Cytotoxic Cardenolides from Woods of *Euonymus alata*

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Three cytotoxic cardenolides, acovenosigenin A 3-*O*-α-L-ramnopyranoside (1), euonymoside A (2) and euonymusoside A (3), were isolated from the woods of *Euonymus alata* (Celastraceae). The chemical structure of a new cardenolide, euonymusoside A (3) has been elucidated on the basis of extensive spectral analysis and enzymic hydrolysis to be acovenosigenin A (1β,3β,14β-trihydroxy-5β-cardenolide) 3-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl(1→4)-α-L-rhamnopyranoside. All three showed potent cytotoxic activity against some neoplastic cell lines.

Key words *Euonymus alata*; Celastraceae; cardenolide; euonymusoside A; cytotoxic activity

In the course of our search for cytotoxic antitumor compounds from natural sources, the methanol extract of the wood parts of *Euonymus alata* (THUNB.) SIEB. was found to show cytotoxicity (ED_{50} = 3.08 μg/ml) against KB cells. Twigs of *Euonymus alata* are used as a traditional medicine for stomach ache and as a vermicide in Japan. The constituents of this plant have been investigated and the presence of sesquiterpenes,[1–3] sesquiterpene alkaloids,[4–6] sterols,[7,8] flavonoids,[9,10] and tannins[11] have been reported. This paper describes the isolation and structural elucidation of euonymusoside A and the cytotoxicity of three isolated cardenolide glycosides.

Repeated chromatographic purification with the guidance of bioassay of growth inhibition against KB cells led to the isolation of cardenolide glycosides, compounds **1**—**3**.

Compound **3**, named euonymusoside A, mp 222—225 °C, $[\alpha]_D$ −38.9°, gave its molecular formula $C_{41}H_{64}KO_{19}$ by its pseudo molecular ion at *m/z* 899.3669 (Calcd 899.3678 for $C_{41}H_{64}KO_{19}$) in the positive FAB-MS, together with the NMR spectral data. The UV absorption at 217 nm and the signals of a pair of methylene protons at δ 4.91 (1H, dd, *J* = 18.3, 1.5 Hz, H-21) and 5.01 (1H, dd, *J* = 18.3, 1.5 Hz, H-21) and of an olefinic proton at δ 5.90 (1H, br s, H-22) in the ^{1}H-NMR spectrum suggested the existence of a butenolactone ring of cardenolides. The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of 3 revealed the presence of three methyl, eleven methylenes, twenty-two methine groups and five quarternary carbons in addition to the characteristic signals at δ 178.4 (C-20), 75.3 (C-21), 117.8 (C-22) and 177.2 (C-23) due to the butenolactone ring, a tertiary carbinol carbon at δ 86.2 (C-14) and three anomeric carbons at δ 99.6 (C-1′), 104.9 (C-1‴) and 105.2 (C-1″) (Table 1). The heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum of **3** showed ^{1}H–^{13}C long-range correlations between methine protons at δ 4.20 (H-3) and carbons at δ 32.6 (C-2) and 74.2 (C-1), and also between a methine carbon at δ 74.2 (C-1) and protons at δ 1.09 (H-19) and 4.20 (H-3). So, the presence of two *O*-functional groups at C-1 and C-3 were shown. From these data, **3** displayed a structure due to the 1,3,14-trihydroxycard-20(22)-enolide and three glycosyl units, indicating three anomeric protons at δ 4.52 (d, *J* = 1.6 Hz), 4.41 (d, *J* = 7.7 Hz) and 4.18 (d, *J* = 7.7 Hz) and three anomeric carbons. On enzymatic hydrolysis of **3** with β-glucosidase, **3** afforded glucose and two prosapogenins, which were identified as compounds **1** and **2**. Compound **2** was identified as euonymoside A [acovenosigenin A 3-*O*-[β-D-glucosyl-(1→4)-a-L-rhamnoside]][12] from the bark of *E. sieboldianus* by comparison of the ^{1}H- and ^{13}C-NMR spectral data. In the ^{13}C-NMR spectrum of **3**, the C-6″ (δ 70.2) is shifted downfield by +7.5 ppm, compared with that of **2** (Table 1). So, a terminal glucose of **3** was attached to the C-6″ of the inner glucose. The same result was obtained from the HMBC experiment, that is, long-range correlations between H-1‴ at δ 4.18 and C-6″ were observed. Therefore, the structure of **3** was characterized as acovenosigenin A 3-*O*-[β-D-glucosyl-(1→6)-β-D-glucosyl-(1→4)-α-L-rhamnoside].

Compound **1**, mp 163—165 °C, $[\alpha]_D$ −33.7° showed a molecular ion peak at *m/z* 536 in its electron impact-mass spectra (EI-MS), the composition of which was determined to be $C_{29}H_{44}O_9$ by its molecular ion measarment in the high resolution EI-MS analysis at *m/z* 536.3020 (Calcd 536.2985 for $C_{29}H_{44}O_9$). Compound **1** was identified as acovenosigenin A 3-α-L-rhamnopyranoside,[12] which has been known as a partial hydrolyzate of euonymoside A, by NMR analysis. The isolation of **1** is the first report from natural sources.

