Cytotoxic Alkaloids and a Flavan from the Bulbs of *Crinum asiaticum* var. *japonicum*

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A new pyrrolophenanthridone alkaloid, criasiaticidine A (1), was isolated from the bulbs of *Crinum asiaticum* var. *japonicum*, together with pratorimine (2), lycorine (3) and 4′-hydroxy-7-methoxyflavan (4). The structure of the new alkaloid was determined to be 4,5-etheno-9,10-dihydroxy-6-phenanthridone by spectroscopic means. The cytotoxicity of the isolated compounds 1—4 was evaluated *in vitro* against Meth-A (mouse sarcoma) and Lewis lung carcinoma (mouse lung carcinoma) tumor cell lines. Furthermore, 3 was examined for *in vivo* antitumor activity with LLC tumor cells.

Key words *Crinum asiaticum* var. *japonicum*; alkaloid; criasiaticidine A; cytotoxicity; lycorine; antitumor activity

In the continuing search for antitumor compounds from natural sources, EtOAc- and alkaline EtOAc-soluble fractions of the bulbs of *Crinum asiaticum* var. *japonicum* Baker (Amaryllidaceae) showed significant cytotoxicity, with ED_{50} values of 1.1 and 0.9 μg/ml, respectively, against Lewis lung carcinoma (LLC) tumor cells. *C. asiaticum* var. *japonicum* grows wild only in Japan and Korea.[1)] As regards phytochemical studies on this plant, the isolation of phenanthridine alkaloids, fatty acids, sterols and triterpene alcohols have been previously reported.[2—4)] Alkaloids isolated from the bulbs of the tribe Amaryllidaceae showed various pharmacological and microbiological effects, such as antiviral,[5)] antimalarial,[6)] cytotoxic[6—9)] and antineoplastic activities,[10—12)] as well as effects on diseases of the nervous system.[13)]

An EtOAc-soluble fraction of *C. asiaticum* var. *japonicum* was separated by column chromatography to afford a new pyrrolophenanthridone alkaloid, criasiaticidine A (**1**). Two known alkaloids, pratorimine (**2**) and lycorine (**3**), and 4′-hydroxy-7-methoxyflavan (**4**) were also isolated. The characterization of the new alkaloid **1** and the cytotoxic activity of **1**—**4**, as well as the *in vivo* antitumor activity of **3**, are reported in this paper.

Results and Discussion

From EtOAc- and alkaline EtOAc-soluble fractions of the MeOH extract of the bulbs of *C. asiaticum* var. *japonicum*, three phenanthridine alkaloids and a flavan were isolated by a combination of silica gel column chromatography and reversed phase medium pressure liquid chromatography (MPLC), followed by crystallization (Chart 1). The known compounds were identified as pratorimine (**2**),[14—17)] lycorine (**3**),[6,18)] and 4′-hydroxy-7-methoxyflavan (**4**)[19)] by comparison of the spectral properties with those reported previously.

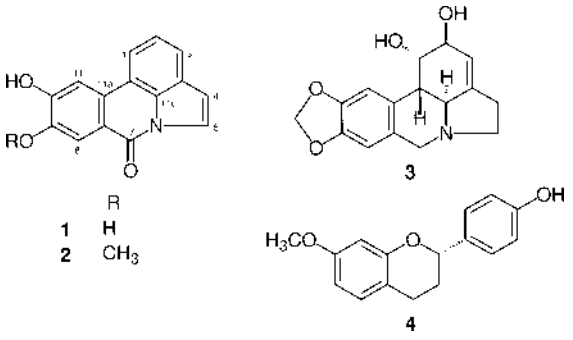

Chart 1. Structures of Compounds Isolated from the Bulbs of *Crinum asiaticum* var. *japonicum*

