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Steroid transformations with *Exophiala jeanselmei* var. *lecanii-corni* and *Ceratocystis paradoxa*☆

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Abstract

The fungi *Exophiala jeanselmei* var. *lecanii-corni* [IMI (International Mycological Institute) 312989, UAMH (University of Alberta Microfungus Collection and Herbarium) 8783] and *Ceratocystis paradoxa* (IMI 374529, UAMH 8784) have been examined for their potential in steroid biotransformation. The study has determined that *E. jeanselmei* var. *lecanii-corni* effected overall anti-Markovnikov hydration on dehydroisoandrosterone, and side-chain degradation on a variety of pregnanes. Both ascomycetes were found to carry out redox reactions of alcohols and ketones as well as 1,4 reduction of α,β-unsaturated carbonyl systems.

Keywords: *Exophiala jeanselmei* var. *lecanii-corni*; *Ceratocystis paradoxa*; Biotransformation; Hydration; Steroid; Ascomycete

1. Introduction

Fungi belonging to the little known genus *Exophiala* are described as black yeasts because of their yeast-like growth and production of melanin [1]. The chemistry of the genus has been little studied until recently [2]. In this regard, strains of *Exophiala jeanselmei* have been found to degrade styrene [3] and the involvement of a P-450 monooxygenase has been confirmed [4]. In other studies, the fungus has been used in the breakdown of syringic acid [5] and cyclohexanol [6]. *E. jeanselmei* var. *lecanii-corni* (Benedek et Specht) de Hoog was first isolated from the soft scale insect *Lecanius corni* (European fruit lecanium). This subspecies is synonymous with *Torula lecanii-cornii* and *Pullularia fermentans* var. *benedekii*. Our isolate was a contaminant in an in vitro culture where the strain was observed to enhance the growth of *Zingiber officinale* plantlets. The mechanism by which this occurs is yet to be determined.

Ceratocystis paradoxa (Dale) C. Moreau boasts an extensive worldwide distribution. It is otherwise known as *Ceratosomella paradoxa*, *Ophiostoma paradoxa*, and *Thielaviopsis paradoxa* and causes widespread damage to many crops, such as sugar cane, pineapple, banana, plantain, and coconut, among others [7,8]. While no secondary metabolites have been reportedly isolated from *C. paradoxa*, isocoumarins have been isolated from *Ceratocystis minor* [9] and *Ceratocystis fimbriata* [10]. Novel sesquiterpene alcohols, cerapicol and ceratopicanol, were isolated from cultures of *Ceratocystis piceae* [11]. *Ceratocystis* spp. have been used in stereoselective ketone reduction [12,13] and resolution of ketoprofen [14].

Based on the forgoing and our interest in the chemistry of fungi [15], we decided to investigate these ascomycetes for biocatalytic utility on some steroid substrates.

2. Materials and methods

^{1}H and ^{13}C NMR spectra were determined at 200 and 50 MHz, respectively, on a Bruker AC 200 instrument. The multiplicities of the carbon atoms were determined

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Table 1
C-13 NMR resonances of transformed steroids determined in $CDCl_3$

	2[a]	3	3[a]	6	7	8[a]	9	11	12	14	15	16	17	18[a]	20	21[a]	22[a]	24[a]
1	35.0	37.1	37.0	37.0	38.6	35.0	36.1	34.5	34.4	156.0	154.6	160.3	35.7	32.8	38.5	36.7	34.8	126.4
2	26.0	31.3	27.7	20.9	38.2	26.8	30.3	31.2	31.1	127.3	127.7	126.7	33.9	26.0	38.1	33.9	34.0	118.6
3	72.9	71.0	73.9	66.5	211.0	74.1	71.3	71.4	71.1	186.8	186.0	199.7	199.2	69.9	211.9	73.6	199.4	148.4
4	30.4	42.1	38.1	135.4	44.7	37.0	35.2	36.4	36.4	124.3	124.7	38.8	124.1	32.8	44.6	27.4	124.6	121.5
5	44.9	139.6	139.8	132.3	46.8	41.8	41.7	42.7	42.8	167.6	165.7	41.4	170.3	40.0	46.6	44.6	168.2	138.2
6	74.0	120.8	122.2	33.1	28.8	26.5	26.8	26.5	26.3	33.6	32.0	25.7	32.6	28.0	28.8	28.4	33.7	29.5
7	31.5	31.3	31.4	30.2	31.3	25.8	25.2	26.5	25.7	32.3	31.8	25.3	31.3	30.8	31.6	31.9	32.3	27.0
8	31.0	31.7	31.7	37.0	35.5	35.5	35.2	37.7	36.3	36.5	35.9	35.6	35.1	35.0	35.3	35.4	36.7	38.2
9	40.6	50.0	50.0	56.2	54.0	40.4	40.6	51.7	52.2	59.9	60.7	58.3	53.8	54.3	53.6	54.0	62.5	44.0
10	34.3	36.4	36.6	39.7	35.9	34.5	34.6	34.1	34.2	42.5	42.2	37.6	38.6	35.9	35.6	35.4	38.1	137.9
11	19.7	20.4	20.5	19.6	21.1	20.2	19.9	210.5	209.2	73.0	207.3	207.9	20.3	20.0	21.4	21.1	209.3	26.0
12	31.5	36.4	36.7	34.7	36.7	32.1	31.6	54.7	50.5	51.3	50.3	49.7	30.7	31.5	38.9	39.0	51.4	36.8
13	47.7	42.5	42.4	44.6	43.0	42.6	47.7	47.1	50.6	51.4	49.9	50.1	47.5	47.8	44.1	44.2	51.4	42.9
14	51.2	51.1	51.0	51.1	50.9	50.8	51.3	50.4	50.5	48.1	49.4	49.4	50.8	51.5	56.4	56.6	48.6	49.8
15	21.7	23.2	23.6	23.4	23.4	23.4	21.6	22.7	21.5	23.4	21.5	21.5	21.7	21.7	24.4	24.3	23.3	23.3
16	35.7	30.1	27.5	28.6	30.5	27.5	35.8	30.8	36.0	33.6	35.8	36.0	35.7	35.8	22.8	22.7	32.2	27.8
17	220.0	81.2	82.8	82.2	81.9	82.7	221.3	80.0	217.5	84.1	216.2	217.0	220.3	221.4	63.7	63.8	83.5	82.7
18	13.8	10.8	11.9	12.9	11.1	12.0	13.6	11.7	14.5	15.6	14.6	14.9	13.7	13.8	13.4	13.4	15.4	12.0
19	24.7	19.2	19.3	19.6	11.5	23.2	23.1	23.1	23.1	18.7	18.8	20.5	17.4	11.3	11.4	12.2	17.2	
20										211.1					210.0	209.1	73.9	
21										64.2					31.5	31.5	63.6	
Ac	21.2		21.2			21.1								21.5		21.4	20.7	21.1
Ac	21.5		21.4			21.3											21.0	21.2
Ac	170.0		170.5			170.5								170.7		171.3	169.4	170.0
Ac	170.2		171.2			171.0										170.8	171.3	

[a] Denotes that the data on the acetate is presented.

