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European Journal of Medicinal Chemistry 43 (2008) 107–113
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

Neurosteroid analogues. 12. Potent enhancement of GABA-mediated chloride currents at $GABA_A$ receptors by *ent*-androgens

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Received 24 July 2006; received in revised form 15 February 2007; accepted 26 February 2007
Available online 12 March 2007

Abstract

Allopregnanolone (**1**) and pregnanolone (**2**), steroids containing a 17β-acetyl group, are potent enhancers of GABA (γ-aminobutyric acid) action at $GABA_A$ receptors. Their effects are enantioselective with the non-naturally occurring enantiomers (*ent*-**1** and *ent*-**2**) being less potent. Androsterone (**3**) and etiocholanolone (**4**), steroids with a C-17 carbonyl group, are weak enhancers of GABA action at $GABA_A$ receptors. Unexpectedly, their enantiomers (*ent*-**3** and *ent*-**4**) have been found to have enhanced, not diminished, activity at $GABA_A$ receptors. Furthermore, the C-17 spiro-epoxide analogues (*ent*-**5** and *ent*-**6**) of *ent*-**3** and *ent*-**4**, respectively, have activities comparable to those of steroids **1** and **2**. The results indicate that some *ent*-steroids are potent modulators of $GABA_A$ receptors and might have clinical potential as GABAergic drugs of the future.

Keywords: Neuroactive steroids; $GABA_A$ receptors; Steroid enantiomers; *ent*-Androgens

1. Introduction

Allopregnanolone (**1**, (3α,5α)-3-hydroxypregnan-20-one) and pregnanolone (**2**, (3α,5β)-3-hydroxypregnan-20-one) are endogenous neurosteroid modulators of $GABA_A$ receptor function and have anesthetic, anticonvulsant and anxiolytic activity (Chart 1) [1]. These neurosteroids bind to multiple sites on this receptor either to directly activate chloride currents in the absence of GABA or to allosterically enhance GABA-mediated chloride currents [2–4]. Recently, mutagenesis studies have identified two different transmembrane sites on the receptor that are important for the direct activation and potentiation effects of these neurosteroids [5].

The actions of neurosteroids **1** and **2** are enantioselective [6,7]. *ent*-Allopregnanolone (*ent*-**1**) and *ent*-pregnanolone (*ent*-**2**) are both weaker allosteric modulators of $GABA_A$ receptor function than neurosteroids **1** and **2**. Additionally, the enantioselectivity observed for the allopregnanolone enantiomers (**1**, *ent*-**1**) is greater than that observed for the pregnanolone enantiomers (**2**, *ent*-**2**). The molecular basis for the observed differences within and between pairs of enantiomers has not been determined. However, since biophysical measurements failed to identify any significant enantioselectivity for the interactions of either allopregnanolone or pregnanolone enantiomers with

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doi:10.1016/j.ejmech.2007.02.017

