Fungal Products. Part XVII.[1] Microbiological Hydroxylation of Gibberellin A_9 and its Methyl Ester

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Gibberellin A_9 (1) is metabolised by re-suspended cultures of *Gibberella fujikuroi*, mutant B1-41a, principally to gibberellins A_{20} (17) and A_{40} (18). Other metabolites, detected by g.l.c.–mass spectrometry include gibberellin A_{10} (2) and A_{11} (24); two didehydrogibberellin A_9 derivatives (12) and (14); 16α,17-dihydro-16,17-dihydroxygibberellin A_9 (26); 3-*epi*-gibberellin A_4 (4); and a monohydroxygibberellin A_9, possibly (19).

Gibberellin A_9 is partially metabolised by cultures of *Rhizopus nigricans* to give only gibberellin A_{10} (2). Gibberellin A_9 methyl ester, however, is converted into the methyl esters of the 16α- and 16β-epimers (26) and (27) of 16,17-dihydro-16,17-dihydroxygibberellin A_9, gibberellin A_{20} (17), gibberellin A_{40} (18), gibberellin A_{45} (20), and a monohydroxygibberellin A_9, possibly (19).

In a previous publication [2] we determined metabolic pathways to the fungal gibberellins (GAs) in mutant B1-41a [3] of *Gibberella fujikuroi* by following the metabolism of added substrates which are produced beyond the biosynthetic block by wild-type strains. In this paper we confirm that GA_9 (1) is not a terminal fungal GA in the mutant B1-41a, and its metabolism by this mutant is described. Since hydroxy-derivatives of GA_9, apart from the accessible fungal GA_4 (3), were required for metabolic studies in higher plants, we have also investigated the metabolism of GA_9 by some steroid-hydroxylating fungi.

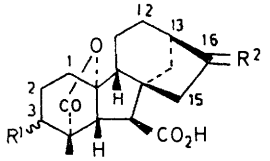
(1) *GA_9 Metabolism by* G. fujikuroi, *Mutant B1-41a.*—Gibberellin A_9 (1) is a minor metabolite of wild-type strains of *G. fujikuroi*. In cultures of the mutant B1-41a it is derived [2] from added *ent*-kaurenoic acid (6) *via* GA_{12} aldehyde (9) and GA_{12} (10). The further metabolism of GA_9 (1) by cultures of the wild-type strain ACC 917 was investigated by Cross *et al.*[4,5] These investigators found that GA_9 (1) was converted in low yield into GA_{10} (2) and the dihydro-derivative (14), but was not a precursor of GA_3 (11), the major GA produced by this strain.

In a preliminary small-scale experiment, GA_9 (1) was incubated for 20 h with pigmented mycelium of the mutant B1-41a, re-suspended in nitrogen-free medium buffered at pH 3.5. Although g.l.c. of the methylated (Me) extract of the culture filtrate showed that 70% of the GA_9 was unmetabolised, three metabolites were detected. These metabolites were identified by g.l.c.–mass spectrometry of the Me and methyl ester trimethylsilyl ether (MeTMSi) derivatives as GA_{10} (2) (2%), GA_{20} (17) (7%), and a new monohydroxy-GA_9 (20%), subsequently characterised as GA_{40} (18) [6] (see later). Smaller amounts of the seco-ring-B di- and tri-acids (21) and (22) and GA_{13} (23) were also identified by g.l.c.–mass spectrometry of the Me and MeTMSi derivatives, respectively; these last three compounds are clearly endogenous metabolites, produced by leakage at the metabolic block in the mutant (see later).

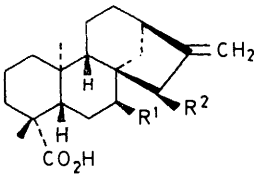
Before undertaking preparative-scale incubations to isolate the relatively inaccessible higher plant GA_{20} (17) and the new GA_{40} (18), the effects of substrate–mycelium ratio, pH of the culture medium, temperature, and length of incubation were investigated. The results showed that, with 1 mg of substrate per 100 ml of resuspension culture, GA_9 (1) was 75% metabolised at pH 4.8 after 7 days at 25 °C. From a large-scale incuba-

[1] Part XVI, J. R. Bearder, V. M. Frydman, P. Gaskin, J. MacMillan, C. M. Wels, and B. O. Phinney, preceding paper.
[2] J. R. Bearder, J. MacMillan, and B. O. Phinney, *J.C.S. Perkin I*, 1975, 721.
[3] J. R. Bearder, J. MacMillan, C. M. Wels, B. Chaffey, and B. O. Phinney, *Phytochemistry*, 1974, **13**, 911.
[4] B. E. Cross, R. H. B. Galt, and J. R. Hanson, *J. Chem. Soc.*, 1964, 295.
[5] B. E. Cross, R. H. B. Galt, and K. Norton, *Tetrahedron*, 1968, **24**, 231.
[6] I. Yamaguchi, M. Miyamoto, H. Yamane, N. Murofashi, N. Takahashi, and K. Fujita, *J.C.S. Perkin I*, 1975, 996.

