

Exploring the Chemistry of Alkaloids from Malaysian *Mitragyna speciosa* (Kratom) and the Role of Oxindoles on Human Opioid Receptors

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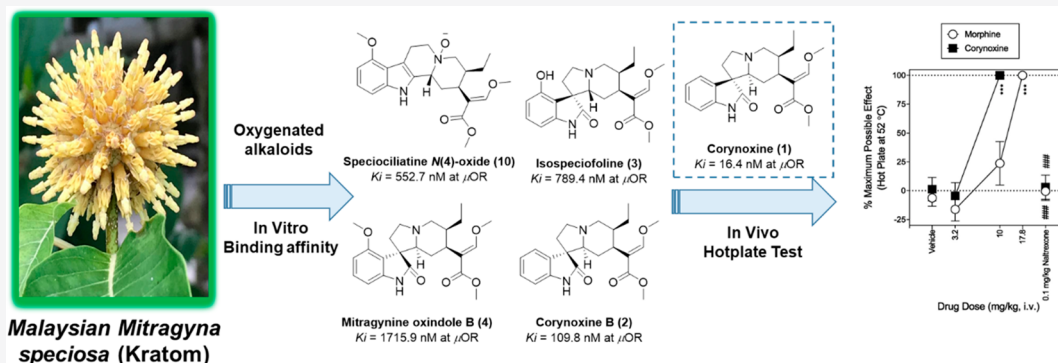
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ABSTRACT: Ten indole and oxindole alkaloids (1–10) were isolated from the freshly collected leaves of Malaysian *Mitragyna speciosa* (Kratom). The chemical structures of these compounds were established on the basis of extensive 1D and 2D NMR and HRMS data analysis. The spectroscopic data of mitragynine oxindole B (4) are reported herein for the first time. The spatial configuration of mitragynine oxindole B (4) was confirmed by single-crystal X-ray diffraction. Simultaneous quantification of the isolated alkaloids in the *M. speciosa* leaf specimens collected from different locations in the northern region of Peninsular Malaysia was also performed using UPLC-MS/MS. The oxindole alkaloids (1–4) and the indole alkaloid (10) were assessed for binding affinity at opioid receptors. Corynoxine (1) showed high binding affinity to μ -opioid receptors with a K_i value of 16.4 nM. Further, corynoxine (1) was 1.8-fold more potent than morphine in rats subjected to a nociceptive hot plate assay. These findings have important implications for evaluating the combined effects of the minor oxindole alkaloids in the overall therapeutic activity of *M. speciosa*.

Mitragyna speciosa Korth. (Rubiaceae), known also as kratom (English), krathum (Thai), or ketum and biak biak (Malay), is a psychoactive plant indigenous to Southeast Asia, particularly in southern Thailand and the northern states of Peninsular Malaysia. Currently, the plant is widely cultivated in Southeast Asian countries such as Indonesia, Malaysia, and Thailand.¹ Indole and oxindole alkaloids are the primary components of *M. speciosa*. The corynanthe-type indole mitragynine (6) exists as the major alkaloid, constituting up to 66% of the total alkaloid content.² Additional significant alkaloids are specioyngine (7) and paynantheine (8), which combined account for approximately 16% of the total alkaloid fraction.²

The concentrations of individual alkaloids in commercialized *M. speciosa* products appear to be largely defined by their origin and environmental factors, although most often their origin is unknown. Nonetheless, there is a widely distinct alkaloid profile between *M. speciosa* grown in the United States (U.S.)

and that grown in Southeast Asia.³ In fact, *M. speciosa* samples cultivated in the south-central U.S. have a concentration of approximately 0.04% mitragynine (6) (dry sample), several fold lower than concentrations present in the Southeast Asian samples.^{2,3} This variance could be attributed to several factors, such as different chemotypes, climates, soil types, and environmental pressures. Recently, an alkaloid profile and simultaneous quantification of 10 different *M. speciosa* alkaloids were published, providing the first step toward standardization

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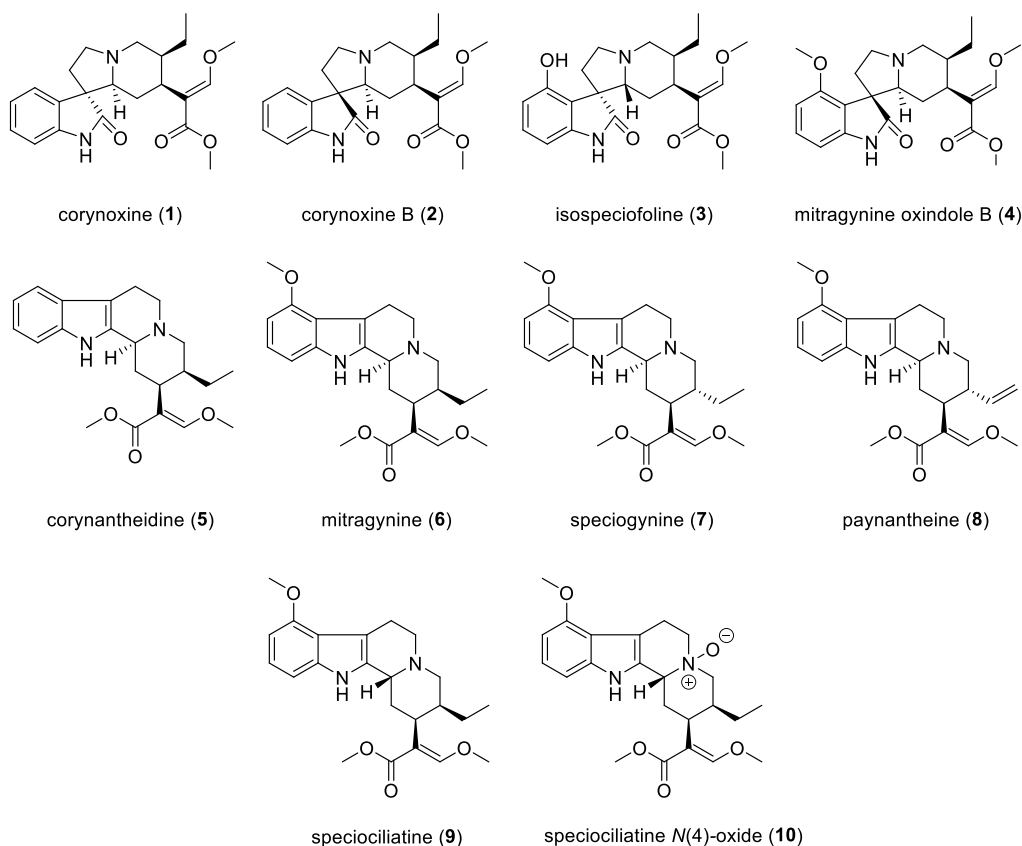


Figure 1. Alkaloids (1–10) isolated from the Malaysian samples of *Mitragyna speciosa*.

and quality control of marketed preparations and for analysis of wild and/or cultured plant material.⁴

Mitragynine (6) and its epimer speciociliatine (9) (2–4% of the total alkaloid content) together with a minor alkaloid, 7-hydroxymitragynine (11) (<2% of total alkaloid content), are currently known as the primary opioid-like components in *M. speciosa*.^{5,6} These alkaloids are partial agonists at the human μ -opioid receptor (MOR) with K_i values of 161.0, 54.5, and 7.2 nM, respectively.⁶ Efforts to reveal the roles of other minor *M. speciosa* alkaloids are ongoing.^{6,7}

This report describes 10 alkaloids (1–10) (Figure 1) isolated from the leaves of Malaysian *M. speciosa* and the alkaloid profile of leaf samples collected from different locations in the northern states of Peninsular Malaysia. Additionally, this study reports *in vitro* μ -opioid receptor binding affinity for the following low-abundance Malaysian *M. speciosa* oxindole alkaloids: corynoxine (1), corynoxine B (2), isospeciofoline (3), mitragynine oxindole B (4), and the oxygenated indole alkaloid speciociliatine N(4)-oxide (10). Corynoxine (1) was also compared to morphine in a rodent (rat) hot plate assay of antinociception.

