243. *Gibberellic Acid. Part V.* The Relation between Gibberellin A_1 and Gibberellic Acid.*

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Gibberellin A_1 is a dihydro-derivative of gibberellic acid since one of two dihydro-derivatives obtained by controlled hydrogenation of methyl gibberellate is shown to be identical with gibberellin A_1 methyl ester. The isolation of some additional metabolites of *Gibberella fujikuroi* is described.

The fungus *Gibberella fujikuroi* has been reported to produce several monobasic acidic compounds which promote plant growth. Gibberellic acid,[1] $C_{19}H_{22}O_6$, m. p. 233—235° (decomp.), $[\alpha]_D$ +92°, is produced by several strains (and in particularly high yield by Akers Laboratories no. 917) on the medium described by Borrow *et al.*[2] Working with strains different from the above and using a medium with a higher nitrogen : carbon ratio Takahashi *et al.*[3] obtained a mixture, gibberellin A, from which gibberellin A_1, $C_{19}H_{24}O_6$, m. p. 255—258° (decomp.), $[\alpha]_D$ +36°,† and gibberellin A_2, $C_{19}H_{26}O_6$,‡ m. p. 235—237° $[\alpha]_D$ +11·7°, were isolated. Using the high-nitrogen medium of the Japanese workers but a different strain of *G. fujikuroi* (N.R.R.L. 2284), Stodola *et al.*[5] obtained a mixture, separated by chromatography on Celite[6] into gibberellin A_1 § and gibberellic acid. Gibberellic acid, gibberellin A_1, and gibberellin A_2 contain respectively two, one, and no ethylenic double bond and differ only in their hydrogen content. The possibility arises therefore that gibberellins A_1 and A_2 may be reduction products of gibberellic acid. This was also considered by Kitamura *et al.*[4] but their evidence is inconclusive. We have now

* Part IV, *Chem. and Ind.*, 1956, 954.

† Takahashi *et al.* gave m. p. 232—235°, $[\alpha]_D$ +42·3°, but these constants require correction.

‡ Takahashi *et al.* gave $C_{20}H_{28}O_6$, subsequently altered [4] to $C_{19}H_{26}O_6$.

§ The name gibberellin A used [5, 6] for this component of Northern Regional Research Laboratories (N.R.R.L.) gibberellin is discontinued by agreement with Dr. F. H. Stodola.

[1] Cross, *J.*, 1954, 4670.

[2] Borrow, Brian, Chester, Curtis, Hemming, Henehan, Jefferys, Lloyd, Nixon, Norris, and Radley, *J. Sci. Food Agr.*, 1955, **6**, 340.

[3] Takahashi, Kitamura, Kawarada, Seta, Takai, Tamura, and Sumiki, *Bull. Agric. Chem. Soc. Japan*, 1955, **19**, 267.

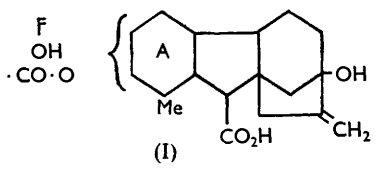
[4] Kitamura, Seta, Takahashi, Kawarada, and Sumiki, *ibid.*, 1957, **21**, 71.

[5] Stodola, Raper, Fennell, Conway, Sohns, Langford, and Jackson, *Arch. Biochem.*, 1955, **54**, 240.

[6] Stodola, Nelson, and Spence, *ibid.*, 1957, **66**, 438.

shown gibberellin A_1 to be a dihydro-derivative of gibberellic acid, but the relation between gibberellic acid and gibberellin A_2 requires further investigation.

Separation of the products of the catalytic reduction of gibberellic acid was difficult and better results were obtained with the methyl ester. Hydrogenation of the latter in the presence of a palladium–carbon catalyst resulted in rapid absorption of 1·1—1·2 mol. of hydrogen followed by further slow absorption. Stopping the reaction after absorption of 0·94 mol. of hydrogen gave a mixture from which an acidic and a neutral fraction were isolated. Crystallisation of the latter gave a mixture of methyl dihydrogibberellates, $[\alpha]_D$ +55° (in EtOH), which was resolved with difficulty by chromatography into α- and β-isomers, $[\alpha]_D$ +46° and +74° respectively. The former was identical with gibberellin A_1 methyl ester. It follows that gibberellin A_1 is a dihydro-derivative of gibberellic acid.



