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Bioorganic & Medicinal Chemistry 16 (2008) 3831–3838
Bioorganic & Medicinal Chemistry

Synthesis and $GABA_A$ receptor activity of oxygen-bridged neurosteroid analogs

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Received 27 December 2007; revised 18 January 2008; accepted 23 January 2008
Available online 30 January 2008

Abstract—Three analogs of neuroactive steroids were prepared (**4**–**6**) in which 1,11- or 11,19-oxygen bridges give a constrained conformation. Their 3D structures were obtained by ab initio calculations and in the case of 3α-hydroxy-11,19-epoxypregn-4-ene-20-one (**4**), confirmed by X-ray analysis. Biological activity of the synthetic steroids was assayed in vitro using *t*-[^{3}H]butylbicycloorthobenzoate as radiolabeled ligand for the $GABA_A$ receptor. The activity of compound **4** was similar to that of allopregnanolone (**1**). 1α,11α-Epoxypregnanolone (**6**) was more active than pregnanolone (**2**).

1. Introduction

γ-Aminobutyric acid (GABA) is one of the most prevalent neurotransmitters in the mammalian central nervous system. As 20–50% of all central synapses use GABA as their neurotransmitter, it controls the activity of a large percentage of neurons. Neuroactive steroids modulate neurotransmission through specific, positive allosteric interaction with a steroid recognition site on the $GABA_A$ receptor–ion-channel complex and it is this action that could be crucial to their potential therapeutic utility.[1–3] The naturally occurring neurosteroid allopregnanolone (3α-hydroxy-5α-pregnan-20-one, **1**) and its 5β-isomer pregnanolone (**2**) are among the most potent positive allosteric modulators. Synthetic entities with improved oral bioavailability have been developed and data from preclinical and clinical studies support the potential efficacy of neuroactive steroids as a novel class of drugs for the therapeutic management of epilepsy, anxiety, insomnia, migraine and drug dependence.[4] The structure/activity requirements for neurosteroids interacting with the neurosteroid binding site on the $GABA_A$ receptor have been studied also using binding, electrophysiological, and behavioral assays. While the neurosteroids may accept chemical modification at some carbon atoms, the 3α-hydroxyl configuration is required for binding and for activity in all assays.[5–7] Although the effect of conformational changes in the biological activity of steroidal hormones has been the subject of several studies,[8,9] little is known about their effect in the activity of neuroactive steroids. The incorporation of oxygen bridges involving selected carbons of the steroid nucleus, provides conformationally restricted analogs that can mimick or change in a controlled way the molecular shape of neurosteroids. Thus, in a previous publication we prepared the 6,19-epoxysteroid **3**, an analog of **2**, in which ring A is highly torsioned towards the α-face.[10] At 0.1 μM concentration, **3** significantly increased GABA induced $^{36}Cl^-$ influx in hamster cerebral cortex synaptoneurosomes, while at 20 mg/kg it decreased the percentage of hamsters showing seizures induced by 3-mercaptopropionic acid. We now report the synthesis and $GABA_A$ receptor activity

Keywords: Steroids; Neurosteroids; γ-Aminobutyric acid; GABA; $GABA_A$ receptor.
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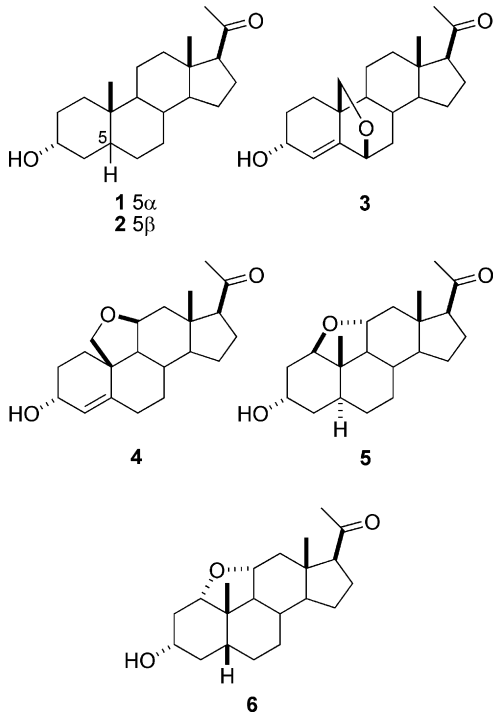
doi:10.1016/j.bmc.2008.01.048

of three neurosteroid analogs, compounds **4**, **5** and **6**, in which the presence of oxygen bridges results in constrained conformations.



2. Chemistry

11,19-Epoxyprogesterone (**7**) was used as starting material for the preparation of compound **4** (Scheme 1).[11] Compound **7** was reduced with $NaBH_4$ to give the 3β-hydroxy-steroid **8**, followed by inversion of the alcohol at C-3 using the Mitsunobu reaction.[12,13] Thus treatment of **8** with $DEAD/Ph_3P/HCO_2H$ gave the 3α-formyloxy pregnane **9** with good stereoselectivity (3α/3β 4:1). The stereochemistry at position 3 was initially inferred from the ^1H NMR spectra of **9** and its 3β isomer, obtained as a by product. The pseudo equatorial H-3 in compound **9** appeared as a narrow unresolved multiplet ($W_{1/2}$ = 15 Hz) at δ 5.27, while for the 3β-stereoisomer (pseudoaxial H-3) it was observed as a broader multiplet ($W_{1/2}$ = 22 Hz) at δ 5.45. Oxidation of compound **9** with pyridinium chlorochromate in the presence of barium carbonate, followed by mild hydrolysis with potassium bicarbonate in MeOH gave **4** in 30% yield from **7**. The structure of compound **4** was confirmed by single crystal X-ray analysis; Figure 1 shows the pseudoaxial orientation of the 3α-hydroxyl, the overall conformation being coincident with that predicted by ab initio calculations (see below).

