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INVESTIGATION OF GIBBERELLINS AND OTHER GROWTH SUBSTANCES IN THE SEED OF *PHASEOLUS MULTIFLORUS* AND OF *PHASEOLUS VULGARIS* BY GAS CHROMATOGRAPHY AND BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY*

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Abstract—By GC–MS the following acids were identified in the seed of *P. multiflorus*: Gibberellins A_1, A_3, A_5, A_6, A_8, A_{17}, A_{19}, A_{20} and a presumed, but incompletely characterized, gibberellin; phaseic acid; two incompletely characterized compounds, C-α and C-β; the saturated straight chain C_{14}-, C_{15}-, C_{18}-, C_{20}-, C_{22}- and C_{24}-acids; and α-hydroxypalmitic acid. β-Sitosterol was identified in the neutral fraction. A GLC peak corresponding to *ent*-kaurene was not positively identified. In the seed of *P. vulgaris*, gibberellins A_6 and A_8 were identified by GC–MS. GLC analyses of the amounts of gibberellins A_1, $A_{5/20}$, A_6 and A_8 in the developing seed of *P. multiflorus* and *P. vulgaris* are presented.

IN A SERIES of preliminary publications we have reported the conclusive identification of known gibberellins[1–3] and the detection of new plant growth substances[4,5] by GC–MS of crude or partly-purified extracts of immature seed of *Phaseolus multiflorus*. We now amplify these preliminary results and describe the application of GLC and GC–MS to the analysis of gibberellins in the developing seed of *P. multiflorus* and *P. vulgaris*. We also report preliminary feeding experiments of 2-[^{14}C]-mevalonic acid to seed pods of *P. vulgaris*.

CONSTITUENTS OF *P. MULTIFLORUS* SEED

The seed were extracted with 70% aq. ethanol and the crude extract was partitioned into strong acid, weak acid, and neutral fractions. After derivatization the strong acid fraction was examined by GC–MS. Our preliminary paper[2] presented the total ion current (TIC) traces of the methylated fraction before and after trimethylsilylation; the peaks with retention times corresponding to gibberellin A_5 methyl ester (MeA_5) and its trimethylsilyl ether (MeA_5TMSi), to MeA_6 and MeA_6TMSi, to MeA_1 TMSi, and to MeA_8TMSi gave MS identical to those of the authentic derivatives[6] when scanned from *m/e* 10–700 in 4 sec. However the MS of peaks, tentatively assigned to MeA_{13} and $MeA_{13}TMSi$ and to MeA_4 and MeA_4TMSi by GLC alone,[7] showed that these peaks were the derivatives of gibberellins, isomeric with gibberellins A_{13} and A_4. These isomers of gibberellins A_{13} and A_4 and

* Part XI in the series "Plant Hormones"; for Part X, see *Tetrahedron* **25**, 5903 (1969).

[1] J. MACMILLAN, R. J. PRYCE, G. EGLINTON and A. MCCORMICK, *Tetrahedron Letters* 2241 (1967).
[2] R. J. PRYCE, J. MACMILLAN, and A. MCCORMICK, *Tetrahedron Letters* 5009 (1967).
[3] J. MACMILLAN and R. J. PRYCE, *Tetrahedron Letters* 1537 (1968).
[4] J. MACMILLAN and R. J. PRYCE, *Tetrahedron Letters* 4173 (1967).
[5] J. MACMILLAN and R. J. PRYCE in *Plant Growth Regulators Soc. Chem. Ind. Monograph*, No. 31, 36 (1968).
[6] R. BINKS, J. MACMILLAN and R. J. PRYCE, *Phytochem.* **8**, 271 (1968).
[7] B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6**, 867 (1967).

other unidentified peaks, were further examined after partial purification of the crude strong acid fraction by column chromatography on celite–charcoal. Sixty-eight fractions were collected by gradient elution with increasing concentrations of acetone in water. Aliquots of these fractions were methylated and monitored by GLC; selected fractions were examined in more detail by GC–MS. The results are summarized in Table 1; the concentration of each compound was calculated from the GLC peak areas of the methyl esters. The presence of gibberellins A_1, A_5, A_6 and A_8 in the seed of *P. multiflorus* has been previously established by isolation.[8,9] The new compounds in Table 1 are discussed below.

TABLE 1. GROWTH SUBSTANCES DETECTED IN SEED OF *P. multiflorus* BY GLC AND GC–MS

Fraction*	Eluant (% Me_2CO in H_2O)	Total wt. (mg)	Compounds	Concentration by GLC (mg/kg fr. wt. seed)	
35	39–40	766	A_8		
40	47	371	A_1, Phaseic acid	A_1	8·0
45	53	366	A_6	A_5	1·5
51	60	148	A_5, A_{20}	A_6	2·5
52	61	142	A_5, A_{19}	A_8	30·0
53	62	144	A_5, A_{17}, A_{19}, A_{20}	A_{17}	2·0
54	62–63	128	A_{17}, A_{19}	A_{19}	0 5
55	63–64	117	A_{17}, A_{19}	A_{20}	0·5
57	65–66	95	A_{17}	C-α	0·35
59	67–68	60	A_{17}	C-β	0·15
66	86–88	74	C-α, C-β	Phaseic acid	0·30

* From celite-charcoal column of crude strong acid fraction.

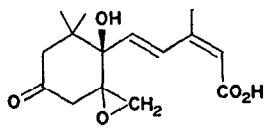
Phaseic Acid

The isolation of phaseic acid from fraction 40, and the spectroscopic evidence for structure (I) was described earlier[10,11] in the meantime Milborrow[12] has found that phaseic acid is formed readily from metabolite C of abscisic acid (II) and has presented irrefutable evidence for the formulation of phaseic acid as (III) and of metabolite C as (IV); we therefore withdraw structure (I).

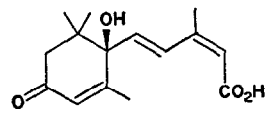
Gibberellin A_{17}

The isomer of gibberellin A_{13}, detected by GC-MS in the crude strong acids, was shown to be present in fractions 53–59 from the celite–charcoal column by GLC and GC–MS. This new gibberellin, numbered A_{17},[4,13] was isolated from fraction 55 by column chromatography on celite–silica gel, followed by TLC. Purification was followed by GLC. Gibberellin A_{17}, obtained as an amorphous solid, m.p. 140–150° with a purity of 90% by GLC, was shown to possess structure (V) by the following spectroscopic data. The gummy methyl ester, obtained with diazomethane, showed a parent ion in the MS with the composition $C_{23}H_{32}O_7$ and was clearly a trimethyl ester from the NMR spectrum (Table 2). The IR

[8] J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **11**, 60 (1960).
[9] J. MACMILLAN, J. C. SEATON and P J. SUTER, *Tetrahedron* **18**, 349 (1962).
[10] J. MACMILLAN and R. J. PRYCE, *Tetrahedron* **25**, 5893 (1969).
[11] J. MACMILLAN and R. J. PRYCE, *Tetrahedron* **25**, 5903 (1969).
[12] B. V. MILBORROW, *Chem. Commun.* 966 (1969).
[13] J. MACMILLAN and N. TAKAHASHI, *Nature* **217**, 170 (1968).



