Triterpene Glycosides from the Leaves of *Pittosporum angustifolium*

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- *Pittosporum angustifolium*
- Pittosporaceae
- A_1-barrigenol
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- pittangretosides

Abstract

Phytochemical investigation of the leaves of *Pittosporum angustifolium* resulted in the isolation and structural elucidation of nine new triterpene saponins, named pittangretosides A-I (**1**–**9**), together with a known compound (**10**). Mainly by NMR and HRESIMS experiments, eight compounds were identified as A_1-barrigenol glycosides (**1**–**7, 10**), whereas two compounds exhibited an unusual 17,22-seco-backbone of oleanolic acid (**8, 9**). All compounds were evaluated for their *in vitro* cytotoxicities against human urinary bladder carcinoma cells (5637). Only compounds with an angeloyl-residue at C-22 of the aglycone (**1**–**4** and **10)** showed antiproliferative effects with IC_{50} values of 4.1, 5.2, 2.1, 17.9, and 2.4 µM, respectively.

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Introduction

Pittosporum angustifolium Lodd. (Pittosporaceae) is a small tree that occasionally occurs in inland areas of most states of Australia and is colloquially referred as "gumby gumby" or "weeping pittosporum" [1,2]. In ethnomedicine, the leaves are traditionally used by Aboriginals for various medical applications [1] and recently also in the field of complementary medicine where beneficial effects as a supportive agent for cancer treatment have been observed [3]. Cytotoxic effects are well known and often reported as biological effects of saponins [4,5].

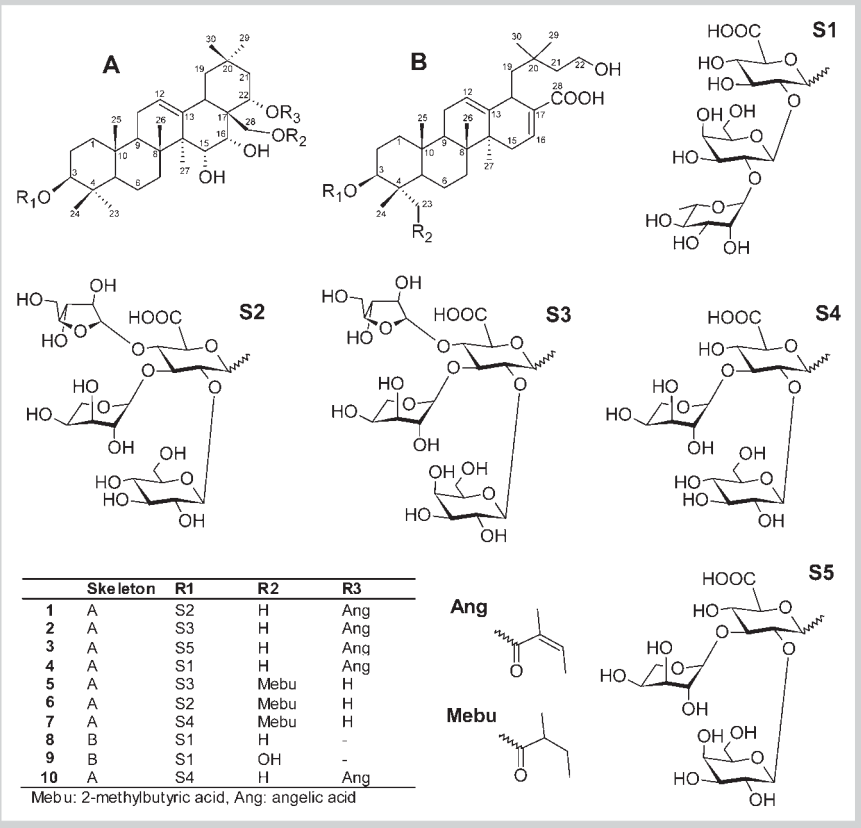
Phylogenetic studies described *P. angustifolium* as a distinct species which previously was considered as a variety of *P. phillyreoides* (syn. *P. phillyraeoides* var. *microcarpa*) [1]. Under this taxonomic classification, antiviral effects have been reported [6]. Earlier publications on *P. phillyreoides* indicated the presence of triterpene aglycones obtained after hydrolysis [7,8], and in other species of the genus *Pittosporum*, triterpene saponins seem to play a major role in respect to secondary metabolites [9–11]. This is the first report dealing with the characterization of triterpene glycosides isolated from the leaves of *P. angustifolium* under this name.

Results and Discussion

To purify and fractionate the crude 80% (v/v) ethanol extract, column chromatography steps using Sephadex LH20, silica gel, and partially RP-SPE separations were carried out successively. The subfractions obtained (A_1, B, and C_1) were subjected to semipreparative HPLC in order to isolate compounds **1**–**10**. Their structures are shown in ◉ **Fig. 1**. The novel compounds **1**–**9** were named pittangretosides A–I.

According to its spectroscopic data, glycoside **10** was identified as the known 22α-angeloyloxy-3β-[β-D-glucopyranosyl-(1 → 2)]-[α-L-arabinopyranosyl-(1 → 3)]-β-D-glucuronopyranosyloxyolean-12-ene-15α,16α,28-triol [12].

Pittangretoside A (**1**) displayed in its ESIMS spectrum quasimolecular ions $[M + Na]^+$ at *m/z* 1197.5643 (pos. mode) and $[M - H]^-$ at *m/z* 1173.5714 (neg. mode) consistent with a molecular formula of $C_{57}H_{90}O_{25}$. As the ^1H and ^{13}C NMR data of the aglycone moiety of **1** were nearly identical to those of **10** (◉ **Tables 1** and **2**), it could be identified as a derivative of A_1-barrigenol, too. The difference of 132 mass units compared to **10** implied an additional pentose unit. Furthermore, the characteristic signals of an angeloyl residue attached to the hydroxyl group at C-22 were also observed (◉ **Tables 1** and **2**). In contrast to **10**, the



	Skeleton	R1	R2	R3
1	A	S2	H	Ang
2	A	S3	H	Ang
3	A	S5	H	Ang
4	A	S1	H	Ang
5	A	S3	Mebu	H
6	A	S2	Mebu	H
7	A	S4	Mebu	H
8	B	S1	H	-
9	B	S1	OH	-
10	A	S4	H	Ang

Mebu: 2-methylbutyric acid, Ang: angelic acid

Fig. 1 Chemical structures of isolated compounds (**1**–**10**).

^{1}H NMR of **1** showed four anomeric protons at δ 5.14 (*br s*), 5.01 (*d, J* = 7.5 Hz), 4.91 (*d, J* = 7.0 Hz), 4.50 (*d, J* = 6.0 Hz) (**Table 3**), corresponding to δ_C 107.7, 102.3, 103.3, and 105.2 ppm in the HMQC (**Table 1**). The chemical shift of C-3 at δ 91.5 as well as an HMBC cross-peak between H-3 and one of the anomeric carbons (δ_C 105.2) indicated the attachment of the oligosaccharide chain to C-3 of the aglycone. Based on extensive H-H-COSY, HMQC, and HMBC experiments, all ^{1}H and ^{13}C resonances of the oligosaccharide moiety could be assigned, revealing the occurrence of a β-glucoronopyranosic acid (GlcA), a β-glucopyranose (Glc), an α-arabinopyranose [Ara(p)], and an α-arabinofuranose [Ara(f)] residue. The absolute configuration of sugars as thiazolidine carboxylates was determined by GC-MS signals at t_R 36.519 min (L-Ara), t_R 39.905 min (D-Glc), and t_R 41.093 min (D-GlcA). HMBC cross-peaks between H-1 of the glucose and δ_C 78.9 (GlcA-2), H-1 of the arabinopyranose and δ_C 79.0 (GlcA-3), and H-1 of the arabinofuranose and δ_C 74.5 (GlcA-4) indicated a trisubstituted glucoronopyranosic acid moiety. The same oligosaccharide unit, but linked to different aglycones, has been previously described for a number of triterpene saponins found in other species of the genus *Pittosporum* [9,10]. The structure of pittangretoside A (**1**) was thus established as 22α-angeloyloxy-3β-[β-D-glucopyranosyl-(1 → 2)]-[α-L-arabinopyranosyl-(1 → 3)]-[α-L-arabinofuranosyl-(1 → 4)]-β-D-glucuronopyranosyloxyolean-12-ene-15α,16α,28-triol.

