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Biosynthetic origins of the isoprene units of gaudichaudianic acid in *Piper gaudichaudianum* (Piperaceae)

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Abstract

The biosynthesis of (2*S*)-2-methyl-2-(4′-methyl-3′-pentenyl)-8-(3″-methyl-2-butenyl)-2*H*-1-benzopyran-6-carboxylic acid (gaudichaudianic acid), the major metabolite in leaves and roots of *Piper gaudichaudianum* Kunth (Piperaceae), has been investigated employing [1-^{13}C]-D-glucose as precursor. The labelling pattern in the isolated gaudichaudianic acid was determined by quantitative ^{13}C NMR spectroscopy analysis and was consistent with involvement of both mevalonic acid and 2-*C*-methyl-D-erythritol-4-phosphate pathways in the formation of the dimethylallyl- and geranyl-derived moieties. The results confirmed that both plastidic and cytoplasmic pathways are able to provide isopentenyl diphosphate units for prenylation of *p*-hydroxybenzoic acid.

Keywords: *Piper gaudichaudianum*; Piperaceae; Biosynthesis; Chromene; Gaudichaudianic acid; Mevalonate pathway; 2-*C*-Methyl-D-erythritol-4-phosphate pathway; Isopentenyl diphosphate units

1. Introduction

The isoprenoids, when considered together with the steroids, constitute the largest class of secondary metabolites comprising more than 30,000 known compounds (Dictionary of Natural Products, 2000). The common biosynthetic building block of this group, the so-called isoprene unit, is derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The biosynthesis of IPP can proceed via two different pathways, namely the long-established mevalonic acid (MVA) and the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathways, the reaction sequence of which has been characterized (Adam et al., 1999). In plants, the MEP pathway appears generally operative for formation of monoterpenoids, diterpenoids, phytols and carotenoids (Hirai et al., 2000; Umlauf et al., 2004; Bouvier et al., 2005).

The key regulatory step of the MVA pathway involves reduction of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, with this being catalysed by the cytosolic enzyme HMG-CoA reductase (HMGR; Fig. 1). Plastidic IPP is derived, however, from MEP formed via the condensation of pyruvate and glyceraldehyde-3-phosphate (G3P) catalysed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS; Fig. 1) (Bouvier et al., 2005; Enfissi et al., 2005). The two pathways to IPP are thus compartmentalized in the cytosol and the plastid, respectively; however, they may function in the formation of certain isoprenoids, in a cooperative process that results from metabolic cross-talk within the plastidic envelope membrane (Enfissi et al., 2005).

A quantitative assessment of the differential contribution of the two IPP pathways in the biosynthesis of an individual isoprenoid may be deduced from the ^{13}C-labelling pattern, as determined by ^{13}C NMR spectroscopy, following incorporation of [1-^{13}C]-D-glucose into product (Rohmer et al., 1993; Umlauf et al., 2004). As shown in

