Steroids 74 (2009) 256–263

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Synthesis of C3, C5, and C7 pregnane derivatives and their effect on NMDA receptor responses in cultured rat hippocampal neurons

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ARTICLE INFO

Article history:
Received 23 September 2008
Received in revised form 31 October 2008
Accepted 15 November 2008
Available online 24 November 2008

Keywords:
Pregnane derivatives
NMDA receptor
Structure–activity relationship
Patch-clamp recording

ABSTRACT

The synthesis of several novel 5α- and 5β-20-oxo-pregnane derivatives substituted in the position 3 and 7 of the steroid skeleton is described. Activity of synthesized compounds was studied in voltage-clamped cultured rat hippocampal neurons. Substituted derivatives inhibited NMDA-elicited neuronal activity. The relationship between biological activity and structure is discussed.

1. Introduction

Neurosteroids are synthesized in the nervous tissue from cholesterol and/or modified to neuroactive compounds from circulating precursors [1]. They do not act through classic steroid hormone nuclear receptors, but through neuronal membrane receptors for essential transmitters (e.g. γ-amino-butyric acid (GABA) and the *N*-methyl-D-aspartate (NMDA) receptors), connected with ion-channels. Such neurosteroids have been proposed to control neuronal excitability by modulating ligand and/or voltage-gated ion channels [2,3] and this action has been shown to affect a lot of physiological processes (learning [4], aging [5], stress [6], etc.) as well as certain neurological and psychiatric disorders (Alzheimer's disease [7], epilepsy [8], etc.).

Defining a binding site of neurosteroids and the knowledge of the molecular mechanism present a molecular template for design and development of novel therapeutic entities. The molecular mechanism by which neurosteroids affect ligand-gated ion channels is still not clearly understood. In general, neurosteroids influence the frequency of single channel openings and the average channel open duration [9,10]. Similarly, much attention has been devoted to recognition of the corresponding binding sites and some of them were identified for the $GABA_A$ receptor [11], but not for NMDA receptor. The results of current studies indicate that neurosteroids have their specific binding sites, independent of particular agonists or other allosteric modulators [12,13].

The knowledge of NMDA receptor's binding site is not as all-embracing as for the GABA receptor; nevertheless, the effect of neurosteroids on NMDA receptor seems to be mediated by independent binding sites located at the extracellular domain of the NMDA receptor [14,15]. The experiments with chimeric receptors have shown crucial role of an extracellular loop between the third and fourth transmembrane domains of the NR2 subunit in the mechanism of potentiating and inhibitory effects of 20-oxo-pregn-5-en-3β-yl sulfate (PS) and 20-oxo-5β-pregnan-3α-yl sulfate (3α5βS) [15,16].

Without doubt, a lot of other aspects of the receptor structure will influence the nature and extent of the neurosteroid modulation: the stereochemistry of the neurosteroids should be mentioned. Previous structure–activity studies have reported several features which are important for activity of neurosteroids at NMDA receptors: the structure should include a sulfonyloxy group at the position 3 and a 20-oxo group as well [17]. The potentiating effect is maintained if the sulfate group is replaced by another negatively charged group, e.g. hemioxalate, hemisuccinate or hemiglutarate [12,18].

Our research is focused on the synthesis of pregnane derivatives as possible inhibitors of the NMDA receptor. This synthesis

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0039-128X/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.steroids.2008.11.011

is a part of a research project searching for the binding site of the given receptor via medicinal chemistry—exploring the structure–activity-relationship. In the present report, we describe the synthesis of some 3α,7α-derivatives of 5α- and 5β-pregnan-20-one [(3α5α7AcHS (**5**), 3α5α7AcS (**6**), 3α5α7NicS (**11**), 3α5β7AcHS (**17**), 3α5β7AcS (**18**), and 3α5β7NicS (**23**)] that were tested as inhibitors of the NMDA receptor by the patch-clamp technique.

2. Experimental

2.1. Chemistry

2.1.1. General

Melting points were determined on a micro-melting point apparatus Hund/Wetzlar (Germany) and are uncorrected. Optical rotations were measured using an Autopol IV (Rudolf Research Analytical, Flanders, USA), $[\alpha]_D$ values are given in $10^{-1}\,^\circ\,cm^2\,g^{-1}$, IR spectra were recorded on a Bruker IFS 88 spectrometer (wavenumbers in cm^{-1}). Proton NMR spectra were measured on a FT NMR spectrometer Varian UNITY-200 (at 200 MHz) or on a FT NMR spectrometer Bruker AVANCE-400 (at 400 MHz) in $CDCl_3$ with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) and width of multiplets (*W*) are given in Hz. Mass spectra were obtained with spectrometers ZAB-EQ (at 70 eV) or LCQ Classic (Thermo Finnigan). Thin-layer chromatography (TLC) was performed on silica gel (ICN Biochemicals), preparative TLC (PLC) was carried out on 200 mm × 200 mm plates coated with a 0.4 mm thick layer of the same material. For column chromatography, silica gel 60–120 μm was used. Analytical samples were dried over phosphorus pentoxide at 50 °C/100 Pa. Pyridine was dried by distillation over potassium hydroxide and chloroform by distillation over phosphorus pentoxide.

Whenever aqueous solution of citric acid was used, the concentration was always 5%. Aqueous solution of potassium hydrogen carbonate was used as a saturated solution. Before evaporation on a rotary evaporator *in vacuo* (bath temperature 50 °C, pressure 1.5 kPa), solutions of organic solvents were dried over anhydrous sodium sulfate.

2.1.2. Synthesis of 5α-pregnane derivatives

*2.1.2.1. 20-Oxo-5α-pregnane-3α,7α-diol (**1**).* This compound was prepared according to the literature [19].

*2.1.2.2. 20-Oxo-5α-pregnane-3α,7α-diyl diacetate (**2**) and 7α-hydroxy-20-oxo-5α-pregnan-3α-yl acetate (**3**).* To a solution of dihydroxy derivative **1** (100 mg, 0.3 mmol) in pyridine (5 mL), acetic anhydride (0.42 mL, 4.4 mmol) was added and the mixture was heated for 6 h at 50 °C. After standing at room temperature for 2 days, the reaction mixture was poured into ice-water and extracted with ethyl acetate (50 mL). Extract was washed with solution of potassium hydrogen carbonate, water, and dried. Solvent was evaporated and the oily residue purified by plate thin layer chromatography (3 plates) in a mixture of acetone/petroleum ether (1:1) to give diacetate **2** (67 mg, 54%) and monoacetate **3** (9 mg, 8%) as a side product.

Diacetate **2**: m.p. 112–114 °C, $[\alpha]_D$ +21.8 (c 0.363, $CHCl_3$). IR spectrum ($CHCl_3$): 1726 (C=O, acetate); 1702 (C=O, ketone); 1259, 1241, 1030 (C—O). ^{1}H NMR (200 MHz): 0.60 (s, 3H, 3 × H-18); 0.80 (s, 3H, 3 × H-19); 2.05 (s, 3H, C(3)–OAc); 2.08 (s, 3H, C(7)–OAc); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, *J* = 8.8, H-17); 4.92 (q, 1H, *J* = 2.9, H-7); 5.03 (m, 1H, H-3). FAB MS: 399 (12%, M+Na), 359 (8%, M+1−H_2O), 299 (80%, M+1−H_2O, AcOH). $C_{25}H_{38}O_5$: calcd. C, 71.74; H, 9.15. Found. C, 71.98; H, 9.27.

Monoacetate **3**: m.p. 174–178 °C, $[\alpha]_D$ + 30.6 (c 0.513, $CHCl_3$). IR spectrum ($CHCl_3$): 3617 (O—H); 1726 (C=O, acetate); 1702 (C=O, ketone); 1263, 1220 (C—O). ^{1}H NMR (200 MHz): 0.60 s, 3H (3 × H-18); 0.79 (s, 3H, 3 × H-19); 2.05 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.56 (t, 1H, *J* = 8.8, H-17); 3.87 (m, 1H, H-7); 5.03 (quintet, 1H, *J* = 2.9, H-3). FAB MS: 399 (12%, M+Na), 359 (8%, M+1−H_2O), 299 (80%, M+1−H_2O, AcOH). $C_{23}H_{36}O_4$: calcd. C, 73.37; H, 9.64. Found. C, 73.17; H, 9.76.

