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Bioorganic & Medicinal Chemistry 14 (2006) 659–665
Bioorganic & Medicinal Chemistry

Steroidal saponins from *Calamus insignis*, and their cell growth and cell cycle inhibitory activities

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Received 26 July 2005; revised 23 August 2005; accepted 23 August 2005
Available online 7 October 2005

Abstract—Three novel spirostanol-type (**2**, **3**, and **5**) and a furostanol-type (**4**) steroidal saponins were isolated from the stems of *Calamus insignis* (Palmae), along with a known steroidal saponin (**1**) by bioassay guided purification. The chemical structures of **1**–**5** were established on the basis of spectroscopic analysis and the result of acidic hydrolysis. Compounds **1**, **2**, **3**, and **5** showed cell growth inhibitory activity against HeLa cells at a concentration of less than 10 μM, and **1** exhibited a cell cycle inhibitory effect at the G2/M stage at concentrations of 1.5 and 2.9 μM by flow cytometric analysis. This effect seems to be correlated with large expression of p21 and inhibition of dephosphorylation of cdc2 according to the result of Western blotting analysis.

1. Introduction

The genus *Calamus* (Palmae) is a kind of rattan and widely grows in Southeast Asia and southern China and western Pacific. *Calamus insignis*, one of the genera, is used in making fine basketware and as a medicinal agent. During our search for bioactive natural products such as cell growth inhibitory activity and cell cycle inhibitory activity from tropical plants,[1] we investigated the chemical constituents of stems of *C. insignis* collected in Thailand. Here, we describe the isolation and structure determination of four new steroidal glycosides, three spirostanols (**2**, **3**, and **5**) and a furostanol (**4**), along with a known steroidal glycoside (**1**). The isolated spirostanol glycosides (**1**, **2**, **3**, and **5**) showed cell growth inhibitory activity against HeLa cells at low concentration (IC_{50} value: <10 μM). Effects of compounds **1** and **2** on the cell cycle progression of HeLa cells were also investigated to reveal that compound **1** arrested the cell cycle at the G2/M phase at concentrations of 1.5 and 2.9 μM. It was demonstrated that this effect resulted from expression of p21 and inhibition of dephosphorylation of cdc2 (see Fig. 1).

2. Results and discussion

The stems of *C. insignis* were extracted with MeOH, and the extract showed high cytotoxicity against HeLa cells. The MeOH extract was partitioned between hexane and 10% aqueous MeOH, and the aqueous phase was further extracted with EtOAc and *n*-BuOH to give four fractions. Since the most potent activity was found in the *n*-BuOH-soluble fraction (IC_{50} values, the *n*-BuOH-soluble fraction: 24.1 μg/mL; the other fractions: >50 μg/mL), the *n*-BuOH-soluble fraction was subjected to repeated ODS column chromatography, followed by final purification with reversed-phase HPLC on ODS to give five steroidal saponins (**1**–**5**). Among them, compounds **2**–**5** proved to be new compounds, while compound **1** was identified as a known compound, Causiaroside I, by comparison of the spectral data with those reported in the literature.[2]

Compound **2**, $[\alpha]_D^{24}$ −73.1° (*c* 0.78, pyridine), was obtained as a white amorphous solid, and its molecular formula was suggested to be $C_{57}H_{92}O_{26}$ by the

Keywords: Palmae; *Calamus insignis*; Steroidal saponin; Cell growth inhibition; Cell cycle arrest.
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doi:10.1016/j.bmc.2005.08.059

