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TRITERPENOID SAPONINS FROM *BELLIUM BELLIDIOIDES*. STRUCTURES OF THE MINOR DEACYLSAPONINS

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Key Word Index—*Bellium bellidioides*; Asteraceae; chemotaxonomy; triterpenoid saponins; bellidioside B_4; bellissaponin BS2; polygalacic acid.

Abstract—A new deacylsaponin of polygalacic acid, desacylbellidioside B_4, was obtained from the whole plants of *Bellium bellidioides* L. The structure has been elucidated by a general strategy involving mass spectrometry (ESI-MS, including tandem MS, and GC-MS) and high-field one- and two-dimensional NMR spectroscopy (^1H and ^{13}C NMR, COSY-45, HMQC, HMBC) as 3-*O*-α-L-rhamnopyranosyl-2β, 3β, 16α, 23-tetrahydroxyolean-12-en-28-oic acid 28-*O*-α-L-rhamnopyranosyl(1 → 3)-β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-[α-L-arabinofuranosyl(1 → 3)-β-D-fucopyranoside. Moreover, bellissaponin BS2 and besysaponin C_{12} have also been isolated, demonstrating the close chemical relationship of *B. bellidioides* to the *Bellis* genus.

INTRODUCTION

Bellium bellidioides L. is a small perennial herb which is native to the islands in the western part of the Mediterranean. The external appearance is similar to the *Bellis* genus. In previous papers, we reported the isolation and structure elucidation of a number of triterpenoid saponins from plants belonging to the *Bellis* genus [1–6]. In the course of these studies, we have also determined the major saponins of *B. bellidioides* [7] which show both similarities and differences in their saponin composition to those of the *Bellis* genus. The present paper describes the isolation and structure elucidation of minor saponins of *B. bellidioides*, among which is a new compound possessing L-arabinofuranose as a sugar constituent. Currently, several possible strategies exist for the efficient structure elucidation of natural products by modern spectroscopic methods. For the saponins, in situations where sufficient material is available (5–15 mg), we find a combination of mass spectrometric and nuclear magnetic resonance techniques to be most useful. The strategy used in the present paper is summarized as follows:
(1.) ESI-mass spectrometry of underivatized material to establish the molecular mass and MS-MS to determine sugar chains from the fragmentation pattern;
(2.) GC and methanolysis to identify sugars present and their absolute configurations;
(3.) GC-mass spectral analysis of methylated alditol acetates to identify sugar substitution patterns;
(4.) One and two-dimensional and $^1H/^{13}C$ NMR spectroscopy, using through-bond correlations, to confirm molecular fragments and to finally establish their sequence.

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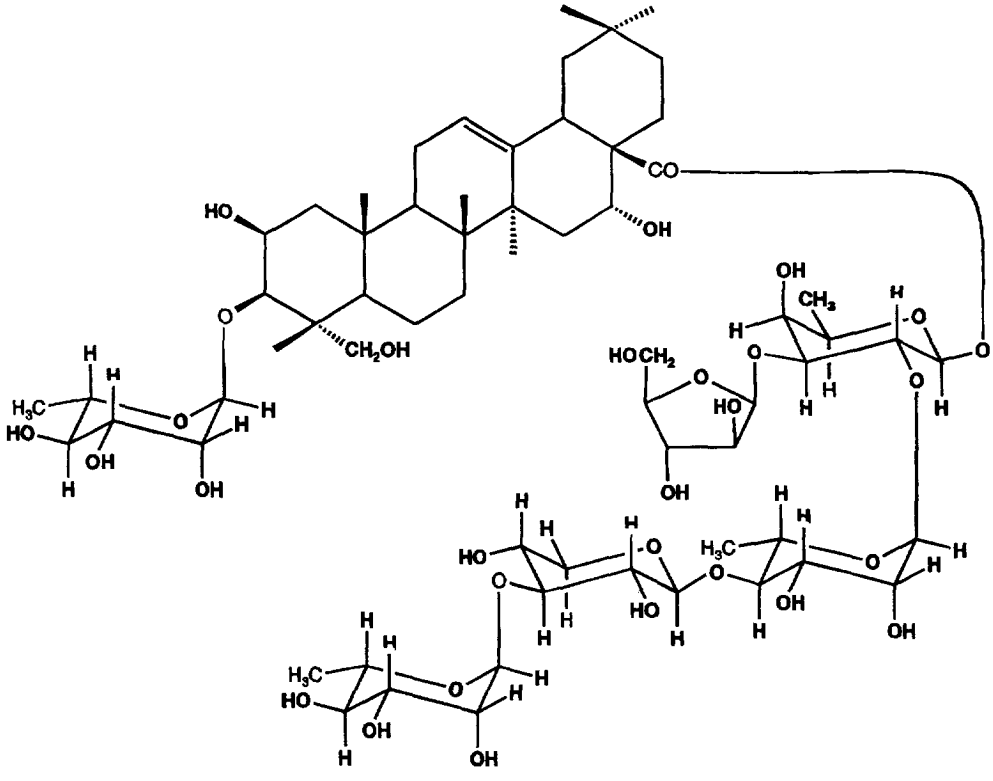
RESULTS AND DISCUSSION

