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Synthesis of deuterium labeled NMDA receptor inhibitor – 20-Oxo-5β-[9,12,12-$^2H_3$]pregnan-3α-yl-L-glutamyl 1-ester

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ABSTRACT

20-Oxo-5β-[9,12,12-$^2H_3$]pregnan-3α-yl-L-glutamyl 1-ester **11** was synthesized as an internal standard for quantification of a neuroprotective NMDA receptor ligand, 20-oxo-5β-pregnan-3α-yl-L-glutamyl 1-ester **18** and its metabolites, in plasma and tissue. 11α-Hydroxy-progesterone (**1**) was reduced under basic conditions to yield the corresponding 5β-steroid. Protection of the 3- and 20-oxo groups and oxidation of the 11α-hydroxy group was then followed by a deuterium exchange, conducted under basic conditions using deuterated methanol. Next, the carbonyl moiety at C-11 was reduced and the 11α-hydroxyl group removed through utilization of the Barton–McCombie reaction. Subsequent deprotection of the 3- and 20-acetals and stereoselective reduction of the 3-oxo group gave the desired trideuterated pregnanolone (**8**). This was coupled with protected glutamic acid, which was then deprotected to yield [9,12,12-$^2H_3$]-pregnanolone glutamate (**11**) with >99% isotopic purity.

1. Introduction

Over the last 20 years, there has been a growing interest in neurosteroids. These steroids are synthesized either *de novo* in the brain or through the metabolic transformation of circulating precursors. Neurosteroids exert their effects through modulation of the synaptic and/or extrasynaptic neurotransmitter–receptors, and are utilized by the brain for fine-tuning the function of the neural network [1]. Often, excitatory transmissions are mediated by L-glutamic acid, namely through the *N*-methyl-D-aspartate receptor (NMDA) receptor. This receptor forms a Ca^{2+} permeable ion channel, and under physiological conditions the activation of this receptor is essential for long-term potentiation and basic cognitive function, including learning and memory [2]. However, overexcitation of the NMDA receptor can also induce cell death. This excitotoxicity is often seen in conjunction with ischemia injuries in the brain, and is also thought to contribute to the neurodegeneration associated with various forms of dementia [2]. While a number of clinical studies have shown that NMDA receptor antagonists exhibit a neuroprotective effect in several human disorders [3–6], their clinical utility is rather limited due to the severity of their side effects [7].

Of interest however, is the fact that progesterone and its reduced metabolites also behave as endogenous neuroprotectives [8,9]. In particular, 3α,5β-pregnanolone sulfate has been shown to be an effective, use-dependent antagonist of the NMDA receptor with a high neuroprotective potential [10]. Unfortunately, this ligand is susceptible to the fast metabolic deactivation caused by sulfatases [11]. Moreover, the bioavailability of this, and other steroid sulfates is low. On the other hand, derivatives wherein the sulfate moiety is substituted by glutamate or another charged substituent have shown pharmacological promise, as they have displayed neuroprotective effects in behavioral tests [12]. Therefore, our focus is on the development of novel synthetic steroidal inhibitors of the NMDA receptor that are resistant to sulfohydrolase activity. As such, 3α,5β-pregnanolone glutamate **18** was selected as our lead structure [12]. In order to examine its bioavailability and pharmacokinetics, it was also necessary to synthesize an isotopically labeled inner standard. This would allow for the quantitative assessment of steroid **18** and its metabolites in tissues and body fluids through the use of LC/MS. Theoretically, two stable isotopes, ^{13}C or deuterium (^2H) could be used. The synthesis of ^{13}C-labeled compounds is rather complex and expensive. In contrast, the incorporation of a deuterium label is generally less difficult and relatively inexpensive. Furthermore, an inner standard

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molecule having deuterium labels differs by only 3–4 mass units from the tested molecule [13], and therefore would not affect chromatographic separation nor would its MS peak overlap with the natural isotopic steroid peaks. While a deuterated L-glutamic acid is commercially available, its deuterium labels are adjacent to a carbonyl group, and are consequently susceptible to deuterium/proton exchange. Moreover, the isotopic labels are unsuitably placed on a hydrolyzable substituent. However, deuterium labels that are incorporated directly into the steroid core will remain intact during chemical processes, analytical procedures, or metabolic degradation.

The following paper describes an efficient synthesis of a trideuterated [9,12,12-$^2H_3$]-3α,5β-pregnanolone glutamate (3α5βP-Glu-d_3, **11**), containing deuterium atoms in positions 9α, 12α, and 12β from the commercially available 11α-hydroxyprogesterone (**1**). Importantly, all three deuterium labels are located in specific positions such that both the chemical and metabolic stability is guaranteed.

2. Experimental

2.1. General methods

Melting points were determined on a micro-melting point apparatus Hund/Wetzlar (Germany) and are uncorrected. Optical rotations were measured in chloroform using an Autopol IV (Rudolf Research Analytical, Flanders, USA), $[\alpha]_D$ values are given in $^\circ$ (10^{-1} deg cm^2 g^{-1}). IR spectra were recorded on a Bruker IFS 55 spectrometer (wavenumbers in cm^{-1}). Proton and carbon NMR spectra were measured on a FT NMR spectrometer Bruker AVANCE-400 (400, 100 MHz) in $CDCl_3$ with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (delta 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 (prep-TLC) was carried out on 200 × 200 mm plates coated with a 0.4 mm thick layer of the same material. For column chromatography, neutral silica gel 60 μm (Merck) was used. Analytical samples were dried over phosphorus pentoxide at 50 °C/100 Pa. Anhydrous THF was prepared by distillation with benzophenone/Na immediately prior to use. An aqueous solution of potassium bicarbonate was used as a saturated solution.

