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Isolation and structure elucidation of flavonoid and phenolic acid glycosides from pericarp of hot pepper fruit *Capsicum annuum* L.

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Abstract

Hot pepper fruits (*Capsicum annuum* L.) var. Bronowicka Ostra have been studied with regard to content of flavonoids and other phenolics. Nine compounds were isolated from pericarp of pepper fruits by preparative HPLC. Their structures were identified by chromatographic (analytical HPLC) and spectroscopic (UV, NMR) techniques. Two of the identified compounds, *trans-p*-feruloylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside and luteolin-7-*O*-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl)-glucopyranoside were found for the first time in the plant kingdom. Additionally compounds: *trans-p*-feruloyl-β-D-glucopyranoside, *trans-p*-sinapoyl-β- D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside, luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside, apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside and luteolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside] were found for the first time in pepper fruit *Capsicum annuum* L.

Keywords: *Capsicum annuum*; Flavonoids; Phenolic acid

1. Introduction

Hot pepper, genus *Capsicum*, belongs to the great family of tropical plants Solanaceae (Somos, 1984). This taxon also includes both sweet cultivars eaten mainly as vegetables and hot ones, often used as a spice. The basic chemical composition of pepper fruit had been studied fairly well, mainly with respect to vitamin (C, E), β-carotene and carotenoid pigments content (Davis et al., 1970; Minquez-Mosquera and Hornero-Mendez, 1994; Palevitch and Craker, 1995; Daood et al., 1996; Perucka, 1996b; Hornero-Mendez et al., 2000). In case of hot cultivars of pepper fruits the capsaicinoids were also studied. Capsaicinoids are alkaloids important for pharmaceutical industry for their neurological effectiveness. When used at low levels in the diet they significantly decrease serum, myocardial and aortic total cholesterol levels (Govindarajan and Sathyanarayana, 1991). The main component, capsaicin (8-methyl-*N*-vanillyl-6-noneamide), derives from phenylpropanoid pathway like flavonoids and lignins (Bennet and Kirby, 1968; Iwai et al., 1979; Fujiwake et al., 1980; Suzuki et al., 1981; Lindsey and Yeoman, 1983; Hall et al., 1987; Holland, 1989; Ochoa-Alejo and Salgado-Garciglia, 1992). Many studies have been carried out on the accumulation of capsaicinoids (Sudhakar Johnson et al., 1992; Perucka, 1996a; Estrada et al., 1997; Minami et al., 1998; Hertog et al., 1992; Sukrasno and Yeoman, 1993; Lee et al., 1995; Howard, 2000; Formica and Regelson, 1995). However, research on flavonoid and phenolic acid content in pepper fruit is scarce. Most of studies concentrated only on flavonoid aglycones (quercetin and luteolin) obtained after hydrolysis (Hertog et al., 1992; Lee et al., 1995; Howard et al., 2000). Sukrasno and Yeoman (1993) reported the presence in pepper fruits of coumaric, caffeic and 3,4-dimethoxy cinnamic acid glucosides, quercetin 3-*O*-rhamnoside and luteolin 7-*O*-glucoside. They also indicated presence of two other unidentified flavonoids. However, complete characterization of flavonoids in pepper fruit has

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never been performed. Due to the fact that flavonoids show different kinds of biological activity (Formica Regelson, 1995; Rice-Evans et al., 1997; Harborne and Williams, 2000), we deemed it of interest to identify phenolic compounds in this widely consumed vegetable.

Thus, the aim of the present work was isolation and structure elucidation of flavonoid and phenolic acid derivatives occurring in pericarp of hot pepper fruits by HPLC, MS, ^{13}C and ^{1}H NMR methods.

