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Synthesis of human long-term metabolites of dehydrochloromethyltestosterone and oxymesterone

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ABSTRACT

We herein report the synthesis of the long-term metabolites "M4" (IUPAC: 4-chloro-17-hydroxymethyl-17-methyl-18-norandrosta-4,13-dien-3-ol) of dehydrochloromethyl-testosterone (DHCMT, Oral Turinabol) and "Oxy M9" (4-hydroxy-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one) of oxymesterone (Oranabol). Both compounds were derived from a common synthetic route starting from dehydroepiandrosterone acetate. Four different stereoisomers were evaluated for metabolite M4. The previously assigned structure could be corrected regarding the C-3 and C-17 stereocenters.

1. Introduction

Dehydrochloromethyltestosterone (DHCMT, **1**) is an anabolic-androgenic steroid (AAS), first patented by Stachowiak and coworkers [1] at Jenapharm in 1961. As outlined in the patent specification the goal was an increased anabolic effect relative to the androgenic effect. The disconnection of these two effects was novel at this point in time [2,3] and provides a clear incentive for illicit use. The drug was approved for clinical use in 1965 and marketed under the name Oral Turinabol. The metabolism of the drug was first intensively investigated by Schubert et al. [4]. In 1995 Schänzer et al. reported the first synthesis of one of the metabolites, 6β-hydroxy-DHCMT [5].

From 1974 to 1989 DHCMT was one of the doping agents used in state-sponsored doping efforts of the GDR [6]. More recently, up until 2010, the number of adverse analytical findings (AAF) reported by the World Anti-Doping Agency (WADA) was small, seldom exceeding single-digit figures [7]. Testing of athletes' samples is based on the available metabolites, which offer variable detection windows. It is therefore of interest to find new metabolites which can extend this time frame. In 2012 Sobolevsky and Rodchenkov published an article outlining a number of new metabolites of DHCMT, many of them long-term metabolites, which can be detected weeks after the last administration [8]. GC/MS-MS was used for detection and identification of target compounds. After new methods adapted from this work were introduced for testing at WADA the number of AAF increased dramatically to 87 in 2013.

One of these metabolites, named "M4", was assigned the structure **2a** depicted in Fig. 1. It is reportedly detectable for roughly 20 days after administration and is most probably formed by hydrogenation of the 1,2-double bond, reduction of the ketone and Wagner-Meerwein-rearrangement followed by an enzymatic hydroxylation (or *vice versa*). Since information about new stereocenters introduced during the metabolic transformations cannot be deduced from MS data exclusively, it is often necessary to synthesize a reference material for comparison. In previous work on a different metabolite of DHCMT ("M3") the assigned stereochemistry from Sobolevsky and Rodchenkov could be corrected and the metabolite unambiguously identified [9]. In 2018 and 2020 synthetic efforts towards the M4 metabolite were reported by Kozyrkov and coworkers [10,11]. The focus of this work was put on the proposed structure by Sobolevsky, namely the 3α-hydroxy-17β-hydroxymethyl isomer **2a** but no validation of the structure by comparison with the *in vivo* metabolites was carried out.

Oxymesterone (**2**) is a less known AAS first patented by "Societa Farmaceutici Italia" in the early 1960s [12]. It was marketed in Spain, the UK and Japan under the brand names: Anamidol, Oranabol, Balnimax and Theranabol [13]. Abuse of this steroid has not nearly been as wide-spread as for other anabolic agents like DHCMT and metandienone. As oxymesterone contains a 17-methyltestosterone substructure it can form rearranged metabolites as well. One of these was identified in 2017 by Van Eenoo et al. to be 4-hydroxy-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one (Oxy M9, **3**) [14]. Recently, Zhabinskii, Hurski and coworkers synthesized **3** in 14 steps through a photochemical approach towards the D-ring pattern [15].

In continuation of our efforts towards the synthesis of long-term

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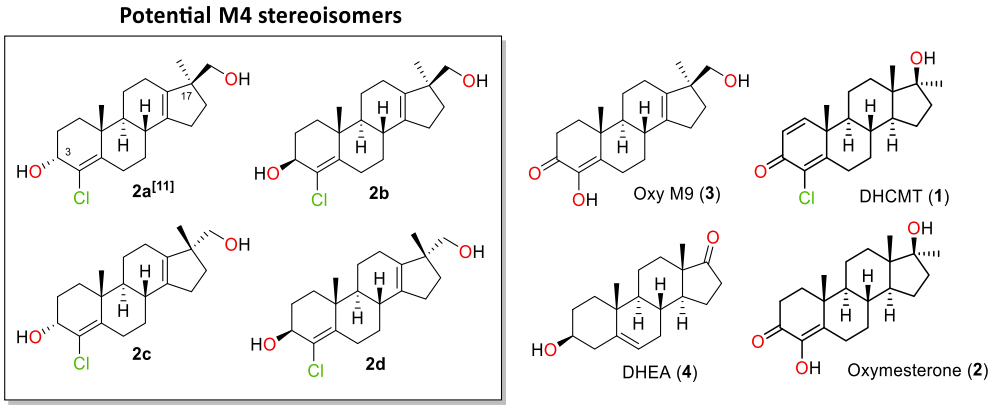


Fig. 1. Molecular structures of relevant compounds.

metabolites [9] with the 18-nor-17-hydroxymethyl-17-methyl-13-ene fragment we herein report the structural correction and synthesis of the DHCMT metabolite "M4" as well as the synthesis of the oxymesterone metabolite "Oxy M9". Focus of this work was on synthesizing the possible stereoisomers concerning C-3 and C-17 using our established Wagner-Meerwein rearrangement strategy. Two different starting materials are needed to access both C-17 epimers. One can be derived from the parent drug **1**, the other from commercially available DHEA (**4**). Initially, synthesis of the proposed compound 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3α-ol (**2a**, see Fig. 1) from **4** was attempted.

