MICROBIAL DEGRADATION OF THE PHYTOSTEROL SIDE-CHAIN TO 24-OXO PRODUCTS

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ABSTRACT

A mutant of the potent sterol degrader *Mycobacterium fortuitum* (ATCC 6842) has been isolated which is defective in its ability to degrade both the steroid nucleus and sterol side-chains that are branched at the 24-position. Bioconversions of phytosterol mixtures by this mutant resulted in the accumulation of the novel 24-oxo intermediates 9-hydroxy-27-nor-4-cholestene-3,24-dione (II) and 9-hydroxy-26,27-dinor-4-cholestene-3,24-dione (III). Under the same conditions, cholesterol is degraded mainly to 9-hydroxy-4-androstene-3,17-dione (I) by this organism.

INTRODUCTION

Microbial degradation of the 17-alkyl side-chain of cholesterol has been shown by Sih *et al.* (1,2) to occur in a manner that is apparently analogous to β-oxidation of fatty acids. This involves the sequential cleavage of 2- and 3-carbon fragments as acetic and propionic acids, and the generation of C-22 and C-24 carboxylic acids as side-chain intermediates. In the case of the phytosterols, (24R)-24-ethyl-5-cholesten-3β-ol (sitosterol), (24R)-24-ethyl-5,22-cholestadien-3β-ol (stigmasterol), and (24R)-24-methyl-5-cholesten-3β-ol (campesterol), in which the side-chain is branched at the 24-position, it has been observed that the rate of side-chain cleavage by *Arthrobacter simplex* is significantly reduced relative to that experienced with cholesterol (3). This may be due to a unique, and perhaps rate-limiting, enzymatic reaction required for degradation of side-chains with that configuration. Thus, we have isolated a mutant of *Mycobacterium fortuitum* which completely degrades the side-chain of cholesterol

but accumulates 24-oxo compounds when incubated with sterols that are branched at the 24-position. This report deals with the isolation and characterization of these novel side-chain intermediates.

EXPERIMENTAL

Microorganisms

The isolation of a mutant of *M. fortuitum* (ATCC 6842), designated NRRL B-8119, has been described elsewhere (4). Although a metabolic block prevented degradation of the steroid nucleus, it effectively cleaved the 17-alkyl side-chains of cholesterol and phytosterols with the resultant accumulation of 9-hydroxy-4-androstene-3,17-dione as the major product. The mutant microorganism [SCM-1] that carried out the bioconversion described in this report was isolated by treating NRRL B-8119 with N-methyl-N'-nitro-N-nitrosoguanidine, and then screening the survivors for their ability to degrade the side-chain of sitosterol/campesterol mixtures.

Fermentation and Extraction Procedures

Fermentation conditions were as previously described (4). The progress of the bioconversion was followed qualitatively by extracting aliquots of the whole beer with three volumes of methylene chloride, and chromatographing the extracts along with appropriate standards on pre-coated thin-layer chromatographic plates (Silica Gel GF; Analtech), developed in cyclohexane/ethyl acetate (1:1 v/v). The spots were visualized by spraying the plates with 50% sulphuric acid and heating at 160° to char.

Instrumental Techniques

Optical rotations were measured in chloroform solution at 25°. Ultraviolet spectra were measured in ethanol solution using a Carey model 219 spectrophotometer, and infrared spectra as Nujol mulls on a Digilab Model FTS-14 fourier transform spectrometer. The nmr spectra were obtained in deuterochloroform solution on a Varian EM-390 nmr spectrometer, and the shift values quoted are in ppm (δ) downfield from tetramethylsilane. Mass spectra were obtained using a Varian MAT-CH7 single focussing mass spectrometer with a PDP8-I based data system. Chromatography was performed on E. Merck (Lobar) prepacked silica columns (size B).

Isolation of 9-Hydroxy-27-nor-4-cholestene-3,24-dione (II) and 9-Hydroxy-26,27-dinor-4-cholestene-3,24-dione (III)

A sitosterol/campesterol mixture [60 g; conc 20 g/liter] was incubated with the SCM-1 culture as described in Ref. 4. The whole beer was then extracted with methylene chloride, and the extract filtered through Celite and evaporated to dryness. The residual oil was taken up in hot acetone, and water added until it became slightly cloudy. On cooling, a crop of unchanged sitosterol crystals separated (16.9 g),

and a further crop (6.74 g) was obtained by concentration of the mother liquors. The filtrate from the second crop was concentrated on a rotary evaporator under reduced pressure to remove the acetone, and the resulting aqueous suspension extracted with ethyl acetate several times. The pooled extracts were filtered through silicone-treated paper to remove water, and concentrated under nitrogen on a steam bath while hexane was slowly added. When the solution became cloudy it was set aside to cool, giving a third crystalline crop (9.32 g) consisting mainly of the two ketones II and III. Further concentration gave a yellow oil (24 g) containing the bulk of the other known 9-hydroxy-steroids.

