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Inhibitable Photolabeling by Neurosteroid Diazirine Analog in the $\beta 3$ -Subunit of Human Heteropentameric Type A GABA Receptors

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Abstract

Pregnanolone and allopregnanolone-type ligands exert general anesthetic, anticonvulsant and anxiolytic effects due to their positive modulatory interactions with the GABA_A receptors in the brain. Binding sites for these neurosteroids have been recently identified at subunit interfaces in the transmembrane domain (TMD) of homomeric $\beta 3$ GABA_A receptors using photoaffinity labeling techniques, and in homomeric chimeric receptors containing GABA_A receptor α subunit TMDs by crystallography. Steroid binding sites have yet to be determined in human, heteromeric, functionally reconstituted, full-length, glycosylated GABA_A receptors. Here, we report on the synthesis and pharmacological characterization of several photoaffinity analogs of pregnanolone and allopregnanolone, of which 21-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoxy]allopregnanolone (21-pTFDBzox-AP) was the most potent ligand. It is a partial positive modulator of the human $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$ GABA_A receptors at sub-micromolar concentrations. [³H]21-pTFDBzox-AP photoincorporated in a pharmacologically specific manner into the α and β subunits of those receptors, with the $\beta 3$ subunit photolabeled most efficiently. Importantly, photolabeling by [³H]21-pTFDBzox-AP was inhibited by the positive steroid modulators alphaxalone, pregnanolone and allopregnanolone, but not by inhibitory neurosteroid pregnenolone sulfate or by two potent general anesthetics and GABA_AR positive allosteric modulators, etomidate and an anesthetic barbiturate. The latter two ligands bind to sites at subunit

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DECLARATION OF INTEREST

The authors declare no competing financial interests.

interfaces in the GABA_AR that are different from those interacting with neurosteroids. 21-pTFDBzox-AP's potency and pharmacological specificity of photolabeling indicate its suitability for characterizing neurosteroid binding sites in native GABA_A receptors.

INTRODUCTION

Neurosteroids such as pregnanolone (**1**, Chart 1), allopregnanolone (**2**) and tetrahydrodeoxycorticosterone (THDOC, **3**) are potent positive allosteric modulators of GABA type-A receptors (GABA_AR), producing sedative, anxiolytic and anticonvulsant effects.^{1–5} The synthetic allopregnanolone derivative alphaxalone (**4**) acts as a general anesthetic,⁶ but was withdrawn from clinical use due to anaphylactoid side effects associated with its ethoxylated castor oil formulation.⁷ Currently, alphaxalone is used in veterinary medicine⁸ and there is strong interest in restoring the clinical use of this and structurally related drugs.^{9,10} More broadly, analogs of endogenous neurosteroids are of interest as potential drugs for treatment of epilepsy, anxiety, mood disorders and depression, and as probes for interrogation of GABA_AR pharmacology.¹¹

Pharmacological studies show the GABA_AR binding site(s) for neuroactive steroids differ from those of GABA and benzodiazepines, which bind to homologous sites at subunit interfaces in the extracellular domain (ECD), or of other intravenous general anesthetics including propofol, barbiturates, and etomidate, which bind to homologous sites at subunit interfaces in the extracellular third of the transmembrane domain (TMD).^{4,12–14} In recent crystal structures of chimeric homo–pentameric receptors containing GABA_AR α subunit TMDs, pregnanolone and THDOC bound to a subunit interface site, whereas the inhibitory steroid pregnenolone sulfate (PS) bound to a distinct intrasubunit site.^{15,16} Both the latter sites are near the cytoplasmic end of the TMD and in close proximity to a residue previously photolabeled by 6-azipregnanolone (**5**) in homomeric $\beta 3$ GABA_ARs.¹⁷

An advantage of photolabeling with tritiated photoprobes followed by microsequencing with Edman degradation is the ability to both identify the photolabeled amino acids and to quantify photoincorporation which enables assessment of pharmacological specificity and allosteric interactions with other ligands. For example, tritium-labeled photoreactive analogs of etomidate, mephobarbital, and propofol have identified multiple binding sites in purified $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$ GABA_ARs (Figure 1),^{18–20} and were useful in photolabeling protection studies that compared other drug's relative affinity for these sites. Multiple steroid photolabels **5–11**, incorporating photoreactive groups at various positions on the steroid backbone, have been tested in GABA_ARs.^{17,21} However, to date no tritiated steroid photolabels have proven useful in such protection studies at steroid binding sites. Previously, we developed tritiated allopregnanolone analogs **12** and **13** containing photoreactive substituents at the 11-position, which were potent anesthetics and GABA_AR modulators, but their photoincorporation in GABA_ARs was not inhibited by other steroid modulators.²²

Our goal in this study was to develop additional anesthetic steroid photolabels suitable for photolabeling the modulatory binding sites in typical heteropentameric receptors. We hypothesized that the upper edge of the steroid framework, where our C-11 photolabels were located, projected out of the binding site, perhaps facing towards the lipids.²² Consequently,

in this work, we placed the photoreactive residues on the opposite edge of the steroid (C-6), where previous studies on the $\beta 3$ homomer had been successful,^{17,21} and on the steroid side-chain (C17 of the framework). Here, we report the synthesis of three neurosteroid analogs equipped with diazirines located at C-6 or C-21 (**14-16**) that act as positive allosteric modulators of heteropentameric $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$ receptors. We found that tritiated compound **16** (21-pTFDBzox-AP) photoincorporated with pharmacological specificity into full-length human $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$ GABA_ARs. In addition, photolabeling was inhibited by other neurosteroid enhancers at concentrations close to their modulatory EC₅₀ values, but not by the inhibitory neurosteroid pregnenolone sulfate, nor by etomidate, or a potent barbiturate anesthetic.

RESULTS

Synthesis of 6-azi-3 α -hydroxy-5 β -pregnane-11,20-dione (**14**, 6-azi-OP), 6-azi-3 α -hydroxy-5 α -allopregnane-11,20-dione (**15**, 6-azi-OAP) and 21-(p-trifluoromethyldiazirynylbenzoxy)allopregnanolone (**16**, 21-pTFDBzox-AP).

The 10-step synthesis of compounds **14** and **15** shown in Scheme 1 started from the commercially available 11 α -hydroxyprogesterone **17**. 11 α -Hydroxyprogesterone **17** was first protected at both the 3- and 20-positions to form the diketal **18**. During this process, under strong acid catalysis, the double bond migrated to the position 5. The double bond was hydroborated with BH₃-THF²³ and the product oxidized in two steps, first with hydrogen peroxide and then with pyridine chlorochromate to form the diketone **19**. The 6-ketone moiety was converted into a diazirine using the one-pot, five-step diazirination procedure suitable for sterically hindered ketones, as described earlier.²² In the course of this procedure, the original 5 β -6-ketone **19** was partially isomerized into a 5 α -isomer, and hence the product constituted the mixture of pregnanolone and allopregnanolone diazirines (**20** and **21**, respectively). The subsequent deprotection step provided the mixture of triketones **22** and **23** that was separated by flash chromatography, and each isomer subjected to the reduction at the 3-position. To choose a stereoselective reducing agent, we needed to establish configurations of the substrates **22** and **23** at carbon 5, however, the complexity of their NMR spectra made that assignment unreliable. We, therefore, obtained the crystals of the compound **22** and determined its structure by x-ray crystallography. The structure clearly shows the 5 β -configuration and the *cis*-fusion of rings A and B for this compound (Figure 2, we were unable to obtain well-diffracting crystals of compound **23**, **14** and **15**).

Due to steric hindrance in compound **22**, the stereoselective reduction of its 3-keto group into the 3 α -product **14** (6-azi-OP) was possible using sodium borohydride. In contrast, the reduction of allopregnanolone diazirine **23** into a 3 α -product could be carried out using the sterically hindered reducing agent, K-selectride,²⁴ to provide the final photoprobe **15** (6-azi-OAP). Reductions of diketones **22** and **23** occurred exclusively at the 3-position, due to the highly hindered environment at the 11-position.²² The configurations of the final compounds **14** and **15** at C-3 were ascertained based on their ¹H NMR spectra.

The synthesis of the pTFD-benzoyl derivative of THDOC **16** is shown in Scheme 2. THDOC (**3**), synthesized by oxidation of the silyl enol ether derived from pregnanolone **1** by

m-chloroperbenzoic acid, as described earlier,²⁵ was coupled with 4-(trifluoromethyldiazirynyl)benzoic acid (**24**) using EDC and DMAP in methylene chloride to provide the ester **16**. In order to obtain the tritiated photoprobe **16**, THDOC was coupled with 3-iodo-4-TFD-benzoic acid (**25**), and the product **26** was reduced with hydrogen gas over palladium catalyst²⁶ to produce **16** (21-pTFDBzox-AP). Using the same methodology, reduction of **26** with tritium gas was then performed by ViTrax Company (Placenta, CA) to afford compound ³H-**16** with 15 Ci/mmol specific radioactivity.

Anesthetic properties of 21-pTFDBzox-AP.

