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Neurosteroid Analogues. 16. A new explanation for the lack of anesthetic effects of Δ^{16}-alphaxalone and identification of a Δ$^{17(20)}$ analogue with potent anesthetic activity

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Abstract

This study addresses the hypothesis that the lack of anesthetic activity for (3α,5α)-3-hydroxypregn-16-ene-11,20-dione (Δ^{16}-alphaxalone) is explained by the steroid Δ^{16} double bond constraining the steroid 20-carbonyl group to a position that prevents it from favorably interacting with γ-aminobutyric acid type A ($GABA_A$) receptors. A series of Δ^{16} and Δ$^{17(20)}$ analogues of Δ^{16}-alphaxalone was prepared to evaluate this hypothesis in binding, electrophysiological and tadpole anesthesia experiments. The results obtained failed to support the hypothesis. Instead, the results indicate that it is the presence of the C-21 methyl group in Δ^{16}-alphaxalone, not the location of the constrained C-20 carbonyl group, which prevents Δ^{16}-alphaxalone from interacting strongly with the $GABA_A$ receptor and having anesthetic activity. Consistent with this conclusion, a Δ$^{17(20)}$ analogue of Δ^{16}-alphaxalone without a C-21 methyl group was found to be very similar to the anesthetic steroid (3α,5α)-3-hydroxypregnane-11,20-dione (alphaxalone) with regard to time of onset and rate of recovery from anesthesia when administered to mice by tail vein injection.

Introduction

It is widely accepted that the intravenous anesthetic alphaxalone[a] (**1**, Chart 1) causes general anesthesia in humans because it allosterically increases chloride currents mediated by the inhibitory neurotransmitter γ-aminobutyric acid (GABA) acting at γ-aminobutyric acid type

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Supporting Information Available: Elemental analyses results for steroids **5a–c, 6a–d, 9** and **12a,b**. X-ray crystallographic date for steroid **6a**. This material is available free of charge via the Internet at http://pubs.acs.org. The crystallographic files for steroid **6a** have also been deposited at The Cambridge Crystallographic Data Centre (CCDC 815191.)

A ($GABA_A$) receptors in the brain.[1,2] By contrast, Δ^{16}-alphaxalone (**2**), has greatly diminished allosteric activity at $GABA_A$ receptors and is not an intravenous general anesthetic in mammals.[3,4] This striking effect that the Δ^{16} double bond has on anesthesia has attracted the attention of many investigators.[5–20] Recently, we determined that the effect the Δ^{16} double bond has on anesthetic activity depends on the group attached at C-17.[21] Thus, steroids **3** and **4** were shown to be similar to each other and to steroid **1** in their GABAergic and anesthetic actions. We proposed that the Δ^{16} double bond found in steroid **4** had minimal pharmacological effect because it did not displace the compound's C-17 carbonitrile group to either side of a vector that passes through the mid-point of the C-14,C-15 bond and C-17. This proposal is consistent with results from prior studies which demonstrated that the orientation of hydrogen bond accepting groups on the steroid D-ring critically affects the activity of anesthetic steroids acting at $GABA_A$ receptors. [22,23]

However, the previous results obtained with steroid **4** did not directly probe the structural features present in steroid **2** that prevent this compound's C-20 carbonyl group from favorably interacting with the $GABA_A$ receptor. Is it, as has been proposed previously,[20] that the carbonyl group of steroid **2** cannot favorably interact with the receptor because it is constrained to an unfavorable location in the steroid, or is it that the location of the carbonyl is allowed and another unsuspected structural feature, i.e., the presence of the constrained 21-methyl group in steroid **2**, explains its low biological activity? To distinguish between these possibilities we synthesized and evaluated a series of Δ^{16} and $\Delta^{17(20)}$ steroids (Chart 2). These analogues have allowed us to establish that constraints on the C-21 methyl group rather than constraints on the C-20 carbonyl group explain the low activity of steroid **2**. Additionally, by comparing behavioral responses of steroid **6a** with those of anesthetic steroid **1** in a mouse model of anesthesia, we identify steroid **6a** as an analogue whose utility as an intravenous anesthetic merits further study.

Chemistry

The steroids shown in Chart 1 were prepared using methods reported previously.[21] Steroid **8** (Scheme 1) was prepared from (3α,5α)-3-hydroxyandrostane-11,17-dione (**7**) as described previously.[21] Reduction of the carbonitrile group of steroid **8** with DIBALH also reduced the C-11 carbonyl group. This diol intermediate was not characterized and was instead oxidized using PCC to intermediate **9** in an overall yield of 48% for the two step procedure. Removal of the MOM protecting group gave analogue **5a** in 85% yield. Hydrogenation of analogue **5a** using Lindlar's catalyst gave analogue **5b** in 70% yield.

Analogues **6a** (25%) and **6b** (44%) were prepared as an isomeric mixture from steroid **7** (Scheme 2) by a Wittig-Horner reaction and separated by preparative TLC. The *Z* stereochemistry for the carbonitrile substituent in compound **6a** was established by a crystal structure determination (Figure 1). Hydrogenation of steroid **6b** gave analogue **5c** in 57% yield.

Steroid **1** was used as starting material to prepare additional $\Delta^{17(20)}$ analogues (Scheme 3). Steroid **1** was acetylated to yield steroid **10** and this steroid was used to prepare α-cyanohydrin diastereomers **11** following a literature procedure used for the preparation of similar steroids from other 20-ketosteroid precursors.[24] After verifying by NMR that intermediate **11** was formed, it was immediately subjected to a dehydration reaction to yield $\Delta^{17(20)}$ products **12a** and **12b**. Purification by recrystallizations and column chromatography

[a]Abbreviations: alphaxalone, (3α,5α)-3-hydroxypregnane-11,20-dione; GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid type A; Δ^{16}-alphaxalone, (3α,5α)-3-hydroxypregn-16-ene-11,20-dione; [^{35}S]-TBPS, [^{35}S]-*tert*- butylbicyclophosphorothionate; LRR, loss of righting reflex; LSR, loss of swimming reflex.

yielded pure **12a** (5.2%) and pure **12b** (21%). Saponification of the 3α-acetoxy groups of steroid **12a** and **12b** gave the desired analogues **6c** (82% yield) and **6d** (82% yield), respectively. Comparison of the ^{1}H NMR spectra of compounds **6a** and **6b** showed that the C-18 methyl group in compound **6a** was shifted downfield relative to the C-18 methyl group of compound **6b** due to a deshielding effect of the nearby nitrile group. Accordingly, for the steroid **6c, 6d** double bond isomer pair, steroid **6c** was assigned as the *Z* double bond isomer.

[^{35}S]-*tert*-Butylbicyclophosphorothionate ([^{35}S]-TBPS) Displacement Results

Compounds shown in Charts 1 and 2 non-competitively displaced [^{35}S]-TBPS from the picrotoxin binding site on $GABA_A$ receptors with IC_{50} values given in Table 1. Compounds **1–4** are the reference compounds for this study and the binding results reported are those recently published.[21] Steroid **5a** is the unsaturated C-17 aldehyde analogue of steroids **2** and **4**. Comparison of the IC_{50} values for [^{35}S]-TBPS displacement potency for steroids **2** and **5a** shows that changing the C-17 acetyl group to an aldehyde group results in a modest ~ twofold increase in displacement potency. Compound **5b**, which lacks the Δ^{16} double bond, is about equal to steroid **5a** as a displacer of [^{35}S]-TBPS. Thus, it appears that neither the Δ^{16} double bond nor the C-21 methyl group has more than a ~ twofold effect on potency for [^{35}S]-TBPS displacement. With regard to the effect of the Δ^{16} double bond, a comparison of the IC_{50} values for nitriles **3** and **4** leads to a similar conclusion. Comparison of the IC_{50} values for steroids **4** and **5a** shows a ~ threefold difference in potency in favor of steroid **4**.

Steroid **6a** is a close structural analogue of steroid **5a**. The conformation of the steroid D-ring is nearly identical in both compounds and the **6a** nitrile group lies along the axis of the atoms in the carbonyl group of steroid **5a** (Figure 2). Additionally, the nitrile and carbonyl groups are both hydrogen bond acceptors. The [^{35}S]-TBPS displacement results obtained with steroids **2** and **5a** suggest that steroid **6a** should have an IC_{50} value more similar to that of steroid **5a** than to that of steroid **2** since steroid **6a** does not have a C-21 methyl group. Indeed, this was found to be the case with compound **6a** being ~ eightfold more potent than steroid **5a**, but ~ 17-fold more potent than steroid **2**. Surprisingly, steroid **6a** was ~ twofold more potent than the anesthetic steroid **1**.

Comparison of the IC_{50} values of steroids **6a** (*Z* isomer) and **6b** (*E* isomer) indicates that interchanging the relative positions of the C-20 substituents (H, CN) has a large effect on [^{35}S]-TBPS displacement potency. Steroid **6a** was ~ 16-fold more potent at displacing [^{35}S]-TBPS than steroid **6b**. A comparison of the IC_{50} values for compounds **6a, 6b** and **5c** shows the effect that hydrogenation of the Δ^{16} double bond present in steroids **6a** and **6b** has on binding potency. The change in conformation of the D-ring and the loss of the steric restraint imposed by the Δ^{16} double bond increased the IC_{50} value of steroid **5c** ~ eightfold relative to steroid **6a**, and decreased the IC_{50} value ~ twofold relative to steroid **6b**.

Steroid **6c** (*Z* isomer) is an analogue of steroid **2** (the nitrile group replaces the carbonyl group) in its minimum energy conformation and steroid **6d** (*E* isomer) is an analogue of steroid **2** in its high energy U-conformation (i.e., the conformation in which the relative positions of the carbonyl and C-21 methyl groups are interchanged). The compounds differ from the **6a** and **6b** analogues only by the presence of the C-21 methyl group in their structures. A comparison of steroids **6a** and **6c** shows displacement potency is decreased ~fivefold by the C-21 methyl group. By contrast, a comparison of steroids **6b** and **6d** shows no significant effect on displacement potency. Thus, the C-21 methyl group has a negative

effect on the [^{35}S]-TBPS displacement potency of the *Z* isomer (**6c**), but little effect on the [^{35}S]-TBPS displacement potency of the *E* isomer (**6d**).