Aliquots of the ketone mixture (3 x 1 g) were chromatographed on three E. Merck Lobar columns (size B) in series, [which had been preconditioned by pumping 2 bed volumes (about 600 ml) of water-saturated ethyl acetate through them], eluting with ethyl acetate/hexane/methylene chloride 30:40:30 at moderate pressure (60-80 psi) and a flow rate of 6 ml/min. The effluent was monitored with a differential refractometer. Appropriate fractions from each run were pooled on the basis of tlc (40% ethyl acetate in cyclohexane), and mixed fractions were pooled and rechromatographed.

The less polar fraction was recrystallized from methylene chloride/acetone to give 9-hydroxy-27-nor-4-cholestene-3,24-dione (II; 1.3 g), mp 208.9-211.1°, $(\alpha)_D^{25}$ + 83° (c, 0.7835). ν_{max} 3500 cm^{-1} (hydroxyl); 1700 cm^{-1} (sat. ketone); 1650 and 1605 cm^{-1} (α, β-unsat. ketone). λ_{max} 242 nm, (ε, 15350). ^{1}H-nmr: 0.72, (18-Me); 0.94, (d., 21-Me); 1.32, (19-Me); 1.08, (tr., 26-CH_3); 5.84, (4-H). ^{13}C-nmr: 212.0, (24 C=O); 199.1, (3 C=O); 169.2, (C-5); 126.6, (C-4); 76.2, (C-9).

Anal.: Calc. for $C_{26}H_{40}O_3$ (400): C, 77.95; H, 10.10.
Found: C, 78.14; H, 10.20; Mol. wt. 400 (Mass spec.).

The more polar fraction was also crystallized from methylene chloride/acetone, giving 9-hydroxy-26,27-dinor-4-cholestene-3,24-dione (III; 0.59 g) mp 201.7-204.8, $(\alpha)_D^{25}$ + 83° (c, 0.9855). ν_{max} 3500 cm^{-1} (hydroxyl); 1700 cm^{-1}, (sat. ketone); 1650 and 1605 cm^{-1}, (α, β-unsat. ketone). λ_{max} 242 nm, (ε, 15,250). ^{1}H-nmr: 0.72, (18-Me); 0.92, (d., 21-Me); 1.32, (19-Me); 2.12, (25-Me); 5.86, (4-H). ^{13}C-nmr: 209.3, (24 C=O); 199, (3 C=O); 169.1, (C-5); 126.6, (C-4); 76.2, (C-9).

Anal.: Calc. for $C_{25}H_{38}O_3$ (386): C, 77.67; H, 9.91.
Found: C, 77.49; H, 9.94; Mol. wt. 386 (Mass spec.).

Dehydration of 9-Hydroxy-27-nor-4-cholestene-3,24-dione (II)

The ethyl ketone II (260 mg) was suspended in methylene chloride (25 ml) and cooled to -8° in an ice-salt bath. A solution of chlorosulfonic acid (231 mg) in the same solvent was added slowly to the stirred and cooled solution, and stirring was maintained for a further 30 minutes after completion of the addition. Water (0.5 ml) was then added to the mixture, which was allowed to warm to room temperature with constant stirring, by which time the solution had become colorless. The methylene chloride solution was separated and percolated through a small silica gel column, concentrated to a small volume, and

then gently warmed under a current of nitrogen while methanol was added to maintain the volume until crystallization began. The product (120 mg) was further crystallized from methanol/water to give an analytical sample of 27-nor-4,9(11)-cholestadiene-3,24-dione (IV), mp 119.1-120.1°, $(\alpha)_D^{25}$ + 80° (c, 1.0010). ν_{max} 1715 cm^{-1}, (sat. ketone); 1660, 1605 cm^{-1}, (α, β-unsat. ketone). ^{1}H-nmr: 0.66, (18-Me); 0.92, (d., 21-CH_3); 1.32, (19-CH_3); 1.06, (tr., 26-CH_3); 5.46, (11-H); 5.74, (4-H).

Anal.: Calc. for $C_{26}H_{38}O_2$ (382): C, 81.62; H, 10.01.
Found: C, 81.47; H, 9.80; Mol. wt. 382 (Mass spec.).

