Elucidation of the Structure of Gibberellin A_{40} from *Gibberella fujikuroi*

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A new gibberellin (A_{40}) isolated from the culture filtrate of *Gibberella fujikuroi* has been identified as *ent*-2β,10-dihydroxy-20-norgibberell-16-ene-7,19-dioic acid 19-10-lactone (1) on the basis of ^{13}C n.m.r. measurements and conversion into deoxygibberellin A_5 methyl ester (10).

EIGHTEEN fungal gibberellins have been isolated and characterized hitherto. We now report the isolation and identification of a new gibberellin (A_{40}).[1]

Gibberellin A_{40}, m.p. 212—213°, was isolated from a concentrate of the gibberellin A_3 mother liquors of a commercial fermentation. Methylation with ethereal diazomethane gave an amorphous monomethyl ester (2) [δ(C_5D_5N) 3·65 (s, CO_2Me)] of molecular formula $C_{20}H_{26}O_5$ (high resolution mass measurement). Hence the free acid (1) should have the molecular formula $C_{19}H_{24}O_5$.

The ^{13}C n.m.r. spectra (proton noise decoupled and proton off-resonance decoupled) were measured. Although signals for two carbon atoms were not observed in the proton noise-decoupled spectrum measured at 25 °C with 8 s pulse interval, they did appear (at 52·6 and 175·3 p.p.m.) in the spectrum measured at −20 °C with 4 s pulse interval. Signals at 92·8, 175·3, and 179·8 p.p.m. are characteristic of C-10, C-7, and C-19 in a C_{19} gibberellin.[2] Signals at 107·3 and 157·8 p.p.m. are characteristic of an exocyclic methylene system (C-17 and C-16), and that at 64·6 p.p.m., which is collapsed to a doublet in the proton off-resonance decoupled spectrum is expected to be due to a secondary carbinol carbon atom.

These observations are in good agreement with ^1H n.m.r. and i.r. data for gibberellin A_{40} and its methyl ester. The ^1H n.m.r. spectrum of gibberellin A_{40} in C_5D_5N showed a pair of doublets at δ 2·95 and 3·08 due to 5-H and 6-H, characteristic of a C_{19} gibberellin, two broad singlets at δ 4·91 and 5·00 due to the exocyclic methylene group, and a 1H multiplet ($W_{\frac{1}{2}}$ 10 Hz) at δ 4·52 due to a secondary carbinol proton. The i.r. spectrum of the ester (2) showed bands at 3400 (OH), 1770 (γ-lactone), 1728 (ester CO), and 1655 cm^{-1} (exocyclic methylene). Thus, gibberellin A_{40} was shown to be a C_{19} gibberellin possessing a hydroxy-group, *i.e.* an isomer of gibberellin A_4 (6).

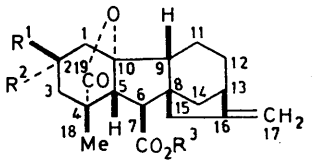
In the Figure, the ^{13}C proton noise-decoupled spectrum of gibberellin A_{40} is compared with those of gibberellins A_4 (6) and A_9 (8). Gibberellin A_{40} shows the same signal pattern for carbon atoms in rings C and D (C-8, C-9, and C-11 to C-17) as gibberellins A_4 and A_9. Therefore the hydroxy-group in gibberellin A_{40} should be located in ring A. Since gibberellin A_{40} was shown not to be identical with the C-3 epimer (7) of gibberellin A_4 by the direct comparison, the hydroxy-group must be at C-1 or C-2. However if it were at C-1, C-2 would be expected to resonate at lower field and C-3 at higher field than the corresponding atoms of gibberellin A_9 (owing to 1,3-interaction [3] in the latter case). In fact the signals due to the two methylene groups in ring A of gibberellin A_{40} appear at lower field than that of C-3 of gibberellin A_9. This signal pattern can only be explained if the hydroxy-group is at C-2; it would then

[1] Preliminary report, I. Yamaguchi, M. Miyamoto, H. Yamane, N. Takahashi, K. Fujita, and M. Imanari, *Agric. and Biol. Chem.* (*Japan*), 1973, **37**, 2453.

