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## Synthesis and Pharmacological Evaluation of Neurosteroid Photoaffinity Ligands

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### Abstract

Neuroactive steroids are potent positive allosteric modulators of GABA<sub>A</sub> receptors (GABA<sub>A</sub>R), but the locations of their GABA<sub>A</sub>R binding sites remain poorly defined. To discover these sites, we synthesized two photoreactive analogs of alphaxalone, an anesthetic neurosteroid targeting GABA<sub>A</sub>R, 11 $\beta$ -(4-azido-2,3,5,6-tetrafluorobenzoyloxy)allopregnanolone, (F4N3Bzoxo-AP) and 11-aziallopregnanolone (11-AziAP). Both photoprobes acted with equal or higher potency than alphaxalone as general anesthetics and potentiators of GABA<sub>A</sub>R responses, left-shifting the GABA concentration – response curve for human  $\alpha$ 1 $\beta$ 3 $\gamma$ 2 GABA<sub>A</sub>Rs expressed in *Xenopus* oocytes, and enhancing [<sup>3</sup>H]muscimol binding to  $\alpha$ 1 $\beta$ 3 $\gamma$ 2 GABA<sub>A</sub>Rs expressed in HEK293 cells. With EC<sub>50</sub> of 110 nM, 11-AziAP is one the most potent general anesthetics reported. [<sup>3</sup>H]F4N3Bzoxo-AP and [<sup>3</sup>H]11-AziAP, at anesthetic concentrations, photoincorporated into  $\alpha$ - and  $\beta$ -subunits of purified  $\alpha$ 1 $\beta$ 3 $\gamma$ 2 GABA<sub>A</sub>Rs, but labeling at the subunit level was not inhibited by alphaxalone (30  $\mu$ M). The enhancement of photolabeling by <sup>3</sup>H-azietomidate and <sup>3</sup>H-*m*TFD-MPAB in the presence of either of the two steroid photoprobes indicates the neurosteroid binding site is different from, but allosterically related to, the etomidate and barbiturate sites. Our observations are consistent with two hypotheses. First, F4N3Bzoxo-AP and 11-aziAP bind to a high affinity site in such a pose that the 11-photoactivatable moiety, that is rigidly attached to the steroid backbone, points away from the protein. Second, F4N3Bzoxo-AP, 11-aziAP and other

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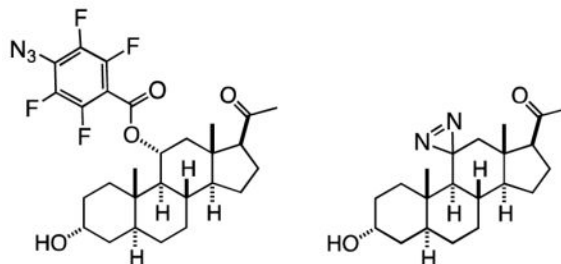
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steroid anesthetics, which are present at very high concentration at the lipid-protein interface due to their high lipophilicity, act via low affinity sites, as proposed by Akk et al. (*Psychoneuroendocrinology* **2009**, *34S1*, S59–S66).

## Graphical Abstract



Azide and diazirine analogs of the neurosteroid general anesthetic drug, alphaxalone, were synthesized and were found to efficiently photolabel heteropentameric GABA<sub>A</sub> receptors.

## INTRODUCTION

Alphaxalone (**1**) is a potent synthetic neurosteroid modulator of GABA<sub>A</sub>R,<sup>1–3</sup> a member of a class of compounds that are analogs of endogenous GABA<sub>A</sub>R modulators, such as certain pregnanolones and corticosterones.<sup>4</sup> Neurosteroids were clinically used as anesthetics in the past, but have been withdrawn due to toxic and other unwanted effects.<sup>5,6</sup> Some of these compounds are still used in veterinary medicine, or remain in clinical evaluations.<sup>7,8</sup> The binding site(s) for neurosteroids is/are different from those of etomidate, propofol, barbiturate and benzodiazepines, and presumably located deeper in the transmembrane domains.<sup>9</sup> The pharmacology of these steroids differs from the other modulators mentioned above, especially with regard to δ-subunit-containing receptors.<sup>9</sup> These intriguing features of their action make them worthwhile targets of investigation.

Our design of a neurosteroid photoprobe is based on the emerging pharmacophore for this class of compounds.<sup>10–14</sup> Briefly, steroids featuring both planar (**1**, allopregnanolone-type, 5α) and kinked (**2**, pregnanolone-type, 5β) scaffold geometry at the A/B ring junction are active,<sup>10</sup> however, 5α-isomers display much larger difference between activities of natural and unnatural enantiomers,<sup>10</sup> potentially indicating different binding sites for 5α- and 5β-isomers. The presence of the 3α-hydroxyl group is essential,<sup>11</sup> whereas the 3β-position can be substituted with a variety of alkyl and aryloxyalkyl appendices.<sup>11</sup> Interestingly, both enantiomers of the steroids are effective ligands, but they seem to bind in different orientations; the natural enantiomers (eg. **1** & **2**, shown above) bind with the upper edge of their steroid framework *pointing out* of the cavity, whereas the unnatural enantiomers (not shown) bind with the upper edge of their steroid framework *pointing into* the cavity.<sup>12</sup> Consistently, the 11-position of the natural enantiomer of the steroid can be functionalized with sterically large groups,<sup>12,13</sup> but in contrast, the 6-position of the natural enantiomer can be modified with only a small group, such as a diazirine.<sup>15,16</sup> The presence of the oxygenated group at the 11-position is nonessential, but if hydroxylated, the 11β-

configuration is preferred.<sup>16</sup> The 17-position can be altered by a replacement of the acetyl group with a keto-group or 17 $\beta$ -nitrile, suggesting that the modulatory activity relies on the presence of a dipole in this part of molecule.<sup>14</sup> The only neurosteroid-based photoprobes reported thus far are 6-azipregnanolone (**3**),<sup>15</sup> and 11 $\beta$ -(*p*-TFD-benzyloxy)pregnanolones **4** and **5**.<sup>17</sup> The photoprobe **3** has photolabeled Phe301 in the M3 helix of the homomeric  $\beta$ 3 receptor. However, it remains unclear if this residue is present at the steroid binding site, since inhibition of photolabeling (or even attempts at) by a pregnanolone drug was not reported.<sup>16</sup>

Given the existing SAR described above and the large size of the steroid scaffold, it should be possible to use photolabels to define not only the steroid site or sites of action, but also their orientation in the binding site. Our strategy in pursuit of this goal is to construct four types of photolabels, where the photoreactive residue is attached at the top (C-11), bottom (C-6), left (C-3) and right sides (C-17) of the steroid framework. In this manuscript, we report the synthesis of the two C-11-modified photoprobes, 11 $\beta$ -(*p*-azidotetrafluorobenzoyloxy)allopregnanolone (**6**, F4N3Bzox-AP), and 11-aziAP (**7**, 11-AziAP), and their use for the photolabeling of the  $\alpha$ 1 $\beta$ 3 $\gamma$ 2 isoform of the GABA<sub>A</sub> receptor.

## RESULTS

**Synthesis of 11 $\beta$ -(4-azido-2,3,5,6-tetrafluorobenzoyloxy)allopregnanolone 6 (F4N3Bzox-AP)**—The 9-step synthesis of the compound **6** shown in Scheme 1 started from the commercially available 11 $\alpha$ -hydroxyprogesterone **8**, which, under mild condition, was selectively converted into the 20-ketal **9**. The double bond at the 4-position was stereoselectively reduced with lithium in liquid ammonia<sup>18</sup> into the 5 $\alpha$ -isomer **10**, and the 11 $\alpha$ -hydroxy group was inverted into 11 $\beta$  orientation by the oxidation-reduction sequence. Toward this goal, the 11 $\alpha$ -hydroxyl was first oxidized with pyridinium dichromate, and the resulting 3,11-diketone **11** was regio- and stereo-selectively reduced at the 3-position with Selectride-K into the 3 $\alpha$ -alcohol and silylated with dimethyl-*tert*-butylsilyl chloride to generate compound **12**. The 11-keto group was subsequently stereoselectively reduced into 11 $\beta$ -alcohol **13** with lithium aluminium hydride, and the 11-hydroxyl group was acylated with pentafluorobenzoyl chloride into the corresponding ester **14**. The silyl and ketal protective groups were removed by acidic methanolysis to afford the penultimate intermediate **15**. In the final step, compound **15** was converted into the azide **6** by the substitution of the fluoride at the *p*-position of pentafluorobenzoate using tetra-*n*-butylammonium azide – TMS azide reagent.

**Tritiation of F4N3Bzox-AP 6**—The synthesis of the tritiated photoprobe <sup>3</sup>H-**6** is shown in Scheme 2. While the simplest way to introduce tritium into **6** would comprise the oxidation-reduction sequence at the 3-position, such procedure with the available highly tritiated agents (*e.g.* NaBT<sub>4</sub>) would provide predominantly an undesirable 3 $\beta$ -isomer.<sup>19</sup> We opted instead for introduction of tritium via saturation of the double bond, that typically also provides higher specific radioactivity due to incorporation of two tritium atoms per probe molecule. Here, the treatment of the intermediate **15a** with TMS iodide in the presence of hexamethyldisilazane produced the corresponding enol silyl ether, and addition of

phenylselenenyl chloride into the double bond afforded the selenide **16**. Oxidation into selenoxide with hydrogen peroxide resulted in a spontaneous elimination, while the deprotection of the 3-silyl group with HCl in methanol yielded the  $\alpha,\beta$ -unsaturated ketone **17**. In a small-scale reaction, hydrogenation of the double bond over palladium catalyst afforded cleanly a 17 $\beta$ -acetyl product **15**, while the subsequent *in situ* azidation produced **6**, that was identical to that produced via Scheme 1. Finally, using this optimized procedure, the reduction of the double bond in **17** with tritium gas followed by azidation was performed by Vitrax Co. to afford  $^3\text{H}$ -**6** with specific radioactivity of 45 Ci/mmol.

**Synthesis of 11-aziallopregnanolone 7 (11-aziAP)**—Starting from the diketone **11** (Scheme 3), the reduction at the 3-position with Selectride-K afforded stereoselectively the  $\alpha$ -alcohol **18**. It is noteworthy that due to very high steric hindrance, the reduction of the 11-keto group was not observed. Due to this steric hindrance, however, application of the typical general procedure used for the installation of the diazirine residue at position-11 was not successful. Instead, we used an alternative method involving transient formation of ketimine<sup>20</sup> applicable to highly hindered ketone substrates. This procedure consisted of five steps carried out sequentially without purification of the intermediates, including formation of the oxime **19**, its nitrosation into the nitrite **20**, formation of the imine **21**, the reaction of the imine with hydroxylamine sulfonic acid to form the diaziridine **22**, and its oxidation with iodine into the diazirine **23** in a very low overall yield. Finally, the hydrolysis of the 20-ketal afforded the ultimate compound **7**.

**Tritiation of the photoprobe 7**—Our tritiation procedure was based on the saturation of the double bond at the 4-position using a modification of Scheme 3, whereby the assembly of a diazirine was performed at the 4-ene analog of **23** and the catalytic saturation of the double bond was carried out just before the ultimate ketal deprotection. The synthesis of tritiated  $^3\text{H}$ -**7** is shown in the Scheme 4.

Here, the intermediate **9** from the above (Scheme 1) was oxidized at the 11-position with chlorochromate in pyridine into a diketone **24**, and the 3-keto group was reduced with sodium borohydride to provide the 3- $\beta$  alcohol **25**. This compound was subjected to diazination as described above to provide the diazirine **26**, and the alcohol function was inverted using the Mitsunobu procedure to provide the 3- $\alpha$ -alcohol **27**. The critical step of stereoselective hydrogenation to provide the 5 $\alpha$ -intermediate **28** was carried out using the homogeneous rhodium catalyst **29**. Lastly, hydrolysis of the ketal group afforded the product **7** that was indistinguishable from that produced via Scheme 3. Saturation of the double bond in compound **27** with a tritium gas, carried out by Vitrax Co., followed by hydrolysis of the ketal group afforded the tritiated photoprobe  $^3\text{H}$ -**7** with an estimated specific radioactivity of 40 Ci/mmol.

**Proof of structures of photoprobes 6 and 7**—Given that  $^1\text{H}$  NMR analyses did not provide the necessary clarity with regard to the stereochemical structures of the final products, we have obtained the structural proof in the form of x-ray structures. Although we were unsuccessful in crystallizing the final products **6** and **7**, we obtained the crystalline

forms of the penultimate intermediates **15** and **28**, and solved their x-ray crystal structures (Figure 1).

The x-ray data prove unequivocally that these compounds, and hence by inference, also the final compounds **6** and **7**, have the desired stereochemical structures.

### Pharmacological Properties

**Anesthetic Potency of F4N3Bzox-AP and 11-AziAP**—The anesthetic potency of F4N3Bzox-AP was studied as loss of righting reflexes (LoRR) in tadpoles, using the concentration range from 30 nM to 10  $\mu$ M, and compared to alphaxalone as a reference. Similarly, 11-aziAP was studied over the 10 nM – 1  $\mu$ M range. Anesthesia with all agents was reversible, and all animals recovered within an hour after transfer to fresh water. The potency of the F4N3Bzox-AP and 11-aziAP were 2.6- and 17-fold greater, respectively, than that of alphaxalone. [Agent, EC<sub>50</sub> ( $\mu$ M), Slope, # of animals: Alphaxalone,  $1.9 \pm 0.36$   $\mu$ M,  $1.7 \pm 0.5$ , 51; F4N3Bzox-AP,  $0.72 \pm 0.09$   $\mu$ M,  $2.1 \pm 0.68$ , 70; 11-aziAP,  $110 \pm 18$  nM,  $1.7 \pm 0.5$ , 70, Errors are standard deviations].