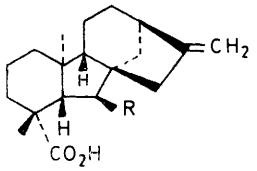
tion under these conditions, the total extract from the culture medium contained mainly GA_9 (1), GA_{20} (17), and GA_{40} (18), identified by g.l.c.–mass spectrometry



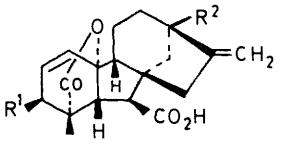
(1) R^1=H, R^2=CH_2
(2) R^1=H, R^2=OH, Me
(3) R^1=β-OH, R^2=CH_2
(4) R^1=α-OH, R^2=CH_2
(5) R^1=H, R^2=O



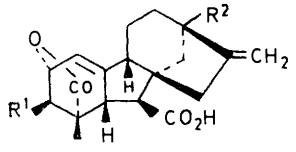
(6) R^1=R^2=H
(7) R^1=OH, R^2=H
(8) R^1=H, R^2=OH



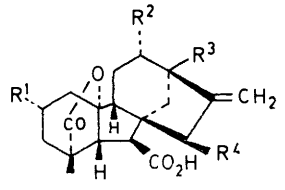
(9) R=CHO
(10) R=CO_2H



(11) R^1=R^2=OH
(12) R^1=R^2=H
(13) R^1=OH, R^2=H



(14) R^1=R^2=H
(15) R^1=R^2=OH
(16) R^1=OH, R^2=H



	R^1	R^2	R^3	R^4
(17)	H	H	OH	H
(18)	OH	H	H	H
(19)	H	OH	H	H
(20)	H	H	H	OH

and shown by g.l.c. to be present in the ratio *ca.* 5 : 2 : 13, respectively. Minor metabolites were also detected but were identified only after the following fractionation procedures.

The total extract from the culture filtrate was separated into a minor fraction A, extracted by ethyl acetate at pH 8.0, and a major fraction B, extracted by ethyl acetate at pH 3.0. A sample of the Me and MeTMSi derivatives of fraction A was shown by g.l.c.–mass spectrometry to contain GA_9 (1), Ga_{11} (24),[7] GA_{40} (18), and a didehydro-GA_9 in the ratio *ca.* 3 : 1 : 2 : 2. A trace of a second didehydro-GA_9, incompletely separated by g.l.c. from GA_9 (1), was also detected. The structures of these didehydro-compounds are discussed later.

The major fraction B was separated by p.l.c. into three zones. From that at R_F 0.45—0.50, GA_{40} (18) was obtained, mixed with a less soluble acid which was separated by repeated crystallisation and identified as succinic acid. By p.l.c. of the mother liquors from the crystallisations, GA_{40} (18) was isolated as a gum containing 1% (g.l.c.) of an unidentified isomer. During our structural studies on GA_{40}, Professor N. Takahashi informed us of his isolation of a new GA assigned[6] structure (18) and the trivial name[8] GA_{40}, as a minor metabolite from a commercial fermentation of *G. fujikuroi*. Direct comparison by n.m.r. and mass spectrometry confirmed that our metabolite was GA_{40} (18).

The less polar zone at R_F 0.5—1.0 from p.l.c. of fraction B was shown by g.l.c.–mass spectrometry of the Me and MeTMSi derivatives to contain GA_{20} (17), the acetonide (25), and an unidentified mono-oxygenated GA_9 which formed a methyl ester but not a MeTMSi derivative. These three compounds were present in about equal proportions. Several other compounds, detected by g.l.c.–mass spectrometry in trace amounts (≤1% yield), included 3-*epi*-GA_4 (4), two unidentified monohydroxy-GA_9s, and a monohydroxy-GA_9 which formed a MeTMSi derivative with a mass spectrum similar to that published[9] for the MeTMSi derivative of dihydro-GA_{31} (19) (an authentic specimen was not available for direct comparison).

The least polar zone at R_F 0.00—0.45 from p.l.c. of fraction B contained 16α,17-dihydro-16,17-dihydroxy-GA_9 (26), GA_3 (11), and the seco-ring-B di-acid (21). Compound (21), as discussed earlier, is clearly produced by leakage at the metabolic block in the mutant. That GA_3 (11) was also produced in this way, and not by metabolism of GA_9 (1), was confirmed as follows. The metabolites from an incubation of [^{3}H]GA_9 were fractionated as before and the fraction containing GA_3 (11) was shown to contain no radioactivity at the retention time of $MeGA_3$ by g.l.c.–radio-counting.[10]

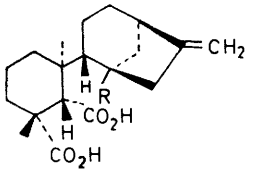
The acetonide (25) is presumably an artefact formed during work-up from the free diol (26) and acetone used as solvent. The formation of this acetonide (25) during g.l.c. of a solution of the diol (26) was also observed. Gaskin and MacMillan [11] have also noted the formation of acetonides during column chromatography of an acidic plant extract with acetone–water mixtures. The acetonide (25) and the diol (26) were identified by direct comparison with authentic samples prepared from GA_9 (1).

