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BIOSYNTHESIS OF GERANIOL AND NEROL IN CELL-FREE EXTRACTS OF *TANACETUM VULGARE**

DEREK V. BANTHORPE, GRAHAM A. BUCKNALL, HILARY J. DOONAN, SHAWN DOONAN and MICHAEL G. ROWAN†

Christopher Ingold Laboratories, University College, London WC1H-0AJ, England

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Key Word Index—*Tanacetum vulgare*; Compositae; cell-free extracts; monoterpenes; biosynthesis; geraniol; nerol; labelling patterns; metabolic pools.

Abstract—Cell-free extracts from leaves of *Tanacetum vulgare* synthesised geraniol and nerol (3,7-dimethylocta-*trans*-2-ene-1-ol and its *cis* isomer) in up to 11·9 and 2·4% total yields from IPP-[4-^{14}C] and MVA-[2-^{14}C] respectively. Optimum preparations were obtained from plant material just before the onset of flowering. The ratio of the monoterpenols varied 28-fold for different preparations under conditions where these products or their phosphate esters were not interconverted. Similar extracts incorporated α-terpineol-[^{14}C] and terpinen-4-ol-[^{14}C] (p-menth-1-en-8- and -4-ol respectively) in 0·05 to 2·2% yields into a compound tentatively identified as isothujone (*trans*-thujan-3-one), and preparations from flowerheads converted IPP-[4-^{14}C] in 2·7% yield into geranyl and neryl β-D-glucosides. Inhibitors of IPP-isomerase had little effect on the incorporation of IPP into the monoterpenols in cell-free systems from which endogenous compounds of low molecular-weight had been removed. The inference that a pool of protein-bonded DMAPP or its biogenetic equivalent was present was supported by the demonstration that geraniol and nerol biosynthesised in the absence of the inhibitors were predominantly (65 to 100%) labelled in the moiety derived from IPP.

INTRODUCTION

Monoterpene biosynthesis in cell-free extracts from higher plants has been extensively investigated, but despite advances in methodology [1–4] the results have been disappointing. The most detailed studies have involved incorporation of MVA or IPP into geraniol, nerol or linalol using preparations from *Pisum sativum* [5], *Pinus radiata* [6, 7] and *Citrus jambhira* [8–10], and of these precursors or GPP and NPP into α-terpineol, limonene and α-pinene in extracts from the last two species [9, 11]. Interconversions of the acyclic and monocyclic monoterpenes of *Mentha piperita* have also been reported [12, 13]. In only a few cases, however, is there conclusive evidence for the products claimed: usually single GLC or TLC fractions have been assayed for radioactivity without further purification and no attempt has been made to recrystallise derivatives of the presumed monoterpenes to constant specific radioactivity. Thus the possibility exists that the chromatographic fractions contained heavily-labelled contaminants [14, 15] e.g. salvage products [16] or higher terpenoids [17]. In any event, the incorporations of tracer into the presumed monoterpenes were, (except for the interconversions in *M. piperita*) uniformly low, and were usually less than 1% of the administered material. We here report the systematic development of cell-free systems from *Tanacetum vulgare* L. (Tansy, Compositae) that synthesise geraniol **1** and nerol **2** (3,7-dimethylocta-*trans*-2-ene-1-ol and its *cis* isomer) in unprecedented high yields, and so constitute effective tools for investigation of the mechanism and stereochemistry of monoterpene biosynthesis in this species.

RESULTS AND DISCUSSION

Intracellular distribution of enzymes for monoterpene biosynthesis. Cell-free extracts from leaves of *T. vulgare* were prepared by modifications of methods reported for *Pinus radiata* [6] and *Pisum sativum* [18]. Results of incubations of these with ^{14}C-labelled substrates are shown in Table 1. Most (>95%) of the MVA was recovered unchanged from the incubation mixtures but the bulk of tracer from IPP in these and in subsequent experiments resided in water-soluble materials that could not be salted out by $(NH_4)_2SO_4$ into organic solvents. The occurrence of these latter products is of great significance for *in vitro* studies of terpene biosynthesis and will be considered in a forthcoming publication. Our results show that (a) the predominantly plastid fraction A or the predominantly membrane/mitochondrial fraction B could not alone effectively sustain monoterpene biosynthesis from MVA but could in combination; (b) addition of a supernatant fraction C to (A + B) or use of the total leaf homogenate (H) gave poor or negligible incorporations from MVA, presumably owing to the presence

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† Present address: School of Pharmacy, University of London, England.

Abbreviations used: MVA, mevalonic acid; MVAP and MVAPP, mevalonic acid 5-phosphate and 5-pyrophosphate; IPP, isopentenyl pyrophosphate; IMP, isopentenyl monophosphate; DMAPP, 3,3'-dimethylallyl pyrophosphate; DMVC-PP, dimethylvinylcarbinyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate.

Table 1. Intracellular distribution of enzymes for monoterpene biosynthesis in *T. vulgare*

System*	Fraction and Type†	Substrate	% C-10‡
1	A-P(12000 *g*)	MVA-[2-^{14}C]	0·0
1	B-P(20000 *g*)	MVA-[2-^{14}C]	0·15
1	A + B-P(12000 *g*) + P(20000 *g*)	MVA-[2-^{14}C]	2·0
1	A + B + C-P(12000 *g*) + P(20000 *g*) + S(20000 *g*)	MVA-[2-^{14}C]	0·07
1	*D*-H	MVA-[2-^{14}C]	0·0
2	E-S(10^6 *g*; $(NH_4)_2SO_4$)	MVA-[2-^{14}C]	0·12
2	E-S(10^6 *g*; $(NH_4)_2SO_4$)	IPP-[4-^{14}C]	3·4
3	A', B', C' as in System 1	MVA-[2-^{14}C]	0·02; 0·001; 0·02§
3	A', B', C' as in System 1	MVAPP-[2-^{14}C]	0·03; 0·001; 0·01§
3	A', B', C' as in System 1	IPP-[4-^{14}C]	2·4; 2·3; 3·5§

* Cell-free systems 1 and 2 are based on methods given in refs. [6] and [18]. System 3 is a modification. For details of methods and incubation procedures, see Experimental.

