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6β,7β-DIHYDROXYKAURENOIC ACID: ITS BIOLOGICAL ACTIVITY AND POSSIBLE ROLE IN THE BIOSYNTHESIS OF GIBBERELLIC ACID*

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Abstract—[17-^{14}C]6β,7β-Dihydroxykaurenoic acid (VIII) has been prepared and fed to *Gibberella fujikuroi*. It did not serve as a precursor of gibberellic acid but it was incorporated (9·7 per cent) into fujenal. Dilution analysis showed that the dihydroxy-acid (VIII) is a metabolite of *G. fujikuroi* and in four bioassays for gibberellins it was more active than kaurenoic acid and the aldehydo-acid (VI) so that the possibility that a derivative of the dihydroxy-acid (VIII), e.g. a 6-pyrophosphate, is a precursor of the gibberellins cannot be excluded.

INTRODUCTION

THE BIOSYNTHESIS[1-3] of the gibberellins, e.g. gibberellic acid (I), by *Gibberella fujikuroi* must involve the oxidation of ring B of (−)-kaurene[2] (II), or its derivatives[4-6] (III), (IV) or (V), followed by ring contraction. If the kaurenoid intermediate is a 6,7-diol it can be predicted[7] that the 6-hydroxyl should be equatorial (β) and it follows[8,9] that the precursor would then be 6β,7β-dihydroxykaurenoic acid (VIII).

RESULTS AND DISCUSSION

6β,7β-Dihydroxykaurenoic acid (VIII) was prepared from the hydroxy-ester[7] (XIV) by an unexceptional route. The hydroxy-ester was converted into its nor-ketone tosylate (XV) and the latter was treated with boiling collidine to give the 6-ene (XVII). Osmylation of the 6-ene afforded a diol, shown to be the 6β,7β-compound (X) because, (*a*) its NMR spectrum (in pyridine) showed the 6-proton as a double doublet at 5·29 τ ($J_{5,6}$ = 11 and $J_{6,7}$ = 2·5 c/s) and the 7-proton as a doublet at 6·08 τ ($J_{6,7}$ = 2·5 c/s), and (*b*) it was different from the known[13]

* Part IV in the series "The Biosynthesis of the Gibberellins"; for Part III, see Ref. 1.

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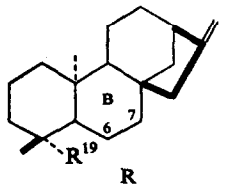
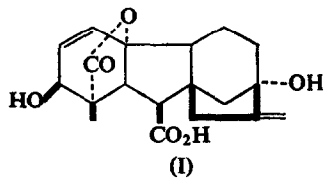
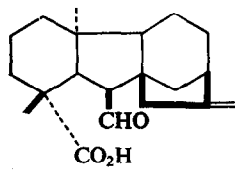


R

(II) Me
(III) CH_2OH
(IV) CHO
(V) CO_2H



(I)



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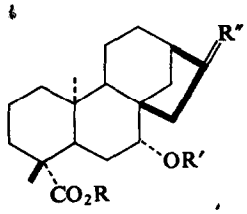
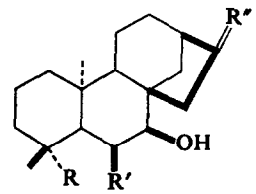


	R	R′	R″
(XIV)	Me	H	CH_2
(XV)	Me	Ts	O
(XVI)	H	H	CH_2



	R	R′	R″
(VII)	CO_2H	H	CH_2
(VIII)	CO_2H	OH	CH_2
(IX)	CO_2H	OH	$^{14}CH_2$
(X)	CO_2Me	OH	O
(XI)	CO_2Me	OH	$^{14}CH_2$
(XII)	CO_2H	OH	O
(XIII)	CH_2OH	OH	$^{14}CH_2$

methyl 6α-7α-dihydroxykaurenoate. Reaction of the diol (X) with the Wittig reagent $^{14}CH_2 = PPh_3$ did not yield the expected ester (XI), but the acid (IX) in very low yield. A higher overall yield of acid (IX) was obtained by demethylating the dihydroxy-ester (X) with lithium iodide in collidine to give the acid (XII) before carrying out the Wittig reaction. The NMR spectrum of the acid (XII) supported the assignment of the 6β,7β-configuration to the diol function.

