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Cytotoxic Constituents from the Roots of *Bryonia alba* L.

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Abstract—Two cucurbitane-compounds were isolated from the roots of *Bryonia alba* L. and the chemical structures were established as 19-norlanost-5-ene-3,11,22-trione-2β, 16α,20β,25-tetrahydroxy-9-methyl (23,24-dihydrocucurbitacin D) and 2-O-β-D-glucopyranosyl 19-norlanost-5-ene-3,11,22-trione-2β,16α,20β,25-tetrahydroxy-9-methyl (arvenin IV), respectively, on the basis of chemical and spectral methods. Both of the compounds showed cytotoxic activity against cancer cell lines, A549, SK-MEL-2, COLO 205 and L1210.

Keywords—*Bryonia alba* L. · 23,24-dihydrocucurbitacin D · arvenin IV · cytotoxicity · 2-D NMR

In a continuing search (Lee *et al.*, 1995) for plant-derived cytotoxic compounds, we found that the MeOH extract obtained from the roots of *Bryonia alba* L. which is distributed in Ukraina and Armenia, and has been used as a traditional medicine for treating of more than 40 diseases in Armenia (Sepetchian, 1948), exhibited significant cytotoxic activity against A549, COLO 205, SK-MEL-2 and L1210. Activity-guided fractionation and repeated column chromatography on the MeOH extract afforded two cytotoxic compounds, which were characterized to be 23,24-dihydrocucurbitacin D and arvenin IV, respectively. The compounds were isolated from this plant for the first time. In this paper, we report the isolation and structural elucidation of the compounds, and their cytotoxic activities against some cancer cell lines.

Experimental

General experimental procedures - Melting points were determined using a Fisher-John apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 instrument. ^{1}H- and ^{13}C-NMR spectra were measured with TMS as internal standard, employing a Brucker AMX 400. The FAB/MS and EI/MS were determined on a VG-VSEQ(EBqQ type) and Varian Mat 212MS, respectively. IR spectra were obtained with a Perkin-Elmer Model 599B.

Plant material - The roots of *B. alba* L. was collected at the east site of Yerevan, Armenia, in September (1993) and identified by Dr. Aprikian, a botanist of Institute of Biochemistry, Armenian National Academy of Sciences, Armenia. A voucher specimen (No. 93-57) representing this collection has been deposited at the laboratory of natural products chemistry in Korea Ginseng & Tobacco Research Institute, Taejeon.

Extraction and isolation - The air-dried and powdered roots of *B. alba* (500 g) was extracted with 80% aqueous MeOH (2 liters x 3) at

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Table 1. ^{13}C-NMR chemical shifts of 23,24-dihydrocucurbitacin D (1), arvenin IV (2) and their acetates (100 MHz)

No of C	1 ($CDCl_3$)	1a ($CDCl_3$)	2 (d_5-Py.)	2a ($CDCl_3$)
1	35.78	31.97	34.97	34.16
2	71.43	74.08	79.02	75.67
3	212.45	205.63	211.45	208.60
4	50.42	51.22	51.50	51.26
5	140.13	139.69	140.61	139.87
6	120.19	120.39	120.27	120.26
7	23.66	23.69	24.01	23.70
8	42.16	42.02	42.59	42.05
9	48.06	47.85	48.55	47.89
10	33.48	34.23	34.08	33.65
11	212.85	211.83	212.68	211.57
12	48.51	48.55	49.05	48.67
13	48.20	48.31	48.60	48.36
14	50.11	49.93	50.84	49.95
15	45.18	43.22	46.14	43.23
16	70.46	73.25	70.13	68.42
17	57.47	53.97	58.54	53.94
18	19.64	19.64	19.67	19.58
19	18.78	18.88	18.69	18.89
20	79.05	78.66	79.94	78.67
21	24.31	24.25	25.22	24.25
22	215.23	213.51	215.88	213.49
23	30.79	30.69	32.60	30.66
24	36.77	37.09	38.36	37.12
25	69.92	69.93	68.95	69.97
26	29.12*	29.43*	29.70*	29.50*
27	28.39*	28.63*	28.63*	28.62*
28	21.05	21.24	21.61	21.44
29	29.76*	29.59*	29.99*	29.51*
30	19.82	19.97	20.13	20.01
1′			104.00	99.42
2′			75.75	71.56
3′			78.57	72.57
4′			71.59	71.27
5′			77.70	74.13
6′			62.37	61.76
acetyl		20.97 20.63		20.97
				20.84 20.61
		170.05 170.05		20.61 20.60
				170.89 170.18 170.04
				169.83 169.41

