Antimicrobially Active Isoquinoline Alkaloids from *Litsea cubeba*

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Key words
- *Litsea cubeba*
- Lauraceae
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- antimicrobial activity
- (+)-*N*-(methoxycarbonyl)-*N*-norboldine
- (+)-isoboldine *β*-N-oxide

Abstract

Bioassay-guided fractionation of the alkaloidal extract of the aerial part of *Litsea cubeba* led to the isolation of two new isoquinoline alkaloids, (+)-*N*-(methoxycarbonyl)-*N*-norboldine (**1**) and (+)-isoboldine *β*-*N*-oxide (**2**), together with 11 known analogues (**3 – 13**). Their structures were established by extensive spectroscopic techniques and by comparing spectroscopic data with those in the literature. Compounds **1** and **4** showed antimicrobial activities. This is the first report on the presence of compounds **1**, **2**, **6**, **8**, **9**, **11**, and **12** in this plant and on the antimicrobial activities of **1** and **4**. The bioactivities of isoquinoline alkaloids are also at least partly responsible for the pharmacological function of the folk medicinal plant *Litsea cubeba*.

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

Introduction

Litsea cubeba (Lour.) Pers. is a 3- to 10-m tree or shrub of the Lauraceae family that is native to China, Indonesia, and other parts of Southeast Asia [1]. It has long been used as a folk remedy in *dai* ethnopharmacy for the treatment of the common cold and stomachache in southwestern China, and its antimicrobial activities against *Staphylococcus aureus*, *Salmonella typhi*, and *Pseudomonas aeruginosa* have been reported [2]. Both essential oils and isoquinoline alkaloids are abundant in *L. cubeba*. However, except for essential oils [3], [4], [5], [6], there have been no literature reports on the antimicrobial activity of the other constituents. This is the first report on the antibacterial activity of isoquinoline alkaloids from *L. cubeba*. According to our continuing research on *dai* ethno-medical materials [7], [8], we have found that not the non-alkaloidal but the alkaloidal extract of *L. cubeba* exerts antimicrobial activity against *Staphylococcus aureus*, which indicates that not only the essential oils but also the alkaloidal part of *L. cubeba* play an important role in its antimicrobial activity. This observation promoted us to perform a detailed chemical investigation on the alkaloidal part of this plant, which led to the isolation of two new isoquinoline alkaloids, (+)-*N*-(methoxycarbonyl)-*N*-norboldine (**1**) and (+)-isoboldine *β*-*N*-oxide (**2**), and 11 known analogues (**3 – 12**) (▶ **Fig. 1**). All compounds were tested for antibacterial activity against *Staphylococcus aureus* and *Mycobacterium tuberculosis* and for antifungal activities against *Alternaria alternata*, *Colletotrichum nicotianae*, *Candida albicans*, *Saccharomyces cerevisiae*, and *Saccharomyces cerevisiae*. Compounds **1** and **4** were found to be active. In this paper, we report the isolation, structure elucidation, and antimicrobial activity of the isoquinoline alkaloids from *L. cubeba*.

Materials and Methods

General experimental procedures

Melting points were obtained on an X-4 micromelting point apparatus. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy using KBr pellets. 1 D and 2 D spectra were run on Bruker DRX-500 and AM-400 spectrometers with TMS as internal standard. Chemical shifts (δ) were expressed in ppm with reference to the

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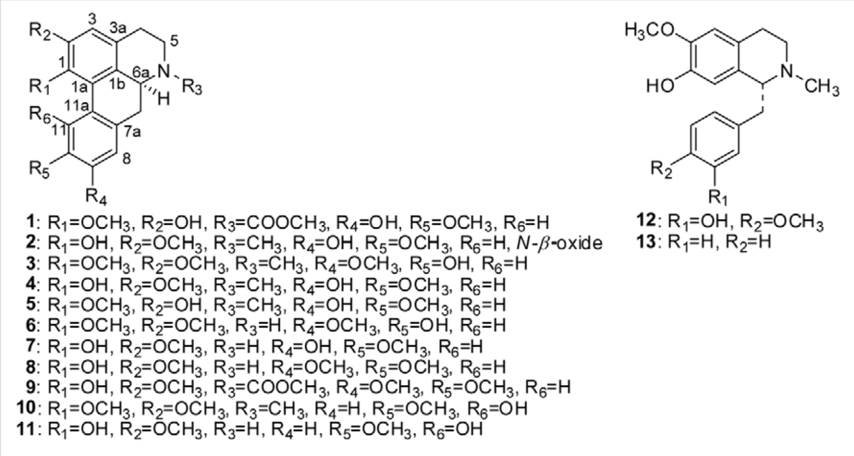

