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PAPER

Hydroxylation of DHEA, androstenediol and epiandrosterone by *Mortierella isabellina* AM212. Evidence indicating that both constitutive and inducible hydroxylases catalyze 7α- as well as 7β-hydroxylations of 5-ene substrates

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The course of transformation of DHEA, androstenediol and epiandrosterone in *Mortierella isabellina* AM212 culture was investigated. The mentioned substrates underwent effective hydroxylation; 5-ene substrates – DHEA and androstenediol – were transformed into a mixture of 7α- and 7β- allyl alcohols, while epiandrosterone was converted into 7α- (mainly), 11α- and 9α- monohydroxy derivatives. Ketoconazole and cycloheximide inhibition studies suggest the presence of constitutive and substrate-induced hydroxylases in *M. isabellina*. On the basis of time course analysis of the hydroxylation of DHEA and androstenediol, the oxidation of allyl C_7-H_α and C_7-H_β bonds by the same enzyme is a reasonable assumption.

Introduction

Recent years have witnessed intensive ongoing research on enzymatic hydroxylation of DHEA and epiandrosterone.[1–12] C-7 Hydroxy derivatives of both the above-mentioned alcohols were identified in numerous mammalian tissues, mostly liver, brain, and skin.[5–11] Investigation of the metabolism of DHEA in the brain tissue of mice provided evidence that almost equivalent quantities of 7α-hydroxy- and 7β-hydroxy-DHEA are formed.[4] Cytochrome P4507B1, present in various human tissues, was found to be responsible for 7α-hydroxylation of DHEA and epiandrosterone.[4] Cytochrome P450 responsible for formation of 7β-hydroxy-DHEA was not identified in any of the investigated mammalian tissues. CYP7B1-deficient mice did not exhibit formation of 7α- or 7β-hydroxy-DHEA, which suggests that both epimeric C-7 alcohols are produced with participation of the same enzyme.[2,11] Studies using rat liver homogenate have shown that the metabolism of DHEA can proceed through the sequence of reactions: 7α-hydroxylation, oxidation of the hydroxyl group at C-7, and subsequent reduction of 7-oxo-DHEA to 7β-hydroxy-DHEA; the listed metabolites partially undergo additional reduction at C-17 to 17β-alcohols.[7] It is proposed that the anti-glucocorticoid effects of 7α-hydroxy-DHEA can be connected with the fact that the same enzyme – 11β-hydroxysteroid dehydrogenase (11β-HSD1) – catalyzes transformations of 7-oxo-DHEA to 7β-hydroxy-DHEA and inactive cortisone into active cortisol.[12] Competition between 7-oxo-DHEA and cortisone may reduce the formation of cortisol. The pathway for the formation of 7α-hydroxy-DHEA and 7β-hydroxy-DHEA in the brain remains unclear. Elucidation of the DHEA metabolism routes in the mammalian brain is important for full explanation of the neuroprotective function of DHEA; enzyme(s) catalyzing the transformation DHEA→7β-hydroxy-DHEA are still not known.[9]

The main metabolites of DHEA in mammalian tissues have also been known for many years as products of microbial transformations of DHEA. A significant proportion of the literature reports on microbial transformations of DHEA is concerned with regioselective oxidation at C-7.[13–25] Cultures of numerous strains were found to carry out stereoselective 7α-hydroxylation of DHEA,[14,18–20,22,25] but generally microbial hydroxylation led more often to a mixture of epimeric 7α- and 7β-alcohols.[13,15–17,21,23,24] Selective formation of 7β-hydroxy-DHEA from DHEA was observed in the transformation carried out by the strain *Botryodiplodia malorum*.[22] Some mixtures of products of microbial transformation of DHEA contained beside the 7-hydroxy derivatives also 7-oxo-DHEA.[13,15,16,18] Reports on microbial hydroxylation of epiandrosterone (EpiA) are scarce.[14,17,26,27] 7β-Hydroxy-EpiA was the main metabolite in transformation by strains of *Cunninghamella elegans* and *Rhizopus nigricans*, and apart from this compound, 7α-hydroxy-EpiA was also isolated.[26,27]

The current study will present results of transformation of DHEA, androstenediol and epiandrosterone, by the fungus *Mortierella isabellina* AM212. The time dependence of transformations of 5-ene substrates was analyzed with the aim of elucidation of the route leading to the formation of 7α- and 7β- allyl alcohols.

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Results and discussion

Transformation of DHEA (1)

After 12 h transformation of 160 mg of DHEA (**1**) the isolates were: 7α-hydroxy-DHEA (**4**), 7β-hydroxy-DHEA (**5**) and 7-oxo-DHEA (**6**).

The structures of products **4** and **6** were determined on the basis of matches between R_t from GC and R_f from TLC with standards obtained by us in our previous studies[20,28] and correspondence of their NMR spectra with the literature data.[16,20,21] The structure of 7β-hydroxy-DHEA (**5**) was determined as a result of comparison of its spectroscopic data with that of the starting material (**1**). The signals at δ_H 3.53–3.58 ppm (m) and δ_H 5.31 ppm (t, J = 1.8 Hz) point to conservation of the 3β-hydroxy-5-ene moiety in molecules of **5**. The appearance of a new signal at δ_H 3.95 ppm suggests monohydroxylation, while the signal shape indicates an equatorial configuration of the introduced hydroxyl group. This observation is supported by the appearance of a new methine carbon signal at δ_C 72.8 ppm which, in combination with the downfield shift for the C-6 (Δ 4.5 ppm) and C-8 (Δ 9 ppm) resonances, is an important confirmation of 7β-hydroxylation. Moreover, the HMBC spectrum shows correlations of H-6 (δ 5.31 ppm) with C-7 (δ 72.8 ppm) and H-7 (δ 3.95 ppm) with C-6 (δ 125.4 ppm), C-8 (δ 40.4 ppm), and C-14 (δ 51.1) ppm. The ^{1}H and ^{13}C NMR data of **6** are in agreement with those reported in the literature.[21]

Transformation of androstenediol (2)

After 24 h transformation of 200 mg of androstenediol (**2**) the following metabolites were isolated: 3β,7α,17β-trihydroxyandrost-5-ene (**7**), 3β,7β,17β-trihydroxyandrost-5-ene (**8**) and 3β,17β-dihydroxyandrost-5-en-7-one (**9**).