2. Experimental

Dehydroepiandrosterone acetate was purchased from FluoroChem. Dry toluene, dichloromethane, dimethylformamide and meta-chloroperoxybenzoic acid (*m*-CPBA) are from Acros Organics. HPLC grade solvents (acetonitrile, methanol, isopropanol) were from VWR. All other non-specified chemicals were from Sigma-Aldrich. Anhydrous tetrahydrofuran was pre-dried using an Innovative Technologies PureSolv system, degassed, and stored over 3 Å molecular sieves. Potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, potassium hydrogen carbonate, potassium carbonate, diphosphorus pentoxide were purchased from Merck. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel, and β -glucuronidase obtained from Roche. Schlenk flasks (cylindrical reaction vessels with an additional ground-glass stopcock on the side) were flame-dried under a constant argon flow before use. Seignette's salt refers to potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$).

NMR spectra were recorded on a Bruker AC400 and AC600 using TMS as internal standard. IR spectra were recorded on a Perkin Elmer Spectrum 65 as thin films (ATR FT-IR). TLC-analysis was performed with pre-coated aluminium-backed plates (Silica gel 60 F254, Merck). Compounds were visualized by submerging in an acidic phosphomolybdic acid/cerium sulphate solution and heating. Melting points were determined with a Kofler hot-stage apparatus.

Sample preparation for GC–MS/MS analysis: To 5 mL of urine 100 µL of methyltestosterone (IS), 1 mL of 0.8 M phosphate buffer pH 7 and 25 µL of β-glucuronidase were added. Hydrolysis was performed at 50 °C for 2 h. After hydrolysis, 1 mL of 20% potassium carbonate buffer pH 9.0 and 7 mL of methyl *tert*-butyl ether (MTBE) were added, and a liquid-liquid extraction was performed by shaking for 10 min. After that, sample was centrifuged for 7 min with 2100 rpm, the organic layer was transferred and evaporated. Subsequently the samples were dried for 15 min over diphosphorus pentoxide. Derivatization was performed by adding 100 µL working solution for derivatization and heating at 60 °C for 20 min. For derivatization, a trimethylsilyl iodide stock solution was prepared by mixing 5 mL of MSTFA with 300 µL of ethanethiol. Subsequently, 100 mg of NH_4I were dissolved in this mixture. A working solution for derivatization was prepared by adding 1 mL of the trimethylsilyl iodide stock solution to 9 mL of MSTFA. Solutions of internal standard (1 µg/mL) and analytes (1 µg/mL) were prepared in methanol and stored at −20 °C.

The GC–MS/MS measurements were performed with a Trace-1300 gas chromatograph coupled to a TSQ-8000 Evo triple quadrupole mass spectrometer, and a TriPlus-100 autosampler (Thermo Fisher, Austin, USA). For chromatographic separation a Rtx-1MS fused silica capillary column, 15 m × 0.25 mm ID, 0.11 µm film thickness was used (Restek, CP-Analytica, Mistelbach, Austria). Injections of 2.5 µL were performed in split mode with an injector temperature of 270 °C, using a split flow of 40 mL/min. The temperature program for the GC run was following: 170 °C initial column temperature, 3 °C/min to 210 °C, held for 1 min, 25 °C/min to 305 °C, held for 3 min. High purity helium gas was used as carrier gas with a constant pressure of 80 kPa. The temperature of the transfer line and ion source was set to 270 °C. Electron impact ionization was used with an electron energy of 70 eV and data were acquired in selected reaction monitoring (SRM) mode.

2.1. (13α)-Spiro[androst-4-en-17β,2′-oxirane]-3-one (6)

To a solution of enone **5** (800 mg, 2.8 mmol) in chloroform (40 mL) and buffer solution (di-sodium hydrogen phosphate/potassium dihydrogen phosphate) pH 6.88 (20 mL) at 0–2 °C was added 70% *meta*-chloroperoxybenzoic acid (680 mg, 2.75 mmol) in portions over 15 min. The reaction was stirred at 2 °C for 7 h and then quenched by addition of saturated $NaHCO_3$ and $Na_2S_2O_3$ solutions (10 mL each). The mixture was extracted twice with CH_2Cl_2 (50 mL) and the pooled organic phases were washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified *via* column chromatography (95 g SiO_2, LP:EtOAc = 7:1) to give 102 mg of recovered starting material **5** (13%), 280 mg 17β-epoxide **6** (33%) as well as 422 mg of undesired 17α-epoxide **7** (50%).

17β-Product (**6**): ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 5.72 (1H, s), 2.67 (2H, dd, J = 36 Hz, 4.67 Hz), 2.35 (4H, m), 2.20 (1H, dd, J = 10.21 Hz, J = 11.38 Hz), 2.12 (1H, m), 2.05 (1H, m), 1.91 (1H, m), 1.83 (1H, m), 1.70–1.78 (2H, m), 1.65 (1H, m), 1.53 (1H, m), 1.45 (1H, m), 1.27–1.39 (3H, m), 1.10–1.18 (1H, m), 1.12 (3H, s), 1.03 (1H, m), 0.92 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 199.92, 171.81, 123.64, 68.38, 53.20, 51.53, 47.64, 41.17, 38.90, 37.37, 35.66, 34.07, 33.09 (2C), 31.68, 30.13, 28.15, 24.57, 22.87, 18.00. IR [cm^{-1}]: 2918, 1672, 1229. $[\alpha]_D^{20}$ = 66.73 (c 1.25, CH_2Cl_2). m.p.: 145–147 °C. HRMS (M + H): Calcd.: 301.2162, found: 301.2185.

