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Mevalonate-independent biosynthesis of bicyclic and tetracyclic diterpenes of *Scoparia dulcis* L.

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Abstract

Mevalonate-independent biosynthesis of bicyclic and tetracyclic diterpenes produced by *Scoparia dulcis* L. has been rigorously established by the feeding experiments with [1-^{13}C]glucose followed by the analysis of their labelling patterns by ^{13}C NMR spectroscopy. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: mevalonate-independent pathway; scopadulcic acid B; scoparic acid A; diterpene; *Scoparia dulcis*.

A biosynthetic pathway starting from acetyl CoA via mevalonate (MVA) was believed to be the sole biosynthetic pathway leading to all terpenoids and steroids for a long time. Rohmer et al.,[1] however, revealed that bacterial hopanoids were biosynthesized by an alternative pathway, which does not involve MVA but 1-deoxy-D-xylulose 5-phosphate, hence designated as an MVA-independent pathway or deoxyxylulose phosphate pathway. Following this finding, the MVA- independent pathway was found not only in eubacteria but also in Actinomycetes, green algae and higher plants.[2] In higher plants, these two biosynthetic pathways of isoprenoids function in separate subcellular loci, i.e., the biosyntheses of steroids, triterpenoids and sesquiterpenoids are carried out in cytoplasm via the MVA pathway, while other terpenoids in plastids such as chloroplast are biosynthesized via the MVA-independent pathway.[2,3]

The leaves of *Scoparia dulcis* L. (Scrophulariaceae) contain bicyclic and tetracyclic diterpenes such as scoparic acid A (SA), scopadulcic acid B (SDB) and scopadulin.[4] Scopadulin possesses the same skeleton as that of aphidicolin which is produced by the filamentous fungi, *Cepharospolium aphidicola* and *Nigrospora sphaerica*.[5] The production of diterpenes of *S. dulcis* is associated with differentiation of green leaves,[6] suggesting involvement of the MVA-independent pathway in their biosynthesis, while aphidicolin was rigorously established to be biosynthesized via the MVA pathway.[7] The involvement of the MVA-independent pathway in the biosynthesis of diterpenoids with similar structures to aphidicolin is important for understanding the diversity of diterpene biosynthesis.

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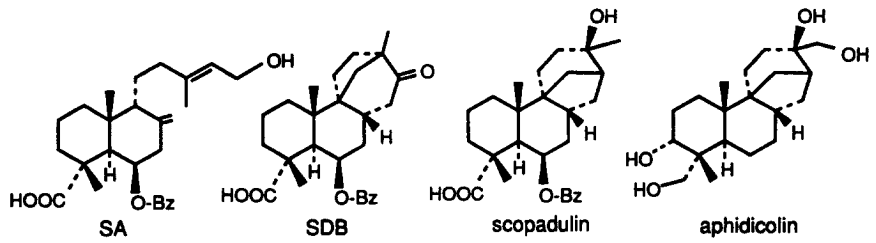


Preliminary incorporation experiments with [2-^{14}C] Na acetate and [2-^{14}C]MVA were carried out to establish feeding conditions and to obtain information on the involvement of the MVA-independent pathway in the biosynthesis of a tetracyclic diterpene SDB. The shoot culture system of *S. dulcis* described previously[6b] was used in the feeding experiments. The incorporation ratios of [2-^{14}C] Na acetate and [2-^{14}C]MVA into SDB were compared with those into β-sitosterol which had been proved to be biosynthesized via the MVA pathway in higher plants. After incubation with ^{14}C labelled compounds for 10 days, SDB and β-sitosterol were extracted from the cultured tissues and purified as methyl ester and acetate ester, respectively. The incorporation ratios of [2-^{14}C] Na acetate and [2-^{14}C]MVA into SDB were 0.00076% from MVA and 0.0031% from Na acetate, while those into β-sitosterol were 0.65% from MVA and 0.38% from Na acetate. These results suggested that the early steps of SDB biosynthesis in *S. dulcis* involve the MVA-independent pathway discovered by Rohmer et al. and are different from those of β-sitosterol.

Direct evidence of the involvement of the MVA-independent pathway in SDB biosynthesis was obtained by feeding experiments with [1-^{13}C]glucose by using the shoot cultures of *S. dulcis*.[8] The labelling patterns of SDB were determined by ^{13}C NMR spectroscopy.[9] In the MVA-independent pathway, the carbon atoms at positions C-1 and C-5 of C_5 isoprenic units are expected to be enriched (Fig. 1). On the other hand, the enrichment of ^{13}C in positions C-2, C-4 and C-5 is expected in the MVA pathway. The labelling patterns of β-sitosterol and phytol obtained from the same shoot cultures were determined as the positive control compounds representing MVA and MVA-independent pathway products. In addition, a bicyclic diterpene, SA, was also examined by using a chemotype of *S. dulcis* producing mainly SA.[10] The relative signal intensities of carbons of each sample were calculated on the basis of the signal intensity of carbon at position C-3 in each C_5 isoprenic unit. As shown in Fig. 2, the ^{13}C enrichments of the carbon atoms were observed at C-2, C-6, C-11, C-14, C-16, C-17, C-19 and C-20 of methylester of SDB (SDB-Me),[9] which correspond to the carbon positions 1 and 5 of the C_5 isoprenic units. The labelling patterns of SA, SDB and phytol were typical of the MVA-independent

