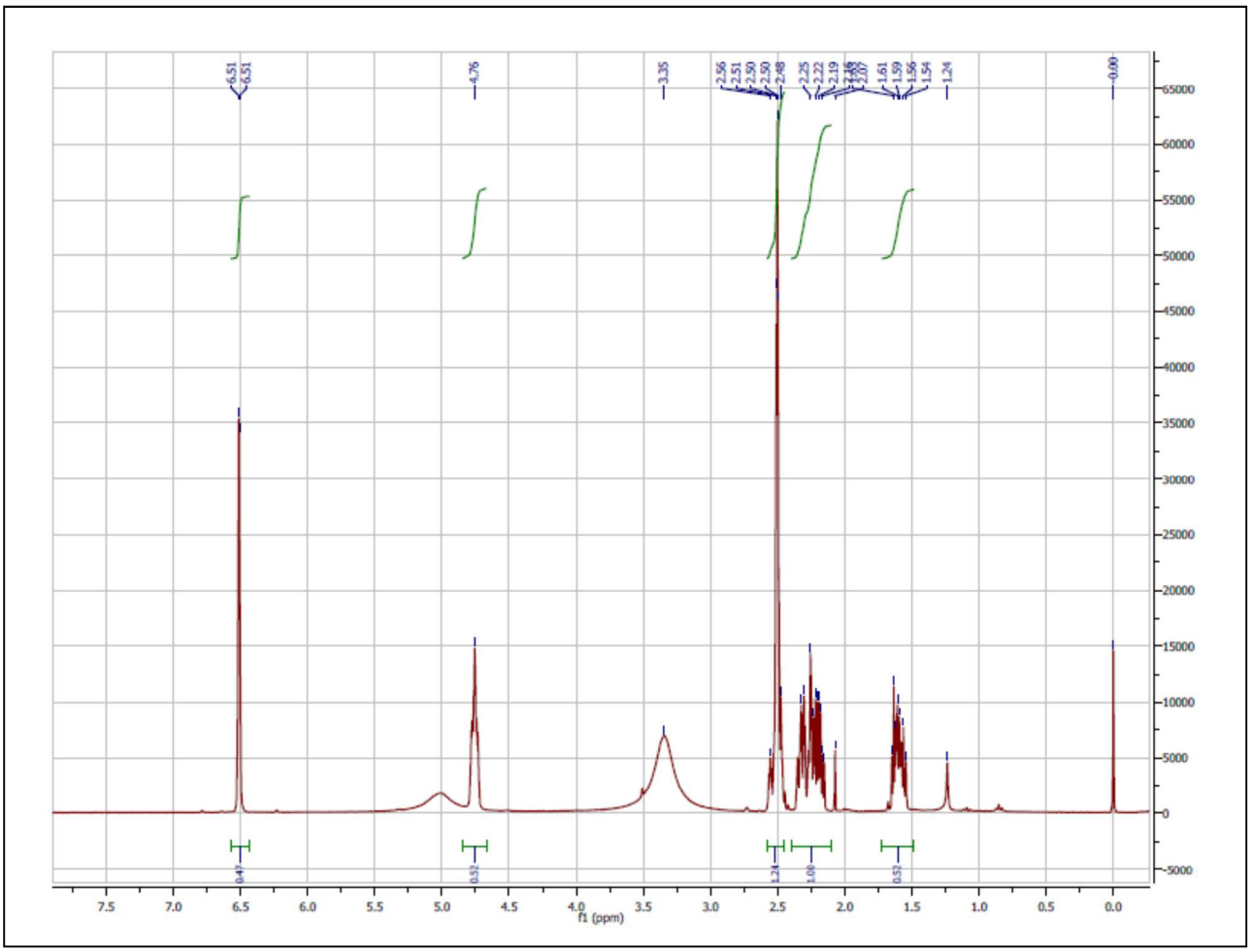


Figure S7. ^{1}H NMR of cold **1**, in DMSO-D_6 (0.1 mL)/H_2O (1 mL), (300 MHz)

