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Acylated flavonol diglucosides from *Lotus polyphyllos*

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Abstract

Three acylated flavonol diglucosides, kaempferol 3-*O*-β-(6″-*O*-*E*-*p*-coumaroylglucoside)-7-*O*-β-glucoside; quercetin 3-*O*-β-(6″-*O*-*E*-*p*-coumaroylglucoside)-7-*O*-β-glucoside; isorhamnetin 3-*O*-β-(6″-*O*-*E*-*p*-coumaroylglucoside)-7-*O*-β-glucoside were isolated from the whole plant aqueous alcohol extract of *Lotus polyphyllos*. The known 3,7-di-*O*-glucosides of the aglycones kaempferol, quercetin and isorhamnetin were also characterized. All structures were established on the basis of chemical and spectral evidence.
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Keywords: *Lotus polyphyllos*; Leguminosae; Acylated flavonol diglucosides; Kaempferol 3-*O*-β-(6″-*O*-*p*-coumaroylglucoside)-7-*O*-β-glucoside; Quercetin 3-*O*-β-(6″-*O*-*p*-coumaroylglucoside)-7-*O*-β-Glucoside; Isorhamnetin 3-*O*-β-(6″-*O*-*p*-coumaroylglucoside)-7-*O*-β-glucoside

1. Introduction

The genus *Lotus* (Leguminosae) includes both acyanogenic and high cyanogenic species (Rizk, 1986). Some of these species are used as a source of lectins. In folk medicine, they are used as contraceptive, prophylactic agents and for treating sexually transmitted disorders, for oral alimentary and for peptic ulcer (Oldham et al., 1995; Oldham and Krivan, 1996; Hirata et al., 1998). Previous phytochemical investigations have proved the isolation of the 3-*O*-β-galactosides of the flavonol gossypetin, its 7- and 8- methyl ethers from *L. corniculatus* (Harborne, 1969; Nielsen, 1970; Jay et al., 1978) and the isolation of kaempferol 3-*O*-β-glucoside together with its 7-*O*-α-rhamnoside derivative from *L. tenuis* (birds trefoil), (Strittmater et al., 1992). Recently, *L. hebranicus* Hochst ex. Brand was reported to contain kaempferol 7-*O*-α-rhamnoside together with its 3-*O*-α-rhamnoside and 3-*O*-sophoroside derivatives. Also kaempferol 3-*O*-α- rhamnoside-7-*O*-sophoroside and isorhamnetin 3-*O*-β-glucoside-7-*O*-β-glucoside have been reported from the same plant (Kassem, 2001). However, there are no reports on the constitutive flavonoids of *Lotus polyphyllos* which grows wild in sand dunes of the western region of the Mediterranean coastal strip of Egypt. In the present communication, we describe the isolation and structure elucidation of the three new acylated flavonol glycoside (**4**–**6**), from the aqueous alcoholic whole plant extract of *Lotus polyphyllos* E. D. Clarke (Syn. *Lotus argenteus* Webb & Berthel). In addition, the known compounds, kaempferol 3,7-di-*O*-glucoside (**1**), quercetin 3,7-di-*O*-glucoside (**2**) and isorhamnetin 3,7-di-*O*-glucoside (**3**) were characterized from the same extract. Also, the ^{13}C NMR data of the known compounds (2 and 3) are recorded and assigned here for the first time.

2. Results and discussion

The concentrated 70% aqueous ethanol extract from a homogenate of the dried whole plant was fractionated by column chromatography over Sephadex LH 20, using water/methanol mixtures of decreasing polarities. Repeated preparative paper chromatography of the 30 and 50% aqueous methanol column fractions afforded pure samples of compounds (**1**–**6**). The known compounds (**1**–**3**) showed chromatographic, UV absorption and hydrolytic data identical with those reported for kaempferol 3,7-di-*O*-glucosides (Egger, 1961), quercetin 3,7-di-*O*-glucoside (Harborne, 1982) and isorhamnetin 3,7-di-*O*-glucoside (Krishnamurti et al., 1965), respectively. The ^{13}C NMR (see Table 2) spectral data of

