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Flavonoids from *Glycyrrhiza pallidiflora* hairy root cultures

Wei Li[a], Kazuo Koike[a], Yoshihisa Asada[b], Masao Hirotani[b], Hekai Rui[b], Takafumi Yoshikawa[b], Tamotsu Nikaido[a,*]

[a]*School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi-city, Chiba 274-8510, Japan*
[b]*School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*

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Abstract

Three flavonoids named licoagrosides D, E and F together with four known flavonoids, medicarpin 3-*O*-glucoside, calycosin 7-*O*-glucoside, formononetin 7-*O*-(6″-malonylglucoside) and 2′-hydroxyformononetin 7-*O*-glucoside were isolated from *Glycyrrhiza pallidiflora* hairy root cultures. Their structures were determined on the basis of spectroscopic evidence. Licoagrosides E and F are the first examples of a 6a-hydroxypterocarpan glycoside and an α-*O*-glycosidic α-hydroxydihydrochalcone, respectively.

Keywords: Licorice; *Glycyrrhiza pallidiflora*; Leguminosae; Hairy root; Flavonoid; Licoagroside; α-Hydroxydihydrochalcone; 6a-Hydroxypterocarpan; Glycoside

1. Introduction

The roots of *Glycyrrhiza* species are one of the oldest plant medicines. In studies on flavonoids produced by hairy root cultures of *Glycyrrhiza* species, we have reported 40 flavonoids, including ten new compounds from *G. glabra* hairy root cultures (Asada et al., 1998, 1999; Li et al., 1998, 2000). We also investigated the flavonoid constituents of *G. pallidiflora* hairy root cultures and reported nine flavonoids, including a new prenylated isoflavone, licoagroisoflavone and a new coumestan glycoside, licoagroside C (Li et al., 2001). Herein, we report the isolation and structural determination of a new pterocarpan glycoside, licoagroside D (**1**), a new α-hydroxypterocarpan glycoside, licoagroside E (**2**) and a new α-hydroxydihydrochalcone glycoside, licoagroside F (**3**) together with 4 known flavonoids, medicarpin 3-*O*-glucoside (**4**), calycosin 7-*O*-glucoside (**5**), formononetin 7-*O*-(6″-malonylglucoside) (**6**), 2′-hydroxyformononetin 7-*O*-glucoside (**7**) from the glycoside fraction of *G. pallidiflora* hairy root cultures (see Fig. 1).

2. Results and discussion

G. pallidiflora hairy root cultures were extracted with methanol and then partitioned between ethyl acetate and water according to the previous paper (Li et al., 2001). The water layer was passed through a diaion HP-20 column and washed with methanol. The methanol eluate was evaporated, then applied to an ODS column and purified by reverse-phase HPLC to give seven compounds. The structures of known compounds **4**–**7** were determined by analysis of the physical and spectroscopic evidence, and confirmed by comparing with the literature data.

Compound **1** was obtained as a powder. The FAB mass spectrum of **1** showed a *pseudo* molecular ion peak at m/z 449 $[M+H]^+$ which is larger by 18 mass units than that of medicarpin 3-*O*-glucoside (**4**). Its molecular formula, $C_{22}H_{24}O_{10}$, was established by the HR-FAB mass spectrum. The ^{1}H NMR spectrum of **1** was similar to that of **4**, except for B ring protons. Namely, the ^{1}H NMR spectrum of **1** showed a set of proton signals [3.73 (1H, *dd*, $J=11.0$, 11.0 Hz, H_{ax}-6), 4.30 (1H, *dd*, $J=11.0$, 5.0 Hz, H_{eq}-6), 3.59 (1H, *ddd*, $J=11.0$, 7.0, 5.0 Hz, H-6a), 5.59 (1H, *d*, $J=7.0$ Hz, H-11a)] characteristic of pterocapan. The spectrum also exhibited the presence of an ABX-type aromatic proton system appearing at δ 7.52 (1H, *d*, $J=9.2$ Hz), 7.06 (1H, *dd*, $J=9.2$, 2.8 Hz)

* Corresponding author. Tel.: +81-474-721391; fax: +81-474-721404.
E-mail address: nikaido@phar.toho-u.ac.jp (T. Nikaido).