2.2. Synthesis of [9,12,12-$^2H_3$]-pregnanolone glutamate (**11**)

2.2.1. 11α-Hydroxy-5β-pregnane-3,20-dione (**2**)

Palladium on $CaCO_3$ (1.5 g, 5%) was added into a solution of compound **1** (15 g, 45.1 mmol) in absolute pyridine (66 mL). The reaction mixture was hydrogenated under slight overpressure of hydrogen. After 8 h, the catalyst was filtered off and washed with chloroform. Combined organic eluents concentrated in vacuo and consecutively evaporated three times with toluene (3 × 20 mL). The residue was then poured into diluted hydrochloric acid (100 mL, 5%) and extracted with chloroform (2 × 60 mL). Combined organic extracts were washed with water (50 mL), solution of sodium bicarbonate (50 mL), and dried over sodium sulfate. Solvents were removed under vacuum and the residue was purified by column chromatography on silica gel (450 g, 50% ethyl acetate in petroleum ether). Crystallization from acetone/*n*-heptane afforded 6.9 g (46%) of compound **2a** and 2.6 g (17%) of compound **2b**. Compound **2a**: mp 124–126 °C (lit. 116–118 °C [14]). ^{1}H NMR δ 0.66 s, 3 H (3 × H-18); 1.14 s, 3 H (3 × H-19); 2.14 s, 3 H (3 × H-21); 2.56 t, 1 H, J = 9 (H-17α); 2.67 t, 1 H, J = 14.5 (H-4α); 3.96–4.04 m, 1 H (H-11β). ^{13}C NMR δ 213.78, 208.82, 68.69, 63.30, 55.61, 50.59, 47.14, 45.84, 44.18, 42.70, 39.98, 38.43, 35.98, 34.42, 31.38, 26.83, 25.60, 24.36, 23.10, 23.03, 14.44. $C_{21}H_{32}O_3$ calcd: C, 75.86; H, 9.70. $C_{21}H_{32}O_3$: calcd. C, 75.86; H, 9.70. Found. C, 75.76; H, 9.81. Compound **2b**: mp 196–198 °C (lit. 198–201 °C [15]). ^{1}H NMR δ 0.66 s, 3 H (3 × H-18); 1.14 s, 3 H (3 × H-19); 2.14 s, 3 H (3 × H-21); 2.55 t, 1 H, J_1 = 9 (H-17α); 2.74–2.79 m, 1 H (2β-H); 3.95–4.03 m, 1 H (H-11β). ^{13}C NMR δ 211.75, 208.79, 68.96, 63.29, 59.80, 55.59, 50.65, 47.35, 45.12, 44.07, 40.14, 38.31, 37.27, 34.51, 31.55, 31.36, 29.33, 24.39, 22.95, 14.41, 11.80. $C_{21}H_{32}O_3$: calcd. C, 75.86; H, 9.70. Found. C, 75.77; H, 9.69.

2.2.2. 3,20-Bis(ethylenedioxy)-5β-pregnan-11α-ol (**3**)

A mixture of dione **2a** (4 g, 12.0 mmol), ethylene glycol (4.8 mL, 86.1 mmol), triethyl orthoformate (10.4 mL, 62.5 mmol), and *p*-toluenesulfonic acid monohydrate (42 mg, 0.21 mmol) in absolute benzene (90 mL) was stirred at room temperature overnight. Then, the reaction mixture was poured into saturated solution of sodium bicarbonate (70 mL) and the product was extracted with ethyl acetate (3 × 50 mL). Combined organic extracts were washed with solution of sodium bicarbonate (2 × 150 mL), water (2 × 150 mL), and dried over sodium sulfate. Solvents were removed under vacuo to afford product **3** (3.8 g, 75%) which was used further without purification: $[\alpha]_D$ +15 (c 0.28, $CHCl_3$) (lit. +21 (c 1.06) [16]). ^{1}H NMR δ 0.76 s, 3 H (3 × H-18); 1.07 s, 3 H (3 × H-19); 1.29 s, 3 H (3 × H-21); 3.86–4.01 m, 4 H (OCH_2CH_2O); 3.93 s, 4 H (OCH_2CH_2O); 4.11–4.15 m, 1 H (H-11β). $C_{25}H_{40}O_5$: calcd. C, 71.39; H, 9.59. Found. C, 71.52; H, 9.42.

2.2.3. 3,20-Bis(ethylenedioxy)-5β-pregnan-11-one (**4**)

Pyridinium chlorochromate on aluminum oxide (2.5 g) was added to a solution of compound **3** (2.5 g, 5.9 mmol) in benzene (30 mL). Then, the reaction mixture was stirred at room temperature. After 24 h, pyridinium chlorochromate on aluminum oxide (3 g) was added [17]. After 12 h, the solids were filtered off and washed with benzene. Combined organic filtrates were evaporated and the residue was crystallized from acetone/*n*-heptane to afford white crystals of compound **4** (2.1 g, 84%): mp 124.5–126.5 °C (lit. 125.4–127 °C [18]). ^{1}H NMR δ 0.70 s, 3 H (3 × H-18); 1.17 s, 3 H (3 × H-19); 1.25 s, 3 H (3 × H-21); 3.84-3.99 m, 4 H (OCH_2CH_2O), 3.92 s, 4 H (OCH_2CH_2O). $C_{25}H_{38}O_5$: calcd. C, 71.74; H, 9.15. Found. C, 71.57; H, 9.13.

2.2.4. 3,20-Bis(ethylenedioxy)-11β-hydroxy-5β-[9,12,12-$^2H_3$]pregnane (**5**)

Potassium *tert*-butoxide (1.3 g, 11.6 mmol) was added to a solution of compound **4** (1 g, 2.4 mmol) in MeOD (490 mmol, 20 mL) and tetrahydrofuran (30 mL). The reaction mixture was refluxed under inert atmosphere for 13 days. Then, solvents were evaporated under vacuum and deuterium oxide (2.5 mL) was added. The supernatant was removed by pipette and the precipitate was washed by another portion of deuterium oxide (2.5 mL). The residue was dried by azeotropic evaporation with toluene (3 × 10 mL) and then dissolved in freshly dry tetrahydrofuran (10 mL). This solution of deuterated steroid was added dropwise to the refluxing mixture of lithium aluminum hydride (1 g, 26.3 mmol) in tetrahydrofuran (20 mL) under inert atmosphere. After 2 h, the reaction mixture was allowed to attain room temperature and saturated aqueous solution of sodium sulfate (4 mL) was added. The mixture was dried over the large excess of anhydrous sodium sulfate. Precipitated aluminum salts were decanted and washed with ether (3 × 10 mL). The combined solvents were removed under reduced pressure and the residue was crystallized from petroleum ether/ether to give white crystals of deuterated steroid **5** (950 mg, 94% yield, 99.4% isotopic purity): mp 130–133 °C, $[\alpha]_D$ +42.5 (c 0.15, $CHCl_3$). ^{1}H NMR δ 0.97 s, 3 H

