Cytotoxic Saponins from the Seeds of *Pittosporum angustifolium*

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Z. Naturforsch. **69c**, 191 – 198 (2014) / DOI: 10.5560/ZNC.2014-0011
Received January 18 / April 23, 2014 / published online June 5, 2014

Three new acylated R_1-barrigenol triterpene glycosides, **1** – **3**, were isolated from the seeds of *Pittosporum angustifolium* Lodd. together with four known glycosides, **4** – **7**, containing R_1- and A_1-barrigenol backbones. On the basis of spectroscopic, spectrometric, and chemical analyses the novel compounds were named pittangretosides N – P and established as 21β-acetoxy-22α-angeloyloxy- (**1**), 21β-acetoxy-22α-(2-acetoxy-2-methylbutyroyloxy)- (**2**), and 21β-(2-methylbutyroyloxy)-22α-acetoxy-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 (**3**). Evaluation of the *in vitro* cytotoxicity against three tumour cell lines and one non-tumourigenic cell line revealed antiproliferative effects with IC_{50} values in a range of 1.74 – 34.1 μM.

Key words: Pittosporum angustifolium, Triterpene Glycosides

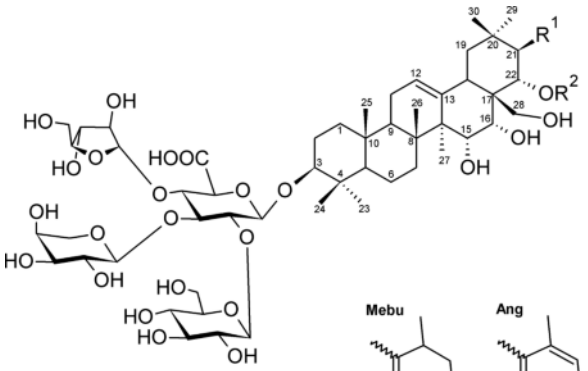
Introduction

Pittosporum angustifolium Lodd. (Pittosporaceae) is a small tree which grows endemically in most inland areas of Australia and is colloquially referred to as "gumby gumby" or "weeping pittosporum" (Cayzer *et al*., 2000; Whittemore and McClintock, 2007). Various preparations of this plant are used by the Aborigines for the treatment of cough, skin diseases, and other disorders (Cayzer *et al*., 2000). In a recent study we have reported the isolation and evaluation for the *in vitro* cytotoxicity of ten A_1-barrigenol glycosides from the leaves of *Pittosporum angustifolium* (Bäcker *et al*., 2013). Also in other species of the *Pittosporum* genus triterpene glycosides seem to be dominant secondary metabolites (Higuchi *et al*., 1983; D'Acquarica *et al*., 2002; Seo *et al*., 2002; Linnek *et al*., 2012; Manase *et al*. 2013). Continuing our phytochemical investigation, we report herewith the isolation of three new and four known A_1- and R_1-barrigenol glycosides from the seeds of *Pittosporum angustifolium* and the characterization of their antiproliferative effects.

Results and Discussion

The crude 80 % ethanolic extract of the seeds of *Pittosporum angustifolium* was purified by chromatography on a Sephadex LH-20 column followed by an RP18 solid phase extraction separation procedure. Fractions obtained were subjected to semipreparative HPLC for the isolation of compounds **1** – **7** (Fig. 1). New natural compounds were named pittangretosides N – P (**1** – **3**).

According to their spectroscopic data, compounds **4** – **7** were determined as the known compounds 21β-angeloyloxy-22α-acetoxy-3β-[β-D-glucopyranosyl-



	R^1	R^2
1	OAc	Ang
2	OAc	AcOMebu
3	OMebu	Ac
4	OAng	Ac
5	H	Mebu
6	H	Ang
7	OAng	Ang

Fig. 1. Triterpene saponins isolated from the seeds of *Pittosporum angustifolium.*

(1→2)]-[α-L-arabinopyranosyl-(1→3)]-[α-L-arabinofuranosyl-(1→4)]-β-D-glucuronopyranosyloxyolean-12-ene-15α,16α,28-triol (**4**) (D'Acquarica *et al.*, 2002), 22α-(2-methylbutyroyloxy)-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 (**5**) (Higuchi *et al.*, 1983), 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 (**6**) (Bäcker *et al.*, 2013), and 21β-angeloyloxy-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 (**7**) (D'Acquarica *et al.*, 2002).

