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Brief Article

Design, Synthesis, and in Vitro Pharmacology of New Radiolabeled γ-Hydroxybutyric Acid Analogues Including Photolabile Analogues with Irreversible Binding to the High-Affinity γ-Hydroxybutyric Acid Binding Sites

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γ-Hydroxybutyric acid (GHB) is a psychotropic compound endogenous to the brain. Despite its potential physiological significance, the complete molecular mechanisms of action remain unexplained. To facilitate the isolation and identification of the high-affinity GHB binding site, we herein report the design and synthesis of the first ^{125}I-labeled radioligands in the field, one of which contains a photoaffinity label which enables it to bind irreversibly to the high-affinity GHB binding sites.

Introduction

γ-Hydroxybutyric acid (GHB[a]) was originally synthesized as a γ-aminobutyric acid (GABA) analogue able to readily cross the blood−brain barrier.[1] Subsequently, GHB was found to occur naturally in the mammalian brain[2] as a metabolite and, under certain conditions, as a precursor of GABA.[3] During the years, GHB has been used for several different scopes. GHB is a registered drug for the treatment of catalepsy and excessive daytime sleepiness associated with narcolepsy (Xyrem)[4] and for ameliorating alcohol withdrawal and reducing alcohol craving in recovered alcoholics (Alcover).[5] On the other hand, GHB is a widely popular drug of abuse because of the euphoria, disinhibition, and heightened sexual awareness associated with its use.[6]

To date, the exact mechanism of action of GHB in the mammalian central nervous system (CNS) is only partially understood. When taken exogenously in high doses, reaching millimolar concentrations in the CNS, many key effects of GHB are mediated by the subtype B of GABA ($GABA_B$) receptors[7] where GHB is a low-affinity weak partial agonist.[8] Apart from the low-affinity binding to $GABA_B$ receptors, GHB also binds to a unique population of high-affinity binding sites in the CNS[9] that are defined by a distinct ontogenesis[10] and distribution[11] and hence suggested to represent a putative GHB receptor. In further support of this view, brains from $GABA_B$ receptor knockout mice have been shown to display intact [^{3}H]GHB binding.[12] Whereas exogenous GHB intake predominantly leads to $GABA_B$ receptor effects, the specific high-affinity site appears critical for the actions of GHB as a neuromodulator and therapeutic drug where concentrations of GHB in the CNS are in the lower micromolar range.[7,13]

Despite substantial recent research and reports of two different GHB receptors in rat[14] and human,[15] the identity of the high-affinity site is debatable[6,16,17] and has not been convincingly linked to a specific protein. Uncovering the identity of the high-affinity GHB binding site would be a major breakthrough in terms of our understanding of the therapeutic and recreational use of GHB and its action as an endogenous signaling molecule.

In continuation of previous work[17,18] and to obtain better pharmacological tools for addressing the neurobiology and pharmacology of GHB, we labeled a high-affinity GHB ligand with a ^{125}I isotope to obtain a higher specific radioactivity than can be achieved with conventional ^{3}H-labeling.[19] Photolabeling is a method that has been widely used to study the interaction of biologically relevant compounds with their target macromolecules.[20,21] An appropriate photoaffinity probe is usually prepared by attachment of a photoreactive group (arylazides, aryldiazirines, or benzophenones) to the ligand molecule. UV irradiation triggers the photoactivating group in the ligand and leads to the conversion of the noncovalent binding into a covalent link between the ligand and amino acidic residues present in the binding pocket in an unspecific fashion.[22]

We here report the development of novel GHB analogues containing a photoreactive azide group, one of which has been labeled with the ^{125}I isotope to generate the first reported GHB photoaffinity radioligand with preference for the GHB high-affinity binding site.

Results and Discussion

We recently reported the synthesis and binding characteristics of a series of high-affinity and selective lipophilic

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[a] Abbreviations: CNS, central nervous system; DMF, dimethylformamide; GABA, γ-aminobutyric acid; GHB, γ-hydroxybutyric acid; HPLC, high performance liquid chromatography; NCS-382, (*E*,*RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid.