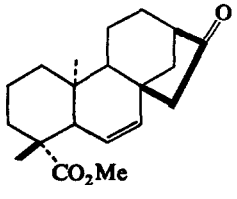
Addition of the labelled dihydroxy-acid (IX) to a fermentation[7] of *Gibberella fujikuroi* and isolation[1,7] of the metabolites afforded inactive methyl gibberellate and 7-hydroxykaurenolide (XVIII) and highly active fujenal (XIX).[14] Although no attempt was made to isolate all of the fujenal the incorporation was 9·7 per cent. Repetition of the fermentation gave almost identical results. Ozonolysis of the fujenal and isolation of the formaldehyde as its dimedone derivative showed that 91 per cent of the radioactivity resided in the terminal methylene group. Similarly, the [17-^{14}C]triol (XIII), prepared by reducing the ester (XI) with lithium aluminium hydride, was not incorporated by *G. fujikuroi* into gibberellic acid whilst its incorporation into fujenal was low (0·36 per cent).

The failure of *G. fujikuroi* to incorporate the 6β,7β-dihydroxy-acid (IX) into gibberellic acid was disappointing, particularly since (*a*) both 7β-hydroxykaurenoic acid (VII)[8,9] and the aldehydo-acid (VI)[1] are known to act as precursors of gibberellic acid and (*b*) the 6β-7β-dihydroxy-acid possesses considerable biological activity (see below). It is possible that once

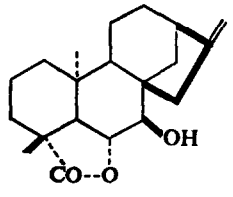
[14] B. E. Cross, R. H. B. Galt and J. R. Hanson, *J. Chem. Soc.* 5052 (1963).

the dihydroxy-acid has left the enzyme surface it can be cleaved by oxygenases to give fujenal, but cannot undergo ring contraction via, for example, a 6-pyrophosphate. If this is so, the dihydroxy-acid should be present in the culture filtrate. This has been demonstrated by adding [17-^{14}C]kaurene to a fermentation of *G. fujikuroi* and isolating the 6β,7β-dihydroxy-acid after dilution analysis. The dihydroxy-acid was active, showing a low incorporation of 0·006 per cent compared to methyl gibberellate (4·6%) (cf. Ref. 2). Ozonolysis of the dihydroxy-acid gave the norketone (XII), which was inactive, and formaldehyde. The latter was isolated as its dimedone derivative and contained 99 per cent of the activity. Thus, the incorporation is specific and the dihydroxy-acid is a metabolite of *G. fujikuroi*.

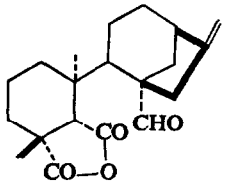
A number of precursors of gibberellic acid have been shown[9–11] to be biologically active in gibberellin bioassays, consequently we determined the activity of the 6β,7β-dihydroxy-acid (VIII) and of the triol (XIII) and the aldehydo-acid (VI).[1] 7α-Hydroxykaurenoic acid[7] was also tested as an example of a closely related compound which would not be expected to be a precursor of gibberellic acid in *G. fujikuroi*.*



(XVII)



(XVIII)



(XIX)

All compounds tested showed a relatively low order of activity in the various bioassay systems (Table 1), the most effective [6β,7β-dihydroxykaurenoic acid (VIII)] being a thousand times less potent than gibberellic acid. Slight differences could be seen in the bioassay spectra, particularly in the case of the 7α-hydroxy-acid (XVI) which was less active than the triol (XIII) in the d5 maize assay but more effective in the lettuce hypocotyl system.

