SYNTHESIS OF 11α-HYDROXYPROGESTERONE HAPTENS

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Received November 19, 1987
Revised June 6, 1988

ABSTRACT

C-4 and C-6 bridged haptens of 11α-hydroxyprogesterone, an anti-androgen, were respectively synthesized via 4,5 and 5α,6α epoxide ring openings using 3-mercaptopropanoic acid. Substitution of 6-bromo derivative of progesterone using the same reagent unexpectedly afforded a C-4 substituted product instead of the normal C-6 substituted product previously reported in the literature.

INTRODUCTION

11α-Hydroxy-4-pregnene-3,20-dione(11α-hydroxyprogesterone; I), having anti-androgenic properties, has been applied in the treatment of androgenic alopecia and acne vulgaris (1-2). Specific analysis of (I) is required to determine the chemical's concentration in biological fluids. The present paper is mainly concerned with preparation of the most desirable hapten of (I) for enzyme immunoassay.

EXPERIMENTAL

Melting points are uncorrected. Infrared (IR) spectra were recorded on a Shimazu IR-435. Nuclear magnetic resonance (NMR) spectra were obtained in chloroform-d with tetramethylsilane as the internal standard, on a JEOL JNM-PMX60SI or an FX-200 spectrometer. Electron impact mass spectra were recorded on a Shimazu GCMS-6020. High performance liquid chromatography (HPLC) was carried out using a Waters Model 510 connected with a Shimazu SPD-6AV. Merck silica gel (Mesh 70-230) was

used in column chromatography. Solvents used were purified by standard methods.

4,5-Epoxy-11α-hydroxypregnane-3,20-dione(II)

Ten percent NaOH aqueous solution (2.9 mL) and 30% H_2O_2 aqueous solution (3.2 mL) were added to a solution containing (I) (0.8 g, 2.42 mmol) dissolved in MeOH (48 mL) at 0°C. The mixture was stirred at 0°C for 3 h. After careful neutralization with AcOH, the resulting solution was concentrated to one-third volume under reduced pressure. The residue was diluted with EtOAc. The solution was washed with H_2O and a saturated NaCl aqueous solution, then dried over anhydrous $MgSO_4$. The dried organic layer was concentrated at reduced pressure. The residue was subjected to column chromatography on silica gel. Elution with EtOAc/n-hexane (3:1) yielded the epimeric 4,5-epoxides (II) as a white amorphous powder (0.766 g, 2.21 mmol; yield: 91.3%). (II): IR(KBr) 3450(OH), 1710 and 1690(C=O), and 1020 cm^{-1} (epoxide-ring). ^{1}H-NMR($CDCl_3$)δ 0.66(6H, s, 18-CH_3), 1.23(3H, s, 19-CH_3), 2.13(3H, s, 21-CH_3), 2.98 and 3.02(total 1H, s, 4α-H and s, 4β-H), and 3.50-4.45 ppm(1H, m, 11β-H).

4-(2'-Carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (III)

