Terpene Biosynthesis. Part IV.[1] Biosynthesis of (+)-Pulegone in *Mentha pulegium* L.

By **D. V. Banthorpe,* B. V. Charlwood,** and **M. R. Young,** Christopher Ingold Laboratories, University College, 20 Gordon Street, London W.C.1

Feeding of 3,3-dimethyl[1-^{14}C]acrylic acid to *Mentha pulegium* L. led to a tracer pattern in (+)-pulegone [*p*-menth-4(8)-en-3-one] that indicated extensive degradation of the additive and probable incorporation as acetate units. A previous report of intact incorporation of the acid could not be substantiated.

[2-^{14}C]Mevalonic acid was incorporated into (+)-pulegone to give asymmetric labelling whereby almost all the tracer was associated with the isopentenyl pyrophosphate portion. The pattern also showed that if (as is believed on the basis of time-incorporation studies) terpinolene [*p*-mentha-1,4(8)-diene] was a precursor of pulegone, oxidation at the two positions α to the exocyclic double bond occurred at comparable rates.

THE role of 3,3-dimethylacrylic acid (3-methylcrotonic acid) in terpene biosynthesis has never been defined. The labelled compound is reported [2] to be incorporated more effectively than acetate into cholesterol of rat liver, and a route from leucine to 3,3-dimethylacrylic acid and thence to mevalonic acid (MVA) has been proposed [3] that is consistent with the reported incorporation of leucine into carotenoids [4] and of 3,3-dimethylacrylic acid into 3-hydroxy-3-methylglutaric acid.[5] Also, an unprecedentedly high (*ca.* 7·8%) incorporation (for C_5 or C_6 acids) of the acid into pulegone [*p*-menth-4(8)-en-3-one] (I), biosynthesised by *Mentha pulegium* L. (pennyroyal) has been claimed,[6] and the results of partial degradation of the product were held to indicate the incorporation of two intact acid units. However, tracer from the acid was incorporated into the terpenoid portion of certain fungal metabolities only after extensive degradation,[7,8] and the acid was ineffective as a precursor of monoterpenes in *M. piperita* L.[9] or in germinating peas.[5]

We here report experiments to elucidate the role of this compound and of MVA in the biosynthesis of pulegone in *M. pulegium* (fam. Labiatae).

RESULTS

Time Course of Incorporation.—The oil of our group of specimens of *M. pulegium* contained menthol (30%); (+)-pulegone, $[\alpha]_D^{20}$ +23° (*c* 5 in EtOH) (24%); menthone (21%); β-pinene (5%); α-terpineol (5%); and menthyl acetate (and possibly isopulegol: these compounds were unresolved in our chromatographic systems) (5%). The pattern of tracer in products isolated from three-month-old specimens at various times after feeding [2-^{14}C]MVA (1 μCi) is shown in the Table. The optimum time of harvesting for the experiments on pulegone was indicated and the maximum incorporations into β-carotene, pulegone, xanthophylls, and chlorophylls were 0·4, 0·02, 0·18, and 0·18%, respectively. In later experiments incorporations of 0·002—0·01% MVA and 0·007% 3,3-dimethylacrylic acid were achieved after feeding tracer.

Autoradiography showed that 20 h after feeding [2-^{14}C]MVA, the tracer was confined to the young leaves and growing shoots. Thus translocation to the presumed [10] biosynthetic sites appeared to be unrestricted.

Time course of incorporation of tracer in *M. pulegium*

	Radioactivity [a]			
t/h [b]	β-Carotene	Pulegone	Xanthophylls	Chlorophylls
5	1254	80	568	2684
23	1386	204	713	2360
46	8207	286	2696	3071
72	2537	380	1825	2823
93	3132	439	3634	3610
119	2435	349	1875	2231

[a] In disintegrations min^{-1}. [b] Time after uptake of tracer.

Labelling Patterns.—The degradation procedure is shown in Scheme 1. Four feeding experiments were carried out and the counts are recorded in disintegrations min^{-1} $mmol^{-1}$.

(i) April 1968: precursor [2-^{14}C]MVA (100 μCi); metabolism period 127 h. Pulegone (I) (19,300 ± 400) gave 3-methyladipic acid (II) (19,300 ± 170) and acetone (III) (550 ± 150). Degradation of (II) gave the diamine (IV) (17,100 ± 600) and carbon dioxide (133 ± 4).

(ii) July 1968: precursor [2-^{14}C]MVA (100 μCi); metabolism period 120 h. Pulegone (4860 ± 120) gave the acid (II) (4640 ± 130) and acetone (190 ± 80). Degradation of (II) gave the diamine (IV) (4780 ± 530) and carbon dioxide (119 ± 5).

