Phytochemistry, 1977, Vol. 16, pp. 1555–1560. Pergamon Press Printed in England

BIOSYNTHESIS OF MONOTERPENES BY *CERATOCYSTIS MONILIFORMIS*

ELAINE LANZA* and JAMES K. PALMER†

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

(*Revised received* 22 *April* 1977)

Key Word Index—*Ceratocystis moniliformis*; monoterpenes; biosynthesis; geraniol; labelling patterns; mevalonate; acetate; leucine.

Abstract—*Ceratocystis moniliformis* produced and excreted monoterpenes when grown on potato-dextrose broth. Geraniol, nerol, citronellol, linalol, α-terpineol, geranial and neral were identified by GC–MS. Their production commenced with the depletion of nitrogen in the growth medium and their combined concentration peaked at about 50 μg/ml on the 5th day of growth. The pathway for the biosynthesis of the identified monoterpenes was studied by supplying the radioactive precursors mevalonic acid-[2-^{14}C], L-leucine-[4,5-^{3}H(N)], and acetate-[2-^{14}C] to *C. moniliformis*. For each precursor, the extent of incorporation into the above monoterpenes and the distribution of radioactivity in geraniol was determined. It was concluded that monoterpenes were formed via the mevalonate pathway, previously established for higher terpenes in other organisms. This represents the first information available on the biosynthetic pathway for free monoterpenes in a microbial system.

INTRODUCTION

Considerable research on the biosynthesis of monoterpenes in higher plants has been undertaken in recent years [1–3]. The results from these studies have been largely inconclusive. Typically, the incorporation of radioactivity from mevalonic acid (MVA), the presumed precursor, has been only 0.001 to 0.1% [1–4]. In addition, the incorporated radioactivity has typically been asymmetrically distributed in the monoterpene skeleton, inconsistent with the MVA pathway established for higher terpenes. For example, Banthorpe *et al.* [5] found that MVA was incorporated into thujone, isothujone and sabinene, but that over 90% of the incorporated label was located in the IPP-derived moiety of the monoterpene skeleton. Similar results have been reported by Banthorpe and LePatourel [6] for α-pinene, by Croteau and Loomis [7] for pulegone and by Suga and Shishibori [8] for citronellol and geraniol. All of these studies were done with leaf and stem tissues of higher plants.

The exception to the above mentioned results was reported by Francis *et al.* [9–11]. For example, they found 1% incorporation from MVA into free monoterpenes and 11% into monoterpene glucosides in 1 hr in rose petals [10]. The label was distributed symmetrically in geraniol and nerol.

The conflicting results regarding the MVA pathway for monoterpene biosynthesis in higher plants have generally been attributed to structural [3, 12–14] or metabolic factors [3, 15] in these tissues. Alternatively, the DMAPP utilized in monoterpene biosynthesis may not originate from MVA.

The recent identification of monoterpenes in cultures of the fungus, *Ceratocystis variospora* [16] offered another experimental system for studying monoterpene biosynthesis. Microorganisms possess certain experimental advantages in studying biosynthetic pathways, including rapid growth, more control over the physiological state of the organism, easier introduction of potential precursors, and excretion of secondary metabolites into the growth media. The present report provices evidence that monoterpenes are biosynthesized via MVA in *C. moniliformis*.

RESULTS

Production of monoterpenes

Earlier studies on the fruit-like aroma of *C. moniliformis* revealed that this organism produced the monoterpenes citronellol and geraniol [17]. Further investigation of the Freon 11 extract of the fungal growth medium has now shown *C. moniliformis* also produces geranial, nerol, neral, linalol and α-terpineol. These monoterpenes were separated by GLC utilizing an improved method developed in our laboratories (Fig. 1). GC–MS was used to establish identity and purity in every analysis which involved separation via GLC, since the validity of biosynthetic experiments is dependent on establishing the identity and purity of the compound being studied.

