Microbiological Degradation of Bile Acids

FURTHER DEGRADATION OF A CHOLIC ACID METABOLITE CONTAINING THE HEXAHYDROINDANE NUCLEUS BY *CORYNEBACTERIUM EQUI*

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1. The further degradation of a cholic acid (I) metabolite, (4*R*)-4-[4 α -(2-carboxyethyl)-3 α -hexahydro-7 α β -methyl-5-oxoindan-1 β -yl]valeric acid (II*a*), by *Corynebacterium equi* was investigated. This organism effected ring-opening and gave (4*R*)-4-[2 α -(2-carboxyethyl)-3 β -(3-carboxypropionyl)-2 β -methylcyclopent-1 β -yl]valeric acid (VI). The new metabolite was isolated as its trimethyl ester and identified by partial synthesis. It was not utilized by *C. equi*. 2. (4*R*)-4-[4 α -(2-Carboxyethyl)-3 α -decahydro-8 α β -methyl-5-oxa-6-oxoazulen-1 β -yl]valeric acid (IV*a*), which is a hypothetical initial oxidation product in the above degradation, was not converted by *C. equi* into the expected metabolite (VI), but into 3-{2 β -[(2*S*)-tetrahydro-5-oxofur-2-yl]-1 β -methyl-5-oxocyclopent-1 α -yl}-propionic acid (VIII), the structure of which was established by partial synthesis. 3. Both the possible precursors of the metabolite (VI), an isomer of the ϵ -lactone (IV*a*), the γ -lactone (XI*a*), and the open form of these lactones, the hydroxytricarboxylic acid (V), were also not utilized by *C. equi*. 4. Under some incubation conditions, *C. equi* also converted compound (II*a*) and 3-(3 α -hexahydro-7 α β -methyl-1,5-dioxoindan-4 α -yl)propionic acid (II*b*) into 5-methyl-4-oxo-octane-1,8-dioic acid (III), (4*R*)-4-(2,3,4,6,6 α β ,7,8,9,9 α ,9 β -decahydro-6 α β -methyl-3-oxo-1*H*-cyclopenta[*f*]quinolin-7 β -yl)valeric acid (VII) and probably a monohydroxy derivative of compound (II*a*) and compound (III), respectively. 5. The possibility that an initial step in the degradation of compound (II*a*) by *C. equi* is oxygenation of the Baeyer-Villiger type, yielding compound (IV*a*), is discussed. Metabolic pathways of compound (II*a*) to compounds (III), (VI), (VII) and (VIII) are also considered.

Numerous studies from our own and other laboratories have provided evidence for the microbiological degradation of a variety of steroids to form the corresponding hexahydroindane derivatives (cf. Smith, 1974; Hayakawa *et al.*, 1976*a*). However, comparatively little progress has been made in identifying the intermediates involved in the further degradation of these derivatives: Schubert *et al.* (1967) and Lee & Sih (1967) have proposed certain pathways for the degradation of the hexahydroindane nucleus, but studies on the isolation of the expected intermediates which would support their proposed pathways are still lacking.

We previously reported that a hexahydroindane-nucleus-containing metabolite formed from 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid) (I) by *Arthrobacter simplex*, (4*R*)-4-[4 α -(2-carboxyethyl)-3 α -hexahydro-7 α β -methyl-5-oxoindan-1 β -yl]valeric acid (II*a*), was not utilized by this organism, but it was utilized by *Corynebacterium equi* and converted into conjugates with amino acids or their derivatives and other unidentified metabolites (Hayakawa *et al.*, 1976*c*) (Scheme 1). Our continued in-

terest in defining the intermediates involved in the degradation of cholic acid (I) has prompted us to isolate and identify these unidentified metabolites.

The present paper describes the isolation and identification of the metabolites formed from compound (II*a*) by *C. equi* and also the reaction sequence involved in this metabolism.

Results and Discussion

Isolation, structural assignments and metabolic pathways of the degradation products

*Degradation of the oxodicarboxylic acid (II*a*) into the oxotricarboxylic acid (VI).* Exposure of compound (II*a*) to *C. equi* resulted in the formation of the conjugates of this acid with amino acids or their derivatives and other unidentified metabolites, as previously reported (Hayakawa *et al.*, 1976*c*). In the present study the isolation of two of the unidentified metabolites was attempted, which corresponded to two main spots on t.l.c. between compound (II*a*) and its conjugate with L-alanine. One of them was isolated as its oily methyl ester, but the other could not be obtained

Scheme 1. Microbiological degradation of cholic acid (I), the oxodicarboxylic acid (IIa), the dioxocarboxylic acid (IIb) and the hydroxytricarboxylic acid ϵ -lactone (IVa)

in a pure form. The i.r. spectrum of this ester showed the presence of both ketone and ester carbonyl groups at 1713 (shoulder) and 1735 cm^{-1} respectively. The n.m.r. (nuclear-magnetic-resonance) spectrum showed signals at δ 0.85 (singlet; 3H; one tertiary methyl group), 0.97 (doublet; 3H; J 6.0 Hz; one secondary methyl group), 2.63 (multiplet; 4H; two methylene groups) and 3.67 (singlet; 9H; three ester methyl groups) p.p.m. The corresponding free acid, obtained after alkaline hydrolysis, could not be induced to crystallize, but its *p*-bromophenacyl ester was obtained as crystals and the elementary analysis consistent with $\text{C}_{42}\text{H}_{43}\text{Br}_3\text{O}_{10}$, suggesting that this metabolite was a mono-oxotricarboxylic acid, $\text{C}_{18}\text{H}_{28}\text{O}_7$. The free acid existed in a mixture of metabolites before esterification, coinciding on t.l.c. with the product obtained after the hydrolysis of the trimethyl ester. Further, a multiplet at δ 2.63 (4H) p.p.m. in the n.m.r. spectrum could be assigned as four methylene protons of the type $-\text{CO}-[\text{CH}_2]_2-\text{CO}_2\text{Me}$ from its chemical shift and multiplicity. Thus the structure was tentatively assigned as (4*R*)-4-[2 α -(2-carboxyethyl)-3 β -(3-carboxypropionyl)-2 β -methylcyclopent-1 β -yl]valeric acid (VI) and then established by partial synthesis as described below. The results show that, in the degradative sequence, the original stereochemistry of compound (IIa) remains undisturbed.

