Biotransformation of Testosterone and Pregnenolone Catalyzed by the Fungus *Botrytis cinerea*

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Testosterone (**1**), a male sex hormone, and pregnenolone (**2**), a precursor of many steroidal hormones, were oxidized by fermentation with the fungus *Botrytis cinerea*. The fermentation of **1** yielded 7*β*,17*β*-dihydroxyandrostan-3-one (**3**) (73%) in a yield comparable to chemical transformations. Fermentation of **2** by the same fungus afforded a major metabolite 3*β*,11α,16*β*-trihydroxypregn-5-en-20-one (**4**) (39%) along with a minor metabolite 11α,16*β*-dihydroxypregn-4-ene-3,20-dione (**5**) (6%). The metabolites are characterized by detailed physical and spectroscopic studies.

The plant pathogenic fungus, *Botrytis cinerea*, is a gray powdery mold producing botrydial and related terpenoids, which enhance the pathogenicity of the fungus.[1−5] Metabolism of some clovanes, caryophyllene oxide, and patchoulol sesquiterpenes by this fungus has previously been reported.[6−8] Our previous research on metabolism of a number of prenylated flavonoids and related phytoalexins by *B. cinerea* revealed that the fungus could epoxidize the double bond of a prenyl side chain.[9] It is, therefore, reasonable to conclude that the fungus possesses constitutive epoxidases and hydroxylases responsible for detoxification of xenobiotics, a process synonymous to drug metabolism in mammalian cells, as was nicely reviewed by Clark and Hufford[10] and Abourasheed et al.[11] It has been shown that the fungus can epoxidize acyclic double bonds, but not the rigid double bond of the ring system of terpenes. We were interested in exploring the epoxidation possibility of steroidal ring double bonds, and to determine the preference of hydroxylation over epoxidation and the effect of regiochemical and stereochemical disposition of the directing groups toward hydroxylation. Testosterone (**1**) and pregnenolone (**2**) were, therefore, chosen as substrates, which have previously been transformed by *Cephalosporium aphidicola*.[12] Testosterone (**1**) on fermentation with *B. cinerea* (AHU 9424) yielded 7*β*,17*β*-dihydroxy-androstan-3-one (**3**) in a good yield as a single metabolite, while **2** afforded a major metabolite 3*β*,11α,16*β*-trihydroxypregn-5-en-20-one (**4**) along with a minor metabolite 11α,16*β*-dihydroxypregn-4-ene-3,20-dione (**5**) (Table 1). This proved that the constitutive epoxidase enzymes of the fungus are unable to epoxidize double bonds of steroidal rings and that hydroxylation predominates over epoxidation. The C-17 hydroxyl group in androstane directs hydroxylation at 7*β*, while an acetyl-group at C-17 of pregnenolone directs hydroxylation at the C-11α and C-16*β* positions, according to the three-site attachment rule of steroidal hydroxylations.

Results and Discussion

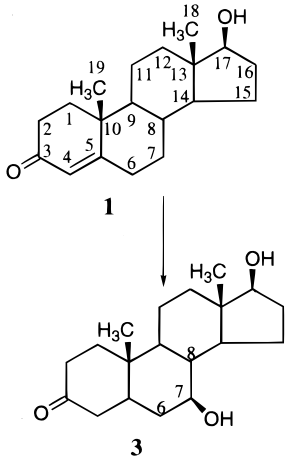
Fermentation of the male sex hormone testosterone (**1**) with *B. cinerea* (AHU 9424) for 10 days yielded a single oxidized metabolite 7*β*,17*β*-dihydroxyandrostan-3-one (**3**) in a good yield (73%) (Scheme 1). The IR spectrum of **3** showed hydroxy and ketonic absorptions at 3320 and 1705 cm^{-1}, respectively. The EIMS of **3** had a molecular ion peak at *m*/*z* 306, which was confirmed by recording the FDMS. The HREIMS of **3** showed *m*/*z* 306.2206, corresponding to the molecular formula $C_{19}H_{30}O_3$. The ^1H NMR spectrum of **3** was very similar to that of **1**, with the disappearance of the olefinic proton and the appearance of a new hydroxyl-bearing methine proton at *δ* 3.65. The ^{13}C NMR spectrum of **3** displayed resonances for 19 carbons, while DEPT spectra showed the presence of two methyl, eight methylene, six methine, and three quaternary carbons. Hydroxylation of a methylene carbon was further indicated by a lowfield hydroxyl-bearing methine signal at *δ* 70.1. The position of the newly introduced hydroxyl (C-7) was established by unambiguous assignments of all carbons and protons through combined use of HMQC, HMBC, and COSY 45 spectra. The HMBC spectrum of **3** showed

