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Synthesis of 6-thia analogs of the natural neurosteroid allopregnanolone

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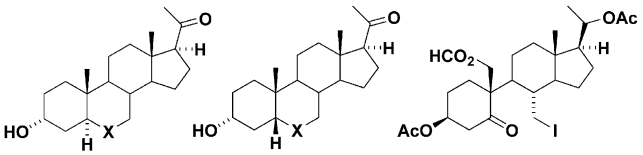
Abstract—A procedure is described for the preparation of 6-thiapregnanes in five steps from pregnenolone via a 5-oxo-7-iodo-secopregnane intermediate. The 6-thiasteroid obtained was converted into 6-thia-allopregnanolone and its sulfoxide and sulfone derivatives. The trans stereochemistry at the A/B ring junction was accomplished by stereoselective reduction of an intermediate hemithioketal with triethylsilane/$BF_3 \cdot Et_2O$. The compounds synthesized are analogs of natural neurosteroids, and exhibited $GABA_A$ receptor activity comparable to allopregnanolone.

1. Introduction

Neuroactive steroids are positive allosteric modulators of the $GABA_A$ receptor that interact with a specific steroid recognition site on the receptor–ion channel complex. They have potential therapeutic interest as anticonvulsants, anesthetics and for the treatment of several neurological and psychiatric disorders.[1] Endogenous neuroactive steroids (neurosteroids) such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, **1**) and its 5β isomer (pregnanolone, **2**), are rapidly biotransformed when administered exogenously and several synthetic analogs of these compounds with improved bioavailability have been developed.[2] An important advance in this search was the finding that substituents conferring water-solubility to these lipophilic steroids could be incorporated at a number of different positions of the steroid nucleus, without loss of anesthetic activity.[3] Analogs obtained by isosteric replacement of carbon atoms of the steroid nucleus by heteroatoms (e.g., O) exhibit localized changes in hydrophobicity and hydrogen bonding acceptor capacity; however, at variance with the presence of additional polar substituents, these modifications do not produce major changes in the overall conformation of the steroid nucleus or increased steric bulk.

We have described a synthetic approach to 5α-H and 5β-H 6-oxapregnanes (**3** and **4**),[4] analogs of the endogenous neuroactive steroids **1** and **2**, via stereospecific cyclizations of secosteroid **5**.[5,6] These compounds were 100-fold less active than **1** in their in vitro activity on the $GABA_A$ receptor, suggesting a significant involvement of the lipophilic character of ring B in modulating ligand binding to neighboring sites. A recent report on the activity of 6-aza-allopregnanolone points to the same conclusion.[7] The replacement of oxygen by sulfur (other characteristics being equal) often generates marked changes in the biological activity of a compound.[8] These biological and physicochemical properties are mostly attributed to the different electronic properties (electronegativity) of the sulfur and oxygen atoms. The larger size of the sulfur atom, and its more disperse electron density, result in a higher lipophilicity and diminished hydrogen bond acceptor capacity. Also, the C–S bond is longer (ca. 1.8 Å) and the C–S–C angle (ca. 95–100°) is smaller than the corresponding oxygen counterparts, thus some changes in flexibility and conformation may be expected. Additionally, the soft sulfur atom can be selectively oxidized to the sulfoxide or sulfone moieties, giving rise to new derivatives with different steric bulk and dipolar moments stereospecifically directed towards the α- and/or β-faces. With these guidelines in mind, we decided to synthesize the 6-thia analog **6**. To our knowledge, only a few procedures for the synthesis of 6-thiasteroids have been reported,[9] none of which correspond to analogs of neuroactive steroids.



Keywords: 6-Thiasteroids; *S*-Oxo-thiasteroids; *S*,*S*-Dioxo-thiasteroids; Neurosteroid analogs; Allopregnanolone; $GABA_A$ receptor.
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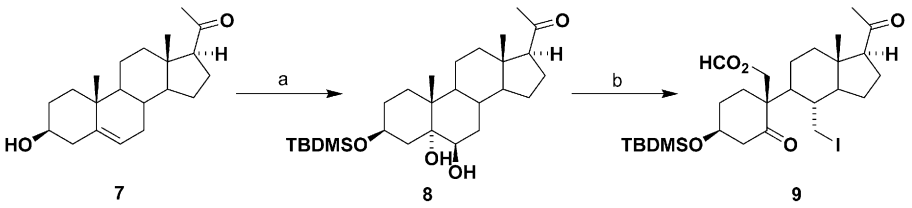
2. Results and discussion

2.1. Chemistry

By analogy with the approach used in the synthesis of the oxa-analogs **3** and **4**,[5,6] we focused our attention on the iodo substituent present in secosteroid **5**. This could be replaced by sulfur nucleophiles, giving rise to thioketals that may be transformed into the desired cyclic thioether. In this case however, the acetate group at C-3 in the 5-oxo secosteroid **5** proved to be too labile, giving elimination by-products throughout the synthesis; thus a silylated derivative was used.[10] Secosteroid **9** was synthesized from commercially available pregnenolone (**7**) in four steps (Scheme 1). Thus, pregnenolone was transformed into the 3β-*tert*-butyldimethylsilyloxy-5α,6β-diol **8** by epoxidation and cleavage (hydrogen peroxide, formic acid) followed by deformylation (methanolic base) to the 3β,5α,6β-trihydroxypregnane (one-pot)[11] and subsequent regioselective silylation of the less hindered equatorial 3β-hydroxy moiety. Treatment of **8** with HgO/I_2 (CCl_4, *hν*) gave the precursor secosteroid **9** in 42% yield. The structure of **9** was confirmed by comparison of its ^1H and ^{13}C NMR spectra with those of the acetylated analog **5** previously synthesized by us.[5]

Stereospecific reduction of the hemithioketal moiety in **11** to the 5α-H-cyclic thioether was accomplished in two steps by protection of the hydroxyl group at C-19 as a formate, followed by selective reduction of the hemithioketal moiety with Et_3SiH and $BF_3\cdot Et_2O$ in Cl_2CH_2. The attack of the hydride on the thiocarbenium intermediate occurred, as expected, from the less hindered α-face with stereoselective formation of the 5α-H-6-thiasteroid **12**. The pyranosic thiocarbenium ion preferently accepts nucleophiles from the α (axial) side due to the anomeric effect of the sulfur atom; this preference is magnified, relative to the tetrahydropyran system, because 1,3-diaxial repulsions are smaller as a result of the longer C–S bond.[12] The silyl protecting group was also cleaved in this step. The coupling pattern of the 3α-H resonance that appeared as a broad multiplet ($W_{1/2}$=15.3 Hz) typical of an axial hydrogen, was consistent with the α orientation of 5-H. Confirmation of that stereochemistry came from the observation of a strong NOE correlation between H-5 and H-3 in the NOESY spectrum of **12**, thus indicating the α orientation for H-5.