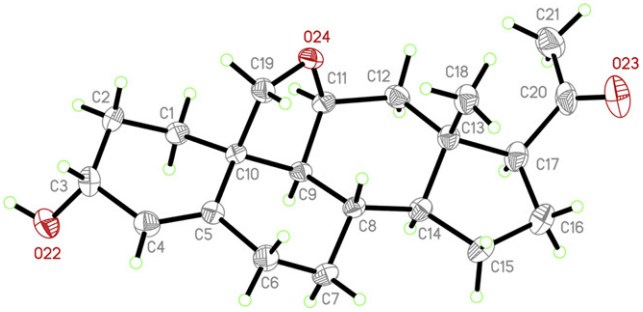
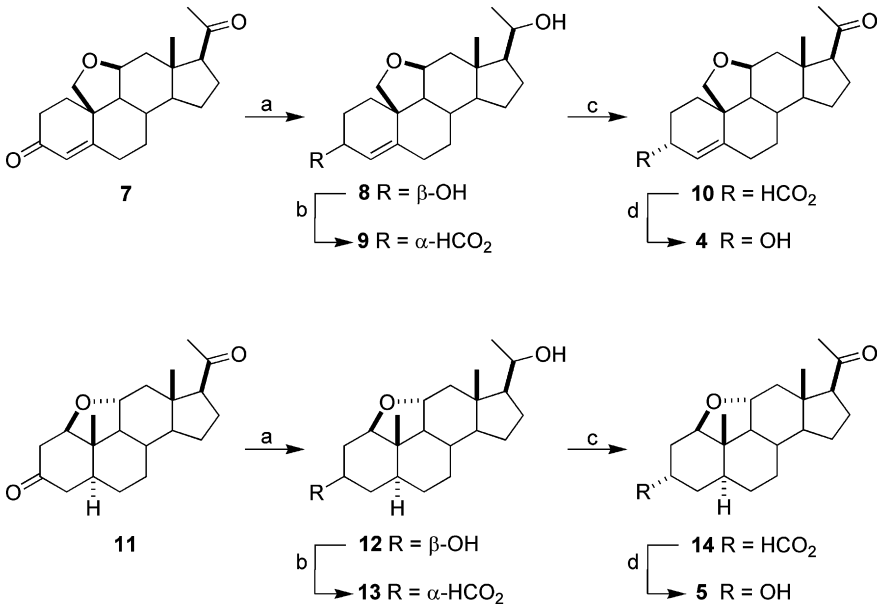
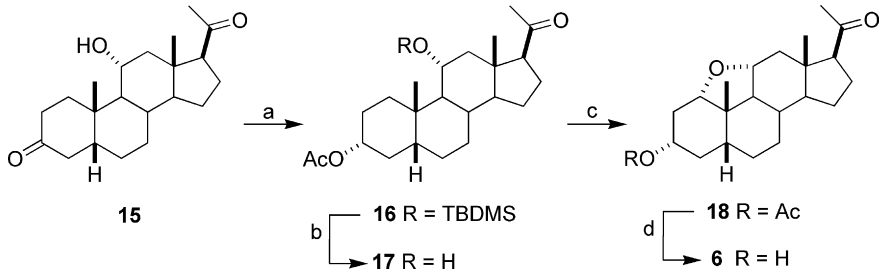


Figure 1. Molecular diagram[22] of 3α-hydroxy-11,19-epoxypregn-4-ene-20-one (**4**), showing the numbering scheme used. Displacement ellipsoids drawn at a 30% probability level.

1,11-Epoxysteroids **5** and **6** were obtained using an intramolecular remote functionalization reaction recently developed by us,[14] involving the photolysis of 11α-



Scheme 1. Reagents and conditions: (a) $NaBH_4$, MeOH, CH_2Cl_2, 25 °C, 90 min; (b) Ph_3P, HCOOH, DEAD, THF, 25 °C, 14 h; (c) PCC, $BaCO_3$, MS, CH_2Cl_2, 25 °C, 30 min; (d), $KHCO_3$, MeOH, 25 °C, 15 min.



Scheme 2. Reagents and conditions: (a) 1—TBDMSCl, imidazole, DMF, 50 °C, 3 h; 2—$NaBH_4$, MeOH, CH_2Cl_2, 0 °C, 30 min; 3—Ac_2O, py, 25 °C, 14 h; (b) HF 40%, THF, acetonitrile, 25 °C, 20 min; (c) DIB, I_2, CH_2Cl_2, *hv*, 25 °C, 20 min; (d) K_2CO_3, MeOH, 25 °C, 30 min.

hydroxysteroids in the presence of diacetoxyiodobenzene (DIB) and iodine. Compound **5** was prepared from 1β,11α-epoxy-5α-pregnane-3,20-dione[14] (**11**) in 27% yield, following the same procedure described above for the preparation of **4** (Scheme 1). In the ^{1}H NMR spectrum of **5**, the H-3 resonance was observed as a narrow unresolved multiplet at δ 4.42 ($W_{1/2}$ = 8.5 Hz), the absence of axial–axial couplings confirmed the equatorial orientation of H-3.

Scheme 2 outlines the synthesis of compound **6** from 11α-hydroxy-5β-pregnane-3,20-dione (**15**), which was obtained by catalytic hydrogenation of 11α-hydroxyprogesterone. Protection of the 11-hydroxyl as the *t*-butyldimethylsilyl (TBDMS) ether, followed by regio- and stereoselective reduction of the C-3 carbonyl with $NaBH_4$ gave, after acetylation, the 3α-acetate **16**. In this case the cis fusion of the A and B rings hinders the α-face of the steroid nucleus and reduction of the C-3 carbonyl occurs from the β-face giving the required α orientation of the hydroxyl at C-3. The TBDMS group was removed with HF to give the 11α-hydroxy intermediate **17**; photolysis in the presence of DIB and iodine, followed by deacetylation at C-3 (K_2CO_3/MeOH) gave **6** in 20% yield from **15**. The ^{1}H NMR spectrum of compound **6** showed a broad multiplet at δ 3.59 for H-3 ($W_{1/2}$ = 30 Hz) characteristic of an axial (β) orientation for this hydrogen. The double doublet at δ 3.95 assigned to H-1 (J = 9.3 and 7.5 Hz) confirmed its axial (β) orientation and the stereochemistry of the oxygen bridge. All structures were unambiguously determined with the aid of 2D NMR experiments.