*Signals which in any vertical column may be interchanged.

room temperature and filtered. The combined filtrates were evaporated at less than 40°C to give a MeOH extract (76 g). This residue was suspended in H_2O (500 ml) and extracted with EtOAc (300 ml x 3) and *n*-BuOH (300 ml x 3), successively, to afford, on drying, an EtOAc soluble fraction (12 g) and *n*-BuOH soluble fraction (21 g), respectively. The EtOAc soluble fraction was applied over silica gel column using *n*-hexane-EtOAc (10:1→3:2) as eluents to give thirteen sub-fractions, whose ninth one was chromatographed with silica gel column eluting with $CHCl_3$-MeOH (30:1) to afford 23,24-dihydrocucurbitacin D (1, 458 mg). 23,24-dihydrocucurbitacin D (1) : colorless fine crystals (*n*-hexane-EtOAc), mp 170-171°C, $[\alpha]_D$ +78.2°(c, 1.6 in $CHCl_3$) {Lit. (Vande and Lavie, 1983) mp 168°C, $[\alpha]_D$ +83°(EtOH)}; IR ν_{max} 3380, 1715 and 1680 cm^{-1}; EI/MS, m/z(rel. int.) 518 (0.4, M^+), 500 (1.2), 482 (11), 166 (12), 113 (100), HR-EI/MS : Found 518.3224, Calc. for $C_{30}H_{46}O_7$ 518.3245; ^1H-NMR (400 MHz, $CDCl_3$) δ: 5.79 (1H, br. s, H-6), 4.42 (1H, dd, *J*=5.6, 13.6, H-2), 4.32 (1H, br. t, *J*=7.6, H-16), 3.25 (1H, d, *J*=14.8, H-12_a), 2.94 (1H, m, H-23_a), 2.73 (1H, H-10), 2.69 (1H, d, *J*=14.8, H-12_b), 2.62 (1H, d, *J*=6.9, H-17), 2.61 (1H, m, H-23_b), 2.42 (1H, m, H-7_a), 2.32 (1H, m, H-1_a), 2.00 (1H, m, H-7_b), 1.97 (1H, H-8), 1.85 (1H, H-15_a), 1.83 (2H, H-24), 1.39 (1H, H-15_b), 1.27 (1H, H-1_b), 1.43 (3H, s, H-21), 1.37 (3H, s, H-19), 1.33 (3H, s, H-28), 1.29, 1.23, 1.20 (each 3H, all s, H-26, 27, 29), 1.07 (3H, s, H-30), 0.96 (3H, s, H-18); ^{13}C-NMR : Table 1.

The *n*-BuOH soluble fraction was applied over silica gel column eluting with $CHCl_3$-MeOH (10:1) and $CHCl_3$-MeOH-H_2O (7:3:1) to produce six sub-fractions. The third one was column chromatographed with silica gel [$CHCl_3$-MeOH (8:1) and EtOAc-*n*-BuOH (5:1)] and ODS [MeOH-H_2O (8:7)], repeatedly, to afford arvenin IV (2, 158 mg). Arvenin IV (2) : white powder (EtOH-H_2O), mp 164-165°C, $[\alpha]_D$ +33.5°(c, 1.2 in EtOH) {Lit. (Yamada *et al.*, 1978) mp 160-161°C, $[\alpha]_D$ +37.5°(c, 0.7 in EtOH)}; IR ν_{max} 3420, 1720 and 1690 cm^{-1}; pos. FAB/MS, m/z : 681 $[M+1]^+$ HR-FAB/MS : Found 681.4228, Calc. for $C_{36}H_{57}O_{12}$ 681.3851; ^1H-NMR (400 MHz, d_5-Py.) δ: 5.54 (1H, br. s, H-6), 5.37 (1H, dd, *J*=5.6, 13.0, H-2), 5.01 (1H, d, *J*=7.7, H-1′), 4.76 (1H, br. t, *J*=7.6, H-16), 1.42, 1.36, 1.30, 1.27 (x3), 1.04, 0.96 (each 3H, all s, H-18, 19, 21, 26, 27, 28, 29, 30); ^{13}C-NMR : Table 1.

