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

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Abstract

Plaunotol is an acyclic diterpene alcohol, which is accumulated in Plaunoi, *Croton stellatopilosus* Ohba.(Euphorbiaceae). Feeding of [U-^{13}C] glucose and [1-^{13}C] glucose into cut shoots showed that the labels from glucose were incorporated into skeleton of plaunotol. The incorporation of [U-^{13}C]glucose and [1-^{13}C]glucose into plaunotol were analyzed by NMR spectroscopy. Data from NMR analysis indicated that the four-isoprenoid moieties of the diterpene showed identical labeling patterns. The labeling patterns were observed as predicted according to the deoxyxylulose phosphate pathway. From this *in vivo* feeding experiments with [U-^{13}C] glucose and [1-^{13}C] glucose indicated that the novel mevalonate-independent or deoxyxylulose phosphate pathway is the dominant metabolic route for plaunotol biosynthesis in *Croton stellatopilosus* Ohba.

Keywords: *Croton stellatopilosus*, Euphorbiaceae, terpenoid biosynthesis, feeding experiment, plaunotol

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More than decade ago, the diterpenoids in the higher plants have been proposed that their isoprene biosynthetic origins proceed via two distinct biosynthetic pathways, which are mevaloate pathway and deoxyxylulose phosphate pathway or methylerythritol phosphate pathway. Two pathways locate in different compartment where sterol biosynthesis appears in cytoplasm and monoterpenes, diterpenes and tetraterpenes biosynthesis appears in the plastids[1]. It, however, is more complexed in higher plants due to the inter-change of intermediates, isopentenyl diphosphate and farnesyl diphosphate, between compartments that occurs as described by "Cross-Talk Theory"[2]. This discovery was the starting point for re-investigating of isoprenoid biosynthesis in higher plants.

Various labeled precursors such as acetate and glucose were studied in *Scenedesmus obliquus*, *Lemna gibba*, *Hordeum vulgare*, *Daucus carota* and cell cultures of *Taxus chinensis* and *Mentha x piperita*[3]. These results showed that green algae and plants possess two biosynthetic routes. Evidence for this hypothesis was the incorporation of 1-deoxy-D-xylulose into isoprenoid side chain into *Ginkgo biloba* and into ferruginol of *Salvia milthiorrhiza*[2]. Subsequent studies in different research groups confirmed the incorporation of isotope-labeled 1-deoxy-D-xylulose into

terpenoids in green cell culture of *Catharanthus roseus*, in the plants *Populus nigra, Chelidonium majus, Salix viminalis, Lemna gibba, Liriodendron tulipifera* [4].

Plaunotol, an antiulcer agent, is a diterpenoid compound that is accumulated in *Croton stellatopilosus* Ohba. (Euphorbiaceae). It is formed from the hydroxylation at position 18 of geranylgeraniol catalyzed by geranylgeraniol 18-hydroxylase[5]. Recently, the biosynthesis of phytosterols in green callus culture of *C. stellatopilosus* has been reported. The IPP building block of phytosterols (β-sitosterol and stigmasterol) proceeds via mixed origin of both pathways[6]. However, the artificial culture could not determine the real biosynthetic in the plants. In addition, the biosynthesis of the pharmacologically compound, plaunotol has not been reported. Therefore, the [U-^{13}C]glucose and [1-^{13}C]glucose were fed into cut shoot of *C. stellatopilosus* to reveal the origin of the isoprene unit of diterpenoid. In this paper, we describe the origin of the carbon skeleton of plaunotol by feeding experiments with ^{13}C-labeled glucoses and analyzed by NMR spectroscopy.

The biosynthetic study on feeding experiment into cut shoot of *Croton stellatopilosus* with [U-^{13}C] glucose and [1-^{13}C] glucose was investigated following previously established method[7, 10]. Plaunotol (**1**, Fig. 1A) was then isolated by silica gel column chromatography[8]. The structures of unlabeled and labeled products were analyzed by ^{1}H and ^{13}C NMR spectroscopy[9]. ^{1}H and ^{13}C NMR spectra of isolated compound was identical as described previously[11].

