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BIOSYNTHESIS OF MONOTERPENES IN PLANTS FROM ^{14}C-LABELLED ACETATE AND CO_2*

DEREK V. BANTHORPE, OLUSEGUN EKUNDAYO, JOHN MANN† and KEITH W. TURNBULL

Christopher Ingold Laboratories, University College, London WC1H-OAJ, England

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Key Word Index—*Tanacetum vulgare*; *Thuja plicata*; *Pelargonium graveolens*; *Mentha pulegium*; biosynthesis; monoterpenes; labelling patterns.

Abstract—Degradation of (+)-isothujone biosynthesized by *Tanacetum vulgare* or *Thuja plicata* from acetate-[1-^{14}C], -[2-^{14}C] and -[2-$^{3}H_3$] or from CO_2-[^{14}C] at physiological concentration revealed a pattern of asymmetric labelling whereby tracer predominantly (72–98%) resided in that part of the skeleton derived from IPP. This is similar to the patterns previously obtained for uptake of MVA-[2-^{14}C] but differed from those reported in other species with acetate-[^{14}C] as precursor. Within the IPP-derived moiety the 3 parts derived from acetate units were not equivalently labelled. Partial degradations of geraniol and (+)-pulegone formed in *Pelargonium graveolens* and *Mentha pulegium* after uptake of ^{14}C-labelled acetate or CO_2 showed that the C-2 units of the skeletons of these monoterpenes were also labelled to widely differing extents and these patterns persisted over a range of feeding and seasonal conditions. These results suggest that metabolic pools of acetyl-CoA and/or acetoacetyl-CoA exist in these plants. The general occurrence of such pools and the consequent nonequivalent labelling patterns in secondary metabolism could invalidate biosynthetic conclusions drawn from partial degradations of labelled natural products.

INTRODUCTION

Chemical degradation of several monoterpenes [1–7] and sesquiterpenes [8, 9] that had been biosynthesized from MVA-[2-^{14}C] in various species of higher plants revealed that the tracer was predominantly (up to 99%) in the portion(s) derived from IPP and was located at a single carbon atom (this indicating that the C-5 unit had been incorporated intact). Such "asymmetric" labelling does not occur in the higher terpenoids that have been investigated [10, 11], but the findings can be understood [12] if metabolic pools of different sizes containing IPP and DMAPP (or their biogenetic equivalents) were present perhaps as a result of compartmentation effects [9, 13] such as have been postulated to occur in other contexts [14] in carotenoid biosynthesis. Equivalent incorporation of tracer from MVA-[2-^{14}C] into the IPP and DMAPP-derived moieties (henceforth termed the I and D-units, respectively‡) of certain monoterpenes has been reported [15–17], but these may be special cases (see Discussion). The above labelling patterns seem well-established: all degradations involved purification of products to constant specific radioactivity, the establishment of isotope balances for substrates and degradation products, and the location of tracer at specific carbon atoms.

Biosynthesis of thujone [18], α-pinene [19], menthol [20], thymol [21] and citronellal [22] from acetate-[1-^{14}C] or [2-^{14}C] in various plant

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† Present address: Dyson Perrins Laboratory, University of Oxford, England.

Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; DNPH, 2,4-dinitrophenylhydrazone.

‡ This notation may be readily extended to higher terpenoids. Thus the three head to tail "isoprene" units of sesquiterpenes may be represented D–I_1–I_2 and the skeleton of triterpenes as D–I_1–I_2–I'_2–I'_1–D'.

Table 1. Tracer patterns in (+)-isothujone biosynthesized from various [^{14}C]-precursors

Expt	Plant	Precursor*	† (hr)	‡ Sp. act.	%I§	Product (%)‖
1	*Thuja plicata*	I	90	13·9	0·031	**2** (7); **3** (93); **7** (2) **6** (75)
2	*Tanacetum vulgare*¶	I	100	3·83	0·040	**2** (35); **3** (63)
3	*T. vulgare***	I	100	6·25	0·034	**2** (5); **3** (94); **7** (28) **10** (30)
4	*T. vulgare*¶	II	100	8·65	0·010	**2** (1); **3** (99); **10** (1) **7** (5)
5	*T. vulgare*¶	III	125	12·6	0·018	**8** (93); **11** (20); **12** (3)
6	*T. vulgare*¶	IV	30	20·1	0·023	**2** (34); **3** (64); **7** (16) **5** (38); **6** (15)

