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Haemolytic acylated triterpenoid saponins from *Harpullia austro-caledonica*

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Abstract

Eight new acylated triterpenoid saponins were isolated from the stem bark of *Harpullia austro-caledonica* along with the known harpuloside (**9**). Their structures were established using 1D and 2D NMR and mass spectrometry as 3-*O*-β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylbarringtogenol C (**1**), 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloyl barringtogenol C (**2**), 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylbarringtogenol C (**3**), 3-*O*-α-L-arabinofuranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin (**4**), 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloyl protoaescigenin (**5**), 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin (**6**), 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin (**7**), 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin (**8**).

The EtOH extract of the stem bark showed in vitro cytotoxic activity against KB cells (90% at 10 μg/ml). At a concentration of 5 μg/ml, the saponin mixture showed haemolytic activity and caused 100% haemolysis of a 10% suspension of sheep erythrocytes.

Keywords: *Harpullia austro-caledonica*; Sapindaceae; Acylated saponins; Protoaescigenin; Barringtogenol C; Haemolysis

1. Introduction

In a continuation of our study on saponin constituents of plants of the Sapindaceae family and particularly on the chemotaxonomy of the genus *Harpullia*, we have examined the stem bark of *Harpullia austro-caledonica* Baillon (Sapindaceae). The genus *Harpullia* consists at least of 37 species distributed in Indo-Malaysia, Australia and the Pacific islands (Mabberley, 1997). Three species have been studied previously, *Harpullia pendula* (Khong and Lewis, 1976), *Harpullia ramiflora* (Dizes et al., 1998) and *Harpullia cupanioides* (Voutquenne et al., 1998). *H. austro-caledonica* is a tree or a shrub originating from New Caledonia and growing in the tropical rain forest (Morat et al., 2001). In the phylogenetic and taxonomic systems of this genus, this isolated species is next to the most primitive species *Harpullia pendula* and *H. arborea* (Leenhouts, 1985). The leaves consist of four to seven pairs of leaflets and the inflorescence is composed of yellow unisexual flowers that show an unusually wide range of variability (Leenhouts and

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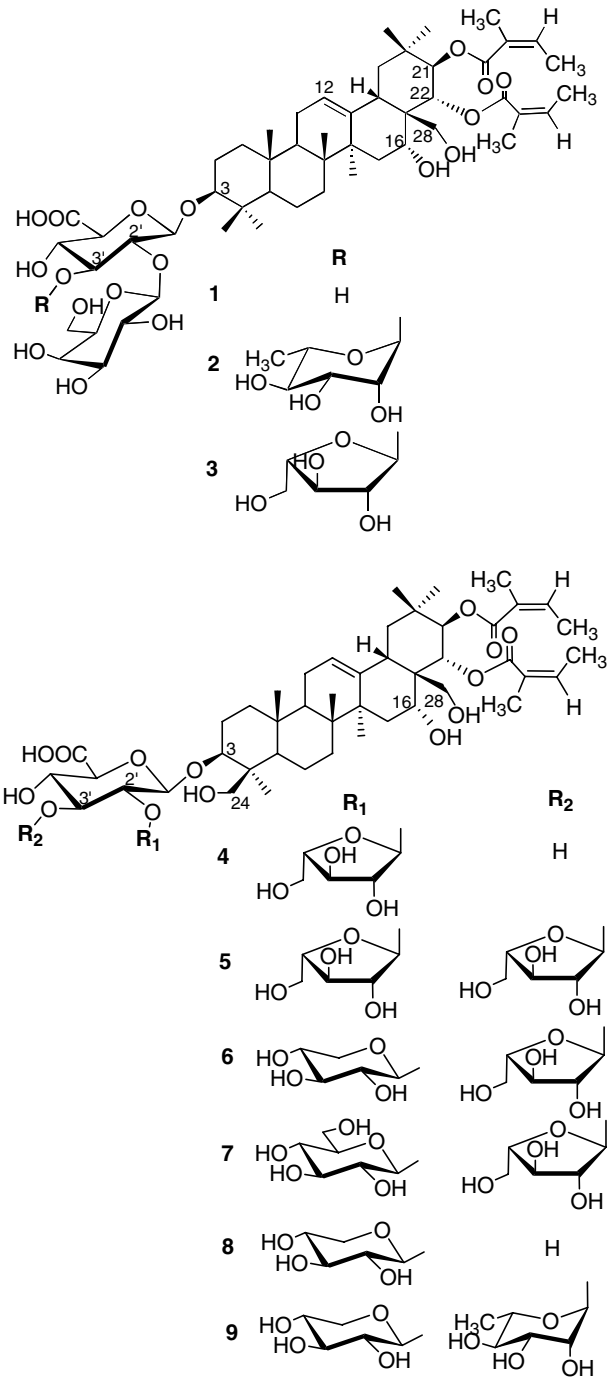
Vente, 1982). This species was selected as a part of a screening program for potential cytotoxic compounds from plants collected in New Caledonia. In a previous chemical study, we isolated three unusual bidesmosidic saponins, along with three prosapogenins, obtained after acid hydrolysis (Voutquenne et al., 2002a). This paper reports on the isolation and structural elucidation of eight new monodesmosidic saponins (**1**–**8**) from the stem bark of this plant along with the known saponin, harpuloside **9**, previously isolated from *H. ramiflora* (Dizes et al., 1998). The ethanolic extract from the stem bark of *H. austro-caledonica* exhibited in vitro cytotoxic activity against KB cells (90% at 10 μg/ml). The haemolytic activity of the saponin mixture was tested and showed an activity 10 fold higher than the dialysed reference saponin Sigma®. Pure saponins **2**, **3** and the mixture of saponins **6**, **7** were tested and are highly haemolytic.



2. Results and discussion

H. austro-caledonica was collected in the special fauna reserve of Amieu Pass and Table Unio in New Caledonia. Dried and powered stem bark was extracted with boiling 80% methanol and the methanolic extract was concentrated and precipitated in acetone. The crude saponin precipitate was dialysed, chromatographed on a silica gel column and purified by reversed phase C-18 column chromatography. Eight new compounds **1**–**8** were obtained, accompanied by impure harpuloside **9** (Dizes et al., 1998). Acid hydrolysis of the saponin extract gave the previously isolated mixture of prosapogenins (Voutquenne et al., 2002a) and sugars identified by TLC and by measurement of optical rotation after separation by preparative TLC as D-glucose, D-galactose, L-rhamnose, L-arabinose D-xylose and D-glucuronic acid.

Saponin **9**, molecular formula $C_{57}H_{88}O_{22}$ (ESI–MS: *m/z* 1123 $[M - H]^-$) was identified as harpuloside, 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β,22α-di-*O*-angeloylprotoaescigenin, on the basis of its spectral data. This compound was previously isolated from *H. ramiflora* (Dizes et al., 1998).

The positive ESI–LC–MS spectrum of compounds **1**, **2** and **3** gave the same ion fragment at *m/z* 677 $[\text{prosapogenin} + Na]^+$, attributed to the loss of the glycosidic chain at position 3 and in agreement with a molecular formula of $C_{40}H_{62}O_7Na$. The prosapogenin was identified as 21β, 22α-di-*O*-angeloylbarringtogenol C from analysis of its ^1H and ^{13}C NMR spectra (Table 1) and from observation of connectivities in COSY, HSQC and HMBC spectra. The set of data was in full agreement with those reported in the literature (Tuntiwachwuttikul et al., 1997; Sati and Rana, 1987).