Inversion of the configuration at C-3, a requisite for agonistic $GABA_A$ receptor activity,[13] was accomplished in a straightforward way using the Mitsunobu reaction. Adequate choice



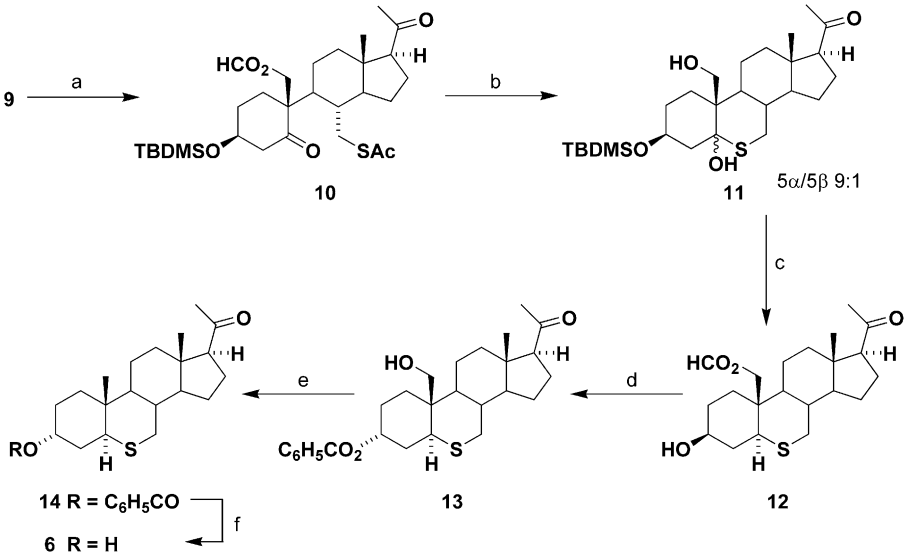
Scheme 1. Reagents and conditions: (a) i. HCO_2H, then 30% H_2O_2; ii. 40% NaOH, MeOH; iii. TBDMSCl, imidazole, DMF; (b) HgO, I_2, Cl_2CH_2–Cl_4C, *hν*, 25 °C, 4.5 h.

Reaction of **9** with potasium thioacetate gave the sulfur substituted secosteroid **10** in 70% yield (Scheme 2). The presence of the sulfur moiety was evident in the NMR and mass spectra. Particularly diagnostic was the shift of the 7-CH_2 hydrogen and carbon resonances (3.14/3.45 and 17.4 ppm for **9**; 2.95/3.05 and 31.5 ppm for **10**), consistent with the change of iodine by thioacetyl at that position. Treatment of **10** with base, simultaneously cleaved the C-19 formate and the C-7 thioacetate, with concomitant cyclization of the intermediate 7-sulfanyl anion to give the hemithioketal **11** as a 9:1 5α/5β mixture (78% yield).[†] Absence of the characteristic signal of a carbonyl group at C-5 in the ^{13}C NMR spectrum of **11** and the presence of a resonance at 81.9 ppm assigned to the hemithioketalic carbon of the 5-α isomer, indicated the success of this transformation. Attempts to purify the diastereomeric mixture on silica gel resulted in partial decomposition and isomerization of the α-epimer into the β-epimer giving rise to a new diastereomeric ratio of ca. 1:1.

[†] Determined in the ^1H NMR spectrum of the mixture, from the intensity ratio of the 13-CH_3 resonances for the α (0.68 ppm) and β (0.62 ppm) isomers. The coupling pattern of the 3α-H resonance of the major isomer, that appeared as a broad multiplet ($W_{1/2}$=22.3 Hz) typical of an axial hydrogen, was consistent with the α orientation of the 5-hydroxyl.

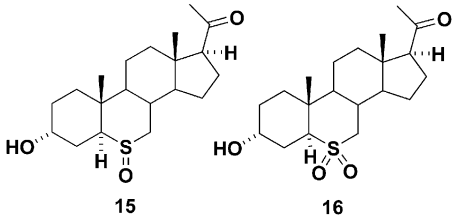
of the acid for this reaction was mandatory, taking into account that the following step involved the regioselective deprotection of the formate group at C-19. The 3α-benzoate in **13** proved to be adequate for this purpose. The success of the Mitsunobu reaction was evident in the ^1H NMR spectrum of the reaction product that showed the equatorial orientation of H-3 ($W_{1/2}$=7.5 Hz, at variance with the same hydrogen in **12**, vide supra). Aromatic hydrogens, belonging to the incorporated benzoyl moiety, were also observed. The 19-hydroxy derivative **13** was obtained by regioselective deprotection of the 19-ester moiety with hydrochloric acid in methanol; under these mild and controlled conditions, the 3α-benzoate was not affected.

Deoxygenation of the primary hydroxyl group attached at C-19 was then carried out using the Barton–Mc Combie procedure, by formation of the 19-imidazoylthionocarbonate derivative and reduction with diphenylsilane to give **14**; attempts to obtain this compound free of unreacted diphenylsilane were unsuccessful and so it was used directly in the next step. The low reactivity of the axial benzoate at C-3 required the use of strong base for its removal, which led to partial isomerization of the side chain at C-17. Smooth debenzoylation was achieved under nucleophilic conditions with sodium methylthiolate in DMF, to give 6-thia-allopregnanolone **6** as single product (61% yield from **13**).



Scheme 2. Reagents and conditions: (a) KSAc, acetone, 3 h, 25 °C; (b) NaOH, MeOH, 3 h, 25 °C; (c) i. formic acetic anhydride, py, 2 h, 25 °C; ii. Et_3SiH, $BF_3{\cdot}Et_2O$, Cl_2CH_2, 1 h, −15 °C; (d) i. benzoic acid, Ph_3P, DEAD, THF, 18 h, 25 °C; ii. 6 N HCl, MeOH, 1 h, 25 °C; (e) i. thiocarbonyldiimidazole, DMAP, Cl_2CH_2, 5 h, reflux; ii. Ph_2SiH_2, AIBN, toluene, 4.5 h, 115 °C; (f) $NaSCH_3$, DMF, 2 h, 100 °C.

