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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 1567–1573

Nuphar alkaloids with immediately apoptosis-inducing activity from *Nuphar pumilum* and their structural requirements for the activity

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Received 18 August 2005; revised 22 November 2005; accepted 9 December 2005
Available online 18 January 2006

Abstract—The methanolic extract and its alkaloid fraction from the rhizomes of *Nuphar pumilum* showed cytotoxic effects on human leukemia cell (U937), mouse melanoma cell (B16F10), and human fibroblast (HT1080). Dimeric sesquiterpene thioalkaloids with the 6-hydroxyl group (6-hydroxythiobinupharidine, 6,6′-dihydroxythiobinupharidine, 6-hydroxythionuphlutine B) showed substantial cytotoxic activity at a concentration of 10 μM, but dimeric sesquiterpene thioalkaloids lacking the 6-hydroxyl group (thiobinupharidine, thionuphlutine B, 6′-hydroxythionuphlutine B, neothiobinupharidine, thionuphlutine B β-sulfoxide, and neothiobinupharidine β-sulfoxide) and monomeric sesquiterpene alkaloids (nupharidine, 7-epideoxynupharidine, and nupharolutine) showed weak activity. Next, apoptosis-inducing activity of a principal active constituent, 6-hydroxythiobinupharidine, on U937 was examined using morphological observation and DNA fragmentation assay (TUNEL method). Apoptosis of U937 was immediately observed within 1 h after treatment of 6-hydroxythiobinupharidine at 2.5–10 μM.

Nupharis rhizoma, the dried rhizomes of *Nuphar japonicum* DC. and *Nuphar pumilum* (TIMM.) DC., has been prescribed for tonic, hemostatic, and diuretic purposes in Japanese and Chinese traditional medicines. Chemical studies of this natural medicine have been carried out and a number of sesquiterpene alkaloids such as nupharidine (**10**) and deoxynupharidine have been identified from *Nuphar japonicum*.[1] In the course of our studies of natural medicines originating from aquatic plants,[2] we isolated thiohemiaminal-type dimeric sesquiterpene thioalkaloids such as 6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), 6-hydroxythionuphlutine B (**5**), and 6′-hydroxythionuphlutine B (**6**) from the rhizomes of *Nuphar pumilum* and found a new rearrangement reaction of the thiaspiran ring in thiohemiaminal-type alkaloids with the 6-hydroxyl group.[3] In addition, new thiaspiran sulfoxide-type dimeric sesquiterpene alkaloids designated as nupharpumilamines A–D were characterized.[4] As pharmacological studies of nuphar alkaloids, the central paralysis effect of deoxynupharidine, and immunosuppressive, anti-invasion, and/or anti-metastatic activities of **2**, **3**, and **5** has been reported to date.[5]

In the present study, cytotoxic effects of the methanolic extract and alkaloid fraction from the rhizomes of *Nuphar pumilum* on human leukemia cell (U937), mouse melanoma cell (B16F10), and human fibroblast (HT1080) were examined. To investigate the cytotoxic effects of the MeOH extract from *Nuphar pumilum* and its active constituents, the viabilities of test substances-treated U937, B16F10, and HT1080 cells were evaluated by WST-8 or MTT assay. The methanolic extract (yield: 10.3% from the dried rhizomes) at 6 μg/mL exhibited significant cytotoxic effects in each cell line. The MeOH extract was then partitioned into a $CHCl_3$/1 M aqueous HCl (1:1) mixture. The 1 M aqueous HCl phase was made to a pH ca. 10 with concd NH_4OH and then extracted with EtOAc, and the EtOAc-soluble fraction (=the alkaloid fraction, 0.21%) and $CHCl_3$-soluble fraction (1.4%) were obtained. The alkaloid fraction showed substantial cytotoxic effects (Table 1), and the $CHCl_3$-soluble fraction showed weaker cytotoxic effects (data not shown). Thus, cytotoxic effects of dimeric sesquiterpene thioalkaloids [6-hydroxythiobinupharidine (**2**),[6] 6,6′-dihydroxythiobinupharidine (**3**),[7] 6-hydroxythionuphlutine B (**5**),[7] 6′-hydroxythionuphlutine B (**6**),[7] neothiobinupharidine (**7**),[8] thionuphlutine B β-sulf-

