HETEROCYCLES, Vol. 74, 2007, pp. 931 - 936. © The Japan Institute of Heterocyclic Chemistry
Received, 15th March, 2007, Accepted, 25th April, 2007, Published online, 27th April, 2007. COM-07-S(W)2

CELL GROWTH AND CELL CYCLE INHIBITORY ACTIVITIES OF 20-EPIDIOSGENYL SAPONIN FROM *CALAMUS INSIGNIS*

Takashi Ohtsuki,[1] Noriko Kaneko,[1] Takashi Koyano,[2] Thaworn Kowithayakorn,[3] Nobuo Kawahara,[4] Yukihiro Goda,[4] and Masami Ishibashi*[,1]

[1]Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan, [2]Temko Corporation, 4-27-4 Honcho, Nakano, Tokyo 164-0012, Japan, [3]Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand, and [4]Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Abstract – A new 20 *epi*-diosgenyl saponin (**1**) was isolated from the stems of *Calamus insignis* (Palmae) by bioassay guided purification. The chemical structure of **1** was established on the basis of spectroscopic analysis and chemical means. Compound **1** showed cell growth inhibitory activity against HeLa cells (IC_{50}; 5.1 μM) and exhibited a cell cycle inhibitory effect at the G2/M stage at the concentration of 2.9 μM by flow cytometric analysis.

INTRODUCTION

During our search for bioactive natural products such as cell growth inhibitory and cell cycle inhibitory activity from tropical plants,[1,2] we investigated the chemical constituents of stems of *Calamus insignis* collected in Thailand. In recent paper, we reported the steroidal saponins from this plant and their cell growth and cell cycle inhibitory activity.[3] We further investigated constituents of this plant and here we report the isolation and structure determination of a new steroidal saponin, 20-epidiosgenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**1**). This compound showed cell growth inhibitory activity against HeLa cells (IC_{50}; 5.1 μM). Effects of **1** on the cell cycle progression of HeLa cells were also examined to reveal that **1** arrested the cell cycle at the G2/M stage at the concentration of 2.9 μM.

This paper is dedicated to Prof. Dr. Ekkehard Winterfeldt on the occasion of his 75th birthday.

RESULTS AND DISCUSSION

We have previously isolated five steroidal saponins from the *n*-BuOH soluble fraction of the MeOH extract of *C. insignis* collected in September 2001. In this study, we investigated the same plant newly collected in June 2005. The MeOH extracts of newly collected materials showed the same characteristics on TLC as before. The extract was partitioned between ethyl acetate and water, and the aqueous phase was further extracted with *n*-BuOH. Further separation of *n*-BuOH-soluble fraction using ODS column chromatography, followed by purification with reversed-phase HPLC gave compound **1**.