*2.1.2.3. 3α-Hydroxy-20-oxo-5α-pregnan-7α-yl acetate (**4**).* A solution of diacetate **2** (90 mg, 0.22 mmol) in benzene (10 mL) was treated with a solution of potassium hydroxide (15.4 mg, 0.27 mmol) in methanol (1 mL). After standing overnight at room temperature, the mixture was poured into water and extracted with ethyl acetate (50 mL). The extract was washed with water, dried and the solvent was evaporated. The residue was purified by plate thin layer chromatography (2 plates) in a mixture of acetone/petroleum ether (1:1) to give compound **4** (69 mg, 85%): m.p. 148–150 °C (ether/petroleum ether), $[\alpha]_D$ + 64.5 (c 0.346, $CHCl_3$). IR spectrum ($CHCl_3$): 3616 (O—H); 1717 (C=O, acetate); 1701 (C=O, ketone); 1256 (C—O). ^{1}H NMR (200 MHz): 0.59 (s, 3H, 3 × H-18); 0.78 (s, 3H, 3 × H-19); 2.07 (s, 3H, OAc); 2.11 (s, 3H, 3 × H-21); 2.55 (t, 1H, *J* = 8.8, H-17); 4.06 (quintet, 1H, *J* = 2.4, H-3); 4.91 (q, 1H, *J* = 2.9, H-7). FAB MS: 399 (17%, M+Na), 317 (8%, M+1−AcOH), 299 (9%, M+1−AcOH, H_2O). $C_{23}H_{36}O_4$: calcd. C, 73.37; H, 9.64. Found. C, 73.70; H, 9.99.

*2.1.2.4. 20-Oxo-5α-pregnane-3α,7α-diyl 3-hemisuccinate 7-acetate (**5**, 3α5α7AcHS).* A mixture of compound **4** (100 mg, 0.27 mmol) and succinic anhydride (100 mg, 1 mmol) was dried in vacuo (25 °C, 100 Pa) for 30 min. Dry pyridine (10 mL) and 4-dimethylaminopyridine (20 mg, 0.17 mmol) were added. The mixture was heated for 6 h at 140 °C. Additional succinic anhydride (200 mg, 2 mmol) and 4-dimethylaminopyridine (20 mg, 0.17 mmol) were added and the mixture was heated for 10 h at 140 °C. The reaction mixture was poured into water and extracted with ethyl acetate (50 mL). Aqueous phase was extracted again with ethyl acetate (50 mL), and the collected extracts dried. The solvent was evaporated and the residue crystallized from hot ethyl acetate to give hemisuccinate **5** (71 mg, 35%): m.p. 193–195 °C, $[\alpha]_D$ + 43.3 (c 0.246, $CHCl_3$). IR spectrum ($CHCl_3$): 3516, 3100 broad (COOH); 1717 (C=O, acetate); 1701 (C=O, ketone); 1379 (CH_3); 1257, 1248 (C—O, acetate). ^{1}H NMR (200 MHz): 0.60 (s, 3H, 3 × H-18); 0.80 (s, 3H, 3 × H-19); 2.08 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, *J* = 8.8, H-17); 2.60–2.71 (m, 4H, $OOCCH_2CH_2COO$); 4.92 (m, 1H, H-3); 5.06 (q, 1H, *J* = 2.4, H-7). FAB MS: 499 (100%, M+Na), 417 (13%, M+1−AcOH), 299 (55%, M−AcOH, $C_4H_5O_4$). $C_{27}H_{40}O_7$: calcd. C, 68.04; H, 8.46. Found. C, 67.83; H, 8.46.

*2.1.2.5. 20-Oxo-5α-pregnane-3α,7α-diyl 3-sulfate 7-acetate pyridinium salt (**6**, 3α5α7AcS).* The mixture of compound **4** (200 mg, 0.53 mmol) and a sulfur trioxide pyridine complex (400 mg, 2.5 mmol), dried in vacuo (25 °C, 100 Pa) for 1 h, was dissolved in dry chloroform (10 mL). The reaction mixture was stirred for 4 h at room temperature under argon. After standing overnight at −20 °C, the undissolved sulfur trioxide pyridine complex was filtered off. The solvent was evaporated and the residue was dissolved in absolute methanol (1 mL). The absolute ether (15 mL) was added and the mixture was concentrated to almost one-half. After standing overnight at −20 °C, the crystals were collected and dried in a desiccator (over potassium hydroxide) to afford compound **6** (230 mg, 82%): m.p. 156–160 °C, $[\alpha]_D$ + 29.3 (c 0.288, $CHCl_3$). IR spectrum ($CHCl_3$): 3072 (pyridinium); 1716 (C=O, acetate); 1702 (C=O, ketone); 1258, 1220 (C—O, acetate); 1258 (S–O). ^{1}H NMR (400 MHz): 0.59 (s, 3H, 3 × H-18); 0.80 (s, 3H, 3 × H-19); 2.06 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.56 (t, 1H, *J* = 9.2, H-17); 4.78 (quintet, 1H, *J* = 2.6, H-3); 4.89 (q, 1H, *J* = 2.9, H-7); 7.98 (m, 2H,

H-3 and H-5, pyridinium); 8.48 (tt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, pyridinium); 8.94 (m, 2H, H-2 and H-6, pyridinium). ESI MS: 574 (17%, M+K), 494 (14%, M+K−pyridinium), 478 (21%, M+Na−pyridinium), 412 (15%, M−CH_3CO, pyridinium), 341 (47%, M+Na−$OSO_3C_5H_6N$, CH_3CO). HR-MS (+ESI) calcd. for $C_{28}H_{41}NNaO_7S$ [M^++Na] 558.2496, found 558.2502.

*2.1.2.6. 3α-(tert-Butyldimethylsilyloxy)-20-oxo-5α-pregnan-7α-yl acetate (**7**).* Alcohol **4** (528 mg, 1.4 mmol) and imidazole (570 mg, 8.37 mmol) were dissolved in *N*,*N*-dimethylformamide (11 mL) and the mixture was cooled to 0 °C. Then, *tert*-butyldimethylsilyl chloride was added (528 mg, 3.5 mmol). The reaction mixture was allowed to attain room temperature and stirred. After 2 h, the reaction was diluted with ethyl acetate (150 mL) and washed with solution of citric acid, potassium hydrogen carbonate, and water. The organic layer was dried and evaporated in vacuo. Crystallization from ethyl acetate gave 687 mg (99%) of compound **7**: m.p. 120–121 °C, $[\alpha]_D$ + 29.0 (c 0.369, $CHCl_3$). IR spectrum ($CHCl_3$): 2897 (($CH_3)_2$Si); 1715 (C=O, acetate); 1701 (C=O, ketone); 1472, 1463 (($CH_3)_3$C); 1258 (C—O, acetate); 1053 (C—OSi). ^{1}H NMR (200 MHz): 0.02 (s, 6 H, 6 × ($CH_3)_2$Si); 0.59 (s, 3H, 3 × H-18); 0.76 (s, 3H, 3 × H-19); 0.89 (s, 9 H, ($CH_3)_3$C); 2.03 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, J = 8.7, H-17); 3.97 (quintet, 1H, J = 2.9, H-3); 4.89 (q, 1H, J = 2.4, H-7). ESI MS: 513 (83%, M+Na), 457 (20%, M+Na−($CH_3)_3$C), 353 (42%, M+Na−($CH_3)_3$C($CH_3)_2$SiO, CH_3CO, AcOH). $C_{29}H_{50}O_4Si$: calcd. C, 70.97; H, 10.27. Found. C, 70.85; H, 10.41.