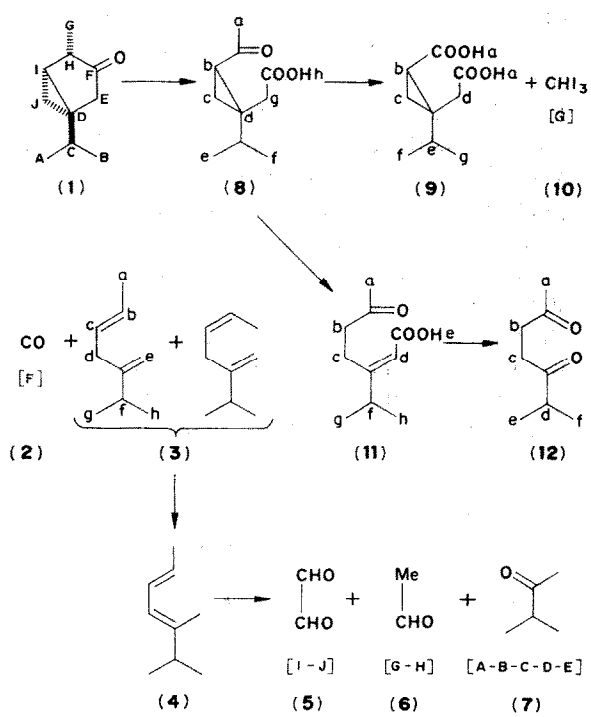
* I Acetate-[2-^{14}C]; II Acetate-[1-^{14}C]; III Acetate-[$^{3}H_3$]; IV CO_2-[^{14}C]. For feeding conditions see Experimental.
† Metabolic period after uptake of tracer.
‡ Specific radioactivity of isothujone × 10^{-3} (dpm mmol^{-1}). Values cannot be compared in different experiments as different quantities of tracer and/or carrier were usually used.
§ % incorporation of ^{14}C into isothujone.
‖ Degradation products (cf Scheme 1); and % of tracer in these. Each value is an independent determination.
¶ Specimens ex U.K. source.
** Specimens ex U.S.A. source.

species was claimed to result in patterns whereby alternate carbons were equivalently labelled, whereas pulegone [13] and geraniol [23] with asymmetric labelling patterns were inferred to be formed from CO_2-[^{14}C]. These results are difficult to assess as the above-mentioned precautions for handling degradation products were not followed and partial degradations often led to the unambiguous location of part only of the incorporated tracer. In addition, in some cases labelling patterns were deduced from the results of partial degradations using the assumption (which now seems questionable) that the I and D units were equivalently labelled.

We here report labelling patterns for an acyclic, a monocyclic and a bicyclic monoterpene in order to establish the extent of asymmetric labelling with precursors other than MVA-[^{14}C]. The use of CO_2-[^{14}C] is especially significant as only this precursor can be administered at physiological concentrations of the gas to allow the metabolism of the unperturbed plant to be studied.

RESULTS

(+)-Isothujone (**1**: *trans*-thujan-3-one) biosynthesized in *huja plicaya L.* (Cupressaceae) and *Tanacetum vulgare L* (Compositae) was degraded as in Scheme 1 and radioactivities of products are given in Table 1. Controls showed that conversion



Scheme 1. Degradation of (+)-isothujone. Carbons in (+)-isothujone (**1**) and its degradation products are lettered in capitals in accordance with the corresponding carbons in the parent geraniol (**13**). The bracketed figures below certain degradation products refer to the carbons of isothujone (and hence of geraniol) contained in the product. Lower case letters identify hydrogen atoms referred to in the discussion of the NMR spectra.

Table 2. Time course of incorporation of [^{14}C] into geraniol in *P. graveolens*

Expt 7: 10 VI 1972: [CO_2-^{14}C]-fed					
Time (hr)*	3	6	9	12	24
Sp. act.†	1·2	30·80	5·0	0·96	31·5
% Incorp.	0·012	0·32	0·05	0·01	0·33
Expt 8: 12 X 1972: [CO_2-^{14}C]-fed					
Time (hr)	3	6	20	24	
Sp. act.	1·3	0·9	6·8	40	
% Incorp.	0·001	0·0006	0·004	0·025	
Expt 9: Various dates: 24 hr metabolism period: [CO_2-^{14}C]-fed					
Date	10.VI.72	12.X.72	12.XII.72	12.III.73	1.VI.73
Sp. act.	31·5	40	2·3	132	67
% Incorp.	0·33	0·025	0·001	0·03	0·30
Expt 10: Various dates: 24 hr metabolism period: Acetate-[1-^{14}C]-fed					
Date	12.I.73	10.VI.73			
Sp. act.	7·3	6·0			
% Incorp.	0·15	0·09			

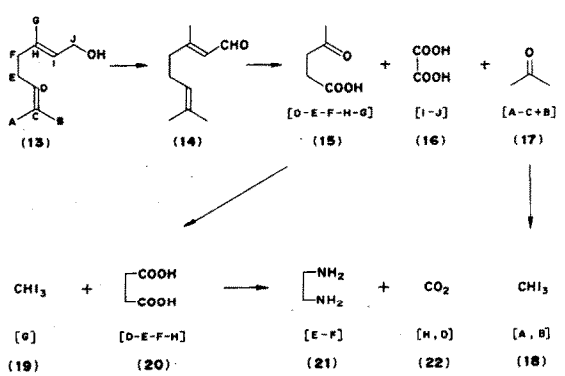
* Metabolic period.

