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THE EFFECT OF PLANT GROWTH RETARDANTS ON THE BIOSYNTHESIS OF DITERPENES BY *GIBBERELLA FUJIKUROI*

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Abstract—Addition of CCC (11 mg/l) and Amo-1618 (10 mg/l) to growing cultures of *Gibberella fujikuroi*, at the beginning of the gibberellic acid production phase, almost completely suppresses the biosynthesis of gibberellic acid and of the diterpenes (−)-kaurene, 7-hydroxykaurenolide and 7,18-dihydroxykaurenolide.

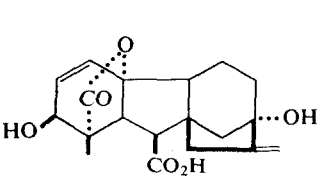
THE EFFECT of the plant growth retardants (2-chloroethyl)-trimethylammonium chloride (CCC or cycocel) and 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl-piperidine-1-carboxylate (Amo-1618) on the production of gibberellins by *Gibberella fujikuroi* has been investigated by Lang and his co-workers.[1–3] They found that these compounds did not affect the growth of the fungus (strain Lilly M-45-339), but that the biosynthesis of gibberellins was suppressed. Gibberellin production, which was low in their controls, was estimated by a bioassay which depended for its validity on the separation of the gibberellins from residual growth retardant by partition techniques. Nevertheless, Lang's results are not unconvincing.[4, 5] In a paper which became available whilst our work was in progress, Adamiec[6] reported a detailed study with *G. fujikuroi* which showed conclusively that CCC suppresses the formation of gibberellic acid (I).

Harada and Lang[3] suggested that CCC prevents the biosynthesis of the gibberellins by blocking a step in their formation from (−)-kaurene (II)[7] by *G. fujikuroi*. West and his co-workers[8] working with an isolated enzyme system from *Echinocystis macrocarpa* also obtained evidence suggesting that CCC did not prevent the biosynthesis of (−)-kaurene. However, more recently, both Anderson and Moore[9] and West's group[10] have shown that high doses of CCC do inhibit the biosynthesis of (−)-kaurene by higher plant enzyme systems. The action of Amo-1618, however, may be different, since even low doses almost completely prevent the conversion of mevalonate into (−)-kaurene by some plant enzyme systems.[8–10]

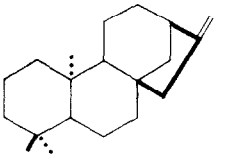
In view of the importance of Lang's conclusions it seemed desirable to investigate the effect of CCC and Amo-1618 on *G. fujikuroi* grown under fermentation conditions which

[1] H. KENDE, H. NINNEMANN and A. LANG, *Naturwiss.* **50**, 599 (1963).
[2] H. NINNEMANN, J. A. D. ZEEVAART, H. KENDE and A. LANG, *Planta* **61**, 229 (1964).
[3] H. HARADA and A. LANG, *Plant Physiol.* **40**, 176 (1965).
[4] P. W. BRIAN, *Intern. Rev. Cytol.* **19**, 247 (1966).
[5] B. E. CROSS, *Progr. Phytochem.* **1**, 195 (1968).
[6] A. ADAMIEC, *Acta. Soc. Botan. Polon.* **35**, 489 (1966).
[7] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).
[8] D. T. DENNIS, C. D. UPPER and C. A. WEST, *Plant Physiol.* **40**, 948 (1965).
[9] J. D. ANDERSON and T. C. MOORE, *Plant Physiol.* **42**, 1527 (1967).
[10] C. A. WEST, M. OSTER, D. ROBINSON, F. LEW and P. MURPHY, personal communication.

would give higher yields of gibberellic acid. Since such fermentations normally produce[11] the diterpenes (−)-kaurene (II), 7-hydroxykaurenolide (III), and 7,18-dihydroxykaurenolide (IV), they could readily be used to study the point at which CCC acts. Furthermore if it were confirmed that CCC blocks a "post-kaurene" reaction in the biosynthesis of gibberellic acid, fermentations containing CCC might be expected to accumulate a precursor of the gibberellins.