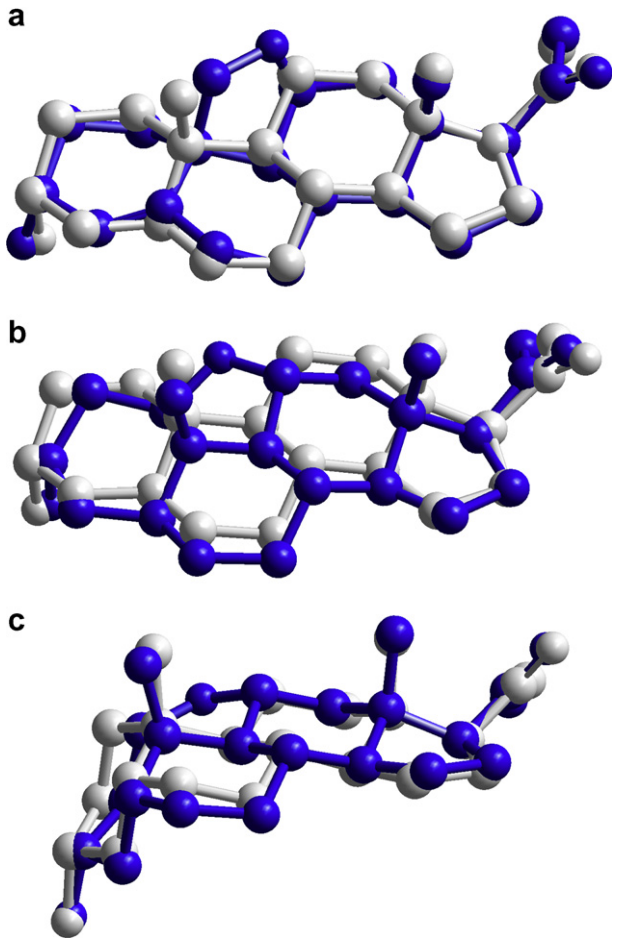


Figure 2. Superposition of calculated structures (HF/6-31G**) of (a) compound **4** (blue) and allopregnanolone (**1**) (white), (b) compound **5** (blue) and allopregnanolone (**1**) (white) and (c) compound **6** (blue) and pregnanolone (**2**) (white). Overlay corresponds to best fit for O(3), C(3), C(17) and C(20); O(3)–C(20) distances are **1**: 10.04 Å; **2**: 9.61 Å;**4**: 10.45 Å; **5**: 9.40 Å; **6**: 9.32 Å.

3. Conformation of compounds 4–6

In the case of compound **4**, ab initio (HF/6-31G**) calculations predicted a conformation similar to that of allopregnanolone (**1**) (Fig. 2a), in agreement with that obtained by single crystal X-ray analysis. The calculated structure of **4** had a normal A ring (1α-halfchair) although flatter, with the 3-hydroxyl displaced from the axial position, resulting in a larger distance between this hydroxyl and the C-20 carbonyl. This effect was slightly larger in the X-ray structure probably due to crystal packing forces and intermolecular interactions.

C-11/C-1 epoxy bridges shift the A ring towards the C ring of the steroid nucleus with C-3 moving to a position close to that of C-2 in a normal non-bridged steroid. The A ring is locked in a fixed conformation by the additional ring. Ab initio calculations (HF/6-31G**) show that in spite of this shift, the overall conformation as well as the conformation of ring A is similar to the corresponding non-bridged pregnanes. Thus, the conformation of 1β,11α-epoxy-5α-pregnane **5** is closely related to that of allopregnanolone (**1**) (Fig. 2b), while the conformation of 1α,11α-epoxy-5β-pregnane **6** is similar to that of pregnanolone (**2**) (Fig. 2c). In both cases, the shift of the C-3 position (and of the 3α-hydroxyl) however, results in a shorter distance between the 3α-hydroxyl and the C-20 carbonyl. The O-3/O-20 distance as well

as the spacial relationship between the 3α-hydroxyl and the D-ring hydrogen bond acceptor (side chain carbonyl) has been considered important parameters for $GABA_A$ receptor activity.[15,16]

4. Biological activity

$GABA_A$ receptor activity was evaluated by assaying the effect of the synthetic analogs (**4**–**6**) on the binding of *t*-[^{3}H]butylbicycloorthobenzoate ([^{3}H]TBOB) and comparison with the effects of the typical neurosteroid allopregnanolone (**1**) and its 5β isomer (**2**). It has been suggested that binding of the cage convulsant *t*-^{35}S butylbicylophosphorothionate ([^{35}S]TBPS) in the presence of GABA closely reflects the functional state of $GABA_A$ receptors and may be useful for characterization of allosteric interactions between various sites on the receptor.[17,18] Studies using *t*-[^{3}H]butylbicycloorthobenzoate ([^{3}H]TBOB) to label a chloride ionophore associated binding site within the $GABA_A$ receptor complex are less common than those using [^{35}H]TBPS, although the use of this radiolabeled ligand is more convenient due to the longer half-life of tritium compared to ^{35}S. Since both ligands are supposed to label the same population of binding sites, studies using the two radioligands may well be compared.[19] Crude membrane receptors from male rat's cerebellum were used to test the capacity of the steroids to displace the specific binding of the radioactive ligand. Allopregnanolone (**1**) was used as a positive control to check the viability of the method. As shown in Figure 3a, compound **1** inhibited [^{3}H]TBOB binding with an IC_{50} of 329 ± 13 nM (mean ± SEM, $n = 3$). By comparison, compound **4** structurally related to **1**, showed a similar activity (IC_{50} = 518 ± 68 nM) but compound **5**, structurally related to **1** and **4**, was less active (IC_{50} = 741 ± 300 nM). On the other hand compound **6** (IC_{50} = 420 ± 48 nM), structurally related to pregnanolone (**2**), was more active than the latter steroid (IC_{50} = 830 ± 11 nM) with values similar to those of **1** (Fig. 3b). Thus in this case, the more compact and rigid structure produced by the additional ring, resulted in a more favorable spatial arrangement for binding to the pregnanolone site.

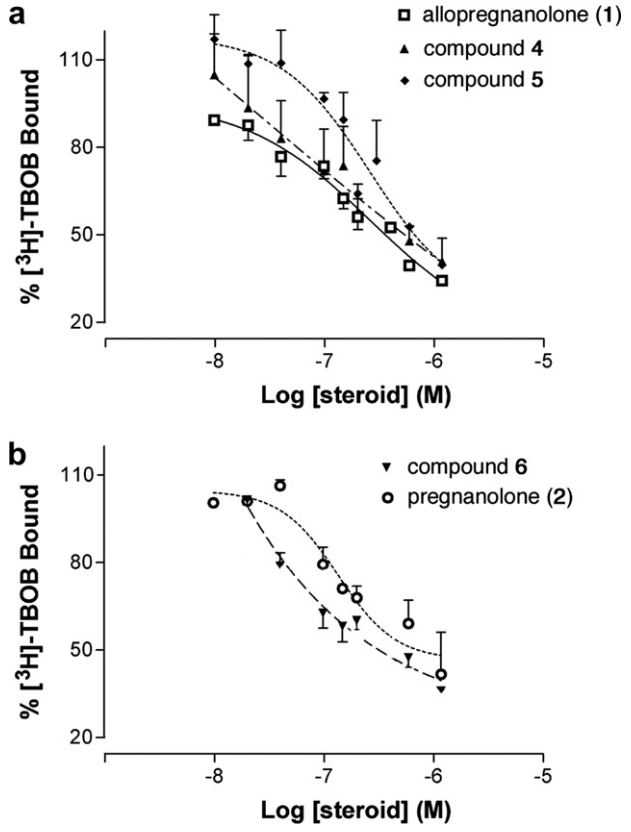


Figure 3. Inhibition of binding of *t*-[^{3}H]butylbicycloorthobenzoate ([^{3}H]TBOB) to crude membranes from male rat cerebral cortex and cerebellum by (a) allopregnanolone (**1**) and structurally related steroids (compounds **4** and **5**) and (b) pregnanolone (**2**) and its structurally related steroid, compound **6**. Membrane preparations were incubated with 10 nM [^{3}H]TBOB in the absence (100% binding) or presence of increasing concentrations of the steroids (10 nM–1.2 μM). Picrotoxin (1 mM) was used to determine non-specific binding. Assays were carried out at 25 °C for 2 h in the presence of 13.5 μM GABA. Calculated IC_{50} values were: **1**, 329 ± 13 nM; **2**, 830 ± 11 nM; **4**, 518 ± 68 nM; **5** 741 ± 300 nM; **6**, 420 ± 48 nM.

