Note

Anti-inflammatory Activity of Pyrrolizidine Alkaloids from the Leaves of *Madhuca pasquieri* (Dubard)

Le Son Hoang,^a Manh Hung Tran,^a Joo Sang Lee,^a Dao Cuong To,^a Van Thu Nguyen,^a Jeong Ah Kim,^b Jeong Hyung Lee,^c Mi Hee Woo,^a and Byung Sun Min^{*,a}

^a College of Pharmacy, Drug Research and Development Center, Catholic University of Daegu; Gyeongbuk 712–702, Korea; ^b College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University; Daegu 702–701, Korea; and ^c College of Natural Science, Kangwon National University; Kangwon 200–701, Korea.
Received December 17, 2014; accepted March 16, 2015

A novel pyrrolizidine alkaloids, madhumidine A (1), and two known alkaloids, lindelofidine benzoic acid ester (2) and minalobine B (3) were isolated from the leaves of *Madhuca pasquieri* (Dubard) H. J. LAM. The chemical structures of these alkaloids were established mainly by NMR techniques and mass spectrometry. Their anti-inflammatory activity was evaluated against lipopolysaccharide-induced nitric oxide production in macrophage RAW264.7 cell. In addition, the cytotoxic activity of all isolated compounds was tested against a panel of cancer cell lines.

Key words *Madhuca pasquieri*; Sapotaceae; lindelofidine-1-hydroxymethyl pyrrolizidine

The genus *Madhuca* (Sapotaceae) includes more than 800 tree species, some of which are used in the production of latex. These species are known to be a rich source of pentacyclic triterpenoid saponins because, in most cases, they possess sugar residues attached at both C-3 and C-28 to an oxygenated oleanolic acid skeleton.^{1–3} In addition, these species contain other secondary metabolites including tannins, alkaloids, and cyanogens.^{1–3} *Madhuca pasquieri* (Dubard) H. J. LAM, a species belonging to the *Madhuca* genus, is a perennial herb that grows throughout the Northern provinces of Vietnam.^{4,5} In Vietnam, it has been used as a medicinal herb to treat bacterial infection and diabetes.⁴ However, biological and phytochemical studies on this plant have not yet been reported. In our continued efforts to find new metabolites from Vietnamese medicinal plants, we have isolated one novel pyrrolizidine alkaloid, madhumidine A (1) and the already known alkaloids, lindelofidine benzoic acid ester (2) and minalobine B (3), from the leaves of *M. pasquieri* (Fig. 1). Here, we describe the isolation and identification of these compounds as well as their anti-inflammatory and cytotoxic activities.

Results and Discussion

Repeated column chromatography (silica gel, RP-18, and semi-preparative HPLC) of the the CHCl₃-soluble fraction from the leaves of *M. pasquieri* resulted in the isolation of three compounds (1–3). The chemical structures of the known

compounds were identified as lindelofidine benzoic acid ester (2) and minalobin B (3) by comparing their spectroscopic data with those reported in the literature.^{6,7)}

Compound 1 was isolated as a yellow oil with the molecular formula C₂₀H₃₃NO₄ as determined by the high resolution-electrospray ionization (HR-ESI)-MS [M]⁺ *m/z* 351.2409 (Calcd for 351.2412), and was positive to Dragendorff's reagent. Its UV spectrum showed absorption maximum at 219nm with the optical rotation as [α]_D²⁵ –26.6 (c 0.32; methanol (MeOH)). Part of the ¹H- and ¹³C-NMR spectra, in addition to the distortionless enhancement by polarization transfer (DEPT) spectrum, of 1 presented similar properties with lindelofidine.⁸⁾ The additional spectra signals corresponding to necic acid moiety properties include δ_H 6.91 (1H, dd, *J*=7.0, 6.5 Hz, H-13), 4.27 (2H, ol, H-14), 2.40 (1H, m, H-17), 1.66 (3H, d, *J*=7.0 Hz, H-21), 1.86 (1H, m, H-18), 1.13 (3H, d, *J*=7.0 Hz, H-22), 1.67 (1H, ol, H-19a), 1.45 (1H, m, H-19b), 1.86 (3H, s, H-23), and 0.89 (3H, t, *J*=7.5 Hz, H-20), which correlate to the carbon signals from the ¹H-detected heteronuclear multiple quantum coherence (HMQC) experiment at δ_C 137.9 (C-13), 62.9 (C-14), 40.7 (C-17), 14.7 (C-21), 16.4 (C-18), 18.8 (C-22), 25.7 (C-19), 16.4 (C-23), and 11.3 (C-20), respectively. This unit was further established by analysis of the ¹³C-NMR and DEPT spectra, which displayed the remaining signals of the two carbonyl carbons at δ_C: 175.9 (C-16) and 162.0 (C-11), and a quaternary carbon at δ_C: 128.5 (C-12). In the