Compounds II, III and V showed less activity generally than the group with ring B hydroxylation (VIII), (XIII) and (XVI). A direct comparison of the 6β,7β-dihydroxy-acid (VIII) and the triol (XIII) also indicates that the presence of a C19 carboxyl may have some significance in terms of biological activity. In the pigment-retention assays the carboxyl group had opposite effects in the presence and absence of ring B hydroxylation. The 6β,7β-dihydroxy-acid (VIII) gave almost a 50 per cent retardation of chlorophyll loss in the *Rumex* leaf discs whilst kaurenoic acid significantly accelerated this process. At the 10 μg level kaurenol was slightly promotive and kaurene had no significant effect.

* It must be remembered, however, that the biosynthetic pathway to gibberellic acid in higher plants may differ from that in the fungus.

Table 1. Comparative activities of the aldehydo-acid (VI) and some kaurene derivatives in three gibberellin bioassay systems

	d5 maize		Lettuce hypocotyl		Rumex leaf disc	
	10·0 μg	1·0 μg	10·0 μg	1·0 μg	1·0 μg	0·1 μg
6β,7β-Dihydroxykaurenoic acid (VIII)	324†	220†	190†	115	147†	141†
6β,7β,19-Trihydroxykaurene (XIII)	177†	140†	130†	111	122*	106
Aldehydo-acid (VI)	120	104	116	100	109	100
7α-Hydroxykaurenoic acid (XVI)	131*	92	140†	107	112	97
Kaurene (II)	110	101	100	100	96	100
Kaurenol (III)	118	100	104	98	92	105
Kaurenoic acid (V)	130*	108	112*	100	87*	84*
Gibberellic acid 0·1 μg	490		341		148	
0·01 μg	275		221		112	

All activities expressed as a percentage of control and based upon the means of three assays.
* Significantly different from control, $p = 0.05$.
† Significantly different from control, $p = 0.01$.

Both Katsumi *et al.*[10] and Brian *et al.*[11] reported a promotive effect of kaurenol and kaurenoic acid on leaf sheath extension in dwarf 5 mutant maize and the data reported here confirm these observations, the activities being of a similar order to those quoted by Brian *et al.*[11] In addition, a slight but significant promotive effect was shown in the lettuce hypocotyl assay by kaurenoic acid at the 10 μg level.

Activity estimates based upon 24-hr incubations in the barley endosperm assay (Table 2) indicated that none of the test compounds had any appreciable effect when compared with gibberellic acid although compounds VI, VIII, XIII and XVI were, as a group, generally more potent than kaurene and the derivatives lacking ring B hydroxylation. The dihydroxy-acid (VIII) and the aldehydo-acid (VI) showed similar activities whilst the triol (XIII) and the 7α-hydroxy-acid (XVI) were less effective.

Table 2. Activities of the aldehydo-acid (VI) and some kaurene derivatives in the barley endosperm amylase-release assay during 24- and 48-hr incubations

Compound	Dosage (μg)	Incubation time 24 hr	48 hr
6β,7β-Dihydroxykaurenoic acid (VIII)	1	38	304
6β,7β,19-Trihydroxykaurene (XIII)	1	16	160
Aldehydo-acid (VI)	1	40	240
7α-Hydroxykaurenoic acid (XVI)	1	26	56
Kaurene (II)	5	6	90
Kaurenol (III)	5	5	208
Kaurenoic acid (V)	5	10	275
Gibberellic acid	0·1	175	630
	0·001	80	325

Activities expressed as units of α-amylase released. All figures are means of duplicate assays. Blank values subtracted.