Selective oxidation of **6** with potassium peroxymonosulfate (Oxone®) gave either sulfoxide **15** or sulfone **16**, depending on the product–reagent ratio and temperature.[14] Thus, treatment with Oxone® (1.3 equiv) in methanol at 0 °C gave sulfoxide derivative **15** as single product (87% yield) whereas excess of the same reagent (3 equiv) at room temperature gave sulfone **16** in 90% yield. Configuration of the sulfur atom in the sulfoxide **15** was inferred from the ^1H NMR spectrum, considering the shift in the 10-CH_3 resonance in sulfone **16** compared to **6** (+0.2 ppm) due to the axial oxygen on the sulfur. A negligible shift was observed in the transformation of **6** to the sulfoxide **15** (ca. +0.05 ppm) indicating that the oxygen was equatorially oriented. This is the expected stereochemistry for **15** considering attack of the reagent from the sterically less demanding α-face.



2.2. $GABA_A$ receptor activity

Biological activity of the three synthetic steroids was assayed by in vitro tests using [^{35}S]-*tert*-butylbicyclo[2,2,2]-phosporothionate (TBPS), [^{3}H]-flunitrazepam, and [^{3}H]-muscimol as radiolabelled ligands to γ-aminobutyric acid receptors ($GABA_A$). Crude membrane receptors from male rats' cerebellum were used to test the capacity of the steroids to displace the specific binding of the radioactive ligands.[13] Allopregnanolone was used as a positive control to check the viability of the methods. Preliminary results revealed that the synthetic steroids show a displacement pattern similar to that of allopregnanolone with an IC_{50} for [^{35}S]-TBPS binding in the 10^{-7} M range (Fig. 1). All three steroids

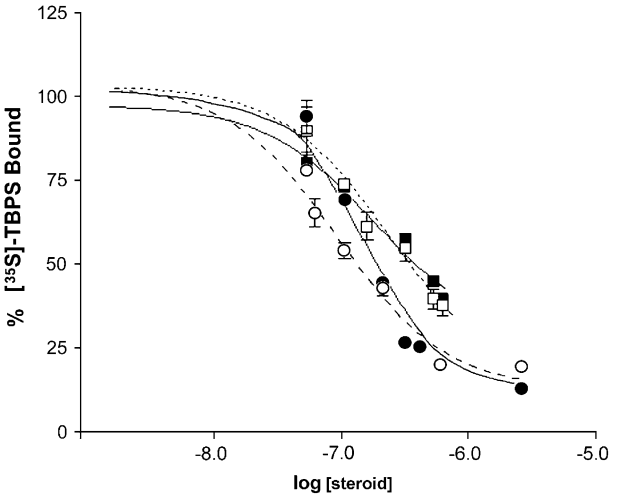


Figure 1. Inhibition of binding of [^{35}S]-*tert*-butylbicyclo-phosporothionate ([^{35}S]-TBPS) to membranes from rat cerebellum by allopregnanolone (**1**, ○), 3α-hydroxy-6-thia-5α-pregnan-20-one (6-thia-allopregnanolone, **6**, ●), *S*-oxo-3α-hydroxy-6-thia-5α-pregnan-20-one (**15**, □), and *S*,*S*-dioxo-3α-hydroxy-6-thia-5α-pregnan-20-one (**16**, ■). Cerebellum membrane preparations were incubated with 10 nM [^{35}S]-TBPS in the absence (100% binding) or presence of increasing concentrations of the steroids (50–600 nM). Picrotoxin (2 mM) was used to determine non-specific binding. Assays were carried out at 22 °C for 2 h in the presence of 5 μM GABA. Calculated IC_{50} values are: **1**, 92.8±14.1 nM; **6**, 171.2±39.2 nM; **15**, 241.1±97.0 nM; **16**, 200.3±37.1 nM.

stimulated in a similar way the binding of [^{3}H]-flunitrazepam and affected [^{3}H]-muscimol binding as well, rendering comparable results to allopregnanolone. Complete biological results will be published in a separate paper.

3. Conclusions

The first 6-thia analog of a neurosteroid and its oxidized derivatives 6-sulfoxide and 6-sulfone, were synthesized from

pregnenolone in 8.1, 7.0, and 7.3% yield, respectively. The key reduction of the intermediate hemithioketal to give the trans-fused A/B rings in the steroidal framework was accomplished stereospecifically, its stereochemical outcome being consistent with the intermediacy of a pyranosic thiocarbenium ion. Furthermore compound **13** is a synthetic precursor of C-19 substituted analogs. $GABA_A$ receptor activity for 6-thia-allopregnanolone (**6**) was similar to that of allopregnanolone (**1**) giving further support to our assumption that the decrease in activity observed for the 6-oxa and 6-aza analogs is related to their hydrogen bonding acceptor and/or donor properties at position 6. The small change in activity observed upon oxidation to the sulfoxide and sulfone analogs (**15** and **16**) indicates that lipophylicity and electrostatic potential changes in the vicinity of position 6 are not critical for $GABA_A$ receptor activity.

4. Experimental

4.1. General experimental procedures

Melting points were determined on a Fisher Johns apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions in a Bruker AC-200 NMR spectrometer at 200.13 (^1H) and 50.32 (^{13}C) MHz or a Bruker AM-500 at 500.13 (^1H) and 125.77 (^{13}C) MHz. NMR assignments are based on multiplicity determinations (DEPT) and 2D spectra (COSY, HETCOR, HMBC, HSQC, and NOESY) are obtained using standard Bruker software. Chemical shifts are given in parts per million (δ) downfield from TMS internal standard. IR spectra were measured as thin films (from dichloromethane solution) on KBr disks, using a FT-IR Nicolet Magna 550 spectrophotometer, wave-numbers are given in cm^{-1}. The electron impact mass spectra were obtained at 70 eV by direct inlet in a Shimadzu QP 5000 mass spectrometer. Thin layer chromatography was performed on silica gel 60 plates with fluorescent indicator. The plates were visualized with a 3.5% solution of phosphomolybdic acid in ethanol. Column chromatography was conducted on silica gel (230–400 mesh) or octadecyl-functionalized silica gel. All solvents were distilled and stored over 4 Å molecular sieves before use. Solvents were evaporated at 45 °C under vacuum. High-resolution mass spectra were measured at the Mass Spectrometry Facility of the Chemistry Department, University of California at Riverside. Pregnenolone was purchased from Steraloids Inc. (USA). All new compounds were determined to be >95% pure by ^1H NMR spectroscopy.