Fig. 1. Chemical Structures of 1–3

* To whom correspondence should be addressed. e-mail: bsmin@cu.ac.kr

1

Fig. 2. Selected COSY, HMBC and NOE Correlations of **1**Table 1. ^1H - (500 MHz) and ^{13}C - (125 MHz) NMR Spectral Data of **1**

	Compound 1		
	^{13}C	$^1\text{H}^a$	COSY
1	39.8	2.79 (1H, m)	9, 8, 2 ^b
2	26.6	1.78 (1H, ol)	2 ^b , 3 ^b
		1.70 (1H, ol)	
3	53.1	3.84 (1H, m)	3b
		2.75 (1H, m)	2a
5	55.2	3.62 (1H, m)	6
		3.08 (1H, m)	6
6	26.3	1.78 (2H, ol)	7 ^a
7	25.9	1.97 (1H, m)	
		1.83 (1H, ol)	8
8	67.9	4.38 (1H, m)	1, 7 ^b
9	62.7	4.23 (1H, dd, 8.5, 11.0)	1
		4.16 (1H, dd, 8.0, 11.0)	1
11	162.0		
12	128.5		
13	137.9	6.91 (1H, dd, 6.5, 7.0)	14, 23
14	62.9	4.27 (2H, ol)	14
16	175.9		
17	40.7	2.40 (1H, m)	18, 21
18	16.4	1.86 (1H, ol)	17, 22, 19
19	25.7	1.67 (1H, ol)	18, 20
		1.45 (1H, m)	18, 20
20	11.3	0.89 (1H, t, 7.5)	19
21	14.7	1.66 (3H, d, 7.0)	17
22	18.8	1.13 (2H, d, 7.0)	18
23	16.4	1.86 (3H, s)	13

a) J in Hz; b) Recorded in pyridine- d_5 .

correlation spectroscopy (COSY) spectra, the correlated signals between H-13/H-14 as well as H-17/H-18/H-19 and H-20 were observed. In the nuclear Overhauser effect spectroscopy (NOESY) experiment, the positive nuclear Overhauser effect (NOE) cross-peaks between δ_{H} 4.38 (H-8) and δ_{H} 4.23 (H-9a); H-9a and H-23; H-23 and H-14/H-21 were observed (Fig. 2). These data together with the heteronuclear multiple bond connectivity (HMBC) correlations observed from δ_{H} 4.27 (2H, ol, H-14) to δ_{C} 128.5 (C-12), 137.9 (C-13), and 175.9 (C-16) demonstrated linkage of this side chain, thereby establishing the necic acid of **1** as (*E*)-13-((17,18-dimethylpentanoyl)-oxy)-12-methylbut-12-enoic acid. The other NOEs signal gave positive cross-peaks for H-21/H-23 and H-23/H-8 but no NOE cross-peaks for H-22/H-8 (Fig. 2), these data merge

with the two methyl proton signals at δ_{H} 1.66 ppm (H-21) and 1.13 ppm (H-22) presented as a respective doublet form (7.0 Hz) (Table 1); thus, these two methyls were in the typical of *erythro* form and the orientation of 17,18-dimethylpentanoyl moiety could be assigned as 17*R*,18*R* which was similar to that of 2*R*,3*R*-dimethylpentanoic acid (δ_{H} 1.13, d, $J=7.0$ Hz) and (δ_{H} 0.94, s, $J=7.0$ Hz)).⁹ In addition, the NOESY spectrum showed positive NOE cross-peaks between δ_{H} 2.79 (1H, m, H-1) and δ_{H} 4.38 (1H, m, H-8), indicating *cis*-configuration for these protons.⁸ Furthermore, HMBC correlations from δ_{H} 4.23/4.16 (H-9) to the carbonyl carbon at δ_{C} 162.0 (C-11) indicated the position of the ester attachment at C-9 (Fig. 2). Thus, **1** was confirmed as (*E*)-13-((17,18-dimethylpentanoyl)-oxy)-12-methylbut-12-enoic acid ester of lindelofidine, named madhumidine A.

