Phytochemistry, Vol. 20, No. 5, pp. 1011–1015, 1981.
Printed in Great Britain.

0031–9422/81/051011–05 $02.00/0

KAURENOLIDE BIOSYNTHESIS IN A CELL-FREE SYSTEM FROM *CUCURBITA MAXIMA* SEEDS*

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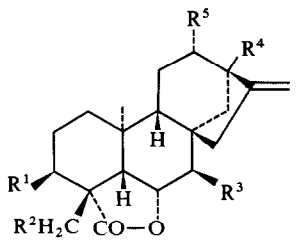
(*Received* 25 *July* 1980)

Key Word Index—*Cucurbita maxima*; Cucurbitaceae; biosynthesis; cell-free system; kaurenolides; gibberellins; *ent*-kaura-6,16-dien-19-oic acid.

Abstract—A new product obtained by incubation of [2-^{14}C]-mevalonic acid with a cell-free system from *Cucurbita maxima* endosperm was identified by GC–MS as *ent*-kaura-6,16-dien-19-oic acid. When this compound was re-incubated with the microsomal fraction it was converted to 7β-hydroxykaurenolide and hence to 7β,12α-dihydroxykaurenolide. The dienoic acid was also obtained by incubation of *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal and *ent*-kaurenoic acid, but not *ent*-7α-hydroxykaurenoic acid, with the microsomal fraction. Thus, in the *C. maxima* cell-free system, the kaurenolides are formed by a pathway which branches from the GA pathway at *ent*-kaurenoic acid and proceeds via the dienoic acid.

INTRODUCTION

The kaurenolides are by-products of GA biosynthesis without any known function. 7β-Hydroxykaurenolide (**1**), 3β,7β-dihydroxykaurenolide (**2**), 7β,18-dihydroxykaurenolide (**3**) and some minor dihydroxykaurenolides have been identified from cultures of *Gibberella fujikuroi* [1–5]. In higher plants 7β,13-dihydroxykaurenolide (**4**) and 7β,12α-dihydroxykaurenolide (**5**) have been identified from seeds of *Phaseolus coccineus* [6] and *Cucurbita pepo* [7], respectively. In addition a dihydroxy-kaurenolide was obtained as a product in a cell-free system from immature seeds of *Cucurbita maxima* [8]. From the mass spectrum presented in ref. [8] this product can now be identified as 7β-12α-dihydroxykaurenolide.



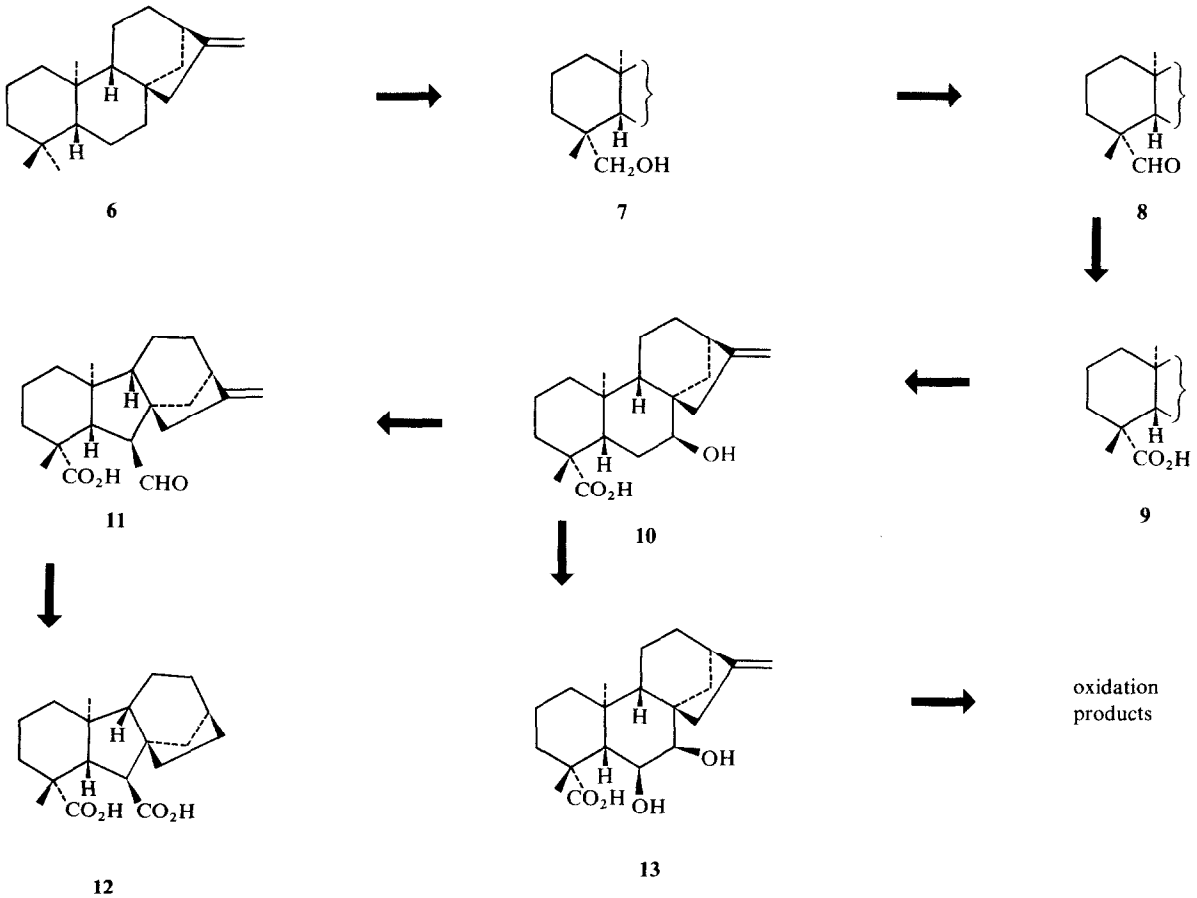
	R^1	R^2	R^3	R^4	R^5
1	H	H	OH	H	H
2	OH	H	OH	H	H
3	H	OH	OH	H	H
4	H	H	OH	OH	H
5	H	H	OH	H	OH
17	H	H	H	H	H