† Fraction A is predominantly plastid; B is the mitochondrial and membrane fraction; C is the supernatant from the centrifugation; D is the debris-free supernatant obtained by centrifuging the homogenate (H) at 200 *g*; E is from ammonium sulphate fractionation. P(x) and S(x) are preparations from pellet and supernatant after centrifugation H is total homogenate. Details in Experimental.

‡ Percentage tracer (±0·1%) incorporated into geraniol + nerol under standard conditions. Yields from MVA are based on 3R-isomer.

§ Incorporations for fractions A', B', C' prepared as for system 1.

of inhibitors or phosphatases in the supernatant, whereas (c) particulate or supernatant fractions converted IPP into monoterpenes with similar facility. A mixture of particulate and soluble fractions of a cell-free extract was found necessary for the synthesis of sesqui- and di-terpenes from MVA by *Pisum sativum* [18]. One possibility for the varying effectiveness of incorporation of MVA is that use of an excess of the metabolite or the presence of the unphysiological 3S isomer in the commercially-available racemate could inhibit one of the enzyme systems necessary for monoterpene formation from this precursor. However, variation of MVA concentration by 50-fold or addition of up to 100-fold excess of 3S-MVA provided no evidence for such an effect.

Seasonal variation of prenyltransferase activity. As a consequence of these pilot studies, attention was confined to the use of IPP as substrate and to the optimisation of prenyltransferase activity [DMAPP:IPP-dimethylallyl transferase; E.C. 2.5.1.1.]. A new cell-free system henceforth referred to as the standard system (CFS-4; see Experimental) was developed for this purpose, a feature of which was a gel filtration step at a late stage which removed low molecular-weight compounds and so enabled percentage incorporations of tracer to be evaluated without complications due to possible pools of IPP and DMAPP or their biogenetic equivalents. This treatment also removed additives introduced during the extraction procedure and eliminated any enzyme inhibitors of low molecular weight.

Assay of prenyl transferase from a particular batch of foliage was highly reproducible: in two sets of experiments carried out in June and July, 1972, the incorporations under the standard incubation conditions into monoterpenes (>96% tracer in geraniol + nerol; see later sub-section) were 1·59 ± 0·05 and 3·21 ± 0·08% (both means of 7 independent determinations). These results reflect the marked seasonal variation in activity that was presumably due to the concentration and type of phenols present, the activity of endogenous proteases and phosphatases, and the physiological state of the plant. The variation across a growing season of prenyl transferase activity in cell-free preparations from clones of *T. vulgare* cultivated under similar outdoor conditions is shown in Figure 1: results in subsequent seasons were essentially the same. Maximum activity occurred for a short period before the onset of flowering: similar results have been obtained for the formation of monoterpenes in cell-free preparations from *Lavandula* species [19] and for formation and metabolic turnover *in vivo* of monoterpenes in *Mentha piperita* [20–21].

Optimisation of cell-free systems. In view of this seasonal variation, meaningful comparisons of the activities of cell-free systems prepared at different times could only be made if each 'experimental' set was accompanied by a control (each comprising 5 independent assays) carried out on the same batch of foliage. The standard system was always used as control and its activity as measured by uptake of tracer from IPP-[4-^{14}C] into geraniol and nerol under defined conditions was assigned as 100 for *each* set (i.e. each pair of experimental and control). The

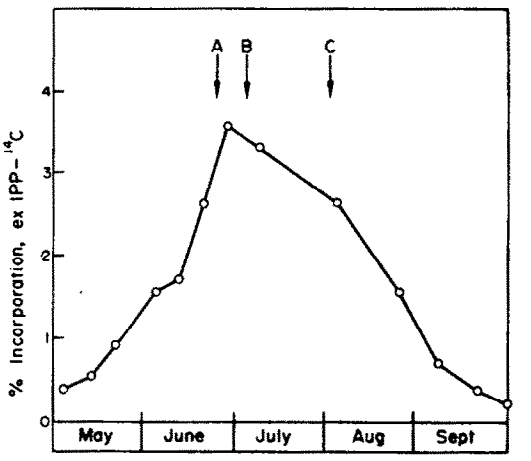

Fig. 1. Seasonal variation in the incorporation of IPP-[4-^{14}C] into monoterpenols of *T. vulgare*: (May–Sept 1972): Established 1 yr-old-plants were used that had been cut to ground level, March, 1972: A = Longest day; B = onset of flowering; C = maximum mean daily temp.

Table 2. Effects of variation in homogenisation conditions on activity of prenyltransferase in cell-free systems from *T. vulgare*

Procedure*	Buffer (0·1M)	Activity†
Ground in liq. N_2, *ca* 3 mins	MES (pH 7·0)‡	100
Ground in liq. N_2, *ca* 3 mins	MES (pH 6·0)	72
Ground in liq. N_2, *ca* 3 mins	Tris (pH 7·0)	30
Ground in liq. N_2, *ca* 3 mins	Tris (pH 8·0)	17
Ground in liq. N_2, *ca* 3 mins	phosphate (pH 7·0)	88
Ground in solid CO_2, *ca* 3 mins	MES (pH 7·0)	83
Mechanical blender; 90 sec at 0°	MES (pH 7·0)	31

* Following infiltration and pulverisation, the tissue was extracted with the buffer containing additives, or the tissue was homogenised in a mechanical blender with the buffer and additives. For details of additives see Experimental, (prepn. of CFS-4). † Prenyltransferase activity with IPP as substrate (assay procedure; see Experimental) relative to that of the standard system (=100); SD activity values ±5. No activity was found unless the standard additives were used. ‡ Procedure for the standard system (see text).

effect of a particular treatment on the activity of the standard system could then be assigned irrespective of the actual percentage incorporation of precursor. In the experiments summarised in Tables 2–8, the recovery of ^{14}C as extractable material (after cleavage of phosphate esters) was in the range 10–80% (typically 15–20%) and the incorporation into the monoterpene fraction varied from 0·8–3·7%. The bulk of the tracer usually resided in water-soluble compounds (see above).