(3 × H-18); 1.20 s, 3 H (3 × H-19); 1.30 s, 3 H (3 × H-21); 3.74–4.00 m, 4 H (OCH_2CH_2O); 3.94 s, 4 H (OCH_2CH_2O); 4.14 s, 1 H (H-11α). ^{13}C NMR δ 111.90, 109.92, 68.48, 64.98, 64.25, 64.09, 63.26, 58.66, 57.85, 44.61, 43.24, 41.25, 35.77, 34.14, 30.83, 30.63, 26.76, 26.43, 25.95, 24.47, 23.76, 22.82, 15.73. IR ($CHCl_3$) 1048 (C-OH), 1181 (CH_2, OCH_2CH_2O), 1048, 1078, 1096 (ring, OCH_2-CH_2O), 2101, 2198 (CD_2), 1365, 1374, 1383 (CH_3). MS (ESI): 446.3 (99.4%, M+Na); 445.3 (0.2% M(D_2)+Na); 444.3 (0.3% M(D_1)+Na); 443.3 (0.1% M(D_0)+Na). $C_{25}H_{37}D_3O_5$: calcd. C, 70.88; H, 8.80. Found. C, 70.98; H, 9.12.

2.2.5. 3,20-Bis(ethylenedioxy)-5β-[9,12,12-$^2H_3$]pregnan-11β-yl-O-(S-methyldithiocarbonate) (**6**)

A solution of *n*-butyllithium (1.6 M in hexane, 1.5 mL, 2.4 mmol) was added dropwise to a stirred solution of alcohol **5** (832 mg, 2.0 mmol) in dry tetrahydrofuran (10 mL) at 0 °C (ice-bath). The reaction mixture was allowed to attain room temperature. After 1 h, carbon disulfide (>99.9%, 0.365 mL, 6.04 mmol) was added and the reaction mixture was stirred overnight at room temperature. Then, methyl iodide (0.25 mL, 4.02 mmol) was added. After 2 h, the reaction mixture was quenched with saturated sodium bicarbonate (50 mL). The product was extracted with diethyl ether (3 × 15 mL), combined organic extracts were washed with brine, dried over sodium sulfate, and the solvent was removed in vacuo. Chromatography on silica gel (30 g) in petroleum ether-ethyl acetate-triethylamine (80:20:1) gave xanthate **6** as a yellow foam (854 mg, 85%): $[\alpha]_D$ +15.0 (c 0.11, $CHCl_3$). ^{1}H NMR δ 0.70 s, 3 H (3 × H-18); 0.94 s, 3 H (3 × H-19); 1.13 s, 3 H (3 × H-21); 2.58 s, 3 H (3 × H-MeS); 3.73-3.90 m, 4 H (OCH_2CH_2O); 3.93 s, 4 H (OCH_2CH_2O); 6.03 s, 1 H (H-11α). ^{13}C NMR δ 214.23, 111.62, 109.68, 80.56, 65.05, 64.25, 64.09, 63.32, 58.59, 57.57, 44.69, 43.17, 40.98, 35.73, 34.35, 31.58, 30.32, 26.46, 26.0, 25.80, 24.40, 23.66, 22.75, 18.88, 15.24. IR ($CHCl_3$) 1245 (C—O—C, xanthate), 1054 (C═S), 1180 (CH_2, OCH_2CH_2O), 2100, 2191 (CD_2), 1373 (CH_3). MS (ESI): 536.3 (99.4%, M+Na); 535.3 (0.2% M(D_2)+Na); 534.3 (0.3% M(D_1)+Na); 533.3 (0.1% M(D_0)+Na). $C_{27}H_{39}D_3O_5S_2$: calcd. C, 63.12; H, 7.65; S, 12.48. Found. C, 62.98, H, 7.88; S, 12.72.

2.2.6. 5β-[9,12,12-$^2H_3$]Pregnane-3,20-dione (**7**)

Tributyltin hydride (0.67 mL, 2.49 mmol) was added to a refluxing solution of compound **6** (848 mg, 1.7 mmol) and 1,1′-azobis(cyclohexanecarbonitrile) (81 mg, 0.33 mmol) in dry toluene (50 mL) under argon atmosphere. After 4 h of reflux, tetrahydrofuran was removed in vacuo and the oily residue was dissolved in acetone (50 mL). Water (5 mL) and HCl (36%, 2 mL) were added and the reaction mixture was stirred for 15 min. Then, the solvents were partly evaporated (1/2) and the residue was poured into the solution of sodium bicarbonate (100 mL). The product was extracted with ethyl acetate (3 × 20 mL), washed with brine (2 × 30 mL), and dried over magnesium sulfate. Solvent was removed in vacuo and the residue was purified by a column chromatography on a silica gel (40 g, 10% ether in petroleum ether) to afford 646 mg (quant.) of compound **7** which was contaminated with traces of inseparable tributyltin residues. Compound **7** was used without further purification. ^{1}H NMR δ 0.64 s, 3 H (3 × H-18); 1.03 s, 3 H (3 × H-19); 2.13 s, 3 H (3 × H-21); 2.55 t, 1 H, J = 9 (H-17α). ^{13}C NMR δ 212.95, 209.33, 63.75, 56.61, 44.17, 44.11, 42.30, 37.17, 36.96, 35.45, 34.85, 31.48, 26.52, 25.76, 24.40, 22.58, 22.57, 20.91, 13.37. IR ($CHCl_3$) 1703 (C═O), 1358 (CH_3), 2100, 2191 (CD_2), 1384, 1358 (CH_3). MS (ESI): 342.3 (99.4%, M+Na); 341.3 (0.2% M(D_2)+Na); 340.3 (0.3% M(D_1)+Na); 339.3 (0.1% M(D_0)+Na).

