

Bioactive Isoquinoline Alkaloids from *Corydalis saxicola*

Authors

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Key words

- *Corydalis saxicola* Bunting
- Fumariaceae
- isoquinoline alkaloids
- acetylcholinesterase

Abstract

Twelve isoquinoline alkaloids including two new nitro-containing tetrahydroprotoberberines, (–)-2,9-dihydroxy-3,11-dimethoxy-1,10-dinitrotetrahydroprotoberberine (**1**) and (+)-4-nitroisopocavidine (**2**), were isolated from the whole plant of *Corydalis saxicola* Bunting. The structures of the new compounds were established by spectroscopic analysis and chemical evidence. The inhibitory activity of these isolates against cholinesterase and canine parvovirus were evaluated. Compounds **1** and **1a**, (+)-1-nitroapocavidine (**5**),

berberine (**8**), palmatine (**9**), dehydrocavidine (**10**), and sanguinarine (**11**) showed potent inhibitory activity against acetylcholinesterase with IC₅₀ values of less than 10 μM, while only compound **1** possessed weak activity against canine parvovirus. Structure-activity studies demonstrated that the nitro substituents at ring A in the tetrahydroprotoberberines led to an increase in the anti-acetylcholinesterase activity.

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Introduction

Corydalis saxicola Bunting (Fumariaceae) is a perennial herb distributed mainly in southwest China [1]. As a traditional Chinese folk medicine, it is used for the treatment of hepatitis, diarrhea, stomachaches, and bleeding hemorrhoids [2]. The plant is rich in isoquinoline alkaloids with activities of anti-hepatitis B virus and DNA topoisomerase I inhibition [3–7]. In our continuing study to find bioactive alkaloids from medicinal plants [8–11], the chemical constituents of *C. saxicola* were investigated, which led to the isolation of 12 isoquinoline alkaloids including two new nitrotetrahydroprotoberberines: 2,9-dihydroxy-3,11-dimethoxy-1,10-dinitrotetrahydroprotoberberine (**1**) and 4-nitroisopocavidine (**2**). Isoquinoline alkaloids from *Corydalis* were known for their antiviral and anti-acetylcholinesterase activities [6, 12]. Therefore, all of these isolates were evaluated for the activity against canine parvovirus (CPV), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE). The structural elucidation of the new compounds and bioassay results are reported.

Materials and Methods

General experimental procedures

Melting points were determined using an X-4 melting point apparatus (Yingyu Yuhua Apparatus Factory) and were not corrected. Optical rotations were determined on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu double-beam 210A spectrometer. CD spectra were measured in a Chirascan circular dichroism spectrometer. IR spectra were recorded on a Bio-Rad FTS-135 infrared spectrophotometer with KBr disks. 1D and 2D NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers with TMS as an internal standard. ESIMS and HRESI analyses were carried out on an API Qstar Pulsar 1 instrument. EIMS and HREIMS were carried out on a Waters Autospec Premier P776 mass spectrometer. Column chromatography was performed over silica gel G (80–100, 200–300, and 300–400 mesh), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB), HPLC (Agilent 1200, chromatography column: Zorbax SB-C₁₈, φ 9.4 × 250 mm, 5 μm), C-18 silica gel (40–75 μm; Fuji Silysia Chemical Ltd.), and MCI gel CHP 20P (polystyrene type, 75–150 μm; Mitsubishi Chem-

ical Corporation). TLC was conducted on precoated silica gel plates GF 254 (Qingdao).

Plant material

The whole plants of *C. saxicola* were collected from Jingxi County, Guangxi Zhuang Autonomous Region in June 2007. The plant was identified by Professor Chun-Lin Long (Kunming Institute of Botany, Chinese Academy of Sciences), and a voucher specimen (No. JX0701) was deposited at the Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany.