RESULTS AND DISCUSSION

Chromatographic purification of the enriched alkaloidal fraction from the methanol extract of the freshly collected Malaysian *M. speciosa* leaves yielded four known oxindole alkaloids, corynoxine (1),⁸ corynoxine B (2),⁸ isospeciofoline (3),⁹ and mitragynine oxindole B (4).¹⁰ Also obtained were the known indole alkaloids corynantheidine (5),⁴ mitragynine (6),⁴ speciogynine (7),⁴ paynantheine (8),⁴ speciociliatine (9),⁴ and speciociliatine N(4)-oxide (10). The chemical

structures of mitragynine oxindole B (4) and speciociliatine N(4)-oxide (10) were confirmed by spectroscopic analysis, with that of mitragynine oxindole B (4) also examined by X-ray crystallography. The structures of the previously described indole alkaloids (5–9) were determined by comparison of their spectroscopic data (MS and ¹H and ¹³C NMR) with previously reported values.

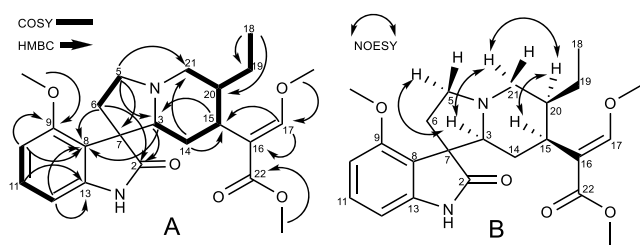
Compound 4 (mitragynine oxindole B) was isolated as colorless needle crystals and determined by HRESIMS to have a molecular formula of C₂₃H₃₀N₂O₅ (m/z 415.2227 [M + H]⁺, calcd for C₂₃H₃₁N₂O₅, 415.2155). This is consistent with 10 degrees of unsaturation. The ¹H and ¹³C NMR data (Table 1) resembled those of corynanthe-type oxindole alkaloids.^{8,9,11} The ¹H NMR spectrum (Table 1) indicated the presence of an AMX trisubstituted aromatic system at δ_H 7.06 (dd, J = 8.0, 8.0 Hz, H-11), 6.48 (d, J = 8.0 Hz, H-10), and 6.42 (d, J = 8.0 Hz, H-12). The spectrum also showed a methoxy substituent attached to an aromatic ring at δ_H 3.78 (s, OCH₃-9), an ethyl group at δ_H 1.73 and 1.10 (m, H₂-19) and 0.80 (t, J = 7.38 Hz, CH₃-18), and an indolic NH proton at δ_H 7.79 (s, N(1)-H). Additionally, signals at δ_H 7.24 (s, H-17), 3.57 (s, OCH₃-17), and 3.56 (s, OCH₃-22) suggested the presence of a β -methoxy acrylate moiety. The ¹³C NMR spectrum (Table 1) confirmed 4 to have 23 carbon atoms, including a carbonyl (δ_C 180.5), an ester carbonyl (δ_C 168.2), three aromatic carbons (δ_C 128.0, 104.3, and 101.6), three aromatic quaternary carbons (δ_C 154.6, 141.1, and 117.5), three methines (δ_C 69.9, 39.5, and 39.2), five methylenes (δ_C 53.9, 53.4, 30.0, 24.2, and 18.1), and one quaternary carbon (δ_C 55.3). Two-dimensional (2D) NMR experiments, namely, homonuclear ¹H–¹H COSY and heteronuclear ¹H–¹³C HSQC and HMBC, helped assign the

Table 1. ^1H and ^{13}C NMR Data of Mitragynine Oxindole B (4) and Speciociliatine N(4)-Oxide (10)

position	compound 4 ^a		compound 10 ^b	
	^1H δ_{H} , mult. (J in Hz)	^{13}C δ_{C} , mult.	^1H δ_{H} , mult. (J in Hz)	^{13}C δ_{C} , mult.
2		180.5, C		127.2, C
3	2.49, br d (10.8)	69.9, CH	5.09 br d (12.7)	65.95, CH
4				
5	3.16, br t (7.7)	53.4, CH ₂	3.69–3.64, m	65.93, CH ₂
	2.42, m		3.57, m ^c	
6	2.23, m ^c	30.0, CH ₂	3.57, m ^c	19.71, CH ₂
			3.18, m	
7		55.3, C		105.0, C
8		117.5, C		116.7, C
9		154.6, C		154.3, C
10	6.48, d (8.0)	104.3, CH	6.46, d (8.1)	98.8, CH
11	7.06, dd (8.0, 8.0)	128.0, CH	6.96, dd (8.1)	121.7, CH
12	6.42, d (8.0)	101.6, CH	6.90, d (8.1)	104.3, CH
13		141.1, C		138.1, C
14	2.23, m ^c	24.2, CH ₂	α , 2.66, ddd (14.5, 12.8, 6.5) β , 2.17, dt (14.4, 2.1)	28.6, CH ₂
15	2.59, dt (13.0; 3.0)	39.2, CH	3.56, m ^c	29.9, CH
16		110.6, C		110.6, C
17	7.24, s	159.4, CH	7.67, s	161.1, CH
18	0.80, t (7.4)	12.4, CH ₃	0.94, t (7.8)	10.8, CH ₃
19	1.73, m	18.1, CH ₂	1.28, m	22.9, CH ₂
	1.1, m			
20	1.41, br d (10.8)	39.5, CH	2.79, ddd (12.4, 7.3, 5.0)	33.6, CH
21	3.20, br d (9.2)	53.9, CH ₂	β , 4.10 br t (12.0)	69.0, CH ₂
	2.04, br d (9.2)		α , 3.18, m	
22		168.2, C		169.8, C
NH	7.79, s			
OMe-9	3.78, s	60.6, CH ₃	3.88, s	54.1, CH ₃
OMe-17	3.57, s	54.2, CH ₃	3.93, s	60.9, CH ₃
OMe-22	3.56, s	50.2, CH ₃	3.75, s	50.7, CH ₃

^aCDCl₃. ^bCD₃OD. ^cOverlapped.

