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9,10-*seco*-9,19-Cyclolanostane arabinosides from the roots of *Actaea podocarpa*

Zulfiqar Ali [a], Shabana I. Khan [a], Frank R. Fronczek [b], Ikhlas A. Khan [a,c,*]

[a] *National Center for Natural Products Research, University of Mississippi, MS 38677, USA*
[b] *Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA*
[c] *Department of Pharmacognosy, School of Pharmacy, University of Mississippi, MS 38677, USA*

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Abstract

Seven 9,10-*seco*-9,19-cyclolanostane arabinosides, named podocarpasides A–G (**1**–**7**), were isolated from the roots of *Actaea podocarpa* DC., a species closely related to black cohosh (a well known dietary supplement). Their structures were determined with the help of spectroscopic data including extensive 2D NMR spectroscopy. The isolates were found inactive, when tested for cytotoxic, estrogenic, and antioxidant activities in cell based assays. They were also tested for anticomplement activity against the classical pathway of complement system and only podocarpaside C (**3**) inhibited modest complement activity with an IC_{50} value of 200 μM.

Keywords: *Actaea podocarpa*; Ranunculaceae; Podocarpasides A—G; Seco-cyclolanostane arabinosides; X-ray crystallography; Anticomplement activity

1. Introduction

Actaea is a small genus in the family Ranunculaceae and consists of about 28 species distributed throughout East Asia, Europe, and North America. Predominantly, *Actaea podocarpa* is found in woods of the high Appalachians in the east. It is also known as *Actaea americana*, *Actaea cordifolia*, *Cimicifuga americana*, *Cimicifuga cordifolia*, mountain bugbane, or summer cohosh (Compton et al., 1998; Rickett, 1967). Black cohosh (*Actaea racemosa*) is one of the important *Actaea* species and has become a well known herbal medicine, with health benefits in treating painful menstrual periods and menopausal disorders not only in the United States but also in European countries. It has also been used for the treatment of diarrhea and sore throat (Watanabe et al., 2002). A number of cyclolanostane type triterpenes and their glycosides (Shao et al., 2000; Hamburger et al., 2001; Wende et al., 2001; Kusano et al., 2001; He et al., 2000) and phenylpropenoids and their glycosides (Kruse et al., 1999; Chen et al., 2002a) have been reported from black cohosh. It is usually collected from the wild in North America where its range overlaps with the closely related summer cohosh (*A. podocarpa*) (Zerega et al., 2002). To address the issues of adulteration/misidentification and bio-equivalence of *A. podocarpa* in comparison with black cohosh, we investigated the chemical composition of this plant. This paper deals with the isolation and structure elucidation of seven novel 9,10-*seco*-9,19-cyclolanostane arabinosides named as podocarpasides A–G (**1**–**7**) from the roots of *A. podocarpa* DC. A combination of normal as well as reverse phase (RP-18) silica gel column chromatography was used for their purification. The structures of all podocarpasides were elucidated with the help of spectroscopic methods including extensive 2D NMR techniques.

* Corresponding author. Tel.: +1 662 9157821; fax: 1 662 915 7989.
E-mail address: ikhan@olemiss.edu (I.A. Khan).

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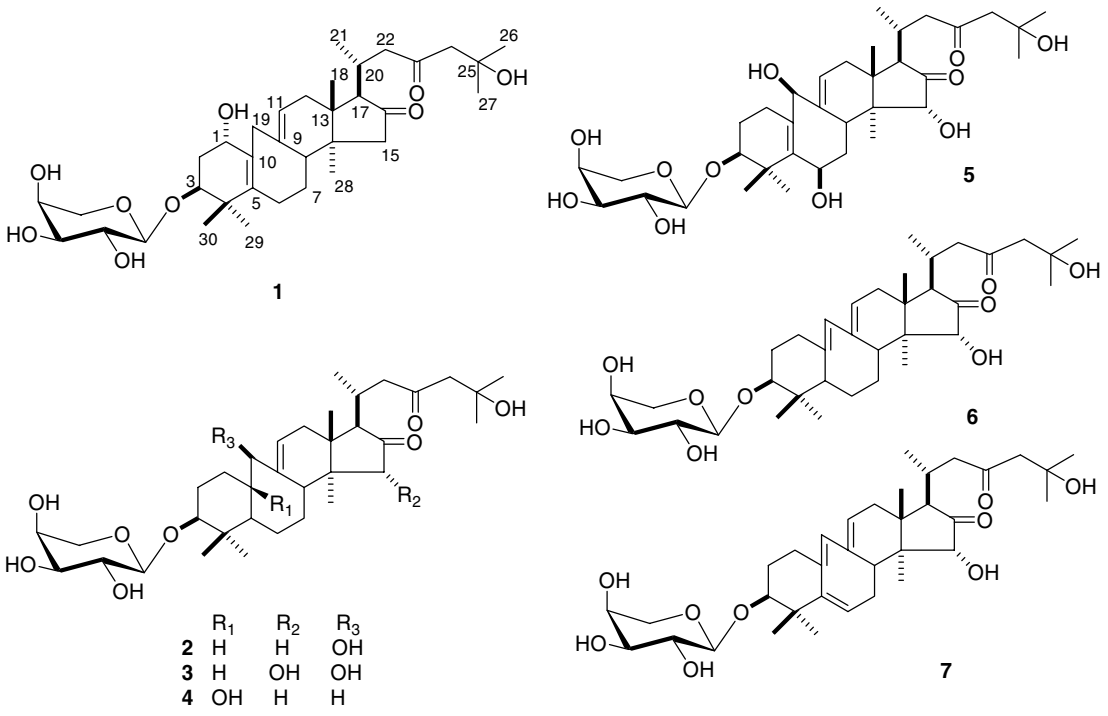

Podocarpasides A–G (**1–7**)

2. Results and discussion

Podocarpasides A (**1**, 63.2 mg), B (**2**, 201.9 mg), C (**3**, 25.2 mg), D (**4**, 119.0 mg), E (**5**, 91.4 mg), F (**6**, 20.6 mg), and G (**7**, 22.1 mg) were obtained from the methanolic extract of the roots of *A. podocarpa* by column chromatography over normal and reverse phase silica (RP-18). Their structural assignments were made by analysis of spectroscopic data including extensive 2D NMR techniques.