Keywords: Nuphar alkaloids; 6-Hydroxythiobinupharidine; Apoptosis-inducing activity; Cytotoxic effect; Caspases; U937; B16F10; HT1080.
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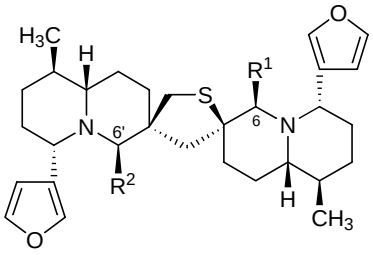
doi:10.1016/j.bmcl.2005.12.032

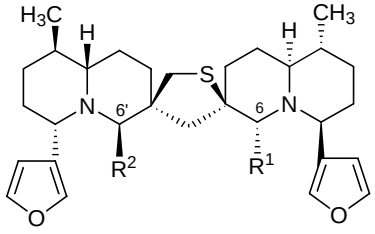
Table 1. Cytotoxic effects of the alkaloid fraction from *Nuphar pumilum* in U937, B16F10, and HT1080 cells

Cell line	Incubation time (h)	Inhibition (%)				
		0 (μg/mL)	0.25 (μg/mL)	0.5 (μg/mL)	0.75 (μg/mL)	1 (μg/mL)
U937	24	0.0 ± 2.0	4.7 ± 3.2	27.4 ± 4.0**	63.5 ± 2.0**	86.4 ± 0.7**
	48	0.0 ± 0.8	10.7 ± 3.4**	40.7 ± 3.0**	88.1 ± 2.5**	96.5 ± 0.2**
	72	0.0 ± 1.6	−4.4 ± 1.7	26.7 ± 3.5**	96.1 ± 1.8**	99.2 ± 0.1**
B16F10	24	0.0 ± 3.6	−6.5 ± 6.3	−19.5 ± 13.9	20.8 ± 6.0	88.3 ± 1.2**
	48	0.0 ± 1.3	16.4 ± 1.0**	46.0 ± 2.3**	70.6 ± 0.8**	97.2 ± 0.5**
	72	0.0 ± 0.7	10.9 ± 1.4**	50.6 ± 3.2**	84.7 ± 1.2**	98.5 ± 0.2**
HT1080	24	0.0 ± 0.6	3.6 ± 2.7	15.4 ± 1.6**	44.7 ± 2.2**	79.6 ± 1.1**
	48	0.0 ± 1.7	9.0 ± 2.0	23.6 ± 1.7**	57.6 ± 1.7**	90.7 ± 1.0**
	72	0.0 ± 1.3	7.6 ± 1.2	28.3 ± 1.6**	67.4 ± 1.6**	94.4 ± 0.6**

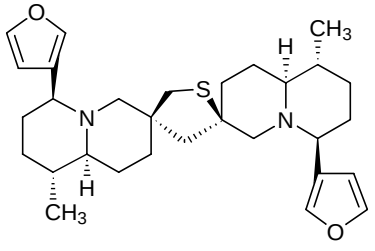
HT-1080 (Cell No. JCRB9113) and U937 (Cell No. JCRB9021) cells were obtained from Health Science Research Resources Bank (Osaka, Japan) and B16F10 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. U937 cells (5×10^3 cells/well) were seeded onto 96-well microplates in 100 μL of RPMI medium containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). After incubation at 37 °C for 20, 44, and 68 h with or without test compound, the viability of the cells was determined using Cell Counting Kit-8 (CCK-8, Dojindo Co. Ltd.) according to manufacturer's instruction. Similarly, B16F10 (2.5×10^3 cells/100 μL of RPMI/well) and HT1080 (5×10^3 cells/100 μL of MEM/well) were seeded onto 96-well microplates in 100 μL of each medium containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). After incubation at 37 °C for 20, 44, and 68 h with test compound, 10 μL of MTT (5 mg/mL in PBS) was added to each well. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced by the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). The test compound was dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO in the medium was 0.1%. Results are expressed as percentage inhibition of cell proliferation. Each value represents the means ± SEM ($N = 4$–6). Significantly different from the control.
** $p < 0.01$.



thiobinupharidine (**1**): $R^1 = R^2 = H$
6-hydroxythiobinupharidine (**2**): $R^1 = OH$, $R^2 = H$
6,6'-dihydroxythiobinupharidine (**3**): $R^1 = R^2 = OH$



thionuphlutine B (**4**): $R^1 = R^2 = H$
6-hydroxythionuphlutine B (**5**): $R^1 = OH$, $R^2 = H$
6'-hydroxythionuphlutin B (**6**): $R^1 = H$, $R^2 = OH$