The growth broth of *C. moniliformis* was examined daily for the presence of monoterpenes (Figs. 2 and 3). Geraniol was the first monoterpene to appear in the broth (day 1), and its concentration increased steadily for 2.5 more days and then declined. By the second day, citronellol, nerol, geranial, and neral were present.

* Present address: USDA–ARS, Nutrition Institute, Nutrient Composition Laboratory, Beltsville, MD20705, U.S.A.

† Present address: Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA24061, U.S.A.

Abbreviations used: MVA, mevalonate; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate.

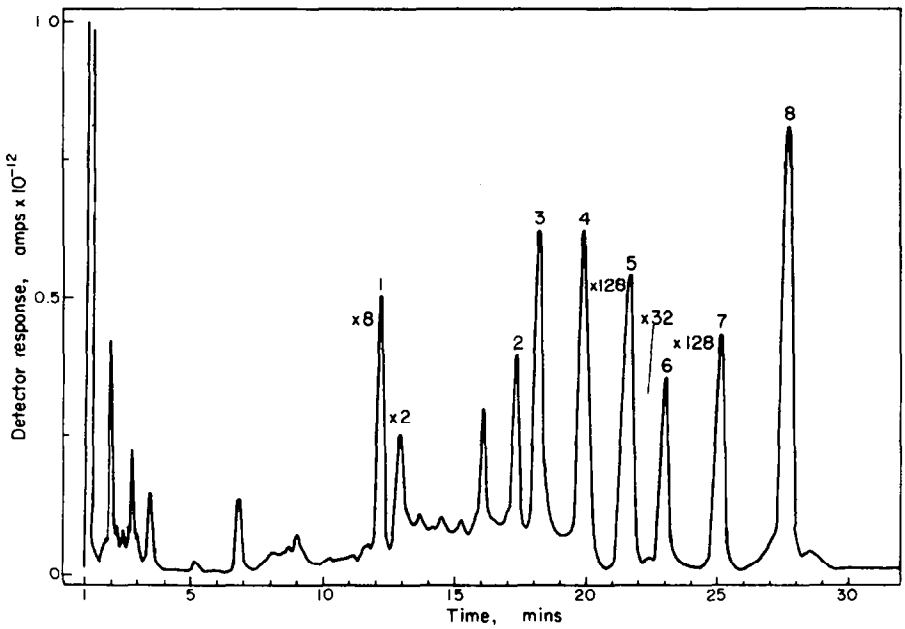

Fig. 1. Gas chromatography of monoterpenes produced by *C. moniliformis*. A Freon extract of 100 ml of a 4 day old culture was prepared and analyzed as described under Experimental. Peak identity: 1. linalol 2. neral 3. α-terpineol 4. geranial 5. citronellol 6. nerol 7. geraniol 8. phenethyl alcohol (internal standard). Attenuation was × 1 unless otherwise indicated.

Linalol and α-terpineol did not appear until the 4th day. The maximum combined concentration of the extracellular monoterpenes (51 μg/ml of broth) occurred on day 5 and then declined rapidly.

To learn more about the relation between monoterpene biosynthesis and the general metabolism of *C. moniliformis*, the time course of growth, carbohydrate utilization and nitrogen utilization of this organism were determined (Fig. 4). Cultures were grown as usual on 3% potato-dextrose broth. Mycelia were harvested daily and dry weights were determined. Portions of the culture broth were examined for carbohydrate and nitrogen utilization and the remainder of the broth was analyzed for monoterpenes as described above. Correlating the information on the daily accumulation of monoterpenes in the growth broth (Figs. 2 and 3) with

