Notes

Sylviside, a Diterpene Glucoside Derivative from *Gnaphalium sylvaticum*[⊥]

Mira M. Konopleva,[†] Irena Matławska,[‡] Małgorzata Wojcińska,[‡] Ahmed A. Ahmed,[*,§] Maria Rybczyńska,[||] Anna Paszel,[||] Shinji Ohta,[∇] Toshifumi Hirata,[○] Wiesława Bylka,[‡] Tom J. Mabry,[*,#] and John F. Cannon[¶]

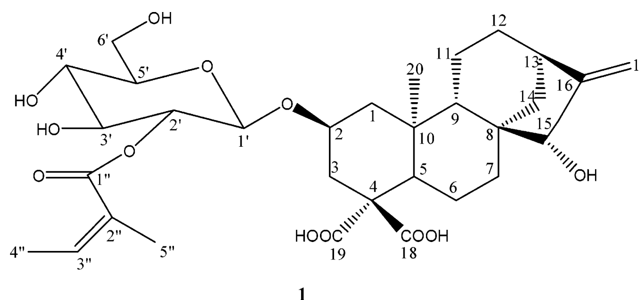
Department of Pharmacognosy and Botany, Vitebsk State University, Vitebsk, Republic of Belarus, Department of Pharmacognosy, Poznań University of Medical Sciences, 61-781 Poznań, Poland, Department of Chemistry, Faculty of Science, El-Minia University, El-Minia 61519, Egypt, Department of Clinical Chemistry, Poznań University of Medical Sciences, 60-357 Poznań, Poland, Laboratory of Environmental Molecular Ecology, Nagahama Institute of Bio-Science and Technology, Nagahama, Shiga 526-0829, Japan, Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, 739-8526, Japan, Molecular Cell and Developmental Biology, The University of Texas at Austin, Austin, Texas 78712-7398, and Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602-5700

Received April 27, 2005

A new diterpene glucoside (**1**), named sylviside, was isolated from the aerial parts of *Gnaphalium sylvaticum*. Its structure was elucidated as 2*β*,15α,20α-trihydroxy-19,20-dicarboxy-*ent*-kaur-16-ene 2*β*-*O*-(2′-angelate)-*β*-D-glucopyranoside, on the basis of spectroscopic analysis (^{1}H NMR, ^{13}C NMR, HMQC, HMBC, NOESY), and was confirmed by X-ray crystallographic analysis. Sylviside (**1**) displayed weak cytotoxicity against HeLa WT (human epitheloid cervical carcinoma) cells and was also evaluated for its effects on reversing multidrug resistance in HeLa cells overexpressing MDR1.

Gnaphalium species (Asteraceae, tribe Inuloidae), known as cudweeds, are annual, biennial, or perennial plants distributed worldwide.[1] Many of these are used in folk medicine, mostly for respiratory and gastrointestinal diseases. Previous studies have shown phenolic compounds (mainly flavonoids and phenolic acids) and diterpenes (*ent*-kaurane type) to be the major secondary metabolite components of these plants.[2] Biological studies have demonstrated antidiabetic, antifeedant, antifertility, anti-inflammatory, antimicrobial, choleretic, cytotoxic, and larvicidal activities of the constituents of *Gnaphalium* species.[3−8] We report herein on the isolation, structural elucidation, and cytotoxicity of sylviside (**1**), a new diterpene glucoside derivative from *G. sylvaticum* L. [syn. *Omalotheca sylvatica* (L.) Sch. Bip et Schultz], a perennial plant native to Europe, Asia, and North America.[9] Its inflorescence is used in folk medicine as a diuretic and vermifuge.[10] Previous phytochemical investigation showed the presence of flavonoids (apigenin, isoquercitrin, luteolin, quercetin, quercimeritrin, tricin) in the aerial parts of this plant.[11,12]

Compound **1** was isolated as white crystal needles with $[\alpha]^{25}_D$ −54.0° (*c* 3.4, $CHCl_3$−MeOH, 1:1). Its molecular formula was deduced to be $C_{31}H_{44}O_{12}$ on the basis of elemental analysis and from the negative HRFABMS that showed an ion peak [M − H] + at *m/z* 607.2743. Intense absorption bands at 3505, 1717, 1705, 1698, and 1645 cm^{-1} were observed in the IR spectrum of **1**, in



