Acetophenone Diglycosides from *Erythroxylum cambodianum*

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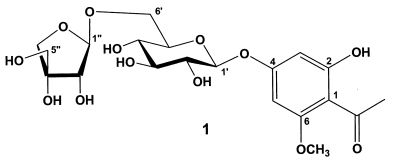
Two new acetophenone diglycosides, erythroxylosides A and B, were isolated from the aerial portion of *Erythroxylum cambodianum* together with (+)-catechin, (−)-epicatechin, quercetin 3-*O*-rutinoside, (3*S*,5*R*,6*R*,7*E*,9*S*-megastigman-7-ene-3,5,6,9-tetrol 3-*O*-β-D-glucopyranoside and citroside A. The structural elucidations were based on analyses of chemical and spectroscopic data.

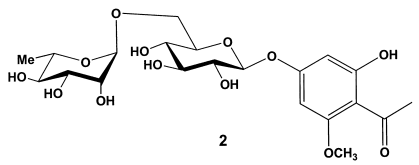
Key words *Erythroxylum cambodianum*; Erythroxylaceae; megastigmane glycoside; acetophenone glycoside; erythroxyloside A; erythroxyloside B

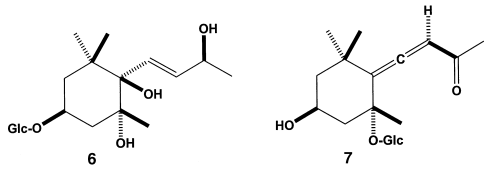
Erythroxylum cambodianum PIERRE (Erythroxylaceae, Thai name: Huun-Hai) is a shrub that grows up to 1.5 m high, distributed in Northeastern Thailand. It is used in Thai traditional medicine for anti-fever purposes, as well as an anti-inflammatory agent. In the course of our continuing studies on Thai medicinal plants, the constituents of this plant were investigated. To the best of our knowledge, no phytochemical study has been carried out on this species. However, previous phytochemical investigation of plants in this genus showed the presence of alkaloids, flavonoids and triterpenoids.[1—4] The present paper deals with the isolation and structural determination of seven compounds, including two new acetophenone glycosides (**1**, **2**), two known flavans (**3**, **4**), one known flavonol glycoside (**5**), and two known megastigmane glucosides (**6**, **7**) from the aerial portion of this plant.

Results and Discussion

The methanolic extract was suspended in H_2O and defatted with Et_2O. The *n*-BuOH soluble fraction was subjected to a Diaion HP-20 column, using H_2O, 50% aqueous MeOH, MeOH and Me_2CO successively. The portion eluted with MeOH was repeatedly chromatographed on columns of silica gel, RP-18, and preparative HPLC-ODS to afford 7 compounds. Five were identified as the known compounds (+)-catechin (**3**),[5] (−)-epicatechin (**4**),[5] quercetin-3-*O*-rutinoside (**5**),[6] (3*S*,5*R*,6*R*,7*E*,9*S*-megastigman-7-ene-3,5,6,9-tetrol 3-*O*-β-D-glucopyranoside (**6**),[7] and citroside A (**7**),[8] by comparison of physical data with values reported in the literature and from spectroscopic evidence.







Erythroxyloside A (**1**) was isolated as an amorphous powder, and determined to be $C_{20}H_{28}O_{13}$ by negative high-resolution (HR)-FAB mass spectrometry. The ^{1}H-NMR spectrum revealed the presence of the signals of a pair of doublets with *meta*-splitting pattern at δ_H 6.19 and 6.17 (J=2.2 Hz), two doublet anomeric signals at δ_H 4.92 (J=2.4 Hz) and 4.90 (J=7.8 Hz), one singlet methoxyl group at δ_H 3.85, and one singlet acetyl group at δ_H 2.53. The ^{13}C-NMR spectrum displayed 20 carbon signals, of which nine were assignable to three oxy-aryl carbons at δ_C 167.7, 165.4 and 164.6, two aryl-methines at δ_C 97.7 and 93.1, one non-protonated aryl carbon at δ_C 107.8, one methoxyl group at δ_C 56.4, and two carbon signals of an acetyl group at δ_C 204.9 and 33.2 for the aglycone part, indicative of an acetophenone derivative.[9] The remaining carbon signals belonged to the sugar part, and were identified as β-apiofuranosyl-(1→6)-β-glucopyranosyl unit by comparing chemical shifts with the reported data.[9] All protonated carbons were assigned by the results from heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond connectivity (HMBC) spectra. Enzymatic hydrolysis of **1** with crude hesperidinase gave 2,4-dihydroxy-6-methoxy-acetophenone (**1a**), identified by ^{1}H- and ^{13}C-NMR spectral analysis,[10] in addition to the sugar fraction from the aqueous layer. The stereochemistry of an apiose could be assigned as D-form by the coupling of H-5″ at δ_H 3.54, which showed the magnetic equivalent.[11] The absolute configuration of a glucose was determined as D-form by treating the sugar fraction with L-cysteine methyl ester in pyridine to provide the thiazolidine derivatives of sugars, and then comparing the *Rf* values with the standard sample of thiazolidine derivative of D-glucose [methyl 2-(D-*gluco*-pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate], which was prepared by the previously reported method (see Experimental).[12,13] Two spots of the derivative from D-glucose were found on TLC (*Rf*: 0.42, 0.49), due to the C-2 epimers of thiazolidine derivatives. The location of the sugar moiety was