4.1.1. 3β-*tert*-Butyldimethylsilyloxy-5α,6β-dihydroxypregnan-20-one (8). A suspension of pregnenolone (**7**, 3.0 g, 9.48 mmol) in 88% formic acid (33 mL) was heated to 70–80 °C with stirring for 5 min and then cooled to 25 °C. The resulting thick slurry containing the 3-formate, was treated with 30% hydrogen peroxide (3.4 mL) keeping the temperature at 40 °C by cooling. After 20 min a pink solution resulted and stirring was continued at ca. 20 °C for 20 h. The reaction mixture was treated with boiling water (50 mL) with stirring, allowed to cool and the white solid was collected and dried. The solid was dissolved in methanol (100 mL) and the solution was treated with 40% sodium hydroxide (5.5 mL) at 0 °C and allowed to reach 25 °C. After 45 min the solution was neutralized with 2 N HCl (27 mL) and diluted with cold water (50 mL). 3β,5α,6β-Trihydroxypregnan-20-one precipitated as a white solid after cooling; it was collected, washed with cold water and dried under vacuum (3.34 g). The triol was dissolved in anhydrous DMF (45 mL) under nitrogen, imidazole (1.94 g, 28.50 mmol) and *tert*-butyldimethylsilyl chloride (2.87 g, 19.03 mmol) were added at 0 °C and the mixture was allowed to reach 25 °C. After 2 h the solution was poured into saturated aqueous NaCl (60 mL), extracted with diethyl ether (3×20 mL), dried with sodium sulfate and evaporated under vacuum. The white solid obtained was purified by flash chromatography on silica gel using hexane/ethyl acetate (90:10), to give the title compound **8** (3.17 g, 72% from **7**). Mp 202–203 °C (hexane/ethyl acetate) [found: C, 69.9, H, 10.5. $C_{27}H_{48}O_4Si$ requires: C, 69.78, H, 10.41]; ν_{max} 3479, 2937, 2860, 1697, 1466, 1359, 1251, 1077, 871, 834, 776; δ_H (500 MHz) 0.05 (6H, s, TBDMS–H), 0.63 (3H, s, 18-H), 0.87 (9H, s, TBDMS–H), 1.18 (3H, s, 19-H), 2.11 (3H, s, 21-H), 2.53 (1H, t, J=8.9 Hz, 17-H), 3.52 (1H, br s, 6-H), 4.07 (1H, m, 3-H); δ_C (50 MHz) −4.6 (CH_3Si), −4.5 (CH_3Si), 13.5 (C-18), 16.8 (C-19), 18.1 ($SiC(CH_3)_3$), 21.1 (C-11), 22.8 (C-16), 24.2 (C-15), 25.9 ($C(CH_3)_3$), 30.3 (C-8), 31.1 (C-2), 31.5 (C-21), 32.4 (C-1), 34.3 (C-7), 38.3 (C-10), 39.0 (C-12), 41.3 (C-4), 44.3 (C-13), 45.7 (C-9), 56.1 (C-14), 63.7 (C-17), 68.1 (C-3), 75.9 (C-6), 76.1 (C-5), 209.6 (C-20); *m/z* (EI) 465 (M+H, 0.1), 447 (M+H−H_2O, 0.2), 389 (2), 315 (24), 297 (30), 279 (8).

4.1.2. 3β-*tert*-Butyldimethylsilyloxy-19-formyloxy-7-iodo-6-nor-5,7-secopregnane-5,20-dione (9). To a solution of diol **8** (1.00 g, 2.15 mmol) in dry dichloromethane (85.0 mL) in a water-jacketed vessel, were added recently distilled carbon tetrachloride (85.0 mL), mercury(II) oxide (1.22 g, 4.73 mmol), and iodine (1.77 g, 7.00 mmol) under nitrogen. The solution was vigorously stirred and irradiated with two 300 W tungsten lamps (5000 lm each) at 25 °C. Three additional portions of mercury(II) oxide (1.22 g, 4.73 mmol) and iodine (1.77 g, 7.00 mmol) were added at intervals of 1 h. After 4.5 h the solution was diluted with dichloromethane (85.0 mL) and filtered. The organic layer was washed with a saturated solution of sodium thiosulfate (60 mL) and water (60 mL), dried, and evaporated under reduced pressure. The oily residue was purified by flash chromatography on octadecyl-functionalized silica gel using methanol/water (70:30) to give the title compound as a foamy yellow solid (0.85 g, 65%). ν_{max} 3488, 2950, 2887, 2858, 1726, 1700, 1467, 1360, 1179, 1052, 837, 778, 738, 700; δ_H (500 MHz) 0.03 (3H, s, TBDMS–H), 0.06 (3H, s, TBDMS–H), 0.69 (3H, s, 18-H), 0.86 (9H, s, TBDMS–H), 0.90 (1H, m, 8-H), 1.13 (1H, m, 15β-H), 1.39 (1H, m, 14-H), 1.48 (1H, m, 12α-H), 1.69 (1H, m, 2β-H), 1.75 (2H, m, 15α-H and 16α-H), 1.81–1.88 (4H, m, 11β-H, 2α-H, 9-H and 11α-H), 2.04 (1H, m, 1α-H), 2.09 (1H, m, 12β-H), 2.11 (1H, m, 1β-H), 2.12 (3H, s, 21-H), 2.15 (1H, m, 16β-H), 2.41 (1H, dt, J=13.9, 2.8 Hz, 4α-H), 2.57 (1H, t, J=9.2 Hz, 17-H), 3.14 (1H, dd, J=10.8, 2.8 Hz, 7a-H), 3.45 (1H, dd, J=10.8, 1.8 Hz, 7b-H), 3.49 (1H, dd, J=13.9, 3.5 Hz, 4β-H), 4.32 (1H, d, J=11.8 Hz, 19a-H), 4.45 (1H, m, 3α-H), 4.45 (1H, d, J=11.8 Hz, 19b-H), 8.09 (1H, s, formate); ^{13}C NMR (125 MHz) δ: −5.0 (C–CH_3Si), −4.9 (C–CH_3Si), 14.0 (C-18), 17.4 (C-7), 17.9 (C–CSi), 22.4 (C-16), 23.3 (C-11), 23.6 (C-15), 25.6 (C–CH_3), 27.4 (C-1), 28.8 (C-2), 31.3 (C-21), 37.6 (C-8), 38.6

(C-12), 39.6 (C-9), 43.1 (C-13), 49.1 (C-4), 53.7 (C-10), 55.4 (C-14), 63.2 (C-17), 65.2 (C-19), 70.4 (C-3), 160.8 (C-formate), 208.8 (C-20), 212.9 (C-5); *m/z* (FAB) 627 (M+Na$^+$, 11), 477 (9), 461 (100), 329 (40); HRMS (FAB) found 627.1980, $C_{27}H_{45}IO_5SiNa$ requires 627.1979.

