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Studies on neurosteroids XV. Development of enzyme-linked immunosorbent assay for examining whether pregnenolone sulfate is a veritable neurosteroid

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

An enzyme-linked immunosorbent assay (ELISA) of pregnenolone sulfate (PREGS) has been developed for examining whether it is a veritable neurosteroid. 11α-Hemiglutaryloxy-PREGS was newly synthesized and conjugated with bovine serum albumin (BSA), which was injected to rabbits for the production of anti-PREGS antibodies. A bridge-heterologous ELISA system employing the sequential saturation method exhibited a high sensitivity with a midpoint of 30 pg. Although the antibody showed some cross-reactivity with PREG (4.4%), it easily discriminated other related steroids reported to exist in the mammalian brain. The rat brain homogenate was treated with hexane and subjected to an OASIS HLB cartridge, which was washed with AcOEt to remove the unconjugated steroids, and then the desired sulfate was eluted with EtOH. The recovery rate of PREGS through the pretreatment was satisfactory, but its brain levels in the preliminary experiments were much lower than those previously measured by gas chromatography (GC)–mass spectrometry (MS) after solvolysis. These results practically agreed with our previous results by liquid chromatography (LC)–electrospray ionization (ESI)–MS without deconjugation.

Keywords: Pregnenolone sulfate; Enzyme-linked immunosorbent assay; Neurosteroid; Rat brain

1. Introduction

In the 1980s, Baulieu and co-workers demonstrated that some steroids, such as pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfates and lipoidal esters, are present in higher concentrations in the brain than in blood using radioimmunoassay (RIA) [1–3]. Furthermore, it was found that the steroids are biosynthesized from cholesterol in the brain without the aid of peripheral sources [4]. Such steroids of nervous origins are now universally referred to as neuro-

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steroids, and this term is implicitly used for the steroids found in large amounts in the brain than in the circulation. These neurosteroids may act as modulators of several membrane receptors, such as γ-aminobutyric acid, *N*-methyl-D-aspartate and sigma receptors, either as stimulators or inhibitors [5,6].

For these reasons, there is a considerable interest in determining the location and levels of different neurosteroids in the brain. The levels of the sulfated neurosteroids have conventionally been evaluated by measuring their genins formed after solvolysis by gas chromatography (GC)–mass spectrometry (MS) [7]. However, the method has some shortcomings, such as requirement of complicated pretreatment and solvolysis steps, loss of information on the conjugation position and decline in recovery rate of the steroid. To overcome these problems, the authors employed liquid chromatography (LC)–electrospray ionization (ESI)–tandem MS to determine PREG sulfate (PREGS) in rat brain without deconjugation [8], but contrary to our expectations, its levels were much lower than those previously reported, which aroused our suspicion regarding whether PREGS fulfils the strict formal criteria of a neurosteroid.

Immunoassay based on an antibody having a high specificity and affinity is an attractive methodology for determination of the sulfated steroids in the brain. Although there are several reports in which the brain levels of sulfates are determined by RIA [1–3], they also required time-consuming separation and solvolysis steps, because they employed antibodies that had been prepared using haptenic derivatives of unconjugated steroids but not those of sulfated steroids. The use of a hapten–carrier conjugate exposing both the A-ring (3β-sulfonic acid residue) and the side chain is expected to provide antibodies having much higher specificity, and the C-11α position of the steroid molecule is an attractive coupling site with the carrier protein. For this reason, the authors synthesized the haptenic derivative of DHEA sulfate (DHEAS) having the bridge at the C-11α position, which produced antibodies having useful properties [9].

In the present paper, the preparation of a rabbit polyclonal antibody to PREGS by immunization of a new haptenic derivative, 11α-hemiglutaryloxy-PREGS (PREGS-HG), linked with bovine serum albumin (BSA) was described. A sensitive bridge-heterologous enzyme-linked immunosorbent assay (ELISA) was then developed using the antibody and applied to the preliminary study on the rat brain PREGS levels.

2. Experimental

2.1. Materials and reagents

PREG, DHEA, epiandrosterone (EA) and progesterone (PROG) were purchased from the Tokyo Kasei Kogyo Co. (Tokyo, Japan). 3β-Hydroxy-5α-pregnan-20-one (3β-AP) and 3α-hydroxy-5α-pregnan-20-one (3α-AP) were kindly donated by Teikoku Hormone Mfg. (Tokyo). PREGS, DHEAS, 3β-AP sulfate (3β-APS), 3α-AP sulfate (3α-APS) and EA sulfate (EAS) were synthesized in our laboratories by known methods [10]. PREG stearate was that used in the previous study [11]. 11α-Hydroxydehydroepiandrosterone [11α(OH)DHEA], poly(vinyl alcohol) (PVA; average mw 3000), BSA, Freund's complete adjuvant, β-galactosidase (β-GAL) (EC 3.2.1.23; from *E. coli*; 600–950 units/mg protein), Sephadex G-25 and goat anti-rabbit immunoglobulin G (IgG) antibody (H+L) were those used in the previous study [9]. The protein G column (HiTrap Protein G HP) was purchased from Amersham Japan Co. (Tokyo). Column chromatography and flash column chromatography were carried out with a Merck (Darmstadt, Germany) silica gel (60–200 mesh) and a Wakogel FC-40 (20–40 mesh; Wako Pure Chemical Co., Osaka, Japan), respectively. An EIA/RIA plate (flat bottom, high binding) purchased from the Corning Costar Co. (Cambridge, MA, USA) was used for the 96-well microtiter plate. OASIS HLB cartridges (60 mg; Waters Assoc., Milford, MA) were successively washed with AcOEt (1 ml), EtOH (2 ml) and H_2O (2 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. Buffers