(I)



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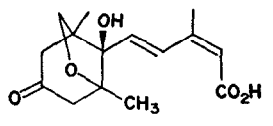

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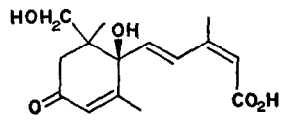

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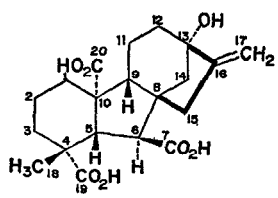

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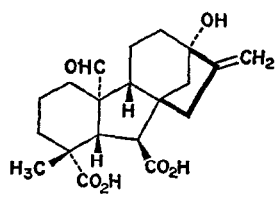

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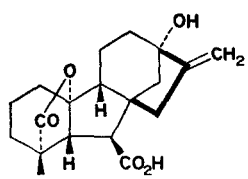

(VII)

TABLE 2. NMR SIGNALS (τ-VALUES) FOR GIBBERELLIN A_{17} AND METHYL ESTER

Acid	Me ester			
$(CD_3)_2CO$	$CDCl_3$	C_5D_5N	Intensity	Assignment
8·84	8·91	8·77	3	$CH_3—C$
6·19	6·28	5·95	1	C—H
4·97	4·94	4·58	1	$CH_2=C$
5·24	5·17	5·05	1	
—	6·36	6·43	3	CH_3OC
	6·41	6·43	3	
	6·64	6·52	3	

spectrum of this trimethyl ester in $CDCl_3$ solution showed broad hydroxyl absorption at *ca.* 3500 cm^{-1} as well as a broad composite carbonyl (1723 cm^{-1}) and exocyclic methylene (1663 and 906 cm^{-1}) absorption. Gibberellin A_{17} was thus characterized as a tribasic hydroxy-acid $C_{20}H_{26}O_7$. Structure (V) follows from the NMR data in Table 2 which shows the presence of a tertiary methyl group and of exocyclic methylene protons. Thus gibberellin A_{17} contains five extra nuclear carbon atoms ($3 \times CO_2H$; $-\overset{\diagdown}{\underset{\diagup}{C}}-CH_3$; $C{=}CH_2$) leaving 15 skeletal carbon atoms and suggesting an *ent*-gibberellane skeleton.* This skeleton was supported by the presence of an AB-quartet in the NMR spectra (Table 2) characteristic of the 5- and 6-protons in gibberellins and by the fragmentation patterns in the MS of the trimethyl ester and its TMSi ether (see below and Ref. 6). As in gibberellins A_{13},[14] A_{19},[15] and A_{24}[16] the 6-proton in A_{17} and MeA_{17} occurs at low field deshielded by the axial carbonyl functions at positions -4 and -10. The presence of a 13-hydroxyl group is revealed by the large difference in the chemical shift of the two 17-protons in C_5D_5N solution.[17] Also the MS of $MeA_{17}TMSi$ shows an intense molecular ion and fragment ions at *m/e* 207/208, both being characteristic of a 13-hydroxy gibberellin TMSi ether.[6] It is noteworthy that gibberellin A_{17} was detected and isolated without the guidance of bio-assay; it was subsequently shown to have a relatively low activity in fourteen bio-assay systems.[18] Gibberellin A_{17} has recently been isolated in crystalline form, m.p. 170–171°, from seed of *Calonyction aculeatum* by Takahashi *et al.*;[19] its identity with our amorphous material was confirmed by GC–MS of the Me ester and of the MeTMSi ether, prepared from a specimen kindly supplied by Professor N. Takahashi.

During the purification of gibberellin A_{17} by TLC on silica gel, gibberellin-like biological activity was detected in a zone of higher R_f value than gibberellin A_{17} from which it was separated by a biologically-inactive region. However GC–MS of methylated material and its TMSi derivative, from this zone, showed that the major component in this region was in fact gibberellin A_{17}, despite the apparently biologically inactive region between the two biologically-active zones. Whatever the reason, this result emphasises the caution with which TLC fractions should be interpreted in terms of the number of bio-active substances.

Gibberellin A_{19}

The close chemical, and possible biosynthetic, relationship between gibberellin A_{17} (V) and gibberellin A_{19} (VI) suggested that gibberellin A_{19} might also be present in seed of *P. multiflorus*. A search for gibberellin A_{19} by GLC and GC–MS in fractions from the celite-charcoal chromatography of the crude strong acid fraction revealed its presence in fractions 52–55. The TIC trace of methylated fraction 53, shown in a preliminary paper,[2] contained three peaks which were scanned by GC–MS from *m/e* 10–500 in 4 sec to give mass spectra, identical to the reference GC–MS spectra[6] of MeA_5, MeA_{17} and MeA_{19}.

* The name *ent*-gibberellane, is adopted for the gibberellin skeleton (see M. F. BARNES, R. C. DURLEY and J. MACMILLAN, *J. Chem. Soc.* (C) 1341 (1970), with the numbering shown in structure (V).

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[16] D. M. HARRISON, J. MACMILLAN and R. H. B. GALT, *Tetrahedron Letters* 3137 (1968).
[17] J. R. HANSON, *J. Chem. Soc.* 5036 (1965).
[18] G. V. HOAD and C. C. KUO, *Can. J. Botany* **48,** 1423 (1970).
[19] N. TAKAHASHI private communication.

In their isolation of gibberellin A_{19} from *Phyllostachys edulis*, Murofushi *et al.*[15] detected two unidentified gibberellin-like substances one of which may be gibberellin A_{17}.

Gibberellin A_{20}

The isomer of gibberellin A_4, detected in the crude strong acid fraction and originally thought to be gibberellin A_4,[1,7] could not be detected by GLC in any of the fractions from the celite-charcoal column until a specimen of gibberellin A_{20} (VII)[20] was obtained. It was then found that this isomer of gibberellin A_4 could not be separated as the Me ester or MeTMSi ether from the corresponding derivatives of gibberellin A_5 under our standard GLC conditions.[7] Gibberellin A_{20} was therefore looked for in fractions containing GLC peaks originally assigned to gibberellin A_5 derivatives. The major sharp peak from a methylated aliquot of fraction 51 was examined by GC–MS in two ways. First, scans from *m/e* 10–500 in 2 sec were taken at four points through the peak and a comparison of the resultant mass spectra with reference spectra of MeA_5 and MeA_{20} established that the peak was an unresolved mixture of MeA_5 and MeA_{20}. Secondly, the peak was examined by the method of accelerating voltage alternation (AVA) focussing the molecular ions *m/e* 344 and 346 of MeA_5 and MeA_{20} respectively. The AVA trace which clearly demonstrated the presence of MeA_5 and MeA_{20} has already been published[3,5] together with the line diagrams for the four separate scans through the peak and these data are not reproduced here. The presence of gibberellin A_{20} in fraction 53 from the celite-charcoal column was likewise established by GC–MS scans at the lower retention side of the MeA_5 peak. The identification of MeA_{20} and MeA_5 in an unresolved GLC peak underlines the effectiveness of GC–MS. Subsequently GLC conditions were developed which separate MeA_5 and MeA_{20} using a 1% XE-60 column (Table 3). Although MeA_{10} and MeA_{20} are not resolved on this column, their TMSi ethers are well separated on 1% QF-1.[5] MeA_{20} (11·5 min) and MeA_5 (12·3 min) can also be separated with a 50% valley between peaks on a column (3 m × 4 mm i.d.) of 1% QF-1 at 206° with a N_2-flow rate of 10 ml/min.

Compounds -α and -β

The two non-gibberellins, compounds α(C-α) and β(C-β) were detected in fraction 66 from the celite-charcoal column. The T.I.C. trace of a methylated aliquot is shown in Fig. 1 and shows two main peaks. When examined, peak 1 gave the MS of MeC-α shown in Fig. 2a, and peak 3 the MS of MeC-β shown in Fig. 2b. Using GLC to follow the isolation, C-α and C-β were obtained in total amounts of 2·8 and 1·2 mg respectively as gums, of over 90% purity, by TLC of the early fractions from a celite–silica gel column. GLC of fractions

TABLE 3. GLC DATA FOR THE METHYL ESTERS OF GIBBERELLINS A_4, A_5, A_7, A_{10} AND A_{20} ON A 1% XE-60 COLUMN (3 m × 4 mm i.d.)

	MeA_4	MeA_7	MeA_5	MeA_{20}	MeA_{10}
Ret. time (min)	10·6	13·9	9·65	8·7	8·55
Kovat's Index	3525	3615	3490	3460	3447

Conditions: Col. temp. 206°; Inject. temp. 230°; Detector temp. 260°. N_2 flow rate, 20 ml/min. Theor. plates for 5α-cholestane 2250.

[20] N. MUROFUSHI, N. TAKAHASHI, T. YOKOTA and S. TAMURA, *Agri. Biol. Chem.* **32**, 1239 (1968).

