BAEYER–VILLIGER OXIDATION OF SOME STEROIDS BY *Aspergillus tamarii* MRC 72400

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Biotransformations of epiandrosterone (**1**), dehydroepiandrosterone (**2**), testosterone (**3**), progesterone (**4**) and pregnenolone (**5**) by *Aspergillus tamarii* MRC 72400 for 5 days have been reported and the results of these incubations have been compared with previously published data obtained with *Aspergillus tamarii* QM 1223. *A. tamarii* MRC 72400 showed higher Bayer–Villiger monooxygenase activities than *A. tamarii* QM 1223 did. Apart from pregnenolone (**5**), *A. tamarii* MRC 72400 metabolized these steroids in different ways. Incubation of epiandrosterone (**1**) afforded 3β,11β-dihydroxy-5α-androstan-17-one (**6**) (3%) and 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**7**) (9.5%). Incubation of dehydroepiandrosterone (**2**) afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) (28%), testolactone (**9**) (6%), 3β,7β-dihydroxyandrost-5-en-17-one (**10**) (13%) and 3β,7α-dihydroxyandrost-5-en-17-one (**11**) (24%). Incubation of testosterone (**3**) afforded testolactone (**9**) (58%). Incubation of progesterone (**4**) also afforded testolactone (**9**), however in higher yield (86%). Incubation of pregnenolone (**5**) afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) (25%) and testolactone (**9**) (27%).

Keywords: Steroids; Biotransformation; Biocatalysis; *Aspergillus tamarii*; Baeyer–Villiger oxidation.

A number of investigations involving microbial biotransformations of a wide range of steroidal substrates have been carried out due to their potential for the preparation of more valuable and functionalized compounds such as steroid drugs and hormones[1–3]. There are still enormous efforts to increase the efficiency of microbial steroid biotransformations and to find new useful microorganisms and reactions[1]. For example, some of these steroid biotransformations have been carried out using *Aspergillus wentii*[4], *Penicillium digitatum*[5] and *Aspergillus terreus*[6]. *A. wentii* showed some high hydroxylase activities whereas *P. digitatum* and *A. terreus* showed some low 5α-reductase and Baeyer–Villiger monooxygenase (BVMO) activities, respectively.