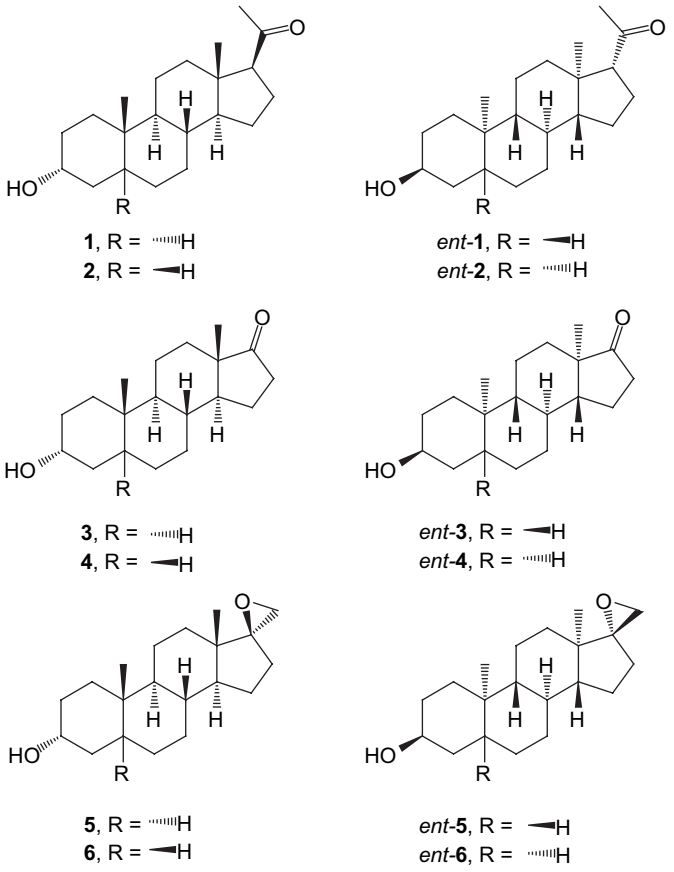
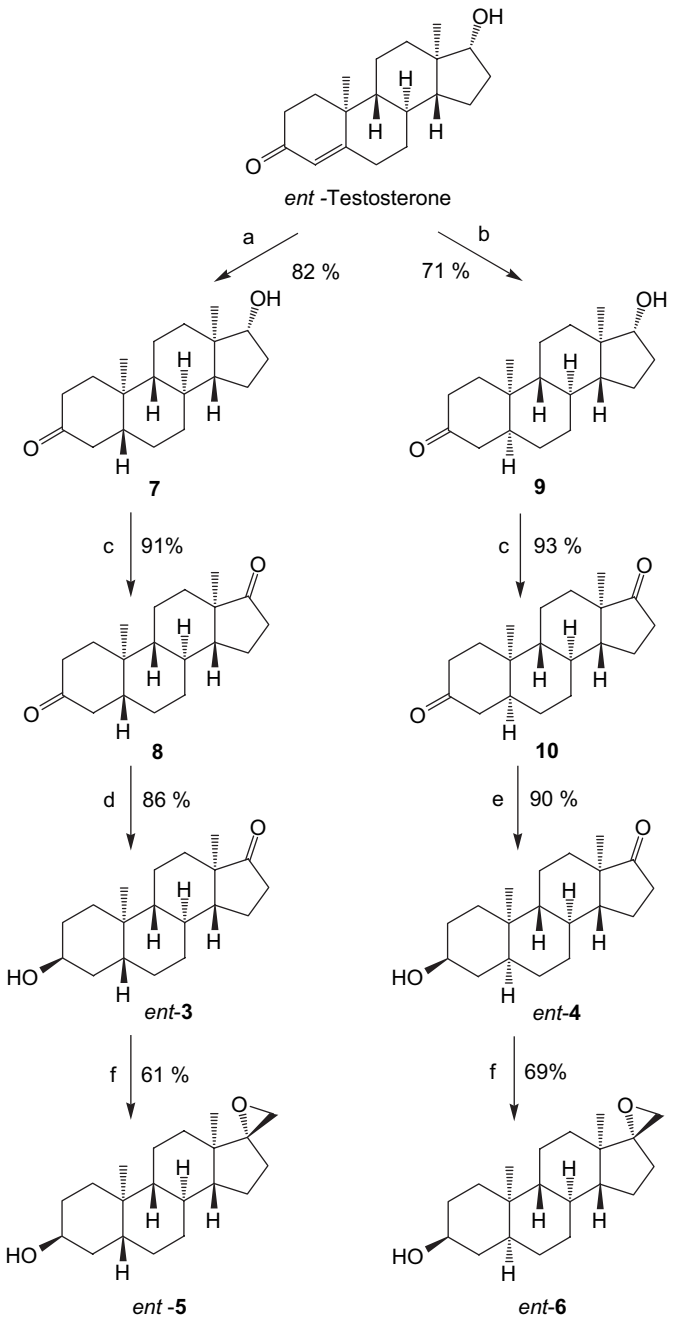


Chart 1.

membrane lipids, it is highly likely that differences in the interactions of the allopregnanolone and pregnanolone enantiomer pairs with the receptor underlie the enantioselectivity [8].

As a continuation of our enantioselectivity studies we have examined the actions of androgen enantiomers at $GABA_A$ receptors. The androgens, androsterone (**3**) and etiocholanolone (**4**), are weak potentiators of $GABA_A$ receptor function [9–15]. We report here that the unnatural enantiomers of steroids **3** and **4** (*ent*-**3** and *ent*-**4**, respectively) are significantly more active modulators of $GABA_A$ receptors than their natural mirror images. We also report that conversion of the C-17 carbonyl group of *ent*-**3** and *ent*-**4** into a spiro-epoxide group yields steroids (*ent*-**5** and *ent*-**6**) with activities comparable to those of the endogenous neurosteroids **1** and **2**.



Reagents: (a) Li/NH_3, ^tBuOH, -78ºC; (b) Pd/C, H_2 (45 psi), KOH, ^iPrOH; (c) Jones reagent, acetone; (d) K-Selectride, THF, -78ºC; (e) $Li(^tBuO)_3AlH$, THF, -42ºC; (f) $Me_3S^+I^-$, KO^tBu, DMF.

