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NOTE

Two new acetylated flavonoid glycosides from *Centaurium spicatum* L

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Abstract Two new acetylated flavonol glycosides, quercetin 3-*O*-[(2,4-diacetyl-α-L-rhamnopyranosyl)-(1→6)]-2,4-diacetyl-*β*-D-galactopyranoside (**1**) and quercetin 3-*O*-[(2,4-diacetyl-α-L-rhamnopyranosyl)-(1→6)]-3,4-diacetyl-*β*-D-galactopyranoside (**2**), in addition to two known acetylated quercetin glycosides quercetin 3-*O*-[(2,3,4-triacetyl-α-L-rhamnopyranosyl)-(1→6)-*β*-D-galactopyranoside (**3**) and quercetin 3-*O*-[(2,3,4-triacetyl-α-L-rhamnopyranosyl)-(1→6)-3-acetyl-*β*-D-galactopyranoside (**4**), were isolated from the aerial part of *Centaurium spicatum* (L.) Fritsch (Gentianaceae). Structure elucidation, especially the localization of the acetyl groups, and complete ^{1}H and ^{13}C NMR assignments of these biologically active compounds were carried out using one- and two-dimensional NMR measurements, including ^{1}H- and ^{13}C-NMR, DEPT-135, H–H COSY, HMQC and HMBC, in addition to HR-FAB/MS experiments.

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Introduction

Centaurium spicatum (L.) Fritsch (Gentianaceae) is an erect small branched annual herb, widespread in Southern Europe and North Africa, where it is used together with other *Centaurium* species such as *C. pulchellum* in traditional medicine for the treatment of abdominal pain, hypertension, gallstones, kidney and ureter stones, renal colic, wounds, and diabetes [1]. The Arabic name is 'Kantaryoun'. A survey of the current literature revealed the isolation and identification of secoiridoids (sweroside, swertiamarin and gentiopicrin) and polyoxygenated xanthones from the plant [1–4]. Alkaloids of the pyridine type (e.g. gentianine), spicatine and the series of amides derived from the secoiridoid glucoside swertiamarin, and kantaurin were also shown to be present [3]. In the present work we report the isolation and structure elucidation of two new acetylated flavonol glycosides **1** and **2**, together with two other known compunds **3** and **4**. The general methodology used for the structure elucidation and the spectral assignments of the two new acetylated flavonol glycosides from *C. spicatum* is discussed in this paper.

Results and discussion

Compound **1** was obtained as yellowish green amorphous powder (2.0 mg), soluble in methanol with $[\alpha]_D^{29.1}$ −39° ($c = 0.333$, MeOH). The UV spectrum (in MeOH) exhibited absorption maxima at 253 nm (band-II) and 356 nm (band-I), indicating a flavonol type. In addition, a

