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Isopentenyl Diphosphate is the Terminal Product of the Deoxyxylulose-5-Phosphate Pathway for Terpenoid Biosynthesis in Plants.

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Abstract: Secretory cells specialized for monoterpene biosynthesis via the deoxyxylulose-5-phosphate pathway for terpenoid biosynthesis were isolated from peppermint (*Mentha piperita*) leaves and used to identify the terminal metabolite of this novel pathway. Incorporation of both [^{13}C]- and [^{14}C]pyruvic acid into monoterpenes in the presence of 2-(dimethylamino)ethyl diphosphate, an inhibitor of isopentenyl diphosphate isomerase, results in accumulation of isopentenyl diphosphate, and not dimethylallyl diphosphate.

Keywords: terpenoid biosynthesis, isopentenyl diphosphate isomerase, deoxyxylulose-5-phosphate, isopentenyl diphosphate, dimethylallyl diphosphate, 2-(dimethylamino)ethyl diphosphate

Until recently, the classical mevalonic acid pathway employed for cholesterol biosynthesis in animals was widely assumed to be universally responsible for the production of terpenoids throughout Nature. However, [^{13}C]-labeling studies, initially with eubacteria, and subsequently with higher plants, have unambiguously established the existence of a non-mevalonate pathway for terpenoid biosynthesis [1]. In higher plants, this mevalonic acid-independent pathway is responsible for the synthesis of monoterpenes, diterpenes and tetraterpenes (including phytol and carotenoids) [1]. The initial enzymatic step of this newly discovered pathway is the condensation of glyceraldehyde-3-phosphate with a C_2-unit derived from pyruvic acid, to yield deoxyxylulose-5-phosphate (DXP) [2] (Figure 1). A subsequent reductoisomerase step yields 2-*C*-methylerythritol-4-phosphate [3]. The intermediates and enzymatic steps beyond 2-*C*-methylerythritol-4-phosphate are still uncharacterized and there is no reason to assume *a priori* that isopentenyl diphosphate (IPP), and not dimethylallyl diphosphate (DMAPP), is the terminal product of the pathway. IPP isomerase is a reversible enzyme which interconverts IPP and DMAPP [4], and is the first enzyme in common between the mevalonic acid and DXP pathways. 2-(Dimethylamino)ethyl diphosphate (DMAEPP)[5], a transition-state analog for IPP isomerase, was used to perturb the flux of intermediates through the DXP pathway in order to force accumulation of the terminal metabolite which immediately precedes IPP isomerase.

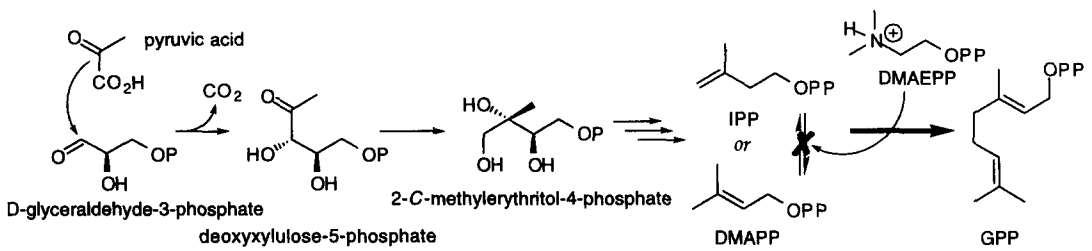

Figure 1. The DXP pathway for terpenoid biosynthesis via geranyl diphosphate (GPP) showing inhibition of IPP isomerase by DMAEPP.