The effects of variation of initial homogenisation procedures and of additives or treatments at the preincubation stage are shown in Tables 2 and 3. Polyvinylpyrrolidone and borate were employed to remove phenols [1–4; 22] and the chelating agents were to inhibit phenol oxidases [23–25]. Here, as in subsequent experiments, variation of a particular parameter could have subtle effects on the complex protein–cofactor–metal ion systems involved in terpene biosynthesis and attempts at rationalisation of the changes in activity as a result of particular treatments is, in most cases, neither possible nor worthwhile.

Attempts to optimise the incubation step are summarised in Table 4. The phospholipids were screened as such compounds have been claimed [26] appreciably to enhance the incorporation of MVA-[2-^{14}C] into monoterpenes formed from cell-free extracts from *Pinus radiata*:

Table 3. Effects of variations in the preincubation step on activity of prenyl transferase in cell-free systems from *T. vulgare*

Treatment/Additive	Activity§	Treatment/Additive	Activity§
[Standard system]	100	1:10-Phenanthroline	
PVP (soluble; 1% w/v)	202	(20 mM)	136
PVP (insoluble; 1% w/v)	119	2-CEPA (50 mM)‡	120
SDDC*	302	Mepyropone (1 mM)	88
Borate†	21	37°/30 min	123
Bovine serum albumin		4°/pH 5·0/1 hr	432
(1% w/v)	99	Omission of Biogel-2, step	82
EDTA (1 mM)	140		

* Vacuum infiltration of leaves with 0·1M phosphate (pH 7·0) containing sodium diethyldithiocarbamate (SDDC; 1 mM) + 2-thioethanol (1 mM) before pulverisation with liquid N_2; † As described in ref. [22]; ‡ 2-chloroethylphosphonic acid; § Substrate, assay conditions etc as in Table 2.

Table 4. Effects of variations in the incubation step on activity of prenyltransferase in cell-free systems from *T. vulgare*

Treatment/Additive	Activity§	Treatment/Additive	Activity§
[Standard system]	100	EDTA (1 mM)	125
Anaerobic conditions*	66	Cofactors‡	64
Triton X100 (1% v/v)	68	Soil extract¶	67
Tween 80 (1% v/v)	97	Cellulose phosphate (0·01% w/v)	31**
Beef brain extract (0·01% w/v)†	106	Fructose-1-phosphate (35 mM)	13**
α-Lecithin (0·01% w/v)	110	Sodium fluoride (35 mM)	50, 48**
Lysolecithin (0·01% w/v)	93	Sodium tungstate (35 mM)	8**

* Assay carried out under N_2 in Thunberg tubes; † Type II ex Sigma Chemicals Ltd., London; ‡ Bovine serum albumin (0·5% w/v); NAD (50 μM), NADP (5 μM), NADH (60 μM), NADPH (60 μM), Coenzyme A (50 μM); ¶ Aqueous extract (100 ml) of soil (100 g) from cultivated plot of *T. vulgare* added (1:1 v/v) to cell-free extract; § Substrate, assay conditions etc. as in Table 2; ** MVA-[2-^{14}C] as precursor.

Table 5. Effects of variations in pH [ATP] and $[M_8^{2+}]$ on activity of prenyltransferase in cell-free systems from *T. vulgare*

pH	5·0	6·0	6·5	7·0†	7·5	8·0
Activity*	161	168	125	100	100	136
Mg^{2+}, mM	0	10	25	40†	60	100
Activity*	95	53	75	100	110	92
ATP, mM	0	2	3	10†	20	50
Activity*	32	72	87	100	94	68

* Substrate, assay conditions etc, as in Table 2. † Standard system: pH 7·0; $[Mg^{2+}]$ 40 mM; [ATP] 10 mM.

Tween 80 and other emulsifiers are known to reverse certain inhibitions caused by tannins [27–28]. The last four additives are either potential or known [29] inhibitors of phosphatases in *T. vulgare*, but they also all inhibited monoterpene biosynthesis. The effects of variation of pH and concentration of ATP and magnesium ion are given in Table 5.

Many of the variations in conditions here used have been previously reported to improve the yields from cell-free systems that can sustain terpenoid biosynthesis (for leading references see [14] but, in our hands, most were ineffective and some caused inhibition. No one treatment led to a spectacular increase in activity of the standard system from *T. vulgare* but on the basis of our present results optimum conditions could be chosen and these are described in the Experimental section. Batches of leaf tissue (at different stages of seasonal development) which yielded standard cell-free systems that incorporated 1·9 ± 0·1 and 3·6 ± 0·1% IPP into monoterpenols could be processed under optimum conditions to give incorporations of 6·8 ± 0·3 and 11·9 ± 0·2% respectively. Such incorporations are unprecedented for monoterpene biosynthesis from C_5 or C_6 precursors but were far from a sum of the individual improvement factors for each parameter that was optimised.

Products from cell-free systems. The fractions separated and assayed in the preceding sections were shown by radioscanning of TLC chromatograms prepared with several solid phases and eluant systems to be almost entirely (>96%) nerol and geraniol. It was not feasible to derivatise and purify to constant specific radioactivity the products from each experiment, but in three cases (two using the standard system and one the optimum system) in which incorporations of tracer (as assayed after purification by GLC and TLC, see Experimental) were 1·9, 2·6 and 11·9%, the purified monoterpenes were converted into aldehydes and solid derivatives of these were recrystallised to constant specific radioactivity. In each example the specific activity of the highly purified products was >98% of that of the routinely-purified materials. Thus significant contamination of the geraniol and nerol obtained in our routine assays can be ruled out.

The proportions of geraniol and nerol formed in cell-free systems prepared at different times in the growing season are in Table 6. The ratio of products varied up to 28-fold under conditions where controls showed no detectable interconversion of the alcohols or their phosphate and pyrophosphate esters. Thus geraniol and nerol were formed directly from C-5 precursors rather than nerol arising by isomerisation of geraniol, as has been presumed in some biogenetic schemes [14], and the varying proportions of products suggest the occurrence of two distinct prenyltransferases rather than one enzyme that can bind the two C-5 moieties in different relative orientations at one or more active sites [cf. 30–31].