5. Experimental

Mps were taken on a Fisher–Johns apparatus and are uncorrected. IR spectra were recorded in thin films using KBr disks on a Nicolet Magna 550 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 500 at 500.13 and 125.77 MHz or on a Bruker AC 200 at 200.13 and 50.32 MHZ, respectively, in deuterochloroform. Chemical shifts are given in ppm downfield from TMS as internal standard, *J* values are given in Hz. Multiplicity determinations and 2D spectra were obtained using standard Bruker software. The electron impact mass spectra (MS) were collected on a Shimadzu QP-5000 mass spectrometer at 70 eV by direct inlet. Electron impact high resolution mass spectra (HRMS) were measured on a VG-ZAB mass spectrometer. Single crystal X-ray measurements were performed on a Bruker SMART CCD diffractometer, with graphite monochromated Mo K_α radiation. The structure was solved by direct methods with SHELXS97[20] and refined by full matrix least squares in F^2 using SHELXL97.[21] Hydrogen atoms were idealized at their expected positions (C–H: 0.93 Å) and allowed to ride. Molecular plots were drawn with XP, in the SHELXTL-PC package.[22] Crystallographic data (excluding structure factors) for the structure reported in this paper has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 669309. The geometries of compounds **1**, **2**, **4**, **5** and **6** were optimized using the ab initio quantum chemistry program Gaussian 03[23] and the HF/6-31G** basis set.

Vacuum liquid chromatography (VLC) and column flash chromatography were carried out on Kieselgel 60-G (Merck) and Kieselgel S 0.040–0.063 mm, respectively. Thin layer chromatography (tlc) analysis was per-

formed on silica gel 60 F254 (0.2 mm thick). The homogeneity of all compounds was confirmed by tlc. 11α-Hydroxy-5β-pregnan-3,20-dione (**15**) was obtained by catalytic hydrogenation (10% Pd–C/ethyl acetate) of 11α-hydroxyprogesterone (Steraloids Inc.). 11,19-Epoxyprogesterone (**7**) and 1β,11α-epoxy-5α-pregnan-3,20-dione (**11**) were obtained from 11α-hydroxyprogesterone following essentially the procedures described previously by us.[11,14]

5.1. 3α-Formyloxy-11,19-epoxypregn-4-en-20-ol (9)

$NaBH_4$ (53 mg, 1.39 mmol) was added to a solution of 11,19-epoxyprogesterone (**7**, 265 mg, 0.81 mmol) in dichloromethane (5.5 mL) and methanol (5.5 mL) at room temperature, and stirring was continued for 90 min. The reaction mixture was acidified with 1 M HCl (to pH 6) and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 7:3) to give compound **8** (216 mg, 80%); ^{1}H NMR (500.13 MHz, $CDCl_3$): 5.54 (1H, s, H-4); 4.27 (1H, br s, H-11); 4.22 (1H, m, H-3); 3.75 (1H, m, H-20); 3.87 (1H, d, J = 8.4 Hz, H-19b); 3.68 (1H, dd, J = 8.4 and 1.5 Hz, H-19a); 1.13 (3H, d, J = 6.2 Hz H-21), 0.94 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 140.0 (C-5), 129.0 (C-4), 78.5 (C-11), 70.2 (C-20), 69.5 (C-19), 67.3 (C-3), 59.2 (C-17), 57.4 (C-9), 52.2 (C-14), 47.4 (C-10), 43.0 (C-13), 42.3 (C-12), 42.8 (C-2), 34.7 (C-8), 34.6 (C-1), 32.7 (C-6), 28.2 (C-7), 25.6 (C-16), 23.6 (C-15), 23.5 (C-21), 14.7 (C-18); MS, m/z 332 (M^+, 13), 302 (100), 284 (6), 269 (4); 255 (8), 239 (7), 224 (5).

To a solution of compound **8** (216 mg, 0.65 mmol) in anhydrous THF (7.8 mL) containing triphenylphosphine (340 mg, 1.29 mmol) and formic acid (59.3 mg, 1.29 mmol) was added a solution of diethylazodicarboxylate (225 mg, 1.29 mmol) in anhydrous THF (1.2 mL). The reaction mixture was stirred for 14 h at room temperature and evaporated to dryness. Purification by flash chromatography (ethyl acetate–hexane 1:1) gave compound **9** (140 mg, 60%) as a white solid: mp 160–161 °C (from methanol); IR (KBr) 3420, 2865 1725,1428, 1210 cm^{-1}; ^{1}H NMR (500.13 MHz, $CDCl_3$): 8.06 (1H, s, H-formate); 5.64 (1H, dd, J = 4.3 and 1.9 Hz, H-4); 5.27 (1H, br s, $W_{1/2}$ 15.0 Hz, H-3); 4.33 (1H, br s, H-11); 3.80 (1H, dd, J = 8.4 and 1.8 Hz, H-19a); 3.75 (1H, m, H-20); 3.70 (1H, d, J = 8.4 Hz, H-19b); 2.42 (1H, m, H-12β); 2.32 (2H, m, H-6); 1.87 (1H, m, H-1β); 1.87 (2H, m, H-2); 1.68 (1H, m, H-16α); 1.67 (1H, m, H-7a), 1.61 (2H, m, H-15), 1.55 (1H, m, H-8); 1.51 (1H, m, H-12α); 1.49 (1H, m, H-1α); 1.36 (1H, m, H-17); 1.29 (1H, m, H-9); 1.19 (1H, m, H-16β), 1.18 (1H, m, H-14); 1.14 (3H, d, J = 6.0 Hz H-21), 1.08 (1H, m, H-7b); 0.94 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 160.0 (formate), 145.4 (C-5), 121.8 (C-4), 78.3 (C-11), 70.8 (C-19), 70.3 (C-20), 66.8 (C-3), 59.3 (C-17), 52.2 (C-14), 57.6 (C-9), 46.6 (C-10), 43.0 (C-13), 42.4 (C-12), 34.7 (C-8 and C-6), 28.7 (C-7 and C-1), 25.6 (C-16), 24.8 (C-2), 23.6 (C-15), 23.6 (C-21), 14.7 (C-18); MS, m/z 360 (M^+, 1), 330 (2), 314 (5), 284 (59); 266 (4); 253 (4); 91 (100); HRMS m/z [M^+] 360.2304 (calcd 360.2301).

