microbial biotechnology

The catalytic activity of mycelial fungi towards 7-oxo-DHEA – an endogenous derivative of steroidal hormone dehydroepiandrosterone

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Summary

Seventeen species of fungi belonging to thirteen genera were screened for the ability to carry out the transformation of 7-oxo-DHEA (7-oxo-dehydroepiandrosterone). Some strains expressed new patterns of catalytic activity towards the substrate, namely 16β-hydroxylation (*Laetiporus sulphureus* AM498), Baeyer–Villiger oxidation of ketone in D-ring to lactone (*Fusicoccum amygdali* AM258) and esterification of the 3β-hydroxy group (*Spicaria divaricata* AM423). The majority of examined strains were able to reduce the 17-oxo group of the substrate to form 3β,17β-dihydroxy-androst-5-en-7-one. The highest activity was reached with *Armillaria mellea* AM296 and *Ascosphaera apis* AM496 for which complete conversion of the starting material was achieved, and the resulting 17β-alcohol was the sole reaction product. Two strains of tested fungi were also capable of stereospecific reduction of the conjugated 7-keto group leading to 7β-hydroxy-DHEA (*Inonotus radiatus* AM70) or a mixture of 3β,7α,17β-trihydroxy-androst-5-ene and 3β,7β,17β-trihydroxy-androst-5-ene (*Piptoporus betulinus* AM39). The structures of new metabolites were confirmed by MS and NMR analysis. They were also examined for their cholinesterase inhibitory activity in an enzymatic-based assay *in vitro* test.

Introduction

Microbial transformations constitute an important methodology in organic chemistry for the preparation of many natural products and their derivatives (Hegazy *et al.*, 2015). The multi-enzymatic systems of a variety of organisms, such as bacteria or fungi with usually broad specificities towards modified substrates, are able to catalyse regio- and stereoselective reactions in virtually every site of a molecule. These reactions proceed even at non-activated C-H bonds which are difficult to reach chemically. In this way, biotransformations can provide novel compounds or better yields of known compounds of natural origin enabling their biological studies. They are often the source of derivatives with enhanced biological activity and/or with improved pharmacodynamic profile relative to the parent molecules (Ibrahim *et al.*, 2020). Moreover, enzymatic-catalysed reactions in association with conventional organic synthesis can produce novel valuable molecules for the development of novel pharmaceuticals (Abdelraheem *et al.*, 2019). On the other hand, catalytic systems of fungi or bacteria can also imitate the mammalian metabolism. Many microbial metabolites formed from xenobiotics are similar to those identified in mammals, mainly due to similarities in their cytochrome P450 systems. For that reasons, microbial-mediated transformations can be used for *in vitro* drug metabolic studies (Osorio-Lozada *et al.*, 2008; Patil *et al.*, 2014; Fan *et al.*, 2017; Ma *et al.*, 2019).

One of the best examples of the successful applications of biotransformation is the steroid drug industry (Fernández-Cabezón *et al.*, 2018). However, finding the appropriate microorganism to perform the desired new biotransformation reactions is still a significant challenge. Therefore, traditional microbial strain screening remains the most useful practice (Nassiri-Koopaei and Faramarzi, 2015). Thus, biotransformations have become an effective tool for the synthesis of libraries of compounds with potential biological activity.

7-Oxo-dehydroepiandrosterone (7-oxo-DHEA) (**1**) is an endogenous metabolite of DHEA – one of the most abundant steroids circulating in the human body, and which concentrations progressively decrease with age. It is produced from DHEA by 11β-hydroxysteroid dehydrogenase type I (11β-HSD1) *via* oxidation of other DHEA

metabolites – 7α-hydroxy- (mainly) and 7β-hydroxy-DHEA (El Kihel, 2012). For nearly four decades since its identification in human urine, 7-oxo-DHEA has not been associated with any physiological activity (Sosvorova *et al.*, 2015). Nowadays, there are substantial evidence that some of the biological functions originally attributed to DHEA are associated with the activity of its metabolites. So, 7-oxo-DHEA (**1**) is an inducer and regulator of thermogenic enzymes with much higher activity than DHEA (El Kihel, 2012). It was even recommended as a drug preventing Raynaud's syndrome attacks (Kazihnitková *et al.*, 2007). 7-Oxo-DHEA may serve as natural antiglucocorticoid (Muller *et al.*, 2006), and it may be involved in the development of an effective immune response (Arsenou *et al.*, 2003; Vecchione *et al.*, 2020). It has been suggested that 7-oxo-DHEA as an antagonist of γ-aminobutyric acid subtype A receptor (GABA$_A$) exerts a beneficial effect for memory retention in mice. Its antagonizing activity towards GABA$_A$ partially eliminated the cholinergic dysfunction in induced amnesia in young mice (Shi *et al.*, 2000). This compound also appears to have benefits to improve symptoms of depression, anxiety and stress disorders (Sageman *et al.*, 2012). Unlike DHEA, 7-keto-DHEA has no androgenic activity and it cannot be converted to androgens and oestrogens known to enhance the risk of hormone-dependent diseases. For this reason and because of a causal link between declining DHEA levels and age-related loss of cognitive function, 7-oxo-DHEA was suggested as a safe alternative to DHEA supplementation. In some countries, it is commercially available and used also as nutritional supplement in sport (Kazihnitková *et al.*, 2007).

As reported in the available scientific literature, 7-oxo-DHEA (**1**) metabolism in humans is mainly concerned with the reduction of C-17 ketone by 17β-hydroxysteroid dehydrogenase (17β-HSD) generating 3β,17β-dihydroxy-androst-5-en-7-one (**2**) or/and reduction of the ketone at C-7 position by 11β-HSD1 leading to the formation of epimeric 7β- (mainly) and 7α- alcohols (7α- and 7β-hydroxy-DHEA (**3**)), and their reduction to 3β,7α,17β- and 3β,7β,17β-triols (3β,7α,17β-trihydroxy-androst-5-ene (**4**) and 3β,7β,17β-trihydroxy-androst-5-ene (**5**)) (Nashev *et al.*, 2007). These metabolites are multifunctional compounds implicated in a broad range of biological processes, mainly attributed to immune system modulation, anti-inflammatory, antiglucocorticoid and neuroprotective actions (El Kihel, 2012; Stárka, 2017; Stárka *et al.*, 2018). Changes in the ratio of 7α/7β-hydroxy-DHEA and both isomeric triols were detected in patients with various disorders including vascular and Alzheimer dementia (Stárka, 2017). The derivatives of 7-oxo- or 7-hydroxy-DHEA with an additional 16α-hydroxy group were isolated from urine of the patient with adrenal carcinoma

(Pouzar *et al.*, 2005). More recently, numerous new reduced and hydroxylated metabolites of 7-oxo-DHEA (**1**) were detected in human urine, but the structures of these compounds need to be confirmed, due to, among other things, the lack of adequate reference materials (Martinez-Brito *et al.*, 2019; Piper *et al.*, 2020).