4.1.3. 3β-*tert*-Butyldimethylsilyloxy-19-formyloxy-7-acetylsulfanyl-6-nor-5,7-seco-pregnane-5,20-dione (10). A mixture of secosteroid **7** (0.65 g, 1.08 mmol) and potassium thioacetate (0.37 g, 3.23 mmol) in dry acetone (33 mL) was stirred at room temperature for 3 h under nitrogen. The reaction mixture was diluted with dichloromethane (70 mL), filtered and the solvent was evaporated. The residue was purified by flash chromatography on silica gel with an hexane/ethyl acetate gradient to give thioacetate **10** (0.42 g, 70%) as a vitreous solid; ν_{max} 2955, 2932, 2894, 2857, 1725, 1700, 1464, 1357, 1174, 1128, 1041, 835; δ_H (500 MHz) 0.03 (3H, s, TBDMS–H), 0.05 (3H, s, TBDMS–H), 0.65 (3H, s, 18-H), 0.85 (9H, s, TBDMS–H), 1.23 (1H, m, 15β-H), 1.41-1.43 (2H, m, 14-H and 12α-H), 1.62 (1H, m, 15α-H), 1.67–1.69 (3H, m, 2β-H, 16α-H and 11β-H), 1.81 (1H, m, 11α-H), 1.88–1.91 (2H, m, 9-H and 2α-H), 1.98–1.99 (2H, m, 8-H and 1α-H), 2.08 (2H, m, 1β-H and 12β-H), 2.11 (3H, s, 21-H), 2.12 (1H, m, 16β-H), 2.30 (3H, s, CH_3COS), 2.36 (1H, dt, *J*=13.8, 2.8 Hz, 4α-H), 2.50 (1H, t, *J*=9.2 Hz, 17-H), 2.95 (1H, dd, *J*=13.9, 2.8 Hz, 7a-H), 3.05 (1H, dd, *J*=13.9, 3.0 Hz, 7b-H), 3.14 (1H, dd, *J*=13.8, 3.5 Hz, 4β-H), 4.36 (1H, d, *J*=11.8 Hz, 19a-H), 4.43 (1H, m, 3α-H), 4.48 (1H, d, *J*=11.8 Hz, 19b-H), 8.08 (1H, s, formate). δ_C (125 MHz) −5.0 (CH_3Si), −4.9 (CH_3Si), 13.1 (C-18), 17.9 (Si*C*$(CH_3)_3$), 22.6 (C-16), 23.7 (C-11), 24.4 (C-15), 25.6 (C(*C*$H_3)_3$), 27.5 (C-1), 28.8 (C-2), 30.9 (*C*H_3COS), 31.2 (C-21), 31.5 (C-7), 36.7 (C-8), 38.8 (C-12), 41.1 (C-9), 43.5 (C-13), 48.0 (C-4), 53.5 (C-14), 54.0 (C-10), 63.4 (C-17), 65.6 (C-19), 70.4 (C-3), 160.8 (formate), 193.8 (CH_3*C*OS), 208.8 (C-20), 211.9 (C-5); *m/z* (FAB) 575 (M+Na$^+$, 100), 494 (30), 493 (83), 441 (9), 419 (35), 361 (26); HRMS (FAB) found 575.2810, $C_{29}H_{48}O_6SSiNa$ requires 575.2839.

4.1.4. 3β-Hydroxy-19-formyloxy-6-thia-5α-pregnan-20-one (12). To a solution of thioacetate **10** (0.40 g, 0.72 mmol) in methanol (40.0 mL) was added 10% aqueous sodium hydroxide (10 mL) at 0 °C under nitrogen. The reaction mixture was warmed to 25 °C; after 3 h the solution was neutralized with 1 N hydrochloric acid (20 mL) and evaporated under vacuum to a fifth of its original volume. The remaining solution was diluted with dichloromethane (50 mL), washed with brine (20 mL), and evaporated to dryness. Purification on a silica gel column using a gradient of hexane/ethyl acetate gave hemithioketal **11** (5α/5β 9:1; 0.318 g, 91%). Data for the 5α-hydroxy isomer: δ_H (200 MHz) 0.05 (6H, s, TBDMS–H), 0.68 (3H, s, 18-H), 0.87 (9H, s, TBDMS–H), 2.11 (3H, s, 21-H), 2.35 (1H, dd, *J*=12.8, 4.0 Hz, 7α-H), 2.53 (1H, t, *J*=8.8 Hz, 17-H), 2.84 (1H, t, *J*=12.8 Hz, 7β-H), 3.71 (1H, d, *J*=12.6 Hz, 19a-H), 4.10 (1H, m, 3α-H), 4.25 (1H, d, *J*=12.6 Hz, 19b-H); δ_C (50 MHz) −4.7 and −4.6 (C–CH_3Si), 13.8 (C-18), 18.1 (Si*C*$(CH_3)_3$), 22.1 (C-16), 22.1 (C-11), 22.4 (C-15), 25.8 (C(*C*$H_3)_3$), 28.1 (C-2), 29.1 (C-1), 31.4 (C-21), 31.6 (C-7), 37.1 (C-8), 39.4 (C-12), 43.5 (C-13), 44.5 (C-10), 45.4 (C-9), 45.8 (C-4), 56.2 (C-14), 63.6 (C-17), 64.1 (C-19), 67.0 (C-3), 81.9 (C-5), 209.2 (C-20).

