



An easy stereoselective synthesis of 5(10)-estrene-3 β ,17 α -diol, a biological marker of pregnancy in the mare



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ABSTRACT

5(10)-Estrane-3 β ,17 α -diol is an essential reference material for doping analysis in horse-racing laboratories. It is used to detect misuse, for doping purpose, of the pregnancy status in the mare. Its stereoselective synthesis from 17 β -estradiol-3-methyl ether (prepared from estrone or 17 β -estradiol) was performed in four steps: (1) Mitsunobu inversion of the 17 β -alcohol; (2) Birch reduction of the aromatic ring; (3) stereoselective reduction of the 3-ketone via Noyori asymmetric transfer hydrogenation; (4) chemoenzymatic purification.

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1. Introduction

The endogenous production of the potent anabolic steroid testosterone is known to increase during pregnancy in the mare. In order to avoid any misuse of the pregnant status for doping purpose, Horseracing Authorities have stated that pregnant mares may compete under definite conditions of date. The pregnant mare is not authorized to compete up to 250 days after successful insemination.

Equine pregnancy is routinely evidenced by an enzyme-linked immunosorbent assay of gonadotrophin (eCG) in plasma. This analytical method allows for eCG detection between Day 40 and 120. The period between Day 70 and 250 is covered by gas chromatography–mass spectrometry monitoring of two urinary pregnancy markers: estrone and 5(10)-estrene-3 β ,17 α -diol **6** [1]. Estrone is a readily available estrogen, but 5(10)-estrene-3 β ,17 α -diol **6** is no longer commercially available. In a pioneer work, **6** was prepared by Birch reduction of 17 α -estradiol [2]. A mixture of 5(10)-estrene-3 α ,17 α -diol and its 3 β epimer was obtained (yield and 3 α /3 β ratio were not reported) and subsequently submitted to a tedious chromatographic purification in order to recover pure **6**. In spite of specific interest (the configuration of the two asymmetric secondary alcohols being unambiguously established by X-ray radiocrystallography), this synthetic method is not convenient for

large scale preparation of **6** which is needed for routine screening by many horseracing antidoping laboratories all over the world. Therefore we decided to set up a stereoselective synthesis of **6**.

2. Experimental

Estradiol, estrone, iodomethane, sodium borohydride, benzoic acid, diphenyl-(2-pyridyl)-phosphine (PyPPH₂), lithium, Novozym 435® (*Candida antarctica* Lipase B immobilized on acrylic resin), anhydrous dioxane, toluene and diethyl ether were purchased from Sigma–Aldrich. Ethanol and isopropanol were from VWR. Hexane and ethyl acetate were from Carlo Erba. Diisopropylazodicarboxylate (DIAD) was from Alfa Aesar. Ammonia was from Air Products. Chloro[[[(1S,2S)-(+)-2-amino-1,2-diphenylethyl](4-toluenesulfonyl)amido](*p*-cymene)ruthenium(II)] was from Strem. Silica gel (0–63 μ m, 60 Å) for chromatography was from MP Biochemicals. 5(10)-estrene-3 β ,17 α -diol reference material was a generous gift from the Hong Kong Jockey Club Racing Laboratory [2]. All reactions were performed in dry nitrogen atmosphere.

GC/MS analyses were performed using Agilent GC5890/MSD5973 instrument equipped with a 25 m DB-5MS column (J&W Scientific). Analytical samples were derivatized with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (Fluka) before GC/MS analyses. NMR spectra were recorded on a Bruker ARX250 instrument. Chemical shifts are quoted relative to residual CHCl₃ in CDCl₃. HRMS spectra were recorded on a Q-Exactive instrument (Thermo Scientific). TLC experiments were performed using

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Whatman silica gel coated plates. Melting points were measured with a Reichert apparatus.

2.1. 17 β -Estradiol-3-methyl ether **1**

2.1.1. Starting from 17 β -estradiol

A mixture of 17 β -estradiol (2.86 g, 10.5 mmol), sodium carbonate (11.1 g, 105 mmol) and iodomethane (17 mL, 38.8 g, 273 mmol) in methanol (50 mL) was heated under reflux with stirring. After 16 h, iodomethane (1 mL, 2.28 g, 16 mmol) was added and the mixture was heated for 48 h. This suspension was then filtered and the inorganic salts were washed with methanol (150 mL). The methanolic solution was distilled under vacuum to give a white solid which was redissolved in boiling methanol (50 mL). Water (250 mL) was slowly added to the boiling solution of methanol with vigorous stirring, leading to the precipitation of a white solid. After 2 h stirring at room temperature, the precipitate was recovered by filtration, washed with water (250 mL) and dried under vacuum at 90 °C to give 2.92 g (yield: 97%) of 17 β -estradiol-3-methyl ether **1**.

