# *Letters*

## Activity-Based Probe for Specific Photoaffinity Labeling γ-Aminobutyric Acid B ($GABA_B$) Receptors on Living Cells: Design, Synthesis, and Biological Evaluation

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**Abstract:** A trimodular activity-based probe was designed, synthesized, characterized, and applied to photoaffinity label the $GABA_B$ receptors transiently expressed in Chinese hamster ovary (CHO) cells. The probe exhibits specific binding activity at the ligand-binding pocket of GB1 subunits and high specificity of photoaffinity labeling, which makes the probe valuable for studying the localization and function of $GABA_B$ receptors on living cells.

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system and exerts its effects through two ligand-gated channels, $GABA_A$ and $GABA_C$ receptors, and the metabotropic $GABA_B$ receptors. $GABA_B$ receptors are widely expressed in the brain or spinal cord during development and are located in both pre- and postsynaptic compartments.[1] They mediate slow synaptic inhibition and are involved in numerous types of nociception, cognitive impairment, epilepsy, spasticity, and drug addiction.[2] $GABA_B$ receptors belong to the family 3 of G-protein-coupled receptors (GPCRs). Structurally, $GABA_B$ receptors possess an extracellular domain so-called Venus flytrap module that binds GABA and a heptahelical domain responsible for the recognition and activation of heterotrimeric G-proteins. Functional $GABA_B$ receptors were reported to repress $Ca^{2+}$ influx[3] and trigger $K^+$ channels opening[4,5] via coupling to $G_{i/o}$-type protein.[6,7] $GABA_B$ receptors can also modulate the level of cyclic adenosine monophospate (cAMP)[8] and the phosphorylation of $ERK_{1/2}$ and CREB.[a,9] $GABA_B$ receptors form heterodimers composed of two subunits: GB1 and GB2.[10] The GB1 subunit has been shown to bind GABA but is unable to activate G-proteins.[11] It also fails to reach the cell surface when expressed alone because of an IRS located in its C-terminal tail.[12] In contrast, the GB2 subunit can activate G-proteins upon assembly of the heterodimer with GB1 at the cell surface but cannot directly bind GABA.[13] Interestingly, the GB2 subunit is also required for high affinity of GABA to the GB1 subunit.[14] Moreover, GB2 facilitates GB1 targeting to the cell surface by masking its IRS when forming heterodimers. However, the mechanisms that regulate $GABA_B$ receptors oligomerization at the plasma membrane remain largely unknown.

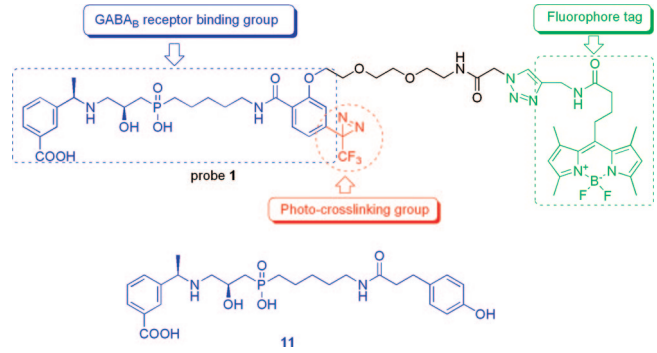


**Figure 1.** Structures of probe **1** and known GB1 antagonist **11** (CGP64213).

The development of small molecular fluorescent probes specifically targeting unmodified or native $GABA_B$ receptors represents a major challenge. Such molecules will be powerful tools to probe the localization and function of $GABA_B$ receptors in living cells, as previously described for other proteins.[15] Activity-based protein profiling (ABPP) is a chemical strategy that utilizes activity-based probes (ABPs) to profile the functional state of proteins in complex proteomes.[16] To date, ABPs have been developed for many enzyme classes and produced global portraits of enzyme activity that depict specific physiological and pathological processes.[17] Nonetheless, the approach has not been applied to study the location of important receptor proteins in living cells.

In this paper, we report the design, synthesis, and biological application of an activity-based fluorescent probe for photoaffinity labeling $GABA_B$ receptors on living cells. The probe, which is termed probe **1**, is designed based on **11**, a GB1 selective high-affinity antagonist.[18] The structure template of probe **1** consists of the main structure moiety in **11** for binding $GABA_B$ receptors, a photolabile diazirine group that effectively generates a covalent, irreversible linkage between the probe and the $GABA_B$ receptors after UV irradiation and a fluorescent reporter tag for visualizing the localization of the labeled proteins, as outlined in Figure 1. The photoinduced cross-linking of probe **1** to the receptor is designed to dramatically increase labeling efficacy. The trifluoromethylaryldiazirine moiety was incorporated as the photochemically reactive functional group because of the advantage of its excellent chemical stability prior to photolysis.[19] We selected 4, 4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) as the fluorophore because of its many attractive spectral characteristics and its hydrophobic nature.[20]

The synthesis of probe **1** is outlined in Scheme 1. The key intermediates **3**−**5** (Figure 2) were prepared individually via procedures similar to those reported previously with some

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[a] Abbreviations: ERK, extracellular signal-regulated kinase; cAMP, cyclic adenosine monophosphate; CREB, cAMP-responsive element binding protein; IRS, intracellular retention signal.