Structures of the products **7** and **9** were determined on the basis of their ^{1}H NMR characteristic shift values being identical to the literature data of the standards.[16,20,28] ^{1}H NMR spectrum of the product **8** with characteristic multiplet at δ_H 3.37–3.48 ppm, triplets at δ_H 3.56 ppm and δ_H 5.25 ppm indicates the presence of 3β- and 17β- hydroxyl groups (present also in the substrate **2**) and double bond at C_5-C_6. Signals at δ_C 72.2 ppm, δ_C 82.3 ppm, δ_C 144.1 ppm and δ_C 127.4 ppm, visible in the ^{13}C NMR spectrum of this metabolite, confirm this analysis. Additionally, the NMR spectra of **8** show new downfield signals for the oxygen-bearing methine proton at δ_H 3.72 (1H, dt, J = 2.1 Hz, J = 7.8 Hz) and methine carbon, δ_C 74.0 ppm. Shape of the signal at δ_H 3.72 ppm is analogous to the signal at δ_H 3.95 ppm in **5**, indicating introduction of a C-7β hydroxyl group in **8**. This is also supported by the downfield shifts for C-6 (Δ 5.1 ppm) and C-8 (Δ 7.8 ppm) observed in ^{13}C NMR spectra.

Transformation of epiandrosterone (EpiA) (3)

After 21 h transformation of 200 mg EpiA (**3**) the following compounds were isolated: 7α-hydroxy-EpiA (**10**), 11α-hydroxy-EpiA (**11**) and 9α-hydroxy-EpiA (**12**).

NMR spectra of **10** show new downfield signals for the oxygen-bearing methine proton at δ_H 3.93–3.96 ppm and the methine carbon at δ_C 66.7 ppm, which indicate introduction of an axial hydroxyl group. This is supported by downfield shifts for C-6 (Δ 7.3 ppm) and C-8 (Δ 4.1 ppm), and γ-*gauche* upfield shifts for C-9 (Δ 8.4 ppm) and C-14 (Δ 5.6 ppm) signals which were observed in the ^{13}C NMR spectra. The spectral data for **10** are in agreement with those reported in the literature for 7α-hydroxy-EpiA.[27,29] The metabolite **11** has a resonance at δ_H 3.52–3.63 ppm (m), confirming that the 3β-OH group is maintained, and new resonances at δ_C 68.6 ppm and δ_H 3.96 ppm (dt, J = 5.2 Hz, J = 10.4 Hz), which suggest hydroxylation at an equatorial position of the steroid molecule. In comparison to the spectrum of the substrate, the C-19 methyl signal of the product demonstrates a significant downfield shift (Δ 0.13 ppm) indicating that hydroxylation occurred at the 11α-position. This is further supported by the ^{13}C NMR of **11**, which shows downfield shifts for C-9 (Δ 6.0 ppm) and C-12 (Δ 11.3 ppm) that are consistent with reference shift values.[30] All these results confirm that the product is 3β,11α-dihydroxy-5α-androstan-17-one. The spectroscopic data of this compound correspond to those described in the literature.[29] In the ^{1}H-NMR spectrum of **12** having visibly higher polarity on TLC than **3**, the C*H*OH signal is lacking. New resonances at δ_C 75.7 ppm, and the downfield shift of signals: C-10 (Δ 4.5 ppm), C-11 (Δ 6.4 ppm), C-8 (Δ 2.2 ppm), and γ-*gauche* upfield shifts of C-5 (Δ 8.6 ppm), C-7 (Δ 6.4 ppm), C-12 (Δ 3.7 ppm) and C-14 (Δ 6.5 ppm) signals of this metabolite in comparison to the spectrum of substrate **3**, indicate hydroxylation at the 9α position. The proposed structure, 9α-hydoxy-EpiA (**12**), is supported by the downfield shift of the δ_H C-19 methyl signal by 0.13 ppm, with respect to the substrate **3**; the ^{1}H-NMR and ^{13}C-NMR spectral data are in agreement with the literature.[31]

Time course analysis of the transformation of substrates

Time course analysis of the transformation of DHEA (**1**), androstenediol (**2**) and epiandrosterone (**3**) exhibits some similarities for all the substrates: hydroxyl derivatives are identified in the mixtures already after 0.5 h incubation of **1**, **2** or **3** (Table 1); the initial period of incubation is marked by small, but systematic growth of the amount of the hydroxyl derivatives, and their share in the mixtures rises sharply in the following incubation stages: 1–2 h for **1**, 3–4 h for **2** and 2–3 h for **3**. All the isolated mixtures of metabolites obtained from the 5-ene substrates **1** and **2** contained both epimeric 7α- and 7β-hydroxyl derivatives; apart from the C-7 allyl alcohols, small amounts of 7-oxo products **6** and **9** were also identified (Fig. 1). The ratios of 7α- *vs.* 7β-hydroxy derivatives differed between transformation of DHEA (**1**) and androstenediol (**2**), and additionally for each substrate the contents of both allyl alcohols in the metabolite mixture varied depending on the incubation time. The mixture of hydroxyl derivatives after 1 h incubation of DHEA contained more 7β-hydroxy-DHEA (**5**) (7α-OH/7β-OH ≈ 0.5 : 1). The mixtures isolated after subsequent incubation periods exhibited a growing proportion of 7α-hydroxy-DHEA (**4**); an especially high (close to 4-fold) increase in the amount of **4** was observed in the 1–2 h incubation period. During the same period, there was a steep increase in the hydroxylating activity of the strain culture (Table 1).