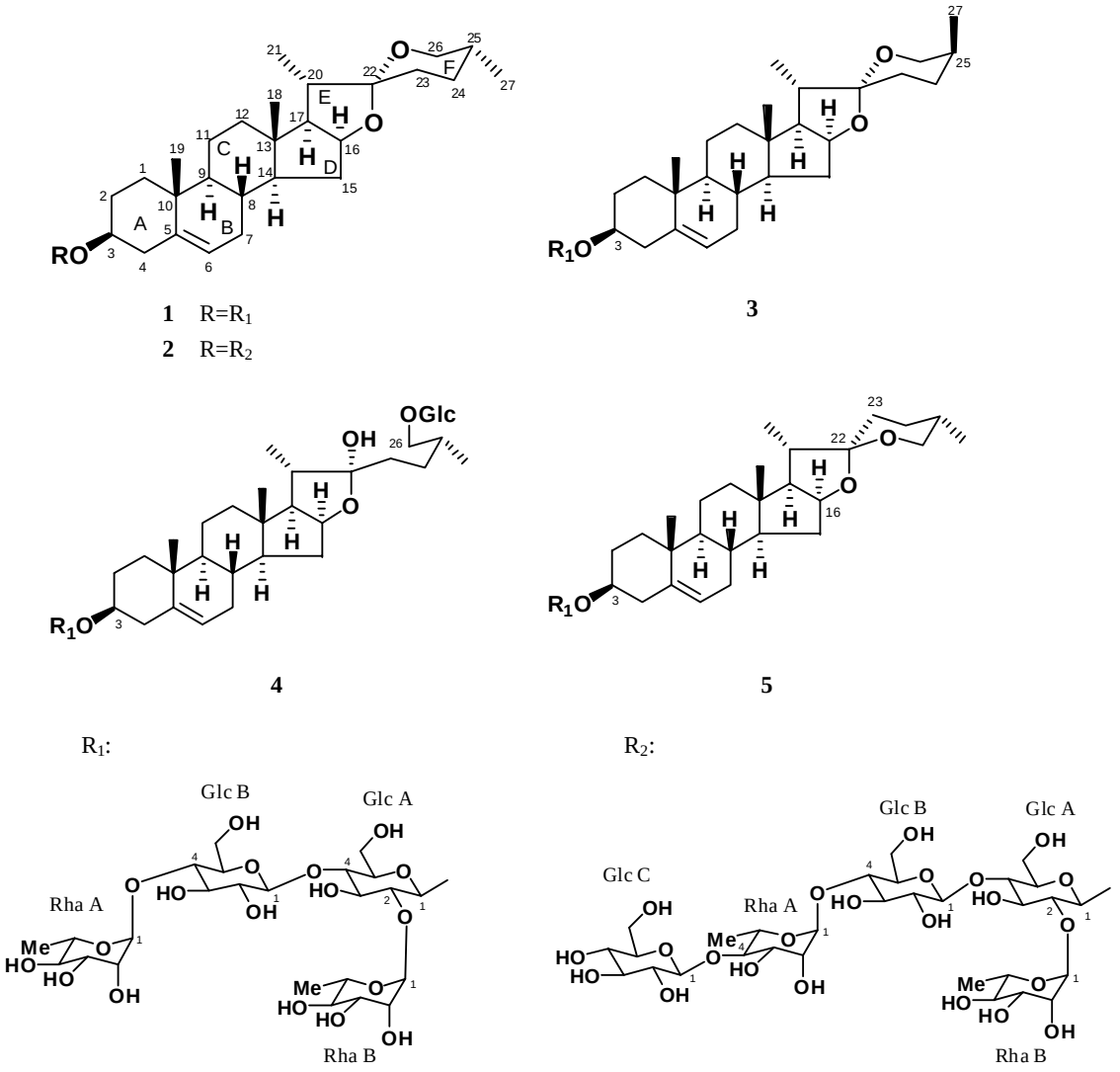

Figure 1. Structures of compounds **1**–**5**.

