Ying-Rui Wu[1, 2]
Yun-Bao Ma[1]
You-Xing Zhao[1]
Shu-Ying Yao[1, 2]
Jun Zhou[1]
Ying Zhou[3]
Ji-Jun Chen[1]

Two New Quaternary Alkaloids and Anti-Hepatitis B Virus Active Constituents from *Corydalis saxicola*

Abstract

Two new quaternary isoquinoline alkaloids, saxicolalines A (**1**) and *N*-methylnarceimicine (**2**), together with sixteen known alkaloids (**3 – 18**) were isolated from *Corydalis saxicola* Bunting. The structures of saxicolalines A (**1**) and N-methylnarceimicine (**2**) were established based on spectral methods including 1D, 2D NMR (COSY, HMQC, HMBC) and HR-ESI-MS. The anti-HBV activities of ten main alkaloids were evaluated. Among the tested compounds, dihydrochelerythrine (**8**) exhibited the most potent activity against HBsAg and HBeAg secretions with $IC_{50} < 0.05\ \mu M$, SI > 3.5, respectively.

Key words
Corydalis saxicola Bunting · Fumariaceae · quaternary isoquinoline alkaloids · anti-HBV activity

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Introduction

Corydalis saxicola Bunting (Fumarioideae) is a perennial herb distributed mainly in calcareous mountain regions of southwest China, and has been used clinically as a folk medicine to treat hepatitis for a long time [1], [2]. The alkaloid extracts of the herb demonstrated pharmacological activities, including anti-virus [3], and eleven alkaloids haveeen isolated from this plant so far [4], [5]. In our preliminary study, the total extract of the roots of *C. saxicola* exhibited significant anti-HBV (hepatitis B virus) activity against HBsAg (hepatitis B virus surface antigen) secretion of Hep G 2.2.15 cells with an IC_{50} = 0.16 mg/mL, and against HBeAg (hepatitis B virus e antigen) secretion with an IC_{50} < 0.04 mg/mL, respectively, which prompted us to further investigate the anti-HBV constituents of this plant. The chemical investigation resulted in the isolation of two new quaternary isoquinoline alkaloids, saxicolaline A (**1**) and *N*-methylnarceimicine (**2**), together with sixteen known alkaloids **3 – 18** (Fig. **1**). Herein, we present the isolation, structure elucidation of saxicolaline A and *N*-methylnarceimicine, and the anti-HBV activities of compounds **1**, **2**, **7**, **8**, **10 – 13**, **15** and **18** *in vitro*.

Materials and Methods

General

Column chromatography: silica gel (200 – 300 mesh; H, Qingdao Marine Chemical Inc.; Qingdao, P. R. China); Sephadex LH-20 (Amersham Biosciences; Uppsala, Sweden); reverse-phase C-8 silica gel (40 – 63 μm; Merck; Darmstadt, Germany). Melting points: XRC-1 apparatus (Sichuan University, Sichuan, China); optical rotations were carried out on a Jasco P-1020 Polarimeter

Affiliation
[1] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P. R. China
[2] Graduate School of Chinese Academy of Sciences, Beijing, P. R. China
[3] Guangxi Institue of Chinese Medicine & Pharmaceutical Science, Nanning, P. R. China

Correspondence
Prof. Dr. Ji-Jun Chen · State Key Laboratory of Phytochemistry and Plant Resources in West China · Kunming Institute of Botany · Chinese Academy of Sciences · Heilongtan · Kunming 650204 · People's Republic of China · Phone/Fax: +86-871-522-3265 · E-mail: chenjj@mail.kib.ac.cn

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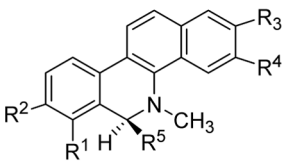
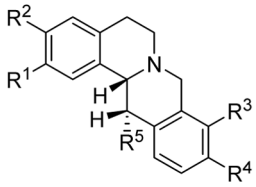
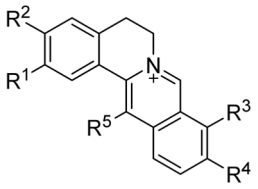