No LoRR was observed when tadpoles were exposed to the following concentrations of 21-pTFDBzox-AP (μM, number of animals): 1, 5; 2, 15; 3, 15 and 10, 5. The concentration-response curve of alphaxalone was determined alone and in the constant presence of 2.5 or 10 μM 21-pTFDBzox-AP (55, 25 and 40 animals, respectively). The response to alphaxalone stabilized between 30 and 60 minutes, and was constant thereafter. Responses were recorded after 60 minutes equilibration. The EC₅₀ of alphaxalone alone was found to be 1.4 ± 0.3 μM and the slope of the curve was 3.1 ± 1.2. This is consistent with the published EC₅₀s of 1 to 2 μM and slopes 1.7 to 3.4.^{22,27} 21-pTFDBzox-AP at 2.5 μM caused a small shift of anesthetic EC₅₀ of alphaxalone from 1.4 ± 0.3 to 0.92 ± 0.3 μM, which was significant (F-test, F=8.174, p=0.005). However, the shift with 10 μM to 1.3 ± 0.3 μM was not significant (F=0.062, p=0.798). When the data from the two sets were combined, the shift to 1.2 ± 0.2 μM was not significant (F=2.152, p=0.145). Overall, it was clear that there is no significant antagonism of alphaxalone's action by 21-pTFDBzox-AP and that a sub-anesthetic contribution to alphaxalone's anesthetic effect could not be established with confidence. Due to very small amounts of 6-azi-OP and 6-azi-OAP available, these compounds were not tested for their anesthetic properties.

Enhancement of GABA_A Receptor Currents by 6-azi-OP, 6-azi-OAP and 21-pTFDBzox-AP.

In voltage clamp electrophysiology studies of human α1β3γ2L GABA_AR expressed in *Xenopus* oocytes, EC₅ GABA-activated chloride currents were potentiated by 6-azi-OAP and 6-azi-OP up to 10.1 ± 0.67 and 8.0 ± 1.4 - fold, respectively (Figure 3). The maximum concentration tested for both compounds was 100 μM, which appeared to establish a plateau effect for 6-azi-OAP, but not for 6-azi-OP. Fits of the data to Eq. 1 (see Materials and Methods) were difficult. Figure 2B shows a lower limit estimate of the EC₅₀s by assuming a Hill coefficient of 1.45 (based on the analysis of [³H]muscimol binding modulation data below). With this assumption, the lower limit estimates of the EC₅₀ values are 22 ± 1.3 and 13 ± 1.1 μM for 6-azi-OP and 6-azi-OAP, respectively. Consistent with this analysis, 10 μM 6-azi-OAP produced significantly higher enhancement than 6-azi-OP (p = 0.048).

In contrast, our third photolabel, 21-pTFDBzox-AP, only weakly enhanced GABA EC₅ and EC₁₀ activation of α1β3γ2L and α1β3 receptors, respectively (Figure 4). At the highest concentration tested, 10 μM, 2.1 ± 0.4-fold enhancement of EC₁₀ GABA-activated α1β3 currents was observed (Figure 4A). Because no enhancement plateau was identified at 10 μM, the Hill coefficient was constrained to 1.7 in logistic fits (based on [³H]muscimol binding data), resulting in a lower limit EC₅₀ of 2.7 ± 0.5 μM. In α1β3γ2L receptors activated with EC₅ GABA, 10 μM 21-pTFDBzox-AP (Figure 3) produced similar

enhancement (2.6 ± 0.3 -fold; $n = 6$) to that in $\alpha 1\beta 3$ receptors. In comparison, THDOC at 10 μM enhanced $\alpha 1\beta 3\gamma 2\text{L}$ receptor activation 16 ± 2.1 -fold (Figure 4B), whereas alphaxalone at 10 μM enhanced $\alpha 1\beta 3$ receptor activation 10.6-fold (not shown). Incorporation of the $\alpha 1\text{Q}242\text{W}$ mutation, which is known to ablate neurosteroid modulation in $\text{GABA}_\text{A}\text{Rs}$,^{4,28} eliminated positive modulation by both THDOC and 21-pTFDBzox-AP (Figure 4B). In spontaneously gating $\alpha 1264\text{T}\beta 3\gamma 2\text{L}$ receptors, 2 μM 21-pTFDBzox-AP directly increased inward current in the absence of GABA (Figure 4C) by about 50%.

Left-Shifts of the GABA Dose-Response Curves.

Both 6-azi-OAP and 6-azi-OP photoprobe interactions with $\alpha 1\beta 3\gamma 2\text{L}$ receptors produced leftward shifts of the GABA concentration – response curve (Figure 5). The GABA EC_{50} decreased approximately 3-fold in the presence of 15 μM 6-azi-OAP (Figure 5A) or 30 μM 6-azi-OP (Figure 5B). The effect of 15 μM 6-azi-OAP on GABA dose-response curves was similar in the case of $\alpha 1\beta 3$ receptor (not shown). Consistent with the low efficacy of 21-pTFDBzox-AP enhancement of $\text{GABA}_\text{A}\text{R}$ currents, this reagent at 5 μM did not reduce the GABA EC_{50} (Figure 5C).

Modulation of Muscimol Binding to GABA_A Receptors by 6-azi-OP, 6-azi-OAP and 21-pTFDBzox-AP.

To additionally characterize the allosteric action, we compared the abilities of the three agents to modulate the binding of 2 nM [^3H]muscimol to $\alpha 1\beta 3\gamma 2$ and $\alpha 1\beta 3$ $\text{GABA}_\text{A}\text{Rs}$ (Figure 6). Displaceable binding of [^3H]muscimol to $\alpha 1\beta 3\gamma 2$ $\text{GABA}_\text{A}\text{Rs}$ was enhanced in a concentration – dependent manner by both 6-azi-OP and 6-azi-OAP between 1 μM and 100 μM , above which it leveled off (Figure 6A). The potencies of 6-azi-OAP, 6-azi-OP were similar, with $\text{EC}_{50} = 25 \pm 2.5$ and 37 ± 1.7 , respectively, and the Hill coefficients were $n_\text{H} = 1.3 \pm 0.15$ and 1.6 ± 0.1 , respectively. The efficacies of the modulation were $330 \pm 9\%$ and $241 \pm 3\%$ for 6-azi-OAP and 6-azi-OP, respectively. The enhancement by 21-pTFDBzox-AP was studied in more detail using both $\text{GABA}_\text{A}\text{Rs}$ in membranes and in detergent/lipid micelles after purification (Figure 6B and 6C, respectively). In $\alpha 1\beta 3\gamma 2\text{L}$ $\text{GABA}_\text{A}\text{Rs}$, 21-pTFDBzox-AP was a more potent enhancer than either 6-azi-OP or 6-azi-OAP, with $\text{EC}_{50} = 0.52 \pm 0.25$ and 0.50 ± 0.06 μM in membranes and in detergent/lipid micelles, respectively, with corresponding Hill coefficients $n_\text{H} = 1.09 \pm 0.22$ and 1.15 ± 0.36 , respectively. For $\alpha 1\beta 3$ $\text{GABA}_\text{A}\text{R}$, $\text{EC}_{50} = 0.36 \pm 0.06$ and 0.16 ± 0.03 μM in membranes and in detergent/lipid micelles, respectively, with corresponding Hill coefficients $n_\text{H} = 1.71 \pm 0.10$ and 1.45 ± 0.51 , respectively. In addition, the maximal enhancement registered in membranes and in detergent/lipid micelles was 199 ± 30 and $135 \pm 1\%$ for $\alpha 1\beta 3\gamma 2$ $\text{GABA}_\text{A}\text{R}$, respectively, whereas it was 271 ± 7 and $172 \pm 6\%$, respectively, for $\alpha 1\beta 3$ $\text{GABA}_\text{A}\text{R}$. The data shown in Table 1 indicate that in terms of potency and maximal effect, the modulatory property of 21-pTFDBzox-AP was in line with those of most other neurosteroid enhancers. As 6-azi-OP and 6-azi-OAP were both low potency ligands, whereas 21-pTFDBzox-AP was significantly more potent, we tritiated 21-pTFDBzox-AP and used this ligand in the photolabeling experiments.

GABA_A Receptor Photolabeling by 21-pTFDBzox-AP and Its Inhibition by Neurosteroid Modulators.

