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# Synthesis and evaluation of photoaffinity labeling reagents for identifying binding sites of sulfated neurosteroids on NMDA and GABA<sub>A</sub> receptors†

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The endogenous neurosteroids dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate (PS) are allosteric modulators of  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) and *N*-methyl-D-aspartate (NMDA) type glutamate receptors. Analogues of these endogenous steroid sulfates can be either positive or negative allosteric modulators (PAMs or NAMs, respectively) of these receptors, but there is limited information about the steroid-protein binding interactions that mediate these effects and photoaffinity labeling reagents (PALs) of sulfated steroids have not been reported previously. The synthesis of a panel of ten sulfated steroid analogues containing a diazirine group, five of which also contain an alkyne group for click chemistry reactions, for use in photoaffinity labeling studies to identify binding sites for steroid sulfates that are either positive or negative allosteric modulators is reported. Electrophysiological measurements on cultured rat hippocampal neurons were made to determine the modes of allosteric modulation in comparison to those of PS on both receptors. PALs with the activity profile of PS (NMDA PAM, GABA<sub>A</sub> NAM) were identified. Unexpectedly, PALs with PAM activity at both receptors were also found. Photolabeling of both receptors by two of the PALs was performed to demonstrate their utility, and by inference those of the other PALs, for future studies to identify binding sites for endogenous steroid sulfates on both receptors.

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## Introduction

The endogenous steroids pregnenolone sulfate (PS) and dehydroepiandrosterone sulfate (DHEAS) (Fig. 1) are allosteric modulators of *N*-methyl-D-aspartate receptors (NMDARs) and  $\gamma$ -aminobutyric acid type-A receptors (GABA<sub>A</sub>Rs). PS is a positive allosteric modulator (PAM) of NMDARs containing GluN2A or GluN2B subunits and a negative allosteric modulator (NAM) of NMDARs containing GluN2C or GluN2D subunits.<sup>1–3</sup> DHEAS is only a weak PAM at NMDAR.<sup>4</sup> PS and DHEAS are NAMs at GABA<sub>A</sub>Rs.<sup>5,6</sup> A cryo-EM study has shown binding of both PS and

DHEAS in the pore of GABA<sub>A</sub>Rs.<sup>7</sup> Additional information on modulation of these receptors by PS and DHEAS has been presented in recent reviews.<sup>8,9</sup> Although site-directed mutagenesis and structural methods (cryo-EM) have been useful for gaining information on the locations of sulfated steroid binding sites on both receptors, another method for identifying these binding sites, photoaffinity labeling, has not been applied, and to our knowledge no photoaffinity labels (PALs) of sulfated steroid modulators of these receptors have been reported. Given limitations of sample preparation, overfitting, and other caveats of cryo-EM, we embarked on a complementary chemical biology effort. A similar effort has been instructive in delineating binding sites for non-sulfated steroid modulators of GABA<sub>A</sub>Rs.<sup>10–13</sup> Herein, we report the synthesis of a panel of sulfated steroid PALs (Fig. 2). The PALs were screened using electrophysiological methods to determine their NAM and PAM actions at both NMDARs and GABA<sub>A</sub>Rs. Selected PALs were also shown to covalently modify NMDARs or GABA<sub>A</sub>Rs. Of particular interest was the finding that three PALs containing the tri-fluoromethylphenyl diazirine (TPD) photolabeling group (MQ235, MQ236, MQ237) had the novel profile of NAM activity at NMDARs and PAM activity at GABA<sub>A</sub>Rs. Another compound containing the TPD photolabeling group (KK238), an analogue of PS, had the novel profile of PAM activity at both receptors.

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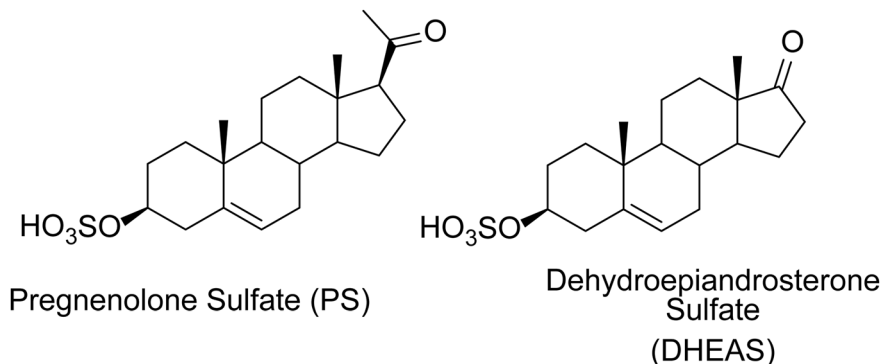


Fig. 1 Structures of PS and DHEAS.

## Results and discussion

### Chemistry

The syntheses of **KK238**, **MQ231** and **YX33** are shown in Scheme 1. **KK238** and **MQ231** were prepared by sulfation of the

previously reported steroids **KK226** (ref. 14) and **MQ182** (ref. 15) followed by conversion to their ammonium salts. **YX33** was prepared from pregnenolone (1) by reaction with cyclohexylamine to yield intermediate enamine 2 which was then

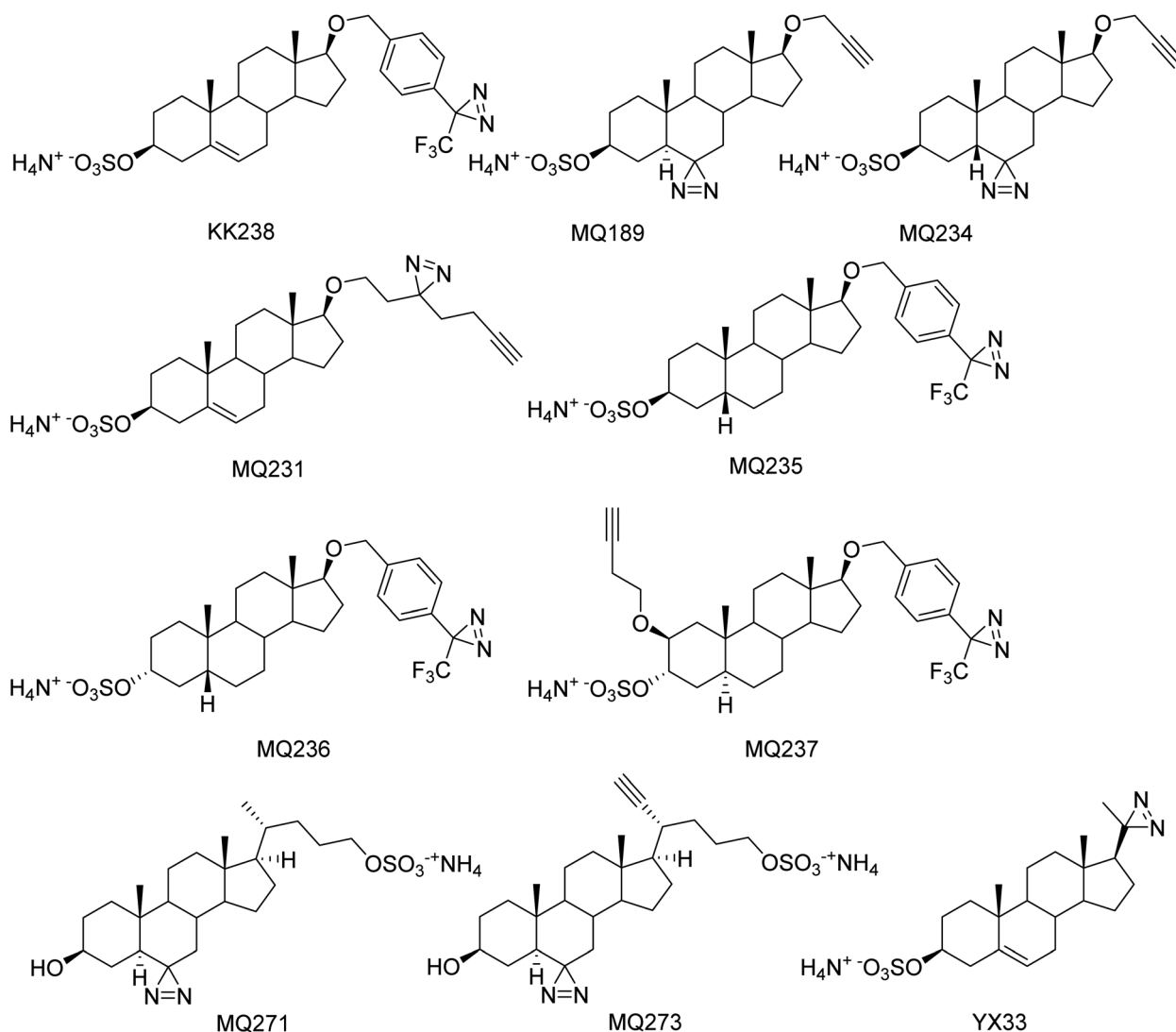
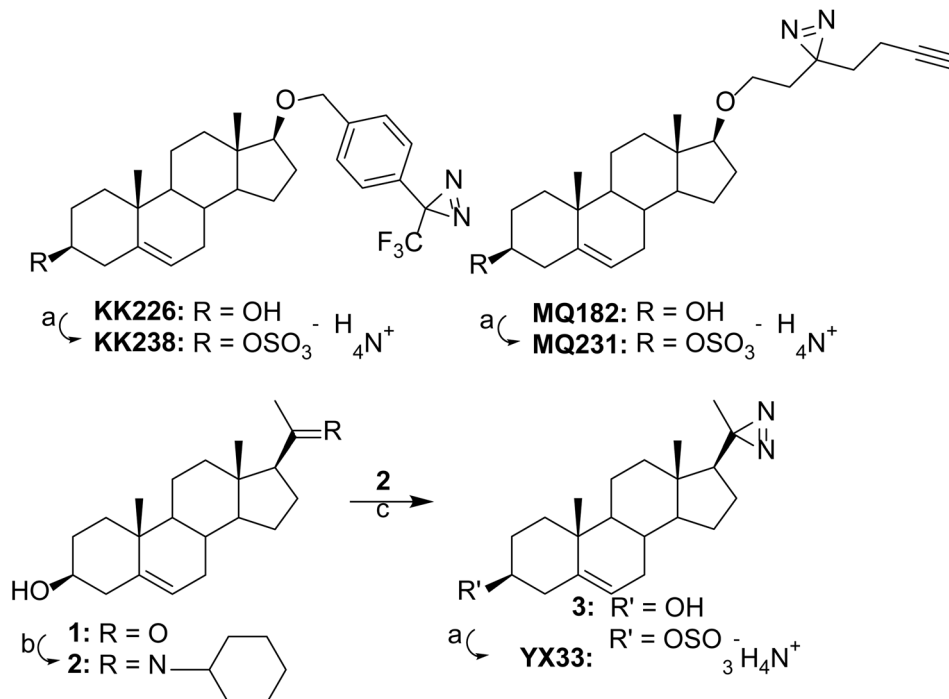


Fig. 2 Structures of synthesized sulfated steroid PALs for photoaffinity labeling studies of NMDARs and GABA<sub>A</sub>Rs.



**Scheme 1** Synthesis of **KK238**, **MQ231** and **YX33**. Reagents: (a) (1)  $\text{SO}_3\text{--Me}_3\text{N}$ , pyridine, 23 °C, 15 h; (2) 7 N  $\text{NH}_3$  in MeOH, (**KK238**, 99%), (**MQ231**, 64%), (**YX33**, 47%); (b)  $\text{CF}_3\text{CO}_2\text{H}$ , cyclohexylamine, reflux under  $\text{N}_2$ , 15 h; (c) (1)  $\text{NH}_2\text{OSO}_3\text{H}$ ,  $\text{NH}_3$ , MeOH, 23 °C, 72 h; (2)  $\text{NH}_3$ ,  $\text{I}_2$ , 0 °C then 23 °C, 20 min; (3)  $\text{SO}_3\text{--Me}_3\text{N}$ , pyridine, 23 °C, 15 h; (4) 7 N  $\text{NH}_3$  in MeOH, (19%, from **1**).

converted to diazirine **3** and then similarly sulfated and converted to its ammonium salt to yield **YX33**.

The syntheses of **MQ189** and **MQ234** are described in Scheme 2. The hydroxyl group of previously reported steroid **4** (ref. 15) was protected to yield steroid **5** which was then hydroborated to give a mixture of steroids **6** and **7** which were separated by flash column chromatography. The newly formed 6-hydroxyl group in separated steroids **6** and **7** was oxidized to yield 6-ketosteroids **8** and **9**, respectively. The hydroxyl protecting group of steroid **8** was then removed to obtain steroid **10** which was then converted by a two-step procedure to diazirine **12**. Propargylation of the 17-hydroxyl group of steroid **12** with subsequent hydrolysis of the hydroxyl protecting group gave propargylated steroid **14**. Sulfation and salt formation gave **MQ189**.

Using a similar, but slightly different reaction sequence steroid **9** was converted to diazirine **11** and then the hydroxyl protecting group was removed to yield steroid **13**. Propargylation as described for the preparation of steroid **14** converted steroid **13** to steroid **15**. Hydrolysis of the hydroxyl protecting group in steroid **15** yielded steroid **16** and then sulfation and salt formation converted steroid **16** to **MQ234**.

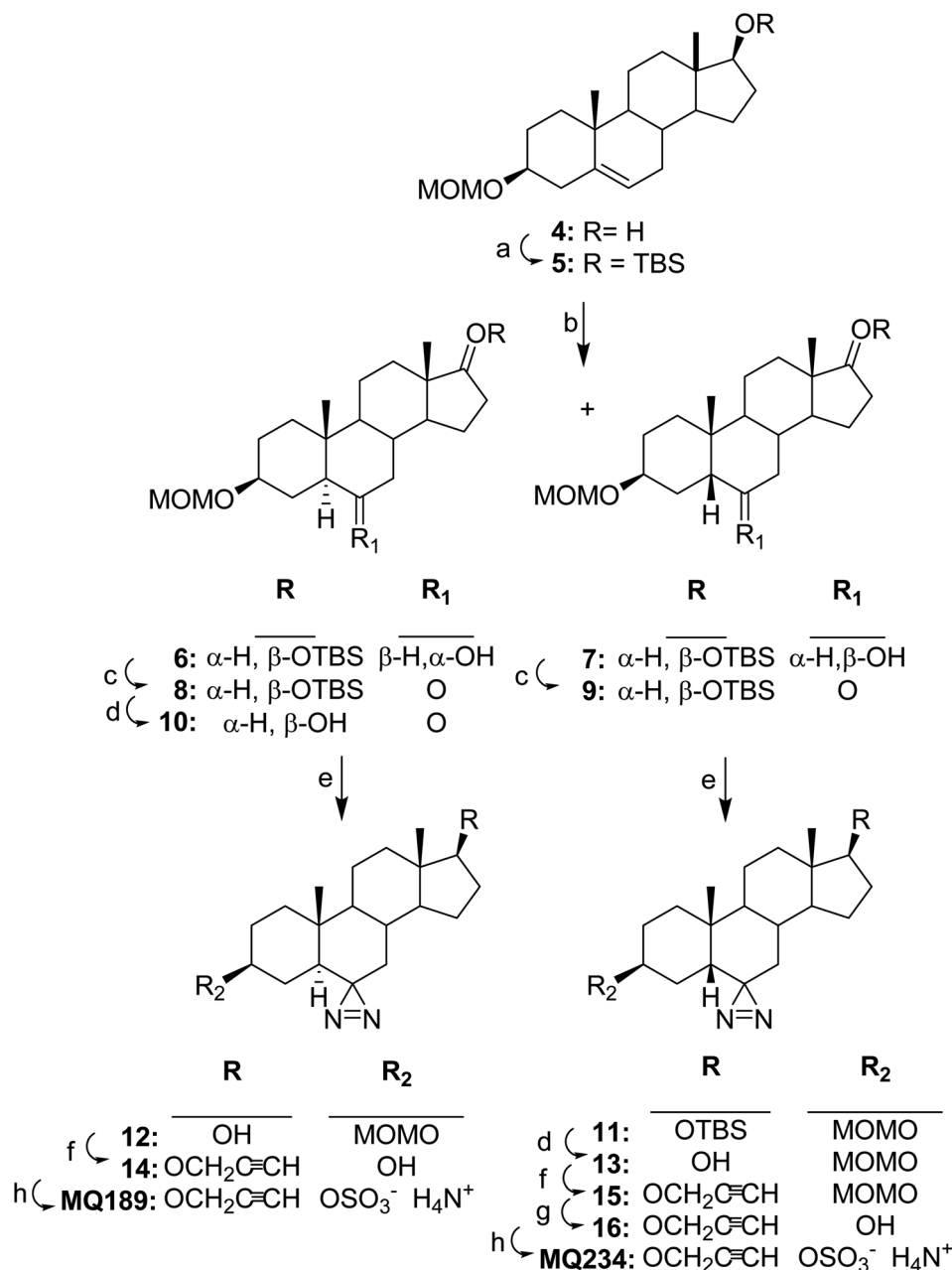
The syntheses of **MQ235** and **MQ236** are described in Scheme 3. The TPD group was attached to the hydroxyl group of previously reported steroid **17** (ref. 16) to give steroid **18**. Hydrolysis of the hydroxyl protecting group in steroid **18** converted it to steroid **19** and sulfation followed by salt formation converted steroid **19** to **MQ235**. A similar reaction sequence (**20** → **21** → **22** → **MQ236**) converted previously reported steroid **20** (ref. 17) to **MQ236**.

