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Brief Article

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New Synthesis and Tritium Labeling of a Selective Ligand for Studying High-Affinity γ-Hydroxybutyrate (GHB) Binding Sites

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Supporting Information

ABSTRACT: 3-Hydroxycyclopent-1-enecarboxylic acid (HOCPCA, **1**) is a potent ligand for the high-affinity GHB binding sites in the CNS. An improved synthesis of **1** together with a very efficient synthesis of [^{3}H]-**1** is described. The radiosynthesis employs in situ generated lithium trimethoxyborotritide. Screening of **1** against different CNS targets establishes a high selectivity, and we demonstrate in vivo brain penetration. In vitro characterization of [^{3}H]-**1** binding shows high specificity to the high-affinity GHB binding sites.

INTRODUCTION

γ-Hydroxybutyrate (GHB, Figure 1) is an endogenous substance that is present in micromolar concentrations in the mammalian brain. It is a metabolite of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Figure 1) but is also believed to be a neuromodulator on its own[1] although the specific function of GHB remains to be elucidated. GHB is administered orally for the treatment of narcolepsy (sodium oxybate)[2] and alcoholism.[3] GHB is tasteless and odorless, and combined with its central nervous system (CNS) effects (mild euphoria, sedation, respiratory depression, and eventually coma in high doses) it is a hazardous drug of abuse (fantasy). In the CNS, GHB binds to both low-affinity and high-affinity binding sites.[1] The low-affinity site is believed to be identical to the GABA orthosteric site at the metabotropic $GABA_B$ receptor, as high dose effects of GHB (micromolar in the brain) are prevented by preincubating with a $GABA_B$ receptor antagonist[3] and several prominent GHB-induced effects are absent in $GABA_B$ knockout mice.[4] However, when comparing the effect of GHB with that of the $GABA_B$ agonist baclofen (Figure 1) in, e.g., c-Fos expression studies[5] and behavioral studies,[6] it is clear that the two compounds have different pharmacological profiles. Thus, GHB effects may also be mediated by non-$GABA_B$ receptors. The fact that GHB high-affinity binding sites are preserved in brains of $GABA_B$ knockout mice[4] make them likely receptor candidates and has prompted the uncovering of their identity. Using photoaffinity labeling combined with proteomic analysis and molecular pharmacology studies, we recently reported that $\alpha_4\beta_{1-3}\delta$ ionotropic $GABA_A$ receptors correlate with the GHB high-affinity sites.[7] To acquire a more complete understanding of the physiological relevance of GHB high-affinity binding sites in the CNS, and particularly the nanomolar effects mediated by GHB at the $\alpha_4\beta_1\delta$ receptors, better tool compounds and further pharmacological investigations are needed.

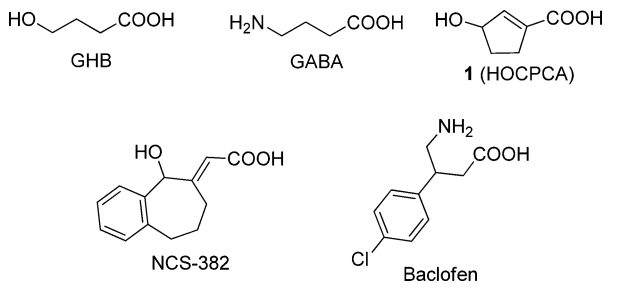

Figure 1. Chemical structures of the two neuroactive substances GHB and GABA. HOCPCA (**1**) and NCS-382 bind selectively to the high-affinity GHB binding site. Baclofen is a selective $GABA_B$ agonist.

