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Steroidal saponins from the roots of *Trillium erectum* (Beth root)

Patricia Y. Hayes [a], Reg Lehmann [b], Kerry Penman [b], William Kitching [a], James J. De Voss [a,*]

[a] *Chemistry, SMMS, The University of Queensland, Cooper Rd., Brisbane, Queensland 4072, Australia*
[b] *MediHerb Research Laboratories, Brisbane Technology Park, Queensland 4113, Australia*

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ABSTRACT

Eleven steroidal saponins including three previously unreported saponins **1–3**, two known ecdysteroids and one fatty acid, have been isolated from the roots of *Trillium erectum* (Beth root) by RP-HPLC and characterized by spectroscopic (1D and 2D NMR experiments) and spectrometric (LCMS) methods.

1. Introduction

Trillium (Melanthiaceae family) is a genus composed of about 40–50 species of perennial herbaceous flowering plants found in North America and Eastern Asia. Beth root is the rhizome of *Trillium erectum* (genus: Trillium and family: Melanthiaceae), a plant found in Eastern North America, from Quebec to Ontario and Michigan and south to Tennessee. It is a medicinal herb traditionally used by various native North American Indian tribes as a woman's herb to aid childbirth (hence the name "Beth root", a corruption of "birth root"), and as a treatment for irregular menstrual periods (Chevallier, 1996). Modern uses (Foster and Duke, 1990) include as a treatment for a wide range of ailments including haemorrhage from the uterus, urinary tract and lungs, and also for excessive menstruation. There are some reports of the roots being an antiseptic, aphrodisiac, astringent and a uterine tonic (Mills, 1997).

Reports from 1942 to 1947 (Lieberman et al., 1942; Marker et al., 1943, 1947) indicated the presence of a number of sapogenins in the roots of *T. erectum*: pennogenin, nologenin, fesogenin, bethogenin, trillogenin and kryptogenin. There is no information available on the nature of the sugars and/or site of glycosylation for all of these sapogenins. Only one steroidal saponin has recently been reported, hypoglaucin A, possessing a yamogenin aglycone not reported in the earlier studies (Ondeyka et al., 2005).

In the present work, we isolated and elucidated the structure of three new steroidal saponins **1–3** along with the eight known saponins **4–11**, two known ecdysones, **12–13** (polypodine A and B) and linoleic acid, **14**. The structure and stereochemistry of the new compounds **1–3** were elucidated by NMR, LCMS and chemical techniques.

2. Results and discussion

Semi preparative RP-HPLC of the methanolic extract of *T. erectum* roots furnished 14 different compounds, including eleven steroidal saponins (**1–11**), two ecdysteroids (**12** and **13**) and one fatty acid (**14**). Compounds **1–3** were found to be new saponins and their structures elucidated by 1D and 2D NMR in combination with MS studies. Compounds **4–11** were known saponins and identified by comparison of their NMR data with those reported in the literature. Their structures were identified as pennogenin 3-O-rhamnosyl-β-chacotrioside **4** (Nohara et al., 1975; Miyamura et al., 1982), pennogenin 3-O-β-chacotrioside **5** (Nohara et al., 1975; Miyamura et al., 1982), polygonatoside B3 **6** (Strigina, 1983; Nohara et al., 1975), dioscin **7** (Zou et al., 2003), prosapogenin A of dioscin **8** (Zou et al., 2003), (3β,22α,25R)-26-(β-D-glucopyranosyloxy)-17,22-dihydroxyfurost-5-en-3-yl-O-α-L-rhamnopyranosyl-(1 → 2)-O-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside **9** (Fukuda et al., 1981), 26-O-β-D-glucopyranosyl-25-R-furost-5-ene-3β,17α,22, 6-tetraol-3-O-rhamnosyl-β-chacotrioside **10** (Fukuda et al., 1981) and protodioscin **11** (Ikeda et al., 2004), respectively. The two ecdysteroids **12** and **13** were found to be polypodine A or

* Corresponding author. Tel.: +61 7 3365 3825; fax: +61 7 3365 4299.
E-mail address: j.devoss@uq.edu.au (J.J. De Voss).

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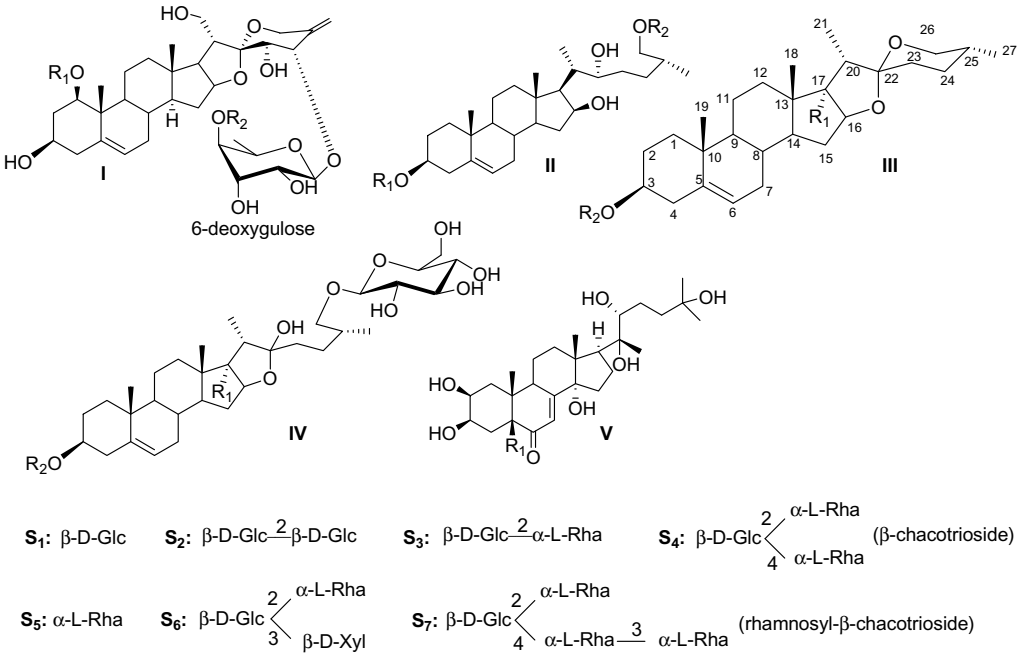
20*R* hydroxyecdysone (Vokac et al., 1998), and polypodine B (Wang et al., 1984), respectively (Fig. 1).