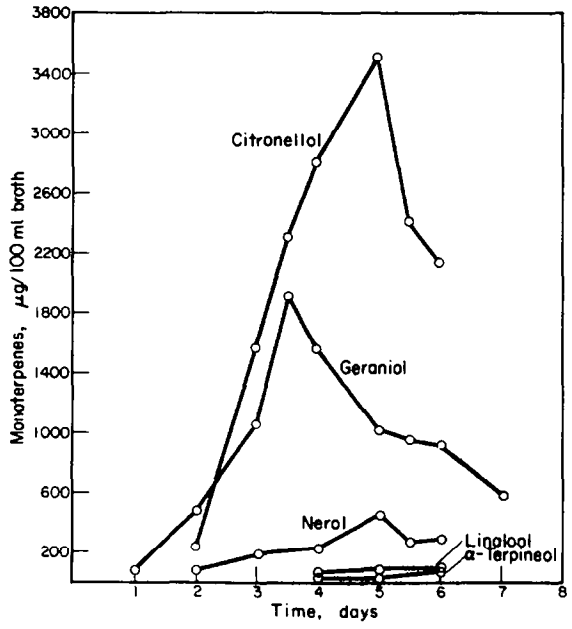

Fig. 2. Production of monoterpene alcohols by *C. moniliformis*. Composite data from a series of runs over a 2 year period.

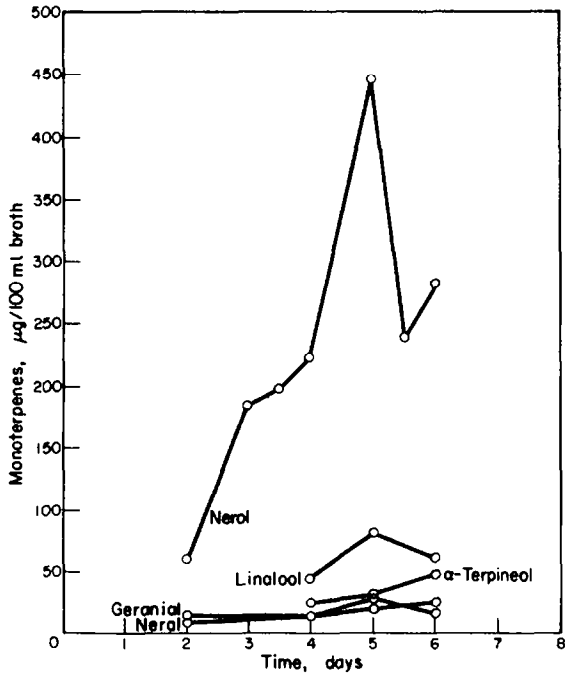

Fig. 3. Production of geranial and neral by *C. moniliformis*; nerol, linalol, α-terpineol also plotted to show relationship to Fig. 2. Composite data from a series of runs over a 2 year period.

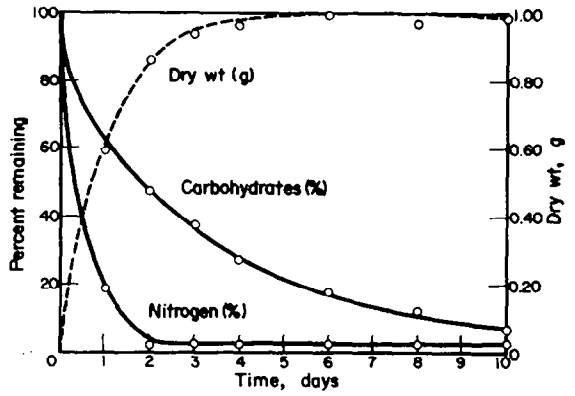

Fig. 4. Growth and nutrient utilization of *C. moniliformis*.

the data in Fig. 4 showed that the monoterpenes were typical secondary metabolites [18], in that they were first produced when the nitrogen source was nearing exhaustion.

The accumulation of monoterpenes inside the fungal cells was also measured on days 1, 2, 3, 4 and 6. None were detected when analyzed with a procedure capable of detecting individual monoterpenes at the 60 ppb level. The *C. moniliformis* cultures were also free of intracellular and extracellular monoterpene pyrophosphates at the 60 ppb limit of detection. Based on these results, only the free extracellular monoterpenes were considered in subsequent biosynthesis studies.

