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Derivatives of 16α-hydroxy-dehydroepiandrosterone with an additional 7-oxo or 7-hydroxy substituent: Synthesis and gas chromatography/mass spectrometry analysis☆

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Abstract

Derivatives of 16α-hydroxy-dehydroepiandrosterone, which have an additional oxygen substituent at position 7 (oxo or hydroxy group), were synthesized. Firstly, 17,17-dimethoxyandrost-5-ene-3β,16α-diyl diacetate was prepared and then oxidized with a complex of chromium(VI) oxide and 2,5-dimethylpyrazole to the respective 7-oxo derivative. This key intermediate was both deprotected or reduced by L-Selectride® or sodium borohydride in the presence of cerium(III) chloride and then deprotected to give 7-oxo, 7α-hydroxy and 7β-hydroxy derivatives of 16α-hydroxy-dehydroepiandrosterone. The target compounds were characterized by ^{1}H and ^{13}C NMR spectra and in the form of *O*-methyloxime-trimethylsilyl derivatives, by gas chromatography/mass spectrometry methods.

Keywords: Steroid; Synthesis; Dehydroepiandrosterone; NMR spectra; GC–MS

1. Introduction

Metabolites of dehydroepiandrosterone (DHEA), hydroxylated in position 7 such as 7α-hydroxy-DHEA, its 7β-epimer, and the respective androst-5-ene-3β,7ξ,17β-triols, are now believed to possess immunomodulatory and immunoprotective effects, as demonstrated by a number of reports published in the last two decades [1]. These compounds are formed in various mammalian tissues by the action of two 7-hydroxylating enzymes of the cytochrome P_{450} series, differing in their characteristics and tissue- and species localization; see ref. [1] and other references therein. Of particular importance are the actions of these steroids in the brain [2]. All of these steroids are present in nanomolar concentrations in human blood, together with another 7-oxygenated DHEA metabolite 3β-hydroxyandrost-5-ene-7,17-dione (7-oxo-DHEA). The latter steroid was shown to act as a potent thermogenic agent by influencing oxidative metabolism in mitochondria in a similar way as thyroid hormones; see e.g., [3] and other references of these authors. 7-oxo-DHEA is also an intermediate in the epimerization of 7α-hydroxy-DHEA to the 7β-isomer and vice versa, which proceeds in mammalian liver and also in a non-enzymatic way in a mild acidic milieu [4].

On the other hand, 16α-hydroxy-DHEA represents a product of a concurrent enzymatic reaction. 16α-Hydroxylation of DHEA and other C19- and C18 steroids occurs not only in liver, but in many other tissues. 16α-Hydroxy-DHEA is the precursor of fetal estrogens and has been detected in the umbilical blood of newborns [5]. Since the development of its first immunoassay, 16α-hydroxy-DHEA has been considered a normal constituent of human serum where it is present in a low nanomolar range [6]. Increased levels of 16-hydroxylated estrogens have been associated with an increased risk of cancer of the female reproductive system and, more recently, with some systemic autoimmune diseases. Because 16α-

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hydroxy-DHEA is a precursor of the latter steroids, we hypothesized that increased formation of 16α-hydroxy-DHEA could be a risk factor in the development of these diseases; in other words, it may counteract the beneficial effects of 7-hydroxylated DHEA metabolites; for review see [5].

Since both 7ξ- and 16α-hydroxylations proceed as concurrent reactions in the same tissues, one may expect that metabolites containing both 7- and 16α-hydroxy groups are formed and are also present in blood. Their ratio in blood and eventually in various tissues (liver, brain, sex organs), may differ in healthy subjects and patients with autoimmune diseases or disorders of brain function.

The isolation of the derivatives of 16α-hydroxy-DHEA, which have an additional oxygen substituent at position 7 (oxo or hydroxy group), was reported from urine of the patient with adrenal carcinoma [7]. In this work, however, only the respective 7-oxo derivative was unambiguously synthesized; for the 7α-hydroxy derivative, the chemical synthesis was unsuccessful. To our knowledge, the corresponding 7β-hydroxy derivative was nor isolated, nor synthesized.

Published synthetic pathways for the preparation of 16α-hydroxy-7-oxo-DHEA differed in the order of introduction of oxygen substituents into positions 7 and 16 [3,7]. During the synthesis of 7,16-dihydroxy derivatives, the situation is more complicated, not only due to the smooth mutual isomerization of the 7-hydroxy derivatives in acid medium [4], but also due to the possible isomerization of 16α-hydroxy-17-oxo derivatives to 17β-hydroxy-16-oxo derivatives in alkaline medium [8,9]. This had to be respected in the process of selecting protecting groups for the synthesis.

As follows from the above analysis, the chemical synthesis of derivatives of 16α-hydroxy-DHEA with an additional oxygen substituent at position 7 is a challenging task. Consequently, the main aim of the present work is to develop their reliable synthesis and unambiguously prove their structure. The importance of this type of compounds consists in their use as standards in the broader study of steroid metabolism disorders.

2. Experimental

2.1. General

Melting points were determined on a Boetius micro melting point apparatus (Germany). Optical rotations were measured at 25 °C on an AUTOPOL IV polarimeter (Rudolph Research Analytical, USA), and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Infrared spectra (wavenumbers in cm^{-1}) were recorded on a Bruker IFS 88 spectrometer. ^{1}H NMR spectra were taken on Brucker AVANCE-400 and AVANCE-500 instruments (400 and 500 MHz, FT mode) at 23 °C in $CDCl_3$ (unless stated otherwise) with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ-scale); coupling constants (*J*) and widths of multiplets (*W*) are given in Hz. ^{13}C NMR spectra were taken on Bruker AVANCE-500 instruments (^{13}C at 125.7 MHz) under the above conditions; secondary referencing was performed using the solvent signal at position $\delta(CDCl_3) = 77.0$ and $\delta(CD_3OD) = 49.0$.

Thin-layer chromatography (TLC) was performed on silica gel G (ICN Biochemicals). Detection was accomplished by spraying with concentrated sulfuric acid followed by heating. Preparative TLC was done on plates (200 mm × 200 mm) with 0.4 mm layer thickness. For silica gel G, spraying with 0.1% morine in methanol and UV light (360 nm) visualization were used for detection. Conjugate ketones **8** and **9** were purified on TLC-Silica gel 60 GF254 (Merck, detection by 254 nm UV light). For column chromatography, neutral silica gel Kieselgel 60 (Merck) was used. Prior to evaporation on a rotary evaporator in vacuo (0.25 kPa, bath temperature 40 °C), solutions in organic solvents were dried over anhydrous sodium sulfate.

16α-Bromoandrost-5-en-3β-ol (**2**) was prepared according to ref. [9].

2.2. Derivatives of 16α-hydroxy-DHEA

2.2.1. 17,17-Dimethoxyandrost-5-ene-3β,16α-diol (3)

(a) Modification of the known procedure [10]. A hot solution of bromo ketone **2** (2.20 g, 6.0 mmol) in methanol (65 ml) was added to a refluxed solution of sodium (4.4 g, 191 mmol) in methanol (130 ml), and the refluxing was continued for 10 min. The reaction mixture was then poured on ice (ca. 600 g). Methanol and part of the water were evaporated in vacuo, and the residue was allowed to stand in the refrigerator overnight. A separated crude product was collected by suction and washed with water until the filtrate was neutral. Crystallization from a small volume (35 ml) of hot acetone afforded 1.43 g (68%) of hydroxy acetal **3**, m.p. 178–181 °C, $[\alpha]_D$ −67 (*c* 0.59, acetone). Literature [10] gives m.p. 177–179 °C (acetone). ^{1}H NMR (400 MHz, $CDCl_3 + CD_3COOD$): 5.342 (1 H, bd, $J \approx 5$, H-6); 4.285 (1 H, dd, $J = 8.9$, $J' = 2.0$, H-16β); 3.528 (1 H, m, $W = 32$, H-3α); 3.459 (3 H, s, OCH_3); 3.367 (3 H, s, OCH_3); 0.993 (3 H, s, 3 × H-19); 0.794 (3 H, s, 3 × H-18).