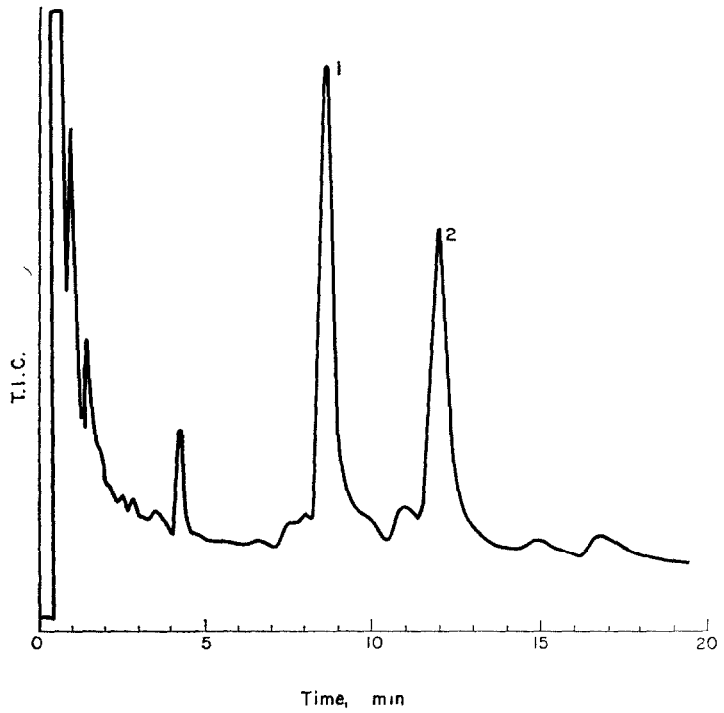


FIG. 1. T.I.C. TRACE OF METHYLATED FRACTION 66 (1% QF-1, 2 m × 3 mm column, HELIUM CARRIER GAS 30 ml/min, TEMPERATURE PROGRAMME 170–225° AT 5°/min).

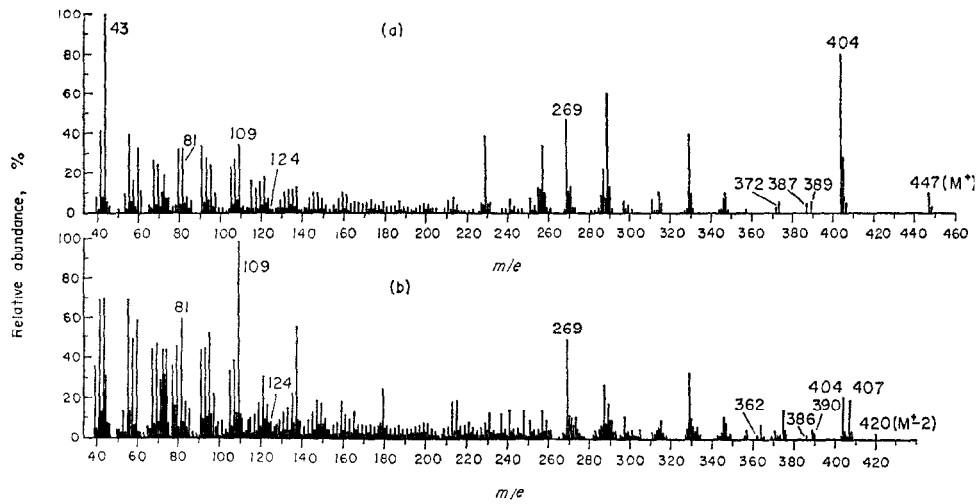


FIG. 2. GC–MS SPECTRA (a) OF MeC-α AND (b) MeC-β.

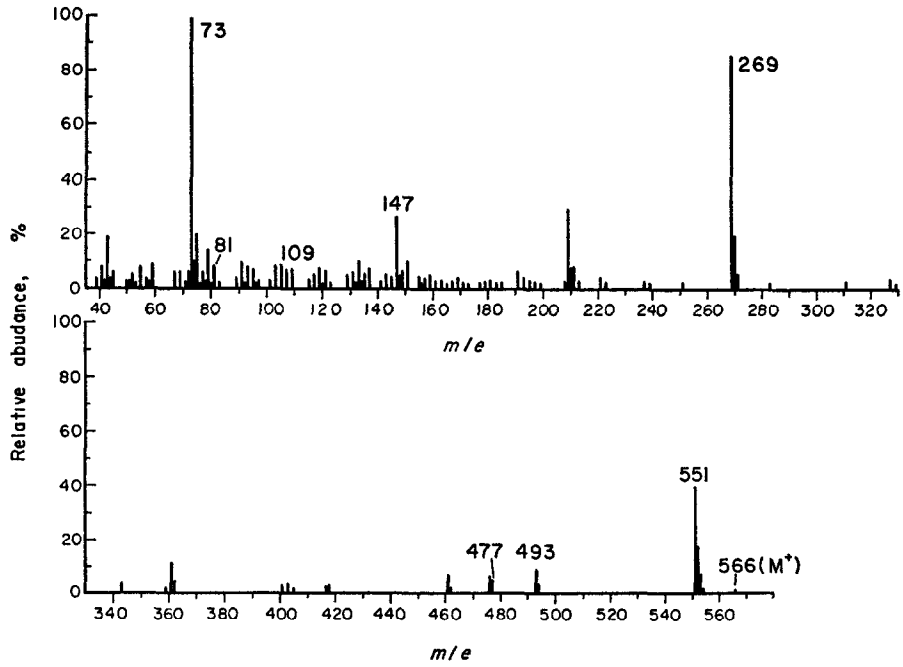


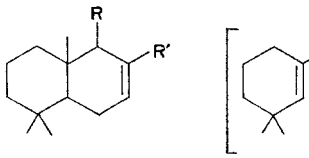
FIG. 3. GC–MS SPECTRUM OF MeCβ-BIS-TMSi.

around 66 indicated the total amounts of C-α and C-β, shown in Table 1. With the quantities available, the structures of C-α and C-β have not yet been established.

The molecular formula $C_{25}H_{37}NO_6$ was assigned to MeC-α by high resolution MS. MeC-α did not form a TMSi ether and the low resolution MS of the free acid showed a strong parent ion at *m/e* 433. Thus C-α appears to be a monocarboxylic acid $C_{24}H_{35}NO_6$ containing no other hydroxyl groups. The parent ions in the MS of both C-β and MeC-β were ill-defined and could not be mass matched, but fragmentations similar to those observed for C-α and MeC-α suggested that the parent ions were at *m/e* 408 and *m/e* 422 for C-β and MeC-β. This conclusion was supported by GC–MS (Fig. 3) for the bis-TMSi ether of MeC-β which showed a parent ion at *m/e* 566. Mass matching of the fragment ions *m/e* 407 ($C_{23}H_{35}O_6$) and *m/e* 404 ($C_{24}H_{36}O_5$) in the MS of the methyl ester suggested that these ions were formed by loss of water and methyl from a parent ion of molecular composition $C_{24}H_{38}O_6$. It was therefore concluded that C-β is a dihydroxy monocarboxylic acid $C_{23}H_{36}O_6$. The GC–MS spectrum (Fig. 2b) showed a small ion at *m/e* 420, probably the result of dehydrogenation in the molecular separator of the LKB 9000 instrument (cf. Ref. 6).

A close structural relationship between C-α and C-β is suggested by the similarity of the MS (Figs. 2a and 2b) of their methyl esters. The MS of MeC-α, after the loss of 43 amu, is very similar to that of MeC-β after the loss of 18 amu. The common starting point of this similarity is therefore the ion *m/e* 404 which had the molecular composition $C_{24}H_{36}O_5$ in both cases and which corresponds to the loss of HCNO from MeC-α and of H_2O from MeC-β. This MS similarity together with the absence of hydroxyl groups in MeC-α and the presence of two hydroxyl groups in MeC-β suggests that C-α is a derivative of C-β in

which both hydroxyl groups are incorporated with one carbon and one nitrogen atom in a cyclic function. The base peak in the MS of MeC-α has *m/e* 43 and is perhaps an HCNO ion; no acetyl group was observed in the NMR or MeC-α. The MS of MeC-α, MeC-β and MeC-βTMSi show a common ion *m/e* 269 which, in the case of MeC-β, had the molecular composition $C_{19}H_{25}O$. The ions at *m/e* 387 in MeC-α and at *m/e* 362 in MeC-β could arise by loss of methyl formate from the methoxy carbonyl group in the parent ion. The loss of 32 amu from parent and fragment ions can be assigned to the loss of methanol from this ester grouping; for example, the ion *m/e* 390 in MeC-α corresponds to the loss of 32 amu from the parent ion and the ion *m/e* 372 to the parent ion less HCNO and CH_3OH. The MS of MeC-α and MeC-β show ions due to the loss of methyl; for example, the ion *m/e* 389 in the MS of MeC-α corresponds to the parent ion less HCNO and CH_3 and the ion *m/e* 407 in MeC-β to the loss of CH_3 from the parent ion. While the loss of the elements of water from parent and fragment ions are observed in the MS of MeC-β, no such losses are evident in the MS of MeC-α. The ion *m/e* 386 in the MS of MeC-β may represent the loss of two molecules of water from the parent ion.



