Bisbenzylisoquinoline Alkaloids from *Nelumbo nucifera*

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From the embryos of the seeds of *Nelumbo nucifera*, three bisbenzylisoquinoline alkaloids, nelumboferine and nelumborines A and B, were isolated along with four known compounds, neferine, liensinine, isoliensinine and anisic acid. The structures of the new alkaloids were determined mainly by spectroscopic methods.

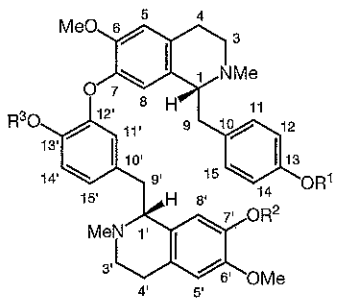
Key words *Nelumbo nucifera*; Nelumbonaceae; bisbenzylisoquinoline alkaloid; nelumboferine; nelumborine

Lotus (*Nelumbo nucifera* GAERTNER, Nelumbonaceae) has been widely used from root to flower as an ingredient for herbal cuisine since ancient times. In particular, "Lian zi xin," embryo loti (green embryo of mature seed), has been used for Chinese traditional medicine as an antifebrile, sedative, and hemostat agent.[1] Previous phytochemical studies of the plant materials led to the isolation of some bisbenzylisoquinoline alkaloids[2] as well as benzylisoquinoline alkaloids[3,4] and aporphine and proaporphine alkaloids.[5] In recent years, bisbenzylisoquinoline alkaloids of embryo loti have received much attention because of their pharmacological effects such as antihypertensive activity,[4] anti-pulmonary fibrosis[6] and anti-human immunodeficiency virus (HIV) activity.[7] On account of our interest in the sedative activity of this herbal medicine, we evaluated the locomotor activities of the extract of embryo loti and found a main bisbenzylisoquinoline alkaloid, neferine, as an active component.[8] We also demonstrated that antidepressant-like effects of neferine are mediated by the 5-HT_{1A} receptor.[9] These biological activities of the alkaloids prompted us to further investigate the minor alkaloidal constituents of embryo loti to isolate three novel alkaloids **1**—**3** together with four known compounds. This paper deals with the isolation and the elucidation of the structure of the new compounds.

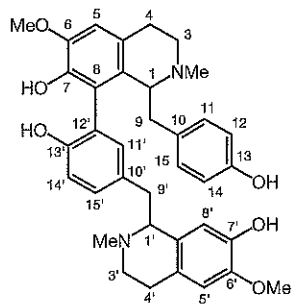
Results and Discussion

The embryos of the seeds of *Nelumbo nucifera* were extracted with hot MeOH. The MeOH extracts were extracted with aqueous tartaric acid solution to obtain an alkaloidal fraction. The fraction was subjected to silica gel column chromatography with $CHCl_3$–MeOH–NH_4OH and then purified by a combination of chromatographic procedures, affording alkaloids **1**—**6** (Fig. 1) and anisic acid. Alkaloids **4**—**6** were identified by their spectroscopic properties as neferine,[2] liensinine[10—13] and isoliensinine,[14] respectively. Anisic acid[15] was isolated for the first time from this species.

Compound **1** was isolated as a colorless amorphous powder, $[\alpha]_D$ −64°. The high-resolution chemical ionization mass spectrum (HR-CI-MS) of **1** exhibited a pseudomolecular ion $[M+H]^+$ at *m/z* 597.2951, indicating a molecular formula of $C_{36}H_{40}N_2O_6$. It showed UV maxima at 225 and 284 nm and IR bands at 3381, 1614 and 1510 cm^{-1}. Its ^{1}H-NMR spectrum (Table 1) showed signals due to two *N*-methyl groups at δ 2.54 and 2.52, four singlets due to aromatic protons at δ 6.67, 6.56, 6.31, 5.98, an aromatic AA′BB′ spin system at δ 6.93 and 6.75, and an AMX spin system at δ 6.75, 6.73 and 6.47. These spectral features demonstrated that **1** was structurally similar to neferine (**4**), a main alkaloid of this plant material. However, there were marked differences in the ^{1}H-NMR spectrum with two methoxy signals at δ 3.89 and 3.84 in **1** instead of four