The two didehydro-derivatives of GA_9 (1), detected as metabolites in the present study, were also shown by g.l.c.–mass spectrometry to be present in a sample, provided by Professor N. Takahashi and shown by him by n.m.r. spectroscopy to contain both GA_9 (1) and 1,10-didehydroiso-GA_9 (14). The second didehydro-compound is possibly 1,2-didehydro-GA_9 (12), which

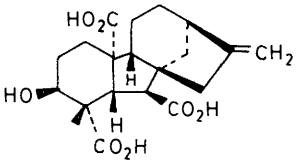
[7] J. C. Brown, B. E. Cross, and J. R. Hanson, *Tetrahedron*, 1967, **23**, 4095.
[8] J. MacMillan and N. Takahashi, *Nature*, 1968, **217**, 170.
[9] I. D. Railton, R. C. Durley, and R. P. Pharis, *Plant Physiol.*, 1974, **54**, 6.
[10] J. MacMillan and C. M. Wels, *Phytochemistry*, 1974, **13**, 1413.
[11] P. Gaskin and J. MacMillan, *Phytochemistry*, 1975, **14**, 1575.

may, however, be a g.l.c. artefact of 1,10-didehydroiso-GA_9 (14), since GA_3 (11) and GA_7 (13) are converted into iso-GA_3 (15) and iso-GA_7 (16), respectively, during g.l.c. of their Me and MeTMSi derivatives.

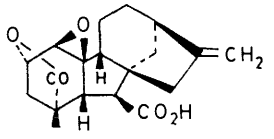
Gibberellin A_{40} (18), the major metabolite of GA_9 (1), was not metabolised when re-fed to cultures of the mutant **B1-41a**. It does not therefore appear to be a precursor of the didehydro-compounds (12) and (14) or



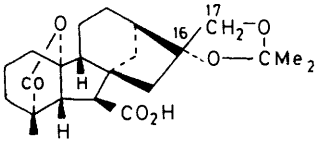
(21) R = CHO
(22) R = CO_2H



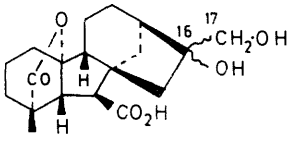
(23)



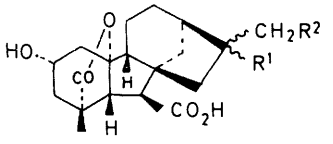
(24)



(25)



(26) 16α-OH
(27) 16β-OH



(28) R^1 = α-H, R^2 = OH
(29) R^1 = α-OH, R^2 = H
(30) R^1 = β-OH, R^2 = H

of GA_{11} (24). An alternative route from GA_9 (1) to GA_{11} (24) *via* the 1,2- and 1,10-didehydro-compounds (12) and (14) could not be tested directly since neither of the didehydro-compounds could be obtained free from GA_9 (1) or prepared by alkaline treatment of GA_{40} (18). However this pathway is consistent with the absence of GA_{11} (24) in incubations of GA_9 (1) with the mutant cultures at pH 3.5 and its presence in incubations at pH 4.8 since the conversion (12) ⟶ (14) should be more favoured at the higher pH by analogy with the base-catalysed conversion [12] of GA_3 (11) into iso-GA_3 (15).

The conversion of GA_9 (1) into the higher plant GA_{20} (17) by the mutant B1-41a was low and this method was abandoned as a preparative route to GA_{20} after a more efficient microbiological route was developed.[1]

We have previously discussed [2] the relevance of metabolic studies with the mutant to the endogenous GA metabolism in wild-type strains of *G. fujikuroi*. The present results provide further evidence that the metabolic pathways from *ent*-kaurenoic acid are similar in the mutant and wild-type strains. Thus GA_{40} (18) and GA_{11} (24) are produced by wild-type strains [6,7] and GA_{10} (2) and the didehydro-compound (14) are known metabolites [4,5] of GA_9 (1) in wild-type strain ACC 917.

[^{3}H]Gibberellin A_9 was prepared from GA_9 norketone (5), [^{3}H]methyltriphenylphosphonium bromide,[1] and potassium t-butoxide. Scrambling of the label was shown by oxidation to [^{3}H]GA_9 norketone containing 41% of the activity which was lost on treatment of the norketone with potassium t-butoxide in t-butyl alcohol.

(2) *GA_9 Metabolism by* Rhizopus nigricans.—The microbiological hydroxylation of steroids, particularly by cultures of *Aspergillus ochraceus*, *Calonectria decora*, and *Rhizopus nigricans*, has been studied extensively.[13,14] These fungi and *Aspergillus niger* have recently been used [15,16] to hydroxylate tetracyclic diterpenes related to biosynthetic precursors of the GAs. However no previous studies on the microbiological hydroxylation of GAs by fungi other than *G. fujikuroi* have been reported. Such studies with the simplest of the C_{19} gibberellins, GA_9 (1), are described below.