Podocarpaside A (**1**) was obtained as white powder, which showed a pseudomolecular ion at *m*/*z* 641 $[M+Na]^+$ in the positive ESIMS. The HRESIMS afforded an ion at *m*/*z* 641.3669 $[M+Na]^+$ (calc. for $C_{35}H_{54}NaO_9$, 641.3666). The ^{13}C NMR spectrum showed 35 signals, which together with the mass data helped in deducing the molecular formula as $C_{35}H_{54}O_9$. The DEPT experiment resolved the 35 carbon signals as 7 methyls, 9 methylenes, 10 methines and 9 quaternary carbons. The signals in the ^1H NMR spectrum (see Table 1) for six *tert*-methyls at δ_H 0.82 (H-18), 1.49 (H-26), 1.50 (H-27), 0.87 (H-28), 1.37 (H-29), and 1.17 (H-30) and a *sec*-methyl at δ_H 1.03 (H-21) indicated a 9,19-cyclolanostane type triterpene skeleton (Shao et al., 2000; Hamburger et al., 2001; Wende et al., 2001; Kusano et al., 2001; He et al., 2000). However, the signals for cyclopropane methylene protons (2H-19) and two quaternary carbons (C-9 and C-10) were not observed at characteristic high magnetic field indicating a 9,10-*seco*-9,19-cyclolanostane derivative (Li et al., 1993; Kadota et al., 1995). The ^1H NMR spectrum also showed the signals for an olefinic methine [δ_H 5.48 (*br* s, H-11)], an anomeric methine [δ_H 4.79 (*d*, $J = 7.2$ Hz, H-1′)] and three geminally coupled methylenes [δ_H 2.14/2.06 (each *d*, $J = 18.0$ Hz, 2H-15)], [δ_H 3.27/3.09 (each *d*, $J = 13.6$ Hz, 2H-19)], [δ_H 2.85/2.80 (each *d*, $J = 14.8$ Hz, 2H-24)]. No vicinal ^1H–^1H COSY correlations were observed for these three methylenes (C-15, C-19 and C-24) which indicated the quaternary centers adjacent to them. Two most downfield chemical shift values in the ^{13}C NMR spectrum of **1** at δ_C 219.0 (C-16) and 211.1 (C-23) as well as the absorption bands at 1715, 1642 cm^{-1} in its IR spectrum, indicated the presence of two ketone groups in the molecule. The ^{13}C NMR spectrum of **1** (see Table 2) also showed four olefinc carbons at δ_C 140.3 (C-5), 138.2 (C-9), 131.8 (C-10) and 121.0 (C-11), two oxygen bearing signals at δ_C 68.9 (C-1) and 69.8 (C-25), in addition with five oxygenated carbons assignable to α-L-arabinopyranose (Watanabe et al., 2002; Chen et al., 2002b) at δ_C 107.8 (C-1′), 73.2 (C-2′), 74.9 (C-3′), 69.9 (C-4′) and 67.2 (C-5′). The following HMBC correlations confirmed the positions of sugar at C-3 [$\delta_{H/C}$ 4.29 (H-3)/107.8 (C-1′), 24.7 (C-29), 20.2 (C-30); $\delta_{H/C}$ 4.79 (H-1′)/82.9 (C-3)], a hydroxyl atC-1 [$\delta_{H/C}$ 4.42 (H-1)/82.9 (C-3), 140.3 (C-5), 131.8 (C-10); $\delta_{H/C}$ 3.09, 3.27 (2H-19)/68.9 (C-1)], another hydroxyl at C-25 [$\delta_{H/C}$ 2.80, 2.85 (2H-24), 1.49 (3H-26), 1.50 (3H-27)/69.8 (C-25)], ketones at C-16 and C-23 [$\delta_{H/C}$ 2.06, 2.14 (2H-15), 2.36 (H-17)/219.0 (C-16) and $\delta_{H/C}$ 2.70, 3.54 (2H-22), 2.80, 2.85 (2H-24)/211.1 (C-23)]. Furthermore, the positions of two double bonds (C_5=C_{10} and C_9=C_{11}) were also

Table 1
^{1}H NMR data for compounds **1**–**7**

Position	1	2	3	4	5	6	7
1	4.42	2.04 1.69	2.08 1.72	1.78 *br d* (14.4) 1.61 *dt* (14.0, 3.6)	2.35 2.14	2.36 2.14	2.28 2.28
2	2.76, 2.26	2.29, 1.84	2.35, 1.87	2.43, 2.21	2.24, 2.12	2.33, 1.87 *ddd* (17.6, 12.0, 4.8)	2.38, 2.03 *ddd* (17.6, 11.6, 5.2)
3	4.29 *dd* (12.0, 3.2)	3.43 *dd* (11.2, 4.0)	3.44 *dd* (12.0, 4.0)	3.47 *dd* (11.6, 3.6)	3.75 *dd* (11.6, 5.6)	3.52 *dd* (12.0, 4.8)	3.59 *dd* (11.6, 4.4)
5		2.05	2.07	1.09 *dd* (12.0, 3.6)		2.02	
6	2.46 *br d* (16.4) 2.26	2.19 1.22	2.21 1.27	1.99 1.84	5.06 *d* (6.8)	2.54 1.36	6.05 *dd* (8.8, 6.0)
7	1.58 1.45	2.3 1.31	2.58 2.38	1.42 1.3	2.60 *dd* (12.8, 6.8) 2.56 *br d* (12.0)	2.02 1.29	3.13 br *dd* (13.2, 9.6) 2.17
8	2.3	2.29	2.61	2.69	2.76	2.61	2.72
10		1.53	1.58				
11	5.48 *br s*	5.62 *br s*	5.68 *br s*	5.28 *br s*	5.68 *br s*	5.86 *br s*	5.61 *br s*
12	2.18 1.97 *dd* (16.8, 4.4)	2.27 2.06	2.22 2.11	2.17 1.97	2.65 2.01 *dd* (17.6, 4.8)	2.25 2.21	2.3 2.12
15	2.14 *d* (18.0) 2.06 *d* (18.0)	2.28 *d* (18.0) 2.14 *d* (18.0)	4.48 *s*	2.25 *d* (17.6) 2.12 *d* (18.0)	4.37 *s*	4.48 *s*	4.48 *s*
17	2.36 *d* (8.4)	2.35 *d* (9.2)	2.18 *d* (8.4)	2.34 *d* (8.0)	2.21 *d* (8.8)	2.18 *d* (8.8)	2.18 *d* (8.8)
18	0.82 *s*	0.93 *s*	1.09*s*	0.90 *s*	0.99 *s*	1.04 *s*	1.04 *s*
19	3.27 *d* (13.6) 3.09 *d* (13.6)	4.25 *dd* (14.0, 3.6)	4.29 *br d* (12.4)	2.61 *brd* (17.2) 2.33 *d* (16.4)	4.87 *s*	6.04 *s*	6.19 *s*
20	2.58	2.59	2.65	2.54	2.51	2.65	2.62
21	1.03 *d* (6.4)	1.04 *d* (6.4)	1.08 *d* (6.4)	1.01 *d* (6.8)	1.04 *d* (6.0)	1.05 *d* (6.8)	1.03 *d* (7.6)
22	3.54 *dd* (17.6, 2.8) 2.70 *dd* (17.6, 8.8)	3.58 *d* (17.6, 2.4) 2.70 *d* (17.2, 8.8)	3.55 *dd* (17.6, 3.6) 2.76 *dd* (17.6, 8.4)	3.56 *dd* (17.6, 2.8) 2.68 *dd* (17.6, 8.8)	3.49 *dd* (17.6, 7.6) 2.71 *dd* (18.0, 3.6)	3.56 *dd* (18.0, 3.6) 2.74 *dd* (17.6, 8.4)	3.54 *dd* (18.0, 3.6) 2.74 *dd* (18.0, 8.0)
24	2.85 *d* (14.8) 2.80 *d* (14.8)	2.83 *d* (14.8) 2.78 *d* (14.8)	2.82 *d* (14.4) 2.76 *d* (14.4)	2.80 *d* (14.4) 2.77 *d* (14.4)	2.78 *br s* 2.78 *br s*	2.81 *d* (14.8) 2.75 *d* (14.8)	2.80 *d* (14.4) 2.74 *d* (14.4)
26	1.49 *s*	1.47 *s*	1.45 *s*	1.46 *s*	1.48 *s*	1.45 *s*	1.45 *s*
27	1.50 *s*	1.49 *s*	1.46 *s*	1.47 *s*	1.48 *s*	1.46 *s*	1.45 *s*
28	0.87 *s*	0.93 *s*	1.06 *s*	0.86 *s*	1.10 *s*	0.96 *s*	1.00 *s*
29	1.37 *s*	1.38 *s*	1.35 *s*	1.39 *s*	1.32 *s*	1.26 *s*	1.44 *s*
30	1.17 *s*	0.91 *s*	0.93 *s*	1.37 *s*	1.24 *s*	0.87 *s*	1.08 *s*
1′	4.79 *d* (7.2)	4.69 *d* (7.2)	4.70 *d* (6.4)	4.82 *d* (7.2)	4.70 *d* (6.8)	4.73 *d* (7.6)	4.72 *d* (7.2)
2′	4.42	4.40 *dd* (8.8, 6.8)	4.40 *dd* (8.4, 6.8)	4.44 *dd* (8.8,6.8)	4.28 *dd* (8.6, 6.8)	4.40 *dd* (9.2, 7.2)	4.42 *dd* (8.4, 7.6)
3′	4.09 *dd* (9.2, 3.6)	4.11 *dd* (9.2, 3.2)	4.11 *dd* (8.4, 3.2)	4.17 *dd* (8.8, 3.2)	4.07 *dd* (9.2, 3.6)	4.13 *dd* (8.8, 3.2)	4.12 *dd* (8.8, 2.8)
4′	4.25 *br s*	4.27 *br s*	4.27 *br s*	4.32 *br s*	4.27 *br s*	4.29 *br s*	4.29 *br s*
5′	4.23 *br d* (11.6) 3.68 *br d* (11.2)	4.25 *dd* (14.0, 3.6) 3.75 *br d* (10.8)	4.26 *br d* (11.2) 3.75 *br d* (11.2)	4.31 *dd* (10.4, 2.8) 3.81 *br d* (10.4)	4.29 *br d* (12.0) 3.78 *br d* (10.8)	4.28 *dd* (10.4, 2.4) 3.78 *dd* (12.8, 2.0)	4.27 *dd* (11.0, 2.4) 3.76 *br d* (10.8)