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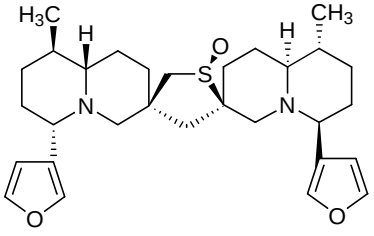
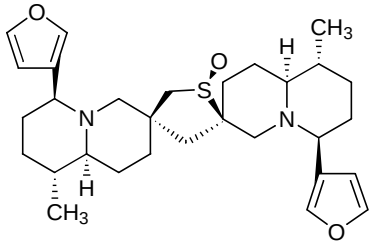
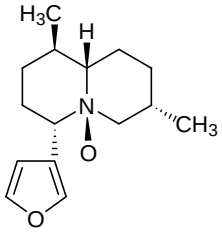


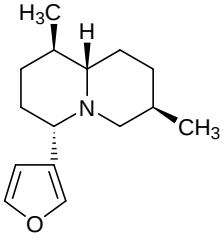
thionuphlutine B β-sulfoxide (**8**)



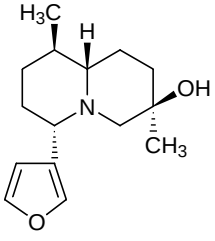
neothiobinupharidine β-sulfoxide (**9**)



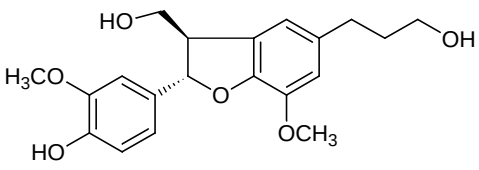
nupharidine (**10**)



7-epideoxynupharidine (**11**)



nupharolutine (**12**)



dihydrodehydrodiconifery alcohol (**13**)

Figure 1. Nuphar alkaloids (**1**–**12**) and dihydrodehydrodiconiferyl alcohol (**13**) isolated from the rhizomes of *Nuphar pumilum*.

Table 2. Cytotoxic effects of alkaloid constituents (**1**–**12**) from *Nuphar pumilum* in U937, B16F10, and HT1080 cells