In contrast to DHEA, 7-oxo-DHEA (**1**) has not been the subject of systematic research on the possibility of its structural modifications using microorganisms. So far, to the best of our knowledge, only *Syncephalastrum racemosum* AM105 was used for this type of transformation. As a result, 1β-, 9α- and 12β-hydroxy derivatives of 7-oxo-DHEA were obtained (Świzdor *et al.*, 2016). The synthesis of 11α-hydroxy-7-oxo-DHEA was reported in *Beauveria bassiana* and *Beauveria caledonica* cultures, but this metabolite was directly derived from DHEA transformation (Kozłowska *et al.*, 2018).

All things were considered, and it was justified to conduct studies on the possibilities of formation of novel 7-oxo-DHEA metabolites with potential biological activity as a result of microbial transformations. For many years, our team has conducted research on microbial functionalization of steroids and other important compounds of natural origin. In the presented manuscript, we describe the structural elucidation of these novel 7-oxo-DHEA metabolites and evaluation of their inhibitory activity against AChE (acetylcholinesterase) and BChE (butyrylcholinesterase), in the context of studying structure of compounds–biological activity relationship. The main function of AChE and BChE inhibitors is to boost the cholinergic systems of an organism by increasing the endogenous level of acetylcholine. This system has been associated with a number of cognitive functions, including memory and emotional processing. To date, a number of *in vitro* studies on inhibitory effects of various steroidal molecules have been carried out, and some of them have been identified as weak or strong inhibitors of these cholinesterases (Richmond *et al.*, 2013; Zafar *et al.*, 2013; Yusop *et al.*, 2020).

Results and discussion

The incubation of 7-oxo-DHEA (**1**) with seventeen strains belonging to thirteen genera of fungi resulted in seven products of transformation (Table 1). The structure of metabolites **2-5** (Fig. 1) was confirmed by comparison of their R_t data from GC and their R_f data from TLC with those of authentic standards. The products **6-8** (Fig. 2) were isolated and purified using column chromatography and finally identified by NMR spectroscopy. The obtained results allowed to establish that the potential of tested microorganisms towards 7-oxo-DHEA (**1**) included four basic metabolic steroidal pathways: reduction, hydroxylation, Baeyer–Villiger oxidation and esterification.

Table 1. The catalytic activity of fungi towards 7-oxo-DHEA (**1**) and taxonomy of the used strains.

Division	Class	Order	Family	Genus	Species	Time (days)	Conv. (%)	Metabolites[a] (%)
Basidiomycota	Agaricomycetes	Agaricales	Physalacriaceae	Armillaria	*A. mellea* AM296	2	100	**2** (100)
		Gloeophyllales	Gloeophyllaceae	Gloeophyllum	*G. odoratum* AM58	3	98	**2** (98)
		Hymenochaetales	Hymenochaetaceae	Inonotus	*I. radiatus* AM70	3	94	**2** (67); **3** (22)
		Polyporales	Fomitopsidaceae	Piptoporus	*P. betulinus* AM39	1	90	**4** (30); **5** (49)
				Laetiporus	*L. sulphureus* AM498	4	64	**2** (18); **6** (31)
				Poria	*P. placenta* AM36	3	90	**2** (81)
			Polyporaceae	Trametes	*T. versicolor* AM536	3	90	**2** (80)
Ascomycota	Eurotiomycetes	Ascosphaerales	Ascosphaeraceae	Ascosphaera	*A. apis* AM496	3	100	**2** (100)
		Eurotiales	Trichocomaceae	Penicillium	*P. camembertii* AM83	3	43	**2 (**43)
				Spicaria	*S. divaricata* AM423	3	92	**8** (79)
					S. fusispora AM136	6	48	**2** (48)
					S. violacea AM439	3	57	**2** (39); **7** (7)
	Sordariomycetes	Hypocreales	Cordycipitaceae	Beauveria	*B. bassiana* KCH1065	2	90	**2** (90)
			Nectriaceae	Fusarium	*F. culmorum* AM282	6	33	**2** (25)
					F. oxysporum AM21	6	33	**2** (33)
			Ophiocordycipitaceae	Penicillium	*P. lilacinum* AM111	4	10	**2** (10)
	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Fusicoccum	*F. amygdali* AM258	7	100	**7** (96)

[a]Conversion (Conv) and contents of metabolites in crude extracts determined by GC analysis. Data are the arithmetic means of three independent experiments. Relative differences between the border values have not exceeded 10%. Metabolites are numbered as follows: 3β,17β-dihydroxy-androst-5-en-7-one **2**; 3β,7β-dihydroxy-androst-5-en-7,17-dione **3**; 3β,7α,17β-trihydroxy-androst-5-ene **4**; 3β,7β,17β-trihydroxy-androst-5-ene **5**; 3β,16β-dihydroxy-androst-5-en-7,17-dione **6**; 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **7**; 3β-acetoxy-androst-5-en-7,17-dione **8**.

1. R=O

2. R=βOH, αH

3

4. R₁=H; R₂=OH

5. R₁=OH; R₂=H
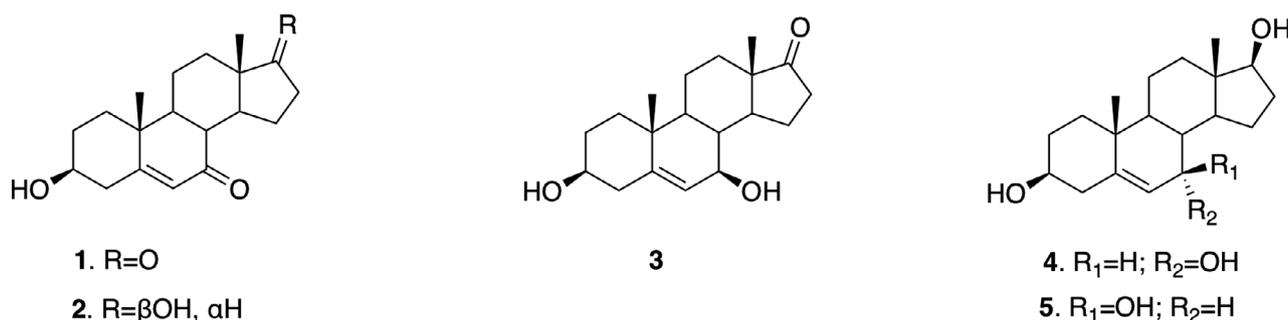
Fig. 1. The products formed in the reductive metabolic pathway of 7-oxo-DHEA (**1**) in tested microorganisms. The obtained compounds are numbered as follows: 3β,17β-dihydroxy-androst-5-en-7-one **2**; 3β,7β-dihydroxy-androst-5-en-7,17-dione **3**; 3β,7α,17β-trihydroxy-androst-5-ene **4**; 3β,7β,17β-trihydroxy-androst-5-ene **5**