**Enhancement of the GABA<sub>A</sub> Receptor Currents by F4N3Bzox-AP and 11-AziAP**—In human  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor expressed in *Xenopus* oocytes, EC<sub>5-10</sub> GABA chloride currents were dramatically potentiated by alphaxalone, F4N3Bzox-AP and 11-aziAP (30-, 17- and 30-fold, respectively) (Figure 2) with EC<sub>50</sub> values of  $2.2 \pm 0.8$   $\mu$ M,  $0.5 \pm 0.2$   $\mu$ M and  $0.2 \pm 0.1$   $\mu$ M, respectively.

**Modulation of the GABA Dose-Response Curves by F4N3Bzox-AP and 11-AziAP**—Interactions of  $\alpha 1\beta 3\gamma 2L$  receptor with alphaxalone, F4N3Bzox-AP and 11-aziAP, resulted in a profound left-shifts of the GABA concentration – response curves consistent with positive allosteric modulation of the agonist site (Figure 3). Thus, the GABA EC<sub>50</sub> = 4.5  $\mu$ M in the absence of the modulator decreased to 0.5  $\mu$ M in the presence of 2.5  $\mu$ M alphaxalone, 70 nM in the presence of 1.4  $\mu$ M F4N3Bzox-AP and 1.23  $\mu$ M in the presence of 1.4  $\mu$ M 11-aziAP.

**Modulation of Agonist-Binding to GABA<sub>A</sub> Receptors by F4N3Bzox-AP and 11-AziAP**—To further assess the allosteric action, the abilities of alphaxalone, F4N3Bzox-AP and 11-aziAP to modulate the binding of 2 nM [<sup>3</sup>H]muscimol to  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>Rs membranes were compared (Figure 4). Displaceable binding of [<sup>3</sup>H]muscimol was enhanced in a concentration-dependent manner by all three agents between 30 nM and 3  $\mu$ M, above which it plateaued. The potencies of alphaxalone, F4N3Bzox-AP, and 11-aziAP were comparable with EC<sub>50</sub> =  $450 \pm 62$ ,  $770 \pm 14$ , and  $250 \pm 43$  nM, respectively. The Hill coefficients were  $1.0 \pm 0.1$ ,  $1.9 \pm 0.1$  and  $1.1 \pm 0.2$ , respectively. The efficacies of the modulation were comparable at  $235 \pm 2$ ,  $228 \pm 4$  and  $227 \pm 2$  % for alphaxalone, F4N3Bzox-AP, and 11-aziAP, respectively.

**F4N3Bzox-AP and 11-AziAP Enhance [<sup>3</sup>H]Azietomidate and [<sup>3</sup>H]R-mTFD-MPAB Photolabeling of Purified Human  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> Receptor**—For GABA<sub>A</sub>Rs photolabeled in the absence of GABA, F4N3Bzox-AP at 1 or 10  $\mu$ M enhanced [<sup>3</sup>H]azietomidate or [<sup>3</sup>H]R-mTFD-MPAB photoincorporation by ~50% and 75%,

respectively, i.e. to the same extent as alphaxalone at 30  $\mu\text{M}$  or GABA at 300  $\mu\text{M}$  (Figure 5A), concentrations that produce maximal enhancement of photolabeling.<sup>22</sup> Similarly, 11-aziAP at 0.3 and 1  $\mu\text{M}$  enhanced photolabeling by both probes to the same extent as GABA or alphaxalone (Figure 5B). F4N3Bzox-AP and 11-aziAP potentiation of [<sup>3</sup>H]azietomidate and [<sup>3</sup>H]*R*-*m*TFD-MPAB photolabeling established that when bound to the GABA<sub>A</sub>R, it also does not prevent [<sup>3</sup>H]azietomidate binding in the pocket near the extracellular end of the transmembrane domain between the  $\beta\text{M2}/\beta\text{M3}$  helices and  $\alpha\text{M2}/\alpha\text{M1}$  helices or [<sup>3</sup>H]*R*-*m*TFD-MPAB binding to the homologous pocket between the  $\alpha\text{M2}/\alpha\text{M3}$  (or  $\gamma\text{M2}/\gamma\text{M3}$ ) and  $\beta\text{M2}/\beta\text{M1}$  helices. The enhancement rather than inhibition of photolabeling indicate that the photoreactive steroids bind to sites distinct from either the etomidate or barbiturate site, and that the latter sites interact allosterically with the steroid binding sites.

#### **GABA<sub>A</sub> Receptor Photolabeling by [<sup>3</sup>H]F4N3Bzox-AP and [<sup>3</sup>H]11-aziAP—**

Purified  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>Rs were photolabeled in the absence of other drugs, or in the presence of bicuculline (100  $\mu\text{M}$ , resting state), GABA (300  $\mu\text{M}$ , desensitized state), or GABA (300  $\mu\text{M}$ ) and alphaxalone (30  $\mu\text{M}$ ), (Figure 6). Photoincorporation was characterized by SDS-PAGE, with <sup>3</sup>H incorporation determined by fluorography (Fig. 6A) or by liquid scintillation counting of excised gel bands containing GABA<sub>A</sub>R subunits (Figs. 6B and C). Based upon fluorography, [<sup>3</sup>H]F4N3Bzox-AP and [<sup>3</sup>H]11-aziAP were each incorporated into the three gel bands revealed by Coomassie Blue stain, a band of 56-kDa, containing by sequence analysis primarily the  $\alpha 1$  subunit, and bands of 59- and 61-kDa containing primarily  $\beta 3$  subunits<sup>22</sup>. The  $\gamma 2$  subunit is broadly distributed in this gel region and present in all three stained bands. Based upon liquid scintillation counting, photoincorporation of either photoreactive steroid at the subunit level in the absence or presence of GABA or bicuculline differed by less than 10%, and in the presence of GABA, alphaxalone altered photolabeling by <10%. Based upon the level of <sup>3</sup>H cpm incorporation into the subunits and the pmols of GABA<sub>A</sub>R loaded on the gel, at 0.7  $\mu\text{M}$  [<sup>3</sup>H]F4N3Bzox-AP photolabeled ~10% and 6% of  $\beta 3$  and  $\alpha 1$  subunits, while [<sup>3</sup>H]11-aziAP photolabeled ~0.4% and 0.2% of  $\beta 3$  and  $\alpha 1$  subunits, respectively. Based upon the  $\alpha$ -subunit photolabeling by [<sup>3</sup>H]azietomidate (Figure 5A) and by [<sup>3</sup>H]F4N3Bzox-AP (Figure 6B) and the radiochemical specific activities of the ligands, [<sup>3</sup>H]azietomidate at 3.5  $\mu\text{M}$  and [<sup>3</sup>H]F4N3Bzox-AP at 0.7  $\mu\text{M}$  photolabeled 10% and 6% of subunits, respectively. This suggests that when photolabeled at the same concentrations, they incorporate with similar efficiency.

## **DISCUSSION AND CONCLUSIONS**

In the past decade, binding sites of several allosteric modulators on GABA<sub>A</sub> receptor have been determined, including those for volatile halogenated ether anesthetics,<sup>24–25</sup> propofol,<sup>26–27</sup> etomidate<sup>28–29</sup> and barbiturates.<sup>22,30</sup> Most of these modulators serve as popular clinically used general anesthetics, sedatives or anticonvulsants. Determining the location of the modulator binding sites, and the conformational state of the receptor targeted (open, closed or desensitized), could prove useful to the understanding of the mechanism of allosteric modulation of GABA<sub>A</sub>R, and ultimately help in development of more efficient and safer GABA-ergic drugs. Despite much therapeutic success with the above listed drugs, the

location of the neurosteroid binding site on the heteropentameric GABA<sub>A</sub>R remains an open question.<sup>9,16,17</sup> Both naturally occurring and synthetic neurosteroids, such as pregnanolones and allopregnanolones, are known potent GABA<sub>A</sub>R modulators,<sup>3,4,9,31</sup> and could potentially be used as general anesthetics and anticonvulsants. The mixture of alphaxalone and alfadolone (21-hydroxylated alphaxalone) has been previously used as a general anesthetic, althesin.<sup>32</sup> Although it has been withdrawn from the clinic due to potentially fatal side effects, these side effects have been later attributed to the solubilizing lipid excipient used in the drug formulation,<sup>6</sup> rather than the pharmacologically active constituent. Another neurosteroid, ganaxalone (3-methyl-allopregnanolone), is in phase 3 clinical studies as an antiepileptic.<sup>33,34</sup> Given the potential broad clinical applications of neurosteroids and their different type of structure as compared to other GABA<sub>A</sub>R ligands, determining the binding site of neurosteroids and understanding their mode of action remains an important biomedical goal.

As with other general anesthetics, our approach toward this objective has been to synthesize photoreactive analogs of alphaxalone, compare their pharmacological properties with those of the parent anesthetic, and if they bear close resemblance, to photolabel the receptor and determine the site of the probe's photoincorporation. Similar efforts have been previously carried out by others, whereby photoactivatable pregnanolones and allopregnanolones have been used in an analogous fashion, most notably, 6-azipregnanolone **3**<sup>15</sup> and 11-[4-(TFD-benzyloxy)]pregnanolone **4** (and its corresponding allo-isomer **5**).<sup>17</sup> Compound **3** was found to photoincorporate into Phe301 in the third transmembrane domain (M3) of the  $\beta$ 3-homomeric receptor.<sup>16</sup> This area would constitute a novel modulator binding site located closer to the cytoplasmic face of the membrane, as compared to the other known intersubunit binding sites for general anesthetics that are located closer to the extracellular domain.<sup>22,27</sup> However, these workers did not establish the pharmacological specificity of the photolabeling at Phe301, so this interpretation should be regarded with caution. On the other hand, compounds **4** and **5** were found to replicate pharmacology of their parent compounds, and to photolabel GABA receptors in rat brain membranes, however their subunit specificities and the sites of photoincorporation were not determined.<sup>17</sup>

In our work, we also chose to modify the position-11 of alphaxalone to synthesize photoreactive anesthetics incorporating either a bulky azidotetrafluorophenyl residue, which produces a photoreactive nitrene, or a smaller diazirine, similar to the photoreactive group in azietomidate. Structure-activity studies of naturally and unnaturally configured neurosteroid analogs<sup>12</sup> suggest that neurosteroids featuring a natural stereochemical structure interact with the GABA<sub>A</sub> receptor using the lower edge of their steroid framework,<sup>12</sup> thereby permitting large scale structural modifications to the upper edge. An uncertainty related to this mode of receptor – modulator interaction was that if correct, a photoreactive group linked to the upper edge might be exposed to interactions with membrane lipids, and hence would not be an efficient protein photolabel, but instead would predominantly modify the membrane lipids.

### F4N3Bzox-AP and 11-aziAP Display Pharmacological Properties Similar to Alphaxalone

This work involves extensive pharmacological characterization of the F4N3Bzox-AP and 11-aziAP photoprobes. In all assays performed, F4N3Bzox-AP and 11-aziAP closely reproduced the pharmacology of the parent compound, alphaxalone, except in most assays they were more potent (Figures 2–4). Thus, they were more potent general anesthetics than alphaxalone in the LORR assay; they were also strong and efficacious positive allosteric modulators of the heteropentameric  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>R in oocytes, potentiating the ion currents 17- and 30-fold, respectively. Likewise, both left-shifted the GABA dose-response curve, and modulated the binding of agonist muscimol by GABA<sub>A</sub>R similarly to alphaxalone.

### Neurosteroid Site Is Distinct From Those of Either Etomidate or Barbiturate

Similar to alphaxalone,<sup>22</sup> F4N3Bzox-AP and 11-aziAP each enhanced photolabeling by the two site-selective photoprobes, [<sup>3</sup>H]azietomidate and [<sup>3</sup>H]*R*-*m*TFD-MPAB (Figure 5). This result indicates that these steroid anesthetics do not compete for the binding to the intersubunit sites we recently located at the extracellular end of the transmembrane domain at the  $\beta^+-\alpha^-$  (etomidate<sup>28</sup>) and at the  $\alpha^+-\beta^-$  and  $\gamma^+-\beta^-$  subunit interfaces (barbiturate).<sup>22</sup> This result is also consistent with the earlier ones showing an enhancement of anesthetic potency of etomidate by neurosteroids<sup>35</sup> and of chromaffin cell's responses to natural neurosteroids in the presence of a variety of barbiturates.<sup>36</sup> Our results confirm that these sites (etomidate, barbiturate and neurosteroid) are nonidentical, but their occupancies are allosterically coupled.<sup>22</sup>

### Photolabeling of the GABA<sub>A</sub> Receptor by F4N3Bzox-AP and 11-AziAP

[<sup>3</sup>H]F4N3Bzox-AP photoincorporated into the GABA<sub>A</sub> receptor  $\alpha$  and  $\beta$  subunits at an efficiency similar to that seen for [<sup>3</sup>H]azietomidate. The major difference between the photoprobes is that 80% of [<sup>3</sup>H]azietomidate subunit photolabeling was pharmacologically specific, i.e. inhibitable by etomidate, while alphaxalone at 30  $\mu$ M did not inhibit [<sup>3</sup>H]F4N3Bzox-AP photolabeling at the subunit level. [<sup>3</sup>H]11-AziAP also photoincorporated into GABA<sub>A</sub>R  $\alpha$  and  $\beta$  subunits, but at only ~5% the efficiency of [<sup>3</sup>H]F4N3Bzox-AP, and also with no inhibition by 30  $\mu$ M alphaxalone.