† Specific activity of geraniol $\times 10^{-3}$ (dpm mmol^{-1}). Values are only comparable within a series run on 1 day, as different quantities of radioactive precursor and different amounts of carrier were used in experiments on different occasions.

of **1** into **8** involved no enolization (and hence possible loss of ^{3}H in experiment 5), whereas complete enolization occurred during the conversions **8** → **11** and **11** → **12**. Dilution factors are not recorded as these have little meaning if large and variable pools of biosynthetic intermediates are present [24]. The specific activities quoted are relative for each experiment as different amounts of carrier were used on different occasions. Standard deviations were usually *ca* ±2%, always less than ±5%. This set of feeding experiments was carried out during April–August in 1970–1973.

Profiles of incorporation of both CO_2-[^{14}C] administered at atmospheric concentration and of acetate-[1-^{14}C] into geraniol (**13**; 3,7-dimethylocta-trans-2,6-dien-1-ol) in *Pelargonium graveolens Ait.* (Geraniaceae) are given in Table 2. Products of some of these experiments were partially degraded using the routes shown in Scheme 2 to give the radioactive fragments listed in Table 3. In all these experiments the incorporation of tracer into the "hexane fraction" (see Experimental) was in the range 1–12%.

Table 4 records results of partial degradation (Scheme 3) of (+)-pulegone [**23**; *p*-menth-4(8)-en-3-one] biosynthesized from CO_2-[^{14}C] in *Mentha pulegium L* (Labiatae).



Scheme 2. Partial degradation of geraniol. The brackets beneath certain degradation products refer to the carbons of geraniol contained in that product.

DISCUSSION

Metabolic and degradation products obtained in this work were all rigorously purified to constant specific radioactivity, if possible by repeated recrystallization from at least two solvents. Suitable solid derivatives could not be obtained for a few liquids or gases (CO, CO_2), and these were purified to criteria specified in the Experimental. Care was also taken to obtain isotope balances in the degradation schemes. It is not sufficient to cleave a portion from a molecule and assume that the balance of tracer remains in the truncated part that is not further investigated. The procedures described here are now known to be absolutely

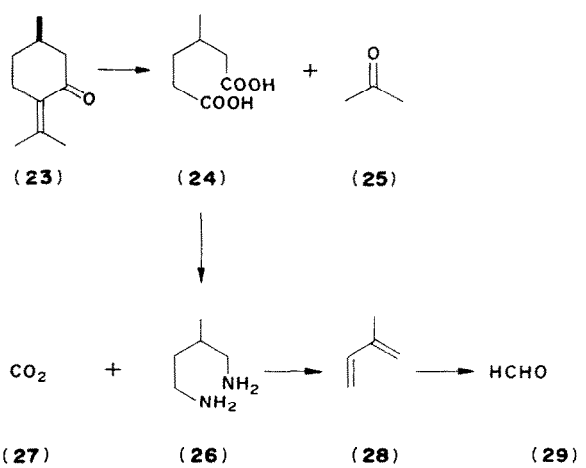
Table 3. Tracer patterns from partial degradation of geraniol biosynthesized in *P. graveolens* from [^{14}C]-precursors*

Expt*	Date*	t* (hr)	Products (% tracer)†
7	VI.72	3	**15** (100); **17** (1); **16** (0)
	VI.72	6	**15** (92); **17** (6); **16** (1)
	VI.72	9	**15** (86); **17** (14); **16** (2)
	VI.72	12	**15** (82); **17** (14); **16** (7)
	VI.72	24	**15** (80); **17** (19); **16** (3)
8	X.72	20	**15** (80); **17** (15); **16** (2)
	X.72	24	**15** (80); **17** (20); **16** (0)
9	III.73	24	**15** (58); **17** (27); **16** (12); **19** (11)
			20 (48); **21** (27); **22** (21); **18** (11)
	VI.73	6	**15** (87); **17** (12); **16** (2); **19** (6)
			20 (79); **21** (37); **22** (35); **18** (5)
10	I.73	24	**15** (59); **17** (22); **16** (16)

* Geraniol-[^{14}C] formed in experiments 7–10 (Table 2) was used in this series of degradation experiments.
† Degradation products (cf. Scheme 2); percentage distribution of tracer in these. All values are independently determined.

essential as conclusions based on the (usually low) radioactivity of GLC or TLC fractions can be invalidated by the presence of heavily-labelled but unsuspected contaminants that are almost invariably present in such preparations [12, 25].

