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On the Metabolism of Fusidic Acid in Man

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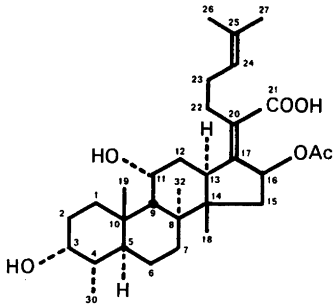
The metabolism of fusidic acid in man has been studied by investigating bile from patients receiving the antibiotic. The three main metabolites have been isolated and characterized and the structures of two of these have been established.

Fusidic acid is an antibiotic formed by fermentation of the fungus *Fusidium coccineum*.[1] Chemically it belongs to the group of tetracyclic triterpenes, and it has recently been shown [2–5] to possess the structure depicted below.



Although this antibiotic has been used extensively in the treatment of staphylococcal infections in man since 1962 little is known about its metabolic fate apart from the observation [6] that high concentrations are present in bile, whereas traces only can be found in the urine.

It has previously been demonstrated that after oral administration of ^{14}C-labeled fusidic acid (prepared by growing the fungus in the presence of 2-^{14}C-mevalolactone) to rats and rabbits the compound is to a very large extent excreted by the liver into the bile, whereas only very little of the radioactivity can be detected in the urine. A comparison between the radioactivity and antibiotic activity of bile indicated that the major part (more than 95 %) was excreted in an antibiotically inactive form.[7]

Through the courtesy of Dr. Marcus Ottsen we have now got the opportunity to investigate bile from patients treated with fusidic acid, and in the

following the result of this investigation will be presented. The investigation was performed with unlabeled fusidic acid. For this reason, and because only an unknown part of the bile production was collected the results are not quantitative.

EXPERIMENTAL

All m.p.'s are corrected. Optical rotations, unless otherwise stated, were measured in chloroform (c = 1), and UV-spectra in 96 % ethanol solution. The IR-spectra were obtained with a Perkin-Elmer Model 21 spectrophotometer with a sodium chloride prism. NMR-spectra were obtained with a Varian HA-100 (100 Mc) high resolution spectrometer, deuteriochloroform being used as solvent. The line positions are given in δ-values and with tetramethylsilane as internal reference. For characterization of the signals the following abbreviations are used: s (singlet), d (doublet), t (triplet), m (multiplet), b (broad, ill-defined signal). The microanalyses were performed by Mr. G. Cornali and Mr. W. Egger. For thin-layer chromatography Silica Gel HF_{254} (Merck, A.G., Darmstadt, West Germany) and glass plates (0.4 × 10 × 20 cm) were used. A saturated solution of $SbCl_3$ in chloroform was used as spray reagent, unless otherwise stated.

Fucidin * (3 × 1 g daily for three consecutive days) was given orally to a patient on whom cholecystectomy had been performed two days before the medication started. During this period part of the bile production was collected through a drain-pipe installed in connection with the operation. Bile taken from the same patient before the fucidin treatment was used as control.

After filtration through Dicalite, the pooled bile (1.3 l) was adjusted to pH 2 with dilute hydrochloric acid and extracted with ethyl acetate (500 + 250 + 250 ml). The combined extracts were washed with water, dried, and evaporated *in vacuo*. The dark residue (5.4 g) was subjected to paper- and thin-layer chromatography together with a corresponding extract of the control bile.

Bioautography of paper chromatograms on agar plates inoculated with *Corynebacterium xerosis* (Fig. 1) revealed that in addition to small amounts of unchanged fusidic

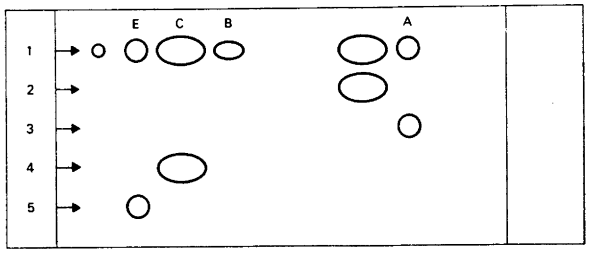


Fig. 1. Paper chromatography of fusidic acid metabolites. Solvent system: Benzene-methanol-water (2:1:1). Whatman No. 1. Temp. 34°. Test organism: *Corynebacterium xerosis*. 1. Crude extract of bile; 2. Fusidic acid; 3. 3-Ketoderivative of fusidic acid; 4. Metabolite C; 5. Metabolite E.

acid at least four antibiotically active compounds were present. None of these could be detected in the control, and must therefore be assumed to be metabolites of fusidic acid.