(b) Aqueous KOH (0.4 M, 0.5 ml) was added to a solution of acetate **4** (44 mg, 0.1 mmol) in tetrahydrofuran (1 ml) and methanol (0.5 ml) mixture. After stirring at room temperature for 3 h, the reaction mixture was poured into ethyl acetate (70 ml) and washed with saturated aqueous NaCl (2 × 25 ml). The solvent was evaporated in vacuo, and the residue was crystallized from acetone. The yield of diol **3**, identical with the above described sample, was 27 mg (76%).

2.2.2. 17,17-Dimethoxyandrost-5-ene-3β,16α-diyl diacetate (4)

Diol **3** (701 mg, 2.0 mmol) was dissolved in pyridine (6.5 ml), and the solution was cooled in an ice bath. Acetic anhydride (0.97 ml, 10 mmol) was added, and the mix-

ture was left at room temperature overnight. The reaction mixture was then poured onto ice (100 g), and the separated product was filtered off and dissolved in benzene (100 ml). This solution was washed successively with ice cold 5% aqueous citric acid, water, saturated aqueous $KHCO_3$, and water. The solvent was evaporated in vacuo, and the residue was chromatographed on ten preparative silica gel plates in a mixture of benzene/ether (90:10). The yield of diacetate **4** was 738 mg (85%), m.p. 177–179 °C (ether), $[\alpha]_D$ −110 (*c* 0.51, $CHCl_3$). IR spectrum (CCl_4): 1736 (C=O, acetate); 1670 (C=C); 1246, 1033 (C–O, acetate); 1167, 1133, 1088, 1069, 1046 (C–O–C–O–C). ^{1}H NMR (400 MHz, $CDCl_3$): 5.355 (1 H, bd, $J \approx 5$, H-6); 5.218 (1 H, dd, $J = 9.7$, $J' = 3.1$, H-16β); 4.602 (1 H, m, $W = 32$, H-3α); 3.488 (3 H, s, OCH_3); 3.284 (3 H, s, OCH_3); 2.092 (3 H, s, CH_3COO); 2.031 (3 H, s, CH_3COO); 1.011 (3 H, s, 3 × H-19); 0.908 (3 H, s, 3 × H-18). Analysis calculated for $C_{25}H_{38}O_6$ (434.6): C, 69.10; H, 8.81. Found: C, 69.39; H, 8.93.

2.2.3. 3β,16α-Dihydroxyandrost-5-en-17-one (*5*)

A solution of 4-toluenesulfonic acid monohydrate (3.9 mg, 0.021 mmol) in water (0.3 ml) was added to a solution of acetal **3** (35 mg, 0.1 mmol) in acetone (2.0 ml), and the reaction mixture was stirred at room temperature for 30 min. The solvents were then evaporated in vacuo, and the residue was dissolved in ethyl acetate (5 ml) and water (1 ml). The aqueous phase was extracted with ethyl acetate (3 ml), and the combined organic phases were washed with water (2 × 2 ml). The solvent was evaporated in vacuo, and the residue was chromatographed on a preparative silica gel plate in a benzene/acetone (80:20) mixture. The yield of **5** was 19 mg (63%), m.p. 186–188 °C (methanol/water). Literature [11] gives 185–188 °C. ^{1}H NMR (400 MHz, $CDCl_3 + CD_3COOD$): 5.377 (1 H, bd, $J \approx 5$, H-6); 4.377 (1 H, bd, $J = 8.2$, H-16β); 3.527 (1 H, m, $W = 32$, H-3α); 1.036 (3 H, s, 3 × H-19); 0.981 (3 H, s, 3 × H-18). The product was identical (TLC, IR, ^{1}H NMR) with the authentic sample [11].

2.2.4. 17-Oxoandrost-5-ene-3β,16α-diyl diacetate (*6*)

A solution of 4-toluenesulfonic acid monohydrate (3.9 mg, 0.021 mmol) in water (0.3 ml) was added to a solution of acetal **4** (43 mg, 0.1 mmol) in acetone (2.0 ml), and the reaction mixture was stirred at room temperature for 120 h. The solvents were then evaporated in vacuo, and the residue was dissolved in ether (5 ml) and water (1 ml). The aqueous phase was extracted with ether (3 ml), and the combined organic phases were washed with water (2 × 2 ml). The solvent was evaporated in vacuo, and the residue was chromatographed on a preparative silica gel plate in a mixture of benzene/ether (90:10). The yield of **6** was 22 mg (57%), m.p. 167–169 °C (ethyl acetate/hexane). The literature [11] gives 164–167 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 5.398 (1 H, bd, $J \approx 5$, H-6); 5.425 (1 H, dd, $J = 9.1$, $J' = 1.1$, H-16β); 4.608 (1 H, m, $W = 32$, H-3α); 2.121 (3 H, s, CH_3COO); 2.038 (3 H, s, CH_3COO); 1.050 (3 H, s, 3 × H-19); 0.989 (3 H, s, 3 × H-18). The product was identical (TLC, IR, ^{1}H NMR) with an authentic sample [11].

2.3. Derivatives of 16α-hydroxy-7-oxo-DHEA

2.3.1. 7,17-Dioxoandrost-5-ene-3β,16α-diyl diacetate (*7*)

A suspension of chromium(VI) oxide (1.50 g, 15.0 mmol) in dichloromethane (12 ml) was stirred at −25 °C under argon with 3,5-dimethylpyrazole (1.50 g, 15.6 mmol). After 15 min, a solution of olefin **6** (389 mg, 1.0 mmol) in dichloromethane (2 ml) was added dropwise, and stirring was continued at −20 °C for 4 h. The reaction mixture was then diluted with a mixture of benzene/ethyl acetate (7:3, 30 ml) and filtered through a short column of silica gel (10 g) layered with celite. The column was washed with the same solvent mixture, and the solvents were evaporated in vacuo. Chromatography on a column of silica gel (30 g) in a mixture of benzene/ethyl acetate (95:5 to 90:10) afforded 283 mg (70%) of ketone **7**, m.p. 199–202 °C (methanol), $[\alpha]_D$ −73 (*c* 0.57, $CHCl_3$). The literature [7] gives m.p. 198–200 °C (methanol), $[\alpha]_D$ −65.3 ($CHCl_3$). IR spectrum ($CHCl_3$): 1733 (C=O, 17-ketone and acetate); 1671 (C=O, 7-ketone); 1631 (C=C); 1246, 1036 (C–O, acetate); 1018 (C–O). ^{1}H NMR (400 MHz, $CDCl_3$): 5.760 (1 H, bd, $J = 1.8$, H-6); 5.434 (1 H, dd, $J = 9.1$, $J' = 1.3$, H-16β); 4.728 (1 H, m, $W = 32$, H-3α); 2.115 (3 H, s, CH_3COO); 2.056 (3 H, s, CH_3COO); 1.242 (3 H, s, 3 × H-19); 1.001 (3 H, s, 3 × H-18).