^1H and ^{13}C NMR data (Figure 2). The location of the β -methoxy acrylate moiety was confirmed by the long-range HMBC correlations observed for H-15 with C-16 and C-17 (Figure 2A). The methoxy group position was determined by long-range ^1H – ^{13}C HMBC correlation of the methoxy protons

**Figure 2.** (A) COSY and HMBC correlations and (B) selected NOESY correlations of mitragynine oxindole B (4).

with C-9 in the aromatic ring. From these observations it was concluded that the planar structure is likely to be a tetracyclic oxindole, similar to corynoxine A/B but with an extra methoxy group in the aromatic ring at the C-9 position.

The relative configuration of 4 was deduced from a NOESY experiment (Figure 2B), which indicated correlations of H-20 (δ_{H} 1.41) and H-15 (δ_{H} 2.59), clearly showing that these protons are in the same plane, while H-3 (δ_{H} 2.49) correlated with the H-21 α (δ_{H} 3.20) and H-5 α (δ_{H} 3.16) protons. The H-21 α proton showed correlation with H-20, suggesting that the protons of the three aliphatic methines at position C-3, C-15, and C-20 are on the same face (3S, 15S, and 20S). This postulated structure was further validated by a single-crystal X-ray diffraction analysis (Figure 3), which also indicated an R configuration at the spiro-carbon, C-7. Prior to this isolation from *M. speciosa* leaves, compound 4 had previously been synthesized by oxidative conversion of mitragynine and assigned the name “mitragynine oxindole B”.¹⁰ We identified only one other report of the natural occurrence of a mitragynine oxindole, in a Thai *M. speciosa* specimen, which used TLC to identify mitragynine oxindoles A and B.¹¹ However, in neither of these previous studies had spectroscopic data been used to confirm the structure. The current report is the first to fully characterize mitragynine oxindole B (4), isolated from an authentic Malaysian *M. speciosa* specimen.

Speciociliatine N(4)-oxide (10) was obtained as a white, amorphous solid, and the molecular formula was confirmed by the analysis of the positive-ion HRESIMS (m/z 415.2591, calcd for C₂₃H₃₁N₂O₅, 415.2233). Compound 10 has the same molecular formula as 4 and similar NMR spectra (Table 1). Both compounds exhibit a trisubstituted aromatic AMX system at δ_{H} 6.96 (dd, J = 8.1, 8.1 Hz, H-11), 6.90 (d, J = 8.0 Hz, H-10), and 6.46 (d, J = 8.0 Hz, H-12), a methoxy substituent attached to an aromatic ring at δ_{H} 3.88 (s, OCH₃-9), an ethyl group at δ_{H} 1.23 (m, H₂-19) and 0.94 (t, J = 7.8 Hz, CH₃-18), and signals for the β -methoxy acrylate moiety at δ_{H} 7.67 (s, H-17), 3.93 (s, OCH₃-17), and 3.75 (s, OCH₃-22). However, compound 10 lacks a lactam carbonyl and a quaternary spiro-carbon, which suggests it has an indole alkaloid skeleton.^{12,13}

The differences in the NMR spectra between 10 (Table 1) and more typical mitragynine-type alkaloids are the aliphatic proton resonances in rings C and D were shifted to lower field, e.g., H-3 at δ_{H} 5.09 (br d, J = 12.7 Hz), H-21 β at δ_{H} 4.10 (br d, J = 12.0 Hz), H-21 α at δ_{H} 3.18 (m), H-4 β at δ_{H} 3.67 (m), and H-4 α at δ_{H} 3.57 (m). These shifts are consistent with the presence of an N-oxide moiety.¹² Homonuclear ^1H – ^1H COSY and heteronuclear ^1H – ^{13}C HSQC and HMBC experiments indicated a structure similar to a mitragynine (6)- or speciociliatine (9)-type indole alkaloid with an N-oxide functionality at N(4).^{12,14} A 2D NOESY experiment failed to establish the configuration of the methine protons at H-3, H-15, and H-20, although 1D selective NOE studies confirmed the proton configurations. Irradiation of H-20 at δ_{H} 2.79 correlated with the signals at δ_{H} 2.66 (ddd, H-14 α) and 3.18 (occurring as a double of doublets (dd) only in the 1D NOE experiment, H-21 α). Moreover, the δ_{H} 3.56 (H-15) signal showed a broad triplet (br t) but only in the 1D NOE experiment, suggesting that H-20 and H-15 are on the same face of the molecule. Irradiation of the signal assigned for H-3 at δ_{H} 5.09 (d) showed a clear correlation of the signals at δ_{H} 2.17 (d, H-14 β) and 4.10 (br t, H-21 β), being on the opposite face of the molecule in comparison with H-15 (Figure 4).

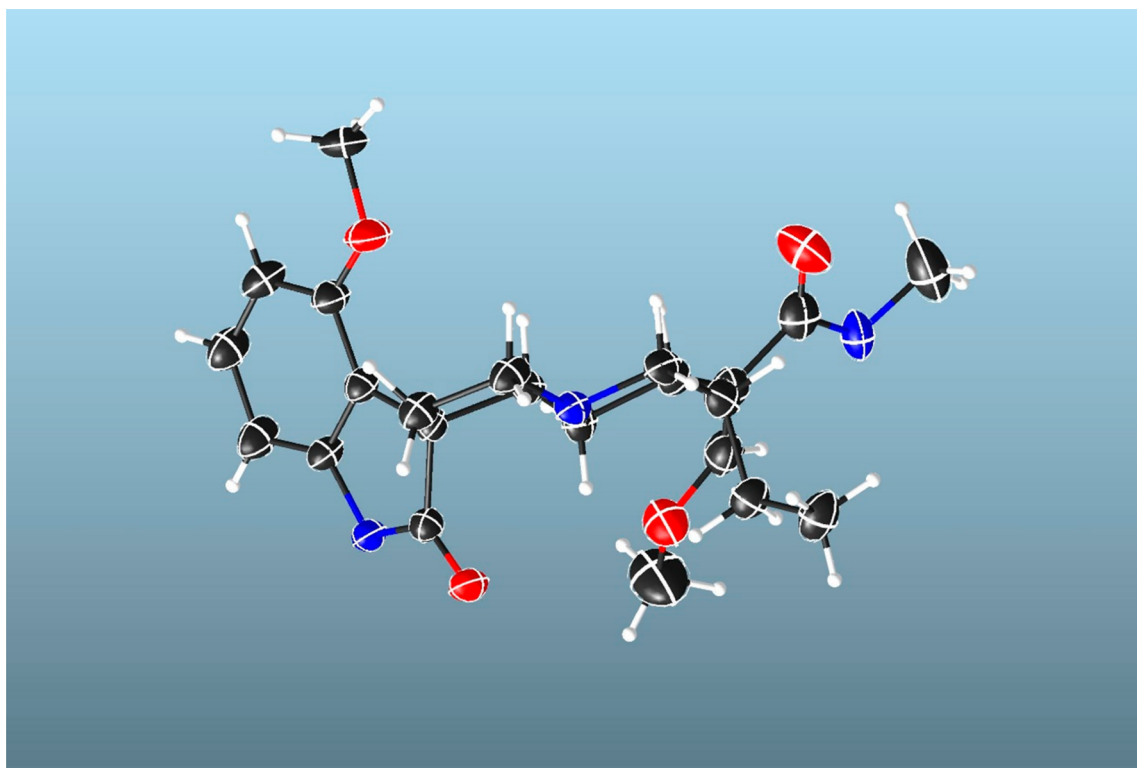


Figure 3. Projection of mitragynine oxindole B (4), with the displacement ellipsoids drawn at the 50% probability level.