Scheme 1.

2. Chemistry

Steroids **3** and **4** are commercially available. The steroid *ent*-**3** was prepared from *ent*-testosterone via intermediates **7** and **8** (Scheme 1) as described by us previously [16]. The steroid *ent*-**4** was prepared from *ent*-testosterone by (1) catalytic hydrogenation (5% Pd/C) under basic conditions (KOH/^{i}PrOH) to yield compound **9**, (2) Jones oxidation to obtain compound **10**, and (3) stereo- and regiospecific reduction of the C-3 carbonyl group using $LiAl[OC(CH_3)_3]_3H$ in THF. The spiro-epoxides **5** and **6** were prepared by treatment of steroids **3** and **4**, respectively, with $Me_3S^+I^-$ and KO^tBu according to the literature [9]. Using the same method of preparation, the compounds *ent*-**5** and *ent*-**6** were prepared from compounds *ent*-**3** and *ent*-**4**, respectively.

3. Results and discussion

The binding interactions of steroids **1**–**6**, and their corresponding enantiomers to $GABA_A$ receptors were determined

Table 1
Inhibition of [^{35}S]-TBPS binding by steroid and *ent*-steroid enantiomer pairs

Compound	IC_{50} (μM)[a]	n_{Hill}
5α-Steroids and their enantiomers		
1[b]	0.074 ± 0.007	0.89 ± 0.06
ent-**1**	1.91 ± 0.27	0.93 ± 0.11
3	0.41 ± 0.13	0.89 ± 0.20
ent-**3**	0.31 ± 0.04	1.00 ± 0.10
5	0.11 ± 0.01	1.32 ± 0.16
ent-**5**	0.47 ± 0.09	4.73 ± 1.90
5β-Steroids and their enantiomers		
2[b]	0.071 ± 0.018	0.57 ± 0.06
ent-**2**	0.28 ± 0.03	0.80 ± 0.07
4	4.15 ± 1.00	1.04 ± 0.16
ent-**4**	0.38 ± 0.06	0.98 ± 0.12
6	0.22 ± 0.02	1.75 ± 0.32
ent-**6**	0.39 ± 0.04	2.29 ± 0.45

[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 [17].
[b] Values reported are from Ref. [17].

by measuring the non-competitive displacement of [^{35}S]-TBPS from the picrotoxin site found on the heterogeneous $GABA_A$ receptors present in rat brain membranes as described by us previously [17]. The results are shown in Table 1. For the 5α-reduced allopregnanolone enantiomers **1** and *ent*-**1**, steroid **1** is a much more potent displacer of [^{35}S]-TBPS than its enantiomer. For the androsterone enantiomers **3** and *ent*-**3**, the *ent*-steroid is slightly more potent than the steroid. Relative to steroid **1**, androsterone and its enantiomer are weak displacers of [^{35}S]-TBPS. For the spiro-epoxide enantiomers **5** and *ent*-**5**, the steroid **5** is nearly as potent a displacer of the radioligand as steroid **1**, which is a result consistent with a literature result using a similar [^{35}S]-TBPS binding assay [9]. However, whereas converting the carbonyl group of steroid **3** to the spiro-epoxide group of steroid **5** produced an increase in potency for [^{35}S]-TPBS displacement, a similar effect was not observed when enantiomer *ent*-**3** was converted into compound *ent*-**5**. In this case, potency was hardly changed, but the Hill coefficient was increased markedly.

The [^{35}S]-TBPS binding results for the 5β-reduced pregnanolone enantiomers **2** and *ent*-**2** are also shown in Table 1. In this case, the steroid is four-fold more potent than the *ent*-steroid. In contrast, for the etiocholanolone enantiomers **4** and *ent*-**4**, the *ent*-steroid is ten-fold more potent than the steroid, consistent with the literature [9]. Converting the carbonyl group of steroid **4** into the spiro-epoxide of steroid **6** markedly enhances potency for [^{35}S]-TBPS displacement. It also increases the Hill coefficient of binding. Once again, as was observed for the carbonyl to spiro-epoxide conversion for the 5α-reduced enantiomers (*ent*-**3** to *ent*-**5**), this conversion (*ent*-**4** to *ent*-**6**) had no significant effect on potency, but did increase the Hill coefficient of binding of spiro-epoxide *ent*-**6**.