Whole plants of *B. bellidioides* were extracted and worked up as described previously [7]. A part of the deacylated saponin mixture was subjected to preparative, centrifugal accelerated radial TLC on silica gel (solvent $CHCl_3$–MeOH–H_2O 13:3:1, lower layer) and gave three fractions, A to C. Compounds **1** (deacylbellidioside B_3) and **2** (deacylbellidioside B_4) were obtained by preparative HPLC of fraction C on LiChrosorb® RP-18 using MeOH–H_2O (31:19).

Compound **1** showed the same R_f value and colour reaction by spraying with anisaldehyde–sulphuric acid on TLC as bellissaponin BS2; it also had the same retention time on HPLC. Inspection of the ^{13}C NMR data proved that deacylbellidioside B_3 was identical to bellissaponin BS2. Consequently, compound **1** is 3-*O*-β-D-glucopyranosyl-2β, 3β, 16α,23-tetrahydroxyolean-12-en-28-oic acid 28-α-L-rhamnopyranosyl(1 → 3)-β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-fucopyranoside.

The second isolate, compound **2**, proved to be a new saponin and its structure was determined using the strategy outlined above. ESI-mass spectrometry gave ions at *m/z* 1353 $[M + H]^+$ and 1375 $[M + Na]^+$, which are compatible with the presence of polygalacic acid



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(2β,3β,16α,23-tetrahydroxyolean-12-en-28-oic acid) as aglycone, and four molecules of deoxyhexose and two molecules of pentose as sugar components. The tandem mass spectrum of the sodium adduct yielded an ion at *m/z* 725 [dhex + pent + dhex + pent + dhex − H_2O + Na]$^+$ as base peak, indicating that **1** possessed a pentasaccharide unit bound acylglycosidically. Rhamnose, xylose, fucose and arabinose were found as sugar components of **2** from methanolysis and GC identification. Hydrolysis and GC identification of the products obtained from reaction with L-cysteinmethylester hydrochloride according to ref. [8] showed that fucose and xylose were present as D-enantiomers, and rhamnose and arabinose as the L-enantiomer in a ratio of 1:1:3:1.

GC-mass spectral analysis of the methylated alditol acetates yielded 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabitol, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-xylitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol and 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-fucitol identified from their retention times and characteristic fragmentation patterns [9]. Peak areas indicate the presence of two terminal rhamnoses and one terminal arabinose. The one-dimensional ^{1}H NMR spectrum (Table 2) showed characteristic signals of one olefinic proton, six anomeric protons, six methyl singlets and four methyl doublets that are characteristic of polygalacic acid with six sugar moieties. From these combined data it appears probable that **2** is a derivative of bellissaponin BS1 carrying an arabinose at C-3 of fucose.

