Neolamarckines A and B, New Indole Alkaloids from *Neolamarckia cadamba*

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Received October 20, 2010; accepted November 29, 2010; published online December 1, 2010

Two new indole alkaloids, neolamarckines A and B (1, 2) were isolated from the leaves of *Neolamarckia cadamba* (Rubiaceae). Structural elucidation of 1 and 2 was performed by combination of 2D-NMR and circular dichroism (CD) spectra, and chemical correlations. Neolamarckine A (1) showed inhibition of inducible nitric oxide synthase (iNOS) dose dependently.

Key words neolamarckine; indole; *Neolamarckia cadamba*; Rubiaceae; inducible nitric oxide synthase activity

Neolamarckia cadamba (Rubiaceae), locally known as Laran in Malaysia, is a tree of moderate size found in India, China, and Malaysia.[1)] The bark and leaves of the plant are reported to possess various medicinal properties such as astringent, antihepatotoxic,[2)] antidiuretic,[3)] and anthelmintic properties.[4)] Chlorogenic acid isolated from the leaves has been reported to have hepatoprotective activity *in vitro* and to cause lipid peroxidation in liver microsomes *in vivo*.[5)] There has been no report of other phytochemical study and medicinal value of *N. cadamba* so far.

In continuation of our search for structurally and biologically interesting alkaloids from tropical plants found in Malaysia,[6—10)] we have embarked on studies of the dichloromethane extract on the leaves of *N. cadamba* and two new indoles, neolamarckines A and B (**1**, **2**) were isolated. This paper describes the structural elucidation of neolamarckines A and B (**1**, **2**), and the inhibitory activity of inducible nitric oxide synthase (iNOS) production of neolamarckine A (**1**).

Neolamarckine A (**1**), a brown amorphous solid, has the molecular formula $C_{22}H_{27}N_3O_4$, by high resolution-electrospray ionization-time-of-flight-mass spectrometry (HR-ESI-TOF-MS) [*m*/*z* 398.2089 $(M+H)^+$, Δ +0.9 mmu]. In the ^{1}H-NMR spectrum, the presence of four aromatic protons, one $-CH_2-CH_2-N-$ group, and a downfield –CH– aliphatic proton were observed, suggesting a tetrahydro-β-carboline type of skeleton.[11)] Two of the four aromatic protons appeared as doublets at δ_H 7.40 and 7.36, and the others as two triplets at 7.01 and 7.09 were attributed to H-9, H-12, H-10, and H-11, respectively. In addition, a downfield signal of H-17 (δ_H 7.45, broad singlet) characteristic of cadamine group type of alkaloids was observed.[12)] Two methoxy signals, one of which is attached to a carbonyl carbon at δ_H 3.65, and another resonating more upfield at δ_H 3.16, which is attached to an sp^3 carbon adjacent to an *N* atom, were observed. The ^{13}C-NMR spectrum showed 22 carbon resonances, which were in agreement with the molecular formula. The signals at δ_C 56.1, 57.0, 79.9, 42.4, and 27.0 could be assigned as the resonances of C-19, C-3, C-21, C-20, and C-15, respectively. The ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) spectral assignments, with the aid of extensive 2D-NMR experiments (correlation spectroscopy (COSY), rotating frame Overhauser enhancement spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC)) are summarized in Table 1. Selected 2D-NMR correlations for neolamarckine A (**1**) were shown in Fig. 1. The position of the 21-OMe was confirmed by the HMBC correlations of the 21-OMe and H-17 to C-21 (δ_C 79.9).

The phase-sensitive ROESY spectrum showed cross-peaks as shown in the 3D drawing of **1** (Fig. 2). The relative stereochemistry at C-3, C-15, C-19, C-20, and C-21 were assigned by detecting the ROESY cross-peaks between H-3/H-5a, H-5b/H-18b, H-15/H-19, H-14a/H-20, and H-21/H-18a, respectively. Hence, the configurations were deduced as 3*R**, 15*S**, 19*R**, 20*R** and 21*S**, respectively, as depicted in Fig. 2.

