# Involvement of 2-*C*-Methyl-D-erythritol-4-phosphate Pathway in Biosynthesis of Aphidicolin-Like Tetracyclic Diterpene of *Scoparia dulcis*

Marguerite Kasidimoko NKEMBO, Jung-Bum LEE, Takeshi NAKAGIRI, and Toshimitsu HAYASHI*

*Faculty of Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.*
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**Specific inhibitors of the MVA pathway (pravastatin) and the MEP pathway (fosmidomycin) were used to interfere with the biosynthetic flux which leads to the production of aphidicolin-like diterpene in leaf organ cultures of *Scoparia dulcis*. Treatment of leaf organs with fosmidomycin resulted in dose dependent inhibition of chlorophylls, carotenoids, scopadulcic acid B (SDB) and phytol production, and no effect on sterol production was observed. In response to the pravastatin treatment, a significant decrease in sterol and pertubation of SDB production was observed.**

**Key words** *Scoparia dulcis* L.; diterpene; scopadulcic acid B; 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway; pravastatin; fosmidomycin

Isoprenoids constituting the most diverse group of natural products are synthesized ubiquitously among prokaryotes and eukaryotes through the condensation of five-carbon intermediate isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). For many years, it has been believed that the mevalonate pathway (MVA), which depends on the condensation of three molecules of acetyl CoA, was the sole biosynthetic pathway leading to all terpenoids and sterols. Recently, the involvement of an alternative route in the pathway of IPP biosynthesis, designated as mevlonate-independent or 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway, led to a reappraisal of isoprenoids biosynthesis in various organisms including eubacteria[1] and several photosynthetic organisms such as green algae and higher plants.[2,3] In the MEP pathway, IPP is biosynthesized from pyruvate and glyceraldehyde 3-phosphate *via* a novel intermediate, 1-deoxy-D-xylulose-5-phosphate (DOXP). Furthermore, it has been demonstrated that higher plants can synthesize IPP *via* these two pathways in different subcellular compartments. Thus, the MVA pathway provides the cytosolic isoprenoids such as sesquiterpenes, triterpenes and sterols, while the MEP pathway leads to plastid-related isoprenoids such as monoterpenes, diterpenes, carotenoids and prenyl side-chains of chlorophyll and plastoquinones.

*Scoparia dulcis* L. (Scrophulariaceae) is a perennial herb used as a folk medicine in tropical and subtropical regions. This plant was found to produce bicyclic and tetracyclic diterpenes such as scoparic acid A (SA), scopadulcic acid B (SDB) and scopadulin (SD) (Chart 1).[4] SD possesses the same skeleton as that of aphidicolin isolated from filamentous fungi *Cephalosporium aphidicola* and *Nigrospora sphaerica*.[5] SDB was determined to be a tetracyclic diterpene with a novel type skeleton termed scopadulan,[6] and was estimated to be biosynthesized from geranylgeranyl diphosphate (GGPP) through a bicyclic diterpene with similar enzyme reaction systems to those of aphidicolin biosynthesis.[7,8] Previous studies on diterpenes of *S. dulcis* revealed that these compounds are mainly accumulated in young leaves[9] and their production is closely related to the differentiation level of green leaves.[8,10] These observations indicated the involvement of MEP pathway in the biosynthesis of diterpenes in *S. dulcis*. It was supported by feeding experiments with $^{13}C$ labeled compound using shoot cultures of *S. dulcis*.[11] For further confirmation, the effects of specific inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, pravastatin,[12] and DOXP reductoisomerase (DOXR), fosmidomycin [3-(*N*-formyl)-*N*-hydroxyaminopropyl]phosphonic acid,[13] on the biosynthesis of isoprenoids in *S. dulcis* were studied.