*2.1.2.7. 3α-(tert-Butyldimethylsilyloxy)-7α-hydroxy-5α-pregnan-20-one (**8**).* The method followed that described for compound **4** but using acetate **7** (650 mg, 1.32 mmol) in benzene (50 mL) and potassium hydroxide in ethanol (0.89 M, 60 mL) at 60 °C for 16 h. Compound **8** (527 mg, 88%): m.p. 135–140 °C (ethyl acetate), $[\alpha]_D$ + 63.3 (c 0.232, $CHCl_3$). IR spectrum ($CHCl_3$): 3615 (O—H); 1699 (C=O, ketone); 1472, 1463 (($CH_3)_3$C); 1253 (($CH_3)_2$Si); 1052 (C—OSi). ^{1}H NMR (200 MHz): 0.02 (s, 6 H, ($CH_3)_2$Si); 0.59 (s, 3H, 3 × H-18); 0.88 (s, 12H, 3 × H-19 and ($CH_3)_3$C); 2.11 (s, 3H, 3 × H-21); 2.56 (t, 1H, J = 8.8, H-17); 3.83 (m, 1H, H-7); 3.98 (m, 1H, H-3). ESI MS: 919 (100%, 2M+Na), 471 (50%, M+Na). $C_{27}H_{48}O_3Si$: calcd. C, 72.26; H, 10.78. Found. C, 72.20; H, 10.91.

*2.1.2.8. 3α-(tert-Butyldimethylsilyloxy)-20-oxo-5α-pregnan-7α-yl nicotinate (**9**).* Compound **8** (450 mg, 1.0 mmol) and 4-dimethylaminopyridine (10 mg, 0.09 mmol) were dissolved in pyridine (15 mL) and the solution was cooled to 0 °C. Nicotinoyl chloride hydrochloride (900 mg, 5.0 mmol) was slowly added to a stirred mixture in small portions. The reaction mixture was stirred at room temperature for 3 h. Then, it was poured into water (50 mL) and, after standing overnight at −20 °C, the precipitate was separated by suction and subsequently dried in a desiccator (over potassium hydroxide) overnight to yield compound **9** (498 mg, 89%): m.p. 147–151 °C, $[\alpha]_D$ + 14.5 (c 0.391, $CHCl_3$). IR spectrum ($CHCl_3$): 2897 (($CH_3)_2$Si); 1714 (C=O, nicotinate); 1703 (C=O, ketone); 1286 (C—O, nicotinate); 1053 (C—OSi). ^{1}H NMR (400 MHz): 0.05 (s, 6 H, ($CH_3)_2$Si); 0.64 (s, 3H, 3 × H-18); 0.72 (s, 9 H, ($CH_3)_3$C); 0.83 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.51 (t, 1H, J = 8.8, H-17); 3.96 (quintet, J = 2.4, 1H, H-3); 5.22 (q, 1H, J = 2.6, H-7); 7.40 (ddd, 1H, J_1 = 7.8, J_2 = 4.8, J_3 = 0.7, H-5, nicotinate); 8.29 (dt, 1H, J_1 = 8, J_2 = 2, H-4, nicotinate); 8.79 (dd, 1H, J_1 = 4.8, J_2 = 1.8, H-6, nicotinate); 9.25 (m, 1H, H-2, nicotinate). FAB MS: 554 (36%, M+1), 496 (8%, M−($CH_3)_3$C), 299 (4%, M−($CH_3)_3$C($CH_3)_2$SiO, $OCOC_5H_4N$), 255 (79%, M−($CH_3)_3$($CH_3)_2$SiO, $OCOC_5H_4N$, CH_3CO). $C_{33}H_{51}NO_4Si$: calcd. C, 71.56; H, 9.28; N, 2.53. Found. C, 71.40; H, 9.41; N, 2.25.

*2.1.2.9. 3α-Hydroxy-20-oxo-5α-pregnan-7α-yl nicotinate (**10**).* Compound **9** (120 mg, 0.21 mmol) was treated with a methanolic solution of *p*-toluenesulfonic acid (0.005 M, 72 mL). After standing at room temperature for 10 days, the mixture was neutralized with 10% potassium carbonate solution, extracted with ethyl acetate (100 mL), organic layer was washed with water and dried. The solvent was evaporated and the crude product purified by plate thin layer chromatography (3 plates) in a mixture of petroleum ether/acetone (8:2) to give **10** (83 mg, 87%): m.p. 183–187 °C (acetone/heptane), $[\alpha]_D$ + 15.0 (c 0.169, $CHCl_3$). IR spectrum ($CHCl_3$): 3615 (O—H); 1715 (C=O, nicotinate); 1703 (C=O, ketone); 1287 (C—O, nicotinate); 1002 (C—OH). ^{1}H NMR (200 MHz): 0.72 (s, 3H, 3 × H-18); 0.86 (s, 3H, 3 × H-19); 2.25 (s, 3H, 3 × H-21); 2.53 (t, 1H, J = 8.8, H-17); 4.05 (quintet, 1H, J = 2.4, H-3); 5.25 (q, 1H, J = 2.8, H-7); 7.44 (m, 1H, H-5, nicotinate); 8.30 (dt, 1H, J_1 = 7.8, J_2 = 1.9, H-4, nicotinate); 8.78 (dd, 1H, J_1 = 4.8, J_2 = 1.4, H-6, nicotinate); 9.26 (m, 1H, H-2, nicotinate). ESI MS: 901 (100%, 2M+Na), 462 (93%, M+Na), 440 (24%). $C_{27}H_{37}NO_4$: calcd. C, 73.77; H, 8.48; N, 3.19. Found. C, 73.66; H, 8.67; N, 3.04.

*2.1.2.10. 20-Oxo-5α-pregnane-3α,7α-diyl 3-sulfate 7-nicotinate pyridinium salt (**11**, 3α5α7NicS).* The method followed that described for compound **6** but using alcohol **10** (60 mg, 0.13 mmol) and a sulfur trioxide pyridine complex (120 mg, 0.75 mmol) in dry chloroform (10 mL) afforded compound **11** (71 mg, 87%) as a foam: $[\alpha]_D$ + 6.0 (c 0.204, $CHCl_3$). IR spectrum ($CHCl_3$): 3457, 3139 (pyridinium); 1715 (C=O, nicotinate); 1702 (C=O, ketone); 1289 (C—O, nicotinate); 1243, 1046 (SO_3). ^{1}H NMR (400 MHz): 0.63 (s, 3H, 3 × H-18); 0.87 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.54 (t, 1H, J = 8.7, H-17); 4.79 (quintet, J = 2.7, 1H, H-3); 5.25 (q, 1H, J = 2.5, H-7); 7.74 (dd, 1H, J_1 = 7.8, J_2 = 5.5, H-5, nicotinate); 7.83 (m, 2H, H-3 and H-5, pyridinium); 8.30 (tt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, pyridinium); 8.62 (dt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, nicotinate); 8.84 (m, 2H, H-2 and H-6, pyridinium); 8.93 (m, 1H, H-6, nicotinate); 9.29 (m, 1H, H-2, nicotinate). FAB MS: 554 (3%, M−CH_3CO), 440 (0.5%, M−2 × C_5H_6N), 422 (10%, M+1−$OSO_3C_5H_6N$). HR-MS (+ESI) calcd. for $C_{32}H_{42}N_2NaO_7S$ [M^++Na] 621.2605, found 621.2609.

*2.1.2.11. 3α-Hydroxy-5α-pregnan-20-one (**12**).* This compound was prepared according to the literature [20].

*2.1.2.12. 20-Oxo-5α-pregnan-3α-yl hemisuccinate (**13**, 3α5αHS).* The method followed that described for compound **5** but using alcohol **12** (150 mg, 0.47 mmol) and succinic anhydride (150 mg, 1.5 mmol) in dry pyridine (15 mL) at reflux. After 7 h the reaction was completed. Compound **13** (105 mg, 53%): m.p. 76–79 °C (toluene), $[\alpha]_D$ + 77.7 (c 0.229, $CHCl_3$). IR spectrum ($CHCl_3$): 1727 (C=O, ester); 1716 (C=O, COOH dimer); 1700 (C=O, ketone); 1188 (C—O, ester). ^{1}H NMR (400 MHz): 0.60 (s, 3H, 3 × H-18); 0.79 (s, 3H, 3 × H-19); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, J = 9, H-17); 2.64–2.69 (m, 4H, $OOCCH_2CH_2COO$); 5.05 (m, 1H, H-3). ESI MS: 457 (3.4% M+K), 441 (100% M+Na), 418 (7.78%, M). HR-MS (+ESI) calcd. for $C_{25}H_{39}O_5$ [M^++1] 419.2792, found 419.2790. $C_{25}H_{38}O_5$: calcd., 71.74; H, 9.15. Found. C, 71.59; H, 9.32.

