MICROBIAL HYDROXYLATION OF SOME STEROIDS BY *Aspergillus wentii* MRC 200316

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Received September 12, 2010
Accepted October 19, 2010
Published online November 26, 2010

Biotransformations of epiandrosterone (**1**), dehydroepiandrosterone (**2**) and pregnenolone (**3**) by *Aspergillus wentii* MRC 200316 for 5 days have been reported. Incubation of epiandrosterone (**1**) afforded 11α-hydroxy-5α-androstane-3,17-dione (**4**) and 3β,11α-dihydroxy-5α-androstan-17-one (**5**). Incubation of dehydroepiandrosterone (**2**) afforded 3β,7β-dihydroxyandrost-5-en-17-one (**6**) and 3β,7α-dihydroxyandrost-5-en-17-one (**7**). Incubation of pregnenolone (3) afforded only 11α-hydroxypregn-4-ene-3,20-dione (**8**).
Keywords: Steroids; Biotransformation; Hydroxylation; *Aspergillus wentii*.

Microbial biotransformation of steroids has found worldwide application for the preparation of more valuable and functionalized compounds such as steroid drugs and hormones due to its high regio- and stereoselectivity[1]. For example, a number of investigations involving microbial biotransformations of a wide range of steroidal substrates have been reported in recent years[1–3]. There are still enormous efforts to increase the efficiency of microbial steroid biotransformations and to find new useful microorganisms and reactions[1]. Some of these steroid biotransformations have recently been carried out using *Penicillium digitatum*[4] and *Aspergillus terreus*[5]. *Penicillium digitatum* and *Aspergillus terreus* showed some low 5α-reductase and Baeyer–Villiger monooxygenase activities, respectively.

Aspergillus wentii Wehmer is a toxigenic, ubiquitous, soil-inhabiting fungus usually found on decayed vegetation and moist grains[6,7]. It produces several toxic metabolites such as aflatoxins[8], emodin[6] and ochratoxin A [9] as well as other secondary metabolites like kojic acid[10], 1-amino-2-nitrocyclopentanecarboxylic acid[11], hexadecylcitraconic acid[12], 3-nitropropionic acid[13], and wentilactone A and B [14].

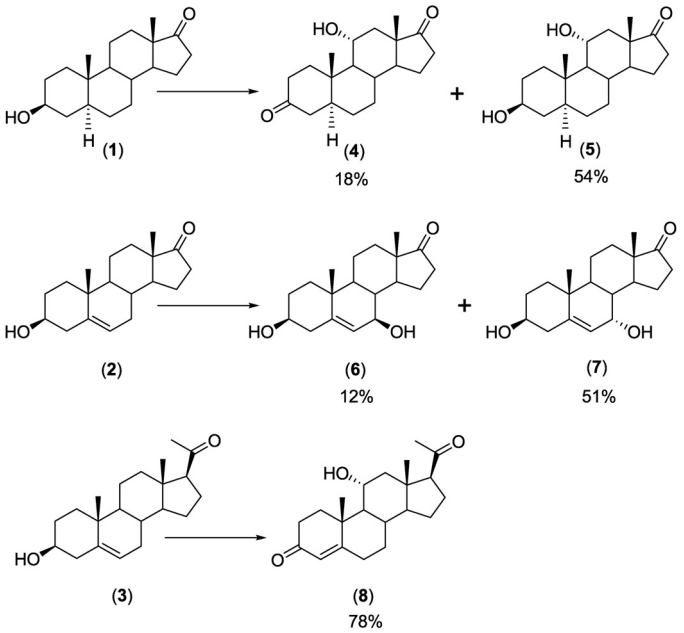
As far as biotransformations by *A. wentii* are concerned, the only steroid incubations with this fungus have been reported recently[15]. In the men-

doi:10.1135/cccc2010112

tioned incubations, *A. wentii* MRC 200316 has hydroxylated testosterone and progesterone in high yields. In this work, the biotransformations of epiandrosterone (**1**), dehydroepiandrosterone (**2**) and pregnenolone (**3**) by *A. wentii* MRC 200316 for 5 days were investigated. *A. wentii* also showed some high hydroxylase activities on these steroids.

