γ-Lactone Carbazoles from *Clausena anisata*

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Received November 17, 2008

The study of chemical constituents of the stems of *Clausena anisata* collected in Thailand led to the isolation and identification of eight known and two new carbazole alkaloids named furanoclausamines A (**1**) and B (**2**). Clausamine E (**3**) was found to exhibit cytotoxicity against the human leukemia cell line HL-60.

Our previous studies of the constituents of plants of the genus *Clausena* have resulted in the isolation of some carbazole alkaloids.[1−5] It has been reported that carbazole alkaloids possess various biological activities such as antitumor, antioxidative, antimutagenic, and antiinflammatory activities.[6−8] In a primary screening for novel antitumor agents, we found that several carbazole alkaloids isolated from *Clausena* potently inhibited Epstein−Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells.[4,5] We have demonstrated that some of the carbazole alkaloids isolated from *Murraya* plants (Rutaceae) display cytotoxicity against a variety of tumor cell lines.[9] In addition, we have shown that some of the carbazole alkaloids isolated from *M. koenigii* induce apoptosis in the human leukemia cell line (HL-60).[10]

In this paper, we report the isolation and structural elucidation of two new carbazole alkaloids from the stems of *Clausena anisata* (Willd.) Oliv. collected in Thailand. Furthermore, in the course of our continuing study, eight known carbazole alkaloids isolated from this plant were tested for cytotoxicity against the human leukemia cell line HL-60.

The acetone extract of the stems of *C. anisata* was fractionated by silica gel column chromatography and preparative TLC to obtain eight known and two new carbazole alkaloids, named furanoclausamines A (**1**) and B (**2**).

Furanoclausamine A (**1**) was obtained as a colorless oil, $[\alpha]^{25}_{D}$ −33, and its molecular formula was determined as $C_{19}H_{17}NO_4$ by HRMS. The UV spectrum was similar to that of clausamine B (**9**),[3] which we also isolated from the same plant. The IR spectrum showed absorption bands due to an amino group and a five-membered lactone carbonyl group at ν_{max} 3464 and 1749 cm^{-1}, respectively. The ^1H NMR ($CDCl_3$) spectrum revealed a D_2O-exchangeable signal at δ 8.79, a methoxy singlet at δ 4.10, a set of four coupled aromatic protons at δ 8.56 (1H, d, J = 7.8 Hz, H-5), 7.38 (1H, t, J = 7.8 Hz, H-6), 7.52 (1H, t, J = 7.8 Hz, H-7), and 7.55 (1H, d, J = 7.8 Hz, H-8), and a singlet at δ 7.33 (1H, s, H-2). An NOE enhancement between the methoxy protons (δ 4.10) and H-2 (δ 7.33) was observed. These data, coupled with the results of an HMBC experiment (see below) and biogenetic considerations,[11−13] suggested the presence of a 1-methoxy-3,4-disubstituted carbazole skeleton with an unsubstituted A-ring.[4−6] Furthermore, in the ^1H NMR spectrum, AB-type signals at δ 5.59 (1H, d, J = 8.0 Hz, H-1′) and 2.84 (1H, d, J = 8.0 Hz, H-2′) and two three-proton singlets at δ 1.74 and 1.41 were observed. The ^1H NMR chemical shift values of the two three-proton singlets and the methine doublet (δ 2.84) coupled with ^{13}C NMR chemical shift values of the methine carbon at δ_C 63.3 (C-2′) and a fully substituted carbon at δ_C 59.2 (C-3′) suggested the presence of a trisubstituted oxirane ring. The linked arrangement of these partial structural units was elucidated by HMBC spectroscopy. Long-range correlations of carbon signals at δ_C 136.6 (C-3), 117.7 (C-4a), and 59.2 (C-3′) with a proton signal at δ_H 5.59 (H-1′), together with correlations of the lactone carbonyl carbon at δ_C 171.6 with δ_H 5.59 (H-1′) and 7.33 (H-2), allowed the linkage of structural units as shown in **1**. These data, together with the ^{13}C NMR spectrum (Table 1) and observed significant mass fragment ions (EIMS) at *m*/*z* 252 resulting from cleavage at C-1′/C-2′, led to the assignment of structure **1** to furanoclausamine A.