We have previously described 3-hydroxycyclopent-1-enecarboxylic acid (HOCPCA, **1**, Figure 1) as a selective ligand for the high-affinity GHB binding site (27 times higher affinity than GHB itself) and, importantly, with no affinity for the $GABA_B$ receptor. Given the close structural relationship to GHB and its relatively small size, **1** is a highly attractive compound to investigate GHB-like pharmacology. We therefore decided to explore synthetic routes for incorporation of hydrogen and carbon isotopes into **1** and at the same time provide a more feasible and preparative synthesis of **1**, which is needed to facilitate in vivo studies. At present, [^{3}H](*E*,*RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid ([^{3}H]-NCS-382) is the standard radioligand in binding experiments for the high-affinity GHB binding site.[8] NCS-382 (Figure 1) is a putative antagonist[8] at the high-affinity GHB

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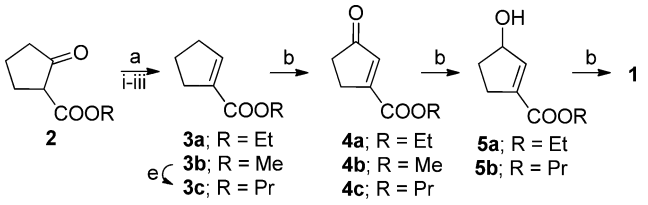
binding site with a K_i 14 times lower than that of GHB.[9] The higher affinity of **1** compared to NCS-382[9] and closer structural relationship to GHB underscores the relevance of developing a better preparative and radiolabeling procedure. This will provide a better alternative to the only available radiolabeled agonist [^{3}H]GHB, which has several caveats, both in terms of selectivity due to its affinity for the $GABA_B$ receptor and in terms of its susceptibility to metabolism and uptake.[10]

Here, we describe a highly improved preparative synthesis of **1** and the synthesis of tritium labeled **1**. Furthermore, the stability of **1** and [^{3}H]-**1** are evaluated, binding competition studies using [^{3}H]-**1** are presented, and the selectivity profile of **1** is investigated. Finally, the brain penetration of GHB, **1**, and NCS-382 is investigated in vivo.

RESULTS AND DISCUSSION

Improved Synthesis of 1. The previously[9] reported synthesis of **1** is outlined in Scheme 1. Reduction of the starting material (**2**) followed by mesylation and elimination gave α,β-unsaturated ethyl ester (**3a**) in 53% yield.

Scheme 1. Previous Synthesis Route to 1 and Its Carbonyl Precursor 9[a]

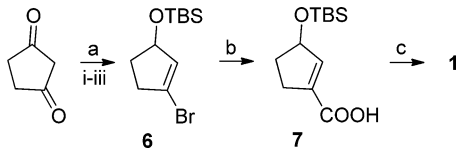


[a]Reagents and conditions: (a) (i) $NaBH_4$, EtOH, 0 °C − rt, 2.5 h, (ii) CH_3SO_2Cl, Py, rt, 3 h, (iii) Et_3N, $CHCl_3$, reflux, 23 h; (b) CrO_3, Ac_2O, AcOH, rt, 30 min; (c) $NaBH_4$, $CeCl_3$, MeOH, 0 °C, 30 min; (d) Na_2CO_3, 50 °C, 16 h; (e) 1-propanol, H_2SO_4, reflux, 1 h.

Oxidation of **3a** with 3 equiv of CrO_3 gave **4a** in varying yields below 39%. Successful Luche reduction gave **5a**, and final deprotection with sodium carbonate gave the product **1** as hygroscopic crystals. The overall yield of the six-step synthesis was 18%. However, this route posed several problems as a preparative synthesis, i.e., varying yields, tedious purification in particular in the oxidation of **3a**, and a hygroscopic end product that is unstable during storage.

In light of the potential of **1** as a pharmacological tool for future in vivo and ex vivo studies, we wanted to develop a more suitable synthesis of **1** that could circumvent these obstacles and generate large quantities of **1**. The new synthetic route to **1** is outlined in Scheme 2. First, 1,3-cyclopentanedione was brominated with dibromo-triphenylphosphine and triethylamine.[11] It was crucial for a high yield to use freshly distilled triethylamine dried over $LiAlH_4$, whereas sublimation (110 °C, 0.2 mbar) of the commercial brownish 1.3-cyclopentanedione to white crystals did not improve the yield. Luche reduction with $NaBH_4$ in methanol gave the alcohol that was immediately TBS-protected to give **6** in 86% overall yield.