by the attached proton test (APT) and distortionless enhancement by polarization transfer (DEPT) experiments. The solvents used were deuterated chloroform with tetramethylsilane as the internal standard. ^{13}C NMR data can be found in Table 1. Mass spectral data was obtained at an ionizing voltage of 70 eV on a Kratos AEI MS-50 instrument for high resolution electron impact (EI) ionization and an MS-12 for chemical ionization (CI) spectrometry. Infrared data was obtained using a Perkin Elmer Fourier transform spectrophotometer and are for KBr discs. Melting points were obtained on a Thomas-Hoover apparatus with open capillary tubes. Ultraviolet spectra were recorded on a Hewlett Packard HP 8452A diode array spectrophotometer. Optical rotations were carried out on a Perkin Elmer 241 mc polarimeter. Thin-layer chromatography was effected on Merck ($HF_{254+366}$) glass- or polyester-backed plates (0.25-mm thick). Visualization of both fed steroids and metabolized products was done by spraying the plates with a mixture of methanol-conc. sulfuric acid (1:1) and heating in an oven at 60°C for 2 min until the colors developed. In some instances, visualization was effected with the aid of a Spectroline ultraviolet lamp model ENF-24. Flash chromatography was done using Merck Kieselgel silica (40–63 μm dia). Petrol refers to the petroleum fraction boiling between 60° and 80°C. Steroids were obtained from Steraloids, Wilton, NH. Fermentations were performed on a Gallenkamp Orbital Shaker. A Janke and Kunkel Ultra-Turrax T25 homogenizer was used to break up the fungal cells. The pH readings were taken with a Schott-Gerate pH meter. Acetylation of some metabolites was performed to improve their solubility in the nuclear magnetic resonance (NMR) solvent.

2.1. *Exophiala jeanselmei var. lecanii-corni*

The fungus was isolated from contaminated ginger (*Z. officinale*) in vitro plantlets. Dr D.W. Minter, International Mycological Institute (IMI), Egham, Surrey, U.K. recognized the organism as belonging to the genus *Exophiala* (IMI 312989). Dr Lynne Sigler, University of Alberta Microfungus Collection & Herbarium (UAMH), Edmonton, Alberta, Canada, identified the strain as *E. jeanselmei* var. *lecanii-corni* (UMAH 8783). The ascomycete was maintained on potato dextrose agar slants. The optimal growth medium, per liter, consisted of: sucrose 10 g, yeast extract 4 g, bactopeptone 5 g, $MgSO_4.7H_2O$ 1 g, and KH_2PO_4 1 g. In a typical fermentation, 20 2-L Erlenmeyer flasks each containing 500 ml of medium were used, and inoculation was effected with one 14-d-old slant/two flasks. Cultures were shaken at 90 rpm at 27°C. After 6.5 d, the mycelial mass was filtered from the broth and was resuspended in ten 2-l flasks each containing 500 ml of sterile water. Nine flasks were fed with steroid (1 g) dissolved in hot ethanol (9 ml). The single control was fed ethanol (1 ml) only. The flasks were shaken for a further 7 d, after which the fungus was harvested. The broth was extracted with chloroform (×3). The mycelium was homogenized in the solvent. The

extracts were combined, dried with sodium sulfate, and the solvent was removed in vacuo. The mycelial residue was dried at 90°C in an oven for 12 h.

2.2. 3β-Hydroxyandrost-5-en-17-one **(1)**

The fermentation of **1**, 1 g, yielded 1.05 g of organic extract and 55.31 g of dry mycelium on harvesting. The extract was subjected to column chromatography using increasing concentrations of ethyl acetate in petrol. The fraction eluting in 10% ethyl acetate was untransformed steroid (55 mg). Further elution using 20% ethyl acetate in petrol afforded 3β,17β-dihydroxyandrost-5-ene (**3**), 115 mg, which crystallized from chloroform as cubes, mp 169–171°, $[\alpha]_D$ − 47° (c = 0.11, EtOH); lit. [16] mp 177–179°, $[\alpha]_D$ − 55° (c = 0.4, ipa); IR ν_{max} 3381, 3308, 2972, 1467 cm^{-1}; HRMS (EI) m/z (%) 290.2248 (100) $[M]^+$ $[C_{19}H_{30}O_2]$, 275 (18), 272 (62), 257 (41), 205 (58), 145 (54), 107 (80); ^1H NMR ($CDCl_3$) δ 0.75 (3H, s, H-18), 1.05 (3H, s, H-19), 3.50 (1H, m, w/2 = 14 Hz, H-3β), 3.63 (1H, t, J = 8.5 Hz, H-17β), 5.30 (1H, d, J = 5.6 Hz, H-6). Further elution of the transformed products with 50% ethyl acetate in petrol gave 3β,6β-dihydroxy-5β-androstan-17-one [17] (**2**), 93 mg, which crystallized from chloroform as cubes, mp 226–230°, $[\alpha]_D$ + 104° (c = 0.25, EtOH); IR ν_{max} 3479, 3397, 1735 cm^{-1}; high resolution mass spectrometry (HRMS) (EI) m/z (%) 306.2182 (5) $[C_{19}H_{30}O_3]$, 288 (12), 270 (2), 233 (100), 138 (2), 94 (9); Acetylation of **2**, 80 mg, followed by column chromatography in 20% ethyl acetate in petrol enabled the purification of 3β,6β-diacetoxy-5β-androstan-17-one (71.3 mg), which resisted crystallization; IR ν_{max} 2944, 1738, 1366, 1236 cm^{-1}; MS (CI) m/z (%) 390 (0.1) $[C_{23}H_{34}O_5]$, 331 (0.1), 272 (2.5), 270 (100); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.01 (3H, s, H-19), 2.00 (3H, s, CH_3CO_2), 2.04 (3H, s, CH_3CO_2), 4.65 (1H, m, w/2 = 18 Hz, H-3α); 4.75 (1H, m, w/2 = 9 Hz, H-6α).

2.3. 3β-Hydroxypregn-5-en-20-one **(4)**

The fermentation of **4**, 1 g, yielded an organic extract of 1.01 g and a dry mycelial weight of 43.6 g. The organic extract was subjected to acetylation. This was then purified by column chromatography using increasing concentrations of ethyl acetate in petrol. The fraction eluting in 15% ethyl acetate in petrol contained 3β,17β-diacetoxyandrost-5-ene, 142 mg, which crystallized from methanol as plates, mp 150–153°, $[\alpha]_D$ − 48° (c = 0.11, EtOH); lit. [18] mp 158–159°, $[\alpha]_D$ − 56°; IR ν_{max} 2939, 1734, 1364, 1246 cm^{-1}; MS (CI) m/z (%) 374 (0.6), 314 (9), 270 (100), 254 (2.7); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.05 (3H, s, H-19), 2.05 (6H, s, 2 × CH_3CO_2), 4.60 (2H, m, w/2 = 16 Hz, H-3α, 17α), 5.38 (1H, d, J = 8 Hz, H-6).