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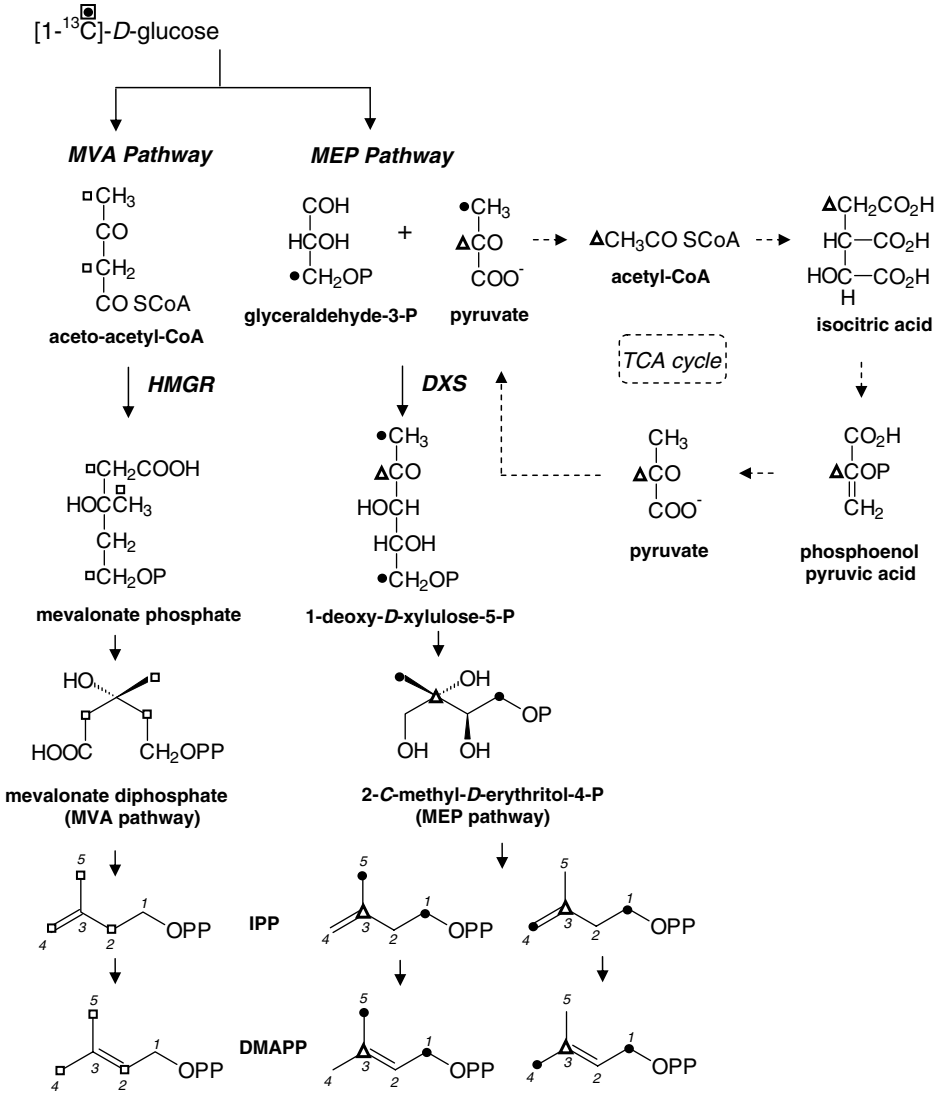

Fig. 1. Predicted labelling patterns in isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) units derived from [1-^{13}C]-D-glucose assuming biosynthesis via the mevalonate (MVA) pathway (□), the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (●), and the latter with additional incorporation through the tricarboxylic acid cycle (TCA) (△). (HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; DXS: 1-deoxy-D-xylulose-5-phosphate synthase.)

Fig. 1, label derived from [1-^{13}C]-D-glucose by glycolysis should be present at C-2 of acetyl CoA and at C-3 of G3P and pyruvate. Thus, DMAPP arising from the MVA pathway, involving three C-2 labelled acetyl CoA molecules, should be labelled at C-2, C-4 and C-5. In the MEP pathway, label from the C-3 positions of G3P and pyruvate would give rise to DMAPP labelled at C-1 and C-5. Involvement of C-2 labelled acetyl CoA in the tricarboxylic acid cycle (TCA) could, however, lead to the presence of an additional label at C-2 of pyruvate and, subsequently, at C-3 of DMAPP. An understanding of the correlations between isotopic fractionations and biochemical processes is, therefore, essential in the elucidation of biosynthetic pathways and for the discrimination among alternative pathways (Chikaraishi et al., 2004). Moreover, mixed biosynthesis with contributions from both pathways may be determined from such studies (Adam et al., 1998; Bergamo et al., 2005).

In continuation of previous studies concerning IPP biosynthesis in plant isoprenoids (Schwender et al., 1996; Knoss et al., 1997; Thiel et al., 1997; Adam and Zapp, 1998; Adam et al., 1998, 1999; Hirai et al., 2000; Barlow et al., 2001; Hertewich et al., 2001; Thiel and Adam, 2002; Umlauf et al., 2004; Massé et al., 2004; Wungsintaweekul and De-Eknamkul, 2005; Bergamo et al., 2005), the metabolism of the powerful antifungal compound (2*S*)-2-methyl-2-(4′-methyl-3′-pentenyl)-8-(3″-methyl-2-butenyl)- 2*H*-1-benzopyran-6-carboxylic acid (**1**; gaudichaudianic acid), the major constituent of leaves and roots of *Piper gaudichaudianum* Kunth (Piperaceae) (Lago et al., 2004), was investigated. Chromene **1** is considered formally to be biosynthesised by the prenylation of

p-hydroxybenzoic acid, derived from the shikimate/phenylpropanoid pathway (Yamamoto et al., 2000), with DMAPP and geranyl diphosphate (GPP) originating from the MVA and/or MEP pathways. With the aim of determining the biosynthetic origin of the isoprene moieties of gaudichaudianic acid, precursor administration experiments with leaves of *P. gaudichaudianum* using [1-^{13}C]-D-glucose as precursor were carried out.