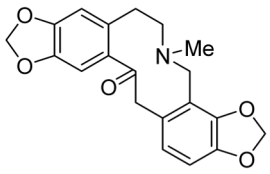
3: R^1, R^2 = OCH_2O, R^3, R^4 = OCH_2O, R^5 = H
7: R^1, R^2 = OCH_2O, R^3, R^4 = OCH_2O, R^5 = CH_2COCH_3
8: R^1, R^2 = OCH_3, R^3, R^4 = OCH_2O, R^5 = H



4: R^1, R^2 = OCH_3, R^3, R^4 = OCH_3, R^5 = CH_3
5: R^1, R^2 = OCH_3, R^3, R^4 = OCH_2O, R^5 = CH_3
6: R^1, R^2 = OCH_2O, R^3, R^4 = OCH_2O, R^5 = H
9: R^1, R^2 = OCH_3, R^3, R^4 = OCH_3, R^5 = H



12: R^1, R^2 = OCH_3, R^3, R^4 = OCH_3, R^5 = H
14: R^1, R^2 = OCH_2O, R^3, R^4 = OCH_3, R^5 = H
15: R^1, R^2 = OCH_2O, R^3, R^4 = OCH_2O, R_5 = H
16: R^1 = OCH_3, R_2 = OH, R^3, R^4 = OCH_2O, R^5 = H
17: R^1 = OH, R^2 = OCH_3, R^3, R^4 = OCH_2O, R^5 = CH3



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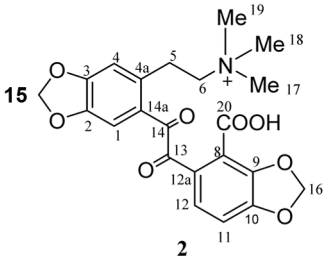

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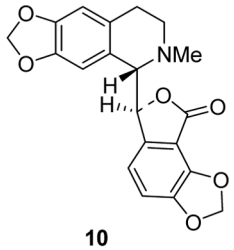

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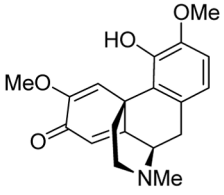

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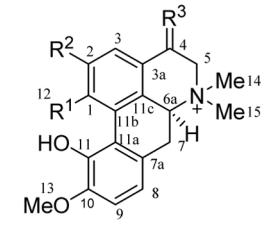

1: R^1 = OCH_3, R^2 = OH, R^3 = =O
18: R^1 = OH, R^2 = OCH_3, R^3 = H_2

Fig. **1** Structures of compounds **1**–**18**.

(JASCO Inc.; Easton MD, USA); UV spectra were obtained in a Shimadzu UV 2401 PC spectrometer (Shimadzu; Kyoto, Japan); IR spectra were measured on a Bruker Tensor 27 spectrometer (Bruker Optics Inc.; Ettlingen, Germany) with KBr pellets, ν in cm^{-1}; MS were recorded on a VG Autospec-3000 or API QSTAR PULSAR LC-Q-TOF spectrometer (VG; Manchester, England); NMR spectra were recorded on a Bruker AM-400 (400 MHz/100 MHz) or DRX-500 (500 MHz/125 MHz) spectrometer (Bruker BioSpin AG; Fällanden, Switzerland) and chemical shifts are given in δ with TMS as internal reference; Fractions were monitored by TLC and spots were visualized by spraying the silica gel plates with Dragendorff's reagent.

Plant material

Corydalis saxicola Bunting was collected in Donglan region, Guangxi Zhuang Municipality, People's Republic of China, in May 2005, and was authenticated by Prof. Zhou Jun. A voucher specimen (ZJ 2005-05-1) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