Reduced metabolites of 7-oxo-DHEA

The results of conducted experiments showed a widespread distribution of enzymes with 17β-HSD activity in mycelial fungi – the ability to perform reduction of 17-keto group of 7-oxo-DHEA (**1**) to 17β-alcohol was observed for almost all tested genera (Table 1). This reaction is one of the more commonly recognized in the transformation of steroids by microorganisms. It often accompanies different steroidal conversions (most often hydroxylations) and plays a role in the regulation of reducing equivalent pool and in detoxification of exogenic C_{19} steroids (Donova et al., 2005). Fungal 17β-HSD activity was first reported in *Cochliobolus lunatus* and *Cylindrocarpon radicicola*. 17β-HSD from *C. lunatus* (17β-HSDcl) was shown to be constitutive as opposed to 17β-HSD from *C. radicicola* (17β-HSDcr) that was inducible. It has been proven that

both these enzymes can act as a reductase as well as an oxidase, although 17β-HSDcr preferred oxidation with 17β-hydroxysteroids as the substrates, and 17β-HSDcl preferentially catalysed the reduction of C-17 keto group (Kristan and Lanišnik Rižner, 2012). Constitutive 17β-HSD activity has also been determined in *Rhizopus nigricans*, *Fusarium lini*, *Pleospora herbarum*, *Trichoderma viride*, *Mucor spinosus* and *Pleutotus ostreatus* (Lanišnik Rižner and Žakelj-Mavrič, 2000). However, only two fungal enzymes, 17β-HSDcr and 17β-HSDcl, have been purified, but only dehydrogenase from *Cochliobolus lunatus* has been cloned and overexpressed in *Escherichia coli* (Donova et al., 2005; Kristan and Lanišnik Rižner, 2012) and *Mycobacterium smegmatis* (Fernández-Cabezón et al., 2017).

A minor 17β-reduction pathway of 7-oxo-DHEA (**1**) to 3β,17β-dihydroxy-androst-5-en-7-one (**2**) was observed
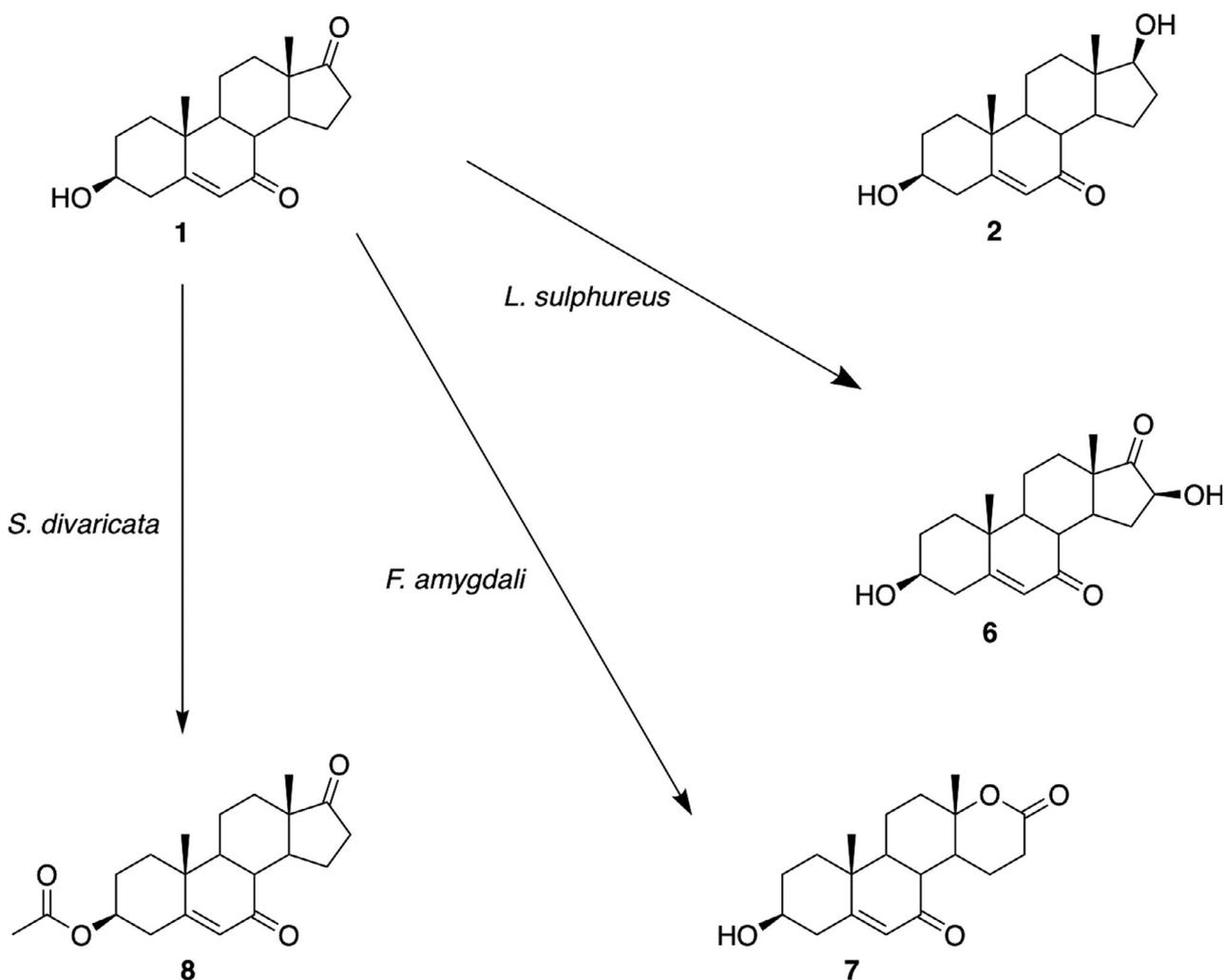
Fig. 2. Metabolites of 7-oxo-DHEA (**1**) isolated following transformation by *L. sulphureus* AM498, *F. amygdali* AM258 and *S. divaricata* AM423. The obtained compounds are numbered as follows: 3β,16β-dihydroxy-androst-5-en-7,17-dione **6**; 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **7**; 3β-acetoxy-androst-5-en-7,17-dione **8**

