Oppositinines A and B: New Vasorelaxant β-Carboline Alkaloids from *Neisosperma oppositifolia*

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A phytochemical study on the bark of *Neisosperma oppositifolia* (Apocynaceae) yielded two new β-carboline indole alkaloids, oppositinines A (1) and B (2), together with five known alkaloids, isoreserpiline, isocarapanaubine, vobasine, 10-methoxydihydrocorynantheol-*N*-oxide, and ochropposinine oxindole. Structural elucidation of 1 and 2 was performed using 2D NMR methods. Oppositinines A (1) and B (2) showed potent vasorelaxant effects on the rat aorta.

Key words β-carboline; indole; *Neisosperma oppositifolia*; Apocynaceae; vasorelaxant activity

Malaysia, one of the 12 mega diversity countries in the world, contains at least 60% of the world's known plant species. The flora of Malaysia are exceedingly rich, and the country is conservatively estimated to be the home of about 15000 species of flowering plants and more than 1170 species of ferns, and over 26% of the tree species in Peninsular Malaysia are endemic.[1)] The richness of the Malaysian flora thus provides opportunities for the discovery of many novel natural products, some of which might possess useful bioactivities such as secoalkaloid rhazinilam from *Kopsia singapurensis*[2—6)] and the cytotoxic limonoids erythrocarpines A—E from *Chisocheton erythrocarpus*.[7)]

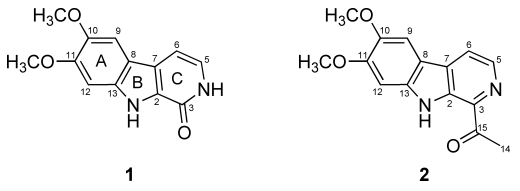
Our continuing interest in alkaloids from Malaysian Apocynaceae plants has led to the study of unique alkaloids from *Neisosperma oppositifoilia*. The genus *Neisosperma* comprises about 25 species that are distributed throughout the Western Pacific Islands, tropical Asia, and Polynesia.[8)] Only one species is found in Malaysia: *Neisosperma oppositifolia*, previously known as *Ochrosia oppositifolia*. The chemotaxonomic criteria that differentiate between the genus *Ochrosia* and the closely related *Neisosperma* is the presence of ellipticine and/or 10-methoxyellipticine in the former and its or their absence in the latter.[9,10)] The bark is used to treat diabetes and as a therapeutic bath to relieve sores. Parts of this plant are also used medicinally as an energizer, and the fragrant flowers are used in perfumes and deodorants. The soft wood of this species is used for light construction and firewood.

With the aim of isolating structurally interesting alkaloids with biological activities from Malaysian Apocynaceae,[7,11,12)] purification of the extracts from the bark of *Neisosperma oppositifolia* gave two new β-carboline alkaloids, oppositinines A (**1**) and B (**2**), together with five known indoles, isoreserpiline,[13)] isocarapanaubine,[13)] vobasine,[14,15)] 10-methoxydihydro-corynantheol-*N*-oxide,[9)] and ochropposinine oxindole.[9)] Oppositinine A (**1**) exhibited potent vasorelaxant activity on the rat aorta, while oppositinine B (**2**) had moderate activity. Their structures were elucidated using spectroscopic techniques such as 1D and 2D NMR, and IR, UV, and MS spectra.

Oppositinine A (**1**) was isolated as a yellowish oil. High-resolution electronspray ionization-mass spectroscopy (HR-ESI-MS) of **1** gave a pseudo-molecular ion peak $(M+H)^+$ at *m*/*z* 245.0938 corresponding to the molecular formula of $C_{13}H_{13}N_2O_3$ (Calcd *m*/*z* 245.0926). The UV spectrum showed maximal absorptions at 210, 252, 286, 325, 345, and 370 nm, resembling the absorption of β-carboline chromophore.[16)] The IR spectrum exhibited a broad band at 3342 cm^{-1} due to N–H stretching and strong absorption of a highly conjugated carbonyl group at 1665 cm^{-1}.[17)]

^1H- and ^{13}C-NMR data (Table 1) indicated the presence of four sp^2 methines, seven sp^2 quaternary carbons, and two methyl groups. Two of the sp^2 quaternary carbons (δ 135.6, 136.6) were attached to the nitrogen atom (*N*-1).[18,19)] These assignments were made using the heteronuclear multiple-bond correlation (HMBC) technique, where H-9 and H-12 was observed to correlate with C-13 (δ 135.6), while H-5 and H-6 interacted with C-2 (δ 135.6). Another nitrogen atom (*N*-4) was attached to one sp^2 quaternary carbon (δ 169.2) and one sp^2 methine (δ 137.0), respectively.