1

accord with the presence of hydroxyl, carbonyl, carboxylic acid, and olefinic functions, respectively. The ^{1}H NMR spectrum of **1** ($CDCl_3$−CD_3OD, 5:1) exhibited signals for a glucopyranosyl moiety in the structure. The anomeric proton appeared at δ_H 4.52 (1H, d, J = 8.1 Hz, H-1′), which correlated in the ^{1}H−^{1}H COSY spectrum with a downfield double doublet signal at δ_H 4.66 (1H, dd, J = 9.5, 8.1 Hz, H-2′). In the same ^{1}H−^{1}H COSY experiment, the H-3′, H-4′, H-5′, and H-6' signals could be assigned unambiguously (Table 1). Additionally, signals of an angelate moiety were observed at δ_H 5.93 (1H, qq, J = 7.3, 1.5 Hz, H-3″), 1.82 (3H, dq, J = 7.3, 1.5 Hz, H-4″), and 1.74 (3H, q, J = 1.5, 1.5 Hz, H-5″). The ^{13}C NMR signals of both moieties, the sugar and angelate, were determined from a combination of the HMQC and HMBC spectra of **1**.

The structure of the aglycon and positions of the sugar and the angelate moieties in **1** could be deduced from a series of 2D NMR measurements, including its ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC spectra. The structure of the aglycon was shown to be a diterpene from the presence of 20 carbon signals in the ^{13}C NMR spectrum in addition to the carbon signals of the sugar and angelate moieties. Moreover, the ^{1}H NMR spectrum showed only one methyl signal, at δ_H 0.84, while the ^{13}C NMR spectrum showed two carboxylic carbon signals at δ_C 174.3 and 174.1. Two oxygenated carbons found at δ_C 71.5 and 82.1 were correlated in the HMQC spectrum with the proton signals at δ_H 4.03 (1H, m, H-2) and 3.66 (1H, br s, H-15), respectively. An additional olefinic group was

[⊥] Dedicated to Dr. Norman R. Farnsworth of the University of Illinois at Chicago for his pioneering work on bioactive natural products.

* To whom correspondence should be addressed. Tel: +2086-234-5267. Fax: +2086-234-2601. E-mail: abdellaahmed@yahoo.com (A.A.A.). Tel: +512-471-1900. Fax: +512-232-3402. E-mail: mabry@mail.utexas.edu (T.J.M.).

[†] State University, Republic of Belarus.

[‡] Department of Pharmacognosy, Poznañ University of Medical Sciences.

[§] El-Minia University.

[||] Department of Clinical Chemistry, Poznań University of Medical Sciences.

[∇] Nagahama Institute of Bio-Science and Technology.

[○] Hiroshima University.

[#] The University of Texas at Austin.

[¶] Brigham Young University Provo.

10.1021/np050144x CCC: $33.50 © 2006 American Chemical Society and American Society of Pharmacognosy
Published on Web 09/27/2005

Table 1. 1D and 2D NMR Data for Sylviside (**1**) [500 MHz: δ_C, δ_H $CDCl_3$−CD_3OD (5:1); TMS]

position	δ_C	DEPT	δ_H		coupling (J in Hz)	J^2, J^3 (H−C) coupling
1	46.5	CH_2	2.09	dd	12.2, 3.4	C-2, C-3, C-5,
			0.71	t	12.2	C-10, C-20
2	71.5	CH	4.03	m		C-1
3	39.2	CH_2	2.49	dd	12.2, 3.7	C-1, C-2, C-4,
			1.15	dd	12.2, 11.7	C-5
4	57.7	C				
5	50.4	CH	1.67	br d	12.2	C-1, C-6, C-7, C-20
6	22.6	CH_2	1.71	m		C-4, C-5, C-7,
			1.51	m		C-8
7	34.5	CH_2	1.51	br d	10.3	C-5, C-6, C-9,
			1.29	m		C-14
8	47.3	C				
9	52.9	CH	0.98	br d	7.6	C-1, C-8, C-10, C-11, C-12, C-14, C-15, C-20
10	39.8	C				
11	17.9	CH_2	1.44	m		C-8, C-10, C-12
			1.31	m		
12	32.1	CH_2	1.31	m		C-11, C-13,
			1.44	m		C-14
13	41.9	CH	2.59	br s		C-8, C-11, C-12, C-15
14	35.9	CH_2	1.71	br d	11.7	C-7, C-9, C-12,
			1.24	dd	11.7, 4.6	C-13
15	82.1	CH	3.66	br s		C-9, C-13, C-14
16	158.6	C				
17	108.4	CH_2	5.06	br s		C-13, C-15
			4.94	br s		
18	174.3	C				
19	174.1	C				
20	16.7	CH_3	0.84	s		C-1, C-5, C-6, C-9, C-10
O-glucose						
1′	99.0	CH	4.52	d	8.1	C-2, C-2′, C-5′
2′	73.1	CH	4.66	dd	9.5, 8.1	C-3′
3′	74.8	CH	3.44	t	9.5	C-2′, C-4′, C-5′
4′	70.1	CH	3.36	t	9.5	C-5′, C-6′
5′	75.8	CH	3.22	m		C-3′, C-4′
6′	61.3	CH_2	3.63	dd	12.2, 4.9	C-4′, C-5′
			3.74	dd	12.2, 2.7	
O-angelate						
1″	167.3	C				
2″	127.3	C				
3″	138.2	CH	5.93	qq	7.3, 1.5	C-1″, C-4″, C-5″
4″	15.4	CH_3	1.82	dq	7.3, 1.5	C-1″, C-2″, C-5″
5″	19.9	CH_3	1.74	q	1.5	C-1″, C-2″, C-3″