2.4. 17β-Hydroxyandrost-4-en-3-one **(5)**

The biotransformation of **5**, 1 g, yielded 49.4 g of dried mycelium and 1.91 g of organic extract on harvesting. Column chromatography on the organic extract using increasing concentrations of ethyl acetate in petrol gave 228 mg of fed and 713 mg of transformed steroid. The fraction eluting in 20% ethyl acetate was 3β,17β-dihydroxyandrost-4-ene (**6**), 15 mg, which resisted crystallization; IR ν_{max} 3418, 2954, 1457 cm^{-1}; HRMS (EI) m/z (%) 290.2238 (100) $[C_{19}H_{30}O_2]$, 272 (13), 231 (50), 107 (27); ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, H-18), 0.90 (3H, s, H-19), 3.98 (1H, m, w/2 = 18 Hz, H-17α), 5.20 (1H, m, w/2 = 12 Hz, H-3α), 6.25 (1H, d, J = 6 Hz, H-4). Further elution with 20% ethyl acetate in petrol enabled the purification of 17β-hydroxy-5α-androstan-3-one (**7**), 4 mg, which crystallized from methanol as cubes, mp 184–186°, $[\alpha]_D$ + 37° (c = 0.02, EtOH); lit. [19] mp 181°, $[\alpha]_D$ + 32° (EtOH); IR ν_{max} 3434, 2925, 1738, 1467 cm^{-1}; HRMS (EI) m/z (%) 290.2244 (83) $[C_{19}H_{30}O_2]$, 272 (24), 231 (27); ^1H NMR ($CDCl_3$) δ 0.75 (3H, s, H-18), 1.00 (3H, s, H-19), 3.65 (1H, t, J = 6 Hz, H-17α). Increasing the polarity of the solvent system to 40% then to 50% ethyl acetate in petrol gave 3α-hydroxy-5β-androstan-17-one (**9**), 104 mg. Crystallization from chloroform afforded cubes, mp 171–173°, $[\alpha]_D$ = +84° (c = 0.3, EtOH); lit. [20] mp 175–176°, $[\alpha]_D$ + 89° (MeOH); IR ν_{max} 3404, 2932, 1738, 1465 cm^{-1}; HRMS (EI) m/z (%) 290.2247 (100) $[C_{19}H_{30}O_2]$, 272 (56), 257 (50), 244 (50); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 0.94 (3H, s, H-19), 3.62 (1H, m, w/2 = 16 Hz, H-3β). Increasing the polarity to 80% ethyl acetate in petrol gave 3α,17β-dihydroxy-5β-androstane (**8**), 268 mg, which crystallized from chloroform as plates, mp 234–236°, $[\alpha]_D$ = +18° (c = 0.25, EtOH); lit. [21] mp 236°, $[\alpha]_D$ + 25° (c = 0.07, EtOH); IR ν_{max} 3632, 3346, 2937, 1451 cm^{-1}; HRMS (EI) m/z (%) 292.2390 (16) $[C_{19}H_{32}O_2]$, 274 (100), 259 (25), 256 (68), 241 (33), 215 (96). Compound **8**, 100 mg, was acetylated in the usual manner. Column chromatography on the acetylated mass, 114 mg, using 10% ethyl acetate in petrol gave pure 3α,17β-diacetoxy-5β-androstane, 99 mg, which resisted crystallization; IR ν_{max} 2934, 1736, 1378, 1027 cm^{-1}; MS (CI) m/z (%) 316 (20), 256 (33); ^1H NMR ($CDCl_3$) δ 0.75 (3H, s, H-18), 0.91 (3H, s, H-19), 2.0 (6H, s, 2 × CH_3CO_2), 4.55 (1H, t, J = 8 Hz, H-17α), 4.70 (1H, m, w/2 = 16 Hz, H-3β).

2.5. 17α,21-Dihydroxypregn-4-ene-3,11,20-trione **(10)**

Fermentation of **10**, 1 g, yielded 21.2 g of dry mycelium and 2.19 g of organic extract at harvest. Column chromatography on the extract with 30% ethyl acetate in petrol gave 3α-hydroxy-5β-androstane-11,17-dione (**12**), 47 mg, which crystallized from methanol as cubes, mp 189–192°, $[\alpha]_D$ + 141.3° (c = 0.23, EtOH); lit. [22] mp 188–189°, $[\alpha]_D$ + 144° (EtOH); IR ν_{max} 3428, 2926, 1742, 1705, 1452, 1076 cm^{-1}; HRMS (EI) m/z (%) 304.2041 (64) $[C_{19}H_{28}O_3]$, 286 (62), 271 (51), 232 (100), 199 (76); ^1H NMR ($CDCl_3$)

δ 0.80 (3H, s, H-18), 1.21 (3H, s, H-19), 3.60 (1H, m, w½ = 15 Hz, H-3β). Increasing the polarity to 80% ethyl acetate in petrol gave 3α,17β-dihydroxy-5β-androstan-11-one (**11**), 85 mg, which resisted crystallization; IR ν_{max} 3375, 2923, 1695, 1449, 1081 cm^{-1}; HRMS (EI) m/z (%) 306.2205 (43) [$C_{19}H_{30}O_3$], 288 (56), 237 (49), 232 (90), 193 (43), 93 (46); ^{1}H NMR ($CDCl_3$) δ 0.70 (3H, s, H-18), 1.10 (3H, s, H-19), 3.65 (1H, m, w½ = 15 Hz, H-3β), 3.85 (1H, t, J = 6 Hz, H-17α).

*2.6. 17α,21-Dihydroxypregna-1,4-diene-3,11,20-trione **(13)***

The fermentation of **13**, 1 g, yielded 42.9 g of dried mycelium and 1.7 g of organic extract on harvest. The extract was subjected to column chromatography using increasing concentrations of ethyl acetate in petrol. The fraction eluting in 30% ethyl acetate was 5β-androst-1-ene-3,11,17-trione (**16**), 100 mg. Crystallization from chloroform afforded cubes, mp 173–175°, $[\alpha]_D$ + 190.6°; (c = 0.25, EtOH); lit. [23] mp 174.5–175.5°, $[\alpha]_D$ + 205°; ($CHCl_3$); IR ν_{max} 2929, 1742, 1707, 1680 cm^{-1}; UV (MeOH) λ_{max} 224 nm, (log ε = 4.2); HRMS (EI) m/z (%) 300.1721 (72) [$C_{19}H_{24}O_3$], 285 (100), 122 (25), 109 (20); ^{1}H NMR ($CDCl_3$) δ 0.81 (3H, s, H-18), 1.30 (3H, s, H-19), 5.75 (1H, d, J = 8 Hz, H-2), 7.40 (1H, d, J = 8 Hz, H-1).

Further elution with 50% ethyl acetate in petrol gave androsta-1,4-diene-3,11,17-trione (**15**), 64 mg, which crystallized from ethanol as cubes, mp 194–196°, $[\alpha]_D$ + 251° (c = 0.18, EtOH); lit. [24] mp 195–196°, $[\alpha]_D$ + 235° (c = 0.1, acetone); IR ν_{max} 2937, 1742, 1708, 1663, 1621, 1456, 1225 cm^{-1}; UV (MeOH) λ_{max} 240 nm, (log ε = 3.14); HRMS (EI) m/z (%) 298.1562 (100) [$C_{19}H_{22}O_3$], 280 (14); ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.40 (3H, s, H-19), 6.06 (1H, s, H-4), 6.18 (1H, d, J = 8 Hz, H-2), 7.60 (1H, d, J = 8 Hz, H-1).