Norris & Trudgill (1971) and Donoghue & Trudgill (1975) have reported that, in the metabolism of cyclohexanol by bacteria, its initial oxidation product, cyclohexanone, is degraded stepwise into 6-oxohexanoic acid via 2-oxacycloheptanone and 6-hydroxyhexanoic acid. A microbiological ring cleavage of this Baeyer-Villiger type has also been found to occur with 3 β -hydroxy-24-methylene-8-lanosten-21-oic acid (eburicoic acid) (Laskin *et al.*, 1964). It is therefore conceivable that compound (IIa) is initially oxidized by *C. equi* to produce (4*R*)-4-[4 α -(2-carboxyethyl)-3 α -decahydro-8 $\alpha\beta$ -methyl-5-oxa-6-oxoazulen-1 β -yl]valeric acid (IVa) and then further converted into compound (VI) via the hydroxytricarboxylic acid (V). However, the oxidation of compound (V) by *C. equi* into compound (VI) could not be demonstrated, because of the inability of this organism to grow on compound (V); this may be due to the limited ability of this acid to penetrate into the organism.

Degradation of the C₁₈ hydroxytricarboxylic acid ϵ -lactone (IVa) into the C₁₃ hydroxyoxodicarboxylic acid γ -lactone (VIII). A metabolite formed from compound (IIa) by *C. equi*, compound (VI), was exposed to this organism with the hope of identifying its further degradation products, but it was not utilized by this organism. *A. simplex*, which is a producer, but not a utilizer, of compound (IIa) (Hayakawa *et al.*, 1976a), was similarly used instead of *C. equi*, but this organism, like *C. equi*, could not utilize this acid. Therefore the following hypothetical precursors of

compound (VI) were synthesized and similarly submitted to this utilization test: the ϵ -lactone (IVa), its isomer, the γ -lactone (XIa), and the open form of both these lactones, the hydroxytricarboxylic acid (V). The latter two compounds, like compound (VI), were not utilized by *C. equi*, whereas the former ϵ -lactone (IVa) was utilized by this organism and converted into a new metabolite.

The metabolite was isolated as its crystalline methyl ester and its elementary analysis was consistent with $\text{C}_{14}\text{H}_{20}\text{O}_5$. Support for this formula was provided by the presence of approximately 20 protons calculated from proton integration in the n.m.r. spectrum. The i.r. and n.m.r. spectra showed bands at 1743 and 1776 cm^{-1} and signals at δ 1.04 (singlet; 3H), 3.64 (singlet; 3H) and approx. 4.47 (unresolved multiplet; 1H) p.p.m. respectively. The presence of a methyl ester group was indicated by a singlet at δ 3.64 (3H) p.p.m. in the n.m.r. spectrum and by an i.r. absorption at 1743 cm^{-1} . By comparing with the i.r. and n.m.r. spectra of the methyl ester of compound (XIa), a band at 1776 cm^{-1} in the i.r. spectrum and an unresolved multiplet at δ approx. 4.47 (1H) p.p.m. in the n.m.r. spectrum could be assigned as a γ -lactone carbonyl group and a methine proton attached to the oxygen atom consisting of this γ -lactone ring respectively. Further, a more intensive i.r. absorption at 1743 cm^{-1} compared with that at 1776 cm^{-1} suggested that the function of the remaining one oxygen atom in the formula would be a five-membered-ring carbonyl group, which was newly introduced by the cleavage of the valeric acid side chain of compound (IVa). This side-chain shortening was also supported by the presence of only one tertiary methyl group, indicated by a singlet at δ 1.04 (3H) p.p.m. in the n.m.r. spectrum. Thus the structure of this metabolite was assigned as 3-{2 β -(2*S*)-tetrahydro-5-oxofur-2-yl]-1 β -methyl-5-oxocyclopent-1 α -yl}propionic acid (VIII), and then established by partial synthesis as described below, indicating that, in the degradative sequence, the original stereochemistry of compound (IVa) remains undisturbed.

As well as the methyl ester of compound (VIII), the presence of the dimethyl esters of the unaltered substrate, compound (IVa), and its isomer, compound (XIa), in the methylation mixture of products were also detected by t.l.c. This suggests that a pathway for the degradation of compound (IVa) may be: (IVa) $\rightarrow$ (XIa) $\rightarrow$ (VIII). However, it is also possible that the valeric acid side chain of compound (IVa) is at first degraded to produce 3-(3 $\alpha\alpha$ -decahydro-8 $\alpha\beta$ -methyl-5-oxa-1,6-dioxoazulen-4 α -yl)propionic acid (IVb), which is then non-enzymically isomerized to yield compound (VIII) during the extraction and preparative t.l.c. processes under the acidic conditions that were made acid with HCl and acetic acid respectively. Support for this possibility is provided by evidence that such acid-catalysed non-enzymic iso-

merization occurred with the ϵ -lactones (IVa) and (Xb) as described below. Thus an actual metabolite formed from compound (IVa) by *C. equi* is probably not compound (VIII), but compound (IVb). Similarly, compound (XIa), detected as its dimethyl ester as described above, may be also an artifact formed from compound (IVa) by acid-catalysed isomerization.

From our previous viewpoint that not all the metabolites formed from cholic acid (I) by a certain micro-organism can be utilized by the same organism (Hayakawa *et al.*, 1976a), the inability of *C. equi* to grow on compound (VI) does not mean that this compound is not an intermediate metabolite involved in the degradation of compound (IIa) by this organism. Although not conclusive, however, evidence that of four compounds (IVa), (V), (VI) and (XIa) only compound (IVa) was utilized and converted by *C. equi* into compound (IVb) as discussed above suggests the following two possibilities: (1) compound (IVa) is at least one of the intermediates involved in the degradation of compound (IIa) by this organism and it is further degraded by a pathway yielding compound (IVb), which is probably subjected to further degradation; (2) as discussed above, compound (IVa) is also metabolized into compound (VI), which may not be further degraded.