Fig. 1 Chemical structures of compounds **1 – 13**.

solvent signals. EI-MS was tested on a VG Autospec-3000 spectrometer. HR-ESI-MS was performed on an API QSTAR Pulsar 1 spectrometer. Column chromatography was performed on silica gel (200 – 300 mesh; Qingdao Haiyang Chemical Co., Ltd.), RP-18 gel (20 – 45 μm; Fuji Silysia Chemical, Ltd.), and Sephadex LH-20 (Pharmacia Fine Chemical Co.). Fractions were monitored by TLC (GF 254; Qingdao Haiyang Chemical Co., Ltd.), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH and Dragendorff's reagent. Strains (purity > 95 %) were provided by Yunnan Agricultural Academy, Yunnan Province, P.R. China. Rifampicin (purity > 92 %) and nystatin (purity > 95 %) were bought from Beijing Keyuan Huada Biotechnology Developing Center.

Plant material

The aerial parts of *L. cubeba* were collected in the Yunnan Province, P.R. China, in April 2007 and were identified by Mr. Jing-Yun Cui, Xishuangbanna Tropic Botanical Garden, Chinese Academy of Sciences. A voucher specimen (No. 20 070 428) has been deposited in the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

An ethanol extract of the aerial parts of *L. cubeba* (10 kg) was concentrated to dryness, dissolved in 5‰ HCl (2 L), and filtered. The filtration was basified using 1 % ammonia water to pH 9 – 10, and then the basic solution was partitioned with EtOAc to give a total alkaloidal fraction (47 g). The latter was chromatographed on a silica gel column (1 kg, 140 × 7 cm, chloroform:methanol, 1 : 0 → 1 : 1) to give three fractions (1 – 3). After repeated column chromatography (silica gel, 400 g, 120 × 4 cm, petroleum ether: acetone, 20 : 1 → 1 : 1), fraction 1 (17.8 g) afforded **3** (129 mg), **4** (977 mg), **5** (2.1 g), **9** (361 mg), and **10** (22 mg). Fraction 2 (7.4 g) was further applied to silica gel (250 g, 90 × 4 cm) eluted with chloroform:acetone (15 : 1 → 1 : 1) to give fractions 2a-2c. Fraction 2a (3.6 g) was further purified by RP-18 (100 g, 50 × 2.5 cm, methanol:water, 5 : 5 → 7 : 3) to afford **1** (74 mg), **6** (29 mg), and **8** (20 mg). Fraction 2b (1.1 g) was applied to Sephadex LH-20 (100 g, 140 × 2.0 cm) eluted with chloroform:methanol (1 : 1) to afford **11** (11 mg) and **12** (13 mg). Fraction 3 (3.1 g) was repeatedly chromatographed on silica gel (150 g, 60 × 2.8 cm, chloroform:methanol, 20 : 1 → 10 : 1) to provide fractions 3a, 3b, and 3c. Fraction 3a (1.2 g) was purified by RP-18 (30 g, 25 × 2.2 cm, methanol:water, 4 : 6) to afford **7** (103 mg). Fraction 3b (833 mg) was purified by Sephadex LH-20 column (70 g, 100 × 1.8 cm, methanol) to afford **13** (65 mg). Compound **2** (8 mg) was obtained as an amorphous powder from fraction 3c (309 mg).

