Microbial Modification of Diosgenin and Hecogenin

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On incubation with an unidentified bacterium diosgenin [(25*R*)-spirost-5-en-3β-ol] (1) was converted into (25*R*)-9-oxode-AB-spirostane-8α-propionic acid (3). Incubation with *Nocardia globerula* produced the same oxo-acid (3) and also a small amount of its 17α-hydroxy-derivative. Surprisingly, hecogenin [(25*R*)-3β-hydroxy-5α-spirostan-12-one] (2) was also converted into the oxo-acid (3) by the bacterium: thus in the course of metabolism a 12-oxo-group had been replaced by a methylene group. A metabolic pathway is suggested for that replacement, based on the isolation of the minor metabolite (25*R*)-12α-hydroxyspirost-4-en-3-one.

SEVERAL metabolites are known to be produced by the action of micro-organisms on diosgenin [(25*R*)-spirost-5-en-3β-ol] (1) but no metabolites of hecogenin [(25*R*)-3β-hydroxy-5α-spirostan-12-one] (2) have been reported. Oxygenation of diosgenin has been observed at one or more of the positions 7β, 11α, 11β, and 12β,[1,2] the 5-en-3β-ol system has been converted into 4-en-3-one and 1,4-dien-3-one systems,[3] and rings E and F have been removed.[3,4] We have found that an unidentified bacterium (ACC 3660) removed ring A and modified ring B to give (25*R*)-9-oxode-AB-spirostane-8α-propionic acid (3), an oxo-acid of the same type as that (4) produced microbiologically from androst-4-ene-3,17-dione [5,6] and that from progesterone.[7]

Bacterium (ACC 3660) † was isolated by culturing local soil on an agar plate containing diosgenin. Preliminary experiments with test-tube cultures showed that diosgenin was completely transformed by the bacterium to a more polar major product, $C_{21}H_{32}O_5$, isolated in 55% yield from larger fermentations, and shown to be the oxo-acid (3). N.m.r. and mass spectra [8] show that rings E and F and the 13-methyl group are intact. The methyl ester formed a 2,4-dinitrophenylhydrazone. Reduction of the oxo-acid with sodium borohydride gave the lactone (5) directly. The n.m.r. spectrum showed a signal centred at τ 6·12 (1H, six-line pattern, *J* 5, 10, and 10 Hz), which was not present in the spectrum of the oxo-acid, and which indicates that the C-9 proton is axial. A similar conclusion was reached by Wang and Sih [9] for the signal at τ 6·10 from the similarly placed proton in the lactone derived from the dioxo-acid (4).

When the bacterium was grown in a buffered solution containing diosgenin as the sole carbon source, conversion was slow and only the oxo-acid (3) accumulated. In a nutrient medium which provided conditions convenient for production of the oxo-acid in quantity, the neutral ketones (25*R*)-spirost-4-en-3-one and (25*R*)-spirosta-1,4-dien-3-one also accumulated. These are the expected intermediates for a metabolic pathway from diosgenin (1) to the oxo-acid (3) similar to that from androst-4-ene-3,17-dione to the dioxo-acid (4), which involves 1,2-dehydrogenation and 9α-hydroxylation.[5,6] Addition of 8-hydroxyquinoline or αα′-bipyridyl to the diosgenin fermentation inhibited the formation of the oxo-acid (3) and caused (25*R*)-spirost-4-en-3-one and (25*R*)-spirosta-1,4-dien-3-one to accumulate. This provided support for the similarity of the pathways, for these chelating agents inhibit the introduction into a steroid 1,4-dien-3-one of the 9α-hydroxy-group which is necessary for the ring B opening process.[3]

A screening programme revealed that the organism *Nocardia globerula* also converted diosgenin into the oxo-acid (3). A minor acidic product, $C_{21}H_{32}O_6$, has been assigned the structure (6) [the 17α-hydroxy-derivative of (3)] on n.m.r. and mass spectral evidence. In particular, the signal due to the 16-proton of (6) was a triplet, whereas in (3) it was a broad multiplet. Further, the characteristic ion *m/e* 139 ($C_9H_{15}O$) (7) from rings E and F of diosgenin [8] was not present in the mass spectrum of (6). An ion *m/e* 153 ($C_9H_{13}O_2$) suggested that hydroxylation had occurred in the region of rings D, E, and F.

