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ROLE OF GERANIOL AND NEROL IN THE BIOSYNTHESIS OF ARTEMISIA KETONE*

DEREK V. BANTHORPE, BARRY V. CHARLWOOD†, GLEN M. GREAVES and CAROL M. VOLLER
Christopher Ingold Laboratories, University College, London WC1H 0AJ. England

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Key Word Index—*Artemisia annua*; Compositae; monoterpene biosynthesis; artemisia ketone; geraniol metabolism.

Abstract—Tracer from geraniol-[2-^{14}C] or nerol-[2-^{14}C] was incorporated into artemisia ketone (an irregular monoterpene) in *Artemisia annua* with extensive scrambling although most (*ca* 60%) was located at C-2. In contrast, the ^{14}C-labelled compounds were incorporated in essentially position-specific fashions (≥96%) into the co-occurring regular monoterpenes isothujone, pinocamphone and borneol. These results support previous proposals that geraniol or nerol are not obligatory precursors of artemisia ketone. The labelling in the latter could not be rationalized by a pathway of degradation of the ^{14}C-labelled compounds proceeding stepwise from C-1 similar to that found in micro-organisms, and oxidative breakdown from the distal end may be involved.

INTRODUCTION

Most monoterpenes are constructed from C_5 units linked head-to-tail and have geraniol, nerol, or possibly linalol (or their biogenetic equivalents) as obligatory precursors. Artemisia ketone (**1**; 3,3,6-trimethylheptan-1,5-dien-4-one) is the most common [1] irregular monoterpene that is not formed from 'head-to-tail-linkage'. There is no obvious route for its formation from the usual C_{10} precursors, and it may be formed by direct condensation of C_5 units. This is unusual as although many types of irregular higher terpenes are known, they are believed to be derived from the same (regular) parents as the regular members of their class [2]. Recent studies have been consistent with the above view of the origin of artemisia ketone; thus tracer from geraniol was incorporated into the ketone in *Artemisia annua* with extensive scrambling of position; and geranyl, neryl and linalyl pyrophosphates were not used as precursors by enzyme extracts that could incorporate MVA, IPP and DMAPP [3–5]. However, it cannot be ruled out that these results reflect compartmentation effects or the inability for the C_{10} compounds to intervene in an ordered multi-enzyme synthetic complex.

We now compare the role of ^{14}C-labelled geraniol and nerol as precursors for artemisia ketone and for the regular monoterpenes borneol (**15**; bornan-2-ol), isothujone (**18**; *trans*-thujan-3-one) and pinocamphone (**19**; *trans*-pinan-3-one) that co-occur in *Artemisia annua*. In the event, extensive scrambling of tracer was again found in the irregular compound and we extended the previous preliminary investigations [3, 4] by assaying radioactivity at each chemically-distinguishable carbon to determine the pathways of degradation of the substrates and the sizes of the reincorporated fragments.

RESULTS AND DISCUSSION

Degradation schemes

Our clone of *A. annua* yielded a steam-volatile oil (*ca* 2.7% wt/wet wt foliage) comprising *inter alia*: artemisia ketone 38%, isothujone $[\alpha]_D^{20}$ +74.2, 9% borneol $[\alpha]_D^{20}$ −29, 20%; and pinocamphone $[\alpha]_D^{20}$ −15.3, 15%. The last two compounds were not optically pure [6] but earlier studies on the biosynthesis of the pinane [7] and bornane [8, 9] compounds have shown that the labelling pattern from ^{14}C-MVA and geraniol was the same for each enantiomer; our present results confirmed this.