Acetylation of 23,24-dihydrocucurbitacin D (1) - A mixture of 1 (15 mg), pyridine (5 ml), and acetic anhydride (4 ml) was stirred for 15 hrs at room temperature. The reaction mixture was poured into cold water (50 ml) and extracted with EtOAc (30 ml x 3). The organic fraction was washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, followed by drying over anhydrous $MgSO_4$. The crude acetylated product was purified by silica gel column chromatography, using *n*-hexane-EtOAc (2:3) as eluent, to give 1a (14.4 mg), the pure diacetate of 1. 1a : ^1H-NMR (400 MHz, $CDCl_3$) δ: 5.78 (1H, br. s, H-6), 5.48 (1H, dd, *J*=5.6, 13.6, H-2), 5.14 (1H, br. t, *J*=7.6, H-16), 3.24 (1H, d, *J*=14.7, H-12_a), 2.15, 1.94 (each 3H, both s, acetyl-methyl), 1.46 (3H, s, H-21), 1.32 (3H, s, H-29), 1.31 (3H, s, H-19), 1.29 (3H, s, H-28), 1.27 (3H, s, H-30), 1.26 (3H, s, H-27), 1.10 (3H, s, H-26), 1.02 (3H, s, H-18); ^{13}C-NMR : Table 1.

Acetylation of arvenin IV (2) - Compound 2 (15 mg) was treated same as 23,24-dihydrocucurbitacin D (1). The crude acetylated product was purified by silica gel column chromatography, using *n*-hexane-EtOAc (1:1) as eluent, to give 2a (11.2 mg), the pure pentaacetate of 2. 2a : ^1H-NMR (400 MHz, $CDCl_3$) δ: 5.75 (1H, br. s, H-6), 5.15 (1H, br. t, *J*=7.6, H-16), 4.71 (1H, d, *J*=7.9, H-1′), 4.56 (1H, dd, *J*=5.7, 13.2, H-2), 2.13, 2.12, 2.02, 2.01, 1.94 (each 3H, all s, acetyl-methyl), 1.45, 1.30, 1.26 (x2), 1.24, 1.08, 1.03, 1.01 (each 3H, all s, methyl); ^{13}C-NMR : Table 1.

Cytotoxicity test - A549 (Lung carcinoma, Human), COLO 205 (Colon, adenocarcinoma, Human) and SK-MEL-2 (Malignant melanoma, metastasis to skin of thigh, Human) and L1210 (Lekemia, Murine) cells were grown in RPMI 1640 medium supplemented with glutamine, sodium bicarbonate, and 5% fetal bovine serum. For growth inhibition studies in A549, COLO 205 and SK-MEL-2 cell lines, 1 x 10^5 cells/ml of the media were seeded into each well of 24 well plates, and preincubated for 24 hrs at 37°C under 5% CO_2, followed by incubation with the compounds for 48 hrs. In the case of L1210, 5 x 10^4 cells/ml of the media were seeded in the 24 well plates, and incubated for 48 hrs with the compounds. Followingly, the plates were centrifuged at 3000 rpm for 10 min. The experiment was carried out according to the sulforhodamin B method (Skehan *et al*, 1990). Cytotoxicity of the compounds at various concentrations was evaluated as the net growth inhibition (%) of cells as compared with that of control. The 50% inhibitory concentration (ED_{50} value) of compounds was determined graphically from the dose-response curve.

Results and Discussion

When the MeOH extract of this plant was fractionated with the guidance of cytotoxicity assay against A549 cell line, activity was shown in the EtOAc and *n*-BuOH soluble fractions. From these fractions, two cytotoxic triterpenoid-compounds, 23,24-dihydrocucurbitacin D (1), and its glucoside, arvenin IV (2), were isolated by a combination of silica gel and ODS column chromatography.

Compound 1, mp 170-171°C, was obtained as colorless fine crystals from *n*-hexane-EtOAc and showed strong absorptions at 3380, 1715, and 1680 cm^{-1} in the IR spectrum, indicating the presence of hydroxy, carbonyl, and alkene groups. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of 1, the signals due to one olefinic (δ

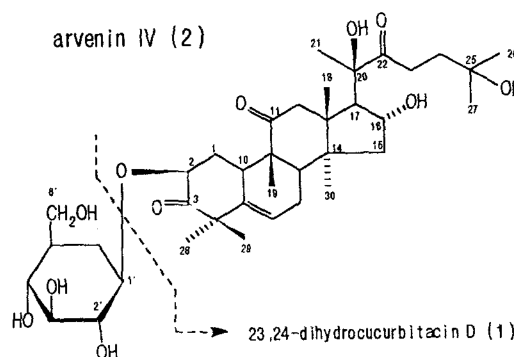

Fig. 1. Chemical structures of compounds isolated from *Bryonia alba* L.