The following buffers were used. PB: 0.05 M NaH_2PO_4–Na_2HPO_4 (pH 7.3). Buffer A, PB containing 0.9% NaCl, 0.1% NaN_3, and 0.1% gelatin; Buffer B, PB containing 0.9% NaCl, 0.02% NaN_3 and 0.05% Tween 20 (v/v). Buffer C: buffer A containing 0.5% PVA.

2.3. Apparatus

^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a JEOL (Tokyo) JNM-EX-270 (270 MHz) spectrometer using tetramethylsilane as the internal standard. Fast-atom bombardment (FAB) mass spectra were determined with a JEOL JMS-DX-303 spectrometer using *m*-nitrobenzylalcohol as the matrix. Melting points (m.p.) were recorded with a Yanagimoto (Kyoto, Japan) micro melting point apparatus and are uncorrected. A Tosoh (Tokyo) MPR A4i microplate reader was used for the absorbance measurement at 415 nm. HPLC was performed on a Shimadzu LC-10AT chromatograph (Kyoto) equipped with a Shimadzu SPD-10A UV (223 nm) detector. The flow rate and the column temperature were set at 1 ml/min and 40 °C, respectively.

2.4. Synthesis of haptenic derivatives

2.4.1. [17Z(20)]-Pregna-5,17(20)-diene-3β,11α-diol (2)

Ethyltriphenylphosphonium bromide (3.80 g, 10.2 mmol) was added to a stirred suspension of potassium *tert*-butoxide (1.20 g, 10.7 mmol) in tetrahydrofuran (THF) (12 ml) at room temperature. The resulting mixture was further stirred at 70 °C for 40 min. After addition of a solution of 11α(OH)DHEA (**1**) (1.00 g, 3.29 mmol) in THF (9 ml), the mixture was refluxed for 2 h. The resulting mixture was cooled, diluted with AcOEt, washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (32×2 cm i.d.) [hexane–AcOEt (1:1, v/v)] to give compound **2** (0.92 g, 88.6%) as colorless prisms. M.p. 180–182.5 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, s, H-18), 1.18 (3H, s, H-19), 3.54 (1H, m, H-3α), 4.11 (1H, m, H-11β), 5.14 (1H, m, H-20), 5.43 (1H, d, $J = 5.6$ Hz, H-6).

2.4.2. [17Z(20)]-3β,11α-Bis(tert-butyldimethylsilyloxy)pregna-5,17(20)-diene (3)

A mixture of **2** (0.89 g, 2.82 mmol), *tert*-butyldimethylsilylchloride (TBSCl) (2.20 g, 14.6 mmol), and imidazole (0.95 g, 14.0 mmol) in *N*,*N*-dimethylformamide (DMF) (7 ml) was stirred at room temperature for 13.5 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (32×2 cm i.d.) [hexane–AcOEt (5:1, v/v)] to give compound **3** (1.44 g, 94.0%) as colorless needles. M.p. 122–123.5 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, 3β-Si(Me)$_2$], 0.11, 0.13 [3H each, s, 11α-Si(Me)$_2$], 0.89 (24H, s, *tert*-Bu, H-18, H-21), 1.12 (3H, s, H-19), 3.49 (1H, m, H-3α), 4.18 (1H, m, H-11β), 5.12 (1H, m, H-20), 5.36 (1H, d, $J = 5.3$ Hz, H-6).

2.4.3. (20S)-3β,11α-Bis(tert-butyldimethylsilyloxy)pregn-5-en-20-ol (4)

A solution of 9-borabicyclo[3.3.1]nonane (9-BBN) in THF (0.5 M, 12 ml) was added to **3** (1.40 g, 2.57 mmol), and the mixture was stirred at room temperature for 18 h. After addition of 3 M NaOH (12 ml) and 30% H_2O_2 (7.5 ml) under ice-cooling, the mixture was stirred at room temperature for 4 h. The resulting solution was diluted with AcOEt, washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (29×2 cm i.d.) [hexane–AcOEt (5:1, v/v)] to give compound **4** (1.21 g, 83.7%) as colorless needles. M.p. 195–195.5 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, 3β-Si(Me)$_2$], 0.08, 0.09 [3H each, s, 11α-Si(Me)$_2$], 0.69 (3H, s, H-18), 0.89 (18H, s, *tert*-Bu) 1.11 (3H, s, H-19), 1.25 (3H, d, $J = 6.3$ Hz, H-21), 3.49 (1H, m, H-3α), 3.67 (1H, m, H-20), 4.10 (1H, m, H-11β), 5.34 (1H, d, $J = 6.1$ Hz, H-6).