We first determined the effect of 30 μ M allopregnanolone on [³H]21-pTFDBzox-AP (0.8 μ M) photolabeling of purified α 1 β 3 and α 1 β 3 γ 2 GABA_ARs in the absence or presence of bicuculline (BIC, 100 μ M, resting state) or GABA (300 μ M, desensitized state). Photoincorporation was characterized by SDS-PAGE, with ³H incorporation determined by fluorography (Figure 7A, α 1 β 3, lanes 1,3–6; α 1 β 3 γ 2L, lanes 2, 7–10) or by liquid scintillation counting of excised gel bands containing GABA_AR subunits (Figure 7B). In both α 1 β 3 and α 1 β 3 γ 2L GABA_ARs, [³H]21-pTFDBzox-AP was incorporated into the three gel bands revealed by Coomassie Blue stain (lanes 1 and 2), a band of 56-kDa, containing (by sequence analysis) primarily the α 1 and γ 2 subunits, and bands of 59- and 61-kDa containing primarily β 3 subunits.¹⁹ The levels of photoincorporation were similar in the presence of GABA and bicuculline, and were strongly reduced by addition of allopregnanolone. Based upon liquid scintillation counting (Figure 6B), 30 μ M allopregnanolone reduced β subunit photolabeling by 60% and 70% in α 1 β 3 and α 1 β 3 γ 2 GABA_ARs, respectively. The 600 ³H cpm of inhibitable β subunit photolabeling indicates photolabeling of ~2% of β subunits, based upon the ~ 2 pmol of GABA_AR loaded on each gel lane and the radiochemical specific activity of [³H]21-pTFDBzox-AP (15 Ci/mmol). This efficiency of photolabeling is close to the 9% efficiency seen for the photoreactive mephobarbital analog [³H]*R*-mTFD-MPAB that contains the same photoreactive group.²⁹ Inhibitable photolabeling by allopregnanolone in the α / γ -subunit gel band was less than 15% the amount in the β subunit gel bands.

To determine the pharmacological specificity of the steroid binding site identified by [³H]21-pTFDBzox-AP photolabeling of α 1 β 3 GABA_ARs, we examined the effects of other anesthetic and non-anesthetic steroids as well as etomidate and the mephobarbital analog *R*-mTFD-MPAB, anesthetics and GABA_AR positive allosteric modulators that bind with high affinity and selectivity to homologous sites at β^+ - α^- , the γ^+ - β^- and α^+ - β^- subunit interfaces in the α 1 β 3 γ 2L GABA_AR transmembrane domain.¹⁹ Two anesthetic steroids, pregnanolone (3 α ,5 β -P) and 3 α ,5 α -THDOC, at high concentrations, inhibited photolabeling to the same extent (by 60%) as 30 μ M allopregnanolone, with the concentration dependence of inhibition characterized by IC₅₀s of 0.7 $\pm$ 0.13 μ M and 2.3 $\pm$ 0.35 μ M, respectively (Figure 8A and Table 1). In contrast, no inhibition of photolabeling was seen in the presence of the antagonist steroid pregnenolone sulfate (PS) at 100 μ M, nor in the presence of *R*-mTFD-MPAB at 60 μ M or etomidate at 1 mM (Figure 7A). Non-radioactive 21-pTFDBzox-AP itself was a potent inhibitor of photolabeling (IC₅₀ = 0.15 $\pm$ 0.02 μ M), and alphaxalone (IC₅₀ = 6 $\pm$ 1 μ M) bound to this site with higher affinity than 6-azi-OAP (IC₅₀ = 44 $\pm$ 6 μ M), while 6-azi-OP inhibited with only low potency (IC₅₀ > 100 μ M) (Figure 8B). For each steroid, the concentration dependence of inhibition was characterized by a Hill coefficient close to 1 (not shown).

DISCUSSION

In our previous work we have described two allopregnanolone derivatives, 11-F4N3Bzox-AP (**12**) and 11-azi-AP (**13**), that displayed potent positive modulatory properties and

efficiently photolabeled GABA_ARs.²² However, the photolabeling by these reagents was not inhibited by other neurosteroids, and hence was not site-specific. The most important result of this report is the successful design of a photoreactive neurosteroid analog 21-pTFDBzox-AP (**16**) that meets the following criteria: (i) It is a potent, albeit low-efficacy, allosteric modulator of human heteromeric GABA_A receptors. (ii) It photolabels GABA_ARs in a pharmacologically specific manner, as evidenced by the observed inhibition of photolabeling at the subunit level by other positive steroid modulators, but not by steroid antagonists nor by ligands such as etomidate and R-mTFD-MPAB, which act at separate sites. (iii) Its photoincorporation is not strongly dependent on the presence or absence of GABA, which is consistent with the low efficacy with which it positively modulates currents induced by low concentrations of GABA. (iv) Lastly, the modulation of GABA currents by 21-pTFDBzox-AP is completely eliminated by the $\alpha 1$ Q242W mutation suggesting that this ligand binds in the same site as that of other neurosteroids.^{4,28,30}

We compared the potency of common steroids to inhibit the photoincorporation of [³H]21-pTFDBzox-AP into its site (IC₅₀) with their potency for enhancing the binding of the agonist [³H]muscimol (EC₅₀), a measure of desensitization (Table 1). These experiments were carried out with heteromeric, human full-length, glycosylated GABA_ARs with subunit composition $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$. In neither case, did the presence of the $\gamma 2$ -subunit exert a major influence on the results, so we pursued more detailed studies with $\alpha 1\beta 3$ receptors. For $3\alpha, 5\alpha$ -steroids: THDOC (**3**), alphaxalone (**4**), 6-Azi-OAP (**6**) and 21-pTFDBzox-AP itself (**16**), $3\alpha, 5\beta$ -steroid (pregnanolone, **1**) and 6-azi-OP (**5**), the agreement between IC₅₀ and EC₅₀ was very good. Furthermore, pregnenolone sulfate, a 3β -steroid that inhibits GABA currents,³¹ neither bound to the 21-pTFDBzox-AP site nor enhanced desensitization. Considering that the binding studies were carried out in much more dilute suspensions than the photolabeling studies, we regard the pharmacological evidence establishing that photoincorporation was occurring in the site that was responsible for enhancing desensitization to be strong.

Photoincorporation of 21-pTFDBzox-AP occurred predominantly in the $\beta 3$ subunit, very modestly in the $\alpha 1$ subunit and not at all in the $\gamma 2$ subunit. These observations taken together with the sensitivity of 21-pTFDBzox-AP enhancement of GABA currents to the $\alpha 1$ M1 mutation Q242W⁴ suggest that the 21-pTFDBzox-AP binding site is in the $\beta 3^+ - \alpha 1^-$ subunit interface. Support for steroid interaction on the β^+ side of the interface comes from two studies. First, Chen et al,¹⁷ using $\beta 3$ homopentamers and the photolabel **5** (6-AziP) identified $\beta 3$ F301 on the third transmembrane helix M3, on the plus side of the $\beta - \beta$ interface, as a photoincorporation site, but were unable to establish its pharmacological specificity. Second, a recent substituted cysteine and modification of protection (SCAMP) protocol mapped alphaxalone-contacting residues to the cytoplasmic end of the $\beta 3$ -subunit's M3, including $\beta 3$ F301 in $\alpha 1\beta 3\gamma 2L$ receptors.³⁰ Unfortunately, $\alpha 1$ M1 Q242W, on the minus side of the $\beta 3^+ - \alpha 1^-$ subunit interface, is not amenable to SCAMP studies, so evidence for this interface in heteromeric receptors rests largely on the original mutagenesis study.

However, two recent crystallography studies probed interactions of a number of natural steroid ligands with the transmembrane domain of $\alpha 1$ and $\alpha 5$ subunits in the unnatural

homomeric chimeras consisting of GLIC ECD – human $\alpha 1$ TMD¹⁵ and human $\beta 3$ ECD – $\alpha 5$ TMD.¹⁶ These studies show that steroids bind at the cytoplasmic end of the transmembrane domain in the $\alpha^+ - \alpha^-$ subunit interface. Of particular interest, the 3 α hydroxyls of THDOC (**3**) and pregnanolone (**1**) form a hydrogen bond with $\alpha 1$ Q242 and $\alpha 5$ Q245 on M1 on the minus side of the interface.^{15,16} This glutamine is conserved in all six α -subunits but is a tryptophan in the $\beta 3$ and $\gamma 2$, and indeed in all other GABA_AR subunits. This suggests that the α^- interaction may be required for high affinity steroid interaction, but the $\beta 3$ homopentamer photolabeling study implies that this requirement is not absolute. The effective concentrations could not be established in the structural studies because the THDOC enhancement curve for the GLIC ECD – human $\alpha 1$ TMD chimera does not saturate, and the $\beta 3$ ECD – $\alpha 5$ TMD receptor was spontaneously open. Finally, in the GLIC ECD – human $\alpha 1$ TMD study,¹⁵ the inhibitory steroid pregnanolone sulfate interacted only with the transmembrane domain of the $\alpha 1$ subunit and its binding pocket did not overlap with that of THDOC, an observation that is consistent with our finding that pregnenolone sulfate does not bind to the 21-pTFDBzox-AP site.

In our previous steroid photolabeling study,²² we found that photolabeling of $\alpha 1\beta 3\gamma 2$ receptor by ligands **12** and **13** was not inhibited by other steroid ligands. We noted that the photoactive moieties had limited mobility relative to the steroid backbone, leading us to hypothesize that the steroid backbone of those photolabels binds with its upper edge pointing out of the binding site, as previously suggested.³² In this work, we found that substitutions on the lower side of the ring (compounds **14** and **15**) caused a significant loss of potency, suggesting that the lower edge of the steroid backbone binds with its receptor site so that even modest substitutions in the 6-position disrupt binding. Consequently, compounds **6** and **14**, which have a diazirine on the upper and lower side of the steroid ring system respectively, modulate agonist binding with potencies of 0.25 and 12 μ M, respectively. The crystallography studies referred to above are quite consistent with this binding pose of steroids in their site.