Pittangretoside B (**2**), which was hard to separate from glycoside **1**, displayed a strong structural similarity to **1**. ESIMS revealed quasimolecular ions $[M + Na]^+$ at *m/z* 1197.5643 (pos. mode) and $[M - H]^-$ at *m/z* 1173.5714 (neg. mode) indicating the same molecular formula as **1** ($C_{57}H_{90}O_{25}$). Again, in the ^{1}H NMR, four anomeric protons at δ 5.15 (br s), 4.93 (*d, J* = 7.5 Hz), 4.88 (d, *J* = 7.0 Hz), and 4.49 (d, *J* = 6.0 Hz) (**Table 3**) were observed. The corresponding carbons were found at δ_C 108.1, 103.3, 103.6, and 105.5 ppm in the HMQC (**Table 1**). Detailed examination of the two-dimensional NMR spectra revealed the occurrence of one β-glucoronopyranosic acid, one α-arabinopyranose, and one α-arabinofuranose residue as in **1**, whereas the fourth sugar unit was identified as β-galactopyranose. HMBC cross-peaks between H-1 of the galactose and δ_C 80.0 (GlcA-2), H-1 of the arabinopyranose and δ_C 79.7 (GlcA-3), and H-1 of the arabinofuranose and δ_C 75.1 (GlcA-4) indicated the same sugar linkage as in **1**. These findings were supported by an examination of the hydrolyzed sugar fraction of **2** by TLC (solvent B, det. reagent B) and GC-MS, establishing the presence of galactose, arabinose, and finally glucuronic acid that was recovered exclusively by GC-MS. The absolute configuration was identified by GC-MS signals at t_R 36.567 min (L-Ara), t_R 40.303 min (D-Gal), and t_R 41.094 min (D-GlcA). The novel chemical structure of pittangretoside B (**2**) was thus determined as 22α-angeloyloxy-3β-[β-D-galactopyranosyl-(1 → 2)]-[α-L-arabinopyranosyl-(1 → 3)]-[α-L-arabinofuranosyl-(1 → 4)]-β-D-glucuronopyranosyloxyolean-12-ene-15α,16α,28-triol.

Pittangretoside C (**3**) showed ESIMS (pos. mode) quasimolecular ion peaks $[M + Na]^+$ at *m/z* 1065.5194 and $[M - H]^-$ at *m/z* 1041.5299 in the negative mode, predictive of a molecular formula of $C_{52}H_{82}O_{21}$, which is identical with that of compound **10**. Therefore, a close structural relationship with **10** was expected, which was supported by the similarity of the NMR spectra. The ^{1}H NMR spectrum revealed the occurrence of three sugar moieties with anomeric protons at δ 4.86 (*d, J* = 7.5 Hz), 4.61 (d, *J* = 7.5 Hz), and 4.49 (d, *J* = 7.5 Hz) (**Table 3**), two of them were identified as glucoronopyranosic acid and arabinopyranose as in **10**, whereas the third sugar turned out to be galactose instead of glucose. Indeed, compounds **3** and **10** and compounds **1** and **2** represent two pairs of saponins differing just in the occurrence

Table 1 ^{13}C (125 MHz) NMR spectroscopic data of pittangretosides A–I (**1–9**) in CD_3OD[a].

Carbon	1	2	3	4	5	6	7	8	9
1	39.4	39.7	39.5	39.0	38.3	39.1	39.2	38.9	38.8
2	26.4	26.8	26.3	24.2	n.d.	25.9	26.2	25.9	25.6
3	91.5	91.3	91.1	91.1	90.6	90.5	91.4	91.2	83.6
4	40.0	40.3	39.5	39.5	39.6	39.5	39.4	39.5	43.0
5	55.9	56.1	56.0	55.7	55.6	55.6	55.6	56.4	48.6
6	19.0	19.4	19.3	19.4	18.4	18.9	18.2	18.0	18.4
7	36.5	36.6	36.5	36.4	36.0	36.1	36.4	32.8	32.2
8	42.2	42.1	41.6	41.2	41.8	40.8	41.0	39.9	39.7
9	47.4	47.8	47.7	47.3	48.7	47.2	47.0	47.8	47.6
10	37.6	37.7	36.7	36.4	36.6	36.5	36.4	36.9	36.9
11	24.4	24.7	24.0	24.1	24.6	23.9	23.7	23.6	23.0
12	125.8	126.2	125.9	125.5	125.3	125.2	125.3	121.9	121.6
13	144.1	144.4	143.4	143.4	143.0	143.4	143.5	143.4	143.0
14	48.3	48.6	47.6	47.4	47.6	47.3	47.5	44.0	43.7
15	68.4	68.4	67.8	67.4	66.9	67.4	67.8	32.5	32.4
16	74.4	74.9	74.8	74.3	74.3	74.2	72.9	141.0	141.0
17	44.9	44.2	47.7	44.2	n.d.	44.4	44.4	137.6	137.3
18	41.9	42.2	40.9	41.5	42.5	41.8	42.0	42.1	42.2
19	47.0	46.8	46.5	46.5	47.0	46.7	46.7	45.8	45.6
20	32.4	32.6	31.9	31.5	31.2	31.5	31.5	32.6	32.7
21	41.5	41.9	41.2	40.9	42.3	42.2	45.2	44.0	44.0
22	72.5	73.1	72.8	72.7	69.4	69.5	68.8	58.5	58.6
23	27.8	28.2	27.7	27.9	27.9	27.7	27.5	27.7	64.8
24	16.3	16.7	16.4	16.0	16.1	16.0	16.1	15.8	12.4
25	15.6	15.9	15.7	15.7	15.6	15.5	15.8	15.7	15.9
26	17.2	17.7	17.3	17.0	16.7	16.9	16.9	16.9	17.0
27	19.9	20.1	20.3	19.8	19.9	20.0	19.6	27.6	27.9
28	63.3	63.5	63.3	63.1	65.2	65.2	65.4	169.1	169.4
29	33.1	33.2	32.8	32.5	32.3	32.3	32.7	27.8	27.9
30	24.5	24.9	24.5	23.9	24.0	24.0	23.9	27.8	27.9
C-3	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA
1	105.2	105.5	105.3	104.9	105.0	105.1	104.8	104.9	103.4
2	78.9	80.0	78.7	77.6	78.5	79.3	77.3	77.7	78.1
3	79.0	79.7	85.8	76.8	78.0	79.0	85.5	77.7	77.3
4	74.5	75.1	71.7	73.6	74.9	74.5	71.5	73.1	73.2
5	77.8	78.5	76.7	76.5	78.5	77.8	76.2	76.2	75.9
6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	172.5	173.7	n.d.
	Glc	Gal	Gal	Gal	Gal	Glc	Glc	Gal	Gal
1	102.3	103.3	103.7	102.0	103.1	102.8	102.0	101.7	101.6
2	75.6	73.0	73.4	76.0	72.7	75.8	75.4	76.0	77.0
3	77.6	75.6	75.1	76.2	75.1	76.7	77.2	75.5	75.5
4	72.3	72.4	71.4	71.3	72.6	71.7	71.7	71.8	70.8
5	77.7	76.9	76.1	76.0	76.5	78.0	77.4	75.4	75.8
6	62.9	62.4	61.7	62.0	61.9	62.7	62.6	62.0	61.7
	Ara(p)	Ara(p)	Ara(p)	Rha	Ara(p)	Ara(p)	Ara(p)	Rha	Rha
1	103.6	103.6	104.4	100.9	103.3	103.4	104.1	101.0	101.2
2	72.6	73.0	71.9	71.3	72.5	72.0	71.5	71.4	71.7
3	73.2	74.2	73.1	71.6	75.2	73.4	73.6	71.3	70.9
4	70.0	70.4	69.3	73.4	69.3	69.3	69.1	73.4	73.3
5	67.0	67.1	67.1	68.5	66.6	66.8	66.6	68.8	68.4
6				17.5				17.0	17.3
	Ara(f)	Ara(f)			Ara(f)	Ara(f)			
1	107.7	108.1			107.0	106.8			
2	81.0	81.5			80.7	81.4			
3	78.2	79.4			78.6	78.7			
4	86.7	87.2			86.8	86.6			
5	62.8	62.5			62.6	62.6			
C-22/-28	Ang	Ang	Ang	Ang	Mebu	Mebu	Mebu		
1	169.2	169.8	168.3	168.4	177.2	177.2	176.3		
2	129.8	130.0	129.1	129.2	42.3	42.5	41.7		
3	137.5	137.6	137.1	137.3	26.7	26.9	27.0		
4	15.5	16.0	15.6	15.3	10.2	10.2	10.5		
5	20.2	20.2	20.2	20.0	16.2	16.3	16.3		

