PREPARATION OF STEROID SULFAMATES AND THEIR INTERACTION WITH $GABA_A$ RECEPTOR

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Sulfamoyl chloride was used for the preparation of 3α- and 3β-sulfamates of steroids: 20-oxo-5α-pregnan-3α-yl sulfamate (**4**), 20-oxo-5α-pregnan-3β-yl sulfamate (**5**), 20-oxo-5β-pregnan-3α-yl sulfamate (**7**), and 20-oxo-5β-pregnan-3β-yl sulfamate (**9**) from the corresponding 3α- and 3β-alcohols. Sulfur trioxide–pyridine complex was employed for the preparation of 20-oxo-5α-pregnan-3α-yl pyridinium sulfate (**10**). The specific steroid binding was detected by the decrease of the specific [^{35}S]-*tert*-butylbicyclo[2.2.2]phosphorothionate binding after application of the tested compounds.

Keywords: Sulfamates; Neurosteroids; $GABA_A$ receptors; Pregnane.

The ability of steroids to penetrate the blood-brain barrier and their activity on the receptors for neurotransmitters allow them to alter neurotransmission and influence events in the nervous system via non-genomic mechanisms resulting in fast behavioral effects. The fastest inhibitory synaptic transmission in the vertebrate central nervous system is mediated by GABA (gamma-aminobutyric acid), which opens Cl^- selective ion channels of the $GABA_A$ receptor subtype.

There are more binding sites for the neuroactive steroids on the $GABA_A$ receptors that differ from the site for GABA and also from the sites for barbiturates, diazepines, and ethanol. Isomeric pregnanolone sulfates operate as allosteric modulators of $GABA_A$ receptor function. The activity of steroid sulfates on the $GABA_A$ receptor significantly changes with the configuration of the C-3 substituent; 3α-pregnanolone sulfates potentiate, whereas 3β-pregnanolone sulfates inhibit $GABA_A$-induced uptake of Cl^- in neurons[1].

The major excitatory neurotransmitter in the mammalian brain is L-glutamic acid. The most frequent receptors activated by L-glutamic acid

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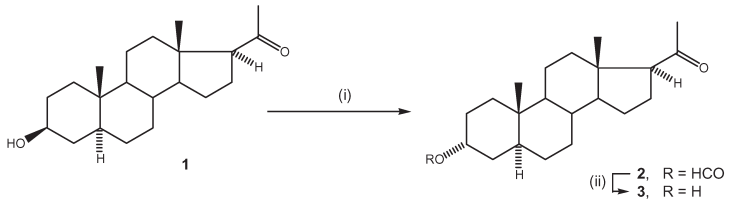
are ionotropic receptors that can be opened also by NMDA (*N*-methyl-D-aspartic acid). Steroid 3-sulfate esters modulate NMDA receptors as well. The direction of their effect also depends on the configuration on C-3 and C-5: 3α-Hydroxysteroid sulfate esters inhibit NMDA-induced increases in intracellular Ca^{2+}. 20-Oxo-5β-pregnan-3α-yl sulfate inhibits more than 20-oxo-5α-pregnan-3α-yl sulfate. 20-Oxo-5β-pregnan-3β-yl sulfate is a very week inhibitor, while 20-oxo-5α-pregnan-3β-yl sulfate potentiates the NMDA-invoked Ca^{2+} uptake[2].

Steroids that positively modulate the $GABA_A$ receptor induce analgesia, anaesthesia, anxiolysis, and sleep[3]. The consequence of a negative modulation of excited NMDA receptors is neuroprotection[4]. In general, the endogenous sulfated neurosteroids play important physiological roles in the brain. Nevertheless, their relatively low stability in neutral and acidic conditions and fast metabolic deactivation render their pharmacological utilization difficult.

On the contrary, steroid sulfamates are chemically and metabolically more resistant, and can also act as steroid sulfatase inhibitors[5]. We expected that the sulfamates of neurosteroids could simulate the action of the corresponding sulfates on the neuronal receptors. Notably, neuroactive steroids are effective at lower concentrations than barbiturates or benzodiazepines[6].



		R^1	R^2
1	5α-H	H	HO
3	5α-H	HO	H
6	5β-H	HO	H
8	5β-H	H	HO

		R^1	R^2
5	5α-H	H	H_2NO_2SO
4	5α-H	H_2NO_2SO	H
7	5β-H	H_2NO_2SO	H
9	5β-H	H	H_2NO_2SO

SCHEME 1

(i) HCOOH, Ph_3P, diethyl azodicarboxylate, benzene; (ii) K_2CO_3, methanol; (iii) sulfamoyl chloride, *N*,*N*-diisopropylethylamine, CH_2Cl_2

Moreover, the potency of the neurosteroids strongly depends on the subunit composition of the receptor, which may be used in the medication of selected neuronal disorders (Scheme 1).