Anesthetic effects of the enantiomer pairs were determined by measuring the loss of righting response (LRR) and loss of swimming response (LSR) in *Xenopus laevis* tadpoles as described by us previously [17]. The results are shown in Table 2. The previously reported differences in potency in this bioassay are two-fold for the allopregnanolone enantiomers (**1** > *ent*-**1**) [6]. For the androsterone enantiomers, *ent*-**3** is three-fold more potent than **3** for LRR, and only *ent*-**3** causes LSR below a concentration of 10 μM. For the spiro-epoxide enantiomers **5** and *ent*-**5**, potency differences are minor for LRR and insignificant for LSR. Except for the finding that the conversion of steroid **3** to steroid **5** confers LSR activity at a concentration below 10 μM on the latter compound, the carbonyl to spiro-epoxide transformation does not markedly alter the EC_{50} for either LRR or LSR for either the steroids or the *ent*-steroids.

As reported previously, the pregnanolone enantiomers **2** and *ent*-**2** have the same potency in the tadpole behavioral bioassays [7]. For the etiocholanolone enantiomers **4** and *ent*-**4**, only the *ent*-steroid is able to cause either LRR or LSR at a concentration below 10 μM. This striking potency difference parallels that found in the [^{35}S]-TBPS binding assay for these enantiomers. The carbonyl to spiro-epoxide conversion that converts steroid **4** to steroid **6**, confers LSR and LRR activity on the latter steroid. However, the analogous transformation for compound *ent*-**4** into compound *ent*-**6**, affects potency for LRR and LSR in only a minor way.

Finally, the functional actions of the compounds at $GABA_A$ receptors were determined using electrophysiological methods and rat $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus laevis* oocytes as described by us previously [17]. Concentration-response information was obtained for each compound. In addition, compounds were compared with each other or with reference steroids **1** or **2** on the same oocyte. The concentration-response information for steroids **1**–**6** and their corresponding enantiomers is shown in Table 3. The direct comparison results are shown in Fig. 1.

Table 3 shows that unlike steroid **1**, which is both potent and efficacious as an enhancer of $GABA_A$ receptor function, its enantiomer *ent*-**1** has neither of these properties. Steroid **3**, although not a potent compound, does appear to be an efficacious compound because its effect on chloride current increased with increasing concentration. Its enantiomer, *ent*-**3** appears to be both more potent and more efficacious. However, the conclusion regarding potency can only be made qualitatively based on the results reported in Table 3 since different oocytes, which can differ in sensitivity to GABA within and between batches of ooctyes, were used for the evaluation of the compounds. As shown in Fig. 1 (panel A), when enantiomers **3** and *ent*-**3** are both compared on the same oocyte, the steroid is clearly less potent than the *ent*-steroid. For the enantiomers **5** and *ent*-**5**, it appears from Table 3 and Fig. 1 (panel B) that both steroids have similar activities across a range of concentrations as well as when evaluated at 0.5 μM on the same oocytes. Fig. 1 (panel B) also shows that enantiomers **5** and *ent*-**5** give a larger response than allopregnanolone under the conditions reported.

In Table 3, the pregnanolone enantiomers **2** and *ent*-**2** qualitatively appear to have similar potency and efficacy. Because these enantiomers were not evaluated on the same oocytes, the three-fold difference in potency for $GABA_A$ receptor modulation previously reported by us for these enantiomers [7] is not apparent; however, this difference is observed when both enantiomers are evaluated on the same oocyte (Fig. 1 (panel D)). A similar difficulty does not apply for a qualitative evaluation of the potency and efficacy differences for etiocholanolone enantiomers **4** and

Table 2
Effects of steroid and *ent*-steroid enantiomer pairs on tadpole righting and swimming responses

Compound	Tadpole LRR[a] EC_{50} (μM)	Tadpole LRR n_{Hill}	Tadpole LSR[b] EC_{50} (μM)	Tadpole LSR n_{Hill}
5α-Steroids and their enantiomers				
1[c]	0.42 ± 0.04	−1.83 ± 0.32	5.5 ± 0.5	−7.5 ± 1.1
ent-**1**	0.97 ± 0.15	−1.30 ± 0.28	>5	–
3	3.38 ± 0.90	−2.83 ± 2.66	None[d]	–
ent-**3**	1.42 ± 0.18	−2.17 ± 0.48	5.48 ± 0.12	−33.3 ± 0.1
5	1.35 ± 0.01	−3.69 ± 0.08	2.76 ± 0.01	−21.1 ± 0.7
ent-**5**	1.08 ± 0.01	−18.1 ± 1.3	2.71 ± 0.01	−21.8 ± 1.0
5β-Steroids and their enantiomers				
2[c]	0.06 ± 0.01	−1.54 ± 0.12	0.30 ± 0.01	−6.9 ± 0.5
ent-**2**	0.07 ± 0.02	−1.06 ± 0.31	0.89 ± 0.02	−6.3 ± 1.2
4	None[d]	–	None[d]	–
ent-**4**	1.97 ± 0.13	−2.14 ± 0.22	8.9 ± 0.0	−20.6 ± 0.0
6	0.99 ± 0.00	−20.9 ± 0.7	1.73 ± 0.04	−36.4 ± 0.1
ent-**6**	1.73 ± 0.01	−5.36 ± 0.0	2.76 ± 0.01	−20.8 ± 0.9

