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NUPHARIC ACID, A NEW SESQUITERPENE ALKALOID FROM *Nuphar japonicum*

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Abstract – A new sesquiterpene alkaloid named nupharic acid (**1**) was isolated from the aerial part of *Nuphar japonicum* together with known alkaloids nupharidine (**2**) and deoxynupharidine (**3**). Alkaloid **1** is the first example of Nuphar alkaloids that lack the common furan moiety.

INTRODUCTION

Nuphar japonicum DC. is widely distributed in Japan and Korea and is a well-known plant used in Chinese traditional medicine. Nuphar rhizoma, the dried rhizome of *Nuphar japonicum* DC. and *Nuphar pumilum* (Timm.) DC., has been prescribed for its tonic, hemostatic, and diuretic effects in Japanese and Chinese traditional medicine.[1] Chemical studies of *N. japonicum* have been carried out and a number of sesquiterpene alkaloids, such as nupharidine (**2**) and deoxynupharidine (**3**)[2], as well as thio-containing dimeric alkaloids[3] have been identified (Figure 1). We investigated the chemical constituents in the aerial part of this plant for the first time and isolated a new alkaloid called nupharic acid (**1**).

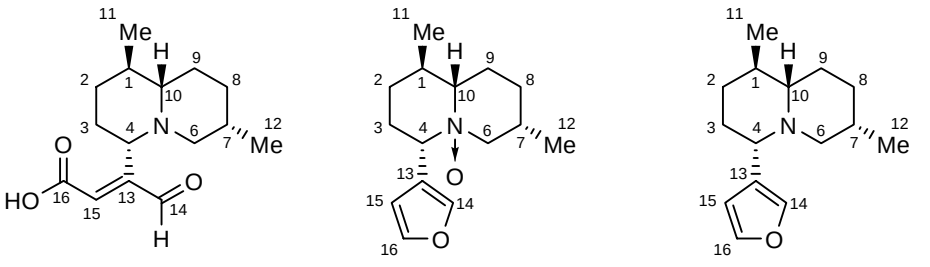

Figure 1. Alkaloids isolated from *N. japonicum*

RESULTS AND DISCUSSION

The molecular formula of new alkaloid **1** was established as $C_{15}H_{23}NO_3$ from HRFABMS [*m/z* 266.1743 (MH^+)], which showed that **1** possessed two oxygens more than deoxynupharidine (**3**).

The ^{1}H-NMR data (Table 1) showed significant signals characteristic of an aldehyde group [(δ 9.51, H-14)], an olefinic or an aromatic proton [(δ 6.96, H-15)], and two tertiary methyl groups [δ 1.19 (3H, d, H_3-12), δ 0.99 (3H, d, H_3-11)]. The ^{13}C-NMR spectrum revealed the presence of two carbonyl carbons [δ_C 193.3 (C-14), δ_C 167.6 (C-16)], two sp^2 carbons [δ_C 150.5 (C-15), δ_C 142.4 (C-13)], three low-field sp^3 carbons bearing an oxygen or a nitrogen atom [δ_C 70.2 (C-10), δ_C 58.8 (C-4), δ_C 58.1 (C-6)], and eight sp^3 carbons. Comparing the NMR data of compound **1** with those of **2** and **3** (Table 1), **1** possessed a quinolizidine ring but lacked a furan residue that commonly exists in Nuphar alkaloids.

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1-3**.