*2.1.2.13. 20-Oxo-5α-pregnan-3α-yl sulfate pyridinium salt (**14**, 3α5αS).* The method followed that described for compound **6** but using alcohol **12** (200 mg, 0.6 mmol) and a sulfur trioxide pyridine complex (400 mg, 2.5 mmol) in dry chloroform (5 mL). Compound **14** (254 mg, 85%): m.p. 181–183 °C (methanol/ether) (lit. 184 °C [21]), $[\alpha]_D$ + 69.0 (c 0.21) (lit. + 70.0 [21]). IR spectrum ($CHCl_3$): 3139 (pyridinium); 1699 (C=O); 1261, 1253, 1237, 1194, 1171 (SO_3 and pyridinium). ^{1}H NMR (400 MHz): 0.58 (s, 3H, 3 × H-18); 0.91 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.52 (t, 1H, J = 8.7, H-17); 4.45 (m, W = 30 Hz, H-3); 8.02 (m, 2H, H-3 and H-5, pyridinium); 8.50

(tt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, pyridinium); 9.00 (m, 2H, H-2 and H-6, pyridinium). FAB MS: 478 (11%, M+1). HR-MS (+ESI) calcd. for $C_{26}H_{39}NNaO_5S$ [M^++Na] 500.2441, found 500.2437.

2.1.3. Synthesis of 5β-pregnane derivatives

*2.1.3.1. 20-Oxo-5β-pregnane-3α,7α-diyl diacetate (**15**).* This compound was prepared according to the literature [22].

*2.1.3.2. 3α-Hydroxy-20-oxo-5β-pregnan-7α-yl acetate (**16**).* A solution of diacetate **15** (100 mg, 0.24 mmol) in methanol (8 mL) was treated with a solution of potassium hydrogen carbonate in water (0.7 mL, 0.2 M) at 70 °C. After 5 h, the mixture was poured into water and extracted with ethyl acetate (70 mL). The organic layer was washed with water, dried and the solvents were evaporated in vacuo. Crystallization from hot ether gave compound **16** (40 mg, 45%): m.p. 143–145 °C, $[\alpha]_D$ + 40.3 (c 0.303, $CHCl_3$). IR spectrum ($CHCl_3$): 3610, 3527 (O—H); 1726 (C=O, acetate); 1703 (C=O, ketone); 1252 (C—O, acetate); 1047 (C—O). ^{1}H NMR (200 MHz): 0.61 (s, 3H, 3 × H-18); 0.93 (s, 3H, 3 × H-19); 2.06 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.55 (t, 1H, J = 8.8, H-17); 3.52 (m, 1H, W = 32.7, H-3); 4.89 (q, 1H, J = 2.4, H-7). FAB MS: 377 (5%, M+1), 317 (12%, M+1−AcOH), 299 (40%, M+1−AcOH, H_2O), 283 (32%, M−AcOH, H_2O, CH_3), 255 (17%, M−AcOH, H_2O, CH_3CO), 159 (30%), 145 (34%), 131 (33%), 119 (37%). $C_{23}H_{36}O_4$: calcd. C, 73.37; H, 9.64. Found. C, 73.49; H, 9.93.

*2.1.3.3. 20-Oxo-5β-pregnane-3α,7α-diyl 3-hemisuccinate 7-acetate (**17**, 3α5β7AcHS).* The method followed that described for compound **5** but using alcohol **16** (100 mg, 0.27 mmol), succinic anhydride (200 mg, 2 mmol) and 4-dimethylaminopyridine (20 mg, 0.17 mmol) in dry pyridine (20 mL) at reflux. After 4 h the reaction was completed. The residue purified by plate thin layer chromatography (3 plates) in a mixture of petroleum ether/acetone (9:1) to give compound **17** (44 mg, 35%): m.p. 131–134 °C, $[\alpha]_D$ + 50.3 (c 0.218, $CHCl_3$). IR spectrum ($CHCl_3$): 3517, 2676 broad (COOH); 1756 (C=O, COOH); 1726 (C=O, acetate); 1720 (C=O, hemisuccinate); 1703 (C=O, ketone); 1252 (C—O, acetate); 1173 (C—O, hemisuccinate). ^{1}H NMR (200 MHz): 0.61 (s, 3H, 3 × H-18); 0.94 (s, 3H, 3 × H-19); 2.06 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.54–2.69 (m, 5 H, H-17 and $OOCCH_2CH_2COO$); 4.62 (m, 1H, W = 31.8, H-3); 4.89 (q, 1H, J = 2.7, H-7). FAB MS: 499 (69%, M+Na), 417 (18%, M+1−AcOH), 299 (28%, M−AcOH, $C_4H_5O_4$). HR-MS (+ESI) calcd. for $C_{27}H_{40}NaO_7$ [M^++Na] 499.2666, found 499.2664. $C_{27}H_{40}O_7$: calcd. C, 68.04; H, 8.46. Found. C, 68.40; H, 8.60.

*2.1.3.4. 20-Oxo-5β-pregnan-3α,7α-diyl 3-sulfate 7-acetate pyridinium salt (**18**, 3α5β7AcS).* The method followed that described for compound **6** but using alcohol **16** (110 mg, 0.3 mmol) and a sulfur trioxide pyridine complex (220 mg, 1.4 mmol) in dry chloroform (4 mL). The crystals of **18** were collected and dried in a desiccator (over potassium hydroxide) for 5 days (155 mg, 99%): m.p. 155–158 °C, $[\alpha]_D$ + 45.7 (c 0.285, $CHCl_3$). IR spectrum ($CHCl_3$): 3140 (pyridinium); 1725 (C=O, acetate); 1702 (C=O, ketone); 1253 (C—O); 1363, 1171, 960 (O–SO_3). ^{1}H NMR (200 MHz): 0.60 (s, 3H, 3 × H-18); 0.92 (s, 3H, 3 × H-19); 2.04 (s, 3H, OAc); 2.56 (t, 1H, J = 8.7, H-17); 4.34 (m, 1H, W = 31, H-3); 4.88 (q, 1H, J = 2.9, H-7); 7.97 (m, 2H, H-3 and H-5, pyridinium); 8.48 (tt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, pyridinium); 8.95 (m, 2H, H-2 and H-6, pyridinium). EI MS: 558 (0.5%, M+Na), 455 (0.5%, M−pyridinium), 256 (0.5%, M−pyridinium, OSO_3, CH_3CO, AcOH), 230 (3%), 80 (100%). HR-MS (+ESI) calcd. for $C_{28}H_{41}NNaO_7S$ [M^++Na] 558.2496, found 558.2501.

*2.1.3.5. 3α-(tert-Butyldimethylsilyloxy)-20-oxo-5β-pregnan-7α-yl acetate (**19**).* The method followed that described for compound **7** but using hydroxy derivative **16** (70 mg, 0.18 mmol), imidazole (76 mg, 1.1 mmol) and *tert*-butyldimethylsilyl chloride (84 mg, 0.56 mmol) in *N*,*N*-dimethylformamide (1.4 mL). After 1 h the reaction was completed. Compound **19** (50 mg, 55%): m.p. 110–112 °C (ethyl acetate), $[\alpha]_D$ + 30.5 (c 0.333, $CHCl_3$). IR spectrum ($CHCl_3$): 2955, 2906 ((CH_3)$_3$C); 1725 (C=O, acetate); 1702 (C=O, ketone); 1254, 1021 (C—O, acetate); 1090, 1076 (C—OSi); 853, 837 ((CH_3)$_2$Si). ^{1}H NMR (200 MHz): 0.05 (s, 6 H, (CH_3)$_2$Si); 0.6 (s, 3H, 3 × H-18); 0.88 (s, 9 H, (CH_3)$_3$C); 0.91 (s, 3H, 3 × H-19); 2.04 (s, 3H, OAc); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, J = 8.7, H-17); 3.45 (m, 1H, W = 32, H-3); 3.87 (q, 1H, J = 2.4, H-7). FAB MS: 433 (2%, M−57, (CH_3)$_3$C), 373 (18%, M−(CH_3)$_3$C, AcOH), 299 (100%, M−(CH_3)$_3$(CH_3)$_2$Si, AcOH), 255 (12%, M−(CH_3)$_3$(CH_3)$_2$SiO, AcOH, CH_3CO). $C_{29}H_{50}O_4Si$: calcd. C, 70.97; H, 10.27. Found. C, 70.65; H, 10.40.