In exploratory experiments using methods similar to those described by Blunt *et al.*,[17] neither GA_9 (1) nor the methyl ester was metabolised by *A. niger* or *A. ochraceus*. When incubated with cultures of *R. nigricans*, GA_9 was partially converted into GA_{10} (2), the only product. On the other hand the methyl ester was completely metabolised to many products.

For more detailed investigations two strains of *R. nigricans* were used. One (Z 14a) was obtained from the Botany Department, Bristol University; the other (DP 1563) from Sir Ewart Jones. Each strain was cultured in unbuffered medium at initial pH values of 3.5 or 5.5 in the presence of GA_9 (1) or its methyl ester. Under all four sets of conditions GA_9 was largely unmetabolised; the only product was GA_{10} (2), which was formed in 15 and 2% yield at pH 3.5 and 5.5, respectively, from strain Z 14a and in 8 and 18% yield at pH 3.5 and 5.5, respectively, from strain DP 1563. The yield of GA_{10} (2), a known product from the treatment of GA_9 (1) with acid,[18] is therefore not related to the pH of the culture medium.

The metabolites from GA_9 methyl ester (Table) were present in the culture filtrate as methyl esters. They were identified by g.l.c.–mass spectrometry before and after trimethylsilylation of the total extract. The yields were estimated by g.l.c. The methyl esters of GA_9 (1), GA_{20} (17), GA_{40} (18), and the 16α,17-diol (26) were identified by direct comparison of their mass spectra with reference spectra. The identity of the

[12] B. E. Cross, J. F. Grove, and A. Morrison, *J. Chem. Soc.*, 1961, 2498.
[13] See W. Charney and H. Herzog, 'Microbiological Transformations of Steroids—A Handbook,' Academic Press, New York, 1967.
[14] See E. R. H. Jones, *Pure Appl. Chem.*, 1973, **33**, 39.
[15] J. P. Beilby, E. L. Ghisalberti, P. R. Jeffries, M. A. Sefton, and P. N. Sheppard, *Tetrahedron Letters*, 1973, 2589.
[16] A. B. Anderson, R. McCrindle, and J. K. Turnbull, *J.C.S. Chem. Comm.*, 1973, 143.
[17] J. W. Blunt, I. M. Clark, J. M. Evans, Sir E. R. H. Jones, G. D. Meakin, and J. J. Pinhey, *J. Chem. Soc.* (*C*), 1971, 1136.
[18] J. F. Grove, *J. Chem. Soc.*, 1961, 3545.

methyl ester of the 16β,17-diol (27) was deduced from the mass spectra of the MeTMSi derivatives of the two diols (26) and (27). The methyl ester of 15β-hydroxy-GA_9 was identified by g.l.c.–mass spectrometric comparison with GA_{45} (20), recently identified [19] in seed of *Pyrus communis*. The presumed 12-hydroxy-GA_9 methyl ester was characterised by the close similarity of the mass spectrum of the MeTMSi ether with that published [9] for the MeTMSi derivative of 2,3-dihydro-GA_{31} (19). The structure of the second major product is unknown; it was characterised as the methyl ester of a dihydro-dihydroxy-GA_9 from the mass spectrum of the MeTMSi derivative. This spectrum was similar to that of the MeTMSi derivative of GA_{40} (18) but different from that of the TMSi derivative of the hydroboration product (28) of GA_{40} methyl ester and from that of the MeTMSi derivatives of the hydration products (29) and (30), prepared by treatment of GA_{40} with aqueous acid. Among the other unidentified products were three monohydroxy-GA_9 methyl esters two of which may be the 1α- and 1β-hydroxy-compounds since the mass spectrum of the MeTMSi derivatives showed *m/e* 129 fragments.[20] One di- and two tri-hydroxy-derivatives of GA_9 methyl ester were also detected by g.l.c.–mass spectrometry.

The product distribution at each pH value was similar for both strains of *Rhizopus*. There were, however, minor differences. For example the presumed 12-hydroxylation occurred with strain Z 14a only and 15β-hydroxylation was observed only with strain DP 1563. However the major metabolites were the same for both strains. The inability of both strains to

Products (%) from GA_9 Me ester and *R. nigricans*

	pH 5.5		pH 3.5	
	Strain		Strain	
Me ester	Z 14a	DP 1563	Z 14a	DP 1563
GA_9		4	7	2
2α-OH-GA_9 (GA_{40})	5		9	2
12ξ-OH-GA_9	2		7	
13-OH-GA_9 (GA_{20})	5	7	6	5
15β-OH-GA_9 (GA_{45})		5		2
16α,17-$(OH)_2$-16,17-dihydro-GA_9	48	39	32	46
16β,17-$(OH)_2$-16,17-dihydro-GA_9	9	9	7	9
x,y-$(OH)_2$-dihydro-GA_9	17	17	12	17
Others	14	19	20	17

metabolise GA_9 (1) contrasts with the rapid metabolism of the methyl ester. This difference is not readily explained in terms of permeability but may be due to the adsorption of the less soluble methyl ester, which is then solubilised by induced hydroxylases.[21]

The metabolism of GA_9 (1) by *G. fujikuroi* mutant B1-41a and of GA_9 methyl ester by *R. nigricans* show qualitative similarities and quantitative differences. Thus monohydroxylation at the 2α-, 12-, and 13-positions, and 16,17-dihydroxylation occurs with both fungi. However 2α-hydroxylation of GA_9 (1) is the main process in cultures of the mutant B1-41a whereas 16,17-dihydroxylation of GA_9 methyl ester predominates in cultures of *R. nigricans*. It is difficult to account for the observed products by a simple model of the type suggested [14] for the microbiological hydroxylation of steroids.