Thin-layer chromatography in two different solvent systems (Fig. 2) revealed the presence of seven well-defined spots (in addition to a spot corresponding to fusidic acid) which could not be detected in the control. These were abitrarily designated A, B, C,

* Fucidin is the trade name for the sodium salt of fusidic acid. It was administered in the form of capsules each containing 250 mg.

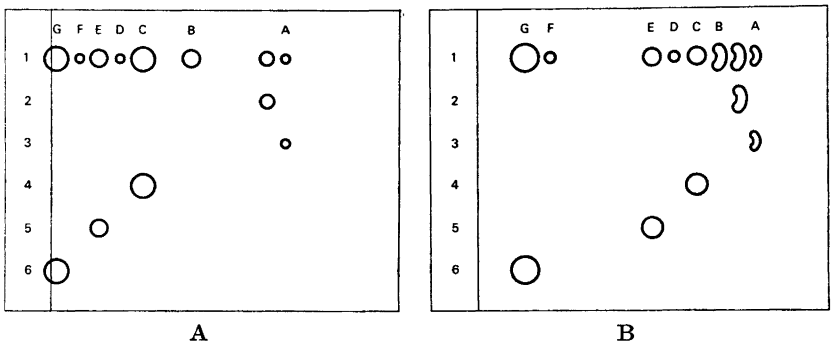


Fig. 2. Thin-layer chromatography of fusidic acid metabolites. Adsorbent: Silica gel HF_{254} (Merck). Solvent systems: A. Cyclohexane-chloroform-methanol-acetic acid(10:80:2.5:10). B. Chloroform-acetic acid-methanol (8:1:1). Spray reagent: Saturated solution of $SbCl_3$ in chloroform. 1. Crude extract of bile; 2. Fusidic acid; 3. 3-Ketoderivative of fusidic acid; 4. Metabolite C; 5. Metabolite E; 6. Metabolite G.

D, E, F, and G according to increasing polarity. Judging from the intensity of the colours the compounds G, C, and E were predominating.*

The dark residue (5.4 g) was partitioned between pentane (100 ml), methanol (80 ml), and water (20 ml). Evaporation of the pentane phase gave a yellow semicrystalline residue (1.6 g) which according to thin-layer chromatography did not contain significant amounts of any of the metabolites. Two recrystallizations from methanol afforded 1.25 g of colourless crystals, m.p. 147—148°, which could be identified as cholesterol. (Identical IR-spectra; no mixed melting point depression).

The methanolic phase was evaporated *in vacuo* and the moist residue (4.0 g) dissolved in dil. aq. sodium bicarbonate (50 ml). The resulting solution was adjusted to pH 6.0 and extracted with ethyl acetate (2 × 25 ml). The combined extracts were washed with water, dried, and evaporated *in vacuo* to leave 600 mg of a yellow-brown residue, which according to thin-layer chromatography contained fusidic acid and the metabolites A, B, C, D, and E, whereas traces only of the compounds F and G were present. This mixture is in the following called mixture I.

The aqueous phase was now adjusted to pH 4.0 and again extracted with ethyl acetate (2 × 25 ml). The combined extracts were washed with water, dried, and evaporated to leave 2.1 g of a very dark residue which according to thin-layer chromatography consisted mainly of the metabolites F and G in addition to traces of C and E. This mixture is in the following called mixture II.

Finally, the aqueous phase was acidified to pH 1.5 and again extracted with ethyl acetate. After washing and drying the extract was evaporated to leave 500 mg of a dark residue containing minor amounts of metabolite G in addition to other, even more polar compounds. This residue was discarded.

Further separation of mixture I and II was achieved by "dry-column" chromatography.[8] As adsorbent silica gel, 100 mesh (Mallinckrodt) was used, the ratio of sample and adsorbent being 1:100.