A solution of 3-mercaptopropanoic acid (0.2 mL, 2.4 mmol) and 25% KOH aqueous solution (0.7 mL) was stirred at room temperature for 10 min. A solution containing (II) (0.5 g, 1.44 mmol) dissolved in EtOH (4.2 mL) was added to the mixture. The mixture was stirred overnight at room temperature. 3-Mercaptopropanoic acid (0.2 mL, 2.4 mmol) was added to the mixture, which was then stirred at 80°C for 5 h. Additional 3-mercaptopropanoic acid (0.2 mL, 2.4 mmol) was added to the mixture, which was stirred overnight at 80°C. The solution was allowed to come to room temperature. H_2O (0.5 mL) was added to the solution, which was then adjusted to pH 4 with diluted HCl aqueous solution at 0°C. The acidified solution was extracted with EtOAc. The organic layer was washed with H_2O and saturated NaCl aqueous solution and then dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with EtOAc/n-hexane (3:1) as eluent. (III) was obtained as colorless needles after recrystallization from EtOAc/n-hexane. Yield: 56% (0.351 g, 0.808 mmol) mp 144-145°C. IR(KBr) 3430(OH), 1720,1700, and 1660 cm^{-1}(C=O). ^{1}H-NMR($CDCl_3$)δ 0.70(3H, s, 18-CH_3), 1.35(3H, s, 19-CH_3), 2.14(3H, s, 21-CH_3), 3.73(1H, t of d, $J_{H6\alpha,H6\beta}$=14.2 Hz, $J_{H6\alpha,H7}$=3.2 Hz, 6α-H), 3.69-3.79 ppm(1H, m, 11β-H). ^{13}C-NMR($CDCl_3$)δ 14.5(q,C-18), 19.7(q,C-19), 23.2 (t,C-15), 24.2(t, C-16), 28.8(t,-S-CH_2-), 30.7(q,C-21), 31.3(t,C-7), 31.9(t,C-6), 34.6(t,C-1), 34.7(d,C-8), 35.1(t,C-2), 35.9(t,-CH_2-COOH), 42.9(s,C-10), 44.2(s,C-13), 50.4(t,C-12), 55.2(d,C-14), 58.9(d,C-9), 63.1(d,C-17), 68.7(d,C-11), 128.4(s,C-4), 175.9 (s,C-5), 176.5(s,-COOH), 195.9(s,C-3), and 209.0 ppm(s,C-20).

6β-Bromo-4-pregnene-3,20-dione (IVa)

4-Pregnene-3,20-dione (1 g, 3.18 mmol), N-bromosuccinimide (850 mg, 4.78 mmol), and 50 mL of absolute CCl_4 was placed in a

100 mL round-bottomed flask equipped with a reflux condenser with a dry tube. The mixture was refluxed for 30 min, then allowed to come to room temperature. The precipitate formed was filtered off. The filtrate was concentrated under reduced pressure, and the residue was subjected to column chromatography on silica gel with EtOAc/n-hexane (2:3) as eluent. The following compounds were obtained. 6β-Bromo-4-pregnene-3,20-dione (IVa): white flat plates (696 mg, 1.47 mmol) after recrystallization from acetone/n-hexane. Yield: 46%. mp 160-162°C. Beilstein test (+). IR(KBr) 1700 and 1660 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.72(3H, s, 18-CH_3), 1.52(3H, s, 19-CH_3), 2.10(3H, s, 21-CH_3), 4.93(1H, m, 6α-H), and 5.87 ppm(1H, s, 4-H). 6β,17α-Dibromo-4-pregene-3,20-dione: white flat plates (339 mg, 0.86 mmol) after recrystallization from acetone/n-hexane. Yield: 27%. mp 155-156°C. Beilstein test (+). IR(KBr) 1680 and 1650 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.89(3H, s, 18-CH_3), 1.53(3H, s, 19-CH_3), 2.38(3H, s, 21-CH_3), 5.00(1H, m, 6α-H), and 5.87 ppm(1H, s, 4-H).

Reaction of (IVa) with 3-mercaptopropanoic acid

(IVa) (200 mg, 0.50 mmol), 3-mercaptopropanoic acid (90 μL, 1.0 mmol) and 8 mL of 1% solution of KOH in MeOH were placed in a 50 mL round bottomed flask equipped with a reflux condenser with a dry tube. The mixture was refluxed for 3 h. The reaction mixture was concentrated under reduced pressure. H_2O was added to it and the pH of the solution was acidified with diluted HCl aqueous solution. The acidified solution was then extracted with Et_2O and the organic layer was dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with EtOAc/n-hexane (1:1) and then $CHCl_3$/MeOH (15:1) as eluents. 4-(2'-Carboxyethylthio)-4-pregnene-3,20-dione (V) was obtained as colorless granules after recrystallization from EtOAc/ petroleum ether. Yield: 22% (46 mg, 0.11 mmol). mp 164-166°C. Beilstein test (-). IR(KBr) 3100(OH), 2850(SCH_2), 1735(COOH), 1700 and 1675 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.68(3H, s, 18-CH_3), 1.23(3H, s, 19-CH_3), 2.13(3H, s, 21-CH_3), 2.93(2H, t, J=6.8 Hz, -S-CH_2-) and 3.69 ppm(1H, t of d, $J_{H6\alpha,H6\beta}$=10.8 Hz, $J_{H6\alpha,H7}$=3.2 Hz, 6α-H). ^{13}C-NMR($CDCl_3$) δ 13.4(q,C-18), 18.2(q,C-19), 21.3(t,C-11), 23.1(t,C-15), 24.4(t,C-16), 28.9(t, -S-CH_2-), 30.8(q,C-21), 31.4(t,C-7), 32.1(t,C-6), 34.4(t,C-2), 34.7(d,C-8), 35.4(t,C-1), 38.5(t,-CH_2-CO_2H), 38.8(t,C-12), 41.5(s,C-10), 43.9(s,C-13), 54.3(d,C-9), 56.1(d,C-14), 63.5(d,C-17), 127.3(s,C-4), 175.3(s,-COOH), 175.8(s,C-5), 195.1(s,C-3), and 209.1 ppm(s,C-20). cf. 4-Pregnene-3,20-dione: ^{1}H-NMR($CDCl_3$) δ 0.70(3H, s, 18-CH_3), 1.05(3H, s, 19-CH_3), 2.12(3H, s, 21-CH_3), 0.83-2.63(20H, m, other protons), and 5.73 ppm(1H, s, 4-H).

