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New chlorogenin hexasaccharide isolated from *Agave fourcroydes* with cytotoxic and cell cycle inhibitory activities

Takashi Ohtsuki,[a] Takashi Koyano,[b] Thaworn Kowithayakorn,[c] Shinobu Sakai,[d] Nobuo Kawahara,[d] Yukihiro Goda,[d] Naoto Yamaguchi[a] and Masami Ishibashi[a,*]

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

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Abstract—A new chlorogenin hexasaccharide (**1**) was isolated from leaves of *Agave fourcroydes* (Agavaceae). The structure of the new saponin was elucidated as chlorogenin 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-{β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)}-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (**1**) by spectroscopic analysis and the result of acidic hydrolysis. The new saponin (**1**) as well as known hexasaccharides (**3** and **5**) isolated here showed cytotoxicity against HeLa cells, and **1** exhibited a cell cycle inhibitory effect at the G2/M stage at the concentration of 7.5 and 10 μg/mL.

1. Introduction

Agave fourcroydes (Agavaceae) is a fiber plant, which is employed in the manufacture of agricultural twine, sacks, carpets, and other products.[1] Previous chemical investigations of the genus *Agave* led to the isolation of several steroidal saponins.[2,3] During our search for bioactive natural products from tropical plants,[4] we investigated the chemical constituents of leaves of *A. fourcroydes* collected in Thailand. Here we describe the isolation and structure elucidation of a new steroidal saponin, chlorogenin 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-{β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)}-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (**1**), along with four known saponins (**2**–**5**). Compound **1** as well as hexasaccharides (**3** and **5**) having the same sugar sequence as **1** showed cytotoxic activity against HeLa cells, while diglucoside (**2** and **4**) did not show cytotoxicity. Effects of compound **1** on the cell cycle progression of HeLa cells were also examined to reveal that compound **1** arrested the cell cycle at the G2/M stage at the concentration of 7.5 and 10 μg/mL.

2. Results and discussion

The leaves of *A. fourcroydes* were extracted with MeOH, and the extract showed high cytotoxicity against HeLa cells. After removal of chlorophylls by Diaion HP-20 column chromatography, the extract was partitioned between ethyl acetate and water, and the aqueous phase was further extracted with *n*-BuOH. Since the cytotoxic activity was found in the *n*-BuOH-soluble fraction, 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, compound **1** proved to be a new compound, while four compounds were known and they were identified as 23*S*,25*R*,5α-spirostane-3β,6α,23β-triol 3,6-di-*O*-glucopyranoside (**2**),[5] furcreastatin (**3**),[6] chlorogenin 3,6,-di-*O*-β-D-glucopyranoside (**4**),[7] and tigogenin 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-{β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)}-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (**5**),[8]

Keywords: Agavaceae; *Agave fourcroydes*; Steroidal saponin; Cytotoxicity; Cell cycle.
* Corresponding author. Tel./fax: +81-43-290-2913; e-mail: mish@p.chiba-u.ac.jp

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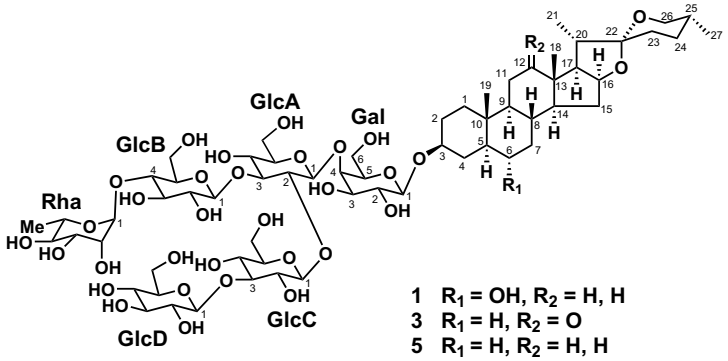
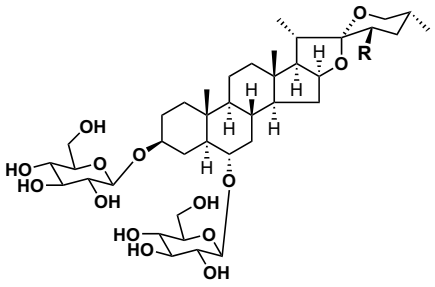




respectively, by comparison of the spectral data with those reported in the literature.

