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deoxyxylulose phosphate pathway**



สำนักงานสภาวิจัย

Biosynthesis of plaunotol in *Croton stellatopilosus* proceeds via the deoxyxylulose phosphate pathway

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Received 25 November 2004; revised 5 January 2005; accepted 21 January 2005

Abstract—Plaunotol, an acyclic diterpene alcohol that accumulates in the chloroplasts of *Croton stellatopilosus* leaves, was studied for its origin of isoprene units. Separate feedings of [U-¹³C]glucose and [1-¹³C]glucose into cut shoots of the plant showed that the labels from glucose were incorporated into plaunotol. NMR analysis of the purified plaunotol revealed that all four isoprene units of the molecule had the same labeling pattern which is consistent with exclusive acquisition via the deoxyxylulose phosphate pathway. The results of in vivo feeding experiments suggested that there was no significant metabolite exchange taking place between the mevalonate pathway and the deoxyxylulose pathway during the biosynthesis of plaunotol in this *Croton stellatopilosus*.
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For the biosynthesis of terpenoids in higher plants, it has been proposed for more than a decade that their constituent isoprene units can originate via two distinct biosynthetic pathways: the mevalonate (MVA) pathway and the deoxyxylulose phosphate (DXP) pathway.^{1,2} These two pathways are believed to have different sub-cellular localization and different contributions to the biosynthesis of various terpenoid groups.^{3–7} The MVA route has been reported to operate in the cytoplasm for the formation of sterols, triterpenes, and sesquiterpenes whereas the alternate DXP pathway occurs in the chloroplasts for the formation of terpenoids required for photosynthetic machinery (carotenoids, phytol, prenyl chain of plastoquinone) and various groups of mono- and diterpenoids.⁸ This compartmentation is, however, not always clear cut based on observations in various labeling experiments.^{9–11} It has been suggested that exchange of common intermediates such as isopentenyl diphosphate, geranyl diphosphate, and farnesyl diphosphate might occur between the two compartments as described by the ‘Cross-Talk Theory’.^{12,13} For this theory, clear data are still needed on the sites

of biosynthesis of plant terpenoids and the degree of metabolite exchange between the two sites.

Among various plant diterpenoids, plaunotol (1, Fig. 1A) which accumulates in the leaf of *Croton sublyratus* Kurz¹⁴ (subsequently identified as *Croton stellatopilosus* Ohba¹⁵) is considered as a good model for studying the degree of exchange of intermediates between the MVA and the DXP pathways in plants. Plaunotol is a simple

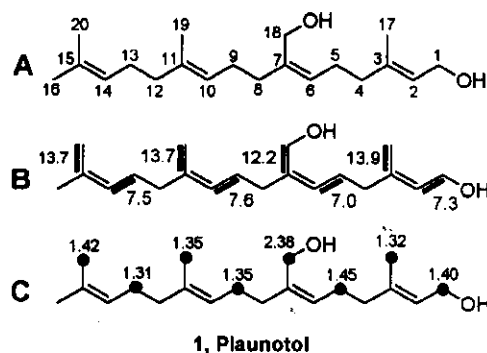


Figure 1. (A): the structure of plaunotol; (B): ¹³C-labeling patterns of plaunotol obtained from the [U-¹³C]glucose feeding experiment, bold lines indicate ¹³C-labeled with adjacent ¹³C atoms; (C): ¹³C-labeling patterns of plaunotol obtained from the [1-¹³C]glucose feeding experiment, filled circles indicate ¹³C-enrichment (>1.30% ¹³C).

Keywords: *Croton stellatopilosus*; Euphorbiaceae; Terpenoid biosynthesis; Feeding experiments; Plaunotol.

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linear acyclic diterpene alcohol in which its basic structure of four isoprene monomers is still maintained as original individual isoprene units. This makes interpretation on the origin of the isoprene units very simple based on the results of labeling patterns in the compound. In addition, plaunotol has been previously shown to accumulate mainly in chloroplasts, which is the same location as the DXP pathway.¹⁶ With this close connection in the same organelle, it was of interest to perform feeding experiments and observe the degree of intermediate exchange during plaunotol biosynthesis in this plant.