2.3.2. 3β,16α-Dihydroxyandrost-5-ene-7,17-dione (*8*)

(a) Sulfuric acid (3 M, 2.8 ml, 8.4 mmol) was added to a suspension of diacetate **7** (201 mg, 0.5 mmol) in methanol (28 ml) and stirred at room temperature for 48 h. Methanol was then evaporated in vacuo, and the residue was treated with ethyl acetate (50 ml) and water (10 ml). The aqueous phase was extracted with ethyl acetate (30 ml), and the combined organic phases were washed with water (2 × 20 ml). The solvent was evaporated in vacuo, and the residue was chromatographed on two preparative silica gel plates in a mixture of chloroform/acetone (70:30). The yield of hydroxy ketone **8** was 124 mg (78%), m.p. 238–243 °C (decomposition, methanol), $[\alpha]_D$ −67 (*c* 0.40, ethanol). Literature [7] gives m.p. 236–243 °C (methanol), $[\alpha]_D$ −92 (ethanol); literature [3] gives m.p. 235–239 °C. ^{1}H NMR (400 MHz, $CDCl_3 + CD_3COOD$): 5.749 (1 H, bd, $J = 1.8$, H-6); 4.402 (1 H, bd, $J = 8.5$, H-16β); 3.699 (1 H, m, $W = 32$, H-3α); 1.254 (3 H, s, 3 × H-19); 0.996 (3 H, s, 3 × H-18). For ^{13}C NMR, see Table 1.

(b) A solution of 4-toluenesulfonic acid monohydrate (3.9 mg, 0.021 mmol) in water (0.3 ml) was added to a suspension of acetal **10** (36 mg, 0.1 mmol) in acetone (2.0 ml), and the reaction mixture was stirred at room

Table 1
^{13}C NMR chemical shifts of 16α-OH-DHEA derivatives

Carbon	**8**[a]	**13**[a]	**16**[b]
1	36.16	36.57	37.95
2	30.46[c]	31.01[d]	32.08[e]
3	69.78	71.10[f]	71.91[g]
4	38.31	36.76	42.87
5	166.95	143.29	146.70
6	125.57	125.60	124.75
7	200.79	72.26[f]	64.93
8	43.07[h]	48.10[i]	43.71[j]
9	44.04[h]	48.11[i]	43.83[j]
10	41.70	41.45	[k]
11	20.14	19.92	20.83
12	33.50	32.94	32.39[e,l]
13	47.67	47.67	48.21
14	49.98	39.78	38.57
15	30.73[c]	31.24[d]	32.39[e,l]
16	70.87	70.98[f]	72.29[g]
17	219.13	219.98	221.07
18	14.07	13.88	14.24
19	17.37	19.09	18.65

[a] In $CDCl_3$; [b] in CD_3OD; [c–j] tentative assignment, values in columns may be interchanged; [k] undeterminable value; [l] signal of two carbons.

temperature for 30 min. The solvents were evaporated in vacuo, and the residue was dissolved in ethyl acetate (5 ml) and water (1 ml). The aqueous phase was extracted with ethyl acetate (3 ml), and the combined organic phases were washed with water (2 × 2 ml). The solvent was evaporated in vacuo, and the residue was chromatographed on a preparative silica gel plate in a mixture of chloroform/acetone (70:30). The yield of hydroxy ketone **8**, identical with the above described sample, was 21 mg (67%).

*2.3.3. 17,17-Dimethoxy-7-oxoandrost-5-ene-3β,16α-diyl diacetate (**9**)*

A suspension of chromium(VI) oxide (1.50 g, 15.0 mmol) in dichloromethane (12 ml) was stirred at −25 °C under argon with 3,5-dimethylpyrazole (1.50 g, 15.6 mmol). After 15 min, a solution of olefin **4** (435 mg, 1.0 mmol) in dichloromethane (3 ml) was added dropwise, and stirring was continued at −20 °C for 4 h. The reaction mixture was then diluted with a mixture of benzene/ethyl acetate (30 ml, 7:3) and filtered through a short column of silica gel (10 g) layered with celite. The column was washed with the same solvent mixture, and the solvents were evaporated in vacuo. The residue was chromatographed on eight preparative silica gel plates in a mixture of benzene/ether (80:20). The yield of ketone **9** was 308 mg (69%), m.p. 214–215 °C (ether), $[\alpha]_D$ −167 (*c* 0.25, $CHCl_3$). IR spectrum ($CHCl_3$): 1732 (C=O, acetate); 1671 (C=O, 7-ketone); 1632 (C=C); 1249, 1039 (C–O, acetate). ^{1}H NMR (400 MHz, $CDCl_3$): 5.701 (1 H, bd, J = 1.6, H-6); 5.260 (1 H, dd, J = 9.4, J' = 3.2, H-16β); 4.716 (1 H, m, W = 32, H-3α); 3.478 (3 H, s, CH_3O); 3.285 (3 H, s, CH_3O); 2.092 (3 H, s, CH_3COO); 2.053 (3 H, s, CH_3COO); 1.202 (3 H, s, 3 × H-19); 0.914 (3 H, s, 3 × H-18). Analysis calculated for $C_{25}H_{36}O_7$ (448.6): C, 66.94; H, 8.09. Found: C, 66.71; H, 7.87.

*2.3.4. 3β,16α-Dihydroxy-17,17-dimethoxyandrost-5-en-7-one (**10**)*

Aqueous KOH (0.4 M, 1 ml) was added to a solution of acetate **9** (90 mg, 0.2 mmol) in tetrahydrofuran (2 ml) and stirred at room temperature for 3 h. The reaction mixture was then poured into ethyl acetate (100 ml) and washed with saturated aqueous NaCl (2 × 30 ml). The solvent was evaporated, and the residue was crystallized from an acetone/hexane mixture. The yield of diol **10** was 58 mg (79%), m.p. 210–212 °C, $[\alpha]_D$ −205 (*c* 0.30, $CHCl_3$). IR spectrum ($CHCl_3$): 3611 (O–H, free); 3518 broad (O–H, bonded); 1671 (C=O); 1631 (C=C); 1169, 1054 (C–O–C–O–C). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.696 (1 H, bd, J = 1.7, H-6); 4.284 (1 H, dd, J = 9.5, J' = 2.2, H-16β); 3.669 (1 H, m, W = 32, H-3α); 1.186 (3 H, s, 3 × H-19); 0.795 (3 H, s, 3 × H-18). Analysis calculated for $C_{21}H_{32}O_5$ (364.5): C, 69.20; H, 8.85. Found: C, 69.17; H, 9.04.

