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Radiosynthesis and Evaluation of [^{11}C]3-Hydroxycyclopent-1-enecarboxylic Acid as Potential PET Ligand for the High-Affinity γ-Hydroxybutyric Acid Binding Sites

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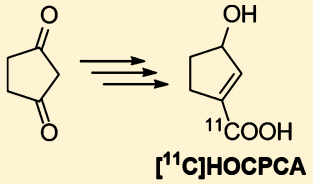
Supporting Information

ABSTRACT: γ-Hydroxybutyric acid (GHB) is an endogenous neuroactive substance and proposed neurotransmitter with affinity for both low- and high-affinity binding sites. A radioligand with high and specific affinity toward the high-affinity GHB binding site would be a unique tool toward a more complete understanding of this population of binding sites. With its high specific affinity and monocarboxylate transporter (MCT1) mediated transport across the blood-brain barrier in pharmacological doses, 3-hydroxycyclopent-1-enecarboxylic acid (HOCPCA) seems like a suitable PET radiotracer candidate. Here, we report the ^{11}C-labeling and subsequent evaluation of [^{11}C]HOCPCA in a domestic pig, as a PET-radioligand for visualization of the high-affinity GHB binding sites in the live pig brain. To investigate the regional binding of HOCPCA in pig brain prior to in vivo PET studies, in vitro quantitative autoradiography on sections of pig brain was performed using [^{3}H]HOCPCA. In vivo evaluation of [^{11}C]HOCPCA showed no brain uptake, possibly due to a limited uptake of HOCPCA by the MCT1 transporter at tracer doses of [^{11}C]HOCPCA.



KEYWORDS: *GHB, HOCPCA, GHB binding site distribution, PET, in vitro autoradiography*

γ-Hydroxybutyric acid (GHB, Figure 1) is an endogenous neuroactive substance that is present in micromolar concentrations in the mammalian brain.[1] The primary source of GHB is believed to be metabolic derivation from the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Figure 1).[2] Additionally, GHB is a recreational drug (Fantasy or liquid ecstacy), but also a clinically prescribed drug for treatment of alcohol dependence (Alcover)[3] and narcolepsy (Xyrem, sodium oxybate).[4] In the central nervous system (CNS), GHB binds to specific high-affinity binding sites that are abundantly expressed and conserved through evolution.[5] The functional role of these has been a matter of thorough investigation for several years.[5] Although many of the observed in vivo pharmacological effects of GHB are mediated by the metabotropic $GABA_B$ receptors, the high-affinity GHB binding sites are preserved in brains of $GABA_{B(1)}$ knockout mice, providing evidence that the high-affinity GHB binding sites are molecularly distinct from the $GABA_B$ receptor complex.[6] Efforts to uncover their molecular identity are of high interest and have so far been largely driven by the advent of nanomolar affinity and highly selective compounds and radioligands.[7−10] Recently, the $\alpha_4\beta_{1-3}\delta$ ionotropic $GABA_A$ receptors have been identified as potential high-affinity GHB targets in vitro,[11] which however remains to be validated by in vivo studies.[12] α_4 knockout mice have ∼40% high-affinity GHB binding sites of wild-type mice, which leave the identity of the remaining 60% of the high-affinity binding sites elusive.[5,11] So far, all these binding studies have exclusively been performed in vitro.

Positron emission tomography (PET) is a noninvasive technology and has been highly useful to quantify neuroreceptor binding in vivo.[13] In order to attain a more complete understanding of the identity and physiological relevance of high-affinity GHB binding sites in the CNS, the availability of a suitable PET radiotracer for visualization of these sites would be of significant value.

We have previously described the conformationally restricted GHB analogue 3-hydroxycyclopent-1-enecarboxylic acid (HOCPCA, **1**, Figure 1) as a highly selective ligand for the high-affinity GHB binding site (K_i 16 μM)[10] (27 times higher affinity than GHB itself), and, more importantly, with no

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affinity for the $GABA_B$ receptor and 45 other neurotargets.[14] Additionally, it has recently been shown that **1** penetrates into the brain by means of the monocarboxylate transporter MCT1.[14,15] Given the close structural relationship to GHB, its selectivity and in vivo brain penetration, **1** is a highly attractive compound to investigate GHB pharmacology mediated by high-affinity binding sites. Recently, we reported the successful tritium labeling of **1** in high radioactive yield.[14] 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 (Figure 1), which makes [^{3}H]**1** a promising radio-pharmacological tool to study exclusively the high-affinity GHB binding sites.[14] Additionally, we reported a new preparative synthetic route to **1** that can be scaled up with a high and reproducible overall yield.[14] The synthetic route can be developed further for isotopic carbon labeling.