EXPERIMENTAL

For general details see Part V.[22] G.l.c.–mass spectrometry conditions were as in Part VII [23] except for g.l.c. columns which were: (A) silanised glass column (152.5 × 0.32 cm), 2% QF-1 on demineralised and silanised Gas-Chrom A (80—100 mesh), He flow rate 30 ml min^{-1}; (B) 2% SE-33 as in (A); (C) 2% SE-33, glass column (215 × 0.32 cm), otherwise as in (A).

Fungal Cultures.—The mutant B1-41a of *Gibberella fujikuroi* has been described.[3] *Rhizopus nigricans* Ehrenb culture DP 1563 was provided by Professor Sir Ewart Jones, Oxford University; it was originally obtained from the Centralbureau voor Schimmelcultures, Baarn, under the synonym *R. stolonifer* (*Ehrenb. ex Fr.*) *Lind.* (Baarn Culture No. 382.52). *R. nigricans* culture Z 14a was provided by Dr. M. F. Madelin, Department of Botany, Bristol University.

Incubation of GA_9 with G. fujikuroi, *Mutant B1-41a.*—(a) *Small-scale.* The mutant B1-41a was grown in shake culture at 25 °C for 3 days on 50% I.C.I. medium.[24] The pigmented mycelium was collected by filtration and re-suspended in the original volume of medium from which the ammonium nitrate was omitted but which contained potassium dihydrogen phosphate (13.6 g l^{-1}) and adjusted to pH 3.5 with 2N-sulphuric acid. GA_9 (1 mg) in the minimum of acetone was added to hot autoclaved water (5 ml) in a conical flask (100 ml). To the cooled flask re-suspended mycelium (20 ml) was added and the culture was incubated at 25 °C for 20 h on an orbital shaker. After centrifugation for 20 min at 4 000 rev. min^{-1} the supernatant was collected, acidified to pH 2.5, and extracted with ethyl acetate. The gum recovered from the ethyl acetate extract was methylated (CH_2N_2), trimethylsilylated,[25] and subjected to g.l.c.–mass spectrometry with column (A) at 215 °C (Me derivatives) and column (B) at 210 °C (MeTMSi). GA_9 (1) was identified as the Me ester, and GA_{10} (2) and GA_{20} (17) were identified as the Me and the TMSi derivatives, from published mass spectra.[26] Characterisation of GA_{40} (18) is described later.

(b) *Large-scale.* GA_9 (10 mg) was added to conical flasks (30 × 250 ml) each containing pigmented mycelium re-suspended in the original volume (100 ml) of 40% I.C.I. medium from which the ammonium nitrate was omitted but containing potassium dihydrogen phosphate (5 g l^{-1}). After incubation at 25 °C for 7 days with shaking the

[19] J. R. Bearder, F. G. Dennis, J. MacMillan, G. Martin, and B. O. Phinney, *Tetrahedron Letters*, 1975, 669.
[20] J. R. Bearder, J. MacMillan, and B. O. Phinney, *Phytochemistry*, 1973, **12**, 2173.
[21] O. Hanc and A. Cizinska, *Abhandl. deut. Akad. Wiss. Berlin, Klasse Med.*, 1968, No. 2, p. 89; E. R. H. Jones, personal communication.
[22] J. MacMillan and T. J. Simpson, *J.C.S. Perkin I*, 1973, 1487.
[23] J. R. Bearder, J. MacMillan, and B. O. Phinney, *Phytochemistry*, 1973, **12**, 2635.
[24] T. A. Geissman, A. J. Verbiscar, B. O. Phinney, and G. Cragg, *Phytochemistry*, 1966, **5**, 933.
[25] C. C. Sweeley, R. Bentley, M. Makita, and M. M. Wells, *J. Amer. Chem. Soc.*, 1963, **85**, 2497.
[26] R. Binks, J. MacMillan, and R. J. Pryce, *Phytochemistry*, 1969, **8**, 271.

combined contents of the flasks were filtered. A methanolic extract of the mycelium contained no GA_9 (1) or metabolites as judged by g.l.c. A sample of the culture filtrate was acidified to pH 3.0 and extracted with ethyl acetate. The gum recovered from the extract was methylated and shown by g.l.c.–mass spectrometry with column (A), temperature programmed from 210 to 230 °C at 2° min^{-1}, to contain GA_9 (1), GA_{20} (17), GA_{40} (18), and GA_{11} (24), identified by comparison with reference spectra,[26] and a didehydro-GA_9, characterised as described below. The main bulk of the culture filtrate was then adjusted to pH 8.0 and extracted with ethyl acetate, which gave fraction A (118 mg). The aqueous layer, adjusted to pH 3.0, was re-extracted with ethyl acetate, which gave fraction B (314 mg).