[a] LRR = loss of righting response. Error limits are calculated as standard error of the mean ($N = 10$ animals at each of five or more different concentrations).
[b] LSR = loss of swimming response. Error limits are calculated as standard error of the mean ($N = 10$ animals at each of five or more different concentrations). Methods were as reported previously [17].
[c] Values reported are from Ref. [17].
[d] None is defined as no loss of behavioral response at the highest concentration tested (10 μM).

ent-**4**. In this case, Table 3 suggests that the steroid is clearly less potent and likely less efficacious than the *ent*-steroid. The potency difference is verified quantitatively in Fig. 1 (panel C). Finally, the transformation of the carbonyl group of the etiocholanolone enantiomers to the spiro-epoxide enantiomers **6** and *ent*-**6** qualitatively improves the potency and efficacy of both compounds (Table 3). Fig. 1 (panel D) shows that compound *ent*-**6** is as active, and steroid **6** is less active, than steroid **2** when these compounds are compared directly on the same oocyte.

4. Conclusion

Results from this study indicate that *ent*-androsterone (*ent*-**3**) and *ent*-etiocholanolone (*ent*-**4**) enhance GABA-mediated currents at $GABA_A$ receptors more than androsterone and etiocholanolone. Similar to what is observed with androsterone and etiocholanolone, the conversion of the C-17 carbonyl group in the two *ent*-steroids to a spiro-epoxide group enhances potency and efficacy for $GABA_A$ receptor modulation. The enhanced GABAergic actions of compounds *ent*-**3** and *ent*-**4** relative to steroids **3** and **4**, respectively, suggest that the carbonyl groups in the *ent*-steroids have favorable interactions with the receptors that cannot be attained by this group in the steroids. Furthermore, the increase in Hill coefficient observed for compounds *ent*-**5** and *ent*-**6** may indicate that there are additional binding sites for these compounds on $GABA_A$ receptors. Mutagenesis studies on the $GABA_A$ receptor are in progress to address this possibility. Additionally, new analogue studies are planned to further delineate the structure–activity relationships of

Table 3
Modulation of rat $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor function by steroid and *ent*-steroid enantiomer pairs

Compound	Oocyte electrophysiology[a]			
	0.1 μM	1 μM	10 μM	(Gating) 10 μM
5α-Steroids and their enantiomers				
1[b]	1.26 ± 0.14	3.89 ± 1.34	9.65 ± 3.87	0.37 ± 0.07
ent-**1**	0.95 ± 0.04	0.92 ± 0.05	1.17 ± 0.07	0.02 ± 0.02
3	0.97 ± 0.02	1.41 ± 0.01	5.44 ± 0.19	0.02 ± 0.01
ent-**3**	1.27 ± 0.29	3.66 ± 0.89	18.87 ± 2.38	0.03 ± 0.21
5	3.11 ± 0.17	21.92 ± 1.30	33.73 ± 2.04	0.22 ± 0.02
ent-**5**	2.62 ± 0.29	15.89 ± 3.89	26.28 ± 7.90	0.10 ± 0.02
5β-Steroids and their enantiomers				
2[b]	1.20 ± 0.10	2.82 ± 0.51	9.77 ± 2.15	0.06 ± 0.03
ent-**2**	1.28 ± 0.08	2.82 ± 0.53	7.92 ± 1.80	0.06 ± 0.15
4	0.89 ± 0.02	0.87 ± 0.02	1.39 ± 0.05	0.00 ± 0.02
ent-**4**	1.40 ± 0.16	2.34 ± 0.34	14.09 ± 4.58	0.09 ± 0.05
6	1.28 ± 0.03	5.17 ± 0.17	25.42 ± 0.59	0.03 ± 0.07
ent-**6**	1.86 ± 0.09	10.49 ± 0.70	20.83 ± 0.97	0.12 ± 0.03

[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 [17].
[b] Values are from Ref. [17].