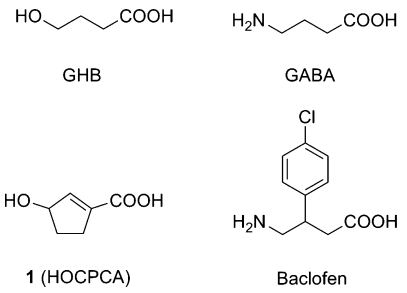


Figure 1. Chemical structures of the two neuroactive substances GHB and GABA. HOCPCA (**1**) binds selectively to the high-affinity GHB binding site. Baclofen is a selective $GABA_B$ receptor agonist.

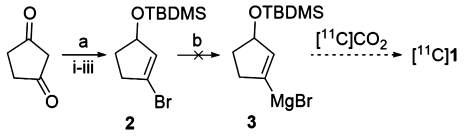
Here, we report the ^{11}C-labeling of **1** using Cu-mediated [^{11}C]carbon dioxide fixation, examine [^{3}H]**1** in in vitro autoradiography, and subsequently evaluate [^{11}C]**1** as a potential PET radioligand in a domestic pig.

RESULTS AND DISCUSSION

A high yielding and reproducible synthesis of **1** has recently been reported by Vogensen et al.,[14] where the carboxylic group was incorporated by halogen-metal exchange followed by CO_2(g). This synthetic strategy seems applicable for late-stage isotopic carbon labeling of **1**, since [^{11}C]CO_2(g) can be employed. Carboxylation of Grignard or organolithium reagents with [^{11}C]CO_2(g) represents a direct route to ^{11}C-labeled carboxylic acids. However, applications of this approach have several caveats due to the high reactivity of the organometallic reagents. Rigorous exclusion of atmospheric moisture and CO_2 during storage and manipulations is necessary to obtain radiolabeled products with consistently high radiochemical yields and specific activities.[16] Initially, the more stable Grignard reagent was pursued rather than the lithium species using the previously reported strategy.[14] Bromination of 1,3-cyclopentanedione followed by Luche reduction provided the alcohol, which was immediately protected as the *tert*-butyldimethylsilyl ether (TBDMS) to give **2** (Scheme 1).

Compound **2** was treated with several commercial Grignard reagents. No halogen−metal exchange was observed in any of the cases. Neither higher temperature, change in solvent, nor prolongation of the reaction time afforded any halogen−metal exchange either. The addition of lithium chloride to Grignard reagents has been reported to increase halogen−metal exchange rate.[17] However, based on the total absence of halogen-metal exchange we decided to try out other strategies.

Scheme 1. Synthesis of [^{11}C]1 Using an Organometallic Approach[a]

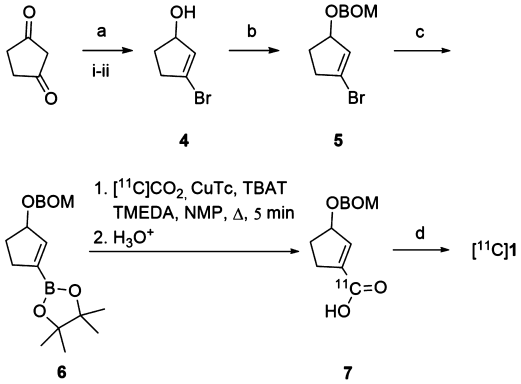


[a]Reagents and conditions: (a) (i) Br_2PPh_3, benzene, Et_3N, rt, 18 h, (ii) $NaBH_4$, $CeCl_3$, MeOH, 0 °C to rt, 2.5 h, (iii) TBDMS-Cl, DMAP, imidazole, CH_2Cl_2, rt, 16 h; (b) *i*PrMgCl, *i*PrMgBr, or EtMgBr, THF, Et_2O, 1,4-dioxane or HMPA, 0 °C to 50 °C, 2−24 h.

Recently, a method for Cu-mediated [^{11}C]CO_2 fixation for the [^{11}C]carboxylation of boronic acid esters was reported.[16,18] Compared to organolithium and Grignard reagents, boronic acid esters are in general less sensitive to air and moisture and therefore attractive as precursors for ^{11}C-carboxylation.[18] In order to use this approach, the pinacol boronic ester **6** was synthesized as outlined in Scheme 2.