Criasiaticidine A (**1**) was isolated as pale brown needles (CH_3CN–H_2O), mp 277—279 °C. The molecular formula of **1** was assigned to be $C_{15}H_9NO_3$ from its molecular ion peak at *m/z* 251.0567 in the high-resolution electron impact mass spectrum (HR-EI-MS). The UV spectrum showing absorption bands at 225, 235, 253, 258, 296 and 345 nm indicated the presence of a pyrrolophenanthridone derivative.[20)]

The proton nuclear magnetic resonance spectrum (^{1}H-NMR) and carbon-13 nuclear magnetic resonance spectrum (^{13}C-NMR) data of **1** were similar to those for **2**, except for the absence of a methoxy group in **1** (Table 1). Information for all of the functional groups and their locations in the molecule was obtained from the heteronuclear multiple-bond correlation spectrum (HMBC) (Fig. 1). Significant HMBC correlations between signals at δ_H 7.76 (H-11) and δ_C 146.8

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2** (DMSO-d_6, δ ppm; *J* in Hz; 400 MHz, ^{1}H; 100 MHz, ^{13}C)

	1		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	118.1	8.04 (d, 7.7)	118.5	8.09 (d, 7.7)
2	124.0	7.48 (t, 7.7)	124.1	7.50 (t, 7.7)
3	121.3	7.78 (d, 7.7)	122.3	7.82 (d, 7.7)
3a	127.8		127.9	
4	110.6	7.03 (d, 3.5)	110.7	7.04 (d, 3.5)
5	123.3	8.06 (d, 3.5)	123.5	8.10 (d, 3.5)
7	157.4		157.4	
7a	118.8		118.6	
8	114.1	7.79 s	109.0	7.85 s
9	146.8		148.7	
10	151.7		152.4	
11	108.8	7.76 s	110.1	7.82 s
11a	127.8		129.1	
11b	118.1		116.2	
11c	130.3		130.4	
OCH_3			55.8	3.96 s (3H)

δ values in ppm and coupling constants (in parentheses) in Hz.

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(C-9), as well as between those at δ_H 7.79 (H-8) and δ_C 151.7 (C-10), confirmed the presence of two hydroxyl groups at C-9 and C-10, as shown in **1**. This was further supported by the chemical shift difference of C-7 to C-11 of **1**, compared with those of **2** (Table 1), and eliminated the possibility of a methoxy substituent at either C-9 or C-10. An sp^2 methine carbon signal at δ_C 110.6 (C-4) showed long-range correlations with signals at δ_H 7.78 (H-3) and 8.06 (H-5). The former proton was further coupled with another quaternary carbon at δ_C 130.3 (C-11c). This suggested the presence of a nuclear indole at C-3a, 4, 5, 11c and N-6. The placement of a carbonyl carbon at C-7 was supported by the observation of long-range correlations of signals at δ_H 7.76 (H-11) and 7.79 (H-8) with a carbon resonance at δ_C 157.4. Consequently, the structure of **1** was determined to be 4,5-etheno-9,10-dihydroxy-6-phenanthridone.

Compounds **1**—**4** were tested for their cytotoxicity against Meth-A (mouse sarcoma) and Lewis lung carcinoma (mouse carcinoma) tumor cell lines. Compound **1** showed moderate cytotoxic effects with ED_{50} values of 3.2 and 4.2 μg/ml, respectively, on Meth-A and LLC cells, and **3** showed strong cytotoxicity (ED_{50}, 0.3 and 0.5 μg/ml) against both tumor cells. Compound **2** exhibited moderate activity against only Meth-A cells (ED_{50}, 4.1 μg/ml), while **4** was inactive.