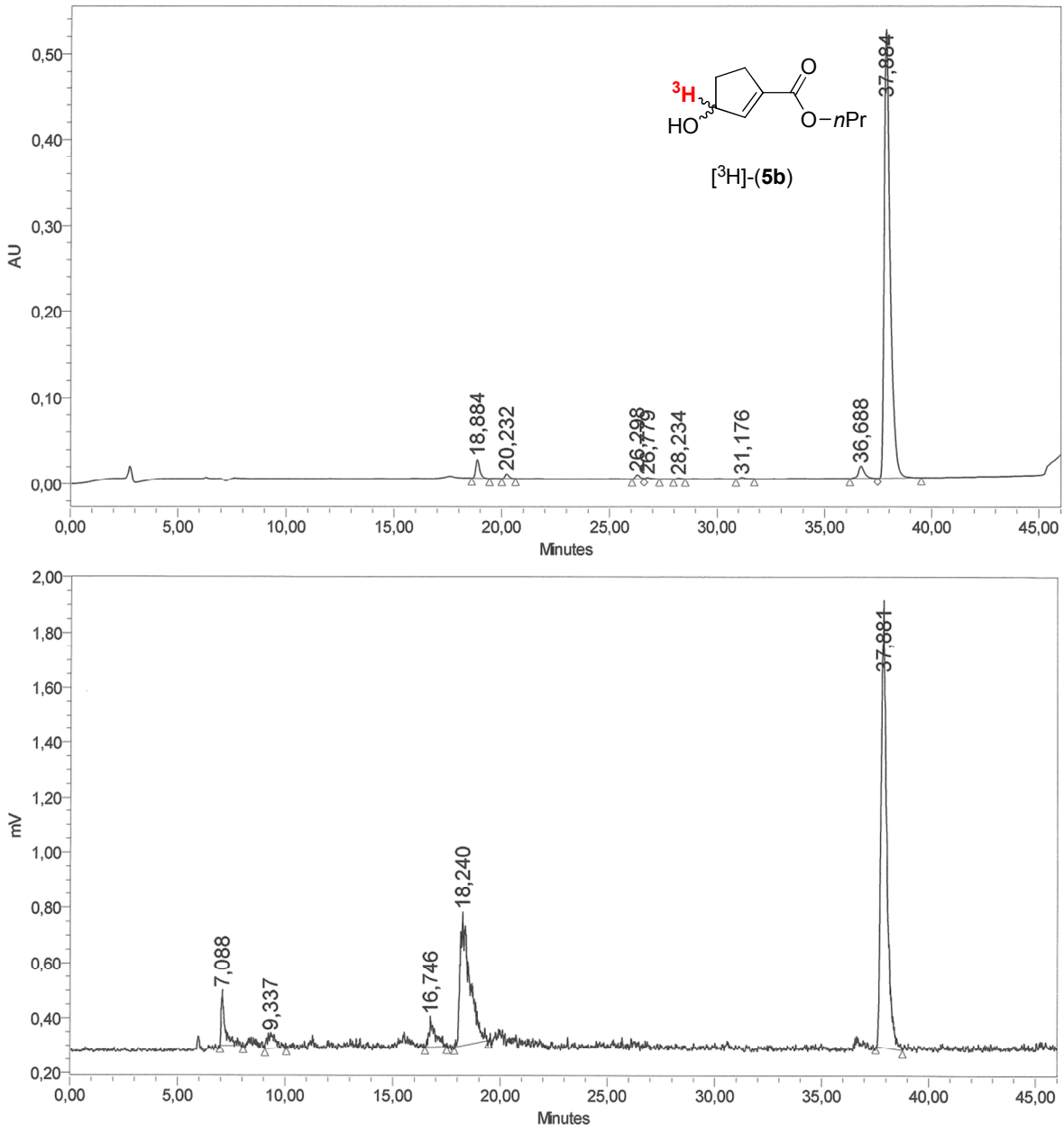


Figure S8. The HPLC chromatograms of the crude mixture of [^{3}H]-**5b**. Upper chromatogram: UV detector (245 nm). Lower chromatogram: Radiodetector.