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Chart 1. Structures of Biarylic Selective GHB Ligands

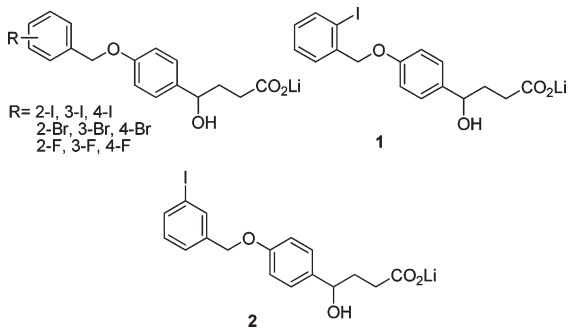
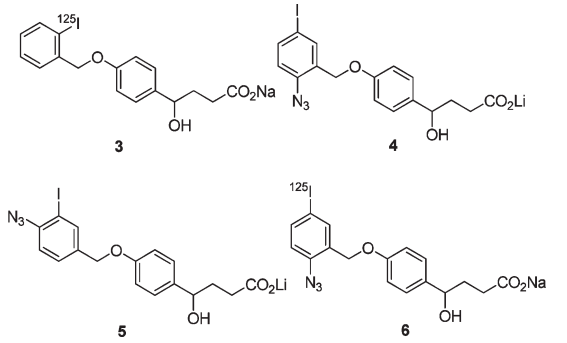


Chart 2. Novel Synthesized Photoaffinity-Labeled and Radiolabelled GHB Analogues



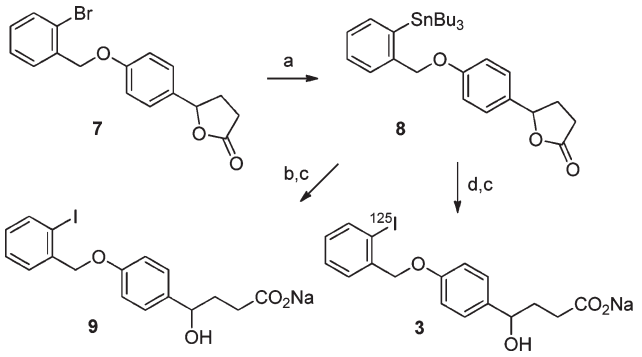
4-diarylic derivatives of GHB (Chart 1) notably devoid of affinity for $GABA_A$ and $GABA_B$ receptors.[18]

For the purpose of developing labeled GHB analogues, we considered the possibility of substituting functions on the terminal aromatic ring of these biarylic GHB ligands. In particular (*R*,*S*)-4-hydroxy-4-[4-(2-iodobenzyloxy)phenyl]-butanoate (**1**) and (*R*,*S*)-4-hydroxy-4-[4-(3-iodobenzyloxy)-phenyl]butanoate (**2**) (Chart 1) seemed like excellent templates because of their high affinity binding in our [^{3}H]NCS-382 binding assay (K_i of 60 and 75 nM, respectively)[18] and their already incorporated, well-tolerated iodine group.

Starting from these structures, we developed the ^{125}I-radiolabeled compound [^{125}I]4-hydroxy-4-[4-(2-iodobenzyloxy)-phenyl]butanoate (**3**) to introduce an azido group and the strategies to generate the photoaffinity radioligand [^{125}I]4-hydroxy-4-[4-(2-azido-5-iodobenzyloxy)phenyl]butanoate (**6**) (Chart 2). Herein we will describe in detail the chemistry leading to **3**, **4**, **5**, and **6**, details on the radiochemistry to reach **3** and **6**, and the initial in vitro pharmacological characterization of the ligands. The detailed pharmacological analysis of **3** and further characterization of **6** will be published elsewhere.