In order to check whether the change of amount of 7α- and 7β-hydroxy derivatives of DHEA in the isolated mixtures is a result of **4** ⇆ **5** interconversion, the following metabolites of DHEA were also subjected to the transformation by the strain: 7α-hydroxy-DHEA (**4**), 7β-hydroxy-DHEA (**5**) and 7-oxo-DHEA (**6**). The

Table 1 Composition of crude mixtures obtained in transformations of **1–3** by *Mortierella isabellina* AM212

Substrate	R_t (min)	Compounds present in the mixture (%)[a]	Time of transformation (h)					
			0.5	1	2	3	4	6
DHEA (**1**)	3.46	DHEA (**1**)	97	86	62	34	10	—
	6.43	7α-Hydroxy-DHEA (**4**)[b]	0.8	4.8	19.5	39	58	63
	7.43	7β-Hydroxy-DHEA (**5**)[b]	2.2	8.2	17	24	28	30
	5.33	7-Oxo-DHEA (**6**)	—	—	—	1.1	1.8	2.4
Androstenediol (**2**)	3.39	Androstenediol (**2**)	99.5	96.5	93.5	88	69	14
	4.87	3β,7α,17β-Trihydroxyandrost-5-ene (**7**)	trace	0.5	2.8	6.7	20.6	63
	4.96	3β,7β,17β-Trihydroxyandrost-5-ene (**8**)	trace	0.5	1.3	3.1	6.5	19.5
	5.55	3β,17β-Dihydroxyandrost-5-en-7-one (**9**)	—	—	—	—	—	1.4
Epiandrosterone (**3**)	3.39	Epiandrosterone (**3**)	99.5	97.0	93	81	65	13
	5.11	3β,7α-Dihydroxy-5α-androstan-17-one (**10**)	trace	1.5	4.6	11.8	23	63
	5.02	3β,11α-Dihydroxy-5α-androstan-17-one (**11**)	—	0.4	1.1	2.8	5.2	14
	4.90	3β,9α-Dihydroxy-5α-androstan-17-one (**12**)	—	0.3	1	2.5	4.7	12

[a] Determined by GC analysis. [b] Determined by GC analysis of their acetates.

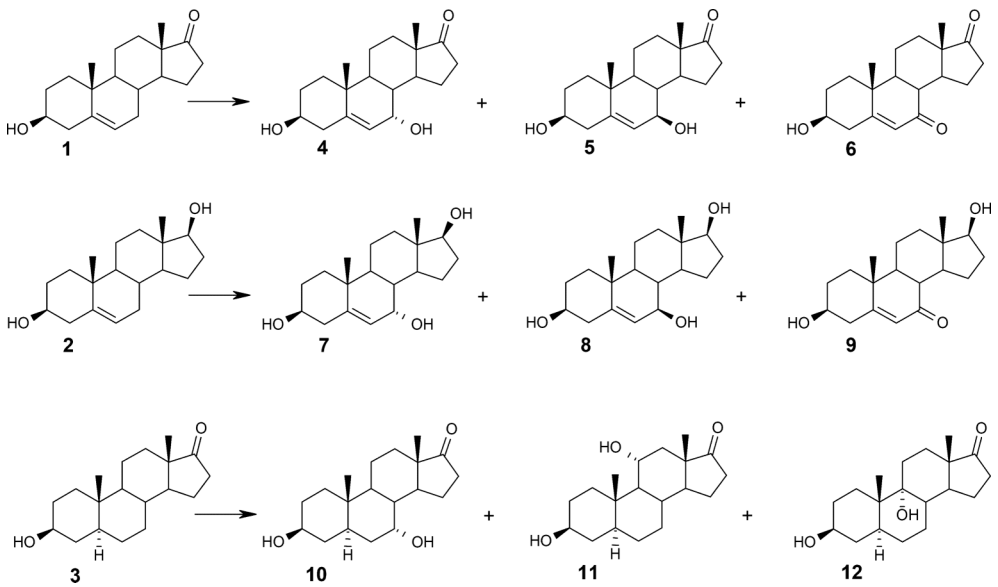


Fig. 1 Transformation of DHEA, androstenediol and epiandrosterone by *Mortierella isabellina*.

mixtures after 12 h incubation of either **4** or **5** contained, apart from the introduced alcohol, only 7-oxo-DHEA (**6**) – the other, epimeric C-7 alcohol was not identified. The sole metabolite of 7-oxo-DHEA (**6**) was 3β,17β-dihydroxyandrost-5-en-7-one (**9**) (a product of reduction of the C-17 carbonyl group); no reduction of the conjugated 7-ketone was observed (Table 2). As a check whether the absence of product of C-7 carbonyl group reduction in **6** might be a consequence of the fact that active inducers of the proper enzymes are only substrates **1** or **2**, transformation of a mixture (1 : 1 w/w) of androstenediol (**2**) and 7-oxo-DHEA (**6**) was carried out. In this metabolite mixture, after 12 h incubation there were identified 7α- and 7β-hydroxy derivatives of androstenediol (**7** and **8**), but the alcohols **4** and **5** – products of reduction of the conjugated carbonyl group of **6** (Table 2) – were absent. The strain culture, in which the 7α- and 7β-hydroxy derivatives of androstenediol are formed, does not exhibit 7-oxo dehydrogenase activity. Therefore, during DHEA transformation the interconversion 7α-hydroxy-DHEA ⇆ 7β-hydroxy-DHEA does not occur; 7-hydroxy derivatives are not formed through reduction of the C-7 ketone.

On the basis of the results of experiments described above, it is reasonable to assume that:

• The same enzyme catalyzes oxidation of C-7–Hα as well as C-7–Hβ bonds in 5-ene substrates,

• The strain culture contains a constitutive enzyme with low activity, which prefers oxidation of the C-7–Hβ bond in DHEA,

• During the 1–2 h DHEA incubation period, there is an increase in the hydroxylating activity of the culture, due to induction of hydroxylase carrying out 7α- and 7β-hydroxylation, but preferring oxidation of the C-7–Hα bond.

The assumption that the same enzyme catalyzes hydroxylation of DHEA (**1**) in positions 7α- and 7β- is supported by the presence of both the hydroxy derivatives after 0.5 h incubation of **1**, and by the fact that during the 1–2 h incubation period, along with the sharp increase in the hydroxylating activity of the culture, there is a significant increase in the amount of both the epimeric hydroxy derivatives (Table 1). The presence of two hydroxylases (constitutive and inducible) is suggested by the time evolution of 7α- to 7β-hydroxy derivative ratio in the mixtures resulting from the DHEA incubation: in the 0–1 h range the

Table 2 Composition of crude mixtures obtained after 12 h transformations of **4–6** and mixture **2** and **6** by *Mortierella isabellina* AM212

