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Microbiological Degradation of Bile Acids

NITROGENOUS HEXAHYDROINDANE DERIVATIVES FORMED FROM CHOLIC ACID BY *STREPTOMYCES RUBESCENS*

By SHOHEI HAYAKAWA, SHIGERU HASHIMOTO and TADAMASA ONAKA*
Shionogi Research Laboratory, Shionogi and Co. Ltd., Fukushima-ku, Osaka, 553 Japan

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The metabolism of cholic acid (I) by *Streptomyces rubescens* was investigated. This organism effected ring A cleavage, side-chain shortening and amide bond formation and gave the following metabolites: (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II*a*) and its mono-amide (valeramide) (II*b*); and 2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-1*H*-cyclopenta[*f*]quinoline-3,7-dione (III*e*) and its homologues with the β-oriented side chains, valeric acid, valeramide, butanone and propionic acid, in the place of the oxo group at C-7, i.e. compounds (III*a*), (III*b*), (III*c*) and (III*d*) respectively. All the nitrogenous metabolites were new compounds, and their structures were established by partial synthesis except for the metabolite (III*c*). The mechanism of formation of these metabolites is considered. A degradative pathway of cholic acid (I) into the metabolites is also tentatively proposed.

Our approach to defining the intermediates and reaction sequence involved in the degradation of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid) by micro-organisms is to identify the further degradation products of a cholic acid metabolite formed by the metabolite-producing organism(s) and to establish the precursor–product relationship between the products. However, it was suggested in a previous paper (Hayakawa *et al.*, 1976*a*) that not all the metabolites formed from cholic acid by a certain organism could be utilized by the same organism. Therefore we searched for other approaches to this problem, and we believe that one of them is to identify as many degradation products as possible formed from cholic acid by cholic acid-utilizing organisms, even though the yield may be quite low. Another one is to identify the further degradation products of a cholic acid metabolite by using the metabolite-utilizing organisms, which, in some cases, may be different from the metabolite-producing organisms. We used *Streptomyces rubescens* for the former approach and *Corynebacterium equi* for the latter. In our earlier studies, *S. rubescens* was shown to utilize cholic acid as the sole source of carbon, and, after the approx. 5-day incubation, produced the following metabolites: 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid, 7α-hydroxy-3,12-dioxochol-4-en-24-oic acid, 12α-hydroxy-3-oxochola-4,6-dien-24-oic acid, 3,12-dioxochola-4,6-dien-24-oic acid and some unidentified products (Hayakawa *et al.*, 1958*a*,*b*). We expected therefore that the incubation of this organism with cholic acid for periods up to more than a week would result in the formation of more degraded metabolites than those isolated previously.

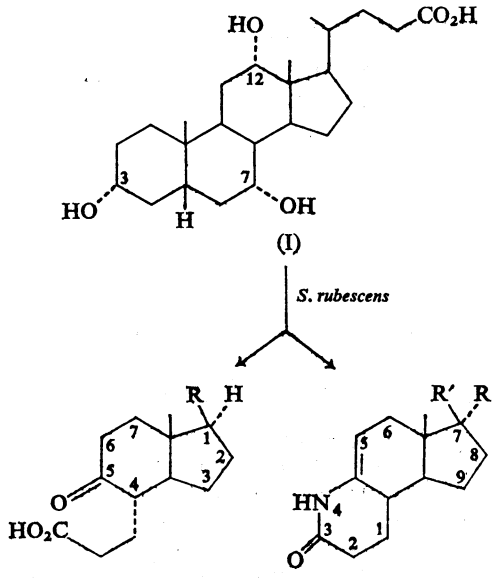
The present paper deals with the isolation and identification of the degradation products formed from cholic acid (I) by *S. rubescens* (Scheme 1); a preliminary note of this work has already appeared (Hayakawa *et al.*, 1969*a*). The metabolism of (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II*a*) by *C. equi* is reported in the following paper (Hayakawa *et al.*, 1976*b*).

* Deceased 23 December 1972. Visiting Scientist from Research Foundation, Itsuu Laboratory, Setagaya-ku, Tokyo, 158 Japan.

Results

Isolation of the metabolites and their structure assignments

The course of the degradation of cholic acid and of the formation of metabolites was monitored by t.l.c. as described for the degradation of cholic acid by *Arthrobacter simplex* (Hayakawa *et al.*, 1969*b*). The result of t.l.c. showed that, after the 4-day incubation, most of cholic acid was converted into the metabolites containing the cholane nucleus isolated previously (Hayakawa *et al.*, 1958*a*,*b*) and some unidentified products, although these products were not isolated for their definite identification. On continued incubation, several metabolites were newly produced in parallel with the disappearance of the above cholane-nucleus-containing products. The incubation was usually carried out for more than 1 week and the



(II) (*a*) R = $CH(CH_3)$-$[CH_2]_2$-CO_2H;
(*b*) R = $CH(CH_3)$-$[CH_2]_2$-$CONH_2$

(III) (*a*) R = H, R' = $CH(CH_3)$-$[CH_2]_2$-CO_2H;
(*b*) R = H;
R' = $CH(CH_3)$-$[CH_2]_2$-$CONH_2$;
(*c*) R = H, R' = $CH(CH_3)$-CO-CH_3;
(*d*) R = H, R' = $CH(CH_3)$-CO_2H;
(*e*) R, R' = =O

Scheme 1. *Microbiological degradation of cholic acid (I) to form the C_{18} oxodicarboxylic acid (IIa) and the nitrogenous hexahydroindane derivatives (IIb), (IIIa), (IIIb), (IIIc), (IIId) and (IIIe)*

resulting incubation mixture used for the isolation of metabolites.

C_{18} enaminolactam amide (IIIb). This was isolated from the neutral fraction of metabolites as crystals and its elementary analysis was consistent with $C_{18}H_{28}N_2O_2$. The u.v.-absorption spectrum was similar in both position and intensity to that of the C_{13} enaminolactam (IIIe) described below and indicated the presence of an enaminolactam structure: this was also indicated by an intensive absorption at 1677 cm^{-1} in the i.r. spectrum and by an unresolved multiplet at δ 4.82 (1H; vinylic proton) and a broad peak at δ 8.27 (1H; CONH) p.p.m. in the n.m.r. spectrum. A broad peak at δ 5.77 (2H) p.p.m. in the n.m.r. spectrum could be assigned to two protons of a primary amide group: this was also indicated by an absorption at 1594 cm^{-1} (amide II band) in the i.r. spectrum. The n.m.r. spectrum indicated also the presence of both secondary and tertiary methyl groups at δ 0.95 (doublet; 3H) and 0.73 (single; 3H) p.p.m. respectively. Thus the structure of this metabolite was assigned as (4*R*)-4-(2,3,4,6,6aβ,7,8,9,9aα,-9bβ-decahydro-6aβ-methyl-3-oxo-1*H*-cyclopenta[*f*]-quinolin-7β-yl)valeramide (IIIb).