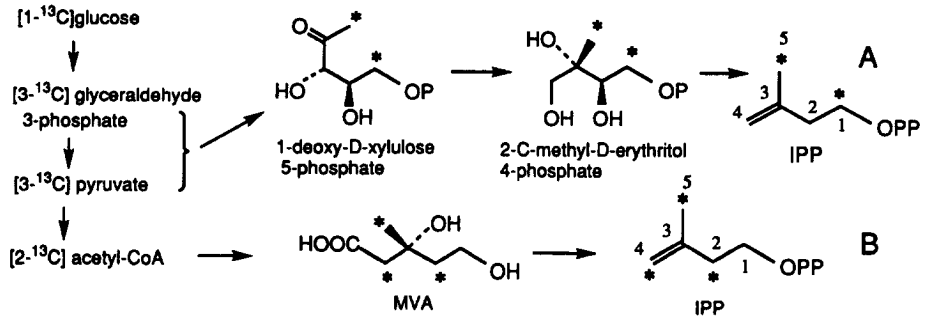


Figure 1. Labelling of isopentenyl diphosphate (IPP) from [1-^{13}C] glucose via the MVA-independent pathway (A) and MVA pathway (B). ^{13}C labelling positions are marked with asterisks

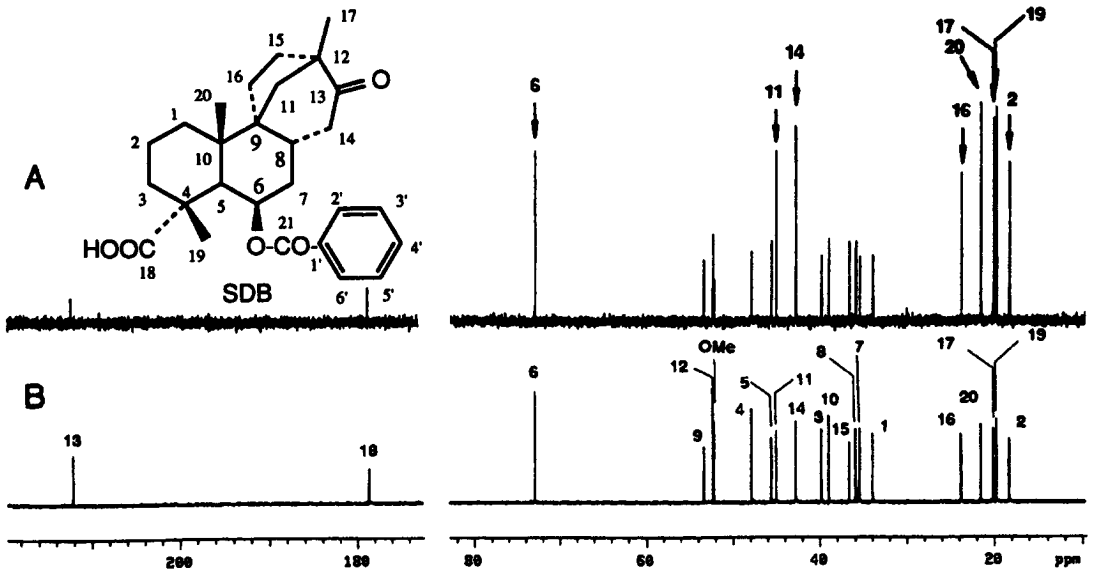
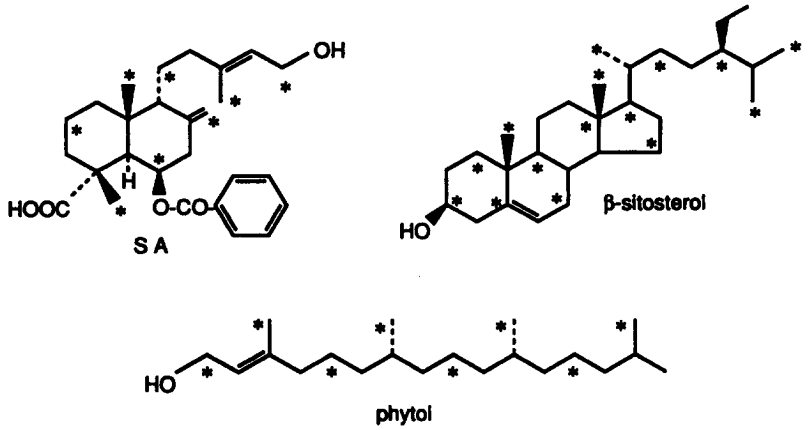