(iii) July 1971: precursor [2-^{14}C]MVA (50 μCi); metabolism period 120 h. Pulegone (29,990 ± 900) gave the acid (II) (28,420 ± 550) and acetone (790 ± 60). Degradation of (II) gave the diamine (IV) (26,070 ± 300) and carbon dioxide (140 ± 10); and the diamine (IV) yielded formaldehyde (V) (16,340 ± 1500).

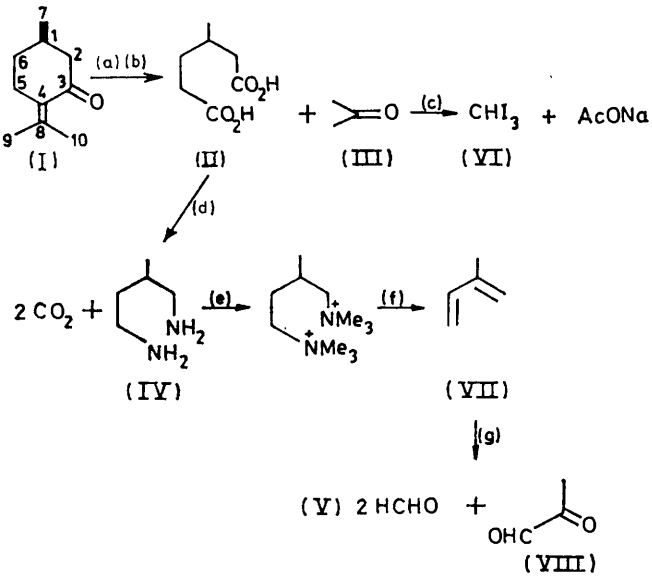
(iv) July 1971: precursor 3,3-dimethyl[1-^{14}C]acrylic acid (45 μCi); metabolism period 120 h. Pulegone (4372 ± 240) gave the acid (II) (3266 ± 200) and acetone (923 ± 70). Degradation of (II) gave the diamine (IV) (2321 ± 120) and carbon dioxide (793 ± 50).

[1] Part III, ref. 12.
[2] K. Bloch, L. C. Clark, and I. Harary, *J. Biol. Chem.*, 1954, **211**, 689.
[3] M. J. Coon, F. P. Kupiecki, E. E. Dekker, M. L. Schlesinger, and A. del Campillo in Ciba Foundation Symposium, 'Biosynthesis of Terpenes and Sterols,' eds. G. E. W. Wolstenholme and M. O'Connor, Churchill, London, 1959, p. 62.
[4] T. W. Goodwin in 'Biosynthetic Pathways in Higher Plants,' eds. J. B. Pridham and T. Swain, Academic Press, London, 1965, p. 37.
[5] D. J. Baisted and W. R. Nes, *J. Biol. Chem.*, 1963, **238**, 1947.
[6] W. Sandermann and H. Stockmann, *Chem. Ber.*, 1958, **91**, 930.
[7] A. J. Birch, R. J. English, R. A. Massy-Westropp, and H. Smith, *Proc. Chem. Soc.*, 1957, 233.
[8] A. J. Birch, R. J. English, R. A. Massy-Westropp, and H. Smith, *J. Chem. Soc.*, 1958, 369.
[9] J. Battaile and W. D. Loomis, *Biochim. Biophys. Acta*, 1961, **51**, 545.
[10] D. V. Banthorpe, B. V. Charlwood, and M. J. O. Francis, *Chem. Rev.*, 1972, **72**, 115.

In these degradations the diene (VII) and methylglyoxal (VIII) were not isolated, and the radioactivity in the iodoform (VI) was too low for meaningful measurement.

DISCUSSION

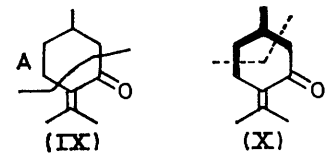
The degradation of (+)-pulegone obtained after feeding 3,3-dimethyl[1-^{14}C]acrylic acid indicated that *ca.* 21% of the incorporated tracer was at C(8)–C(9)–C(10) and 18% at C(3)–C(4). Incorporation of intact C_5 units would have resulted, as previously claimed,[6] in the tracer being exclusively located at and equally divided between C(3) and C(5). Our results suggest that tracer is only incorporated after extensive degradation of the precursor, probably to acetate units. Our low incorporations (0·007%) are in contrast to the high (*ca.* 7·8%) value obtained by the previous workers. However, such low incorporations are usual for the incorporation of C_5 or C_6 acids into monoterpenes in leaf



SCHEME 1

tissue,[10] and the sample of pulegone obtained previously [6] may have been contaminated with a heavily labelled impurity; extensive purification of product was not carried out in these latter experiments and the degradation schemes were recognised to lead to non-homogeneous products.