Similar to **2**, 1,3-cyclopentanedione was brominated followed by a Luche reduction. Because of the short half-life ($t_{1/2}$ = 20.4

Scheme 2. Synthesis of [^{11}C]1 and Optimization of the Reaction Conditions for the Carbonylation of 6[a]



Entry[b]	CuTc [equiv]	TBAT [equiv]	T [°C]	6 [M]	RCY [%][c]
1[d]	0.17	0.17	100	0.1	9
2[d]	0.17	0.17	120	0.1	4
3	0.34	0.34	100	0.1	5
4	0.17	0.17	80	0.1	7
5	0.17	0.17	80	0.2	13
6	0.34	0.34	100	0.2	28

[a](a) Reagents and conditions: (a) (i) Br_2PPh_3, benzene, Et_3N, rt, 18 h, (ii) $NaBH_4$, $CeCl_3$, MeOH, 0 °C to rt, 2.5 h; (b) BOM-Cl, DIPEA, CH_2Cl_2, rt, 16 h; (c) *t*-BuLi, THF, −78 to −50 °C, 2 h, − 78 °C, isopropyl pinacol boronate, 1 h; (d) conc. HCl/EtOH 1:1, rt, 3 min. (b) Unless otherwise noted, [^{11}C]CO_2 was trapped in the reaction mixture at −10 °C. (c) Determined by radioHPLC integration of peaks from product, byproducts, and unreacted [^{11}C]CO_2 complex. (d) [^{11}C]CO_2 was collected in the reaction mixture at rt. Abbreviations: RCY, radiochemical yield; CuTc, copper(I)-thiophene-2-carboxylate; TBAT, tetrabutylammonium difluorotriphenylsilicate; TMEDA, tetramethylethylenediamine; NMP, *N*-methyl-2-pyrrolidone; M, molar; equiv, equivalent.

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min) of carbon-11, an easily removable protecting group for the alcohol moiety was needed. Benzyloxymethyl acetal (BOM), which readily cleaves under acidic conditions,[19] was considered as a suitable protection group for this purpose. Halogen-metal exchange using *tert*-BuLi followed by addition of isopropyl pinacol boronate provided **6**.

The synthesis of [^{11}C]**1** was initially attempted using the conditions reported by Riss et al.[18] Cyclotron generated [^{11}C]CO_2 was bubbled into a reaction vial containing the pinacol boronic ester precursor **6**, *N*,*N*-dimethylformamide (DMF), copper(I) iodide (CuI), potassium fluoride (KF), 4,7,13,16,21,24-hexaoxa-1,10-diazobicyclo[8.8.8]hexacosane (Crypt-222), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA). Unfortunately, all attempts to produce the desired [^{11}C]**1** using this approach were unsuccessful, and only unlabeled **6** and unreacted [^{11}C]CO_2 were observed.

Second, we attempted ^{11}C-labeling of **1** using the conditions reported by Rotstein et al.[16] (Scheme 2, entry 1). By using the more soluble copper(I) thiophene-2-carboxylate (CuTC) and replacing the KF/Crypt-222 with the bench-stable tetrabutylammonium difluorotriphenylsilicate (TBAT), formation of the radiolabeled carboxylic acid **7** was observed. Furthermore, to improve the catalyst and precursor solubility, the solvent was changed from DMF to *N*-methylpyrrolidinone (NMP).

However, radiochemical yield remained suboptimal (Scheme 2, entry 1), so we turned to alternative conditions to improve the ^{11}C-labeling of **6**. Higher temperature (Scheme 2, entry 2) was unable to afford greater yield as the release of [^{11}C]CO_2 presumably increased. Lowering the temperature (Scheme 2, entry 4) only markedly lowered the yield. Significant improvements were realized by increasing the concentration of the precursor (Scheme 2, entry 5) in the reaction mixture from 0.1 to 0.2 M, which led to a moderate conversion to the labeled product **7**. Furthermore, an increased precursor concentration combined with an increase in the addition of catalyst (CuTC) and additive (TBAT) (Scheme 2, entry 6) provided an analytical radiochemical yield of 28% determined by radioHPLC.