RESULTS AND DISCUSSION

Incubation of epiandrosterone (**1**) with *A. wentii* for 5 days afforded two metabolites (Scheme 1). The first metabolite was identified as 11α-hydroxy-5α-androstane-3,17-dione (**4**). The metabolite had characteristic resonances[16,17] at δ_H 3.98 ppm (1 H, dt, J = 5.0 and 10.0 Hz) and δ_C 68.66 ppm, which were consistent with the hydroxylation of 11α-equatorial position. The ^{13}C NMR spectrum of the metabolite demonstrated β-carbon downfield shifts for C-9 (Δ 5.47 ppm) and C-12 (Δ 11.36 ppm), and a γ-carbon upfield shift for C-8 (Δ 0.92 ppm), all of which consistent with 11α-hydroxylation[17]. The metabolite lacked the 3α-H resonance of **1** at δ_H 3.53 (1 H, tt, J = 5.0 and 12.0 Hz) and had a new resonance at δ_C 212.72 ppm, suggesting that the 3β-hydroxy group was oxidized to a 3-keto group.



SCHEME 1
Biotransformation of steroidal substrates by *A. wentii* MRC 200316

The second metabolite was identified as 3β,11α-dihydroxy-5α-androstan-17-one (**5**). The metabolite had new resonances at δ_H 3.88 ppm (1 H, dt, J = 5.0 and 10.0 Hz) and δ_C 68.43 ppm, which were in accordance with the presence of an 11α-hydroxyl group. The ^{13}C NMR spectrum of the metabolite showed downfield shifts for C-9 (Δ 6.02 ppm) and C-12 (Δ 11.34 ppm), and a γ-carbon upfield shift for C-8 (Δ 0.84 ppm), which were further in accordance with 11α-hydroxylation[17]. The metabolite had the 3α-H resonance of **1** at δ_H 3.50 ppm (1 H, tt, J = 5.0 and 10.0 Hz), suggesting that the 3β-hydroxy group was maintained.

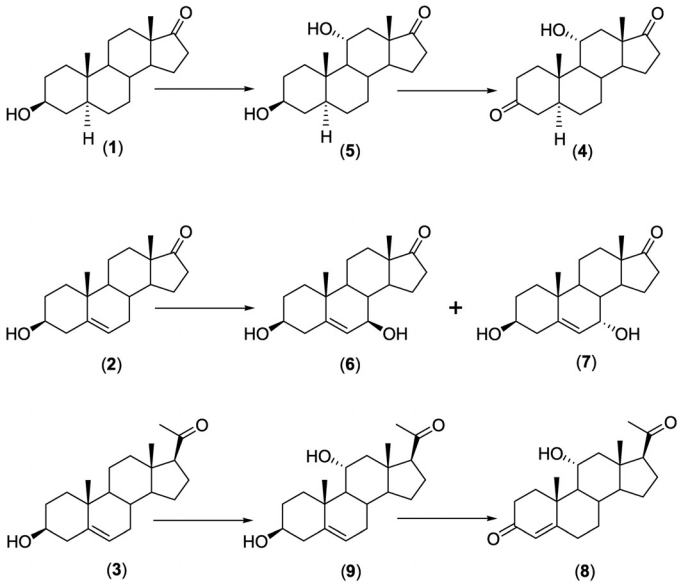
During the time course experiment for epiandrosterone (**1**), the 19-methyl resonance of **1** shifted from 0.84 to 0.92 ppm and the comparison of the methyl group integrations in the ^{1}H NMR spectra indicated that 75% of the substrate was converted into 3β,11α-dihydroxy-5α-androstan-17-one (**5**) and the hydroxylation had been completed by 72 h. At 96 h, the 19-methyl resonance of **1** shifted from 0.84 to 0.89 and 0.92 ppm, and the comparison of the methyl group integrations in the ^{1}H NMR spectra indicated that almost 30% of 3β,11α-dihydroxy-5α-androstan-17-one (**5**) was converted into 11α-dihydroxy-5α-androstane-3,17-dione (**4**). Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions took place after 96 h. Time course experiment results indicated that epiandrosterone (**1**) was first converted into 3β,11α-dihydroxy-5α-androstan-17-one (**5**) by a 11α-hydroxylase and some of this diol was then converted into 11α-hydroxy-5α-androstan-3,17-dione (**4**) via an oxidation at C-3 (Scheme 2).