The absolute configuration of the sugar components was determined by enantioselective gas chromatography, after acid hydrolysis of the different saponins and comparison with authentic standards (6-deoxy-L-gulose and D-rhamnose are not commercially available and were obtained by synthesis from methyl α-D-mannopyranoside (Mori et al., 1986)). Each new saponin (**1–3)** was subjected to: (1) methanolysis followed by trifluoroacetylation (TFAA derivatives) and (2) acid hydrolysis to the corresponding peracetylated derivatives. The sugar derivatives were analysed by GC using enantioselective stationary phases: Chirasil-L-Val (TFAA derivatives, König et al., 1981) and cyclo-β dextrin (peracetylated derivatives). Analysis of the peracetylated derivatives indicated the presence of glucose, rhamnose, xylose and 6-deoxygulose for compound **1** and glucose only for compounds **2** and **3**. The absolute configuration of the sugar residues was determined as D-xylose, D-glucose, L-rhamnose and D-6-deoxygulose for **1** and D-glucose for **2** and **3** from the TFAA derivatives. Despite investing significant time in method development, we were unable to separate the enantiomers of the acetate derivatives by GC but the TFAA derivatives were resolved nicely (see Section 3).

Compound **1** was isolated as an amorphous solid, and positive-ion HRESIMS provided an ion at 1231.5345 ([M+Na$^+$]), corresponding to a molecular formula of $C_{56}H_{88}O_{28}$. The presence of five anomeric protons signals at δ 4.39 (*d*, *J* 8.0 Hz), δ 4.40 (*d*, *J* 8.0 Hz), δ 4.81 (*d*, *J* 8.2 Hz), δ 4.85 (*d*, *J* 1.4 Hz) and δ 5.41 (*d*, *J* 1.4 Hz) and three tertiary methyl groups at δ 1.17 (*d*, *J* 6.4 Hz), 1.23 (*d*, *J* 6.1 Hz) and 1.24 (*d*, *J* 6.1 Hz) suggested the presence of five monosaccharides including three deoxyhexoses. The presence of two broad singlets at δ 5.00 and 5.10 ppm in the ^{1}H NMR spectrum (both correlated to a carbon signal at δ 114.2 ppm in the HSQC



	Type	R_1	R_2	Common name
1	I	S_6	S_5	Bethoside A
2	II	S_2	S_1	Bethoside B
3	II	S_2	H	Bethoside C
4	III	OH	S_7	Pennogenin 3-O-rhamnosyl-β-chacotrioside
5	III	OH	S_4	Pennogenin 3-O-β-chacotrioside
6	III	OH	S_3	Polygonatoside B3
7	III	H	S_4	Dioscin
8	III	H	S_3	Prosapogenin A of dioscin
9	IV	OH	S_4	Protopennogenin 3-O-β-chacotrioside
10	IV	OH	S_7	Protopennogenin 3-O-rhamnosyl-β-chacotrioside
11	IV	H	S_4	Protodioscin
12	V	H		Polypodine A
13	V	OH		Polypodine B

Fig. 1. Structures of furostanol and spirostanol glycosides **1**–**11**; ecdysteroids **12–13** isolated from *Trillium erectum* roots.

Table 1
^{1}H (750MHz) and ^{13}C (188 MHz) spectral data (δ in ppm, *d4*-methanol) for compound **1**.

	Aglycone					Sugars	
	^{1}H [δ, *mult*, *J* (Hz)]	^{13}C	HMBC (H → C)	ROESY		^{1}H [δ, *mult*, *J* (Hz)]	^{13}C
1	3.51 *dd* (4.3, 10.4)	84.8	C-2, C-1′	H-2α, H19	1-O−β-D-Glc[b]		
2a	1.71 *q* (12.6)	37.5	C-1, C-3	H-19	**1′**	4.39 *d* (8.0)	100.4
2b	2.07 *dd* (4.3, 10.6)		C-1, C-3	H-1, H-3	**2′**	3.51 *dd* (8.0, 9.0)	77.1
3	3.37 *m*[a]	69.2	C-2	H-4α, H-2α	**3′**	3.65 *dd* (9.0, 9.0)	88.9
4a	2.19 *brt* (12.6)	43.4	C-10, C-6	H-3α	**4′**	3.33 *dd* (9.0, 9.0)	70.7
4b	2.24 *dd* (5.8, 12.6)		C-10, C-6	H-6, H-19	**5′**	3.25 *m*	77.6
5		139.6			**6a′**	3.61 *dd* (6.1, 11.3)	63.6
6	5.56 *d* (5.6)	126.1	C-8, C-10	H-4β, H-8	**6b′**	3.90 *dd* (3.1, 11.3)	
7a	1.55 *m*[a]	32.7			2′-O-α-L-Rha[b]		
7b	1.96 *m*[a]				**1″**	5.41 *brd* (1.4)	101.5
8	1.55 *m*[a]	34.2	C-6	H-18, H-19	**2″**	3.93 *dd* (1.4, 3.5)	72.3
9	1.35 *m*[a]	51.1	C-19	H-14, H-1	**3″**	3.66 *dd* (3.5, 9.4)	71.7
10		43.5			**4″**	3.40 *dd* (9.4, 9.4)	73.8
11a	1.42 *brd* (13.2)	24.8			**5″**	4.08 *dq* (6.1, 9.4)	69.7
11b	2.46 *brd* (13.2)				**6″**	1.25 *d* (6.1)	18.5
12a	1.20 *m*[a]	41.0	C-13, C-17, C-18		3′-O-β-D-Xyl[b]		
12b	1.71 *m*[a]		C-13, C-17, C-18		**1‴**	4.40 *d* (8.0)	105.4
13		41.8			**2‴**	3.24 *dd* (8.0, 9.0)	75.0
14	1.22 *m*[a]	58.0	C-18	H-9, H-17, H-16	**3‴**	3.33 *dd* (9.0, 9.0)	78.1
15a	1.47 *td* (6.3, 12.0)	33.2	C-13		**4‴**	3.52 *m*	71.0
15b	2.03 *ddd* (6.3, 7.6, 12.0)		C-13		**5a‴**	3.24 *m*	67.8
16	4.56 *ddd* (6.3, 6.3, 8.6)	84.6	C-13	H-14, H-17	**5b‴**	3.92 *dd* (5.7, 12.0)	
17	1.84 *dd* (6.6, 8.6)	58.8	C-13, C-18, C-20	H-14, H-16, H-21	24-O-6-deoxy-β-D-Gul[b]		
18	0.93 *s*	17.2	C-12, C-13, C-14, C-17	H-8, H-20	**1⁗**	4.81 *d* (8.3)	103.5
19	1.10 *s*	15.4	C-1, C-3, C-9, C-10	H-8, H-2β, H-4β	**2⁗**	3.61 *dd* (8.3,3.2)	70.3
20	2.73 *brdd* (6.6, 6.6)	46.6	C-23	H-18, H-23	**3⁗**	4.12 *dd* (3.2, 3.2)	69.1
21a	3.52 *dd* (6.6, 11.0)	62.9	C-13, C-17, C-20, C-22	H-14, H-16, H-17	**4⁗**	3.53 *brd* (3.2)	77.5
21b	3.64 *dd* (7.1, 11.0)		C-13, C-17, C-20, C-22	H-14, H-16, H-17	**5⁗**	4.05 *dq* (1.3, 6.4)	70.1
22		112.0			**6⁗**	1.17 *d* (6.4)	16.9
23	3.75 *d* (4.3)	72.1	C-20,C-24	H-20, H-24	4⁗-O-α-L-Rha[b]		
24	4.29 *d* (4.3)	83.9	C-22, C-23, C-27, C-1⁗	H-23, H-27b	1′′′′′	4.85 *brd* (1.4)	99.4
25		144.4			2′′′′′	3.81 *dd* (2.4, 3.3)	72.8
26a	3.74 *brd* (12.0)	62.1	C-22, C-25	H-25a	3′′′′′	3.69 *dd* (3.3, 9.4)	72.3
26b	4.47 *brd* (12.0)		C-22, C-25		4′′′′′	3.39 *dd* (9.4, 9.4)	73.5
27a	5.00 *brs*	114.2	C-24		5′′′′′	3.69 *dq* (6.1, 9.4)	70.7
27b	5.10 *brs*		C-24		6′′′′′	1.24 *d* (6.1)	17.7