CONCLUSIONS

We have synthesized and characterized the first steroid photolabel, 21-pTFDBzox-AP, to photoincorporate into heteromeric human GABA_ARs with pharmacological specificity. It neither binds in the etomidate site nor the R-mTFD-MPAB site, which are at the extracellular end of the transmembrane domain $\beta^+ - \alpha^-$ and $\gamma^+ - \beta^-$ subunit interfaces, respectively. It photolabels almost exclusively into the β -subunit, and based on the evidence discussed above likely binds in the $\beta^+ - \alpha^-$ interface. Most importantly, [³H]21-pTFDBzox-AP is well positioned to quantitatively probe binding of ligands to the steroid site in human GABA_ARs of any subunit composition (see for example Table 1) in a way that crystallography and mass spectrometry (a least to date) would find difficult.

MATERIALS AND METHODS

Materials.

11 α -Hydroxy-progesterone **16** was from Santa Cruz Biotechnology, Inc. Allopregnanolone [5 α -pregnan-3 α -ol-20-one (3 α ,5 α -P)], alphaxalone (5 α -pregnan-3 α -ol-11,20-dione), and

etomidate were from Tocris Bioscience, and 5 α -pregnan-3 α , 21-diol-20-one (3 α ,5 α -THDOC) was from Steraloids, Inc. Pregnanolone [5 β -pregnan-3 α -ol-20-one (3 α ,5 β -P)] was from Research Plus and pregnenolone sulfate (PS) was from Santa Cruz Biotechnology. Bicuculline methochloride was from Abcam. Human α 1 β 3 and 3 α ,21-Dihydroxy-5 α -pregnan-20-one **3**, TFD-benzoic acids **21** and **22** were prepared according to the known methods.^{25,26} Anhydrous grade solvents were from Aldrich, and were not further dried or purified. *R*-*m*TFD-MPAB was synthesized as described previously.²⁹ α 1 β 3 γ 2 GABA_ARs with a FLAG epitope on the N-terminus of α 1 subunit were expressed in tetracycline-inducible HEK293S cells and purified from detergent extracts as described^{18,19,33,34} by use of an anti-FLAG antibody column. After elution from columns in the presence of 0.1 mM FLAG peptide, purified GABA_ARs were stored at -80°C until use in elution buffer containing 5 mM CHAPS and 0.2 mM asolectin.

Analytical Chemistry.

¹H, ¹³C and ¹⁹F NMR spectra were recorded on a Bruker Avance spectrometer at 400 MHz, 100 MHz and 376 MHz, respectively, unless otherwise noted. NMR chemical shifts were referenced indirectly to TMS for ¹H and ¹³C, and CFCl₃ for ¹⁹F NMR. HRMS experiments were performed with Q-TOF-2TM (Micromass). TLC was performed with Merck 60 F254 silica gel plates. Purity of the final compounds was assessed by HPLC analysis with a Synergy Hydro-RP column (4 μ m, 4.60 $\times$ 150 mm) using methanol and methanol-water (1:1); the gradient applied was from 1% methanol at the start to 99% methanol over 32 min, followed by isocratic elution. Elution was monitored by UV at 254 nm. These HPLC analyses indicated purity greater than 96%.

Anesthetic Properties.

Studies of anesthetic properties of the synthesized ligands were conducted in tadpoles according to an animal protocol preapproved by the Massachusetts General Hospital (MGH) Subcommittee on Research Animal Care (Protocol #2006N000124). Tris buffer (2.5 mM) was used for these experiments – 100 mL of buffer (2.5 mM) per 5 tadpoles per glass beaker. Drugs were added from DMSO stock solutions (final DMSO 0.2%). The tadpoles were tested for loss of righting reflexes (LoRR) after being exposed to the drug solution for 1 hour, and responses were recorded as having LoRR (1) or not having LoRR (0). A bent Pasteur pipette was used to flip the animal over. If the animal remained inverted for longer than 5 seconds, it was considered to have LoRR. The tadpoles were then transferred to recovery beakers containing de-chlorinated water for 24 hours. In these experiments all tadpoles were alive after 24 hours. Tadpoles were killed immediately at the end of each protocol by immersion in a lethal concentration of pentobarbital (1 mM).

Electrophysiology of GABA_A Receptors.

Female *Xenopus laevis* frogs were housed in a veterinarian-supervised facility and used with MGH IACUC approval (Protocol #2010N000002) as a source of oocytes for electrophysiology. Stage V oocytes were harvested via mini-laparotomy from frogs anesthetized in 0.15% tricaine. Efforts were made to minimize animal suffering.

GABA_AR coding DNA sequences for $\alpha 1$, $\beta 3$, $\gamma 2L$, and mutant $\alpha 1L264T$ and $\alpha 1Q242W$ subunits were cloned into pCDNA3.1 plasmids, which were linearized before use as templates in synthesizing capped mRNAs, as previously described.³⁵ Up to 10 ng total messenger RNA mixtures (1:1 ratio for $\alpha\beta$ and 1:1:3 ratio for $\alpha\beta\gamma$) were injected into oocytes, which were incubated for at least 24 hours before use in electrophysiological experiments.

Electrophysiological studies on oocyte-expressed GABA_ARs were performed at 20–22°C, as previously described.³⁰ Oocytes were placed in a custom low-volume (30 μ L) flow chamber, impaled with two borosilicate microelectrodes filled with 3 M KCl (resistance < 1 M Ω), and voltage clamped at –50 mV (OC-725, Warner Instruments). Oocyte superfusion at 2–3 mL/min with ND96-based solutions (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 0.8 MgCl₂, 10 HEPES, pH 7.4) containing GABA or steroids alone or in combination was controlled using computer-actuated valves (VC-8, Warner Instruments) and polytetrafluoroethylene micro-manifold (MP-8, Warner Instruments) and tubing. Currents (OC-725) were filtered at 1 kHz, digitized at 100 Hz (Digidata 1322, Molecular Devices) and recorded on a PC running ClampEx 8.0 software (Molecular Devices). Amplitudes of recorded currents were calculated as peak – baseline.

Drug-dependent gating enhancement in spontaneously-gating $\alpha 1L264T\beta 3\gamma 2L$ receptors was studied using without GABA. In $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2L$ receptors, enhancement at EC₅ or EC₁₀ GABA was assessed. Oocytes were pre-exposed to drug for 30 s prior to exposure to GABA + drug for an additional 30–60 s. Washout in ND96 between steroid exposures was for up to 10 minutes.

GABA-dependent receptor activation in the absence vs. presence of steroids was assessed in the same set of oocytes. Current activations by GABA concentrations ranging from 0.1 μ M to 1 mM were recorded, with every second or third recording a 1 mM maximal control for normalization. Current activations at the same GABA concentrations combined with a fixed concentration of steroid drug were then recorded in the same oocyte, with frequent normalizing 1 mM GABA + drug controls.

Analysis of Electrophysiological Data.

Results from repeated measures are reported as mean $\pm$ sem. Data from drug-dependent enhancement of EC₅–10 GABA responses was analyzed by non-linear least-squares fitting to a logistic function of the form:

$$\text{Enhancement Ratio} = \frac{\text{Maximum} - 1.0}{1 + \left(10^{(\log EC_{50} - X) \times n_H}\right)} + 1.0 \quad (1)$$

where X = log(Drug), EC₅₀ = half-maximal concentration, and n_H = Hill slope. EC₅₀ is reported as mean with 95% confidence interval for these fits.

Normalized GABA-dependent responses were analyzed by non-linear least-squares fitting to a logistic function of the form:

$$\text{Normalized Current} = \frac{\text{Maximum}}{1 + \left(10^{(\log EC_{50} - X) \times nH}\right)} \quad (2)$$

Statistical comparisons of repeated measures in two sets of results or two ratios of paired experiments were based on Student's t-tests. Fitted EC₅₀s in the absence vs. presence of drug were compared using sum-of-squares F-tests. All statistical calculations were performed in Prism 5.0. Statistical significance was set at $p < 0.05$.

Allosteric modulation of agonist binding to GABA_A receptors.

Human $\alpha 1\beta 3\gamma 2$ GABA_ARs were expressed in HEK293 TetR cells. Homogenized cell membranes were prepared as described previously³³ and 200 μg of $\alpha 1\beta 3\gamma 2$ GABA_AR membrane protein was resuspended in 500 μL of assay buffer (10 mM phosphate buffer, pH 7.4, 200 mM KCl, and 1 mM EDTA). The suspension was equilibrated with 2 nM [³H]muscimol and various concentrations of steroid at 4°C for 1 h. The nonspecific binding was determined in the presence of 1 mM GABA. The suspension was filtered on GF/B glass fiber filters (Whatman, Schleicher & Schuell, Maidstone, U.K.) that were pretreated in 0.5% w/v polyethyleneimine for 1 h. After receptor application, filters were washed under vacuum with 7 mL of cold assay buffer and dried under a lamp for 30 min. Subsequently, they were equilibrated in Liquiscint (Atlanta, GA) and counted (Tri-Carb 1900, liquid scintillation analyzer, Perkin-Elmer/Packard, Waltham, MA).