To demonstrate this unequivocably, two-dimensional COSY-45, HMQC (^{1}H-detected multiple quantum coherence) [11] and HMBC (^{1}H-detected multiple-quantum multiple-bond coherence) [12] spectra were recorded. The cross-peaks in these spectra confirmed the presence of polygalacic acid and the sugar moieties and allowed the assignment of all the ^{1}H and ^{13}C NMR signals (Tables 1 and 2). In addition to establishing the assignments of quaternary carbons, cross peaks in the HMBC spectrum unambiguously established the fragment connectivities from three-bond correlations. Thus, the correlation between H-1 (δ 4.90) and C-1 (δ 104.6) of one rhamnose and C-3 (δ 82.5) and H-3 (δ 3.73) of the aglycone confirmed the position of this sugar. The structure of the pentasaccharide chain was confirmed by the connectivities between H-1/C-1 (δ 5.18/102.5) of the second terminal rhamnose and C-3/H-3 (δ 84.2/3.51) of xylose, between H-1/C-1 (δ 4.53/107.0) of xylose and C-4/H-4 (δ 84.3/3.58) of rhamnose, between H-1/C-1 (δ 5.20/101.9) of rhamnose and C-2/H-2 (δ74.0/3.97) of fucose and between H-1/C-1 (δ 5.14/111.6) of arabinose and C-3/H-3 (δ 84.4/3.78) of fucose. A cross peak between H-1 of fucose (δ 5.42) and C-28 of the aglycone (δ 177.3) indicated that the pentasaccharide was bound acylglycosidically. The presence of arabinose in the furanose form was taken from the ^{13}C NMR chemical shifts and from the appearance of C-5 as a hydroxymethylene carbon in the DEPT spectrum. Vicinal coupling constants of the anomeric protons of 7.7 and 8.1 Hz

Table 1. ^{13}C NMR chemical shifts of compound **2** in MeOH-d_4

Aglycone		Sugar		
C-1	45.1	Rha 1	C-1	102.5
C-2	71.8		C-2	72.3†
C-3	82.5*		C-3	72.2†
C-4	43.5		C-4	74.0
C-5	48.0		C-5	70.4
C-6	19.1		C-6	18.5
C-7	33.7	Xyl	C-1	107.0
C-8	40.9		C-2	76.4
C-9	48.3†		C-3	84.2‡
C-10	37.8		C-4	69.9
C-11	24.7		C-5	67.2
C-12	123.6	Rha 1,4	C-1	101.9
C-13	144.7		C-2	72.8
C-14	43.0		C-3	72.3†
C-15	36.5		C-4	84.3‡
C-16	74.5		C-5	69.1
C-17	50.2		C-6	18.0
C-18	42.4	Fuc	C-1	95.1
C-19	48.0		C-2	74.0
C-20	31.3		C-3	84.4
C-21	36.5		C-4	72.3†
C-22	31.9		C-5	72.3†
C-23	65.7*		C-6	16.5
C-24	14.9	Ara	C-1	111.6
C-25	18.0		C-2	83.2
C-26	17.9		C-3	78.7
C-27	27.2		C-4	86.5
C-28	177.3		C-5	63.2
C-29	33.3	Rha 1§	C-1	104.2
C-30	24.9		C-2	72.4†
			C-3	72.3†
			C-4	74.0
			C-5	70.0
			C-6	17.8

* Data obtained from the HMBC-spectrum.
†, ‡ Assignments may be interchanged.
§ Sugar bound to the aglycone.

established the β-glycosidic linkage for xylose and fucose and a coupling constant of 1.2 Hz the α-glycosidic linkage of arabinose, respectively. H1-C1 coupling constants of *ca.* 170 Hz demonstrated the α-glycosidic linkage of rhamnose. Thus **2**, deacylbellidioside B_4, has the structure of 3-*O*-α-L-rhamnopyranosyl-2β, 3β, 16α, 23-tetrahydroxyolean-12-en-28-oic acid 28-*O*-α-L-rhamnopyranosyl(1 → 3)-β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-[α-L-arabinofuranosyl(1 → 3)]β-D-fucopyranoside.