In this study, separate-feeding of [U-¹³C]glucose and [1-¹³C]glucose into cut shoots with 3–4 leaves of *C. stellatopilosus* were carried out according to a method described previously.^{17,18} The shoots were cut from a six-month old plant and immediately immersed into one of the following solutions: solution 1 contained 1% (w/v) unlabeled glucose; solution 2 contained 0.95% (w/v) unlabeled glucose and 0.05% (w/v) [U-¹³C]glucose (99% ¹³C enrichment); solution 3 contained 0.5% (w/v) unlabeled glucose and 0.5% (w/v) [1-¹³C]glucose (99% ¹³C enrichment). The plant segments were incubated at 25 ± 2 °C under controlled photoperiods of 16 h/day for 10 days. Small sections of the stems were removed using a razor blade every 24 h.

Procedure for the isolation of plaunotol: the plant material (ca. 10 g dry weight) was refluxed with 200 ml 95% (v/v) ethanol for 1 h. The extract was filtered and concentrated to dryness under vacuum. The residue was then re-dissolved in 70% (v/v) ethanol containing 1% (v/v) NaOH and heated at 60 °C for 30 min. After cooling, the mixture was partitioned with *n*-hexane (5 × 100 ml). The *n*-hexane fractions were pooled and evaporated to dryness under vacuum. The crude residue was then isolated by column chromatography. The mixture was applied to a column of silica gel 60 (Scharlau Chemie; 0.04–0.06 mm; column size 2.5 × 25 cm) which was eluted with chloroform: *n*-propanol; 24:1. Fractions were analyzed using TLC on silica gel 60 F₂₅₄ (Merck) using chloroform: *n*-propanol; 24:1 as mobile phase and a mixture of anisaldehyde/H₂SO₄ as visualization reagent. The *R_F* value of plaunotol was 0.38. The fractions containing plaunotol were combined and evaporated to dryness (32 mg).

The structures of unlabeled and labeled products were then analyzed by ¹H and ¹³C NMR spectroscopy. The ¹H and ¹³C NMR spectra of the isolated compound were identical to those of plaunotol as described previously.¹⁴ Quantitative ¹³C NMR measurements of an unlabeled and a labeled sample of plaunotol were recorded and labeled carbons were determined by integration of the two spectra.¹⁹

In the experiment with [U-¹³C]glucose, the ¹³C NMR spectra showed that the [U-¹³C]glucose was incorporated into plaunotol biosynthesis. The carbon blocks from the proffered glucose could be detected via ¹³C-¹³C coupling satellites accounting for approximately 7–14% of the central signal intensity of some car-

bon atoms with coupling constants between 40 and 50 Hz of their adjacent carbons (Fig. 1B). In addition, the ¹³C NMR signals at C-1, C-5, C-9, and C-13 coupled through space to C-17, C-18, C-19, and C-20, respectively. The results indicated that the four-isoprene units in the molecule of plaunotol were attached to each other through head-to-tail connection. In the experiments with proffered [1-¹³C]glucose and unlabeled glucose, the ¹³C NMR spectra of labeled plaunotol and of unlabeled plaunotol were recorded under identical conditions. Relative ¹³C-abundance of each position of labeled species was calculated as described previously.¹⁸ The results showed very clearly that eight carbon atoms of plaunotol acquired an average relative ¹³C-abundance of 1.49 ± 0.36, whereas the others position had an average ¹³C-abundance of 1.08 ± 0.09 (Fig. 1C). Those eight carbon atoms were C-1, C-5, C-9, C-13, C-17, C-18, C-19, and C-20 corresponding to the C-1 and C-5 of isopentenyl diphosphate (IPP) (Table 1).

Based on the glycolysis metabolism, labeling patterns of various intermediates can be predicted for IPP and DMAPP formed via the deoxyxylulose phosphate (DXP) and the mevalonate (MVA) pathways (Fig. 2). From our data, the labeling pattern of plaunotol is clearly in agreement with the deoxyxylulose phosphate pathway prediction without significant contribution from the mevalonate pathway. Therefore, it was concluded that the biosynthesis of plaunotol in *C. stellatopilosus* proceeds exclusively via the deoxyxylulose phosphate pathway.