The absolute configuration of neolamarckine A (**1**) could be assigned by comparing its experimental circular dichroism (CD) spectrum with the calculated CD spectrum (CD calculation was performed by Turbomole 6.1[13)] using Time Dependent Density Functional Theory with Resolution of Identity Approximation (RI-TD-DFT)-BP86/aug-cc-pVDZ[14—18)] level of theory on RI-DFT-BP86/SVP[14—17,19)] optimized geometries). The calculated CD spectrum of the isomer with 3*R*, 15*S*, 19*R*, 20*R* and 21*S* configurations showed similar CD pattern compared to those of **1** as shown in Fig. 3. Therefore, its absolute stereochemistries were deduced as shown in Fig. 2.

The molecular formula of neolamarckine B (**2**) was larger than that of neolamarckine A (**1**) by 16 mass units. ^{1}H- and

1 **2**

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Table 1. ^{1}H- (J, Hz) and ^{13}C-NMR Data of Neolamarckines A and B (**1**, **2**) in $CDCl_3/CD_3OD$ (1 : 1) at 300 K

	1		**2**	
	δ_H	δ_C	δ_H	δ_C
2		131.5		129.8
3	4.60, br s	57.0	4.65, br s	74.6
5a	3.12, m	47.7	3.80, m	66.3
5b	3.71, m		4.36, dd (12.8, 4.5)	
6a	2.86, br d (13.2)	17.1	3.04, m	20.0
6b	2.62, dd (4.6, 13.2)		3.13, m	
7		107.9		106.2
8		127.6		126.6
9	7.40, d (7.2)	118.0	7.48, d (7.8)	118.5
10	7.01, dd (7.2, 7.2)	119.3	7.11, dd (7.8, 7.8)	120.1
11	7.09, dd (7.2, 7.2)	121.1	7.21, dd (7.8, 7.8)	122.9
12	7.36, d (7.2)	111.7	7.44, d (7.8)	112.9
13		136.7		138.0
14a	1.62, ddd (4.6, 13.3, 12.2)	31.6	2.51, ddd (4.6, 13.5, 12.9)	27.3
14b	3.34, m		3.38, m	
15	2.29, dd (12.2, 11.0)	27.0	2.43, dd (12.9, 12.4)	26.5
16		98.1		98.2
17	7.45, s	143.2	7.50, s	143.3
18a	3.61, br d (11.5)	57.7	3.81, br d (12.5)	58.6
18b	4.05, br d (11.5)		4.45, br d (12.5)	
19	2.86, br d (11.0)	56.1	3.46, br d (12.3)	65.1
20	1.77, dd (11.3, 11.0)	42.4	2.85, dd (12.5, 12.4)	37.8
21	4.46, s	79.9	4.51, s	79.7
22		169.2		169.2
21-OCH_3	3.16, s	54.2	3.15, s	54.2
22-OCH_3	3.65, s	50.9	3.71, s	51.0

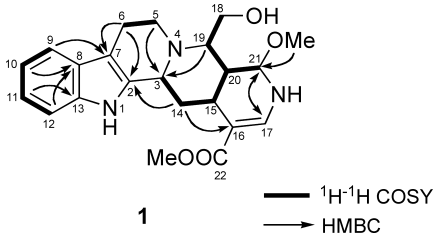

Fig. 1. Selected 2D-NMR Correlations for Neolamarckine A (**1**)

^{13}C-NMR data (Table 1) of **2** with two singlets at δ_H 3.15 and 3.71 for two methoxy groups were analogous to those of **1**. In addition, downfield signals observed in neolamarckine B (**2**) at C-3 (δ_C 74.6), C-5 (δ_C 66.3) and C-19 (δ_C 65.1) around *N*-4 suggested the presence of an *N*-oxide functionality.[20] Treatment of neolamarckine B (**2**) with Na_2SO_3 in aqueous MeOH afforded the reductive derivative, whose spectroscopic data and specific rotation were identical with those of neolamarckine A (**1**). Thus, the configuration of **2** was confirmed.