The products from experiment 2, Table 6, were completely analysed. The 'free lipid' and 'pyrophosphate' fractions (see Experimental) comprised 42 and 10% of the tracer from IPP-[4-^{14}C] and the balance was water soluble. The recovered tracer (after esterification and combination of the fractions) was distributed in isopentenol (40·7%), 3,3-dimethylallyl alcohol (0·3%), dimethylvinylcarbinol (0·4%), nerol (2·5%) and geraniol (0·4%). No linalol could be detected and little (<0·4%) tracer occurred in the chromatographic fractions containing sesqui- and di-terpenes. (Control experiments showed that there was negligible (<0·1%, if any) conversion of GPP and NPP in the presence of IPP into sesqui- or di-ter penes in these cell-free extracts.) This lack of formation of higher terpenes was general in our experiments and may reflect the small quantities (<0·2% of the essential

Table 6. Proportions of geraniol and nerol formed in standard cell-free systems from *T. vulgare*

System	1	2	3	4	5	6	7	8
% C-10*	3·1	2·9	1·6	0·19	0·11	0·15	0·13	0·19
Nerol/geraniol	7·0	6·3	1·3	0·34	0·97	0·85	0·32	0·25

* Incorporation of IPP-[4-^{14}C] into geraniol + nerol (total).

Table 7. Effect of inhibitors of IPP-isomerase on incorporation of IPP into geraniol and nerol in standard cell-free systems from *T. vulgare*

Inhibitor	CFS-1†		CFS-2†	
	+(Biogel-P2)	−(Biogel-P2)	+(XAD-4)	−(XAD-4)
SKF 525A (1 mM)*	120	105	146	133
SKF 3301A (1 mM)*	124	123	—	—
Iodoacetate (5 μM)	97	93	111	100
V_2O_5 (5 to 500 μM)	66	85	—	—

* Kindly donated by Smith Kline & French Ltd., Welwyn Garden City, Herts. † Two cell-free systems were prepared using the standard procedure. One (CFS-1) was divided and the Biogel-2 column step was either used or omitted. The other (CFS-2) was similarly treated for a Dowex XAD-4 column step. The figures quoted are activities relative to controls (≡100) that were similarly treated except that the inhibitor was omitted. Details of substrate, incubation, assay etc as in Table 2.

Table 8. Degradation of monoterpenols produced from IPP-[4-^{14}C] by cell-free systems from *T. vulgare*

System*	% Inc†	Product‡ dpm/mmol	Degradation products§ dpm/mmol
CFS-2	1·3	Geraniol 17810	Levulinic acid 11560 (65); Acetone 6080 (34); Oxalic acid 0 (0)
CFS-2	1·0	nerol 18600	Levulinic acid 12630 (68); Acetone 6150 (33); Oxalic acid 0 (0)
CFS-4 ("standard")	1·8	nerol 1275	Levulinic acid 1251 (98); Acetone 0 (0); Oxalic acid 0 (0)

* For preparation of cell-free systems, see Experimental. † Percentage incorporation of tracer into geraniol + nerol. ‡(±5%): products were purified as semicarbazones of the corresponding aldehydes. Values of specific activities are not comparable in different experiments as different quantities of precursor and carrier were used in each case. §(±5%) Products, see Fig. 2 and in brackets, % tracer in each. All values are independent determinations. Degradations carried out by Drs. G. N. J. Le Patourel and O. Ekundayo (UCL).

oil) of sesquiterpenes or diterpenes in *T. vulgare*. Prenyltransferases purified from yeast [32], liver [33–34], pumpkin [35] and microorganisms [36] formed considerable amounts of farnesol and geranylgeraniol from C-5 and C-10 precursors, sometimes to the virtual exclusion of geraniol and nerol. About 6% of the administered tracer in this experiment, and up to 15% in others, was located in an extractable polar compound that cochromatographed on GLC and TLC with isopentenol oxide (3-methyl-3,4-epoxy-butan-1-ol). We have evidence [37] that a salvage mechanism exists in a variety of Compositae which, in its first step, epoxidises unphysiological amounts of applied precursors and foreign molecules: the operation of this process under natural conditions may explain the numerous terpene oxides that have been reported [38].

Effect of inhibitors of IPP-isomerase. The SKF drugs 525A and 3301A [39–41], vanadium pentoxide [42–43] and iodoacetate [43–44] are potent inhibitors of IPP-isomerase (E.C. 5.3.3.2.) in many plant and animal systems, but they had little effect (and that often stimulatory) on incorporation of tracer from IPP into geraniol and nerol in our standard cell-free system (Table 7), even when any endogenous DMAPP (or its biogenetic equivalent) was removed by passage through gel or ion-exchange columns. One explanation of these observations is that IPP-isomerase in *T. vulgare* is not susceptible to these additives. A more attractive possibility (see next subsection) is that a protein-bonded pool of DMAPP (or its equivalent) exists in the cell-free system, and this condenses with exogenous IPP in the presence of prenyltransferase to form monoterpenes.

Labelling patterns in biosynthetically-produced geraniol and nerol. Geraniol and nerol produced from IPP-[4-^{14}C] using the standard cell-free system and also cell-free system-2 [Table 1] were converted into aldehydes **3**, derivatised, and purified to constant specific radioactivity. The aldehydes were then partially degraded (Fig. 2) to locate the distribution of tracer. All degradation products were purified to constant specific radioactivity (by means of solid derivatives, if necessary) and an isotope balance in starting material and products was achieved (Table 8). The actual location of tracer was not revealed by this degradation scheme, but as it is extremely unlikely that breakdown of IPP and reincorporation of labelled fragments into monoterpenes could have occurred it is reasonable to assume that the C-5 unit(s) was incorporated intact. (This is supported by the absence of tracer in oxalic acid obtained on degradation of the monoterpenols, Table 8.) With this proviso, the results indicate a marked asymmetry of labelling, and in particular the nerol produced in the standard cell-free system contained essentially *all* (98%) the incorporated tracer in the IPP-derived moiety. This type of pattern has been found for the biosynthesis of many different types of monoterpenes *in vivo* from MVA, acetate and even carbon dioxide in a variety of plant species [14, 45] and has been attributed to the occurrence of (a) an endogenous pool of DMAPP or its biogenetic equivalent, (b) nonmevalonoid origin of the DMAPP-derived fragment, or (c) the existence of specific compartmentation effects [14]. The last factor is probably ruled out by the occurrence of the phenomenon in cell-free systems where structural organisation is largely absent, and our results were consistent with the first explanation embracing a protein-bonded pool of DMAPP (cf. the previous subsection: the use of a gel-filtration step in the preparation of the cell-free system would have removed free DMAPP or its biogenetic equivalent). The hemiterpenoid component of the pool could be of either mevalonoid origin or not.