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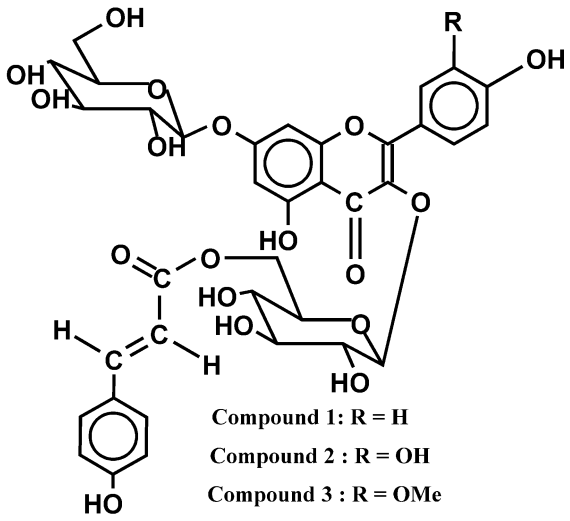
compounds **2** and **3** were recorded and assigned here for the first time.



Compound **4**, a yellow amorphous powder, showed chromatographic properties (dark purple spot on paper chromatogram under UV light, turning lemon yellow when fumed with ammonia vapour, moderate migration in aqueous and organic solvents) and colour reactions (a lemon yellow colour with Naturstoffe and a bright-yellow colour with 2% $ZrOCl_2$ which disappeared on addition of 2% citric acid and water), (Liu et al., 1997) characteristic of a 4′-oxygenated flavonol bearing a free hydroxyl at the 5-position and a substituted one at the 3-position. UV spectral analysis (see Table 1) in methanol and on addition of shift reagents (Harborne and Williams, 1975; Mabry et al., 1969) confirmed the presence of a free hydroxyl at 4′-position (stable MeONa spectrum) and a substituted hydroxyl at the 7-position (no shift with NaOAc). Normal acid hydrolysis of 1 (2 N aqueous HCl, 1 h, 100 °C) yielded glucose (comparative paper chromatography Co-PC), kaempferol and *p*-coumaric acid (Co-PC, UV spectral and ^{1}H NMR). On β-glucosidase enzymatic hydrolysis (incubation together with 0.5 ml of the enzyme in acetate buffer, pH 5.1 at 37 °C, for 24 h) **4** yielded kaempferol 3-*O* - β - (6″ - *p* - coumaroylglucoside) (chromatographic properties and ^{1}H NMR), (Kumar et al., 1985). Consequently, **4** is kaempferol 3-*O*-(*p*-coumaroyl)glucoside)-7-*O*-glucoside. The result of negative ESI–MS analysis of **4** supported this, indicating a molecular ion peak at $[M-H]^-$ 755, corresponding to a molecular weight (M_r) of 756. In order to determine unambiguously the structure of **4**, specially the site of attachment of the *p*-coumaroyl moiety to the 3-*O*-glucoside moiety, it was necessary to apply NMR spectroscopic analysis. The ^{1}H NMR spectrum of **4** (DMSO-d_6, room temperature) revealed two distinct anomeric hexose proton resonances at δ ppm 5.02 and 5.55 (each *d*, $J = 8.5$ Hz) attributed to the anomeric glucoside moieties at the 7- and 3-positions in **4**.

The spectrum also showed a pair of glucose proton resonances at δ 4.26 (*dd*, $J = 12$ and 4.5 Hz) and 4.12 (*m*), assignable to the two methylenic glucose protons whose hydroxyl group is acylated by the *p*-coumaroyl moiety. Resonances of the two methylenic protons in the second glucose moiety, together with all of the remaining glucose protons appeared more upfield in the region from δ 3.3 to 3.9, overlapped with water protons signal. In this spectrum the characteristic protons of the *E*-*p*-coumaroyl moiety resonated at δ 6.15 (*d*, $J = 16$ Hz, H-8‴), 6.8 (*d*, $J = 8$ Hz, H-3‴ and H-5‴),7.55 (*d*, $J = 16$ Hz, H-7‴), 7.6 (*d*, $J = 8$ Hz, H-2‴ and H-6‴). The spectrum showed in addition, the presence of a 7-*O*-substituted kaempferol moiety by the proton resonances at δ 6.32 (*d*, $J = 2.5$ Hz) and 6.72 (*d*, $J = 2.5$ Hz), assignable to the H-6 and H-8 of this moiety. These two chemical shift values were closely similar to those reported for the corresponding proton resonances in the spectrum of kaempferol-3,7-di-*O*-glucoside **1** (Markham and Geiger, 1994) In this spectrum the chemical shifts of the B ring proton resonances (see Experimental) were found to be in close agreement with the proposed structure.