17α-Product (**7**): ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 5.71 (1H, s), 2.79 (2H, dd, J = 20.3 Hz, J = 4.84 Hz), 2.25–2.45 (4H, m), 2.00–2.11 (4H, m), 1.89 (1H, m), 1.68 (1H, dddd, J = 13.84 Hz, J = 13.7 Hz, J = 4.98 Hz). 1.48–1.61 (2H, m), 1.34 (2H, m), 1.15–1.26 (2H, m),

1.09 (3H, s), 0.98 (3H, s), 0.91–1.15 (4H, m). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 199.57, 171.02, 123.84, 66.54, 55.22, 53.17, 51.61, 40.87, 38.72, 37.63, 35.54, 33.95, 32.96, 32.93, 31.93, 29.48, 26.34, 24.13, 22.11, 17.87. IR [cm^{-1}]: 2917, 1674, 1229. $[\alpha]_D^{20}$: 68.03 (c 0.96, CH_2Cl_2). m.p.: 156 °C. HRMS (M + H): Calcd.: 301.2162, found: 301.2180.

2.2. (13α)-4-Chlorospiro[androst-4-en-17β,2′-oxirane]-3-one (8)

To a solution of spiroepoxide **6** (132 mg, 0.42 mmol) in dry pyridine (4 mL) at 0 °C was added dropwise freshly distilled sulfuryl chloride (180 mg, 0.11 mL, 1.33 mmol). The solution was stirred for 30 min and then diluted by addition of toluene (20 mL). A precipitate formed which was filtered off and the solids were thoroughly washed with ethyl acetate. The filtrate was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and the solvent evaporated. There were obtained 140 mg orange crystals of **8** which were immediately used in the next step.

2.3. 4-Chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one (9)

A Schlenk flask was charged with dry CH_2Cl_2 (4 mL) and 2,6-lutidine (1.22 mmol). The solution was chilled to −78 °C at which point TMSOTf (0.18 mL, 1.01 mmol) was added dropwise followed by a solution of the crude product **8** (141 mg, 0.42 mmol) in CH_2Cl_2 (2 mL). The reaction was stirred at that temperature for 20 min followed by addition of methanol (3 mL) and 2 M HCl (4 mL) and removal of the acetone/$N_{2(l)}$ bath. The reaction was further stirred for 15 min followed by phase separation and extraction with EtOAc. Combined organic phases were washed with saturated $NaHCO_3$ solution and brine. After drying with $MgSO_4$ and evaporating under reduced pressure 160 mg crude product was obtained. Purification *via* column chromatography (10 g SiO_2, LP:EtOAc = 15:1 to 5:1) yielded 77 mg **9** (55%) as colorless crystals.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 3.41 (2H, dd, *J* = 60.7 Hz, *J* = 10.39 Hz), 3.31 (1H, m), 2.56–2.65 (2H, m), 2.07–2.38 (6H, m), 1.95–2.05 (2H, m), 1.84–1.94 (2H, m), 1.77 (1H, m), 1.57 (1H, m), 1.28–1.39 (2H, m), 1.24 (1H, m), 1.20 (3H, s), 1.15 (1H, m), 0.95 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 190.79, 164.46, 139.17, 137.69, 127.82, 68.89, 51.87, 51.55, 41.41, 36.16, 34.29, 34.01, 33.94, 30.46, 30.22, 29.49, 22.57, 22.55, 21.78, 17.41. IR [cm^{-1}]: 3475, 2922, 1679. $[\alpha]_D^{20}$: 84.05 (c 1.04, CH_2Cl_2). m.p.: 124–127 °C. HRMS (M + H): Calcd.: 335.1773, found: 335.1771.

2.4. 4-Chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3ξ-ol (2a, 2b)

Dry CH_2Cl_2 (5 mL) and enone **9** (57 mg, 0.17 mmol) were charged in a Schlenk flask and the mixture was chilled to −78 °C in an acetone/$N_{2(l)}$ bath. At this temperature there was added 1 M DIBAL-H in hexane (0.58 mL, 0.58 mmol) and the reaction was stirred for 1 h at −78 °C followed by 1 h at room temperature. The reaction was quenched by addition of methanol, followed by addition of Seignette's salt solution (10 mL) and stirring for 30 min. The biphasic mixture was then extracted with CH_2Cl_2 (2 ×) and washed with brine. Pooled organics were dried over $MgSO_4$ and evaporated under reduced pressure. The products were isolated *via* column chromatography (5 g SiO_2, LP:EtOAc = 5:1), giving 5 mg diol **2a** as well as 40 mg diol **2b** as light yellow oils.

3α-Product (**2a**): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H = 4.15 (1H, m), 3.48 (1H, d, *J* = 10.77 Hz), 3.32 (1H, d, *J* = 10.7 Hz), 3.00 (1H, m), 2.11–2.35 (3H, m), 1.99 (3H, m), 1.89 (3H, m), 1.71 (1H, m), 1.54 (2H, m), 1.48 (1H, m), 1.20–1.35 (3H, m), 1.06–1.17 (2H, m), 1.02 (3H, s), 0.93 (3H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ_C = 143.36, 140.26, 137.02, 127.69, 69.93, 69.03, 52.25, 51.66, 40.61, 36.63, 33.98, 31.39, 30.63, 30.60, 27.46, 27.24, 23.16, 22.65, 21.87, 17.78. IR [cm^{-1}]: 3360, 2909, 1444. $[\alpha]_D^{20}$: 84.42 (c 0.3, CH_2Cl_2). HRMS (M + H): Calcd.: 335.1783, found: 335.1741.