Electrophysiology Results

Each compound was evaluated for its ability to potentiate chloride currents mediated by 2 μM GABA at rat $\alpha_1\beta_2\gamma_{2L}$ type $GABA_A$ receptors expressed in *Xenopus laevis* oocytes (Table 2). This concentration of GABA, on average, gates ~ 4% of the maximum response of a cell. However, the sensitivity to GABA of the receptors, which determines the degree of steroid potentiation that can be measured, varied from one batch of oocytes to another. Hence, it is not possible to confidently compare the absolute potentiation among analogues shown in Table 2, for which different oocyte batches were used. However, comparison of concentration-response data for individual compounds in the table can distinguish highly active from weakly active compounds. Highly active compounds cause increasing potentiation of the 2 μM GABA-mediated response as the concentration of the compound is increased (0.1, 1 and 10 μM). Compounds that are weak potentiators yield flat concentration–response data and frequently only augment GABA-mediated currents at the highest concentration tested (10μM).

As reported previously, steroid **1** is a strong potentiator and its Δ^{16} analogue **2** is not.[21] Steroid **3** and its Δ^{16} analogue **4** are both strong potentiators.[21] Steroid **5a**, the Δ^{16} analogue in which the C-17 substituent is an aldehyde, yielded concentration-dependent potentiation, with a strong increment in potentiation at the highest concentration (10 μM). These results differ from those obtained with steroid **2**, the Δ^{16} analogue containing the C-17 acetyl substituent, whose degree of measured potentiation increased minimally when the concentration was increased from 1 μM to 10 μM. As a potentiator, steroid **5a** has a profile more similar to that of steroid **4** than to that of steroid **2**. These results correlate well with the [^{35}S]-TBPS displacement results where steroid **5a** was found to be intermediate between steroids **2** and **4** for potency of [^{35}S]-TBPS displacement.

Hydrogenation of the Δ^{16} double bond in steroid **5a** had little effect on activity since steroids **5a** and **5b** similarly potentiated GABA-mediated currents when their concentrations were increased from 1 μM to 10 μM. The result is in striking contrast to the effect this structural difference has on the GABAergic actions of steroids **1** and **2**, but similar to the effect it has on the GABAergic actions of steroids **3** and **4**. All of these results correlate with those found for potency of [^{35}S]-TBPS displacement (see Table 1).

Table 2 qualitatively suggests that steroid **6a** is a strong potentiator, steroid **6b** is not and steroid **5c**, the hydrogenation product of either **6a** or **6b**, has intermediate activity. In order to quantitatively distinguish enhancement differences for compounds **6a, 6b** and **5c**, these three compounds were directly compared at a concentration of 10 μM on the same oocytes (Figure 3). Steroid **6a** strongly enhanced GABA-mediated currents and steroid **6b** did not. The hydrogenation product, steroid **5c**, enhanced currents more than steroid **6b**, but less than steroid **6a**. These functional results correlate with the order of the IC_{50} values for [^{35}S]-TBPS displacement by these compounds.

Neither steroid **6c** nor steroid **6d**, the two steroids containing the C-21 methyl group, was found to be a strong potentiator. To quantitate activity differences between these two steroids and to compare their electrophysiological activities to those of steroids **1**, **6a** and **6b**, all five compounds were directly compared at a concentrations of 1 and 10 μM on the same oocytes (Figure 4). Steroid **6c** was found to be two to threefold stronger than steroid **6d**. These results again correlate with the IC_{50}s of these two compounds for [^{35}S]-TBPS displacement (see Table 1). Fig. 4 also shows that steroids **6c** and **6d** differ less from each

other in their activities than do steroids **6a** and **6b**. Lastly, Figure 4 shows that steroids **1** and **6a** potentiate to the same level when directly compared to each other on the same oocyte.

Tadpole Loss of Righting Reflex (LRR) and Loss of Swimming Reflex (LSR) Results

The anesthetic effects of the compounds in tadpoles are summarized in Table 3. The results for steroids **1–4** were published previously.[21] Unlike steroid **2**, which did not cause LRR, steroid **5a** does cause LRR with an EC_{50} of ~ 3 μM. Relative to steroid **4**, steroid **5a** is ~ threefold less potent at causing LRR, and only steroid **4** caused LSR. The concentration–response curve found for steroid **5a** is very steep and unique among the compounds in this study. The reason for this phenomenon is not known. Steroid **5b**, the compound produced by hydrogenation of steroid **5a**, had LRR and LSR EC_{50} values comparable to those of steroid **5a**. Steroid **5c** was ~ fivefold less potent at causing LRR than steroid **3** and unlike steroid **3**, it did not cause LSR.

Steroid **6a** is ~ eightfold and ~ threefold more potent than steroid **6b** at causing LRR and LSR, respectively. Steroids **6c** and **6d** have similar tadpole LRR EC_{50} values, but only steroid **6c** causes LSR with an EC_{50} below 10 μM. Comparison of tadpole LRR EC_{50} values for the Z isomers **6a** and **6c** shows that the presence of the C-21 methyl group in the steroid decreases the potency of the *Z* isomer for tadpole LRR slightly more than twofold. The same comparison for the *E* isomers **6b** and **6d** shows that the C-21 methyl group increases the potency of the *E* isomer slightly more than fourfold. Steroid **5c**, the hydrogenation product of either steroid **6a** or **6b**, is less potent than steroid **6a** and more potent than steroid **6b** at causing tadpole LRR, and it does not cause LSR at concentrations ≤ 10 μM. Figure 5 shows a rank order correlation for the [^{35}S-TBPS] IC_{50} values and tadpole LRR EC_{50} values for all 11 compounds. The correlation coefficient is 0.8 ($p < 0.05$) indicating a strong correlation between these parameters. Based on the [^{35}S-TBPS] IC_{50} values, steroid **6a** was less potent and steroid **6d** was more potent at causing tadpole LRR than expected.

Anesthesia in Mice Results

An assessment of the potency, rate of onset and rate of recovery of steroid **6a** relative to these parameters for anesthetic steroid **1** was made using tail vein injections in mice. The duration of anesthesia, defined as loss of righting reflex, observed for the two steroids is shown in Figure 6. Steroid **1** caused very brief anesthesia at a dose of 8 mg/kg. At a dose of 16 mg/kg, steroid **1** caused an immediate loss of righting reflex that lasted for ~ 4 min. Recovery was characterized by a rapid progression over a period of 1–2 min from an initial return of leg movement followed by righting and subsequent walking around the cage.

Steroid **6a** was an anesthetic at a dose of 8 mg/kg. Loss of movement and righting reflex occurred in 10–25 sec and lasted for ~ 1.5 min. At a dose of 16 mg/kg, steroid **6a** caused an immediate loss of righting reflex that lasted on average ~ 4 min. The behavioral pattern of recovery for mice injected with either dose of steroid **6a** was not distinguishable from the pattern observed for the mice injected with 16 mg/kg of anesthetic steroid 1.

Discussion

Our recent study of steroid **4**, the 17-carbonitrile analogue of steroid **2**, confirmed an earlier hypothesis which stated that a change in the conformation of the steroid D-ring caused by introduction of the Δ^{16} double bond was not the reason for the reduction in GABAergic and anesthetic actions of steroid **2**.[21] The goal of this study was to gain an increased understanding of the other structural features of steroid **2** that are responsible for its

diminished actions. We approached this problem by assuming that the rings of steroids **2** and **4** are superimposed when bound to the same binding site(s) on $GABA_A$ receptors. With this assumed alignment, it is easy to recognize that the C-20 carbonyl and C-21 methyl groups of steroid **2** are located to the left and right, respectively, of the nitrile group of steroid **4**. Thus, the inactivity of steroid **2** could result from the fact that the C-20 carbonyl group, the C-21 methyl group, or both groups are placed in positions that prevent favorable interactions of steroid **2** with the receptor. We then prepared analogues to address these possibilities.

Steroid **5a** allowed us to test the possibility that the C-21 methyl group of steroid **2** had a negative effect on the compound's activity. We found that this was indeed the case with differences in activity being largest in the electrophysiological and tadpole bioassays. Compound **5b** was made to address the possibility that the position of the aldehyde group in steroid **5a** was in a favorable, not an unfavorable position. Because compounds **5a** and **5b** had very similar activities in all three bioassays we could not conclude that the steric restraint imposed by the $\Delta^{17(20)}$ double bond had either a favorable or unfavorable effect. We could only conclude that it had a minimal effect on activity in the absence of a C-21 methyl group. Overall, these results suggest that it is the presence of the C-21 methyl group in steroid **2**, not the location of the C-20 carbonyl group, which is responsible for the diminished pharmacological actions of steroid **2a**. The result further implies that compounds having other hydrogen bond acceptor groups located where the carbonyl group of steroids **2** and **5a** is located would have significant pharmacological activity provided that the new analogues did not have a C-21 methyl group.

To reinforce the above conclusions, we prepared analogues **6a–6d**. Steroid **6a** is a close structural analogue of steroid **5a**, and steroid **6b** is a close structural analogue of the high energy U conformation (i.e., the conformation of the steroid in which the positions of the carbonyl group and hydrogen atom on C-20 are interchanged) of steroid **5a**. Based on the results obtained with steroid **5a**, steroid **6a** was expected to be more active than steroid **2**. This was indeed the case as steroid **6a** had activities comparable to those of anesthetic steroid **1**. On the other hand, steroid **6b**, which places the cyano group in the position of the 21-methyl group of steroid **2**, had much lower activity than steroid **5a**. Steroid **5c**, in which the cyano group can freely rotate, has an activity intermediate between the activities of steroids **6a** and **6b**. These results reinforce the conclusion that the location of the 20-carbonyl group in steroid **2** is not the major structural reason for the inactivity of this steroid. The results further suggest that analogues which mimic the conformation of steroid **2** in its minimal energy conformation are likely to be more active that those that mimic the high energy U conformation of steroid **2**.

Steroids **6c** and **6d** were made to probe the effect that adding a C-21 methyl group would have on the actions of steroids **6a** and **6b**. A comparison of the results obtained with steroids **6a–d** clearly demonstrates that the presence of a C-21 methyl group negatively affects compound activity. The unfavorable effect of the C-21 methyl group is evident for both the *Z* stereoisomers (compare steroids **6a** and **6c**) and the *E* stereoisomers (compare steroids **6b** and **6d**).