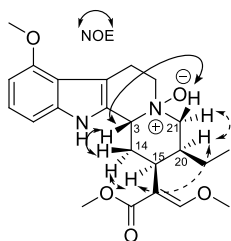


Figure 4. Selective NOE correlations observed for speciociliatine N(4)-oxide (10).

Additional irradiation of the isolated signals confirmed the structure as speciociliatine N(4)-oxide (10). The same low shift for H-3 has also been reported for the β configuration of isopaynantheine N(4)-oxide, a major secondary metabolite found in *Mitragyna diversifolia*.¹²

Owing to the high polarity of speciociliatine N(4)-oxide (10), the R_f values on normal silica gel TLC were much lower than those of speciociliatine (9) in various solvent systems, giving indirect evidence for the N-oxide assignment. Speciociliatine and mitragynine N-oxides were first described as oxidative products formed in a harsh acid medium.¹⁰ However, Takayama et al. also derived mitragynine N-oxide from mitragynine using *m*-chloroperbenzoic acid.¹⁴ During the preparation of this article, speciociliatine N(4)-oxide (10) was identified by Flores-Bocanegra et al. in a commercial *M. speciosa* product.¹⁵

The reported alkaloidal concentrations in *M. speciosa* leaf samples collected in Malaysia and Thailand are significantly different from each other,¹⁶ and specimens grown in the United States are even more distinctive.^{3,16} The precise reasons for these distinctions are unclear, but potentially include the influence of growing conditions such as seasonal

variance and photoperiod lengths, age of the plant at harvest, and sample handling (e.g., whether the leaves are fresh or dried). Postharvest environmental factors, such as storage, transportation, humidity, and contaminating microorganisms, may also significantly affect the alkaloid content of the leaf material. This suggests the opportunity to use authentic *M. speciosa* leaves from known origins to develop chemical fingerprints that can define biomass.

The specimens used in the current study were collected from different locations in Kedah and Penang, Malaysia (Table S1 and Figure S1, Supporting Information). Interestingly, while mitragynine (6) was the most abundant alkaloid in T1, -2, and -5–16, two samples (T3, T4) did not contain quantifiable levels of mitragynine (6), similar to plants reported that were grown in the south-central U.S.³ Trees T1–T5 were of similar age and located in the same Penang plantation, and the leaves were collected on the same day. Interestingly, mitragynine (6) was below the lower limit of quantification in T3 and T4, and speciogynine (7), and paynantheine (8) concentrations were also low, but speciociliatine (9) was in a higher concentration than in any of the other samples (Table 2). This variance in T3 and T4 suggests that *M. speciosa* exhibits chemotypes, which could also explain the alkaloidal profile found in *M. speciosa* growing in the U.S.³ and the recent report from Oberlies' group that outlined clear differences in the alkaloidal composition on marketed *M. speciosa* specimens in the U.S.¹⁵ This difference in alkaloidal profiles demonstrates that the historical literature should not be taken as universal. It is worth noting that plants with multiple chemotypes are not unusual. Indeed *Cannabis sativa* is a prime example; certain chemotypes produce high levels of Δ^9 -trans-tetrahydrocannabinol (THC), while hemp varieties produce little to no measurable amounts of THC.¹⁷

Table 2. Alkaloid Content in Freshly Collected *M. speciosa* Leaf Specimens from Malaysia

state	place	code	mg compound/1000 mg dried leaves													
			1 ^a	2 ^a	3 ^a	4 ^a	5 ^a	6 ^a	7 ^a	8 ^a	9 ^a	10 ^a	11 ^b	12 ^b	13 ^b	14 ^b
Penang	Kubang Semang	T1	0.37	0.12		0.10	0.08	10.89	1.12	1.17	3.51		0.05	0.07		
		T2	0.27	0.10	0.03	0.18	0.24	12.12	1.05	1.07	6.38	0.02	0.06	0.17		
		T3	0.46	0.15		0.11	0.08				13.49	0.06		0.09		
		T4	0.28	0.09		0.08	0.12			0.01	14.76	0.06		0.10		
		T5				0.16	0.09	18.85	1.23	0.93	2.04		0.15	0.10		
Penang	Bukit Mertajam (Pak Teh Plantation)	T6	0.03			0.14	0.03	9.38	0.99	1.01	1.65		0.08	0.07		
		T7	0.13	0.05		0.19	0.05	13.00	1.53	1.47	1.81		0.11	0.07		
		T8	0.10	0.05		0.05	0.03	12.22	1.74	1.63	0.43		0.06	0.04		
		T9				0.05	0.22	18.45	1.95	1.90	1.64		0.04	0.05		
		T10	0.25	0.12		0.07	0.03	9.73	1.25	1.40	1.12		0.03	0.05		
Kedah	Kulim (Halim Plantation)	T11	0.15	0.06		0.09	0.03	17.29	1.99	1.78	1.00		0.06	0.05		
	Baling	T12				0.18	0.06	15.68	1.33	1.09	1.25		0.11	0.05		
		T13				0.16	0.13	11.02	0.97	0.88	1.94		0.12	0.03		
		T14				0.14	0.42	12.42	1.23	1.09	1.39		0.09	0.12		
		T15	0.07			0.21	0.16	16.52	1.55	1.18	0.97		0.15	0.04		
		T16	0.16	0.06		0.17	0.23	13.28	1.12	1.12	2.22		0.11	0.07		

^aIsolated alkaloids from *M. speciosa*: 1, corynoxine; 2, corynoxine B; 3, isospeciofoline; 4, mitragynine oxindole B; 5, corynantheidine; 6, mitragynine; 7, speciogynine; 8, paynantheine; 9, speciociliatine; 10, speciociliatine N(4)-oxide. ^bOther alkaloid standards: 11, 7-hydroxymitragynine; 12, isocorynantheidine; 13, ajmalicine; 14, mitraphylline. Non value = below lower limit of quantification (LLOQ) (<0.1%)

Mitragynine oxindole B (4) was present in all the specimens examined in the current study, but in highly variable concentrations (0.05–0.21 mg/g; Table 2). Similarly, the other oxindoles examined, corynoxine (1) (0–0.42 mg/g) and its epimer, corynoxine B (2) (0 to 0.17 mg/g), were inconsistent in their representation with previously investigated specimens. In *Uncaria* species, the oxindole content is known to be affected by environment and seasonal factors such as total precipitation and plant water content.^{18,19} It is therefore possible that oxindole alkaloid biosynthesis from their indole precursors requires certain levels of water and oxygen. A short discussion about the biosynthesis of the kratom alkaloids (Figure S2) is provided in the Supporting Information.