Kaurene has been shown by Jones[15] to initiate α-amylase release in the barley system with incubation periods longer than 24 hr and this finding is clearly supported by the 48-hr data quoted in Table 2. All test compounds exhibited amylase-inducing properties at the 1 or 5 μg level, the highest activity again being associated with the 6β,7β-dihydroxy-acid (VIII) followed by the aldehydo-acid (VI) and the triol (XIII). The 7α-hydroxy-acid (XVI) gave the smallest response, suggesting that hydroxylation in the 6 and 7 positions may partly determine the level of biological activity of kaurene derivatives in the barley system.

The comparatively high biological activity of the 6β,7β-dihydroxy-acid suggests that this compound may be a precursor of gibberellins in higher plants.

EXPERIMENTAL

A. Chemical Section

Chromatographic materials and general experimental methods were as described in Parts II[7] and III[1] except that radioactivities were measured on a Packard Tri-carb Scintillation Spectrometer Model 3310.

Preparation of methyl 7α-hydroxy-16-oxo-17-norkauran-19-oate. Powdered $NaIO_4$ (6·5 g) was added, in small portions during 15 min, to a stirred ice-cold soln. of the hydroxy-ester (XIV)[7] (4·55 g) and OsO_4 (50 mg) in THF (100 ml) and water (80 ml). The soln. was stirred overnight at room temp. and then conc. *in vacuo*. Recovery in AcOEt gave a gum which was chromatographed on silica gel (80 g). Elution with AcOEt–light petroleum (3:7) gave the *nor-ketone* which crystallized from acetone–light petroleum in needles (2·75 g), m.p. 181–182° (Found: C, 71·4; H, 8·85. $C_{20}H_{32}O_4$ required: C, 71·8; H, 9·0%) ν_{max} 3595 and 1729 cm^{-1}.

The *tosylate* (XV), prepared with tosyl chloride in pyridine at room temp., crystallized from acetone–light petroleum in plates, m.p. 169–172° (Found: C, 66·45; H, 7·55. $C_{27}H_{36}O_6S$ required: C, 66·4; H, 7·4%) ν_{max} 1744, 1727, 1600 cm^{-1}, τ 9·11 (3H, s, 20-Me), 9·0 (3H, s, 18-Me), 7·55 (3H, s, Ar-Me), 5·61 (m, 7-H), 6·36 (3H, s, CO_2Me), 2,42 (AB quartet, aromatic H).

Preparation of methyl 16-oxo-17-norkaur-6-en-19-oate (XVII). The tosylate (XV) (4·18 g) was heated overnight under reflux with dry collidine (30 ml). The mixture was poured into excess iced dil. H_2SO_4 and the product was recovered in AcOEt and chromatographed on alumina (100 g). Elution with AcOEt–light petroleum (1:9) gave the *6-ene* (XVIII) which crystallized from acetone–light petroleum in prisms (1·2 g), m.p. 168–170° (Found: C, 75·9; H, 8·65. $C_{20}H_{28}O_3$ required: C, 75·9; H, 8·9%) ν_{max} 1744, 1730, 1680 cm^{-1}, τ (pyridine) 9·12 (3H, s, 20-Me), 8·78 (3H, s, 18-Me), 6·38 (3H, s, CO_2Me), 4·56 (dd, $J_{5,6}$ 3 c/s, $J_{6,7}$ 10 c/s, 6-H), 3·76 (dd, $J_{6,7}$ 10 c/s, $J_{5,7}$ 2 c/s, 7-H).

Osmylation of the 6-ene (XVII). The 6-ene (206 mg) in pyridine (5 ml) was treated with OsO_4 (200 mg) at room temp. overnight. $NaHSO_3$ (900 mg), pyridine (12·5 ml) and water (15 ml) were added and the mixture left to stand for 30 min. Recovery in AcOEt gave *methyl 6β,7β-dihydroxy-16-oxo-17-norkauran-19-oate* (X) which crystallized from acetone–light petroleum in prisms (150 mg), m.p. 180–181° (Found: C, 68·6; H, 8·6. $C_{20}H_{30}O_5$ required: C, 68·5; H, 8·6%) ν_{max} 3565, 1744, 1695 cm^{-1}, τ (pyridine) 9·05 (3H, s, 20-Me), 8·31 (3H, s, 18-Me), 6·30 (3H, s, CO_2Me), 6·08 (d, J 2·5 c/s, 7-H), 5·28 (dd, $J_{5,6}$ 11 c/s, $J_{6,7}$ 2·5 c/s, 6-H).