The absence of pyrophosphates was surprising, since these compounds are generally considered immediate precursors of free monoterpenes [19]. Probably the pyrophosphates are precursors of monoterpenes in *C. moniliformis*, but there is no significant accumulation because of their rapid turnover. However, biosynthesis via a pathway not involving pyrophosphates remains a possibility.

Precursors of monoterpenes

Since the rate of accumulation of monoterpenes in the growth broth was highest at 3–5 days, MVA-[2-^{14}C] was added to 4 day old cultures and incorporation into monoterpenes measured (Table 1). The low level of incorporation (approximately 0.1% in 48 hr) of MVA-[2-^{14}C] into the identified monoterpenes is similar to the levels typically reported for monoterpene studies in higher plants [1–4]. The level of incorporation of MVA-[2-^{14}C] into monoterpenes in *C. moniliformis* was lower than expected since previous studies with fungi had shown considerably more incorporation of MVA into higher terpenes [20–22].

In an attempt to increase incorporation, MVA [2-^{14}C] was added at the time of inoculation and incubation continued for up to 96 hr. The incorporation of MVA-[2-^{14}C] was still low. For example, during the 0–96 hr growth period, 33.3 μmoles of monoterpenes were produced, yet only 0.0023 μmoles (0.36%) of the 0.642 μmoles of added MVA-[2-^{14}C] was incorporated into monoterpenes. Similar results were obtained when labelled MVA was added after two days of growth.

It was assumed that the poor incorporation of MVA resulted from permeability barriers. Precursors of MVA were therefore tested to see if these could cross the barriers and result in MVA production at the site of monoterpene synthesis.

Leucine, a known precursor of MVA [23], was tested but the incorporation of L-leucine-[4,5-^{3}H(N)] into monoterpenes was also low (0.013% in 18 hr).

Acetate-[2-^{14}C] was tested next and found to be more rapidly incorporated. In one experiment 0.74% was incorporated into monoterpenes (in 4 day old cultures) in 5 hr; in another, 1% was incorporated in only 2 hr. In the latter experiment, the incorporation dropped to 0.5% at 22 hr and to 0.2% at 44 hr, suggesting further metabolism of the synthesized monoterpenes. Because there are numerous pathways of acetate metabolism, the rapid incorporation of acetate-[2-^{14}C] (relative to MVA and leucine) into monoterpenes was unexpected.

The incorporation data showed that all three precursors were being converted to monoterpenes, acetate much more efficiently than MVA or leucine. To obtain information on the pathway(s) involved for each conversion, samples of geraniol labelled from each of these radioactive precursors were degraded sufficiently to locate the label in the monoterpene skeleton.

Distribution of label in geraniol

Cultures of *C. moniliformis* were grown in the presence of radioactive MVA, leucine or acetate and the labelled geraniol was collected by repeated trapping during GC separations. The identity and purity (99 + %) of the geraniol was established by GC–MS, and the geraniol was degraded. This was done twice for each precursor.

Geraniol was degraded by periodate–permanganate oxidation. The products of this reaction are: levulinic acid (derived from C-3, C-4, C-5, C-6, C-9), acetone (C-7, C-8, C-10), and formic acid (C-1). Iodoform was formed by a further reaction from the C-9 of levulinic acid and the C-8 and C-10 of acetone. The distribution of radioactivity (Table 2) conformed in all cases to that theoretically expected for formation of geraniol from MVA.

DISCUSSION

The primary objective of this research was to determine if monoterpenes can be biosynthesized via the MVA pathway established for higher terpenes. The low levels of incorporation from MVA and the asymmetric labelling of monoterpenes from labelled MVA in higher plants could mean that the monoterpenes are formed via a different pathway.

