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MICROBIOLOGICAL OXIDATION OF STEROLS BY *CORIOLUS HIRSTUS*

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Key Word Index—*Coriolus hirstus* Polyporaceae, fungus, 3β-hydroxycholest-5-en-7-one; cholest-5-en-3β, 7α-diol, cholest-5-en-3β, 7β-diol, 6α-hydroxycholest-4-en-3-one, 6β-hydroxycholest-4-en-3-one

Abstract—Fermentation of cholesterol with a culture of *Coriolus hirstus* yielded a mixture containing 7-oxo-cholesterol and hydroxylated-cholesterol derivatives Preparation of a possible precursor of antheridiol, 7-oxofucosterol, by this fungus was also examined

INTRODUCTION

OUR ATTENTION has recently been focused on the biosynthesis of steroidal hormones, ecdysones[1] and antheridiol, a fungal sex hormone from the water mold, *Achlya bisexualis*.[2,3] We now report the results obtained during the course of a study on the metabolism of cholesterol by the microorganism *Coriolus hirstus* (IFO 4917).

RESULTS

Aerobic incubation of cholesterol added to a 3-day-old *C. hirstus* culture gave after 5 days three hydroxyketone metabolites, A, B, C and two dihydroxy compounds, D-1 and D-2. The main metabolite, C, m.p. 170–172·5°, $C_{27}H_{44}O_2$, presented the characteristic bands of an α,β-unsaturated ketone [IR(CCl_4): 1676 and 1634 cm^{-1}, UV (EtOH): 239 nm] and of a hydroxyl group [IR(CCl_4): 3460 cm^{-1}]. In the NMR spectrum of compound C there were singlet signals due to the protons of the angular Me groups at C-18 (δ 0·69) and at C-19 (δ 1·21), which agree well with the values [C-18 (δ 0·68) and C-19 (δ 1 17)] calculated[4] for 3β-hydroxycholest 5-en-7-one(**1**). Finally, the metabolite C proved to be identical with 3β-hydroxycholest-5-en-7-one(**1**), by comparison with the physical properties [m.p., $(\alpha)_D$, UV] of an authentic sample [5] The other hydroxyketone metabolities, A, m.p. 191–195° and B, m.p. 161–162° were identified, from their physical data, as 6β-hydroxycholest-4-en-3-one and 6α-hydroxycholest-4-en-3-one, respectively.[6] The metabolites, D-1 and D-2, showed a characteristic blue colour on TLC after spraying with 50% H_2SO_4 and were inseparable by PTLC and column chromatography. The MS of the mixture had a

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[3] POPPLESTONE, C R and UNRAU, A M (1973) *Phytochemistry* **12**, 1131
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[6] MIYAKE, A and TOMOEDA, M (1972) *J Chem Soc Perkin I* 663

peak at *m/e* 384 (M^+-18), indicating that these metabolites were monohydroxy-cholesterol derivatives The mixture was benzoylated and separated by PTLC to give two steryl dibenzoates. The less polar dibenzoate (D-1 dibenzoate), m p 151- 154° and the polar one (D-2 dibenzoate), m p. 172- 174 were confirmed to be identical, by comparison with the physical properties [m.p., $(\alpha)_D$, UV] of authentic samples,[7] with cholest-5-en-3β,7α-diol dibenzoate and cholest-5-en-β,7β-diol dibenzoate, respectively Thus, the metabolites D-1 and D-2 were proved to be cholest-5-en-3β,7α-diol (**2**) and cholest-5-en-3β,7β-diol (**3**), respectively Only 7-hydroxycholesterol among these metabolites has previously been reported as a microbial transformation product of cholesterol [8]

Presumably the 7-hydroxycholesterols were obtained from cholesterol by an enzymatic process in *C hirstus* However cholesterol and Δ^4-3-ketosteroids can also be hydroxylated by autooxidation [9,10] Therefore we attempted to verify that the above metabolites were formed by enzymatic processes The extract obtained after the aerobic incubation of cholesterol in *C hirstus* culture at 30 for 5 days was purified by PTLC to remove the residual cholesterol and so give the metabolite fraction This material was then trimethylsilylated and analysed by GLC, which showed five peaks corresponding to compounds A, B, C, D-1 and D-2 (see Table 1) Aerobic incubations of cholesterol in the malt medium and

TABLE 1 GLC ANALYSIS OF STEROIDAL MLTABOLITES

Steroid	RR_1	Yield of Steroids (%)
A-TMS (6β-hydroxycholest-4-en-3-one)	5 03	1 50
B-TMS (6α-hydroxycholest-4-en-3-one)	8 42	0 41
C-TMS (3β-hydroxycholest-5-en-7-one)	6 12	2 68
D-1-TMS (cholest-5-en-3β, 7α-diol)	1 00*	1 29
D-2-TMS (cholest-5-en-3β 7β-diol)	1 39	0 96