*2.1.3.6. 3α-(tert-Butyldimethylsilyloxy)-7α-hydroxy-5β-pregnan-20-one (**20**).* The method followed that described for compound **4** but using acetate **19** (40 mg, 0.08 mmol) and solution of potassium hydroxide in ethanol (0.89 M, 4 mL) in benzene (5 mL) at reflux. After 3 h the reaction was completed. The residue was purified by plate thin layer chromatography (2 plates) in a mixture of petroleum ether/acetone (8:2) to give alcohol **20** (20 mg, 55%): m.p. 131–136 °C, $[\alpha]_D$ + 47.6 (c 0.29, $CHCl_3$). IR spectrum ($CHCl_3$): 3621 (O—H); 1699 (C=O); 1472, 1463, 1385 ((CH_3)$_3$C); 1361 (Ac, (CH_3)$_3$C); 1254 ((CH_3)$_2$Si); 1098,1082 (C—OSi). ^{1}H NMR (200 MHz): 0.05 (s, 6 H, (CH_3)$_2$Si); 0.60 (s, 3H, 3 × H-18); 0.88 (s, 12H, 3 × H-19 and (CH_3)$_3$C); 2.12 (s, 3H, 3 × H-21); 2.53 (t, 1H, J = 9, H-17); 3.41 (m, 1H, W = 32.7, H-3); 3.86 (m, 1H, H-7). FAB MS: 449 (11%, M+1), 317 (10%, M−(CH_3)$_3$(CH_3)$_2$SiO), 299 (26%, M+1−(CH_3)$_3$(CH_3)$_2$SiO, H_2O), 283 (13%, M+1−(CH_3)$_3$(CH_3)$_2$SiO, H_2O, O), 255 (14%, M−(CH_3)$_3$(CH_3)$_2$SiO, H_2O, CH_3CO). $C_{27}H_{48}O_3Si$: calcd. C, 72.26; H, 10.78. Found. C, 72.20; H, 10.84.

*2.1.3.7. 3α-(tert-Butyldimethylsilyloxy)-20-oxo-5β-pregnan-7α-yl nicotinate (**21**).* The method followed that described for compound **9** but using alcohol **20** (240 mg, 0.53 mmol), 4-dimethylaminopyridine (10 mg, 0.09 mmol) and nicotinoyl chloride hydrochloride (720 mg, 4.0 mmol) in pyridine (10 mL). After 10 h the reaction was completed. The crude product was purified by plate thin layer chromatography (6 plates) in a mixture of petroleum ether/acetone (9:1) to give compound **21** (208 mg, 70%): m.p. 57–59 °C, $[\alpha]_D$ + 54.9 (c 0.387, $CHCl_3$). IR spectrum ($CHCl_3$): 2907 (CH_3, (CH_3)$_3$(CH_3)$_2$SiO); 1714 (C=O, nicotinate); 1703 (C=O, ketone); 1286, 1108 (C—O, nicotinate); 1092 (C—OSi). ^{1}H NMR (400 MHz): 0.05 (s, 6 H, (CH_3)$_2$Si); 0.63 (s, 3H, 3 × H-18); 0.77 (s, 9 H, (CH_3)$_3$C); 0.96 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.52 (t, 1H, J = 9.1, H-17); 3.43 (m, 1H, W = 32, H-3); 5.21 (q, 1H, J = 2.8, H-7); 7.44 (ddd, 1H, J_1 = 7.8, J_2 = 7.8, J_3 = 0.7, H-5, nicotinate); 8.30 (dt, 1H, J_1 = 8.3, J_2 = 2, H-4, nicotinate); 8.78 (dd, 1H, J_1 = 4.8, J_2 = 1.7, H-6, nicotinate); 9.27 (dd, 1H, J_1 = 2, J_2 = 0.7, H-2, nicotinate). FAB MS: 579 (6%, M+Na), 554 (22%, M+1), 496 (7%, M−(CH_3)$_3$C), 299 (4%, M−(CH_3)$_3$(CH_3)$_2$SiO, $OCOC_6H_4N$), 255 (79%, M−(CH_3)$_3$C(CH_3)$_2$SiO, $OCOC_6H_4N$, CH_3CO). $C_{33}H_{51}NO_4Si$: calcd. C, 71.56; H, 9.28; N, 2.53. Found. C, 71.33; H, 9.37; N, 2.31.

*2.1.3.8. 3α-Hydroxy-20-oxo-5β-pregnan-7α-yl nicotinate (**22**).* A solution of protected compound **21** (100 mg, 0.18 mmol) in freshly distilled THF (10 mL) was cooled to 0 °C and solution of tetrabutylammonium fluoride (1 M in THF, 0.3 mL, 0.3 mmol) was added. The mixture was stirred at room temperature. After 2 days, further tetrabutylammonium fluoride solution (1 M in THF, 0.1 mL, 0.1 mmol) was added and the mixture was stirred for another 3 days. The mixture was diluted with ethyl acetate (100 mL), organic layer was washed solution of citric acid, potassium hydrogen carbonate, water, and dried. The solvent was evaporated and the crude product purified by plate thin layer chromatography (2 plates) in a mixture of petroleum ether/acetone (9:1) to give **22** (58 mg, 74%): m.p. 70–73 °C (acetone/heptane), $[\alpha]_D$ + 29.2 (c 0.279, $CHCl_3$). IR

spectrum ($CHCl_3$): 3613; 3451 (O—H); 1715 (C=O, nicotinate); 1705 (C=O, ketone); 1287 (C—O, nicotinate); 1034, 1026 (C—OH); 1592, 1421 (nicotinate). ^{1}H NMR (400 MHz): 0.64 (s, 3H, 3 × H-18); 0.98 (s, 3H, 3 × H-19); 2.12 (s, 3H, 3 × H-21); 2.54 (t, 1H, J = 9.2, H-17); 3.49 (m, 1H, W = 31, H-3); 5.20 (q, 1H, J = 2.7, H-7); 7.45 (ddd, 1H, J_1 = 7.8, J_2 = 4.8, J_3 = 0.7, H-5, nicotinate); 8.31 (dt, 1H, J_1 = 7.8, J_2 = 1.7, H-4, nicotinate); 8.80 (dd, 1H, J_1 = 4.8, J_2 = 1.7, H-6, nicotinate); 9.26 (dd, 1H, J_1 = 2, J_2 = 0.7, H-2, nicotinate). FAB MS: 440 (12%, M+1), 145 (7%), 124 (100%), 105 (35%). $C_{27}H_{37}NO_4$: calcd. C, 73.77; H, 8.48; N, 3.19. Found. C, 73.67; H, 8.80; N, 2.98.

*2.1.3.9. 20-Oxo-5β-pregnane-3α,7α-diyl 3-sulfate 7-nicotinate pyridinium salt (**23**, 3α5β7NicS).* The method followed that described for compound **6** but using alcohol **22** (45 mg, 0.1 mmol) and a sulfur trioxide pyridine complex (90 mg, 0.56 mmol) in freshly distilled chloroform (5 mL). After 4 h the reaction was completed to afford **23** (40 mg, 74%) as a white foam: $[\alpha]_D$ + 42.0 (c 0.321, $CHCl_3$). IR spectrum ($CHCl_3$): 3139, 2652 (pyridinium); 1714 (C=O, nicotinate); 1703 (C=O, ketone); 1287 (C—O, nicotinate); 1175, 978, 958 (O–SO_3). ^{1}H NMR (400 MHz): 0.63 (s, 3H, 3 × H-18); 0.98 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.56 (t, 1H, J = 8.7, H-17); 4.32 (m, 1H, W = 32, H-3); 5.26 (m, 1H, H-7); 7.74 (dd, 1H, J_1 = 7.6, J_2 = 5.3, H-5, nicotinate); 7.82 (m, 2H, H-3 and H-5, pyridinium); 8.33 (t, 2H, J = 7.8, H-4, pyridinium); 8.60 (d, 1H, J = 7.6, H-4, nicotinate); 8.88 (m, 2H, H-2 and H-6, pyridinium); 8.93 (d, 1H, J = 5, H-6, nicotinate); 9.33 (m, 1H, H-2, nicotinate). FAB MS: 564 (10%), 542 (20%), 519 (12%, M+1−pyridinium), 444 (18%), 422 (10%, M+1−$OSO_3C_5H_6N$). HR-MS (+ESI) calcd. for $C_{32}H_{42}N_2NaO_7S$ [M^++Na] 621.2605, found 621.2608.