Photolabeling of $\alpha 1\beta 3\gamma 2$ and $\alpha 1\beta 3$ GABA_ARs.

To determine subunit level [³H]21-*p*-TFDBzox-AP incorporation and the pharmacological specificity of photolabeling, GABA_AR were photolabeled on an analytical scale (50 μL per gel lane, ~2 pmol [³H]muscimol binding sites). Appropriate volumes of [³H]21-*p*-TFDBzox-AP were dried down under a gentle stream of argon gas and resuspended in GABA_ARs in elution buffer for 30 min, on ice, such that the final concentration range of [³H]21-*p*-TFDBzox-AP was between 0.5 to 1 μM , and 300 μM GABA was added. GABA_ARs equilibrated with [³H]21-*p*-TFDBzox-AP (40 μL) were then aliquoted into glass vials (CERT5000–69LV, ThermoFisher Scientific) already containing 10 μL of GABA_AR in elution buffer. The appropriate volume of competitor solution required for the desired final concentration was delivered using a 1 μL syringe (Hamilton 86200). Samples were vortexed and incubated on ice for 45 min. Aliquots were then transferred to 96 well plates (Corning catalog no. 2797) and irradiated using a Spectroline model EN-280L 365nm lamp for 30 min on ice at <1 cm distance from the lamp. Solutions of 3 α ,5 α -P, 3 α ,5 β -P, 3 α ,5 α -THDOC, and alphaxalone dilutions were prepared from 60 mM stock solutions in ethanol. 21-*p*-TFDBzox-AP, 6-AziOAP and 6-AziP dilutions were prepared from 20 mM stocks in DMSO. During labeling, ethanol was present at 0.5 % (v/v). DMSO was present at 0.2 % (v/v) for 21-*p*-TFDBzox-AP and at 0.5 % (v/v) for 6-AziP and 6-AziOAP. GABA_AR subunits were separated by SDS-PAGE and the α/γ (56 kDa) and β (59/ 61 kDa) subunit gel bands were identified by Coomassie Blue stain. Gel bands were then either excised to determine subunit incorporation of [³H] 21-*p*-TFDBzox-AP by scintillation counting or analyzed by fluorography as described previously.¹⁹ The concentration dependence of inhibition of [³H]21-*p*-TFDBzox-AP photolabeling was fit to the equation:

$$B(x) = \frac{B_0 - B_{ns}}{1 + \left(\frac{x}{IC_{50}}\right)^{n_H}} + B_{ns}$$

where $B(x)$ is the [^3H] cpm incorporated in the $\beta 3$ subunit (59/61 kDa gel bands) at a total inhibitor concentration of x , B_0 the incorporation in the absence of inhibitor, B_{ns} the non-specific incorporation in the presence of $30 \mu\text{M}$ $3\alpha,5\alpha\text{-P}$, IC_{50} the total drug concentration that reduces the specific incorporation by 50%, and n_H is the Hill coefficient. Data were fit for individual experiments using Sigma Plot (v11, Systat Software Inc.) with IC_{50} and n_H as adjustable parameters. To combine data from individual experiments, ^3H incorporation for each experiment at each inhibitor concentration was normalized to incorporation in the absence of inhibitor (as %), and the combined data were fit to the equation. For all fits, the best fit values ($\pm$ S.E) of the variable parameters are tabulated.

Chemical Synthesis.

3,20-Diethylenedioxy-11 α -hydroxy-pregn-5-ene (18).—A mixture of 11 α -hydroxyprogesterone **17** (4.96 g, 15 mmol), ethylene glycol (10 mL) and *p*-toluenesulfonic acid monohydrate (10.0 mg) in benzene (70 mL) was refluxed for 5 h. The reaction mixture was cooled to room temperature and quenched with triethylamine (1.0 mL). The reaction mixture was evaporated and recrystallized from methanol, giving bis-ketal **18** as a white solid (4.105 g, 65%). ^1H NMR (400 MHz, CDCl_3) δ 5.40 (d, $J = 5.6$ Hz, 1H), 4.13–3.80 (m, 9H), 2.66–2.47 (m, 2H), 2.36 (dd, $J = 11.9, 4.9$ Hz, 1H), 2.11 (dd, $J = 14.0, 3.0$ Hz, 1H), 2.02–1.91 (m, 1H), 1.87–1.59 (m, 7H), 1.48–1.33 (m, 2H), 1.29 (s, 3H), 1.26–1.14 (m, 4H), 1.14–1.03 (m, 3H), 0.95–0.88 (m, 1H), 0.79 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 140.7, 122.0, 111.8, 109.4, 69.2, 65.2, 64.5, 64.3, 63.4, 58.0, 56.8, 55.9, 51.1, 42.7, 42.5, 38.5, 38.2, 31.8, 31.5, 31.3, 24.7, 23.9, 23.3, 18.7, 14.1. HRMS: calculated for $\text{C}_{25}\text{H}_{39}\text{O}_5$ $[\text{M}+\text{H}]^+$: 419.2797; found: 419.2793.

3,20-Diethylenedioxy-5 α -pregnane-6,11-dione (19).—A solution of borane-THF complex (8.0 mL, 1.0 M borane in THF) was added dropwise at room temperature to a stirred solution of 3,20-diketal **18** (837 mg, 2.0 mmol) in THF (16 mL). The resulting mixture was stirred at room temperature for 3 h. After cooling to 0°C in an ice-water bath, 3 N aqueous sodium hydroxide (6.7 mL) was added dropwise and formed white precipitate. Aqueous hydrogen peroxide (50%, 4.0 mL) was then added, and the cloudy dispersion clarified. The ice-water bath was removed and the mixture was stirred at room temperature for 1.5 h. Ethyl acetate (40 mL) was added and layers were separated, and the aqueous layer was extracted with ethyl acetate (40 mL $\times$ 3). The combined organic layer was dried by anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (pretreated with triethylamine) using hexane - ethyl acetate (1:2) to give 6,11-diol as white solid. A mixture of pyridinium chlorochromate (1.191 g, 5.5 mmol) and celite (1.191 g) in dichloromethane (15 mL) was stirred at room temperature for 20 min. Sodium acetate (906 mg, 11 mmol) was added and the above 6,11-diol in dichloromethane (4.0 mL) was added dropwise. The resulting mixture was stirred at room temperature for 4 h. The brown suspension was filtered with celite and concentrated

under vacuum. The crude product was purified by flash chromatography on silica gel (treated with triethylamine) using hexane and ethyl acetate (1:1) to give 6,11-diketone **19** as white solid (446 mg, 52%). ¹H NMR (400 MHz, CDCl₃) δ 4.06–3.80 (m, 8H), 2.79 (d, *J* = 11.5 Hz, 1H), 2.73–2.58 (m, 2H), 2.43–2.21 (m, 4H), 2.18–2.01 (m, 2H), 1.96–1.78 (m, 4H), 1.77–1.59 (m, 2H), 1.57–1.50 (m, 2H), 1.43–1.30 (m, 1H), 1.31–1.13 (m, 4H), 1.07 (s, 3H), 0.72 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 211.3, 209.7, 111.0, 107.5, 64.8, 64.4, 63.2, 58.9, 57.7, 56.8, 56.0, 49.8, 46.3, 42.5, 37.2, 37.1, 34.8, 32.6, 30.7, 24.3, 23.5, 23.1, 14.1. HRMS: calculated for C₂₅H₃₆NaO₆ [M+Na]⁺: 455.2410; found: 455.2390.