Previous studies have established that secretory cells isolated from glandular trichomes of peppermint (*Mentha piperita*) are capable of efficiently synthesizing the monoterpenes and sesquiterpenes characteristic of the essential oil of this plant from radiolabeled substrates such as [^{14}C]pyruvic acid, that the cytoplasmic MVA pathway is inoperative in these cells, and that the IPP used for monoterpene biosynthesis is derived exclusively from a plastid-localized biosynthetic pathway [6]. Subsequent labeling experiments with [^{13}C]glucose established that the monoterpenes produced in peppermint, as well as a variety of other essential oil-producing plants, are derived from the plastid-localized DXP pathway [7]. Several features of peppermint secretory cells make this an ideal model system for identifying potential intermediates of this novel pathway. The secretory cells are terminally differentiated and

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highly specialized for the synthesis of monoterpenes. The isolated cells are non-specifically permeable to low molecular weight (<~1000 Da) water-soluble species as a consequence of the anatomy of the oil glands, and the method of their isolation [8]. As a result, the cytoplasm of the isolated cells is depleted of endogenous metabolites, and the composition of cofactors and substrates in the cytoplasm is determined by the buffer in which the cells are suspended. This feature consequently facilitates uptake of metabolic inhibitors into the cytoplasm, although uptake into the plastid may still be restricted.

DMAEPP was synthesized by a literature method as previously described [5]. Secretory cells were isolated from peppermint leaves and incubated with cofactors as previously described with only minor modifications [6]. Under these conditions, [^{14}C]pyruvic acid is very efficiently incorporated into monoterpenes. Isolated secretory cells were incubated with DMAEPP for 15 min at room temperature in an assay volume of 1 ml. The cells were subsequently washed into fresh buffer containing the same concentration of cofactors and DMAEPP, and pyruvic acid was added to initiate the reaction. The cells were aerated for 45 min at room temperature before quenching the assay with 9 M urea. After urea extraction, phosphorylated metabolites were adsorbed onto an anion exchange resin [9], the resin was washed with water to remove uncharged species, and anionic metabolites were subsequently eluted from the resin with 1 M triethylammonium (TEA) bicarbonate. The resulting eluate was passed through a cation exchange resin that was pre-equilibrated with TEA, in order to remove any residual Na^+ or K^+ which would interfere with subsequent analysis by atmospheric pressure ionization electrospray mass spectrometry (API/MS). The samples were concentrated to dryness under vacuum at room temperature, redissolved in a minimum volume of water and analyzed by high performance liquid chromatography (HPLC) using a TEA bicarbonate/acetonitrile gradient on a C_{18} column interfaced with an API/MS operated in the negative ion mode. Mass isotopomer analysis of the prenyl diphosphates was carried out by selected ion monitoring (SIM) for each prenyl diphosphate (e.g. DMAPP/IPP at 245, 246, 247). The remaining sample was overlaid with pentane and enzymatically hydrolyzed to the corresponding prenyl alcohols with alkaline phosphatase and apyrase. The pentane extract was subsequently analyzed either by radio-gas chromatography (GC) with a wide-bore capillary column [6] or by GC/MS.

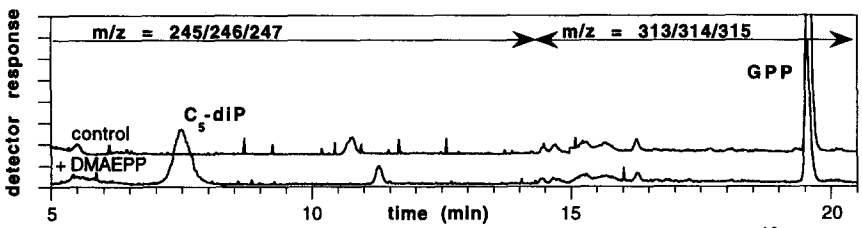

Figure 2. HPLC/API/MS (SIM) analysis of prenyl diphosphates derived from incubation with [2-^{13}C]pyruvic acid.