5.2. 3α-Hydroxy-11,19-epoxypregn-4-ene-20-one (4)

A suspension of pyridinium chlorochromate (114.5 mg, 0.61 mmol), barium carbonate (48 mg, 0.34 mmol) and 3 Å molecular sieves (92 mg) in anhydrous dichloromethane (4.6 mL) was stirred for 5 min under a nitrogen atmosphere. A solution of the 3α-formate **9** (47 mg, 0.13 mmol) in anhydrous dichloromethane (3.3 mL) was added and stirring continued at room temperature for 30 min. The reaction mixture was diluted with ether and percolated through Florisil with ether and ethyl acetate–hexane (8:2). Evaporation of the solvent afforded the 20-ketone **10** (39.0 mg, 84%); ^{1}H NMR (500.13 MHz, $CDCl_3$): 8.10 (1H, s, H-formate); 5.66 (1H, d, J = 4.4 Hz, H-4); 5.28 (1H, br s, H-3); 4.40 (1H, br s, H-11); 3.79 (1H, dd, J = 8.4 and 1.8 Hz, H-19a); 3.70 (1H, d, J = 8.4 Hz, H-19b); 2.13 (3H, s, H-21), 0.80 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 209.6 (C-20), 160.8 (C-formate), 144.9 (C-5), 122.1 (C-4), 77.8 (C-11), 70.8 (C-19), 66.7 (C-3), 64.4 (C-17), 57.3 (C-9), 52.9 (C-14), 46.5 (C-10), 44.4 (C-13), 41.3 (C-12), 34.8 (C-8), 34.6 (C-6), 31.2 (C-21), 28.7 (C-7), 28.7 (C-1), 24.8 (C-2), 22.6 (C-16), 23.4 (C-15), 15.7 (C-18).

Compound **10** (24.3 mg, 0.068 mmol) was dissolved in methanol (1.9 ml) and a solution of $KHCO_3$ (70.7 mg) in methanol (1.0 mL) was added. After stirring at room temperature for 15 min, the reaction mixture was neutralized with 1 M HCl and extracted with dichloromethane. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 7:3) to give compound **4** (16.8 mg, 75%) as a white solid: mp 175–176 °C (from methanol); IR (KBr) 3428, 2879, 1720, 1425, 1024 cm^{-1};^{1}H NMR (500.13 MHz, $CDCl_3$): 5.68 (1H, d, J = 4.4 Hz, H-4); 4.41 (1H, br s, H-11); 4.10 (1H, br s, $W_{1/2}$ 12.5 Hz, H-3); 3.76 (1H, dd, J = 8.2 and 1.8 Hz, H-19a); 3.68 (1H, d, J = 8.2 Hz, H-19b); 2.52 (1H, m, H-17); 2.41 (1H, m, H-12β); 2.29 (2H, m, H-6); 2.13 (3H, s, H-21); 2.07 (2H, m, H-16); 1.82 (2H, m, H-7); 1.72 (1H, m, 12α); 1.67 (1H, H-2a); 1.66 (1H, m, H-15a); 1.51 (2H, m, H-1); 1.50 (1H, m, H-8); 1.32 (1H, m, H-15b); 1.08 (1H, m, H-2b); 0.80 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 209.0 (C-20), 141.9 (C-5), 126.5 (C-4), 77.8 (C-11), 70.8 (C-19), 64.7 (C-17), 63.4 (C-3), 57.4 (C-9), 53.0 (C-14), 46.7 (C-10), 44.4 (C-13), 41.5 (C-12), 34.9 (C-8), 34.7 (C-6), 31.2 (C-21), 28.9 (C-2), 28.3 (C-1), 27.8 (C-7), 23.9 (C-15), 22.6 (C-16), 15.7 (C-18); MS, m/z 330 (M^+, 8), 312 (7), 300 (60), 282 (8), 257 (7), 239 (6), 110 (100); HRMS m/z [M^+] 330.2204 (calcd 330.2195).

5.2.1. Crystallographic data and data collection parameters. Colorless prismatic crystals recrystallized from methanol: mp 175–176 °C. $C_{21}H_{30}O_3$, M = 330.45, orthorhombic, space group $P2_12_12_1$ (No. 19); cell constants a = 7.641(3) Å, b = 13.945(6) Å, c = 17.570(7) Å;

$V = 1872.0(13)$ Å^3, D_c ($Z = 4$) = 1.173 g cm^{-3}; crystal dimensions $0.32 \times 0.14 \times 0.12$ mm, reflections measured: 14832, reflections unique: 2348, reflections observed ($I > 2\sigma(I)$): 1738; $R = 0.063$ and $R_w^2 = 0.092$.

5.3. 3α-Formyloxy-1β,11α-epoxy-5α-pregnan-20-ol (13)

The 3β-hydroxysteroid **12** was obtained from 1β,11α-epoxy-5α-pregnan-3,20-dione (**11**, 73 mg, 0.22 mmol), following the procedure described for compound **8**. The resulting solid was purified by flash chromatography (dichloromethane–methanol 20:1) to give **12** (59 mg, 80%); ^{1}H NMR (500.13 MHz, $CDCl_3$): 4.00 (1H, m, H-3); 3.79 (1H, m, H-11); 3.68 (1H, m, H-20); 3.60 (1H, dd, J = 12.1 and 5.7 Hz, H-1), 2.60 (1H, m, H-12β); 2.10 (1H, m, H-2β); 1.77 (1H, s, H-4β); 1.66 (1H, m, H-2α); 1.43 (1H, m, H-17); 1.35 (1H, m, H-4α); 1.15 (1H, m, H-12α); 1.14 (3H, d, J = 6.2 Hz H-21); 0.94 (3H, s, H-19), 0.78 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 84.9 (C-1), 75.3 (C-11), 70.1 (C-20), 68.3 (C-3), 64.4 (C-9), 58.1 (C-14), 56.7 (C-17), 47.5 (C-13), 44.0 (C-12), 41.2 (C-5), 38.0 (C-10), 36.2 (C-2 or C-4), 34.3 (C-4 or C-2), 32.4 (C-6), 31.4 (C-8), 26.8 (C-7 or C-16), 26.7 (C-16 or C-7), 23.8 (C-21), 22.8 (C-15); 16.2 (C-19); 13.6 (C-18).

