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Neuroactive steroids with perfluorobenzoyl group

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ABSTRACT

During an initial study in searching for the alternative derivatives suitable for photolabeling of neuroactive steroids, perfluorobenzoates and perfluorobenzamides in position 17 of 5β-androstan-3α-ol were synthesized from the corresponding 17-hydroxy and 17-amino derivatives. After transformation into glutamates or sulfates, 17α-epimers had comparable inhibitory activity at NMDA receptors to the natural neurosteroid (20-oxo-5β-pregnan-3β-yl sulfate), however, were more potent (2- to 36-fold) than their 17β-substituted analogs. In one case, fluorine in position 4′ of perfluorobenzoate group was substituted with azide and activity of the final glutamate was retained comparing with the corresponding perfluorobenzoate. The series was expanded with perfluorobenzoyl derivatives of pregnanolone: Perfluorobenzamide of glutamate and perfluorobenzoate of 11α-hydroxy pregnanolone were prepared and tested. From nine tested compounds, four of them exhibit very good inhibition activity and can serve as promising leads for photolabeling experiments.

1. Introduction

Azidoperfluorophenyl group has been designed as a source of nitrenes for photoaffinity labeling and crosslinking due to their favorable photochemical properties, suitable storage stability, and ease of preparation [1,2]. The reagents for introducing this group in form of substituted benzoate are commercially available [3]. In the steroidal field, use of diazirine label is the most frequent [4-7]. Iodine substituted azidobenzoates, however, were used for labeling of DHEA [8]. During a process of searching for suitable photoaffinity label for neuroactive steroids, we have decided to prepare several models with perfluorobenzoyl group attached to various parts of base skeleton of neuroactive steroids and study the activity of modified compounds in inhibition tests on *N*-Methyl-D-aspartate (NMDA) receptors. We were encouraged with our previous findings [9] which proved the possibility of replacing 20-keto pregnane side chain of pregnanolone with azide without disturbing the inhibitory activity on the NMDA receptor.

2. Experimental

2.1. General

Melting points were determined on a Boetius micro melting point apparatus (Germany) and are uncorrected. Optical rotations were measured at 25 °C on a AUTOPOL IV polarimeter (Rudolph Research Analytical, USA), and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Infrared spectra (wavenumbers in cm^{-1}) were recorded on a Bruker IFS 88 spectrometer in chloroform. ^{1}H, ^{13}C, and ^{19}F NMR spectra were taken on Bruker AVANCE-400 instrument at 23 °C and referenced to tetramethylsilane as the internal standard. For referencing of ^{19}F spectra, hexafluorobenzene (δ = 162.9) was used. ^{13}C NMR spectra for selected derivatives (Tables 1a and b) were measured on Bruker AVANCE-600 instrument under the above conditions, secondary referencing was performed using the solvent signal at position δ($CDCl_3$) = 77.0. In addition to 1D proton and carbon NMR spectra, homonuclear 2D-spectra (H,H-PFG-COSY, and ROESY) together with heteronuclear 2D-spectra (H,C-PFG-HSQC and H,C-PFG-HMBC) were used for complete structural assignment of signals. Chemical shifts are given in ppm (δ-scale); coupling constants (*J*) are given in Hz.

Thin-layer chromatography (TLC) was performed on silica gel G (ICN Biochemicals), detection by spraying with concentrated sulfuric acid followed by heating. For column chromatography, Silica gel 60 (Merck, 63–100 μm) was used. Prior to evaporation on a rotary evaporator in vacuo (0.25 kPa, bath temperature 40 °C), solutions in organic solvents were dried over anhydrous sodium sulfate.

17β-Hydroxy-5β-androstan-3-one (**1a**) [10], 17α-hydroxy-5β-androstan-3-one (**1b**) [11,12], 3α-hydroxy-5β-androstan-17-one oxime (**9**) [13], 17α-azido-5β-androstan-3α-ol (**11**) [9], and 11α-hydroxy-5β-pregnane-3,20-dione (**18**) [14,15] are known compounds and were prepared according to literature procedures.

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Table 1a
Carbon-13 chemical shifts of selected perfluorobenzoyl steroids[a]: Skeletal carbon atoms.

Carbon	5a	6b	8	14a	14b[b]	17	21
1	34.88	35.31	34.85	34.85	35.81	34.83	37.79
2	26.46	27.59	26.44	26.37	27.56	26.43	27.89
3	76.74	79.52	76.95	76.52	78.52	76.65	79.00
4	31.92	33.09	31.89	31.96	33.23	31.97	33.62
5	41.85	41.97	41.82	41.81	43.30	41.78	43.26
6	26.77	26.78	26.74	26.77	28.05	26.80	27.05
7	25.81	26.57	25.79	25.93	27.93	26.22	26.32
8	40.42	40.19	40.43	40.51	41.78	40.39	34.96
9	35.55	35.87	35.54	35.92	37.58	35.73	43.67
10	34.63	34.45	34.62	34.61	35.99	34.57	35.67
11	20.25	20.17	20.24	20.34	21.54	20.83	74.92
12	36.82	31.92	36.82	37.07	33.94	39.08	45.18
13	42.98	45.09	42.96	43.93	46.64	44.32	43.86
14	50.42	50.15	50.42	52.44	51.58	56.61	55.15
15	23.55	24.49	23.54	23.58	25.85	24.38	24.32
16	27.53	30.12	27.54	27.58	30.17	22.86	23.00
17	85.67	85.28	85.49	59.84	60.66	63.82	63.10
18	12.11	16.49	12.11	11.92	18.42	13.41	14.05
19	23.22	23.11	23.20	23.14	23.75	23.21	23.49
20	-	-	-	-	-	209.90	208.52
21	-	-	-	-	-	31.53	31.39

[a] Measured in $CDCl_3$ or in a mixture of $CDCl_3/CD_3OD$ (10:1) (**5a**, **8**, and **14a**).
[b] Hydrochloride.

Table 1b
Carbon-13 chemical shifts of selected perfluorobenzoyl steroids: Substituents.

Carbon	5a	6b[a]	8	14a	14b[b]	17[a]	21
Perfluorobenzoyl group							
1	158.56	158.61	159.31	157.71	158.80	157.36	158.51
1′	108.56	109.00	108.14	112.23	113.94	110.85	108.66
2′,6′	145.33	145.12	145.57	143.78	145.02	142.53	143.12
3′,5′	137.62	137.69	141.41	141.89	138.87	137.66	137.60
4′	142.99	142.96	123.02	137.44	143.20	144.24	143.00
Glutamate							
1	170.26	-	169.92	171.34	169.81	170.43	-
2	53.05	-	52.86	59.84	53.39	52.52	-
3	26.38	-	26.09	27.41	26.73	26.96	-
4	33.04	-	32.09	32.67	30.24	29.57	-
5	177.65	-	176.93	176.96	175.40	176.99	-

[a] Data for sulfates (pyridinium nucleus) - **6b**: 142.23 (2,6), 127.14 (3,5), 145.72 (4); **21**: 142.20 (2,6), 127.12 (3,5), 145.74 (4).
[b] Data for hydrochloride.

2.2. Chemical synthesis

2.2.1. 3-Oxo-5β-androstan-17β-yl pentafluorobenzoate (*2a*)

Pentafluorobenzoyl chloride (1 mL, 6.94 mmol) was added dropwise to a stirred solution of 17β-Hydroxy-5β-androstan-3-one (**1a**) (1 g, 3.44 mmol) in pyridine (10 mL) cooled with ice-bath. The mixture was allowed to attain the room temperature and the stirring continued for 1 h. Then, after pouring into water with ice, the product was extracted with ethyl acetate (200 mL). The ethyl acetate extract was washed with 5% hydrochloric acid, saturated aqueous potassium hydrogen carbonate (2×), water, and dried. Solvents were evaporated and the crude pentafluorobenzoate **2a** (1.7 g) was chromatographed on a silica gel column (20 mL) in a mixture of petroleum ether/acetone (20:1). Resulting foamy product **2a** (1.56 g, 93%) was used without further purification. Analytical sample crystallized after addition of ether, m.p. 126-128 °C, $[\alpha]_D$ + 49 (c 0.30, chloroform). IR: 1732 (C=O, ester); 1709 (C=O, ketone); 1390, 1381 (CH_3); 1242, 1010, 1000 (C–O). ^{1}H NMR ($CDCl_3$): 4.89 dd, 1H, J = 9.0, J' = 7.8 (H-17α); 2.69 dd, 1H, J = 14.6, J' = 13.7 (H-4α); 1.04 s, 3H 3× H-19); 0.87 s, 3H (3 × H-18). Calcd. for $C_{26}H_{29}F_5O_3$ (484.5): C, 64.45; H, 6.03; F, 19.61. Found: C, 64.34; H, 5.88; F, 19.89.