Isolation of metabolites C and E. Mixture I (600 mg) was chromatographed on a dry silica gel column (27 × 270 mm). Solvent system: Chloroform, acetic acid, cyclohexane, methanol (80:10:10:2.5). The column was divided into sections which were extracted with methanol. The fractions, which according to TLC contained metabolite C contaminated with small amounts of metabolite B were combined, decolorized with charcoal (50 mg), and evaporated to dryness. The oily residue (180 mg) crystallized from ethyl acetate

* Extract of bile from four other patients given fucidin were examined in the same manner. Although quantitative differences were observed they all showed the same general pattern.

to yield 110 mg of colourless crystals, m.p. 210—211°. Recrystallization from acetone-pentane raised the m.p. to 211.5—212°. UV: λ_{max} 220 mμ (ε 19 400). IR (KBr): 3400 (OH), 2600 (COOH), 1730 and 1695 (CO), 1635 (C=C), 1265 and 1250 cm^{-1} (acetate). (Found: C 67.72; H 8.50. Calc. for $C_{31}H_{46}O_8$: C 68.10; H 8.48).

The fractions, which according to TLC contained metabolite E contaminated with small amounts of metabolite D, were combined, decolorized with charcoal, and evaporated to yield 120 mg of amorphous, purple-coloured material. This was rechromatographed, and in this way 80 mg of an amorphous, but homogeneous product (metabolite E) was obtained. UV: λ_{max} 222 mμ (ε 8100). IR (KBr): 3400 (OH), 1715 and 1735 (CO), 1260 cm^{-1} (acetate).

Methyl ester of metabolite C. Metabolite C (230 mg) in ether (5 ml) was treated with ethereal diazomethane until the yellow colour persisted. The almost clear solution was filtered and evaporated to yield an oil which was chromatographed on a dry silica gel column (14 × 140 mm). Solvent system: Ethyl acetate, cyclohexane (70:30). The column was divided into sections which were eluted with ethyl acetate. The fractions, which according to TLC were homogeneous were combined and evaporated to yield 190 mg of a colourless amorphous product. $[\alpha]_D^{20}$ −10.6°. UV: λ_{max} 222 mμ (ε 19 300). IR (CCl_4): 3620 and 3420 (OH), 1735 and 1720 (CO), 1650 (C=C), 1255 and 1235 cm^{-1} (acetate). NMR-spectrum, see Fig. 3 and Table 1. Mass-spectrum: peak at *m/e* = 496 (M−60−18), M^+ = 574 absent. (Found: C 68.45; H 8.78. Calc. for $C_{33}H_{50}O_8$: C 68.96; H 8.77).

Methyl ester of metabolite E. To a solution of metabolite E (70 mg) in ether (2 ml) ethereal diazomethane was added until the yellow colour persisted. Evaporation gave an oil which was chromatographed on a dry silica gel column (14 × 140 mm). Solvent system: Ethyl-acetate, cyclohexane (85:15). The fractions, which according to TLC were homogeneous, were combined and evaporated to yield 45 mg of colourless, amorphous material. UV: λ_{max} 222 mμ (ε 8200). IR (KBr): 3460 (OH), 1720 and 1735 (shoulder) (CO), 1255 and 1240 (acetate). NMR-spectrum, see Table 1. (Found: C 66.85; H 9.08. Calc. for $C_{32}H_{50}O_9$: C 66.41; H 8.71). Mass-spectrum: peaks at *m/e* = 474, 449, 442, 427, 416, 415, 399, 381, 358, 314, 298, 277, 275, 249, and 233.

Ozonolysis of metabolite C methyl ester. Ozonized oxygen was bubbled through a solution of metabolite C methyl ester (30 mg) in dry CH_2Cl_2 (3 ml) containing dry pyridine (30 μl) at −70° for 10 min (about 1.6 mole equivalents of ozone was used). Zn dust (40 mg) and acetic acid (60 μl) were added, and after shaking for 10 min at 30° the precipitate was filtered off and washed with CH_2Cl_2. The combined filtrate and washings were