2. Results and discussion

A sample of ^{13}C-enriched gaudichaudianic acid (**1**) was isolated by CC fractionation of a $CHCl_3$–MeOH extract derived from young leaves of *P. gaudichaudianum* that had been administrated with an [1-^{13}C]-D-glucose solution for 72 h. The ^{13}C NMR spectra of **1** enriched with ^{13}C and that of a reference sample of the chromene (^{13}C natural abundance) were measured in $CDCl_3$ under identical conditions, and the relative enrichment at each position was calculated by assuming an abundance of 1.1% for the carbon with the lowest ^{13}C-enrichment.

The results presented in Table 1 demonstrated that increases in the signals of the prenyl moieties occurred at all positions except C-4′ and C-3″. As shown in Fig. 2, incorporation of label into **1** at C-3′, C-5′, C-6′, C-2″, C-4″ and C-5″ is consistent with the operation of the MVA pathway, whilst enhancement of signals at C-2′, C-5′, C-1″ and C-5″ would be as expected from the MEP pathway. Administering [1-^{13}C]-D-glucose thus resulted in increases in isotopic labeling, at positions 2, 4 and 5, as well as at 1 and 5, of IPP indicating simultaneous operation of the MVA and MEP pathways, respectively (Kuzuyama and Seto, 2003).

Additionally, significant ^{13}C-enrichment was observed in the pyran ring of **1** at position C-2, corresponding to position 3 of IPP. Such enrichment could derive from [2-^{13}C]-pyruvate formed from [2-^{13}C]-acetyl CoA returning through the tricarboxylic acid cycle (TCA) (Fig. 1). If both [2-^{13}C]- and [3-^{13}C]-pyruvate enter the MEP pathway, IPP units labelled at C-1, C-3 and C-5 would be formed. Moreover, since C-4 and C-5 of DMAPP are chemically equivalent, if the stereo-control of the IPP/DMAPP isomerase is imperfect, labelling of this isoprene unit at C-4 could also be observed as shown previously for abscisic acid (Hirai et al., 2000). The increases in signal intensities at positions C-4a, C-5 and C-8 of the *p*-hydroxybenzoic acid moiety presumably result from scrambling of label from [1-^{13}C]-D-glucose after glycolysis.

Table 1
Relative ^{13}C-abundance in gaudichaudianic acid (**1**) isolated following incorporation of [1-^{13}C]-D-glucose into leaves of *Piper gaudichaudianum*

Carbons	δ (ppm)[a]	Relative enrichment[b] (%)
C-2	79.9	12.1
C-3	129.5	1.1
C-4	122.5	5.5
C-4a	120.1	6.6
C-5	126.7	8.8
C-6	120.8	3.3
C-7	131.8	8.8
C-8	128.9	1.1
C-8a	155.8	9.9
C-9	27.1	1.1
C-1′	41.8	5.5
C-2′	22.7	8.8
C-3′	123.9	7.7
C-4′	131.8	1.1
C-5′	17.6	8.8
C-6′	25.6	9.9
C-1″	28.1	5.5
C-2″	121.9	11.0
C-3″	132.6	2.2
C-4″	25.7	9.9
C-5″	17.8	11.0
COOH	172.1	1.1

[a] Referenced to $CDCl_3$.
[b] Calculated by comparison of relative intensities of signals in the ^{13}C NMR spectra of ^{13}C-labelled **1** and a reference standard with ^{13}C at natural abundance.