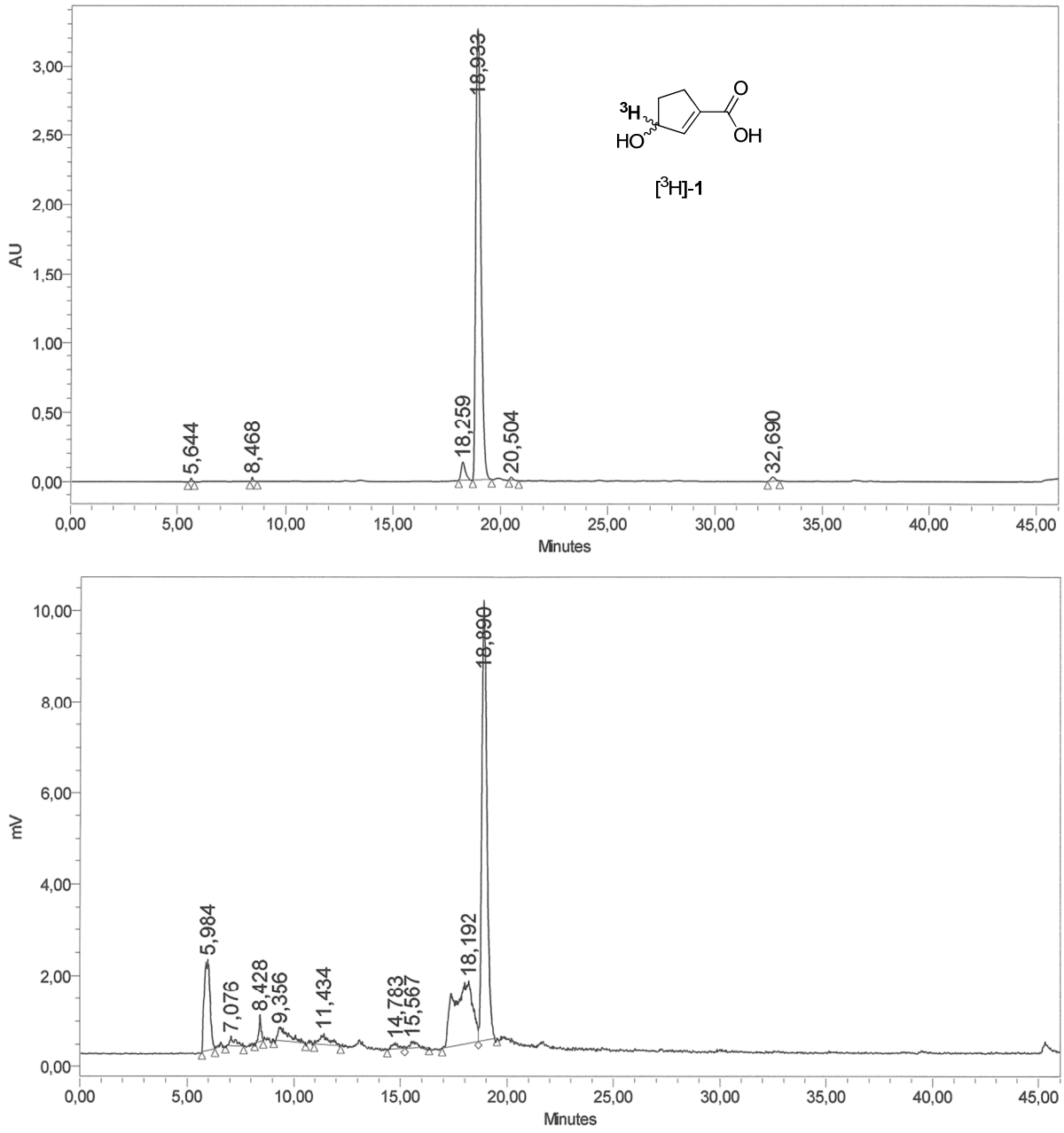


Figure S9. Preparative HPLC chromatograms of crude mixture of [^{3}H]-**1**. Upper chromatogram: UV detector (245 nm). Lower chromatogram: radiodetector.