To a solution of the hemithioketal **11** obtained above (0.318 g, 0.659 mmol) in dry pyridine (14.2 mL) was added recently prepared formic acetic anhydride[15] (8.25 mL) at 25 °C under nitrogen. After 2 h the solution was poured into cold 2 N HCl (50 mL), extracted with dichloromethane (3×15 mL), dried with sodium sulfate, and the solvent was evaporated. The residue was dissolved in dry dichloromethane (50 mL) and triethylsilane (1.05 mL, 6.6 mmol) and $BF_3\cdot Et_2O$ (0.85 mL, 6.6 mmol) were added at −15 °C under nitrogen. After 1 h, cold water (20 mL) was added followed by solid sodium bicarbonate until neutral. The solution was washed with saturated sodium bicarbonate (20 mL) and brine (20 mL), dried with sodium sulfate, and the solvent was evaporated. Purification by column chromatography on silica gel with a gradient of hexane/ethyl acetate gave the 6-thiapregnane **12** (0.157 g, 64% from **10**). Mp 161–162 °C (hexane/ethyl acetate); ν_{max} 3407, 2937, 2869, 1715, 1360, 1174, 1060; δ_H (500 MHz) 0.64 (3H, s, 18-H), 0.89 (1H, td, *J*=11.4, 3.7 Hz, 9-H), 0.98 (1H, td, *J*=13.9, 3.6 Hz, 1α-H), 1.18 (1H, m, 14-H), 1.26–1.33 (2H, m, 15β-H and 12α-H), 1.38 (1H, m, 2β-H), 1.50 (1H, m, 11β-H), 1.55 (1H, m, 4α-H), 1.67–1.70 (2H, m, 16α-H and 15α-H), 1.77 (1H, ddd, *J*=13.9, 7.0, 3.7 Hz, 11α-H), 1.86–1.88 (1H, m, 8-H and 2α-H), 2.00–2.02 (1H, m, 4β-H and 12β-H), 2.10 (3H, s, 21-H), 2.19 (1H, m, 16β-H), 2.33 (1H, dt, *J*=14.0, 3.5 Hz, 1β-H), 2.41 (1H, dd, *J*=13.2, 11.3 Hz, 7α-H), 2.49 (1H, t, *J*=9.0 Hz, 17-H), 2.55 (1H, dd, *J*=13.2, 3.6 Hz, 7β-H), 2.72 (1H, dd, *J*=13.4, 3.6 Hz, 5α-H), 3.69 (1H, m, 3α-H), 4.48 (1H, d, *J*=12.8 Hz, 19a-H), 4.75 (1H, d, *J*=12.8 Hz, 19b-H), 8.12 (1H, s, formate); δ_C (125 MHz) 13.4 (C-18), 22.3 (C-11), 22.7 (C-16), 24.3 (C-15), 31.2 (C-1), 31.3 (C-2), 31.3 (C-21), 34.5 (C-7), 37.3 (C-4), 37.3 (C-8), 38.5 (C-10), 39.1 (C-12), 44.0 (C-13), 48.8 (C-5), 54.5 (C-9), 55.8 (C-14), 62.4 (C-19), 63.5 (C-17), 69.9 (C-3), 160.9 (formate), 208.9 (C-20); *m/z* (EI) 380 (M$^+$, 33), 335 (M−HCOOH, 24), 321 (4), 251 (8), 93 (55), 79 (82); HRMS (EI) found 380.2035, $C_{21}H_{32}O_4S$ requires 380.2021.

4.1.5. 3α-Benzoyloxy-19-hydroxy-6-thia-5α-pregnan-20-one (13). To a solution of 6-thiapregnane **12** (0.125 g, 0.328 mmol) in dry THF (5.0 mL), were added triphenylphosphine (0.258 g, 0.984 mmol), benzoic acid (0.094 g, 0.770 mmol), and DEAD (0.090 mL, 0.659 mmol) at 25 °C under nitrogen. The mixture was stirred for 18 h and the THF was evaporated under vacuum. The residue was purified by column chromatography on silica gel (cyclohexane/ethyl acetate), dissolved in methanol (26 mL) and 6 N HCl (5.9 mL, 35.8 mmol) added at 0 °C. The solution was allowed to reach 25 °C under nitrogen, after 1 h a saturated solution of potassium bicarbonate was added until neutral, the methanol was evaporated to a fifth of the original volume and the mixture was poured into dichloromethane (30 mL), washed with brine (1 mL), dried with sodium sulfate, and the solvent was evaporated. The light yellow solid was purified by column chromatography on silica gel using a gradient of hexane/ethyl acetate, to give the title compound **13** (0.140 g, 93%) as a white solid. Mp 86–88 °C (hexane/ethyl acetate) [found: C, 70.8, H, 8.2. $C_{27}H_{36}O_4S$ requires: C, 71.02, H, 7.95]; ν_{max} 3496, 2945, 2873, 1707, 1449, 1353, 1112, 715; δ_H (500 MHz) 0.72 (3H, s, 18-H), 0.99 (1H, td, *J*=11.4, 3.7 Hz, 9-H), 1.21 (1H, m, 14-H), 1.28–1.35 (2H, m, 15β-H and 1α-H), 1.43 (1H, td, *J*=12.8, 4.1 Hz,

12α-H), 1.67–1.72 (3H, m, 16α-H, 15α-H and 11β-H), 1.79–1.82 (2H, m, 2β-H and 11α-H), 1.93 (2H, m, 1β-H and 2α-H), 2.06–2.07 (2H, m, 4β-H and 12β-H), 2.11 (1H, m, 4α-H), 2.12 (3H, s, 21-H), 2.20 (1H, m, 16β-H), 2.28 (1H, qd, J=10.9, 3.8 Hz, 8-H), 2.50 (1H, dd, J=13.1, 11.4 Hz, 7α-H), 2.52 (1H, t, J=9.2 Hz, 17-H), 2.61 (1H, dd, J=13.1, 3.8 Hz, 7β-H), 3.16 (1H, dd, J=13.1, 3.8 Hz, 5α-H), 3.83 (1H, dd, J=12.2, 7.0 Hz, 19a-H), 4.34 (1H, d, J=12.2 Hz, 19b-H), 5.35 (1H, m, 3β-H), 7.46 (2H, m, *meta*-ArH), 7.59 (1H, m, *para*-ArH), 8.06 (2H, m, *ortho*-ArH); δ_C (50 MHz) 13.6 (C-18), 21.6 (C-11), 22.5 (C-16), 24.0 (C-15), 26.3 (C-2), 29.3 (C-1), 31.3 (C-21), 32.1 (C-4), 34.2 (C-7), 38.1 (C-8), 39.0 (C-12), 39.5 (C-10), 44.2 (C-13), 45.8 (C-5), 54.7 (C-9), 56.3 (C-14), 62.5 (C-19), 63.4 (C-17), 69.5 (C-3), 128.3 (*meta*-phenyl), 129.4 (*ortho*-phenyl), 130.5 (*ipso*-phenyl), 132.9 (*para*-phenyl), 165.5 (Ph*C*OO), 209.2 (C-20); *m*/*z* (EI) 456 (M^+, 10), 334 (61), 316 (5), 316 (5), 304 (25), 303 (24), 105 (41).