[a] *m*: multiplet, indicates overlapping signals.
[b] Glc: glucose, Rha: rhamnose, Xyl: xylose, and Gul: gulose.

spectrum and a carbon signal at δ 144.4 ppm in the HMBC spectrum) indicated the presence of one *exo* double bond (Table 1).

Acid hydrolysis and gas chromatographic analysis of the peracetylated derivatives of the sugars indicated the presence of rhamnose, glucose, xylose and one unknown sugar in a 2:1:1:1 ratio. The ^{1}H–^{1}H-COSY and 1D TOCSY experiments (using increasing mixing time) starting from the anomeric proton of the non-identified sugar [δ 4.81 (*d*, *J* 8.2 Hz), H-1] indicated spin coupling links with the protons located at δ 3.61 (*dd*, *J* 8.2, 3.2 Hz, H-2), δ 4.12 (*t*, *J* 3.2 Hz, H-3), δ 3.53 (*brd*, *J* 3.2 Hz, H-4), δ 4.05 (*dq*, *J* 6.4, 1.3 Hz, H-5) and finally with δ 1.17 (*d*, *J* 6.4 Hz, H-6). The analysis of the different coupling constants indicated that this sugar was a 6-deoxy-β-gulopyranose (or antiarose), rarely encountered in steroidal saponins (Mimaki et al., 1997a,b; Kuroda et al., 2006). The ^{1}H NMR spectrum (in d_4-methanol) revealed the presence of two methyl groups (δ 0.93 and 1.10 ppm) attached to quaternary carbons, corresponding to the angular methyl groups of a steroidal saponin, and the absence of any methyl group on a tertiary carbon. Assignment of the carbons of the A/B-rings of the aglycone was achieved by careful examination of the HSQC/HMBC and COSY spectra. Correlations in the HMBC spectrum were found between the olefinic proton at δ 5.56 ppm (H-6) and a quaternary carbon at δ 43.6 ppm and a methyl singlet at δ 15.4 ppm attributable to C-10 and C-19, respectively. Further, cross-peaks between C-10 and a proton located at δ 3.51 ppm (H-1, with cross-peak to a carbon at δ 84.8 ppm in the HSQC spectra) as well as between a methylene group at δ 37.5 ppm (C-2, δ 1.71 and 2.07 ppm for H-2) and H-1 and a proton at δ 3.37 ppm (H-3, δ 69.2 ppm for C-3) indicated di-hydroxylation of the A-ring at C-1 and C-3 with glycosylation at C-3 only.

The different ring junctions and configurations at C-1, C-3 and C-20 were deduced from a 2D ROESY experiment. Intense correlations were observed between H-19/H-8/H-18, H-18/H-20 and between H-9/H-14/H-16/H-17 showing the usual *trans* ring fusion for the rings B/C and C/D and *cis* junction for the rings D/E as well as the 20α configuration. Further correlations between H-9/H-1/H-2β/H-3/H-4α and between H-4β/H-19 indicated the 1β and 3β configurations (Table 1).

^{1}H–^{1}H-COSY correlations were observed between H-20 at δ 2.73 ppm and three different proton signals at δ 1.85 ppm (H-17), δ 3.74 and 4.47 ppm (CH_2, H-21) and HMBC cross-peaks between C-22 (δ 112.0 ppm) and H-21 suggesting hydroxylation at C-21 (δ 62.9 ppm). Other HMBC correlations observed between C-22 and proton signals at δ 4.30 ppm (CH, H-24) and δ 3.74 and 4.47 ppm (CH_2, H-26) indicated a highly hydroxylated F-ring. Furthermore, the proton at δ 4.30 ppm (CH, H-24) exhibited several cross-peaks in the HMBC spectrum with carbon signals at δ 144.4 (C, C-25), δ 114.2 (CH_2, C-27), δ 112.0 (C, C-22) and δ 77.1 (CH, C-23) and a correlation in the COSY spectrum with δ 3.75 ppm (CH, H-23) demonstrating the hydroxylation of the F-ring at both positions 23 and 24 as well as the presence of an *exo* double bond at C-25. The 10 ppm downfield shift observed for C-24 (δ 83.9 ppm) relative to a free hydroxyl group indicated glycosylation at this position (corroborated by a cross-peak in the HMBC spectrum between C-24 and

the anomeric proton of the 6-deoxy-β-gulopyranose at δ 4.81 ppm). The small coupling constant (4.3 Hz) observed between H-23 and H-24 is consistent with the reported values (3.0–4.5 Hz) for similar structures with 23*S* and 24*S* configurations (Mimaki et al., 2003; Watanabe et al., 2003; Watanabe et al., 2002; Takaashi et al., 1995). Correlations in the 2D ROESY experiment between H-20/H-23 and between H-23/H-24 corroborated the (*S*) configuration at C-23 and C-24.

The connectivity of the sugar units was deduced from the HMBC spectrum. The presence of cross-peaks between the proton signals of the glucose unit at δ 4.39 ppm (H-1′, Glc), δ 3.51 ppm (H-2′, Glc), δ 3.65 ppm (H-3′, Glc) and the carbon signals at δ 84.8 ppm (C-1, aglycone), δ 101.5 ppm (C-1″, Rha) and δ 105.4 ppm (C-1‴, Xyl), respectively, indicated a 1,2,3-tri-substituted glucopyranose. Furthermore, glycosylations at C-24 of the aglycone and C-4 of the 6-deoxygulose unit were demonstrated by the presence of a cross-peak between C-24 (aglycone, δ 83.9 ppm) and the anomeric proton at δ 4.81 ppm (H-1⁗, 6-deoxygulose), and by a cross-peak between the carbon signal at δ 77.5 ppm (H-3⁗, 6-deoxygulose) and the proton located at δ 4.85 ppm (H-1⁗′, Rha). Compound **1** was elucidated as (1β, 3β, 23S, 24S)-1-[O-α-L-rhamnopyranosyl (1 → 2)-O-(β-D-xylopyranosyl (1 → 3)]-O-β-D-glucopyranosyl]-3,23-dihydroxyspirosta-5,25-dienyl-24-[O-α-L-rhamnopyranosyl (1 → 4)-β-D-6-deoxygulopyranoside (Bethoside A) (shifts summarized in Table 1).