Table 1. NMR Data of Furanoclausamines (**1** and **2**) in $CDCl_3$[a]

	furanoclausamine A (**1**)			furanoclausamine B (**2**)		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1		147.1	OMe		146.7	OMe
1-OMe	4.10 (3H, s)	56.1		4.08 (3H, s)	56.0	
2	7.33 (s)	100.7		7.32 (s)	100.7	
3		136.6	H-2, H-1′		139.0	H-2, H-1′
4		117.4			118.2	
4a		117.7	H-5, H-1′		117.1	
4b		121.8	H-6, H-8		121.1	H-6
5	8.56 (d, 7.8)	123.0	H-7	7.79 (d, 8.0)	122.4	H-7
6	7.38 (t, 7.8)	121.3		7.28 (t, 8.0)	120.8	H-8
7	7.52 (t, 7.8)	127.0	H-5	7.49 (t, 8.0)	126.5	H-5
8	7.55 (d, 7.8)	111.3	H-6	7.55 (d, 8.0)	111.5	H-6
8a		139.3	H-5, H-7		139.2	H-5, H-7
9a		134.2	H-2		133.9	H-2
NH	8.79 (br s)			8.74 (br s)		
1′	5.59 (d, 8.0)	80.3		6.48 (d, 9.5)	77.3	
2′	2.84 (d, 8.0)	63.3	H-4′, H-5′	5.29 (br d, 9.5)	120.0	
3′		59.2	H-1′		143.3	H-1′
4′	1.74 (3H, s)	19.5		2.18 (3H, s)	19.0	H-2′
5′	1.41 (3H, s)	24.4		1.88 (3H, s)	25.9	H-2′
C=O		171.6	H-2, H-1′		172.1	H-2

[a] Values in (δ_H and δ_C) ppm. All signals correspond to one proton, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hz.

Furanoclausamine B (**2**) was isolated as a colorless oil, $[\alpha]^{25}_{D}$ −20. The molecular formula, $C_{19}H_{17}NO_3$, a difference of one oxygen compared with **1**, was established by HRMS. The UV and IR spectra were similar to those of **1**. The ^1H NMR spectrum (Table 1) showed a similar signal pattern to that of **1**, except for a trisubstituted oxirane ring. In the NOE experiment, an enhancement between the three-proton singlet (δ 4.08) and the one-proton singlet at δ 7.32 was observed. Further, AB-type signals at δ 6.48 (1H, d, J = 9.5 Hz, H-1′) and 5.29 (1H, br d, J = 9.5 Hz, H-2′) and two three-proton singlets at δ 2.18 and 1.88 assignable to two allyl methyl groups were evident. These results, coupled with the results of an HMBC experiment (Table 1) and the presence of a significant fragment ion at *m*/*z* 253 resulting from cleavage at C-1′/C-2′ with

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10.1021/np800735m CCC: $40.75
Published on Web 05/21/2009

Table 2. Effect of Carbazole Alkaloids on Cell Viability of HL-60 Cells[a]

compound	cell viability (30 μM)
clausamine E (**3**)	47.3 ± 2.9^b
clausamine B (**4**)	80.3 ± 1.5^b
clausamine C (**5**)	79.7 ± 0.9^b

[a] Values are mean ±SE for three independent experiments, in which each measurement was made in triplicate. [b] $P < 0.05$ compared to controls.

hydrogen transfer in the EIMS, indicated that the structure of furanoclausamine B was **2**.

Thus compounds **1** and **2** were determined to be 1-methoxy 3,4-disubstituted carbazoles containing a γ-lactone moiety. This is the first isolation of γ-lactone carbazole alkaloids from natural sources. The absolute and relative configurations of these alkaloids remain to be determined.

Eight known carbazole alkaloids were also isolated and identified by comparison of physical data with published data.[1−5] These were mukonal,[14] glycosinine,[15] mukonidine,[16] clausine F,[1] clausamine D,[4] clausamine E (**3**),[4] clausamine B (**4**),[3] and clausamine C (**5**).[3]

We are continuing primary screening of various plant sources against human leukemia cell lines *in vitro* in order to discover novel cytotoxic compounds. We thus examined the cytotoxic activity of the eight known carbazole alkaloids at a concentration of 30 μM for 24 h on the growth of HL-60 cells. As shown in Table 2, clausamine E (**3**) exhibited cytotoxic activity (cell viability 47.3%) against HL-60. Clausamine B (**4**) and clausamine C (**5**) were less cytotoxic (cell viability about 80%) than **3**. The other five compounds did not affect cell viability.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a DIP-370 (JASCO) in $CHCl_3$ at 25 °C. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in MeOH. IR spectra were recorded on an IR-230 (JASCO) in $CHCl_3$. ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC (J = 8 Hz), and NOE were measured on JNM A-400, A-600, and/or ECP-500 (JEOL) NMR spectrometers. Chemical shifts are shown in δ (ppm) with tetramethylsilane (TMS) as an internal reference. All mass spectra were recorded under EI conditions, unless otherwise stated, using a HX-110 (JEOL) and/or JMS-700 (JEOL) spectrometer having a direct inlet system. Preparative TLC was performed on Kieselgel 60 F_{254} (Merck).