HRFABMS data [*m*/*z* 1215.5760, $(M+Na)^+$, Δ −1.4 mmu]. The ^1H NMR spectrum of **2** (Table 1) showed characteristic proton signals due to one olefinic proton at δ_H 5.27 (1H, br d, J = 6.0 Hz; H-6), two secondary methyl groups at δ_H 1.12 (3H, d, J = 7.0 Hz; H_3-21) and 0.68 (3H, d, J = 6.5 Hz; H_3-27), and two tertiary methyl groups at δ_H 1.03 (3H, s; H_3-19) and 0.81 (3H, s; H_3-18) of the steroidal nucleus, two methyl groups of 6-deoxyhexapyranose at δ_H 1.75 (d, J = 6.0 Hz), 1.74 (d, J = 6.0 Hz), and five anomeric protons at δ_H 6.19 (1H, s), 5.86 (1H, s), 5.25 (1H, d, J = 8.0 Hz), 5.06 (1H, d, J = 7.5 Hz), and 4.92 (1H, d, J = 7.5 Hz). Also, the ^{13}C NMR spectrum of **2** showed 57 carbon signals including those of five anomeric carbons at δ_C 106.7, 105.0, 101.8, 101.6, and 99.9, suggesting **2** to be a steroidal glycoside having five sugar units. The ^{13}C NMR chemical shifts for the aglycone moiety of **2** were very similar to those of **1**, suggesting that the aglycone of **2** was diosgenin, which is the same as that of **1**. On acidic hydrolysis with 5% sulfuric acid, **2** gave a crude sugar mixture. The ^1H and ^{13}C NMR data (Table 1) of **2** suggested that **2** contained three glucose and two rhamnose residues, which was confirmed by the HPLC analysis (Capcell Pak NH_2 UG80, 85%CH_3CN) of the crude sugar mixture obtained by acid hydrolysis. The absolute configurations of the sugar residues were determined to be D-glucose and L-rhamnose, respectively, on the basis of HPLC analysis by comparison with the authentic samples using a combination of the RI and optical rotation detectors. The configurations of the anomeric positions of two glucoses were assigned as β by judging from their large coupling constants between H-1 and H-2 of the sugar ring protons ($J_{1,2}$ values: Glc A, 7.5 Hz; Glc B, 7.5 Hz; Glc C, 8.0 Hz). The anomeric configurations for the two rhamnoses were deduced as α from the one-bond coupling constant between C-1 and H-1 (Rha A, $J_{C1,H1}$ values: 169.5 Hz, Rha B, $J_{C1,H1}$ values: 172.3 Hz; literature values[3]: α-anomer, 169 Hz; β-anomer, 160 Hz). The ^{13}C NMR chemical shifts for Glucose A, Glucose B, and Rhamnose B of **2** were very similar to those of **1**, whereas C-4 of Rhamnose A of **2** (δ_C 85.0) significantly shifted to downfield by ca. 11.0 ppm from that of **1** (δ_C 73.9), indicating that Glucose C was attached on C-4 of

Table 1. ^{1}H NMR data for compounds **2**–**5** in pyridine-d_5

	Position	**2**	**3**	**4**	**5**
Aglycone	1	0.98 m	0.96 m	0.94 m	0.96 m
		1.73 m	1.72 m	1.72 m	1.71 m
	2	1.85 m	1.83 m	1.85 m	1.84 m
		2.07 m	2.07 m	2.07 m	2.07 m
	3	3.86 m	3.86 m	3.84 m	3.87 m
	4	2.73 m	2.73 m	2.72 m	2.73 m
	5				
	6	5.27 br d (6.0)	5.27 br d (6.0)	5.26 br d (3.6)	5.26 br d (5.5)
	7	1.41 m	1.40 m	1.49 m	1.44 m
		1.87 m	1.84 m	1.84 m	1.85 m
	8	1.55 m	1.51 m	1.55 m	
	9	0.87 m	0.88 m	0.89 m	0.88 ddd (4.6, 11.6, 11.6)
	10				
	11	1.40 m	1.41 m	1.43 m	1.21 m
					1.48 m
	12	1.05 m	1.07 m	1.06 m	1.06 m
		1.64 m	1.66 m	1.72 m	1.65 m
	13				
	14	1.02 m	1.39 m	1.07 m	0.95 m
	15	1.48 m	1.51 m	1.43 m	1.44 m
		2.02 ddd (6.0, 7.0, 13.0)	2.00 ddd (6.0, 7.0, 13.0)	2.00 ddd (6.0, 7.0, 13.0)	2.00 ddd (6.0, 7.0, 13.0)
	16	4.54 q-like (7.5)	4.50 q-like (7.5)	4.96 m	4.29 q-like (7.5)
	17	1.76 m	1.76 m	1.91 m	1.56 m
	18	0.81 s	0.80 s	0.88 s	0.94 s
	19	1.03 s	1.03 s	1.04 s	1.01 s
	20	1.94 dq (7.0,7.5)	1.89 m	2.22 dq (6.5, 7.3)	2.31 dq (7.0, 7.3)
	21	1.12 d (7.0)	1.13 d (6.6)	1.32 d (6.5)	0.99 d (7.2)
	22				
	23	1.63 m	1.40 m	1.99 m	1.66 m
		1.66 m	1.88 m		
	24	1.52 m	1.34 m	1.67 m	1.41 m
			2.13 m	2.04 m	1.50 m
	25	1.54 m	1.57 m	1.92 m	1.61 m
	26	3.49 br d (12.0)	3.34 br d (11.0)	3.61 dd (6.0, 10.0)	3.67 m
		3.57 dd	4.04 m	3.93 m	
	27	0.68 d (6.5)	1.06 d (6.6)	0.97 d (6.6)	0.67 d (6.5)
Glc A	1	4.92 d (7.5)	4.93 d (8.0)	4.92 d (7.8)	4.93 d (7.8)
	2	4.18 m	4.20 m	4.19 m	4.19 m
	3	4.12 m	4.14 m	4.12 m	4.13 m
	4	4.14 m	4.16 m	4.15 m	4.16 m
	5	3.78 m	3.80 m	3.80 m	3.80 m
	6	4.03 br d (12.0)	4.04 m	4.06 m	4.07 br d (12.0)
		4.17 m	4.17 m	4.17 m	4.18 m
Glc B	1	5.06 d (7.5)	5.06 d (8.5)	5.06 d (7.8)	5.06 d (7.8)
	2	3.96 br t (8.0)	3.98 br t (7.8)	3.98 br t (7.8)	3.98 br t (7.8)
	3	4.15 m	4.14m	4.13 m	4.13 m
	4	4.49 m	4.45 m	4.45 m	4.46 m
	5	3.70 m	3.73 m	3.73 m	3.71 m
	6	4.42 m	4.43 m	4.42 m	4.43 br d (11.5)
		4.48 m	4.49 m	4.50 m	4.50 m
Glc C	1	5.25 d (8.0)			
	2	4.13 m			
	3	4.28 t (9.1)			
	4	4.21 t (9.1)			
	5	3.80 m			
	6	4.35 dd (5.0, 12.8)			
		4.42 m			
Rha A	1	5.86 s	5.87 s	5.87 s	5.87 s
	2	4.64 br s	4.66 br s	4.65 br s	4.65 br s
	3	4.67 dd (2.7, 9.4)	4.51 m	4.50 m	4.51 br d (9.5)
	4	4.45 t (9.5)	4.34 t (9.5)	4.33 t (9.0)	4.34 t (9.5)
	5	5.04 dq (6.0, 9.5)	4.99 m	4.98 m	4.99 dq (6.0, 9.5)
	6	1.74 d (6.4)	1.71 d (6.0)	1.70 d (6.0)	1.71 d (6.0)