1: R^1=R^2=R^3=H
4: R^1=R^2=Me, R^3=H
5: R^1=R^3=H, R^2=Me
6: R^1=Me, R^2=R^3=H
7: R^1=R^2=R^3=Me



2 and **3**

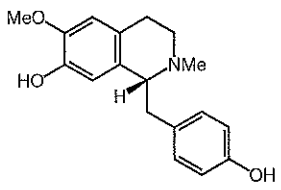


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Fig. 1. Structures of Compounds **1**—**7**

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds **1**, **2** and **3**

H/C	**1**[a]				**2**[b]				**3**[b]			
	δ_H		δ_C	HMBC	δ_H		δ_C	HMBC	δ_H		δ_C	HMBC
1	3.52 dd	(9.5, 2.0)	65.3	C-3, 10, NMe	3.92 dd	(9.5, 2.5)	63.8	C-3, 4a, 8, 8a, 9, 10, NMe	3.9 m		64.0	C-3, 8a
3	2.75 dt	(12.0, 6.0)	47.6	C-1, 4, 4a, NMe	2.70 m		45.2	C-1, 4, 4a	2.73—2.80 m		45.0	C-1
	3.20 m	(13.0, 7.0, 5.5)		C-1, 4, NMe	3.34 m			NMe	3.42 m			
4	2.83 m		26.3	C-3, 4a, 5	2.58 m		24.9	C-4a, 5	2.63 m		24.1	
	2.83 m			C-3, 4a, 5	2.96 ddd	(16.5, 11.0, 7.0)		C-3, 4a, 8a	3.00 m			
4a			129.5				125.7				124.6	
5	6.67 s		112.2	C-4, 6, 7, 8a	6.69 s		111.8	C-4, 6, 7, 8a	6.71 s		112.7	C-4, 6, 7, 8a
6			148.3				147.7				148.3	
7			143.27[c]				143.9				143.8	
8	6.31 s		118.2	C-1, 4a, 6, 7			125.5				125.3	
8a			130.6				130.0				129.8	
9	2.69 dd	(13.5, 9.5)	39.8	C-1	2.59 m		39.5	C-1, 10, 11, 15	2.60 m		40.6	C-1, 11, 15
	3.19 dd	(13.5, 2.0)		C-1	2.64 m			C-1, 10, 11, 15	2.60 m			C-1, 11, 15
10			130.4				132.9				132.0	
11	6.93 d	(8.0)	130.9	C-9, 12, 13, 14	6.41 d	(8.5)	130.9	C-9, 13	6.38 d	(8.5)	131.0	C-9, 13
12	6.75 d	(8.0)	116.6	C-10, 13	6.50 d	(8.5)	115.8	C-10, 13	6.50 d	(8.5)	115.9	C-10, 13
13			155.5	C-10, 13			156.3	C-10, 13			156.5	C-10, 13
14	6.75 d	(8.0)	116.6		6.50 d	(8.5)	115.8		6.50 d	(8.5)	115.9	
15	6.93 d	(8.0)	130.9	C-9, 12, 13, 14	6.41 d	(8.5)	130.9	C-9, 13	6.38 d	(8.5)	131.0	C-9, 13
NMe	2.54 s		42.6	C-1, 3	2.21 s		43.1	C-1, 3	2.32 s		42.5	C-1, 3
6-OMe	3.89 s		56.0	C-6	3.86 s		56.3	C-6	3.88 s		56.5	C-6
1′	3.67 dd	(9.0, 3.0)	64.2	C-3′	3.72 t	(6.0)	66.5	C-3′, 4a′, 8′, 8a′, 9′, 10′, N′Me	3.74 br t	(6.5)	65.8	C-3′, 4a′, 8′, 8a′, 9′, 10′, N′Me
3′	2.96 m		44.7	C-1′, 4′, 4a′	2.70 m		48.0	C-1′, 4′, 4a′	2.63 m		47.4	
	3.40 m			N′Me	3.18 ddd	(12.5, 9.0, 5.0)		N′Me	3.09 m			
4′	2.62 m		22.2	C-4a′	2.61 m		25.9	C-3′, 4a′	2.58 m		25.6	
	2.93 m			C-3′, 4a′	2.82 ddd	(16.5, 9.0, 6.0)		C-3′, 4a′	2.73—2.80 m			
4a′			123.3				125.7				125.6	
5′	6.56 s		110.8	C-4′, 6′, 7′, 8a′	6.61 s		112.7	C-4′, 6′, 7′, 8a′	6.58 s		112.0	C-4′, 6′, 7′, 8a′
6′			145.6				147.8				147.9	
7′			143.31[c]				145.4				145.4	
8′	5.98 s		114.4	C-1′, 4a′, 6′, 7′	6.49 s		115.5	C-1′, 4a′, 6′, 7′	6.36 s		115.6	C-1′, 4a′, 6′, 7′
8a′			127.8				131.2				130.5	
9′	2.67 dd	(13.5, 9.0)	42.3	C-11′, 15′	2.88 dd	(14.0, 6.0)	41.3	C-1′, 10′, 11′, 15′	2.84 dd	(14.0, 6.5)	41.0	C-1′, 10′, 11′, 15′
	3.09 dd	(13.5, 3.0)			3.12 dd	(14.0, 6.0)		C-1′, 10′, 11′, 15′	3.00 dd	(14.0, 6.0)		C-1′, 10′, 11′, 15′
10′			130.5				132.7				132.3	
11′	6.73 d	(2.0)	121.3	C-9′, 13′, 15	6.87 d	(2.0)	133.9	C-8, 9′, 13′	6.90 d	(2.0)	135.2	C-8, 9′, 13′, 15′
12′			143.9				124.4				123.8	
13′			146.7				154.9				153.9	
14′	6.75 d	(8.0)	115.6	C-10′, 12′	6.88 d	(8.5)	117.2	C-10′, 12′, 15′	6.89 d	(8.0)	116.9	C-10′, 12′
15′	6.47 dd	(8.0, 2.0)	126.9	C-9′, 11′, 13′	7.14 dd	(8.5, 2.0)	130.8	C-9′, 11′, 13′	6.95 dd	(8.0, 2.0)	131.3	C-11′, 13′
N′Me	2.52 s		40.6	C-1′, 3′	2.42 s		42.9	C-1′, 3′	2.36 s		42.5	C-1′, 3′
6′-OMe	3.84 s		55.8	C-6′	3.79 s		56.4	C-6′	3.78 s		56.4	C-6′