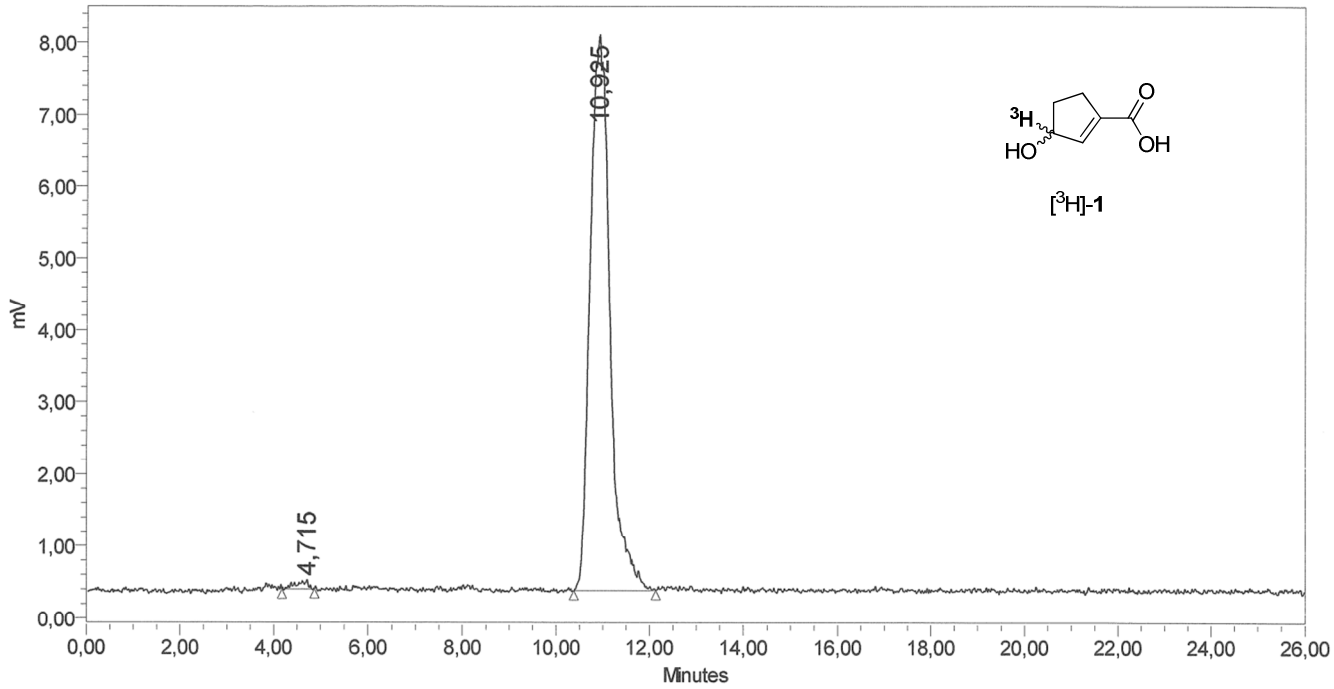


Figure S10. Analytical HPLC radiochromatogram of the final formulation of [^{3}H]-**1** (1 mCi/mL in EtOH 30 days after formulation), injection volume diluted 1:10 with water.

