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Flavanone 8-dimethylallyltransferase in *Sophora flavescens* cell suspension cultures

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Abstract

Dimethylallyl diphosphate: naringenin 8-dimethylallyltransferase (EC 2.5.1) was characterized. The enzyme was enantiospecific for (−)-(2*S*)-naringenin and utilized 3,3-dimethylallyl diphosphate as sole prenyl donor. It required Mg^{2+} (optimum concentration, 10 mM), and has an optimum pH of 9–10. The apparent K_m values for 3,3-dimethylallyl diphosphate and naringenin were 120 and 36 μM, respectively. The microsomal fraction prenylated several other flavanones at the C-8 position less effectively as compared with naringenin. Interestingly, when 2′-hydroxynaringenin was used as a prenyl acceptor, the 8-lavandulyl (sophoraflavanone G) and the 6-dimethylallyl derivatives were formed, together with the 8-dimethylallyl derivative, leachianone G. These results suggest that the 2′-hydroxy group of naringenin plays an important role for the formation of a lavandulyl group.

Keywords: *Sophora flavescens*; Leguminosae; Cell suspension cultures; Biosynthesis; Dimethylallyltransferase; Naringenin; Prenylated flavanones; Sophoraflavanone B; Sophoraflavanone G

1. Introduction

Cultured cells of *Sophora flavescens* Aiton produce prenylated flavanones, such as sophoraflavanone G (SFG) and lehmannin (7,2′,4′-trihydroxy-8-lavandulylflavanone) (Yamamoto et al., 1991). The cells also contain kushenol F, leachianone G (LG), 5,7,2′,4′-tetrahydroxy-6-dimethylallylflavanone and 7,2′,4′-trihydroxy-8-dimethylallylflavanone, in extremely small quantities, as well as lupalbigenin [6,3′-di-(3,3-dimethylallyl)-genistein] which has not yet been isolated from the original plant (Yamamoto et al., in preparation). A hydroxy group at C-2′ and either a 3,3-dimethylallyl or a lavandulyl group, an irregular monoterpenoid unit, at C-8 or C-6 are structural characteristics common to these flavanones. The production of these prenylated flavanones with a 2′-hydroxy group was stimulated by addition of elicitors, such as Cu^{2+}, glucomannans in yeast extracts and pectin (Yamamoto et al., 1995). Recently, we reported that cork tissues stimulated production of flavanones without any inhibitory effects on cell growth (Yamamoto et al., 1996). The studies on the mechanism for this stimulation by cork tissues prompted us to investigate the enzymes catalyzing the prenylation of flavanones. Several studies have been conducted on prenyltransferases of aromatic substrates, such as flavonoids of some types (Dhillon and Brown, 1976; Schröder et al., 1979; Biggs et al., 1987; Welle and Grisebach, 1991; Laflamme et al., 1993; Yamamoto et al., 1997), coumarins (Hamerski and Matern, 1988), *p*-hydroxybenzoate (Heide and Tabata, 1987; Mühlenweg et al., 1998) and olivetolate (Fellermeier and Zenk, 1998). However, nothing has so far been reported concerning the lavandulylation of flavanones. The following two possible

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mechanisms for the formation of a lavandulyl group are presumed: one is the direct lavandulylation of the flavanone skeleton and the other is the two-step dimethylallylation, the first to the flavanone skeleton and the second to the resultant prenyl side chain.

In order to clarify the mechanism for the attachment of a lavandulyl moiety onto flavanone skeleton, the prenyltransferases in *S. flavescens* cell suspension cultures were investigated and the results are discussed in this paper.