### Possible modes of interactions of F4N3Bzox-AP and 11-aziAP with GABA<sub>A</sub>R

Both F4N3Bzox-AP and 11-aziAP are potent allosteric effectors of GABA<sub>A</sub> receptors whose binding site is distinct from, but allosterically coupled to, those of both etomidate and *R*-*m*TFD-MPAB. The fact that a large substitution at the 11-position of the steroid framework does not compromise its interactions with the GABA receptor is consistent with the established SAR.<sup>10–14</sup> Nonetheless, the observed photolabeling was not pharmacologically specific. The simplest explanation for these observations is that both F4N3Bzox-AP and 11-aziAP bind with the photoreactive moieties oriented away from the binding site as suggested by Krishnan et al.<sup>12</sup> Inspection of Figure 1 shows that in F4N3Bzox-AP, the photoactive moiety has extremely limited mobility relative to the rigid steroid backbone. Thus, if the latter is tightly bound in certain orientations, the



photoactivated group would be incapable of photoincorporation. This is consistent with the suggestion that such agents bind with the upper edge pointing out of the binding site.<sup>12</sup>

If the above hypothesis is correct, the GABA<sub>A</sub> receptor photolabeling we observed must result primarily from nonspecific interactions. The high lipophilicity of neuroactive steroids in general, including our new photoprobes, as well as compounds **3–5**, means that the lipid phase magnifies the concentration of the ligand.<sup>31</sup> For example, alphaxalone, with a partition coefficient of  $\log P = 3.1$ ,<sup>31</sup> reaches a near millimolar concentration in the membrane at bulk phase concentrations close to the potentiation EC<sub>50</sub> (0.6  $\mu$ M).<sup>9,31</sup> Due to even higher lipophilicity of F4N3Bzox-AP (by ca. 1.5 log P units as compared to alphaxalone), its membrane concentration is likely even higher. At such high concentration of ligand in proximity to the receptor, nonspecific photolabeling is probably unavoidable, and it is possible that a pharmacologically specific (inhibitible) portion of the overall (specific plus nonspecific) photolabeling is too small to be discerned at the subunit level, but perhaps could be detected at the amino acid level. For example, the hydrophobic photoprobe [<sup>3</sup>H]diazofluorane appeared to photolabel the nicotinic acetylcholine receptor (nAChR) in a pharmacologically non-specific manner at the subunit level, whereas state-dependent, pharmacologically specific photolabeling was identified at the amino acid level within the ion channel by sequence analysis of appropriate subunit fragments.<sup>37</sup> However, preliminary results from sequencing GABA<sub>A</sub> receptors photolabeled with F4N3Bzox-AP, have not yet revealed such sites.<sup>38</sup>

We cannot rule out that our results are consistent with the hypothesis developed by Akk et al.<sup>31</sup> that in spite of submicromolar potencies of neurosteroid anesthetics, specific binding of these ligands to the receptor protein occurs with much lower, possibly millimolar, intrinsic affinity for the GABA<sub>A</sub>R because, as noted above, the actual concentration at the lipid-receptor interface is millimolar.<sup>31</sup>

Development of the additional neurosteroid photoprobes proposed in the introduction will be required to resolve these issues in a more definitive manner. The known structure–activity relationships suggest it may be difficult to achieve high potency with substitutions larger than the diazirine residue. The limitation of aliphatic diazirines is that they photolabel only a limited number of amino acids bearing nucleophilic side chains. In any event, it may be advantageous for such agents to have lower membrane partition coefficients to decrease their membrane concentration and to have smaller photoreactive residues located closer to the steroid backbone or other positions of the steroid scaffold to enhance the chance of photoincorporation into the binding site and to limit potential lipid photolabeling.

## MATERIALS AND METHODS

### Analytical Chemistry

<sup>1</sup>H, <sup>13</sup>C and <sup>19</sup>F NMR spectra were recorded on Bruker Avance spectrometer at 400 MHz, 100 MHz and 376 MHz, respectively, unless otherwise noted. NMR chemical shifts were referenced indirectly to TMS for <sup>1</sup>H and <sup>13</sup>C, and CFCl<sub>3</sub> for <sup>19</sup>F 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  $\mu\text{m}$ , 4.60  $\times$  150 mm) using methanol-water with 0.05% trifluoroacetic acid; the gradient applied was from 50% MeOH at the start to 100% MeOH over 18 min, followed by isocratic elution. Elution was monitored by UV at 254 nm. The analysis indicated purity greater than 96%. Preparative Shimadzu LC-20 HPLC system was used for all preparative and analytical separations, unless otherwise stated.

## Materials

11 $\alpha$ -Hydroxyprogesterone was from Santa Cruz Biotechnology, Inc. Anhydrous grade solvents were from Aldrich, and were not further dried or purified. [ $^3\text{H}$ ]F4N3Bzox-AP (45 Ci/mmol) and [ $^3\text{H}$ ]-11-aziAP (40 Ci/mmol) were synthesized by ViTrax Company (Placentia, CA) by tritiation of compounds **15** and **25**, respectively, using the procedure described below. [ $^3\text{H}$ ]Azietomidate (12 Ci/mmol) and [ $^3\text{H}$ ]R-*m*TFD-MPAB (38 Ci/mmol) were prepared as described.<sup>30,40</sup> Human  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>Rs with a FLAG epitope on the N-terminus of the  $\alpha 1$  subunit was purified as described<sup>41</sup> on an anti-FLAG antibody column from detergent extracts of membrane fractions from a tetracycline-inducible HEK293S cell line. GABA<sub>A</sub>R was eluted from the column with 0.1 mM FLAG peptide in elution buffer containing 5 mM CHAPS, 0.2 mM asolectin and then stored at  $-80^\circ\text{C}$  until use.

## Determination of the x-ray structures of compound 15 and 23

A crystal measuring roughly 10  $\times$  10  $\times$  30  $\mu\text{m}$  was selected for data collection. It was encased in silicon oil, drawn into a nylon loop, and cooled to 100 K. Data collection was carried out at beam line 21-ID-D, LS-CAT, Advanced Photon Source, Argonne National Laboratory, using a MAR 300mm CCD detector, with a wavelength of 0.72849  $\text{\AA}$  and a crystal-to-detector distance of 95 mm; 180 images were collected, each from a 2 $^\circ$  rotation with an exposure of 1 s. Integration of the data with XDS resulted in a total of 67,769 reflections, and an average of 6090 unique reflections to a resolution of 0.76  $\text{\AA}$ . The unit cell was identified as hexagonal, and subsequent analysis led to the selection of the space group as P6<sub>2</sub> (No. 171) or P6<sub>4</sub> (No. 172). The structure was solved with SHELXS, and refined with SHELXL-2013. All hydrogen atoms were evident in the difference electron density map, and placed in calculated positions; hydrogen atoms on the methyl and hydroxyl groups were restrained to ride on the adjacent atom at fixed bond lengths. The inverted structure was also refined, and resulted in the selection of space group as P6<sub>4</sub> (No. 172). A water molecule is present in the structure along the *c*-axis, and the hydrogen atoms were not evident in the electron density difference map. Since the water could not be adequately modeled, it was removed from the refinement model. After convergence, SQUEEZE was run to modify the structure factors. Thus, we assign a water molecule to this structure, at half occupancy along the *c*-axis, but it is not included in the final reported model. There is substantial libration with the fluoroaromatic ring, and the fluorine atoms were modeled as single atoms with large librational motion perpendicular to the plane of the aromatic ring. We were unable to determine the absolute configuration of the structure with this data. The final agreement is good, with RF(all) = 0.0819, RF(2 $\sigma$ ) = 0.0795, Rw(all) = 0.2071, goodness-of-fit = 1.046.

## Electrophysiology of GABA<sub>A</sub> Receptors

With prior approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, oocytes were obtained from adult, female *Xenopus laevis* (Xenopus One, Dextor, Michigan) and prepared using standard methods as described below. *In vitro* transcription from linearized cDNA templates and purification of subunit specific cRNAs was carried out using Ambion mMessage Machine RNA kits and spin columns. Oocytes were injected with ~100 ng total mRNA ( $\alpha 1$ ,  $\beta 2$ ,  $\gamma 2L$ ) mixed at a ratio of 1:1:2 transcribed from human GABA receptor subunit cDNAs in pCDNA3.1.<sup>42</sup> All two-electrode voltage clamp experiments were done at room temperature, with the oocyte transmembrane potential clamped at  $-50$  mV and with continuous oocyte perfusion with ND96 (100 mM NaCl, 2 mM KCl, 10 mM Hepes, 1 mM EGTA, 1 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, pH 7.5) at ~2 mL/min. Barbiturate stock solutions were prepared in DMSO at a concentration of 100 mM for storage at  $-20^{\circ}\text{C}$ . Compounds were further diluted in ND96 to achieve the desired concentration (the highest final DMSO concentration was 1%). All agents were applied for 15–25 s; oocytes were washed ~3 min between each application. Currents were amplified using an Oocyte Clamp OC-725C amplifier (Warner Instrument Corp), digitized using a Digidata 1322A (Axon Instruments, Foster City, CA), and analyzed using Clampex/Clampfit 8.2 (Axon Instruments) and OriginPro 6.1 software.

Concentration response data were fit by nonlinear least squares regression to the Hill (logistic) equation (1) of the general form:

$$I_X/I_{\text{GABA,max}} = (I_{X,\text{max}}/I_{\text{GABA,max}}) \times (1/(1 + (\text{EC}_{50}/[X])^{n_H})) \quad (1)$$

where X is the concentration of the activating ligand,  $I_{\text{GABA,max}}$  is the maximally evoked current,  $\text{EC}_{50}$  is the concentration of X eliciting half of its maximal effect, and  $n_H$  is the Hill coefficient of activation. Inhibition experiments were fit with logistic equations of the form:

$$I = 1 - ([X]^{n_H}/(\text{IC}_{50}^{n_H} + [X]^{n_H})) \quad (2)$$

## Allosteric regulation of agonist binding to GABA<sub>A</sub> receptors

Human  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptors were expressed in HEK293 TetR cells. Homogenized cell membranes were prepared as described previously,<sup>40</sup> and 200  $\mu\text{g}$  of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptor membrane protein was resuspended in 500  $\mu\text{L}$  of assay buffer (10 mM phosphate buffer, pH 7.4, 200 mM KCl, and 1 mM EDTA). The suspension was equilibrated with 2 nM [<sup>3</sup>H]muscimol and various concentrations of steroid at  $4^{\circ}\text{C}$  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 $\alpha 1\beta 3\gamma 2$ GABA<sub>A</sub>Rs

Aliquots of purified GABA<sub>A</sub>R in elution buffer were photolabeled on an analytical scale (35–75  $\mu$ L/aliquot containing  $\sim 3$  pmol [<sup>3</sup>H]muscimol binding sites) to determine subunit photoincorporation and the effects of non-radioactive anesthetics on photolabeling. An appropriate volume of a tritiated, photoreactive anesthetic solution in methanol was transferred into a glass tube. After solvent was evaporated to near dryness under a stream of argon gas, freshly thawed GABA<sub>A</sub>R in elution buffer was added to the tube, and the radioligand was resuspended at 4°C by gentle agitation for 30 min to final concentrations of 0.4 – 0.7  $\mu$ M [<sup>3</sup>H]F4N3-alphaalone or [<sup>3</sup>H]11-aziAP, 3  $\mu$ M [<sup>3</sup>H]azietomidate, or 1  $\mu$ M [<sup>3</sup>H]*R*-mTFD-MPAB. Aliquots were then transferred to glass vials, and appropriate amounts of non-radioactive drugs were added using a 1  $\mu$ L glass syringe (Hamilton #7101) from stock solutions prepared in methanol such that the final methanol concentration was 0.5% (v/v). After another 30 min incubation (4 °C), the samples were transferred to the wells of plastic microtiter plates and irradiated on ice for 30 min at 365 nm with a Spectroline model EN-280L lamp at a distance of < 1 cm. After photolysis, GABA<sub>A</sub>R subunits were resolved by SDS-PAGE, and <sup>3</sup>H incorporation into GABA<sub>A</sub>R subunits was determined by liquid scintillation counting or fluorography as described.<sup>22</sup>

**20-Ethylenedioxy-11 $\alpha$ -hydroxy-pregn-4-ene-3-one (9)**—A suspension of finely powdered 11 $\alpha$ -hydroxyprogesterone (**8**, 2.00 g, 6.06 mmol) in dry ethylene glycol (50 mL) was vigorously stirred for 30 min at room temperature and pyridinium *p*-toluenesulfonate (0.80, 1.58 mmol) was added at once, followed by trimethyl orthoformate (1.0 mL, 9.14 mmol). The reaction mixture was left stirred at room temperature for 48 h (control – TLC in ethyl acetate – hexane, 7:3). The reaction mixture was neutralized with saturated solution of sodium bicarbonate (10 mL), the newly formed precipitate was filtered off and washed with water (2 $\times$ 10 mL), dried on air and recrystallized from the mixture of methanol and triethylamine (100:1), filtered, washed with small amount of MeOH (2  $\times$  1 mL) and dried on air to give a white solid (1.023 g, 45%). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  5.73 (s, 1H, -CH=), 4.07 – 3.83 (m, 5H, OCH<sub>2</sub>CH<sub>2</sub>O + 11 $\alpha$ -CH), 2.67 (dt, 1H, *J* = 13.8 Hz, 6.4 Hz), 2.50 – 2.22 (m, 5H), 2.01 (td, 1H, *J* = 13.8 Hz, 4.5 Hz), 1.90 – 1.70 (m, 4H), 1.70 – 1.60 (m, 1H), 1.60 – 1.44 (m, 1H), 1.32 (s, 3H, CH<sub>3</sub>), 1.30 (s, 3H, CH<sub>3</sub>), 1.28 -1.00 (m, 5H), 0.84 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  200.2, 171.3, 124.4, 111.5, 68.9, 65.1, 63.2, 59.2, 57.8, 55.1, 51.4, 42.6, 39.9, 37.5, 34.5, 34.2, 33.7, 24.5, 23.6, 23.0, 18.3, 14.2. HR MS: predicted for C<sub>23</sub>H<sub>33</sub>O<sub>4</sub> [M-H]<sup>+</sup>: 373.2379; found 373.24.