11α-Hydroxy-5-pregnene-3,20-dione-di-(ethylene ketal) (VI)

A solution containing (I) (1.0 g, 3.03 mmol) dissolved in absolute benzene (50 mL) was refluxed for 2 h with 6.2 mL of ethylene glycol and p-toluenesulfonic acid monohydrate (128 mg, 0.67 mmol) in a 100 mL flask fitted with an H_2O separator, and was then allowed to come to room temperature. The mixture was diluted with Et_2O. The solution was washed with H_2O, saturated

$NaHCO_3$ aqueous solution, H_2O, and saturated NaCl aqueous solution and then dried over anhydrous $MgSO_4$. The dried organic layer was concentrated at reduced pressure. The residue was subjected to column chromatography on silica gel with $CHCl_3$/MeOH (30:1) as eluent. Compound (VI) was obtained as colorless needles after recrystallization from EtOH. Yield: 89%(1.13 g, 12.9 mmol). mp 184°C. IR($CHCl_3$) 3450 cm^{-1}(OH). ^{1}H-NMR($CDCl_3$) δ 0.80(3H, s, 18-CH_3), 1.17(3H, s, 19-CH_3), 1.28(3H, s, 21-CH_3), 3.88(8H, s, two ethylenedioxy groups), 3.55-4.21(1H, m, 11β-H), and 5.21-5.43 ppm(1H, m, 6-H).

Epoxidation of (VI)

Compound (VI) (2.6 g, 6.2 mmol), m-chloroperbenzoic acid (1.6 g, 9.4 mmol) and 100 mL of absolute benzene were placed in a 200 mL flask. The mixture was stirred at room temperature for 2 h. Et_2O was added to the mixture. The organic layer was washed with 10% Na_2SO_3 aqueous solution, H_2O, 5% $NaHCO_3$ aqueous solution, and saturated NaCl aqueous solution and then dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with EtOAc/n-hexane (2:3) as eluent. After recrystallization from EtOH, 5α,6α-epoxy-11α-hydroxypregnane-3,20-dione-di-(ethylene ketal) (VII-α) and 5β,6β-epoxy-11α-hydroxypregnane-3,20-dione-di-(ethylene ketal) (VII-β) were obtained as colorless needles (0.97 g, 2.20 mmol; yield: 36%) and colorless needles (1.36 g, 3.10 mmol; yield: 50%), respectively. (VII-α): mp 203°C. IR($CHCl_3$) 3450 cm^{-1}(OH). ^{1}H-NMR($CDCl_3$) δ 0.71(3H, s, 18-CH_3), 1.18(3H, s, 19-CH_3), 1.27(3H, s, 21-CH_3), 2.80(1H, d, J=4.0 Hz, 6β-H), 3.85 and 3.90(4H each, s, two ethylenedioxy groups), and 3.50-4.15 ppm(1H, m, 11β-H). (VII-β): mp 187-189°C. IR($CHCl_3$) 3470 cm^{-1}(OH). ^{1}H-NMR($CDCl_3$) δ 0.75(3H, s, 18-CH_3), 1.17(3H, s, 19-CH_3), 1.27(3H, s, 21-CH_3), 3.02(1H, d, J=2.4 Hz, 6α-H), 3.83(8H, s, two ethylenedioxy groups), and 3.30-4.15 ppm(1H, m, 11β-H).