The ^{13}C NMR analysis confirmed the structure of **4**. As expected, the spectrum (DMSO-d_6, room temperature) exhibited twelve distinct glucose carbon resonances (see Table 2). The two β-anomers were recognized from the downfield resonances at δ 99.8 and 100.6, while the most upfield resonances at δ 60.1 and 63.0 were assigned to the methylenic C-6‴ glucose carbon bearing a free

Table 1
Chromatographic and UV data of the new flavonoids (**4**–**6**)

Compound	Chromatographic properties (R_fs×100)			UV spectral data λ_{max} mn				
	H_2O	HOAc 15%	BAW	MeOH	NaOAc (a)	(a) + H_3BO_3	$AlCl_3$	MeONa
4	52	58	44	268, 316, 360[a]	268, 317, 366	268, 318	275, 312, 392	277, 362
5	50	55	40	256, 265[a], 297[a], 313, 354[a]	256[a], 265, 300, 297[a], 312, 374	273, 299, 313, 438[a]	243[a], 274, 367	
6	48	52	43	253, 266[a], 300[a], 313, 354	255, 266, 302[a], 313, 376[a]	257, 265[a], 314, 355	267, 314[a], 398	269, 365
Kaempferol-3-*O*-β-(6″-*p*- coumaroyl)-glucoside	28	42	57	269, 295[a], 312, 356[a]	278, 302[a], 313, 366[a]	277, 300[a], 312, 405	277, 312, 379	

[a] Inflection.

Table 2
^{13}C-NMR chemical shifts (ppm) of the flavonoid (**2**–**6**) of *Lotus polyphyllos*

Carbon	**2**	**4**	**3**	**5**	**6**
2	156	156.9	156.1	156.5	156.2
3	133.6	133.4	133.5	133.1	133.2
4	177.6	177.6	177.5	177.6	177.5
5	160.9	160.8	160.6	160.8	160.3
6	99.3	99.3	99.5	99.2	99.4
7	162.8	162.8	163.2	162.4	162.9
8	94.3	94.4	94.6	94.2	94.5
9	156	155.9	156.9	156	157
10	105.7	105.5	105.8	105.6	105.8
1′	121	120.9	122.2	122.1	122.4
2′	115.4	130.3	113	115.3	113,5
3′	144.7	115.9	149.5	144.8	149.6
4′	148.6	159.8	146.9	148.8	147
5′	116.3	115.9	115.9	115.9	115.7
6′	121.6	130.3	122.8	121	122,0
1″	100.5	100.6	105.8	100.7	106.8
2″	74.1	74.1	74.2	74.1	74.3
3″	77.6	77.2	77.7	77.5	77.6
4″	69.9	69.8	69.9	69.9	69.9
5″	76.4	74.5	76.5	74.4	74.3
6″	60.9	63.5	60.6	63.7	63.3
1‴	99.6	99.9	99.7	99.7	99.8
2‴	73.1	73.1	73.2	73	73.2
3‴	76.4	76.4	76.6	76.3	76.5
4‴	69.5	69.7	69.8	69.6	70
5‴	76.5	76.4	76.8	76.9	76,5
6‴	60.6	60.1	60.3	60,4	60.5
1⁗		124.6		124,8	124.8
2⁗		130		130.3	130.2
3⁗		115.9		116.1	116
4⁗		159.9		159.9	159.8
5⁗		115.9		116.1	116
6⁗		130		130.3	130.2
7⁗		144		144.3	144.2
8⁗		114.7		115	116
9⁗		166.1		165.9	166
Ome			55.9		55.8

hydroxyl (located at the 7-position of the kaempferol moiety) and to the methylenic C-6″ (at the 3-position), respectively.