Column conditions 5% OV-210 on Gaschrom Q, 3 mm × 100 cm glass column, column temp 230 The steroids were analysed as their TMS derivatives

* 3 30 min

in a *C hirstus* culture pretreated by heat (100 , 5 min) were performed as controls. The extracts of both control incubations were treated as above, but showed no peaks on GLC analysis corresponding to any of the metabolites This indicates that the above metabolites must be formed by enzymatic reactions As an extention of these studies we investigated the preparation of a possible precursor of antheridiol, 7-oxofucosterol, by fermentation of fucosterol [stigmasta-5,*E*-24(28)-dien-3β-ol] with *C hirstus* The fermentation of *C hirstus* culture with fucosterol gave products which showed a similar pattern of spots on TLC to that of the cholesterol metabolites There were three UV absorbing spots (A', B' and C') and one spot (D') showing the blue colour after spraying with 50% H_2SO_4 These metabolites were separated by PTLC The MS of metabolites A', B' and C' had the parent peak at *m/e* 426 and a strong peak at *m/e* 328 (M^+-98) which probably occurred by a McLafferty rearrangement of the side chain due to the 24-ethylidene group [11] The metabolite

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[8] KRÄMLL A and HORVATH, J (1948) *Nature* **162**, 619
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D′ had the parent peak at *m/e* 428 and a strong peak at *m/e* 330 (M^+-98). From these findings, the metabolites of fucosterol are assumed to be 7-oxofucosterol, 7-hydroxyfucosterols and 6-hydroxyfucost-4-en-3-ones.

EXPERIMENTAL

M.Ps were measured on a Kofler hot-stage apparatus and are uncorrected. MS were measured with a JEOL JMS-D 100 and NMR spectra were recorded on a JEOL MH-100 in $CDCl_3$ with TMS as an internal standard.

Conversion of cholesterol by C. hirstus. The fermentation medium consisted of malt extract (20 g), dextrose (20 g), peptone (1 g) and water (1 litre). *C. hirstus* was inoculated into 100 ml of the medium contained in a 500 ml Sakaguchi flask and incubated at 30° for 3 days on a reciprocal shaker. To this culture was added cholesterol (50 mg) in dioxane (1 ml) and the incubation continued for an additional 5 days at 30°. The contents of 55 Sakaguchi flasks were filtrated, the combined broth saturated with NaCl, and then extracted twice with EtOAc (5 l.) to give 1.017 g of the broth extract (be). The mycelium was extracted by repeated soaking in Me_2CO and decantation. After evaporation of the Me_2CO, the residue was partitioned between EtOAc and saturated NaCl soln. The organic layer afforded the mycelium extract. The crystallization of the mycelium extract gave 366 mg of cholesterol and 2.151 g of the residual mycelium extract (me). The combined extract (be + me) was subjected to silica gel column (3 × 27 cm) chromatography. Elution of the column with 7.5% (v/v) EtOAc in *n*-hexane (1 litre) yielded cholesterol (553 mg). After elution with 10% (v/v) EtOAc in *n*-hexane (1 litre), the column was eluted with 20% (v/v) EtOAc in *n*-hexane (2 l.) to give fraction I (325 mg) containing three metabolites A, B and C. This fraction was further purified by PTLC (silica gel 60 PF_{254}, developed with EtOAc–*n*-hexane (1:1)) to give compound A (58 mg, R_f = 0.38), compound B (16 mg, R_f = 0.31) and compound C (95 mg, R_f = 0.24). The column was then eluted with 40% (v/v) EtOAc in *n*-hexane (1:5 l.) to give fraction II (112 mg) containing D-1 and D-2. Fraction II was benzoylated to give a mixture of the dibenzoates, which were separated by PTLC (silica gel 60 PF_{254}, developed with $CHCl_3$) to afford D-1 dibenzoate (28 mg, R_f = 0.78) and D-2 dibenzoate (26 mg, R_f = 0.72).

Metabolite A (*6β-hydroxycholest-4-en-3-one*). Recryst. from *n*-hexane, needles, m.p. 191–195°, $(\alpha)_D^{20}$ +29.4° (*c*, 0.462 $CHCl_3$) [lit.[6] m.p. 192–195°, $(\alpha)_D^{22}$ +31.8°]. (Found: C, 80.94; H, 11.07. Calc. for $C_{27}H_{44}O_2$: C, 80.83; H, 10.98%). NMR ($CDCl_3$): δ 0.74 (3H, *s*, C-18 Me), 1.36 (3H, *s*, C-19 Me), 4.32 (1H, *t*, *J* 4.4 Hz, C-6), 5.76 (1H, *s*, C-4 H). IR (CCl_4) max (cm^{-1}): 3625, 1680, 1615 [lit.[6] IR, Nujol (cm^{-1}): 3360, 1670, 1612]. UV (EtOH): 238 nm (ϵ = 13000) [lit.[6] UV (95% EtOH): 239 nm (ϵ = 13000)]. MS: M^+, 400, M^+-15, 385, M^+-18, 382, M^+-side chain, 287, M^+-side chain-41, 246, M^+-side chain-42, 245.

