Microbial Transformation of β-Sitosterol: Accumulation of 3-(5α-Hydroxy-7aβ-methyl-1-oxo-3aα*H*-hexahydroindan-4α-yl)propionic Acid and the *X*-Ray Structural Identification of Its δ-Lactone

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β-Sitosterol is degraded by a mutant of *Nocardia* sp. M29 to 3-(5α-hydroxy-7aβ-methyl-1-oxo-3aα*H*-hexahydroindan-4α-yl)propionic acid (VI), the δ-lactone of which (VII) was identified by *X*-ray structural analysis. The ratio of the acid to its lactone is found to be dependant on the pH of the medium and is not the result of enzymatic interaction. The characteristics of the β-sitosterol degradation are discussed.

THE synthesis of steroid hormones and their precursors for the convergent synthesis of pharmaceutically interesting retrosteroids may be achieved by means of fermentation processes. Phytosterols, as potential cheap sub-

strates, are often employed for microbial transformations.[1-4] Perhydroindanylpropionic acid derivatives are obtained as intermediates of microbial steroid oxidation and are useful precursors for convergent synthesis. As previously reported, they may be obtained from progesterone and other C_{21} steroids.[5-7] The first isolation of a ring-CD δ-oxo-acid was reported in 1961 by Schubert *et al.*,[8] who incubated *Mycobacterian smegmatis* in the presence of progesterone as part of their investigations of the mechanisms of steroid degradation. They were able to elucidate the degradation pathway of the steroid skeleton and the C-17 side-chain of cholesterol and to demonstrate it as a common reaction scheme for microbial transformations.[9] In a screening for microorganisms capable of degrading the C-17 side-chain of β-sitosterol we undertook mutation experiments on *Nocardia* sp. M29, a strain which is capable of total and rapid oxidation of β-sitosterol to CO_2, H_2O, and cellular material. Here we report the transformation of β-sitosterol to 3-(5α-hydroxy-7αβ-methyl-1-oxo-3aα*H*-hexahydroindan-4α-yl)propionic acid (VI) by the mutant *Nocardia* M29–40.

EXPERIMENTAL

Materials—*Nocardia* sp. M29, a laboratory strain capable of rapid assimilation of β-sitosterol was mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The present results were made on the mutant M29–40. Casamino acids (technical) were purchased from Difco Laboratories, Detroit, Michigan, corn-steep liquor from Kellogg AG, Bremen, 5α-cholestane from Fluka AG, Neu-Ulm/Donau, Tween 80 and Amberlite XAD-2 from Serva Feinbiochemica, Heidelberg, t.l.c. plates Woelm, silica gel F254/366 from ICN Pharmaceutics. All other chemicals were purchased from Merck, Darmstadt.

Methods.—Cultures were incubated at 30 °C on a rotary shaker at 100 r.p.m. in 500 ml Erlenmayer flasks filled with 100 ml of a medium which contained casamino acids (0.5 g), corn-steep solids, $Na_2HOP_4{\cdot}2H_2O$ (0.1 g), KH_2PO_4 (0.05 g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.3 g), $CaCO_3$ (0.3 g), β-sitosterol (for enzyme induction) (20 mg), and glucose (1.0 g). The pH was adjusted to 6.8 with NaOH before autoclaving. The medium was inoculated with a 24 h old culture (1 ml) of the mutant *Nocardia* M29–40 contained in the same medium; 24 h after incubation, β-sitosterol or other steroids (progesterone, testosterone, or androsta-1,4-diene-3,17-dione) were added to give a final concentration of 2 g l^{-1}. The cultures were incubated for a further 48 h. The β-sitosterol (or other steroid) was taken from an autoclaved and sonicated stock suspension containing β-sitosterol (or other steroid) (4.0 g) and Tween 80 (2.0 g) in distilled water (100 ml).