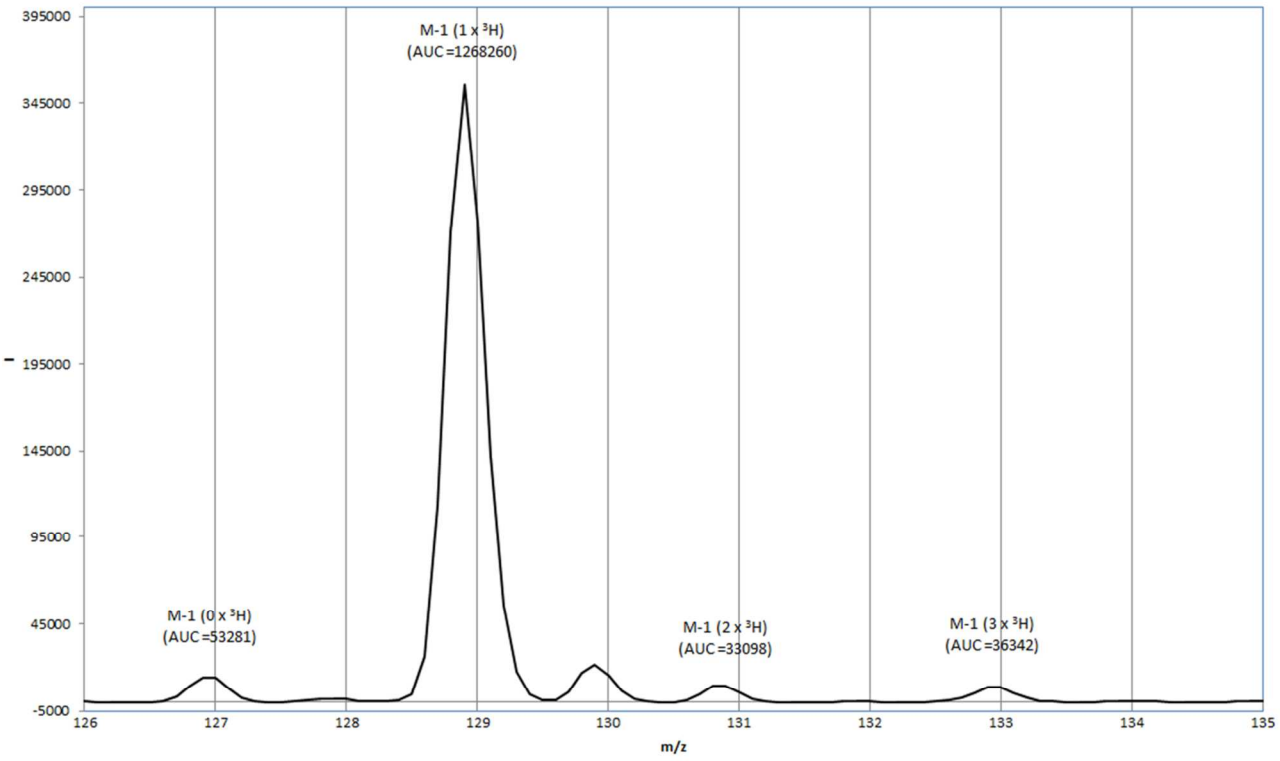


Figure S11. Mass spectra of [^{3}H]-**1** showing the intensity of isotopomers. The specific activity was determined to 28.9 Ci/mmol.

Pharmacology

Materials

GHB and GABA were purchased from Sigma-Aldrich. NCS-382 and gabazine were purchased from Tocris Bioscience (Bristol, UK). Baclofen was purchased from Ascent Scientific (Cambridge, UK). **1** (free acid and Na^+-salt) were prepared as described above.

Membrane Preparation and [^{3}H]-1 Binding Assay

For binding assays, synaptic membranes of cerebral cortex were prepared from adult male Sprague-Dawley rats with tissue preparation as described previously.[2] For the assay, the membrane preparation was thawed, suspended in 40 volumes of ice-cold assay buffer (50 mM KH_2PO_4 buffer, pH 6.0) using an UltraTurrax homogenizer (IKA Works, Inc., Wilmington, NC), and centrifuged at 48,000*g* for 10 min at 4 °C. This wash was repeated four times to remove endogenous GHB. The final pellet was resuspended in the assay buffer.

The [^{3}H]-**1** binding assay was performed in 96-well plate format using the same general assay setup as described previously with [^{3}H]NCS-382[3]: Membrane suspension (15-35 µg protein) was incubated with 10 nM [^{3}H]-**1** in assay buffer, with or without test compound (1 mM GHB, 100 µM NCS-382, 1 mM **1**, 1 mM GABA, 100 µM gabazine and 100 µM baclofen) for 1 h in 200 µL assay buffer per well (triplicates). For determination of IC_{50} values, increasing concentrations of GHB and **1** were applied, and nonspecific binding was determined with 1 mM GHB. The reaction was terminated by rapid filtration through Whatman GF/C unifilters (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) followed by three washes with assay buffer. The dried filters were added 30 µL scintilliation liquid (Microscint 0, PerkinElmer) and radioactivity was measured on a Packard TopCount NXT Microplate Scintillation Counter (PerkinElmer). Using these conditions, specific binding was in average 98.3% and no ligand depletion was seen. Data analysis was done using GraphPad Prism by nonlinear regression (ver. 5.0, San Diego, CA, USA).

Screening of 1 against other targets

1 was tested at concentration of 10 µM. At 44 out of 45 targets, **1** was found to be inactive, defined as < 50% inhibition of the radioligand binding (n = 4) or < 50% change in efficacy or inhibition of agonist (Hist H4, n = 4). At the µ opioid receptor, 54.5% inhibition was found and a K_i value was therefore determined (10.0 µM average). Obtained values are summarized in Table S2.

Table S2 Summary of **1** tested at 45 different receptors and transporters.