2.1.2. Starting from estrone

A mixture of estrone (2.0 g, 7.4 mmol), sodium carbonate (9.0 g, 84.9 mmol) and iodomethane (10 mL, 22.8 g, 160.6 mmol) in methanol (50 mL) was heated under reflux with stirring. After 16 h, this mixture was cooled to room temperature and water (100 mL) was slowly added with vigorous stirring, leading to the precipitation of a white solid. After 1 h stirring, the precipitate was recovered by filtration, washed with water (300 mL) and dried under vacuum to give 2.049 g of estrone-3-methyl ether (yield: 97.6%). A solution sodium borohydride (0.5 g, 13.2 mmol) in water (10 mL) was added to a suspension of estrone-3-methyl ether (2.049 g, 7.2 mmol) in ethanol (350 mL) and stirred at room temperature. After 3 days, hydrochloric acid (1 N, 20 mL) was slowly added, the volume was reduced under vacuum to 50 mL (a crystalline precipitate appeared) and water (250 mL) was slowly added under vigorous stirring. The precipitate was collected by filtration, washed with water (150 mL) and dried under vacuum to give β -estradiol-3-methyl ether **1** as a white solid (1.982 g, 96%).

These 17 β -estradiol-3-methyl ether preparations were identical to a reference standard, as evidenced by GC/MS.

2.2. 3-Methoxy-estra-1,3,5(10)-trien-17 α -ol-17-benzoate **2**

A solution of diisopropyl-azodicarboxylate (1.6 mL, 7.86 mmol) in toluene (10 mL) was added dropwise to a solution of 17 β -estradiol-3-methyl ether **1** (1.50 g, 5.24 mmol), benzoic acid (1.0 g, 8.18 mmol) and diphenyl-(2-pyridyl)-phosphine (2.0 g, 7.59 mmol) in toluene (50 mL) in an ice bath. After 24 h heating at 80 °C, the mixture was cooled at room temperature and washed with hydrochloric acid (1 N, 4 $\times$ 100 mL), saturated aqueous sodium hydrogencarbonate solution (3 $\times$ 100 mL), dried with brine (100 mL) and anhydrous sodium sulfate.

After distillation of the solvent under vacuum, the residual brown gum was purified by chromatography on a silica gel column (5 $\times$ 50 cm, elution: hexane/ethyl acetate, 95:15, v/v) to give a light yellow solid which was recrystallized from boiling ethanol (10 mL) to give white crystals (1.168 g, yield: 57.1%).

TLC: R_f = 0.5 (hexane/ethyl acetate, 8:1, revelation: UV 254 nm). Melting point: 115 °C (Litt.: 114–115 °C [6]). MS (EI), *m/z* (%): 390 (100), 268 (20), 253 (6), 241 (12), 239 (12), 225 (12), 211 (7), 186 (14), 174 (44), 160 (27), 147 (23), 105 (64). HRMS (APCI, MH⁺) calc.: 391.22677, found: 391.22696. ¹H NMR ppm: 8.23 (d, *J* = 8 Hz, 2H benzoate o-H), 7.57 (t, *J* = 6 Hz, 1H benzoate p-H), 7.49 (t, *J* = 8.5 Hz, 2H benzoate m-H), 7.24 (d, *J* = 8 Hz 1H 1-H), 6.74 (dd, *J* = 2.75 Hz, *J* = 8.5 Hz, 1H 2-H), 6.68 (d, *J* = 3 Hz, 1H 4-H), 5.15 (d, *J* = 6 Hz, 1H 17 β -H), 3.81 (s, 3H, -OCH₃), 0.89 (s, 3H 18-

CH₃), complex multiplets between 1.0 and 3.0 ppm. ¹³C NMR ppm: 166.13 (C=O), 157.50 (3-C), 137.95, 132.76, 132.58, 130.91, 129.54, 128.37, 126.39, 113.85, 111.53, 82.65 (17-C), 55.21 (-OCH₃), 49.55, 45.43, 43.71, 39.16, 32.18, 30.29, 29.95, 28.12, 26.23, 24.49, 16.82 (18-C).