5.79, br. s), eight singlet methyls (δ 1.43, 1.37, 1.33, 1.29, 1.23, 1.20, 1.07, 0.96), and two methines bearing oxygen [δ 4.42 (dd, *J*=5.6, 13.6), 4.32 (br. t, *J*=7.6)] protons were observed. In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of 1, thirty carbon signals were observed, among which the presence of three ketones (δ 215.23, 212.85, 212.45), two methines (δ 71.43, 70.46) and two quaternaries (δ 79.05, 69.92) carbons bearing oxygen, one double bond (δ 140.13, 120.19), and eight methyls (δ 29.76, 29.12, 28.39, 24.31, 21.05, 19.82, 19.64, 18.78) was confirmed by their chemical shifts and DEPT experiment. From above results and molecular weight, which was determined by EI/MS (*m/z* 518 $[M]^+$) to be 518, compound 1 was supposed to be a cucurbitane-triterpenoid. Meanwhile, in the ^{1}H- and ^{13}C-NMR of 1, it was clear the absence of signals due to acetyl and singlet methine proton signal linked to oxygen, which are typical in cucurbitacin B-type or isocucurbitacin-type (Arisawa *et al.*, 1984), respectively, and the presence of signals due to only one double bond. Finally, the chemical structure of compound 1 was established as 19-norlanost-5-en-3,11,22-trione-2β,16α,20β,25-tetrahydroxy-9-methyl (23,24-dihydrocucurbitacin D) (Vande and Larvie, 1983). Most proton and carbon signals of 1 were assigned by ^{1}H-^{1}H COSY and HMQC, respectively.

Acetylation of 1 with acetic anhydride in

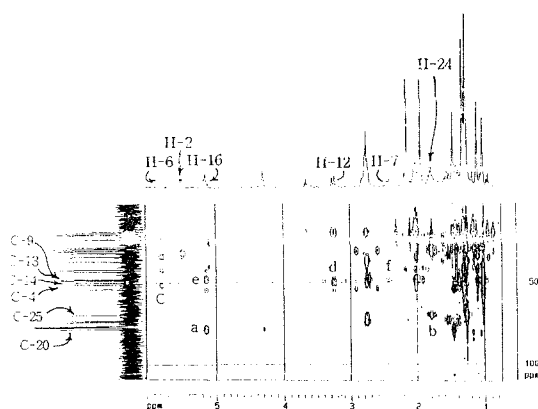

Fig. 2-1. HMBC Cpectrum of 23.24-dihydrocucurbitacin D-diacctaic (1a)

pyridine produced 1a, diacetate of 1, which was confirmed from two acetyl signals observed at δ2.15, 1.94 (eath 3H, both s) in the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) and δ 170.05 (x2), 20.97, 20.63 in the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$). By the way, though it is usually difficult to assign quaternary carbon signal in the ^{13}C-NMR spectrum, all of that in 1a were assignable by use of HMBC experiment. In the HMBC spectrum of 1a (Fig. 2-1), peak a (C-20/H-16), peak b (C-25/H-24), peak c (C-4/H-6), peak d (C-14/H-12), peak e (C-13/H-16), and peak f (C-9/H-7) made quaternary carbons of 1a, C-20, C-25, C-4, C-14, C-13, and C-9, be assigned as signals observed at δ78.66, 69.93, 51.22, 49.93, 48.31, 47.85, respectively. And then, in the ^{1}H-NMR spectrum, singlet methyl proton signal is not assignable by ^{1}H-^{1}H COSY. However, eight singlet methyl proton signals of 1a were also assigned by HMBC experiment. In the HMBC spectrum of 1a (Fig. 2-2), peak a and b showed long-range carbon-proton coupling of a singlet methyl proton signal observed at δ1.46 with C-20 and C-17, respectively, indicating the signal was assignable to H-21. By the same way, H-29 [peak c (H-29/C-4), peak d (H-29/C-28)], H-28 [peak e (H-28/C-4)], H-19 [peak f (H-19/C-8)], H-27 [peak g (H-27/C-25)], H-26 [peak h (H-26/C-27)], and H-18

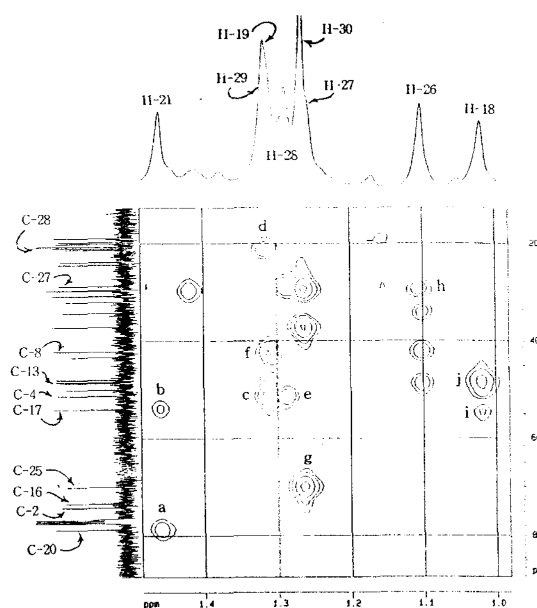

Fig. 2-2. HMBC Cpectrum of 23.24-dihydrocucurbitacin D-diacctaic (1a)