Compound **1,** $[\alpha]_D^{24}$ -26.4 (*c* 0.4, pyridine), was obtained as a white amorphous solid, and its molecular formula was suggested to be $C_{51}H_{82}O_{21}$ by the HRFABMS data [*m/z* 1069.5052, $(M+K)^+$, Δ +6.7 mmu]. The ^{1}H NMR spectrum of **1** (Table 1) showed characteristic proton signals due to one olefinic proton at δ_H 5.26 (1H, br d, *J*=4.8 Hz; H-6), two secondary methyl groups at δ_H 1.11 (3H, d, *J*=7.8 Hz; H_3-21) and 0.69 (3H, d, *J*=6.0 Hz; H_3-27) and two tertiary methyl groups at δ_H 1.04 (3H, s; H_3-19) and 0.95 (3H, s; H_3-18) of the steroidal nucleus, two methyl groups of 6-deoxyhexapyranose at δ_H 1.76 (d, *J*=6.0 Hz), 1.71 (d, *J*=6.6 Hz) and four anomeric protons at δ_H 6.20 (1H, s), 5.87 (1H, s), 5.06 (1H, d, *J*=8.4 Hz), and 4.92 (1H, overlapped). Also, the ^{13}C NMR spectrum of **1** showed 51 carbon signals including those of two olefinic carbons at δ_C 140.7 and 121.7 and four anomeric carbons at δ_C 104.9, 102.6, 101.6, and 99.9, suggesting **1** to be an olefinic steroidal glycoside having four sugar units. Upon the acid hydrolysis with 5% sulfuric acid, the monosaccharide units were determined as two D-glucose and two L-rhamnose on the basis of HPLC analysis by comparison with the authentic samples using a combination of the RI and optical rotation detectors and ^{1}H and ^{13}C NMR spectra. The configurations of the anomeric positions of two D-glucoses were assigned as β by judging from their large coupling constants between H-1 and H-2 (Glc B, $J_{1,2}$= 8.4 Hz) and the small one-bond coupling constants between C-1 and H-1 (Glc A, $^1J_{C1,H1}$= 160.9 Hz; Glc B, $^1J_{C1,H1}$= 162.8 Hz ; literature values[4]: α-anomer, 169-171 Hz; β-anomer, 158-162 Hz). The anomeric configurations for the two L-rhamnoses were deduced as α from the large one-bond coupling constant between C-1 and H-1 (Rha A, $^1J_{C1,H1}$=166.6 Hz; Rha B, $^1J_{C1,H1}$= 170.4 Hz).[4] The ^{13}C NMR spectrum of aglycone moiety of **1** was very similar to those of Cauialoside I (**2**),[3] a tetraglycoside isolated from *C. insignis* previously, except for the signals of the D- and E-ring parts of aglycone. In particular, C-21(δ_C 11.4) of **1** shifted to up-field from that of **2** (δ_C 15.0),[3] while C-20 (δ_C 46.6) of **1** shifted to downfield from that of **2** (δ_C 41.9).[3] Similarly, H-20 (δ_H 2.68) of **1** shifted to downfield from that of **2** (δ_H 1.94)[3] in the ^{1}H NMR spectrum, suggesting that **2** had the opposite configuration at C-20 with 21β-methyl group. This was confirmed by the ROESY correlations between H_3-21 (δ_H 1.11) and H_3-18 (δ_H 0.95), H-20 (δ_H 2.68) and H-17 (δ_H 1.98), H_3-21 (δ_H 1.11) and H-23 (δ_H 1.80). The linkages among aglycone and sugars moieties were determined on the basis of HMBC experiments. The HMBC

spectrum showed the ^{1}H-^{13}C long-range correlations between H-1 of Glc A (δ_H 4.92) and C-3 of aglycone (δ_C 78.1), H-1 of Glc B (δ_H 5.06) and C-4 of Glc A (δ_C 81.8), H-1 of Rha A (δ_H 5.87) and C-4 of Glc B (δ_C 77.1), H-1 of Rha B (δ_H 6.20) and C-2 of Glc A (δ_C 77.1).

Consequently, the structure of **1** was concluded as 20-epidiosgenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside.

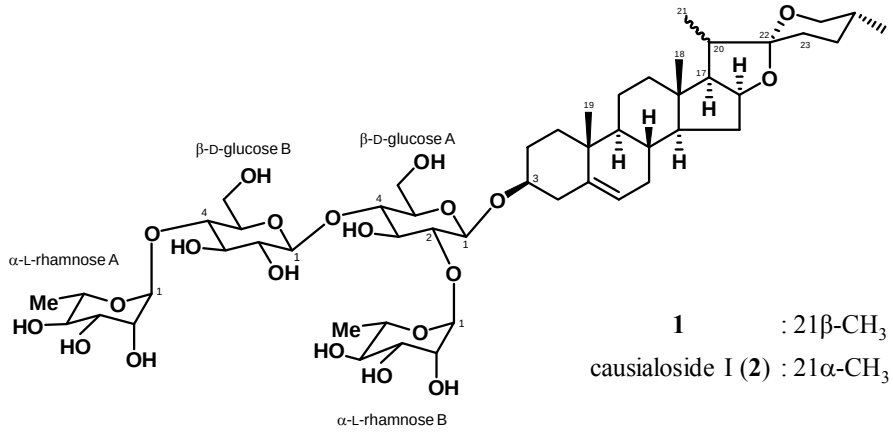

Fig.1 Structures of compounds **1** and **2**

Table 1. ^{1}H and ^{13}C NMR data for Compound **1** in pyridine-d_5

position	^{1}H	^{13}C
1	0.96 m	37.5
	1.73 m	
2	1.85 m	30.1
	2.07 m	
3	3.85 m	78.1
4	2.69 m	38.9
5		140.7
6	5.26 br d (4.8)	121.7
7	1.44 m	32.0
	1.83 m	
8	1.53 m	31.3
9	0.85 m	50.2
10		37.0
11	1.38 m	20.6
12	1.05 m	39.9
	1.77 m	
13		41.9
14	0.89 m	57.5
15	1.49 m	32.4
	2.03 m	
16	4.59 m	81.2
17	1.98 dd (6.4, 9.0)	60.9
18	0.95 s	16.1
19	1.04 s	19.3
20	2.68 m	46.6
21	1.11 d (7.8)	11.4
22		108.4
23	1.80 m	30.8
24	1.56 m	28.9
25	1.64 m	30.9
26	3.60 dd (3.6, 10.5)	68.1
	3.62 brd (10.5)	
27	0.69 d (6.0)	17.3