The positive HR-MS of saponin **1** gave a quasi-molecular ion peak at *m/z* 1015.5258 $[M + Na]^+$ and in the negative ESI–LC–MS a molecular ion was detected at *m/z* 991 $[M - H]^-$ in agreement with an M_r of 992 amu ($C_{52}H_{80}O_{18}$). The positive ESI–MS experiment showed a quasi-molecular ion peak at *m/z* 1037 $[(M - H + Na) + Na]^+$ and the MS^2 experiment of this ion gave positive fragments at *m/z* 937 $[(M - H + Na) + Na - 100]^+$, 853 $[M + Na - 162]^+$ and 677 $[M + Na - 338]^+$ attributed to the losses of an angeloyl group ($C_5H_8O_2$), a terminal hexose and a disac-

Table 1
^{1}H and ^{13}C NMR data of saponins **1**, **2** and **3**

	1			**2**			**3**		
	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C
Barringtogenol C									
3	3.22	*dd* (9.4–5.4)	91.0	3.22	*dd* (11.2–4.7)	92.2	3.22	*dd* (11.5–3.8)	91.9
12	5.42	*m* ($w_{1/2}$ = 10.3)	125.3	5.41	*brt* (3.5)	125.3	5.41	*brt* (3)	125.3
13	–		143.0	–		143.0	–		143.0
15	1.39	*m*	35.0	1.39	*dd* (15–1)	34.9	1.39	*brd* (12.5)	34.9
	1.72	*m*		1.72	*dd* (15–3.7)		1.72	*brd* (12.5)	
16	4.02	*m* ($w_{1/2}$ = 6.6)	69.7	4.02	*m* ($w_{1/2}$ = 6.9)	69.7	4.01	*m* ($w_{1/2}$ = 6.6)	69.8
18	2.66	*dm* (12.4)	40.9	2.66	*dm* (11.8)	40.8	2.66	*dd* (12.3–3.6)	40.9
19	1.22	*dm* (12.4)	47.9	1.21	*dd* (11.8–3.1)	47.8	1.22	*dd* (12.3–3.6)	47.8
	2.72	*t* (12.4)		2.72	*t* (11.8)		2.72	*t* (12.3)	
21	6.03	*d* (10.3)	79.8	6.02	*d* (10.1)	79.8	6.03	*d* (10.1)	79.8
22	5.61	*d* (10.3)	74.3	5.60	*d* (10.1)	74.3	5.61	*d* (10.1)	74.4
23	1.11	*s*	28.5	1.10	*s*	28.4	1.12	*s*	28.4
24	0.90	*s*	17.0	0.90	*s*	16.9	0.90	*s*	16.9
25	1.01	*s*	16.2	1.01	*s*	16.2	1.00	*s*	16.2
26	0.97	*s*	17.4	0.97	*s*	17.3	0.97	*s*	17.3
27	1.52	*s*	27.8	1.52	*s*	27.7	1.43	*s*	27.7
28	2.98	*d* (11.2)	64.5	2.98	*d* (11.2)	64.5	2.98	*d* (11.1)	64.5
	3.30	*d* (11.2)		3.29	*d* (11.2)		3.30	*d* (11.1)	
29	0.90	*s*	29.7	0.90	*s*	29.7	0.90	*s*	29.7
30	1.12	*s*	20.3	1.11	*s*	20.3	1.12	*s*	20.3
21-Angeloyl									
1	–		169.1	–		169.2	–		169.2
2	–		129.4	–		129.4	–		129.4
3	6.08	*qq* (7.3–1.4)	138.9	6.08	*qq* (7.3–1.5)	138.8	6.08	*qq* (7.2–1.2)	138.8
4	1.93	*dq* (7.3–1.4)	16.0	1.93	*dq* (7.3–1.5)	16.0	1.93	*dq* (7.2–1.2)	16.0
5	1.84	q^t (1.4)	20.9	1.84	q^t (1.5)	20.9	1.84	q^t (1.2)	20.9
22-Angeloyl									
1	–		169.7	–		169.7	–		169.7
2	–		129.2	–		129.2	–		129.2
3	6.10	*qq* (7.3–1.5)	139.5	6.10	*qq* (7.3–1.5)	139.5	6.10	*qq* (7.2–1.2)	139.5
4	1.95	*dq* (7.3–1.5)	16.0	1.95	*dq* (7.3–1.5)	16.0	1.95	*dq* (7.2–1.2)	16.0
5	1.86	q^t (1.5)	20.9	1.86	q^t (1.5)	20.9	1.86	q^t (1.2)	20.9
3-β-D-GlcA									
1′	4.50	*d* (7.1)	105.5	4.58	*d* (7.4)	105.4	4.51	*d* (7.5)	105.5
2′	3.57	*t* (7.1)	83.0	3.80	*dd* (8.7–7.4)	78.7	3.77	*m*	79.1
3′	3.63	*m*	78.0	3.72	*t* (8.7)	85.9	3.7	*t* (7.5)	86.5
4′	3.51	*m*	73.6	3.66	*t* (8.7)	72.2	3.60	*m*	72.5
5′	3.85	*m*	76.8	3.78	*d* (8.7)	76.8	3.60	*m*	77.0
6′	–		na[a]	–		175.2	–		na[a]
2′-β-D-Gal									
1″	4.56	*d* (7.6)	106.2	4.58	*d* (7.4)	104.4	4.69	*d* (7.6)	104.5
2″	3.63	*t* (7.7)	74.1	3.57	*dd* (9.8–7.4)	73.0	3.56	*dd* (9.8–7.7)	73.4
3″	3.51	*dd* (7.8–5)	74.8	3.51	*dd* (9.8–3.3)	74.8	3.50	*dd* (9.8–3.3)	74.8
4″	3.89	*m*	69.7	3.83	*dm* (3.3)	70.2	3.85	*m*	70.2
5″	3.49	*m*	76.8	3.47	*ddm* (7.5–5)	77.2	3.49	*m*	77.0
6″	3.72	*brd* (12.2)	62.7	3.66	*dd* (11.7–5)	62.7	3.67	*dd* (11.6–5.2)	62.6
	3.75	*brd* (12.2)		3.80	*dd* (11.7–7.5)		3.77	*brd* (11.6)	
				3′-α-L-Rha			*3′-α-L-Ara(f)*		
1‴				5.09	*brd* (1.8)	103.4	5.29	*d* (2.3)	110.6
2‴				4.08	*dd* (3.3–1.8)	72.1	4.13	*m*	83.4
3‴				3.70	*dd* (9.5–3.3)	72.2	3.85	*m*	78.0
4‴				3.44	*t* (9.5)	73.8	4.13	*m*	85.2
5‴				3.99	*dq* (9.5–6.2)	70.6	3.78	*dd* (12.3–4.2)	63.0
							3.63	*dd* (12.3–6)	
6‴				1.27	*d* (6.2)	17.9			

[a] na, not assigned.

charide moiety $C_{12}H_{18}O_{11}$, consisting of a hexosuronic acid and a hexose, respectively.