Transformation of the oxodicarboxylic acid (IIa) into the C₉ acid (III), the enaminolactam (VII) and an unidentified product. *C. equi*, cultured in a medium containing both 0.1% compound (IIa) and 0.05% yeast extract in a mineral solution that was used in the degradation of cholic acid (I) by *A. simplex* (Hayakawa *et al.*, 1969), rapidly degraded this acid without the apparent accumulation of metabolites, whereas under other incubation conditions, it could accumulate the metabolites: the conjugates of this acid with amino acids or their derivatives were isolated and identified as the metabolites, when the organism, precultured in this medium or its modified one, was cultured in a medium containing 0.3% compound (IIa) only in this mineral solution (Hayakawa *et al.*, 1976c). As well as the conjugates, compound (VI) was also isolated from the latter culture. In another study, *C. equi* was cultured in a medium containing 0.3% compound (IIa) and 0.03% yeast extract in the above mineral solution with the hope of decreasing the rapid degradation of compound (IIa), and a mixture of metabolites was obtained, which mainly consisted of other metabolites rather than the conjugates. One of the metabolites was isolated as the methyl ester of (4R)-4-(2,3,4,6,6a β ,7,8,9,9a α ,9b β -decahydro-6a β -methyl-3-oxo-1H-cyclopenta-[f]quinolin-7 β -yl)valeric acid (VII) by direct comparison with an authentic sample. This transformation of compound (IIa) into compound (VII) provides support for our previous presumption that, in the degradation of cholic acid (I) by *Streptomyces rubescens*, its metabolites, compound (IIa) and its

C₁₆ and C₁₂ hexahydroindane homologues, are converted by this organism into the corresponding enaminolactam derivatives such as compound (VII) (Hayakawa *et al.*, 1976b). *C. equi* probably converts compound (IIa) into compound (VII) in the same manner as *S. rubescens*. As well as these enaminolactams in *S. rubescens*, however, compound (VII) is probably not an intermediate involved in the degradation of compound (IIa) by *C. equi*, but is a side product.

The second metabolite could not be isolated in a pure form, but the characteristic coloration on t.l.c. suggested that it was 5-methyl-4-oxo-octane-1,8-dioic acid (III), which we had isolated and identified as one of the intermediates involved in cholic acid degradation by micro-organisms (Hayakawa & Hashimoto, 1969; Hayakawa & Fujiwara, 1969). That this metabolite was the expected one was established by direct comparison of the g.l.c.-mass spectrum with that of an authentic sample, although it could not be determined whether it was optically active.

The third metabolite was isolated as its oily methyl ester. The i.r. and n.m.r. spectra indicated that the structure of this metabolite would be a monohydroxy derivative of compound (IIa) (see under 'Isolation of the degradation products' in the Experimental section). The elementary analysis of the corresponding crystalline free acid was consistent with C₁₈H₂₈O₆.

Degradation of the dioxocarboxylic acid (IIb) to the C₉ acid (III). We have isolated and identified compound (III) as a metabolite formed from 3-(3a α -hexahydro-7a β -methyl-1,5-dioxoindan-4 α -yl)-propionic acid (IIb) by *S. rubescens* (Hayakawa & Hashimoto, 1969). We expected, therefore, that compound (IIb) might be one of the intermediates involved in the above degradation of compound (IIa) by *C. equi* into compound (III). Exposure of compound (IIb) to *C. equi* resulted in the formation of compound (III) as expected. It was isolated as its optically active dimethyl ester and identified by comparison of the behaviour on t.l.c. and the i.r. and n.m.r. spectra with those of an authentic sample.

Compound (III) has been isolated as a common metabolite involved in the respective degradations of cholic acid (I) and compound (IIb) by *A. simplex* (Hayakawa & Fujiwara, 1969) and *S. rubescens* (Hayakawa & Hashimoto, 1969). Therefore the degradation of both compounds (IIa) and (IIb) by *C. equi* into compound (III) described here suggests that this C₉ acid is of general importance in the microbiological degradation of cholic acid and its metabolites. Further, the above results suggest that the degradative sequence of compound (IIa) by *C. equi* into compound (III) probably proceeds through the following pathway: (IIa) $\rightarrow$ (IIb) $\rightarrow$ (III), which is the same as that postulated for cholic acid degradation by *S. rubescens* (Hayakawa & Hashimoto, 1969;

Hayakawa *et al.*, 1976b). The possibility that a catabolic pathway, (IIa) $\rightarrow$ (IVa) $\rightarrow$ (IVb) $\rightarrow$ (III), is involved in this degradation cannot be excluded, since as discussed above compound (IIa) may be initially oxidized by *C. equi* to yield compound (IVa).

Partial syntheses of the degradation products (VI) and (VIII)

Because the above structural assignments are only indicative, we sought a more definitive proof of the structure of the new degradation products by partial synthesis (Scheme 2).

Oxotricarboxylic acid (VI). The Baeyer–Villiger oxidation (Hassall, 1957) of methyl (4*R*)-4-[3 α -hexahydro-4 α -(2-methoxycarbonylethyl)-7 $\alpha\beta$ -methyl-5-oxoindan-1 β -yl]valerate (IXa) with *m*-chloroperbenzoic acid gave the corresponding seven-membered-ring lactone (Xa). Treatment with alkali and subsequently with dilute HCl converted this ϵ -lactone into the hydroxytricarboxylic acid γ -lactone (XIa) as a main product, probably owing to the higher stability of this γ -lactone compared with the corresponding ϵ -lactone (IVa). The former was converted by aqueous alkali into the corresponding trisodium salt and the aqueous solution was then oxidized with ruthenium tetroxide to yield the oxotricarboxylic acid (VI). The trimethyl ester was identical in all respects (t.l.c., i.r. and n.m.r.) with a sample isolated as its trimethyl ester from growing cultures.

Hydroxyoxodicarboxylic acid γ -lactone (VIII). Exactly as described above for compound (IXa), 3-(1 β -acetoxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-

oxoindan-4 α -yl)propionic acid (IXb) was oxidized with *m*-chloroperbenzoic acid to obtain the corresponding ϵ -lactone (Xb). As well as this ϵ -lactone, after purification by chromatography, its isomer, the γ -lactone (XIc), was also isolated. This γ -lactone is probably an artifact formed from the ϵ -lactone (Xb) by isomerization, which was catalysed by acetic acid used in the chromatographic process, since similar acid-catalysed isomerization occurred in the Baeyer–Villiger oxidation of compound (IIa) in acetic acid containing toluene-*p*-sulphonic acid as a catalyst as described below.

Kondo *et al.* (1969) have synthesized the ϵ -lactone (Xb) by the Baeyer–Villiger oxidation of compound (IXb) in acetic acid containing toluene-*p*-sulphonic acid as a catalyst. However, the physical data such as m.p. and i.r. and n.m.r. spectra did not coincide with those of our sample of the ϵ -lactone (Xb), but resembled closely those of the γ -lactone (XIc). It seems likely that the structure of the sample of Kondo *et al.* (1969) has been erroneously assigned and it is not the ϵ -lactone (Xb), but the γ -lactone (XIc). If so, their biochemical conclusion deduced from the results using compound (XIc) instead of compound (Xb) may be also modified.