a) Measured in $CDCl_3$. *b*) Measured in CD_3OD. *c*) Assignments may be interchanged.

methoxy signals in **4**. Methylation of **1** with trimethylsilyldiazomethane afforded a methylated compound **7**, which was identical to *O*-methylneferine derived from neferine (**4**).[16] These findings demonstrated that the new compound **1** was a desmethylated compound of neferine (**4**).

The location of two methoxy groups in **1** was confirmed by 2D-NMR experiments with **1**. From ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) correlations with C-4 and C-4′ as well as nuclear Overhauser effect spectroscopy (NOESY) interactions with H_2-4 and H_2-4′, two singlets at δ 6.67 and 6.56 were assigned to H-5 and H-5′, respectively. Important NOESY cross peaks between H-5 and the methoxy signal at δ 3.89, and between H-5′ and the methoxy signal at δ 3.84, indicated the presence of methoxy groups at C-6 and C-6′ (Fig. 2). Further support was obtained from comparison of ^{13}C-NMR spectral data of **1** with those of liensinine (**5**) and isoliensinine (**6**). The chemical shifts of C-12, 13 and 14 in **1** (δ 116.6, 155.5, 116.6) agreed well with those of liensinine (**5**) (δ 116.9, 155.7, 116.9), but differed from those of isoliensinine (**6**) (δ 113.5, 157.8, 113.5). On the other hand, carbon signals of C-5′, 6′, 7′ and 8′ in **1** (δ 110.8, 145.6, 143.3, 114.4) resonated at nearly the same frequencies as those of isoliensinine (**6**) (δ 110.6, 145.1, 143.5, 113.7) rather than those of liensinine (**5**) (δ 111.5, 147.8, 146.3, 111.4). Thus, compound **1** was elucidated to be 13,7′-*O*-didesmethylneferine (=7′-*O*-desmethylliensinine or 13-*O*-desmethyisoliensinine). Desmethylliensinine and desmethyisoliensinine have recently been detected by HPLC analysis as metabolic products of neferine (**4**) in rat liver but their structures remained to be confirmed.[17] On the other hand, novel metabolites of neferine after incubation with dog hepatic microsomes were identified as 6-*O*-desmethylneferine, 2′-*N*-desmethylneferine, 2′-*N*-6-*O*-didesmethylneferine and 6,13-*O*-didesmethylneferine by HPLC and data-dependent electrospray ionization (ESI)-MS/MS with diode-array detection (DAD).[18] The present study constitutes the first isolation of 13,7′-*O*-didesmethylneferine as a natural product and the new alkaloid designated nelumboferine.