We have now studied further the biosynthesis of kaurenolides in the cell-free system from *C. maxima*. This system catalyses a complete pathway from mevalonic acid (MVA) to C_{20}- and C_{19}-GAs [9, 10]. The portion of the pathway that includes the steps from *ent*-kaurene (**6**) to GA_{12} (**12**) via GA_{12}-aldehyde, as well as a major branch leading to *ent*-6α,7α-dihydroxykaurenoic acid (**13**) and its oxidation products (Scheme 1), is catalysed by microsomal enzymes. It can therefore be studied separately from the remainder of GA biosynthesis, which in this system is catalysed by soluble enzymes [9]. The formation of the kaurenolides is part of the microsomal system.

RESULTS

Analysis of the products obtained from an incubation of [2-^{14}C]-MVA with the complete cell-free system (15000 *g* supernatant fraction) from *C. maxima* endosperm revealed a previously unidentified component. This component migrated with *ent*-kaurenoic acid (**9**) when the products were subjected to TLC on Si gel developed in $CHCl_3$–EtOAc–HOAc (70:30:1). However, after re-chromatography in toluene–EtOAc (17:3), a radioactive substance migrating slightly more slowly than *ent*-kaurenoic acid separated. This component was identified by GC–MS as *ent*-kaura-6,16-dien-19-oic acid (**14**) by comparison of its mass spectrum with the spectrum of the synthesized compound [11]. The mass spectrum of the biosynthetic product clearly showed ^{14}C isotope peaks demonstrating that the identified compound originated from [2-^{14}C]-MVA.

* A part of this work was reported at the 197th Conference of the Society for Experimental Biology held at Imperial College, London, in March 1980. The manuscript is published in the British Plant Growth Regulator Group, Monograph 5, 1980.



Scheme 1. Known reactions of the *Cucurbita maxima* microsomal system.

^{14}C-Labelled **14** was also produced when labelled *ent*-kaurene (**6**), *ent*-kaurenol (**7**), *ent*-kaurenal (**8**) or *ent*-kaurenoic acid (**9**) were incubated with the 200 000 *g* pellet in the presence of NADPH and $MgCl_2$ but it was not observed when *ent*-7α-hydroxykaurenoic acid (**10**) was used as a substrate (Table 1). Confirmation that [^{14}C]-*ent*-kauradienoic acid was produced from each substrate except *ent*-7α-hydroxykaurenoic acid was obtained by GC–MS. Thus *ent*-kaurenoic acid is the immediate precursor of *ent*-kauradienoic acid and the enzyme activity for this conversion is situated in the microsomal fraction.