In the present study, incorporation of MVA-[2-^{14}C] into monoterpenes was also relatively low (maximum 0.4%). However, the label in the monoterpene alcohol geraniol was found to be symmetrically distributed between the IPP and DMAPP derived moieties of the monoterpene skeleton, as predicted from the MVA pathway. Acetate-[2-^{14}C] and leucine-[4,5-^{3}H(N)] were also shown to be incorporated into geraniol via MVA, as indicated by the same type of symmetrical labelling. This symmetrical labelling has been the primary evidence for the MVA pathway to higher terpenes in animals, microorganisms, and higher plants [24].

The labelling patterns found in this study provide direct evidence for the formation of monoterpenes from MVA. Earlier studies have shown that acetate [25–27] and leucine [28] can be incorporated into monoterpenes

Table 1. Incorporation of MVA-[2-^{14}C] into monoterpenes in 4 day old cultures of *C. moniliformis*

Incubation time (hr)	MVA-[2-^{14}C] added* (μmol)	Incorporation into monoterpenes (%)+
0.2	1.28	0
1	4.60	0.001
6	2.70	0.030
24	4.60	0.070
48	1.01	0.124

* MVA-[2-^{14}C](sp. act. = 8.64 × 10^6 dpm/μmol) was added to 100 ml cultures. + Calculated as the sum of radioactivity found in the individual monoterpenes geraniol, nerol, citronellol, neral, geranial, linalol and α-terpineol, divided by the amount of labelled precursor added to the culture.

in higher plants via the MVA pathway, although Suga *et al.* [28] indicated that DMAPP could also originate from leucine via a non-mevalonoid pathway.

The incorporation data (Table 1 and text) indicated that acetate-[2-^{14}C] was more rapidly incorporated into monoterpenes than was MVA-[2-^{14}C]. Comparison of the rate of uptake and the efficiencies of incorporation of entering acetate-[2-^{14}C] and MVA-[2-^{14}C] into monoterpenes indicate a compartmentalization of monoterpene synthesis within the *C. moniliformis* cells.

The results in the present study with a fungal system cannot be compared directly with those obtained with higher plants. However, the demonstration that monoterpenes can be formed from MVA are consistent with the conclusion of Allen *et al.* [29] that another pathway is unlikely and that the asymmetric labelling of monoterpenes in higher plants can be explained by metabolic factors.

EXPERIMENTAL

Materials. Ceratocystis moniliformis ATCC 12861 was supplied by Dr. Ralph Collins, University of Connecticut, Storrs, CT. All monoterpene standards were a gift of Dr. Robert Erickson, Givaudan Corp., Clifton, New Jersey. Na acetate-[2-^{14}C] sp. act. 2.0 mCi/mmol; DL-mevalonic acid-[2-^{14}C] (dibenzylethylenediamine salt), sp. act. 7.16 mCi/mmol; L-leucine-[4,5-^{3}H(N)], sp. act. 44.2 Ci/mmol were purchased from New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

Cultural techniques. C. moniliformis was maintained on 5% potato-dextrose agar slants. The mycelia were harvested by flooding each slant with 5 ml H_2O. The resultant suspension of mycelia was inoculated into 100 ml 3% potato-dextrose broth [17], contained in 250 ml Erlenmyer flasks. The inoculated seed flasks were plugged with cheesecloth-wrapped non-absorbent cotton and incubated on a rotary shaker at 25° for 72 hr. For production of monoterpenes, a 2 ml portion of the seed culture was transferred to a 250 ml Erlenmyer flask containing 100 ml 3% potato-dextrose broth. The production flasks were incubated on a rotary shaker at 25°.

Growth of C. moniliformis. Growth of cultures was measured from the dry wt of harvested mycelia. The ninhydrin method for estimation of amino acids [30] was used to measure utilization of nitrogen. Carbohydrate (starch + glucose) utilization was determined by first converting starch to glucose with amyloglucosidase, and then measuring glucose by the Glucostat procedure [31].

