Phytochemistry, Vol. 22, No. 11, pp. 2465–2468, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00

METABOLISM OF LINALOYL, NERYL AND GERANYL PYROPHOSPHATES IN *ARTEMISIA ANNUA*

DEREK V. BANTHORPE, PAUL N. CHRISTOU, CHRISTOPHER R. PINK* and DAVID G. WATSON

Department of Chemistry, University College, London WC1H OAJ, U.K.

(*Revised received* 14 *April* 1983)

Key Word Index—*Artemisia annua*; Compositae; biosynthesis; monoterpenes; monoterpenyl pyrophosphates.

Abstract—The effectiveness of incorporation of radioactivity from ^{14}C-labelled monoterpenyl pyrophosphates into isothujone, 1,8-cineol and α-pinene formed in excised leaves of *Artemisia annua* was: linaloyl pyrophosphate (LPP) > neryl pyrophosphate (NPP) > geranyl pyrophosphate (GPP). In cell-free extracts that sustained synthesis of α-pinene the same order occurred, but this was an artefact as inhibition of endogenous phosphate-cleaving enzymes led to the reverse sequence. Consequently, previous conclusions drawn from measurements of the relative efficiencies of the esters as precursors for cyclic monoterpenes (which indicated LPP to be the preferred substrate) may be invalid. Simple, but effective, modifications of previous procedures have led to the preparation of hemi- and mono-terpenyl phosphates and pyrophosphates in good (20–50%) yields in excellent (> 99%) purity. Loss of ^{3}H from C-1 of GPP and NPP occurred on storage—reasons for this unexpected exchange are discussed.

INTRODUCTION

GPP* and NPP have been generally considered to be obligatory precursors for the biosynthesis of cyclic monoterpenes in higher plants. The role of LPP, although often discussed ([1] and refs. cited therein), has not been defined until recently. In this latter work, LPP has been convincingly demonstrated as a direct precursor of bornyl pyrophosphate in *Salvia* species as a result of detailed enzymic studies [2]; and in certain *Mentha*, *Perilla* and *Citrus* species the preferential incorporation LPP > NPP > GPP both *in vivo* and in cell-free extracts into various cyclic monoterpenes was considered to support the direct incorporation of LPP into products without the necessary formation of either GPP or NPP [3]. However, in *Salvia* species, phosphatases are present which show differing activities towards monoterpenyl pyrophosphates and phosphates [4] and these, if of general occurrence, may interfere both *in vivo* and *in vitro* with the efficiencies of incorporations of these precursors into derived monoterpenes. In the present work, we measured the relative efficiencies of incorporations of the three monoterpenyl pyrophosphates into certain monoterpenes by excised shoots and cell-free extracts of *Artemisia annua* L. in order to determine if the incorporation pattern found in the *Mentha* and other species could be repeated.

RESULTS AND DISCUSSION

Relative incorporation of monoterpenyl pyrophosphates, etc.

Our specimens of *A. annua* yielded a volatile oil (2.8%, wt:wet wt) comprising artemisia ketone (39%; 3,3,6-trimethylheptan-1,5-dien-4-one), borneol (15%; boran-2-ol), pinocamphone (15%; *trans*-pinan-3-one), 1,8-cineol (12%; *p*-menthan-1,8-oxide); α-pinene (8%; pin-2-ene), β-pinene (1%; pin-2(10)-ene) and isothujone (10%; *trans*-thujan-3-one). Percentage incorporations of tracer from ^{14}C-labelled mono- and pyro-phosphates into isothujone, 1,8-cineol and α-pinene in excised shoots are recorded in Table 1. All products were rigorously purified to constant specific radioactivity by recrystallization (of solid derivatives, if necessary) and all feeding conditions were carefully standardized for comparison purposes. The incorporations were typical of those found in comparable experiments with other species. Incorporations into the main component of the oil, artemisia ketone, were not determined, as this irregular monoterpene has been shown not to be derived from the usual regular C_{10}-precursors [5]. Position-specific incorporation of tracer from precursor to products was not demonstrated, but previously under very similar conditions of feeding excised shoots of *Artemisia annua* with 2-^{14}C-labelled geraniol or nerol, such specificity of incorporation into isothujone, pinocamphone and borneol was proved [5].

A cell-free system based on that previously developed for the synthesis of artemisia ketone by *A. annua* [6] was also employed. This only effectively synthesized α- and β-pinenes from the C_{10}-pyrophosphates with less than 0.01% incorporation into the other monoterpenes: the incorporations are recorded for the former isomer. β-Pinene was not purified to constant specific activity, but incorporations were at least some 3-fold lower.