Labelling patterns in (+)-isothujone. Detailed studies on several species, including *Tanacetum vulgare* and *Thuja plicata*, have shown that (+)-isothujone is biosynthesized such that the I and D-units contribute as shown in **30** [1]: consequently the degradation product **7** (Scheme 1) contained all the tracer from the D-unit. The specific activity of **7** obtained by degradation of isothujone formed from acetate-[^{14}C] in experiments 1, 3 and 4 with metabolism times near optimum for passage of tracer into this compound [26] was low and showed that tracer was predominantly (98, 72, 95%) incorporated into the I-unit. Further degradations (experiments 1–3) showed that the three parts of this unit derived from different acetate molecules were not equivalently labelled. The I-unit is constructed from methyl (m) and carboxyl (c) carbons of acetate linked (1 + 2 + 3) as in **31**: and these make up part of the thujane skeleton as shown: the lettering in this skeleton corresponds to that in the parent geraniol, **13**. In experiment 1, uptake of methyl (m) labelled acetate resulted in the sub-units (m_1 + c_1), (m_2 + c_2) and (m_3) possessing 75%, 16% and 7% of the tracer. Degradations were less complete in experiments 2 and 3 but here 35% and 5% respectively of tracer incorporated from (m)-labelled acetate was at (m_3). Experiment 4 involved carboxyl (c)-labelled acetate: here

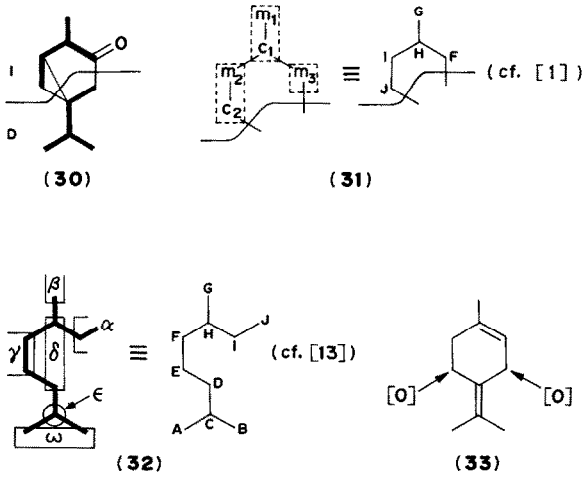


Scheme 3. Partial degradation of (+)-pulegone. In this case carbon to carbon correlation with geraniol is not made as the biosynthetic situation is here more complicated than for isothujone (see text).

Table 4. Tracer pattern from partial degradation of (+)-pulegone biosynthesized in *M. pulegium* from CO_2-[^{14}C]

Expt	Date	t (hr)	Sp. act.	% Incorp.*	Products (% tracer)
11	VI.73	6	40·8 $\times 10^{-3}$	0·12	**24** (88); **25** (8)
					26 (55); **27** (31); **29** (25)

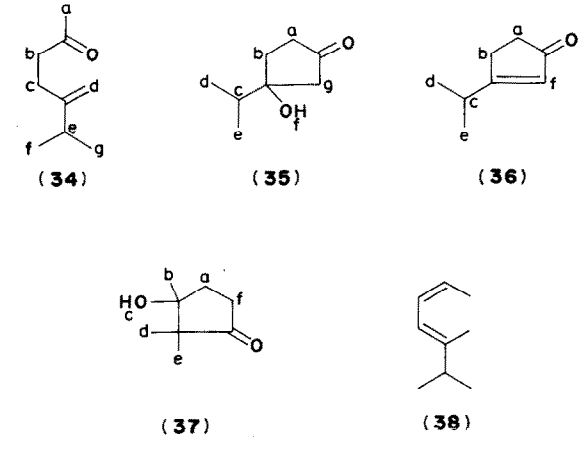
* In contrast, a feeding carried out in December 1973, gave only 0·01% incorporation into (+)-pulegone.



Scheme 4.

the predominantly labelled (95% of incorporation) I-unit contained very little (*ca* 1%) tracer at either (m_3) or (m_1) which is consistent with little c → m scrambling of label before incorporation.

Asymmetric labelling was also found in experiments 5 and 6. In experiment 5, incorporation of acetate-[2-$^3H_3$] led to the methyls of the *iso*propyl group (A, B in **1**; hypothetically-derived from the m-group of acetate containing only 3% tracer. In the latter, administration of CO_2-[^{14}C] under physiological conditions led to the I-unit containing 84% of the incorporated tracer divided *ca* 1:2:2 in the sub-units (m_1 + c_1), (m_2 + c_2) and m_3.



Scheme 5. Degradation products of (+)-isothujone. Letters identify hydrogen atoms referred to in the discussion of NMR spectra.

These asymmetric labelling patterns and also the nonequivalence of labelling within the I-unit in products biosynthesized from acetate-[^{14}C] differ from those previously reported for other monoterpenes and, in particular, from that for thujone; *sic*: probably (+)-isothujone [1]; biosynthesized in *Thuja occidentalis* [18].

