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Antiproliferative Cardenolide Glycosides of *Elaeodendron alluaudianum* from the Madagascar Rainforest[1]

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Abstract

Bioassay-guided fractionation of an ethanol extract of a Madagascar collection of *Elaeodendron alluaudianum* led to the isolation of two new cardenolide glycosides (**1** and **2**). The ^{1}H and ^{13}C NMR spectra of both compounds were fully assigned using a combination of 2D NMR experiments, including ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY sequences. Both compounds **1** and **2** were tested against the A2780 human ovarian cancer cell line and the U937 human histiocytic lymphoma cell line assays, and showed significant antiproliferative activity with IC_{50} values of 0.12 and 0.07 μM against the A2780 human ovarian cancer cell line, and 0.15 and 0.08 μM against the U937 human histiocytic lymphoma cell line, respectively.

1. Introduction

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Groups (ICBG) program,[2] we obtained an extract of the stems of a plant initially identified as a *Hippocratea* sp. from Madagascar. The extract had significant antiproliferative activity against the A2780 ovarian cancer cell line, and it was thus selected for bioassay-guided fractionation. While the work was in progress the plant was reidentified as *Elaeodendron alluaudianum* H. Perrier (Celastraceae). As noted previously,[3] there are about forty species in the genus *Elaeodendron* from the Mexican coast, Bermuda, Africa, Madagascar (incl. the Mascarenes), India, Melanesia, and Australia.[4] The plants in this genus are usually glabrous trees or shrubs,[4] and flavonoids,[5] terpenoids,[6] and cardenolides[7] have been isolated from them. The cytotoxicities and cardiac activities of cardenolides have been widely studied.[8]

The extract of *E. alluaudianum* had an IC_{50} value of 3.3 μg/mL against the A2780 human ovarian cancer cell line. The crude extract afforded two new cardenolide glycosides, designated elaeodendroside V (**1**) and W (**2**), after solvent partitioning and reversed-phase C18 HPLC. Herein we report the structural elucidation of the two new cardenolide

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glycosides and their bioactivities against the A2780 human ovarian cancer cell line and the U937 human histiocytic lymphoma cell line.

2. Results and Discussion

Elaeodendroside V (**1**) was obtained as a white amorphous solid. Its molecular formula was established as $C_{35}H_{54}O_{16}$ on the basis of a protonated molecular ion peak at *m/z* 731.3496 in its HRFAB mass spectrum. Its ^{1}H NMR spectrum in CD_3OD showed characteristic signals of an α,β-unsaturated γ-lactone (δ_H 5.01, dd, *J* = 18.4, 1.6 Hz, H-21a; δ_H 4.92, dd, *J* = 18.4, 1.6 Hz, H-21b; and δ_H 5.91, s, H-22) (Table 1). Its ^{13}C NMR spectrum contained 35 signals (Table 2), which were assigned as two methyls, eleven methylenes (including three oxymethylenes), sixteen methines (including twelve oxymethines and one olefinic carbon), and six quaternary carbons (including two oxyquaternary carbons, one olefinic carbon and one carbonyl carbon) based on ^{13}C NMR (Table 2) and HSQC spectra.

The complete ^{1}H and ^{13}C NMR signal assignments and connectivity were determined from a combination of COSY, TOCSY, HSQC and HMBC data and comparison with the spectra of known cardenolides.[9]

COSY and TOCSY correlations established three spin systems, which were H_2-1–H_2-2–H-3–H_2-4 in ring A, H_2-6–H_2-7–H-8–H–9–H-11–H_2-12 in rings B and C, and H_2-15–H_2-16–H-17 in ring D (Figure 1). Further assembly of rings A-D and the α,β-unsaturated γ-lactone of the aglycone was determined on the basis of HMBC correlations. HMBC correlations of H_2-19 to C-1, C-5 and C-9, H_2-1 to C-9, and H_2-6 to C-5 established the connectivity of rings A and B. Correlations of H_2-12, H_2-16, H-17 and H_3-18 to C-13 and of H_2-16 and H_3-18 to C-14 indicated the connectivity of rings C and D. In the meantime, an HMBC correlation of H_2-16 to C-20 suggested the location of the lactone ring at C-17 (Figure 1).