Extraction.—Unless noted otherwise, the cultures were acidified to pH 2.0 with 10% H_2SO_4, extracted with chloroform and subsequently separated. The concentrated extracts were spotted on thin-layer plates with fluorescent indicator and developed in diethyl ether–chloroform (1 : 4) or toluene–dioxan–glacial acetic acid (30 : 10 : 1). The spots were made visible by u.v., sprayed with concentrated H_2SO_4, and then heated at 120 °C for 5 min. G.l.c. was performed on a Packard 427 with 2.0 m glass columns (2 mm i.d.) filled with 3% OV 17 on Chromosorb G (80—100 mesh), with N_2 carrier gas. The oven was heated for 7 min at 250 °C, raised to 290 °C at 11° min^{-1}, then held at this temperature for 24 min. 5α-Cholestane was used as internal standard. Yields are expressed as % theoretical.

Purification and Identification.—Fermentation products were isolated from the raw extract by preparative t.l.c. in the solvent systems mentioned or by column chromatography on Amberlite XAD 2 (100—200μ) eluted with a water gradient with an increasing methanol content and 5% dichloromethane. Fractions containing the lactone (VII) were evaporated to dryness and recrystallised from light petroleum (b.p. 40—80 °C)–methanol. The m.p. was determined on a Kofler apparatus. I.r. spectra were recorded on a Perkin-Elmer 521 spectrometer, mass spectra on an A.E.I. MS 30 spectrometer, and ^1H and ^{13}C n.m.r. spectra on Varian XL 100 12 and CFT 20 spectrometers respectively, in the Fourier-transform mode.

X-*Ray Analyses of* (VII).—Intensities from an elongated prismatic crystal were collected on a Syntex $P2_1$ four-circle diffractometer with graphite-monochromated Mo-K_α radiation (λ = 0.710 69 Å). Measurements were carried out in the θ—2θ mode ($3.0 \leqslant 2\theta \leqslant 50.0°$) at scan speeds varying linearly between 2.93 and 11.72° min^{-1}. Scan and background times were equal. Three standard reflexions, monitored at regular intervals, did not display any significant variations. Lorentz and polarisation but no absorption corrections (μ(Mo-K_α) 0.51 cm^{-1}) were applied. After application of the acceptance criterion $F \geqslant 3.0\sigma(F)$, where $\sigma(F)$ is based on the counting statistics, 1 314 unique reflexions were retained for use in the structure analysis. Cell dimensions were determined by a least-squares fit to the settings for 15 reflexions ($\pm$ *hkl*) on the diffractometer.

Crystal data for VII. $C_{13}H_{18}O_3$, M = 222.3. Orthorhombic, a = 16.721(7), b = 19.914(9), c = 7.023(4) Å, U = 2338.5(19) Å^3, Z = 8, D_c = 1.26 g cm^{-3}. Space group $P2_12_12_1$.

Structure determination. The unit cell contains two independent molecules. The structure was solved with some difficulty by non-centrosymmetric direct methods. After attempts to solve the structure with various starting sets for E_{min} 1.2 had failed, it was found that reduction of the number of phases by increasing E_{min} to 1.3 yielded an E map which included 28 of the 32 non-hydrogen atoms. Hydrogen atoms were located in subsequent difference syntheses. Refinement, with anisotropic temperature factors for all non-hydrogen atoms and group isotropic temperature factors for the hydrogen atoms, was by blocked full-matrix least-squares, $\Sigma w\Delta^2$ being minimised. The terminal value of R_G [$= (\Sigma\omega\Delta^2/\Sigma wF_o^2)^{1/2}$] was 0.071, with R' ($= \Sigma w^{1/2}\Delta/\Sigma w^{1/2}F_o$) 0.065, and R 0.074. The weights were given by $w = k[\sigma^2(F_o) + gF_o^2]^{-1}$ where k and g refined to 1.595 4 and 0.001 120. Calculations were carried out with

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the program SHELX 76 (G. M. Sheldrick, Cambridge) and local ancillary programs (W. S. S.). Complex neutral-atom scattering factors [10,11] were employed. Tables of hydrogen-atom positions, carbon, and oxygen anisotropic temperature factors, and observed and calculated structure factors are deposited as Supplementary Publication No. SUP 22178 (11 pp., 1 microfiche).*