RESULTS AND DISCUSSION

The Mitsunobu reaction[7] of 3β-hydroxy-5α-pregnan-20-one (**1**) with formic acid afforded the 3α-formate **2**. Hydrolysis of the formyl group gave 3α-alcohol **3** in overall yield 58%. The inversion of configuration of the 3-hydroxy group was confirmed by the chemical shift (δ 4.05 ppm) and half-width ($W_{1/2}$ = 7 Hz) of the 3β-H signal in the ^{1}H NMR spectrum. The other substrates were prepared according to published procedures[8].

Sulfamates were prepared by sulfamoylation[9] of the corresponding steroid alcohols. The reactions of sulfamoyl chloride with the steroid alcohols were performed in the presence of *N*,*N*-diisopropylethylamine. Sulfamates **4** and **5** of the 5α-series were prepared from the corresponding alcohols **3** and **1** in the yields of 64 and 43%, respectively. Sulfamoylation of steroid alcohols **6** and **8** afforded the desired 3α-sulfamate **7** and 3β-sulfamate **9** in the yields of 56 and 40%, respectively. The structures of the sulfamates were confirmed by the presence of the characteristic signals of NH_2 group (ca. 4.7 ppm) in the ^{1}H NMR spectra and characteristic bands in the IR spectra.

The ability of steroid ligands to potentiate GABA effect on the Cl^- channel can be screened as inhibition of the binding of the convulsant [^{35}S]-*tert*-butylbicyclo[2.2.2]phosphorothionate ([^{35}S]-TBPS), a high affinity ligand for the picrotoxin site of $GABA_A$ receptors[6].

The values in Table I showed that 3α-sulfamate-5β-pregnanolone **7** reduced the binding of TBPS to the $GABA_A$ receptor by 23.2%. It means that its $GABA_A$ potentiating activity is comparable with 3α-sulfate-5α-pregnanolone **10** (25.1% reduction), which is the most active compound in the sulfate series, while 3α-sulfamate-5α-pregnanolone **4** is, according to this screening, a moderate inhibitor of the $GABA_A$ receptor function. 5α-H-3β-sulfamate **5** exerts a similar, very low inhibiting activity, but 5β-H-3β-sulfamate **9** was a very weekly positive modulator of the $GABA_A$ receptor function. However, the activity of all three compounds was too week for further utilization.

In our search for pharmacologically interesting neuroactive steroids, we focused on positive modulators of $GABA_A$ receptors and inhibitors of the NMDA receptor. The promising binding properties of compound **7** were the reason for the evaluation of concentration–activity dependence (Table II).

Neuroactive steroids[10] are among the most potent and efficacious modulators of $GABA_A$ receptors known. The potency, expressed as IC_{50}, is an empirical measure reflecting the concentration of a drug required to yield half the maximum response that a saturating concentration of the drug is capable of producing. Efficacy (expressed as I_{max}) is a measure of the maximum effect produced by a saturating drug concentration[11]. Sulfamate **7** potency is 2 times higher than that of the most active sulfate epimer **10**. The efficacy of sulfamate **7** was slightly lower in comparison with that of sulfate **10**.

The obtained data cannot unambiguously explain why in the sulfate series, the 3α,5β-epimer mostly increases the effect of the neurotransmitter on the $GABA_A$ receptor, while the most active sulfamate is the 3α,5β-analogue. The data do not explain other differences in the activity of both sets, either.

TABLE I
Modulatory effect of steroid sulfamates **4**, **5**, **7**, **9** and sulfate **10** on $GABA_A$ receptors

Compound	[^{35}S]-TBPS binding E_{100nM}, %
4	112.0
5	108.5
7	76.8
9	93.5
10[a]	74.9

[a] **10** = 20-Oxo-5α-pregnan-3α-yl sulfate.

TABLE II
Modulatory effect of steroid sulfamate **7** and sulfate **10** on $GABA_A$ receptors

Compound	[^{35}S]-TBPS binding	
	I_{max}[a], %	IC_{50}[b], nM
7	40	50
10[c]	31	100

[a] The maximum suppression of binding. [b] Steroid concentration producing half of the maximum inhibition. [c] **10** = 20-Oxo-5α-pregnan-3α-yl sulfate.