(*continued on next page*)

Table 1 (*continued*)

	Position	2	3	4	5
Rha B	1	6.19 s	6.20 s	6.19 s	6.20 s
	2	4.70 br s	4.72 br s	4.72 br s	4.71 br s
	3	4.57 dd (2.7, 9.4)	4.56 dd (3.0, 9.0)	4.57 m	4.58 dd (3.0, 9.0)
	4	4.33 t (9.5)	4.34 t (9.0)	4.33 t (9.0)	4.34 t (9.0)
	5	4.93 m	4.94 m	4.92 m	4.93 m
	6	1.75 d (6.4)	1.76 d (6.0)	1.75 d (6.0)	1.75 d (6.0)
26-*O*-Glc	1			4.80 d (7.8)	
	2			4.06 m	
	3			4.23 m	
	4			3.96 m	
	5			3.94 m	
	6			4.38 dd (6.0, 12.6)	
				4.57 m	

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

Rhamnose A. The structure was finally confirmed by the HMBC technique. 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 77.8), 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 5.86) and C-4 of Glc B(δ_C 76.7), H-1 of Glc C (δ_H 5.25) and C-4 of Rha A (δ_C 85.0), and H-1 of Rha B (δ_H 6.19) and C-2 of Glc A (δ_C 77.1). Therefore, the structure of **2** was deduced as diosgenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

Compound **3**, $[\alpha]_D^{23}$ −80.4° (*c* 1.45, pyridine), was isolated as a white amorphous solid. The molecular formula was suggested to be $C_{51}H_{82}O_{21}$ by the HRFABMS data [*m*/*z* 1053.5201, $(M+Na)^+$, Δ −4.5 mmu]. Acid hydrolysis of **3** afforded D-glucose and L-rhamnose as sugar residues, being identified by HPLC analysis. The ^{13}C NMR chemical shifts of **3** were similar to those of **1**, except for the signals of the F-ring part. In particular, the methyl signal of C-27 was observed at δ_C 16.3, suggesting that the absolute configuration of C-25 was *S*, which was yamogenin (literature[4]: 25*S*, δ_C 16.2 ± 0.2; 25*R*, δ_C 17.1 ± 0.1). In addition, the ^{1}H NMR chemical shifts of the F-ring of **3** were very similar to average values of those of 25*S* spirostane-type steroidal sapogenins in the literature[5] (H_2-23: δ_H 1.40, 1.89; H_2-24: δ_H 1.39, 2.06; H-25: δ_H 1.71; H_2-26: δ_H 3.35, 4.04; H_3-27: δ_H 1.10). Thus, the structure of **3** was determined as yamogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyrano side.