HPLC of fraction B gave deacylbellidioside B (= bellissaponin BS1, 3-*O*-α-L-rhamnopyranosyl-2β, 3β, 16α, 23-tetrahydroxyolean-12-en-28-oic acid 28-α-L-rhamnopyranosyl(1 → 3)-β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-fucopyranoside) [7], while a mixture of compounds was obtained by HPLC separation of fraction A (fraction A–1). TLC investigation of fraction A–1 indicated that it consisted of two compounds with similar R_f values and colour reactions to those of bellissaponin BS1 and besysaponin C_{12} (3-*O*-α-L-rhamnopyranosyl-2β,3β,16α,23-tetrahydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-fucopyranoside [5]). To prove the presence of besysaponin C_{12} in *B. bellidioides* an ESI-mass spectrum of the mixture was taken, giving molecular ions at *m/z* 1243 $[M + Na]^+$ and *m/z* 1097 $[M + Na]^+$ in an intensity ratio which was in an agreement with the ratio seen on TLC. While the molecular mass of 1242 Da is identical to that of bellissaponin BS1, the molecular weight of 1096 agrees with besysaponin C_{12}. The tandem mass spectra of the ion at *m/z* 1243 gave a prominent daughter ion at *m/z* 593 $[dhex + pent + dhex + dhex - H_2O + Na]^+$, which is in agreement with the tetrasaccharide of bellissaponin BS1. An intense ion at *m/z* 1097 was not obtained showing that this ion is a molecular ion of a second compound. Tandem mass spectra showed a prominent daughter ion at *m/z* 447 $[pent + dhex + dhex - H_2O + Na]^+$ which is in agreement with the trisaccharide unit of besysaponin C_{12}. From the chromatographic behaviour and the results obtained by mass spectrometry, the presence of besysaponin C_{12} in *B. bellidioides* is unequivocable.

Deacylbellidioside B_4 is a new triterpenoid saponin that is very similar to the saponins present in species of the *Bellis* genus. The only difference from bellissaponin BS1 is the presence of an additional arabinofuranosyl unit. In addition, the occurence of bellissaponin BS2 and besysaponin C_{12} in *Bellium bellidioides* confirm that, from a chemical point of view, it is very closely related to the *Bellis* genus.

EXPERIMENTAL

General. 1D and 2D NMR spectra of **1** and **2** and HMQC and COSY spectra of compound **2** were recorded in CD_3OD at 300 K on a Bruker ARX 400 NMR spectrometer (^1H: 400.1 MHz; ^{13}C: 100.6 MHz), HMBC spectra of **2** on a Bruker DMX 600 NMR spectrometer (^1H: 600.1 MHz; ^{13}C: 150.9 MHz). Mass spectra were obtained on a Finnigan TSQ 700 equipped with a Finnigan electrospray source (ESI-MS and MS–MS) and a Kratos MS 50 FS connected to a Carlo Erba Mega Series gas chromatograph (GC–MS). $[\alpha]_D$ were measured on a Perkin-Elmer 241 C polarimeter; TLC was carried out on silica gel 60 plates or foils (Merck) using anisaldehyde–sulphuric acid as spraying reagent, prep., centrifugal accelerated radial TLC on a Chromatotron model 8924 (Harrison Research) using silica gel 60 PF_{254} (Merck) as sorbent and HPLC on a Hitachi/Merck, model D-6000 equipped with a L-4000 UV detector. GC separations were performed on a Hewlett Packard HP 5890 Series II gas chromatograph.

Isolation. The deacylsaponin mixture of *B. bellidioides* [7] (700 mg) was subjected to prep. centrifugal accelerated radial TLC on silica gel using $CHCl_3$–MeOH–H_2O (13: 3: 1, lower layer) as solvent giving frs A (33 mg), B (430 mg) and C (33 mg). HPLC of fraction A on LiChrosorb® RP-18 (7 μm, 250 mm × 10 mm i.d., MeOH–H_2O, 32: 18, 6 ml min^{-1}, UV 206 nm) gave 4 mg of fr.