The ESI mass spectrum of pittangretoside N (**1**) displayed a quasimolecular ion $[M-H]^-$ at m/z 1231.5810 (negative mode) consistent with a molecular formula of $C_{59}H_{92}O_{27}$. Since the ^1H and ^{13}C NMR data of **1** were almost identical to those of compound **7**, the aglycone backbone could also be identified as R_1-barrigenol. Furthermore, the ^1H NMR spectrum showed four anomeric protons at δ_H 5.14, 5.01, 4.91, and 4.51 ppm (Table I) corresponding to δ_C 108.1, 102.5, 104.0, and 105.4 ppm in the HMQC spectrum (Table I). The HMBC crosspeak between H-3 of the aglycone and one of the anomeric carbon atoms (δ_C 105.4 ppm) implied an attachment of the oligosaccharide chain to C-3 (δ_C 91.4 ppm, Table II). Extensive ^1H-^1H-COSY, HMQC, and HMBC experiments led to the assignment of all sugar units, which turned out to be a β-glucuronopyranosic acid (GlcA), a β-glucopyranose (Glc), an α-arabinopyranose [Ara (p)], and an α-arabinofuranose [Ara (f)]. This was also supported by thin-layer chromatography (TLC) and GC-MS analyses of the hydrolyzate, revealing signals for glucuronopyranosic acid, glucose, and arabinose. The absolute configuration of the corresponding thiazolidine carboxylates of sugars was determined as L-Ara (t_R 36.517 min), D-Glc (t_R 39.901 min), and D-GlcA (t_R 41.091 min). The HMBC spectrum revealed crosspeaks between H-1 of the glucose moiety and δ_C 80.2 ppm (GlcA-2), H-1 of the arabinopyranose and δ_C 79.4 ppm (GlcA-3), and H-1 of the arabinofuranose and δ_C 74.4 (GlcA-4) indicating a 2,3,4-trisubstituted glucuronopyranosic acid unit. The same oligosaccharide moiety has been described as the predominant sugar moiety of triterpene saponins isolated from several *Pittosporum* species (Higuchi *et al.*, 1983; D'Acquarica *et al.*, 2002; Seo *et al.*, 2002; Bäcker *et al.*, 2013; Manase *et al.*, 2013). Moreover, ^1H and ^{13}C NMR data showed signals for two additional acyl substituents that were identified as angeloyl and acetyl moieties. The downfield shifts of the protons H-21 (5.84 ppm) and H-22 (5.58 ppm) implied the corresponding ester linkage to the aglycone. Unequivocal evidence for the acyl substitution pattern was obtained from HMBC data displaying a long-range correlation of H-21 and the acyl carbon atom of the acetyl moiety, while H-22 showed a long-range correlation with the acyl carbon atom of the angeloyl residue. Thus, the new natural product pittangretoside N (**1**) was established as 21β-acetoxy-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 O (**2**) showed a quasimolecular ion peak $[M-H]^-$ at m/z 1291.5968 in its ESI mass spectrum, predictive of a molecular formula of $C_{61}H_{96}O_{29}$. The ^1H and ^{13}C NMR data were almost identical to those of **1**, leading to the assumption of a structural similarity of both compounds. Again, in the ^1H NMR spectrum four anomeric protons at

Table I. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectroscopic data of the sugar moieties of compounds **1** – **3** in CD_3OD (*J* in Hz)[a].