Artemisia ketone was recovered after feeding geraniol-[2-^{14}C] and nerol-[2-^{14}C] (**13**, **14**) and was degraded using two novel procedures. Firstly, reduction to artemisia alcohol (**2**) and pyrolysis led to a retro-ene cleavage to give **3**, **4**, **5** and **6** (Scheme 1). Compound **6** is known to be formed on photolysis of **4** [10] but thermal cyclization is forbidden by orbital symmetry and presumably occurs here as the reaction is surface-catalysed. Compound **6** slowly ($t_{\frac{1}{2}}$ *ca* 2 days) reverted to **4** at room temperature. Product **5** occurs in *Artemisia* and *Santolina* spp. [1, 11] perhaps as an artefact of isolation. It is possible that *iso*-artemisia ketone (**7**) which is claimed [1] to occur naturally may be formed (artefactually or otherwise) by a similar type of rearrangement (cf. Scheme 1). A second route for degradation employed a photolytic rearrangement of **1** to **9**, presumably via the radical pair **8**, which on alumina rearranged to **10** (Scheme 2). This type of 1,3-sigmatropic rearrangement is novel for ketones [12, 13] although an example has been reported for 1-cyano-2,6-dimethylhepta-1,5-diene [14]. The generality of this reaction was explored using the ketone **11**; photolysis yielded bicyclic products [15] but an analogous

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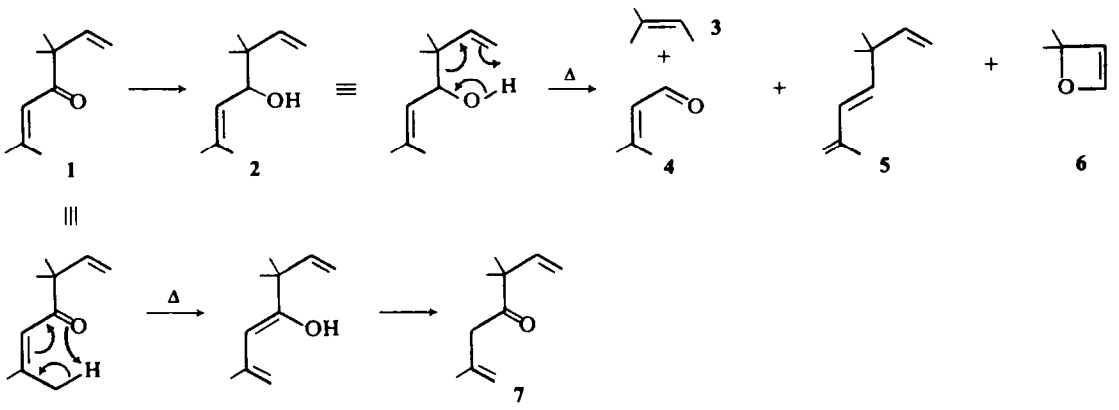
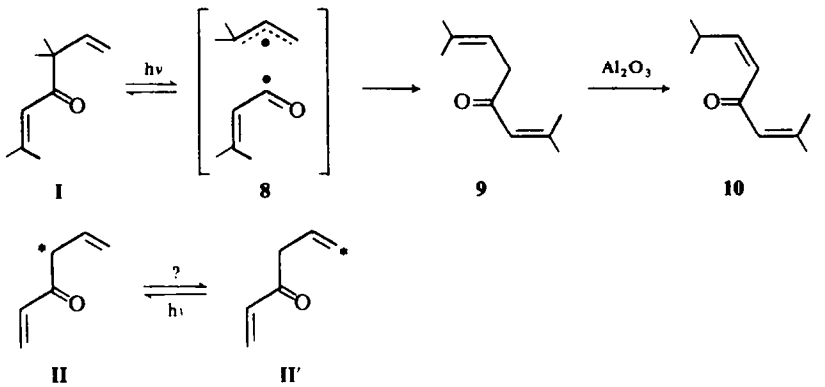
† Present address: Department of Plant Sciences, University of London King's College, 68 Half Moon Lane, London SE24 9JF, England.