	^{1}H-NMR in $CDCl_3$				^{13}C-NMR in $CDCl_3$		
position	**1**	**2**	**3**	position	**1**	**2**	**3**
1	1.95 (m)	2.08 (overlapped)	1.50-1.43 (overlapped)	1	33.6	30.3	35.6
2	1.82 (dq, 13.7, 3.3)	1.93 (dq, 13.4, 3.3)	1.68 (overlapped)	2	31.6	32.7	33.8
	1.27 (m)	1.45 (m)	1.12 (m)				
3	2.12 (m)	2.08 (2H, overlapped)	1.64 (2H, overlapped)	3	28.7	27.2	34.9
	1.76 (m)						
4	3.82 (ddd, 12.3, 6.2, 3.8)	4.25 (t, 7.9)	2.92 (m)	4	58.8	74.0	60.0
6	2.54 (ddd, 11.9, 7.6, 2.9)	2.76 (t, 11.5)	2.65 (br-d, 12.1)	6	58.1	58.1	58.1
	2.99 (d, 12.2)	2.60 (dd, 11.5, 4.2)	1.81 (br-d, 10.7)				
7	2.10 (m)	2.46 (m)	1.72 (overlapped)	7	27.0	25.8	28.4
8	1.76 (m)	1.54 (dd, 14.3, 3.6)	1.50 (overlapped)	8	29.0	26.0	30.4
	1.69 (m)	1.06 (qd, 13.7, 3.6)	1.43 (overlapped)				
9	1.99 (br-dd, 14.0, 3.6)	2.90 (tt, 14.3, 3.9)	1.71 (overlapped)	9	22.9	21.1	25.5
	1.69 (m)	1.72 (br-d, 14.3)	1.43 (overlapped)				
10	2.25 (br-qd, 10.6, 2.7)	2.99 (br-d, 10.9)	1.59 (overlapped)	10	70.2	79.3	69.5
11	0.99 (3H, d, 6.4)	0.99 (3H, d, 6.7)	0.88 (3H, d, 6.4)	11	18.4	18.9	19.0
12	1.19 (3H, d, 7.3)	0.83 (3H, d, 6.7)	0.99 (3H, d, 7.0)	12	17.5	19.1	17.4
13				13	142.4	119.6	129.9
14	9.51 (s)	7.40 (t, 1.5)	7.25 (m)	14	193.3	142.3	139.0
15	6.96 (s)	6.61 (br-d, 0.9)	6.38 (m)	15	150.5	111.8	109.5
16		7.63 (br-s)	7.32 (br-t, 1.7)	16	167.6	143.6	142.6

^{1}H-^{1}H COSY and HMQC analyses (Figure 2) indicated the presence of a long carbon chain consisting of eleven carbons. The three low-field carbons (C-4, C-6, and C-10) are all connected to a common nitrogen atom and form a quinolizidine ring. This idea was supported by HMBC correlations from H-6 to C-4 and C-10. The IR spectrum indicates Bohlmann absorption at 2850-3000 cm^{-1} due to the *trans*-quinolizidine ring.[4] HMBC correlations from a low-field olefin proton (δ_H 6.96, H-15) to two carbonyl carbons [δ_C 193.3 (C-14), δ_C 167.6 (C-16)] and from an aldehyde proton (δ_H 9.51, H-14) to an olefin carbon (δ_C 142.4

C-13) revealed the existence of a β-formyl acrylic acid residue. Furthermore, HMBC crosspeaks from H-14 and H-15 to C-4 demonstrated that the β-formyl acrylic acid residue was attached to C-4 on the quinolizidine ring at β-position (C-13).

NOE correlations of H-10/H-4, H-4/H-6, and H-6/H-7 suggested that H-4, H-6, H-7, and H-10 have syn configuration (Figure 3). NOE correlation between H-1 and H-3α indicated that the methyl group at C-1 should have equatorial orientation. The geometry of the C13-15 double bond was deduced to be *E* by NOE experiments.

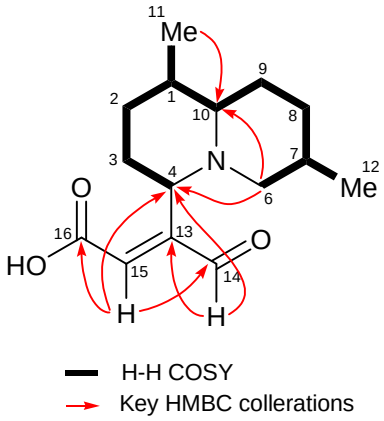

Figure 2. Key 2D-NMR colerations of **1**.