Treatment with alkali and subsequently with acid converted both the acetoxy γ - and ϵ -lactones (XIc) and (Xb) into the same product, the hydroxy γ -lactone (XIb), as discussed above in the hydrolysis of compound (Xa). Oxidation with Jones reagent (Curtis *et al.*, 1953) afforded the hydroxyoxodicarboxylic acid γ -lactone (VIII). The methyl ester was identical

Scheme 2. Partial syntheses of the degradation products (VI) and (VIII)

in all respects (t.l.c., m.p., mixed m.p., $[\alpha]_D$, i.r. and n.m.r.) with a sample isolated as its methyl ester from growing cultures.

Partial synthesis of the hydroxytricarboxylic acid ϵ -lactone (IVa)

As described above, the ester ϵ -lactone (Xa) was not hydrolysed to the corresponding free acid ϵ -lactone (IVa), but to the γ -lactone (XIa). Compound (IIa) in acetic acid was directly subjected to Baeyer-Villiger oxidation by using *m*-chloroperbenzoic acid and toluene-*p*-sulphonic acid as a catalyst, and yielded compound (IVa). In this oxidation the formation of its isomer, the γ -lactone (XIa), was also observed, probably owing to acid-catalysed isomerization.

Experimental

General

The methods of incubation, physical measurements and adsorption column chromatography and the micro-organisms, *Arthrobacter simplex* and *Corynebacterium equi*, were as described by Hayakawa *et al.* (1969, 1976c), except where otherwise indicated. Reversed-phase partition chromatography was performed as reported by Sjövall (1964) with Hostalen S [Farbwerke Hoechst A.-G., Frankfurt (M), Germany] as support and both the solvent systems F1 and F2 were used [for composition of solvents, see Sjövall 1964]. Unless otherwise stated, optical rotations and i.r.-absorption spectra were determined in chloroform solution.

T.l.c.

Except where otherwise stated, the glass plates were as described by Hayakawa *et al.* (1976b), and cyclohexane/ethyl acetate/acetic acid (5:5:1, by vol.) was used as a developing solvent system for both analytical and preparative t.l.c. In preparative t.l.c., bands were revealed by a water spray or by exposure to I_2 vapour, scraped from the plates and then extracted with chloroform/methanol (1:1, v/v). Continuous t.l.c. was performed as reported by Truter (1964), except that development for making a so-called 'hair-line origin' was omitted.

G.l.c.-mass spectrometry

Samples dissolved in acetone were analysed directly on a Neva 1740 gas chromatograph (Nippon Electric Varian, Tokyo, Japan) which was interfaced, via a glass-jet-type molecular separator, made by Dr. Y. Nakagawa in this laboratory, with a Hitachi RMU-6 single-focus mass spectrometer (Hitachi, Tokyo, Japan.)

The g.l.c. separations were performed on a coiled-glass column (length 1.5 m, internal diameter 2 mm) packed with Gas-Chrom Q (100–120 mesh; Applied

Science Laboratory Inc., State College, PA, U.S.A.) coated with SE-30 [3% (w/w); Applied Science Laboratory Inc.] at 158°C and a flow rate of 18 ml of helium/min. The g.l.c.-mass spectrometer molecular-separator temperature was kept at 230°C. The mass spectra were measured at 70 eV ionizing voltage and at 230°C ion-source temperature. Data were recorded by Honeywell 1508 Visicorder (Honeywell, Denver, CO, U.S.A.) on a Kodak photographic paper (Eastman Kodak Co., Rochester, NY, U.S.A.).

Other materials

As substrates for incubation, authentic samples for identification and starting materials for partial synthesis, the following compounds were prepared by our own methods previously reported: (4*R*)-4-[4 α - (2 - carboxyethyl) - 3 $\alpha\alpha$ - hexahydro - 7 $\beta\beta$ - methyl-5-oxoindan-1 β -yl]valeric acid (IIa) and its dimethyl ester (IXa) (Hayakawa & Fujiwara, 1969; Hayakawa *et al.*, 1969); ($\pm$)-5-methyl-4-oxo-octane-1,8-dioic acid (Hayakawa & Hashimoto, 1969) and its dimethyl ester (Hayakawa & Fujiwara, 1969); (4*R*) - 4 - (2,3,4,6,6 $\alpha\beta$,7,8,9,9 $\alpha\alpha$,9 $\beta\beta$ - decahydro-6 $\alpha\beta$ -methyl - 3 - oxo - 1*H* - cyclopenta[*f*]quinolin-7 β -yl)-valeric acid (VII) and its methyl ester (Hayakawa *et al.*, 1976b); 3-(1 β -acetoxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IXb) (Hayakawa *et al.*, 1976b). (4*R*)-4-[2 α -(2-Carboxyethyl)-3 β -(3-carboxypropionyl) - 2 β - methylcyclopent - 1 β - yl]-valeric acid (VI) and (4*R*)-4-[2 α -(2-carboxyethyl)-3 β - [(2*S*) - tetrahydro - 5 - oxofur - 2 - yl] - 2 β - methylcyclopent-1 β -yl]valeric acid (XIa) were obtained in the partial syntheses of the degradation products as described below. (4*R*)-4-[2 β -(2-Carboxyethyl)-3 β - [(1*S*) - 3 - carboxy - 1 - hydroxypropyl] - 2 β - methylcyclopent-1 β -yl]valeric acid (V) was obtained by the alkaline hydrolysis of compound (XIa) as an aqueous solution of its trisodium salt. (4*R*)-4-[4 α -(2-Carboxyethyl) - 3 $\alpha\alpha$ - decahydro - 8 $\alpha\beta$ - methyl - 5 - oxa - 6 - oxoazulen-1 β -yl]valeric acid (IVa) was synthesized as follows: a solution of *m*-chloroperbenzoic acid [15 g; approx. 80% (w/w) purity; FMC Corp., New York, NY, U.S.A.] in acetic acid (150 ml) was added dropwise to a solution of compound (IIa) (5 g) in acetic acid (100 ml) at approx. 20°C, with stirring. After addition of toluene-*p*-sulphonic acid (100 mg), the mixture was kept at the same temperature for 17 h. The crystals of *m*-chlorobenzoic acid, precipitated on diluting the mixture with water (3 litres), were filtered off, and the filtrate was saturated with NaCl and extracted with 5 $\times$ 1 litre of diethyl ether/ethyl acetate (2:1, v/v). The combined extract was evaporated *in vacuo* to yield a residue (15.9 g), which still contained *m*-chlorobenzoic acid as indicated by t.l.c. On treatment with acetone/light petroleum, it gave crystals (2.96 g). The mother liquor was evaporated *in vacuo* to dryness and chromatographed on silicic acid (60 g). Elution with chloroform gave *m*-chloroben-

