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DIMETHYLALLYL DIPHOSPHATE: KAEMPFEROL 8-DIMETHYLALLYL TRANSFERASE IN *EPIMEDIUM DIPHYLLUM* CELL SUSPENSION CULTURES

HIROBUMI YAMAMOTO, JUNKO KIMATA, MASAYUKI SENDA and KENICHIRO INOUE

Department of Pharmacognosy, Gifu Pharmaceutical University, 6-1 Mitahora-higashi 5 chome, Gifu 502, Japan

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Key Word Index—*Epimedium diphyllum*; Berberidaceae; dimethylallyl transferase; cell suspension cultures; biosynthesis; des-*O*-methylanhydroicaritin; kaempferol; epimedoside A.

Abstract—The enzymatic formation of des-*O*-methylanhydroicaritin from dimethylallyl diphosphate and kaempferol was investigated in the cell-free extract of *Epimedium diphyllum* cell suspension cultures. The enzyme catalysing the dimethylallyl group transfer to C-8 position of kaempferol (EC 2.5.1.) was membrane-bound and required divalent cations such as Mg^{2+} and Mn^{2+}. Mg^{2+} was the most effective for the reaction. The enzyme showed a very broad pH optimum in the alkaline region, pH 7.5–11.0. It required dimethylallyl diphosphate as a sole prenyl donor, but had a rather broad substrate specificity for the prenyl acceptor. Not only kaempferol, but also quercetin (39%), apigenin (60%) and luteolin (34%) were prenylated, whereas kaempferol glycosides, naringenin and genistein were not prenylated. These results suggested that the prenylation reaction of kaempferol precedes the glycosylation step in epimedoside A biosynthesis. The apparent K_m values for dimethylallyl diphosphate and kaempferol were 0.58 and 0.13 mM, respectively. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Prenylated polyphenols are widely distributed in higher plant families such as the Berberidaceae, Boraginaceae, Compositae, Guttiferae, Leguminisae, Moraceae Rutaceae and Umbelliferae. However, reports of prenyltransferases for aromatic substrates are still rare [1–7]. On the other hand, we have reported that cell suspension cultures of *Epimedium diphyllum* produced a large amount of des-*O*-methylanhydroicaritin glycosides such as epimedoside A [8, 9]. The structural features of epimedoside A include one dimethylallyl group bound to the C-8 position of kaempferol, and rhamnosyl and glucosyl moieties bound to its C-3 OH and C-7 OH, respectively. Both the glycosylation and the prenylation reactions were regarded as later steps of its biosynthetic pathway [10], but the relationship of each step remained unknown. To clarify the order of prenylation and glycosylation, we investigated the prenyltransferase in *E. diphyllum* cell suspension cultures.

RESULTS AND DISCUSSION

Detection and stability of the prenyltransferase activity

The crude cell-free extract of *E. diphyllum* cell cultures prepared according to the procedure described in the Experimental were incubated with 1 mM kaempferol (5,7,4′-trihydroxyflavonol), 2 mM dimethylallyl diphosphate (DMAPP) and 10 mM $MgCl_2$ for 1 hr at 25°. After the termination of the reaction with HCl, phenolic compounds were extracted with EtOAc and analysed by a reverse phase HPLC-photodiode array system. In the chromatogram, a new peak whose retention time and UV absorption pattern were in complete agreement with those of des-*O*-methylanhydroicaritin was detected. To confirm the structure of this enzymatic reaction product, 35 μmol of kaempferol were incubated with DMAPP, Mg^{2+} and the cell-free extract, and the product was isolated by HPLC. The product was confirmed as 8-prenylated kaempferol, des-*O*-methylanhydroicaritin, by direct comparisons of EI-MS and ^{1}H NMR spectra with the authentic sample. This result demonstrated the presence of dimethylallyl diphosphate: kaempferol dimethylallyl transferase.