2.2.2. 3-Oxo-5β-androstan-17α-yl pentafluorobenzoate (*2b*)

To a stirred solution of 17α-hydroxy-5β-androstan-3-one (**1b**) (960 mg, 3.31 mmol) in dichloromethane (8 mL) under argon atmosphere, pentafluorobenzoic acid (770 mg, 3.63 mmol) and 4-dimethylaminopyridine (100 mg, 0.82 mmol) were added. Then, *N*,*N*′-diisopropylcarbodiimide (570 μL, 3.68 mmol) was added dropwise and stirring continued for 1 h. The reaction mixture was cooled with ice bath, solids were filtered off and the solution was applied on the column of silica gel (50 mL) in petroleum ether. The product was eluted with a mixture of petroleum ether/acetone (10:1) yielding 298 mg (81%) of ester **2b**. Analytical sample crystallized from ethanol, m.p. 144-145 °C, $[\alpha]_D$ -12 (c 0.29, chloroform). IR: 1730 (C=O, ester); 1710 (C=O, ketone); 1382 (CH_3); 1245, 1238, 1107 (C–O). ^{1}H NMR ($CDCl_3$): 5.23 bd, 1H, J = 6.2 (H-17β); 2.81 dd, 1H, J = 15.0, J' = 13.4 (H-4α); 1.04 s, 3H (3 × H-19); 0.83 s, 3H (3 × H-18). Calcd. for $C_{26}H_{29}F_5O_3$ (484.5): C, 64.45; H, 6.03; F, 19.61. Found: C, 64.38; H, 6.11; F, 19.39.

2.2.3. 3α-Hydroxy-5β-androstan-17β-yl pentafluorobenzoate (*3a*)

A solution of cerium(III) chloride heptahydrate (713 mg, 1.91 mmol) in methanol (10 mL) was added to a stirred solution of ketone **2a** (845 mg, 1.74 mmol) in ethyl acetate (4 mL), and starting immediately, sodium borohydride (66 mg, 1.74 mmol) was added in portions over 5 min at room temperature. After additional stirring for 10 min, the reaction mixture was poured into ice-cold 5% hydrochloric acid, extracted with ethyl acetate (100 mL), and organic layer was washed with saturated aqueous potassium hydrogen carbonate (2×), water, and dried. Solvents were evaporated and crude product (820 mg, 97%) was crystallized from acetone. The yield of ester **3a** was 490 mg (58%); additional 140 mg (16%) were obtained by column chromatography of the mother liquors on silica gel (70 mL) in a mixture of petroleum ether/acetone (10:1). M.p. 163-164 °C, $[\alpha]_D$ + 40 (c 0.24, chloroform). IR: 3609 (OH); 1732 (C=O); 1389, 1377 (CH_3); 1243, 1009, 998 (C-O); 1033 (C-OH). ^{1}H NMR ($CDCl_3$): 4.87 dd, 1H, J = 9.0, J' = 7.7 (H-17α); 3.64 tt, 1H, J = 10.9, J' = 4.6 (H-3β); 0.94 s, 3H (3× H-19); 0.83 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −139.5 m, 2 F; −150.4 m, 1 F; −161.7 m, 2 F. Calcd. for $C_{26}H_{31}F_5O_3$ (486.5): C, 64.19; H, 6.42; F, 19.53. Found: C, 64.21; H, 6.30; F, 19.80.

2.2.4. 3α-Hydroxy-5β-androstan-17α-yl pentafluorobenzoate (*3b*)

Ketone **2b** (1.3 g, 2.68 mmol), ethyl acetate (5 mL), cerium(III) chloride heptahydrate (1.1 g, 2.95 mmol) in methanol (15 mL), and sodium borohydride (100 mg, 2.67 mmol) were processed as described in Section 2.2.3. Crude product (1.3 g) was chromatographed on a silica gel column (100 mL) in a mixture of petroleum ether/acetone from (20:1) to (10:1). The main fractions contained foamy hydroxy derivative **3b** (1.2 g, 92%), which did not crystallize. $[\alpha]_D$ -16 (c 0.33, chloroform). IR: 3609 (OH); 1730 (C=O); 1381 (CH_3); 1245, 1108 (C–O); 1032 (C–OH). ^{1}H NMR ($CDCl_3$): 5.08 bd, 1H, J = 5.7 (H-17β); 3.65 tt, 1H, J = 11.0, J' = 4.7 (H-3β); 0.93 s, 3H (3× H-19); 0.79 s, 3H (3 × H-18). Calcd. for $C_{26}H_{31}F_5O_3$ (486.5): C, 64.19; H, 6.42; F 19.53. Found: C, 64.23; H, 6.51; F, 19.64.

2.2.5. 5β-Androstane-3α,17β-diyl 17-(pentafluorobenzoate) 3-[O^5-tert-butyl-N-(tert-butoxycarbonyl)-L-glutamate] (*4a*)

To a stirred solution of ester **3a** (200 mg, 0.41 mmol) in dichloromethane (2 mL), O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl) -L-glutamate (136 mg, 0.45 mmol) and 4-dimethylaminopyridine (20 mg, 0.16 mmol) were added. Then, N,N′-diisopropylcarbodiimide (64 μL, 0.41 mmol) was added dropwise under argon atmosphere and stirring continued for 1 h. The reaction mixture was cooled with ice bath, solids were filtered off and the solution was applied on the column of silica gel (10 mL) in petroleum ether. The product was eluted with a mixture of petroleum ether/acetone (20:1) yielding 298 mg (94%) of foamy ester **4a**, which was used

further without additional purification. IR: 3437 (NH); 2980, 1475, 1392, 1369, 1158 (CH_3 *tert*-butyl); 1728 (C=O, ester); 1710 (C=O, amide); 1381 (CH_3); 1230, 1008 (C–O).^{1}H NMR ($CDCl_3$): 5.08 d, 1H, J = 8.5 (NH); 4.88 dd, 1H, *J* = 9.0, *J′* = 7.7 (H-17α); 4.78 tt, 1H, *J* = 11.0, *J′* = 4.6 (H-3β); 4.25 m, 1H (CH-NH); 1.45 s, 9H and 1.44 s, 9H (2× $(CH_3)_3C$); 0.95 s, 3H (3× H-19); 0.84 s, 3H (3 × H-18).

2.2.6. 5β-Androstane-3α,17α-diyl 17-(pentafluorobenzoate) 3-[O^5-tert-butyl-N-(tert-butoxycarbonyl)-L-glutamate] (**4b**)

Ester **3b** (300 mg, 0.61 mmol) in dichloromethane (2 mL) was treated with O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate (204 mg, 0.67 mmol), 4-dimethylaminopyridine (30 mg, 0.25), and *N*,*N′*-diisopropylcarbodiimide (96 μL, 0.62 mmol) as described in Section 2.2.5. The crude product (430 mg, 90%) was chromatographed on a silica gel column (80 mL) in a mixture of petroleum ether/acetone from (50:1) to (20:1) to give foamy ester **4b** (340 mg, 71%). IR: 3437 (NH); 2980, 1469, 1393, 1369, 1165 (CH_3 *tert*-butyl); 1726 (C=O, ester); 1710 (C=O, amide); 1382 (CH_3); 1238, 1006 (C-O). ^{1}H NMR ($CDCl_3$): 5.08 d, 1H, J = 8.8 (H-17β); 5.06 bd, 1H (NH); 4.77 tt, 1H, *J* = 11.0, *J′* = 4.6 (H-3β); 4.23 m, 1H (CH-NH); 1.44 s, 9H and 1.43 s, 9H (2× $(CH_3)_3C$); 0.95 s, 3H (3 × H-19); 0.80 s, 3H (3 × H-18).