(I)

One of the ethylenic bonds in gibberellic acid is in a terminal methylene group, the other in ring A of the partial structure (I).[7] A terminal methylene group is also present in methyl α-dihydrogibberellate and we have confirmed the production of formaldehyde on ozonolysis reported by Seta *et al.*[8] Methyl α-dihydrogibberellate therefore arises by reduction of the double bond in ring A of methyl gibberellate.

Although Akers Laboratories strain no. 917 produced gibberellic acid and no other gibberellins under the conditions of Borrow *et al.*[2], it gave gibberellin A_1 in low yield (and no gibberellic acid) on the high-nitrogen medium of Stodola *et al.*[5] Succinic acid and 5-hydroxymethylfuran-2-carboxylic acid [9] were the only other acidic metabolic products isolated from strain no. 917.

Using the strain and culture conditions described by Stodola *et al.*[5] we have confirmed the production of gibberellin A_1 and gibberellic acid. The separation of gibberellin A_1 and gibberellic acid on Celite is laborious and separation of the methyl esters by chromatography on alumina is more satisfactory.

Stodola *et al.*[5] also recorded the isolation of fusaric acid, m. p. 106—107°: we have obtained this acid with m. p. 100—101° and also dehydrofusaric acid,[10] m. p. 118—119°, separable by chromatography on Celite. In our view pure fusaric acid has m. p. 100—101°, which is raised by admixture with dehydrofusaric acid, and it seems probable that many of the earlier specimens of fusaric acid [11,12,13] isolated from *G. fujikuroi* contained dehydrofusaric acid. Similar views have been expressed by Gaumann.[14] The infrared spectrum of dehydrofusaric acid showed a strong band at 908 cm.$^{-1}$ absent from the spectrum of fusaric acid: both compounds had bands near 1000 cm.$^{-1}$ but the intensity of a band at 993 cm.$^{-1}$ was greater in dehydrofusaric than in fusaric acid. This evidence suggests the presence of a $\cdot CH{:}CH_2$ group in dehydrofusaric acid, and, taken in conjunction with the ultraviolet absorption of dehydrofusaric acid, which showed the absence of an ethylenic double bond conjugated with the pyridine nucleus, supports the 5-but-3′-enylpyridine-2-carboxylic acid structure for this compound proposed by Stoll and Renz.[15]

Experimental

M. p.s are corrected. Microanalyses are by Messrs. W. Brown and A. G. Olney. Celite 545 and alumina, Grade II, pH 4 were used in chromatography.

Separation of Gibberellin A_1 and Gibberellic Acid.—Culture filtrates (200 l.) of *Gibberella*

[7] Cross, Grove, MacMillan, and Mulholland, *Chem. and Ind.*, 1956, 954.
[8] Seta, Kitamura, Takahashi, and Sumiki, *Bull. Agric. Chem. Soc. Japan*, 1957, **21**, 73.
[9] Kawarada, Takahashi, Kitamura, Seta, Takai, and Sumiki, *ibid.*, 1955, **19**, 84.
[10] Stoll, *Phytopath. Z.*, 1954, **22**, 233.
[11] Yabuta, Kambe, and Hayashi, *J. Agric. Chem. Soc. Japan*, 1934, **10**, 1059.
[12] Plattner, Keller, and Boller, *Helv. Chim. Acta*, 1954, **37**, 1379.
[13] Nakashima, *J. Pharm. Soc. Japan*, 1955, **75**, 1010.
[14] Gaumann, *Phytopath. Z.*, 1957, **29**, 1.
[15] Stoll and Renz, *ibid.*, p. 380.

fujikuroi (*Fusarium monoiliforme*) N.R.R.L. 2284 were extracted by the method of Borrow *et al.*,[2] giving crude gibberellin (2·30 g.), m. p. 235—238° (decomp.), $[\alpha]_D^{20}$ +53° (11·5 mg./l.) and, on evaporation of the ethyl acetate mother-liquor, a gum (*B*) (3·44 g.).