Nitric oxide (NO) is an important intracellular and intercellular signaling molecule that functions as a mediator in cardiovascular, nervous, and immune systems.[21] NO is involved in various biological reactions including vasorelaxation,[22] inhibition of platelet aggregation,[23] neurotransmission,[24] inflammation,[25] and immunoregulation.[26] In mammalian cells, NO is synthesized from L-arginine (L-Arg) by NO synthase (NOS), which is classified into three homologues: inducible-NOS (iNOS), endothelial-NOS (eNOS), and neuronal-NOS (nNOS).[27] iNOS produces large amounts of NO in macrophages stimulated by lipopolysaccharide

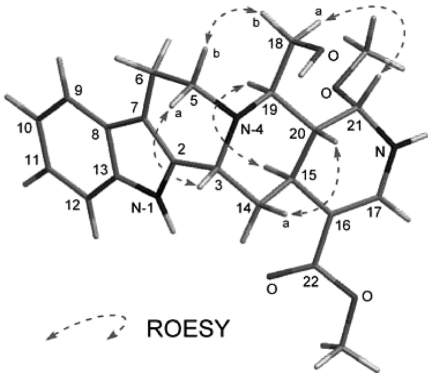

Fig. 2. Selected ROESY Correlations of a Minimum Energy Conformer of Neolamarckine A (**1**)

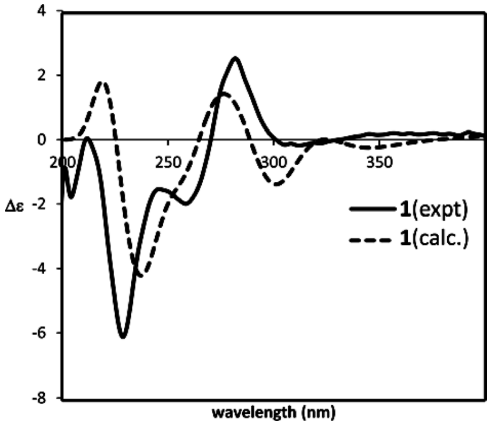

Fig. 3. CD Spectra of Neolamarckine A (**1**)
Dotted lines indicated simulated CD curves of **1**.

(LPS) and proinflammatory cytokines such as tumor necrosis factor (TNF) and interferon-γ (IFN-γ).[28] Therefore, inhibition of excess NO production by iNOS might have potential therapeutic value for the oxidative stress-induced inflammatory diseases and septic shock.[29]

Inhibition ratios at 50 μg/ml of **1** and **2** were 76 and 49%, respectively; although their cell viability was above 95% at each concentration. Neolamarckine A (**1**) exhibited moderate NO production inhibitory activity in J774.1 stimulated by LPS, dose-dependently at high cell viability as shown in Fig. 4, whereas, neolamarckine B (**2**) was found to be scarcely active.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 spectrophotometer and IR spectra on a JASCO FT/IR-4100 spectrophotometer. CD spectra were recorded on a JASCO J-820 polarimeter. High-resolution ESI-MS were obtained on a LTQ Orbitrap XL (Thermo Scientific). ^{1}H- and 2D-NMR spectra were recorded on a Bruker AV 500 spectrometer, and chemical shifts were referenced to the residual solvent peaks (δ_H 7.26 and δ_C 77.0 for $CDCl_3$). Standard pulse sequences were employed for the 2D-NMR experiments.

Material The leaves of *Neolamarckia cadamba* was collected at Piah Reserve Forest, Sungai Siput, Perak, Malaya, in 2007. The botanical identification was made by Mr. Teo Leong Eng, Faculty of Science, University of Malaya. The voucher specimen (Herbarium No. KL5501) was deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

Extraction and Isolation Dried and ground leaves of *N. cadamba* (2.0 kg) were defatted with hexane, and the plant material was dried and

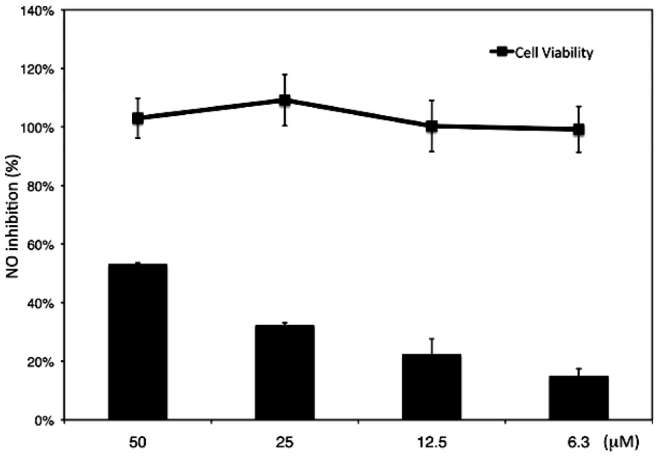

Fig. 4. NO Inhibition Ratio and Cell Viability, (—■—) of Neolamarckine A (**1**) in J774.1 Stimulated by LPS