	position	^{1}H	^{13}C
Glc A	1	4.92 *ol.* *	99.9
	2	4.19 m	77.1
	3	4.12 m	76.3
	4	4.18 m	81.8
	5	3.81 m	76.1
	6	4.07 m	60.8
		4.20 m	
Glc B	1	5.06 d (8.4)	104.9
	2	3.98 brt (8.4)	75.1
	3	4.14 m	77.0
	4	4.46 m	77.1
	5	3.73 m	76.3
	6	4.43 brd (10.0)	61.6
		4.50 m	
Rha A	1	5.87 s	102.6
	2	4.65 br s	72.5
	3	4.51 dd (3.0, 9.0)	72.7
	4	4.33 t (9.2)	73.9
	5	4.95 m	70.3
	6	1.71 d (6.6)	18.4
Rha B	1	6.20 s	101.6
	2	4.71 br s	72.4
	3	4.55 dd (3.0, 9.2)	72.7
	4	4.33 t (9.2)	74.0
	5	4.92 m	69.4
	6	1.76 d (6.0)	18.6

Values in parentheses indicate coupling constants (*J* in Hz). *, overlapped

Cell growth inhibitory activitiy of **1** was examined by fluorometric microculture cytotoxicity assay (FMCA).[5] Compound **1** exhibited considerable inhibitory activity against HeLa cells with IC_{50} value of 5.4 μM. Although **1** was less active than **2** (IC_{50}; 1.5 μM), it showed almost the same activity comparing to that of either dioscin, representative diosgenyl glycoside (IC_{50}; 4.5 μM)[6] or mitomycin C (IC_{50}; 5.1 μM) as positive control.

The effects of **1** on the cell cycle distribution of HeLa cells were examined by flow cytometric analysis. As shown in Table 2, the cells treated with **1** induced an appreciable accumulation of cells of the G2/M phase at 2.9 μM after 24 h of incubation (50±5.1% against 12±0.5% of the control). However, treatment with concentration of 4.9 μM remarkably increased Sub-G1 and decreased G1 phase cells. We showed previously that treatment of HeLa cells with **2** for 24 h increased G2/M phase cell population as a result of expression of p21 and inhibition of dephosphorylation of cdc2. Since the structures of **1** and **2** are close, quantitative and qualitative changes of p21 and cdc2 might be concerned with the effect of G2/M phase arrest of **1** as that of **2**.

Table 2. Effect of compound **1** on the cell cycle distribution (%) of HeLa cells.

	Sub-G1	G1	S	G2/M
control	13±0.5	64±0.3	11±0.4	12±0.5
1.5 μM	11±1.2	60±1.7	13±0.9	15±0.5*
2.9 μM	15±2.3	23±0.6**	10±1.1	50±5.1**
4.9 μM	38±0.9**	15±0.4**	15±0.1**	33±0.6**

Data are given as the percentage of the total number of cells.

The values are mean±s.e.m. (** $p<0.01$, * $p<0.05$ vs. Control, $n=3$)

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured in JASCO FT-IR 230 spectrophotometer. NMR spectra were recorded on JEOL JNM ECA 800 and ECP600 spectrometers. High-resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JMS HX-110 mass spectrometer.

Plant Material. Stems of *Calamus insignis* were collected in Khon Kaen, Thailand, in June 2005 and were identified by T.K. A voucher specimen (6-696) is maintained at Faculty of Agriculture, Khon Kaen University.