2.4. Derivatives of 7,16α-dihydroxy-DHEA

*2.4.1. 17,17-Dimethoxy-7α-hydroxyandrost-5-ene-3β,16α-diyl diacetate (**11**)*

L-Selectride® (1 M solution in tetrahydrofuran, 0.6 ml) was added under argon at −78 °C to a solution of ketone **9** (224 mg, 0.5 mmol) in tetrahydrofuran (6 ml), and the mixture was stirred at −78 °C for 5 h. Water (0.35 ml) was then added, and the mixture was warmed to 0 °C. An aqueous 6 M NaOH (0.65 ml, 3.9 mmol) was added, followed by 30% hydrogen peroxide (0.65 ml), and stirring was continued at room temperature for 30 min. The mixture was diluted with ether (200 ml), and the organic layer was washed successively with ice cold 5% aqueous citric acid, water, saturated aqueous $KHCO_3$, and water. The solvent was evaporated, and the residue was chromatographed on four preparative silica gel plates in a mixture of benzene/ethyl acetate (60:40). The yield of the hydroxy derivative **11** was 138 mg (61%), amorphous foam, $[\alpha]_D$ −143 (*c* 0.41, $CHCl_3$). IR spectrum ($CHCl_3$): 3603 (O–H, free); 3529, 3444 (O–H, bonded); 1730 (C=O); 1665 (C=C); 1252 (C–O, acetate); 1192, 1134, 1070, 1037 (C–O–C–O–C). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.606 (1 H, dd, J = 5.1, J' = 1.5, H-6); 5.233 (1 H, dd, J = 9.9, J' = 3.2, H-16β); 4.637 (1 H, m, W = 32, H-3α); 3.796 (1 H, bt, $J \approx$ 4.5, H-7β); 3.490 (3 H, s, CH_3O); 3.297 (3 H, s, CH_3O); 2.079 (3 H, s, CH_3COO); 2.031 (3 H, s, CH_3COO); 1.000 (3 H, s, 3 × H-19); 0.911 (3 H, s, 3 × H-18). Analysis calculated for $C_{25}H_{38}O_7$ (450.6): C, 66.64; H, 8.50. Found: C, 65.51; H, 8.68.

*2.4.2. 17,17-Dimethoxyandrost-5-ene-3β,7α,16α-triol (**12**)*

Aqueous 0.4 M KOH (1.25 ml, 0.5 mmol) was added to a solution of acetate **11** (113 mg, 0.25 mmol) in tetrahydro-

furan (3 ml) and stirred at room temperature for 48 h. The reaction mixture was then poured into ethyl acetate (100 ml), washed with water (3 × 50 ml), and the solvent was evaporated in vacuo. The yield of chromatographically (TLC) pure product **12** was 79 mg (86%). The analytical sample was obtained by crystallization from ether, m.p. 225–228 °C, decomposition, $[\alpha]_D$ −153 (*c* 0.33, $CHCl_3$). IR spectrum (KBr): 3640, 3415 shoulder, 3310 shoulder (O–H); 1668 (C=C); 1054 (C–O); 1192, 1132, 1037 (C–O–C–O–C). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.567 (1 H, dd, J = 5.4, J' = 1.6, H-6); 4.319 (1 H, dd, J = 9.1, J' = 2.2, H-16β); 3.816 (1 H, bt, $J \approx 4.5$, H-7β); 3.569 (1 H, m, W = 32, H-3α); 3.468 (3 H, s, CH_3O); 3.365 (3 H, s, CH_3O); 0.973 (3 H, s, 3 × H-19); 0.799 (3 H, s, 3 × H-18). Analysis calculated for $C_{21}H_{34}O_5$ (366.5): C, 68.82; H, 9.35. Found: C, 68.97; H, 9.36.

2.4.3. 3β,7α,16α-Trihydroxyandrost-5-en-17-one (13)

A solution of 4-toluenesulfonic acid monohydrate (3.9 mg, 0.021 mmol) in water (0.3 ml) was added to a solution of acetal **12** (37 mg, 0.1 mmol) in acetone (2.0 ml) and stirred at room temperature for 30 min. The reaction mixture was then poured into ethyl acetate (150 ml), washed with water (2 × 30 ml), and the solvent was evaporated in vacuo. The residue was chromatographed on a preparative silica gel plate in a mixture of chloroform/acetone (50:50). The yield of **13** was 23 mg (71%), m.p. 211–213 °C (methanol), $[\alpha]_D$ −60 (*c* 0.31, dimethyl sulfoxide). IR spectrum (KBr): 3430 broad (O–H); 1747 (C=O); 1664 (C=C); 1055, 1034, 1010 (C–O). ^{1}H NMR (500 MHz, CD_3OD): 5.579 (1 H, dd, J = 5.3, J' = 1.7, H-6); 4.355 (1 H, d, J = 7.9, H-16β); 3.870 (1 H, m, W = 11, H-7α); 1.038 (3 H, s, 3 × H-19); 0.961 (3 H, s, 3 × H-18). For ^{13}C NMR see Table 1. Analysis calculated for $C_{19}H_{28}O_4$ (320.4): C, 71.22; H, 8.81. Found: C, 71.03; H, 8.66.

2.4.4. 17,17-Dimethoxy-7β-hydroxyandrost-5-ene-3β,16α-diyl diacetate (14)

A 0.4 M solution of cerium(III) chloride heptahydrate in methanol (1.25 ml, 0.5 mmol) was added to a stirred solution of ketone **9** (224 mg, 0.5 mmol) in tetrahydrofuran (3 ml). Sodium borohydride (18 mg, 0.48 mmol) was added to this mixture in small portions over 3 min, and stirring was continued for 10 min. The mixture was then poured into ether (60 ml) and washed with ice cold 5% aqueous citric acid (60 ml). The aqueous phase was extracted with ether (60 ml), and the collected organic layers were washed successively with ice cold, 5% aqueous citric acid, water, saturated aqueous $KHCO_3$, and water. The solvent was evaporated, and the residue was chromatographed on four preparative silica gel plates in a mixture of benzene/ethyl acetate (70:30). The yield of the hydroxy derivative **14** was 163 mg (72%), m.p. 177–179 °C (ether-hexane), $[\alpha]_D$ −36 (*c* 0.27, $CHCl_3$). IR spectrum ($CHCl_3$): 3626 shoulder (O–H, free); 3597 (O–H, bonded); 1726 (C=O); 1675 (C=C); 1253 (C–O, acetate); 1188, 1128, 1084, 1069 (C–O–C–O–C). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.289 (1 H, bs, H-6); 5.258 (1 H, dd, J = 9.9, J' = 2.9, H-16β); 4.619 (1 H, m, W = 32, H-3α); 3.867 (1 H, dt, J = 8.2, J' = 2.0, H-7α); 3.487 (3 H, s, CH_3O); 3.289 (3 H, s, CH_3O); 2.094 (3 H, s, CH_3COO); 2.041 (3 H, s, CH_3COO); 1.053 (3 H, s, 3 × H-19); 0.917 (3 H, s, 3 × H-18). Analysis calculated for $C_{25}H_{38}O_7$ (450.6): C, 66.64; H, 8.50. Found: C, 66.72; H, 8.46.

2.4.5. 17,17-Dimethoxyandrost-5-ene-3β,7β,16α-triol (15)

Aqueous 0.4 M KOH (1.25 ml, 0.5 mmol) was added to a solution of acetate **14** (113 mg, 0.25 mmol) in tetrahydrofuran (3 ml) and stirred at room temperature for 48 h. The reaction mixture was then poured into saturated aqueous NaCl (25 ml); the separated product was collected on a filter and dried in vacuo. Crystallization from methanol afforded 58 mg (63%) of hydroxy derivative **15**, m.p. 253–255 °C, $[\alpha]_D$ −22 (*c* 0.31, DMSO). IR spectrum (KBr): 3401, 3340 shoulder, 3287 shoulder (O–H); 1056, 1019 (C–O); 1190, 1126, 1083 (C–O–C–O–C). ^{1}H NMR (400 MHz, DMSO-d_6): 5.123 (1 H, bs, H-6); 3.549 (1 H, bt, $J \approx 8$, H-16β); 4.084 (1 H, m, W = 20, H-7α); 3.382 (3 H, s, CH_3O); 3.225 (3 H, s, CH_3O); 0.941 (3 H, s, 3 × H-19); 0.755 (3 H, s, 3 × H-18). Analysis calculated for $C_{21}H_{34}O_5$ (366.5): C, 68.82; H, 9.35. Found: C, 68.76; H, 9.27.