C_{13} enaminolactam (IIIe). This was also isolated from the neutral fraction of metabolites as crystals and its elementary analysis was consistent with $C_{13}H_{17}NO_2$ (mol.wt. 219.27). Support for this formula was provided by the mol.wt. (212) and by the presence of 17 protons calculated from proton integration in the n.m.r. spectrum. The n.m.r. spectrum showed signals at δ 0.95 (singlet; 3H), 4.95 (unresolved multiplet; 1H) and 8.54 (broad peak; 1H) p.p.m. The presence of a lactam group was indicated by bands at 3390 and 3190 (NH) and 1667 (C=O) cm^{-1} in the i.r. spectrum: this was also indicated by a broad peak at δ 8.54 (1H; CONH) p.p.m. in the n.m.r. spectrum. The presence of only one methyl group, indicated by a singlet at δ 0.95 (3H; tertiary Me) p.p.m. in the n.m.r. spectrum, suggested that the cholic acid side chain did not remain undisturbed, since a doublet corresponding to the secondary methyl group at C-20 was

not observed. Therefore an i.r. absorption at 1740 cm^{-1} could be assigned to a five-membered-ring carbonyl (17-one in the steroid skeleton). Further, a strong u.v.-light absorption at 230.5 nm (ε 13 800) was similar in both position and intensity to that of several steroids containing a Δ^5-4-aza-3-one structure (cf. Doorenbos *et al.*, 1961). The presence of a double bond, which corresponds to this 5-ene, was also indicated by a multiplet at δ 4.95 (1 H; vinylic proton) p.p.m. in the n.m.r. spectrum. From its source and these physical data, the structure of this metabolite was assigned as 2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-1 *H*-cyclopenta[*f*]quinoline-3,7-dione (III*e*).

C_{18} oxodicarboxylic acid (IIa). This was isolated from the acidic fraction of metabolites as its methyl ester. The structure of this ester and its corresponding free acid was established by direct comparison with the respective authentic samples of (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II*a*) and its dimethyl ester.

All the metabolites (II*b*), (III*a*), (III*c*) and (III*d*) described below were also isolated from the acidic fraction as crystals.

Enaminolactam methyl ketone (IIIc). The elementary analysis was consistent with $C_{17}H_{25}NO_2$ (mol.wt. 275.38). The mol.wt. (280) also supported this formula. As described above for compound (III*e*), the presence of an enaminolactam structure was indicated by the i.r., u.v. and n.m.r. spectra (see under 'Isolation of the degradation products' in the Experimental section). Further, the n.m.r. spectrum showed signals at δ 0.75 (singlet; 3 H; one tertiary methyl group), 1.12 (doublet; 3 H; one secondary methyl group) and 2.11 (singlet; 3 H; one acetyl group) p.p.m. On the basis of these data, the structure of this metabolite was assigned as (3*S*)-3-(2,3,4,6,6aβ,-7,8,9,9aα,9bβ-decahydro-6aβ-methyl-3-oxo-1 *H*-cyclopenta[*f*]quinolin-7β-yl)butan-2-one (III*c*), which is not acidic but neutral, although it was isolated from the acidic fraction.

C_{18} enaminolactam acid (IIIa) and C_{16} enaminolactam acid (IIId). The elementary analysis of these metabolites was consistent with $C_{18}H_{27}NO_3$ and $C_{16}H_{23}NO_3$ respectively. The presence of an enaminolactam structure was indicated by the u.v. and i.r. spectra (see under 'Isolation of the degradation products' in the Experimental section). Thus the structure of these metabolites was assigned as (4*R*)-4-(2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-3-oxo-1 *H*-cyclopenta[*f*]quinolin-7β-yl)-valeric acid (III*a*) and its dinor homologue, (2*S*)-2-(2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-3-oxo-1 *H*-cyclopenta[*f*]quinolin-7β-yl)propionic acid (III*d*) respectively.

C_{18} mono-amide (IIb). The elementary analysis was consistent with $C_{18}H_{29}NO_4$. In contrast with the other metabolites containing an enaminolactam structure described above, the u.v.-absorption spectrum showed no absorption in the wavelength region 210–360 nm. The i.r. spectrum showed the presence of both carboxyl and primary amide groups at approx. 2200–2800 and 1711 and 3450, 3330, 3240, 1649 and 1590 cm^{-1} respectively. On the hydrolysis of the amide bond with alkali, the metabolite yielded the oxodicarboxylic acid (II*a*). Accordingly the structure of this metabolite was assigned as either (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeramide (II*b*) or its position isomer with respect to the amide bond.

Partial syntheses of the metabolites

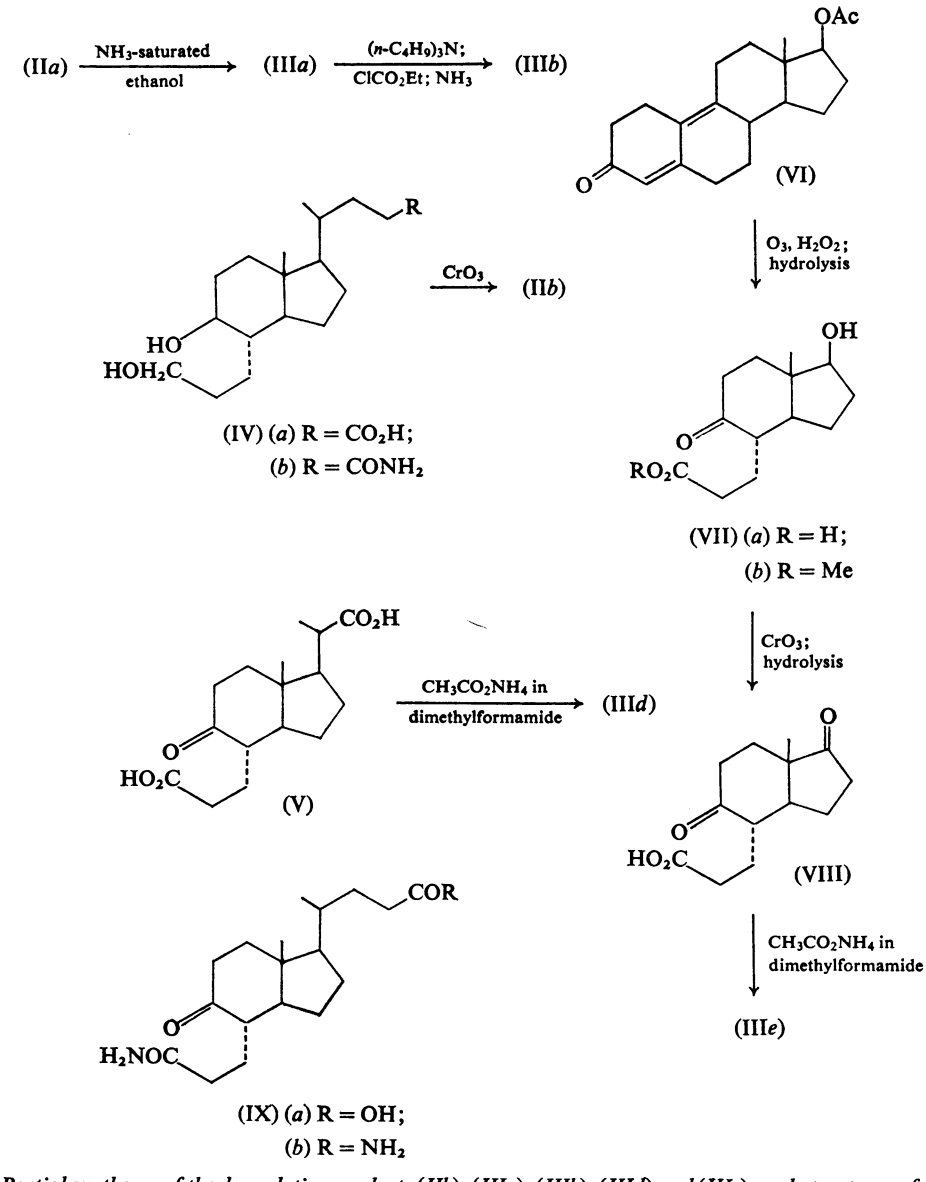
Since the above assignments were only indicative except for the metabolite (II*a*), that they were correct was ascertained by the partial syntheses of all the nitrogenous metabolites, except for the metabolite (III*c*) (Scheme 2).