Substrate	Compounds present in the mixture	(%)[a]
7α-Hydroxy-DHEA (**4**)	7α-Hydroxy-DHEA (**4**)[b]	88.4
	7β-Hydroxy-DHEA (**5**)[b]	—
	7-Oxo-DHEA (**6**)	9.7
7β-Hydroxy-DHEA (**5**)	7β-Hydroxy-DHEA (**5**)[b]	62
	7α-Hydroxy-DHEA (**4**)[b]	—
	7-Oxo-DHEA (**6**)	32
7-Oxo-DHEA (**6**)	7-Oxo-DHEA (**6**)	87
	7α-Hydroxy-DHEA (**4**)[b]	—[c]
	7β-Hydroxy-DHEA (**5**)[b]	—[c]
	3β,17β-Dihydroxyandrost-5-en-7-one (**9**)	9
Mixture of androstenediol (**2**) and 7-oxo-DHEA (**6**)	3β,7α,17β-Trihydroxyandrost-5-ene (**7**)	30
	3β,7β,17β-Trihydroxyandrost-5-ene (**8**)	12
	7-Oxo-DHEA (**6**)	52
	7α-Hydroxy-DHEA (**4**)[b]	—
	7β-Hydroxy-DHEA (**5**)[b]	—
	3β,17β-Dihydroxyandrost-5-en-7-one (**9**)	3.5

[a] Determined by GC analysis. [b] Determined by GC analysis of their acetates. [c] Not present also in the mixtures after 24 h incubation.

7β-hydroxy-DHEA prevails, whereas at the time of the rapid increase in the hydroxylating ability of the culture (the inducible hydroxylase) the amount of 7β-hydroxy-DHEA grows twofold, while for the 7α-isomer – almost 4-fold. In the subsequently isolated mixtures, the 7α-OH/7β-OH product ratio grows systematically; the mixture after complete transformation of DHEA contained over two times more 7α-hydroxy-DHEA than the 7β-epimer (Table 1).

The C-7-Hα and C-7-Hβ bonds can be located in analogous positions with respect to the oxidating center of the hydroxylase in the enzyme–substrate complexes with participation of the oxygen functions at C-3 (normal binding complex) or C-17 (reverse inverted binding complex) (Fig. 2). Such influence of substituents in the substrate molecule on the formation of the enzyme–substrate complex was proposed for the first time to explain 6β- and 11α-hydroxylation of steroids by *Aspergillus tamarii*.[32] This model was confirmed by studies on transformation of numerous steroids in cultures of other strains.[33–37] The distances between the oxygen atom participating in the proposed enzyme–substrate complexes (Fig. 2) and the hydrogen atom in the hydroxylated position, calculated on the basis of X-ray structures of DHEA and androstenediol, have quite similar values (falling into the range 6.21 Å–6.58 Å) (Fig. 3) which supports the assumption that both 7α- and 7β-hydroxylation can be catalyzed by the same hydroxylase.

The time evolution of the androstenediol (**2**) transformation confirms the conclusions based on the analysis of the DHEA transformations. Also during the transformation of androstenediol, both 7-epimeric hydroxyl derivatives are identified already after 0.5 h incubation; the hydroxylating activity of the culture increases sharply during the 3–4 h period; at this time the amount of 7α- as well as 7β-hydroxy derivatives is growing. The above observations indicate that the oxidation of both allyl C-7–H bonds can possibly be catalyzed by the same enzyme. The lower content of the 7β-hydroxy derivative in the mixture of androstenediol metabolites, in comparison to DHEA (Table 1), corresponds with the assumption that the C-7–Hβ bond oxidation occurs in the inverted reverse complex in which the oxygen atom at C-17 participates, because 17β-hydroxyl group (androstenediol) exhibits a weaker directing effect than the C-17 carbonyl (DHEA). The stronger directing effect of the carbonyl group is indicated by *e.g.* results of transformation of 5α-androstan-17-one and 5α-androstan-17β-ol by *Calonectria decora*; the 17-oxo substrate yielded 33% of 1β,6α-dihydroxy product, whereas the 5α-androstan-17β-ol gave only 11%.[33] Literature reports claim that in many microbial cultures the DHEA was transformed to a mixture of 7α- and 7β-hydroxy derivatives[21–23] *e.g.* among 64 strains capable of hydroxylation at C-7, 37 metabolized DHEA to both allyl alcohols.[23]

The main product of epiandrosterone (**3**) transformation is 7α-hydroxy-EpiA (**10**), besides which also 11α- and 9α-hydroxy derivatives were isolated. After 0.5 h incubation of EpiA only trace amounts of 7α-hydroxy-EpiA were identified; the mixture after 1 h reaction time contained all the isolated hydroxyl derivatives of EpiA (Table 1); the hydroxylating activity of the culture sharply increased in the period 2–3 h of incubation of **3**. In the metabolite mixtures isolated after various EpiA incubation periods, ratios of the formed hydroxyl derivatives were similar – 7α-hydroxy-EpiA/11α-hydroxy-EpiA/9α-hydroxy-EpiA ≈ 4 : 1 : 0.9.

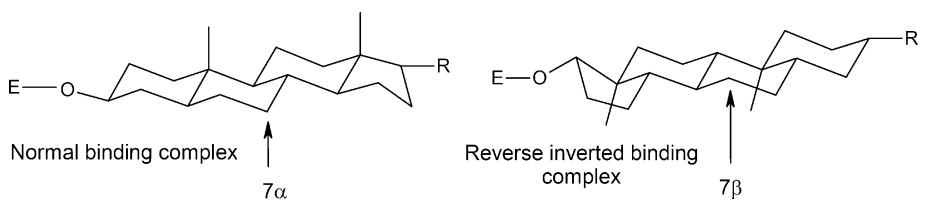
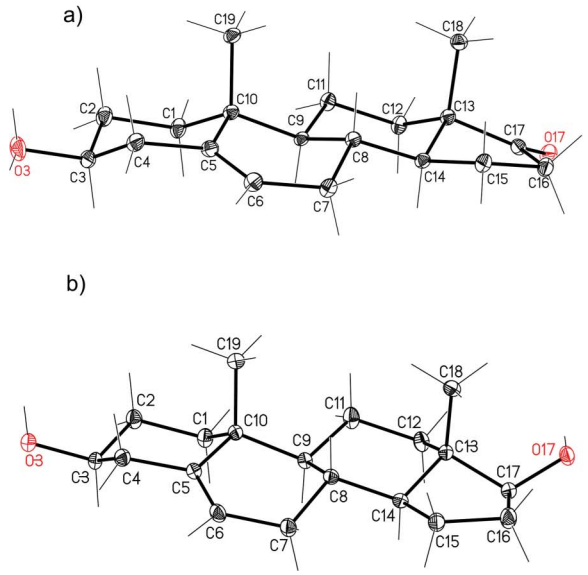


Fig. 2 Proposed enzyme–substrate complexes for 7α- and 7β-hydroxylation.