2. Results and discussion

2.1. Detection of naringenin 8-prenyltransferase activity

In examining the flavanone prenyltransferases in *S. flavescens* cells, naringenin and 2′-hydroxynaringenin were assumed to serve as prenyl acceptors, and 3,3-dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP) and lavandulyl diphosphate (LPP) as prenyl donors. Taking into account the occurrence of leachianone G (LG) and 7,2′,4′-trihydroxy-8-dimethylallylflavanone in *Sophora* cells, we chose DMAPP as a prenyl donor in the present study. We also adopted naringenin as a prenyl acceptor because the presence of hydroxy groups on flavonoid skeletons was reported not to be essential for the recognition of substrates by prenyltransferases (Schröder et al., 1979; Laflamme et al., 1993; Biggs et al., 1987; Yamamoto et al., 1996). Thus, the crude cell-free extract of *S. flavescens* cultured cells was incubated with 1 mM naringenin, 2 mM DMAPP and 10 mM $MgCl_2$ for 2 h at 25°C. After termination of the reaction with dil. HCl, the phenolic compounds extracted with EtOAc were analyzed by a reversed phase HPLC-photodiode-array system. A new peak whose UV absorption pattern was in complete agreement with that of 8-(3,3-dimethylallyl)-naringenin (sophoraflavanone B; 8-DMAN) was detected on the chromatogram. In the fractionation of the crude cell-free extract by the ultracentrifugation, more than 85% of the total prenylation activity was found in the twice-washed 100,000 $\times g$ pellets, indicating that the prenyltransferase was tightly bound to the membrane fraction of the cells (data not shown). The time course of the enzymatic reaction was linear for at least 180 min. After storage of the enzyme solution at −85°C for 2 weeks, more than 90% of the activity was retained (data not shown). To confirm the structure of the prenylated product, the microsomal fraction obtained from 300 g of the cultured cells was incubated with DMAPP, Mg^{2+} and commercially available naringenin which was optically inactive. Purification of the EtOAc extracts of the reaction mixture by prep. HPLC gave the product (14 mg), which was identified as 8-(3,3-dimethylallyl)-naringenin (8-DMAN) by comparison of its EIMS and ^{1}H NMR spectra with the data reported previously (Shirataki et al., 1988). Interestingly, 8-DMAN obtained, whose absolute configuration was determined to be 2*S* from its optical rotation, ($[\alpha]_D$ − 28°; Lit. −26° both in MeOH) (Shirataki et al., 1988). These results demonstrate that *S. flavescens* cultured cells contain dimethylallyl diphosphate: naringenin 8-dimethylallyltransferase, which is stereospecific for (2*S*)-naringenin.

2.2. Properties of prenyltransferase

The optimum pH of the enzymatic reaction was about 9–10 in Tris–HCl buffer and 10 in Glycine–NaOH buffer. The following experiments were carried out at pH 9, because, in alkaline solution, naringenin was liable to be converted into the corresponding chalcone. The enzyme required a divalent metal ion for the reaction and Mg^{2+} was most effective for activity. Mn^{2+} was also effective, though to a lesser extent (32% of Mg^{2+}), but addition of Ca^{2+} (6%), Co^{2+} (5%), Zn^{2+} (2%) or Cu^{2+} (1%) gave only very low activities (data not shown). Saturation for Mg^{2+} was reached at 10 mM. Optimal substrate concentrations were investigated using the microsomal fraction. Maximal conversion rates were observed at 10 mM of DMAPP 1 mM of naringenin, respectively and apparent $K_m$values for DMAPP and naringenin were determined to be 120 and 36 μM, respectively, from the Lineweaver-Burk plot (Fig. 1, insert).

2.3. Substrate specificity for prenyl diphosphate

Incubation of the microsomal fraction with Mg^{2+}, naringenin and IPP at pH 9 gave 8-dimethylallylnaringenin (8-DMAN) at a yield of only 1.3% of that obtained when DMAPP was used as prenyl donor. Incubation of the cell-free extracts or the microsomal fraction with LPP gave no prenylated product (data not shown). Accordingly, at pH 9, this prenyltransferase utilizes only DMAPP as the prenyl donor and introduces one dimethylallyl group solely into C-8 of naringenin.

2.4. Substrate specificity for flavanones

The prenylation activities of the twice-washed microsomal fraction for several flavanones were also investigated (Table 1). When liquiritigenin (5,7-dihydroxyflavanone), hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) and taxifolin (3,5,7,3′,4′-pentahydroxylfavanone) were used as substrates, the microsomal fraction catalyzed the transfer of a 3,3-dimethylallyl group to their C-8 positions less effectively compared with the case of naringenin, suggesting that the presence of hydroxy groups at 3-, 5- and 3′-