2.2.7. 3α-Hydroxy-5β-[9,12,12-$^2H_3$]pregnan-20-one (**8**)

An ice-cold solution of sodium hydroxide (44 mg, 1.1 mmol) in water (0.4 mL) and methanol (12 mL) was added to a vigorously stirred ice-cold solution of compound **7** (506 mg, 1.6 mmol) in methanol (37 mL). Then, an ice-cold solution of sodium borohydride (80 mg, 2.1 mmol) in pyridine (11 mL) was added. The progress of the reaction was continuously checked by TLC. Once the reaction was complete (15 min), it was quenched with 5% HCl (pH checked). Resulting mixture was extracted with diethyl ether (3 × 25 mL), and combined organic extracts were consecutively washed with solution of sodium bicarbonate (25 mL) and brine. Organic phase was dried with sodium sulfate and solvent evaporated in vacuo. Chromatography on silica gel (20% ether in petroleum ether) gave desired 3α-alcohol **8** (253 mg, 50%): mp 128–130 °C, $[\alpha]_D$ +86.0 (c 0.1, $CHCl_3$). ^{1}H NMR δ 0.59 s, 3 H (3 × H-18); 0.93 s, 3 H (3 × H-19); 2.11 s, 3 H (3 × H-21); 2.56 t, 1 H, J = 9.1 (H-17α); 3.61–3.68 m, 1 H (H-3). ^{13}C NMR δ 209.60, 71.74, 63.86, 56.71, 44.16, 42.01, 36.42, 35.76, 35.35, 34.54, 31.48, 30.53, 27.07, 26.39, 24.44, 23.26, 22.89, 20.53, 13.34. IR ($CHCl_3$) 1036 (C—OH), 1698 (C═O), 2101, 2191 (CD_2), 1384, 1378 (CH_3). MS (ESI): 344.3 (98%, M+Na); 343.3 (1.9% M(D_2)+Na); 342.3 (0.3% M(D_1)+Na); 341.3 (0.1% M(D_0)+Na). $C_{21}H_{31}D_3O_2$: calcd. C, 78.45; H, 9.72. Found. C, 78.78; H, 10.10.

2.2.8. 20-Oxo-5β-[9,12,12-$^2H_3$]pregnan-3α-yl (2S)-5-(benzyloxy)-2-[(tert-butoxycarbonyl)amino]-5-oxo-pentanoate (**9**)

Compound **8** (245 mg, 0.8 mmol) and Boc-Glu(OBzl)-OH (257 mg, 0.8 mmol) were dissolved in dry benzene (8.5 mL). Then, 4-dimethylaminopyridine (10 mg, 0.1 mmol) and dicyclohexylcarbodiimide (1 M in benzene, 0.84 mL, 0.84 mmol) were added under inert atmosphere and the reaction mixture was stirred at room temperature. After 12 h, the reaction mixture was poured into saturated solution of sodium bicarbonate (50 mL) and the product was extracted with ethyl acetate (3 × 15 mL). Combined organic extracts were washed with water (2 × 15 mL). Precipitated *N,N′*-dicyclohexylurea was filtered off, filtrate was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. Chromatography on silica gel (15 g, 10% ether in petroleum ether) gave oily conjugate **9** (456 mg, 93%): $[\alpha]_D$ +103 (c 0.12, $CHCl_3$). ^{1}H NMR δ 0.60 s, 3 H (3 × H-18); 0.93 s, 3 H (3 × H-19); 1.42–1.45 m, 9 H (3 × CH_3-Boc); 2.12 s, 3 H (3 × H-21); 2.43–2.52 m, 2 H (CH_2-4′); 2.54 t, 1 H, J = 8.8 (H-17α); 4.29 dd, 1 H, J_1 = 8.1, J_2 = 12.8 (H_a, CH-2′); 4.73–4.80 m, 1 H (H-3β); 5.10–5.12 m, 1 H (NH); 5.13–5.16 m, 2 H (CH_2-benzyl); 7.34–7.38 m, 5 H (phenyl). ^{13}C NMR δ 209.40, 170.50, 170.15, 155.36, 135.22, 128.57 (2 × C), 128.26 (2 × C), 128.17, 80.13, 75.69, 66.46, 63.84, 56.61, 50.76, 44.14, 41.84, 35.71, 34.95, 34.54, 32.12, 31.47, 30.32, 28.32 (3 × C), 28.02, 26.86, 26.57, 26.26, 24.41, 23.18, 22.93, 20.56, 13.34. IR ($CHCl_3$) 1704 (C═O, acetate), 1357 (C—H, acetate), 2100, 2192 (CD_2), 1731 (C═O, glutamate), 1714 (C═O, -NHBoc), 1499 (amide, -NHBoc), 1232 (C—O, glutamate); 1384 (CH_3). MS (ESI): 663.4 (98%, M+Na); 662.4 (1.9% M(D_2)+Na); 661.4 (0.3% M(D_1)+Na); 660.4 (0.1% M(D_0)+Na). $C_{38}H_{52}D_3NO_7$: calcd. C, 71.22; H, 8.19; N, 2.19. Found. C, 71.31; H, 8.23; N, 2.16.