MVA and various C-5 compounds as substrates for the cell-free system. Certain other potential substrates were screened as precursors of monoterpenols in a standard cell-free system (Table 9). Endogenous IPP and DMAPP were removed in the preparation of this system and so the negligible incorporation of DMAPP is consistent with the absence or inactivity of IPP-isomerase. However

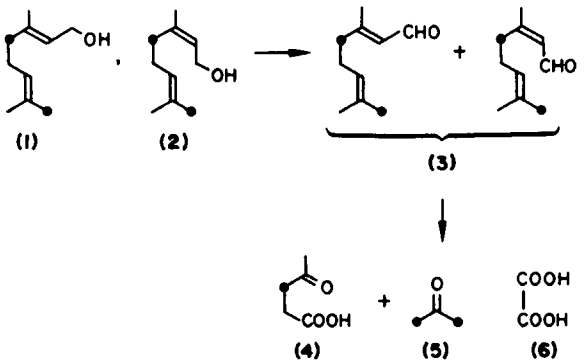

Fig. 2. Degradation of geraniol and nerol. ● = position of tracer expected if IPP-[4-^{14}C] is incorporated without scrambling.

Table 9. Screening of some potential substrates using the standard cell-free system from *T. vulgare*

Substrate (0·1 mM)*	% C-10†	Substrate (0·1 mM)*	% C-10†
IPP-[4-^{14}C]	2·1	DMVC-[Me-^{14}C]‡	4·6
DMAPP-[4-^{14}C]	0·0	IPP-[4-^{14}C] + DMVC-[Me-^{14}C]‡	3·1
IPP-[4-^{14}C] + DMAPP-[4-^{14}C]*	4·2	3R-MVA-[2-^{14}C]	0·2

* Mixtures of terpenes, 1:1 v/v. † Percentage incorporation into geraniol + nerol (±0·1%). Incubation and assay, as in Table 2. ‡ Dimethylvinylcarbinol (3-methyl-3-hydroxy-but-1-ene). Solubilised with Triton X-100 (1:1 v/v).

a mixture of DMAPP with IPP was incorporated more effectively than the latter and so exogenous DMAPP may be able to become part of the protein-bonded pool of DMAPP. Dimethylvinylcarbinol was two-fold more efficiently incorporated than IPP, under the same conditions and a mixture of this and IPP was also more effective as a precursor than was the latter alone. These intriguing results raise the question, that has never previously been defined, as to the role, if any, of dimethylvinylcarbinol in monoterpene biosynthesis. 3R-MVA is significantly incorporated into monoterpenols in the cell-free system designed for IPP as substrate, and so MVA-activating enzymes must be active in this preparation.

Optimum conditions for the use of MVA as substrate were not worked out but a standard cell-free system prepared just before the onset of flowering (i.e. conditions that gave most active preparations for utilisation of IPP) gave incorporations of 2·4% into geraniol and nerol. Addition of nonradioactive DMAPP and DMVC-PP to these systems strongly inhibited the uptake of 3R-MVA (Table 10), and a product analysis revealed three significant points. (a) The 3- to 4-fold reduction of incorporation into IPP suggests that MVA-activating enzymes were inhibited. (b) IPP-isomerase occurs in these systems, although it generally played no significant role in the biosynthesis of monoterpenols from IPP (see previous sections). The high IPP:DMAPP ratios in the inhibited systems may indicate that the additives inhibited this enzyme or may be a consequence of displacement of an equilibrium in favour of IPP. (c) There is a marked change in the proportions of geraniol and nerol due largely to inhibition of the synthesis of the former (inhibition of prenyltransferase by monoterpene pyrophosphates has been previously reported [46–47]). This is further evidence for the existence of two prenyltransferases in our system and may indicate that the geraniol synthesising enzyme is a control point in terpenoid synthesis.

Cell-free systems from flowerheads of T. vulgare. Tracer from MVA-[2-^{14}C] was incorporated into monoterpene-β-D-glucosides *in vivo* in flowerheads of *T. vulgare* considerably more effectively than into monoterpenes in leaves [48]: in general, petals may be very efficient sites of monoterpene synthesis [14, 49]. Results given in Table 11 compare the incorporations of IPP-[4-^{14}C] into monoterpenes in standard cell-free systems prepared from flowerheads and leaves of the same plants at 5 days after the onset of flowering: the bulk of the tracer (total 4·0%) incorporated into monoterpenes by the petal systems was in geranyl- and neryl-β-D-glucosides and the rest was in the free alcohols or their pyrophosphates. Total uptake was some 10-fold greater than in the corresponding leaf system.

Cell-free systems from etiolated specimens of T. vulgare. In order to determine if the activities of enzymes of monoterpene biosynthesis were enhanced during the greening period following etiolation, a specimen of *T. vulgare* was cut to ground level, maintained in darkness for 3 weeks, and the translucent shoots (10–15 cm.) produced were exposed to natural illumination and harvested at intervals for the preparation of the standard cell-free system. No significant differences from controls were found in the incorporations of IPP into monoterpenols (Table 12). The total recovery of tracer did con-

Table 10. Effect of pyrophosphates upon the incorporation of 3R-MVA into monoterpenes in the standard cell-free system from *T. vulgare*

Additive (1 mM)§	Activity recovered (%)*						
	DMAPP	IPP	DMVC-PP	G	N	Unidentified	Total
None	12·2	9·0	2·8	2·2	0·2	6·6	33
DMAPP	1·0	3·4	0·2	0·16	0·12	0·4	5·3
DMVC-PP	0·8	2·2	0·14	0·2	0·2	0·6	4·1

* G, N = geraniol, nerol. § For incubation methods, assay etc. see Table 2.