2.4.6. 3β,7β,16α-Trihydroxyandrost-5-en-17-one (16)

A solution of 4-toluenesulfonic acid monohydrate (3.9 mg, 0.021 mmol) in water (0.3 ml) was added to a solution of acetal **15** (37 mg, 0.1 mmol) in acetone (2.0 ml) and stirred at room temperature for 30 min. The reaction mixture was then poured into ethyl acetate (150 ml), washed with water (2 × 30 ml), and the solvent was evaporated in vacuo. The residue was chromatographed on a preparative silica gel plate in a mixture of chloroform/acetone (50:50). The yield of **16** was 25 mg (77%), m.p. 214–217 °C, decomposition (ether-acetone), $[\alpha]_D$ +118 (*c* 0.12, $CHCl_3$). IR spectrum (KBr): 3417 broad (O–H); 1745 (C=O); 1054, 1045, 1028, 1013 (C–O). ^{1}H NMR (500 MHz, $CDCl_3$ + CD_3COOD): 5.297 (1 H, bt, $J \approx 2$, H-6); 4.351 (1 H, bd, J = 8.2, H-16β); 3.944 (1 H, dt, J = 8.1, J' = 2.2, H-7α); 3.540 (1 H, m, W = 32, H-3α); 1.078 (3 H, s, 3 × H-19); 0.984 (3 H, s, 3 × H-18). For ^{13}C NMR see Table 1. Analysis calculated for $C_{19}H_{28}O_4$ (320.4): C, 71.22; H, 8.81. Found: C, 71.36; H, 8.57.

2.5. Derivatives 7-hydroxy-DHEA

2.5.1. 7α-Hydroxy-17-oxoandrost-5-en-3β-yl acetate (17)

A sample was prepared from DHEA according to the literature [12] in the 41% yield: ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.676 (1 H, dd, J = 5.3, J' = 1.7, H-6); 4.649 (1 H, m, W = 32, H-3α); 3.975 (1 H, bt, $J \approx 4$, H-7β); 2.045 (3 H, s, CH_3COO); 1.037 (3 H, s, 3 × H-19); 0.887 (3 H, s, 3 × H-18).

*2.5.2. 7β-Hydroxy-17-oxoandrost-5-en-3β-yl acetate (**18**)*

A sample was prepared from DHEA according to the literature [12] in the 43% yield: ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.344 (1 H, bt, $J \approx 2$, H-6); 4.628 (1 H, m, $W = 32$, H-3α); 3.973 (1 H, dt, $J = 8.2$, $J' = 2.1$, H-7α); 2.044 (3 H, s, CH_3COO); 1.096 (3 H, s, 3 × H-19); 0.907 (3 H, s, 3 × H-18).

*2.5.3. 3β,7α-Dihydroxyandrost-5-en-17-one (**19**)*

Hot 5% aqueous Na_2CO_3 (2.0 ml, 1.0 mmol) was added to a refluxed solution of acetate **17** (173 mg, 0.5 mmol) in methanol (10 ml), and the refluxing was continued for 75 min. Methanol and part of the water were then evaporated in vacuo, and the mixture was diluted with water and extracted with dichloromethane (3 × 20 ml). The collected extracts were washed with water (2 × 50 ml), dried, and the solvent was evaporated in vacuo. Crystallization of the residue from a mixture of acetone-hexane afforded 135 mg (89%) of diol **19**, m.p. 178–180 °C, $[\alpha]_D$ −80 (*c* 0.14, $CHCl_3$). Literature [13] gives m.p. 183–184 °C, $[\alpha]_D$ −73 (*c* 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.653 (1 H, dd, $J = 5.3$, $J' = 1.7$, H-6); 3.986 (1 H, bt, $J \approx 4$, H-7β); 3.582 (1 H, m, $W = 32$, H-3α); 1.025 (3 H, s, 3 × H-19); 0.890 (3 H, s, 3 × H-18).

*2.5.4. 3β,7β-Dihydroxyandrost-5-en-17-one (**20**)*

Hot 5% aqueous Na_2CO_3 (2.0 ml, 1.0 mmol) was added to a refluxed solution of acetate **18** (173 mg, 0.5 mmol) in methanol (10 ml), and the refluxing was continued for 75 min. Methanol and part of the water were then evaporated in vacuo, and the mixture was diluted with water and extracted with dichloromethane (3 × 20 ml). The collected extracts were washed with water (2 × 50 ml), dried, and the solvent was evaporated in vacuo. Crystallization of the residue from a mixture of acetone-hexane afforded 127 mg (83%) of diol **20**, m.p. 213–215 °C, $[\alpha]_D$ +63 (*c* 1.3, $CHCl_3$). Literature [13] gives m.p. 214–215 °C, $[\alpha]_D$ +71 (*c* 0.4, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3COOD): 5.321 (1 H, bt, $J \approx 2$, H-6); 3.976 (1 H, dt, $J = 8.1$, $J = 2.0$, H-7α); 3.586 (1 H, m, $W = 32$, H-3α); 1.081 (3 H, s, 3 × H-19); 0.905 (3 H, s, 3 × H-18).

2.6. GC–MS analysis

2.6.1. Chemicals

The analytical grade solvents were from Merck (Darmstadt, Germany). The derivatization agents Sylon BFT and *O*-methylhydroxylamine hydrochloride were from Supelco (Bellefonte, PA, USA) and Sigma (St. Louis, MO, USA).

2.6.2. Instruments

The GC–MS system by Shimazdu (Kyoto, Japan), consisted of GC17A gas chromatograph equipped with an automatic flow control, an AOC-20 autosampler and a QP5050A mass spectrometer equipped with a quadrupole electron impact detector and a fixed electron voltage of 70 eV.

2.6.3. Derivatization

In the first step, 10 μg of the steroids under investigation were derivatized with 50 μl of a 2% solution of *O*-methylhydroxylamine hydrochloride in pyridine at 60 °C for 1 h to deactivate the oxo groups. Then, the mixtures were dried under a stream of nitrogen. To deactivate the hydroxy groups, the dried residues were derivatized with 50 μl Sylon BFT (99% BSTFA + 1% TMCS) at 90 °C for 45 min. Subsequently, the derivatization agent was evaporated under a stream of nitrogen and the steroid derivatives were dissolved in 1 ml of isooctane. Then, 4 μl portions of the mixtures corresponding to 40 ng of the steroids were injected into the GC–MS system.

2.6.4. GC–MS analysis

GC separation was carried out using a Zebron ZB-50 capillary column (15 m × 0.25 mm) with 0.15 μm film thickness, Cat. no. 7EG-G004-05, (Phenomenex, St. Torrance, CA, USA). The temperature of the injection port was 300 °C.

The following protocol was used: Splitless high-pressure injection for 1 min at 100 kPa, 1 min delay at 120 °C and 30 kPa, then, a steep gradient 40 °C/min and 8.5 kPa/min up to 220 °C and 51 kPa, a gentle gradient 2.9 °C/min and 0.5 kPa/min up to 240 °C and 54.5 kPa, and a steep gradient 40 °C/min and 9 kPa/min up to 310 °C and 70 kPa followed by a 2 min plateau. The duration of the analysis was 14.2 min.