detected from the two carbon signals at δ_C 158.6 and 108.4. The latter signal was correlated in the HMQC spectrum with two proton resonances at δ_H 5.06 (1H, br s) and 4.94 (1H, br s). The positions of the functional groups and the sugar and angelate moieties in **1** were determined from the HMBC spectrum. In this experiment, the carbon signal at δ_C 71.5 (C-2) showed a cross-peak with the anomeric proton resonance (δ_H 4.52) as well as the proton signals at δ_H 2.09 (H-1a), 0.71 (H-1b), 2.49 (H-3a), and 1.15 (H-3b), thereby giving the partial structure, C-1−C-2 (*O*-sugar)−C-3. This result was supported by NOESY and NOE difference spectra in which the anomeric proton (δ_H 4.52, H-1′) was correlated with two protons at δ_H 4.03 (m, H-2) and 2.49 (dd, H-3a). The methyl signal at δ_H 0.84 in the HMBC spectrum showed a correlation with C-1 (δ_C 46.5), C-5 (δ_C 50.4), and C-9 (δ_C 52.9). The second carbon-bearing oxygen was placed at C-15 on the basis of the HMBC spectrum. Finally, the structure and relative stereochemistry of **1** were confirmed as 2β,15α,20α-trihydroxy-19,20-dicarboxy-*ent*-kaur-16-ene 2β-*O*-(2′-angelate)-β-D-glucopyranoside (Figure 1).

The cytotoxicity of sylviside (**1**) was tested against two tumor cell lines. Initial screening showed that the IC_{50} value of sylviside for HeLa WT cells was 325.3 μmol, whereas the results obtained

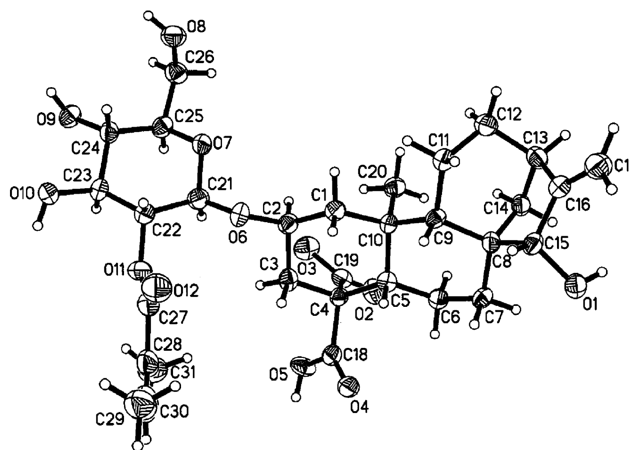


Figure 1. ORTEP drawing of **1**.

Table 2. Cytotoxic Activity of Sylviside (**1**) aganist HeLa WT and HeLa MDR1-G185 Cells

	IC_{50}	
compound	HeLa WT	HeLa MDR1-G185
sylviside (**1**)	325.3 (μmol)	1.70 (mmol)
adriamycin	231.0 (nmol)	25.65 (μmol)

from the HeLa MDR1 cell line indicated that this compound had no ability to reverse multidrug resistance in cells overexpressing MDR1 (IC_{50} value of 1.7 mmol). Sylviside (**1**) was considerably less effective than adriamycin (used as a positive control) against both HeLa WT and HeLa MDR1-G185 cells (Table 2).