The reaction was dependent on the presence of intact enzyme, kaempferol, DMAPP and Mg^{2+} (Table 1). At 25°, the reaction was linearly dependent on time for 90 min (Fig. 1). In the buffer at 4°, the enzyme lost about 90% of its activity after 48 hr. However, after its storage at −80° for 2 weeks, more than 90% of the activity was retained.

pH optimum

As shown in Fig. 2, the crude enzyme exhibited an

Table 1. Formation of des-*O*-methylanhydroicaritin in incubations with crude cell-free extract from *Epimedium diphyllum* cultured cells

Incubation mixture	Des-*O*-methylanhydroicaritin (pkat/mg protein)	(%)
Complete assay	23.3	100.0
Cell-free extract	n.d.	0.0
Kaempferol	n.d.	0.0
DMAPP	0.04	0.2
Mg^{2+}	0.04	0.2
Heat-denaturated extract	0.03	0.1

n.d. = not detected.

unusually broad optimum pH in the alkaline region. The activity reached its maximum at pH 7.5, and was nearly constant in the higher pH conditions (to pH 11.0). In the following experiments, the enzymatic reaction was carried out in Tris–HCl buffer at pH 7.5 because of the instability of kaempferol and des-*O*-methylanhydroicaritin in the strong alkaline condition.

Substrate dependence

Optimal substrate concentrations were investigated using the crude cell-free extract. The optimal concentration for DMAPP was between 2 and 5 mM. Higher concentrations inhibited the reaction slightly (Fig. 3), as shown in the case of the geranylation reaction of *p*-hydroxybenzoic acid by geranyltransferase in the cell-free extract of cultured cells of *Lithospermum erythrorhizon* [7]. Welle and Grisebach revealed that dimethylallyl diphosphate: trihydroxypterocarpan dimethylally transferase in *Glycine max* was competitively inhibited by IPP [1]. The inhibition observed in *E. diphyllum* cells was also thought to be due to the same reason, because IPP could be formed from DMAPP by IPP isomerase present in the soluble enzyme fraction during incubation. The apparent K_m value for DMAPP was calculated as 0.58 mM from the Lineweaver–Burk plot with varying concentrations (0.01–3 mM; Fig. 3, insert). With kaempferol as the acceptor substrate, the enzyme activity increased up to a concentration of 5 mM. However, kaempferol started to precipitate at this concentration, and therefore 1 mM was routinely used in the following experiments (Fig. 4). The apparent K_m value for kaempferol was calculated as 0.13 mM based on the calculation in the above manner (0.01–1 mM; Fig. 4, insert).

Metal ion requirements

Table 2 shows the effects of several metal ions (10 mM) on the dimethylallyl transferase activity. Mg^{2+} was the most effective for the activity. Mn^{2+} (59% of Mg^{2+}) and to a lesser extent, Zn^{2+} (35%) and Co^{2+} (29%) were also effective in the reaction. Ca^{2+} (5%) and Cu^{2+} (2%) gave only minor activity. Saturation for Mg^{2+} was reached at 10 mM.

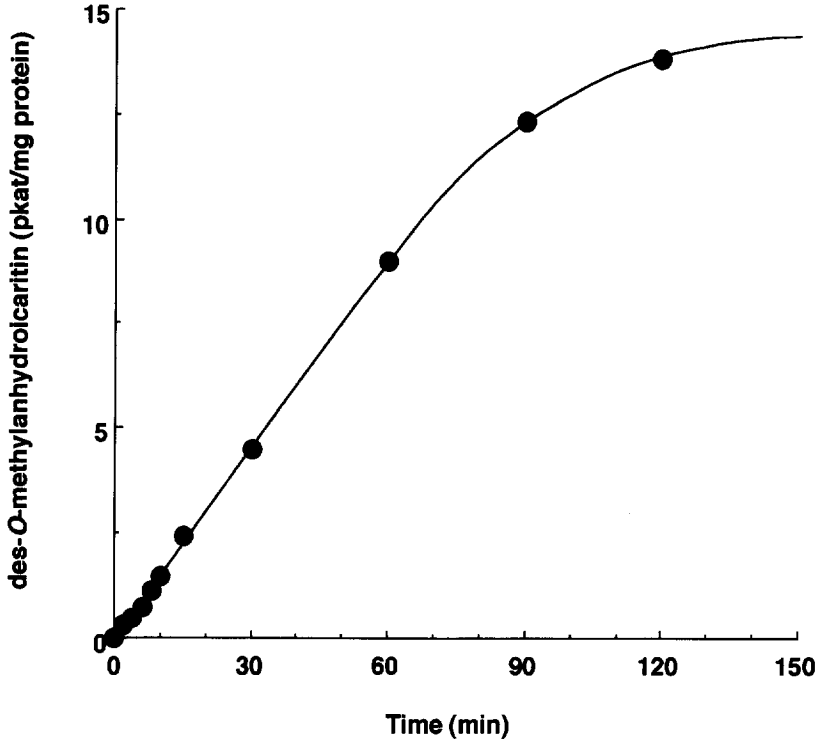

Fig. 1. Time-course of kaempferol 8-dimethylallyl transferase in crude cell-free extract of *E. diphyllum* cell suspension cultures measured under standard assay conditions.