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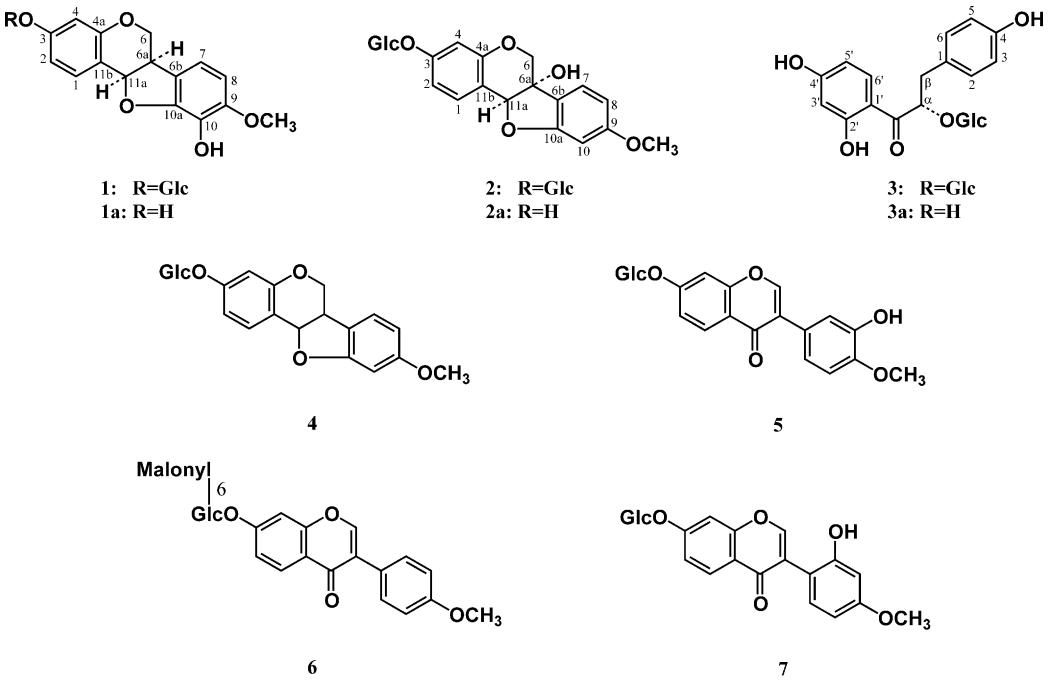

Fig. 1. Flavonoids from *Glycyrrhiza pallidiflora* hairy root cultures.

and 7.08 (1H, *d*, $J=2.8$ Hz) due to A ring protons, an AB-type aromatic proton signals resonating at δ 6.82 (1H, *d*, $J=7.8$ Hz) and 6.22 (1H, *d*, $J=7.8$ Hz) due to B ring protons as well as a methoxyl proton signal at δ 3.78 and the protons of a β-glucosyl group with the anomeric proton signal resonating at δ 5.66 (1H, *d*, $J=7.3$ Hz). The positions of the methoxyl and β-glucopyranosyloxy groups in **1** were determined from the NOESY spectrum as shown in Fig. 2. Namely, NOE's observed between the methoxy and C-8 protons, anomeric proton of β-glucopyranosyloxy groups and C-2, C-4 protons suggested that the methoxy and β-glucopyranosyloxy groups are located at C-9 and C-3, respectively. The proton and carbon signals were further assigned as shown in Table 1 by the analysis of HMQC and HMBC spectra (Fig. 3). The vicinal coupling constants of 7.0 Hz between protons of C-6a and C-11a indicated they are *cis*- configuration (Pachler et al., 1967) and the absolute configuration was determined as 6a-*R* and 11a-*R* by CD spectrum analysis: a negative Cotton effect at 228 nm and a positive Cotton effect at 284 nm were observed (Kitakawa et al., 1994). Enzymatic hydrolysis of **1** with naringinase gave an aglycone **1a** and glucose. **1a** was confirmed to be 3, 10-dihydroxy-9-methoxypterocarpan by its ^{1}H NMR spectrum (Kurosawa et al., 1978). Glucose was determined as having a D- form by HPLC analysis of its 1-[(*S*)-*N*-acetyl - α - methylbenzylamino] - 1 - deoxyalditol acetate derivative (Oshima and Kumanotani, 1981; Oshima et al., 1982). Thus, the structure of compound **1** was determined as (6a-*R*, 11a-*R*)-3-*O*-β-D-glucopyranosyl-10-hydroxy-9-methoxypterocarpan, named as licoagroside D.

