Phytochemistry, 1977, Vol. 16, pp. 355–358 Pergamon Press. Printed in England.

EPOXIDATION AND HYDRATION OF PRENYL PYROPHOSPHATES BY PLANT EXTRACTS*

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(*Received* 15 *May* 1976)

Key Word Index—*Tanacetum vulgare*; *Artemisia annua*; *Santolina chamaecyparissus*; Compositae; cell-free extracts; prenyl pyrophosphates; epoxidation and hydration.

Abstract—Cell-free extracts of *Tanacetum vulgare, Artemisia annua,* and *Santolina chamaecyparissus* contain seasonally-dependent enzyme systems that convert IPP§, DMAPP, GPP and NPP into water-soluble products in up to 96% yield. 3-Methyl-3,4-oxidobutan-1-ol; 3-methylbutan-1,3,4-triol; 3,7-dimethyl-6,7-oxido-octa-*trans*-2-en-1-ol; and 3,7-dimethylocta-*trans,trans*-2,5-dien-1,7-diol were major products when IPP and GPP respectively were substrates and several other terpene epoxides and their ring-opened products were tentatively identified. 2-CEPA blocked formation of diols from the epoxides. The occurrence of these enzymes accounts for some hitherto puzzling observations that have arisen in studies of monoterpene biosynthesis both *in vivo* and *in vitro*.

INTRODUCTION

The preparation of cell-free extracts from higher plants that can sustain monoterpene synthesis is difficult and rarely has more than 1% of tracer from presumed C_5 or C_{10} precursors been proved to be incorporated into expected products [1]. The fate of the balance of tracer has usually passed without comment but perusal of procedures suggests that often much was converted into water-soluble products that were not phosphate esters and which could not be easily extracted into organic solvents [cf. 2]. During studies of monoterpene biosynthesis using extracts of *Tanacetum vulgare, Artemisia annua,* and *Santolina chamaecyparissus* (Compositae) we have frequently found that tracer from IPP-[^{14}C] and GPP-[^{14}C] passed into such products and we here report preliminary studies on the hitherto unstudied enzyme systems that mediate these processes. For brevity, we shall refer to these systems as 'salvage' enzymes; a possible rationalisation of this term is given below.

RESULTS AND DISCUSSION

Seasonal dependence and specificity. The seasonal variation in salvage enzyme activity using clonal material for each species is shown in Fig. 1. Reproducibility of the cell-free preparation was excellent; four independent preparations from *T. vulgare* (April 1973) gave 42, 38, 39 and 38% incorporation into water-soluble materials. Boiled-enzyme controls over the yearly period gave less than 2% (if any) such incorporation. This activity profile and recently reported variations in activity of geraniol (3,7-dimethyl-octa-*trans,trans*-2,6-dien-1-ol) synthetase [1], phosphatases [3] and alcohol oxidase [4] in *T. vulgare* illustrate the importance of selection of the correct season for enzyme studies on higher plants.

Acetone powders from *T. vulgare* retained their activity with IPP as substrate after storage at −20° for several months, but activity towards GPP (which in freshly-prepared extracts followed a time-course similar to that in Fig. 1) was lost within a few days.

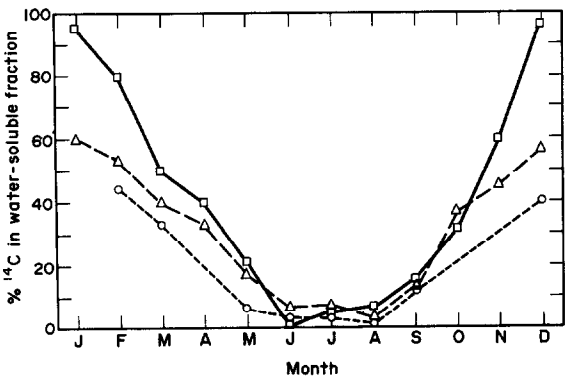

Fig. 1. Seasonal Variation of Salvage Enzymes, IPP as substrate, J to D represents January to December. The % in the water-soluble fraction is the tracer that can not be extracted into organic solvents after the initial incubation with the cell-free preparation and subsequent incubation with phosphatases (see Experimental). % incorporation is ±*ca* 2% for *T. vulgare*, and ± 6% for *A. annua* and *S. chamaecyparissus*. —△— *T. vulgare*; ---○--- *A. annua*; —□— *S. chamaecyparissus*.