Incubation of dehydroepiandrosterone (**2**) with *A. wentii* for 5 days afforded two metabolites (Scheme 1). The first metabolite was identified as 3β,7β-dihydroxyandrost-5-en-17-one (**6**). The ^{1}H NMR spectrum of **6** had a new resonance at δ_H 3.95 ppm (1 H, dt, J = 3.0 and 8.0 Hz) and experienced an upfield shift for the 6-H resonance (Δ 0.03 ppm), which was consistent with the presence of a 7β-hydroxy group. Its ^{13}C NMR spectrum, in comparison with **2**, showed downfield shifts for C-6 (Δ 4.54 ppm) and C-8 (Δ 8.93 ppm), whereas it showed a γ-gauche upfield shift for C-9 (Δ 2.01 ppm), which are comparable with literature values[18]. 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 retained.

The second metabolite was identified as 3β,7α-dihydroxyandrost-5-en-17-one (**7**). The ^{1}H NMR spectrum of **7** had a new resonance at δ_H 3.96 ppm (1 H, bs, W_h = 12.0 Hz) and experienced a downfield shift for the 6-H resonance (Δ 0.28 ppm), which was in accordance with a 7α-hydroxy group. Its ^{13}C NMR spectrum, in comparison with **2**, showed downfield shifts for C-6

(Δ 2.40 ppm) and C-8 (Δ 5.80 ppm), whereas it showed a γ-gauche upfield shift for C-9 (Δ 8.53 ppm), which are comparable with literature values[18]. The resonance at δ_H 3.56 ppm (1 H, m, W_h = 24.0 Hz) was still present in the product ^{1}H NMR spectrum, indicating that the 3β-hydroxy group maintained.

During the time course experiment, the 19-methyl signal of dehydroepiandrosterone (**2**) moved from 1.01 to 1.08 and 1.03 ppm, respectively, and the comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 75% of dehydroepiandrosterone (**2**) had been converted into 3β,7β-dihydroxyandrost-5-en-17-one (**6**) and 3β,7α-dihydroxyandrost-5-en-17-one (**7**), and the hydroxylation had been completed by 72 h. Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions were observed after that time. According to time course experiment results for dehydroepiandrosterone (**2**), *A. wentii* showed both 7α- and 7β-hydroxylase activities (Scheme 2). This fungus had a major 7α-hydroxylase activity and a minor 7β-hydroxylase activity on dehydroepiandrosterone (**2**) as in the incubations of the same substrate with *Rhizomucor tauricus*[19] and *Mucor racemosus*[20].



SCHEME 2
Metabolism of steroidal substrates by *A. wentii* MRC 200316

Incubation of pregnenolone (**3**) with *A. wentii* for 5 days afforded only 11α-hydroxypregn-4-ene-3,20-dione (**8**) (Scheme 1). The ^{1}H NMR spectrum of the metabolite demonstrated a significant downfield shift (Δ 0.30 ppm) for the 19-methyl group. The metabolite had characteristic resonances[16,17] at δ_H 3.98 ppm (1 H, dt, J = 5.0 and 10.0 Hz) and δ_C 68.63 ppm, suggesting that an 11α-hydroxylation had taken place. The ^{1}H NMR spectrum of the metabolite lacked the resonance of starting material at δ_H 3.45 ppm (1 H, tt, J = 5.0 and 12.0 Hz, 3-H), and the double bond signal of **3** at δ_H 5.32 ppm (1 H, d, J = 5.0 Hz, 6-H) had an important downfield shift to 5.69 ppm (Δ 0.37 ppm), suggesting that the 5-ene-3β-hydroxy moiety of **3** was converted into the 4-ene-3-keto moiety.