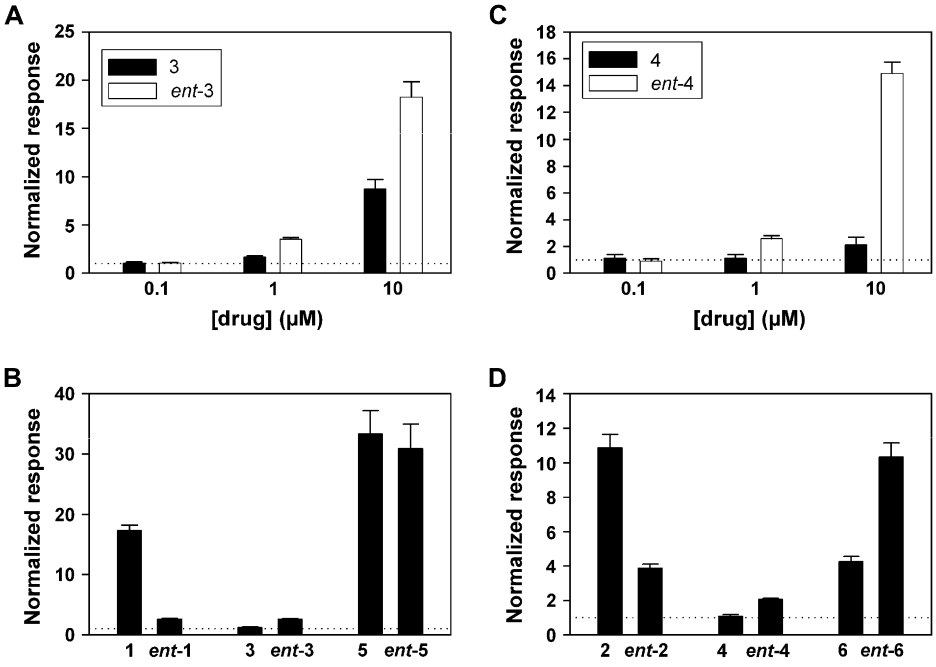


Fig. 1. Within-cell electrophysiological comparisons of androgens and their enantiomers. (A) A three-point concentration-response evaluation of compounds **3** and *ent*-**3**, evaluated on the same four oocytes (a different set of cells from those evaluated in Table 3). For all panels in this figure, the dotted horizontal line at $y = 1$ indicates the normalized GABA response in the absence of any steroid. (B) A comparison of the reference steroid **1** (allopregnanolone) and its enantiomer *ent*-**1** with compounds **3**, *ent*-**3**, **5**, and *ent*-**5**. All compounds were compared at 0.5 μM on four oocytes. (C) Summary of a three-point concentration-response relationship for compounds **4** and *ent*-**4** on five oocytes. (D) Summary of an evaluation at 0.5 μM for steroid **2** (pregnanolone) and its enantiomer *ent*-**2** with compounds **4**, *ent*-**4**, **6**, and *ent*-**6** on five oocytes.

ent-androgens at $GABA_A$ receptors. In a wider context, *ent*-androgens may be useful pharmacological tools for mechanistic studies of androgen action in neuronal tissues [18,19]. It is also possible that *ent*-androgens might have clinical potential as GABAergic drugs of the future.

5. Experimental

5.1. Chemistry

Melting points were determined on a Kofler micro hot stage and are uncorrected. NMR spectra were recorded in $CDCl_3$ at 300 MHz (^1H) or 75 MHz (^{13}C). IR spectra were recorded as films on a NaCl plate. Elemental analyses were carried out by M-H-W Laboratories, Phoenix, AZ. Solvents were used either as purchased or dried and purified by standard methodology. Flash chromatography was performed using silica gel (32–63 μm) purchased from Scientific Adsorbents, Atlanta, GA.