**11 $\alpha$ -Hydroxy-20-ethylenedioxy-5 $\alpha$ -pregnane-3-one (10).**<sup>18</sup>—A mixture of freshly distilled ammonia (50 mL), dry toluene (20 mL) and absolute THF (5mL) was cooled to –78°C under argon with stirring. Lithium metal (50 mg, 7.2 mmol) was added at once and stirring continued until full dissolution of the metal ( $\sim$  1 hour at –78°C). The ketal **9** (1.00 g, 2.67 mmol) was dissolved in boiling toluene (10 mL) under the stream of argon, cooled with stirring and redissolved in dry THF (10 mL). The resulted solution was added dropwise over 5 min into vigorously stirred Li-NH<sub>3</sub>-THF-toluene mixture at –78°C. After one hour, the reaction mixture was quenched by addition of solid NH<sub>4</sub>Cl (5 g, stirred at –78°C until the disappearance of the blue color) and left to warm up to room temperature overnight while the ammonia gas evaporated. The resulting mixture was partitioned between ethyl acetate

and water (50 mL ea.), the organic phase was washed with brine and water (20 mL ea.), dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum. Chromatography on silica gel in ethyl acetate – hexane (1:1, 0.2% of triethylamine) afforded saturated hydroxyketone **10** as a slowly crystallizing white solid (734 mg, 73%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.06 – 3.83 (m, 5H), 2.78 (ddd, *J* = 13.8 Hz, 6.4 Hz, 2.2 Hz, 1H), 2.48 – 2.24 (m, 4H), 2.10 (ddd, *J* = 15.0, ~4.1 Hz, 2.4 Hz), 1.85 (dd, *J* = 10.7 Hz, 8.4 Hz, 1H), 1.81 – 1.48 (m, 6H), 1.48 – 1.30 (m, 3H), 1.30 (s, 3H, CH<sub>3</sub>), 1.27 – 1.07 (m, 2H), 1.15 (s, 3H, CH<sub>3</sub>), 1.04 – 0.91 (m, 2H), 0.89 – 0.80 (m, 1H), 0.81 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 212.1, 111.6, 69.0, 65.1, 63.2, 60.0, 58.0, 55.4, 51.6, 47.4, 45.2, 42.5, 40.2, 38.4, 37.2, 34.1, 31.5, 29.4, 24.5, 23.8, 23.0, 14.1, 11.8. HR MS: predicted for C<sub>23</sub>H<sub>37</sub>O<sub>4</sub> [M+H]<sup>+</sup>: 377.2692; found 377.2694.

**20-Ethylenedioxy-5α-pregnane-3,11-dione (11)**—A suspension of CrO<sub>3</sub> (2.00 g, 20 mmol) in dry dichloromethane (10 mL) was cooled with stirring to –20°C under argon, and dry pyridine (5 mL, 63 mmol) was added dropwise with vigorous stirring. The resulted dark suspension was stirred 30 min at –20°C, then 30 min at 0°C, and the solution of hydroxyketone **10** (700 mg, 1.86 mmol) in DCM (5 mL) was added dropwise. The reaction mixture was stirred for 1 h on ice bath and 2 h at 20°C. The mixture was cooled to –10°C and quenched with a mixture of ice and saturated aqueous NaHCO<sub>3</sub> (5 mL each). The resulted heavy and dark suspension was stirred for 5 h with the mixture of ethyl acetate, aqueous NaHCO<sub>3</sub> (20 mL ea.) and EDTA (6 g, ~20 mmol). The layers were separated, the aqueous phase was extracted with ethyl acetate (2×20 mL), the combined organic phases were washed with water (2×20 mL), brine (10 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. Chromatography on silica gel (eluent hexane – ethyl acetate 4:1 + 0.1% NEt<sub>3</sub>) afforded pure 3,11-dione **11** as colorless needles (571 mg, 82%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.05 – 3.83 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub>O), 2.80 (ddd, *J* = 13.1 Hz, 6.4 Hz, 2.0 Hz, 1H), 2.64 (d, *J* = 12.4 Hz, 1H), 2.46 (td, *J* = 14.8 Hz, 6.5 Hz), 2.35 – 2.20 (m, 3H), 2.15 – 1.97 (m, 2H), 1.95 – 1.60 (m, 7H), 1.55 – 1.42 (m, 1H), 1.41 – 1.30 (m, 2H), 1.26 (s, 3H, CH<sub>3</sub>), 1.22 (s, 3H, CH<sub>3</sub>), 1.25 – 1.05 (m, 1H), 0.75 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 211.5, 211.0, 111.2, 64.8, 63.7, 63.2, 57.7, 56.8, 55.3, 47.0, 46.0, 44.3, 38.0, 37.1, 36.3, 35.1, 32.2, 28.3, 24.3, 23.5, 23.4, 14.6, 11.1. HR MS: predicted for C<sub>23</sub>H<sub>35</sub>O<sub>4</sub> [M+H]<sup>+</sup>: 375.2535; found: 375.2525.

**3α-(tert-Butyldimethylsilyloxy)-20-ethylenedioxy-5α-pregnane-11-one (12)**—Regio- and stereo-selective reduction of 3,11-diketone **11** (555 mg, 1.48 mmol) at the 3 position with Selectride-K in THF at –78°C was performed as described previously,<sup>18</sup> except urea – hydrogen peroxide complex was used instead of pure aqueous H<sub>2</sub>O<sub>2</sub>. The crude 3α-alcohol from the previous step (yield was nearly quantitative) was stirred with the solution of TBDMSCl (452 mg, 3.00 mmol), imidazole (408 mg, 6.00 mmol) in DMF (2 mL) for 2 days at room temperature. A usual work-up and chromatography on silica gel (eluent hexane – ethyl acetate, 9:1 + 0.1% NEt<sub>3</sub>) afforded 3α-silyloxyketone **12** as colorless crystals (559 mg, 77% in two stages). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.03 – 3.83 (m, 5H), 2.59 (d, *J* = 12.2 Hz, 1H), 2.29 (d, *J* = 12.2 Hz, 1H), 2.15 (dt, *J* = 12.7, 3.4 Hz, 1H), 2.04 (t, *J* = 9.3 Hz, 1H), 1.91 – 1.65 (m, 7H), 1.65 – 1.50 (m, 1H), 1.50 – 1.38 (m, 1H), 1.32 – 1.12 (m, 6H), 1.25 (s, 3H, CH<sub>3</sub>), 1.00 (s, 3H, CH<sub>3</sub>), 0.90 (s, 9H, *tert*-Bu), 0.73 (s, 3H, CH<sub>3</sub>), 0.03 (s, 6H, Si-CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 211.7, 111.3, 66.7, 64.9, 64.4, 63.2, 57.9, 57.0, 55.6, 46.0,

39.0, 36.4, 36.4, 35.6, 32.7, 31.1, 29.5, 28.1, 25.9, 24.3, 23.5, 23.3, 18.1, 14.1, 11.1, -4.8, -4.9. HR MS: predicted for C<sub>29</sub>H<sub>51</sub>O<sub>4</sub>Si [M+H]<sup>+</sup>: 491.3557; found: 491.3548.

**3 $\alpha$ -(*tert*-Butyldimethylsilyloxy)-20-ethylenedioxy-11 $\beta$ -hydroxy-5 $\alpha$ -pregnane (13)**—A solution of silyloxyketone **12** (530 mg, 1.08 mmol) in absolute ether (10 mL) under argon was stirred with lithium aluminium hydride (40 mg, 1.05 mmol). After two hours, the reaction mixture was cooled to -78°C, quenched with acetone (1 mL), and added with 10% aq. NH<sub>4</sub>Cl (5 mL) and EDTA-sodium hydroxide buffer with pH 7 (~10% EDTA, 10 mL). The resulted suspension was left stirred while warming up and partitioned between ethyl acetate (50 mL) and water (20 mL). An extraction and chromatography (eluent hexane – ethyl acetate 9:1 to 4:1) afforded pure 11 $\beta$ -hydroxysteroid **13** as colorless crystals (392 mg, 74%). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  4.34 -4.28 (m, 1H, 11 $\alpha$ -CH), 4.04 - 3.83 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub>O + 3 $\beta$ -CH), 2.21 (dd, *J* = 14.1, 2.2 Hz, 1H), 1.84 – 1.62 (m, 6H), 1.62 – 1.38 (m, 7H), 1.31 (s, 3H, CH<sub>3</sub>), 1.27 – 1.10 (m, 4H), 1.10 – 0.95 (m, 2H), 1.02 (s, 3H, CH<sub>3</sub>), 0.99 (s, 3H, CH<sub>3</sub>), 0.90 (s, 9H, *tert*-Bu), 0.80 (d, *J* = 7.0, 3.0 Hz), 0.03 (s, 6H, Si-CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  111.9, 68.4, 66.7, 65.0, 63.2, 58.7, 58.2, 57.9, 48.4, 41.3, 39.9, 36.2, 36.1, 32.3, 32.1, 30.7, 29.4, 28.1, 25.9, 24.5, 23.7, 22.7, 15.8, 14.6, -4.9.

**3 $\alpha$ -(*tert*-Butyldimethylsilyloxy)-20-ethylenedioxy-11 $\beta$ -(pentafluorobenzoyloxy)-5 $\alpha$ -pregnane (14)**—A solution of 11 $\beta$ -hydroxysteroid **13** (190 mg, 0.385 mmol) in anhydrous THF (5 mL) under argon was cooled with stirring to -78°C, and butyllithium (0.29 mL, 1.6 M in hexane) was added dropwise. The reaction mixture was stirred for additional 10 min, and pentafluorobenzoyl chloride (0.067 mL, 0.46 mmol) was added dropwise, followed by *N,N*-diisopropylethylamine (0.1 mL, 0.57 mmol). After an overnight stirring, a usual work-up and chromatography (hexane – ethyl acetate 24:1), the pentafluorobenzoyl derivative **14** was crystallized from methanol (190 mg, 72%). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  5.71 (q, *J* = 2.5 Hz, 1H, 11 $\alpha$ -CH), 4.04 - 3.80 (m, 5H, OCH<sub>2</sub>CH<sub>2</sub>O + 3 $\beta$ -CH), 2.47 (dd, *J* = 14.9, 1.8 Hz, 1H), 1.86 – 1.65 (m, 6H), 1.62 – 1.45 (m, 6H), 1.45 -1.32 (m, 2H), 1.28 (s, 3H, CH<sub>3</sub>), 1.27 – 1.05 (m, ~8H), 1.02 (s, 3H, CH<sub>3</sub>), 0.90 (s, 9H, *tert*-Bu), 0.87 (s, 3H, CH<sub>3</sub>), 0.81 (d, *J* = 7.0, 3.0 Hz), 0.03 (s, 6H, Si-CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  158.7, 144.5 (dm, *J* ~ 255.8 Hz), 142.7 (dm, *J* ~ 258.7 Hz), 137.6 (dm, *J* ~ 250.7 Hz), 111.6, 74.2, 66.5, 65.0, 63.2, 58.5, 57.4, 57.0, 44.5, 41.0, 40.2, 36.2, 35.8, 32.2, 32.1, 31.4, 29.7, 29.2, 27.8, 25.9, 24.5, 23.6, 22.7, 18.1, 14.8, 13.9, -4.9. <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  -138.6 (m, 2F, *o*-CF), -149.9 (tt, *J* = 17.5, 3.3 Hz, 1F, *p*-CF), -160.2 (m, 2F, *m*-CF). HR MS: predicted for C<sub>36</sub>H<sub>52</sub>O<sub>5</sub>F<sub>5</sub>Si [M+H]<sup>+</sup>: 687.3504; found: 687.3530.

**3 $\alpha$ -Hydroxy-11 $\beta$ -(pentafluorobenzoyloxy)-pregnane-20-one (15) and 3 $\alpha$ -(*tert*-butyldimethylsilyloxy)-11 $\beta$ -(pentafluorobenzoyloxy)-pregnane-20-one (15a)**—A stirred solution of silyloxy-ketal **14** (168 mg, 0.245 mmol) in the MeOH-DCM mixture (2:1, 3 mL) was treated with 0.5 M solution of hydrogen chloride in methanol at room temperature. After 1h (control – TLC), the reaction mixture was divided between aq. NaHCO<sub>3</sub> and ethyl acetate (10 mL each). Organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuum. Chromatography on silica gel afforded hydroxyketone **15** (36 mg, 28%) and silyloxy-ketone **15a** (110 mg, 70%) as colorless crystalline solids.

**15:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.78 (q, *J*=2.6 Hz, 1H, 11α-CH) 4.06 (s, 1H, 3β-CH<sub>3</sub>), 2.51 (t, *J*=9.3 Hz, 1H), 2.46 (dd, *J*=14.5, 2.2 Hz, 1H), 2.25 – 2.12 (m, 1H), 2.12 (s, 3H, COCH<sub>3</sub>), 1.90 – 1.35 (m, 14H), 1.35 – 1.26 (m, 6H), 1.13 (dd, *J*=10.9, 3.1 Hz, 1H), 1.08 – 0.95 (m, 1H), 0.82 (s, 3H, CH<sub>3</sub>), 0.73 (s, 3H, CH<sub>3</sub>). <sup>19</sup>F NMR (CDCl<sub>3</sub>): δ -138.2 (m, 2F, *o*-CF), -149.1 (tt, *J*=21.0, 3.2 Hz, 1F, *p*-CF), -160.0 (m, 2F, *m*-CF). HR MS: calculated for C<sub>28</sub>H<sub>33</sub>O<sub>4</sub>F<sub>5</sub>Na [M+Na]<sup>+</sup>: 551.2197; found: 551.2202.