During the time course experiment, the 19-methyl signal of pregnenolone (**3**) shifted from 0.98 to 1.17 ppm [21] and the comparison of the methyl group integrations in the ^{1}H NMR spectrum indicated that 80% of pregnenolone (**3**) was converted into 3β,11α-dihydroxypregn-5-en-20-one (**9**) and hydroxylation had been completed by 72 h. At 96 h, the presence of the 19-methyl signal of pregnenolone (**3**) shifted from 0.98 to 1.28 ppm, and the lack of the 19-methyl resonance of 3β,11α-dihydroxypregn-5-en-20-one (**9**) at δ_H 1.17 ppm indicated that all of 3β,11α-dihydroxypregn-5-en-20-one (**9**) had been converted into 11α-hydroxypregn-4-ene-3,20-dione (**8**). Further comparison of the methyl group integrations in the ^{1}H NMR spectra suggested that no more reactions took place after 96 h. Time course experiment results demonstrated that *A. wentii* first had an 11α-hydroxylase activity on pregnenolone (**3**) and this afforded 3β,11α-dihydroxypregn-5-en-20-one (**9**). All of this diol was then converted into 11α-hydroxypregn-4-ene-3,20-dione (**8**) by an isomerase enzyme (Scheme 2). This type of isomerization of a few 11α-hydroxylated metabolites was also observed during the incubations of pregnenolone (**3**) with *Botrytis cinerea*[22] and *Cunninghamella elegans*[23].

This study has demonstrated that *A. wentii* MRC 200316 is capable to hydroxylate a range of steroids including epiandrosterone (**1**), dehydroepiandrosterone (**2**) and pregnenolone (**3**). *A. wentii* showed high 11α-hydroxylase activities on epiandrosterone (**1**) and pregnenolone (**3**) as in the incubation of progesterone[13]. A range of fungi including *Rhizopus nigricans*[24], *Aspergillus ochraceus*[25] and *Cephalosporium aphidicola*[26] are capable to functionalize at the 11α position which is influenced by the presence of a C-17 side chain. Interestingly, the C-17 side chain had no influence on hydroxylation at this position by *A. wentii*.

Uniquely in this study, dehydroepiandrosterone (**2**) was hydroxylated at C-7 by *A. wentii*. This suggested that the introduction of a Δ^5-double bond

completely altered the results from the incubation of epiandrosterone (**1**) by *A. wentii* leading to only non-stereospesific allylic hydroxylation at C-7 in place of the attack at C-11α. Its hydroxylase activity at the axial proton was higher. Apart from a minor hydroxylation at C-14α, *A. wentii* also hydroxylated testosterone mainly at the allylic, 6β-axial position, although it did not hydroxylate pregnenolone (**3**) and progesterone at any allylic position[15]. This suggested that the presence of a Δ^5- or Δ^4-double bond and the lack of a side chain at C-17 resulted in mainly allylic hydroxylations by *A. wentii*.

In conclusion, it has been shown that *A. wentii* has the ability to hydroxylate steroidal substrates and it is a good 11α-hydroxylator for epiandrosterone (**1**) and pregnenolone (**3**). It has also been shown that the presence of a double bond in ring A or B and the lack of a side chain at C-17 resulted in allylic hydroxylations by *A. wentii*. Our work on steroid biotransformation by *A. wentii* and some other fungi is in progress.

EXPERIMENTAL

Epiandrosterone, dehydroepiandrosterone and pregnenolone were purchased from Fluka (Istanbul, Turkey). Aspergillus wentii MRC 200316 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 ingredients 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. TLC plates were dipped into an anisaldehyde–H_2SO_4 reagent and heated to 120 °C for 3 min in order to visualize the spots. Infrared spectra (wavenumbers in cm^{-1}) were recorded using a Shimadzu IR Prestige-21 apparatus. Optical rotation mesurements were carried out on a WXG-4 polarimeter. Elemental analysis was performed using a Thermo Finnigan Flash EA 1112 elemental analyser. ^{1}H NMR spectra were recorded in deuteriochloroform with tetramethylsilane as an internal standard 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*) and width multiplets (*W*) 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 5 days of incubation, all controls were also harvested and analysed by TLC. No metabolites were detected in controls.