2. Results and discussion

Preliminary analysis of 40% MeOH fraction isolated from hot pepper fruits showed its fairly complex composition. After preparative separation and purification nine pure compounds were obtained as indicated in HPLC profile (Fig. 1). The phenolic acids *trans-p*-feruloyl-β-D-glucopyranoside (**1**), *trans-p*-sinapoyl-β-D-glucopyranoside (**2**) and flavonoids quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (**3**), luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside (**5**), apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside known as schaftoside (**6**), lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside] (**7**) and quercetin 3-*O*-α-L-rhamnopyranoside (**8**) were isolated and identified based on their ^{1}H and ^{13}C NMR spectral data compared to those in the literature (Harborne, 1967; Markham, 1982; Ossipov et al., 1995; Ryan et al., 1999; Steeves et al., 2001). These compounds had been ealier identified in other plants. Glucoside ester of *trans-p*-ferulic acid (**1**) occurs e.g. in *Salvia officinalis* (Lu and Fuo 2000), and of *trans-p*-sinapic acid (**2**) in *Bunias orientalis* (Dietz and Winterhalter, 1996). Apigenin and luteolin *C*-glycosides (**5**, **6**) were identified by Raffaelli et al. (1997) in *Passiflora incarnata*. Luteolin apiosylglucoside (**7**) occurs also in the seeds of parsley and celery, and quercetin 3-*O*-rhamnoside (**8**) among others in *Quercus tinctoria, Vitis vinifera, Prunus salicina* and *P. tomentosa* (Harborne, 1967). These compounds except **8** were found for the first time in pepper fruit.