7-Hydroxymitragynine (11) was present in small amounts in all samples (0.03–0.15 mg/g) except for T3 and T4, which supports recent reports suggesting 7-hydroxymitragynine could be a postharvest artifact of mitragynine.⁵ Two pentacyclic alkaloids, mitraphylline (13) and ajmalicine (14) (Figure S2, Supporting Information), have previously been reported in *M. speciosa*,² but were below the lower limit of quantification in all *M. speciosa* samples in the present study (Table 2).

Oxindoles (1–4) and the N-oxide (10) were screened at concentrations of 0.1 and 10 μ M for their capacity to displace tritiated opioid agonists from cultured cells expressing recombinant human δ -, κ -, and μ -opioid receptors (Table 3). In addition, each compound's binding displacement constant (K_i) for μ -opioid receptors was accessed over a more complete concentration range (Table 4). At 10 μ M, all compounds except isospeciofoline (3) displaced >50% [³H]DADLE at δ -opioid receptors (53.5–87.1%), and all except isospeciofoline (3) and mitragynine oxindole B (4) displaced >50% [³H]U69,593 (51.8–80.9%) from κ -opioid receptors (Table 3). At human μ -opioid receptors (hMOR), corynoxine (1)

Table 3. Percent Displacement of Radioligands by Selected *M. speciosa* Alkaloids at Opioid Receptor Subtypes in a Screening Profile^a

<i>M. speciosa</i> alkaloid concentration	δ -opioid	κ -opioid	μ -opioid
corynoxine (1)			
100 nM	−3.9	3.8	80.1
10 000 nM	73.8	75.3	97.8
corynoxine B (2)			
100 nM	−4.4	4.5	45.4
10 000 nM	65.5	51.8	94.4
isospeciofoline (3)			
100 nM	0.0	0.1	13.1
10 000 nM	27.1	37.3	83.3
mitragynine oxindole B (4)			
100 nM	10.4	5.0	7.3
10 000 nM	87.1	23.0	66.5
speciociliatine N(4)-oxide (10)			
100 nM	5.1	6.6	10.5
10 000 nM	53.5	80.9	84.6

^aFor subtype δ , human recombinant Chem-1 (RBL) cells. [³H]DADLE at 0.5 nM was used as a ligand. Naltrexone (10 μ M) was used as a nonspecific binder, with 60 min incubation. For subtype κ , human recombinant RBL cells. [³H]U69,593 at 0.5 nM was used as a ligand. Naloxone (10 μ M) was used as a nonspecific binder, with 60 min incubation. For subtype μ , human recombinant HEK-293 cells. [³H]DAMGO at 0.5 nM was used as a ligand. Naloxone (10 μ M) was used as a nonspecific binder, with 120 min incubation. All assays were performed at room temperature. ^bBold font in body of the table represents >50% displacement.

exhibited the highest affinity with a K_i value of 16.4 ± 1.4 (mean $\pm$ SEM) nM. Interestingly, corynoxine B (2), which differs only in its configuration at C-7, demonstrated about 6 times less affinity ($K_i = 109.8 \pm 8.1$ nM; Table 5). The O-

Table 4. K_i Values of Alkaloids 1–10 at the Human μ -Opioid Receptor

alkaloid	μ -opioid, $K_i \pm$ SEM (nM)	average abundance in Malaysian samples (% w/w) (except T3 and T4)
corynoxine (1)	16.4 $\pm$ 1.4	0.18
corynoxine B (2)	109.8 $\pm$ 8.1	0.06
Isospeciofoline (3)	789.4 $\pm$ 293.8	0.03
mitragynine oxindole B (4)	1715.9 $\pm$ 12.0	0.13
corynantheidine (5) ⁶	1910 $\pm$ 50.0	0.22
mitragynine (6) ⁶	161 $\pm$ 10.0	14.11
speciogynine (7) ²⁸	728 $\pm$ 61.0	1.48
paynantheine (8) ²⁸	410 $\pm$ 15.2	1.39
speciociliatine (9) ⁶	54.5 $\pm$ 4.4	3.4
speciociliatine N(4)-oxide (10)	552.7 $\pm$ 22.5	0.01
DAMGO	0.403 $\pm$ 0.008	
morphine	4.19 (2.03–11.1) ^a	

^a95% confidence limit.

methyl group at C-9 in mitragynine oxindole B (4) also greatly reduced human μ -opioid receptor affinity (1715.9 $\pm$ 912.0 nM), and was 16-fold lower than corynoxine B (2) (Table 4). In a recent study to evaluate the μ -opioid affinity for some selected *M. speciosa* alkaloids, corynoxine B (2) was found to have an affinity approximately 15-fold lower than that found in the present study.⁷ Since corynoxine (1) had the highest affinity for human μ -opioid receptors (Table 4), its in vivo activity was compared with that of morphine in a rat hot plate antinociception assay. Both compounds were administered via the intravenous (i.v.) route in order to minimize differences in bioavailability and time to maximum effect. Morphine dose-dependently increased antinociception, with an ED₅₀ value (95% confidence interval) of 12.1 (10.2–14.4) mg/kg, while the ED₅₀ value of corynoxine (1) was 6.75 (5.90–7.62) mg/kg (Figure 5, Table 5). Both morphine and corynoxine (1) antinociception was fully reversed by 0.1 mg/kg (i.v.) naltrexone (opioid receptor antagonist). These data demonstrate that corynoxine (1) is as antinociceptive as morphine in the hot plate assay, despite having slightly less affinity in the in vitro studies (Table 4). It is unclear whether this reflects a difference in the affinity of corynoxine for rat vs human opioid receptors or a difference in the efficacy of morphine and corynoxine (1) to produce antinociception in rats.

This study has revealed that the oxindole alkaloids corynoxine (1) and corynoxine B (2) have high binding affinities toward μ -opioid receptors. Since these oxindoles are found in very low concentrations within *M. speciosa* leaves, they