These correlations established the flat structure of the aglycone of **1**. The structures of the sugar moieties of **1** were determined by 1D TOCSY, COSY and HMBC data. Two sugar units was clearly shown by COSY and TOCSY correlations of two spin systems, H-1′–H-2′–H-3′–H-4′–H-5′–H_3-6′ and H-1″–H-2″–H-3″–H-4″–H-5″–H_2-6″ (Figure 1). An HMBC correlation of H-3 to C-1′ indicated the sugars were connected to the aglycone at C-3. In the meantime, HMBC correlations of H-1″ to C-4′ and of H-4′ to C-1″ established that the two sugars were connected from C-1″ to C-4′ (Figure 1).

The relative configuration of the aglycone of **1** was established by analysis of its ROESY correlations and calculation of coupling constants (Figure 2, Table 1). The ROESY correlations of H_3-18 to H-22, H_2-21, H-8 and H-11, and of H_2-19 to H-8 and H-11 indicated that the C-17 side chain, H-8, H-11 and the oxymethylene at C-10 were all β-oriented. Calculation of coupling constants of H-11 (td, *J* = 9.8, 4.2 Hz) suggested that the adjacent H-9 occupied the α-orientation. The above assignments indicated that the B/C ring junction was *trans*-fused. ROESY spectra of **1** obtained in pyridine-d_5 showed a correlation of C-14-OH to H_3-18 that suggested a *cis*-fused ring junction of rings C and D. Determination of the relative configuration of the remaining portions of **1** was carried out by comparison with literature data.[9,8l] These comparisons indicated that rings A and B were connected by *cis*-fused ring junctions and that the C-3 side chain (sugar moiety) existed in the β-orientation. The relative configuration of the sugar moiety was established mainly by calculation of coupling constants and was further proved by ROESY correlations (Table 1, JFigure 2). Coupling constants of H-1′ (d, = 8.0 Hz), H-3′ (t, *J* = 3.0 Hz) and H-4′ (dd, *J* = 9.6, 3.0 Hz) indicated that H-1′, H-2′, H-4′ and H-5′ were in the axial orientation, and that H-3′ was in the equatorial orientation. Coupling constants of H-1″ (d, *J* = 7.6 Hz), H-3″ (t, *J*

= 2.9 Hz) and H-4″ (dd, *J* = 9.2, 2.9 Hz) suggested that H-1″, H-2″, H-4″ and H-5″ were in the axial orientation, and that H-3″ was in the equatorial orientation. Those assignments were proved by ROESY correlations of H-1′ to H-5′, H-1″ to H-5″ and H-2″ to H-4″. In addition, the structure of the sugar moiety of **1** was confirmed by comparison with the known compounds sarmentogenin-3β-*O*-[β-allosyl-(1→4)-β-6-deoxyalloside and securigenin-3β-*O*-[β-allosyl-(1→4)-β-6-deoxyalloside.[8l] The sugar moieties of **1** had identical ^{1}H and ^{13}C NMR data to those of the reported compounds. Therefore, the structure and configuration of **1** was determined as sarmentologenin-3β-*O*-[β-allosyl-(1→4)-β-6-deoxyalloside.

Elaeodendroside W (**2**) was obtained as a white amorphous solid. Its molecular formula was established as $C_{35}H_{52}O_{16}$, which was two units less than that of **1**, on the basis of a sodiated molecular ion peak at *m/z* 751.311 in its MALDI-TOF/TOF mass spectrum. The ^{1}H NMR spectrum of **2** showed characteristic signals of a α,β-unsaturated γ-lactone (δ_H 5.00, br d, *J* = 18.4 Hz, H-21a, δ_H 4.92, br d, *J* = 18.4 Hz, H-21b, and δ_H 5.91, s, H-22) (Table 1). Comparison of the ^{1}H NMR and ^{13}C NMR spectra of compounds **1** and **2** showed that they were very similar, but that the oxymethylene resonances (δ_H 4.18, d, *J* = 11.2 Hz, H-19a, δ_H 3.80, m, H-19b and δ_C 65.9, C-19) that appeared in the spectra of **1** were absent in the spectra of **2**, and that the aldehyde resonances (δ_H 9.97, s, H-19 and δ_C 211.1, C-19) that appeared in the spectra of **2** were absent in the spectra of **1** (Tables 1 and 2). Those data indicated that **2** had a similar structure to **1** except that **2** has an aldehyde instead of a hydroxymethyl group at C-19. Further comparison of the ^{1}H and ^{13}C NMR spectra of **2** and the known cardenolide glycoside, sarmentosigenin-3β-*O*-β-6-deoxyguloside, confirmed the assignments, since the ^{1}H NMR and ^{13}C NMR data of the aglycone of **2** were identical to the literature data for sarmentosigenin. The structure of **2** was further confirmed by analysis of 2D NMR spectra including COSY, HMQC, HMBC and ROESY spectra. Therefore, the structure and configuration of **2** was determined as sarmentosigenin-3β-*O*-[β-allosyl-(1→4)-β-6-deoxyalloside].