Increasing the polarity of the solvent to 60% ethyl acetate in petrol allowed the isolation of 22.8 mg of untransformed steroid. Further elution with 90% ethyl acetate in petrol gave a fraction which contained 11α,17α,21-trihydroxypregna-1,4-diene-3,20-dione (**14**), 53.6 mg, which resisted crystallization; IR ν_{max} 3400, 2937, 1702, 1659, 1617, 1046 cm^{-1}; UV (MeOH) λ_{max} 238 nm (log ε = 2.99); HRMS (EI) m/z (%) 360.1921 (20) [$C_{21}H_{28}O_5$], 342 (14), 299 (64), 281 (12), 121 (100); ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, s, H-18), 1.42 (3H, s, H-19), 4.10 (1H, q, J = 8 Hz, H-11β), 6.05 (1H, s, H-4), 6.20 (1H, d, J = 8 Hz, H-2), 7.70 (1H, d, J = 8 Hz, H-1).

2.7. Ceratocystis paradoxa

This isolate was obtained from the Coconut Industry Board, Kingston, Jamaica. Rejuvenation of the fungus, by inoculation of the host (young *Cocos nucifera* nuts, 8–10 cm in length) and reisolation, became necessary after about 12 months in culture. The ascomycete was identified by Dr P.L. Coates Beckford, Botany Department University of the West Indies and this was confirmed by Dr P.M. Kirk, International Mycological Institute, Egham, Surrey, U.K. (IMI 374529). A specimen was deposited with the University of Alberta Microfungus Collection & Herbarium (UAMH 8784). The microorganism was maintained on potato dextrose agar slants. The growth medium was a modification of the Salmink medium [25]: sucrose 20 g, corn steep solids 4 g, yeast extract 2 g, KH_2PO_4 6 g, $MgSO_4.7H_2O$ 4 g, $ZnSO_4.7H_2O$ 8 mg, $FeCl_3.6H_2O$ 6.6 mg/l. The pH was not adjusted. In a typical fermentation, 20 500-ml flasks, each containing 250 ml of medium, were used, and inoculation was effected with one 14-d-old slant/flask. Flasks were shaken at 200 rev./min at 27°C. The steroid (1 g) was pulse fed over a 4-day period to all flasks except the single control: 0.1 g on day 8, 0.2 g on day 9, 0.3 g on day 10, and 0.4 g on day 11. The steroid was dissolved in about 10 ml of ethanol for all feedings. The contents of the flasks were harvested on day 14, and the broth was separated from the mycelium by suction filtration. The broth was extracted with ethyl acetate (×2) while the mycelial cells were disrupted by homogenization in the solvent. The extracts were combined, dried with sodium sulfate, and the solvent was removed in vacuo. The mycelial residue was dried at 90°C in an oven for 12 h.

*2.8. 3β-Hydroxyandrost-5-en-17-one **(1)***

The biotransformation of **1**, 1 g, yielded 30.06 g of dried mycelium and 0.90 g of organic extract on harvest. Column chromatography on the organic extract using 15% ethyl acetate in petrol gave 3β,17β-dihydroxyandrost-5-ene (**3**), 30 mg, which was identified by comparison with an authentic sample.

*2.9. 17β-Hydroxyandrost-4-en-3-one **(5)***

Fermentation of **5**, 1 g, yielded 40.36 g of dried mycelium and 0.89 g of organic extract on work up. Column chromatography of the acetylated organic extract was conducted with increasing proportions of ethyl acetate in petrol (7–25%). Elution in 10% ethyl acetate in petrol gave an inseparable mixture of two diacetylated steroids that could not be unambiguously identified. Further elution of the transformed products in 10% ethyl acetate in petrol enabled the purification of 3α-acetoxy-5α-androstan-17-one, 23 mg, which crystallized from methanol as needles, mp 160–162.5°, $[\alpha]_D$–213° (c = 0.8, $CHCl_3$), lit. [26] mp 161–162°; IR ν_{max} 1746, 1730 cm^{-1}; UV ($CHCl_3$) λ_{max} 204, 220 nm (log ε = 2.72, 2.20); HRMS (EI) m/z (%) 332.2354 (20) $[M]^+$ [$C_{21}H_{32}O_3$], 272 (100), 257 (30), 218 (26), 108 (41), 93 (24), 79 (21); ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, s, H-18), 0.88 (3H, s, H-19), 2.15 (3H, s, CH_3CO_2), 5.02 (1H, br s, H-3β).

Elution with 10% ethyl acetate in petrol delivered the fed steroid as its 17β-acetoxy ester, 280 mg, followed by an-

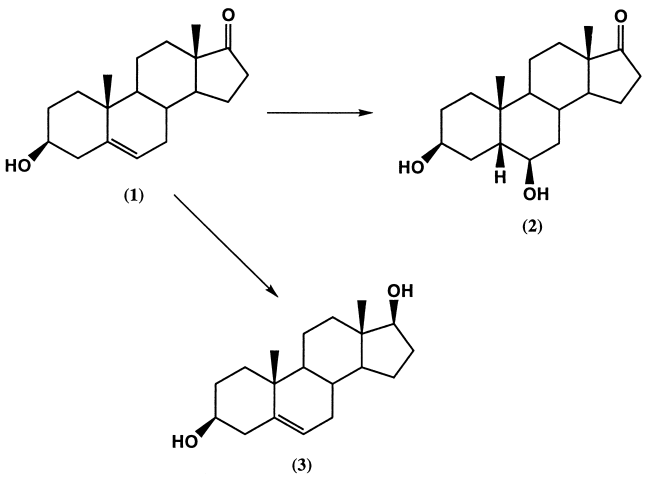
drost-4-ene-3,17-dione (**17**), 189 mg, which crystallized from methanol as cubes, mp 167–168°, $[\alpha]_D$ + 135° (c = 0.5, $CHCl_3$), lit. [27] mp 169–170°, $[\alpha]_D$ + 205°; IR ν_{max} 1708, 1667 cm^{-1}; UV ($CHCl_3$) λ_{max} 246 nm (log ε = 2.82); HRMS (EI) m/z (%) 286.1931 (100) $[M]^+$ $[C_{19}H_{26}O_2]$, 244 (58), 201 (15), 150 (16), 124 (72), 91 (31), 79 (31); ^1H NMR ($CDCl_3$) δ 0.85 (3H, s, H-18), 1.16 (3H, s, H-19), 5.72 (1H, s, H-4).