All spectra were recorded in pyridine-d_5 at 400 MHz. Chemical shifts are given in ppm. *J* values in parentheses are in Hz.

supported by the HMBC correlations [$\delta_{H/C}$ 4.42 (H-1)/140.3 (C-5), 131.8 (C-10); $\delta_{H/C}$ 3.09, 3.27 (2H-19)/140.3 (C-5), 131.8 (C-10); $\delta_{H/C}$ 1.37 (3H-29), 1.17 (3H-30)/140.3 (C-5) and $\delta_{H/C}$ 3.09, 3.27 (2H-19), 1.97, 2.18 (2H-12)/138.2 (C-9), 121.0 (C-11)]. The assignment of NMR data was done by analyzing the HMQC, HMBC (see Fig. 1) and ^{1}H–^{1}H COSY spectra. The relative stereochemistry was assigned on the basis of coupling constants and ROESY spectrum (see Fig. 2). Axial orientation (α) of H-3 was well supported by its coupling constants (12.0, 3.2 Hz). The coupling constants of H-1 were not clear due to its overlapping with one of the sugar proton (H-2′). The overlap was still there when the ^{1}H NMR spectrum was recorded in $CDCl_3$. A HOMO 2D-J resolved spectrum helped to find the coupling constants for H-2′ (9.0, 7.0 Hz) as similar in podocarpasides B–G (**2**–**7**) and for H-1 (7.7 Hz). In ROESY spectrum, H-1 did not show correlation with axially (α) orientated H-3 but did correlate with both methylene protons at C-19. The characteristic coupling constant (7.7 Hz) of H-1, its correlations with both methylene protons (2H-19) and no correlation with axially (α) oriented H-3 revealed that H-1 is at equatorial (β) posi-

Table 2
^{13}C NMR data for compounds **1**–**7**

Position	1	2	3	4	5	6	7
1	68.9 *d*	32.9 *t*	32.9 *t*	42.1 *t*	25.3 *t*	39.1 *t*	36.0 *t*
2	37.4 *t*	30.5 *t*	30.5 *t*	27.1 *t*	26.3 *t*	32.3 *t*	30.5 *t*
3	82.9 *d*	88.0 *d*	88.0 *d*	89.0 *d*	84.7 *d*	87.7 *d*	85.5 *d*
4	41.4 *s*	40.3 *s*	40.3 *s*	40.9 *s*	38.4 *s*	42.5 *s*	42.9 *s*
5	140.3 *s*	44.8 *d*	45.0 *d*	56.8 *d*	139.3 *s*	51.2 *d*	146.3 *s*
6	27.0 *t*	32.9 *t*	33.0 *t*	28.3 *t*	76.7 *d*	25.4 *t*	126.1 *d*
7	25.1 *t*	26.2 *t*	24.9 *t*	30.3 *t*	32.0 *t*	30.3 *t*	27.4 *t*
8	44.6 *d*	47.8 *d*	48.6 *d*	48.1 *d*	39.2 *d*	49.9 *d*	48.9 *d*
9	138.2 *s*	144.8 *s*	145.7 *s*	140.0 *s*	139.8 *s*	140.0 *s*	141.2 *s*
10	131.8 *s*	41.6 *d*	41.6 *d*	72.6 *s*	128.0 *s*	136.6 *s*	133.7 *s*
11	121.0 *d*	125.2 *d*	125.1 *d*	121.8 *d*	126.2 *d*	129.1 *d*	127.6 *d*
12	36.6 *t*	37.1 *t*	38.4 *t*	37.3 *t*	37.7 *t*	40.0 *t*	37.6 *t*
13	44.9 *s*	44.3 *s*	40.5 *s*	44.5 *s*	41.2 *s*	40.3 *s*	41.0 *s*
14	41.6 *s*	42.7 *s*	45.8 *s*	42.6 *s*	44.2 *s*	45.6 *s*	44.7 *s*
15	49.0 *t*	48.9 *t*	81.8 *d*	49.1 *t*	81.4 *d*	81.8 *d*	81.9 *d*
16	219.0 *s*	219.1 *s*	220.5 *s*	219.2 *s*	219.8 *s*	220.4 *s*	220.0 *s*
17	59.7 *d*	59.8 *d*	57.4 *d*	59.9 *d*	57.5 *d*	57.4 *d*	58.3 *d*
18	16.1 *q*	16.8 *q*	17.5 *q*	17.0 *q*	16.9 *q*	17.5 *q*	17.4 *q*
19	40.3 *t*	82.7 *d*	82.9 *d*	53.9 *t*	85.5 *d*	130.3 *d*	132.2 *d*
20	27.6 *d*	27.7 *d*	27.8 *d*	27.9 *d*	27.8 *d*	27.8 *d*	27.8 *d*
21	20.8 *q*	20.6 *q*	21.5 *q*	20.7 *q*	21.6 *q*	21.4 *q*	21.4 *q*
22	51.4 *t*	51.5 *t*	50.9 *t*	51.5 *t*	50.8 *t*	50.9 *t*	50.8 *t*
23	211.1 *s*	211.0 *s*	211.0 *s*	211.2 *s*	210.8 *s*	211.0 *s*	211.0 *s*
24	56.1 *t*	56.2 *t*	56.1 *t*	56.2 *t*	56.1 *t*	56.1 *t*	56.2 *t*
25	69.8 *s*	69.9 *s*	69.7 *s*	69.8 *s*	69.8 *s*	69.8 *s*	69.8 *s*
26	30.4 *q*	30.4 *q*	30.4 *q*	30.4 *q*	30.5 *q*	30.4 *q*	30.4 *q*
27	30.7 *q*	30.8 *q*	30.8 *q*	30.8 *q*	30.7 *q*	30.8 *q*	30.8 *q*
28	18.5 *q*	17.7 *q*	10.4 *q*	17.7 *q*	11.3 *q*	10.6 *q*	10.0 *q*
29	24.7 *q*	26.6 *q*	26.6 *q*	27.7 *q*	25.9 *q*	24.9 *q*	24.3 *q*
30	20.2 *q*	15.3 *q*	15.2 *q*	16.6 *q*	22.1 *q*	15.3 *q*	24.0 *q*
1′	107.8 *d*	107.7 *d*	107.6 *d*	107.8 *d*	107.5 *d*	107.7 *d*	107.7 *d*
2′	73.2 *d*	73.3 *d*	73.3 *d*	73.3 *d*	73.3 *d*	73.3 *d*	73.3 *d*
3′	74.9 *d*	74.9 *d*	74.9 *d*	75.0 *d*	75.3 *d*	75.0 *d*	75.0 *d*
4′	69.9 *d*	69.9 *d*	69.8 *d*	69.9 *d*	70.0 *d*	70.0 *d*	70.0 *d*
5′	67.2 *t*	67.1 *t*	67.1 *t*	67.1 *t*	67.4 *t*	67.3 *t*	67.4 *t*