The [^{14}C]-*ent*-kauradienoic acid was re-incubated with the 200000 *g* pellet in the presence of NADPH and $MgCl_2$. Separation of the products by TLC using $CHCl_3$–EtOAc–HOAc (70:30:1) revealed two radioactive metabolites at R_f 0.17 and 0.74 as well as unconverted substrate at R_f 0.90. The less polar product was identified by GC–MS as 7β-hydroxykaurenolide (*ent*-6β,7α-dihydroxykaur-16-en-19-oic acid 19-6 lactone) (**1**). The

Table 1. Incorporation of ^{14}C-labelled *ent*-kaurenoid precursors into *ent*-kauradienoic acid and other products by the microsomal system

	Radioactivity in the following products (dpm × 10^{-3})				
Substrate	*ent*-Kauradienoic acid (**14**)	*ent*-Kaurenoic acid (**9**)	*ent*-7α-OH Kaurenoic acid (**10**)	GA_{12}-Aldehyde (**11**)	*ent*-6α,7α-$(OH)_2$-Kaurenoic acid (**13**)
ent-Kaurene (**6**)	3.6	12.2	24.7	52.1	44.7
ent-Kaurenol (**7**)	7.9	4.9	39.0	108	75.6
ent-Kaurenal (**8**)	11.7	7.5	33.9	133	101
ent-Kaurenoic acid (**9**)	12.8	3.7	25.8	139	98.3
ent-7α-OH-kaurenoic acid (**10**)	0.3	0.5	2.8	96.7	92.7

more polar product was found to be 7β,12α-dihydroxykaurenolide (**5**). In both cases the mass spectra showed the presence of ^{14}C in the products proving that the identified compounds originated from [^{14}C]-*ent*-kauradienoic acid. Re-incubation of the ^{14}C-labelled 7β-hydroxykaurenolide with the 200000 g pellet resulted in its conversion to the dihydroxykaurenolide whereas this latter product was not further converted.

Our results contrast those obtained with the fungus *Gibberella fujikuroi* for which data has been presented indicating that *ent*-7α-hydroxykaurenoic acid is a precursor of the kaurenolides [12, 13]. In order to be certain that an alternative pathway to the kaurenolides via *ent*-7α-hydroxykaurenoic acid (**10**) was not operating in the *C. maxima* cell-free system it was necessary to compare the incorporation of label from [^{14}C]-*ent*-kaurenoic acid and [^{14}C]-*ent*-7α-hydroxykaurenoic acid into 7β,12α-dihydroxykaurenolide very critically. This was done by using substrates of higher specific radioactivity than normal (88 Ci/mol instead of 44 Ci/mol) and calculating the ^{14}C-specific radioactivity in the resulting dihydroxykaurenolide from its mass spectrum. The higher specific radioactivity was necessary because the microsomes contain significant amounts of *ent*-kaurene (**6**), which is metabolized under the incubation conditions leading to dilution of the label. Endogenous 7β,12α-dihydroxykaurenolide is present in the endosperm but remains with the 200000 *g* supernatant when the microsomes are prepared. Table 2 lists the intensities of the ions at *m/e* 296, 298, 300 and 304 in the mass spectrum of the dihydroxykaurenolide–diTMSi ether prepared from incubations with [^{14}C]-*ent*-kaurenoic acid, [^{14}C]-*ent*-7α-hydroxykaurenoic acid or without added substrate. The fragment ion with *m/e* 296 (**15**), which is due to the loss of both trimethylsilyl groups as TMSiOH and which contains the complete carbon skeleton, was chosen because of its high relative intensity in this molecule and because it contains no silicon. The presence of silicon with its attendant minor isotopes would have complicated the calculation of specific radioactivity. The relative intensities of the ions from the dihydroxykaurenolide resulting from the incubation with [^{14}C]-*ent*-kaurenoic acid indicate that label has been incorporated. The specific radioactivity of the dihydroxykaurenolide is calculated to be *ca* 27 Ci/mol by the method of Bowen *et al.* [14], which shows that the labelled product has been diluted in a ratio of 1:2.3 by product synthesized from endogenous precursors. The dihydroxykaurenolide from the incubation with [^{14}C]-*ent*-7α-hydroxykaurenoic acid contains no ^{14}C. Therefore we conclude that the dihydroxykaurenolide (**5**) is synthesized solely from *ent*-kaurenoic acid (**9**) via *ent*-kauradienoic acid (**14**).