Table 11. Incorporation of IPP into monoterpenes in standard cell-free systems from flowers and leaves of *T. vulgare*

Product	% Incorporation*	
	Leaf system	Flower system
Geraniol + nerol	0·2	0·87
Geranyl + neryl pyrophosphates	0·2	0·44
Geranyl + neryl-β-D-glucosides	0·0	2·70

* Percentage incorporation of tracer under standard conditions. Assays, incubations etc. as in Table 2.

Table 12. Incorporation of IPP into monoterpenes in standard cell-free systems from etiolated specimens of *T. vulgare*

No. hr Greening	0	24	48	72	96	120	Control‡
% C-10*	3·8	4·1	2·7	2·5	3·2	3·7	3·6
%-extractable†	90	96	65	95	85	84	80

* % incorporation into geraniol + nerol; incubation, assay etc as in Table 2. † total % extractable tracer. ‡ Control: standard cell-free system.

Table 13. Cyclase activity in cell-free systems from *T. vulgare*

Substrate*	CFS†	Activites‡ (%) in Compound 7	8	9
α-Terpeneol-[^{14}C]	1-Fraction A	—	—	2·2
α-Terpeneol-[^{14}C]	1-(B + C)	—	—	1·8
α-Terpeneol-[^{14}C]	1-(A + B + C)	—	6·4	2·2
α-Terpeneol-[^{14}C]	2	94	0·0	0·0¶
Terpinen-4-ol-[^{14}C]	1-(A + C)	0·2	48	0·05
α-Terpineol-[^{14}C]	4("standard")	94	0·0	0·0
α-Terpineol-[^{14}C]	4(– Biogel-2 step)§	96	0·5	0·5
α-Terpineol-[^{14}C]	5	99	0·6	0·4
α-Terpineol-[^{14}C]	6	95	0·7	1·1
Terpinen-4-ol-[^{14}C]	6	0·5	88	0·4

* Substrates (0·1 μCi) solubilised with Triton X-100 (1:1:v/v) or DMSO (15% v/v). The solubilising agents did not effect the activity of controls. † For details of prep. of cell-free system, incubations and assays, see Experimental. ‡ % incorporations: α-terpineol (**7**); terpinen-4-ol (**8**); isothujone (**9**) (not all assayed for each system). ¶ Terpin hydrate (*cis*-1,8-dihydroxymenthane) (1% incorporation) was formed. § Gel filtration on Biogel-2 omitted in preparation of cell-free system.

siderably vary: detailed product analyses were not made but radioscanning of chromatograms indicated that a large portion (*ca* 40–60%) comprised unchanged IPP.

Formation of bicyclic monoterpenes in cell-free systems. Cyclase activity was monitored in a variety of cell-free preparations using α-terpineol-[9, 10-$^{14}C_2$] (*p*-menth-1-en-8-ol; **7**) and terpinen-4-ol-[7-^{14}C] (*p*-menth-1-en-4-ol; **8**) as substrates. Radioactivity passed into many products and only isothujone (*trans*-thujan-3-one, **9**; *trans* here refers to the disposition of the 1-isopropyl and 4-methyl groups [50]) and the two monocyclic alcohols were assayed. The ketone is the major monoterpene of *T. vulgare* whereas the latter (which are hypothetical precursors) are present in small quantities in the leaf oil [51]. In most incubations (Table 13), 0·5–2·0% of administered tracer was incorporated into isothujone and cyclase activity appeared to be associated with a particulate or subcellular fraction (cf. a similar location for the enzymes responsible for interconverting water-insoluble menthol derivatives in cell-free preparations from *Mentha piperita* [12]. The lower efficiency of terpinen-4-ol than α-terpineol as a precursor, despite it being further along the hypothetical but generally-accepted pathway to bicyclic terpenes [14], may indicate that the bicyclisation takes place at the site for binding of α-terpineol (or its biogenetic equivalent) and its interconversion to terpinen-4-ol: thus exogenously-supplied terpinen-4-ol may not be able to intervene effectively (Fig. 3).

The monocyclic alcohols and isothujone produced in these incubations were purified, after addition of carrier, on two TLC systems followed by GLC, but were not derivatised and purified to constant specific activity. Thus we claim their identification as tentative only, on par with claims for the formation of the bicyclic monoterpene α-pinene from cell-free systems of *Pinus* species [9, 11].

Methods for the preparation of IPP-[4-^{14}C]. The IPP used in these studies was obtained from 3RS-MVA-[2-^{14}C] by preparations from pig liver in which IPP-isomerase was either largely removed [52] or inhibited [34], or using latex from *Hevea brasiliensis* which does not contain this enzyme [53]. The methods are compared in Table 14: the last was the best and gave a 66% yield based on the utilisable isomer of MVA; the second provided an acceptable route to DMAPP, and all could be used to obtain quantitative recoveries of 3S-MVA.

EXPERIMENTAL

Materials. T. vulgare was grown from seed outdoors in Central London. 1-yr-old plants were cut to the ground in March and subsequently pruned at intervals during the growing season (March–October). For the determination of the seasonal

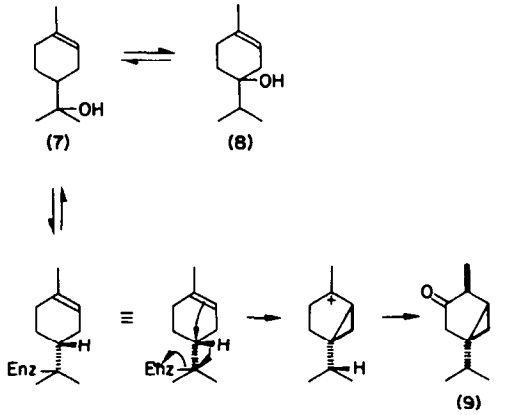

Fig. 3. Hypothetical biogenetic scheme of monoterpenes in *T. vulgare*.