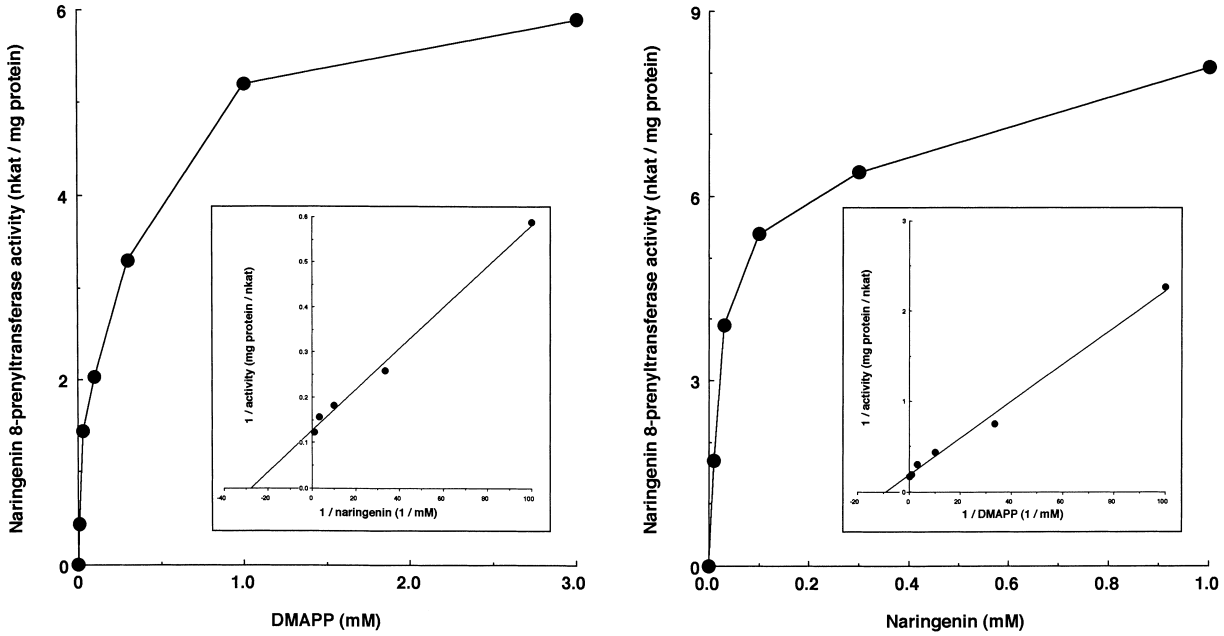


Fig. 1. Dependency of naringenin 8-dimethylallyltransferase activity on the concentration of dimethylallyl diphosphate and naringenin measured with a microsomal fraction of *S. flavescens* cultured cells. Insert: Lineweaver-Burk plot with varying concentrations (0.01–3.0 mM) of dimethylallyl diphosphate and varying concentrations (0.01–1.0 mM) of naringenin to calculate the apparent K_m value for DMAPP and naringenin, respectively.

positions were not essential for substrate recognition. When 2′-hydroxynaringenin was used, 8-dimethylallyl derivative, LG, was detected only in a very small quantity (3% of the yield of the corresponding derivative formed from naringenin). However, 8-lavandulyl-2′-hydroxynaringenin (SFG), along with the 6-dimethylallyl derivative (6-DMAHN), were produced four times more effectively than LG. This observation was quite different from that with the other flavanones mentioned above.

2.5. Substrate specificity for flavonoids other than flavanones

The specificity of the prenyltransferase for prenyl acceptors was examined with several flavonoids at 0.3

Table 1
Prenylation of flavonoids by microsomal fraction from *S. flavescens* cultured cells

Substrate (0.3 mM)	Relative activity (%)		
	8-Dimethylallyl	8-Lavandulyl	6-Dimethylallyl
Flavanones			
Naringenin	100.0[a]	–[b]	–
Liquiritigenin	35.3	–	–
Hesperetin	57.1	–	–
Taxifolin	42.2	–	–
2′Hydroxynaringenin	3.1	12.8	16.1
Other flavonoids			
Kaempferol	16.5	–	–
Quercetin	18.7	–	–
Apigenin	1.8	–	–
Genistein	–	–	80.6
Maackiain	–	–	11.6[c]

[a] Naringenin 8-prenyltransferase activity was 5.94 nkat/mg protein.
[b] Not detected.
[c] C-6 and C-3′ were prenylated.

mM concentration and pH 9 (Table 1). In this experiment, the microsomal fraction washed twice with Tris–HCl buffer were used to remove the different biotransformation of the substrates and any further metabolism of the products. The formation of prenylated flavonoids was monitored using a HPLC-photodiode-array system. Not only naringenin, but also kaempferol (5,7,4′-trihydroxyflavonol), quercetin (5,7,3′,4′-tetrahydroxyflavonol), apigenin (5,7,4′-trihydroxyflavone) were prenylated at their C-8 position. Genistein (5,7,4′-trihydroxyisoflavone) was also prenylated to afford wighteone (6-dimethylallylgenistein) and lupalbigenin [6,3′-di(dimethylallyl)-genistein], but maackiain, a pterocarpan was not prenylated. Taking into account that the microsomal fraction used in this experiment recognized the stereochemistry of C-2 of naringenin rigidly as mentioned above and the previous observation that kaempferol 8-prenyltransferase obtained from *Epimedium diphyllum* cultured cells did not accept naringenin as substrate (Yamamoto et al., 1997), it seems to be unlikely that naringenin 8-prenyltransferase catalyzes the prenylation reaction for all flavonoids examined. It is probable that the microsomal fraction contains more than one prenyltransferase, such as naringenin and kaempferol 8-prenyltransferases.