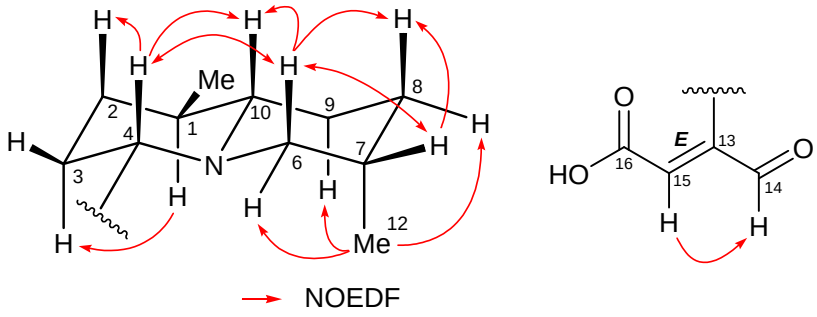

Figure 3. NOE correlations of **1**.

Nupharic acid (**1**) is the first example of Nuphar alkaloids lacking a furan residue. Biogenetically, **2** and **3** were derived from *trans, cis*-farnesyl pyrophosphate *via* the formation of a quinolizidine ring and a furan ring. In contrast, **1** would be derived from *cis, cis*-farnesyl pyrophosphate *via* the formation of a quinolizidine ring and oxidation at C-14 and C-16 positions (Figure 4).

The MeOH extract and its alkaloid fraction from the rhizomes of *Nuphar pumilum* are known to show cytotoxic effects on human leukemia cell (U937), mouse melanoma cell (B16F10), and human fibroblast (HT1080)[1e], however, compound **1** did not show cytotoxic activity against tumor cell lines (A549 and HT29).

EXPERIMENTAL

General Procedure

^{1}H- and ^{13}C-NMR spectra: JEOL JNM A-500 at 500 MHz (^{1}H-NMR) and at 125 MHz (^{13}C-NMR). UV: JASCO V-560. IR: JASCO FT/IR-230. FAB-MS: JEOL JMS-AX500. HR-FAB-MS: JEOL JMS-HX110. Optical rotation: JASCO P-1020. CD: JASCO J-720WI. TLC: Precoated silica gel 60 F_{254} plates (Merck, 0.25 mm thick). Column chromatography: Silica gel 60 (Merck, 70-230 mesh). Flash column chromatography: Silica gel 60N (Kanto Chemical, 40-50 μm).