The synthesis of **MQ237** is described in Scheme 4. The hydroxyl group of epiandrosterone (**23**) was converted into mesylate **24** and then steroid **24** was converted into an inseparable mixture of the  $\Delta^2$  and  $\Delta^3$  (5 : 1 ratio) steroids **25**. The 17-ketone group of steroid mixture **25** was reduced to give steroid mixture **26** which was then converted into epoxide mixture **27**. The hydroxyl groups of epoxy steroid mixture **27** were protected to obtain steroid mixture **28** and then the epoxides were opened using 3-butyne-1-ol in tetracyanoethylene to give a mixture of products from which pure steroid **29** was obtained after flash column chromatography. Protection of the hydroxyl group of steroid **29** gave steroid **30** and removal of the hydroxyl protecting group from steroid **30** gave steroid **31**. Using reactions already shown in Scheme 3 for addition of the TPD group, *i.e.*, removal of the hydroxyl protecting group, sulfation and salt formation; the reaction sequence **31** → **32** → **33** → **MQ237** completed the synthesis of **MQ237**.

The initial steps for the synthesis of **MQ271** and **MQ273** are shown in Scheme 5 and the final steps are shown in Scheme 6. In Scheme 5, the ketone group of steroid **10** was protected as a cyclic ketal to give steroid **34** and oxidation of the hydroxyl group of steroid **34** gave steroid **35**. The 3-ketone group of steroid **35** was selectively reduced and the resultant 3-hydroxyl group was subsequently protected to obtain steroid **36**. A previously reported procedure was used to introduce a side chain at the C17 position with the required stereochemistry.<sup>18</sup> Thus, a Wittig reaction converted steroid **36** to steroid **37** and subsequent hydrogenation converted steroid **37** to steroid **38**.

In Scheme 6, alkylation of steroid **38** with ((3-iodopropoxy)methyl)benzene converted steroid **38** to steroid **39** and



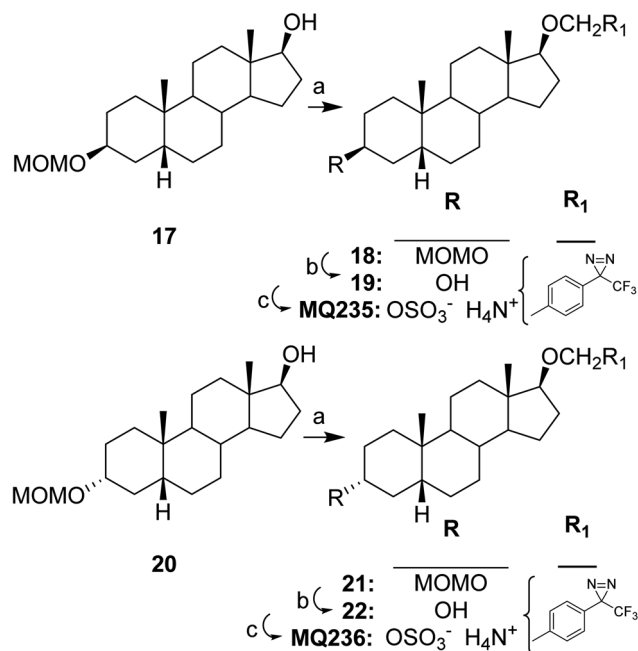


**Scheme 2** Synthesis of **MQ189** and **MQ234**. Reagents, conditions and yields: (a) TBSCl, imidazole, DMF, 23 °C, 16 h, (96%); (b) (1) BH<sub>3</sub>-THF, 23 °C, 2 h, (2) 3 N NaOH, 23 °C, 1 h, (6, 73% + 7, 13%); (c) Dess–Martin reagent, NaHCO<sub>3</sub>, 23 °C, 1 h, (8, 88%), (9, 98%); (d) TBAF, THF, 23 °C, 16 h, (8 → 10, 96%), (11 → 13, 74%); (e) (1) 7 N NH<sub>3</sub> in MeOH, 23 °C, 24 h followed by NH<sub>2</sub>OSO<sub>3</sub>H, 23 °C, 72 h; (2) NH<sub>3</sub>, I<sub>2</sub>, 23 °C, 20 min, (9 → 11, 4% + 12, 43%), (10 → 12, 86%); (f) (1) NaH, THF, reflux, 1 h; (2) propargyl bromide in toluene, reflux 16 h, (13 → 15, 83%), (12 → 14, 62% after removal of MOM group as described for steroid 16 in following reaction (g)); (g) 6 N HCl, THF, 23 °C, 2 h, (16, 74%); (h) (1) SO<sub>3</sub>-Me<sub>3</sub>N, pyridine, 23 °C, 15 h; (2) 7 N NH<sub>3</sub> in MeOH, (**MQ189**, 89%), (**MQ234**, 93%).

reduction of its carboethoxy group yielded steroid **40**. Mesylation of steroid **40** followed by displacement of the mesylate gave steroid **41** and hydrogenation of steroid **41** yielded steroid **42**. Removal of the ketal group in steroid **42** gave steroid **43**. The ketone group of steroid **43** was then converted to the diazirine group in steroid **44**. Protecting group removal followed by sulfation and salt formation converted steroid **44** to **MQ271**.

In Scheme 6, alkylation of steroid **38** with (3-iodopropoxy)(-triisopropyl)silane converted steroid **38** to steroid **45** and

reduction of its carboethoxy group yielded steroid **46**. Oxidation of the hydroxyl group of steroid **46** gave an intermediate aldehyde that was immediately converted to the alkyne containing steroid **47**. Removal of the TIPS protecting group converted steroid **47** to steroid **48** and removal of the ketal group in steroid **48** converted this compound to steroid **49**. The ketone group of steroid **43** was then converted to the diazirine group in steroid **50** and removal of the hydroxyl protecting group, sulfation and salt formation converted steroid **44** to **MQ273**.



**Scheme 3** Syntheses of MQ235 and MQ236. Reagents, conditions and yields: (a) 3-[4-(iodomethyl)phenyl]-3-(trifluoromethyl)-3H-diazirine, Bu<sub>4</sub>NI, NaH, THF, reflux 16 h, (**18**, 59%), (**21**, 63%); (b) 6 N HCl, THF, 23 °C, 2 h, (**19**, 57%), (**22**, 91%); (c) (1) SO<sub>3</sub>-Me<sub>3</sub>N, pyridine, 23 °C, 15 h; (2) 7 N NH<sub>3</sub> in MeOH, (MQ235, 74%), (MQ236, 93%).

### Electrophysiologic evaluation

To determine the allosteric mode of action of the PAL reagents the compounds were evaluated using electrophysiological methods on cultured rat hippocampal neurons. The PAL reagents were screened at 10 μM at NMDARs and at 5 μM at GABA<sub>A</sub>Rs (Fig. 3). The lower concentration for the GABA<sub>A</sub>R determinations was chosen to avoid saturation (complete inhibition) observed by some of the PALs at 10 μM and thereby facilitate meaningful comparisons. We were also interested in how test compounds compared with the published reference sulfated neurosteroid PS. NMDA and GABA agonist concentrations were set below EC<sub>50</sub> to facilitate detection of either inhibition or potentiation. The screening protocol was designed to compare activity across compounds on either receptor class at equimolar modulator concentration but not to identify differences in PAL potency or efficacy. Future photolabeling studies of either receptor will require additional dose-response determinations. At 10 μM, and based on a criterion of a ratio of *t* test *p* value < 0.05, five PALs (MQ235, MQ236, MQ237, MQ271, MQ273) have NMDAR NAM activity, three PALs (KK238, MQ189, MQ231) have PAM activity and two PALs do not have significant NAM or PAM activity (MQ234, YX33). At 5 μM, six PALs have GABA<sub>A</sub>R NAM activity (MQ189, MQ231, MQ234, MQ271, MQ273, YX33), three compounds have clear PAM activity (MQ235, MQ236, MQ237) and KK238 has slight but statistically consistent GABA<sub>A</sub>R PAM activity. The control comparator PS was reported previously to have NMDAR PAM activity and GABA<sub>A</sub>R NAM activity.<sup>4,5</sup> At the screening concentration of 10 μM, PS NMDAR activity was expected to be at the threshold of

biological activity; also expected, the GABA<sub>A</sub> NAM activity was more potent.<sup>4</sup> MQ234 and YX33 may also have the PS activity profile as each has the same PS activity at one of the two receptors, but not the PS activity at the other receptor under the chosen screening conditions. In general, GABA<sub>A</sub>R NAM activity of neurosteroids exhibit apparent greater potency than either effect on NMDARs.<sup>4</sup> Therefore, actions of MQ231, with little or no GABA<sub>A</sub>R activity but strong NMDAR activity, or like MQ237, with potentiating GABA<sub>A</sub> and robust NMDAR NAM activity, may be particularly surprising.

MQ231, YX33 and KK238, like PS, have a 5,6-double bond and the 3β-sulfate group. MQ231 has the same reported activity profile as PS even though the substituents at the C-17 position on the steroid D-ring are different. The lack of significant activity at the screening concentrations chosen in this study makes it impossible to conclude that YX33 has the PS activity profile. However, the similarity of the C-17 substituent in YX33 to that of it in PS suggests that their activity profiles are similar. By contrast, the C-17 substituent (TPD group) in KK238 is the same as that of MQ235, MQ236 and MQ237 and these four compounds do not have the PS activity profile, as unlike PS, these PALs are GABA<sub>A</sub>R PAMs. In results not shown, KK238 also was found to have greater GABA<sub>A</sub>R PAM activity when evaluated at 10 μM (71 ± 7% increase, *n* = 4) thereby confirming the statistical results shown in Fig. 3. It appears that the TPD group is responsible for the GABA<sub>A</sub>R PAM activity since this activity is found regardless of other structural differences in the four PALs. The finding that these four steroids have GABA<sub>A</sub>R PAM activity indicates that unlike PS and DHEAS these analogues are unlikely to bind in the ion channel pore as previously reported in a cryo-EM study<sup>7</sup> because binding within the ion channel would be expected to produce NAM, not PAM, activity.

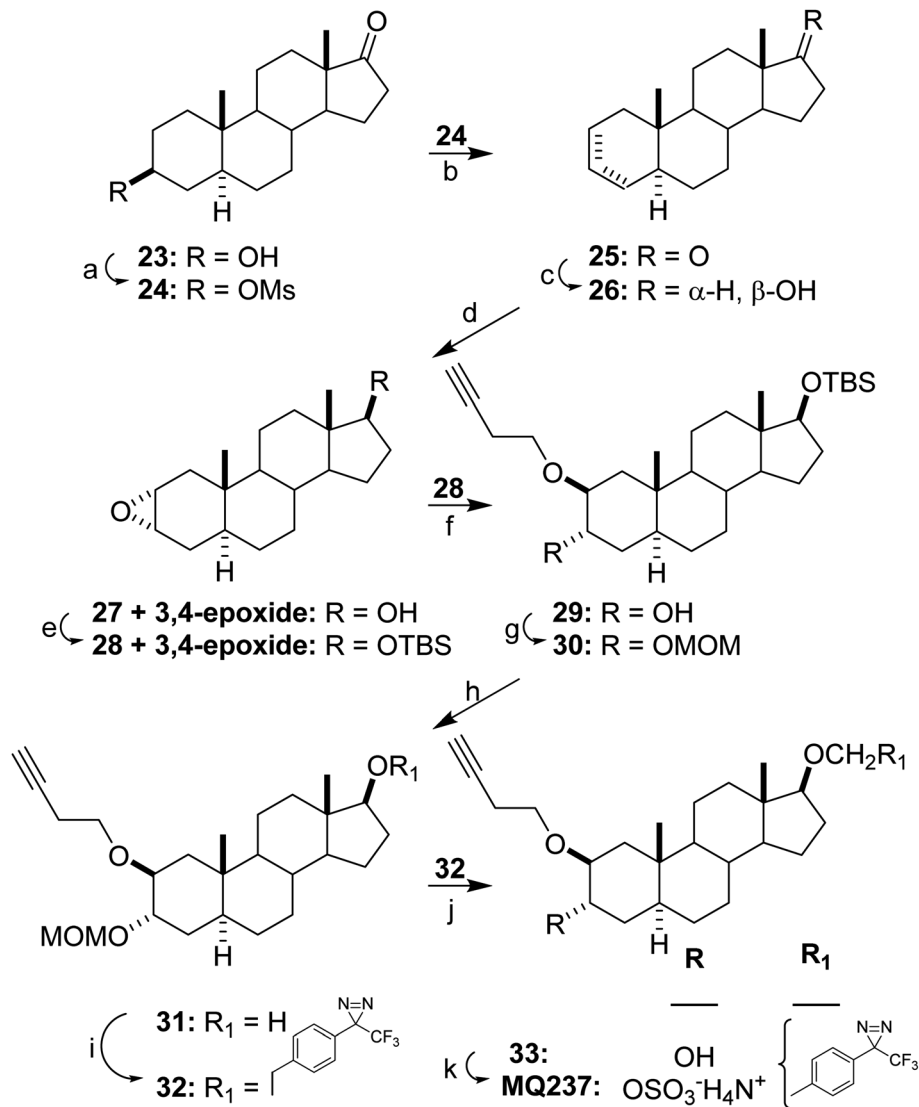
MQ271 and MQ273 have the sulfate group in the C-17 side chain. As screened, both are NMDAR NAMs but only MQ271 showed GABA<sub>A</sub>R NAM activity at 5 μM. In a previous study, we reported that a series of non-photolabeling androstane analogues containing a sulfate group in the same 17β-side-chain found in MQ271 and MQ273 had a variety of actions at GABA<sub>A</sub>Rs and NMDARs depending on the stereochemistry at the steroid 3 and 5 positions.<sup>19</sup> One of those analogues (MQ221) was a GABA<sub>A</sub>R PAM and NMDAR NAM. Since MQ221 does not contain the 17β-TPD group found in KK238, MQ235, MQ236 and MQ237, it may be possible that different PAL analogues of MQ271 and MQ273 could also have GABA<sub>A</sub>R PAM and NMDAR NAM activity. The binding orientation of steroids having the sulfate group in the 17β-side-chain may be opposite to that of the C-3 sulfated steroid modulators of these receptors and this may explain why GABA<sub>A</sub>R PAM and NMDAR NAM activity exists for both series of sulfated steroids.