DHEA (**1**)	Distance
Relationship between	
3β–O ↔ $H_{7α}$	6.58 Å
C_{17}=O ↔ $H_{7β}$	6.21 Å

Androstenediol (**2**)	Distance
Relationship between	
3β–O ↔ $H_{7α}$	6.54 Å
17β–O ↔ $H_{7β}$	6.32 Å

Fig. 3 X-Ray structures with distance determination between hydroxylated hydrogen atoms at C-7 and: (a) oxygen atom of 3β-alcohol (O3) or C-17 keto oxygen (O17) in DHEA; (b) oxygen atom of 3β- (O3) or 17β- (O17) alcohols in androstenediol.

Time course evaluation of the DHEA transformation following cycloheximide and/or ketoconazole treatment

DHEA transformations by the strain cultures were carried out in the presence of cycloheximide, ketoconazole and both of these compounds together. This inhibition study was carried out to provide additional data helpful in testing one of the major hypotheses of this work, namely, whether the 7α- and 7β-hydroxylation can be carried out by one and the same enzyme. In the presence of cycloheximide, an inhibitor of *de novo* protein synthesis, the substrate was hydroxylated with a slower rate; after 24 h incubation there was nearly 60% DHEA left, while in the standard transformations after 6 h incubation the substrate was already not identifiable (Table 1 and Table 3). The mixtures isolated after various incubation DHEA periods in the presence of cycloheximide contained the 7β-hydroxy product **5** as the major component, and the ratios of the epimeric C7-alcohols were very close (7α-OH/7β-OH ≈ 0.55/1) (Table 3). The hydroxylating activity of the cultures treated with cycloheximide was decreased by 15–80% under ketoconazole concentrations 0.375–1.5 mM; complete inhibition occurred at the concentration of ketoconazole equal to 2.25 mM.

Inhibition of the hydroxylating activity of the strain in the presence of ketoconazole as a sole inhibitor turned out to be dependent on the incubation time. In the concentration range of 0.375–2.25 mM a slight lowering of the activity (data not shown), comparable to that of observed in a control after addition of the solvent (DMF), was noticeable during the incubation period 0–1 h. At the inhibitor concentration of 3.0 mM, the mixture of metabolites after 4 h incubation contained 35% more substrate than in the corresponding experiments without ketoconazole, but the substrate became almost totally transformed after an additional 2 h (Table 4). Also the ratios of 7α- *vs.* 7β-hydroxy-DHEA in the metabolite mixtures formed in the presence of ketoconazole varied depending on the incubation time; the growth profile of the share of the 7α-hydroxy derivative **4** was similar to that observed in the standard, uninhibited, transformations (for comparison see Table 1 and Table 4). The lack of inhibitory effect of ketoconazole in the cultures without added cycloheximide can result from the fact, that after the inhibitor was added, some enzymes become induced, whose action subsequently deactivates the inhibitor.

Aiming at determination of the influence of ketoconazole on the induced hydroxylase(s), we carried out a series of experiments, in

Table 3 Percentage composition[a] of the reaction mixtures after DHEA transformation by *M. isabellina* in the presence of 1.0 mg of cycloheximide and varying concentration of ketoconazole

	Ketoconazole concentration (mM)														
	None (0.05 ml DMF)[b]			0.375			0.750			1.125			1.50[c]		
Time (h)	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH
1	97	1.0	1.8	98	0.7	1.2	98.5	0.45	0.75	98.6	0.5	0.85	99	0.4	0.6
3	91.5	3.1	5.4	93	2.5	4.4	95	1.7	2.9	96	1.3	2.3	98.5	0.5	0.8
6	81	6.5	11.5	84	5.5	10	89	3.7	7	92	2.8	5	97.5	0.7	1.2
24	56	14.5	28	64.5	12.5	21.5	72	9.5	17	80	6.5	11.5	92.0	2.5	4.5

[a] Determined by GC analysis. [b] pH after 1 h incubation was 4.1; after 6 h, 4.2. [c] pH after 1 h incubation was 4.1; after 6 h, 4.2.

Table 4 The effect of ketoconazole on transformation of DHEA by *M. isabellina*

	Ketoconazole concentration (mM)					
	None (0.05 ml DMF)[b]			3.0		
	% of steroid in reaction mixture[a]			% of steroid in reaction mixture[a]		
Time of transformation (h)	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH
1	97	1.2	1.8	99.7	0.1	0.2
2	60.5	25	14.5	80	10.5	9.5
4	12	57	30.5	46	32.5	21
6	—	64	33	5.5	59.5	32.5

[a] Determined by GC analysis of their acetates. [b] pH after 1 h incubation was 4.1; after 6 h, 4.2.

Table 5 The effect of ketoconazole and cycloheximide[a] on transformation of DHEA in *M. isabellina* culture pre-incubated for 1 h with DHEA

	Ketoconazole concentration (mM)					
	2.25			3.0[b]		
	% of steroid			% of steroid		
Time of transformation (h)	DHEA	7α-OH	7β-OH	DHEA	7α-OH	7β-OH
1[c]	89.5	4.5	5.7	90	4.2	5.6
2	66	19.5	13.5	74.5	15	10.5
4	18.5	54.5	26	40	38.5	21.5
6	—	66.5	33	10.5	58	30.5
8				—	66.5	31.5

[a] Ketoconazole was introduced simultaneously with cycloheximide into the *M. isabellina* cultures. [b] pH after 1 h incubation was 4.1; after 6 h, 4.2. [c] Moment of addition of ketoconazole and cycloheximide.

which this compound was introduced simultaneously with cycloheximide into the microorganism cultures after 1 h transformation of the substrate. At inhibitor concentrations used in this series of experiments (2.25 or 3.0 mM), the constitutive hydroxylase was inactive. In the presence of cycloheximide and 2.25 mM ketoconazole a slight decrease in the hydroxylating activity of the culture (*ca.* 5%) was observed; with 3.0 mM ketoconazole the activity decreased by almost 25% (Table 5). The increase in the percentage of 7α-hydroxy- (**4**) and 7β-hydroxy-DHEA (**5**) in the mixtures isolated after successive incubation periods – Δ of **4**/Δ of **5** was rather stable (*ca.* 2.1) (Table 5).