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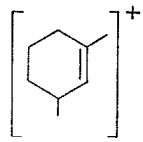

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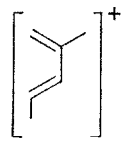

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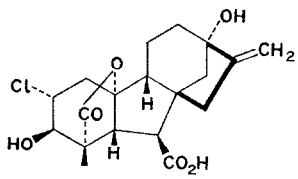

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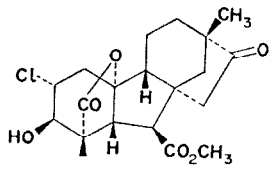

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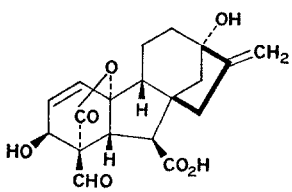

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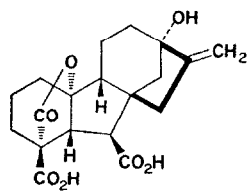

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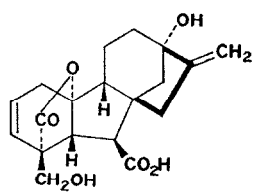
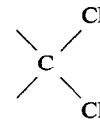
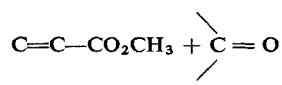
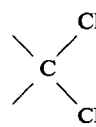


(XVI)

TABLE 4. FEATURES OF THE INFRA-RED SPECTRA ($CHCl_3$) OF C-α AND C-β AND THEIR METHYL ESTERS TOGETHER WITH ASSIGNMENTS

C-α (cm^{-1})	Assignments	C-β (cm^{-1})
3510 and down to *ca.* 2400	– COOH	3670 and down to *ca.* 2400
1730		1728
1693 1605	C=C—CO_2H	1700 sh.* 1600
1381 1370		† 1380 1370
Methyl C-α		**Methyl C-β**
v. weak br.* *ca.* 3600–3100	Impurity?	R—OH 3670 and 3600 to *ca.* 3200
1720 1600		1725 1603
1670 sh.*	C=C *trans* di-, tri-, or tetra-substituted	1670 sh.*
1382 1371		† 1382 1372

* sh. = Shoulder; br. = broad.
† Equal intensity doublet.

The MS of MeC-α, MeC-β and MeC-βTMSi contain prominent ions at *m/e* 109 (C_8H_{13} for MeC-β) and at *m/e* 81 together with a weaker ion at *m/e* 124 in the MS of MeC-α and MeC-β. Enzell and Ryhage[21] have suggested that the appearance of an *m/e* 109 ion is a characteristic feature of the MS of diterpenes containing the structural unit (VIII) which fragments in the following manner. The ion-radical of (VIII) is cleaved by a retro-Diels Alder process to give the ion (IX, *m/e* 124) which by loss of CH_3, followed by a second retro-Diels Alder cleavage, gives successively the ions (X, *m/e* 109) and (XI, *m/e* 81). Thus it is tentatively concluded that both C-α and C-β contain the structural unit (VIII) or can readily give rise to it.

The presence of a *gem*-dimethyl group in C-α and C-β is supported by the IR spectra of the acids and their methyl esters. The IR data and possible assignments are shown in Table 4. The UV and NMR data were not very helpful. The NMR spectra were weak but consistent with the MS and IR data indicating the presence of three or four tertiary methyl

[21] C. R. ENZELL and R. RYHAGE, *Arkiv. Kemi.* **23**, 367 (1965).

groups in both MeC-α and MeC-β and of methanol protons of a secondary vicinal diol in MeC-β. The presence of a *vic*-diol in C-β is also supported by the MS of the bis TMSi ether of MeC-β which shows an ion at *m/e* 147, characteristic[6] of a *vic*-bis TMSi ether.

Compounds C-α and C-β may have biological significance. In a single experiment in the oat mesocotyl bioassay (Dr. L. C. Luckwill, personal communication) C-α showed growth inhibition (73% control at 6 μg/ml) while C-β showed promotion (144% control at 6 μg/ml). Because of their apparently close structural relationship and opposite biological activities C-α and C-β could, by loss or gain, of a small nitrogenous protecting group represent an economic plant growth regulatory system.

Weak Acids

The weak acids from the large-scale extraction was examined by GLC after methylation, and after methylation and trimethylsilylation. Gibberellins A_9, A_{10}, A_{12} and *ent*-kauren-19-oic acid were specifically looked for but no evidence of their presence was obtained. The weak acid fraction consisted mainly (35%) of a neutral oil which has been identified[22] as the plasticizer, acetyl tri-*n*-butylcitrate.

Neutral Fraction

The neutral fraction from the extraction of seed of *P. multiflorus* was examined for the presence of *ent*-kaur-16-ene and *ent*-kaur-en-19-ol by GLC and GC–MS but neither diterpene could be positively identified. TLC of the unfractionated neutral fraction provided no evidence of the presence of *ent*-kaurene. The following partially purified fractions of the extract were then examined by GLC: (a) a sublimate obtained at 100° under water pump vacuum; (b) fractions from dry column chromatography[21] on silica gel; (c) ether and *n*-hexane soluble material. In each case except for the sublimate, GLC revealed the presence of β-sitosterol (identified as described below) which had the same retention time as *ent*-kaurene on 2% SE-33. On the 2% QF-1 column β-sitosterol and *ent*-kaurene were well resolved and in all three partially purified neutral fractions, a peak corresponding to *ent*-kaurene was present. However conclusive identification by GC–MS was not possible owing to the low intensity of the peak. It is known[24] that *ent*-kaurene and *ent*-atisirene are not resolved on several columns including QF-1 and SE-33. The *ent*-kaurene-like peak was also observed in GLC traces of the unfractionated neutral extract in amounts of about 0·25 mg/kg fresh weight of seed.

β-Sitosterol was present in the neutral fraction in amounts of about 1 mg/kg fr. wt. of seed. It was isolated by dry column chromatography of the unfractionated and the hexane-soluble portion, of the neutral extract. β-Sitosterol was identified by its m.p., molecular formula as determined by high resolution MS, and by comparison of the MS with the published MS.[24] GC–MS revealed an impurity in the β-sitosterol with a presumed molecular ion at *m/e* 412 but with a fragmentation pattern, quite unlike that[25] published for stigmasterol which commonly occurs[26] with β-sitosterol. Palmitic acid was also isolated from the neutral fraction.

[22] R. BINKS, R. J. GOODFELLOW, J. MACMILLAN and R. J. PRYCE, *Chem. & Ind.* 565 (1970).
[23] B. LOEV and K. M. SNADER, *Chem. & Ind.* 15 (1965).
[24] R. A. APPLETON, A. J. MCALEE, A. MCCORMICK, P. MCCRINDLE and R. D H. MURRAY, *J. Chem. Soc* (C), 2319 (1966); S. S. FRIEDLAND, C. H. LANE, JR., R. T. LONGMAN, K. E. TRAIN and H. J. O'NEAL, JR., *Anal. Chem.* **31,** 169 (1959).
[25] H. J. M. FITCHES, *Advan. Mass Spect.* **2,** 428 (1963).
[26] L. F. FIESER and M. FIESER, *Steroids*, Reinhold Publishing, pp. 346–352 (1959); D. F JONES, *Nature* **202,** 1309 (1964).

GIBBERELLINS IN DEVELOPING SEED OF *P. MULTIFLORUS*

As a preliminary to biosynthetic studies, the gibberellin content of seed of *P. multiflorus* at various stages of development was analysed by GLC and GC–MS.

