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BIOSYNTHESIS OF CARVONE IN *MENTHA SPICATA**

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Key Word Index—*Mentha spicata*; Labiatae; biosynthesis; monoterpenes; carvone; limonene.

Abstract—Degradation of, and measurement of isotope ratios in, (−)-carvone that had been biosynthesized in *Mentha spicata* from ^{3}H- and ^{14}C-labelled geraniol and mevalonate indicate that (a) oxidation of limonene or its biogenetic equivalent to form carvone involves shift of the endocyclic double bond; (b) (+)-limonene and (−)-carvone are biogenetically related and are probably formed on divergent pathways from a common intermediate; and (c) the exocyclic double bond of carvone is not formed regiospecifically. These results enable the mechanisms for the introduction of the carbonyl group and for the formation of the isopropenyl side-chain to be delimited.

INTRODUCTION

The biosynthesis of carvone (**6**; menth-6,8(9)dien-2-one) in higher plants probably involves the sequential formation of the biogenetic equivalents of geraniol and nerol (**1** and **2**; Scheme 1) but nothing is known about the later steps [1]. Three main problems are apparent. First, whether limonene (**5**) which co-occurs with carvone in all species where the latter is found, is an obligatory precursor (i.e. routes **2**→**3**→**5**→**6** or **4**→**5**→**6**) or whether it and carvone are derived from a common intermediate (i.e **3**→**6** and **3**→**5**). Secondly, which of four likely routes (enumerated later) is adopted for the introduction of the carbonyl group. Thirdly, by what mechanism is the double bond introduced into the isopropenyl side-chain bearing in mind that this type of side-chain is most unusual in monoterpenes.

We now report attempts to solve these problems by the feeding of suitably labelled precursors for the biosynthesis of (−)-carvone in *Mentha spicata* (spearmint; Labiatae). The ketone occurs in all aerial parts of the plant and this species is thus more convenient for study than other sources of carvone, i.e. *Carum carvi* (carraway) and *Anethum graveolens* (dill) where synthesis of the (+)-isomer occurs in the seeds. These are the three main sources of carvone and in no case is the optically-pure compound produced.

RESULTS AND DISCUSSION

Preparation of biosynthetic precursors

In order to elucidate the problems outlined, it was necessary to supply exogenous geraniol that was labelled with ^{14}C, or in some cases ^{3}H, at C-2, C-4 and C-10 (**7**) or to generate such compounds or their equivalents *in vivo* from exogenously-supplied mevalonate (MVA). In general, geraniol (and monoterpenes derived therefrom) formed in higher plants from MVA-[2-^{14}C] is predominantly (>95%) labelled at C-4, i.e. only the moiety derived from isopentenyl pyrophosphate (IPP) is appreciably labelled. This is believed to be due to the presence of a metabolic pool of 3,3-dimethylallyl pyrophosphate (DMAPP) [1]. This asymmetry of labelling was also found (see later) for carvone in *Mentha spicata*. Consequently, MVA could not be used to generate carvone adequately labelled at C-10. However, geraniol labelled at C-10 was obtained by use of petals of *Rosa dilecta* which incorporated MVA-[2-^{14}C] into geraniol and its β-glucoside in high yield (up to 22% of the *R*-isomer) and in which both the C-5 moieties were specifically and equally labelled at positions C-4 and C-10 [2]. This approach was used to prepare geraniol from MVA-[2-^{14}C] for use in experiments 4, 5 and 8 (Table 1) and from MVA-[2-$^{3}H_2$] for use in experiment 1. Geraniol-[U-^{14}C] for use as a marker for isotope ratios (experiment 1) was obtained by exposure of *Pelargonium graveolens* to CO_2-[^{14}C] and work-up of the radioactive essential oil.