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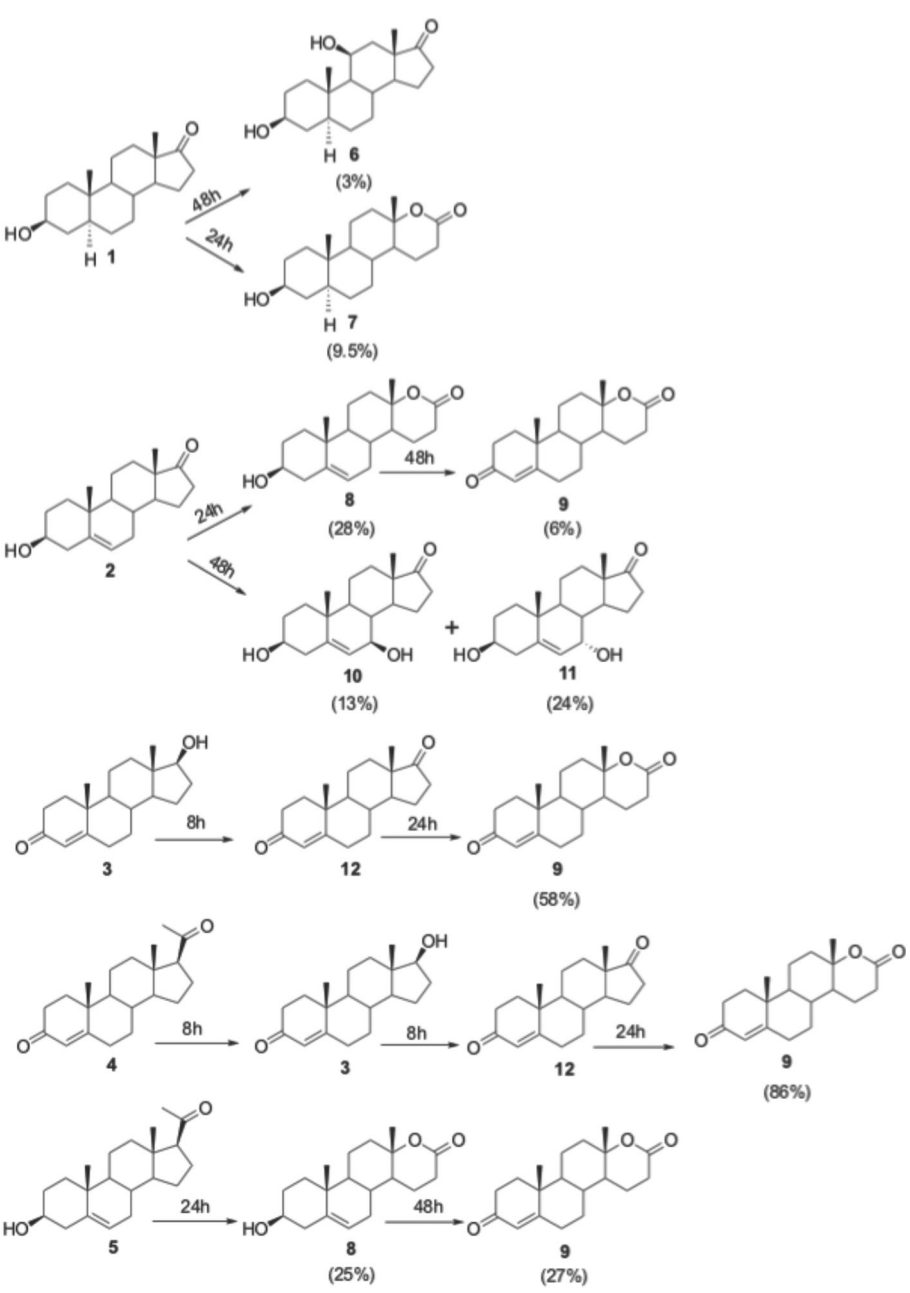
Baeyer–Villiger oxidations of some steroids afford steroidal lactones, which are important compounds because of their anticarcinogenic[7,8], antiandrogenic[9,10] and antihypercholestrolemic activites[11]. Although chemical syntheses of these lactones are possible, the microbial biotransformations are much more convenient due to environmental concerns[12]. The degradation of 17β-acetyl side chain of some C_{21} steroids and ring D oxidation of some androgens are carried out by BVMOs [13]. Many bacteria and fungi have these enzymes and they catalyze the insertion of an oxygen atom next to a keto function and convert different ketones to corresponding esters and lactones[14].

Aspergillus tamarii Kita is a dark greenish-brown fungus usually found on substrates yielding isolates of *Aspergillus flavus* Link, *Aspergillus parasiticus* Speare, and related fungi[15]. It produces some toxic metabolites such as aflatoxins B_1 and B_2, and cyclopiazonic acid[15,16].

As far as biotransformations by *A. tamarii* are concerned, *A. tamarii* QM 1223 has been the only strain of this fungus used for steroid biotransformation[17–25]. This strain mainly showed BVMO activities on different steroidal substrates and has attracted considerable interest due to its ability to transform progesterone (**4**) into testolactone (**9**) in high yield. In this study we have investigated the biotransformation of epiandrosterone (**1**), dehydroepiandrosterone (**2**), testosterone (**3**), progesterone (**4**) and pregnenolone (**5**) by *A. tamarii* MRC 72400 in order to extend previous results on steroid biotransformation by *A. tamarii*. *A. tamarii* MRC 72400 showed higher BVMO activities on these steroids than *A. tamarii* QM 1223 did.