3α-Formate **13** was obtained from compound **12** (59 mg, 0.176 mmol), following the procedure described for compound **9**. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 4:6) to give **13** (38 mg, 60%) as a white solid: mp 156–157 °C (from ethyl acetate–hexane); IR (KBr) 3418, 2862 1722,1413, 1207 cm^{-1}; ^{1}H NMR (500.13 MHz, $CDCl_3$): 8.00 (1H, s, formate); 5.44 (1H, m, H-3); 3.91 (1H, dd, J = 11.8 and 5.7 Hz, H-1), 3.79 (1H, m, H-11); 3.69 (1H, m, H-20); 2.61 (1H, dd, J = 11.4 and 4.9 Hz, H-12β); 2.16 (1H, m, H-2β); 2.06 (1H, s, H-4β); 1.67 (1H, m, H-2α); 1.42 (1H, m, H-17); 1.24 (1H, m, H-4α); 1.15 (1H, m, H-12α); 1.14 (3H, d, J = 6.2 Hz, H-21); 0.92 (3H, s, H-19), 0.77 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 160.0 (formate), 82.6 (C-1), 74.7 (C-11), 70.9 (C-3), 70.1 (C-20), 64.3 (C-9), 58.1 (C-14), 56.6 (C-17), 47.4 (C-13), 44.0 (C-12), 42.7 (C-5), 38.0 (C-10), 32.4 (C-6), 31.4 (C-8), 31.2 (C-2 and C-4), 26.8 (C-7 or C-16), 26.6 (C-16 or C-7), 23.8 (C-21), 22.8 (C-15); 15.5 (C-19); 13.6 (C-18); MS, m/z 362 (M^+, 1), 316 (7), 283 (5); 270 (8); 229 (19); 43 (100); HRMS m/z [M^+] 362.2451 (calcd 362.2457).

5.4. 3α-Hydroxy-1β,11α-epoxy-5α-pregnan-20-one (5)

20-Ketosteroid **14** was obtained from compound **13** (38.0 mg, 0.105 mmol), following the same procedure described for compound **10**. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 1:1) to give **14** (38 mg, 60%):^{1}H NMR (500.13 MHz, $CDCl_3$) δ 8.00 (1H, s, formate); 5.45 (1H, m, H-3); 3.93 (1H, dd, J = 11.8 and 6.0 Hz, H-1), 3.77 (1H, m, H-11); 2.64 (1H, t, J = 9.0 Hz, H-17); 2.41 (1H, dd, J = 11.0 and 5.0 Hz, H-12β); 2.16 (1H, m, H-2β); 2.13 (3H, s, H-21); 2.06 (1H, s, H-4β); 1.66 (1H, m, H-2α); 1.23 (1H, m, H-4α); 1.15 (1H, m, H-12α); 0.92 (3H, s, H-19), 0.66 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 209.2 (C-20), 160.4 (formate), 82.8 (C-1), 74.1 (C-11), 70.9 (C-3), 64.4 (C-9), 61.5 (C-17), 58.8 (C-14), 48.4 (C-13), 43.0 (C-12), 42.7 (C-5), 38.0 (C-10), 32.3 (C-6), 31.9 (C-8), 31.2 (C-2 or C-4), 31.1 (C-4 or C-2), 26.5 (C-7), 24.4 (C-16), 23.8 (C-21), 22.8 (C-15); 15.5 (C-19); 14.5 (C-18).

3α-Hydroxy-1β,11α-epoxy-5α-pregnan-20-one (**5**) was obtained from compound**14** (28.5 mg, 0.079 mmol), following the same procedure described for compound **4**. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 4:6) to give **5** (19.6 mg, 75%) as a white solid mp 168–169 °C (from methanol); IR (KBr) 3410, 2890, 1715, 1425, 1029 cm^{-1}; ^{1}H NMR (500.13 MHz, $CDCl_3$): 4.42 (1H, br s, $W_{1/2}$ 8.5 Hz, H-3); 4.03 (1H, dd, J = 11.8 and 5.7 Hz, H-1), 3.77 (1H, ddd, J = 11.0, 10.5 and 4.8 Hz, H-11); 2.65 (1H, t, J = 8.2 Hz, H-17); 2.42 (1H, dd, J = 11.0 and 4.8 Hz, H-12β); 2.24 (1H, m, H-16β); 2.12 (3H, s, H-21); 2.08 (1H, m, H-2α); 1.94 (1H, m, H-4β); 1.93 (2H, m, H-6), 1.87 (1H, m, H-5); 1.71 (2H, m, H-15α and H-16α); 1.65 (1H, m, H-8); 1.63 (1H, m, H-2β); 1.54 (2H, m, H-7), 1.35 (1H, m, H-12α); 1.27 (1H, m, H-14); 1.23 (1H, m, H-15β), 1.19 (1H, m, H-4 α); 0.97 (1H, m, H-9); 0.94 (3H, s, H-19), 0.64 (3H, s, H-18); ^{13}C NMR (125.77 MHz, $CDCl_3$): 209.2 (C-20), 83.3 (C-1), 73.9 (C-11), 68.2 (C-3), 64.4 (C-9), 61.6 (C-17), 58.8 (C-14), 48.4 (C-13), 43.3 (C-5), 43.2 (C-12), 38.1 (C-10), 34.8 (C-2), 33.6 (C-4), 32.4 (C-6), 31.9 (C-21), 31.3 (C-8), 26.7 (C-7), 24.4 (C-16), 22.8 (C-15); 15.6 (C-19); 14.6 (C-18); MS, m/z 332 (M^+, 3), 314 (5), 299 (2), 271 (2); 244 (8); 159 (12); 43 (100); HRMS m/z [M^+] 332.2353 (calcd 332.2351).

5.5. 3α-Acetoxy-11α-hydroxyy-5β-pregnan-20-one (17)

Imidazole (470 mg, 6.90 mmol) and *t*-butyldimethylsilyl-chloride (700 mg, 4.64 mmol) were added successively to a solution of 11α-hydroxy-5β-pregnan-3,20-dione (**15**, 390 mg, 1.18 mmol) in anhydrous DMF (4 ml) and the solution was stirred for 3 h at 50 °C under a nitrogen atmosphere. The reaction mixture was allowed to cool to room temperature and then extracted with ether. The organic layer was washed successively with brine and water and dried with sodium sulfate. Evaporation of the solvent followed by flash chromatography (ethyl acetate–hexane 3:7) gave the 11-silyl ether (262 mg, 50%); ^{1}H NMR (200.13 MHz, $CDCl_3$): 4.08 (1H, m, H-11); 2.60 (1H, m, H-17), 2.12 (3H, s, H-21); 1.11 (3H, s, H-19), 0.89 (9H, s, *t*-butyl-Si);0.63 (3H, s, H-18); 0.09 (3H, s, CH_3–Si) and 0.10 (3H, s, CH_3–Si). $NaBH_4$ (22.4 mg, 0.59 mmol) was added to a solution of the silyl ether obtained above (262 mg, 0.59 mmol) in dichloromethane (5.3 mL) and methanol (5.3 mL) at 0 °C, and stirring was continued for 30 min. The reaction mixture was acidified (pH 6) with 1 M HCl and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum. Acetylation of the residue with acetic anhydride (1.87 mL) and pyridine (1.87 mL) gave compound **16** (212 mg, 73%); ^{1}H NMR (200.13 MHz, $CDCl_3$) δ 4.78

(1H, m, H-3); 3.94 (1H, m, H-11); 2.60 (1H, m, H-17), 2.13 (3H, s, H-21); 2.04 (3H, s, acetate); 1.08 (3H, s, H-19), 0.89 (9H, s, *t*-butyl-Si); 0.64 (3H, s, H-18); 0.09 (3H, s, CH_3-Si) and 0.10 (3H, s, CH_3-Si).