Isotope studies

The reason for the particular choice of labelled precursors becomes apparent when possible mechanisms for the biosynthesis of carvone are considered. The exocyclic double bond could arise from a carbonium ion-like intermediate (**3**) in which the *gem*-methyls of geraniol may have lost their stereochemical identity (i.e. **3**→**6**; or **3**→**5**→**6**) or it may have arisen from a concerted elimination–cyclization that is regiospecific (i.e. **4**→**5**→**6**). Thus the fate of C-10 of geraniol used as precursor is of crucial interest. In fact,

* Part 24 in the series "Terpene Biosynthesis". Reprints of this paper are not available. For Part 23 see Banthorpe, D. V. and Poots, I. (1979) *Phytochemistry* **18**, 1297.

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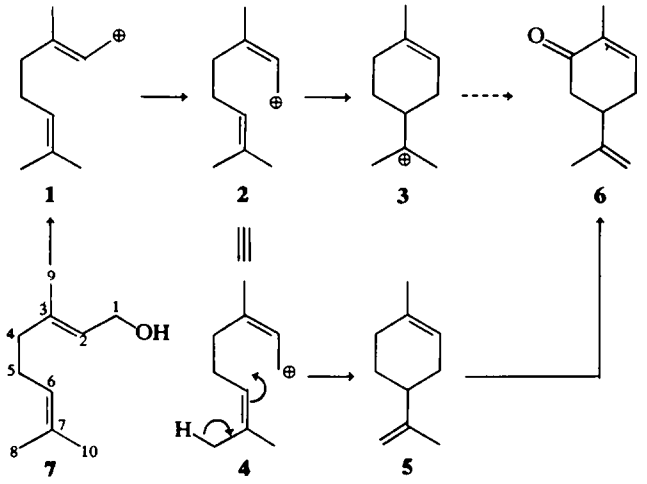


Scheme 1. Biogenetic routes to limonene and carvone. Intermediates are formally represented as carbonium ions; the actual species may be phosphate esters, glucosides or may be enzyme-bonded, amongst other possibilities.

labelling at C-8 of geraniol would be more instructive, but it is difficult to introduce tracer at this position by our method owing to the unavailability of the appropriately labelled MVA. The presumed enzymic introduction of the carbonyl group of carvone could involve one (or more) of four possible routes, all of which are well known in preparative organic chemistry. Thus, carvone may be formed (Scheme 2) by (a) radical-type oxidation, (b) allylic oxidation at the γ-position, (c) 'ene'-oxidation with migration of the double bond or (d) diol formation (presumably via an epoxide) followed by dehydration and oxidation. Hence the need for geraniol labelled at C-4 and C-10. Feeding of appropriate precursors was carried out and metabolism times (40–48 hr) were chosen which preliminary experiments had shown were optimum for the incorporation of tracer from MVA and geraniol into carvone. The ketone, and in one case limonene, were isolated and rigorously purified to chemical homogeneity (>99% by GLC, TLC) and to constant specific radioactivity. In some experiments (Table 1) isotope ratios were measured and in the remainder the purified biosynthetic product was degraded to locate tracer and the fragments in turn were rigorously purified (by recrystallization of solids or solid derivatives where possible) and isotope balances were achieved. Routes for degradation are shown in Scheme 3. It is worth noting that the apparently obvious device of reducing carvone to limonene by the Wolf–Kishner method (and then opening up the ring at the double bond) could not be employed as it is thought that this reaction involves formation of an intermediate carbanion which in this example would be mesomeric. Thus the position of the double bond of carvone would not be retained in the product formed after reduction.