2.2.9. 20-Oxo-5β-[9,12,12-$^2H_3$]pregnan-3α-yl N-(tert-butoxycarbonyl)-L-glutamyl 1-ester (**10**)

Palladium on $CaCO_3$ (45 mg, 5%) was added to the solution of compound **9** (449 mg, 0.7 mmol) in absolute MeOH (8 mL). The reaction mixture was hydrogenated under slight overpressure of hydrogen. After 8 h, the reaction mixture was filtered to remove the catalyst, which was washed with chloroform. Combined filtrates were evaporated in vacuo. The residue was dissolved in ether. Evaporation in vacuo gave white foam of compound **10** (379 mg, 98%): $[\alpha]_D$ +140 (c 0.22, $CHCl_3$). ^{1}H NMR δ 0.60 s, 3 H (3 × H-18); 0.94 s, 3 H (3 × H-19); 1.43–1.46 m, 9 H (3 × CH_3-Boc); 2.12 s, 3 H (3 × H-21); 2.44–2.48 m, 2 H (CH_2-4′); 2.55 t, 1 H, J = 8.8 (H-17α); 4.30 dd, 1 H, J_1 = 7.8, J_2 = 12.4 (H_a, CH-2′); 4.76–4.82 m, 1 H (H-3β); 5.20 d, 1 H, J = 7.8 (NH). ^{13}C

NMR δ 209.58, 176.58, 171.52, 151.61, 80.23, 75.86, 63.84, 56.62, 52.90, 44.16, 41.86, 35.72, 34.95, 34.55, 32.13, 31.46, 30.03, 28.31 (3 × C), 28.19, 26.88, 26.57, 26.26, 24.42, 23.19, 22.94, 20.57, 13.35. IR ($CHCl_3$) 1739 (C═O, COOH), 1701 (C═O, acetate), 1357 (C—H, acetate), 2101, 2192 (CD_2), 1730 (C═O, glutamate), 1712 (C═O, NHBoc), 1501 (amide, NHBoc), 1378, 1384 (CH_3). MS (ESI): 573.3 (98%, M+Na); 572.3 (1.9%, M(D_2)+Na); 571.3 (0.3%, M(D_1)+Na); 570.3 (0.1%, M(D_0)+Na). $C_{31}H_{46}D_3NO_7$: calcd. C, 67.61; H, 8.44; N, 2.54. Found. C, 67.75; H, 8.39; N, 2.63.

2.2.10. 20-Oxo-5β-[9,12,12-$^2H_3$]pregnan-3α-yl L-glutamyl 1-ester (**11**)

Trifluoroacetic acid (0.83 mL, 11.2 mmol) was added dropwise to a stirred solution of compound **10** (363 mg, 0.7 mmol) in dichloromethane (6 mL). After 2 h of stirring at room temperature and then 12 h at 5 °C, solvents were removed by azeotropic distillation with benzene. The residue was dissolved in pyridine (1 mL) and MeOH (1 mL), solvents were evaporated. The crude product was dissolved in chloroform (15 mL), washed with water, and dried with anhydrous sodium sulfate. Solvent was removed in vacuo and the oily residue was transformed into a white foam of pregnanolone glutamate **11** (245 mg, 82%) by dissolving in ether and evaporating under reduced pressure at low temperature of the rotary evaporator bath (15 °C): $[\alpha]_D$ +108 (c 0.1, $CHCl_3$). ^1H NMR δ 0.61 s, 3 H (3 × H-18); 0.96 s, 3 H (3 × H-19); 2.14 s, 3 H (3 × H-21); 2.49 t, 2 H, J = 8.8 (CH_2-4′); 2.59 t, 1 H, J = 8.8 (H-17α); 3.73 dd, 1 H, J_1 = 3.8, J_2 = 7.8 (H_a, CH-2′); 4.78–4.88 m, H (H-3β). ^{13}C NMR δ 209.43, 177.53, 171.37, 75.82, 63.85, 56.66, 55.49, 44.29, 41.82, 40.46, 39.16, 35.71, 34.91, 34.61, 32.10, 31.47, 26.85, 26.53, 26.25, 24.40, 23.21, 22.93, 20.86, 13.40. IR ($CHCl_3$) 1739 (C═O, COOH), 1700 (C═O, acetate), 1359 (C—H, acetate), 2099, 2192 (CD_2), 1729 (C═O, glutamate), 3375 (NH_2). MS (ESI): 473.4 (98%, M+Na); 472.4 (1.9% M(D_2)+Na); 471.4 (0.3% M(D_1)+Na); 470.4 (0.1% M(D_0)+Na). $C_{26}H_{38}D_3NO_5$: calcd. C, 69.30; H, 8.51; N, 3.11. Found. C, 69.31; H, 8.53; N, 3.02.

2.3. Synthesis of pregnanolone glutamate (**18**)

2.3.1. 3,20-Bis(ethylenedioxy)-5β-pregnan-11β-ol (**12**)

Lithium aluminum hydride (80 mg, 2.1 mmol) was added in portions to a solution of compound **4** (100 mg, 0.2 mmol) in tetrahydrofuran (6 mL) and the reaction mixture was refluxed. After 2 h, saturated solution of sodium sulfate (2 mL) was added. The mixture was dried over large excess of anhydrous sodium sulfate. The solvent was decanted from aluminum salts, washing with ether (3 × 10 mL). The solvents were removed under reduced pressure and crystallized from petroleum ether/diethyl ether to give white plates of compound **12** (96 mg, 96%): mp 138–139.5 °C (lit. 138.5–139.5 °C [18]). ^1H NMR δ 0.97 s, 3 H (3 × H-18); 1.00–1.08 m, 1 H (H-9α); 1.2 s, 3 H (3 × H-19); 1.29 s, 3 H (3 × H-21); 1.38–1.50 m, 1 H (H-12 α); 2.18-2.23 dd, 1 H, J_1 = 2.5, J_2 = 14.1 (H-12β); 3.85–3.99 m, 4 H (OCH_2CH_2O); 4.16–4.19 m, 1 H (H-11α). $C_{25}H_{40}O_5$: calcd. C, 71.39; H, 9.59. Found. C, 71.17; H, 9.65.