Position	**1**		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
C-3	GlcA		GlcA		GlcA	
1	105.4	4.51 d (6.9)	106.6	4.47 d (7.1)	106.8	4.49 d (6.8)
2	80.2	3.92	80.6	3.93	80.7	3.92
3	79.4	3.89	80.4	3.90	80.5	3.89
4	74.4	3.81	74.4	3.83	74.4	3.82
5	79.4	3.77	79.5	3.76	80.3	3.75
6	n.d.	–	n.d.	–	n.d.	–
	Glc		Glc		Glc	
1	102.5	5.01 d (7.9)	103.8	5.03 d (7.9)	103.7	5.01 d (7.6)
2	75.7	3.20 t (8.7)	77.1	3.21 t (8.3)	76.8	3.20 t (8.9)
3	78.0	3.38 t (9.0)	79.0	3.39 t (9.0)	78.8	3.37 t (8.9)
4	72.3	3.10 t (9.2)	72.3	3.12 t (9.2)	72.7	3.09 (9.5)
5	78.0	3.28	77.8	3.31	78.2	3.29
6	63.2	3.64 dd (11.3, 5.0), 3.83	64.1	3.64 dd (11.3, 5.1), 3.82 dd (11.0, 4.4)	64.1	3.67 dd (11.3, 5.0), 3.82
	Ara (p)		Ara (p)		Ara (p)	
1	104.0	4.91 d (7.8)	104.7	4.93 d (7.8)	104.8	4.90
2	72.9	3.58	74.1	3.60	74.1	3.58
3	73.8	3.50	74.7	3.52	74.4	3.50
4	70.1	3.76	70.7	3.77	70.7	3.75
5	67.2	3.51 dd (9.6, 2.9), 3.84	67.9	3.50 dd (9.6, 3.4), 3.85	67.8	3.50 dd (9.7, 3.5), 3.83
	Ara (f)		Ara (f)		Ara (f)	
1	108.1	5.14 br s	108.7	5.18	108.6	5.15
2	81.4	3.96 br s	82.5	3.97	82.6	3.95
3	79.3	3.77	80.1	3.77	80.3	3.76
4	87.1	4.44 q (4.3)	87.7	4.44	87.6	4.43
5	63.2	3.69 dd (12.1, 4.8), 3.57	64.5	3.67 dd (12.1, 4.5), 3.57	64.4	3.72 dd (12.2, 4.2), 3.58

[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; GlcA, β-glucuronopyranosic acid; Glc, β-glucopyranose; Ara (p), α-arabinopyranose; Ara (f), α-arabinofuranose.

δ_H 5.18, 5.03, 4.93, and 4.47 ppm (Table I) were observed with the corresponding carbon atoms at δ_C 108.7, 103.8, 104.7, and 106.6 ppm in the HMQC spectrum. Further examination of the two-dimensional NMR spectra indicated the presence of one β-glucuronopyranosic acid (GlcA), one β-glucopyranose (Glc), one α-arabinopyranose [Ara (p)], and one α-arabinofuranose [Ara (f)] which was confirmed by the results of TLC and GC-MS analyses. Establishing the absolute configuration of sugars, corresponding thiazolidine carboxylates were determined as L-Ara (t_R 36.537 min), D-Glc (t_R 39.914 min), and D-GlcA (t_R 41.094 min). The sugar branching turned out to be the same as in **1**, HMBC crosspeaks between H-1 (Gal) and δ_C 80.6 ppm (GlcA-2), H-1 [Ara (p)] and δ_C 80.4 ppm (GlcA-3), and finally H-1 [Ara (f)] and δ_C 74.4 ppm (GlcA-4) were observed. Concerning the aglycone part the NMR data revealed an R_1-barrigenol skeleton, too. The additional molecular part of $C_2H_4O_2$, compared with the molecular composition of **1**, had to be due to a different acyl composition which was also supported by HRMS data. Indeed, in addition to an acetyl group whose acyl carbon atom showed a long-range correlation (HMBC) with the downfield shifted proton H-21 (5.77 ppm), a 2-acetoxy-2-methylbutyroyl residue was determined. Its acyl carbon atom displayed a long-range correlation with H-22, which was also shifted downfield at 5.46 ppm. The novel structure of pittangretoside O (**2**) was thus established as 21β-acetoxy-22α-(2-acetoxy-2-methylbutyroyloxy)-3β-[β-D-glucopyranosyl-(1 → 2)]-[α-L-arabinopyranosyl-(1 → 3)]-[α-L-arabinofuranosyl-(1 → 4)]-β-D-glucuronopyranosyloxy-olean-12-ene-15α,16α,28-triol.

Table II. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectroscopic data of the aglycone moieties of compounds **1** – **3** in CD_3OD (J in Hz)[a].