Thus, the analogues prepared allowed us to achieve our goal of gaining a better understanding of why introducing a Δ^{16} double bond into anesthetic steroid **1** results in a major decrease of anesthetic activity. An additional outcome of the study was the identification of steroid **6a** as an experimental intravenous steroid anesthetic. We have shown that steroid **6a** is comparable in potency to anesthetic steroid **1** as an intravenous anesthetic in mice and, that like anesthetic steroid **1**, this compound has a short onset of action. We also observed that mice anesthetized with either steroid **6a** or steroid **1** displayed similar behaviors upon rapid recovery from anesthesia. It is hoped that steroid **6a** also will

have the other favorable anesthetic properties of anesthetic steroid **1**. Future studies are planned to examine the effects of steroid **6a** on heart rate, respiration and intracranial pressure.

Conclusion

We have shown that a previous hypothesis that explains the lack of GABAergic and anesthetic actions of Δ^{16}-alphaxalone is inadequate. We determined that the C-21 methyl group location, not the previously proposed location of the C-20 carbonyl group, in Δ^{16}-alphaxalone is likely the major reason for the loss of activity for this Δ^{16} steroid. We prepared a series of $\Delta^{17(20)}$ analogues and identified anesthetic steroid **6a** as a candidate for future investigation.

Experimental Section

General Methods

Solvents were either used as purchased or dried and purified by standard methodology. Extraction solvents were dried with anhydrous Na2SO4 and after filtration, removed on a rotary evaporator. Flash chromatography was performed using silica gel (32–63 μm) purchased from Scientific Adsorbents (Atlanta, GA). Melting points were determined on a Kofler micro hot stage and are uncorrected. FT-IR spectra were recorded as films on a NaCl plate. NMR spectra were recorded in CDCl3 at ambient temperature at 300 MHz (^{1}H) or 74 MHz (^{13}C). Purity was determined by combustion analysis for C,H and N (when present) and was performed by M-H-W Laboratories (Phoenix, AZ). Steroids **1** and **7** were purchased from Steraloids (Newport, RI). Steroids **2, 3** and **4** were prepared as described previously[12].

(3α,5α)-3-Hydroxy-11-oxoandrost-16-ene-17-carboxaldehyde (5a)

Steroid **9** (110 mg, 0.31 mmol) dissolved in EtOH (8 mL) and 6 N HCl (2 mL) were stirred at room temperature for 6 h. The reaction was adjusted to basic pH by adding aqueous NaHCO3 and solvents were removed under reduced pressure to give a residue. Water was added and the product was isolated by extraction with CH_2Cl_2. The combined extracts were dried and concentrated to give a white solid, which was purified by passing through a short column of silica gel (eluted with 50% EtOAc in hexanes) to give product **5a** as a white solid (83 mg, 85%): mp 164–167 °C; $[\alpha]_D^{20}$ = +71.2 (*c* = 0.11, $CHCl_3$); IR ν_{max} 3392, 2922, 1702, 1677, cm^{-1}; H NMR δ 9.70 (s, 1H), 6.85 (br s, 1H), 4.05 (br s, 1H), 2.98 (d, 1H, *J* = 12.6 Hz), 2.44 (d, 1H, *J* = 12.6 Hz), 1.02 (s, 3H), 0.84 (s, 3H);^{13}C NMR δ 209.6, 189.1, 154.6, 152.3, 66.2, 66.0, 56.2, 53.6, 47.5, 39.1, 36.1, 35.3, 35.2, 32.4, 32.1, 30.9, 28.9, 27.8, 17.3, 10.9. Anal. ($C_{20}H_{28}O_3$) C, H.

(3α,5α,17β)-3-Hydroxy-11-oxoandrostane-17-carboxaldehyde (5b)

A mixture of the unsaturated aldehyde **5a** (31 mg, 0.1 mmol), Lindlar's catalyst (60 mg) and EtOAc (10 mL) was hydrogenated in a Parr hydrogenation apparatus (H_2, 60 psi) for 4 h. The reaction mixture was then passed through a short silica gel column eluted with EtOAc. After solvent removal, the product was isolated as a solidified foam with a low melting point that could not be accurately determined. Product **5b** (26 mg, 70%) had: $[\alpha]_D^{20}$ = +61.9 (*c* = 0.11, $CHCl_3$); IR ν_{max} 3391, 2921, 1706 cm^{-1}; H NMR δ 9.71 (d, 1H, *J* = 1.9 Hz), 4.05 (br s, 1H), 1.01 (s, 3H), 0.71 (s, 3H);^{13}C NMR δ 209.5, 203.2, 66.2, 64.3, 61.1, 56.1, 55.5, 47.8, 38.9, 36.3, 35.8, 35.2, 32.6, 30.8, 28.8, 27.8, 24.1, 21.5, 14.6, 10.9. Anal. ($C_{20}H_{30}O_3$) C, H.

(3α,5α)-3-Hydroxy-11-oxopregnan-21-carbonitrile (5c)

A solution of steroid **6b** (90 mg, 10.3 mmol) in EtOAc (45 mL) and EtOH was hydrogenated in the presence of Pd/C (10%, 10 mg) overnight at 60 psi. The next day

additional Pd/C (10 mg) was added and the hydrogenation was continued for an additional 12 h. The catalyst was removed by filtration through a short column of silica gel eluted with CH_2Cl_2 and the solvent was removed to yield a white solid. Crystallization from Et2O/EtOAc/hexanes afforded product **5c** (52 mg, 57%): mp 176–178 °C; $[\alpha]_D^{20}$ = +24.1 (*c* = 0.25, $CHCl_3$); IR ν_{max} 3400, 2922, 2249, 1703 cm^{-1}; H NMR δ 4.03 (1H, m), 1.00 (3H, s), 0.58 (3H, s); ^{13}C NMR δ 209.9, 119.2, 66.4, 64.5, 55.4, 54.9, 46.5, 45.8, 39.1, 37.2, 35.9, 35.4, 32.7, 31.0, 29.0, 28.6, 27.9, 23.9, 17.7, 13.2, 11.1. Anal. ($C_{21}H_{31}NO_2$) C, H, N.

[3α,5α,17(20)*Z*]-3-Hydroxy-11-oxopregn-17(20)-ene-21-nitrile (6a) and [3α,5α,17(20)*E*]-3-Hydroxy-11-oxopregn-17(20)-ene-21-nitrile (6b)

To a suspension of NaH (60% dispersion in oil, 0.55 mmol, 14 mg) in dry THF (5 mL) at 0 °C under N_2, diethyl(cyanomethyl)phosphonate (0.6 mmol, 0.1 mL) was added dropwise. After disappearance of the sodium hydride, steroid **7** (147 mg, 0.48 mmol) in dry THF (10 mL) was added dropwise. This mixture was allowed to attain room temperature and stirred overnight. The reaction mixture was then poured into an aqueous solution of NH4Cl and the product was extracted with EtOAc. The combined extracts were washed with brine and dried. After solvent evaporation, the residue was purified on preparative TLC (4 plates) developed with EtOAc/hexanes (1:1) to obtain product **6b** (65 mg) and an unseparated mixture of product **6a** and unreacted steroid **7** (56 mg). The latter mixture was again treated as just described with NaH (7 mg, 60% dispersion in oil, 0.17 mmol) and diethyl(cyanomethyl)phosphonate (0.03 mmol, 0.2 mL) to convert the unreacted steroid **7** in the mixture to products **6a** and **6b**. Purification by preparative TLC provided product **6a** (40 mg) and additional product **6b** (5 mg).

Product **6a** (40 mg, 25%) had: mp 219–221 °C (Et2O/hexanes), $[\alpha]_D^{20}$ = +10.0 (*c* = 0.18, $CHCl_3$); IR ν_{max} 3391, 2922, 2215, 1704, 1636 cm^{-1}; H NMR δ 5.15 (1H, t, *J* = 2.4 Hz), 4.02 (1H, br s), 3.16 (1H, d, *J* = 12 Hz), 0.99 (3H, s), 0.89 (3H, s);^{13}C NMR δ 208.7, 175.7, 115.8, 89.4, 66.3, 64.6, 54.6, 53.3, 49.6, 39.0, 35.9, 35.7, 35.4, 32.7, 32.5, 30.9, 29.0, 27.8, 23.5, 18.2, 11.1. Anal. ($C_{21}H_{29}NO_2$) C, H, N.

Product **6b** (70 mg, 44%) had: mp 166–168 °C (Et2O/hexanes), $[\alpha]_D^{20}$ = −5.9 (*c* = 0.36, $CHCl_3$); IR ν_{max} 3435, 2923, 2217, 1705, 1638 cm^{-1}; H NMR δ 4.95 (1H, t, *J* = 2.7 Hz), 4.02 (1H, br s), 2.71–2.83 (2H, m), 0.99 (3H, s), 0.79 (3H, s);^{13}C NMR δ 208.6, 177.7, 116.8, 89.2, 66.2, 64.7, 53.5, 53.2, 49.5, 39.0, 36.2, 36.0, 35.4, 32.5, 30.9, 30.7, 28.9, 27.8, 23.6, 19.2, 11.1. Anal. ($C_{21}H_{29}NO_2$) C, H, N.

[3α,5α,17(20)*Z*]-3-Hydroxy-11-oxopregn-17(20)-ene-20-carbonitrile(6c)

Steroid **12a** (20 mg, 0.053 mmol) and K_2CO_3 (25 mg) in MeOH (3 mL) were refluxed for 2 h. After cooling to room temperature, the MeOH was removed under reduced pressure to give a residue. Water (25 mL) was added and the product was extracted with CH_2Cl_2. Solvent removal gave a solid which was purified by column chromatography on silica gel (eluted with 50% EtOAc in hexanes) to give the product **6c** (15 mg, 82%): mp 203–205 °C; $[\alpha]_D^{20}$ = +4.0 (*c* = 0.08, $CHCl_3$); IR ν_{max} 3368, 2923, 2210, 1704, 1594, 1453 cm^{-1}; H NMR δ 4.05 (br s, 1H), 3.26 (d, 1H, *J* = 12.4 Hz) 2.60–2.20 (m, 4H), 1.83 (br s, 3H), 1.01 (s, 3H), 0.88 (s, 3H); ^{13}C NMR δ 208.9, 167.2, 118.5, 98.2, 66.2, 64.6, 54.9, 53.5, 49.0, 38.9, 35.8, 35.5, 35.2, 32.5, 31.1, 30.8, 28.9, 27.7, 23.2, 18.2, 18.0, 10.9. Anal. ($C_{22}H_{31}NO_2$) C, H, N.