Compound **2** was isolated as a white solid, and positive-ion HRESIMS provided an ion at 943.4873 ([M+Na$^+$]), corresponding to a molecular formula of $C_{45}H_{76}O_{19}$. The ^{1}H NMR spectrum (in d_5-pyridine) revealed the presence of two methyl groups (δ 1.02 and 1.15 ppm) attached to quaternary carbons, corresponding to the angular methyl groups of a steroidal saponin, two methyl groups [δ 1.00 (*J* 6.8 Hz) and δ 1.19 (*J* 7.1 Hz)] on tertiary carbons as well as three anomeric protons signals at δ 4.82 (*d*, *J* 7.8 Hz), δ 5.06 (*d*, *J* 7.8 Hz), δ 5.29 (*d*, *J* 7.8 Hz) and a broad doublet at δ 5.35 (*d*, *J* 5.2 Hz) suggesting the presence of three monosaccharides and one double bond (Tables 2 and 3). Acid hydrolysis and examination of the vicinal coupling constants for the sugars (determined through 1D TOCSY experiments) indicated the presence of three β-D-glucopyranoses (see Table 3). The ^{13}C NMR spectrum displayed 45 signals including four methyl groups, 13 methylenes, 25 methines and only three quaternary carbons located at δ 37.0, 42.7 and 141.1 ppm, respectively, suggestive of a non-furostanol or spirostanol type saponin for **2** with the absence of the usual acetal type carbon at C-22. Examination of the COSY, HSQC and HMBC spectra revealed the presence of three secondary hydroxyl groups at the positions C-3 (δ 79.4), C-16 (δ 71.4) and C-22 (δ 75.3) of the aglycone, a primary hydroxyl group at C-26 and the unusual cholestene-type skeleton for **2** equivalent to opening the E-ring of the steroidal saponin (Zhu et al., 2001; Yahara et al., 1989). The absolute stereochemistries of C-22 and the different ring junctions of the aglycone were determined by acid hydrolysis followed by derivatisation to the corresponding tetra-acetate **2a** (Yahara et al., 1989) and comparison of the NMR data with those reported in the literature (Yahara et al., 1989). The ^{1}H NMR spectrum of the

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) spectral data (δ in ppm, in d_5-pyridine) for the algycone of **2–3**.

	2			**3**		
	^{1}H [δ, *mult*, *J* (Hz)]	^{13}C	HMBC	^{1}H [δ, *mult*, J (Hz)]	^{13}C	HMBC
1a	0.92 *m*[a]	37.5	C-19	0.92 *m*[a]	37.5	C-19
1b	1.71 *m*[a]			1.71 *m*[a]		
2a	1.83 *m*[a]	30.3		1.81 *m*[a]	30.3	
2b	2.15 *m*[a]			2.15 *m*[a]		
3	3.83 *m*[a]	79.4	C-4, C-1′	3.83 *m*[a]	79.4	C-4, C-1′
4a	2.82 *brdd* (4.7, 12.3)	39.3	C-6	2.83 *ddd* (2.1, 4.8, 13.0)	39.3	C-6
4b	2.68 *brt* (12.3)			2.70 *brd* (13.0)		
5		141.1			141.1	
6	5.35 *d* (5.2)	121.8	C-4, C-10, C-8	5.35 *d* (4.9)	121.8	C-4, C-10, C-8
7a	1.49 *m*[a]	32.2		1.40 *m*[a]	32.3	
7b	1.87 *m*[a]			1.87 *m*[a]		
8	1.50 *m*[a]	31.9	C-6	1.51 *m*[a]	31.9	C-6
9	0.89 *m*[a]	50.5	C-19	0.90 *m*[a]	50.6	C-19
10		37.0	C-6		37.1	C-6
11a	1.42 *m*[a]	21.1		1.42 *m*[a]	21.2	
11b	1.48 *m*[a]			1.48 *m*[a]		
12a	1.13 *m*[a]	40.4		1.14 *m*[a]	40.5	
12b	2.03 *m*[a]			2.04 *m*[a]		
13		42.7	C-18		42.7	C-18
14	0.90 *m*[a]	55.0	C-18	0.90 *m*[a]	55.1	C-18
15a	1.50 *td* (6.2, 13.0)	37.2		1.51 *td* (5.4, 13.5)	37.2	
15b	2.28 *td* (7.5, 13.0)			2.27 *td* (7.1, 13.5)		
16	4.74 *ddd* (4.4, 7.4, 7.4)	71.4	C-13	4.75 *m*[a]	71.5	C-13
17	1.63 *dd* (6.9, 11.3)	58.1	C-18, C-20	1.64 *dd* (7.0, 10.7)	58.2	C-18, C-20
18	1.15 *s*	13.5	C-12, C-14, C-13, C-17	1.16 *s*	13.5	C-12, C-14, C-13
19	1.02 *s*	19.6	C-1, C-9	1.03 *s*	19.6	C-1, C-9
20	2.56 *td* (6.2, 7.4)	36.1	C-17, C-21, C-22	2.61 *m*[a]	36.1	C-17, C-21, C-22
21a	1.19 *d* (7.1)	15.3	C-20, C-22	1.21 *d* (7.1)	15.3	C-20, C-22
21b						
22		75.3	C-21		75.6	C-21
23a	1.78 *m*[a]	31.9		1.87 *m*[a]	32.1	
23b	1.78 *m*[a]			1.87 *m*[a]		
24a	1.32 *m*[a]	31.6	C-27	1.42 *m*[a]	31.6	C-27
24b	2.10 *m*[a]			2.22 *m*[a]		
25		34.3	C-27		37.0	C-27
26a	3.63 *dd* (6.0, 9.4)	75.3	C-27	3.71 *m*[a]	67.7	C-27
26b	3.93 *dd* (7.3, 9.4)			3.80 *m*[a]		
27	1.00 *d* (6.8)	17.7	C-26, C-25, C-24	1.12 *d* (6.7)	17.7	C-26, C-25, C-24

[a] m: multiplet, indicates overlapping signals.