Position	**1**		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	39.2	1.03, 1.66	39.7	1.00, 1.65	38.9	1.00, 1.68
2	26.1	1.74, 1.91	26.4	1.72, 1.93	25.8	1.73, 1.89
3	91.4	3.20	91.0	3.19	91.2	3.23
4	39.4	–	39.4	–	39.6	–
5	55.7	0.80 d (11.9)	55.8	0.78 d (11.4)	55.7	0.82 (11.7)
6	18.8	1.43, 1.57	19.0	1.45, 1.57	19.5	1.43, 1.56
7	36.1	1.76, n.d.	36.2	1.74, n.d.	35.5	1.75, 1.80
8	41.0	–	41.1	–	41.2	–
9	47.2	1.62	47.1	1.61	47.3	1.60
10	36.6	–	36.7	–	36.6	–
11	23.7	1.96	23.7	1.92	23.6	1.96
12	125.9	5.48 br t (3.7)	126.1	5.45 br t (3.6)	126.3	5.49 br t (3.4)
13	143.0	–	142.5	–	142.3	–
14	47.4	–	47.4	–	47.4	–
15	67.6	3.87	67.4	3.85	67.2	3.76
16	73.7	3.92	73.5	3.90	72.5	3.86
17	47.7	–	n.d.	–	47.8	–
18	40.5	2.63	40.9	2.52 br d (14.1)	40.8	2.55 br d (12.6)
19	46.3	1.20 d (9.1), 2.61	46.6	1.17 dd (13.5, 4.5), 2.61 t (13.5)	46.5	1.18 dd (12.2, 3.4), 2.62 t (12.6)
20	35.8	–	36.4	–	35.9	–
21	79.6	5.84 d (10.1)	80.5	5.77 d (10.1)	78.9	5.74 d (10.2)
22	72.9	5.58 d (10.1)	72.8	5.46 d (10.1)	73.9	5.50 d (10.2)
23	27.5	1.08 s	27.3	1.08 s	27.6	1.10 s
24	15.9	0.88 s	15.9	0.87 s	16.0	0.89 s
25	15.3	0.99 s	15.2	0.98 s	15.3	1.00 s
26	16.4	1.02 s	16.6	1.01 s	16.6	1.03 s
27	20.0	1.42 s	19.9	1.39 s	19.8	1.42 s
28	62.8	3.04 d (11.0) 3.31	63.4	3.04 d (11.1), 3.27 d (11.1)	62.3	3.07 d (11.2), 3.28 d (11.2)
29	28.3	0.87 s	28.6	0.90 s	28.7	0.88 s
30	18.9	1.08 s	18.9	1.04 s	18.9	1.07 s
C-21		Ac		Ac		Mebu
1	171.6	–	172.5	–	177.8	–
2	19.8	1.97 s	20.5	2.06 s	42.2	2.37 m
3	–	–	–	–	26.7	1.49, 1.66
4	–	–	–	–	11.0	0.94 t (7.3)
5	–	–	–	–	16.2	1.14 s
C-22		Ang		AcOMebu		Ac
1	168.2	–	172.6	–	171.9	–
2	128.2	–	81.4	–	20.2	2.02 s
3	138.5	6.06 q (7.4)	30.9	1.75, 1.98	–	–
4	15.6	1.97	6.7	0.94 t (7.9)	–	–
5	20.2	1.91	20.5	1.53 s	–	–
1′	–	–	170.6	–	–	–
2″	–	–	19.6	2.02 s	–	–

[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; Ac, acetic acid; Mebu, 2-methylbutyric acid; Ang, angelic acid; AcOMebu, 2-acetoxy-2-methylbutyric acid.

Pittangretoside P (**3**) revealed in its ESI mass spectrum quasimolecular ion peaks $[M + Na]^+$ at m/z 1257.6064 (positive mode) and $[M - H]^-$ at m/z 1233.5810 (negative mode) indicating a molecular formula of $C_{59}H_{94}O_{27}$, with just two hydrogen atoms more than compound **1**. A presumed structural relation to **1** and also **2** was again substantiated from the similarity of the NMR spectra. In the ^{1}H NMR spectrum