[a] Assignments were made using ^1H-^1H COSY, HMBC, and HMQC experiments; n.d.: not determined; Mebu: 2-methylbutyric acid, Ang: angelic acid; (f)/(p): furanose/pyranose

Table 2 ^{1}H (500 MHz) NMR spectroscopic data of the acylated aglycones of pittangretosides A–I (**1–9**) in CD_3OD (*J* in Hz) [a].

Proton	1	2	3	4	5	6	7	8	9
1	0.99, 1.65	0.98, 1.63	1.01, 1.63	1.04, 1.69	1.03, 1.65	1.02, 1.65	1.01, 1.69	1.05, 1.65	0.99, 1.60
2	1.72, 1.90	1.72, 1.91	1.72, 1.90	1.75, 2.00	1.73, 1,93	1.72, 1.91	1.74, 1.93	1.74, 1.98	1.77, 1.98
3	3.17 dd (5.0, 11.5)	3.17 dd (4.5, 12.0)	3.18 dd (7.5, 12.0)	3.22 dd (5.5, 10.0)	3.18	3.19	3.24 dd (4.5, 12.0)	3.22 dd (4.5, 12.0)	3.65 dd (4.5, 12.0)
5	0.79 d (11.1)	0.78 d (11.0)	0.78 d (11.5)	0.81 brd (11.5)	0.79 d (10.5)	0.80 d (12.0)	0.81 d (11.0)	0.83 d (10.0)	1.22 d (12.0)
6	1.40, 1.55	1.42, 1.56 brd (11.0)	1.40, 1.55	1.42, 1.58	n. d., 1.57	1.42, 1.56	1.45, 1.58	1.49, 1.64	1.47, 1.61
7	1.71, 1.76	1.71, 1.75	1.71, 1.76	1.56, 1.73	n. d., 1.76	n. d., 1.76	1.56, 1.75	1.55, 1.58	1.52, 1.63
9	1.60	1.60	1.61	1.63	1.57	1.59	1.61	1.70	1.64
11	1.87, 1.90	1.91, 1.94	1.90, 1.93	1.95, 1.98	1.58, 1.98	1.88, 1.91	1.60, 1,98	1.85, 2.04 m	1.84, 2.01 m
12	5.43 br t (4.0)	5.43 br t (4.0)	5.43 br t (4.0)	5.45 br t (4.0)	5.36 brs	5.37 brs	5.36 brs	5.51 brs	5.49 brs
15	3.79 d (4.5)	3.78 d (4.5)	3.78 d (4.5)	3.80 d (4.0)	3.87	3.87	3.87	1.94 d (18.6), 2.55 dd (18.6, 7.6)	1.91 d (18.5), 2.53 dd (18.5, 7.5)
16	3.90 d (4.5)	3.93 d (4.5)	3.93 d (4.5)	3.95 d (4.0)	3.91	3.90	3.97	6.99 brd (7.2)	6.97 brd (7.2)
18	2.53 dd (3.5, 14.0)	2.53 dd (4.0, 13.5)	2.53 dd (3.5, 14.5)	2.55 brd (15.5)	2.43	2.44	2.43	3.57 t (6.5)	3.54 t (6.5)
19	1.05, 2.44 t (13.5)	1.05 dd (4.0, 13.5), 2.44 t (13.5)	1.05, 2.45 t (12.5)	1.10, 2.46 t (12.0)	1.06, 2.45	1.09, 2.44	1.05, 2.45	1.37 dd (6.5, 11.5), 1.55	1.33, 1.63
21	1.59, 2.23 t (12.0)	1.59, 2.23 dd (11.0, 12.0)	1.60, 2.22 t (12.0)	1.64, 2.25 t (12.0)	1.50, 2.12 t (12.0)	1.47, 2.12 t (12.0)	1.50, 2.12 t (11.0)	1.57, 1.67	1.59, 1.66
22	5.45 dd (6.0, 12.8)	5.45 dd (5.7, 13.0)	5.45 dd (5.5, 12.8)	5.48 dd (5.5, 12.0)	4.06 dd (6.0, 12.0)	4.06 dd (6.0, 12.0)	4.06 dd (6.0, 11.0)	3.61, 3.67	3.6, 3.60
23	1.07 s	1.08 s	1.07 s	1.14 s	1.10 s	1.10 s	1.10 s	1.14 s	3.84 d (10.6), 3.35 d (10.6)
24	0.86 s	0.87 s	0.87 s	0.91 s	0.90 s	0.90 s	0.90 s	0.90 s	0.76 s
25	0.98 s	0.98 s	0.98 s	1.01 s	1.00 s	1.00 s	1.01 s	0.93 s	0.93 s
26	1.02 s	1.02 s	1.02 s	1.05 s	1.04 s	1.04 s	1.04 s	0.61 s	0.59 s
27	1.41 s	1.40 s	1.40 s	1.43 s	1.38 s	1.39 s	1.40 s	1.34 s	1.32 s
28	3.13 d (11.0), 3.31 d (11.0)	3.13 d (11,5), 3.31 d (11.5)	3.13 d (10.6), 3.31 d (10.6)	3.15 d (11.0), 3.33 d (11.0)	3.95	3.94	3.97	–	–
29	0.92 s	0.91 s	0.92 s	0.94 s	0.93 s	0.94 s	0.93 s	0.99 s	0.97 s
30	1.04 s	1.04 s	1.04 s	1.07 s	1.03 s	1.03 s	1.02 s	0.97 s	0.94 s
C22/28	Ang	Ang	Ang	Ang	Mebu	Mebu	Mebu		
2					2.42 m	2.42 m	2.42 m		
3	6.05 q (7.0)	6.06 q (7.0)	6.06 q (7.0)	6.08 dq (7.0, 1.0)	1.55, 1.66	1.52, 1.67	1.55, 1.68		
4	1.97 d (7.0)	1.96 d (7.0)	1.97 d (7.0)	2.00 d (7.0)	1.03 t (7.5)	1.03 t (7.5)	1.04 t (7.0)		
5	1.90 s	1.90 s	1.91 s	1.94 s	1.19 d (7.5)	1.19 d (7.5)	1.18 d (7.0)		

[a] Assignments were made using ^{1}H-^{1}H COSY, HMBC, and HMQC experiments; overlapped ^{1}H signals are reported without designated multiplicity; n. d.: not determined; Ang: angelic acid, Mebu: 2-methylbutyric acid

of galactose instead of glucose in the oligosaccharide moiety. The determination of the absolute configuration showed signals at t_R 36.488 min (L-Ara), 40.357 min (D-Gal), and 41.065 min (D-GlcA). Pittangretoside C (**3**) was thus characterized as 22*α*-angeloyloxy-3*β*-[*β*-D-galactopyranosyl-(1 → 2)]-[*α*-L-arabinopyranosyl-(1 → 3)]-*β*-D-glucuronopyranosyloxyolean-12-ene-15*α*,16*α*, 28-triol.