We have isolated three cardenolides, acovenosigenin A 3-α-L-rhamnopyranoside, euonymoside A and euonymusoside A (**3**), from the cytotoxic fractions of the wood part of *E. alata*. The *in vitro* cytotoxicity activity of each was

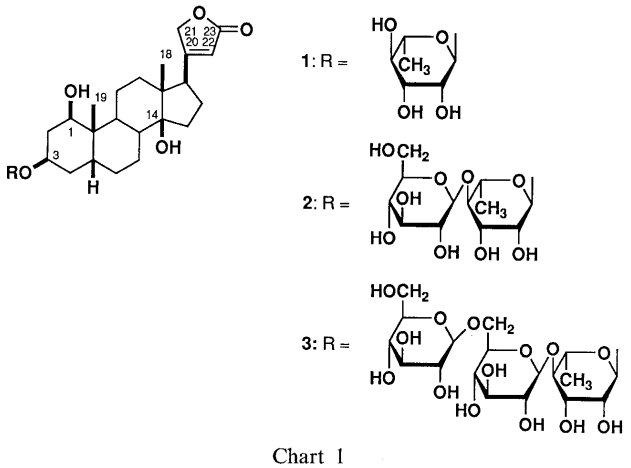

Chart 1

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Table 1. ^{13}C-NMR Data for Compounds **1**—**3** (in CD_3OD)

	1	2	3
1	74.2	74.2	74.2
2	32.6	32.6	32.6
3	73.4	73.7	73.6
4	29.2	29.3	29.3
5	32.1	32.1	32.0
6	27.3	27.3	27.3
7	22.3	22.3	22.3
8	42.8	42.7	42.7
9	38.6	38.5	38.5
10	41.4	41.4	41.3
11	22.2	22.2	22.2
12	40.8	40.7	40.7
13	50.9	50.9	50.9
14	86.3	86.2	86.2
15	33.4	33.3	33.3
16	28.1	28.0	28.0
17	52.1	52.0	52.0
18	16.4	16.4	16.4
19	19.3	19.2	19.2
20	178.4	178.4	178.4
21	75.4	75.3	75.3
22	117.9	117.8	117.8
23	177.3	177.2	177.2
1′	98.7	98.7	99.6
2′	72.5[a)]	72.5[a)]	72.5[a)]
3′	72.6[a)]	72.4[a)]	72.4[a)]
4′	73.9	83.1	82.7
5′	70.6	69.1	69.0
6′	18.1	18.2	18.3
1″		105.6	105.2
2″		76.0	75.2
3″		78.2[b)]	78.1[b)]
4″		71.5	71.7
5″		78.0[b)]	77.2
6″		62.7	70.2
1‴			104.9
2‴			75.9
3‴			78.0[b)]
4‴			71.5
5‴			77.9[b)]
6‴			62.8

a, b) Assignments may be interchanged in each column.

Table 2. Cytotoxicity (IC_{50} in μg/ml) of Compounds **1**—**3** against Tumor Cell Lines *in Vitro*

Tumor cell	1	2	3
KB	0.10	0.33	1.63
HL-60	0.02	0.5	1.25
A549	0.04	0.2	1
Hela	0.20	0.25	1.25
L1210	>10	>10	>10
P388	>10	>10	>10

examined (Table 2). Compounds **1**—**3** showed significant cytotoxicity against KB, HL-60, A549, and Hela tumor cells, while these compounds were almost inactive against mouse leukemia cells (L1210, P388). By a comparison of acovenosigenin A 3-α-L-rhamnopyranoside, and euonymusoside A, the cytotoxic activities seem to decrease against the number of sugars in the three glycosides.

Experimental

All melting points were determined with a Yanagimoto micro-melting-point apparatus and were corrected. The UV spectra were recorded on a Hitachi 200-10 spectrophotometer. The NMR spectra were taken on a JEOL JNM GX-400 instrument (400 MHz for ^{1}H-NMR); the chemical shifts were given in ppm relative to internal tetramethylsilane (TMS). The MS were obtained on a JEOL JMS-SX102 spectrometer.

Extraction and Isolation The powdered woods (5.8 kg) of *Euonymus alata* (THUNB.) SIEB. were extracted with MeOH (12 l × 4). The MeOH extract was concentrated *in vacuo* to give a brown mass (117 g), which was then dissolved in H_2O. The solution was extracted with Et_2O, AcOEt and 1-BuOH, successively. The AcOEt solution was concentrated to give a dark mass (20.0 g), which was subjected to SiO_2 column chromatography with 1—5% MeOH–$CHCl_3$ twice. The cytotoxic fraction against KB cells (228 mg) was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was concentrated to give a colorless powder (54.5 mg), which was purified over Sephadex LH-20 column chromatography with $CHCl_3$–MeOH (1 : 1) to give a colorless powder (25.8 mg). The powder was chromatographed over Toyopearl to give compound **1** (16.5 mg). The 1-BuOH fraction (16.45 g) was chromatographed on a Mitsubishi Diaion HP 20 with MeOH–H_2O (0—100%) to give an active fraction (4.76 g). The fraction was subjected to silica gel column chromatography with MeOH–$CHCl_3$. The active fraction was further purified on Sephadex LH-20 column chromatography with MeOH and then reversed phase column chromatography (YMC ODS-AM, 16—18% MeCN–H_2O) to give **2** (33.8 mg) and **3** (70.0 mg).