3β-Product (**2b**): ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 4.15 (1H, dd, *J* = 6.5 Hz, 9.5 Hz), 3.47 (1H, d, *J* = 10.44 Hz), 3.30 (1H, d, *J* = 10.54 Hz), 2.98 (1H, m), 2.08–2.32 (4H, m), 1.92–2.05 (4H, m), 1.79–1.89 (3H, m), 1.74 (1H, m), 1.67 (1H, m), 1.55 (1H, m), 1.18–1.42 (3H, m), 1.07 (3H, s), 0.94–1.14 (2H, m), 0.93 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 142.24, 140.27, 137.04, 128.96, 69.01, 52.12, 51.63, 40.54, 36.61, 34.02, 33.72, 30.81, 30.56, 28.09, 27.67, 22.78, 22.64, 21.86, 18.91. IR [cm^{-1}]: 3384, 2936, 1452, 731. $[\alpha]_D^{20}$: 41.64 (c 0.5, CH_2Cl_2). HRMS (M + H): Calcd.: 335.1783, found: 335.1742.

2.5. 4-Hydroxy-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one (3)

A round bottom flask was charged with starting material **9** (57 mg, 0.17 mmol), which was dissolved in freshly distilled, degassed *t*-BuOH (3 mL). KOH (30 mg, 0.5 mmol) was added and the mixture stirred at reflux for 30 min. After cooling to room temperature, the solution was neutralized by addition of saturated NH_4Cl solution (5 mL) and the mixture was diluted with water (5 mL). Extraction with CH_2Cl_2 (3 ×) and drying the extracts over $MgSO_4$ was followed by evaporation of the solvent. The residue was purified *via* column chromatography (5 g SiO_2, LP:acetone = 10:1) and then crystallized from MTBE /*n*-hexane to give 27 mg of ketone **3** (50%) as off-white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 6.09 (1H, s), 3.49 (1H, d, *J* = 10.53 Hz), 3.33 (1H, d, *J* = 10.53 Hz), 3.07 (1H, m), 2.47–2.60 (2H, m), 2.18–2.38 (2H, m), 1.97–2.14 (4H, m), 1.83–1.94 (2H, m), 1.70 (1H, m), 1.57 (1H, m), 1.24–1.34 (2H, m), 1.05–1.21 (4H, m), 1.14 (3H, s), 0.95 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 193.77, 141.60, 140.09, 139.57, 137.30, 69.07, 52.23, 51.71, 37.91, 36.36, 34.66, 34.03, 31.90, 30.67, 30.20, 23.60, 22.60, 22.56, 21.87, 16.88. IR [cm^{-1}]: 3400, 2943, 1666, 1171. $[\alpha]_D^{20}$: 23.35 (c 0.54, CH_2Cl_2). m.p.: 76–78 °C HRMS (M + H): Calcd.: 317.2111, found: 317.2106.

EI fragmentation MS:

The full scan mass spectrum of the tri-TMS derivative of **3** is shown below. (Fig. 2) The most abundant fragment 371.4 *m/z* is presumably formed by loss of two TMS groups and a methyl group (Δ = 161 u) from the trisylilated M + 532.2 *m/z*. The signal at 459.4 *m/z* describes the loss of one TMS group (Δ = 73 u).

2.6. 4-Chloro-17α-methylandrost-4-en-17β-ol-3-one (10)

In a Schlenk flask ethanol (25 mL), CH_2Cl_2 (5 mL) and Wilkinson's catalyst (845 mg, 0.91 mmol) were charged. To the resulting solution starting material **1** (1.08 g, 3.23 mmol) was added under argon flow and the atmosphere was exchanged for hydrogen. The solution was further stirred at room temperature over 18 h, concentrated and directly loaded onto a silica gel column (50 g SiO_2, LP:EtOAc = 5:1 to 2:1) to give 1.01 g of hydrogenated product **10** (93%) as off-white solid.

Analytical data is in accordance to literature [16].

2.7. 4-Chloro-17-methylenandrost-4-en-3-one (11) and 4-Chloro-17-methylandrosta-4,16-dien-3-one (12)

To a Schlenk flask containing starting material **10** (496 mg, 1.47 mmol) was added dry toluene (25 mL) and Burgess' reagent (1.05 g, 4.4 mmol). The solution was heated to 70 °C for 1 h, quenched with water, and extracted three times with EtOAc. The pooled organic phases were washed with brine and dried over $MgSO_4$. After evaporation of the solvent 459 mg of crude product was isolated as a 2:1 mixture of regioisomers, favoring 17-*exo*-methylene compound **11**. The product was used directly in the next step.