RESULTS AND DISCUSSION

The analyzed metabolites resulting from incubations of steroids with *A. tamarii* MRC 72400 are shown in Scheme 1. Incubation of epiandrosterone (**1**) with *A. tamarii* afforded two metabolites. The first metabolite had a new resonance at δ_H 4.39 ppm (1 H, bs), which was in accordance with the characteristic 11α-H resonance[26]. Its ^{13}C NMR spectrum, on comparison to **1**, showed downfield shifts for C-18 (Δ 2.11 ppm), C-19 (Δ 3.3 ppm), C-9 (Δ 4.18 ppm), and C-12 (Δ 9.09 ppm) whereas it showed γ-gauche upfield shifts for C-8 (Δ 4.12 ppm) and C-13 (Δ 0.98 ppm), which was consistent with 11β-hydroxylation[27]. The metabolite had a resonance at δ_H 3.55 ppm (1 H, tt, J = 5.0 and 12.0 Hz) confirming that the 3β-hydroxy group was maintained. All these results suggested that the metabolite was 3β,11β-dihydroxy-5α-androstan-17-one (**6**).



SCHEME 1
Metabolism of steroidal substrates by *Aspergillus tamarii* MRC 72400

The ^{1}H NMR spectrum of the second metabolite showed an important downfield shift (Δ 0.45 ppm) for the 18-methyl resonance at δ_H 0.84 ppm of the starting material. The ^{13}C NMR spectrum of the metabolite demonstrated important downfield shifts for C-13 (Δ 35.49 ppm) and C-18 (Δ 6.34 ppm) whereas it demonstrated an important upfield shift for C-14 (Δ 5.06 ppm), which was in accordance with heteroatom insertion into ring D via Baeyer–Villiger oxidation. The metabolite had a resonance at δ_H 3.53 ppm (1 H, tt, J = 5.0 and 11.0 Hz), indicating that the 3β-hydroxy group was retained. These results indicated that the metabolite was 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**7**).

During the time course experiment, the 18-methyl signal of epiandrosterone (**1**) shifted from 0.84 to 1.29 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 10% of epiandrosterone (**1**) had been converted to 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**7**) by 24 h. At 48 h, the 18-methyl signal of epiandrosterone (**1**) shifted from 0.84 to 1.29 and 1.10 ppm, and comparison of the methyl group integrations in the ^{1}H NMR spectrum suggested that lactonization had been completed and that 5% of remaining epiandrosterone (**1**) had been converted to 3β,11β-dihydroxy-5α-androstan-17-one (**6**). Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that hydroxylation had been completed by 72 h and no more reactions were observed after that time. Time course experiment results indicated that epiandrosterone (**1**) was mainly converted to the corresponding lactone **7** by a BVMO. In addition, an independent minor 11β-hydroxylation pathway took place (Scheme 1). *A. tamarii* QM 1223 only showed a 11β hydroxylase activity on the same substrate and the yield[18] was higher than that from *A. tamarii* MRC 72400.

Incubation of dehydroepiandrosterone (**2**) with *A. tamarii* afforded four metabolites. The ^{1}H NMR spectrum of the first metabolite showed a significant downfield shift (Δ 0.47 ppm) for the 18-methyl resonance at δ_H 0.87 ppm of the starting material. The ^{13}C NMR spectrum of the metabolite had significant downfield shifts for C-13 and C-18 (Δ 35.86 ppm and Δ 6.24 ppm, respectively) whereas it had an important upfield shift for C-14 (Δ 5.1 ppm), which was consistent with steroidal D lactone formation. This metabolite had resonances at δ_H 3.54 ppm (1 H, tt, J = 5.0 and 11.0 Hz) and δ_H 5.35 ppm (1 H, d, J = 5.0 Hz), indicating that the 3β-hydroxy group and the double bond at C5-C6 was maintained. All these results suggested that the metabolite was 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**).

