A CONVENIENT ENZYMATIC PREPARATION OF
SPECIFICALLY-LABELLED GERANIOL

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SUMMARY

Geraniol was formed in high (up to 36%) yield when equi-molar quantities of isopentenyl and 3,3-dimethylallyl pyrophosphates (1 mmole) together with inhibitors of the isomerase that interconverts them were incubated with a strictly defined cell-free extract from flowerheads of *Rosa dilecta* cv. Lady Seaton. The C_5 units were incorporated *in toto* with essentially (>98%) complete position specificity. A simple route is thus available, given the appropriately labelled C_5 precursors, for the preparation of geraniol and thence by standard chemical routes of its isomers nerol and linalool and their pyrophosphates with any chosen carbon atom specifically labelled at isotope abundances suitable for biosynthetic studies. Similarly, any desired ^2H or ^3H labelled geraniol or its relatives could in principle be prepared with both regio- and stereo-specificity.

The roles of geranyl pyrophosphate (1a) and the corresponding esters of nerol (2) and linalool (3) as parents of the various classes of regular acyclic and cyclic monoterpenes, and that of the first as being in addition the initial building unit for the higher terpenoids, has led to numerous tracer studies involving these compounds as precursors. Usually $[1-^{14}C]$- and $[2-^{14}C]$- geraniol (1b) and nerol or their pyrophosphates have been employed as the alcohols are conveniently prepared thus labelled, e.g. by Reformatski or Wittig coupling of a radioactive C_2 unit with the appropriate C_8 ketone: but frequently the $[1-^{3}H]$- derivatives of 1b and 2 have been prepared (sometimes stereospecifically) by reduction of the corresponding aldehydes. However, it would often be convenient

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CHART 1

1a R = PP
1b R = H

2

3

4

5

6
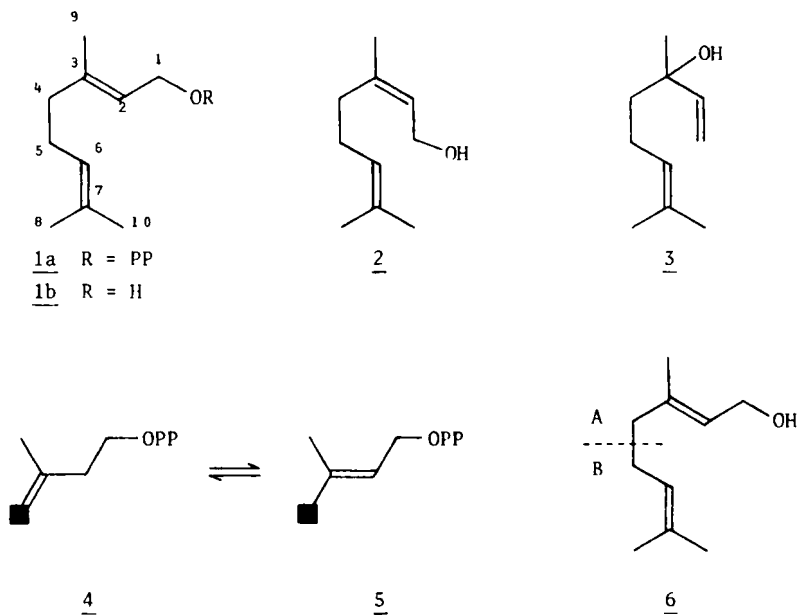
to have available other specifically-labelled precursors to allow particular
carbons to be followed through biosynthetic pathways, and enzymatic preparations
would seem the best approach to this end. Cell-free extracts from higher plants
generally convert mevalonate or the derived C_5 precursors, isopentenyl
pyrophosphate (4; IPP) and 3,3-dimethylallyl pyrophosphate (5; DMAPP), into geraniol
in very low (<1%) yields and preparations from mammalian liver similarly are
unsatisfactory as they result largely in sesquiterpenoids and higher homologues.
3R-Mevalonate is however incorporated in excellent (up to 24%) yields into geraniol
and nerol in flower-heads of *Rosa* species[1-3] when the C_5 moieties of the latter
derived from IPP and DMAPP (6; A and B respectively) are equally labelled with
tracer in a position-specific manner. Thus feeding of [2-^{14}C]-mevalonate led to
the isolation of [4,10-^{14}C$_2$]-geraniol that could be used in biosynthetic
investigations[4,5]. In order to obtain geraniol uniquely labelled at any *one* carbon
atom, the obvious approach would be to feed a 1:1 mixture of IPP and DMAPP (one of
which is appropriately labelled with tracer) in the presence of inhibitors of IPP
isomerase (isopentenyldiphosphate Δ-isomerase; EC 5.3.3.2), the enzyme that
catalyses the conversion 4 → 5 to give a mol. ratio (4:5) of approx. 1:9 at

equilibrium. However, preliminary studies involving feeding of $[1-^{14}C]$-IPP + DMAPP in the presence of such known inhibitors (see later) gave negligible incorporations into geraniol, no doubt owing to the failure of the ionic species to penetrate membranes to reach the sites of biosynthesis. Similarly, feeding of a mixture of the free C_5-alcohols, either in solution (solubility ca. 6 g/100 cm^3 water at 20°C) or emulsified, failed: here, the appropriate kinases required to generate the pyrophosphate esters may have been absent.