Demethylation of the ester (X). The ester (200 mg) was heated overnight under reflux with LiI (1·6 g) and collidine (16 ml). The soln. was poured into excess iced dil. HCl and the products were recovered in AcOEt and separated into acidic and neutral fractions with $NaHCO_3$ soln. The acidic product crystallized from acetone in needles (70 mg) of *6β,7β-dihydroxy-16-oxo-17-norkauran-19-oic acid* (XII), m.p. 253–254° (Found: C, 67·4; H, 8·1. $C_{19}H_{28}O_5$ required: C, 67·8; H, 8·4%) ν_{max} 3480, 3300, 1740, 1688 cm^{-1}, τ (pyridine) 8·77 (3H, s, 20-Me), 8·18 (3H, s, 18-Me), 6·10 (d, J 2 c/s, 7-H), 5·10 (dd, $J_{5,6}$ 11 c/s, $J_{6,7}$ 2 c/s, 6-H).

Wittig reaction on the nor-ketone (XII). A suspension of [^{14}C]triphenylmethylphosphonium iodide (2·9 g; 200 μc) in dry THF (20 ml) was stirred under N_2 with *n*-butyl lithium (1·1 N; 2·6 ml) until the solid dissolved. The nor-ketone (300 mg) in THF (30 ml) was added and the mixture was stirred for 15 min. Unlabelled triphenylmethylphosphonium iodide was then added to react with any excess of butyl lithium. The mixture was refluxed overnight, the solvent was removed *in vacuo*, water was added to the residue and the soln. was acidified with dil. HCl and extracted with AcOEt. The extract was washed with $NaHCO_3$ soln. and the aqueous layer was acidified with dil. HCl and extracted with AcOEt. Recovery gave a gum (317 mg) which crystallized from acetone in needles (190 mg; 13·07 μc, $7{\cdot}471 \times 10^7$ counts/100 sec/mmole) of [17-^{14}C]*6β,7β-dihydroxykaur-16-en-19-oic acid* (IX), m.p. 233–237° (Found: *m/e* 334·2146. $C_{20}H_{30}O_4$ required: *M*, 334·2144) ν_{max} 3550, 3400, 1690, 1680, 890 cm^{-1}, τ (pyridine) 8·82 (3H, s, 20-Me), 8·21 (3H, s, 18-Me), 6·14 (d, J 2·5 c/s, 7-H), 5·21 br (6-H), 5·09 br ($C=CH_2$).

[15] K. C. Jones, *Planta*, **78**, 366 (1968).

Wittig reaction on the nor-ketone ester (X). Reaction of the nor-ketone (400 mg) with [^{14}C]triphenylmethylphosphonium iodide (2·65 g; 200 μc) as in the preceding experiment gave an acidic gum (147 mg) which was chromatographed on silica gel (4 g). Elution with AcOEt–light petroleum (1:4) and (1:1) gave the dihydroxy-acid (X) (15 mg; 1·32 μc, 6·61 × 10^7 counts/100 sec/mmole), m.p. 233–237°, identical with the sample prepared in the preceding experiment.

Preparation of the [17-^{14}C]*triol (XIII).* The acid (IX) (65 mg) was methylated with CH_2N_2 and the crude ester was refluxed overnight with excess $LiAlH_4$ in THF (20 ml). AcOEt followed by potassium sodium tartrate soln. was added and the product was recovered in AcOEt. It crystallized from acetone–light petroleum in needles of [17-^{14}C]6β,7β,19-*trihydroxykaur*-16-*ene* (XIII), m.p. 195–196° (46 mg; 3·27 μc, 7·75 × 10^7 counts/100 sec/mmole) (Found: C, 75·0; H, 10·3. $C_{20}H_{32}O_3$ required: C, 75·0; H, 10·1%) ν_{max} 3370, 1658, 870 cm^{-1}.