* Part 18 of series 'Terpene Biosynthesis'. For part 17, see Banthrope, D. V., Doonan, S. and Gutowski, J. (1977) *Phytochemistry* **16**, 85.

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§ Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; LPP, linalyl pyrophosphate; 2-CEPA, 2-chloroethylphosphonic acid.

At the time of maximum salvage activity (Jan. 1974), preparations from *T. vulgare* gave the following incorporations of tracer from [^{14}-C]-labelled substrates into the water-soluble fraction under standard conditions: IPP 96; DMAPP 88%; GPP 80%; NPP 72%; LPP 51%; 3-methylbutan-1-ene-3-ol 3%; *cis-trans*-chrysanthemyl pyrophosphates (esters of *cis* and *trans*-2,2-dimethyl-3(2-methylpropenyl)cyclopropylmethanol) 0; artemisyl alcohol (3,3,6-trimethylheptan-1,5-dien-4-ol) 0; α-terpineol (*p*-menth-1-en-8-ol) 0: terpinen-4-ol (*p*-menth-1-en-4-ol) 0%. Isopentenol (3-methylbutan-3-ene-1-ol) and geraniol were only metabolised 25 and 15% under comparable conditions. Less extensive assays on systems from the other plant species gave similar patterns of specificity. The observations have only qualitative significance as IPP-isomerase (E.C. 5.3.3.2) together with kinases and phosphatases of undetermined specificity were present in the crude preparations.

Products. Isolation of the extremely hydrophilic products from IPP presented difficulty (see Experimental) but incubations using extracts of *T. vulgare* prepared in the period near maximum salvage activity resulted in the recovery of considerable tracer (*ca* 40%) in salvage products and the bulk of this (60%) was in 3-methyl-3,4-oxidobutan-1-ol and 3-methylbutan-1,3,4-triol (9%). These products were purified and characterised (IR, NMR, GC–MS). Part of the remaining products (8%) was tentatively identified as 3-methyl-2,3-oxidobutan-1-ol, 3-methylbutan-1,2,3-triol and 3-methylbutan-1,3-diol by comparison (TLC) with products of epoxidation and subsequent ring-opening of 3-methylbutan-2-en-1-ol. The same pattern (TLC) of salvage products resulted when extracts from the other plant species were employed.

Incubation of GPP with extracts from *T. vulgare* yielded salvage products that were partially extractable from salt-saturated aqueous solutions. In a large-scale incubation (30% incorporation into salvage products), the bulk of the water-soluble fraction comprised 3,7-dimethylocta-*trans,trans*-2,5-dien-1,7-diol (40%) and 6,7-oxidogeraniol (22%; both identified by IR, NMR, GC–MS). Over a dozen other metabolites were detected and tentative identifications (TLC) were made of 2,3-oxidogeraniol; 2,3-6,7-dioxidogeraniol and products of their ring-scission (total 23%).

Reincubation of mixtures of salvage products with various cell-free preparations from *T. vulgare* [cf. 1] provided no evidence that any of the remaining unidentified fractions were protein-bonded intermediates of terpenoid biosynthesis and likewise there was no evidence for terpenoid-sugar conjugates that could be dissociated by pyrogallol or dimethylsulphoxide [cf. 5,6]. Up to 5% (but typically <1%) of the water-soluble tracer obtained after initial incubations could be cleaved on treatment with β-glucosidase [E.C. 3.2.1.2.1] to liberate labelled material with the characteristics (GLC, TLC) of monoterpenes. α-Glucosidase was uneffective under similar conditions. Terpene-β-glucosides occur in petals of *T. vulgare* [1, 7] but have not been reported in foliage.