TABLE 1

Atom positional parameters for (VII)

	x/a	y/b	z/c
C(1′)	0.978 7(5)	0.174 8(4)	0.316 2(12)
C(2′)	0.960 0(5)	0.247 6(5)	0.293 7(15)
C(3′)	0.880 4(5)	0.263 5(5)	0.203 0(17)
C(4)	0.814 5(5)	0.218 7(3)	0.278 3(13)
C(5)	0.837 4(5)	0.114 8(5)	0.242 2(16)
O(5)	0.918 8(3)	0.129 6(2)	0.317 3(8)
C(6)	0.783 0(5)	0.094 3(5)	0.336 0(18)
C(7)	0.761 7(5)	0.108 5(5)	0.543 1(17)
C(7)a	0.731 3(4)	0.180 5(4)	0.563 2(13)
C(3a)	0.796 7(4)	0.227 7(3)	0.489 6(12)
C(3)	0.770 4(5)	0.297 6(5)	0.560 4(17)
C(2)	0.736 6(6)	0.283 0(6)	0.753 4(16)
C(1)	0.718 2(5)	0.206 8(5)	0.762 6(15)
O(1′)	0.046 0(3)	0.153 9(2)	0.348 4(9)
O(1)	0.698 8(4)	0.176 4(4)	0.901 2(11)
C(7aβ)	0.649 2(5)	0.188 4(5)	0.465 1(16)
C(1′)′	0.502 4(5)	0.337 2(4)	0.181 2(12)
C(2′)′	0.591 6(5)	0.347 0(4)	0.165 9(16)
C(3′)′	0.618 3(5)	0.410 5(4)	0.071 1(16)
C(4)′	0.569 1(5)	0.471 3(4)	0.137 1(14)
C(5)′	0.481 4(5)	0.457 0(4)	0.091 4(16)
O(5)′	0.453 9(3)	0.391 1(2)	0.166 2(9)
C(6)′	0.422 8(5)	0.508 2(4)	0.174 6(17)
C(7)′	0.436 4(5)	0.526 0(4)	0.381 9(16)
C(7a)′	0.523 8(4)	0.545 5(3)	0.411 8(14)
C(3a)′	0.577 4(5)	0.486 8(3)	0.345 9(12)
C(3)′	0.659 6(5)	0.506 0(5)	0.430 9(16)
C(2)′	0.639 0(6)	0.531 9(5)	0.623 9(15)
C(1)′	0.550 9(5)	0.555 9(4)	0.617 1(14)
O(1′)′	0.472 1(3)	0.283 9(2)	0.217 9(10)
O(1)′	0.513 8(3)	0.576 6(3)	0.749 1(10)
C(7aβ)′	0.541 7(6)	0.613 4(4)	0.310 1(17)

TABLE 2

Bond lengths (Å)

C(2′)–C(1′)	1.491(13)	O(5)–C(1′)	1.346(10)
O(1′)–C(1′)	1.220(11)	C(2′)–C(3′)	1.509(14)
C(3′)–C(4)	1.515(13)	C(4)–C(5)	1.542(13)
C(3a)–C(4)	1.524(13)	C(5)–O(5)	1.492(10)
C(5)–C(6)	1.507(14)	C(6)–C(7)	1.524(18)
C(7)–C(7a)	1.527(13)	C(3a)–C(7a)	1.532(11)
C(1)–C(7a)	1.511(14)	C(7a)–C(7aβ)	1.544(13)
C(3)–C(3a)	1.542(13)	C(2)–C(3)	1.497(16)
C(1)–C(2)	1.549(17)	C(1)–O(1)	1.192(13)
C(2′)′-C(1′)′	1.507(13)	O(5)′–C(1′)′	1.349(10)
O(1′)′–C(1′)′	1.204(11)	C(2′)′–C(3′)′	1.498(14)
C(3′)′–C(4)′	1.535(13)	C(4)′C(5)′	1.529(13)
C(3a)′–C(4)′	1.505(13)	C(5)′O(5)′	1.487(11)
C(5)′–C(6)′	1.531(14)	C(6)′–C(7)′	1.516(16)
C(7)′–C(7a)′	1.527(12)	C(3a)′–C(7a)′	1.544(11)
C(1)′–C(7a)′	1.525(14)	C(7a)′–C(7aβ)′	1.558(13)
C(3)′–C(3a)′	1.547(13)	C(2)′–C(3)′	1.491(16)
C(1)′–C(2)′	1.550(13)	C(1)′–O(1)′	1.189(12)

* See Notice to Authors No. 7 in *J.C.S. Perkin II*, 1977, Index issue.