zoic acid, and elution with acetone/chloroform (1:9, v/v) afforded a fraction (1.44 g), which, after treatment with acetone/light petroleum, yielded crystals (1.093 g). The crystalline samples were combined and recrystallized from methanol/diethyl ether to give (4*R*) - 4 - [4 α - (2 - carboxyethyl) - 3 $\alpha\alpha$ - decahydro - 8 $\alpha\beta$ - methyl - 5 - oxa - 6 - oxoazulen - 1 β - yl]valeric acid (IVa) as prisms (1.23 g), m.p. 146–146.5°C, $[\alpha]_D^{25} - 35.8 \pm 0.7^\circ$ (c 1.050), i.r. max. at approx. 2600–3100 (OH of CO₂H) and 1714 (C=O of CO₂H and ϵ -lactone) cm⁻¹ and n.m.r. absorptions at 0.88 (3H; singlet; 8 $\alpha\beta$ -Me), 0.96 (3H; superimposable doublet; Me in the side chain), approx. 4.33 (1H; unresolved multiplet; 4 β -H) and 9.83 (2H; broad peak; 2 CO₂H) p.p.m. (Found: C, 63.3; H, 8.3; C₁₈H₂₈O₆ requires C, 63.5; H, 8.3%). The mother liquor yielded another crop of crystals (738 mg; m.p. 90–97°C), but it was contaminated with the γ -lactone (XIa) as indicated by an i.r. absorption at 1770 cm⁻¹ characteristic of five-membered-ring lactones.

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

Degradation of the oxodicarboxylic acid (IIa) into the oxotricarboxylic acid (VI). As previously described (Hayakawa *et al.*, 1976c), the acid (60 g) was incubated with *C. equi* for 44–52 h and gave both a chloroform-soluble and an ethyl acetate-soluble fraction. The former fraction (892 mg) mainly contained unaltered compound (IIa) and the latter (3 g) consisted of the amino acid conjugates previously isolated by Hayakawa *et al.* (1976c) and other unidentified metabolites as indicated by t.l.c. The main components of these unidentified metabolites were detected as two spots (*R_F* 0.29 and 0.37) between compound (IIa) and its conjugate with L-alanine. Part (2.2 g) of the latter was subjected to reversed-phase partition chromatography (Hostalen S, 300 g; solvent system F1) and fractions of 17 ml were collected. Fractions 5–13, which were similar in behaviour on t.l.c. to each other, were combined and evaporation of the solvents *in vacuo* left an oily residue (1.133 g). This contained the two desired metabolites, as well as the conjugate of compound (IIa) with glutamic acid previously isolated (Hayakawa *et al.*, 1976c) and other more polar products. Part (700 mg) of the residue was then further fractionated by reversed-phase partition chromatography (Hostalen S, 90 g; solvent system F2; fractions of 10 ml were collected). Fractions 6–17 (569 mg) rich in the above two metabolites were obtained, but all were still contaminated with the relatively large amounts of other polar products. It was then esterified with ethereal diazomethane and subjected to preparative t.l.c. (25 plates,

0.5 mm thick). Two bands (*R_F* 0.48 and 0.56) separated, which moved faster than that of the trimethyl ester of the glutamic acid conjugate. The faster-moving band gave the *trimethyl ester of (4R)-4-[2 α -(2-carboxyethyl) - 3 β - (3 - carboxypropionyl) - 2 β - methylcyclopent-1 β -yl]valeric acid* (VI) as an oily mass (37 mg). It could not be induced to crystallize, but showed i.r. max. at 1735 (ester) and 1713 (shoulder) (C=O) cm⁻¹ and n.m.r. absorptions at 0.85 (3H; singlet; 2 β -Me), 0.97 (3H; doublet; *J* 6.0 Hz; Me in the side chain), 2.63 (4H; multiplet; CO-[CH₂]₂-CO₂Me) and 3.67 (9H; singlet; 3 CO₂Me) p.p.m.

The slower-moving band yielded an oily product (51 mg) consisting mainly of a spot (*R_F* 0.48) on t.l.c., but the product exhibited several n.m.r. signals in the region of δ 0.7–1.5 p.p.m., indicating the presence of impurities. It was not further treated.

The above trimethyl oxotricarboxylate (30 mg) was dissolved in a mixture of methanol (1 ml) and 1 M-NaOH (0.5 ml) and kept overnight at approx. 20°C. The mixture was diluted with water, acidified with dilute HCl, saturated with NaCl and thoroughly extracted with diethyl ether. The ethereal extract was washed with saturated NaCl, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to afford the corresponding *free acid* (VI) as an oily mass, which could not be induced to crystallize. The *R_F* (0.29) on t.l.c. was the same as in its crude state, indicating no occurrence of side reactions in both the esterification and the alkaline hydrolysis used for purification. The free acid was converted into the corresponding *p*-bromophenacyl ester: the acid (21 mg) was dissolved in ethanol (3 ml) and adjusted to approx. pH 6 with 0.1 M-NaOH. *p*-Bromophenacyl bromide (150 mg) was added to the ethanolic solution and the mixture was heated under reflux for 4 h. The expected tri-(*p*-bromophenacyl) ester could not be isolated directly from the mixture as crystals, but was extracted with ethyl acetate and the extract subjected to preparative t.l.c. (six plates, 0.5 mm thick). The extract, obtained from the faster-moving band (*R_F* 0.63), crystallized from diethyl ether to give the *tri-(p-bromophenacyl) ester* (18 mg), m.p. 104–108°C and i.r. max. at 1748 (ester), 1706 (C=O) and 1589 and 1486 (weak) (C₆H₄) cm⁻¹ (Found: C, 53.0; H, 4.6; Br, 24.8; C₄₂H₄₃Br₃O₁₀ requires C, 53.2; H, 4.6; Br, 25.3%).

The i.r.-absorption spectrum of the extract (14 mg) obtained from the slower-moving band (*R_F* 0.55) indicated the presence of a free carboxyl group at approx. 2600–3100 cm⁻¹. This product might be the di-(*p*-bromophenacyl) ester of compound (VI), but it was not further treated.