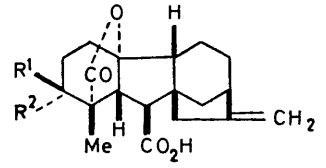
[2] I. Yamaguchi, K. Fumita, and N. Takahashi, preceding paper.

[3] J. D. Roberts, F. J. Weigert, J. I. Krochwitz, and H. J. Reich, *J. Amer. Chem. Soc.*, 1970, **92**, 1338.

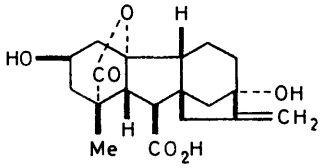
deshield both C-1 and C-3 (the signals at 39·1 and 45·3 p.p.m. are assigned to C-1 and C-3, respectively).



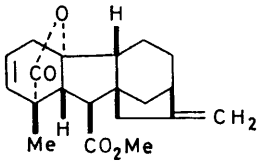
(1) $R^1=R^3=H, R^2=OH$
(2) $R^1=H, R^2=OH, R^3=Me$
(3) $R^1R^2=O, R^3=H$
(4) $R^1=OH, R^2=R^3=H$
(5) $R^1=H, R^2=O\cdot SO_2\cdot C_6H_4Me\ p, R^3=Me$



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



(9)



(10)

The ^1H n.m.r. spectrum of the ester (2) showed a C*H*OH signal at δ 4·50 (t, *J* 5·0 Hz) in C_5D_5N and at δ 4·28 (ill-defined m, $W_{\frac{1}{2}}$ 10 Hz) in $CDCl_3$, suggesting that this proton is equatorial. Hence the C-2 hydroxy-group should have the α-configuration (axial), and the structure (1) is indicated for gibberellin A_{40}.

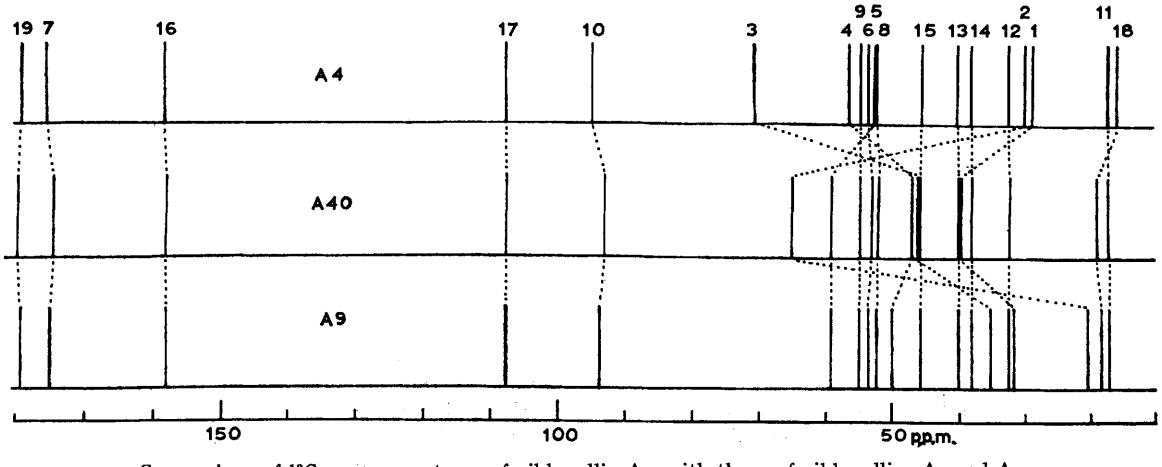
This structure was confirmed by chemical conversions of gibberellin A_{40} and its methyl ester. Treatment of gibberellin A_{40} with Jones reagent gave the keto-acid (3), which was converted into gibberellin A_{40} and its C-2 epimer (4) by reduction with sodium borohydride. The epimer (4) showed a C*H*OH signal (m, $W_{\frac{1}{2}}$ 20 Hz) at δ 4·30 in C_5D_5N The chemical shift and the $W_{\frac{1}{2}}$ value agreed well with the corresponding data for gibberellin A_{29} (9),[4] indicating the β-configuration of the 2-hydroxy-group.