In the experiment with [U-^{13}C] glucose, the labeled precursor was diluted 20-fold with natural abundance glucose. ^{13}C-NMR spectra showed that the [U-^{13}C] glucose was metabolized into the plaunotol biosynthesis. The carbon blocks from the proffered glucose could be detected via ^{13}C^{13}C coupling satellites accounting for approximately 7-14% of the central signal intensity of some carbon atoms with the coupling constants between 40-50 Hz of their adjacent carbons (Fig. 1B). In addition, ^{13}C-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 were observed (Fig. 2). The data indicated that a molecule of plaunotol comprised of 4 isoprene units, which were attached together with head-to-tail connection. In the plaunotol sample from the experiment with the proffered [1-^{13}C] glucose comparison to unlabeled glucose. ^{13}C NMR spectra of the labeled plaunotol and of the unlabeled plaunotol were recorded under identical condition. Absolute ^{13}C-enrichment of each position of labeled species was calculated as described previously[12]. The results showed that 8 carbon atoms of plaunotol acquired ^{13}C-enrichment with an average ^{13}C-abundance of 1.49±0.36% ^{13}C, whereas the others position had ^{13}C-enrichment with an average ^{13}C-abundance of 1.08±0.09% ^{13}C (Fig. 1C). Those 8 carbon atoms were 1, 5, 9, 13, 17, 18, 19, 20 which correspond to the carbon atoms 1 and 5 of IPP (Table 1).

On the basis of glycolysis metabolism, labeling patterns of intermediates can be predicted for IPP and DMAPP formed via the deoxyxylulose phosphate (DXP) and the mevalonate (MVA) pathways as shown in Fig. 3. The incorporation rate of isotopically labeled glucoses can be assumed by the experiment of [U-^{13}C]glucose. However, the resulted isotopomers could not distinguish between two pathways. With [1-^{13}C]glucose as a carbon source, enrichments at C-1 and C-5 of IPP are expected from the deoxyxylulose phosphate pathway whereas the enrichments at C-2, C-4 and C-5 of IPP from the mevalonate pathway (Fig. 3). From our data, the labeling patterns

of plaunotol is in agreement with the deoxyxylulose phosphate pathway prediction and is clearly distinct from the labeling pattern predicted via mevalonate. From this study, concluded that the deoxyxylulose phosphate pathway is the dominant metabolic route for plaunotol biosynthesis in *C. stellatopilosus* Ohba.

It has been reported that in *C. stellatopilosus*, plaunotol was biosynthesized from the molecule of geranylgeraniol by hydroxylation at position 18 and storage in the leaf chloroplast as oil globule[5,13]. The geranylgeraniol diphosphate was derived from three molecules of IPP with one molecule of DMAPP to give a C20 compound, a precursor of diterpenes. IPP and DMAPP, however, can be derived from 2 distinct biosynthetic pathways, DXP and MVA, which are compartmentalized in plastid and cytoplasm, respectively. The evidence of *C. stellatopilosus* geranylgeraniol diphosphate synthase contains the putative chloroplast targeting sequence confirmed that enzymes involved in plaunotol biosynthesis located in the plastids[13]. To confirm the plaunotol biosynthesis metabolized via the deoxyxylulose phosphate pathway, the feeding of intermediates such as deoxyxylulose into plant can be suggested. Genes and enzymes involved this pathway should be studied in detail in order to understanding of regulation of plaunotol biosynthesis in this plant.