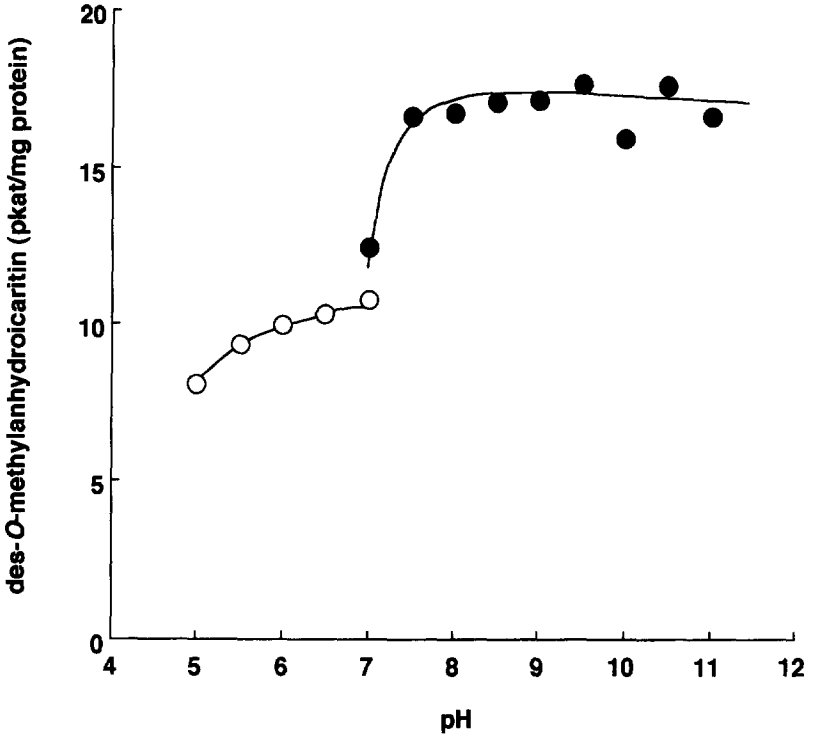

Fig. 2. pH dependency of kaempferol 8-dimethylallyl transferase activity. Crude cell-free extracts were used, and 100 mM sodium phosphate (○-○; pH 5–7) and 100 mM Tris–HCl (●-●; pH 7.5–11) were chosen to obtain the required pH conditions.

