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

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ABSTRACT

5(10)-Estrene-3β,17α-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 17β-estradiol-3-methyl ether (prepared from estrone or 17β-estradiol) was performed in four steps: (1) Mitsunobu inversion of the 17β-alcohol; (2) Birch reduction of the aromatic ring; (3) stereoselective reduction of the 3-ketone via Noyori asymmetric transfer hydrogenation; (4) chemoenzymatic purification.

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-3β,17α-diol **6** [1]. Estrone is a readily available estrogen, but 5(10)-estrene-3β,17α-diol **6** is no longer commercially available. In a pioneer work, **6** was prepared by Birch reduction of 17α-estradiol [2]. A mixture of 5(10)-estrene-3α,17α-diol and its 3β epimer was obtained (yield and 3α/3β 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 ($PyPPh_2$), 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{[(1*S*,2*S*)-(+)-2-amino-1,2-diphenylethyl](4-toluenesulfonyl)amido}(*p*-cymene)ruthenium(II) was from Strem. Silica gel (0–63 m, 60 Å) for chromatography was from MP Biochemicals. 5(10)-estrene-3β,17α-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 $CHCl_3$ in $CDCl_3$. 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. 17β-Estradiol-3-methyl ether **1**

2.1.1. Starting from 17β-estradiol

A mixture of 17β-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 17β-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, 72 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 17β-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-17α-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 17β-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 × 100 mL), saturated aqueous sodium hydrogencarbonate solution (3 × 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 × 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: Rf = 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. ^{1}H 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 17β-H), 3.81 (s, 3H, $-OCH_3$), 0.89 (s, 3H 18-CH_3), complex multiplets between 1.0 and 3.0 ppm. ^{13}C 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 ($-OCH_3$), 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)-Estren-17α-ol,3-one **3**

A solution of 3-methoxy-estra-1,3,5(10)-trien-17α-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 × 300 mL). The organic fractions were combined, washed with water (2 × 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 × 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. ^{1}H NMR ppm: 3.77 (d, *J* = 6 Hz, 1H 17β-H), 0.68 (s, 3H 18-CH_3), complex multiplets between 1.0 and 3.0 ppm. ^{13}C 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)-Estrene-3β,17α-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)-estren-17α-ol,3-one **3** (392 mg, 1.43 mmol) and chloro{[(*1S,2S*)-(+)-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 × 200 mL), saturated sodium hydrogen carbonate solution (3 × 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 SiO_2, loaded onto a silica gel column (3 × 25 cm) and eluted by hexane/ethyl acetate mixture (80/20, v/v). Three fractions were collected: the first one, containing pure 5(10)-estrene-3β,17α-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 3α 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

room temperature. After 22 h, the mixture was filtered on a Celite® pad, the catalyst was rinsed with diethyl ether and the solvent was evaporated to give a yellow gum. This gum was dissolved in dichloromethane, adsorbed on SiO_2, loaded onto a silica gel column (3 × 15 cm) and eluted by hexane/diethyl ether mixture (50/50, v/v) to give 96.6 mg of pure **6** (white solid, yield: 98.6%). This compound was recrystallized from boiling hexane / ethyl acetate mixture (80/20, v/v) (69 mg, yield: 71.5%).

TLC: Rf = 0.5 (hexane/ethyl acetate, 1:1, revelation: phosphomolybdic acid in ethanol). Melting point: 162–163 °C. MS (EI), bis-TMS derivative, *m/z* (%): 420 (3), 405 (3), 330 (100), 315 (5), 240 (58), 225 (42), 212 (11), 199 (29), 185 (11), 183 (13), 159 (11), 145 (18), 129 (28), 117 (13), 105 (11). HRMS (APCI, MH^+) calc.: 277.21621, found: 277.21637. ^{1}H NMR ppm: 4.06 (s, broad, 1H 3α-H), 3.76 (d, *J* = 5.75 Hz, 1H 17β-H), 0.68 (s, 3H 18-CH_3), complex multiplets between 1.0 and 2.5 ppm. ^{13}C NMR ppm: 129.88 (10-C), 124.60 (5-C), 80.15 (17-C), 66.10 (3-C), 47.79, 46.30, 46.01, 39.46, 39.32, 32.67, 32.04, 31.52, 30.11, 27.81, 25.28, 24.25, 22.66, 17.54 (18-C).

3. Results and discussion

Initially, the preparation of 5(10)-estrene-3β,17α-diol **6** was attempted starting from readily available 5(10)-estrene-3,17-dione. The reduction of this compound with sterically hindered lithium tri-*tert*-butoxyaluminum hydride gave exclusively the 17β alcohol with a 9/1 mixture of 3α/3β epimers.