The assays were performed $n=3$. Error bars represent S.D.

then soaked in 25% NH_4OH for 2 h. They were then soaked and macerated with CH_2Cl_2 twice over a period of 3 d. The supernatant obtained was concentrated to give crude alkaloids (3.5 g). The crude alkaloids (3.0 g) were subjected to column chromatography over silica gel using dichloromethane and methanol as solvents (100 : 0→4 : 1) and the column was finally flushed with methanol. Further purification of the 7th and 8th fractions was done by a preparative thin layer chromatography (92 CH_2Cl_2 : 8 MeOH, saturated with NH_4OH) yielded neolamarckines A (**1**) (5.2 mg) and B (**2**) (10.3 mg). Neolamarckines A (**1**) and B (**2**) could also be detected from the crude extract by HPLC and both of them should be considered as natural products.

Neolamarckine A (**1**): Brown amorphous solid; $[\alpha]_D^{27}$ −30 (c=0.2, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.70), 220 (sh, 4.65), 270 (4.39), and 347 (sh, 3.70) nm; CD (MeOH) λ_{max} 203 ($\Delta\varepsilon$ −2.18), 228 (−6.28), 258 (−2.03), 270 (0), 282 (2.61) nm; IR (KBr) ν_{max} 3399, 1668, 1621, and 1096 cm^{-1}; ^{1}H- and ^{13}C-NMR data (Table 1); ESI-MS m/z 398 $(M+H)^+$; HR-ESI-TOF-MS m/z 398.2089 (M+H; Calcd for $C_{22}H_{28}N_3O_4$, 398.2080).

Neolamarckine B (**2**): Brown amorphous solid; $[\alpha]_D^{27}$ −73 (c=0.3, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.73), 220 (4.78), and 271 (4.55) nm; CD (MeOH) λ_{max} 202 ($\Delta\varepsilon$ −7.22), 227 (−15.98), 259 (−0.42), 269 (0), 282 (7.73) nm; IR (KBr) ν_{max} 3394, 1671, 1624, and 1075 cm^{-1}; ^{1}H- and ^{13}C-NMR data (Table 1); ESI-MS m/z 414 $(M+H)^+$; HR-ESI-TOF-MS m/z 414.1979 (M+H; Calcd for $C_{22}H_{28}N_3O_5$, 414.2029).

Chemical Transformation of Neolamarckine B (2) into Neolamarckine A (1) To a solution of neolamarckine B (**2**, 0.2 mg) in aqueous MeOH (0.2 ml) was added Na_2SO_3 (1.0 mg) and the mixture was kept at room temperature for 30 min. After evaporation, the residue was applied to a silica gel column ($CHCl_3$/MeOH, 4 : 1) to give a compound (0.15 mg), whose spectroscopic data including $[\alpha]_D^{27}$ −28 (c=0.1, MeOH) was identical to that of natural neolamarckine A (**1**).

NO Production Assay by J774.1 Cell Lines J774.1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. J774.1 cells were seeded onto a 96-well microtiter plate at 1×10^5 cells in 100 μl solution per well and were preincubated for 12 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the medium containing LPS (5 μg/ml) with or without the test sample at different concentrations for 24 h. NO production was then determined by the Griess assay. One hundred microliters of the supernatant of the cultured medium was transferred to a 96-well microtiter plate, and then 100 μl of Griess reagent (1% sulfanilamide, 0.1% *N*-L-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4 was added). After incubation at room temperature for 15 min, the absorbance at 540 nm and 620 nm was measured with a microplate reader (Benchmark Plus microplate spectrometer, Bio-Rad).

Cell Viability Assay The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Fifteen microliters of MTT solution (5 mg/ml) was added into each well of the cultured medium. After a further 2 h incubation period, the medium was removed, and then 50 μl of dimethyl sulfoxide (DMSO) was added to resolve the formazan crystals. The optical density measurements were made using a microplate reader equipped with a two wavelength system at 550 nm and 700 nm. In each experiment, three replicates were prepared for each sample. The ratio of the living cells was determined on the basis of the difference of the absorbances between those of samples and controls.

Acknowledgment This work was partly supported by a University Malaya Research grant, UMRG011/09BIO, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Grant from the Open Research Center Project.

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