The sugar part of **1** consisted of two residues with anomeric carbons at δ 105.5 and 106.2 in the ^{13}C NMR spectrum, attached to proton doublets at δ 4.50 and 4.56, respectively (HSQC). The proton system of each sugar was completely assigned on the basis of COSY and TOCSY experiments (Table 1). The sugar with its anomeric proton at δ 4.56 (J = 7.6 Hz) corresponded to a β-D-galactose with a hydroxymethyl carbon at δ 62.7 and characterised by an equatorial proton H-4 at δ 3.89 ($J_{3,4}$ = 5 Hz). The second sugar with its anomeric proton at δ 4.50 (J = 7.1 Hz) was identified as a β-D-glucuronic acid based on its carbon resonances in accordance with a β-D-glucuronic acid (Agrawal, 1992; Crublet et al., 2002) (Table 1). The deshielding of C-2′ (δ 83.0) of glucuronic acid suggested the point of linkage of the galactose (Crublet et al., 2002). Sequencing of the disaccharidic chain was achieved by analysis of a ROESY experiment which showed ROE interactions between H-3 (δ 3.22) of barringtogenol C and H-1′ of the glucuronic acid (δ 4.50), and between H-2′ (δ 3.57) of the glucuronic acid and H-1″ (δ 4.56) of the galactose unit. Thus, saponin **1** is 3-*O*-β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloyl barringtogenol C.

The positive HR-MS of saponin **2** exhibited a molecular ion peak at *m/z* 1161.5806 $[M + Na]^+$ in agreement with an M_r of 1138 amu ($C_{58}H_{90}O_{22}$). The negative ESI–MS experiment showed a $[M - H]^-$ ion peak at *m/z* 1137 and the MS^2 experiment of this ion gave negative fragment at *m/z* 991 $[M - H - 146]^-$ suggesting an additional 6-desoxy-hexose relative to saponin **1**. The three anomeric proton doublets of **2** were detected in the ^1H NMR spectrum at δ 5.09 and 4.58 (2H) and had correlations with their anomeric carbons at δ 103.4, 105.4 and 104.4, respectively, in the HSQC experiment. The sugar with its anomeric proton as a broad doublet at δ 5.09 (J = 1.8 Hz) was identified as a rhamnose, with a methyl proton doublet at δ 1.27 (J = 6.2 Hz) which correlated in HSQC spectrum with a methyl carbon at δ 17.9. The observation of a ROE interaction between H-1′ and H-2‴ of rhamnose, and of the deshielded chemical shift for its anomeric proton beyond 5 ppm (δ_H 5.09, J_{1-2} = 1.8 Hz) allowed us to propose an equatorial position for the two protons and subsequently an α configuration of the L-rhamnose (Agrawal, 1992). This was confirmed by the absence of any ROE interaction between the anomeric proton H-1‴ and neither H-3‴ nor H-5‴, in ROE spectrum. In addition, the chemical shift of ^1H and ^{13}C were in full agreement with those reported in the literature for α-L-rhamnopyranose (Agrawal, 1992). The two sugars with their anomeric protons at δ 4.58 (J = 7.4 Hz) corresponded to a β-D-galactose and a β-D-glucuronic acid as in saponin **1** (Table 1). The downfield shifts of C-2′ (δ 78.7) and C-3′ (δ 85.9) of the glucuronyl moiety suggested the points of linkage of the trisaccharide chain (Table 1). The HMBC spectrum showed cross peaks between H-3 (δ_H 3.22) of barringtogenol C and C-1′ of the glucuronic acid (δ_C 105.4) and between C-2′ and C-3′ of this glucuronic acid and H-1″ (δ_H 4.58) of the galactose unit and H-1‴ (δ_H 5.09) of the rhamnose unit, respectively. This sequence was confirmed by the observation of ROE interactions between the protons involved in the interglycosidic linkages. Thus, saponin **2** is 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylbarringtogenol C.

Saponin **3** ($C_{57}H_{88}O_{22}$) exhibited a $[M - H]^-$ molecular ion peak at *m/z* 1123 in the negative ESI–MS. The MS^2 experiment gave negative fragments at *m/z* 1023 $[M - H - C_5H_8O_2]^-$, 991 $[M - H - 132]^-$ and 923 $[M - H - 200]^-$ due to the losses of two angeloyl groups, and of a terminal pentose. These results indicated that saponin **3** contained a supplementary pentose unit relative to saponin **1**. The ^{13}C NMR spectrum revealed the presence of three anomeric carbons at δ 104.5, 105.5 and 110.6, and their corresponding proton doublets were detected at δ 4.69, 4.51 and 5.29, respectively, in the HSQC experiment. Analysis of 2D experiments (COSY, TOCSY and HSQC) revealed the presence of a terminal β-D-galactose and a disubstituted β-D-glucuronic acid with anomeric protons at δ 4.69 (J = 7.6 Hz) and 4.51 (J = 7.5 Hz), respectively (Table 1). The deshielded chemical shifts of the anomeric proton (δ_H 5.29) and carbon (δ_C 110.6) of the third glycosidic unit indicated a furanosyl ring. This third sugar was identified as an α-L-arabinose as shown by the ^{13}C NMR data, which were in good agreement with those reported for α-L-arabinofuranoside (Tezuka et al., 2000). The cross peaks observed in the HMBC experiment between C-3 (δ 91.9) of aglycone and H-1′ of glucuronic acid and between C-2′ (δ 79.1) and C-3′ (δ 86.5) of this glucuronic acid and H-1″ of galactose and H-1‴ of arabinofuranose, respectively, showed that saponin **3** is 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylbarringtogenol C.

As observed with saponins **1**–**3**, the positive ESI–LC–MS experiments of compounds **5**–**7** gave a common ion fragment at *m/z* 693 $[prosapogenin + Na]^+$ attributed to the loss of the glycosidic part. In addition, in the negative ESI–MS–MS of saponins **4**–**8**, the MS^2 experiment of the $[M - H]^-$ ion gave fragment at *m/z* 669 $[M - H - glycosidic\ part]^-$, indicating a molecular formula of $C_{40}H_{62}O_8$ for the prosapogenin moiety, which was identified as 21β, 22α-di-*O*-angeloylprotoaescigenin, previously isolated after acidic hydrolysis (Dizes et al., 1998; Voutquenne et al., 2002a), from analysis of ^1H and ^{13}C NMR, COSY, HSQC and HMBC spectra (Table 2).