anomeric protons of four sugar units were assigned at δ_H 5.15, 5.01, 4.90, and 4.49 ppm (Table I). Extensive NMR as well as TLC and GC-MS analyses of the sugar components obtained by hydrolysis led to the identification of the same oligosaccharide chain as that found in compounds **1** and **2**. Thiazolidine carboxylates of the corresponding sugars were determined at t_R 36.532 min (L-Ara), t_R 39.934 min (D-Glc), and at t_R 41.097 min (D-GlcA). The acylation pattern was quite similar to that of compound **1**, but a detailed investigation of the NMR data revealed the occurrence of one acetyl and one 2-methylbutyroyl residue instead of an angeloyl moiety as in **1**. Direct evidence for their linkage to the R_1-barrigenol aglycone was derived from HMBC correlations between H-21 (5.74 ppm) and the acyl carbon atom of the 2-methylbutyroyl moiety and H-22 (5.50 ppm) and the acyl carbon atom of the acetyl unit. So, pittangretoside P (**3**) was unambiguously elucidated as 21β-(2-methylbutyroyloxy)-22α-acetoxy-3β-[β-D-glucopyranosyl-(1$\rightarrow$2)]-[α-L-arabinopyranosyl-(1$\rightarrow$3)]-[α-L-arabinofuranosyl-(1$\rightarrow$4)]-β-D-glucuronopyranosyloxyolean-12-ene-15α,16α,28-triol.

From a structural point of view, seven acylated triterpene glycosides, thereof two with an A_1-barrigenol aglycone – **5** and **6** – and five with an R_1-barrigenol (one additional OH group at C-21) aglycone – **1**–**4** and **7** – were successfully isolated and their structures elucidated. While all compounds contain the same oligosaccharide moiety, the composition and/or linkage of attached acyl groups varies. The known compounds **4** and **7** have been found in the fruits of *Pittosporum tobira* (D'Acquarica *et al*., 2002) and compound **6** in the leaves of *Pittosporum angustifolium* (Bäcker *et al*., 2013). Saponin **5** was described once as a constituent of the leaves of *Pittosporum undulatum*, but Higuchi and co-workers (1983) were not able to isolate a pure compound, but rather a mixture of **5** and a similar compound.

Several studies in the past indicated a cytotoxic or antiproliferative potential of mono- or diacylated saponins from *Pittosporum* species (D'Acquarica *et al*., 2002; Seo *et al*., 2002; Bäcker *et al*., 2013; Manase *et al*., 2013). Based on those findings, compounds **1**–**7** were tested against three cancer cell lines: 5637 (urinary bladder carcinoma), MCF7 (breast cancer), LN18 (glioblastoma, grade IV). Additionally, a non-tumourigenic human keratinocyte cell line, HaCaT, was used. All investigated cell lines have been recently used for various cytotoxicity tests (Qiu *et al*. 2009; Hossain *et al*., 2012; Bäcker *et al*., 2013; Mitra *et al*., 2014). The results are shown in Table III. Facing the fact, that the sugar part and also the non-acylated aglycone part of the structures are identical – except **5** and **6** with a missing OH group at C-21 (A_1-barrigenol) – the differences between the IC_{50} values must have been caused by the acyl-linked region of the molecules (C-21, C-22). 5637 and LN18 cells were comparably sensitive to the compounds while the IC_{50} values for the MCF7 and HaCaT cells were higher. Of all compounds, the one possessing two angeloyloxy residues at C-21 and C-22, compound **7**, was the most potent or among the most potent ones, respectively, with IC_{50} values of less than 10 μM for the tested cell lines. A replacement of the angeloyloxy group at C-21 by an acetyloxy residue like in compound **1** caused a decrease of antiproliferative potential against all cell lines. The complete loss of the functional (acylated) OH group at C-21 like in compound **6** also reduced cytotoxic-

Table III. *In vitro* cytotoxicity of the isolated triterpene saponins **1**–**7** aganist four cell lines[a].

Compound	Substituent at		IC_{50} [μM]			
	C-21	C-22	5637	LN18	MCF7	HaCaT
1	OAc	OAng	10.6 ± 0.9	10.4 ± 2.7	28.8 ± 1.7	34.1 ± 2.3
2	OAc	OAcOMebu	6.9 ± 1.0	4.4 ± 0.7	22.9 ± 1.8	19.2 ± 1.4
3	OMebu	OAc	4.5 ± 1.0	4.9 ± 0.8	23.2 ± 0.8	9.0 ± 0.5
4	OAng	OAc	6.2 ± 1.3	13.4 ± 0.8	19.8 ± 0.2	15.9 ± 1.2
5	H	OMebu	n.t.[b]	3.9 ± 0.1	n.t.[b]	n.t.[b]
6	H	OAng	4.1 ± 1.0[c]	6.4 ± 0.5	21.8 ± 0.8	12.9 ± 0.5
7	OAng	OAng	1.7 ± 0.1	4.6 ± 0.5	8.9 ± 0.8	2.2 ± 0.5
Etoposide[d]	–	–	0.6 ± 0.1[c]	0.6 ± 0.1	1.0 ± 0.2	1.5 ± 0.2