2.2.7. 5β-Androstane-3α,17β-diyl 17-(pentafluorobenzoate) 3-(L-glutamate) (**5a**)

Ester **4a** (240 mg, 0.31 mmol) was treated with trifluoroacetic acid (0.85 mL) at room temperature for 10 min. Solvent was evaporated, the residue was dissolved in a solution of pyridine (0.6 mL) in methanol (4 mL) and the mixture was poured into ice cold water (50 mL). After overnight standing at 5 °C, the product was filtered off and dried. The yield of glutamate **5a** was 177 mg (93%). ^{1}H NMR ($CDCl_3$/CD_3OD, 10:1): 4.88 dd, 1H, *J* = 8.7, *J′* = 8.0 (H-17α); 4.83 tt, 1H, *J* = 11.5, *J′* = 5.0 (H-3β); 4.00 bdd, 1H, J = 8.4, *J′* = 5.0 (CH-NH_2); 0.96 s, 3H (3× H-19); 0.84 s, 3H (3× H-18). For ^{13}C NMR data see Tables 1a and b. Calcd. for $C_{31}H_{38}F_5NO_6.1/2\ H_2O$ (624.6): C, 59.61; H, 6.29; F, 15.21; N, 2.24. Found: C, 59.51; H, 6.44; F, 15.36; N, 2.07.

2.2.8. 5β-Androstane-3α,17α-diyl 17-(pentafluorobenzoate) 3-(L-glutamate) (**5b**)

Ester **4b** (200 mg, 0.26 mmol) was treated with trifluoroacetic acid (0.80 mL) as described in Section 2.2.7. The yield of glutamate **5b** was 153 mg (96%). ^{1}H NMR ($CDCl_3$/CD_3OD, 10:1): 5.08 d, 1H, *J* = 6.2 (H-17α); 4.82 tt, 1H, *J* = 11.4, *J′* = 4.6 (H-3β); 3.85 bdd, 1H, *J* = 7.8, *J′* = 3.2 (CH-NH_2); 0.96 s, 3H (3× H-19); 0.80 s, 3H (3× H-18). Calcd. for $C_{31}H_{38}F_5NO_6.H_2O$ (633.6): C, 58.76; H, 6.36; F, 14.99; N, 2.21. Found: C, 58.79; H, 6.13; F, 14.83; N, 2.02.

2.2.9. 17β-(Pentafluorobenzoyl)oxy-5β-androstan-3α-yl sulfate pyridinium salt (**6a**)

To a stirred solution of hydroxy derivative **3a** (200 mg, 0.41 mmol) in pyridine (3 mL), sulfur trioxide - pyridine complex (196 mg, 1.23 mmol) was added and stirring continued at room temperature for 30 min. Then, the solvent was evaporated and the residue was dissolved in methanol (0.5 mL) and water (1.5 mL) was added and the solution was applied to semi-preparative column of Lichrosorb RP-18 (75 mL) pretreated with water. Polar impurities were washed out with water and the product was eluted with 80% aqueous methanol. The main fraction contained oily sulfate **6a**, 120 mg, (45%), which did not crystallize. ^{1}H NMR ($CDCl_3$): 8.92 m, 2 H (H-2′, H-6′); 8.49 tt, 1H, *J* = 1.6, *J′* = 7.8 (H-4′); 8.00 m, 2H (H-3′, H-5′); 4.85 dd, 1H, *J* = 7.9, *J′* = 8.9 (H-17α); 4.50 tt, 1H, *J* = 4.8, *J′* = 11.1 (H-3β); 0.94 s, 3H (3× H-19); 0.83 s, 3H (3 × H-18). MS (negESI): Calcd. for $[C_{26}H_{30}F_5O_6S]^-$: 565.57. Found: 565.2.

2.2.10. 17α-(Pentafluorobenzoyl)oxy-5β-androstan-3α-yl sulfate pyridinium salt (**6b**)

Hydroxy derivative **3b** (200 mg, 0.41 mmol) was processed as described in Section 2.2.9. After chromatography, the sulfate **6b** (240 mg) was crystallized from a mixture of ethanol/diethyl ether. The yield was 175 mg (66%), m.p. 164-166 °C, $[\alpha]_D$ -4 (c 0.28, chloroform). IR: 3200-1950 (N^+–H); 1638, 1548, 1490 (pyridine ring); 1382 (CH_3); 1238, 1171, 1049 (SO_3). ^{1}H NMR ($CDCl_3$): 8.94 m, 2 H (H-2′, H-6′); 8.48 tt, 1H, *J* = 1.6, *J′* = 8.0 (H-4′); 7.99 m, 2 H (H-3′, H-5′); 5.07 bd, 1H, J = 5.9 (H-17β); 4.48 tt, 1H, *J* = 4.9, *J′* = 11.2 (H-3β); 0.92 s, 3H (3× H-19); 0.78 s, 3H (3× H-18). Calcd. for $C_{31}O_{36}F_5NO_6S$ (645.7): C, 57.67; H, 5.62; F, 14.71; N, 2.17; S, 4.97. Found: C, 57.37; H, 5.59; F, 14.56; N, 2.11; S, 4.78.

2.2.11. 3α-Hydroxy-5β-androstan-17β-yl 4-azidotetrafluorobenzoate (**7**)

Ester **3a** (300 mg, 0.62 mmol) in acetone (1.5 mL) was stirred and heated to 55 °C with water (75 μL) and sodium azide (80 mg, 0.69 mmol) for 5 h. Then, the reaction mixture was poured into water with ice, extracted with ethyl acetate (40 mL), and organic layer was washed with saturated aqueous potassium hydrogen carbonate (2×), water, and dried. Solvents were evaporated and the crude product (300 mg) was chromatographed on silica gel (50 mL) in a mixture of petroleum ether/acetone (10:1). The yield of foamy azido derivative **7** was 240 mg (76%). IR: 3609 (OH); 2131 (N_3); 1728 (C=O); 1389, 1378 (CH_3); 1261, 1008 (C-O); 1032 (C–OH). ^{1}H NMR ($CDCl_3$): 4.85 dd, 1H, *J* = 10.9, *J′* = 7.8 (H-17α); 3.64 tt, 1H, *J* = 10.9, *J′* = 4.7 (H-3β); 0.94 s, 3H (3× H-19); 0.83 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −139.9 m, 2 F; −152.3 m, 2 F. Calcd. for $C_{26}H_{31}F_4N_3O_3$ (509.5): C, 61.29; H, 6.13; F, 14.91; N, 8.25. Found: C, 61.53; H, 6.30; F, 14.80.

2.2.12. 5β-Androstane-3α,17β-diyl 17-(4-azidotetrafluorobenzoate) 3-(L-glutamate) (**8**)

Compound **7** (200 mg, 0.39 mmol) was processed in dichloromethane (2 mL) with O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate (131 mg, 0.43 mmol), 4-dimethylaminopyridine (20 mg, 0.16 mmol), and *N*,*N′*-diisopropylcarbodiimide (62 μL, 0.39 mmol) as described in Section 2.2.5. The same work-up gave 290 mg (93%) of protected glutamate and deprotection with trifluoroacetic acid (Section 2.2.7) gave 190 mg (76% overall from **7**) of glutamate **8**. ^{1}H NMR ($CDCl_3$/CD_3OD, 10:1): 4.86 dd, 1H, *J* = 8.7, *J′* = 8.0 (H-17α); 4.84 m (H-3β); 4.02 m 1H, (CH–NH_2); 0.96 s, 3H (3× H-19); 0.84 s, 3H (3× H-18). For ^{13}C NMR data see Tables 1a and b. Calcd. for $C_{31}H_{38}F_4N_4O_6.H_2O$ (656.7): C, 56.70; H, 6.14; F, 11.57; N, 8.53. Found: C, 56.52; H, 5.93; F, 11.77; N, 8.29.

