Microbiological Degradation of Bile Acids

METABOLITES FORMED FROM 3-(3 α -HEXAHYDRO-7 $\alpha\beta$ -METHYL-1,5-DIOXOINDAN-4 α -YL)PROPIONIC ACID BY *STREPTOMYCES RUBESCENS*

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1. The metabolism of 3-(3 α -hexahydro-7 $\alpha\beta$ -methyl-1,5-dioxoindan-4 α -yl)propionic acid (III), which is a possible precursor of 2,3,4,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9b β -decahydro-6 $\alpha\beta$ -methyl-1H-cyclopenta[*f*]quinoline-3,7-dione (II) formed from cholic acid (I) by *Streptomyces rubescens*, was investigated by using the same organism. 2. This organism effected amide bond formation, reduction of the carbonyl groups, *trans* $\alpha\beta$ -desaturation and *R*-oriented β -hydroxylation of the propionic acid side chain and skeleton cleavage, and the following metabolites were isolated as these forms or their derivatives: compound (II), 1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9b β -dodecahydro-6 $\alpha\beta$ -methylcyclopenta[*f*][1]benzopyran-3,7-dione (IVa), (1*R*)-1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9b β -dodecahydro-1-hydroxy-6 $\alpha\beta$ -methylcyclopenta[*f*][1]benzopyran-3,7-dione (IVb), (*E*)-3-(3 α -hexahydro-5 α -hydroxy-7 $\alpha\beta$ -methyl-1-oxo-indan-4 α -yl)prop-2-enoic acid (V), (+)-(5*R*)-5-methyl-4-oxo-octane-1,8-dioic acid (VI), 3-(4-hydroxy-5-methyl-2-oxo-2*H*-pyran-6-yl)propionic acid (VII) and 3-(3 α -hexahydro-1 β -hydroxy-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (VIII). The metabolites (IVb), (V), (VI) and (VII) were new compounds, and their structures were established by chemical synthesis. 3. The question of whether these metabolites are true degradative intermediates is discussed, and a degradative pathway of compound (III) to the possible precursor of compound (VII), 7-carboxy-4-methyl-3,5-dioxoheptanoyl-CoA (IX), is tentatively proposed. The further degradation of compound (IX) to small fragments is also considered.

No evidence to suggest the occurrence of carbon-carbon bond cleavage of bile acids in the mammalian body has been reported so far, except by Ferrari (1967), who demonstrated the aerobic degradation of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) (I) *in vitro* to unidentified non-steroidal compounds by human intestinal flora, and by Dirscherl & Pelzer (1970), who reported the presence of deoxybilianic acid (12-oxo-3,4-seco-5 β -cholane-3,4,24-trioic acid) in normal human urine. However, it seems likely that bile acids excreted in the faeces of vertebrates and also originating from their carcasses are degraded by micro-organisms in Nature. We believe that defining the intermediates and reaction sequence involved in this biodegradation is an interesting object of steroid biochemistry, and we have continued to work on this subject (cf. Hayakawa, 1973).

During the course of our studies on bile-acid degradation with growing cultures, we have been forced to conclude that not all the bile-acid metabolites formed by a certain organism can be utilized by that organism (Hayakawa *et al.*, 1976a). These anomalous observations prevented our attempts to degrade further the once-formed cholic acid metabol-

ites such as (4*R*)-4-[4 α -(2-carboxyethyl)-3 α -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-1 β -yl]valeric acid and 5-methyl-4-oxo-octane-1,8-dioic acid (VI) by the metabolite-producing organism *Arthrobacter simplex*, and to identify the degradation products. This was true in the degradation of cholic acid (I) by *Streptomyces rubescens*; we found that the organism could convert cholic acid into the several metabolites, but could not utilize 2,3,4,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9b β -decahydro-6 $\alpha\beta$ -methyl-1H-cyclopenta[*f*]quinoline-3,7-dione (II), which is the most degraded product among the metabolites identified (Scheme 1). The inability of this organism to grow on compound (II) supported our previous proposal that an actual degradative intermediate involved in cholic acid degradation by this organism would probably not be compound (II) but its possible precursor, 3-(3 α -hexahydro-7 $\alpha\beta$ -methyl-1,5-dioxoindan-4 α -yl)-propionic acid (III) (Hayakawa *et al.*, 1976b). Thus we studied the degradation of compound (III) by *S. rubescens*; this compound was utilized well by the organism, and it was readily prepared as described in the preceding paper (Hayakawa *et al.*, 1977).

The present paper describes the isolation and identification of the metabolites formed from com-

Scheme 1. Metabolites formed from cholic acid (I) and 3-(3 α -hexahydro-7 α -methyl-1,5-dioxindan-4 α -yl)propionic acid (III) by *S. rubescens*

pound (III) by *S. rubescens*; a preliminary account of portions of this work has already appeared (Hayakawa & Hashimoto, 1969).

Results

Isolation and structural assignments of the metabolites

When compound (III) instead of cholic acid (I) was exposed to *S. rubescens* in the same manner as previously described (Hayakawa *et al.*, 1976b), this organism could utilize this compound as its sole source of carbon, and accumulated metabolites that were detectable by t.l.c. Except for the C₁₃ enaminolactam (II), they were isolated as crystals from an acidic fraction of the metabolites in the form of these metabolites or of their derivatives.

C₁₃ enaminolactam (II). This metabolite was isolated in a preliminary study as to whether *S. rubescens* can utilize compound (III) and accumulate metabolites. Identity with an authentic sample of compound (II) was established by comparison of the melting points and the behaviour on t.l.c.

Hydroxy-oxo acid δ -lactone (IVa). The elementary analysis of this metabolite was consistent with C₁₃H₁₈O₃, and the i.r. spectrum was quite consistent with that of 1,2,3,4 α β ,5,6,6 α β ,7,8,9 α ,9 β -dodecahydro - 6 α β - methylcyclopenta[*f*][1]benzopyran - 3,7-dione (IVa) reported by Wang & Sih (1963). Identity with the δ -lactone (IVa), which was synthesized from compound (III) by the method of Sih & Wang (1963) and Wang & Sih (1963), was established by mixed m.p. and by comparison of the [α]_D and the i.r. and n.m.r. (nuclear-magnetic-resonance) spectra.

β -Hydroxy δ -lactone (IVb). The formula $C_{13}H_{18}O_4$ was given to this metabolite by elementary analysis and molecular-weight determination by mass spectrometry. The i.r. spectrum showed the presence of hydroxyl and carbonyl groups at 3400 and 1736 and 1701 cm^{-1} respectively. The acetate, prepared with acetic anhydride/pyridine (1:1, v/v), showed n.m.r. absorptions at δ 2.10 (singlet; 3H; OAc) and 5.30 (multiplet; 1H; CHOAc) p.p.m. The other feature of this n.m.r. spectrum was similar to that of compound (IVa). These results indicated that the metabolite was a monohydroxy derivative of compound (IVa). Oxidation of the metabolite with Jones reagent (Curtis *et al.*, 1953) gave a crystalline product corresponding to $C_{13}H_{16}O_4$, which showed the bathochromic shift in u.v. spectrum on passing from neutral to alkaline solution expected for a β -dioxo compound. This u.v. spectrum resembled that of a β -oxo six-membered-ring lactone reported by Johnston *et al.* (1966). From these physical data, the structure of this metabolite was tentatively assigned as either (1*R*)-1,2,3,4a β ,5,6,6a β ,7,8,9,9a α ,9b β -do-decahydro - 1 - hydroxy - 6a β - methylcyclopenta - [f][1]benzopyran-3,7-dione (IVb) or its (1*S*)-isomer (XIa).

