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Dalcochinin-8′-*O*-β-D-glucoside and its β-glucosidase enzyme from *Dalbergia cochinchinensis*

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Abstract

An isoflavonoid glucoside was isolated from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre), and the aglycone was shown to be 12-dihydroamorphigenin, which we named dalcochinin. Dalcochinin-8′-*O*-β-D-glucoside is well hydrolyzed by Thai Rosewood β-glucosidase (K_m 1.68 mM), but not by other β-glucosidases, such as cassava stem linamarase, almond β-glucosidase, or mustard seed myrosinase.

Keywords: *Dalbergia cochinchinensis*; Leguminosae; Thai Rosewood; ^{1}H-^{13}C NMR; enzymology; isoflavonoids; dalcochinin; β-glucosidase

1. Introduction

β-Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of β-*O*-glucosidic linkages formed between D-glucose and an aglycone or another sugar. There are many β-glucosidase enzymes with different substrate specificities and different functions (Esen, 1993), including hydrolysis of cyanogenic glycosides (Fan & Conn, 1985; Kuroki & Poulton, 1986; Eksittikul & Chulavatnatol, 1988; Pocsi, Kiss, Hughes, & Nanasi, 1989; Keresztessy, Kiss, & Hughes, 1994; Sermsuvityawong, Svasti, Sawangareetrakul, Kisamanonta, & Chulavatnatol, 1995), cellobiose (Ferreira & Terra, 1983), gentiobiose (Sano, Amemura, & Harada, 1975), phenolic glucosides (Podstolski & Lewak, 1970) and thioglucoside (Durham & Poulton, 1989).

Earlier, we reported the purification and properties of an enzyme with both β-glucosidase and β-fucosidase activities from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre) (Srisomsap, Svasti, Surarit, Champattanachai, Boonpuan, Sawangareetrakul, Subhasitanont, & Chokchaichamnankit, 1996) using P-nitrophenyl (P-NP) glycosides as substrates. Good hydrolysis was observed with both P-NP-β-D-glucoside and P-NP-β-D-fucoside (Srisomsap et al., 1996), and mixed kinetic studies (Surarit, Matsui, Chiba, Svasti, & Srisomsap, 1997) indicated that both activities originate from the same active site. However, commercially available natural substrates, such as cyanogenic glucosides, glucose disaccharides, and thioglucoside were poorly hydrolyzed, with none of the tested compounds showing a reaction rate higher than 5% of the reaction rate with P-NP-β-D-glucoside, when tested at 5 mM concentration (Srisomsap et al., 1996). In the present paper, we report the purification and structural characterization of a novel isoflavonoid β-glucoside from Thai Rosewood seeds, together with enzymatic studies which confirm that this compound is likely to be the natural substrate of the β-glucosidase enzyme previously described.

2. Results and discussion

Extraction of the ground seeds (10 g) of *Dalbergia cochinchinensis* Pierre with EtOH, followed by column chromatography on silica gel using absolute EtOH and preparative C_{18} reversed phase TLC using 70% aqueous MeOH gave compound **1** (0.171 g). Compound **1** was shown to be a β-glucoside by enzymatic hydrolysis with purified *D. cochinchinensis* β-glucosidase to give the aglycone **2** and D-glucose (analyzed by TLC and HPLC).

Positive FAB-Mass data of the aglycone **2** showed the signal at m/z 413 corresponding to $[M+1]^+$, hence a molecular weight of 412. The UV spectrum showed only a benzenoid chromophore (λ_{max}^{MeOH} 285 nm, ε 4000). ^{1}H

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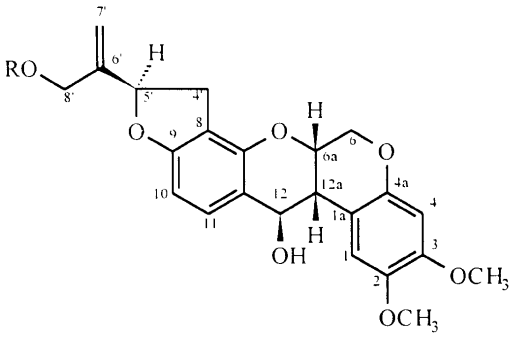