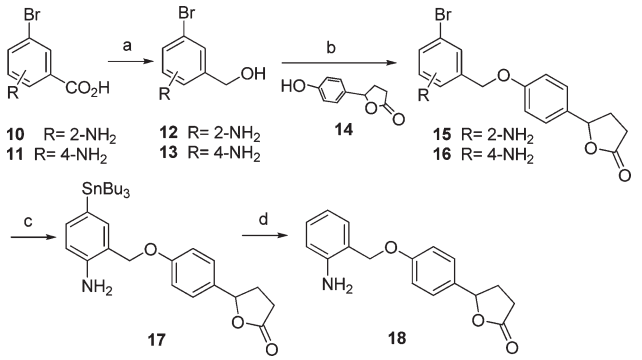
Chemistry. The synthesis of the stannyl precursor **8** for the radioligand **3** was performed as depicted in Scheme 1. 5-(4-(2-Bromobenzyloxy)phenyl)dihydrofuran-2(3*H*)-one (**7**) was prepared by Mitsunobu reaction between 2-bromobenzyl alcohol and 5-(4-hydroxyphenyl)tetrahydrofuran-2-one as previously reported.[18] The obtained aryl bromide **7** was then treated with hexabutyldistannane in the presence of tetrakis(triphenylphosphine)Pd(0) in refluxing toluene to furnish the stannylated derivative **8**. To test the efficiency of the iodination reaction, **8** was initially converted into the cold ligand **9** by reaction with NaI under oxidative conditions with chloramine-T in a mixture of EtOAc, DMF, AcOH, and H_2O, followed by hydrolysis of the lactone with NaOH. As this strategy proved functional, the radioligand **3** was generated in a similar fashion. Thus, radioiodination was carried out by treatment with Na^{125}I using the chloramine-T method[23] followed by hydrolysis with aqueous NaOH without isolation of the intermediate protected lactone. The radioligand **3** was purified by HPLC and analyzed to have a radiochemical purity of 97% and a total activity of 2.48 mCi (83% based on [^{125}I]NaI).

Scheme 1[a]



[a] Reagents and conditions: (a) $Pd(PPh_3)_4$, $(SnBu_3)_2$, toluene, reflux, 22 h; (b) NaI, chloramine-T, 94% EtOAc, 4% DMF, 1% AcOH, 1% H_2O, room temp, 30 min, $Na_2S_3O_5$; (c) NaOH, 1 h; (d) 3 mCi Na^{125}I and chloramine-T in a mixture of 94% EtOAc, 4% DMF, 1% AcOH, and 1% H_2O, room temp, 30 min, $Na_2S_3O_5$.

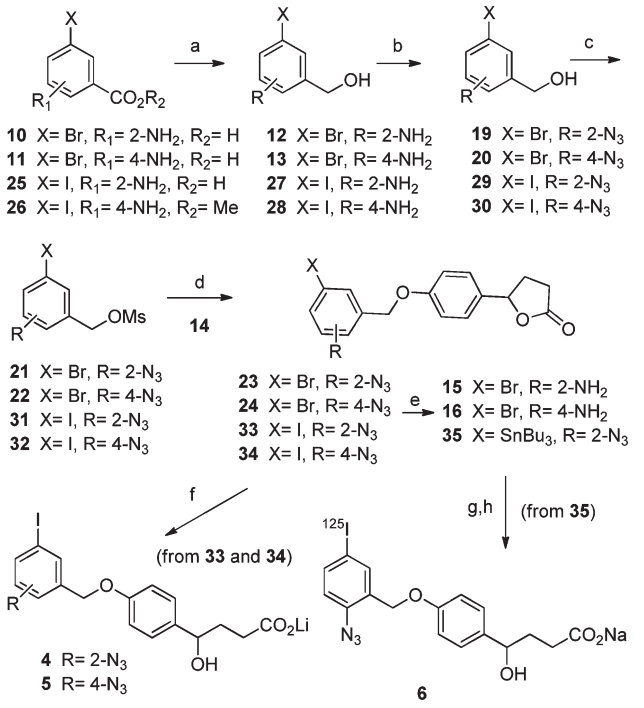
Scheme 2[a]



[a] Reagents and conditions: (a) $LiAlH_4$, Et_2O, room temp, 2 h; (b) PPh_3, DEAD, THF, reflux, 18 h; (c) $(SnBu_3)_2$, tetrakis(PPh_3)Pd(0), toluene, reflux, 14 h; (d) $NaNO_2$, NaN_3, CF_3CO_2H, 0 °C, 10 min.