Compound **2** was found to be an isomer of **1** with a molecular formula of $C_{36}H_{40}N_2O_6$. The ^{1}H-NMR spectral data of **2** with signals for two methoxy and two *N*-methyl groups, an aromatic AA′BB′ spin system and an AMX spin system were similar to those of **1**. However, only three singlets due to aromatic protons were observed in the ^{1}H-NMR spectrum of **2** (Table 1). With the aid of ^{1}H–^{1}H shift correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC), HMBC and NOESY experiments, the aromatic singlets at δ 6.69, 6.61 and 6.49 could be assigned to H-5, H-5′ and H-8′, respectively, and the signal due to H-8 was missing in **2**. Two methoxy groups were located at C-6 and C-6′ by the NOESY cross peaks between H-5 and the methoxy signal at δ 3.86, and between H-5′ and the methoxy signal at δ 3.79 (Fig. 2). The doublet at δ 6.87 (J=2.0 Hz) was assigned to H-11′ by HMBC correlation with C-9′ and NOESY cross peak with H-1′. The signal of H-11′ revealed a significant HMBC interaction with a quaternary aromatic carbon signal at δ 125.5, which could be assigned to C-8 by HMBC correlation from H-1. These observations allowed determination of the structure in which two units of *N*-methylcoclaurine were coupled through the C–C linkage of C-8 and C-12′.

Compound **3** was isomeric with **1** and **2** and its spectral features showed close similarity to those of **2**. Differences between **2** and **3** were recognized only in the ^{1}H-NMR chemical shifts of H-8′, H-15′ and *N*-methyl groups (Table 1). Detailed 2D-NMR experiments indicated that **3** had the same planar structure as **2**. It could be assumed that the novel compounds **2** and **3**, designated nelumborines A and B, were atropisomers due to rotating hindrance of the biaryl system or diastereoisomers with respect to the chiral centers of C-1 and/or C-1′.

The absolute configuration at C-1 and C-1′ of neferine (**4**),[2] liensinine (**5**)[12] and isoliensinine (**6**)[14] had been elucidated to be *R*. (*R*)-*N*-Methylcoclaurine (**8**) has already been isolated from the leaves of *N. nucifera*.[7] From a biogenetic perspective, the chiral centers at C-1 and C-1′ of **2** and **3** were supposed to possess the same configuration *R* as in **1**, **4**—**6** and **8**. This assumption, together with important NOESY correlation between H-11′ and H-1, which was observed in **2** but not in **3**, suggested that these compounds might be atropisomers with axial chirality of *S*- and *R*-configurations, respectively, as shown in Fig. 3. However, the possibility that the compounds were (1*S*)- or (1′*S*)-diastereoisomers could not be excluded. The stereochemistry of **2** and **3** remained to be elucidated.