All of the isolated compounds were tested for antiproliferative activity against the A2780 human ovarian cancer cell line and the U937 human histiocytic lymphoma cell line. It was found that both **1** and **2** showed significant antiproliferative activity, with IC_{50} values of 0.12 and 0.07 μM against the A2780 human ovarian cancer cell line and 0.15 and 0.08 μM against the U937 human histiocytic lymphoma cell line, respectively. The antiproliferative activities of **1** and **2** do not appear to be correlated with the oxidation status of C-19 because their IC_{50} values were very close to each other. The known cardenolide glycoside, sarmentosigenin-3β-*O*-β-6-deoxyguloside, which possesses the same aglycone as **2** but contains a 6-deoxygulose, also showed significant cytotoxicity with an IC_{50} value of 0.074 μM against a KB cell line,[8l] while another cardenolide with an identical aglycone to **2** but a glycosylated with rhamnose instead of allose also showed significant cytotoxicity with an IC_{50} value of 0.049 μM (0.028 μg/mL) against the HSG cell line.[10] Those data suggested that compounds with the same skeletons as **1** and **2** might show significant activities against cultured cancer cells. The cytotoxicity and antiproliferative activities of many structurally diverse cardenolide glycosides against cultured tumor cells have been widely investigated. Some recent reports are cited.[8m,8n,11,12] This class of compounds has not however found any clinical applications as anticancer agents, in part because of unfavorable toxicity profiles.

3. Experimental Section

General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. IR and UV spectra were performed on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on JEOL Eclipse 500, Varian Inova 400, and

Varian Unity 400 spectrometers. Mass spectra were obtained on a JEOL-JMS-HX-110 and an Applied Biosystems 4800 MALDI TOF/TOF instruments.

Chemical shifts are given in δ (ppm), and coupling constants (*J*) are reported in Hz. HPLC was performed Shimadzu LC-10A pumps coupled with a Varian Dynamax semipreparative C18 column (250 × 10 mm). Both HPLC instruments employed a Shimadzu SPD-M10A diode array detector.

Antiproliferative Bioassay

The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.[13] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[14]

The U937 human histiocytic lymphoma cell line assay was performed at Eisai Research Institute. The cells were cultured in 96-well plates in the absence or continuous presence of 0.005 to 10 ug/ml extract for 96 hours. Cell growth was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to manufacturer's recommendations. Luminescence was read on the EnVision 2102 Multilabel Reader (Perkin-Elmer). IC_{50} values were determined as the concentration of an extract at which cell growth was inhibited by 50% compared to untreated cell population. Two independent repeating experiments were preformed.

Plant Material

Root, stem, and leaf samples of *Elaeodendron alluaudianum* H. Perrier (Celastraceae) were collected in the forest of Bemosa, a dense humid forest, in orthern Madagascar, at an elevation 200 m, at 13.14.17 S, 49.37.50 E, on November 2, 2005. The tree was 10 m high with diameter at breast height of 12 cm and white flowers. It was identified by R. H. Archer (South African National Biodiversity Institute); its assigned collector number is Randrianaivo et al. 1281.