in our previous study with *Syncephalastrum racemosum* AM105 (Świzdor *et al.*, 2016). In the present work, among tested Basidiomycota, the maximum 17β-HSD activity towards 7-oxo-DHEA (**1**) was found in *Armillaria mellea* AM296 for which complete conversion of **1** to **2** was observed (Table 1). Similar activity among Ascomycota was demonstrated in *Ascosphaera apis* AM496. The results of preliminary studies on the character of both enzymes suggest that 17β-HSD(s) from *A. mellea* AM296 has a constitutive nature. After inhibition of the cultures of this fungus by cycloheximide (CHI) (inhibitor of *de novo* protein synthesis), only a slight reduction (from 17% to 15% after 12 h of reaction) in the effectiveness of the transformation compared to standard incubation was recorded (Fig. 3A). This trend continued until the end of the transformation process. Simultaneously, in a parallel experiment, in which 7-oxo-DHEA (**1**) was

added to the *A. mellea* culture induced by this substrate 6 h earlier (a culture after the same period of incubation with **1** exhibited 17β-HSD activity), only slight enhancement of transformation (from 17% to 20% after 12 h reaction) was detected. The reduction of 17-keto group of **1** was significantly inhibited in the presence of CHI in the culture of *A. apis* AM496 (Fig. 3B). The reaction mixture after three days of transformation contained 11% of **2**, compared to total conversion substrate in the standard experiment. This result suggested that the responsible enzyme(s) was present at a low constitutive level in the fungus, but it can be induced by steroid molecule through protein synthesis. So, the reaction mixture after 24 h in the standard incubation of **1** contained 2% of 3β,17β-dihydroxy-androst-5-en-7-one (**2**), and after further 12 h, its contents grew to 20% and successively to 44% with completed conversion after 72 h. In the

substrate-induced culture, 7-oxo-DHEA (**1**) was reduced with a faster rate; after 48 h incubation, there was 75% of conversion, while in the standard transformations it was below 50%. The obtained results demonstrated that 7-oxo-DHEA induces 17β-HSD activity in *A. apis* AM496.

Two strains of tested fungi were also able to reduce the conjugated 7-keto group of the substrate. These were *Inonotus radiatus* AM70 and *Piptoporus betulinus* AM39 (Table 1). In the culture of *I. radiatus,* we observed stereospecific reduction of this group leading to 7β-hydroxy-DHEA (**3**) (Fig. 2). Reduction of 7-keto group by *P. betulinus* was non-stereospecific, and as a result, both 7-hydroxisomers – 3β,7α,17β-trihydroxy-androst-5-ene (**4**) and 3β,7β,17β-trihydroxy-androst-5-ene (**5**) (in a 3:5 ratio), were formed (Fig. 1, Table 1). The reducing metabolic pathway of both carbonyl groups of 7-oxo-DHEA observed in the case of these fungi reveals similarities with the metabolism of this steroid in mammals – it relates to the nature of compounds which were formed and the clear preference in the stereochemistry of reduction of 7-oxo group to 7β-alcohol (Nashev *et al.*, 2007). Therefore, this fungi can be considered as potential microbial models of mammalian metabolism in the future.

Oxygenated metabolites of 7-oxo-DHEA

Bioconversion of 7-oxo-DHEA (**1**) with *Laetiporus sulphureus* AM498 generated two main products (Table 1, Fig. 2). Purification on silica gel yielded a known metabolite **2** and a new compound **6**. Mass spectrometry (MS) data (Fig. S1) of this metabolite revealed an [M]$^+$ at

m/z 318.5, which is 16 amu (atomic mass units) higher than the parent compound **1**, and suggest the presence of an additional hydroxyl group. The ^{13}C NMR spectrum of **6** was quite similar to that of **1** with the exception of signals of the D-ring carbons. A new oxygen-bearing methine carbon signal at δ_C 75.4 ppm and C*H*(OH) signal in the ^1H NMR spectrum of this metabolite at δ_H 3.94 ppm confirmed secondary hydroxylation of the substrate. The position and stereochemistry of the newly introduced hydroxyl group were assigned as 16β by multiplicity (*t*, *J* = 8.5 Hz) of the C*H*(OH) signal and the downfield shift signal of C-15 (Δ10.2 ppm). These values were similar to those characteristic of other 16β-hydroxy-17-oxo steroids (Świzdor *et al.*, 2017). Correlation between H-16 signal and downfield H-15α signal (δ_H 3.14-3.18 ppm) and its lack between H-16 and C-18 methyl group protons in NOESY spectrum of **6** were an important confirmation of 16β-hydroxylation (Fig. 4). The spectroscopic data (Fig. S1-S6) led to the identification of this metabolite as 3β,16β-dihydroxy-androst-5-en-7,17-dione (**6**). An interesting connection to mammalian metabolism is provided by recent studies suggesting the presence of multihydroxy compounds with 16β-alcohol group in human urinary metabolic profile of 7-oxo-DHEA after oral administration of this steroid (Martinez-Brito *et al.*, 2019).

The biotransformation of 7-oxo-DHEA (**1**) by *Fusicoccum amygdali* AM258 yielded only one metabolite (Fig. 2). Preliminary MS analysis (Fig. S7) indicated that the product had an M + 16 in comparison with the molecular weight of substrate. There were no major changes observed in the ^1H NMR spectrum of this compound except downfield shifts of the methyl groups, in
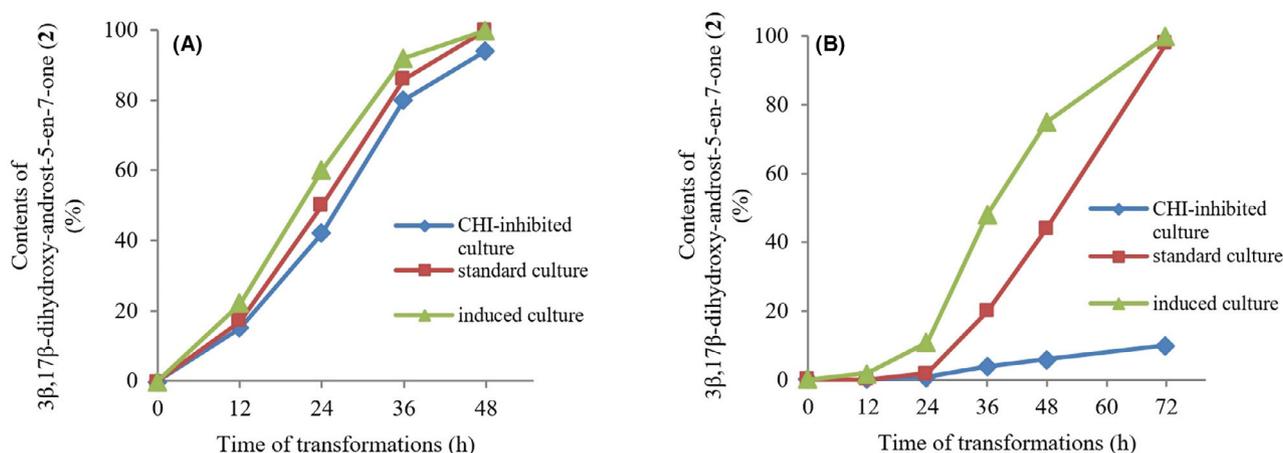
Fig. 3. Comparison of percentage of 3β,17β-dihydroxy-androst-5-en-7-one (**2**) in the mixtures after transformation of 7-oxo-DHEA (**1**) by (A) *A. mellea* AM296, (B) *A. apis* AM496. Reactions were carried out as described for the screening procedure. CHI was added to the growth culture of the fungi as DMF solution, in final concentration of 0.1 mg mL⁻¹ of medium, simultaneously with the substrate. In the induced cultures, **1** was added in two doses: one as an inducer (1 mg) and then the remaining substrate after 6 h of transformation in *A. mellea* culture, and after 12 h of transformation by *A. apis*
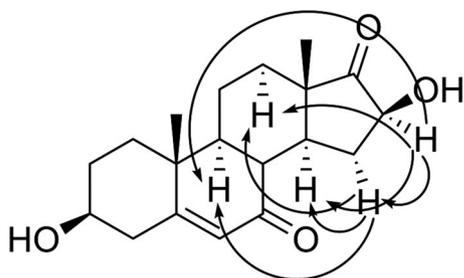
Fig. 4. Key NOESY correlations for metabolite **6**