*2.4.4. 3β,11α-Bis(tert-butyldimethylsilyloxy)pregn-5-en-20-one (**5**)*

A mixture of **4** (1.15 g, 2.05 mmol) and pyridinium chlorochromate (PCC) (0.90 g, 4.18 mmol) in CH_2Cl_2 (12 ml) was stirred at room temperature for 2.5 h. The crude product was purified by column chromatography (34 × 2 cm i.d.) [hexane–AcOEt (10:1, v/v)] to give compound **5** (1.04 g, 90.8%) as colorless needles. M.p. 184–187 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, 3β-Si(Me)$_2$], 0.10, 0.11 [3H each, s, 11α-Si(Me)$_2$], 0.63 (3H, s, H-18), 0.89 (18H, s, *tert*-Bu), 1.11 (3H, s, H-19), 2.12 (3H, s, H-21), 3.49 (1H, m, H-3α), 4.13 (1H, m, H-11β), 5.34 (1H, d, J = 5.4 Hz, H-6).

*2.4.5. 3β,11α-Dihydroxypregn-5-en-20-one (**6**)*

Five percent HCl (0.5 ml) was added to a solution of **5** (1.00 g, 1.79 mmol) in MeOH–THF (4:1, v/v, 25 ml), and the mixture was stirred at room temperature for 3 h. The resulting solution was diluted with AcOEt, washed (5% $NaHCO_3$ and brine) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (31 × 2 cm i.d.) [$CHCl_3$–MeOH (20:1, v/v)] to give compound **6** (0.58 g, 97.9%) as colorless needles. M.p. 185–186 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, H-18), 1.17 (3H, s, H-19), 2.14 (3H, s, H-21), 3.53 (1H, m, H-3α), 4.08 (1H, m, H-11β), 5.41 (1H, d, J = 5.7 Hz, H-6).

*2.4.6. 3β-(tert-Butyldimethylsilyloxy)-11α-hydroxypregn-5-en-20-one (**7**)*

A mixture of **6** (201 mg, 0.605 mmol), TBSCl (150 mg, 1.00 mmol) and imidazole (105 mg, 1.54 mmol) in DMF (3 ml) was stirred at room temperature for 5.5 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (22 × 2 cm i.d.) [$CHCl_3$–MeOH (50:1, v/v)] to give compound **7** (229 mg, 84.8%) as colorless prisms. M.p. 178–180 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, Si(Me)$_2$], 0.65 (3H, s, H-18), 0.89 (9H, s, *tert*-Bu), 1.16 (3H, s, H-19), 2.14 (3H, s, H-21), 3.47 (1H, m, H-3α), 4.07 (1H, m, H-11β), 5.38 (1H, d, J = 5.6 Hz, H-6).

*2.4.7. 3β-(tert-Butyldimethylsilyloxy)-20-oxopregn-5-en-11α-yl hemiglutarate (**8**)*

A mixture of compound **7** (80 mg, 0.179 mmol), glutaric anhydride (307 mg, 2.69 mmol) and dimethylaminopyridine (DMAP) (2 mg, 16.4 μmol) in pyridine (0.3 ml) was stirred at room temperatute for 5.5 h. H_2O was added to the mixture, which was stirred for a further 10 min. The mixture was then extracted with AcOEt, and the organic layer was washed (5% HCl, 5% $NaHCO_3$ and brine) and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography (36 × 1 cm i.d.) [$CHCl_3$–MeOH (50:1, v/v)] to give compound **8** (91 mg, 90.6%) as colorless needles. M.p. 166.5–167.5 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, Si(Me)$_2$], 0.70 (3H, s, H-18), 0.89 (9H, s, *tert*-Bu), 1.08 (3H, s, H-19), 2.11 (3H, s, H-21), 3.47 (1H, m, H-3α), 5.30 (1H, m, H-11β), 5.38 (1H, d, J = 5.6 Hz, H-6).

*2.4.8. 3β-(tert-Butyldimethylsilyloxy)-20-oxopregn-5-en-11α-yl hemisuccinate (**9**)*

A mixture of compound **7** (121 mg, 0.271 mmol), succinic anhydride (683 mg, 6.83 mmol) and DMAP (4 mg, 32.7 μmol) in pyridine (0.8 ml) was stirred at 50 °C for 6 days. After the addition of H_2O, the mixture was treated in the same way as compound **8** to give compound **9** (124 mg, 83.7%) as colorless needles. M.p. 165–167 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.06 [6H, s, Si(Me)$_2$], 0.69 (3H, s, H-18), 0.89 (9H, s, *tert*-Bu), 1.07 (3H, s, H-19), 2.11 (3H, s, H-21), 3.48 (1H, m, H-3α), 5.32 (1H, m, H-11β), 5.37 (1H, d, J = 5.3 Hz, H-6).