Several routes were investigated in order to obtain the photoaffinity-labeled derivatives **4** and **5** (Chart 2). Initially we applied a synthetic strategy similar to the one reported for the other substituted biarylic GHB analogues,[18] as outlined in Scheme 2. Commercially available 2-amino-5-bromobenzoic acid **10** and 4-amino-3-bromobenzoic acid **11** were first reduced with $LiAlH_4$ in 87% and 91% yield, respectively, to give the corresponding benzyl alcohols **12** and **13**. These were refluxed overnight with 5-(4-hydroxyphenyl)tetrahydrofuran-2-one **14**[18] in the presence of triphenylphosphine and diethylazodicarboxylate to furnish the Mitsunobu products **15** and **16** in 36% and 32% yield, respectively, upon purification. The following stannylation reaction proceeded very slowly, and while **17** could be isolated only in 23% yield, no stannylated product was obtained from **16**. Compound **17** was then treated with $NaNO_2$ and NaN_3 in trifluoroacetic acid with the aim of converting the arylamine moiety into an arylazido group. Unfortunately, no azide formation was detected and the acidic condition used for the Sandmeyer reaction led to loss of the stannyl group, to furnish the unstannylated derivative **18**.

Scheme 3[a]



[a] Reagents and conditions: (a) $LiAlH_4$, THF, room temp, 4 h; (b) $NaNO_2$, NaN_3, CF_3CO_2H, 0 °C, 10 min; (c) MsCl, Et_3N, DMAP, CH_2Cl_2, 0 °C, 1 h, room temp, 4 h; (d) 5-(4-hydroxyphenyl)tetrahydrofuran-2-one (**14**), K_2CO_3, DMF, 70 °C, 14 h; (e) $(SnBu_3)_2$, tetrakis-$(PPh_3)Pd(0)$, toluene, reflux, 14 h; (f) LiOH, THF, H_2O, room temp, 24 h; (g) 3 mCi$Na^{125}I$ and chloramine-T in a mixture of 94% EtOAc, 4% DMF, 1% AcOH, and 1% H_2O, room temp, 30 min, $Na_2S_3O_5$; (h) NaOH, 1 h.

Consequently, we decided to introduce the azido moiety before the stannylation step in an alternative synthetic strategy as shown in Scheme 3. With this strategy, 2-amino-5-bromobenzyl alcohol **12** and 4-amino-3-bromobenzyl alcohol **13** were first transformed into the corresponding arylazide derivatives **19** and **20** in 88% and 82% yield, respectively, by reaction with $NaNO_2$ and NaN_3 in trifluoroacetic acid at 0 °C. The benzyl alcohol was then transformed into the mesyl derivatives **21** and **22** by treatment with mesyl chloride and triethylamine in 90% and 92% yield, respectively. The following reaction between **21** and **22** with the tetrahydrofuran-2-one **14**[18] in DMF in the presence of 1.5 equiv of K_2CO_3 gave the desired derivatives **23** and **24** in 70% and 77% yield, respectively. Unfortunately, the following stannylation reaction did not work as expected. No transformation was observed when the reaction was performed under mild conditions (bis-triphenylphosphinopalladium-(II) chloride, dioxane, 50 °C), while stronger conditions (bis-triphenylphosphinopalladium(II) chloride, dioxane, reflux or $(SnBu_3)_2$, tetrakis(triphenylphosphine)Pd(0), toluene, reflux) led to the degradation of the azido group to furnish **15** and **16**. Since it was not possible to transform the aryl bromides **23** and **24** into the corresponding tributyltin derivatives without the reduction of the azido group, we decided to replace the bromine atom with a more reactive iodine atom that has been reported to allow transformation into the corresponding arylstannane derivative in the presence of an azido group, in ortho and in para positions.[24]