particular C-18 (Δ0.41 ppm), as compared to **1**. However, there were significant differences in the ^{13}C NMR spectrum with the disappearance of the carbonyl group signal at δ_C 220.4 ppm, the appearance of a lactone carbonyl signal at δ_C 171.7 ppm, and downfield shifts of the C-13 (Δ 34.5 ppm) and the C-18 (Δ7.1 ppm) signals. All these data confirm insertion of an oxygen atom into the ring-D of the molecule. Thus, metabolite **7** was identified as 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione (Fig. S7-S10). This compound was previously obtained with very low yield (below 10%) as one of the three metabolites in biotransformation of DHEA by *Beauveria bassiana* KCh BBT (Kozłowska *et al.*, 2018). The spectroscopic data of **7** were in agreement with this earlier study. Steroidal lactones are important compounds due to their anticancer and antiandrogenic activity (Świzdor, 2013). As aromatase inhibitors they were used to study the role of oestrogen in age-related changes in humans (Séralini and Moslemi, 2001). DHEA lactone derivatives were also evaluated *in vivo* and *in vitro* as potential therapeutic antiandrogens. Some of them exhibited similar or higher inhibiting activity towards steroidal 5α-reductase and low affinity to the androgen receptor as compared to finasteride (Garrido *et al.*, 2011). The ability to oxidize ketosteroids to lactones was detected in fungi of different taxonomic classes, especially *Apergillus*, *Fusarium* and *Penicillium* (Świzdor *et al.*, 2012; Świzdor *et al.*, 2018; Panek *et al.*, 2020a). The formation of hydroxylactones from C_{19} steroids was demonstrated for *Beauveria bassiana* (Świzdor *et al.*, 2011; Świzdor *et al.*, 2014) and *Isaria fumosorosea* (previously classified as *Spicaria fumosorosea*) (Lobastova *et al.*, 2015; Kozłowska *et al.*, 2017). To the best authors' knowledge, this is the first report on Baeyer–Villiger oxidation activity in *Fusiccocum amygdali*. This activity is induced by the presence of the substrate (Fig. 5A). After two days of transformation, the content of lactone **7** in the reaction mixture was 10%, reaching 83% after further two days. Nearly complete 7-oxo-DHEA conversion was achieved after three days of reaction, when the microbial culture was induced by the substrate. Contrary to these results,

after inhibition of *F. amygdali* by CHI, only low enzyme activity (4% of lactone **7**) after four days of transformation was detectable. Interestingly, the improvement in the transformation efficiency (96% of lactone **7** yield) was achieved by using a higher substrate concentration (1 g l^{-1}) with a simultaneous extension of the transformation time to 7 days (Panek *et al.*, 2020b). Thus, the possibility of the effective microbial oxidation using *F. amygdali* AM258 enabled us to evaluate this strain as promising for further practical use in the preparation of potential bioactive steroidal lactones.

Other metabolites

Fermentation of 7-oxo-DHEA (**1**) with *Spicaria divaricata* AM423 generated one major product **8** (Fig. 2). The structure of this metabolite was readily determined by a new methyl signal in the ^{1}H NMR spectrum at δ_H 2.05 ppm which is consistent with the presence of an acetate group. A downfield shift in the 3α-H multiplet from δ_H 3.65-3.73 ppm to δ_H 4.69–4.74 ppm indicated that the acetylation occurred on the 3β-alcohol. New signals in the ^{13}C NMR spectrum of **8** at δ_C 170.3 ppm (C-20) and δ_C 21.2 ppm (C-21) further supported the presence of the acetate. The spectroscopic data (Fig. S11-S14) of this compound are consistent with 3β-acetoxy-androst-5-en-7,17-dione (Coutts *et al.*, 2005). In the available scientific literature, capacity to acetylation (or reversible acetylation) of steroidal secondary alcohols was demonstrated only for a few microorganisms. These were the species of yeast: *Saccharomyces fragilis*, *S. lactis*, *Candida pseudotropicalis*, *Torulopsis sphaerica* (Čapek *et al.*, 1964) and fungi: *Penicillum* sp., *Spicaria* sp. (Kraychy et al., 1971), *Myceliophthora thermophila* (Hunter *et al.*, 2009) and *Aspergillus nidulans* (Savinova *et al.*, 2019). Although some strains belonging to the *Spicaria* species were able to acetylate 3β- and 17β-hydroxy groups of steroids, two other strains tested by our team, *S. fusispora* AM136 and *S. violacea* AM439, catalysed the reduction of 7-oxo-DHEA (**1**) to 3β,17β-dihydroxy-androst-5-en-7-one (**2**) and did not exhibit acylating activity against the substrate. As shown by the obtained results (Fig. 5B), the enzyme from *S. divaricata* AM423 is induced by the presence of a steroid substrate. The 3-acetates of steroids are useful products both because of their valuable pharmacological properties and the fact that they serve as intermediates in synthesis of pharmacologically significant compounds.