The deshielding of the second resonance is obviously due to the acylation by *p*-coumaric acid. Assignment of the remaining glucose carbons was aided by comparison with the reported ^{13}C chemical shifts of kaempferol 3,7-di-*O*-glucoside (Markham et al., 1978). The presence of only one *p*-coumaroyl moiety in **4** followed from the single carboxyl carbon resonance at δ 166.3 and from the recognized characteristic pattern of the remaining *p*-coumaroyl carbon resonances (see Experimental). The recorded chemical shifts of the kaempferol carbon resonances, in the ^{13}C NMR spectrum, confirmed substitution at the 3- and 7-positions. This followed from the relative upfield shifts of the C-3 and C-7 resonances to δ 133.4 and 162.8, respectively. As expected, these shifts were accompanied by downfield shifts of the resonances of the corresponding *o*- and *p*-carbons all in comparison with the corresponding resonances in the spectrum of the aglycone, kaempferol (see Experimental), (Markham et al., 1978). Furthermore, the measured chemical shift values of the carbon resonances of the two glucose moieties confirmed that the sugar cores exist in the pyranose form. Consequently, **4** is identified as kaempferol 3-*O*-β-(6″-*p*-coumaroylglucopyranoside)-7-*O*-β-glucopyranoside, which has not been reported previously in nature.

Compound **5** was isolated as faint yellow amorphous powder which appeared dull purple on chromatograms under UV light, turning orange when fumed with ammonia vapour. It gave glucose, quercetin and *p*-coumaric acid (CoPC), on normal acid hydrolysis. Compound **5** exhibited a molecular weight (M_r) of 772 in negative ESI–MS ([M–H]$^-$ 771). These data together with R_f values and UV spectral analysis (see Table 1), indicated that **5** is the quercetin analogue of **4**. ^{1}H and ^{13}C NMR (see Table 2) spectroscopic analysis of **5** confirmed its structure to be quercetin 3-*O*-β-(6″-*E*-*p*-coumaroylglucopyranoside)-7-*O*-β-glucopyranoside which has not been reported before as a natural product.

The new compound **6** (dull yellow amorphous powder) was identified as the isorhamnetin analogue of **4** and **5** from chromatographic, UV spectral (see Table 1), hydrolytic, and negative ESI–MS ([M–H]. 785, corresponding to molecular weight (M_r) of 786) data. Its structure was confirmed by ^{1}H and ^{13}C NMR (see Table 2) analysis to be isorhamnetin 3-*O*-β-(6″-*E*-*p*-coumaroylglucopyranoside) - 7 - *O* - β - glucopyranoside, which represnts the third new natural product.

It should be noted that this is the first reported occurrence of acylated flavonol glycosides in *Lotus* species.

3. Experimental

NMR: Jeol EX-270 spectrometer, 270 MHz (^{1}H NMR) and 67.5 MHz (^{13}C NMR), respectively. ^{1}H resonances were measured relative to TMS and ^{13}C NMR resonances to DMSO-d_6 and converted to TMS scale by adding 39.5. ESI–MS: Micromass Quattro-LC triple quadrupole mass spectrometer equipped with a "Z-Spray" electrospray ion source. PC (descending): Whatman no. 1 paper, using solvent systems: (1) H_2O; (2) 15% HOAc; (3) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer); (4) C_6H_6-*n*-BuOH–H_2O–pyridine (1:5:3:3, upper layer). Solvents 3 and 4 were used for sugar analysis and solvent 3 for preparative isolation on Whatman no. 3MM paper.

3.1. Plant material

Flowering specimens of *Lotus polyphyllos* E. D. Clarke, shrubs, were collected from the sand dunes west of Mersa

Metrouh, Egypt, during June 2001 and identified by Dr. M. El Gibali, National Research Centre (NRC), Dokki, Cairo, Egypt. A voucher specimen has been deposited at the herbarium of the NRC.

3.2. Isolation and identification

The ground dried aerial parts (2 kg) of *L. polyphyllos* plants were extracted (×3) by refluxing with 4 l. of EtOH–H_2O (3:1) over a boiling water bath for 8 h. The conc. extract was applied to a Sephadex LH-20 column (100×5 cm inte) and eluted with H_2O followed by H_2O–MeOH mixtures of decreasing polarities to yield, nine fractions, among which two were found to contain flavonoids, eluted by H_2O–MeOH (70:30) and (40:60). They appeared on the column as dark purple bands under UV light. Repeated PPC chromatography, using BAW as an eluant afforded pure samples of **1**, (105 mg), **2** (164 mg), **3** (88 mg), from the 30% column fraction and **4** (76 mg), **5** (90 mg) and **6** (58 mg), from the 60% column fraction.