All spectra were recorded in pyridine-d_5 at 100 MHz. Chemical shifts are given in ppm.

tion, which ultimately assigned the axial (α) orientation of hydroxyl at C-1. The sugar obtained after acid hydrolysis was identified as L-arabinose by comparing its TLC and specific rotation with the standard. On the basis of the evidence above, podocarpaside A (**1**) was elucidated as 1α, 3β,25-trihydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-5(10),9(11)-dien-3-*O*-α-L-arabinopyranoside.

Podocarpaside B (**2**), a white powder, showed a pseudomolecular ion at *m/z* 643 $[M+Na]^+$ in the positive ESIMS. Its ^{13}C NMR and HRESIMS *m/z*: 643.3827 $[M+Na]^+$ helped in determining the molecular formula ($C_{35}H_{56}O_9$). The NMR data (see Tables 1 and 2) of **2** resembled those of podocarpaside A (**1**) except for changes at C-1 (δ_C 32.9), C-5 (δ_C 44.8), C-10 (δ_C 41.6) and C-19 (δ_C 82.7) due to the position of hydroxyl at C-19 instead of at C-1 along with the absence of C_5-C_{10} olefinic bond. The position of hydroxyl at C-19 was confirmed by the correlations in the HMBC spectrum between H-19 (δ_H 4.25) and C-1 (δ_C 32.9), C-5 (δ_C 44.8), C-8 (δ_C 47.8) and C-11 (δ_C 125.2) (see Fig. 1). A correlation in the ROESY spectrum between H-11/H-19 (see Fig. 2) and a coupling constant of 14.0 Hz for H-19 and H-10 were observed, which is only possible if H-10 and H-19 have β and α orientations, respectively. Thus podocarpaside B (**2**) was characterized as 3β,19β,25-trihydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-9(11)-en-3-*O*-α-L-arabinopyranoside.

Podocarpaside C (**3**), white powder, showed a pseudomolecular ion in the positive ESIMS at *m/z* 659 $[M+Na]^+$. Its molecular formula ($C_{35}H_{56}O_{10}$) was deduced from the analysis of ^{13}C NMR and HRESIMS *m/z*: 659.3766 $[M+Na]^+$ data. The DEPT spectrum showed seven methyls, eight methylenes, thirteen methines and seven quaternary carbons. The DEPT spectrum of **3** was similar to that of **2** except for the appearance of an oxygenated methine at δ_C 81.8 instead of a methylene at δ_C 48.9 for C-15. This methine appeared as a singlet in the ^{1}H NMR spectrum at δ_H 4.48 and showed correlations in the HMBC spectrum (see Fig. 1) with the carbons at δ_C 48.6 (C-8), 40.5 (C-13), 45.8 (C-14), 220.5 (C-16) and 10.4 (C-28). These HMBC correlations as well as a characteristic upfield shift (~7 ppm) of the C-28 methyl revealed a hydroxyl at C-15 which was the only a difference found between podocarpaside C (**3**) and B (**2**). The correlation in the ROESY spectrum (see Fig. 2) between H-15 (δ_H

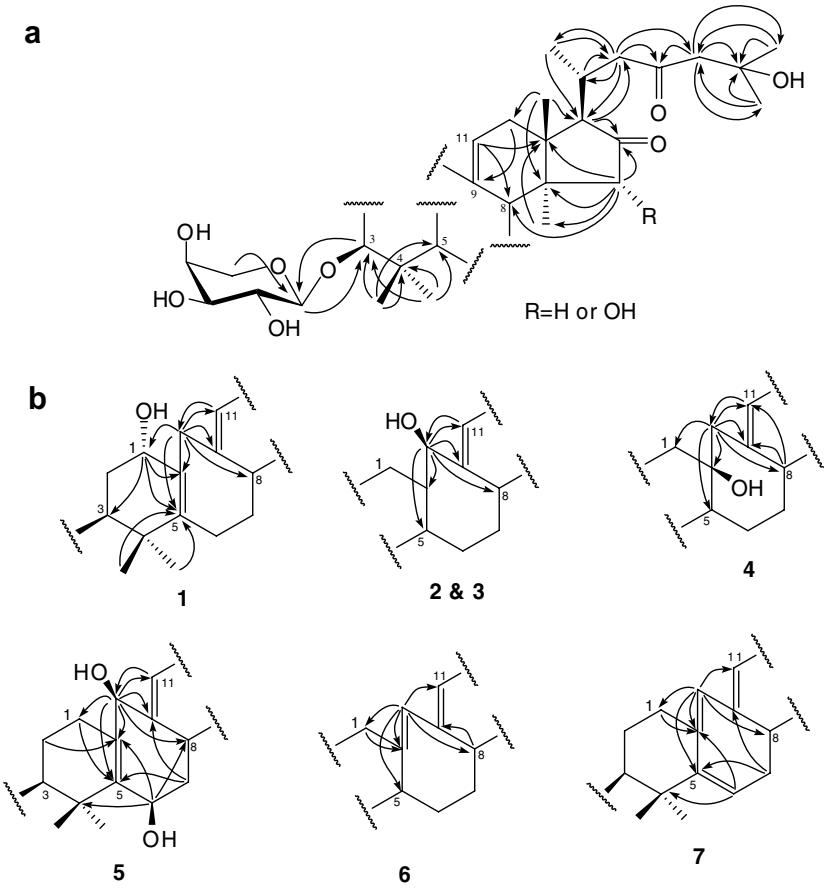

Fig. 1. (a) Common and (b) individual HMBC correlations for **1**–**7**.