Compound **2** was obtained as a powder. The FAB mass spectrum of **2** showed a molecular ion peak at *m*/*z* 448 $[M]^+$. HR-FAB mass spectrum suggested it has the same molecular formula, $C_{22}H_{24}O_{10}$ as **1**. A comparison of the ^{1}H NMR spectrum of **2** with that of **1**, showed that H-6a proton signal of **1** disappeared, and H-11a proton signal changed to a singlet, H-6 protons changed to an AB quartet. Also, in the ^{13}C NMR spectrum, the

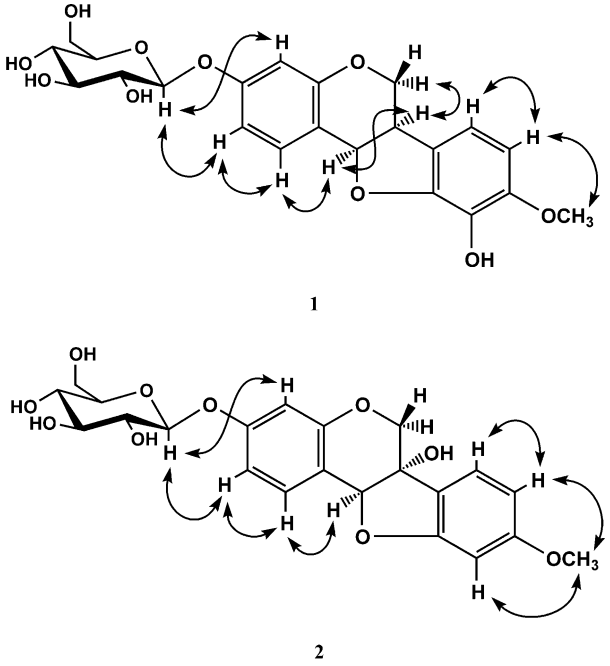

Fig. 2. NOE's detected by the NOESY spectra of flavonoids **1** and **2**.

Table 1
^{1}H and ^{13}C NMR spectral data for licoagroside D (**1**) and E (**2**) in pyridine-d_5

