Phytochemistry, Vol. 22, No. 2, pp. 427–430, 1983.
Printed in Great Britain.

0031–9422/83/020427–04$03.00/0

SYNTHESIS OF [^{2}H]GIBBERELLINS FROM STEVIOL USING THE FUNGUS *GIBBERELLA FUJIKUROI*

THOMAS GIANFAGNA*, JAN A. D. ZEEVAART† and WILLIAM J. LUSK

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.

(*Received* 20 *May* 1982)

Key Word Index—*Gibberella fujikuroi*; fungi; gibberellin biosynthesis; ^{2}H-labelled; GC/MS; GA_1; GA_{18}; GA_{23}; GA_{53}; steviol.

Abstract—[^{2}H]Steviol (ent-13-hydroxykaur-16-en-19-oic acid) was synthesized from steviol acetate norketone (ent-13-acetoxy-16-oxo-17-norkauran-19-oic acid) by the Wittig reaction using (methyl-d_3)triphenylphosphonium bromide. A mixture of steviol analogs was produced containing from one to four ^{2}H/molecule. [^{2}H]Steviol was fed to strain LM-45-399 of the fungus *Gibberella fujikuroi* which was grown on synthetic medium (ICI, 0% N) in the presence of the growth retardant CCC. [^{2}H]GA_1, [^{2}H]GA_{18}, [^{2}H]GA_{23} and [^{2}H]GA_{53} were isolated from the fungal medium after 4 days. This strain converted steviol to 13-hydroxy GAs in the highest yields of the four *Gibberella* strains tested, and in amounts suitable for metabolic studies with higher plants.

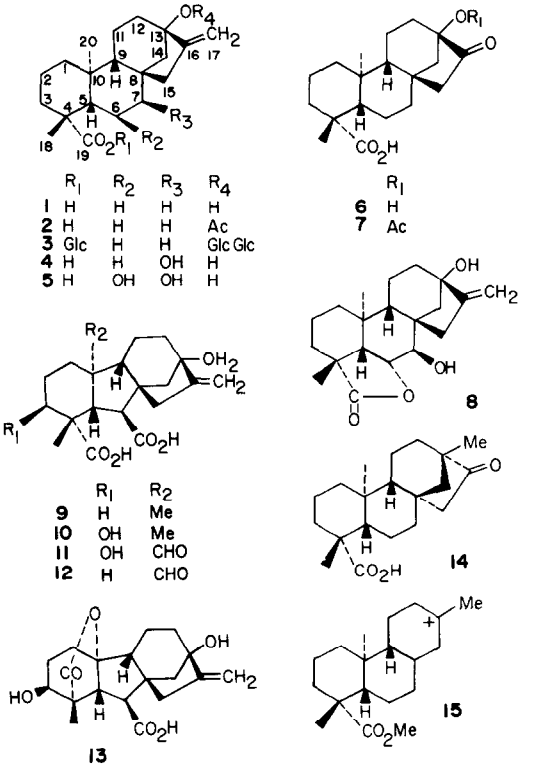
INTRODUCTION

The isolation and identification of GAs from higher plants have proceeded rapidly in recent years with the identification of the endogenous GAs by GC/MS in a wide range of species, including *Pisum sativum* [1], *Phaseolus vulgaris* [2], *Vicia faba* [3], *Cucurbita maxima* [4], *Spinacia oleracea* [5], *Agrostemma githago* [6] and *Zea mays* [7]. Few of the GAs found in higher plants, however, are available in adequate amounts for metabolic studies. In fact, of the more than 60 GAs identified in both the fungus *Gibberella fujikuroi* and in higher plants, only GA_3 and the $GA_{4/7}$ mixture are readily obtainable. Synthetic methods have been developed [8, 9] to convert GA_3 to a number of less readily available GAs. Furthermore, the microbial metabolism of steviol (**1**), the 13-hydroxy analog of ent-kaurenoic acid, has been suggested as another means of obtaining plant GAs [10]. Based upon the procedures described by Bearder *et al.* [10], we have developed a method to obtain ^{2}H-labelled 13-hydroxy GAs from steviol (**1**) using the fungus *G. fujikuroi*. A similar procedure has been used by Heupel, Hedden and Phinney [personal communication] to synthesize [^{3}H]GA_{53} for metabolic studies in *Zea mays*.