[3α,5α,17(20)*E*]-3-(Acetyloxy)-11-oxopregn-17(20)-ene-20-carbonitrile (6d)

Steroid **12b** (60 mg, 0.053 mmol) was converted into steroid **6d** using the procedure reported for the preparation of steroid **6c**. Product **6d** (45 mg, 82%) had: mp 186–188 °C; $[\alpha]_D^{20}$ = −7.8 (*c* = 0.18, $CHCl_3$); IR ν_{max} 3468, 2924, 2210, 1704, 1639 cm^{-1} ; H NMR δ

4.02 (br s, 1H), 2.76 (d, 1H, *J* = 12.1 Hz), 2.69 (m, 2H), 2.56 (d, 1H, *J* = 12.4 Hz), 2.09 (m, 1H), 1.85 (t, 3H, *J* = 2 Hz), 0.98 (s, 3H), 0.84 (s, 3H); ^{13}C NMR **8** 209.1, 166.8, 119.6, 100.5, 66.0, 64.5, 55.1, 54.7, 49.2, 38.7, 35.7, 35.2, 33.2, 32.2, 30.7, 28.7, 27.6, 23.6, 17.0, 15.0, 10.8. Anal. ($C_{22}H_{31}NO_2$) C, H, N.

(3α,5α)-3-(Methoxymethoxy)-11-oxoandrost-16-ene-17-carboxaldehyde (9)

To a cold (−78 °C) solution of steroid **8** (250 mg, 0.7mmol) in CH_2Cl_2 was added 1 M DIBALH in toluene (2.1 mL, 2.1 mmol) and the reaction was stirred at −78 °C for 90 min. The excess DIBALH was quenched by adding few drops of acetone and then 1 M HCl (15 mL), and the cooling bath was removed. The biphasic mixture was stirred at room temperature for 0.5 h. The CH_2Cl_2 layer was separated and the aqueous phase was extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with brine, dried and concentrated to give a pale yellow oil. This material was subjected to oxidation without any purification or characterization.

The pale yellow oil dissolved in CH_2Cl_2 (10 mL) and PCC (862 mg, 4 mmol) were stirred at room temperature for 3 h and the brown solution was purified by column chromatography (silica gel, eluted with 30% EtOAc in hexanes) to give product **9** (120 mg, 48%): mp 111–114 °C; IR ν_{max} 2923, 1704, 1681, 1594, cm^{-1}; ^{1}H NMR δ 9.69 (s, 1H), 6.85 (b s, 1H), 4.66 (d, 1H, *J* = 6.8 Hz), 4.63 (d, 1H, *J* = 6.9 Hz), 3.82 (s, 1H), 3.36 (s, 3H), 2.97 (d, 1H, *J* = 12.6 Hz), 2.43 (d, 1H, *J* = 12.6 Hz), 1.03 (s, 3H), 0.83 (s, 3H); ^{13}C NMR δ 209.6, 189.1, 154.6, 152.3, 94.6, 71.4, 66.0, 56.3, 55.1, 53.6, 47.5, 39.8, 35.9, 35.2, 33.4, 32.4, 32.1, 31.6, 27.8, 26.0, 17.3, 11.1. Anal. ($C_{22}H_{32}O_4$) C, H.

[3α,5α,17(20)*Z*]-3-(Acetyloxy)-11-oxopregn-17(20)-ene-20-carbonitrile (12a) and [3α,5α,17(20)*E*]-3-(Acetyloxy)-11-oxopregn-17(20)-ene-20-carbonitrile (12b)

The acetylated steroid **10** was prepared from steroid **1** using a standard acetylation procedure (pyridine/Ac_2O). Steroid **10** (281 mg, 0.75 mmol), KCN (325 mg, 5 mmol), AcOH (0.8 mL), EtOH (3 mL) and water (0.2 mL) were stirred at 0 °C for 0.5 h and then allowed to warm to room temperature. Stirring was continued at room temperature for another 60 h. Water (50 mL) was added to the reaction mixture and the resulting white precipitate was filtered. The filter-cake was dried under high vacuum for 6 h. The NMR spectrum of this white solid showed that it was a mixture of diastereomeric cyanohydrins **11** and unreacted starting material. The product mixture was used without purification or further characterization.

The crude product mixture was dissolved in pyridine (3 mL) and $POCl_3$ (0.8 mL) was added at room temperature and the reaction was stirred for 15 hours. It was then cooled to 0 °C and carefully quenched with water and the biphasic solution was extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were dried and concentrated to give a colorless oil. The crude product was purified by column chromatography (silica gel, 15– 35% EtOAc in hexanes).

The *Z*-isomer **12a** (15 mg, 5.3%) eluted second from the column: mp 228–230 °C; IR ν_{max} 2922, 2209, 1731, 1702, 1595 cm^{-1}; ^{1}H NMR δ 5.01 (br s, 1H), 3.27 (d, 1H, *J* = 12.6 Hz), 2.60–2.25 (m, 4H), 2.05 (s, 3H), 1.84 (b s, 3H), 1.03 (s, 3H), 0.89 (s, 3H); ^{13}C NMR δ 208.7, 170.6, 167.1, 118.5, 98.2, 69.8, 64.4, 54.8, 53.5, 49.0, 39.9, 35.5 (2 × C), 32.3 (2 × C), 31.5, 31.1, 27.6, 25.8, 23.2, 21.5, 18.2, 18.0, 11.1. Anal. ($C_{24}H_{33}NO_3$) C, H, N.

The *E*-isomer **12b** (60 mg, 21%) eluted first from the column: mp 166–168 °C; IR ν_{max} 2929, 2209, 1732, 1705 cm^{-1}; ^{1}H NMR δ 4.99 (br s, 1H), 2.78 (d, 1H, *J* = 12.4 Hz), 2.71 (m, 1H), 2.55 (d, 1H, *J* = 12.4 Hz), 2.25 (m, 1H), 2.03 (s, 3H), 1.87 (br s, 3H), 1.00 (s, 3H), 0.86 (s, 3H); ^{13}C NMR δ 208.9, 170.5, 166.7, 119.7, 100.7, 69.7, 64.4, 55.2, 54.7. 49.2, 39.8,

35.4, 35.2, 33.2, 32.3, 32.2, 31.5, 27.5, 25.7, 23.6, 21.5, 17.0, 15.1, 11.0. Anal. ($C_{24}H_{33}NO_3$) C, H, N.

[^{35}S]-TBPS Binding Methods

The methods used were as described previously.[25]

Xenopus Oocyte Electrophysiological Methods

Receptor expression and whole-cell recordings were carried out as described previously.[25]

Tadpole Behavioral Methods

The methods used were as described previously.[25]

Mouse Behavioral Methods

Anesthetic evaluations were performed in 7–8 weeks old BALB/C mice, weighing approximately 20 g. Steroids were dissolved in 22.5% (w/v) 2-hydroxypropyl-β-cyclodextrin solution (Sigma-Aldrich) at a concentration of 1.6 mg/ml or 3.2 mg/ml and injected through the tail vein in volumes of 5μl/g body weight. Doses (8 or 16 mg/kg) were calculated according to body weight. Animals were placed prone as soon as they stopped moving. Loss of LRR was defined as inability of mice to right themselves within 5 seconds after being placed in a prone position. Sleep time was defined as the time from when the mice displayed LRR until they were able to right themselves. Animals were placed on a warming blanket during the time that they were anesthetized. Each dose was administered to three or four mice and the results are presented as mean ± standard error of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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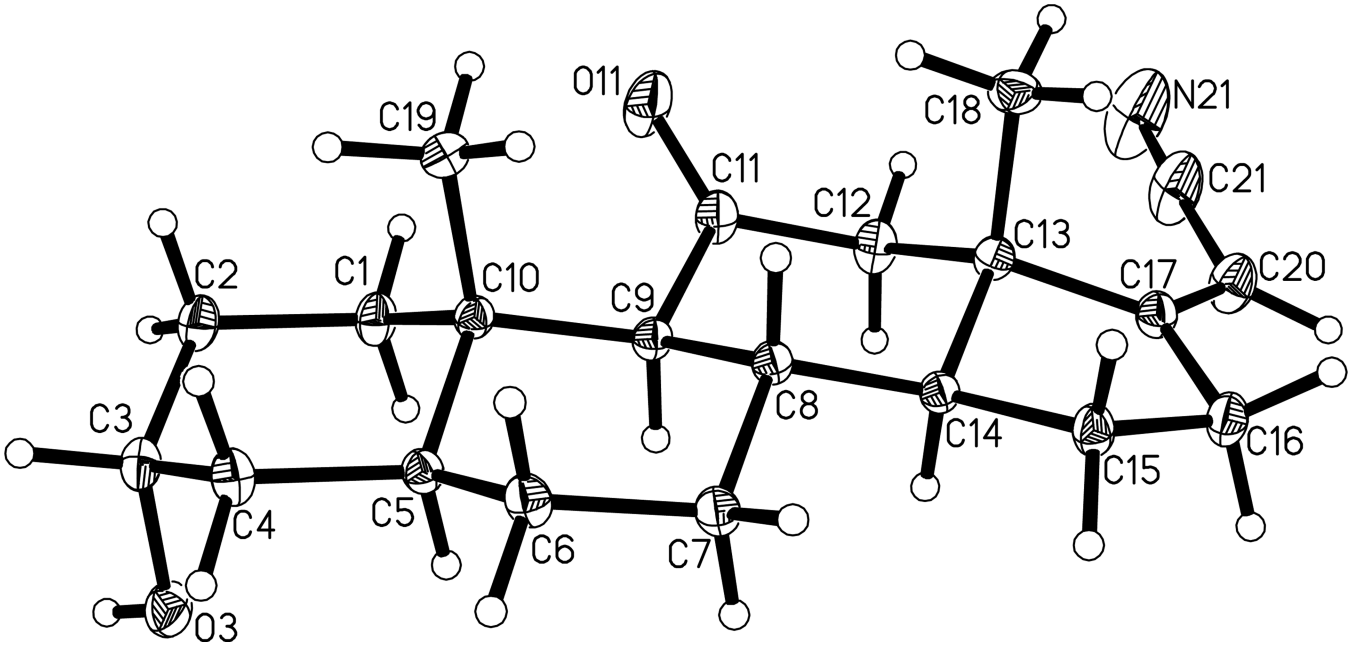


Figure 1.
Projection plot (50% thermal ellipsoids) of the X-ray crystal structure of steroid **6a**.