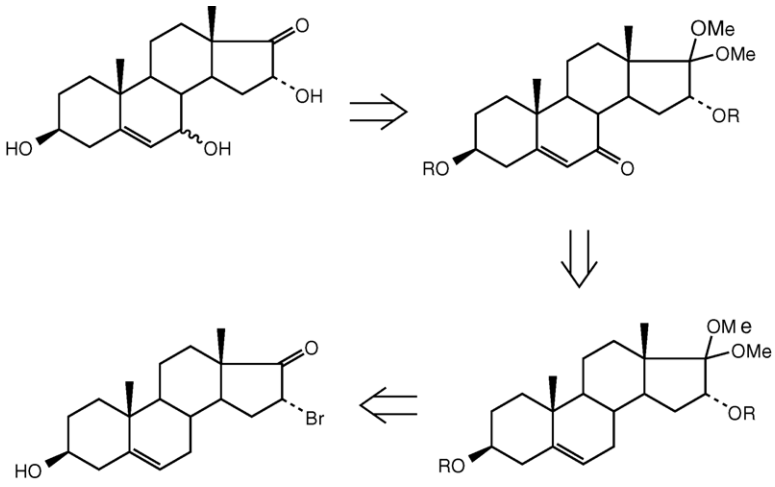
The detector voltage was at 1.65 kV, and the sampling rate was 0.25 s. The temperature of the interface was 320 °C. The response was recorded in total ion current mode recording the mass spectrum from the effective mass 70 amu up to 700 amu at the scan speed 4000 amu/s.

3. Results and discussion

3.1. Synthesis

On the basis of the model experiments, the approach of the "16-substituent first" was selected (Scheme 1). As a starting compound, the fully protected derivative of 16α-hydroxy-DHEA was necessary with hydroxy groups in positions 3 and 16 protected against oxidation and the 17-oxo group protected from reduction. These requirements were fulfilled with the derivative **4**, which had hydroxy groups protected as acetates and a 17-oxo group protected as dimethyl acetal. Moreover, the protected α-ketol moiety in the D ring was resistant against unnecessary rearrangement into the isomeric 17β-hydroxy-16-oxo derivative.

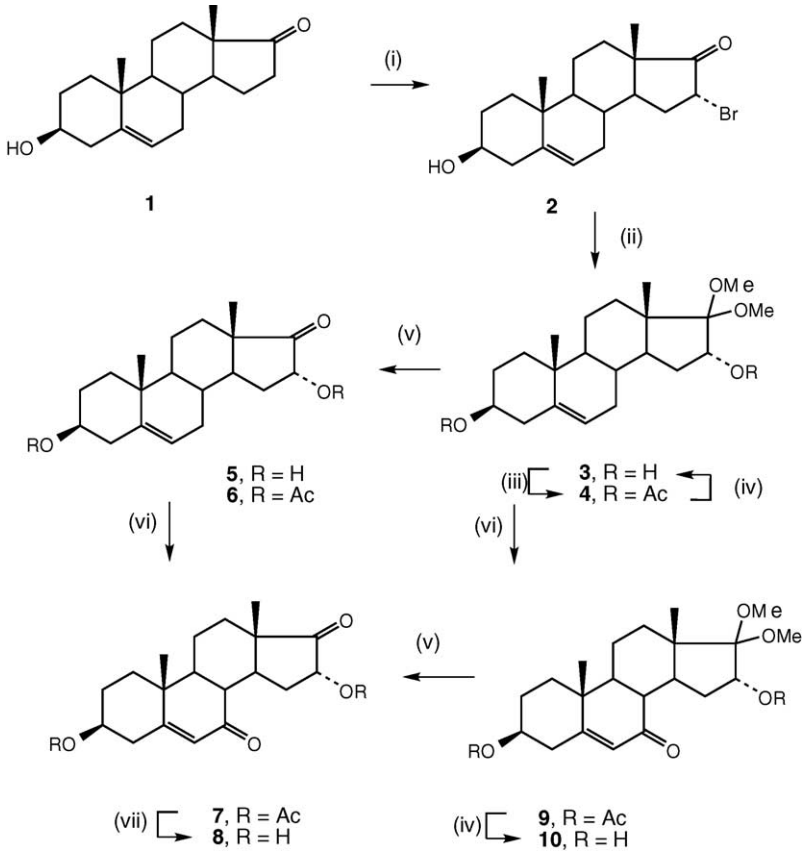
For syntheses of all three target compounds **8**, **13**, and **16**, the same starting compound, 17,17-dimethoxy-16α-hydroxy derivative **3**, was used (Scheme 2). This compound was easily available from the 16α-bromo-17-oxo derivative **2** by modification of the procedure described in the literature [10].



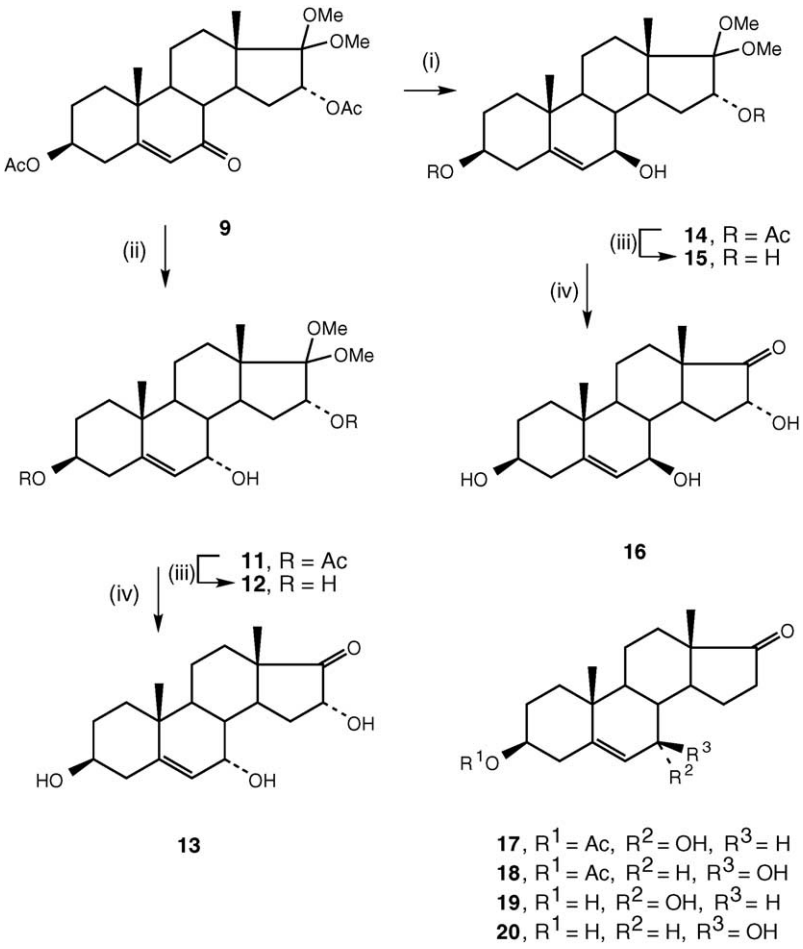
Scheme 1. Retrosynthetic analysis.