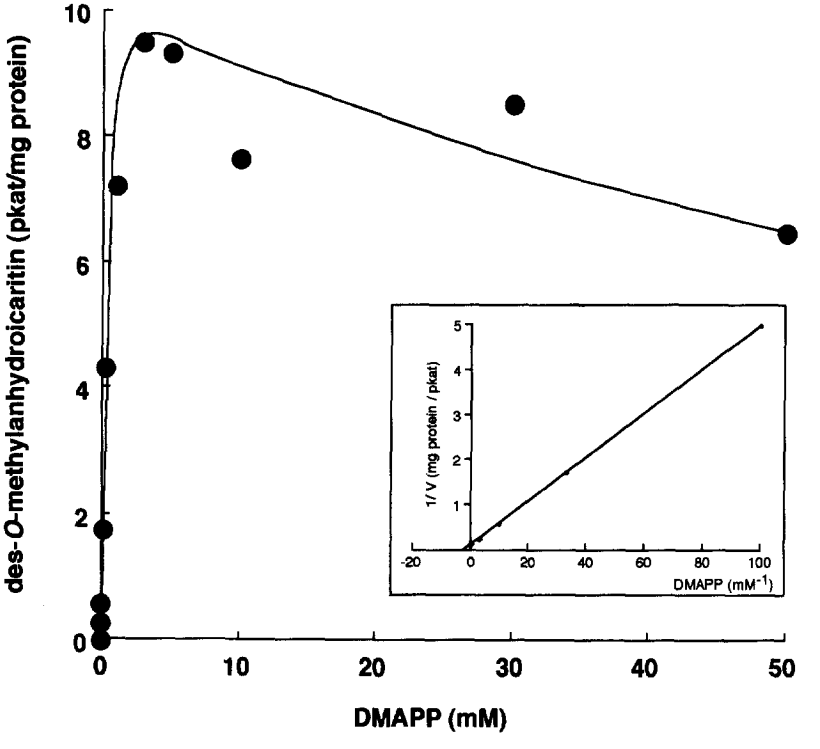

Fig. 3. Dependency of kaempferol 8-dimethylallyl transferase activity on the concentration of dimethylallyl diphosphate measured with crude cell-free extract. Insert: Lineweaver–Burk plot with varying concentrations (0.01–3 mM) to calculate the apparent K_m value for DMAPP.

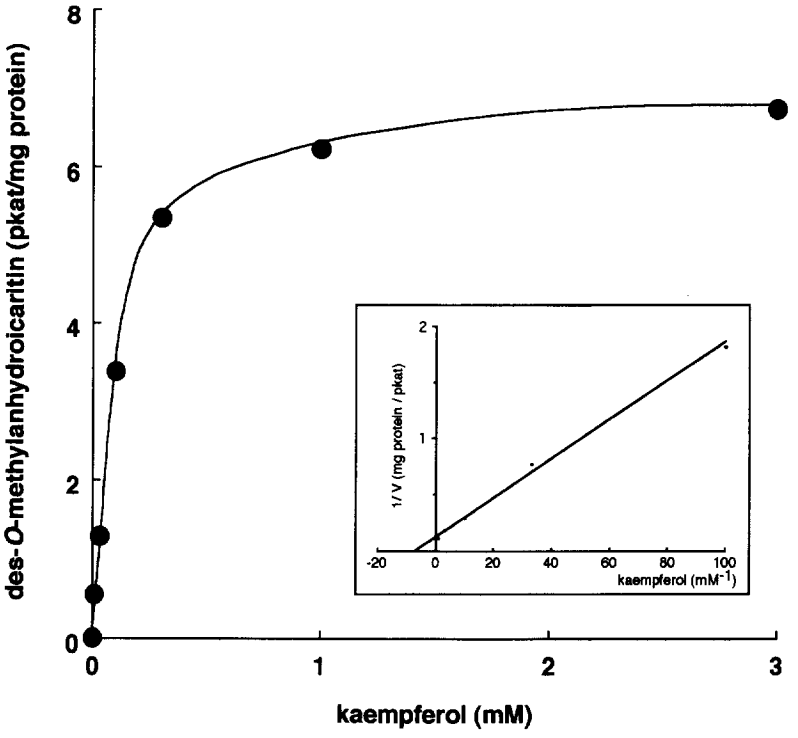

Fig. 4. Dependency of kaempferol 8-dimethylallyl transferase activity on the concentration of kaempferol measured with crude cell-free extract. Insert: Lineweaver–Burk plot with varying concentrations (0.01–1 mM) to calculate the apparent K_m value for kaempferol.

Table 2. Effect of different metal ions on kaempferol 8-dimethylallyltransferase

Metal ion (10 mM)	Des-*O*-methylanhydroicaritin	
	(pkat/mg protein)	(%)
Mg^{2+}	4.85	100.0
Mn^{2+}	2.87	59.1
Zn^{2+}	1.71	35.3
Cu^{2+}	0.10	2.1
Ca^{2+}	0.23	4.9
Co^{2+}	1.42	29.3

Crude cell-free extracts were used for this experiment.

Subcellular localization

As shown in Table 3, kaempferol prenyl transferase was membrane-associated, similar to other polyphenol prenyltransferases previously reported [1–7]. 37% of the total activity (24.9 pkat g-fr.-wt^{-1}) of the crude extract remained in the first supernatant; but after the second ultracentrifugation, the activities in the supernatant and the pellet were 1.6% (1.1 pkat g-fr.-wt^{-1}) and 70% (46.6 pkat g-fr.-wt^{-1}) of the total activity, respectively. The specific activity of the pellet of the second ultracentrifugation was 117 pkat mg-protein^{-1}, which was 8 times higher than that in the crude extract. The result that *ca* 40% of the total activity was recovered from the soluble enzyme fraction suggested that the enzyme may be attached to the membrane by a weak ionic bond, similar to umbelliferone dimethylallyltransferase in *Ruta graveolens* [4].