To a solution of **16** (212 mg, 0.43 mmol) in THF (5 mL) and acetonitrile (5.5 mL) was added 40% hydrofluoric acid (4.4 mL) and the solution was stirred for 20 min at room temperature. The reaction mixture was neutralized with aqueous potassium bicarbonate and extracted with ethyl acetate. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 4:6) to give 11α-hydroxysteroid **17** (140 mg, 85%) as a white solid: mp 140–141 °C (from ethyl acetate–hexane); IR (KBr) 3410, 2880, 1715, 1420, 1130 cm^{-1}; ^1H NMR (200.13 MHz, $CDCl_3$): 4.78 (1H, m, H-3); 3.92 (1H, m, H-11); 2.55 (1H, m, H-17), 2.14 (3H, s, H-21); 2.04 (3H, s, acetate); 1.05 (3H, s, H-19), 0.61 (3H, s, H-18); ^{13}C NMR (50.32 MHz, $CDCl_3$) δ 209.6 (C-20), 171.0 (acetate), 74.5 (C-3), 69.0 (C-11), 63.4 (C-17), 55.6 (C-14), 50.7 (C-12), 47.1 (C-9), 44.2 (C-13), 43.4 (C-5), 37.9 (C-4 or C-1), 35.8 (C-10), 34.7 (C-8), 32.6 (C-1 or C-4), 31.4 (C-21), 27.6 (C-2 or C-6), 27.3 (C-6 or C-2), 26.1 (C-7), 24.4 (C-16), 23.7 (C-15); 22.9 (C-19); 21.4 (acetate), 14.4 (C-18); MS, *m*/*z* 376 (M^+, 2), 358 (1), 316 (6); 298 (36); 283 (15); 255 (23), 43 (100); Anal. Calcd for $C_{23}H_{36}O_4$: C,73.37; H, 9.64. Found: C, 73.76; H 9.90.

5.6. 3α-Hydroxy-1α,11α-epoxy-5β-pregnan-20-one (6)

Compound **17** (140 mg, 0.37 mmol) was dissolved in recently distilled dichloromethane (33 mL) and DIB (145 mg, 0.45 mmol) and iodine (93.8 mg, 0.37 mmol) were added to the solution. The reaction mixture was vigorously stirred at 25 °C in a water jacketed flask, while irradiating with a 300 W tungsten lamp (5000 lm) for 20 min. The solution was washed with aqueous sodium thiosulfate, dried with sodium sulfate and the solvent evaporated. The resulting solid was purified by flash chromatography on Florisil, using hexane–ethyl acetate 60:40 as eluant to give 1α,11α-epoxysteroid **18** (112 mg, 80%); ^1H NMR (200.13 MHz, $CDCl_3$): 4.67 (1H, m, H-3); 3.99 (1H, dd, J = 7 and 8 Hz, H-1); 3.91 (1H, m, H-11); 2.68 (1H, m, H-17), 2.14 (3H, s, H-21); 2.03 (3H, s, acetate); 1.09 (3H, s, H-19), 0.66 (3H, s, H-18).

A solution of compound **18** (112 mg, 0.30 mmol) in methanol (10 mL) containing K_2CO_3 (50 mg) was stirred for 30 min at 25 °C. The reaction mixture was neutralized with 1 M HCl and extracted with dichloromethane. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (ethyl acetate–hexane 4:6) to give compound **6** (80 mg, 80%) as a white solid: mp 150–151 °C (methanol); IR (KBr) 3413, 2856, 1724, 1357, 1035 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$): 3.95 (1H, dd, J = 9.3 and 7.5 Hz, H-1), 3.90 (1H, dt, J = 11.0, 11.0 and 4.3 Hz, H-11); 3.59 (1H, m, $W_{1/2}$ = 30.0 Hz, H-3); 2.63 (1H, t, J = 9.0 Hz, H-17); 2.48 (1H, dd, J = 11.0 and 4.3 Hz, H-12β); 2.30 (1H, m, H-2α); 2.14 (3H, s, H-21); 1.86 (2H, m, H-7b); 1.77 (1H, m, H-16); 1.71 (1H, m, H-15a); 1.67 (1H, m, H-6a); 1.61 (1H, m, H-4), 1.47 (1H, m, H-7); 1.43 (1H, m, H-8); 1.41 (1H, m, H-9); 1.40 (1H, m, H-2β); 1.38 (1H, t, J = 11.0 Hz m, H-12), 1.28 (1H, m, H-14); 1.23 (1H, m, H-15b); 1.07 (3H, s, H-19), 1.01 (1H, m, H-6b); 0.64 (3H, s, H-18);^{13}C NMR (125.77 MHz, $CDCl_3$): 208.0 (C-20), 85.3 (C-1), 75.5 (C-11), 68.3 (C-3), 62.0 (C-17), 58.4 (C-14), 51.3 (C-9), 47.6 (C-13), 45.2 (C-12), 42.0 (C-2), 38.6 (C-5), 38.4 (C-10), 35.3 (C-4), 34.7 (C-8), 31.7 (C-21), 26.8 (C-7), 26.6 (C-6), 24.2 (C-16), 22.5 (C-15), 21.3 (C-19), 15.0 (C-18); MS, *m*/*z* 332 (M^+, 46), 314 (68), 299 (18), 271 (26); 244 (78);159 (100); HRMS *m*/*z* [M^+] 332.2345 (calcd 332.2351).