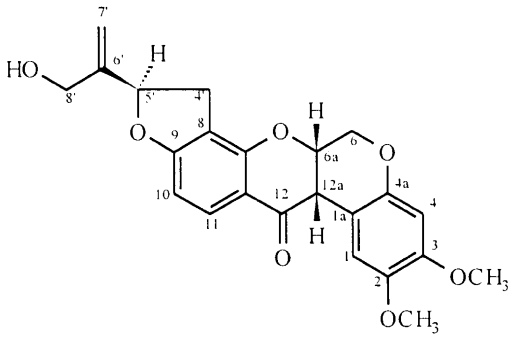

1: R = β-D-glucose
2: R = H

3

Structure 1

and ^{13}C-NMR data (Table 1) are similar to the spectral data of amorphigenin (**3**) (Konoshima, Terada, Kokumai, Kozuka, Tokuda, Ester, Li, Wang, & Lee, 1993). Replacement of the C=O group at C-12 of **3** with an OH group in **2** is reflected in the absence of a signal of the C=O group (at 189.0 ppm) in the ^{13}C-NMR spectrum. This was confirmed by its IR absorptions (KBr) at 3420 cm^{-1} (O-H) and 1090 cm^{-1} (C-O). The ^{1}H NMR spectrum of **2** shows the expected high-field shift of H-11 (δ 7.85 in **3** to 7.06 in **2**), H-12a (δ 3.85 in **3** to 3.35 in **2**) and shows a new signal of H-12 at δ 5.10. This data suggested that **2** is 12-dihydroamorphigenin. Analysis of COSY (H-H correlation and C-H correlation) led to the complete structure of **2**. 12-Dihydroamorphigenin is a novel isoflavonoid compound, which we propose to call dalcochinin after the name of the plant species, *Dalbergia cochinchinensis* Pierre, where it was first found naturally.

Positive FAB-Mass data of the novel β-glucoside **1** showed a signal at m/z 575 corresponding to $[M+1]^+$ and also showed signals at m/z 597, 557 and 395 corresponding to $[M+Na]^+$, $[M+1\text{-}H_2O]^+$ and $[M+1\text{-Glucose}]^+$, respectively. This spectral data suggested that the molecular weight of the β-glucoside **1** should be 574. ^{1}H and ^{13}C-NMR data are similar to **2** except for the presence of a more complicated pattern in the region δ 3.0-5.0. In addition to carbon signals similar to those of **2**, the ^{13}C-NMR spectrum of **1** shows six carbon signals belonging to glucose. This data suggests that **1** is dalcochinin-8′-*O*-β-D-glucoside or 12-dihydroamorphigenin-8′-*O*-β-D-glucoside.

The aglycone (**2**) is an isoflavonoid compound in the rotenoid family (Dewick, 1993). Previous reports of rotenoid-*O*-glycosides include 12a-hydroxyamorphigenin-8′-*O*-β-D-glucoside (dalbin) from the seed of *D. latifolia* (Chibber & Khera, 1979) and from the bark of *D. nitidula* Welw. ex. Bak. (van Heerden, Brandt, & Roux, 1980), dehydrodalpanol-*O*-β-D-glucoside from the seeds of *D. paniculata* (Rao & Rao, 1991), and amorphigenin-8′-*O*-β-D-glucoside from the seeds of *D. monetaria* (Abe, Donelly, Moretti, & Polonsky, 1985). Isoflavonoid compounds have also been found as C-glycosides in *Dalbergia* spp. (van Heerden et al., 1980; Rao & Rao, 1991) and as

Table 1
Hydrolytic activity of β-glucosidases towards various substrates*

Substrate (2 mM)	Thai Rosewood seed β-glucosidase Relative activity	Mustard seed myrosinase Relative activity	Cassava stem linamarase Relative activity	Almond β-glucosidase Relative activity
Gentiobiose	0.2	69.8	<0.1	0.5
Phloridzin	<0.1	72.4	<0.1	<0.1
Prunasin	<0.1	46.9	0.2	158.1
Sinigrin	<0.1	16,211	<0.1	<0.1
Linamarin	<0.1	47.1	53.1	<0.1
Amygdalin	2.0	55.6	<0.1	124.8
Salicin	1.5	58.2	0.5	11.6
Dalcochinin 8′-β-glucoside	184.2	75.0	0.1	0.1
p-NP-β-D-Glucoside	100.0	100.0	100.0	100.0

Reactions were performed in 0.1 M NaOAc, pH 5.0, 30°C for 10 min, and glucose released was quantitated using a glucose oxidase kit. Hydrolytic activities are expressed relative to the activity obtained with p-NP-β-D-glucoside.

various aglycones, including 12A-hydroxyamorphigenin (dalbinol) from *D. latifolia* seeds (Dewick, 1993).