Extraction and Isolation

The stems of the dried plant sample described above (270 g) were extracted with EtOH to give 5.78 g of extract designated MG 3593. A total of 1.63 g of extract was supplied to VPISU, and this had an IC_{50} value of 3.3 μg/mL against A2780 cells. A portion of this extract (96 mg) was suspended in 20 mL of 30% MeOH/CH_2Cl_2 and filtered. The filtrate was evaporated to afford 67 mg residue (IC_{50} 0.14 μg/mL). The residue was suspended in aqueous MeOH (90% MeOH/H_2O, 10 mL), and extracted with n-hexane (3 × 10 mL). The aqueous layer was then diluted to 60% MeOH (v/v) with H_2O and extracted with CH_2Cl_2 (3 × 15 mL). The aqueous MeOH extract (44 mg) was found to be the most active (IC_{50} 0.50 μg/mL) and a portion of this (39 mg) was loaded on a C18 SPE cartridge and eluted with solvent systems of 30% MeOH/H_2O, 70% MeOH/H_2O and MeOH to obtain 3 fractions (I-III). The most active fraction was Fraction I (IC_{50} 0.14 μg/mL), and this was separated via semipreparative HPLC over a C18 column using MeOH-H_2O (75:25) to afford 12 fractions (IV-XV). Fraction XII afforded elaeodendroside V (**1**, 5.3 mg, t_R 35.7 min), and fraction XIV afforded elaeodendroside W (**2**, 2.4 mg, t_R 44.1 min).

Elaeodendroside V (**1**) white amorphous solid; $[\alpha]_D^{23}$ +1.6 (c 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 215 (4.31) nm; IR: ν_{max} 3382, 2944, 2828, 1732, 1028 cm^{-1}; 1H and ^{13}C NMR spectra, see Tables 1 and 2; HRFABMS *m/z* 731.3496 $[M+1]^+$ (calcd for $C_{35}H_{55}O_{16}$, 731.3490).

Elaeodendroside W (2): white amorphous solid; $[\alpha]_D^{23}$ +1.6 (c 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.34) nm; IR: ν_{max} 3382, 2945, 2833, 1731, 1026 cm^{-1}; 1H and ^{13}C NMR spectra, see Tables 1 and 2; MALDI-TOF/TOF-MS *m/z* 751.311 $[M+Na]^+$ (calcd for $C_{35}H_{52}O_{16}Na$, 751.315).

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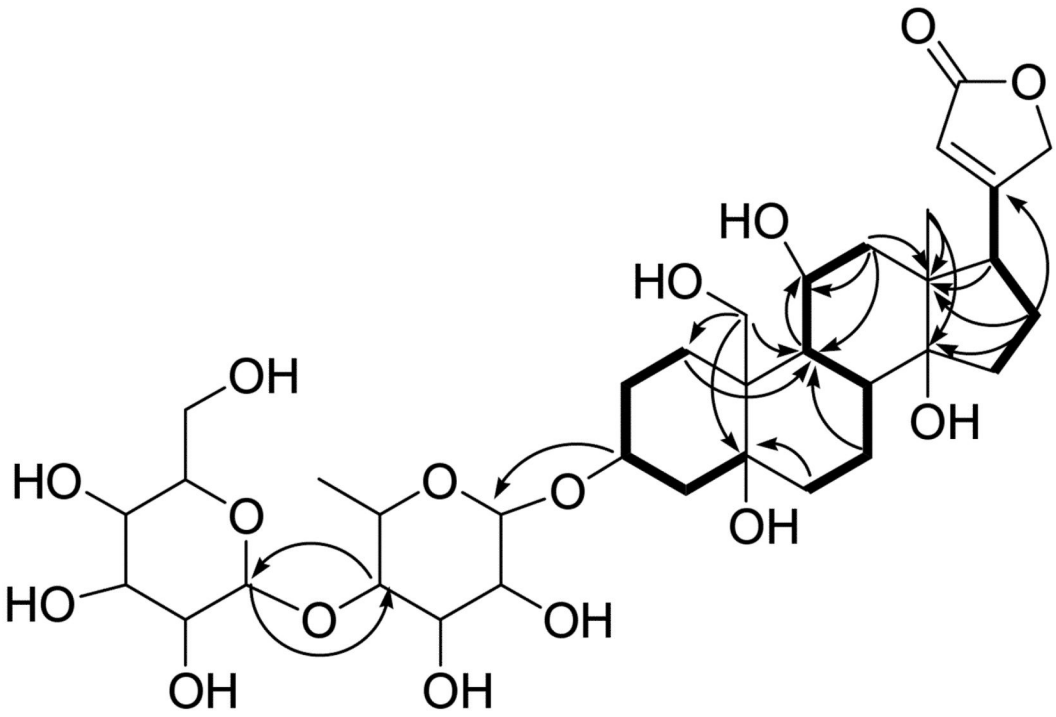


Figure 1.
Key COSY (bold) and HMBC (arrows) correlations of **1**.