Compound **1**, $[\alpha]_D^{24}$ −16.6 (*c* 0.78, pyridine), was obtained as a white amorphous solid, and its molecular formula was suggested to be $C_{63}H_{104}O_{33}$ by the HRFABMS data [*m/z* 1411.6333, $(M+Na)^+$, Δ − 2.5 mmu]. The ^1H NMR spectrum of **1** (Table 1) showed characteristic proton signals for four methyl groups of the steroidal nucleus at δ_H 1.12 (3H, d, $J = 6.5$ Hz, C-21), 0.81 (3H, s, C-18), 0.68 (3H, s, C-19), and 0.67 (3H, d, $J = 6.0$ Hz, C-27), one methyl group of 6-deoxyhexapyranose at δ_H 1.68 (d, $J = 6.0$ Hz), and six anomeric proton signals at δ_H 5.79 (1H, s), 5.53 (1H, d, $J = 7.5$ Hz), 5.17 (1H, d, $J = 8.0$ Hz), 5.11 (1H, d, $J = 7.8$ Hz), 5.08 (1H, d, $J = 7.5$ Hz), and 4.86 (1H, d, $J = 7.7$ Hz), suggesting **1** to be a steroidal glycoside having six sugar units. On acidic hydrolysis with 5% sulfuric acid, **1** gave an aglycone and a crude sugar mixture. The aglycone was identified as chlorogenin (**6**) by comparison of optical rotation ($[\alpha]_D^{22}$ −48.8) and spectral data with those in the literature ($[\alpha]_D^{22}$ −42).[9–11] The ^1H and ^{13}C NMR data (Table 1) of **1** suggested that **1** contained four glucose, one galactose, and one rhamnose residues, which was confirmed by the HPLC analysis of the crude sugar mixture obtained by acid hydrolysis. The absolute configurations of the sugar residues were determined to be D-glucose, D-galactose, and L-rhamnose, respectively, by comparison with the authentic samples using a combination of the RI and optical rotation detectors. The configurations of the anomeric positions of all four glucoses and one galactose were assigned as β by judging

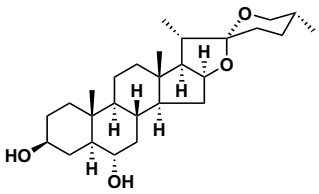
Table 1. ^1H and ^{13}C NMR data for compound **1** in pyridine-d_5

	Position	^1H, δ (*J*, Hz)	^{13}C, δ
Aglycone	1	0.84 m	37.6
		1.49 m	
	2	1.63 m	29.9
		2.01 m	
	3	3.87 m	77.6
	4	1.47 m	29.4
		1.78 br d (12.5)	
	5	1.17 m	52.1
	6	3.55 br d (8.8)	68.3
	7	1.14 m	42.6
		2.18 m	
	8	1.58 m	34.2
	9	0.60 ddd (3.6, 13.1, 13.1)	54.0
	10		36.4
	11	1.15 m	21.1
		1.38 m	
	12	1.01 ddd (4.0, 12.3, 12.3)	40.0
		1.64 br d (12.3)	
	13		40.7
	14	1.01 m	56.2
	15	1.43 m	32.1
		2.07 ddd (6.0, 7.0, 13.0)	
	16	4.51 br d (6.8)	81.0
	17	1.79 dd (6.8, 7.9)	62.9
	18	0.81 s	16.5
	19	0.68 s	13.4
	20	1.94 dq (6.5, 7.8)	41.9
	21	1.12 d (6.5)	14.9
	22		109.1
	23	1.67 m	31.7
	24	1.54 m	29.2
		1.65 m	
	25	1.56 m	30.5
	26	3.48 dd (11.0, 12.0)	66.8
		3.57 dd (4.0, 12.0)	
	27	0.67 d (6.0)	17.2
Gal	1	4.86 d (7.7)	102.2
	2	4.37 br t (9.6)	73.1
	3	4.06 m	75.5
	4	4.54 br s	79.9
	5	3.98 m	75.5
	6	4.16 m	60.4
		4.63 m	
Glc A	1	5.11 d (7.8)	104.7
	2	4.30 br t (9.0)	80.7
	3	4.09 m	88.1
	4	3.72 m	70.5
	5	3.78 m	77.4
	6	3.97 m	62.9
		4.40 m	