Properties of salvage systems. Exploratory studies of the salvage system for IPP in extracts from *T. vulgare* indicated that: (a) the MW range was above 150000 daltons; (b) activity was not located in unique soluble or particulate fractions; (c) optimum activity was at pH 6.8 to 7.8 falling to 50 and 10% at pH 5.0 and 8.5 respectively; (d) Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} (0–100 mM) or (e) EDTA and 1:10 phenanthroline (0.1–10 mM) or (f) KF, KCN, V_2O_5, iodoacetate, mepyrone and the SKF drugs S25A and 3301A (0.1—10 mM) had insignificant effects. Some of the last group are inhibitors of previously studied epoxidases and epoxide hydratases [8–10].

Two treatments did affect the salvage systems. Firstly, addition of 2-CEPA (> 5 mM) to extracts from all three plants completely suppressed ring-opening of epoxides and led to the accumulation of 3-methyl-3,4-oxidobutan-1-ol. 2-CEPA has a variety of physiological effects on higher plants that have been attributed to the ethylene formed from it *in vivo* [11] and may be the result of the chelating attributed properties of the latter. This seems unlikely in the present case [cf. (e) above], and one of the many other decomposition products [11] may be responsible for the effects in our systems. Secondly, large scale incubations (IPP, 0.5 g) under N_2 gave negligible incorporations of tracer into salvage products. This effect was not observed in routine assays (IPP-[^{14}C], *ca* 1 μg) presumably owing to sufficient endogenous O_2 being present (protein or carrier-bonded?) to consume the substrate.

General. The cell-free preparations containing salvage enzymes also converted IPP into GPP and NPP in up to 12% yield [1]. Although the latter synthetase activity was at a maximum [1] when the salvage activity was very low, some attempts were made to inhibit or remove the latter in order to increase synthetase activity in off-peak preparations. Treatment of the crude preparation with $(NH_4)_2SO_4$ or centrifugation (up to 100000*g*) failed to locate salvage activity in any particular fraction. Similar results have been obtained for aldrin epoxidase from *Pisum sativum* [12–14] and mammalian epoxide hydratases [15] and may indicate the existence of several related enzyme systems, partial solubilisation of particulate enzymes or non-specific complexing. More sophisticated separation techniques led to loss of both synthetase and salvage activity.

Addition of 2-CEPA did not increase the percentage incorporation of tracer from IPP into monoterpenes, the 'salvage' fraction was now comprised of essentially only epoxides rather than a mixture of these with diols and triols. More surprisingly, large scale incubations under anaerobic conditions eliminated the salvage activity but had little effect on incorporation into monoterpenes. Thus substrate may have been irreversibly bonded to components of the salvage enzyme complex but not further metabolised under these conditions. A variety of possible competitive inhibitors of the salvage enzymes were explored but were ineffective. However, addition of 3,3-dimethylacrylate (5 mM) almost doubled the percentage conversion of IPP into GPP and NPP but the reason is unknown. On addition to our previous procedure [1] for the optimum preparation of geraniol synthetase this additive ensured a conversion of *ca* 24% of IPP into the monoterpenyl pyrophosphates.

Although salvage enzymes for hemi- and monoterpenes have hitherto not been investigated in plants, epoxides of certain monoterpenes occur naturally [16, 17] and have been proposed as possible intermediates for cyclic monoterpenes [18]. Epoxides of higher terpenoids are involved in cutin biosynthesis [19] and in the violaxanthin cycle [20], and several epoxidases are concerned in the detoxification of pesticides [12]. Enzyme systems that epoxidise and subsequently cleave cytotoxic metabolites are well known in mammalian preparations [21–25] and in microorganisms [9, 18,