C_{18} mono-amide (IIb). We at first synthesized the hexahydroindanylvaleramide (II*b*) and not its position isomer, the hexahydroindanylpropionamide (IX*a*), since a convenient starting material for the former synthesis, (4*R*)-4-[3aα-hexahydro-5β-hydroxy-4α-(3-hydroxypropyl)-7aβ-methylindan-1β-yl]valeric acid (IV*a*), was easily available to us (Hayakawa *et al.*, 1976*a*). The 5β-dihydroxy acid was converted into the corresponding amide (IV*b*) by the usual coupling method of mixed carbonic–carboxylic anhydrides with amino acids (cf. Norman, 1955). The 5β-dihydroxy amide was oxidized with CrO_3 to yield compound (II*b*). Fortunately, the synthetic sample was identical in all respects (t.l.c., m.p., mixed m.p., $[\alpha]_D$ and i.r.) with the metabolite (II*b*) obtained from growing cultures. The result excluded another possible structure for this metabolite, the hexahydroindanylpropionamide (IX*a*).

C_{18} enaminolactam acid (IIIa) and its amide (IIIb). Various methods for the syntheses of enaminolactams from δ-oxo acids, derived from the chemical cleavage of certain steroids, have been reported (cf. Tökés, 1963). One of them, the method of Doorrenbos *et al.* (1961), was used for the synthesis of the former: the oxodicarboxylic acid (II*a*) was heated with NH_3-saturated ethanol in a sealed vessel to yield compound (III*a*). Identity with the metabolite (III*a*) obtained from growing cultures was established by comparison of the behaviour on t.l.c., the optical rotation and the u.v. and i.r. spectra.

Compound (III*a*) was converted into the corresponding primary amide (III*b*) by the method described above in the synthesis of compound (IV*b*). The synthetic sample was identical in all respects (t.l.c., m.p., $[\alpha]_D$, u.v., i.r. and n.m.r.) with a sample obtained from growing cultures.

C_{16} enaminolactam acid (IIId). Exactly as described above in the synthesis of compound (III*a*), (2*S*)-2-



Scheme 2. *Partial syntheses of the degradation products (IIb), (IIIa), (IIIb), (IIId) and (IIIe), and structures of the respective hypothetical intermediates (IXa) and (IXb) in the formation of compounds (IIIa) and (IIIb) from compound (IIa)*

[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]propionic acid (V) was treated with NH_3-saturated ethanol to yield compound (III*d*). Identity of the synthetic sample with the isolated sample was established by comparison of the behaviour on t.l.c., the melting point, the optical rotation and the u.v. and i.r. spectra.

Compared with the above method, the acid (V) was more advantageously converted into compound (III*d*) by the method of Nominé *et al.* (1968), who synthesized an enaminolactam derivative by heating a δ-oxo acid with ammonium acetate in dimethylformamide.

C_{13} enaminolactam (IIIe). Just as described above for compound (V), 3-(3aα-hexahydro-7aβ-methyl-1,5-dioxoindan-4α-yl)propionic acid (VIII), which was prepared as described below, was converted into compound (III*e*) by the method of Nominé *et al.*

(1968). The synthetic sample was identical in all respects (t.l.c., m.p., $[\alpha]_D$, u.v., i.r. and n.m.r.) with a sample isolated from growing cultures.

Dioxocarboxylic acid (VIII). 17β-Acetoxyoestra-4,9-dien-3-one (VI) was treated with ozone and then with H_2O_2 to yield the corresponding des-A compound, which with alkali gave 3-(3aα-hexahydro-1β-hydroxy-7aβ-methyl-5-oxoindan-4α-yl)propionic acid (VII*a*). Oxidation of its methyl ester (VII*b*) with a CrO_3–pyridine complex, prepared by the method of Poos *et al.* (1953), followed by alkali yielded the dioxocarboxylic acid (VIII).

Experimental

General

Micro-organism, *S. rubescens*, culture medium and methods for incubation were as described by Hayakawa *et al.* (1958*a*,*b*), and methods for physical measurements were as described by Hayakawa *et al.* (1969*b*). The molecular weight was determined in methanol by the vapour-pressure osmometric method. Unless otherwise stated, optical rotations and i.r.-absorption spectra were determined in chloroform solution and u.v.-absorption spectra were measured in ethanol.

Chromatography

(*a*) *Adsorption column chromatography.* Details were given by Hayakawa *et al.* (1969*b*).

(*b*) *T.l.c.* The glass plates [5cm×20cm (20cm×20cm for preparative t.l.c.), 0.25mm thick] coated with silica gel GF_{254} (nach Stahl; E. Merck A.-G., Darmstadt, Germany) and ethyl acetate/iso-octane/acetic acid (15:5:1, by vol.) as a developing solvent system were used for both analytical and preparative t.l.c., except where otherwise indicated.

(*c*) *Reversed-phase partition chromatography.* This was carried out essentially as described by Sjövall (1964) with the use of Hostalen S [Farbwerke Hoechst A.-G., Frankfurt (M), Germany] as support and the solvent system F1 [for a ratio of solvents, see Sjövall (1964)].

Other materials

As authentic samples for identification and starting materials for partial synthesis, the following compounds were prepared by published methods: (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II*a*) and its dimethyl ester (Hayakawa *et al.*, 1969*b*); (4*R*)-4-[3aα-hexahydro-5β-hydroxy-4α-(3-hydroxypropyl)-7aβ-methylindan-1β-yl]valeric acid (IV*a*) and (2*S*)-2-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]propionic acid (V) (Hayakawa *et al.*, 1976*a*); 17β-acetoxyoestra-4,9-dien-3-one (VI) (Perelman *et al.*, 1960; Perelman & Farkas, 1963). Other materials were obtained from commercial sources and where necessary recrystallized or distilled before use. Light petroleum refers to the fraction of b.p. 40–60°C.

Isolation of the degradation products

The culture filtrate (approx. 2 litres), resulting from the incubation of sodium cholate (6g) with *S. rubescens* for 9 days, was concentrated *in vacuo* to approx. 200ml below 45°C. The concentrate was acidified with dilute HCl to pH approx. 2 and thoroughly extracted with ethyl acetate. The acidic aqueous layer was not treated any further. The organic layer was extracted with 5% (w/v) $NaHCO_3$. The alkaline extract was acidified with dilute HCl and extracted with ethyl acetate. Evaporation of the solvent left an acidic fraction (540mg). The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield a neutral fraction (790mg).