Hecogenin (2) was incubated with the bacterium

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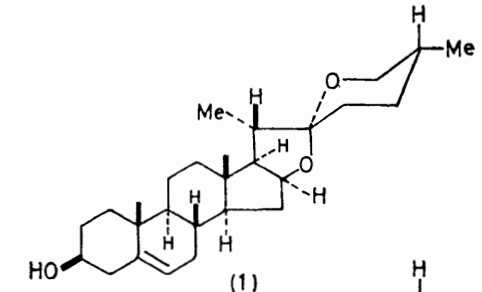
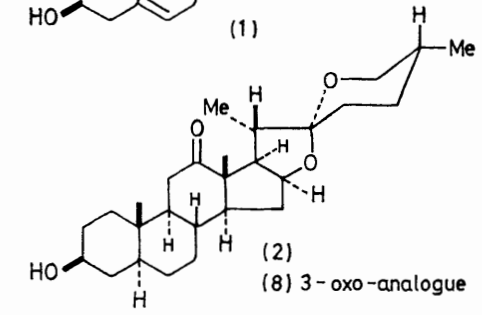
(ACC 3600) in an attempt to obtain the 9,12-dioxo-analogue of the 9-oxo-acid (3). Surprisingly, the oxo-acid (3) was obtained, and also in a longer fermentation

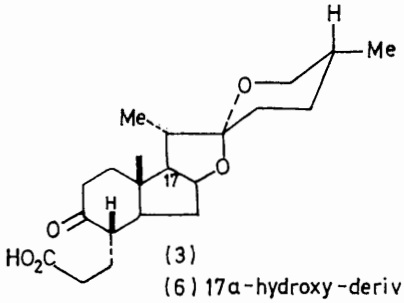


(1)



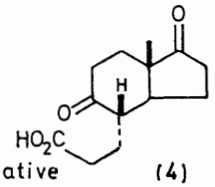
(2)

(8) 3-oxo-analogue

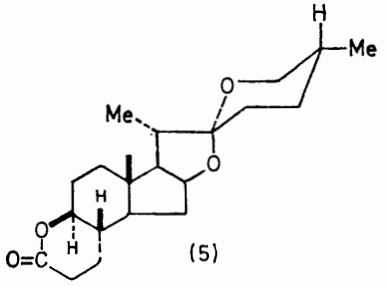


(3)

(6) 17α-hydroxy-derivative



(4)



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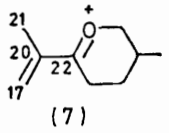

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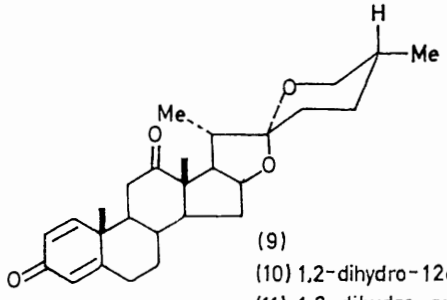

(9)

(10) 1,2-dihydro-12α-hydroxy-analogue

(11) 1,2-dihydro-analogue

the related δ-lactone (5). The hecogenin had been purified *via* its acetate and contained no diosgenin. The low m.p. of the lactone (5) is attributed to the presence of a small amount of the 9β-epimer, but not enough to alter significantly the mass or n.m.r. spectra. Wang and Sih have reported a similar mixture of 9-epimeric lactones formed by the action of *Nocardia restrictus* on 17β-hydroxyandrost-4-en-3-one.[9]