$\alpha\beta$ -Unsaturated hydroxy-oxo acid (V). The acid was isolated as its methyl ester acetate (XIII) in a pure form. Both the mass spectrometry (m/e 294) and the elementary analysis were consistent with $C_{16}H_{22}O_5$. The presence of a *trans* double bond conjugated with a methoxycarbonyl group was indicated by a λ_{max} . at 209 nm (ϵ 14400), i.r. bands at 1657 and 981 cm^{-1} and n.m.r. absorptions at δ 5.93 (doublet; J 15.8 Hz; 1H) and 6.82 (doublet-doublet; J 15.8 and 8.3 Hz; 1H) p.p.m. Catalytic reduction of this double bond and subsequent hydrolysis converted this acetate into compound (IVa). These results indicated that the structure of this metabolite was (*E*)-3-(3a α -hexahydro - 5 α - hydroxy - 7a β - methyl - 1 - oxoindan - 4 α -yl)-2-propenoic acid (V). This metabolite was also isolated from another run as a free acid form.

C_9 oxo dicarboxylic acid (VI). This metabolite was shown to have the formula $C_9H_{14}O_5$ by elementary analysis and molecular-weight determination by mass spectrometry (m/e 202). The i.r. spectrum showed the presence of a carboxyl group at approx. 2500–3000 and 1706 cm^{-1} . The n.m.r. spectrum exhibited a three-proton doublet at δ 1.13 p.p.m. and a two-proton broad peak at δ 10.3 p.p.m., indicating the presence of one secondary methyl and two carboxyl groups respectively. The function of a remaining oxygen atom in the formula was assigned as a carbonyl group from the positive 2,4-dinitrophenylhydrazine test. From these data the structure of this metabolite was tentatively assigned as 5-methyl-4-oxo-octane-1,8-dioic acid (VI). This structure was confirmed by the synthesis of the racemate of this acid as described below. The (5*R*)-configuration

was deduced from a negative Cotton effect in the optical-rotatory-dispersion curve (Djerassi & Geller, 1959).

Pyronylpropionic acid (VII). The i.r. spectrum of this metabolite, obtained only in small quantities, showed the presence of double bonds, hydroxyl and carboxyl groups at 1650, 1623, 1572 and 1524, 3400 and approx. 2400–3200 and 1695 cm^{-1} respectively. These complicating but sharp i.r. bands in the wavelength region corresponding to double bonds and the above i.r. band at 1695 cm^{-1} , which was assigned to a carboxyl group, indicated the presence of an α -pyrone ring: this was further indicated by a λ_{max} . at 289.5 nm ($A_{1\%}^{1\text{cm}}$ 330) (cf. Mors *et al.*, 1962, p. 141). Treatment with diazomethane converted this metabolite into a mixture of two methylated products. One of them was isolated as crystals, but the other could not be induced to crystallize. The former had a molecular ion of 226 mass units and the other fragment ions of 195 (M -OMe), 167 (M -CO₂Me) and 139 (M -[CH₂]₂-CO₂Me) in the mass spectrum, indicating the presence of a propionic acid side chain in the original metabolite. The i.r. spectrum showed the presence of an α -pyrone ring at 1700 (C=O) and 1649 and 1566 (C=C) cm^{-1} : this was further indicated by a λ_{max} . at 285.5 nm (ϵ 6600). The latter showed a λ_{max} . at 246 nm and i.r. bands at 1668 (C=O) and 1600 (C=C) cm^{-1} characteristic of γ -pyrones (cf. Mors *et al.*, 1962, p. 141), but further work on this product was prevented by lack of material. Since a 4-hydroxy- α -pyrone was converted by diazomethane into a mixture of the corresponding methoxylated α - and γ -pyrone derivatives (cf. Mors *et al.*, 1962, p. 150), the above results were best explained by assuming that the structure of this metabolite is 3-(4-hydroxy-5-methyl-2-oxo-2H-pyran-6-yl)propionic acid (VII), which corresponds to a product formed by a ring closure of 7-carboxy-4-methyl-3,5-dioxoheptanoyl-CoA (IX).

1 β -Hydroxy-5-oxo acid (VIII). The elementary analysis of this metabolite was consistent with $C_{13}H_{20}O_4$, indicating that it is a dihydro derivative of the substrate (III). It was identified as 3-(3a α -hexahydro - 1 β - hydroxy - 6a β - methyl - 5 - oxoindan - 4 α -yl)propionic acid (VIII) by mixed m.p. and by comparison of the behaviour on t.l.c. and the i.r. spectrum with an authentic sample.

Chemical syntheses of the metabolites and their derivatives

For more definitive proof of the structure of the new metabolites (IVb), (V), (VI) and (VII) and their later use as substrates, these compounds or their derivatives were synthesized.

Hydroxy-oxo acid δ -lactone (IVa). This compound was prepared as a starting material for the partial syntheses of the metabolites or their derivatives. The synthetic route was the same as that reported by Sih & Wang (1963) and Wang & Sih (1963), except

Scheme 2. Syntheses of the oxidation product (XIb) of the metabolite (IVb), the ester acetate (XIII) of the metabolite (V), the racemate of the metabolite (VI) and the methylated derivative (XVI) of the metabolite (VII)

that the methods of the enol lactonization of compound (III) and of the separation of the catalytic-reduction products of the resultant enol lactone were modified.

Stereoisomer (XIa) of the metabolite (IVb) and its oxidation product (XIb). Johnston *et al.* (1966) reported that 4,4-dimethyl-15-oxa-D-homo-5 α -androstan-16-one is dehydrogenated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to yield the corresponding $\alpha\beta$ -unsaturated lactone, and also that, after treatment with alkali and subsequently with acid, this $\alpha\beta$ -unsaturated lactone yields the 17 α -hydroxy lactone only, instead of the expected free acid. This route was used for our synthetic work (Scheme 2); compound (IVa) was converted into the only one hydration product via the corresponding $\alpha\beta$ -unsaturated lactone (X), indicating that this addition,

like the hydration reported by Johnson *et al.* (1966), appeared to be stereoselective. This selectivity is probably due to steric hindrance by the γ -substituted groups. The hydroxyl group, newly introduced by the addition of water from the less-hindered β -side of the perhydrobenzopyran nucleus, was then assigned as the (1*S*)-configuration, as shown in the formula (XIa). This synthetic sample differed in physical data from the metabolite (IVb), but its oxidation product, the β -oxo lactone (XIb), was identical in all respects (mixed m.p., $[\alpha]_D$ and u.v. and i.r. spectra) with that of the metabolite (IVb). Therefore the structure of this metabolite was assigned as the (1*R*)-isomer (IVb).

Ester acetate (XIII) of the metabolite (V). Compound (X) was converted by alkaline hydrolysis and subsequent treatment with diazomethane into the

cis-unsaturated hydroxy-oxo ester (XII), which was then converted by the photochemical isomerization method of double bonds (Hammond *et al.*, 1961) into the corresponding *trans* isomer. It was then acetylated with acetic anhydride/pyridine (1:1, v/v) to yield the ester acetate (XIII). Identity with the ester acetate of the metabolite (V) isolated from growing cultures was established by mixed m.p. and by comparison of the u.v. and i.r. spectra.

