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BIOSYNTHESIS OF IRREGULAR MONOTERPENES IN EXTRACTS FROM HIGHER PLANTS*

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Key Word Index *Artemisia annua; Santolina chamaecyparissus;* Compositae; biosynthesis; enzyme extracts; irregular monoterpenes.

Abstract Extracts from *Artemisia annua* and *Santolina chamaecyparissus* converted ^{14}C-labelled IPP‡, DMAPP‡ and DMVC‡ into artemisia ketone, its corresponding alcohol, lavandulol and *trans*-chrysanthemyl alcohol with up to 12.0% incorporation of tracer. DMVC was the most effective precursor under standard conditions and led to unequal distribution of tracer in the C–5 moieties. The same extracts interconverted *cis* and *trans*-chrysanthemyl alcohols and their pyrophosphates, artemisia ketone, and artemisyl alcohol in up to 10·4% yields, but geraniol, nerol and linalol or their pyrophosphates were not precursors of any of these compounds. Formation of artemisia ketone and its alcohol from C–5 intermediates was enhanced by NAD^+ and $NADP^+$ but was unaffected by absence of oxygen. These co-factors did not affect the yields of lavandulol or *trans*-chrysanthemyl alcohol. These observations suggest closely related biogenetic pathways to the three irregular skeltons that do not involve the usual C–10 intermediates of monoterpene biosynthesis: *i.e.* the biogenetic isoprene rule is not obeyed.

INTRODUCTION

Biogenetic speculations [1, 2] suggest that ring scissions of an ion **1** with the chrysanthemyl (2,2-dimethyl-3-[2-methylpropenyl] cyclopropylmethyl) skeleton can lead to the ions **2**, **3** and **4** with the artemisyl (3,3,6-trimethylheptyl), santolinyl (2,5-dimethyl-3-ethylhexyl) and lavandulyl (2,3,6-trimethylheptyl skeletons that are the formal parents of the monoterpenes artemisia ketone (**5**), santolinatriene (**6**) and lavandulol (**7**). These last compounds are 'irregular' in that they obey neither the classical isoprene rule nor its biogenetic extension [3] in any obvious manner. Compounds with the skeletons **1**–**4** however rarely co-occur in nature, eg **1** and **2** are typically found in a few families of the Compositae whereas **4** has been found solely in the Labiatae and Umbelliferae [1].

Feeding of whole plants of *Artemisia annua L.* and *Santolina chamaecyparissus L.* (Compositae) with *cis*- and *trans*-chrysanthemyl alcohol and the corresponding chrysanthemates (**8**, **9**) provide no evidence that these are precursors of artemisia ketone [4], but this may merely reflect the inability of the exogenous metabolites to reach the sites of monoterpene synthesis. We here describe the preparation of cell-free extracts from these species that can effect monoterpene synthesis *in vitro* and in which such limitations hopefully are minimised.

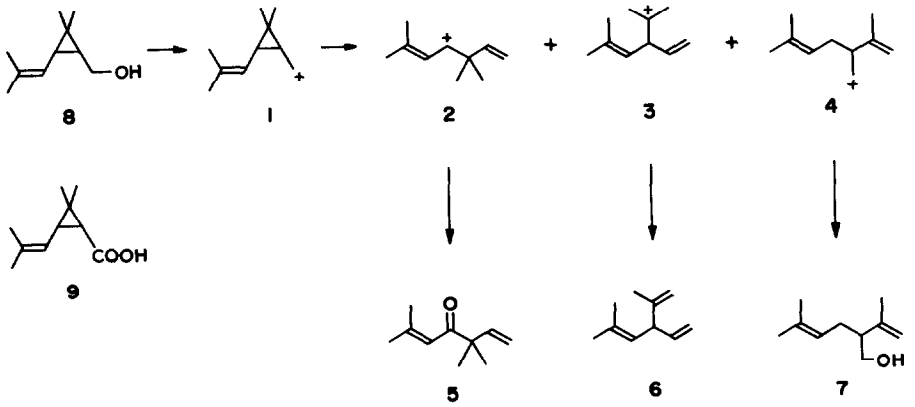

Fig. 1. Hypothetical schemes for the biogenesis of irregular monoterpenes.

* Part 17 of the series "Terpene Biosynthesis". For Part 16 see: K. G. Allen, D. V. Banthorpe, B. V. Charlwood and C. M. Voller (1977) *Phytochemistry* **16**, 79.

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‡ Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; DMVC (PP), dimethylvinylcarbinol (≡ 3-methylbut-1-en-3-ol) and its pyrophosphate.

RESULTS AND DISCUSSION

General

Specimens of *A.annua* were from our previously used stock [4] but new varieties of *S.chamaecyparissus* were used (see experimental). An analysis of these latter made in June, but typical of that obtained across the growing season, yielded oil (0.7% w/w) comprising: artemisia ketone 16.7; β-phellandrene 13.9; myrcene 10.3; β-pinene 17.1; camphor 1.1; artemisyl alcohol 0.8; α-pinene 0.4; camphene 0.1; unidentified monoterpenes 3.2; α-curcumene 6.2; β-bisabolene 1.1; α-humulene 1.1; sesquiterpene ketones and hydrocarbons (4 in number) to 100%. This analysis refers to greenhouse material which gave oils of more uniform composition over the season than outdoor specimens. Our previous variety produced oils containing 85–90% artemisia ketone [4] whilst others had recorded 50–60% of this component [5–7].