2.3.2. 3,20-Bis(ethylenedioxy)-5β-pregnan-11β-yl-O-(S-methyl-dithiocarbonate) (**13**)

Compound **13** was prepared in the same manner as compound **6** (Section 2.2.5). Starting from compound **12** (250 mg, 0.6 mmol), compound **13** (252 mg, 84%) was obtained as an oily material after column chromatography on silica gel (petroleum ether/ethyl acetate/triethylamine, 80:20:1): $[\alpha]_D$ +11.1 (c 0.27, $CHCl_3$). ^1H NMR δ 0.74 s, 3 H (3 × H-18); 0.94 s, 3 H (3 × H-19); 1.13 s, 3 H (3 × H-21); 1.51–1.60 m, 1 H (H-12 α); 1.73–1.79 m, 1 H (H-9α); 2.31–2.34 dd, 1 H, J_1 = 2.5, J_2 = 14.6 (H-12β); 2.55 s, 3 H (MeS); 3.72–3.88 m, 4 H (OCH_2CH_2O); 5.87 s, 1 H (H-11α). ^{13}C NMR δ 214.49, 111.53, 109.09, 79.81, 64.27, 64.13, 63.82, 63.71, 57.88, 56.31, 43.12, 43.08, 42.94, 42.89, 35.71, 34.32, 32.05, 31.41, 30.34, 26.53, 25.97, 25.71, 24.29, 23.43, 22.66, 19.02, 15.48. IR ($CHCl_3$) 1244 (C—O—C, xanthate), 1054 (C═S), 1179 (CH_2, OCH_2-CH_2O), 1373 (CH_3). $C_{27}H_{42}O_5S_2$: calcd. C, 63.49; H, 8.29. Found. C, 63.86; H, 8.48.

2.3.3. 5β-Pregnane-3,20-dione (**14**)

Compound **14** was prepared in the same manner as compound **7** (Section 2.2.6). Starting from compound **13** (850 mg, 1.7 mmol), compound **14** (808 mg, 95%) was obtained as a white solid: mp 118.6–119 °C (ethyl acetate/*n*-heptane) (lit. 118.5–120 °C [19]). ^1H NMR δ 0.64 s, 3 H (3 × H-18); 1.03 s, 3 H (3 × H-19); 2.13 s, 3 H (3 × H-21); 2.55 t, 1 H, J = 9 (H-17α). $C_{21}H_{32}O_2$: calcd. C, 79.70; H, 10.19. Found. C, 79.73; H, 10.42.

2.3.4. 3α-Hydroxy-5β-pregnan-20-one (**15**)

Compound **15** was prepared in the same manner as compound **8** (Section 2.2.7). Starting from compound **14** (800 mg, 2.5 mmol), compound **15** (408 mg, 50%) was obtained as a white solid after column chromatography on silica gel (20% ether in petroleum ether): mp 144–148 °C (lit. 146–148 °C [20]). ^1H NMR δ 0.60 s, 3 H (3 × H-18); 0.93 s, 3 H (3 × H-19); 2.11 s, 3 H (3 × H-21); 2.53 t, 1 H, J = 9.1 (H-17α); 3.60–3.68 m, 1 H (H-3). $C_{21}H_{34}O_2$: calcd. C, 79.19; H, 10.76. Found. C, 79.10; H, 10.71.

2.3.5. 20-Oxo-5β-pregnan-3α-yl (2S)-5-(benzyloxy)-2-[(tert-butoxycarbonyl)amino]-5-oxo-pentanoate (**16**)

Compound **16** was prepared in the same manner as compound **9** (Section 2.2.8). Starting from compound **15** (250 mg, 0.8 mmol), compound **16** (456 mg, 93%) was obtained as an oily material after column chromatography on silica gel (10% ether in petroleum ether): $[\alpha]_D$ +38.0 (c 0.11, $CHCl_3$). ^1H NMR δ 0.60 s, 3 H (3 × H-18); 0.93 s, 3 H (3 × H-19); 1.42–1.45 m, 9 H (3 × CH_3-Boc); 2.12 s, 3 H (3 × H-21); 2.43–2.47 m, 2 H (CH_2-4′); 2.53 t, 1 H, J = 8.8 (H-17α); 4.3 dd, 1 H, J_1 = 8.1, J_2 = 12.8 (H_a, CH-2′); 4.73–4.78 m, 1 H (H-3β); 5.08–5.10 m, 1 H (NH); 5.13–5.18 m, 2 H (CH_2-benzyl); 7.35–7.40 m, 5 H (phenyl). ^{13}C NMR δ 209.60, 170.70, 170.35, 155.36, 135.42, 128.55 (2 × C), 128.38 (2 × C), 128.31, 80.02, 75.89, 66.71, 63.84, 56.62, 50.19, 44.29, 41.77, 40.38, 39.14, 36.94, 35.74, 34.89, 34.57, 31.98, 31.53 (2 × C), 28.30 (3 × C), 26.82, 26.32, 26.25, 24.38, 23.23, 22.87, 20.82, 13.40. IR ($CHCl_3$) 1704 (C═O, acetate), 1357 (CH_3, acetate), 3435 (NH, NHBoc), 1704 (C═O, NHBoc), 1499 (amide, NHBoc), 2979 (CH_3, NHBoc), 1731 (C═O, glutamate), 1452 (ring, benzyl), 1330 (CH_2, benzyl), 1386 (CH_3). $C_{38}H_{55}NO_7$: calcd. C, 71.55; H, 8.69; N, 2.20. Found. C, 71.62; H, 9.01; N, 2.37.