2.3. 5(10)-Estrone-17 α -ol,3-one **3**

A solution of 3-methoxy-estra-1,3,5(10)-trien-17 α -ol-17-benzoate **2** (1.42 g, 3.63 mmol) in 1,4-dioxane/diethyl ether (1:1, 100 mL) was added to a solution of lithium (1.42 g, 204.6 mmol) in ammonia (250 mL). Ethanol (30 mL) was added dropwise over a period of 20 min (until decoloration), ammonia was allowed to evaporate and water (200 mL) was added. This mixture was extracted with diethyl ether (2 $\times$ 300 mL). The organic fractions were combined, washed with water (2 $\times$ 300 mL) dried with brine (300 mL), sodium sulfate and evaporated under vacuum to give a white solid.

This solid was dissolved in methanol (120 mL) and a solution of oxalic acid dihydrate (1.4 g, 11.1 mmol) in water (20 mL) was added. After 2 h stirring at room temperature, the volume was reduced under vacuum at 40 °C to 50 mL. Diethyl ether (200 mL) was added and washed with sodium hydroxide solution (1 N, 200 mL). The aqueous layer was back extracted with diethyl ether (200 mL) and the combined organic fractions were washed with sodium hydroxide solution (1 N, 3 $\times$ 200 mL) and water (200 mL). The organic layer was dried with brine (200 mL), sodium sulfate and evaporated to give a white solid. Recrystallization from a hexane/ethyl acetate mixture gave 443.6 mg of **3** (white crystals, yield: 44.5%).

MS (EI), bisTMS derivative, *m/z* (%): 418 (100), 403 (9), 327 (3), 285 (4), 259 (7), 246 (6), 233 (4), 220 (7), 195 (8), 182 (38). HRMS (APCI, MH⁺) calc.: 275.20056, found: 275.20076. ¹H NMR ppm: 3.77 (d, *J* = 6 Hz, 1H 17 β -H), 0.68 (s, 3H 18-CH₃), complex multiplets between 1.0 and 3.0 ppm. ¹³C NMR ppm: 211.43 (3-C), 131.12 (10-C), 126.45 (5-C), 79.91 (17-C), 47.32, 45.87, 44.68, 39.35, 39.13, 32.55, 31.81, 30.83, 27.56, 27.30, 25.04, 24.05, 17.31 (18-C). Melting point: 149–150 °C (Litt.: 144–150 °C [5]).

2.4. 5(10)-Estrone-3 β ,17 α -diol **6**

A solution of potassium hydroxide (19.5 mg, 0.35 mmol) in isopropanol (1.95 mL) was added to a solution of 5(10)-estrone-17 α -ol,3-one **3** (392 mg, 1.43 mmol) and chloro-[(1*S*,2*S*)-(+)-2-amino-1,2-diphenylethyl](4-toluenesulfonyl)amido}(p-cymene)ruthenium(II) (204 mg, 0.32 mmol) in isopropanol (40 mL). After 20 h stirring at room temperature, the solution was diluted with ethyl acetate (300 mL) and washed with hydrochloric acid (1 N, 200 mL). The aqueous phase was back extracted with ethyl acetate (200 mL). The organic extracts were combined and washed with hydrochloric acid (1 N, 3 $\times$ 200 mL), saturated sodium hydrogen carbonate solution (3 $\times$ 200 mL), dried with brine (250 mL) and sodium sulfate.

Evaporation of the solvent under vacuum gave a black solid. This residue was dissolved in ethyl acetate (50 mL), adsorbed on SiO₂, loaded onto a silica gel column (3 $\times$ 25 cm) and eluted by hexane/ethyl acetate mixture (80/20, v/v). Three fractions were collected: the first one, containing pure 5(10)-estrone-3 β ,17 α -diol **6** (166 mg), the second one, containing target compound **6** contaminated with 2% of undesired epimer (147.1 mg) and the third one, containing a small amount of a 1/1 mixture of the two epimers (this fraction was discarded). Combined: 313.1 mg, yield: 79.7%.

Chemo-enzymatic purification: 100 mg of the second fraction (containing 2% of the 3 α epimer) was dissolved in a solution of vinyl acetate (1 mL, 10.8 mmol) in anhydrous toluene (35 mL). Novozym 435[®] (3 g) was added and this mixture was stirred at

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