Racemate of the metabolite (VI). Since cyclic β -diketones that are completely substituted at the common α -position are extremely prone to ring cleavage by alkali (Wendler *et al.*, 1951), 3-(1-methyl-2,5-dioxocyclopent-1-yl)propionic acid (XIV) was treated with alkali and ($\pm$)-5-methyl-4-oxo-octane-1,8-dioic acid was obtained as expected. The i.r., n.m.r. and mass spectra were identical with those of the metabolite (VI) obtained from growing cultures.

Methylated derivative (XVIa) of the metabolite (VII). Douglas & Money (1968) have reported a method of synthesis of 4-methoxy-2-oxo-2H-pyran-6-ylacetic acid (XVIa) and its acid chloride starting from 4-methoxy-6-methyl-2H-pyran-2-one (XVa). This sequence of reactions was carried out by using the 5-methyl derivative (XVb) of compound (XVa): compound (XVb), which is one of the products prepared from 4-hydroxy-5,6-dimethyl-2H-pyran-2-one (XVc) with diazomethane (cf. Mors *et al.*, 1962, p. 150), was condensed with dimethyl oxalate to yield the enol form (XVIb) of the corresponding methyl α -pyronylpyruvate. After acidic hydrolysis, the resulting free acid was oxidized with H₂O₂ to give the α -pyronylacetic acid (XVIc). This compound was not obtained in a pure form, but the structure was confirmed by the spectral data of its analytically pure methyl ester. It was then converted by Arndt-Eistert reaction (cf. Bachmann & Struve, 1942) into the corresponding homoester (XVIa). Identity with one of the methylated derivatives of the metabolite (VII) was established by comparison of the behaviour on t.l.c., the m.p. and the u.v., i.r. and mass spectra.

Experimental

General

The micro-organism, *S. rubescens*, and methods for incubation were as described by Hayakawa *et al.* (1976b). The special care needed in preparing toxic and explosive diazomethane from the carcinogen, *N*-nitrosomethylurea, and methods for crystallization or recrystallization from a mixture of two miscible solvents such as acetone/light petroleum were as described by Hayakawa *et al.* (1977). Methods for physical measurements were as described by Hayakawa & Hashimoto (1969) and Hayakawa *et al.* (1969). 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

Details of adsorption column chromatography were as described by Hayakawa *et al.* (1969), except that silicic acid which had been previously deactivated by the addition of water (15%, w/w) was used.

Except where otherwise indicated, glass plates (5 cm $\times$ 20 cm, 0.25 mm thick) coated with silica gel GF₂₅₄ (nach Stahl; E. Merck A.-G., Darmstadt, Germany) and glass plates (20 cm $\times$ 20 cm, 0.75 mm thick) coated with silica gel GF₂₅₄ or silica gel HF₂₅₄ (E. Merck A.-G.) were used for analytical and preparative t.l.c. respectively. The following mixtures were used as developing solvent systems for both analytical and preparative t.l.c.: (a) ethyl acetate/iso-octane/acetic acid (15:5:1, by vol.); (b) ethyl acetate/iso-octane (3:1, v/v). In analytical t.l.c., spots were rendered visible by the methods described by Hayakawa *et al.* (1969), and, in preparative t.l.c., bands were revealed by exposure to I₂ vapour, a u.v.-scanner or water spray, and were extracted with chloroform/methanol (1:1, v/v), unless otherwise specified. Continuous t.l.c. was as described by Hayakawa & Fujiwara (1977).

Medium

A synthetic medium was prepared by the addition of the sodium salt of compound (III) (3 g) to a mineral solution (1 litre) which contained (g/l): (NH₄)₂SO₄, 2; K₂HPO₄, 1; MgSO₄·7H₂O, 0.5; FeCl₃·6H₂O, 0.01 [for compound (III), see under 'Other materials' below], and the pH was adjusted to 7.2. The 100 ml amounts of medium in a 500 ml shake-flask were sterilized by autoclaving (10 kPa for 20 min).

Other materials

As a substrate for incubation, 3-(3 α -hexahydro-7 β -methyl-1,5-dioxoindan-4 α -yl)propionic acid (III) was synthesized as described in the preceding paper (Hayakawa *et al.*, 1977). As authentic samples for identification, the following compounds were prepared by our own methods (Hayakawa *et al.*, 1976b): 2,3,4,6,6 β ,7,8,9,9 α ,9 β -decahydro-6 α β -methyl-1H-cyclopenta[*f*]quinoline-3,7-dione (II), the methyl ester of compound (III) and 3-(3 α -hexahydro-1 β -hydroxy-5-oxoindan-4 α -yl)propionic acid (VIII).

1,2,3,4 α β ,5,6,6 α β ,7,8,9,9 α ,9 β - Dodecahydro - 6 α β - methylcyclopenta[*f*][1]benzopyran - 3,7 - dione (IVa) was prepared as a starting material for partial synthesis. The reagent A (15 ml) of Edwards & Rao (1966), which contains HClO₄ and acetic anhydride in ethyl acetate, was added to a suspension of compound (III) (2 g) in dry ethyl acetate (15 ml). The mixture was left for a few minutes at approx. 20°C, then the resultant clear solution was diluted with

ethyl acetate (approx. 100ml), washed with 5% (w/v) NaHCO_3 and then with water, and dried over anhydrous Na_2SO_4 . Evaporation of the solvent *in vacuo* left a residue (1.80g), which crystallized from acetone/light petroleum to yield 1,2,3,6,6a β ,7,8,9,9a α ,9b β - decahydro - 6a β - methylcyclopenta[*f*][1] - benzopyran-3,7-dione in prisms (1.15g, m.p. 128.5–133°C). A sample further recrystallized from acetone/light petroleum had m.p. 132–132.5°C (Found: C, 71.2; H, 7.3. Calc. for $\text{C}_{13}\text{H}_{16}\text{O}_3$: C, 70.9; H, 7.3%). Sih & Wang (1963) give m.p. 137–138.5°C for this compound. The product was catalytically reduced by the method reported by Wang & Sih (1963), and a mixture of products, obtained from this enol lactone (1g), crystallized from acetone/light petroleum to give crystals (432mg, m.p. 124.5–128.5°C). The mother liquor was subjected to preparative continuous t.l.c. (silica gel HF₂₅₄, six plates; diethyl ether; 2.5h) and two bands were separated. The extract, obtained from the slower-moving band, crystallized from acetone/light petroleum to give compound (IVa) in needles (75mg), m.p. 119–121°C, $[\alpha]_D^{23} +123.9 \pm 0.5^\circ$ (c 0.977), i.r. max. at 1735 (C=O and δ -lactone) cm^{-1} and n.m.r. absorptions at 0.92 (3H; singlet; 6a β -Me) and 4.54 [1H; multiplet; full width at half-height (*WH*) 5Hz; 4a β -H] p.p.m. (Found: C, 70.0; H, 8.2. Calc. for $\text{C}_{13}\text{H}_{18}\text{O}_3$: C, 70.2; H, 8.2%). Wang & Sih (1963) give m.p. 124–127°C and $[\alpha]_D^{27} +23.6^\circ$ (in chloroform) for this compound.