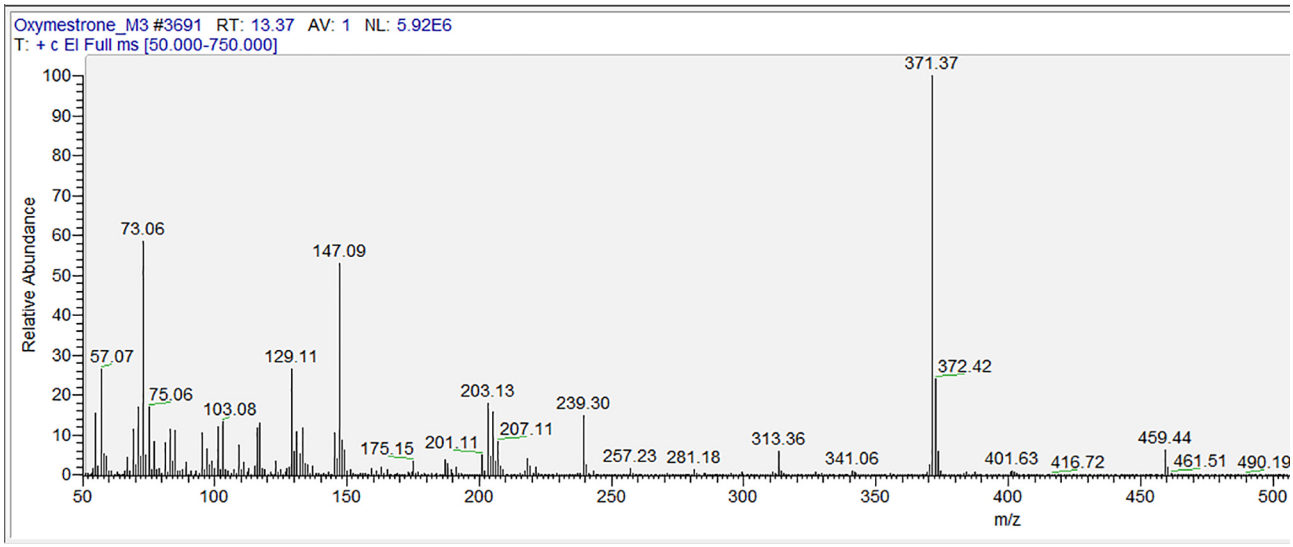


Fig. 2. EI fragmentation spectrum of **3**.

2.8. 4-Chlorospiro[androst-4-en-17α,2′-oxiran]-3-one (13)

A round bottom flask containing crude olefin mixture **11** and **12** (306 mg, 0.91 mmol) was chilled to 0 °C and dissolved in acetone (5 mL). To this solution 15 mL dimethyldioxirane in acetone (ca. 0.06 M, 0.9 mmol) was added and the reaction stirred for 30 min. The solution was evaporated to dryness under reduced pressure and purified via column chromatography (20 g SiO_2, LP:Et_2O = 5:1) to give 101 mg epoxide **13** (31%) as a white solid. Isomeric epoxides were not isolated in a pure form and thus not characterized.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 3.26 (1H, ddd, J = 15.02 Hz, 4.05 Hz, 2.67 Hz), 2.76 (1H, d, J = 4.40 Hz), 2.67 (1H, d, J = 4.40 Hz), 2.53–2.59 (2H, m), 2.31 (1H, m), 2.16 (1H, m), 1.93–2.07 (2H, m), 1.89 (1H, m), 1.69 (1H, m), 1.52–1.59 (2H, m), 1.32–1.43 (3H, m), 1.23 (3H, s), 1.16–1.30 (3H, m), 1.12 (1H, m), 1.03 (1H, m), 0.86 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 190.78, 164.65, 127.52, 69.76, 53.87, 52.10, 47.41, 41.52, 41.45, 35.35, 34.57, 34.10, 30.97, 29.93, 29.06, 24.01, 20.34, 17.94, 16.16. IR [cm^{-1}]: 2938, 1690, 886. $[\alpha]_D^{20}$: 131.88 (c 1.5, CH_2Cl_2). m.p.: 150–152 °C. HRMS (M + H): Calcd.: 335.1773, found: 335.1785.

2.9. 4-Chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3-one (14)

A round bottom flask was charged with epoxide **13** (202 mg, 0.6 mmol), the solid dissolved in glacial acetic acid (5 mL) and stirred over night at room temperature. The reaction was then diluted with water (25 mL) and neutralized by addition of $NaHCO_3$. The resulting suspension was extracted with CH_2Cl_2 (3 ×) and the pooled organic phases were washed with brine. After drying over $MgSO_4$ and evaporating the solvent under reduced pressure the crude product was purified via column chromatography (15 g SiO_2, LP:Et_2O = 5:1) to give 144 mg ketone **14** (71%) as waxy solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 3.39 (1H, d, J = 10.66 Hz), 3.31 (1H, d, J = 10.66 Hz), 3.31 (1H, m), 2.60 (2H, m), 2.20–2.35 (3H, m), 2.03–2.19 (4H, m), 1.96 (1H, m), 1.71–1.91 (3H, m), 1.59 (1H, m), 1.10–1.36 (4H, m), 1.21 (3H, s), 0.99 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 190.79, 164.37, 139.59, 137.38, 127.99, 69.15, 52.05, 51.63, 41.44, 36.55, 34.36, 34.20, 34.07, 30.63, 30.51, 29.54, 22.75, 22.72, 21.81, 17.47. IR [cm^{-1}]: 2922, 2860, 1681, 1031. $[\alpha]_D^{20}$: 92.30 (c 0.4, CH_2Cl_2). m.p.: 87–92 °C. HRMS (M + H): Calcd.: 335.1773, found: 335.1795.

2.10. 4-Chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3α-ol (2c) and 4-Chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3β-o (2d)

The starting material **14** (59 mg, 0.18 mmol) was charged in a flame-dried Schlenk flask. There was added CH_2Cl_2 (3 mL) and the solution was chilled to −78 °C. DIBAL-H (1 M, 0.6 mL, 0.6 mmol) was added dropwise at that temperature and the reaction was stirred at −78 °C for 1 h and then 1 h at room temperature. The reaction was quenched by addition of methanol, followed by Seignette's salt solution (15 mL) and stirring for 30 min. The biphasic mixture was then extracted with CH_2Cl_2 (3 ×) and washed with brine. Pooled organics were dried over $MgSO_4$ and evaporated under reduced pressure. The products were isolated *via* column chromatography (5 g SiO_2, *n*-hexane:EtOAc = 5:1), giving 6 mg diol **2c** (10%) and 37 mg diol **2d** (62%) as white solids.