Position	**1**		**2**	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1	132.5	7.52 (1H, *d*, *J* = 9.2 Hz)	132.8	7.59 (1H, *d*, *J* = 8.5 Hz)
2	110.9	7.06 (1H, *d*, *J* = 9.2, 2.8 Hz)	111.4	7.08 (1H, *dd*, *J* = 8.5, 2.2 Hz)
3	159.6		160.0	
4	105.0	7.08 (1H, *d*, *J* = 2.8 Hz)	105.1	7.07 (1H, *d*, *J* = 2.2 Hz)
4a	157.0		156.8	
6	66.8	3.73 (1H, *dd*, *J* = 11.0, 11.0 Hz, Hax) 4.30 (1H, *dd*, *J* = 11.0,5.0 Hz, Heq)	70.7	4.42 (1H, *d*, *J* = 11.0 Hz) 4.55 (1H, *d*, *J* = 11.0 Hz)
6a	40.6	3.59 (1H, *ddd*, *J* = 11.0, 7.0, 5.0 Hz)	76.3	
6b	121.7		123.1	
7	114.2	6.82 (1H, *d*, *J* = 7.8 Hz)	124.9	7.54 (1H, *d*, *J* = 8.5 Hz)
8	105.7	6.22 (1H, *d*, *J* = 7.8 Hz)	107.6	6.65 (1H, *dd*, *J* = 8.5, 2.2 Hz)
9	150.2		162.6	
10	133.3		97.4	6.66 (1H, *d*, *J* = 2.2 Hz)
10a	148.3		161.7	
11a	78.7	5.59 (1H, *d*, *J* = 7.0 Hz)	86.2	5.80 (1H, *s*)
11b	114.7		115.5	
OCH_3	56.4	3.78 (3H, *s*)	55.5	3.64 (3H, *s*)
Glc-1	101.8	5.66 (1H, *d*, *J* = 7.3 Hz)	102.1	5.62 (1H, *d*, *J* = 7.4 Hz)
Glc-2	74.8	4.29∼4.43 (4H, *m*, Glc-2,3,4-H and Glc-6-H_a)	74.9	4.29∼4.43 (4H, *m*, Glc-2,3,4-H and Glc-6-H_a)
Glc-3	78.3		78.5	
Glc-4	71.0		71.1	
Glc-5	78.8	4.10 (1H, *ddd*, *J* = 10.1, 5.5, 2.0 Hz)	78.9	4.08 (1H, *m*)
Glc-6	62.1	4.50 (1H, *dd*, *J* = 12.0, 2.0 Hz, Glc-6-H_b)	62.2	4.49 (1H, *dd*, *J* = 12.0, 2.3 Hz, Glc-6-H_b)

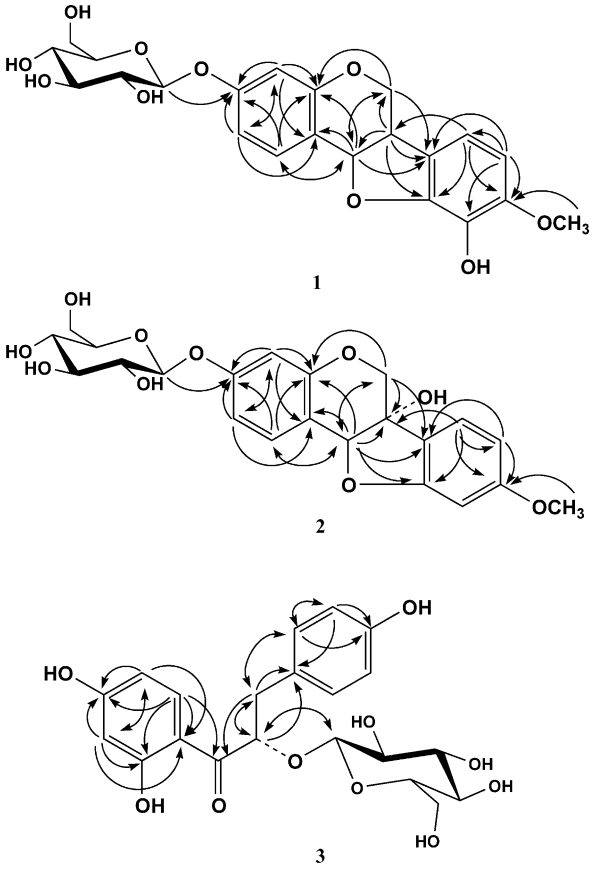

Fig. 3. ^{1}H–^{13}C long-range correlations by the HMBC spectra of flavonoids **1**–**3**.

