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GIBBERELLINS AND SUBSTANCES RELATED TO THEM

IV. Comparative Study of the Formation of Neutral Metabolites by Fusarium moniliforme on Various Fermentation Media

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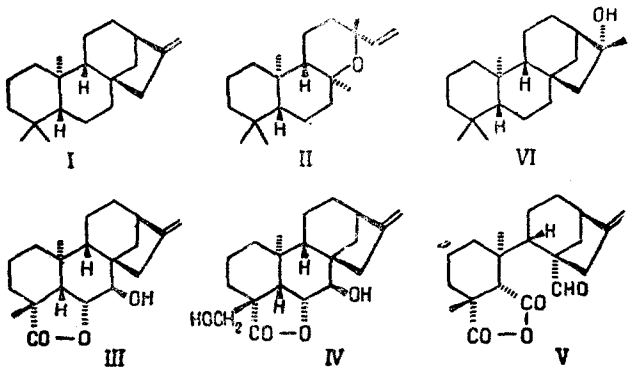
We have previously [1-5] shown that the deep cultivation of the fungus Fusarium moniliforme on media containing vegetable oil or other fats and fatty acids (source of C) and with ammonium nitrate (source of N), considerably enhances the formation of gibberellins. Thus, the yield of gibberellin A_3 under these conditions amounted to not less than 650 mg/*l*, and the yield of gibberellins A_4 and A_7 (combined) was about 80-90 mg/*l*. We have attempted to elucidate the course of the observed stimulation of the biosynthesis of the gibberellins on a medium containing fats. For this purpose we have carried out a comparative study of the formation of neutral diterpenoids by F. moniliforme on three different media.

The first medium (medium 1) was Fusca's medium, which is considered extremely suitable for the microbiological synthesis of the gibberellins [6]. The second medium (medium 2) was a medium with sunflowerseed oil and ammonium nitrate (proposed by us) [7]; it gives a considerably higher yield of gibberellins. Since the dynamics of fermentation on media 1 and 2 are different, we investigated a third variant—with the fractional addition of sucrose. The dynamics of fermentation in this medium are extremely similar to those in medium 2. The yield of gibberellins in this medium exceeded the yield in Fusca's medium although it was less than for the fatty medium. With such a selection of media, we hoped to find how the nature of the substrate and the dynamics of its consumption affect the biosynthesis of the gibberellins.

The F-6 strain of F. moniliforme was cultivated in shaken flasks at 27° C until the maximum formation of gibberellic acid was achieved. After the usual treatment of the culture filtrate [4], an acidic and a neutral fraction of the metabolites were obtained. The neutral fractions were chromatographed on columns of silica gel, and the substances so isolated were identified by known methods. The course of the separation of the substances of the columns was monitored by thin-layer chromatography in silica gel, and the purity of the products obtained was checked by the same method. On fermentation in Fusca's medium (7 days) we isolated (−)-kaurene (I), 13-epi-(−)-manoyl oxide (II), 7β-hydroxykaurenolide (III), 7β,18-dihydroxykaurenolide (IV), and fujenal (V); on fermentation in the fatty medium (12 days) we isolated (−)-kaurene (I), 13-epi-(−)-manoyl oxide (II), (−)-kauran-16α-ol (VI), and a very small amount of 17β,18-dihydroxykaurenolide (IV). In addition, the presence of at least two unassimilated components of the substrate was found in the neutral fraction, one of them being β-sitosterol. Finally, the cultivation of the F-6 strain in a medium with fractionally added sucrose (15 days) led to the production of (−)-kaurene (I), 13-epi-(−)-manoyl oxide (II), (−)-kauran-16α-ol (VI), 7β-hydroxykaurenolide (III), 7β,18-dihydroxykaurenolide (IV), and fujenal (V).



On comparing the three variants of the media, appreciable quantitative differences were found in the contents of several neutral metabolites up to the moment of the maximum accumulation of gibberellic acid in the culture liquid (table).