Cell line	Incubation time (h)	Inhibition (%)			
		0 (μM)	0.1 (μM)	1.0 (μM)	10 (μM)
*Thiobinupharidine (**1**)*					
U937	72	0.0 ± 2.1	—	6.9 ± 2.2	10.9 ± 3.1**
B16F10	72	0.0 ± 1.7	6.8 ± 1.2	7.9 ± 0.7	4.4 ± 1.5
HT1080	72	0.0 ± 1.1	9.9 ± 1.4	6.3 ± 1.1	−11.7 ± 1.9**
*6-Hydroxythiobinupharidine (**2**)*					
U937	24	0.0 ± 0.3	—	3.6 ± 2.4	83.2 ± 0.2**
	48	0.0 ± 0.6	—	1.3 ± 1.0	92.5 ± 0.0**
	72	0.0 ± 0.4	—	−1.0 ± 0.8	94.3 ± 0.1**
B16F10	24	0.0 ± 2.8	—	—	88.6 ± 0.3**
	48	0.0 ± 5.4	10.3 ± 2.4	27.3 ± 3.9**	97.3 ± 0.4**
	72	0.0 ± 1.2	11.1 ± 1.7**	20.8 ± 3.5**	98.7 ± 0.2**
HT1080	24	0.0 ± 2.3	1.8 ± 2.3	14.0 ± 2.3**	96.1 ± 0.1**
	48	0.0 ± 2.7	1.6 ± 2.7	8.3 ± 2.7	97.5 ± 0.3**
	72	0.0 ± 1.3	5.4 ± 1.6	15.7 ± 2.3**	99.2 ± 0.6**
*6,6′-Hydroxythiobinupharidine (**3**)*					
U937	24	0.0 ± 2.2	—	5.3 ± 1.1	54.7 ± 0.6**
	48	0.0 ± 0.7	—	2.3 ± 1.7	80.0 ± 0.6**
	72	0.0 ± 1.1	—	1.3 ± 1.2	81.6 ± 3.1**
B16F10	24	0.0 ± 2.0	—	—	16.6 ± 4.1**
	48	0.0 ± 2.2	−8.0 ± 1.3	−15.0 ± 1.8	51.8 ± 3.1**
	72	0.0 ± 1.4	−3.5 ± 2.0	−6.0 ± 2.6	54.8 ± 2.3**
HT1080	24	0.0 ± 2.7	—	—	19.3 ± 2.7**
	48	0.0 ± 2.7	9.0 ± 3.3	0.5 ± 0.6	75.0 ± 1.2**
	72	0.0 ± 1.4	0.1 ± 1.6	−0.9 ± 1.6	75.3 ± 2.1**
*Thionuphlutine B (**4**)*					
U937	72	0.0 ± 2.1	—	9.1 ± 0.9	7.8 ± 2.4
B16F10	72	0.0 ± 0.9	1.7 ± 1.0	−2.2 ± 1.4	2.2 ± 1.7
HT1080	72	0.0 ± 1.1	−10.8 ± 1.3**	0.9 ± 2.3	−12.0 ± 2.8**
*6-Hydroxythionuphlutine B (**5**)*					
U937	24	0.0 ± 2.2	—	74.6 ± 0.6**	80.0 ± 0.3**
	48	0.0 ± 0.7	—	89.0 ± 0.7**	91.3 ± 0.1**
	72	0.0 ± 1.1	—	85.3 ± 3.6**	94.1 ± 0.1**
B16F10	24	0.0 ± 3.9	—	−22.9 ± 9.8	91.5 ± 0.6**
	48	0.0 ± 2.8	0.3 ± 2.0	65.0 ± 0.4**	98.2 ± 0.1**
	72	0.0 ± 1.3	0.9 ± 3.4	69.3 ± 2.0**	99.1 ± 0.2**
HT1080	24	0.0 ± 0.6	6.3 ± 3.1	42.0 ± 3.1**	95.9 ± 0.6**
	48	0.0 ± 1.4	8.6 ± 1.4	53.3 ± 2.8**	98.0 ± 0.1**
	72	0.0 ± 1.1	1.0 ± 1.6	63.5 ± 2.2**	98.7 ± 0.1**
*6′-Hydroxythionuphlutine B (**6**)*					
U937	72	0.0 ± 1.1	—	0.1 ± 1.1	0.5 ± 0.3
B16F10	72	0.0 ± 1.4	1.7 ± 1.2	−1.6 ± 1.2	−4.8 ± 1.2
HT1080	72	0.0 ± 2.1	−9.8 ± 3.5	−7.3 ± 3.1	−9.1 ± 2.8
*Thionuphlutine B β-sulfoxide (**7**)*					
U937	24	0.0 ± 2.2	—	5.2 ± 1.7	50.7 ± 3.0**
	48	0.0 ± 0.7	—	5.4 ± 1.6	65.6 ± 1.7**
	72	0.0 ± 1.1	—	−1.2 ± 1.0	40.1 ± 3.8**
B16F10	48	0.0 ± 10.5	4.4 ± 2.0	−8.9 ± 2.6	24.7 ± 0.6**
	72	0.0 ± 1.5	8.9 ± 1.0	4.0 ± 1.9	30.2 ± 0.8**
HT1080	48	0.0 ± 2.5	−1.0 ± 2.1	−4.1 ± 2.5	7.5 ± 2.1
	72	0.0 ± 2.0	−3.4 ± 1.7	−0.8 ± 1.7	38.0 ± 3.1**
*Neothiobinupharidine (**8**)*					
U937	72	0.0 ± 1.4	—	4.7 ± 1.0	6.3 ± 1.5
B16F10	72	0.0 ± 1.0	10.4 ± 1.8**	3.2 ± 1.3	10.8 ± 1.1**
HT1080	72	0.0 ± 1.6	−6.1 ± 2.0	−8.1 ± 2.3	−4.4 ± 2.0
*Neothiobinupharidine β-sulfoxide (**9**)*					
U937	72	0.0 ± 1.4	—	5.8 ± 1.3	10.0 ± 1.0**
B16F10	72	0.0 ± 2.0	−3.7 ± 3.6	7.7 ± 2.0	9.1 ± 1.2
HT1080	72	0.0 ± 2.9	−0.4 ± 2.6	−1.8 ± 1.9	6.8 ± 4.2

** $p < 0.01$.

oxide (**8**),[9] and neothiobinupharidine β-sulfoxide (**9**)[10]] and monomeric sesquiterpene alkaloids [nupharidine (**10**),[1] 7-epideoxynupharidine (**11**),[11] and nupharolutine (**12**)[12]] from the alkaloid fraction,[3] and a lignan, dihydrodehydrodiconiferyl alcohol (**13**),[13] from the $CHCl_3$-soluble fraction[3] (Fig. 1) on the cells were examined.