The ESIMS spectrum (neg. mode) of pittangretoside D (**4**) displayed a quasimolecular ion peak at *m/z* 1055.5626 for $[M - H]^-$, compatible with the molecular formula of $C_{53}H_{84}O_{21}$. Concerning the aglycone part, the proton and carbon NMR spectra of pittangretoside D (**4**) were again quite similar to those of **1**–**3** and **10** indicating the same acylated A_1-barrigenol backbone. The glycosidic part, on the other hand, displayed striking differences. The ^{1}H NMR spectrum showed three anomeric protons at *δ* 5.21 (*s*), 4.84 (d, *J* = 7.0 Hz), and 4.48 (d, *J* = 7.0 Hz). No characteristic arabinose signals were observed, instead, the singlet at *δ* 5.21 in combination with a methyl doublet at *δ* 1.29 (*J* = 6.0 Hz) suggested the occurrence of one rhamnopyranose unit. The structures of the oligosaccharide moieties were deduced using two-dimensional NMR experiments, which indicated that one *β*-glucuronopyranose, one *β*-galactopyranose, and one *α*-rhamnopyranose were present (**Tables 1** and **3**), while analogous thiazolidine carboxylates of the hydrolyzate gave signals at t_R 37.625 min (L-Rha), t_R 40.359 min (D-Gal), and t_R 41.067 min (D-GlcA). Direct evidence for the sugar sequence and their linkage site to the aglycone was derived from the results of the HMBC experiment that showed unequivocal correlations between resonances at *δ* 4.48 and 91.1 ppm (H-1glcA–C-3), *δ* 4.84 and 77.6 ppm (H-1gal-C-2glcA), and *δ* 5.21 and 76.0 ppm (H-1rha-C-2gal). The same glycoside chain, but linked to different aglycones, has been described previously [13]. Thus, the structure of compound **4** was established as 22*α*-angeloyloxy-3*β*-[*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-gal-

Table 3 ^{1}H (500 MHz) NMR spectroscopic data of the sugar moieties of pittangretosides A–I (**1–9**) in CD_3OD (*J* in Hz) [a].

Proton	1	2	3	4	5	6	7	8	9
C-3	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA	GlcA
1	4.50 d (7.0)	4.49 d (7.0)	4.49 d (7.5)	4.48 d (7.0)	4.51 d (7.5)	4.50 d (7.5)	4.55 d (7.5)	4.48 d (7.6)	4.50 d (7.6)
2	3.93	3.93	3.80	3.71	3.92	3.93	3.84	3.72	3.69
3	3.90	3.92	3.75	3.64	3.91	3.90	3.77	3.65	3.67
4	3.89	3.93	3.63	3.48	3.91	3.90	3.65	3.50 t (9.0)	3.47
5	3.76	3.78	3.67	3.45	3.78	3.74	3.77	3.45	3.43
	Glc	Gal	Gal	Gal	Gal	Glc	Glc	Gal	Gal
1	5.01 d (7.5)	4.88 d (7.5)	4.86 d (7.5)	4.84 d (7.0)	4.89 d (7.5)	5.03 d (7.5)	4.99 d (7.5)	4.84 d (7.0)	4.87 d (6.5)
2	3.19 dd (9.0, 7.5)	3.57	3.50	3.68	3.58	3.20	3.16 t (8.0)	3.67	3.66
3	3.38 t (9.0)	3.49	3.47	3.53	3.50	3.39 t (9.0)	3.38 t (8.0)	3.50	3.48
4	3.11 t (9.0)	3.64	3.66	3.66	3.62	3.14 t (8.2)	3.10 t (9.0)	3.67	3.62
5	3.29	3.47	3.46	3.50	n. d.	3.30	3.33	3.60	3.63
6	3.57, 3.83	3.67, 3.76	3.65, 3.78	3.63, 3.80	3.70, 3.78	3.59, 3.85		3.63, 3.81	3.63, 3.75
	Ara(p)	Ara(p)	Ara(p)	Rha	Ara(p)	Ara(p)	Ara(p)	Rha	Rha
1	4.91 d (7.0)	4.93 d (7.0)	4.61 d (7.5)	5.21 s	4.95 d (7.5)	4.94 d (7.5)	4.61 d (7.0)	5.20 brs	5.17 brs
2	3.58	3.61	3.60	3.94 brs	3.61	3.59	3.64	3.94 brs	3.94 brs
3	3.50	3.48	3.50	3.78	3.50	3.50	3.52	3.78	3.78
4	3.76	3.76	3.82	3.42	3.77	3.74	3.84	3.42	3.42
5	3.55, 3.84	3.58, 3,78	3.61, 3.92	4.17 dq (9.0, 6.0)	3.60, 3.87	3.57, 3.84	3.63, 3.94	4.17 dq (9.0, 6.0)	4.12 dq (9.0, 6.0)
6				1.29 d (6.0)				1.28 d (6.0)	1.30 d (6.0)
	Ara(f)	Ara(f)			Ara(f)	Ara(f)			
1	5.14 brs	5.15 brs			5.17 brs	5.17 brs			
2	3.96 brs	3.96 brs			3.97 brs	3.98 brs			
3	3.77	3.76			3.77	3.78 brs			
4	4.44 q (4.5)	4.43 q (4.5)			4.45 q (4.5)	4.45 q (4.5)			
5	3.68	3.66			3.67	3.69			

[a] Assignments were made using ^{1}H-^{1}H COSY, HMBC, and HMQC experiments; overlapped ^{1}H signals are reported without designated multiplicity; n. d.: not determined; (f)/(p): furanose/pyranose

actopyranosyl-(1 → 2)]-*β*-D-glucuronopyranosyloxyolean-12-ene-15*α*,16*α*,28-triol.

Pittangretoside E (**5**) was recovered from silica gel fraction B, while the closely eluting substance pittangretoside F (**6**) was found mainly in fraction A. Because of the poor separability of these compounds by HPLC, this partition step was an important relief for semipreparative working. Its ESIMS spectrum (neg. mode) displayed a quasimolecular ion peak $[M - H]^-$ at *m/z* 1175.6005 predicting a molecular formula of $C_{57}H_{92}O_{25}$, which meant two additional mass units compared to **1** and **2**. A detailed look at the NMR spectroscopic data confirmed a common tetrasaccharide moiety with compound **2** but a different acyl substitution at the aglycone. Instead of the angeloyl moiety, characteristic signals for a 2-methylbutyroyl residue were observed: two methyl groups at *δ* 1.03 (*t, J* = 7.5 Hz) and 1.19 (*d, J* = 7.5 Hz), one methylene group at *δ* 1.55 (*m*) and 1.68 (*m*), and one methine proton at *δ* 2.42 (*m*) ppm. The ^{13}C NMR additionally displayed a carboxyl group at δ_C 177.2 ppm (**Tables 1** and **2**). Compared to **2**, the characteristic downfield proton signal of the acylated C-22 at *δ* 5.45 (*dd, J* = 5.7; 13.0 Hz) was also missing, instead H-22 appeared at *δ* 4.06 ppm (**Table 2**), implying a different substitution pattern of the aglycone. Indeed, the signal for H_2-28 was suspiciously shifted downfield (**1**: *δ* 3.13 and 3.31 ppm; **5**: *δ* 3.95 ppm), suggesting an acylation in this position. GC-MS signals at t_R 36.563 min (L-Ara), t_R 40.308 min (D-Gal), and t_R 41.094 min (D-GlcA) confirmed the absolute configuration. So, pittangretoside E (**5**) was established as 28-(2-methylbutyroyloxy)-3*β*-[*β*-D-galactopyranosyl-(1 → 2)]-[*α*-L-arabinopyranosyl-(1 → 3)]-[*α*-L-arabinofuranosyl-(1 → 4)]-*β*-D-glucuronopyranosyloxyolean-12-ene-15*α*,16*α*,22*α*-triol.