Crude gibberellin (300 mg.) in ether (200 ml.) was run on to a Celite column (53 × 8 cm.) buffered at pH 7·0, and elution continued with ether previously saturated with water. When solid began to appear in the eluate, aliquot parts (200 ml.) were collected.

(*a*) Eluates 1—52 gave a gum (102 mg.) which on trituration with ethyl acetate and recrystallisation of the resulting solid furnished gibberellin A_1 (15 mg.), prisms, m. p. 255—258° (decomp.), $[\alpha]_D^{20}$ +38° ± 3 (*c* 0·39 in EtOH), p*K* 4·3 (Found: C, 65·3; H, 6·9%; equiv., 354. Calc. for $C_{19}H_{24}O_6$: C, 65·5; H, 6·9%; *M*, 348). The infrared spectrum was identical with that of gibberellin A_1[3] and with the spectrum of a specimen of N.R.R.L. " gibberellin A," m. p. 258° (decomp.), $[\alpha]_D^{20}$ +36·5° (*c* 0·4 in EtOH), supplied by Dr. F. H. Stodola.

The m. p. of gibberellic acid was raised on admixture with gibberellin A_1. Gibberellin A_1 did not give the characteristic red colour and blue fluorescence in concentrated sulphuric acid given by gibberellic acid; it gave a straw yellow solution which slowly developed a weak greenish fluorescence during several days. R_F values (Whatman No. 1 paper; descending chromatograms) for gibberellin A_1 run in 4 : 5 : 1 butanol–water–ammonia solution (*d* 0·88) (bromocresol-green spray) and in 20 : 4 : 2 : 1 chloroform–ethanol–water–formic acid (19 : 1 ethanol–sulphuric acid spray) were identical with those found for gibberellic acid, *viz.*, 0·31 and 0·80 respectively. The fluorescent spot developed by spraying with ethanolic sulphuric acid was very much less intense than that produced by gibberellic acid.

The methyl ester, prepared in methanol with ethereal diazomethane, formed needles, m. p. 234—235° [from ethyl acetate–light petroleum (b. p. 60—80°)], $[\alpha]_D^{20}$ +46° (*c* 0·41 in EtOH), +36·5° (*c* 0·41 in acetone) (Found: C, 66·4; H, 7·4. Calc. for $C_{20}H_{26}O_6$: C, 66·3; H, 7·2%). The infrared spectrum was identical with those of gibberellin A_1 methyl ester [3] and of the methyl ester prepared from N.R.R.L. " gibberellin A."

(*b*) Eluates 53—74 gave a gum (54 mg.), $[\alpha]_D$ +58°, which was a mixture of gibberellin A_1 and gibberellic acid.

(*c*) Eluates 75—154 gave a solid (145 mg.) which on crystallisation from ethyl acetate–light petroleum (b. p. 60—80°) furnished gibberellic acid (134 mg.), m. p. 232—235° (decomp.), $[\alpha]_D$ +90°, identified by its infrared spectrum.

It is not claimed that the above method of separation, worked out independently of that of Stodola *et al.*,[6] has any advantages over the latter: indeed, the recovery of gibberellin A_1 is lower than that achieved by the N.R.R.L. workers.

The gum (*B*) was chromatographed in ether (250 ml.) on Celite (45 × 3 cm.; pH 7·0). Elution was effected with ether (500 ml. portions). All eluates were free from gibberellin A_1 and gibberellic acid, as shown by the absence of an absorption band at 1784 $cm.^{-1}$ (lactone C=O) in dioxan solution.

Eluate 3 gave material (845 mg.) which on crystallisation from ethyl acetate yielded fusaric acid (102 mg.), m. p. 95—98°, purified by sulimation at 90°/10^{-4} mm. and further crystallisation from ethyl acetate. It formed prisms, m. p. 100—101° (Found: C, 66·7; H, 7·3. Calc. for $C_{10}H_{13}O_2N$: C, 67·0; H, 7·3%), identical (mixed m. p. and infrared spectrum) with an authentic specimen. The m. p. of fusaric acid was raised on admixture with dehydrofusaric acid (see below).