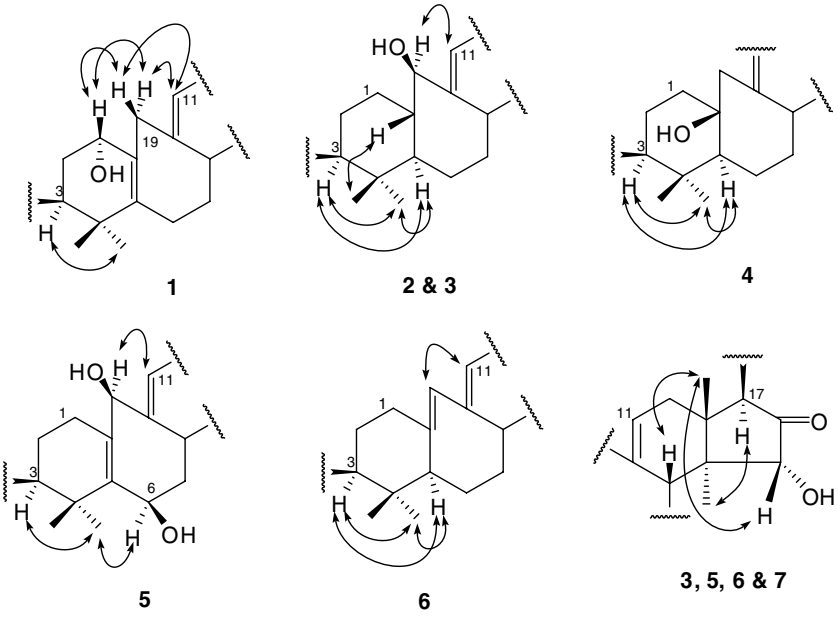

Fig. 2. Selected ROESY or NOESY correlations of podocarpasides A–G (**1**–**7**).

4.48) and the biogenetically β oriented methyl Me-18 (δ_H 1.09) helped in assigning the α position of hydroxyl at C-15. Finally, podocarpaside C (**3**) was characterized as 3β,15α,19β,25-tetrahydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-9(11)-en-3-*O*-α-L-arabinopyranoside.

Podocarpaside D (**4**) was isolated as powder and formed crystals in methanol. The pseudomolecular ion was observed in the positive ESIMS at *m/z* 643 $[M+Na]^+$ and in the HRESIMS at *m/z* 643.3821, which in association with the ^{13}C NMR helped in determining the molecular formula ($C_{35}H_{56}O_9$), found identical to podocarpaside B (**2**). The DEPT spectrum of **4** differed from **2** in that two methines were transformed into a methylene (δ_C 53.9) and an oxygenated quaternary carbon (δ_C 72.6) in **4**. The comparative ^1H NMR study with **2** showed resemblance except for the appearance of a pair of geminal coupled protons at δ_H 2.33 (*d*, $J = 16.4$ Hz, H-19a), and δ_H 2.61 (*br d*, $J = 17.2$ Hz, H-19b) instead of two methines as in **2** at δ_H 1.53 (H-10) and δ_H 4.25 (*dd*, $J = 14.0$, 3.6 Hz, H-19). A methylene at C-19 position was confirmed by the correlations in the HMBC spectrum (see Fig. 1) between its protons and C-1 (δ_C 42.1), C-5 (δ_C 56.8), C-8 (δ_C 48.1), C-9 (δ_C 140.0), C-10 (δ_C 72.6) and C-11 (δ_C 121.8). The hydroxyl at C-10 was well supported by the HMBC correlation (see Fig. 1) between H-19/C-10, as well as by the downfield shift of adjacent carbons (C-1, C-5) about 10-11 ppm as compared to podocarpaside B (**2**). The identity of compound **4** was confirmed by X-ray crystallography which showed the β orientation of hydroxyl at C-10 and confirmed that podocarpaside D (**4**) was 3β,10β,25-trihydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-9(11)-en-3-*O*-α-L-arabinopyranoside.

Podocarpaside E (**5**) was purified as white powder. The positive ESIMS showed a pseudomolecular ion at *m/z* 673 $[M+Na]^+$, leading to the molecular formula $C_{35}H_{54}O_{11}$. Its NMR data resembled those of **1** and **4** (Tables 1 and 2). A number of signals in the ^{13}C NMR spectrum at δ_C 84.7 (C-3), 76.7 (C-6), 81.4 (C-15), 85.5 (C-19), 219.8 (C-16), 210.8 (C-23), and 69.8 (C-25), in addition to the signals for arabinose revealed high oxygenation in the molecule. Identical to **1**, the signals for two olefinic bonds were observed at δ_C 139.3 (C-5), 139.8 (C-9), 128.0 (C-10), 126.2 (C-11). Besides the signals for arabinose, four other oxygenated methines were observed in the ^1H NMR spectrum at δ_H 3.75 (*dd*, $J = 11.6$, 5.6 Hz, H-3), 5.06 (*d*, $J = 6.8$ Hz, H-6), 4.37 (*s*, H-15), and 4.87 (*s*, H-19). The appearance as singlets and also the absence of any correlation in the ^1H–^1H COSY spectrum for the signals at δ_H 4.37 (H-15) and 4.87 (H-19) indicated the quaternary centers adjacent to them. The correlations in the HMBC spectrum (see Fig. 1) between $\delta_{H/C}$ 5.06 (H-6)/38.4 (C-4), 139.3 (C-5), 39.2 (C-8), 128.0 (C-10); $\delta_{H/C}$ 4.37 (H-15)/39.2 (C-8), 44.2 (C-14), 219.8 (C-16), 11.3 (C-28); $\delta_{H/C}$ 4.87 (H-19)/25.3 (C-1), 139.3 (C-5), 39.2 (C-8), 139.8 (C-9), 128.0 (C-10), 126.2 (C-11) revealed the hydroxyls at C-6, C-15 and C-19, respectively. The interactions in the ROESY spectrum (see Fig. 2) between H-5 (δ_H 5.06)/Me-29 (biogenetically α oriented, δ_H1.32); H-15 (δ_H 4.37)/Me-18 (biogenetically β oriented, δ_H 0.99); H-19 (δ_H 4.87)/H-11 (δ_H 5.68) assigned the relative stereochemistry of hydroxyls as β, α, and β at C-6, C-15 and C-19, respectively. Therefore, podocarpaside E (**5**) was characterized as 3β,5β,15α,19β,25-pentahydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-5(10),9(11)-dien-3-*O*-α-L-arabinopyranoside.