The cytotoxic effects of nuphar alkaloids (**1**–**12**), **13**, and a reference compound, campthothecin,[14] on the cells are summarized in Tables 2 and 3. Camptothecin showed strong cytotoxic effects in a concentration- and time-dependent manner. However, the effect of camptothecin on HT1080 was weaker than those on other cells, and camptothecin could not show complete cell death (inhibition of cell viability: 60.6–75.0%) after incubation for 24 h, even if at a high concentration (10 μM). The dimeric sesquiterpene thioalkaloids lacking the 6-hydroxyl group [6′-hydroxythionuphlutine B (**6**), neothiobinupharidine (**7**), thionuphlutine B β-sulfoxide (**8**), neothiobinupharidine β-sulfoxide (**9**)], monomeric sesquiterpene alkaloids [nupharidine (**10**), 7-epideoxynupharidine (**11**), and nupharolutine (**12**)], and dihydrodehydrodiconiferyl alcohol (**13**) showed weak activity at 10 μM after incubation for 72 h. On the other hand, dimeric sesquiterpene thioalkaloids with the 6-hydroxyl group in the quinolizidine ring [6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), and 6-hydroxythionuphlutine B (**5**)] were found to show substantial cytotoxic effects at 10 μM, and their cytotoxic effects at 24 h were almost equivalent to that of camptothecin. Furthermore, thiobinupharidine (**1**)[15] and thionuphlutine B (**4**)[15] derived from **2** and **5** also lacked the activity. These findings suggested that the thiohemiaminal structure with the 6-hydroxyl group is essential for strong activity.

Apoptosis plays an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells. On the other hand, induction of apoptosis of cancer cells is also recognized to be useful for cancer treatment, since cytotoxic drugs (e.g., etoposide, cisplatin, and paclitaxel) used in chemotherapy of leukemias and solid tumors are known to cause apoptosis in target cells.[16] Therefore, apoptosis-inducing effect of 6-hydroxythiobinupharidine (**2**) in U937 cells was examined.

To determine the apoptosis-inducing effect of a principal dimeric sesquiterpene thioalkaloid **2**, we examined the morphological changes in U937 after treatment of **2**. As shown in Figure 2, characteristic breakdown of the cells into smaller units (apoptotic bodies) was observed in U937 at 5 and 10 μM, and in 1 h after treatment of **2**. On the other hand, a reference compound, camptothecin, even if at a high concentration (10 μM), exhibited the characteristic changes 4 h after the treatment.

Table 3. Cytotoxic effects of alkaloid constituents (**10**–**12**) and dihydrodehydrodiconiferyl alcohol (**13**) from *Nuphar pumilum* in U937, B16F10, and HT1080 cells

Cell line	Incubation time (h)	Inhibition (%)				
		0 (μM)	0.1 (μM)	1.0 (μM)	10 (μM)	
*Nupharidine (**10**)*						
U937	72	0.0 ± 1.4	—	8.0 ± 1.2	11.5 ± 0.8	
B16F10	72	0.0 ± 1.0	0.6 ± 2.1	5.3 ± 1.2	4.1 ± 1.4	
HT1080	72	0.0 ± 1.3	−3.3 ± 1.3	−9.0 ± 1.0	−9.0 ± 1.0	
*7-Epideoxynupharidine (**11**)*						
U937	72	0.0 ± 1.4	—	6.4 ± 1.5	8.0 ± 0.8	
B16F10	72	0.0 ± 1.9	−1.5 ± 1.7	−9.8 ± 1.5	−7.9 ± 1.5	
HT1080	72	0.0 ± 1.1	—	−6.6 ± 1.4	−10.1 ± 2.3**	
*Nupharolutine (**12**)*						
U937	72	0.0 ± 1.4	—	12.5 ± 7.2**	2.8 ± 1.6	
B16F10	72	0.0 ± 1.7	−3.1 ± 1.2	−1.2 ± 1.5	−0.9 ± 1.2	
HT1080	72	0.0 ± 1.5	−12.9 ± 1.5**	−13.2 ± 1.5**	−16.7 ± 1.2**	
Dihydrodehydrodiconiferyl alcohol						
U937	72	0.0 ± 2.1	—	9.3 ± 1.4	13.4 ± 1.7**	
B16F10	72	0.0 ± 1.6	−2.4 ± 1.6	−5.3 ± 2.2	−6.5 ± 1.8	
HT1080	72	0.0 ± 2.5	8.9 ± 0.9	5.2 ± 1.4	11.9 ± 1.2**	
Cell line	Incubation time	0 (μM)	0.03 (μM)	0.1 (μM)	1 (μM)	10 (μM)
Camptothecin						
U937	24	0.0 ± 1.2	7.1 ± 1.8	46.0 ± 0.7**	75.8 ± 0.7**	75.0 ± 0.8**
	48	0.0 ± 1.1	61.9 ± 0.9**	90.6 ± 0.4**	94.6 ± 0.4**	98.2 ± 0.1**
	72	0.0 ± 2.9	78.5 ± 0.8**	95.3 ± 0.3**	98.2 ± 0.1**	98.0 ± 0.1**
B16F10	24	0.0 ± 4.8	−1.9 ± 18.4	16.2 ± 9.3	47.8 ± 2. 0**	60.6 ± 1.7**
	48	0.0 ± 5.8	7.5 ± 7.7	39.1 ± 1.5**	83.2 ± 1.6**	94.7 ± 0.3**
	72	0.0 ± 1.9	33.4 ± 1.3**	76.9 ± 0.8**	93.5 ± 0.1**	98.1 ± 0.1**
HT1080	24	0.0 ± 1.5	−3.8 ± 1.0	−0.4 ± 2.5	49.5 ± 1. 9**	62.0 ± 1.8**
	48	0.0 ± 1.9	−7.1 ± 11.7	−11.7 ± 4.6	77.0 ± 0.8**	93.1 ± 0.6**
	72	0.0 ± 5.5	−3.2 ± 3.7	5.4 ± 1.8	65.8 ± 2.1**	93.8 ± 0.6**