An unexpected problem was the difficulty of purification of the terpene pyrophosphates needed as substrates for the enzyme systems. The pyrophosphate esters of geraniol (3,7-dimethylocta-*trans* 2,6-dien-1-ol), nerol (the corresponding *cis*-2-isomer), linalol (3,7-dimethylocta-1,6-dien-3-ol), chrysanthemyl alcohol and DMVC were prepared by a standard method [8] but previously described methods of purification, involving recrystallisation [9, 10] or chromatography [11–17], in our hands gave impure (typically *ca* 80%) products contaminated with mono- and tri-phosphates of the required alcohol together with phosphates of isomeric alcohols. Others have commented on the inadequacy of the available methods [7, 14, 18–20] and analytical, spectroscopic and chromatographic identifications of products have invariably been glossed over or omitted. The methods finally adopted here resulted in satisfactorily pure (>98%) products (see Experimental).

Cell-free preparations

The simple procedure for the preparation of cell-free extracts resulted from detailed studies involving variation of techniques, buffers, additives etc carried out along the lines previously used for the preparation of extracts of geraniol synthetase [21] and by the experience of others [22, 23]: full details of our screening procedures are available [24, 25]. Some of the methods adopted were quite empirical; e.g. *Tris–acetate* (pH 7.0), although just off its buffer range at 5°, was used in the maceration process as it gave more active systems than those obtained with other buffers. In these multi-enzyme, multi-component preparations, factors other than pH are obviously of great importance (e.g. anion-inhibition, differential stimulation or inhibition of kinases and phosphatases by buffer components, complexing effects etc.) as shown by the fact that the use of phosphate or MES buffers at pH 7.0 gave preparations in which the amounts of terpenes synthesised were severely reduced. Where possible, especially in the early stages of the preparation, speed was preferable to refinements in technique.

Most (>90%) of the monoterpene synthetase (IPP as substrate) was located in the 10^5 *g* supernatant and the residual activity in the pellet was variable probably due to contamination by soluble enzymes. Using the soluble preparation in the presence of ATP, $NADP^+$, NAD, $MgCl_2$ $MnCl_2$ and dithiothreitol, it was found that artemisia ketone, (**5**) artemisyl alcohol and *trans*-chrysanthemyl alcohol (**8**) were formed from IPP, DMAPP and DMVC. In each case the products were characterised by the recrystallisation of solid derivatives to constant specific radioactivity. Santolinatriene was unavailable as a standard and was not assayed: the activity of the unpurified hydrocarbon fraction (TLC) probably indicates an upper limit to its yield.

Sodium diethyldithiocarbamate was added to the original extract to inhibit polyphenol oxidase [26], although phenolic levels were lower (*ca* 10-fold less) in the greenhouse plants than in material cultivated outdoors [27]. Dithiothreitol presumably kept sulphydryl enzymes in reduced from [28], and/or acted as a general anti-oxidant and quinone scavenger [29]. Inhibitors of endogenous phosphatases [30] were omitted as they also inhibited monoterpene formation. Addition of Mg^{2+}, Mn^{2+}, Ca^{2+} or Zn^{2+} singly or in combination or EDTA (all 0–100 mM) had no effect on efficiency of tracer incorporation.

Routine assays were made at pH 7.0 which is thought to simulate that of plant cytoplasm [31]. Preparations from both plant species showed maximum activity at between pH 6.0 and 8.0 for formation of artemisia ketone, its alcohol and the hydrocarbon fraction and a similar optima was found for the formation of chrysanthemyl alcohol by extracts of *A.annua*. In contrast, preparations from *S.chamecyparissus* showed maximum synthesis of the last alcohol at between pH 5.0 and 6.5; at pH 7.0 only 70% of the maximum activity was achieved, so our standard assay underestimated incorporations into this compound. Percentage incorporations were also found to be inversely dependent on the initial concentration of substrate (Table 1).

Table 1. Effect of substrate concentration on incorporation in preparations from *A.annua**

	% incorporation†			
[IPP] nmol	HC	AK	AA	CA
1.6	2.4	1.5	2.5	5.4
3.5	2.2	0.8	1.4	6.7
7.0	0.8	0.5	0.7	3.8
14.1	0.4	0.2	0.3	2.2

* Incubation conditions as in Exptl. One cell-free preparation was used as a control in all these experiments. † Incorporation (%) of tracer into products that had been purified to constant specific radioactivity. AK, AA and CA are artemisia ketone (**5**), artemisyl alcohol and *trans*-chrysanthemyl alcohol (**8**). HC is the "hydrocarbon" fraction (TLC).

The extractable monoterpene synthetase varied widely in activity over the growing season for outdoor specimens of either plant, the maximum occuring in June–August as found for geraniol synthetase from *Tanacetum vulgare* [21]. Indoor specimens showed little variation in activity during May–September, and this material was used in all the studies reported here. The reproducibility of activity in preparations from *A.annua* over this period was good (Table 2:) and results for the other species were similar. Nevertheless, each set of experiments

dealing with the effects of variable was carried out using a single preparation.

The influence of added coenzymes on the standard preparation from *A.annua* is shown in Table 3: closely similar results wee obtained with *S.chamaecyparissus*. As can be seen, NADPH and NADH did not have much effect but the oxidised forms of both coenzymes both increased incorporation into the hydrocarbon fraction, artemisia ketone and artemisyl alcohol. However, there was no stimulation of the synthesis of *trans*-chrysanthemyl alcohol. Thus it appears likely that a dehydrogenase system is involved in the formation of the artemisyl compounds. Incorporations of tracer from IPP into the products in Table 1 by cell-free preparations from either plant were unaffected by conducting the reactions under N_2 or in air containing 10 or 50% CO. These observations, together with the unimportance of NADPH, indicate that a mixed-function oxidase dependent on cytochrome P450 was not involved in the formation of the oxygenated products.