A short fermentation of hecogenin provided one major and two minor neutral products. The major product was (25*R*)-5α-spirostane-3,12-dione (8) (hecogenone).[10] The less polar of the minor products, *m/e* 424 ($C_{27}H_{36}O_4$), was (25*R*)-spirosta-1,4-diene-3,12-dione (9), the identity of which was confirmed by producing it from the 3,12-dione (8) by oxidation with dichlorodicyanoquinone.[11] The more polar minor product, *m/e* 428 ($C_{27}H_{40}O_4$), had a u.v. spectrum characteristic of a Δ^4-3-one and was assigned the structure (25*R*)-12α-hydroxyspirost-4-en-3-one (10). An n.m.r. signal at τ 4·27 was attributed to the olefinic proton at C-4, and a triplet at τ 6·22, not present in the spectrum of (25*R*)-spirost-4-en-3-one, had position and pattern characteristic of a 12β-proton.[12] Oxidation of the metabolite (10) with chromium trioxide in acetic acid, under conditions previously used for oxidation of a steroidal 12α-hydroxy-group,[13] gave (25*R*)-spirost-4-ene-3,12-dione (11),[14] confirming the presence and position of the hydroxy-group in (10).

The isolation of the 12α-hydroxy-metabolite (10) suggests that removal of the 12-oxo-group of hecogenin may proceed by dehydration and then reduction of a 12α-hydroxy-derivative of the oxo-acid (3). Removal of a 12α-hydroxy-group by a similar sequence has been postulated in the transformation of cholic acid by *Corynebacterium simplex*.[15]

EXPERIMENTAL

Light petroleum had b.p. 60—80° unless stated otherwise. N.m.r. spectra were determined for solutions in $CDCl_3$. T.l.c. was carried out using silica gel GF_{254} (Merck) plates developed in solvent systems (1) ethyl acetate, (2) ethyl acetate–formic acid (98%) (99 : 1), (3) ethyl acetate–benzene–formic acid (98%) (33 : 66 : 1), or (4) ethyl acetate–toluene–formic acid (98%) (33 : 66 : 1). Products were detected by spraying with anisaldehyde–sulphuric acid (*d* 1·84)–ethanol (0·5 : 2 : 97·5 v/v) and then heating.

Organisms able to grow in the presence of diosgenin were isolated from local soil samples by culturing on an agar plate containing medium (*a*), composed of potassium dihydrogen phosphate (0·2%), ammonium sulphate (0·1%), magnesium sulphate heptahydrate (0·04%), minor elements

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concentrate (0·1%),[16] agar (1·5%), and diosgenin (0·05%). Diosgenin (10 mg ml^{-1}) was added as a solution in ethanol to sterile molten agar immediately before preparation of the plates. Particular organisms were isolated from the resulting microbial growth by culturing on nutrient agar (*b*), consisting of Lab-Lemco beef extract (Oxoid Ltd.) (0·1%), yeast extract (Oxoid L20) (0·2%), peptone (Oxoid L37) (0·5%), sodium chloride (0·5%), and agar (1·5%), adjusted to pH 7·4.

Screening for metabolites derived from the added substrate was carried out using submerged shaken cultures of the organisms isolated or organisms selected from the I.C.I. collection, in either test tube or conical flask. Typically the substrate (200—400 μg) in ethanol (0·1 ml) was added to a culture of the organism grown at 25° in a suitable medium (3 ml) in test tubes (3 × 5/8 in) on a rotary shaker for 48 h. The cultures plus substrate were incubated for 3—5 days. Cultures were then adjusted to pH 2 and extracted with ethyl acetate. The extracts were compared by t.l.c. with control extracts from the organism and substrate incubated separately in the nutrient medium. A suitable medium (*c*) consisted of Lab-Lemco beef extract (0·1%), yeast extract (0·7%), peptone (0·5%), and sodium chloride (0·5%), adjusted to pH 7·4. Subsequent larger scale conversions were carried out in conical flasks (500 ml) containing medium (200 ml), or in stirred and aerated fermenters containing nutrient medium (5 or 80 l) at 25°. Metabolites were isolated by extracting culture filtrates and mycelia with ethyl acetate at pH 2. The organic extracts were separated conventionally into acidic, phenolic, and neutral fractions.