*2.10. Pregn-4-ene-3,20-dione **(19)***

The biotransformation of **19**, 1 g, yielded 48.47 g of dried mycelium and 0.87 g of organic extract on harvest. The extract was acetylated and subjected to column chromatography using 12% ethyl acetate in petrol. Elution in this solvent system delivered 3β-acetoxy-5α-pregnan-20-one, 37 mg, which crystallized from acetone-petrol as plates, mp 141–143°, $[\alpha]_D$ + 200° (c = 0.2, $CHCl_3$), lit. [28] mp 139–144°; IR ν_{max} 1729, 1711, 1385, 1368, 1264 cm^{-1}; HRMS (EI) m/z (%) 360.2671 (23) $[M]^+$ $[C_{23}H_{36}O_3]$, 342 (18), 300 (100), 285 (24), 215 (55), 107 (44), 93 (40), 84 (58); ^1H NMR ($CDCl_3$) δ 0.62 (3H, s, H-18), 0.84 (3H, s, H-19), 2.02 (3H, s, H-21), 2.10 (3H, s, CH_3CO_2), 4.64 (1H, m, w/2 = 17 Hz, H-3α). Further elution gave 5α-pregnane-3,20-dione (**20**), 26 mg, which crystallized from acetone-petrol as cubes, mp 198–201°, $[\alpha]_D$ + 144° (c = 1.6, $CHCl_3$), lit. [29] mp 197–200°; IR ν_{max} 1709 (br), 1385, 1354 cm^{-1}; HRMS (EI) m/z (%) 316.2671 (29) $[M]^+$ $[C_{21}H_{32}O_2]$, 298 (33) $[M\text{-}H_2O]^+$, 259 (56), 139 (76), 97 (41), 84 (42), 45 (100); ^1H NMR ($CDCl_3$) δ 0.65 (3H, s, H-19), 0.95 (3H, s, H-18), 2.10 (3H, s, H-21). The fed steroid was then eluted (244 mg) followed by a more polar metabolite. The quantity of the latter was insufficient for identification.

*2.11. 17α,21-Dihydroxypregn-4-ene-3,11,20-trione **(10)***

Fermentation of **10**, 1 g, yielded 54.43 g of dried mycelium and 0.60 g of organic extract. Column chromatography of the acetylated extract in 25% ethyl acetate in petrol led to the elution of the acetate of the fed steroid (228 mg). Further elution with 30% ethyl acetate in petrol enabled the purification of 20,21-diacetoxy-17α-hydropregn-4-ene-3,11-dione, 31 mg, which crystallized from methanol as cubes, mp 215–220°, $[\alpha]_D$ − 50° (c = 0.6, $CHCl_3$); IR ν_{max} 3415, 1747, 1732, 1697, 1672, 1385, 1367, 1245, 1257 cm^{-1}; UV ($CHCl_3$) λ_{max} 246 nm (log ε = 3.80); HRMS (EI) m/z (%) 446.2307 (78) $[M]^+$ $[C_{25}H_{34}O_7]$, 402 (39), 386 (35), 342 (62), 326 (47), 300 (27), 272 (36), 256 (33), 122 (100); ^1H NMR ($CDCl_3$) δ 0.75 (3H, s, H-18), 1.42 (3H, s, H-19), 2.00 (3H, s, CH_3CO_2), 2.14 (3H, s, CH_3CO_2), 4.25 (1H, dd, J = 8.3, 12 Hz, H-21), 4.50 (1H, dd, J = 4, 12 Hz, H-21), 5.30 (1H, dd, J = 4, 8.3 Hz, H-20), 5.75 (1H, s, H-4).



Scheme 1. Transformation of 3β-hydroxyandrost-5-en-17-one by *E. jeanselmei*.

*2.12. 3-Hydroxyestra-1,3,5(10)-trien-17-one **(23)***

The fermentation of **23**, 1 g, yielded 34.88 g of dried mycelium and 0.62 g of organic extract on work up. The acetylated extract was subjected to column chromatography to enable the purification of 3,17β-diacetoxyestra-1,3,5(10)-triene, 22 mg, which crystallized from methanol as cubes, mp 122–123°, $[\alpha]_D$ + 125° (c = 0.8, EtOH), lit. [30] mp 123–125°; IR ν_{max} 1766, 1733, 1494, 1262, 1197, 1039 cm^{-1}; UV ($CHCl_3$) λ_{max} 242, 260, 270, 302 nm (log ε = 3.46, 3.36, 3.41, 2.99); HRMS (EI) m/z (%) 356.1990 (13) $[M]^+$ $[C_{22}H_{28}O_4]$, 314 (100), 172 (14), 159 (11), 146 (11), 133 (8); ^1H NMR ($CDCl_3$) δ 0.85 (3H, s, H-18), 1.35 (3H, s, H-19), 2.08 (3H, s, CH_3CO_2), 2.38 (3H, s, CH_3CO_2), 4.70 (1H, t, J = 8.5 Hz, H-17α).

3. Results and Discussion

3.1. Exophiala jeanselmei var. lecanii-corni

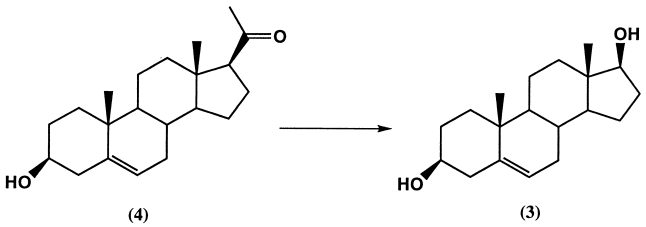
The protocol for biotransformation was determined using dehydroisoandrosterone (**1**) as the substrate.

3.2. 3β-Hydroxy-Δ^5-Steroids

*3.2.1. Bioconversion of 3β-hydroxyandrost-5-en-17-one **(1)** (Scheme 1)*

3β,17β-Dihydroxyandrost-5-ene (**3**): High-resolution mass spectrometry indicated a molecular ion at m/z 290.2248, which corresponded to a formula of $C_{19}H_{30}O_2$. Fragments at m/z 275, 272, and 257 resulted from the loss of either a methyl group, or water, or both, respectively. The NMR data revealed that the carbonyl had been reduced [31].

The transformed product below was converted to its acetate for complete characterization. NMR data was compared with that for the 3β-acetate of **1**. The 5α or β con-



Scheme 2. Transformation of 3β-hydroxypregn-5-en-20-one by *E. jeanselmei*.

figuration in this compound and other steroids was determined by comparison of the ^{13}C NMR resonance values for C-5, -7, -9, and -19 with those reported by Blunt and Stothers [32].

3β,6β-Diacetoxy-5β-androstan-17-one: HRMS data from **2** indicated a molecular formula of $C_{19}H_{30}O_3$. MS (EI) of the acetylated compound gave a molecular ion at m/z 390, which indicated the presence of two acetate groups. The ^{13}C NMR spectrum denoted 23 carbons, two of which were assignable to acetate carbonyls (170.0 and 170.2 ppm) and a ketone at 221 ppm. There were no resonances for olefinic carbons, implying that the double bond had been hydrated to produce the 6β-hydroxy-5β-steroid. The bioconversion of dehydroisoandrosterone (**1**) yielded 3β,17β-dihydroxyandrost-5-ene (**3**) and 3β,6β-dihydroxy-5β-androstan-17-one (**2**). Microbial hydration of alkenes to produce alcohols is rare, but has been reported previously. The steps are believed to involve oxidation to the 5,6-epoxide followed by isomerization to a 6-ketone [33]. The latter would then be reduced to the 6β-alcohol. The actions of a monooxygenase, epoxide isomerase, and dehydrogenase have been implicated previously in the conversion of styrene to phenylacetic acid by *E. jeanselmei* [4].