**15a:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.77 (q, *J*=2.8 Hz, 1H, 11α-CH) 3.96 (s, 1H, 3β-CH<sub>3</sub>), 2.51 (t, *J*=9.0 Hz, 1H), 2.45 (dd, *J*=14.4, 2.2 Hz, 1H), 2.25 – 2.12 (m, 1H), 2.12 (s, 3H, COCH<sub>3</sub>), 1.90 – 1.72 (m, 4H), 1.72 – 1.45 (m, 6H), 1.45 – 0.98 (m, 11H), 1.28 (s, 3H, CH<sub>3</sub>), 1.27 – 1.05 (m, ~8H), 0.90 (s, 9H, *tert*-Bu), 0.80 (s, 3H, CH<sub>3</sub>), 0.74 (s, 3H, CH<sub>3</sub>), 0.04 (s, 3H, Si-CH<sub>3</sub>), 0.03 (s, 3H, Si-CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 208.7 (COMe), 66.4, 63.8, 57.7, 57.0, 43.9, 42.8, 40.2, 36.1, 35.9, 32.3, 32.2, 31.8, 31.3, 29.1, 27.7, 25.9, 24.2, 22.5, 22.3, 18.1, 15.1, 14.0, -4.9 (Si-CH<sub>3</sub>). <sup>19</sup>F NMR (CDCl<sub>3</sub>): δ -138.2 (m, 2F, *o*-CF), -149.1 (tt, *J*=21.0, 3.2 Hz, 1F, *p*-CF), -160.0 (m, 2F, *m*-CF). HR MS: calculated for C<sub>34</sub>H<sub>47</sub>F<sub>5</sub>NaO<sub>4</sub>Si [M+Na]<sup>+</sup>: 665.3061; found: 665.3068.

### 11β-(4-Azido-2,3,5,6-tetrafluorobenzoyloxy)-3α-hydroxy-5α-pregnane-20-one

**(6)**—A solution of an azidation reagent was prepared by dissolving tetra-*n*-butylammonium azide (10 mg, 0.035 mmol) and trimethylsilyl azide (0.05 mL, 0.37 mmol) in dry deuterated acetone. A solution of pentafluorobenzoyloxy derivative **15** (35.3 mg, 0.0668 mmol) in dry deuterated acetone (0.6 mL), was mixed with 0.05 mL of the reagent solution at room temperature. After 1h, <sup>19</sup>F NMR showed a complete and clean conversion of pentafluorobenzoyl derivative into the 4-azido-2,3,5,6-tetrafluorobenzoyl derivative. The reaction mixture was diluted with methanol, evaporated in the stream of air, dissolved in methanol – ethyl acetate mixture (1 mL ea.) and treated with methanolic HCl (0.1 mL, 0.5M). After 1 h at room temperature, the reaction mixture was divided between ethyl acetate (10 mL) and water (5 mL), washed with brine and water (5 ml ea.), dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum. Chromatography on silica gel in ethyl acetate – hexane 2:3 to 1:1 v/v afforded pure target steroid **6** as white colorless solid (35.2 mg, 96%).

**6:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.77 (q, *J*=2.7 Hz, 1H, 11α-CH) 4.06 (s, 1H, 3β-CH<sub>3</sub>), 2.51 (t, *J*=9.2 Hz, 1H), 2.45 (dd, *J*=14.5, 2.4 Hz, 1H), 2.25 – 2.12 (m, 1H), 2.12 (s, 3H, COCH<sub>3</sub>), 1.93 – 1.15 (m, 24H), 1.12 (dd, *J*=11.0, 3.2 Hz, 1H), 1.08 – 0.96 (m, 1H), 0.82 (s, 3H, CH<sub>3</sub>), 0.74 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 208.6, 159.0, 144.9 (d, *J*=~252 Hz), 140.6 (d, *J*=~251 Hz), 72.9, 66.1, 63.8, 57.9, 57.0, 44.0, 42.8, 40.1, 36.0, 35.2, 32.2, 32.0, 31.8, 31.3, 28.6, 27.6, 27.0, 24.2, 22.5, 15.1, 13.7. <sup>19</sup>F NMR (CDCl<sub>3</sub>): δ -138.4 (m, 2F), -150.5 (m, 2F). HR MS: calculated for C<sub>28</sub>H<sub>33</sub>F<sub>4</sub>N<sub>3</sub>NaO<sub>4</sub> [M+Na]<sup>+</sup>: 574.2305; found: 574.2308.

### 3α-(tert-Butyldimethylsilyloxy)-11β-(pentafluorobenzoyloxy)-17-phenylselenylpregnane (16) and 3α-Hydroxy-11β-(pentafluorobenzoyloxy)-pregn-17-ene

**(17).**<sup>39</sup>—The solution of the silyl ether **15a** (114 mg, 0.177 mmol) in dry pentane (5 mL) under argon was cooled with stirring to -25°C. Hexamethyldisilazane (44 μL, 0.212 mmol) was added at once, followed by trimethylsilyl iodide (28 μL, 0.195 mmol). The reaction mixture was stirred at -20°C for 30 min, then left to warm up overnight. The mixture was

cooled with stirring to  $-25^{\circ}\text{C}$  and quenched with saturated aqueous  $\text{NaHCO}_3$  (1 mL), left to warm up to room temperature, and stirred with water (1 mL) until full dissolution of solids ( $\sim 30$  min). This mixture was cooled to  $-30^{\circ}\text{C}$ , the organic layer was decanted, solid layer was washed with pentane ( $2 \times 1$  mL), the combined organic phases were dried over  $\text{Na}_2\text{SO}_4$  and concentrated in the stream of argon. The resulted colorless oil was dissolved in absolute THF and cooled with stirring under argon to  $-78^{\circ}\text{C}$ , and dry pyridine (20  $\mu\text{L}$ , 0.25 mmol) was added, followed by a solution of phenylselenenyl chloride (40.6 mg, 0.212 mmol) in THF (0.5 mL). The mixture was stirred for 30 min at  $-78^{\circ}\text{C}$  and quenched with addition of aqueous HCl (1 M, 1 mL) and ethyl acetate (5 mL). The reaction mixture was allowed to warm up to room temperature, phases were separated, organic layer was washed with water ( $2 \times 2$  mL) and dried over  $\text{Na}_2\text{SO}_4$ . The resulted crude solution was stirred with a mixture of pyridine (25  $\mu\text{L}$ , 0.31 mmol), urea-hydrogen peroxide complex (150 mg, 1.6 mmol) and water (2 drops) on ice bath. After 1 h, the resulted mixture was partitioned between ethyl acetate (10 mL) and water (5 mL). The organic layer was washed with water and brine (5 mL each), dried and evaporated under vacuum. Chromatography on silica gel (eluent hexane – ethyl acetate 30:1 to 24:1) afforded crystalline phenylselenenyl derivative **16**, starting material **14**, and the unsaturated product **17**. The recovered phenyl selenide **16** was dissolved in THF (1 mL) and stirred with pyridine (20  $\mu\text{L}$ ), urea-hydrogen peroxide (100 mg) and water (3 drops) on ice bath for 2 hours. After similar work-up and chromatography, the desired unsaturated steroid **17** was isolated as a colorless solid (24.3 mg, 21% from **15a**, 81% from the phenyl selenide **16**).

**16:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.50 – 7.25 (m, 5H, Ph), 5.84 (s, 1H, 11 $\alpha$ -CH), 3.98 (s, 1H, 3 $\beta$ -CH<sub>3</sub>), 2.55 – 2.40 (m, 1H), 2.43 (s, 3H, COCH<sub>3</sub>), 2.38 – 2.18 (m, 2H), 1.95 – 1.05 (m, 17H), 0.98 (s, 3H, CH<sub>3</sub>), 0.94 (s, 9H, tert-Bu) 0.74 (s, 3H, CH<sub>3</sub>).  $^{19}\text{F}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  -138.4 (m, 2F), -149.0 (t,  $J$  = 21.0 Hz, 1F), -159.8 (m, 2F). HR MS: calculated for  $\text{C}_{40}\text{H}_{52}\text{F}_5\text{O}_4\text{SeSi}$   $[\text{M}+\text{H}]^+$ : 799.2720; found: 799.2723.

**17:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  6.71 (q,  $J$  =  $\sim 1.5$  Hz, 1H, -CH=), 5.77 (unres. q,  $J$  = 2.0 Hz, 1H, 11 $\alpha$ -CH), 4.06 (q, 1H, 3 $\beta$ -CH<sub>3</sub>), 2.79 (dd,  $J$  = 15.1, 2.0 Hz, 1H), 2.40 (ddd,  $J$  = 17.0, 6.6, 3.4 Hz, 1H), 2.26 (s, 3H, COCH<sub>3</sub>), 2.17 – 1.97 (m, 2H), 1.92 – 1.83 (m, 1H), 1.74 – 1.36 (m, 9H), 1.36 – 1.20 (m, 8H), 1.16 (dd,  $J$  = 11.1, 4.0 Hz, 1H), 1.06 (s, 3H, CH<sub>3</sub>), 0.93 – 0.86 (m, 3H), 0.85 (s, 3H, CH<sub>3</sub>).  $^{19}\text{F}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  -138.2 (m, 2F), -149.4 (t,  $J$  =  $\sim 21.0$  Hz, 1F), -160.1 (m, 2F). HR MS: calculated for  $\text{C}_{28}\text{H}_{31}\text{F}_5\text{NaO}_4$   $[\text{M}+\text{Na}]^+$ : 549.2040; found: 549.2046.

### Conversion of alkene **17** into F4N3Bzox-AP (**6**)

The alkene **17** was hydrogenated (tritiated) using hydrogen (tritium) gas under atmospheric pressure over 10% Pd/C in ethyl acetate. The catalyst was filtered off, the solution concentrated, the residue dissolved in a minimal amount of DMSO and treated with  $\sim 1\%$  tetra-*n*-butylammonium azide in DMSO for 1 minute. The product was isolated by extraction with ethyl acetate and purified by chromatography on silica gel using ethyl acetate - hexane 1:1 for elution or by HPLC. The tritiated compound was purified by HPLC using Eclipse XDB-C18 3.5  $\mu\text{m}$ , 4.6–150 mm column and linear gradient of acetonitrile in isocratic 0.05% TFA- $\text{H}_2\text{O}$  for elution (60% at  $t=0$ , 90% at  $t=10$  min) with 1.0 mL/min flow



rate. The identity of the tritiated product was verified by MS and by HPLC co-elution of the tritiated product with a genuine sample of **6** obtained via Scheme 1. The product had 45 Ci/mmol specific radioactivity.

**20-Ethylenedioxy-3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-11-one (18).**<sup>18</sup>—Selective reduction of 3,11-diketone **11** (555 mg, 1.48 mmol) with Selectride-K in THF at  $-78^{\circ}\text{C}$  was achieved as described<sup>18</sup>, except urea-hydrogen peroxide complex was used instead of aqueous  $\text{H}_2\text{O}_2$ . While the yield was nearly quantitative, the crude product contained up to 10% of the 3 $\beta$ -OH-isomer.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  4.04 (brs, 1H, 3 $\beta$ -CH), 4.02 - 3.83 (m, 4H,  $\text{OCH}_2\text{CH}_2\text{O}$ ), 2.59 (d,  $J = 12.3$  Hz, 1H), 2.31 - 2.20 (m, 2H), 2.03 (t,  $J = 9.7$  Hz, 1H), 1.93 - 1.70 (m, 7H), 1.70 - 1.61 (m, 2H), 1.61 - 1.48 (m, 3H), 1.44 - 1.31 (m, 2H), 1.26 (s, 3H,  $\text{CH}_3$ ), 1.30 - 1.09 (m, 5H), 1.02 (s, 3H,  $\text{CH}_3$ ), 0.72 (s, 3H,  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  211.6 (C=O), 111.3 (O-C-O), 66.3, 64.8, 64.2, 63.2, 57.8, 56.9, 55.6, 46.0, 39.0, 36.3, 35.7, 35.3, 32.5, 30.9, 28.9, 27.9, 24.3, 23.5, 23.3, 14.1, 10.9.

**11-Azi-20-ethylenedioxy-3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane (23)**—*Formation of an oxime 19.* A suspension of the ketone **18** (947 mg, 2.51 mmol), hydroxylamine hydrochloride (1.72 g, 25 mmol) and sodium hydroxide (1.10 g, 27.5 mmol) in methanol (10 mL) was refluxed with stirring under nitrogen atmosphere. After 36 h, the reaction mixture was cooled, and additional portion of hydroxylamine hydrochloride and NaOH (same amounts as above) was added, and the resulted heavy colorless suspension was refluxed for another 48 h. The reaction mixture was filtered, extracted with water (20 mL), and divided between water (20 mL) and ethyl acetate (50 mL). The phases were separated, the organic phase was washed with brine (10 mL), dried over  $\text{MgSO}_4$  and evaporated under vacuum. *Nitroization of the oxime 19.* The foregoing crude oxime **19** and finely powdered sodium nitrite were dispersed in dry dichloromethane (40 mL) with vigorous stirring. Glacial acetic acid (2.17 mL, 38 mmol) was added dropwise during 2 h with slow nitrous oxide evolution, and the resulted colored reaction mixture was stirred at room temperature for extra 2 h. The reaction mixture was washed with 5% sodium bicarbonate (40 mL) and brine (20 mL), dried over  $\text{MgSO}_4$ , and concentrated under vacuum. *Formation of the imine 21.* The crude product from the above step was cooled on ice bath under argon, and ammonia in methanol (~7 M, 5 mL) was added dropwise. The reaction flask was sealed with a septum and warmed up to room temperature. After 5 h of stirring, the resulted light-orange suspension was concentrated and dried under high vacuum. *Formation of the diaziridine 22.* A suspension of hydroxylaminesulfonic acid (1.819 g, 16.1 mmol) in dry methanol (5 mL) under argon was cooled to  $-70^{\circ}\text{C}$  and diisopropylethylamine (3.08 mL, 17.7 mmol) was added dropwise with vigorous stirring. The resulted mixture was slowly warmed up to room temperature (during 1 h) and added via syringe to the cooled ( $-50^{\circ}\text{C}$ ) crude imine **21** with stirring under argon. After 3 h of stirring at  $0^{\circ}\text{C}$ , the reaction mixture was left in a refrigerator for one week with occasional stirring. *Formation of the diazirine 23.* The reaction mixture from the previous step was divided between the solution of  $\text{Na}_2\text{CO}_3$  [1.0 g in water (15 mL)] and triethylamine (2 mL) and EtOAc (20 mL). Phases were separated and the organic layer was washed with  $\text{H}_2\text{O}$  (10 mL), diluted with MeOH (10 mL) and cooled to  $-5^{\circ}\text{C}$  with stirring. The excess of iodine (634 mg, 2.5 mmol) was added portionwise, the mixture stirred for 10 min on ice bath, then 5 min at room temperature. The resulted brown solution was washed with 5%

aqueous sodium sulfite, water and brine (10 mL each), dried over MgSO<sub>4</sub>, concentrated under vacuum and chromatographed on silica gel using ethyl acetate – hexane 1:4 to 2:3, with 0.1% of trimethylamine, for elution. The product was recrystallized from methanol (1 mL). The product was also isolated from the mother liquor by reverse phase HPLC using Phenomenex C18 column: 250 mm × 21.2 mm and Macherey-Nagel guard column 16×10 mm, both with 5 μm grain. Eluent A: methanol-water 1:1, eluent B: pure methanol; 10 mL/min. Elution: 0–2 min 1%B, 2–44 min gradient 1% B to 99% B, then 99%B (44–64 min). The elution of the product (retention time 48.1 min) was monitored by UV at 254 and 350 nm. The combined yield of **23** was 62 mg (6.3% from **18**). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.94 (brs, 3β-H), 3.98 – 3.78 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub>O), 1.88 – 1.66 (m, 8H), 1.54 – 1.12 (m, 10H), 1.18 (s, 3H), 1.09 (s, 3H), 1.08 – 0.92 (m, 1H), 0.92 – 0.77 (m, 1H), 0.68 (d, *J* = 13.7 Hz), 0.48 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 111.5 (O-C-O), 65.8, 64.8, 63.2, 57.3, 56.5, 53.3, 48.7, 43.5, 39.4, 35.5, 35.3, 35.0, 31.7, 31.6, 30.3, 28.4, 28.2, 24.2, 23.8, 23.0, 14.0, 11.1. HR MS: predicted for C<sub>23</sub>H<sub>37</sub>N<sub>2</sub>O<sub>3</sub> [M+H]<sup>+</sup>: 389.2804; found: 389.2557.