These observations show that, for both feeding to excised shoots and to cell-free extracts, the order of efficiency of precursors was: LPP > NPP > GPP. The corresponding phosphates appeared to show a similar order from the limited data available. Incorporations of the free alcohols were irregular and irreproducible in their order but were generally some 10-fold lower than the

*Abbreviations: LPP, linaloyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; LP, linaloyl phosphate; GP, geranyl phosphate; NP, neryl phosphate.

Table 1. Relative efficiencies of C_{10}-phosphate esters as precursors of monoterpenes in *A. annua*

Material	Precursor*	Monoterpene (%)†		
		Isothujone	1,8-Cineol	α-Pinene
Excised shoots	LPP	0.8	6.3	3.2
	NPP	0.3	2.1	0.8
	GPP	0.1	1.3	0.5
Excised shoots	LP	0.4	1.3	1.5
	NP	—	—	0.8
	GP	0.1	0.4	—
		α-Pinene	α-Pinene + F^-‡	
Cell-free extracts	LPP	9.3	7.0	
	NPP	2.1	21.3	
	GPP	0.3	26.5	
Cell-free extracts	LP	0.1	0.1	
	GP	0.1	0.0	

*Concentrations, incubation conditions, etc. as in Experimental.
† %Incorporation of ^{14}C from precursor (s.d. ± 5% in duplicate matched runs).
‡F^- (2 mM) added, conditions as in Experimental.

incorporations of the phosphates. Thus the pattern of relative incorporations obtained by Suga *et al.* [3] was obtained. Some believe that isoprenoid phosphate esters cannot be transported into plant cells and that hydrolysis precedes cellular uptake of the terpenoid moiety. There is no experimental evidence whatsoever on this point, but if it were true, results of *in vivo* studies of relative incorporations of monoterpenyl (and other isoprenoid) phosphate esters would provide no index of the efficiency of these compounds as precursors (however, in practical terms, the use of the water-soluble esters rather than the insoluble alcohols as prospective precursors has obvious advantages). This possible conceptual objection has no relevance to the *in vitro* studies.

However, a set of experiments was carried out using the cell-free extracts as before but with the addition of F^-. This has been shown to be the best reversible inhibitor of many studied for endogenous phosphatases and pyrophosphatases from *Tanacetum vulgare* and at 2 mM in a standard extract reduced (by > 95%) phosphatase activities towards a variety of hemi- and mono-terpenyl phosphate esters, without, however, affecting the other enzymes of terpenoid metabolism [7]. The presence of F^- (2 mM) gave the results in Table 1, and at 50 mM essentially (variation ± 10%) the same results were obtained: thus confirming the lack of effect of the additive on the terpene synthetase. The order of efficiency of incorporation of the C_{10}-pyrophosphates was now reversed to: GPP > NPP > LPP. Results with the monophosphates were not clear-cut. An obvious explanation of this reversal is that the plants and their extracts contained phosphatases and/or pyrophosphatases that selectively, perhaps specifically, destroyed NPP and GPP rather than LPP, and that the 'true' order of efficiency of precursors could only be determined when those systems were inhibited: i.e. in cell-free extracts. The presence of these phosphatases in our crude extracts was demonstrated by direct assay. Thus, apyrase-type activity (to cleave the terminal phosphate from pyrophosphate) was present with relative values for hydrolysis of substrates: GPP (100), NPP (83), LPP (9): and direct phosphatase activities were: GP (100), NP (93), LP (80). All these activities were effectively (> 90%) inhibited by F^- (2 mM).

Thus, the observation that LPP is the most effective precursor of monoterpenes in uninhibited systems may lead to biosynthetic deductions that are not valid in plant systems generally. Indeed (especially for *in vivo* studies) the situation is so complex with different (albeit related) substrates perhaps possessing both differing ease of access to the biosynthetic sites and different susceptibilities with respect to phosphatase and kinase systems, and also being accepted to different extents by terpene synthetases and cyclases, that the drawing of valid general conclusions from simple measurements of relative efficiencies of incorporation seems unlikely.