The ^{1}H NMR spectrum of the second metabolite also had a significant downfield shift (Δ 0.50 ppm) for the 18-methyl resonance at δ_H 0.87 ppm of the starting material. The ^{13}C NMR spectrum of the metabolite showed important downfield shifts for the C-13 and C-18 (Δ 35.01 ppm and Δ 6.52 ppm, respectively) whereas it showed an important upfield shift for C-14 (Δ 6.08 ppm), which was in accordance with insertion of an oxygen atom adjacent to this position on the ring D. The metabolite lacked the resonances of **2** at δ_H 3.46 ppm (1 H, tt, J = 5.0 and 12.0 Hz) and δ_H 5.35 ppm (1 H, d, J = 5.0 Hz) and had a new resonance at δ_H 5.78 ppm (1 H, bs), suggesting that the conversion of a 5-ene-3β-hydroxy steroid into a 4-ene-3-keto steroid had taken place. These results suggested that the metabolite was testolactone (**9**).

The ^{1}H NMR spectrum of the third metabolite had the 7α-H resonance at δ_H 3.95 ppm (1 H, dt, J = 4.0 and 8.0 Hz) and showed an upfield shift (Δ 0.03 ppm) for the 6-H resonance (1 H, d, J = 5.0 Hz) at δ_H 5.35 ppm of the starting material, which was consistent with 7β-hydroxylation[28]. Its ^{13}C NMR spectrum, in comparison to **2**, showed downfield shifts for C-6 (Δ 4.93 ppm) and C-8 (Δ 8.69 ppm) whereas it showed a γ-gauche upfield shift for C-9 (Δ 1.93 ppm), which are comparable with literature values[29]. The metabolite had a resonance at δ_H 3.55 ppm (1 H, tt, J = 5.0 and 10.0 Hz), indicating that the 3β-hydroxy group was maintained. All these results indicated that the metabolite was 3β,7β-dihydroxyandrost-5-en-17-one (**10**).

The ^{1}H NMR spectrum of the fourth metabolite had the 7β-H resonance signal at δ_H 3.96 ppm (1 H, bs, W_h = 11.0 Hz) and showed a significant downfield shift (Δ 0.27 ppm) for the 6-H resonance (1 H, d, J = 5.0 Hz) at δ_H 5.35 ppm of the starting material, which was in accordance with 7α-hydroxylation[28]. Its ^{13}C NMR spectrum, in comparison to **2**, showed downfield shifts for C-6 (Δ 2.12 ppm) and C-8 (Δ 5.88 ppm) whereas it showed a γ-gauche upfield shift for C-9 (Δ 8.37 ppm), which are comparable with literature values[29]. The metabolite had a resonance at δ_H 3.56 ppm (1 H, m, W_h = 22.0 Hz) suggesting that the 3β-hydroxy group retained. These results suggested that the metabolite was 3β,7α-dihydroxyandrost-5-en-17-one (**11**).

During the time course experiment, the 18-methyl signal of dehydroepiandrosterone (**2**) shifted from 0.87 to 1.34 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 35% of dehydroepiandrosterone (**2**) had been converted to 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) after 24 h. By 48 h, the 18-methyl signals of dehydroepiandrosterone (**2**) shifted from 0.87 to 1.34, 1.37, 0.89 and

0.90 ppm, and further comparison of the methyl group signals suggested that 5% of 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) had been oxidized to testolactone (**9**) and that most of the remaining dehydroepiandrosterone (**2**) had been hydroxylated at C-7β and C-7α. Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions took place after 72 h. According to time course experiment results, *A. tamarii* MRC 72400 first showed a BVMO activity on dehydroepiandrosterone (**2**) and this was followed by oxidation of some of the initial product and independent minor hydroxylations at C-7 (Scheme 1). Both 7α- and 7β-hydroxylase activities of *A. tamarii* MRC 72400 were independent from its BVMO activity and its 7α-hydroxylase activity was higher than its 7β-hydroxylase one. *A. tamarii* QM 1223 showed the same activities[23]. However, its 7α-hydroxylase activity was fully concomitant with its BVMO one and afforded a 7α-hydroxylated lactone. The yields of **8**, **9** and **10** from *A. tamarii* MRC 72400 were higher than those from *A. tamarii* QM 1223. The yield of **9** was lower than that of **8** and it was thought that this was due to possible inhibitory effects of allylic hydroxylations at C-7 on the isomerase enzyme as in the literature[23].