18a]. The corresponding enzymes in *T. vulgare* may be a salvage system to regulate unphysiological levels of monoterpene precursors or (in view of the seasonal variation) to convert monoterpenes into water-soluble products for transport and subsequent degradation [cf. 26, see also 18, 18a, 26a]. The general occurrence of such salvage systems could account for hitherto puzzling observations obtained for monoterpene biosynthesis in a variety of higher plants incubated *in vivo* with ^{14}C-labelled MVA, IPP etc. Thus it was found [18, 27, 28] that monoterpenes biosynthesised in such systems and purified by GLC (and in some cases TLC) were heavily contaminated with unidentified labelled compound(s) and repeated recrystallisation of appropriate solid derivatives to constant specific radioactivity was essential to obtain meaningful results [28]. We suggest that this contamination arises from breakdown on GLC of labelled epoxides, diols etc., and in support of this we have found that both ^{14}C-labelled 3-methyl-3,4-oxidobutan-1-ol and 6,7-oxidogeraniol thus decompose in typical purification conditions to give products that bleed from the column over a range of retention volumes encompassing those of many monoterpenes while the epoxides themselves bleed from the column in a broad plateau. The suggestion that epoxides and their derived products are salvage products from exogenous precursors added in unphysiological amounts is supported by our finding that isothujone (*trans*-thujan-3-one; the main monoterpene of *T. vulgare*) produced *in vivo* from ^{14}C-labelled MVA, IPP, DMAPP, GPP or NPP is heavily contaminated by extraneous tracer after purification by GLC, whereas such contamination is negligible when the compound is biosynthesised from CO_2-[^{14}C] supplied at the physiological concentration of the gas.

EXPERIMENTAL

Materials and methods. Plants were cultivated outdoors during April to November and indoors for the remainder of the year under natural light at 20° in a bright south-facing aspect. Sources of plants, preparation of ^{14}C-labelled substrates, radiochemical and chromatographic techniques etc. were as previously described [1, 29].

Preparation of cell-free systems. Leaves were stored (*ca* 1 hr) at 0° after harvesting before further processing and all subsequent operations were performed at 4°. Typically a sample (10 g) was washed (1% aq. EDTA; H_2O), pulverised in liquid N_2 and then stirred into 20 ml MES (0.1 M; pH 7.0) containing sodium metabisulphate (0.01 M) and sucrose (0.3 M). After the mixture had thawed at 4° (*ca* 0.5 hr), it was passed through glass wool and filtrate was centrifuged (10000 *g*; 20 min). The supernatant was desalted (Biogel P2 column, 25 g) and equilibrated with MES (0.01 M pH 7) containing 2-mercaptoethanol (1 mM) to give a soln (15 ml) containing 5–10 mg ml^{-1} protein [30] that was used in subsequent assays. Large-scale preparations for the isolation of products utilised leaves (250 g) homogenised as above in (500 ml Pi buffer; 0.1 M; pH 7.0) with addition of sodium metabisulphite (0.01 M), sucrose (0.4 M) and $MgSO_4$ (40 mM). Me_2CO powders were prepared in a few cases by standard procedures [31] and stored at −20°; they were reconstituted by stirring the powder (12 g) into 25 ml MES (0.05 M; pH 7.0) containing sucrose (0.6 M), sodium metabisulphite (0.01 M), EDTA (0.2 mM) and 2-mercaptoethanol (1 mM) and after leaving at 4° (1 hr), filtering and centrifuging (10000 *g*; 20 min). The acetone powders contained particulate fragments etc. It was found by chance that sucrose in the reconstituting medium consistently gave solns of improved (up to 50%) enzymatic activity.