5.7. Membrane preparation

Whole cerebella from male Sprague–Dawley rats (200–250 g) were homogenized in 5 vol (v/w) of ice-cold 0.32 M sucrose, using a Teflon-glass homogenizer at 1200 rpm. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was carefully decanted and centrifuged for 20 min at 15,000*g* at 4 °C. The pellet (P_2) was washed twice with 50 mM Tris–HCl buffer (pH 7.4) followed by centrifugation for 20 min at 15,000*g* at 4 °C. The final pellet was suspended in 1.2 ml of the same buffer and frozen at −20 °C. On the days of the assays, membranes were thawed, centrifuged for 20 min at 15,000*g* at 4 °C, and the pellet was washed twice with 30 vol of the incubation ice-cold buffer followed by centrifugation (15,000*g*, 20 min). The final pellet was resuspended in the incubation buffer (50 mM Tris–citrate, pH 7.4, containing 200 mM NaCl) to a protein concentration of approximately 8 mg/ml.[24]

5.8. *t*-[^{3}H]Butylbicycloorthobenzoate ([^{3}H]TBOB) binding assays

The effects of the steroids **1**, **2**, **4**–**6** on [^{3}H]TBOB binding were evaluated in cerebellar P_2 homogenates using essentially a previously described protocol with some modifications.[25] One hundred microliter aliquots of cerebellum membrane preparation P_2 were incubated with 10 nM [^{3}H]TBOB (21.0 Ci/mmol Amersham Biosciences) in the presence or absence of different concentrations of the steroids (10 nM–1.2 μM). All steroids were dissolved in DMSO (Sigma–Aldrich Corp., St. Louis, MO) and diluted with the incubation buffer (1:1000) immediately before use. 1 mM picrotoxin was used as non-specific binding. Assays were carried out at 25 °C for 2 h in the presence of 13.5 μM GABA (Sigma–Aldrich Corp.) and terminated by rapid filtration through glass microfiber filters (Whatman GF/C). Filter bound radioactivity was quantified by liquid scintillation spectrophotometry. IC_{50} (concentration at which half-maximal inhibition of control binding occurs) values were determined by linear computerized regression analysis after logit/log[26] transformation. All IC_{50} values are expressed in nanomolar.

Acknowledgments

We thank Agencia Nacional de Promoción Científica y Tecnológica (PICT 10962), Universidad de Buenos Aires and CONICET (Argentina) PIP 5508, for financial support. M.T.G. and R.F.B. thank the Spanish Research Council (CSIC) for providing a free-of-charge license to the CSD system.

Supplementary data

Supporting information available: complete crystallographic data for **4** (in .cif format). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.01.048.

References and notes

1. Baulieu, E. E. *Psychoneuroendocrinology* **1998**, *23*, 963.
2. Dubrosvsky, B. O. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2005**, *29*, 162.
3. Belelli, D.; Lambert, J. J. *Nat. Rev. Neurosci.* **2005**, *6*, 565.
4. Gasior, M.; Carter, R. B.; Witkin, J. M. *Trends Pharmacol. Sci.* **1999**, *20*, 107.
5. Souli, Ch.; Avlonitis, N.; Calogeropoulou, T.; Tsotonis, A.; Maksay, G.; Bíró, T.; Politi, A.; Mavromoustakos, T.; Makriyannis, A.; Reis, H.; Papadopoulos, M. *J. Med. Chem.* **2005**, *48*, 5203.
6. Suñol, C.; Garcia, D. A.; Bujons, J.; Kristofikova, Z.; Matyas, L.; Babot, Z.; Kasal, A. *J. Med. Chem.* **2006**, *49*, 3225, and references cited therein.
7. Scaglione, J. B.; Manion, B. D.; Benz, A.; Taylor, A.; DeKoster, G. T.; Rath, N. P.; Ever, s A. S.; Zorumski, C. F.; Mennerick, S.; Covey, D. F. *J. Med. Chem.* **2006**, *49*, 4595, and references cited therein.
8. Burton, G.; Galignana, M. D.; de Lavallaz, S.; Brachet-Cota, A. L.; Sproviero, E. M.; Ghini, A. A.; Lantos, C. P.; Damansco, M. C. *Mol. Pharmacol.* **1995**, *47*, 535.
9. Veleiro, A. S.; Pecci, A.; Monteserin, M. C.; Baggio, R.; Garland, M. T.; Lantos, C. P.; Burton, G. *J. Med. Chem.* **2005**, *48*, 5675.
10. Veleiro, A. S.; Rosenstein, R. E.; Jaliffa, C. O.; Grilli, M. L.; Speroni, F.; Burton, G. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 343.
11. Brachet-Cota, A. L.; Burton, G. *Z. Naturforsch.* **1990**, *45b*, 711.
12. Bose, A. K.; Hoffman, W. A.; Manhas, M. S. *Tetrahedron Lett.* **1973**, 1619.
13. Herr, R. J. *Tech. Rep.* **1999**, *3*, 1.
14. Veleiro, A. S.; Taich, P. J.; Alvarez, L. D.; DiChenna, P. H.; Burton, G. *Tetrahedron Lett.* **2005**, *46*, 4235.
15. Jiang, X.; Manion, B. D.; Benz, A.; Rath, N. P.; Evers, A. S.; Zorumski, C. F.; Mennerick, S.; Covey, D. F. *J. Med. Chem.* **2003**, *46*, 5334–5348.
16. In Figure 2, the O(3)–C(20) distance has been used for comparison, as the O(3)–O(20) distance may be affected by minor (non-relevant) changes in side chain position due to rotation around the C(17)–C(20) bond.
17. Im, W. B.; Blakeman, D. P. *Mol. Pharmacol.* **1991**, *39*, 394.
18. Lawrence, L. J.; Palmer, C. J.; Gee, K. W.; Wang, X.; Yamamura, H. I.; Casida, J. E. *J. Neurochem.* **1985**, *45*, 798.
19. VanRijn, C. M.; Dirksen, R.; Willems-vanBree, E.; Maksay, G. *J. Recept. Signal Transduct. Res.* **1995**, *15*, 787.
20. Sheldrick, G. M. *SHELXS-97. Program for Structure Resolution*; University of Göttingen: Germany, 1997.
21. Sheldrick, G. M. *SHELXL-97. Program for Structure Refinement*; University of Göttingen: Germany, 1997.
22. Sheldrick, G.M. SHELXTL-PC version 5.0. Siemens Analytical X-ray Instruments, Inc., Madison, Wisconsin, USA, **1994**.
23. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria G. E.; Robb, M.A.; Cheeseman, J.R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G.A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D.K.; Rabuck, A.D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J.V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A.; Gaussian 03, Revision B.05, Gaussian, Inc.: Pittsburgh, PA, **2003**.
24. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
25. Gee, K. W.; Bolger, M. B.; Brinton, R. E.; Coirini, H.; McEwen, B. S. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 803.
26. Rodbard, D.; Lewalds, J. E. In *Steroid Assay by Protein Binding: Karolinska Symposia on Research Methods in Reproductive Endocrinology*; Diczfalusi, E., Ed.; Karolinska Institute: Stockholm, 1979; pp 79–103.