Time course experiments[27] 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 be-

tween 6 flasks (each containing 100 ml of medium). One flask was harvested and extracted after 8 h. Then one flask was harvested and extracted every 24 h. 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. wentii*

Spores freshly obtained from PDA slopes were transferred aseptically into 10 Erlenmeyer flasks of 250 ml, containing 100 ml of a sterile synthetic medium[28] (1.5% sucrose, 1.5% glucose, 0.5% polypeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% K_2HPO_4 and 0.001% $FeSO_4 \cdot 7H_2O$, pH 7.2) in a biological safety cabinet. After cultivation at 27 °C for 2 days on a rotary shaker (150 rpm), epiandrosterone (**1**; 500 mg, 1.72 mmol) dissolved in 10 ml of DMF was evenly distributed aseptically among the flasks. The biotransformation of the substrate was carried out in 10 flasks for 5 days under the same conditions. The fungal mycelium was separated from the broth by filtration under the vacuum and the mycelium was rinsed with ethyl acetate (500 ml). The broth was saturated with NaCl and then extracted 3 × each with 1 l of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate and the solvent was evaporated in vacuo to give a brown gum (735 mg), which was then chromatographed on a silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (60 mg, 12%), 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 11α-hydroxy-5α-androstane-3,17-dione (**4**; 95 mg, 18%), which was crystallized from petroleum ether as needles, m.p. 194–195 °C, $[\alpha]_D^{20}$ +63, *c* 0.1, $CHCl_3$ (lit.[29] gives m.p. 194–195 °C, $[\alpha]_D^{20}$ +66, *c* 1, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.41) calculated: 74.96% C, 9.27% H; found: 74.35% C, 9.73% H. IR: 3335 (O–H), 1719 (C=O), 1735 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.89 s, 3 H (H-18); 1.14 s, 3 H (H-19); 3.98 dt, 1 H, *J*(11β,12β) = 5.0, *J*(11β,9) = *J*(11β,12α) = 10.0 (H-11). ^{13}C NMR (75 MHz, $CDCl_3$): 219.82, 212.72, 68.66, 59.72, 50.09, 47.96, 47.21, 44.96, 42.75, 39.83, 38.21, 37.30, 35.79, 34.01, 30.21, 29.02, 21.75, 14.50, 11.73.

Elution with 60% ethyl acetate in hexane afforded 3β,11α-dihydroxy-5α-androstan-17-one (**5**; 285 mg, 54%), which was crystallized from acetone as cubes, m.p. 104–106 °C, $[\alpha]_D^{20}$ +48, *c* 0.1, $CHCl_3$ (lit.[29] gives m.p. 93–103 °C, $[\alpha]_D^{20}$ +53, *c* 1, $CHCl_3$). For $C_{19}H_{30}O_3$ (306.43) calculated: 74.47% C, 9.87% H; found: 74.50% C, 9.65% H. IR: 3580 (O–H), 1740 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.83 s, 3 H (H-19); 0.92 s, 3 H (H-18); 3.50 tt, 1 H, *J*(3,2) = 5.0, *J*(3,4) = 10.0 (H-3); 3.88 dt, 1 H, *J*(11β,12β) = 5.0, *J*(11β,9) = *J*(11β,12α) = 10.0 (H-11). ^{13}C NMR (75 MHz, $CDCl_3$): 219.90, 70.65, 68.43, 60.27, 50.19, 47.94, 44.97, 42.73, 38.42, 38.35, 37.25, 35.81, 34.09, 31.55, 30.65, 28.80, 21.72, 14.44, 12.64.