4.1.6. 3α-Hydroxy-6-thia-5α-pregnan-20-one (6). To a solution of 3α-benzoate **13** (0.127 g, 0.279 mmol) in dry dichloromethane (6.4 mL), were added thiocarbonyldiimidazole (0.253 g, 1.40 mmol) and 4-dimethylaminopyridine (0.002 g, 0.015 mmol) and the solution was refluxed under nitrogen for 5 h. The reaction mixture was evaporated to dryness and purified by column chromatography on silica gel with hexane/ethyl acetate (8:2) to give the intermediate 19-imidazoylthionocarbonate as a yellow solid (0.136 g). The solid was dissolved in anhydrous toluene (6.6 mL) and heated to 115 °C under nitrogen, diphenylsilane (0.265 mL, 1.450 mmol) was added followed by 18 aliquots (0.05 mL each) of a solution of AIBN in anhydrous toluene (0.158 g/mL, 1.8 equiv) at 15 min intervals. The solvent was evaporated and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate as eluent to give an oily fraction of **14** containing residual diphenylsilane that could not be separated; δ_H (500 MHz) 0.65 (3H, s, 18-H), 1.09 (3H, s, 19-H), 2.11 (3H, s, 21-H), 2.44 (1H, dd, J=13.2, 12.2 Hz, 7α-H), 2.51 (1H, t, J=9.3 Hz, 17-H), 2.53 (1H, dd, J=13.2, 3.8 Hz, 7β-H), 3.09 (1H, dd, J=13.4, 3.5 Hz, 5α-H), 5.29 (1H, m, 3β-H), 7.47 (2H, m, *meta*-ArH), 7.58 (1H, m, *para*-ArH), 8.06 (2H, m, *ortho*-ArH); δ_C (125 MHz) 11.5 (C-19), 13.2 (C-18), 21.0 (C-11), 22.7 (C-16), 24.2 (C-15), 25.9 (C-2), 31.3 (C-21), 31.9 (C-1), 31.9 (C-4), 34.3 (C-7), 36.7 (C-8), 36.9 (C-10), 38.8 (C-12), 43.9 (C-13), 46.6 (C-5), 54.1 (C-9), 55.5 (C-14), 63.5 (C-17), 69.7 (C-3), 128.3 (*meta*-phenyl), 129.5 (*ortho*-phenyl), 130.7 (*ipso*-phenyl), 132.8 (*para*-phenyl), 165.5 (Ph*C*OO), 209.0 (C-20).

To a solution of the crude fraction obtained above containing **14**, in dry DMF (5.0 mL), was added sodium methanethiolate (0.254 g, 3.63 mmol) and the mixture was heated for 2 h at 100 °C under nitrogen. The resulting solution was cooled, poured into brine (10 mL), and extracted with ethyl ether (30 mL). The organic layer was washed with brine (2×10 mL), dried with sodium sulfate, and the solvent was evaporated. The yellowish solid was purified by column chromatography on silica gel using hexane/ethyl acetate (8:2 to 1:1) to give the title compound **6** as a white solid (0.057 g, 61% from **13**). Mp 174–175 °C (hexane/ethyl acetate); ν_{max} 3430, 2939, 2875, 1696, 1426, 1359, 1010, 752; δ_H (500 MHz) 0.63 (3H, s, 18-H), 0.90 (1H, m, 9-H), 1.03 (3H, s, 19-H), 1.17 (1H, m, 14-H), 1.25 (1H, m, 15β-H), 1.33 (1H, m, 11β-H), 1.40 (2H, m, 1α-H and 12α-H), 1.64–1.65 (3H, m, 1β-H, 4α-H and 4β-H), 1.66–1.67 (1H, m, 16α-H and 2α-H), 1.70–1.74 (4H, m, 15α-H, 2β-H, 11α-H and 8-H), 2.06 (1H, br d, J=11.6 Hz, 12β-H), 2.11 (3H, s, 21-H), 2.17 (1H, m, 16β-H), 2.45 (1H, dd, J=13.1, 11.2 Hz, 7α-H), 2.55 (1H, dd, J=13.1, 3.8 Hz, 7β-H), 2.56 (1H, t, J=9.3 Hz, 17-H), 3.12 (1H, dd, J=10.4, 6.7 Hz, 5α-H), 4.08 (1H, m, 3β-H); δ_C (125 MHz) 11.4 (C-19), 13.2 (C-18), 21.0 (C-11), 22.7 (C-16), 24.3 (C-15), 28.5 (C-2), 31.0 (C-1), 31.4 (C-21), 34.3 (C-7), 34.9 (C-4), 36.9 (C-8), 37.1 (C-10), 38.8 (C-12), 44.0 (C-13), 45.5 (C-5), 54.1 (C-9), 56.7 (C-14), 63.6 (C-17), 65.8 (C-3), 209.2 (C-20); *m*/*z* (EI) 336 (M^+, 5), 318 ($M-H_2O$, 10), 303 (2), 251 (2), 207 (1); HRMS (EI) found 336.2131, $C_{20}H_{32}O_2S$ requires 336.2123.

4.1.7. *S*-Oxo-3α-hydroxy-6-thia-5α-pregnan-20-one (15). To a solution of 6-thiapregnane **6** (0.009 g, 0.027 mmol) in methanol (1.0 mL) cooled to 0 °C, was added a suspension of Oxone® (0.011 g, 0.018 mmol) in water (0.8 mL). After 5 min, a saturated solution of sodium bisulfite (1.0 mL) was added, the methanol was evaporated and the resulting mixture was extracted with ethyl ether (10 mL). The residue obtained after evaporation of the solvent was purified by preparative TLC (dichloromethane/methanol 20:1) to give sulfoxide **15** (0.0082 g, 87%). Mp 182–183 °C (hexane/ethyl acetate); ν_{max} 3382, 2940, 2863, 1701, 1429, 1359, 1014, 756; δ_H (500 MHz) 0.64 (3H, s, 18-H), 0.95 (3H, s, 19-H), 1.15 (1H, td, J=11.4, 4.1 Hz, 9-H), 1.30 (1H, m, 11β-H), 1.31 (1H, m, 15β-H), 1.39 (1H, m, 14-H), 1.42 (1H, m, 12α-H), 1.50 (1H, m, 1α-H), 1.57 (1H, dd, J=13.1, 4.0 Hz, 1β-H), 1.64 (1H, m, 2β-H), 1.72–1.74 (4H, m, 2α-H, 11α-H, 16α-H and 15α-H), 1.78–1.79 (1H, m, 4β-H and 8-H), 2.05 (1H, br d, J=12.2 Hz, 12β-H), 2.12 (3H, s, 21-H), 2.19 (1H, m, 16β-H), 2.35 (1H, t, J=12.2 Hz, 7α-H), 2.36 (1H, m, 4α-H), 2.55 (1H, t, J=8.9 Hz, 17-H), 2.89 (1H, dd, J=13.2, 3.7 Hz, 5α-H), 3.41 (1H, dd, J=11.6, 2.8 Hz, 7β-H), 4.23 (1H, m, 3β-H); δ_C (125 MHz) 13.1 (C-19), 13.2 (C-18), 20.7 (C-11), 22.7 (C-16), 24.3 (C-15), 27.6 (C-2), 29.1 (C-4), 31.4 (C-21), 32.0 (C-1), 33.5 (C-8), 38.3 (C-12), 38.9 (C-10), 43.9 (C-13), 53.4 (C-9), 55.4 (C-14), 56.4 (C-7), 63.3 (C-17), 64.3 (C-3), 64.7 (C-5), 208.7 (C-20); MS (EI) *m*/*z* (%): 352 (M^+, 0.5), 318 (1), 298 (1), 173 (4), 121 (13); HRMS (EI) found 352.2070, $C_{20}H_{32}O_3S$ requires 352.2072.