Compound **4**, $[\alpha]_D^{24}$ −150.5° (*c* 1.57, pyridine), was obtained as a white amorphous solid. The HRFABMS of **4** showed a peak due to $(M+Na)^+$ at 1233.5833 (Δ −4.8 mmu), suggesting that its molecular formula was $C_{57}H_{92}O_{26}$. It showed a positive coloration with the Ehrlich reagent by TLC analysis, indicating that **4** was furostanol saponin.[6] In the ^{13}C NMR spectrum, chemical shifts of **4** were similar to those of **1**, except for the signals of C-20 to C-26 and additional signals corresponding to a β-D-glucopyranose unit. In addition, the HMBC spectrum displayed long-range correlation between H-1 of this β-D-glucopyranose (δ_H 4.80) and C-26 of aglycone (δ_C 75.1), indicating that **4** was 3, 26-di-*O*-glycosylated diosgenyl saponin. The configuration of 22-OH was deduced as α from the ROESY correlation between H-20 and H-23, and the ^{13}C NMR chemical shifts of C-22 (δ_C 112.0) in CD_3OD (literature values[7]: α-configuration, δ_C 112.0, β-configuration, δ_C 115.5). From these results, the structure of **4** was confirmed as 26-*O*-β-D-glucopyranosyl(25*R*)-furost-5-ene-3β,22α, 26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

Compound **5**, $[\alpha]_D^{24}$ −36.9° (*c* 1.0, pyridine), was isolated as a white amorphous solid. The HRFABMS of **5** exhibited a quasi-molecular ion peak at *m*/*z* 1053.5201, [$(M+Na)^+$, Δ −4.5 mmu], indicating the molecular formula as $C_{51}H_{82}O_{21}$. The ^{13}C NMR chemical shifts of **5** were similar to those of **3**, except for the signals of C-21 to C-27. The ^{1}H NMR chemical shifts of the E- and F-rings of **5** were different from that of **3**. In particular, H-16 (δ_H 4.29) and H_3-21 (δ_H 0.99) of the aglycone part of **5** shifted to up-field from that of **3** (H-16: δ_H 4.50, H_3-21: δ_H 1.13), while H-20 (δ_H 2.31) of the aglycone part of **5** resonated in lower-field than that of **3** (δ_H 1.89), suggesting that the absolute configuration of C-22 of **5** was *S*. The absolute configuration of C-22 was confirmed to be *S* by the ROESY correlation between H-16 and H-23α, and comparison with ^{13}C NMR data of C-21 to C-27 (Table 2) of 22-epiyamogenin glycoside (literature[8]: C-20, δ_C 42.1; C-21, δ_C 17.0; C-22, δ_C 110.6; C-23, δ_C 28.3; C-24, δ_C 28.1; C-25, δ_C 30.7; C-26, δ_C 69.6; C-27, δ_C 17.3). The absolute configuration of C-25 was determined by agreement with NMR data of H_2-26 (δ_H 3.67), H_3-27 (δ_H 0.67), C-25 (δ_C 30.6), and C-27 (δ_C 17.2) of 22-epiyamogenin glycoside (literature[8]: H_2-26, δ_H 3.67; H_3-27, δ_H 0.69; C-25, δ_C 30.7; C-27, δ_C 17.3). Consequently, the structure of **5** was concluded as 22-epiyamogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

Cell growth inhibitory activitiy of isolated compounds (**1**–**5**) was examined by fluorometric microculture cytotoxicity assay (FMCA).[9] As shown in Table 3, steroidal