It has been reported that in *C. stellatopilosus*, plaunotol is biosynthesized from geranylgeraniol by hydroxylation at position 18 and storage in the leaf chloroplast as an oil globule.²⁰ The geranylgeraniol diphosphate was derived from three molecules of IPP with one molecule of DMAPP to give a C-20 compound, a precursor of diterpenes. IPP and DMAPP, however, can be derived from two distinct biosynthetic pathways, DXP and MVA, which are compartmentalized in plastids and cytoplasm, respectively. The evidence that *C. stellatopilosus* geranylgeranyl diphosphate synthase contains the putative chloroplast targeting sequence confirmed that the enzymes involved in plaunotol biosynthesis are located in the plastids.¹⁶ To confirm plaunotol biosynthesis via the deoxyxylulose phosphate pathway, the feeding of intermediates such as deoxyxylulose to the plant can be suggested. Genes and enzymes involved in this pathway should be studied in detail in order to understand the regulation of plaunotol biosynthesis.

Table 1. Relative ¹³C-abundance of labeled plaunotol

Unit	Relative ¹³ C-abundance				
	C-1	C-2	C-3	C-4	C-5
IPP 1	1.40	1.07	1.14	1.18	1.32
IPP 2	1.45	1.12	1.17	0.99	2.38
IPP 3	1.35	1.07	1.10	1.10	1.31
IPP 4	1.31	1.12	1.10	0.81	1.42

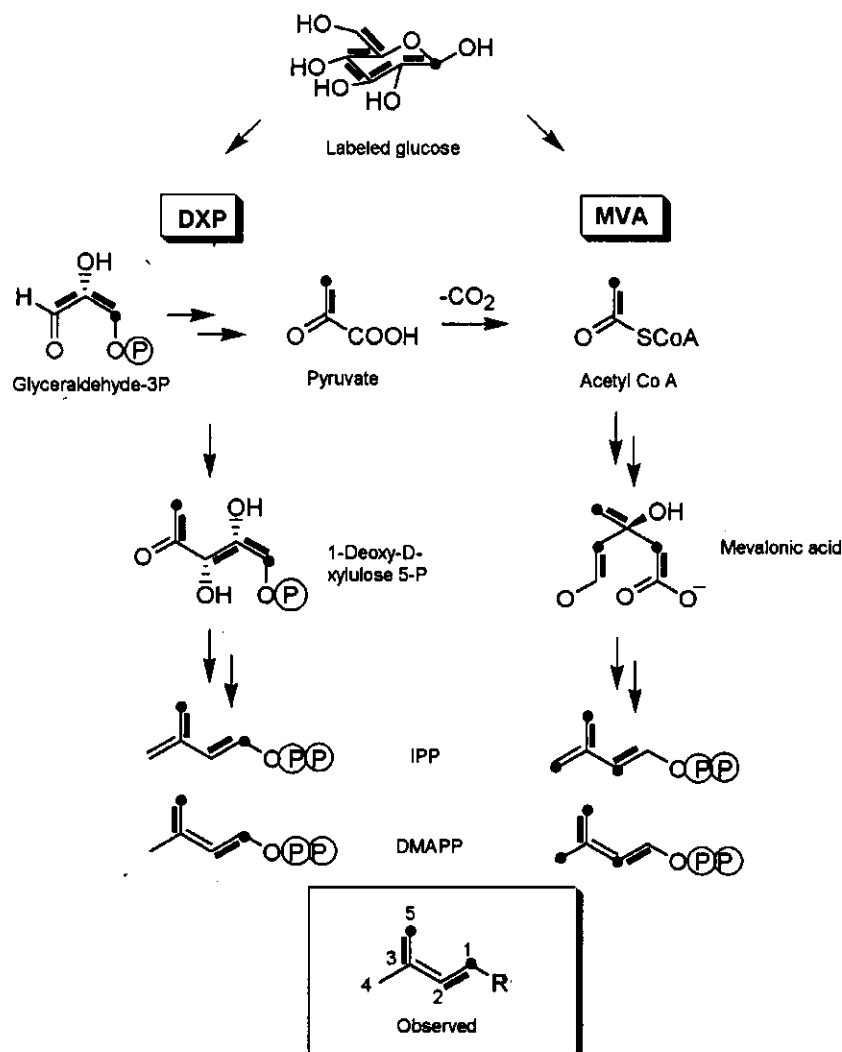


Figure 2. Predicted different labeling pattern for IPP and DMAPP from the experiments with [U - ^{13}C]glucose (bold line) and [1 - ^{13}C]glucose (filled circle) via the deoxyxylulose phosphate pathway (DXP) and via the mevalonate pathway (MVA).

Acknowledgements

This work was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the Thailand Research Fund (TRF) (grant no. TRG4580040).

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