Its acetylation with a mixture of acetic anhydride and pyridine gave a fully protected derivative **4**. Using this derivative, the possibility of selective removal of the protecting groups for the hydroxy group and oxo group was checked. Acid deprotection under controlled conditions afforded the ketone-diacetate **6**, whereas alkaline hydrolysis gave the diol-ketal **3**.

For introduction of a 7-oxo group into a molecule of fully protected derivative **4**, oxidation with a complex of chromium(VI) oxide and 2,5-dimethylpyrazole [14] was used, and ketone **9** was prepared. The removal of acetyl groups from compound **9** in alkaline medium yielded diol **10**, which subsequently gave the known 16α-hydroxy-7-oxo-DHEA **8** [3,7] by acidic splitting of the acetal moiety. An



Scheme 2. (i) $CuBr_2$, MeOH; (ii) MeONa, MeOH; (iii) Ac_2O, Py; (iv) KOH, H_2O/THF; (v) TsOH, Me_2CO/H_2O; (vi) 3,5-diMepyrazole-CrO_3, CH_2Cl_2, −20 °C; (vii) 3 M H_2SO_4, MeOH.



Scheme 3. (i) $NaBH_4$, $CeCl_3$, MeOH/THF; (ii) 1. L-Selectride®, THF, −78 °C, 2. NaOH, H_2O_2; (iii) KOH, H_2O/THF; (iv) TsOH, Me_2CO/H_2O.

alternative synthesis started from the known [10,11] 3β,16α-diacetoxy-17-oxo derivative **6**, which was transformed by oxidation with a complex of chromium(VI) oxide – 2,5-dimethylpyrazole [14] to 7,17-dione **7**. After removal of the acetyl protecting groups, the known target compound 16α-hydroxy-7-oxo-DHEA **8** [11] was prepared by an independent way.

Synthesis of epimeric 7,16α-dihydroxy derivatives DHEA **13** and **16** started from the 7-oxo derivative **9** mentioned above (Scheme 3). In the synthesis of the first isomer, the 7α-hydroxy derivative **11** was prepared from **9** by controlled stereoselective reduction with L-Selectride® [15]. Alkaline hydrolysis removed the acetyl protecting groups to give triol **12**. Finally, a ketal moiety was split off by 4-toluenesulfonic acid, and 7α,16α-dihydroxy-DHEA **13** was obtained.

The second isomer 7β,16α-dihydroxy derivative **16** was prepared by reduction of **9** with sodium borohydride in the presence of cerium(III) chloride [12] and successive deprotection of the obtained 7β-hydroxy derivative **14** via intermediate **15** analogously as above. Both isomeric derivatives **13** and **16** differed on TLC, so any mutual isomerization at position 7 during the synthesis could be monitored. Both syntheses were sufficiently stereoselective, and no apparent isomerization was observed. The structure of both isomers was assigned mainly on the basis of ^1H NMR spectroscopy (see below).

3.2. NMR spectra of 7- and 16-hydroxy derivatives of DHEA

The observed shape (apparent multiplicity) of the proton signals in positions substituted by the hydroxy group depends on the measurement conditions for both the 7-hydroxy [15,16] and 16α-hydroxy [11] derivatives of DHEA. This is the reason why there are differences among published values given in the literature, especially for the various 7-hydroxy derivatives of DHEA. In the present study, the spectra of the model derivatives of DHEA were acquired under the same conditions, including those of the 7-oxo compounds **5** and **6** and the 7-hydroxy derivatives with acetylated (**17** and **18**), and free (**19** and **20**) 3β-hydroxy groups. The spectra in deuteriochloroform were measured with additional perdeuterioacetic acid (1%) in order to achieve exchange of protons in the hydroxy groups and consequently to simplify the spectra. Acetic acid at this concentration and during a time period of tens of minutes did not cause marked cleavage of the acetal

protecting group in position 17. The 5% aqueous solution of citric acid, used in the work-up of reaction mixtures, also did not cleave this protecting group. For its removal, a stronger acid such as 4-toluenesulfonic acid was necessary.

The spectra of all 17-oxo derivatives with 16α-hydroxy and 16α-acetoxy groups contained a characteristic doublet of 16β-H with a coupling constant J(15β,16β) of 8–9 Hz. The coupling of 16β-H with 15α-H was close to zero, and it was observable only for some compounds in the series. The corresponding 17,17-dimethoxy derivatives had different geometry at ring D and the coupling constant J(15α,16β) was slightly higher beeing about 2–3 Hz. The isomeric 17-oxo derivatives with a 16β-acetoxy group differed significantly, giving the 16α-H as a characteristic triplet with a coupling constant of about 8.5 Hz [3].

For the 7-hydroxy derivatives, it was possible to assign the configuration of a hydroxy group on the basis of a chemical shift value of H-6, which differed significantly for both isomers [12]. For the 7α-isomer, the value of H-6 was about 5.6 ppm, and for the 7β-isomer, it was about 5.3 ppm. The value of J(6,7) was also different: for the 7α-hydroxy derivative, it amounted to about 5 Hz, and for the 7β-hydroxy derivative, it was about 2 Hz. The shape of the signal was further influenced by the allylic interaction ^{4}J(4β,6) amounting to about 2 Hz. Signal H-7 was further split by the interaction with H-8, and again, the value was different for both isomers; it was about 4 Hz for the 7α- and 8 Hz for the 7β-isomer. For the 7β-hydroxy derivative, an additional coupling ^{5}J(4β,7α) of about 2 Hz was discernible. The final shape of the signals depended on particular coupling constants and could be different for any combination of substituents.

The configurations of the hydroxy groups at position 7, assigned on the basis of the ^{1}H NMR spectra, were in

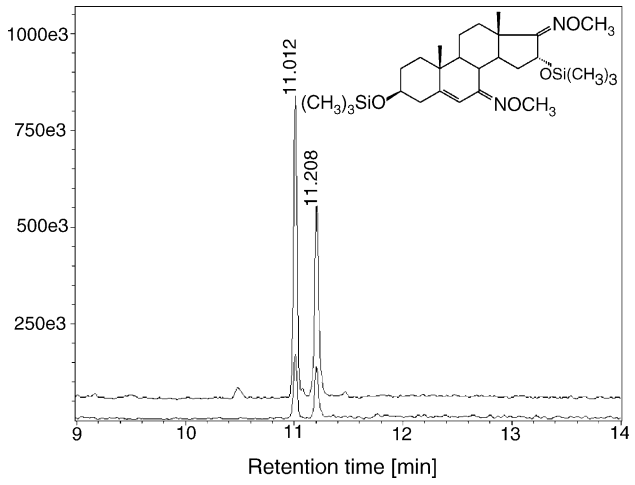


Fig. 1. Chromatogram of the MO-TMS derivative of 3β,16α-dihydroxyandrost-5-ene-7,17-dione (**8**) as recorded on the effective masses 117 and 129.

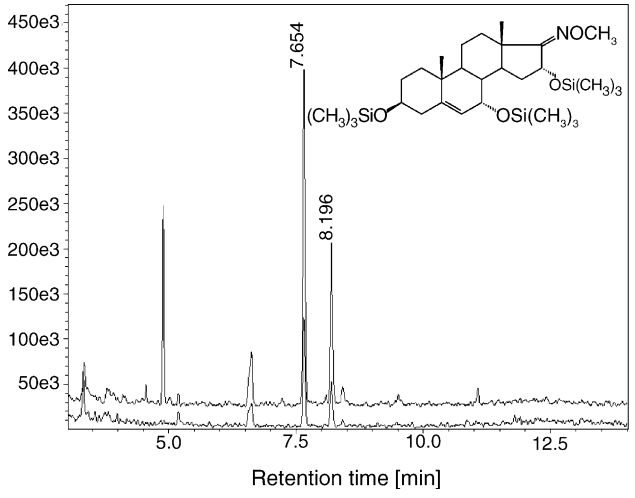


Fig. 2. Chromatogram of the MO-TMS derivatives of 3β,7α,16α-trihydroxyandrost-5-en-17-one (**13**) as recorded on the effective masses 117 and 129.