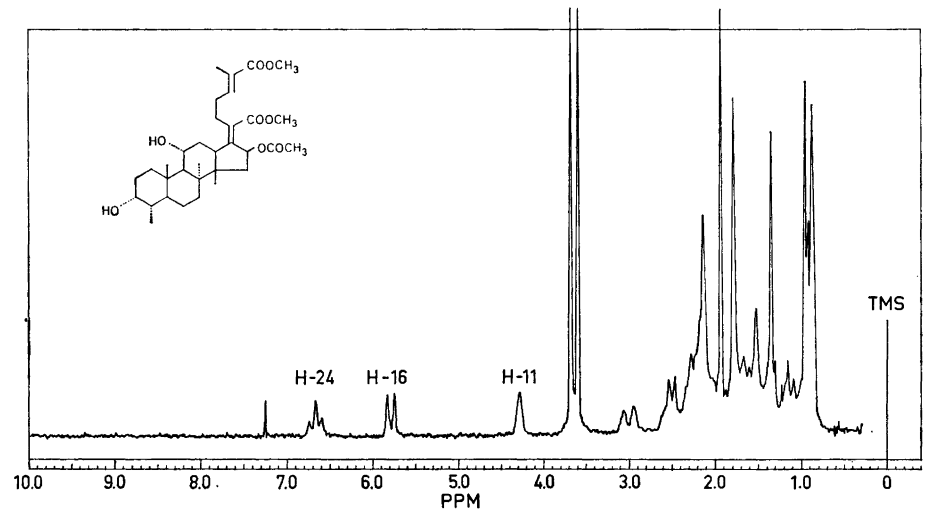


Fig. 3. 100 Mc NMR-spectrum of metabolite C dimethyl ester.

Table 1. NMR Values (in δ) of the methyl esters of fusidic acid and the metabolites C and E.

Fusidic acid methyl ester* (*1b*)	Metabolite C dimethyl ester (*3b*)	Metabolite E methyl ester	Assignment
0.917 (s)	0.88 (s)	0.90 (s)	CH_3-18 + CH_3-19
0.984 (s)	0.94 (s)	0.96 (s)	
0.933 (d) J = 7 cps	0.87 (d) J = 7 cps	0.89 (d) J = 7 cps	CH_3-30
1.392 (s)	1.34 (s)	1.35 (s)	CH_3—32
1.601 (d) $J \cong$ 1 cps	1.82 (d) J = 1.3 cps	1.24 (s)	CH_3—26
1.685 (d) $J \cong$ 1 cps	—	1.35 (s)	CH_3—27
1.982 (s)	1.94 (s)	1.94 (s)	CH_3—C(=O)—O—
3.10 (m)	3.05 (m)	3.02 (m)	CH-13
3.630 (s)	3.60 (s)	3.62 (s)	CH_3—O—C(21)=O
—	3.68 (s)	—	CH_3—O—C(27)=O
3.75 (m)	3.68 (m)	3.70 (m)**	CH-3
4.360 (m)	4.30 (m)	4.31 (m)	CH-11
5.860 (d) J = 8.5 cps	5.80 (d) J = 8.5 cps	5.81 (d) J = 8.5 cps	CH-16
5.124 (m)	6.68 (m)	—	CH-24

* The values for fusidic acid methyl ester are taken from Ref. 11.

** This signal represents two protons.

washed with water, dried, and evaporated *in vacuo*. The residue was subjected to TLC in two solvent systems (ethyl acetate-cyclohexane, 7:3 and CH_2Cl_2-methanol, 9:1). In both cases the R_F-value of the main spot corresponded exactly to that of the aldehyde *6* obtained on ozonolysis of fusidic acid methyl ester in the same manner as described above.