Consequently, a similar synthetic strategy was used starting from 2-amino-5-iodobenzoic acid **25** and methyl 4-amino-3-iodobenzoate **26** (Scheme 3). After reduction with $LiAlH_4$, the resulting alcohols **27** and **28** were treated with $NaNO_2$ and NaN_3 in trifluoroacetic acid to obtain the desired arylazides **29** and **30** in 28% and 60% yield. The transformation of the alcohols **29** and **30** into a mesyl group gave **31** and **32** in 90% and 60% yield respectively. The nucleophilic reaction between the tetrahydrofuran-2-one **14**[18] and the mesyl derivatives **31** and **32**, respectively, furnished the key intermediates **33** and **34** in 50% and 88% yield. Treatment with 1.1 equiv of LiOH led to the photoaffinity-labeled GHB analogues **4** and **5** in 41% and 25% yield, respectively. Starting from **33**, a stannylation reaction was performed ($(SnBu_3)_2$, tetrakis(triphenylphosphine)Pd(0), toluene, reflux, overnight) to obtain the desired stannyl derivative **35** as a pure pale-yellow oil in 34% yield. The tributylstannyl derivative **35** was then used as precursor for the radioiodination step. Treatment with [^{125}I]NaI using the chloramine-T method[23] followed by hydrolysis with aqueous NaOH furnished the photoaffinity-labeled radioligand **6**. Compound **6** was purified by HPLC and had a radiochemical purity of 96% and a total activity of 1.66 mCi (83% based on [^{125}I]NaI).

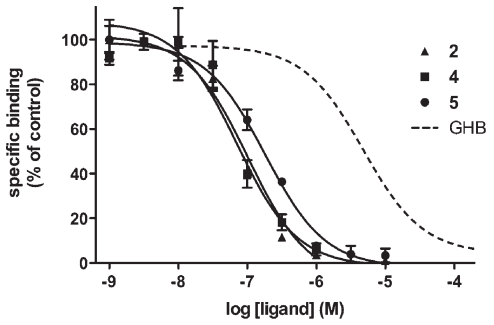


Figure 1. Concentration-dependent inhibition of [^{3}H]NCS-382 binding to rat cerebrocortical membranes by GHB and the GHB analogues **2**, **4**, and **5**. Results are expressed as the mean ± SD of a single representative experiment performed in triplicate. Average K_i values for GHB (4.3 μM) and **2** (75 nM) have been reported earlier.[17] Novel **4** and **5** gave average K_i of 120 and 186 nM, respectively (n = 2).

In Vitro Pharmacological Evaluation. To evaluate the affinity of our two novel photoaffinity-labeled GHB analogues **4** and **5** (Scheme 3) for the high-affinity GHB binding sites in mammalian brain tissue, we studied their ability to compete for binding with the commercially available GHB radioligand (*E*,*RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid, [^{3}H]NCS-382,[25] in our previously reported binding assay using rat brain cortical membranes.[16] As shown in Figure 1, **4** and **5** are able to inhibit [^{3}H]NCS-382 binding in a concentration-dependent manner. Both compounds bind with affinities several orders of magnitude higher than GHB itself and with K_i (120 nM for **4** and 186 nM for **5**) only slightly below that of the previously reported analogue **2** without the azido group (K_i = 60 nM),[18] thus rendering them both suitable GHB high-affinity ligands.

To develop a protocol for studying the photolinking properties of **4** and **5** indirectly, we initially found that whereas [^{3}H]NCS-382 could be dissociated from membranes labeled to equilibrium with cold GHB or NCS-382, compounds **4** and **5** were not able to induce dissociation of the radioligand in the same time frame. This excluded evaluation of the photoproperties against [^{3}H]NCS-382 within the same time period.

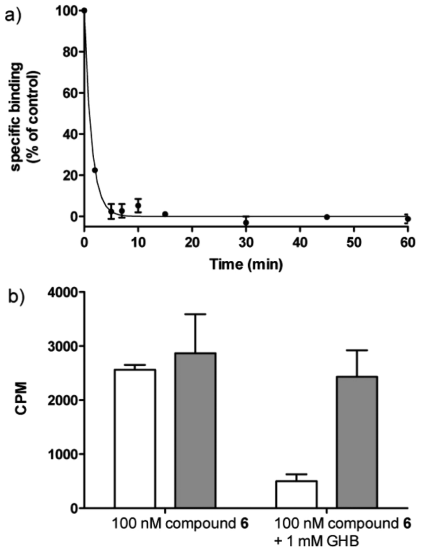


Figure 2. (a) Dissociation of 100 nM **6** from rat cortical membranes. After equilibrium binding, dissociation was time-dependently initiated by the addition of 1 mM GHB. Data shown are the mean ± SD of two independent experiments performed in triplicate. (b) Photoincorporation dissociation experiments showing the effect of UV irradiation on the ability of 1 mM GHB to initiate dissociation of 100 nM **6**. Samples (gray bars) were subjected to UV irradiation (302 nm, 25 W) for 1 min and compared to samples with no irradiation (white bars). Data shown are representative of two experiments carried out in duplicate.

