Antitussive Indole Alkaloids from *Kopsia hainanensis*

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**Key words**

- *Kopsia hainanensis*
- Apocynaceae
- aspidofractinine type
- chanofruticosinate type
- antitussive activity

**Abstract**

Three new indole alkaloids, named kopsihainains A–C (1–3), and two known compounds, kopsinine (4) and methyl demethoxycarbonylchanofruticosinate (5), were isolated from the stems of *Kopsia hainanensis*. Their structures were determined using extensive spectroscopic methods. The two main constituents 4 and 5 exhibited significant antitussive activity in a citric acid induced guinea pig cough model. The antitussive effect of 4 was demonstrated to interact with the δ-opioid receptor. This is the first report of antitussive effects of aspidofractinine type and chanofruticosinate type alkaloids.

**Supporting information** available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

**Introduction**

The opioid receptor is the primary centrally acting target for the treatment of cough. Current available opioid-derived antitussives mainly exert their actions on the μ-opioid receptor [1], one of the four well-established opioid receptor subtypes (μ-, δ-, κ- and ORL₁-receptors) [2]. Codeine is probably the most commonly prescribed and reliable antitussive drugs. However, due to its μ-opioid receptor antagonist effect, codeine has undesirable side effects similar to other opiates, such as dependence, respiratory depression, constipation, and euphoria. Recently, targeting δ-opioid receptors, which are free from the μ-opioid receptor antagonist side effects, has been proposed as a new concept for antitussive treatment [3]. Paradoxically, both δ-opioid receptor agonist SB 227122 [4] and antagonist naltrindole [5] are reported to show marked antitussive effect (SB 227122 in guinea pigs and naltrindole in mice and rats). Such conflicting results may be explained by the different pharmacological roles of the two δ₁ (inhibitory effects) and δ₂ (synergistic effects) opioid receptor subtypes [1,3] and the pharmacology of the δ/μ receptor heteroligomerization [6,7]. In two recent articles, naltrindole analogs TRK-850 and -851 were reported to show promising potential for the development of a novel class of antitussive agents [8,9].

Since the indole moiety in indolomorphinans like naltrindole is fundamental for the δ receptor selectivity [10–12], we argue that some natural occurring indole alkaloids might also have similar pharmacological effects due to the great chemical diversity of indole alkaloids. In our continuous effort to study the antitussive alkaloids in medicinal plants [13–17], we particularly focused on the investigation of the indole alkaloids from *Kopsia hainanensis* (Apocynaceae). The *Kopsia* genus, which is widely distributed in the Asian tropical areas, is well known for its diversified indole alkaloids with different intriguing carbon skeletons [18–28]. However, biological activities of these alkaloids reported so far are mainly interested in their cytotoxic and anti-multidrug resistant effects against some tumor cell lines. Herein, we report the isolation and structure elucidation of three new compounds, kopsihainains A–C (1–3), as well as the antitussive effects of two major alkaloids from this plant, kopsinine (4) and methyl demethoxycarbonylchanofruticosinate (5) (Fig. 1).

Our result revealed that compounds 4 and 5 exhibited significant antitussive activity in citric acid induced guinea pig cough mode. Such activity was associated with the δ-opioid receptor.
Materials and Methods

General experimental procedures

UV spectra were detected using a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were recorded using a Nicolet Magna 750 FTIR (KBr) spectrophotometer. Optical rotations were measured using a Perkin-Elmer M341 polarimeter. MS data were obtained using a MAT-95 mass spectrometer. NMR spectra were recorded using a Bruker AM-400 instrument with TMS as an internal standard; the chemical shift values are reported in ppm (δ) and coupling constants (J) in Hz. Silica gel 100–200, 200–300 mesh and silica gel GF254 for precoated plates (produced by Qingdao Haiyang Chemical Group Co.) were used for column chromatography and for preparative TLC, respectively. Codeine phosphate (purity > 99%) was purchased from Macfarlan Smith Limited. Naloxone hydrochloride (purity > 99%) was purchased from Tocris Bioscience, Inc. All experiments were conducted under the approval of Animal Experimentation Ethics Committees of The Chinese University of Hong Kong (06/046/MIS).

Plant material

The stems of *K. hainanensis* were collected in Hainan province, P.R. China, and identified by Prof. Jin-Gui Shen of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. A voucher specimen (20020014B) was deposited in the herbarium of the Institute.