The cytotoxic properties of **3** agreed with those previously reported for several human and mouse tumor cell lines with ED_{50} values of 0.3—1.8 μg/ml.[6,9,21—22] Compound **4** has been reported to show an inhibitory effect on the incorporation of ^{3}H-thymidine in Molt 4 cells (child T-cell leukemia), with an IC_{50} value of less than 10 μg/ml, as well as a cytotoxic effect on Molt 4 cells (ED_{50}, 42 μg/ml).[7]

Compound **3** was also reported to inhibit the *in vivo* growth of a murine tranplantable ascites tumor.[20] Therefore, we examined the *in vivo* antitumor activity of lycorine with Lewis lung carcinoma tumor cells in BDF-1 mice. When the mice were treated for 2 weeks, **3** showed antitumor activity with a T/C value (tumor inhibition rate, as described in Experimental) of 80.5% at a dose of 10 mg/kg on day 19 (Table 3 and Fig. 2). Compound **3** at a dose of 10 mg/kg maximally decreased the body weight by approximately 5% of the control, but the body weight loss was gradually improved to the range of the control after the termination of administration (Table 3). On considering the antitumor activity and toxicity, **3** might be used as a lead to develop a potential anticancer agent.

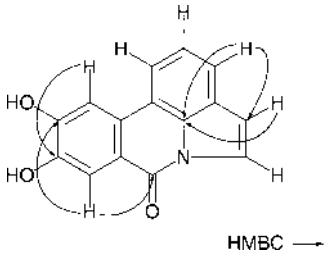

Fig. 1. Long-Range Correlations Observed in the HMBC Spectrum of **1**

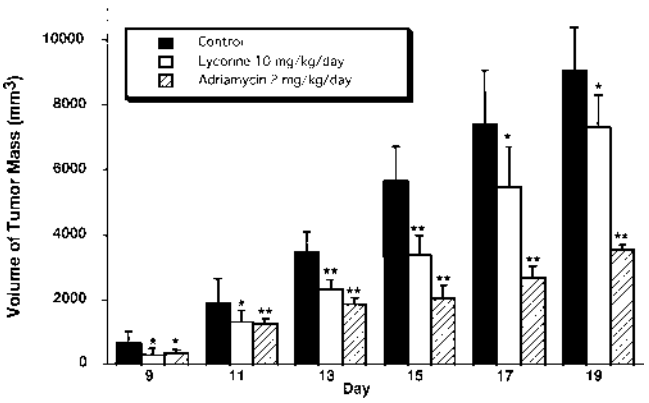

Fig. 2. *In vivo* Antitumor Activity of Lycorine (**3**) against LLC Tumor Cells.

Statistical Significance: $*p<0.05$, $**p<0.001$, *vs.* control. Points, Mean±S.E. (Lycorine: 10 mg/kg/d, Adriamycin: 2 mg/kg/d)

Experimental

Melting points were measured on a Yanagiomoto micro hot-stage melting point apparatus and are not corrected. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co.). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu Co.). IR spectra were measured on a FT/IR-230 infrared spectrometer (Jasco Co.). ^{1}H- and ^{13}C-NMR spectra were measured with Jeol JNA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; Jeol Co.) spectrophotometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. HR-EI-MS and EI-MS were measured with a JMX-AX 505 HAD mass spectrophotometer (Jeol Co.). Column chromatography was carried out on silica gel (Kieselgel 60, 70—230 mesh, Merck Co.). MPLC was carried out on a LiChroprep RP-18 (size A, Merck Co.). Thin layer chromatography (TLC) was carried out on pre-coated Silica gel 60 F_{254} plates (0.25 mm, Merck Co.) and RP-18 F_{254} S (0.25 mm, Merck Co.), and spots were detected under a UV light and by spraying with Dragendorff reagent.

Plant Material The bulbs of *C. asiaticum* var. *japonicum* Baker (Amaryllidaceae) were collected during October 1999 at Cheju-island,

Table 2. Cytotoxicity of Compounds (**1**—**4**) against Meth-A and LLC Tumor Cells

Compound	ED_{50} (μg/ml)	
	Meth-A	LLC
Criasiaticidine A (**1**)	3.2	4.2
Pratorimine (**2**)	4.1	>10
Lycorine (**3**)	0.3	0.5
4′-Hydroxy-7-methoxyflavan (**4**)	>10	>10
Adriamycin[a]	<0.09	0.1

a) Positive control.