Podocarpaside F (**6**) exhibited a pseudomolecular ion in the positive ESIMS at *m/z* 641 $[M+Na]^+$, and was isolated as a white powder. Its ^{13}C NMR and HRESIMS *m/z*: 639.3503 (calc. 639.3509) helped in finding the molecular formula ($C_{35}H_{54}O_9$). The ^1H and ^{13}C NMR spectra showed close resemblance with those of podocarpaside C (**3**) except for the appearance of signals for an olefinic bond at δ_H 6.04 (H-19), δ_C 136.6 (C-10),δ_C 130.3 (C-19) instead of the signals observed for two methines (C-10 and C-19) in **3**. This indicated the existence of a C_{10}–C_{19} double bond in **6** as a result of dehydrogenation of **3**. The correlations observed in the HMBC spectrum (see Fig. 1) between the olefinic methine H-19 (δ_H 6.04) and C-1 (δ_C 39.1), C-5 (δ_C 51.2), C-9 (δ_C 49.9), and C-11 (δ_C 129.1) confirmed the position of olefinic bond at C-10. The relative stereochemistry of the C-15 hydroxyl was found to be identical to that in **3** (see Fig. 2). Ultimately podocarpaside F (**6**) was elucidated as 3β,15α,25-trihydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-9(11),10(19)-dien-3-*O*-α-L-arabinopyranoside.

Podocarpaside G (**7**), white powder, gave the pseudomolecular ion at *m/z* 639 $[M+Na]^+$, corresponding to the molecular formula $C_{35}H_{52}O_9$. The ^1H and ^{13}C NMR spectra of **7** resembled those of **6** except for the appearance of signals for another olefinic bond at δ_H 6.05 (*dd*, $J = 8.8$, 6.0 Hz, H-6), δ_C 146.3 (C-5) and δ_C 126.1 (C-6) instead of signals observed for a methine (H-5) and a methylene (C-6) in **6**. The correlations in the HMBC spectrum (see Fig. 1) between H-6 (δ_H 6.05)/C-4 (δ_C 42.9), C-8 (δ_C 48.9), C-10 (δ_C 133.7); H-19 (δ_C 6.19)/ C-5 (δ_C 146.3) confirmed the position of this double bond between C-5 and C-6. Similar to podocarpasides C (**3**), E (**5**), and F (**6**), the relative stereochemistry of hydroxyl at C-15 was found to be α (see Fig. 2) and the structure of podocarpaside G (**7**) was characterized as 3β,15α,25-trihydroxy-16,23-dione-9,10-*seco*-9,19-cyclolanost-5(6),9(11),10(19)-trien-3-*O*-α-L-arabinopyranoside.

The important common facts in all the podocarpasides are six *tert*-methyls (C-18, C-26 - C-30), a *sec*-methyl (C-21), a hydroxyl at C-25, β oriented α-L-arabinose at C-3, ketones at C-16 and C-23, and a C_9-C_{11} olefinic bond. The assignment of NMR data (Tables 1 and 2) for all compounds was done with the help of extensive 2D-NMR experiments like ^1H–^1H COSY, HMQC, and HMBC (see Fig. 1). The relative stereochemistry was determined with the help of coupling constant values, molecular models, X-ray crystallography, NOESY or ROESY experiments (see Fig. 2). A possible biogenetic pathway is proposed for the formation of the seven member ring as a result of opening of cycloartane ring (see Fig. 3). The monosaccharide moiety in all podocarpsides was obtained by acid

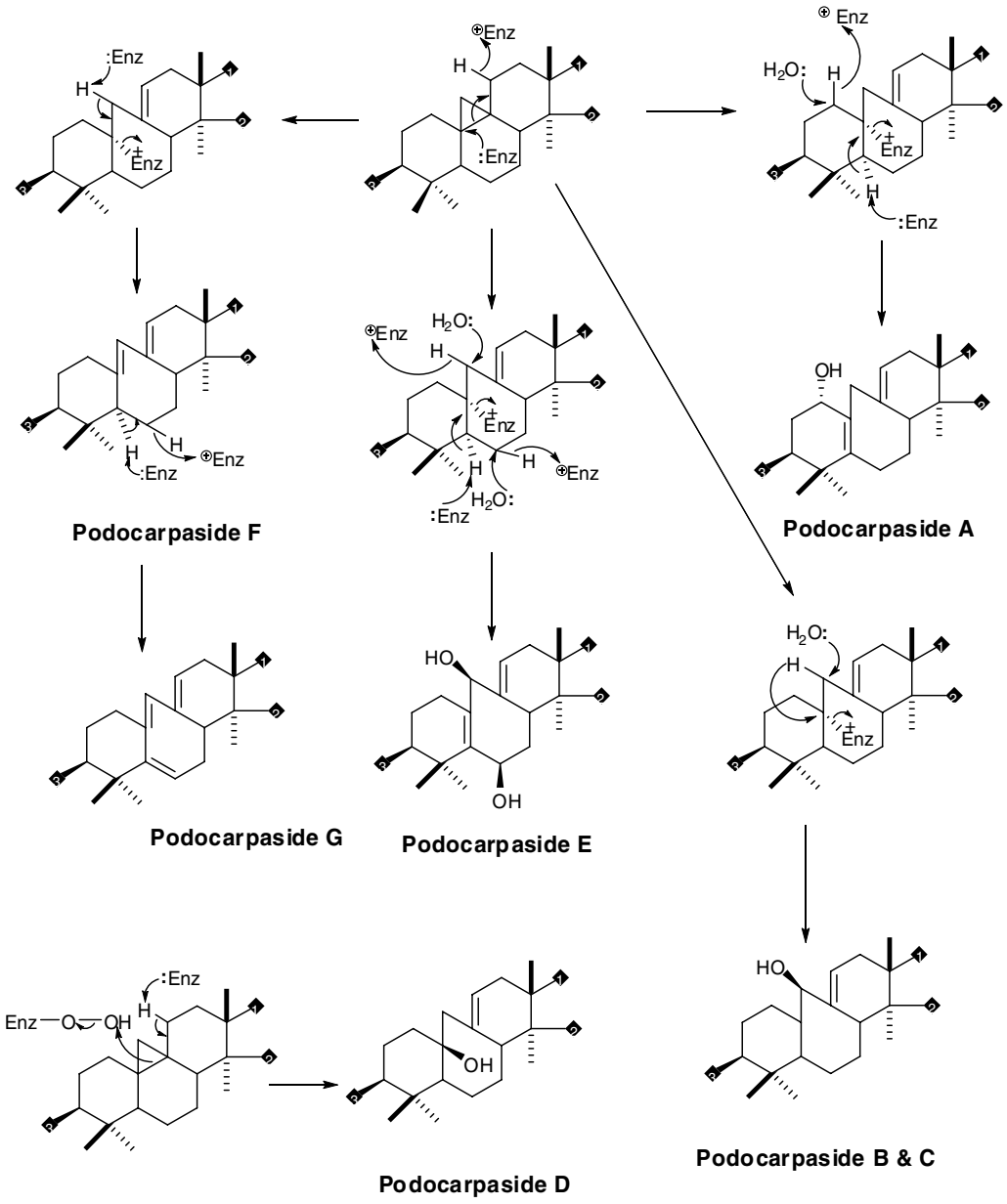

Fig. 3. Proposed biogeneic pathways for the construction of podocarpasides A–G from cycloartane.

hydrolysis and identified as L-arabinose by comparing its TLC and specific rotation with the standard one.