(+)-N-(Methoxycarbonyl)-N-norboldine (**1**): Colorless crystals (CH_3OH); m. p. 140 – 142 °C, $[\alpha]_D^{20}$: + 269.2 (*c* 0.19, CH_3OH); UV (CH_3OH): λ_{max} (log ε) = 303 (3.88), 283 (3.89), 216 (4.36), 194 (4.19) nm; IR (KBr): ν_{max} = 3429, 1668, 1598, 1520, 1463, 1208, 1020 cm^{-1}; EI-MS: *m/z* = 371, 356, 310, 296, 284, 283, 269; positive HR-ESI-MS: *m/z* = 372.1451 $[M + H]^+$, calcd.: 372.1447; ^1H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) data, see ▶ **Table 1**.

(+)-Isoboldine β-N-oxide (**2**): Brown powder (CH_3OH); m. p. 147 °C, $[\alpha]_D^{20}$: + 86.1 (*c* 0.18, CH_3OH); UV (CH_3OH): λ_{max} (log ε) = 304 (3.71), 284 (3.70), 217 (4.13), 201 (4.14) nm; IR (KBr): ν_{max} = 3440, 1567, 1210, 1073, 1026, 860 cm^{-1}; positive HR-ESI-MS: *m/z* = 344.1504 $[M + H]^+$, calcd.: 344.1497; ^1H-NMR (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) data, see ▶ **Table 1**.

The specific rotation of the known compounds was also tested (see Supporting Information).

Antimicrobial activity bioassay

All compounds (purity > 90 %) were screened for their antimicrobial activity *in vitro* using the disk-diffusion method as described in the literature with minor modifications [9]. Strains including two species of bacteria (*Staphylococcus aureus, Mycobacterium tuberculosis*), six species of fungi (*Gibberella pulicaris*, *Streptomyces scabies*, *Alternaria alternata*, *Colletotrichum nicotianae*, *Phytophthora capsici*, *Gonatopyricularia amomi*), and two species of yeast (*Candida albicans, Saccharomyces cerevisiae*) were used. Rifampicin and nystatin were used as positive controls for antibacterial and antifungal activities, respectively. A disk containing only DMSO was used as the negative control. The sterile filter paper disks (6-mm diameter) were soaked with 1 – 10 % (v/v) solution of the test compounds in DMSO and placed onto nutrient agar medium plates for testing of antibacterial activity or onto sabouraud dextrose broth (SDB) agar plates for testing of antifungal activity. The plates were inoculated with a standardized suspension (0.5 McFarland scale unit, 0.1 mL) of the tested strains, which were incubated at 37 °C for testing of antibacterial activity and at 24 – 28 °C for testing of antifungal activity. The diameter of the inhibition zone was measured after 18 h and 24 h for testing of antibacterial and antifungal activities, respectively. Experiments were performed in triplicate, and the results are presented as the mean values of the diameters of the inhibitory zones from three runs.

Table 1 ^{1}H- and ^{13}C-NMR data of **1** and **2** in CD_3OD.

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1		144.7 s		143.3 s
1a		128.3 s		121.7 s
1b		131.5 s		122.4 s
2		150.3 s		149.1 s
3	6.55 (s)	115.2 d	6.70 (s)	109.8 d
3a		125.1 s		122.1 s
4	2.71 (m) 2.51 (d, 15.2)	30.6 t	2.74 (m) 3.55 (m)	25.3 t
5	2.88 (m) 4.27 (ddd, 11.2, 4.0, 2.4)	40.1 t	3.57 (m) 3.72 (m)	65.5 t
6a	4.54 (dd, 13.4, 4.2)	53.1 d	4.41(dd, 13.6, 3.6)	72.5 d
7	2.63 (m) 2.74 (m)	35.2 t	3.05 (dd, 13.6, 4.3) 3.17 (t, 13.6)	30.1 t
7a		124.5 s		124.9 s
8	6.68 (s)	116.0 d	6.77 (s)	115.9 d
9		147.2 s		146.7 s
10		147.5 s		147.4 s
11	7.99 (s)	113.4 d	8.11 (s)	114.0 d
11a		131.3 s		128.3 s
1-OCH_3	3.55 (s)	60.0 q		
2-OCH_3			3.89 (s)	56.4 q
10-OCH_3	3.84 (s)	56.5 q	3.84 (s)	56.5 q
N-CH_3			3.36 (s)	58.1 q
$\underline{C}OOCH_3$		157.5 s		
$COO\underline{CH_3}$	3.70 (s)	53.3 q		

[a] No activity.