*2.4.9. 3β-Hydroxy-20-oxopregn-5-en-11α-yl hemiglutarate (**10**)*

A solution of tetrabutylammonium fluoride (TBAF) in THF (1 M, 1.6 ml) was added to a solution of **8** (91 mg, 0.162 mmol) in THF (0.5 ml), and the mixture was stirred at room temperature for 4.5 h. The resulting solution was diluted with $CHCl_3$ (5 ml) and purified by column chromato-

graphy (37 × 1 cm i.d.) [$CHCl_3$–MeOH (20:1, v/v)] to give compound **10** (71 mg, 98.0%) as colorless needles. M.p. 145–148 °C (from hexane–AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, H-18), 1.09 (3H, s, H-19), 2.12 (3H, s, H-21), 3.52 (1H, m, H-3α), 5.31 (1H, m, H-11β), 5.41 (1H, d, $J = 6.1$ Hz, H-6).

2.4.10. 3β-Hydroxy-20-oxopregn-5-en-11α-yl hemisuccinate (*11*)

A solution of TBAF in THF (1 M, 1.8 ml) was added to a solution of **9** (101 mg, 0.185 mmol) in THF (0.5 ml), and the mixture was stirred at room temperature for 5.5 h. The resulting solution was diluted with $CHCl_3$ (5 ml) and treated in the same way as compound **10** to give compound **11** (67 mg, 83.9%) as a colorless solid. ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, H-18), 1.08 (3H, s, H-19), 2.11 (3H, s, H-21), 3.49 (1H, m, H-3α), 5.31 (1H, m, H-11β), 5.41 (1H, d, $J = 5.9$ Hz, H-6).

2.4.11. 11α-Hemiglutaryloxypregnenolone 3-sulfate (PREG-HG) (*12*)

The freshly prepared sulfur trioxide–pyridine complex (ca. 30 mg) was added to a solution of compound **10** (29 mg, 65.0 μmol) in pyridine (0.5 ml) under ice-cooling and then stirred at room temperature for 1.5 h. The mixture was neutralized with 5% $NaHCO_3$, diluted with H_2O (200 ml) and subjected to an Amberlite XAD-2 column (21 × 2 cm i.d.). The column was washed with H_2O and the steroid was eluted with MeOH. After removal of the solvent under reduced pressure, the residue was purified by flash column chromatography (27 × 1 cm i.d.) [$CHCl_3$–MeOH (3:1, v/v)] to give compound **12** (22 mg, 64.3%) as a colorless solid. M.p. 185.5–188 °C. ^{1}H-NMR [$(CD_3)_2SO$] δ: 0.60 (3H, s, H-18), 1.00 (3H, s, H-19), 2.04 (3H, s, H-21), 3.83 (1H, m, H-3α), 5.17 (1H, m, H-11β), 5.37 (1H, d, $J = 5.1$ Hz, H-6). FAB-MS m/z: 547 $[M-H+Na]^-$ (29%), 524 $[M-H]^-$ (54%), 153 (100%).

2.4.12. 11α-Hemisuccinyloxypregnenolone 3-sulfate (PREG-HS) (*13*)

The freshly prepared sulfur trioxide-pyridine complex (ca. 80 mg) was added to a solution of compound **10** (52 mg, 0.120 mmol) in pyridine (0.8 ml) under ice-cooling and then stirred at 50 °C for 5 h. The resulting mixture was treated in the same way as compound **12** to give compound **13** (47 mg, 76.3%) as a colorless solid. M.p. 170–176.5 °C. ^{1}H-NMR [$(CD_3)_2SO$] δ: 0.60 (3H, s, H-18), 0.99 (3H, s, H-19), 2.04 (3H, s, H-21), 3.83 (1H, m, H-3α), 5.15 (1H, m, H-11β), 5.37 (1H, d, $J = 4.2$ Hz, H-6). FAB-MS m/z: 533 $[M-H+Na]^-$ (17%), 510 $[M-H]^-$ (58%), 153 (100%).

2.5. Preparation of hapten–BSA conjugate (BSA–PREGS) (*16*)

The hapten PREGS-HG (**12**) (10 mg, 19.0 μmol) was converted to its *N*-succinimidyl ester (**14**) (11 mg, 92.9%) by the method previously reported [9], which was then dissolved in pyridine (0.4 ml). A solution of BSA (28 mg, 0.424 μmol) in PB (0.4 ml) was added to the solution of the ester (**14**). The resulting mixture was stirred at 4 °C for 1 day and dialyzed against cold H_2O for 1 day. After the addition of acetone (10 ml) and a small amount of NaCl, the resulting suspension was centrifuged (1500 × g) at 4 °C for 15 min, and then the supernatant was discarded. The procedure was repeated two more times. The precipitate was dissolved in 20% pyridine (5 ml) and dialyzed against chilled 0.9% NaCl at 4 °C for 1 day to give the desired conjugate **16** (23.1 mg) as a solution in 0.9% NaCl.