Metabolism of Diosgenin by Unidentified Bacterium ACC 3660.—(a) Thirty conical flasks (500 ml) containing nutrient broth (*c*) (200 ml) were inoculated with a 24 h culture of the organism (1 ml) and then incubated at 25° on a rotary shaker. After 24 h, diosgenin (0·2 g) in ethanol (10 ml) was added to each flask and 120 h later the fermentation was stopped. The acid fraction (4·3 g) contained one major product, R_F 0·45 [system (3)] and several trace components. Chromatography on silica (50 g) and elution with chloroform (1500 ml) gave a mixture (67 mg) which was discarded. Elution with chloroform–methanol (99 : 1) gave (25R)-9-*oxode*-AB-*spirostane*-8α-*propionic acid* (3) (3·1 g) as needles, m.p. 110—111° (from light petroleum) (Found: C, 69·2; H, 8·6. $C_{21}H_{32}O_5$ requires C, 69·2; H, 8·85%), $[\alpha]_D^{21}$ −61·8° (*c* 1·0 in MeOH), *m/e* 364 (M^+, $C_{21}H_{32}O_5$) and 139 [$C_9H_{15}O$ (7)],[8] τ 5·4—5·7 (m, 16-H), 6·5—6·9 (m, 26-H), 7·2—8·7 (19H, m), 8·92 (s, 18-H_3), 9·04 (d, 27-H_3), and 9·22 (d, 21-H_3). Treatment with diazomethane gave the methyl ester as a gum, R_F 0·73 [system (2)], which gave a 2,4-*dinitrophenylhydrazone*, m.p. 166—167° (from methanol) (Found: C, 60·3; H, 6·8; N, 10·0. $C_{28}H_{38}N_4O_8$ requires C, 60·2; H, 6·9; N, 10·0%).

The neutral fraction (0·9 g) was chromatographed on neutral alumina (10 g). Elution with light petroleum (b.p. 40—60°) at first gave fatty material which was discarded. Continued elution gave solid (32 mg) as needles, m.p. 177° (from light petroleum), $\lambda_{max.}$ (MeOH) 241 nm (ε 16,000), *m/e* 412 ($C_{27}H_{40}O_3$) and 410 ($C_{27}H_{38}O_3$), shown by t.l.c. to be a mixture of (25*R*)-spirost-4-en-3-one, R_F 0·65, and (25*R*)-spirosta-1,4-dien-3-one, R_F 0·60 [system (3)].

(b) *Effect of* 8-*hydroxyquinoline and* αα′-*bipyridyl*. Two flasks containing the growing organism were set up as in (a). To one flask was added diosgenin (100 mg) and 8-hydroxyquinoline (29 mg) in ethanol (10 ml) and to the other (the control) was added 8-hydroxyquinoline (29 mg) in ethanol (10 ml). Samples (2 ml) taken after 31, 97, 121, 144, and 288 h during the incubation were extracted and the extracts examined by t.l.c. [system (3)]. (25*R*)-Spirost-4-en-3-one, R_F 0·65, and (25*R*)-spirosta-1,4-dien-3-one, R_F 0·60, were the major components in all samples from the flask to which disogenin had been added. No sign of the oxo-acid (3), R_F 0·45, was seen in any sample.

The same results were obtained when αα′-bipyridyl (31 mg) was used in place of 8-hydroxyquinoline.