Table 2. ^{1}H NMR data of compound **2** in MeOH-d_4

Aglycone			Sugar			
H-Atom	Chemical shift	Coupling constant (Hz)	Sugar	H-Atom	Chemical shift	Coupling constant (Hz)
H-1A	2.07	3.5, 14.0	Rha 1	H-1	5.18	1.8
H-1B	1.24	4.0, 14.0		H-2	3.99	3.5, 1.8
H-2	4.25	ca. 3.5		H-3	3.77	
H-3	3.73	3.5		H-4	3.4	
H-5	†			H-5	4.04	6.2, 9.5
H-6A/B	1.52			H_3-6	1.29	6.2
H-7A	1.66		Xyl	H-1	4.53	7.7
H-7B	1.47			H-2	3.42	ca. 8.0
H-9	1.68			H-3	3.51	8.7, 8.7
H-11A	2.03			H-4	3.57	
H-11B	1.98			H-5A	3.94	
H-12	5.38	3.5, 3.5		H-5B	3.28	
H-15A	1.76	3.5, 15.0	Rha 1,4	H-1	5.20	1.5
H-15B	1.52	3.0, 15.0		H-2	3.96	
H-16	4.52			H-3	3.86	
H-18	3.00	4.0, 14.5		H-4	3.58	
H-19A	2.34	13.6, 14.5		H-5	3.72	
H-19B	1.09	4.0, 13.6		H_3-6	1.35	6.4
H-21A	1.98		Fuc	H-1	5.42	8.1
H-21B	1.22			H-2	3.97	
H-22A	2.00			H-3	3.78	
H-22B	1.83			H-4	3.68	
H-23A	3.38			H-5	3.77	
H-23B	3.32			H_3-6	1.26	6.5
H_3-24	0.94		Ara	H-1	5.14	1.2
H_3-25	1.36			H-2	4.18	1.2, 3.5
H_3-26	0.83			H-3	3.81	
H_3-27	1.43			H-4	4.12	
H_3-29	0.92			H-5A	3.78	
H_3-30	0.96			H-5B	3.68	
			Rha 1*	H-1	4.90	1.6
				H-2	3.96	3.4, 1.7
				H-3	3.83	9.5, 3.4
				H-4	3.42	9.5, 9.5
				H-5	3.85	9.5, 6.3
				H_3-6	1.28	6.5

* Sugar bound to the aglycone.
† Assignment impossible.

A–1; 80 mg of fr. B under the same conditions gave 47 mg of bellissaponin BS1 (= deacylbellidiastroside B [7]). HPLC of fraction C under conditions above yielded 3 mg of compound **1** and 12 mg of compound **2**.

Identification of the component monosaccharides. The determination was performed according to ref. [10] using 0.5 mg of each compound. GLC conditions: column J&W Scientific DB-17 (30 m × 0.25 mm i.d., film thickness 0.25 μm), oven temp.: 170° for 10 min, then increasing by 2° min^{-1}, injection port and detector temperature 250°, carrier gas He (0.4 ml sec^{-1}). Retention times: arabinose 8.07, 8.44, 8.83 and 9.19 min, rhamnose 8.77 min, fucose 9.90 and 10.59 min, xylose 11.61 min.

Determination of the absolute configuration of the sugars. The determination was performed according to ref. [8] using about 0.5 mg of compound **1**. GLC conditions: column J&W Scientific DB-17 (30 m × 0.25 mm i.d., film thickness 0.25 μm), oven temperature 250°, injection port and detector temperature 280°, carrier gas He (22.3 l hr^{-1}). Retention times: D-xylose 8.95 min (L-xylose 9.44 min), L-arabinose 8.95 min (D-arabinose 9.49 min), L-rhamnose 9.72 min, D-fucose 10.26 min (L-fucose 10.93 min). As documented above, the retention times of D-xylose and L-arabinose are identical. However, the differentiation between D- and L-arabinose was clear as no peak appeared at the retention time of D-arabinose. In addition, derivatives of bellidiastroside U_{D1} and U_{D3} were prepared and analysed in an identical manner giving peak area ratios of 1:1 between D-xylose (R_t 8.95 min) and D-fucose (R_t 10.26 min). In contrast **2** gave a peak area ratio of 2: 1, proving that the peak at R_t 8.95 min is created by two monosaccharide units.