Table 2. ^{13}C NMR data for compounds **2**–**5** in pyridine-d_5

	Position	2	3	4	5
	1	37.4	37.4	37.4	37.4
	2	30.1	30.1	30.1	30.0
	3	77.8	78.1	78.1	78.0
	4	38.8	38.8	38.9	38.8
	5	140.7	140.7	140.7	140.7
	6	121.7	121.7	121.8	121.7
	7	32.2	32.2	32.4	32.3
	8	31.6	31.6	31.6	31.4
	9	50.2	50.2	50.3	50.3
	10	37.0	37.1	37.0	37.0
	11	21.0	21.0	21.0	21.1
	12	39.8	39.7	39.8	40.2
	13	40.4	40.3	40.6	40.9
	14	56.5	56.5	56.5	55.7
	15	32.1	32.1	32.3	33.2
	16	81.0	81.1	81.0	80.7
	17	62.8	62.6	63.8	62.4
	18	16.2	16.2	16.4	16.6
	19	19.3	19.3	19.3	19.3
	20	41.9	42.4	40.7	42.0
	21	15.0	14.8	16.4	16.6
	22	109.2	109.7	110.6	110.5
	23	31.7	26.3	37.1	28.1
	24	29.2	26.1	28.3	28.0
	25	30.5	27.5	34.2	30.6
	26	66.8	65.0	75.1	69.6
	27	17.2	16.3	17.4	17.2
Glc A	1	99.9	99.9	99.9	99.9
	2	77.1	77.1	77.1	77.5
	3	76.3	76.3	76.3	76.1
	4	81.8	81.8	81.8	81.8
	5	78.4	76.1	76.1	77.5
	6	60.8	60.8	60.8	60.8
Glc B	1	105.0	105.0	105.0	105.0
	2	75.2	75.1	75.1	75.1
	3	77.5	77.6	77.6	77.0
	4	76.7	77.6	77.5	77.1
	5	77.0	77.1	77.0	76.3
	6	61.6	61.7	61.7	61.6
Glc C	1	106.7			
	2	76.4			
	3	71.2			
	4	78.6			
	5	76.1			
	6	62.5			
Rha A	1	101.8	102.6	102.6	102.6
	2	71.9	72.5	72.5	72.5
	3	72.4	72.7	72.7	72.7
	4	85.0	73.9	73.9	73.9
	5	68.4	70.3	70.3	70.3
	6	18.3	18.5	18.5	18.5
Rha B	1	101.6	101.6	101.6	101.6
	2	72.5	72.4	72.4	72.4
	3	72.6	72.7	72.7	72.7
	4	74.0	74.0	74.0	74.0
	5	69.4	69.4	69.4	69.4
	6	18.6	18.6	18.6	18.6
26-*O*-Glc	1			104.9	
	2			75.2	
	3			78.5	
	4			71.6	
	5			78.4	
	6			62.8	

Table 3. Cell growth inhibitory activities of compounds **1**–**5**, diosgenin, and mitomycin C against HeLa cells

Compound	IC_{50} (μM)
1	1.5
2	2.1
3	4.5
4	13.9
5	7.7
Diosgenin	17.5
Mitomycin C	5.1

saponins isolated from *C. insignis* exhibited considerable activity against HeLa cells with IC_{50} values ranging from 1.5 to 13.9 μM. Spirostanol saponins (**1**, **3**, and **5**) with the same sequence of the sugar chains were particularly more active than furostanol saponin (**4**). Compounds **1** and **2** showed stronger activity against HeLa cells than dioscin, representative diosgenyl glycoside (literature[10]: IC_{50} 4.5 μM), and mitomycin C (IC_{50} 5.1 μM) as positive control. Additionally, it is found that the sugar moiety of steroidal saponin had a crucial role in this effect against HeLa cells since **1** and **2** possessed more potent activity than their aglycone, diosgenin (IC_{50} 17.5 μM).

The effects of compounds **1** and **2** on the cell cycle distribution of HeLa cells were examined by flow cytometric analysis. As shown in Table 4, the cells treated with compound **1** induced an appreciable accumulation of cells of the G2/M phase at 2.9 μM after 24 h of incubation (38 ± 2.1% against 13 ± 2.2% of the control), and this effect took place in a dose-dependent manner. In contrast, treatment of HeLa cells with compound **2** for 24 h remarkably increased Sub-G1 and decreased G1 phase cells.

To define the mechanism of G2/M arrest by compound **1**, the expression of cell cycle regulatory proteins in the G2/M phase was evaluated by Western blotting analysis. Compound **1** treatment against HeLa cells caused up-regulation of p21 and phosphorylated cdc2 levels in a dose-dependent manner for 24 h, while cyclin B1 and p53 levels showed no change (Fig. 2). Accordingly, compound **1** inhibited the proliferation of HeLa cells through arresting in the G2/M phase by inhibiting dephosphorylation of cdc2 and inducing expression of p21. Also, the number

Table 4. Effect of compounds **1** and **2** on the cell cycle distribution (%) of HeLa cells