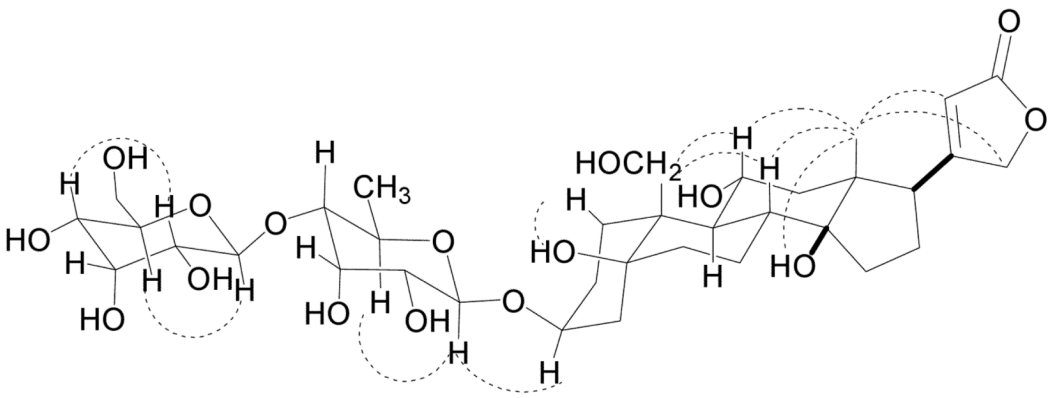


Figure 2.
Key ROESY correlations of 1

Table 1

^{1}H NMR Data of Compounds **1** and **2**[c]

no.	1[a]	1[b]	2[b]
1	3.03 m	2.24 m	2.47 m
	2.85 td (13.8, 2.5)	2.19 m	2.24 m
2	2.45 m	1.94 m	1.92 m
	2.25 m	1.82 m	1.85 m
3	4.48 m	4.15 m	4.19 br s
4	2.21 m	2.10 dd (15.4, 3.0), 1.64 m	2.09 m
	1.84 m		1.72 m
6	2.34 m	1.87 m	1.89 m
	1.67 br d	1.47 m	1.68 m
7	2.44 m	1.99 m	2.06 m
	1,49 m	1.25 m	1.28 m
8	2.40 m	1.81 m	2.00 m
9	2.19 m	1.78 m	1.73 m
11	4.52 m	3.93 td (9.8, 4.2)	3.94 dd (9.8, 4.7)
12	1.97 m	1.66 m	1.67 m
	1.89 m	1.54 dd (13.2, 9.8)	1.49 dd (13.2, 10.8)
15	2.31 m	2.17 m	2.15 m
	1.97 m	1.72 m	1.68 m
16	2.09 m	2.17 m	2.14 m
	2.00 m	1.91 m	1.89 m
17	3.00 m	2.93 t (7.2)	2.94 t (7.2)
18	1.13 s	0.91 s	0.90 s
19	4.66 d (10.7)	4.18 d (11.2)	9.97 s
	4.38 m	3.80 m	
21	5.28 br d (18.2)	5.01 dd (18.4, 1.6), 4.92 dd (18.4, 1.6)	5.00 br d (18.4)
			4.92 br d (18.4)
	5.03 m		
22	6.11 s	5.91 s	5.91 s
1′	5.37 d (8.0)	4.72 d (8.0)	4.73 d (8.0)
2′	3.90~4.60[d]	3.35 m	3.35 m
3′	3.90~4.60[d]	4.33 t (3.0)	4.33 t (2.8)
4′	3.90~4.60[d]	3.28 dd (9.6, 3.0)	3.28 m
5′	3.90~4.60[d]	3.85 m	3.85 m
6′	1.60 d (6.0)	1.30 d (6.0)	1.30 d (6.0)
1″	5.49 d (7.7)	4.73 d (7.6)	4.73 d (8.0)
2″	3.90~4.60[d]	3.33 m	3.33 m
3″	3.90~4.60[d]	4.05 t (2.9)	4.05 t (2.8)
4″	3.90~4.60[d]	3.53 dd (9.2, 2.9)	3.52 dd (9.2, 2.9)