The ability of *D. cochinchinensis* β-glucosidase and other β-glucosidases to hydrolyze the isolated dalcochinin-8′-*O*-β-D-glucoside (2 mM) was measured, and compared to their hydrolytic activities towards p-NP-β-D-glucoside (100%) and other natural substrates at 2 mM Table 1. *D. cochinchinensis* β-glucosidase shows high activity towards dalcochinin-8′-*O*-β-D-glucoside, with a relative rate of 184% compared to p-NP-β-D-glucoside, in contrast to the rates of <2% obtained with the other substrates. This relative rate of hydrolysis of the natural glycoside by *D. cochinchinensis* β-glucosidase is higher than the relative rates obtained for cassava stem linamarase with linamarin (53%) or for almond β-glucosidase with prunasin (158%) and amygdalin (125%). Moreover, dalcochinin-8′-*O*-β-D-glucoside is not well hydrolyzed by other β-glucosidases, giving relative hydrolysis rates of <0.2% for cassava linamarase and almond β-glucosidase. Although the rate of hydrolysis of dalcochinin-8′-*O*-β-D-glucoside with mustard seed myrosinase was 75% of the rate of hydrolysis of p-NP-β-glucoside, it is actually very low compared to the rate of hydrolysis of the natural substrate sinigrin, which gave a rate more than 160-fold higher than the rate of hydrolysis of p-NP-β-D-glucoside.

Kinetic studies yielded a K_m value of *D. cochinchinensis* β-glucosidase for dalcochinin-8′-β-D-glucoside (1.68 mM) that was intermediate between that for p-NP-β-D-glucoside (5.37 mM) and p-NP-β-D-fucoside (0.54 mM) (Srisomsap et al., 1996). Moreover, the V_{max} value for dalcochinin-8′-*O*-β-D-glucoside (452 nkat/ml) was slightly higher than that (423 nkat/ml) obtained with p-NP-β-D-glucoside, which was previously found to have a k_{cat} value about double that of p-NP-β-D-fucoside (Srisomsap et al., 1996). We have so far been unable to detect the presence of the β-fucoside derivative of **2**.

We estimate the content of dalcochinin-8′-*O*-β-D-glucoside in the seed to be as high as 3.5% (0.35 g per 10 g seed), based on the losses during purification and on quantitation of the compound in the crude extract by scanning of TLC plates. These values are much higher than the content of isoflavonoid compounds in most species, with the exception of dalbin and amorphigenin-8′-*O*-β-D-glucoside, which were reported in *D. monetaria* (Abe et al., 1985) at levels of 3.7% and 2.2% respectively. At the same time, we have also shown that *D. cochinchinensis* seed contains high levels (3 mg enzyme per 10 g seed) of a β-glucosidase enzyme active against dalcochinin-8′-O-β-D-glucoside. Neither the glycoside (**1**) nor the aglycone (**2**) could be detected in other tissues of the plant, such as stem, leaf, petiole or shoot tip (data not shown), and the levels of β-glucosidase in these other tissues is 1% or less than the level found in the seed (Srisomsap et al., 1996). The high levels of both enzyme and substrate in the seed suggest that both play important biological roles in the seed, possibly as a defence against insect or other predators.

3. Experimental

3.1. Materials and reagents

Seeds of *D. cochinchinensis* Pierre (Thai Rosewood) were obtained from the ASEAN-Canada Forest Tree Seed Center, Muaklek, Saraburi, Thailand. Thai Rosewood glucosidase/β-fucosidase was purified from seeds by $(NH_4)_2SO_4$ fractionation, preparative isoelectric focusing pH 4-6, and Sephadex G-150 chromatography (Srisomsap et al., 1996). Cassava linamarase was purified from cassava stem by $(NH_4)_2SO_4$ fractionation and Sepharose 4B chromatography (Dewick, 1993). p-NP-Glycosides, gentiobiose, linamarin, amygdalin, phloridzin, salicin, sinigrin, mustard seed myrosinase, and almond β-glucosidase were from Sigma Chemical Co., USA.