The occurrence of these new alkaloids gives interesting in-

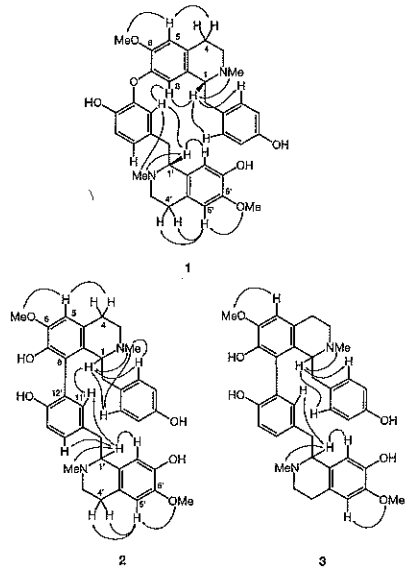

Fig. 2. Selected NOESY Correlations of **1**—**3**

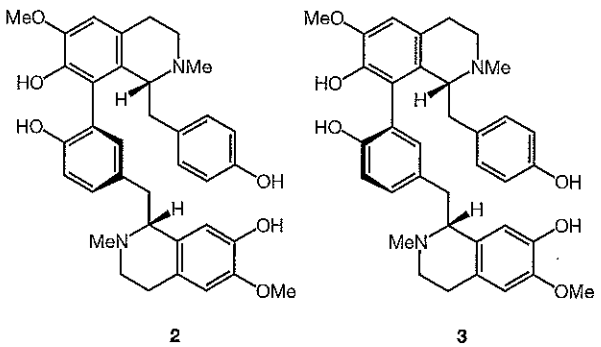

Fig. 3. Possible Stereochemistry of **2** and **3**

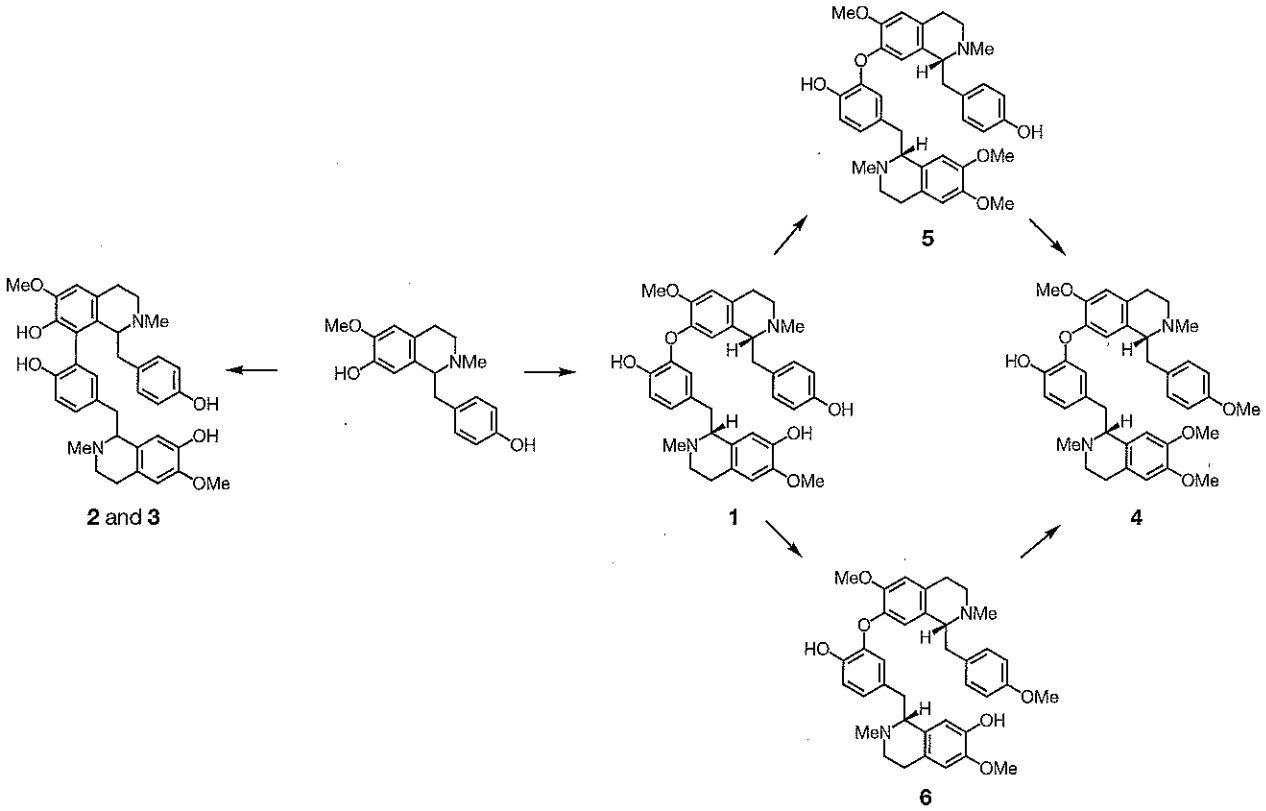

Chart 1. Biogenetic Scheme from *N*-Methylcoclaurine to Bisbenzylisoquinoline Alkaloids **1**—**6** in *Nelumbo nucifera*