The extract (170mg), obtained from the faster-moving band, crystallized from acetone/light petroleum to give crystals (117mg). This crystalline sample was combined with the above crystalline sample obtained by direct crystallization and recrystallized from acetone/light petroleum to yield an isomer of compound (IVa), 1,2,3,4a α ,5,6,6a β ,7,8,9,9a α ,9b β - dodecahydro - 6a β - methylcyclopenta - [*f*][1]benzopyran-3,7-dione, as needles (529mg), m.p. 128.5–129.5°C and $[\alpha]_D^{23} +31.9 \pm 1.1^\circ$ (c 0.670) (Found: C, 70.4; H, 8.2. Calc. for $\text{C}_{13}\text{H}_{18}\text{O}_3$: C, 70.2; H, 8.2%). Wang & Sih (1963) give m.p. 128.5–130.5°C and $[\alpha]_D^{27} +52.6^\circ$ (in chloroform) for this compound.

3 - (1 - Methyl - 2,5 - dioxocyclopent - 1 - yl)propionic acid (XIV) (Brown *et al.*, 1966) and 4-hydroxy-5,6-dimethyl-2H-pyran-2-one (XVc) (Harris *et al.*, 1968) were also prepared by published methods as starting materials for chemical synthesis. The former had m.p. 122.5–124°C (Found: C, 58.7; H, 6.6. Calc. for $\text{C}_9\text{H}_{12}\text{O}_4$: C, 58.7; H, 6.6%). Brown *et al.* (1966) give m.p. 126°C for this compound. The latter had m.p. 196–198°C (decomp.) (Found: C, 60.3; H, 5.6. Calc. for $\text{C}_7\text{H}_8\text{O}_3$: C, 60.6; H, 5.8%). Harris *et al.* (1968) give m.p. 204–206°C for this compound. Compound (XVc) (6.94g) was treated with ethereal diazomethane to give the methylation mixture, which was subjected to fractional crystallization from cyclohexane to yield two types of crystals: the first crop

was obtained as prisms (2.56g) and the second crop as needles (0.75g). The mother liquor was chromatographed on silica gel (214g). Elution with diethyl ether/methanol (from 93:7, v/v, 24:1, v/v) gave a fraction (2.14g) having R_F 0.25 (solvent *b*) on t.l.c. This fraction was combined with the above first crop and recrystallized from water and then from cyclohexane to give 4-methoxy-5,6-dimethyl-2H-pyran-2-one (XVb) as prisms (3.47g), m.p. 97–98.5°C, λ_{max} 286nm (ϵ 6460), i.e. max. (in KBr) at 1707 (C=O) and 1650 and 1563 (C=C) cm^{-1} (Found: C, 62.1; H, 6.5; $\text{C}_8\text{H}_{10}\text{O}_3$ requires C, 62.3; H, 6.5%). Further elution with diethyl ether/methanol (from 23:2, v/v, to 1:1, v/v) gave a fraction (2.16g) having R_F 0.08 (solvent *b*). This fraction was combined with the above second crop and recrystallized from cyclohexane to give 2-methoxy-5,6-dimethyl-4H-pyran-4-one as needles (2.16g), m.p. 81–81.5°C, λ_{max} 244nm (ϵ 17400), i.r. max. (in KBr) at 1671 (C=O) and 1626 and 1599 (C=C) cm^{-1} (Found: C, 62.2; H, 6.4; $\text{C}_8\text{H}_{10}\text{O}_3$ requires C, 62.3; H, 6.5%).

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.

Preliminary test for the degradation of compound (III)

When *S. rubescens* was inoculated into a medium (see under 'Medium' above) (400ml) and incubated, this organism grew well and produced several metabolites that were detected by t.l.c. (solvent *a*). After the 48h incubation, the mycelium-free culture filtrate was acidified with dilute HCl and extracted with ethyl acetate to give a gummy mixture (239mg) of metabolites. The extract, after esterification with ethereal diazomethane, was subjected to preparative t.l.c. (silica gel HF₂₅₄, five plates; solvent *b*) and gave three discrete bands as revealed by spraying with water. The syrup (164mg) obtained from the fastest band (R_F 0.5) showed an i.r. spectrum identical with that of the methyl ester of compound (III). The extract (6mg) obtained from the slowest band (R_F 0.1) crystallized from diethyl ether, but the crystals contained impurities, as indicated by t.l.c., and were not further treated. The mother liquor, on crystallization from acetone/light petroleum, gave the C_{13} enaminolactam (II) in prisms [0.6mg, m.p. 225–236.5°C (decomp.)]. Identity with an authentic sample was established by comparison of the melting point and the behaviour on t.l.c. (solvent *b*).

Fractionation of the metabolites

Compound (III) (30g) was incubated with *S. rubescens* for 90h, and the culture filtrate (approx. 10 litres) was concentrated *in vacuo* to approx. 600ml, acidified with dilute HCl to approx. pH2 and extracted with ethyl acetate. The organic layer was

extracted with 5% (w/v) NaHCO_3 , washed with water and evaporated *in vacuo* to give a neutral fraction (496 mg). This fraction contained compounds (II), (IVa) and (IVb), as indicated by t.l.c. (solvent *a*), but it was not treated further. The alkaline extract was combined with the washings, acidified with dilute HCl and saturated with NaCl. Recovery in ethyl acetate gave an acidic fraction (5.62 g), which was chromatographed on silicic acid (224 g). Elution with 560 ml portions of solvents was performed and part of each fraction collected was examined by t.l.c. Fractions that were similar to each other in behaviour on t.l.c. (solvent *a*) were combined, and evaporation of the solvents *in vacuo* gave the following pattern: pool *A* (fraction 1, eluted with dichloromethane), 3 mg; pool *B* [fraction 2, eluted with dichloromethane, and fraction 3, eluted with acetone/dichloromethane (1:99, v/v)], 719 mg; pool *C* [fractions 4 and 5, eluted with acetone/dichloromethane (1:49, v/v and 3:97, v/v respectively) and fractions 6–8, eluted with acetone/dichloromethane (1:24, v/v)], 2854 mg; pool *D* [fractions 9–10, eluted with acetone/dichloromethane (1:19, v/v)], 157 mg; pool *E* [fractions 11–12, eluted with acetone/dichloromethane (1:19, v/v) and fraction 13, eluted with acetone/dichloromethane (3:17, v/v)], 342 mg; pool *F* [fraction 14, eluted with acetone/dichloromethane (3:17, v/v)], 188 mg; pool *G* [fraction 15, eluted with acetone/dichloromethane (1:4, v/v)], 115 mg; pool *H* [fraction 16, eluted with acetone/dichloromethane (1:4, v/v)], 339 mg; pool *I* [fractions 17–18, eluted with acetone/dichloromethane (3:7, v/v)], 417 mg; pool *J* [fraction 19, eluted with acetone/dichloromethane (1:1, v/v)], 83 mg; and pool *K* (fraction 20, eluted with acetone), 99 mg.

Isolation of the metabolites

Pool *C* containing mainly the unaltered compound (III) was treated with methanol/conc. H_2SO_4 (approx. 50:1, v/v; 120 ml) to give the methylation mixture, which was chromatographed on alumina (48 g, grade III). Elution with light petroleum/benzene (from 2:1, v/v, to 1:1, v/v) gave the methyl ester of compound (III), which was then hydrolysed to give compound (III) (549 mg, m.p. 104–107°C). Since pools *A*, *E*, *G*, *I* and *K* were an intractable complex mixture, they were not treated further.