Scheme 2. New Synthesis Route to 1[a]

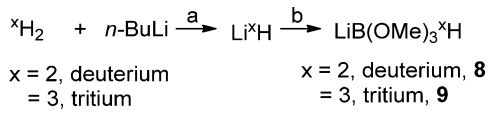


[a]Reagents and conditions: (a) (i) Br_2PPh_3, benzene, Et_3N, rt, 18 h, (ii) $NaBH_4$, $CeCl_3$, MeOH, 0 °C − rt, 2.5 h, (iii) TBS-Cl, DMAP, imidazole, DCM, rt, 16 h; (b) *t*-BuLi, THF, −78 to −50 °C, 2 h, −78 °C, CO_2(g), 20 min; (c) H_2SiF_6 (aq 23%), CH_3CN, rt, 1 h.

Metal−halogen exchange of **6** using *t*-BuLi followed by addition of CO_2(g) afforded the carboxylic acid **7** in 78% yield. Finally, deprotection of the TBS-protected alcohol using an aqueous solution of H_2SiF_6 as described by Pilcher and DeShong[12] gave **1** as white crystals in 75% yield upon recrystallization. The product was stable as the free acid as well as the sodium salt. This new synthetic route is high yielding (56% overall), includes more simple purifications, and produces a nonhygroscopic stable product without the use of chromic acid. The route is also applicable for late-stage isotopic carbon labeling of **1** because labeled CO_2(g) can be employed when incorporating the carboxylic acid group. Alternatively, the bromide **6** can be transformed to the corresponding pinacol boronic ester and treated with [^{11}C]CO_2(g) in the presence of CuI/crypt-222/KF as recently described by Riss et al.[13] On the other hand, the route is not stereoselective and provide both enantiomers.

Tritium Labeling. The previously reported synthesis of **1** outlined in Scheme 1 was a good starting point for a route to tritium labeled **1** as reduction of the α,β-unsaturated ketone (**4**) enabled introduction of tritium late in the synthesis. Still, some optimization of this route was feasible. **3b** is commercially available, but volatility of both **3b** and **4b** compromised the yield in the first step and **3b** was therefore transesterified to the propylester **3c** in 70% yield. Attempts to replace the excess amount of chromic acid with $KMnO_4$[14] or TBHP/Pd(OH)C[15] were unsuccessful. The use of catalytic amounts of $Mn(OAc)_2\cdot 4H_2O$[16] was at first glance successful (75% yield), but unfortunately an unidentified impurity not visible by NMR affected the course of the following reduction negatively and the oxidation was therefore performed with chromic acid to afford **4c**. The Luche reduction (1 equiv of $NaBH_4$ in MeOH in the presence of $CeCl_3$) gave the alcohol **5b** in 98% yield (Supporting Information (SI), Table S1). To prepare [^{3}H]-**1** with a maximal specific activity, we decided to screen the reaction conditions using borodeuterides prepared in situ from deuterium gas instead of using commercial deuterides. In the literature, a broad spectrum of in situ prepared borodeuterides[17,18] and tritides,[19,20] respectively, are described. To suppress the risk of reduction of the double bond and ester group, we were especially interested in less reactive borodeuterides. As demonstrated by Zippi et al.,[19] $LiB(OMe)_3{}^2H$ (**8**) can be readily generated from Li^2H by addition of trimethoxyborate (Scheme 3). Lithium deuteride is formed by the treatment of a *n*-BuLi-TMEDA solution under gaseous $^2H_2$ atmosphere[17] (Scheme 3).

Scheme 3. Synthesis of the Lithiumboronic Reducing Reagent[a]



[a]Reagents and conditions: (a) TMEDA, 2 h, room temperature; (b) $B(OMe)_3$, 30 min, room temperature.

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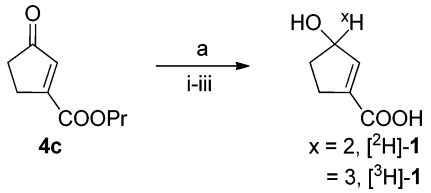
The yield of in situ generated borodeuteride (**8**) was determined indirectly using 4-anisaldehyde as a trapping agent in independent reactions. The reaction was carried out under various ratios (1:1, 2:1, and 1:2) of (**8**) (theoretically generated amounts based on *n*-BuLi) and anisaldehyde, and the yield of **8** was determined by HPLC and ^{1}H NMR to be in the range of 40−47% in all three cases. That is in accordance with the yield of Li^{3}H (35%) reported by Zippi et al.[19] For radiation safety reasons during the ^{3}H-labeling of **1**, it was desirable to perform the reduction with borotritide and the ester hydrolysis of **4c** in one pot. Therefore, upon lyophilization of the solvent, aq NaOH was added and hydrolysis of the propylester gave [^{2}H]-**1** (Scheme 4). The lyophilization was crucial as the yield dropped from 88% to 15% if omitted. Next the reaction conditions for the reduction of **4c** (Scheme 4) was investigated (see SI Table S1).