C_{18} enaminolactam amide (IIIb) and C_{13} enaminolactam (IIIe). The above neutral fraction (790mg), on recrystallization from ethyl acetate, yielded crystals (518mg), which were subjected to column chromatography on silicic acid (20g). Elution with dichloromethane/acetone (3:2, v/v) gave (4*R*)-4-(2,3,4,6,6aβ,7,8,9,9aα,9bβ-*decahydro-6aβ-methyl-3-oxo-1H-cyclopenta[f]quinolin-7β-yl)valeramide* (III*b*), which crystallized from methanol in prisms (228mg), m.p. 218.5–221°C (decomp.), $[\alpha]_D^{22}$ +156.0 ±3.6° (*c* 0.545), $\lambda_{max.}$ 232nm (ε13200), i.r. max. at 3500, 3400 and 3190 (NH) and 1677, 1660 (shoulder) and 1594 ($CONH_2$ and enaminolactam) cm^{-1} and n.m.r. absorptions at 0.73 (3H; singlet; 6aβ-Me), 0.95 (3H; doublet; *J* 4.4Hz; Me in the side chain), 4.82 (1H; unresolved multiplet; 5-H), 5.77 (2H; broad peak; $CONH_2$) and 8.27 (1H; broad peak; CONH) p.p.m. (Found: C, 70.8; H, 9.3; N, 9.1; $C_{18}H_{28}N_2O_2$ requires C, 71.0; H, 9.3; N, 9.2%).

The mother liquor, after removal of the above crystals (518mg), was evaporated *in vacuo* to give a residue (272mg), which contained two major and a few minor components as indicated by t.l.c. The residue was subjected to preparative t.l.c. (nine plates, 0.75mm thick). Two distinct bands (R_F, 0.30 and 0.37) due to the major components, as detected by the u.v. scanner, were separately extracted with chloroform/methanol (9:1, v/v). The behaviour on t.l.c. and the i.r. spectrum of the extract (72mg) obtained from the slower-moving band were identical with those of compound (III*b*). The extract (78mg) obtained from the faster-moving band crystallized from methanol to yield an analytical sample of *2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-1H-cyclopenta[f]quinoline-3,7-dione* (III*e*) in prisms (25mg), m.p. 255°C (decomp., sintered at 238°C),

$[\alpha]_D^{23}$ +340.5±10.0° (*c* 0.385), $\lambda_{max.}$ 230.5 nm (ε 13800), i.r. max. at 3390 and 3190 (NH), 1740 (C=O at C-7) and 1667 (C=O of lactam) cm^{-1} and n.m.r. absorptions at 0.95 (3H; singlet; 6aβ-Me), 4.95 (1H; unresolved multiplet; 5-H) and 8.54 (1H; broad peak; CONH) p.p.m. (Found: C, 71.2; H, 7.8; N, 6.7; $C_{13}H_{17}NO_2$ requires C, 71.2; H, 7.8; N, 6.4%; mol.wt. 212).

The neutral fraction (1.962g), obtained from the second run as described below, was treated in a manner similar to that described above for the first run and yielded compounds (III*b*) (341 mg) and (III*e*) (100mg).

C_{18} oxodicarboxylic acid (IIa). The above acidic fraction (540mg) obtained from the first run was chromatographed on silicic acid (20g) and eluted with dichloromethane with progressively increasing amounts of acetone. Elution with dichloromethane/acetone (9:1, v/v) gave the crude acid (II*a*) (78mg), which, after esterification with ethereal diazomethane, was subjected to column chromatography on alumina (2.5g; grade III). Elution with light petroleum/benzene (9:1, v/v) gave the corresponding dimethyl ester as an oil (64mg), which ran as a practically single spot on t.l.c. The i.r. spectrum was identical with that of an authentic sample. On hydrolysis with methanolic 2.5% (w/v) K_2CO_3 (2ml) in the usual manner, the ester yielded a pale-yellowish solid (50mg). Recrystallization from acetone gave an analytical sample of the corresponding free acid (II*a*) (13mg), m.p. 164.5–167°C and $[\alpha]_D^{24}$ +22.6±0.7° (*c* 0.852) (Found: C, 66.6; H, 8.7. Calc. for $C_{18}H_{28}O_5$: C, 66.6; H, 8.7%). Identity with an authentic sample was established by mixed m.p. and by comparison of the behaviour on t.l.c., the $[\alpha]_D$ and the i.r. spectrum.

Enaminolactam methyl ketone (IIIc), C_{16} enaminolactam acid (IIId) and C_{18} enaminolactam acid (IIIa). After elution of the metabolite (II*a*) described above, continued elution with dichloromethane/acetone (from 4:1, v/v, to 3:2, v/v) gave a mixture of products (98mg), which mainly consisted of two metabolites having the respective R_F values 0.57 and 0.51. Since attempts to isolate them from the mixture in a pure form were unsuccessful, the following second run was carried out: sodium cholate (13.80g) was incubated with *S. rubescens* for 14 days, and both the acidic (795 mg) and neutral (1.962g) fractions were obtained in a manner similar to that described above in the first run. There was no significant difference in t.l.c. patterns between the respective fractions of both the runs.

The second acidic fraction was chromatographed on silicic acid (32g) and afforded the mentioned metabolites as follows. Elution with dichloromethane/acetone (from 9:1, v/v, to 4:1, v/v) gave a mixture of products (66mg). It was mainly composed of compound (II*a*) and a compound having R_F 0.55, and then subjected to preparative t.l.c. (two plates, 0.5mm thick). Distinct separation of a main band, as detected by the u.v. scanner, was achieved by developing with ethyl acetate/iso-octane/methanol (15:5:2, by vol.). The band was scraped from the plates and extracted with chloroform/methanol (1:1, v/v). On crystallization from methanol, the extract gave (3*S*)-3-(2,3,4,6,6a*β*,7,8,9,9a*α*,9b*β*-*decahydro*-6a*β*-*methyl*-3-*oxo*-1*H*-*cyclopenta*[*f*]*quinolin*-7*β*-*yl*)*butan*-2-*one* (III*c*) in prisms (13mg), m.p. 242–242.5°C (decomp.), $[\alpha]_D^{24}$ +122.1±13.3°C (*c* 0.122 in ethanol), $\lambda_{max.}$ 233nm (ε 12800), i.r. max. at 3380 and 3200 (NH), 1704 (C=O) and 1662 (enaminolactam) cm^{-1} and n.m.r. absorptions at 0.75 (3H; singlet; 6aβ-Me), 1.12 (3H; doublet; *J* 6.7Hz; Me at C-3 in the butan-2-one side chain), 2.11 (3H; singlet; COMe in the butan-2-one side chain), 4.80 (1H; unresolved multiplet; 5-H) and 7.79 (1H; broad peak; CONH) p.p.m. [Found: C, 74.4; H, 9.0; N, 4.7; $C_{17}H_{25}NO_2$ (mol.wt. 275.38) requires C, 74.1; H, 9.2; N, 5.1%; mol.wt. 280].