In addition to the incorporation given in Tables 1–3 (in which all products were rigorously purified to constant specific radioactivity) some 20 cell-free preparations were made from each plant species where preliminary values of incorporations had been obtained from assay of otherwise unpurified TLC fractions. These fractions were pooled into three groups: A and B (both ex *Santolina*) and C (ex *Artemisia*) and the products from each were rigorously purified. The incorporations for A and B were: artemisia ketone 1.2, 1.3%; artemisyl alcohol 1.9, 2.4%; *trans*-chrysanthemyl alcohol 3.3 and 7.2%. Corresponding values for Group C were 1.6, 2.5 and 5.3%. Lavandulol (7) and 3,3-dimethyallyl alcohol were also rigorously assayed in these fractions: the former was produced 0.3, 0.6 and 0.2% in Groups A to C and the latter 6.0, 5.5 and 7.1%. The formation of lavandulol is noteworthy as the alcohol could not be detected in the volatile oils of our species and indeed has never been recorded in the *Compositae* [1]: *trans*-chrysanthemyl alcohol [8] was similarly not present in the oils. The formation of 3,3-dimethylallyl alcohol from IPP indicated the existence of IPP- isomerase in the preparations: DMVC was not detected. In a few cases, it was shown that incubations directly formed terpenols and their phosphate esters in approximately equimolar amounts, so endogenous phosphates were also present.

Table 2. Reproducibility of cell-free preparations from *A.annua*

Substrate*	Products (%)†		
	AK	AA	CA
IPP-[4-^{14}C]	0.5, 0.5, 0.6, 0.6	0.8, 0.8, 1.0, 1.1	2.6, 2.2, 2.5, 2.1
DMAPP-[4-^{14}C]	0.5, 0.4, 0.6, 0.6	0.8, 0.6, 0.8, 1.0	3.2, 1.7, 2.4, 2.7
DMVC-[Me-^{14}C]	0.9, 0.6, 0.8, 0.5	1.6, 1.4, 1.4, 0.9	8.5, 6.9, 5.5, 4.9

* Incubation conditions as in the Exptl. Section: [^{14}C]-substrates, 3.5 nmol. 4 separate experiments were carried out (all substrates used in each) in the period May–Sept. † Products as in Table 1. Percentage incorporation of tracer. The hydrocarbon fraction contained, variously, 0.0–0.4% tracer.

Table 3. Effect of coenzymes on cell-free preparations from *A.annua*

Coenzyme added	Products (%)†			
	HC	AK	AA	CA
None	0.2	0.4	0.6	3.9
NADPH	0.3	0.2	0.8	2.7
NADH	0.2	0.3	0.5	3.2
$NADP^+$	0.5	1.5	2.6	3.9
NAD^+	0.6	1.1	2.8	3.7
$NADP^+ + NAD^+$	0.5	1.5	2.8	3.9

* Coenzyme, 15 μmol; IPP-[4-^{14}C], 3.5 nmol. Conditions for incubation similar to those in Table 1 and 2. † Percentage incorporation of tracer from IPP-[4-^{14}C]. Products as in Table 1.

Utilisation of C–5 and of C–10 substrates

Some of the monoterpene alcohols and corresponding pyrophosphates were compared with the hemiterpenes as substrates in a standard cell-free preparation and the results are shown in Table 4. Lavandulol was not assayed in this series. All the substrates used except DMAPP

Table 4. Interconversion of C–10 compounds in cell-free preparations

Products* (%)	Substrate†										
	ex. *S.chamaecyparissus*								ex. *A.annua*		
	A	B	C	D	E	F	G	H	E	I	J
AK	0.8	0.7	1.3	6.9	2.9	1.4	1.4	1.4	2.8	2.9	3.6
AA	1.5	1.5	1.9	‡	3.8	1.9	2.5	1.9	2.9	4.5	3.4
CA	3.2	2.8	8.8	3.5	‡	‡	§	‡	‡	§	‡

* % incorporation into products purified as before. AK, AA and CA represent artemisia ketone, artemisyl alcohol and *trans*-chrysanthemyl alcohol. † Precursors (all ^{14}C-labelled; feeding conditions in Experimental Section applied to preparations from *S.chamaecyparissus* and *A.annua*. A represents IPP; B DMAPP; C DMVC; E (±)-*cis*, *trans*-chrysanthemyl pyro phosphate; D artemisyl alcohol; F(±)-*cis*, *trans*-chrysanthemyl alcohol; G(±)-*cis*-chrysanthemyl alcohol; H (±)-*trans*-chrysanthemyl alcohol; I (±)-*cis*-chrysanthemyl pyrophosphate; J (±)-*trans*-chrysanthemyl pyrophosphate. ‡ Usually >80% recoveries of substrates were recovered unchanged. § Not assayed.

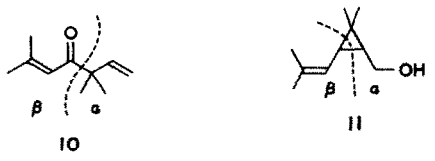
gave solutions from which, after incubation with enzyme to cleave the phosphates, 90–100% of the applied tracer could be extracted into ether or hexane. However, only 40–56% recoveries could be achieved for DMAPP, even when extractions were made from salt-saturated ($(NH_4)_2SO_4$) solutions, although boiled enzyme controls gave 95–100% recoveries. Presumably DMAPP is particularly susceptible to the epoxidase–hydratase systems that remove unphysiological excesses of exogenous metabolites [4]. We intend to pursue these matters in subsequent work.