### Photolabeling of GABA<sub>A</sub>Rs and NMDARs

Having determined the activity profiles of the PAL reagents we next selected two of them for a preliminary study of receptor photolabeling. To determine if the sulfated neurosteroid analogue PAL reagents labeled GABA<sub>A</sub> receptors, we used MQ189, a GABA<sub>A</sub> NAM containing a diazirine moiety for



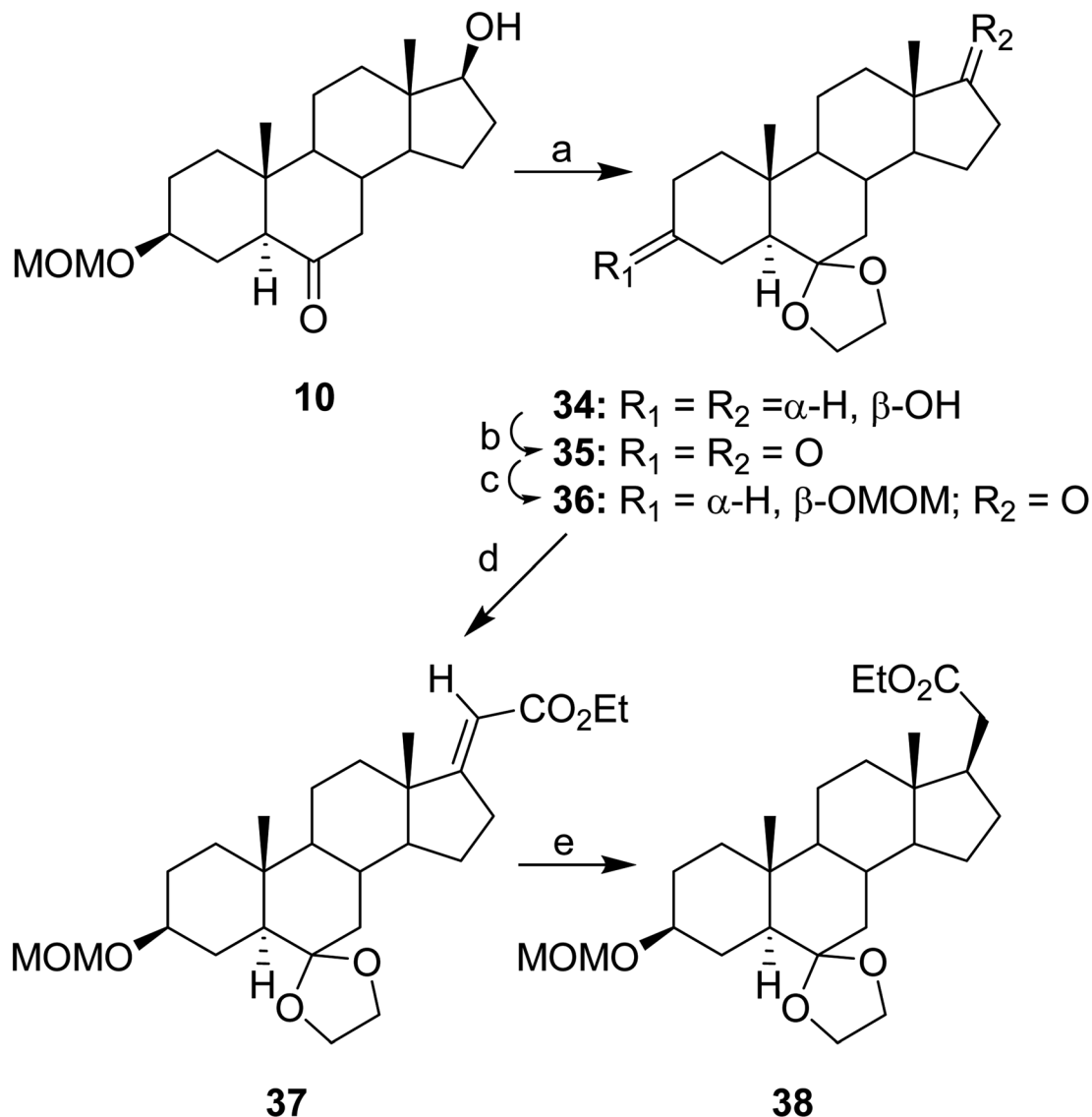




**Scheme 4** Synthesis of **MQ237**. Reagents, conditions and yields: (a) MsCl, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 2 h, (98%); (b) LiBr, DMF, 130 °C, 90 min, (91%); (c) NaBH<sub>4</sub>, EtOH, 23 °C, 1 h, (95%); (d) HCO<sub>2</sub>H, 30% H<sub>2</sub>O<sub>2</sub>, 23 °C, 2 h, (81%); (e) TBSCl, imidazole, DMF, 23 °C, 16 h, (86%); (f) C<sub>2</sub>(CN)<sub>4</sub>, 3-butyn-1-ol, 23 °C, 72 h, (44%); (g) ClCH<sub>2</sub>OMe, (i-Pr)<sub>2</sub>NEt, CH<sub>2</sub>Cl<sub>2</sub>, 23 °C, 16 h, (78%); (h) TBAF, THF, 23 °C, 16 h, (71%); (i) 3-[4-(iodomethyl)phenyl]-3-(trifluoromethyl)-3H-diazirine, Bu<sub>4</sub>Nl, NaH, THF, reflux 16 h, (52%); (j) 6 N HCl, THF, 23 °C, 2 h, (68%); (k) (1) SO<sub>3</sub>-Me<sub>3</sub>N, pyridine, 23 °C, 15 h; (2) 7 N NH<sub>3</sub> in MeOH, (**MQ237**, 30%).

photolabeling and an alkyne that could be used for enrichment of photolabeled proteins. Membranes containing human 8x His-α<sub>1</sub>FLAGβ<sub>3</sub> GABA<sub>A</sub>Rs were photolabeled with either **MQ189** or as a positive control, **KK123**, a non-sulfated neurosteroid PAL previously shown to label the α<sub>1</sub> and β<sub>3</sub> subunits of GABA<sub>A</sub>Rs.<sup>10</sup> Photolabeled proteins in the membranes were solubilized, clicked to a cleavable biotin-azide linker, immobilized on streptavidin beads and eluted by chemical cleavage of the linker. Photolabeled GABA<sub>A</sub>Rs were then identified by the FLAG-tag on the α<sub>1</sub> subunit. Fig. 4 shows a western blot illustrating that the α<sub>1</sub> subunit of the GABA<sub>A</sub>R was identified as a ~53 kDa band and that **MQ189**- and **KK123**-labeled FLAG-tagged proteins of the same molecular weight. These data indicate that **MQ189** (100 μM) and **KK123** (30 μM) label the α<sub>1</sub> subunit of GABA<sub>A</sub>Rs to a similar extent.

To determine if the sulfated neurosteroid analogue PAL reagents labeled NMDARs, we used two sulfated neurosteroid PAL reagents, **MQ189** and **MQ234**, both containing a diazirine photolabeling group and an alkyne for enrichment but differing by their stereochemistry at C5. **MQ189** is an NMDAR PAM and **MQ234** has neither NMDAR NAM nor PAM activity under the chosen screening concentrations. As a negative control, we also used **MQ181** [(3β,17β)-17-ethoxyandrost-5-en-3-ol, 3-sulfate, ammonium salt], a sulfated neurosteroid that acts as an NMDAR PAM but contains neither a diazirine nor an alkyne moiety. We photolabeled membranes containing GluN1<sub>FLAG</sub>/GluN2b NMDA receptors with either 15 μM **MQ189**, **MQ234** or **MQ181**. The photolabeled proteins were then attached to either TAMRA dye or to **MQ112**,<sup>10</sup> a cleavable biotin-azide linker molecule, using a cycloaddition reaction. TAMRA-labeled



**Scheme 5** Initial steps in synthesis of **MQ271** and **MQ273**. (a)  $\text{HO}(\text{CH}_2)_2\text{OH}$ , benzene, PTSA, reflux, 16 h, (57%); (b) Dess–Martin reagent,  $\text{NaHCO}_3$ , 23 °C, 1 h, (98%); (c) (1)  $\text{Li}(t\text{-BuO})_3\text{AlH}$ , THF,  $-40$  °C, 1 h; (2)  $\text{ClCH}_2\text{OMe}$ ,  $(i\text{-Pr})_2\text{NEt}$ ,  $\text{CH}_2\text{Cl}_2$ , 23 °C, 16 h, (84%, 2 steps); (d) triethyl phosphonoacetate, EtOH, NaOEt, under  $\text{N}_2$ , reflux 16 h, (74%); (e)  $\text{H}_2$ ,  $\text{PtO}_2$ , EtOH, 23 °C, 3 h, (87%).

proteins were analyzed by SDS-PAGE with fluorescent imaging. The fluorescent images show that **MQ189** and **MQ234** label multiple protein bands, with similar but non-identical labeling patterns. Both reagents label a band at  $\sim 135$  kDa (red arrow) with the approximate mass of the  $\text{GluN1}_{\text{FLAG}}$  subunit. No photolabeled proteins were observed with **MQ181** due to its lack of the functional groups required for photolabeling and cycloaddition (Fig. 5A). The **MQ112**-coupled proteins were analyzed by western blot with anti-FLAG antibody. The protein lysate (prior to enrichment) shows that  $\text{GluN1}_{\text{FLAG}}$  migrates to the same location as the TAMRA-labeled 135 kDa protein (red arrow in Fig. 5A), and that each of the labeled samples contain an equivalent amount of  $\text{GluN1}_{\text{FLAG}}$  subunit. Following enrichment on streptavidin beads, the  $\text{GluN1}_{\text{FLAG}}$  band is observed for the **MQ189** and **MQ234**, but not the **MQ181** labeled samples

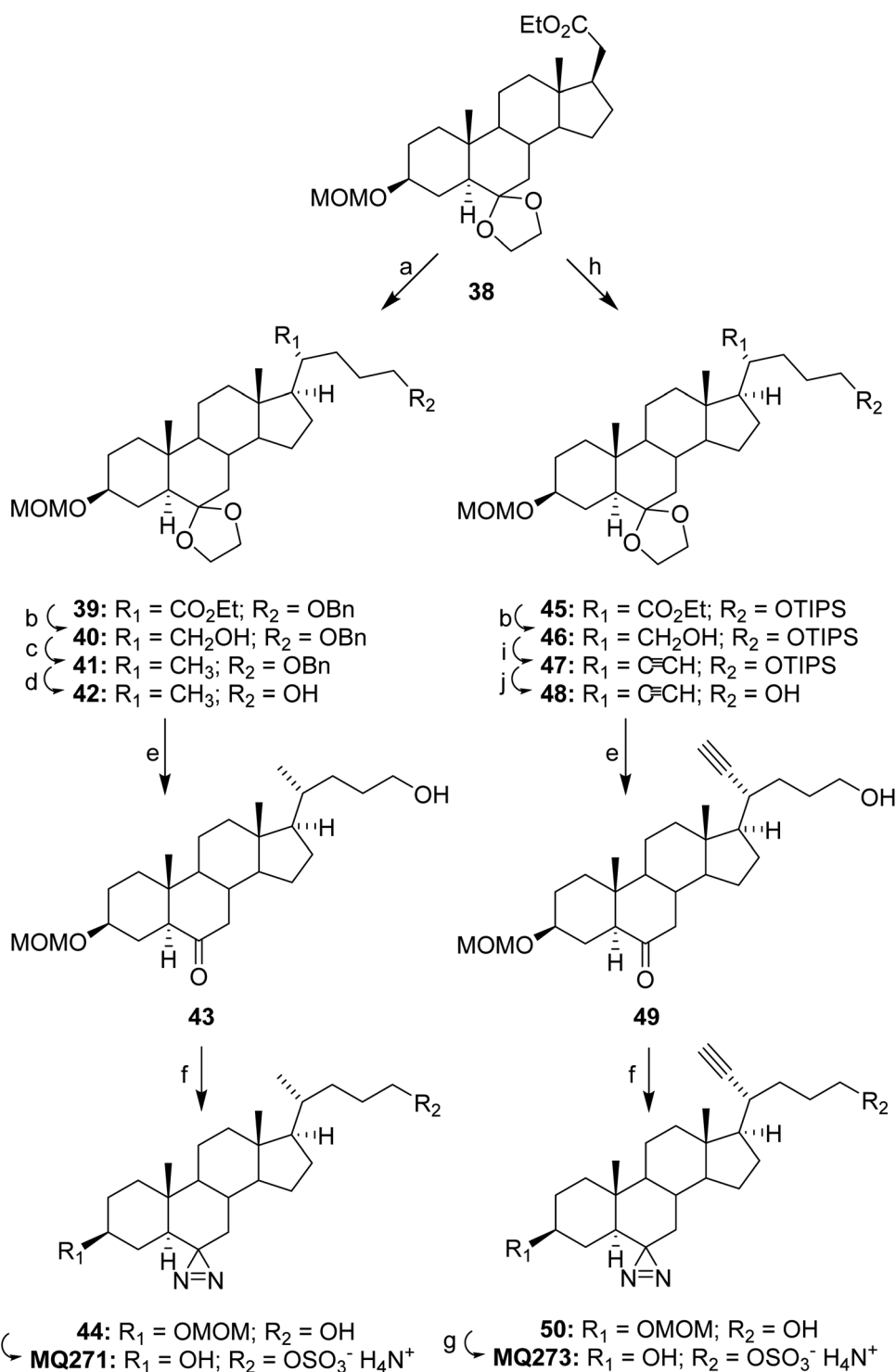
(Fig. 5C). These data indicate that **MQ189** and **MQ234** photolabel the  $\text{GluN1}_{\text{FLAG}}$  subunit of the NMDAR.

### Conclusions and future directions

In conclusion, we report the synthesis and electrophysiological evaluation for a panel of sulfated steroid PALs for photolabeling of NMDARs and  $\text{GABA}_\text{A}$ Rs. To our knowledge, these are the first sulfated PALs reported for use in photolabeling experiments to identify binding sites for DHEAS and/or PS on either of these receptors. Some of these PALs may also find use in identifying sulfated steroid binding sites on other type of receptors. For example, transient receptor potential melastatin 3 (TRPM3) receptors are activated by PS.<sup>20</sup>

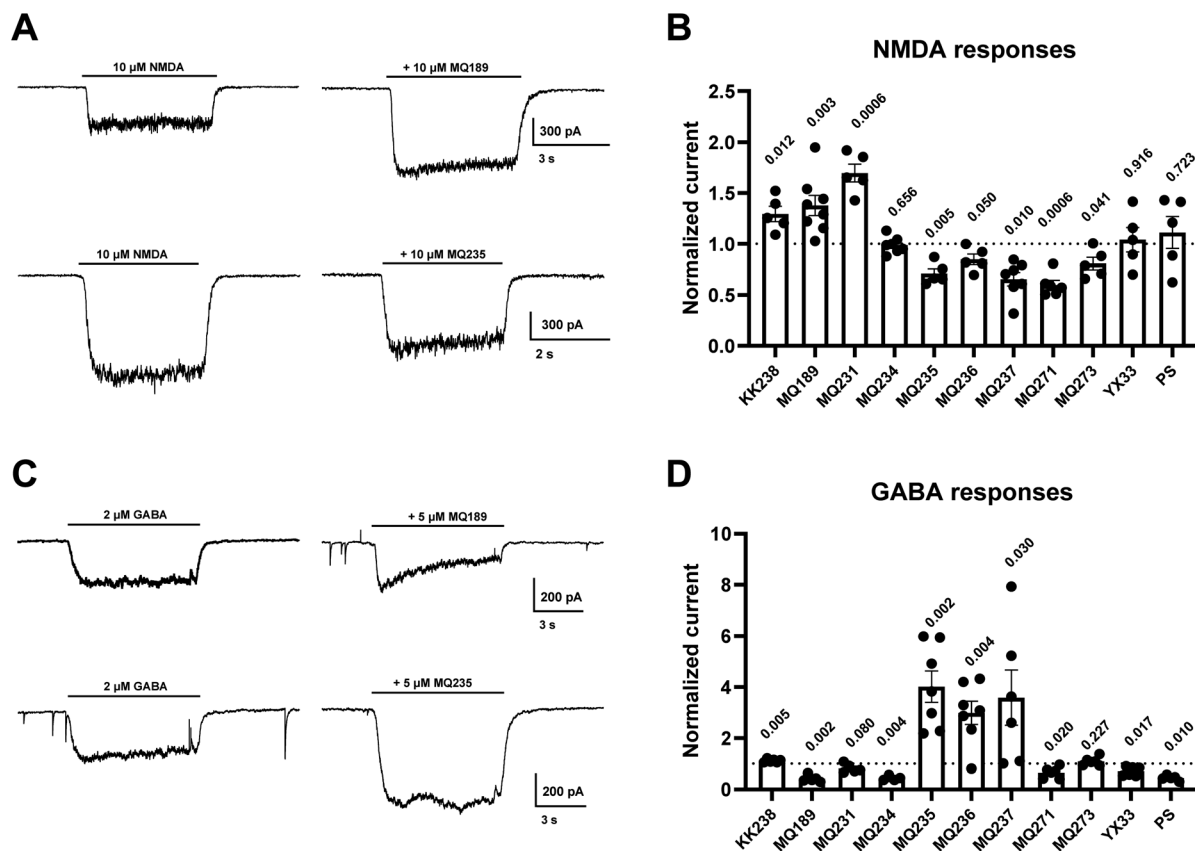
The finding of PAM activity on  $\text{GABA}_\text{A}$ Rs for PALs containing the TPD group on the steroid D-ring is particularly noteworthy as only one sulfated steroid has previously been shown to have this





**Scheme 6** Final steps in synthesis of MQ272 and MQ273. Reagents: (a) ((3-iodopropoxy)methyl)benzene, LDA, HMPA, THF,  $-78^{\circ}\text{C}$ , 45 min then  $23^{\circ}\text{C}$ , 16 h, (98%); (b)  $\text{LiAlH}_4$ ,  $\text{Et}_2\text{O}$ ,  $23^{\circ}\text{C}$ , 2 h, (40, 82%; 46, 96%); (c) (1)  $\text{MsCl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $0^{\circ}\text{C}$  2 h; (2)  $\text{LiAlH}_4$ ,  $\text{Et}_2\text{O}$ , 45 min,  $23^{\circ}\text{C}$ , (97%); (d)  $\text{Pd/C}$ ,  $\text{H}_2$  (50 psi),  $\text{EtOAc}$ , 1 h, (93%); (e)  $\text{PTSA}$ , acetone/ $\text{H}_2\text{O}$ ,  $23^{\circ}\text{C}$ , 3 h, (43, 84%; 49, 96%); (f) (1)  $\text{NH}_2\text{OSO}_3\text{H}$ ,  $\text{NH}_3$ ,  $\text{MeOH}$ ,  $23^{\circ}\text{C}$ , 72 h; (2)  $\text{NH}_3$ ,  $\text{I}_2$ ,  $0^{\circ}\text{C}$  then  $23^{\circ}\text{C}$ , 20 min, (44, 84%; 50, 73%); (g) (1)  $\text{SO}_3\text{-Me}_3\text{N}$ , pyridine,  $23^{\circ}\text{C}$ , 15 h; (2) 6 N  $\text{HCl}$ , THF,  $23^{\circ}\text{C}$ , 2 h; (3) 7 N  $\text{NH}_3$  in  $\text{MeOH}$ , (MQ271, 79%; MQ273, 83%); (h) (3-iodopropoxy)(triisopropyl)silane, LDA, HMPA, THF,  $-78^{\circ}\text{C}$  then  $23^{\circ}\text{C}$ , 16 h, (95%) (i) (1) Dess–Martin reagent,  $\text{NaHCO}_3$ ,  $23^{\circ}\text{C}$ , 2 h; (2) (dimethyl-1-diazo-2-oxopropyl)phosphonate,  $\text{K}_2\text{CO}_3$ ,  $\text{MeOH/THF}$ ,  $23^{\circ}\text{C}$ , 48 h, (64%); (j) TBAF, THF,  $23^{\circ}\text{C}$ , 4 h, (~100%).