Labelling patterns in geraniol. Profiles of incorporation of CO_2-[^{14}C] at physiological concentration into geraniol in *Pelargonium graveolens* (Table 2) led to several conclusions. First, a probable diurnal variation occurred that was consistent with recent views [12, 27] that monoterpenes, far from being inert metabolic wastes, generally undergo rapid turnover in higher plants. Secondly, there was a seasonal effect which was in accord with general experience [12] (practically no quantitative data are available concerning this). Thirdly CO_2-[^{14}C] was incorporated *ca* four-fold more effectively than acetate-[^{14}C] in June, but *ca* 150-fold less effectively in mid-winter (experiments 9, 10) under comparable feeding conditions. Carbon dioxide has generally been found to be a more efficient precursor of monoterpenes than MVA or acetate [12, 27–9] under summer conditions, but our results suggest that this order depends on the level of photosynthesis. Finally, CO_2-[^{14}C] was incorporated into geraniol in *P. graveolens* some 150-fold greater than it or acetate-[^{14}C] was incorporated into *iso*thujone in *T. vulgare* under comparable conditions for optimum uptake. This probably reflects the status of geraniol and isothujone as "early" and "very late" products of biosynthesis: the former (as its pyrophosphate) being additionally a parent of higher terpenoids.

Partial degradations of geraniol formed from CO_2-[^{14}C] under a variety of conditions (Table 3) showed that the carbons were not equivalently labelled although the regimen chosen (continuous rather than pulse-feeding in a closed system; metabolism time up to 24 hr) would have favoured attainment of such a pattern. The scheme used did not allow the relative radioactivities of the I and D-units to be deduced, but the labelling of Me_2CO (**17**; part of the D-unit) increased from 1 to 19% of the total over a 24 hr. metabolism period, whereas (except for experiments 9 and 10 carried out in winter) that in oxalic acid (**16**; part of the I-unit) remained at near 0% over the same period. Previous workers [23] using the same plant and pre-

cursor have assumed that although the D- and I-units might be labelled to differing extents, the carbons of each were equivalently labelled and so have calculated the ratio of tracer in these units from measurements of the radioactivity of the Me_2CO cleaved on degradation. Our results show that this assumption (which was clearly stated as such in Ref. [23]) is invalid. Although our results and those of the previous workers are consistent with a pattern of asymmetric labelling in which the I-unit is predominantly labelled, this pattern cannot be proved from the results of the present and previous partial degradations as these do not determine the labelling of the carbons (D, E) of geraniol derived from C_1 and C_2 of DMAPP. In view of the extreme asymmetry of labelling within the I-unit (as indicated by the negligible radioactivity of oxalic acid obtained on degradation, see above) any assumption as to the degree of labelling of these two carbons must be highly speculative.

The further degradations (of material from experiment 9) give the relative labelling of the sub-units α to ω in **32**: these sub-units comprise the carbons of the skeleton (cf. [**1**]) as shown. In particular, sub-unit γ was the most labelled (37%, 27%) and the ratios of tracer in ω and ϵ were 5:1 and 4:1. Thus the supposition [23] (*vide supra*) that the carbons of the D-unit biosynthesized from CO_2[^{14}C] were equivalently labelled is demonstrably invalid. Degradation of product from experiment 10 reveals that the labelling pattern is less asymmetric when acetate-[1-^{14}C] is precursor under the same seasonal conditions as those used for CO_2-[^{14}C]. Now Me_2CO and oxalic acid contain 22% and 16% of the incorporated tracer compared with 25% in each expected if acetate were incorporated equally and equivalently into the D- and I-units with no (c)→(m) randomization of label.

Labelling pattern in (+)-pulegone. Determination of the labelling pattern in the geranyl pyrophosphate which is the presumed precursor of pulegone is complicated as the former is believed on the basis of previous tracer studies to be converted into the menthadiene **33** which is oxygenated with equal facility at the positions shown [6] before reduction to the ketone. However, partial degradation of pulegone biosynthesized from CO_2-[^{14}C] in *Mentha pulegium* did indicate a highly asymmetric pattern of tracer: *e.g.* the Me_2CO (**25**) contained little (*ca* 8%) of the total. The pattern observed resembled that for (+)-pulegone biosynthesized in *Mentha piperita* [13] but again deduction of the relative labelling of the D- and I-units (cf. ref [13]) is not permissible from such restricted data.

Generality of asymmetric labelling. These findings can be compared with those for the same monoterpenes formed from MVA-[2-^{14}C] in *Tanacetum vulgare*, *Thuja plicata*, *Mentha pulegium* and *Pelargonium graveolens* using clonal material [1, 6, 15]. The products from the first 3 species were asymetrically labelled (70–98% of incorporated tracer in the I-unit whereas geraniol from *P. gravelons* was symmetrically labelled in the D- and I-units. It may be significant that geraniol [15], nerol [15] and linalol [17] were formed with symmetrical labelling from MVA-[2-^{14}C] in other species. Geranyl pyrophosphate is a parent of higher terpenes and if the biosynthetic sites for mono and higher terpenes are segregated, conditions at the former might result in asymmetric labelling of the C-10 parent and its readily interconvertible isomers whereas those at the latter could lead to symmetric labelling [24]. The observed patterns may then result from mixing the pools, the second being the larger, during extraction. Despite these exceptions, the occurrence of asymmetric labelling after uptake of tracer from MVA and acetate at concentrations typical of biosynthetic studies or from CO_2 at atmospheric concentrations now seems general for monoterpene biosynthesis. The factors controlling this will be discussed in a future paper.