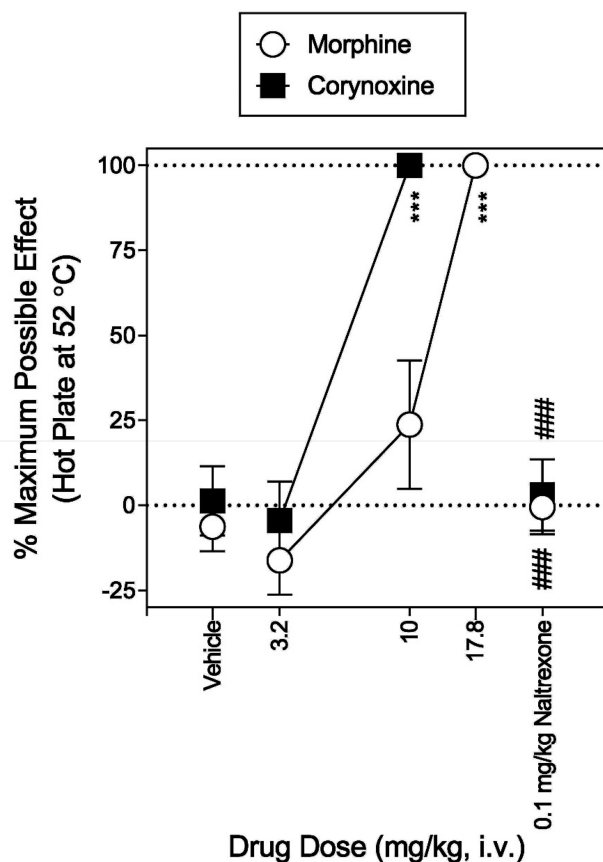


Figure 5. Effects of i.v. injection of morphine and corynoxine (1) on hot plate latency in catheterized rats. Abscissae: Vehicle, cumulative drug dose in mg/kg (i.v., log scale), and 0.1 mg/kg naltrexone (i.v.). Ordinates: Percentage of maximum possible effect (MPE). The hot plate was maintained at 52 $\pm$ 0.1 $^{\circ}$ C. Each point represents the mean $\pm$ SEM (n = 6, three subjects per sex). Following vehicle, cumulative doses were administered, followed by naltrexone (0.1 mg/kg, i.v.) at 5 min intervals. The antinociceptive effects of morphine and corynoxine (1) were antagonized by naltrexone. *** p < 0.001 compared with vehicle. ### p < 0.001 compared with morphine (17.8 mg/kg) or corynoxine (1, 10 mg/kg). Details for statistical analyses are shown in Table 5.

may not exert a significant pharmacological effect in the overall translation to human consumption of *M. speciosa*. However, these oxindole alkaloids are reported as major components in other medicinal plants of the *Uncaria* genus, including *Uncaria rhynchophylla*, *U. macrophylla*, and *U. tomentosa*, popularly known as Cat's Claw or Guo Teng.^{20–22}

In conclusion, 10 alkaloids were isolated from freshly collected Malaysian *M. speciosa* leaves including mitragynine oxindole B (4) and speciociliatine N(4)-oxide (10) and fully

Table 5. Statistical Analyses of Dose–Effect Functions for the Antinociceptive Effects of Test Compounds (i.v.) as Shown in Figure 5^a

drug	sex	dose (mg/kg, i.v.)	interaction	post hoc test
morphine + 0.1 mg/kg naltrexone	$F_{1,16} = 0.0129$; $p = 0.915$	$F_{4,16} = 14.3$; $p < 0.001$	$F_{4,16} = 0.205$; $p = 0.932$	17.8 mg/kg vs vehicle ($t = 6.05$, $p < 0.001$), 17.8 mg/kg vs naltrexone ($t = 5.72$, $p < 0.001$)
corynoxine (1) + 0.1 mg/kg naltrexone	$F_{1,20} = 1.73$; $p = 0.259$	$F_{5,20} = 54.3$; $p < 0.001$	$F_{5,20} = 0.882$; $p = 0.478$	17.8 mg/kg vs vehicle ($t = 10.2$, $p < 0.001$), 17.8 mg/kg vs naltrexone ($t = 10.2$, $p < 0.001$)

^aThe sample sizes are described in each figure legend. Comparisons were made using a two-way repeated-measures ANOVA followed by post hoc Bonferroni t tests with results shown when effects were statistically significant. Significant differences are in bold.

characterized. Additionally, a UPLC-MS method was described and demonstrated to simultaneously quantify 14 different *M. speciosa* alkaloids from 16 samples collected in Peninsular Malaysia. A high variability in the composition of the alkaloid fractions taken from different leaf specimens was observed, suggesting the existence of at least two different chemotypes, one with high content of mitragynine (6) and another with high concentrations of speciociliatine (9). This is also the first report to demonstrate that the oxindole alkaloid corynoxine (1) exhibits a high affinity for human μ -opioid receptors (16.4 ± 1.4 nM) and opioidergic efficacy in an antinociception assay comparable to that of morphine. This series of compounds may be valuable leads in the development of new analgesics.

EXPERIMENTAL SECTION

General Experimental Procedures. ^1H and ^{13}C NMR spectra were obtained using Bruker Avance NEO 600 NMR and Bruker Avance II 600 MHz NMR spectrometers operating at 600 MHz in ^1H and 151 MHz in ^{13}C (Bruker Co., Billerica, MA, USA). The chemical shift values were reported in parts per million units (ppm) from tetramethylsilane (TMS) using known solvent chemical shifts. Coupling constants were recorded in hertz (Hz). Standard pulse sequences were used for COSY, HMQC, HMBC, TOCSY, NOESY, and DEPT. HRMS and purity ($\geq 98\%$) were determined using an Agilent 1290 Infinity series ultraperformance liquid chromatograph (UPLC) system equipped with a photodiode array detector and quadrupole-time-of-flight (QTOF) Agilent 6540 mass spectrometer (Agilent Co., Santa Clara, CA, USA). Column chromatography was carried out on silica gel (70–230 mesh, Merck) and Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). TLC (silica gel 60 F254, Sorbtech, Norcross, GA, USA) was used to monitor obtained fractions from column chromatography. Preparative TLC was carried out on silica gel 60 PF254 + 366 plates (20×20 cm, 1 mm thick). Visualization of the TLC plates was achieved with a UV lamp ($\lambda = 254$ and 365 nm) and anisaldehyde/acid spray reagent (MeOH–acetic acid–anisaldehyde–sulfuric acid, 85:9:1:5). All solvents and reagents used for plant extraction and compound isolation were of analytical grade (Fisher Scientific, Fair Lawn, NJ, USA). Corynoxine (1), corynoxine B (2), isospeciofoline (3), mitragynine oxindole B (4), corynantheidine (5), mitragynine (6), paynantheine (7), speciogynine (8), speciociliatine (9), and speciociliatine N(4)-oxide (10) (purity $\geq 98\%$) used for UPLC-MS analysis were extracted and purified from the leaves of *M. speciosa* following the procedures described below. 7-Hydroxymitragynine (11) ($\geq 98\%$), used for UPLC-MS analysis, was synthesized according to the method described by Maxwell et al.²³ Another three alkaloid standards, ($\geq 98\%$) isocorynantheidine (12), ajmalicine (13), and mitraphylline (14), were obtained from an in-house alkaloid library. LC-grade MeOH, acetonitrile, acetic acid, ammonium acetate, and water were purchased from Fisher Scientific. Corynoxine (1) was converted to its hydrochloride salt for animal studies.

Plant Material. Fresh leaves of *M. speciosa* were collected on Permatang Rawa, Seberang Perai Tengah ($5^\circ 22' 17''$ N; $100^\circ 27' 1''$ E), Penang, Malaysia, for the extraction and purification of alkaloids. The plant was authenticated by a botanist, Dr. Rosazlina Binti Rusly, from the School of Biological Sciences, Universiti Sains Malaysia (USM), and a voucher specimen (NEL-(K)-2019(02)) was deposited at the herbarium of the School of Biological Sciences, USM. The additional 16 samples (T1–T16) were collected from several kratom plantations (Kubang Semang, Bukit Mertajam, Kulim, and Baling areas) in the states of Kedah and Penang, Malaysia. The exact location and dates of collection are summarized in Table S2 and Figure 1S (Supporting Information).