^1H–^1H correlation spectroscopy (COSY) of **1** showed a connection between H-5 and H-6. This was confirmed by the existence of two sets of doublets (J=5 Hz) in the ^1H-NMR spectrum at δ 8.30 and 7.94 which corresponded to H-5 and H-6, respectively. Furthermore, two singlets were observed at δ 7.48 and 7.02 attributable to H-9 and H-12, which showed correlation with C-10 and C-11 in ring A.[16)] In addition, an-



1 **2**

Chart 1

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Table 1. ^{1}H-NMR [400 MHz, δ_H (J, Hz)] and ^{13}C-NMR [100 MHz, δ_C] of Oppositinines A (**1**) and B (**2**) in $CDCl_3$

Position	**1**		**2**	
	^{1}H (J, Hz)	^{13}C	^{1}H (J, Hz)	^{13}C
2		135.6		135.4
3		169.2		135.7
4				
5	8.30 (*d*, 5 Hz)	137.0	8.45 (*d*, 5 Hz)	137.9
6	7.94 (*d*, 5 Hz)	117.0	8.00 (*d*, 5 Hz)	118.1
7		131.6		131.8
8		112.4		112.5
9	7.48 (*s*)	103.0	7.51 (*s*)	103.2
10		145.2		145.9
11		152.2		152.3
12	7.02 (*s*)	94.4	7.04 (*s*)	94.7
13		136.6		136.7
14			2.86 (*s*)	26.0
15				203.6
OCH_3	3.98 (*s*)	56.2	4.00 (*s*)	56.6
OCH_3	3.98 (*s*)	56.5	3.99 (*s*)	56.3
NH	7.99 (*s*)			
NH	10.16 (*s*)		10.18 (*s*)	

other singlet was also present at δ 3.98 integrated for two methoxyl groups. Finally, the presence of two broad singlets at δ 7.99 and 10.16 were attributed to the proton attached to *N*-1 and *N*-4.

The complete assignments were further substantiated by heteronuclear multiple-quantum correlation (HMQC) and HMBC spectra. One of the interactions was given by H-9, which showed long-range heteronuclear interactions with C-11 and C-13. The ^{1}H–^{1}H COSY and selected HMBC correlations are shown in Fig. 1.

The optically inactive oppositinine B (**2**) was obtained as a colorless oil. The UV and IR spectra showed similarity with those of **1**. The UV spectrum exhibited absorption maxima at 212, 245, 292, 335, and 360 nm. The IR spectrum revealed bands at 3378 and 1665 cm^{-1} characteristic of NH and a highly conjugated carbonyl absorption, respectively.[17] The HR-ESI-MS of **2** gave a pseudo-molecular ion peak $(M+H)^+$ at *m*/*z* 271.1092 corresponding to the molecular formula of $C_{15}H_{15}N_2O_3$ (Calcd *m*/*z* 271.1083), which differed from **1** by the addition of 26 mass units, thus suggesting the presence of two additional carbons. This was further supported by the ^{1}H- and ^{13}C-NMR spectral data (Table 1) and HMQC spectrum, which showed a marked resemblance to those of **1** except for some notable differences. For example, the existence of a singlet at δ 2.86 in the ^{1}H-NMR spectrum and one carbon signal at δ 26.0 in the ^{13}C-NMR spectrum were consistent with the presence of one methyl group attached to an sp^2 carbon. The correlations between methyl groups with two quaternary carbons, C-15 (δ 203.6) and C-3 (δ 135.7), in the HMBC spectra were also observed. These observations were explained by the existence of one methyl ketone in **2**. Other long-range correlations are presented in Fig. 1. Finally, all the assignments from the 2D NMR data confirmed that oppositinine B (**2**) was a related derivative of **1** with a methyl ketone group at C-3 (Fig. 1).