**11-Aziallopregnanolone (7)**—Diazirine ketal **23** (5.0 mg, 0.0129 mmol) was dissolved in a mixture of methanol (2 mL), water (1 mL) and 1M aqueous hydrochloric acid (0.2 mL) and stirred for 10 min. The dissolution of the substrate occurred after 10 min, and the reaction was complete after 30 min. The product was purified by HPLC (using the same chromatography conditions as described above) to afford the ketone **7** in a nearly quantitative yield. The product was eluted at the retention time of 42.2 min. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.95 (brq, *J* = 2.7 Hz, 1H, 3β-H), 2.50 (t, *J* = 9.1 Hz, 1H), 2.25 – 2.12 (m, 1H), 2.03 (d, *J* ~13 Hz), 1.88 – 1.66 (m, 4H), 1.53 – 1.28 (m, 9H), 1.28 – 1.05 (m, 3H), 1.05 – 0.97 (m, 1H), 0.95 (s, 3H), 0.86 (dt, *J* = 13.2, 3.5 Hz), 0.67 (d, *J* = 13.2 Hz), 0.48 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 208.5 (C=O), 65.7, 62.7, 56.8, 53.2, 47.9, 45.0, 39.4, 35.5, 35.4, 35.3, 31.8, 31.5, 31.1, 30.1, 28.4, 28.0, 24.3, 22.7, 14.2, 11.0. HR MS: predicted for C<sub>21</sub>H<sub>33</sub>N<sub>2</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 345.2540; found: 345.2537.

**3,11-Dioxo-20-ethylenedioxy-pregn-4-ene (24)**—Compound **24** was obtained in 83% yield by PCC oxidation of ketal **9**, analogously to the synthesis of the diketone **11**. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.71 (s, 1H, -CH=), 4.03 – 4.82 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub>O), 2.77 (ddd, *J* = 13.6, 5.0, 3.3 Hz, 1H), 2.67 (d, *J* = 12.6 Hz, 1H), 2.53 – 2.35 (m, 2H), 2.35 – 2.23 (m, 2H), 2.03 (t, *J* = 9.6 Hz, 1H), 2.00 – 1.75 (m, 6H), 1.72 – 1.56 (m, 2H), 1.41 (s, 3H), 1.36 – 1.22 (m, 2H), 1.25 (s, 3H), 0.77 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 209.6 (C=O), 199.7 (C=O), 168.9, 124.4 (=CH-), 111.1 (O-C-O), 64.8, 63.2, 62.7, 57.7, 56.7, 54.7, 38.2, 36.5, 34.7, 33.7, 32.3, 32.1, 24.3, 23.5, 23.4, 17.2, 14.1. HR MS: predicted for C<sub>23</sub>H<sub>33</sub>O<sub>4</sub> [M+H]<sup>+</sup> 373.2379; found: 373.2373.

**20-Ethylenedioxy-3β-hydroxy-11-oxo-pregn-4-ene (25)**—A suspension of enedione **24** (2.586 g, 6.94 mmol) in MeOH (50 mL) and NaOH (28 mg, 0.7 mmol) was cooled on ice bath with stirring. NaBH<sub>4</sub> (263 mg, 6.94 mmol) was added in small portions over the period of 1 h. The reaction mixture was partitioned between ethyl acetate (200 mL) and water (100 mL), the upper layer was washed with water and brine (50 mL each), dried over MgSO<sub>4</sub> and concentrated under vacuum. Chromatography on silica gel using methyl *tert*-butyl ether as eluent and subsequent crystallization afforded pure **25** as colorless crystals, yield 527 mg

(20%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  5.34 (brd,  $J = 1.2$  Hz, 1H,  $-\text{CH}=\text{}$ ), 4.13 (br. s, 1H,  $3\alpha\text{-H}$ ), 4.03 – 4.82 (m, 4H,  $\text{OCH}_2\text{CH}_2\text{O}$ ), 2.63 (d,  $J = 12.5$  Hz, 1H), 2.50 (ddd,  $J = 13.7, 4.5, 2.8$  Hz, 1H), 2.30 – 2.18 (m, 2H), 2.10 – 1.98 (m, 2H), 1.97 – 1.72 (m, 7H), 1.68 – 1.58 (m, 1H), 1.53 – 1.40 (m, 1H), 1.37 (d,  $J = 6.6$  Hz, 1H), 1.35 – 1.25 (m, 1H), 1.28 (s, 3H), 1.26 (s, 3H), 1.24 – 1.06 (m, 2H), 0.75 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  210.9 ( $\text{C}=\text{O}$ ), 145.7, 125.0 ( $=\text{CH}-$ ), 111.2 ( $\text{O}-\text{C}-\text{O}$ ), 67.7, 64.9, 63.9, 63.2, 57.8, 56.8, 55.1, 45.8, 36.9, 36.8, 34.4, 33.4, 31.8, 29.0, 24.3, 23.5, 23.4, 18.9, 14.1.

**11-Azi-20-ethylenedioxy-3 $\beta$ -hydroxy-pregn-4-ene (26)**—The diazirine **26** was synthesized in 5% yield from 11-ketone **25** using the analogous procedure as described above.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  5.24 (brq,  $J \sim 1.4$  Hz, 1H,  $-\text{CH}=\text{}$ ), 4.09 (br. s, 1H), 3.99 – 3.80 (m, 4H,  $\text{OCH}_2\text{CH}_2\text{O}$ ), 2.17 (tm,  $J = 14.0, \sim 2.3$  Hz), 1.99 (ddd,  $J = 13.9, 4.1, 2.5$  Hz, 1H), 1.92 (dd,  $J = 11.2, 3.6$  Hz, 1H), 1.89 – 1.68 (m, 7H), 1.39 (d,  $J = 4.0$  Hz, 1H), 1.36 (s, 1H), 1.35 – 1.18 (m, 2H), 1.19 (s, 3H), 1.16 – 1.12 (m, 2H), 1.11 (s, 3H), 0.94 (qd,  $J \sim 12.0, 4.2$  Hz), 0.79 (s, 3H), 0.70 (d,  $J = 13.6$  Hz).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  146.2, 124.3 ( $-\text{CH}=\text{}$ ), 111.4 ( $\text{O}-\text{C}-\text{O}$ ), 67.2, 64.8, 63.2, 57.3, 56.0, 53.1, 48.7, 43.4, 36.5, 35.4, 34.7, 32.8, 32.0, 30.2, 28.3, 24.2, 23.8, 23.0, 20.0, 13.9.

**11-Azi-20-ethylenedioxy-3 $\alpha$ -hydroxy-pregn-4-ene (27)**—The flask (3 mL) containing 3 $\beta$ -alcohol **26** (20 mg, 0.052 mmol) and  $\text{PPh}_3$  (27 mg, 0.104 mmol) was purged with argon, and the solids dissolved in THF (1 mL). Neat formic acid (4  $\mu\text{L}$ , 0.104 mmol) was added at once, followed by diethyl azodicarboxylate (16  $\mu\text{L}$ , 0.104 mmol). The resulted light-yellowish solution was left stirred at room temperature for 2 h. The reaction mixture was divided between methyl *tert*-butyl ether (3 mL) and aqueous  $\text{NaHCO}_3$  (2%, 1 mL), the organic phase washed with water (1 mL), added with methanolic  $\text{NaOH}$  (1M, 0.2 mL) and left for for 20 min. The reaction mixture was washed with water and brine (1 mL each), dried over  $\text{MgSO}_4$  and concentrated under vacuum. Chromatography on silica (ethyl acetate – hexane 1:4, 0.1%  $\text{NEt}_3$ ), afforded diastereomerically pure **27**, contaminated with 33 mol. % of bis-(ethoxycarbonyl)hydrazine ( $\text{DEAD-H}_2$ ). This product was used for the reduction in the presence of the rhodium catalyst without further purification, as described below. Yield 18 mg (78%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.43 (dd,  $J = 4.8, 1.2$  Hz, 1H,  $-\text{CH}=\text{}$ ), 3.98 (br. s, 1H, 3 $\beta$ -OH), 3.98 – 3.80 (m, 4H,  $\text{OCH}_2\text{CH}_2\text{O}$ ), 2.18 (t,  $J \sim 13.7, 2.3$  Hz), 1.99 (ddd,  $J = 13.8, 4.0, 2.5$  Hz, 1H), 1.92 (dd,  $J = 11.2, 3.6$  Hz, 1H), 1.89 – 1.70 (m, 6H), 1.62 – 1.48 (m, 2H), 1.47 (d,  $J = 11.0$  Hz), 1.45 – 1.40 (m, 1H), 1.40 – 1.19 (m, 3H), 1.19 (s, 3H), 1.25 (s, 3H), 1.06 – 0.91 (m, 2H), 0.72 (d,  $J = 13.6$  Hz), 0.69 (s, 3H). HR MS: calculated for  $\text{C}_{23}\text{H}_{33}\text{N}_2\text{O}_3$  [ $\text{M}-\text{H}$ ] $^-$ : 385.2491; found: 385.2631.

**Rhodium Catalyst  $\text{Rh}^+(\text{dppf})(\text{NBD})\text{SbF}_6^-$  (29)**—The solution of bis-acetonitrile-[norbornadiene]-rhodium(I) hexafluoroantimonate (51.3 mg, 100  $\mu\text{mol}$ ) in dry THF (1 mL) was stirred under argon and 1,1'-bis(diphenylphosphino)ferrocene (61 mg, 110  $\mu\text{mol}$ ) was slowly added. The resulted bright-red solution was stirred at room temperature for 30 min and concentrated using a stream of argon until crystallization of the product has started. Absolute ether (0.5 mL) was added and the resulting suspension was stirred for another 30 min, filtered under positive argon pressure and washed with ether (1 mL). Yield 86 mg (87%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.77 – 7.68 (m, 8H, *o*-Ph), 7.65 – 7.55 (m, 12H, *m*-, *p*-Ph),

4.44 (br s, 4H), 4.39 (s, 4H), 4.35 (s, 4H), 4.00 (br s, 2H), 1.57 (s, 2H, -CH<sub>2</sub>-). ESHR MS: (positive-ion mode): predicted for C<sub>41</sub>H<sub>36</sub>FeP<sub>2</sub>Rh [cation]<sup>+</sup>: 749.07; found: 749.07, (negative-ion mode), predicted for SbF<sub>6</sub><sup>-</sup> [anion]<sup>-</sup>: 234.89, found: 234.87.

### 11-Azi-20-ethylenedioxy-allopregnanolone (28) and 11-aziallopregnanolone (7)

All the glassware was flame-dried and cooled under argon. The reaction was done in a small, custom made piece of glassware with a very small stirring bar, in order to maintain strictly anaerobic and anhydrous condition during the reaction. Unsaturated alcohol **27** (1.0 mg, 2.6 μmol) was dissolved in anhydrous THF (0.2 mL), and catalyst **29** was added. The solution was purged by a stream of hydrogen gas under slightly positive pressure. After one hour, the resulted dark red-brown solution was diluted with mixture of aqueous HCl (1 M) and methanol (1:9, 0.1 mL) and stirred for 5 min. The reaction mixture was diluted with methanol-water 1:1 (0.3 mL), filtered through a submicron filter and chromatographed on a semi-prep HPLC column: Phenomenex Luna (2), 21mm × 250mm, 5 μm, C18 with Macherey-Nagel Nucleodur guard column, 10 x16 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 38 min. Detection: UV at 254 and 350 nm wavelength, the pure diazine fractions were identified by the characteristic absorption at 350 nm, that is ~2 times greater than that at 254 nm. Under those conditions the retention times were 42.2 min for 20-ketone **7**, 48.1 min for 20-ketal **28**, and 47.5 min for substrate **27**. The combined yield of **28** and **7** was nearly quantitative. TLC and <sup>1</sup>H NMR spectra of the products were identical with those of the product obtained via Scheme 3. The tritiation was performed by Vitrox Inc. using analogous procedure to that described above. [<sup>3</sup>H]11-azi-AP was purified by HPLC using Eclipse XDB-C18, 5 mm, 4.6×150 mm column using linear gradient of acetonitrile in isocratic 0.05% TFA-H<sub>2</sub>O (50% ACN at t=0, 90% CAN at t=10 min) at 1.0 mL/min flow rate. The product had 30 Ci/mmol specific radioactivity.