Treatment of the ester (2) with toluene-*p*-sulphonyl chloride and dry pyridine gave a monotosylate (5), which was refluxed in collidine to give deoxygibberellin A_5 methyl ester (10).[5]

EXPERIMENTAL

Analytical t.l.c. plates were viewed in visible or u.v. light after spraying with 70% sulphuric acid and heating at 120 °C. ^{13}C N.m.r. spectra were measured for solutions in C_5D_5N containing 0·5% Me_4Si as internal standard with a JNM PS 100 unit by use of the pulse Fourier transform technique.[2] JNM PS 100 and JNM MH 100 instruments were used for ^1H n.m.r. spectra, a JASCO-S KC1 spectrometer for i.r. spectra, an RMU-H2 instrument for the high resolution mass spectrum, and an RMU-6L spectrometer for low resolution mass spectra.

Isolation of Gibberellin A_{40}.—(a) *Charcoal chromatography.* A concentrate of the gibberellin A_3 mother liquors from an acidic ethyl acetate fraction of a commercial fermentation of *Gibberella fujikuroi* was supplied by Kyowa Hakko Kogyo Ltd. A portion (*ca.* 700 g) was adsorbed on Celite and placed on a column of charcoal (2·0 kg), which was eluted with water–acetone mixtures containing an increasing acetone concentration (5% steps in 10 l fractions). Fractions eluted with 50 and 55% acetone–water were shown to contain a new gibberellin by t.l.c.



Comparison of ^{13}C n.m.r. spectrum of gibberellin A_{40} with those of gibberellins A_4 and A_9

(b) *Counter-current distribution.* An acidic gum (*ca.* 100 g) containing the new gibberellin was subjected to

[4] T. Yokota, N. Murofushi, N. Takahashi, and S. Tamura, *Agric. and Biol. Chem.* (*Japan*), 1971, **35**, 583.

[5] N. Murofushi, T. Yokota, A. Watanabe, and N. Takahashi, *Agric. and Biol. Chem.* (*Japan*), 1973, **37**, 1101.

counter-current distribution with 500 ml each of ethyl acetate and 1M-phosphate buffer (pH 5·5). After 10 transfers, 11 fractions were obtained; no. 1 contained the most polar and no. 11 the least polar substances. The new gibberellin was found in fractions 6—11.

(c) *Silica gel adsorption chromatography.* Fractions 6—10 were combined and the concentrate (18·2 g) was chromatographed on a column of silica gel (400 g). The column was eluted with benzene–ethyl acetate mixtures containing an increasing ethyl acetate concentration (5% steps in 4 l fractions). The fractions eluted with ethyl acetate–benzene in the ratios 20 : 80, 25 : 75, 30 : 70, and 35 : 65 contained the new gibberellin. Fraction 11 obtained in (b) was separately chromatographed on a column of silica gel (700 g), which was eluted with benzene–ethyl acetate mixtures in 5 l fractions. The fractions eluted with ethyl acetate–benzene in the ratios 20 : 80 to 35 : 65 contained the new gibberellin. All fractions containing the new gibberellin (from both column separations) were combined to give an acidic gum (37·7 g).

(d) *Charcoal re-chromatography.* The acidic gum (37·7 g) obtained in (c) was re-chromatographed by the procedure described in (a). The column of charcoal (100 g) was eluted with water–acetone containing an increasing acetone concentration (5% steps in 1 l fractions). The fractions eluted by 40, 45, and 50 acetone–water were combined to give an acidic gum (21 g).

(e) *Silica gel adsorption re-chromatography.* The acidic gum (21 g) obtained in (d) was re-chromatographed in the manner described in (c). The column of silica gel (100 g) was eluted with benzene–ethyl acetate mixtures in 2 l fractions. The new gibberellin was found in the fractions eluted with ethyl acetate–benzene in the ratios 30 : 70, 35 : 65, 40 : 60, 45 : 55, and 50 : 50, which were combined to give an acidic gum (6·8 g).