Isolation of metabolite G. Mixture II (2.1 g) was dissolved in boiling ethyl acetate (21 ml). Upon cooling to room temp. a dark tar deposited. Decantation followed by evaporation *in vacuo* gave 1.7 g of an orange amorphous residue, which was chromatographed on a dry silica gel column (27 × 270 mm). Solvent system: Chloroform, acetic acid, methanol (80:10:10). The column was divided into sections which were eluted with methanol. The fractions, which according to TLC contained metabolite G contaminated with metabolite F were combined and evaporated *in vacuo*. The dark residue was dissolved in ethyl acetate, treated with decolorizing carbon, filtered, and the pink-coloured filtrate evaporated *in vacuo*. The residue (1.0 g) was dissolved in hot acetonitrile (10 ml). The resulting solution was slowly cooled to room temp. whereby a purple oil separated. The supernatant was filtered and crystallization induced by scratching. After standing 320 mg of crystals, m.p. 156—160° (decomp.) was collected. Repeated extractions of the purple oil with hot acetonitrile gave a further 130 mg of crystals, m.p. 156—160° (decomp.). The combined crystalline fractions were recrystallized from acetonitrile to yield 300 mg of pure metabolite G, m.p. 158—162°. $[\alpha]_D^{20}$ −32.3° (c = 2, ethanol). UV: λ_{max} 230 mμ (ε 9100). IR (KBr): 3420 (OH), 2600 (COOH), 1730 (CO), 1650 (C=C), 1265 cm^{-1} (acetate). Electrometric titration in 50 % (v/v) ethanol gave a pK-value of 4.40 (corresponding to a pK of approximately 3.4 in water) and an equiv. wt. of 728. (Found: C 61.57; H 8.27; H_2O (Carl Fischer) 3.5 %. Calc. for $C_{37}H_{56}O_{12}$, 1.5 H_2O: C 61.73; H 8.26; H_2O 3.7 %; M = 719.84).

Enzymatic cleavage of metabolite G. To a solution of metabolite G (230 mg) in phosphate buffer (100 ml, pH 6; I = 0.05) β-glucuronidase bacterial powder (Sigma No. 105—10)

(500 mg) and two drops of chloroform were added, whereafter the solution was kept at 37° for 4 h. The resulting milky mixture was adjusted to pH 3.0 with dil. hydrochloric acid and extracted with ethyl acetate (2 × 20 ml). The combined extracts were washed with water, dried, and evaporated *in vacuo*. The residue (180 mg) was dissolved in ether (10 ml), the resulting solution filtered, and the solvent removed *in vacuo*. The residue crystallized from benzene to yield 175 mg of fusidic acid benzene solvate, identical in every respect (IR, TLC, antibacterial activity) with an authentic sample.

The aqueous phase was concentrated *in vacuo* to 20 ml and subjected to TLC (solvent system: acetone-acetic acid-methanol, 6:2:2; spray reagent: naphthoresorcinol*) which revealed the presence of glucuronic acid and glucuronolactone.

RESULTS AND DISCUSSION

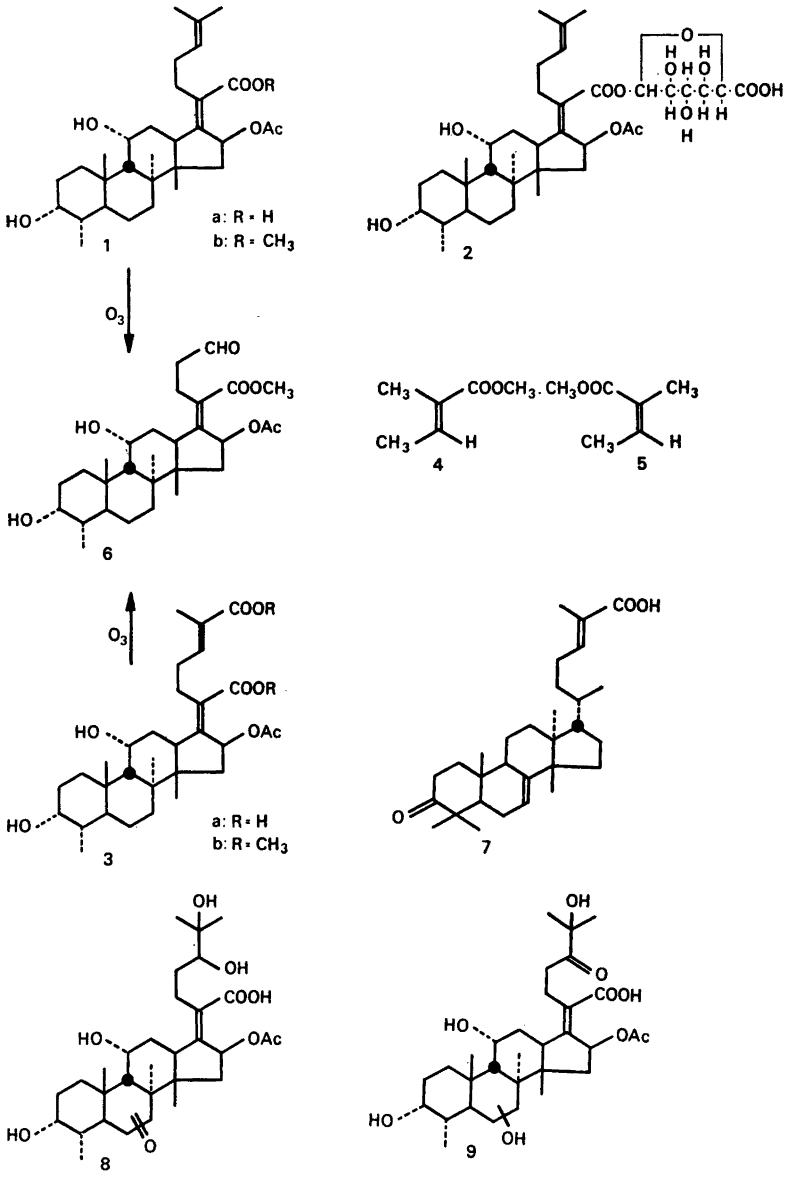
Thin-layer and paper chromatography of an ethyl acetate extract of bile from patients receiving fusidic acid shows, in accordance with previous evidence from animal experiments [7] that only a small amount of the antibiotic is excreted unchanged. Of the seven metabolites, which could be detected by thin-layer chromatography of the extract, the three most abundant (G, C, and E) have been isolated and characterized. The remaining metabolites A, B, D, and F are present in small amounts only, and have not been obtained in a pure state.