Hydroxy-oxo acid δ -lactone (IVa). Pool *B*, which mainly consisted of a metabolite having R_F 0.22 (solvent *a*) on t.l.c., crystallized from acetone/light petroleum to give 1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ -dodecahydro - 6 $\alpha\beta$ - methylcyclopenta[*f*] [1]benzopyran-3,7-dione (IVa) as needles (130 mg), m.p. 118.5–120°C and $[\alpha]_D^{23} +119.4 \pm 1.6^\circ$ (*c* 1.005) (Found: C, 70.4; H, 8.3. Calc. for $\text{C}_{13}\text{H}_{18}\text{O}_3$: C, 70.2; H, 8.2%). This metabolite was identical in all respects (m.p., mixed m.p., $[\alpha]_D$, i.r. and n.m.r.)

with an authentic sample. The mother liquor gave also compound (IVa) (324 mg, m.p. 118–120°C).

β -Hydroxy δ -lactone (IVb). Pool *D*, which gave a main spot (solvent *a*; R_F 0.16) on t.l.c., crystallized from acetone/light petroleum and then from acetone to give (1*R*)-1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ -dodecahydro - 1 - hydroxy - 6 $\alpha\beta$ - methylcyclopenta[*f*] [1] - benzopyran-3,7-dione (IVb) as prisms (38 mg), m.p. 236.5–238°C, $[\alpha]_D^{23} +121.0 \pm 4.0^\circ$ [*c* 0.378 in chloroform/methanol (1:1, v/v)] and i.r. max. (in KBr) at 3400 (OH), 1736 (C=O) and 1701 (δ -lactone) cm^{-1} [Found: C, 65.5; H, 7.6; $\text{C}_{13}\text{H}_{18}\text{O}_4$ (238.27) requires C, 65.5; H, 7.6%; mol.wt. 238 by mass spectrometry].

The acetate, prepared with acetic anhydride/pyridine (1:1, v/v; 16 h, at room temperature), could not be induced to crystallize, but showed i.r. max. at 1736 (C=O, acetate and δ -lactone) cm^{-1} and n.m.r. absorptions at 0.94 (3H; singlet; 6 $\alpha\beta$ -Me), 2.10 (3H; singlet; OAc), 4.53 (1H; multiplet; *WH* 5.5 Hz; 4 $\alpha\beta$ -H) and 5.30 (1H; multiplet; 1-H) p.p.m.

The above β -hydroxy δ -lactone (11 mg) in acetone (1 ml) was treated with Jones reagent (13.5 μl) at approx. 5°C for 1 h. The amorphous powder that was precipitated on dilution with water (10 ml) was collected by filtration, washed with water and dried under reduced pressure. Crystallization from acetone gave 1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ -dodecahydro-6 $\alpha\beta$ - methylcyclopenta[*f*] [1]benzopyran-1,3,7-trione (XIb) as prisms (9 mg), m.p. 204–208°C, $[\alpha]_D^{28} -30.3 \pm 3.1^\circ$ [*c* 0.228 in chloroform/methanol (1:1, v/v)], λ_{max} 244.5 nm (ϵ 10700) and λ_{max} 270.5 nm [ϵ approx. 20000 in ethanol/1M-NaOH (approx. 50:1, v/v)] and i.r. max. (in KBr) at 3420 and approx. 2400–2800 (enolic OH), 1743 (C=O), 1668 (δ -lactone) and 1610 and 1598 (enolic C=C) cm^{-1} [Found: C, 66.1; H, 6.6; $\text{C}_{13}\text{H}_{16}\text{O}_4$ (236.26) requires C, 66.1; H, 6.8%; mol.wt. 236 by mass spectrometry].

$\alpha\beta$ -Unsaturated hydroxy-oxo acid (V). Pool *F* gave a main spot (solvent *a*; R_F 0.38) on t.l.c. that was also detected by a u.v.-scanner. On trituration with light petroleum, this pool gave a yellowish powder (84 mg), which was recrystallized from acetone/light petroleum to yield a crude sample of (*E*)-3-(3 α -hexahydro - 5 α - hydroxy - 7 $\alpha\beta$ - methyl - 1 - oxoindan-4 α -yl)-2-propenoic acid (V) (52 mg, m.p. 160–161°C). Since attempts to purify by recrystallization were unsuccessful, part (29 mg) of the sample was treated with ethereal diazomethane and subjected to preparative t.l.c. (silica gel GF₂₅₄, two plates; solvent *a*). The methyl ester (21 mg), obtained from the major band (R_F 0.48), could not be induced to crystallize, but it showed i.r. max. at 3590 and 3452 (OH), 1726 (C=O and ester) and 1654 and 987 (C=C) cm^{-1} . The ester acetate (XIII), prepared from the ester with acetic anhydride/pyridine (1:1, v/v; 16 h, at room temperature), crystallized from diethyl ether/light petroleum in needles (18 mg), m.p. 118–120°C, $[\alpha]_D^{24} +27.8 \pm 1.5^\circ$

(c 0.467 in ethanol), $\lambda_{\max}$ 209 nm (ϵ 14400), i.r. max. at 1736 (C=O, ester and acetate) and 1657 and 981 (C=C) cm^{-1} and n.m.r. absorptions at 0.94 (3H; singlet; 7 α -Me), 2.07 (3H; singlet; OAc), 3.75 (3H; singlet; CO₂Me), 5.10 (1H; multiplet; WH 5Hz; 5 β -H), 5.93 (1H; doublet; J 15.8Hz; 2-H of the side chain) and 6.82 (1H; doublet-doublet; J 15.8 and 8.3Hz; 3-H of the side chain) p.p.m. [Found: C, 65.3; H, 7.5; C₁₆H₂₂O₅ (294.34) requires C, 65.3; H, 7.5%; mol.wt. 294 by mass spectrometry].

The above ester acetate (12mg) in methanol (3.5ml) was hydrogenated over 10% (w/w) palladium on charcoal (Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; 7mg) at approx. 20°C and atmospheric pressure. After 30min, the uptake of hydrogen ceased. The catalyst was filtered off and the filtrate was evaporated *in vacuo* to yield a syrup (11mg), which showed i.r. bands at 1737 (shoulder) and 1721 (C=O, ester and acetate) cm^{-1} . Treatment with methanolic 1M-KOH and subsequently with dilute HCl converted this syrup into a product (6mg, m.p. 115–118°C) that was identical with the δ -lactone (IVa).

The faster-moving band, which is described under 'Isolation of the metabolites, 1 β -hydroxy-5-oxo acid (VIII)' below, was extracted with chloroform/methanol/0.01M-HCl (100:20:1, by vol.). The extract crystallized from acetone/light petroleum to give (*E*)-3-(3 α -hexahydro-5 α -hydroxy-7 α -methyl-1-oxoindan-4 α -yl)-2-propenoic acid (V) in prisms (33mg), m.p. 168–172°C, $[\alpha]_D^{27} +54.1 \pm 1.4^\circ$ (c 0.481), $\lambda_{\max}$ 212nm (ϵ 13800) and i.r. max. at 3400 (broad) (OH), approx. 2600–3200 (OH of CO₂H), 1738 (C=O), 1699 (C=O of CO₂H) and 1653 and 987 (C=C) cm^{-1} (Found: C, 65.5; H, 7.6; C₁₃H₁₈O₄ requires C, 65.5; H, 7.6%).