chemical shift of C-6a was deshielded by 35.7 ppm relative to that of **1**. All these data indicated that **2** is a 6a-hydroxypterocarpan derivative. In the ^{1}H NMR spectrum, **2** also showed two ABX-type aromatic proton signals appearing at δ 7.59 (1H, *d*, *J* = 8.5 Hz), 7.08 (1H, *dd*, *J* = 8.5, 2.2 Hz) and 7.07 (1H, *d*, *J* = 2.2 Hz) due to A ring protons, and *δ* 7.54 (1H, *d*, *J* = 8.5 Hz), 6.65 (1H, *dd*, *J* = 8.5, 2.2 Hz) and 6.66 (1H, *d*, *J* = 2.2 Hz) due to B ring protons, a methoxy proton signal at *δ* 3.64 and an anomeric proton signal at *δ* 5.62 (1H, *d*, *J* = 7.4 Hz), suggesting a β-D-glucopyranoside. The positions of the methoxy and β-glucopyranosyloxy groups in **2** were determined from the NOESYspectrum as shown in Fig. 2. The absolute configuration was determined as 6a-*S* and 11a-*S* by CD spectrum analysis: a negative Cotton effect at 228 nm and a positive Cotton effect at 286 nm were observed (van Aardt et al., 2001). Furthermore, enzymatic hydrolysis gave an aglycone which was confirmed to be 3, 6a-dihydroxy-9-methoxypterocarpan (**2a**) by its ^{1}H NMR spectrum (Bilton et al., 1976). The β-glucose was determined as having a D- form by HPLC analysis performed as described for **1**. Thus, the structure of compound **2** was determined as (6a-*S*, 11a-*S*)-3-*O*-β-D-glucopyranosyl-6a-hydroxy-9-methoxypterocarpan, named as licoagroside E.

Compound **3** was obtained as a powder. The FAB mass spectrum of **3** showed a pseudo molecular ion peak at *m*/*z* 437 $[M+H]^+$ which is less by 12 mass units than that of **1**. Its molecular formula, $C_{21}H_{24}O_{10}$, was

established by the HR-FAB mass spectrum. The UV spectrum of **3** showed maximum absorption at 214 (4.27), 283 (4.11), 327 (3.93) nmwhich suggested **3** is a dihydrochalcone derivative (Mabry et al., 1970). The ^{1}H NMR spectrum of **1** showed methylene proton signals appearing at δ 3.49 (1H, *dd*, $J=16.0$, 7.1 Hz), 3.51 (1H, *dd*, $J=16.0$, 7.1 Hz) and an oxymethine proton resonating at δ 5.98 (1H, *dd*, $J=7.1$, 7.1 Hz). In addition, ABX-type aromatic proton signals appearing at δ 6.68 (1H, *dd*, $J=8.9$, 2.3 Hz), 6.70 (1H, *d*, $J=2.3$ Hz) and 8.37 (1H, *d*, $J=2.3$ Hz), AA′BB′-type aromatic proton signals appearing at δ 7.01 (1H, *d*, $J=8.3$ Hz) and 7.26 (1H, *d*, $J=8.3$ Hz), and the protons of a β-glucosyl group with the anomeric proton signal appearing at δ 5.10 (1H, *d*, $J=7.6$ Hz) indicated that **3** is an α-hydroxydihydrochalcone glucoside. Comparing the ^{13}C NMR spectra of **3** and kanzonol Y (Li et al., 2000; Fukai et al., 1996), an α-hydroxydihydrochalcone isolated from *G. glabra* hairy root cultures, the signal of C-3 showed a 5.2 ppm down-field shift, suggesting that the α-hydroxy was glycosylated. This was further confirmed by analysis of the HMBC spectrum. Stereochemistry of C-α was determined as *R* by enzymatic hydrolysis, and comparing the value of the $[\alpha]_D$ of its aglycone **3a** with the literature (Ferrari et al., 1983; Bezuidenhoudt et al., 1987). The glucose subunit was confirmed as having a D- form by the same method as **1** and **2**. Thus, the structure of **3** was determined as α-R, α-*O*-β-D-glucopyranosyl-4, 4′, 7′-trihydroxydihydrochalcone, and named as licoagroside F.

In conclusion, we have isolated three new flavonoids and four known flavonoids from the hairy roots of *G. pallidiflora*. To our knowledge, licoagroside E (**2**) is the first example of the glycoside of 6a-hydroxypterocarpan and licoagroside F (**3**) is the first example of α-hydroxydihydrochalcone which is glycosylated at the α-position. The known flavonoids, calycosin 7-*O*-glucoside (**5**), formononetin 7-*O*-(6″-malonylglucoside) (**6**) have been isolated from the hairy root cultures of *G. glabra*, but none of the known flavonoids has been reported from roots of *G. pallidiflora*.