5α,11α-Dihydroxy-6β-(2'-carboxyethylthio)-pregnane-3,20-dione-di-(ethylene ketal) (VIII)

Absolute EtOH (150 mL) and 3.5 g of metal Na were placed in a 200 mL round-bottomed flask. When the reaction was complete, 3-mercaptopropanoic acid (5 mL, 38.7 mmol) was added to the mixture on cooling. The mixture, allowed come to room temperature, was stirred for 20 min. Absolute EtOH solution containing (VII-α) (955 mg, 2.20 mmol) was added to the mixture, which was then refluxed for 2 h. The mixture was allowed to come to room temp. and was then concentrated to one-third volume under reduced pressure. H_2O (100 mL) was added to the condensed solution, which was then adjusted to pH 4 with diluted HCl aqueous solution. The acidified solution was extracted with EtOAc and dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with EtOAc/n-hexane (1:1) and then $CHCl_3$/MeOH (3:1) as eluents. (VIII) was obtained as colorless needles after recrystallization from EtOAc/n-hexane. Yield: 89% (1.05 g, 1.95 mmol). mp 132-133°C. IR($CHCl_3$)

3450(OH) and 1710 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.78(3H, s, 18-CH_3), 1.17(3H, s, 19-CH_3), 1.27(3H, s, 21-CH_3), 3.90(8H, s, two ethylenedioxy groups), 3.40-4.22(1H, m, 11β-H), and 5.63 ppm(1H, m, 5α-OH).

5α,11α-Dihydroxy-6β-(2'-carboxyethylthio)-pregnane-3,20-dione (IX)

Absolute acetone solution (15 mL) containing (VIII) (147 mg, 0.27 mmol) was stirred with p-toluenesulfonic acid monohydrate (14 mg, 0.074 mmol) in a 30 mL round bottomed flask at room temperature for 1 h. The mixture was concentrated to one-third volume and diluted with EtOAc. The organic layer was washed repeatedly with H_2O, further washed with saturated NaCl aqueous solution, and then dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The obtained colorless powder (IX) was used in the next reaction without further purification. (IX): mp 178-180°C. Colorless granules (recrystallization from EtOH/Et_2O). IR($CHCl_3$) 3480(OH), 1720(COOH), and 1700 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.67(3H, s, 18-CH_3), 1.23(3H, s, 19-CH_3), 2.10(3H, s, 21-CH_3), 3.17-4.35(2H, m, 6α-H and 11β-H), and 5.00 ppm(1H, m, 5α-OH).

6β-(2'-Carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (X) and 6α-(2'-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (XI)

A typical example is described below. While a stream of dry HCl was bubbled into the absolute acetone solution containing (IX), the solution was stirred in an ice bath for 20 min. Stirring at this temperature was continued for an additional 30 min. The solution was concentrated under reduced pressure below 30°C. EtOAc and H_2O were added to the residue on cooling, and the solution was neutralized with $KHCO_3$ aqueous solution. The organic layer was separated from the neutralized solution. The water layer was adjusted to pH 4 with diluted HCl solution and then extracted again with EtOAc. The total organic layer was washed with H_2O and saturated NaCl aqueous solution and then dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was purified by HPLC. HPLC: detector, 263nm; mobile phase, $CHCl_3$:MeOH:H_2O=97:3:3 (lower phase); flow rate, 2.5 ml/min; column, Φ 7.5 mm x 300 mm packed with Nucleosil 50-5; (X), Rt=25.5 min, (XI), Rt=29.0 min .