RESULTS

GAs and their ent-kaurenoid precursors have frequently been labelled by the Wittig reaction in the C-17-exomethylene position after oxidation to the 17-nor-16-ketone [11]. Attempts to synthesize steviol norketone (**6**) directly from stevioside (**3**) as described by Bearder *et al.* [12] were unsuccessful. The crystalline product obtained had the following mass spectrum after methylation with diazomethane: *m/z* (rel. int.): 332 $[M]^+$ (58), 317 (4), 304 (3), 291 (100), 272 (24), 231 (16), 161 (10), 121 (30), 109 (13) and 41 (29). This compound was most likely the acid catalysed rearrangement product isosteviol (**14**). The base peak at *m/z* 291, which is absent in steviol (**1**), may have the structure **15** following the loss of ketene.

An alternate route to the norketone was developed via the intermediate steviol acetate (**2**). Steviol (**1**) was obtained by enzymatic hydrolysis of stevioside (**3**) and the



*Present address: Department of Horticulture, Rutgers University, New Brunswick, NJ 08903, U.S.A.

†To whom correspondence should be addressed.

product acetylated with acetic anhydride–pyridine under reflux. The resulting acetate (**2**) was oxidized with osmium tetroxide–sodium periodate to steviol acetate norketone (**7**). Labelled steviol acetate (**2**) was produced by the Wittig reaction from **7**, using [^{14}C], [^{3}H] or [^{2}H](methyl)triphenylphosphonium halide. Labelled steviol acetate (**2**) was hydrolysed in base to steviol (**1**) for the fungal feeding experiments. [^{2}H]Steviol (**1**) was composed of a mixture of analogs containing 1–4 ^{2}H/molecule (Table 1). This resulted most likely from scrambling of the label between the C-15 and C-17 positions [12]. In an attempt to incorporate four deuterium atoms into steviol (**1**), the norketone (**7**) was refluxed in acetone-d_6–D_2O (2:1). Methylation of this product with diazomethane in MeOD and subsequent GC/MS indicated the incorporation of 1–5 deuterium atoms, presumably by exchange with the C-15 hydrogens as well as those of the C-13 acetate. After the Wittig reaction with [^{2}H]phosphonium halide and subsequent hydrolysis of steviol acetate (**2**) there was, however, no increase in the final deuterium content. The D_2O exchange reaction with **7** was, therefore, omitted in subsequent experiments.

Steviol (**1**) was fed to resuspended cultures of *G. fujikuroi* in the presence of CCC [(2-chloroethyl)trimethylammonium chloride] which was added to the medium to block endogenous GA production [13, 14]. After purification and fractionation by HPLC of the fungal medium, four zones with GA-like activity were found (Fig. 1). In fractions 11–13 GA_1 (**13**), GA_{18} (**10**) and GA_{23} (**11**) were identified by GC/MS. GA_{18} (**10**) was isolated from fractions 14–16. Fractions 17–19 contained ent-7α,13-dihydroxykaurenoic acid (**4**), ent-6α,7α,13-trihydroxykaurenoic acid (**5**), and 7β,13-dihydroxykaurenolide (**8**). GA_{53} (**9**) was found in fractions 20–22. Each compound was identified by comparison of the mass spectrum obtained with that of published spectra [10, 15].

The major product of steviol (**1**) feeds to the fungal strain GF-1a was 7β,13-dihydroxykaurenolide (**8**) (57% of the total metabolites). A trihydroxykaurenolide was also found with the following mass spectrum of the TMSi derivative: *m/z* (rel. int.): 564 $[M]^+$ (39), 451 (18), 371 (38), 357 (64), 327 (37), 281 (96), 267 (57), 235(57), 208 (100), 207 (50), 193 (45), 167 (32), 147 (48), 109 (36), 103(61).

Table 1. Incorporation of deuterium into: (A) steviol after the Wittig reaction of **7** with (methyl-d_3)triphenylphosphonium bromide, and (B)GA_{53} after feeding [^{2}H]steviol to *Gibberella fujikuroi*

m/z	% [^{2}H] abundance	^{2}H	% total
(A) [^{2}H]Steviol-Me. Av. MW 334.56; *m/z*: 123(100)			
332	1.97	0	2.5
333	10.47	1	13.0
334	23.32	2	29.4
335	28.65	3	36.1
336	15.01	4	18.9
(B) [^{2}H]GA_{53}-MeTMSi. Av. MW 450.60; *m/z*: 210(100)			
448	1.70	0	2.2
449	8.63	1	11.2
450	23.42	2	30.4
451	28.12	3	36.5
452	15.14	4	19.7