C₉ oxo dicarboxylic acid (VI). Pool *H* was re-chromatographed on silicic acid (12g), and a fraction (96mg) eluted with acetone/dichloromethane (7:43, v/v) was subjected to preparative t.l.c. (silica gel HF₂₅₄, four plates, 0.5mm thick; solvent *a*). An upper area (R_F 0.38, approx. 1cm wide) of the narrow band, which consists of an unknown metabolite as detected by a u.v. scanner, was extracted with chloroform/methanol/0.01M-HCl (100:20:1, by vol.). The extract crystallized from diethyl ether/light petroleum to give (+)-(5*R*)-5-methyl-4-oxo-octane-1,8-dioic acid (VI) as prisms (12mg), m.p. 66–69.5°C, $[\alpha]_D^{23} +13.5 \pm 1.3^\circ$ (c 0.430), i.r. max. at approx. 2500–3000 (OH of CO₂H) and 1706 (C=O and C=O of CO₂H) cm^{-1} , n.m.r. absorptions at 1.13 (3H; doublet; J 6.8Hz; 5-Me) and 10.3 (2H; broad peak; 2 CO₂H) p.p.m., mass peaks at m/e 202 (*M*), 184 (*M* - H₂O), 101 (base peak; CO-[CH₂]₂-CO₂H) and 60 [CH₂=C(OH)₂] and optical rotatory dispersion (c 0.355) $[\phi]_{400} +57$, $[\phi]_{303} -365$ and $[\phi]_{260} +1106$ (Found: C, 53.4; H, 7.1; C₉H₁₄O₅ requires C, 53.5; H, 7.0%). Although

the chemical shift of two carboxyl protons was erroneously reported previously as 7.02p.p.m. (Hayakawa & Hashimoto, 1969), it should be revised as described here.

Pyronylpropionic acid (VII). Pool *J* crystallized from acetone to yield 3-(4-hydroxy-5-methyl-2-oxo-2H-pyran-6-yl)propionic acid (VII) in rhombic crystals (1.5mg), m.p. 212–226.5°C (decomp.), $\lambda_{\max}$ 289.5nm ($A_{1\text{cm}}^{1\%}$ 330) and i.r. max. (in KBr) at 3400 (OH), approx. 2400–3200 (OH of CO₂H), 1695 (C=O and C=O of CO₂H) and 1650, 1623, 1572 and 1524 (C=C) cm^{-1} . The product gave a single spot (solvent *a*; R_F 0.21) on t.l.c., but, after treatment with ethereal diazomethane, the methylation mixture gave two spots (solvent *b*; R_F 0.29 and 0.08). It was then subjected to preparative t.l.c. (silica gel GF₂₅₄, one plate, 5cm×20cm, 0.25mm thick; solvent *b*). The extract obtained from the upper band crystallized from diethyl ether/light petroleum to yield methyl 3-(4-methoxy-5-methyl-2-oxo-2H-pyran-6-yl)propionate (XVIa) in needles (0.4mg), m.p. 93.5–94.5°C, $\lambda_{\max}$ 285.5nm (ϵ 6600), i.r. max. at 1731 (ester), 1700 (C=O) and 1649 and 1566 (C=C) cm^{-1} and mass peaks at m/e 226 (*M*), 195 (*M* - OMe), 167 (base peak; *M* - CO₂Me) and 139 (*M* - [CH₂]₂-CO₂Me).

The extract obtained from the lower band was a syrup (approx. 1mg), which was believed to be methyl 3-(2-methoxy-5-methyl-4-oxo-4H-pyran-6-yl)propionate from its $\lambda_{\max}$ 246nm and i.r. max. at 1740 (ester) and 1668 (C=O) and 1600 (C=C) cm^{-1} .

1 β -Hydroxy-5-oxo acid (VIII). As described above, compound (III) (30g) was exposed to *S. rubescens* for 75h and gave a mixture of acidic metabolites (13.17g), which was chromatographed on silicic acid (600g). A fraction (194mg), which was eluted with acetone/dichloromethane (3:17, v/v; 1.3 litres) and gave two major spots (solvent *a*; R_F 0.38 and 0.28) on t.l.c., was subjected to preparative t.l.c. (silica gel HF₂₅₄, five plates; solvent *a*). The faster-moving band was used for the isolation of the metabolite (V) [see under ' $\alpha\beta$ -Unsaturated hydroxy-oxo acid (V)' above]. The slower-moving band gave 3-(3 α -hexahydro-1 β -hydroxy-7 α -methyl-5-oxoindan-4 α -yl)propionic acid (VIII) (22mg), which was further purified by preparative t.l.c. and recrystallization from acetone/light petroleum to give an analytical sample with m.p. 145–147.5°C (Found: C, 64.7; H, 8.0. Calc. for C₁₃H₂₀O₄: C, 65.0; H, 8.4%). Identity with an authentic sample was established by mixed m.p. and by comparison of the behaviour on t.l.c. (solvent *a*) and the i.r. spectrum.

Chemical syntheses of the metabolites and their derivatives

(1*S*)-Epimer (XIa) of the metabolite (IVb) and its oxidation product (XIb). (a) Dehydrogenation of the lactone (IVa). A solution of the lactone (663mg) and

2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.43 g) in dioxan (66 ml) and acetic acid (14.3 ml) was heated for 8 days under reflux. The crystals that were precipitated on cooling were filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in dichloromethane/diethyl ether (approx. 1:5, v/v; 120 ml), washed with 5% (w/v) NaHCO_3 and then with water and dried over anhydrous Na_2SO_4 . Evaporation of the solvents left a reddish syrup (571 mg), which was subjected to preparative continuous t.l.c. (silica gel GF₂₅₄, 19 plates; diethyl ether; 2 h). The extract (195 mg), obtained from a major band revealed by a u.v. scanner, crystallized from acetone/light petroleum to give 3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ - dodecahydro - 6 $\alpha\beta$ - methylcyclopenta-[f][1]benzopyran-3,7-dione (X) in needles (160 mg, m.p. 160–161°C). An analytical sample, further recrystallized from acetone/light petroleum, showed m.p. 161.5–164°C, $[\alpha]_D^{25} -184 \pm 3.5^\circ$ (c 0.163 in ethanol), λ_{max} 203 nm (ϵ 10200), i.r. max. at 1740 (shoulder) (C=O), 1725 (δ -lactone) and 1623 (very weak) (C=C) cm^{-1} and n.m.r. absorptions at 0.94 (3H; singlet; 6 $\alpha\beta$ -Me), 4.61 (1H; multiplet; *WH* 5Hz; 4 $\alpha\beta$ -H), 6.07 (1H; doublet; *J* 9.7Hz; 2-H) and 7.01 (1H; doublet-doublet; *J* 9.7 and 6.0Hz; 1-H) p.p.m. (Found: C, 71.2; H, 7.4; $\text{C}_{13}\text{H}_{16}\text{O}_3$ requires C, 70.9; H, 7.3%).

(b) Hydration of compound (X). A solution of this compound (90 mg) in dioxan (5 ml) and 1M-NaOH (5 ml) was heated for 70 h under reflux. The mixture was acidified with dilute HCl and extracted with chloroform. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a syrup (80 mg), which was subjected to preparative t.l.c. (silica gel GF₂₅₄, two plates; solvent *a*). The extract (63 mg) obtained from a broad band (R_F 0.15) crystallized from acetone/light petroleum to give (1*S*)-1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ - dodecahydro - 1 - hydroxy - 6 $\alpha\beta$ - methylcyclopenta[*f*][1]benzopyran-3,7-dione (XIa) in prisms (55 mg), m.p. 138–138.5°C, $[\alpha]_D^{24} +101.9 \pm 0.9^\circ$ (c 0.586 in methanol) and i.r. max. at 3600 (weak) and 3460 (OH) and 1737 (C=O and δ -lactone) cm^{-1} (Found: C, 65.6; H, 7.7; $\text{C}_{13}\text{H}_{18}\text{O}_4$ requires C, 65.5; H, 7.6%).