Table 14. Comparison of enzymic preparations of IPP from 3RS MVA

Product	Method 1*	Method 2‡	Method 3§
MVA-lactone	9·8	2·1	3·4
3S-MVA	50·0	50·2	50·1
MVAP	37·5	20·9	10·2
MVAPP	0·3	4·7	0·6
IMP	0·0	0·0	2·2
IPP	4·3	2·0	33·0
DMAPP	0·0	19·1	0·0

* ex pig liver (ref. [52]), % yield. ‡ ex pig liver (ref. [34]), % yield. § ex *Hevea* latex (ref. [53]), % yield.

variation in enzyme activity (Fig. 1) a group of unpruned plants were used: otherwise, batches of young leaflets (10–25 cm) from different pruned plants were destalked and pooled to provide material for each enzyme preparation. Geraniol, nerol and isothujone (all > 99% pure by GLC and TLC on several systems) were available [45]. IPP-[4-^{14}C] was prepared from MVA-[2-^{14}C] (5 μmol; 50 μCi) using preparations from pig liver [34, 52] or serum solids from latex of *Hevea brasiliensis* [53]: the latter was kindly supplied by Dr. D. Barnard (Malaysian Rubber Producers Research Assocn, Hertford, England). These preparations also provided ^{14}C-labelled 3S-MVA, MVAP and DMAPP for use as enzyme substrates or inhibitors, but DMAPP was best prepared (80% yield) from IPP-[4-^{14}C] (10 μCi) using another fraction from pig liver homogenate [52]. This compound was unstable and was stored at −20° on the paper on which it had been purified by PC: when required it was eluted with 1% aq. NH_4OH. Dimethylvinylcarbinol-[Me-^{14}C] was synthesised by reaction of $(Me)_2$CO-[1.3-^{14}C] (0·1 mCi) with vinyllithium [54] and was pyrophosphorylated with Et_3N^+H $H_2PO_4^-$ and Cl_3CCN [55]. α-Terpineol-[^{14}C] and terpinen-4-ol-[^{14}C] were prepared as described previously [51, 56]. Apyrase was prepared from potatoes [57] and was assayed by a standard method [58]. Isopentenol oxide was made by treatment of isopentenol with 3-chloroperbenzoic acid–CH_2Cl_2 at −4° and was purified by TLC on Si gel H with EtOAc–C_6H_6 (1:5): R_f 0·19. NMR (60 MHz; 10% $CDCl_3$; TMS standard) τ 8·6 (*s*, 3H); 7·9 (*t*, 2H); 7·4 (*s*, 2H), 6·6 (*t*, 2H), 6·5 (*s*, 1H; removed by D_2O). MS and elem. analysis were consistent with structure proposed. Buffers were pH 5·0–6·0, succinate; 6·0–7·0 MES; 7·0–8·0 Pi or tris–HCl. Other compounds were the purest commercially available: unusual sources are given in the legends to the Tables.

Preparation of cell-free systems. In the following, leaves (50 g) were washed (1% aq. EDTA: H_2O) and stored at 0° for transport (1 hr) to the laboratory. All subsequent operations were performed at 4°. Protein assays were routinely made by a UV method [59] or more accurately by a modified Folin technique [60]. Chlorophyll was determined by a UV method [61]. Incubations were generally carried out at 25°/3 hr by which time the plateau of incorporation had almost always been achieved: reactions were then quenched, either by heating (100°/2 min) or by adding $HClO_4$ (1N; 0·5 ml) at 0° and extracting with hexane. Water-insoluble substrates were emulsified with Triton X-100 (1:1 v/v).

Cell-free system (CFS) 1. After homogenisation (90 sec) with phosphate buffer (pH 7·4; 0·1M; 100 ml) the product (fraction H) was filtered through muslin and centrifuged (200 *g*; 5 min) to give fraction D as the supernatant. This was centrifuged (1000 *g*; 15 min) and the pellet was suspended in the buffer (20 ml), centrifuged (1000 *g*; 15 min) and finally suspended in tris–HCl (pH 8·0; 0·067M; 200 ml) to give fraction A. The S^{1000} supernatant was recentrifuged (20000 *g*; 30 min) and the pellet was resuspended as above to give fraction B, and the S^{20000} supernatant was taken as fraction C. The protein concentrations of each fraction was 5 to 10 mg ml^{-1} and the chlorophyll concentration of fraction A was 2 to 5 mg ml^{-1}. *Aliquots of these fractions* (1·0 ml) were incubated (25°/3 hr) following a previous procedure [62] in tris–HCl (pH 8·0; 0·067M; 1·5 ml) with the ^{14}C-substrate (0·1 μCi), ATP (10 mM) coenzyme A (50 μM), NADPH (10 μM), glucose-1-phosphate (1 mM), $KHCO_3$ (30 mM) and $MnCl_2$ (2 mM).

CFS-2. Leaves were ground with solid CO_2 and then triturated with phosphate buffer (pH 7·0; 0·1M; 100 ml) containing 2-thioethanol (1 mM) and EDTA (0·2 mM). The product was filtered through glass wool and the filtrate was centrifuged (10^6 *g*; 90 min) and the supernatant was taken to 80% saturation with $(NH_4)_2SO_4$ and the ppt. spun off (4000 *g*; 20 min). After dialysis of the ppt. (vs Pi buffer, 24 hr), an aliquot (1·5 ml) was incubated with the ^{14}C-substrate (0·1 μCi), ATP (2 mM) and $MgCl_2$ (2·5 mM) in tris–HCl (pH 7·8; 0·050M; 2 ml).

CFS-3. Following the first step of CFS-2, the product was filtered to give a homogenate (fraction A') and this was then centrifuged to obtain supernatants S^{100000} and S^{100000} (fractions B' and C'). These were incubated as in CFS-2.