5.1.1. Synthesis of ent-(3α,5β)-3-hydroxyandrostan-17-one (ent-4)

To a dry flask compound **10** (460 mg, 1.59 mmol) dissolved in dry THF (30 mL) was added. While under N_2, the solution was cooled to −42 °C in a bath of acetonitrile and dry ice. Lithium tri-*tert*-butoxyaluminohydride (1.99 mL, 1.98 mmol, 1 M in THF) was added dropwise and the solution was stirred for 2 h at −42 °C under N_2. After determining by TLC that the reaction was not complete, the solution was allowed to warm to −20 °C for 2 h. The reaction was quenched with 3 N HCl. EtOAc (250 mL) was added and the organic solution was washed with saturated aqueous $NaHCO_3$ (150 mL) and brine (150 mL). The organic layer was dried over Na_2SO_4, and filtered, then the solvent was removed *in vacuo* to give a white solid. Column chromatography (silica gel, 20% EtOAc/hexanes to 50% EtOAc/hexanes) yielded product *ent*-**4** (414 mg, 90%) as a white solid, which was recrystallized from a mixture of acetone/hexanes (1:1) to give white crystals: mp 149–150 °C; (natural enantiomer, **4**, literature [20] mp 150–151 °C); $[\alpha]_D^{25} = -108.2$ ($c = 0.4$, ethanol); (natural enantiomer, **4**, literature [21] $[\alpha]_D^{24} = 111$ ($c = 0.5$, ethanol)); IR 3401, 1739 cm^{-1}; ^1H NMR δ 3.67–3.58 (m, 1H), 2.48 (dd, 1H, $J = 10.5$, 8.7 Hz), 0.99 (s, 3H), 0.84 (s, 3H); ^{13}C NMR δ 221.40, 71.58, 51.44, 47.83, 41.97, 40.71, 36.28, 35.90, 35.37, 35.29, 34.71, 31.68, 30.45, 26.85, 25.33, 23.25, 21.78, 20.03, 13.75. Anal. Calcd. for $C_{19}H_{30}O_2$: C, 78.57; H, 10.41. Found: C, 78.67; H, 10.13.

5.1.2. Synthesis of ent-(3α,5α,17β)-spiro[androstane-17,2′-oxiran]-3-ol (ent-5)

To a flask compound *ent*-**3** (200 mg, 0.69 mmol) dissolved in DMF (5 mL) was added. While stirring under N_2, trimethylsulfonium iodide (212 mg, 1.04 mmol) and potassium-*t*-butoxide (117 mg, 1.04 mmol) were added. The solution was stirred at room temperature for 2 h after which water was added to precipitate a beige solid. The solid was collected by filtering and further washed with water. This product was purified by column chromatography (silica gel, 5% EtOAc/hexanes to

30% EtOAc/hexanes) to give a white solid, which was recrystallized from acetone/hexanes (1:1) to yield product *ent*-**5** (127 mg, 61%) as fine white needles: mp 212–214 °C; (natural enantiomer, **5**, literature [9] mp 227–231 °C; natural enantiomer prepared according to the literature [9] mp 210–213 °C); $[\alpha]_D^{25} = -8.5$ ($c = 0.3$, $CHCl_3$); (natural enantiomer, **5**, literature [9] $[\alpha]_D = 7.0$ ($c = 0.7$, $CHCl_3$)); IR 3435 cm^{-1}; ^1H NMR δ 4.04–4.03 (m, 1H), 2.89 (d, 1H, $J = 4.8$ Hz), 2.59 (d, 1H, $J = 5.1$ Hz), 0.86 (s, 3H), 0.78 (s, 3H); ^{13}C NMR δ 70.58, 66.48, 54.35, 53.67, 52.87, 40.13, 39.14, 36.17, 35.82, 35.59, 33.97, 32.18, 31.43, 29.02, 28.99, 28.37, 23.46, 20.11, 14.37, 11.15. Anal. Calcd. for $C_{20}H_{32}O_2$: C, 78.90; H, 10.59. Found: C, 79.00; H, 10.43.

5.1.3. Synthesis of ent-(3α,5β,17β)-spiro[androstane-17,2′-oxiran]-3-ol (ent-6)

Using compound *ent*-**4** (125 mg, 0.43 mmol) and a procedure similar to the one used to make compound *ent*-**5**, compound *ent*-**6** (90 mg, 69%) was obtained as a white solid after recrystallization from acetone/hexanes (1:1): mp 159–161 °C; (natural enantiomer, **6**, literature [9] mp 155–158 °C); $[\alpha]_D^{25} = -18.6$ ($c = 0.6$, $CHCl_3$); (natural enantiomer, **6**, literature [9] $[\alpha]_D = 18.1$ ($c = 0.6$, $CHCl_3$)); IR 3376 cm^{-1}; ^1H NMR δ 3.66–3.57 (m, 1H), 2.89 (d, 1H, $J = 4.8$ Hz), 2.60 (d, 1H, $J = 5.1$ Hz), 0.92 (s, 3H), 0.85 (s, 3H); ^{13}C NMR δ 71.66, 70.55, 53.58, 52.82, 42.00, 40.56, 40.18, 36.32, 35.94, 35.32, 34.65, 34.11, 30.43, 29.07, 26.97, 25.88, 23.51, 23.27, 20.14, 14.30. Anal. Calcd. for $C_{20}H_{32}O_2$: C, 78.90; H, 10.59. Found: C, 78.78; H, 10.52.