Incubation of testosterone (**3**) with *A. tamarii* afforded only testolactone (**9**), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with that of an authentic sample.

During the time course experiment, the 18-methyl signal of testosterone (**3**) shifted from 0.77 to 0.93 ppm [26] and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 60% of testosterone (**3**) was converted to androst-4-ene-3,17-dione (**12**) after 8 h of incubation. After 24 h, the presence of the 18-methyl signal of testosterone (**3**) shifted from 0.77 to 1.37 ppm and further comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 100% of androst-4-ene-3,17-dione (**12**) had been converted to testolactone (**9**). Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions took place after 48 h. Time course experiment indicated that testosterone (**3**) was first converted to androst-4-ene-3,17-dione (**12**) and this compound was then converted to testolactone (**9**) by a BVMO activity (Scheme 1) as it was in the incubation of this substrate with *Penicillium lilacinum*[30]. *A. tamarii* QM 1223 showed the same activities accompanied by an independent minor 11β-hydroxylation pathway and the yield[17] of **9** was lower than that from *A. tamarii* MRC 72400.

Incubation of progesterone (**4**) with *A. tamarii* also afforded testolactone (**9**), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with that of an authentic sample.

During the time course experiment, the 18-methyl signal of progesterone (**4**) shifted from 0.66 to 0.77 and 0.93 ppm [26], and comparison of the methyl group integrations in ^{1}H NMR spectrum indicated that 90% of progesterone (**4**) had been converted to testosterone (**3**) and androst-4-ene-3,17-dione (**12**) at 8 h. After 24 h, the presence of the 18-methyl signals at δ_H 0.66 ppm and δ_H 1.37 ppm, and further comparison of the methyl group integrations in ^{1}H NMR spectrum indicated that all of testosterone (**3**) had been converted to androst-4-ene-3,17-dione (**12**), which was then fully transformed to testolactone (**9**). By 48 h, further comparison of the methyl group integrations in the ^{1}H NMR spectrum suggested that lactonization had been completed during the first 24 h. No further changes were observed in the methyl group integrals of the ^{1}H NMR spectra after 72 h. According to time course experiment results for progesterone (**4**), a BVMO first converted progesterone (**4**) to testosterone acetate, which was not observed during the time course experiment due to the presence of high levels of an esterase enzyme activity[31]. This ester was then hydrolyzed to give testosterone (**3**). The oxidation of testosterone (**3**) at C-17 gave androst-4-ene-3,17-dione (**12**). Finally, **12** was fully converted to testolactone (**9**) by BVMO activity (Scheme 1). Apart from a minor 11β-hydroxylation pathway, *A. tamarii* QM 1223 showed the same activities on the substrate and the yield[17] of **9** was lower than that from *A. tamarii* MRC 72400.

Incubation of pregnenolone (**5**) with *A. tamarii* afforded two metabolites. The first metabolite was 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with that of an authentic sample. The second metabolite was testolactone (**9**), which was also identified by comparison of its ^{1}H and ^{13}C NMR spectra with that of an authentic sample.

During the time course experiment, the 18-methyl signal of pregnenolone (**5**) shifted from 0.60 to 1.34 ppm and comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 50% of pregnenolone (**5**) had been converted to 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) in 24 h. By 48 h, the presence of the double bond signal at δ_H 5.78 (1 H, bs), three 18-methyl signals at δ_H 0.60, 1.34 and 1.37 ppm and further comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 55% of 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) had been converted to testolactone (**9**). After 72 h, no further changes were observed in the methyl group integrals of the ^{1}H NMR spectra. Time course experiment results for pregnenolone (**5**) suggested that 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**) occured as the first lactone and that some of it was then converted to testolactone (**9**)

(Scheme 1). *A. tamarii* QM 1223 showed the same activities with lower yields[23].