Table 2
^{1}H and ^{13}C NMR data of the prosapogenin part of saponins **4–8**

	4			**5**			**6**			**7**			**8**		
	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C
Protoaescigenin															
3	3.45	*m*	91.7	3.42	*m*	92.8	3.34	*m*	92.2	3.42	*m*	92.8	3.37	*m*	91.7
12	5.42	*m* ($w_{1/2}$ = 11.5)	125.2	5.41	*m* ($w_{1/2}$ = 11.5)	125.2	5.41	*brt* (3.4)	125.1	5.41	*m* ($w_{1/2}$ = 11.5)	125.2	5.41	*brt* (3.5)	125.2
13	–		143.0	–		143.1	–		143.0	–		143.0	–		143.0
15	1.38	*dd* (11–3.8)	34.8	1.39	*brd* (14.5)	34.8	1.39	*brd* (15.2)	34.8	1.39	*brd* (15)	34.8	1.39	*brd* (14.5)	34.8
	1.71	*dm* (11)		1.71	*brd* (14.5)		1.72	*m*		1.72	*m*		1.71	*dd* (14.2–4.3)	
16	4.01	*m* ($w_{1/2}$ = 7.6)	69.7	4.01	*m*	69.7	4.01	*m* ($w_{1/2}$ = 6.7)	69.7	4.02	*m* ($w_{1/2}$ = 6.6)	69.7	4.02	*m* ($w_{1/2}$ = 7.4)	69.7
18	2.66	*dm* (11.8)	40.8	2.66	*dm* (13.7)	40.8	2.66	*dd* (13.7–2.8)	40.8	2.66	*dd* (12–4.2)	40.8	2.66	*dm* (12.1)	40.8
19	1.22	*dm* (11.8)	47.8	1.22	*dm* (13.7)	47.9	1.22	*m*	47.8	1.22	*m*	47.8	1.22	*dd* (12.1–11.4)	47.8
	2.72	*t* (11.8)		2.72	*t* (13.7)		2.72	*t* (13.3)		2.72	*t* (12)		2.72	*t* (11.4)	
21	6.02	*d* (10.1)	79.8	6.02	*d* (10)	79.8	6.03	*d* (10.2)	79.8	6.02	*d* (10.1)	79.8	6.02	*d* (10.2)	79.8
22	5.61	*d* (10.1)	74.3	5.61	*d* (10)	74.3	5.60	*d* (10.2)	74.3	5.60	*d* (10.1)	74.3	5.61	*d* (10.2)	74.3
23	1.26	*s*	23.1	1.26	*s*	23.1	1.21	*s*	22.6	1.21	*s*	22.8	1.21	*s*	22.7
24	3.37	*d* (11.7)	64.5	3.36	*d* (11.7)	64.4	3.23	*d* (11.4)	63.8	3.24	*d* (11.1)	64.1	3.25	*d* (11.8)	63.8
	4.09	*d* (11.7)		4.10	*d* (11.7)		4.09	*d* (11.4)		4.09	*d* (11.1)		4.09	*d* (11.8)	
25	0.96	*s*	16.3	0.95	*s*	16.3	0.90	*s*	16.1	0.91	*s*	16.2	0.91	*s*	16.1
26	0.96	*s*	17.2	0.95	*s*	17.2	0.95	*s*	17.2	0.96	*s*	17.2	0.96	*s*	17.2
27	1.52	*s*	27.7	1.52	*s*	27.7	1.52	*s*	27.7	1.52	*s*	27.7	1.52	*s*	27.7
28	2.98	*d* (11.2)	64.5	2.98	*d* (11.7)	64.5	2.98	*d* (11.1)	64.5	2.98	*d* (11.2)	64.5	2.98	*d* (11.2)	64.5
	3.29	*d* (11.2)		3.29	*d* (11.7)		3.29	*d* (11.1)		3.29	*d* (11.2)		3.29	*d* (11.2)	
29	0.90	*s*	29.7	0.89	*s*	29.7	0.90	*s*	29.7	0.90	*s*	29.7	0.90	*s*	29.7
30	1.12	*s*	20.3	1.11	*s*	20.3	1.11	*s*	20.3	1.11	*s*	20.3	1.11	*s*	20.3
21-Angeloyl															
1	–		169.2	–		169.2	–		169.3	–		169.3	–		169.2
2	–		129.4	–		129.4	–		129.4	–		129.4	–		129.4
3	6.07	*qq* (7.3–1.5)	138.8	6.08	*qq* (7.4–1.4)	138.8	6.08	*qq* (7.3–1.5)	138.8	6.08	*qq* (7.3–1.5)	138.8	6.08	*qq* (7.2–1.5)	138.8
4	1.93	*dq* (7.3–1.5)	16.0	1.93	*dq* (7.4–1.4)	16.0	1.93	*dq* (7.3–1.5)	16.0	1.93	*dq* (7.3–1.5)	16.0	1.92	*dq* (7.2–1.5)	16.0
5	1.84	q^t (1.5)	20.9	1.84	q^t (1.4)	20.9	1.84	q^t (1.5)	20.9	1.84	q^t (1.5)	20.9	1.84	q^t (1.5)	20.9
22-Angeloyl															
1	–		169.7	–		169.6	–		169.7	–		169.7	–		169.7
2	–		129.2	–		129.2	–		129.2	–		129.2	–		129.2
3	6.10	*qq* (7.3–1.5)	139.5	6.10	*qq* (7.4–1.5)	139.5	6.10	*qq* (7.3–1.5)	139.5	6.10	*qq* (7.3–1.5)	139.5	6.10	*qq* (7.3–1.5)	139.5
4	1.95	*dq* (7.3–1.5)	16.0	1.95	*dq* (7.4–1.5)	16.0	1.95	*dq* (7.3–1.5)	16.0	1.95	*dq* (7.3–1.5)	16.0	1.95	*dq* (7.3–1.5)	16.0
5	1.86	q^t (1.5)	20.9	1.86	q^t (1.5)	20.9	1.86	q^t (1.5)	20.9	1.86	q^t (1.5)	20.9	1.86	q^t (1.5)	20.9

Saponin **4** was found to have the molecular formula $C_{51}H_{78}O_{18}$ as deduced from the $[M + Na]^+$ molecular ion at *m/z* 1001.5098 (M_r 978 amu) in the positive HR-MS spectrum. The MS^2 experiment of the $[M - H]^-$ ion at *m/z* 977 in the negative ESI–MS spectrum gave fragments at *m/z* 877 $[M - H - C_5H_8O_2]^-$, 845 $[M - H - 132]^-$ and 669 $[M - H - 308]^-$ corresponding to the losses of an angeloyl group, a terminal pentose and a disaccharide chain ($C_{11}H_{16}O_{10}$) consisting of a pentose and a hexosuronic acid. The ^1H NMR spectrum of saponin **4** showed two anomeric protons at δ 4.53 (*d*, *J* = 7.2 Hz) and 5.42 (*brs*) which correlated in the HSQC experiment with the anomeric carbons at δ 105.0 and 109.6, respectively. Analysis of 2D experiments (COSY, TOCSY and HSQC) permitted the identification of a terminal α-L-arabinofuranose (δ_H 5.42) linked to a β-D-glucuronic acid (δ_H 4.53) monosubstituted in position 2′ (δ_C 80.6) (Table 3). Thus, saponin **4** is 3-*O*-α-L-arabinofuranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin.

Comparison of the ^1H and ^{13}C NMR spectra of saponins **5** and **4** indicated that compound **5** possessed one supplementary glycosidic unit. The molecular ion peak observed at *m/z* 1109 $[M - H]^-$ in the negative ESI–MS experiment, in agreement with an M_r of 1110 amu ($C_{56}H_{86}O_{22}$), and the negative fragment at *m/z* 977 $[M - H - 132]^-$ showed that this sugar was a pentose. In the ^1H NMR and ^{13}C NMR spectra, the detection of two anomeric protons at δ_H 5.19 (*brs*) and 5.30 (*d*, *J* = 3.6 Hz) with anomeric carbons at δ_C 110.9 and 110.1 (HSQC), and of two CH_2OH at δ_C 63.0 indicated the presence of two terminal α-L-arabinofuranosyl moieties (Table 3). The sugar attached to the genin was identified as a β-D-glucuronic acid (δ_H 4.56, δ_C 105.0) disubstituted in positions 2′ (δ_C 80.9) and 3′ (δ_C 86.5) as in saponins **2**and **3**. The observation of ROE connectivities between H-3 of protoaescigenin and H-1′ of glucuronic acid and between H-2′ and H-3′ of this glucuronic acid and H-1″ (δ_H 5.3) of the first arabinofuranose and H-1‴ (δ_H 5.19) of the second arabinofuranose identified saponin **5** as 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin.

Compounds **6** and **7** were difficult to obtain in a pure state. The best separation was obtained by semi-preparative HPLC with an isocratic elution with 47–48% MeCN in H_2O at pH 2.4 (TFA). The retention times were 24.9 min for saponin **6** and 25.8 min for saponin **7**.