6-Azi-5α-pregnane-3,11,20-trione (22) and 6-Azi-5α-pregnane-3,11,20-trione (23).—

The reaction mixture of hydroxylamine hydrochloride (174 mg, 2.5 mmol) and sodium bicarbonate (252 mg, 3.0 mmol) in methanol (5.0 mL) was stirred at room temperature for 0.5 h. 6,11-Diketone **19** (433 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. The mixture was filtered and concentrated under vacuum giving oxime. The foregoing crude oxime was dissolved in dichloromethane (5.0 mL) and sodium nitrite (414 mg, 6.0 mmol) was added, followed by glacial acetic acid (0.43 mL). After 1 h, another portion of glacial acetic acid (0.43 mL) was added. The reaction mixture was stirred at room temperature overnight. The reaction mixture was quenched with 5% sodium bicarbonate (10 mL) and extracted with dichloromethane (20 mL×3). The combined organic phase was dried by anhydrous sodium sulfate, concentrated under vacuum, giving nitroso-oxime as a pale yellow solid. The above nitroso-oxime was dissolved in methanol (4.0 mL), cooled to –50°C, and ammonia in methanol (7 M, 4.5 mL) was added dropwise. The reaction was stirred at 0°C for 1 h, and the resulting yellow solution was concentrated and dried under vacuum, giving an 11-imine. A solution of hydroxylaminesulfonic acid (1.03 g, 9.1 mmol) in methanol (6.0 mL) under argon was cooled to –70°C and diisopropylethylamine (1.6 mL, 9.1 mmol) was added dropwise. The resulting mixture was added dropwise to the above imine at –30°C. After complete addition, the resulting yellow solution was left in a refrigerator for one week. The reaction mixture was first added with sodium bicarbonate (1.00 g) and triethylamine (2.5 mL) followed by finely ground iodine at 0°C until brown color persisted. The excess iodine was reduced with 10% aqueous sodium sulfite, and the aqueous layer was extracted with ethyl acetate (40 mL×3). The combined organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel using hexane and ethyl acetate (5:1) to give a mixture of diazirines **20** and **21** (130 mg, 29%). Treatment of the mixture of diazirines (83 mg, 0.19 mmol) with *p*-toluenesulfonic acid monohydrate (72 mg, 0.38 mmol) in acetone (4 mL) gave crude 6-azi-triketone products. The individual 6-azi-triketone products were isolated after flash chromatography on silica gel using dichloromethane - ethyl acetate (10:1) giving 6-azi-5β-triketone **22** (17 mg, 7.5% from compound **19**) and 6-azi-5α-triketone **23** (12 mg, 5.3% from compound **19**). 6-Azi-5β-pregnane-3,11,20-trione (**22**) was a white solid. ¹H NMR (400 MHz, CDCl₃) δ 2.82–2.66 (m, 3H), 2.66–2.49 (m, 3H), 2.38 (dd, *J* = 15.8, 4.5 Hz, 1H), 2.33–2.17 (m, 4H), 2.14 (s, 3H), 2.01–1.80 (m, 3H), 1.79–1.65 (m, 1H), 1.65–1.53 (m, 1H), 1.49 (s, 3H), 1.38–1.28 (m, 1H), 0.69 (s, 3H), 0.55 (ddd, *J* = 18.9, 14.0, 4.2 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 209.7, 208.2, 207.7, 62.1, 56.8, 55.4, 52.0, 50.7, 47.3, 37.4,

36.8, 36.5, 35.3, 35.2, 33.3, 31.4, 27.9, 23.8, 23.6, 22.5, 14.5. HRMS: Calculated for $C_{21}H_{29}N_2O_3$ $[M+H]^+$: 357.2178; Found: 357.2164.

6-Azi-5 α -pregnane-3,11,20-trione (**23**) was a white solid. 1H NMR (400 MHz, $CDCl_3$) δ 2.96–2.81 (m, 1H), 2.79–2.66 (m, 2H), 2.56 (d, J = 12.0 Hz, 1H), 2.50–2.35 (m, 1H), 2.34–2.17 (m, 3H), 2.16–2.08 (m, 4H), 2.00–1.63 (m, 5H), 1.55 (s, 3H), 1.37–1.15 (m, 4H), 0.68 (s, 3H), 0.66–0.59 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.3, 208.2, 207.7, 63.0, 62.1, 56.7, 55.3, 47.3, 46.9, 39.5, 37.8, 37.7, 37.4, 36.5, 35.1, 31.4, 28.4, 23.8, 23.5, 14.5, 12.3. HRMS: calculated for $C_{21}H_{29}N_2O_3$ $[M+H]^+$: 357.2178; found: 357.2164.

6-Azi-3 α -hydroxy-5 β -pregnane-11,20-dione (14).—Sodium borohydride (1.3 mg, 0.034 mmol) in 3.0 mL of 0.1 M sodium hydroxide solution was added into a stirred solution of 6-azi-5 β -triketone **22** (9.0 mg, 0.034 mmol) in ethanol (3.0 mL) at 0°C. The reaction was stirred at 0°C for 3 h. The reaction was quenched with a drop of acetic acid and extracted with ethyl acetate (15 mL $\times$ 3). The combined organic layer was dried by anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel using hexane - ethyl acetate (1:2) to give 6-azi-3 α -hydroxy-5 β -dione (**14**) as a colorless oil (8.0 mg, 67%). 1H NMR (400 MHz, $CDCl_3$) δ 3.60–3.46 (m, 1H), 2.85–2.64 (m, 3H), 2.58 (t, J = 11.8 Hz, 2H), 2.31–2.15 (m, 2H), 2.13 (s, 3H), 2.01–1.78 (m, 4H), 1.78–1.63 (m, 4H), 1.40 (s, 3H), 1.25–1.17 (m, 2H), 0.99 (td, J = 14.4, 3.2 Hz, 1H), 0.64 (s, 3H), 0.43 (dd, J = 14.4, 3.8 Hz, 1H), 0.15 (dd, J = 13.2, 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.6, 207.7, 70.1, 62.0, 56.8, 55.5, 50.7, 49.2, 47.3, 36.0, 35.2, 33.7, 33.4, 31.6, 31.3, 30.5, 28.5, 23.6, 23.3, 23.0, 14.3. HRMS: Calculated for $C_{21}H_{31}N_2O_3$ $[M+H]^+$: 359.2335; Found: 359.2330.

6-Azi-3 α -hydroxy-5 α -pregnane-11,20-dione (15).—Into the solution of 6-azi-5 α -triketone **23** (29 mg, 0.08 mmol) in tetrahydrofuran (3.0 mL) was added K-selectride (96 μ L, 1.0 M in tetrahydrofuran, 0.096 mmol) at –78°C, and the reaction mixture stirred for 40 min. The reaction was quenched with water and extracted with ethyl acetate (10 mL $\times$ 3). The combined organic phase was dried by anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel using hexane and ethyl acetate (1:2) to produce the mixture of 3 α -hydroxy-5 α -diketone and 3 β -hydroxy-5 α -diketone (23 mg). The mixture of 3 α - and 3 β -isomers was resolved via HPLC: Phenomenex Luna (2) C18 column, 250 $\times$ 21.2 mm, 5 μ m, with Macherey-Nagel Nucleodur C18 guard column, 16 $\times$ 10 mm, 5 μ m, using 10 mL/min flow-rate. Gradient conditions: phase A, methanol-water 1:1, phase B, methanol; gradient: 1% B, 0–2 min, then linear gradient to 99% B at 44 min, followed by washing with 99% B for the next 20 min. Fraction collector: 3.0 mL each fraction, starting from 23 min. Detection: UV at 254 and 350 nm wavelength, the pure diazirine fractions were identified by the characteristic absorption at 350 nm, that is about 2 times greater than that at 254 nm. Under those conditions the retention times were 28.0 min for 3 β -hydroxy-5 α -diketone and 33.6 min for 3 α -hydroxy-5 α -diketone. 6-Azi-3 α -hydroxy-5 α -pregnane-11,20-dione (**15**) was a white solid (10.0 mg, 34%). 1H NMR (400 MHz, $CDCl_3$) δ 3.94 (s, 1H), 2.72 (t, J = 9.4 Hz, 1H), 2.63 (d, J = 11.9 Hz, 1H), 2.53 (d, J = 12.0 Hz, 1H), 2.31–2.12 (m, 4H), 2.10 (s, 3H), 1.97 (d, J = 11.4 Hz, 1H), 1.87–1.62 (m, 5H), 1.55–1.46 (m, 1H), 1.38–1.29 (m, 4H), 1.28–1.17 (m, 2H),

0.70–0.58 (m, 5H), 0.54 (dd, J = 13.8, 4.4 Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 208.6, 207.8, 64.9, 63.3, 62.0, 56.7, 55.4, 47.3, 39.2, 37.9, 34.9, 31.3, 30.3, 30.2, 29.7, 28.8, 28.0, 23.5, 23.3, 14.3, 11.8. HRMS: calculated for $\text{C}_{21}\text{H}_{31}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$: 359.2335; found: 359.2318.

21-[4-(3-(Trifluoromethyl)-3H-diazirin-3-yl)benzoxy]allopregnanolone (16).—A reaction mixture of 3 α ,21-dihydroxy-5 α -pregnan-20-one (**3**, 34 mg, 0.10 mmol), TFD-benzoic acid **24** (28 mg, 0.12 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl) (27 mg, 0.14 mmol) and 4-dimethylaminopyridine (18 mg, 0.15 mmol) in dichloromethane (4.0 mL) was stirred at room temperature for 18 h. The reaction mixture was quenched with saturated sodium bicarbonate (10 mL) and extracted with dichloromethane (20 mL $\times$ 3). The combined organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The product was purified by flash chromatography on silica gel using hexane - ethyl acetate (4:1) to give compound **16** as a white solid (33 mg, 59%). ^1H NMR (400 MHz, CDCl_3) δ 8.10 (d, J = 8.3 Hz, 2H), 7.25 (d, J = 6.4 Hz, 2H), 4.97 (d, J = 16.7 Hz, 1H), 4.75 (d, J = 16.7 Hz, 1H), 4.04 (s, 1H), 2.56 (t, J = 8.5 Hz, 1H), 2.21 (dd, J = 19.8, 10.7 Hz, 1H), 2.07 (d, J = 11.0 Hz, 1H), 1.80–1.55 (m, 7H), 1.50–1.12 (m, 12H), 1.04–0.89 (m, 1H), 0.86–0.74 (m, 4H), 0.68 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 203.5, 165.0, 134.2, 130.7, 130.4, 126.5, 122.0 (q, $J_{\text{C-F}}$ = 273.2 Hz), 70.0, 66.6, 59.6, 57.1, 54.3, 45.2, 39.2, 39.0, 36.3, 36.0, 35.7, 32.3, 32.1, 29.1, 28.6, 28.6 (q, $J_{\text{C-F}}$ = 40.4 Hz), 24.6, 23.0, 20.9, 13.5, 11.3. ^{19}F NMR (376 MHz, CDCl_3) δ –64.90. HRMS: Calculated for $\text{C}_{30}\text{H}_{38}\text{F}_3\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$: 547.2784; Found: 547.2784.