The seed of *P. multiflorus* were divided into groups (A) to (I) according to seed length (Table 5) and each group was processed as described for the large scale extraction. The crude strong acids from each group were methylated with diazomethane, then passed through a short column of charcoal. This purification step prevented deterioration of the GLC columns which otherwise occurred at the point of on-column injection after a few injections of the crude methylated fractions. The methylated fractions from each group were analysed by GLC isothermally on a 2% QF-1 column then, after trimethylsilylation of the methylated fraction, on a 2% SE-33 column. The amounts of A_1, A_5, A_6 and A_8 were estimated by comparison of the peak areas with those of standards. Under the conditions used, gibberellins A_5 and A_{20} were not resolved and these two gibberellins were estimated

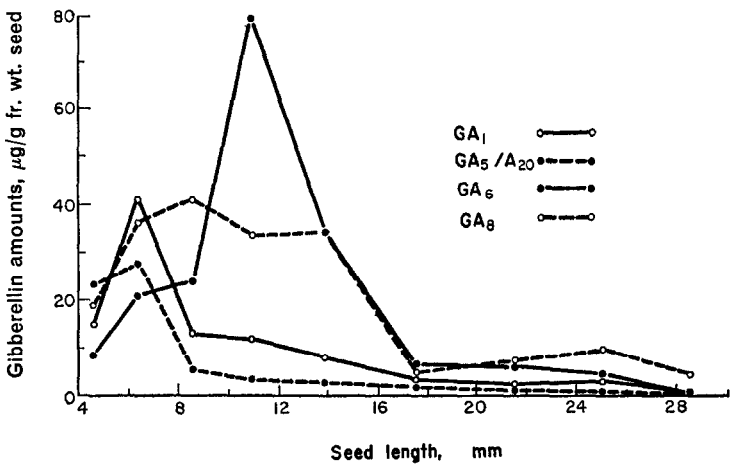


FIG. 4. GIBBERELLIN CONTENT OF SEED OF *Phaseolus multiflorus* AT VARIOUS STAGES OF SEED GROWTH.

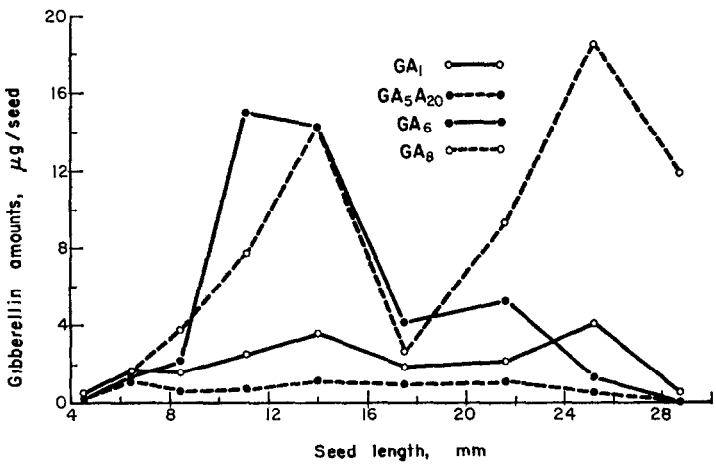


FIG. 5. GIBBERELLIN CONTENT OF SEED OF *Phaseolus multiflorus* AT VARIOUS STAGES OF SEED GROWTH.

Table 5. GLC analysis of gibberellins in seed of *P. multiflorus*

Group	Seed length (mm)	Total wt. (g)	Mean seed wt. (g)	Crude acid fraction (mg)	Gibberellin (μg/g fr. wt. seed)				Gibberellin (μg/seed)			
					A_1	$A_{5/20}$	A_6	A_8	A_1	$A_{5/20}$	A_6	A_8
A	4–5	8·52	0·016	8	15·4	21·8	8·1	19·1	0 25	0 35	0·13	0·3
B	6–7	12·80	0 036	13	40·3	28·5	20·8	36·7	1·5	1·1	1·0	1·4
C	8–9	20·34	0·094	28	12·8	5·7	22·6	40 4	1·2	0·52	2·1	3·8
D	10–12	44·69	0 216	55	11·9	3·1	79·0	33·4	2·6	0 67	15 2	7·2
E	13–15	42·58	0·422	41	8·2	2·3	33·9	34·1	3 4	0 96	14·3	14·4
F	16–19	24·70	0 677	13	2 9	1·4	6·2	4·3	1·9	0·44	4·1	2·9
G	20–23	37·66	1·30	21	1·7	0·76	5·8	6·8	2·1	1·0	5·9	8·9
H	24–26	35·22	1·85	23	2·2	0·12	0·36	9·9	4·0	0·39	0·67	18·3
I	27–30	22·12	2·72	7	0·21	0·00	0·00	4·3	0·57	0·00	0·00	12·0

Table 6. GLC analysis of seed of *P. vulgaris*

Group	Seed length (mm)	Total wt. (g)	Mean seed wt. (g)	Crude acid fraction (mg)	Gibberellin (μg/g fr. wt. seed)				Gibberellin (μg/seed)			
					A_1	$A_{5/20}$	A_6	A_8	A_1	$A_{5/20}$	A_6	A_8
X	4–7	1·93	0·023	8·0	1·3	0·8	6·7	41·9	0·03	0·018	0·15	0·97
Y	9–12	2·33	0·097	3·6	0·7	0·03	4·5	23·6	0·07	0 029	0·44	2·29
Z	18–23	4·48	0·747	1·4	0·2	0·00	0·3	5·7	1·49	0 00	0 22	4·26

together. Although peaks due to the appropriate derivatives of gibberellins A_{17} and A_{19} were observed, they were often obscured by other peaks and so weak that reliable estimates could not be made. The best GLC traces for estimation of gibberellins A_1, $A_{5/20}$, A_6 and A_8 were obtained on SE-33 for both derivatives and these results are shown in Table 5; these values were in good agreement with those obtained from the methylated fractions on 2% QF-1. The results in Table 5 are expressed diagrammatically in Figs. 4 and 5. They are in approximate agreement with those obtained by Jones[26] using TLC although there are some differences. The amounts of total and individual gibberellins [with the possible exception of gibberellin $A_{5/20}$ show two distinct maxima (Fig. 5)]. In agreement with the result of Jones,[26] gibberellin $A_{5/20}$ was found to be produced at an early stage and group A seed contained more gibberellin $A_{5/20}$, per seed or per g fr. wt., than the other gibberellins. Gibberellins A_1 and $A_{5/20}$ reached a maximum concentration (Fig. 4) earlier than gibberellins A_6 and A_8. The amount and concentration of gibberellin A_6 reached a maximum in group D seed and these maximum values contrast sharply with the maximum quantity of 0·5 μg/seed reported by Jones.[26] However Jones detected a compound *b* which reached a maximum at the same developmental stage as gibberellin A_6 and then decreased in amount in a similar way. Jones suggested that compound *b* was the chlorohydrin (XII). We have confirmed this structure in the following way.[3]

The only extant specimen of compound *b* was provided by Dr. D. F. Jones. It gave one major GLC peak, after methylation, with retention times of 17·5 min on 2% QF-1 and of 9·5 min on 2% SE-33 under the conditions described previously.[7] The MS obtained by GC–MS of this peak was virtually identical to that of the methyl ester of gibberellin A_6 and contained no ^{37}Cl ions. However the MS, obtained by GC–MS of the methylated and trimethyl silylated compound *b*, showed the formation of a bis-TMSi ether with M^+540 and a ^{37}Cl-isotope parent at M^+542. These peaks were consistent with the structure (XII) for compound *b*. Ions at *m/e* 481/483 and *m/e* 525/527 corresponded to the loss of 59 amu from the methoxycarbonyl group and of 15 amu from the trimethylsilyl group. The molecular ion was the base peak supporting[6] the presence of a 13-0-TMSi ether.

This mass spectral data confirms structure (XII) for compound *b*. The loss of hydrogen chloride from the methyl ester in the mass spectrometer was supported by examination of the chlorohydrin (XIII), prepared by rearrangement[9] of gibberellin A_6 with aqueous hydrochloric acid. The MS of the chlorohydrin (XIII), obtained by GC–MS with the LKB 9000 at a molecular separator temperature of *ca.* 250° or by direct insertion with an AEI MS9 at a source temperature of *ca.* 300°, were essentially identical showing no ^{37}Cl-containing ions and a parent ion at *m/e* 360 corresponding to the molecular ion of the epoxide derived from the chlorohydrin (XIII) by loss of hydrogen chloride. Trimethylsilylation of the chlorohydrin (XIII) gave a mixture of compounds, the composition of which depended upon the silylating agent. However GC–MS of the main component obtained with BSA, showed a parent ion at M^+468, with the appropriate ^{37}Cl-ion, corresponding to the TMSi ether of (XIII). The loss of hydrogen chloride from the *trans(a,a)*-chlorohydrins (XII) and (XIII), but not in their TMSi ethers, indicates that hydrogen is lost from the hydroxyl adjacent to the chlorine. It is not known whether this loss is thermal or by electron-impact.