2.3.6. 20-Oxo-5β-pregnan-3α-yl N-(tert-butoxycarbonyl)-L-glutamyl 1-ester (**17**)

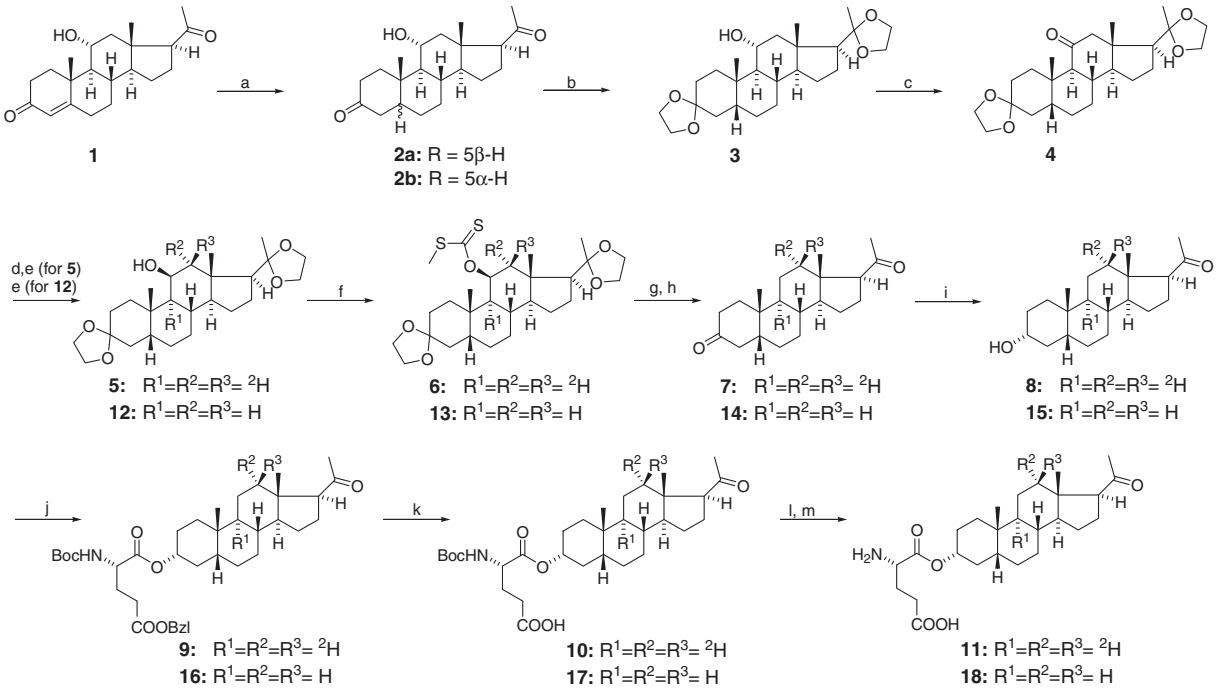
Compound **17** was prepared in the same manner as compound **10** (Section 2.2.9). Starting from compound **16** (200 mg, 0.3 mmol), compound **17** (171 mg, 99%) was obtained as white foam: $[\alpha]_D$ +62.9 (c 0.08, $CHCl_3$). ^1H NMR δ 0.60 s, 3 H (3 × H-18); 0.94 s, 3 H (3 × H-19); 2.12 s, 3 H (3 × H-21); 2.46 t, 2 H, J = 8.8 (CH_2-4′); 2.55 t, 1 H, J = 8.8 (H-17α); 4.3 dd, 1 H, J_1 = 8.0, J_2 = 12.4 (H_a, CH-2′); 4.76–4.81 m, 1 H (H-3β); 5.20 d, 1 H, J = 7.8 (NH). ^{13}C NMR δ 209.83, 175.22, 170.31, 155.45, 80.20, 75.67, 63.86, 56.63, 49.99, 44.33, 41.80 (2 × C), 39.13, 36.61, 35.76, 34.91, 34.60, 32.0, 31.51, 28.30 (3 × C), 26.86, 26.39, 26.26, 25.25, 24.40, 23.25, 22.88, 20.83, 13.40. IR ($CHCl_3$) 1710 (C═O, acetate), 1357 (CH_3, acetate), 3434 (NH, NHBoc), 2979 (CH_3, NHBoc), 1736 (C═O, glutamate, COOH), 3511 (OH, COOH), 1736, 1710 (C═O, COOH), 1486 (CH_3). $C_{31}H_{49}NO_7$: cacld: C, 67.98; H, 9.02; N, 2.56. Found. C, 67.62, H, 8.72; N, 2.49.

*2.3.7. 20-Oxo-5β-pregnan-3α-yl L-glutamyl 1-ester (**18**)*

Compound **18** was prepared in the same manner as compound **11** (Section 2.2.10). Starting from compound **17** (200 mg, 0.4 mmol), compound **18** (159 mg, 98%) was obtained as white foam: $[\alpha]_D$ +106.7 (c 0.12, $CHCl_3$). ^{1}H NMR δ 0.61 s, 3 H (3 × H-18); 0.96 s, 3 H (3 × H-19); 2.13 s, 3 H (3 × H-21); 2.46 t, 2 H, J = 8.8 (CH_2-4′); 2.59 t, 1 H, J = 8.8 (H-17α); 3.61 dd, 1 H, J_1 = 3.8, J_2 = 8.8 (H_a, CH-2′); 4.78–4.86 m, 1 H (H-3β). ^{13}C NMR δ 210.95, 177.67, 170.59, 76.43, 63.59, 56.35, 53.08, 44.18, 41.56, 40.15, 38.78, 35.50, 34.54, 34.31, 33.25, 31.72, 31.14, 26.97, 26.56, 26.19, 25.96, 24.08, 22.85, 22.54, 20.56, 13.03. IR ($CHCl_3$) 3516 (OH, COOH), 1739 (C=O, COOH), 1702 (C=O, acetate), 1359 (C—H, acetate), 1729 (C=O, glutamate), 3376 (NH_2). $C_{26}H_{41}NO_5$: calcd. C, 69.77; H, 9.19; N, 3.11. Found. C, 69.56; H, 9.19; N, 2.99.