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### Abbreviations Used

<b>ACh</b>	acetylcholine
<b>DMF</b>	dimethylformamide
<b>DMSO</b>	methyl sulfoxide
<b>TBDMS</b>	<i>tert</i> -butyldimethylsilyl
<b>THF</b>	tetrahydrofuran

<b>EC<sub>50</sub></b>	concentration required for 50% of full effect
<b>EC<sub>5</sub></b>	concentration required for 5% of full effect
<b>GABA</b>	$\gamma$ -aminobutyric acid
<b>GABA<sub>A</sub>R</b>	GABA <sub>A</sub> -type receptor
<b>IC<sub>50</sub></b>	concentration required for 50% of full inhibitory effect
<b>LoRR</b>	loss of righting reflexes
<b>nH</b>	Hill coefficient
<b>SD</b>	standard deviation
<b>TFD</b>	trifluoromethylidiazirine
<b>THF</b>	tetrahydrofurane

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### Highlights

Two photoreactive 5- $\alpha$ -neurosteroid modulators of GABA<sub>A</sub>R were synthesized

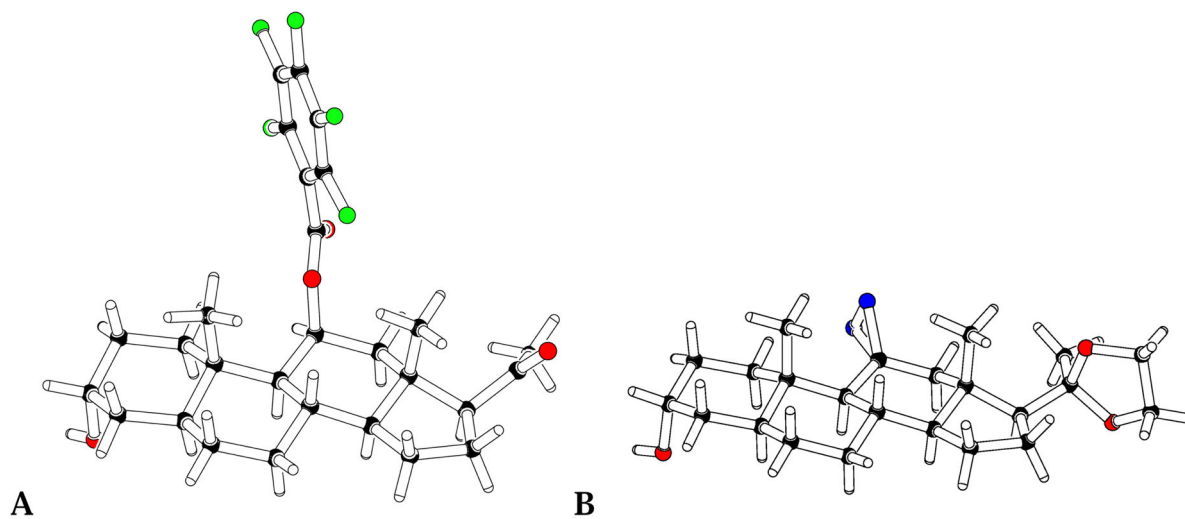
Both ligands are potent general anesthetics and GABA<sub>A</sub>R receptor modulators

Both ligands interact with GABA<sub>A</sub>R at sites distinct from etomidate or barbiturate site

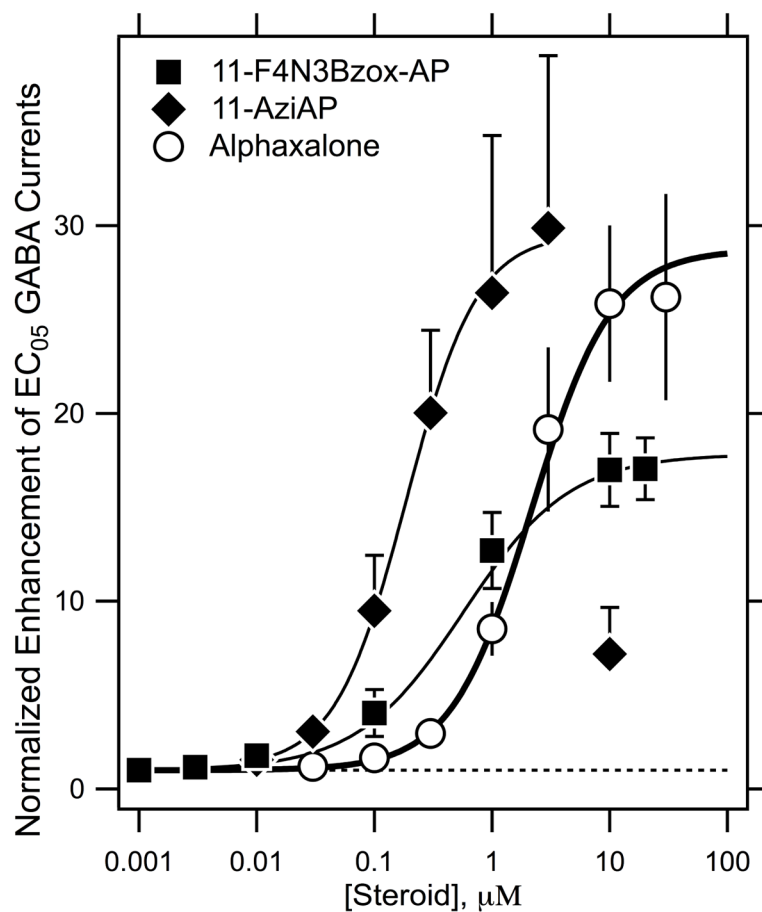
<sup>3</sup>H-Ligands photolabel  $\alpha$ - and  $\beta$ -subunits in  $\alpha 1\beta 3\gamma 2$ -GABA receptor

Photolabeling is not inhibited by alphaxalone or the unlabeled ligands

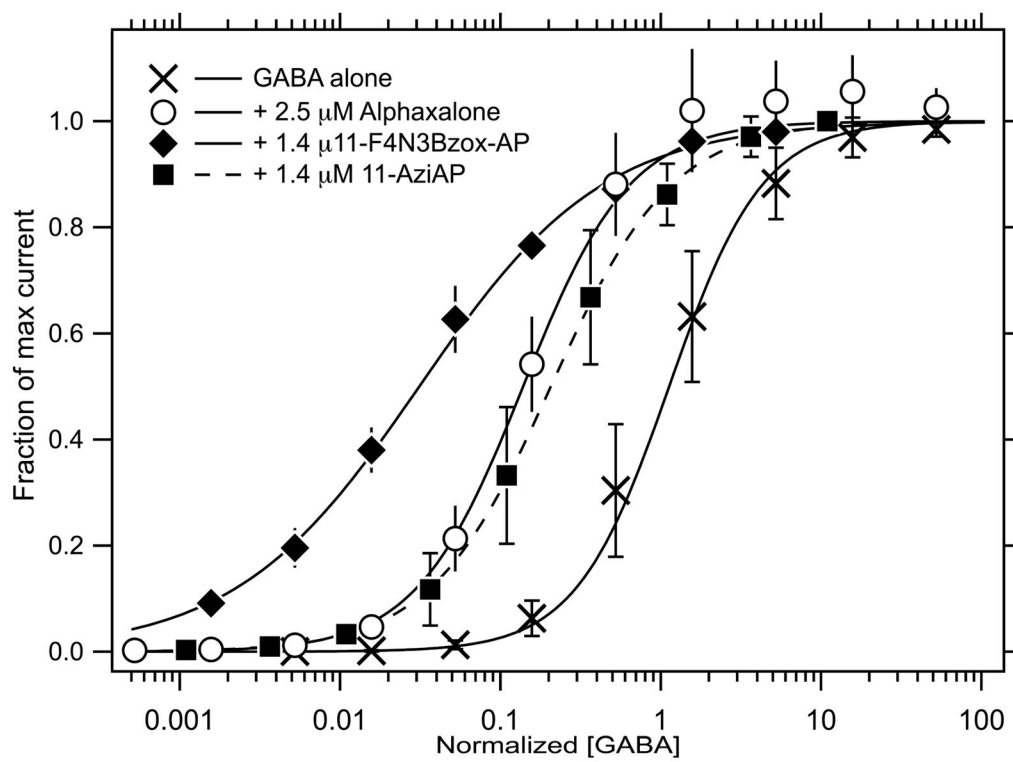




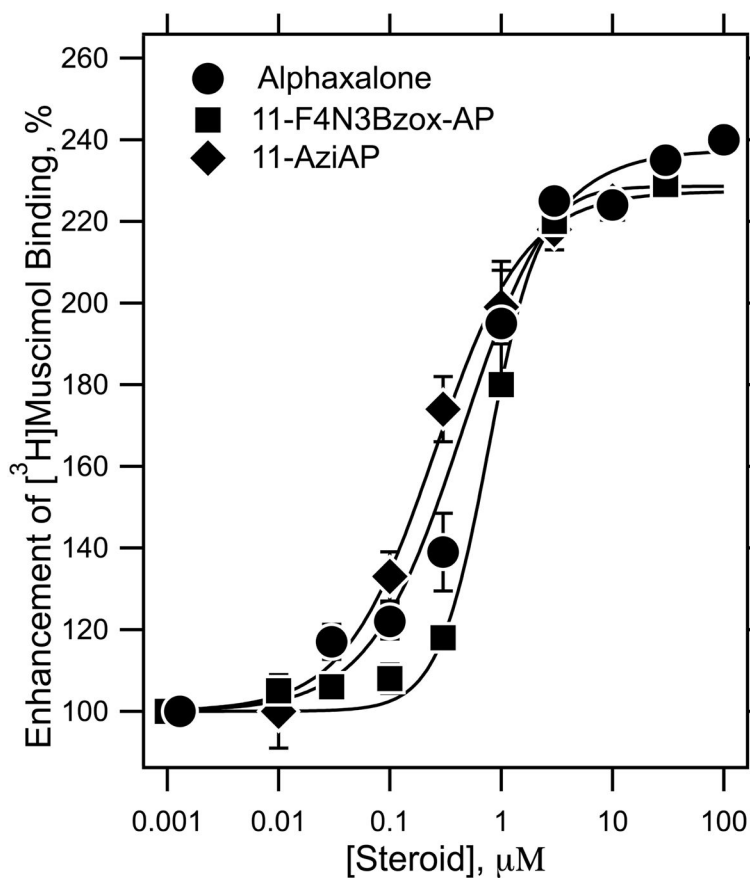
**Figure 1.**  
X-ray structures of compounds **15** (A) and **28** (B), the penultimate intermediates in the syntheses F4N3Bzox-AP **4** and 11-azi-allopregnanolone **7**, respectively.



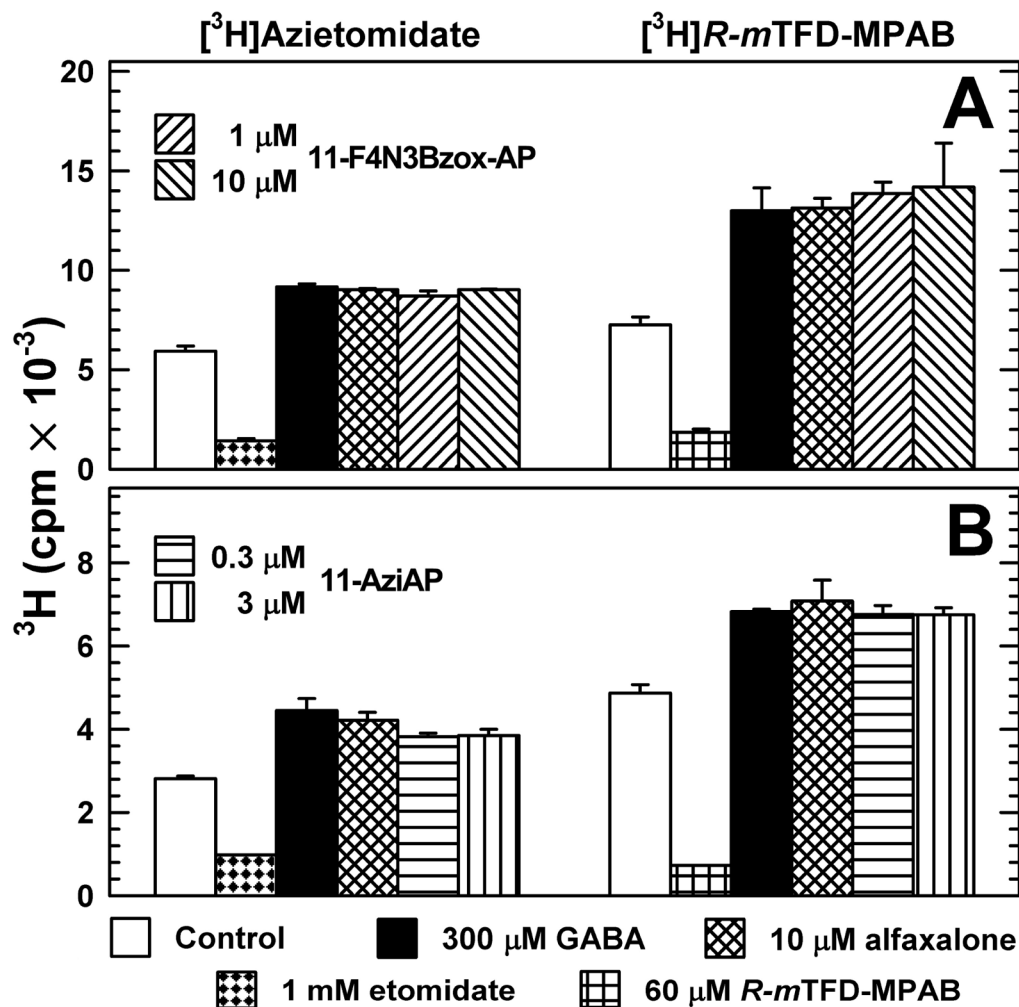
**Figure 2.** F4N3Bzox-AP and 11-aziAP are potent and efficacious positive modulators of GABA-induced currents.