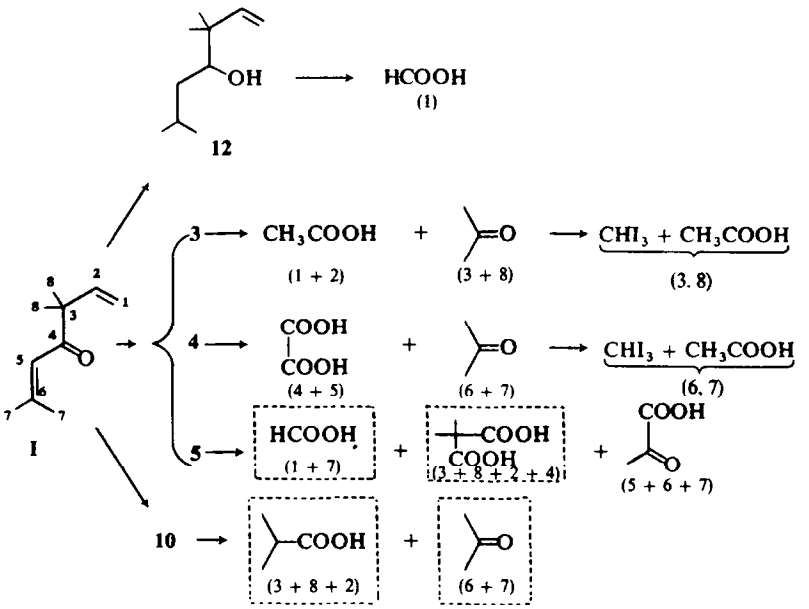
Abbreviations used: MVA, mevalonate; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethallyepyrophosphate.



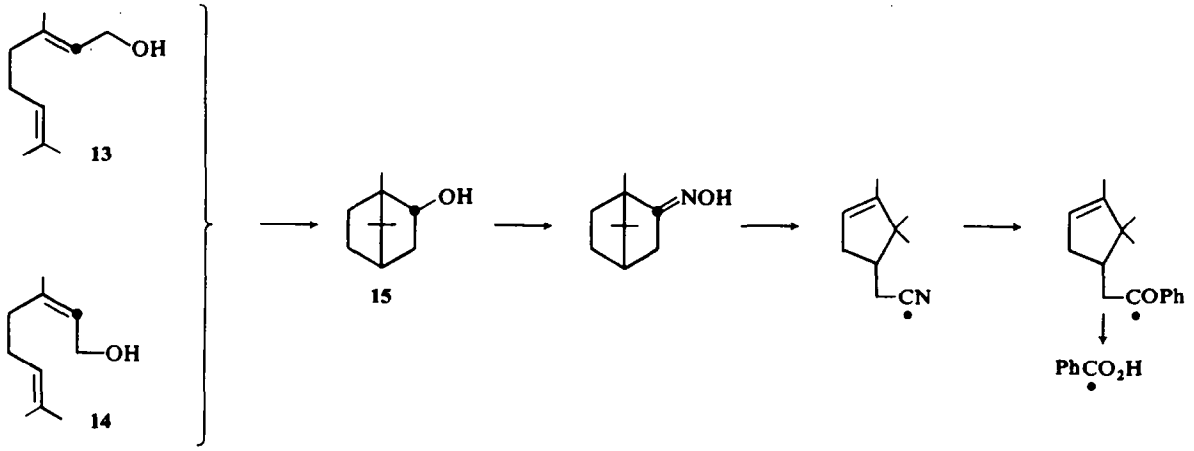
Scheme 1. Thermal degradation of artemisia alcohol.



Scheme 2. Photochemical rearrangement of artemisia ketone.



Scheme 3. Complete degradation of artemisia ketone. Chemically-distinguishable carbons in 1 are numbered 1 to 8. the degradations shown yield a pattern of products from which the labelling at each carbon may be deduced. The 'boxed' compounds provide an independent check on combination involving every carbon except C-5. The numbers in brackets appended to degradation products indicate the carbons contained.