Scheme 4. Deuterium and Tritium Labeling of 1[a]



[a]Reagents and conditions: (a) (i) **8** or **9**, THF, room temperature, 2 h, room temperature, lyophilized, (ii) 1 M NaOH, 1 h, room temperature, (iii) 1 M HCl.

The protic solvent MeOH of the original experiment could not be exchanged with EtOH in the presence of $CeCl_3$. Actually, the presence of $CeCl_3$ apparently resulted in an increased amount of hydrolyzed **4c** and could successfully be omitted. In the absence of $CeCl_3$, both DMF and even the easily lyophilizable solvent THF could be used.

Lowering of the reaction temperature to 0 or −78 °C did not improve the deuterated yield nor suppressed the hydrolysis of the ketone. For comparison, in situ synthesized Li^{2}H and LiB^{2}H$_4$ (see SI) were also attempted but resulted in lower yields and more complex reaction mixtures.

Finally, the best conditions (LiB(OMe)$_3{}^2$H, THF, room temperature) were used to prepare [^{3}H]-**1** (Scheme 4). To achieve full conversion of **4c**, excess amounts (1.8 equiv) of LiB(OMe)$_3{}^3$H were used. Reduction of **4c** gave the ^{3}H-labeled propylester in 95% yield according to HPLC (245 nm). Subsequent hydrolysis gave the desired product [^{3}H]-**1** (97%, 245 nm). We determined the amounts of prepared [^{3}H]-**1** to 637 mCi with a specific activity of 28.9 Ci/mmol (determined by MS and HPLC/LSC, which accounts for close to 1 tritium/molecule) and a radio chemical purity (RCP) and chemical purity >99% after purification (HPLC, SI Figure S11).

Stability. Both **1** and [^{3}H]-**1** was subjected to stability testing by HPLC (area%, 245 nm). The rate of decomposition of **1** was tested at both room temperature and −30 ± 2 °C in Milli-Q water (2 mg/mL). pH of this solution was 3.5. At −30 °C, compound **1** proved completely stable (1 year and 4 months after formulation) and at room temperature only a minor decomposition of **1** was observed (100% at day 0 to 96% at day 35 and 88% at day 99). The stability of [^{3}H]-**1** was investigated in four different formulations for the protection against radiolysis (see SI). The best stability of [^{3}H]-**1** was found to be a solution of 1 mCi/mL in ethanol or in an aqueous solution of gentisic acid (50 mM).

Binding Studies. Binding of [^{3}H]-**1** to rat cortical membranes was investigated using assay conditions previously described for [^{3}H]NCS-382[9] with GHB (1 mM) for determining nonspecific binding (Figure 2A). IC_{50} [pIC_{50} ± SEM] values for **1**, sodium salt of **1**, and GHB were, respectively, 0.073 μM [7.14 ± 0.02], 0.116 μM [6.93 ± 0.01], and 1.14 μM [5.94 ± 0.06]. This is in relative accordance with values obtained in the comparable [^{3}H]NCS-382 binding assay (0.16 and 4.3 μM, respectively).[9] As expected, [^{3}H]-**1** binding was inhibited by the GHB-selective ligand NCS-382 and the $GABA_A$ antagonist gabazine[7,21] but notably not by baclofen and GABA at high concentrations.[7,9] These data suggest that [^{3}H]-**1** selectively labels the high-affinity GHB binding site. The selectivity was further explored in The National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH-PDSP) (SI Table S2), where **1** was found to be more than 100 times selective for the [^{3}H]-**1**