Pittangretoside F (**6**) displayed in the ESIMS (negative mode) a quasimolecular ion peak $[M - H]^-$ at *m/z* 1175.5995 indicating the same molecular formula as compound **5** ($C_{57}H_{92}O_{25}$). A comparison of the NMR spectra with those of compounds **1** and **2** revealed that galactose was again replaced by glucose in the case of **6**. Corresponding thiazolidine carboxylates of the sugar hydrolyzate showed signals at t_R 36.564 min (L-Ara), 39.976 min (D-Glc), and 41.093 min (D-GlcA). Compound **6** was thus identified as 28-(2-methylbutyroyloxy)-3*β*-[*β*-D-glucopyranosyl-(1 → 2)]-[*α*-L-arabinopyranosyl-(1 → 3)]-[*α*-L-arabinofuranosyl-(1 → 4)]-*β*-D-glucuronopyranosyloxyolean-12-ene-15*α*,16*α*,22*α*-triol.

Pittangretoside G (**7**) exhibited a molecular formula of $C_{52}H_{84}O_{21}$, substantiated by its ESIMS spectrum showing a quasimolecular ion peak $[M - H]^-$ at *m/z* 1043.5495 (neg. mode). This is a difference of two mass units compared to compound **10**. Indeed, the NMR spectra of **7** (**Tables 1**, **2**, and **3**) were quite similar to those of **10** concerning the aglycone moiety and the oligosaccharide chain. Instead of the angeloyl residue, a 2-methylbutyroyl residue (δ_H 1.04 (3H, *t, J* = 7.5 Hz), 1.18 (3H, *d, J* = 7.5 Hz), 1.55 (1H, *m*), 1.68 (1H, *m*), 2.42 (1H, *m*), δ_C 16.3, 10.5, 27.0, 41.7, 176.3 ppm) was present, which had to be attached to the hydroxyl at C-28 due to its suspected downfield shift (*δ* 3.97 ppm). The absolute configuration of the sugar composition was determined by thiazolidine carboxylates to be L-Ara (t_R 36.484 min), D-Glc (t_R 39.933 min), and D-GlcA (t_R 41.065). The chemical structure of **7** consequently was elucidated as 28-(2-methylbutyroyloxy)-3*β*-[*β*-D-glucopyranosyl-(1 → 2)]-[*α*-L-arabinopyranosyl-(1 → 3)]-*β*-D-glucuronopyranosyloxyolean-12-ene-15*α*,16*α*,22*α*-triol.

Pittangretoside H (**8**) was the main component of the two saponins **8** and **9** obtained from subfraction C_1. During HPLC, an ab-

sorption maximum at 223 nm was observed. Facing a weaker absorption of the A_1-barrigenol derivatives **1**-**7** and **10**, an extended chromophoric structure of the molecule was presumed. The ESIMS spectrum displayed quasimolecular ion peaks at *m/z* 955.4931 $[M - H]^-$ (neg. mode) and *m/z* 979.4912 $[M + Na]^+$ (pos. mode) generating a calculated molecular formula of $C_{48}H_{76}O_{19}$. On acid hydrolysis, **8** gave rhamnose, galactose, and glucuronic acid which were assigned by TLC (solvent B, det. reagent B) and GC-MS results as well as by the corresponding thiazolidine carboxylates [t_R 37.633 min (L-Rha), 40.396 min (D-Gal), 41.067 min (D-GlcA)]. This was supported by the NMR data (**Tables 1** and **3**), revealing the same oligosaccharide side chain as in **4**. Analysis of the NMR spectra of the aglycone moiety (**Tables 1** and **2**) revealed some similarities with oleanolic acid, but also striking differences. Seven quaternary methyls at δ_H 0.61, 0.90, 0.93, 0.97, 0.99, 1.14, and 1.34, a hydroxymethine proton at δ_H 3.22 (*dd, J* = 4.5, 12.0 Hz), and an olefinic signal at δ_H 5.51 (*br s*), a normal shift for Δ^{12}-oleanenes, on the ^{1}H-NMR spectrum suggested **8** to be an oleanolic acid derivative. Nevertheless, the carboxyl group at δ_C 169.1 ppm was high-field shifted compared to other oleanolic acid-type saponins [14] implying an α,β-unsaturation. A one proton resonance at δ 6.99 (*br d, J* = 7.2 Hz) corresponded to H-16, an olefinic proton deshielded by the CO_2H group. H-18 resonated at δ 3.57, an unusually low shift caused by the bis-allylic position of this proton. In particular, the long-range correlations between H-18 (δ_H 3.57) and C-17 (δ_C 137.6), C-13 (δ_C 143.4), C-12 (δ_C 121.9), C-19 (δ_C 45.8), and C-28 (δ_C 169.1) were supportive of the unusual 17,22-seco-skeleton. The deshielded protons of the hydroxymethylene group in position 22 appeared at δ_H 3.61 and 3.67 (δ_C 58.5) and were assigned by H-H-COSY correlations with the methylene group in position 21. Additionally, HMBC correlations were observed between H-15 (δ_H 2.55) and C-16 (δ_C 141.0), C-17 (δ_C 137.6), and C-8 (δ_C 39.9), as well as between H-16 (δ_H 6.99) and C-28 (δ_C 169.1). H–H COSY and HMBC led us to the plane structure of the aglycone moiety of **8** as 22-hydroxy-17,22-secoolean-12,16-dien-28-oic acid. The same aglycone, named steganogenin, has been described once as a constituent of a saponin isolated from *Steganotaenia araliacea,* Apiaceae [15], and the NMR data of the aglycone moiety of **8** are fully in agreement with steganogenin. The structure of pittangretoside H (**8**) was thus elucidated as 3β-[α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranosyl(1 → 2)]-β-D-glucuronopyranosyloxy-22-hydroxy-17,22-secoolean-12,16-dien-28-oic acid.

Pittangretoside I (**9**), the more polar of the two glycosides obtained from subfraction C_1, also displayed an absorption maximum at 223 nm during HPLC. Quasimolecular ion peaks at *m/z* 995.4815 $[M + Na]^+$ (pos. mode) as well as *m/z* 971.4894 $[M - H]^-$ and *m/z* 485.2353 $[M - 2H]^{2-}$ (neg. mode) became apparent in its ESIMS spectrum, which gave a molecular formula of $C_{48}H_{76}O_{20}$, just one oxygen more than in compound **8**. The ^{1}H and ^{13}C NMR data of **9** and **8** were also quite similar suggesting the same sugar chain and also a similar seco-skeleton. In contrast to **8**, the ^{1}H NMR of **9** (**Table 2**) showed only six three-proton singlets at high-field, leading to the assumption that one of the methyl groups had to be oxidized. Indeed, signals for an additional hydroxymethylene group were observed (δ_H 3.85, d, *J* = 11.0 Hz, 3.36, d, *J* = 11.0 Hz, δ_C 64.8). The position of the hydroxymethylene group was determined as C-23 by HMBC correlations between H-24 at δ 0.76 ppm and C-3 (δ_C 83.6), C-4 (δ_C 43.0), C-5 (δ_C 48.6), and C-23 (δ_C 64.8). Furthermore, the chemical shift (δ_C 12.4) of C-24 was characteristic for a β-orientation of the methyl group, since otherwise it should be around δ_C 23 ppm [16]. Again, the absolute configuration of sugars was determined as L-Rha (t_R 37.633 min), D-Gal (t_R 40.355 min), and D-GlcA (t_R 41.068 min). The structure of pittangretoside I (**9**) was thus determined as 3β-[α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranosyl(1 → 2)]-β-D-glucuronopyranosyloxy-22,23-dihydroxy-17,22-secoolean-12,16-dien-28-oic acid.