As can be seen (Table 4), both *cis* and *trans*-chrysanthemyl alcohols and their pyrophosphates were converted (up to 7.4% yield) into artemisia ketone and its alcohol in preparations from both plants, and as racemic substrates were used the true incorporations may be double these. Artemisyl alcohol was also effectively incorporated into artemisia ketone (6.9%) and *trans*-chrysanthemyl alcohol (3.5%) in the preparation from *S.chamaecyparissus*. The utilisation of the *cis*-chrysanthemyl compounds at least as efficiently as their *trans*-isomers is noteworthy, as compounds of the former series have never been recorded in nature. The preparations which effected these transformations of C-10 compounds were also very effective (up to *ca* 12% total incorporation Table 1) for the conversion of the C–5 precursors into the irregular monoterpenes.

Our results imply that the artemisyl and chrysanthemyl skeltons are closely related biogenetically. Furthermore, the preparation used for the above interconversions did not effectively convert geranyl, neryl or linalyl pyrophosphates into irregular monoterpenes. Incorporations were negligible or low (0.0–0.02% total) using the standard incubation conditions or for incubations over the range pH 4.0–8.6. It could be argued that the sequence from the C–5 precursors to the irregular monoterpenes occurs on a protein-bound 'conveyor-belt' and that although classical intermediates related to geranyl pyrophosphate are involved, these cannot exchange with exogenous labelled compounds of the same type. However, since exogenous irregular monoterpenes are apparently interconverted, it seems simpler to assume that the classical C–10 intermediates are not involved in the biosynthesis of the former which are presumably directly formed from C–5 species. If this is so, hypothetical mechanisms involving linalyl and carane derivatives as intermediates for artemisia ketone [32–34; cf. 4] can be ruled out. In addition these irregular monoterpenes would abrogate the biogenetic isoprene rule [3] being the only class of compounds within our knowledge to do so.

Asymmetry of labelling in irregular skeletons

Artemisia ketone and *trans*-chrysanthemyl alcohol biosynthesised from DMVC-[Me-^{14}C] by several preparations from *S.chamaecyparissus* were separately pooled and cleaved by ozonolysis. The ketone (3810 dpm mmol^{-1}) yielded acetone (760), formaldehyde (0) and a polymer (3020; calc as the dialdehyde–ketone). *trans*-Chrysanthemyl alcohol (4200 dpm. mmol^{-1}) yielded acetone (1590) and chrysanthemyl alcohol aldehyde (2510). As extensive degradation of DMVC and reincorporation of the fragments into monoterpenes *in vitro* seems extremely unlikely, these results indicate that the two C–5 units of artemisia ketone and chrysanthemyl alcohol (α, β in **10** and **11**) were labelled 80:20 and 62:38 respectively. Similar asymmetry of labelling of the C–5 moieties occurred in artemisia ketone biosynthesised *in vivo* by *A.annua* from MVA-[2-^{14}C] [4] and the phenomenon (which probably reflects existence of an endogenous pool of DMAPP or its biogenetic equivalent) appears to be general for monoterpene biosynthesis *in vivo* and probably *in vitro* [35].



10 11

Fig. 2. C–5 moieties in irregular monoterpenes.

The role of DMVC in monoterpene biosynthesis

DMVC (or its pyrophosphate), although readily derived from DMAPP by allylic rearrangement, has rarely been considered as a biogenetic intermediate. The only experimental study indicated it to be a more effective precursor of geraniol in certain cell-free preparations than was IPP or DMAPP [21]. Results in the previous sections and comparative studies on the hemiterpenes using the same cell-free preparation (Table 5) and in the presence of 10-fold molar excesses of unlabelled hemi- or mono-terpenes (Table 6) all indicate that DMVC was marginally more effective as a precursor of the irregular monoterpenes than was IPP or DMAPP. Unfortunately, DMVC–PP could not be prepared sufficiently pure for a direct comparison with the other phosphate esters, but 3,3-dimethylallyl alcohol and isopentenol were *less* effective precursors than their pyrophosphates by a factor of 2–3, and so the enhanced position of DMVC to DMAPP and IPP is unlikely to be

Table 5. Hemiterpene substrates for cell-free preparations from *S.chamaecyparissus*

Products* (%)	Substrate†					
	IPP	DMAPP	DMVC	IPP + DMAPP	IPP + DMVC	DMAPP + DMVC
AK	0.8	0.7	0.6	1.0	0.9	0.9
AA	1.4	1.0	1.3	1.3	1.6	1.9
CA	1.5	2.5	4.7	2.6	3.5	3.7

* Products, (abbreviations as before), % incorporation of tracer. † Substrates, all ^{14}C-labelled. Introduced at similar molarity and specific activity (see Exptl).

due to its alcoholic nature and its introduction into the incubation mixture with a solubilising agent. Thus DMVC (presumably as its pyrophosphate) may be an obligate intermediate of monoterpene biosynthesis. However, negligible (< 1 %) interconversions of DMAPP and DMVC–PP could be demonstrated in our preparations, and as DMAPP–isomerase, if it exists, did not survive solubilisation.