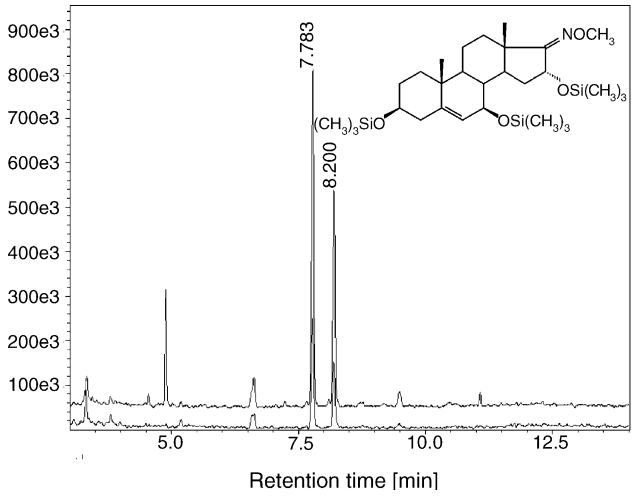


Fig. 3. Chromatogram of the MO-TMS derivatives of 3β,7β,16α-trihydroxyandrost-5-en-17-one (**16**) as recorded on the effective masses 117 and 129.

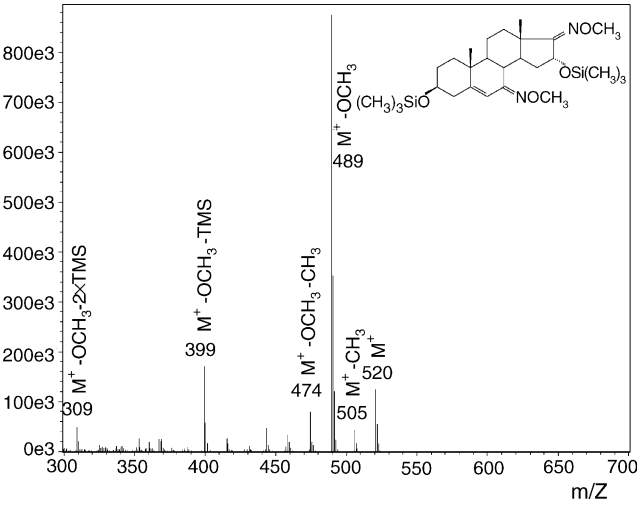


Fig. 4. Mass spectra of the MO-TMS derivatives of 3β,16α-dihydroxyandrost-5-ene-7,17-dione (**8**); for better clarity, only the effective masses greater than 300 amu are shown.

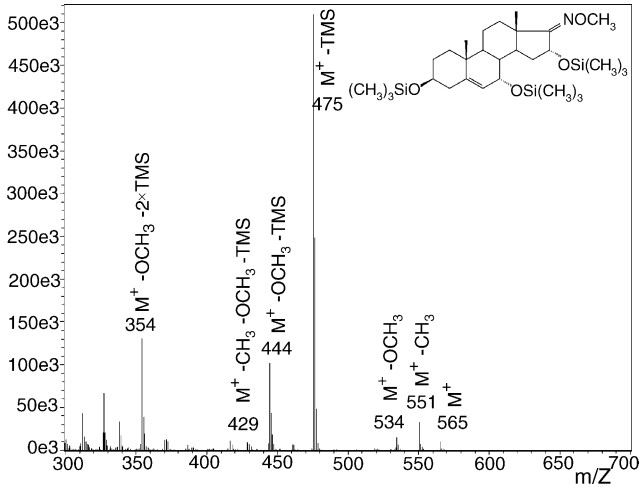


Fig. 5. Mass spectra of the MO-TMS derivatives of 3β,7α,16α-trihydroxyandrost-5-en-17-one (**13**); for better clarity, only the effective masses greater than 300 amu are shown.

accordance with the configurations predicted by the known reaction course of the reduction of a 7-oxo group with the employed reduction reagents [12,15,16].

The ^{13}C NMR spectra for target compounds **8**, **13** and **16** are summarized in Table 1. Chemical shifts of particular carbons were in accordance with the corresponding structures. On the basis of the available spectral data, isomeric 17β-hydroxy-16-oxo derivatives could be excluded due to the absence of a characteristic chemical shift of C-17 at about 85 ppm [17].

3.3. GC–MS analysis

The chromatograms of the MO-TMS derivatives of compounds **8**, **13**, and **16** are shown in Figs. 1–3. The responses were recorded on the effective masses 117 and 129 that are typical for a number of steroids with the 3β-OTMS-5-ene structure, including the compounds evaluated. All measured compounds gave two peaks due to the presence of both E and Z isomers of 17-(O-methyloxime) derivatives. Both peaks displayed nearly the same mass spectra, thus, in the Figs. 4–6, only the mass spectra of the stronger peaks with shorter retention times together with their interpretation were included. The expected fragmentation patterns are shown in Figs. 4–6 and were in accordance with the proposed structures. The acquired retention times and the fragmentation gave a base for the use of GC–MS in the screening of these 16α-hydroxy-DHEA derivatives in biological samples.

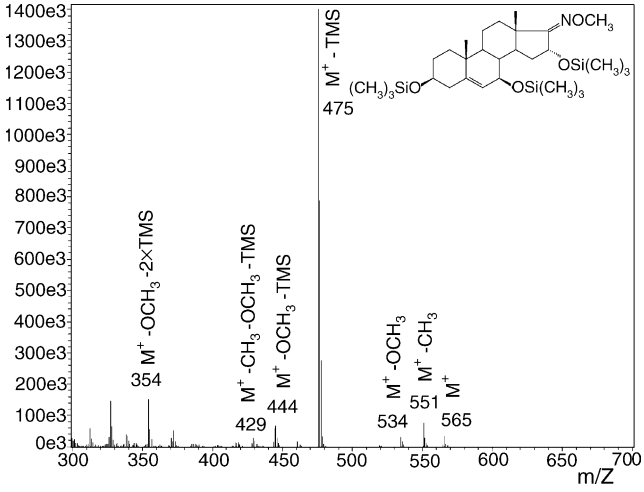


Fig. 6. Mass spectra of the MO-TMS derivatives of 3β,7β,16α-trihydroxyandrost-5-en-17-one (**16**); for better clarity, only the effective masses greater than 300 amu are shown.

4. Conclusions

The synthesis of derivatives of 16α-hydroxy-DHEA with an additional oxygen substituent at position 7, i.e., 7-oxo derivative **8** and both epimeric 7-hydroxy derivatives **13** and **16**, was developed and their structures were proved by NMR spectroscopy. These compounds were designed for further use as standards in gas chromatography/mass spectrometry assays. Monitoring of their occurrence in biological tissues can lead to better knowledge of related steroid metabolism pathways, for example, those associated with brain disorders or autoimmune diseases.

Acknowledgements

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