Instead, for direct evaluation of their photolinking properties of our compounds, we synthesized the photoaffinity radioligand **6**, the ^{125}I-labeled analogue of **4** (Scheme 3). It was chosen to synthesize the labeled analogue of **4** based on the more feasible synthetic route of its stannyl precursor.

To set up the photoaffinity linking experiments using the photoaffinity radioligand **6**, we initially examined the binding kinetics of the radioligand without applying UV light. This served to determine the time needed to achieve binding and unbinding of the radioligand. Preliminary experiments using 100 nM radioligand indicated a fast association time of ~2 min and equilibrium binding after 10 min that was constant for at least 1.5 h (data not shown). Consequently, a 60 min incubation time was used for the assays. Dissociation was fast with a $t_{1/2}$ of ~1 min (Figure 2a). Thus, in photoincorporation experiments we used an initial 60 min preincubation time before UV radiation followed by a secondary 30 min incubation time to potentially allow for full dissociation of the radioligand.

Photoincorporation of **6** with the GHB binding protein was then tested utilizing the demonstrated ability of the radioligand to rapidly dissociate in the absence of photolinking. As shown in Figure 2b, the photoaffinity radioligand **6** covalently links to the high-affinity GHB binding sites, as pre-equilibration of membranes with **6**, irradiation by UV light, and subsequent addition of a high concentration of GHB did not lead to dissociation of the photolinker. By contrast, non-UV-irradiated membrane samples were fully displaced by GHB (Figure 2b). Taken together, this clearly demonstrated that our novel photoaffinity radioligand **6** is able to covalently link with the high-affinity GHB binding site upon UV irradiation. Characterization of the linked protein now awaits.

In conclusion, we have designed and developed two photoaffinity-labeled GHB analogues **4** and **5** and a ^{125}I-labeled GHB radioligand **6** with high affinity for the specific GHB binding sites. The compounds are the first reported ^{125}I-labeled analogues for the GHB binding site and will serve as useful tools for the photolinking and identification of the specific GHB high-affinity binding protein.

Experimental Section

Chemistry. The syntheses of selected compounds are described below. The general chemistry, experimental information, and syntheses of all other compounds are in Supporting Information. Purity of tested compounds was established using HPLC and is more than 95% pure.

Lithium 4-(4-(2-Azido-5-iodobenzyloxy)phenyl)-4-hydroxybutanoate (4). To a solution of **33** (0.05 g, 0.11 mmol) in THF (2 mL), a solution of LiOH (3 mg, 0.12 mmol) in H_2O (0.2 mL) was added all at once. The resulting orange solution was stirred at room temperature for 24 h. Then the solvent was removed in vacuo. The orange residue was recrystallized from EtOH/H_2O to furnish 21 mg (0.045 mmol, 41%) of **4** as a white solid. ^{1}H NMR (D_2O) δ: 1.53−1.62 (2H, m), 1.88−1.97 (2H, m), 4.17−4.19 (2H, m), 4.67 (2H, s), 6.33−6.40 (3H, m), 6.78−6.80 (2H, m), 7.14−7.16 (1H, m), 7.38 (1H, s).

Lithium 4-(4-(4-Azido-3-iodobenzyloxy)phenyl)-4-hydroxybutanoate (5). Compound **5** was prepared according to the same procedure as described for compound **4** starting from compound **34** in 25% yield. ^{1}H NMR (D_2O) δ: 1.66−1.75 (2H, m), 1.92−1.99 (2H, m), 4.27 (1H, bs), 4.44−4.50 (1H, m), 4.67 (2H, s), 6.54 (2H, d, J = 6.9 Hz), 6.67 (1H, d, J = 6.9 Hz), 6.90 (2H, d, J = 6.9 Hz), 6.99 (1H, d, J = 6.9 Hz), 7.44 (1H, s).