It is known that the effect of steroids on the GABA-gated chlorine channel is mediated by at least of two different binding sites on the protein. Another factor is the access of the steroid to the receptor protein, because at least one of the binding sites is in the intramembrane part of the receptor. In addition, the presence or absence of a charge[10] in the molecule is an important factor in the activity of neurosteroids. In the article cited above it has been postulated that the negative charge is the main reason for the modulation of GABA activity by the pregnanolone sulfate epimers. But later findings, and our experiments showed that the interaction of steroids with the $GABA_A$ receptor is very complex.

Evaluation of the activity of the synthesized compounds in the voltage-clamped cultured rat hippocampal neurons to assess their ability to act as NMDA receptor inhibitors was also studied. This test is based on monitoring the currents elicited by 100 μM NMDA in cultured hippocampal neurons, voltage-clamped at a holding potential of –60 mV in the presence of 100 μM solution of the steroid[12]. Unfortunately, the low solubility of the sulfamoyl analogues of pregnanolone sulfate epimers in the buffer disabled the determination of their electrophysiological effect.

However, the sulfamate epimers extend the scope[10] of $GABA_A$ receptor steroid ligands.

EXPERIMENTAL

Melting points were determined on a Boetius (Germany) melting point microapparatus and are uncorrected. Analytical samples were dried over phosphorus pentoxide at 50 °C/100 Pa. Optical rotations were measured in chloroform ($[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}) on an Autopol IV polarimeter (Rudolf Research Analytical, Flanders, USA). IR spectra of chloroform solutions or KBr pellets were recorded on a Bruker IFS 55 spectrometer; wavenumbers are given in cm^{-1}. ^1H NMR spectra were measured on an FT NMR spectrometer Bruker AVANCE-400 (at 400 MHz) in $CDCl_3$ with tetramethylsilane as internal reference. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) and multiplet half-widths ($W_{1/2}$) in Hz. The data were interpreted as first-order spectra. Thin layer chromatography (TLC) was performed on silica gel (MP Biomedicals). Preparative TLC (PLC) was carried out on 200 × 200 mm plates coated with a 0.7 mm thick layer of the same material. For column chromatography, 60 μm silica gel (Fluka) was used. For TLC detection, spraying with concentrated sulfuric acid with subsequent heating was used, whereas PLC plates were sprayed with 2% morine solution in methanol and visualized in UV light (254 nm). Extraction from the treated plates was done with diethyl ether. The solvents were evaporated on a rotary evaporator in vacuum (bath temperature 50 °C). Benzene was dried by distillation from sodium, dichloromethane and chloroform by distillation from P_2O_5. Sulfamoyl chloride was prepared according to the literature[9].

Receptor binding assay[6]. Membranes were isolated from the whole brains of adult male Wistar rats. The membranes were re-suspended in a buffer (20 mM KH_2PO_4, 200 mM KCl, pH

7.4). Aliquots were incubated with 2 nM [^{35}S]-TBPS, 1 μM GABA and steroids at 37 °C for 1 h. The non-specific binding was estimated using 200 μM picrotoxinin. The results were related to the control samples containing GABA and DMSO and expressed in %. Screening experiments were carried out with 100 nM solutions of the steroids (see Table I). More precise experiments were performed with the 1 nM to 1 μM solutions of the steroids (see Table II).

20-Oxo-5α-pregnan-3α-yl Formate (**2**)

A mixture of hydroxy derivative **1** (478 mg, 1.5 mmol) and triphenylphosphine (642 mg, 2.4 mmol) was dissolved with gentle warming in dry benzene (35 ml) and anhydrous formic acid (0.105 ml, 2.78 mmol) was added. Diethyl azodicarboxylate (0.46 ml, 3.0 mmol) was added dropwise under stirring as a solution in benzene (6 ml) and the mixture was stirred overnight at room temperature. The reaction mixture was then poured into water (100 ml), extracted with ethyl acetate (50 ml) and subsequently washed with aqueous hydrochloric acid (5%, 100 ml), saturated $NaHCO_3$ (100 ml), and with water to neutral reaction. The organic layer was dried with anhydrous Na_2SO_4 and evaporated in vacuo. Chromatography on a column of silica gel (20 g) with petroleum ether–acetone (92:8) gave the pure compound, which was crystallized from acetone, yielding white crystals of **2** (370 mg, 71%). M.p. 184–186 °C, $[\alpha]_D$ +122.3 (*c* 0.25). IR ($CHCl_3$): 1714 (C=O formate); 1706 (C=O ketone); 1197, 1154 (C–O). ^{1}H NMR: 8.07 s, 1 H (HCOO); 5.17 m, 1 H, $W_{1/2}$ = 7 (H-3); 2.53 t, 1 H, *J* = 8.9 (H-17); 2.12 s, 3 H (3 × H-21); 0.81 s, 3 H (3 × H-19); 0.61 s, 3 H (3 × H-18). For $C_{22}H_{34}O_3$ (346.5) calculated: 76.26% C, 9.89% H; found: 76.09% C, 9.58% H.