bathochromic shift (58 nm) with a high intensity of band-I was observed in the NaOMe spectrum which indicated the presence of a free OH group at position C-4′. The presence of a free OH group at position C-5 was proved through the $AlCl_3$ spectrum where there is a bathochromic shift (47 nm) in band-I relative to MeOH spectrum. The presence of an ortho-dihydroxy system in ring B was confirmed from the hypsochromic shift when HCl was added to the $AlCl_3$. The presence of a bathochromic shift (14 nm) in band-II with NaOAc indicated the presence of a free OH group at C-7. The IR spectrum of **1** (in $CHCl_3$) indicated the presence of hydroxyl (3,440 cm^{-1}), carbonyl (1,780 cm^{-1}), and phenyl (2,980, 1,640, 1,530 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **1** indicated the presence of a quercetin moiety, two sugar moieties (hexoses) and four acetyl groups. The ^{1}H-NMR spectrum showed a pair of doublets at δH 6.21 (H-6) and δH 6.42 (H-8), and a three-spin system with the typical coupling pattern of a 1′,3′,4′-trisubstituted benzene ring [δH 7.63 (H-2′), 6.86 (H-5′) and 7.67 (H-6′)], which are two features characteristic of a flavonol with phenolic hydroxyl groups at positions 5, 7, 3′ and 4′. The ^{13}C-NMR and DEPT spectra (showing the multiplicities of the carbon atoms) were in agreement with a 3-substituted quercetin moiety. Substitution of quercetin in position C-3 was evident from the chemical shift of C-2 (δC 159.5), whereas in flavonols with an unsubstituted hydroxyl functionality at this position C-2 is expected around δC 147 [5]. The typical doublet of the C-6 secondary methyl group of rhamnose was found at δH 0.96 (1H, d, $J = 6.4$ Hz) and δC 17.5 in the ^{1}H- and ^{13}C-NMR spectra, respectively. The anomeric hydrogens showed characteristic doublets in ^{1}H-NMR spectrum at δH 5.30 for galactose, with a coupling constant of 7.7 Hz, indicating a β-configuration, and at δH 4.53 for rhamnose, with a coupling constant of 1.7 Hz, indicating α-configuration [6]. In addition, optical rotation values of both D-galactopyranose and L-rhamnopyranose tetrabenzoate derivatives of the acid hydrolysis products of **1** were $[\alpha]_D^{31.5}$ +54.5 ($c = 0.019$, $CHCl_3$) for β-D-galactopyranose tetrabenzoate and $[\alpha]_D$ $^{31.8}$ +76.9 ($c = 0.002$, $CHCl_3$) for α-L-rhamnopyranose tetrabezoate, which were identical with the optical rotation of the synthetic models of both [7, 8]. A long-range correlation, observed in the HMBC experiment, between C-3 of quercetin (δC 135.1) and the anomeric proton of galactose (δH 5.30) confirmed that this was the site of glycosylation, and that galactose was the first sugar. The only methylene functionality of the molecule, C-6 of galactose, was found at δC 68.0 in the ^{13}C NMR spectrum, after establishing its multiplicity in the DEPT-135 experiment. This chemical shift was comparable to the value observed for C-6 of glucose in rutin, a rhamnosyl-(1–6)-glucoside, and therefore indicated substitution of galactose in position C-6 with rhamnose [5]. This was obvious from the long-range correlation between C-1 of rhamnose (δC 99.2) and both H-6 hydrogens of galactose (δH 3.58 and 3.30, as assigned by their one-bond C–H correlation, observed in an HMQC experiment, with C-6 of galactose at δC 68.0). All ^{1}H- and ^{13}C-NMR signals of the quercetin moiety and both sugar residues could be assigned by analyzing the H–H COSY, HMQC and HMBC spectra (Table 1). Long-range correlations observed between the carbonyl signal at δC 171.4 and H-2 of galactose (δH 4.99), at δC 172.6 and H-4 of galactose (δH 5.26), at δC 171.5 and H-2 of rhamnose (δH 5.05) and at δC 171.8 and H-4 of rhamnose (δH 4.84) unequivocally allowed us to locate the acetyl groups at positions C-2, C-4 of galactose and at C-2, C-4 of the terminal rhamnose residue. This was confirmed by four-bond correlations observed between the corresponding carbons of galactose (C-2 at δC 70.7 and C-4 at δC 71.9 and the methyl signals at δH 1.87 and δH 2.18, respectively) as well as the four-bond correlations observed between the corresponding carbons of rhamnose (C-2 at δC 70.6 and C-4 at δC 71.8 and the methyl signals at δH 1.97 and δH 2.05, respectively). The molecular weight of 778 with a molecular formula $C_{35}H_{38}O_{20}$, which was evident from an $[M+H]^+$ peak at *m/z* 779 in the positive ion FAB mass spectrum, and also from $[HR\text{-}FAB]^+$/mass at 779.2031, was in agreement with a tetracetylated quercetin rhamnosyl galactoside. Hence **1** could unequivocally be identified as quercetin 3-*O*-[(2,4-diacetyl-α-L-rhamnopyranosyl)-(1→6)]-2,4-diacetyl-β-D-galactopyranoside, a new compound.