The isolated compounds were tested for cytotoxicity *in vitro* by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in three human tumor cell lines: A549 lung cancer cells, PC-3 prostate cancer cells, and MCF-7 breast cancer cells.¹⁰ Compounds **1–3** showed weak cytotoxic activity against all cell types, with half maximal inhibitory concentration (IC_{50}) values greater than 100 μM . In addition, these alkaloids were tested for their effect against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophage RAW264.7 cells.¹¹ The cytotoxic effects of **1–3** were first evaluated by using the MTT assay, and these compounds did not affect the cell viability of RAW264.7 cells in either the presence or absence of LPS even at a dose of 50 μM lasting 24 h (data not shown). NO production was determined by the percent inhibition compared to blanks containing LPS, but neither compounds nor positive control treatments. To assess the effects of **1–3** on LPS-induced NO production in RAW264.7 cells, cell culture medium was harvested, and NO production was measured by using the Griess reaction. During the incubation time of 24 h, RAW264.7 macrophages produced very low levels of NO in the resting state. After LPS (1 $\mu\text{g}/\text{mL}$) stimulation, NO production increased dramatically to 100% baseline levels. However, compounds **1–3** reduced the NO production 24 h after LPS stimulation in a dose-dependent manner. Compound **2**, lindelofidine benzoic acid ester, exhibited the most potent inhibition of NO production with an IC_{50} value as $25.5 \pm 3.9 \mu\text{M}$, followed by madhumidine A (**1**) (IC_{50} value $38.6 \pm 5.7 \mu\text{M}$) (Table 2). Minalobin B (**3**) showed weak NO production inhibition with IC_{50} value over than 50 μM .

Pyrrolizidine alkaloids (PAs) are potentially hepatotoxic, secondary natural products commonly found in the Senecione-

Table 2. Cytotoxicity and NO Production Inhibition of **1**–**3**

Compounds	IC ₅₀ (μM) ^{a)}			
	A549	MCF-7	PC-3	NO production
1	>30	>30	>30	38.6±5.7
2	>30	>30	>30	25.5±3.9
3	>30	>30	>30	>50
AMT ^{b)}	—	—	—	0.022
Adriamycin ^{c)}	2.4±2.5	3.4±1.3	3.4±3.6	—

^{a)} The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC₅₀) relative to the vehicle control. ^{b)} AMT (2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride); positive control for NO production inhibition. ^{c)} Adriamycin; positive control for cytotoxicity. (—) not tested.

ae, Eupatorieae, Fabaceae, and especially Boraginaceae plant families.¹²⁾ Each PA consists of two parts, the naine base and the nenic acid(s). The structural discussions on PA are well reported, and are not limited to 1-azabicyclo[3.3.0]-octane derivatives isolated from plants or sequestered from them in higher organisms as bacterial and fungal sources, as well as those that bear little biosynthetic relationship to the plant pyrrolizidines. Skvortsov published several reviews compiling important findings on the stereochemical and conformational properties of both natural and synthetic pyrrolizidines. The *cis*–*trans* conversion in pyrrolizidines, and the deciding effect of steric factors on their conformational equilibrium have been discussed.^{13,14)} In addition, NMR assignment studies showed that shift values for base ring protons are very distinctive and allow for the immediate recognition of important features, particularly any unsaturation and the oxygenation pattern. The usual oxygenation pattern is either mono-oxygenation at C-9 or the other PA with multi-oxygenation at C-2, C-7, and C-9. Recently, analysis of the alkaloids in other sources led to the discovery of several new PAs showing unprecedented functional groups varying in their substitution pattern or stereochemistry around the pyrrolizidine core. These compounds showed antifungal and antibacterial activities. Some of them exhibited selective inhibitory glycosidase and telomerase activities. One of the novel PAs, pochonicine, showed potent inhibition of a variety of β -*N*-acetylglucosaminidases (GlcNAcases), at a level comparable to that of nagstatin, a natural inhibitor; the authors state that this is the first report of GlcNAcase inhibition by a pyrrolizidine.¹⁵⁾ However, PAs and their derivative compounds normally show weak cytotoxicity against various cancer cell lines.^{16,6)} In accordance with our present work, the cytotoxicity of the isolated PAs is also weak against cancer cell lines such as A549, PC-3, and MCF-7. However, their ability to potentially inhibit NO production suggests that the PA class may serve a beneficial role in other pharmacological applications.

Experimental

Chemicals and Reagents Solvents were purchased from Samchun Chemicals Company (Korea). The cancer cell lines LH-60, MCF-7, and HeLa were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin, and streptomycin were bought from GIBCO, Inc. (NY, U.S.A.).