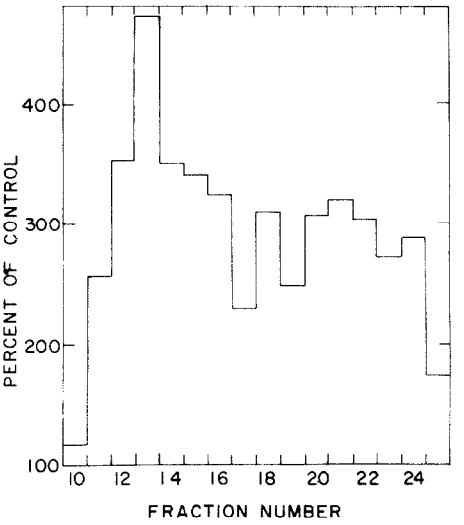


Fig. 1. Growth response of the d_5 mutant of *Zea mays*. Length of first + second leaf sheath in control: 42 mm (100%). HPLC fractions of extract from *Gibberella fujikuroi* to which steviol had been fed in the presence of CCC.

In the strain LM-45-399 the major metabolite of steviol (**1**) was ent-7α,13-dihydroxykaurenoic acid (**4**); the trihydroxykaurenolide was not found. The compounds 7β,13-dihydroxykaurenolide (**8**), ent-7α,13-dihydroxykaurenoic acid (**4**) and GA_{53} (**9**) were each re-fed to strain LM-45-399. None of these compounds were efficiently metabolized over a period of 3 days; ent-7α,13-dihydroxykaurenoic acid (**4**) was partially converted to ent-6α,7α,13-trihydroxykaurenoic acid (**5**). GA_{53} (**9**), GA_{18} (**10**) and GA_1 (**13**). No kaurenolide was found. GA_{53} (**9**) (3 mg feed) was essentially quantitatively recovered from the fungal medium after 3 days. Small amounts of GA_1 (**13**) (73 μg), GA_{18} (**10**) (57 μg) and GA_{19} (**12**) (16 μg) were also found. The kaurenolide was not metabolized by this strain.

[^{14}C]Steviol (**1**) was fed to four fungal strains and the efficiency of metabolism and distribution of radioactivity determined (Table 2). Of the four strains of *G. fujikuroi* used, LM-45-399 most efficiently converted [^{14}C]steviol (**1**) into acidic ethyl acetate soluble compounds. This strain was, therefore, used in subsequent experiments to maximize GA production. The maximal level of acidic ethyl acetate soluble radiolabelled material was found 2 days after feeding [^{14}C]steviol (**1**). The level of these compounds remained constant for 7 days after the addition of [^{14}C]steviol and declined thereafter. The maximum concentration of GA_{53} (**9**) was found after 4 days. The yield of GA_{53} (**9**) was strongly dependent upon the concentration of steviol (**1**) in the medium. Dilute steviol (**1**) feeds (10 μg/ml) resulted in the synthesis of GA_{53} (**9**) in up to 1.2% yield. Concentrations of steviol (**1**) in excess of 200 μg/ml resulted in yields of less than 0.1%. In contrast, when [^{14}C]steviol acetate (**2**) was fed to strain GF-1a and LM-45-399, 80% of the radioactivity was found in the neutral ethyl acetate fraction of the mycelium after 4 days. The radioactivity co-chromatographed with authentic steviol acetate (**2**) by TLC and there was no evidence for the conversion of steviol acetate (**2**) to steviol (**1**).

[^{2}H]Steviol (**1**) was fed to strain LM-45-399 to synthesize [^{2}H]GAs for metabolic studies (Table 3). The

Table 2. Distribution of radioactivity after feeding [^{14}C]steviol (0.5 μCi) to four strains of the fungus *Gibberella fujikuroi* for 4 days

Fungal strain	Radioactivity in medium (cpm × 10^{-4})		Radioactivity in mycelium (cpm × 10^{-4})	
	Neutral EtOAc	Acidic EtOAc	Neutral EtOAc	Acidic EtOAc
LM-45-399	27.0	38.0	0.9	0.6
GF-1a	17.0	1.6	39.0	0.3
M419	1.0	0.1	11.0	0.2
$P_1A_1S_5$	0.5	0.2	*	*

*Not measured.