Deacylbellidioside B_3 **(1)**. Amorphous powder; TLC: R_f 0.38 ($CHCl_3$–MeOH–H_2O 7:4:1); ^1H NMR: aglycone 0.83, 0.92, 0.99, 1.01, 1.35, 1.42 (*s*, $6 \times H_3$), 1.09 (*dd*, $J \approx 3.5$, 14 Hz, H-19B), 1.22 (*dd*, $J \approx 3.5$, 14 Hz, H-1B), 1.50 (*dd*, $J \approx 3.5$, 15 Hz, H-15B), 1.74 (*dd*, $J = 3.5$, 15.0 Hz, H-15A), 1.65 (H-9B), 1.68 (H-9A), 1.96 (H-11B), 1.98 (H-11A), 2.13 (*dd*, $J = 2.5$, 14.3 Hz, H-1A). 2.34 (*t*, $J = 13.9$ Hz, H-19A), 2.98 (*dd*, $J = 4.0$, 13.9 Hz, H-18), 3.68 (H-3), 4.38 (H-2), 4.53 (H-16), 5.33 (*t*, $J \approx 3$ Hz, H-12); fucose 5.34 (*d*, $J = 8.6$ Hz, H-1), 3.84 (H-2), 3.58 (H-3), 3.60 (H-4), 3.70 (H-5), 1.26 (*d*, $J = 6.5$ Hz, H_3-6), rhamnose (1,4-linked) 5.42 (*d*, $J = 1.6$ Hz, H-1), 3.98 (H-2), 3.87 (H-3), 3.70 (H-4), 3.82 (H-5), 1.29 (*d*, $J = 6.2$ Hz, H_3-6), xylose 4.52 (*d*, $J = 7.6$ Hz, H-1), 3.43 (H-2), 3.32 (H-3), 3.71 (H-4), 3.86 (H-5A), 3.32 (H-5B), rhamnose (terminal) 5.18 (*d*, $J = 1.5$ Hz, H-1), 3.99 (H-2), 3.78 (H-3), 3.44 (H-4), 4.04 (*dd*, $J = 6.2$, 9.6 Hz, H-5), 1.37 (*d*, $J = 6.2$ Hz, H_3-6), glucose 4.47 (*d*, $J = 7.7$ Hz, H-1), 3.31 (H-2), 3.27 (H-5), 3.91 (*dd*, $J = 5.3$, 11.4 Hz), 3.59 (H-6B); ^{13}C NMR: aglycone 44.5 (C-1), 71.1 (C-2), 84.2 (C-3), 43.2 (C-4), 19.0 (C-6), 33.7 (C-7), 40.9 (C-8), 48.0 (C-9), 37.8 (C-10), 24.7 (C-11), 123.6 (C-12), 144.7 (C-13), 43.0 (C-14), 36.4 (C-15), 74.8 (C-16), 50.4 (C-17), 42.4 (C-18), 48.0 (C-19), 31.3 (C-20), 36.5 (C-21), 32.1 (C-22), 65.5 (C-23), 14.9 (C-24), 17.9 (C-25), 17.8 (C-26), 27.2 (C-27), 177.3 (C-28), 33.4 (C-29), 24.8 (C-30), fucose 95.1 (C-1), 76.5 (C-2), 74.6 (C-3), 73.6 (C-4), 72.0 (C-5), 16.5 (C-6), rhamnose (1,4-linked) 101.4 (C-1), 72.4* (C-2), 72.3* (C-3), 84.7 (C-4), 69.9 (C-5), 18.1 (C-6), xylose 107.2 (C-1), 76.5 (C-2), 84.3 (C-3), 68.8 (C-4), 67.2 (C-5), rhamnose (terminal) 102.6 (C-1), 72.7* (C-2), 72.2* (C-3), 74.1 (C-4), 70.0 (C-5), 18.4 (C-6), glucose 105.5 (C-1), 75.4 (C-2), 77.8 (C-3), 71.1 (C-4), 78.2 (C-5), 62.3 (C-6).

Deacylbellidioside B_4 **(2)**. Amorphous powder; TLC: R_f 0.38 ($CHCl_3$–MeOH–H_2O, 7: 4: 1); $[\alpha]_D - 41.6°$ ($c = 0.63$, MeOH); ^1H NMR: see Table 1; ^{13}C NMR: see Table 2.

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