RESULTS

Cultures of the mutant strain incubated with β-sitosterol accumulated a rather polar substance, which was not u.v.-active and was identified after extraction as the δ-lactone (VII) of 3-(5α-hydroxy-7aβ-methyl-1-oxo-3aα*H*-hexahydroindan-4α-yl)propionic acid (VI). Figure 1 shows the degradation of β-sitosterol and the accumulation of (VI) in the culture. It shows that degradation of (VI) commences when a high percentage of β-sitosterol is still present. About 20% of the original substrate remained even when (VI) was completely assimilated. Elution of the culture extract on an Amberlite XAD 2 column by water–methanol–dichloromethane (75 : 24 : 1) yielded (VII) and traces of androsta-1,4-diene-3,17-dione. By use of these solvents at (50 : 47.5 : 2.5) 17-hydroxyandrosta-1,4-dien-3-one was first eluted, followed by large quantities of residual β-sitosterol and stigmast-4-en-3-one with solvent concentrations (20 : 76 : 4). Androsta-1,4-diene-3,17-dione and 17-hydroxyandrosta-1,4-dien-3-one were identified by comparison with authentic substances. After recrystallisation the following characteristic data were obtained for (VII): m.p. 123—124 °C; $[\alpha]_D^{20}$ +118° (*c* 1.43 g l^{-1} in CH_3OH); *m/e* 222 (M^+) (Found: C, 70.30; H, 8.10. Calc. for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16%). The structure of (VII) is confirmed by the *X*-ray analysis (Figure 2). There can be no doubt concerning the structure at O(5), C(5), and C(6), in view of the fact that all protons could be located and refined with sensible group isotropic temperature factors, and in view of the O(5)–C(1′) [1.346(10) and 1.349(10) Å], O(5)–C(5) [1.492(10) and 1.487(11) Å], and C(5)–C(6) [1.507(14) and 1.531(14) Å] bond distances. All C–C bond distances lie within the range 1.491—1.558 Å

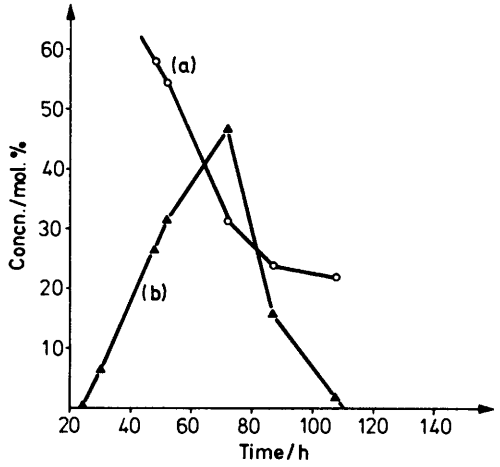

FIGURE 1 Degradation of (a) β-sitosterol and (b) formation of (VI) in a culture of *Nocardia* M29–40 (incubation temperature 30 °C, 2 g l^{-1} β-sitosterol added after 24 h cultivation)

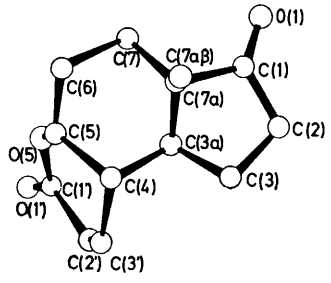

FIGURE 2 Perspective drawing of (VII) showing the numbering system used in the crystallographic analysis

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and are thereby typical for a single bond, as are the C(1′)–O(1′) and C(1)–O(1) distances (range 1.189—1.220 Å) for a double bond. Both independent molecules in the unit cell display the same absolute configuration. Although no attempt was made to confirm this by use of the anomalous dispersion of oxygen, on the justified assumption that C(7a) is in the β-configuration, the ring conformations are as follows: B chair, C chair, and D 3aα envelope. The ring junction B/C is *cis*, whereas C/D is *trans*. The ring lettering is here based on steroid nomenclature.