The negative ESI–MS experiment of compound **6** ($C_{56}H_{86}O_{22}$) gave a molecular ion at *m/z* 1109 $[M - H]^-$. The MS^2 of this ion gave the same negative fragments at *m/z* 977 $[M - H - 132]^-$ and 669 $[M - H - 440]^-$ observed for saponin **5** suggesting that **5** and **6** were isomers. Analysis of COSY and TOCSY experiments identified an α-L-arabinofuranose (δ_H 5.28, *d*, *J* = 2.2 Hz), a β-D-xylose (δ_H 4.65, *d*, *J* = 7.8 Hz), and a disubstituted β-D-glucuronic acid (δ_H 4.51, *d*, *J* = 7.6 Hz) (Table 3).

The sequencing of the triglycosidic chain was achieved by analysis of HMBC correlations observed between H-1′ of the glucuronic acid (δ_H 4.51) and C-3 (δ_C 92.2) of the genin, between H-1″ of the arabinofuranose (δ_H 5.28) and C-3′ (δ_C 86.8) of the glucuronic acid, and between H-1‴ of the xylose (δ_H 4.65) and C-2′ (δ_C 78.5) of the glucuronic acid (Table 3). Thus, saponin **6** is 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin.

The ESI–MS–MS experiments and the ^1H and ^{13}C NMR spectra showed for saponin **7** a set of signals corresponding to the major saponin (75%) and signals of smaller intensity (25%) due to the occurrence of residual saponin **6**. The negative ESI–MS experiment gave an intense molecular ion at *m/z* 1139 $[M - H]^-$ for compound **7** ($C_{57}H_{88}O_{23}$) accompanied by a minor ion at *m/z* 1109 $[M' - H]^-$ for the residue of **6**. This result indicated that the difference corresponded to the presence of one hexose instead of a pentose in the glycosidic chain of saponin **7**. The MS^2 of $[M - H]^-$ at *m/z* 1139 gave negative fragments at *m/z* 1007 $[M - H - 132]^-$ due to the loss of a terminal pentose, and the MS^3 of this ion gave fragment at *m/z* 845 $[M - H - 132 - 162]^-$ suggesting a loss of a terminal hexose. Analysis of COSY, TOCSY and HSQC experiments showed the presence of a terminal α-L-arabinofuranose (δ_H 5.24), a terminal β-D-glucose (δ_H 4.81, *d*, *J* = 7.9 Hz), and a disubstituted β-D-glucuronic acid (δ_H 4.49) (Table 3). The analysis of HMBC correlations showed that saponin **7** is 3-*O*-α-L-arabinofuranosyl-(1 → 3)-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucuronopyranosyl-21β, 22α-di-*O*-angeloylprotoaescigenin.

As observed with ESI–MS and HR-MS experiments, saponins **4** and **8** ($C_{51}H_{78}O_{18}$) were isomers. The fragmentation showed the presence of a terminal pentose identified by NMR as a β-D-xylose (δ_H 4.66), and of a hexosuronic acid identified as a β-D-glucuronic acid (δ_H 4.50) monosubstituted in position 2′ (δ_C 80.8). The ROE correlations showed that saponin **8** is 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl-21β,22α-di-*O*-angeloylprotoaescigenin.

The haemolytic activity of the decoloured saponin mixture and of pure saponins **2**, **3** and the mixture of **6**, **7** was assessed on sheep erythrocytes (10% suspension in phosphate buffer saline) using the method previously described (Voutquenne et al., 2002b). The saponin mixture was more active than the dialysed reference saponin mixture from Sigma® (Sigma® D) and than the pure tested saponins. 25% Haemolysis was obtained at 1 μg/ml. The HD_{100} was determined at 5 μg/ml and the HD_{50} was estimated at 2 μg/ml. Saponins

Table 3
^{1}H and ^{13}C NMR data of the glycosidic part of saponins **4–8**

	4			**5**			**6**			**7**			**8**		
	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C
3-β-D-*GlcA*															
1′	4.53	*d* (7.2)	105.0	4.56	*d* (7.2)	105.0	4.51	*d* (7.6)	105.0	4.49	*d* (7.8)	105.0	4.50	*d* (7.2)	105.0
2′	3.44	*m*	80.6	3.55	*dd* (8.5–7.2)	80.9	3.64	*dd* (8.9–7.7)	78.5	3.70	*m*	78.6	3.52	*t* (7.9)	80.8
3′	3.58	*m*	78.6	3.67	*m*	86.5	3.72	*t* (8.7)	86.8	3.70	*m*	87.0	3.65	*t* (8.1)	78.3
4′	3.50	*m*	73.6	3.63	*m*	72.6	3.69	*m*	72.7	3.58	*t* (9)	72.5	3.53	*m*	73.3
5′	3.89	*m*	77.8	3.57	*m*	na[a]	3.59	*m*	78.0	3.61	*d* (9)	77.9	3.87	*m*	77.8
6′	–		na[a]	–		na[a]	–		176.3	–		176.3	–		na[a]
2′-α-L-*Ara*(*f*)				*2′*-α-L-*Ara*(*f*)			*2′*-β-D-*Xyl*			*2′*-β-D-*Glc*			*2′*-β-D-*Xyl*		
1″	5.42	*brs*	109.6	5.30	*d* (3.6)	110.1	4.65	*d* (7.8)	104.6	4.81	*d* (7.9)	104.0	4.66	*d* (7.7)	105.0
2″	4.05	*m*	83.0	4.02	*dd* (6–3.6)	83.3	3.19	*dd* (9.2–7.8)	75.4	3.19	*t* (8.1)	75.4	3.22	*dd* (9.3-7.9)	75.5
3″	3.85	*t* (6.1)	77.3	3.88	*t* (6)	76.9	3.30	*t* (9.2)	78.0	3.38	*t* (8.7)	78.0	3.30	*t* (8.8)	78.1
4″	4.08	*m*	85.0	4.07	*ddd* (6–5–2.3)	84.6	3.53	*ddd* (10.7–9.2–5.5)	70.9	3.53	*t* (8.9)	70.0	3.52	*ddd* (9.9–8.8–5.1)	70.8
5″	3.59	*dd* (11.8–4.8)	63.1	3.58	*dd* (12.4–5)	63.0	3.17	*t* (11.1)	66.8	3.23	*m*	78.2	3.15	*t* (11)	66.9
	3.75	*dd* (11.8–2.4)		3.76	*dd* (12.4–2.3)		3.84	*dd* (11.5–5.5)					3.84	*dd* (11–5.3)	
6″										3.78	*m*	61.5			
										3.80	*m*				
3′-α-L-*Ara*(*f*)															
1‴				5.19	*brs* ($w_{1/2}$ = 5)	110.9	5.28	*d* (2.2)	110.9	5.24	*d* (2.2)	110.8			
2‴				4.13	*m*	83.2	4.13	*dd* (4.6–2.2)	83.3	4.13	*dd* (4.5–2.2)	83.4			
3‴				3.88	*t* (6)	77.8	3.86	*dd* (6.7–4.6)	77.8	3.85	*dd* (7–4)	77.9			
4‴				4.13	*m*	85.5	4.12	*m*	85.4	4.09	*m*	85.3			
5‴				3.65	*dm* (11.5)	63.0	3.80	*dd* (11.7–2.3)	63.0	3.78	*dd* (12–2.5)	62.9			
				3.79	*dm* (11.5)		3.62	*dd* (11.7–6.3)		3.63	*dd* (12–4)				

[a] na, not assigned.