Table 3
^{1}H (500 MHz) and ^{13}C (125 MHz) spectral data (δ in ppm, in d_5-pyridine) for the sugar units of **2–3**.

	2				**3**			
	^{1}H δ (ppm)	*mult*	*J* (Hz)	^{13}C δ (ppm)	^{1}H δ (ppm)	*mult*	*J* (Hz)	^{13}C δ (ppm)
3-O-β-D-Glucose								
1′	5.06	*d*	7.8	101.5	5.07	*d*	7.8	101.6
2′	4.15	*dd*	7.8, 9.1	84.8	4.16	*dd*	7.8, 9.2	84.9
3′	4.37	*dd*	9.1, 9.1	77.9	4.37	*dd*	9.2, 9.0	77.9
4′	4.24	*dd*	9.1, 9.1	71.5	4.24	*dd*	9.0, 9.0	71.5
5′	3.91	*ddd*	2.6, 5.4, 9.1	78.3	3.91	*ddd*	2.3, 5.1, 9.1	78.3
6a′	4.37	*dd*	5.4, 12.0	62.6	4.37	*dd*	5.1, 12.0	62.6
6b′	4.52	*dd*	2.7, 12.0		4.52	*dd*	2.3, 12.0	
2′-O-β-D-Glucose								
1″	5.29	*d*	7.8	106.7	5.28	*d*	8.0	106.8
2″	4.14	*dd*	7.8, 9.1	77.1	4.14	*dd*	8.0, 9.0	77.2
3″	4.25	*dd*	9.1, 9.1	78.1	4.25	*dd*	9.0, 9.0	78.1
4″	4.32	*dd*	9.1, 9.1	71.6	4.33	*dd*	9.0, 9.0	71.6
5″	3.99	*ddd*	3.3, 4.7, 9.1	78.8	3.98	*ddd*	2.8, 4.6, 9.0	78.9
6a″	4.47	*dd*	4.7, 11.7	62.8	4.47	*dd*	4.6, 11.7	62.8
6b″	4.58	*dd*	3.3, 11.7		4.58	*dd*	2.8, 11.7	
26-O-β-D-Glucose								
1‴	4.82	*d*	7.8	104.9				
2‴	4.03	*dd*	7.8, 9.0	75.3				
3‴	4.24	*dd*	9.0, 9.0	78.7				
4‴	4.22	*dd*	9.0, 9.0	71.8				
5‴	3.94	*ddd*	2.5, 5.0, 9.0	78.5				
6a‴	4.38	*dd*	5.0, 11.8	62.9				
6b‴	4.55	*dd*	2.5, 11.8					

tetra-acetate derivative **2a** was identical to the one reported for the (*R*) configuration at C-22 and a *trans* junction between the rings B/C and C/D (see Table 4).

Intense correlations in the 2D ROESY spectrum of **2** between H-16/H-17/H-14/H-15α and between H-18/H-15β indicated the 16β configuration for the hydroxyl group (Fig. 2).

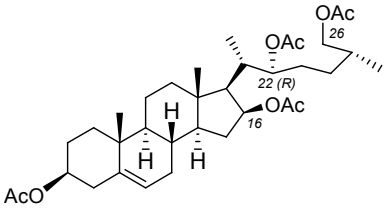
In the HMBC spectrum of **2**, a cross-peak between the proton signal at δ 5.06 ppm (H-1′, 3-O-β-D-glucose) and the carbon signal at δ 79.4 ppm (C-3, aglycone) and between the proton at δ 4.82 ppm (H-1‴, 26-O-β-D-glucose) and the carbon signal at δ 75.3 ppm (C-26, aglycone) indicated glycosylation of the aglycone at both C-3 and C-26 positions. The downfield chemical shift by ≈10 ppm of the C-2 (δ 84.8 ppm) of the 3-O-β-D-glucose unit and the presence of a cross-peak in the HMBC spectrum between this carbon and the anomeric proton at δ 5.29 ppm (H-1″, 2′-O-β-D-glucose) indicated glycosylation at C-2 of this glucose unit (see Tables 2 and 3 for data). Therefore, the structure of compound **2** was elucidated as 3-O-β-D-glucopyranosyl-[β-D-glucopyranosyl(1 → 2)]-26-O-(β-D-glucopyranosyl)-(3β,16β,22R,25R)-16,22-dihydroxycholest-5-ene (Bethoside B).

The cholest-5-ene-3β,16β,22,26-tetrol skeleton of the aglycone of **2** has been found in *Solanum lyratum* and *Solatum genera* (Solanaceae) (Zhu et al., 2001; Yahara et al., 1989) with a similar glycosylation pattern. It has also been found in *Allium tuberosum* L. (Alliaceae) but with glycosylation at C-16 (Sang et al., 2003).

The NMR spectra of compound **3** were very similar to that of compound **2** except in the those regions of the spectra derived from the sugars, suggesting the presence of the same skeleton but with differences in the nature and/or number of sugar presents in the molecule. Compound **3** was isolated as a white solid, and positive-ion HRESIMS provided an ion at 781.435 ([M+Na$^+$]), corresponding to a molecular formula of $C_{39}H_{66}O_{14}$ and a molecule possessing one less glucose unit in comparison to compound **2**. The examination of the ^{1}H NMR spectrum (in d_5-pyridine) revealed the presence of only two anomeric protons located at δ 5.07 (*d*, *J* 7.8 Hz), δ 5.28 (*d*, *J* 8.0 Hz). In the HMBC spectrum, a cross-peak between the proton signal at δ 5.07 ppm (H-1′, 3-O-β-D-glucose) and the carbon signal at δ 79.4 ppm (C-3, aglycone) indicated glycosylation of the aglycone at the C-3 position. Another cross-peak between the second anomeric proton at δ 5.28 ppm and the carbon at δ 84.9 ppm (C-2′, 3-O-β-D-glucose) as well as the upfield chemical shift observed for C-26 (from 75.3 ppm in saponin **2** to 67.7 ppm in saponin **3**) corroborated the presence of a free primary hydroxyl group at C-26 of saponin **3** (see Tables 2 and 3 for data). Acid hydrolysis followed by derivatisation to the corresponding tetra-acetate **3a** (Yahara et al., 1989) and comparison of the NMR data with **2a** indicated that these two compounds were identical. Therefore, **3** was established as 3-O-β-D-glucopyranosyl-[β-D-glucopyranosyl(1 → 2)]-(3β,16β,22R,25R)-16,22-dihydroxycholest-5-ene (Bethoside C).

This work describes the first phytochemical profile of the saponin contents of the roots of *T. erectum*. Our investigation led to the isolation of several known saponins and ecdysteroids previously reported in different *Trillium* species. Although ecdysteroids are more commonly found in the Silene genus (family: Caryophyllaceae) they have previously been found in the underground parts

Table 4
Selected NMR spectral data (δ ppm and multiplicity, $CDCl_3$, 400 MHz) for the tetra-acetate derivatives **2a** and **3a** and literature data.