Table 4 *In vitro* cytotoxicity of isolates **1**–**10** against 5637 cells[a].

Compound	IC_{50} [µM]	Compound	IC_{50} [µM]
1	4.1 ± 1.0	**6**	n. d.
2	5.5 ± 0.5	**7**	n. d.
3	2.1 ± 0.1	**8**	n. d.
4	17.9 ± 1.0	**9**	n. d.
5	n. d.	**10**	2.5 ± 0.3
Etoposide	0.6 ± 0.1		

[a] Data expressed as mean value ± SD (duplicate); etoposide: positive control; n. d.: not determined (IC_{50} > 125 µg/mL)

In support of the taxonomic distinction made between *P. angustifolium* and *P. phillyreoides* [1], none of the isolated compounds and the corresponding aglycones was found in former studies of *P. phillyreoides* [8]. Seco-structures are rarely observed among triterpene saponins and were found at attached sugar moieties [17–19] or at different aglycone types [20] such as lupane [21, 22], ursane [23–25], dammarane [26], cycloartane [27], and at the oleanolic acid backbone [15] as it is present in pittangretosides H (**8**) and I (**9**).

In order to substantiate reported biological activities, isolated compounds (**1**-**10**) were tested for their cytotoxic potential against the human urinary bladder carcinoma cell line 5637, which has been used for test screenings of natural and synthetic compounds [28, 29]. The neutral red uptake (NRU) assay was used with etoposide as the positive control. As presented in **Table 4**, only saponins **1–4** and **10** showed antiproliferative effects. As a common feature, all of them display the same acylation pattern with angelic acid at C-22 of the A_1-barrigenol aglycone. Seco-glycosides (**8, 9**) and compounds possessing a 2-methylbutyroyl residue at C-28 (**5–7**) revealed no cytotoxic impact within the investigated concentration range. These results support recent studies in which the necessity of angeloyl attachments for cytotoxicity in this region (C-22) of triterpene aglycones has been confirmed [5, 30]. Among the effective angeloyl-acylated compounds **1–4** and **10**, an influence of the sugar linkage seems to play an additional role, since the lack of a pentose unit in compounds **3** and **10** increased the cytotoxicity in comparison to the tetrasaccharide chain of **1** and **2**. Saponin **4** and its relatively weaker antiproliferative effect can also indicate that the sugar units affect cytotoxicity, because glycoside **4** possesses a differently composed trisaccharide chain than compounds **1–3** and **10**, while the aglycone part is completely identical.

Material and Methods

General

NMR spectra were recorded in CD_3OD on a Bruker DRX 500 apparatus. For analytical and semipreparative HPLC, a Shimadzu system (LC-10 ATVP dual plunger pump, SPD-M10AVP diode array detector, FCV-M10AVP low pressure gradient unit, SCL-10AVP system controller) together with an RP18 column (250 mm × 4.6 mm, 4 µm; Phenomenex) was used. GC-MS techniques were

carried out on an Agilent system (gas chromatograph G1530N, mass selective detector MSD G2588A) using a DB-5MS column (30 m × 0.25 mm × 0.25 µm; J&W Scientific). Injection was performed with an Agilent G2613A series injector [split 1 : 25 at 230 °C, carrier gas helium 1 mL·min^{-1} (60 kPa) at 110 °C, pressure rise 6 kPa·min^{-1}]. Initial oven temperature of 70 °C was retained for one minute, then increased by 1.5 °C·min^{-1} to 76 °C, followed by an increase by 5 °C·min^{-1} to 330 °C maintained for ten minutes. For ionization in the electron impact mode, an energy of 70 eV was used. Derivatization procedure of hydrolyzed sugars was carried out according to Liebeke et al. [31]. For identification of detected compounds, mass spectral data were compared with NIST database 2.0 d (National Institute of Standards and Technology) data obtained from authentic samples and further by comparison of retention times of the TIC (total ion chromatograms). Comparisons were executed with authentic samples of D-glucose (Aldrich, purity > 99%), D-galactose (Aldrich, purity > 99%), L-rhamnose (Applichem, purity > 99%), L-arabinose (Fluka, purity > 99%), D-arabinose (Alfa Aesar, purity 99%), and D-glucuronic acid (Sigma, purity > 98%). LC-MS measurements were performed with a Shimadzu system (LCMS-IT-TOF) using a Chromolith SpeedRod RP18 column (50 mm × 4.6 mm; Merck) and electrospray ionization (ESI). The LC system consisted of a solvent delivery module (LC-20AD), auto sampler (SIL-20AC HT), column oven (CTO-20A), system controller CBM-20A, and a photodiode array detector (SPD-M20A). CD spectra were recorded on a Jasco J-710 spectropolarimeter. Optical rotation values were determined by a Perkin Elmer 241 polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 spectrophotometer. ATR-IR spectra were recorded using a Thermo Scientific Nicolet IR 200 FT-IR spectrometer. TLC examinations were performed on precoated silica gel 60 plates (Merck) with a mixture of EtOAc/HCOOH/HOAc/H_2O (6.7 : 0.75 : 0.75 : 1.8; solvent A) and 20% H_2SO_4 as the detection reagent (A) for triterpene saponins and further EtOAc/*iso*-PrOH/HOAc/H_2O (4 : 2 : 2 : 1; solvent B) and spray reagent B (thymol 0.25 g, H_2SO_4 2.5 mL, EtOH 47.5 mL) for sugars. Plates were heated for 5 minutes at 110 °C (triterpene glycosides) or at 135 °C (sugar fraction of the hydrolyzate) and analyzed in the VIS. A vacuum manifold and RP18-cartridges (Strata C18E, 20 g/120 mL; Phenomenex) were applied for SPE fractionations. Melting points were recorded with a Kofler heating block (uncorr.).

Plant material

Leaves of *Pittosporum angustifolium* were collected in June 2008 on the ground of Central Queensland GG foundation (K. A. Amato and the Trustee for the Milner Krasser Family Trust) in the surroundings of Mount Morgan, Rockhampton, Queensland, Australia and a gift of Dr. Kornelia Krasser and Mr. Klaus von Glyszcinsky, Yeppon, Australia. Plant material was identified by Dr. Peter König, Curator of the botanic garden of Greifswald. A voucher specimen (No. 20110013PA) and samples of air-dried material were deposited at the Institute of Pharmacy, Department of Pharmaceutical Biology at Ernst-Moritz-Arndt-University, Greifswald, Germany.

Extraction and isolation

Ground leaves (140 g) were defatted with CH_2Cl_2 by Soxhlet apparatus (700 mL, 10 h) and then extracted three times with 80% (v/v) EtOH under reflux (each 1000 mL, 10 h) to give a lyophilized residue (50 g). 3 g of the crude extract were applied to a column of Sephadex LH20 gel (Sigma-Aldrich) eluting with methanol (1000 mL). TLC-based screening (solvent A, det. reagent A) led to the recovery of a purified triterpene glycoside fraction (TTG; 2.2 g). For further partition, 0.9 g of the TTG amount were applied to a silica gel (60–40 µm; Merck) column eluting with a stepwise gradient of CH_2Cl_2/MeOH 9 : 3 (350 mL), CH_2Cl_2/MeOH/H_2O 8 : 4 : 0.5 (400 mL), and CH_2Cl_2/MeOH/H_2O 6 : 4 : 1 (900 mL). Followed by a TLC (solvent A, det. reagent A) and HPLC checkup, three subfractions were subjected to further analysis (A: 218 mg, B: 163 mg, and C: 63 mg). A and C were submitted to an additional SPE-C18 procedure to remove high polarity impurities by eluting with H_2O and 30% MeOH (each 200 mL). By flushing the cartridge with MeOH (200 mL), saponins were recovered as subfractions A_1 (160 mg) and C_1 (36 mg), respectively. Compounds **1** (t_R 29.0 min, 20 mg), **2** (t_R 28.4 min, 6.7 mg), **6** (t_R 26.4 min, 3.0 mg), and **7** (t_R 30.3 min, 3.2 mg) were isolated mainly from subfraction A_1 and compounds **3** (t_R 32.2 min, 1.9 mg), **4** (t_R 29.5 min, 5.2 mg), **5** (t_R 26.0 min, 2.0 mg), and **10** (t_R 32.8 min, 3.8 mg) from subfraction B by semipreparative HPLC (CH_3CN/0.05% HCOOH in H_2O 36 : 64, detection 206 nm, flow rate 1 mL·min^{-1}). Seco-structures **8** (t_R 12.7 min, 4.8 mg) and **9** (t_R 8.3 min, 2.0 mg) were obtained from subfraction C_1 using semipreparative HPLC (CH_3CN/0.05% HCOOH in H_2O 30 : 70, detection 227 nm and 206 nm, flow rate 1 mL·min^{-1}).