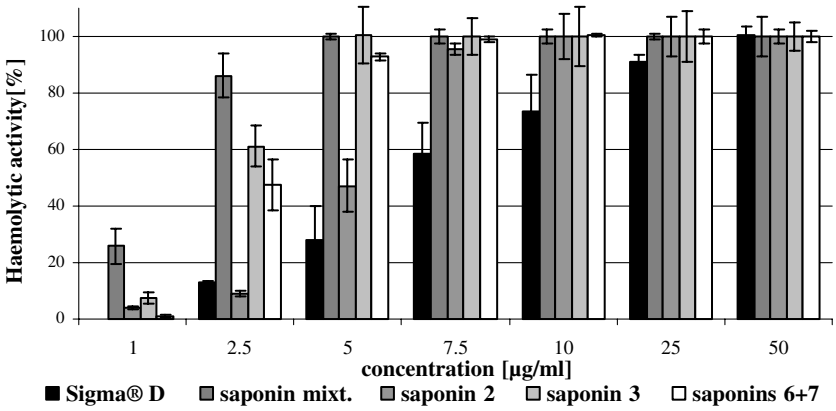


Fig. 1. Haemolytic activity of the saponin mixture of *H. austro-caledonica* relative to the pure saponins **2**, **3** and the mixture of **6**, **7**, and the commercial saponin from Sigma®.

2, **3** and the mixture **6**, **7** showed HD_{100} at 10, 5 and 10 μg/ml and HD_{50} at 5, 2.5 and 3 μg/ml, respectively (Fig. 1). The haemolytic activity of saponin **3** was twice that of saponin **2**, suggesting that an arabinofuranosyl moiety attached to position 3 of glucuronic acid is more effective than a rhamnosyl moiety. More saponins need to be tested to confirm this result. The quantities of the isolated pure saponins **1**, **4**, **5**, and **8** were insufficient to allow the measurement of their individual haemolytic activities in order to complete structure–activity relationships.

3. Experimental

3.1. General experimental procedures

^1H and ^{13}C NMR spectra were recorded on Bruker Avance DRX 500 in CD_3OD (^1H at 500 MHz and ^{13}C at 125 MHz); 2D experiments were performed using standard Bruker microprograms. High Resolution Mass Spectra (HR-MS) were obtained on an Applied Biosystemes MALDI-TOF Voyager DE STR (LesUlis-France). ESI–LC–MS, ESI–MS and MS–MS experiments were recorded on a Finningan LCQ deca ion trap mass spectrometer (Finnigan MAT, San Jose, USA); For MS^n experiments, the samples were introduced by direct infusion of a methanolic solution at a flow rate of 5 μl min^{-1}. Optical rotations were measured in MeOH with a Perkin–Elmer 241 Polarimeter. CC was carried out on Kieselgel 60 (63–200 mesh) Merck or LiChroprep RP-18 (40–63 μm) Merck. HPLC was performed on a DIONEX apparatus equipped with an ASI-100 autosampler, a P580 pump, a diode array detector UVD 340S (212 nm) and Chromeleon® software. A C-18 DIONEX VYDAC (201SP510, 250 × 10 mm, 5 μm) was used for semi-preparative HPLC with a binary eluent (solvent A, H_2O (pH 2.4 with 0.025% TFA); solvent B, MeCN) and a flow rate of 3 ml min^{-1}.

3.2. Plant material

Stem bark of *H. austro-caledonica* was collected in the rain forest at an elevation of 600 m in the special fauna reserve of Amieu Pass and Table Unio, New Caledonia, in March 1997. The specimen of the plant (LIT 0250) is deposited in the herbarium of the Botany and Plant Ecology Department at the Research Institute for the Development (IRD) of Noumea (New Caledonia).

3.3. Extraction and isolation

Dried and powered stem bark (1110 g) was macerated in 20% aq. MeOH (10 l) for 17 h and boiled for 3 h. The hydromethanolic extract was filtered, evaporated and freeze-dried to give a residue (106 g) which was suspended in MeOH (400 ml). The methanolic solution was added to 2 l of Me_2CO and the precipitate was filtered and dried over KOH in vacuo. This dried precipitate (57 g) was dissolved in pure H_2O and dialysed against H_2O in seamless cellulose tubing under agitation for 48 h. The contents of the tubes were freeze-dried to afford 32 g of a saponin mixture (yield 3%).

Two aliquots of the saponin mixture (1 and 3 g) were fractionated on a silica gel CC, using a gradient of $CHCl_3$–MeOH–H_2O (8:2:0 to 15:10:1) for the first sample and (7:3:0 to 12:8:1) for the second sample.

Frs. [11–14] (90 mg) of the first column and frs. [8–9] (200 mg) of the second column eluted with $CHCl_3$–MeOH (7:3) were similar on TLC ($CHCl_3$–MeOH–H_2O, 12:8:1) and then were purified on a reversed-phase RP-18 CC using a gradient of MeOH–H_2O (55:45 to 8:2). Frs. [10–25] eluted with MeOH–H_2O (65:35) were purified by silica gel CC, eluting with a gradient of $CHCl_3$–MeOH–HCOOH (90:10:1 to 70:30:1) and then finally purified by semi-preparative HPLC with a linear gradient of 50–51% B in 20 min to give saponins **9** (rt = 15.1 min, 4 mg), **6**, **7** (rt = 15.6 min, 3.2 mg) and **5** (rt = 17.9 min, 1.5 mg); Frs. [33–38] eluted with

MeOH–H_2O (65:35) were purified by semi-preparative HPLC, using the same conditions, to give saponin **2** (rt = 13.9 min, 2.2 mg).

Frs. [15–22] (280 mg) of the first column and frs. [10–21] (1.16 g) of the second column, eluted with $CHCl_3$–MeOH (7:3), were similar on TLC ($CHCl_3$–MeOH–H_2O, 12:8:1) and were then purified on a reversed-phase RP-18 CC using a gradient of MeOH–H_2O (55:45 to 8:2). Frs. [14–16] eluted with MeOH–H_2O (55:45) were purified by preparative TLC in $CHCl_3$–MeOH–H_2O (12:8:1) to give a mixture of saponins **6**, **7** (5 mg); Frs. [42–47] (272 mg), eluted with MeOH–H_2O (6:4), were purified by reversed-phase RP-18 CC, eluting with MeOH–H_2O (85:15), and followed by semi-preparative HPLC using various elution prog.: 62% B (0–20 min) for frs. [9–11] to give saponin **2** (rt = 8 min, 7.9 mg), 56–57% B (0–15 min) for frs. [12–20] to give saponins **6**, **7** (rt = 9.4 min, 13.2 mg), and 49–50% B (0–30 min) for frs. [49–64] to give saponins **5** (rt = 22.9 min, 1.5 mg) and **4** (rt = 24.8 min, 2 mg); Frs. [48–63] (272 mg), eluted with MeOH–H_2O (7:3), were purified by reversed-phase RP-18 CC and then by semi-preparative HPLC with a linear gradient of 65–68% B in 20 min to give 2.5 mg of saponin **2** (rt = 10.2 min) or by preparative TLC in $CHCl_3$–MeOH–HCOOH (65:35:1) to give 6.7 mg of saponin **3**.

Frs. [22–30] (302 mg) of the second column, eluted with $CHCl_3$–MeOH–H_2O (30:20:1), were purified by silica gel CC, eluting with a gradient of $CHCl_3$–MeOH–HCOOH (90:10:1 to 60:40:1). Frs. [49–57], [62–68] and [76–88], eluted with (85:15:1), were purified by semi-preparative HPLC with 49% B in 30 min to give saponins **6** (rt = 19.7 min, 3.2 mg), **7** (rt = 20.1 min, 3.2 mg), **8** (rt = 21 min, 2 mg), **3** (rt = 21.8 min, 1.2 mg), **1** (rt = 25.9 min, 2 mg) and **2** (rt = 27.9 min, 2 mg).