Table 3. The metabolites isolated from the medium 4 days after feeding 30 mg [^{2}H]steviol to the fungus *Gibberella fujikuroi*, strain LM-45-399

Compound	mg	% yield
Ent-7α,13-dihydroxykaurenoic acid (**4**)	15.0	50.0
7β,13-Dihydroxykaurenolide (**8**)	2.5	8.3
Ent-6α,7α,13-trihydroxykaurenoic acid (**5**)	0.9	3.0
GA_{18} (**10**)	0.42	1.4
GA_1 (**13**)	0.37	1.2
GA_{53} (**9**)	0.35	1.2
GA_{23} (**11**)	Trace	—

relative abundance of the d_1, d_2, d_3 and d_4 analogs of [^{2}H]GA_{53} (**9**) was similar to that found for [^{2}H]steviol (**1**) (Table 1). This indicates that isotope effects did not play a role in the biological conversion of [^{2}H]steviol to [^{2}H]GA_{53}.

DISCUSSION

It is not clear why the synthesis of steviol norketone (**6**) from stevioside (**3**) was not successful. However, protection of the 13-hydroxy group as steviol acetate (**2**) does provide a means for preventing epimerization of the C/D ring, which would have occurred during the Wittig reaction with steviol norketone (**6**) [12], thus significantly reducing the yield of labelled steviol (**1**). It is also unclear why the D_2O exchange reaction with steviol acetate norketone (**7**) did not result in a greater final deuterium content after the Wittig reaction. Nevertheless, the constant ratio of the $[M+2]^+$, $[M+3]^+$ and $[M+4]^+$ ions provides a useful marker by which labelled metabolites may be identified by mass spectral data.

The results for the GA_{53} (**9**) re-feeding experiments were similar to those of Bearder *et al.*[10] with GA_{53}, and of Hedden *et al.* [16] for refeeds of GA_{14} to the fungus. Both compounds were poorly metabolized and can, therefore, not be used as starting material for more polar GAs. It was hoped that refeeding GA_{53} (**9**) to the fungus would provide a route to GA_{19} (**12**), but the yield was too low to make this practical.

Bearder *et al.* [10] used the GA-less mutant B1-41a in which GA biosynthesis is blocked between ent-kaurenal and ent-kaurenoic acid to demonstrate the production of 13-hydroxy GAs from steviol (**1**). If CCC is used to block endogenous GA production, any fungal strain can be used for synthesis of labelled GAs. Strain LM-45-399 was the most useful in a limited survey of fungal strains generating enough [^{2}H]GAs for metabolic studies with higher plants. Strain GF-1a would be a good source of 7β,13-dihydroxykaurenolide (**8**), a compound from which GAs can be synthesized [16].

EXPERIMENTAL

Steviol (**1**). Stevioside (**3**) (5 g) was hydrolysed at 45° with crude pectinase (Pectinol AC, Rohm & Haas) in 0.1 M Pi buffer, pH 4.5, for 24 hr. The ppt was collected by filtration and chromatographed on a Si gel 60 (70 230 mesh) column (40 g) using a 5% step gradient of EtOAC in hexane. Steviol (**1**) eluted at 45–50% EtOAc in hexane. The material was crystallized from boiling MeOH to give **1** as needles in 20% yield. Mp 208–212°. MS, methyl ester, *m/z* (rel. int.): 332[M]$^+$ (75), 317 (6), 314 (8), 299 (19), 274 (25), 273 (22), 254 (15), 255 (16), 146 (26), 121 (96) and 40 (100).

Steviol acetate (**2**). Steviol (**1**) (600 mg), Ac_2O (30 ml) and C_5H_5N (10 ml) were refluxed for 16 hr. The brown gum obtained after evaporation was chromatographed on Si gel 60 (50 g). Steviol acetate (**2**) eluted with 20–25% EtOAc in hexane and was crystallized as long needles from Me_2CO–H_2O in 72% yield. Mp 195–197°. IR ν_{max}^{KBr}cm^{-1}: 1735, 1695, 1665 and 880. MS, methyl ester, *m/z* (rel. int.): 374 [M]$^+$ (18), 342 (4), 332 (66), 314 (55), 299 (31), 276 (27), 273 (29), 255 (27), 146 (33), 121 (70) and 43 (100).