The first experiment utilized a mixture of [2-^{14}C]- and [2-^{13}C]pyruvic acid (0.4 mM each) as tracer. Control incubations in the absence of DMAEPP demonstrated a substantial accumulation of the monoterpene precursor geranyl diphosphate (GPP), but no IPP or DMAPP was detected (Figure 2). Isotopic enrichment of GPP was assessed by comparing the ion intensities at M, M+1, and M+2 with the corresponding ions measured with an authentic standard of unlabeled GPP. Significant enrichment of both the M+1 and M+2 ions was observed, demonstrating incorporation of the labeled pyruvic acid (Table 1). Enrichment of the M+2 peak is due to the presence of two ^{13}C-labeled C_5 units derived from IPP (see below), and to the presence of an isotopomer derived from the [2-^{14}C]pyruvic acid. Parallel incubation in the presence of 1.3 mM DMAEPP resulted in a 50% decrease in the total amount of GPP accumulated, with a pronounced accumulation of a putative C_5 diphosphate exhibiting significant labeling. Comparison with authentic standards indicated that chromatographic resolution under these conditions was insufficient to unambiguously identify the product as either IPP or

DMAPP. Hydrolysis of the remaining sample, with subsequent analysis of the ^{14}C-labeled products by radio-GC, revealed radiolabeled isopentenol, but not dimethylallyl alcohol (results not shown), thereby confirming that the product observed by HPLC/MS at m/z 245-247 was IPP and not DMAPP.

Table 1. Isotopomer intensities for IPP and GPP determined by HPLC/API/MS with SIM analysis.

	IPP @ m/z 245/246/247[1]		GPP @ m/z 313/314/315	
Substrate	control	+ DMAEPP	control	+ DMAEPP
[2-^{14}C]+[2-^{13}C]pyruvate	ND[2]	100:30.7:11.3	100:46.5:21.8	100:47.6:21.7
[^{12}C]pyruvate	-[3]	100:6.0:2.7	100:13.4:3.4	100:13.0:3.4
[1-^{13}C]pyruvate	-	100:7.4:4.0	100:11.3:4.3	100:10.5:3.3
[2-^{13}C]pyruvate	-	100:66.0:7.0	100:100:42.6	90.1:100:46.1
[3-^{13}C]pyruvate	-	100:69.0:7.1	93.2:100:46.2	88.7:100:43.3

[1] Natural abundance for IPP is 100:6.0:2.0, natural abundance for GPP is 100:10.1:2.1

[2] ND: not detectable

[3] detectable, but not quantifiable due to low abundance and the presence of overlapping peaks

The second experiment utilized either [^{12}C], [1-^{13}C], [2-^{13}C], or [3-^{13}C]pyruvic acid as substrate (1.6 mM) in the presence of 1.9 mM DMAEPP. With the higher concentration of pyruvate used in this experiment, low levels of IPP and/or DMAPP could be detected by HPLC/API/MS in control incubations, although the insufficient chromatographic resolution and low amount prevented accurate quantification of the isotopomer ratios. In the presence of DMAEPP, large amounts of IPP accumulated. As expected, little enrichment was observed from [1-^{13}C]pyruvate, as C1 of pyruvate is lost during biosynthesis of DXP, whereas both [2-^{13}C] and [3-^{13}C]pyruvate gave equivalent levels of enrichment. In the case of GPP, the much higher enrichment observed at M+1 and M+2 in the second experiment, compared to the first, can be attributed to the higher total concentration of labeled substrate and the lack of dilution of the ^{13}C-isotopomer by either the ^{12}C or ^{14}C isotopomers present in the [^{14}C]pyruvate. Unlike GPP, the IPP which accumulates shows much lower enrichment of the M+2 peak, demonstrating the incorporation of only a single labeled pyruvate molecule into IPP, as expected for the DXP pathway [10]. After HPLC/API/MS analysis, the samples were enzymatically hydrolyzed and subjected to GC/MS analysis (Figure 3). Although quantification of the relative amounts of isopentenol and geraniol was not possible due to significant evaporative losses during sample preparation, dimethylallyl alcohol was not observed in the reaction products as evaluated by GC/MS. Product identification by both retention time and spectral matches demonstrated elevated levels of isopentenol in the enzymatic hydrolysates from DMAEPP-treated cells.