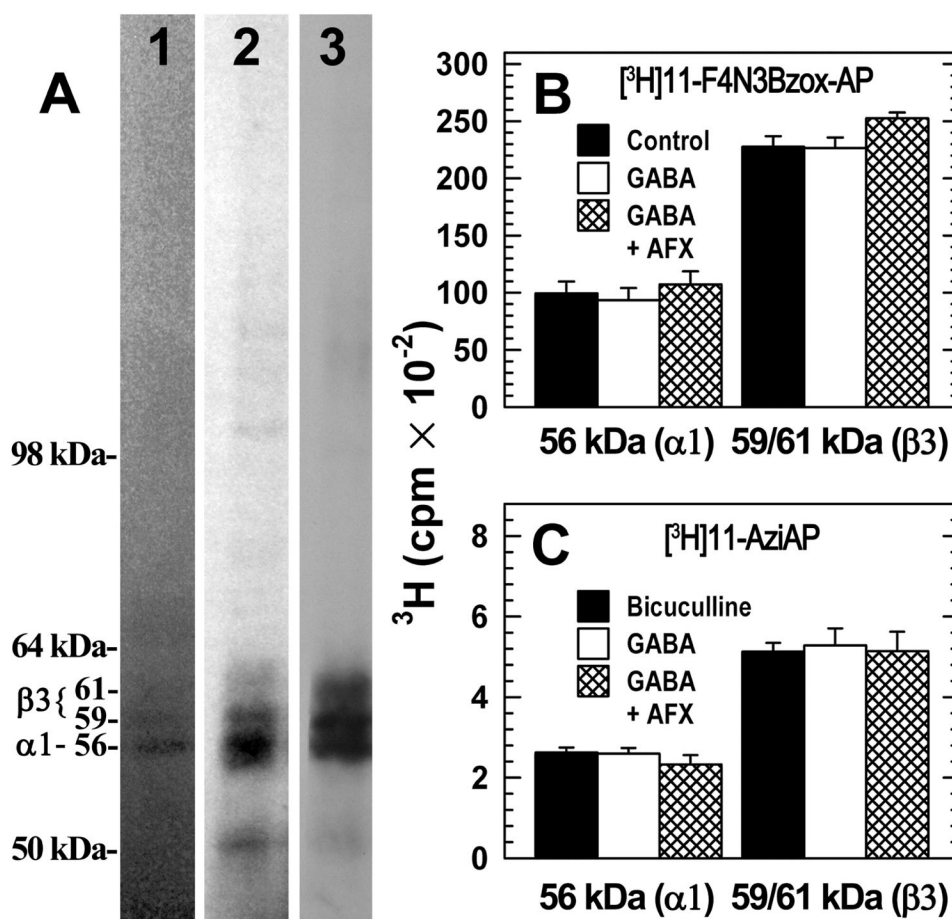
**Figure 3.** F4N3Bzox-AP, 11-aziAP and alphaxalone shift the GABA dose-response curve to the left.



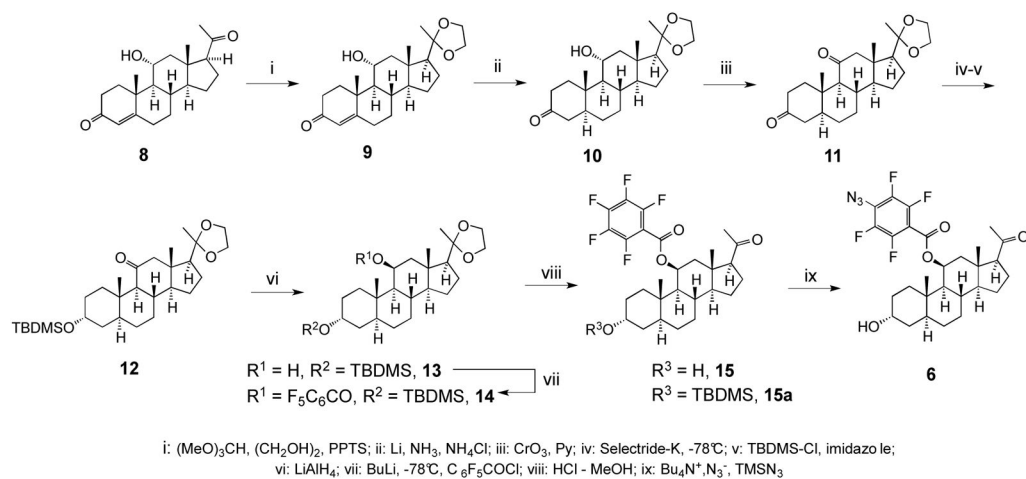
**Figure 4.** F4N3Bzox-AP, 11-aziAP and alphaxalone modulate agonist binding to human  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptors. The specific binding of 2 nM [<sup>3</sup>H]muscimol to membrane suspensions of GABA<sub>A</sub> receptors heterogeneously expressed in HEK293 cells was evaluated as a function of steroid concentration. Each data point was determined in triplicate and is plotted as the mean  $\pm$  standard deviation.



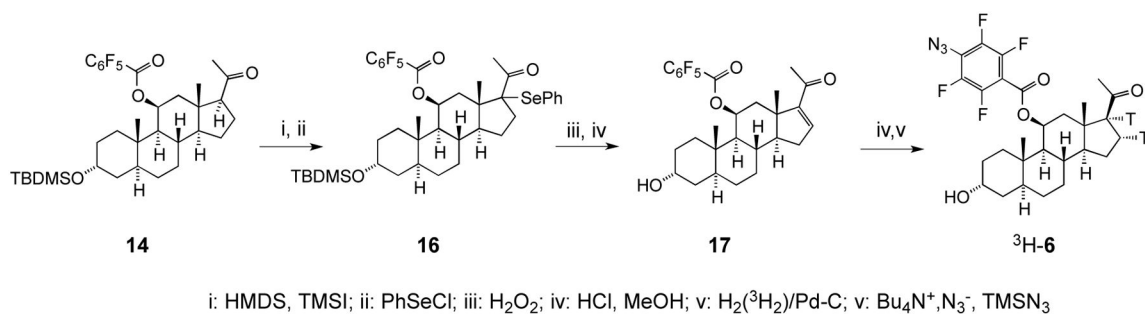
**Figure 5.** 11-F4B3Bzox-AP and 11-AziAP potentiate  $[\text{H}]$ azietomidate and  $[\text{H}]R\text{-}m\text{TFD-MPAB}$  photolabeling of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>R similar to alfaxalone or GABA. Affinity-purified  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptors ( $\sim 2$  pmol of muscimol sites in 35  $\mu\text{L}$ /lane) were equilibrated with 3  $\mu\text{M}$   $[\text{H}]$ azietomidate or 1  $\mu\text{M}$   $[\text{H}]R\text{-}m\text{TFD-MPAB}$  in the absence or presence of various drugs. After irradiation, the samples (in duplicate except for etomidate and *R-mTFD-MPAB* in B) were separated by SDS-PAGE and the stained GABA<sub>A</sub>R subunits were excised for  $^3\text{H}$  quantification by liquid scintillation counting. Means and ranges for the duplicate samples are plotted.



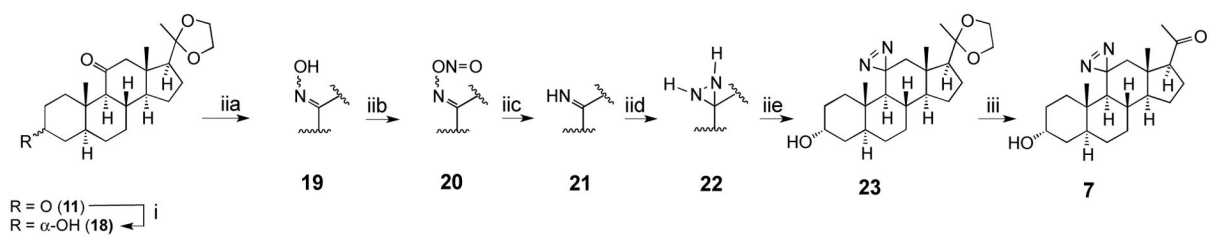
**Figure 6.** [<sup>3</sup>H]F4N3Bzox-AP and [<sup>3</sup>H]11-AziAP photolabeling of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>Rs. **A**, Aliquots of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>R (4 pmol [<sup>3</sup>H]muscimol sites in 80  $\mu$ l) were equilibrated with 300  $\mu$ M GABA and 0.7  $\mu$ M [<sup>3</sup>H]11-aziAP (lane 2) or 0.4  $\mu$ M [<sup>3</sup>H]F4N3Bzox-AP (lane 3). After irradiation, the aliquots were separated by SDS-PAGE. The gel was stained with Coomassie blue (lane 1, a representative lane) and the stained gel was prepared for fluorography, dried, and exposed to X-ray film for 11 days (lane 3) or 42 days (lane 2). Denoted on the left are the mobilities of the molecular mass markers and the Coomassie Blue stained bands that contain GABA<sub>A</sub>R subunits. **B** and **C**, In separate experiments, aliquots of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>R (3.0 pmol [<sup>3</sup>H]muscimol sites in 80  $\mu$ l) were equilibrated with either 0.7  $\mu$ M [<sup>3</sup>H]F4N3Bzox-AP (**B**) or 0.7  $\mu$ M [<sup>3</sup>H]11-AziAP (**C**) in the absence of other drugs or in the presence of 100  $\mu$ M bicuculline, 300  $\mu$ M GABA, or 300  $\mu$ M GABA and 30  $\mu$ M alphaxalone. After irradiation the aliquots were separated by SDS-PAGE, and the stained gel bands containing  $\alpha 1$  and  $\beta 3$  subunits were excised and counted for tritium. The means and standard deviations from 3 ([<sup>3</sup>H]F4N3Bzox-AP) or 4 ([<sup>3</sup>H]11-aziAP) parallel samples are plotted. The efficiency of incorporation of [<sup>3</sup>H]F4N3Bzox-AP was ~20-fold greater than that of [<sup>3</sup>H]11-AziAP, with no evidence of inhibition by alphaxalone of photolabeling at the subunit level.



**Scheme 1.**  
 Synthesis of F4N3Bzox-AP **6**.

**Scheme 2.**

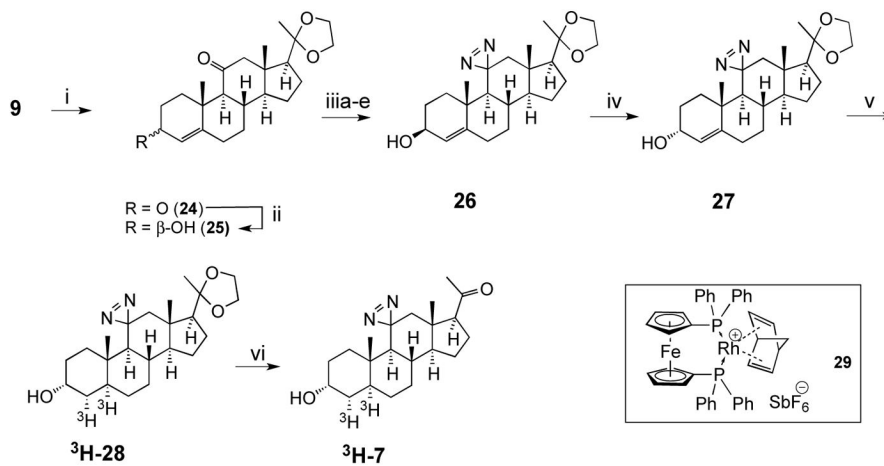




i: Selectride-K, -78°C; ii: (a) H<sub>2</sub>NOH, NaOH; (b) NaNO<sub>2</sub>, AcOH; (c) NH<sub>3</sub>; (d) H<sub>2</sub>NOSO<sub>3</sub>H, DIPEA, -20°C; e: NEt<sub>3</sub>, I<sub>2</sub>; iii: HCl, MeOH

**Scheme 3.**

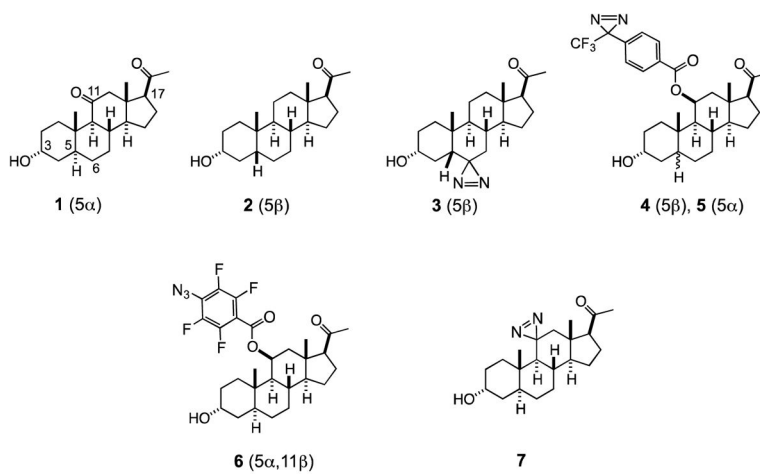
Synthesis of 11-aziallopregnanolone **7** (11-aziAP).



i: PCC/Py; ii: NaBH<sub>4</sub>; iii: (a) H<sub>2</sub>NOH, NaOH; (b) NaNO<sub>2</sub>, AcOH; (c) NH<sub>3</sub>; (d) H<sub>2</sub>NOSO<sub>3</sub>H, DIPEA, -20°C; e: NEt<sub>3</sub>, I<sub>2</sub>; iv: (a) HCO<sub>2</sub>H, PPh<sub>3</sub>; (b) NaOH, v: catalyst **29**, H<sub>2</sub> or <sup>3</sup>H<sub>2</sub>; vi: HCl, MeOH

**Scheme 4.**

Synthesis of tritiated 11-aziAP <sup>3</sup>H-7.

**Chart 1.**