(f) *Partition chromatography.* The acidic gum (6·8 g) obtained in (e) was adsorbed on Celite and placed on a column prepared as follows. Silica gel (500 g), previously treated with 1M-phosphate buffer (pH 5·6; 400 ml), was suspended in benzene and packed in a glass column. The column was eluted with benzene–butan-1-ol mixtures. Beginning with 0·2% butanol in benzene, the concentration of butanol was increased by 0·2% steps in 1 l fractions. Gibberellin A_{36} [6] (*ca.* 1 g) was crystallized from the fraction eluted by 0·4% butanol in benzene, and the mother liquor was concentrated to give an acidic gum (3·8 g).

(g) *Silica gel adsorption re-chromatography.* The acidic gum (3·8 g) was re-chromatographed on a column of silica gel (80 g), which was eluted with benzene–ethyl acetate mixtures containing an increasing concentration of ethyl acetate (2·5% steps) and beginning with 20% ethyl acetate in 1 l of benzene. Fractions eluted by 22·5—27·5% ethyl acetate–benzene contained the new gibberellin.

(h) *Preparative t.l.c.* Each fraction containing the new gibberellin obtained in (g) was separately subjected to t.l.c. on silica gel GF_{254} in ethyl acetate–chloroform–acetic acid (20 : 8 : 1; R_F 0·32) to give a new gibberellin. Finally, *gibberellin* A_{40} (150 mg) was obtained as needles (from ethyl acetate–hexane), m.p. 212—213°, $\nu_{max.}$ (Nujol) 3380, 1754, 1735, and 1650 cm^{-1}; δ_H (C_5D_5N) 1·34 (s, 18-H_3), 2·95 (d, *J* 11 Hz, 5-H), 3·08 (d, *J* 11 Hz, 6-H), 4·52 (m, $W_{\frac{1}{2}}$ 10 Hz, 2-H), and 4·91br (s) and 5·00br (s) (17-H_2); δ_C (C_5D_5N) 16·6 (C-11), 18·4 (C-18), 31·8 (C-12), 37·3 (C-14), 39·1 (C-1), 39·4 (C-13), 45·0 (C-15), 45·3 (C-3), 46·3 (C-4), 51·7 (C-8), 52·6 (C-6), 54·2 (C-9), 58·7 (C-5), 64·6 (C-2), 92·8 (C-10), 107·3 (C-17), 157·8 (C-16), 175·3 (C-7), and 179·8 (C-19).

Gibberellin A_{40} *Methyl Ester* (2).—Treatment of gibberellin A_{40} (8 mg) with ethereal diazomethane yielded the *methyl ester* (2) as a homogeneous amorphous solid (t.l.c. and g.l.c.) (Found: M^+, 346·1772. $C_{20}H_{26}O_5$ requires *M*, 346·1778); *m/e* 346 (M^+, 11%), 328 (0·5), 314 (71), 302 (55), 300 (9), 286 (70), 284 (25), 268 (23), 259 (90), and 242 (100); δ_H (C_5D_5N) 1·26 (s, 18-H_3), 2·83 (d, *J* 11 Hz, 5-H), 2·95 (d, *J* 11 Hz, 6-H), 3·68 (s, CO_2Me), 4·50 (t, *J* 5 Hz, 2-H), and 4·90br (s) and 4·98br (s) (17-H_2).

Oxidation of Gibberellin A_{40}.—Gibberellin A_{40} (40 mg) was treated with a small amount of Jones reagent in acetone (0·5 ml) for 5 min at 0 °C. Ethyl acetate (5 ml) was added to the mixture, which was washed with water (5 × 1 ml). The organic phase was dried (Na_2SO_4) and concentrated to give a gum (38 mg), which was purified by preparative t.l.c. in ether–hexane (5 : 1; R_F 0·3), to give ent-10-*hydroxy*-2-*oxo*-20-*norgibberell*-16-*ene*-7,19-*dioic acid* 19,10-*lactone* (3) as fine needles (24 mg), m.p. 151—153°; $\nu_{max.}$ (Nujol) 3050br, 1753, 1735, and 1657 cm^{-1}; *m/e* 330 (M^+, 100%), 312 (25), 302 (10), 287 (25), 285 (30), 284 (60), and 239 (90) (Found: M^+, 330·1468. $C_{19}H_{22}O_5$ requires *M*, 330·1466); δ_H (C_5D_5N) 1·22 (s, 18-H_3), 2·52br (s, 1-H_2), 2·73 (d, *J* 9 Hz, 5-H), 3·01 (d, *J* 9 Hz, 6-H), and 4·82br (s) and 4·92br (s) (17-H_2).