TABLE 3

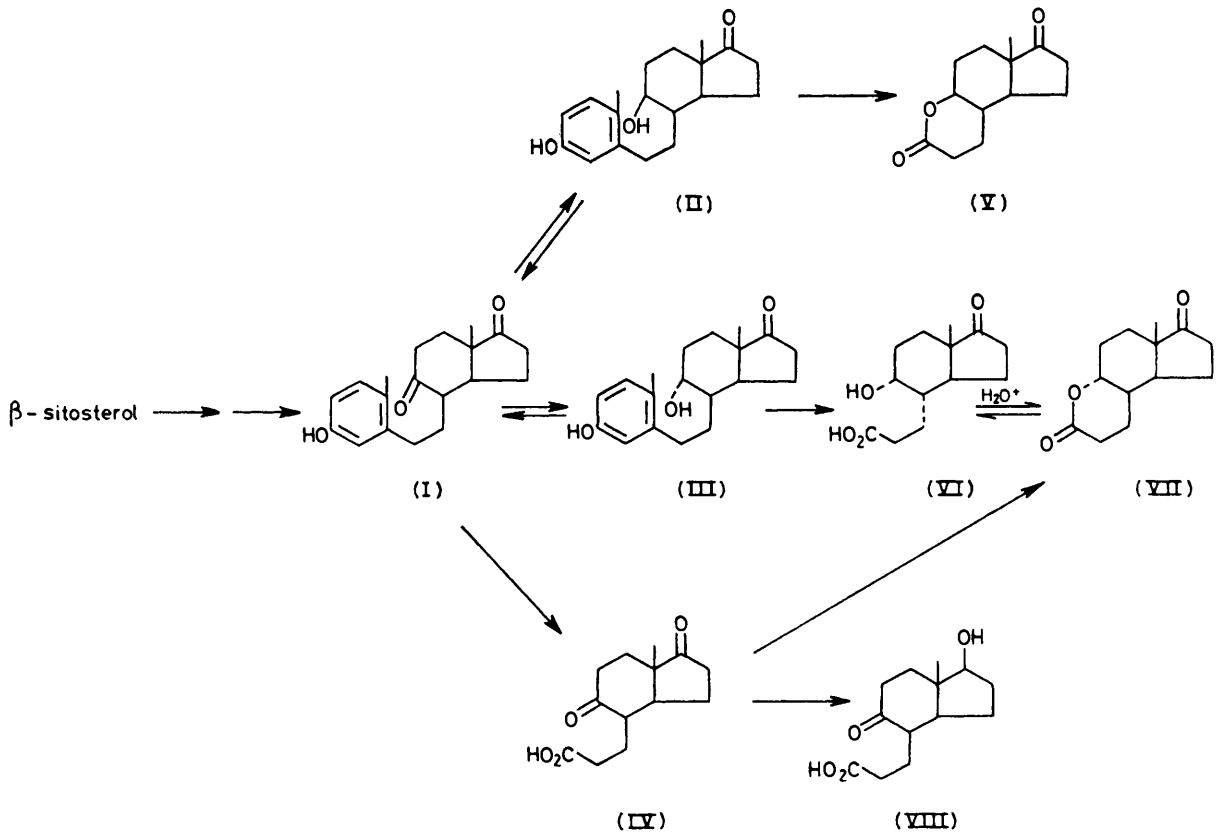
^{13}C Chemical shifts (p.p.m.) of (VII) in $CDCl_3$ to low field of the internal standard tetramethylsilane

Shift	Assignment *
218.66	C(1)
172.06	C(1′)
77.81	C(5)
47.46	C(7a)
41.75	C(3a)
35.20	C(2)
32.52	+
26.60	+
26.41 (×2)	+
21.34	+
21.04	+
12.69	C(7aβ)

* See text. Resonances of C(3), C(4), C(6), C(7), C(11), and C(12) were not unambiguously assigned.