This mixture was then converted, via a Mitsunobu reaction, into a 9/1 mixture of 5(10)-estrene-3α,17α-diol and 5(10)-estrene-3β,17α-diol. Unfortunately, the reaction produced of a large amount of by-products. We assume this might be attributed to rearrangement of homo-allylic steroidal alcohols in the course of Mitsunobu reaction [3]. This synthetic route was not further investigated and it was decided to prepare **6** via a stereoselective reduction of the 3-keto group (Scheme 1).

Readily available 3-methoxy-estra-1,3,5(10)-trien-17β-ol (17β-estradiol-3-methyl ether, **1**) was the starting material, prepared from estrone or 17β-estradiol with up to 97% yield [4,5].

The first step of this synthesis consists in the well known 17β inversion of 3-methoxy-estra-1,3,5(10)-trien-17β-ol **1** into its 17α epimer via a Mitsunobu reaction [6]. 3-methoxy-estra-1,3,5(10)-trien-17α-ol-17-benzoate **2** was obtained with 57,1% yield.

5(10)-Estren-17α-ol,3-one **3** was prepared by Birch reduction of **2** according to a published procedure used for its preparation from 3-methoxy-estra-1,3,5(10)-trien-17α-ol [5]. Benzoate ester saponification prior to Birch reduction was not necessary, according to the synthesis of 5α-estrane-3β,17α-diol [7]. This 5(10)-estren-17α-ol-3-one was obtained with 44.5% yield after recrystallization.

The stereoselective reduction of the 3-keto group of **3** into the 3β alcohol is the key step of this synthetic approach, but the 3-keto reduction in the 5(10)-estrene series has been scarcely investigated. It was reported that reduction using lithium aluminum hydride [8], sodium borohydride [9,10], di-isobutylaluminum hydride [9,11] and lithium tri-*tert*-butoxyaluminum hydride [8,9,12] gave mainly 3α alcohol (62–100% depending on the substrate and the reducing agent). These data prompted us to undertake a systematic investigation of 5(10)-estren-17β-ol-3-one reduction (the 17β epimer was used for investigative purpose). Reduction of this substrate with various stoichiometric reagents gave rise to mixtures of 3-hydroxy epimers with 3α/3β ratio ranging from 16/84 (with lithium tri-*sec*-butyl-borohydride) to 90/10 (with lithium tri-*tert*-butoxyaluminum hydride) (Table 1).

In order to improve the 3α/3β ratio by increasing 3β yield, it was decided to perform 3-keto reduction by using a chiral reagent. For cost reason and ease of product recovery, a catalytic reduction was preferred instead of stoichiometric asymmetric reducing agents. We postulated that a spatial distortion in steroidal A ring induced by the 5(10) double bond, might lead to strong discrimination between the axial and the equatorial site for the action of the chiral catalyst during hydrogen addition.

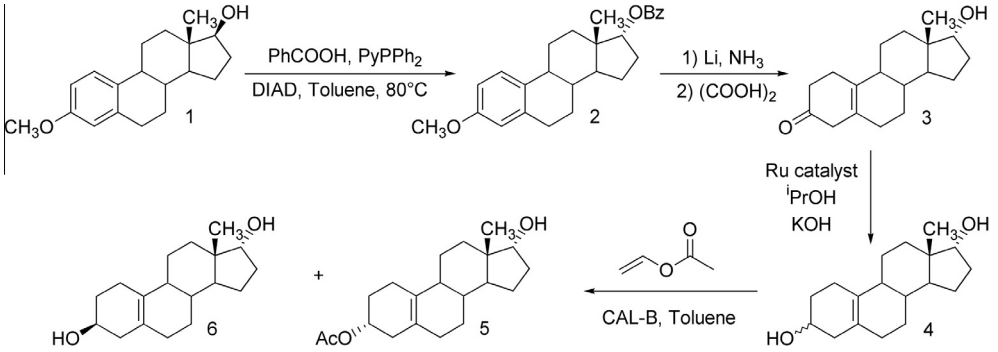
The reduction was performed under asymmetric transfer hydrogenation (ATH) conditions, because this method has high efficiency and proceeds under very mild conditions [13,14]. We were encouraged by the results of ruthenium catalyzed ATH of 2-tetralone (3,4-dihydro-2(1*H*)-naphthalenone): the corresponding alcohol was obtained with up to 82% ee [15]. We assumed that the spatial configuration of the cyclohexene rings in **3** and 2-tetralone were similar.

Results on ATH reduction of various 3-ketosteroids catalyzed with RuCl[(*S*,*S*)-Tsdpen](*p*-cymene) (Noyori catalyst [16]) are summarized Table 2. In the case of 5α-androstan-17β-ol-3-one, the diastereoisomeric excess obtained after the ATH is moderate. The conformation of A ring allows for a weak 3α/3β discrimination by the chiral catalyst. On the opposite, the presence of a double bond in the A ring at the C-4 or C-5(10) position resulted in a strong increase of 3α/3β discrimination.