To refine the involvement of the MEP pathway in the biosynthesis of diterpenes in *S. dulcis*, leaf organs were cultured in Murashige–Skoog (MS) liquid medium containing 0.1 $\mu$M of 4-pyridylurea (4-PU) with either pravastatin or fosmidomycin. Mevinolin, which is known as one of HMG-CoA reductase inhibitors, has been reported to inhibit tobacco BY-2 cell growth at a range of 0.1 to 5 $\mu$M.[16] When the leaf organs were treated with pravastatin or fosmidomycin at 1 $\mu$M, the tissue growth was significantly suppressed to 32.4% and 44.4%, respectively (data not shown). Thus, we set the drug concentration range from 1 to 100 nM to avoid the influence of toxicity. Treatment with pravastatin did not significantly inhibit tissue growth in the tested range (Table 1), while it resulted in darkening of leaf organs (data not shown). This color change was confirmed by a decrease in the contents of chlorophylls and carotenoids in the cultured tissues (Table 1). As indicated in Fig. 1A, pravastatin inhibited the production of $\beta$-sitosterol, the MVA-derived isoprenoid, in the cultured tissues in a dose-dependent manner.

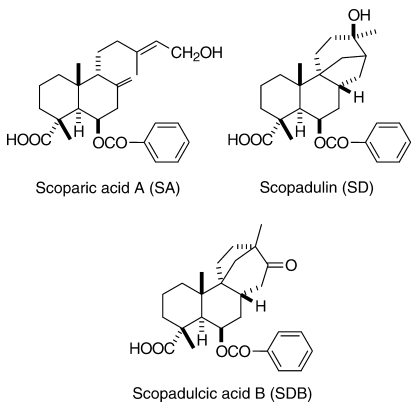


Chart 1

* To whom correspondence should be addressed. e-mail: hayashi9@ms.toyama-mpu.ac.jp

Table 1. Effect of Pravastatin on Tissue Growth and Content of Photosynthetic Pigments

| Concentration (nM) | Growth (mg fresh weight) | Chlorophylls ($\mu$g/g fresh weight) | Carotenoids ($\mu$g/g fresh weight) |
|---|---|---|---|
| 0 | 946.7±83.3 | 494.8±95.7 | 79.3±24.3 |
| 1 | 873.7±133.3 | 469.4±25.0 | 60.2±4.5 |
| 10 | 886.7±26.7 | 425.0±17.6 | 54.9±0.9 |
| 25 | 826.7±103.3 | 413.5±7.5 | 63.5±0.9 |
| 50 | 826.7±63.3 | 403.7±46.6 | 48.8±5.8 |
| 100 | 793.3±176.7 | 231.9±0.4* | 46.0±0.4 |

Leaf organ cultures treated for 10 d with different concentrations of pravastatin as indicated. Data are expressed as mean±S.E.; $n=3$. Significant difference was calculated from the control, $*p<0.05$.

Table 2. Effect of Fosmidomycin on Tissue Growth and Content of Photosynthetic Pigments

| Concentration (nM) | Growth (mg fresh weight) | Chlorophylls ($\mu$g/g fresh weight) | Carotenoids ($\mu$g/g fresh weight) |
|---|---|---|---|
| 0 | 946.7±83.3 | 494.8±95.7 | 79.3±24.3 |
| 1 | 893.3±43.3 | 355.4±37.8 | 78.5±11.3 |
| 10 | 676.7±33.3 | 265.9±7.8 | 67.5±0.6 |
| 25 | 723.3±63.3 | 222.9±23.1* | 64.3±18.5 |
| 50 | 683.3±76.0 | 212.8±36.7** | 64.2±0.6 |
| 100 | 710.0±50.0 | 199.7±11.1** | 42.4±2.9 |

Leaf organ cultures treated for 10 d with different concentrations of pravastatin as indicated. Data are expressed as mean±S.E.; $n=3$. Significant difference was calculated from the control, $*p<0.05$, $**p<0.01$.

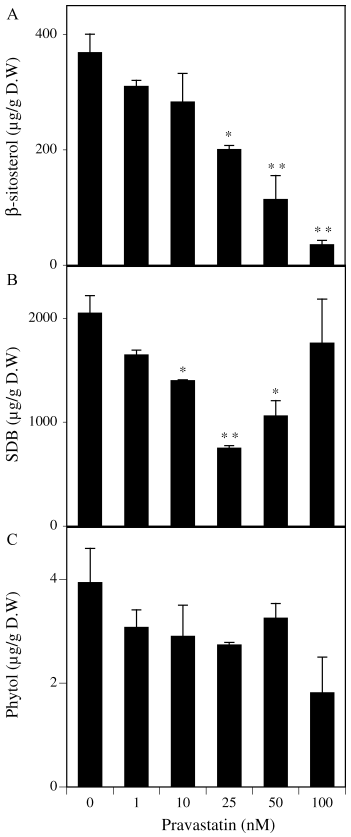


Fig. 1. Effect of Pravastatin on Production of Isoprenoids in Leaf Organ Cultures