Consequently, attention was concentrated on developing a cell-free extract from flowerheads of *Rosa dilecta* cv. Lady Seaton that could efficiently carry out the desired condensations. The only related work is a report that a simple preparation from petals of *R. damascena* converted phenylalanine into 2-phenylethanol in ca. 30% yield[6]. Establishment of a successful cell-free system was dependent on the following methodology and procedures: (a) The physiological state of the flowerhead required to achieve maximum biosynthetic ability was crucial[3] (see Experimental). (b) Three days before harvesting, the flowerheads were removed and infiltrated with mevinolin (10^{-5} M). This known inhibitor of mevalonate synthesis[7] largely destroys the pool of this precursor and thus prevents formation of an appreciable pool of DMAPP (protein bonded?) that could dilute label in the DMAPP-derived moiety of geraniol, cf. refs 8,9. (c) One hour before preparation of the extract, the flowerheads were vacuum-infiltrated with sodium diethyldithiocarbamate (1 mM) and 2-thioethanol (1 mM) to maintain the prenyltransferase that catalyses the condensation in an active form. (d) Soluble PVP (polyvinylpyrrolidone)was added to the extract to complex phenolics and Amberlite XD-4 resin was employed to remove endogenous monoterpenes and pigments. And, (e), equimolar quantities of IPP and DMAPP were incubated in the presence of the drugs SKF-525A or SKF-3301A or of iodoacetate (all 1 mM) that are known to be potent inhibitors of IPP isomerase from pig liver[10].

Preparations based on the above protocol were used in incubations with different combinations of labelled precursors as shown in Table 1. The choices of locations of tracer in substrates were to ensure easy confirmation of position-specificity of incorporation. C-1 of IPP becomes C-1 of geraniol (or its pyrophosphate) and C-4 (in the E-methyl group) of DMAPP (which is known to be

derived from C-4 of IPP; see marked atoms in 4 and 5) becomes C-10 of geraniol[4];

and both C-1 and C-10 of geraniol are easily excised for radiochemical assay by

previously developed methods (see Experimental). Incorporations into geraniol

were 32-36% which resulted under our conditions in product of activity ca.

0.01 mCi mmole[-1]; and as expected, inhibition of the isomerase was essentially

complete and incorporation of tracer was position-specific. Advantages of the use

of cell-free systems rather than *in vivo* methods were that no monoterpenyl-β-D-

glucosides were generated in the former conditions (cf. refs 1,3); neither were

citronellol (the $\Delta^{2,3}$ reduction product of geraniol) nor higher terpenoids

produced. The products (after hydrolysis to cleave phosphate esters) comprised

solely geraniol (ca. 95%) and nerol (ca. 5%); it is not clear whether the latter

resulted from isomerisation of its first-formed isomer *in situ* or whether its

production was catalysed by a separate transferase.

Several chemical and enzymatic methods are available for step-wise synthesis

of the C_5-compounds[11-13], and given the availability of the appropriately-labelled

(^{14}C or ^{13}C) IPP or DMAPP use of our cell-free system will yield the

correspondingly labelled geraniol with position-specific incorporation of tracer.

Geraniol can be readily isomerised to nerol[14] and linalool[15], and all three

monoterpenes can in turn be efficiently (60-70%) converted into their

pyrophosphates[16]. Our system can thus be used for the preparation of a variety

of biosynthetic intermediates. In addition, it could be employed for the

stereospecific introduction of ^{3}H or ^{2}H from IPP or DMAPP derived from the

appropriate (R) or (S)-labelled mevalonate. The correlations between the pro-(R)

and -(S) hydrogens of mevalonate, IPP and DMAPP and those of geraniol have been

previously established[3,17].

EXPERIMENTAL

Materials. [1-^{14}C]-Isopentenol (ca. 0.5 mCi mmole[-1]) was prepared[18] by

decomposition of the Grignard reagent from 3-bromo-2-methylprop-1-ene with

[^{14}C]-CO_2 and was converted into the pyrophosphate in 52% yield[19].

(E)-[4-^{14}C]-DMAPP (labelled as in 5; ca. 0.1 mCi mmole[-1]) was prepared

enzymatically using a liver preparation[20] from [4-^{14}C]-IPP that had been prepared

in turn from $[2-^{14}C]$-mevalonate by means of a fraction from latex of *Hevea brasiliensis*[21].

Table 1:   Incorporation of C_5-precursors into geraniol in cell-free extracts of *R. dilecta*

Substrate[b]	% Incorporation[a,c]	Location[d]
$[1-^{14}C]$-IPP + DMAPP	32	99% at C_1
IPP + $[4-^{14}C]$-DMAPP	36	98% at C_{10}
Boiled control	0	-

(a)   S.D. for incorporation ±3% (actual value), for location ±1% (ditto).