** $p < 0.01$.

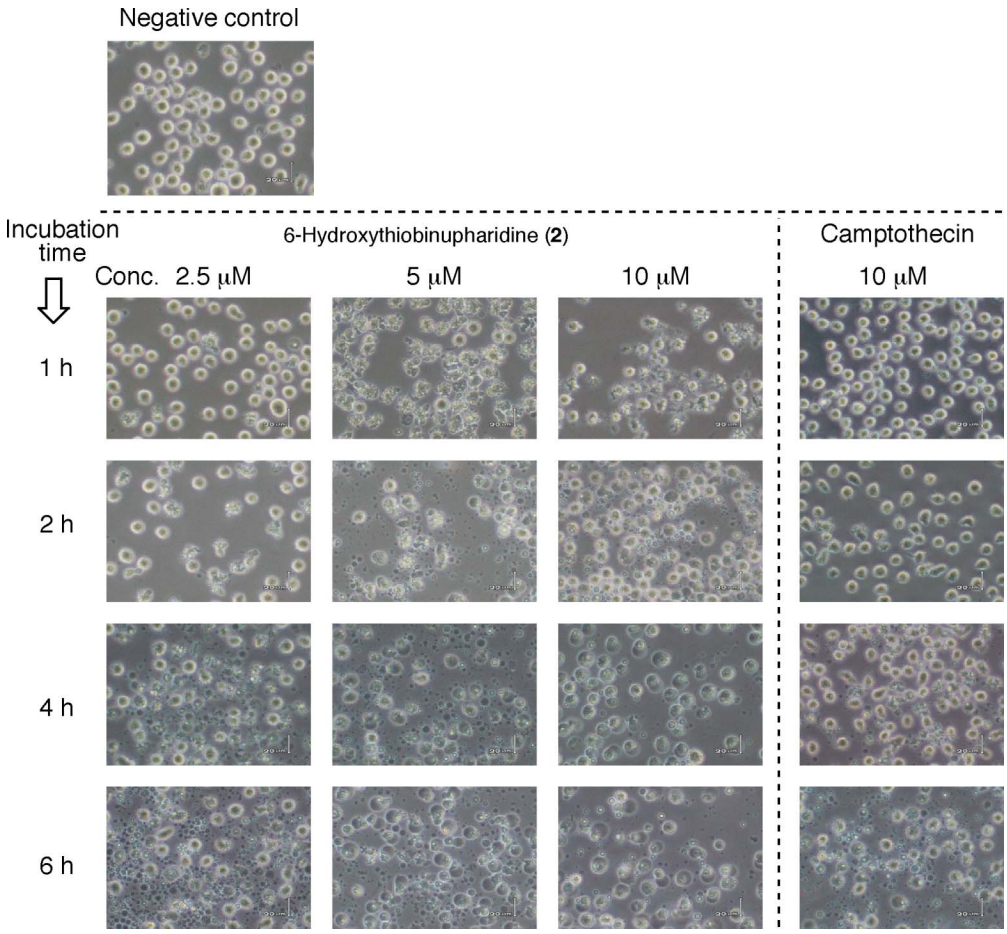


Figure 2. Morphological changes of U937 cells by 6-hydroxythiobinupharidine (**2**). U937 cells (2×10^5 cells/well) were seeded onto 24-well microplates in 1 mL of RPMI1640 medium containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). After incubation with 6-hydroxythiobinupharidine (**2**) at 37 °C for 1–6 h with or without of test compound, the morphological changes were observed using a microscope.