2.6. Activity for the formation of lavandulyl group in microsomal fraction

In the above-mentioned experiments, we adjusted the pH of the reaction mixture to 9 because it was the optimum for naringenin 8-prenyltransferase. However, it is reported that the optimum pH of many prenyltransferases from a variety of sources were around 7.5 (Dhillon and Brown, 1976; Heide and Tabata, 1987; Laflamme et al., 1993; Schröder et al., 1979; Biggs et al., 1987; Welle and Grisebach, 1991; Hamerski and Matern, 1988; Fellermeier and Zenk, 1998; Mühlenweg et al., 1998). Thus, we re-examined the prenylation activity for naringenin and 2′-hydroxynaringenin at pH 7.5. As shown in Fig. 2, when naringenin was used as the prenyl acceptor, only 8-DMAN was formed whereas when 2′-hydroxynaringenin was used, three products, LG, 6-DMAHN and SFG, were obtained. These observations, at pH 7.5, were similar to those at pH 9 except for the ratio of products. The total amounts of the prenylated derivatives formed from 2′-

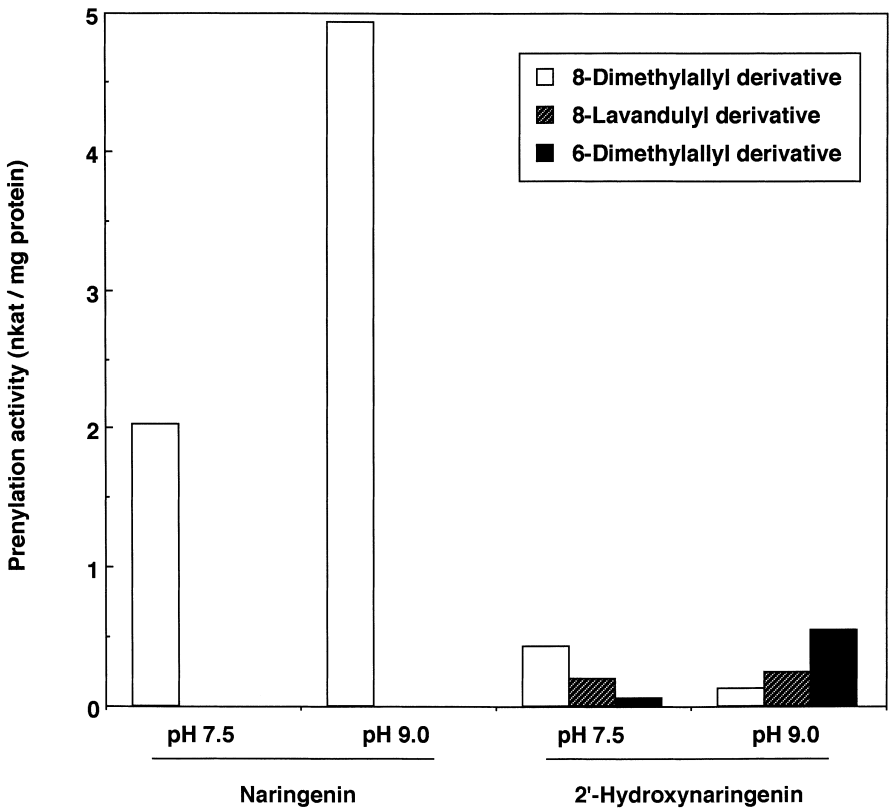


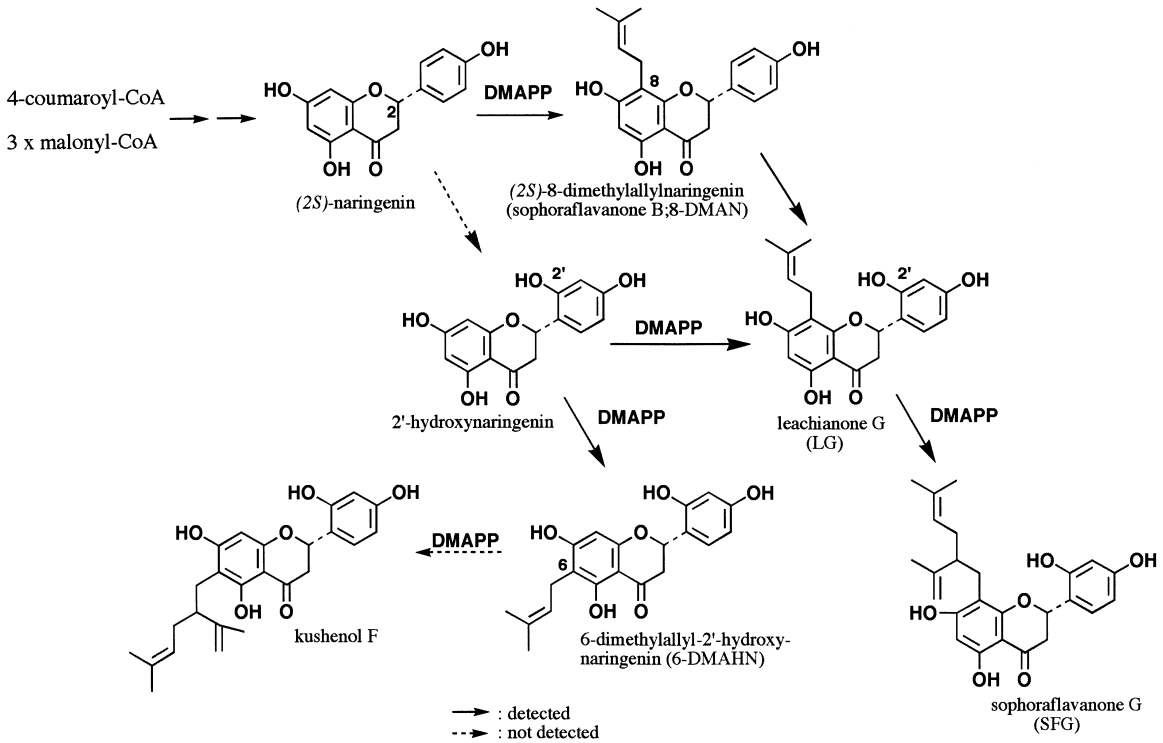
Fig. 2. Formation of 8-dimethylallyl-, 8-lavandulyl- and 6-dimethylallyl flavanones by a microsomal fraction of *S. flavescens* cultured cells. 0.3 mM of naringenin or 2′-hydroxynaringenin were incubated with the enzyme at pH 7.5 or 9 in the presence of 10 mM Mg^{2+} and 2 mM DMAPP.

hydroxynaringenin at pH 7.5 were almost equal to those at pH 9. However, at pH 7.5, the amount of the 8-prenylated (8-dimethylallyl and 8-lavandulyl) derivatives was superior to that of the 6-dimethylallyl derivative. On the contrary, at pH 9, the latter was predominantly formed. The