[a] Data expressed as mean ± SD (triplicate).
[b] n.t., not tested.
[c] According to Bäcker *et al.* (2013).
[d] Etoposide, positive control.

ity. The additional acetyloxy group at C-21 in compound **1** led to an attenuation of cytotoxicity compared to compound **6**. Furthermore, the 2-acetoxy-2-methylbutyroyloxy attachment at C-22 in compound **2** manifested higher cytotoxic effects than the angeloyloxy residue found in **1**. Comparing **1** and **4** which both possess the same acetyloxy and angeloyloxy group linked to C-21 and C-22 in **1** and vice versa in **4**, the substitution pattern of compound **4** resulted in lower IC_{50} values in 5637, MCF7, and HaCaT cells. A selective activity only against tumourigenic cells could not be observed, since all investigated compounds had a moderate cytotoxic potential against the non-tumourigenic HaCaT cells as well.

Experimental

General

NMR spectra were recorded in CD_3OD on a Bruker DRX 500 device (Billerica, MA, USA). For semipreparative HPLC a Shimadzu system (Kyoto, Japan) with a diode array detector together with an RP18 column (column A) or an ether-linked phenyl phase column (column B) (each 250 mm × 10 mm, 4 μm; Phenomenex, Torrance, CA, USA) were used. For GC-MS analysis an Agilent system (gas chromatograph, G1530N; mass selective detector, MSD G2588A; Santa Clara, CA, USA) with a DB-5MS column (30 m × 0.25 mm × 0.25 μm; J & W Scientific, Folsom, CA, USA) was used. Further conditions are described in Bäcker *et al.* (2013). Detected compounds were identified by mass spectral data compared with those in the NIST database 2.0 d (National Institute of Standards and Technology, Gaithersburg, MD, USA) and data obtained from comparison of retention times of the TIC (total ion chromatograms) of authentic samples of D-glucose (Sigma-Aldrich, St. Louis, MO, USA), D-galactose (Sigma-Aldrich), L-rhamnose (Applichem, Darmstadt, Germany), L-arabinose (Fluka, St. Louis, MO, USA), D-arabinose (Alfa Aesar, Ward Hill, MA, USA), and D-glucuronic acid (Sigma-Aldrich). LC-MS measurements were performed on a Shimadzu system (LC-MS-IT-TOF) using a Chromolith SpeedRod RP18 column (50 mm × 4.6 mm; Merck, Darmstadt, Germany) and electrospray ionization (ESI). Optical rotation was determined by a Perkin Elmer 241 polarimeter (Waltham, MA, USA). ATR-IR spectra were recorded on a Thermo Scientific Nicolet IR 200 FT-IR spectrometer (Waltham, MA, USA). Thin-layer chromatography (TLC) examinations were performed on pre-coated silica gel 60 plates (Merck) with a mixture of EtOAc/*iso*-PrOH/HOAc/H_2O (4:2:2:1) and detection reagent [0.25 g thymol (Sigma-Aldrich), 2.5 mL H_2SO_4, 47.5 mL EtOH] for sugars. Plates were heated for 5 min at 135 °C (sugar fraction of the hydrolyzate). A vacuum manifold and RP18-cartridges (Strata C18E, 200 g/120 mL; Phenomenex) were applied for solid-phase extraction (SPE).

Plant material

Seeds of *Pittosporum angustifolium* were collected in June 2008 on the grounds of the Central Queensland GG foundation (K. A. Amato and the Trustee for Milner Krasser Family Trust) near Mount Morgan, Rockhampton, Queensland, Australia and were a gift of Dr. Cornelia Krasser and Mr. Klaus von Gliszczynski, Yepoon, Australia. Plant material was authenticated by the Curator of the Botanical Garden of Greifswald, Germany, Dr. Peter König. A voucher specimen (No. 20110013PA-S) was deposited at the Institute of Pharmacy, Department of Pharmaceutical Biology at Ernst Moritz Arndt University, Greifswald, Germany.