Extraction and Isolation. *M. speciosa* leaves (500 g) were extracted with hot MeOH (5 L) in a circulating water bath (45°C) for 12 h followed by maceration at room temperature for another 12 h. The same extraction procedure was repeated three times with fresh MeOH until the solution became colorless. After evaporation in

vacuo, the crude extract was partitioned between hexane (3×4 L) and 10% CH_3COOH (4 L). The water-soluble portion was then adjusted to pH 9.0 with 25% NH_4OH and extracted with CHCl_3 (3×4 L). The concentrated CHCl_3 -soluble portion was lyophilized to yield a crystalline alkaloid fraction (6.8 g). The alkaloid fraction was subjected to a passage over silica gel column chromatography (CC) (hexane–EtOAc–MeOH, 100:0:0 to 0:0:100, v/v) to give seven fractions (Fr. 1–Fr. 8). Fr. 2 (2.0 g) was purified by a silica gel CC (hexane–EtOAc, 80:20, v/v) to afford corynantheidine (5) (14 mg) and mitragynine (6) (1.72 g). Fr. 3 (0.35 g) was subjected to flash silica gel CC (hexane–EtOAc, 90:10 to 20:80, v/v) to afford paynantheine (8) (20 mg). Fr. 4 (0.42 g) was further separated by silica gel CC (hexane–EtOAc, 70:30 to 0:100, v/v) to yield three subfractions (SFr. 4a, 4b, and 4c). SFr. 4a was repeatedly purified by silica gel CC (hexane–EtOAc, 40:60, v/v) to yield compound corynoxine (1) (30 mg). SFr. 4b was further purified by a normal-phase preparative TLC (CHCl_3 –EtOAc, 85:15 v/v) to afford speciogynine (7) (22 mg). Fr. 5 (0.43 g) was separated by silica gel CC and eluted with CHCl_3 –EtOAc/MeOH mixtures (10:0 to 0:10) to afford three subfractions (SFr. 5a, 5b, and 5c). SFr. 5a was recrystallized to yield corynoxine B (2) (23 mg), while SFr. 5b was purified using preparative TLC (CHCl_3 –MeOH, 95:5, v/v), to obtain isospeciofoline (3) (10 mg). SFr. 6 (0.82 g) was separated by silica gel CC (CHCl_3 –EtOAc, 10:1 to 5:5, v/v) to give two subfractions (SFr. 6a and 6b). SFr. 6a was recrystallized using a mixture of CHCl_3 –EtOAc (5:5, v/v) to give a white needle, mitragynine oxindole B (4) (20 mg). Speciociliatine (9) (48 mg) was obtained from SFr. 6b through silica gel CC (EtOAc–MeOH, 10:0 to 0:100, v/v). Speciociliatine N(4)-oxide (25 mg) (10) was obtained from the Fr. 7, as a white solid precipitate, which was cleaned by washing with a mixture of hexane–EtOAc (1:1) and filtered.

Mitragynine oxindole B (4). Transparent crystals (EtOAc); mp 167 – 169°C ; UV (MeOH) λ_{max} (log ϵ) 220 (3.9), 245 (3.2), 290 (1.2) nm; IR (film, NaCl) ν_{max} 3236, 2948, 2791, 1728, 1707, 1615, 1466, 1450, 1251, 1152, 1098, 773 cm^{-1} ; ^1H NMR and ^{13}C NMR data, see Table 1; positive HRESIMS m/z 415.2227 [$\text{M} + \text{H}$] $^+$ (calcd for $\text{C}_{23}\text{H}_{30}\text{N}_2\text{O}_5$, 415.2155).

X-ray Crystallography of Mitragynine Oxindole B (4). Colorless crystals of compound 4 were obtained in ethyl acetate at room temperature. X-ray intensity data were collected at 100 K on a Bruker DUO diffractometer using Cu $K\alpha$ radiation ($\lambda = 1.54178 \text{ \AA}$), from an ImuS power source, and an APEXII CCD area detector. Raw data frames were read by the program SAINT²⁴ and integrated using 3D profiling algorithms. The resulting data were reduced to produce hkl reflections and their intensities and estimated standard deviations. The data were corrected for Lorentz and polarization effects, and numerical absorption corrections were applied based on indexed and measured faces. The structure was solved and refined in SHELXTL-2014, using a full-matrix least-squares refinement.²⁵ The non-H atoms were refined with anisotropic thermal parameters, and all of the H atoms were calculated in idealized positions and refined riding on their parent atoms. The correct stereochemistry of the molecule was refined evidenced by the following parameters: Flack $x = 0.01(9)$, Parsons $z = 0.06(9)$, Hooft $y = 0.06(8)$. Additionally, the $P2$ and $P3$ for refining the true stereochemistry are 1.000, while the $P3$ (rac-twin) is 0.1×10^{-6} and $P3$ (false) is 0.1×10^{-31} . In the final cycle of refinement, 3220 reflections (of which 3137 are observed with $I > 2\sigma(I)$) were used to refine 279 parameters, and the resulting R_1 , wR_2 , and S (goodness of fit) were 4.51%, 12.52%, and 1.031, respectively. The refinement was carried out by minimizing the wR_2 function using F^2 rather than F values. R_1 is calculated to provide a reference to the conventional R value, but its function is not minimized. Crystallographic data for 4 were deposited at the Cambridge Crystallographic Data Centre (deposition number: CCDC 2021386). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: + 44(0)-1233-336033 or e-mail: deposit@ccdc.cam.ac.uk].

Sample Preparation. The leaves collected from the 16 different *M. speciosa* trees (T1–16) (Table S1, Supporting Information) were used for the sample preparation. A total of 25 g of powder of air-dried

leaves from each sample was extracted with 250 mL of hot MeOH (45 °C) for 12 h, followed by maceration at room temperature for another 12 h. The same procedure was repeated three times to ensure all the alkaloids were extracted as indicated by Mayer's reagent. The extracts (3 × 250 mL) from each sample were filtered through Whatman filter paper (No. 4, Fisher Scientific). The filtrate from each sample was evaporated in vacuo and freeze-dried to give a lyophilized powder. The powdered extracts were kept at −80 °C prior to UPLC-MS analysis.

Instrumentation and Analytical Conditions for Quantitative Analysis. A UPLC-MS/MS method reported by Sharma et al.⁴ was implemented for the simultaneous quantification of 14 alkaloids in the collected *M. speciosa* samples. In brief, a Waters Acquity Class I plus UPLC coupled with a Xevo TQ-S Micro triple quadrupole mass spectrometer (Milford, MA, USA) was used for the analysis. Chromatographic separation of the analyzed alkaloids was achieved on a Waters Acquity BEH C₁₈ column (1.7 μm, 2.1 × 100 mm) along with the mobile phase consisting of 10 mM aqueous ammonium acetate buffer of pH 3.5 (A) and acetonitrile (B) using a gradient method over 22.5 min. The column was equilibrated initially with 80% A, which was held for 1 min, then linearly decreased to 72% until 19.5 min, then further linearly decreased to 10% reaching 21.5 min. The conditions were returned back to the initial conditions by sharply increasing A up to 80% by 21.5 min and maintaining them until 22.5 min to re-equilibrate the column.