The ketoconazole inhibition study indicates the presence of hydroxylases: a constitutive one, whose activity was noticeably inhibited at 0.375 mM of ketoconazole, and an inducible one, much less susceptible to the presence of the inhibitor (visible inactivation at the inhibitor concentration of 3.0 mM). The presence of the constitutive hydroxylase(s) is confirmed by hydroxylation of DHEA in the cultures simultaneously fed with the substrate and cycloheximide. The mixtures of the hydroxy derivatives, independently of the incubation time and ketoconazole concentration, exhibited very similar ratios of **4**:**5** (Table 3), which indicates that the synthesis of both epimers was inhibited to the same degree. The obtained results support the assumption that the same enzyme catalyzes oxidation of both C-7–H bonds in DHEA. Hydroxylation with two participating selective enzymes would be quite unlikely to result in the lack of difference in the **4**:**5** ratios in transformations carried out by cultures of such diverse degree of inhibition of the hydroxylating activity.

The analogous growth of the amount of both epimeric alcohols (Δ of **4**/Δ of **5**) in the mixtures formed in the experiments with cycloheximide and ketoconazole added after 1 h incubation of DHEA also supports the assumption that the inducible hydroxylase catalyzes 7α- and 7β-hydroxylation of DHEA.

Conclusion

Analysis of the composition of the metabolite mixtures isolated after varying substrate incubation periods, together with the dynamics of DHEA hydroxylation in the presence of cycloheximide and ketoconazole, indicate that the strain *M. isabellina* AM212 produces constitutive and substrate-inducible hydroxylases. The strain *Fusarium moniliforme* was also shown to produce constitutive and inducible enzymes catalyzing hydroxylation of C-7–Hα as well as C-7–Hβ bonds in DHEA. The inducible hydroxylating activity of *F. moniliforme* was markedly higher than the constitutive one.[19] The hydroxylases of *F. moniliforme* exhibited selectivity – the major product was the axial 7α-hydroxy derivative of DHEA, whereas the inducible hydroxylase of *M. isabellina* catalyzed hydroxylation of both C-7–H bonds. In the 4–6 h period of androstenediol incubation, together with an abrupt increase in the hydroxylating activity, the amount of both 7α- and 7β-alcohol increased threefold.

The collected results suggest that 7α- and 7β-allyl alcohols, as well as 7α-hydroxy-EpiA, can be formed with participation of the same hydroxylase; there are no indications of participation of two selective hydroxylases. The 7α- and 7β- hydrogen atoms can occupy similar positions with respect to the hydroxylase active center in the corresponding enzyme–substrate complexes. C-7 hydroxylation of DHEA and androstenediol is favored by the activating vicinity of π electrons.

Microbial transformations can be used as models in studies on enzymatic hydroxylation; they are suitable tools for investigating the metabolic fate of the substrates tested and hydroxylase capability.[38,39] Although no X-ray structure of a fungal origin hydroxylase has been determined so far, the research on hydroxylation of steroids by whole-cell biocatalysts provided ample information on this group of enzymes, *e.g.* on relations between structures of the substrates and regioselectivity of hydroxylation.[40] The results presented in the current study indicate similarities

of catalytic properties of fungal hydroxylases and mammalian P450 enzymes known in the literature – the strain *M. isabellina* AM212, similarly to human cytochrome P4507B1, catalyzes 7α-hydroxylation of DHEA as well as epiandrosterone.[4,6] It is reasonable to consider the hypothesis stating that the mammalian hydroxylase catalyzes oxidation of C-7–Hα and C-7–Hβ bonds in DHEA. This assumption is supported by results of studies which had proved that DHEA is transformed by yeast-expressed human CYP7B1 to a mixture of 7α-hydroxy- and 7β-hydroxy derivatives,[4,6] and by the absence of either 7α-hydroxy or 7β-hydroxy-DHEA in mice with a disrupted Cyp7b gene.[11]

Experimental section

Substrates

The substrates: dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, DHEA) (**1**), androstenediol (3β,17β-dihydroxyandrost-5-ene) (**2**), epiandrosterone (3β-hydroxy-5α-androstan-17-one, EpiA) (**3**) were purchased from Sigma–Aldrich Chemical Co, were of the highest purity commercially available and were used without further purification. 7α-Hydroxy-DHEA (3β,7α-dihydroxyandrost-5-en-17-one) (**4**), 7β-hydroxy-DHEA (3β,7β-dihydroxyandrost-5-en-17-one) (**5**) and 7-oxo-DHEA (3β-hydroxyandrost-5-en-7,17-dione) (**6**) were the metabolites isolated after transformation of DHEA by the *M. isabellina* AM212 strain. They were found to be in excess of 99.3%, 99.3% and 99.5% purity following GC and elemental analysis.

Microorganism

The fungal strain *Mortierella isabellina* AM212 used in this study was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław. The fungi were maintained on Sabouraud 4% dextrose–agar slopes at 4 °C and freshly subcultured before use in the transformation experiments.

Conditions of cultivation and transformation

General experimental and fermentation details have been described previously in our paper.[41] Each experiment was performed with at least three replications. All transformations within a given series of experiments were carried out with equal amounts of biomass.

Isolation and identification of the products

The products of biotransformation were extracted three times with chloroform. The organic extract was dried over anhydrous magnesium sulfate, concentrated *in vacuo* and analyzed by TLC and GC. Transformation products were separated by column chromatography on silica gel with acetone–ethyl acetate–methylene chloride (0.5 : 1.5 : 1 v:v:v) for DHEA (**1**), acetone–chloroform (1 : 1 v:v) for androstenediol (**2**) and hexane–acetone–chloroform (1 : 1.5 : 1.25 v:v:v) for EpiA (**3**) as eluents. TLC was carried out with Merck Kieselgel 60 F_{254} plates using the same eluents. In order to develop the image, the plates were sprayed with solution of methanol in concentrated sulfuric acid (1 : 1) and heated to 120° C for 3 min. GC analysis was performed using Hewlett Packard 5890A Series II GC instrument (FID, carrier gas H_2 at flow rate of 2 ml min^{-1}) with a HP-1 column cross-linked Methyl Siloxane, 30 m × 0.53 mm × 1.5 μm film thickness. The following program was used in the GC analysis: 220 °C/1 min, gradient 4 °C min^{-1} to 300 °C/4 min; injector and detector temperatures were 300 °C. Retention times of the identified compounds are given in Table 1. The structure of the compounds was confirmed on the basis of the NMR spectra recorded on a DRX 300 MHz Bruker Avance spectrometer with TMS as internal standard. The NMR spectra were measured in $CDCl_3$, or, when the solubility of metabolites in chloroform was low, in CD_3OD. Characteristic ^1H and ^{13}C NMR shift values in comparison to the starting compounds were used to determine structures of metabolites, in combination with DEPT analysis to identify the nature of the carbon atoms. Melting points (uncorrected) were determined on a Boetius apparatus. Elemental analysis was performed on a Vario EL III analyzer.