Proton	22R[a]	22S[a]	**2a**	**3a**
OAc	1.99 *s*	2.03 *s*	1.99 *s*	1.98 *s*
OAc	2.03 *s*	2.03 *s*	2.01 *s*	2.00 *s*
OAc	2.04 *s*	2.06 *s*	2.02 *s*	2.01 *s*
OAc	2.06 *s*	2.11 *s*	2.03 *s*	2.02 *s*
H-26	3.85 *dd*, 3.89 *dd*	3.88 *d*	3.84 *dd*, 3.86 *dd*	3.83 *dd*, 3.86 *dd*
H-22	4.61 *m*	4.70 *m*	4.58 *m*	4.58 *m*
H-16	4.83 *t*	5.07 *m*	4.80 *t*	4.80 *t*

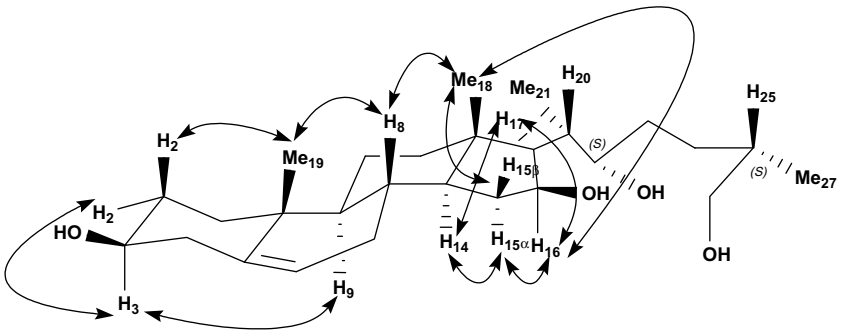


Fig. 2. ROESY-correlations for aglycone of saponin **2**.

of *Trillium kamtschaticum* Pall (Fukuda et al., 1981 and Ono et al., 2007). Three novel steroidal saponins containing a rare steroidal skeleton (Bethoside A, **1**) or an unusual fission of the E/F-rings (Bethosides B, **2** and C, **3**) were isolated and fully characterized. Bethoside A (**1**) was found to possess a polyhydroxylated skeleton (with hydroxylation at C-1, C-3, C-21, C-23 and C-24) previously reported only in *Nolina recurvata* (Recurvosides A–E, family: Agavacea) and *Helleborus orientalis* (family: Ranunculaceae). A similar hydroxylation pattern (albeit with a different aglycone and different stereochemistry at C-23/C-24) has been found in trillenogenin from *T. kamtschaticum* Pall (Ono et al., 2003). Another interesting feature of Bethoside A is the presence at C-24 of a 6-deoxygulopyranose (or antiarose) unit rarely encountered in nature and in steroidal saponins. Interestingly, the presence of antiarose has been reported from two plants from the liliaceae family, *Brodiaea californica* (Mimaki et al., 1997a,b) and from *Ornithogalum thyrsoides* (Kuroda et al., 2006) and to date has been always found at the C-24 position of the aglycone.

The isolation of the complex of saponins reported here allows one to postulate a unified biosynthetic scheme that explains the origins of most of these compounds (**2**–**11**, Fig. 3). The saponin **3** can arise from C-3 glucosylation and hydroxylation at C-16, C-22 and C-26 of a cholesterol precursor. Glucosylation at C-26 of **3** then yields **2**, which importantly has the nucleophilic C-26 hydroxyl masked so that it can no longer interact with a carbonyl at C-22. The next step in the proposed biogenesis of these compounds is oxidation at C-22 to provide a carbon at the same oxidation state as seen in **4–11**. The next likely key intermediate is the furanostanol protobioside (Fig. 3) that, although not observed in this work, has been reported from a related species *T. kamtschaticum*. To form protobioside from **2** requires not only C-22 oxidation but also a change in glycosylation at C-3, with the 2′ glucose replaced by a 2′ rhamnose moiety. Whether this change in glycosylation actually occurs or whether **2** and **3** represent unprocessed (and thus accumulated) equivalents of the real (unobserved) intermediates **2a**/**3a** with a C-3 2′-rhamnosyl glucoside moiety remains to be determined. Protobioside (Fig. 3) represents a branch point in the proposed biogenic pathway. Cleavage of the C-26 glucose and spontaneous cyclisation would yield the spirostanolic **8**, whilst addition of a 4′ rhamnose to the C-3 glucose moiety leads to **11**.

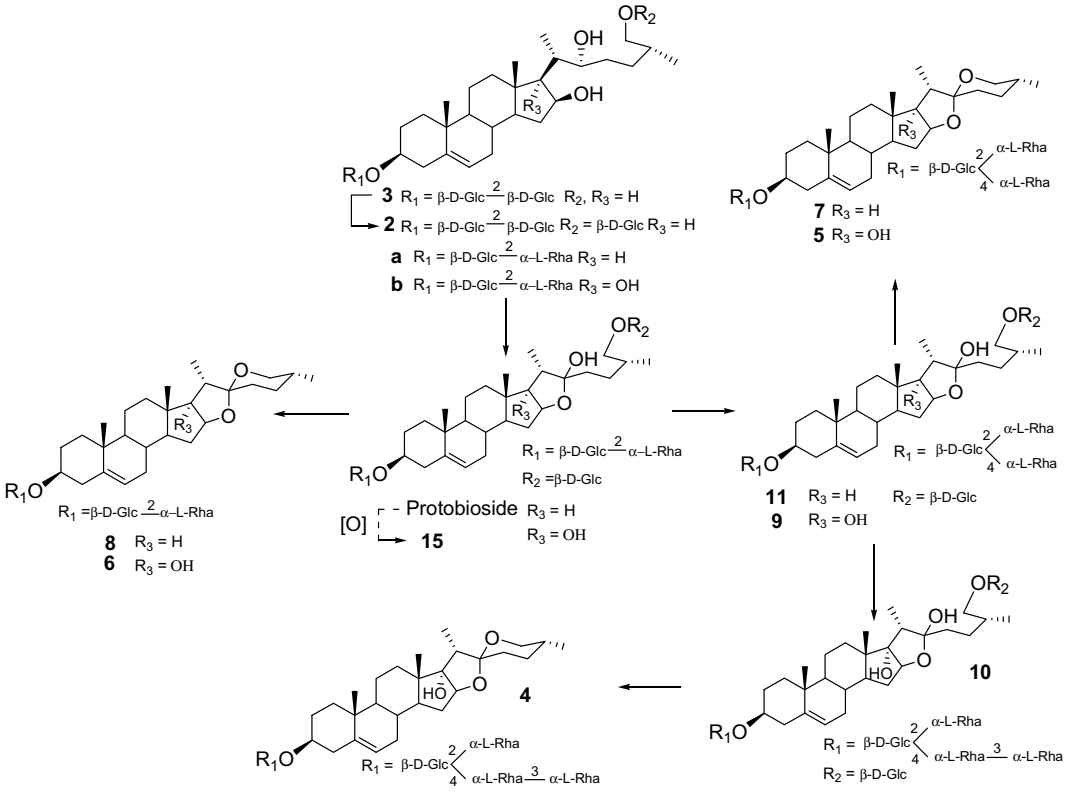


Fig. 3. Postulated biosynthesis of furanostanol and spirostanol stereoidal saponins isolated from *T. erectum*.