Target	Radioligand	Compound **1** % inhibition (K_i μM)[a]
5-HT1A	[^{3}H]8-OH-PDAT	-4.9
5-HT1B	[^{3}H]GR125743	-2.5
5-HT1D	[^{3}H]GR125743	-5.3
5-HT1e	[^{3}H]5-HT	49.4
5-HT2A	[^{3}H]ketanserin	0.2
5-HT2B	[^{3}H]LSD	-18.9
5-HT2C	[^{3}H]mesulergine	-2.3
5-HT3	[^{3}H]LY278584	17.4
5-HT5a	[^{3}H]LSD	9.8
5-HT6	[^{3}H]LSD	10.2
5-HT7	[^{3}H]LSD	17.0
Alpha1A	[^{3}H]prazosine	9.9
Alpha1B	[^{3}H]prazosine	20.1
Alpha1D	[^{3}H]prazosine	11.1
Alpha2A	[^{3}H]rauwolscine	17.4
Alpha2B	[^{3}H]rauwolscine	16.4
Alpha2C	[^{3}H]rauwolscine	5.9
Beta1	[^{125}I]pindolol	28.0
Beta2	[^{3}H]CGP12177	31.2
Beta3	[^{3}H]CGP12177	3.8
BZP rat brain	[^{3}H]Flunitrazepam	-5.8
Dopamine D1	[^{3}H]SCH23390	-0.1
Dopamine D2	[^{3}H]N-methylspiperone	10.6
Dopamine D3	[^{3}H]N-methylspiperone	-29.4
Dopamine D4	[^{3}H]N-methylspiperone	-4.6
Dopamine D5	[^{3}H]SCH23390	37.6
DAT	[^{3}H]WIN35428	24.5
DOR	[^{3}H]DADLE	0.2
$GABA_A$ rat brain	[^{3}H]muscimol	16.8
Histamine H1	[^{3}H]pyrilamine	10.4
Histamine H3	[^{3}H]alpha-methylhistamine	3.5
Histamine H4	N.B. functional assay	1.4 ago; 4.9 antag
KOR	[^{3}H]U69593	-12.7
Muscarinic M1	[^{3}H]QNB	-8.4
Muscarinic M2	[^{3}H]QNB	-3.3
Muscarinic M3	[^{3}H]QNB	-7.7
Muscarinic M4	[^{3}H]QNB	-0.8

Muscarinic M5	[^{3}H]QNB	5.3
MOR	[^{3}H]DAMGO	54.0 (10.0 ave)
NET	[^{3}H]nisoxetine	46.8
PBR kidney	[^{3}H]PK11195	-4.4
SERT	[^{3}H]citalopram	28.8
Sigma1	[^{3}H]pentazocine(+)	7.0
Sigma2	[^{3}H]DTG	41.0

[a] K_i determinations, agonist and/or antagonist functional data and receptor binding profiles were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA.

In vivo brain penetration studies

Male C57 mice (20-25 g) received an oral dose of 10 mg/kg of GHB, **1** or NCS-382 dissolved in water (n=3 for each compound). 30 minutes after drug administration, cardiac blood was obtained under isoflurane anaesthesia followed by decapitation and removal of the brain. Plasma samples were harvested after centrifugation of the blood samples and brain samples were prepared by homogenizing the brains using 70% acetonitrile (1:4 v/v) followed by centrifugation and collection of the supernatant. Brain and plasma exposure was determined quantitatively by LC-MS/MS. The brain to plasma distribution ratio was expressed as brain concentration (ng/g) divided by plasma concentration (ng/ml). For GHB, the results were corrected for interference from endogenous levels of GHB in plasma and brain matrices.

Reference List

1. Zippi, E. M. Lithium Hydride and Lithium Trimethoxyborohydride: A Comparative Study of Their Reducing Power. *Synthetic commun.* **1994**, *24*, 2515-2523.

2. Ransom, R. W.; Stec, N. L. Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J. Neurochem.* **1988**, *51*, 830-836.

3. Wellendorph, P.; Høg, S.; Greenwood, J. R.; de Lichtenberg, A.; Nielsen, B.; Frølund, B.; Brehm, L.; Clausen, R. P.; Bräuner-Osborne, H. Novel Cyclic γ-Hydroxybutyrate (GHB) Analogs with High Affinity and Stereoselectivity of Binding to GHB Sites in Rat Brain. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 346-351.