Table 1. Percentage Yields of the Metabolites of Testosterone (**1**) and Pregnenolone (**2**)

substrate	metabolite	yield (%)[a]
testosterone (**1**)	7*β*,17*β*-dihydroxyandrostan-3-one (**3**)	73
pregnenolone (**2**)	3*β*,11α,16*β*-trihydroxypregn-5-en-20-one (**4**)	39
	11α,16*β*-dihydroxypregn-4-ene-3,20-dione (**5**)	6

[a] Substrate amount = 500 mg each.

Scheme 1



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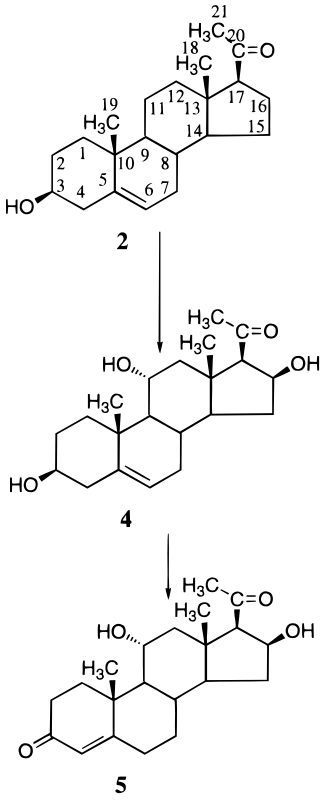
Scheme 2



correlations of H-7 (δ 3.65, dt, J = 4.6, 10.4) with C-6 (δ 30.4) and C-8 (δ 38.0), and the COSY 45 spectrum exhibited correlations of H-7 (δ 3.65, dt, J = 4.6, 10.4) with H-6 (δ 1.45) and H-8 (δ 1.86). The β-stereochemistry of the hydroxyl group at C-7 was established by the multiplicity pattern of H-$7_{eq\ (\alpha)}$ (δ 3.65, dt, J 7_{ax}, 6_{ax} = 7_{ax}, 8_{ax} = 10.4, and J 7_{ax}, 6_{eq} = 4.6 Hz) and the NOESY correlations of H-7α (δ 3.65) with H-9α (δ 2.14), H-5α (δ 1.81), and H-14α (δ 1.20). Thus, **3** was characterized as 7β,17β-dihydroxyandrostan-3-one, probably formed either by reduction of **1** to afford 4,5-dihydrotestosterone followed by hydroxylation at C-7, or by hydroxylation at C-7 to yield 7β-hydroxytestosterone followed by reduction of the $\Delta^{4,5}$ double bond (Scheme 1).

Two new oxidized metabolites of pregnenolone (**2**) obtained from biotransformation by *B. cinerea* were characterized as 3β,11α,16β-trihydroxypregn-5-en-20-one (**4**) and 11α,16β-dihydroxypregn-4-ene-3,20-dione (**5**) (Scheme 2). The IR spectrum of **4** displayed absorptions at 3337, 1698, and 1628 cm^{-1}. The FDMS and EIMS of **4** displayed the molecular ion peak at *m*/*z* 348, indicating two additional oxygen atoms. The molecular formula of **4** was deduced as $C_{21}H_{32}O_4$ from HREIMS (*m*/*z* 348.2326). The ^{1}H NMR spectrum of **4** showed two downfield signals for oxygen-bearing methine protons at δ 4.21 (dt, J = 4.7, 10.1 Hz) and 4.29 (dt J = 4.6, 10.0 Hz) suggesting hydroxylation at any two of the 11α-, 15β-, or 16β-positions. The ^{13}C NMR of **4** exhibited resonances for 21 carbons, while DEPT spectra revealed three methyl, six methylene, eight methine, and four quaternary carbons. The two additional downfield methine carbons resonated at δ 71.8 and 70.4 (11-*C*HOH and 16-*C*HOH, respectively). The C-11 hydroxyl group was established from HMBC correlations of H-11 (δ 4.21) with C-9 (56.5) and COSY 45 correlations of H-11 (δ 4.21) with H-12 (δ 1.61, 1.63) and H-9 (δ 1.73). The α-stereochemistry of OH-11 was confirmed from multiplicity of the 11-βH (δ 4.21, dt, $J_{11ax,9ax}$ = $J_{11ax,12ax}$ = 10.1 and $J_{11ax,12eq}$ = 4.7 Hz) and the NOESY correlations of H-11β (δ 4.21) and H-18 (δ 1.38). The new hydroxyl at C-16 was assigned based on HMBC correlations of H-16 (δ 4.29) with C-17 (δ 62.5), and COSY correlations of H-16 (δ 4.29) with H-17 (δ 2.47) and H-15 (δ 1.48). The multiplicity of the H-16 (δ 4.29, dt, $J_{16\alpha,15\alpha}$ = $J_{16\alpha,17\alpha}$ = 10.0 and $J_{16\alpha,15\beta}$ = 4.6 Hz) and the NOESY correlations of H-16α (δ 4.29) with H-14α (δ 1.45) and H-17α (δ 2.47) indicated β-stereochemistry of OH-16.