[b] Not tested.

Supporting information

NMR and MS data for compounds **1** and **2**, specific rotation data for compounds **3 – 13**, and pictures of the antimicrobial assay are available as Supporting Information.

Results and Discussion

▼

A total alkaloidal extract from the aerial parts of *L. cubeba* showed antimicrobial activity against *Staphylococcus aureus*. Further purification of this extract led to the isolation of the new compounds **1** and **2**, together with the 11 known analogues lirioferine (**3**) [10], isoboldine (**4**) [11], boldine (**5**) [12], norlirioferine (**6**) [13], laurelliptine (**7**) [14], wilsonirine (**8**) [10], *N*-(methoxycarbonyl)-*N*-norisoboldine (**9**) [15], isocorydine (**10**) [12], muricinine (**11**) [16], reticuline (**12**) [10], and *N*-methylcoclaurine (**13**) [17]. The structural elucidations of all compounds were based on spectroscopic evidence and comparison with data reported in the literature.

Compound **1** was obtained as colorless crystals (CH_3OH). The EI-MS afforded a molecular weight at $m/z = 371$, and its HR-ESI-MS revealed the $[M+H]^+$ peak at $m/z = 372.1451$ (calcd. for $C_{20}H_{22}NO_6$: 372.1447), corresponding to the molecular formula $C_{20}H_{21}NO_6$. Its UV absorption at $\lambda_{max} = 303$, 283, and 216 nm showed an aporphine alkaloid skeleton with substituents at C-1, C-2, C-9, and C-10 [18], [19]. The ^{1}H-NMR spectrum of **1** showed three aromatic singlets at $\delta = 7.99$ (H-11), 6.68 (H-8), and 6.55 (H-3) and three methoxys at $\delta_H = 3.84$, 3.70, and 3.55. Its ^{13}C-NMR spectrum indicated 20 carbons, three methoxy groups ($\delta_C = 60.0$, 56.5, 53.3), three sp^2 methine carbons ($\delta_C = 115.2$, 116.0, 113.4), one sp^3 methine ($\delta_C = 53.1$), three sp^3 methylenes ($\delta_C = 30.6$, 40.1, 53.1), and 10 sp^2 quaternary carbons ($\delta_C = 144.7, 128.3, 131.5, 150.3, 125.1, 124.5, 147.2, 147.5, 131.3, 157.5$). An IR band at 1668 cm^{-1} and a signal at $\delta_C = 157.5$ in the ^{13}C-NMR spectrum evidenced the presence of a carbamate moiety [20]; the mass fragment at $m/z = 284$ $[M-(CH_2\text{-}N\text{-}COOCH_3)]^+$ in the EI-MS further supported the *N*-carbamate group [21]; and the HMBC correlations (▶ **Fig. 2**) of $\delta_C = 157.5$ with $\delta_H = 3.70$ (3H, s, OMe), 4.54 (1H, dd, $J = 13.4$, 4.2 Hz, H-6a), 4.27 (1H, ddd, $J = 11.2$, 4.0 and 2.4 Hz, H-5a), and 2.88 (1H, m, H-5b) confirmed the above argument. Meanwhile, in the ^{13}C-NMR spectra of **1**, signals of C-7 and –CO-appeared as broad peaks due to the stereo-hindrance effect of methyl ester with C-7 on the NMR time scale at room temperature, and signals sharpened when measured under heating conditions (50 °C). The positions of two other methoxys were assigned based on the ROESY spectrum (▶ **Fig. 2**).

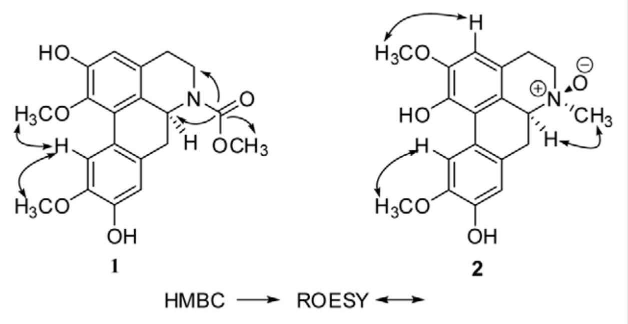

Fig. 2 Key HMBC and ROESY correlations of **1** and key ROESY correlations of **2**.