DISCUSSION

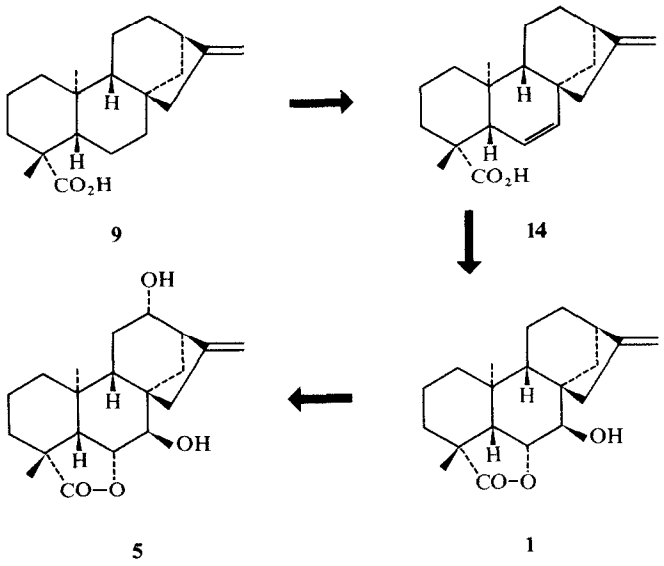
The results show that in the *C. maxima* cell-free system the kaurenolides are biosynthesized from *ent*-kaurenoic acid (**9**) via *ent*-kaura-6,16-dienoic acid (**14**) and not via *ent*-7α-hydroxykaurenoic acid (**10**). Since the dienoic acid is not a precursor of GAs, the kaurenolide pathway branches from the GA pathway at *ent*-kaurenoic acid (Scheme 2). The end-product, 7β,12α-dihydroxykaurenolide (**5**) is present endogenously in the *C. maxima* endosperm (MacMillan *et al.*, unpublished results) so the same pathway probably operates *in vivo*.

Cross *et al.* [15] suggested a kaurenoid compound with a 6,7-double bond as a common precursor of the kaurenolides and the GAs. They fed [17-^{14}C]-*ent*-kaura-6,16-dien-19-ol to cultures of *Gibberella fujikuroi* and found that it was not a precursor of GAs but did not report on incorporation of radioactivity into the kaurenolides. They also suggested the intermediacy of the 6,7-epoxide. Indeed, compounds with unsaturated carbon atoms are known to react with hydroxylases to give epoxides. Thus the 7β-hydroxylase, the enzyme for which *ent*-kaurenoic acid is a substrate, might be expected to convert *ent*-kaura-6,16-dienoic acid to *ent*-6α,7α-epoxykaurenoic acid (**16**), which would be a logical intermediate in kaurenolide biosynthesis. However, we failed to find this epoxide and it is possible that it is converted too quickly to 7β-hydroxykaurenolide to reach detectable levels. Alternatively, the free epoxide is not formed and 7β-hydroxykaurenolide is produced directly from the dienoic acid by a concerted reaction involving the 19-oic acid group.

Table 2. Data for calculation of incorporation of label from [^{14}C]-*ent*-kaurenoic acid and [^{14}C]-*ent*-7α-hydroxykaurenoic acid into dihydroxykaurenolide

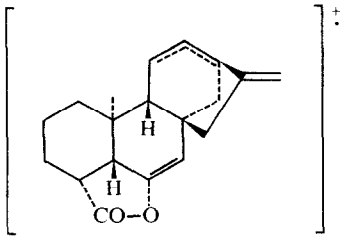
	Relative intensity in dihydroxykaurenolide from		
m/e	*ent*-Kaurenoic acid	*ent*-7α-Hydroxykaurenoic acid	No added substrate
296	100.0	100.0	100.0
298	14.4	3.1	2.7
300	11.4	0.6	0.3
302	5.0	0.3	0.1
304	1.1	0.4	0.1

The relative intensities of the ions F, F + 2, F + 4, F + 6 and F + 8 were measured for the fragment ion F = *m/e* 296 in the mass spectrum of the diTMSi ether of the product dihydroxykaurenolide.



Scheme 2. Biosynthesis of kaurenolides in the *Cucurbita maxima* microsomal system.