no.	1[a]	1[b]	2[b]
5″	3.90~4.60[d]	3.67 m	3.67 m
6″	4.37 m	3.84 m	3.84 m
	4.30 m	3.68 m	3.69 m
5-OH	5.88 s		
11-OH	5.77 d (4.7)		
14-OH	5.53 s		

[a] in pyridine-d_5.

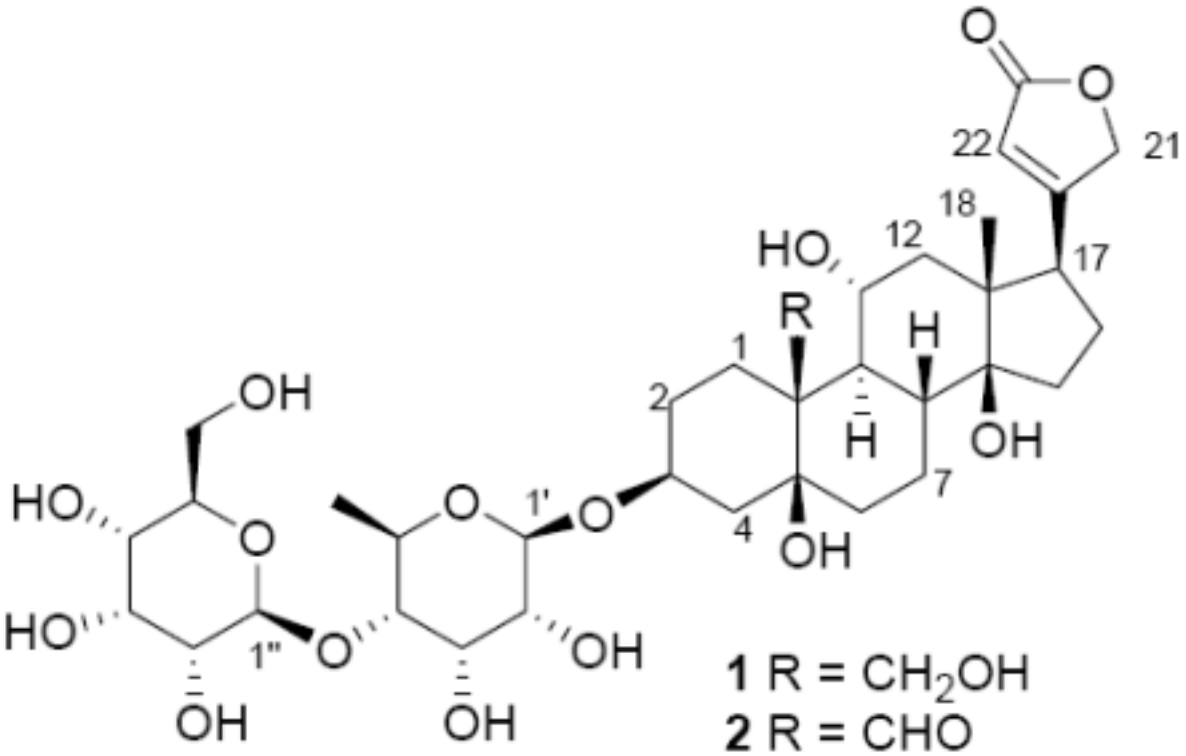
[b] in CD_3OD.

[c] δ (ppm) 500 MHz.