Pittangretoside A (**1**): Colorless amorphous powder, $C_{57}H_{90}O_{25}$, mp 235–239 °C (decomp.), $[\alpha]_D^{20}$ – 30.0 (c 0.23, MeOH), CD (*c* 4.3×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 203 (– 31.46). UV (MeOH) λ_{max} (log ε) 218 (3.82), ATR-IR ν_{max} 3376, 2921, 1685, 1456, 1386, 1243, 1160, 1068, 1041, 1015, 915 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 1S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 749.4513 (2.6) [(M + H)-Glc-2Ara]$^+$, 631.3865 (16.2) [(M + H)-Glc-2Ara-Ang-2H_2O]$^+$, 613.3741 (9.8) [(M + H)-Glc-2Ara-Ang-3H_2O]$^+$, 555.4056 (8.0) [(M + H)-GlcA-Glc-2Ara-H_2O]$^+$, 537.3964 (9,9) [(M + H)-GlcA-Glc-2Ara-2H_2O]$^+$, 519.3635 (3.5) [(M + H)-GlcA-Glc-2Ara-3H_2O]$^+$, 455.3529 (71.0) [(M + H)-GlcA-Glc-2Ara-Ang-2H_2O]$^+$, 437.3427 (100) [(M + H)-GlcA-Glc-2Ara-Ang-3H_2O]$^+$, negative mode: 1173.5751 (20.0) [M – H]$^-$ (calcd. for $C_{57}H_{89}O_{25}$, 1173.5698 monoisotopic mass).

Pittangretoside B (**2**): Colorless amorphous powder, $C_{57}H_{90}O_{25}$, mp 263–266 °C (decomp.), $[\alpha]_D^{20}$ – 22.2 (c 0.22, MeOH), CD (*c* 4.3×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 198 (– 27.94), UV (MeOH) λ_{max} (log ε) 218 (3.77), ATR-IR ν_{max} 3348, 2924, 1688, 1605, 1454, 1376, 1244, 1159, 1072, 1040, 999 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 2S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 1197.5643 (14.0) [M + Na]$^+$ (calcd. for $C_{57}H_{90}O_{25}Na$, 1197.5663 monoisotopic mass), 631.3882 (14.3) [(M + H)-Gal-2Ara-Ang-2H_2O]$^+$, 555.3534 (7.1) [(M + H)-GlcA-Gal-2Ara-H_2O]$^+$, 537.3989 (8.3) [(M + H)-GlcA-Gal-2Ara-2H_2O]$^+$, 455.3539 (62.6) [(M + H)-GlcA-Gal-2Ara-Ang-2H_2O]$^+$, 437.3410 (100) [(M + H)-GlcA-Gal-2Ara-Ang-3H_2O]$^+$, 419.3497 (17.6) [(M + H)-GlcA-Gal-2Ara-Ang-4H_2O]$^+$, negative mode: 1173.5714 (3.25) [M – H]$^-$.

Pittangretoside C (**3**): Colorless amorphous powder, $C_{52}H_{82}O_{21}$, mp 259–263 °C (decomp.), $[\alpha]_D^{20}$ – 28.8 (c 0.20, MeOH), CD (*c* 4.8×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 213 (– 23.78), UV (MeOH) λ_{max} (log ε) 218 (3.91), ATR-IR ν_{max} 3353, 2955, 1684, 1454, 1388, 1242, 1158, 1076, 1043, 1008 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 3S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 1065.5194 (14.4) [M + Na]$^+$ (calcd. for $C_{52}H_{82}O_{21}Na$, 1065.5241 monoisotopic mass), 749.4500 (2.8) [(M + H)-Gal-Ara]$^+$, 631.3860 (14.7) [(M + H)-Gal-Ara-Ang-2H_2O]$^+$, 613.3756 (9.6) [(M + H)-Gal-Ara-Ang-3H_2O]$^+$, 595.3663 (3.1) [(M + H)-Gal-Ara-Ang-4H_2O]$^+$, 555.4052 (8.5) [(M

+ H)-GlcA-Gal-Ara-H_2O]$^+$, 537.3966 (8.7) [(M + H)-GlcA-Gal-Ara-2H_2O]$^+$, 455.3520 (52.8) [(M + H)-GlcA-Gal-Ara-Ang-2H_2O]$^+$, 437.3427 (100) [(M + H)-GlcA-Gal-Ara-Ang-3H_2O]$^+$, negative mode: 1041.5299 (10.0) [M – H]$^-$.

Pittangretoside D (**4**): Colorless amorphous powder, $C_{53}H_{84}O_{21}$, mp 235–239 °C (decomp.), $[\alpha]_D^{20}$ – 38.6 (c 0.11, MeOH), CD (*c* 4.7×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 214 (– 15.95), UV (MeOH) λ_{max} (log ε) 217 (3.88), ATR-IR ν_{max} 3347, 2930, 1686, 1465, 1387, 1242, 1142, 1070, 1041, 980 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 4S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 631.3864 (22.8) [(M + H)-Gal-Rha-Ang-2H_2O]$^+$, 613.3757 (12.7) [(M + H)-Gal-Rha-Ang-3H_2O]$^+$, 555.4062 (6.4) [(M + H)-GlcA-Gal-Rha-H_2O]$^+$, 537.3984 (6.7) [(M + H)-GlcA-Gal-Rha-2H_2O]$^+$, 455.3627 (48.0) [(M + H)-GlcA-Gal-Rha-Ang-2H_2O]$^+$, 437.3426 (100) [(M + H)-GlcA-Gal-Rha-Ang-3H_2O]$^+$, negative mode: 1055.5626 (100) [M – H]$^-$ (calcd. for $C_{53}H_{83}O_{21}$, 1055.5432 monoisotopic mass).

Pittangretoside E (**5**): Colorless amorphous powder, $C_{57}H_{92}O_{25}$, mp 255–259 °C (decomp.), $[\alpha]_D^{20}$ – 36.0 (c 0.08, MeOH), CD (*c* 4.2×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 208 (– 21.00), UV (MeOH) λ_{max} (log ε) 218 (3.42), ATR-IR ν_{max} 3359, 2938, 1719, 1603, 1454, 1386, 1363, 1241, 1145, 1076, 1044, 1003 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 5S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 733.4560 (10.5) [(M + H)-Gal-2Ara-H_2O]$^+$, 715.4435 (7.3) [(M + H)-Gal-2Ara-2H_2O]$^+$, 539.4125 (100) [(M + H)-GlcA-Gal-2Ara-2H_2O]$^+$, 521.4017 (42.9) [(M + H)-GlcA-Gal-2Ara-3H_2O]$^+$, 437.3433 (61.6) [(M + H)-GlcA-Gal-2Ara-Mebu-3H_2O]$^+$, 419.3322 (35.5) [(M + H)-GlcA-Gal-2Ara-Mebu-4H_2O]$^+$, negative mode: 1175.5995 (100) [M – H]$^-$ (calcd. for $C_{57}H_{91}O_{25}$, 1175.5855 monoisotopic mass).