Further elution with dichloromethane/acetone (from 4:1, v/v, to 3:2, v/v) gave a mixture of products (132mg) rich in the above two metabolites, which were present in the acidic fraction obtained from the first run but could not be isolated. It was then subjected to reversed-phase partition chromatography (Hostalen S; 26g), and the 2.2ml amounts of each fraction were collected, yielding 140 fractions. A 0.5ml portion of each fraction was titrated with 0.02M-NaOH, and the titration curve gave three peaks centred at the effluent volumes of 31, 108 and 220ml. The titrated solution was concentrated by passage of a stream of N_2 and then applied to t.l.c. Fractions (from fraction no. 10 to no. 32), which corresponded to the first peak, contained a complex mixture of more polar products than the metabolites (III*a*) and (III*d*) and were not further treated. Fractions (from fraction no. 35 to no. 53) were all combined, which corresponded to the second peak and mainly contained a substance having R_F 0.51. Evaporation of the solvents *in vacuo* yielded a residue (25mg), which was chromatographed on silicic acid (1.1g). Elution with dichloromethane/acetone (4:1, v/v) gave (2*S*)-2-(2,3,4,6,6a*β*,7,8,9,9a*α*,-9b*β*-*decahydro*-6a*β*-*methyl*-3-*oxo*-1*H*-*cyclopenta*[*f*]-*quinolin*-7*β*-*yl*)*propionic acid* (III*d*), which crystallized from acetone in prisms (3mg), m.p. 243.5–249.5°C (decomp.), $[\alpha]_D^{24}$ +172.1±26.7° (*c* 0.079) and i.r. max. (in KBr) at 3200 (NH), approx. 2600–3000 (OH of CO_2H) and 1723 (shoulder), 1678 and 1628 (C=O of CO_2H and enaminolactam) cm^{-1} (Found: C, 69.4; H, 8.3; N, 5.2; $C_{16}H_{23}NO_3$ requires C, 69.3; H, 8.4; N, 5.1%). Another analytically pure sample [5mg, m.p. 241–247.5°C (decomp.)] was also obtained from the third run, in which sodium cholate (13.2g) was incubated with *S. rubescens* for 11 days. This sample showed $[\alpha]_D^{24}$ +151.0±12.8° (*c* 0.149 in ethanol) and $\lambda_{max.}$ 231.5nm (ε 13000).

Fractions (from fraction no. 80 to no. 113) corresponding to the third peak, on standing at room temperature for a few days, yielded crystals (6.7mg), which were filtered and dried. Recrystallization from methanol gave an analytical sample of (4*R*)-4-(2,3,4,6,-6aβ,7,8,9,9aα,9bβ-*decahydro-6aβ-methyl-3-oxo-1H-cyclopenta[f]quinolin-7β-yl)valeric acid* (III*a*) as prisms (5mg), m.p. 224–233°C (decomp.), $[\alpha]_D^{24}$ +149.0±37.4° (*c* 0.051 in ethanol), $\lambda_{max.}$ 231nm (ε13300) and i.r. max. at 3400 and 3200 (NH), approx. 2300–2800 (OH of CO_2H) and 1702 (shoulder), 1677, 1661 (shoulder) and 1631 (shoulder) (C=O of CO_2H and enaminolactam) cm^{-1} (Found: C, 70.7; H, 8.6; N, 4.4; $C_{18}H_{27}NO_3$ requires C, 70.8; H, 8.9; N, 4.6%).

C_{18} mono-amide (IIb). The silicic acid column charged with the acidic fraction of the first run, after elution with dichloromethane/acetone (from 4:1, v/v, to 3:2, v/v), was further eluted with the same solvent system (3:2, v/v) to yield (4*R*)-4-[4α-(2-*carboxyethyl*)-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-1β-*yl]valeramide* (II*b*), which crystallized from acetone in prisms (29mg), m.p. 183–185°C, $[\alpha]_D^{25}$ +24.8±2.0° (*c* 0.323 in ethanol) and i.r. max. (in Nujol) at 3450, 3330 and 3240 (NH), approx. 2200–2800 (OH of CO_2H), 1711 (C=O and C=O of CO_2H) and 1649 and 1590 ($CONH_2$) cm^{-1} (Found: C, 66.8; H, 9.0; N, 4.6; $C_{18}H_{29}NO_4$ requires C, 66.8; H, 9.0; N, 4.3%).

The *methyl ester*, prepared with ethereal diazomethane, could not be induced to crystallize. It showed i.r. bands at 3530 and 3440 (NH), 1732 (ester), 1704 (C=O) and 1680 and 1594 ($CONH_2$) cm^{-1}.

The above mono-amide (II*b*) (2mg) was heated with 20% (w/v) KOH (approx. 0.05ml) on a boiling-water bath for 1h and yielded the corresponding free acid. Identity with an authentic sample of compound (II*a*) was established by comparison of t.l.c.

Partial syntheses of the metabolites

C_{18} mono-amide (IIb). (*a*) Amidation of the 5β-dihydroxy acid (IV*a*). The acid (400mg) in anhydrous dioxan (2ml) containing tri-*n*-butylamine (0.34ml) was cooled to approx. 12°C and ethyl chloroformate (0.135ml) was added, with stirring. After 30min of the stirring, 28% (w/w) NH_3 (0.5ml) was added and the mixture was stirred vigorously for 90min. Evaporation of the solvent *in vacuo* left a residue, which was suspended in a small volume of water and extracted with chloroform. The extract was successively washed with dilute HCl, 5% (w/v) $NaHCO_3$ and water and dried over anhydrous Na_2SO_4. Evaporation of the chloroform *in vacuo* left a syrup, which crystallized from acetone to yield (4*R*)-4-[3aα-*hexahydro*-5β-*hydroxy*-4α-(3-*hydroxypropyl*)-7aβ-*methylindan*-1β-*yl]valeramide* (IV*b*) (207mg), m.p. 124.5–125.5°C, $[\alpha]_D^{25}$ +37.3±0.9° (*c* 0.820 in ethanol) and i.r. max. (in Nujol) at 3350 and 3200 (OH and NH) and 1668 and 1609 ($CONH_2$) cm^{-1} (Found: C, 69.9; H, 10.9; N, 4.2; $C_{18}H_{33}NO_3$ requires C, 69.4; H, 10.7; N, 4.5%). The mother liquor yielded also compound (IV*b*) (115mg, m.p. 121–125°C).