Cleavage of the C-26 glucose from **11** in turn yields **7** via cyclisation. Alternatively, hydroxylation of protobioside at C-17 yields **15** of the pennogennin series. Again, **15** is unobserved in this work but has previously been found in *T. kamtschaticum* (Nohara et al., 1975). Furanostanol **15** could also arise via a pathway in which C-17 hydroxylation occurs at a much earlier point to give intermediates **2b**/**3b** analogous to **2**/**3** as proposed in the formation of **7**, **8** and **11**. In either case, **15** could then be processed in a manner entirely analogous to that proposed for protobioside. Thus, cleavage of the C-26 glucose and spontaneous cyclisation yields **6**, while successive additions of rhamnose moieties to the C-3 glucose yields **9** and **10**. Cleavage of the C-26 glucose of **9** and **10** would then yield the corresponding spirostanols **5** and **4**, respectively. Clearly, a number of other pathways can be postulated to explain the biosynthesis of these compounds but the present postulate explains simply the occurrence of the observed metabolites in *T. erectum* and related species. For example the kryptogenin (C-16 oxo) analogs of protobioside (Fig. 3) and **11** have recently been reported from *T. kamtschaticum* (Ono et al., 2003). They could arise via C-16 and C-22 oxidation of **2a** followed by glycosylation as postulated for the biosynthesis of protobioside and **11**.

This work will hopefully facilitate future investigation into the biological activity of Beth root and provides a biosynthetic framework for these saponins against which future results can be compared.

3. Experimental

3.1. General

Optical rotations were measured at 20 °C on a Perkin–Elmer 241 MC polarimeter. ^{1}H NMR spectra were recorded on Bruker AV500 or AV750 MHz spectrometers with the residual protonated signal in the CD_3OD solvent (δ 3.31 ppm) or in the d_5-pyridine solvent (δ 8.71 ppm) as internal standards. ^{13}C NMR spectra were recorded at 125 or 188 MHz with either the central peak of the CD_3OD septet (δ 49.00 ppm) or the broad singlet of pyridine (δ 149.9 ppm) as internal standards. *J* values are reported in Hz. Mass spectra were recorded on a Shimadzu LCMS-QP8000 alpha equipped with a SPD-M10Avp diode array detector and an Adsorbosphere HS C18 7 micron column (150 mm × 4.6 mm ID, Alltech). High resolution mass spectra were recorded on a Kratos MS-25RFA spectrometer. Compounds **1–10** were purified by semi preparative HPLC using an LC-10AT Shimadzu Liquid Chromatograph (acetonitrile/water gradient from 8% to 100% of acetonitrile in 90 min, flow rate of 2 mL/min) using a ELSD-LT Shimadzu detector (52 °C, P 200 kPa) and Varian HPLC column (Dynamax HPLC column, 250 × 10 mm, OmniSpher 5 C18).

3.2. Plant material

The roots of *T. erectum* were harvested in October 2005 in Yellow Creek, Graham County, North Carolina and were identified by Botanical Liaisons, Crestmoor Drive, Boulder, Colorado. A voucher specimen (Ref.: NCM06-013) was deposited at the Medicinal Plant Herbarium of the Southern Cross University, Lismore, Australia.

3.3. Extraction and isolation

Powdered roots (25 g) of *T. erectum* were extracted (90% methanol/water, 250 mL), assisted by sonication. After filtration and removal of the solvent under vacuum, the residue was diluted in 10 mL (90% methanol/water), filtered and purified by semi preparative RP-HPLC (acetonitrile/water gradient from 8% to 100% of acetonitrile in 90 min, flow rate of 2 mL/min). Twelve different fractions (Fr **I–XII**, in order of elution**)** were obtained. Fractions **IV–V** and **VII–XII** were pure and corresponded to compounds **2** (Rt: 22.8 min; 10.7 mg), **11** (Rt: 24.0 min; 19.2 mg, protodioscin), **4** (Rt: 34.8 min; 3.1 mg, pennogenin 3-O-rhamnosyl-β-chacotrioside), **5** (Rt: 35.7 min; 7.9 mg, pennogenin-β-chacotrioside), **6** (Rt: 37.8 min; 0.1 mg, polygonatoside B3), **7** (Rt: 44.4 min; 30 mg, dioscin), **8** (Rt: 54.8 min; 0.1 mg, prosapogenin A of dioscin) and **14** (Rt: 72.0 min; 2.7 mg, linoleic acid), respectively. Fractions **I–III** and **VI** were subjected to further fractionation by RP-HPLC. Fraction **I** (Rt: 19.1 min; isocratic conditions, 18% acetonitrile in water) gave one pure major sub-fraction, Fr **Ia** (Rt: 26.5 min; **1**, 0.75 mg). Fraction **II** (Rt: 20.0 min; isocratic conditions, 18% acetonitrile in water) gave two sub-fractions, Fr **IIa** (Rt: 19.7 min; **12**, 1.5 mg, polypodine A) and Fr **IIb** (Rt: 21.7 min; **13**, 0.5 mg, polypodine B). Fraction **III** (Rt: 21.8 min; isocratic conditions, 20% acetonitrile in water) yielded two sub-fractions, Fr **IIIa** (Rt: 52.4 min; **9**, 8.2 mg, pennogenin-β-chacotrioside) and Fr **IIIb** (Rt: 54.6 min; **10**, 3.5 mg, pennogenin 3-O-rhamnosyl-β-chacotrioside). Finally,fraction **VI** (Rt: 34.8min; isocratic conditions, 22% acetonitrile in water) afforded one pure major sub-fraction, Fr **VIa** (Rt: 15.9 min; **3**, 9.7 mg).