(I)



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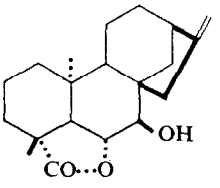
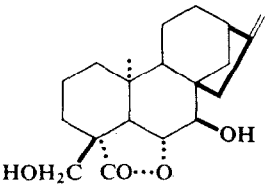


(III)



(IV)

G. fujikuroi ACC. 917 was grown in the dark[12] on a glucose-ammonium tartrate medium[13] in stirred aerated fermenters. When all the ammonium nitrogen had been consumed, i.e. at the beginning of gibberellic acid production,[13] the growth retardant was added in sterile aqueous solution and the fermentation was continued for about 90 hr. The metabolites were extracted and separated into crude acid and neutral fractions as previously described,[14] and the gibberellic acid was isolated by chromatography and purified as its methyl ester.[14] The neutral fractions, which contained antifoam, were subjected to a preliminary column chromatography and the (−)-kaurene, 7-hydroxykaurenolide and 7,18-dihydroxykaurenolide in the column fractions were determined by gas-liquid chromatography (GLC). The results obtained are shown in Table 1.

Lang and his co-workers[1,2] found that Amo-1618 at concentrations of 100 and 300 mg/l and CCC at concentrations of 3–300 mg/l, when present throughout the fermentation, almost completely suppressed the low yield (~10 μg/ml in the control) of gibberellic acid given by a weak growth (~4 mg dry weight of mycelium/ml) of *G. fujikuroi*, although the dry weight of the mycelium was not significantly affected. The results in the Table show that biosynthesis of gibberellic acid (~50 μg/ml in the control*) in vigorously growing cultures (25 mg dry weight of mycelium/ml) of fungus (strain ACC. 917) is also effectively inhibited by Amo-1618 and CCC. Furthermore concentrations of only 10 mg/l and 1 mg/l of Amo-1618 caused complete suppression and 63 per cent inhibition of gibberellic acid production respectively.

* This figure, which is based upon the amount of pure methyl gibberellate isolated, would be much higher if it had been determined by a bioassay as used by Lang and his co-workers.[1,2]

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[12] cf. D. MERTZ and W. HENSON, *Physiol. Plantarum* **20**, 187 (1967).

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TABLE 1. INHIBITION OF DITERPENOID PRODUCTION IN *Gibberella fujikuroi* BY CCC AND AMO -1618*

Additions	Dry weight of mycelium g/l	Crude acids g/10 l	Methyl gibberellate mg/10 l	% control‡	7-Hydroxy-kaurenolide (III) mg/10 l	% control‡	7,18-Dihydroxy-kaurenolide (IV) mg/10 l	% control‡	Kaurene (II) mg/10 l	% control‡
None†	25	2·5	532	100	26	100	115	100	37	100
Amo-1618 10 mg/l	23	0·41	5	1	0·7	6	2	4	0	0
Amo-1618 1 mg/l	—	1·52	130	37	3	15	34	34	—	—
CCC 11 mg/l	26	0·38	15	3	2	8	6	5	0·6	1·6
CCC 1·1 mg/l	—	1·44	114	24	—§	—	—§	—	4	4

* Crude neutral fractions were not weighed because they contained antifoam.

† Control fermentations were run for every addition but only 1 representative experiment is recorded here.

‡ % Control refers to the yield of metabolite from the fermentation containing inhibitor expressed as a percentage of the yield from the control fermentation which was run simultaneously.

§ This experiment gave poor GLC data and accurate measurements of the amounts of kaurenolides present could not be made. However the results showed that the content of both 7-hydroxykaurenolide and 7,18-dihydroxykaurenolide was much less than in the control.

In these experiments the growth retardant was added when the ammonium nitrogen was exhausted, i.e. after cell division of the mycelium had ceased and when gibberellic acid production was beginning.[13] This ensured that the retardant did not affect in any way the growth and condition of the mycelium needed for gibberellic acid production.

Amo-1618 also caused a similar inhibition of the production of the diterpenes (−)-kaurene (II), 7-hydroxykaurenolide (III) and 7,18-dihydroxykaurenolide (IV). This suggests that Amo-1618 inhibits the formation of all tetracyclic diterpenes by *G. fujikuroi*, and that its site of action in the biosynthetic pathway to (−)-kaurene may be the same in the fungus as it is[8,9] in isolated higher plant enzyme systems. If this is so, addition of Amo-1618 to fermentations would not be likely to lead to the accumulation of interesting biosynthetic precursors of the gibberellins.