Leaf organs were cultured in the presence of pravastatin as indicated. Tissues were harvested after 10 d and analyzed for their $\beta$-sitosterol (A), SDB (B), and phytol (C) contents. Data are expressed as mean±S.E., $n=3$. Significant differences were calculated from control, $*p<0.05$, $**p<0.01$.

The level of $\beta$-sitosterol in the presence of 50 and 100 nM of pravastatin decreased to about 30.7% and 12.7% respectively compared to the control. On the other hand, the content of SDB was pertubated in the tested range (Fig. 1B), while phytol content was not significantly affected (Fig. 1C). SDB production decreased in a dose-dependent manner at 1 to 25 nM in the presence of pravastatin although the content of SDB was recovered at 100 nM. Furthermore, the SDB content increased *ca.* 2- or 4-fold compared to the control in the presence of pravastatin at 1 and 10 $\mu$M, although tissue growth was severely suppressed (data not shown). It is uncertain why pertubation of SDB biosynthesis was observed with the addition of the HMG-CoA reductase inhibitor, pravastatin, which is not directly associated with diterpene biosynthesis. However, it has been reported that paclitaxel and baccatin III productions were inhibited by the addition of mevinolin, which is a HMG-CoA reductase inhibitor.[14] Furthermore, the crosstalk between the cytosolic MVA and the plastidial MEP pathways in *A. thaliana* and tobacco BY-2 cells have been reported.[15,16] Therefore, there might be a possibility that the plastidial IPP produced *via* MEP pathway could complement the biosynthesis of sterols and, as a result, the suppression of SDB production was observed. In contrast to above conditions, when pravastatin is added to the medium at a higher concentration than 50 nM, the strong inhibitory effect of pravastatin induced the depletion of the cytosolic IPP which could not be complemented by the plastidial IPP. In the presence of 100 nM pravastatin, the recovery of SDB production was observed. This might be explained by the results that the contents of phytol as well as chlorophylls relatively decreased compared to the control at this concentration. Phytol is an important component to survive the plants since it is a structural part of chlorophylls and carotenoids, which contribute to photosynthesis. Thus, we speculated that the significant inhibition of phytol production by pravastatin was not found in contrast to the case of SDB.

Culture of leaf organs in a medium containing fosmidomycin led to leaf bleaching (data not shown), which was confirmed by analysis of photosynthetic pigments accumulation. As indicated in Table 2, in the presence of fosmidomycin (100 nM), the level of chlorophylls decreased to about 40.4% of the control, and that of carotenoids decreased to about 53.5% of the control. Moreover, quantitative analyses of isoprenoids revealed a dose-dependent inhibition of SDB and phytol production. In comparison to untreated tissues, fosmidomycin-treated tissues (50, 100 nM) decreased in their SDB content to about 31.7% and 25.3%, respectively (Fig. 2B). The same tendency was observed when the phytol content was examined in the presence of the inhibitor (Fig. 2C), whereas the addition of fosmidomycin did not significantly affect the production of $\beta$-sitosterol (Fig. 2A). The etiolation of leaf organs and decrease in production of phytol and SDB in the tissues suggested the depletion of plastidial IPP consecutive to the addition of fosmidomycin. Together with previous results,[11] this finding provides evidence for an MEP-pathway acting as a biosynthetic route by which SDB is synthesized in *S. dulcis*. However, it was suggested that the biosynthetic routes of isoprenoids could not be clearly separated into cytosolic MVA- and plastidial MEP-pathways because treatment of leaf organ cultures of *S. dulcis* with