3α-Hydroxy-5α-pregnan-20-one (**3**)

Formate **2** (230 mg, 0.66 mmol) was dissolved in methanol (10 ml) and 10% solution of K_2CO_3 in methanol (10 ml) was added while stirring. After 10 min, the conversion was complete according to TLC. Methanol was partially evaporated from the reaction mixture, the residue was poured into water (50 ml), extracted with chloroform (30 ml), washed with 5% HCl (50 ml), saturated $NaHCO_3$ (50 ml), and finally with water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. Crystallization from acetone and ethyl acetate gave white crystals of **3** (172 mg, 82%). M.p. 169–172 °C, $[\alpha]_D$ +92.4 (*c* 0.26). Literature[13] gives 168–170 °C (ether–acetone), $[\alpha]_D$ +96 (*c* 0.5). ^{1}H NMR: 4.05 m, 1 H, $W_{1/2}$ = 7 (H-3); 2.53 t, 1 H, *J* = 9.1 (H-17); 2.11 s, 3 H (3 × H-21); 0.78 s, 3 H (3 × H-19); 0.60 s, 3 H (3 × H-18).

Preparation of Steroid Sulfamates **4**, **5**, **7** and **9**. General Procedure

A steroid alcohol (1.0 mmol) was dissolved in anhydrous dichloromethane (4 ml), *N*,*N*-diisopropylethylamine (0.35 ml, 2.0 mmol) was added and, the mixture was cooled to 0 °C. A solution of sulfamoyl chloride in benzene (1 M, 1 ml) was added dropwise and the reaction mixture was stirred overnight. The mixture was poured into water (50 ml) and extracted with dichloromethane (50 ml). The organic phase was washed with 5% aqueous ammonia (50 ml) and water to neutral reaction. The extract was dried with anhydrous magnesium sulfate and concentrated in vacuo.

20-Oxo-5α-pregnan-3α-yl sulfamate (**4**). Hydroxy derivative **3** (100 mg, 0.314 mmol) was converted into **4** according to General Procedure. The reaction mixture was poured into water (100 ml), precipitated crystals were filtered off, washed with 5% aqueous ammonia and water to neutral reaction, and subsequently dried in a desiccator over potassium

hydroxide pellets to afford compound **4** (79 mg, 64%). M.p. 154–156 °C, $[\alpha]_D$ was not measured due to a low solubility of the sample. IR (KBr): 3347, 3239 (NH_2); 1696 (C=O); 1385, 1367, 1191 (SO_2); 929 (C–O); 898 (N–S). ^{1}H NMR: 4.89 m, 1 H, $W_{1/2}$ = 4 (H-3); 4.64 s, 2 H (NH_2); 2.54 t, 1 H, J = 8.8 (H-17); 2.11 s, 3 H (3 × H-21); 0.79 s, 3 H (3 × H-19); 0.60 s, 3 H (3 × H-18). For $C_{21}H_{35}NO_4S$ (397.6) calculated: 63.44% C, 8.87% H, 3.52% N, 8.07% S; found: 62.42% C, 9.11% H, 3.57% N, 8.22% S.

20-Oxo-5α-pregnan-3β-yl sulfamate (**5**). Hydroxy derivative **1** (150 mg, 0.471 mmol) was converted into **5** according to General Procedure. After workup, the product was separated on a column of silica gel (12 g), which was prewashed with 2% triethylamine in benzene. Benzene with an increasing amount of ethyl acetate (2–12%) was used as the eluent. Crystallization from petroleum ether and acetone yielded **5** as white crystals (80 mg, 43%). M.p. 158–160 °C, $[\alpha]_D$ +46.1 (*c* 0.25). IR ($CHCl_3$): 3443, 3351 (NH_2); 1698 (C=O); 1382, 1361, 1182 (SO_2); 947, 939 (C–O); 916 (N–S). ^{1}H NMR: 4.71 s, 2 H (NH_2); 4.53 m, 1 H, $W_{1/2}$ = 19 (H-3); 2.52 t, 1 H, J = 9.1 (H-17); 2.12 s, 3 H (3 × H-21); 0.83 s, 3 H (3 × H-19); 0.60 s, 3 H (3 × H-18). For $C_{21}H_{35}NO_4S$ (397.6) calculated: 63.44% C, 8.87% H, 3.52% N, 8.07% S; found: 63.47% C, 9.2% H, 3.37% N, 7.71% S.