Material (659 mg.) from eluates 5—18, on crystallisation from ethyl acetate, sublimation at 80—100°/10^{-4} mm., and two recrystallisations from ethyl acetate, furnished dehydrofusaric acid (243 mg.), prismatic needles, m. p. 118—119° (Found: C, 67·6; H, 6·8; N, 8·1%; equiv., 167. Calc. for $C_{10}H_{11}O_2N$: C, 67·8; H, 6·3; N, 7·9%; *M*, 177), $\lambda_{max.}$ (in EtOH) 230, 270 mμ (log ε 4·00, 3·71), identical (mixed m. p. and infrared spectrum) with an authentic specimen kindly provided by Dr. C. Stoll, E.T.H., Zurich.

Material (421 mg.) from eluate 4 furnished prisms (136 mg.) (from ethyl acetate), m. p. 104—105°, shown by the infrared spectrum to be a mixture of fusaric and dehydrofusaric acid.

Separation of Gibberellin A_1 Methyl Ester and Methyl Gibberellate.—Crude gibberellin, $[\alpha]_D^{20}$ +53° (385 mg.), from strain N.R.R.L. 2284, in methanol (10 ml.) was treated with ethereal diazomethane in ether. After 24 hr. the excess of diazomethane and solvents were removed and the residual gum (400 mg.) was chromatographed in 200 : 1 benzene–methanol (75 ml.) on alumina (15·5 × 2 cm.), fractional elution (50 ml. eluates) being used. Eluates 8—14 [benzene–methanol (200 : 1)], yielded gibberellin A_1 methyl ester (157 mg.), m. p. 225—232°. Eluates

15—17 [benzene–methanol (200 : 1)] and 18—21 [benzene–methanol (50 : 1)] yielded methyl gibberellate (150 mg.), m. p. 200—203°. Both products were identified by their infrared spectra.

In our hands the method of Takahashi *et al.*[3] using ethyl acetate–benzene as eluant was less successful.

Investigation of the Metabolic Products of G. fujikuroi *strain* 917.—(a) *Cultured on the medium of Borrow* et al.[2] In the standard procedure [16] for isolating gibberellic acid the final ethyl acetate extract is concentrated until crystals of gibberellic acid begin to appear and when crystallisation is complete the gibberellic acid is filtered off; for this experiment one-tenth of the final ethyl acetate extract of the culture filtrate (5 l.) was evaporated completely *in vacuo* at 20°, giving a sticky solid (85 mg.), $[\alpha]_D$ +52°. This was chromatographed in ether (100 ml.) on Celite (25 × 1·5 cm.; pH 7), elution being with 100 ml. portions of ether. Material from eluates 3—8 had $[\alpha]_D^{22}$ +88°, m. p. 233—238° (decomp.) (15 mg.) and consisted of gibberellic acid (30 mg./l.). Material (20 mg.) from eluates 9—12 deposited prisms, m. p. 175—180°, of succinic acid on crystallisation from ethyl acetate–light petroleum (b. p. 60—80°).

(b) *Cultured on the medium of Stodola* et al.[5] After extraction of the culture filtrate (67 l.) by the method of Borrow *et al.*[2] the ethyl acetate was removed *in vacuo*, furnishing a gum (101 mg.). This was chromatographed in ether (100 ml.) on Celite as described above. Material (27 mg.), $[\alpha]_D^{20}$ +49°, from eluates 3—5 yielded gibberellin A_1 (4 mg.; 0·06 mg./l) as prisms, m. p. 233—237° (decomp.), identified by the infrared spectrum. Material (18 mg.) from eluates 9—13 gave succinic acid (2 mg.).

A result almost identical with this was obtained by using the methanolic ammonia extraction procedure of Stodola *et al.*[5]

(c) *Isolation of* 5-*hydroxymethylfuran*-2-*carboxylic acid.* On one occasion the crude gibberellic acid from strain no. 917 had an abnormally low m. p. of 155—175° (decomp.). The crude material (10 g.) was heated under reflux with ethyl acetate (900 ml.; charcoal) and, after filtration, boiling light petroleum (b. p. 60—80°; 1 l.) was added. After 2 hr. at room temperature and 24 hr. at 0° the crystalline material was removed, giving gibberellic acid (3·80 g.), m. p. 218—220° (decomp.), $[\alpha]_D^{17}$ +82°. Concentration of the mother-liquors afforded crude 5-hydroxymethylfuran-2-carboxylic acid (4·40 g.), m. p. 155—160° (decomp.), purified by further crystallisation from ethyl acetate. It formed prisms, m. p. 163—165° (decomp.), p*K* 3·5 (Found: C, 50·9; H, 4·6%; equiv., 143. Calc. for $C_6H_6O_4$: C, 50·7; H, 4·3%; *M*, 142), $\lambda_{max.}$ (in EtOH) 258 mμ (log ε 4·13), identical (mixed m. p. and infrared spectrum) with a specimen provided by Dr. H. W. B. Reed, Imperial Chemical Industries Limited, Billingham Division.