Extraction and isolation

Pulverized seeds (3 g) were extracted three times with 80% (v/v) EtOH under reflux. Of the residue of 730 mg obtained, 500 mg were applied to a column chromatographic purification step using Sephadex LH-20 gel (Sigma-Aldrich) eluting with methanol. A triterpene glycoside-enriched fraction of 325 mg was recovered. For further partition, the whole fraction was separated by an RP18-SPE procedure using H_2O, 35, 60, 80, and 100% (v/v) MeOH as washing solvents. The 60% (190 mg) and 80% MeOH (55 mg) subfractions were used for subsequent semipreparative isolation by HPLC. Compounds **1**–**6** were obtained from the 60% MeOH fraction [column A; CH_3CN (solvent B) and H_2O (solvent A), each with 0.05% HCOOH; gradient (time:concentration of solvent B), 0:36, 20:36, 27:45, 28:100, 29:36, 35:36; detection, 206 nm; flow rate, 4 mL/min] at t_R 20.67 min (**1**; 19.3 mg), t_R 21.70 min (**2**; 12.4 mg), t_R 22.60 min (**4**; 5.3 mg), t_R 24.57 min (**3**; 11.6 mg), t_R 28.58 min (**5**; 3.0 mg), and t_R 29.65 min (**6**; 5.8 mg). To remove impurities in compound **3**, a further separation using column B (isocratic 39% solvent B) was necessary yielding a pure isolate **3** at t_R 10.39 min (7.9 mg). Com-

pound **7** (4.3 mg) was obtained from subfraction 80% MeOH (SPE) at t_R 14.54 min (column A, isocratic 50% solvent B).

Pittangretoside N (**1**): Colourless amorphous powder. – $[\alpha]_D^{20}$ – 37.3 (*c* 0.37, MeOH). – ATR-IR: ν_{max} = 3374, 2920, 1720, 1606, 1458, 1369, 1250, 1072, 1022, 1004 cm^{-1}. – ^1H and ^{13}C NMR: see Tables I and II. – HRESI-IT-TOFMS: *m/z* (rel. int., positive mode) = 571.3973 (31.4) $[(M+H) - GlcA - Glc - 2Ara - Ac - H_2O]^+$, 553.3893 (100) $[(M+H) - GlcA - Glc - 2Ara - Ac - 2H_2O]^+$, 535.3772 (36.0) $[(M+H) - GlcA - Glc - 2Ara - Ac - 3H_2O]^+$, 453.3361 (43.6) $[(M+H) - GlcA - Glc - 2Ara - Ac - Ang - 3H_2O]^+$, 435.3269 (35.7) $[(M+H) - GlcA - Glc - 2Ara - Ac - Ang - 4H_2O]^+$, 417.3066 (2.9) $[(M+H) - GlcA - Glc - 2Ara - Ac - Ang - 5H_2O]^+$; (negative mode) 1231.5810 (68.0) $[M-H]^-$ (calcd. for $C_{59}H_{91}O_{27}$, 1231.5753 monoisotopic mass).

Pittangretoside O (**2**): Colourless amorphous powder. – $[\alpha]_D^{20}$ –32.3 (*c* 0.22, MeOH). – ATR-IR: ν_{max} = 3404, 2930, 1738, 1597, 1458, 1371, 1258, 1131, 1073, 1052, 1031 cm^{-1}. – ^1H and ^{13}C NMR: see Tables I and II. – HRESI-IT-TOFMS: *m/z* (rel. int., positive mode) = 531.3789 (4.2) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - H_2O]^+$, 513.3581 (100) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - 2H_2O]^+$, 495.3470 (54.9) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - 3H_2O]^+$, 471.3615 (2.3) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - Ac - 2H_2O]^+$, 453.3362 (67) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - Ac - 3H_2O]^+$, 435.3244 (30.0) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - Ac - 4H_2O]^+$, 417.3123 (4.9) $[(M+H) - GlcA - Glc - 2Ara - AcOMebu - Ac - 5H_2O]^+$; (negative mode) 1291.5968 (100) $[M-H]^-$ (calcd. for $C_{61}H_{95}O_{29}$, 1291.5965 monoisotopic mass).