Figure 2. ^{13}C NMR spectra of SDB-Me obtained from the cultured tissues of *S. dulcis* after feeding with [1-^{13}C]glucose(A) and non-labelled SDB-Me (B) in pyridine-d_5

biosynthesis, whereas β-sitosterol was labelled as expected by the MVA pathway (Table 1). These data clearly demonstrate that diterpenes SDB and SA were biosynthesized via the MVA-independent pathway in contrast to the biosynthesis of fungal tetracyclic diterpene aphidicolin with similar structure.



Recent studies on the biosynthesis of Ginkgo diterpenes and Chamomile sesquiterpenes showed a possible cross-talk between the two different pathways.[11] In these terpenes, heterogeneous labelling patterns of isoprenic units were observed. These observations are explained by the exchange of metabolic intermediates between the cytoplasm and the chloroplasts. The present data suggested that phytol and cyclic diterpenes SDB and SA as well as their linear precursor, geranylgeranyl diphosphate (GGPP) are biosynthesized in the chloroplasts via the MVA-independent pathway, consistent with our earlier observation that the production of SDB and SA is closely associated with green leaf differentiation.[6] Recently, we cloned a gene for GGPP synthase from *S. dulcis*, which contains a chloroplasts-targetting signal sequence at the N-end and successfully demonstrated its localization in the chloroplasts.[12] For

Table 1
Average ^{13}C-isotopic abundances measured in isoprenic units of isoprenoids produced by *S. dulcis* after feeding with [1-^{13}C]glucose

terpenoid	^{13}C-abundances in C-atoms of C_5 unit				
	C-1	C-2	C-3	C-4	C-5
SDB-Me	1.98	0.80	1.00	0.89	2.60
SA-Me	1.59	0.78	1.00	1.03	1.88
phytol-Ac	2.09	0.93	1.00	1.21	2.21
β-sitosterol-Ac	1.27	3.08	1.00	2.85	3.54

further understanding of the biosynthesis of SDB and SA, studies on the enzymes involved in the formation of the diterpenes including cyclases will be required.

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8. In the feeding experiments, the MS liquid medium was supplemented with a mixture of [1-^{13}C]glucose and sucrose (1:4) as a carbon source. Four 100 ml flasks, each containing 15 ml medium, were inoculated with 2 g each of cultured tissues of *S. dulcis* (SDB type). The tissues were incubated on a rotary shaker (120 rpm) under continuous illumination at 26°C for 10 days. After extraction of the cultured tissues with acetone, the extract was dissolved in $CHCl_3$ and subjected to column chromatography on silica gel (10×45 mm). The column was eluted with 50 ml of $CHCl_3$ and 100 ml of 10% MeOH in $CHCl_3$ successively. The eluate with 10% MeOH in $CHCl_3$ was then subjected to preparative thin layer chromatography (PLC) on silica gel by using a solvent system of $CHCl_3$:EtOAc:AcOH (60:40:1). The fraction containing SDB was dissolved in MeOH (80 ml) and benzene (292 ml), and treated with trimethylsilyldiazomethane (28 ml) for 1 h to give SDB-Me (1.8 mg). From the eluate with $CHCl_3$, phytol and β-sitosterol were isolated and purified as corresponding acetate esters (phytol-Ac, 1.1 mg; β-sitosterol-Ac, 1.6 mg). SA was obtained as a methyl ester (SA-Me, 6.2 mg) from the cultured tissues of SA type plant by the same procedure as described in the purification of SDB-Me.
9. The ^{13}C NMR spectra of the samples were recorded in pyridine-d_5 (SDB-Me) or $CDCl_3$ (SA-Me, phytol-Ac and β-sitosterol-Ac) with a Varian Unity 500 spectrometer. The following ^{13}C NMR assignment of SDB-Me was made based on the experiments including HMQC, HMBC and COSY. 18.30 (C-2), 19.78 (C-19), 20.12 (C-17), 21.57 (C-20), 23.79

(C-16), 33.93 (C-1), 35.47 (C-7), 35.90 (C-8), 36.65 (C-15), 39.00 (C-10), 39.8 (C-3), 42.77 (C-14), 45.04 (C-11), 45.63 (C-5), 47.88 (C-4), 52.15 (OMe), 52.32 (C-12), 53.35 (C-9), 72.97 (C-6), 129.13 (C-3′,5′), 130.0 (C-2′,6′), 131.30 (C-1′), 133.54 (C-4′), 166.20 (C-21), 178.65 (C-18), 212.11 (C-13).

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