Table 1 (*continued*)

	Position	^{1}H, δ (J, Hz)	^{13}C, δ
Glc B	1	5.17 d (8.0)	104.1
	2	3.95 m	75.5
	3	4.07 m	76.4
	4	4.36 t (9.5)	77.7
	5	3.77 m	77.1
	6	4.04 m	61.0
		4.21 m	
Glc C	1	5.53 d (7.5)	103.9
	2	4.05 m	74.6
	3	4.02 m	88.1
	4	4.07 m	69.3
	5	3.75 m	78.0
	6	4.26 m	62.0
		4.45 br d (11.0)	
Glc D	1	5.08 d (7.5)	105.6
	2	3.97 br t (8.5)	75.5
	3	4.15 m	77.9
	4	4.16 m	71.4
	5	3.88 m	78.4
	6	4.24 m	62.4
		4.48 br d (11.6)	
Rha	1	5.79 s	102.5
	2	4.63 br s	72.5
	3	4.52 br d (9.0)	72.6
	4	4.33 d (8.5)	73.9
	5	4.95 dq (6.0, 8.5)	70.3
	6	1.68 d (6.0)	18.4

from their large coupling constants between H-1 and H-2 of sugar ring protons ($J_{1,2}$ values: Gal 7.7 Hz; Glc A, 7.8 Hz; Glc B, 8.0 Hz; Glc C, 7.5 Hz; Glc D, 7.5 Hz). The anomeric configuration for the rhamnose was deduced as α from the one-bond coupling constant between C-1 and H-1 ($J_{C1,H1}$ = 168.2 Hz; literature values:[12] α-anomer, 169 Hz; β-anomer, 160 Hz), and the ^{13}C NMR chemical shifts of C-3 (δ_C 72.6) and C-5 (δ_C 70.3) of rhamnose of **1** (literature values:[13] α-anomer, $\delta_{C\text{-}3}$ 72.5 and $\delta_{C\text{-}5}$ 69.4; β-anomer, $\delta_{C\text{-}3}$ 75.4 and $\delta_{C\text{-}5}$ 73.5). The ^{13}C NMR chemical shifts for the sugar moieties of **1** were very similar to those of **3** and **5** in the literature,[6,8] suggesting that the sugar sequence of **1** was the same as that of **3** and **5**, which was also suggested by the following observations. The C-3 of the aglycone part of **1** (δ_C 77.6) was significantly shifted to downfield by ca. 7.0 ppm from that of chlorogenin (**6**, δ_C 70.9), indicating that the sugar sequence was attached on C-3 of the aglycone. Evidence for these sugar–sugar and sugar–aglycone connectivities was also provided by the HMBC spectrum of **1**, which afforded the ^{1}H–^{13}C long-range correlations between H-1 of Gal (δ_H 4.86) and C-3 of aglycone (δ_C 77.6), H-1 of Glc A (δ_H 5.11) and C-4 of Gal (δ_C 79.9), H-1 of Glc B (δ_H 5.17) and C-3 of Glc A (δ_C 88.1), H-1 of Rha (δ_H 5.79) and C-4 of Glc B (δ_C 77.7), H-1 of Glc C (δ_H 5.53) and C-2 of Glc A (δ_C 80.7), H-1 of Glc D (δ_H 5.08) and C-3 of Glc C (δ_C 88.1). From all of these results, the structure of compound **1** was determined to be chlorogenin 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)-{β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)}-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside].



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Cytotoxic activity of isolated compounds (**1**–**5**) was examined by fluorometric microculture cytotoxicity assay (FMCA)[14] against HeLa cells.[15] As shown in Table 2, hexasaccharides (compounds **1**, **3**, and **5**) with the same sequence of sugar chains showed cytotoxic activity with IC_{50} values of 13.1, 5.2, and 4.8 μg/mL, respectively, while diglucosides (compounds **2** and **4**) were inactive. These results suggested that the sequence and/or the number of sugar moieties are important for cytotoxic activity.