Analysis of monoterpenes. Cultures were filtered to remove the mycelia and the filtrate was extracted with an equal vol. of Freon 11 (trichlorofluoromethane). Phenethyl alcohol was added to the filtrate prior to extraction as an internal standard. The Freon extract was conc to about 3 ml in a round bottom flask fitted with a 470 mm Vigreux column, by placing the apparatus in a 4° cold room and heating the flask containing the Freon extract to 30°. Hexane (1 ml) was added to the concentrate and the remaining Freon 11 was removed with a stream of N_2. (Freon 11 was removed since it tends to corrode the flame ionization detector.) The monoterpene concentrate was separated on a 3.7 m × 2 mm (i.d.) stainless steel column packed with 5% Carbowax 20 M. The column temp. was initially 102° and was programmed at 2°/min to 165°. The carrier gas was N_2 at 25 ml/min.

Identity and purity via GC–MS. In every experiment involving GC separation of monoterpenes, the separated components were analysed by GC–MS. Identity was established by comparison with retention data and MS of standards under identical conditions. Purity was established by repeated scanning (1 scan

Table 2. Distribution of radioactivity in geraniol labelled from MVA-[2-^{14}C], leucine-[4,5-^{3}H(N)] and acetate-[2-^{14}C]

	MVA			Leucine			Acetate		
Component counted	Sp. act dpm/μmol	Found %	Theory %	Sp. act dpm/μmol	Found %	Theory %	Sp. act dpm/μmol	Found %	Theory %
Geraniol	1417*	100	100	5667‡	100	100	6060‖	100	100
Levulinic acid (C-3, C-4, C-5, C-6, C-9)	694	49	50	2834	50	50	3151	52	52
Acetone (C-7, C-8, C-10)	751	53	50	3060	54	50	2121	35	33
Formic acid (C-1)	0	0	0	0	0	0	0	0	0
Iodoform from acetone (C-8, C-10)	368†	52	50	—§	—	—	939	31	33
Iodoform from levulinic acid (C-9)	8	0.6	0	—	—	—	909	15	17

* About 1.2 mg geraniol collected from cultures incubated with MVA-[2-^{14}C] from 0 to 96 hr after inoculation.
† 2 moles of iodoform are formed from every mole of acetone, and only the idioform from C-10 is labelled from MVA-[2-^{14}C].
‡ About 0.7 mg geraniol collected from cultures incubated with L-leucine-[4,5-^{3}H(N)] 96 hours after inoculation.
§ Iodoform reaction could not be carried out since tritium is lost in this reaction.
‖ About 1 mg geraniol collected from cultures incubated with labelled acetate 96 hours after inoculation.

every 7 seconds) of a component as it emerged from the GC, and showing that the MS were identical throughout the period of elution.

Determination of monoterpene pyrophosphates and intracellular monoterpenes. Monoterpene pyrophosphates were converted to free monoterpenes by bacterial alkaline phosphatase [32], and then analyzed as free monoterpenes. To determine intracellular monoterpenes and intracellular monoterpene pyrophosphates, the fungal cells were disrupted with a Braun Rotary Homogenizer. These compounds were then analyzed by the same procedures described for extracellular compounds.

Determination of radioactivity. Radioactivity was measured on a scintillation counter. Counting efficiencies were typically 95% for ^{14}C and 55% for ^{3}H in Econofluor. Samples were always counted until the counting error was less than $\pm 2\%$.

Incorporation of radioactive precursors into monoterpenes. The radioactive precursors MVA-[2-^{14}C], acetate-[2-^{14}C], and L-leucine-[4,5-^{3}H(N)] were sterilized by passing through a 0.2 μm pore diameter filter, and added to growing cultures. Monoterpenes were extracted from the cultures as previously described.