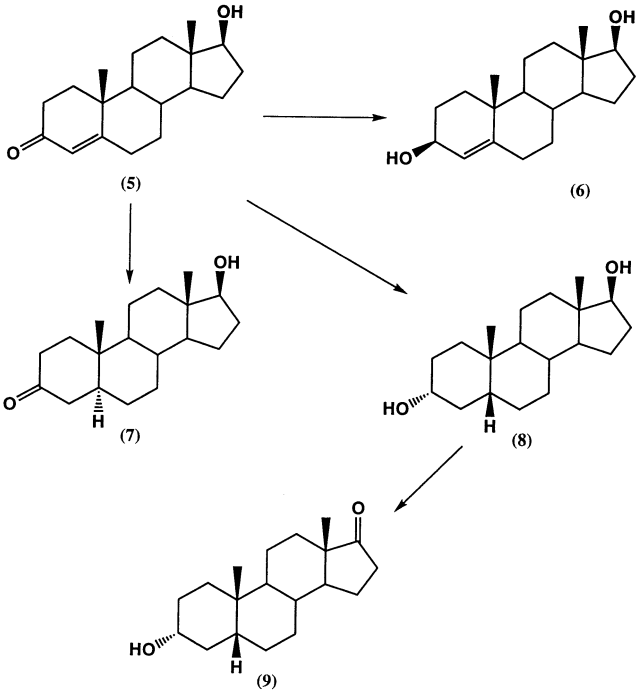
3.2.2. Bioconversion of 3β-hydroxypregn-5-en-20-one (4) (Scheme 2)

The transformed compound (**3**) was purified and characterized as the acetate and was compared with the 3β-acetate of **4**.

3β,17β-Diacetoxyandrost-5-ene: MS (CI) gave a molecular ion at m/z 374. Other major peaks at 314 and 254 were due to the sequential loss of two molecules of acetic acid. This would then imply a molecular formula of $C_{23}H_{34}O_4$. ^{13}C NMR data supported the proposed formula. It was noted that the carbonyl at δ 208.3 (C-20) had disappeared and an acetoxy-bearing carbon appeared at 82.8 ppm (C-17). It was thus concluded that the fungus had cleaved the C-17 side chain and inserted a 17β-hydroxyl group. The side chain cleavage of pregnenolone by fungi to produce compounds containing a 17β-hydroxyl group has been reported [34,35].

3.3. 3-Keto-Δ⁴-Steroids

3.3.1. Bioconversion of 17β-hydroxyandrost-4-en-3-one (5) (Scheme 3)



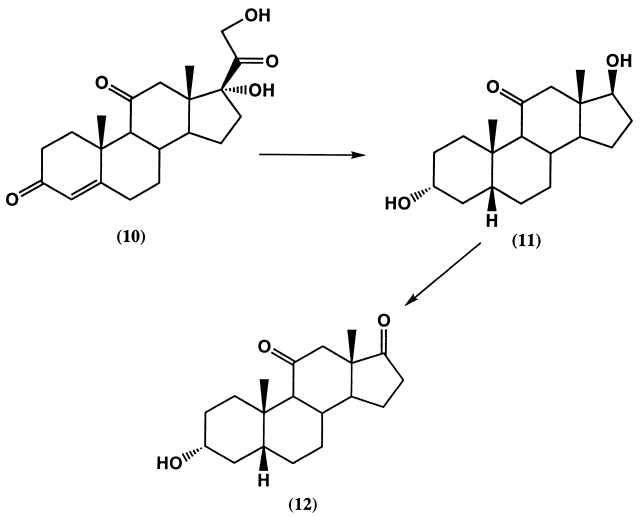
Scheme 3. Transformation of 17β-hydroxyandrost-4-en-3-one by *E. jeanselmei*.

3β,17β-Dihydroxyandrost-4-ene (**6**): The HRMS had a molecular ion at m/z 290.2238 corresponding to a formula of $C_{19}H_{30}O_2$. In the ^{13}C NMR spectrum, the carbonyl at 198 ppm of the fed steroid had disappeared, and an additional methine appeared at δ 66.5. The chemical shifts associated with the olefinic carbons C-4 and C-5 had moved upfield by 11.2 and 18.5 ppm, respectively. A comparison of the ^{13}C NMR data with that in the literature [32] supported the observation that the fungus had reduced the 3-carbonyl to produce **6**.

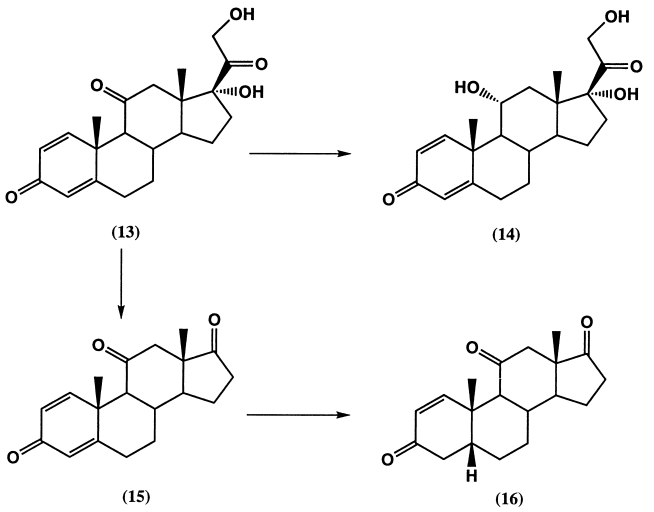
17β-Hydroxy-5α-androstan-3-one (**7**): The ^{13}C NMR spectrum showed that there was one carbonyl and no carbon-carbon double bond in this compound. The proposed conversion was supported by HRMS data which gave M^+ at 290.2243, corresponding to the formula $C_{19}H_{30}O_2$. Fungal reduction of C-4,5 double bond in the presence of a 3-carbonyl has been reported [36].

3α,17β-Dihydroxy-5β-androstane (**8**): HRMS gave a molecular mass of 292.2390, which corresponded to a formula of $C_{19}H_{32}O_2$. The polar nature of the compound required its conversion to the diacetate for NMR characterization. By comparison of the spectra with those of 17β-acetoxyandrost-4-en-3-one, it was observed that the unsaturated ketone had been completely reduced. The signal at δ 74.1 was therefore assigned to C-3. Effects of the presence of acetoxy groups as reported by Blunt and Stothers [32] led to the conclusion that the conjugated system had been reduced from the β face to produce the 3α-hydroxy-5β-steroid.

3α-Hydroxy-5β-androstan-17-one (**9**): A comparison of the ^{13}C NMR spectrum of this compound with that of the



Scheme 4. Transformation of 17α,21-dihydroxypregn-4-ene-3,11,20-trione by *E. jeanselmei*.