Table 3. Antitumor Activity of Lycorine (**3**) against LLC Implanted s.c. in Mouse

Compound	Dose (mg/kg/d)[a]	No. of mice	Tumor		*p* value *versus* Control	Body wt. (T/C, %)
			Size (mm^3)[b]	T/C (%)		
Control		6	9089±545			100
Lycorine (**3**)	10	6	7321±587	80.5	<0.05	99.5
Adriamycin[c]	2	6	3566±168	39.2	<0.001	68.9

Listed are the data on day 19. *a*) Drugs were administered intraperitoneally once daily for 2 consecutive weeks. *b*) Mean±S.E. *c*) Positive control.

Korea, and dried at room temperature. A voucher specimen (CNU1005) is deposited in the herbarium of the College of Pharmacy, Chungnam National University, Taejon, Korea.

Tumor Cells Meth-A (mouse sarcoma) and LLC (mouse lung carcinoma) cells were purchased from RIKEN Cell Line Bank (Tsukuba, Japan). The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 7% fetal bovine serum, sodium bicarbonate (2 g), penicillin G (100000 units) and streptomycin (100 mg).

Isolation Procedure The minced bulbs of *C. asiaticum* var. *japonicum* (3 kg) were extracted with MeOH (10 l×3) to give an extract (1091 g). A part of the MeOH extract (1 kg) was suspended in H_2O (2000 ml) and extracted with hexane (1000 ml×3) to give a hexane soluble fraction (21 g). The H_2O layer was extracted with EtOAc (1000 ml×3, 6.9 g). The resulting H_2O layer was basified with NH_4OH to pH 9.0, and then extracted with EtOAc (40.2 g) and BuOH (26.7 g), respectively. Each fraction was then evaluated for cytotoxic activity against LLC tumor cells. The EtOAc-fraction (ED_{50}, 1.1 μg/ml) and the basic EtOAc-fraction (ED_{50}, 0.9 μg/ml) were selected for further investigation. The EtOAc-soluble fraction (6.9 g) was chromatographed on a column of silica gel (300 g). The column was eluted with a stepwise gradient of $CHCl_3$ and MeOH to give 4 fractions (Fr. A—D; 2.9, 1.1, 0.6 and 1.1 g, respectively). Repeated column chromatography of Fr. A on silica gel (hexane–acetone, 4 : 1) and MPLC on RP-18 (50 and 75% aq. CH_3CN) afforded **1** (1.5 mg), **2** (11 mg) and **4** (7.2 mg). The crude basic-EtOAc fraction was dissolved in MeOH, kept overnight at room temperature, and lycorine (**3**, 1.5 g) was crystallized as colorless prisms.

4,5-Etheno-8,9-dihydroxy-6-phenanthridone (**1**, Criasiaticidine A): Pale brown needles (CH_3CN–H_2O, 1.5 mg), mp 277—279 °C. UV λ_{max} nm (log ε, MeOH): 225 (4.10), 235 (4.19), 253 (4.30), 258 (4.30), 287 (sh), 296 (4.26), 345 (3.89), 355 (sh). IR ν_{max} cm^{-1}: 3410, 1671, 1613, 1509, 1459, 1328. EIMS *m/z* (rel. int.): 251 $[M]^+$ (100). HR-EIMS *m/z*: 251.0567 (M^+, Calcd for $C_{15}H_9NO_3$: 251.0583). ^{1}H- and ^{13}C- NMR: see Table 1.

Pratorimine (**2**): Pale brown needles (CH_3CN–H_2O, 11 mg), mp 224—226 °C. UV λ_{max} nm (log ε, MeOH): 203 (4.29), 225 (4.31), 235 (4.30), 252 (4.44), 257 (4.30), 287 (sh), 296 (4.34), 336 (sh), 345 (4.02), 358 (3.92). IR ν_{max} cm^{-1}: 3330, 1650, 1613, 1502, 1439, 1320. EI-MS *m/z* (rel. int.): 265 $[M]^+$ (100).