*2.1.3.10. 3α-Hydroxy-5β-pregnan-20-one (**24**).* This compound was prepared according to the literature [20].

*2.1.3.11. 20-Oxo-5β-pregnan-3α-yl hemisuccinate (**25**, 3α5βHS).* The method followed that described for compound **5** but using alcohol **24** (100 mg, 0.27 mmol), succinic anhydride (250 mg, 2.5 mmol) and 4-dimethylaminopyridine (25 mg, 0.21 mmol) in dry pyridine (10 mL) at reflux. After 4 h the reaction was completed. Compound **25** (63 mg, 48%): m.p. 156–159 °C (ether), $[\alpha]_D$ + 101.6 (c 0.309, $CHCl_3$). IR spectrum ($CHCl_3$): 1726 (C=O, ester); 1717 (C=O, COOH-dimer); 1701 (C=O, ketone); 1176 (C—O, ester); 1175, 978, 958 (O–SO_3). ^{1}H NMR (400 MHz): 0.60 (s, 3H, 3 × H-18); 0.93 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.53 (t, 3H, J = 9, H-17); 2.56–2.69 (m, 4H, $OOCCH_2CH_2COO$); 4.76 (m, 1H, W = 32, H-3). EI MS: 418 (1.5%, M), 400 (12%, M−H_2O), 300 (100%, M+1−HOOC-CH_2CH_2–COO), 285 (19%, M−HOOC–CH_2CH_2–COO, O), 267 (15%, M−HOOC–CH_2CH_2–COO, $COCH_3$). HR-MS (+ESI) calcd. for $C_{25}H_{38}NaO_5$ [M^++Na] 441.2611, found 441.2609. $C_{25}H_{38}O_5$: calcd. C, 71.74; H, 9.15. Found. C, 71.50; H, 9.36.

*2.1.3.12. 20-Oxo-5β-pregnan-3α-yl sulfate pyridinium salt (**26**, 3α5βS).* The method followed that described for compound **6** but using alcohol **24** (200 mg, 0.6 mmol) and a sulfur trioxide pyridine complex (400 mg, 2.5 mmol) in dry chloroform (5 mL). After 4 h the reaction was completed. Compound **26** (240 mg, 80%): m.p. 173–175 °C (methanol/ether) (lit. 170 °C [21]), $[\alpha]_D$ + 100.0 (c 0.28, $CHCl_3$) (lit. + 103.0 [21]). IR spectrum ($CHCl_3$): 3139 (pyridinium); 1698 (C=O, ketone); 1270, 1257, 1236, 1179, 1166 (SO_3 and pyridinium). ^{1}H NMR (400 MHz): 0.60 (s, 3H, 3 × H-18); 0.78 (s, 3H, 3 × H-19); 2.11 (s, 3H, 3 × H-21); 2.52 (t, 1H, J = 8.7, H-17); 4.45 (m, 1H, W = 30); 8.03 (m, 2H, H-3 and H-5, pyridinium); 8.50 (tt, 1H, J_1 = 7.8, J_2 = 1.5, H-4, pyridinium); 9.00 (m, 2H, H-2 and H-6, pyridinium). FAB MS: 478 (22%, M+1), 440 (22%, M+1−C_5H_6N), 301 (17%, M+1−$OSO_3C_5H_6N$). HR-MS (+ESI) calcd. for $C_{26}H_{39}NNaO_5S$ [M^++Na] 500.2441, found 500.2436.

2.2. Biological assays

2.2.1. Cell culture

Primary dissociated hippocampal cultures were prepared from 1- to 2-day-old postnatal rats. Animals were decapitated and the hippocampi dissected. Trypsin digestion, followed by mechanical dissociation, was used to prepare cell suspension. Single cells were plated at a density of 500,000 cells/cm^2 on 31 mm or 12 mm polylysine-coated glass cover slips. Neuronal cultures were maintained in Neurobasal™-A (Invitrogen, Carlsbad, USA) medium supplemented with glutamine (0.5 mM) and B-27 Serum-Free Supplement (Invitrogen) at 37 °C and 5% CO_2.

2.2.2. Electrophysiology

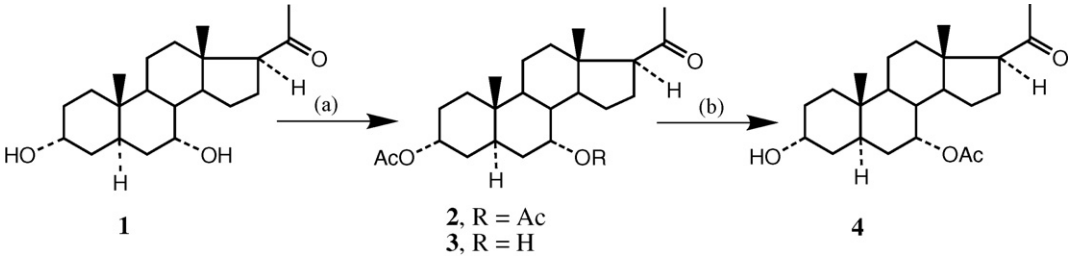
For the electrophysiological experiments, neurons cultured for 5–10 days were used. Whole-cell voltage-clamp recordings were made with a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Inc., Foster City, USA) after capacitance and series resistance (<10 MΩ) compensation of 80–90%. Agonist-induced responses were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, USA), digitally sampled at 5 kHz and analyzed using pClamp software version 9 (Axon Instruments). Patch pipettes (3–6 MΩ) pulled from borosilicate glass were filled with Cs^+-based intracellular solution containing (in mM): 125 gluconic acid, 15 CsCl, 5 EGTA, 10 HEPES, 3 $MgCl_2$, 0.5 $CaCl_2$, and 2 ATP-Mg salt (pH-adjusted to 7.2 with CsOH). Extracellular solution (ECS) contained (in mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 0.2 EDTA and 0.7 $CaCl_2$ (pH-adjusted to 7.3 with NaOH). Glycine (10 μM) and TTX (0.5 μM) were present in the control and test solutions. Steroids were dissolved in dimethyl sulfoxide (DMSO) to make a fresh stock solution (20 mM) before each experiment. The same concentration of DMSO was maintained in all extracellular solutions. A microprocessor-controlled multibarrel fast perfusion system, with a time constant of solution exchange around cells of ~10 ms, was used to apply test and control solutions.

3. Results and discussion

3.1. Chemistry

The starting compound for the synthesis of 5α-derivatives, diol **1**, was prepared from protected (20R)-pregn-5-ene-3β,20-diol [19]. An oxygen-containing substituent was incorporated into the structure by allylic oxidation and subsequently, the 5(6)-double bond was reduced by catalytic hydrogenation to afford the desired 5α-isomer **1**. Acetylation afforded diacetate **2** and by selective hydrolysis of less stable acetate group, 7-monoacetate **4**, the starting compound for the synthesis of all derivatives of the 5α-serie was obtained in the yield of 85% (Scheme 1). Its structure was confirmed by comparison of 3β-H and 7β-H chemical shifts in ^{1}H NMR spectrum.