Radiochemistry. [^{125}I]4-Hydroxy-4-[4-(2-iodobenzyloxy)-phenyl]butanoic Acid (3). Radiolabeling was achieved without isolation of the intermediate protected lactone. Chloramine-T trihydrate (40.8 mg, 145 μmole) was suspended in a mixture of EtOAc (4.75 mL), DMF (0.25 mL), acetic acid (50 μL), and a small amount of water was added (50 μL) to dissolve the compound. This solution (1000 μL) was added to the stannyl precursor **8** (1.19 mg, 2.1 μmol) in a small HPLC vial. The vial was capped and shaken to dissolve the compounds. [^{125}I]NaI (30 μL, 3 mCi) was added to the vial which was quickly reclosed. The vial was shaken and the contents left to react for 30 min. Sodium metabisulfite (9.70 mg) in water (350 μL) was added to quench excess chloramine-T, and the reaction mixture was washed with purified water (3 × 300 μL). The organic layer was evaporated to dryness under an argon flow. Subsequently, the reaction mixture was dissolved in acetonitrile (500 μL) and NaOH (20 μL, 4 M), the latter to hydrolyze the lactone. The sample was shaken and left overnight at 5 °C. Then it was neutralized with HCl (80 μL, 1 M) and immediately subjected to preparative HPLC for purification. The desired product **3** was collected at 24.7 min and analyzed to have a radiochemical purity of 97% and a total activity of 2.48 mCi (83%). The product was diluted 5 times with ultrapurified water and loaded onto a preactivated C-18 Sep-PAK (160 mg). It was then washed with ultrapurified water (5 mL), eluted with EtOH (5 mL), and subsequently diluted to ~5 MBq/mL with a dilute NaOH solution (0.001 M).

[^{125}I]4-Hydroxy-4-[4-(2-azido-5-iodobenzyloxy)phenyl]butanoic Acid (6). Radiolabeling was achieved without isolation of the intermediate protected lactone as described for **3**. As the compound is light sensitive, all experiments were performed protected from light. The desired product **6** was collected at 26.7 min and analyzed to have a radiochemical purity of > 95% (Figure 2) and a total activity of 1.75 mCi (88% based on Na^{125}I). The product was diluted 5 times with ultrapurified water and loaded onto a preactivated C-18 Sep-PAK (160 mg). It was then washed with ultrapurified water (5 mL), eluted with EtOH (5 mL), and subsequently diluted to a concentration of ~5 MBq/ml in a NaOH solution (0.001 M).The use of slightly basic conditions in the final product matrix eliminated the potential lactone

formation. The total yield of formulated and final product was 1.66 mCi (83% based on [^{125}I]NaI) with a radioactive purity of 96% and a moderate specific activity of approximately 2 Ci/mmol. The moderate specific activity was due to traces of the cold iodide compound used in the preparation of the stannyl precursor.

Pharmacology. Rat brain (cerebral cortex) synaptic membrane preparation, [^{3}H]NCS-382 binding assay, and data analysis were carried out exactly as previously described.[16] Dissociation experiments for **6** were carried out using a modified [^{3}H]NCS-382 assay protocol. In brief, well-washed membranes (10 μg of protein per sample) were preincubated with 100 nM **6** in 50 mM phosphate buffer, pH 6, for 1 h at room temperature in a total volume of 2 mL. To initiate dissociation, 20 μL of GHB (final concentration of 1 mM) was added. At the indicated time points, the reactions were terminated by rapid filtration through Whatman GF/C filters (PerkinElmer Life and Analytical Sciences, Waltham, MA) using a Brandel M-48R cell harvester, followed by washing with 3 $\times$ 3 mL of ice-cold assay buffer. The amount of filter-bound radioactivity was quantified by adding 3 mL of scintillation fluid (Opti-Fluor, PerkinElmer) to the dried filters and counting in a Packard Tricarb 2100 liquid scintillation counter. Data were fitted using the model for exponential decay in the program Graphpad Prism 5.0, yielding the $t_{1/2}$ as the time for half of the radioligand to dissociate. Total bound radioligand accounted for less than 10% of the total amount of radioactivity in the reaction.

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Supporting Information Available: Zipped file containing details for the synthesis of intermediates **8** and **12**−**35**, radiolabeling and photoincorporation, HPLC chromatograms, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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