Extraction and isolation

Air-dried stems of *K. hainanensis* (10 kg) were ground and percolated with 95% EtOH. After evaporation of the solvent, the residue was acidified with dilute HCl (5%) to pH 1–2 and filtered. The filtrate was basified successively with aq. NH3 to pH 7 and then to pH 9–10, and subsequently extracted with CH2Cl2 (1 L) to yield crude alkaloids RM1 (50 g) and RM2 (49 g), respectively. The aq. residue was then partitioned with n-BuOH (1 L) to give an n-BuOH extract (90 g). The crude alkaloid RM2 (45 g) was then subjected to column chromatography (CC) over silica gel eluted with petroleum ether (PE)-acetone gradients (9:1, 3:1, 2:1, 1:2). Compound 4 (5.2 g) was crystallized from Fr. 2 (10.5 g) in a mixture of PE/petroleum ether (10 g) to give yield crude alkaloids RM1 (50 g) and RM2 (49 g), respectively. The aq. residue was then partitioned with n-BuOH (1 L) to give an n-BuOH extract (90 g). The crude alkaloid RM2 (45 g) was then subjected to column chromatography (CC) over silica gel eluted with petroleum ether (PE)-acetone gradients (9:1–2:1) to give seven major fractions (Fr. 1–Fr. 7). Compound 4 (5.2 g) was crystallized from Fr. 2 (10.5 g) in a mixture of PE/petroleum ether (8:1). Compound 5 (4.1 g) was obtained by crystallization from Fr. 3 (9.5 g) in a mixture of PE/acetone (7:1). Fr. 4 (3.0 g) was subjected to CC over silica gel eluted with cyclohexane-acetone (9:1–2:1) to give four subfractions (Fr. 4.1–Fr. 4.5). Compound 1 (14 mg) from Fr. 4.4 (190 mg) was purified by repeated silica gel CC (PE-EtOAc 9:1). The n-BuOH extract was subjected to macro-porous resin CC eluting in a step manner with H2O, 30%, 60%, and 95% EtOH to yield four fractions (Bu. 1–Bu. 4). Bu. 2 (32 g) was subjected to normal phase silica gel CC with gradient elution (the low layer of CHCl3-MeOH-H2O 20:3:1, 10:3:1, 65:35:10) to give 6 fractions (Fr. 8.1–Fr. 8.4). Compounds 2 (81 mg) from Fr. 8.3 and 3 (14 mg) from Fr. 8.4 were purified by CC over Sephadex LH-20 (CHCl3-MeOH 1:1), respectively.

Isolates

*Kopsihainin A* (1): white amorphous powder; UV (CH3CN): λ<sub>max</sub> = 243, 281 nm; [α]<sub>D</sub><sup>25</sup> = +47 (c 0.120, MeOH); IR (KBr): ν<sub>max</sub> (cm<sup>-1</sup>) = 3433, 3352, 2951, 1738, 1709, 1483, 1435, 1352, 1323, 1259, 1232, 1213, 1013, 754 cm<sup>-1</sup>; HR-EIMS: m/z = 398.1841 (calcld. for C<sub>22</sub>H<sub>26</sub>N<sub>2</sub>O<sub>5</sub> [M]+, 398.1842); 12C- and 1H-NMR: see Tables 1 and 2.

*Kopsihainin B* (2): yellow amorphous powder; UV (CH3CN): λ<sub>max</sub> = 232, 281 nm; [α]<sub>D</sub><sup>25</sup> = −64 (c 0.160, MeOH); IR (KBr): ν<sub>max</sub> (cm<sup>-1</sup>) = 3427, 3294, 2955, 2819, 1720, 1606, 1479, 1458, 1389,
Table 2  ^1^H-NMR data of compounds 1–3 in CDCl\textsubscript{3} (δ in ppm, J in Hz).

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<td>3.42 m</td>
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<td>5</td>
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<td>6</td>
<td>2.12 d (15.7); 3.24 d (15.8)</td>
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<td>3.06 d (4.1)</td>
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<td>3.161 (9.6)</td>
<td>3.10</td>
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<td>17</td>
<td>2.02 d (16.2); 3.04 d (16.0)</td>
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<td>1.48 m, 1.88 m</td>
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<td>1.27 m, 1.64 m</td>
<td>1.88 m, 2.02 m</td>
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<td>21</td>
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CO\textsubscript{2}Me 3.62 s