Extraction and Isolation. The air-dried stems (316 g) were extracted with MeOH. The MeOH extract (48 g) was partitioned between hexane (500 mL x 3) and 10% aqueous MeOH (500 mL), and the

aqueous phase was further extracted with EtOAc (500 mL x 3) and *n*-BuOH (500 mL x 3) to give four fractions (hexane phase, 581 mg; EtOAc phase, 1.6 g; *n*-BuOH phase, 19.4 g; aqueous phase, 29.4 g). A part of the *n*-BuOH-soluble fraction (13.8 g) was subjected to ODS column chromatography (40 x 350 mm) eluted with gradient mixtures of 50-100% MeOH in H_2O to give seven fractions: A (7.5 g), B (501 mg), C (5.3 g), D (108 mg), E (874 mg), F (284 mg), and G (290 mg). Fraction E eluted with 80% MeOH was purified by preparative HPLC (Develosil ODS HG-5, 10 x 250 mm; eluent, 85% MeOH; flow rate, 1.5 mL/min) to afford compound **1** (7.5 mg, t_R 36 min).

20-Epidiosgenin3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (1): white amorphous solid, $[\alpha]_D^{24}$ -26.4 (*c* 0.4, pyridine); IR ν_{max} (film) 3367 and 1053 cm^{-1}; ^1H and ^{13}C NMR data in Table 1 and 2; FABMS *m/z* 1053 $(M+Na)^+$ and *m/z* 1069 $(M+K)^+$; HRFABMS *m/z* 1069.5052, calcd for $C_{51}H_{82}O_{21}K$, 1069.4985.

Acid Hydrolysis of Compound 1 and Determination of the Absolute Configuration of Sugars. Compound **1** (6.0 mg) in 1, 4-dioxane (4.5 mL) and 5% aqueous H_2SO_4 (3 mL) was heated at 95 ˚C for 2.5 h. After cooling to rt, water was added to the reaction mixture, and the mixture was partitioned with EtOAc. The aqueous layer containing the saccharide mixture was neutralized by passage through an Amberlite IRA-96SB column, then analyzed by HPLC (Capcell Pak NH_2 UG80, 4.6 x 250 mm; eluent, 85% MeCN; flow rate, 0.7 mL/min; column temperature, 40 ˚C; detection, RI and optical rotation (JASCO OR-1590)) according to the literature conditions[7] to identify L-rhamnose (t_R 8.33 min, negative peak in optical rotation detector) and D-glucose (t_R 14.49 min, positive peak in optical rotation detector).

Cell Growth Inhibitory Activity. The procedure of assay was the same as previously described.[3] Briefly, HeLa cells (6 x 10^3 cells) were treated with different concentrations of each isolated compound for 24 h at 37 °C. After the medium containing the isolated compounds was removed, cell growth inhibitory activity was determined by the FMCA method[5] using a fluorescence platereader.

Cell Cycle Analysis. Cell cycle analysis was measured the same as previously described.[3] Briefly, HeLa cells (5 x 10^5 cells) were treated with different concentrations of the samples at 37 °C for 24 h, were then fixed with 70% EtOH at 4 °C for 60 min, resuspended in 100 μg/mL of RNAs and 100 μg/mL propidium iodide to stain DNA and analyzed for DNA contents using a flow cytometer.

ACKNOWLEDGEMENTS

We thank Professor H. Takayama (Chiba University) for generous use of the optical rotation detector. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, from the Kampou Science Foundation, and from the Shorai

Foundation for Science and Technology.

REFERENCES

1. T. Ohtsuki, T. Koyano, T. Kowithayakorn, N. Yamaguchi, and M. Ishibashi, *Planta Med.*, 2004, **70**, 1170.
2. T. Ohtsuki, T. Koyano, T. Kowithayakorn, S. Sakai, N. Kawahara, Y. Goda, N. Yamaguchi, and M. Ishibashi, *Bioorg. Med. Chem.*, 2004, **12**, 3841.
3. T. Ohtsuki, M. Sato, T. Koyano, T. Kowithayakorn, N. Kawahara, Y. Goda, and M. Ishibashi, *Bioorg. Med. Chem.*, 2006, **14**, 659.
4. K. Bock, I. Lundt, and C. Pedersen, *Tetrahedron Lett.*, 1973, **13**, 1037.
5. R. Larsson, J. Kristensen, C. Sandberg, and P. Nygren, *Int. J. Cancer*, 1992, **50**, 177.
6. Z. Wang, J. Zhou, J. Yong, S. Yao, and H. Zhang, *Tsinghua Science and Technology*, 2001, **6**, 239.
7. M. Kuroda, Y. Mimaki, F. Hasegawa, A.Yokosuka, Y. Sashida, and H. Sakagami, *Chem. Pharm. Bull.*, 2001, **49**, 726.