3. Conclusion

The observation of mixed biosynthesis, with contributions from both the MVA and the MEP pathways, implies that certain biosynthetic steps proceed in different compartments, and that specific intermediates traverse the chloroplast boundary. In the case of the formation of **1**, it would appear that prenylation of *p*-hydroxybenzoic acid by GPP occurs in the plastid with the prenyl moiety having been synthesised from IPP units derived from each pathway. Two alternatives routes to **1** should be considered. In the first, initial C-prenylation with DMAPP derived from either the MVA or MEP pathways would be followed by a second C-prenylation of the aromatic nucleus with a GPP unit thus forming the benzopyran ring. The alternative pathway would involve initial prenylation with GPP followed by a second prenylation with DMAPP. A plausible biosynthetic pathway of the isoprene units in *P. gaudichaudianum* described herein is shown in Fig. 3. This report thus constitutes the second example of the participation of DMAPP/IPP moieties derived from both MVA and MEP in the prenylation of benzoic acid derivatives (Bergamo et al., 2005).

4. Experimental

4.1. General

NMR: Varian 500 ($CDCl_3$ ^{1}H δ 7.24; ^{13}C δ 77.0). [1-^{13}C]-D-glucose (99% isotopic abundance) was purchased from Sigma–Aldrich.

4.2. Plant material

Specimens of *P. gaudichaudianum* were cultivated from seed under greenhouse conditions at the Institute of

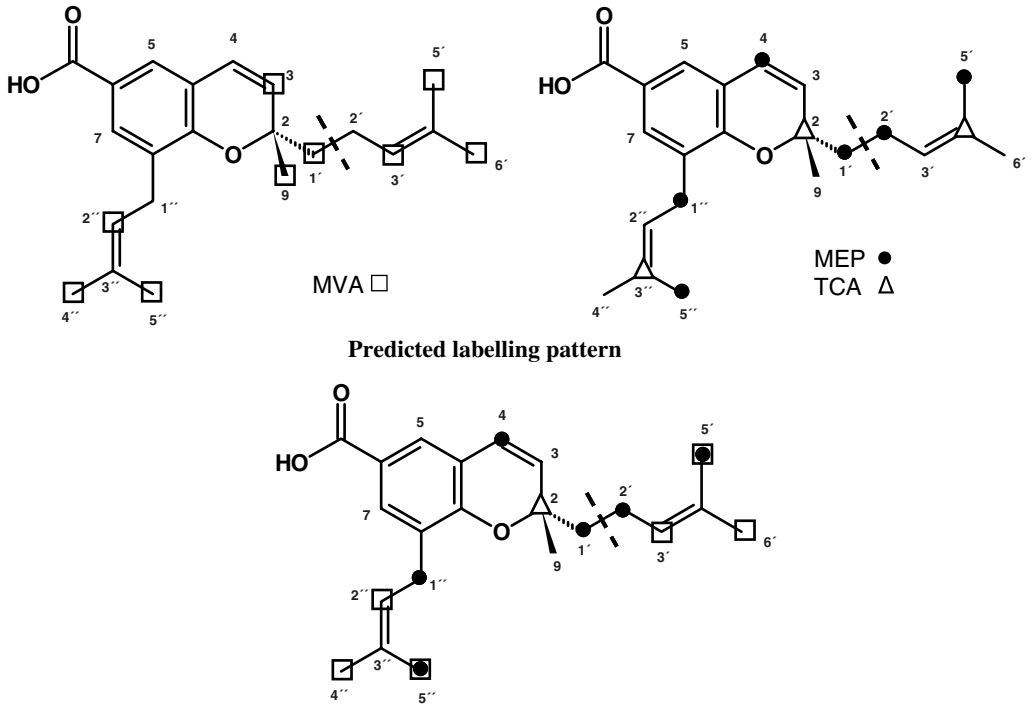

Fig. 2. Predicted labelling patterns in gaudichaudianic acid derived from [1-^{13}C]-D-glucose assuming biosynthesis via the mevalonate (MVA) pathway (□), the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (●), and the latter with additional incorporation through the tricarboxylic acid cycle (TCA) (△). The experimentally observed labelling pattern and the presumed origin of label at each position are also shown.