Oppositinines A (**1**) and B (**2**) belong to the β-carboline type of alkaloids. This is the second report on the occurrence of these simple indole alkaloids in *Neisosperma* species. The

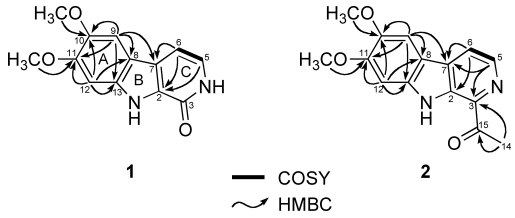

Fig. 1. Selected 2D NMR Correlations for Oppositinines A (**1**) and B (**2**)

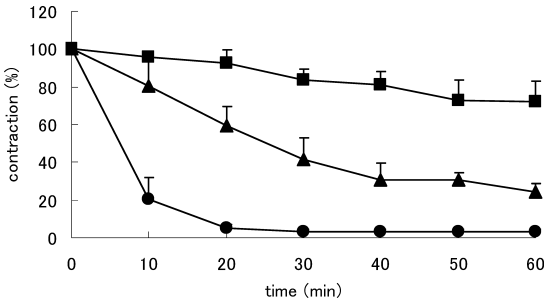

Fig. 2. Relaxation Responses Induced by Oppositinine A in Aortic Rings Precontracted with 3×10^{-7} M Phenylephrine (PE); Symbols: —●—, at 3×10^{-5} M; —▲—, at 10^{-5} M; —■—, at 3×10^{-6} M

Values are means±S.E. (n=3).

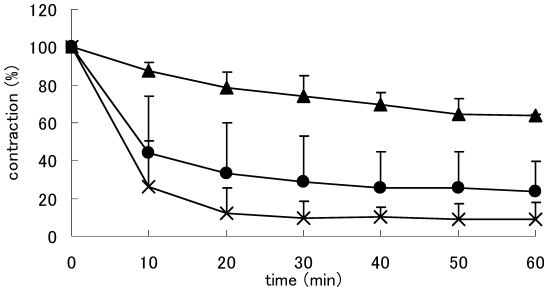

Fig. 3. Relaxation Responses Induced by Oppositinine B in Aortic Rings Precontracted with 3×10^{-7} M Phenylephrine (PE); Symbols: —×—, at 10^{-4} M; —●—, at 3×10^{-5} M; —▲—, at 10^{-5} M

Values are means±S.E. (n=3).

first β-carboline communicated was isolated from *Neisosperma kilneri* collected in Queensland, Australia.[20]

Vasodilators are useful for treatment of cerebral vasospasm and hypertension and for improvement of peripheral circulation.[21] Many natural products evoke vasodilatation through multiple mechanisms such as the nitric oxide system, potassium channel, and calcium channel functions.[22] Some β-carboline alkaloids such as harmine and harmaline were reported to inhibit phenylephrine-induced contraction in endothelium-intact aortic rings.[23] When phenylephrine (PE) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response, we added oppositinines A (**1**) and B (**2**). Oppositinine A (**1**) showed potent vasorelaxant activity (97% relaxation at 3×10^{-5} M), whereas oppositinine B (**2**) showed moderate vasorelaxant action (76% relaxation at 3×10^{-5} M). These vasorelaxant activities were observed in a concentration-dependent manner, as shown in Figs. 2 and 3. Treatment with N^G-monomethyl-L-arginine (L-NMMA, 10^{-4} M), an inhibitor of nitric oxide (NO) synthase, inhibited oppositine-induced vasorelaxation and the vasorelaxant effects were also attenuated by endothe-

lium removal. The vasodilator effects of **1** and **2** may be mediated through the increased release of NO from endothelial cells. Vasodilation appears to be influenced by hydrophobicity and the substituent pattern of the six-membered ring C. Efforts are currently underway to determine the precise mode by which oppositinines A (**1**) and B (**2**) exert vasorelaxant activity.

Experimental

General Experimental Procedures UV spectra were obtained on a Shimadzu UV-250 UV–visible spectrophotometer, and IR spectra were recorded on a Perkin Elmer 1600 spectrophotometer. ^{1}H- and 2D-NMR spectra were recorded on a JEOL ECA400 spectrometer, and chemical shifts were referenced to the residual $CDCl_3$ (δ_H 7.26 and δ_C 77.0). Standard pulse sequences were employed for the 2D NMR experiments. HR-ESI-MS were obtained on a LTQ Orbitrap XL (Thermo Scientific).

Plant Material The bark of *Neisosperma oppositifolia* was collected in Pangkor Island, Perak, Malaysia, in 1990. Identification was made by Mr. Teo Leong Eng, University of Malaya. Voucher specimens (KL 3813) were deposited in the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia, and in the Herbarium of the Forest Research Institute, Kepong, Malaysia.