Table 2 Diameters of the inhibitory zones of compounds **1** and **4** (50 μg/disk).

Strain	1	Rifampicin	4	Nystatin
Staphylococcus aureus	9 mm	40 mm	NA[a]	NT[b]
Alternaria alternata	NA	NT	8 mm	12 mm
Colletotrichum nicotianae	NA	NT	7 mm	10 mm
Saccharomyces cerevisiae	NA	NT	11 mm	22 mm

[a] No activity.

[b] Not tested.

The NOE correlations of H-11 with the signals of two OCH_3 ($\delta_H = 3.84$ and 3.55, respectively) positioned two methoxys at C-1 and C-10. The HMBC of C-10 ($\delta_C = 147.5$) with OCH_3 ($\delta_H = 3.84$) and H-11 ($\delta_H = 7.99$) indicated that this OCH_3 should be connected to C-10 (▶ **Fig. 2**), and thus the other OCH_3 ($\delta_H = 3.55$) should be connected to C-1. These data showed similarities to those of boldine (**5**) [12]. The stereochemistry of C-6a was determined to be *S* by its positive specific rotation ($[\alpha]_D^{20}$: + 269.2 °) [22]. Therefore, compound **1** was identified as (+)-*N*-(methoxycarbonyl)-*N*-norboldine.

The HR-ESI-MS of **2** gave a $[M + H]^+$ peak at $m/z = 344.1504$ (calcd. for $C_{19}H_{22}NO_5$: 344.1497 $[M + H]^+$), which afforded a molecular formula of $C_{19}H_{21}NO_5$. The UV spectrum showed absorptions at $\lambda_{max} = 304$, 284, 217, and 201 nm, characteristic of a 1, 2, 9, 10-substituted aporphine. Its ^{1}H-NMR spectrum showed a very similar pattern to those of (–)-isoboldine β-*N*-oxide [23], including three aromatic singlets at $\delta_H = 8.11$ (H-11), 6.77 (H-8), and 6.70 (H-3); two OCH_3 singlets at $\delta_H = 3.89$ and 3.84; and three highly downfield shifts of *N*-CH_3 at $\delta_H = 3.36$ (3H, s), H-6a at $\delta_H = 4.41$(1H, dd, $J = 13.6$, 3.6 Hz), and H-5 at $\delta_H = 3.57$ and 3.72 (each 1H, m) due to the *N*-oxide. In addition, the regiochemistry of methoxys was determined by the NOE correlations of H-3 with OCH_3 ($\delta_H = 3.89$, s) and H-11 with OCH_3 ($\delta_H = 3.84$, s) (▶ **Fig. 2**), which indicated that the substituents of OCH_3 were at C-2 and C-10, respectively. The positive specific rotation ($[\alpha]_D^{20}$: + 86.1 °) of **2** indicated an *S* configuration of C-6a [22], compared with the *R* configuration of that in (–)-isoboldine β-*N*-oxide ($[\alpha]_D^{20}$: –90.32 °) [23]. Furthermore, the key NOE correlations of *N*-CH_3 with H-6 indicated the β-*N*-oxide in **2** (▶ **Fig. 2**). Thus, compound **2** was determined to be (+)-isoboldine β-*N*-oxide.

The presence of compounds **1**, **2**, **6**, **8**, **9**, **11**, and **12** in *Litsea cubeba* is reported for the first time. All compounds were tested for antibacterial and antifungal activities at 50 μg/disk. Compounds **1** and **4** were found to be active (▶ **Table 2**). It should be noted that compound **1** showed significant antibacterial activity against *Staphylococcus aureus*, which may validate the antimicrobial activity of the alkaloidal extract against *Staphylococcus aureus*.

In conclusion, both the alkaloidal extract of *L. cubeba* and some isoquinoline alkaloids showed antimicrobial activity; thus, the indicated isoquinoline alkaloids might contribute, at least in part, to the pharmacological functions of *Litsea cubeba.*

Acknowledgements

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