Compound **2** was obtained as yellowish green amorphous powder (1.5 mg), soluble in methanol with $[\alpha]_D^{29.8}$ −34.6° ($c = 0.333$, MeOH). The UV spectrum (in MeOH) exhibited absorption maxima at 256 nm (band-II) and 358 nm (band-I), indicating a flavonol type. In addition, a bathochromic shift (54 nm) with a high intensity of band-I was observed in the NaOMe spectrum, which indicated the presence of a free OH group at position C-4′. The presence of a free OH group at position C-5 was proved through the $AlCl_3$ spectrum where there was a bathochromic shift (43 nm) in band-I relative to MeOH spectrum. The presence of an ortho-dihydroxy system in ring B was confirmed from the hypsochromic shift when HCl was added to the $AlCl_3$. The presence of a bathochromic shift (12 nm) in band-II with NaOAc indicated the presence of a free OH group at C-7. The IR spectrum of **2** (in $CHCl_3$) indicated the presence of hydroxyl (3026 cm^{-1}), carbonyl (1747 cm^{-1}), and phenyl (2927, 1610, 1550 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **2** looked very similar to those of **1**, as it has a molecular weight of 778 with the same molecular formula, which was evident from an $[M+H]^+$ peak at *m/z* 779 in the positive ion FAB mass spectrum, and also from $[HR\text{-}FAB]^+$/mass at 779.2031 but with a small difference in the distribution of the acetyl groups. Based on the assignments, a long-range C–H correlation was

Table 1 ^{13}C- and ^{1}H-NMR assignments for compounds **1** and **2** recorded in CD_3OD

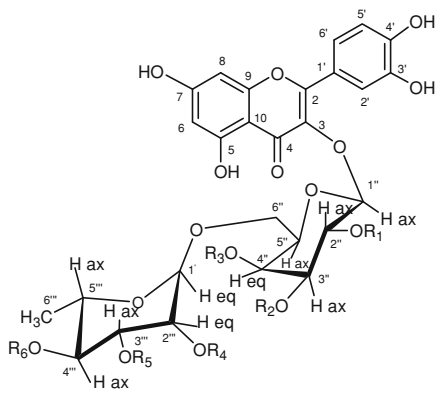
Position	**1**		**2**	
	^{13}C-NMR (δ, mult)	^{1}H-NMR [δ, mult, J (Hz)]	^{13}C-NMR (δ, mult)	^{1}H-NMR [δ, mult, J (Hz)]
2	159.5,s		158.5,s	
3	135.1,s		135.1,s	
4	179.4,s		179.3,s	
5	163		163.8,s	
6	99.9,d	6.19,d, 2.2	99.3,d	6.21,d, 2.4
7	165.9,s		166.2,s	
8	94.8,d	6.36,d, 2.2	94.9,d	6.42,d, 2.4
9	158.5,s		158.7,s	
10	105.7,s		105.6,s	
1′	123.2,s		122.6,s	
2′	117.5,d	7.63,d, 2.2	117.8,d	7.89,d, 2.4
3′	145.9,s		145.8,s	
4′	149.7,s		150.1,s	
5′	115.9,d	6.86,d, 8.7	116.2,d	6.86,d, 8.5
6′	123.4,d	7.67,dd, 8.7, 2.2	122.9,d	7.64,dd, 8.5, 2.4
Galactopyranosyl				
1″	104.3,d	5.30,d, 7.7	105.6,d	5.23,d, 7.9
2″	70.7,d	4.99, overlapped	70.8,d	4.01,dd, 10.3, 7.9
3″	73.6,d[a]	3.82,dd, 10.0, 3.4	77.4,d	4.82,dd, 10.3, 3.4
4″	71.9,d	5.26,d, 3.4	72.0,d	4.05,d, 3.4
5″	73.2,d[a]	3.86, overlapped	75.6,d	3.86, overlapped
6″	68.0,t	3.58, overlapped	68.0,d	3.58, overlapped
		3.30, overlapped		3.30, overlapped
2-Acetyl			3-Acetyl	
C=O	171.4,s		C=O	171.4,s
–CH_3	20.6,q	1.87,s	–CH_3	20.6,q	1.87,s
4-Acetyl			4-Acetyl	
C=O	172.6,s		C=O	172.4,s
–CH_3	21.0,q	2.18,s	–CH_3	21.0,q	2.18,s
Rhamnopyranosyl				
1	99.2,d	4.53,d, 1.7	100.0,d	4.60,d,1.7
2	70.6,d	5.05,dd, 6.8, 1.7	70.6,d	5.01,dd, 6.8, 1.7
3	73.1,d	3.78, overlapped	75.3,d	3.78,dd, 6.8, 2.7
4	71.8,d	4.84, overlapped	71.1,d	4.84,dd, 10.0, 2.7
5	67.5,d	3.66,dd, 10.0, 6.4	67.8,d	3.66,dd, 10.0, 6.4
6	17.5,q	0.96,d, 6.4	17.5,q	0.96,d, 6.4
2-Acetyl			2-Acetyl	
C=O	171.5,s[b]		C=O	171.5,s[d]	
–CH_3	20.6,q[c]	1.97,s	–CH_3	20.7,q	1.97,s
4-Acetyl			4-Acetyl	
C=O	171.8,s[b]		C=O	171.7,s[d]	
–CH_3	20.7,q[c]	2.05	–CH_3	20.7,q	2.05