No evidence for the presence of the chlorohydrin (XII) in seeds of groups A–I was obtained by GLC. Also a reexamination of the GLC traces of the fractions from charcoal: celite chromatography of the strong acids from the large-scale extraction of seed of *P. multiflorus* revealed none of the chlorohydrin (XII). We conclude that the chlorohydrin (XII) is an artefact, derived from gibberellin A_6. We have observed on several occasions

that the amount of gibberellin A_6 in crude acidic extracts decreases over a period of weeks whereas gibberellins A_1, A_5 and A_8 are relatively stable in the crude extracts.

The identity of the peaks assigned to MeA_1TMSi, MeA_5TMSi, MeA_6TMSi and MeA_8-TMSi in group B seed by GLC was confirmed by GC–MS. In temperature-programmed runs, the TIC trace also showed a small peak with a slightly longer retention time than MeA_1TMSi. This peak gave an MS, identical to that of the MeA_3TMSi. A total of 2·1 μg gibberellin A_3 in group B seed was estimated. Little or no gibberellin A_3 was found in the other groups although it was very difficult to reliably detect the appropriate peak which was overshadowed by the strong adjacent peaks of MeA_1 or MeA_1TMSi.

GC–MS of group H seed as the MeTMSi derivative showed three major peaks consisting of MeA_8TMSi, an unknown, and MeA_1TMSi in decreasing order of magnitude. The MS of the unknown contained a very strong *m/e* 73 ion typical of a TMSi ether. Other ions included M^+518 (26·5%), M^+-18 (9·0%), M^+-28 (55%), M^+-44 (9%), M^+-60 (44%), M^+-(60+28) (45%), M^+-90 (60%), *m/e* 208 (100%) and *m/e* 207 (77%). The last two ions are characteristic[6] of the TMSi ether of a 13-hydroxy gibberellin and the strong M^+-28 ion suggests the presence of an aldehyde group. The loss of 44 amu supports the presence of a lactone ring and the M^+-60 indicates a methoxyl carbonyl group. Structure (XIV) would satisfy the MS data and is reminiscent of gibberellins A_{21} (XV) and A_{22} (XVI)[27] but confirmation of structure (XIV) would require isolation of this unidentified compound.

GC–MS of the neutral fraction from group A seed after methylation showed the presence of the methyl esters of the C_{14}, C_{15}, C_{16}, C_{18} and C_{20}-straight chain saturated acids. No *ent*-kaurene or other diterpenes were detected. GC–MS of the methylated weak acids showed the presence of the methyl esters of the C_{16}, C_{18}, C_{22} and C_{24} fatty acids and of the α-hydroxy-C_{16}-acid. Unaccountably the ethyl ester of the C_{16}-acid was also detected. No *ent*-kaurenoic acid or gibberellin A_9 was detected in the weak acid fraction.

GIBBERELLINS IN DEVELOPING SEED OF *P. VULGARIS*

The above work on immature seed of *P. multiflorus* was performed with plants grown out of doors. An attempt to continue the study during the following winter with plants grown under artificial light met with an unexpected difficulty when the plants failed to flower. However, *P. vulgaris* under the same conditions flowered and set fruit in the normal way. Preliminary analyses of the gibberellin content of developing seed of *P. vulgaris* were therefore made from plants grown in the greenhouse.

Less seed of *P. vulgaris* were available. They were graded into three groups (Table 6) and the gibberellin content determined by GLC as before. The results are shown in Table 6. The peaks assigned to MeA_6 and MeA_8 in group X were examined by GC–MS and gave MS identical with those of the authentic derivatives.[6] The occurrence of gibberellins A_1 and A_5 in seed of *P. vulgaris* has been established by West and Phinney.[28] The present identification of gibberellins A_1 and A_5 establishes that the major gibberellins in seed of both *P. vulgaris* and *P. multiflorus* are the same. Gibberellins A_6 and A_8 are present in relatively larger amounts than gibberellins A_1 and A_5. Indeed the concentration of gibberellins $A_{5/20}$ decrease to zero as the seed matures.

The number of stages sampled were too few to allow a comparison of the results with those obtained by Skene and Carr[29] by paper chromatography and bioassay. However the

[27] N. MUROFUSHI, N. TAKAHASHI, T. YOKOTA and S. TAMURA, *Agri. Biol. Chem.* **33**, 598 (1969).
[28] C. A. WEST and B. O. PHINNEY, *J. Am. Chem. Soc.* **81**, 2424 (1959).
[29] K. G. M. SKENE and D. J. CARR, *Australian J. Biol. Sci.* **14**, 13 (1961).

results obtained above for *P. multiflorus* seed are in close agreement with those obtained by Skene and Carr[29] for developing seed of *P. vulgaris*.

It was decided that application of radio-active precursors should be made just before development of the seed to group X size. An aqueous solution of potassium [2-^{14}C]-mevalonate was fed to immature pod of *P. vulgaris* at this developmental stage by two methods: (a) petiole uptake and (b) by direct injection at the junction of the stem and pod. After 100 hr the pods were collected and the seed were extracted as before. GLC traces of the crude acid fraction were similar to those obtained for group X seed. By method (a) the crude strong acid fraction from the seed contained 0·039% of the total applied radioactivity and the crude strong acid fraction from the pod contained 1·33%. By method (b) the corresponding figures were 0·011 and 0·30%. Feeding method (a), although not very efficient, is superior to method (b). The distribution of [^{14}C]-label between gibberellins A_1, A_5, A_6 and A_8 is at present under investigation.

EXPERIMENTAL

General. M.ps, determined on a Kofler block, are corrected. The following chromatographic materials were used for column chromatography: activated charcoal (BDH), silica gel MFC (Hopkin and Williams), celite 545 (Johns–Manderville) and neutral alumina (Woelm, grade I). In preparative TLC plates (0·9 mm) were prepared with Mallinckrodt 'silicAR' and were pre-eluted with ethyl acetate. For analytical TLC, plates (0·3 mm) were prepared with Kiesel gel G (Merck) or Mallinckrodt 'silicAR'; after development they were viewed either in UV light after spraying with 5% H_2SO_4 in EtOH and heating or in visible light after spraying with 4% $Ce_2(SO_4)_3$ in 10% H_2SO_4 then heating. IR spectra were determined using a Unicam SP200 or Perkin–Elmer 257 instruments. NMR spectra were obtained for solutions containing tetramethylsilane as internal standard with a Varian HA100 instrument. MS as probe samples were obtained using an AEI MS9 instrument. Radio-activity was measured in NE220 liquid scintillator on an IDL scintillation counter, type 6012 using [1-^{14}C]-hexadecane as internal standard.

Gas–liquid chromatography. Me esters and MeTMSi ethers were prepared as described in Part V.[7] To avoid rapid deterioration of GLC columns in repetitive analysis of crude derivatized extracts, the methylated extract in MeOH was passed through a column of charcoal (1·5 × 0·4 cm) before injection or trimethylsilylation. Standard conditions for gibberellin derivatives on 2% QF-1 and 2% SE-33 columns were as described in Part V.[7]

Standard conditions for *ent*-kaurene and derivatives were: 2% QF-1, 128°, N_2-flow, 60 ml/min; and 2% SE-33, 208°, N_2-flow, 60 ml/min *ent*-Kaurene, *ent*-kauren-19-ol and methyl *ent*-kauren-19-oate had the respective retention times 5·2, 15·0 and 12·1 min on QF-1 and 4·4, 10·9 and 9·3 min on SE-33.

Combined gas chromatography–mass spectrometry. All GC–MS spectra were obtained on a LKB9000 instrument operating at 70 eV using GLC conditions specified for each run. GLC traces were given by the TIC (Total Ion Current) trace at 20 eV. Background peaks from the stationary phases were subtracted from each mass spectrum.