3.4. Acid hydrolysis for sugar analysis

3.4.1. Sugar analysis

The saponins **1–3** (0.5–1 mg) were heated with 90% formic acid (0.4 mL) for 1 h at 100 °C cooled and concentrated. The residue was then heated with 2 M TFA (0.3 mL) for 2 h at 120 °C, and after cooling the solution was evaporated to dryness and rinsed twice with methanol (2 × 0.5 mL). The resulting product was reduced at room temperature for 30 min using a solution of 0.25 M $NaBH_4$ in NH_4OH (0.3 mL) and the reaction was quenched with a solution of 10% of acetic acid in MeOH (4 × 0.5 mL). The reduced product was then acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 1 h. This mixture was diluted with water and extracted with ethyl acetate and the extract analyzed by GCMS (cyclo-β dextrin 0.25 μm, 25 m × 0.22 mm, Alltech) FID detection, carrier gas: helium, injector: 200 °C, detector: 200 °C, column flow: 1.32 mL/min, split ratio: 56.0, pressure: 80 kPa. Temperature program: initial temperature 100 °C for 2 min, raised at 10 °C/min to a final temperature of 220 °C for 30 min. The standards were prepared following the same procedure. Under these conditions, the retention times for the standards were: Glc (29.49 min), Gal (28.87 min), Rha (17.99 min), Fuc (18.30 min), Xyl (20.06 min), Ara (18.96 min) and L-6-deoxygulose (18.86 min). For saponins **2** and **3**, only one peak was observed at 29.36 min (**2**) and 29.34 min (**3**) (Glc). For saponin **1**, peaks were observed at 17.97 min (Rha), 18.82 min (L-6-deoxygulose), 20.01 (Xyl) and finally at 29.47 (Glc). During co-injection studies, identical retention times were observed between the different hydrolysates and authentic standards.

3.4.2. Determination of the sugar absolute configurations

About 1 mg of the each pure saponin (**1–3**) was heated for 2 h at 100 °C with 10% HCl in methanol (0.5 mL). After cooling, the solution was concentrated under a stream of nitrogen and then partitioned between water and $CHCl_3$ (500 μL). The aqueous layer was separated and concentrated under a stream of nitrogen. The methanolysis product was dissolved in a mixture of trifluoroacetic anhydride (TFAA)/CH_2Cl_2 (1/1, 200 μL) and heated at 100 °C for 10 min, cooled and then concentrated under a stream of nitrogen. The residue was redissolved in CH_2Cl_2 (1.5 mL) and then analyzed by enantioselective GC (Chirasil-L-Val capillary column (25 m × 0.32 mm × 0.20 μm), FID detection, carrier gas: helium, injector: 200 °C, detector: 200 °C, column flow: 1.32 mL/min, split ratio: 56.0, and pressure: 40 kPa. Temperature program: initial tempera-

ture 50 °C for 6 min, raised at 4 °C/min to a final temperature of 160 °C for 5 min. The standards were prepared following the same procedure. Under these conditions, the retention times for the standards were: L-Glc (25.11 and 28.54 min), D-Glc (25.16 and 28.40 min), L-Rha (16.95 and 22.40 min), D-Rha (17.12 and 22.63 min), L-Xyl (17.02, 18.78 and 21.33 min), D-Xyl (17.16, 18.78 and 21.33 min) and L-6-deoxygulose (17.46 and 18.04 min). For saponins **2** and **3**, peaks were observed at 25.15 and 28.40 min (D-Glc). For saponin **1**, peaks were observed at 17.18, 18.72, 21.29 min (D-Xyl), 16.97 and 22.40 min (L-Rha), 25.15 and 28.39 (D-Glc) and finally at 17.73 and 18.26 min (D-6-deoxygulose). During co-injection studies, identical retention times were observed between the different hydrolysates and authentic standards (D-Glc, L-Rha and D-Xyl) whereas co-injection of the hydrolysate with L-6-deoxygulose gave peaks with different retention times (17.73/18.26 min for D-6-deoxygulose and 17.46/18.04 min for L-6-deoxygulose). The multiple peaks observed are a result of the formation of α and/or β anomers of pyranose and/or furanose forms and coincide with the number previously reported for these or analogous sugars (König et al., 1981).

3.4.3. Determination of the aglycone absolute configuration for saponins **2** and **3**

Following an experimental procedure adapted from literature (Yahara et al., 1989), saponin **2** (5 mg) was dissolved in 1 M HCl in dioxane–H_2O (1–1, 1.5 mL) and refluxed for 1 h. After cooling, the reaction mixture was neutralized with 1 M NaOH and the precipitate removed by filtration. The solvent was then removed under vacuum and the residue dissolved in acetic anhydride (0.5 mL) and pyridine (1 mL) and left at room temperature overnight. The solution was then concentrated to dryness under a stream of nitrogen and the residue purified by flash chromatography (silica, gradient from pure hexane to 10% ether in hexane) to afford the aglycone tetra-acetate **2a** as a colorless oil. $[\alpha]_D$ −52.3 (*c* 0.06, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): see Table 4. Under similar conditions, saponin **3** gave the corresponding tetra-acetate **3a**, identical to **3a**. $[\alpha]_D$ −54.8 (*c* 0.04, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): see Table 4. Lit. $[\alpha]_D$ −61.1 (*c* 0.53, $CHCl_3$) (Yahara et al., 1989).

3.5. Bethoside A **1**

Amorphous solid, $[\alpha]_D$ −45.3 (*c*, 0.07, CH_3OH). HRESIMS: Calcd for $C_{56}H_{88}NaO_{28}$ (M+Na): 1231.5360. Found: 1231.5345. Negative ion ESI *m/z*: 1207 [M−1]. Positive ESI-MS *m/z*: 1209, 1047, 914, 900, 767, 751, 605, 569, 553, 537, 501, 441, 411, 393, 375, 287, 269, and 251. ^1H (d_4-methanol, 750 MHz) and ^{13}C NMR (d_4-methanol, 188 MHz), see Table 1.

3.6. Bethoside B **2**

Amorphous solid, $[\alpha]_D$ −60.0 (*c*, 0.12, CH_3OH). HRESIMS: Calcd for $C_{45}H_{76}NaO_{19}$ (M+Na): 943.4878. Found: 943.4873. Negative ion ESI *m/z*: 919 [M−1]. Positive ESI-MS *m/z*: 922, 760, 597, 579, 561, 543, 417, 399, 381, 283, 271, and 253. ^1H (d_5-pyridine, 500 MHz) and ^{13}C NMR (d_5-pyridine, 125 MHz), see Tables 2 and 3.

3.7. Bethoside C **3**

White solid, m.p. 180 °C (decomp.). $[\alpha]_D$ −39.6 (*c*, 0.31, pyridine). HRESIMS: Calcd for $C_{39}H_{66}NaO_{14}$ (M+Na): 781.4350. Found: 781.4345. Negative ion ESI *m/z*: 757 [M−1]. Positive ESI-MS *m/z*: 759, 579, 561, 429, 417, 411, 299, 381, 283, 271, and 253. ^1H (d_5-pyridine, 500 MHz) and ^{13}C NMR (d_5-pyridine, 125 MHz), see Tables 2 and 3.

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