Extraction and Isolation Extraction of the bark of *N. oppositifolia* (3.3 kg) was carried out by extracting exhaustively with hexane using a soxhlet extractor for 17 h to remove non-polar organic compounds, waxes, and fats. Then the extract was dried using a rotary evaporator. The plant material was dried and wetted with 10% ammonia solution and left overnight. It was then re-extracted successively with dichloromethane (CH_2Cl_2) and methanol (MeOH). After removal of the solvents, the crude extracts of hexane (0.7 g), dichloromethane (50.0 g), and methanol (3.55 g) were obtained.

Dichloromethane crude extract was dissolved in CH_2Cl_2 500 ml and re-extracted with 5% hydrochloric acid (HCl) until a negative result was formed with Mayer's reagent. The combined extracts were then basified with 25% NH_3 solution (pH 11) and re-extracted with CH_2Cl_2 until a negative Mayer's test was obtained and later washed with distilled water and sodium chloride solution and dried with sodium sulfate anhydrous. Finally, the extract was evaporated to dryness to give an alkaloid crude extract (5.1 g).

The alkaloid crude extract (5.0 g) was subjected to column chromatography (diameter: 2.5 cm, length: 40.0 cm) over silica gel (70—230 mesh ASTM, Merck 7734). The column was eluted with solvent mixtures (250 ml) of increasing polarity (CH_2Cl_2, CH_2Cl_2/MeOH and MeOH) to give 249 fractions. Fractions having spots with the same *Rf* value were grouped into four series of fractions (monitored by TLC Merck-1.05554.0001). Each series of fractions was then treated separately by extensive column chromatography and preparative TLC to purify the alkaloids. Seven alkaloids were obtained after the extensive isolation and purification procedures. Fraction A was further purified by preparative TLC (Merck 1.05715.0001) eluted with hexane/ethyl acetate (6 : 4) and afforded oppositinine A (**1**, 11.0 mg), oppositinine B (**2**, 3.3 mg), and isoreserpiline (0.12 g). The work-up procedure on fraction B with preparative TLC yielded isocarapanaubine (74.0 mg, hexane/ethyl acetate, 7 : 3) and vobasine (8.4 mg, hexane/ethyl acetate, 5 : 5); preparative TLC on fraction C gave 10-methoxydihydrocorynantheol-*N*-oxide (29.9 mg, hexane/ethyl acetate, 3 : 7). Finally, preparative TLC on fraction D, which was eluted with ethyl acetate, afforded ochropposinine oxindole (14.0 mg).

Oppositinine A (**1**): Yellowish oil; UV (MeOH) λ_{max} 210, 252, 286, 325, 345, and 370 nm; IR (liquid film) ν_{max} 3342 (NH), 3020, 2924, 1665 (C=O), 1213, 1053, and 751 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS *m/z* 245.0938 [(M+H)$^+$; Calcd for $C_{13}H_{13}N_2O_3$, 245.0926, Δ +1.2 mmu].

Oppositinine B (**2**): Colorless oil; UV (MeOH) λ_{max} 212, 245, 292, 335, and 360 nm; IR (liquid film) ν_{max} 3378 (NH), 2934, 1665 (C=O), 1207, 1053, 1161, and 750 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS *m/z* 271.1092 [(M+H)$^+$; Calcd for $C_{15}H_{15}N_2O_3$, 271.1083, Δ +0.9 mmu].

Vasodilation Assay[21] A male Wistar rat weighting 260 g was sacrificed by bleeding from the carotid arteries under anesthesia. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs–Henseleit solution (KHS: NaCl 118.0 mM, KCl 4.7 mM, $NaHCO_3$ 25.0 mM, $CaCl_2$ 1.8 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, and glucose 11.0 mM). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 5 ml of KHS solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time, the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with PE 3×10^{-7} M. The presence of functional endothelial cells was confirmed by demonstrating relaxation in response to acetylcholine 10^{-5} M, and aortic ring in which 80% relaxation occurred was regarded as tissue with endothelium. When the PE-induced contraction reached a plateau, each sample (**1**, **2**, 3×10^{-7}—10^{-4} M) was added.

The animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Acknowledgments We gratefully acknowledge the financial support provided by the University of Malaya (Vote PS142/2007B) and the Center for Natural Product Research and Drug Discovery (CENAR, Vote TA039/2008A). This work was also supported by a Grant from the Open Research Center Project of Hoshi University, Japan.

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