2.6. Preparation of enzyme-labeled PREGS (β-GAL-PREGS) (*17*)

The hapten PREGS-HS (**13**) (5 mg, 9.77 μmol) was converted to its *N*-succinimidyl ester (**15**) (5 mg, 84.1%) by the method previously reported [9]. A portion of the ester (22.6 μg) in dioxane (100 μl) was mixed with a solution of β-GAL (1 mg) in PB (100 μl) and then the mixture was stirred at 4 °C for 4 h. The resulting mixture was introduced into a Sephadex G-25 column (11.5 × 1 cm i.d.) and eluted with PB–EtOH (4:1, v/v). The enzymic active fractions were collected and dialyzed overnight against cold PB. The resulting fraction was adjusted to produce the β-GAL-labeled antigen (**17**) solution (ca. 100 μg/ml) containing NaCl (0.9%), NaN_3 (0.1%), and gelatin (0.1%) (buffer A), which was then stored at 4 °C until used.

2.7. Production of antisera and their immunoglobulin G fractions

Three domestic albino rabbits (3 months old, female) were used for immunization with the BSA-PREGS conjugate (**16**). The suspension of **16** (1.5 mg) in isotonic saline (1.5 ml) was emulsified with complete Freund's adjuvant (1.5 ml), and a portion of the emulsion (ca. 1.0 ml) was injected into each rabbit subcutaneously at multiple sites along the back. This procedure was repeated biweekly for 4 months using the conjugate (0.75 mg).

Blood was collected 9 days after the last immunization and allowed to stand at room temperature for 2 h and then at 4 °C overnight. Centrifugation at $1500 \times g$ (4 °C, 20 min) afforded the desired antisera. A portion of the antisera (100 μl) was applied to the protein G column, and the IgG fractions obtained, which was expressed as **Ab1**, **Ab2**, and **Ab3** based on the immunized rabbit numbers, respectively (the recovery of IgG was not determined), were diluted with buffer C (8 ml; corresponding to 1:80), and stored at 4 °C before use.

2.8. ELISA procedure

An ELISA plate coated with anti-rabbit IgG antibody was prepared as previously reported [9]. The anti-PREGS antibody diluted with buffer C (1:300 000, 100 μl) and a series of standard PREGS (0–1000 pg) or brain sample dissolved in EtOH (25 μl) was added to an ELISA plate, and the mixture was incubated overnight at 4 °C. After the addition of the β-GAL-PREGS (**17**) (25 ng) in buffer A (100 μl), the mixture was incubated at 4 °C for an additional 2 h (sequential saturation). The plate was washed three times with 200 μl of buffer B and then *o*-nitrophenyl-β-D-galactoside (0.06%) in buffer A (100 μl) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol was added. After incubation at 37 °C for 1.5 h, the enzymic reaction was terminated by the addition of 100 μl of 1 M Na_2CO_3 and the β-GAL activities of the bound fractions were measured by colorimetry (415 nm) with the microplate reader.

2.9. Cross-reaction study

The cross-reaction of the ELISA system was examined with 11 kinds of compounds having structures closely related to that of PREGS. The relative amounts (mol) required to reduce the initial binding of β-GAL-labeled PREGS by half, where the mole of unlabeled PREGS was arbitrarily taken as 100%, were calculated from a standard curve.

2.10. Separation of PREGS and PREG by solid phase extraction cartridge

A solution of PREGS and PREG (each 5 μg) in PB (0.5 ml) was applied to an OASIS HLB cartridge. After the cartridge was washed with H_2O (2 ml), the steroids were first eluted with AcOEt (1 ml), and then with EtOH (1.5 ml). The respective fractions eluted with AcOEt and EtOH were evaporated and the residues were dissolved in each mobile phase (100 μl), parts of which (10 μl) were subjected to HPLC. The columns, mobile phases and retention times for PREGS and PREG were as follows: PREGS; J'sphere ODS-M80 (150×4.6 mm i.d., YMC, Kyoto), MeCN-0.5% $(NH)_2CO_3$ (1:2, v/v) and 6.7 min, PREG; YMC-Pack C_8 (150×4.6 mm i.d., YMC), MeCN–H_2O (1:1, v/v) and 11.7 min.

2.11. Pretreatment of brain sample

Wistar strain rats (7-week-old, male) obtained from Japan S.L.C. (Hamamatsu, Japan) were decapitated. The whole brain (ca. 1.5 g) was homogenized in MeOH containing 1% CH_3COOH (8 ml) using an ultrasonic homogenizer under ice-cooling and further sonicated in an ultrasonic bath for 30 min [12]. The volume of the homogenate was adjusted to 10 ml with MeOH containing 1% CH_3COOH, and aliquots corresponding to 200 mg of brain tissue were pipetted into tubes. The sample was centrifuged at $1500 \times g$ (4 °C, 10 min) and the supernatant was saved. The precipitate was further extracted with MeOH containing 1% CH_3COOH (0.5 ml) and centrifuged. The supernatant was combined and concentrated to about half volume under an N_2 gas stream.