Individual monoterpenes were collected as they were eluted from a GC column, by using an 8 port micro-volume valve (Carle Instruments, Fullterton, Calif.). Valve exits were connected to V shaped capillaries (1.6 mm i.d.) filled with *ca* 2.5 cm of 100/120 mesh glass beads. The lower halves of these traps were immersed in liquid N_2. The efficiency of trapping was $92 \pm 5\%$. The traps were crushed and dropped into scintillation vials containing 10 ml Econofluor for measurement of radioactivity as described above.

Collection and degradation of labelled geraniol. Monoterpene concentrates were injected 5–20 times (about 3 μl/injection) onto the GC column and the geraniol trapped. The conc and sp. act. of geraniol was determined.

Particular care was taken to establish the identity and purity of the collected geraniol. First, it was shown that the geraniol gave a single peak in a GLC system which provides excellent separation from other monoterpenes (Fig. 1). Second, the MS of the collected geraniol was identical to that of authentic geraniol. Third, a computer comparison of the geraniol MS with 41000 mass spectra using the Probability Based Matching System [33] showed that the only compounds likely to be confused with geraniol were nerol and the formate, acetate and propionate esters of geraniol and nerol. These are all well separated from geraniol in the GLC system employed (Fig. 1). Overall, the GC–MS data indicate that an impurity greater than 1% in the collected geraniol would have been detected in the GC separation, in the MS or both.

Geraniol was degraded by modification of the permanganate–periodate oxidation of von Rudloff [34]. A typical oxidation consisted of *ca* 1 mg geraniol in 0.20 ml C_5H_5N, to which was added a soln of 26 mg $NaIO_4$ and 1.3 g $KMnO_4$ in 0.60 ml H_2O, and 3 mg Na_2CO_3 to produce a pH of 8–9 in the final soln. The reaction mixture was stirred at room temp. for 48 hr. To stop the reaction, a few drops of 0.1 N H_2SO_4 were added to acidify the soln, followed by enough Na metabisulfite to reduced the excess oxidant. Levulinic acid, HCOOH and Me_2CO were formed. Levulinic acid and Me_2CO were further degraded by the iodoform reaction [35].

Analysis of degradation products from labelled geraniol. A 1 m × 2 mm (i.d.) GLC column packed with Porapak Q was used to separate Me_2CO and HCOOH. The column temp. was 160° and the carrier gas flow rate 24 ml/min. Repeated injections were used to collect enough of these compounds so that sp. act. and purity could be determined. The collected Me_2CO was then diluted 5 fold with carrier Me_2CO before being subjected to the iodoform reaction. Iodoform was filtered off, purified by sublimation under red. press., identified by its mp and counted. Another portion of the periodate–permanganate reaction mixture was injected onto a 60 cm × 2 mm (i.d.) GLC column packed with 5% EGSP-Z. The column temp. was 130° and programmed 2°/min to 160°; N_2 carrier flow 30 ml/min. The separated levulinic acid was trapped, identified and counted as described for Me_2CO and HCOOH. The collected levulinic acid was then diluted 5-fold with carrier levulinic acid and further degraded by the iodoform reaction. Commercially available levulinic acid was purified via TLC [36] to obtain pure carrier levulinic acid. Iodoform was identified and counted as previously described.

Acknowledgement—The authors are indebted to R. Croteau for advice during the course of this research.