Furthermore, the apoptosis-inducing effect of **2** in U937 cells was examined using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay (APO-BRDU™, BD Biosciences). As shown in Figure 3, the TUNEL-positive cells (number of cells in M2 area >57%) were observed 1 h after treatment of **2** at 2.5–10 μM, but the TUNEL-positive cells were not observed at 1 μM after 24 h (cells in M2 area <1.2%). These results indicated that DNA fragmentation, which is characteristic of apoptotic cells, was observed after treatment of **2**. On the other hand, camptothecin at 5 μM induced apoptosis of the cells by ca. 70% after 24 h similar to the result of cytotoxic assay.

Caspases play a central role in the apoptotic signaling pathway and contribute to the overall apoptotic morphology by cleavage of various cellular substances. Caspase-8 is the initial caspase activated in response to receptors with a death domain that interacts with FADD. The mitochondrial stress pathway begins with the release of cytochrome c from mitochondria, which then interacts with Apaf-1, causing self-cleavage and activation of caspase-9. The effector caspase-3 is downstream of the activator caspases and acts cleaving various cellular targets.[17] Therefore, we examined the effects of a caspase-3 inhibitor [benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-FMK), BD Biosciences], a caspase-8 inhibitor [benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone (Z-IETD-FMK), BD Biosciences], and a caspase-9 inhibitor [benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone (Z-LEHD-FMK), BD Biosciences] at 20 μM on apoptotic DNA fragmentation of U937 by **2**. As shown in Figure 4, the DNA fragmentation induced by **2** was markedly reduced by treatment with the inhibitors of caspase-3 and -8, but not with the inhibitor of caspase-9. This result indicates that compound **2**-induced apoptotic cell death is largely dependent on activation of caspase-3 and -8, suggesting that the pathway through the activation of caspase-8 is involved in the apoptosis-inducing effect of **2**.

In conclusion, the methanolic extract and its alkaloid fraction from the rhizomes of *Nuphar pumilum* showed cytotoxic effects against U937, B16F10, and HT1080 cells. Among the constituents, dimeric sesquiterpene thioalkaloids with the 6-hydroxyl group [6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), and 6-hydroxythionuphlutine B (**5**)] showed potent activity. The principal dimeric sesquiterpene thioalka-