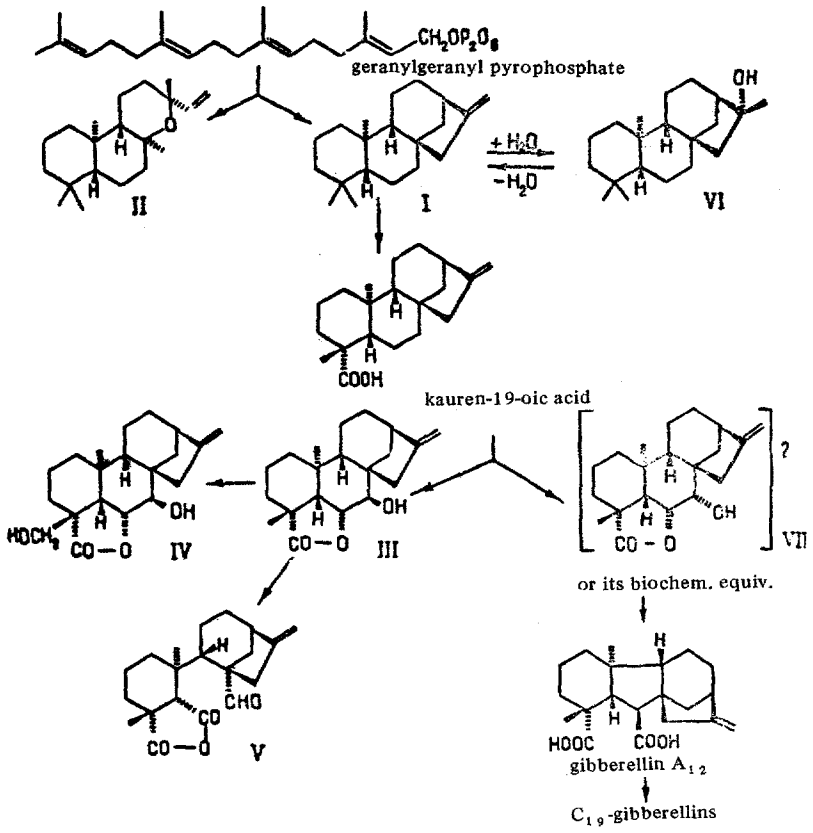
Thus, on media in which the source of C is a carbohydrate, the formation of the kaurenolides III and IV is more pronounced than in a fatty medium. It can also be seen that in carbohydrate media the total concentration of highly oxidized diterpenes—the two kaurenolides III and IV and fujenal (V) is higher than in a fatty medium. At the same time, in media in which the source of carbon is consumed gradually (the variants with sunflowerseed oil and with the fractionally added sucrose), far more kaurene (I) is formed than in Fusca's medium. A completely similar situation has been observed for the PG-7 strain of F. moniliforme (see part V).

Concentration of Neutral Metabolites in the Culture Liquid

Metabolites	Content in the culture liquid, mg/l		
	medium 1	medium 2	medium 3
Kaurene (I)	2—3	12	20
Epimanoyl oxide (II)	1—2	2	1—2
Kauran-16α-ol (VI)	—	2	3
17β-Hydroxykaurenolide (III)	1—2	—	1—2
17β,18-Dihydroxykaurenolide (IV)	3	0.5	30—32
Fujenal (V)	20	16—17	9—10

In experiments with the gradual consumption of the substrate, in addition to an increase in the total yield of gibberellins a considerable increase in the concentration of kaurene (I) and of isokaurene in the culture liquid was observed. Furthermore, the product of the hydration of kaurene—kauran-16α-ol (VI)—is formed in such media, while it has not been possible to detect it in Fusca's medium. The concentration of epimanoyl oxide (II) is small and approximately the same in all variants.