Thin-layer chromatography (TLC) of the crude acid fraction from the fermentation to which Amo-1618 (10 mg/l) had been added revealed a strong spot running just in front of gibberellic acid; there were no other spots indicating isolable amounts of metabolites. Subsequently a weak spot, at a similar R_f, was observed for the control fermentation. Column chromatography of the crude acids led to the isolation of the compound responsible for this TLC spot; it was identified as 5-hydroxymethyl-2-furoic acid, a known[15] metabolite of *G. fujikuroi*.

The Table shows that CCC is as effective as Amo-1618 in inhibiting the production by *G. fujikuroi*, not only of gibberellic acid, but also of (−)-kaurene, 7-hydroxykaurenolide and 7,18-dihydroxykaurenolide. Thus the hope (cf. Ref. 3) that addition of CCC to a fermentation would block a "post-kaurene" step in the biosynthesis of gibberellic acid was not realised. The potency of CCC against *G. fujikuroi* is in contrast to its relative ineffectiveness in inhibiting the biosynthesis of (−)-kaurene by higher plant enzyme systems.[9,10]

Comparison by TLC of the crude acid fractions from the fermentations to which CCC had been added, with those of the controls, did not reveal spots corresponding to any new metabolites. Thin-layer chromatograms of the crude neutral fractions are usually too complex to be used to distinguish new metabolites. However column chromatography of the neutral fraction from the fermentation containing CCC (1·1 mg/l), followed by examination of the fractions by TLC, failed to reveal any evidence for the presence of compounds not produced by the control fermentations.

EXPERIMENTAL

The following chromatographic materials were used: silica gel (Whatman Chromedia SG 31) and alumina (Woelm neutral alumina, grade II). Light petroleum refers to the fraction b.p. 60–80°.

TLC was carried out on silica gel G (E. Merck, A.-G., Darmstadt).

Gas-Liquid Chromatography

Instrument and column. Retention times were determined with a Varian 1527B gas chromatograph fitted with a flame-ionization detector. Silanized glass columns 150 × 0·3 cm i.d. were packed with 2% QF-1 on silanized Anakrom and stabilized at 240° overnight. N_2 was used as the carrier gas.

Organism and Inoculation

G. fujikuroi (Saw.) Wr. strain ACC. 917 was grown for 7–14 days in 8 oz medicine bottles on isolation medium.[16] The mycelium from 1 bottle was used to inoculate 4 × 500 ml conical flasks each containing 150 ml of the following medium: glucose, 50 g/l; ammonium tartrate, 2·5 g/l; KH_2PO_4, 5 g/l; $MgSO_4, 7H_2O$, 1 g/l; and minor elements concentrate,[17] 1 ml/l. The flasks were shaken at ~30° for ~40 hr in natural light.[12]

[15] A. KAWARADA, N. TAKAHASHI, H. KITAMURA, Y. SETA, M. TAKAI and S. TAMURA, *Bull. Agric. Chem. Soc. Japan* **19**, 84 (1955).

[16] E. G. JEFFERYS, P. W. BRIAN, H. G. HEMMING and D. LOWE, *J. Gen. Microbiol.* **9**, 314 (1953).

[17] P. W. BRIAN, P. J. CURTIS and H. G. HEMMING, *Trans. Brit. Mycol. Soc.* **29**, 173 (1946).

Stirred Fermentations

10 l. of a medium (cf. Ref. 13) containing (g/l): glucose, 100; ammonium tartrate, 4·6; KH_2PO_4, 5; $MgSO_4$, $7H_2O$, 1; and minor elements concentrate,[17] 2 ml was placed in each of two identical stainless steel fermenters (Taylor Rustless Fittings Co. Ltd., Leeds). Each fermenter was inoculated with the contents of 2 × 500 ml conical flasks (from above), stirred at 600 rev/min, and aerated at 5 l/min. Arachis oil or polypropylene glycol (M.W. 1025) was added as antifoam. The fermenters were immersed in a constant temperature water-bath, maintained at 25°, and samples were removed periodically. When (after ~30 hr) analysis showed that the concentration of ammonium ion in the filtered medium was approximately zero, an aqueous solution of the growth retardant, previously sterilized by means of a Seitz filter, was added to one of the fermenters. The other fermenter was run as the control. The fermentations were continued for 88–94 hr before harvest.