Reduction of the Oxo-acid (3).—Sodium borohydride (30 mg) was added to a solution of the oxo-acid (60 mg) in methanol (20 ml). After 16 h, glacial acetic acid (1 ml) was added, the solvent was evaporated off, and the organic material was extracted into chloroform. The extract gave (25R)-9β-*hydroxyde*-AB-*spirostane*-8α-*propionic acid lactone* (5) as needles (35 mg), m.p. 172—174° [from acetone–light petroleum (b.p. 40—60°)], R_F 0·42 [system (3)] (Found: C, 72·4; H, 9·3%. $C_{21}H_{32}O_4$ requires C, 72·4; H, 9·3%), $[\alpha]_D^{21}$ −100·6° (*c* 0·9 in EtOH), *m/e* 348 (M^+), τ 5·40—5·67 (m, 16-H), 6·0—6·24 (six-line pattern, *J* 5, 10, and 10 Hz, 9-H), 6·4—6·78 (m, 26-H_2), and 7·28—7·50 (m, 6-H_2).

Metabolism of Diosgenin by Nocardia globerula (N.C.I.B. 9158).—A fermenter containing nutrient broth (*c*) (80 l) was inoculated with a 50 h culture (2·1 l) of *N. globerula* grown in conical flasks (500 ml). After 45 h growth at 25° (210 rev. min^{-1}; >10 l min^{-1} air), diosgenin (40 g) in ethanol (1·5 l) was added. After a further 51 h the fermentation was harvested and centrifuged to remove the cells. The supernatant was adjusted to pH 2 and extracted with ethyl acetate. The acid fraction (3 g) and the phenolic fraction (2·6 g), which showed similar t.l.c. behaviour, were combined and chromatographed on silica (70 g). Elution with chloroform and then with chloroform–methanol (199 : 1) gave an oil (A) (2·9 g) which contained the oxo-acid (3), R_F 0·45, and a minor component (6), R_F 0·3 [system (3)]. Continued elution with chloroform–methanol (99 : 1) gave an oil (B) (240 mg) containing the same components as (A), but enriched in component (6). The oil (A) was re-chromatographed on silica (40 g). Elution with chloroform gave the oxo-acid (3) (1·5 g), m.p. and mixed m.p. 110—111°. The oil (B) was triturated with chloroform–light petroleum to give a solid (243 mg). This was purified by preparative t.l.c. [system (3)] and the new component, (25R)-17α-*hydroxy*-9-*oxode*-AB-*spirostane*-8α-*propionic acid* (6), R_F 0·3, was isolated as plates, m.p. 156—157° (76 mg) (from chloroform–light petroleum) (Found: C, 65·9; H, 8·3. $C_{21}H_{32}O_6$ requires C, 66·3; H, 8·5%), *m/e* 380 ($C_{21}H_{32}O_6$), 153 ($C_9H_{13}O_2$, base peak), and 126 ($C_8H_{14}O$, 2-ethyl-5-methyltetrahydropyrylium), τ 5·98 (t, 16-H), 6·3—6·8 (m, 26-H_2), 7·2—8·7 (18H, m), 8·88 (s, 18-H_3), 9·08 (d, 27-H_3), and 9·2 (d, 21-H_3).

Metabolism of Hecogenin by Unidentified Bacterium ACC 3660 (*a*).—The fermentation was carried out as for diosgenin (a) except that hecogenin (6 g) was used and the fermentation was carried out for 168 h after the addition of substrate. During separation of the total organic extract into acidic, phenolic, and neutral fractions, hecogenin separated from the organic phase (total 2·2 g), m.p. and mixed m.p. 264°. The acidic fraction (3·0 g) and the phenolic fraction (0·4 g) were combined and chromatographed on silica (40 g). Elution with chloroform–light petroleum (1 : 1) and with chloroform gave combined fractions (2 g), which on further

[16] P. W. Brian, P. J. Curtis, and H. G. Hemming, *Trans. Brit. Mycol. Soc.*, 1946, **29**, 173.

purification by t.l.c. [system (3)] gave the oxo-acid (3) as needles, m.p. and mixed m.p. 110—111° (0·6 g), identical (n.m.r. and mass spectrometry) with the sample produced from diosgenin.