Dried roots of *C. saxicola* (5.5 kg) were powdered and extracted with 95% EtOH (3×10 L) under reflux. The EtOH extract was soaked with 2% HCl solution (8.0 L), then filtered. The aqueous phase was basified to pH 10 with 25% NH_3/H_2O and extracted with $CHCl_3$ (0.5 L×4) to give extract A (93.6 g). The aqueous layer was further basified with 10% NaOH solution to pH 12 and extracted with *n*-BuOH (0.5 L×4) to afford extract B (63.6 g). Extract B (63.6 g) was submitted to dry column chromatography on silica gel (10×120 cm, 200–300 mesh, 2500 g) eluting with $CHCl_3/CH_3OH/H_2O$ (7:3:0.5, 4.5 L) to give fractions E–I. Fraction H (2.9 g) was subjected to vacuum liquid chromatography (VLC) (2×15 cm, silica gel H, 50 g) in a step gradient manner with $CHCl_3/CH_3OH/H_2O$ from 8:2:0.2 to 6:4:0.4 (650 mL) to give compounds **18** (540 mg) and **1** (89 mg). Compound **2** (16 mg) was obtained from fraction I (8.2 g) by repeated VLC (sintered glass funnel, 250 mL, silica gel H, 100 g) eluted with $EtOH/H_2O$ (5:1, 1.5 L), column chromatography (3×45 cm, reverse-phase C-8 silica gel, 70 g) with $EtOH/H_2O$ (from H_2O to $EtOH/H_2O$, 2:8, each 500 mL) and purified by Sephadex LH-20 column chromatography (1.5×45 cm) eluted with H_2O (300 mL).

Isolates

Saxicolaline A (**1**): yellow crystalline solid (MeOH); m.p. 223–225 °C; $[\alpha]_D^{27}$: 718.8 (*c* 0.16, C_5H_5N); UV (MeOH): λ_{max} (log ε) = 205 (4.39), 231 (4.23), 289 (4.09), 358 (4.05) nm; IR (KBr): ν_{max} = 3425, 2935, 1654, 1607, 1576, 1466, 1442, 1408, 1039 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table **1**; FAB-MS (neg. ion mode): *m/z* = 355 ($[M-H]^-$), 339 ($[M-CH_3]^-$, 100), 325 ($[M-OCH_3]^-$, 77), 311 ($[M-NMe_2]^-$, 37), 293, 265, 154, 97, 80; HR ESI-MS (pos. ion mode): *m/z* (%) = 356.1484 $[M]^+$ (calcd. for $C_{20}H_{22}NO_5$: 356.1497).

N-Methylnarceimicine (**2**): yellow crystalline solid (MeOH); m.p. 194–196 °C; UV (MeOH): λ_{max} (log ε) = 207 (4.48), 331 (4.02)

Table **1** ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) data of compounds **1** and **2** in DMSO-d_6

Position	1		2	
	^{13}C	$^{1}H^{a}$	^{13}C	$^{1}H^{a}$
1	152.3 (s)	–	113.0 (d)	7.71 (1H, s)
2	165.3 (s)	–	145.3 (s)	–
3	103.8 (d)	7.16 (1H, s)	149.8 (s)	–
3a	111.5 (s)	–	–	–
4	182.6 (s)	–	111.5 (d)	7.01(1H, s)
4a	–	–	133.8 (s)	–
5	67.5 (t)	4.45 (1H, d, 15.4), 4.16 (1H, d, 15.4)	27.3 (t)	3.02 (2H, dd, 10.2, 3.1)
6	–	–	65.5 (t)	3.50 (2H, m)
6a	68.9 (d)	4.77 (1H, dd, 14.0, 3.1)	–	–
7	29.5 (t)	3.23 (1H, dd, 14.4, 3.1), 2.81 (14.4, 14.0)	–	–
7a	124.1 (s)	–	–	–
8	116.2 (d)	6.62 (1H, d, 7.8)	124.8 (s)	–
9	110.2 (d)	6.73 (1H, d, 7.8)	145.4 (s)	–
10	149.9 (s)	–	150.5 (s)	–
11	148.1 (s)	–	107.8 (d)	6.90 (1H, d, 7.9)
11a	122.0 (s)	–	–	–
11b	111.5 (s)	–	–	–
11c	131.4 (s)	–	–	–
12	55.8 (q)	3.72 (3H, s)	122.7 (d)	6.96 (1H, d, 7.9)
12a	–	–	132.9 (s)	–
13	55.1 (q)	3.69 (3H, s)	191.9 (s)	–
14	52.8 (q)	3.42 (3H, s)	188.0 (s)	–
14a	–	–	127.5 (s)	–
15	44.9 (q)	3.06 (3H, s)	102.0 (t)	6.11 (2H, s)
16	–	–	101.6 (t)	6.06 (2H, s)
17	–	–	52.2 (q)	3.09 (3H, s)
18	–	–	52.2 (q)	3.09 (3H, s)
19	–	–	52.2 (q)	3.09 (3H, s)
20	–	–	166.0 (s)	–