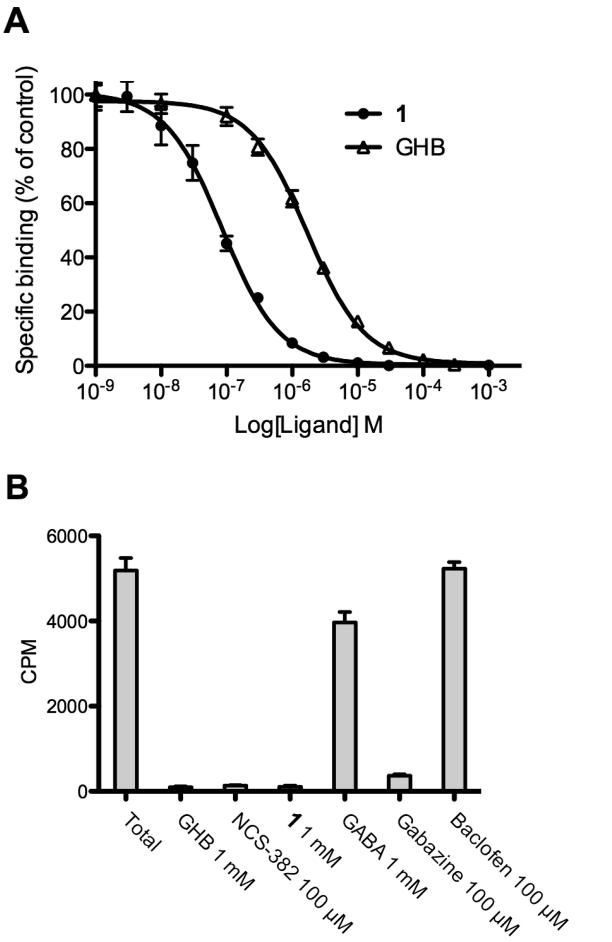


Figure 2. (A) Concentration-dependent inhibition of [^{3}H]-**1** binding (10 nM) to rat cerebrocortical membranes by GHB and **1**. Results are expressed as mean (SD of a single representative experiment performed in triplicate). Three additional experiments gave similar results. (B) Displacement of 10 nM [^{3}H]-**1** binding to rat cerebrocortical membranes by 1 mM GHB, 100 μM NCS-382, 1 mM **1**, 1 mM GABA, 100 μM gabazine, and 100 μM baclofen. Results are expressed as mean CPM (SD of a single representative experiment performed in triplicate) and were repeated at least twice with similar results.

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binding site over 45 different receptors and transporters. The high selectivity of **1** combined with a ligand efficiency[22] of 1.05 kcal/mol establish **1** as a highly interesting compound for further studying the high-affinity GHB binding site.

Brain Penetration Studies. The brain to plasma distribution ratio of GHB, **1**, and NCS-382 was investigated acutely in mice 30 min after oral administration of 10 mg/kg. All three compounds were found to be brain penetrant, reflected by their brain to plasma ratios of 0.20, 0.37, and 0.38 for GHB, **1**, and NCS-382, respectively. Considering the carboxylic acid group shared by all three compounds, often associated with restricted passive brain penetration, these results may suggest an involvement of active transport across the blood–brain barrier. This hypothesis was supported by assessment of the in vitro permeability using MDCK-MDR1 cells[23] which showed that GHB, **1**, and NCS-382 all exhibited very low passive permeability. Thus, after spiking of test compounds on either the apical or basal side of the cell monolayers, no compound could be detected at the respective receiver sides.

CONCLUSION

In conclusion, we have developed a new preparative synthetic route to **1** that can be scaled up with a high and reproducible overall yield. The new synthesis can readily be developed further for carbon labeling. Compound **1** is stable during storage and has a favorable selectivity profile when tested against other relevant targets. Furthermore, compound **1** was successfully tritium labeled using in situ generated lithium trimethoxyborotritide. The incorporation of one tritium atom proceeded in high radioactive yield, with close to theoretical specific activity and with high radiochemical purity. Binding of [^{3}H]-**1** could be displaced by GHB and **1** in a concentration-dependent manner and not by the $GABA_B$ receptor agonist baclofen. This new radioligand will be useful for further understanding the molecular mechanism of GHB at its high-affinity binding site and $\alpha4\beta\delta$ $GABA_A$ receptors and for elucidating the pharmacology of the GHB system in the mammalian CNS, given the brain permeability of **1**, for instance through ex vivo or in vivo labeling of the binding sites with positron-emitting or other isotopes.