Reduction of the Keto-Acid (3) *with Sodium Borohydride.*—The keto-acid (3) (20 mg) was treated with sodium borohydride (20 mg) in ethanol (5 ml) for 6 h. A small amount of acetic acid was added to the mixture to decompose the excess of borohydride, and the mixture was extracted with ethyl acetate after addition of water (20 ml). An acidic gum (18 mg) was recovered from the ethyl acetate fraction, and subjected to preparative t.l.c. in ethyl acetate–chloroform–acetic acid (20 : 8 : 1) to give 2-*epi*-gibberellin A_{40} (9 mg; R_F 0·41) and gibberellin A_{40} (8 mg; R_F 0·32). The 2-epimer (4) crystallized as needles (from ethyl acetate–hexane), m.p. 190—193°; $\nu_{max.}$ (Nujol) 3300, 1743, 1704, and 1650 cm^{-1}; δ_H (C_5D_5N) 1·38 (s, 18-H_3), 3·04 (s, 5- and 6-H), 4·28 (m, $W_{\frac{1}{2}}$ 20 Hz, 2-H), and 4·80br (s) and 4·90br (s) (17-H_2); *m/e* (for *methyl ester*) 346 (M^+, 10%), 328 (5),314 (100), 302 (3), 286 (35), 284 (50), and 268 (100) (Found: M^+, 346·1807. $C_{20}H_{26}O_5$ requires *M*, 346·1778).

Tosylation of Gibberellin A_{40} *Methyl Ester* (2).—The methyl ester (2) (7 mg) was treated with toluene-*p*-sulphonyl chloride (2·0 equiv.) in dry pyridine (20 μl) for 48 h at room temperature. Ethyl acetate (20 ml) was added to the mixture, which was washed with N-sulphuric acid (5 ml), aqueous sodium hydrogen carbonate, and water three times each in succession. A gum (11·2 mg) was recovered from the organic phase and subjected to preparative t.l.c. in ether–benzene (3 : 1; R_F 0·60) to give a monotosylate (5) (5·3 mg); δ_H ($CDCl_3$) 0·92 (s, 18-H_3), 2·32 (s, $ArCH_3$), 2·24 (d, *J* 11 Hz, 5-H), 2·30 (d, *J* 11 Hz, 6-H), 3·62 (s, CO_2Me), 4·78br (s) and 4·92br (s) (17-H_2), 5·08 (m, $W_{\frac{1}{2}}$ 10 Hz, 2-H), and 7·32 (d) and 7·74 (d) (ArH_4).

Preparation of Deoxygibberellin A_5 *Methyl Ester* (10).[5]—The tosylate (5) (5·3 mg) was boiled in 2,4,6-collidine for 3·5 h. The mixture was purified by the same procedure as for isolation of the tosylate (5) to give deoxygibberellin A_5 methyl ester (10) as rods (0·8 mg), identical (mass spectral fragmentation pattern and g.l.c. retention times)

[6] J. R. Bearder and J. MacMillan, *J.C.S. Perkin I*, 1973, 2824.

with an authentic sample;[5] m.p. 148—149°; *m/e* 328 (M^+, 5%), 296 (12), 284 (70), 269 (10), 256 (6), 252 (8), 243 (8), 241 (8), 225 (65), and 224 (100); t_R 4·5 min (2% OV-1 on Chromosorb W, 3 mm × 2 m glass column, oven temp. 186 °C, N_2 flow 34 ml min^{-1}) and 14·6 min (2% QF-1 on Chromosorb W, 3 mm × 1 m glass column, oven temp. 186 °C, N_2 flow 38 ml min^{-1}).

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