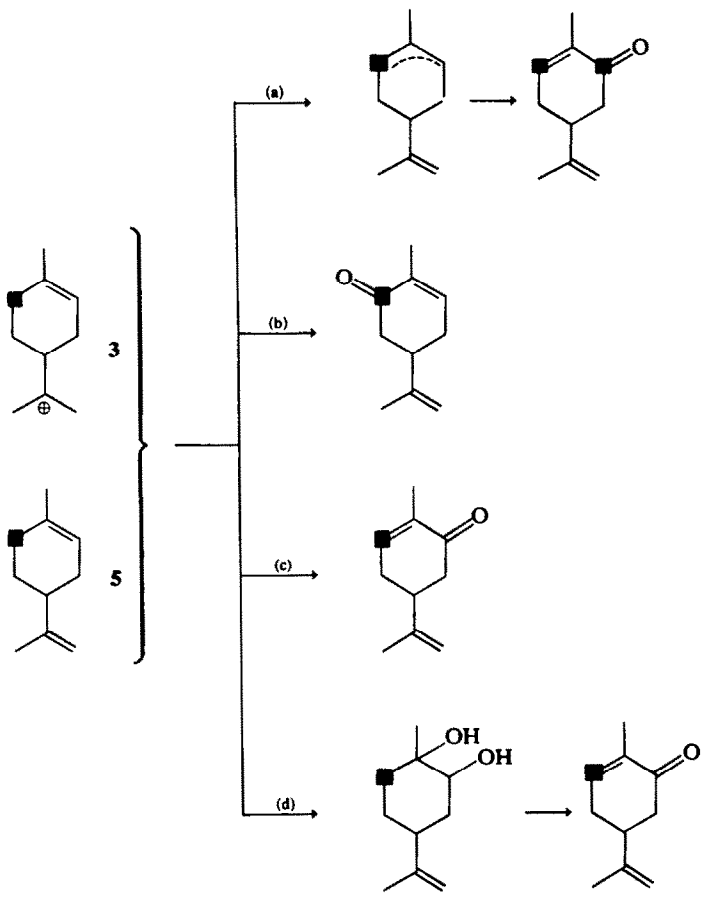
Table 1. Incorporation of labelled precursors into carvone by *Mentha spicata*

Expt.	Precursor	%*	Degradation procedure†	Isotope ratios‡ Precursor	Limonene	Carvone
1	Geraniol-[4, 10-$^3H_4$, U-^{14}C]	0.001 0.05	—	1.51	1.49	1.12
2	MVA-[4*R*-$^3H_1$, 2-^{14}C]	0.004	—	1.70	—	0.17
3	MVA-[4*S*-$^3H_1$, 2-^{14}C]	0.003	—	1.70	—	0.12
				Sp. act. (dpm/mmol) Carvone	Degradation products	
4	Geraniol-4, 10-$^{14}C_2$]	0.05	A	417	**12**, 219	
5	Geraniol-[4, 10-$^{14}C_2$]	0.05	B	565	**17**, 42; **16**, 518	
6	MVA-[2-^{14}C]	0.004	A	440	**12**, 427	
7	MVA-[2-^{14}C]	0.004	B	352	**17**, 22; **16**, 323	
8	Geraniol-[4-10-$^{14}C_2$]	0.04	C	398	**19**, 293; **20**, 112	
9	MVA-[2-^{14}C]	0.004	C	363	**19**, 332; **20**, 17	

* For feeding conditions, etc., see Experimental. Percentage incorporation of tracer (from (3*R*) MVA where appropriate and of ^{14}C in isotope ratio experiments).

† See Scheme 3.