EXPERIMENTAL SECTION

[^{3}H]-3-Hydroxycyclopent-1-enecarboxylic Acid ([^{3}H]-1). Carrier free $^3H_2$ (13.8 Ci) trapped on a uranium bed (as uranium tritide) was released by heating (500 °C) and directed in a tritium manifold into a dried reaction vial (1 mL two-necked round-bottom flask). *N,N,N′,N′*-Tetramethylethylenediamine (50 μL) was added. *n*-Butyl lithium (100 μL, 160 μmol, 1.6 M in hexane) was added dropwise, and within 10 min a white precipitate of Li^{3}H was formed. After 2 h of vigorous stirring, trimethyl borate (18 μL, 160 μmoles) was added and the reaction was stirred for 30 min to generate $LiB(OMe)_3{}^3H$. A solution of propyl 3-oxo-cyclopent-1-en carboxylate (**4c**) (15 mg, 89 μmol) in THF (300 μL) was added dropwise, and the reaction was stirred for 2 h. Solvents were lyophilized. Water (500 μL) was added, and a sample showed predominantly [^{3}H]-**5b** by HPLC analysis (95%, 245 nm). Aqueous NaOH (2 M, 100 μL) was added, and the reaction was stirred for 1 h. The reaction mixture was neutralized with 1N HCl to reach pH 6. Water was lyophilized and the solid residue dissolved in accurate amounts of water. Then 1/10 of the crude mixture was used for qualitative and quantitative analysis (see SI). Preparative HPLC gave [^{3}H]-**1** ((97%, 245 nm), 637 mCi, 28.9 Ci/mmol) as white solid. ^{3}H NMR (320 MHz, DMSO-d_6/H_2O 10:1): δ 4.71 (s). MS (ESI): 128.9 (100, M − 1).

3-Hydroxycyclopent-1-enecarboxylic Acid (1). 3-((*tert*-Butyldimethylsilyl)oxy)cyclopent-1-enecarboxylic acid (3.17 g, 13.08 mol) was dissolved in acetonitrile (130 mL) in a polypropylene vial, and H_2SiF_6 (aq, 20–25% wt, 2.3 mL, 3.9 mmol) was added. The solution was stirred for 1 h at room temperature. Satrated Na_2CO_3 (100 mL) and water (50 mL) were added, and the aqueous phase was washed with diethyl ether (2 × 20 mL). The aqueous phase was made acidic by the addition of HCl (aq, 4M) and extracted with ethyl acetate (4 × 150 mL). The combined organic phase was dried ($MgSO_4$) and the solvent removed in vacuo to give a white solid. CC (2:1 heptane/ethyl acetate + 2% AcOH) gave a white solid. Recrystallization (acetonitrile) gave the product as white crystals (1.26 g, 75%); mp 134–136 °C. ^{1}H NMR (CD_3OD): δ 1.71 (m, 1H), 2.30–2.48 (m, 2H), 2.62–2.70 (m, 1H), 4.86–4.91 (m, 1H), 6.63–6.65 (m, 1H). ^{13}C NMR (CD_3OD): δ 30.81, 34.23, 77.65, 139.93, 144.72, 168.62. Anal. Calcd for $C_6H_8O_3$: C, 56.24; H, 6.29. Found: C, 56.34; H, 6.17. LC-MS (system B): $(M - H_2O)H^+$: 111.1.

ASSOCIATED CONTENT

Supporting Information

Experimental data for compounds (**3c**, **4c**, **6**, **7**, [^{2}H]-**5b**, [^{2}H]-**1**), description of stability studies, in vitro pharmacological methods, and NIMH-PDSP screening results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GHB, γ-hydroxybutyrate; HOCPCA, 3-hydroxycyclopent-1-enecarboxylic acid; NCS-382, (2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen)-6-ylidene ethanoic acid

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