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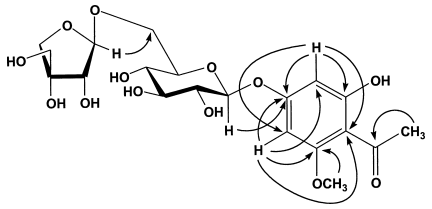

Fig. 1. Significant HMBC Correlations of Erythroxyloside A (**1**)

assigned by nuclear overhauser effect spectroscopy (NOESY) experiment. Since the signals of two aryl protons appeared at δ_H 6.17 and 6.19 (in CD_3OD), it was quite difficult to observe the NOESY correlations. The NMR spectrum of compound **1** was re-measured in C_5D_5N to provide the clear signals of two aryl protons at δ_H 6.27 and 6.69. The NOESY correlation was found between δ_H 5.60 (H-1′ Glc) and δ_H 6.27 (H-3), 6.69 (H-5), indicating that the sugar part was attached at C-4. Moreover, HMBC spectrum provided further confirmation of the structure including location of an apiose residue as illustrated in Fig. 1. Consequently, the structure of compound **1** was concluded to be 2,4-dihydroxy-6-methoxy-acetophenone 4-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside.

Erythroxyloside B (**2**) was obtained as an amorphous powder. Its molecular formula was determined as $C_{21}H_{30}O_{13}$ by negative HR-FAB mass spectrometry. The ^{1}H- and ^{13}C-NMR spectral data were very similar to those of erythroxyloside A (**1**), except for the difference in the chemical shifts of the terminal sugar, which was identified as *α*-L-rhamnopyranose (Tables 1, 2). This sugar moiety was located at C-6′ of the glucosyl moiety due to the downfield shift of this carbon signal at *δ* 67.7, and was confirmed by the HMBC spectrum. Enzymatic hydrolysis of **2** with crude hesperidinase also gave 2,4-dihydroxy-6-methoxy-acetophenone (**1a**) together with a sugar fraction from the aqueous layer. The absolute configurations of sugars were determined by comparison of the *Rf* values of the treated sugar fraction with standard samples of thiazolidine derivatives of D-glucose and L-rhamnose, which were prepared in the same way as described above. Four spots were observed, and identified to be methyl 2-(D-*gluco*-pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate (*Rf*: 0.42, 0.49), and methyl 2-(L-rhamno-tetrahydroxypentyl)-thiazolidine-4(*R*)-carboxylate (*Rf*: 0.61, 0.68), both cases due to the C-2 epimers of thiazolidine derivatives. Therefore, the structure of erythroxyloside B (**2**) was elucidated as 2,4-dihydroxy-6-methoxy-acetophenone 4-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside.

Table 1. ^{1}H-NMR Spectral Data of Erythroxylosides A (**1**) and B (**2**) (400 MHz, CD_3OD)