(X): mp 130°C colorless granules (recrystallization from EtOAc/n-hexane). IR(KBr) 3430, 3250(OH), 1720(COOH), 1690, and 1655 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.73(3H, s, 18-CH_3), 1.58(3H, s, 19-CH_3), 2.14(3H, s, 21-CH_3), 3.60-3.62(1H, m, 6α-H), 4.02-4.14(1H, m, 11β-H), and 5.68 ppm(1H, s, 4-H). ^{13}C-NMR($CDCl_3$) δ 14.5(q, C-18), 21.5(q, C-19), 23.1(t, C-15), 24.3(t, C-16), 26.8(t, -S-CH_2-), 30.5(q, C-21), 31.2(d, C-8), 33.8(t, C-7), 34.3(t, C-1), 36.4 (t, C-2), 39.7(t, -CH_2-COOH), 39.8(s, C-10), 44.2(s, C-13), 48.7 (s, C-6), 50.4(t, C-12), 55.2(s, C-14), 59.1(s, C-9), 63.2(s, C-17), 68.8(s, C-11), 126.7(s, C-4), 167.4(s, C-5), 175.5(s, -COOH), 200.2(s, C-3), and 208.5 ppm(s, C-20). High-resolution EI-MS m/z found, 434.2250 (calcd $C_{24}H_{34}O_5S$ 434.2127).

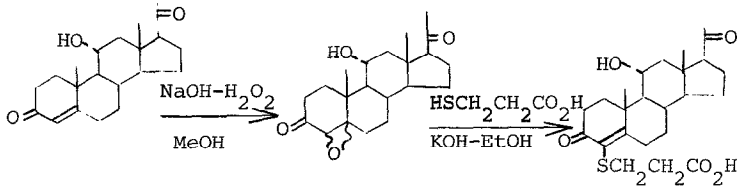
(XI): mp 132-134°C. Colorless powder (recrystallization from EtOH/Et_2O). IR(KBr) 3400(OH), 1720(COOH), 1700, and 1655 cm^{-1} (C=O). ^{1}H-NMR($CDCl_3$) δ 0.71(3H, s, 18-CH_3), 1.32(3H, s, 19-CH_3), 2.14 (3H, s, 21-CH_3), 3.56-3.65(1H, m, 6β-H), 3.94-4.08(1H, m, 11β-H), and 6.42 ppm(1H, d, $J_{H4,H6\beta}$=1.47 Hz, 4-H). ^{13}C-NMR($CDCl_3$) δ 14.5 (q, C-18), 19.7(q, C-19), 23.2(t, C-15), 24.2(t, C-16), 26.1(t, -S-CH_2-), 31.3(q, C-21), 34.1(t, C-1), 34.5(t,C-7), 35.4(d, C-8), 37.0(t, C-2), 40.1(t, -CH_2-COOH), 41.4(s, C-10), 44.2 (s, C-13), 46.7(d, C-6), 50.3(t, C-12), 55.0(d, C-14), 57.9 (d, C-9), 63.0 (d, C-17), 68.6(d, C-11), 124.0(d, C-4), 168.6(s, C-5), 175.6(s, -COOH), 200.5(s, C-3), and 208.6 ppm(s, C-20). High-resolution EI-MS m/z, found, 434.2126 (calcd $C_{24}H_{34}O_5S$ 434.2127).

RESULTS AND DISCUSSION

It has generally been said that conjugates prepared through a position distal to structurally unique regions would lead to the production of more specific antibodies (3). In order to prepare an antibody specific to (I), an antibody must be prepared that can recognize the 11α-hydroxy group. This requires preparation of a (I) hapten possessing the bridge at the C-4, C-6, or C-7 position, because 4-pregnene-3,20-dione having an 11α-hydroxy group, that is, (I), does not exist in nature, as does that having an 11β-hydroxy group. Preparation of the antibody most specific to (I) requires preparation of a (I) hapten possessing the bridge at the C-6 or C-7 position of a B-ring having no functional groups, since the prepared antibody must recognize not only an 11α-hydroxy group but also a 4-ene-3-one region. Therefore we first prepared a (I) hapten possessing the bridge at the C-4 position, and then a (I) hapten possessing the bridge at the C-6 position.

The (I) hapten possessing the bridge at the C-4 position, 4-(2'-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (III), was prepared in accordance with the method of Hosoda et al (4),

as shown in Figure 1.



(I) (II) (III)

Figure 1. Synthesis of 4-(2'-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (III).