	Sub-G1	G1	S	G2/M
Compound **1**				
Control	16 ± 2.2	57 ± 1.7	14 ± 1.7	13 ± 2.2
0.73 μM	8 ± 1.4	56 ± 3.3	17 ± 0.5	19 ± 3.0
1.5 μM	11 ± 1.2	45 ± 0.9**	19 ± 1.0	25 ± 0.2**
2.9 μM	17 ± 1.0	26 ± 1.3**	19 ± 0.8	38 ± 2.1**
Compound **2**				
Control	16 ± 2.2	57 ± 1.7	14 ± 1.7	13 ± 2.2
1.0 μM	20 ± 1.9	45 ± 0.9**	20 ± 0.5	15 ± 0.9
2.1 μM	31 ± 3.6*	29 ± 1.0**	20 ± 1.1	20 ± 1.7
4.2 μM	40 ± 4.0**	25 ± 1.2**	18 ± 1.4	17 ± 2.2

Data are given as the percentage of the total number of cells. The values are means ± SEM ($^{**}p < 0.01$, $^{*}p < 0.05$ vs. Control, $n = 5$).

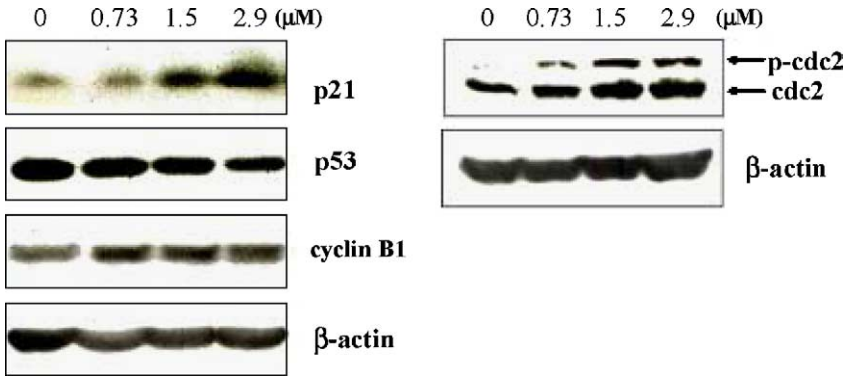

Figure 2. Effect of compound **1** on cell cycle regulatory proteins, p21, p53, cyclin B1, cdc2, and phosphorylated cdc2. Total protein was separated on 10% (p21, p53, and cyclin B1) or 12.5% SDS–PAGE (cdc2) ($n = 3$).

and/or sequence of the sugar part seemed to be important for the G2/M phase arrest against HeLa cells because the cell cycle inhibitory effect of compound **1** was quite different from that of compound **2**.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured on KBr disks 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.

3.2. Plant material

Stems of *C. insignis* were collected in Khon Kaen, Thailand, in September 2001 and were identified by T.K. A voucher specimen (6-397) is maintained at Department of Horticulture, Faculty of Agriculture, Khon Kaen University.

3.3. Extraction and isolation

The air-dried stems (315 g) were extracted with MeOH. The MeOH extract (62 g) was partitioned between hexane (500 mL × 3) and 10% aqueous MeOH (500 mL), and the aqueous phase was further extracted with EtOAc (500 mL × 3) and *n*-BuOH (500 mL × 3) to give four fractions (hexane phase, 1.2 g; EtOAc phase, 2.8 g; *n*-BuOH phase, 19.1 g; aqueous phase, 35.5 g). A part of the *n*-BuOH-soluble fraction (6.7 g) was subjected to ODS column chromatography (column A: 50 × 200 mm) eluted with gradient mixtures of 50–100% MeOH in H_2O to give 10 fractions: A (4.8 g), B (349.6 mg), C (1.1 g), D (86.2 mg), E (50.2 mg), F (13.2 mg), G (39.1 mg), H (194.8 mg), I (238.0 mg), and J (152.9 mg). Fraction H (194.8 mg) eluted with 80% MeOH was purified by preparative HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 80% MeOH; flow rate, 1.8 mL/min) to afford compound **2** (1.9 mg, t_R 39 min) and compound **1** (10.0 mg, t_R 42 min), and a crude fraction (18.1 mg, t_R 45 min), which was further purified by HPLC (YMC-pack pro C18, 10 × 250 mm; eluent, 85% MeOH; flow rate, 1.5 mL/min) to give compound **3** (3.2 mg, t_R 47 min). A part of fraction C (37.4 mg) eluted with 70% MeOH was further separated by HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 25% MeCN; flow rate, 1.5 mL/min) to afford compound **4** (14.1 mg, t_R 50 min). Fraction G (39.1 mg) eluted with 80% MeOH was purified by preparative HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 80% MeOH; flow rate, 1.5 mL/min) to yield compound **5** (1.9 mg, t_R 40 min).