[peak i (H-18/C-17), peak j (H-18/C-13)] were assigned unambiguously.

Compound 2, white powder (EtOH-H_2O), mp 164-165°C, showed IR absorption at 3420, 1720 and 1690 cm^{-1}, indicating the presence of hydroxy, carbonyl and alkene. In the ^{1}H-NMR spectrum (400 MHz, d_5-Py.), besides all signals of 23,24-dihydrocucurbitacin D (1), additional one anomeric proton and some signals due to sugar moiety were observed between 4.88 and 3.48 ppm. From the molecular weight, 680, determined by FAB-MS and chemical shifts of sugar moieties in the ^{13}C-NMR spectrum (100 MHz, d_5-Py.), compound 2 was supposed to be a glycoside of 23,24-dihydrocucurbitacin D with a D-glucopyranose. The position of the sugar linkage was determined to be the C-2 hydroxy group of 1, from the fact that the glycosidation shift (+ 7.59 ppm) in the ^{13}C-NMR spectrum of 2 was observed at the C-2 position of the aglycone moiety. The anomeric configuration of D-glucopyranose was determined as β from coupling constant (J=7.7 Hz) at δ5.01 in the ^{1}H-NMR

Table 2. The cytotoxic activities of two cucurbitane-compounds on some cancer cell lines

	ED_{50} values (μg/ml)			
	A549	SK-MEL-2	COLO 205	L1210
23,24-dihydrocucurbitacin D (1)	1.5	0.87	1.6	0.49
arvenin IV (2)	2.7	2.6	4.8	1.9

spectrum of 2. These above results clearly established the structure of 2 as 2-O-β-D-glucopyranosyl 19-norlanost-5-ene-3,11,22-trione-2β,16α,20β,25-tetrahydroxy-9-methyl (arvenin IV) (Yamada *et al.*, 1978).

The treatment of 2 with acetic anhydride and pyridine afforded a pentaacetate of 2 (2a), which was confirmed from the signals due to five acetyls in the ^{1}H-NMR (400 MHz, $CDCl_3$ δ 2.13, 2.12, 2.02, 2.01, 1.94) and ^{13}C-NMR (100 MHz, $CDCl_3$, δ 170.89, 170.18, 170.04, 169.83, 169.41, 20.97, 20.84, 20.61, 20.61, 20.60) spectra.

Although there were some reports on cytotoxic activities (Konopa *et al.*, 1974a) and chemical constituents (Panosyan *et al.*, 1978) of *B. alba*, these two cytotoxic compounds, 23,24-dihydrocucurbitacin D (1) and arvenin IV (2), have not been yet isolated from this plant, previously. Such a difference in the composition of cucurbitacins existing in *B. alba* may be due to the collection period, site and the age of the plant, because biological activities of the extract from this plant were reported to be different dependent upon above mentioned conditions for collection (Konopa *et al.*, 1974b). Meanwhile, it has been well known that a highly oxygenated tetracyclic triterpenoid group, like cucurbitane-triterpenoids, possessed a wide range of biological activities (Hylands and Magd, 1986)), and cytotoxic effect of the compounds on cancer cell lines *in vitro* (Ryu *et al.*, 1994) or *in vivo* (Kupchan *et al.*, 1972). Cytotoxic activity of compound 1 and 2 on A549, SK-MEL-2, COLO 205, and L1210 were evaluated by the procedure of the SRB methods (Skehan *et al.*, 1990). As shown in Table 2, ED_{50} values of compound 1 were 1.5, 0.87, 1.6 and 0.49 μg/ml on A549, SK-MEL-2, COLO 205 and L1210, respectively, whereas those of compound 2 were 2.7, 2.6, 4.8 and 1.9 μg/ml, respectively. Comparison of the values with those reported previously (Ryu *et al.*, 1994) lead the conclusion that elimination of a double bond at C-23,24 and acetyl group at C-25 in cucurbitane-triterpenoids caused to reduce the cytotoxicity, remarkably.

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