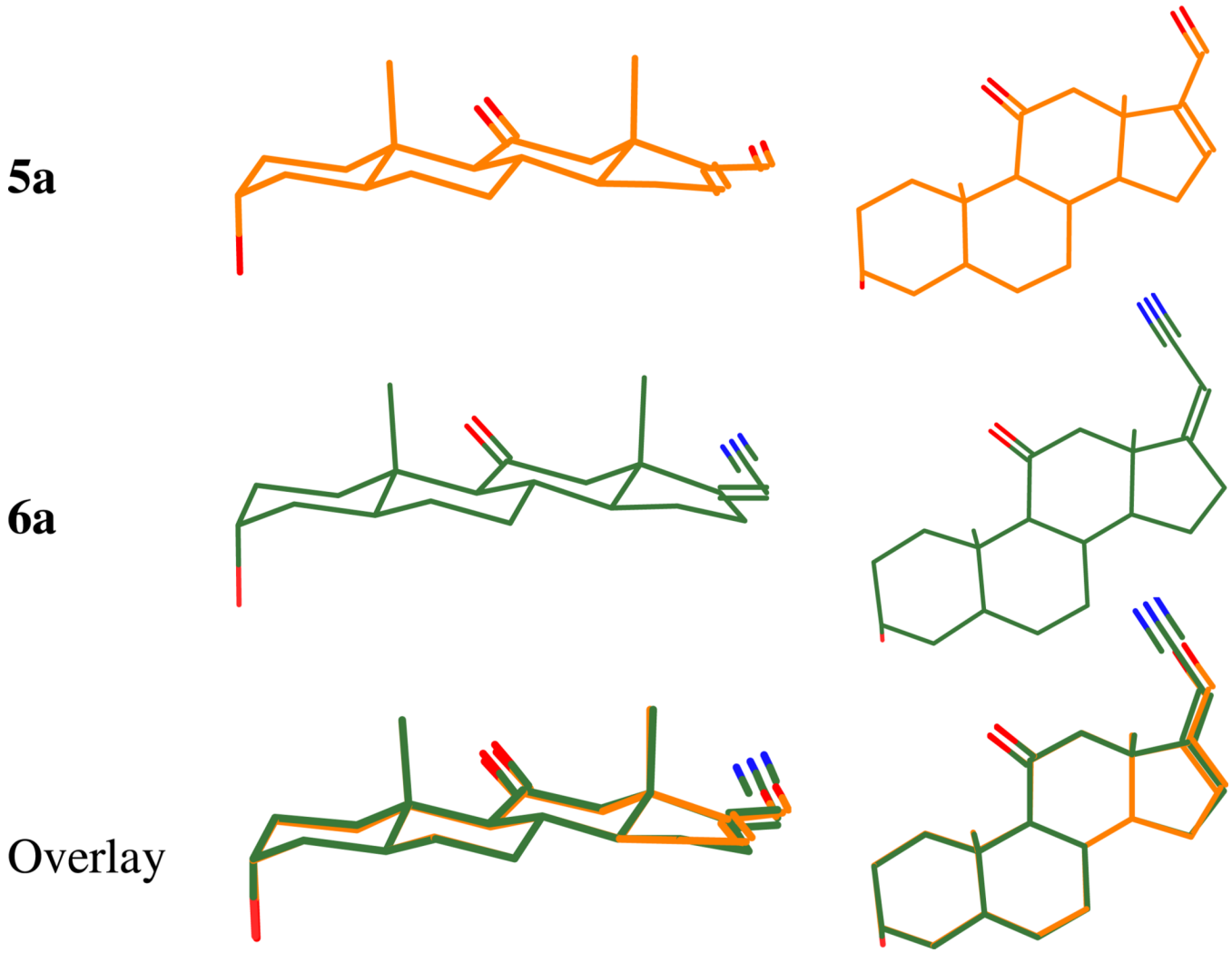


Figure 2.
Molecular models showing steroids **5a** (top) and **6a** (middle) and their overlay (bottom). Left column, edge view. Right column, top view.

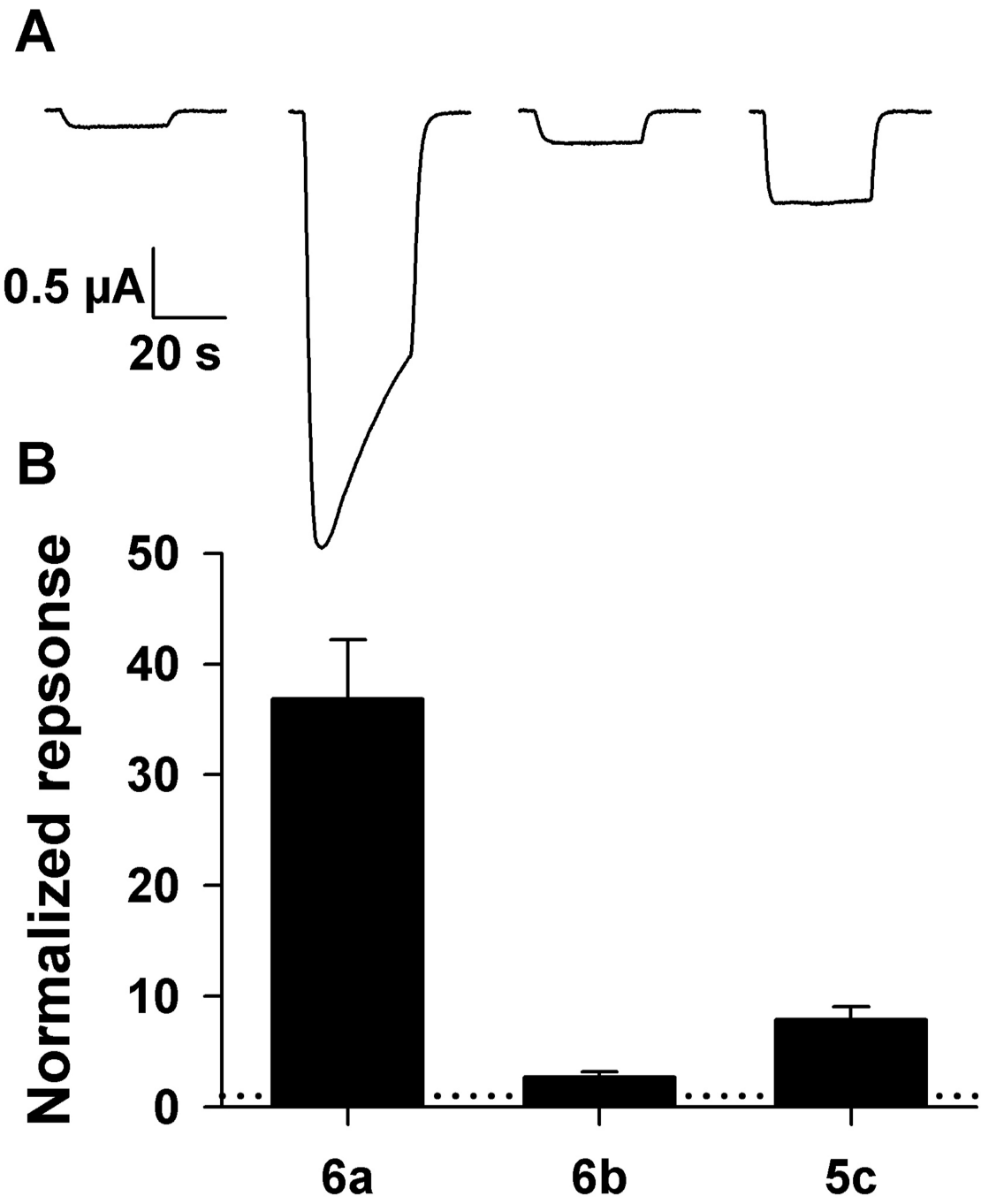


Figure 3.

Direct quantitative comparison of steroids **6a**, **6b** and **5c** at 10 μM on responses to GABA in *Xenopus* oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor subunits. **A**. Responses of to 2 μM GABA alone (left trace) and to GABA co-applied with 10 μM each of **6a** (second trace), **6b** (third trace) and **5c** (right trace). All responses are from the same oocyte. **B**. Summary of responses in which **6a**, **6b** and **5c** were tested within oocytes, normalized to the response of GABA alone in each oocyte. The normalizing response is indicated with a dotted horizontal line at y = 1 (N = 4). Error bars are SEM.

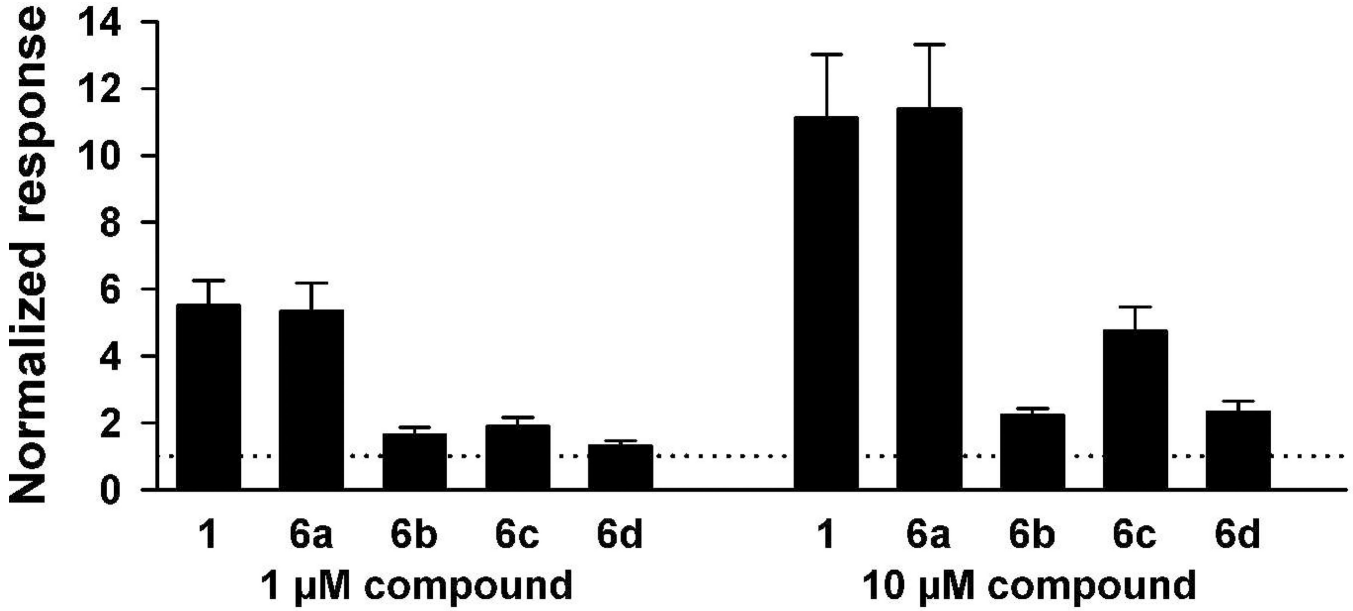


Figure 4.