3.2. General chemical techniques

Analytical and preparative TLC were carried out on precoated silica gel (Merck 60 F-254) and C_{18} reverse phase plates (Merck RP-18). Components were detected by spraying with conc. H_2SO_4. HPLC was performed on a Waters 625LC instrument: separation of aglycone and glycoside employed UV detection using a C_{18} (Lichrosphere WP 300A) column (244 × 4 mm) eluted with a water:MeOH gradient from 70:30 to 30:70, while sugar analysis employed refractive index detection using a Biorad HPX-87C column (300 × 7.8 mm) eluted with water. All mp's were taken on a Fisher-Jones apparatus and are uncorrected. IR and UV spectra were recorded on Perkin-Elmer System 2000 and Lambda 6 spectrometers, respectively. ^1H and ^{13}C-NMR spectra were obtained on a 400 MHz Bruker Aspect 3000 spectrometer. All chemical shifts are reported in δ (ppm) from TMS. Mass was determined on a Finnigan MAT-90 mass spectrometer.

3.3. Extraction of natural substrate from Thai Rosewood seeds

Dried *D. cochinchinensis* Pierre seeds (10 g) were blended at low speed for 3 s in a Waring Blender. Seed coats were removed, and ground seeds were extracted with 20 ml of absolute EtOH overnight at room temp. The EtOH extract was centrifuged at 10,000 × *g* at 25°C for 10 min to remove solids and yield the crude extract. The natural substrate was identified as a compound which, after incubation with purified Thai Rosewood β-glucosidase, had increased mobility on silica gel TLC with $CHCl_3$:MeOH:water (15:3:1) as solvent. The remainder of the crude extract (0.97 g) was concentrated to 3.6 ml by rotary evaporation and then chromatographed on a

silica gel 60 (230-400 mesh, Merck) column (1.3 × 37 cm) using absolute EtOH as an eluent. Fractions from the column were analyzed by silica gel TLC, and those containing natural substrate were pooled (0.314 g), concd by rotary evaporation, and further purified by prep C_{18} reversed phase TLC (RP-18, 1 mm) using 70% aqueous MeOH as solvent to yield compound (**1**) (0.171 g). The purity of the final product (**1**) was checked by TLC on silica gel 60 F-254 using $CHCl_3$:MeOH:water (15:3:1 by vol.) and *n*-BuOH:EtOH:water (5:3:2 by vol.). Compound **2** was prepared by incubating compound **1** (30 mg) with purified *D. cochinchinensis* β-glucosidase at 37°C for 15 h in 0.1 M NaOAc buffer, pH 5.0. The reaction mixture was extracted with EtOAc, and the organic extract was washed, dried and evaporated. The EtOAc residue was purified by prep TLC (silica gel 60 G, 2 mm) to give the aglycone **2**.

3.4. Enzyme assays

Hydrolytic activity of various β-glucosidases was studied by incubating each enzyme with various substrates (2 mM) in 0.1 ml of 0.1 M NaOAc buffer, pH 5.0 at 30°C. Reactions were stopped after 10 min by adding 0.1 ml of 2 M Tris-HCl buffer, pH 7.0, and glucose released was estimated by adding 1.0 ml of a glucose oxidase reagent kit (BM Laboratories, Bangkok, Thailand), followed by measuring A_{505}. DMSO (5% v/v, final concentration) was used in assays of less soluble substrates, namely the natural substrate and phloridzin. Kinetic studies were carried out in a similar manner except that substrate concentrations were varied between 0.2 mM to 4 mM for the natural substrate and between 0.5 mM to 16 mM for p-NP-β-D-glucoside. Kinetic constants were determined by using the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.).