The effects of compounds **1** and **3** on the cell cycle progression of HeLa cells were examined by flow cytometry. Although the effects on the cell cycle progression of aglycone part had been described previously,[16] such effects of steroidal glycosides have not been described to the best of our knowledge. As shown in Table 3, compound **1** induced an appreciable accumulation of cells of the G2/M phase at 7.5 μg/mL after 24 h of incubation (20% against 15% of the control), and this effect was enhanced at 10 μg/mL of **1** (25% against 15% of the control). However, on treatment with **1** at 25 μg/mL, dead cells (sub-G1 phase) increased and the number of cells at G1 and G2/M phase was markedly reduced. In the case of compound **3**, cell cycle inhibitory effect was not observed, but increment of sub-G1 cells was induced in a dose-dependent manner.

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 FTIR 230 spectrophotometer. NMR spectra were recorded on JEOL JNM ECA 800 and A500 spectrometers. High-resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JMS HX-110 mass spectrometer.

Table 2. Cytotoxic activities of compounds **1**–**5** against HeLa cells

Compounds	IC_{50} (μg/mL)
1	13.1
2	>200
3	5.2
4	>200
5	4.8

Table 3. Cell cycle distributions of HeLa cells after treatment with compounds **1** and **3** for 24 h

		1 μg/mL		5 μg/mL		7.5 μg/mL		10 μg/mL		25 μg/mL	
	Control	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
Sub-G1	7	N.T.	14	5	24	6	N.T.	9	91	43	N.T.
G1	63	N.T.	55	61	43	56	N.T.	47	4	28	N.T.
S	15	N.T.	19	19	18	18	N.T.	19	2	22	N.T.
G2/M	15	N.T.	12	15	15	20	N.T.	25	3	7	N.T.

Data are given as the percentage of the total number of cells. N.T.: not tested.

3.2. Plant material

Leaves of *A. fourcroydes* were collected in Khon Kaen, Thailand, in July 1999. A voucher specimen (6-245) is maintained at Department of Horticulture, Faculty of Agriculture, Khon Kaen University.

3.3. Extraction and isolation

The air-dried leaves (341 g) were extracted with MeOH. After removal of chlorophylls from the extract by Diaion HP 20 column chromatography (100 × 300 mm), the fraction (72.0 g) eluted with 100% MeOH was partitioned between EtOAc (1 L × 3) and 10% aqueous MeOH (1 L), and the aqueous phase was further extracted with *n*-BuOH (1 L × 3) to give three fractions (EtOAc phase, 3.2 g; *n*-BuOH phase, 17.0 g; aqueous phase, 37.3 g). A part of the *n*-BuOH-soluble fraction (6.9 g) was subjected to ODS column chromatography (column A: 50 × 200 mm) eluted with gradient mixtures of 50–100% MeOH in H_2O. The fraction eluted with 80% MeOH was further separated by ODS column (column B: 18 × 320 mm) eluted with gradient mixtures of 60–75% MeOH in H_2O. The fraction (46 mg) of column B eluted with 70–75% MeOH was then purified with HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 75% MeOH; flow rate, 1.8 mL/min) to afford compound **1** (6.0 mg, t_R 57 min) and compound **4** (8.2 mg, t_R 60 min). The fraction (120 mg) of column A eluted with 70% MeOH was further separated by ODS column (18 × 320 mm, 65% and 75% MeOH). A fraction (72 mg) eluted with 65% MeOH was partially (30 mg) purified with HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 60% MeOH; flow rate, 1.8 mL/min) to give compound **2** (14.1 mg, t_R 32 min). The fraction (201 mg) of column A eluted with 70% MeOH was separated by Sephadex LH-20 (13 × 500 mm) eluted with MeOH to give compound **3** (35.1 mg). The fraction (155 mg) of column A eluted with MeOH was separated by ODS column (18 × 320 mm, 60–80% MeOH), followed by separation with HPLC (Develosil ODS HG-5, 10 × 250 mm; eluent, 85% MeOH; flow rate, 1.5 mL/min) to yield compound **5** (6.1 mg, t_R 42 min).