Fermentations with ^{14}C-labelled substrates. General fermentation conditions and isolation procedures have been described.[7]

a. [17-^{14}C]6β,7β-*Dihydroxykaur*-6-*en*-19-*oic acid.* The fermentation was continued for 96 hr after adding the labelled acid (IX) (44 mg; 3·0 μc). The crude acidic fraction was crystallized from AcOEt giving gibberellic acid (496 mg) which was methylated with ethereal CH_2N_2 and chromatographed on alumina (20 g). Elution with AcOEt–light petroleum (2:3) gave methyl gibberellate which crystallized from MeOH–benzene in needles (348 mg; 128 counts/100 sec/mmole; 0·00% incorporation).

The gibberellic acid mother liquor was chromatographed on silica gel (150 g). Elution with chloroform gave fujenal (XIX) which crystallized from AcOEt–light petroleum in prisms (80 mg; 3·84 × 10^6 counts/100 sec/mmole; 9·7% incorporation), m.p. 169–170°.

The neutral fraction was diluted with unlabelled 7-hydroxykaurenolide (102 mg) and chromatographed on alumina (200 g). Elution with AcOEt–light petroleum (1:4) gave 7-hydroxykaurenolide which crystallized from AcOEt–light petroleum in needles (80 mg; 50 counts/100 sec/mmole; 0·00% incorporation), m.p. 187–189°.

b. [17-^{14}C]6β,7β,19-*Trihydroxykaur*-16-*ene* (XIII). The fermentation was run for 96 hr after adding the triol (46 mg; 3·27 μc). The crude acidic fraction (4·41 g) was chromatographed on silica gel (150 g). Elution with chloroform gave fujenal (39 mg) which was diluted with unlabelled material (18 mg). It crystallized from AcOEt–light petroleum in needles (40 mg; 2·08 × 10^6 counts/100 sec/mmole; 0·36% incorporation), m.p. 169–170°. Elution with AcOEt–light petroleum (1:1) gave crude gibberellic acid which was methylated and purified as above, to give methyl gibberellate (839 mg; 57 counts/100 sec/mmole; 0·00% incorporation), m.p. 208–210°.

c. [17-^{14}C]*Kaurene.* The fermentation was continued for 24 hr after adding the [17-^{14}C]kaurene (351 mg; 12 μc). The crude acidic fraction was diluted with unlabelled 6β,7β-dihydroxykaurenoic acid (85 mg) and chromatographed on silica gel (50 g). Elution with AcOEt–light petroleum (1:10) gave a band (200 mg) which was rechromatographed on kieselgel G (4 × 5 cm) in AcOEt–AcOH (99:1). A band (100 mg) was obtained which crystallized from acetone–light petroleum in needles of 6β,7β-dihydroxykaurenoic acid [41 mg; 8·42 × 10^3 counts/100 sec/mmole, 0·0057% incorporation (calc. for 85 mg)], m.p. 230–234°. Elution of the silica gel column with AcOEt–light petroleum (1:1) afforded gibberellic acid (306 mg) which was purified as its methyl ester (268 mg; 2·34 × 10^6 counts/100 sec/mmole, 4·62% incorporation).