Hydrogenation of Methyl Gibberellate.—A solution of methyl gibberellate (3·30 g.) in ethyl acetate (180 ml.) was shaken with 10% palladium–carbon (1·0 g.; prepared from nitric acid-washed carbon [17]) in hydrogen at 19°. When 0·94 mol. of hydrogen had been absorbed (21 min.) the catalyst was filtered off and the filtrate was extracted with sodium carbonate, washed with water, dried (Na_2SO_4), and evaporated, giving a neutral solid (1·83 g.).

The acidified alkaline extract was re-extracted with ethyl acetate, giving on recovery a semicrystalline acidic product (0·68 g.) which will be discussed in a later paper.

The combined neutral products (3·74 g.) from two experiments were fractionally crystallised from ethyl acetate. Fractions with m. p. between 225° and 234° (decomp.) (3·10 g.), $[\alpha]_D^{20}$ +55° ± 3°, were chromatographed in benzene (1·5 l.) on alumina (30 × 5·0 cm.). Elution with 200 : 1 benzene–methanol (500 ml. aliquot parts) gave, after a forerun, (1) 0·35 g., (2) 0·41 g., (3) 0·37 g., (4)—(14), 1·62 g. Further elution with 100 : 1 benzene–methanol gave (15)—(19) 0·15 g.

Recrystallisation of fractions (1) and (2) from ethyl acetate–light petroleum gave *methyl α-dihydrogibberellate* as needles, m. p. 233—235° (decomp.), $[\alpha]_D^{24}$ +46° ± 3° (*c* 1·04 in EtOH), $[\alpha]_D^{24}$ +31° ± 3° (*c* 0·93 in acetone) (Found: C, 66·6, 66·3; H, 7·4, 7·5. $C_{20}H_{26}O_6$ requires C, 66·3; H, 7·2%). In acetic acid in the presence of palladium black it took up 0·7 mol. of hydrogen.

The infrared absorption spectrum was identical with that of gibberellin A_1 methyl ester prepared (as above) by methylation of gibberellin A_1 obtained from *G. fujikuroi*. Mixed m. p. determinations showed no depression.

[16] Curtis and Cross, *Chem. and Ind.*, 1954, 1066.
[17] Vogel, " Practical Organic Chemistry," Longmans, Green and Co., London, 1948, p. 989.

Fraction (3), $[\alpha]_D$ +46°, contained traces (infrared spectrum) of the β-isomer (see below) which was present in increasing amounts in fractions (4)—(14).

Fractions (15)—(19) crystallised from ethyl acetate–light petroleum in needles of *methyl β-dihydrogibberellate*, m. p. 227—230° (decomp.), $[\alpha]_D^{17}$ +74° (*c* 1·02 in EtOH) (Found: C, 66·2; H, 7·3%). Microhydrogenation showed 0·9 ethylenic bond.

Ozonolysis of Methyl α-Dihydrogibberellate.—Ozonised oxygen (100 ml./min., 4·8 mg. of O_3/min.) was passed into a solution of the ester (107 mg.) in acetic acid (10 ml.) at 20° until absorption ceased (10 min.). The solution was diluted with water (10 ml.) and steam-distilled. The distillate was treated with an equal volume of saturated dimedone solution and set aside for 48 hr. Needles, m. p. 182—185° (53·4 mg., 0·6 mol.), of the dimedone derivative of formaldehyde separated from the first 100 ml. of distillate. Recrystallisation from 50% ethanol gave needles (46·2 mg.), m. p. 188—189°, identical (mixed m. p. and infrared spectrum) with an authentic specimen. The non-steam-volatile products will be discussed in a later paper.

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