Summary of normalized responses of oocytes to co-applied GABA (2 μM) plus 1 μM (left set of bars) or 10 μM (right set of bars) of the indicated compounds, tested within oocytes. The normalizing response of GABA alone is indicated by the horizontal dotted line at y = 1. Bars represent mean responses of 6 to 7 oocytes for each compound. Error bars are SEM.

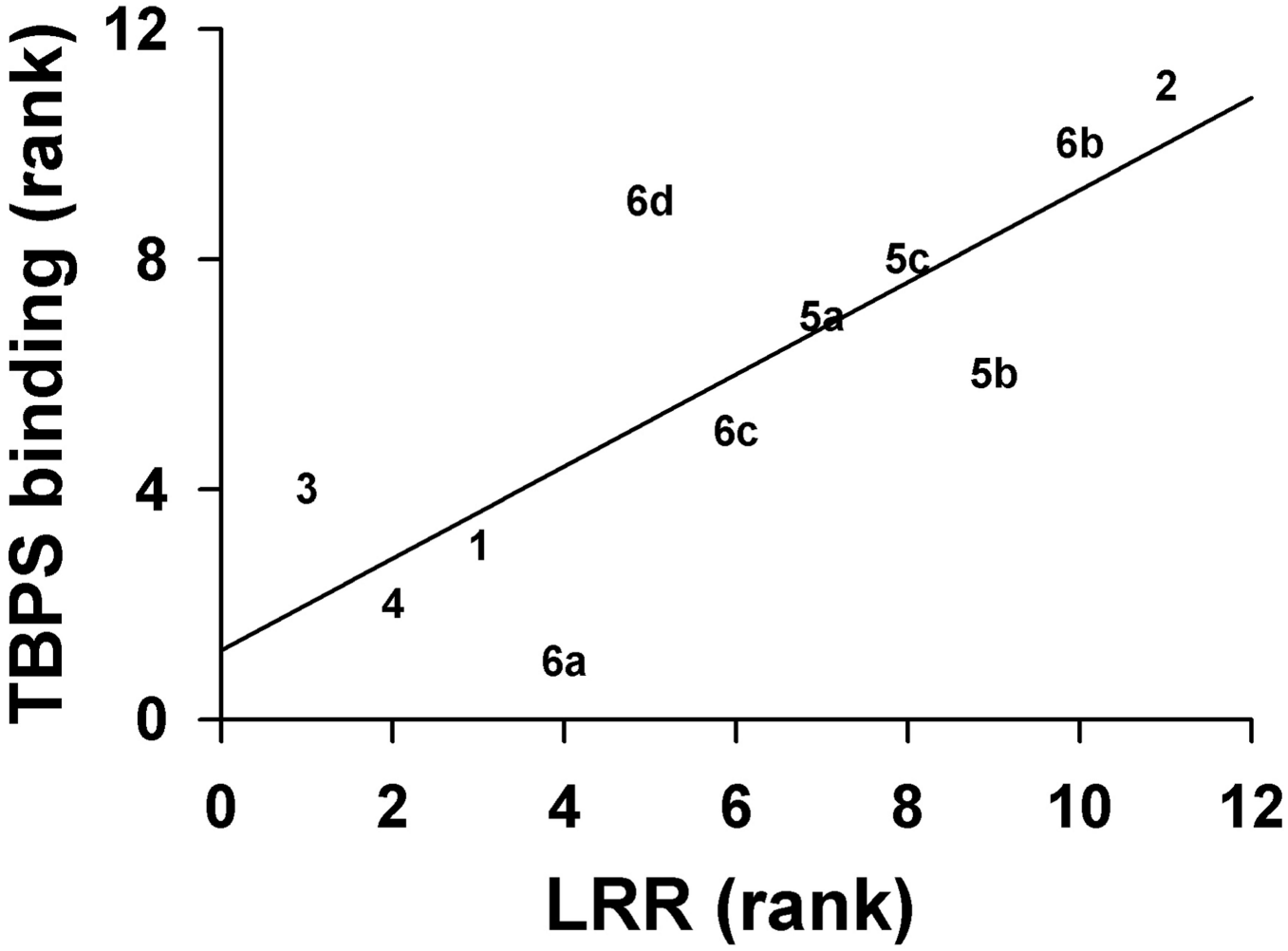


Figure 5.
A rank order correlation plot of the analogue [^{35}S]-TBPS IC_{50} values with their corresponding tadpole LRR EC_{50} values. Compound numbers are used to represent data points on the plot. The correlation is significant, $r = 0.8$, ($p < 0.05$).

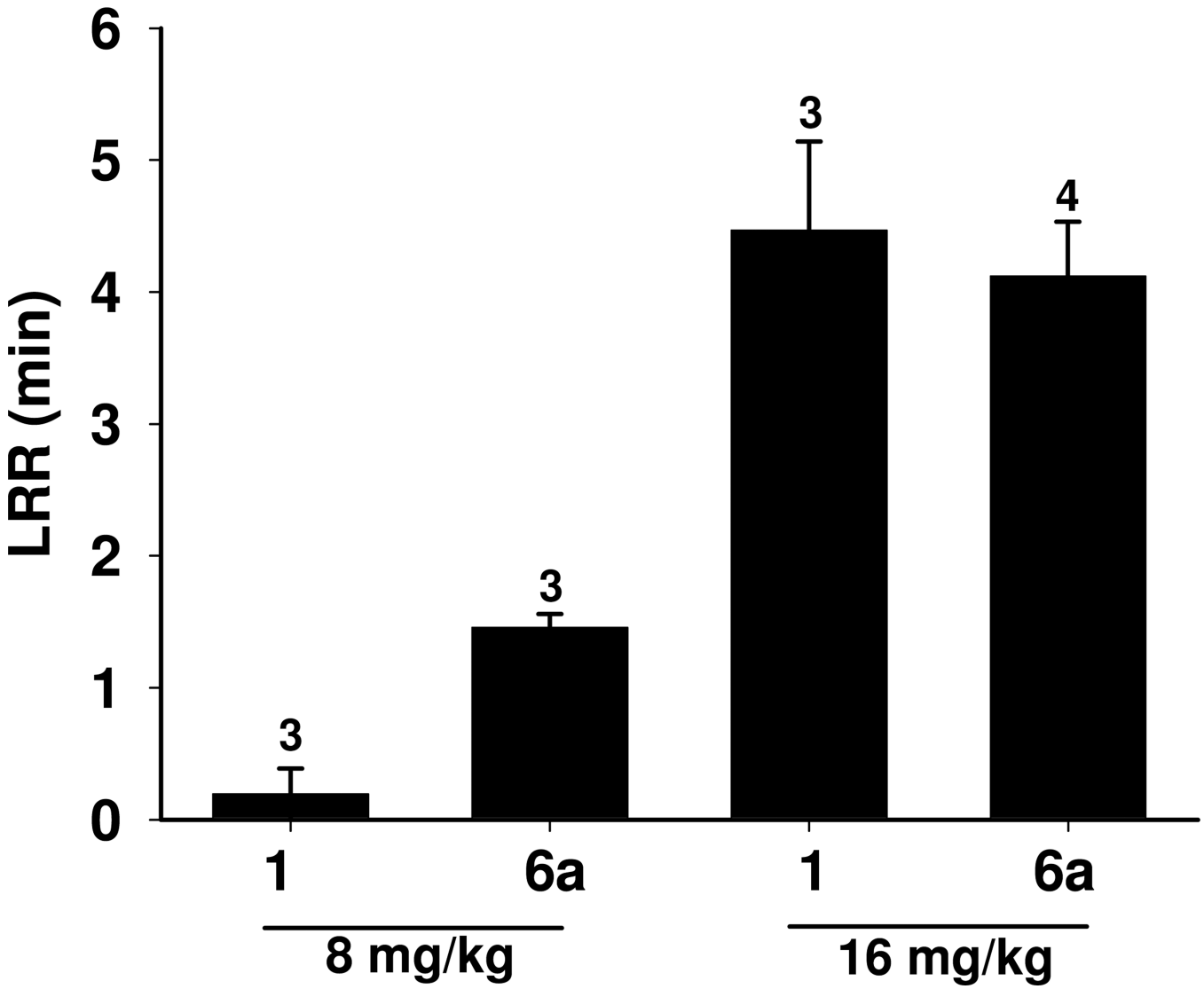
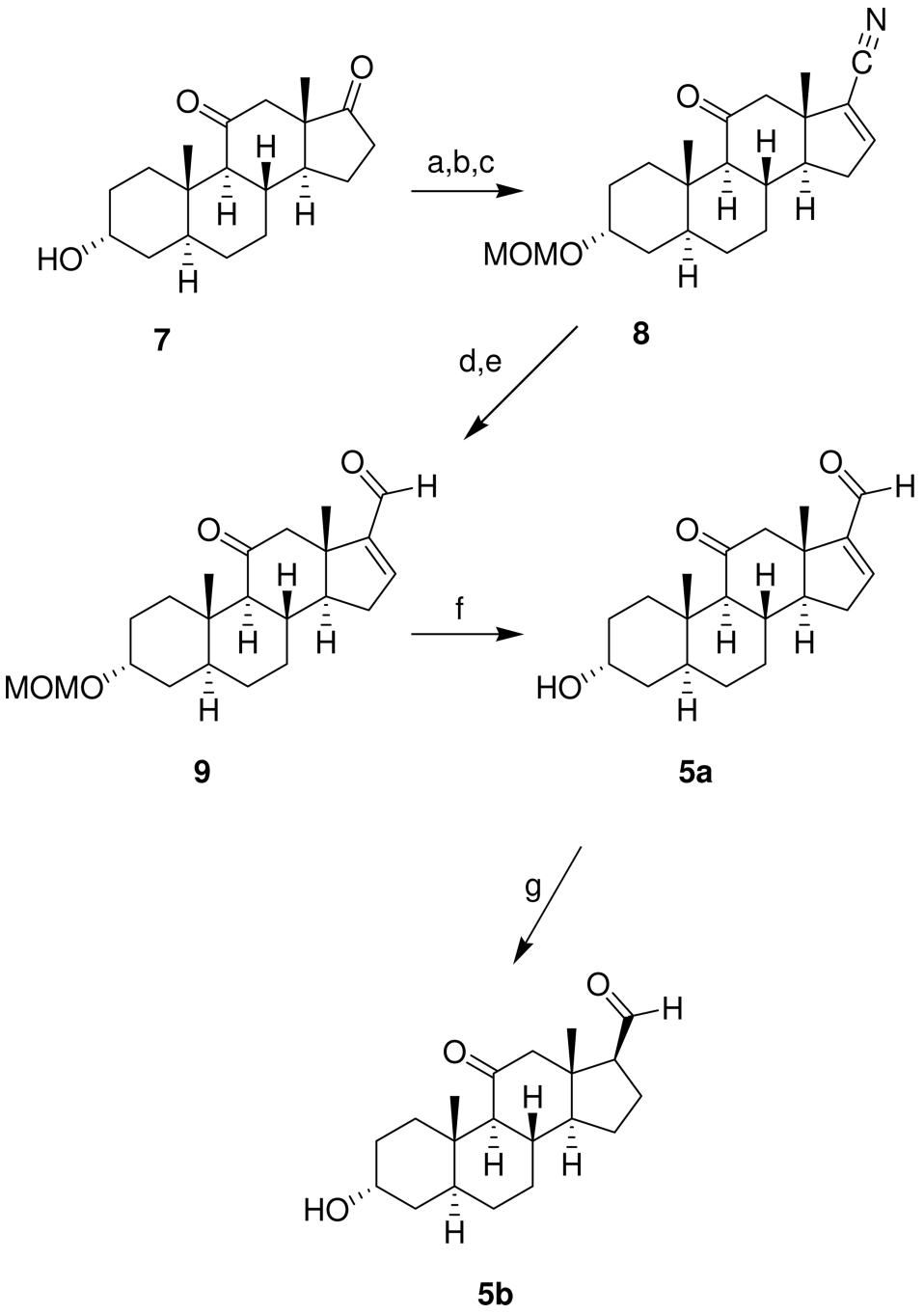
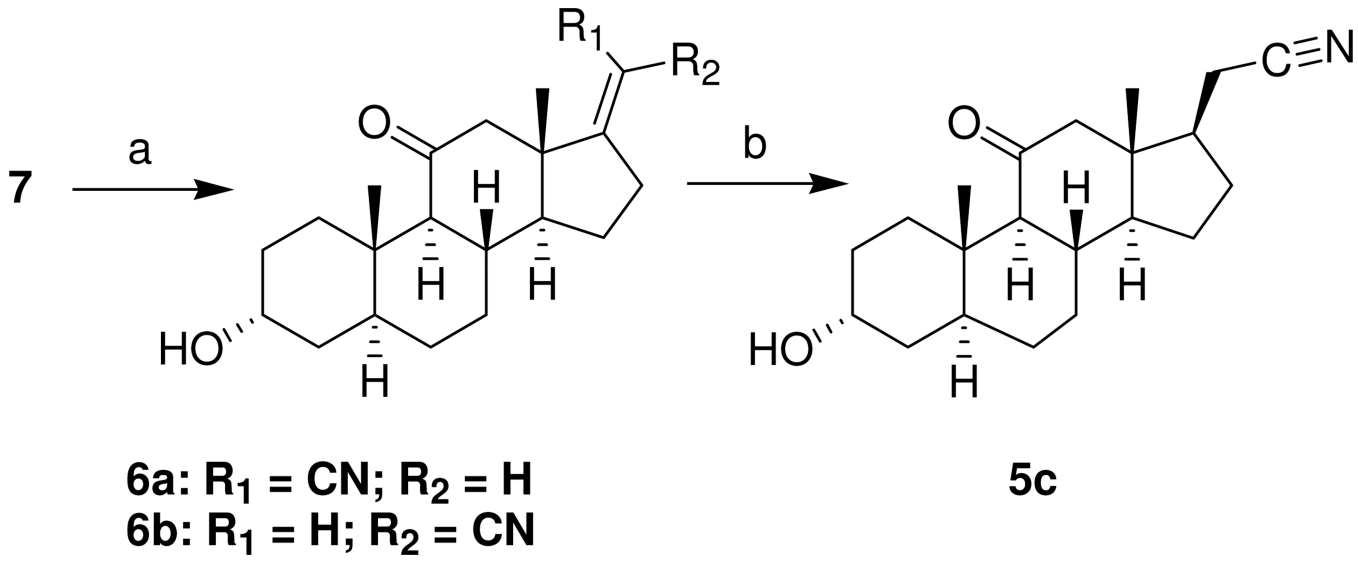