3.3.1. Diosgenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (2). White amorphous solid, $[\alpha]_D^{24}$ −73.1° (*c* 0.82, pyridine); IR ν_{max} (KBr) 3420 and 1051 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; FABMS *m/z* 1215 $(M+Na)^+$ and *m/z* 1231 $(M+K)^+$; HRFABMS *m/z* 1215.5760, calcd for $C_{63}H_{104}O_{33}Na$, 1215.5774.

3.3.2. Yamogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (3). White amorphous solid, $[\alpha]_D^{23}$ −80.4° (*c* 1.45, pyridine); IR ν_{max} (KBr) 3401 and 1066 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; FABMS *m/z* 1053 $(M+Na)^+$ and *m/z* 1069 $(M+K)^+$; HRFABMS *m/z* 1053.5201, calcd for $C_{63}H_{104}O_{33}Na$, 1215.5246.

3.3.3. 26-*O*-β-D-Glucopyranosyl(25*R*)-furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (4). White amorphous solid, $[\alpha]_D^{24}$ −150.5° (*c* 1.57, pyridine); IR ν_{max} (KBr) 3392 and 1065 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; FABMS *m/z* 1233 $(M+Na)^+$ and *m/z* 1249 $(M+K)^+$; HRFABMS *m/z* 1233.5833, calcd for $C_{63}H_{104}O_{33}Na$, 1233.5881.

3.3.4. 22-Epiyamogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (5). White amorphous solid, $[\alpha]_D^{24}$ −36.9° (*c* 1.0, pyridine); IR ν_{max} (KBr) 3403 and 1058 cm^{-1}; ^1H and ^{13}C NMR data in Tables 1 and 2; FABMS *m/z* 1053 $(M+Na)^+$ and *m/z* 1069 $(M+K)^+$; HRFABMS *m/z* 1053.5201, calcd for $C_{63}H_{104}O_{33}Na$, 1053.5246.

3.4. Acid hydrolysis of compounds 2–5 and determination of the absolute configuration of sugars

Compound **2** (3.8 mg) in 1,4-dioxane (4.5 mL) and 5% aqueous H_2SO_4 (3 mL) was heated at 95 °C for 1.5 h. After cooling to room temperature, 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, and then analyzed by HPLC (Capcell Pak NH_2 UG80, 4.6×250 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,[11] to identify L-rhamnose (t_R 9.29 min, negative peak in optical rotation detector) and D-glucose (t_R 14.52 min, positive peak in optical rotation detector). Sugar residues of compound **3**–**5** were also analyzed by the same procedures as above to give the same results (L-rhamnose and D-glucose).

3.4.1. Cell growth inhibitory activity. The procedure of assay was the same as previously described.[1,12] Briefly, HeLa cells (6×10^3 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[9] using a fluorescence platereader.

3.4.2. Cell cycle analysis. Cell cycle analysis was measured the same as previously described.[1,12] Briefly, HeLa cells (5×10^5 cells) were treated with different concentrations of the samples at 37 °C for 24 h, were then fixed with 70% ethanol at 4 °C for 60 min, resuspended in 100 μg/mL RNAs and 100 μg/mL propidium iodide to stain DNA, and analyzed for DNA contents using a flow cytometer.

3.4.3. Western blotting analysis. HeLa cells treated and untreated with compound **1** or **2** for 24 h were washed with ice-cold PBS and then lysed in lysis buffer containing 1% SDS, 10 mM Tris–HCl (pH 7.4), and 1 mM sodium orthovanadate on ice. The lysate was centrifuged for 30 min at 4 °C, and the supernatants were frozen until analysis. Equal amounts of protein were loaded and separated by SDS–PAGE, and transferred electrophoretically onto Immun-blot PVDF membrane (Bio-Rad). After blocking with TBST (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk, the membrane was incubated at room temperature for 1 h with mouse monoclonal anti-p53 (1:1600), mouse monoclonal anti-p21 (1:400), mouse monoclonal anti-β-actin (1:1600), rabbit anti-cyclin B1 (1:2000), and rabbit anti-cdc2 (1:2000) as primary antibody. β-Actin was used as internal control. After washing with TBST, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (1:4000) or anti-rabbit IgG antibodies (1:4000) as secondary antibody. After washing with TBST, immunoreactive bands were detected by the ECL system (Amersham biosciences). The secondary antibodies were obtained from Amersham Biosciences. All the other antibodies were obtained from Sigma.

Acknowledgments

This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid from the Keimeikai Foundation.

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