[a,b,c,d] Assignments bearing the same superscript may be reversed

observed in an HMBC experiment between the carbonyl group of the first acetyl functionality (δC 171.4) and one of the two remained hydrogens of the galactosyl residue at δH 4.82, but there were two possible locations of the first acetyl group, at C-2 or C-3 of galactose. However, the ^{1}H-NMR signal at δH 4.82 was a doublet of doublets with

coupling constants of 10.3 and 3.4 Hz, which obviously corresponded to an axially and equatorially coupled hydrogen atom [9]. In galactopyranose, which is the C-4 epimer of glucopyranose, all hydrogens except H-4 are axial. Since H-4 in galactose is equatorial, it has one H_{ax}–H_{eq} coupling with H-3. On the other hand, H-2 has two H_{ax}–H_{ax} couplings. Thus, in **2** only H-3 of the galactoside residue could show an H_{ax}–H_{eq} (with H-4) and an H_{ax}–H_{ax} (with H-2) coupling, and the doublet of doublets at δH 4.82 could be assigned to this hydrogen, corresponding to a ^{13}C-NMR signal at δC 77.4 (C-3 of galactose) in HMQC experiment. Hence **2** could unequivocally be identified as quercetin 3-*O*-[(2,4-diacetyl-α-L-rhamnopyranosyl)-(1→6)]-3,4-diacetyl-β-D-galactopyranoside, a new compound. In addition, two known acetylated quercetin glycosides, quercetin 3-*O*-[(2,3,4-triacetyl-α-L-rhamnopyranosyl)-(1→6)-β-D-galactopyranoside (**3**) (27.0 mg) and quercetin 3-*O*-[(2,3,4-triacetyl-α-L-rhamnopyranosyl)-(1→6)-3-acetyl-β-D-galactopyranoside (**4**) (1.5 mg), previously isolated from the same plant [10], were also isolated and the data were compared with those previously published. (Fig. 1).