Evaluation of the acetylcholinesterase inhibitory activity

Evaluation of inhibitory activity of new metabolites of 7-oxo-DHEA (compounds **6-8**) was carried out by standard *in vitro* AChE and BuChE inhibition assays (Ellman's
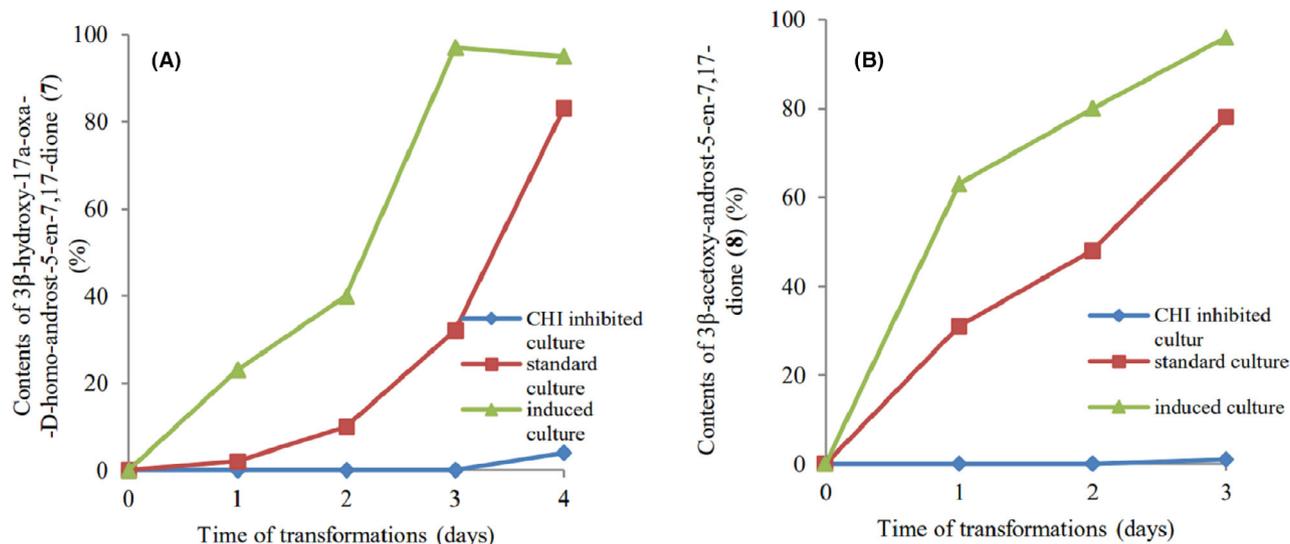
Fig. 5. Comparison of percentage of (A) 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione (**7**), (B) 3β-acetoxy-androst-5-en-7,17-dione in the mixtures after transformation of 7-oxo-DHEA (**1**) by (A) *F. amygdali* AM258, (B) *S. divaricata* AM423. Reactions were carried out as described in the Legend of Fig. 3

assay method). The percentage inhibition was calculated and compared to that of **1**. Both the substrate and its metabolites did not exhibit any significant inhibitory activity against any of the enzymes. 7-Oxo-DHEA (**1**) at a maximum concentration of 500 μM inhibited AChE at $11.12 \pm 0.15\%$ and BChE at $13.24 \pm 0.11\%$. Results at lower concentrations revealed a mild linear decrease in inhibition. The introduction of the acetyl group into the substrate (metabolite **8**) or oxidation of the ketone in the D-ring in the Baeyer–Villiger reaction with the formation of δ D-lactone (metabolite **7**) resulted only in a 27% activity increase against AChE and a 23% increase against BChE at the same concentration of both compounds. The metabolite **6** with an additional 16β-hydroxyl group exhibited, regardless of its concentration, a lower inhibition effect for both enzymes than the substrate (8% and 11%, respectively).

Conclusions

In conclusion, seventeen species of fungi were screened for the ability to carry out the transformation of 7-oxo-DHEA. The potential of microorganisms included three basic metabolic pathways of steroid compounds: reduction, hydroxylation and Baeyer–Villiger oxidation. Two metabolites, not previously reported (3β,16β-dihydroxy-androst-5-en-7,17-dione (**6**)) or obtained previously with very low yield (3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione (**7**)), were described. Because a detailed description of the pharmacology of 7-oxo-DHEA and DHEA itself depends on an understanding of the pharmacology of their metabolome, obtaining such derivatives in amounts that allow further investigations is of continuous interest to researchers. In future, these compounds can be used as standards in a broad study of steroid metabolism disorders or be subjected to other tests for their biological activity. They can also form the basis for the synthesis of new steroid pharmaceuticals. The acylating activity of *S. divaricata* AM423 disclosed in the described studies will be a potential phenomenon to be tested in the context of its regioselectivity in the esterification of steroid diols and triols.

Experimental procedures

Materials

7-Oxo-DHEA (**1**) was obtained by the chemical conversion of DHEA according to the procedure described earlier (Świzdor *et al.*, 2016). Chemical standards: 3β,17β-dihydroxy-androst-5-en-7-one (**2**), 7β-hydroxy-DHEA (**3**), 3β,7α,17β-trihydroxy-androst-5-ene (**4**) and 3β,7β,17β-trihydroxy-androst-5-ene (**5**) were prepared in our previous work (Kołek *et al.*, 2011). AChE (EC 3.1.1.7) from electric eel and BChE (EC 3.1.1.8) from horse serum, acetylthiocholine iodide, butyrylthiocholine iodide, 5,5-dithiobis-[2-nitrobenzoic acid] (DTNB) and eserine were purchased from Sigma-Aldrich Co. Seventeen strains of fungi (Table 1) used for screening experiments were obtained from the collection of the Department of Pharmaceutical Biology and Botany of the Wrocław Medical University, Poland. Fungi were maintained on Sabouraud 4% dextrose agar slopes and freshly subcultured before use in the transformation experiments.

Culture conditions and biotransformations

The cultures in the screening studies were shaken at 180 rpm in 100 ml Erlenmeyer flasks with 30 ml of the medium consisting of glucose (30 g l^{-1}) and aminobak (10 g l^{-1}), and in 300 ml Erlenmeyer flasks with 100 ml of this medium in the analytical scale transformations. The cultivation time ranged from 3 to 7 days depending on the growth rate of the strain. Fungi were grown at 25 °C. In the screening test, a solution of 7-oxo-DHEA (**1**) (10 mg in 0.2 ml of acetone) was added to the cultures. The progress of conversion was monitored by TLC. After biotransformations, the metabolites and remaining substrate were extracted with methylene chloride. The organic solutions were dried with anhydrous magnesium sulphate, filtered, concentrated *in vacuo* and analysed by GC. In the analytical scale biotransformations using selected strains, 0.2 g of **1** dissolved in 2 ml of acetone was equally distributed among flasks with fungal cultures. The reactions were carried out under the same conditions as in screening tests and continued until the substrate was metabolized. The progress of conversion was monitored by TLC. When the transformation completed, mycelia and broth were extracted three times with methylene chloride. The organic extracts were combined, dried over anhydrous magnesium sulphate and filtered, and the solvent was evaporated *in vacuo*. These crude extracts were analysed by TLC and GC and then chromatographed on a column of silica gel.