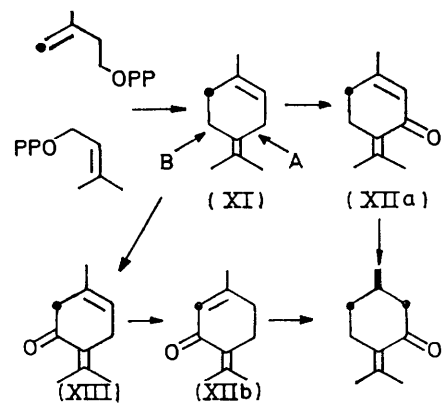
The percentage incorporation of MVA into pulegone was similar, but the labelling pattern was completely different and indicated that MVA was an obligate precursor. In three experiments carried out at different times of the year(s) degradation showed that C(8)–C(9)–C(10) contained only *ca.* 3·1%, and C(3)–C(4) contained 0·5—2·4% of the incorporated tracer. This is consistent with a pattern of asymmetric labelling such that the part of the skeleton derived from isopentenyl pyrophosphate (IPP) (A, IX) was predominantly labelled. A similar pattern has been found in several other species,[11–13] and rationalisations have been proposed.[11] Recently a symmetric labelling pattern,



SCHEME 2

whereby the portions derived from both IPP and 3,3-dimethylallyl pyrophosphate (DMAPP) were equally labelled, has been found in leaves of *Cinnamomum camphora*,[14] although the products were not rigorously purified.

Further degradation showed that 54% of the tracer located in the IPP-derived portion resided on C(2)–C(5), since ozonolysis cleaved the isoprene residue obtained in step (f) at the positions indicated (X). Continued degradation to locate the tracer in the keto-aldehyde (VIII) or to distinguish the extents of labelling at C-2 and C-5 was not possible with the materials available. The simplest explanation consistent with these results is that the tracer (54%) was at C-2 and the balance (40% of the total incorporated) was on C-6. This pattern could result if pulegone was derived from terpinolene (XI), formed from [2-^{14}C]MVA with asymmetric labelling, followed by oxidation at positions A and B (Scheme 3) α to the exocyclic double bond. Piperitenone [*p*-mentha-1,4(8)-dien-3-one] (XII) is a common constituent of *Mentha* oils: the dienone (XIII) has never been detected in these systems but would probably rapidly isomerise to (XII), and stereospecific



SCHEME 3

reduction of (XIIa) or (XIIb) could lead to (+)-pulegone. Terpinolene and piperitenone have been implicated in the biosynthesis of menthane derivatives by several

[11] D. V. Banthorpe, J. Mann, and K. W. Turnbull, *J. Chem. Soc.* (*C*), 1970, 2689.
[12] D. V. Banthorpe and D. Baxendale, *J. Chem. Soc.* (*C*), 1970, 2694.
[13] D. V. Banthorpe and B. V. Charlwood, *Nature New Biology*, 1971, **231**, 285; R. Croteau and W. D. Loomis, *Phytochemistry*, 1972, **11**, 1055.
[14] T. Suga, T. Shishibori, and M. Bukeo, *Phytochemistry*, 1971, **10**, 2725.

studies [15-24] of the time course of passage of tracer into the oils of *Mentha* species.

The experiments with 3,3-dimethylacrylic acid provide no evidence that the part of the menthane skeleton derived formally from DMAPP is actually derived from this acid: however negative conclusions may be invalidated by difficulties of translocation of the additive, here and in other cases, to the biosynthetic site.

In view of the known pattern of interconversions of menthane derivatives,[15-24] our present results can be expected to apply to the synthesis of the major (related) monoterpenoids of other *Mentha* species.

EXPERIMENTAL

All compounds had m.p. or b.p. in agreement with previous reports, and spectra were consistent with the proposed structures. All solids were recrystallised to constant specific activity.

Materials.—Specimens of *M. pulegium* were obtained from the Chelsea Physic Gardens, London, and were cultivated in a south-facing aspect over the period of the feeding experiments. 3,3-Dimethyl[1-^{14}C]acrylic acid was prepared [25] by treatment of 2-methylallylmagnesium chloride (1·0 g) with [^{14}C]carbon dioxide (3 mCi) followed by isomerisation of the resulting acid and reduction. The product (5%), m.p. 65°, was chemically (t.l.c., g.l.c.) and radiochemically (autoscanner) pure.

Feeding Experiments.—In the preliminary experiment to give the time-incorporations, [2-^{14}C]MVA (1 μCi) was fed to cut stems of sets of six matched plants (6 cm; three-month-old) which were maintained on sterile nutrient medium.[11] Such plant material was viable for up to 14 days. Products were extracted [11,12] and purified and chromatograms were scanned for radioactivity. Autoradiographs of leaves and stems were made (97 h exposure) with Kodak Kodirex Estar-base *X*-ray film.