NCO\textsubscript{2}Me 3.88 s

1211, 758 cm\textsuperscript{-1}; HR-ESIMS: m/z = 323.1759 (calcd. for C\textsubscript{20}H\textsubscript{22}N\textsubscript{2}O\textsubscript{3} [M + H]+ 338.1616 (calcd. for C\textsubscript{20}H\textsubscript{22}N\textsubscript{2}O\textsubscript{3} by HRESIMS). The \(^{13}\)C and \(^{1}H-NMR: see Tables 1 and 2. Kopsihainin C (3): yellow amorphous powder; UV (CH\textsubscript{3}CN): \(\lambda_{\text{max}} = 233, 281 \text{ nm} \); [\(\alpha\)]\textsubscript{D}\textsuperscript{25} = −97 (c 0.185, MeOH); IR (KBr): \(v_{\text{max}}\) (cm\textsuperscript{-1}) = 3344, 2960, 1718, 1657, 1632, 1591, 1481, 1460, 1392, 1313, 1028, 762 cm\textsuperscript{-1}; HR-ESIMS: m/z = 338.1616 (calcd. for C\textsubscript{20}H\textsubscript{22}N\textsubscript{2}O\textsubscript{3} [M]+ 338.1602); \(^{13}\)C- and \(^{1}H-NMR: see Tables 1 and 2.

Antitussive activity of compounds 4 and 5

With the availability of adequate amounts of isolated compounds, the main alkaloids 4 and 5 were tested for the antitussive activity and the mechanism of cough suppression was further investigated with the most abundant compound 4 only. A well-developed citric acid-induced guinea pig cough model [13, 14, 16, 17, 29, 30] was adopted for testing antitussive activity. Briefly, unrestrained conscious Dunkin-Hartley guinea pigs were randomly divided into different groups. A single dose was given intraperitoneally (4: 20 [0.06], 45 [0.13], 70 [0.21] mg/kg [mmol/kg]; 5: 70 [0.20], 150 [0.43], 250 [0.71] mg/kg [mmol/kg]), or orally (70 mg/kg for both 4 and 5). For the investigation of the mechanism of antitussive action, a nonselective opioid receptor antagonist naloxone (5 mg/kg) [31] or a selective \(\delta\)-opioid receptor antagonist naltrexone (6 mg/kg) [32] was given subcutaneously at 10 min prior to the intraperitoneal administration of 4 (45 mg/kg), respectively. Codeine (30 mg/kg [0.1 mmol/kg] i.p.) as the positive antitussive control and the vehicle control were also conducted in parallel. Treated animals were individually placed into a transparent Perspex air-tight chamber. At 30 min after the alkaloid treatment, each animal was exposed to 0.5 M citric acid aerosols for 8 min with a flow rate of 0.5 mL/min. During the aerosol exposure, the animal was continuously monitored, and cough sounds were recorded and analyzed by Cool Edit 2000 software (Syntrillium). Cough episodes were determined using our previously developed software CoughCount-CHHK (2003 Copyright) [13, 14, 16, 17]. Antitussive activity was evaluated by the comparison of numbers of cough episodes between the treated and the corresponding vehicle control group. Statistical analyses were conducted using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests. Statistical analyses of differences between i.p. and oral effect (weighted mean differences) were calculated using z-tests. P values less than 0.05 were considered significant. Half maximal inhibitory dose (ID\textsubscript{50}) values were calculated by linear regression analysis.

Supporting information

NMR data of compounds 1–5 are available as Supporting Information.

Results and Discussion

Kopsihainin A (1) was isolated as a white amorphous powder. The HR-ESIMS gave a possible molecular formula of C\textsubscript{22}H\textsubscript{26}N\textsubscript{2}O\textsubscript{5} (m/z = 398.1841 [M]+), indicating 11 degrees of unsaturation. The IR absorption showed absorption bands corresponding to amino (3433 cm\textsuperscript{-1}), carbonyl (1738, 1709 cm\textsuperscript{-1}), and phenyl (1597 cm\textsuperscript{-1}) groups. The \(^{13}\)C-NMR spectrum (Fig. 3A) displayed a total of 22 signals, including 3 characteristic quaternary carbons (\(\delta\)c 78.0, 53.4, 34.5; C-2, C-7, C-20) and one characteristic methine (\(\delta\)c 62.0; C-21). Consistent with the \(^{13}\)C-NMR data, its \(^{1}H-NMR\) spectrum (Fig. 3B) exhibited a characteristic singlet of H-21 at \(\delta\)H 2.48 and 4 aromatic protons of an unsubstituted indole ring. A detailed analysis of its NMR data (Tables 1 and 2) revealed that 1 possessed a daniuylphill-type skeleton similar to that of the known 11,12-de(methylenedioxy)danuphyllin [25], except for the absence of a formyl at N-4. Such elucidation was further confirmed by the HMBC experiment. Therefore, compound 1 was determined as N(4)-deformyl-11,12-de(methylenedioxy)danuphyllin.