Steviol acetate norketone (**7**). Steviol acetate (**2**) (400 mg) in THF (4 ml) and H_2O (3.5 ml) was oxidized with OsO_4 (20 mg; 4% aq. soln). After 10 min $NaIO_4$ (840 mg) was added slowly with stirring and the reaction mixture kept at room temp. for 15 hr and partitioned against EtOAc. The EtOAc fraction was chromatographed on Si gel 60 (35 g) and eluted with 35–40% EtOAc in hexane. The norketone (**7**) was crystallized as plates from Me_2CO–H_2O in 70% yield. Mp 221–223°. MS, methyl ester, *m/z* (rel. int.): 376 [M]$^+$ (14), 344 (9), 334 (23), 316 (30), 302 (7), 291 (100), 275 (21), 257 (11), 121 (30), 43 (82).

Preparation of [^{14}C]*steviol.* [^{14}C](Methyl)triphenylphosphonium-I (325 mg; 1 mCi) and potassium *t*-butoxide (125 mg) were dried for 2 hr *in vacuo* over P_2O_5, dissolved in THF (1.5 ml, distilled from CaH_2) and stirred for 10 min under N_2. Steviol acetate norketone (**7**) (40 mg) was also dried *in vacuo* over P_2O_5 and added to the yellow-colored solution. The mixture was stirred at room temp for 2 days under N_2. The soln was poured into 0.5 M KH_2PO_4 buffer, pH 4.5 (40 ml), and partitioned × 3 against EtOAc (20 ml). The EtOAc was dried *in vacuo* at 35°. The residue was taken up in Me_2CO and chromatographed by prep. TLC on Si gel G plates (20 × 20 × 0.25 cm) with $CHCl_3$–EtOAc–HOAc (80:20:1). A zone at R_f 0.29–0.57 was removed and eluted with H_2O-satd EtOAc. The eluate was re-chromatographed under the same conditions and the zone at R_f 0.36–0.50 removed and eluted as before. TLC in $CHCl_3$–EtOAc–HOAc (80:20:1) gave one radioactive spot at the same R_f as authentic steviol acetate (**2**) after spraying with 20%

H_2SO_4 and heating at 120° for 30 min. The yield of [^{14}C] steviol acetate (**2**) was 20 μCi; sp. act. 1.2 mCi/mmol. [^{14}C]Steviol acetate (**2**) was dissolved in 2 M KOH (10 ml) and refluxed for 1 hr. The soln was acidified with 3 M HCl to pH 2.5 and partitioned × 3 with EtOAc. TLC on Si gel G in $CHCl_3$–EtOAc–HOAc (80:20:1) gave a single radioactive spot at R_f 0.12 which corresponded to the R_f of steviol (**1**).

Preparation of [^{2}H]*steviol* (**1**). (Methyl-d_3)triphenylphosphonium-Br (1.5 g) and potassium *t*-butoxide (609 mg) were dissolved in freshly distilled THF (8 ml) and stirred for 10 min under N_2. Steviol acetate norketone (**7**) (248 mg) was added and the reaction mixture was stirred for 2 days at room temp. under N_2. After partitioning with EtOAc, the brown gum was chromatographed on Si gel 60 (20 g) and the resulting [^{2}H]steviol acetate (**7**) (90 mg) was refluxed for 1 hr in 2 M KOH. [^{2}H]Steviol (80 mg) was recovered after acidification and partitioning against EtOAc.

Culture maintenance and growth. G. fujikuroi strains were maintained on potato dextrose agar slants at 4°. Mycelium was transferred to potato dextrose medium (PDM) (50 ml) [13] and cultured for 2 days. A 1 ml suspension was then used to inoculate 50 ml ICI medium (40% N, pH 4.8) [17] which contained 1.9 mM CCC. The mycelium was harvested by filtration after 4 days and resuspended in ICI medium with 0% N at pH 4.8 and 1.9 mM CCC. All cultures were kept at 30° and shaken continuously.

Steviol feeding. Steviol (**1**) (30 mg) and related compounds were dissolved in EtOH (3 ml) and 1 mg added to hot, autoclaved medium (100 ml) in 250 ml Erlenmeyer flasks. The EtOH was allowed to evaporate and the mycelium was added when the medium had cooled. Flasks were incubated at 30° with shaking for 4 days.