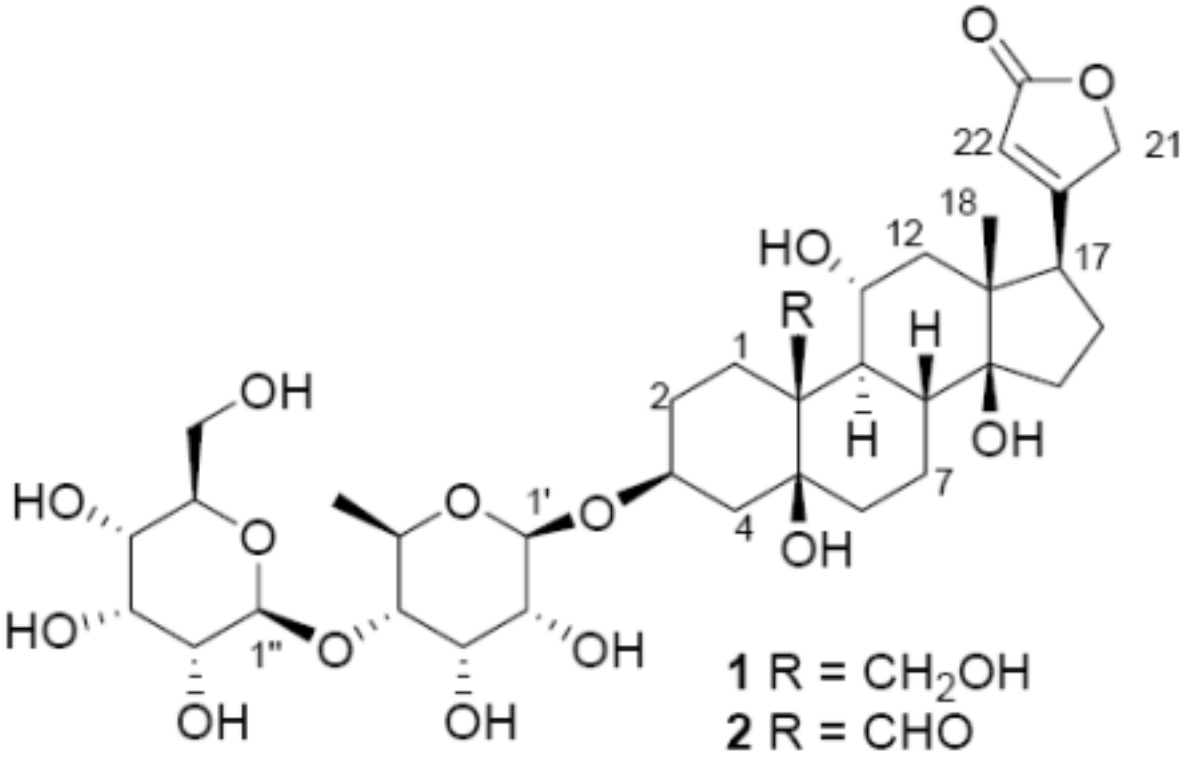
[d] overlapped resonances not assigned.

Table 2

^{13}C NMR Data of Compounds **1**-**2**[c]



no.	1[a]	1[b]	2[b]
1	22.7	22.4	21.3
2	27.6	27.7	27.1
3	74.8	76.2	75.5
4	35.3	35.9	35.7
5	76.3	77.5	75.5
6	37.3	37.0	38.7
7	25.0	25.1	25.5
8	40.8	41.1	42.2
9	45.1	45.2	46.1
10	45.4	45.7	56.7
11	68.7	69.1	68.5
12	50.7	50.6	50.1
13	51.0	51.8	51.0
14	85.0	85.9	85.4
15	33.5	33.5	33.1
16	28.0	28.0	28.0
17	51.4	51.1	51.5



no.	1[a]	1[b]	2[b]
18	18.2	17.8	17.6
19	65.6	65.9	211.1
20	174.8	177.2	177.2
21	74.0	75.5	75.5
22	118.1	118.1	118.1
23	175.8	177.7	177.5
1′	99.3	99.5	99.5
2′	65~80[d]	72.3	72.4
3′	65~80[d]	72.2	72.2
4′	83.8	84.0	84.0
5′	65~80[d]	69.9	69.9
6′	18.7	18.3	18.3
1″	104.3	103.7	103.7
2″	65~80[d]	72.4	72.4
3″	65~80[d]	73.3	73.3
4″	65~80[d]	68.6	68.6
5″	65~80[d]	75.4	75.4
6″	63.0	62.8	62.8

[a] in pyridine-d_5.

[b] in CD_3OD.

[c] δ (ppm) 100 MHz.

[d] resonances not assigned because of overlapping

^1H NMR resonances in the HMBC spectrum.