2.2.13. N-(3α-Hydroxy-5β-androstan-17β-yl)-pentafluorobenzamide (**12a**)

To a stirred solution of 3α-hydroxy-5β-androstan-17-one oxime (**9**) (1 g, 3.27 mmol) in methanol (73 mL), nickel(II) chloride hexahydrate (1.55 g, 6.52 mmol) was added and after dissolution, sodium borohydride (1.24 g, 32.80 mmol) was added in portions over 30 min at room temperature. After additional 15 min stirring, the reaction mixture was cooled in ice bath and 1 M sulfuric acid (40 mL) was added under stirring. The mixture was poured into 25% aqueous ammonia, diluted with ice water (1:1, 150 mL), and extracted with chloroform (150 mL). The extract was washed with ice water, dried and the solvent was evaporated. The crude mixture of amines **10a** and **10b** (1 g) was dissolved in dichloromethane (12 mL) while stirring under argon and *N*,*N*-diisopropylethylamine (1.2 mL, 6.89 mmol) was added. Active ester solution was added to this mixture, prepared separately from pentafluorobenzoic acid (763 mg, 3.60 mmol), 1-hydroxybenzotriazole (620 mg, 3.96 mmol), and *N*,*N′*-diisopropylcarbodiimide (557 μL, 3.60 mmol) in

dichloromethane (15 mL) by 20 min stirring at room temperature, cooling with ice bath, and filtering into syringe. After 30 min stirring at room temperature, the reaction mixture was diluted with chloroform (100 mL), washed with 5% hydrochloric acid, saturated aqueous potassium hydrogen carbonate (2×), water, and dried. Solvents were evaporated and the crude mixture of perfluorobenzoates (1.9 g) was chromatographed on a silica gel column (200 mL) in a mixture of petroleum ether/acetone from (20:1) to (10:1). Less polar fractions contained 17α-isomer **12b** (275 mg, 17% from oxime **9**), see below. Further elution gave 1.04 g of amide **12a** (66% from oxime **9**). Analytical sample was crystallized from methanol, m.p. 243–244 °C, $[\alpha]_D$ + 16 (c 0.22, chloroform). IR: 3609 (OH); 3432 (NH); 1685, 1505 (CONH); 1388, 1377 (CH_3); 1033 (C-OH). ^{1}H NMR ($CDCl_3$): 5.72 d, 1H, *J* = 8.9 (NH); 4.09 q, 1H, *J* = 9.2 (H-17α); 3.63 m, 1H (H-3β); 0.94 s, 3H (3× H-19); 0.73 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −142.1 m, 2 F; −152.8 tt, 1 F, *J* = 20.7, *J′* = 2.8; −161.7 m, 2 F. Calcd. for $C_{26}H_{32}F_5NO_2$ (485.5): C, 64.32; H, 6.64; F, 19.56; N, 2.88. Found: C, 64.36; H, 6.72; F, 19.79; N, 2.74.

2.2.14. N-(3α-Hydroxy-5β-androstan-17α-yl)-pentafluorobenzamide (**12b**)

To a stirred solution of 17α-azido-5β-androstan-3α-ol (**11**) (500 mg, 1.57 mmol) in a mixture of dioxane (5 mL) and ethanol (15 mL), sodium borohydride (200 mg, 5.29 mmol) was added followed by a solution of nickel(II) chloride hexahydrate in methanol (0.1 mol.L^{-1}, 1.5 mL, 0.15 mmol). Stirring continued for 45 min at room temperature. Then, the reaction mixture was cooled in ice bath and 1 M sulfuric acid (9 mL) was added under stirring. The mixture was poured into 25% aqueous ammonia diluted with ice water, (1:1, 90 mL) and extracted with chloroform (60 mL). The extract was washed with ice water, dried and the solvent was evaporated. The crude amine (490 mg) was processed as described in Section 2.2.13. The yield was 627 mg (82% to azide **11**) of foamy amide **12b**, $[\alpha]_D$ + 8 (c 0.24, chloroform). IR: 3609 (OH); 3431 (NH); 1682, 1504 (CONH); 1383 (CH_3); 1038 (C-OH). ^{1}H NMR ($CDCl_3$): 5.76 d, 1H, *J* = 8.4 (NH); 4.20 ddd, 1H, *J* = 8.9, *J′* = 7.8, *J″* = 1.4 (H-17β); 3.65 tt, 1H, *J* = 10.9, *J′* = 4.8, (H-3β); 0.93 s, 3H (3× H-19); 0.85 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −141.4 m, 2 F; −152.4 tt, 1 F, *J* = 20.8, *J′* = 2.7; −161.1 m, 2 F. Calcd. for $C_{26}H_{32}F_5NO_2$ (485.5): C, 64.32; H, 6.64; F, 19.56; N, 2.88. Found: C, 64.58; H, 6.70; F, 19.78; N, 2.81. The product was identical with the less polar product from the chromatography of amide **12a** (Section 2.2.13).

2.2.15. 17β-(Pentafluorobenzoyl)amino-5β-androstan-3α-yl O^5-tert-butyl-N-(tert-butoxycarbonyl)-L-glutamate (13a)

Amide 12a (400 mg, 0.82 mmol) in dichloromethane (4 mL) was processed as described in Section 2.2.5 with O5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate (272 mg, 0.90 mmol), 4-dimethylaminopyridine (40 mg, 0.33 mmol), and *N*,*N′*-diisopropylcarbodiimide (128 μL, 0.83 mmol). The yield of foamy protected glutamate **13a** was 630 mg (99%). ^{1}H NMR ($CDCl_3$): 5.74 d, 1H, *J* = 8.9 (NH); 5.08 d, 1H, *J* = 8.4 (NH Boc); 4.77 tt, 1H, *J* = 11.0, *J′* = 4.6 (H-3β); 4.25 m, 1H (CH–NH Boc); 4.11 q, 1H, J = 9.2 (H-17α); 1.45 s, 9H and 1.44 s, 9H (2× $(CH_3)_3C$); 0.95 s, 3H (3× H-19); 0.73 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −141.7 m, 2 F; −152.3 m, 1 F; −161.2 m, 2 F.

2.2.16. 17α-(Pentafluorobenzoyl)amino-5β-androstan-3α-yl O^5-tert-butyl-N-(tert-butoxycarbonyl)-L-glutamate (**13b**)

Amide **12b** (264 mg, 0.54 mmol) in dichloromethane (2.5 mL) was processed as described in Section 2.2.5. with O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate (182 mg, 0.60 mmol), 4-dimethylaminopyridine (25 mg, 0.20 mmol), and *N*,*N′*-diisopropylcarbodiimide (85 μL, 0.55 mmol). The yield of foamy protected glutamate **13b** was 370 mg (89%). ^{1}H NMR ($CDCl_3$): 5.80 d, 1H, J = 9.1 (NH); 5.06 d, 1H, J = 8.4 (NH Boc); 4.76 tt, 1H, *J* = 11.2, *J′* = 4.6 (H-3β); 4.23 m, 2H (CH-NH Boc, H-17α); 1.44 s, 9H and 1.43 s, 9H (2× $(CH_3)_3C$); 0.95 s, 3H (3× H-19); 0.86 s, 3H (3× H-18).

2.2.17. 17β-(Pentafluorobenzoyl)amino-5β-androstan-3α-yl L-glutamate (**14a**)

Protected glutamate **13a** (400 mg, 0.52 mmol) was dissolved in dioxane (0.5 mL) and hydrogen chloride in dioxane (4.0 M solution, 1.5 mL, 6 mmol) was added while stirring at room temperature. After 24 h standing, the solvent was evaporated, the residue was coevaporated twice with ethanol and triturated with ether. Solid product was filtered off, dissolved in a mixture of methanol (4 mL) and pyridine (40 μL, 0.49 mmol) and added dropwise into water with ice (20 mL). After standing at 5 °C overnight, the solid glutamate **14a** was filtered of and dried. The yield was 190 mg (60%). ^{1}H NMR ($CDCl_3$/CD_3OD, 10:1): 4.83 tt, 1H, *J* = 11.2, *J′* = 4.5 (H-3β); 4.07 t, 1H, *J* = 9.4 (H-17α); 3.73 dd, 1H (CH–NH_2); 0.96 s, 3H (3× H-19); 0.73 s, 3H (3× H-18). For ^{13}C NMR data see Tables 1a and b. Calcd. for $C_{31}H_{39}F_5N_2O_5.H_2O$ (632.7): C, 58.85; H, 6.53; F, 15.01; N, 4.43. Found: C, 58.94; H, 6.22; F, 15.20; N, 4.38.

2.2.18. 17α-(Pentafluorobenzoyl)amino-5β-androstan-3α-yl L-glutamate (**14b**)

Protected glutamate **13b** (350 mg, 0.46 mmol) was processed as described in Section 2.2.17. Trituration with ether gave crystalline hydrochloride, 250 mg (83%). ^{1}H NMR (CD_3OD): 4.87 tt, 1H, *J* = 11.2, *J′* = 4.5 (H-3β); 4.08 dd, 1H, *J* = 6.2, *J′* = 1.5 (H-17β); 4.07 t, 1H, *J* = 6.8 (CH–NH_2); 1.01 s, 3H (3× H-19); 0.88 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −141.4 m, 2 F; −153.6 m, 1 F; −161.7 m, 2 F. For ^{13}C NMR data see Tables 1a and b. Solid product was filtered off and processed as described in Section 2.2.17. The glutamate **14b** had rather geloid structure and the yield was 90 mg (32% from **13b**) due to mass loss during filtration. Calcd. for $C_{31}H_{39}F_5N_2O_5.H_2O$ (632.7): C, 58.85; H, 6.53; F, 15.01; N, 4.43. Found: C, 58.52; H, 6.53; F, 14.97; N, 4.20.