A multiple reaction monitoring (MRM) mode was utilized for the detection of analytes using a triple-quadrupole mass spectrometer. All the analyzed alkaloids showed an adequate response with electrospray ionization (ESI) in the positive-ion mode. The monitored parent to corresponding daughter ion mass transitions (*m/z*) for 14 alkaloids and internal standards along with instrument parameters are shown in Table S2, Supporting Information. Collected *M. speciosa* samples were analyzed along with freshly prepared calibration and quality control standards. Control of UPLC/MS-MS, acquisition, and analysis of data was performed using MassLynx XS software version 4.2.

Radioligand Receptor Binding Assays. The alkaloids from *M. speciosa* were screened at Eurofins Cerep (Celle l'Evescault, France) for their in vitro receptor-binding affinities at opioid receptor subtypes. Briefly, each cell membrane homogenate was incubated with a radioligand in the absence or presence of one of the isolated alkaloids in a buffer. Nonspecific binding was determined in the presence of a specific agonist or antagonist at the target receptor. Following incubation, each sample was filtered rapidly under vacuum through glass fiber filters presoaked in a buffer and rinsed several times with an ice-cold buffer using a 48- or 96-sample cell harvester. The experimental conditions that were used for the binding assay are summarized in Table 3.

In Vivo Functional Studies. Salt and enantiomeric forms of the compounds used in the present study were as follows: (−)-morphine sulfate pentahydrate (National Institute on Drug Abuse, Drug Supply Program, Rockville, MD, USA), (−)-naltrexone hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA), and corynoxine (1) hydrochloride (see General Experimental Procedures). Doses of the compounds used in the present study are expressed as the weight of the salt or free base form listed above. All the compounds were dissolved in a vehicle consisting of sterile water (HyClone water for injection, 1 L, Fisher Scientific, Waltham, MA, USA) containing 5% Tween 80 (polyoxyethylenesorbitanmonooleate, Fisher Scientific) and 5% propylene glycol (Sigma-Aldrich Co.). All the compounds and the vehicle were administered i.v. Each solution was filtered with a 0.2 μm pore size syringe filter (Millex-LG, 0.20 μm, SLLG025SS, Sigma-Aldrich Co.) prior to injections. The injection volumes were 1.0 mL/kg of subject body weight. The dose ranges and pretreatment times were referenced to our preliminary data and the literature.²⁶

Animals. Adult, naive female and male (*n* = 6 per sex) Sprague–Dawley rats (Envigo, Indianapolis, IN, USA; age 10 weeks) were preimplanted with a catheter into a right external jugular vein upon receipt and singly acclimated in ventilated cages for at least 3 days to a temperature- (21.9 ± 1.9 °C) and humidity-controlled (53 ± 14%) vivarium with a 12 h light/dark cycle (lights on at 07:00 h) during

which food (2918 Teklad Global 18% protein rodent diets, Envigo) was available in their housing cages at all times and reverse osmosis water was available in the vivarium at all times. The animal protocol (#201710059) was approved by the Institutional Animal Care and Use Committee at the University of Florida and was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All experiments were conducted in the light cycle (08:00 to 17:00 h) using drug naïve subjects.

Hot-Plate Testing. The hard black anodized aluminum plate with built-in digital thermometer used in the present study (hot plate analgesia meter, 1440 analgesia hot plate with RS-232 port and software, Columbus Instruments, Columbus, OH, USA) was 33.1 cm long, 28.8 cm wide, and 9.9 cm high. A clear acrylic cage (26.7 cm long, 26.7 cm wide, 31.0 cm high) surrounded the square plate to confine an animal on the plate (total height: 40.9 cm). The size of the heated surface was 25.4 cm long, 25.4 cm wide, and 1.9 cm high, while the surface temperature range was 30 to 79.9 °C across the plate. The temperature accuracy was ±0.1 °C. The temperature, humidity, and light/dark cycle in an experimental room were equivalent to those in the vivarium. Prior to commencement of each experiment, the body weight of each animal was measured. The sample size of each experiment group per treatment was six (three subjects per sex). Following a baseline measurement for hot plate latency in each subject (see below for more details), vehicle and then cumulative injections of ascending doses of a test compound were administered i.v. at an interval of 5 min to assess the effects of compounds alone. Following are the baseline values for hot plate latency [mean (standard error of mean; SEM) seconds]: morphine [21.0 (3.11)] and corynoxine (1) [16.5 (3.31)]. For the baseline hot plate latency, two-way analysis of variance (ANOVA) indicated no significant difference in sex ($F_{1,8} = 1.94$, $p = 0.201$), group ($F_{1,8} = 1.16$, $p = 0.312$), or their interaction ($F_{1,8} = 1.86$, $p = 0.210$). Because there was no significant difference in baseline values and sex, data from the hot plate assay were combined per treatment (see data analysis below).

Following the baseline measurement for hot plate latency, vehicle, cumulative injections of morphine or corynoxine (1), and naltrexone (0.1 mg/kg) were administered i.v. every 5 min. The measurement of each baseline was repeated five times at a 5 min interval. Immediately following assessment of the effects of vehicle, the cycle of injections of each test compound was repeated until the hot plate latency was maximized at 60 s in all subjects to avoid tissue damage, or any deemed toxicity (i.e., seizure) or death was observed. Finally, naltrexone was injected to see reversibility of the antinociceptive effects of both test compounds.

Data Analysis. The hot plate latencies were normalized to the percentage of the maximal possible effect (MPE), using the following formula: % MPE = 100(postinjection latency − preinjection baseline latency)/(maximum latency 60 s − preinjection baseline latency). All data per treatment group in Figure 5 were presented as means (±SEM). Using the SigmaPlot software (ver. 14.0; Systat Software Inc., San Jose, CA, USA), the significance of effects on each variable above was assessed by ANOVA, with post hoc Bonferroni *t* tests. A two-way (repeated-measures) ANOVA was used as appropriate (factors were compound dose and sex). Outcomes were considered significant at $p < 0.05$. The ED₅₀ values (95% confidence intervals) for hot plate latency were calculated by linear regression²⁷ of the dose–effect functions using the GraphPad Prism software (ver. 8; GraphPad Software, San Diego, CA, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01055>.

Map and table describing the sampling locations of *M. speciosa* specimens in Peninsular Malaysia; description of the putative biosynthesis of main alkaloids in *M. speciosa*;

^1H and ^{13}C NMR for compounds **1–3** and **10** and ^1H , ^{13}C , and 2D NMR spectra of compound **4**; ESI-TOF-MS/MS spectra of compounds **4** and **10**; MRM chromatograms of test compounds **1–14** and internal standard; tables summarizing the compound parameter for tested alkaloids **1–14**, internal standard for UPLC/MS/MS analysis (PDF)

Crystallographic data of compound **4** (CIF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Dr. Hampson was substantially involved with this work, consistent with his role as Scientific Officer of UG3 DA048353. He had no substantial involvement in the other cited grants.

Notes

The authors declare no competing financial interest.

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