(b) DMVC or its pyrophosphate may be an important C–5 intermediate. (c) A metabolic pool of DMAPP, probably protein-bonded, exists that contributes one C–5 moiety of the irregular monoterpene skeleton. (d) Artemisyl and chrysanthemyl compounds probably occur on divergent pathways from C–5 precursors (cf. experiments with NAD^+ etc). (e) Artemisia ketone is formed by a dehydrogenase system rather than by a

Table 6. Hemiterpene substrates for cell-free preparations from *S.chamaecyparissus*: with unlabelled metabolites

	Substrate†							
		IPP-[^{14}C] +		DMVC-[^{14}C] +				
Products* (%)	DMAPP-[^{14}C] + DMVC	DMAPP	DMVC	DMAPP	AA	AK	CA	CPP[c]
AK	0.7	0.5	0.6	0.9	1.3	1.0	0.5	0.7
AA	1.2	0.9	0.9	1.4	1.9	1.7	0.9	1.2
CA	1.4	1.5	1.5	4.3	3.9	2.6	3.3	3.3

* See Table 5. † ^{14}C-substrates all at same concentration and total activity. Unlabelled metabolites in *ca* 10–fold excess. ‡ CPP = (+)-*trans*-chrysanthemyl pyrophosphate.

Evidence for involvement of sulphydryl enzymes

Addition of thiols or reducing agents (5mM) to incubation mixtures gave the following efficiencies of incorporation of IPP-[^{14}C] into irregular monoterpenes under standard conditions: dithiothreitol (100), redistilled 2-mercaptoethanol (95), metabisulphite, glutathione (90), cysteine, ascorbate (88) and control (72). All values are estimated ± 3 and use of DMVC or DMAPP as substrate gave essentially the same results. The inference that a sulphydryl enzyme was possibly involved was supported by a series of experiments with DMVC-[^{14}C] or substrate. Here the reducing reagents were omitted from the incubation medium but Ag^+ or Hg^+ (5 μM; EDTA used in the initial extraction had been removed by gel filtration before incubation), iodoacetate, iodoacetamide or *p*-chloromercuribenzoate (1–5 μM) were individually added. All additives reduced monoterpene biosynthesis to 65–75 % of its optimum value and higher concentrations of the inhibitors (up to 20 μM) caused insignificant further changes. This is consistent with about 70 % of the reaction to monoterpene involving a sulphydryl enzyme that is protected by a C–5 moiety, and which comprises the metabolic pool of DMAPP (see previous sections) that had survived extraction from the plant. Similar experiments involving IPP-[^{14}C] and DMAPP-[^{14}C] as substrates gave a pattern of results with the different inhibitors that could not be rationalized: this may indicate that the inhibitors were now interfering with a IPP–DMAPP isomerase system.

Biosynthetic pathways

Previous suggestions [2] that the chrysanthemyl skeleton is an obligatory precursor of the other three irregular types (**2**–**4**), and speculations concerning more restricted interconversions of these skeletons in plants based on studies with so-called model systems [36–40] have unfortunately not been backed by any experimental evidence relevant to the situation *in vivo*. Our present results for *in vitro* plant systems may be summarised: (a) A sulphydryl-enzyme system(s) is probably involved. mixed-function oxidase. (f) Enzyme systems exist that interconvert artemisyl compounds and *trans*-chrysanthemyl alcohol. *cis*-Chrysanthemyl alcohol is not formed under these conditions but is converted into the other irregular monoterpenes. (g) Lavandulol is also formed from the C–5 precursors (assays for santolinatriene were not carried out). (h) Geranyl, neryl and linalyl pyrophosphates are insignificantly converted into irregular monoterpenes. (i) In over twenty experiments involving IPP, DMAPP and DMVC as substrates, the ratio of artemisyl alcohol to its ketone in products was 1.60 $\pm$ 0.17, whereas that of *trans*-chrysanthemyl alcohol to the ketone varied over the range 2.3–11.3; this suggests that the redox system reached an equilibrium in these extracts, although the ketone invariably greatly predominated in oils from whole plants [1].

Figure 3, which accommodates some of the speculations of earlier workers [36–40] and in which many details are adjustable, is consistent with our observations. Nothing is known of the nature of the groups at the active sites of the enzymes that couple C–5 units to give terpenoids in plants cf. [41] although there have been suggestions [42–43b] that sulphonium centres are involved that undergo SN2-type reactions and Stevens rearrangements. Our present results indicate that a sulphydryl group may be implicated and we tentatively suggest the occurrence of a species **12** (E = enzyme; R = alkyl; Me, C_5H_9) as we have some independent evidence that the DMAPP-pool in other members of the Compositae is protein bonded [35]. An ylide form of this species is chemically reasonable and permits a direct route to the artemisyl skeleton that by-passes the formation of chrysanthemyl compounds. An ylide intermediate is not considered to occur in the hypothetical route [35] to regular monoterpenes: here the DMAPP (or its biogenetic equivalent) presumably acts as an electrophilic reagent towards IPP. Direct electrophilic addition of the 3,3-dimethylallyl residue from the pool (i.e. the protonated form of **12**) to IPP or DMAPP (or their biogenetic equivalents) in an *anti*-Markownikoff fashion would lead to 'free' (i.e. not bonded to protein)

3,3,6-trimethylheptan 1,5-diene: this could only reasonably lead to artemisyl alcohol and its ketone by the intervention of a mixed function oxidase, and we have found no evidence for the intervention of such a system.

Comparisons of interconversions and incorporations achieved with our newly-developed systems and those from *in vivo* studies of the same plants and precursors [4] illustrate the advantage of the former. Fractionation of the multi-enzyme extracts is necessary to elucidate further mechanistic details, but our attempts at this have proved largely unsuccessful so far.