2.2.19. 20-Oxo-5β-pregnan-3α-yl L-glutamate hydrochloride (**16**)

3α-Hydroxy-5β-pregnan-20-one (**15**) (500 mg, 1.57 mmol) in dichloromethane (5 mL) was processed with O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate (524 mg, 1.72 mmol), 4-dimethylaminopyridine (50 mg, 0.41 mmol), and *N*,*N′*-diisopropylcarbodiimide (250 μL, 1.61 mmol) as described in Section 2.2.5. Crude foamy protected glutamate (935 mg) was treated in dioxane (1.5 mL) with hydrogen chloride in dioxane (4.0 M solution, 3.0 mL, 12 mmol) overnight. The solvents were evaporated, the residue was coevaporated twice with ethanol and crystallized from minimum ethanol by adding of ether. The yield of hydrochloride **16** was 460 mg (61% from pregnanolone **15**). Calcd. for $C_{26}H_{42}ClNO_5$ (484.1): C, 64.51; H, 8.75; Cl, 7.32; N, 2.89. Found: C, 64.24; H, 8.77; Cl, 7.07; N, 2.73.

2.2.20. 20-Oxo-5β-pregnan-3α-yl N-(pentafluorobenzoyl)-L-glutamate (**17**)

Hydrochloride **16** (250 mg, 0.52 mmol) was suspended in dichloromethane (2 mL) and while stirring under argon *N*,*N*-diisopropylethylamine (0.4 mL, 2.30 mmol) was added. Active ester solution was added to this mixture, prepared separately from pentafluorobenzoic acid (120 mg, 0.57 mmol), 1-hydroxybenzotriazole (89 mg, 0.57 mmol), and *N*,*N′*-diisopropylcarbodiimide (90 μL, 0.58 mmol) in dichloromethane (1.5 mL) by 20 min stirring at room temperature, cooling with ice bath, and filtering into syringe. After 1 h stirring at room temperature, the reaction mixture was diluted with chloroform (100 mL), washed with 10% sulfuric acid, water (2 ×), and dried. Solvents were evaporated and the

crude product (380 mg) was chromatographed on a silica gel column (75 mL) in a mixture of benzene/ethyl acetate from (10:1) to (5:1). The yield of foamy perfluorobenzoyl glutamate **17** was 300 mg (91%), $[\alpha]_D$ + 66 (c 0.32, chloroform). IR: 3513 (OH); 3091, 2649 (OH, dimer); 3407 (NH); 1728 (C=O, ester); 1713 (C=O, dimer); 1695, 1505 (CONH); 1386, 1357 (CH_3). ^{1}H NMR ($CDCl_3$): 6.90 d, 1H, *J* = 7.5 (NH); 4.83 tt, 1H, *J* = 11.2, *J′* = 4.5 (H-3β); 4.79 dt, 1H, *J* = 7.5, *J′* = 4.8 (C**H**–NH); 2.12 s, 3H (3× H-21); 0.95 s, 3H (3× H-19); 0.61 s, 3H (3× H-18). ^{19}F NMR ($CDCl_3$): −141.3 m, 2 F; −151.1 tt, 1 F, *J* = 20.9, *J′* = 3.2; −161.0 m, 2 F. Calcd. for $C_{33}H_{40}F_5NO_6$ (641.7): C, 61.77; H, 6.28; F, 14.80; N, 2.18;. Found: C, 61.89; H, 6.06; F, 15.10; N, 1.95.

*2.2.21. 3,20-Dioxo-5β-pregnan-11α-yl pentafluorobenzoate (**19**)*

A solution of 11α-hydroxy-5β-pregnane-3,20-dione (**18**) (900 mg, 2.71 mmol) in dichloromethane (9 mL) was stirred with 4-dimethylaminopyridine (90 mg, 0.74 mmol) and pentafluorobenzoic acid (630 mg, 2.97 mmol) at room temperature and *N*,*N′*-diisopropylcarbodiimide (465 μL, 3.00 mmol) was added dropwise. After 1 h stirring, the reaction mixture was cooled with ice bath, filtered into syringe and applied to silica gel column (50 mL) pretreated with petroleum ether. The product was eluted with a mixture of petroleum ether/acetone (10:1); the yield was 1.3 g (91%). Analytical sample of **19** was crystallized from a mixture of acetone/hexanes, m.p. 156-157 °C, $[\alpha]_D$ + 67 (c 0.37, chloroform). IR: 1732 (C=O, ester); 1706 (C=O, ketone); 1391, 1382, 1357 (CH_3); 1234, 1109 (C–O). ^{1}H NMR ($CDCl_3$): 5.50 dt 1H, *J* = 5.2, *J′* = 10.5 (H-11β); 2.13 s, 3H (3× H-21); 1.19 s, 3H (3× H-19); 0.75 s, 3H (3× H-18). Calcd. for $C_{28}H_{31}F_5O_4$ (526.5): C, 63.87; H, 5.93; F, 18.04. Found: C, 64.34; H, 5.88; F, 19.89.

*2.2.22. 3α-Hydroxy-20-oxo-5β-pregnan-11α-yl pentafluorobenzoate (**20**)*

Diketone **19** (830 mg, 1.58 mmol) was dissolved in tetrahydrofuran (33 mL), stirred and cooled to -70 °C and lithium tri(*tert*-butoxy)aluminum hydride (600 mg, 2.36 mmol) was added during 5 min. The reaction mixture was stirred 30 min at −70 °C, then allowed to attain −30 °C during 45 min and stirred additional 1 h. Saturated aqueous solution of ammonium chloride (2 mL) was added and about one half of the solvent was evaporated. The mixture was poured into 5% hydrochloric acid and extracted with ethyl acetate (80 mL). The extract was washed with saturated aqueous potassium hydrogen carbonate (2×), water, and dried. The solvents were evaporated and the crude product (860 mg) was chromatographed on a silica gel column (160 mL) in a mixture of petroleum ether/acetone from (10:1) to (5:1). The main fractions contained hydroxy derivative **20** (580 mg, 70%) with spots of less polar 3β-isomer. Crystallization from a mixture of acetone/hexanes gave pure compound (450 mg, 55%), m.p. 174–175 °C, $[\alpha]_D$ +77 (c 0.26, chloroform). IR: 3610 (OH); 1731 (C=O, ester); 1703 (C=O, ketone); 1390, 1360 (CH_3); 1245, 1235, 1107 (C–O); 1037 (C-OH). ^{1}H NMR ($CDCl_3$): 5.42 dt 1H, *J* = 5.4, *J′* = 10.5 (H-11β); 3.63 m, 1H (H-3β); 2.12 s, 3H (3× H-21); 1.09 s, 3H (3× H-19); 0.70 s, 3H (3× H-18). Calcd. for $C_{28}H_{33}F_5O_4$ (528.6): C, 63.63; H, 6.29; F, 17.97. Found: C, 63.52; H, 6.48; F, 17.81.

*2.2.23. 20-Oxo-11α-(pentafluorobenzoyl)oxy-5β-pregnan-3α-yl sulfate pyridinium salt (**21**)*

Hydroxy derivative **20** (200 mg, 0.38 mmol) in pyridine (3 mL), sulfur trioxide - pyridine complex (180 mg, 1.13 mmol) was processed as described in Section 2.2.9. The crude sulfate after chromatography (250 mg) was crystallized from a mixture of ethanol/diethyl ether. The yield of **21** was 145 mg (51%), m.p. 187-189 °C, $[\alpha]_D$ + 78 (c 0.23, chloroform). ^{1}H NMR ($CDCl_3$): 8.92 m, 2 H (H-2′, H-6′); 8.48 tt, 1H, *J* = 1.6, *J′* = 7.9 (H-4′); 7.98 m, 2 H (H-3′, H-5′); 5.42 dt, 1H, *J* = 5.3, *J′* = 10.4 (H-11β); 4.45 m, 1H, (H-3β); 2.59 t, 1H, *J* = 8.9 (H-17α); 2.51 dd, 1H, *J* = 5.3, *J′* = 11.7 (H-12α); 2.11 s, 3H (3× H-21); 0.92 s, 3H (3× H-19); 0.78 s, 3H (3× H-18). Calcd. for $C_{33}H_{38}F_5NO_7S$ (687.7): C, 57.63; H, 5.57; F, 13.81; N, 2.04; S, 4.66. Found: C, 57.46; H, 5.53; F, 14.04; N, 1.97; S, 4.79.