Experimental Section

General Experimental Procedures. The melting point (uncorrected) was obtained on a Reichert apparatus. The optical rotation was recorded on a JASCO-20C automatic recording spectropolarimeter ($CHCl_3$−MeOH, 1:1). The IR spectrum ($CHCl_3$) was recorded on a Perkin-Elmer FT-IR spectrometer. ^{1}H NMR (500 MHz, $CDCl_3$−CD_3OD, 5:1) and ^{13}C NMR (125 MHz, $CDCl_3$−CD_3OD, 5:1) spectra were measured on a JEOL 500 MHz Lambda spectrometer with TMS as an internal standard (at 25 °C). Mass spectra were recorded on a Finnigan TSQ-700 triple-stage quadrupole mass spectrometer. TLC was performed using Merck precoated plates (silica gel 60 F_{254}) of 0.25 mm thickness. A Sephadex LH-20 (Fluka) column was used for column chromatography.

Plant Material. Aerial parts of *Gnaphalium sylvaticum* L. were collected in full blossom in the Vitebsk area (Republic of Belarus) and dried at room temperature. The plant material was identified by Prof. W. N. Reschnikov, Botanical Garden of the Belarussian Academy of Sciences, Minsk, and a voucher specimen (WNR 12) was deposited at the Department of Pharmacognosy and Botany, Vitebsk State Medical University, Vitebsk, Republic of Belarus.

Extraction and Isolation. The air-dried plant material (1 kg) was extracted under reflux with EtOH (3 L × 1 h). The extracts were combined and concentrated in vacuo to dryness. The dried residue (150 g) was suspended in hot water and let stand overnight. A chlorophyll precipitate obtained was removed by filtration, and the filtrate was partitioned between solvents of increasing polarity (Et_2O, followed by EtOAc, and finally BuOH). The ethyl acetate fraction was further chromatographed on a polyamide column eluted with water, followed by a step gradient H_2O−MeOH solvent system. The H_2O−MeOH (40:60) fraction (120 mg) was subjected to passage over a Sephadex LH-20 column, which was eluted with *n*-hexane−CH_2Cl_2−MeOH (7:4:0.5, 7:4:1, 7:4:2). Sixty fractions of 50 mL each were collected and monitored by TLC using CH_2Cl_2−MeOH (7:2), visualized by heating the plates after spraying with vanillin reagent. Crystalline needles of sylviside (**1**, 50 mg) were obtained from fraction 56 using a mixture of methanol and water.

Sylviside (1): white crystals; mp 164−165.5 °C; $[\alpha]^{25}_D$ −54.0° (*c* 3.4, $CHCl_3$−MeOH, 1:1); IR (KBr) ν_{max} 3505, 1717, 1705 1698, 1645 cm^{-1}; ^{1}H NMR, DEPT, and ^{13}C NMR spectroscopic data, together with

principal HMBC correlations, Table 1; FABMS (negative mode) m/z 607 $[M - H]^+$, 563 $[M - COOH]^+$; HRFABMS (negative mode) m/z 607.2743 (calcd for $C_{31}H_{43}O_{12}$, 607.2755).

X-ray Crystallography of Sylviside (1). Crystal data: $C_{31}H_{44}O_{12}$, formula wt 608.275, crystal dimensions 0.10 × 0.01 × 0.01 mm, orthorhombic, space group $P2_12_12_1$, a = 10.3683(4) Å, b = 15.2072(7) Å, c = 23.6901(10) Å, V = 3735.3(3) Å^3, Z = 4, D_{calc} = 1.307 g/cm^3, F(000) = 1584, GOF = 1.036. The reflection data were collected on a Bruker diffractometer with a CCD area detector using phi and omega scans with graphite-monochromated Cu Kα radiation (λ = 1.54178 Å). The structure was solved, refined, and displayed using the program package SHELTXL.[13] The final R and R_w factors for data with $I > 2\sigma$ were 0.0408 and 0.0851, respectively. Crystallographic data for this structural analysis have been deposited with the Cambridge Crystallographic Data Center (CCDC 258907). These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