3.4.1. Compound *1*

Crystallized from MeOH as an amorphous solid, mp 121–123°C; FAB-MS (*m*/*z*): 575 $(M+1)^+$, 597 $[M+Na]^+$, 557 $[M+1\text{-}H_2O]^+$and 395 [M + 1-Glucose]$^+$; ^{1}H NMR: δ [$CDCl_3$] 6.66 (1H, *s*, aromatic =C*H*), 6.39 (1H, *s*, aromatic =C*H*), 4.23 (1H, *d*, *J* = 12 Hz, O-CH_2), 4.56 (1H, *dd*, *J* = 3 Hz, O-CH_2), 4.79 (1H, broad *t*, OC*H*CH_2), 6.34 (1H, *d*, *J* = 8 Hz, aromatic =C*H*), 7.06 (1H, *d*, *J* = 8 Hz, aromatic =C*H*), 5.10 (1H, *d*, *J* = 2 Hz, HO-C*H*), 3.35 (1H, broad *s*, HOCHC*H*), 2.99 (1H, *dd*, *J* = 8,15 Hz, aromatic -CH_2), 3.30 (1H, *dd*, *J* = 8,15 Hz, aromatic -CH_2), 5.27 (1H, *t*, *J* = 8 Hz, aromatic -OC*H*CH_2), 5.21 (2H, *s*, =CH_2), 4.17 (2H, broad *s*, HO-CH_2), 3.75 (3H, *s*, OCH_3), 3.78 (3H, *s*, OCH_3), 4.45 (1H, D, *J* = 7.7 Hz, *H*-1 glucose). ^{13}C NMR: δ [$CDCl_3$] 110.5 (*C*-1), 143.3 (*C*-2), 148.9 (*C*-3), 100.8 (*C*-4), 148.2 (*C*-4a), 67.2 (*C*-6), 65.7 (*C*-6a), 160.8 (*C*-7a), 114.0 (*C*-8), 176.2 (*C*-9), 102.3 (*C*-10), 130.0 (*C*-11), 65.7 (*C*-12), 37.7 (*C*-12a), 102.3 (*C*-1a), 32.2 (*C*-4′), 84.3 (*C*-5′), 147.6 (*C*-6′), 111.4 (*C*-7′), 67.2 (*C*-8′), 55.7 (OCH_3), 56.6 (OCH_3), 102.1 (C-1 D-glucosyl), 73.4 (C-2 D-glucosyl), 76.3 (C-3 D-glucosyl), 70.0 (C-4 D-glucosyl), 75.7 (C-5 D-glucosyl), 69.5 (C-6 D-glucosyl).

3.4.2. Compound *2*

Crystallized from EtOAc-MeOH as needles, mp 171-172°C; UV (λ_{max}^{MeOH} 285 nm); FAB-MS (*m*/*z*): 413 $(M+1)^+$; FT-IR (KBr): cm^{-1} 3420 (O-H) and 1090 (C-O); ^{1}H NMR: δ [$CDCl_3$] 6.66 (1H, *s*, aromatic =C*H*), 6.39 (1H, *s*, aromatic =C*H*), 4.23 (1H, *d*, *J* = 12 Hz, O-CH_2), 4.56 (1H, *dd*, *J* = 3 Hz, O-CH_2), 4.79 (1H, broad *t*, OC*H*CH_2), 6.34 (1H, *d*, *J* = 8 Hz, aromatic =C*H*), 7.06 (1H, *d*, *J* = 8 Hz, aromatic =C*H*), 5.10 (1H, *d*, *J* = 2 Hz, HO-C*H*), 3.35 (1H, broad *s*, HOCHC*H*), 2.99 (1H, *dd*, *J* = 8,15 Hz, aromatic -CH_2), 3.30 (1H, *dd*, *J* = 8,15 Hz, aromatic -CH_2), 5.27 (1H, *t*, *J* = 8 Hz, aromatic -OC*H*CH_2), 5.21 (2H, *s*, =CH_2), 4.17 (2H, broad *s*, HO-CH_2), 3.75 (3H, *s*, OCH_3), 3.78 (3H, *s*, OCH_3). ^{13}C NMR: δ [$CDCl_3$] 110.5 (*C*-1), 143.3 (*C*-2), 148.9 (*C*-3), 100.8 (*C*-4), 148.2 (*C*-4a), 67.2 (*C*-6), 65.7 (*C*-6a), 160.8 (*C*-7a), 114.0 (*C*-8), 176.2 (*C*-9), 102.3 (*C*-10), 130.0 (*C*-11), 65.7 (*C*-12), 37.7 (*C*-12a), 102.3 (*C*-1a), 32.2 (*C*-4′), 84.3 (*C*-5′), 147.6 (*C*-6′), 111.4 (*C*-7′), 67.2 (*C*-8′), 55.7 (OCH_3), 56.6 (OCH_3).

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