(*b*) A repeat fermentation proceeded more slowly and was harvested 260 h after addition of substrate. The acidic fraction (2·2 g) yielded the oxo-acid (3) (0·3 g). The neutral fraction (0·8 g) was triturated with light petroleum and gave solid which formed needles (34 mg), m.p. 154—156° (from light petroleum), R_F 0·42 [system (3)]. N.m.r. and mass spectra were almost identical with those of the lactone (5) prepared by reduction of the oxo-acid (3).

(*c*) A further fermentation was harvested only 46 h after addition of substrate. The neutral fraction (5·3 g) was chromatographed on silica (50 g), and fractions were monitored by t.l.c. Elution with chloroform–light petroleum (3 : 7) gave (25*R*)-5α-spirostane-3,12-dione (8) (2·0 g), m.p. and mixed m.p. 241° (from acetone) (Found: C, 75·4; H, 8·9. Calc. for $C_{27}H_{40}O_4$: C, 75·7; H, 9·4%), R_F 0·6 [system (1)], *m/e* 428. Continued elution with the same solvent gave a fraction (86 mg) which yielded (25R)-*spirosta*-1,4-*diene*-3,12-*dione* (9) (20 mg) as needles, m.p. and mixed m.p. 235—236° (from light petroleum), R_F 0·43 [system (4)], *m/e* 424, $\lambda_{max.}$ (MeOH) 244 nm (ε 12,900), τ 3·22 (d, *J* 10 Hz, H-1), 3·82 (dd, *J* 10 and 2 Hz, H-2), and 3·96br (s, H-4) (Found: C, 75·1; H, 8·3. $C_{27}H_{36}O_4,0·5H_2O$ requires C, 74·8; H, 8·5%). Elution with chloroform–light petroleum (1 : 1) gave a fraction (110 mg) which after repeated preparative t.l.c. [system (4)] gave (25R)-12α-*hydroxyspirost*-4-*en*-3-*one* (10) (4 mg), m.p. 205—207° (not crystallised), *m/e* 428 ($C_{27}H_{40}O_4$), 314 ($C_{21}H_{30}O_2$), and 139 ($C_9H_{15}O$), τ 4·27 (s, 4-H), 5·5—5·75 (m, 16-H), 6·27 (t, 12β-H),[15] 6·5—6·8 (m, 26-H_2), 8·8 (s, 19-H_3), 9·02 (d, 27-H_3), 9·13 (s, 18-H_3), and 9·2 (d, 21-H_3).

(25R)-*Spirosta*-1,4-*diene*-3,12-*dione* (9).—(25*R*)-5α-Spirostan-3,12-dione (200 mg), dichlorodicyanoquinone (318 mg), and dioxan (5 ml) were heated under reflux for 18 h. Light petroleum (20 ml) was added and the mixture was filtered. The filtrate contained two products, R_F 0·40 and 0·43 [system (4)], which could not be separated cleanly by column chromatography on alumina. The material R_F 0·43, isolated by repeated preparative t.l.c., was (25*R*)-spirosta-1,4-diene-3,12-dione (9), needles, m.p. and mixed m.p. 234° (from acetone–light petroleum) (Found: C, 75·0; H, 8·6. Calc. for $C_{27}H_{36}O_4,0·5H_2O$: C, 74·8; H, 8·5%).

Oxidation of (25R)-12α-*Hydroxyspirost*-4-*en*-3-*one* (10).—A solution of the 12α-hydroxy-compound (2·8 mg) in glacial acetic acid (0·2 ml) was added to a solution of chromium trioxide (1·1 mg) in glacial acetic acid (0·4 ml) at room temperature. After 50 min water (10 ml) was added and the mixture was extracted with ether. The extract was washed with water, dried, and evaporated to dryness. The solid (25*R*)-spirost-4-ene-3,12-dione formed needles, m.p. and mixed m.p. 235° (from acetone–light petroleum), R_F 0·55 [system (1)], identical with a sample prepared by the method of Djerassi *et al.*[14] which gave the following analytical figures (Found: C, 76·3; H, 8·7. Calc. for $C_{27}H_{38}O_4$: C, 76·0; H, 9·0%).

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