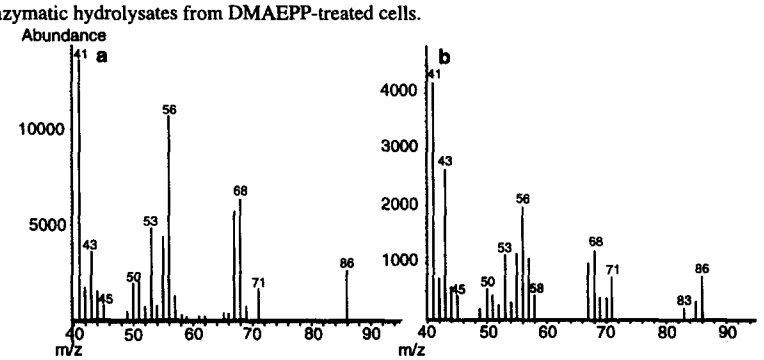

Figure 3. GC/MS characterization of isopentenol accumulated in response to treatment with DMAEPP. **a.** Mass spectrum of authentic isopentenol at retention time 9.51 min. **b.** Isopentenol at 9.49 min derived from [^{12}C]pyruvate.

Under the conditions of these experiments, the balance between GPP synthesis by the DXP pathway and consumption by

monoterpene biosynthesis allows the accumulation of a pool of GPP which is heavily labeled by exogenous pyruvate. The glyceraldehyde-3-phosphate required for DXP synthesis is formed from sucrose and glucose-6-phosphate in the incubation buffer [6]. Inhibition of IPP isomerase by the transition state analog inhibitor DMAEPP results in a large increase in the levels of the terminal metabolite of the DXP biosynthetic pathway immediately preceding IPP isomerase. The IPP isomerase of peppermint is assumed to kinetically resemble the enzyme from other plant sources in which equilibrium lies in the direction of DMAPP formation [4]. Although DMAEPP is effective as an inhibitor *in vitro* at sub-nanomolar concentrations [5], the elevated concentrations required to effect inhibition of IPP isomerase in this study are likely due to the limited uptake into the plastid of this substrate analog (assuming the IPP isomerase of peppermint is a typical enzyme of this class). The accumulation of [^{13}C]-enriched GPP observed indicates that IPP isomerase is only partially inhibited by DMAEPP, allowing continued synthesis of GPP, albeit at a reduced rate. Preliminary software modeling [11] of carbon flux through IPP isomerase, GPP synthase and limonene synthase (the major enzyme responsible for conversion of GPP to monoterpenes in peppermint) using available kinetic data for these enzymes [4,12,13] indicates that, under conditions in which all three enzymes have similar levels of flux, both IPP and DMAPP remain at very low levels while GPP can accumulate to significant levels. Thus, inhibition of IPP isomerase can result in accumulation of IPP, without greatly perturbing GPP levels. If DMAPP is the final product of the DXP pathway, the only conditions in which IPP would accumulate in response to treatment with DMAEPP would be if the equilibrium of IPP isomerase lay in the direction of IPP formation, and the inhibitor was acting primarily on GPP synthase. This possibility is considered unlikely. Detailed time-course analysis of labeling of endogenous pools of IPP and GPP, and comparison with kinetic models, should further clarify carbon flux control through the DXP pathway. The kinetic details not withstanding, analysis of the products from DMAEPP-treated cells clearly establishes IPP and not DMAPP as the terminal product of the DXP pathway.

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10. Assuming no significant gluconeogenic flux from pyruvate to glyceraldehyde-3-phosphate, both C2 and C3 of pyruvate will contribute 1 carbon each to IPP via the DXP pathway. Incorporation via the mevalonic acid pathway would result in C2 of pyruvate contributing 2 carbons, and C3 contributing 3 carbons to IPP.
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