Hexane (1 ml) was added to the solution and vortex-mixed. The hexane layer was discarded, and the MeOH layer was evaporated to dryness following an additional washing with hexane (1 ml). The resulting residue was dissolved in PB (0.5 ml) and applied to an OASIS HLB cartridge. After the cartridge was washed with H_2O (2 ml) and AcOEt (1 ml), PREGS was eluted with EtOH (1.5 ml). The collected solvent was evaporated under an N_2 gas stream and the residue was dissolved in EtOH (100 μl). Three 25 μl aliquots of the sample were submitted for ELISA.

3. Results and discussion

3.1. Preparation of BSA–PREGS conjugate

It is well-known that the specificity of antibodies for small molecules, such as steroids, is significantly influenced by the position on the compound used for conjugation to carrier proteins. Generally, the antigen-binding site of antibodies is complementary to the hapten portion remote from the position used for attachment to the carrier proteins. Therefore, hapten-carrier conjugates linked though a position on the C-ring seemed promising for the production of antibodies with higher specificity, because both the A-ring and side chain of the steroid are expected to be well exposed. Based on this concept, the authors synthesized the haptenic derivative DHEAS having the bridge at the C-11α position, which produced antibodies having useful properties [9]. In this study, to develop a simple and specific immunoassay system for PREGS, the new haptenic derivative, PREGS-HG (**12**), was synthesized from 11α(OH)DHEA (**1**) in ten steps with an overall yield of 30% as shown in Fig. 1.

The construction of the PREG-type side chain was a major problem in synthesizing the hapten (**12**) starting with the precursor (**1**). The approach started with the conversion of a 17-oxosteroid to a [17*Z*(20)]-ethylidene steroid by the Wittig reaction [13]. The hydroboration of **3** with 9-BBN followed by the oxidation with NaOH and H_2O_2 produced 20(*S*)-alcohol (**4**) [14], which was then treated with PCC to convert the desired side chain (**5**). A bridge structure and a sulfonic acid residue were successively introduced into the 11α-position and 3β-position of **5**, respectively, to give the hapten (**12**), which exhibited satisfactory ^{1}H-NMR and mass spectral data.

The BSA–PREGS conjugate (**16**) was prepared by the *N*-succinimidyl ester method, as outlined in Fig. 1. The hapten/BSA molar ratio of the obtained conjugate was determined to be 25 by the treatment with H_2SO_4 followed by the measurement of the absorbance at 600 nm, which was judged to be sufficient for its use as an immunogen.

3.2. Preparation of enzyme-labeled PREGS

It is well recognized that in the hapten enzyme immunoassay, the bridge-heterologous system, in which the labeled antigen has a shorter bridge length than the immunogen, is useful to obtain higher sensitivity [9]. In this respect, the labeled antigen, β-GAL-PREGS (**17**), was synthesized by reacting a 20-fold excess of the *N*-succinimidyl ester of the hapten (**15**) with β-GAL (Fig. 1). The obtained labeled antigen satisfactorily retained its enzymic activity (77% of the native enzyme) and showed sufficient immunoreactivity with the anti-PREGS antibody for use in ELISA.

3.3. Production of polyclonal antibodies

The properties of the antibodies were characterized by ELISA, in which the sequential saturation method was employed to obtain higher sensitivity [9]. All of the three rabbits immunized with the conjugate (**16**) repeatedly produced antibodies (**Ab1**, **Ab2** and **Ab3**) reacting with the β-GAL-labeled antigen (**17**). The optimum dilution of the antibody was determined by the following criteria: the enzymic activity (absorbance at 415 nm) at B_0 (absence of PREGS) should be approximately 0.3 and should exceed by 30-fold the activity of the non-specific binding. All of the antibodies gave satisfactory values (**Ab1**; 1:300 000, **Ab2**; 1:680 000 and **Ab3**; 1:1 000 000) in the ELISA system. The midpoint (the amount of PREGS which causes 50% displacement) of the systems using **Ab1** was 30 pg per well and much smaller than those using