Extraction of the Mould Metabolites

At harvest the mycelium was removed by filtration, washed with water, and rejected. In those experiments where the dry weight of mycelium was determined 1 l. of the culture was filtered and the pad of mycelium was washed with water (200 ml) and dried at 80° for 24 hr.

The filtrate and washings from the mycelium were combined and acidified to pH = ~2 with conc. HCl and extracted with AcOEt (3 × 0·1 volume). The combined AcOEt extracts were extracted 3 times with $NaHCO_3$ soln. and then washed with water. Evaporation of the AcOEt gave the crude neutral metabolites contaminated with much of the antifoam. The $NaHCO_3$ extracts were combined, acidified with conc. HCl and extracted with AcOEt. Evaporation of the AcOEt extracts afforded the crude acid fraction.

Isolation of Gibberellic Acid as its Methyl Ester

The crude acid fractions were chromatographed on silica gel, e.g. 2·5 g acids on a column (20 × 3 cm), and eluted with AcOEt. The combined gibberellic acid fractions were methylated with ethereal CH_2N_2 and the product chromatographed on silica gel. Elution with AcOEt-light petroleum (4:1) and AcOEt gave methyl gibberellate which was crystallized from benzene-methanol, m.p. 207–211°.

*Isolation of 5-Hydroxymethyl-2-Furoic Acid from the Fermentation Treated with Amo-*1618 (10 *mg/l*)

TLC of the crude acids in AcOH-di-isopropyl ether (5:95) gave, after spraying with EtOH-H_2SO_4 (95:5) and heating to 100–150° for ~5 min, an intense black spot running in front of the gibberellic acid. Elution of the acids (0·41 g) from silica gel with AcOEt-$CHCl_3$ (4:1) gave 5-hydroxymethyl-2-furoic acid (35 mg) which crystallized from AcOEt-light petroleum as needles, m.p. 164·5–165·5°, (decomp.) identified by its i.r. spectrum.

Determination of (−)-*Kaurene* (*II*), 7-*Hydroxykaurenolide* (*III*) *and* 7,18-*Dihydroxykaurenolide* (*IV*) *in the Neutral Fractions*

The crude neutral fractions were chromatographed on alumina (20–25 × 1·5 cm). The first fraction, which was eluted with light petroleum (500 ml), contained the (−)-kaurene and some of the 7-hydroxykaurenolide. It was evaporated to dryness to give residue (A).

The second fraction, eluted with AcOEt (500 ml), contained most of the 7-hydroxykaurenolide and the 7,18-dihydroxykaurenolide. Evaporation to dryness gave residue (B).

Residue (A) was dissolved in $CHCl_3$ and the solution was made up to 5 ml. Samples (0·5 and/or 1·0 μl) were analysed by GLC using a column temperature of 142°. Comparison of the peak area (measured by peak height × width at half-height) due to (−)-kaurene (retention time 6·5 min) with those given by standard solutions of (−)-kaurene enabled the concentration of (−)-kaurene to be determined.

The $CHCl_3$ solution of residue (A) was combined with residue (B) and the solution made up to 10 ml. Aliquots (0·5 μl) were analysed by GLC (column temperature 209°). The concentrations of 7-hydroxykaurenolide (retention time 21 min) and 7,18-dihydroxykaurenolide (retention time 25 min) were calculated by comparison of peak areas (determined from the weight of cut-out peaks) with those given by standard solutions of the two kaurenolides.

Chromatography of the Neutral Fraction from the Fermentation treated with CCC (1·1 *mg/l*)

The neutral fraction was eluted from alumina (20 × 1·5 cm) with AcOEt-light petroleum to give the following fractions (100 ml) (percentage AcOEt in parentheses): (i) (0), (ii) (0), (iii) (10), (iv) (20), (v) (30), (vi) (40), (vii) (50), (viii) (60), and (ix) (100). Examination of the fractions by TLC in EtOH-benzene (1:9) failed to reveal any spots corresponding to new metabolites.

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