Preparation and assay of terpene pyrophosphates and phosphates

Almost all reports of the preparation of terpenyl pyrophosphates use di-triethylamine phosphate in trichloroacetonitrile as the phosphorylation agent but there is a bewildering variety of methods for working up and purifying the crude products. Analytical, spectroscopic and chromatographic characterizations of products have almost always been glossed over or omitted and the situation for particular compounds has often been considered unsatisfactory [8–12]. The C_{10}-pyrophosphates used in our assays were prepared by the usual phosphorylation [13, 14] followed by a four-stage purification involving paper chromatography, electrophoresis and crystallization [6]: this gave acceptable (typically 25%) yields of satisfactorily pure LPP, GPP and NPP and the corresponding monophosphates. In our hands this procedure was superior to recipes (particular for various terpenyl esters) involving different phosphorylating agents [15] or purifications on silica gel [16, 17], amberlite resins [17], DEAE-cellulose or Sephadex [18, 19]. However, our methods were time-consuming and, by small, but vital modifications of published procedures for conditions of

phosphorylation, recrystallization of the mono- and pyrophosphates, and ion exchange on Dowex X-8 (the last step has previously been applied to the purification of farnesyl pyrophosphate [20]) we have found a simpler procedure for certain C_5-, C_{10}- and C_{15}-alcohols that is probably widely applicable. Details are in the Experimental: yields were excellent (for the type of reactions); thus for pyro- and mono-phosphates of geraniol (35, 45%); nerol (48, 30); linalool (42, 40); citronellol (50, 22); farnesol (35, 30); isopentenol (30, 26); and 3,3-dimethylallyl alcohol (30, 25). All products were characterized by homogeneity on PC and TLC; by C, H, P and phosphate analyses; and by ^{1}H NMR and ^{31}P NMR spectroscopy. All were highly (> 99%) pure, save the esters of linalool and the pyrophosphates of the two C_5-alcohols which were less pure (> 97%). An excellent method for assay of mixtures of mono- and pyro-phosphates was by ^{31}P NMR at 80.98 MHz with proton-decoupling. The former esters gave a singlet at *ca* δ 4.4 (80% aqueous H_3PO_4 as external standard; δ 0.0), whereas the pyrophosphates gave doublets (J = 22 Hz) at *ca* δ 5.5 and 9.5. Spectra were pH-dependent and optimum resolution was at 8.6. In contrast, the phosphorylating agent (di-triethylamine phosphate) gave a signal at δ 0.72.

The ^{14}C-labelled C_{10}-pyrophosphates prepared in these studies are known to decompose in the solid state and in frozen solutions [18] and are best stored in dilute ammonia at 4°: under these conditions they are claimed to be stable for several weeks and the specific radioactivity is practically unchanged. Specimens of initially highly purified [1-^{3}H$_1$]GPP that had been prepared from chemically and radiochemically pure (> 99%) [1-^{3}H$_1$]geraniol were found, after storage for 2–3 months under the latter conditions, to be contaminated with up to 25% NPP, and also to have suffered a loss of specific radioactivity by up to 90%. The same phenomenon was found for NPP and here GPP was the contaminant. There is a precedent for these unexpected results: [1-^{3}H$_1$]farnesyl pyrophosphate was found to lose up to 50% of its tracer (as indicated by a change in ^{3}H/^{14}C ratio) on storage, and this 'perplexing' result was tentatively attributed to capture of the allylic cation by a nucleophile other than phosphate by a process exhibiting a negative ^{3}H-isotope effect [20]. Another explanation that accommodates both isotope loss from C-1 and the isomerization is that the allylic ion cyclized to a cyclopropyl carbocation that could undergo hydride shift, elimination to a cyclopropene derivative, or ring-opening, all in equilibrium. Such a scheme has been suggested to account for isomerization of 2-*trans*-farnesyl pyrophosphate into its 2-*cis*-isomer in the course of sesquiterpene biosynthesis [21].

EXPERIMENTAL

Materials. A. annua was cultivated as described [5]. [2-^{14}C]Geraniol (126 μCi/mmol), [2-^{14}C]nerol (126 μCi/mmol) and [1,2-^{14}C$_2$]linalool (150 μCi/mmol) were available [5].

Preparation of pyrophosphates and phosphates. The standard phosphorylation method [13, 14] was used with the following modifications: (a) The reagents were in the ratio substrate–trichloroacetonitrile-di-triethylamine phosphate, 1:9:3. (b) The first two were stirred (0°, 1 hr) before addition of the phosphorylating agent. (c) The latter was added (4 hr) and the reaction continued for 24–48 hr. Longer reaction times led to the formation of predominately polyphosphates. Progress of the reaction was monitored by ^{31}P NMR spectroscopy. The purification procedure was: (a) rotary evaporation (35°) to give a slurry; then 30 min further (b) fractional crystallization of the cyclohexylamine salts (cf. [22]); (c) conversion into Li salts (LiCl) and ion-exchange chromatography on Dowex 50W-X8 (NH_4^+ form; 20–50 mesh) with 0.08 M NH_4^+ formate–MeOH. Elution times were irreproducible in different runs on the same product, and the retention position was determined by assaying the eluant from the column [PC, Whatman 3M with *i*-PrOH–NH_4OH–H_2O (8:1:1) with detection by acid molybdate spray (5% aq. soln)]. Storage properties of products are given in Discussion. Products had the expected elemental analysis (C, H, P) phosphate content [23] and showed the expected ^{1}H NMR and ^{31}P NMR spectra (see Discussion). The latter spectra were recorded on a Varian XL200 spectrometer at 80.98 MHz.