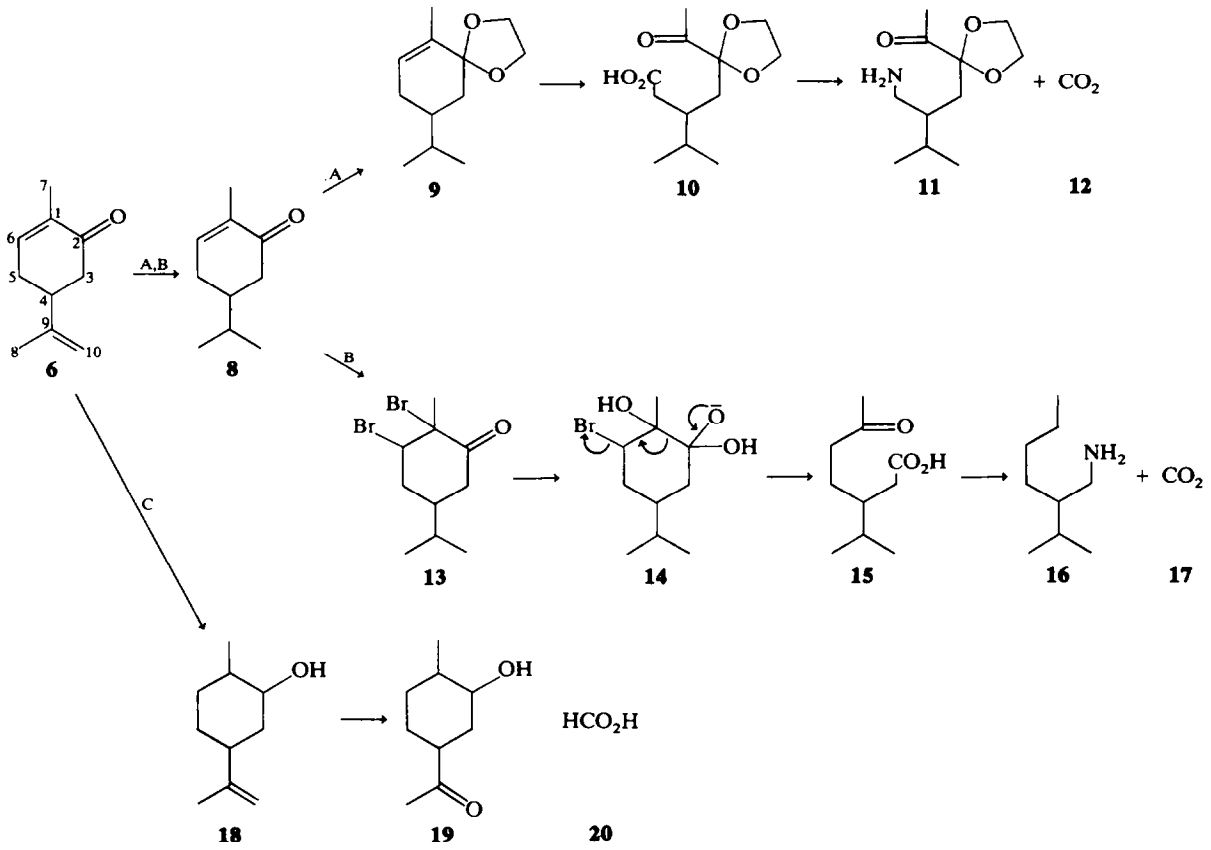
‡ ^3H:^{14}C. Standard error (estimated) for ratio, ±0.02. ^{14}C-Radioactivity was typically 10^3–10^4 dpm. All experiments were duplicated.



Scheme 2. Introduction of carbonyl group of carvone; ■ indicates carbon initially at C-4 of geraniol.

The only experiment in which appreciable radioactivity was recovered in limonene was No. 1 carried out in March 1977. The rest (in June–August 1978) used the same clone of *M. spicata* but incorporations into the hydrocarbon were $<10^{-5}$%. Incorporations of MVA and geraniol, although low, were as good as is generally found for this type of *in vivo* experiment [3]. The normalized isotope ratios (^3H:^{14}C) in experiment 1 for precursor, limonene, and carvone were 4.0, 3.9, and 3.0 respectively. These values are consistent with migration of the endocyclic double bond during introduction of oxygen by a route as shown in Scheme 4. If the tracer in the isopropenyl group is equally scrambled between the methyl and methylene groups (as we shall show later is approximately the case) and if there is no isotope effect involved in the loss of hydrogen to form the double bond in this group, the three normalized ratios should be 4.0, 3.7 and 2.7 respectively but any isotope effect could increase them towards the values 4.0, 4.0 and 3.0 respectively. The ratios from experiments 2 and 3 also support this interpretation, if it is assumed that labelling only occurs in the IPP-derived moiety of carvone, as in this case all ^3H incorporated in a position-specific manner would be removed by oxidation. The expectation of asymmetric labelling was proved for the concomitantly performed experiments 6 and 9 (>94% of tracer into the IPP-derived unit) and was indicated for experiment 2 by the finding that conversion of carvone thus formed into **19** resulted in a compound which contained <3% exchangeable tracer. The double bond shift that was suggested by the above work was confirmed by the use of ^{14}C-labelled precursors (Table 1, experiments 4–9). Both MVA-[2-^{14}C] and geraniol-[4-^{14}C] label the same atom in carvone and degradation routes A and B (Scheme 3) allow a decision as to whether this atom is C-2 or C-6. The results show that oxygen is introduced into the presumed monocyclic precursor with 94±5% of double bond shift. These results eliminate mechanisms (a) and (b), Scheme 2. Consequently, mechanisms (c) or (d) probably occur. There is evidence for a photosensitized 'ene' oxidation analogous to the former in *Tanacetum vulgare* [4]. Moreover, epoxides necessary for route (d) are known to occur in certain *Mentha* species [5–7] although we have not been able to detect them in *M. spicata*.