2.3. Biological assays

For the electrophysiological experiments on NMDA receptors, human embryonic kidney cells (HEK293) were transfected with GluN1–1a/GluN2B/GFP plasmids as described previously [16]. Experiments were performed 24–48 h after the end of transfection. HEK293 cells were voltage-clamped at a holding potential of −60 mV and currents elicited were recorded. Whole-cell voltage-clamp recordings were made with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvate, CA). Patch pipettes (3–5 M Ω) pulled from borosilicate glass were filled with Cs^+-based intracellular solution (Cs-ICS) containing the following (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 the following: (in mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 0.2 EDTA, and 0.7 $CaCl_2$ (pH-adjusted to 7.3 with NaOH). Glycine (10 μM), a NMDA receptor co-agonist, was present in the control and test solutions. All steroids solutions were made from freshly prepared 20 mM stock in dimethyl sulfoxide (DMSO). The same concentration of DMSO was added in all extracellular solutions (see [17] for details).

3. Results and discussion

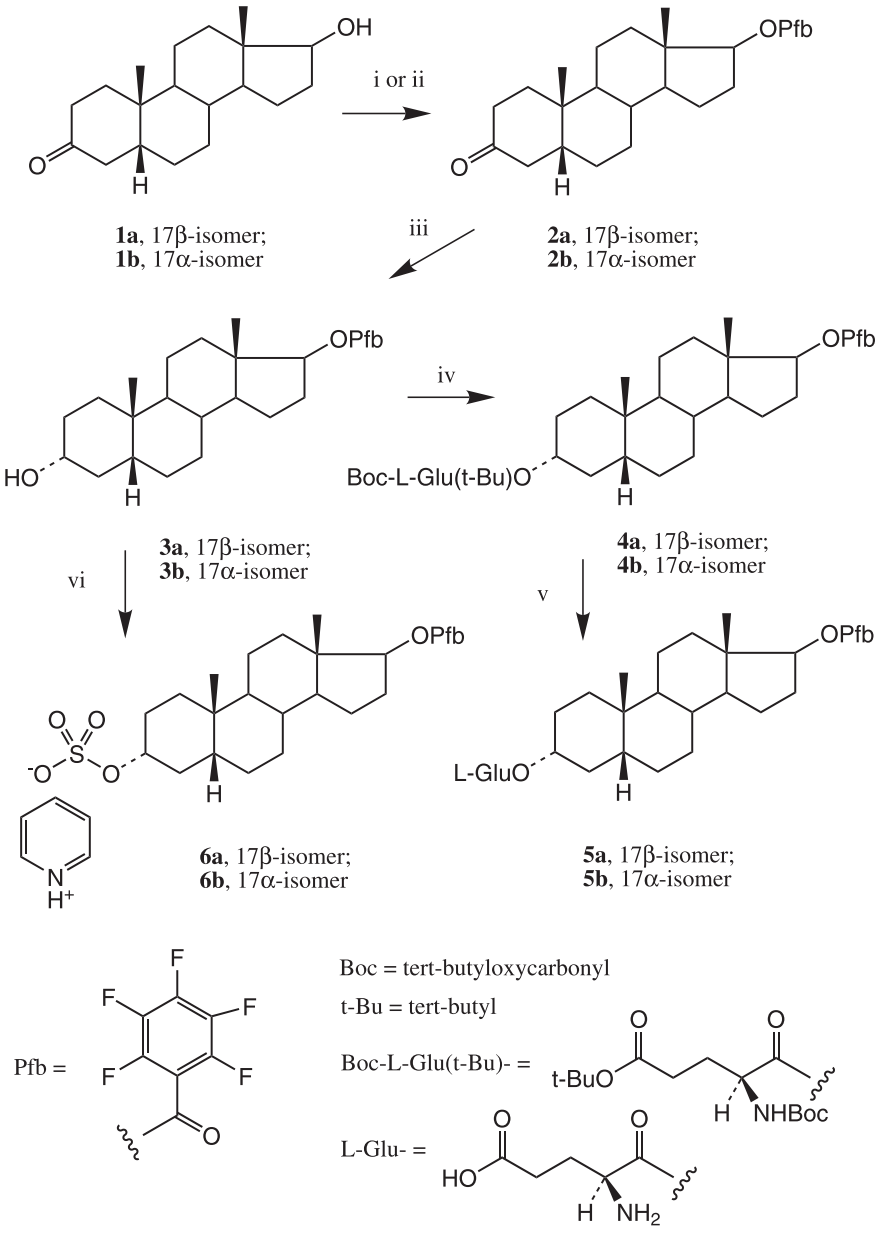
3.1. Syntheses of perfluorobenzoylated compounds

The initial experiments were performed on the 5β-androstan-3α-ol skeleton with perfluorobenzoyl group attached in position 17 (Scheme 1). Starting compound was easily available 17β-hydroxy-5β-androstan-3-one (**1a**) [10], which was reacted with perfluorobenzoyl chloride in pyridine and resulting ester **2a** was selectively reduced with sodium borohydride in presence of cerium(III) chloride heptahydrate [18]. Thus obtained 3α-hydroxy derivative **3a** was transformed both into a glutamate **5a** and to a sulfate **6a**. For a preparation of glutamate, an indirect method was used. In the first step, protected glutamate **4a** was prepared by the reaction of **3a** with O^5-*tert*-butyl hydrogen *N*-(*tert*-butoxycarbonyl)-L-glutamate and *N*,*N′*-diisopropylcarbodiimide in presence of 4-dimethylaminopyridine. Then, both protective groups were removed with trifluoroacetic acid. Corresponding sulfate was prepared in form of pyridinium salt by reaction **3a** with sulfur(VI) oxide - pyridine complex.

The preparation of 17α-isomer **3b** was similar, except the first step, where the reaction of 17α-hydroxy-5β-androstan-3-one (**1b**) [11,12] with perfluorobenzoic acid, *N*,*N′*-diisopropylcarbodiimide, and 4-dimethylaminopyridine was used. The yield of perfluorobenzoate **2b** was slightly lower than for 17β-isomer (81%), but the acid is cheaper and more easily handled in comparison with corresponding acid chloride. The ketone reduction to **3b**, preparation of glutamate **5b** via protected intermediate **4b** and preparation of sulfate **6b** were identical to procedures used previously.

In the case of more accessible 17β ester **3a**, conversion into 4-azidoperfluorobenzoate was performed (Scheme 2). Nucleophilic substitution with sodium azide in acetone at 55 °C took 5 h and it was accompanied in some extent with thermal degradation. Nevertheless, it was easy way to azide **7**, which was then transformed into glutamate **8** by the indirect procedure mentioned previously.

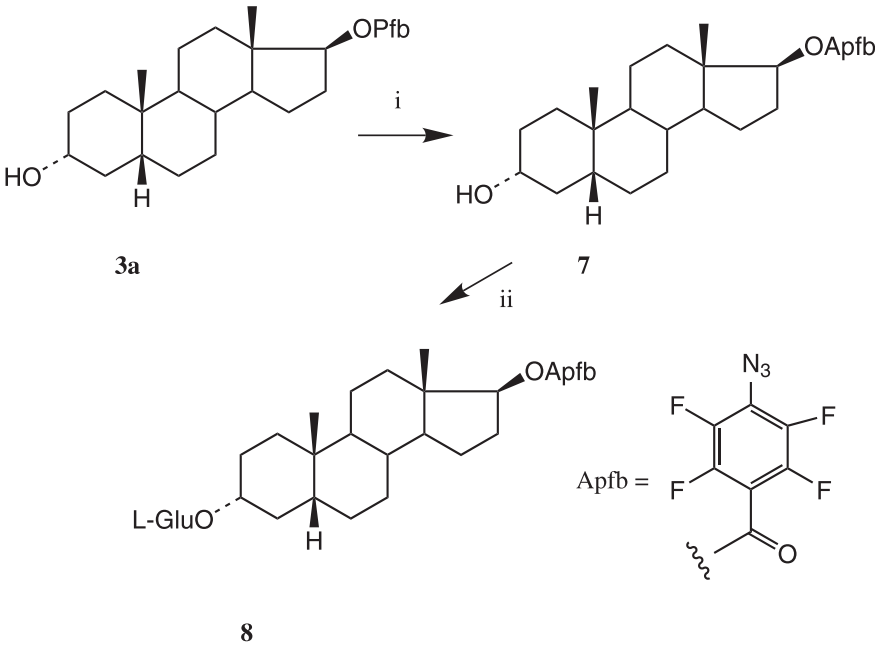
For the synthesis of analogous amides, firstly the corresponding amino derivatives were prepared (Scheme 3). As a starting com-