Plant Materials. *Clausena anisata* (Willd.) Oliv. plants were collected during January and February 1996 in Kanchanaburi Province, Thailand. Authentication was done by comparison with the herbarium specimen at the Royal Forest Department, Ministry of Agriculture and Cooperative, Thailand. A voucher specimen has been deposited at the Faculty of Pharmacy, Meijo University, under number MUY0113.

Extraction and Isolation. The dried stems (986 g) of *C. anisata* were extracted with acetone at room temperature, and the solvent was evaporated under reduced pressure to give the acetone extract (10.6 g). The residue was further extracted with MeOH under reflux to give the MeOH extract (12.4 g). The acetone extract was subjected to silica gel column chromatography eluted with *n*-hexane−acetone (10:1, 4:1, 3:1, 2:1, 1:1), acetone, and MeOH, successively to give seven fractions. For each fraction, normal-phase column chromatography on silica gel and preparative TLC using appropriate combinations of solvents (*n*-hexane, EtOAc, $CHCl_3$, CH_2Cl_2, Et_2O, acetone, iPr_2O, benzene, and MeOH) resulted in isolation of the following compounds: from fraction 1 (*n*-hexane−acetone, 10:1), clausamine D (2.2 mg) and **3** (1.0 mg); from fraction 2 (*n*-hexane−acetone, 4:1), mukonal (3.0 mg) and glycosinine (2.2 mg); from fraction 3 (*n*-hexane−acetone, 3:1), **1** (3.0 mg), **2** (1.4 mg), mukonidine (2.2 mg), clausine F (92.1 mg), and **5** (2.3 mg); and from fraction 4 (*n*-hexane−acetone, 2:1): **4** (9.5 mg).

Furanoclausamine A (1): colorless oil; $[\alpha]^{25}_D$ −33 (*c* 0.042, MeOH); HRMS *m/z* 323.1140 (calcd for $C_{19}H_{17}NO_4$, 323.1158); EIMS *m/z* 323 (M^+, 51%), 279 (15), 252 (100), 237 (19), 224 (10), 209 (10); UV (MeOH) λ_{max} 222, 238, 248, 266, 312, 320, 334 nm; IR ($CHCl_3$) ν_{max} 3464, 1749, 1594 cm^{-1}; for ^{1}H, ^{13}C NMR and HMBC see Table 1.

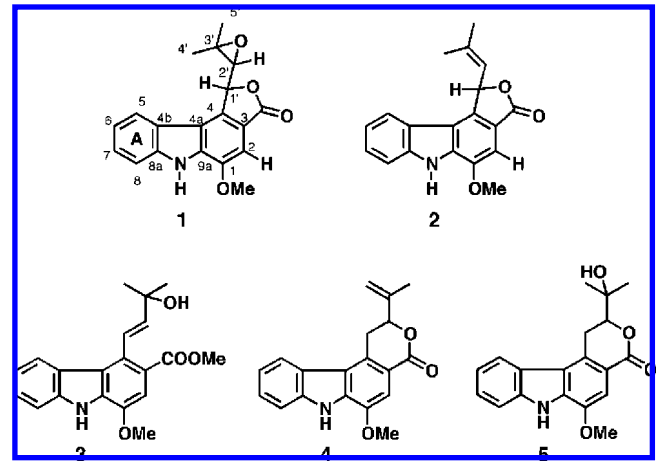

Figure 1. Structures of carbazole alkaloids from *Clausena anisata*.

Furanoclausamine B (2): colorless oil; $[\alpha]^{25}_D$ −20 (*c* 0.075, MeOH); HRMS *m/z* 307.1230 (calcd for $C_{19}H_{17}NO_3$, 307.1208); EIMS *m/z* 307 (M^+, 67%), 292 ($M^+ - CH_3$, 32), 267 (28), 265 (32), 253 (23), 237 (17), 224 (100); UV (MeOH) λ_{max} 224, 236, 248, 268, 304sh, 320, 334 nm; IR ($CHCl_3$) ν_{max} 3463, 1742, 1604 cm^{-1}; for ^{1}H, ^{13}C NMR and HMBC see Table 1.