**Fig. 3** Electrophysiological activity of PAL reagents at NMDARs and GABA<sub>A</sub>Rs. (A) Representative traces of neuroactive steroid analogues that potentiate (top) and inhibit (bottom) responses to 10  $\mu$ M NMDA elicited from rat hippocampal neurons. Left panels show responses from two different neurons to NMDA alone; each NMDA response is matched to a response from the same cell to co-application of NMDA plus 10  $\mu$ M modulator (right panels). Note that **MQ189** potentiated and **MQ235** inhibited the response to NMDA. (B) Summary of multiple sulfated neurosteroids screened on NMDA responses ( $n = 3-5$ ). The dashed line illustrates the initial NMDA response, to which co-application responses were normalized. (C) The same paradigm was used to examine modulation of GABA<sub>A</sub>R responses. Left panels are representative traces of the response to 2  $\mu$ M GABA elicited from two different rat hippocampal neurons. This response was inhibited by co-application of 5  $\mu$ M **MQ189** at the steady-state (upper panel). In contrast, the GABA response was enhanced by co-application of 5  $\mu$ M **MQ235** (lower panel). (D) Summary of sulfated neurosteroids screened for GABA<sub>A</sub>R modulation ( $n = 5$ ). The dashed line illustrates the initial GABA response to which co-application responses were normalized. The  $p$  values for SEM differences from control are shown above each compound.

activity.<sup>19</sup> No previous structural studies have characterized a binding site for sulfated steroids with PAM activity on GABA<sub>A</sub>Rs. Sulfated steroids with modified D-rings have been prepared and shown to be NAMs at GluN1/GluN2A-D types of NMDARs and when a subset of the reported compounds was evaluated on GABA<sub>A</sub>Rs, the compounds were found to be GABA<sub>A</sub>R NAMs.<sup>16,21</sup>

Herein we showed that selected PALs photolabeled both receptors. These neurosteroid PALs will now be used to identify PAM and NAM binding sites for sulfated steroids on exogenously expressed GABA<sub>A</sub> and NMDA receptors using photolabeling coupled with mass spectrometric sequencing followed by validation of functional significance using site-directed mutagenesis of residues in the putative binding sites coupled with electrophysiologic analysis of ion channel function. We anticipate that the full panel of sulfated steroid PALs will be useful, particularly since the finding of both PAM and NAM activity suggests the possibility of more than one binding site for sulfated steroids on the both GABA<sub>A</sub> and NMDA receptors

and different PALs may discriminate between multiple binding sites as was found for non-sulfated GABA<sub>A</sub>R PALs.<sup>10-12</sup>

## Experimental

### Chemistry

**General methods.** Extraction solvents were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and removed under aspirator vacuum on a rotary evaporator. <sup>1</sup>H NMR and <sup>13</sup>C NMR were recorded at 400 MHz and 100 Mhz, respectively, in CDCl<sub>3</sub> unless indicated otherwise.

(3 $\beta$ ,17 $\beta$ )-17-[[4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-5-en-3-ol, 3-sulfate ammonium salt (**KK238**). To stirred (3 $\beta$ ,17 $\beta$ )-17-[[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-5-en-3-ol<sup>14</sup> (**KK226**, 28 mg, 0.057 mmol) in pyridine (4 mL) was added trimethylamine sulfur trioxide complex (139 mg, 1 mmol) and the reaction was stirred at 55  $^{\circ}$ C for 15 hours. The reaction was cooled, pyridine was removed under reduced pressure and the residue was acidified with 6 N HCl in MeOH (4 mL) to pH-3. The acidic solution was



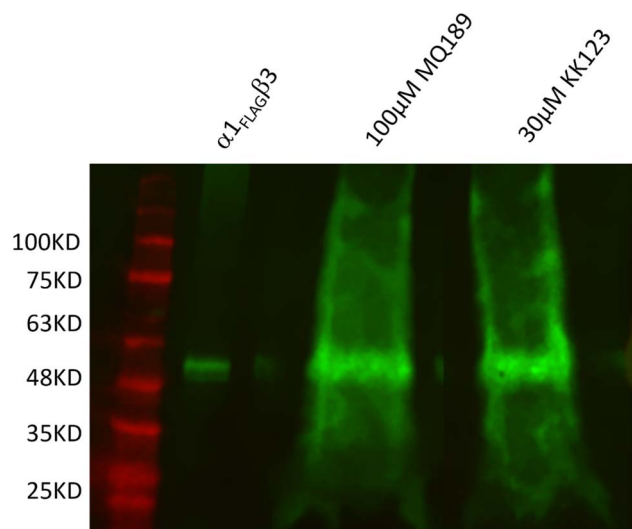


Fig. 4 Enrichment of  $\alpha 1_{\text{FLAG}}\beta 3$  GABA<sub>A</sub>Rs following photolabeling with **MQ189**, a sulfated NAM neurosteroid PAL, and **KK123**, a non-sulfated PAM neurosteroid PAL. The alkyne moiety of the covalently attached PAL reagent was coupled to a cleavable biotin-azide linker **MQ112** using click chemistry. Photolabeled receptors were then enriched on streptavidin-agarose beads and eluted by cleavage of the linker. The figure shows an anti-FLAG-western demonstrating enrichment of photolabeled GABA<sub>A</sub>Rs. Lane 1 lysate of membranes expressing  $\alpha 1_{\text{FLAG}}\beta 3$  GABA<sub>A</sub>Rs and shows the mass (~52 kDa) of the  $\alpha 1_{\text{FLAG}}$  subunit. The bands at 52 kDa indicates that both **MQ189** (Lane 2) and **KK123** (Lane 3) photolabel the  $\alpha 1_{\text{FLAG}}$  subunit of the GABA<sub>A</sub>R.

extracted with  $\text{CH}_2\text{Cl}_2$  ( $5 \times 30$  mL). Solvent was removed, and to the residue was added 7 N  $\text{NH}_3$  in MeOH (6 mL) at room temperature. After 1 h, MeOH was removed under reduced pressure. The resulting crude ammonium salt was purified by flash column chromatography (silica gel eluted with 5–20% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to yield **KK238** (34 mg, 99%):  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.43 (d, 2H,  $J = 8.2$  Hz), 7.21 (d, 2H,  $J = 8.2$  Hz), 5.38–5.37 (m, 1H), 4.56 (s, 2H), 4.16–4.11 (m, 1H), 3.46 (t, 1H,  $J = 8.1$  Hz), 2.60–0.92 (m, 23H), 1.03 (s, 3H), 0.83 (s, 3H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  143.2, 141.7, 129.1 ( $2 \times \text{C}$ ), 128.9, 127.6 ( $2 \times \text{C}$ ), 123.3, 90.3, 80.0, 72.0, 52.8, 51.8, 44.2, 40.5, 39.0, 38.6, 37.8, 33.2, 32.7, 30.1, 29.0, 24.6, 22.0, 19.9, 12.3.

( $3\beta,17\beta$ )-17-(2-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)-androst-5-en-3-ol, 3-sulfate ammonium salt (**MQ231**). Using the sulfation and salt formation procedure described for the preparation of **KK238**, ( $3\beta,17\beta$ )-17-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)-androst-5-en-3-ol<sup>15</sup> (**MQ182**, 52 mg, 0.126 mmol) was converted to **MQ231** (41 mg, 64%). Purification was by flash column chromatography (silica gel eluted with 10% MeOH in  $\text{CH}_2\text{Cl}_2$ ):  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}/\text{CDCl}_3$ )  $\delta$  5.43–5.16 (m, 1H), 4.23–4.14 (m, 1H), 3.70–3.31 (m, 3H), 2.42–0.93 (m, 28H), 1.08 (s, 3H), 0.85 (s, 3H), 0.50–0.39 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}/\text{CDCl}_3$ )  $\delta$  141.7, 123.2, 90.8, 83.8, 79.9, 70.2, 65.7, 52.8, 51.8, 44.0, 40.5, 39.0, 38.5, 37.8, 34.6, 34.2, 33.1, 32.7, 30.0, 28.9, 28.2, 24.5, 21.9, 19.9, 14.0, 12.2.

( $3\beta,17\beta$ )-(20-(Cyclohexylimino))-pregn-5-en-3ol (**2**). To a solution of pregnenolone (**1**, 500 mg, 1.58 mmol) in cyclohexylamine (9.2 mL) was added  $\text{CF}_3\text{CO}_2\text{H}$  (8  $\mu\text{L}$ ) at 23 °C. The mixture was

refluxed 16 h under  $\text{N}_2$ . The solvent was removed to give steroid **2** as a light-brown semi-solid (620 mg) which was converted to steroid **3** without purification or characterization.

( $3\beta,17\beta$ )-17-(3-Methyl-3H-diazirin-3-yl)-androst-5-en-3-ol (**3**). To a solution of crude compound **2** (620 mg) in MeOH (30 mL) was added  $\text{NH}_3$  in MeOH solution (7 N, 20 mL).  $\text{NH}_2\text{OSO}_3\text{H}$  (1.0 g) in MeOH (3 mL) was slowly added and the reaction was stirred for 72 h. The solvent was removed under reduced pressure and water was added to the residue. The steroid was extracted into EtOAc (50 mL). The organic layer was washed with water (20 mL), brine (20 mL), dried and filtered. The solvent was removed to yield the intermediate diaziridine product as a light yellow solid residue (520 mg) which was converted to steroid **3** without purification or characterization.

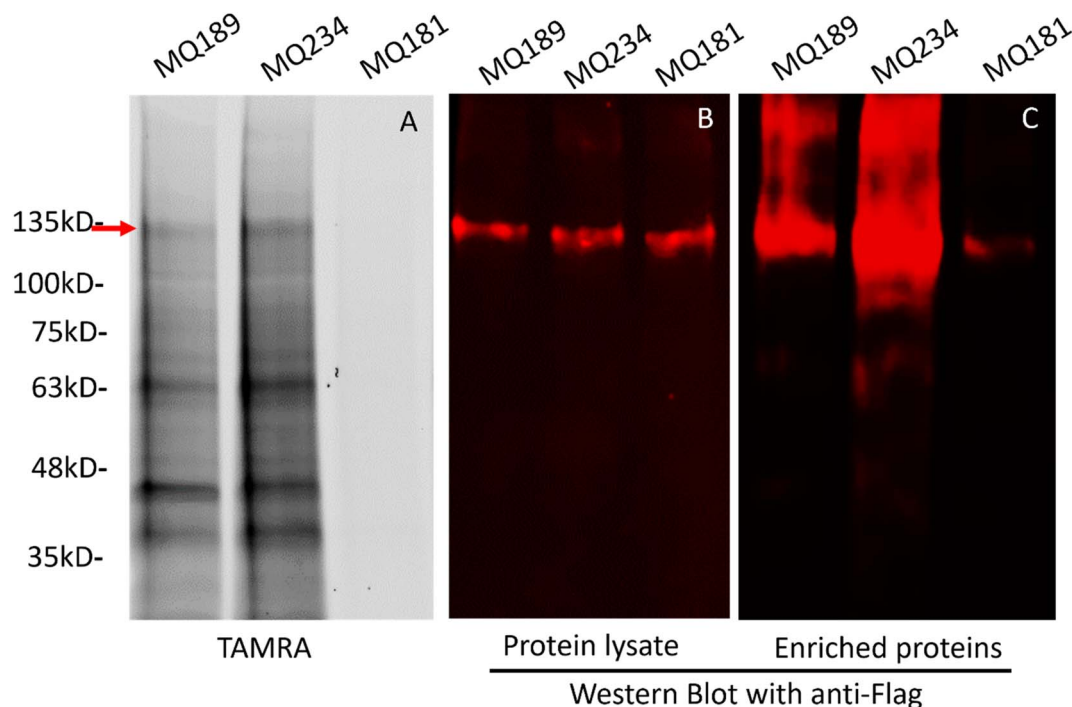
To a solution of the crude diaziridine intermediate (520 mg) in dry MeOH (20 mL) was added  $\text{Et}_3\text{N}$  (3 mL) at 23 °C. The mixture was cooled 0 °C.  $\text{I}_2$  was dissolved in MeOH and added dropwise until a brown color persisted and the reaction was stirred at 23 °C for 20 min. Aqueous  $\text{Na}_2\text{S}_2\text{O}_3$  was added and the steroid product was extracted into EtOAc ( $2 \times 50$  mL). The combined organic layers were dried, filtered, the solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give steroid **3** (100 mg, 19%, 3 steps):  $^1\text{H}$  NMR  $\delta$  5.32 (s, 1H), 3.52–3.48 (m, 1H), 2.26–0.62 (m, 21H), 1.00 (s, 3H), 0.98 (s, 3H), 0.85 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  140.7, 121.3, 71.5, 55.8, 53.6, 50.0, 43.8, 42.1, 38.6, 37.2, 36.4, 31.6, 31.5, 31.4, 26.0, 23.9, 23.4, 21.2, 20.6, 19.3, 13.0.

( $3\beta,17\beta$ )-17-(3-Methyl-3H-diazirin-3-yl)-androst-5-en-3-ol, 3-sulfate ammonium salt (**YX33**). Using the sulfation procedure described for the preparation of **KK238**, steroid **3** (65 mg, 0.2 mmol) was converted to **YX33** (40 mg, 47%). Purification was by flash column chromatography (silica gel eluted with 10% MeOH in  $\text{CH}_2\text{Cl}_2$ ) followed by HPLC purification on a C18 reverse phase column:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  5.36–5.35 (m, 1H), 4.11–4.09 (m, 1H), 2.53–2.49 (m, 1H), 2.35–2.32 (m, 1H), 2.06–0.91 (m, 22H), 1.02 (s, 3H), 0.96 (s, 3H), 0.86 (s, 3H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  140.2, 121.7, 78.3, 55.7, 53.6, 50.1, 43.6, 38.9, 38.5, 37.0, 36.3, 31.4, 31.4, 28.6, 25.3, 23.6, 23.0, 20.4, 20.1, 18.3, 12.2.

(1,1-Dimethylethyl)[[( $3\beta,17\beta$ )-3-(methoxymethoxy)-androst-5-en-17-yl]oxy]dimethylsilane (**5**). To a solution of ( $3\beta,17\beta$ )-3-(methoxymethoxy)-androst-5-en-17-ol<sup>15</sup> (**4**, 7.33 g, 21.9 mmol) in DMF (50 mL) was added imidazole (2.8 g, 40 mmol) and TBSCl (4.0 g, 26.3 mmol) at 23 °C. After 16 h, water (50 mL) was added the product was extracted into EtOAc ( $2 \times 150$  mL). The combined organic layers were dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give steroid **5** (9.44 g, 96%):  $^1\text{H}$  NMR  $\delta$  5.31–5.30 (m, 1H), 4.63 (s, 2H), 3.54 (t,  $J = 8.2$ , 1H), 3.40–3.30 (m, 1H), 3.32 (s, 3H), 2.30–0.88 (m, 19H), 0.98 (s, 3H), 0.85 (s, 9H), 0.69 (s, 3H), –0.02 (s, 6H);  $^{13}\text{C}$  NMR  $\delta$  140.7, 121.3, 94.5, 81.7, 76.7, 54.9, 50.9, 50.4, 43.0, 39.5, 37.2, 36.9, 36.7, 31.9, 31.5, 30.8, 28.8, 25.8 ( $3 \times \text{C}$ ), 23.5, 20.6, 19.3, 18.0, 11.1, –4.6, –4.9.