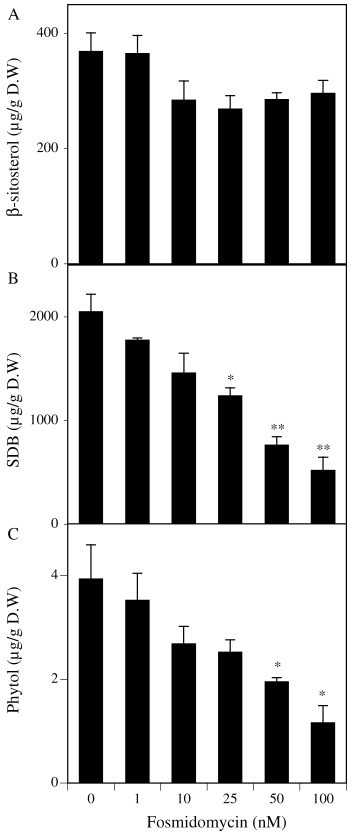


Fig. 2. Effect on Fosmidomycin on Production of Isoprenoids in Leaf Organ Cultures

Leaf organs were cultured in the presence of fosmidomycin as indicated. Tissues were harvested after 10 d and analyzed for their $\beta$-sitosterol (A), SDB (B), and phytol (C) contents. Data are expressed as mean±S.E., $n=3$. Significant differences were calculated from the control, $*p<0.05$, $**p<0.01$.

pravastatin influenced the production of SDB. Further exploration is required to elucidate the presence of IPP cross-talk between plastids and cytosol.

## Experimental

**Materials** 4-PU was kindly donated by Professor Kouichi Shudo, University of Tokyo. Pravastatin was donated by Professor Ushio Sankawa and fosmidomycin was obtained from Molecular Probes, Inc (Eugene, OR, U.S.A.). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

**General Procedures** Gas chromatography was performed using a GL-Science GC-353 gas chromatograph equipped with a FID. The GC column was a fused silica capillary column (SPB-1: 30 m×0.32 mm×0.25 $\mu$m film thickness) obtained from Supelco Inc. (Bellefonte, PA, U.S.A.). High performance liquid chromatography (HPLC) was performed on a HPLC system equipped with a L-7100 HPLC pump and a L-4000 UV spectrometric detector (Hitachi, Tokyo, Japan); a Cosmosil column $5C_{18}$-AR (4.6×150 mm, Nacalai Tesque, Kyoto, Japan).

**Plant and Tissue Culture** Seeds of *S. dulcis* were obtained from SDB-type plants grown in the herbal garden of University of Toyama. Seed germination, culture of seedlings and leaf organ cultures were performed as described by Hayashi *et al.*[8] In all experiments, about 400 mg of fresh leaf organs were transferred into a 100 ml culture flask containing 50 ml MS medium supplemented with 0.1 $\mu$M 4-PU and incubated on a rotary shaker at 140 rpm at 26 °C under continuous illumination (6000 lux). A known volume of filter-sterilized solutions of inhibitors was added into the culture media prior to inoculation of the leaf organs. Tissues were harvested after 10 d for the quantitative analyses.

**Quantification of Diterpene and Sterol Contents** In the quantitative analyses of SDB and $\beta$-sitosterol in *S. dulcis*, the cultured tissues were freeze-dried and extracted twice with $CHCl_3$–MeOH (3 : 1, 5 ml) under sonication for 20 min. The combined extract was dried and the residue was dissolved in $CHCl_3$ (2 ml). The resulting solution was subjected to solid phase extraction using a Bondesil-SI cartridge (500 mg, Varian Inc., Palo Alto, CA, U.S.A.). The column was eluted successively with $CHCl_3$ and MeOH. SDB eluted with MeOH was determined by HPLC method.[7] Phytol and $\beta$-sitosterol present in $CHCl_3$ eluate were measured using a GC method.[17]

**Quantitative Analyses of Chlorophylls and Carotenoids** The quantification of Chlorophylls (a+b) and total carotenoids was performed spectrometrically using the extinction coefficients reported previously.[18] The harvested tissues (100 mg) were homogenized with 80% acetone and pigments were extracted with an equal volume of hexane. The organic part was evaporated to dryness under reduced pressure and the residue was dissolved in 80% acetone, and spectrometrical analyses of pigments in the tissue were performed at 663.2, 646.8 and 470 nm.

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