The FDMS and EIMS of **5** displayed the molecular ion peak at *m*/*z* 346 and HREIMS corresponded to molecular formula $C_{21}H_{30}O_4$. The IR spectrum of **5** displayed absorptions at 3348, 1706, 1651, and 1611 cm^{-1}. The ^{1}H NMR spectrum was very similar to that of **4**, except that the signal for H-3α disappeared due to oxidation of the 3-hydroxyl group to a keto group. Resonances for 21 carbons were observed in the ^{13}C NMR spectrum of **5**, while DEPT spectra revealed three methyl, six methylene, seven methine, and five quaternary carbons. Two lowfield hydroxyl-bearing methine signals resonated at δ 73.9 and 68.8 and a ketonic resonance at δ 199.9 confirmed the introduction of two hydroxy groups and oxidation of the 3-OH. The C-11 position of the newly introduced hydroxyl group was deduced from the HMBC correlations of C-11 (δ 73.9) with H-12 (δ 1.51, 2.31) and H-9 (δ 1.19), and COSY interactions of H-11 (δ 3.97) with H-12 (δ 1.51, 2.31) and H-9 (δ 1.19). The α-sterochemistry of OH-11 was established from the coupling pattern of 11-βH (δ 3.97, dt, $J_{11\beta,12\alpha}$ = $J_{11\beta,9\alpha}$ = 10.6 Hz and $J_{11\beta,12\beta}$ = 4.8 Hz) and the NOESY correlations of H-11β with CH_3-18β (δ 0.76), CH_3-19β (δ 1.35). The second hydroxyl group was placed at 16β based on the HMBC correlations of H-16 (δ 3.31) with C-17 (δ 62.3) and C-15 (δ 27.1) and COSY couplings of H-16 (δ 3.31, dt, $J_{16\alpha,17\alpha,15\alpha}$ = 9.5 Hz, $J_{16\alpha,15\beta}$ = 5.1 Hz) with H-17 (δ 2.59), H-15 (δ 1.55, 2.34), multiplicity pattern of the H-16α and NOESY correlations of H-16α (δ 3.31) with H-14α (1.44) and H-17α (δ 2.59). Thus, **5** was characterized as 11α,16β-dihydroxypregn-4-ene-3,20-dione probably formed by the oxidation of 3-OH of the **4** (Scheme 2).

Experimental Section

General Experimental Procedures. The Si gel 60 mesh 230−400 was used for column chromatography. Purity of the samples was checked on Merck Kieselgel 60 F_{254}, 0.2-mm thick TLC plates, while the spots were viewed under 254 and 365 nm UV light and sprayed with EtOH−H_2SO_4 (1:1). Melting points were recorded on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. Optical rotations were carried out on a JASCO DIP-370 polarimeter. The IR spectra were recorded in $CHCl_3$ on a Perkin-Elmer 2000 FTIR. A JEOL JMS-SX 102 A mass spectrometer was used to record the mass spectra. ^{13}C NMR spectra were recorded on a JEOL EX-270 spectrometer, while collecting at 65 MHz, and the ^{1}H and 2D NMR spectra were recorded on a Bruker AMX500 spectrometer.