Kopsihainin B (2), a yellow amorphous powder, was found to have a molecular formula of C\textsubscript{20}H\textsubscript{22}N\textsubscript{2}O\textsubscript{3} from HREIMS with 11 degrees of unsaturation. The \(^{1}H\)- and \(^{13}\)C-NMR data of 2 (Tables 1 and 2) suggested it to be an aspidofractineline type alkaloid with a similar structure to venalostine [18], except for the absence of a carboxylic methyl. H-16 was deduced to be \(\beta\)-oriented from the observed NOE interactions between H-16 and H-18 in the NOESY spectrum. Such orientation was further confirmed by the absence of W coupling between H-16/H-18 [20]. Thus, the structure of 2 was established as shown.

Kopsihainin C (3) was obtained as a yellow amorphous powder (C\textsubscript{20}H\textsubscript{22}N\textsubscript{2}O\textsubscript{3} by HREIMS). The \(^{13}\)C- and \(^{1}H-NMR\) data of 3 were in good agreement with those of 2, except for the presence of an epoxy group rather than an olefinic bond at C-14 and C-15. The structure of 3 was further confirmed by the HMBC correlations of C-15/H-3, C-15/H-17, C-15/H-14, and C-21/H-15. The orientation of the 14,15-epoxy group was determined to be \(\alpha\) from the observed NOE correlation between H-17 and H-15. Thus, 3 was deduced to be a 14,15-\(\alpha\)-epoxy derivative of 2.

Compounds 4 and 5 were identified as kopsinine and methyl demethoxycarbonylchanofruticosinate, respectively, by comparing their NMR and ESIMS data with those in the literature [19, 21]. Both alkaloids via i.p. administration suppressed citric acid-induced coughing in the guinea pig dose-dependently. At the highest dose tested, 4 and 5 produced 88% and 76% cough inhibition (Fig. 2A), and 4 was more potent (ID\textsubscript{50}: 0.08 mmol/kg, 95% confidence intervals [CI]: 0.03 to 0.19) than 5 (ID\textsubscript{50}: 0.45 mmol/kg, CI: 0.06 to 0.98). Compound 4 was also orally active but with a markedly lower potency comparing with the i.p. route at the same dose of 70 mg/kg (49 vs. 67% inhibition). No significant cough suppression was observed after oral administration of 5 at 70 mg/kg (Fig. 2B). With 10 min pretreatment of the nonselective opioid receptor antagonist naloxone, the inhibition of cough by both codeine and 4 was abolished (Fig. 3A), suggest-
ing that the mechanism of antitussive action of 4 was similar to codeine via interaction of the opioid receptor. Furthermore, the selective δ-opioid receptor antagonist naltrindole also significantly reversed the antitussive effect of 4 (Fig. 3B), indicating that the activation was specific toward the δ-opioid receptor. Portoghese proposed a “message-address concept” during his development of the indolomorphinan δ-selective antagonists [10–12]. The δ-opioid receptor was envisaged to contain two major recognition subsites: a message subsite (the morphinan skeleton) which recognizes the pharmacophore, and an address subsite (the indole moiety) that is unique for δ receptor selectivity (Fig. 4A). Nevertheless, the δ-opioid receptor agonist effect of compound 4 in this study appeared to be contrary to the antagonist effects of indolomorphinans. This could be explained that the indole moiety of compounds 4 and 5 was most likely to be served as a “message” component for pharmacophore recognition rather than as an “address” component for δ receptor selection. As illustrated in Fig. 4B and 4C, compounds 4 and 5, similarly to codeine, possess all the essential elements for opioid receptor agonists [2,33]. However, on account of the great complexity of opioid receptor pharmacology, further investigation is needed to elucidate the real role of these classes of compounds in modulating δ-opioid receptor activity.

Our study revealed that aspidofractinine type alkaloids (compound 4) and chanofruticosinate type (compound 5) alkaloids are two new classes of antitussive compounds that are involved with the δ-opioid receptor agonist effect. Furthermore, our finding may provide evidence for the development of a novel lead for the treatment of other ailments that are related with the δ-opioid receptor such as depression [34,35].

### Acknowledgements

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### Fig. 2

Antitussive activity of 4 and 5 in guinea pigs treated with a single i.p. (A) and oral (B) dose. Data are expressed as mean ± SEM (n = 4). * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the vehicle control.

### Fig. 3

Effects of naloxone (Nal) (A) and naltrindole (Nalt) (B) on the antitussive activity of codeine (0.1 mmol/kg, i.p.) and 4 (0.13 mmol/kg, i.p.). Data are expressed as mean ± SEM (n = 4). * P < 0.05, ** P < 0.01 compared with the vehicle control. ## P < 0.01 compared with the corresponding treatment without antagonist.
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Fig. 4 A Functional components of nitridinol for δ-opioid receptor selectivity based on the “message-address concept”. B Essential elements for opioid receptor agonists. C Essential functional groups of codeine, 4, and 5 for opioid receptor agonists.
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