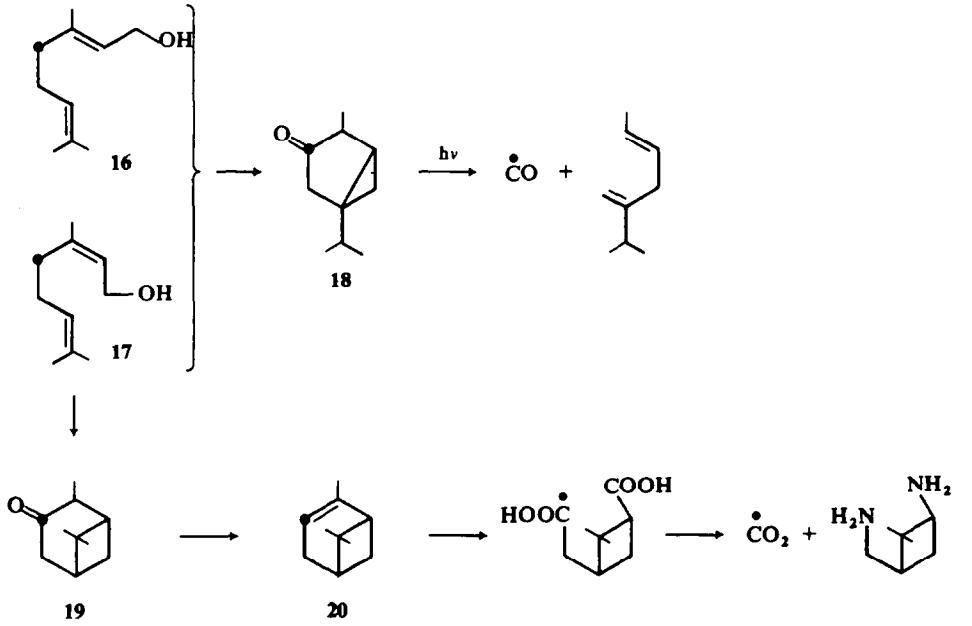


Scheme 4. Degradation of borneol biosynthesized from geraniol-[2-^{14}C] and nerol-[2-^{14}C]. • Represents position of ^{14}C in substrate and in product if the former is directly incorporated.

reaction to **1**→**9** would be degenerate and so undetected. Our attempts to demonstrate the rearrangement using **11** dideuteriated at the starred position failed (rearrangement here could be followed from the ^{1}H-NMR of 'unreacted' substrate; cf. **11**⇌**11'**) and the conversion of **1**→**9** may depend on one component of the intermediate radical pair being a delocalized tertiary radical.

The degradation products **3**, **4**, **5** and the reduction product **12** were then cleaved to give compounds (Scheme 3) from which the tracer at every chemically-distinguishable carbon of artemisia ketone could be deduced and isotope balances determined. In addition, use of the rearrangement product **10** allowed independent checks to be made on the tracer in combinations of every carbon except C-5. Borneol (**15**) was also recovered from the ^{14}C-labelled oil and was degraded (Scheme 4) to locate tracer incorporated directly from geraniol- or nerol-[2-^{14}C] [9].

Knowledge of the biosynthesis of isothujone and α-pinene in other species [7, 16, 17] indicated that location of tracer directly incorporated into isothujone (**18**) or pinocamphone (**19**) from **13** and **14** would be difficult to achieve, and for these compounds geraniol-[4-^{14}C] (**16**) and the correspondingly labelled nerol (**17**) were used as precursors. Isothujone was then degraded by simple procedures (Scheme 5) to locate tracer that had been directly incorporated [16] and pinocamphone was converted into α-pinene (**20**) which was treated similarly [7]. The reaction **19**→**20** involved reduction and dehydration (with I_2) but any rearrangement leading to



Scheme 5. Degradations of isothujone and pinocamphone biosynthesized from geranol-[4-^{14}C] and nerol-[4-^{14}C]. •Represents position of ^{14}C in substrate and in products if the former is directly incorporated.