[a] δ in ppm and J in Hz.

nm; IR (KBr): ν_{max} = 3500 – 2500, 2906, 1661, 1607, 1586, 1505, 1487, 1446, 1373, 1360, 1251, 1030, 921 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table **1**; ESI-MS (pos. ion mode): *m/z* (%) = 450 ([100, M + Na – H]$^{+}$, 100), 428 ([M]$^{+}$, 14); HR-ESI-MS (pos. ion mode): *m/z* = 428.1345 [M]$^{+}$ (calcd. for $C_{22}H_{22}NO_8$: 428.1333).

The anti-HBV assays were performed according to the method in our previous report [18]. An antivirus agent, lamivudine (3TC; Glaxosmithkline; Suzhou, China), was used as positive control.

Supporting information

A detailed description of the extraction and isolation of **3 – 18** and the structures, physicochemical data and spectral data for compounds **3 – 12** and **15 – 18** are available as Supporting Information.

Results and Discussion

Saxicolaline A (**1**) was obtained as a yellow crystalline solid and was positive to Dragendorff's reagent. Its molecular formula was determined as $C_{20}H_{22}NO_5$ based on HR-ESI-MS at m/z = 356.1484 ([M]+, calcd.: 356.1497), indicating that compound **1** possessed eleven degrees of unsaturation. The ^{1}H-NMR spectrum displayed three aromatic protons including two *o*-coupled signals at δ = 6.73 and 6.62 (d, J = 7.8 Hz), two methoxy proton groups at δ = 3.72 and 3.69, and two N-methyl proton groups at δ = 3.42, 3.06 respectively. The ^{13}C-NMR spectrum revealed the presence of twenty carbon atoms including two benzene rings, four methyl groups, two methylene groups, one methine group and one carbonyl corresponding to the IR absorption at 1654 cm^{-1}. The ^{1}H- and ^{13}C-NMR features were closely similar to those of magnoflorine (**18**) [15] except for the absence of two aliphatic protons and one methylene carbon in an aporphine skeleton. The

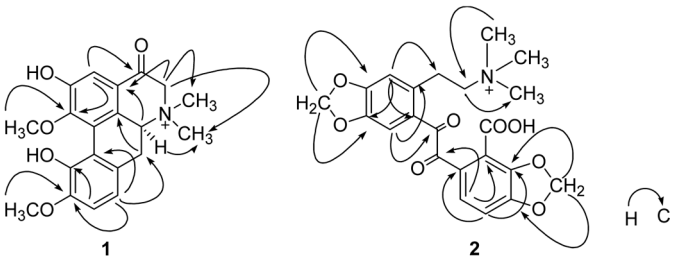

Fig. **2** Key HMBC correlations of compounds **1** and **2**.

Table **2** Inhibitory activities of main alkaloids against HBsAg and HBeAg secretions in Hep G 2.2.15 cell line

Compound	CC_{50}(μM)		*HBsAg*[b]			*HBeAg*[c]		
			IC_{50}(μM)		*SI*[d]	IC_{50}(μM)		*SI*
1	>2.81	>2.81	2.19	2.19	>1.3	>2.81	>2.81	1.0
2	2.05	2.02	1.24	1.22	1.6	1.86	1.84	1.0
7	>2.54	>2.54	6.64	6.55	>1.5	>2.54	>2.54	1.0
8	0.16	0.16	<0.05	<0.05	>3.5	<0.05	<0.05	>3.5
10	>2.73	>2.73	1.35	1.36	>2.0	>2.73	>2.73	1.0
11	0.51	0.56	0.27	0.26	2.1	0.45	0.43	1.3
12	1.97	1.94	>4.26	>4.26	<0.5	>4.26	>4.26	<0.5
13	0.24	0.23	2.63	2.61	0.1	>4.25	>4.25	<0.1
15	>1.02	>1.02	2.74	2.74	>1.2	3.20	3.19	1.0
18	3.60	3.20	>4.39	>4.39	<0.7	>4.39	>4.39	<0.7
3TC[a]	42.83	43.00	15.37	14.72	2.9	44.85	42.93	1.0
Total extract of the roots of *C. saxicola*	0.23 mg/mL	0.21 mg/mL	0.17 mg/mL	0.16 mg/mL	1.3	<0.04 mg/mL	<0.04 mg/mL	>5.3