Fraction A was shown by g.l.c.–mass spectrometry of a methylated sample [column (B) temperature programmed from 187 °C at 6° min^{-1}] to contain: GA_9 (1) and GA_{11} (24), identified by reference mass spectra;[26] GA_{40} (18), characterised as described later; a didehydro GA_9 [(12) or (14)], *m/e* for Me ester 328 (M^+, 13%), 296 (38), 284 (20), 282 (13), 268 (24), 223 (100), 155 (37), 143 (22), and 105 (40); and a second didehydro-GA_9 [(14) or (12)], *m/e* for Me ester 328 (M^+, 9%), 296 (36), 284 (28), 282 (11), 268 (23), 223 (100), 209 (23), 195 (18), 181 (32), 155 (42), 143 (31), and 105 (51).

Fraction B was strip-loaded on 8 silica gel plates (each 40 × 40 × 0.04 cm) and developed with ethyl acetate–chloroform–acetic acid (15 : 5 : 1). Silica from R_F 0.00—0.45, 0.45—0.50, and 0.50—1.00 was eluted with moist ethyl acetate to give fractions B-1, B-2, and B-3, respectively. Fraction B-2 (33 mg) was rechromatographed on 2 silica HF plates (each 40 × 40 × 0.04 cm) developed twice with light petroleum–ethyl acetate–acetic acid (70 : 30 : 1). Elution of silica from R_F 0.14—0.18 gave a gum (15.6 mg) which was crystallised from acetone–light petroleum to give succinic acid (7 mg) (Found: C, 40.4; H, 5.6. Calc. for $C_4H_6O_4$: C, 40.7; H, 5.1%), identified by the mass spectrum of the di-TMSi ester. The material recovered from the mother liquors was purified by p.l.c. on silica HF plates (20 × 20 × 0.04 cm), developed (× 2) with light petroleum–ethyl acetate–acetic acid (50 : 50 : 1). The eluate from R_F 0.15—0.19 gave GA_{40} as a gum (3.1 mg); τ ($CDCl_3$) 8.87 (s, 4-Me), 7.40 and 7.23 (each d, *J* 11 Hz, 5- and 6-H), 5.70 (m, $W_{\frac{1}{2}}$ 10 Hz, 2-H), and 5.13br and 5.00br (17-H_2); *m/e* for Me ester 346 (M^+, 2), 328 (2), 314 (95), 302 (31), 286 (78), 259 (88), 242 (100), 233 (48), and 225 (47); *m/e* for MeTMSi 418 (M^+, 0), 403 (2), 371 (100), 343 (82), 299 (74), 284 (70), 225 (48), 223 (29), 199 (11), and 143 (20). The isomer of GA_{40} had *m/e* (MeTMSi) 418 (M^+, 8), 371 (100), 343 (31), 299 (16), 284 (11), 225 (22), 223 (17), 195 (19), and 143 (13) and *m/e* (Me ester) 346 (M^+, 91), 328 (20), 296 (70), 268 (100), 259 (80), and 105 (80).

Fraction B-3 was examined by g.l.c.–mass spectrometry of the Me [column (A), 188—240 °C at 3° min^{-1} and column (B), 212 °C] and MeTMSi [column (A), 183—240 °C at 3° min^{-1} and column (B), 212 °C] derivatives. The following major components were identified: (*a*) GA_{20} (17) by comparison with reference spectra;[26] (*b*) the acetonide (25) by direct g.l.c.–mass spectrometric comparison with the Me and MeTMSi derivatives of an authentic sample prepared as described later; and (*c*) an unidentified metabolite, *m/e* for Me ester 346 (M^+, 5), 318 (100), 303 (25), 290 (32), 262 (25), 243 (15), 217 (15), 149 (45), and 111 (41). Minor components: (*a*) 16α,17-dihydro-16,17-dihydroxy-GA_9 (26) identified by comparison of the mass spectra of the Me and MeTMSi derivatives with those of authentic samples, prepared as described later; (*b*) 3-*epi*-GA_4 (4), identified from the mass spectrum of the MeTMSi derivative of an authentic specimen,[27] *m/e* 418 (M^+, 16), 400 (5), 386 (11), 371 (17), 328 (8), 289 (74), 233 (33), 225 (27), 129 (58), and 73 (100); (*c*) GA_{40} (18); and (*d*) the presumed 12ξ-hydroxy-GA_9 by comparison with the published mass spectrum of the MeTMSi derivative of dihydro-GA_{31} (19).

Fraction B-1 was examined by g.l.c.–mass spectrometry of the MeTMSi derivative on column (B), programmed from 183 °C at 3° min^{-1}. The major component was 16α,17-dihydro-16,17-dihydroxy-GA_9 (26), with a trace of GA_3 (11).