Instruments Optical rotations were measured with a JASCO DIP-370 polarimeter. UV spectra were measured with a Thermo 9423AQA2200E UV spectrometer. IR spectra were measured with a Bruker Equinox 55 FT-IR spectrometer.

High pressure liquid chromatography (HPLC, Waters, U.S.A.) was used for purification and isolation with an YMC-Pack ODS-A HPLC column. NMR spectra were recorded on Varian Unity Inova 400 (¹H, 400MHz; ¹³C, 100MHz) spectrometers. Conventional pulse sequences were used for COSY, HMBC and HMQC spectroscopy. All chemical shifts are given in ppm units with reference to tetramethylsilane (TMS) as an internal standard and the coupling constants (*J*) are in Hz. HR-ESI-MS was measured on a JMS-700 MStation mass spectrometer. Thin layer chromatography (TLC) was carried out on precoated silica gel 60 F254 (Merck). Chromatography suppliers were used for isolation: silica-gel (Kieselgel 60, 63–200 mesh, Merck) or reverse phase silica-gel (LiChroprep® RP-18, 40–63mm, Merck). Optical density (OD) values in the cytotoxic activity evaluation by MTT assays were read on a TECAN-ELISA Microplate Reader.

Materials and Methods The leaves of *M. pasquieri* were collected from the Northern Vietnam in March 2013. The sample was identified by Prof. Byung Sun Min, Natural Product Chemistry and Pharmacognosy Division, College of Pharmacy, Catholic University of Daegu, Korea. The dried leaves (2.08 kg) were extracted by MeOH reflux. After evaporation of the solvent under reduced pressure, the crude MeOH extract (517.33 g) was obtained. This extract was suspended in water (2 L) and partitioned with *n*-hexane (2 L×3 times), CHCl₃ (2 L×3 times), and EtOAc (2 L×3 times) to afford the *n*-hexane (83.28 g), CHCl₃ (10.7 g), and EtOAc soluble fraction (126.89 g), respectively. The CHCl₃-soluble fraction was chromatographed on a silica-gel column chromatography eluting with a gradient of *n*-hexane–EtOAc (99:1–50:50) and CHCl₃–MeOH (100:1–5:95) to afford thirteen fractions (C1–C13) based on TLC pattern. Subfraction C11 was chromatographed by medium pressure liquid chromatography (MPLC-ODS, 11 mm ×300 mm, 2 mL/min) with the mobile phase MeOH–H₂O (1:1) to give six subfractions. From fraction C11.3, compound **3** (1.5 mg) was separated by MPLC (MPLC-IS, 11 mm ×300 mm, 2 mL/min) with CHCl₃–MeOH (95:5). Fraction C12 (2.16 g) was subjected to a silica gel column chromatography eluting with a gradient of CHCl₃–acetone (3:1–1:1) and CHCl₃–MeOH (5:1–1:1) to afford fourteen subfractions C12.1–C12.14. The subfraction C12.13 (325.4 mg) was subjected to a silica-gel column chromatography eluting with a gradient of CHCl₃–acetone (18:1–15:1) to afford five fractions (C12.13.1–C12.13.5). The subfraction C12.13.4 (57.9 mg) was purified by HPLC [using mobile phase acetonitrile (ACN)–H₂O (40:60–30:70)] over 90 min; flow rate: 5 mL/min; UV detection at λ₁ 210 nm and λ₂ 230 nm; column COSMOSIL (20×250 mm; 5 mm)] to obtain compounds **2** (3.5 mg, *t*_{r1}=44.28 min) and **1** (7.5 mg, *t*_{r2}=55.05 min).

Madhumidine A (**1**): yellow oil; $[\alpha]_D^{25}$ –26.6 (*c* 0.32; MeOH); UV $\lambda_{\max}$ (MeOH) nm: 219; IR (KBr): $\nu_{\max}$ 3415, 2964, 2922, 2854, 1719, 1631, 1602, 1436 cm^{-1} ; positive HR-ESI-MS m/z 351.2409 $[\text{M}]^+$ (Calcd for $\text{C}_{20}\text{H}_{33}\text{NO}_4$ 351.2412); ^1H (Pyridine- d_5 , 500 MHz) and ^{13}C -NMR (Pyridine- d_5 , 125 MHz) data, see Table 1.