Acylated flavonoid glycosides are less common than flavonoid glycosides in the plant kingdom; acetic acid is only one of the acylating acids of the sugar hydroxyl groups that have been described. Acylation changes the solubility properties of the original flavonoid glycoside, converting it into a lipophilic substance [11], from which acylation of anthocyanins in aqueous media using phenolic acids as acyl donors can be achieved in vitro using natural



	R_1	R_2	R_3	R_4	R_5	R_6
1	$COCH_3$	H	$COCH_3$	$COCH_3$	H	$COCH_3$
2	H	$COCH_3$	$COCH_3$	$COCH_3$	H	$COCH_3$
3	H	H	H	$COCH_3$	$COCH_3$	$COCH_3$
4	H	$COCH_3$	H	$COCH_3$	$COCH_3$	$COCH_3$

Fig. 1 Structures of compounds **1**–**4** from *C. spicatum*

plant enzymes [12]. There are also a number of reports of the use of lipases to acylate flavonoid glycosides [13, 14]. Most of these studies used *Candida antarctica* lipase B (Novozym 435®) to acylate common flavonoid glycosides, e.g. naringin, with fatty acids, in organic solvents at low water activity. The acylation reactions appear to be selective for primary sugar residue hydroxyl groups, where present, and for the secondary 4-hydroxyl of rhamnose residues [15]. In conclusion, the structure of two new acetylated flavonoid glycosides from *C. spicatum* could unequivocally be established, and their ^{1}H- and ^{13}C-NMR spectra completely assigned, by 1D- and 2D-NMR techniques. To further elucidate the beneficial activities of the acetylated flavonoid glycosides, we are currently investigating biological activities such as anti-inflammatory or anti-melanogenesis of these acetylated flavonoid glycosides, comparing them with the corresponding non-acylated compounds.

Experimental

Spectroscopic methods

Optical rotations were determined with a Horiba SEPA-3000 high-sensitivity polarimeter. UV spectra were measured on a Shimadzu UV–Vis spectrometer. IR spectra were recorded on a Shimadzu IR-460 IR spectrophotometer. NMR spectra were recorded on a JOEL EC-600 spectrometer operating at 600.17 MHz for ^{1}H and at 150.92 MHz for ^{13}C at 20.1°C in CD_3OD (99.5% D) in 5-mm sample tubes. The solvent peaks at δH 3.30 in the ^{1}H- NMR spectra, and at δC 49.00 in ^{13}C-NMR spectra, respectively, were used as internal references downfield of tetramethylsilane (TMS) at 0 ppm. Spectral widths were 9008 Hz (26K acquisition points) and 37878 Hz (26K acquisition points) for ^{1}H- and ^{13}C-NMR, respectively. Chemical shifts are presented in ppm downfield of TMS. For CH_n groups, n was determined in DEPT-135 yielding a 180° phase difference between $–CH_2–$ signals on the one hand and –CH– and $–CH_3$ signals on the other). Proton–proton chemical shift correlations were obtained in a COSY experiment. Proton–carbon chemical shift correlations were obtained in inversely detected HMQC and HMBC experiments. Fast atom bombardment (FAB) mass spectra were recorded on a MStation instrument in the positive ion mode, using glycerol as the liquid matrix.

Plant material

C. spicatum (L.) Fritsch (Gentianaceae) aerial parts were collected in May 2009 from New Valley, 200 km southwest of Assiut City, Egypt. The plant was identified and

authenticated by Prof. Dr. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University. A voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy Assiut University, Assiut, Egypt and at the Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan.