Cytotoxicity Assay. The human leukemia cell line HL-60 was provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Miyagi, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The carbazole samples were dissolved in DMSO and were added to culture medium to give a final DMSO concentration of 0.5% v/v. This concentration of DMSO had no significant effect on the growth of the cell line tested (data not shown). Cell viability was determined using CellTiter 96 Aqueous Assay kit (Promega, Madison, WI). Cells were seeded in 96-well plates at a density of 1×10^5 cells per well. The cells were maintained for 24 h at 37 °C, and then each sample to be tested (30 μM) was added to the culture medium. MTS solution was added to the 96-well plates, and the cells were incubated for 1 h at 37 °C. The absorbance was measured at a wavelength of 490 nm with a Wallac 1420 ARVOsx microplate counter (Applied Biosystems, Foster City, CA).

Acknowledgment. This work was supported in part by grants-in-aid (C.I., 2007) for scientific research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and Notes

(1) Wu, T.-S.; Huang, S.-C. *Chem. Pharm. Bull.* **1992**, *40*, 1069–1071.
(2) Ito, C.; Ohta, H.; Tan, H. T.-W.; Furukawa, H. *Chem. Pharm. Bull.* **1996**, *44*, 2231–2235.
(3) Ito, C.; Katsuno, S.; Ruangrungsi, N.; Furukawa, H. *Chem. Pharm. Bull.* **1998**, *46*, 344–346.
(4) Ito, C.; Katsuno, S.; Itoigawa, M.; Ruangrungsi, N.; Mukainaka, T.; Okuda, M.; Kitagawa, Y.; Tokuda, H.; Nishino, H.; Furukawa, H. *J. Nat. Prod.* **2000**, *63*, 125–128.
(5) Ito, C.; Itoigawa, M.; Katsuno, S.; Omura, M.; Tokuda, H.; Nishino, H.; Furukawa, H. *J. Nat. Prod.* **2000**, *63*, 1218–1224.
(6) Ramsewak, R. S.; Nair, M. G.; Strasburg, G. M.; DeWitt, D. L.; Nitiss, J. L. *J. Agric. Food Chem.* **1999**, *47*, 444–447.
(7) Tachibana, Y.; Kikuzaki, H.; Lajis, N. H.; Nakatani, N. *J. Agric. Food Chem.* **2001**, *49*, 5589–5594.
(8) Nakahara, K.; Trakoontivakorn, G.; Alzoreky, N. S.; Ono, H.; Onishi-Kameyama, M.; Yoshida, M. *J. Agric. Food Chem.* **2002**, *50*, 4796–4802.
(9) Itoigawa, M.; Kashiwada, Y.; Ito., C.; Furukawa, H.; Tachibana, Y.; Bastow, K. F.; Lee, K.-H. *J. Nat. Prod.* **2000**, *63*, 893–897.
(10) Ito, C.; Itoigawa, M.; Nakao, K.; Murata, T.; Tsuboi, M.; Kaneda, N.; Furukawa, H. *Phytomedicine* **2006**, *13*, 359–365.

(11) Chakraborty, D. P. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Grisebach. H., Kirby. G. W., Ed.; Springer-Verlag: New York, 1977; Vol. 34, pp 299−371.
(12) Bhattacharyya. P.; Chakraborty. D. P. In *Progress in the Chemistry of Organic Natural Products*; Herz. W., Grisebach, H., Kirby. G. W., Ed.; Springer-Verlag: New York, 1987; Vol. 52, pp 159−209.
(13) Chakraborty, D. P.; Roy. S. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Grisebach, H., Kirby, G. W., Ed.; Springer-Verlag: New York, 1991; Vol. 57, pp 71−152.
(14) Bhattacharyya, P.; Chakraborty, A. *Phytochemistry* **1984**, *23*, 471–472.
(15) Jash, S. S.; Biswas, G. K.; Bhattacharyya, S. K.; Bhattacharyya, P.; Chakraborty, A.; Chowdhury, B. K. *Phytochemistry* **1992**, *31*, 2503–2505.
(16) Wu, T.-S.; Huang, S.-C.; Lai, J.-S.; Teng, C.-M.; Ko, F.-N.; Kuoh, C.-S. *Phytochemistry* **1993**, *32*, 449–451.

NP800735M