The results obtained may be interpreted with the aid of the scheme of the metabolism of diterpenes in a culture of F. moniliforme given below. This scheme follows from the work of Cross et al [8–12] and of a group of American authors [13–15] using labelled precursors of the gibberellins, and it also takes into account the results of Galt and Hanson on the influence of the stereochemistry at C_7 in the epimeric 7-hydroxykaurenolides on the possibility of the contraction of ring B and the passage from the kaurane system to the gibbane system. The proposed scheme contains several points where the metabolic flow branches.



It is natural to assume that the conditions of fermentation (osmotic pressure, redox potential, and pH of the medium, rate of assimilation of the substrate, etc.) are capable of interfering with the direction of this flow even if only at some points of branching. The first such point is the stage of the cyclization of geranylgeranyl pyrophosphate into kaurene (I) or into epimanoyl oxide (II). Since the concentration of the latter is approximately the same in all cases and the concentration of kaurene in the media gradually rises with the consumption of the substrate, it may be considered that these media displace the metabolism in the direction of the formation of the tetracyclic structure. The stimulation of the biosynthesis of the gibberellins in a medium with a slowly assimilated substrate (starch) or with the fractional addition of glucose observed by Darken et al [17] is apparently due to the same displacement. The second point of branching may

be the competing formation of 7β-hydroxykaurenolide (III) and its 7β-epimer (VII) or, probably, the biochemical equivalent of this epimer, since only the latter is capable of the contraction of ring B with the formation of derivatives of gibberellin A_{12} with the "required" stereochemistry. In carbohydrate media (1 and 3) the total concentration of kaurenolides III and IV and also of fujenal V is higher than in fatty media. This means that the fraction of the metabolic flow directed towards the biosynthesis of the gibberellins is relatively smaller in carbohydrate media than in fatty media.

Thus, the maximum yield of gibberellins in fatty media is possibly due to the conditions of fermentation which makes it possible to displace the metabolic flow in the direction of the formation of gibberellins at both the first and the second points of branching, i.e., both at the cyclization stage and at the stage of the contraction of ring B in the kaurene system.

Experimental

Column chromatography was carried out with type KSK silica gel (100-150 mesh) and thin-layer chromatography with the same silica gel having grain dimensions <250 mesh. The IR spectra were recorded on a UR-10 instrument in molded KBr tablets. Gas-liquid chromatography was carried out in a column containing 10% of SE-30 on silanized Chromosorb W with the addition of 0.5% of neopentyl glycol succinate (length 2 m); the mobile phase was a mixture of nitrogen and hydrogen (consumption 33/4 ml/min, pressure at the inlet 2.3-2.5 atm, flame detector). The column temperature was 175-180° C. The melting points were determined on a "Boetius" stage, and the angles of rotation in a "Hilger" polarimeter.

Fermentation and the separation of the acid and neutral metabolites. A) The F-6 strain was fermented on rotary shakers in flasks with a capacity of 0.7 *l* (volume of the medium 0.15 *l*) at a constant rate of shaking of 210 rpm at 26° C. The concentration of gibberellins in the culture liquid was determined photocolorimetrically with the Folin-Ciocalteu reagent. Fermentation was stopped when the maximum accumulation of gibberellins had been achieved.

Medium 1 (Fusca). Composition: sucrose, 50 g; $(NH_4)_2HPO_4$, 0.4 g; K_2CO_3, 0.4 g; $MgCO_3$, 0.4 g; $(NH_4)_2SO_4$, 0.17 g; $ZnSO_4$, 0.05 g; $FeSO_3$, 0.05 g; soya bean flour 10 g; distilled water 1 *l*. Initial pH, 3.5. Time of fermentation, 7 days. Concentration of gibberellins, 400 mg/*l*; final pH, 2.5.