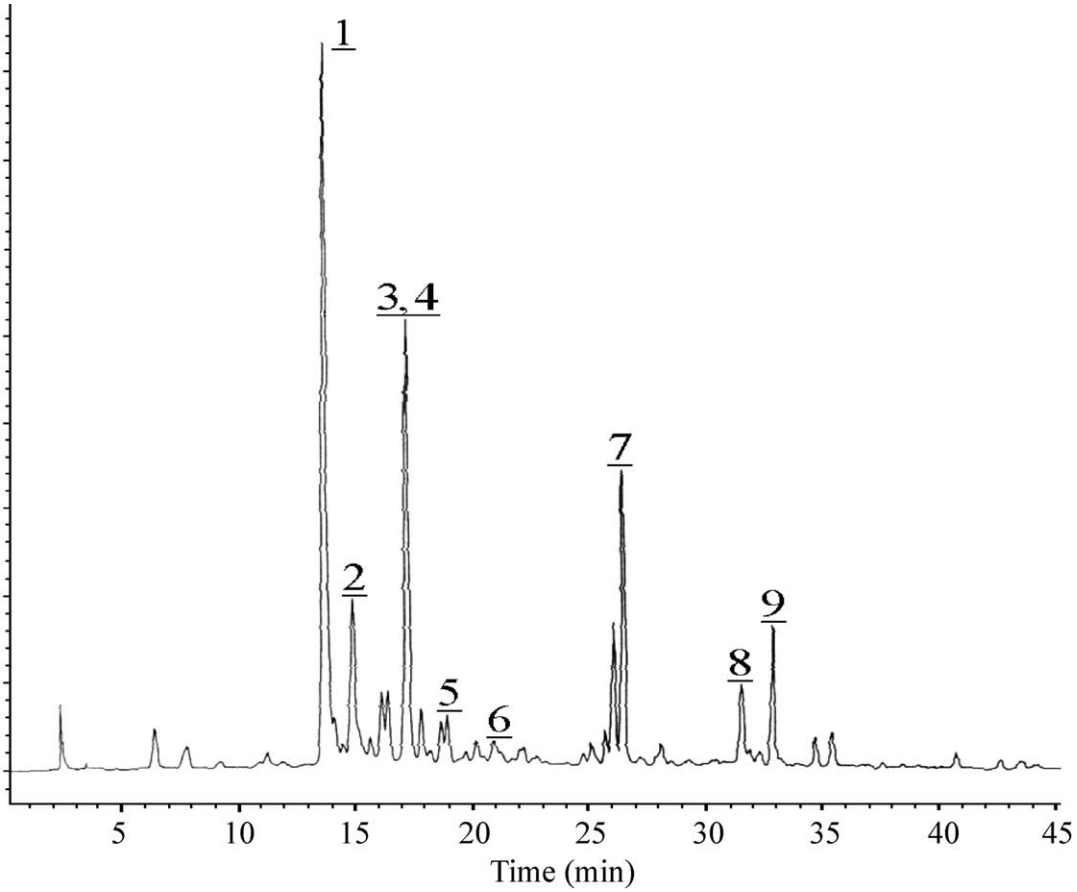


Fig. 1. HPLC–UV chromatogram of phenolic compounds isolated from hot pepper fruits *Capsicum annuum* L. **1**: *trans-p*-feruloyl-β-D-glucopyranoside, **2**: *trans-p*-sinapoyl-β-D-glucopyranoside, **3**: quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside, **4**: *trans-p*-ferulylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside, **5**: luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside, **6**: apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside, **7**: lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside], **8**: quercetin 3-*O*-α-L-rhamnopyranoside, **9**: luteolin 7-*O*-[2-(β-D-apiofuranosyl)-4-(β-D-glucopyranosyl)-6-malonyl]-β-D-glucopyranoside.

Compounds **4** and **9** had not been identified earlier. In the case of compounds **3** and **4** the UV spectrum was recorded for their mixture. In the spectrum presence of three maxima of absorption was found, at 210, 257 and 347 nm. A spectroscopic analysis of compound **4** showed presence of a positive molecular ion $[M+H]^+$ at $m/z=429.5$ and a negative ion $[M-H]^-$ at $m/z=427.4$. The obtained results suggest that the empirical formula of the compound is $C_{20}O_{10}H_{28}$. The ^{13}C NMR analysis confirmed presence of 20 carbon atoms in the molecule, 14 of which belonged to the non-sugar part (Table 1), and the aglycone of the molecule contained 10 carbon atoms. A comparison of the ^1H and ^{13}C NMR spectra with the literature data (Steeves et al., 2001) points to its great similarity to coniferin (feruloyl alcohol 4-*O*-β-D glucoside). However, there were some differences: in compound **4** additional resonances were found in spectrum ^{13}C NMR that pointed to presence of 4 carbon atoms and a ^1H NMR analysis confirmed presence of a 2-methyl-3-hydroxypropionyl substituent. A downfield shift at C-6′ (δ 64.4) of the glucopyranosyl residue in the ^{13}C NMR spectrum proved that this substituent was located at C-6′ (Table 1). Hence, compound **4** is *trans*-*p*-ferulylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside (coniferin 6′-*O*-2-methyl-3-hydroxypropionyl) (Fig. 2).

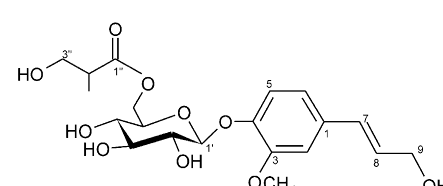
The UV spectrum of compound **9** had three absorption maxima, at 205, 257 and 347 nm. A mass spectrometry analysis showed the empirical formula of compound **9** is $C_{35}O_{23}H_{46}$. The ^{13}C NMR spectrum (Table 1) of the compound revealed presence of 17 carbon atoms belonging to 3 sugar residues, 15 carbon resonances corresponding to the aglycone and three further carbons suggesting the occurrence of a malonyl substituent. The aglycone in this compound was identified as luteolin by comparing the ^1H and ^{13}C NMR data with literature (Markham, 1982; Stochmal et al., 2001). The structure of the oligosaccharide unit was achieved using 1D-TOCSY and 2D NMR experiments. The results of 1D-TOCSY and DQF-COSY experiments allowed the sequential assignments of all proton resonances within each sugar residue, starting from the well isolated anomeric proton signals (Experimental). Thus on the basis of the chemical shifts, the multiplicity of the signals, the absolute values of the coupling constants, the three sugar residues were identified as two β-D-glucopyranosyl units and one β-D-apiofuranosyl unit. The D-configuration of the apiose moiety was proven by the fact that the pair of protons in position 5‴ showed isochronic signals and β-configuration of the anomeric carbon was proven by the ^{13}C NMR shift value measured for C-1‴ ($\delta_c=110.2$) and by comparing these findings with literature data of the three other possible apiofuranosyl-isomers (Ishii and Yanagisawa, 1998; Zidorn et al., 2002).