Feeding experiments. Feeding of precursors to excised shoots of *A. annua* were as described [5]. Cell-free preparations were obtained as have been described in great detail [6] for the formation of irregular monoterpenes from C_5-precursors. Work-up and preliminary preparation of isothujone, α-pinene and 1,8-cineol were as before [5, 6]. Partially purified isothujone was recrystallized to constant specific radioactivity as its 4-phenylsemicarbazone, mp 184° (*ex* Me_2CO; aq. EtOH); 1,8-cineol was purified similarly as its adduct [24] with 2-chlorophenol, mp 58° (*ex* petrol, bp 60–80°); and α-pinene was converted into its adduct with 2-mercaptoacetic acid and this was derivatized as the *S*-benzylisothioronium chloride, mp 162° (*ex* Me_2CO; MeOH). Radiochemical assays were conventional using butyl-PBD in toluene as scintillant. Typically, aliquots (5–10 mg) containing 5 $\times 10^2$–10^4 dpm were assayed. 4×10^4 disintegrations were accumulated so that 2σ was ± 1%.

Acknowledgements—We thank the SRC for grants to C.R.P, D.G.W. and P.N.C., and also Dr. Stephanie A. Branch for help with some experiments.

REFERENCES

1. Charlwood, B. V. and Banthorpe, D. V. (1979) *Prog. Phytochem.* **5**, 65.
2. Croteau, R. and Karp, F. (1979) *Arch. Biochem. Biophys.* **198**, 512.
3. Suga, T., Shishibori, T. and Morinaka, H. (1980) *J. Chem. Soc. Chem. Commun.* 167.
4. Croteau, R. and Karp, F. (1979) *Arch. Biochem. Biophys.* **198**, 523.
5. Banthorpe, D. V., Charlwood, B. V., Greaves, G. M. and Voller, C. M. (1977) *Phytochemistry* **16**, 1387.
6. Banthorpe, D. V., Doonan, S. and Gutowski, J. A. (1977) *Phytochemistry* **16**, 85.
7. Banthorpe, D. V., Chaudhry, A. R. and Doonan, S. (1975) *Z. Pflanzenphysiol.* **76**, 143.
8. Crombie, L., Firth, P. A., Houghton, R. P., Whiting, D. A. and Woods, D. K. (1972) *J. Chem. Soc. Perkin Trans.* 1, 642.
9. Heinstein, P. L., Herman, D. L., Tove, S. B. and Smith, F. H. (1970) *J. Biol. Chem.* **245**, 4658.
10. George-Nascimento, C. and Cori, O. (1971) *Phytochemistry* **10**, 1803.
11. Battersby, A. R., Brown, R. T., Knight, J. A. and Plunkett, A. D. (1966) *J. Chem. Soc. Chem. Commun.* 346.
12. Tidd, B. K. (1971) *J. Chem. Soc. B* 1168.
13. Cramer, F., Ruttersdorf, W. and Bohm, W. (1962) *Annalen* **654**, 180.
14. Popjak, G., Cornforth, J. W., Cornforth, R. H. C., Ryhage, R. and Goodman, D. S. (1962) *J. Biol. Chem.* **237**, 56.
15. Rapi, G., Chelli, M. and Ginanneschi, M. (1978) *J. Chem. Res. (Suppl.)* 461.

16. Gafin, Y. and Schecter, I. (1979) *Analyt. Biochem.* **92**, 248.
17. Cornforth, R. H. and Popjak, G. (1976) *Methods Enzymol.* **15**, 359.
18. Vial, M. V., Rojas, C., Pontilla, G., Chayet, L., Perez, L. M., Cori, O. and Bunton, C. A. (1981) *Tetrahedron* **37**, 2351.
19. Dugan, R. E., Rasson, E. and Porter, J. W. (1968) *Analyt. Biochem.* **22**, 249.
20. Mackie, H. and Overton, K. H. (1977) *Eur. J. Biochem.* **77**, 101.
21. Evans, R. and Hanson, J. R. (1976) *J. Chem. Soc. Perkin Trans.* 1, 326.
22. Edmonds, J., Popjak, G., Wong, S. M. and Williams, V. P. (1971) *J. Biol. Chem.* **246**, 6254.
23. Marsh, B. B. (1959) *Biochim. Biophys. Acta* **32**, 357.
24. Dodge, F. D. (1933) *J. Am. Pharm. Soc.* **22**, 20.