formation about the biosynthesis of bisbenzylisoquinoline alkaloids in *Nelumbo nucifera*, in which *N*-methylcoclaurine could be recognized as a common intermediate. Two molecules of (*R*)-*N*-methylcoclaurine (**8**) are assumed to be coupled *via* C–O oxidative coupling of phenolic compounds to form **1**, which could be transformed to liensinine (**5**) and isoliensinine (**6**) and further to neferine (**4**) by *O*-methylation. On the other hand, two molecules of *N*-methylcoclaurine might suffer oxidative C–C coupling to construct **2** and **3** (Chart 1).

Phytochemical re-examination of embryo loti (embryos of the seeds of *N. nucifera*), led to the isolation and structural elucidation of three new bisbenzylisoquinoline alkaloids **1**—**3**. It is noteworthy that nelumborines A (**2**) and B (**3**) are dimeric alkaloids with a C–C linkage of two molecules of *N*-methylcoclaurine, whereas nelumboferine (**1**) is a C–O coupled dimer that is closely related to a bioactive component neferine (**4**). Biological activities of these minor alkaloids and stereochemistry of nelumborines are of great interest. Their total syntheses are now under investigation.

Experimental

Genaral Procedures Optical rotations were measured on a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2500PC spectrophotometer and IR spectra on a Shimadzu FTIR-8200 spectrophotometer. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectra were recorded on a Varian VXR-500 spectrometer with tetramethylsilane (TMS) as an internal standard. MS were obtained with a Hitachi M-4100 mass spectrometer. Glycerol was used as the matrix for secondary ion mass spectrum (SI-MS). TLC was performed on precoated Kieselgel 60F_{254} plates (Merck).

Plant Material Embryos of the seeds of *Nelumbo nucifera* were purchased from Longhua Hospital, Shanghai University of Traditional Chinese Medicine, in Shanghai, China. A voucher specimen (KPUY-031) is deposited in the laboratory of Kobe Pharmaceutical University.

Extraction and Isolation The embryos of the seeds of *Nelumbo nucifera* (13 kg) were extracted with hot MeOH. The MeOH extracts were concentrated *in vacuo* and the resulting residue (7.36 kg) was exhaustively extracted with 3% aqueous tartaric acid solution. The acidic aqueous solutions were made basic with 28% NH_4OH and extracted with $CHCl_3$. Concentration of the $CHCl_3$ layers gave a residue (84.7 g), which was subjected to flash column chromatography (CC) on Wakogel FC40. Elution with $CHCl_3$–MeOH–NH_4OH (95 : 4.5 : 0.5) gave fraction I (32.7 g) consisting of nearly pure neferine (**4**) and fraction II (9.84 g). Elution with $CHCl_3$–MeOH–NH_4OH (90 : 9 : 1) gave fractions III (16.2 g), IV (10.3 g) and V (2.06 g). Frs. II—IV further purified by a combination of CC on Wakogel FC40 ($CHCl_3$–MeOH, C_6H_6–AcOEt–Et_2NH) yielded liensinine (**5**) (II: 2.03 g, III: 8.31 g, IV: 5.33 g) and isoliensinine (**6**) (II: 5.00 g, III: 3.02 g, IV: 0.27 g). Fr. V was further purified by a combination of CC on Wakogel FC40 ($CHCl_3$–MeOH, C_6H_6–AcOEt–Et_2NH) and preparative TLC ($CHCl_3$–MeOH–NH_4OH, 90 : 9 : 1, 17 : 3 : 0.3, 40 : 10 : 1, 17 : 5 : 1), which gave **1** (47.9 mg), **2** (30.0 mg), **3** (35.9 mg) and anisic acid (18.8 mg).