HSQC experiments, which correlated all proton resonances with those of each corresponding carbon, allowed the assignments of the interglycosidic linkages. The absence of any ^{13}C NMR glycosidation shift for one β-D-glucopyranosyl and the β-D-apiofuranosyl residues suggested these sugars to be terminal. Glycosidation shifts were observed for C-2″ (δ 78.2) and C-4″ (δ 80.4) of the glucose residue with the anomeric proton signal at δ 5.21.

The HMBC experiment which showed long-range correlations between C-7 (δ 64.5) of the aglycon and H-$1_{glc'}$ (δ 5.21), C-$2_{glc'}$ (δ 78.2) and H-1_{api} (δ 5.52), C-$4_{glc'}$ (δ 80.4) and H-$1_{glc''}$ (δ 4.45) C=O of the malonyl unit (δ 168.7) and H-$6_{glc'}$ (δ 4.48 and 4.64), allowed to deter-

Table 1
^{13}C NMR data of the compounds **4** and **9**

Aglycones				Sugars				Other			
C	4	C	9	C	4	C	9	C	4	C	9
1	133.8	2	166.8	1′	101.8	1″	101.0	1″	176.1	1^{V}	168.7
2	111.0	3	104.1	2′	74.2	2″	78.2	2″	43.2	2^{V}	42.1
3	150.7	4	184.1	3′	77.4	3″	76.8	3″	64.4	3^{V}	170.3
4	147.0	5	162.9	4′	70.7	4″	80.4	4″	12.8		
5	117.3	6	101.3	5′	74.9	5″	77.8				
6	120.5	7	164.5	6′	64.4	6″	64.6				
7	131.0	8	96.1			1‴	110.2				
8	128.7	9	158.9			2‴	78.1				
9	63.2	10	107.3			3‴	80.6				
OMe	56.2	1′	123.1			4‴	75.5				
		2′	114.3			5‴	65.9				
		3′	147.1			6‴					
		4′	151.2			1^{IV}	103.5				
		5′	116.8			2^{IV}	74.9				
		6′	120.5			3^{IV}	77.8				
						4^{IV}	69.8				
						5^{IV}	77.8				
						6^{IV}	62.0				



4

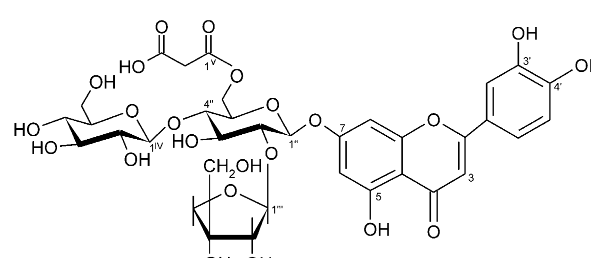


Fig. 2. Structure of the new compounds identified in red pericarp *Capsicum annuum* L. **4**: *trans-p*-Ferulylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl)glucopyranoside). **9**: Luteolin-7-*O*-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl)-glucopyranoside.

mine the position of each sugar residue and to locate the malonyl residue at C-6′ of the glucose residue with the anomeric proton signal at δ 5.21. Hence compound **9** is luteolin 7-*O*-[2-(β-D-apiofuranosyl)-4-(β-D-glucopyranosyl)-6-malonyl]-β-D-glucopyranoside (Fig. 2).

In the present work it was shown that quercetin is found in pericarps of red fruit in two compounds: not only quercetin 3-*O*-rhamnoside as mentioned earlier (Sukrasno and Yeoman, 1993), but also as quercetin 3-*O*-rhamnoside-7-*O*-glucoside, whereas luteolin in the form of three compounds: luteolin 6-*C*-glucoside-8-*C*-arabinoside, luteolin 7-*O*-[2-(apiosyl)-glucoside] and luteolin 7-*O*-[2-(apiosyl)-4-(glucosyl)-6-malonyl]-glucoside.