possibility of the direct transfer of the lavandulyl group to 2′-hydroxynaringenin also was re-examined at pH 7.5 using LPP as the prenyl donor, but the 8-lavandulyl derivative was not detected (data not shown). These findings suggest that a lavandulyl group at C-8 of naringenin is formed by two-step dimethylallylations and that subsequent dimethylallylation at the prenyl group of C-8 and direct dimethylallylation at C-6 of flavanone skeleton requires hydroxylation at C-2′. Thus, the activities of hydroxylation at C-2′ of naringenin and 8-DMAN in the microsomal fraction of *S. flavescens* cultured cells were tentatively investigated using NADPH, NADH, FAD and FMN as cofactor. The hydroxylation activity of 8-DMAN was observed (data not shown), indicating the presence of a route from naringenin via 8-DMAN (naringenin–8-DMAN–LG–SFG) for the biosynthesis of sophoraflavanone G. However, the activity for naringenin was not detected although the microsomal fraction had the activities of prenylations at C-8 or C-6 of 2′-hydroxynaringenin. Nevertheless, it seems to be likely that the activity of hydroxylation at C-2′ of naringenin is present in *S. flavescens* cells, although very low, because of the low content of 6-prenylated 2′-hydroxynaringenin in *S. flavescens* cell cultures (Yamamoto et al., in preparation) and of the neccesity for the hydroxy group at C-2′ for prenylation at C-6. The possible biosynthetic pathways from naringenin to sophoraflavanone G and the enzymatic reactions detected in the present paper were summarized in Scheme 1. Detailed experiments concerning the hydroxylation at C-2′ in flavanones are now in progress.