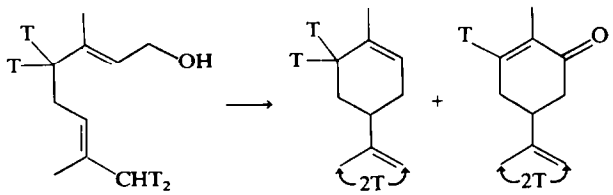
Experiments 8 and 9 (Table 1) give information concerning the construction of the exocyclic double bond at the distal end. The latter experiment indicates that over 90% of the incorporated tracer from MVA resides as expected in the IPP-derived moiety. However, the former experiment reveals that C-8 and C-10 (which together we can presume carry half the incorporated tracer) are labelled in the ratio 1.00:1.22, thus, if subsequent isomerization of the intially formed double bond does not occur, these results rule out the



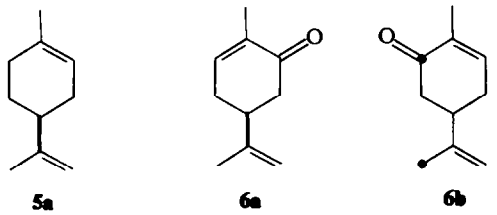
Scheme 3. Degradation of carvone. Routes A, B and C locate respectively tracer present at C-6, C-2 and C-10 of carvone.

concerted cyclization–elimination (Scheme 1; **4**→**5**→**6**) and indicate that dehydrogenation of some intermediate formally represented by **3** occurs with a lack of regiospecificity. A similar scrambling of diastereotropic terminal methyl groups in various monoterpenes of the iridane type has previously been demonstrated [8, 9] and the same situation has been found for the isopropenyl side-chain of certain sesquiterpenes [10, 11].

The carvone produced by *M. spicata* was a mixture of the (−)- and (+)-isomers (86:14) but the tracer results show that both isomers have been produced by the same mechanism involving shift of the double bond. This implies that (+)-limonene (**5a**) was structurally correlated with (−)-carvone (**6a**) and (−)-limonene with (+)-carvone. However our (−)-carvone



Scheme 4. Interpretation of isotope ratios in limonene and carvone. T = ^{3}H. ^{14}C (uniformly positioned) not shown. Normalized isotope ratios may be 4.0:3.7:2.7 rather than 4.0:4.0:3.0 (as indicated here for clarity): see text for details.



5a **6a** **6b**

was accompanied by (−)-limonene [(−), (+):60,40%] in the plant oil. This pattern of optical isomers is better explained by a route in which carvone and limonene were formed by divergent routes from a common intermediate rather than by one where limonene was an obligatory precursor of carvone. However, the common intermediate must be before the hypothetical, species (**3**) (Scheme 1) as the latter has the asymmetric centre established. Thus, two different stereoisomers of **3** may be required for the synthesis of (−)-carvone and (−)-limonene in the same plant. Attempts to elucidate the importance of the two routes in *C. carvi* and *M. spicata* by measurement of uptake of $^{14}CO_2$ into the two monoterpenes were inconclusive [12, 13].