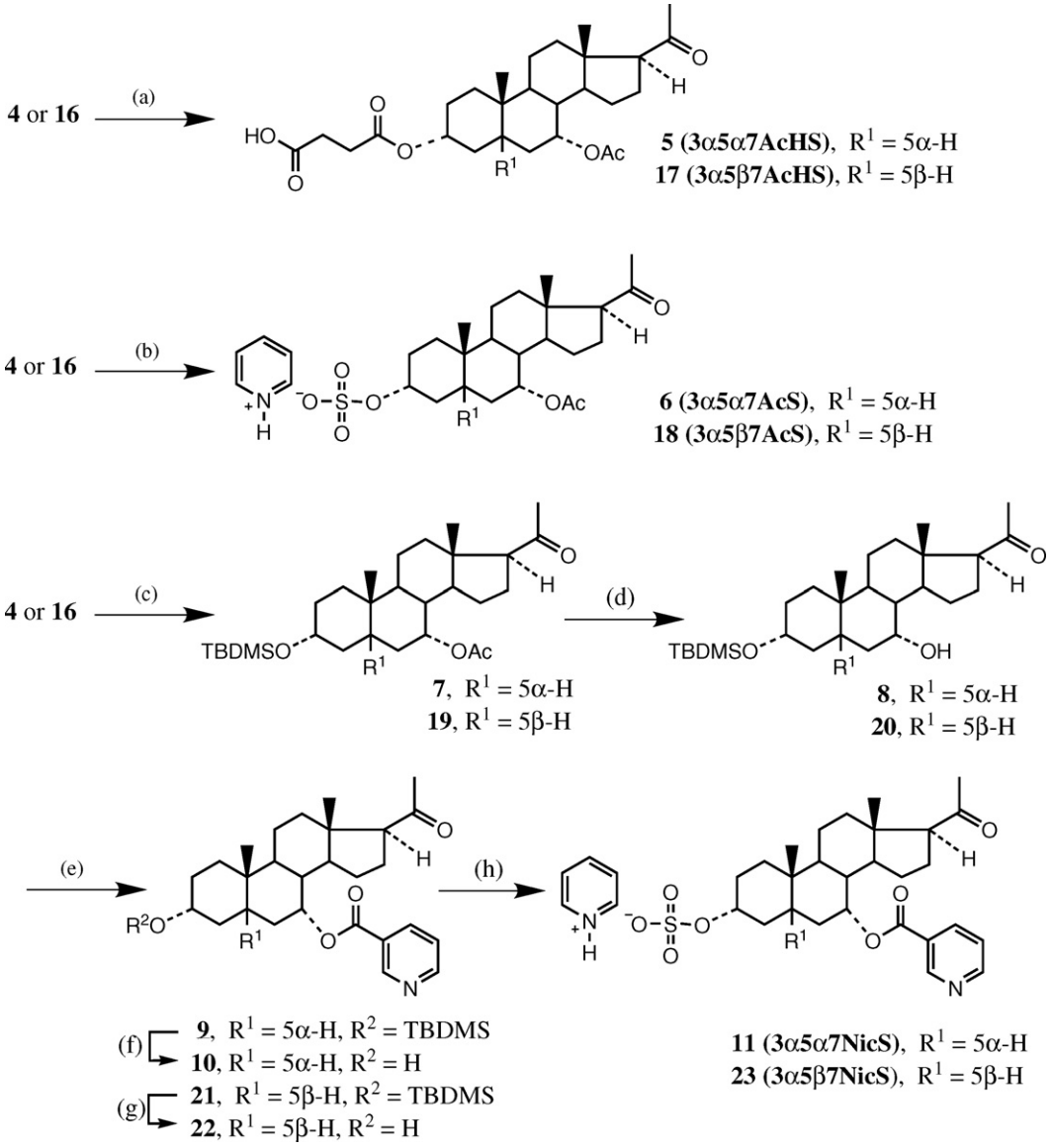
Treatment of monoacetate **4** with succinic anhydride in pyridine under catalysis by 4-dimethylaminopyridine (DMAP) gave hemisuccinate **5** in the yield of 35% (Scheme 2). 3-Sulfate **6**, the next derivative with a polar substituent in the position 3, was obtained by treatment of monoacetate **4** with a sulfur trioxide pyridine complex in chloroform. Preservation of 7-acetate group was confirmed by ^{1}H NMR spectrum. The synthesis of compound **10** (Scheme 2), derivative with a nicotinate group in the position 7, involved firstly protecting of a 3-hydroxy group as a *tert*-butyldimethylsilyl derivative by the reaction of monoacetate **4** with *tert*-butyldimethylsilyl chloride and subsequently selective hydrolysis of acetate protecting group by treating with methanolic solution of potassium hydroxide. Secondly, the free 7-hydroxy group was converted into nicotinate ester and finally, the *tert*-butyldimethylsilyl group of compound



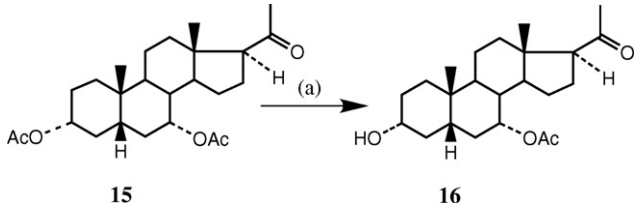
Scheme 1. Synthesis of compounds **2**–**4**. Reaction conditions: (a) Ac_2O, pyridine, 50 °C; (b) KOH, MeOH, benzene.

9 was selectively cleaved by solution of *p*-toluenesulfonic acid in methanol. Using a solution of tetrabutylammonium fluoride, the reagent comfortable for cleavage of the *tert*-butyldimethylsilyl derivatives was not successful, because lead to cleavage both *tert*-butyldimethylsilyl group and nicotinate group. On the other hand, this reagent was successfully used in 5β-serie to afford desired compound **22**. The structure of compound **10** was confirmed by chemical shifts in ^{1}H NMR spectrum. Compound **10** was converted into desired sulfate pyridinium salt **11** in the total yield of 59% from compound **4** (Scheme 2).

For the synthesis of 5β-derivatives, the chenodeoxycholic acid was used, because it contains the required 5β-configuration and suitable substituents in positions 3 and 7. Diacetate **15** was prepared by degradation [22] of a part (carbons 22-24) of the side chain. All



Scheme 2. Synthesis of compounds **5**–**11** and **17**–**23**. Reaction conditions: (a) succinic anhydride, pyridine, 4-dimethylaminopyridine, 140 °C; (b) sulfur trioxide pyridine complex, $CHCl_3$; (c) *tert*-butyldimethylsilyl chloride, imidazol, DMF; (d) KOH, EtOH, benzene, 60 °C; (e) nicotinoyl chloride hydrochloride, 4-dimethylaminopyridine, pyridine; (f) *p*-toluenesulfonic acid, MeOH, rt; (g) tetrabutylammonium fluoride solution, THF, rt; (h) sulfur trioxide pyridine complex, $CHCl_3$.



Scheme 3. Synthesis of compound **16**. Reaction conditions: (a) $NaHCO_3$, H_2O, MeOH, 70 °C.

target compounds of 5β-serie were prepared analogously to the 5α-serie. Partial hydrolysis of diacetate **15** gave 7-acetate **16** (Scheme 3). Hemisuccinate **17** and pyridinium salt of sulfate **18** were prepared in the yield of 35% and 99%, respectively. Compound **23** was prepared in the total yield of 12% from compound **16**. The structures of all derivatives in both series were confirmed by ^1H NMR spectra.

For the synthesis of hemisuccinates (**13**, **25**) and sulfates (**14**, **26**), the 3α-hydroxy pregnane derivatives (**12**, **24**) were used, previously prepared by selective reduction with $NaBH_4$ of 3-keto group [20]. The hemisuccinates were prepared by a common procedure of treating the steroid alcohol in pyridine with succinic anhydride and the sulfates by treating the steroid alcohol with sulfur trioxide pyridine complex (Scheme 4). Hemisuccinate **13** and **25** were prepared in the yield of 53% and 48%, respectively. Pyridinium salt of sulfate **14** and **26** were prepared in the yield of 85% and 80%, respectively.

3.2. Biological activity

Currents elicited by 100 μM NMDA were recorded in cultured hippocampal neurons voltage-clamped at a holding potential of −60 mV. In accordance with previous results, pregnanolone sulfate diminished the amplitude of NMDA-induced currents (Fig. 1) [14,16]. At 100 μM pregnanolone sulfate, the mean inhibitory effect was 71.3 ± 5.0% (n = 5). Synthetic analogs of pregnanolone sulfate had also negative modulatory effect on NMDA-induced responses with the mean inhibition induced by 100 μM steroid that ranged from 17% (**5**, 3α5α7AcHS) to 71% (**26**, 3α5βS). The relative degree of steroid-induced inhibition was used to estimate IC_{50}. IC_{50} was calculated from the logistic equation $RI = 1 - (1/(1 + ([steroid]/IC_{50})^h)$; where RI is relative degree of steroid-induced inhibition and h is the apparent Hill coefficient (1.2; see Ref. [16]). The values of relative inhibition and estimated IC_{50} values of steroids **5**, **6**, **11**, **13**, **14**, **17**, **18**, **25**, and **26** are listed in Table 1.