20-Oxo-5β-pregnan-3α-yl sulfamate (**7**). Hydroxy derivative[8] **6** (320 mg, 1.0 mmol) was converted into **7** according to General Procedure. After workup, chromatography on a silica gel column (15 g) in petroleum ether–acetone (8:2) yielded compound **7** (225 mg, 56%). M.p. 170–173 °C (heptane–dichloromethane), $[\alpha]_D$ +92.8 (*c* 0.23). IR ($CHCl_3$): 3347, 3351 (NH_2); 1700 (C=O); 1380, 1362, 1181 (SO_2); 943, 933 (C–O); 912 (N–S). ^{1}H NMR: 4.71 s, 2 H (NH_2); 4.58 m, 1 H, $W_{1/2}$ = 19 (H-3); 2.54 t, 1 H, J = 9.0 (H-17); 2.12 s, 3 H (3 × H-21); 0.94 s, 3 H (3 × H-19); 0.60 s, 3 H (3 × H-18). For $C_{21}H_{35}NO_4S$ (397.6) calculated: 63.44% C, 8.87% H; 3.52% N, 8.07% S; found: 61.84% C, 8.82% H; 3.22% N, 7.91% S.

20-Oxo-5β-pregnan-3β-yl sulfamate (**9**). Hydroxy derivative[8] **8** (306 mg, 0.96 mmol) was converted into **9** according to General Procedure. After workup, the product was separated on a silica gel column (18 g), which was prewashed with 2% triethylamine in benzene. Benzene with an increasing amount of ethyl acetate (2–12%) was used as the eluent. Crystallization from ethanol and water yielded **9** as white crystals, which were dried in a desiccator over sodium hydroxide pellets (0.153 g, 40%). M.p. 169–172 °C, $[\alpha]_D$ +70.4 (*c* 0.27). IR ($CHCl_3$): 3341, 3350 (NH_2), 1698 (C=O); 1385, 1365, 1183 (SO_2); 924 (C–O); 913 (N–S). ^{1}H NMR: 4.97 m, 1 H (H-3); 4.71 m, 2 H (NH_2); 2.53 t, 1 H, J = 9.1 (H-17); 2.11 s, 3 H (3 × H-21); 0.98 s, 3 H (3 × H-19); 0.61 s, 3 H (3 × H-18). For $C_{21}H_{35}NO_4S$ (397.6) calculated: 63.44% C, 8.87% H, 3.52% N, 8.07% S; found: 62.81% C, 9.13% H, 3.30% N, 7.99% S.

20-Oxo-5α-pregnan-3α-yl Sulfate Pyridinium Salt (**10**)

Hydroxy derivative **3** (159 mg, 0.5 mmol) and sulfur trioxide–pyridine complex (398 mg, 2.5 mmol) in freshly distilled chloroform (10 ml) were stirred at room temperature under argon for 4 h. The mixture was cooled to –18 °C, filtered, and the solvent was removed under reduced pressure. The resulting crude product was dissolved in chloroform (2 ml), chilled in a freezer for a short time, and the precipitate of sulfur trioxide–pyridine complex was removed. Light petroleum (7 ml) was added to the filtrate and after keeping at room temperature for 2 h, steroid pyridinium sulfate was collected. M.p. 181–183 °C, $[\alpha]_D$ +69.0 (*c* 0.21). Literature[14] gives m.p. 184 °C, $[\alpha]_D$ +70.0. IR ($CHCl_3$): 3139 (pyridinium); 1698 (C=O, ketone); 1270, 1257, 1236, 1179, 1166 (SO_3 and pyridinium). ^{1}H NMR: 9.00 m, 2 H (H-2 and H-6, pyridinium); 8.50 tt, 1 H, J_1 = 7.8, J_2 = 1.5 (H-4, pyridinium); 8.03 m, 2 H

(H-3 and H-5, pyridinium); 4.76 m, 1 H (H-3); 2.52 t, 1 H, *J* = 8.7 (H-17); 2.11 s, 3 H (3 × H-21); 0.78 s, 3 H (3 × H-19); 0.60 s, 3 H (3 × H-18). For $C_{26}H_{39}NO_5S$ (477.6) calculated: 65.38% C, 8.32% H, 2.93% N, 6.71% S; found: 65.11% C, 8.02% H, 2.99% N, 6.85% S.

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