Substrate specificity for prenyl diphosphate

Incubation of the crude cell-free extract with Mg^{2+}, kaempferol and isopentenyl diphosphate (IPP) gave des-*O*-methylanhydroicaritin at a yield of 30% of that

Table 3. Subcellular localization of kaempferol 8-dimethylallyltransferase activity. Cells (5 g) were homogenized, and the microsomal preparation of the first centrifugation was resuspended in fresh buffer and was then recentrifuged

Fraction	Protein (mg g fr. wt^{-1})	Total activity (pkat g fr. wt^{-1})	(%)	Specific activity (pkat mg protein^{-1})	Purification factor
Crude cell-free extract	4.4	67.7	100.0	15.4	1.0
156 000 *g* Supernatant 1	3.9	24.9	36.8	6.4	0.4
156 000 *g* Supernatant 2	0.2	1.1	1.6	5.5	0.4
156 000 *g* Pellet 2	0.4	46.6	68.8	117.0	7.6

obtained with DMAPP. When the microsomal preparation were incubated with IPP, low amounts of des-*O*-methylanhydroicaritin were also formed, indicating that a soluble DMAPP-IPP isomerase was present in the crude cell-free extract. Incubation of the cell-free extract or microsomal preparation with lavandulyl diphosphate gave no prenylated products. Accordingly, this prenyltransferase utilizes only DMAPP as substrate.

Substrate specificity for flavonoids

Several flavonoids were examined as putative prenyl acceptors at 1 mM concentration. In this experiment, microsomal enzyme preparations were used to avoid the decomposition of the substrates or any further metabolism of the products. The formation of prenylated compounds was monitored using an HPLC-photodiode array system. When a new peak was observed, the corresponding compound was isolated by HPLC and examined for the presence of a prenyl group by EI-MS spectroscopy. The enzyme was also active with quercetin (5,7,3′,4′-tetrahydroxyflavonol), apigenin (5,7,4′-trihydroxyflavone) and luteolin (5,7,3′,4′-tetrahydroxyflavonol) as prenyl acceptor, although the position of prenylation is still unknown. The prenylation activities for quercetin, apigenin and luteolin were 39, 60 and 34%, respectively, of that obtained with kaempferol. Naringenin (5,7,4′-trihydroxyflavanone), genistein (5,7,4′-trihydroxyisoflavone) and *p*-hydroxybenzoic acid were not prenylated (data not shown). It is unlikely that different prenyltransferases exist in the microsomal preparation because neither quercetin nor apigenin derivatives have been isolated from the intact plant and from cell cultures of *E. diphyllum*. One prenyltransferase might catalyse the dimethylallyl moiety transfer to both flavonol and to flavone skeletons as shown in the case of flavonoid 7-*O*-glucosyltransferase in parsley cultured cells [11]. The existence of a hydroxyl group at C-3 or C′-3 positions in both skeletons might not be important for this prenyltransferase to recognize the substrate.

Substrate specificities for kaempferol glycosides were also investigated using kaempferol 3-*O*-galactoside, 3-*O*-rhamnoside (afzelin) and 3,7-*O*-dirhamnosides (kaempferitrin), but no prenylated products were detected (data not shown). It is well known that methylation, glycosylation and prenylation reactions usually take place at the later steps of flavonoid biosynthesis [10, 12]. Methylation reaction could occur either before or after glycosylation [12], but the order of glycosylation and prenylation has not yet been clarified. In *E. diphyllum* cells, the glycosyltransferases for des-*O*-methylanhydroicaritin have not been identified yet, but the above evidence demonstrates that the prenylation reaction precedes glycosylation.

EXPERIMENTAL

Chemicals. Dimethylallyl diphosphate and lavandulyl diphosphate were synthesized according to the method of ref. [13]. Isopentenyl diphosphate was a kind gift from Dr K. Yazaki, Faculty of Pharmaceutical Sciences, Kyoto University. Des-*O*-methylanhydroicaritin as the authentic sample was isolated from *Sophora flavescens* roots according to the procedure of ref. [14].