A rough estimate of the amounts of unchanged fusidic acid and the metabolites C, E, and G in the extract was obtained by paper and thin-layer chromatography of the crude extract together with known amounts of the pure compounds. The paper chromatograms were subjected to bioautography on agar plates inoculated with *Corynebacterium xerosis*, and evaluated by measuring the zones of inhibition. The thin-layer chromatograms were sprayed with chloroformic $SbCl_3$ and the spots evaluated visually. According to these admittedly rough determinations the dry residue of the ethyl acetate extract contained about 15 % of metabolite G, 10 % of metabolite C, and 2—4 % of metabolite E, whereas the content of fusidic acid amounted to about 0.3 %.

Metabolite G, which was obtained in a crystalline state, is an acid considerably stronger ($pK = 3.4$) than fusidic acid ($pK = 5.35$). Under the influence of the enzyme β-glucuronidase the compound is cleaved to fusidic acid (isolated in high yield as its benzene solvate) and glucuronic acid (identified by thin-layer chromatography). The titration curve indicates that only one carboxylic group is present (equiv. wt. *ca.* 730), and the compound can therefore be assigned structure *2*.

Metabolite C, which also was obtained in a crystalline state, is a dicarboxylic acid, the elementary analysis of which corresponds to the formula $C_{31}H_{46}O_8$. This formula is consistent with the mass-spectrum of the corresponding dimethyl ester which shows a peak at *m/e* 496 (M—60—18; loss of acetic acid and water) whereas no molecular ion peak is present. The UV-spectrum of the dimethyl ester shows an absorption maximum at the same wavelength (222 mμ) as 24,25-dihydrofusidic acid methyl ester, but the high extinction coefficient ($\varepsilon = 19\,300$) indicates that a new chromophore, most likely

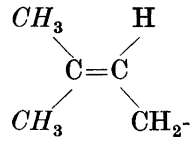
* This reagent was a 1:1 mixture of 0.2 % ethanolic naphthoresorcinol and 20 % aqueous trichloroacetic acid.



a tri- or tetrasubstituted α,β-unsaturated ester has been introduced into the molecule.