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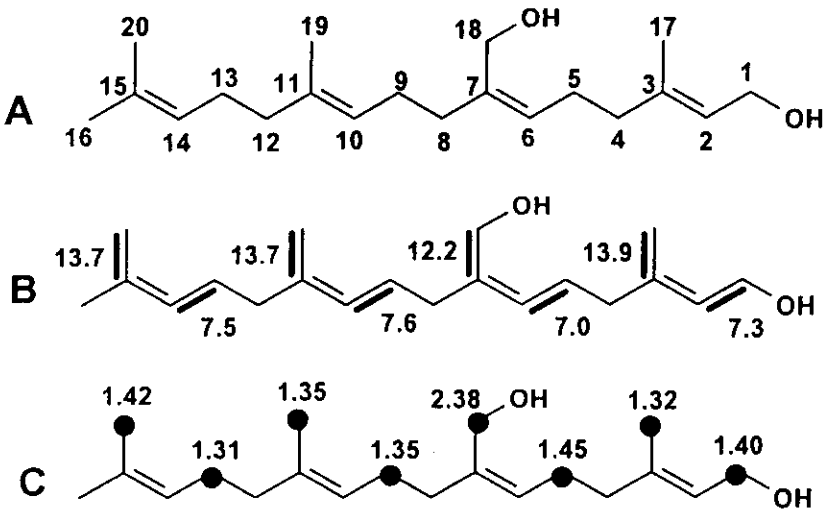

1, Plaunotol

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

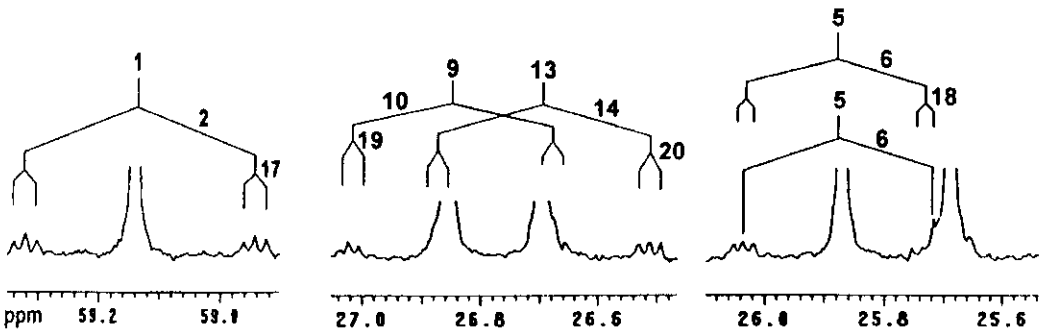

Figure 2
Expanded view of ^{13}C NMR signals of C-1, C-5, C-9 and C-13 of plaunotol