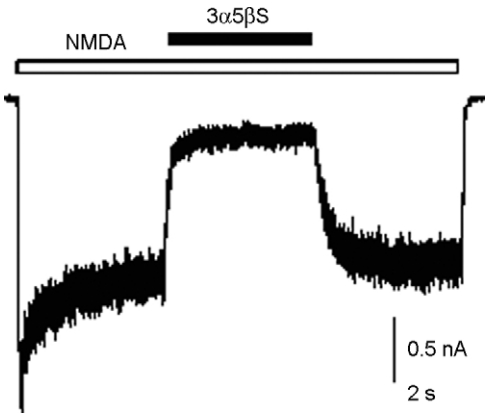


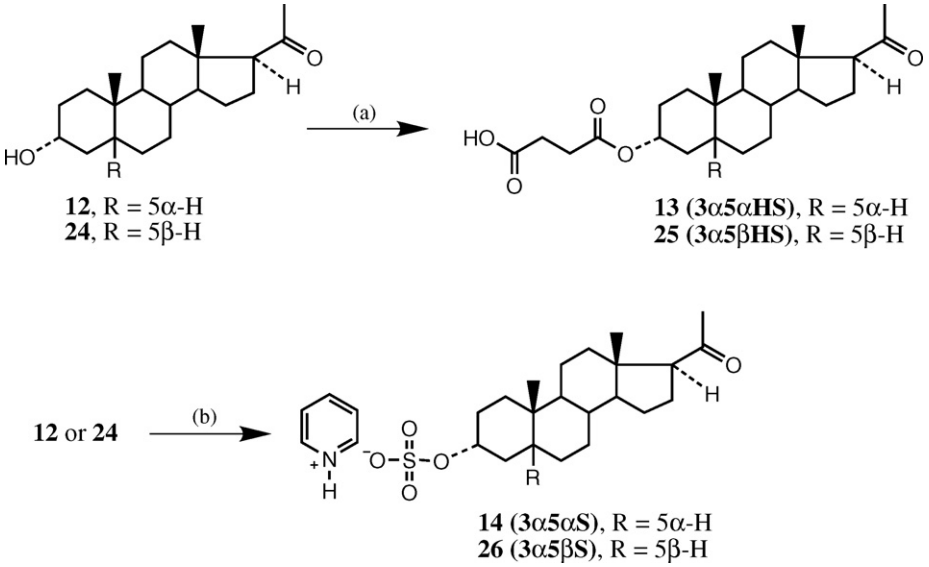
Fig. 1. Biological activity of the steroid—example of the text.

Table 1
Inhibition of NMDA-induced response in cultured hippocampal neurons by pregnanolone sulfate and its synthetic analogs.

Compound	Inhibition (%)	IC_{50} (μM)	n
3α5βS (**26**)	71.3 ± 5.0	47.2 ± 9.9	5
3α5βHS (**25**)	69.2 ± 7.0	51.9 ± 14.2	6
3α5β7AcHS (**17**)	21.4 ± 2.7	299.9 ± 40.9	6
3α5β7AcS (**18**)	34.1 ± 10.2	185.8 ± 61.1	5
3α5β7NicS (**23**)	46.9 ± 9.7	116.0 ± 36.5	5
3α5αS (**14**)	56.8 ± 2.3	79.8 ± 6.1	6
3α5αHS (**13**)	35.1 ± 2.6	167.6 ± 15.5	5
3α5α7AcHS (**5**)	17.4 ± 4.3	383.5 ± 93.5	5
3α5α7AcS (**6**)	35.2 ± 2.7	167.2 ± 16.3	5
3α5α7NicS (**11**)	37.8 ± 4.7	153.8 ± 25.0	6

The first column shows relative degree of steroid (100 μM) inhibition of current responses induced in cultured hippocampal neurons by fast application of 100 μM NMDA. In the second column calculated IC_{50} values are listed. Results are expressed as mean ± S.D., with n indicating the number of cells studied.

Current response induced in a cultured hippocampal neuron by 100 μM NMDA and its simultaneous application with 100 μM 3α5βS (duration of 3α5βS and NMDA application is indicated by filled and open bar, respectively).



Scheme 4. Synthesis of compounds **13**, **14**, **25** and **26**. Reaction conditions: (a) succinic anhydride, pyridine, 4-dimethylaminopyridine, 140 °C; (b) sulfur trioxide pyridine complex, $CHCl_3$.

4. Discussion

Pregnanolone sulfate acts as an inhibitor of NMDA receptor channels and may function as endogenous neuromodulator [14]. Our recent results have shown that pregnanolone sulfate is a use-dependent but voltage-independent inhibitor of NMDA receptors, with more potent action at tonically than at phasically (synaptically) activated receptors [16]. In this study, we examined the effect of newly synthesized C3 and C7-substituted analogs of pregnanolone sulfate on ionotropic glutamate receptors expressed in cultured hippocampal neurons and found structural correlates that determine steroid inhibitory efficacy at NMDA receptors.

In our experiments, the synthetic homologues of naturally occurring 5β-pregnanolone sulfate (**26**, 3α5βS) reversibly inhibited NMDA receptor responses with the relative effect at a concentration of 100 μM varying from 71.3% to 69.2% for pregnanolone sulfate and pregnanolone hemisuccinate (**25**, 3α5βHS) to 17.4% for compound **5** (3α5α7AcHS). While the effect of pregnanolone sulfate was similar to that observed in cultured chicken spinal cord neurons [14], the effect of 3α5βHS (**25**) and 3α5αS (**14**) observed in our experiments was more pronounced than that observed earlier [14]. Several reasons could account for this difference including subunit composition of the receptor, use-dependent action of neurosteroids, species and neuronal type-dependent differences in the steroid affinity [16].

To infer on the structural–functional correlates of the newly synthesized neurosteroids molecular models (see Experimental) of steroids were constructed and the IC_{50} calculated from the single dose inhibition assuming monotonous dose–response relation with the Hill coefficient 1.2. Structural–functional analysis of 10 synthesized steroids indicate that substitution at the steroid A-ring affects substantially IC_{50}, suggesting involvement of these residues with the binding site at the NMDA receptor channel complex. We have drawn following conclusions: (i) neurosteroids β-substituted at C5 are more potent inhibitors than those α-substituted; (ii) succinate substitution for the C3-sulfate of the 3α5βS has no effect for the steroid affinity at NMDA receptors, however, similar substitution of 3α5αS reduces its affinity; (iii) binding site for the inhibitory neurosteroids at NMDA receptors can adopt rather large substituents at C3 (-hemisuccinate) and at C7 (-acetate, -nicotinate); (iv) substituents at C3 and C7 affect steroid binding to the receptor; and (v) C7 substituents inhibit NMDA receptor channel activity.

Pregnanolone sulfate and its homologues have been shown to exert beneficial role in various models of human diseases such as NMDA-induced seizures, formalin-induced pain and neuroprotective effect, both in *in vitro* and *in vivo* models [23,24]. Based on our findings in the present study we speculate that homologues of pregnanolone sulfate may have a therapeutic value in the treatment of human diseases of the central nervous system. Derivatives substituted in the position 7 conserve inhibitory activity of primary compounds, which can help in the study of the NMDA receptor.

Acknowledgements

We are indebted to Dr. S. Vašíčková for taking the IR spectra and Dr. P. Fiedler for interpreting them. Optical rotations were measured and elemental analyses were carried out in the Analytical Laboratory (Dr. S. Matějková, Head). This work was supported by the Grant Agency of the Czech Republic (309/07/0271; 203/08/1498), Research Project of the AS CR AV0Z 50110509, Z4 005 905, EC FP6 PHOTOLYSIS (LSHM-CT-2007-037765) and Ministry of Education, Youth and Sports of the Czech Republic (1M0002375201 and LC554).

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