Table 1. Labelling patterns in monoterpenes of *Artemisia annua*

Substrate	Product	% Incorporation	Location of tracer
Geraniol-[2-^{14}C]	Borneol	0.32	C-2,98*
Nerol-[2-^{14}C]	Borneol	0.41	C-2,99
Geraniol-[4-^{14}C]	Pinocamphone	0.05	C-2 and C-3,97
Nerol-[4-^{14}C]	Pinocamphone	0.07	C-2 and C-3,96
Geraniol-[4-^{14}C]	Isothujone	0.10	C-3,99
Nerol-[4-^{14}C]	Isothujone	0.15	C-3,97
Geraniol-[2-^{14}C]	Artemisia ketone	0.02	C-1,6; C-2,56; C-3,7; C-4,3; C-5,10; C-6,5; C-7,6; C-8,7.
Nerol-[2-^{14}C]	Artemisia ketone	0.01	C-1,7; C-2,61; C-3,3; C-4,2; C-5,6; C-6,8; C-7,7; C-8,3.

* Percentage of ^{14}C at indicated position. C-2 of borneol was recovered as PhCOOH (Scheme 4); C-3 of isothujone was obtained as CO (Scheme 5); C-2 and C-3 of pinocamphone were recovered as CO_2 (Scheme 5). The percentage of label at individual carbons are estimated to be within ±3% (of the actual value) for major labelled positions and ±30% (of the actual value) for portions labelled to less than 10% af the total.

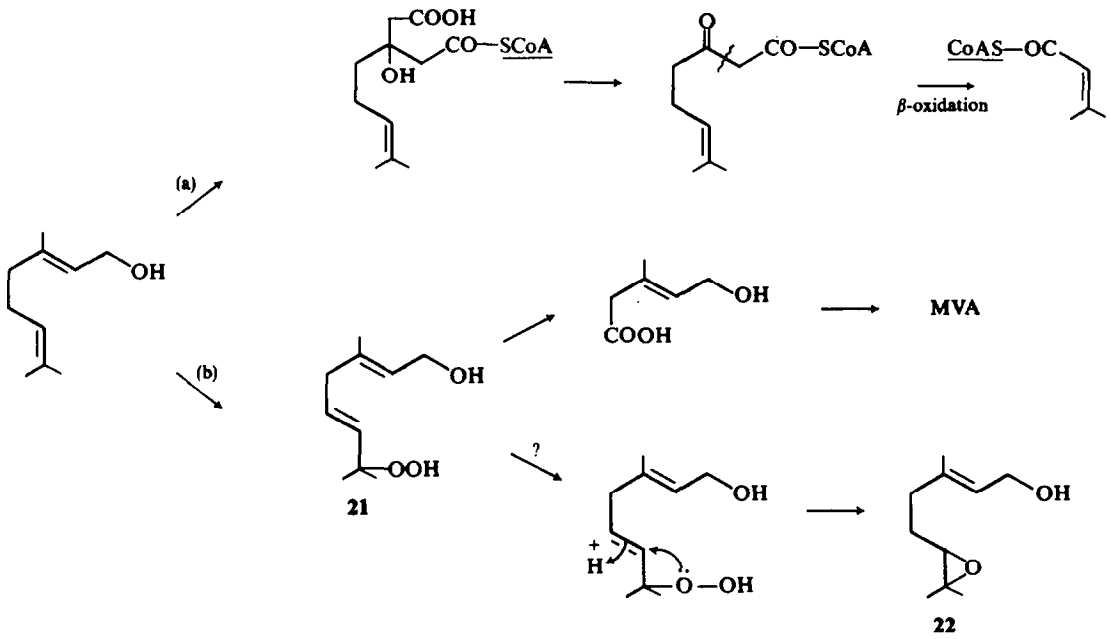
scrambled tracer in the last step was excluded by controls which showed that the cycle (+)-**20**→**19**→(+)-**20** did not involve racemization and hence there was no rearrangement. This conclusion was confirmed by the labelling pattern obtained in the biosynthetic studies (see below). Isotope balances in substrates and products were established in all the degradations and the products, as solid derivatives where necessary, were recrystallized to constant specific radioactivity. CO and CO_2 were purified to previously outlined criteria [16].