3.3. Kaempferol 3-O-β-(6″-E-p-coumaroylglucopyranoside)-7-O-β-glucopyranoside (4)

M_r 756, ESI–MS: negative ion: *m*/*z* (rel. int.): 755(52), $[M–H]^-$, 447 (30), [M - *p*-coumaroyl glucose]$^-$, 285(39), [kaempferol, 145(24),[*p*-coumaroyl]$^-$. R_f-values: Table 1. UV λ_{max}^{MeOH} nm: Table 1. Normal acid hydrolysis gave glucose (Co-PC), kaempferol and *p*-coumaric acid [Co-PC, UV spectral data (Table 1), ^{1}H NMR of kaempferol: ppm 6.2 (*d*, J = 2.5 Hz, H-6), 6.42 (*d*, *J* = 2.5 Hz, H-8), 6.98 (*d*, *J* = 7.5 Hz, H-3′ and H-5′), 8.03 (*d*, *J* = 7.5 Hz, H-2′ and H-6′); ^{1}H NMR of *p*-coumaric acid: ppm 6.3 (*d*, *J* = 16 Hz, H-8‴), 6.8 (*d*, *J* = 7.5 Hz, H-3′ and H-5′), 7.46 (*d*, = 7.5 Hz, H-2′ and H-6′), 7.55 (*d*, *J* = 16 Hz, H-2′ and H-6′). β-Glucosidase enzymatic hydrolysis yielded kaempferol 3-*O*-β-(6″-*p*-coumaroylglucoside): R_f-values: Table 1. ^{1}H NMR: *p*-coumaroyl moiety: ppm 6.2 (*d*, *J* = 16 Hz, H-8), 6.84 (*d*, *J* = 7.5 Hz, H-3′ and H-5′), 7.4 (*d*, *J* = 16 Hz, H-7), 7.45 (*d*, *J* = 7.5 Hz, H-2′ and H-6′); kaempferol moiety: 6.18 (*d*, *J* = 2.5 Hz, H-6), 6.38 (*d*, *J* = 2.5 Hz, H-8), 6.88 (*d*, *J* = 7.5 Hz, H-3′ and H-5′), 8.0 (*d*, *J* = 7.5 Hz, H-2′ and H-6′), 12.2 (*s*, OH-5); β-glucoside moiety: 5.5 (*d*, J = 8.5 Hz, H-1), 4.35 (*dd*, *J* = 12 and 3.5 Hz, one proton H-6), 4.18 (*m*, one proton H-6), 3.3–3.85 (*m*, glucose protons overlapped with water protons). ^{1}H NMR of **4**: *p*-coumaroyl moiety: ppm 6.15 (*d*, *J* = 16 Hz, H-8‴), 6.8 (*d*, *J* = 8 Hz, H-3‴ and H-5‴), 7.55 (*d*, *J* = 16 Hz, H-7‴), 7.6 (*d*, *J* = 8 Hz, H-2‴ and H-6‴); Kaempferol moiety: ppm 6.32 (*d*, *J* = 2.5, H-6), 6.72 (*d*, *J* = 2.5 Hz, H-8), 6.86 (*d*, *J* = 7.5 Hz, H-3′ and H-5′), 8.0 (*d*, *J* = 7.5 Hz, H-2′ and H-6′), 11.8 (*s*,OH-5); β-glucoside moieties: ppm 5.02 (*d*, *J* = 8.5 Hz, H-1‴), 5.55 (*d*, *J* = 8.5 Hz, H-1″), 4.26 (*d*, *d*, *J* = 12 and 4.5 Hz, one methylenic H-6″ proton), 4.12 (m, one methylenic H-6″ proton), 3.3–3.9 (*m*, glucose protons overlapped by water protons). ^{13}C NMR of **4**: Table 2.