( $3\beta,5\alpha,6\alpha,17\beta$ )-17-((tert-Butyldimethylsilyl)oxy)-3-(methoxymethoxy)-androst-6-ol (**6**) and ( $3\beta,5\beta,6\beta,17\beta$ )-17-((tert-butyldimethylsilyl)oxy)-3-(methoxymethoxy)-androst-6-ol (**7**). To





**Fig. 5** Photolabeling of GluN1<sub>FLAG</sub>/GluN2b NMDAR by **MQ189** (a PAM PAL analogue) and **MQ234** (a neutral NAM PAL analogue at the screening concentration). Membranes containing GluN1<sub>FLAG</sub>/GluN2b NMDA receptors were photolabeled with either **MQ189**, **MQ234** or **MQ181** [(3 $\beta$ ,17 $\beta$ )-17-ethoxyandrost-5-en-3 $\alpha$ -ol, 3-sulfate ammonium salt, a non-diazirine containing NMDAR PAM analogue] and lysed in SDS. Photolabeled receptors were then coupled via click chemistry to either TAMRA (a fluorescent dye; panel A) or to a cleavable biotin-azide linker (panels B and C). (A) Fluorescent image of an SDS-PAGE gel showing TAMRA-coupled photolabeled proteins. **MQ234** labeled multiple proteins including a band at ~135 kDa (arrow) corresponding to the GluN1<sub>FLAG</sub> subunit. No TAMRA staining was observed with **MQ181** indicating that the diazirine is required for photolabeling. (B) Anti-FLAG western blot of lysate of photolabeled membranes showing that the **MQ189**, **MQ234** and **MQ181** lysates contain equal amounts of GluN1<sub>FLAG</sub> subunit. (C) Photolabeled receptors were enriched on streptavidin-agarose beads, eluted by cleavage of the linker and analyzed by SDS-PAGE and anti-FLAG western blot. **MQ189**- and **MQ234**-photolabeled GluN1<sub>FLAG</sub> subunits were strongly enriched, whereas no enrichment was observed with **MQ181**.

a solution of steroid **5** (9.44 g, 21.1 mmol) in THF (150 mL) was slowly added BH<sub>3</sub>-THF (1.0 M in THF, 40 mL, 40 mmol) at 23 °C. After 2 h, 3 N NaOH (40 mL) and H<sub>2</sub>O<sub>2</sub> (20 mL) were added. The reaction was stirred for 1 h at 23 °C and the mixture of steroids **6** and **7** was extracted into EtOAc (2 × 150 mL). The combined extracts were washed with brine (2 × 100 mL), dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give the major product, steroid **6** (6.89 g, 73%) and the minor product steroid **7** (1.2 g, 13%).

**Steroid 6:** <sup>1</sup>H NMR  $\delta$  4.70 (q, 2H,  $J$  = 6.7 Hz), 3.56–3.39 (m, 3H), 3.36 (s, 3H), 2.25–0.60 (m, 21H), 0.87 (s, 9H), 0.81 (s, 3H), 0.68 (s, 3H), 0.00 (s, 6H); <sup>13</sup>C NMR  $\delta$  94.3, 81.6, 76.0, 69.3, 55.1, 54.1, 51.7, 50.4, 43.3, 41.2, 37.3, 36.9, 36.4, 34.3, 30.8, 29.2, 28.4, 25.8 (3 × C), 23.5, 20.7, 18.0, 13.4, 11.3, –4.6, –4.9.

**Steroid 7:** <sup>1</sup>H NMR  $\delta$  4.65 (s, 2H), 3.86 (s, 1H), 3.69 (s, 1H), 3.58 (t, 3H,  $J$  = 8.6 Hz), 1.88–0.91 (m, 22H), 1.14 (s, 3H), 0.87 (s, 9H), 0.72 (s, 3H), 0.00 (d, 6H,  $J$  = 1.2 Hz); <sup>13</sup>C NMR  $\delta$  94.4, 81.8, 73.2, 71.1, 55.2, 50.7, 44.4, 43.4, 40.6, 37.3, 34.7, 34.0, 31.5, 30.9, 30.8, 30.7, 26.1, 25.8 (3 × C), 24.8, 23.5, 20.5, 18.1, 11.4, –4.5, –4.9.

(3' $\beta$ ,5' $\alpha$ ,17' $\beta$ )-17-((*tert*-Butyldimethylsilyl)oxy)-3-(methoxymethoxy)-androst-6-one (**8**). To a solution of steroid **6** (4.4 g, 9.44 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (60 mL) was added Dess–Martin

periodinane (6.36 g, 15 mmol) and NaHCO<sub>3</sub> (8.0 g) at 23 °C. After 1 h, water (50 mL) was added and the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (150 mL × 3) and washed with brine (50 mL × 2). The organic layer was dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give steroid **8** (3.86 g, 88%): <sup>1</sup>H NMR  $\delta$  4.71 (q,  $J$  = 6.7, 2H), 3.59 (t, 1H,  $J$  = 8.2 Hz), 3.52–3.36 (m, 1H), 3.45 (s, 3H), 2.32–1.02 (m, 20H), 0.86 (s, 9H), 0.75 (s, 3H), 0.69 (s, 3H), –0.00 (s, 6H); <sup>13</sup>C NMR  $\delta$  210.4, 94.3, 81.4, 75.1, 56.8, 55.1, 54.2, 51.0, 46.2, 43.7, 41.0, 37.9, 36.7, 36.6, 30.7, 28.1, 26.9, 25.8 (3 × C), 23.3, 21.1, 18.0, 13.1, 11.3, –4.5, –4.9.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-17-((*tert*-Butyldimethylsilyl)oxy)-3-(methoxymethoxy)-androst-6-one (**9**). Using the procedure described for the preparation of 5 $\alpha$ -steroid **8**, the 5 $\beta$ -steroid **7** (830 mg, 1.78 mmol) was converted to steroid **9**. Purification was by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes). Steroid **9** (811 mg, 98%): <sup>1</sup>H NMR  $\delta$  4.61 (s, 2H), 3.88 (s, 1H), 3.60 (t, 1H,  $J$  = 7.8 Hz), 3.34 (s, 3H), 2.43–1.02 (m, 20H), 0.86 (s, 12H), 0.68 (s, 3H), –0.01 (s, 6H); <sup>13</sup>C NMR  $\delta$  214.8, 94.4, 81.4, 69.4, 55.2, 55.0, 51.2, 43.8, 42.5, 39.8, 37.9, 37.0, 36.9, 30.7, 30.4, 29.3, 25.8 (3 × C), 25.2, 23.8, 23.2, 20.8, 18.0, 11.3, –4.6, –4.9.





(3' $\beta$ ,5' $\alpha$ ,17' $\beta$ )-17-Hydroxy-3-(methoxymethoxy)-androstan-6-one (**10**). To a solution of steroid **8** (3.86 g, 8.32 mmol) in THF (50 mL) was added TBAF (1.0 M in THF, 15 mL, 15 mmol) at 23 °C. The reaction was refluxed for 16 h. THF was removed and the residue was purified by flash column chromatography (silica gel eluted with 30% EtOAc in hexanes) to give steroid **10** (2.80 g, 96%):  $^1\text{H}$  NMR  $\delta$  4.70 (q, 2H,  $J$  = 6.6 Hz), 3.69 (t, 1H,  $J$  = 7.4 Hz), 3.51–3.43 (m, 1H), 3.36 (s, 3H), 2.34–1.11 (m, 21H), 0.77 (s, 3H), 0.74 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  210.4, 94.4, 81.5, 75.1, 56.8, 55.2, 54.0, 51.3, 46.2, 43.4, 41.0, 37.9, 36.7, 36.3, 30.3, 28.1, 26.9, 23.1, 21.1, 13.1, 11.1.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-17'-((*tert*-Butyldimethylsilyl)oxy)-3'-(methoxymethoxy)-spiro[3H-diazirine-3,6'-androstan] (**11**).  $\text{NH}_3$  (6 mL, 7.0 N in MeOH, 42 mmol) was added to a solution of steroid **9** (710 mg, 1.53 mmol) in MeOH (20 mL) at 23 °C. After 24 h, hydroxylamine-*O*-sulfonic acid (234 mg, 1.68 mmol) in MeOH (5 mL) was added and the reaction was stirred for 72 h. The reaction was filtered and the precipitate was washed with EtOAc (100 mL). Solvents were removed and the residue was dissolved in MeOH (10 mL).  $\text{Et}_3\text{N}$  (1.0 mL) was added followed by  $\text{I}_2$  in MeOH until a brown color persisted. Aqueous  $\text{Na}_2\text{S}_3\text{O}_3$  (50 mL) was added and the product was extracted into  $\text{CH}_2\text{Cl}_2$  (2  $\times$  100 mL) and the combined extracts were washed with brine (2  $\times$  50 mL), dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 5% EtOAc in hexanes) to give steroid **11** (30 mg, 4%) and steroid **12** (310 mg, 43%). Steroid **11** had:  $^1\text{H}$  NMR  $\delta$  4.58 (s, 2H), 3.91 (s, 1H), 3.58 (t, 1H,  $J$  = 8.7 Hz), 3.31 (s, 3H), 1.88–0.94 (m, 18H), 1.20 (s, 3H), 0.88 (s, 9H), 0.73 (s, 3H), 0.52–0.47 (m, 1H), 0.28–0.24 (m, 1H), 0.01 (d, 6H,  $J$  = 4.3 Hz);  $^{13}\text{C}$  NMR  $\delta$  94.5, 81.6, 70.3, 55.2, 50.6, 44.1, 43.5, 39.6, 37.1, 36.7, 34.0, 32.7, 30.8, 29.7, 29.3, 26.6, 25.8 (2  $\times$  C), 24.8, 23.6, 23.2, 20.6, 18.1, 11.3, –4.5, –4.8.

(3' $\beta$ ,5' $\alpha$ ,17' $\beta$ )-3'-(Methoxymethoxy)-spiro[3H-diazirine-3,6'-androstan]-17'-ol (**12**). Using the procedure described for the preparation of diazirine **11**, steroid **10** (2.8 g, 8 mmol) was converted to steroid **12** (2.5 g, 86%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes):  $^1\text{H}$  NMR  $\delta$  4.53 (q, 2H,  $J$  = 7.4 Hz), 3.57 (t, 1H,  $J$  = 8.6 Hz), 3.33–3.27 (m, 1H), 3.23 (s, 3H), 2.05–0.72 (m, 19H), 1.08 (s, 3H), 0.70 (s, 3H), 0.43–0.33 (m, 2H);  $^{13}\text{C}$  NMR  $\delta$  94.2, 81.3, 75.5, 54.9, 53.7, 50.5, 45.1, 42.9, 37.6, 36.8, 36.3, 36.2, 33.8, 30.2, 30.1, 29.1, 27.8, 22.9, 20.6, 12.8, 11.0.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-3'-(Methoxymethoxy)-spiro[3H-diazirine-3,6'-androstan]-17'-ol (**13**). Using the procedure described for the preparation of compound **10**, steroid **11** (30 mg, 0.063 mmol) was converted into steroid **13** (23 mg, 74%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes):  $^1\text{H}$  NMR  $\delta$  4.57 (s, 2H), 3.90 (s, 1H), 3.66 (t, 1H,  $J$  = 8.2 Hz), 3.29 (s, 3H), 2.06–1.00 (m, 19H), 1.20 (s, 3H), 0.76 (s, 3H), 0.52–0.47 (m, 1H), 0.28–0.23 (m, 1H);  $^{13}\text{C}$  NMR  $\delta$  94.5, 81.7, 70.3, 55.2, 50.9, 44.0, 43.2, 39.5, 36.7, 36.6, 34.0, 32.7, 30.4, 29.6, 29.2, 26.6, 24.8, 23.6, 23.0, 20.5, 11.1.

(3' $\beta$ ,5' $\alpha$ ,17' $\beta$ )-17'-(*Prop*-2-yn-1-yloxy)-spiro[3H-diazirine-3,6'-androstan]-3'-ol (**14**). To a solution of steroid **12** (2.5 g, 6.9 mmol) in THF (60 mL) was added NaH (2.24 g, 56 mmol) at 23 °C. The mixture was refluxed for 1 h and then propargyl bromide (10

mL, 80% in toluene) was added. The mixture was refluxed 16 h. After cooling to 23 °C, water was added and the product was extracted into EtOAc (3  $\times$  100 mL). The combined extracts were dried, filtered and the solvents removed. The residue was dissolved in MeOH (50 mL) and acetyl chloride (4 mL) was added at 23 °C. After 1 h, water was added and the product was extracted into  $\text{CH}_2\text{Cl}_2$  (3  $\times$  100 mL). The combined extracts were washed with aqueous  $\text{NaHCO}_3$  (2  $\times$  50 mL), dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give steroid **14** (1.52 g, 62%, 2 steps):  $^1\text{H}$  NMR  $\delta$  4.18–4.08 (m, 2H), 3.53 (t, 1H,  $J$  = 8.2 Hz), 3.47–3.40 (m, 1H), 2.38 (t, 1H,  $J$  = 2.4 Hz), 2.02–0.71 (m, 19H), 1.12 (s, 3H), 0.78 (s, 3H), 0.45–0.36 (m, 2H);  $^{13}\text{C}$  NMR  $\delta$  87.8, 80.4, 73.8, 70.8, 57.1, 53.7, 50.7, 45.2, 42.9, 37.5, 37.3, 36.8, 36.3, 33.6, 33.0, 30.6, 29.1, 27.4, 23.0, 20.8, 12.9, 11.6.

(3' $\beta$ ,5' $\alpha$ ,17' $\beta$ )-17'-(*Prop*-2-yn-1-yloxy)-spiro[3H-diazirine-3,6'-androstan]-3'-ol, 3'-sulfate ammonium salt (**MQ189**). Using the sulfation procedure described for the preparation of **KK238**, steroid **14** (250 mg, 0.70 mmol) was converted to **MQ189** (283 mg, 89%). Purification was by flash column chromatography (silica gel eluted with 10% MeOH in  $\text{CH}_2\text{Cl}_2$ ):  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  4.22–4.11 (m, 3H), 3.63 (t, 1H,  $J$  = 7.9, Hz), 3.38 (s, br, 1H), 2.82 (s, 1H), 2.09–0.92 (m, 21H), 1.20 (s, 3H), 0.84 (s, 3H), 0.50–0.39 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  89.2, 81.4, 79.4, 75.4, 58.1, 55.0, 51.9, 46.4, 44.3, 38.7, 38.6, 38.0, 37.5, 35.1, 31.6, 30.2, 29.2, 28.5, 24.2, 22.0, 13.4, 12.3.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-3'-(Methoxymethoxy)-17'-(*prop*-2-yn-1-yloxy)-spiro[3H-diazirine-3,6'-androstan] (**15**). Using the procedure described for the preparation of compound **3**, steroid **13** (23 mg, 0.063 mmol) was converted to steroid **15** (21 mg, 83%). Purification was by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes):  $^1\text{H}$  NMR  $\delta$  4.57 (s, 2H), 4.20–4.11 (m, 2H), 3.91 (s, 1H), 3.57 (t, 1H,  $J$  = 8.3 Hz), 3.30 (s, 3H), 2.39 (s, 1H), 2.03–1.02 (m, 18H), 1.20 (s, 3H), 0.79 (s, 3H), 0.52–0.48 (m, 1H), 0.28–0.23 (m, 1H);  $^{13}\text{C}$  NMR  $\delta$  94.5, 87.9, 80.5, 73.8, 70.3, 57.2, 55.2, 51.1, 44.0, 43.1, 39.5, 37.6, 36.7, 33.7, 32.7, 29.6, 29.2, 27.5, 26.6, 24.8, 23.6, 23.1, 20.5, 11.7.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-17'-(*prop*-2-yn-1-yloxy)-spiro[3H-diazirine-3,6'-androstan]-3'-ol (**16**). To steroid **15** (21 mg, 0.053 mmol) in THF (5 mL) was added 6 N HCl (5 mL) at 23 °C. After 2 h, the product was extracted into  $\text{CH}_2\text{Cl}_2$  (2  $\times$  100 mL). Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give steroid **16** (16 mg, 74%):  $^1\text{H}$  NMR  $\delta$  4.20–4.10 (m, 3H), 3.57 (t, 1H,  $J$  = 8.2 Hz), 2.40 (d, 1H,  $J$  = 1.5 Hz), 2.03–1.01 (m, 19H), 1.21 (s, 3H), 0.79 (s, 3H), 0.56–0.52 (m, 1H), 0.27–0.24 (m, 1H);  $^{13}\text{C}$  NMR  $\delta$  87.9, 80.5, 73.8, 73.7, 65.3, 57.2, 51.1, 43.3, 43.1, 39.3, 37.5, 36.9, 33.7, 32.7, 29.2, 29.0, 28.8, 27.5, 23.6, 23.0, 20.5, 11.7.

(3' $\beta$ ,5' $\beta$ ,17' $\beta$ )-17'-(*prop*-2-yn-1-yloxy)-spiro[3H-diazirine-3,6'-androstan]-3'-ol, 3'-sulfate ammonium salt (**MQ234**). Using the sulfation procedure described for the preparation of **KK238**, steroid **16** (16 mg, 0.045 mmol) was converted to **MQ234** (19 mg, 93%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in  $\text{CH}_2\text{Cl}_2$ ):  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  4.71 (s, 1H), 4.21–4.12 (m, 2H), 3.64 (t, 1H,  $J$  = 8.3 Hz), 2.81 (s, 1H), 2.07–1.12 (m, 22H), 1.22 (s, 3H), 0.81 (s, 3H), 0.54–0.49 (m,

1H), 0.25–9.21 (m, 1H); <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 89.3, 81.5, 75.4, 75.2, 75.2, 58.1, 52.1, 45.5, 44.4, 40.8, 38.7, 37.8, 35.2, 33.7, 30.7, 30.1, 28.6, 28.0, 26.2, 24.2, 21.8, 12.2.