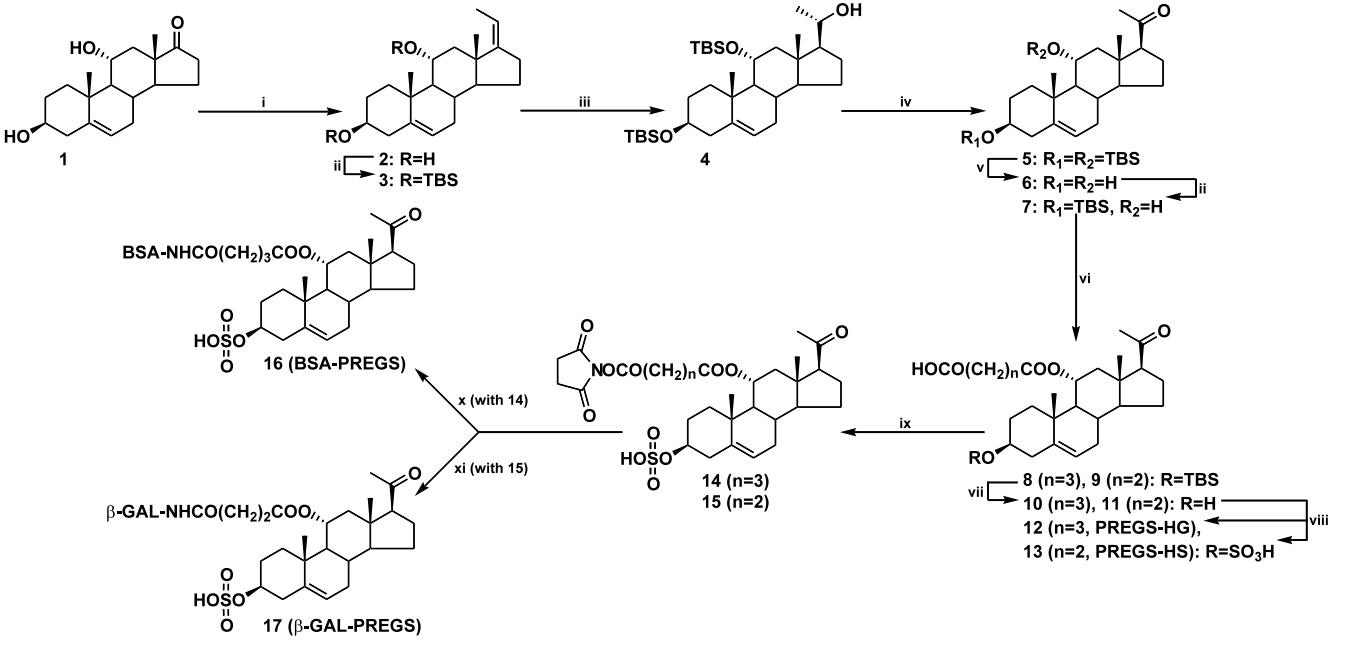

Fig. 1. Preparation of PREGS–BSA conjugate and β-GAL-labeled PREGS. Reagents, (i) Ph_3PEtBr, *tert*-BuOK, THF; (ii) TBSCl, imidazole, DMF; (iii) 9-BBN, THF then NaOH, 30% H_2O_2; (iv) PCC, CH_2Cl_2; (v) 5% HCl, MeOH–THF; (vi) glutaric anhydride or succinic anhydride, DMAP, pyridine; (vii) TBAF, THF; (viii) sulfur trioxide–pyridine complex, pyridine; (ix) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl, *N*-hydroxysuccinimide, 95% dioxane; (x) BSA, PB; (xi) β-GAL, dioxane–PB.

Ab2 and **Ab3** (100 and 300 pg per well, respectively). These results prompted us to use **Ab1** for the following experiments.

A typical standard curve obtained from the ELISA is shown in Fig. 2. This ELISA system afforded a dose-response curve whose measurable range was 1–1000 pg. The values in parentheses in Fig. 2 represent the coefficient of variation (CV) of B/B_0 at each point (%, $n = 5$), which demonstrated that the ELISA was quite reproducible. The minimal detectable amount, that is twice the standard deviation (S.D.) of the zero determination ($n = 6$), was 0.2 pg per well.

3.4. Cross-reaction study

The cross-reactions of the ELISA with 11 kinds of compounds having closely related structures are listed in Table 1. The antibody showed only negligible cross-reactivities with the 17-oxosteroids including their sulfates (DHEA, EA, DHEAS and EAS), indicating that it is extremely specific for the PREG-type side chain. There were no significant cross-reactivities with PROG, 3α-AP and its sulfate, which indicates that the antibody strictly recognizes the 3β-hydroxysteroids. However, contrary to our expectations, the antibody exhibited some cross-reactivity with PREG (4.4%). The authors do not have any plausible explanation

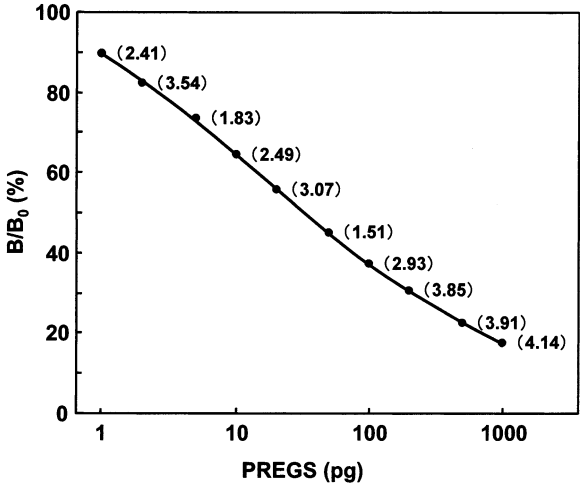

Fig. 2. Dose–response curve for PREGS ELISA. Values in parentheses represent CV (%, $n = 5$).