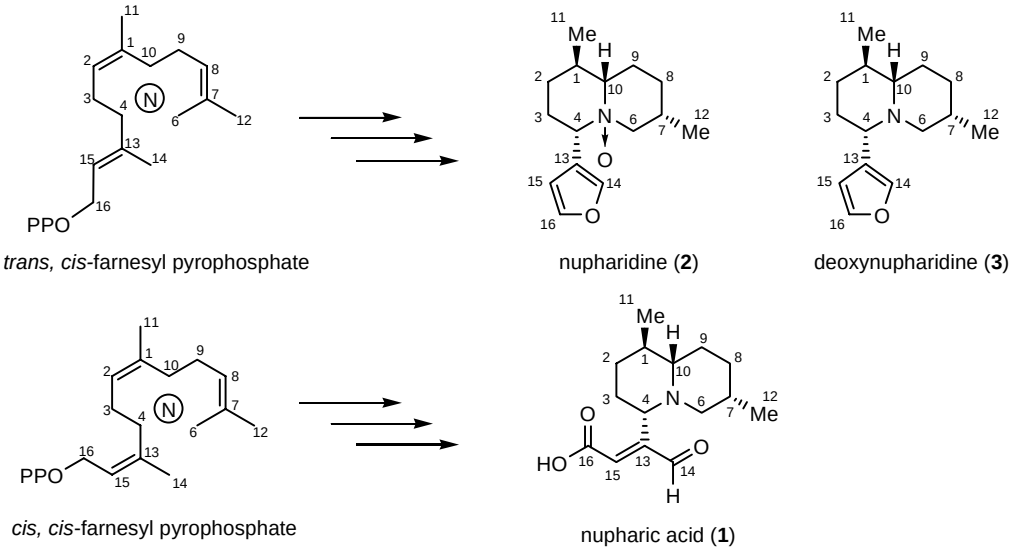

Figure 4. Possible biogenetic route of **1-3**.

Plant Material

Nuphar japonicum DC. was harvested from the medicinal plant garden of Chiba University, Japan. A voucher specimen (No. 20060501) was deposited at the Faculty of Pharmaceutical Sciences, Chiba University, Japan. The plant was identified by Professor Dr. F. Ikegami and a voucher specimen was deposited in the herbarium of our department.

Extraction and Isolation of Nupharic Acid (1)

The aerial part of *N. japonicum* DC. (2751 g, dry weight) was extracted with MeOH (5 L, three times at room temperature and three times under reflux) to give the extract (152.9 g). The MeOH extract (152.9 g) was dissolved in H_2O (0.66 L) containing a small amount of MeOH (20 mL) and extracted with *n*-hexane (0.27 L x 4) to give the *n*-hexane extract (20.15 g). The aqueous layer was successively extracted with AcOEt (0.35 L x 3), 5% MeOH/$CHCl_3$ (0.4 L x 4), and *n*-BuOH (0.35 L x 4) to give the AcOEt extract (3.23 g), the 5% MeOH/$CHCl_3$ extract (1.75 g), and the *n*-BuOH extract (10.95 g), respectively. The 5% MeOH-$CHCl_3$ extract (1.75 g) was separated by SiO_2 flash column chromatography with MeOH/$CHCl_3$ gradient to give 6 fractions: fr. A $CHCl_3$ (143.4 mg); fr. B 1% MeOH/$CHCl_3$ (33.7 mg); fr. C 5% MeOH/$CHCl_3$ (627.6 mg); fr. D 10-20% MeOH/$CHCl_3$ (578.3 mg); fr. E 20-30% MeOH/$CHCl_3$ and MeOH (466.4 mg); and fr. F 5% AcOH/MeOH (3.9 mg). Fr. C was purified successively by SiO_2 flash column chromatography (MeOH/AcOEt gradient and MeOH/$CHCl_3$ gradient) to afford nupharic acid (**1**, 34.3 mg). Nupharic acid (**1**): FAB-MS (NBA) *m/z*: 266 ($M+H^+$), HR-FAB-MS (NBA/PEG) *m/z*: 266.1743 ($M+H^+$, Calcd for $C_{15}H_{24}NO_3$: 266.1756). ^{1}H-NMR: see Table 1. ^{13}C-NMR: see Table 1. UV (MeOH) λ_{max} nm: 206.0. CD (*c* = 0.358 mmol/L, MeOH, 24 °C) $\Delta\varepsilon$ (nm): 0 (316), +2.3 (259), 0 (234), -2.0 (217). IR ($CHCl_3$, cm^{-1}): 3384, 3021, 2931, 2857, 1760, 1689, 1608. $[\alpha]_D^{24}$ -5.3° (*c* = 0.256, MeOH).

Cell Culture.

Human lung and colorectal cancer cell lines, A549 and HT29, respectively, were obtained from ATCC (Rockville, MD, USA). A549 and HT29 cells were maintained in Dulbecco's modified eagle's medium (D-MEM) (D6046, D6046) and D-MEM/F-12 medium (D8062, Sigma) with 10% heat-inactivated fetal bovine serum (FBS) and 5 mg/mL gentamicin, respectively, at 37 °C in a humidified atmosphere containing 5% CO_2.

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