(c) Oxidation of compound (XIa). This compound (34 mg) in acetone (3 ml) was oxidized with Jones reagent (60 μl) at approx. 5°C for 1 h. The crystals (24 mg) that were precipitated on dilution with water (20 ml) were collected by filtration, washed with water and dried under reduced pressure. Recrystallization from acetone gave 1,2,3,4 $\alpha\beta$,5,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ - dodecahydro - 6 $\alpha\beta$ - methylcyclopenta[*f*][1]benzopyran-1,3,7-trione (XIb) as prisms (22 mg), m.p. 203.5–207°C and $[\alpha]_D^{28} -31.7 \pm 1.2^\circ$ [c 0.596 in chloroform/methanol (1:1, v/v)] (Found: C, 66.4; H, 7.1; $\text{C}_{13}\text{H}_{16}\text{O}_4$ requires C, 66.1; H, 6.8%). Identity with the oxidation product of the metabolite

(IVb) was established by mixed m.p. and by comparison of the $[\alpha]_D$ and the u.v. and i.r. spectra.

Ester acetate (XIII) of the metabolite (V). (a) Hydrolysis and esterification of the $\alpha\beta$ -unsaturated lactone (X). A solution of the lactone (200 mg) in a mixture of methanol (1 ml), water (1 ml) and 1M-NaOH (0.91 ml) was warmed at 80–90°C for approx. 10 min. The mixture was acidified with 10% (v/v) acetic acid under cooling, saturated with NaCl and extracted with dichloromethane. The organic layer, after drying over anhydrous Na_2SO_4 , was immediately treated with ethereal diazomethane. Evaporation of the solvents left a syrup (185 mg), which was subjected to preparative t.l.c. (silica gel HF₂₅₄, four plates; solvent *b*). The extract, obtained from a zone (R_F 0.4) detected by a u.v. scanner, crystallized from diethyl ether/light petroleum to give methyl (Z)-3-(3 $\alpha\alpha$ - hexahydro - 5 α - hydroxy - 7 $\alpha\beta$ - methyl - 1 - oxoindan-4 α -yl)-2-propenoate (XII) in needles (70 mg), m.p. 93–97°C, $[\alpha]_D^{29} +104.5 \pm 3.1^\circ$ (c 0.467), λ_{max} 214 nm (ϵ 10000), i.r. max. at 3630 and 3570 (OH), 1737 (C=O), 1722 (ester) and 1648 (C=C) cm^{-1} and n.m.r. absorptions at 0.96 (3H; singlet; 7 $\alpha\beta$ -Me), 3.72 (3H; singlet; CO_2Me), 4.01 (1H; multiplet; *WH* 6Hz; 5 β -H), 5.94 (1H; doublet; *J* 11.7Hz; 2-H in the side chain) and 6.40 (1H; doublet-doublet; *J* 11.7 and 9.5Hz; 3-H in the side chain) p.p.m. (Found: C, 66.4; H, 7.9; $\text{C}_{14}\text{H}_{20}\text{O}_4$ requires C, 66.6; H, 8.0%).

(b) Photochemical isomerization of compound (XII). A solution of this compound (57 mg) and acetophenone (21.5 mg) in benzene (50 ml) was irradiated in a quartz vessel with a 1 kW mercury lamp for 25 min under bubbling with argon gas. Evaporation of the solvent *in vacuo* gave a syrup, which was subjected to preparative continuous t.l.c. [silica gel HF₂₅₄, two plates; diethyl ether/*n*-pentane (7:5, v/v); 1 h]. The extract (12 mg), obtained from a band that was located below compound (XII), centred at a line 7 cm from the origin and detected by a u.v. scanner, was treated with acetic anhydride/pyridine (1:1, v/v; 16 h, at room temperature). The acetylation mixture crystallized from diethyl ether/light petroleum to give methyl (E)-3-(5 α -acetoxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-1-oxoindan-4 α -yl)prop-2-enoate (XIII) in prisms (5 mg) with m.p. 118–120°C (Found: C, 65.5; H, 7.5; $\text{C}_{16}\text{H}_{22}\text{O}_5$ requires C, 65.3; H, 7.5%). Identity with a sample isolated from growing cultures as its ester acetate was established by mixed m.p. and by comparison of the u.v. and i.r. spectra.

Racemate of the metabolite (VI). A solution of 3-(1-methyl-2,5-dioxocyclopent-1-yl)propionic acid (XIV) (5 g) in cold 0.5M-NaOH (200 ml) was kept at approx. 20°C for 2 h. The pale-purple mixture was acidified with concn. HCl under cooling, saturated with NaCl and extracted with diethyl ether. The ethereal extract (4.424 g) crystallized from diethyl ether to give ($\pm$)-5-methyl-4-oxo-octane-1,8-dioic acid (1.409 g, m.p. 45–50°C). The mother liquor was

chromatographed on silicic acid (120g). Elution with acetone/dichloromethane (from 1:9, v/v, to 1:4, v/v) gave also this racemate (991 mg, m.p. 53–56°C). Both the products were combined and recrystallized from diethyl ether/light petroleum to give an analytical sample as prisms with m.p. 55.5–57°C (Found: C, 53.5; H, 7.0; $C_9H_{14}O_5$ requires C, 53.3; H, 7.0%). The i.r., n.m.r. and mass spectra were identical with those of the optically active metabolite (VI).

Methylated derivative (XVI_d) of the metabolite (VII). (a) Condensation of the methoxy α -pyrone (XV_b) with dimethyl oxalate. A mixture of the α -pyrone (3.32 g), dimethyl oxalate (3.20 g) and atomized metallic sodium (715 mg) in dry benzene (150 ml) was treated in the manner reported by Douglas & Money (1968) to give a mixture of products as a syrup. It was pulverized with diethyl ether to give a crude sample of *methyl 2-hydroxy-3-(4-methoxy-5-methyl-2-oxo-2H-pyran-6-yl)-prop-2-enoate* (XVI_b) (3.45 g, m.p. 143–146°C) as a yellowish powder. Recrystallization from methanol, water and then from ethyl acetate led to an analytical sample as pale-yellowish prisms, m.p. 149.5–151°C and i.r. max. at 3480 (OH), 1738 (shoulder) (ester), 1711 (C=O) and 1638, 1614 (weak) and 1548 (C=C) cm^{-1} (Found: C, 55.3; H, 4.9; $C_{11}H_{12}O_6$ requires C, 55.0; H, 5.0%).

(b) Hydrolysis and oxidative decarboxylation of compound (XVI_b). These reactions were carried out by the method of Douglas & Money (1968); the acidic hydrolysis of this compound (3.40 g) gave a crude sample of the corresponding *free acid* [1.37 g, m.p. 198–204°C (decomp.); 1.40 g, m.p. 184–185°C (decomp.)]. These products were used for the next step without further purification: this free acid (2.68 g) in an ice-cold solution of NaOH (1.70 g) in water (53 ml) was treated with 30% (w/w) H_2O_2 (5.8 ml) and gave a mixture of the products, which crystallized from ethyl acetate to yield *4-methoxy-5-methyl-2-oxo-2H-pyran-6-ylacetic acid* (XVI_c) in prisms (1.39 g) with m.p. 164–165°C. This sample contained a trace of impurities, as indicated by t.l.c. (solvent *a*), but it was used for the next step without further purification.