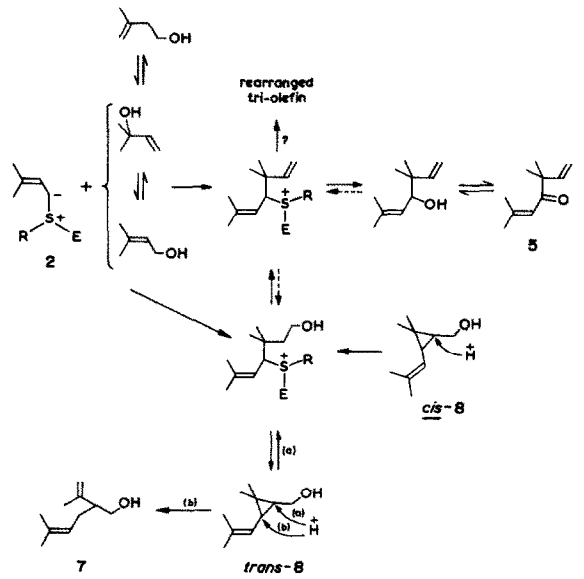


Fig. 3. Tentative scheme for biosynthesis of irregular monoterpenes in *in vitro* systems. **12** is the protein-bonded pool of DMAPP; E is carrier protein (enzyme?); R is an alkyl group to sustain the sulphonium centre. For reasons of clarity alcohols are shown as reaction intermediates: pyrophosphate esters are probably involved except in a few steps (e.g. the oxidation to yield **5**).

EXPERIMENTAL

Methods. Preparative and analytical GLC and TLC techniques and radiochemical methods have been described [4]. All substrates and products had satisfactory elemental analyses and mps in agreement with literature values unless stated, and IR, NMR and MS consistent with their accepted structures. H-NMR of solns. (10% in $CDCl_3$ or $(CD_3)_2CO$) were recorded at 60 MHz with TMS (τ10.00) as internal standard.

Materials. A.annua L. and *S.chamaecyparissus L.* were obtained from Dr. P. Yeo of the Cambridge Botanical Garden and were cultivated in a greenhouse and harvested at the 10–15 cm stage as in the previous study [4]. *S.chamaecyparissus* cv chamaecyparissus (Sincana) and neopolitana (Jord. et Fourr.) Fiori were also used (voucher specimens are kept at Cambridge) and these yielded oils of virtually the same composition and gave cell-free systems with essentially identical properties as those from the main taxon. IPP-[4-^{14}C], DMAPP-[4-^{14}C] (both 17 mCi mmol^{-1}), DMVC-[Me-^{14}C] (1.2 mCi mmol^{-1}), artemisyl alcohol-[G-^{14}C] (1.0 mCi mmol^{-1}), linalol-[2-^{14}C] (72 mCi mmol^{-1}) and [^{14}C]-labelled chrysanthemyl alcohols (0.35–1.0 mCi mmol^{-1}) were prepared as previously described [4]. In some preliminary studies IPP-[1-^{14}C] and DMAPP-[1-^{14}C] were used: isopentenol-[1-^{14}C] (8 mCi mmol^{-1}) was formed by treatment of 2-methylallylmagnesium chloride with CO_2-[^{14}C] [44] and reduction of the product with sodium dihydro-*bis*-(2-methoxyethoxy) aluminate in C_6H_6 followed by purification by GLC on FFAP (10% on G-Cel; 3m × 5 mm; 4.2 l hr^{-1} N_2) at 120°: overall yield 52%, bp 132°. Part of the product from the Grignard step was isomerised to 3,3-dimethylacrylic acid-[1-^{14}C], mp 69°, by 50% aq. KOH (90°/45 min) which was separated by GLC on the same column and reduced to 3,3-dimethylallyl alcohol-[1-^{14}C], yield 32%; bp 137°. A mixture of geraniol-[2-^{14}C] and nerol-[2-^{14}C] was prepared cf [45] by condensation of diethylphosphonomethyl acetate-[2-^{14}C] in a Wittig reaction with 6-methyl-5-hepton-2-one followed by reduction of the products: the phosphorous ester having been obtained from methyl bromoacetate-[2-^{14}C] (500 μCi; 4·7 mCi mmol^{-1}) and triethylphosphite in a Michael–Arbuzov reaction [46]. The mixture was separated by: (a) TLC on silicic acid–Si gel GF 254 (2:1) with EtOAc–C_6H_{12} (15:85; 4 ×) to give nerol (R_f 0.55) and geraniol (R_f 0.47); (b) TLC on Si gel GF 254 impregnated with $AgNO_3$ (12%) developed with EtOAc [47] to give nerol (R_f 0.72) and geraniol (R_f 0.61); or (c) isolating geraniol as its complex with $CaCl_2$ [48]. Recoveries of up to 80% were achieved from the plates and the geraniol–nerol ratio was *ca* 3:1. GLC-analyses (Carbowax 20M or FFAP: 20 m × 0.5 mm capillary; 0.251 hr^{-1} N_2; 100°) of the alcohols and their TMS derivatives indicated >99% purity. Alternative separation of geraniol and nerol involved oxidation of the mixture to aldehydes and separation by TLC on (a) Absorbosil 5 (Applied Science Labs, State College, Pa, USA) Si gel GF 254 (10:1) eluted (5 ×) with EtOAc–C_6H_{12} (1:9) to give neral and geranial (R_f 0.86, 0.79); (b) the same plate eluted (6 ×) with EtOAc–C_6H_{12} (5:95; neral and geranial R_f 0.54 and 0.48); or (c) Absorbosil 5- silicic acid (10:1) eluted (4 ×) with EtOAc–C_6H_{12} (1:9; neral and geranial, R_f 0.75 and 0.68). Purified aldehydes (>98% pure by GLC) were then reduced (sodium dihydro-*bis*-(2-methoxyethoxy)aluminate) to the alcohols. Linalol-[1,2-$^{14}C_2$] was obtained by condensation of 6-methyl-5-hepton-2-one with lithium acetylide-[1,2-$^{14}C_2$] [49–51] followed by reduction over a Lindlar catalyst [52]. The crude material was purified by TLC on Si gel GF 254: $AgNO_3$ (4:1) with EtOAc (2 ×) to give a product (R_f 0.71; yield 65%); 72 mCi mmol^{-1}) that was >99% pure (GLC; TLC). Artemisia ketone for use as carrier was distilled from Santolina oil (Raphael–Carbonel; Vallauris, France) using a Büchi spinning-band column (70 × 1.2 cm). The fraction, bp 180–90°, was subjected to prep. GLC on Carbowax 20M (30% on G-Cel; 6 m × 1.5 cm; 4.5 lh^{-1} N_2; 150°) to give a product, bp 181° that was 99% pure (TLC; GLC). Artemisyl alcohol was obtained from this by reduction (sodium dihydro-*bis*-(2-methoxyethoxy)aluminate) and the product was passed through a column (6 × 2.5 cm) of Si gel H with EtOAc–C_6H_6 (15:85) and purified by TLC on Si gel H254 with $CHCl_3$ at 0° (alcohol and ketone, R_f 0.73 and 0.46) and then by GLC on Carbowax 20M (30% on G-Cel; 4.2 lh^{-1}; 30°) to give product (80% overall; >99% by TLC and GLC), bp 90·2–90·5°/20 mm Hg. Lavandulol was cleaved from its acetate (Givaudan et Cie.; Grasse, France) with sodium dihydro-*bis*-(2-methoxyethoxy) aluminate followed by GLC on FFAP (30% on G-Cel; 6 m × 1.5 cm; 4.2 lh^{-1} N_2; 150°) to give product bp 99–100°/15 mm Hg (>98% pure, GLC, TLC).