Cytotoxicity Assay. HeLa WT (human epitheloid cervix carcinoma) and multidrug-resistant HeLa MDR1 cells (both provided by Prof. J. Hoffmann, Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria) were grown in RPMI-1640 medium (Sigma), supplemented with 10% heat inactivated fetal bovine serum (Sigma) (2 mmol), glutamine (Sigma), and gentamycin (50 μg/mL) (Sigma). The multidrug-resistant MDR1-overexpressing HeLa cell line was obtained by transfection of human HeLa S3 (HeLa WT) with a MDR1 wild-type gene construct (HeLa MDR1) as previously described.[14] Following transfection, the multidrug-resistant stock cultures were grown in the presence of 100 nmol of vinblastine (Sigma). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The MTT proliferation assay was used to estimate the effect of sylviside (**1**) on cell viability in vitro. The MTT test is a colorimetric method based on the cleavage of the reagent, a yellow tetrazolium salt (MTT), to yield purple formazan crystals. This reaction, which is catalyzed by the mitochondrial dehydrogenase of living metabolically active cells, can be measured spectrophotometrically. Logarithmically growing cells were washed once with PBS (Sigma), trypsinized using Tripsin-EDTA (Sigma), and resuspended in a suitable volume of the growth medium. Then, they were seeded at a density of 3000 cells/well in 96-well microtiter plates. A stock solution of sylviside (**1**, 20 mmol) was prepared in DMSO. After 4 h growth at 37 °C in a humidified atmosphere containing 5% CO_2, the cells were exposed for 72 h to various concentrations (1, 5, 10, 50, 100 μmol) of the test compound. Subsequently, a MTT (Sigma) solution was added (final concentration 0.5 mg/mL), and the cells, after a 4 h incubation under standard conditions, were solubilized overnight with a solubilizing solution (10% SDS in 0.01 mol of HCl). The solubilized formazan was quantified spectrophotometrically using a Labsystems Multiscan RC reader, with the absorbance recorded at 570 nm (test wavelength) and at 690 nm (reference wavelength). Adriamycin (Sigma), with a well-documented activity against cancer cell lines, was used as a positive control.[15] All experiments were carried out in duplicate.

Acknowledgment. T.J.M. acknowledges support from the Robert A. Welch Foundation (Grant F 130). A.A.A. thanks the Japan Society for the Promotion of Science (JSPS) for a short visit to Hiroshima University.

References and Notes

(1) *Flora URSS*, Volume XXV; Editio Academiae Scientiarum URSS: Moscow, Leningrad, 1959; p 381.
(2) Meragelman, T. L.; Silva, G. L.; Mongelli, E.; Gil, R. R. *Phytochemistry* **2003**, *62*, 569−572.
(3) Morimoto, M.; Kumeda, S.; Komai, K. *J. Agric. Food Chem.* **2000**, *48*, 1888−1891.
(4) Villagómez-Ibarra, J. R.; Sánchez, M.; Espejo, O.; Zúñiga-Estrada, A.; Torres-Valencia, J. M.; Joseph-Nathan, P. *Fitoterapia* **2001**, *72*, 692−694.
(5) Goun, E. A.; Petrichenko, V. M.; Solodnikov, S. U.; Suhinina, T. V.; Kline, M. A.; Cunningham, G. *J. Ethnopharmacol.* **2002**, *81*, 337−342.
(6) Molokovskii, D. S.; Davydov, V. V.; Khegai, M. D. *Rast. Res.* **2002**, *38*, 15−28.
(7) Rastrelli, L.; Saravia, A.; Hernandez, M.; De Simone, F. *Pharm. Biol.* **1998**, *36*, 315−319.
(8) Tachibana, K.; Okada, Y.; Okuyama, T. *Nat. Med.* **1995**, *49*, 266−268.
(9) Lauber, K.; Wagner, G. *Flora Helvetica*; Verlag Paul Haupt: Bern, 1998; p 1066.
(10) Broda, B.; Mowszowicz J. *Przewodnik do Oznaczania Roślin Leczniczych, Trujących i Użytkowych*; Wydawnictwo Lekarskie PZWL: Warsaw; 1996; p 684.
(11) Konopleva, M. M.; Glyzin, V. I.; Shelyuto, V. L. *Khim. Prir. Soedin.* **1978**, *3*, 402−403.
(12) Konopleva, M. M.; Shelyuto, V. L.; Smirnova, L. P.; Glyzin, V. I. In *Fitokhim. Izuch. Flory BSSR, Biofarm. Issled. Lek. Prep.*; Medvedskii, E. N., Ed.; Leningr. Med. Inst. Im. I.P. Pavlova: Leningrad, 1975; pp 93−95.
(13) Sheldrick, G. M. *SHELXTL PC*, Version 5.03; Bruker Analytical X-ray Systems Inc.: Madison, WI, 1994.
(14) Utz, I.; Spitaler, M.; Rybczyńska, M.; Ludescher, C.; Hilbe, W.; Regenass, U.; Grunicke, H.; Hoffmann J. *Int. J. Cancer* **1998**, *77*, 64−69.
(15) Wang, Y.; Cao. J.; Zeng, S. *World J. Gastroenterol*. **2004**, *10*, 1365−1368.

NP050144X