The nonequivalent labelling of the carbons of the C-2 sub-units when acetate or CO_2 is precursor may also be general and implies that the acetate units comprising MVA came from pools of different isotopic concentrations. The results obtained using CO_2-[^{14}C] suggest that under the experimental conditions the Calvin-Bassham cycle led to hexoses that were generally but not equivalently labelled (cf. discussion in Ref. [30]) and/or the combined effect of the Tricarboxylic acid cycle and the malic enzyme scrambled tracer between the carbons of the acetate units.

Our results thus suggest the occurrence of metabolic pools of acetyl coenzyme A and/or acetoacetyl coenzyme A in these species of plants. The existence of distinct pools of acetyl coenzyme A that influence the labelling patterns of antibiotics

formed in certain micro-organisms [31, 32] and of cholesterol in rat-liver [33] has recently been demonstrated, and an analogous pool may be inferred from the finding [34] that isoprene formed from CO_2-[^{13}C] in poplar leaves was mainly labelled at C_1 and C_5. The generality of such pools in secondary metabolism with the consequent occurrence of asymmetrical and nonequivalent labelling could invalidate the conclusions of many biosynthetic studies in which the tracer content in a (often small) fragment of a molecule is measured and conclusions drawn using conventional hypotheses as to the distribution of tracer in the truncated molecule. Many examples of the latter procedure are mentioned in recent reviews [10–12].

EXPERIMENTAL

Materials. Specimens of *M. pulegium* and *P. graveolens* were obtained by vegetative propagation of previously-used material [6, 15] and cultivated indoors at 20–25° in natural light in a bright south-facing aspect. *T. vulgare* was grown outdoors in Central London (March–October) from seed (ex Royal Botanic Gardens, Kew, U.K.; or H. E. Saier, Dimondale, Michigan, U.S.A.) and was wintered indoors as for *M. pulegium* etc. *T. plicata* was a sapling (5-yr-old; 2 m) cultivated outdoors [1]. For the first 3 species, shoots containing 6 leaflets were excised in sterile conditions for 6 to 12-week-old plants. New shoots (15–25 cm) of *T. plicata* were used. (+)-*Iso*thujone for use as carrier was isolated [1] from oil of tansy. (+)-Pulegone (*ca* 99% pure) was purchased and geraniol (*ca* 90%) was purified (>99%) via its $CaCl_2$-complex [35]. The composition of the plant oils was essentially the same as previously recorded [1, 15, 26, 36a] except that *P. graveolens* now produced geraniol (85%), citronellol (14%) and nerol (1%) whereas clonal material analysed 3 yr before contained proportions of the last 2 components reversed. Isothujones and pulegones had $[\alpha]_D^{20}$ (5%, EtOH) + 76° (ex *T. vulgare*) + 74° (ex *T. plicata*) and + 23° (ex *M. pulegium*).

Administration of tracer. Acetate-[^{14}C], or-[$^3H_3$] (typically 200–500 μCi; 0·1 mg) in sterile H_2O (2 ml) containing ATP (0·2 mg) was stem-fed to foliage (50–150 g) under illumination and forced transpiration [26]. ATP was added to feeding medium as it increased the incorporation of MVA into (+)-isothujone in *T. vulgare* by up to 5-fold over controls without changing the labelling pattern (unpub. results: Dr. K. W. Turnbull). Similar stimulations were recorded for uptake of MVA into other monoterpenes in *T. vulgare* [26] and into rubber in latex of *Hevea brasiliensis* [36b]. Use of this quantity of plant material ensured tracer was introduced at low concn. In initial expts, after tracer uptake (*ca* 0·5–1 hr) plant was maintained on Pfeiffer's soln but sterile tap H_2O was later found to be equally effective. Short-term (<24 hr) runs commenced before 10.00 hr.; others always before 12.00 hr. CO_2-[^{14}C] was generated by treatment of $BaCO_3$-[^{14}C] (53 mCi mmol^{-1}) with H_2SO_4 (6N) or lactic acid to give natural concns (*ca* 0·033% w/w) of the gas in a vessel (10 l.) equipped with a stirrer and a device for introducing nutrient solns. Usually a sealed atmosphere was maintained during expts but venting and replacing with air at 8 or 16 hr had little effect on incorporations or tracer patterns.

Extraction procedures. Pulegone was extracted and purified as described earlier [6].