Products analysis

TLC of crude extracts was carried out with Merck Kieselgel 60 F$_{254}$ plates, visualized by spraying them with a mixture of methanol in concentrated sulphuric acid (1:1 v: v) and heating to 120°C until the colours developed. Metabolites obtained in the analytical transformations were separated by column chromatography on silica gel 60 (230–400 mesh) eluting with the same eluent as for TLC. 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 DB-5MS column (crosslinked phenyl methyl siloxane, 30 m × 0.32 mm × 0.25 μm). The applied temperature program was 220°C 1 min^{-1}, gradient 4°C min^{-1} to 280°C and then 30°C to 300°C 3 min^{-1}; injector and detector temperature were 300 °C (for *L. sulphureus* temperature program was 215°C 1 min^{-1}, gradient 4°C min^{-1} to 280°C and then 30°C to 300°C 3 min^{-1}). MS analyses were performed on Varian CP-3800/Saturn 2000 apparatus with a Zebron ZB-5 MSI (30 m × 0.25 mm × 0.25 μm) column. The following temperature program was used: 220°C 1 min^{-1}, gradient 5°C min^{-1} to 300°C 5 min^{-1}. The NMR spectra were recorded on a Bruker Avance^{TM} 600 MHz spectrometer and measured in CDCl$_3$ or CD$_3$OD. Characteristic shift values in the ^{1}H NMR and ^{13}C NMR spectra in comparison with the starting compounds were used to determine structures of metabolites, in combination with DEPT analysis to identify the nature of the carbon atoms. The stereochemistry of the hydroxyl group was deduced on the basis of NOESY experiment. Melting points were determined on a Boetius apparatus and are uncorrected. GC spectra and TLC of the extracts obtained after transformations, as well as the transformation time course diagrams, are included in the Supporting information (Fig. S15-S26).

Biotransformation with Ascosphaera apis AM496

7-Oxo-DHEA (30 mg) dissolved in 0.6 ml of acetone was evenly distributed among three flasks with 7 days old fungal cultures and incubated for further 3 days. This procedure yielded an extract, which was analysed by GC and TLC. Elution with 50% acetone in hexane afforded the known 3β,17β-dihydroxy-androst-5-en-7-one (**2**) (100% determined by GC analysis; R_t = 12.0 min) (Kołek *et al.*, 2011).

Biotransformation with Inonotus radiatus AM70

7-Oxo-DHEA (30 mg) dissolved in 0.6 ml of acetone was evenly distributed among three flasks with 5 days old fungal cultures and incubated for further 3 days. The standard procedures yielded an extract, which was analysed by GC and TLC. Elution with mixture of acetone: ethyl acetate:methylene chloride (0.5:1.5:1 v:v:v) yielded untransformed 7-oxo-DHEA (**1**) (6%), **2** (67%) and known 7β-hydroxy-DHEA (**3**) (22%, R_t = 10.4 min) according to GC analysis (Kołek *et al.*, 2011).

Biotransformation with Piptoporus betulinus AM39

The standard one day of incubation of 7-oxo-DHEA (30 mg in 0.6 ml of acetone) with 5 days old fungal cultures resulted in two metabolites. Elution with ethyl acetate:methylene chloride:methanol (3:2:0.2 v:v:v) gave three compounds: untransformed 7-oxo-DHEA (**1**) (10%), and two known products: 3β,7α,17β-trihydroxy-androst-5-ene (**4**) (30% R_t = 8.9 min), and 3β,7β,17β-trihydroxy-androst-5-ene (**5**) (49%, R_t = 9.1 min) according to GC analysis (Kołek *et al.*, 2011).

Biotransformation with Laetiporus sulphureus AM498

Incubation of substrate **1** (0.2 g in 2 ml of acetone) with 4 days old fungal cultures for 7 days resulted in two metabolites. Elution with acetone:ethyl acetate:methylene chloride (0.5:1.5:1 v:v:v) yielded the fed substrate **1**

(62 mg; 31% mol.), known 3β,17β-dihydroxy-androst-5-en-7-one (**2**) (30 mg; 15% mol.), and a new product characterized as 3β,16β-dihydroxy-androst-5-en-7,17-dione (**6**) (57 mg; 27% mol., R_t = 19.4 min).

3β,16β-Dihydroxy-androst-5-en-7,17-dione (**6**): white amorphous solid; ^{1}H NMR (CD$_3$OD, 600 MHz) δ: 0.96 (3H, s, H-18), 1.27 (3H, s, H-19), 3.14-3.18 (1H, m, H-15α), 3.54–3.60 (1H, m, H-3α), 3.94 (1H, t, J = 8.5 Hz, H-16α), 5.72 (1H, d, J = 1.7 Hz, H-6). ^{13}C NMR (CD$_3$OD, 151 MHz) δ: 14.9 (CH$_3$, C-18), 17.7 (CH$_3$, C-19), 21.4 (CH$_2$, C-11), 31.9 (CH$_2$, C-2), 32.1 (CH$_2$, C-12), 34.5 (CH$_2$, C-15), 37.4 (CH$_2$, C-1), 39.9 (C, C-10), 41.1 (CH, C-14), 42.8 (CH$_2$, C-4), 44.7 (CH, C-8), 48.2 (C, C-13), 51.6 (CH, C-9), 71.1 (CH, C-3), 75.4(CH, C-16), 126.1 (CH, C-6), 169.6 (C, C-5), 203.3 (C, C-7), 220.7 (C, C-17). EI-MS m/z 318.5 [M]$^+$(27), 290.4 (100), 192.5 (48), 91.5 (66), 77.4 (33).

Biotransformation with Fusicoccum amygdali AM258

7-Oxo-DHEA (0.2 g) dissolved in 2 ml of acetone was evenly distributed among two flasks with 4 days old fungal cultures and incubated for further 7 days. The standard procedure gave extracts, which were purified on silica gel. Elution with acetone:ethyl acetate:methylene chloride (0.5:1.5:1 v:v:v) yielded 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione (**7**) as a sole product of reaction (182 mg, 87% mol., R_t = 12.1 min) (Kozłowska *et al.*, 2018).