(*b*) Oxidation of the hydroxy amide (IV*b*). To a solution of the amide (200mg) in 90% (v/v) acetic acid (3ml) was added dropwise a solution of CrO_3 (140mg) in 80% (v/v) acetic acid (2ml) over a period of 10min. After 3h of the stirring at room temperature, the mixture was treated with a small volume of 5% (w/v) $NaHSO_3$ to destroy the excess of CrO_3, and evaporated *in vacuo* to dryness. The residue was suspended in a small volume of water and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and then evaporated *in vacuo* to yield a residue (165mg), which was chromatographed on silicic acid (7.0g). Elution with dichloromethane/acetone (3:2, v/v) gave (4*R*)-4-[4α-(2-*carboxyethyl*)-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-1β-*yl]valeramide* (II*b*), which crystallized from acetone in prisms (40mg), m.p. 179–182°C and $[\alpha]_D^{24}$ +24.4±0.8° (*c* 0.840 in ethanol) (Found: C, 66.5; H, 9.2; N, 4.1; $C_{18}H_{29}NO_4$ requires C, 66.8; H, 9.0; N, 4.3%). Identity with the metabolite (II*b*) isolated from growing cultures was established by mixed m.p. and by comparison of the behaviour on t.l.c., the $[\alpha]_D$ and the i.r. spectrum.

C_{18} enaminolactam acid (IIIa). The C_{18} oxocarboxylic acid (II*a*) (9.5g) was dissolved in 99.5% (v/v) ethanol (140ml), which had been saturated previously with NH_3 at 0°C, and heated in a sealed vessel at approx, 100°C for 10h. After cooling to room temperature, the dark-yellowish reaction mixture containing crystals was concentrated *in vacuo* to yield a residue, which was dissolved in a mixture of 1M-NaOH (25.5ml) and water (65ml). The solution was extracted with dichloromethane to remove a small amount of neutral by-products. The fine crystals that were precipitated on acidification of the aqueous layer with dilute HCl were collected and dissolved in chloroform (120ml) with the aid of methanol (approx. 4ml). The solution was washed twice with water (approx. 50ml), dried over anhydrous Na_2SO_4 and then filtered through a column of silicic acid (10g) to remove coloured materials. The column was further washed with chloroform (approx. 250ml), and the washings were combined with the filtrate. After concentration *in vacuo* to a small volume, addition of large volumes of methanol to the concentrate yielded (4*R*)-4-(2,3,4,6,6aβ,7,8,9,9aα,9bβ-*decahydro*-6aβ-*methyl*-3-*oxo*-1*H*-*cyclopenta[f]quinolin*-7β-*yl*)-*valeric acid* (III*a*) as prisms (7.99g). Further recrystallization from methanol/water (14:3, v/v) gave an analytical sample, m.p. 223–232°C (decomp.), $[\alpha]_D^{24}$ +146.2±2.0° (*c* 0.978 in ethanol) and $\lambda_{max.}$ 231nm (ε 13100) (Found: C, 71.0; H, 9.1; N, 4.7; $C_{18}H_{27}NO_3$ requires C, 70.8; H, 8.9; N, 4.6%). The behaviour on t.l.c., the $[\alpha]_D$ and the u.v. and i.r.

spectra were identical with those of the metabolite (III*a*) obtained from growing cultures.

The *methyl ester*, prepared with ethereal diazomethane, crystallized from methanol in plates, m.p. 187.5–191.5°C, $[\alpha]_D^{22}$ +146.6±3.8° (*c* 0.444 in ethanol), $\lambda_{max.}$ 230.5nm (ε 13600), i.r. max. at 3400 and 3200 (NH), 1728 (ester) and 1660 (enaminolactam) cm^{-1} and n.m.r. absorptions at 0.72 (3H; singlet; 6aβ-Me), 0.93 (3H; doublet; *J* 4.6Hz; Me in the side chain), 3.66 (3H; singlet; CO_2Me), 4.80 (1H; unresolved multiplet; 5-H) and 8.05 (1H; broad peak; CONH) p.p.m. (Found: C, 71.4; H, 9.2; N, 4.3; $C_{19}H_{29}NO_3$ requires C, 71.4; H, 9.2; N, 4.4%).

C_{18} enaminolactam amide (IIIb). The C_{18} enaminolactam acid (III*a*) (200mg) was treated by the amidation method which was described above for the 5β-dihydroxy acid (IV*a*) and afforded (4*R*)-4-(2,3,4,6,6aβ,7,8,9,9aα,9bβ-*decahydro-6aβ-methyl-3-oxo-1H-cyclopenta[f]quinolin-7β-yl)valeramide* (III*b*), which crystallized from methanol in plates (127mg), m.p. 215.5–218.5°C (decomp.), $[\alpha]_D^{23}$ +156.8±7.4° (*c* 0.266) and $\lambda_{max.}$ 232nm (ε 13500) (Found: C, 70.8; H, 9.3; N, 9.1; $C_{18}H_{28}N_2O_2$ requires C, 71.0; H, 9.3; N, 9.2%). Identity with the metabolite (III*b*) obtained from growing cultures was established by comparison of the behaviour on t.l.c., the m.p., the $[\alpha]_D$ and the u.v., i.r. and n.m.r. spectra.

C_{16} enaminolactam acid (IIId). Exactly as described above for compound (II*a*), the condensation of the C_{16} oxodicarboxylic acid (V) (506mg) with NH_3 in ethanol (80ml) yielded (2*S*)-2-(2,3,4,6,6aβ,7,8,9,9aα,-9bβ-*decahydro-6aβ-methyl-3-oxo-1H-cyclopenta[f]-quinolin-7β-yl)propionic acid* (III*d*), which was recrystallized from methanol and then from chloroform/methanol as prisms (300mg), m.p. 243–247°C (decomp.), $[\alpha]_D^{24}$ +127.6±2.3° (*c* 0.734 in ethanol) and $\lambda_{max.}$ 231nm (ε12100) (Found: C, 69.2; H, 8.4; N, 5.1; $C_{16}H_{23}NO_3$ requires C, 69.3; H, 8.4; N, 5.1%). The behaviour on t.l.c., the m.p., the $[\alpha]_D$ and the u.v. and i.r. spectra were identical with those of the metabolite (III*d*) obtained from growing cultures.

Compound (III*d*) was also prepared as follows: a mixture of the acid (V) (3.00g) and dry ammonium acetate (12.10g) in dimethylformamide (12ml) was heated at approx. 87°C for 5h under a stream of N_2. Addition of water (approx. 200ml) to the cooled mixture precipitated an almost colourless powder, which was filtered, washed with water and then dried. Crystallization from chloroform/methanol (1:1, v/v) afforded compound (III*d*), [2.05g, m.p. 243–248°C (decomp.)].

The *methyl ester*, prepared with ethereal diazomethane, crystallized from acetone in plates, m.p. 250.5–251°C (decomp.), $[\alpha]_D^{25}$ +138.2±2.1° (*c* 0.617 in ethanol), $\lambda_{max.}$ 232nm (ε 12500), i.r. max. at 3390 and 3200 (NH), 1729 (ester) and 1671 and 1660 (shoulder) (enaminolactam) cm^{-1} and n.m.r. absorptions at 0.74 (3H; singlet; 6aβ-Me), 1.19 (3H; doublet; *J* 6.9Hz; Me in the side chain), 3.66 (3H; singlet; CO_2Me), 4.80 (1H; unresolved multiplet; 5-H) and 7.86 (1H; broad peak; CONH) p.p.m. (Found: C, 70.3; H, 8.6; N, 4.8; $C_{17}H_{25}NO_3$ requires C, 70.1; H, 8.7; N, 4.8%).