21-[4-(3-(Trifluoromethyl)-3H-diazirin-3-yl)-3-iodo-benzoxy]allopregnanolone (26).—Compound **26** was prepared using the above procedure and 3-iodo-4-TFD-benzoic acid **25** (43 mg, 0.12 mmol) to give compound **26** as a white solid (34 mg, 51% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.98 (d, J = 8.3 Hz, 1H), 7.70 (d, J = 1.2 Hz, 1H), 7.29 (d, J = 8.3 Hz, 1H), 4.97 (d, J = 16.7 Hz, 1H), 4.78 (d, J = 16.7 Hz, 1H), 4.09–3.99 (m, 1H), 2.55 (t, J = 8.9 Hz, 1H), 2.21 (dd, J = 20.0, 10.8 Hz, 1H), 2.07 (dd, J = 11.6, 3.2 Hz, 1H), 1.81–1.57 (m, 7H), 1.52–1.19 (m, 12H), 1.04–0.88 (m, 1H), 0.85–0.79 (m, 1H), 0.78 (s, 3H), 0.67 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 203.2, 165.1, 139.0, 135.8, 133.9, 131.6, 126.2, 121.7 (q, $J_{\text{C-F}}$ = 273.2 Hz), 94.6, 70.1, 66.6, 59.7, 57.1, 54.3, 45.2, 39.2, 39.0, 36.3, 36.0, 35.6, 32.3, 32.1, 29.1, 28.5, 27.7 (q, $J_{\text{C-F}}$ = 41.1 Hz), 24.6, 23.0, 20.9, 13.6, 11.3. ^{19}F NMR (376 MHz, CDCl_3) δ –64.92. HRMS: calculated for $\text{C}_{30}\text{H}_{37}\text{F}_3\text{IN}_2\text{O}_4$ $[\text{M}+\text{H}]^+$: 673.1750; found: 673.1780.

Hydrodeiodination of compound 26.—A solution of compound **26** (2.0 mg, 0.0030 mmol) in MeOH (1.0 mL) was degassed by bubbling argon gas for 10 min., Pd/C (5 wt. %, 2.0 mg) was added, and the reaction mixture was degassed again by bubbling hydrogen for 10 min. The mixture was stirred at room temperature under atmospheric pressure hydrogen for 3 h. The slurry was filtered through Celite and concentrated under vacuum. The reaction mixture was dissolved in MeOH (1.0 mL), filtered through a submicron filter and chromatographed on a semi-prep HPLC column: Phenomenex Luna (2), 250 $\times$ 21.2 mm, 5 μm , C18 with Macherey-Nagel Nucleodur guard column, 16 $\times$ 10 mm, 5 μm , C18 using 10 mL/min flow rate. Gradient conditions: phase A, methanol-water 1:1, phase B, methanol;

gradient: 1% B, 0–2 min, then linear gradient to 99% B at 44 min, followed by washing with 99% B for the next 20 min. Fraction collector: 1.5 mL each fraction, starting from 49 min. Detection: UV at 254 and 350 nm wavelength, the pure diazine fractions were identified by the characteristic absorption at 350 nm. Under those conditions the retention times were 49.8 min for deiodinated compound **16** and 51.4 min for iodo-precursor **26**. The yield of compound **16** was 63%. The identity of this product with that synthesized earlier was ascertained by ^1H NMR and TLC.

Crystal Structure of Compound 22.—Colorless crystals were grown from methanol-water mixture. Data was collected at the Advanced Photon Source, LS-CAT, 21-ID-D at 100K on a crystal measuring $20 \times 50 \times 50 \mu\text{m}$ with a wavelength of $0.72927 \text{ \AA}$. The unit cell was monoclinic, $a = 6.959(2) \text{ \AA}$, $b = 32.659(8) \text{ \AA}$, $c = 8.135(2) \text{ \AA}$, $\beta = 90.338(10)^\circ$, $V = 1849 \text{ \AA}^3$, space group $P2_1$ (No. 4) with two crystallographically independent molecules in the asymmetric unit. A total of 128,725 intensities were collected to a maximum resolution of $0.83 \text{ \AA}$. The intensities were later scaled and averaged to a total of 9913 independent reflections, $R(\text{merge}) = 0.0525$. Structure solution using SHELXS. Refinement of all atoms, with anisotropic Gaussian displacement parameters for the non-hydrogen atoms. Hydrogen atoms were placed with geometric restraints. The final refinement yielded $R2 = 0.2342$, $R1 = 0.0881$ for 3972 intensities greater than 2σ , and $R1 = 0.0919$ for all 3972 reflections with a $\text{GOF} = 1.036$. The absolute stereochemistry was not well determined; the Flack parameter was estimated by two methods: $-0.3(5)$ by the traditional method, and $-0.3(5)$ by the Parson's method; the Hooft y -parameter was determined to be $-0.5(6)$, G -parameter was determined to $2(1)$, and the probability of correct stereochemical assignment is 0.945.

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ABBREVIATIONS USED

DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	methyl sulfoxide
EC_5	concentration required for 5% of full effect
EC_{50}	concentration required for half-maximal effect
GABA	γ -aminobutyric acid
ECD	extracellular domain
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide

GABA_AR	GABA _A -type receptor
IC₅₀	concentration required for 50% of full inhibitory effect
GLIC	<i>Gloeobacter</i> ligand-gated ion-channel
LoRR	loss of righting reflexes
n_H	Hill coefficient
R-mTFD-MPAB	(R)-5-allyl-1-methyl-5-[m-trifluoromethyldiazirynyl]phenyl]-barbituric acid
THDOC	tetrahydrocorticosterone
THF	tetrahydrofuran
TFD	trifluoromethyldiazirine
TMD	transmembrane domain
3α,5α-P	allopregnanolone
3α,5β-P	pregnanolone
PS	pregnenolone sulfate
SD	standard deviation

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Figure 1.

Locations of binding sites for GABA, benzodiazepines, and general anesthetics in an $(\alpha 1)_2(\beta 3)_2\gamma 2$ GABA_AR. GABA binds in the extracellular domain at the interfaces between the β and α subunits referred to as the $\beta^+ - \alpha^-$ subunit interface, and with that nomenclature continued, the benzodiazepines bind to a homologous site at the $\alpha^+ - \gamma^-$ subunit interface. Depicted in the transmembrane domain are the four transmembrane helices (M1–M4) in each subunit, the homologous binding sites for etomidate and *R-m*TFD-MPAB, a mephobarbital analog, in the extracellular third of the $\beta^+ - \alpha^-$ and $\alpha^+ - \gamma^-$ subunit interfaces, respectively, and binding sites for neurosteroids in the intracellular third of the $\beta^+ - \alpha^-$ subunit interfaces.

Figure 2.
ORTEP drawing of the x-ray structure of compound **22**.

Figure 3.

Modulation of GABA-induced currents in $\alpha 1\beta 3\gamma 2L$ GABA_AR by 6-azi-OP and 6-azi-OAP. (A) 6-Azi-OAP and 6-azi-OP enhance EC₅ GABA (4 μM) currents in WT $\alpha 1\beta 3\gamma 2L$ GABA_AR-expressing *Xenopus* oocytes. Example traces are shown from electrophysiological experiments, each set in a single oocyte. (B) Concentration dependence of the enhancements ratios for 6-azi-OP and 6-azi-OAP. Points represent mean $\pm$ sem (6-azi-OAP; filled circles; $n = 3-4$ and 6-azi-OP; filled squares; $n = 3-5$). Lines through data represent non-linear least-squares fits to Equation (1).

Figure 4.