Based on the optimized conditions, **7** was synthesized and isolated. Briefly, after bubbling [^{11}C]CO_2 into the reaction mixture, the vial was sealed and the mixture was heated to 100 °C for 5 min. The reaction was quenched with 0.1% aqueous phosphoric acid, and radiolabeled **7** was isolated by semi-preparative HPLC followed by trapping on a solid-phase light C-18 Sep-Pak extraction column. Radiolabeled **7** was eluted from the C-18 Sep-Pak using EtOH, which afforded pure **7**.

Preliminary experiments revealed that diluted aqueous solutions of TFA or HCl were sufficiently effective for the removal of the BOM-protecting group. However, under radioactive conditions, TFA appeared insufficient for the deprotection even with the use of concentrated TFA at 80 °C. The use of HCl proved to be more desirable as fast conversion of **7** was observed. Treating **7** with concentrated HCl (12.4 M) at 80 °C for 3 min provided a 4:1 mixture of **7** and byproduct, presumably the eliminated product. Using a 3 M solution of HCl markedly increased the formation of [^{11}C]**1**, and by lowering the temperature to 60 °C, the formation of byproduct was slightly lowered. However, we concluded that the optimal conditions for cleavage of the BOM-protection group were concentrated HCl at room temperature for 3 min at atmospheric pressure which provided a 9:1:0 ratio of [^{11}C]**1**, byproduct and **7**, respectively.

Due to the high polarity of **1**, purification of [^{11}C]**1** was troublesome. Solid phase extraction with neither an ion-exchange light Sep-Pak nor a normal phase Sep-Pak could retain the byproduct in sufficient amount. Only by using two solid-phase light C-18 Sep-Pak extraction column in a series, purification of [^{11}C]**1** was achieved but half of [^{11}C]**1** was lost. The pH was adjusted to 7 with 5 M NaOH. Average specific activity was around 1.5 GBq/μmol (range 1−2 GBq/μmol) (n = 2) with a radiochemical purity above 97%. Typically, an amount of 831−1012 MBq could be isolated by using a cyclotron beam time of 40 min.

To investigate the regional binding of **1** in different brain regions in the pig prior to in vivo PET studies, in vitro autoradiography was performed using [^{3}H]**1** on pig brain sections at both pH 6.0 and pH 7.4 (Table 1 and Figure 2).

Table 1. Quantifications of Specific Binding (fmol/mg) of [^{3}H]1 (4.5 nM) As Determined from Autoradiography in the Pig Brain at pH 6.0 and 7.4

region	pH 6.0	pH 7.4
cortical regions		
prefrontal cortex	165.1	44.94
frontal cortex	224.3	50.11
temporal cortex	217.7	59.44
cingulate cortex	176.2	28.32
occipital cortex	191.5	
striatum	99.6	22.52
lateral septal nucleus	322.7	
thalamus	54.27	12.47
central amygdala	128.6	42.95
hippocampal regions		
CA1	284.9	53.61
CA2/3	233.4	32.72
dentate gyrus	254.8	42.17
parahippocampal gyrus	318.5	52.05
subiculum	383.9	70.06
cerebellum	14.3	6.89

As shown in Figure 2, a high degree of specific binding was obtained with [^{3}H]**1**, which permitted elucidation of binding levels in various brain regions. From this experiment, the distribution of [^{3}H]**1** binding sites in pig brain in vitro indicates that the frontal cortex, septal nucleus, and hippocampus contain a high density of high-affinity GHB binding sites. The thalamic regions, amygdala, and caudate putamen have intermediate levels of binding sites (Table 1 and Figure 2), whereas cerebellum and hypothalamic regions display low levels of binding sites. The regional distribution pattern is very similar to that reported for rodents, using selective high-affinity radioligands [^{3}H]NCS-382 or [^{125}I]BnOPh-GHB as well as for [^{3}H]**1** itself.[7,11,20,21] Thus, the distribution of high-affinity GHB binding in vitro proves to be highly conserved across species. Interestingly, the absolute binding of [^{3}H]**1** to pig brain was 4−5 times higher at pH 6.0 compared with pH 7.4. The binding of GHB to the high-affinity GHB binding sites has been reported to be pH-dependent, with an optimum around pH 6.0.[22] However, both the physiological role of the binding optimum at pH 6.0 and the molecular explanation for this is yet unknown. It could be either a consequence of local changes in the binding pocket or allosteric changes in the protein due to protonation of specific amino acid residues.