The *methyl ester*, prepared with ethereal diazomethane and purified by preparative t.l.c. (silica gel GF₂₅₄; solvent *b*), crystallized from diethyl ether/light petroleum in needles, m.p. 82–84°C, λ_{max} 285 nm (ϵ 6450) and i.r. max. at 1742 (ester), 1703 (C=O) and 1652 and 1566 (C=C) cm^{-1} (Found: C, 56.9; H, 5.9; $C_{10}H_{12}O_5$ requires C, 56.6; H, 5.7%).

(c) Arndt–Eistert reaction (cf. Bachmann & Struve, 1942) of compound (XVI_c). This compound (285 mg) was converted by the method of Douglas & Money (1968) into the corresponding acid chloride, which was then dissolved in dry benzene (25 ml). The solution was reacted with the large excess of dry ethereal diazomethane in the usual manner (cf. Bachmann & Struve, 1942). The mixture was left at approx. 25°C

for 45 min, then evaporated *in vacuo* to give a crude sample (140 mg) of the corresponding diazoketone as a reddish amorphous residue. It was dissolved in dry methanol (25 ml), and silver oxide (Kanto Chemical Co. Tokyo, Japan; 29 mg) was added in small portions with stirring during a period of 2 h with warming at approx. 60°C. Small quantities of charcoal was added to the mixture and filtered. The filtrate was subjected to preparative t.l.c. (silica gel GF₂₅₄, five plates; solvent *b*). The extract (80 mg), obtained from a band (R_f 0.29) revealed by a u.v. scanner, crystallized from diethyl ether to give *methyl 3-(4-methoxy-5-methyl-2-oxo-2H-pyran-6-yl)-propionate* (XVI_d) in prisms (34 mg, m.p. 88–91.5°C). An analytical sample, further recrystallized from diethyl ether, had m.p. 94–96°C and λ_{max} 287 nm (ϵ 6900 in methanol) (Found: C, 58.4; H, 6.2; $C_{11}H_{14}O_5$ requires C, 58.4; H, 6.2%). Identity with one of the methylated products of the metabolite (VII) was established by comparison of the behaviour on t.l.c. (solvent *b*), the m.p. and the u.v., i.r. and mass spectra.

Discussion

From the above chemical investigations there has been no doubt as to the structure of the metabolites (II), (IV_a), (IV_b), (V), (VI), (VII) and (VIII). However, the question has been raised as to whether these structures represent those of the true degradative intermediates formed from compound (III) by *S. rubescens*. First, although the isolation of the enaminolactam (II) provides support for our previous proposal that a precursor of this lactam formed from cholic acid (I) by this organism is compound (III) (Hayakawa *et al.*, 1976b), this lactam is probably not a degradative intermediate but a side product involved in the degradation of compound (III) as well as in that of cholic acid. Although it is not conclusive, the metabolite (VIII) is probably in equilibrium with compound (III) in the culture broth but not a degradative intermediate. Secondly, both the δ -lactones (IV_a) and (IV_b) probably did not exist in the culture broth as these forms but in their open forms, the corresponding δ -hydroxy acids, because most of these lactones were isolated as acidic (NaHCO₃-extractable) products but not as neutral products. This lactonization might occur during the isolation process under acidic conditions. Thirdly, since the present work was accomplished with unlabelled compound (III), no answer to the question is provided as to whether the metabolite (VI) is a product that was formed biogenetically from once-formed small fragments from compound (III), rather than that formed directly from compound (III). Further, non-utilization of the racemate of this metabolite by *S. rubescens* (S. Hashimoto & S. Hayakawa, unpublished work) deepens this question. However,

since we have observed a few examples of such non-utilization of a certain metabolite by the metabolite-producing organism (Hayakawa *et al.*, 1976a), this inability of the organism to grow on the racemate does not mean that the metabolite (VI) is not a degradative intermediate. We believe from the structure of this metabolite that the metabolite (VI) is a degradative intermediate formed from compound (III) in the manner by which the 7 α -methyl group in compound (III) remains undisturbed as the (5*R*)-methyl group in this metabolite. Finally, the metabolite (VII) could be also considered from its structure as an intermediate of biogenetic rather than degradative origin. We think that this metabolite is either a biogenetic product or a chemical artifact formed by cyclization of compound (IX), which corresponds to one of the intermediates involved in the degradation of compound (VI) by the β -oxidation-type mechanism. Support for this consideration is provided by the work of Harris & Harris (1969), who suggested that thiolic acid esters of 3,5-dioxoalkanoic acids with CoA or protein thiols are intermediates involved in the biogenesis of α -pyrones.

From the above considerations and the structure of the metabolites, the metabolic sequence of compound (III) can probably be shown as follows: II and VIII $\rightleftharpoons$ III $\rightarrow$ IVa (as its open form) $\rightarrow$ V $\rightarrow$ IVb (as its open form) $\rightarrow$ VI $\rightarrow$ IX $\rightarrow$ further degradation. Since compound (III) was used as the sole carbon and energy sources for the growth of the organism, the initial step of this degradative sequence must be energetically an oxidative reaction, but it is nevertheless represented as a reductive process. With respect to this question, we have thought of the following two possibilities: (1) compounds (IVa) (as its open form), (V) and (IVb) (as its open form) were probably in equilibrium with the corresponding 5-oxo derivatives in the culture broth, and only 5 α -hydroxy derivatives could be isolated, probably owing to differences in the degradative rate; (2) the initial reductive step could be catalysed by the organism inoculum itself, yielding the open form of compound (IVa). The propionic acid side chain of compound (III) or the open form of compound (IVa) is probably oxidized by the β -oxidation-type mechanism to yield 3 α -hexahydro-7 α -methyl-1,5-dioxoindane-4 α -carboxylic acid (C₁₁ dioxo acid) or 3 α -hexahydro-5 α -hydroxy-7 α -methyl-1-oxoindane-4 α -carboxylic acid (C₁₁ hydroxy-oxo acid) respectively. This possibility is supported by the isolation of compounds (IVb) and (V), which correspond to the intermediates involved in this side-chain shortening.

Even if the above C₁₁ hydroxy-oxo acid or C₁₁ dioxo acid could be formed in the manner described above, the steps between these C₁₁ acids and compound (VI) in the above-postulated sequence remain speculative. However, the stereochemistry of compound (VI), which is the (5*R*)-configuration,

suggests that fission between C-1 and C-7a of these C₁₁ acids must occur to retain the configuration of C-7a. The possible presence of compound (IX) in the culture broth, as discussed above, also suggests that compound (VI) is probably degraded further by the β -oxidation-type mechanism to the acetyl-CoA and 5-carboxy-2-methyl-3-oxopentanoyl-CoA via compound (IX).

Much of the representation of the degradative sequence of compound (III) described here is still largely hypothetical. However, the present results probably give an insight into the mechanism of biodegradation of bile acids in Nature when they are considered together with our previous study (Hayakawa *et al.*, 1976b) dealing with the degradation of cholic acid to compound (III).

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