The different fractions containing the mixture of saponins **6**, **7** were gathered together and purified in twice by semi-preparative HPLC with 47–48% B in 30 min to give saponins **6** (rt = 24.9 min, 3.3 mg) and **7** (rt = 25.8 min, 1.3 mg).

3.4. Saponin *1*

$[\alpha]_D^{21}$ +2.3° (MeOH; *c* 0.13); ^1H and ^{13}C NMR (CD_3OD), see Table 1; HR-MS *m/z* 1015.5258 $[M + Na]^+$ (calcd for $C_{52}H_{80}O_{18}Na$, 1015.5242); ESI–LC–MS (negative ion mode) *m/z* 991 $[M - H]^-$; ESI–LC–MS (positive ion mode) *m/z* 1015 $[M + Na]^+$, 677 $[M + Na - 338]^+$; ESI–MS (positive ion mode) *m/z* 1037 $[(M - H + Na) + Na]^+$, 677 $[M + Na - 338]^+$; ESI–MS–MS: MS^2 (1037) *m/z* 1019 $[(M - H + Na) + Na - H_2O]^+$, 937 $[(M - H + Na) + Na - 100]^+$, 875 $[(M - H + Na) + Na - 162]^+$, 853 $[M + Na - 162]^+$, 699 $[(M - H + Na) + Na - 338]^+$, 677 $[M + Na - 338]^+$; MS^3 (853) *m/z* 753 $[M + Na - 162 - 100]^+$, 577 $[M + Na - 100 - 338]^+$.

3.5. Saponin *2*

$[\alpha]_D^{21}$ −10.0° (MeOH; *c* 0.66); ^1H and ^{13}C NMR (CD_3OD), see Table 1; HR-MS *m/z* 1161.5806 $[M + Na]^+$ (calcd for $C_{58}H_{90}O_{22}Na$, 1161.5821); ESI–LC–MS (positive ion mode) *m/z* 1161 $[M + Na]^+$, 677 $[M + Na - 484]^+$; ESI–MS (negative ion mode) *m/z* 1137 $[M - H]^-$; ESI–MS–MS: MS^2 (1137) *m/z* 991 $[M - H - 146]^-$; ESI–MS (positive ion mode) *m/z* 1183 $[(M - H + Na) + Na]^+$, 677 $[M + Na - 484]^+$; ESI–MS–MS: MS^2 (1183) *m/z* 1083 $[(M - H + Na) + Na - 100]^+$, 1037 $[(M - H + Na) + Na - 146]^+$, 1021 $[(M - H + Na) + Na - 162]^+$, 999 $[M + Na - 162]^+$, 899 $[M + Na - 162 - 100]^+$, 853 $[M + Na - 162 - 146]^+$.

3.6. Saponin *3*

$[\alpha]_D^{21}$ −10.9° (MeOH; *c* 0.53); ^1H and ^{13}C NMR (CD_3OD), see Table 1; HR-MS *m/z* 1147.5677 $[M + Na]^+$ (calcd for $C_{57}H_{88}O_{22}Na$, 1147.5664); ESI–LC–MS (positive ion mode) *m/z* 1147 $[M + Na]^+$, 677 $[M + Na - 470]^+$; ESI–MS (negative ion mode) *m/z* 1123 $[M - H]^-$; ESI–MS–MS: MS^2 (1123) *m/z* 1023 $[M - H - 100]^-$, 991 $[M - H - 132]^-$, 923 $[M - H - 200]^-$; ESI–MS (positive ion mode) *m/z* 1169 $[(M - H + Na) + Na]^+$, 677 $[M + Na - 470]^+$; ESI–MS–MS: MS^2 (1169) *m/z* 1069 $[(M - H + Na) + Na - 100]^+$, 1037 $[(M - H + Na) + Na - 132]^+$, 1007 $[(M - H + Na) + Na - 162]^+$, 985 $[M + Na - 162]^+$, MS^3 (1069) *m/z* 969 $[(M - H + Na) + Na - 200]^+$, 937 $[(M - H + Na) + Na - 100 - 132]^+$, 907 $[(M - H + Na) + Na - 100 - 162]^+$, 675 $[(M - H + Na) + Na - 200 - 294]^+$, 577 $[M + Na - 100 - 470]^+$, MS^3 (1007) *m/z* 907 $[(M - H + Na) + Na - 162 - 100]^+$, 875 $[(M - H + Na) + Na - 162 - 132]^+$, 807 $[(M - H + Na) + Na - 162 - 200]^+$.

3.7. Saponin *4*

$[\alpha]_D^{21}$ −25.5° (MeOH; *c* 0.11); ^1H and ^{13}C NMR (CD_3OD), see Tables 2 and 3; HR-MS *m/z* 1001.5098 $[M + Na]^+$ (calcd for $C_{51}H_{78}O_{18}Na$, 1001.5085); ESI–LC–MS (positive ion mode) *m/z* 1001 $[M + Na]^+$, 693 $[M + Na - 308]^+$; ESI–MS (negative ion mode) *m/z* 977 $[M - H]^-$; ESI–MS–MS: MS^2 (977) *m/z* 877 $[M - H - 100]^-$, 845 $[M - H - 132]^-$, 669 $[M - H - 308]^-$; ESI–MS (positive ion mode) *m/z* 1023 $[(M - H + Na) + Na]^+$, 923 $[(M - H + Na) + Na - 100]^+$, 693 $[M + Na - 308]^+$.

3.8. Saponin *5*

$[\alpha]_D^{21}$ −32.3° (MeOH; *c* 0.13); ^1H and ^{13}C NMR (CD_3OD), see Tables 2 and 3; HR-MS *m/z* 1133.5528 $[M + Na]^+$ (calcd for $C_{56}H_{86}O_{22}Na$, 1133.5508); ESI–LC–MS (positive ion mode) *m/z* 1133 $[M + Na]^+$, 1001

$[M + Na - 132]^+$, 869 $[M + Na - 264]^+$, 693 $[M + Na - 440]^+$; ESI–MS (negative ion mode) *m/z* 1109 $[M - H]^-$, 977 $[M - H - 132]^-$; ESI–MS–MS: MS^2 (1109) *m/z* 977 $[M - H - 132]^-$, 669 $[M - H - 440]^-$; ESI–MS (positive ion mode) *m/z* 1155 $[(M - H + Na) + Na]^+$, 693 $[M + Na - 440]^+$; ESI–MS–MS: MS^2 (1155) *m/z* 1055 $[(M - H + Na) + Na - 100]^+$, 1023 $[(M - H + Na) + Na - 132]^+$, 715 $[(M - H + Na) + Na - 440]^+$, MS^3 (1023) *m/z* 923 $[(M - H + Na) + Na - 132 - 100]^+$, 891 $[(M - H + Na) + Na - 132 - 132]^+$, 693 $[M + Na - 440]^+$.