3α-Product (**2c**): ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 4.15 (1H, m), 3.38 (1H, d, J = 10.45 Hz), 3.28 (1H, d, J = 10.45 Hz), 3.00 (1H, m), 2.30 (1H, m), 2.09–2.24 (2H, m), 1.99–2.08 (3H, m), 1.96 (1H, m), 1.84–1.93 (3H, m), 1.81 (1H, m), 1.71 (1H, m), 1.54–1.65 (2H, m), 1.26–1.34 (2H, m), 1.16 (1H, m), 1.09 (1H, m), 1.02 (3H, s), 0.98 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 143.28, 140.65, 136.61, 127.79, 69.93, 69.16, 52.50, 51.64, 40.61, 36.97, 34.27, 31.41, 30.94, 30.78, 27.46, 27.25, 23.22, 22.75, 21.90, 17.78. IR [cm^{-1}]: 2925, 2853, 1460, 1030. $[\alpha]_D^{20}$: 93.07 (c 0.13, CH_2Cl_2). HRMS (M + H): Calcd.: 335.1773, found: 335.1782.

3β-Product (**2d**): ^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 4.16 (1H, m), 3.37 (1H, d, J = 10.07 Hz), 3.29 (1H, d, J = 10.07 Hz), 2.99 (1H, m), 2.24–2.60 (2H, m), 1.9–2.24 (7H, m), 1.75–1.88 (3H, m), 1.71 (1H, m), 1.59 (1H, m), 1.25–1.40 (2H, m), 0.93–1.12 (3H, m), 1.08 (3H, s), 0.98 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 142.14, 140.68, 136.64, 129.09, 69.79, 69.18, 52.35, 51.64, 40.54, 36.96, 34.27, 33.75, 31.11, 30.73, 28.07, 27.69, 22.86, 22.75, 21.88, 18.93. IR [cm^{-1}]: 2919, 2857, 1068, 1037. $[\alpha]_D^{20}$: 40.42 (c 1.4, CH_2Cl_2). m.p.: 157–159 °C. HRMS (M + H): Calcd.: 335.1773, found: 335.1775.

EI fragmentation MS:

The following full scan spectrum of the bis-TMS derivative of **2d** (Fig. 3) shows the following fragmentation by EI: M + 481.2 *m/z* the fragments 465.3 *m/z* (–CH_3, Δ = 15 u) and 377.3 (-CH_2OTMS, Δ = 103 u) are observed. By an additional loss from 377.3 *m/z* of HCl, the fragment 341.3 *m/z* (Δ = 36 u) is found. By the additional loss from 377.3 *m/z* of the second TMS group, the fragment 287.3 *m/z* (-TMSOH, Δ = 90 u) is formed. By an additional loss of HCl, the fragment

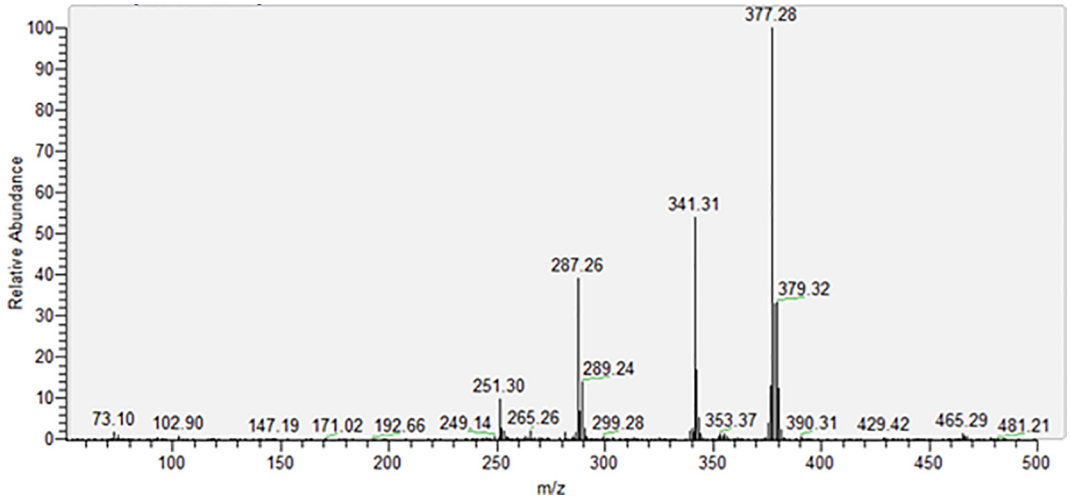
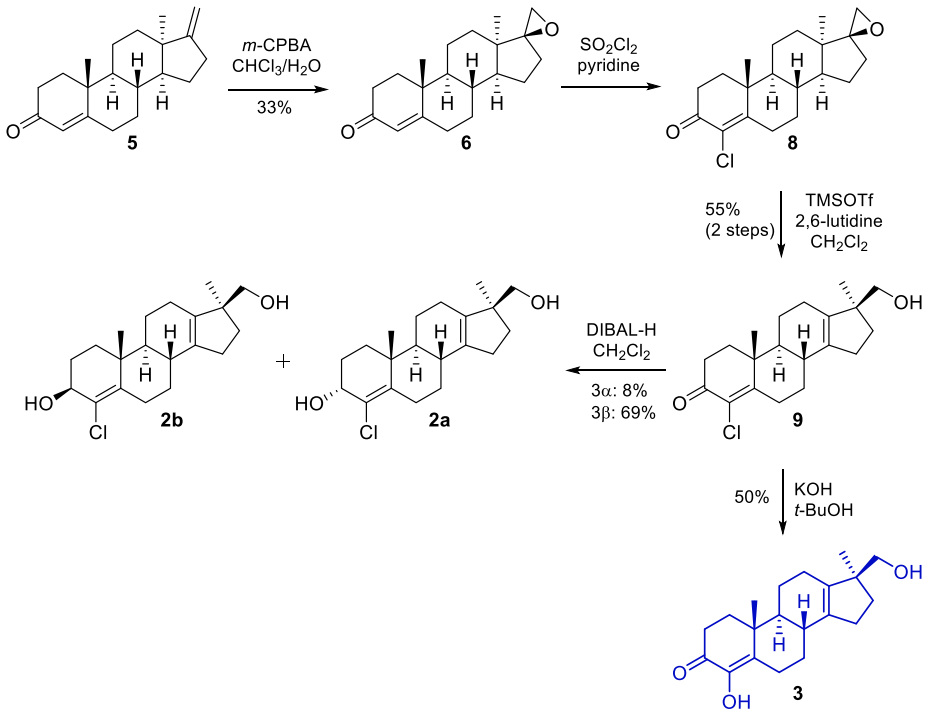