Medium 2. Composition: sunflowerseed oil, 80 g; NH_4NO_3, 3 g; KH_2PO_4, 2 g; K_2SO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; mixture of trace elements, 1 mg; corn extract 1 ml; distilled water 1 *l*. Initial pH 5.5. Time of fermentation, 12 days Concentration of gibberellins, 790 mg/*l*; final pH, 4.9.

Medium 3. Initial composition: sucrose, 40 g; NH_4NO_3, 3 g; KH_2PO_4, 2 g; K_2SO_4, 0.2 g; $MgSO_4 \cdot 7 H_2O$, 0.2 g; mixture of trace elements, 1 mg; corn extract 1 ml; distilled water 1 *l*. Initial pH 5.5. After each period of 48 hr, 2% of sucrose was added to the medium; a total of 18% of sucrose was added, at the start. Time of fermentation, 15 days. Concentration of gibberellins, 700 mg/*l*; final pH 3.5.

The filtrates of the culture liquid were extracted twice with butanol at pH 2.5 (0.2 and 0.1 of the volume of the filtrate), and the extracts were separated into acid and neutral fractions by a published method [3].

Analysis of the metabolites of the F-6 strain. B) Medium 1. The neutral resin (1.06 g) obtained from 5.5 *l* of culture filtrate was chromatographed on a column containing 58 g of silica gel, 100-ml fractions being collected. Fractions 1 and 2 (hexane) gave 17 mg of an oil which crystallized on standing. Recrystallization from methanol yielded 9 mg of pure (−)-Kaurene (I) with mp 50.5-51.5° C, $[\alpha]_D^{21}$ −78° (c 0.55; chloroform). IR spectrum: 2930 cm^{-1}, 2860, 2840, 1660, 1450, 1376 and 890 cm^{-1} (cf. [18]).

Fractions 4 and 5 (hexane) gave 8.5 mg of a semicrystalline mass; crystallization from ethanol yielded 4.5 mg of pure 13-epi-(−)-manoyl oxide (II), identical with the sample obtained previously [4].

Fractions 6-20 (from 5 to 15% of ethyl acetate in hexane) consisted of 53 mg of a chromatographically inhomogeneous resin.

Fractions 21-23 (17.5% of ethyl acetate in hexane) consisted of 51 mg of a semicrystalline residue; the latter was chromatographed on a preparative silica gel plate [22 × 20 × 0.2 cm, chloroform—ehtyl acetate—methanol (90 : 8 : 2) system] to give 6 mg of 7β-hydroxykaurenolide (III) with mp 182-187° C $[\alpha]_D^{20}$ −23° (c 0.45; ethyl acetate). IR spectrum: 3530 cm^{-1}, 1760, 1660, 1465, 1040 and 880 cm^{-1} (Cf. [18]).

Fractions 24-25 (from 20 to 27.5% of ethyl acetate in hexane) consisted of 50 mg of a chromatographically inhomogeneous resin.

Fractions 36-41 (30 and 32.5% of ethyl acetate in hexane) consisted of 193 mg of a resin which partially crystallized on standing; it was chromatographed on two preparative silica gel plates [22 × 20 × 0.2 cm, chloroform—methanol (95 : 5) system], giving 19 mg of crystals with mp 191-203° C. Recrystallization from a mixture of hexane and ethyl acetate gave 15 mg of pure 7β,18-dihydroxykaurenolide (IV) with mp 214-218° C, $[\alpha]_D^{21}$ −39° (c 0.65; ethyl acetate). IR spectrum:

3565 cm^{-1}, 3480, 3220, 1762, 1755 (shoulder), 1660, 1050, and 880 cm^{-1} (cf. [18]). The action on IV of acetic anhydride in pyridine (20° C, 36 hr) gave a diacetate with mp 165–169° C (from a mixture of hexane and benzene); IR spectrum: 2950 cm^{-1}, 1775, 1745, 1660 and 865 cm^{-1} (cf. [19]).