REFERENCES

1. Banthorpe, D. V., Charlwood, B. V. and Francis, M. J. O. (1972) *Chem. Rev.* **72**, 115.
2. Francis, M. J. O. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W. ed.) p. 29. Academic Press, London.
3. Loomis, W. D. and Croteau, R. (1973) in *Recent Advances in Phytochemistry* (Runeckles, V. C. ed.) Vol. 6. Academic Press, New York.
4. Loomis, W. D. (1967) in *Terpenoids in Plants* (Pridham, J. B. ed.) p. 59. Academic Press, London.
5. Banthorpe, D. V., Mann, J. and Turnbull, K. W. (1970) *J. Chem. Soc. C* 2689.
6. Banthorpe, D. V. and Le Patourel, G. N. J. (1972) *Biochem. J.* **130**, 1055.
7. Croteau, R. and Loomis, D. W. (1972) *Phytochemistry* **11**, 1055.
8. Suga, T. and Shishibori, T. (1973) *Bull. Chem. Soc. Japan.* **46**, 3545.
9. Francis, M. J. O., Banthorpe, D. V. and Le Patourel, G. N. J. (1969) *Nature* **228**, 1005.
10. Francis, M. J. O. and O'Connell, M. (1969) *Phytochemistry* **8**, 1705.
11. Banthorpe, D. V., Le Patourel, G. N. J. and Francis, M. J. O. (1972) *Biochem. J.* **130**, 1055.
12. Amelunxen, F. (1964) *Planta Med.* **12**, 121.
13. Amelunxen, F. (1967) *Planta Med.* **15**, 32.
14. Stubbs, J. M. and Francis, M. J. O. (1971) *Planta Med.* **20**, 211.
15. Croteau, R., Burbott, J. A. and Loomis, W. D. (1972) *Phytochemistry* **11**, 2937.
16. Collins, R. and Halim, A. F. (1970) *Lloydia* **33**, 481.
17. Lanza, E., Ko, K. H. and Palmer, J. K. (1976) *J. Agr. Food Chem.* **24**, 1247.
18. Bu'Lock, J. D. (1965) in *The Biosynthesis of Natural Products*, p. 38. McGraw-Hill, London.
19. Moss, G. (1971) *J. Soc. Cosmet. Chem.* **22**, 231.
20. Hallson, I. R. and White, A. F. (1969) *J. Chem. Soc. C* 981.
21. Hemming, F. W. (1967) in *Terpenoids in Plants* (Pridham, J. B. ed.) p. 223. Academic Press, New York.
22. Jones, E. R. H. and Lowe, G. (1960) *J. Chem. Soc.* 3959.
23. Coon, M. J., Kupiecki, F. P., Dekkar, E. E., Schlesinger, M. L. and del Campillo, A. (1959) *Ciba Foundation Symposium* (Woltstenholme, G. W. and O'Connor, M. eds.) p. 62. Churchill, London.
24. Goodwin, T. W. (1971) in *Rodd's Chemistry of Carbon Compounds.* Vol. III, part E, p. 54. (Coffey, S. ed.). Elsevier New York.
25. Birch, A. J., Boulter, D., Fryer, R. I., Thompson, R. J. and Willis, J. L. (1959) *Tetrahedron Letters* 3.
26. Sandermann, W. and Schweens, W. (1962) *Tetrahedron Letters* 259.
27. Yamazaki, M., Usui, T. and Shibata, S. (1963) *Chem. Pharm. Bull* **11**, 363.
28. Suga, T., Hirata, T., Shishibori, T. and Tange, K. (1974) *Chem. Letters* 189.
29. Allen, K. G., Banthorpe, D. V., Charlwood, B. V., Ekundayo, O. and Mann, J. (1976) *Phytochemistry* **15**, 101.
30. Moore, S. and Stein, W. (1954) *J. Biol. Chem.* **211**, 907.
31. Worthington Biochemicals (1968) Bulletin 4–68.
32. Potty, V. H. and Bruemmer, J. H. (1970) *Phytochemistry* **9**, 1229.

33. Pesyna, G. M., Venkataraghavan, R., Dayringer, H. E. and McLafferty, F. W. (1976) *Anal. Chem.* **48**, 1362.
34. Von Rudloff, E. (1965) *Can. J. Chem.* **43**, 2660.
35. Pasto, D. J. and Johnson, C. R. (1969) in *Organic Structure Determination*, p. 334. Prentice Hall Englewood Cliffs, N.J.
36. Dittman, J. (1968) *J. Chromatog.* **34**, 407.