Isothujone. Foliage was frozen (liq. N_2), pulverized and refluxed (10 min) with NaOAc buffer (150 ml; 0·1 M; pH 5·0) and then steam-distilled. Hexane extract (20 ml) was passed through Si gel H (Merck; 15 × 1·0 cm) and isothujone was finally isolated by preparative GLC on Carbowax 20 M (30% w/w on G-cel; 60–80 mesh, acid–alkali washed; 1·2 m × 9 cm) at 150° with N_2 flow rate 12 l. hr^{-1}. Collection was either in a U-tube at −78° with the gas flow reduced to 3 l. hr^{-1} (recovery *ca* 60%); or 2 traps in series, one empty (20°) and the other containing glass wool moistened with MeOH (−78°) with the same gas flow (*ca* 80%). When not in use the GLC-column was continually purged at 150° to reduce radioactive background. Isothujone was then converted into its 4-phenylsemicarbazone by reaction (2 hr at 20°) with 4-phenylsemicarbazide in 60% aq MeOH at pH 5·2 (90% yield) mp 183–184° (ex EtOH–H_2O).

Geraniol. Pulverized extract (as above) was extracted with hexane-CH_2Cl_2 (1:1) and passed through Si gel H with hexane as eluant, followed by petrol (bp 60–80°)–Et_2O (99:1). The product was purified by preparative GLC, oxidized with MnO_2 to form citral (**14**), and derivatized as previously described [15].

Degradation schemes. Many of the steps are well-documented [3] and were scaled down to 100–150 mg. Known products had mps or bps in agreement (±1°) with literature values unless noted and gave elemental analyses, IR, NMR and MS, consistent with accepted structures. ^{1}H-NMR of solns (10% in $CDCl_3$ or D_2O) were recorded at 60 MHz with TMS (τ 10·00) as internal standard. NMR assignments refer to the letterings of hydrogens in Schemes 1 and 5. Relative retention times (RRT) are with respect to isothujone (1·00) on Carbowax 20 M (20 w/w) at 130°.

Degradation of isothujone. (a) Isothujone (**1**) was photolysed [1] to give CO (**2**) and the *trans*- and *cis*-dienes (**3**: 9: 1). The former product (**3**) after purification [1] had: ν 3067, 2950, 1630, 975, 890 cm^{-1}; M$^+$ 124; τ 9·02 (6H; *d J* 7·2 Hz; g + h), 8·39 (3H; *d J* 6 Hz; a), 7·86 (1H; *m*; f), 7·41 (2H; *dd*, *J* 6, 1 Hz; d), 8·48 (2H, *b d*; e), 4·73 (2H; *m* c + b). (b) Oxidation of **1** with aqueous $KMnO_4$ [38] at below pH 9·0 yielded α-thujaketonic acid (**8**; 67%); dec. > 100°; oxime mp 168° (ex EtOH). Sodium salt: ν 3333–2500 (*b*), 1709, 1653, 1020 cm^{-1}; τ 9·5–8·5 (4H; *ms*; d + c + b), 9·06 (6H; *d*, *J* 5 Hz; e + f), 7·77 (3H; *s*; a), 7·56 (2H; *b m*; g). (c) Heating **8** (4 hr at 140–160°/8 mmHg) gave [38] β-thujaketonic acid (**11**, 65%); mp (ex H_2O) 81–82°; semicarbazone mp 189° (ex EtOH–H_2O); (**11**) had: ν 1715, 1690, 1637, 860 cm^{-1}; τ 9·90 (6H; *d J* 6 Hz: g + h), 7·96 (3H; *s*; a), 7·8–7·2 (5H; *m*; b + c + f), 4·52 (1H; *d*, *J* 2 Hz; d), −1·48 (1H; *s*, removed by D_2O treatment; e). A minor product was probably thuja ketone **34** M$^+$ 140; τ 9·00 (6H; *d J* = 7 Hz; g + f), 7·88 (3H; *s*; a), 7·58–7·10 (5H; *m*; b + c + e), 5·48 (2H; *b d*; d). (d) Treatment of **11** aqueous $KMnO_4$ (20° for 4·5 hr) gave [39] ω-dimethylacetonylacetone (**12**; 72%): RRT 1·55; mp 123° (ex EtOH); dioxime mp 133° (ex EtOH), lit. 128–130° [41]; τ 8·92 (6H; *d J* 6·6 Hz; e + f), 7·90 (3H; *s*; a), 7·54 (1H; *m*; d), 7·34 (4H; *m*; b + c) and **35** (20%) M$^+$ 142; τ 9·02 (6H; *J* 8 Hz; d + e), 7·65 (4H; *m*; a + g), 8·4–8·7 (3H; *m*; b + c), 6·2 (1H; *s* removed by D_2O treatment; f). This latter decomposed to form **36**: M$^+$ 124; λ_{max} 223, $10^4\epsilon$ 1·05 (EtOH); τ 9·05 (6H; *J* 8 Hz: d + e), 7·60–7·85 (5H; *m*; a + b + c), 4·15 (1H; *m*; f) on attempted GLC at > 160°, and polymerized on standing. (e) **8** was cleaved by treatment [42] with I_2–NaOH to give iodoform (**10**; 62%) mp 119° (ex H_2O) and the diacid (**9** 50%) mp 142–143° (ex H_2O): ν 3030, 2632, 1712, 1700, 1304, 945, 1000 cm^{-1}; τ 9·05 (6H; *J* 5 Hz; f + g), 9·2–8·1 (4H; *m*; b + c + e), 7·38 (2H; *d J* 5 Hz; d). (f) Enolization during some of these reactions was checked using integrated NMR spectra of reactions in D_2O. Conversion of **1** into its 4-phenylsemicarbazide or **8** involved no deuterium exchange. A simulated conversion of **8** into **11** by treatment