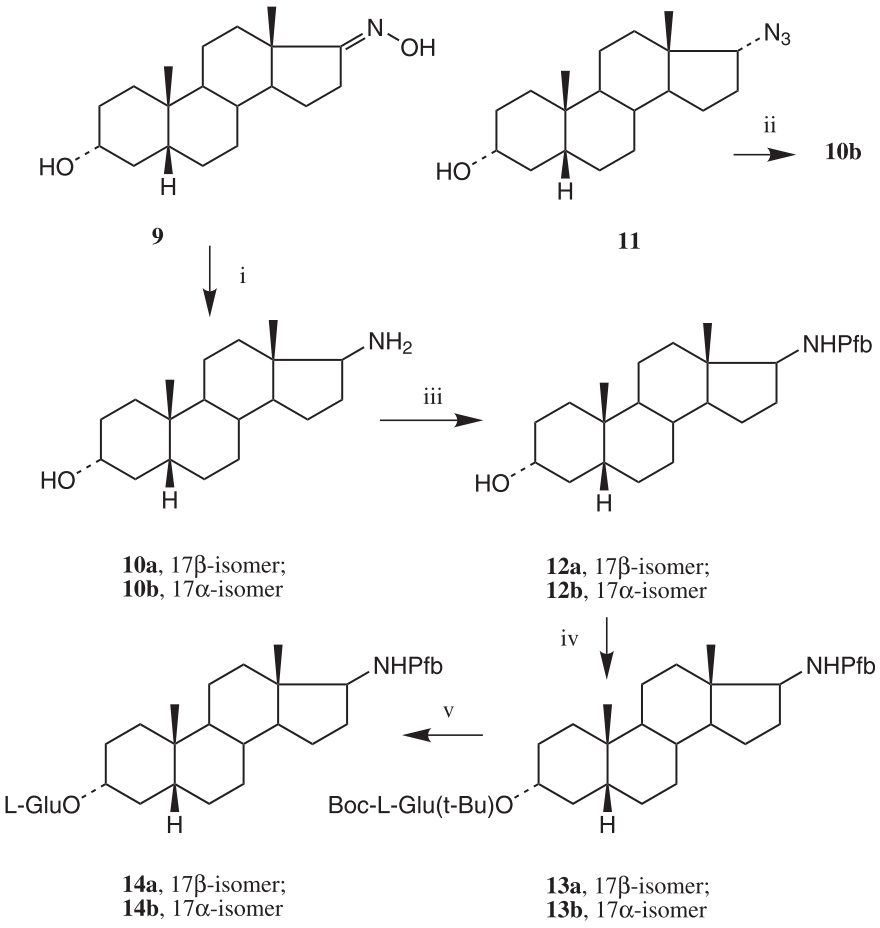
Scheme 1. Synthesis of 17-perfluorobenzoyl derivatives. (i) PfbCl / Py, r.t., 1 h, 93%; (ii) PfbOH, DMAP, DIC / DCM, r.t., 1 h, 81%; (iii) $NaBH_4$, $CeCl_3.7H_2O$ / EtOAc, MeOH, r.t., 15 min, 17β: 74%, 17α: 92%; (iv) Boc-L-Glu(t-Bu)OH, DMAP, DIC / DCM, r.t., 1 h, 17β: 94%, 17α: 71%; and (v) 1. TFA, r.t., 10 min, 2. Py / MeOH, 17β: 93%, 17α: 96%; (vi) SO_3-Py / Py, r.t., 30 min, 17β: 45%, 17α: 66%.

pound, 3α-hydroxy-5β-androstan-17-one oxime (**9**) [13] was used. Reduction with sodium borohydride – nickel(II) chloride [19,20] gave preferently 17β-amino derivative **10a** with an admixture of 17α-isomer **10b**. The crude mixture of amines **10a** and **10b** reacted with active ester, prepared from 1-hydroxybenzotriazole and perfluorobenzoic acid. The resulting mixture of amides **12a** and **12b** was separated by column chromatography. From the isolated yields, ratio of isomers can be estimated roughly as 4:1 in favor of **12a**. For the preparation of 17α-isomer **12b** more suitable synthesis can be alternatively used. 17α-Azido-5β-androstan-3α-ol (**11**) [9] gave with before-mentioned reducing agent pure amine **10b** and subsequently amide **12b** in the 82% overall yield. Both amides **12a** and **12b** were transformed into corresponding glutamates **14a** and **14b** via protected derivatives **13a** and **13b** in the analogous way, as described for the esters **5a** and **5b**. For the final deprotection, hydrogen chloride in dioxane was used and in the case of derivative **14b**, corresponding hydrochloride could be isolated.

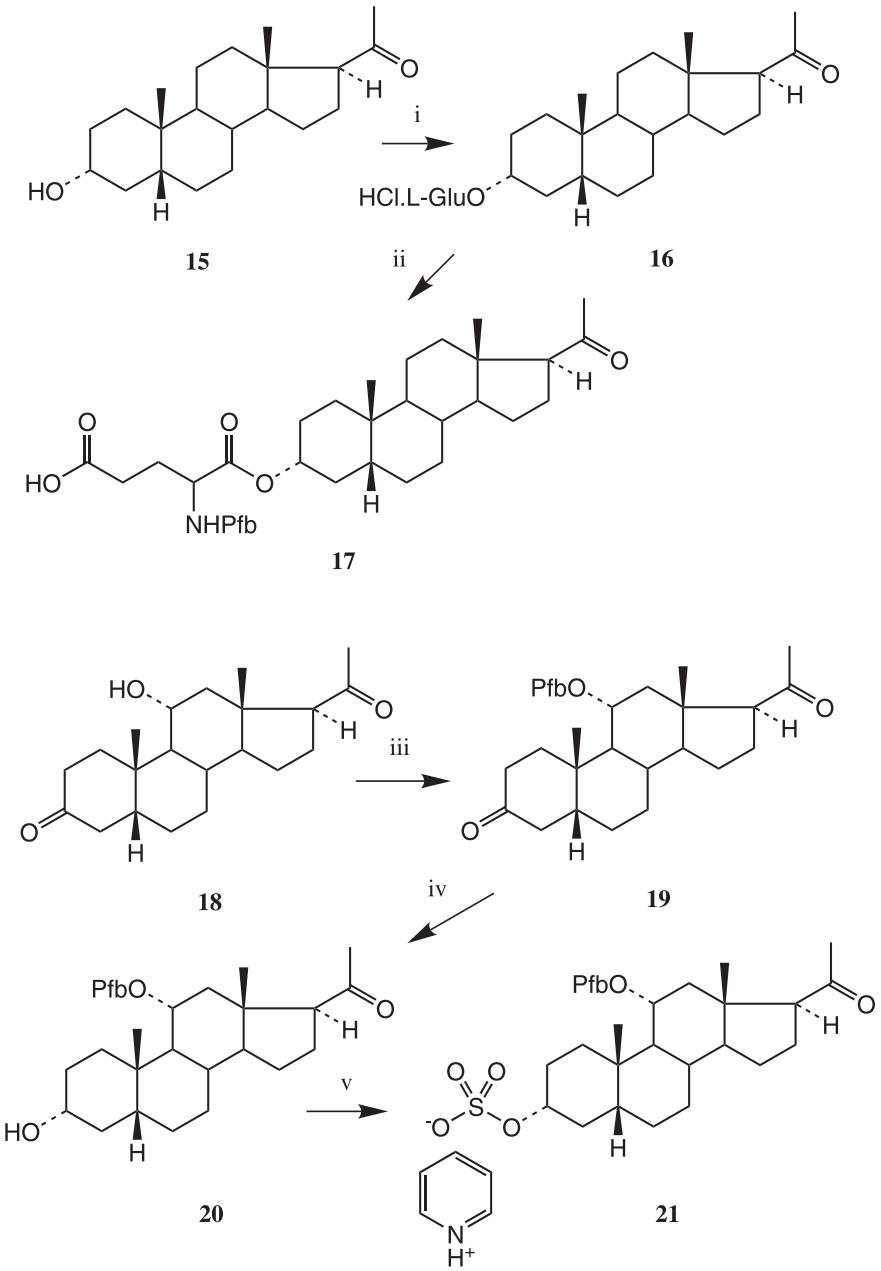
The series was expanded with derivatives of different type (Scheme 4). From 3α-hydroxy-5β-pregnan-20-one **15** (pregnanolone), the corresponding glutamate [21] was prepared in form of hydrochloride **16** and condensation with active ester of perfluorobenzoic acid with procedure mentioned previously gave perfluorobenzamide **17**. Finally, from 11α-hydroxy-5β-pregnane-3,20-dione (**18**) [14,15], perfluorobenzoate **19** was prepared by the method used for 17-hydroxy derivative **3b** and subsequent selective reduction of 3-keto group with lithium tri(*tert*-butoxy)aluminum hydride [22] gave 3α-hydroxy derivative **20**. For transformation into corresponding sulfate **21**, reaction with sulfur(VI) oxide - pyridine complex was used.