3-(4-(((3β,5β,17β)-3-(Methoxymethoxy)-androst-17-yl)oxymethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (**18**). To a solution of (3β,5β,17β)-3-(methoxymethoxy)-androst-17-ol<sup>16</sup> (**17**, 110 mg, 0.327 mmol) in THF (20 mL) was added 3-[4-(iodomethyl)phenyl]-3-(trifluoromethyl)-3H-diazirine (326 mg, 1 mmol) in THF (10 mL), Bu<sub>4</sub>Ni (80 mg) and NaH (160 mg, 60% in mineral oil, 4 mmol) at 23 °C. The reaction was gently refluxed for 16 h. After cooling to 23 °C, water was added and the product was extracted into EtOAc (2 × 150 mL). Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give steroid **18** (104 mg, 59%): <sup>1</sup>H NMR δ 7.41 (d, 2H, *J* = 8.2 Hz), 7.36 (d, 2H, *J* = 8.2 Hz), 4.67 (s, 2H), 4.55 (s, 2H), 3.90 (s, 1H), 3.38 (s, 3H), 2.02–0.84 (m, 23H), 0.98 (s, 3H), 0.82 (s, 3H); <sup>13</sup>C NMR δ 141.3, 127.9, 127.5 (2 × C), 126.4 (2 × C), 94.5, 88.7, 72.1, 71.5, 70.7, 51.3, 43.3, 40.2, 38.2, 37.1, 35.5, 35.0, 31.2, 30.6, 27.9, 26.6, 25.8, 25.3, 23.9, 23.4, 20.7, 11.8.

(3β,5β,17β)-17-[[4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-3-ol (**19**). Using the procedure described for the preparation of steroid **16**, diazirine **18** (104 mg, 0.195 mmol) was converted to diazirine **19** (54 mg, 57%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes): <sup>1</sup>H NMR δ 7.39 (d, 2H, *J* = 8.2 Hz), 7.28 (d, 2H, *J* = 8.2 Hz), 4.55 (s, 2H), 4.12 (s, 1H), 3.41 (t, 1H, *J* = 8.2 Hz), 2.06–0.90 (m, 23H), 0.99 (s, 3H), 0.83 (s, 3H); <sup>13</sup>C NMR δ 141.3, 127.9, 127.5 (2 × C), 126.4 (2 × C), 86.7, 70.7, 67.1, 51.3, 43.3, 40.0, 38.2, 36.6, 35.4, 35.2, 33.5, 30.0, 27.9, 27.8, 26.5, 25.8, 23.9, 23.4, 20.7, 11.8.

(3β,5β,17β)-17-[[4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-3-ol, 3-sulfate ammonium salt (**MQ235**). Using the sulfation procedure described for the preparation of **KK238**, steroid **19** (23 mg, 0.047 mmol) was converted to **MQ235** (32 mg, 74%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in CH<sub>2</sub>Cl<sub>2</sub>): <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 7.45 (d, 2H, *J* = 7.8 Hz), 7.24 (d, 2H, *J* = 7.8 Hz), 4.69 (s, 1H), 4.57 (s, 2H), 3.49 (t, 1H, *J* = 8.2 Hz), 2.07–0.97 (m, 26H), 1.00 (s, 3H), 0.84 (s, 3H); <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 143.3, 129.0 (2 × C), 128.9, 127.6 (2 × C), 90.4, 77.22, 77.20, 72.0, 52.6, 44.6, 41.6, 39.4, 38.5, 37.0, 36.0, 32.6, 31.7, 29.1, 27.7, 27.0, 26.9, 24.6, 24.5, 22.0, 12.4.

3-(4-(((3α,5β,17β)-3-(Methoxymethoxy)-androst-17-yl)oxymethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (**21**). Using the procedure described for the preparation of steroid **18**, (3α,5β,17β)-3-(methoxymethoxy)-androst-17-ol (**20**, 150 mg, 0.45 mmol) was converted to diazirine **21** (151 mg, 63%). Purification was by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes): <sup>1</sup>H NMR δ 7.37 (d, 2H, *J* = 8.6 Hz), 7.17 (d, 2H, *J* = 8.6 Hz), 4.68 (s, 2H), 4.53 (s, 2H), 3.54–3.38 (m, 1H), 3.36 (s, 3H), 1.99–0.82 (m, 23H), 0.93 (s, 3H), 0.80 (s, 3H); <sup>13</sup>C NMR δ 141.3, 127.9, 127.4 (2 × C), 126.4 (2 × C), 94.5, 88.8, 70.8, 55.1, 51.1, 51.2, 43.2, 42.1, 40.6, 38.1, 35.6, 34.8, 33.5, 27.9, 27.7, 27.0, 25.9, 25.3, 23.4, 23.3, 20.4, 11.8.

(3α,5β,17β)-17-[[4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-3-ol (**22**). Using the procedure described for

the preparation of steroid **16**, diazirine **21** (151 mg, 0.28 mmol) was converted to diazirine **22** (125 mg, 91%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes): <sup>1</sup>H NMR δ 7.38 (d, 2H, *J* = 8.6 Hz), 7.18 (d, 2H, *J* = 8.6 Hz), 4.54 (s, 2H), 3.66–3.58 (m, 1H), 3.41 (t, 1H, *J* = 8.2 Hz), 2.04–0.83 (m, 23H), 0.93 (s, 3H), 0.81 (s, 3H); <sup>13</sup>C NMR δ 141.2, 127.9, 127.4 (2 × C), 126.3 (2 × C), 88.7, 71.7, 70.7, 51.2, 43.2, 42.0, 40.6, 38.1, 36.3, 35.6, 35.3, 34.6, 30.4, 27.9, 27.0, 25.9, 23.4, 23.3, 20.4, 11.8.

(3α,5β,17β)-17-[[4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androst-3-ol, 3-sulfate ammonium salt (**MQ236**). Using the sulfation procedure described for the preparation of **KK238**, diazirine **22** (23 mg, 0.047 mmol) was converted to **MQ236** (26 mg, 93%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in CH<sub>2</sub>Cl<sub>2</sub>): <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 7.45 (d, 2H, *J* = 8.2 Hz), 7.23 (d, 2H, *J* = 8.2 Hz), 4.87 (s, 2H), 4.32–4.26 (m, 1H), 3.50 (t, 1H, *J* = 8.2 Hz), 2.07–0.97 (m, 26H), 0.97 (s, 3H), 0.82 (s, 3H); <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 143.3, 129.1 (2 × C), 128.9, 127.6 (2 × C), 90.4, 80.5, 72.1, 52.5, 44.5, 43.8, 42.1, 39.4, 37.2, 36.6, 35.8, 34.7, 29.1, 29.0, 28.3, 27.2, 24.6, 23.9, 21.7, 19.5, 12.4.

(3β,5α)-3-Hydroxyandrost-17-one, 3-mesylate (**24**). To a solution of (3β,5α)-3-hydroxyandrost-17-one (**23**, 1.5 g, 5.2 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (30 mL) was added mesyl chloride (0.62 mL, 8 mmol) and Et<sub>3</sub>N (1.54 mL, 11 mmol) at 0 °C. After 2 h, 5% HCl (20 mL) was added and the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (2 × 100 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give mesylate **24** (1.88 g, 98%): <sup>1</sup>H NMR δ 4.62–4.54 (m, 1H), 2.97 (s, 3H), 2.44–2.42 (m, 1H), 2.08–0.65 (m, 21H), 0.83 (s, 6H); <sup>13</sup>C NMR δ 220.9, 81.7, 54.0, 51.1, 47.6, 44.6, 38.7, 36.6, 35.7, 35.3, 34.9, 34.8, 31.3, 30.6, 28.5, 28.0, 21.6, 20.3, 13.7, 12.0.

(5α)-Androst-2-en-17-one and (5α)-androst-3-en-17-one mixture (**25**). To mesylate **24** (1.88 g, 5.16 mmol) in dry DMF (40 mL) was added LiBr (1.8 g, 20.7 mmol) at 23 °C. The reaction was heated to 130 °C for 90 min. After cooling, water (30 mL) was added and the product was extracted into EtOAc (2 × 150 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give the inseparable Δ<sup>2</sup> and Δ<sup>3</sup> double bond products **25** in a 5 : 1 ratio (1.26 g, 91%). The major Δ<sup>2</sup> product had: <sup>1</sup>H NMR δ 5.61–5.52 (m, 2H), 2.43–0.95 (m, 20H), 0.84 (s, 3H), 0.77 (s, 3H); <sup>13</sup>C NMR δ 221.2, 125.6, 125.6, 54.0, 51.3, 47.5, 41.3, 39.6, 35.7, 35.0, 34.6, 31.5, 30.5, 30.1, 28.3, 21.6, 20.1, 13.6, 11.5.

(5α,17β)-Androst-2-en-17-ol and (5α,17β)-androst-3-en-17-ol mixture (**26**). To a solution of the inseparable steroids **25** (1.26 g, 4.7 mmol) in EtOH (50 mL) was added NaBH<sub>4</sub> (378 mg, 10 mmol) at 23 °C. After 1 h, aqueous NH<sub>4</sub>Cl (30 mL) was added and the reaction was stirred for 10 min. EtOH was removed under reduced pressure and the product was extracted into EtOAc (2 × 150 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give unseparated Δ<sup>2</sup> and Δ<sup>3</sup> double bond products **26** in a 5 : 1 ratio product (1.20 g, 95%): The major Δ<sup>2</sup> product had: <sup>1</sup>H NMR δ 5.63–5.56 (m, 2H), 3.66 (t, 1H, *J* = 8.6 Hz), 2.04–0.68 (m, 21H), 0.78 (s, 3H), 0.75 (s, 3H); <sup>13</sup>C



NMR  $\delta$  125.8, 125.8, 82.0, 54.2, 51.0, 42.9, 41.5, 36.7, 35.6, 31.4, 30.5, 30.3, 28.6, 23.4, 21.0, 20.5, 14.2, 11.7, 11.0.

(2 $\alpha$ ,3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-2,3-Epoxyandrostan-17-ol (**27**). To a solution of the unseparated steroids **26** (1.20 g, 4.44 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (35 mL) was added aqueous formic acid (10 mL) and aqueous 30% H<sub>2</sub>O<sub>2</sub> (10 mL) at 23 °C. After 2 h, the product was extracted into CH<sub>2</sub>Cl<sub>2</sub> (2  $\times$  100 mL) washed with brine (2  $\times$  50 mL), dried, the solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give the 2,3-epoxide **27** which also contained the unseparated 3,4-epoxide (1.04 g, 81%). 2,3-Epoxy **27** had: <sup>1</sup>H NMR  $\delta$  3.59 (t, 1H, *J* = 8.6 Hz), 3.11–3.06 (m, 2H), 1.98–0.54 (m, 21H), 0.72 (s, 3H), 0.67 (s, 3H); <sup>13</sup>C NMR  $\delta$  81.6, 53.7, 52.4, 51.0, 50.7, 38.1, 36.5, 36.2, 35.5, 33.6, 33.1, 31.1, 30.1, 28.9, 28.1, 23.2, 20.3, 12.8, 10.9.

(1,1-Dimethylethyl)[[(2 $\alpha$ ,3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-2,3-epoxyandrostan-17-yl]oxy]dimethylsilane (**28**). Using the procedure described for the preparation of steroid **5**, 2,3-epoxide **27** containing the 3,4-epoxide (1.04 g, 3.61 mmol) was converted to 2,3-epoxide **28** containing the 3,4-epoxide (1.24 g, 86%). Purification was by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes). 2,3-Epoxy **28** had: <sup>1</sup>H NMR  $\delta$  3.54 (t, 1H, *J* = 8.6 Hz), 3.13–3.08 (m, 2H), 1.93–0.56 (m, 20H), 0.87 (s, 9H), 0.75 (s, 3H), 0.67 (s, 3H), 0.00 (s, 3H), –0.01 (s, 3H); <sup>13</sup>C NMR  $\delta$  81.7, 53.9, 52.3, 50.9, 50.4, 43.0, 38.3, 37.0, 36.2, 35.7, 33.6, 31.2, 30.8, 29.0, 28.3, 25.8 (3  $\times$  C), 23.4, 20.5, 18.0, 12.9, 11.2, –4.6, –3.9.

(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-2-(But-3-yn-1-yloxy)-17-[[1,1-dimethylethyl]dimethylsilyl]oxy]-androstan-3-ol (**29**). To a solution of 2,3-epoxide **28** containing the 3,4-epoxide epoxide (856 mg, 2.13 mmol) in 3-butyne-1-ol (3 mL) was added tetracyanoethylene (800 mg) at 23 °C. After 72 h, 3-butyne-1-ol was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to give steroid **29** (440 mg, 44%): <sup>1</sup>H NMR  $\delta$  4.66 (t, 1H, *J* = 5.8 Hz), 3.90 (s, 1H), 3.63–3.43 (m, 3H), 2.79–2.75 (m, 1H), 2.42–2.38 (m, 2H), 2.21–0.59 (m, 21H), 0.92 (s, 3H), 0.86 (s, 9H), 0.67 (s, 3H), –0.02 (s, 6H); <sup>13</sup>C NMR  $\delta$  81.7, 79.0, 75.2, 73.1, 69.1, 68.4, 67.0, 55.2, 50.6, 43.2, 39.0, 37.1, 36.2, 35.9, 34.9, 32.1, 31.5, 30.8, 28.0, 25.8, 23.4, 20.5, 20.1, 19.8, 18.0, 13.3, 11.3, –4.6, –4.8.

(1,1-Dimethylethyl)[[(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-2-(but-3-yn-1-yloxy)-3-(methoxymethoxy)-androstan-17-yl]oxy]dimethylsilane (**30**). To a solution of steroid **29** (440 mg, 0.93 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added ClCH<sub>2</sub>OMe (0.2 mL) and (i-Pr)<sub>2</sub>NEt (0.42 mL, 3 mmol) at 23 °C. The reaction was stirred at 23 °C for 16 h. Water was added and the product was extracted into EtOAc (2  $\times$  150 mL). Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes) to give steroid **30** (378 mg, 78%): <sup>1</sup>H NMR  $\delta$  4.68–4.63 (m, 2H), 3.75 (s, 1H), 3.64–3.45 (m, 4H), 3.36 (s, 3H), 2.43–2.39 (m, 2H), 1.96–1.20 (m, 18H), 0.96–0.84 (m, 3H), 0.93 (s, 3H), 0.87 (s, 9H), 0.68 (s, 3H), 0.00 (s, 6H); <sup>13</sup>C NMR  $\delta$  95.3, 81.8, 81.2 (2  $\times$  C), 77.4, 73.9, 69.1, 67.0, 55.3, 50.7, 43.3, 39.7, 37.2, 36.9, 35.7, 35.0, 31.6, 30.9, 29.8, 28.1, 25.8 (3  $\times$  C), 23.4, 20.5, 20.2, 18.1, 13.3, 11.4, –4.5, –4.8.

(2 $\beta$ ,3 $\alpha$ ,5 $\alpha$ ,17 $\beta$ )-2-(But-3-yn-1-yloxy)-3-(methoxymethoxy)-androstan-17-ol (**31**). Using the procedure described for the preparation of compound **10**, steroid **30** (378 mg, 0.732 mmol)

was converted into steroid **31** (212 mg, 71%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes): <sup>1</sup>H NMR  $\delta$  4.65–4.61 (m, 2H), 3.72 (s, 1H), 3.62–3.56 (m, 4H), 3.35 (s, 3H), 2.41–2.38 (m, 2H), 2.03–0.64 (m, 22H), 0.92 (s, 3H), 0.69 (s, 3H); <sup>13</sup>C NMR  $\delta$  95.3, 81.8, 81.4, 77.3, 73.8, 69.1, 67.0, 55.3, 55.1, 51.0, 42.9, 39.6, 36.7, 36.7, 35.6, 34.9, 31.5, 30.4, 29.7, 28.0, 23.3, 20.4, 20.1, 13.2, 11.1.