3. Experimental

3.1. General experimental procedures

The UV spectra were obtained with a Shimadzu UV-160 spectrophotometer, whereas the IR spectra were measured with a JASCO FT/IR-300E (by a KBr disk method) spectrometer. The optical rotations were measured with a JASCO DIP-370 digital polarimeter in a 0.5 dm length cell, while the CD spectra were recorded on a JASCO J-720W spectropolarimeter. The EI–MS, FAB–MS, HR–EI–MS and HR–FAB–MS were taken on a JEOL JMS-AX505HA. The ^{1}H and ^{13}C NMR spectra were measured with a JEOL ECP-500 spectrometer in pyridine-d_5 solution and chemical shifts are expressed in δ (ppm) referring to TMS. For HPLC, JASCO HPLC system was used. Column chromatography was carried out using Chromatorex DM1020T ODS. TLC was conducted on Kieselgel 60 F_{254} plates (Merck).

3.2. Extraction and separation of flavonoids

The MeOH extract obtained previously was partitioned between EtOAc and H_2O (Li et al., 2001). The water phase was evaporated under reduced pressure below 40 °C to remove EtOAc, and then subjected to a Diaion HP-20 column and further washed with MeOH to give the crude glycoside fraction (GPH, 2.63 g). The GPH was next subjected to ODS chromatography and eluted by aqueous MeOH (0–100%) to give five fractions. Further purification of fractions 3 (283 mg) and 4 (200 mg) was achieved by repeated RP–HPLC (SenshuPak Pegasil ODS, 5 μm C_{18}−120 Å, 20 mm ×150 mm) to give **1** (11 mg), **2** (7 mg), **3** (7 mg), **4** (2 mg), **5** (11 mg), **6** (5 mg) and **7** (1 mg).

3.3. Licoagroside D (*1*)

Powder, $[\alpha]_D$ −117.4° ($c=1.09$, MeOH, 22 °C). CD ($c=2.23\times10^{-4}$, MeOH, 23 °C): $[\theta]_{284}$ 13 409.8, $[\theta]_{228}$−36358.4. UV λ nm (log ε): 227 (4.98), 277 (4.54). IR υ cm^{-1}: 3390, 1621, 1502. FAB–MS (positive) *m*/*z*: 449 [(M + H)$^+$]. HR–FAB–MS (positive) *m*/*z*: Found: 449.1483; Calc. for $C_{22}H_{25}O_{10}$ [(M + H)$^+$]: 449.1447. ^{1}H NMR (500 MHz, pyridine-d_5): see Table 1. ^{13}C NMR (125 MHz, pyridine-d_5): see Table 1.

3.4. Licoagroside E (*2*)

Powder, $[\alpha]_D$ −176.4° ($c=0.66$, MeOH, 22 °C). CD ($c=2.23\times10^{-4}$, MeOH, 23 °C): $[\theta]_{286}$ 28632.8, $[\theta]_{228}$−89228.0. UV λ nm (log ε): 224 (4.95), 283 (4.78). IR υ cm^{-1}: 3394, 1622, 1500. FAB–MS (positive) *m*/*z*: 471 [(M + Na)$^+$], 448 [M$^+$]. HR–FAB–MS (positive) *m*/*z*: Found: 448.1366; Calc. for $C_{22}H_{24}O_{10}$ [M$^+$]: 448.1370. ^{1}H NMR (500 MHz, pyridine-d_5): see Table 1. ^{13}C NMR (125 MHz, pyridine-d_5): see Table 1.

