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

Ahmad Kaleem QURESHI,^a Mat Ropi MUKHTAR,^{*a} Yusuke HIRASAWA,^b Takahiro HOSOYA,^b Alfarius Eko NUGROHO,^b Hiroshi MORITA,^{*b} Osamu SHIROTA,^c Khalit MOHAMAD,^d A. Hamid A. HADI,^a Marc LITAUDON,^e and Khalijah AWANG^a

^aDepartment of Chemistry, Faculty of Science, University of Malaya; ^dDepartment of Pharmacy, Faculty of Medicine, University Malaya; 50603 Kuala Lumpur, Malaysia; ^bFaculty of Pharmaceutical Sciences, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan; ^cFaculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University; 1314–1 Shido, Sanuki, Kagawa 769–2193, Japan; and ^eInstitut de Chimie de la Substances Naturelles, Centre Nationale de la Recherches Scientifique; 91198, Gif-sur Yvette, Cedex, France.

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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 C₂₂H₂₇N₃O₄, 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 ¹H-NMR spectrum, the presence of four aromatic protons, one –CH₂–CH₂–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 ¹³C-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 ¹H- (500 MHz) and ¹³C-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 6.1¹³⁾ 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. ¹H- and

* To whom correspondence should be addressed. e-mail: moritah@hoshi.ac.jp; matropi@um.edu.my

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 ($\Delta\epsilon -2.18$), 228 (-6.28), 258 (-2.03), 270 (0), 282 (2.61) nm; IR (KBr) ν_{max} 3399, 1668, 1621, and 1096 cm^{-1} ; ^1H - and ^{13}C -NMR data (Table 1); ESI-MS m/z 398 ($\text{M}+\text{H}^+$); HR-ESI-TOF-MS m/z 398.2089 ($\text{M}+\text{H}^+$; Calcd for $\text{C}_{22}\text{H}_{28}\text{N}_3\text{O}_4$, 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 ($\Delta\epsilon -7.22$), 227 (-15.98), 259 (-0.42), 269 (0), 282 (7.73) nm; IR (KBr) ν_{max} 3394, 1671, 1624, and 1075 cm^{-1} ; ^1H - and ^{13}C -NMR data (Table 1); ESI-MS m/z 414 ($\text{M}+\text{H}^+$); HR-ESI-TOF-MS m/z 414.1979 ($\text{M}+\text{H}^+$; Calcd for $\text{C}_{22}\text{H}_{28}\text{N}_3\text{O}_5$, 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 ($\text{CHCl}_3/\text{MeOH}$, 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 $\mu\text{g}/\text{ml}$) 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 -1-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.

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