3.3.1. Chlorogenin 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)-{β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)}-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside] (1). White amorphous solid, $[\alpha]_D^{24}$ −16.6 (*c* 0.78, pyridine); IR ν_{max} (KBr) 3398 and 1070 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; FABMS *m/z* 1411 $(M+Na)^+$ and *m/z* 1427 $(M+K)^+$; HRFABMS *m/z* 1411.6333, calcd for $C_{63}H_{104}O_{33}Na$, 1411.6358.

3.3.2. Acid hydrolysis of 1 and determination of the absolute configuration of sugars. A solution of **1** (5.7 mg) in 1,4-dioxne (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 extracted with EtOAc. The organic layer was evaporated to dryness in vacuo. The residue was purified by silica gel column chromatography to give **6** (1.0 mg). The aqueous layer containing the sugar mixture was neutralized by passage through an Amberlite IRA-96SB column, 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,[17] to identify L-rhamnose (t_R 7.8 min, negative peak in optical rotation detector), D-galactose (t_R 14.5 min, positive peak in optical rotation detector), and D-glucose (t_R 15.5 min, positive peak in optical rotation detector).

3.3.3. Chlorogenin (6). Amorphous solid, $[\alpha]_D^{22}$ −48.8 (*c* 0.10, MeOH), ^1H NMR (pyridine-d_5) δ_H 4.65 (1H, q, $J = 7.3$ Hz, H-16), 3.91 (1H, m, H-3), 3.65 (1H, m, H-6), 3.58 (1H, br d, $J = 10.5$ Hz, H-26eq), 3.49 (1H, br d, $J = 10.5$ Hz, H-26ax), 1.13 (3H, d, $J = 6.7$ Hz, H_3-21), 0.89 (3H, s, H_3-18 or H_3-19), 0.86 (3H, s, H_3-18 or H_3-19), and 0.67 (3H, d, $J = 5.2$ Hz, H_3-27); ^{13}C NMR (pyridine-d_5) δ_C 38.0 (C-1), 32.3 (C-2), 70.9 (C-3), 33.7 (C-4), 52.7 (C-5), 68.5 (C-6), 42.8 (C-7), 34.3 (C-8), 54.2 (C-9), 36.5 (C-10), 21.3 (C-11), 40.0 (C-12), 40.8 (C-13), 56.4 (C-14), 32.1 (C-15), 81.0 (C-16), 63.0 (C-17), 16.5 (C-18), 13.7 (C-19), 42.0 (C-20), 14.9 (C-21), 109.1 (C-22), 31.8 (C-23), 29.2 (C-24), 30.5 (C-25), 66.8 (C-26), and 17.2 (C-27); EIMS *m/z* 432 (M^+). These data were identical with those described in the literature.[9–11]

3.3.4. Cytotoxic activity. HeLa cells were seeded onto 96-well microtitre plates at 6×10^3 cells per well, and were pre-incubated for 24 h at 37 °C. The medium was replaced with fresh medium containing different concentrations of each isolated compound. The cells were then incubated at 37 °C for 24 h. After the medium containing the isolated compounds was removed, cell proliferation was determined by fluorometric microculture cytotoxicity assay (FMCA)[14] using a fluorescence platereader. The ratio of the living cells was determined

as the fluorescence in the sample wells expressed as a percentage of that in the control wells, and cytotoxic activity was indicated as an IC_{50} value.

3.4. Cell cycle analysis

Cells were seeded at a density of 1×10^5 cells/mL in 5 mL into a dish and were pre-incubated at 37 °C for 24 h. Next, different concentrations of the samples were added, and the cells were incubated at 37 °C for 24 h. After incubation, the cells were washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol at 4 °C for 60 min. After washing with PBS, the cells were treated with 100 μg/mL of RNAs followed by addition of 100 μg/mL propidium iodide for 15 min to stain DNA and analyzed for DNA contents using a flow cytometer.[18]

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