4.1.8. *S,S*-Dioxo-3α-hydroxy-6-thia-5α-pregnan-20-one (16). To a solution of 6-thiapregnane **6** (0.0116 g, 0.0416 mmol) in methanol (1.3 mL) cooled to 0 °C, was added a suspension of Oxone® (0.038 g, 0.062 mmol) in water (1.0 mL). The reaction mixture was allowed to reach 25 °C and after 5 h a saturated solution of sodium bisulfite (1.3 mL) was added, the methanol was evaporated, and the residue was extracted with ethyl ether (10 mL). The residue obtained after evaporation of the solvent was purified by preparative TLC (dichloromethane/methanol 20:1) to give the sulfone **16** (0.0114 g, 90%). Mp 187–188 °C (hexane/ethyl acetate); ν_{max} 3491, 2947, 2865, 1699, 1289, 1131, 1012, 903; δ_H (500 MHz) 0.67 (3H, s, 18-H), 1.15 (1H, m, 9-H), 1.16 (3H, s, 19-H), 1.31–1.32 (2H, m, 14-H and 15β-H), 1.43 (2H, m, 11β-H and 12α-H), 1.53 (1H, m, 1α-H), 1.59 (1H, m, 1β-H), 1.67 (1H, m, 15β-H), 1.70 (2H, m, 2α-H and 2β-H), 1.73 (1H, m, 16α-H), 1.77 (1H, m, 11α-H),

1.93 (1H, m, 4β-H), 2.07 (1H, m, 12β-H), 2.12 (3H, s, 21-H), 2.20 (2H, m, 8-H and 16β-H), 2.24 (1H, m, 4α-H), 2.54 (1H, t, J=9.2 Hz, 17-H), 2.66 (1H, t, J=13.9 Hz, 7α-H), 3.08 (1H, dd, J=13.9, 3.4 Hz, 7β-H), 3.24 (1H, dd, J=12.9, 2.6 Hz, 5α-H), 4.28 (1H, m, 3β-H); δ_C (125 MHz) 12.2 (C-19), 13.1 (C-18), 20.8 (C-11), 22.6 (C-16), 23.9 (C-4), 24.1 (C-15), 27.7 (C-2), 31.4 (C-21), 32.9 (C-1), 34.2 (C-8), 38.2 (C-12), 39.4 (C-10), 43.8 (C-13), 52.5 (C-9), 54.8 (C-14), 56.2 (C-7), 61.2 (C-5), 63.2 (C-17), 64.2 (C-3), 208.5 (C-20); *m/z* (EI) 368 (M^+, 0.3), 350 ($M-H_2O$, 0.3), 298 (1), 207 (8), 121 (4), 44 (100); HRMS (EI) found 368.2016, $C_{20}H_{32}O_4S$ requires 368.2021.

4.2. Biological activity assays

4.2.1. Membrane preparation. Whole cerebellum from male Sprague–Dawley rats (200–250 g) was rapidly removed after sacrifice and stored at −80 °C. The material was thawed and 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 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 100 vol of the corresponding ice-cold buffer by centrifugation (15,000*g*, 20 min). The final pellet was suspended in the incubation buffer to a protein concentration of approximately 8 mg/mL.[16]

4.2.2. [^{35}S]-*tert*-Butylbicyclo-phosporothionate ([^{35}S]-TBPS) binding. Binding assays were carried out using a previously described protocol with some modifications.[13] Aliquots (100 μL) of cerebellum membrane preparation were incubated with 10 nM [^{35}S]-TBPS (65.13 Ci/mmol, Perking Elmer Life Science Inc., Boston, MA) in the absence or presence of increasing concentration of the steroids (50–600 nM). The synthetic steroids and allopregnanolone, used as control, were dissolved in DMSO and diluted with the incubation buffer (1:1000; 50 mM Tris–HCl, 200 mM NaCl, pH 7.4) immediately before use; 2 mM picrotoxin was used to determine non-specific binding. Assays were carried out at 22 °C for 2 h in the presence of 5 μM GABA (Sigma–Aldrich Corp.) and terminated by rapid filtration through a glass fiber filter (Number 30, Schleicher & Schuell Inc., Keene, NH). 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 transformation.[17]

4.2.3. [^{3}H]-Flunitrazepam ([^{3}H]-FLU) binding. Aliquots (100 μL) of cerebellum membrane preparation were incubated with 3 nM [^{3}H]-FLU (85.2 Ci/mmol, Perking Elmer Life Science Inc., Boston, MA) in the absence or presence of increasing concentration of the steroids (50–600 nM). Allopregnanolone, was used as a standard and 1 mM diazepam (Roemmers Lab, Buenos Aires) was used as non-specific binding.[18] Incubations were carried out at 4 °C for 90 min in 50 mM Tris–HCl buffer (pH 7.4) in the absence of GABA and terminated by rapid filtration through a glass fiber filter as above.

4.2.4. [^{3}H]-Muscimol ([^{3}H]-Mus) binding. Aliquots (100 μL) of washed cerebellum membrane preparation in Tris–acetate 50 mM buffer pH 6.1 were incubated with 10 nM [^{3}H]-Mus (18.0 Ci/mmol, Perkin Elmer Life Science Inc., Boston, MA) in the absence or presence of increasing concentrations of the steroids (50–600 nM); 1 mM GABA (Sigma–Aldrich Corp.) was used to determine non-specific binding. Incubations were carried out at 4 °C for 60 min in the absence of GABA and terminated by rapid filtration through glass fiber filter as above.

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