Attempts to degrade further the oxotricarboxylic acid (VI) with C. equi or A. simplex. The acid (25, 50 and 100 mg) was dissolved in 0.1 M-NaOH (2.11, 4.22 and 8.44 ml) respectively and diluted with water to 10 ml. These three solutions were separately added to a basal medium, which consisted of (NH₂)₄SO₄

(200mg), KH_2PO_4 (100mg), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (50mg), KCl (50mg), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1mg), yeast extract (50mg) and water (90ml) and adjusted with 0.1M-NaOH to pH7.1, and autoclaved ($1\text{kg}/\text{cm}^2$ for 20 min). After inoculation with *C. equi* grown on a nutrient slope, the course of the degradation of compound (VI) was monitored by t.l.c. as previously described (Hayakawa *et al.*, 1969). The growth of the organism was also qualitatively followed by estimating the turbidity of the cultures with the naked eye. Even after the 11-day incubation, no growth of the organism occurred and nor were metabolites detected by t.l.c. at any concentrations tested. *A. simplex* was then used instead of *C. equi* under the same conditions, but as with *C. equi*, no growth was observed.

Utilization test of the hydroxytricarboxylic acid δ -lactone (IVa), its isomeric γ -lactone (XIa) and the hydroxytricarboxylic acid (V) with *C. equi*. Both the lactones (each 50mg) were separately suspended in water (5ml), treated with 0.1M-NaOH (2.94ml) to neutralize their two carboxyl groups and then diluted with water to 10ml. The γ -lactone (50mg) was dissolved in 0.1M-NaOH (4.42ml), heated on a boiling-water bath for approx. 10min to yield the corresponding trisodium salt of compound (V) and then diluted with water to 10ml. These three solutions were sterilized by passing through a Millipore GS filter (pore size $0.22\mu\text{m}$; Millipore Filter Corp., Bedford, MA, U.S.A.). Under sterile conditions, each filtrate was added to the autoclaved basal medium (90ml) in an attempt to induce further degradation of compound (VI). The final pH of all three media was approx. 7.0. After inoculation with *C. equi*, the growth of the organism and the formation of metabolites were observed for 7 days. Neither compounds (V) nor (XIa) were utilized by *C. equi*, and nor was any detectable metabolite of these compounds produced, whereas compound (IVa) was converted into more polar products. One of these was isolated and identified, as described below.

Degradation of the hydroxytricarboxylic acid δ -lactone (IVa) into the hydroxyoxodicarboxylic acid γ -lactone (VIII). The mixture, resulting from the incubation of the lactone (400mg) with *C. equi* for 11 days, was acidified with dilute HCl to approx. pH2, saturated with NaCl and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield a residue (364mg), which mainly contained two components (R_F 0.11 and 0.28) as indicated by t.l.c. After esterification with ethereal diazomethane, it was subjected to preparative t.l.c. (12 plates, 0.75mm thick). The extract (74mg), obtained from the slower-moving band (R_F 0.15), crystallized from acetone/diethyl ether to yield the *methyl ester of 3-[2 β -(2S)-tetrahydro-5-oxofur-2-yl]-1 β -methyl-5-oxocyclopent-1 α -yl]propionic*

acid (VIII) in rods (58mg), m.p. 68–69°C, $[\alpha]_D^{25} +92.1 \pm 1.3^\circ$ (c 1.005), i.r. max. at 1776 (γ -lactone) and 1743 (ester and five-membered-ring C=O) cm^{-1} and n.m.r. absorptions at 1.04 (3H; singlet; 1 β -Me), 3.64 (3H; singlet; CO_2Me) and approx. 4.47 (1H; unresolved multiplet; CH in the γ -lactone) p.p.m. (Found: C, 62.8; H, 7.3; $\text{C}_{14}\text{H}_{20}\text{O}_5$ requires C, 62.7; H, 7.5%).

The extract (203mg), obtained from the wide faster moving band centred at R_F 0.42, was a mixture consisting mainly of approximately equal amounts of both the dimethyl esters of compounds (IVa) and (XIa), as indicated by t.l.c.

Transformation of the oxodicarboxylic acid (IIa) into the C_9 acid (III), the enaminolactam (VII) and an unidentified product. The disodium salt of the acid (3g) and yeast extract (0.3g) was dissolved in a mineral solution (1 litre) containing (g/litre): $(\text{NH}_4)_2\text{SO}_4$, 2; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; KCl, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01. The solution was adjusted with 0.1M-NaOH to pH7.2 and autoclaved. After inoculation with *C. equi*, it was incubated on a rotary shaker (150 rev./min) and the course of the degradation of compound (IIa) was monitored by t.l.c. After incubation for 70h, the result of t.l.c. showed that some compound (IIa) remained but several metabolites had been produced. After the 72h incubation, the mixture was centrifuged to remove cells. The cell-free supernatant was acidified with dilute HCl, saturated with NaCl and then thoroughly 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 an oily residue (280mg), which was chromatographed on silicic acid (12g). Elution with diethyl ether/chloroform (1:4, v/v) gave a crude sample (62mg) of (4R)-4-(2,3,4,6,6a β ,7,8,9,9a α ,9b β -decahydro-6a β -methyl-3-oxo-1H-cyclopenta[*f*]quinolin-7 β -yl)valeric acid (VII). After esterification with ethereal diazomethane, it was recrystallized from diethyl ether and then from methanol to give an analytical sample of the corresponding methyl ester, m.p. 189–192°C (Found: C, 71.6; H, 9.2; N, 4.3. Calc. for $\text{C}_{19}\text{H}_{29}\text{NO}_3$: C, 71.4; H, 9.2; N, 4.4%). Identity with an authentic sample was established by mixed m.p. and by comparison of the behaviour on t.l.c. and the i.r. spectrum.

Elution with diethyl ether/chloroform (3:7, v/v) gave a fraction (9mg), which contained a metabolite that gave a characteristic yellowish-brown-coloured spot on t.l.c. after spraying with concentrated H_2SO_4 and heating. This spot coincided in position and coloration with that of an authentic sample of ($\pm$)-5-methyl-4-oxo-octane-1,8-dioic acid. Although the fraction consisted of the relatively large amounts of other products besides this metabolite, it was esterified with ethereal diazomethane and subjected to g.l.c.–mass spectrometry without further purification. The presence of dimethyl ($\pm$)-5-methyl-4-oxo-octane-

1,8-dioate in the methylation mixture was confirmed by direct comparison with an authentic sample.