Concentration of these determined compounds in pericarps of red fruits of pepper cv. Bronowicka Ostra was various. In 1 g of dry mass of fruits there was most sinapic acid (0.382 ± 0.047 mg) and ferulic acid (0.270 ± 0.012 mg) glucosides as well as luteolin apiosylglucoside (0.231 ± 0.026 mg) and quercetin rhamnoside (0.137 ± 0.02 mg). The contents of the remaining compounds was lower and constituted less than 28% of the mass of the determined derivatives of phenolic acids and flavonoids.

A further stage in our studies will be separation of these compounds in bigger amounts, which will allow the examination of their biological activity.

3. Experimental

3.1. General

The HPLC analysis was made on Waters chromatograph with PDA 996 detector, 616 pump and the Millenium programme. Separations were perfomed on RP-18 (Eurospher 80, Säulentechnik 250×4.6 mm, 5 μm) column and at 1 ml/min solvent delivery. The linear gradient of 0–40% acetonitrile (CH_3CN) in 1% H_3PO_4 was used as a solvent system. The initial identification of phenolic compounds fraction was conducted on the basis of UV spectra recorded on PAD detector during chromatographic analysis.

The spectra ^1H NMR and ^{13}C NMR were recorded in CD_3OD on Bruker DRX-600 spectrometer working at 400 MHz for ^1H and 100 MHz for ^{13}C NMR.

Mass spectra ESI-TOF were recorded on Mariner spectrometer (PerSeptive Biosystems).

3.2. Plant material and isolation

Hot pepper *Capsicum annuum* L. var. Bronowicka Ostra was grown in experiment fields of the Agricultural University of Lublin in the years 1997–1999. The fruits were harvested at the end of August at their full maturity stage and divided into pericarps, placenta and seeds. The pericarps were freeze-dried and stored at −20 °C until they were analyzed.

Freeze-dried material (equivalent to 1 kg fresh fruits) was homogenized with 80% EtOH according to Lee et al. (1995). For initial purification the extract was evaporated under reduced pressure to oily residue. The residue was suspended in water and loaded onto C18 column (3×5 cm, LiChroprep RP-18, 40–63 μm, Merck) equilibrated with water. The column was washed with H_2O and the phenolic compounds were washed out with 40% MeOH. The MeOH fraction was evaporated to dryness, redissolved in H_2O, initially analyzed on HPLC in gradient system (0–40% CH_3CN in 1% H_3PO_4) (Stochmal et al., 2001), then the phenolic fraction was loaded onto preparative column (3×40 cm, LiChroprep RP-18, 25–40 μm, Merck). The column was washed with H_2O and then with a linear gradient of MeOH–H_2O mixture (starting with 0 and ending with 100% MeOH). Fractions of 10 cm^3 were collected with a fraction collector. The obtained fractions were analyzed with TLC (DC-Alufolien Cellulose, Merck) developed in 15% AcOH and observed in UV light. Fractions showing similar TLC patterns were combined, evaporated to dryness and redissolved in a small amount of MeOH. Each fraction was analyzed on HPLC. Fractions containing two or three components were further purified on RP-18 column (0.8×25 cm, 10 μm, Merck), in isocratic system (CH_3CN–1% H_3PO_4) with concentration selected for each fraction on the basis of HPLC separation. Fractions containing one compound were combined, dried and their structures were determined by HPLC, MS, ^{13}C and ^1H NMR methods.

Nine single compounds were isolated.