Further elution with more polar mixtures gave only chromatographically inhomogeneous resins. In this experiment, fujenal (V) appeared in the acid fraction; when the latter was chromatographed on a column of silica gel treated with phosphate buffer at pH 6.2, chloroform elution yielded 108 mg of pure fujenal with mp 166–168° C and $[\alpha]_D^{20}$ −71°, identical with the sample described previously [4].

Medium 2. The neutral resin (8.59 g) obtained from 5 *l* of culture filtrate was chromatographed on a column containing 435 g of silica gel, 500-ml fractions being collected.

Fractions 2 and 3 (hexane) gave 429 mg of a colorless oil which was rechromatographed on 43 g of alumina (activity grade II). The material from the first fractions eluted with hexane were crystallized from methanol, giving 60 mg of pure (−)-kaurene (I) with mp 51–52° C, $[\alpha]_D^{20}$ −79.5° (c. 0.75; chloroform), IR spectrum identical with that of the sample described above.

Fractions 4 and 5 (hexane)—78 mg of chromatographically inhomogeneous resin—were rechromatographed on 5 g of alumina (elution with hexane). After crystallization of the middle fractions from methanol, 10 mg of pure 13-epi-(−)-manoyl oxide (II) was obtained with mp 95–97° C, $[\alpha]_D^{21}$ −36° (c 0.45; chloroform), identical with the sample isolated previously [4].

Fractions 6–8 (5% of ethyl acetate in hexane) were blank.

Fractions 9–14 (7.5 and 10% of ethyl acetate in hexane) consisted of 4.850 g of a viscous yellow oil the main spot of which, according to thin-layer chromatography, was identical with one of the components of the substrate.

Fractions 15 and 16 (12.5% of ethyl acetate in hexane) consisted of 664 mg of a resin which partially crystallized; chromatography on preparative alumina plates [activity grade III/IV, 22 × 20 × 0.2 cm, benzene–ethyl acetate 85 : 15) system] yielded 75 mg of a semicrystalline mass (zone with R_f values of 0.25 to 0.40) from which two crystallizations from ethyl acetate gave 10.2 mg of pure (−)-kauran-16α-ol (VI) with mp 213–215° C (sublimes with melting in the range from 145–165° C), $[\alpha]_D^{20}$ −41.5° (c 0.45; methanol). IR spectrum: 3330 cm^{-1}, 2960, 2930, 2865, 2840, 1465, 1120, 1070 and 935 cm^{-1}. On evaporation, the mother liquor from the crystallization gave another 27 mg of crystals with mp 120–140° C and $[\alpha]_D^{20}$ −41.5°, identical chromatographically with the pure samples. To clinch the matter, the structure of the (−)-kauran-16α-ol isolated was confirmed by its independent sysnthesis from (−)-kaurene (see below, B).

Fractions 17 and 18–20 (12.5 and 15% of ethyl acetate and hexane) consisted of 844 mg of a semicrystalline mass from which, by the method described previously [4], we isolated 230 mg of β-sitosterol with mp 138–140° C and $[\alpha]_D^{21}$ −26°.

Fractions 21–32 (from 17.5 to 25% of ethyl acetate in hexane) consisted in total complexity of 582 mg of chromatographically inhomogeneous resin.

Fractions 33–38 (30–35% of ethyl acetate in hexane) consisted of 217 mg of a resin which was chromatographed on two preparative silica gel plates [22 × 20 × 0.2 cm in the chloroform–methanol (95 : 5) system]; the lower band was eluted, the eluate was evaporated, and the residue was crystallized from a mixture of hexane and ethyl acetate to give 2.3 mg of pure 7β,18-dihydroxykaurenolide (IV) with mp 218–220° C having an IR spectrum identical with that of the sample described above. Further elution of the column with more polar mixtures (up to pure methanol) gave only a chromatographically inhomogeneous resin. When the acid fraction was chromatographed on buffered silica gel, 84 mg of pure fujenal was isolated [elution with a mixture of hexane and chloroform (1 : 3)]; it had mp 166–168° C (from a mixture of hexane and ethyl acetate), identical with that of the sample described previously [4].