No.	**1**	**2**
3	6.19 (1H, d, *J*=2.2 Hz)	6.16 (1H, d, *J*=2.2 Hz)
5	6.17 (1H, d, *J*=2.2 Hz)	6.16 (1H, d, *J*=2.2 Hz)
8	2.53 (3H, s)	2.53 (3H, s)
MeO-6	3.85 (3H, s)	3.83 (3H, s)
1′	4.90 (1H, d, *J*=7.8 Hz)	4.92 (1H, d, *J*=7.3 Hz)
2′	} 3.26—3.42 (3H)[a]	} 3.26—3.57 (4H)[a]
3′		
4′		
5′	3.58 (1H, m)	
6′	3.99 (1H, br d, *J*=9.7 Hz)	3.98 (1H, br d, *J*=9.5 Hz)
	3.60 (1H, dd, *J*=9.7, 3.4 Hz)	3.64 (1H, d, *J*=9.5, 3.4 Hz)
1″	4.92 (1H, d, *J*=2.4 Hz)	4.65 (1H, br s)
2″	3.86 (1H, d, *J*=2.4 Hz)	} 3.53—3.64 (4H)[a]
3″	—	
4″	3.96 (1H, d, *J*=9.8 Hz)	
	3.71 (1H, d, *J*=9.8 Hz)	
5″	3.54 (2H, s)	
6″		1.15 (3H, d, *J*=6.3 Hz)

a) Signal pattern unclear due to overlapping.

Table 2. ^{13}C-NMR Spectral Data of Erythroxylosides A (**1**) and B (**2**) (100 MHz)

No.	**1**[a]	**1**[b]	**2**[a]	**2**[b]
1	107.8	107.3	107.8	107.3
2	167.7	167.5	167.7	167.6
3	97.7	97.7	97.7	97.7
4	165.4	165.1	165.3	165.0
5	93.1	92.4	93.0	92.4
6	164.6	163.6	164.5	163.5
7	204.9	203.7	204.9	203.7
8	33.2	33.1	33.2	33.1
MeO-6	56.4	56.0	56.4	55.9
1′	101.3	101.7	101.2	101.7
2′	74.7	74.8	74.6[c]	74.8[c]
3′	77.8[c]	78.5[c]	77.8[d]	78.5[d]
4′	71.5	71.6	71.3	71.5
5′	77.2[c]	77.6[c]	77.1[d]	77.6[d]
6′	68.9	69.1	67.6	67.7
1″	111.1	111.2	102.1	102.5
2″	78.1	77.9	72.3[e]	72.9[e]
3″	80.5	80.5	72.0[e]	72.2[e]
4″	75.0	75.2	74.0[c]	74.2[c]
5″	65.7	65.6	69.8	70.0
6″			17.9	18.1

a) Measued in CD_3OD. *b*) Measured in C_5D_5N. *c*—*e*) May be interchanged in each column.

Experimental

General Procedures NMR spectra were recorded in CD_3OD and C_5D_5N using a JEOL JNM *α*-400 spectrometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR). MS values were obtained on a JEOL JMS-SX 102 spectrometer. Optical rotations were measured with a union PM-1 digital polarimeter. For column chromatography, silica gel 60 (70—230 mesh, no. 7734, Merck), RP-18 (50 *μ*m, YMC), and Diaion HP-20 (Mitsubishi Chemical Industries Co., Ltd.) were used. Preparative HPLC was carried out on an ODS column (250×20 mm i.d., YMC) with a Toyo Soda RI-8000 refractive index detector. The flow rate was 6 ml/min. The solvent systems were: I) EtOAc–MeOH (9 : 1); II) EtOAc–MeOH–H_2O (40 : 10 : 1); III) EtOAc–MeOH–H_2O (70 : 30 : 3); IV) 10—50% aqueous MeOH; V) 15% aqueous MeCN; VI) 17% aqueous MeCN; and VII) 20% aqueous MeCN. The spraying reagent used for TLC was 10% H_2SO_4 in 50% EtOH.

Plant Material The aerial portion of *Erythroxylum cambodianum* Pierre was collected in August 2003 from Ban Muang Waan, Khon Kaen Province, Thailand. The plant was identified by Dr. Thaweesak Thitimetharoch, Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher specimen (TK-PSKKU-0049) was deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University (PSKKU).