General Fermentation and Extraction Conditions. To distilled water (2 L) were added glucose (80 g), yeast extract (2 g), KH_2PO_4 (10 g), $MgSO_4$ (1 g), $NaNO_3$ (4 g), $FeSO_4$ (20 mg), and $ZnSO_4$ (10 mg) to prepare the fermentation medium for *B. cinerea* (AHU 9424). The fermentation medium thus obtained was distributed equally among 10 flasks of 500-mL capacity (200 mL in each) and autoclaved. Two-day-old seeds of *B. cinerea* (1 mL) from stage I fermentation were inoculated into each flask and incubated on a reciprocal shaker for 3 days. A clear ethanolic solution (10 mL) of the substrate (500 mg) was equally distributed in the culture flasks, and fermentation was carried out for a further 10 days. The mycelium was filtered and washed with H_2O and ethyl acetate, and the broth thus obtained was extracted with ethyl acetate (6 L). The ethyl

acetate extract was washed with brine and dried over anhydrous sodium sulfate. Evaporation of the solvent on a rotary evaporator yielded a brown gum that was adsorbed on an equal quantity of Si gel and subjected to column chromatography.

Biotransformation of Testosterone (1). The brown gum (873 mg) obtained by fermentation of **1** was washed with ethyl acetate, and the colorless solid, obtained by crystallization as a pure compound, was characterized as 7*β*,17*β*-dihydroxyandrostan-3-one (**3**) (391 mg).

7*β*,17*β*-Dihydroxyandrostan-3-one (3): colorless amorphous material; mp 196−197 °C; $[\alpha]^{20}_D$ −94°(*c* 0.10, MeOH); IR ($CHCl_3$) ν_{max} 3320, 1705 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.69 (1H, t, J = 8.5 Hz, H-17), 3.65 (1H, dt, J = 4.6, 10.4 Hz, H-7α), 2.17 (2H, m, H-12), 2.14 (1H, m, H-9α), 1.95 (2H, m, H-2), 1.86 (1H, m, H-8*β*), 1.83 (2H, m, H-4), 1.81 (1H, m, H-5α), 1.76 (2H, m, H-16), 1.62 (2H, m, H-11), 1.45 (2H, m, H-6), 1.20 (1H, m, H-14α), 1.18 (2H, m, H-15), 0.96 (2H, m, H-1), 0.87 (3H, s, H-18), 0.74 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 65 MHz) δ 213.5 (C-3), 81.5 (C-17), 70.1 (C-7), 59.4 (C-9), 51.6 (C-14), 43.5 (C-13), 42.2 (C-12), 40.3 (C-5), 38.0 (C-8), 37.1 (C-1), 36.5 (C-10), 34.8 (C-4), 34.4 (C-2), 30.4 (C-6), 29.9 (C-16), 23.2 (C-15), 23.1 (C-19), 20.5 (C-11), 11.1 (C-18); FDMS *m*/*z* 306 [M^+, 100%); EIMS *m*/*z* 306 [M^+] (100), 288 [$M^+ - 18$] (31), 273 (18), 229 (14), 189 (12), 161 (11), 149 (12), 133 (14), 121 (13), 107 (16), 95 (30), 79 (20), 67 (16), 55 (17), 43 (11), 41 (13); HREIMS *m*/*z* 306.2206 (calcd for $C_{19}H_{30}O_3$, 306.2196).

Biotransformation of Pregnenolone (2). Brown gummy material (1.4 g) obtained by fermentation of **2** was adsorbed on Si gel (1.4 g) and chromatographed. Elution with pure ethyl acetate afforded **2** (starting material, 27 mg). Elution with 2% MeOH in EtOAc yielded 11α,16*β*-dihydroxypregn-4-ene-3,20-dione (**5**) as a colorless powder (35 mg). Elution with 5% MeOH in EtOAc yielded 3*β*,11α,16*β*-trihydroxypregn-5-en-20-one (**4**) (213 mg).