Figure 6.
Duration of anesthesia induced by tail vein injection of steroids **1** and **6a** into mice at two doses. The steroids were dissolved in 22.5% aqueous 2-(hydroxypropyl)-β-cyclodextrin. Numbers of animals tested are given above the bars.



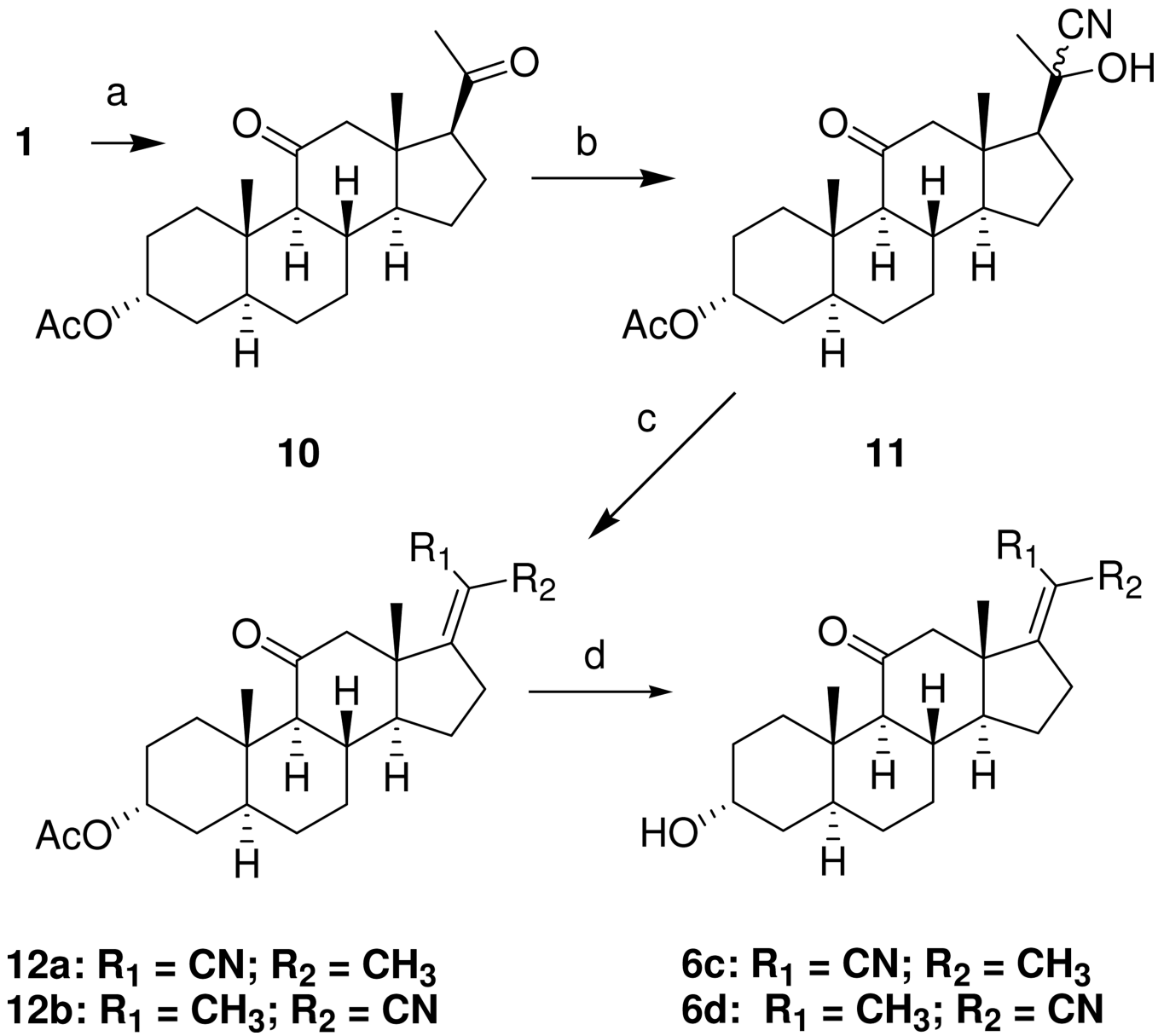
Scheme 1a.

[a]Reagents: a) MOMCl, Hunig's base, CH_2Cl_2; b) $PhN(SO_2CF_3)_2$, KHMDS, THF, –78 °C; c) NaCN, CuI, $Pd(PPh_3)_4$, MeCN; d) DIBALH, toluene, CH_2Cl_2, –78 °C; e) PCC, CH_2Cl_2, f) EtOH, 6 N HCl; g) H_2 (60 psi), Lindlar's catalyst (5%), EtOAc.



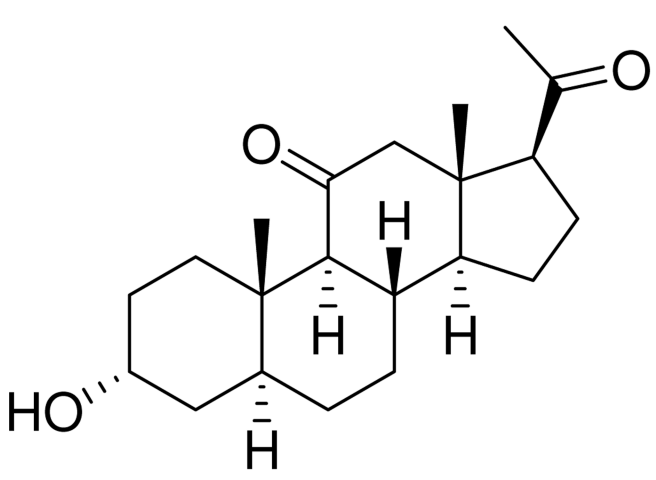
Scheme 2a.