Pittangretoside P (**3**): Colourless amorphous powder. – $[\alpha]_D^{20}$ – 36.0 (*c* 0.17, MeOH). – ATR-IR: ν_{max} = 3358, 2929, 1718, 1603, 1456, 1389, 1262, 1182, 1147, 1072, 1029, 1006 cm^{-1}. – ^1H and ^{13}C NMR: see Tables I and II. – HRESI-IT-TOFMS: *m/z* (rel. int., positive mode) = 1257.6064 $[M+Na]^+$ (3.9), 531.3689 (2.9) $[(M+H) - GlcA - Glc - 2Ara - Mebu - H_2O]^+$, 513.3541 (73.1) $[(M+H) - GlcA - Glc - 2Ara - Mebu - 2H_2O]^+$, 495.3444 (63.9) $[(M+H) - GlcA - Glc - 2Ara - Mebu - 3H_2O]^+$, 477.3342 (16.9) $[(M+H) - GlcA - Glc - 2Ara - Mebu - 4H_2O]^+$, 471.3454 (33.0) $[(M+H) - GlcA - Glc - 2Ara - Mebu - Ac - 2H_2O]^+$, 453.3347 (69.9) $[(M+H) - GlcA - Glc - 2Ara - Mebu - Ac - 3H_2O]^+$, 435.3248 (100) $[(M+H) - GlcA - Glc - 2Ara - Mebu - Ac - 4H_2O]^+$, 417.3159 (77.8) $[(M+H) - GlcA - Glc - 2Ara - Mebu - Ac - 5H_2O]^+$; (negative mode) 1233.5810 (100) $[M-H]^-$ (calcd. for $C_{59}H_{93}O_{27}$, 1233.5910 monoisotopic mass).

Acidic hydrolysis

Amounts of 0.5 – 1.0 mg of each compound were separately added to 2 mL of 2 M trifluoroacetic acid and then heated at 80 °C for 2 h (heating block BT200; Kleinfeld Labortechnik, Gehrden, Germany). After cooling, each mixture was centrifuged at 1600 × *g* for 5 min. The supernatant was removed, dried under reduced pressure, and submitted to TLC and GC-MS analyses, and to the derivatization procedure according to Hara *et al.* (1987) to confirm the absolute configuration of monosaccharides. Assignments were made by GC-MS analysis referring to authentic samples with 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).

Evaluation for cytotoxicity

The human urinary bladder carcinoma cell line 5637 (ACC 35) and the human breast cancer cell line MCF7 (ACC 115) were purchased from the Leibnitz Institute DSMZ, Braunschweig, Germany. The human keratinocyte cell line HaCaT was purchased from CLS, DKFZ, Heidelberg, Germany. Human glioblastoma cells LN18 were obtained from ATCC, LGC, Wesel, Germany. 5637, MCF7, and HaCaT cells were cultivated in RPMI-1640 medium (Bio Whittaker, Verviers, Belgium) supplemented with 10 or 8% (only HaCaT cells) fetal bovine serum (Sigma-Aldrich). MCF7 cells were additionally supplemented with 1% non-essential amino acids (100×) (PAA Laboratories, Cölbe, Germany), 1% sodium pyruvate (100 mM; Sigma-Aldrich) and 0.1% insulin (10 mg/mL; Sigma-Aldrich). LN18 cells were cultivated in DMEM medium (Bio Whittaker) supplemented with 5% fetal bovine serum and 1% non-essential amino acids (100×) (PAA Laboratories). All media contained 1% penicillin/streptomycin (10,000 U/10,000 µg/mL) (Merck). Cells were cultivated at 95% humidity, 5% CO_2, and 37 °C. Using 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red; Merck) and the neutral red uptake (NRU) assay, compounds were tested for

their antiproliferative potential as described recently (Bäcker *et al*., 2013) (cells/well: 5637, $3 \cdot 10^3$; LN18, $6 \cdot 10^3$; HaCaT, $8 \cdot 10^3$; MCF7, $17.5 \cdot 10^3$). IC_{50} values were obtained from dose-response curves and expressed as mean ± SD. Etoposide (Alexis Biochemicals, San Diego, CA, USA) was used as positive control.

Acknowledgement

We sincerely wish to thank Dr. Rudolf Kunze, Berlin, Germany, Dr. Cornelia Krasser and Mr. Klaus von Gliszczynski, Yepoon, Australia, for the collection and provision of the plant material.

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