Large scale extraction of seed of P. multiflorus. The extraction of immature seed (25·8 kg, containing seed of all sizes A–I, shown in Table 1), from varieties *Streamline* and *Prizewinner*, has been briefly described.[8]

The seed were stored at −50° for 9 months, then extracted twice at room temp. for several days with 70% aq. EtOH (12 l.) then pressed out. The combined extract and pressings were concentrated *in vacuo* at 40–45° to about 3 l. (pH 6·6). After adjusting to pH 7·2–7·5 with 2N–Na_2CO_3 (160 ml) the aqueous extract was extracted with EtOAc (7 × 750 ml). A green semi-solid neutral fraction (30·6 g) was recovered from this EtOAc extract. The aqueous layer was then adjusted to pH 3·0–3·2 with 3N–HCl (1·2 l.) then extracted with EtOAc (8 × 1·3 l.), followed by *n*-BuOH (6 × 750 ml). Recovery of the *n*-BuOH extract gave a red viscous gum (170·2 g). After drying with Na_2SO_4, concentration of the EtOAc extract caused precipitation of fumaric acid (10·4 g), identified by IR. After removal of the fumaric acid by filtration the concentrated EtOAc extract (500 ml) was extracted with phosphate buffer, pH 6·3. The extracted EtOAc solution was recovered to give the weak acid fraction as a semi-solid (3·1 g). The phosphate buffer extract was adjusted to pH 3 with 3N-HCl (325 ml); extraction with EtOAc (14 × 250 ml), then recovery, gave the strong acid fraction as a gummy solid (16·1 g).

Charcoal–celite chromatography of the strong acid fraction. The strong acid fraction (15·6 g) was adsorbed on silica gel (35 g) from an acetone solution and placed on top of a column (70 × 5·5 cm) of celite (350 g) and charcoal (175 g). The column was eluted by the method previously described with an increasing gradient of acetone in H_2O, obtained by connecting an aspirator of water (7·9 l.) to one containing acetone (10 l.). Sixty 200 ml fractions were collected, followed by eight 500 ml fractions. The fraction weights and composition of the eluant as determined by refractometry are shown in Table 1.

Succinic acid (415 mg) was isolated by crystallization of fraction 19 from MeOH and was identified by IR. Fumaric acid (54 mg) was isolated similarly from fraction 26 and identified by IR Gibberellin A_1 (160 mg) m.p. 243–250° (decomp.), and phaseic acid (6·4 mg) m.p. 205–207·5, were isolated by further chromatography of fraction 40 on celite–silica gel as previously described.[7,10]

Isolation of gibberellin A_{17}. Fraction 55 (112 mg) from charcoal·celite chromatography of the strong acid extract was adsorbed on silica gel (1·5 g) and placed on a column (10 5 × 1·5 cm) of celite–silica gel (5 g–2·5 g) made up in light petroleum. The column was eluted in 25 ml. fractions of $CHCl_3$ containing EtOAc increasing in 5% steps Fractions 17–23 eluted with 60–80% EtOAc were shown by GLC of methylated aliquots to contain the purest gibberellin A_{17}; they were combined to yield a semi-solid (29·5 mg) and chromatographed on a TLC plate (20 cm × 0·3 mm) with the solvent mixture, EtOAc–$CHCl_3$–AcOH (15:5:1). The region R_f 0 4–0 6, previously determined as the R_f value of gibberellin A_{17} by GLC analysis of methylated aliquots from extracts of zones from an identical TLC plate and also by spraying with $Ce_2(SO_4)_3$, was extracted to yield impure gibberellin A_{17} (16·5 mg). After removal of silica gel by dissolution in EtOAc, followed by filtration, gibberellin A_{17} was precipitated by light petroleum as an amorphous solid (7 mg) m.p. 140–150° of 90% purity by GLC of a methylated portion; ν_{max} (C_5H_5N) *ca.* 3450, 1710, 1670(sh) and 1635 cm^{-1}; NMR see Table 2.

After extraction of gibberellin A_{17} from the region R_f 0·4–0 6, the regions R_f 0 6–0·75 and R_f 0·75–0·85 were extracted. The former showed no activity in the oat mesocotyl bioassay while the latter showed 203% growth over controls at 3 μg/ml The gummy extract (*ca.* 90 mg) from this region contained *ca* 30 μg of a component which was subsequently showed to be gibberellin A_{17} by GC–MS of a methylated portion.

From the celite–silica gel column chromatography of fraction 55, fractions 15 and 16, eluted with $CHCl_3$ containing 50 and 55% EtOAc, fractions 24–26, eluted with $CHCl_3$ containing 90–100% EtOAc, and fractions 27–36 eluted with EtOAc containing 0–100% acetone in 10% steps were found to contain gibberellin A_{17} by GLC. These fractions were combined with the residues from the purification of gibberellin A_{17} by precipitation with light petroleum from EtOAc solution. The combined material was methylated with CH_2N_2 to give a gum (46 5 mg) which was chromatographed on grade II alumina (8·5 × 0 9 cm). The fractions (25 ml) eluted with light petroleum:benzene then benzene–$CHCl_3$ mixtures were monitored by GLC. Elution with benzene–$CHCl_3$ (3:1, 50 ml; 3 7, 25 ml; and 3 5:6·5, 25 ml) gave *gibberellin A_{17} methyl ester* as a gum (7·5 mg), 80% pure by GLC ν_{max} *ca.* 3500, 1723, 1663 and 906 cm^{-1} (M^+ 420 217, $C_{23}H_{32}O_7$ requires 420 215; M^+-32 388 186, $C_{22}H_{28}O_6$ requires 388·189). NMR (see Table 2).

Isolation of Compounds -α and -β. Fraction 66 (69 mg), eluted from the charcoal:celite column chromatography of the strong acid fraction was adsorbed on silica gel (1·5 g) and placed on top of a column of celite–silica gel (2:1, 8·5 × 1·2 cm) Elution with $CHCl_3$ alone (50 ml) and $CHCl_3$ containing 5–10% EtOAc (150 ml) contained C-α and C-β by GLC analysis of methylated aliquots. The combined fractions (12 5 mg) were chromatographed by TLC on silica gel plates (0 3 mm × 20 cm) developed with (*iso*-Pr)$_2$O–AcOH (95:5). C-α, A semi-solid (2·8 mg) was extracted with MeOH from the region R_f 0·65–0·80 and C-β, also a semi-solid (1·2 mg) from the region R_f 0·15–0·25. (The R_f values of C-α and C-β were determined by GLC of methylated aliquots from extraction of the plates at intervals of R_f 0·5.) C-α showed M^+ 433 ($C_{24}H_{35}NO_6$ requires 433). C-β showed M^+ 408 ($C_{23}H_{34}O_5$ requires 408). C-α methyl ester, obtained with CH_2N_2, had retention times of 7·0 min on QF-1 and 30·2 min on SE-33 under the standard GLC conditions. (Found: M^+ 447·265, $C_{25}H_{37}NO_6$ requires 447·262.) C-β methyl ester showed no parent ion (M^+-18 404·257, $C_{24}H_{36}O_5$ requires 404·256; M^+-15 407·245, $C_{23}H_{35}O_6$ requires 407·244). GLC retention times: 14·2 min on QF-1 and 45 0 min on SE-33 under the standard conditions. C-β methyl ester formed a bis-TMSi ether (M^+ 566) with retention times of 10·2 min on QF-1 and 34 6 min on SE-33 under the standard GLC conditions.

Other characterizing spectroscopic data for C-α, C-β, and their methyl esters are presented in the discussion.

Examination of the Neutral Fraction from the Large Scale Extraction of Seed of P. multiflorus

(a) The unfractionated neutral, extract was examined for *ent*-kaurene by TLC on silica gel using 60% EtOAc in benzene or *n*-pentane, followed by GLC of the extracts from zones at intervals of 0 1 R_f units. None was detected.