Extraction and Isolation The dried aerial portion of *E. cambodianum* (3.4 kg) was extracted three times with hot MeOH (8 l for each extraction, under reflux). The MeOH extract was concentrated *in vacuo* to give a brownish powder (380.9 g). This residue was suspended in 1.0 l of H_2O, and then partitioned with 1.0 l each of Et_2O and *n*-BuOH, successively. The *n*-BuOH soluble fraction (114.9 g) was subjected to highly porous synthetic resin column chromatography (Diaion HP-20), and eluted successively with H_2O, 50% aqueous MeOH, MeOH and $(CH_3)_2CO$. The fraction eluted with 50% aqueous MeOH (71.5 g) was subjected to a column of silica gel using solvent systems I, II and III, to provide seven fractions (Fr. a—g). Fraction b (11.6 g) was subjected to a column of RP-18 using solvent system IV to give nine fractions (fr. b-1 to b-9). Fraction b-3 was purified by preparative HPLC-ODS with solvent system V to afford compound **3** (163.3 mg). Frac-

tion b-5 was further purified by preparative HPLC-ODS with solvent system VII to give compound **4** (188.3 mg). Fraction d (10.1 g) was subjected to a column of RP-18 using solvent system V to provide ten fractions (fr. d-1 to d-10). Fraction d-5 was purified by preparative HPLC-ODS with solvent system VI to give compounds **6** (16.4 mg) and **7** (26.3 mg). Similarly, fraction d-6 was purified by preparative HPLC-ODS using solvent system VII to provide compounds **1** (34.2 mg) and **2** (56.5 mg). Compound **5** (64.5 mg) was obtained from fraction d-7 by crystallization.

Erythroxyloside A (**1**): Amorphous powder, $[\alpha]_D^{24}$ −88.0° (c=0.97, MeOH); ^{1}H-NMR (CD_3OD): Table 1, ^{1}H-NMR (C_5D_5N): δ 6.69 (1H, d, J=2.2 Hz, H-5), 6.27 (1H, d, J=2.2 Hz, H-3), 5.67 (1H, d, J=2.7 Hz, H-1″ Api), 5.60 (1H, d, J=7.1 Hz, H-1′ Glc), 3.70 (3H, s, MeO-6), 2.46 (3H, s, H-8); ^{13}C-NMR (CD_3OD, C_5D_5N): Table 2. Negative HR-FAB-MS, m/z: 475.1452 ($C_{20}H_{27}O_{13}$ required 475.1451).

Erythroxyloside B (**2**): Amorphous powder, $[\alpha]_D^{24}$ −78.3° (c=1.07, MeOH); ^{1}H-NMR (CD_3OD): Table 1, ^{1}H-NMR (C_5D_5N): δ 6.69 (1H, d, J=2.2 Hz, H-5), 6.27 (1H, d, J=2.2 Hz, H-3), 5.59 (1H, d, J=2.7 Hz, H-1′ Glc), 5.41 (1H, br s, H-1″ Rha), 3.67 (3H, s, MeO-6), 2.46 (3H, s, H-8); ^{13}C-NMR (CD_3OD, C_5D_5N): Table 2. Negative HR-FAB-MS, m/z: 489.1608 ($C_{21}H_{29}O_{13}$ required 489.1608).

Enzymetic Hydrolysis of Erythroxylosides A (1) and B (2) Each sample of erythroxylosides A (**1**, 10 mg) and B (**2**, 10 mg) was hydrolyzed with crude hesperidinase[14] (22 mg) in 2 ml of H_2O. After stirring at 37 °C for 24 h, the reaction mixtures were extracted with EtOAc, and then evaporated to dryness to provide 2,4-dihydroxy-6-methoxy-acetophenone (**1a**, 2.5, 2.8 mg, respectively). The structure was identified by ^{1}H- and ^{13}C-NMR spectral analysis.[10] The aqueous layer of each experiment was evaporated to give the sugar fraction.

Determination of the Absolute Configurations of the Sugars of Erythroxylosides A (1) and B (2) A reaction mixture of pyridine (1 ml), L-cysteine methyl ester (8 mg), and the sugar fraction from erythroxyloside A (**1**) was heated at 60 °C for 1 h in the same method as described in the literatures.[12,13] After removal of the solvent, the residue was dissolved in water and extracted with *n*-BuOH (1 ml). The organic layer after evaporation was shown to contain methyl 2-(D-*gluco*-pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate, identified by TLC with the solvent system $CHCl_3$–MeOH–H_2O (50 : 20 : 3), *Rf*: 0.42, 0.49, C-2 epimers of thiazolidine. In the same way, the sugar fraction of erythroxyloside B (**2**) contained methyl 2-(D-*gluco*-pentahydroxypentyl)-thiazolidine-4(*R*)-carboxylate (*Rf*: 0.42, 0.49, C-2 epimers of thiazolidine) and methyl 2-(L-rhamno-tetrahydroxypentyl)-thiazolidine-4(*R*)-carboxylate (*Rf*: 0.61, 0.68, C-2 epimers of thiazolidine).

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