Hanson *et al.* [12] found that kaurenolide itself (**17**) is not an intermediate in the formation of hydroxylated kaurenolides in *G. fujikuroi* as it would have been if oxidation had occurred at C-6 before C-7. This is in accord with our data. However, they also showed



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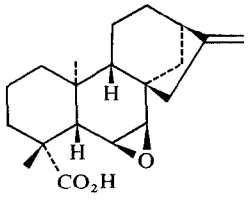

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incorporation of label from [17-^{14}C]-*ent*-7α-hydroxykaurenoic acid into both 7β-hydroxykaurenolide (0.03%) and 7β,18-dihydroxykaurenolide (0.44%). Thus the first step in kaurenolide biosynthesis in *G. fujikuroi* was thought to occur by 6α-hydroxylation of *ent*-7α-hydroxykaurenoic acid [16], which is not in accord with our data from *C. maxima*. Bearder *et al.* [13] also observed formation of 7β-hydroxykaurenolide by a mutant of *G. fujikuroi* after feeding non-labelled *ent*-7α-hydroxykaurenoic acid.

Kaurenolides have been found in very few plants to date. Since the early GA pathway is virtually the same in *G. fujikuroi* and *C. maxima*, one might expect it to be universal and thus kaurenolides to be general by-products of GA biosynthesis. In this case the identification of kaurenolides from many other plant sources can be anticipated.

EXPERIMENTAL

(3*R*, *S*)-[2-^{14}C]-Mevalonic lactone (sp. act. 22 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. The lactone was hydrolysed to the acid by treatment with 2 equivalents of KOH at 30° for 15 min. In most cases the [2-^{14}C]-MVA was diluted with non-radioactive MVA to a sp. act. of 11 mCi/mmol before use. Cell-free extracts were prepared from endosperm of *Cucurbita maxima* as previously described [8].

Preparation and isolation of ent-*kaura*-6,16-*dien*-19-*oic acid.* (3*R*, *S*)-[2-^{14}C]-MVA (22.7 μmol, 11 mCi/mmol) was incubated for 2 hr at 30° with the 15000 *g* supernatant from the *C. maxima* system (30 ml) in the presence of $MgCl_2$ (5 mM), $MnCl_2$ (1 mM), ATP (5 mM), ATP (5 mM), PEP (7.5 mM), NADPH (0.75 mM) in a total vol. of 40 ml. After acidification to pH 3.0 with 5N HCl and the addition of Me_2CO (40 ml), the products were extracted with EtOAc (3 × 30 ml). The combined EtOAc extracts were washed with H_2O (10 ml) and taken to dryness *in vacuo*. The products were separated by TLC on Si gel developed in $CHCl_3$–EtOAc–HOAc (70:30:1), detected by radioscanning and eluted from the Si gel with Me_2CO. The products which migrated together at R_f 0.8 were re-chromatographed in toluene–EtOAc (17:3) and resolved into 3 components. These were identified using GC–MS on the Me esters by comparison of their mass spectra with reference spectra. Thus they were shown to be *ent*-kaur-16-en-19-ol (**7**), *ent*-kaur-16-en-19-oic acid (**9**) and *ent*-kaura-6,16-dien-19-oic acid (**14**) in order of decreasing R_f. The yield of *ent*-kauradienoic acid was 27 nmol or 0.24% of the utilisable MVA. The other ^{14}C-labelled *ent*-kaurenoid products were purified as described previously [8].

Incubation with ent-*kaurenoid GA precursors.* A microsomal fraction was prepared from the cell-free extract by centrifugation of the 15000*g* supernatant at 200000*g* for 90 min and resuspension of the resulting pellet in 50 mM K–Pi buffer (pH 7.6) to give a vol. half that of the original homogenate. For each substrate the resuspended pellet (0.45 ml) containing $MgCl_2$ (5 mM), NADPH (1 mM) and K–Pi buffer (50 mM, pH 7.6) in a total vol. of 0.60 ml was incubated for 5 min at 30°. After this time the substrate (4 nmol) was added in Me_2CO (0.010 ml) and the incubation was continued for a further 15 min. The incubation was stopped by the addition of Me_2CO (0.5 ml) and 1N HCl (0.04 ml) and the products were extracted with EtOAc (3 × 1 ml). After washing with H_2O (1 ml) the combined EtOAc extracts were taken to dryness *in vacuo*. An aliquot of each extract was subjected to 2-dimensional TLC on Si gel developed with toluene–EtOAc (17:3) in the 1st dimension and with $CHCl_3$–EtOAc–HOAc (70:30:1) in the 2nd direction. Where available, non-radioactive standards were co-chromatographed with the extracts in order to facilitate location of the products by visualization in I_2 vapour. Those products for which no standards were available were located by radioscanning. The Si gel associated with each product was removed and counted by liquid scintillation. The remainder of the extracts were methylated (CH_2N_2), trimethylsilylated (TMCS–HMDS–C_5H_5N, 1:2:10) and analysed by GC–MS.