Podocarpasides A–G were tested for their in vitro cytotoxicity towards mammalian kidney fibroblasts (Vero) and kidney epithelial (LLC-PK_1) cells as well as for their in vitro anti-cancer potential in a panel of human solid tumor cells (SK-MEL malignant melanoma; KB epidermal carcinoma, oral; BT-549 ductal carcinoma, breast and SK-OV-3 ovary carcinoma) as described previously (Mustafa et al., 2004). The cells were exposed to test compounds for 48 h. Cell viability was determined by Neutral Red dye. However, they did not show any cytotoxic activity up to a highest concentration of 25 μg/mL in any of the cell lines tested. None of the compounds showed any estrogenic effect up to a highest concentration of 500 μg/mL, in a cell based assay utilizing recombinant yeast cells (*Saccharomyces cerevisiae*), expressing human estrogen receptor alpha as described earlier (Tabanca et al., 2004). No antioxidant activity was seen up to 20 μg/ml, in HL-60 cells induced by PMA. Generation of ROS was determined by DCFH-DA method (Takamatsu et al., 2003). Out of all the isolates, only podocarpaside C (**3**) inhibited complement activity in a dose dependent manner with an IC_{50} of 200 μM, when tested for in vitro complement inhibitory activity. The assay was performed by using sensitized sheep RBCs and hemolysis was determined by measuring the hemoglobin released in the supernatant spectrophotometrically. Intrinsic hemolytic activity of the test compounds (in the absence of complement) was also determined by incubating the test compounds with erythrocytes (Master et al., 2005). None

of the compounds produced any hemolytic effect on RBCs without complement. The activity of compound **3** was compared with the activity of ursolic acid (IC_{50} 55 μM) and oleanolic acid (IC_{50} 78 μM). The anticomplement activity of oleanolic acid and its coumaroyl analogs along with other triterpenoids isolated from *Zizyphus jujuba* (Lee et al., 2004) and its synthetic analogs (Assefa et al., 1999) has been reported earlier. The complement system is a major factor of humoral immunity. Activation of complement is normally beneficial for the host, but can also cause adverse effects depending on the site, extent and duration of activation. The modulation of complement activity is important in the treatment of inflammation as well as during organ transplantation (Lee et al., 2004).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Rudolph Research Auto Pol IV polarimeter. The IR spectra were recorded on a Bruker, Tensor 27 FT-IR spectrometer. The NMR spectra were recorded on Varian AS 400 NMR spectrometers in pyridine-d_5. The ESIMS and HRESIMS data were obtained on a Agilent Series 1100 SL mass spectrometer. Column chromatography was performed using silica gel (JT Baker, 40 μm for flash chromatography) and reverse phase RP-18 silica (Polarbond, JT Baker). TLC analyses were carried out on Silica gel 60 F_{254} plates (Merck, Germany).

3.2. Plant material

The roots of *A. podocarpa* DC. were collected from North Carolina (August 2004) and identified by Gregory Gust, William L. Brown Center Missouri Botanical Garden, Missouri, USA. The voucher specimens have been deposited at the Missouri Botanical Garden, Missouri.

3.3. Extraction and isolation

The lyophilized roots of *A. podocarpa* were ground into powder (543 g), and then extracted with methanol (1.5 L × 24 h × 5). The combined extracts were concentrated under reduced pressure to obtain dry methanolic extract (53 g). A part of methanolic extract (38.0 g) was subjected to vacuum liquid column chromatography over silica gel (600 g) and eluted initially with EtOAc to give three fractions, A (5.0 g), B (6.3 g), and C (0.5 g) and then get final fraction D (21.1 g) with methanol. Fraction B (6.0 g) was divided into twelve fractions (B1-B12) after performing column chromatography (cc) over reverse phase silica (RP-18, 300 g, MeOH/H_2O 7:3). Podocarpasides C (25.2 mg) and E (91.4 mg) were purified from fraction B3 (270.1 mg) as a result of cc (silica gel, $CHCl_3$/MeOH/H_2O 90:10:1). Podocarpaside B (201.9 mg) was obtained from fraction B5 (1.02 g) after double cc over silica gel ($CHCl_3$/MeOH/H_2O 85:15:1 and EtOAc/$CHCl_3$/MeOH/H_2O 15:8:4:1). Podocarpaside A (63.2 mg) was purified as a result of cc of fraction B6 (166.7 mg) (silica gel, EtOAc/$CHCl_3$/MeOH/H_2O 15:8:4:1) and podocarpaside D (119.0 mg) was obtained as undissolved material when methanol was added in room temperature dried fraction B9 (1.13 g). Fraction B10 (413.8 mg) was further fractionated into ten sub-fractions (B10a–B10j) by conducting cc (silica gel, EtOAc/$CHCl_3$/MeOH/H_2O 15:8:4:1). Sub-fraction B10d (83.7 mg) was subjected to double cc, initially over RP-18 silica (MeOH/H_2O 13:7) then over silica gel ($CHCl_3$/MeOH 9:1) to afford podocarpaside F (20.6 mg) and podocarpaside G (22.1 mg) was obtained as undissolved material when chloroform was added in room temperature dried sub-fraction B10g (75.2 mg).

3.3.1. Podocarpaside A (*1*)

White powder; $[\alpha]_D^{26}$ −41.7 (*c* 0.17, MeOH); IR (KBr) ν_{max} 3422, 1715, 1642 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; positive ESIMS *m/z*: 641 $[M+Na]^+$; HRESIMS *m/z*: 641.3669 (calc. for $C_{35}H_{54}NaO_9$, 641.3666).

3.3.2. Podocarpaside B (*2*)

White powder; $[\alpha]_D^{25}$ −52.3 (*c* 0.13, MeOH); IR (KBr) ν_{max} 3405, 1719, 1698 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; positive ESIMS *m/z*: 643 $[M+Na]^+$; HRESIMS *m/z*: 643.3827 (calc. for $C_{35}H_{56}NaO_9$, 643.3822).

3.3.3. Podocarpaside C (*3*)

White powder; $[\alpha]_D^{25}$ −17.3 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3426, 1710, 1644 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR; see Table 2; positive ESIMS *m/z*: 659 $[M+Na]^+$; HRESIMS *m/z*: 659.3766 (calc. for $C_{35}H_{56}NaO_{10}$, 659.3771).

3.3.4. Podocarpaside D (*4*)

White powder; $[\alpha]_D^{25}$ −18.2 (*c* 0.11, MeOH); IR (KBr) ν_{max} 3430, 1721, 1695 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR: see Table 2; positive ESIMS *m/z*: 643 $[M+Na]^+$; HRESIMS *m/z*: 643.3821 (calc. for $C_{35}H_{56}NaO_9$, 643.3822).

3.3.5. Podocarpaside E (*5*)

White powder; $[\alpha]_D^{26}$ +27.3 (*c* 0.11, MeOH); IR (KBr) ν_{max} 3408, 1736, 1692 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR, see Table 2; positive ESIMS *m/z*: 673 $[M+Na]^+$; HRESIMS *m/z*: 673.3560 (calc. for $C_{35}H_{54}NaO_{11}$, 673.3564).