(b)   Conditions as in Experimental Section. Extracts were preincubated with SKF-525A; SKF-3301A and iodoacetate gave similar results.

(c)   % Incorporation of tracer into geraniol (isolated after cleavage of any phosphate esters).

(d)   Location of tracer in geraniol.

Preparation of cell-free systems.   All operations were carried out at 4°C. Flowerheads of *R. dilecta* (3x; 6-10 g per head, including 2 cm stalk) were excised 5 days after initial splitting of the calyx, and were fed by the inverted-tube technique (time of uptake < 5 min) with a solution of mevinolin (10^{-5} M; DMSO-water 1:1 v/v; 0.1 cm^3). They were then maintained on sterile water for a further 3 days (to the "preperfect" stage, cf. Ref.1). The tissue was then vacuum-infiltrated with phosphate buffer (pH 7.0; 0.1 M; 150 cm^3) containing sodium diethyldithiocarbamate (1 mM) and 2-thioethanol (1 mM), and after an hour the whole tissue (stalk, petals, calyx) was harvested, ground in liq. N_2 and the resulting frozen powder was stirred into MES buffer (pH 7.0; 0.1 M; 100 cm^3) containing PVP (soluble form; 1% wt/wt), EDTA (0.2 mM), sucrose (0.6 M) and sodium sulphite (0.1 M). The slurry was allowed to thaw (ca. 30 min) and extracted with Et_2O (2 x 10 cm^3). Amberlite XD-4 resin (Na^+ form; 10 g) was then stirred into the aqueous fraction and after 10 min the mixture was centrifuged (10^3 g; 5 min) and the supernatant was taken to pH 5.0 (1 M HCl) and the

precipitate formed spun off (bench centrifuge) after 30 min. The pH was then

readjusted to 6.0 (0.1 M NaOH) to give a solution (protein conc., ca. 1 mg cm^{-3};

Lowry-Potty method) that showed unimpaired prenyltransferase activity for up to

12 hr when stored at 0°C.

Preparation, purification and assay of products. The above extract was pre-

incubated during a warming-up period from 0°C to 25°C (ca. 15 min) with the IPP-

isomerase inhibitors sodium iodoacetate, SKF-525A (β-diethylaminoethyl-

diphenylpropyl acetate hydrochloride) or SKF-3301A [2,2-diphenyl-1-(β-

dimethylaminoethoxy)pentane hydrochloride] (all 1 mM). An aliquot (20 cm^3) was

then mixed with MES buffer (pH 6.0; 0.1 M; 10 cm^3) at 25°C containing IPP and

DMAPP (154 mg, 0.1 mCi), ATP (10 mM) and MgCl$_2$ (40 mM) and the solution was

incubated at 25°C for 3 hr. After this period, pyrophosphate esters were cleaved

by sequential incubations with apyrase and alkaline phosphatase[9], protein was

precipitated with TCA (4%) and the free alcohols (from products and unreacted

substrates) were extracted with Et$_2$O (2 x 2 cm^3) after addition of geraniol

(500 mg) as carrier. The dried (Na$_2$SO$_4$) ethereal extract was reduced in volume

(to ca. 0.5 cm^3) in a stream of N$_2$ at 0°C (less than 10% of geraniol was lost

during this procedure) and the products (0.1 cm^3 aliquots) were separated by GC on

FFAP (10% wt/wt on 60-80 Chromosorb W; 2 m x 0.5 cm; N$_2$ flow 2 lhr^{-1}) at 100°C for

10 min, followed by a 100°C to 150°C programme over 30 min. Under these conditions

relative retention times of geraniol and nerol were 1.00 and 0.89, and collection

efficiencies (in Et$_2$O at 0°C) were > 80%. The geraniol was shown to be chemically

pure (>99%) by capillary GC on Carbowax 20M and SE-30 (50 m x 0.02 mm) or TLC on

SiGel H with a variety of eluants; and to be radiochemically homogeneous by 2π

radioscanning of appropriate TLC plates.

Tracer location in geraniol was demonstrated by previously described methods.

Thus that at C-10 was determined by SeO$_2$ oxidation of the biosynthesised product

to 10-hydroxygeraniol, oxidation to the C-10 acid and decarboxylation by the

Schmidt reaction[4]. That at C-1 was located by ozonolysis of geranial (derived

from the initial product) to yield oxalic acid, acetone, and laevulinic acid[3].

Tracer was located only in the first fragment and although this gives the total

radioactivity at (C-1 + C-2) of geraniol, there can be no doubt on biogenetic

grounds that the source of any tracer must be the former atom in the context of the present work. ^{14}C was estimated by LSC with butyl-PBD: 4×10^4 disintegrations were routinely accumulated so that 2σ was $\pm 1\%$.

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