CFS-4 ("standard system"). The leaves were ground in liq. N_2 and stirred into MES buffer (pH 7·0; 0·1M; 100 ml) containing sucrose (0·6M), 2-thioethanol (redistilled; 1 mM), $Na_2S_2O_5$ (0·1M) and EDTA (0·2 mM). After being thawed, the mixture was filtered through glass wool and centrifuged (10000 *g*; 20 min). The supernatant was passed through a column (1 × 15 cm) of Biogel P-2 or (less usually) Sephadex G-25 and eluted with MES (pH 7·0; 0·05M). The protein forerun (35 ml; 2 mg ml^{-1}) was collected and a fraction (1·0 ml) was incubated with the ^{14}C-substrate (0·1 μCi), ATP (10 mM), $MgCl_2$ (40 mM), 2-thioethanol (20 mM) in MES (pH 7·0; 0·05M; 0·6 ml).

CFS-4 ("optimum system"). Leaves were vacuum-infiltrated with Pi buffer (pH 7·0; 0·1M, 100 ml) containing sodium diethyldithiocarbamate (1 mM) and 2-thioethanol (1 mM) and were then ground in liq. N_2 and stirred into MES buffer (pH 7·0; 0·1M; 100 ml) containing soluble PVP (1% w/v), EDTA (0·2 mM), sucrose (0·6 M) and $Na_2S_2O_5$ (0·1M). After being thawed and passed through glass wool, the filtrate was centrifuged (10000 *g*; 20 min) and the supernatant was adjusted (HCl; 0·1M) to pH 5·0 and left at 4° for 1 hr. Any ppt. was spun-off (bench centrifuge) and the pH of the supernatant was adjusted (0·1M NaOH) to pH 6·0 and a fraction (1 ml) was incubated with the ^{14}C-substrate (0·1 μCi), ATP (10 mM) and $MgCl_2$ (40 mM) in MES buffer (pH 6·0; 0·1M; 1 ml).

CFS-5. The leaves were ground in liq. N_2 and stirred into MES buffer (pH 7·0; 0·1M; 100 ml); the mixture was filtered and an aliquot (1·0 ml) was incubated with the ^{14}C-substrate (0·1 μCi), ATP (10 mM), $MgCl_2$ (40 mM), coenzyme A (50 μM) and NADPH (65 μM) in MES buffer (pH 7·0; 0·1M; 1·0 ml).

CFS-6. Leaves pulverised as in CFS-5 were stirred into Pi buffer (pH 7·4; 0·1M; 100 ml), the mixture was filtered through glass wool and the filtrate was centrifuged (20000 *g*; 15 min). The pellet, resuspended in the internal buffer as in CFS-1, was fraction A. The supernatant was recentrifuged (20000 *g*; 30 min) to give a pellet which was resuspended to give fraction B, and a supernatant, fraction C. These fractions (1 ml) were incubated with the ^{14}C-substrate (0·1 μCi), ATP (15 mM), $KHCO_3$ (15 mM), and $MgCl_2$ (1 mM).

The system for flowerheads was prepared as for CFS-4 (standard).

Product analysis. After the incubations had been quenched, they were extracted with Et_2O (3 ml × 3) to give the 'free lipid' fraction. The aq residue was boiled (3 min) and adjusted (Na_2CO_3–$NaHCO_3$) to pH 10·5, before being incubated (37°/2 hr) with apyrase and alkaline phosphatase (5 mg, Sigma). After quenching this second incubation (100°/2 min), the mixture was centrifuged (200 *g*; 2 min) and re-extracted with Et_2O (3 × 3 ml) to give the "pyrophosphate fraction". Controls showed that this cleavage procedure hydrolysed >99% of the monoterpene phosphates and pyrophosphates. Cell-free systems from leaves did not form terpene glucosides; those from flowerheads were further incubated with β-glucosidase [48]. Products in the combined ether extracts were routinely separated by a two-step TLC procedure: (1) on MgO with hexane–$(Me)_2$CO (1:1) and hexane which localised the C-5 and

C-10 compounds in the region R_f 0·3 to 0·5, followed by (ii) on Si gel H with 3 consecutive elutions with EtOAc–toluene (1:4) which separated geraniol and nerol (R_f 0·69) from hemiterpenols (R_f 0·62). Phosphomolybdic acid (5% aq) was used as spray and the chromatograms were developed by heating (100°/2 min). Extensive controls using GLC on two capillary columns (Carbowax 20M and SE-30; 50 m × 0·04 mm, both WCOT with FID), TLC on Si gel G and H, cellulose and MgO with various eluants, and 4π-radioscanning of chromatograms showed that the geraniol–nerol band contained essentially only (i.e. >98% chemically and radiochemically pure) these compounds. Usually this band was collected and assayed for incorporation of tracer. When the individual yields of the monoterpenes, or purifications to constant specific radioactivity were required, the mixture was further chromatographed on Si gel H and Si gel HF_{274} (2:1 w/w) with 4 consecutive elutions with EtOAc–hexane (15:85) to give geraniol (R_f 0·53) and nerol (R_f 0·59) which could be separately eluted. Further purification was by GLC with Carbowax 20M and FFAP (both 15% w/w on Chromosorb W; 5 m × 0·5 cm) programmed from 100° to 180° with FID. Controls enabled correction factors to be applied to any losses (usually 20–30% overall; mainly at GLC stage) in these purifications. In a few expts, geraniol and nerol thus purified were converted into aldehydes and these were purified to constant specific radioactivity as the semicarbazones, mp's 162 and 183° (ex H_2O. EtOH) [30].

Other procedures. Nerol and geraniol were partially degraded by methods that have been described in detail [30]. All products were purified to constant specific radioactivity if necessary by recrystallisation of appropriate solid derivatives, viz. levulinic 4-phenylsemicarbazone, mp 185° (ex H_2O–EtOH); acetone thiosemicarbazone, mp 179° (ex H_2O–EtOH); oxalic acid, mp 189° (ex EtOH–C_6H_6).

Radiochemical methods, counting statistics and errors were as described in related studies [45]. Typically, aliquots contained 500–1000 dpm. Reproducibility between duplicate assays was ±5% at most and usually better than ±2%. Thunberg tubes were used for studies of enzyme activities under anaerobic conditions. These were degassed, and refilled with N_2 in a 6-cycle-procedure.

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