Dehydration of 9-Hydroxy-26,27-dinor-4-cholestene-3,24-dione (III)

Using the procedure described above, the methyl ketone III (186 mg) was treated with chlorosulfonic acid (190 mg) in methylene chloride (7 ml) and the product obtained as crystals (110 mg) from methanol which were shown by tlc to contain a little starting material. This was separated by chromatography on a small column of silica gel prepared in a Pasteur pipette and eluted with methylene chloride. Recrystallization of the major fraction from methanol/water gave 26,27-dinor-4,9(11)-cholestadiene-3,24-dione (V; 70 mg), mp 145.1-146.2°, $(\alpha)_D^{25}$ + 81° (c, 0.9680). ν_{max} 1715 cm^{-1}, (sat. ketone); 1660, 1610 cm^{-1}, (α, β-unsat. ketone). ^{1}H-nmr: 0.66, (18-Me); 0.92, (d., -21-Me); 1.34, (19-Me); 2.14, (26-Me); 5.48, (11-H); 5.74, (4-H).

Anal.: Calc. for $C_{25}H_{36}O_2$ (368): C, 81.47; H, 9.85.
Found: C, 81.63; H, 10.14; Mol. wt. 368 (Mass spec.).

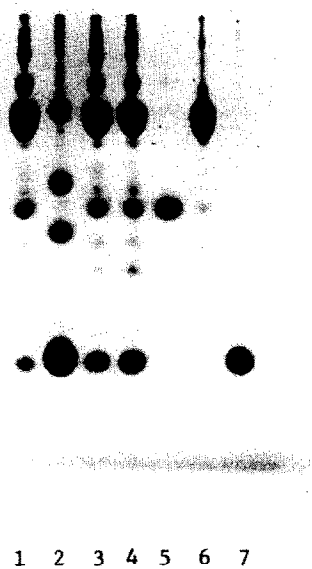


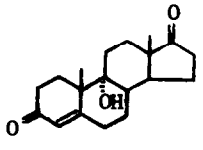
Fig. 1.: Thin layer chromatogram of the products of sterol bioconversions by *M. fortuitum* mutants NRRL B-8119 and SCM-1.

(Tlc conditions as described in the Experimental section and bioconversion procedures as in Ref. 4.)

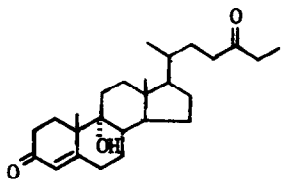
1) Sitosterol/campesterol.
2) 9-hydroxy-4-androsten-3,17-dione (I).
3) 9-hydroxy-3-oxo-23,24-dinor--4-cholen-22-oic acid methyl ester.
4) Bioconversion of sitosterol/campesterol mixture by NRRL B-8119.
5) Bioconversion of cholesterol by NRRL B-8119.
6) Bioconversion of sitosterol/campesterol by SCM-1.
7) Bioconversion of cholesterol by SCM-1.

RESULTS AND DISCUSSION

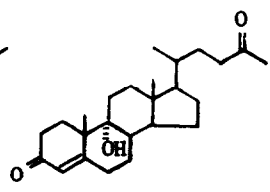
It was previously found (4) that *M. fortuitum* NRRL B-8119, a mutant blocked in its ability to degrade the steroid ring system, efficiently converted cholesterol, campesterol and sitosterol to 9-hydroxy-4-androstene-3,17-dione (I), plus smaller quantities of 9,17β-dihydroxy-4-androsten-3-one; 9,22-dihydroxy-23,24-dinor-4-cholen-3-one; 3-keto-9-hydroxy-23,24-dinor-4-cholen-22-oic acid, and the corresponding methyl ester (Fig. 1, lanes 4 and 5). A culture of this mutant was subsequently treated with N-methyl-N'-nitro-N-nitrosoguanidine, and from it was isolated a new mutant, designated SCM-1, that bioconverted cholesterol as did NRRL B-8119, but which showed quite different product accumulations when incubated with sterols bearing side-chains branched at the C-24 position. Thus, when crude methylene chloride extracts of sitosterol/campesterol bioconversions by SCM-1 were examined by thin layer chromatography, it was found that two new products were present, intermediate in polarity between the unconverted sterols and the 9-hydroxy-ketone (I) (Fig. 1). After removal of the bulk of the residual sterols from such an SCM-1 bioconversion by crystallization from aqueous acetone, the remaining mother-liquor solids were redissolved in ethyl acetate, from which a mixture of the two new compounds could be directly



I



II



III

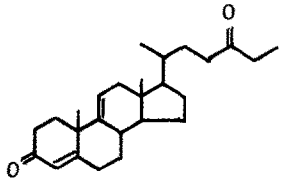
crystallized. The individual components of this mixture were then separated by chromatography on silica gel.