Preparation of pyrophosphates. The pyrophosphate esters of geraniol, nerol, linalol, isopentenol and 3,3-dimenthylallyl alcohol were prepared by a standard procedure [8, 9] and were purified by the following sequence. (a) PC (descending, 50 cm; 70 hr) on Whatman 3M (for washing procedure, ref. [13] or Whatman No 1 (for IPP–DMAPP) with iPrOH–NH_4OH–H_2O (8:1:1). Organic mono-, pyro-, and tri-phosphates and inorganic phosphates had R_f 0.21–0.24; 0.09–0.13; 0.03–0.05; and 0.00 respectively. Sprays were I_2–MeOH (10%); bromocresol purple (0.04% aq.) and ascorbate–molybate [53–4]. Fractions were eluted (30 ml of aq. NH_4OH, 1%) and yields were 10–50% and the ratios of phosphates and pyrophosphates were typically 3:1. (b) Electrophoresis (cooled-plate; Locarte, London) of the separated fractions on Whatman 3M (23 ×

57 cm) in aq. NH_4OH (0.1 M) with gradient 140 V cm^{-1}, 1.5 h. (c) Solution in aq. NH_4OH (0.3 M; 1 ml) and precipitation with $(Me)_2CO$–MeOH (8:1), followed by similar precipitation (4 ×) with $(Me)_2CO$. (d) Solution in aq. NH_4OH (0.1 M; 1 ml) and lyophilisation. Final products were stored at −20° under vacuum desiccation. For chrysanthemyl pyrophosphates, step (a) was replaced by column chromatography (12 × 1.5 cm) on Permutite Deacidite H-1P-SRA-124(OH^-) with aq. NH_4OH (1%). All the purified mono- and pyro-phosphates had the expected ^{1}H-NMR and cleavage with alkaline phosphatase and apyrase gave the expected alcohol (>99% GLC). The monophosphates of geraniol, nerol, linalol and the chrysanthemyl alcohols all analysed (±1%) expected values) as $[C_{10}H_{17}PO_4]^{2-}[NH_4]^+[H^+]3H_2O$ and the corresponding pyrophosphates as $[C_{10}H_{17}P_2O_7]^{3-}[NH_4]_2^+[H^+]2H_2O$. Previously only one set of unsatisfactory analytical data has been reported [8] and the *bis*-cyclohexyl-ammonium lithium salts of pyrophosphates have been found to contain varying amounts of H_2O that could not be reproducibly removed. The pyrophosphates were reasonably stable (<10% decomposition in 6 mths) at −20°. Stability of DMAPP was markedly improved if stored impregnated on paper, whilst that of chrysanthemyl pyrophosphates was much increased (virtually no decomposition at −20°/4 mths) if $(NH_4)_2CO_3$ (0.3 mg) was added to the ester (20 mg) before lyophilisation.