3.4. Quercetin 3-O-β-(6″-E-p-coumaroylglucopyranoside)-7-O-β- gucopyranoside (5)

M_r 772, ESI–MS: negative ion: *m*/*z* (rel. int.): 771(60), $[M–H]^-$, 463 (28), [M - *p*-coumaroyl glucose]$^-$, 301 (36), [quercetin]$^-$, 145 (33), [*p*-coumaroyl]$^-$. R_f-values: Table 1. UV λ_{max}^{MeOH} nm: Table 1. Normal acid hydrolysis gave glucose (CoPC), quercetin and *p*-coumaric acid [CoPC, UV spectral data (Table 1), ^{1}H NMR of quercetin: ppm 6.22 (*d*, *J* = 2.5 Hz, H-6), 6.40 (*d*, *J* = 2.5 Hz, H-8), 6.85 (*d*, *J* = 7.5 Hz, H-5′),. 7.35 (*d*, *J* = 2.5 Hz, H-2′), 7.5 (*dd*, *J* = 7.5 and 2.5 Hz, H-6′), 12.3 (*s*, OH-5)]. ^{1}H NMR of **5**: *p*-coumaroyl moiety: ppm 6.15 (*d*, *J* = 16 Hz, H-8‴), 6.86 (*d*, *J* = 8 Hz, H-3‴ and H-5‴), 7.50 (*d*, *J* = 16 Hz, H-7‴), 7.54 (*d*, *J* = 8 Hz, H-2‴ and H-6‴); quercetin moiety: ppm 6.36 (*d*, *J* = 2.5, H-6), 6.75 (*d*, *J* = 2.5 Hz, H-8), 6.8 (*d*, *J* = 7.5 Hz, H-5′), (*m*, H-2′ and H-6′), 12.6 (*s*, OH-5); β-glucoside moieties: ppm 5.16 (*d*, *J* = 8.5 Hz, H-1‴), 5.50 (*d*, *J* = 8.5 Hz, H-1″), 4.4 (*d*, *d*, *J* = 12 and 4.5 Hz, one methylenic H-6″ proton), 4.24 (*m*, one methylenic H-6″ proton), 3.3–3.9 (*m*, glucose protons overlapped by water protons). ^{13}C NMR of **5**: Table 2.

3.5. Isorhamnetin 3-O-β-(6″-E-p-coumaroyglucopyranoside)-7-O-β-glucopyranoside (6)

M_r 786, ESI–MS: negative ion: *m*/*z* (rel. int.): 785 (63), $[M–H]^-$, 477 (40), [M - *p*-coumaroyl glucose]$^-$, 315 (44), [isorhamnetin aglycone]$^-$, 145 (32), [*p*-coumaroyl]$^-$. R_f-values: Table 1. UV λ_{max}^{MeOH} nm: Table 1. Normal acid hydrolysis gave glucose (CoPC), isorhamnetin and *p*-coumaric acid [CoPC, UV spectral data (Table 1) and ^{1}H NMR, ^{1}H NMR of isorhamnetin: ppm 6.22 (*d*, *J* = 2.5 Hz, H-6), 6.46 (*d*, *J* = 2.5 Hz, H-8), 6.88 (*d*, J = 7.5 Hz, H-5′), 7.7 (*d*, *d*, J = 7.5 and J = 2.5 Hz, H-6′), 7.78 (*d*, J = 2.5 Hz, H-2′), 11.8 (*s*, OH-5)]. ^{1}H NMR of **5**: *p*-coumaroyl moiety: ppm 6.24 (*d*, *J* = 16 Hz, H-8‴), 6.88 (*d*, *J* = 8 Hz, H-3‴ and H-5‴), 7.45 (*d*, *J* = 16 Hz, H-7‴), 7.52 (*d*, *J* = 8 Hz, H-2‴ and H-6‴); isorhamnetin moiety: ppm 6.33 (*d*, *J* = 2.5, H-6), 6.70 (*d*, *J* = 2.5 Hz, H-8), 6.85 (*d*, *J* = 7.5 Hz, H-5′), 7.8 (*dd*, *J* = 2.5 and 7.5 Hz, H-6′), 7.8.5 (*d*, *J* = 2.5 Hz, H-2′), 12.3 (*s*, OH-5); β-glucoside moieties: ppm 5.12 (*d*, *J* = 8.5 Hz, H-1‴), 5.60 (*d*, = 8.5 Hz, H-1″), 4.32 (*dd*, *J* = 12 and 4.5 Hz, one methylenic H-6″ proton), 4.2 (*m*, one methylenic H-6″ proton), 3.22–3.88 (*m*, glucose protons overlapped by water protons). ^{13}C NMR of **5**: Table 2.

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