Labelling patterns

The low incorpoations (Table 1) are typical of those observed for C_5, C_6 or C_{10} precursors of monoterpenes in higher plants [18] and the uniformly higher incorporation of nerol into the cyclic compounds may reflect that it (or its biogenetic equivalent) is a more immediate precursor. Tracer from both geraniol and nerol was incorporated into borneol and isothujone with almost specificity of position, and the same situation almost certainly occurred for pinocamphone although here our degradation scheme could not distinguish between C-2 and C-3 in the product. Previously, geraniol-[2-^{14}C], or geraniol-[1-^{14}C] was demonstrated to be directly incorporated into camphor [9] and 1,8-cineole [19] in *Salvia* and *Eucalyptus* spp. The situation is completely different for artemisia ketone: tracer from either substrate was extensively scrambled in the product. In particular, C-2 was labelled predominantly (*ca* 60% of total) in each case and every other carbon was significantly radioactive; these results are fully consistent with our preliminary study using geraniol-[2-^{14}C] where the labelling pattern was not completely elucidated and the labelling at C-2 and C-4 could not be distinguished because of the limitations of the degradation schemes [3]. The present, and previous, results on the biosynthesis of the co-occurring regular and irregular monoterpenes make it probable that geraniol and nerol cannot act as direct precursors for artemisia ketone and that tracer can only be transferred to the latter by degradation of the substrates followed by reincorporation of fragments. Presumably, such fragments can find their way into the routes to the regular monoterpenes but their effect on the labelling patterns of the latter are swamped by the effect of direct incorporation of the C_{10} precursors (cf. % incorporation, Table 1). Our attempts to check if linalol (3,7-dimethylocta-1,6-dien-3ol) or its biogenetic equivalent was a direct precursor of artemisia ketone were foiled by the negligible incorporations ($<10^{-5}$%) of linalol-[1,2-^{14}C] into total monoterpenes of *A. annua* following either stem-feeding or vacuum-infiltration into excised leaves.

One interpretation of our results is that geraniol, nerol and linalol are not direct precursors of the irregular monoterpene and so the 'biogenetic isoprene rule' [2] does not apply to this compound. Another explanation is that the potential precursors could not, in the special case of artemisia ketone, reach the biosynthetic sites. Such compartmentation effects are certainly found for formation of artemisia ketone in both *A. annua* and *Santolina chamaecyparissus* [3, 4] for tracer from MVA or chrysanthemate was not incorporated *in vivo*. However, it seems significant that both these latter precursors and DMAPP and IPP are effectively utilized for irregular monoterpene biosynthesis by cell-free extracts from these plants. By contrast we found that the same systems would not accept any one of the presently studied C_{10} compounds [5]. Thus we favour the former interpretations; especially as extensive study and development of soluble enzyme extracts from *A. annua* that sustain terpene synthesis provides no evidence for the utilization of the C_{10} compounds as substrates (Dr. J. Gutowski; unpublished work) for irregular monoterpenes.

Degradation of geraniol-^{14}C and nerol-[^{14}C] and reincorporation of the fragments would yield a complex pattern in artemisia ketone depending on the type (i.e. C_1, C_2 or C_5) of units and the size of the metabolic pools that they enter [20]. However, the predominance of tracer at C-2 suggests that a *quasi*-IPP moiety is cleaved from geraniol and nerol and after dilution with IPP built up *in situ* from radioactive C_1 or C_2 units is incorporated into the irregular ketone such as to generate a fragment comprising of C-1, C-2, C-3 and C-8 (Scheme 3) similar to the asymmetric pattern of labelling when the compound is formed both *in vivo* and *in vitro* by *A. annua* [3–5]. It is not known if exogenous (or indeed endogenous) geraniol is degraded in higher plants but a route



Scheme 6. Possible routes for metabolic degradation of geraniol in (a) micro-organisms and (b) in *A. annua*. CoA-SH is coenzyme A.

summarized in Scheme 6 has been elucidated in micro-organisms [21]. If such a process, basically involving β-oxidation from C-1 were to occur in *A. annua*, it is difficult to account for the predominant location of tracer at C-2 in artemisia ketone. A more attractive scheme is that the bulk (or all) of the geraniol (or nerol) is degraded by an oxidative process initiated at the distal end of the molecule to generate a precursor of IPP-[2-^{14}C] that can be incorporated into artemisia ketone. A tentative proposal in which many details are adjustable and which ignores the roles of coenzymes is presented in Scheme 6. 'Ene'-oxidations such as to form **21** are known to occur in cell-free systems from plants [22] and compound **21** could be a precursor of 6,7-oxidogeraniol (**22**) that is synthesized in several spp. of Compositae (23).