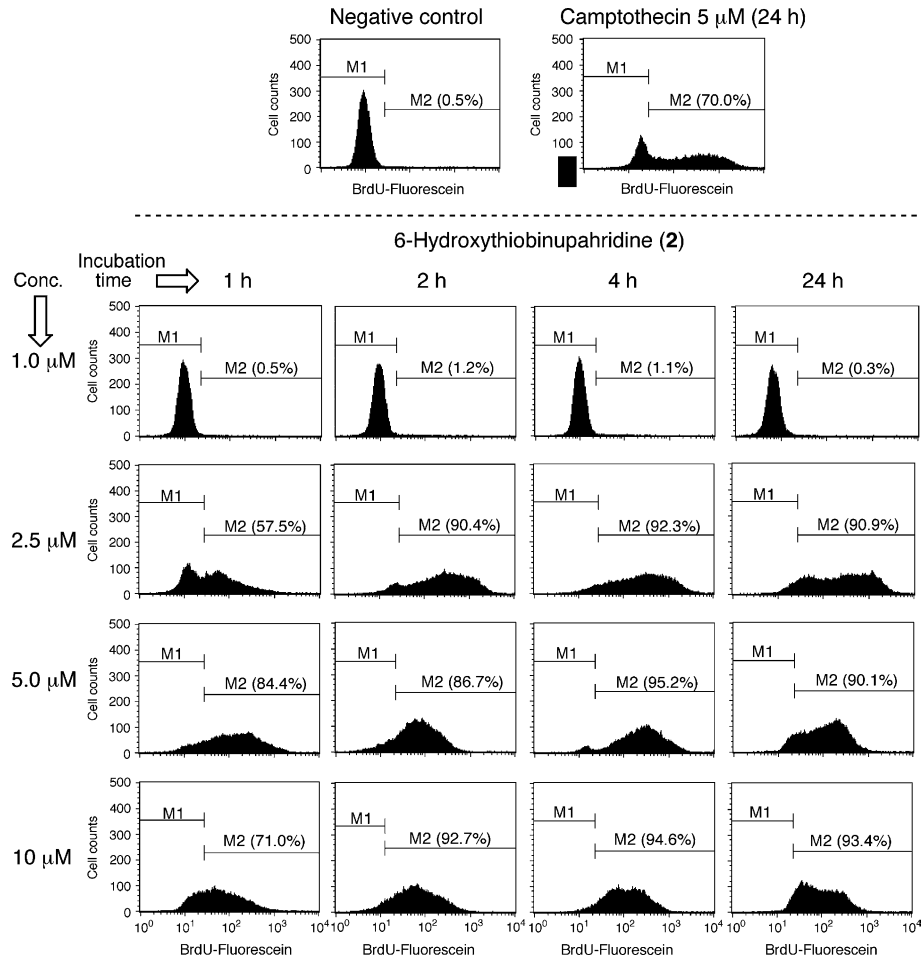


Figure 3. FACS analysis of DNA fragmentation induced by 6-hydroxythiobinupharidine (**2**) in U937 cells. TUNEL assay (APO-BRDU™, BD Biosciences) was performed according to manufacturer's instruction. Briefly, U937 cells (5×10^5 cells/5 mL/culture flask) in RPMI1640 containing 10% FCS with or without best compound were incubated at 37 °C for 1–24 h in 5% CO_2 atmosphere. After incubation, the cells were fixed and stained with fluorescein-labeled BrdU and propidium iodide (PI). After incubation for 30 min at room temperature, the fluorescence intensity of each cell was analyzed by flow cytometry (FACSCalibur, Becton–Dickinson). M1 and M2 indicate TUNEL-negative and -positive cells, respectively.

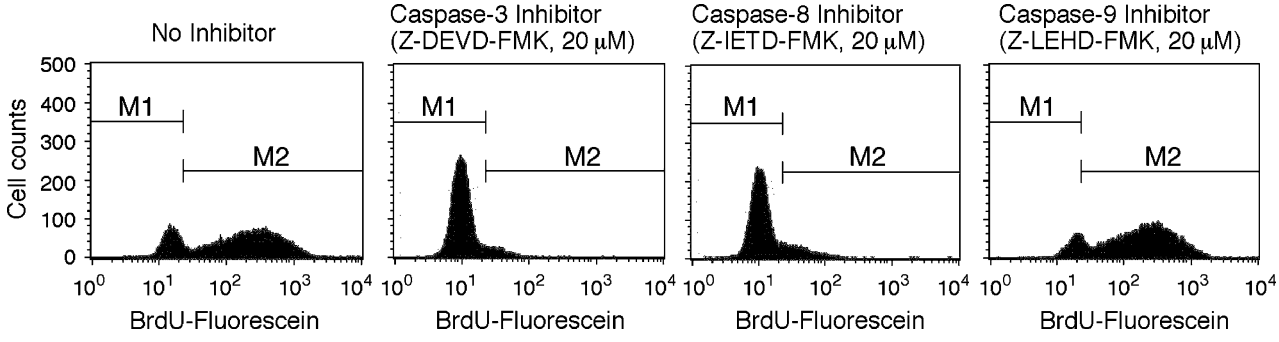


Figure 4. Effects of inhibitors of caspases-3, 8, and 9 on DNA fragmentation induced by 6-hydroxythiobinupharidine (**2**) in U937 cells. The cells were incubated with **2** (5 μM) and each caspase inhibitor (20 μM) for 4 h, and then TUNEL assay was performed. M1 and M2 indicate TUNEL-negative and -positive cells, respectively.

loid **2** induced apoptosis of U973 cells in an hour after treatment of **2** (2.5–10 μM). The apoptotic pathway through caspase-8 to caspase-3 may be involved in the apoptosis-inducing effect of **2**, and the mechanisms of action of **2** need to be studied further. To the best of our knowledge, no compound with the immediate apoptosis-inducing activity within 1 h was reported so far. These active dimeric sesquiterpene thioalkaloids (**2**, **3**, and **5**) may be a new candidate for apoptosis-inducing cancer chemopreventive agents.

Acknowledgments

This research was supported by the 21st COE Program, Academic Frontier Project, and Grand-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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