The mass spectrum of the less polar compound showed a molecular ion at m/e 400, and the distinctive pattern of intense peaks at m/e 124, 136, and 137 characteristic of a 9-hydroxy-4-en-3-one structure (4). The infrared spectrum supported this partial structure, showing peaks at 3540 cm^{-1} (hydroxyl); 1650 and 1605 cm^{-1} (α, β-unsat. ketone); and in addition a peak at 1700 cm^{-1} which revealed the presence of an additional saturated keto-group. The pmr spectrum was quite similar to that of 9,22-dihydroxy-23,24-bisnor-4-cholen-3-one (4), with *tert*-methyl signals at 0.72 and 1.32 and a *sec*-methyl doublet at 0.94, but there was also a sharply-defined triplet due to a primary methyl group at 1.08. The foregoing analytical and spectral evidence points unequivocally to the structure II for this compound, derived by oxidative removal of the terminal isopropyl group from the sitosterol side-chain.

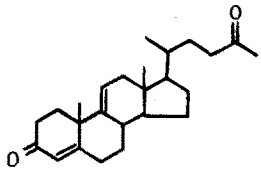
The more polar of the two new metabolites showed an infrared spectrum that was closely similar to that of the 9-hydroxy-dione II, and the mass spectrum exhibited a molecular ion at m/e 386. The high-mass fragments were all 14 mass units less than those shown by the 24-ketone II, while the low-mass fragments were practically the same, which was taken as an indication that the differences lay in the side-chain rather than the steroid nucleus. The pmr spectra were also very similar, except for the fact that the primary-methyl triplet was missing and in its place was a new singlet at 2.14, indicative of an acetyl (CH_3-CO) group. The structure III was therefore assigned to the more polar metabolite, which is evidently derived in the same way as its

homolog II from the campesterol present in the crude sitosterol used in the bioconversion.

These structures were further confirmed by dehydration to the corresponding 9(11)-enes IV and V, using chlorosulfonic acid (5) which avoids the production of double-bond isomers. In each case the distinctive mass spectral peaks at m/e 137, 136, and 124 were no longer of significant intensity. Both products showed intense molecular ions (m/e 367 and 353). In the pmr spectrum of each dehydration product a new signal, due to the olefinic proton at C-11, appeared as a broad doublet at 5.46.



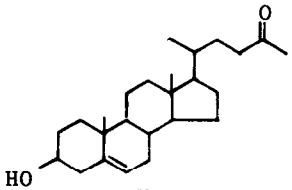
IV



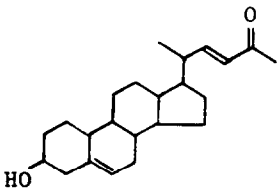
V

The differential product accumulations observed in bioconversions of cholesterol and phytosterols by SCM-1 suggests the involvement of a unique enzyme in the degradation of side-chains branched at C-24. As shown in Fig. 1 (lane 6), this mutant also accumulated significant amounts of the 9-hydroxy-dione I in addition to the 24-oxo compounds II and III when incubated with phytosterols. This suggests that either a) the mutation affecting cleavage at C-24 in SCM-1 is leaky, or b) that there is a secondary pathway not utilizing this enzyme by which phytosterols are converted to 17-keto-steroids by this bacterium. Further bioconversion experiments and studies with cell-free extracts may enable us to determine which of these possibilities is correct, and also the nature of the genetic defect in mutant SCM-1.

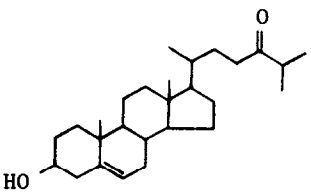
Finally, it is of interest to note that a few steroids with side-chains similar to those of the products reported here have been isolated from natural sources. The 24-ketone VI, for instance, occurs in the sponge *Psammaplysilla purpurea* (6), and both the unsaturated ketone VII and the isopropyl ketone VIII have been found in the sponge *Damiriana hawaiiana* (7). It is not yet known if these steroids are biosynthesized by the organisms themselves or if they originate in their diet, but artifact formation from autoxidation products was definitely ruled out.



VI



VII



VIII

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