Table 1
Percent cross-reaction with related compounds

Compound	Cross-reactivity (%)[a]
PREGS	100
3β-APS	16.6
3α-APS	0.24
DHEAS	0.27
EAS	0.12
PREG stearate	< 0.025
PREG	4.4
3β-AP	1.3
3α-AP	0.12
PROG	0.76
DHEA	< 0.025
EA	< 0.025

[a] Fifty percent displacement method. Values are calculated on a molar basis.

for the reason why the expected specificity could not be obtained using the haptenic derivative having the bridge at the 11α-position in the case of PREGS, although the desired antibody was successfully elicited against DHEAS [9]. This may simply be because of the lack of corresponding B cell clone in the rabbit. According to our data, PREG is present in higher concentration in the brain than its sulfate [8,15]. Therefore, the ELISA using this antibody may overestimate the brain levels of PREGS without removing PREG from the PREGS fraction during the sample pretreatment steps. Although the antibody significantly cross-reacted with 3β-APS (16.6%), this sulfate is not detected in the brain [16]. This insufficient recognition of the C5–6 double bond was also observed in the anti-DHEAS antibody raised against 11α-hemiglutaryloxy-DHEAS–BSA conjugate [9]. PREG-stearate was easily discriminated by the antibody. The authors have reported that this lipoidal ester exists in the brain at a much higher level in comparison with PREGS and that its levels decrease with a rise in the PREG levels after acute stress [11].

3.5. Application of ELISA to determination of PREGS in rat brain

Although the developed ELISA is able to measure PREGS without deconjugation, the

method may overestimate the brain PREGS levels due to the cross-reactivity of the antibody to PREG as mentioned above. Therefore, the procedure for removing PREG is necessary for the ELISA to determine PREGS in the brain more reliably. In the previous study [9], the authors found that DHEAS was retained on an OASIS HLB cartridge, though the cartridge was washed with AcOEt, by which the unconjugated steroids were eluted, and the sulfate was quantitatively recovered with EtOH after the washing step. Based on these data, a preliminary experiment on the separation of PREGS and PREG using this cartridge was carried out. A mixture of standard PREGS and PREG was subjected to the cartridge and the recoveries of these steroids in the AcOEt and EtOH fractions each were determined by HPLC. In consequence, PREGS [91.2 ± 0.6%, mean ± S.D., $n = 3$] and PREG (91.1 ± 6.6%, $n = 3$) were quantitatively recovered in the EtOH and AcOEt fractions, respectively, and PREGS in the AcOEt fraction and PREG in the EtOH fraction were not detected. These results demonstrate that this straightforward procedure avoids the overestimation of the PREGS levels due to the contaminant of PREG.

Although the validation tests of the method are not yet performed, a pilot study on a brain was carried out. According to the previously reported brain concentration of PREGS (more than 5 ng/g tissue) [2,3,12,16], the ELISA is able to determine it with less than 20 mg of tissue. However, anticipating that the concentration is lower [8], 200 mg of tissue was used in the present study. The whole brain was homogenized by the method of Liere et al., by which steroids are reported to be quantitatively extracted from the brain tissue [12]. To determine the recovery rate of PREGS during the washing and solid phase extraction steps, aliquots of the homogenate corresponding to 200 mg of brain tissue were pipetted into tubes, and some were spiked with 100 pg of standard PREGS. These samples were pretreated and subjected to the ELISA. The recovery rate was 60.2 ± 5.3% (mean ± S.D., $n = 5$) and was reasonable, but the PREGS concentrations (50–430 pg/g tissue, $n = 5$, the above recovery rate was used for calculating the brain concentrations) were much lower than those previously measured by GC-MS after solvolysis [12,16]. Similar phenomena were also observed in our previous work measuring DHEAS in the brain using the newly developed ELISA [9]. Although the authors did not sufficiently explain this discrepancy, there may be a possibility that the methods combined with solvolysis co-measured PREG derived from other conjugated forms in the brain. That is, it has been proved that the steroids exist in the brain in the unconjugated form and their sulfates together with fatty acid esters [3] and sulfolipid conjugates [16]. The steroids may also exist in other forms besides the above forms and be solvolyzed to give the unconjugated form as well as the sulfates. Incidentally, the highly sensitive nano-ESI-tandem MS method for PREGS has also been developed, and its potential for determining the brain PREGS levels was proved using the PREGS-spiked sample [17], but the endogenous PREGS was not referred to in the report.

To resolve the problem, it seems profitable to determine the correlation of the values by the ELISA and those by the GC-MS method with solvolysis. Such studies together with validation tests of the ELISA are now in progress in our laboratories.

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