It had been previously claimed that carvone was formed from limonene in *C. carvi* with no migration of the double bond, but no details are available [14]. Full details are available to back the report that uptake of MVA-[2-^{14}C] by *A. graveolens* yields carvone labelled as in **6b** [15, 16]. However, these conclusions can be

disregarded on two counts. First, tracer was not located in the part of the molecule derived from DMAPP, but rather the exocyclic double bond was cleaved and the resulting formaldehyde shown to contain no tracer. The inference that the methyl of the isopropenyl group was thus labelled is erroneous as it is very probable that asymmetric labelling would have resulted in only the IPP-derived moiety being appreciably radioactive. Secondly, the degradation that was purported to locate the tracer at C-2 (the carbonyl group) was ambiguous and it is probable that tracer from C-6 was isolated.

EXPERIMENTAL

Materials. *M. spicata* and *R. dilecta* were obtained from the Botanic Garden of the School of Pharmacy, London. Appropriately-labelled geraniol was obtained by feeding (in May and June) the flower heads of *R. dilecta* with MVA-[2-^{14}C], MVA-[4*R*-$^{3}H_1$], MVA-[4*S*-$^{3}H_1$], and MVA-[$^{2}H_2$] 100 μCi) with a metabolism time of 1 hr after uptake of tracer before harvesting [2]. After cleavage of any glucosides, the geraniol was purified to chemical and radiochemical purity (>99.5%; GLC, TLC) [2]. Incorporations of 3–7.5% of MVA were achieved. The compound was confirmed by oxidative degradation [2] to be equally and specifically labelled in the moieties derived from IPP and DMAPP.

Feeding methods and isolation of products. Young shoots (5–15 cm; 20 g) of *M. spicata* were fed via their stems in sunlight and under forced transpiration with geraniol or MVA (5μCi) that had been emulsified by sonification with Tween 80 (1 mg) in H_2O (1 ml). After 40–48 hr, the foliage was ground in liq. N_2 and the resulting product was extracted with Et_2O (Soxhlet; 200 ml; 16 hr). The solvent was then removed by flash distillation (37°; 1.5 ml/hr). After carvone (500 mg) had been added as carrier, the mixture was chromatographed on a column (15 cm) of MgO with C_6H_{12} (to remove most pigments) followed by a similar column of Si gel. The fraction containing carvone was then subjected to prep.-GLC (20% Carbowax 20 M; 3 m × 0.5 cm;150°) and PLC (Si gel H; EtOAc) to give a product that was chemically and radiochemically pure (for criteria see [2]).

Degradation (*Scheme* 3). These were typically carried out on a 100–500 mg scale. ^{1}H NMR, IR, MS and elemental analysis of intermediates and products were consistent with the accepted structures. *Scheme A:* Carvone (**6**) was converted into carvotanacetone (**8**) either by treatment with Br_2 followed by Zn–MeOH [18, 19] or by reduction over a rhodium catalyst [20]: bp 227–228°, 93%. The ketal (**9**) was then prepared by a standard method [21]; mp 92°, 75% and was oxidized with KIO_4–$KMnO_4$ or O_3 [22] to yield **10**; bp 260.5°/35 mmHg, 70%. This was decarboxylated by a Schmidt reaction [23] to give CO_2 (35%) which was collected in aq. $Ba(OH)_2$. The ketoamine (**11**) polymerized under the conditions of reaction. *Scheme B:* **8** was degraded by treatment [24] with Br_2 and KOH to yield **15**; 220–224°/3 mmHg, 88% which was purified as its semicarbazone; mp 158–159° ex MeOH, 80%. This was reduced by the Wolf–Kishner process and the crude product was subjected to a Schmidt reaction as before to give **16** which was purified as the benzoate; mp 92°, 55%; and CO_2 (60%). *Scheme C:* Carvone was converted [25] into dihydrocarveol (**18**); bp 107°/15 mmHg, 90% and this was ozonized (in MeOH) and worked up with alkaline H_2O_2 [26]. The hydroxyketone (**19**) was recovered by steam distillation and HCO_2H (**20**) was purified as its *S*-benzylisothiuronium salt; mp 151° ex EtOH, 76%.

Radiochemical methods. These were described previously [2]. Radioactive compounds were purified by recrystallization (of appropriate solid derivatives if necessary) to constant sp. act. and all experiments were carried out in duplicate. S.E. (estimated) were ±5%.

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