3.3. trans-p-Ferulylalcohol-4-O-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside (**4**)

UV λ_{max} (nm) 210, 257, 347; MS *m*/*z*: 429.5 $[M+H]^+$, 446.4 $[M+NH_4]^+$, 451.3 $[M+Na]^+$, 427.4 $[M-H]^-$; 1 H NMR δ: 7.11 (1H, *d*, *J* = 1.3 Hz, H-2), 7.09 (1H, *d*, *J* = 8.0 Hz, H-5), 6.97 (1H, *dd*, *J* = 1.3 and 8.0 Hz, H-6), 6.57 (1H, *d*, *J* = 16.0 Hz, H-7), 6.32 (1H, *dd*, *J* = 5.9 and 16.0 Hz, H-8), 4.93 (1H, *d*, *J* = 7.5 Hz, H-1Glc), 4.50 (1H, *dd*, *J* = 2.0 and 12.0 Hz, H-6Glc), 4.25 (1H, *dd*, *J* = 4.5 and 12.0 Hz, H-6Glc), 4.25 (2H, *d*, *J* = 5.9 Hz, H-9), 3.91 (3H, *s*, O-CH_3), 3.85 (1H, *dd*, *J* = 5.5 and 11.0 Hz, H-3″), 3.68 (1H, *dd*, *J* = 9.0 and 9.0 Hz, H-4Glc), 3.68 (1H, *m*, H-5Glc), 3.65 (1H, *dd*, *J* = 5.5 and 11.0 Hz, H-3″), 3.54 (1H, *dd*, *J* = 7.5 and 9.0 Hz, H-2Glc), 3.40 (1H, *dd*, *J* = 9.0 and 9.0 Hz, H-3Glc), 2.64 (1H, *m*, H-2″), 1.14 (3H, *d*, *J* = 6.5 Hz, H-4″). ^{13}C NMR see Table 1.

3.4. Luteolin 7-O-[2-(β-D-apiofuranosyl)-4-(β-D-glucopyranosyl)-6-malonyl]-β-D-glucopyranoside (**9**)

Amorphous yellow powder; UV λ_{max} (nm) 205, 257, 347; MS *m*/*z*: 829.8 $[M+H]^+$, 827.5 $[M-H]^-$; 1H NMR δ: 7.45 (1H, *dd*, *J* = 1.2 and 8.0 Hz, H-6′), 7.44 (1H, *d*,

$J = 1.2$ Hz, H-2′), 6.94 (1H, *d*, $J = 8.0$ Hz, H-5′), 6.81 (1H, *d*, $J = 1.2$ Hz, H-8), 6.63 (1H, *s*, H-3), 6.51 (1H, *d*, $J = 1.2$ Hz, H-6), 5.52 (1H, *d*, $J = 2.0$ Hz, H-1Api), 5.21 (1H, *d*, $J = 7.5$ Hz, H-1Glc_1), 4.64 (1H, *dd*, $J = 2.0$ and 12.0 Hz, H-6Glc_1), 4.48 (1H, *dd*, $J = 4.5$ and 12.0 Hz, H-6Glc_1), 4.45 (1H, *d*, $J = 7.5$ Hz, H-1Glc_2), 4.07 (1H, *d*, $J = 10.0$ Hz, H-4Api), 3.98 (1H, *m*, H-5Glc_1), 3.95 (1H, *d*, $J = 2.0$ Hz, H-2Api), 3.92 (1H, *dd*, $J = 2.0$ and 12.0 Hz, H-6Glc_2), 3.86 (1H, *dd*, $J = 9.0$ and 9,0 Hz, H-3Glc_1), 3.86 (1H, *d*, $J = 10.0$ Hz, H-4Api), 3.79 (1H, *dd*, $J = 7.5$ and 9.0 Hz, H-2Glc_1), 3.71 (2H, *dd*, $J = 9.0$ and 9.0 Hz, H-4Glc_1, H-4Glc_2), 3.71 (1H, *dd*, $J = 4.5$ and 12.0 Hz, H-6Glc_2), 3.57 (2H, *s*, H-5Api), 3.43 (1H, *dd*, $J = 9.0$ and 9.0 Hz, H-3Glc_2), 3.40 (1H, *m*, H-5Glc_2), 3.39 (2H, *s*, H-2^V), 3.26 (1H, *dd*, $J = 7.5$ and 9.0 Hz, H-2Glc_2). ^{13}C NMR see Table 1.

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