Scheme 5. Transformation of 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione by *E. jeanselmei*.

fed steroid (**5**) showed significant differences. The 3-carbonyl had been reduced to a 3α-hydroxyl. There were no olefinic carbons. The 17-hydroxyl was absent and the resonances of carbons in the vicinity of C-17 had all shifted downfield due to the deshielding effect of the new carbonyl. HRMS data supported the proposed structure with M^+ at 290.2247 ($C_{19}H_{30}O_2$) along with other characteristic fragments. The ability of fungi to oxidize the C-17 hydroxyl has been reported [37]. Here the carbonyl and olefin were reduced from the β-face. It is also known that some fungi will reduce the α,β-unsaturated systems of some 3-keto-Δ^4-steroids [34]. Biotransformation of testosterone yielded four compounds, 3β,17β-dihydroxyandrost-4-ene (**6**), 17β-hydroxy-5α-androstan-3-one (**7**), 3α-hydroxy-5β-androstan-17-one (**9**) and 3α,17β-dihydroxy-5β-androstane (**8**). Apparently, there were four distinct enzyme systems operating. The carbonyl of the conjugated system was reduced to give a 3β-hydroxyl group. In addition, hydrogenation of the conjugated double bond occurred from the α face. In another system, testosterone was reduced to the 3α,17β-dihydroxy-5β-androstane which was further oxidized to the 17-ketone.

3.4. 17α,21-Dihydroxypregnene-3,11,20-triones

*3.4.1. Bioconversion of 17α,21-dihydroxypregn-4-ene-3,11,20-trione **(10)** (Scheme 4)*

3α,17β-Dihydroxy-5β-androstan-11-one (**11**): HRMS (EI) gave a molecular ion at m/z 306.2205, corresponding to a formula of $C_{19}H_{30}O_3$. The IR data showed the presence of hydroxyl and carbonyl groups and the ^{1}H NMR spectrum contained signals for two hydroxyl bearing methines. The double bond had been reduced. The data suggested that the unsaturated ketone had been reduced, and that side chain cleavage had occurred.

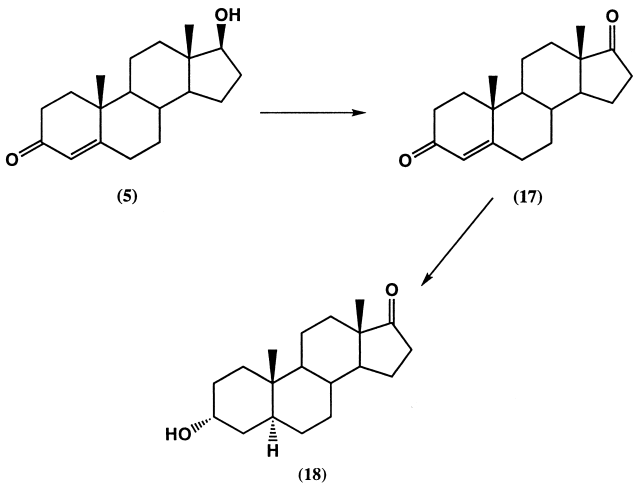
3α-Hydroxy-5β-androstane-11,17-dione (**12**): HRMS gave a molecular formula of $C_{19}H_{28}O_3$, and this was supported by ^{13}C NMR data. It was therefore proposed that the C-17 side chain had been cleaved, and reduction of the double bond had occurred. The ^{13}C NMR spectrum showed the presence of two carbonyls which were assigned to C-11 and C-17. The methine at δ 71.1 was assigned to C-3. It is proposed that a series of enzyme systems were operating, one which effected the reduction of the α,β-unsaturated system to produce a 3α-hydroxy-5β-steroid [34]. Another cleaved the C-17 side chain to produce the 17α-hydroxyl group, which was then oxidized to the 17-ketone [35]. Side chain cleavage of pregnanes to form 17β-hydroxy and 17-ketosteroids has been reported [38].

*3.4.2. Bioconversion of 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione **(13)** (Scheme 5)*

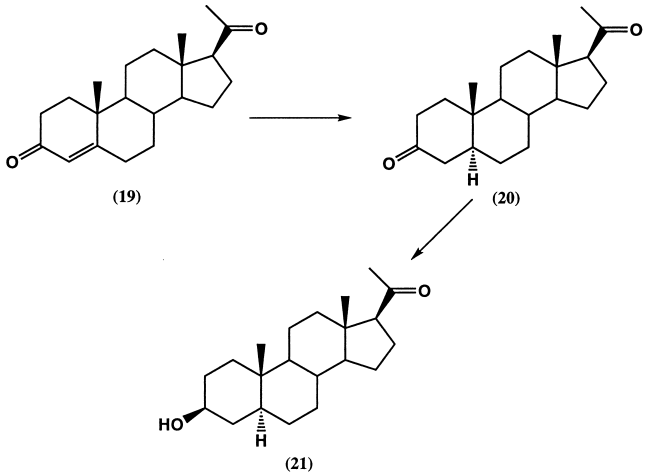
Androsta-1,4-diene-3,11,17-trione (**15**): A comparison of the ^{1}H NMR data with that of the xenobiote showed no change in the olefinic region. Signals for protons associated with C-21 were lost, and the ^{13}C NMR data showed the presence of 19 carbons. This was supported by HRMS, which gave a formula of $C_{19}H_{22}O_3$. It was proposed that side-chain degradation had taken place. The ^{13}C NMR spectrum contained a peak at 216.2 ppm, which was readily assigned to C-17. The proposed biotransformation was confirmed by comparison of the ^{13}C NMR data with that published for androsta-1,4-diene-3,11,17-trione by Hanson and Siverns [39].

5β-Androst-1-ene-3,11,17-trione (**16**): The IR spectrum contained absorptions representing two carbonyls and one α,β-unsaturated carbonyl. HRMS (EI) gave a molecular formula of $C_{19}H_{24}O_3$, and the ^{13}C NMR data showed C-3 at 199.7 ppm. This implied a reduction of the extended chromophore in ring A of **14**. C-17 side chain cleavage had also occurred.

11α,17α,21-Trihydroxypregna-1,4-diene-3,20-dione



Scheme 6. Transformation of 17β-hydroxyandrost-4-en-3-one by *C. paradoxa*.



Scheme 7. Transformation of pregn-4-ene-3,20-dione by *C. paradoxa*.

(**14**): The ^{1}H NMR showed the presence of an additional methine bearing a hydroxyl group at (H-11β). MS (EI) had the molecular ion at m/z 360.1920, corresponding to a formula of $C_{21}H_{28}O_5$. In the ^{13}C NMR spectrum it was observed that the signal for the 11-carbonyl had disappeared, and an oxygen-bearing carbon had appeared. From the magnitude of the coupling constant of the H-11 signal, it was concluded that the alcohol had α stereochemistry. Prednisone had thus been biotransformed to three compounds, 5β-androst-1-ene-3,11,17-trione (**16**), androsta-1,4-diene-3,11,17-trione (**15**) and 11α,17α,21-trihydroxypregna-1,4-diene-3,20-dione (**14**). Here, one saw three distinct enzyme systems operating. There was one that effected the C-17 side chain cleavage to produce the C-17 ketone. Another reduced the C-4,5 double bond from the β face, and a third system reduced the C-11 ketone to the 11α-alcohol.