3.5. Licoagroside F (*3*)

Powder, $[\alpha]_D$ −2.6° ($c=0.91$, MeOH, 22 °C). CD ($c=2.29\times10^{-5}$, MeOH, 23 °C): $[\theta]_{294}$ 4166.6, $[\theta]_{275}$−4324.7, $[\theta]_{237}$ 5447.6. UV λ nm (log ε): 214 (4.27), 283 (4.11), 327 (3.93). IR υ cm^{-1}: 3414, 1619, 1525. FAB–MS (positive) *m*/*z*: 437 [(M + H)$^+$], 459 [(M + Na)$^+$]. HR–FAB–MS (positive) *m*/*z*: Found: 459.1276; Calc. for $C_{21}H_{24}O_{10}Na$ [(M + Na)$^+$]: 459.1267. ^{1}H NMR (500

Table 2
^{1}H and ^{13}C NMR spectral data for licoagroside F (**3**) in pyridine-d_5

Position	^{13}C NMR	^{1}H NMR
C=O	202.6	
α	79.3	5.98 (1H, *dd*, *J* = 7.1, 7.1 Hz)
β	39.4	3.49 (1H, *dd*, *J* = 16.0, 7.1 Hz)
		3.51 (1H, *dd*, *J* = 16.0, 7.1 Hz)
1	127.4	
2 and 6	131.1	7.26 (2H, *d*, *J* = 8.3 Hz)
3 and 5	116.0	7.01 (2H, *d*, *J* = 8.3 Hz)
4	157.5	
1′	113.3	
2′	167.1	
3′	103.6	6.70 (1H, *d*, *J* = 2.3 Hz)
4′	166.6	
5′	109.4	6.68 (1H, *dd*, *J* = 8.9, 2.3 Hz)
6′	134.0	8.37 (1H, *d*, *J* = 8.9 Hz)
Glc-1	103.2	5.10 (1H, *d*, *J* = 7.6 Hz)
Glc-2	75.4	
Glc-3	78.3	
Glc-4	71.5	
Glc-5	78.6	3.90 (1H, *ddd*, *J* = 11.5, 5.5, 2.1 Hz)
Glc-6	62.7	4.34 (1H, *dd*, *J* = 11.5, 5.5 Hz)
		4.53 (1H, *dd*, *J* = 11.5, 2.1 Hz)

MHz, pyridine-d_5): see Table 2. ^{13}C NMR (125 MHz, pyridine-d_5): see Table 2.

3.6. Enzymatic hydrolysis of compounds *1–3*

A solution of **1** (3.5 mg), **2** (3.7 mg) or **3** (3.5 mg) in 0.1 M acetate buffer (pH 4.0, 1.0 ml) was treated with naringinase (Sigma Chemical Co., 3.0 mg) and then the reaction mixture was stirred at 40 °C for 12 h. The reaction mixture was passed through a Sep-Pak C_{18} cartridge using H_2O and MeOH. The MeOH elute was evaporated to give the aglycones **1a** (2.1 mg), **2a** (1.7 mg) and **3a** (2.0 mg), respectively. The H_2O eluate was concentrated and the residue was dissolved in 1 ml H_2O. EtOH solution (1 ml) of L-(−)-α-methylbenzylamine (5 mg) and $NaBH_3CN$ (8 mg) was added to the H_2O solution. The mixture was stirred at 40 °C for 4 h, then acidified by addition of glacial HOAc 0.2 ml and evaporated to dryness. The resulting solid was acetylated with acetic anhydride (0.3 ml) in pyridine (0.3 ml) for 24 h at room temperature. After evaporation, H_2O (1 ml) was added to the residue, and then passed through a Sep-pak C_{18} cartridge and washed with H_2O and CH_3CN. The CH_3CN eluate was analyzed by HPLC. The 1-[(*S*)-*N*-acetyl-α-mzethylbenzylamino]-1-deoxyglucitol acetate was identified by co-HPLC analysis with the derivatives of standard sugars prepared under the same conditions (Koike et al., 1999). HPLC conditions: Column, Senshu Pak Pegasil ODS, 4.6×150 mm; Solvent: 33% aqueous CH_3CN; flow rate, 0.8 ml/min; detection, UV 230 nm. The derivatives of D-glucose were detected with the t_R of 38.3 min.

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