Extraction and isolation

Air-dried *C. spicatum* aerial parts (2 kg) was extracted three times with MeOH (5 L) at room temperature. The solvents were combined and filtered through filter paper (Advantec MFS Incorporated). The solvent was removed under reduced pressure at 40°C to yield the methanol extract (550 g), which was partitioned between distilled water and ethylacetate (1 L of each) to give the aqueous fraction (300 g) and the ethyl acetate fraction (80 g). The aqueous fraction was further partitioned by *n*-butanol to give the *n*-butanol fraction (100 g) and the remaining aqueous fraction (140 g). The ethyl acetate fraction was in turn partitioned between 90% methanol and *n*-hexane to give the 90% methanol fraction (50 g) and *n*-hexane fraction (20 g). The *n*-butanol fraction (100 g) was separated on a Diaion HP-20 column using water (2 L), and ethanol (25, 50, 75, and 100%) (2 L of each). The aqueous fraction was further subjected to Sephadex G-200 (Pharmacia Fine Chemicals, Sweden) using stepwise gradient 25, 50 and 75% methanol solvent to give three fractions. Individual fractions were obtained after removal and evaporation of the respective solvents. A 50% methanol-eluted fraction (33.4 g) was further separated by chromatography on an ODS column (80 × 200 mm) (Cosmosil 140 C_{18} PREP, Nacalai Tesque, Tokyo, Japan) using six concentrations of CH_3CN–H_2O (10, 25, 40, 50, 60, 70, and 90% v/v; elution volume 1.5 L of each) to give six corresponding fractions. The fraction eluted with 40% CH_3CN (3.8 g) was further separated by column chromatography on silica gel and stepwise gradient eluted with $CHCl_3$:MeOH (ratios of 9:1, 6:1, 4:1, 3:1, and 1:1, v/v, elution volume 200 ml each) to give five corresponding fractions. A 150-mg portion of the fraction eluted with 6:1 $CHCl_3$:MeOH was further separated by preparative HPLC, ODS column C30 UG-5, 20 mm × 250 mm, particle size 5 μm, flow rate 3 ml/min (Develosil, Nacalai Tesque) equipped with a UV detector (210 nm). The mobile phase was 25% CH_3CN in H_2O. This resulted in elution of compound **1**. These preparative HPLC conditions were also used to separate the same fraction giving compound **2**. Spectroscopic data for compounds **1** and **2** are available as supplementary materials.

Acid hydrolysis

Acid hydrolysis of the glycosides was carried out by refluxing 5 mg of compound in 5 ml of 6% HCl in MeOH for 3 h. The reaction mixture was partitioned against EtOAc (3 × 10 ml). The aglycone was obtained from the EtOAc layer and identified as quercetin by co-chromatography on silica gel with a reference sample (Sigma-Aldrich, St. Louis, MO, USA). The aqueous layer was evaporated and developed crystal needles with EtOAc–H_2O–MeOH–HOAc (13:3:3:4). Identification of galactose and rhamnose present in the sugar fraction was carried out by comparison with authentic samples, galactose (R_f 0.42), glucose (R_f 0.48), and rhamnose (R_f 0.64) (Sigma-Aldrich), in TLC over silica gel ($CHCl_3$–MeOH–H_2O 8:5:1) using 5% H_2SO_4 in MeOH as spraying reagent followed by heating the plates at 120°C for 15–20 min.

Measurements optical rotation of D-galactopyranose and L-rhamnopyranose tetrabenzoate derivatives

Benzoyl chloride (0.5 ml) was added to each ice-cooled solution of either D-galactopyranose (18.0 mg) or L-rhamnopyranose or (15.0 mg) in dry pyridine (1.0 ml), and each mixture was stirred at room temperature for 15 h. MeOH (1.0 ml) was added dropwise to the reaction mixture, stirred for 30 min, and then diluted with EtOAc and aqueous Na_2CO_3, and the layers were separated. Each organic layer was washed with brine, and the combined aqueous layers for each were extracted with EtOAc. Each combined organic extract was dried over $MgSO_4$ and concentrated, and the corresponding residual dark brown oils were individually purified by silica gel CC (eluting with hexane/EtOAc 5:1) to give either D-galactopyranose tetrabenzoate (40 mg) $[\alpha]_D^{31}$ +53.5 ($c = 1.2$, $CHCl_3$) or L-rhamnopyranose tetrabenzoate (22 mg) $[\alpha]_D^{29.6}$ +75.0 ($c = 1.6$, $CHCl_3$) as colorless oils, respectively [7, 8].

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