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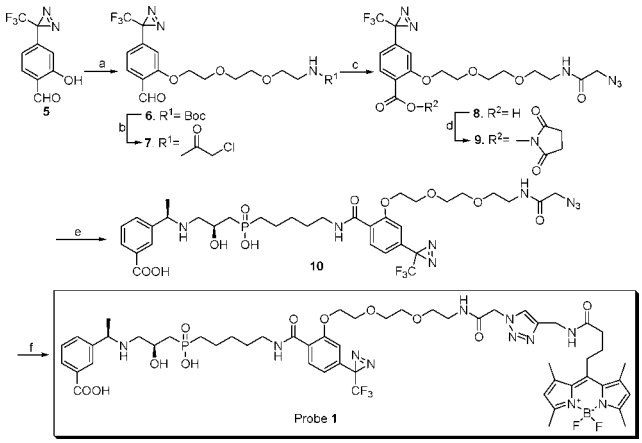
**Scheme 1.** Synthesis of Probe **1**[a]




[a] Reagents and conditions: (a) *tert*-butyl 2-(2-(2-bromoethoxy)ethoxy)-ethylcarbamate, $K_2CO_3$, $(n\text{-}C_4H_9)_4NI$, DMF, 60 °C, 24 h, 70%; (b) (i) HCl/EtOAc, room temp, 1 h, (ii) chloroacetyl chloride, $NaHCO_3$, 1,4-dioxane, room temp, 24 h, 65%, two steps; (c) (i) $NaN_3$, NaI, acetone, 60 °C, 12 h, (ii) $(n\text{-}C_4H_9)_4NMnO_4$, pyridine, room temp, 12 h, 86%, two steps; (d) *N*-hydroxysuccinimide, EDC·HCl, $CH_2Cl_2$, room temp, 12 h, 83%; (e) **3**, *i*-$Pr_2NEt$, MeOH, room temp, 12 h, 89%; (f) **4**, $CuSO_4$, vitamin C, MeOH/$H_2O$, room temp, 1 h, 82%.

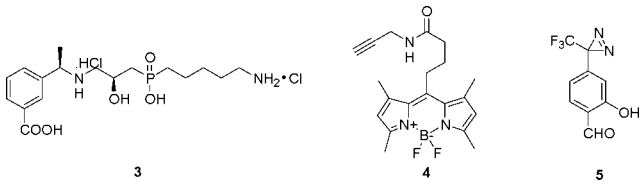
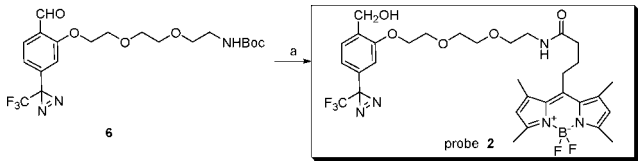



**Figure 2.** Structures of the three elements comprising probe **1**.

**Scheme 2.** Synthesis of Probe **2**[a]




[a] Reagents and conditions: (a) (i) $NaBH_4$, EtOH, room temp, 1 h; (ii) HCl/EtOAc, room temp, 1 h; (iii) *N*-hydroxysuccinimidyl-4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)butyric acid, *i*-$Pr_2NEt$, room temp, 12 h, 82.1%, three steps.

modifications.[21–23] The azide group was attached to the phenyldiazirine **5** via a poly(ethylene glycol) (PEG) linker, and the benzaldehyde was oxidized to the corresponding benzoic acid, which was subsequently activated with *N*-hydroxysuccinimide to give compound **9**. Coupling **9** with the bioactive ligand **3** gave **10**, which was subsequently reacted with the fluorophore **4** to give probe **1** employing the copper-catalyzed [3 + 2] azide−alkyne cycloaddition.[24]

As a control compound, probe **2** (Figure 1), which has no bioactive ligand toward $GABA_B$ receptors but only contains the photoaffinity and fluorophore groups, was also synthesized by reduction and deprotection of **6**, followed by coupling with the activated ester of the fluorophore (Scheme 2).