[a] 3TC (lamivudine): positive control.
[b] HBsAg: hepatitis B virus surface antigen.
[c] HBeAg: hepatitis B virus e antigen.
[d] SI (selective index) is the ratio of CC_{50} and IC_{50}.

carbonyl was assigned to be at C-4 in the B ring by the key HMBC correlation of H-3 (δ = 7.16) and C-4 (δ = 182.5). The optical rotation was dextrorotatory ($[\alpha]_D^{27}$: 718.8), indicating that the stereochemistry of compound **1** at C-6a can be proposed as the *S*-form [16]. The structural deduction of compound **1** was further confirmed by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. Therefore, the structure of saxicolaline A was elucidated in Fig. **2**.

N-Methylnarceimicine (**2**) was obtained as a yellow crystalline solid and was positive to Dragendorff's reagent. The molecular formula was established as $C_{22}H_{22}NO_8$ by HR-EI-MS of *m/z* = 428.1333 ([M]$^+$, calcd.: 428.1345), indicating thirteen degrees of unsaturation in the structure. The IR spectrum showed absorption bands due to a carboxyl group (3500 – 2500 cm^{-1}), carbonyl groups (1661 and 1607 cm^{-1}) and aromatic rings (1586, 1505 and 1446 cm^{-1}). The ^{1}H-NMR spectrum exhibited three N-methyl groups at δ = 3.09 (9H, s), two methylenedioxy groups at δ = 6.11 (2H, s) and 6.01 (2H, s), and four aromatic protons at δ = 7.71 (1H, s), 7.01 (1H, s), 6.96 and 6.90 (1H, d, *J* = 7.9 Hz). The ^{13}C NMR spectrum showed twenty-two carbon atoms which were attributed to two aromatic rings, two methylenedioxy groups, two aliphatic methylene groups, three N-methyl groups and three carbonyl groups, whose chemical shifts were closely similar to those of narceimicine [17], except for one additional methyl linked to nitrogen atom which was confirmed by the correlations between H-17, -18 and -19 (δ = 3.09) with C-6 (δ = 65.5). The deduction was further supported by the analysises of the ^{1}H–^{1}H COSY, HMQC and HMBC spectra. Accordingly, compound **2** was elucidated as *N*-methylnarceimicine.

The other sixteen known alkaloids were also isolated from this plant, and were identified as dihydrosanguinarine (**3**) [6], corydaline (**4**) [7], cavidine (**5**) [8], stylopine (**6**) [9], 6-acetonyl-5,6-dihydrosanguinarine (**7**) [10], dihydrochelerythrine (**8**) [6], tetrahydropalmatine (**9**) [7], adlumidine(**10**) [11]), (–)-salutaridine (**11**) [12], palmatine (**12**) [13], protopine (**13**), berberine (**14**), coptisine **(15)** [7], thalifaurine (**16**) [14], dehydroapocavidine (**17**) [7] and (+)-magnoflorine (**18**) [15] by comparing spectral data with those reported in the literature and of authentic samples. The isolation procedure and spectral data of known compounds are described in the Supporting Information.

The total extract of the roots of *C. saxicola* showed significant inhibition activity *in vitro* on both HBsAg and HBeAg secretions of the Hep G 2.2.15 cell line. Thus, ten pure compounds isolated in large amounts were assayed against HBV. The anti-HBV activities are summarized in Table **2**. New compounds **1**, **2** as well as compounds **7**, **10** and **11** were moderately active while compounds **13** and **18** showed weak inhibitory effects. Interestingly, compound **8** exhibited the most potent activity against HBeAg secretion with IC_{50} < 0.05 μM and SI > 3.5, which prompted us to further investigate its mechanism of action against HBV.

Acknowledgements

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