3*β*,11α,16*β*-trihydroxypregn-5-en-20-one (4): colorless crystals; mp 238−239 °C; $[\alpha]^{20}_D$ −85° (*c* 0.11, MeOH); IR ($CHCl_3$) ν_{max} 3337, 1698, 1628 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ 5.88 (1H, s, H-6), 4.29 (1H, dt, J = 4.6, 10.0 Hz, H-16α), 4.21 (1H, dt, J = 4.7, 10.1 Hz, H-11*β*), 3.83 (1H, tt, J = 5.4, 10.3 Hz, H-3α), 2.52 (2H, m, H-4), 2.47 (1H, d, J = 10.0 Hz, H-17), 2.47 (1H, m, H-8α), 2.34 (2H, m, H-7), 2.06 (2H, m, H-2), 2.03 (3H, s, H-21), 1.73 (1H, m, H-9α), 1.63 (1H, d, J = 10.1 Hz, H-12α), 1.61 (1H, d, J = 4.7 Hz, H-12*β*), 1.48 (2H, m, H-15), 1.45 (1H, ddd, J = 6.1, 10.1, 12.3 Hz, H-14α), 1.38 (3H, s, H-18), 1.31 (2H, m, H-1), 1.26 (3H, s, H-19); ^{13}C NMR (C_5D_5N, 65 MHz) δ 208.2 (C-20), 166.1 (C-5), 127.6 (C-6), 72.2 (C-3), 71.8 (C-11), 70.4 (C-16), 62.5 (C-17), 56.5 (C-9), 54.9 (C-14), 50.2 (C-12), 44.5 (C-13), 42.7 (C-8), 40.5 (C-10), 40.2 (C-4), 37.5 (C-2), 34.5 (C-1), 31.0 (C-21), 27.3 (C-15), 24.0 (C-7), 18.4 (C-19), 16.8 (C-18); FDMS *m*/*z* 348 [M^+, 100%); EIMS *m*/*z* 348 [M^+] (8), 330 [$M^+ - 18$] (100), 315 [$M^+ - (18 + 15)$] (5), 312 (23), 297 (10), 279 (8), 269 (6), 251 (5), 242 (7), 227 (5), 209 (8), 195 (4), 183 (3), 171 (5), 157 (7), 145 (10), 141 (10), 129 (6), 119 (11), 106 (18), 105 (11), 91 (11), 81 (12), 79 (7), 67 (5), 55 (5), 43 (28), 41 (5); HREIMS *m*/*z* 348.2326 (calcd for $C_{21}H_{32}O_4$, 348.2302).

11α,16*β*-Dihydroxypregn-4-ene-3,20-dione (5): colorless amorphous material; mp 229−230 °C; $[\alpha]^{20}_D$ −60°(*c* 0.11, MeOH); IR ($CHCl_3$) ν_{max} 3348, 1706, 1651, 1611 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 5.73 (1H, s, H-4), 3.97 (1H, dt, J = 4.8, 10.6 Hz, H-11*β*), 3.31 (1H, dt, J = 5.1, 9.5 Hz, H-16α), 2.59 (1H, d, J = 9.5 Hz, H-17α), 2.45 (2H, m, H-6), 2.34 (2H, m, H-15), 2.31 (1H, d J = 4.8 Hz, H-12*β*), 2.26 (2H, m, H-2), 2.15 (2H, m, H-7), 2.13 (3H, s, H-21), 2.08 (2H, m, H-1), 1.60 (1H, m, H-8α), 1.51 (1H, d, J = 10.6, H-12α), 1.44 (1H, m, H-14α), 1.35 (3H, s, H-19), 1.19 (1H, d, J = 10.6, H-9α), 0.76 (3H, s, H-18); ^{13}C NMR (CD_3OD, 65 MHz) δ 208.9 (C-20), 199.9 (C-3), 166.7 (C-5), 125.6 (C-4), 73.9 (C-11), 68.8 (C-16), 62.3 (C-17), 56.3 (C-9), 54.6 (C-14), 50.5 (C-12), 44.8 (C-13), 43.1 (C-6), 42.4 (C-8), 39.4 (C-10), 37.2 (C-1), 34.2 (C-2), 31.4 (C-21), 27.1 (C-15), 23.9 (C-7), 18.4 (C-19), 14.6 (C-18); FDMS *m*/*z* 346 [M^+, 100%); EIMS *m*/*z* 346 [M^+] (100), 331 [$M^+ - 15$] (9), 328 (50), 310 (28), 295 (20), 285 (19), 267 (30), 255 (10), 243 (26), 225 (23), 211 (9), 197 (8), 185 (11), 173 (17), 161 (29), 149 (18), 145 (20), 133 (27), 123 (46), 122 (26), 121 (25), 109 (34), 107 (29), 95 (37), 91 (33), 81 (23), 79 (21), 67 (13), 55 (16), 43 (65), 41 (14); HREIMS *m*/*z* 346.2164 (calcd for $C_{21}H_{30}O_4$, 346.2145).

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