In conclusion, we have shown that apart from pregnenolone (**5**), *A. tamarii* MRC 72400 metabolized these steroids in different ways when compared with *A. tamarii* QM 1223[17,18,23]. *A. tamarii* MRC 72400 did not show any 11β-hydroxylase activity on testosterone (**3**) and progesterone (**4**), and its 7α-hydroxylase activity on dehydroepiandrosterone (**2**) was independent from its BVMO activities. *A. tamarii* MRC 72400 showed low BVMO and 11β-hydroxylase activities on epiandrosterone (**1**) whereas *A. tamarii* QM 1223 only showed a high 11β-hydroxylase activity on the same substrate. We have also shown that *A. tamarii* MRC 72400 showed higher BVMO activities than *A. tamarii* QM 1223 did and that this strain may at least be an effective substitute for *A. tamarii* QM 1223 in the production of testolactone (**9**) from progesterone (**4**).

EXPERIMENTAL

Epiandrosterone, dehydroepiandrosterone, testosterone, progesterone and pregnenolone were purchased from Fluka (Istanbul, Turkey). *A. tamarii* Kita MRC 72400 was obtained from TUBITAK (Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit, Kocaeli, Turkey). Stock cultures were maintained at 4 °C on PDA slopes. Solvents were of analytical grade and were purchased from Merck (Istanbul, Turkey). Potato dextrose agar and agar for PDA slopes and malt extract for liquid medium were also purchased from Merck (Istanbul, Turkey).

The steroids were separated by column chromatography on silica gel 60 (Merck 107734) with increasing concentrations of ethyl acetate in hexane as eluent. TLC was carried out with 0.2 mm thick Merck Kieselgel 60 F_{254} TLC plates using ethyl acetate/hexane (1:1, v/v) as eluent. In order to develop the chromatograms, TLC plates were dipped into an anisaldehyde–H_2SO_4 reagent and heated to 120 °C for 3 min. Infrared spectra (wavenumbers in cm^{-1}) were recorded using a Shimadzu IR Prestige-21 apparatus. Optical rotation measurements were carried out on a WXG-4 polarimeter. Optical rotation values are given in 10^{-1} deg cm^2 g^{-1}. Elemental analysis was performed using a Thermo Finnigan Flash EA 1112 elemental analyser. High-resolution mass spectra (HRMS) were determined on a Bruker Daltonics Apex III mass spectrometer operating in the electrospray mode. ^{1}H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard reference at 300 MHz with a Varian Mercury 300 spectrometer. ^{13}C NMR spectra were recorded in deuteriochloroform at 75 MHz with a Varian Mercury 300 spectrometer. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) are given in Hz. Melting points were determined by an Electrothermal IA 9200 melting point apparatus and are uncorrected. Biotransformation experiments were run with control flasks containing non-inoculated sterile medium and one of the substrates. After five days of incubation, all controls were also harvested and analysed by TLC. No metabolites were detected in controls.

Time course experiments[23] were conducted in order to determine the metabolic pathway. Conditions were identical to those in main biotransformation experiments except that each

individual steroidal substrate (300 mg) dissolved in DMF (6 ml) was evenly distributed between 6 flasks (each containing 100 ml of medium). One flask was harvested after 8 h. Then every 24 h one flask was harvested and extracted. TLC analysis was performed immediately on the isolated mixture. Following 6 h under high vacuum, the product ^{1}H NMR spectra were determined in $CDCl_3$ to confirm the steroidal nature of the extracts.