C_{13} enaminolactam (IIIe). (*a*) Ozonolysis of 17β-acetoxyoestra-4,9-dien-3-one (VI). A solution of the dienone (10.00g) in a mixture of ethyl acetate (300ml) and acetic acid (60ml) was oxidized with a stream of 3% (w/w) ozonized O_2 at −15°C for 3.5h. Water (150ml) was added and the mixture was stirred at room temperature for 1h. Then 30% (w/w) H_2O_2 (60ml) was added, and the mixture was kept overnight in a refrigerator. The mixture was diluted with water and extracted with ether. The ethereal solution was extracted several times with 5% (w/v) $NaHCO_3$. The combined alkaline extract was acidified with dilute HCl and extracted with ether to recover an acidic fraction. The ethereal layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and then evaporated *in vacuo* to yield a solid (6.38g), which was recrystallized from ether/light petroleum and then from acetone to yield 3-(1β-acetoxy-3aα-hexahydro-7aβ-methyl-5-oxoindan-4α-yl)propionic acid as prisms (4.56g, m.p. 136–138°C). Further recrystallization from acetone/light petroleum gave an analytical sample, m.p. 140–142°C, $[\alpha]_D^{20}$ +6.8±0.3° (*c* 1.010 in ethanol) and i.r. max. (in Nujol) at approx. 2600–3200 (OH of CO_2H), 1741 (C=O of acetate) and 1714 and 1697 (C=O and C=O of CO_2H) cm^{-1} (Found: C, 63.8; H, 7.9. Calc. for $C_{15}H_{22}O_5$: C, 63.8; H, 7.9%).

The acetate (34.00g), obtained by repeating the above ozonolysis, was dissolved in 10% (w/v) KOH (170ml) and kept overnight at room temperature. The colourless crystals (25.10g, m.p. 150–152°C) that were precipitated on acidification with dilute HCl were collected, washed with water and then dried. Recrystallization from acetone/light petroleum gave the corresponding hydroxy-oxo acid (VII*a*) as prisms (23.51g), m.p. 151–152°C, $[\alpha]_D^{20}$ +10.4±0.7° (*c* 0.454 in ethanol) and i.r. max. (in Nujol) at 3440 (OH), approx. 2600–3100 (OH of CO_2H) and 1720 and 1698 (C=O and C=O of CO_2H) cm^{-1} (Found: C, 64.8; H, 8.3. Calc. for $C_{13}H_{20}O_4$: C, 65.0; H, 8.4%). Velluz *et al.* (1963) give m.p. 153°C and $[\alpha]_D$ +12° (in ethanol) for this compound.

(*b*) Oxidation of the hydroxy-oxo acid (VII*a*). The acid was converted into the corresponding oily methyl ester (VII*b*) with ethereal diazomethane. A solution of the ester (1.90g) in dry pyridine (10ml) was added dropwise to a well-stirred CrO_3–pyridine complex at room temperature, which was prepared from CrO_3 (2.28g) and dry pyridine (22.8ml) by the method of Poos *et al.* (1953). After stirring for 1h at room temperature, the mixture was kept overnight, diluted with water (140ml) and then thoroughly extracted with ether. The extract was washed with dilute HCl to

remove pyridine and then with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford an oily residue (1.527g), which was chromatographed on alumina (45g; grade III). Elution with benzene/light petroleum (from 1:9, v/v, to 1:1, v/v) gave *methyl 3-(3aα-hexahydro-7aβ-methyl-1,5-dioxoindan-4α-yl)propionate* as an oil (1.056g), which could not be induced to crystallize but showed i.r. max. at 1736 (ester and C=O at C-1) and 1708 (C=O at C-5) cm^{-1}. The dioxo ester was dissolved in 60% (v/v) methanolic 5% (w/v) K_2CO_3 (25ml) and heated under reflux for 1h. After neutralization with dilute HCl, the mixture was concentrated *in vacuo* to yield a residue, which was dissolved in a small volume of water. Acidification of the solution with dilute HCl precipitated prismatic crystals (627mg, m.p. 107–108°C), which were filtered, washed with water and then dried. Repeated recrystallization from acetone/light petroleum gave an analytical sample of 3-(3aα-hexahydro-7aβ-methyl-1,5-dioxoindan-4α-yl)propionic acid (VIII), m.p. 107–107.5°C, $[\alpha]_D^{20}$ +100.7±1.2° (*c* 1.004) and i.r. max. at approx. 2600–3200 (OH of CO_2H), 1739 (C=O at C-1) and 1709 (C=O at C-5 and C=O of CO_2H) cm^{-1} (Found: C, 65.4; H, 7.9. Calc. for $C_{13}H_{18}O_4$: C, 65.5; H, 7.6%). Sih & Wang (1963) give m.p. 110–111.5°C and $[\alpha]_D^{25}$ +121° (in chloroform) for this compound.

The above aqueous filtrate was combined with the washings and extracted with ether/dichloromethane (4:1, v/v). The extract was washed with water, dried over anhydrous Na_2SO_4 and then evaporated *in vacuo* to yield a pale-yellowish residue (305mg). Crystallization from acetone/light petroleum yielded also compound (VIII) (264mg, m.p. 106–107°C).

(*c*) Conversion of the acid (VIII) into the C_{13} enaminolactam (III*e*). The acid (3.00g) was treated with ammonium acetate (15.00g) in dimethylformamide (15ml) as described above for compound (V) and yielded the corresponding enaminolactam as slightly pink-coloured prisms (2.40g). Extraction from the mother liquor with chloroform yielded another crop of yellowish crystals (199mg). The combined crystals were recrystallized from chloroform/methanol to afford *2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-1H-cyclopenta[f]quinoline-3,7-dione* (III*e*) as colourless prisms (2.28g), m.p. 265°C (decomp., sintered at 251°C), $[\alpha]_D^{22}$ +320.4±7.1° (*c* 0.515) and $\lambda_{max.}$ 230.5nm (ε 13200) (Found: C, 71.2; H, 7.8; N, 6.3; $C_{13}H_{17}NO_2$ requires C, 71.2; H, 7.8; N, 6.4%). Identity with the metabolite (III*e*) obtained from growing cultures was established by comparison of the behaviour on t.l.c., the m.p., the $[\alpha]_D$ and the u.v., i.r. and n.m.r. spectra.

Discussion

The results of the above chemical investigations show that, in the degradative sequence of cholic acid (I) by *S. rubescens*, the original stereochemistry of the cholic acid (I) molecule remains undisturbed, as observed previously in the degradation of cholic acid (I) and its analogues, 3-oxo-24-nor-5β-cholan-23-oic acid and (20*S*)-3β-hydroxypregn-5-ene-20-carboxylic acid, by *A. simplex* (Hayakawa *et al.*, 1969*b*, 1976*a*). Although the structure of the enaminolactam methyl ketone (III*c*) was not established by partial synthesis, its stereochemistry would be the same as that of the other nitrogenous metabolites. This ketone has no acidic functional groups, but it has been nevertheless isolated from the acidic fraction of metabolites. This suggests that the ketone (III*c*) is probably an artifact formed in the fractionation process of a mixture of acidic metabolites. If so, the corresponding parent metabolite may be a β-oxo derivative of the C_{18} enaminolactam acid (III*a*), (4*S*)-4-(2,3,4,6,6aβ,7,8,-9,9aα,9bβ-decahydro-6aβ-methyl-3-oxo-1*H*-cyclopenta[*f*]quinolin-7β-yl)-3-oxovaleric acid, since such types of compounds usually are readily converted into the corresponding methyl ketones by spontaneous decarboxylation characteristic of β-oxocarboxylic acids.