Scheme 2. Synthesis of 4-azidoperfluorobenzoate. (i) NaN_3 / aq. acetone, 55 °C, 5 h, 76%; and (ii) 1. Boc-L-Glu(t-Bu)OH, DMAP, DIC / DCM, r.t., 1 h, 2. TFA, r.t., 10 min, 3. Py / MeOH, 76%.



Scheme 3. Synthesis of 17-perfluorobenzamido derivatives. (i) $NaBH_4$, $NiCl_2.6H_2O$ / MeOH, r.t., 45 min; (ii) $NaBH_4$, $NiCl_2.6H_2O$ / dioxane - EtOH, r.t., 45 min; (iii) 1. PfbOH, HOBt, DIC / DCM, r.t. 20 min, 2. DIPEA / DCM, r.t., 30 min, 17β: 66% (i) + (ii), 17α: 82% (i) + (iii); (iv) Boc-L-Glu(t-Bu)OH, DMAP, DIC / DCM, r.t., 1 h, 17β: 99%, 17α: 89%; and (v) 1. HCl / dioxane, r.t., 24 h, 2. Py / MeOH, 17β: 60%, 17α: 32%.



Scheme 4. Syntheses of perfluorobenzoyl pregnanolone derivatives. (i) 1. Boc-L-Glu(t-Bu)OH, DMAP, DIC / DCM, r.t., 1 h, 2. HCl / dioxane, r.t., o.n., 61%; (ii) PfbOH, HOBt, DIC / DCM, r.t. 20 min, 2. DIPEA, / DCM, r.t., 1 h, 91%; (iii) PfbOH, DMAP, DIC / DCM, r.t., 1 h, 91%; (iv) Li(tri-t-BuO)AlH / THF, -70 to -30 °C, 2.25 h, 55%; and (v) SO_3-Py / Py, r.t., 30 min, 51%.

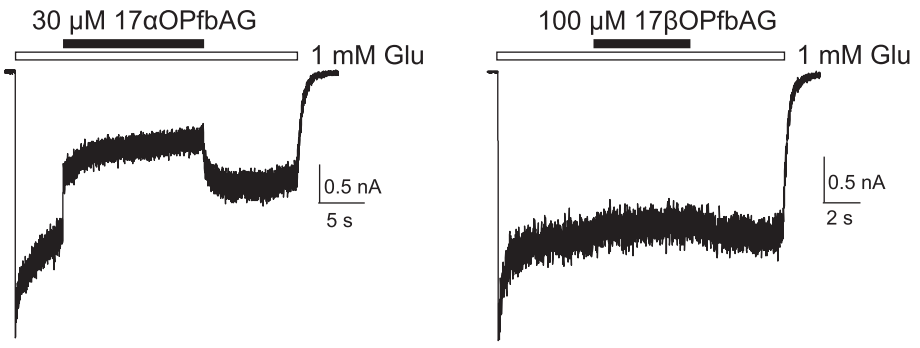


Fig. 1. Example of traces obtained from HEK293 cells transfected with GluN1/GluN2B receptor. Perfluorobenzoates **5a** (17βOPfbAG, 100 μM) and **5b** (17αOPfbAG, 30 μM) were applied simultaneously with 1 mM glutamate (duration of steroid and glutamate application is indicated by filled and open bars, respectively).

Table 2
Inhibition of Glu-induced response in recombinant NMDA receptors cells by perfluorobenzoate substituted steroids.

Compound[a]	Conc. (μM)	% Inhib. ± S.D.[b]	IC_{50} (μM) ± S.D.[c]	n[d]
17βOPfbAG **5a**	100	6.4 ± 3.3	1580 ± 1105	5
17αOPfbAG **5b**	30	39.5 ± 7.0	44.1 ± 11.9	4
17βOPfbAS **6a**	100	76.6 ± 2.0	37.3 ± 3.5	4
17αOPfbAS **6b**	10	76.7 ± 4.9	3.7 ± 0.8	5
17βOApfbAG **8**	100	6.0 ± 1.8	1365 ± 565	5
17βNHPfbAG **14a**	100	9.0 ± 2.2	718 ± 175	5
17αNHPfbAG **14b**	50	53.0 ± 3.2	45.5 ± 4.9	5
NHPfbPG **17**	50	73.2 ± 1.9	22.2 ± 1.7	5
11αOPfbPS **21**	50	82.2 ± 7.8	15.7 ± 2.7	5

[a] Skeleton and position/configuration of perfluorobenzoate group (OPfb – perfluorobenzoate, OApfb – 4-azidoperfluorobenzoate, NHPfb – perfluorobenzamide) were indicated (AG – 5β-androstan-3α-ol glutamate, AS – 5β-androstan-3α-ol sulfate, PG – pregnanolone glutamate, PS – pregnanolone sulfate).

[b] Relative degree of steroid inhibition of current responses induced in GluN1-1a/GluN2B receptors cells by fast application of 100 μM glutamate.

[c] The IC_{50} values were determined from a single dose of steroid using the formula $IC_{50} = [S]\cdot((1-I)/I)^{1/h}$, where I is the relative degree of inhibition, [S] is the steroid concentration used, and h (fixed at 1.2) is the apparent Hill coefficient [17]. Calculations of IC_{50} were made assuming 100% inhibition at saturating steroid concentration. Results are expressed as mean ± standard deviation (S.D.).

[d] Number of cells studied.

3.2. Biological activity

Perfluorobenzoyl derivatives **5a**, **5b**, **6a**, **6b**, **14a**, **14b**, **17**, **21** and azide **8** were tested on responses mediated by glutamate on NMDA receptors (Table 2). Data for compounds with perfluorobenzoyl substitution in position 17 indicate that the values of IC_{50} for 17α-derivatives are in general lower than for 17β-epimers; for an example of response curves for 17-epimers **5a** and **5b** see Fig. 1. Sulfates are more active than glutamates. From derivatives with greater inhibition activity, sulfates **6a**, **6b**, and **21** and substituted glutamate **17** can be named. Azide **8** has approximately similar activity as unsubstituted perfluorobenzoate **5a**. Except 17β-derivatives **5a**, **8**, and **14a**, all other derivatives have inhibition activity comparable or better than pregnanolone sulfate on the same receptors (IC_{50} = 44.4 ± 9.8 μM, [17]) or pregnanolone glutamate on hippocampal neurons (IC_{50} = 69.1 ± 9.9, [16]).

Our recent results suggest that the mechanism of inhibitory steroid action at NMDA receptors is complex and support hypothesis that a steroid accesses the receptor from cell membranes [23]. The results of this study suggest that steroids have different potency at NMDA receptors depending on α or β position of the residue at carbon C-17. This supports a hypothesis of a direct stereo-specific interaction of steroid with the protein structure of the NMDA receptor, and argues against indirect steroid effect induced by e.g. perturbation of the physical membrane properties that may as a consequence affect NMDA receptor channel activity [24].

4. Conclusion

5β-Androstane and 5β-pregnane derivatives with perfluorobenzoyl group attached in various parts of molecule were synthesized. Both ester and amide bonds were used for bindings. Among eight structural types tested, only two 17β-substituted derivatives **5a** and **14a** had significantly low inhibition activity, the others displayed activity sufficient for labeling studies. Interesting results were found for 17α-substituted derivatives **5b**, **6b**, and **14b**. For them significant inhibition activity was found, even if methylketone in position 17 is absent and configuration of new substituent is opposite to the original side chain. To our opinion, compounds **17** and **21**, where the base skeleton of pregnanolone is conserved and activity is high, are the most promising for future studies on NMDA receptor.

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