The ^{1}H and ^{13}C n.m.r. spectra are compatible with the *X*-ray structure of (VII). In the ^{1}H spectrum there is a quartet at 4.5 p.p.m. to low field of tetramethylsilane which is characteristic of a proton with a geminal oxygen function. The quartet structure, containing *J* values of 2.5 Hz, arises from three vicinal couplings of equal magnitude which indicate *gauche* arrangements of the coupled protons.[12–14] The ^{13}C spectral data are given in Table 3. The resonance at 218.66 p.p.m. unambiguously identifies the carbonyl group in the five-membered ring, and signals characteristic of the carbonyl function at C(1′) and the oxygen-carrying C(5) are observed at 172.06 and 77.81 p.p.m. respectively.[13] Single-frequency off-resonance spectra unambiguously identified the resonance of C(7a) at 12.69 p.p.m. while the remaining signals were partially assigned by comparison with the ring D resonances found in androst-4-ene-3,17-dione.[15,16]

As we noticed that the yield of (VII) extracted from cultures depended on the pH of the medium, titration experiments were undertaken. We found that an equilibrium exists between the lactone (VII) and its parent acid form (VI) which is dependent on pH and is not the result of enzymatic interaction. Under culture conditions at pH 6.0—7.5 (VI) will dominate. When we incubated the mutant in the presence of testosterone, androsta-1,4-diene-3,17-dione, or progesterone, we isolated 71, 71, and 60%



SCHEME Degradation pathway of β-sitosterol

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of (VII) respectively, 26 h after substrate addition. That the larger quantity of (VI) was obtained at an earlier stage of cultivation is easily inderstandable, as these derivatives are, or resemble, intermediates in the β-sitosterol degradation. Upon addition of the sodium salt of (VI) to cultures of M29–40 we observed almost no degradation of the substrate within 24 h, whereas the parent was capable of oxidising it completely.

DISCUSSION

The intermediate (VII) has also been isolated by Wang and Sih [17] by exposing *Nocardia restrictus* to testosterone (see Scheme) and characterised by i.r. and ^{1}H n.m.r. spectra. In addition, they also isolated the 5β-hydroxy-epimers (V) and their precursors the secophenols (I), (II), and (III). They demonstrated further that both lactones (V) and (VII) are not obligatory intermediates. On incubating (IV) with *Nocardia corallina* two products (VII) and (VIII) were obtained.[18]

However, we never observed the 5β-hydroxy-epimers (V) or (IV) and (VII) in our fermentations with the mutant M29–40. The main product of β-sitosterol degradation was always (VI). Upon incubating the mutant with its own degradation intermediate androsta-1,4-diene-3,17-dione we again observed an accumulation of (VI), in this case at an even higher rate. The degradation of testosterone and progresterone also led to a large quantity of (VI). So we assume (VI) to be the preferred intermediate in the degradation of the steroid skeleton by our mutant. As we could demonstrate that the parent strain was able to oxidise (VI) rapidly and completely, whereas the mutant produced virtually no degradation within the same time, it is probable that the mutation causes a diminished enzymatic activity in one of the enzymes, which are responsible for steroid C,D-ring degradation. Thus the mutant M29–40 enables the production of a useful precursor for the convenient synthesis of retrosteroids from β-sitosterol, whereas former transformations aiming at perhydroindane derivatives were possible only with the more valuable C-19 or C-21 steroids as substrates.

We thank Frau G. Hoffmann, Drs. L. Grotjahn, G. Rehme, and V. Wray for carrying out the i.r., mass, o.r.d., and n.m.r. spectroscopic measurements, and Frau A. Borkenstein for technical assistance in the *X*-ray analysis. We also thank Drs. S. Lang and V. Wray for helpful discussions on the interpretation of spectra.

[7/911 *Received*, 26*th May*, 1977]

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