Feed of GA_{40} (18) to Mutant B1-41a.—GA_{40} (100 μg) was incubated for 5 days with re-suspended mycelium (10 ml) of the mutant B1-41a under the conditions used for the large-scale incubation of GA_9. The ethyl acetate extract from the culture filtrate contained only unmetabolised GA_{40}, identified by g.l.c.–mass spectrometry of the Me and MeTMSi derivatives. The methanol extract of the mycelium contained neither GA_{40} nor any metabolites.

Attempted Rearrangement of GA_{40} (18).—GA_{40} (100 μg) was heated at 121 °C for 15 min at 15 lb in^{-2} in 0.2M-potassium dihydrogen phosphate (pH 8.5). The ethyl acetate extract from the acidified solution contained only GA_{40} (by g.l.c. of the methyl ester).

[^{3}H]GA_9.—Resublimed potassium t-butoxide in tetrahydrofuran (0.743M; 3.22 ml) was added to a stirred suspension of [^{3}H]methyltriphenylphosphonium bromide (0.7 g; 56.5 mCi $mmol^{-1}$) in tetrahydrofuran (20 ml) under nitrogen. After 1.5 h GA_9 norketone (5) (137 mg) in tetrahydrofuran (5 ml) was added. After 19 h at 20 °C water (20 ml) was added and the tetrahydrofuran was evaporated off. The aqueous residue, acidified to pH 3.0 with 3N-hydrochloric acid, was extracted with ethyl acetate (3 × 30 ml), which afforded a gum (497 mg). P.l.c. of this gum on silica gel G, developed with ethyl acetate–light petroleum–acetic acid (40 : 60 : 1), and recovery from the band at R_F 0.4, gave [^{3}H]GA_9 (108 mg), which was recrystallised to constant specific activity (29 mCi $mmol^{-1}$) and identified by mass spectral comparison of the methyl ester with an authentic specimen. The radioactive purity was established by g.l.c.–radio-counting[10] of the methyl ester on columns (A) and (B) programmed from 180 °C at 3° min^{-1}. In each case, as well as the major mass and radioactive peak at the retention time of GA_9 methyl ester, a minor radioactive peak, containing less than 3% of the activity, was observed with retention times 2.0 and 1.5 times that of GA_9 methyl ester on columns (A) and (B), respectively.

Oxidation of [^{3}H]GA_9.—[^{3}H]GA_9 (19.2 mg; 0.563 mCi $mmol^{-1}$) in tetrahydrofuran (4 ml) was treated with osmium tetraoxide (11 mg). After 10 min sodium periodate (34 mg) in water (7 ml) was added and the solution was stirred for 3 h. The usual work-up gave [^{3}H]GA_9 norketone which was purified by p.l.c. (R_F 0.5) on silica gel G with chloroform–ethyl acetate–acetic acid (10 : 10 : 1) and crystallised from acetone–light petroleum, then ethyl acetate–light petroleum to constant specific activity (11 mg; 0.231 mCi $mmol^{-1}$) and identified by the mass spectrum of the methyl ester. The specific activity was 41% of that of the starting material.

Treatment of [^{3}H]GA_9 Norketone with Base.—The above norketone (10 mg) in t-butyl alcohol (12 ml) containing re-

[27] D. H. Bowen, C. Cloke, D. M. Harrison, and J. MacMillan, *J.C.S. Perkin I*, 1975, 83.

sublimed potassium t-butoxide was refluxed for 1 h under nitrogen. Aqueous potassium dihydrogen phosphate was added and the bulk of the t-butyl alcohol was removed under vacuum. Water was added and the product was recovered in ethyl acetate. Purification as described in the previous experiment gave GA_9 norketone (6.2 mg) containing 0.06% of the specific activity of the starting material.

Incubation of GA_9 and GA_9 Methyl Ester with Rhizopus nigricans.—Cultures were stored on 3% malt agar slopes and maintained by subculturing every 24 days. A small piece of mycelium from these slopes was used to inoculate medium B[17] (40 ml) in conical flasks (500 ml) and adjusted to pH 3.5 or 5.5 with 2N-hydrochloric acid or 2N-potassium hydroxide, respectively. The flasks were shaken at 25 °C for 2 days. Then GA_9 (1 mg) or GA_9 methyl ester (1 mg) in dimethyl sulphoxide (0.4 ml) was added and the cultures were shaken at 25 °C for a further 5 days. The mycelium was collected by filtration and extracted by shaking in acetone (20 ml) overnight; evaporation of the acetone, addition of dilute hydrochloric acid (20 ml), and extraction with ethyl acetate gave no metabolites (by g.l.c. either before or after formation of derivatives). The culture filtrate had pH 8.2 from cultures with an initial pH of 5.5 and pH 7.5 from an initial pH of 3.5. In each case the pH was adjusted to 2.5 by addition of 2N-hydrochloric acid; the solution was saturated with sodium chloride and extracted with ethyl acetate (3 × 20 ml). Recovery from the ethyl acetate extract gave gummy products.