Medium 3. The neutral resin (1.59 g) obtained from 4.4 *l* of culture liquid was chromatographed on a column containing 88 g of silical gel, 200-ml fractions being collected.

Fraction 1 (hexane) gave 39 mg of a semicrystalline mass, and fractions 2 and 3 (hexane) 83 mg of pure crystals with mp 51–52.5° C. According to gas-liquid chromatography, all three fractions consisted of mixtures of (−)-kaurene (i) with (−)-isokaurene in a proportion of 1 : 3.

Fractions 4 and 5 (hexane) consisted of 18 mg of an oil which was chromatographed on 1 g of alumina (activity grade II; elution with hexane); 55 mg of pure 13-epi-(−)-manoyl oxide with mp 94–96° C (from methanol) was isolated. Its IR spectrum was identical with that of a sample isolated previously [4].

Fractions 6–11 (5 and 7.5% of ethyl acetate in hexane) consisted of 110 mg of a yellow resin.

Fraction 12 (10% of ethyl acetate in hexane) consisted of 45.5 mg of crystals contaminated with oil; after recrystallization from ethyl acetate, 14 mg of pure (−)-kauran-16α-ol (VI), with mp 210–213° C, identical with the sample described above, was obtained.

Fractions 13–20 (from 10 to 15% of ethyl acetate in hexane) consisted of 204 mg of a chromatographically inhomogeneous resin.

Fractions 21–23 (17.5% of ethyl acetate in hexane) consisted of 28.5 of a resin which partially crystallized; chromatography on preparative silica gel plates [22 × 8 × 0.2 cm; chloroform–ethyl acetate–methanol (90 : 8 : 2) system] led to the isolation from it of 7 mg of 7β-hydroxykaurenolide (III) with mp 186–189° C (from a mixture of hexane and ethyl acetate) identical with the sample described above.

Fractions 24–30 (from 20 to 25% of ethyl acetate in hexane) consisted of 56 mg of chromatographically inhomogeneous resin.

Fractions 31–33 (25 and 27.5% of ethyl acetate in hexane) consisted of 128 mg of pure crystals with mp 214–218° C; recrystallization from a mixture of hexane and ethyl acetate gave 120 mg of pure 7β, 18-dihydroxykaurenolide (IV) with mp 218–220° C, $[\alpha]_D^{22}$ −40° (c 0.55; methanol), identical with the sample described previously. Further elution of the column with more polar mixtures gave only chromatographically inhomogeneous resins.

In this experiment, fujenal (V) was unexpectedly found in the more polar fractions of the acid resin, from which 40 mg of crystals with mp 162–165° C and $[\alpha]_D$ −57° were isolated. Moreover, an additional amount of 7β, 18-dihydroxykaurenolide (IV) was found in the acid resin.

Identification of the (−)-kaurene derivatives. B) (−)-Isokaurene. One small crystal of iodine was added to a solution of 5 mg of pure (−)-kaurene (retention time 26 min), and the mixture was boiled for 6 hr [21]. Then it was washed with sodium hyposulfite, and the organic layer was concentrated and analyzed by gas-liquid chromatography. The retention time of the peak that had appeared (−)-isokaurene (22.5 min) accurately coincided with the retention time of the first component in mixtures obtained by the chromatography of some neutral fractions of the F-6 strain (medium 3) and the PG-7 strain (medium 1).

(−)-Kauran-16-ol. A misture of kaurene and isokaurene (15 mg) was dissolved in ether and, with cooling to −15° C, a current of dry hydrogen chloride was passed through the solution. This gave 15 mg of kaurene hydrochloride with mp 110–112° C (from ethyl acetate). Literature data: mp 114–115° C [21]. The hydrochloride was dissolved in 15 ml of ether saturated with aqueous sodium bicarbonate solution, and the solution was left at +5° C for 15 days. The solvent was distilled off and the residue was filtered through 0.1 g of alumina (in benzene) to give 2 mg of pure (−)-kauran-16α-ol (VI) with mp 211–213° C (from ethyl acetate), identical with the sample described above.