Epoxidation of (I) with alkaline hydrogen peroxide produced epimeric 4,5-epoxides (II) at a 91% yield. Base-catalyzed cleavage of (II) with 3-mercaptopropanoic acid produced (III) at a 56% yield.

The first synthetic approach to a (I) hapten possessing the bridge at the C-6 position was carried out in accordance with the method of Lindner et al (5), which involved direct substitution of 6β-bromo derivatives (IVa) using 2-mercaptoethanoic acid. However, the reaction of 6β-bromo-11α-acetoxy-4-pregnene-3,20-dione (IVb) with 3-mercaptopropanoic acid did not occur under the same conditions. Therefore we re-examined the results described by Lindner et al (5); that is, the mixture of 6β-bromo-4-pregnene-3,20-dione (IVa) and 3-mercaptopropanoic acid was refluxed in a solution of KOH in MeOH, unexpectedly producing a 4-substituted product (V) (Figure 2).

In the ^{1}H-NMR spectrum of the product (V), the singlet signal (5.73 ppm) attributed to a methine proton at the C-4 position disappeared, and that of 6α-H appeared as doublets of a

triplet at 3.69 ppm, its signal being shifted downfield in comparison with that of 4-pregnene-3,20-dione (6). Further, the ^{1}H-NMR spectrum of (V) was coincident with that of the corresponding moiety of (III), prepared according to method shown in Figure 1.

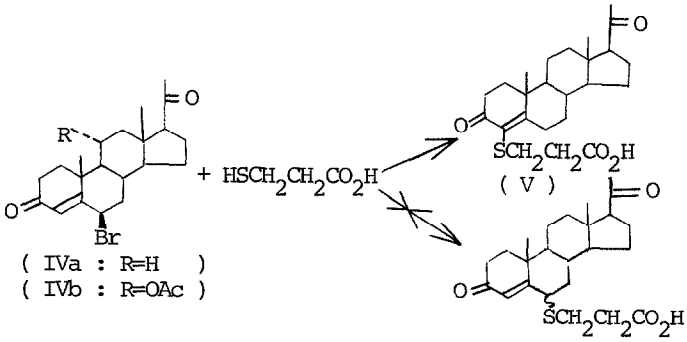


Figure 2. The reaction of 6β-bromo-4-pregnene-3,20-dione (IVa) with 3-mercaptopropanoic acid.

The above results revealed that the product was a 4-pregnene-3,20-dione derivative (V) not at the C-6 position but at the C-4 position. Further, the ^{13}C-NMR spectrum of (V) also led to a (V) structure of 4-(2'-carboxyethylthio)-4-pregnene-3,20-dione.

Curiously, the ^{1}H-NMR spectrum of (V) was coincident with that of the corresponding moiety of 6-carboxymethylthio-4-pregnene-3,20-dione described by Lindner et al (5). As to the disappearance of the proton signal at position 4 in their article (5), they stated, "The proton at position 4 did not appear in the vinylic region of the NMR spectrum. We tend to attribute this to

an intramolecular interaction with the substituent at C-6. A rearrangement of the substituent from C-6 to C-4 seems unlikely in view of the appearance of the broad band between 3.17-3.82 ppm which can be assigned to a methine proton at C-6."

Consequently, Lindner et al have misassigned the ^{1}H-NMR spectrum of their product. Our conclusion is consistent with that described by Tomoeda et al (6), because a methine proton signal should appear at position 4 in 6β-(2'-carboxyethylthio)-4-pregnene-3,20-dione; further, the signal attributed to C6α-H was observed as triplets of a doublet due to spin-spin coupling with the C6β-H at J=10.8 Hz and further to spin-spin coupling with the C-7 hydrogens at J=3.2 Hz.