EXPERIMENTAL

Materials. A. annua was cultivated as described [4]. Geraniol-[2-^{14}C] and nerol-[2-^{14}C] (both 126 μCi/mmol) and linalol-[1,2-^{14}C] (72 mCi/mmol) were available [4, 5]. Geraniol-[4-^{14}C] and the corresponding nerol (50 μCi/mmol) were formed by metabolism of IPP-[4-^{14}C] in cell-free extracts from *Tanacetum vulgare* [24].

Feeding and degradation. Tracer (*ca* 50 μCi; 1–5 μCi/g plant material) was stem fed (or in the case of linalol was applied to the leaves by vacuum-infiltration) as in previous work [3, 4] in June/July. The metabolic period was 40 hr as this was known from preliminary screening (TLC) to give maximum uptake of tracer into the desired products. Artemisia ketone and isothujone were obtained in a pure form by methods previously described [4, 16, 17]. Borneol and pinocamphone were recovered after addition of carrier by preparative GLC (30% Carbowax 20 M on Chromosorb P; 150°) and were recrystallized (EtOH–H_2O) to constant sp. radioact. In the case of pinocamphone this was as the oxime, from which the free ketone could be recovered by refluxing for 2 hr with excess (×10) pyruvic acid. All degradation products were either well-known [3–5] or gave expected MS, IR and ^{1}H-NMR spectra and all were routinely shown to be homogenous by GLC (carbowax 20 m and SE-30; 50 m × 0.2 mm, s.c.o.t) and TLC (Si gel G and H; various solvents). Isothujone and borneol were degraded by standard procedures [9, 16, 17]. Pinocamphone was reduced ($LiAlH_4$) and the crude product dehydrated (trace I_2, C_6H_6: 78°, 30 min) to give α- and β-pinenes (95 : 3%) and the mixture was degraded by a standard procedure [7]. Artemisia ketone was (a) reduced ($LiAlH_4$) to the alcohol which was pyrolysed *in situ* on GLC (uncoated Porapak S; stainless steel column 2 m × 0.4 cm; 285°; N_2 50 ml/min). Virtually complete (96%) cracking occurred to give 3-methylbut-2-ene (38%), 3-methylallyladehyde (18%) and its heterocyclic isomer (16%) and artemisia triene (24%) which spearated on the column and were collected [7] at −80° (30–60% efficiency). These were ozonized (MeOH; −80°) and worked up oxidatively to give the compounds in Scheme 3 which were, in turn, separated, in some cases subjected to the iodoform reaction, and purified to constant sp. radioact. [4, 7]. (b) Reduction of artemisia ketone in two steps (Li-liq. NH_3; $LiAlH_4$) gave 3,3,6-trimethylhept-1-en-4-ol (overall yield 90%) which was purified (3,5-dinitrophthalate) and ozonized to give formic acid (60%) and an intractable polymer. (c) Artemisia ketrone was photolysed (Siemens MB/D 125W Hg lamp with envelope removed) either neat or in C_6H_{12} (20%) in a Si cell (15°; water jacketed) under N_2 (2 hr) to yield 2,7-dimethylocta-2,6-dien-4-one (**9**, 20%) and unidentified products. On passage in hexane (20%) through Al_2O_3 (Brockmann, Grade III), this partly rearranged to 2,7-dimethlocta-2,5-dien-4-one (**10**. 43%). The latter was cleaved by ozonolysis (MeOH, −80°) to give 2-methylpropionic acid (60%) and Me_2CO (30%).

Radiochemical methods. Counting techniques were as described [17]. Typically, aliquots (5–10 mg) containing 5 × 10^2 to 10^4 dpm were assayed.

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