3. Experimental

3.1. Chemicals

Dimethylallyl diphosphate and lavandulyl diphosphate were synthesized according to the method of Cornforth and Popjak (1969). 2′-Hydroxynaringenin was synthesized according to the method of He et al. (1988). Isopentenyl diphosphate was a kind gift from Dr. K. Yazaki, Graduate School of Agriculture, Kyoto University. Liquiritigenin was a kind gift from Dr. H. Hayashi, Niigata College of Pharmacy. Naringenin, hesperetin, taxifolin, kaempferol, apigenin, quercetin, and genistein were purchased from Funakoshi, Japan. Maackiain was isolated from cultured cells of *S. flavescens* (Yamamoto et al., 1991).



Scheme 1. Possible biosynthetic pathways leading to sophoraflavanone G in *S. flavescens* cultured cells.

3.2. Plant material and culture method

The origin and subculturing of callus cultures (Yamamoto et al., 1991) and the establishment of cell suspension cultures (Yamamoto et al., 1996) of *S. flavescens* were described in previous papers.

3.3. Enzyme preparation

For the enzyme preparation, all procedures were carried out at 4°C. *S. flavescens* cells (5 g) cultured for 8–10 days, were homogenized in 100 mM K–Pi buffer (pH 6.5) containing 10 mM DTT and 0.5 g PVPP using a teflon homogenizer. The homogenate was centrifuged at 12,000 $\times g$ for 20 min, then the supernatant was passed through a Sephadex PD-10 (Pharmacia) column equilibrated with 100 mM Tris–HCl buffer (pH 9) containing 10 mM DTT, and the eluate was used as the crude enzyme preparation. For the preparation of the microsomal fraction, the 12,000 $\times g$ supernatant was centrifuged at 100,000 $\times g$ for 20 min. The pellet was washed twice with the same buffer and then resuspended in 5 ml of the same buffer.

3.4. Protein content

Protein contents were determined according to the method of Bradford (1976).

3.5. Enzyme reaction

The incubation mixture contained 0.5 μmol naringenin, 1 μmol DMAPP, 5 μmol $MgCl_2$, and enzyme extract, 200 μl (ca. 0.35 mg protein) in a total vol. of 500 μl. DMAPP and $MgCl_2$ were dissolved in H_2O, respectively, and naringenin was dissolved in EtOH. The reaction was initiated by the addition of naringenin to the mixture and, after the incubation for 60 min at 25°C, terminated by the addition of 50 μl of 6 M HCl. The reaction mixture was extracted with 200 μl of EtOAc containing 0.1 mg/ml naphthalene as an internal standard. The EtOAc extract was analyzed by HPLC.

3.6. HPLC analysis

The amount of 8-prenylnaringenin was determined by HPLC using a Capcellpak C18 AG-120 column (4.6 × 250 mm, Shiseido, Japan) in an oven at 40°C, with CH_3CN/H_2O linear gradient solvent system containing 1% AcOH, from 40 to 58% CH_3CN in 24 min, at a flow rate of 0.9 ml/min, monitoring the absorption at 294 nm or using a photodiode-array SPDM-6A system (Shimadzu). The quantities were calculated from the peak area at 294 nm recorded by Chromatopac C-R4A (Shimadzu).

3.7. Isolation and identification of the reaction product

The microsomal fraction obtained from 300 g of fresh cells was incubated with 400 μmol naringenin, 800 μmol DMAPP and 4 μmol $MgCl_2$ in 100 mM Tris–HCl buffer (pH 9, total vol. 400 ml) at 25°C for 4 h. The reaction was terminated by the addition of 40 ml of 6 M HCl, and the products were extracted with EtOAc (3 × 200 ml). The organic layers were combined and concd. in vacuo. The residue was dissolved in MeOH and purified by use of prep. HPLC using the following conditions: column, Hikarisil C18-2E (20 × 250 mm, Asahi Chemical, Japan); solvent, 45% CH_3CN/H_2O; flow rate, 9.5 ml/min; oven temp., 40°C; detection, 294 nm. The fraction around Rt 49 min was collected and evaporated in vacuo to give 14 mg of sophoraflavanone B, identical with the authentic sample in the MS and ^1H NMR spectra. $[\alpha]_D 25-28^\circ$ ($c = 1.0$, MeOH).

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