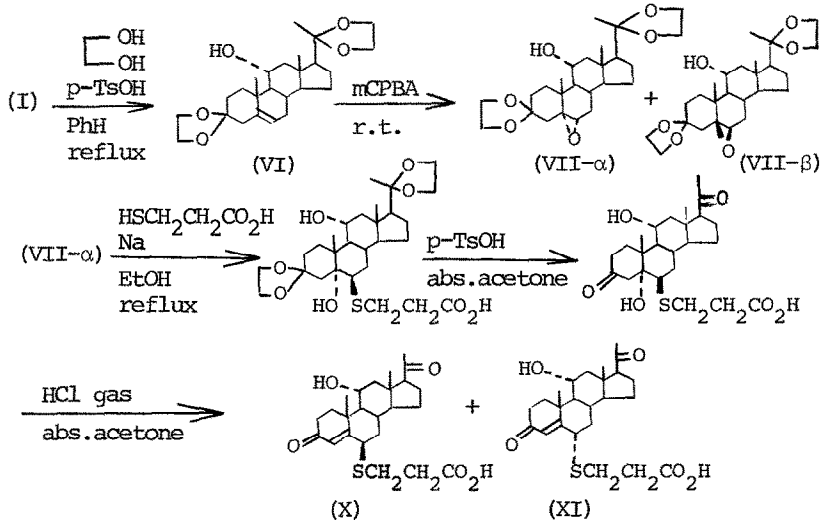


Figure 3. Preparation of 11α-hydroxy-4-pregnene-3,20-dione derivatives at C-6 position.

Finally, we prepared 6β-(2'-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (X) and 6α-(2'-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione (XI), as in Figure 3.

Ketalization of (I) resulted in (VI) accompanied by migration of a double bond. (VI) was epoxidated with m-chloroperbenzoic acid (mCPBA) in absolute benzene at room temperature for 2 h to yield a mixture of α-isomer (VII-α) and β-isomer (VII-β). The reaction of (VII-α) with 3-mercaptopropanoic acid in the presence of sodium ethoxide in refluxing ethanol afforded (VIII) in an 89% yield, whereas under the same conditions the reaction of (VII-β) with 3-mercaptopropanoic acid did not occur. The latter result may be due to steric hindrance. Compound (VIII) was deketalated with p-toluenesulfonic acid in absolute acetone at room temperature to yield (IX), which was treated with dry HCl gas in absolute acetone to afford steric isomers [(X) and (XI)] at a ratio of 2:1. A comparison of the ^{1}H-NMR spectrum of (X) with that of (XI) revealed that the signal due to methyl protons at C-19 position was shifted downfield. Further, in the ^{1}H-NMR spectra, the signal due to a methine proton at the C-4 position of (XI) was observed as a doublet due to allylic coupling between the C-4 and C-6β protons, and was further downfield than that of (X), because of the long-range deshielding effects of polar functions at the C-6α position against the C-4 proton in 6-substituted-4-en-3-oxo-steroid (6). Balent et al report an investigation concerning the dehydration of 6β-acetoxy-5α-hydroxypregnane-3,20-dione (7). Therefore we investigated the dehydration condition of (IX) using modifications of their method

(7). The results are summarized in Table 1.

Table 1. The Dehydration of (IX) under Various Conditions

No.	solvents	isomer ratio (X:XI)	yields (%)
1	abs.acetone	2:1	37
2	abs.EtOH/abs.$CHCl_3$ (1:160)	2:1	45
3	abs.$CHCl_3$	only β	18
4[a]	abs.EtOH	mainly α (β:trace)	39

The dehydration of (IX) was performed with dry hydrogen chloride in the above described solvents (see Experimental).
a) Esterification occurred in this procedure, affording 6α-(2'-ethoxycarbonylethylthio)-11α-hydroxy-4-pregnene-3,20-dione.

The mixture of (X) and (XI) was purified by HPLC separation eluting with a lower phase of chloroform/methanol/water from a normal-phase column (Nucleosile 50-5); the 6β-isomer (X) was obtained, after recrystallization from EtOAc/n-hexane, as colorless granules; the 6α-isomer (XI) was obtained after recrystallization from EtOH/Et_2O as colorless fine crystals. The overall yields of (X) and (XI) were 7.0% and 3.6%, respectively.

Studies are in progress on the preparation of antisera using (III), (X), and (XI) for steroid enzyme immunoassay.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Yamaki, Mukogawa Women's College, for the NMR spectra taken on a JEOL FX-200 spectrometer, and Miss H. Nakashige, Rohto Pharmaceutical Co., Ltd., for the high-resolution mass spectra.

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