3-(4-(((2 $\beta$ ,3 $\alpha$ ,5 $\beta$ ,17 $\beta$ )-2-(But-3-yn-1-yloxy)-3-(methoxymethoxy)-androstan-17-yl)oxymethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (**32**). Using the procedure described for the preparation of compound **18**, steroid **31** (210 mg, 0.52 mmol) was converted to steroid **32** (164 mg, 52%). Purification was by flash column chromatography (silica gel eluted with 10% EtOAc in hexanes): <sup>1</sup>H NMR  $\delta$  7.37 (d, 2H, *J* = 8.8 Hz), 7.17 (d, 2H, *J* = 8.8 Hz), 4.73–4.68 (s, 2H), 4.67 (s, 2H), 4.73 (s, 1H), 3.59–3.39 (m, 4H), 3.36 (s, 3H), 2.44–2.37 (m, 2H), 2.02–0.66 (m, 21H), 0.93 (s, 3H), 0.80 (s, 3H); <sup>13</sup>C NMR  $\delta$  141.3, 127.8, 127.4 (2  $\times$  C), 126.3 (2  $\times$  C), 95.3, 88.7, 81.4, 77.4, 73.9, 70.7, 69.1, 67.0, 55.3, 55.1, 51.2, 43.1, 39.6, 38.0, 36.8, 35.6, 34.7, 31.5, 29.8, 28.0, 27.8, 23.3, 20.5, 20.2, 13.3, 11.8.

(2 $\beta$ ,3 $\alpha$ ,5 $\beta$ ,17 $\beta$ )-2-(But-3-yn-1-yloxy)-17-[[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androstan-3-ol (**33**). Using the procedure described for the preparation of steroid **16**, diazirine **32** (164 mg, 0.327 mmol) was converted to diazirine **33** (104 mg, 68%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes): <sup>1</sup>H NMR  $\delta$  7.38 (d, 2H, *J* = 8.8 Hz), 7.18 (d, 2H, *J* = 8.8 Hz), 4.54 (s, 2H), 4.96 (s, 1H), 3.68–3.38 (m, 3H), 3.36 (t, 1H, *J* = 8.6 Hz), 2.45–2.41 (m, 2H), 2.00–0.66 (m, 22H), 0.95 (s, 3H), 0.81 (s, 3H); <sup>13</sup>C NMR  $\delta$  141.3, 127.9, 127.4 (2  $\times$  C), 126.4 (2  $\times$  C), 99.7, 81.5, 79.1, 70.7, 69.1, 68.5, 67.0, 55.2, 51.2, 43.1, 39.0, 38.0, 36.3, 35.9, 34.7, 32.1, 31.5, 28.0, 27.8, 23.3, 20.5, 20.2, 13.3, 11.9.

(2 $\beta$ ,3 $\alpha$ ,5 $\beta$ ,17 $\beta$ )-2-(But-3-yn-1-yloxy)-17-[[4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl]methoxy]-androstan-3-ol 3-sulfate, ammonium salt (**MQ237**). Using the sulfation procedure described for the preparation of **KK238**, diazirine **33** (60 mg, 0.108 mmol) was converted to **MQ237** (21 mg, 30%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in CH<sub>2</sub>Cl<sub>2</sub>): <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  7.44 (d, 2H, *J* = 7.8 Hz), 7.22 (d, 2H, *J* = 7.8 Hz), 4.55 (s, 2H), 4.41 (s, 1H), 3.61–3.42 (m, 4H), 2.41–0.67 (m, 27H), 0.96 (s, 3H), 0.81 (s, 3H); <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  141.7, 127.5 (2  $\times$  C), 127.3, 126.0 (2  $\times$  C), 88.9, 80.9, 76.7, 74.6, 70.4, 68.8, 67.2, 55.2, 51.0, 42.9, 39.2, 37.7, 36.9, 35.1, 34.7, 31.3, 29.4, 27.6, 27.4, 22.9, 20.2, 19.5, 12.6, 10.9.

(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-Spiro[androstan-3,17-diol-6,2'[1,3]dioxolane] (**34**). To a solution of steroid **10** (1.65 g, 4.7 mmol) in benzene (150 mL) was added ethylene glycol (5.0 mL) and PTSA (200 mg) at 23 °C. The reaction was refluxed in a flask equipped with a Dean–Stark trap for 16 h. After cooling, solid NaHCO<sub>3</sub> (2 g) was added and stirred for 15 min. The product was extracted into EtOAc (250 mL). The organic layer was washed with brine (5  $\times$  100 mL), solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 40% EtOAc in hexanes) to give steroid **34** (934 mg, 57%): <sup>1</sup>H NMR  $\delta$  3.96–3.56 (m, 6H), 1.96–0.67 (m, 22H), 0.95 (s, 3H), 0.75 (s, 3H); <sup>13</sup>C NMR  $\delta$  109.5, 81.8, 71.5, 65.5, 64.3, 53.7, 50.7, 50.6, 43.0, 41.0, 38.2, 36.9, 36.5, 33.4, 31.1, 30.4, 29.1, 23.3, 20.7, 14.2, 11.1.



*(5 $\alpha$ )-Spiro[androstane-3,17-dione-6,2'-[1,3]dioxolane]* (35).

Using the procedure described for the preparation of compound **8**, steroid **34** (164 mg, 0.327 mmol) was converted to steroid **35** (913 mg, 98%). Purification was by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes):  $^1\text{H}$  NMR  $\delta$  3.95–3.76 (m, 4H), 2.51–0.78 (m, 20H), 1.09 (s, 3H), 0.86 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  220.5, 212.0, 108.6, 65.6, 64.2, 53.0, 51.3, 50.7, 47.6, 39.8, 38.8, 37.5, 36.9, 36.2, 35.6, 32.9, 31.2, 21.6, 20.4, 13.7, 13.3.

*(3 $\beta$ ,5 $\alpha$ )-Spiro[3-(methoxymethoxy)-androstane-17-one-6,2'-[1,3]dioxolane]* (36). To a solution of steroid **35** (913 mg, 2.64 mmol) in THF (40 mL) was slowly added lithium tri-*tert* butoxide aluminum hydride (1.0 M in THF, 5 mL, 5 mmol) at  $-40^\circ\text{C}$ . After 1 h, water (30 mL) was added at  $-40^\circ\text{C}$  and then warmed to  $23^\circ\text{C}$ . The product was extracted into EtOAc ( $2 \times 200$  mL). The combined extracts were dried, filtered and the solvents removed. The crude product was dissolved in  $\text{CH}_2\text{Cl}_2$  (50 mL), (*i*-Pr) $_2\text{EtN}$  (1.3 mL, 7.5 mmol) and  $\text{ClCH}_2\text{OMe}$  (0.45 mL, 6.0 mmol) were added and the reaction was stirred at  $23^\circ\text{C}$  for 16 h. Water was added and the product extracted into  $\text{CH}_2\text{Cl}_2$ . The combined extracts were washed with brine, dried and solvent removed to give a viscous liquid that was purified by flash column chromatography (silica gel eluted with 25–35% EtOAc in hexanes) to give steroid **36** as a colorless liquid (859 mg, 2 steps, 84%):  $^1\text{H}$  NMR  $\delta$  4.69 (s, 2H), 3.99–3.81 (m, 4H), 3.54–3.47 (m, 1H), 3.37 (s, 3H), 2.49–0.73 (m, 20H), 0.97 (s, 3H), 0.88 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  221.0, 109.3, 94.4, 76.0, 65.6, 64.4, 55.2, 53.7, 50.9, 50.8, 47.8, 40.3, 38.1, 37.1, 35.8, 33.0, 31.3, 28.2, 26.2, 21.7, 20.3, 14.2, 13.8.

*(3 $\beta$ ,5 $\alpha$ ,17E)-Spiro[3-(methoxymethoxy)-pregn-17(20)-ene-21-oic acid-6,2'-[1,3]dioxolane], ethyl ester* (37). Steroid **36** (859 mg, 2.19 mmol) and triethyl phosphonoacetate (4.4 mL, 22 mmol) in anhydrous EtOH (25 mL) under  $\text{N}_2$  was stirred at  $35\text{--}40^\circ\text{C}$ . NaOEt solution (sodium 460 mg, 20 mmol in ethanol 20 mL) was slowly added dropwise and then the reaction was refluxed for 16 h. EtOH was removed and water was added. The product was extracted into  $\text{CH}_2\text{Cl}_2$  ( $2 \times 150$  mL) and the combined extracts were washed with brine ( $2 \times 50$  mL). The extract was dried, filtered, solvents removed and the residue was purified by flash column chromatography (silica gel eluted with 20% EtOAc in hexanes) to give steroid **37** (748 mg, 74%):  $^1\text{H}$  NMR  $\delta$  5.48 (s, 1H), 4.64 (s, 2H), 4.12–4.07 (q, 2H,  $J = 6.6$  Hz), 3.92–3.74 (m, 4H), 3.46–3.44 (m, 1H), 3.32 (s, 3H), 2.80–2.78 (m, 2H), 2.00–0.68 (m, 21H), 0.92 (s, 3H), 0.79 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  175.9, 167.2, 109.2, 108.4, 94.3, 76.0, 65.4, 64.2, 59.4, 55.0, 53.5, 52.9, 50.5, 46.1, 41.1, 38.0, 36.9, 35.0, 33.0, 30.2, 28.1, 26.1, 24.2, 20.8, 18.4, 14.3, 14.1.

*(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-Spiro[3-(methoxymethoxy)-pregnane-21-oic acid-6,2'-[1,3]dioxolane], ethyl ester* (38). To a solution of steroid **37** (748 mg, 1.62 mmol) in EtOH (100 mL) in a hydrogenation flask was added  $\text{PtO}_2$  (30 mg) at  $23^\circ\text{C}$ . The flask was evacuated and filled three times with  $\text{H}_2$ . The hydrogenation was carried out under 50 psi of  $\text{H}_2$ . After 3 h, the reaction mixture was filtered through Celite and washed with EtOAc (200 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes)

to afford steroid **38** (650 mg, 87%):  $^1\text{H}$  NMR  $\delta$  4.63 (s, 2H), 4.09 (q, 2H,  $J = 7.0$  Hz), 3.93–3.66 (m, 4H), 3.49–3.33 (m, 1H), 3.31 (s, 3H), 2.33–0.63 (m, 26H), 0.90 (s, 3H), 0.56 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  173.6, 109.4, 94.3, 76.2, 65.4, 64.1, 60.0, 55.0, 54.7, 53.7, 50.7, 46.7, 42.1, 41.3, 38.1, 37.1, 36.9, 35.2, 33.4, 28.2, 28.0, 26.2, 24.4, 20.7, 14.1, 14.0, 12.5.

*(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-2R-(3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)-5-((benzyl)oxy)pentanoate, ethyl ester* (39). To a solution of steroid **38** (642 mg, 1.38 mmol) in THF (30 mL) was added LDA (1.5 mL, 2.0 M, 3 mmol) and HMPA (4 mmol, 0.72 mL) at  $-78^\circ\text{C}$ . After 45 min, ((3-iodopropoxy)methyl)benzene (828 mg, 3 mmol) in THF (5 mL) was added and the reaction was warmed to  $23^\circ\text{C}$  for 16 h. Water was added and the product was extracted into EtOAc ( $2 \times 150$  mL). The combined extracts were dried, filtered and the solvents removed. The residue was purified by flash column chromatography (silica gel eluted with 10–25% EtOAc in hexanes) to afford steroid **39** (830 mg, 98%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.35–7.28 (m, 5H), 4.70 (s, 2H), 4.49 (d, 2H,  $J = 1.8$  Hz), 4.13 (q, 2H,  $J = 7.0$  Hz), 3.97–3.77 (m, 4H), 3.51–3.43 (m, 3H), 3.37 (s, 3H), 2.24–0.78 (m, 29H), 0.94 (s, 3H), 0.70 (s, 3H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  175.9, 138.5, 128.3 ( $2 \times \text{C}$ ), 127.5 ( $2 \times \text{C}$ ), 127.4, 109.5, 94.4, 76.3, 72.7 ( $2 \times \text{C}$ ), 69.9, 65.4, 64.2, 59.8, 55.2, 55.1, 53.6, 52.6, 50.7, 47.1, 42.2, 41.3, 38.1, 37.5, 36.9, 33.4, 28.5, 28.3, 27.3, 26.9, 26.2, 23.7, 20.9, 14.2, 12.2.

*(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-2R-(3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)-5-((benzyl)oxy)pentanol* (40). To a solution of steroid **39** (830 mg, 1.36 mmol) in diethyl ether (50 mL) was added  $\text{LiAlH}_4$  (2.0 M in THF, 5 mL, 10 mmol) at  $23^\circ\text{C}$ . After 2 h, 1.2 M NaOH (2 mmol, 1.7 mL) was slowly added. After stirring for 45 min, the mixture was filter through Celite and washed with  $\text{CH}_2\text{Cl}_2$  (200 mL). Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel eluted with 35% EtOAc in hexanes) to afford steroid **40** (637 mg, 82%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.35–7.28 (m, 5H), 4.69 (s, 2H), 4.51 (s, 2H), 3.95–3.45 (m, 9H), 3.37 (s, 3H), 1.99–0.70 (m, 27H), 0.95 (s, 3H), 0.69 (s, 3H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  138.4, 128.3 ( $2 \times \text{C}$ ), 127.6 ( $2 \times \text{C}$ ), 127.5, 109.6, 94.4, 76.2, 72.9, 70.8, 65.4, 64.2, 62.5, 55.8, 55.1, 53.6, 50.6, 50.3, 42.3, 42.1, 41.3, 39.1, 38.1, 36.9, 33.3, 28.2, 27.5, 26.2, 25.9, 25.5, 24.0, 21.0, 14.1, 12.2.

*17-((R)-5-(Benzyl)oxy)pentan-2-yl)-3-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-(methoxymethoxy))-spiro[androstane-6,2'-[1,3]dioxolane]* (41). To a solution of steroid **40** (637 mg, 1.12 mmol) in  $\text{CH}_2\text{Cl}_2$  (30 mL) was added  $\text{MsCl}$  (0.20 mL, 2.5 mmol) and  $\text{Et}_3\text{N}$  (0.56 mL, 4 mmol) at  $0^\circ\text{C}$ . After 1 h, aqueous  $\text{NaHCO}_3$  (50 mL) was added and the product was extracted into  $\text{CH}_2\text{Cl}_2$  ( $2 \times 100$  mL). The combined extracts were dried, filtered and the solvents removed. To a solution of the crude mesylate (724 mg, 1.12 mmol) in diethyl ether (50 mL) was added  $\text{LiAlH}_4$  (2.0 M in THF, 5 mL, 10 mmol) at  $23^\circ\text{C}$ . After 2 h, 1.2 M NaOH (2 mmol, 1.7 mL) was slowly added. After stirring for 45 min, the mixture was filtered through Celite and the Celite was washed with  $\text{CH}_2\text{Cl}_2$  (250 mL). Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to afford steroid **41** (600 mg, 97%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.35–7.25 (m, 5H), 4.69 (s, 2H), 44.50 (s, 2H), 3.95–3.41 (m, 7H), 3.36 (s, 3H), 1.99–0.72 (m, 29H), 0.94 (s, 3H), 0.67 (s, 3H);



$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  138.7, 128.3 (2  $\times$  C), 127.6 (2  $\times$  C), 127.4, 109.7, 94.4, 76.3, 72.8, 71.0, 65.5, 64.2, 56.0, 56.0, 55.1, 53.6, 50.7, 42.6, 41.4, 39.8, 38.2, 37.0, 35.5, 33.4, 32.2, 28.3, 28.1, 26.3, 26.2, 24.1, 21.0, 18.6, 14.2, 12.0.

(*R*)-4-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)pentan-1-ol (**42**). To a solution of steroid **41** (600 mg, 1.08 mmol) in EtOAc (100 mL) in a hydrogenation flask was added 10% Pd/C (300 mg) at 23 °C. The flask was evacuated and flushed three times with  $\text{H}_2$ . Hydrogenation was carried out at 50 psi of  $\text{H}_2$ . After 1 h, the reaction mixture was filtered through Celite and the Celite was washed with EtOAc (200 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 35% EtOAc in hexanes) to afford steroid **42** (470 mg, 93%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.69 (s, 2H), 3.97–3.66 (m, 4H), 3.49–3.46 (m, 7H), 3.37 (s, 3H), 1.99–0.65 (m, 26H), 0.95 (s, 3H), 0.68 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  109.6, 94.4, 76.3, 65.5, 64.2, 63.5, 56.0, 56.0, 55.1, 53.6, 50.7, 42.6, 41.4, 39.7, 38.2, 37.0, 35.5, 33.4, 31.8, 29.4, 28.3, 28.1, 26.2, 24.1, 21.0, 18.6, 14.2, 12.0.

(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-17-((*R*)-5-Hydroxypent-2-yl)-3-(methoxymethoxy)-androstane-6-one (**43**). To a solution of the ketal (470 mg, 1 mmol) in acetone (50 mL) and water (0.5 mL) was added *p*-toluenesulfonic acid monohydrate (50 mg) at 23 °C. After 3 h, solid  $\text{NaHCO}_3$  (200 mg) was added. Acetone was removed and the residue was purified by flash column chromatography (silica gel eluted with 30% EtOAc in hexanes) to afford steroid **43** (325 mg, 84%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.64–4.56 (m, 2H), 3.52–3.37 (m, 3H), 3.29 (s, 3H), 2.26–0.80 (m, 30H), 0.69 (s, 3H), 0.60 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  210.7, 94.2, 75.1, 63.1, 56.6, 56.5, 55.8, 55.0, 53.7, 46.5, 42.8, 40.9, 39.3, 37.7, 36.5, 35.3, 31.7, 29.2, 28.0, 27.9, 26.8, 23.8, 21.3, 18.4, 12.9, 11.9.