with 0·1 N D_2SO_4–D_2O at 110°/13 hr gave exchange at a. Preparation of the oxime of **11** did not cause further exchange but its treatment with 0·1 N D_2SO_4–D_2O at 150°/24 hr completely exchanged at (a + b). **12** did not exchange hydrogen with a neutral medium at 95°/36 hr; treatment with KOD–D_2O (0·15 N; 25°/15 min) effected complete exchange at (a + b + c) but none at d and after 20 min the aldol products **35** and **37** τ 9·02 (6H; *J* 6 Hz; d + e), 8·70 (3H; *s*; b), 8·60 (2H; *m*; a), 7·52 (2H; *m*; f), 4·2 (1H; *s* removed by D_2O; c) were formed. However, treatment of **12** with 0·1 N D_2SO_4–D_2O at 95°/11 hr gave clean exchange of (a + b + c + d). (g). Attempts to ozonize **3** gave intractable products and the diene was isomerized [43] (*t*BuOK DMSO; sealed tube, 55°/60 hr) and the products worked up on Si gel H with CCl_4 to give **4** (90%): RRT 2·06: λ_{max} 238 $10^4\epsilon$ 2·97 (EtOH); τ 9·00 (6H; *d J* 6·6; g + h); 8·33 (6H; brd *d*; e + a), 7·78 (1H; *m*; f), 4·49 (1H; *m*; b), 4·18 (1H; *m*; d), 3·21 (1H; *m*; c) and (probably) **38** (10%); RRT 1·94. The former was then ozonized in CCl_4 at −5°, worked up with Me_2S [44] and the volatiles collected as DNPH derivatives. These were separated by TLC on Si gel H with C_6H_{14}–MeOH (98:2) to give derivatives (ex HOAc) of 3-methyl-butan-2-one (**7**), R_f 0·33, mp 124°; acetaldehyde (**6**), R_f 0·12, mp 169°; and glyoxal (**5**), R_f 0·00, mp 326° in yields 50, 40 and 10%. These were characterized by comparison with authentic samples. **7** could be regenerated from its DNPH derivative by ozonolysis in MeOH at −5° and decomposition of the product with H_2O under reflux. The ozonide derived from **4** was alternatively decomposed with HCO_3H generated *in situ* [45] and **7** was distilled off and collected as its DNPH derivative, hydrazone mp 123–124°, or 4-phenylsemicarbazide mp 104°. The neutralized residue was then worked up, HOAc was distilled off; S-benzylthiuronium salt mp 137° (ex EtOH–H_2O); and oxalic acid and recovered from the involatiles; detoluide mp 267°. Overall yields were similar to those in the alternative method.

Degradation of pulegone and geraniol. These were as previously fully described [5, 15].

Radiochemical techniques. Solids and solid derivatives of liquids were recrystallized or resublimed, usually at least thrice, to obtain a product at constant specific radioactivity. In the few cases where no suitable solid derivative exists; products which had already been purified by TLC or GLC at 2 stages in the previous purification, were tested by the following criteria for radiochemical and chemical purity: (a) tracer (>98%) was located in a single TLC spot (4π-radiochromatography scanner) and several segments across the spot had constant specific activity; (b) tracer (>99%) resided in a single GLC fraction and again the specific activity was constant across the fraction; and (c) these requirements were met on at least 4 different GLC and TLC systems. CO and CO_2 were purified by scrubbing methods previously described [1, 6].

Radiochemical assays used Butyl-PBD [5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole; ex Ciba Ltd.] as scintillant in Na-dried toluene (8 g) l^{-1}). Efficiencies were *ca* 96 and 55% for ^{14}C and ^{3}H respectively and were unaffected by redistillation of the toluene or removal of O_2 in a stream of N_2. Butyl-PBD (5 g) in toluene (500 ml) and MeOH (500 ml) or Bray's solution [46] were used for toluene-insoluble samples. The methodology of counting and quenching corrections was standard [47]. Usually the sample (20–100 mg) containing 500–5000 dpm was assayed to accumulate *ca* 4×10^4 scintillations to ensure 2σ was ±1%. Reproducibility between duplicate experiments and assays was ±5% at worst, and was usually better than ±2%.

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