Plant material and culture method. The origin and subculturing of callus cultures [8] and the establishment of cell suspension cultures [9] of *E. diphyllum* have been described elsewhere.

Enzyme preparation. For enzyme extraction, all procedures were carried out at 4°. 5 g of 1-week-cultured *E. diphyllum* cells were homogenized in 100 mM Na-P_i buffer (pH 6.5) containing 10 mM DTT and 0.5 g PVPP by a Teflon homogenizer. The homogenate was centrifuged at 15 000 *g* for 20 min, then the supernatant was passed through the Sephadex PD-10 (Pharmacia) equilibrated with 100 mM Tris–HCl buffer (pH 7.5) containing 10 mM DTT to remove low M_r compounds and to exchange the buffer. This prepn was used directly for enzyme assay as the crude cell-free extract.

For subcellular localization, the supernatant of 15 000 *g* centrifugation was centrifuged at 156 000 *g* for 20 min. The pellet was resuspended in 10 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 10 mM DTT, and centrifuged again at 156 000 *g*, for 20 min. The pellet resuspended in the same buffer was used as the microsomal prepn.

Protein content. Protein contents were determined according to the method of ref. [15].

Enzyme reaction. The incubation mixt. contained in a total vol. of 500 μl: kaempferol, 0.5 μmol; DMAPP, 1 μmol; $MgCl_2$, 5 μmol; and enzyme extract, 200 μl (*ca* 0.35 mg protein). DMAPP and $MgCl_2$ were dissolved in Tris–HCl buffer, respectively, and kaempferol was dissolved in 0.1 M NaOH. The reaction was started by the addition of kaempferol to the mixt., incubated for 60 min at 25°, and was terminated by addition of 100 μl of 6 M HCl. The reaction mixt. was extracted twice with 500 μl of EtOAc. The EtOAc extracts were combined, concd under a stream of N_2 gas, dissolved in 100 μl MeOH and analysed by HPLC.

HPLC analysis. The amount of des-*O*-methylanhydroicaritin was determined by HPLC using Hikarisil C18 column (4.6 × 250 mm, Asahi Chemical Industry Co. Ltd, Japan) in an oven at 40°, with a 45% CH_3CN/H_2O solvent at a flow rate of 0.9 ml min^{-1}, monitoring the *A* at 272 nm or using a photodiode-array SPDM-6A system (Shimadzu). The quantities were calculated from the peak area at 272 nm recorded by Chromatopac C-R6A (Shimadzu).

Isolation and identification of the reaction product. The crude enzyme solution obtained from 5 g of cells (14 ml) was incubated with 35 μmol kaempferol, 70 μmol DMAPP and 350 μmol $MgCl_2$ in 100 mM Tris–HCl buffer (pH 7.5, total volume 35 ml) at 25° for 2 hr. After the incubation, the reaction was terminated by addition of 7 ml 6 M HCl, and the products were

extracted 3×50 ml EtOAc. The organic frs were combined and concentrated *in vacuo*. The residue was dissolved in MeOH and the product purified by use of HPLC using the following conditions: column, Hikarisil C18 (5 μm, 4.6×250 mm); solvent, 35% CH_3CN/H_2O; flow rate, 1 ml/min^{-1}; oven temp. 40°; detection, 272 nm. The retention time of the product was 48.8 min. The purified product was passed through Sephadex LH-20 short column (0.6×7.5 cm) with MeOH as a solvent. The product containing fraction was evaporated, and 44 μg of des-*O*-methylanhydroicaritin was obtained.

EI-MS. *m/z*; 354 (M^+), 339, 337, 299, 286, 121. ^{1}H NMR (500 MHz; TMS; acetone-d_6) δ 1.67, 1.82 (each *d*, 3H, $J = 1.2$ Hz, H_3-4″ and H_3-5″), 3.58 (broad *d*, 2H, $J = 6.6$ Hz, H_2-1″), 5.29 (*triple septet*, 1H, $J = 6.4$ and 1.4 Hz, H-2″), 6.38 (*s*, 1H, H-6), 7.04 (*d*, 2H, $J = 9.0$ Hz, H-3′, H-5′), 8.19 (*d*, 2H, $J = 9.0$ Hz, H-2′, H-6′), 12.10 (*s*, 1H, C-5 OH).

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