(*R*)-4-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-3-(Methoxymethoxy)-spiro[androstane-6,3'-diazirin]-17-yl)pentan-1-ol (**44**). Using the procedure described for the preparation of compound **11**, steroid **43** (375 mg, 0.89 mmol) was converted to diazirine **44** (325 mg, 84%). Purification was by flash column chromatography (silica gel eluted with 25–40% EtOAc in hexanes):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.56 (s, 2H), 3.59 (q, 2H,  $J$  = 5.2 Hz), 3.54–3.23 (m, 1H), 3.28 (s, 3H), 2.02–0.73 (m, 28H), 1.12 (s, 3H), 0.68 (s, 3H), 0.49–0.36 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  94.3, 75.9, 63.4, 56.0, 55.9, 55.1, 53.6, 45.2, 42.6, 39.6, 37.6, 37.3, 36.3, 35.4, 33.8, 31.7, 30.2, 29.3, 29.3, 28.0, 27.9, 23.8, 21.1, 18.6, 12.8, 12.0.

(*R*)-4-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-3-Hydroxy-spiro[androstane-6,3'-diazirin]-17-yl)pentan-1-ol, 1-sulfate ammonium salt (**MQ271**). Using the sulfation procedure described for the preparation of **KK238**, diazirine **44** (325 mg, 0.75 mmol) was converted to **MQ271** (287 mg, 79%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in  $\text{CH}_2\text{Cl}_2$ ):  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  3.91 (t, 2H,  $J$  = 6.4 Hz), 3.42–2.29 (m, 1H), 2.10–0.64 (m, 32H), 1.08 (s, 3H), 0.68 (s, 3H), 0.37–0.26 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  71.7, 68.8, 57.6, 57.4, 55.1, 46.5, 44.0, 41.2, 38.8, 38.5, 37.6, 36.9, 35.3, 34.1, 33.2, 31.5, 29.2, 27.2, 25.1, 22.4, 19.2, 13.5, 12.6.

(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-2*R*-(3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)-5-((triisopropylsilyl)oxy)pentanoate, ethyl ester (**45**). To a solution of steroid **38** (650 mg, 1.4 mmol) in THF (30 mL) was added LDA (2.1 mL, 2.0 M, 4.2 mmol) and HMPA

(5 mmol, 0.90 mL) at  $-78$  °C. After 45 min, (3-iodopropoxy)-(triisopropyl)silane (1.44 g, 4.2 mmol) in THF (5 mL) was added and the reaction was warmed to 23 °C for 16 h. Water was added and the product was extracted into EtOAc (2  $\times$  150 mL). The combined extracts were dried, filtered, solvents removed and the residue was purified by flash column chromatography (silica gel eluted with 25% EtOAc in hexanes) to afford steroid **45** (902 mg, 95%):  $^1\text{H}$  NMR  $\delta$  4.63 (s, 2H), 4.10 (q, 2H,  $J$  = 7.4 Hz), 3.90–3.81 (m, 3H), 3.78–3.56 (m, 3H), 3.48–3.33 (m, 1H), 3.31 (s, 3H), 2.22–0.61 (m, 32H), 1.00 (s, 18H), 0.88 (s, 3H), 0.66 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  175.9, 109.4, 94.3, 76.2, 65.4, 64.2, 62.8, 59.6, 55.2, 55.0, 53.5, 52.5, 50.6, 47.0, 42.1, 41.3, 38.0, 37.4, 36.9, 33.3, 30.4, 28.2, 26.8, 26.1, 23.6, 20.8, 17.9 (7  $\times$  C), 14.1 (2  $\times$  C), 12.1, 11.8 (3  $\times$  C).

(3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-2*R*-(3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)-5-((triisopropylsilyl)oxy)pentanol (**46**). To a solution of the steroid **45** (902 mg, 1.33 mmol) in diethyl ether (60 mL) was added  $\text{LiAlH}_4$  (2.0 M in THF, 4 mL, 8 mmol) at 23 °C. After 2 h, 1.2 M NaOH (1.6 mmol, 1.33 mL) was slowly added. After stirring for 45 min, the mixture was filter through Celite and the Celite was washed with  $\text{CH}_2\text{Cl}_2$  (200 mL). Solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 40% EtOAc in hexanes) to afford steroid **46** (813 mg, 96%):  $^1\text{H}$  NMR  $\delta$  4.66 (s, 2H), 3.93–3.35 (m, 9H), 1.96–0.64 (m, 30H), 1.04 (s, 21H), 0.91 (s, 3H), 0.67 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  109.6, 94.3, 76.2, 65.4, 64.2, 63.7, 62.8, 55.8, 55.1, 53.6, 50.6, 50.4, 42.3, 42.0, 41.3, 39.1, 38.1, 36.9, 33.3, 28.9, 28.2, 27.4, 26.2, 25.1, 24.0, 21.0, 18.0 (6  $\times$  C), 14.1, 12.2, 11.9 (3  $\times$  C).

Triisopropyl(((*R*)-4-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-3-(methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)hex-5-yn-1-yl)oxy)silane (**47**). To a solution of steroid **46** (813 mg, 1.28 mmol) in  $\text{CH}_2\text{Cl}_2$  (30 mL) was added  $\text{NaHCO}_3$  (800 mg) and Dess–Martin periodinane (1.27 g, 3 mmol) at 23 °C. After 2 h, water was added and the product was extracted into  $\text{CH}_2\text{Cl}_2$  (2  $\times$  100 mL). The solvent was removed and the residue was purified by flash chromatography (silica gel, eluted with 25% EtOAc in hexanes) to give an aldehyde intermediate (687 mg, 1.08 mmol) which was dissolved in MeOH/THF (6 mL/6 mL) and (dimethyl-1-diazo-2-oxopropyl)phosphonate (0.46 mL, 3 mmol) and  $\text{K}_2\text{CO}_3$  (437 mg, 4 mmol) were added at 23 °C. After 48 h, water was added and the product was extracted into EtOAc (3  $\times$  100 mL). The solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10–25% EtOAc in hexanes) to give recovered aldehyde (120 mg) and steroid **47** (438 mg, 64%):  $^1\text{H}$  NMR  $\delta$  4.68 (s, 2H), 3.95–3.37 (m, 7H), 3.35 (s, 3H), 2.23–0.66 (m, 30H), 1.05 (s, 18H), 0.94 (s, 3H), 0.73 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  109.5, 94.3, 87.5, 76.2, 70.4, 65.4, 64.2, 63.1, 55.6, 55.0, 53.7, 53.3, 50.7, 42.7, 41.4, 38.8, 38.1, 36.9, 33.4, 32.7, 30.1, 29.9, 28.3, 27.8, 26.2, 23.7, 20.8, 18.0 (6  $\times$  C), 14.1, 12.3, 11.9 (3  $\times$  C).

(*R*)-4-((3 $\beta$ ,5 $\alpha$ ,17 $\beta$ )-3-(Methoxymethoxy)-spiro[androstane-6,2'-[1,3]dioxolan]-17-yl)hex-5-yn-1-ol (**48**). To a solution of steroid **47** (438 mg, 0.70 mmol) in THF (10 mL) was added TBAF (2 mL, 1.0 M in THF, 2.0 mmol) at 23 °C. After 4 h, THF was removed and the residue was purified by flash column chromatography (silica gel eluted with 30% EtOAc in hexanes) to give steroid **48**



(330 mg, ~100%):  $^1\text{H}$  NMR  $\delta$  4.62 (s, 2H), 3.89–3.31 (m, 7H), 3.29 (s, 3H), 2.48–0.61 (m, 28H), 0.88 (s, 3H), 0.68 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  109.4, 94.2, 87.2, 76.2, 70.5, 65.3, 64.1, 62.3, 55.5, 54.9, 53.5, 53.1, 50.5, 42.6, 41.3, 38.7, 38.0, 36.8, 33.2, 32.7, 30.0, 29.7, 28.1, 27.8, 26.0, 23.6, 14.0, 13.8, 12.2.

( $3\beta,5\alpha,17\beta$ )-17-((*R*)-6-Hydroxyhex-1-yn-3-yl)-3-(methoxymethoxy)-androstane-6-one (**49**). To a solution of the steroid **48** (320 mg, 0.68 mmol) in acetone/water (50 mL/0.5 mL) was added *p*-toluenesulfonic acid (100 mg) at 23 °C. After 3 h, solid  $\text{NaHCO}_3$  (200 mg) was added, solvent was removed and the residue was purified by flash column chromatography (silica gel eluted with 10–25% EtOAc in hexanes) to give steroid **49** (278 mg, 96%):  $^1\text{H}$  NMR  $\delta$  4.65 (q, 2H,  $J = 6.6$  Hz), 3.61 (t, 2H,  $J = 6.2$  Hz), 3.46–3.39 (m, 1H), 3.31 (s, 3H), 2.28–0.66 (m, 28H), 0.71 (s, 3H), 0.68 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  210.7, 94.2, 87.1, 75.1, 70.7, 62.4, 56.6, 56.2, 55.0, 53.9, 53.1, 46.5, 43.0, 40.9, 38.4, 37.7, 36.5, 32.7, 30.0, 29.7, 28.0, 27.7, 26.8, 23.4, 21.1, 12.9, 12.2.

(*R*)-4-(( $3\beta,5\alpha,17\beta$ )-3-(Methoxymethoxy)-spiro[androstane-6,3'-diazirin]-17-yl)hex-5-yn-1-ol (**50**). Using the procedure described for the preparation of compound **11**, steroid **49** (278 mg, 0.65 mmol) was converted to diazirine **50** (203 mg, 73%). Reaction times: step 1, 72 h; step 2, 20 min. Purification was by flash column chromatography (silica gel eluted with 25–40% EtOAc in hexanes):  $^1\text{H}$  NMR  $\delta$  4.59 (s, 2H), 3.69 (t, 2H,  $J = 6.2$  Hz), 3.40–3.33 (m, 1H), 3.31 (s, 3H), 2.47–0.74 (m, 26H), 1.15 (s, 3H), 0.77 (s, 3H), 0.51–0.39 (m, 2H);  $^{13}\text{C}$  NMR  $\delta$  94.4, 87.3, 75.7, 70.8, 62.8, 55.6, 55.1, 53.8, 53.2, 45.3, 42.8, 38.8, 37.7, 37.4, 36.3, 33.8, 32.9, 30.3, 30.0, 29.9, 29.4, 28.0, 27.9, 23.4, 21.0, 12.9, 12.3.

(*R*)-4-(( $3\beta,5\alpha,17\beta$ )-3-Hydroxy-spiro[androstane-6,3'-diazirin]-17-yl)hex-5-yn-1-ol, 1-sulfate ammonium salt (**MQ273**). Using the sulfation procedure described for the preparation of **KK238** followed by removal of the MOM protecting group using 6 N HCl in THF at 23 °C for 2 h, converted diazirine **50** (148 mg, 0.34 mmol) to **MQ273** (137 mg, 83%). Purification was by flash column chromatography (silica gel eluted with 10–25% MeOH in  $\text{CH}_2\text{Cl}_2$ ):  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  3.96 (q, 2H,  $J = 6.2$  Hz), 3.37–2.31 (m, 1H), 2.47–0.60 (m, 30H), 1.09 (s, 3H), 0.74 (s, 3H), 0.36–0.26 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  87.9, 72.6, 71.6, 69.0, 57.1, 55.2, 54.9, 50.0, 46.5, 44.1, 40.4, 38.8, 38.6, 37.6, 35.3, 34.0, 31.5, 31.4, 30.3, 29.0, 27.8, 24.7, 22.3, 13.6, 12.9.

### Physiological methods

**Materials.** GABA, NMDA and salts were purchased from Sigma-Aldrich (St. Louis, MO). Channel blockers NBQX, D-APV and gabazine were from Tocris Bioscience (Minneapolis, MN).

**Rat hippocampal cultures.** Rat primary cultures of hippocampal cells were prepared from 1 to 3 days postnatal Sprague Dawley rats, as described previously.<sup>22</sup> Tissue was prepared according to protocols approved by the Institutional Animal Care and Use Committee.

**Whole-cell patch-clamp recording in cell cultures.** Experiments on hippocampal neurons were conducted using standard whole-cell techniques. The bath solution contained (in mM): 138 NaCl, 4 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 glucose, and 10 HEPES; pH 7.25 with NaOH. Patch pipettes were filled with an internal solution containing (in mM): 130 CsCl, 4 NaCl, 4  $\text{MgCl}_2$ , 0.5  $\text{CaCl}_2$ , 5 EGTA, 10 HEPES, pH 7.25 with CsOH. When filled with this solution, pipette open tip resistance was 3–6 M $\Omega$ . Cells were

clamped at  $-70$  mV. Agonist was applied alone or was co-applied with putative modulator with a multibarrel, gravity-driven local perfusion system. Agonist-alone trials were interleaved with co-applications to ensure reversibility. Culture recordings were performed at room temperature.

Currents were recorded under voltage clamp with an Axopatch 200B amplifier (Molecular Devices, San Jose, CA) and were filtered at 2 kHz and recorded at 5 kHz using pClamp 9.0 software (Clampex, Molecular Devices). Data were analyzed with Clampfit 9.0 (Molecular Devices). The peak current was measured to estimate potentiation, and the ending current was measured to estimate inhibition. Data are presented normalized to the preceding agonist-alone trial and are given in the text and figures as mean  $\pm$  SEM.

### Photolabeling methods

**Cell culture and membrane preparation.** HEK293 cells expressing high density human  $\alpha_1$ -8x His-FLAG and human  $\beta_3$  GABA<sub>A</sub> receptors were generated as previously described.<sup>10</sup> Full-length rat GluN1<sub>FLAG</sub>/GluN2B receptors were expressed in GNTI<sup>−</sup> cells using the BacMam expression system.<sup>23</sup> HEK cell membranes were prepared as previously described (Chen *et al.* 2019). Briefly, HEK cells at 80% confluency were harvested in phosphate-buffered saline (PBS) containing proteinase inhibitors. The cells were homogenized with a glass mortar and Teflon pestle and the cell membranes were collected as a pellet after centrifugation at 34 000g for 30 minutes at 4 °C. The membranes were resuspended in 10 mM potassium phosphate and 100 mM KCl and stored at  $-80$  °C.

**Photolabeling and cycloaddition reactions.** HEK cell membranes were resuspended at 1.25 mg mL<sup>−1</sup> in 10 mM potassium phosphate and 100 mM KCl. Photolabeling reagents were added to the membrane suspensions and incubated on ice for 1 hour before UV light exposure. Photolabeling was performed as previously described.<sup>24</sup> The photolabeled membranes were collected by centrifugation at 21 000g for 15 min. Membranes were then solubilized in 2% SDS/PBS for two hours at room temperature. Following centrifugation at 21 000g for 30 minutes, the supernatant (protein lysate) containing solubilized membrane protein was collected. TAMRA or MQ112 (biotin-azide linker) was attached to the photolabeled proteins by incubation overnight at room temperature in PBS buffer containing 2% SDS, TAMRA or MQ112, 2.5 mM sodium ascorbate, 250  $\mu\text{M}$  Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, and 2.5 mM  $\text{CuSO}_4$ . TAMRA and MQ112 were added to the incubations at a 3 : 1 molar ratio to the concentration of the PAL used for labeling.

**Enrichment of photolabeled proteins.** Photolabeled proteins coupled to **MQ112** were enriched by diluting SDS to 0.5% and then loading the lysate onto a column containing streptavidin agarose beads. The column was washed with 0.5% SDS/PBS and photolabeled proteins were eluted by cleaving the linker with 100 mM sodium dithionite/0.5% SDS/PBS. The yellow color of **MQ112** was eliminated following dithionite elution, indicating complete cleavage of the linker. The eluted proteins were concentrated to 100  $\mu\text{L}$  using a 30 kDa cutoff Centricon filter. The concentrated proteins were analyzed by SDS-PAGE followed by western blot with an anti-Flag antibody.





## Data availability

ESI materials† contain the carbon-13 and proton NMR spectra of the 10 reported photoaffinity labeling reagents.

## Author contributions

DFC and SM conceptualized the study. DFC, SM and ASE designed and had oversight for the experiments. MQ and YX carried out the chemical syntheses and compound characterizations. H-JS performed the electrophysiology experiments. Z-WC and LW performed the photolabeling experiments. DFC wrote the original manuscript. SM, AES and CFZ edited the manuscript. All authors contributed to the final version of the manuscript.

## Conflicts of interest

CFZ is a member of the Scientific Advisory Board of Sage Therapeutics and has equity in the company. DFC has equity in Sage Therapeutics and receives income from a license agreement between Washington University in St. Louis and Sage Therapeutics. Sage Therapeutics was not involved in this study. The other authors have no conflicts to declare.

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