Biotransformation of Dehydroepiandrosterone (**2**) by *A. wentii*

Under similar conditions, the incubation of dehydroepiandrosterone (**2**; 500 mg, 1.73 mmol) with *A. wentii* afforded a brown gum (746 mg), which was then chromatographed on a silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted 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 80% ethyl acetate in hexane afforded 3β,7β-dihydroxyandrost-5-en-17-one (**6**; 63 mg, 12%), which was crystallized from ethyl acetate–petroleum ether as needles, m.p.

208–209 °C, $[\alpha]_D^{20}$ +67, *c* 0.1, $CHCl_3$ (lit.[30] gives m.p. 213–215 °C, $[\alpha]_D^{20}$ +63, *c* 1.3, $CHCl_3$). For $C_{19}H_{28}O_3$ (304.41) calculated: 74.96% C, 9.27% H; found: 74.75% C, 9.03% H. IR: 3237 (O–H), 1736 (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); 3.50 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) = 3.0 (H-7); 5.32 bs (H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 221.33, 143.64, 125.44, 72.79, 71.19, 51.12, 48.16, 47.75, 41.57, 40.38, 36.82, 36.61, 35.96, 31.40, 31.16, 24.16, 20.34, 19.14, 13.55.

Elution with pure ethyl acetate in hexane afforded 3β,7α-dihydroxyandrost-5-en-17-one (**7**; 269 mg, 51%), which was crystallized from ethyl acetate–petroleum ether as needles, m.p. 174–175 °C, $[\alpha]_D^{20}$ –73, *c* 0.1, $CHCl_3$ (lit.[30] gives m.p. 178–180 °C, $[\alpha]_D^{20}$ –80, *c* 0.14, CH_3OH). For $C_{19}H_{28}O_3$ (304.41) calculated: 74.96% C, 9.27% H; found: 74.81% C, 9.10% H. IR: 3347 (O–H), 3163 (O-H), 1734 (C=O), 1622 (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 = 24 (H-3); 3.96 bs, 1 H, W_h = 12 (H-7); 5.63 d, 1 H, *J*(6,7β) = 5.0 (H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 221.89, 145.97, 123.30, 70.75, 63.89, 47.01, 44.69, 42.24, 41.64, 37.25, 36.98, 36.73, 35.65, 30.84 (2 × C), 21.67, 19.84, 18.07, 13.08.

Biotransformation of Pregnenolone (**3**) by *A. wentii*

Under similar conditions, the incubation of pregnenolone (**3**; 500 mg, 1.58 mmol) with *A. wentii* afforded a brown gum (753 mg), which was then chromatographed on a silica gel. Elution with 30% ethyl acetate in hexane afforded the unreacted starting material (60 mg, 12%), 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 11α-hydroxypregn-4-ene-3,20-dione (**8**; 407 mg, 78%), which was crystallized from ethyl acetate as needles, m.p. 163–164 °C, $[\alpha]_D^{20}$ +165, *c* 0.1, $CHCl_3$ (lit.[31] gives m.p. 165–167 °C, $[\alpha]_D^{20}$ +169, $CHCl_3$). For $C_{21}H_{30}O_3$ (330.45) calculated: 76.32% C, 9.15% H; found: 76.21% C, 9.08% H. IR: 3380 (O–H), 1654 (C=O), 1610 (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): 0.65 s, 3 H (H-18); 1.28 s, 3 H (H-19); 2.10 s, 3 H (H-21); 3.98 dt, 1 H, *J*(11β,12β) = 5.0, *J*(11β,9) = *J*(11β,12α) = 10.0 (H-11); 5.69 s, 1 H (H-4). ^{13}C NMR (75 MHz, $CDCl_3$): 209.02, 200.49, 171.40, 124.34, 68.63, 63.02, 58.77, 55.21, 50.18, 44.04, 39.86, 37.32, 34.82, 34.06, 33.51, 31.43, 31.27, 24.12, 22.83, 18.18, 14.38.

The author would like to thank F. Sonmez and H. Genc for running the ^{1}H and ^{13}C NMR spectra.

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