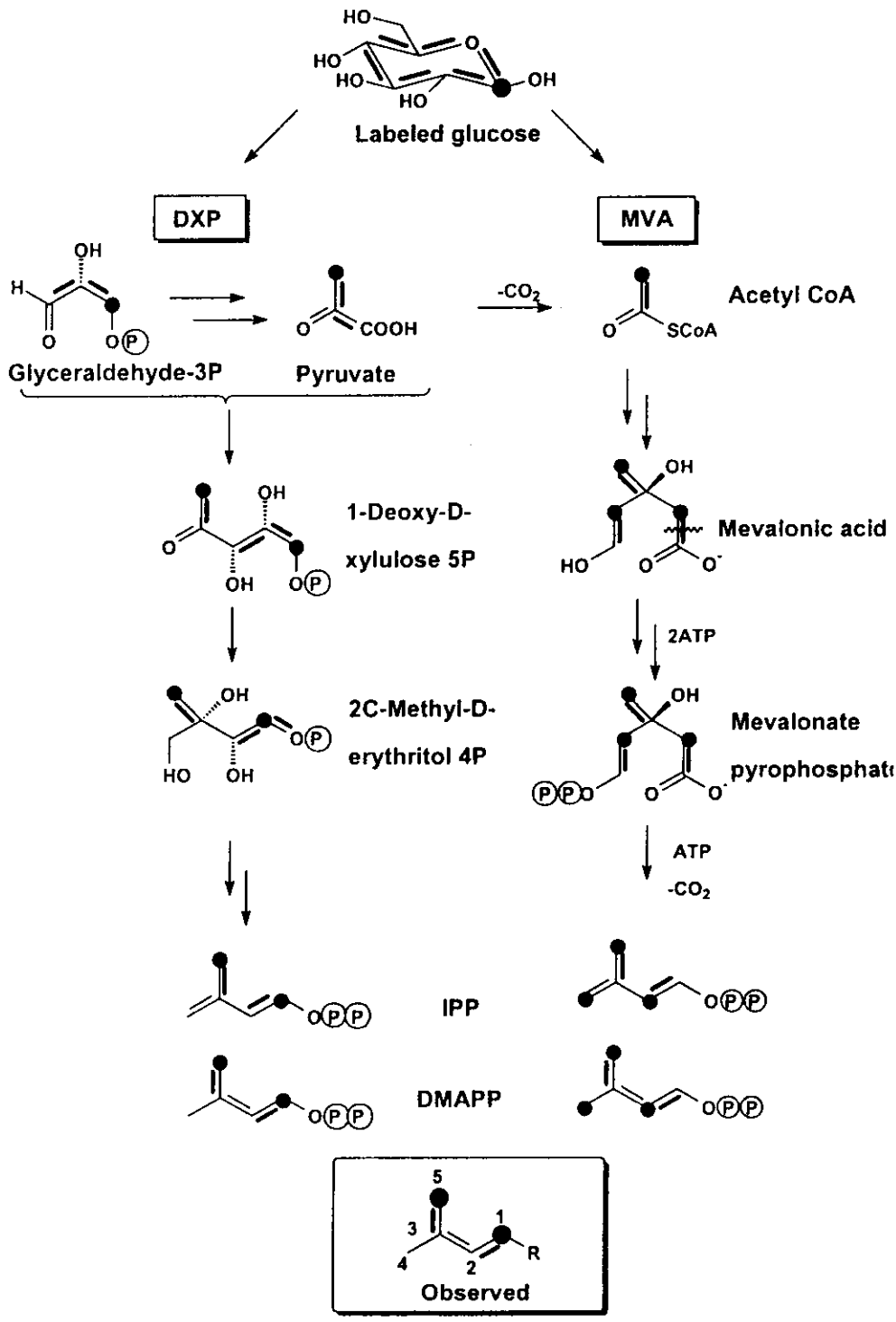

Figure 3
Proposed labeling pattern for IPP and DMAPP from the experiment with [U-^{13}C]glucose (bold line) and [1-^{13}C]glucose (filled circle); Predicted via the deoxyxylulose phosphate pathway (DXP) and via the mevalonate pathway (MVA).

Table 1 Absolute ^{13}C-enrichment of labeled plaunotol

Unit	Absolute ^{13}C-enrichment (%)				
	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**

References and notes

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7. For feeding experiment, ten shoots of *C. stellatopilosus* Ohba., each containing 2 or 3 leaves, were cut from a six-month old plant. The shoots were 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-^{13}C]glucose (99% ^{13}C enrichment). Solution 3 contained 0.5% (w/v) unlabeled glucose and 0.5% (w/v) [1-^{13}C]glucose (99% ^{13}C enrichment). The plant segments were incubated at 25±2 °C under controlled photoperoid of 16 hr/day for 10 days. Small sections of the stems were cut with a razor blade every 24 hr. The optimal time of feeding was investigated with [U-^{13}C]glucose within 10 days comparison to 15 days. The isolated plaunotol was analyzed by quantitative ^{13}C-NMR spectroscopy. ^{13}C-signal of 10-days feeding was higher than ^{13}C-signal of 15-days feeding for 2.0±0.30 times.
8. Isolation of plaunotol, the plant material (*ca.* 10 g dry weight) was refluxed with 200 ml 95% (v/v) ethanol for 1 hr. 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 heat at 60°C for 30 min. After cooling, the mixture was partitioned with 5 times of 100 ml n-hexane. The n-hexane fractions were pooled and evaporated to dryness under vacuum (0.47 g). 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 x 25 cm) which was developed with chloroform: *n*-propanol; 24:1. Fractions were analyzed using TLC on silica gel 60 F_{254} (Merck) using chloroform: *n*-propanol; 24:1 as a mobile phase and a mixture of anisaldehyde/H_2SO_4 as visualization reagent. The R_F value of plaunotol was 0.38. The fractions containing plaunotol were combined and evaporated to dryness (32 mg).
9. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ using a Varian INOVA-500 spectrometer, operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively. Qualitative ^{13}C NMR measurements of an unlabeled and a labeled sample of plaunotol were recorded under the same conditions; labeled carbons were determined by integration of two spectra.

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