Nelumboferine (**1**): Colorless amorphous powder; $[\alpha]_D^{28}$ −64.0° (c=1.0, MeOH); UV λ_{max} (MeOH) nm (log ε): 225 (4.43), 284 (3.95); IR (KBr) cm^{-1}: 3381, 1614, 1510; ^{1}H- and ^{13}C-NMR, HMBC correlations see Table 1; electron ionization (EI)-MS *m/z*: 596 $[M]^+$, 192; HR-CI-MS *m/z*: 597.2951 $[M+H]^+$ (Calcd for $C_{36}H_{41}N_2O_6$: 597.2966).

Nelumborine A (**2**): Colorless amorphous powder; $[\alpha]_D^{27}$ −18.3° (c=0.3, MeOH); UV λ_{max} (MeOH) nm (log ε): 206 (4.86), 284 (4.13); IR (KBr) cm^{-1}: 3407, 1514; ^{1}H- and ^{13}C-NMR, HMBC correlations see Table 1; HR-SI-MS *m/z*: 597.2986 $[M+H]^+$ (Calcd for $C_{36}H_{41}N_2O_6$: 597.2966).

Nelumborine B (**3**): Colorless amorphous powder; $[\alpha]_D^{27}$ −72.5° (c=0.9, MeOH); UV λ_{max} (MeOH) nm (log ε): 220 (4.58), 286 (4.02); IR (KBr) cm^{-1}: 3397, 1510; ^{1}H- and ^{13}C-NMR, HMBC correlations see Table 1; HR-SI-MS *m/z*: 597.2971 $[M+H]^+$ (Calcd for $C_{36}H_{41}N_2O_6$: 597.2966).

Methylation of 1 and 4 A solution of neferine (**4**) (40.8 mg) in MeOH (0.5 ml) and *n*-hexane (1 ml) was methylated with trimethylsilyldiazomethane and purified by preparative TLC ($CHCl_3$–MeOH–NH_4OH, 95 : 4.5 : 0.5) to afford *O*-methylneferine (22.4 mg). *O*-Methylneferine (**7**): White powder; $[\alpha]_D^{23}$ −29.6° (c=0.6, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 225 (4.48), 281.5 (3.94); IR (KBr) cm^{-1}: 1610, 1514, 1510; ^{1}H-NMR ($CDCl_3$) δ: 2.49 (3H, s, N′Me), 2.51 (3H, s, NMe), 2.58 (1H, m, H-4′), 2.61 (1H, m, H-4), 2.73 (1H, dd, J=14.0, 8.0 Hz, H-9′), 2.75 (1H, m, H-3), 2.77 (1H, m, H-3′), 2.80 (1H, dd, J=13.5, 6.5 Hz, H-9), 2.80 (2H, m, H-4, H-4′), 2.99 (1H,

m, H-9), 3.13 (1H, m, H-9′), 3.13 (1H, m, H-3), 3.17 (1H, m, H-3′), 3.57 (3H, s, 7′-OMe), 3.65 (1H, m, H-1′), 3.66 (1H, m, H-1), 3.71 (3H, s, 13-OMe), 3.78 (3H, s, 6-OMe), 3.805 (3H, s, 6′-OMe), 3.808 (3H, s, 13′-OMe), 6.01 (1H, s, H-8′), 6.32 (1H, s, H-8), 6.50 (1H, s, H-5′), 6.58 (1H, d, J=1.5 Hz, H-11′), 6.62 (1H, s, H-5), 6.67 (2H, d, J=8.5 Hz, H-12, 14), 6.71 (1H, dd, J=8.5, 1.5 Hz, H-15′), 6.81 (1H, d, J=8.5 Hz, H-14′), 6.90 (2H, d, J=8.5 Hz, H-11, 15); CI-MS *m/z*: 639 $[M+H]^+$, 206.

In a similar manner, methylation of **1** (10.9 mg) gave **7** (4.0 mg) as a white powder, $[\alpha]_D^{23}$ −24.3° (c=0.4, $CHCl_3$). This compound was identified with *O*-methylneferine.

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