Ozonolyses of [^{14}C]*metabolites.* *a.* Fujenal from the [^{14}C]6β,7β-dihydroxykaurenoic acid fermentation. The fujenal was diluted with unlabelled material. It crystallized from AcOEt–light petroleum in prisms (69 mg; 1·817 × 10^6 counts/100 sec/mmole), m.p. 169–171°. Ozonolysis of this fujenal (63 mg) in AcOH by the literature method[14] gave formaldehyde dimedone (20 mg; 1·655 × 10^6 counts/100 sec/mmole = 91% of the activity of the fujenal), m.p. 188–191°.

b. 6β,7β-Dihydroxykaurenoic acid from the [17-^{14}C]kaurene fermentation. Excess ozonized oxygen was passed through the dihydroxy-acid (27 mg) in AcOH (10 ml) at room temp. for 5 min. Water (10 ml) was added, and the soln. steam-distilled. The distillate (20 ml) was added to a soln. of dimedone (100 mg) in water (5 ml) and left to stand at 0°. After 72 hr formaldehyde dimedone, m.p. 188–190° (6 mg; 8·395 × 10^3 counts/100 sec/mmole = 99% of the activity of the dihydroxy-acid), was collected. Extraction of the aqueous residue from the steam-distillation with AcOEt afforded a gum which crystallized from AcOEt–light petroleum in needles, m.p. 253–254° (9 mg; 0 counts/100 sec/mmole), of the nor-ketone (XII).

B. Bioassay Methods

All compounds were dissolved in either acetone or ethanol to give a concentration of 1 μg/ml and dispensations for bioassay were made with Hamilton syringes. Detailed procedures were as follows:

a. Dwarf 5 maize assay. Seeds were supplied by Professor B. O. Phinney, University of California at Los Angeles, U.S.A. All seedlings were grown to the first leaf stage in John Innes No. 1 compost and the selected dwarfs were transplanted into containers filled with half-strength Hoagland nutrient solution. Test compounds were applied to the partially expanded first leaf and the plants were placed in a 16 hr/20° growth room for 7 days. Activity was assessed by measurement of the first leaf sheath.

b. Lettuce hypocotyl assay. The method used was essentially that of Frankland and Wareing[16] using the cultivar 'Arctic King' supplied by Carters Tested Seeds Ltd., Raynes Park, London, S.W.20. Test solutions were dried onto 2 × 2cm squares of Whatman No. 1 paper to form the substrate for the assay. Hypocotyl length was measured after 48 hr of continuous illumination at 20°.

c. Rumex leaf disc assay. A system similar to that of Whyte and Luckwill[17] was employed. Fully expanded leaves of *Rumex obtusifolius* L., grown under glasshouse conditions, were used as test material. Five 1-cm discs of interveinal tissue were used for each test, being placed on a 3 × 3cm square of filter paper containing the appropriate solution. The squares were placed in 5-cm petri dishes which, in turn, were placed in a large glass dish containing wetted tissue, to maintain the humidity level. After 3–4 days in total darkness the control discs had usually lost almost all their chlorophyll and the assay was terminated at this point. Chlorophyll contents of the discs in the other treatments were determined by measurement of the optical densities of 80% ethanol extracts at 665 nm.

d. Barley half-seed α-amylase release assay. The procedure was a modification of that described by Jones and Varner.[18] Embryoless halves of 'Himalaya' barley grains were sterilized for 20 min in 2% sodium hypochlorite and imbibed with water for a further 3 days on sterile pads of Whatman 3MM paper. Assays were performed, in sterile conditions, in 25-ml conical flasks containing 0·25 ml sodium acetate buffer (0·01 M, pH 4·8), 0·25 ml $CaCl_2$ solution (0·1 M), 0·1 ml chloramphenicol solution (0·5 mg/ml) and 0·4 ml of distilled water. Ten half-grains and 1 μl of test solution were added to each flask and incubations were carried out on a water-bath shaker (Gallenkamp-Towers, Widnes, Lancs.) at 25° for 24 or 48 hr. The incubate and rinsings, making a total volume of 3 ml, were centrifuged for 5 min at 2000 *g* to remove debris and the α-amylase activity of the supernatant was measured by following the reduction in iodine staining of a standard starch reagent. Units of α-amylase were calculated according to the formula of Jones and Varner[18] using a starch factor of 2·4.

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