3β-Hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione (**7**): colourless needles, mp. 226-227 °C. ^{1}H NMR (CDCl$_3$, 600 MHz) δ: 1.19 (3H, s, H-19), 1.31 (3H, s, H-18), 3.63-3.69 (1H, m, H-3α), 5.71 (1H, d, J = 1.6 Hz, H-6). ^{13}C NMR (CDCl$_3$, 151 MHz) δ: 16.8 (CH$_3$, C-19), 20.8 (CH$_3$, C-18), 21.2 (CH$_2$, C-11), 21.6 (CH$_2$, C-15), 28.9 (CH$_2$, C-16), 30.9 (CH$_2$, C-2), 36.1 (CH$_2$, C-1), 37.9 (CH$_2$, C-12), 38.7 (C, C-10), 40.2 (CH, C-14), 41.5 (CH$_2$, C-4), 46.3 (CH, C-8), 48.9 (CH, C-9), 70.3 (CH, C-3), 82.3 (C, C-13), 125.5 (CH, C-6), 165.0 (C, C-5), 171.7 (C, C-17), 199.8 (C, C-7). EI-MS m/z 318.5 [M]$^+$ (100), 301.5 (46), 274.5 (33), 210.5 (26), 192.5 (54), 161.5 (51), 152.4 (82), 134.5 (75), 105.4 (37), 91.5 (95).

Biotransformation with Spicaria divaricata AM423

7-Oxo-DHEA (0.2 g in 2 ml of acetone) was evenly distributed among five flasks with 4 days old fungal cultures and incubated for further three days. The standard procedure yielded crude extract, which was purified on silica gel. Elution with 50% ethyl acetate in methylene chloride gave untransformed 7-oxo-DHEA (**1**) (18 mg, 6% mol.) and 3β-acetoxy-androst-5-en-7,17-dione (**8**) as a main product of reaction (164 mg, 72% mol., R_t = 11.4 min) (Coutts *et al.*, 2005).

3β-Acetoxy-androst-5-en-7,17-dione (**8**): white needles, mp. 187–188°C (186–192°C; Coutts *et al.*, 2005). ^{1}H NMR (CDCl$_3$, 600 MHz) δ: 0.89 (3H, s, H-18), 1.23 (3H, s, H-19), 2.05 (3H, s, OAc), 4.69-4.74 (1H, m, H-3α), 5.75 (1H, d, J = 1.8 Hz, H-6). ^{13}C NMR (CDCl$_3$, 151 MHz) δ: 13.7 (CH$_3$, C-18), 17.3 (CH$_3$, C-19), 20.5 (CH$_2$, C-11), 21.2 (CH$_3$, C-21), 24.1 (CH$_2$, C-15), 27.2 (CH$_2$, C-2), 30.6 (CH$_2$, C-12), 35.6 (CH$_2$, C-16), 35.9 (CH$_2$, C-1), 37.8 (CH$_2$, C-4), 38.4 (C, C-10), 44.3 (CH, C-8), 45.7 (CH, C-14), 47.8 (C, C-13), 49.9 (CH, C-9), 71.9 (CH, C-3), 126.5 (CH, C-6), 164.8 (C, C-5), 170.3 (C, C-20), 200.7 (C, C-7), 220.3 (C, C-17). EI-MS m/z 284.5 (100), 256.7 (55), 241.5 (30), 161.5 (28), 91.5 (35), 43.2(40).

Cholinesterase inhibition assay

Determination of enzyme inhibition was performed according to the spectrophotometric Ellman's method with some modifications (Ellman *et al.*, 1961). The assay was carried out in 96 well-plates in triplicates. All tested compounds were prepared in methanol (concentrations: 5, 0.5, 0.05 mM). The total reaction mixture volume was 200 μl containing 110 μL of phosphate buffer (KH$_2$PO$_4$/Na$_2$HPO$_4$, 50 mM, pH 7.7 with 0.05% Tween 20), 20 μL of the tested compound, 30 μl of 5 mM DTNB, 20 μl of enzyme (AChE 0.033 U/well or BChE 0.02 U/well) and 20 μl of the enzyme substrate (5 mM acetylcholine iodine for AChE, 5 mM butyrylthiocholine iodine for BChE). The control contained 110 μl of phosphate buffer, 20 μl of methanol, 30 μl of 5 mM DTNB, 20 μl of enzyme and 20 μl of the enzyme substrate. Eserine was used as the reference inhibitor for both enzymes. The reaction was prepared in steps. First, the tested compound and DTNB were added to the buffer, and the plate was mixed by 10 min at 25°C. After this, the enzyme was added and the plate was mixed again for 10 min at 25°C. To initiate the reaction, the substrate for the enzyme was added and the plate was mixed and incubated for further 10 min at 25°C. The amount of product formed was measured by using a micro plate reader Epoch (BioTek, Winooski, VT, USA) at 405 nm.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. NMR (^{1}H, ^{13}C, DEPT, NOESY) and MS spectra of the isolated biotransformation products. GC spectra and TLC of extracts obtained after transformations. Time course diagrams for the transformations. **Fig. S1**. MS spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S2**. 1H NMR spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S3**. 13C NMR spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S4**. DEPT spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S5**. HSQC spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S6**. NOESY spectrum of 3β,16β-dihydroxy-androst-5-en-7,17-dione **(6)**. **Fig. S7**. MS spectrum of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **(7)**. **Fig. S8**. 1H NMR spectrum of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **(7)**. **Fig. S9**. 13C NMR spectrum of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **(7)**. **Fig. S10**. DEPT spectrum of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-7,17-dione **(7)**. **Fig. S11**. 1H NMR spectrum of 3β-acetoxy-androst-5-en-7,17-dione **(8)**. **Fig. S12**. 13C NMR spectrum of 3β-acetoxy-androst-5-en-7,17-dione **(8)**. **Fig. S13**. DEPT spectrum of 3β-acetoxy-androst-5-en-7,17-dione **(8)**. **Fig. S14**. MS spectrum of 3β-acetoxy-androst-5-

en-7,17-dione (**8**). **Fig. S15**. (A) GC spectrum of extract after transformation of **1** by *A. apis* AM496 (B) TLC. **Fig. S16**. Time course of transformation of **1** by *A. apis* AM496. **Fig. S17**. (A) GC spectrum of extract after transformation of **1** by *I. radiatus* AM70 (B) TLC. **Fig. S18**. Time course of transformation of **1** by *I. radiatuss* AM70. **Fig. S19**. (A) GC spectrum of extract after transformation of **1** by *P. betulinus* AM39 (B) TLC. **Fig. S20**. Time course of transformation of **1** by *P. betulinus* AM39. **Fig. S21**. (A) GC spectrum of extract after transformation of **1** by *L. sulphureus* AM498 (B) TLC. **Fig. S22**. Time course of transformation of **1** by *L. sulphureus* AM498. **Fig. S23**. (A) GC spectrum of extract after transformation of **1** by *F. amygdali* AM258 (B) TLC. **Fig. S24**. Time course of transformation of **1** by *F. amygdali* AM258. **Fig. S25**. (A) GC spectrum of extract after transformation of **1** by *S. divaricata* AM423 (B) TLC. **Fig. S26**. Time course of transformation of **1** by *S. divaricata* AM423.