Biotransformation of Epiandrosterone (**1**) by *A. tamarii* MRC 72400

Spores freshly obtained from PDA slopes were transferred aseptically into 10 Erlenmeyer flasks of 250 ml containing 100 ml of sterile 3% malt extract medium[17] in a biological safety cabinet. After cultivation at 24 °C for 3 days on a rotary shaker (180 rpm), epiandrosterone (**1**; 500 mg, 1.72 mmol) dissolved in 10 ml of DMF was evenly and aseptically distributed among the flasks. The biotransformation of the substrate was carried out in 10 flasks for five days under the same conditions. The fungal mycellium was separated from the broth by filtration under the vacuum and the mycellium was rinsed with ethyl acetate (500 ml). The broth was then extracted three times each with 1 l of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate and the solvent evaporated in vacuo to give a brown gum (716 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (300 mg, 60%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 40% ethyl acetate in hexane afforded 3β,11β-dihydroxy-5α-androstan-17-one (**6**; 16 mg, 3%), which was crystallized from diethyl ether as needles, m.p. 228–231 °C, $[\alpha]_D^{20}$ +83, *c* 0.1, $CHCl_3$ (lit.[32] gives m.p. 235–238 °C, $[\alpha]_D^{20}$ +84.5, *c* 1, C_2H_5OH). HRMS for $C_{19}H_{30}O_3$: calculated 306.2196, found 306.2206. IR: 3470 (O–H), 1741 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 1.06 s, 3 H (H-19); 1.10 s, 3 H (H-18); 3.55 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 12.0 (H-3); 4.39 bs, 1 H (H-11). ^{13}C NMR (75 MHz, $CDCl_3$): 219.94, 70.96, 68.06, 58.43, 52.90, 46.92, 45.72, 40.48, 37.57, 36.83, 35.74, 35.33, 31.24, 31.09, 30.81, 27.84, 21.65, 15.86, 15.51.

Elution with 50% ethyl acetate in hexane afforded 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one (**7**; 50 mg, 9.5%), which was crystallized from acetone as needles, m.p. 170–173 °C, $[\alpha]_D^{20}$ –43, *c* 0.1, $CHCl_3$ (lit.[6] gives m.p. 173–174 °C, $[\alpha]_D^{20}$ –45, *c* 0.1, $CHCl_3$). HRMS for $C_{19}H_{30}O_3$: calculated 306.2196, found 306.2182. IR: 3438 (O–H), 1724 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.78 s, 3 H (H-19); 1.29 s, 3 H (H-18); 3.53 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 11.0 (H-3). ^{13}C NMR (75 MHz, $CDCl_3$): 171.65, 83.39, 70.99, 53.01, 46.22, 44.13, 39.24, 37.84, 37.73, 36.70, 35.44, 31.24, 30.55, 28.59, 28.21, 21.97, 20.09, 19.73, 12.11.

Biotransformation of Dehydroepiandrosterone (**2**) by *A. tamarii* MRC 72400

Under similar conditions, the incubation of dehydroepiandrosterone (**2**; 500 mg, 1.734 mmol) with *A. tamarii* afforded a brown gum (765 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unchanged starting material (50 mg, 10%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 50% ethyl acetate in hexane afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**; 148 mg, 28%), which was crystallized from ethyl acetate as needles, m.p. 234–235 °C, $[\alpha]_D^{20}$ –92, *c* 0.1, $CHCl_3$ (lit.[6] gives m.p. 234–235 °C, $[\alpha]_D^{20}$ –95, *c* 0.1, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.4) calculated: 74.96% C, 9.27% H; found: 75.11% C, 9.10% H. IR: 3448

(O–H), 1720 (C=O), 1657 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 0.98 s, 3 H (H-19); 1.34 s, 3 H (H-18); 3.54 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 11.0 (H-3); 5.35 d, 1 H, *J*(6,7) = 5.0 (H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 171.97, 140.49, 120.49, 83.35, 71.40, 48.85, 46.57, 41.58, 38.77, 36.79, 36.48, 34.33, 31.17, 30.95, 28.67, 21.86, 19.97, 19.72, 19.19.

Elution with 60% ethyl acetate in hexane afforded testolactone (**9**; 32 mg, 6%), which was crystallized from methanol as needles, m.p. 210–211 °C, $[\alpha]_D^{20}$ +44, *c* 0.1, $CHCl_3$ (lit.[6] gives m.p. 206–207 °C, $[\alpha]_D^{20}$ +48, *c* 0.1, $CHCl_3$). For $C_{19}H_{26}O_3$ (302.4) calculated: 75.46% C, 8.67% H; found: 75.32% C, 8.49% H. IR: 1718 (C=O), 1618 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 1.17 s, 3 H (H-19); 1.37 s, 3 H (H-18); 5.78 bs, 1 H (H-4). ^{13}C NMR (75 MHz, $CDCl_3$): 199.46, 171.43, 169.70, 123.99, 82.80, 52.41, 45.59, 38.87, 38.41, 37.89, 35.39, 33.71, 32.29, 30.33, 28.45, 21.76, 20.00, 19.76, 17.32.