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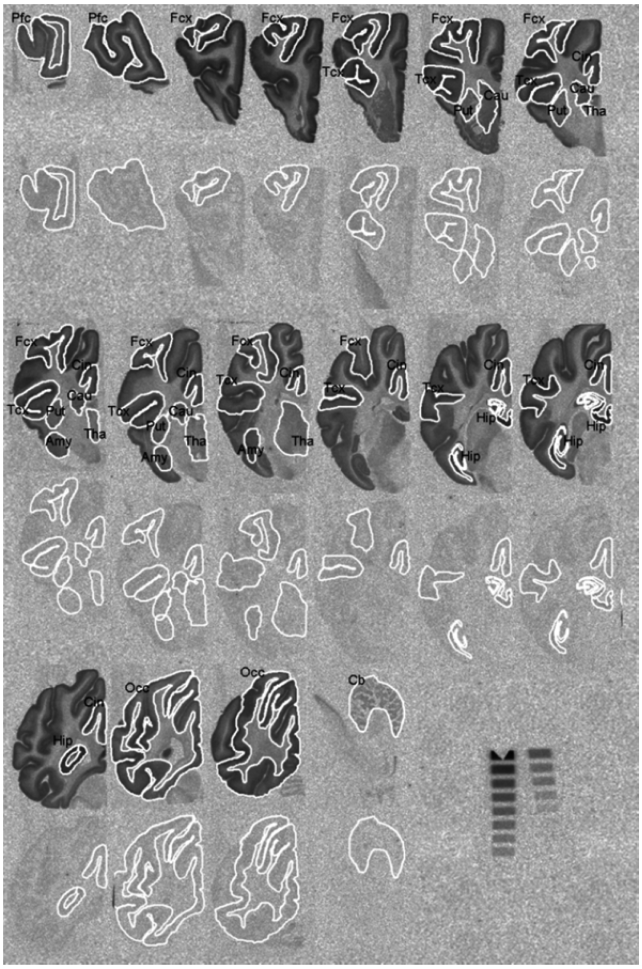

Figure 2. Representative autoradiograms of [^{3}H]**1** (4.5 nM) binding to pig brain sections (20 μm) at pH 6.0 (top). Nonspecific binding was established in the presence of 1 mM GHB (bottom). Coronal sections showing: prefrontal cortex (Pfx), frontal cortex (Fcx), temporal cortex (Tcx), occipital cortex (Occ), cingulate cortex (Cin), putamen (Put), caudate (Cau), thalamus (Tha), amygdala (Amy), and hippocampus (Hip); and sagittal section of cerebellum (Cb) as guided by the stereotaxic atlas of Félix et al.[23]

Using the chemistry delineated in Scheme 2, [^{11}C]**1** was prepared for evaluation in in vivo PET imaging studies in a domestic pig using a high resolution research tomography (HRRT) PET scanner. The summed PET images (Figure 3B) and time−activity curves (Figure 3A) showed that the radioligand [^{11}C]**1** did not enter the pig brain. A tendency to slow rising activity curves can be seen in Figure 3A, which could indicate radiometabolites. However, no information on radiotracer metabolism is available in this specific case.

The lack of detectable brain penetrance of [^{11}C]**1** in the live pig was at first sight rather surprising given the known brain penetrance of **1** into the mouse brain at an oral dose of 10 mg/kg.[14] The first obstacle in terms of getting brain exposure is the blood-brain barrier (BBB). Being 99% charged at physiological pH, little passive diffusion of **1** is expected. This is supported by studies using MDCK-MDR1 cells showing low passive permeability toward **1**.[14] Indeed, it has been reported that both GHB and **1** enter the brain via active transport by MCT1 at the BBB, at least in rodents.[15,24] In the same study, **1** showed a rapid absorption, a B/P ratio of 0.4 and a first order elimination profile with a half-life of approximately 20 min for

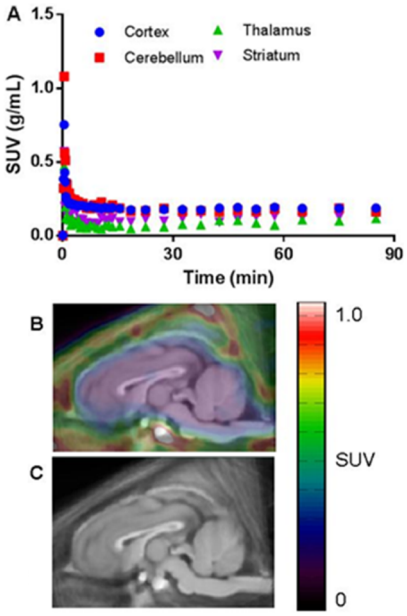

Figure 3. (A) Time−activity curves for [^{11}C]**1** in the indicated regions of the pig brain. (B) Summed PET image (0−90 min, sagittal view) of the pig brain. (C) Corresponding MR-image of the pig brain. SUV: Standardized uptake value (g/mL).