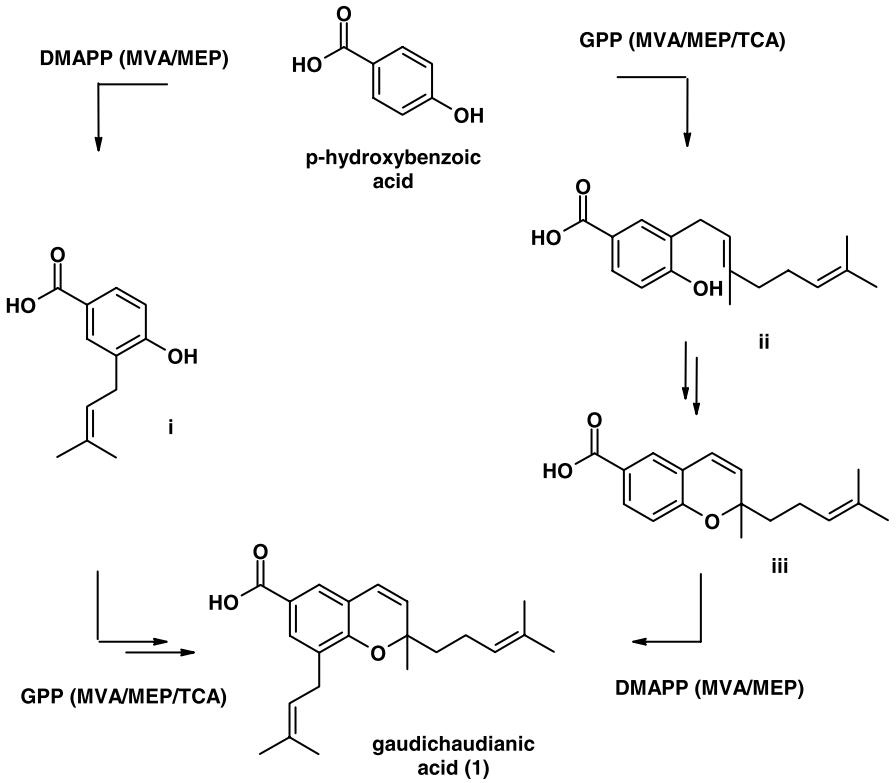

Fig. 3. Proposed biosynthetic pathway for the formation of gaudichaudianic acid (**1**) from *p*-hydroxybenzoic acid, dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) in leaves of *Piper gaudichaudianum*. (MVA: mevalonic acid pathway; MEP: 2-*C*-methyl-D-erythritol-4-phosphate pathway; TCA: tricarboxylic acid cycle.)

Chemistry, UNESP, Araraquara, SP, Brazil. Plant material was authenticated by Dr. Guillermo E.D. Paredes (Universidad Pedro Ruiz Gallo – Peru) and a voucher specimen (Kato 92) is deposited at the Herbarium of Institute of Bioscience – USP, São Paulo, SP, Brazil.

4.3. Administration of [1-^{13}C]-D-glucose into young leaves from P. gaudichaudianum

Eleven leaves, freshly excised from 4 to 5-month-old plants, were placed separately in 2 mL Eppendorf tubes and each administrated 100 μL of a 10% solution of [1-^{13}C]-D-glucose in water at 25 °C for 72 h (Bergamo et al., 2005).

4.4. Extraction and isolation of **1**

After incubation, the leaves were frozen in liquid N_2 and subsequently extracted with 2×25 mL $CHCl_3$–MeOH (2:1). The organic extracts were combined, evaporated to dryness in vacuum, with the resulting extract (0.250 g) fractionated by CC over silica gel (Merck; 70–230 mesh; 20×1.4 cm column) using hexane as eluent, containing increasing amounts of EtOAc (up to 50%) to yield 10 fractions. Fraction 7 (0.080 g) was further separated by CC over reversed phase ODS (Merck; C-18; 2.0×1.0 cm column) with MeOH–H_2O (4:1) to yield 6 fractions, the third of which (0.043 g) was submitted to CC over silica gel (Merck; 230–400 mesh; 5×1.0 cm column) eluted with hexane–EtOAc (4:1) to yield **1** (0.011 g) as an amorphous colourless solid. Relative ^{13}C-enrichment values were obtained by comparison of ^{13}C NMR spectra of **1** derived from incorporation experiments with that of a reference sample of **1** isolated from a field-grown plant.

4.5. Gaudichaudianic acid (*1*)

Rf value 0.60 by TLC (Merck silica gel 60 F_{254}) with hexane–EtOAc (4:1) and visualised using anisaldehyde/H_2SO_4 reagent; ^{1}H NMR spectroscopic data were similar to those for (2*S*)-2-methyl-2-(4′-methyl-3′-pentenyl)-8-(3″-methyl-2-butenyl)-2*H*-1-benzopyran-6-carboxylic acid (Lago et al., 2004); ^{13}C NMR data are presented in Table 1.

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