Metabolite B (*6α-hydroxycholest-4-en-3-one*). Recryst. from MeOH, needles, m.p. 161–162°, $(\alpha)_D^{27}$ +77.2° (*c*, 0.167 $CHCl_3$) [lit.[6] m.p. 163.5–164°, $(\alpha)_D^{14}$ +80.0]. NMR ($CDCl_3$): 0.72 (3H, *s*, C-18 Me), 1.18 (3H, *s*, C-19 Me), 4.35 (1H, *q*, *J* 12.6 Hz, C-6 H), 6.22 (1H, *d*, *J* 2 Hz, C-4 H). IR (CCl_4) max (cm^{-1}): 3400, 1670, 1620 [lit.[6] IR, Nujol (cm^{-1}): 3355, 1665, 1617]. UV (EtOH): 243 nm (ϵ = 16200) [lit.[6] UV (95% EtOH): 243 nm (ϵ = 14700)]. MS: M^+, 400, M^+-15, 385, M^+-18, 382, M^+-side chain, 287, M^+-side chain-41, 246, M^+-side chain-42, 245.

Metabolite C (*3β-hydroxycholest-5-en-7-one*). Recryst. from ether-pentane, needles, m.p. 170–172.5°, $(\alpha)_D^{33}$ −106° (*c*, 0.634 $CHCl_3$) [lit.[5] m.p. 171°, $(\alpha)_D$ −113°]. (Found: C, 80.74; H, 10.93. Calc. for $C_{22}H_{44}O_2$: C, 80.83; H, 10.98%). NMR ($CDCl_3$): δ 0.69 (3H, *s*, C-18 Me), 1.21 (3H, *s*, C-19 Me), 3.71 (1H, *m*, C-3 H), 5.72 (1H, *s*, C-6 H). IR (CCl_4) max (cm^{-1}): 3460, 1676, 1634. UV (EtOH): 239 nm (ϵ = 12700) [lit.[5] UV (EtOH): 237 nm (ϵ = 13400)]. MS: M^+, 400, M^+-18, 382, M^+-18-15, 367, M^+-side chain, 287, M^+-side chain-42, 245.

Metabolite D-1 dibenzoate (*cholest-5-en-3β, 7α-diol dibenzoate*). Recryst. from *n*-hexane, m.p. 151–154°, $(\alpha)_D^{27}$ −96.5° (*c*, 0.707 $CHCl_3$) [lit.[7] m.p. 155°, $(\alpha)_D^{25}$ −114°]. NMR ($CDCl_3$): δ 0.73 (3H, *s*, C-18 Me), 1.13 (3H, *s*, C-19 Me), 4.90 (1H, *m*, C-3 H), 5.25 (1H, *m*, C-7 H), 5.75 (1H, *d*, *J* 5 Hz, C-6 H), 7.46 (6H, *m*), 8.00 (4H, *m*). IR (CCl_4) max (cm^{-1}): 1720, 1604. UV (EtOH) nm (ϵ): 230 (26300), 275 (1520) [lit.[7] UV: 230, 272 nm]. MS: M^+-122, 488, M^+-122 × 2, 366.

Metabolite D-2 dibenzoate (*cholest-5-en-3β, 7β-diol dibenzoate*). Recryst. from *n*-hexane, needles, m.p. 172–174°, $(\alpha)_D^{27}$ + 102° (*c*, 0.144 $CHCl_3$) [lit.[7] m.p. 174°, $(\alpha)_D^{25}$ + 92°]. NMR ($CDCl_3$): δ 0.76(3H, *s*, C-18 Me), 1.18 (3H, *s*, C-19 Me), 4.90 (1H, *m*, C-3 H), 5.35 (1H, *bd*, *J* 8 Hz, C-7 H), 5.40 (1H, *d*, *J* 2 Hz, C-6 H). IR(CCl_4) max (cm^{-1}): 1720, 1605. UV (EtOH) nm (ϵ): 230 (27400), 275 (2120) [lit.[7] UV: 229, 272 nm]. MS: M^+-122, 488, M^+-122 × 2, 366.

Hydrolysis of dibenzoates. Alkaline hydrolysis of the dibenzoates gave the original diols.

Conversion of fucosterol by C. hirstus. Fucosterol (30 mg) was treated according to the procedure described for the conversion of cholesterol. The crude extract (20 mg) was separated by PTLC (silica gel 60 PF_{254}, 20 × 20 cm, 1 plate developed with EtOAc and n-hexane (1:1)) into five fractions (trace amounts of four metabolites and 8 mg of fucosterol).

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