Time course experiments

Time course experiments were conducted in order to determine the metabolic pathways. Conditions were identical to those in main biotransformation experiments. At regular intervals, 5 ml samples of reaction mixture were taken, extracted with chloroform and analyzed by GC as described in the general experimental procedure.

Preparation of acetates

The transformation mixture was dissolved in 0.1 ml of dry pyridine, and 0.5 ml of acetic anhydride was added. The mixture was left overnight at room temperature. Next, the reaction mixture was diluted with water and the products were extracted with chloroform. The extract was washed with water, sodium carbonate solution, and then with water and dried with anhydrous magnesium sulfate.

Time course experiment following cycloheximide and/or ketoconazole treatment

Along with incubation using DHEA alone, several sets of incubations were carried out using cycloheximide and/or ketoconazole. Reactions were carried out in 50 ml Erlenmeyer flasks with 10 ml of medium. Cycloheximide was added to a three-day-old culture of the microorganism as a DMF solution, in final concentration of 0.1 mg ml^{-1} of medium, simultaneously with 2 mg of DHEA dissolved in 0.05 ml of acetone. Ketoconazole was added to a three-day-old culture of the microorganism as a DMF solution (0.05 ml) for 15 min prior to the main DHEA transformation. Reaction mixtures were extracted and analyzed by GC and TLC as described above. pH of the cultures was measured at the moment of substrate introduction, as well as after 1 and 6 h incubation. Determination of pH was carried out for transformations of DHEA, DHEA with 0.05 ml DMF, and DHEA with cycloheximide and ketoconazole.

The presented values in all experiments are arithmetic means of three measurements (Tables 1–5); relative differences between the border values have not exceeded 10%.

Crystal data collection

X-Ray data of DHEA (**1**) and androstenediol (**2**) were collected using a Kuma KM4CCD diffractometer (Mo-Kα radiation;

$\lambda = 0.71073$ Å). The strategy for the data collection was evaluated by using the CrysAlis CCD software. Data reduction and analysis were carried out with the CrysAlis RED program. The structure was solved by direct methods using SHELXS97[42] program and refined using all F^2 data, as implemented by the SHELXL97 program.

Products isolated in the course of transformations

7α-Hydroxy-DHEA (4): (90.3 mg, 53.5%); mp 181–182 °C (from acetone) (lit.,[21] 179.4–181.7 °C); Found: C, 74.88; H, 9.24. $C_{19}H_{28}O_3$ requires C, 74.96; H, 9.27%. IR ν_{max}/cm^{-1} 3360, 2932, 1736, 1620. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.88 (3H, s, 18-Me); 1.01 (3H, s, 19-Me); 3.56–3.61 (1H, m, 3α-H); 3.97 (1H, t, J = 3.6 Hz, 7β-H); 5.64 (1H, d, J = 5.0 Hz, 6-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 13.0 (18-C), 18.0 (19-C), 19.8 (11-C), 21.6 (15-C), 30.8 (2-C), 31.0 (12-C), 35.5 (16-C), 36.6 (10-C), 36.9 (1-C), 37.2 (8-C), 41.6 (4-C), 42.3 (13-C), 44.6 (9-C), 46.8 (14-C), 64.0 (7-C), 70.9 (3-C), 123.3 (6-C), 146.3 (5-C), 220.0 (17-C).

7β-Hydroxy-DHEA (5): (40.5 mg, 24%); mp 215–217 °C (from acetone) (lit.,[21] 216.2–217.6 °C); Found: C, 74.89; H, 9.25. $C_{19}H_{28}O_3$ requires C, 74.96; H, 9.27%. IR ν_{max}/cm^{-1} 3229, 2933, 1733. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.89 (3H, s, 18-Me); 1.07 (3H, s, 19-Me); 3.53-3.58 (1H, m, 3α-H); 3.95 (1H, dt, J = 2.1 Hz, J = 7.8 Hz, 7α-H); 5.31 (1H, t, J = 1.8 Hz, 6-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 13.5 (18-C), 19.1 (19-C), 20.3 (11-C), 24.1 (15-C), 31.1 (2-C), 31.4 (12-C), 35.9 (16-C), 36.6 (10-C), 36.8 (1-C), 40.4 (8-C), 41.5 (4-C), 47.7 (13-C), 48.1 (9-C), 51.1 (14-C), 72.8 (7-C), 71.2 (3-C), 125.4 (6-C), 143.6 (5-C), 221.2 (17-C).

7-Oxo-DHEA (6): (10 mg, 6%); mp 239 °C (from methanol) (lit.,[43] 236–239 °C); Found: C, 75.42; H, 8.65. $C_{19}H_{26}O_3$ requires C, 75.46; H, 8.66%. IR ν_{max}/cm^{-1} 3482, 1728, 1654, 1610. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.90 (3H, s, 18-Me); 1.23 (3H, s, 19-Me); 3.65-3.73 (1H, m, 3α-H); 5.75 (1H, d, J = 1.5 Hz, 6-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 13.7 (18-C), 17.4 (19-C), 20.6 (11-C), 24.2 (15-C), 30.7 (12-C), 31.1 (2-C), 35.6 (16-C), 36.3 (10-C), 38.4 (1-C), 41.8 (4-C), 44.3 (14-C), 45.7 (8-C), 47.8 (13-C), 50.0 (9-C), 70.3 (3-C), 126.0 (6-C), 166.0 (5-C), 201.0 (7-C), 220.4 (17-C).