We reported in a previous paper that cholic acid (I) was degraded to the oxodicarboxylic acid (II*a*) by *A. simplex* (Hayakawa *et al.*, 1969*b*). Accordingly, the isolation and identification of compound (II*a*) in this present work suggests that *S. rubescens* is also able to degrade cholic acid (I) to this compound in a manner analogous to that proposed for the cholic acid (I) degradation by *A. simplex*. This is further supported by the isolation of the following degradative intermediates, which are common in both the organisms: 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid and 12α-hydroxy-3-oxochola-4,6-dien-24-oic acid (Hayakawa *et al.*, 1958*a*,*b*, 1969*b*). Recently we have obtained the evidence that, in the degradation of cholic acid (I) by *A. simplex*, its 12α-hydroxyl group is probably eliminated as water to yield a Δ^{11}-9-oxo (in the steroid skeleton or Δ^{6}-5-oxo in the hexahydroindane skeleton) intermediate, which is further metabolized to compound (II*a*) (cf. Hayakawa, 1973, pp. 176–177). Therefore our earlier proposal (Hayakawa *et al.*, 1958*b*) that one of the significant intermediates involved in the degradation of cholic acid (I) by *S. rubescens* is probably 3,12-dioxochola-4,6-dien-24-oic acid and the dienoic acid further degraded to small fragments must be corrected as follows: in the incubation mixture 7α-hydroxy-3,12-dioxochol-4-en-24-oic acid and 3,12-dioxochola-4,6-dien-24-oic acid are probably in equilibrium with their corresponding 12α-hydroxy derivatives respectively, and *S. rubescens*, like *A. simplex*, degrades the latter, containing the 12α-hydroxyl group, but not the former, to small fragments including compound (II*a*), although these four metabolites have been isolated together.

The isolation and identification of several nitrogenous metabolites in the present work demonstrate

for the first time the occurrence of amide bond formation in the cholic acid degradation by micro-organisms. However, a mechanism for the amide bond formation is unknown, and it is also obscure whether these nitrogenous compounds are the actual intermediates involved in the degradation of cholic acid by *S. rubescens*. As discussed in the following paper, it is suggested that the peptide bond in the conjugates of compound (II*a*) with amino acids produced by *C. equi* is probably formed by either a reaction between an amino acid and an acyl-CoA (or acyl-AMP) derivative or a glutamine synthetase-type reaction (Hayakawa *et al.*, 1976*b*). Such a mechanism appears to function similarly to the amide bond formation in *S. rubescens*, in which ammonia acts in place of amino acids in *C. equi*. Thus two carboxyl groups of compound (II*a*) formed from cholic acid (I) in the manner discussed above may be further converted into the corresponding primary amide groups, yielding the mono-amides, compound (II*b*) and compound (IX*a*), and the di-amide (IX*b*). The occurrence of this enzymic amidation is strongly supported by the isolation of both compounds (II*a*) and (II*b*) as metabolites. In connexion with the formation of compound (IX*a*), the possibility that this compound is directly formed from a precursor of compound (II*a*) such as the 4,5:9,10-diseco intermediate, which is believed to be involved in the cholic acid (I) degradation by *A. simplex* (cf. Hayakawa *et al.*, 1969*b*, p. 255), can be completely excluded, since it is well known that such diseco intermediates react non-enzymically with ammonia to form pyridine carboxylic acids but not primary amides (cf. Ribbons, 1966, pp. 448–453). Both the δ-oxo amides, compounds (IX*a*) and (IX*b*), formed in such a manner are probably further cyclized to yield the corresponding enaminolactams (III*a*) and (III*b*). Although it is unknown whether this ring closure is enzymic or not, it may be enzymic. Support for the occurrence of these transformations is provided by evidence that both the δ-oxocarboxylic acids (VII*a*) and (VIII) were converted into the same enaminolactam (III*e*) by *S. rubescens*, indicating that the formation of the corresponding enaminolactam ring from δ-oxo acids in the presence of NH_4^+ is enzymic (S. Hashimoto & S. Hayakawa, unpublished work). Similarly, the δ-oxocarboxylic acids (II*b*), (V) and (VIII) are perhaps transformed into the corresponding enaminolactams (III*b*), (III*d*) and (III*e*) via the respective propionamide derivatives. Another possible mechanism for the formation of the respective enaminolactams from these δ-oxocarboxylic acids (II*a*), (V) and (VIII) is as follows: the non-enzymically formed δ-lactols (δ-hydroxy δ-lactones), which are tautomeric with these δ-oxo acids, react enzymically with ammonia to produce the corresponding δ-hydroxy δ-lactams, which are further converted into the corresponding enaminolactams by dehydration.

We have considered that the amino acid conjugates, isolated in the following paper, are probably not the intermediates involved in the degradation of compound (II*a*) by *C. equi* (Hayakawa *et al.*, 1976*b*). As well as these conjugates, the nitrogenous metabolites (II*b*), (III*a*) and (III*b*), (III*d*) and (III*e*) are probably not the intermediates involved in the cholic acid degradation by *S. rubescens* but the side products, and they might be further degraded to small fragments via the corresponding δ-oxocarboxylic acids (II*a*), (V) and (VIII), which are presumed to be the precursors of these metabolites as described above. Further, we believe from the above considerations that the actual intermediates are compounds (II*a*), (V) and (VIII) themselves. Although compounds (V) and (VIII) were not isolated, one can expect that compound (II*a*) could be metabolized to these compounds in a manner analogous to the conventional fatty acid β-oxidation mechanism. Support for this presumption is provided by the isolation of compound (III*c*), which corresponds to the enaminolactam form of a decarboxylation product of (4*S*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]-3-oxovaleric acid, which is one of the intermediates in the β-oxidation-type cleavage of compound (II*a*) as described above. However, the possibility that compound (III*a*) may undergo stepwise oxidation to compounds (III*d*) and (III*e*) by the β-oxidation-type mechanism cannot be excluded.

Even if compound (III*d*) could be formed in any manner, it is noteworthy that compound (V), which had been previously synthesized as one of the hypothetical metabolites of cholic acid (I) by micro-organisms (Hayakawa *et al.*, 1976*a*), was isolated as its enaminolactam form in this present work. Other investigators have demonstrated the degradation of certain naturally occurring and structurally different steroids by different species of micro-organisms to the same intermediate, compound (VIII) (cf. Hayakawa, 1973, pp. 185–186). As discussed above, there seems no doubt that cholic acid (I) is also degraded to this compound by *S. rubescens*. Thus further studies on the degradation of compound (VIII) by *S. rubescens* or other organisms should give an insight into the mechanism of degradation of not only cholic acid (I) but also other steroids. Some results of this work have been already reported in a preliminary form (Hayakawa & Hashimoto, 1969).

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