[a]Reagents: a) NaH, diethyl(cyanomethyl)phosphonate, THF, 0 to 20 °C; b) H_2 (60 psi), Pd/C, EtOAc/EtOH.



Scheme 3.

[a]Reagents: a) Ac_2O, pyridine; b) KCN, pyridine, AcOH, EtOH, 0 to 20 °C; c) $POCl_3$ pyridine; d) K_2CO_3, MeOH, reflux.



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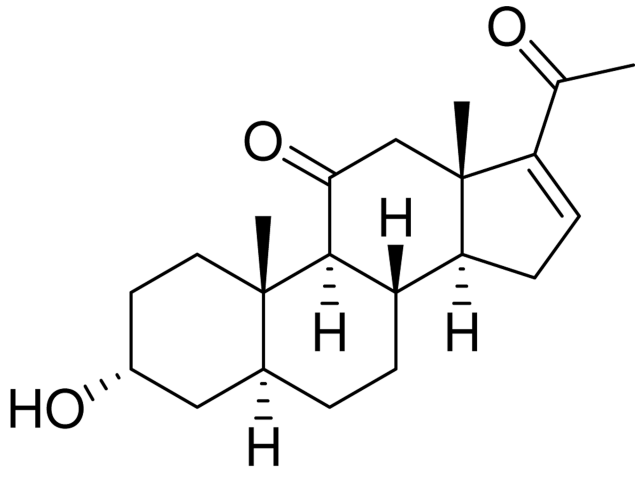

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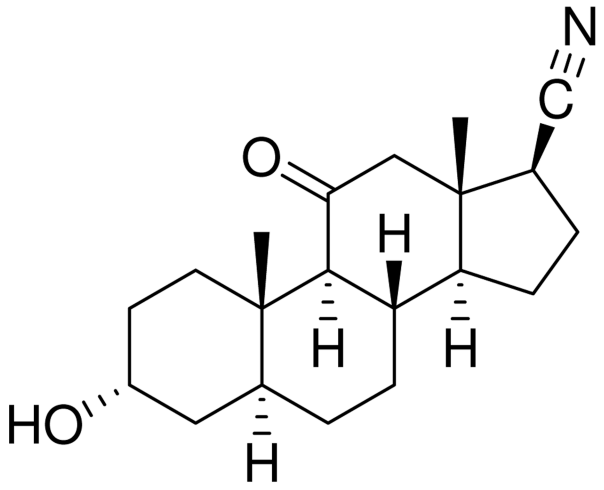

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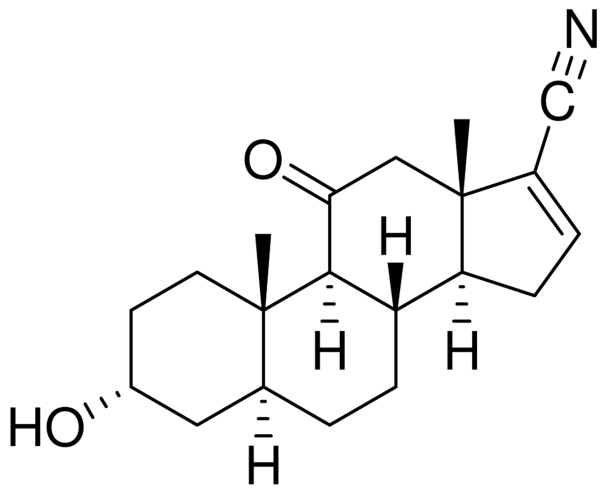
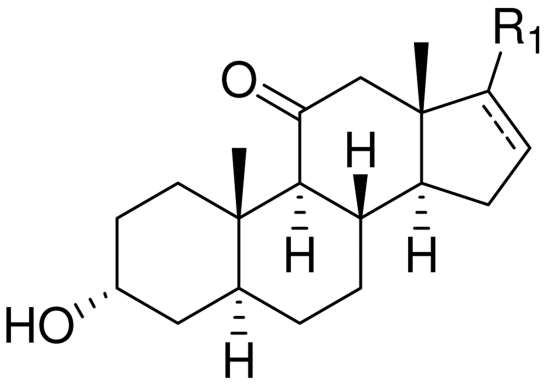
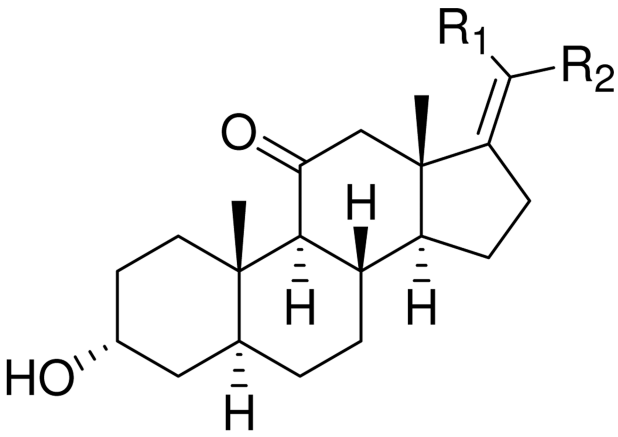


4

Chart 1.



5a: Δ^{16} ; R_1 = CHO
5b: R_1 = 17β-CHO
5c: R_1 = 17β-CH_2CN



6a: R_1 = CN; R_2 = H
6b: R_1 = H; R_2 = CN
6c: R_1 = CN; R_2 = CH_3
6d: R_1 = CH_3; R_2 = CN

Chart 2.

Table 1

Inhibition of [^{35}S]-TBPS Binding by Steroids **1**, **2** and Structural Analogues[a]

Compound	IC_{50} (nM)	n_{Hill}
1[b]	226 ± 24	1.10 ± 0.11
2[b]	2,220 ± 260	1.24 ± 0.14
3[b]	190 ± 18	1.14 ± 0.11
4[b]	361 ± 58	1.00 ± 0.14
5a	997 ± 187	1.50 ± 0.36
5b	770 ± 98	0.97 ± 0.10
5c	1,020 ± 204	0.94 ± 0.14
6a	128 ± 11	1.44 ± 0.15
6b	2,030 ± 810	0.90 ± 0.23
6c	629 ± 89	1.48 ± 0.25
6d	1,840 ± 480	1.46 ± 0.42

[a]Results presented are from duplicate experiments performed in triplicate. Error limits are calculated as standard error of the mean. Methods were as reported previously.[25]

[b]These values are from the literature.[21]

Table 2

Modulation of Rat $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ Receptor Function by Steroids **1**, **2** and Structural Analogues.

Compound	oocyte electrophysiology[a]			
	0.1 μM	1 μM	10 μM	(gating) 10 μM
1[b]	2.91 ± 0.57	4.70 ± 1.11	19.64 ± 4.04	0.11 ± 0.02
2[b]	0.94 ± 0.04	0.97 ± 0.05	1.87 ± 0.14	0.08 ± 0.07
3[b]	1.12 ± 0.03	4.59 ± 0.42	21.14 ± 2.14	0.14 ± 0.03
4[b]	1.49 ± 0.44	4.07 ± 1.09	23.75 ± 3.61	0.21 ± 0.04
5a	0.77 ± 0.06	1.57 ± 0.15	10.78 ± 0.64	0.02 ± 0.01
5b	0.87 ± 0.02	1.44 ± 0.02	8.22 ± 0.14	0.04 ± 0.01
5c	0.91 ± 0.03	1.13 ± 0.08	4.67 ± 0.14	0.01 ± 0.01
6a	1.17 ± 0.04	5.06 ± 0.6	21.51 ± 7.07	0.17 ± 0.00
6b	0.79 ± 0.05	0.77 ± 0.04	1.82 ± 0.21	0.02 ± 0.01
6c	0.90 ± 0.05	1.52 ± 0.24	5.02 ± 0.62	0.04 ± 0.01
6d	0.90 ± 0.01	0.86 ± 0.04	1.53 ± 0.02	0.05 ± 0.02

[a]The GABA concentration used for the control response was 2 μM. Each compound was evaluated on at least four different oocytes at the concentrations indicated, and the results reported are the ratio of currents measured in the presence/absence of added compound. Gating represents direct current gated by 10 μM compound in the absence of GABA, and this current is reported as the ratio of compound only current/2 μM GABA current. Error limits are calculated as standard error of the mean ($N \geq 4$). Methods were as reported previously. [25]

[b]These values are from the literature.[21]

Table 3

Effects of Steroids **1**, **2** and Structural Analogues on Tadpole Righting and Swimming Reflexes[a]

Compound	Tadpole LRR EC_{50} (μM)	Tadpole LRR n_{Hill}	Tadpole LSR EC_{50} (μM)	Tadpole LSR n_{Hill}
1[b]	1.12 ± 0.14	−3.38 ± 2.28	5.48 ± 0.11	−33 ± 0[c]
2[b]	> 10	–	None[d]	–
3[b]	0.72 ± 0.11	−1.49 ± 0.26	5.48 ± 0.12	−33 ± 0
4[b]	1.04 ± 0.14	−1.77 ± 0.38	5.48 ± 0.12	−33 ± 0
5a	3.22 ± 0.03	−16 ± 1.8	None	–
5b	3.98 ± 2.43	−2.76 ± 3.73	None	–
5c	3.58 ± 1.59	−3.21 ± 5.34	None	–
6a	1.44 ± 0.20	−2.84 ± 0.77	5.48 ± 0.20	−33 ± 0
6b	9.15 ± 5.37	−1.70 ± 1.15	17.3 ± 0.17	−36 ± 0
6c	2.71 ± 0.26	−2.47 ± 0.64	5.48 ± 0.20	−33 ± 0
6d	2.09 ± 0.12	−2.42 ± 0.24	> 10	–

[a] The methods are as reported previously. [25] Error limits are calculated as standard error of the mean (N = 10 or more animals at each of five or more different concentrations).

[b] These values are from the literature.[21]

[c] LSR typically has a very steep dose–response curve and the n_{Hill} values reflect the fact that at 3 μM (10 μM for compound **6b**) all or nearly all animals had a swimming response and at 10 μM (30 μM for compound **6b**) the animals did not.

[d] None indicated that all animals had a swimming response at 10 μM of the test compound.