(b) The neutral fraction (6·39 g) was adsorbed on silica gel (20 g) and placed on a dry column of silica gel and 50 ml fractions were collected as follows. Fractions 1–14, eluted with benzene gave gums (105 mg). Fractions 15–20, eluted with 10% EtOAc in benzene, gave crude β-sitosterol (33·5 mg) which was crystallized from MeOH in needles m.p. 123–125°; see below for isolation of a purer sample. Fractions 21–25, eluted with 20% EtOAc in benzene gave gums (22 mg). Fractions 26–28 and 29–32, eluted respectively with 40 and 60% EtOAc in benzene gave a wax (404 mg) from which palmitic acid (54 mg) crystallized in rosettes of plates m.p. 56–60°. (Found: M^+ 256·238, $C_{16}H_{32}O_2$ requires M^+ 256·240, identified by IR, GLC, and MS.) Each set of fractions was examined by GLC under the standard conditions for *ent*-kaurene derivatives. The results are described in the Discussion.

(c) The neutral fraction (3·09 g) was sublimed at 100° and 30 mm for 1·5 hr. The sublimate (8 mg) was examined for *ent*-kaurene as in (b).

(d) The neutral fraction (22·6 g) was stirred under reflux for 2 hr with ether (200 ml). The extracted material (10·33 g) was recovered and stirred under reflux with *n*-pentane (300 ml) for 3 hr. The *n*-pentane solubles (4·75 g) and ether solubles (5·55 g) were examined by GLC as in (b).

(e) The fraction (4·7 g), soluble in *n*-pentane and obtained as in (d), was adsorbed on silica gel (13 g) and placed on a dry column of alumina (38 × 3 cm, Grade I). Fractions (75 ml) were collected as follows. Light petroleum eluted intractable oils (56 mg). Elution with light petroleum containing 0–100% benzene, then benzene containing up to 60% $CHCl_3$ gave intractable gums (16 mg). Elution with benzene containing 60–100% $CHCl_3$, then $CHCl_3$ containing 0–75% EtOAc gave oils (838 mg). The latter were rechromatographed on a dry column of silic gel (26 × 4 cm) which was eluted in 25 ml fractions with $CHCl_3$. Fractions 6–13 gave a semi-solid (78 mg) which was purified by TLC on silica gel, developed with $CHCl_3$. The EtOAc extract from the zone R_f 0·27–0·40 was crystallized from MeOH to give β-sitosterol in plates (6·0 mg) m.p. 134–136°. (Found: M^+ 414·385. $C_{29}H_{50}O$ requires M^+ 414·386) ν_{max} (CCl_4) 3620 and 1670 cm^{-1}. GLC on 2% QF-1 at 199° and N_2-flow rate of 60 ml/min showed 80% β-sitosterol (retention time 16·2 min) and 20% of another compound (retention time 13·6 min, M^+ 412 by GC–MS). The two compounds could not be separated by fractional crystallization.

Examination of the Weak Acid Fraction from Large Scale Extraction of Seed of P. multiflorus

(a) An aliquot of the weak acid fraction was methylated and examined, before and after trimethylsilylation, by GLC on SE-33 and QF-1 under standard conditions for both gibberellins and *ent*-kaurene derivatives. Gibberellins A_{10} and A_{12} and *ent*-kaurenoic acid were not present.

(b) The weak acid fraction (3 g) in EtOAc (1·2 l.) was extracted with 2N-NaOH (9 × 200 ml). Recovery from the EtOAc gave acetyl tri-*n*-butyl citrate (1·48 g).

Analysis of Developing Seed of P. multiflorus (*var.* Prizewinner)

(a) *Extraction.* Seed were graded by size (Table 5) into groups A–I. After being stored at −20° for 3 days, each group was extracted as described above for the large scale extraction. A summary of the extraction is shown in Table 5.

(b) *GLC.* The crude strong acid fractions were dried over $CaCl_2$ for 24 hr under vacuum, then methylated with CH_2N_2. The crude methylation product, in MeOH, was passed through a charcoal column (15 × 4 mm), then recovered. One half of the product was dissolved in a known volume of MeOH and aliquots were examined by GLC on the QF-1 column under standard gibberellin conditions. The other half was dried over solid KOH; aliquots were trimethylsilylated, then examined by GLC on both QF-1 and SE-33 columns under the standard conditions for gibberellins.

The amounts of gibberellins A_1, $A_{5/20}$, A_6, and A_8 were measured by comparing the weights of the peaks cut out from the GLC traces with those, obtained from GLC traces of standards, run under identical conditions. Under the standard conditions, gibberellins A_5 and A_{20} could not be resolved and the unresolved peak was measured as gibberellin A_5. Under the standard isothermal conditions, gibberellin A_3 derivative could not be detected in fractions from groups A–I. In a temperature programmed run from 150° to 220° at 3°/min on the SE-33 column, gibberellin A_3 methyl ester TMSi ether was detected in seed of group B in quantities of 2·1 μg/g fr. wt. seed.

(c) *GC–MS.* An aliquot of the crude strong acids from groups B and H were methylated and silylated then examined by GC–MS of a temperature programmed run on the 2% SE-33 column from 150° to 220° at 3°/min. In the case of group B seeds, peaks assigned to the methyl ester trimethylsilyl ethers of gibberellin A_1, A_5, A_6, and A_8 by GLC retention times were scanned at 4 sec per mass decade and gave mass spectra, identical to those of the authentic derivatives. A similar scan of the peak immediately following the gibberellin A_1 MeTMSi peak established the presence of gibberellin A_3 MeTMSi admixed with traces of the corresponding derivative of gibberellin A_1. Similarly for the group H extract, the peaks assigned to MeTMSi ethers of gibberellins A_1 and A_8 by GLC retention times gave mass spectra identical to the spectra of the authentic derivatives. The unknown gibberellin derivative had a retention time 0·87 times that of gibberellin A_1 under the GLC conditions used and was estimated to be present in amounts of 2·7 μg/g fr. wt. of seed. The mass spectra obtained by scanning other peaks in the extract from group H were too weak for positive identification.

The neutral and weak acid fractions were methylated with CH_2N_2 and examined by GC–MS on the 2% SE-33 column in temperature programmed runs from 150° to 190° at 3°/min.

Analysis of Developing Seed of P. vulgaris

Extraction and *GLC* were performed as described above for *P. multiflorus* for the sizes of seed (Table 6).

GC–MS. The methylated strong acid fraction from group X was examined on 2% QF-1 with temperature programming from 150° to 220° at 3°/min. Peaks were scanned at 4 sec per mass decade. The peaks assigned to Me A_6 and Me A_8 by GLC retention times gave mass spectra identical to those of the authentic compounds. The mass spectra from the peaks assigned to the methyl esters of A_1 and A_5 were too weak for positive identification.

TABLE 7. INCORPORATION OF POTASSIUM [2-^{14}C]-MEVALONATE IN SEED OF *P. vulgaris*

	Seed		Pod	
	Method a	Method b	Method a	Method b
Weight (g)	1·44	0·746	108	61
No.	34	25	—	—
Average weight (mg)	42·3	29·9	—	—
Weight of crude extract (mg)	3·14	1·61	34·3	14·9
Radio-activity of crude extract (μc)	$2{\cdot}66 \times 10^{-2}$	$7{\cdot}55 \times 10^{-3}$	0·978	0·211

Potassium [2-^{14}C]-*mevalonate*. [2-^{14}C]-RS-Mevalonic acid lactone ($4{\cdot}15 \times 10^{-2}$ mM; specific activity, 4·82 mc/mM; total activity 0·2 mc) was dissolved in 0·088N-KOH (0·56 ml) The total volume, after heating at 35° for 30 min, was 0·52 ml.

Feeding experiments with potassium [2-^{14}C]-*mevalonate*. Plants of *P. vulgaris* were grown under artificial light until the pods reached 7–8 mm in length. The estimated seed length was 0·4 mm.

(a) *Petiole uptake*. The solution of K [2-^{14}C]-mevalonate (0·28 ml, 0·108 mc) was divided equally into four phials. Four petioles supplying two or three pods were cut under water and dipped into each of the phials. During uptake over 8 hr each phial was washed with a total of 0·6 ml water. After 100 hr under long day conditions, the seed and pod were separately collected and extracted as described above. The pods from one phial contained no seed. Two pods from the remaining three phials died. The collected seed represented $\frac{11}{15}$th of the total radio-activity fed. The results are summarized in Table 7.

(b) *Direct injection*. The solution of K [2-^{14}C]-mevalonate (0·24 ml, 0·092 mc) was injected directly by syringe into ten seed pods at a point close to the junction of pod and stem. After 100 hr under long day conditions of artificial light, the pods were collected and worked up as described in (a). The results are summarized in Table 7.

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