The gums from each of the four incubations of GA_9 were methylated and trimethylsilylated. In each case GA_{10} (2) was the sole metabolite, identified by g.l.c.–mass spectrometry on column (B) programmed from 196 °C at 3° min^{-1} as the MeTMSi derivative by comparison with a reference spectrum.[26] The yields, estimated by g.l.c., were: 2% (initial pH 5.5) and 15% (initial pH 3.5) from culture Z 14a; and 18% (initial pH 5.5) and 8% (initial pH 3.5) from culture DP 1563.

The gums from each of the four incubations of GA_9 methyl ester gave the same g.l.c.–flame ionisation detector trace on column (B) at 220 °C before and after methylation. The metabolites (Table) were identified by g.l.c.–mass spectrometry of the MeTMSi derivatives on columns (A), (B), and (C), programmed from 180 °C at 3° min^{-1} as follows: (*a*) methyl esters of GA_{20} (17), GA_{40} (18), and GA_{45} (20) from reference spectra;[19,26] (*b*) the presumed methyl ester of 12ξ-hydroxy-GA_9 (19) by comparison with the spectrum published[8] for the MeTMSi derivative of dihydro-GA_{31}; and (*c*) the methyl ester of 16α,17-dihydro-16,17-dihydroxy-GA_9 (26) and of the derived acetonide (25) by g.l.c.–mass spectral comparison with the appropriate derivatives, prepared as described later. The unidentified metabolites were characterised by the following mass spectral data for the MeTMSi derivatives: (i) the presumed 16β,17-dihydro-16,17-dihydroxy-GA_9 methyl ester (27), *m/e* 508 (M^+, 0.2%), 493 (3), 405 (100), 217 (3), and 147 (15); (ii) a dihydro-dihydroxy-GA_9 methyl ester, *m/e* 508 (M^+, 1), 493 (21), 461 (100), 433 (26), 418 (80), 401 (48), 341 (80), 283 (42), 225 (28), 223 (40), 183 (33), 129 (37), and 103 (46); and two monohydroxy-GA_9 methyl esters, *m/e* 418 (M^+, 28), 403 (5), 358 (8), 296 (10), 269 (11), 268 (16), 223 (15), 129 (100), and 73 (>100) and *m/e* 418 (M^+, 100), 403 (11), 358 (15), 296 (17), 269 (18), 268 (18), 245 (25), 223 (20), 215 (30), 157 (40), 129 (90), and 73 (>100).

16α,17-*Dihydro*-16,17-*dihydroxy-GA_9 Methyl Ester* (26).—GA_9 methyl ester (1 mg) in pyridine–chloroform (0.8 ml; 1 : 1 v/v) and a small crystal of osmium tetraoxide were left at 20 °C for 3 days. Sodium disulphite (15 mg) in water (1 ml) and pyridine (1 ml) was added to the solution, which was then shaken for 15 min. After the addition of water (10 ml) the mixture was extracted with ethyl acetate (2 × 10 ml). The product, recovered from the ethyl acetate, was re-methylated with diazomethane, then characterised as the MeTMSi derivative by g.l.c.–mass spectrometry on column (B) programmed from 232 °C at 2° min^{-1}: *m/e* 508 (M^+, 0.5), 493 (2), 405 (100), 217 (4), and 147 (11); the methyl ester of the acetonide (25), prepared from the re-methylated product by heating with acetone and toluene-*p*-sulphonic acid, had *m/e* 404 (M^+, 25), 389 (23), 346 (3), 329 (100), 269 (63), 242 (28), 72 (85), and 43 (38).

Treatment of GA_{40} (18) with Aqueous Acid.—GA_{40} (100 μg) was heated at 75 °C for 2 h with methanol (10 μl) and N-hydrochloric acid (1 ml). Extraction with ethyl acetate, and recovery, gave the 16α- and 16β-hydroxy-isomers (29) and (30) of 16,17-dihydro-GA_{40}, characterised as the MeTMSi derivatives by g.l.c.–mass spectrometry on column (B), programmed from 212 °C at 3° min^{-1}; *m/e* 508 (M^+, 30), 493 (19), 461 (13), 371 (24), 329 (27), 284 (27), 282 (18), 225 (23), 223 (26), 189 (24), 169 (28), 156 (37), 143 (45), and 130 (100) and *m/e* 508 (M^+, 35), 493 (19), 371 (48), 329 (36), 284 (15), 282 (10), 225 (28), 223 (25), 183 (30), 169 (27), 156 (35), 143 (40), and 130 (100).

16α,17-*Dihydro*-17-*hydroxy-GA_{40} Methyl Ester.*—GA_{40} methyl ester (100 μg) in tetrahydrofuran (100 μl) was treated with 0.5M-diborane in tetrahydrofuran (50 μl). After 0.5 h at 20 °C, *N*-sodium hydroxide (100 μl) and 30% hydrogen peroxide (50 μl) were added and, after a further 0.5 h, the solution was extracted with ethyl acetate. The product recovered from the ethyl acetate was characterised by g.l.c.–mass spectrometry as the MeTMSi derivative on column (B) programmed from 215 °C at 4° min^{-1}: *m/e* 508 (M^+, 0), 493 (3), 461 (100), 443 (29), 374 (33), 284 (31), 225 (41), 223 (21), 183 (25), and 143 (30).

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