Further elution with diethyl ether/chloroform (from 2:3 to 1:1, v/v) gave an oily mass (87 mg) which on t.l.c. ran as a single spot. After esterification with ethereal diazomethane, the product moved as two spots on continuous t.l.c. in *n*-pentane/diethyl ether (1:2, v/v) as a developing solvent system. The methylation mixture was subjected to preparative continuous t.l.c. (three plates, 0.75 mm thick) in the same solvent system, but only a wide zone was obtained, which was then divided horizontally in three equal parts. The gummy extract (37 mg) obtained from the lowest part ran as one spot on the above continuous t.l.c. system, and showed i.r. max. at 3520 (OH), 1730 (ester) and 1708 (shoulder) (C=O) and n.m.r. absorptions at approx. 0.94 (3H; superimposable doublet; secondary Me), 1.01 (3H; singlet; tertiary Me), approx. 2.88 (1H; broad peak; OH), 3.63 (3H; singlet; CO₂Me), 3.70 (3H, singlet; CO₂Me) and approx. 4.07 (1H; multiplet; CH bonded to OH) p.p.m. Part (25 mg) of this ester was hydrolysed to give the corresponding free acid, which crystallized from diethyl ether as prisms, m.p. 158–160°C and i.r. max (in KBr) at 3430 (OH) and 1707 (C=O, and C=O of CO₂H) cm⁻¹ (Found: C, 63.6; H, 8.3; C₁₈H₂₈O₆ requires C, 63.5; H, 8.3%).

Degradation of the dioxocarboxylic acid (IIb) into the C₉ acid (III). As described above for the degradation of compound (IIa), the acid (1.5 g) was exposed to *C. equi* for 48 h to yield a mixture of products (65 mg). The main spot on t.l.c. coincided in position and coloration with that of an authentic sample of compound (III). After esterification with ethereal diazomethane, the methylation mixture was subjected to preparative t.l.c. (three plates, 0.75 mm thick). The oily extract (30 mg), obtained from the fastest-moving wide zone, was identical in all respects (t.l.c., and i.r. and n.m.r. spectra) with an authentic sample of dimethyl (±)-5-methyl-4-oxo-octane-1,8-dioate and also showed $[\alpha]_D^{25} + 14.9 \pm 0.6^\circ$ (c 1.027 in methanol).

Partial synthesis of the degradation product (VI)

Baeyer-Villiger oxidation of the dimethyl oxodicarboxylate (IXa). To a solution of the ester (1 g) in benzene (15 ml) a solution of *m*-chloroperbenzoic acid (1 g; see under 'Other materials' above) in benzene (20 ml) at approx. 20°C was added dropwise, with stirring. The mixture was kept overnight at the same temperature, and washed successively with 5% (w/v) Na₂SO₃, 5% (w/v) NaHCO₃ and water, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield an oily residue. This showed an i.r. band only at 1729 cm⁻¹ (ester and ϵ -lactone) in the region of 1700–1800 cm⁻¹, indicating that its main component was the expected ϵ -lactone (Xa), but it could not be induced to crystallize. The residue was then dissolved in 1 M-NaOH (20 ml) by heating at approx. 60°C for a

few minutes and kept at room temperature for 1 h. The mixture was acidified with dilute HCl, saturated with NaCl and then extracted with diethyl ether. Evaporation of the ether *in vacuo* left a residue, which crystallized from ether. Recrystallization from acetone/ether gave (4*R*)-4-[2 α -(2-carboxyethyl)-3 β -(2*S*)-tetrahydro-5-oxofur-2-yl]-2 β -methylcyclopent-1 β -yl]valeric acid (XIa) as needles (480 mg), m.p. 85.5–87°C, $[\alpha]_D^{25} + 31.5 \pm 0.7^\circ$ (c 1.046), i.r. max. at approx. 2600–3100 (OH of CO₂H), 1770 (γ -lactone) and 1712 (C=O of CO₂H) cm⁻¹ and n.m.r. absorptions at 0.91 (3H; singlet; 2 β -Me), 0.99 (3H; superimposable doublet; Me in the side chain), 4.37 (1H; unresolved multiplet; CH in the γ -lactone) and 10.15 (2H; broad peak; 2 CO₂H) p.p.m. (Found: C, 63.6; H, 8.4; C₁₈H₂₈O₆ requires C, 63.5; H, 8.3%).

The *methyl ester*, prepared with ethereal diazomethane, could not be crystallized, but it showed i.r. max. at 1771 (γ -lactone) and 1733 (ester) cm⁻¹ and n.m.r. absorptions at 0.90 (3H; singlet; 2 β -Me), 0.96 (3H; superimposable doublet; Me in the side chain), 3.67 (6H; singlet; 2 CO₂Me) and 4.33 (1H; unresolved multiplet; CH in the γ -lactone) p.p.m.

Oxidation of the hydroxytricarboxylic acid (V). The above γ -lactone (XIa) (800 mg) was converted by heating with 1 M-NaOH (2.35 ml) for a few minutes on a boiling-water bath into the corresponding trisodium salt of the hydroxytricarboxylic acid (V). The solution was diluted with water (100 ml) and chilled to 0°C. A yellowish solution of RuO₄ in carbon tetrachloride, which was prepared from RuO₂ (0.8 g) in carbon tetrachloride (100 ml) and sodium metaperiodate (6.4 g) in water (100 ml) by the method of Nakata (1963) and chilled to 0°C, was added to the chilled solution under cooling at approx. 0°C, with vigorous stirring. After the stirring for 1 h at the same temperature, the black aqueous layer was separated and concentrated *in vacuo* to approx. 50 ml without heating. The concentrate was acidified with dilute HCl, saturated with NaCl and then extracted with chloroform. The organic layer was washed with saturated NaCl, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to give an oily residue. It was dissolved in methanol (approx. 10 ml) and neutralized with 0.1 M-NaOH to the end point of phenolphthalein. The neutralized solution was evaporated *in vacuo* to yield a crystalline residue, which was recrystallized from methanol/acetone to give the *trisodium salt* of (4*R*)-4-[2 α -(2-carboxyethyl)-3 β -(3-carboxypropionyl)-2 β -methylcyclopent-1 β -yl]valeric acid (VI) (410 mg, m.p. >300°C). It was dissolved in water (approx. 5 ml) and the solution was acidified with dilute HCl and saturated with NaCl. Recovery in diethyl ether gave the corresponding *free acid*, which crystallized from ether as prisms (287 mg, m.p. 113–114°C). Further recrystallization from ether gave an analytical sample, m.p. 114–115°C, $[\alpha]_D^{25} + 47.0 \pm 0.8^\circ$ (c 1.053 in ethanol) and i.r. max. (in KBr) at approx. 2600–

3100 (OH of CO₂H) and 1706 (C=O and C=O of CO₂H) cm⁻¹ (Found: C, 60.7; H, 8.2; C₁₈H₂₈O₇ requires C, 60.7; H, 7.9%).