However, the presence of a conjugated double bond slowed down the reaction rate. The reduction of 5α-androstan-17β-ol-3-one and 5(10)-estren-17α-ol-3-one was completed within 2 h but the reduction of testosterone (4-androsten-17β-ol-3-one) and nandrolone (4-estren-3-one-17β-ol) was not completed within 12 h.

To the best of our knowledge, this is the first application of Noyori asymmetric transfer hydrogenation for the reduction of 3-ketosteroids to diastereomeric alcohols.

ATH reduction of 3 in isopropanol at room temperature, catalyzed by RuCl[(*S*,*S*)-Tsdpen](*p*-cymene), gave a mixture of 5(10)-estrene-3,17α-diol with an overall 80% yield, and 93% of 3β-hydroxy epimer (determined by GC/MS analysis of the reaction mixture).



Scheme 1.

Table 1
Reduction of 5(10)-estren-17β-ol-3-one with stoichiometric reagents. The 3α/3β ratio values were determined by GC/MS analysis of the reaction mixture.

Reducing agent	Equivalent	Solvent	Temperature (reaction time)	3α/3β Ratio
Lithium tri-*tert*-butoxyaluminum hydride	3	THF	−78 °C (8 h)	90/10
Sodium borohydride/cerium chloride	5	MeOH	−78 °C (1 h) → RT (1 h)	88/12
Lithium tri-*tert*-butoxyaluminum hydride	3	THF	RT (1 h)	80/20
Sodium borohydride	10	EtOH	RT (1 h)	75/25
Lithium aluminum hydride	15	Et_2O	RT (2 h)	65/35
(*R*)-BINOL(OEt)AlH	6	THF	−78 °C (1 h) → RT (15 h)	40/60
Lithium tri-*sec*-butyl-borohydride	4	THF	−78 °C (1 h)	16/84

Table 2
3-OH α/β ratio obtained after ATH reduction of some 3-ketosteroids with RuCl[(*S*,*S*)-Tsdpen](*p*-cymene). Reagents and conditions: 3-ketosteroid: 0.14 mmol, catalyst: 10 mol%, isopropanol: 2 mL, 2 M KOH in isopropanol: 100 μL, room temperature, reaction time: 12 h. The 3α/3β ratio values were determined by GC/MS analysis of the reaction mixture.

3-Ketosteroid	3α/3β Ratio
5α-Androstan-17β-ol-3-one	78/22
4-Androsten-17β-ol-3-one (testosterone)	2/98
4-Estren-3-one-17β-ol (nandrolone)	1/99
5(10)-Estrene-17α-ol-3-one	7/93
1,4-Androstadien-3-one-17β-ol (boldenone)	No reaction

The purification of target compound **6** was a critical step since in doping analysis an analytical standard, free of any epimeric diol, is requested.

The catalyst was removed by column chromatography. Three fractions were collected: the first one containing pure 5(10)-estrene-3β,17α-diol **6**, the second one containing **6** contaminated with 2% of undesired epimer and in the third one, a small amount of a 1/1 mixture of the two epimers was found and discarded.

As reported previously [2], chromatographic separation of a mixture of 3α and 3β diols is a long and tedious process. So, in order to avoid time-consuming chromatographic purification of the second fraction, it was decided to use a selective chemo-enzymatic transformation of the 3α-epimer. A similar approach was successfully used for the stereoselective preparation of 3-hydroxy metabolites of tibolone (17α-ethynyl-7α-methyl-5(10)-estren-17β-ol-3-one) [11].

Epimeric diol separation was performed by diastereoselective 3α-acetylation catalyzed by *C. antarctica* Lipase B (CAL-B) immobilized on acrylic resin (Novozym 435®). After stirring a solution of the epimer mixture and vinyl acetate in toluene at room temperature in the presence of CAL-B, the separation of target compound **6** from the 5(10)-estrene-3α,17α-diol-3-acetate was easily achieved by column chromatography with high yield (98.8%). The 3α acetate **5** was observed by GC/MS analysis of the reaction mixture (characteristic molecular ion and typical 5(10)-estrene nucleus fragmentation) but it was not isolated. It is noteworthy that the 17α- and 17β-hydroxy groups remained unaffected by this treatment (as determined in preliminary experiments).

The identity of 5(10)-estrene-3β,17α-diol was established by NMR spectrometry and GC/MS data comparison with a reference standard (generous gift from the Hong Kong Jockey Club Racing Laboratory [2]).

In conclusion, a simple and efficient stereoselective synthesis of 5(10)-estrene-3β,17α-diol has been performed by mild asymmetric transfer hydrogenation, which was found to be a powerful tool for the stereoselective synthesis of 3-hydroxy steroids. Furthermore, a chemo-enzymatic purification process has been proven of real practical value.

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