Isolation and purification of metabolites. The medium was filtered, acidified to pH 3.0 and passed through a charcoal–Celite (2:1) column (8 g charcoal/l. medium). Metabolites were eluted with 3 vols. 80% aq. Me_2CO. The eluate was concd *in vacuo* at 35° and an equal vol. 0.5 M Pi buffer, pH 7.5, added and partitioned against EtOAc (× 3; 0.5 vol.) to give the neutral EtOAc fraction. The aq. phase was then adjusted to pH 3.0 with 3 M HCl and partitioned against EtOAc (× 3; 0.5 vol.) to give the acidic fraction. The EtOAc fractions were dried *in vacuo* at 35° and the steviol (**1**) metabolites in the acidic and neutral fractions separated by reverse-phase HPLC according to the method of ref. [18]. Aliquots (0.1%) were removed from each HPLC fraction and tested for GA activity using the d_5 *Zea mays* bioassay [19]. Aliquots of HPLC fractions were methylated with CH_2N_2 and silylated with Trisil (Pierce Chemical Co., 50 μl).

GC and GC/MS. The GC conditions were: 3% SP-2100 on 100–200 mesh Gas Chrom Q in a glass column (180 × 0.2 cm) held at 215° for 6 min, then temp. programmed to 265° at 4°/min: He at 30 ml/min.

For mass spectrometry a Hewlett–Packard 5985 quadrupole GC/MS equipped with a computer data system was used with an ionization potential of 70 eV and the mass range of 60–600 a.m.u. scanned repetitively at 266 a.m.u./sec.

Acknowledgements—This work was supported by the United States Department of Energy under Contract No. DE-AC02-76ER01338. We also acknowledge the use of GC/MS equipment in the MSU-NIH Mass Spectrometry Facility (RR00480). We thank Dr. James Metzger for preliminary feeding studies with steviol, Dr. Maria Tanis for advice with the Wittig reaction and Dr. Gregory Boyer for helpful discussions throughout the course of this work.

REFERENCES

1. Frydman, V. M., Gaskin, P. and MacMillan, J. (1974) *Planta* **118**, 123.
2. Durley, R. C., MacMillan, J. and Pryce, R. J. (1971) *Phytochemistry* **10**, 1891.
3. Sponsel, V. M., Gaskin, P. and MacMillan, J. (1979) *Planta* **146**, 101.
4. Graebe, J. E., Hedden, P. and Rademacher, W. (1980) *British Plant Growth Regulators Group, Monograph* **5**, 31.
5. Metzger, J. D. and Zeevaart, J. A. D. (1980) *Plant Physiol.* **65**, 623.
6. Jones, M. G. and Zeevaart, J. A. D. (1980) *Planta* **149**, 274.
7. Hedden, P., Phinney, B. O., Heupel, R., Fujii, D., Cohen, H., Gaskin, P., MacMillan, J. and Graebe, J. E. (1982) *Phytochemistry* **21**, 391.
8. Beale, M. J., Gaskin, P., Kirkwood, P. S. and MacMillan, J. (1980) *J. Chem. Soc. Perkin Trans.* 1, 885.
9. Duri, Z. J., Fraga, B. M. and Hanson, J. R. (1981) *J. Chem. Soc. Perkin Trans.* 1, 161.
10. Bearder, J. R., MacMillan, J., Wels, C. M. and Phinney, B. O. (1975) *Phytochemistry* **14**, 1741.
11. MacMillan, J. (1980) in *Plant Growth Substances* 1979 (Skoog, F., ed.) p. 161. Springer, Berlin.
12. Bearder, J. R., Frydman, V. M., Gaskin, P., MacMillan, J., Wels, C. M. and Phinney, B. O. (1976) *J. Chem. Soc. Perkin Trans.* 1, 173.
13. Ninnemann, H., Zeevaart, J. A. D., Kende, H. and Lang, A. (1964) *Planta* **61**, 229.
14. Murofushi, N., Nagura, S. and Takahashi, N. (1979) *Agric. Biol. Chem.* **43**, 1159.
15. Binks, R., MacMillan, J. and Pryce, R. J. (1969) *Phytochemistry* **8**, 271.
16. Hedden, P., MacMillan, J. and Phinney, B. O. (1974) *J. Chem. Soc. Perkin Trans.* 1, 587.
17. Geissman, T. A., Verbiscar, A. J., Phinney, B. O. and Cragg, G. (1966) *Phytochemistry* **5**, 933.
18. Jones, M. G., Metzger, J. D. and Zeevaart, J. A. D. (1980) *Plant Physiol.* **65**, 218.
19. Zeevaart, J. A. D. (1966) *Plant Physiol.* **41**, 856.