The trimethyl ester, prepared with ethereal diazomethane, could not be crystallized. Identity with a sample isolated from growing cultures was established by comparison of the behaviour on t.l.c., and the i.r. and n.m.r. spectra.

Partial synthesis of the degradation product (VIII)

Baeyer-Villiger oxidation of the acetoxycyclohexanecarboxylic acid (IXb). To a solution of the acid (2g) in benzene (30ml) a solution of *m*-chloroperbenzoic acid (2g; see under 'Other materials' above) in benzene (40ml) was added dropwise at approx. 20°C, with stirring. After the mixture had been kept overnight at the same temperature, it was evaporated *in vacuo* to yield an oily residue, which was chromatographed on silicic acid (120g). Elution with 20 ml portions of chloroform/methanol/acetic acid (99.5:5:1, by vol.) was performed and part of each fraction collected was examined by t.l.c. Fractions 40–42, which were similar in behaviour on t.l.c. to each other, were combined and evaporation of the solvents *in vacuo* gave 3-(1-β-acetoxy-3α-decahydro-8β-methyl-5-oxa-6-oxoazulen-4α-yl)propionic acid (Xb) as an oil (232mg). It showed i.r. max. at approx. 2600–3200 (OH of CO₂H), 1727 (C=O of acetate and ε-lactone) and 1697 (shoulder) (C=O of CO₂H) cm⁻¹. Kondo *et al.* (1969) give m.p. 151–153°C, [α]_D²⁵+41° (in chloroform) and i.r. max. (in Nujol) at 1770, 1745 and 1696cm⁻¹ for this compound.

The oily methyl ester, prepared with ethereal diazomethane, showed an i.r. band at 1730 (C=O of acetate, ester and ε-lactone) and n.m.r. absorptions at 1.01 (3H; singlet; 8β-Me), 2.04 (3H; singlet; OAc), 3.68 (3H; singlet; CO₂Me) and approx. 4.5 (2H; broad peak; 1α-H and 4β-H) p.p.m.

Fractions 43–69, after evaporation of the solvents *in vacuo*, were separately triturated with acetone/diethyl ether to obtain crystals. The combined crystals were further recrystallized from the same solvents to yield 3-(5β-acetoxy-2β-[(2*S*)-tetrahydro-5-oxofur-2-yl]-1β-methylcyclopent-1α-yl)propionic acid (XIc) (378 mg), m.p. 148.5–150°C [α]_D²⁵+42.7±0.9° (c 0.996), i.r. max. (in Nujol) at approx. 2700–3100 (OH of CO₂H), 1768 (γ-lactone), 1744 (C=O of acetate) and 1694 (C=O of CO₂H) cm⁻¹ and n.m.r. absorptions at 1.01 (3H; singlet; 1β-Me), 2.04 (3H; singlet; OAc), approx. 4.45 (1H; unresolved multiplet; CH in the γ-lactone), 4.82 (1H; unresolved multiplet; 5α-H) and 8.43 (1H; broad peak; CO₂H) p.p.m. (Found: C, 60.4; H, 7.4; C₁₅H₂₂O₆ requires C, 60.4; H, 7.4%).

The acetate (XIc) was hydrolysed with alkali as described above for compound (Xa) to give the corresponding free acid, which was soluble in water. It was then recovered by repeated extraction with ethyl acetate from the NaCl-saturated hydrolysate under

acidic conditions. Recrystallization from acetone/diethyl ether yielded 3-(2β-[(2*S*)-tetrahydro-5-oxofur-2-yl]-5β-hydroxy-1β-methylcyclopent-1α-yl)propionic acid (XIb), m.p. 146–147.5°C, [α]_D²⁴+20.6±0.7° (c 1.032 in acetone), i.r. max. (in Nujol) at 3380 (OH), approx. 2700 (OH of CO₂H), 1759 (γ-lactone) and 1725 (C=O of CO₂H) cm⁻¹ (Found: C, 61.0; H, 7.9; C₁₃H₂₀O₅ requires C, 60.9; H, 7.9%).

The methyl ester, prepared with ethereal diazomethane, could not be crystallized, but it showed i.r. max. at 3500 (OH), 1773 (γ-lactone) and 1731 (ester) cm⁻¹.

Oxidation of the hydroxy γ-lactone (XIb). The lactone (435mg) in acetone (20ml) was treated at approx. 0°C with Jones reagent (0.6ml), which was prepared by the method of Curtis *et al.* (1953). After the addition of a few drops of 5% (w/v) Na₂SO₃ to the mixture, it was concentrated *in vacuo* to approx. 4ml below 0°C, diluted with water, saturated with NaCl and then extracted with ethyl acetate. The extract was washed with saturated NaCl, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield an oily mass (389mg), which was chromatographed on silicic acid (16g). Elution with acetone/chloroform (1:9, v/v) gave 3-(2β-[(2*S*)-tetrahydro-5-oxofur-2-yl]-1β-methyl-5-oxocyclopent-1α-yl)propionic acid (VIII), which crystallized from acetone/ether in prisms, m.p. 76.5–77.5°C, [α]_D^{21.5}+91.6±1.3° (c 1.000 in acetone) and i.r. max. at approx. 2600–3100 (OH of CO₂H), 1775 (γ-lactone), 1744 (five-membered-ring C=O) and 1715 (shoulder) (C=O of CO₂H) cm⁻¹ (Found: C, 61.4; H, 7.1; C₁₃H₁₈O₅ requires C, 61.4; H, 7.1%).

The methyl ester, prepared with ethereal diazomethane, crystallized from acetone/ether as rods, m.p. 70–70.5°C and [α]_D^{21.5}+92.7±1.3° (c 1.046) (Found: C, 62.9; H, 7.5; C₁₄H₂₀O₅ requires C, 62.7; H, 7.5%). The synthetic sample was identical in all respects (t.l.c., m.p., mixed m.p., [α]_D, i.r. and n.m.r.) with a sample isolated as its methyl ester from growing cultures.

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