Lycorine (**3**): Colorless prisms (MeOH, 1.5 g), mp 254—256 °C. $[\alpha]_D^{26}$: −105° (MeOH, c=0.1). UV λ_{max} nm (log ε, MeOH): 205 (4.42), 235 (3.62), 292 (3.70). IR ν_{max} cm^{-1}: 3410, 1671, 1613, 1509, 1459, 1328. EIMS *m/z* (rel. int.): 287 $[M]^+$ (60) with a base peak at 226.

4′-Hydroxyl-7-methoxyflavan (**4**): Colorless prisms (hexane–acetone, 7.2 mg), mp 115–117 °C. $[\alpha]_D^{26}$: −12° (MeOH, c=0.1). UV λ_{max} nm (log ε, MeOH): 234 (4.26), 282 (3.74), 286 (sh). IR ν_{max} cm^{-1}: 3378, 1671, 1617, 1587, 1501, 1152, 832. EI-MS *m/z* (rel. int.): 256 $[M]^+$ (15) with a base peak at 55.

Cytotoxicity Assay LLC cells were cultured with RPMI 1640 medium containing 7% fetal bovine serum (FBS). For sulforhodamine B (SRB) assay,[23] the cells were cultured in RPMI 1640 medium containing 7% FBS. A portion of the cell suspension (4—5×10^4 cells/ml) in the culture medium was inoculated to each well of 96-well microtiter plates. One day after plating, a time zero control plate was made. Compounds were directly treated, and the cells were incubated for a further 48 h in a humidified 5% CO_2 atmosphere at 37 °C. The cells were fixed with 50 μl of 50% trichloroacetic acid (TCA) solution for 1 h at 4 °C, and the plates were washed 5 times with tap water and air-dried. A 50 μl sample of SRB solution (0.4% in 1% acetic acid) was added, and staining was done at room temperature for 30 min. The residual dye was washed out with 1% acetic acid, and the plates were air-dried. To each well, Tris buffer solution (10 mM, pH 10.5) was added. Optical density (OD) was measured with a microtiter plate reader at 540 nm. Growth inhibition was calculated as follows: the OD of the treated well was subtracted with the OD at a time-zero (Tz) plate and divided by a calculated value of untreated control. 50% Growth inhibition of (ED_{50}) was calculated by the Probit method.[24]

Animal Specific pathogen-free female BDF-1 mice (4 weeks of age, weighing 14 to 16 g) were purchased from Japan SLC, Inc. (Shizuoka). The animals were fed a commercial pellet chow (Clea Japan Inc., Tokyo) in a temperature-controlled room at 25±2 °C and water *ad libitum*.

Tumor Transplantation LLC cells were maintained in cell culture. A suspension of 5×10^5 cells in 0.2 ml of 0.9% NaCl solution was inoculated subcutaneously into the left flank of mice for the subcutaneous tumor assay.[25]

Drug Compound **3** was dissolved in 0.1 ml of 0.9 % NaCl solution, and administered intraperitoneally once daily for 2 consecutive weeks to mice. Control animals were given 0.1 ml of a 0.9 % NaCl solution by i.p. injection.

Estimation Tumors were measured on each alternate day using a vernier caliper from the initiation of treatment to the time when gross ulceration of the tumor was developed in control mice. The tumor size was calculated as:

$$\text{tumor vol. } (mm^3) = 0.5 \times a \times b^2$$

where a is the longest diameter and b is the shortest diameter.[26] The effects by treatments were represented by:

$$\text{T/C (\%)} = (\text{mean value of a treated group/mean value of a control group}) \times 100$$

Statistical Analysis The significance of differences between the experimental groups was calculated by Dunnett's t test. $p<0.05$ was considered significant.

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