3.5. Ceratocystis paradoxa

The fungus was grown on the modified Salmink liquid medium [25] rather than the other recommended medium [40], as the former gave better growth.

3.6. 3β-Hydroxy-Δ^5-Steroids

*3.6.1. Bioconversion of 3β-hydroxyandrost-5-en-17-one (**1**)*

3β,17β-Dihydroxyandrost-5-ene (**3**): This compound had been characterized previously.

3.7. 3-Keto-Δ^4-Steroids

*3.7.1. Bioconversion of 17β-hydroxyandrost-4-en-3-one **(5)** (Scheme 6)*

The organic extract recovered from this fermentation was acetylated prior to column chromatography to identify two biotransformed products.

Androst-4-ene-3,17-dione (**17**): The molecular ion of m/z 286.1931 corresponded to a formula of $C_{19}H_{26}O_2$ consistent with seven degrees of unsaturation. Loss of CH_2=C=O resulted in a daughter ion at m/z 244. Due to delocalization effects in the α,β-unsaturated system, the lower infrared absorption of 1667 cm^{-1} was attributed to this system while the unconjugated carbonyl absorbed at 1708 cm^{-1}. Oxidation reactions of this nature have been previously reported [41].

3α-Acetoxy-5α-androstan-17-one: The molecular ion (at m/z 332.2354) in the high resolution mass spectrum corresponded to a molecular formula of $C_{21}H_{32}O_3$, which contained six double bond equivalents. The base peak at m/z 272 resulted from the loss of acetic acid from the C-3 position. A further loss of a methyl substituent yielded a fragment at m/z 257. The carbonyl group of the α,β-unsaturated system was reduced to the corresponding alcohol and the C-17 hydroxyl functionality was oxidized. The reduction of the α,β-unsaturated system in these steroids is a feature of many enzyme-mediated reactions [34].

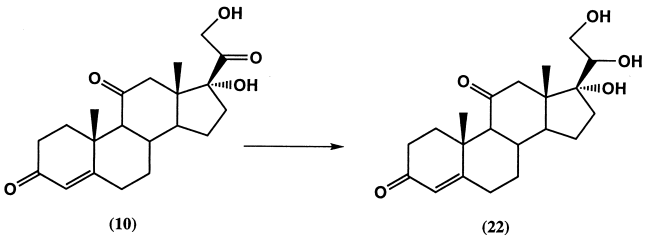
A more polar steroid metabolite [m/z 344.1987, $C_{21}H_{28}O_4$] was also present but the quantity was too small for characterization.

*3.7.2. Bioconversion of pregn-4-ene-3,20-dione **(19)** (Scheme 7)*

The extract was acetylated prior to column chromatography.

5α-Pregnane-3,20-dione (**20**): In the HRMS, the [M]$^+$ peak occurred at 316.2671 ($C_{21}H_{32}O_2$). Reduction of the olefinic moiety was observed.

3β-Acetoxy-5α-pregnan-20-one: A molecular mass of 360.2671 and formula of $C_{23}H_{36}O_3$ was deduced from high resolution mass spectral studies of the acetylated product. Fragmentation of 18 mass units corresponded to dehydration, while the base peak at m/z 300 was due to a loss of acetic acid. Two carbonyl absorption bands appeared corresponding to the 3-ester and C-20 ketone functionalities.



Scheme 8. Transformation of 17α,21-dihydroxypregn-4-ene-3,11,20-trione by *C. paradoxa*.

The peak at δ 2.02 for the C-21 methyl group remained unaffected, hence the C-3 reduction appeared to have occurred with a highly selective enzyme system that targeted only this ketone. Reduction of both olefin and carbonyl functionalities occurred from the α face.

Two more polar steroids could be identified by mass spectrometry [m/z 372.2311, $C_{23}H_{32}O_4$ and m/z 370.2138, $C_{23}H_{30}O_4$], but they were isolated in too minute amounts to enable further characterization.

*3.7.3. Bioconversion of 17α,21-dihydroxypregn-4-ene-3,11,20-trione **(10)** (Scheme 8)*

The transformed product was isolated and characterized as the acetylated derivative.

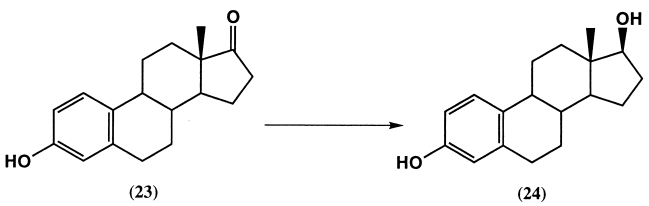
20,21-Diacetoxy-17α-hydroxypregn-4-ene-3,11-dione: A molecular formula of $C_{25}H_{34}O_7$ was deduced from the high resolution mass spectral data of the diacetylated compound. The infrared data accounted for the presence of four carbonyl groups that corresponded to the two esters on C-20 and C-21, the C-11 ketone, and the α,β-unsaturated-3-ketone, respectively. This product resulted from reduction of the carbonyl group at C-20. Selective reduction of this ketone has been reported with other fungal systems [42].

Another product, possibly that of side-chain cleavage [m/z 344.1987, $C_{21}H_{28}O_4$] could be identified in a fraction from chromatographic analysis, but the small quantity isolated was inadequate for characterization.

*3.7.4. Bioconversion of 3-hydroxyestra-1,3,5(10)-trien-17-one **(23)** (Scheme 9)*

This transformed compound was identified after derivatization with acetic anhydride.

3,17β-Diacetoxyestra-1,3,5(10)-triene (25): The acetylated metabolite demonstrated a molecular ion at 356.1990



Scheme 9. Transformation of 3-hydroxyestra-1,3,5(10)-trien-17-one by *C. paradoxa*.

consistent with a formula of $C_{22}H_{28}O_4$. The base peak at m/z 314 resulted from the loss of 42 mass units corresponding to CH_2=C=O. Reduction of the ketone functionality to the 17α-alcohol had occurred.

Thus *E. jeanselmei* var. *lecanii-corni* was found to biotransform five steroids: 3β-hydroxyandrost-5-en-17-one (dehydroisoandrosterone) (**1**), 3β-hydroxypregn-5-en-20-one (pregnenolone) (**4**), 17β-hydroxyandrost-4-en-3-one (testosterone) (**5**), 17α,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) (**10**), and 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione (prednisone) (**13**). These experiments indicate that the fungus is able to effect reduction of enones mainly from the β face to form 3α-hydroxy-5β-steroids. Side chain degradation and, surprisingly, hydration of a Δ^5-steroid were also seen. Of the seven substrates fed to *Ceratocystis paradoxa*, biotransformations were effected with five such steroids, namely, 3β-hydroxyandrost-5-en-17-one (dehydroisoandrosterone) (**1**), 17β-hydroxyandrost-4-en-3-one (testosterone) (**5**), pregn-4-ene-3,20-dione (progesterone) (**19**), 17α,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) (**10**), and 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone) (**23**). Here, reduction of enones **5** and **19** occurred to give 3α and 3β-hydroxy-5α-steroids, respectively. Redox reactions on alcohols and ketones by both microorganisms also took place.

Acknowledgements

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