Elution with 80% ethyl acetate in hexane afforded 3β,7β-dihydroxyandrost-5-en-17-one (**10**; 69 mg, 13%), which was crystallized from ethyl acetate–petroleum ether as needles, m.p. 207–209 °C, $[\alpha]_D^{20}$ +65, *c* 0.1, $CHCl_3$ (lit.[4] gives m.p. 208–209 °C, $[\alpha]_D^{20}$ +67, *c* 0.1, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.4) calculated: 74.96% C, 9.27% H; found: 74.85% C, 9.23% H. IR: 3239 (O–H), 1740 (C=O), 1665 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 0.90 s, 3 H (H-18); 1.08 s, 3 H (H-19); 3.55 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 10.0 (H-3); 3.95 dt, 1 H, *J*(7α,8) = 8.0, *J*(7α,6) = *J*(7α,4β) = 4.0 (H-7); 5.32 bs, 1 H (H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 221.40, 143.16, 125.76, 72.32, 70.74, 51.19, 48.20, 47.65, 41.60, 40.10, 36.85, 36.51, 35.89, 31.37, 31.13, 24.08, 20.26, 18.96, 13.44.

Elution with pure ethyl acetate in hexane afforded 3β,7α-dihydroxyandrost-5-en-17-one (**11**; 127 mg, 24%), which was crystallized from ethyl acetate–petroleum ether as needles, m.p. 173–175 °C, $[\alpha]_D^{20}$ –77, *c* 0.1, $CHCl_3$ (lit.[4] gives m.p. 174–175 °C, $[\alpha]_D^{20}$ –73, *c* 0.1, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.4) calculated: 74.96% C, 9.27% H; found: 74.81% C, 9.20% H. IR: 3422 (O–H), 3325 (O–H), 1736 (C=O), 1672 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 0.89 s, 3 H (H-18); 1.03 s, 3 H (H-19); 3.56 m, 1 H, W_h = 22 (H-3); 3.96 bs, 1 H, W_h = 11 (H-7); 5.62 d, 1 H *J*(6,7β) = 5.0 (H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 221.48, 145.75, 122.95, 70.69, 63.86, 46.97, 44.73, 42.31, 41.76, 37.29, 37.05, 36.75, 35.65, 31.00, 30.88, 21.71, 19.87, 18.08, 13.05.

Biotransformation of Testosterone (**3**) by *A. tamarii* MRC 72400

Under similar conditions, the incubation of testosterone (**3**; 500 mg, 1.734 mmol) with *A. tamarii* afforded a brown gum (738 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unchanged starting material (125 mg, 25%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 60% ethyl acetate in hexane afforded testolactone (**9**; 304 mg, 58%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Biotransformation of Progesterone (**4**) by *A. tamarii* MRC 72400

Under similar conditions, the incubation of progesterone (**4**; 500 mg, 1.59 mmol) with *A. tamarii* afforded a brown gum (782 mg), which was then chromatographed on silica gel. Elution with 60% ethyl acetate in hexane afforded testolactone (**9**; 414 mg, 86%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Biotransformation of Pregnenolone (**5**) by *A. tamarii* MRC 72400

Under similar conditions, the incubation of pregnenolone (**5**; 500 mg, 1.58 mmol) with *A. tamarii* afforded a brown gum (730 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (205 mg, 41%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 50% ethyl acetate in hexane afforded 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**8**; 120 mg, 25%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

Elution with 60% ethyl acetate in hexane afforded testolactone (**9**; 129 mg, 27%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic material.

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