3.9. Saponin **6**

$[\alpha]_D^{21}$ −13.2 (MeOH; *c* 0.25); ^1H and ^{13}C NMR (CD_3OD), see Tables 2 and 3; HR-MS *m/z* 1133.5518 $[M + Na]^+$ (calcd for $C_{56}H_{86}O_{22}Na$, 1133.5508); ESI–LC–MS (positive ion mode) *m/z* 1133 $[M + Na]^+$, 1001 $[M + Na - 132]^+$, 693 $[M + Na - 440]^+$; ESI–MS (negative ion mode) *m/z* 1109 $[M - H]^-$; ESI–MS–MS: MS^2 (1109) *m/z* 977 $[M - H - 132]^-$, 669 $[M - H - 440]^-$; ESI–MS (positive ion mode) *m/z* 1155 $[(M - H + Na) + Na - H)]^+$, 693 $[M + Na - 440]^+$; ESI–MS–MS: MS^2 (1155) *m/z* 1055 $[(M - H + Na) + Na - 100]^+$, 1023 $[(M - H + Na) + Na - 132]^+$, 955 $[(M - H + Na) + Na - 200]^+$, 715 $[(M - H + Na) + Na - 440]^+$, MS^3 (1055) *m/z* 955 $[(M - H + Na) + Na - 200]^+$, 923 $[(M - H + Na) + Na - 100 - 132]^+$, MS^3 (1023) *m/z* 1005 $[(M - H + Na) + Na - 132 - H_2O]^+$, 923 $[(M - H + Na) + Na - 132 - 100]^+$, 715 $[(M - H + Na) + Na - 440]^+$, 693 $[M + Na - 440]^+$.

3.10. Saponin **7**

^1H and ^{13}C NMR (CD_3OD), see Tables 2 and 3; HR-MS *m/z* 1163.5642 $[M + Na]^+$ (calcd for $C_{57}H_{88}O_{23}Na$, 1163.5613); ESI–LC–MS (positive ion mode) *m/z* 1163 $[M + Na]^+$, 693 $[M + Na - 470]^+$; ESI–MS (negative ion mode) *m/z* 1139 $[M - H]^-$; ESI–MS–MS: MS^2 (1139) *m/z* 1007 $[M - H - 132]^-$, MS^3 (1007) *m/z* 907 $[M - H - 132 - 100]^-$, 845 $[M - H - 132 - 162]^-$, 669 $[M - H - 470]^-$; ESI–MS (positive ion mode) *m/z* 1185 $[(M - H + Na) + Na]^+$, 693 $[M + Na - 470]^+$; ESI–MS–MS: MS^2 (1185) *m/z* 1085 $[(M - H + Na) + Na - 100]^+$, 1053 $[(M - H + Na) + Na - 132]^+$, 715 $[(M - H + Na) + Na - 470]^+$, MS^3 (1085) *m/z* 985 $[(M - H + Na) + Na - 200]^+$, 953 $[(M - H + Na) + Na - 100 - 132]^+$, MS^3 (1053) *m/z* 1035 $[(M - H + Na) + Na - 132 - H_2O]^+$, 953 $[(M - H + Na) + Na - 132 - 100]^+$, 891 $[(M - H + Na) + Na - 132 - 162]^+$, 693 $[M + Na - 470]^+$.

3.11. Saponin **8**

$[\alpha]_D^{21}$ −3.76° (MeOH; *c* 0.13); ^1H and ^{13}C NMR (CD_3OD), see Tables 2 and 3; HR-MS *m/z* 1001.5092 $[M + Na]^+$ (calcd for $C_{51}H_{78}O_{18}Na$, 1001.5085); ESI–MS (negative ion mode) *m/z* 977 $[M - H]^-$; ESI–MS–MS: MS^2 (997) *m/z* 877 $[M - H - 100]^-$; 845 $[M - H - 132]^-$; 669 $[M - H - 308]^-$.

3.12. Acid hydrolysis of saponins

The crude saponin mixture (500 mg) was dissolved in 16 ml of a mixture (1:1) of 6.5% $HClO_4$ and H_2SO_4 0.02 N, and heated at 140 °C in a sealed tube for 2 h. After cooling, the sapogenin precipitate was filtered, rinsed with H_2O and dried in vacuo over P_2O_5. The acid aq. layer was neutralised with KOH 0.5 M and freeze-dried. Six sugars were identified with authentic samples by TLC in MeCOEt–*iso*-PrOH–Me_2CO–H_2O (20:10:7:6) as glucuronic acid, glucose, galactose, arabinose, xylose and rhamnose. After preparative TLC of the sugar mixture (100 mg) in this solvent, the optical rotation of each purified sugar was measured.

3.13. Haemolytic activity

This assay was performed as described previously (Voutquenne et al., 2002b). The 10% sheep erythrocyte suspension was obtained by dilution of a commercial 50% suspension from Biomerieux® Lyon with phosphate buffer saline (PBS). Thirty milligrams of the saponin mixture was decoloured by VLC with $CHCl_3$–MeOH–H_2O (70:30:5) to eliminated the tannins. The purified saponin mixture and saponins **2**, **3** and **6**, **7** were dissolved in PBS; the samples were prepared in triplicate with concentrations ranging from 1 to 50 μg/ml. Twenty-five microlitres of erythrocytes diluted suspension were added to 1 ml of the sample and rapidly stirred. Absorbance of the supernatant was measured at 540 nm after 60 min of incubation and 5 min of centrifugation at 3000 rpm. HD_{50} and HD_{100} were the concentrations of sample which cause 50% and 100% of haemolysis, respectively.

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References

Agrawal, P.K., 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31, 3307–3330.

Crublet, M.-L., Pouny, I., Delaude, C., Lavaud, C., 2002. Acylated triterpenoid saponins from the stem bark of *Foetidia africana*. J. Nat. Prod. 65, 1560–1567.

Dizes, C., Gerald, F., Lavaud, C., Elias, R., Faure, R., Massiot, G., 1998. Harpuloside, a triterpenoid saponin from *Harpullia ramiflora*. Phytochemistry 48, 1229–1232.

Khong, P.W., Lewis, K.G., 1976. Chemical constituents of *Harpullia pendula*. Aust. J. Chem. 29, 1351–1364.

Leenhouts, P.W., Vente, M., 1982. A taxonomic revision of *Harpullia* (Sapindaceae). Blumea 28, 1–52.

Leenhouts, P.W., 1985. An attempt towards a natural system of *Harpullia* (Sapindaceae). Blumea 31, 219–234.

Mabberley, D.J., 1997. The Plant-book: a Portable Dictionary of the Vascular Plants, second ed. Cambridge University Press, Cambridge.

Morat, P., Jaffre, T., Veillon, J.-M., 2001. The flora of New Caledonia's calcareous substrates. Bull. Mus. Natl. Hist., Adansonia Ser. 3, 109–127.

Sati, O.P., Rana, U., 1987. Triterpenoids of *Aesculus indica*. Pharmazie 42, 141.

Tezuka, Y., Honda, K., Banskota, A.H., Maung Thet, M., Kadota, S., 2000. Kinmoonosides A–C, three new cytotoxic saponins from the fruits of *Acacia concinna*, a medicinal plant collected in Myanmar. J. Nat. Prod. 63, 1658–1664.

Tuntiwachwuttikul, P., Pancharoen, O., Mahabusarakam, W., Wiriyachitra, P., Taylor, W.C., Bubb, W.A., Towers, G.H.N., 1997. A triterpenoid saponin from *Maesa ramentacea*. Phytochemistry 44, 491–495.

Voutquenne, L., Lavaud, C., Massiot, G., Delaude, C., 1998. Saponins from *Harpullia cupanioides*. Phytochemistry 49, 2081–2085.

Voutquenne, L., Kokougan, C., Lavaud, C., Pouny, I., Litaudon, M., 2002a. Triterpenoid saponins and acylated prosapogenins from *Harpullia austro-caledonica*. Phytochemistry 59, 825–832.

Voutquenne, L., Lavaud, C., Massiot, G., Le Men-Olivier, L., 2002b. Structure–activity relationships of haemolytic saponins. Pharm. Biol. 40, 253–262.