Pittangretoside F (**6**): Colorless amorphous powder, $C_{57}H_{92}O_{25}$, mp 231–236 °C (decomp.), $[\alpha]_D^{20}$ – 34.2 (c 0.11, MeOH), CD (*c* 4.2×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 205 (– 19.01), UV (MeOH) λ_{max} (log ε) 218 (3.55), ATR-IR ν_{max} 3379, 2945, 1716, 1606, 1454, 1386, 1244, 1145, 1076, 1043, 1007 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 6S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 733.4524 (25.6) [(M + H)-Glc-2Ara-H_2O]$^+$, 557.4213 (41.2) [(M + H)-GlcA-Glc-2Ara-H_2O]$^+$, 539.4113 (100) [(M + H)-GlcA-Glc-2Ara-2H_2O]$^+$, 521.4014 (50.4) [(M + H)-GlcA-Glc-2Ara-3H_2O]$^+$, 437.3430 (38.1) [(M + H)-GlcA-Glc-2Ara-Mebu-3H_2O]$^+$, 419.3322 (32.6) [(M + H)-GlcA-Glc-2Ara-Mebu-4H_2O]$^+$, negative mode: 1175.6005 (100) [M – H]$^-$ (calcd. for $C_{57}H_{91}O_{25}$, 1175.5855 monoisotopic mass).

Pittangretoside G (**7**): Colorless amorphous powder, $C_{52}H_{84}O_{21}$, mp 222–226 °C (decomp.), $[\alpha]_D^{20}$ – 5.0 (c 0.20, MeOH), CD (*c* 4.8×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 211 (– 24.26), UV (MeOH) λ_{max} (log ε) 214 (3.59), ATR-IR ν_{max} 3403, 2924, 1716, 1463, 1361, 1237, 1139, 1078, 1044, 994 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 7S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 733.4550 (25.4) [(M + H)-Glc-Ara-H_2O]$^+$, 715.4447 (19.1) [(M + H)-Glc-Ara-2H_2O]$^+$, 631.3915 (2.6) [(M + H)-Glc-Ara-Mebu-2H_2O]$^+$, 539.4121 (100) [(M + H)-GlcA-Glc-Ara-2H_2O]$^+$, 521.4014 (52.9) [(M + H)-GlcA-Glc-Ara-3H_2O]$^+$, 455.3512 (7.1) [(M + H)-GlcA-Glc-Ara-Mebu-2H_2O]$^+$, 437.3428 (38.1) [(M + H)-GlcA-Glc-Ara-Mebu-3H_2O]$^+$, 419.3320 (36.3) [(M + H)-GlcA-Glc-Ara-Mebu-4H_2O]$^+$, negative mode: 1043.5495 (100) [M – H]$^-$ (calcd. for $C_{52}H_{83}O_{21}$, 1043.5432 monoisotopic mass).

Pittangretoside H (**8**): Colorless amorphous powder, $C_{48}H_{76}O_{19}$, mp 229–233 °C (decomp.), $[\alpha]_D^{20}$ – 62.9 (c 0.17, MeOH), CD (*c* 5.2×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 246 (+ 29.18), UV (MeOH) λ_{max} (log ε) 223 (3.76), ATR-IR ν_{max} 3393, 2933, 1681, 1367, 1232, 1133, 1063, 1044, 980 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 8S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 649.3972 (5.6) [(M + H)-Gal-Rha]$^+$, 473.3627 (10.8) [(M + H)-GlcA-Gal-Rha]$^+$, 455.3623 (24.8) [(M + H)-GlcA-Gal-Rha-H_2O]$^+$, 437.3431 (100) [(M + H)-GlcA-Gal-Rha-2H_2O]$^+$, negative mode: 955.4931 (100) [M – H]$^-$ (calcd. for $C_{48}N_{75}O_{19}$, 955.4908 monoisotopic mass).

Pittangretoside I (**9**): Colorless amorphous powder, $C_{48}H_{76}O_{20}$, mp 239–243 °C (decomp.), $[\alpha]_D^{20}$ – 34.3 (c 0.11, MeOH), CD (*c* 5.1×10^{-4} M, MeOH) λ_{max} nm ($\Delta\varepsilon$): 247 (+ 28.71), UV (MeOH) λ_{max} (log ε) 223 (3.89), ATR-IR ν_{max} 3351, 2936, 1682, 1373, 1245, 1140, 1060, 1045, 985 cm^{-1}. For ^{1}H and ^{13}C NMR data, see **Tables 1–3** and **Fig 9S**, Supporting Information. HRESI-IT-TOFMS *m/z* (rel. int.) positive mode: 995.4815 (100) [M + Na]$^+$ (calcd. for $C_{48}H_{76}O_{20}Na$, 995.4822 monoisotopic mass), 665.3811 (2.3) [(M + H)-Gal-Rha]$^+$, 453.3439 (29.7) [(M + H)-GlcA-Gal-Rha-2H_2O]$^+$, 435.3344 (10.9) [(M + H)-GlcA-Gal-Rha-3H_2O]$^+$, negative mode: 971.4894 (23.6) [M – H]$^-$, 485.2353 (100) [M – 2H]$^{2-}$.

Acidic hydrolysis

0.4–2.0 mg of each compound were separately added to 2 mL of 2 M TFA and then heated for 2 hours at 80 °C in a heating block (BT200; Kleinfeld Labortechnik). After cooling, each reaction mixture was centrifuged three times at 4000 RPM for 5 min. The supernatant was removed and dried under reduced pressure. A part of each sugar mix was submitted to TLC and GC-MS analysis, another part was used for a derivatization procedure according to [32] to confirm the absolute configuration of monosaccharides. Assignments were made by GC-MS analysis referring to authentic samples showing retention times of the corresponding thiazolidine carboxylates at t_R 36.538 min (L-Ara), 37.088 min (D-Ara), 37.647 min (L-Rha), 39.969 min (D-Glc), 40.383 min (D-Gal), and 41.109 min (D-GlcA).

Cytotoxicity assay

5637 cells, a human urinary bladder carcinoma cell line, were purchased from the Leibnitz Institute DSMZ (ACC 35), Germany. Cells were cultivated in RPMI-1640 medium (Bio Whittaker) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) at 95% humidity, 5% CO_2, and 37 °C. The cytotoxicities of compounds **1**-**10** against 5637 cells were investigated using 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red; Merck) and the NRU assay. After 24 h cultivation in 96-well plates (3×10^3 cells/well), medium was removed and cells were exposed to various concentrations (max. 125 µg·mL^{-1}) of isolates **1–10** for 72 h. After removal of the medium, wells were washed with 200 µL HBSS (Hanks Balanced Salt Solution; PAA) and cells were incubated with 100 µL/well of a neutral red solution (33 µg·mL^{-1}) for 4 h. Medium was removed, wells were washed two times with 100 µL HBSS, and 100 µL of a 1% acetic acid in 50% EtOH were added for cell lysis. Finally, after 45 min, optical density was determined at 450 nm in a plate reader (Fluostar Omega; BMG Labtech). The IC_{50} values were defined from obtained dose-response curves and expressed in mean ± SD. All compounds were tested in duplicate, and purity (%) was determined by HPLC: **1** (96), **2** (94), **3** (97), **4** (97), **5** (93), **6** (94), **7** (94), **8** (96), **9** (96), **10** (95). Etoposide (Alexis Biochemicals, purity > 98%) was used as a positive control.

Supporting information

^{1}H NMR spectra of new compounds **1**–**9** are available as Supporting Information.

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Conflict of Interest

All involved authors declare that there is no conflict of interest.

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