Fig. 3. EI fragmentation spectrum of **2d.**



Scheme 1. Synthetic route for DHCMT-M4 isomers **2a** and **2b** and Oxy M9 (**3**).

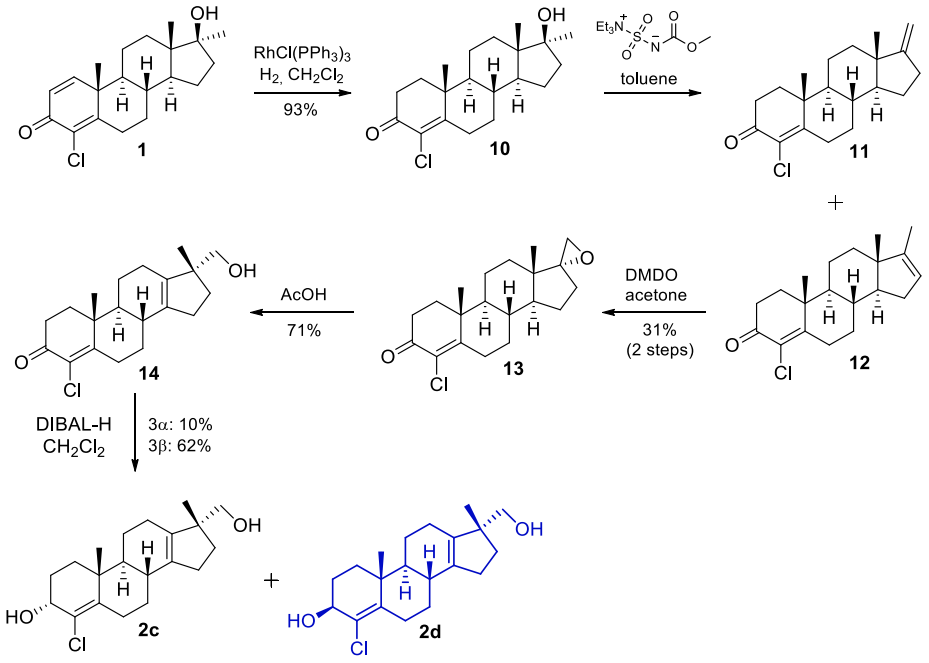
251.3 *m/z* (Δ = 36 u) can be described. The same fragments are described in the literature [8]. Only the fragments 377.3 *m/z* and 287.3 *m/z* have been confirmed by MS/MS measurements.

3. Results and discussion

Synthesis of the first target compound **2a** started from **5** which was synthesized according to a known procedure [9] (6 steps, 46% from **4**, see Scheme 1). In order to introduce chlorine in this compound at the C-4 it is necessary to mask any electron-rich double bond like 17-*exo*-methylene in **5**. This was achieved by epoxidation. The epoxide is later needed to introduce the substituents at C-17 as well as the 13,14-double bond by Wagner-Meerwein rearrangement.

Epoxidation with meta-chloroperoxybenzoic acid (*m*-CPBA) afforded the desired minor 17β-epoxide **6** in 33% yield (13% recovered **5**, 50% major product 17a-epoxide **7** not depicted). With this substrate it was important to use sub stochiometric amounts of oxidant in order to prevent Baeyer-Villiger-oxidation. Now the C-4 chlorination can proceed in pyridine with excess sulfuryl chloride [17]. The chlorinated product **8** is used immediately after work-up as the epoxide is very labile at this stage. Rearrangement is initiated using trimethylsilyl triflate and 2,6-lutidine in dichloromethane at low temperatures [9,11,18] giving chloroenone **9** in 55% yield. This key fragment was then reacted with potassium hydroxide in refluxing *t*-butanol, effecting vinylic substitution of the chloride and yielding target compound **3** directly in 50% yield. Alternatively, reduction with DIBAL-H [19] delivered **2a** in just 8% yield, as well as 69% of the 3β-hydroxy product **2b**. After both compounds showed different retention times than the compound found in excretion studies (GC–MS/MS), the next objectives were the C-17 epimeric 17α-hydroxymethyl-17β-methyl derivatives **2c** and **2d**.