A 100 Mc NMR-spectrum of the dimethyl ester of this metabolite is shown in Fig. 3. This spectrum in connection with the elementary analysis, the mass-spectrum, and the UV-data strongly suggest that metabolite C has the structure depicted in formula *3 a:*

a. The singlets at $\delta = 3.60$ and 3.68 indicate the presence of two carbomethoxyl groups.
b. The doublets centered at $\delta = 1.601$ and $\delta = 1.685$ in the spectrum of *1 b* corresponding to the grouping



(*cf.* Table 1) are absent and replaced by a doublet at $\delta = 1.82$ ($J = 1.3$ cps). The chemical shift and splitting of this signal is similar to that of the α-methyl group in the spectrum of methyl tiglate (*4*).[9]
c. The signal at $\delta = 5.12$ corresponding to the C-24 proton in *1 b* is replaced by a multiplet centered at $\delta = 6.68$. The chemical shift of this signal corresponds well to that of the signal due to the vinylic proton in *4* ($\delta = 6.73$), whereas in the spectrum of methyl anglate (*5*), this signal appears at $\delta = 5.92$.[9] Therefore, a *cis*-relationship between the C-24 proton and the terminal carboxylic group in metabolite C is highly probable, a view which also is supported by the UV-spectrum.[10] Spin-spin decoupling reveals a long range coupling between the C-24 proton and the terminal methyl group: Irradiation of the former causes the signal of the latter to collapse into a singlet, whereas irradiation of the latter converts the signal of the former into a triplet.
d. The chemical shifts and splittings of the remaining signals in the NMR-spectrum of metabolite C methyl ester correspond closely to similar signals in the spectrum of *1 b* (*cf.* Table 1), and it is therefore unlikely that transformations in other parts of the molecule have taken place. To verify this last point, the dimethyl ester was subjected to ozonolysis. The fact that this process afforded the same aldehyde (*6*) as was obtained on ozonolysis of fusidic acid methyl ester (*1 b*) indicates that metabolite C is derived from fusidic acid simply by oxidation of one of the terminal methyl groups of the latter (most likely CH_3-27) to a carboxylic group.
A side-chain similar to that of *3 a* is present in mastica-dienonic acid (*7*), isolated by Barton and Seone [10] from resin gum-mastic.

Metabolite E was obtained in an amorphous but, according to TLC, homogeneous state. The elementary analysis of the corresponding methyl ester, obtained on esterification with diazomethane, suggested the formula $C_{32}H_{48-50}O_9$. In the mass-spectrum of the ester no molecular-ion peak could be detected, the highest peak being at *m/e* 474. The UV-spectrum of the methyl ester, (λ_{max} 222 mμ (ε 8200)) is very similar to that of 24,25-dihydrofusidic acid methyl ester,[2] and suggest that the 24,25-double bond in fusidic acid has been attacked, a view which is confirmed by the NMR-spectrum (*cf.* Table 1): The signal at $\delta = 5.12$ due to the vinylic C-24 proton in *1 b* is absent, and the doublets at $\delta = 1.601$ and 1.685 in the spectrum of fusidic acid methyl ester, due to the vinylic methyl groups at C-26 and C-27 are replaced by singlets at $\delta = 1.24$ and 1.35. The chemical shift of these signals are in accord-

ance with the presence of the grouping $-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{C}}-OH$.[12] It will be seen from Table 1 that all of the methyl groups are intact, and that signals of similar chemical shifts and splittings as the signals due to the protons at C-3, C-11, C-13, and C-16 as well as to the carbomethoxyl group at C-21 and the acetoxyl group at C-16 in the spectrum of fusidic acid methyl ester are present in the spectrum of metabolite E methyl ester. Therefore, it is unlikely that transformations involving these carbon atoms or their neighbours have taken place. The fact that the signal at $\delta = 3.70$ represents two protons indicates the presence of an extra $-O-\overset{\overset{|}{}}{\underset{\underset{|}{}}{C}}-H$ group. A low-intensity band at 283 mμ (ε 40) in the UV-spectrum indicates the presence of an unconjugated keto group.

Although the exact structure of metabolite E cannot be deduced on the basis of the available data they strongly suggest that the 24,25-double bond in fusidic acid has been hydroxylated, and that one additional oxygen function, probably a secondary hydroxyl group, has been introduced in the molecule, most likely in ring A or ring B as illustrated in the tentative formulae *8* and *9*.

A determination of the inhibitory activities of the metabolites C, E, and G against a number of gram-positive and gram-negative bacteriae has revealed that all of them are much weaker antibiotics than fusidic acid. Details of the microbiological work will be published elsewhere.

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