3β,7α,17β-Trihydroxyandrost-5-ene (7): (101.2 mg, 47.9%); mp 205–208 °C (from methanol) (lit.,[44] 205 °C); Found: C, 74.38; H, 9.85. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%. IR ν_{max}/cm^{-1} broad absorption around 3300. ^{1}H-NMR (300 MHz, CD_3OD) δ_H: 0.75 (3H, s, 18-Me); 1.02 (3H, s, 19-Me); 3.44–3.55 (1H, m, 3α-H); 3.61 (1H, t, J = 8.5 Hz, 17α-H); 3.76 (1H, t, J = 3.6 Hz, 7β-H); 5.54 (1H, d, J = 5.0 Hz, 6-H). ^{13}C-NMR (75 MHz, CD_3OD) δ_C: 11.4 (18-C), 18.7 (19-C), 21.5 (11-C), 24.2 (15-C), 30.7 (16-C), 32.2 (2-C), 37.5 (12-C), 38.2 (C-1), 38.5 (10-C), 39.1 (8-C), 42.9 (4-C), 43.6 (13-C), 43.7 (9-C), 45.4 (14-C), 65.5 (7-C), 72.0 (3-C), 82.5 (17-C), 125.0 (6-C), 146.7 (5-C).

3β,7β,17β-Trihydroxyandrost-5-ene (8): (46 mg, 21.8%); mp 231–233 °C (from methanol) (lit.,[45] 236 °C); Found: C, 74.39; H, 9.86. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%. IR ν_{max}/cm^{-1} broad absorption around 3510. ^{1}H-NMR (300 MHz, CD_3OD) δ_H: 0.76 (3H, s, 18-Me); 1.09 (3H, s, 19-Me); 3.37-3.48 (1H, m, 3α-H); 3.56 (1H, t, J = 8.5 Hz, 17α-H); 3.72 (1H, dt, J = 2.1 Hz, J = 7.8 Hz, 7α-H); 5.25 (1H, t, J = 1.8 Hz, 6-H). ^{13}C-NMR (75 MHz, CD_3OD) δ_C: 11.6 (18-C), 19.6 (19-C), 21.9 (11-C), 26.6 (15-C), 30.9 (16-C), 32.3 (2-C), 37.7 (10-C), 37.8 (12-C), 38.3 (1-C), 41.3 (8-C), 42.6 (4-C), 44.3 (13-C), 50.1 (9-C), 52.3 (14-C), 72.2 (3-C), 74.0 (7-C), 82.3 (17-C), 127.4 (6-C), 144.1 (5-C).

3β,17β-Dihydroxyandrost-5-en-7-one (9): (17.1 mg, 8.2%); mp 202–204 °C (from methanol) (lit.,[45] 201 °C); Found: C, 74.88; H, 9.25. $C_{19}H_{28}O_3$ requires C, 74.96; H, 9.27%. IR ν_{max}/cm^{-1} 3304, 1651, 1615. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.75 (3H, s, 18-Me); 1.20 (3H, s, 19-Me); 3.62–3.69 (1H, m, 3α-H); 3.64, t, J = 10.8 Hz, 17α-H); 5.68 (1H, d, J = 1.5 Hz, 6-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 11.0 (18-C), 17.4 (19-C), 20.9 (11-C), 25.8 (15-C), 30.7 (16-C), 31.2 (2-C), 35.6 (12-C), 36.4 (1-C), 38.4 (10-C), 41.8 (4-C), 43.3 (13-C), 44.9 (14-C), 45.2 (8-C), 50.0 (9-C), 70.4 (3-C), 81.0 (17-C), 125.9 (6-C), 165.5 (5-C), 201.9 (7-C).

7α-Hydroxy-EpiA (10): (154 mg, 73%); mp 194 °C (from acetone) (lit.,[46] 194–195 °C); Found: C, 74.41; H, 9.88. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%. IR ν_{max}/cm^{-1} 3600, 3418, 1739. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.83 (3H, s, 19-Me); 0.86 (3H, s, 18-Me); 3.56–3.66 (1H, m, 3α-H); 3.93–3.96 (1H, m, 7β-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 11.2 (19-C), 13.5 (19-C), 20.3 (11-C), 21.3 (15-C), 31.3 (12-C), 31.4 (2-C), 35.7 (6-C), 36.0 (10-C), 36.6 (1-C), 36.6 (16-C), 37.0 (5-C), 37.5 (4-C), 39.1 (8-C), 45.8 (14-C), 46.1 (10-C), 47.5 (13-C), 66.7 (7-C), 70.9 (3-C), 221.1 (17-C).

11α-hydroxy-EpiA (11): (13 mg, 6.9%); mp 103–104 °C (from acetone) (lit.,[47] 103–106 °C); Found: C, 74.40; H, 9.88. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%. IR ν_{max}/cm^{-1} 3470, 1741. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.86 (3H, s, 18-Me); 0.94 (3H, s, 19-Me); 3.52-3.63 (1H, m, 3α-H); 3.96 (1H, dt, J = 5.2, J = 10.4 Hz, 11β-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 12.7 (19-C), 14.5 (18-C), 21.8 (15-C), 28.9 (6-C), 30.7 (7-C), 31.7 (2-C), 34.2 (8-C), 35.8 (16-C), 37.3 (10-C), 38.5 (1-C), 38.5 (4-C), 42.9 (12-C), 45.0 (5-C), 48.0 (13-C), 50.3 (14-C), 60.5 (9-C), 68.6 (11-C), 70.7 (3-C), 219.5 (17-C).

9α-hydroxy-EpiA (12): (19 mg, 9%); mp 191–192 °C (from acetone) (lit.,[31] 192–193 °C); Found: C, 74.40; H, 9.86. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%. IR ν_{max}/cm^{-1} 3344, 1740. ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H: 0.85 (3H, s, 18-Me); 0.95 (3H, s, 19-Me); 3.53-3.64 (1H, m, 3α-H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ_C: 12.7 (18-C), 14.2 (19-C), 24.5 (7-C), 25.6 (15-C), 26.9 (11-C), 27.2 (6-C), 28.1 (12-C), 29.8 (1-C), 31.1 (2-C), 35.8 (16-C), 36.3 (5-C), 37.2 (8-C), 38.0 (4-C), 40.3 (10-C), 44.2 (14-C), 47.6 (13-C), 70.7 (3-C), 75.7 (9-C), 220.8 (17-C).

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