[2-^{14}C]MVA and 3,3-dimethyl[1-^{14}C]acrylic acid were fed [11] to foliage (15 cm; 25 g; three-month-old) in the main series of experiments and harvesting took place after five days. After extraction,[11] pigments were removed by column chromatography on magnesium oxide with hexane as eluant, and pulegone was isolated by preparative g.l.c. and t.l.c. in several systems [11] to give material of constant specific activity: this specific activity was the same as that of appropriate derivatives.

Separation Methods.—Preparative g.l.c. on Carbowax 20 M or FFAP was run at 150—170°, and the effluent was passed into hexane or methanol at 20° to give good (60 to 80%) recovery of pulegone. T.l.c. separations were carried out as previously described.[11] In addition, pulegone was chromatographed on plates of silica gel H (Merck) or Biosil A (silicic acid, *ex* Biorad, Richmond, California). Sprays used were aqueous potassium permanganate, rhodamine B, and phosphomolybdic acid.

Radiochemical Techniques.—These have been previously described [11,12] save that carbon dioxide was trapped in ethanolamine–2-methoxyethanol and counted in a recommended [26] scintillation medium.

Degradation Procedures (*Scheme* 1).—Most reactions are standard.[27] Step (a): use of a three-fold excess of aqueous permanganate at 20° for 3 h yielded 3-methyladipic acid (II), m.p. 92°, $[\alpha]_D^{20}$ +95° (*c* 1·0 in H_2O), 40% yield. Acetone was purified as its 2,4-dinitrophenylhydrazone. Step (b): ozonolysis of pulegone (I) in purified methanol at −40° gave the ozonide corresponding (II) to (60%). The ozonide was decomposed with alkaline hydrogen peroxide at reflux and the acetone was purified as its 2,4-dinitrophenylhydrazone. Step (d): the Schmidt reaction was carried out at 40° in concentrated sulphuric acid [28] and the diamine was purified as the dibenzoyl derivative, m.p. 155° (from aqueous ethanol) or as its tetraphenylboron adduct, m.p. 214° (from water). The amine was regenerated from the latter [29] and converted into the diamine dihydrochloride (70%), m.p. 170° (from water). Step (e) was a conventional methylation with methyl iodide and silver oxide with periodic addition of the base. Step (f) was a Hofmann degradation of an aqueous solution of the 'onium compound at 180—200°. The yield of isoprene was low (*ca.* 10%) because of polymerisation, and the product was distilled out and directly ozonised as in step (b) to give formaldehyde, which was purified as the 2,4-dinitrophenylhydrazone: pyruvaldehyde (VIII) was not recovered.

We thank the S.R.C. for studentships (to B. V. C. and M. R. Y.).

[2/118 *Received*, 19*th January*, 1972]

[15] H. Rothbaecher, *Pharmazie*, 1968, **23**, 389.
[16] R. H. Reitsema, *J. Amer. Pharm. Assoc. Sci. Edn.*, 1958, **47**, 267.
[17] R. G. Battu and H. W. Youngken, *Lloydia*, 1966, **29**, 360.
[18] A. J. Burbott and W. D. Loomis, *Plant Physiol.*, 1967, **42**, 20.
[19] R. H. Reitsema, F. J. Cramer, N. J. Scully, and W. Chorney, *J. Pharm. Sci.*, 1961, **50**, 18.
[20] F. W. Hefendehl, *Planta Med.*, 1962, **10**, 241.
[21] H. Rothbaecher and H. Heltmann, *Pharmazie*, 1968, **23**, 387.
[22] F. H. L. van Os, *Bull. Soc. Pharm. Strasbourg*, 1964, **7**, 49.
[23] F. W. Hefendehl, *Planta Med.*, 1967, **15**, 121.
[24] F. W. Hefendehl, E. W. Underhill, and E. von Rudloff, *Phytochemistry*, 1967, **6**, 823.
[25] D. L. Williams and D. G. Ott in 'Organic Synthesis with Isotopes,' eds. A. Murray and D. Williams, Interscience, New York, 1958, p. 56.
[26] H. Jeffay and J. Alvarez, *Analyt. Chem.*, 1961, **33**, 612.
[27] J. L. Simonsen, 'The Terpenes,' Cambridge Univ. Press, London, vol. 1, 2nd. edn., 1953.
[28] F. Möller in 'Methoden der Organischen Chemie', (Houben-Weyl), Thieme, Stuttgart, 1958, vol. 11 (2), 4th edn., p. 876.
[29] L. Zeidler, *Z. physiol. Chem.*, 1956, **291**, 177.