Conclusions

1. The neutral metabolites elaborated by Fusarium moniliforme (strain F-6) in various medi have been chromatographed and six known diterpenoids biogenetically related to the gibberellins have been identified.

2. The influence of the rate of consumption of the source of C during fermentation and its chemical nature on the course of the biosynthesis of the gibberellins have been noted.

3. Considerations have been put forward concerning the mechanism of the stimulation of the biosynthesis of the gibberellins in media containing fats which are based on modern ideas concerning the metabolism of diterpenes in a culture of F. moniliforme.

REFERENCES

1. G. S. Muromtsev and L. P. Dubovaya, USSR patent no. 165 614 of 27 February 1963; Byulleten isobretenii, no. 19, 1963.

2. G. S. Muromtsev and L. P. Dubovaya, Mikrobiologiya, **33**, 1048, 1964.

3. G. S. Muromtsev, V. N. Agnistikova, L. M. Lupova, L. P. Dubovaya, T. A. Lekareva, V. F. Kucherov, and E. P. Serebryakov, Mikrobiologiya, **35**, 1037, 1966.

4. E. P. Serebryakov, V. F. Kucherov, and G. S. Muromtsev, KhPS [Chemistry of Natural Compounds], **2**, 55, 1966.

5. G. S. Muromtsev, L. P. Dubovaya, and Yu. S. Rakovskii, Prikl. Biokhim. i Mikrobiol. [Applied Biochemistry and Microbiology], **2**, 401, 1966.

6. J. Fusca, I. Kuhr, M. Podojil, and V. Ševcik, Folia microbiol., **6**, 1, 1961.

7. G. S. Muromtsev, Yu. S. Rakovskii, L. P. Dubovaya, G. V. Tomnikova, and A. N. Fedchenko, Prikl. biokhim. i mikrobiol. [Applied Biochemistry and Microbiology], **4**, 398, 1968.

8. E. E. Cross, R. H. B. Galt, and I. R. Hanson, Regulateurs Naturels de la Croissance Vegetale, Paris, 265, 1964.

9. E. E. Cross, R. H. B. Galt, and I. R. Hanson, J. Chem. Soc., 295, 1964.

10. E. E. Cross and K. Norton, Chem. Comm., London, 535, 1965.

11. R. H. B. Galt, J. Chem. Soc., 3143, 1965.
12. B. E. Cross and K. Norton, Tetrah. let., no. 48, 6003, 1966.
13. J. E. Graebe, D. T. Dennis, C. D. Upper, and C. A. West, J. Biol. Chem., 240, 1847, 1965.
14. D. T. Dennis, C. D. Upper, and C. A. West, Plant Physiol., **40**, 948, 1965.
15. T. A. Geissman, A. I. Verbiscor, B. D. Phinney, and G. Cragg, Phytochemistry, **5**, 933, 1966.
16. R. H. B. Galt and I. R. Hanson, J. Chem. Soc., 1565, 1965.
17. M. A. Darken, A. L. Jensen, and P. Shur, Appl. Microbiol., **7**, 301, 1959.
18. B. E. Cross, R. H. B. Galt, J. R. Hanson, P. J. Curtis, J. F. Grove, and A. Morrison, J. Chem. Soc., 2937, 1963.
19. B. E. Cross, R. H. B. Galt, and J. R. Hanson, J. Chem. Soc., 3783, 1963.
20. R. A. Bell, R. E. Ireland, and L. N. Mauder, J. Org. Chem., 31, 2536, 1966.
21. L. H. Briggs and R. W. Cawleg, J. Chem. Soc., 1888, 1948.

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