Prior to the labeling studies, the probes were first evaluated for their bioactivity as $GABA_B$ receptor antagonists. As shown in Figure 3, probe **1** significantly inhibited $GABA_B$ receptors activation induced by GABA with an $IC_{50}$ of 1.03 $\mu$M, while probe **2** had no such inhibitory effect (Figure 3). These results confirmed that probe **1** retains substantial affinity to $GABA_B$

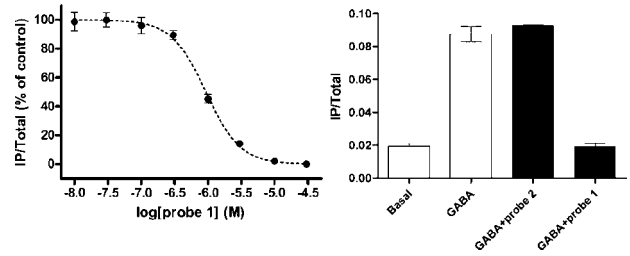



**Figure 3.** Probe **1** inhibits $GABA_B$ receptor activation. HEK293 cells cotransfected with the cDNA of wild-type GB1 and GB2 subunits, and the chimera Gqi9, were incubated with the indicated concentrations of probe **1** for 15 min before stimulation with 10 $\mu$M of GABA for 30 min. IP formation data are expressed as the percentage of the response. $IC_{50}$ value of probe **1** is 1.03 $\mu$M. Inner panel, 10 $\mu$M probe **2** is not able to inhibit the IP production induced by 10 $\mu$M GABA, compared to 10 $\mu$M probe **1**. Values are the mean of triplicates from one representative of three independent experiments.

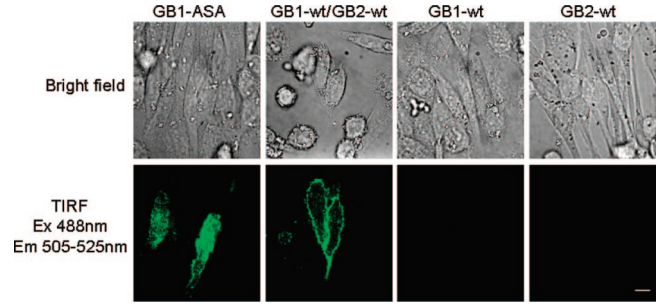



**Figure 4.** Probe **1** specifically labels GB1 subunit expressed on the surface of living cells. CHO cells transiently transfected with the indicated constructs were treated with probe **1** followed by UV irradiation and then observed in TIRF microscopy (OLYMPUS IX-70, oil immersion, ×100 objective) using filter sets for probe **1**. Upper panels are bright field images, while the lower panels represent fluorescent images under probe **1** channel. Scale bar represents 10 $\mu$m.

receptors and represents an activity-based fluorescent probe. The selectivity of the probe **1** on other GABA receptors was not determined.

Probe **1** was further evaluated for its capacity to label the $GABA_B$ receptors in living cells. CHO cells transiently expressing GB1-ASA[14] (a GB1 mutant able to reach the cell surface alone) were incubated with probe **1** for 15 min. After photolysis under UV for 15 min at room temperature, the cells were washed with phosphate buffered saline (PBS) twice and observed by total internal reflection fluorescent (TIRF) microscopy to acquire detailed information about protein expressed on the cell surface.[25] As shown in Figure 4, probe **1** specifically labels GB1 expressed on the cell surface. Similar results were obtained on CHO cells transiently cotransfected with GB1-wt and GB2-wt. However, CHO cells transfected only with wild-type GB1 that remains intracellular, or GB2 with no affinity for **11**, could not be labeled with probe **1**. All these results were in agreement with the previously reported structural properties[11–13] of the $GABA_B$ receptor and illustrate the specificity of the labeling experiment. To show that probe **1** specifically labels the GB1 subunits expressed on the cell surface, we conducted double labeling experiments using fluorescence confocal microscopy. The DsRed markers[26] combined with green fluorescent probe **1** markers for double labeling enable us to optically distinguish and quantitatively analyze the specificity of labeling in living cells. CHO cells transiently cotransfected with DsRed2-tagged GB1-wt and GB2-wt, DsRed2-tagged GB1-ASA, or DsRed2-tagged GB1-wt were labeled with probe **1** as previously