Assay procedure. The enzyme soln (1 ml) was added to 1 ml MES (0.1 M; pH 7.0) containing 2-mercaptoethanol (1 mM), $MgSO_4$ (40 mM) and ATP (2 mM) and was incubated with ^{14}C-labelled substrate (0.1 μCi) at 25° for 3 hr. Water insoluble substrates were solubilised with Triton X-100 (1:1). Reaction was stopped either by heating (100° for 2 min) or by addition of $HClO_4$ (1 N; 0.5 ml) at 0° and products were extracted with Et_2O (3 × 1 ml) and C_6H_{12} (2 × 1 ml); controls showed that these methods of stopping the reaction did not affect the products. The aqueous residue was adjusted (Na_2CO_3–$NaHCO_3$) (31a] to pH 10.5, reincubated with phosphatase and apyrase as previously described [1] and extracted with Et_2O and C_6H_{12} The residual tracer in the aq. fraction represented salvage products: such products from IPP and DMAPP were only difficultly extractable with organic solvents from $(NH_4)_2SO_4$-saturated solns although with GPP as substrate some 80% of tracer could be recovered under these conditions. Autoxidation is a bane of terpene chemistry and for all substrates boiled-enzyme controls were made on incubation mixtures and corresponding controls were made. In no case did a control represent more than 2% of the tracer that remained in the aq. fraction, usually the value was <1%.

Product analyses. Preparations from large batches (250 g) of foliage were incubated with isopentenol or geraniol (0.5 g) to which isopentenol-4-[^{14}C] or geraniol-1-[^{3}H] (5 μg; 0.05 μCi) had been added as tracer together with Triton X-100 (0.5 ml) as solubiliser After solvent extraction and cleavage of phosphates (as described above) different procedures were employed for products in the two cases. For the isopentenol incubation, the aq. residue (*ca* 500 ml) was carefully distilled and the forerun (40 ml) was mixed with a slight excess of Plaster of Paris at 4°. After solidification and pulverisation, the product was extracted with MeOH (48 h) in a Soxhlet apparatus: by this procedure a satisfactory (*ca* 60%) recovery of tracer from the aq. phase was possible. The products were separated by TLC on Si gel H with EtOAc–toluene (1:4). The main components (R_f 0.18 and 0.81) were eluted with D_2O-$(CD_3)_2CO$ (1:1) and identified by comparison of NMR and GC–MS with authentic specimens of 3-methylbutan-1,3,4-triol and 3-methyl-3,4-oxidobutan-1-ol that had been prepared by epoxidation of isopentenol with *m*-chloroperbenzoic acid and ring-opening of this respectively. Three minor components (R_f 0.00, 0.32 and 0.75 with the EtOAc–toluene system) were tentatively identified by their co-chromatography with 3-methylbutan-1,2,3-triol, 3-methylbutan-1,3-diol and 3-methyl-2,3-oxidobutan-1-ol in 6 TLC systems (Si gel H; eluted variously with EtOAc–C_6H_6–toluene). The standards were prepared by epoxidation and subsequent hydrolytic and reductive ring-cleavage of 3-methylbutan-2-en-1-ol: all had spectra consistent with the proposed structures. Products from large-scale incubation of geraniol were more easily extracted (3 × 200 ml, Et_2O) and were separated (TLC) MgO with C_6H_{12}–Me_2CO (1:1) and Si gel H with toluene–EtOAc (1:1). The main components were shown to be 3,7-dimethylocta-*trans*,*trans*-2,5-dien-1,7-diol and 6,7-oxidogeraniol by NMR and GC–MS and in the latter case by comparison with an authentic standard. Using samples of 2,3-oxidogeraniol and 2,3,-6,7-dioxidogeraniol [32] co-chromatography on several TLC systems indicated that these compounds were formed in the incubations as minor products. Several other products co-chromatographed with components of mixtures prepared by hydrolytic and reductive ring-cleavage of the three geraniol epoxides. Attempts to convert the mixtures of epoxides into more-readily identifiable olefins by treatment with 3-methylselenoxobenzothiazole [33] yielded intractable products. Assays for terpene-β-glucosides in the incubation mixtures were as previously described [7].

Acknowledgements—We thank the S.R.C. for awards to G.A.B. and M.G.R. We also thank Dr E. Cardemil, Messrs K. G. Allen and P. Steele for carrying out certain experiments.

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