3.3.6. Podocarpaside F (*6*)

White powder; $[\alpha]_D^{26}$ +29.3 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3430, 1737, 1690 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; positive ESIMS *m/z*: 641 $[M+Na]^+$; HRESIMS *m/z*: 641.3659 (calc. for $C_{35}H_{54}NaO_9$, 641.3666).

3.3.7. Podocarpaside G (*7*)

White powder; $[\alpha]_D^{26}$ +24.6 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3402, 1735, 1699 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see

Table 2; positive ESIMS *m/z*: 639 $[M+Na]^+$; HRESIMS *m/z*: 639.3503 (calc. for $C_{35}H_{52}NaO_9$, 639.3509).

3.4. Acid hydrolysis and identification of sugar in 1–7

The compound (10–15 mg) was refluxed with 0.5 N HCl (3 mL) for 2 h. The reaction mixture was diluted with water and extracted with $CHCl_3$. The water layer was dried under reduced pressure as well as under N_2 to give the monosaccharide which showed the comparable R_f value (EtOAc/$CHCl_3$/MeOH/H_2O 12:8:8:4) and specific rotation $[\alpha]_D^{29}$ 102 ± 0–2 (*c* 0.5 ± 0–0.3, H_2O) with that of L-arabinose.

3.5. X-ray crystallography

The structure of the methanol solvate of podocarpaside D (**4**) was determined, using data collected at $T = 110$ K with Mo Kα radiation on a Nonius Kappa CCD diffractometer. Crystal data: $C_{35}H_{56}O_9 \cdot 2CH_3OH$, orthorhombic space group $P2_12_12_1$, $a = 6.4914(15)$, $b = 12.947(5)$, $c = 44.733(16)$ Å, $V = 3760(2)$ Å^3, $Z = 4$, $R = 0.049$ for 2203 observed data of 3015 unique data. The X-ray crystallographic data can be found in supplementary publication CCDC 295967, available from the Cambridge Crystallographic Data Centre. Fig. 4 shows the structure of the molecule, including the seven-membered ring resulting from the opening of the cycloartane ring at C9–C10, as well as the α-L-arabinopyranose subunit. The C9═C11 distance, 1.332(5) Å, and the C23═O4 distance, 1.214(5) Å, are typical of double bonds. The unusual seven-membered ring has a twisted conformation, with an approximate twofold axis passing through C9 and bisecting C5–C6. However, distortions from an ideal C_2 conformation are significant, with torsion angles across the axis differing by an average of 12.4°. All OH groups, including those of the methanol solvent molecules, are involved in intermolecular hydrogen bonding. ORTEP diagram (see Fig. 4) is showing the molecular structure of **4**. The solvent molecules are not shown.

3.6. Biological study

3.6.1. Assay for in vitro cytotoxicity

Cytotoxicity of the compounds was determined by Neutral Red Assay procedure as described earlier (Mustafa et al., 2004).

3.6.2. Assay for estrogenic activity

The assay was performed as described previously using recombinant Yeast cells *S. cerevisiae*, expressing the human estrogen receptor alpha (Tabanca et al., 2004) The samples were tested up to a highest concentration of 500 μg/mL.

3.6.3. Assay for anti-oxidant activity

Antioxidant activity was determined by the DCFH-DA method in Myelomonocytic HL-60 cells as described previously (Takamatsu et al., 2003).

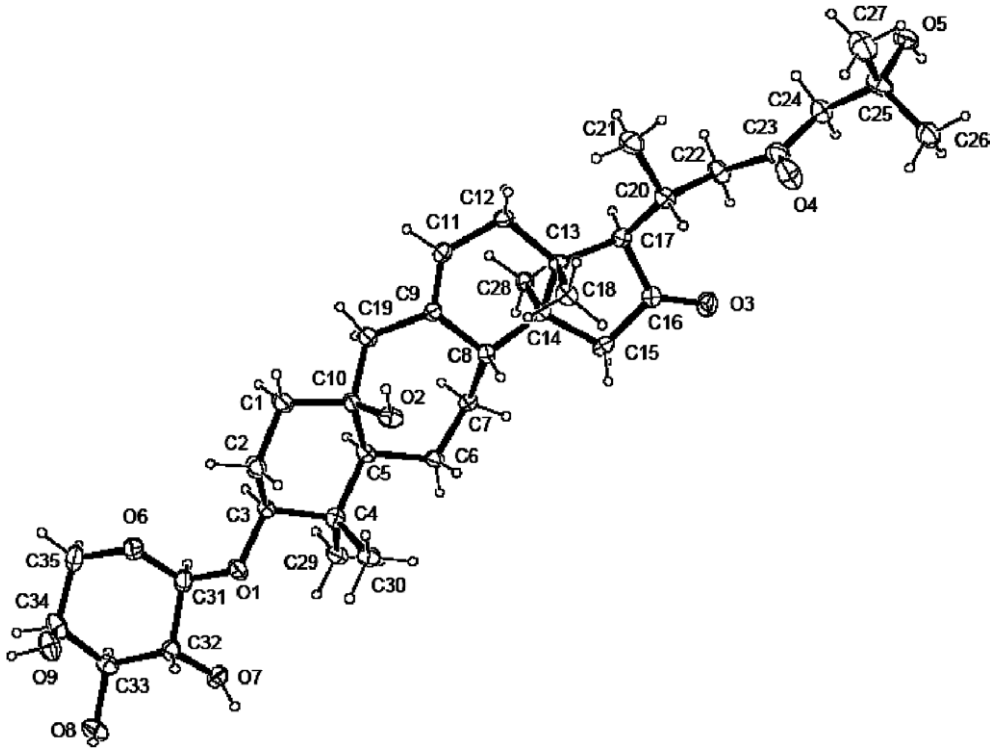

Fig. 4. ORTEP drawing of podocarpaside D (**4**). The solvent molecules are not shown.

3.6.4. Assay for inhibition of complement activity

The compounds were tested in vitro for their inhibition of the classical pathway activation of human complement using antibody-coated sensitized sheep erythrocytes and lyophilized human complement obtained from DiaMedix (Miami, FL). Complement was reconstituted in 300 μL nanopure water and diluted 10 times with ice cold PBS. The assay was performed in 96-well round bottom plates. Complement (10 μL) was first incubated with test samples (diluted in PBS) for 15 min at room temperature in a total volume of 50 μL. Sensitized erythrocytes (150 μL) were added. Plates were incubated at 37 °C for 1 h. After centrifugation (1800 rpm, 10 min), 100 μL supernatant was transferred to a flat bottom 96-well plate and absorbance was read at a dual wavelength of 405–630 nm, on a Biotek EL312 plate reader as a measure of hemoglobin release. DMSO was used as vehicle control. Oleanolic acid and ursolic acid were used as positive controls as mentioned previously (Master et al., 2005) Complement mediated lysis of sensitized erythrocytes in presence of test compounds was compared with the DMSO control. IC_{50} values (the concentration of test compound that caused a 50% inhibition of complement activity) were obtained from dose curves. (Master et al., 2005).

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