Incubations with ent-*kauradienoic acid and its metabolites.* ent-Kauradienoic acid or its metabolic products were incubated with the 200000*g* pellet as described above. The size and duration of the incubations varied but substrate and co-factor concentrations remained constant as given above. After separation by TLC in $CHCl_3$–EtOAc–HOAc (70:30:1), the products were eluted with Me_2CO for re-incubation or analysis by GC–MS.

GC–MS. GC–MS was carried out using a Finnigan 4015 GC–MS-Data System. Methylated (CH_2N_2) and trimethylsilylated (MSTFA) samples were injected in 1 μl aliquots into a SP-2100 glass capillary WCOT column (30 m × 0.24 mm) and chromatographed using the Grob spitless injection method. The column was maintained at 50° for 1 min, then it was programmed at 15°/min to 230° and at 3°/min to 280°. The injector temp. was 280° and the carrier gas (He) flow rate was 2 ml/min. The split (50:1) was opened after 0.5 min. The column effluent was led directly into the ion source which was maintained at 290°. An electron energy of 70 eV and an emission current of 0.25 mA were used.

Mass peak intensities for the determination of sp. act. were measured by scanning over a limited mass range (*m/e* 294–304) with an integration time of 20 msec per ion. Mass fragmentograms were generated for each ion and the peak areas were determined.

Acknowledgements—We thank Mrs. G. Bodtke for able technical assistance, Professor J. MacMillan for supplying mass spectra of *ent*-kauradienoic acid and 7β,12α-dihydroxykaurenolide and the Deutsche Forschungsgemeinschaft for supporting this work.

REFERENCES

1. Cross, B. E., Galt, R. H. B. and Hanson, J. R. (1963) *J. Chem. Soc.* 2944.
2. Cross, B. E., Galt, R. H. B. and Hanson, J. R. (1963) *J. Chem. Soc.* 3783.
3. Serebryakov, E. P., Simoin, A. V., Kucherov, V. F. and Rosynov, B. V. (1970) *Tetrahedron* **26,** 5215.
4. Bateson, J. H. and Cross, B. E. (1972) *J. Chem. Soc. Perkin Trans.* 1, 1117.
5. Hedden, P., MacMillan, J. and Grinstead, M. J. (1973) *J. Chem. Soc. Perkin Trans.* 1, 2773.
6. Gaskin, P. and MacMillan, J. (1975) *Phytochemistry* **14,** 1575.
7. Fukui, H., Nemori, R., Koshimizu, K. and Yamazaki, Y. (1977) *Agric. Biol. Chem.* **41,** 181.
8. Graebe, J. E., Hedden, P., Gaskin, P. and MacMillan, J. (1974) *Phytochemistry* **13,** 1433.
9. Graebe, J. E. and Hedden, P. (1974) in *Biochemistry and Chemistry of Plant Growth Regulators* (Schreiber, K., Schütte, H. R. and Sembdner, G., eds.) p. 1. Acad. Sci. German Democratic Republic, Inst. Plant Biochem., Halle, G.D.R.
10. Graebe, J. E., Hedden, P., Gaskin, P. and MacMillan, J. (1974) *Planta* **120,** 307.
11. Bearder, J. R. (1973) Ph.D. Thesis, University of Bristol.
12. Hanson, J. R., Hawker, J. and White, A. F. (1972) *J. Chem. Soc. Perkin Trans.* 1, 1892.
13. Bearder, J. R., MacMillan, J. and Phinney, B. O. (1975) *J. Chem. Soc. Perkin Trans.* 1, 721.
14. Bowen, D. H., MacMillan, J. and Graebe, J. E. (1972) *Phytochemistry* **11,** 2253.
15. Cross, B. E., Galt, R. H. B. and Norton, K. (1968) *Tetrahedron* **24,** 231.
16. Hanson, J. R. and Sarah, F. Y. (1979) *J. Chem. Soc. Perkin Trans.* 1, 3151.