Acovenosigenin A 3-α-L-Rhamnopyranoside (1) Colorless fine needles, mp 163—165 °C, $[\alpha]_D^{25}$ −33.7° (c=0.40, MeOH). UV λ_{max}^{MeOH} nm (log ε): 217 (4.46). ^{1}H-NMR (MeOH-d_4) δ: 0.89 (3H, s, Me-18), 1.07 (3H, s, Me-19), 1.27 (3H, d, J=5.9 Hz, H-6′), 1.52 (1H, m, H-4), 1.64 (1H, d, J=11.7 Hz, H-15), 1.80 (1H, m, H-2β), 1.93 (1H, dt-like, J=15.0 Hz, H-2α), 2.08 (2H, m, H-15, 16), 2.82 (1H, dd, J=8.8, 5.9 Hz, H-17), 3.19 (1H, dd, J=9.6, 7.0 Hz, H-4′), 3.39 (1H, dd, J=9.6, 3.5 Hz, H-3′), 3.54 (1H, m, H-5′), 3.71 (1H, br s, $\Delta\nu_{1/2}$=3.5 Hz, H-1), 3.67 (1H, dd, J=3.5, 1.6 Hz, H-2′), 4.21 (1H, br s, $\Delta\nu_{1/2}$=6.0 Hz, H-3), 4.65 (1H, d, J=1.6 Hz, H-1′), 4.91 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.06 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.90 (1H, br s, H-22). HR-EI-MS m/z: Calcd for $C_{29}H_{44}O_9$: 536.2985, Found: 536.3020.

Euonymuside A (2) Colorless fine needles, mp 182—185 °C, $[\alpha]_D^{18}$ −35.9° (c=1.58, MeOH). UV λ_{max}^{MeOH} nm (log ε): 217 (4.04). ^{1}H-NMR (MeOH-d_4) δ: 0.88 (3H, s, Me-18), 1.08 (3H, s, Me-19), 1.14 (3H, d, J=5.8 Hz, Me-6′), 2.81 (1H, dd, J=8.3, 5.9 Hz, H-17), 4.19 (1H, br s, H-3), 4.39 (1H, d, J=8.1 Hz, H-1″), 4.59 (1H, d, J=1.6 Hz, H-1′), 4.90 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.02 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.89 (1H, br s, H-22). HR-FAB-MS m/z: Calcd for $C_{35}H_{54}NaO_{14}$: 721.3412, Found: 721.3381.

Euonymusoside A (3) Colorless fine needles, mp 222—225 °C, $[\alpha]_D^{18}$ −38.9° (c=0.81). UV λ_{max}^{MeOH} nm (log ε): 217 (4.02). ^{1}H-NMR (MeOH-d_4) δ: 0.88 (3H, s, Me-18), 1.09 (3H, s, Me-19), 1.13 (3H, d, J=5.9 Hz, Me-6′), 2.82 (1H, dd, J=8.8, 5.9 Hz, H-17), 4.18 (1H, d, J=7.7 Hz, H-1‴), 4.20 (1H, br s, H-3), 4.41 (1H, d, J=7.7 Hz, H-1″), 4.52 (1H, d, J=1.5 Hz, H-1′), 4.91 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.01 (1H, dd, J=18.3, 1.5 Hz, H-21), 5.90 (1H, s, H-22). HR-FAB-MS m/z Calcd for $C_{41}H_{64}KO_{19}$: 899.3678, Found: 899.3669.

Enzymatic Hydrolysis of Euonymusoside A (3) A solution of **3** (26.2 mg) in a phosphoric acid buffer (pH 5.6, 1 ml) was treated with b-glucosidase (17 mg) at 37 °C for 18 h. The reaction mixture was extracted with 1-BuOH, and the 1-BuOH layer was evaporated *in vacuo* to obtain a residue, which was subjected to silica gel column chromatography and eluted with $CHCl_3$–MeOH to afford **1** (7.8 mg) and **2** (6.6 mg).

Bioassay of Cytotoxic Activity HL-60, P388 and L1210 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. A549 lung carcinoma, Hela and KB cells were grown in Dulbeco's modified Eagle's medium supplemented with 10% calf serum. All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded into 96-well microtiter plates (2×10^4 cells/well) and incubated for 24 h. The test sample, dissolved in dimethylsulfoxide (DMSO), was added in serial dilutions. After the addition, the plates were incubated for 72h. For the evaluation of *in vitro* cytotoxicity, a microculture tetrazolium assay (MTT assay) was used. The IC_{50} value was calculated using PROBIT's method.

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