Cytotoxicity Assay The cancer cell lines (A549, PC-3 and MCF-7) were maintained in RPMI 1640, which included L-glutamine with 10% FBS and 2% penicillin–streptomycin. Cells were cultured at 37°C in a 5% CO_2 incubator. Cytotoxicity was measured by using a modified MTT assay. Viable cells were seeded in the growth medium (100 μL) into 96-well microtiter plates (1×10^4 cells per well) and incubated at 37°C in a 5% CO_2 incubator. The test samples were dissolved in dimethylsulfoxide (DMSO) and adjusted to final sample concentrations ranging from 5 to 50 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After standing for 24 h, 10 μL of the test sample was added to each well. The same volume of DMSO was added to the control wells. On removing the medium after 48 h of the test sample treatment, MTT (10 μL) solution was also added to the each well (final concentration, 5 mg/mL). After 4 h incubation, the plates were removed, and the resulting formazan crystals were dissolved in DMSO (150 μL). The OD was measured at 570 nm. The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

Cell Culture The murine macrophage cell line (RAW264.7) was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured in DMEM (GIBCO, Inc., NY, U.S.A.) supplemented with 100 U/mL of penicillin, 100 U/mL of streptomycin and 10% FBS (GIBCO, Inc.). The cells were incubated in an atmosphere of 5% CO_2 at 37°C and were sub-cultured every 3 d.

Determination of NO Production The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants as described previously. Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 $\mu\text{g}/\text{mL}$ LPS (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 24 h in the presence or absence of the test compounds (5–50 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled H_2O). The absorbance at 540 nm was determined with a micro plate reader (Molecular

Devices, Emax, Sunnyvale, CA, U.S.A.), and the absorption coefficient was calibrated by using a sodium nitrite (NaNO_2) solution standard. Cell viability was measured with an MTT-based colorimetric assay. For this experiment, celastrol was used as a positive control.

Acknowledgments This research was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MEST) (KRF-2012R1A1A2003547 and 2012R1A2A2A06046921). We are grateful to the Korean Basic Science Institute (KBSI) for supplying the NMR spectra.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References

- Li X., Liu Y., Wang D., Yang C., Nigam S. K., Misra G., *Phytochemistry*, **37**, 827–829 (1994).
- Jakhmola R. C., Sharma V., Punj M. L., *Int. J. Anim. Sci.*, **2**, 113–126 (1987).
- Yoshikawa K., Tanaka M., Arihara S., Pal B. C., Roy S. K., Matsuura E., Katayama S., *J. Nat. Prod.*, **63**, 1679–1681 (2000).
- Royen P. van, *Blumea*, **10**, 122–125 (1960).
- Chantaranothai P., *Nord. J. Bot.*, **18**, 493–497 (1998).
- Kim K. H., Lee K. H., Choi S. U., Kim K. R., Lee K. R., *Heterocycles*, **81**, 1493–1502 (2010).
- Shakhidoyatov K. M., Abdullaev N. P., Kadyrov C. S., *Chem. Nat. Compd.*, **13**, 65–68 (1977).
- Huang S., Zhou X., Wang C., Wang Y., Xiao F., Shan L. H., Guo Z. Y., Weng J., *Phytochemistry*, **93**, 154–161 (2013).
- Pino P., Rossi R., Bucci P., *Tetrahedron Lett.*, **7**, 3295–3301 (1966).
- Van L. T. K., Hung T. M., Thuong P. T., Ngoc T. M., Kim J. C., Jang H.-S., Cai X. F., Oh S. R., Min B.-S., Woo M. H., Choi J. S., Lee H. K., Bea K. H., *J. Nat. Prod.*, **72**, 1419–1423 (2009).
- Tung N. T., Cuong T. D., Hung T. M., Lee J. H., Woo M. H., Choi J. S., Kim J., Ryu S. H., Min B. S., *Bioorg. Med. Chem. Lett.*, **23**, 1428–1432 (2013).
- Liddell J. R., *Nat. Prod. Rep.*, **19**, 773–781 (2002).
- Skvortsov I. M., *Chem. Heterocycl. Compd.*, **42**, 1247–1266 (2006).
- Skvortsov I. M., *Chem. Heterocycl. Compd.*, **39**, 493–503 (2003).
- Usuki H., Toyo-oka M., Kanzaki H., Okuda T., Nitoda T., *Bioorg. Med. Chem.*, **17**, 7248–7253 (2009).
- Park Y. C., Gunasekera S. P., Lopez J. V., McCarthy P. J., Wright A. E., *J. Nat. Prod.*, **69**, 580–584 (2006).