3. Results and discussion

The synthesis of [9,12,12-$^2H_3$]-3α,5β-pregnanolone glutamate (**11**) and 3α,5β-pregnanolone glutamate (**18**) is summarized in Scheme 1. The starting material for the synthesis was the commercially available 11α-hydroxyprogesterone **1**. The δ^4 double bond was hydrogenated under basic conditions to give the 5β-derivative **2a** (46% yield). In addition, the 5α-derivative **2b** was also obtained as the minor product (2.6 g, 17%). Their configurations at 5-H were determined by comparison of melting points with literature [14,15] as well as the ^{1}H and ^{13}C spectra. Simple discrimination between 5α- and 5β-isomers of 3-oxo-steroids used to be accomplished by circular dichroism spectra [21]. The Cotton effect of 5α-alfa isomers is positive, while that of 5β-isomers is negative. However, we used more common NMR technique. There is a difference between the signals of hydrogen atoms in the position 4: the ^{1}H NMR of the 5β-derivative **2a** displays a characteristic pseudotriplet of the *axial*-4α-H at δ 2.67 ppm with splitting 14.5 Hz. On contrary, the 5α-derivative **2b** is characterized by similar shape of the *axial*-4β-H but shifted to higher fields ($\delta \sim 2.4$ ppm), where it is overlapped by other signals [22]. Carbonyl moieties in positions C-3 and C-20 were protected as acetals to limit the introduction of the deuterium labels to only the C-9 and C-12 positions. As the acetal protecting group is acid-sensitive, the oxidation of the 11-hydroxyl group (**3**) was achieved by treatment with a mild oxidative reagent – pyridinium chlorochromate on aluminum oxide. Compounds **3** and **4** were prepared in yields of 75% and 84%, respectively. The three deuterium labels were then introduced by the refluxing of compound **4** with deuterated methanol and dry THF in the presence of potassium *tert*-butoxide for 13 days. The extensive reaction time was necessary due to the slower introduction of the deuterium label at the more sterically hindered 12β position, as preliminary experiments showed that deuterium exchange in the less sterically hindered 9α and 12α positions was completed within hours. Next, the 11-oxo group was reduced using lithium aluminum hydride to yield compound **5** in 99.4% isotopic purity (94% yield); with the isotopic distribution as follows: 99.4% $^2H_3$ (*m/z* 446.3, M+Na), 0.2% $^2H_2$ (*m/z* 445.3, M+Na), 0.3% $^2H_1$ (*m/z* 444.3, M+Na), $^2H_0$ (*m/z* 443.3, M+Na). Since position C-11 is extremely sterically hindered, it was not surprising that initial attempts to deoxygenate this position by refluxing an 11β-mesylate with LAH, or reducing an 11-tosylhydrazone with sodium borohydride failed. Eventually, the deoxygenation was achieved by the Barton–McCombie reaction [23,24] via xanthate intermediate **6**, using ABCN for initiation and Bu_3SnH as the hydrogen radical source. Then, the deprotection of acetals was achieved by treatment of compound **6** with hydrochloric acid in water-acetone mixture. The 3-oxo group of compound **7** was selectively reduced utilizing sodium borohydride at low temperature under slightly basic conditions that suppress the reduction of sterically more hindered 20-oxo group. The *equatorial*-3-alcohol **8** was formed as the major product in 50% isolated yield. This corresponds to a known preference of sodium borohydride for axial attack of the conformationally-locked cyclohexanone systems. The equatorial configuration was confirmed by ^{1}H NMR spectroscopy (broad multiplet at 3.65 ppm). The last three steps of the synthesis pertain to the introduction of the glutamyl ester moiety to position C-3. First, compound **8** was esterified with Boc-Glu(OBzl)-OH using DCC and DMAP. Secondly, the benzyl ether protecting group was



Scheme 1. (a) H_2, $Pd/CaCO_3$, pyridine; (b) ethylene glycol, triethyl orthoformate, *p*-TsOH, benzene; (c) PCC/Al_2O_3, benzene; (d) *tert*-BuOK, MeOD, THF, rfl; (e) LAH, THF, rfl; (f) *n*-BuLi, CS_2, CH_3I, THF, rt; (g) ABCN, Bu_3SnH, toluene, rfl; (h) HCl, water, acetone; (i) $NaBH_4$, NaOH, MeOH, H_2O, pyridine; (j) Boc-Glu(OBzl)-OH, DCC, DMAP, benzene; (k) H_2, $Pd/CaCO_3$, MeOH; (l) CF_3COOH, DCM; (m) pyridine, MeOH.

removed by palladium-catalyzed hydrogenation. Finally, *tert*-butoxycarbonyl (Boc) deprotection was performed via acid hydrolysis with trifluoroacetic acid in DCM. Compounds **9**, **10**, and **11** were prepared in yields of 93%, 98%, and 82%, respectively.

Excluding the introduction of the three deuterium labels into the steroidal skeleton, the same synthetic strategy was also used for the synthesis of compound **18**. While this was an unusual and somewhat complex synthetic approach for the synthesis of compound **18**, it would provide a model synthesis by which the feasibility of the proposed synthetic strategy for compound **11** could be examined. Thus, the 11-oxo group of compound **4** was reduced by LAH, followed by the Barton–McCombie deoxygenation, which afforded xanthate **13** in 84% yield. Deprotection of acetal protecting groups was achieved by treatment with hydrochloric acid in water-acetone mixture. Compound **14** was prepared in 95% yield. Selective reduction of the 3-oxo group gave the *equatorial*-3-alcohol **15** (50% yield). Finally, esterification with Boc-Glu(OBzl)-OH, followed by deprotection of the benzyl ether and Boc protecting groups, afforded compound **18**. Compounds **16**, **17**, and **18** were prepared in yields of 93%, 99%, and 98%, respectively.

The study herein describes a convenient method for the introduction of three non-exchangeable deuterium atoms to the steroid skeleton. Accordingly, 3α5βP-Glu-d_3 (**11**) was prepared and was successfully used in evaluating 3α5βP-Glu (**18**) levels in plasma and tissue [12]. This approach can also be expanded to the deuterium labeling of positions 6α, 6β, and 8β, by utilizing a steroid molecule with a 7-oxo group instead of an 11-oxo group, resulting in three non-exchangeable deuterium labels (although, in this case the introduction of a cis-A,B-ring fusion would increase the overall number of synthetic steps) [25]. To the best of our knowledge, the successful introduction of deuterium labels into the non-exchangeable 9α, 12α, and 12β positions on the steroid skeleton has not been described in the literature. In turn, this is a particularly attractive strategy for the synthesis of novel deuterium labeled steroids. In addition, this methodology could also provide useful starting materials for investigating various future avenues in the field of steroid chemistry.

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