5.1.4. Synthesis of ent-(5β,17β)-17-hydroxyandrostan-3-one (9)

KOH (0.65 g) and ^{i}PrOH (40 mL) were added to a hydrogenation bottle, and the solution was stirred for 30 min to allow some of the KOH to dissolve. Pd/C (0.16 g, 5%) was added to the hydrogenation bottle followed by the addition of *ent*-testosterone [16] (2.70 g, 9.4 mmol) dissolved in ^{i}PrOH (40 mL). ^{i}PrOH was added so that the total volume in the hydrogenation flask was ~120 mL. The solution was hydrogenated overnight on a Parr hydrogenator for 18 h at 45 psi and then it was filtered through a pad of Celite, eluting with MeOH. After removal of the solvent *in vacuo*, a yellow oil remained. To this oil was added brine (200 mL) and Et_2O (300 mL), and the aqueous phase was neutralized with 6 N HCl. After thorough mixing the aqueous layer was removed, and the organic layer was washed with brine (200 mL × 3) and then dried over Na_2SO_4. This solution was filtered and the solvent was removed *in vacuo* to give a white solid. Column chromatography (silica gel, 20% EtOAc/hexanes to 40% EtOAc/hexanes) of the resulting solid yielded compound **9** (1.93 g, 71%) as a white solid. An analytical sample of compound **9** was recrystallized from acetone/hexanes (1:1): mp 141–143 °C; (natural enantiomer, literature [22] mp 142–144 °C); $[\alpha]_D^{25} = -31.4$ ($c = 0.2$, ethanol); (natural enantiomer, literature [22] $[\alpha]_D^{25} = 31$ ($c = 1.93$, ethanol)); IR 3469, 1705 cm^{-1}; ^1H NMR δ 3.60 (t, 1H, $J = 8.6$ Hz), 2.62 (t, 1H, $J = 14.1$ Hz), 0.98 (s, 3H), 0.71 (s, 3H); ^{13}C NMR δ 213.31, 81.54, 50.85, 44.15, 42.95, 42.13, 40.75, 37.00, 36.88, 36.69, 35.44, 34.82, 30.30, 26.30, 25.23, 23.21, 22.51, 20.64, 11.02. Anal. Calcd. for $C_{19}H_{30}O_2$: C, 78.57; H, 10.41. Found: C, 78.64; H, 10.23.

5.1.5. Synthesis of ent-(5β)-androstane-3,17-dione (10)

Compound **9** (1.93 g, 6.7 mmol) was dissolved in acetone (60 mL) and while stirring Jones reagent was added dropwise to this solution until a yellow color persisted. The solution was stirred at room temperature for 30 min after which time ^{i}PrOH was added dropwise to quench any remaining Jones reagent. The reaction mixture was poured into brine (300 mL) and the aqueous solution was extracted with EtOAc (200 mL × 3). The organic extracts were combined, dried over Na_2SO_4 and filtered. After solvent removal *in vacuo*, a blue solid remained. This solid was passed through silica gel, eluting with 50% EtOAc/hexanes to yield compound **10** (1.79 g, 93%) as a white solid. An analytical sample of compound **10** was recrystallized from acetone/hexanes (1:1): mp 132–133 °C; (natural enantiomer, literature [23] mp 133–134 °C); $[\alpha]_D^{25} = -110.3$ ($c = 0.4$, ethanol); (natural enantiomer, literature [23] $[\alpha]_D^{17} = 110.5$ ($c = 0.8$, ethanol)); IR 1728, 1708 cm^{-1}; ^1H NMR δ 2.60 (t, 1H, $J = 14.1$ Hz), 0.97 (s, 3H), 0.80 (s, 3H). ^{13}C NMR δ 220.64, 212.59, 51.12, 47.58, 43.97, 41.98, 40.74, 36.88, 36.72, 35.64, 34.88, 34.83, 31.42, 26.11, 24.48, 22.36, 21.54, 20.25, 13.58. Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.78. Found: C, 79.49; H, 9.53.

5.2. Bioassays

5.2.1. [^{35}S]-TBPS binding methods

The methods were as described previously [17].

5.2.2. Xenopus oocyte electrophysiological methods

The methods were as described previously [17].

5.2.3. Tadpole behavioral methods

The methods were as described previously [17].

Acknowledgment

This work was supported by NIH grants GM47969 (DFC, AES, CFZ), AA12952 (SM), a grant from the McDonnell Center for Cellular and Molecular Neurobiology (SM) and a grant from the Bantly Foundation (CFZ).

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