Starting from **1** first the 1,2-double bond had to be hydrogenated, which was achieved by hydrogenation using Wilkinson's catalyst [20] in 93% yield giving methylclostebol **10** (see Scheme 2). Next,



Scheme 2. Synthetic route for DHCMT-M4 isomers **2c** and **2d.**

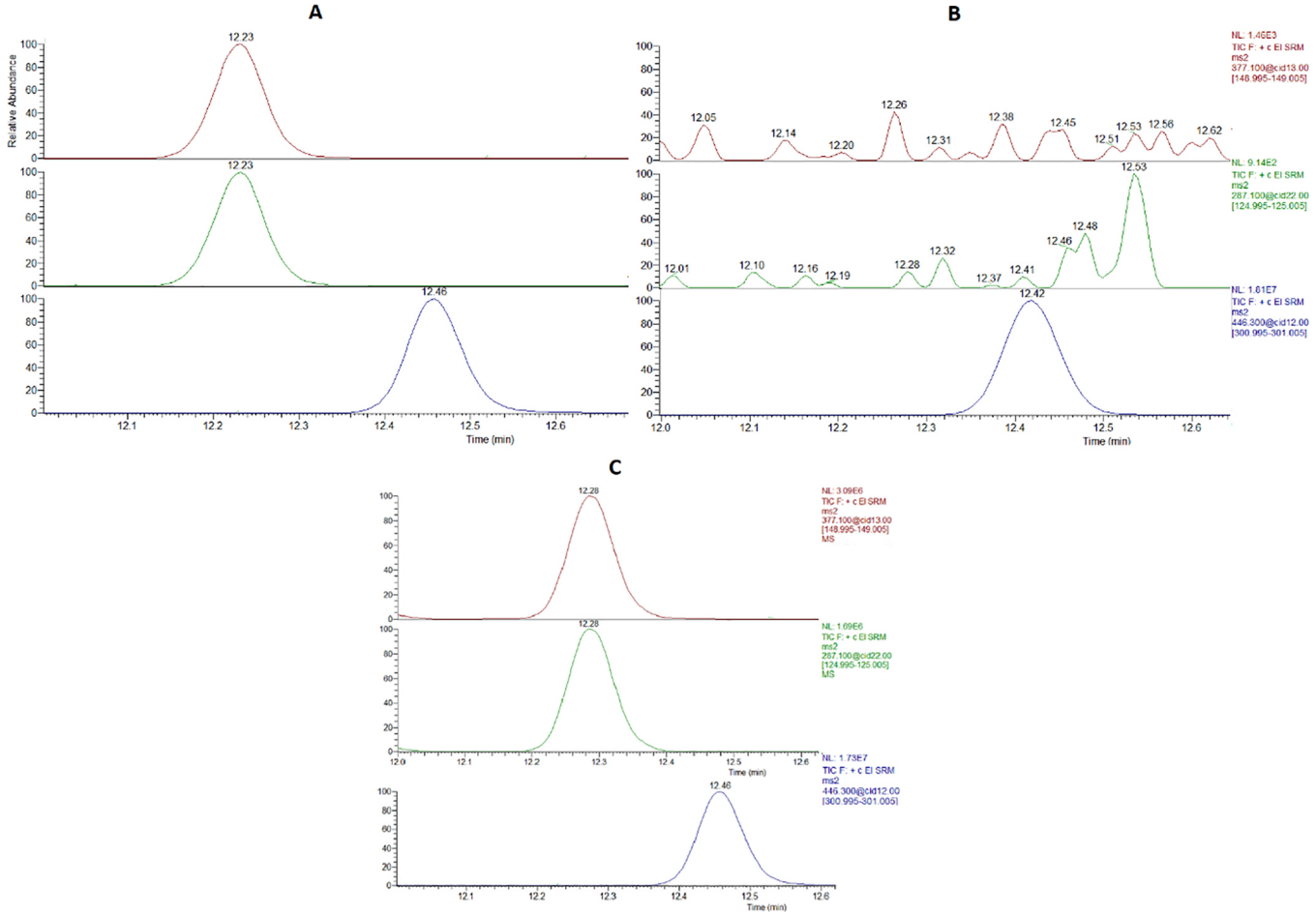


Fig. 4. Detection of metabolite **2d** in positive urine sample by GC–MS/MS; Row 1–2: mass transitions for metabolite **2d** (377 → 149, 287 → 125); Row 3: mass transition for internal standard methyltestosterone (446 → 301); A: compound **2d**; B: blank urine sample; C: positive urine sample.

dehydration of the tertiary alcohol was evaluated with a number of dehydrating agents of which the Burgess' reagent [21] proved most useful giving chloroenones **11** and **12** as a 2/1 mixture (not separated at this stage), which was further epoxidized with dimethyldioxirane (DMDO) in acetone (prepared according to [22]) to give the desired 17α-epoxide **13** in 31% yield. Rearrangement in acetic acid at room temperature provides chloroenone **14** which is reduced again with DIBAL-H affording 3α-alcohol **2c** in 10% yield and 3β-alcohol **2d** in 62% yield.

Comparison with excretion study samples proved identity of compound **2d** (4-chloro-17α-hydroxymethyl-17β-methyl-18-norandrost-4,13-en-3β-ol) with the *in vivo* metabolite "M4" described in the literature. The traces of the excretion study and the synthesized standard are depicted in Fig. 4 (methyltestosterone as internal standard). It is remarkable that this compound contains a 3β-hydroxy as well as 17α-hydroxymethyl moiety, the other long-term metabolite "M3" was shown [9b] to be 3α-hydroxy and 17β-hydroxymethyl suggesting they originate from different metabolic pathways.

4. Conclusion

To summarize, a short and efficient synthesis for four possible stereoisomers of the metabolite "M4" of DHCMT has been developed based on two synthetic routes with 5 and 10 steps respectively. The isomer **2d**, bearing a 17α-hydroxymethyl substituent, was found to be identical to the human metabolite "M4" found in excretion studies. The compound identified as "Oxy M9" (**3**) could be accessed by the same route in 10 steps from DHEA acetate.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2020.108716.

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