both brain and plasma.[15] Given the conserved role for MCT1 in transporting L-lactate in pigs and the localization at the BBB, it is anticipated that **1** is also actively transported in pig cells in a similar fashion. Under this assumption, a primary concern is the K_m of **1** for MCT1 (16.3 mM at pH 7.4 at recombinant MCT1 expressed in *Xenopus* oocytes).[15] Carrier-mediated transport across the BBB follows Michaelis−Menten kinetics plus a nonsaturable component that increases linearly with concentration. Assuming Michaelis−Menten kinetics alone and an estimated plasma concentration of [^{11}C]**1** of 240 nM, it means that only a relative transporter velocity of 0.002% was reached in the current experiment. Thus, the most obvious approach to try to obtain better brain penetrance would be to increase transporter activity. This can be attained either by increasing the dosage of [^{11}C]**1** or by decreasing the extracellular pH (K_m for **1** at MCT1 decreases at more acidic pH values).[15] Increasing the dosage of [^{11}C]**1** is not an option since that would violate tracer kinetics. Acidification is routinely done in vitro, but has also been attempted in vivo by inducing hypercapnia in humans to study the pH dependency of lactate BBB permeability. Here, results showed increased permeability, though this was not significant.[25] Despite active transport into the brain, the total brain exposure of **1** could be adversely affected by transporter-mediated efflux. Various transporters have been found on the BBB that acts as efflux transporters, but no reports have yet addressed this issue for **1**. If transporter-mediated efflux of **1** is involved, inhibition of the relevant efflux transporter could be an alternative approach to enhance brain exposure. Indeed, this approach has been studied for diclofenac and mefenamic acid, where brain penetration seems to be enhanced by inhibition of the organic anion efflux transporter 3.[26] Here, the potential efflux of **1** mediated by organic acid transport carriers was investigated in mice by coadministering **1** with increasing doses of the inhibitor probenecid. As shown in Figure 4, the extent of brain penetration of **1** was not influenced by this inhibitor. Thus, if this translates to pigs, this indicates

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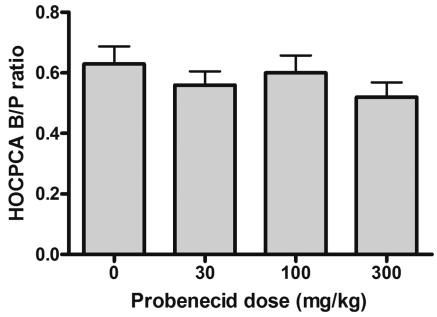

Figure 4. Brain-to-plasma distribution of HOCPCA in the presence of probenecid measured in vivo.

that the brain efflux does not involve a probenecid sensitive transport mechanism.

Structural optimization of **1** could be another option aiming for enhanced transport via MCT1 or, by use of the prodrug concept, targeting alternative transporters, e.g., a peptide transporter. Alternatively, passive diffusion of **1** across the BBB could be attempted by physicochemical optimization.

In summary, using a method for Cu-mediated [^{11}C]CO_2 fixation, we have developed an efficient approach for the synthesis of [^{11}C]**1**. Although [^{3}H]**1** is a good radioligand for in vitro studies, [^{11}C]**1** appears to be a less promising candidate for in vivo imaging of the high-affinity GHB binding sites. However, the labeling procedure illustrates an efficient approach for radiolabeling GHB-type ligands and will be used in further studies aiming for enhanced brain penetrance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00335.

Full experimental details for organic and radiochemical procedures, PET imaging study, and in vitro autoradiography (PDF)

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Author Contributions

C.H.J. performed the synthetic chemistry, C.H.L., S.L., and M.M.H. performed the radiochemistry, H.D.H. performed the autoradiography and imaging study, and C.B. performed the efflux study. The manuscript was written through contributions of all the authors who have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BBB, blood-brain barrier; CNS, central nervous system; GABA, γ-aminobutyric acid; GHB, γ-hydroxybutyric acid; HOCPCA, 3-hydroxycyclopent-1-enecarboxylic acid; MCT1, monocarboxylate transporter 1; PET, positron emission tomography; TLC, thin layer chromatography

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