Preparation of cell-free systems. All procedures were performed at +4° and precautions (stoppered tubes etc.) were used to prevent volatilisation during assay. Boiled enzyme controls were used at all times. Foliage (5 g; the youngest 4 pairs of leaflets and the tuft at the growing tips) were excised under sterile conditions, frozen (liq. N_2) and pulverised. The powder was mixed with Polyclar AT (ex GAF, Inc. Binghampton, N.Y.; pretreated as in ref. [55]; 1:2 v/v) and stirred into *Tris*-MeCOOH (0.1 M; pH 7.0; 40 ml) containing cysteine HCl (10 m M), sodium diethyldithiocarbanate (10 m M), EDTA (1 m M) and sucrose (0·25 M, and after standing (1 hr, occasional stirring) was filtered (glass wool) before centrifugation (500 *g*; 10 min.). Supernatant (10 ml) was passed through Sephadex G-10 (45 × 2.5 cm; 50 ml hr^{-1}) and eluted with H_2O. The excluded protein (*ca* 20 ml) was used for all subsequent experiments. Use of shorter columns or elution with buffers resulted in the protein being contaminated with phenolics. Addition of other reducing agents, e.g. redistilled 2-mercaptoethanol, ascorbate or metabisulphite, to the initial extract gave inferior results. Typically protein concentrations were *ca* 0.06 and 0.13 mg ml^{-1} for *S.chamaecyparissus* and *A.annua* respectively, and these assays were made by either a turbidity procedure [56, 57] or a modified Lowry method [58]. An aliquot of this preparation (1 ml) in maleate buffer (0.1 M; pH 6.5; 2 ml) containing ATP, $NADP^+$, NAD^+, $MgCl_2$, $MnCl_2$ and dithiothreitol (5 m M each), 'soil extract' (100 μl) and the substrate (*ca* 0.5 mg; 1.0 to 2.5 × 10^6 dpm) was chosen as the standard incubation mixture after considerale experimentation (see Discussion). We found that use of a 'soil extract' prepared by shaking (4 hr) material (20 g) from the cultivation pots with water (100 ml), boiling (2 min) and millepore filtration (0.45 μm pore) gave worthwhile (up to 10%) and reproducible increases of incorporation of tracer into monoterpenoids. This fortuitous discovery was used in several sets of runs. The composition of the extract was not determined, but as evaporation and ashing gave a residue with stimulatory properties we presume that trace metals had been involved. After incubation (2 hr/30°) to reach the plateau region, carbonate buffer (0.5 M, pH 10.4, 1 ml) and $MgCl_2$ (30 μmol) were added and the solution was re-incubated (4 hr/37°) with phosphatase (15 mg) and apyrase (10 mg).The latter enzyme was prepared from potatoes [59] and was stored at 4° in $(NH_4)_2SO_4$(3 M) at pH 4.0: under these conditions it lost *ca* 10% activity per month. Attempts to terminate the reaction with TCA (10% aq.) led to extensive polymerisation of artemisia ketone. The final incubation mixture was quenched with $CHCl_3$–EtOH (1:1, 0.1 ml) at 0° and the aq. layer was extracted with Et_2O (1.5 ml), petroleum bp 60–80° (1.5 ml) and Et_2O–petrol (1:1:1 ml) after saturation with NaCl. Boiled enzyme controls showed negligible incorporations (<0.1%) of tracer into final products. Water-insolubles were incubated with Tween 80 (0·5 μl), Triton X-100 (0·5 μl), EtOH (20 μl), or DMSO (20 μl): these solublisers had no significant effect on enzyme activities in controls but Tween 80 produced the best incorporations. Anaerobic runs were conducted in Thunberg tubes that were evacuated in a five-stage cycle to 0.5 mm Hg with intermittent introduction of N_2 to 760 mm. Contamination with bacteria or symbiotic microorganisms was checked by incubation of aliquots with bovine serum albumin. Typically, the number of viable cells (300 ± 50 per field) were unchanged after incubation. pH-Activity profiles used the following buffers: pH 5.0, 0.1 M Na succinate; 6.0–6.5, 0.1 M Na maleate; 7.0–8.0, 0.1 M Tris–Me_3COOH.

Assay of products. Fractions (0.5 ml) of the organic extracts were separated by TLC on Si gel H with EtOAc–C_6H_6 (15:85; 2 ×). The appropriate sectors of the plate (hydrocarbons R_f 0.85–1.00; artemisia ketone 0·79–0.85; artemisyl alcohol 0.55–0.70; chrysanthemyl alcohols 0.43–0.55; lavandulol and C-5 alcohols 0.34–0.43) were eluted with Et_2O to give products which subsequent rigorous purification showed were usually >80% radiochemically pure. Carrier (50 μl) was added to each Et_2O-extract (500 μl) and the terpene was separated by GLC on FFAP at 175° with an unheated collector port to minimise formation of fogs. In preliminary studies, the ^{14}C-products were passed directly from the exit port into the scintillant via a polypropylene tube (4 cm × 1 mm) which was subsequently cut into pieces and added to the vial. By this means up to 96% recovery of hemiterpenols could be achieved. Artemisia ketone was converted [sodium dihydro-*bis*-(2-methoxyethoxy)aluminate] into its alcohol and this and the others were purified as 3,5-dinitrobenzoates which were recrystallised (at least thrice) to constant specific radioactivity from MeOH or aq. $(Me)_2CO$. Mp's of derivatives were: artemisyl alcohol 89–90°; lavandulol 71–2° (lit. 73–4°; ref. 60); *trans*-chrysanthemyl alcohol mp 126° isopentenol mp 55°; 3,3-dimethylallyl alcohol mp 69°. In a few experiments artemisia ketone was purified as the semicarbazone, mp 93°. Loss of ^{14}C from the TLC plates was estimated as *ca* 5% for the monoterpenols, but up to 60% for the hemiterpenols: corrections were made for these losses.

Partial degradations. Artemisia ketone and *trans*-chrysanthemyl alcohol (*ca* 50 mg; *ca* 50 000 dpm) in MeOH (1 ml) were ozonised at −20° and the products decomposed by refluxing (1 h) with H_2O. $(CH_3)_2CO$ and HCHO were distilled off and purified as their 2,4-dinitrophenylhydrazones and the residues were recovered and purified by TLC.

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