(A) 21-pTFDBzox-AP enhances EC₁₀ (1 μM GABA) currents in α1β3 and EC₅ (4 μM GABA) currents in α1β3γ2L GABA_ARs expressed in *Xenopus* oocytes. Enhancement ratio is defined as $I_{EC10}(GABA + 21\text{-pTFDBzox-AP}) / I_{EC10}(GABA)$. The inset shows example traces from a single oocyte. Plotted symbols represent mean ± sem (n = 4). The line through data was generated by fitting to Equation (1). Hill slopes of both dose-response curves were constrained to 1.7 (fitted value from the [³H]muscimol binding assay). Without n_H constraint, maximal enhancement and EC₅₀ were indeterminate. Fitted parameters for α1β3 are: maximal enhancement ratio = 2.1 ± 0.4 ; EC₅₀ = 2.7 ± 0.5 μM. Fitted parameters for α1β3γ2L are: maximal enhancement ratio = 2.6 ± 0.3 ; EC₅₀ = 3.0 ± 0.7 μM. (B) 21-pTFDBzox-AP enhancement of α1β3γ2L receptor gating is eliminated by the steroid site mutation α1Q242W. Bars represent mean ± sem (n = 4 each) enhancement ratios normalized to EC₅ GABA (4 μM) responses in the same oocyte. Tetrahydrodeoxycorticosterone (THDOC) and 21-pTFDBzox-AP were both applied at 10 μM. Both steroids significantly enhanced gating of WT α1β3γ2L receptors (compared to 1.0 in two-tailed t-test; p = 0.0056 for THDOC and p = 0.015 for 21-pTFDBzox-AP), but did not significantly enhance gating of α1Q242Wβ3γ2L receptors (p = 0.54 for THDOC and p = 0.057 for 21-pTFDBzox-AP). Unpaired Student's t-tests showed significantly different enhancement in WT vs. αQ242W

with both THDOC (n=4; p =0.0007) and 21-pTFDBzox-AP (n=4; p= 0.0015). (C) 21-pTFDBzox-AP at 2 μ M directly activated currents in oocytes expressing the spontaneously gating receptor mutant α 1L264T β 3 γ 2L. Bars represent mean $\pm$ sem (n = 4). 21-pTFDBzox-AP at 2 μ M elicited approximately 21% of the maximal GABA response (p = 0.0012 by paired t-test). Picrotoxin at 2 mM produced outward currents averaging about 40% of maximal GABA-induced inward currents.

Figure 5. 6-azi-OAP (A) and 6-azi-OP (B) shift GABA dose-response curve to the left in $\alpha 1\beta 3\gamma 2L$ GABA_AR- expressing *Xenopus* oocytes. 6-azi-OAP (15 μ M) shifted the fitted EC₅₀ value approximately three-fold, from 25 ± 1.0 to 9.1 ± 1.1 μ M (sum-of squares F test for logEC₅₀, $F=53.45$, $p<0.0001$). 6-azi-OP (30 μ M) also shifted the fitted EC₅₀ value approximately 3.5-fold, from 59 ± 1.0 to 17 ± 1.0 μ M. (Sum-of squares F test for logEC₅₀, $F=302.4$, $p<0.0001$). 21-pTFDBzox-AP (C) did not shift the GABA dose response curve in WT $\alpha 1\beta 3\gamma 2L$ -expressing oocytes (sum-of squares F test for logEC₅₀, $F=0.1808$, $p=0.6730$).

Figure 6.

Steroid photolabels enhance agonist binding in a concentration – dependent manner. Typical experiments for steroid modulation of a subsaturating concentration (2 nM) of $[^3\text{H}]$ muscimol binding are shown. (A) Both 6-azi-OP and 6-azi-OP efficaciously modulate $[^3\text{H}]$ muscimol binding to $\alpha 1\beta 3\gamma 2\text{L}$ receptors, but with relatively low potency. 6-Azi-OAP was slightly more efficacious and potent than 6-azi-OP. Nonlinear least squares fitting to the Hill equation yielded EC_{50} values, Hill coefficients and maximum effects that are given in the text. (B) 21-pTFDBzox-AP enhances $[^3\text{H}]$ muscimol binding to $\alpha 1\beta 3$ receptors with higher efficacy but comparable potency to $\alpha 1\beta 3\gamma 2\text{L}$ receptors. (C) A similar pattern is observed in receptors reconstituted into CHAPS:Asolectin (5 mM: 200 μM), although the efficacy is lower.

Figure 7.

Allopregnanolone inhibits [3H]21-pTFDBzox-AP photolabeling of $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ GABA_ARs. (A) Aliquots of $\alpha1\beta3$ (lanes 1, 3–6) and $\alpha1\beta3\gamma2$ GABA_AR (lanes 2, 7–10) were equilibrated with 0.8 μM [3H]21-pTFDBzox-AP in the presence of 100 μM bicuculline (BIC) or 300 μM GABA, with (+) or without (–) 30 μM allopregnanolone (3 $\alpha,5\alpha$ -P). After irradiation, the aliquots were fractionated by SDS-PAGE and the gel was stained with Coomassie Blue (lanes 1 and 2, representative lanes). The stained gel was then prepared for fluorography (lanes 3–10), dried and exposed to film for 21 days. Denoted on the left are the mobilities of the molecular mass markers and the calculated mobilities of the GABA_AR subunit bands ($\alpha1$, 56 kDa; $\beta3$, 59/61 kDa). (B) In a parallel experiment, irradiated samples were fractionated by SDS-PAGE, the stained gel bands containing the $\alpha1$ and $\beta3$ subunits

were excised, and ^3H incorporation was determined by liquid scintillation counting. The means $\pm 1/2$ range are plotted from 2 gels.

Figure 8.

Pharmacological specificity of [^3H]21-*p*TFDBzox-AP photolabeling of $\alpha 1\beta 3$ GABA_AR. Aliquots of GABA_ARs were photolabeled in the presence of the indicated concentrations of anesthetic steroids (3 α ,5 β -P, 3 α ,5 α -THDOC, alphaxalone, 6-AziOP, 6-AziOAP, and 21-*p*TFDBzox-AP), the antagonist steroid pregnenolone sulfate (PS), etomidate, or the mephobarbital analog, *R*-*m*TFD-MPAB. Covalent incorporation of radioactivity was determined by liquid scintillation counting of $\beta 3$ subunits isolated by SDS-PAGE. For each experiment, ^3H cpm incorporation was normalized to total cpm incorporated in the absence of competitor, and the plotted data are the means ($\pm$ SD) from 4 independent experiments. The concentration dependence of inhibition was fit to a single site model with variable Hill

coefficient (see Methods), with the non-specific subunit photolabeling determined in the presence of 30 μ M allopregnanolone (3 α ,5 α -P). Non-specific photolabeling, which varied between experiments with different purifications of GABA_AR, was $38 \pm 3\%$ for the data of Panel A (dotted line), and in Panel B it was $13 \pm 2\%$ for 21-pTFDBzox-AP, $27 \pm 2\%$ for alphaxalone, and $22 \pm 6\%$ for 6-AziOAP and 6-AziOP.

i: (CH₂OH)₂, TsOH; ii: (a) BH₃-THF, (b) NaOH, H₂O₂; iii: PCC, NaOAc; iv: (a) NH₂O-HCl, NaHCO₃; (b) NaNO₂, AcOH; (c) NH₃ - MeOH; (d) NH₂OSO₃H, DIPEA; (e) I₂, Et₃N; v: TsOH-H₂O; vi: NaBH₄; vii: K-selectride

Scheme 1.

Synthesis of photoprobes 6-azi-11-oxo-allopregnanolone (**14**, 6-azi-OAP) and 6-azi-11-oxo-pregnanolone (**15**, 6-azi-OP).

Scheme 2.

Synthesis of 21-(*p*-trifluoromethyldiazirinybenzox)-allopregnanolone (21-pTFDBzox-AP, **16**) and [³H]21-pTFDBzox-AP (**³H-16**).

Chart 1.
Structures of anesthetic steroids (**1-4**) and their photoaffinity analogs (**5-16**) used to probe GABA_AR binding sites.

Table 1.

Comparison of potency of various steroid ligands as modulators of 2 nM [³H]muscimol binding [EC₅₀] and as inhibitors of photolabeling of GABA_ARs with [³H]21-pTFDBzox-AP [IC₅₀].

Steroid ligand	Stereo-chemistry	EC ₅₀ [μM]	SD (±)	IC ₅₀ ^{c,d}	SD (±)
21-pTFDBzox-AP ^{a,b}	3α,5α	0.52	0.25	ND	
21-pTFDBzox-AP ^{a,c}	3α,5α	0.50	0.06	ND	
21-pTFDBzox-AP ^{d,b}	3α,5α	0.36	0.06	ND	
21-pTFDBzox-AP ^{d,c}	3α,5α	0.16	0.03	0.15	0.02
6-Azi-OP ^a	3α,5β	37	1.7	>100	
6-Azi-OAP ^a	3α,5α	25	2.5	44	6
Allopregnanolone ^{d,b}	3α,5α	0.58	0.22		
Pregnanolone ^{d,b}	3α,5β	0.62	0.08	0.7	0.13
Alphaxalone ^{d,b}	3α,5α	0.71	0.09	6	0.1
THDOC ^{d,b}	3α,5α	1.00	0.15	2.3	0.35
Pregnenolone sulfate	3β	>500		No effect	

^a α1β3γ2L receptor;

^b native membranes;

^c purified receptor preparation;

^d α1β3 receptor. The EC₅₀ for alphaxalone was previously reported in reference 22.