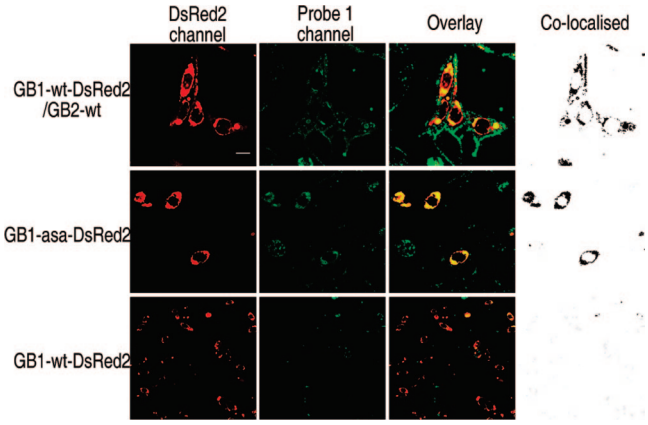



**Figure 5.** Probe **1** does not label intracellular $GABA_B$ receptors in living cells. CHO cells were transiently cotransfected with the indicated constructs. Images were detected by LSCM (OLYMPUS, FV-1000, ×60 objective). The colocalization of the DsRed2-tagged receptor with probe **1** results in the apparition of a third color in the overlay image: yellow for red and green merge. Probe **1** channel was detected as previously, whereas DeRed-tagged constructs were detected after excitation (ex) at 550 nm and emission (em) at 585 nm. Scale bar represents 10 $\mu$m.

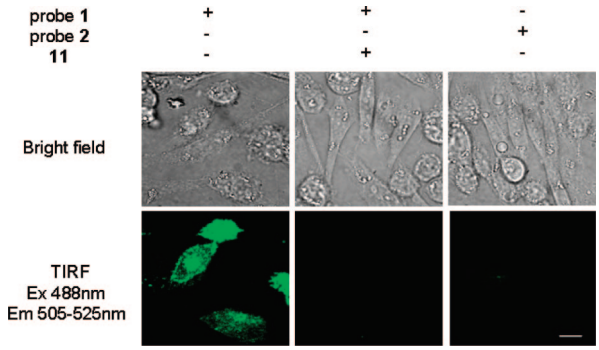



**Figure 6.** The specificity of labeling depends on the active moiety. Pretreatment of CHO cells expressing GB1-ASA with **11** significantly eliminates the fluorescent intensity compared with no pretreatment. Probe **2** treatment produces no labeling. Results were obtained by TIRF microscopy.

described. Only negligible staining was obtained with cells expressing DsRed2-tagged GB1-wt alone (Figure 5), suggesting that probe **1** did not label intracellular $GABA_B$ receptor. Furthermore, competition experiments were conducted to confirm the labeling specificity. CHO cells transiently transfected with GB1-ASA were pretreated with **11** (500 $\mu$M) and then incubated with probe **1** as described previously. Fluorescence microscopy experiments showed an apparent elimination of fluorescence intensity in these conditions (Figure 6). Incubation of CHO cells expressing GB1-ASA with probe **2** produced no labeling (Figure 6). These data demonstrate that the active moiety of probe **1** is essential for its specificity.

We also demonstrated the necessity of a covalent bond between probe **1** and the $GABA_B$ receptor induced by UV irradiation to enhance the recognition. CHO cells transiently transfected with GB1-ASA were treated with probe **1** followed by UV irradiation or not. The fluorescence intensity under no UV irradiation condition was significantly decreased compared with that under UV irradiation condition (Supporting Information Figure 2). These results indicated that UV induced covalent bond formation could increase labeling efficiency, even though the probe has low affinity for its target protein.

In summary, an activity-based trimodular probe was designed, synthesized, and applied to the specific labeling of $GABA_B$ receptors transiently expressed in living cells. Pharmacological studies show that the probe conserved reasonable antagonist potency for $GABA_B$ receptors. Subsequent photoaffinity labeling and different fluorescent microscopy experiments also show that the probe labels the functional $GABA_B$ receptors on the cell surface with high specificity in an activity-based manner. Several lines of evidence support this view. First, only GB1 with its IRS mutated (GB1-ASA) or the heterodimeric functional $GABA_B$ could be labeled but not GB2 expressed alone. Second, the labeling could be significantly completed by pretreatment with the bioactive moiety of the probe. Finally, probe **2**, which has no affinity to $GABA_B$ receptors, failed to label the GB1-ASA transiently expressed on the CHO cells surface. Moreover, the approach has the advantage of forming a permanent covalent bond between the probe and the receptors through photolysis, thus allowing direct study of the dynamics of these receptors in living cells together with its biochemical analysis. Although many fluorescent photoaffinity probes have been reported,[27] it is important to note that our experiments were performed on living cells. The functional multiplicity and the specificity of the labeling of the probe suggest useful applications for this method.

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**Supporting Information Available:** Synthesis and chromatographic data of **6**−**10** and probes **1** and **2**, biological methods, and HPLC conditions and traces for probes **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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