Extraction and isolation

The whole plants of *C. saxicola* (1.5 kg) were extracted three times with MeOH (3 × 5 L) under reflux. The solvent was evaporated under reduced pressure to give a residue (379 g). The extract was separated by silica gel column chromatography (80 × 10 cm, No. 1007388, 80–100 mesh; Qingdao), eluted with CHCl₃–MeOH (30:1, 20:1, 10:1, 5:1, 2:1, and 1:1). Fractions were combined based on TLC analysis, and 6 fractions (A–F) were obtained. Fraction B (38 g) was subjected to C-18 reversed-phase (6 × 50 cm, LiChroprep RP-18; Merck) eluted with MeOH–H₂O (gradient from 1:20 to 95% MeOH) to afford 7 fractions (B₁–B₇). Fraction B₃ (5.4 g) was separated on silica gel CC (50 × 4 cm, No. 0803136, 200–300 mesh; Qingdao) eluted with CHCl₃–MeOH (20:1 to 0:1) to afford 6 fractions (B₃₁–B₃₆). The fraction B₃₆ (468 mg) was applied to Sephadex LH-20 (200 cm, Sephadex LH-20) with MeOH and further purified by column chromatography (40 × 2 cm, No. 0100349, 300–400 mesh; Qingdao) eluted with CHCl₃: MeOH (5:1) to gain **1** (12.0 mg) and **10** (110 mg). The fraction B₃₃ (676 mg) was purified on silica gel CC (40 × 2 cm, No. 0100349, 300–400 mesh; Qingdao) eluted with petroleum ether–Me₂CO (10:1 to 0:1) to afford 5 fractions (B₃₃₁–B₃₃₅), then the fraction B₃₃₃ (50 mg) was purified by preparative TLC (petroleum ether–Me₂CO, 2:1) to gain **11** (5.0 mg) and **12** (6.0 mg). The fraction B₂ (400 mg) was separated by Sephadex LH-20 (200 cm, Sephadex LH-20) with MeOH to get 5 fractions (B₂₁–B₂₅). The fraction B₂₃ (110 mg) was subjected to column chromatography (40 × 2 cm, No. 0100349, 300–400 mesh; Qingdao) eluted with CHCl₃–MeOH–diethylamine (40:2:1) to get 3 fractions (B₂₃₁–B₂₃₄), and the fraction B₂₃₂ (58 mg) was chromatographed on a Sephadex LH-20 column (200 cm, Sephadex LH-20) to obtain **8** (15 mg) and **9** (17 mg). The fraction C (4.0 g), which was subjected to column chromatography over MCI gel CHP 20P (80 × 10 cm, polystyrene type, 75–150 μm) was eluted with MeOH–H₂O (4:1 to 100% MeOH) to obtain 3 fractions (C₁–C₃). The fraction C₁ (3.2 g) was flashed on C₁₈ silica gel (4 × 50 cm, LiChroprep RP-18; Merck) with MeOH–H₂O (gradient from 1:20 to 95% MeOH) to get 6 fractions (C₁₁–C₁₆). The fraction C₁₅ (500 mg) was separated on silica gel CC (40 × 2 cm, No. 0100349, 300–400 mesh; Qingdao) eluted with CHCl₃–ethyl acetate (1:0 to 0:1) to obtain 5 fractions (C₁₅₁–C₁₅₅). The fraction C₁₅₂ (106 mg) was purified by preparative TLC (petroleum ether–ethyl acetate, 2:1) to obtain a mixture and **5** (31 mg). The mixture was then submitted to Sephadex LH-20 (200 cm, Sephadex LH-20) with MeOH and preparative TLC (petroleum ether–ethyl acetate, 2:1) to gain **2** (11 mg). The fraction C₁₅₁ (86 mg) was subjected to preparative TLC (petroleum ether–ethyl acetate, 2:1) to gain **7** (50 mg). The fraction C₁₄ (400 mg) was then submitted to Sephadex LH-20 (200 cm, Sephadex LH-20) with MeOH to obtain 4 fractions (C₁₄₁–C₁₄₄); then the fraction C₁₄₂ (53 mg) was purified by preparative TLC (CHCl₃–MeOH, 6:1) to get **4** (15 mg). The fraction

C₁₄₃ (60 mg) was purified by preparative TLC (CHCl₃–MeOH, 1:1) to gain **3** (27 mg) and **6** (20 mg). The purity of compounds **1**–**12** was greater than 95% as determined by TLC and NMR.

(–)-2,9-Dihydroxy-3,11-dimethoxy-1,10-dinitrotetrahydroprotoberberine (**1**): yellow amorphous powder (MeOH); [α]_D²⁵ – 586.3 (c 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 342 (3.82), 274 (3.73) nm; CD Δε (c 0.0020, MeOH) – 0.60 (240), – 1.52 (207); IR (KBr) ν_{max} 3432, 1629, 1517, 1327 cm⁻¹; ¹H and ¹³C NMR, see ● Table 1; ESIMS *m/z* 418 [M + H]⁺; HRESIMS *m/z* 418.1251 [M + H]⁺ (calcd. for C₁₉H₂₀N₃O₈, 418.1250).

Preparation of **1a**: K₂CO₃ (10.0 mg), Me₂CO (10 mL), and Me₂SO₄ (0.5 mL) were added to a solution of **1** (8.0 mg). The mixture was stirred for 12 h at 50 °C. Then, 25% NH₃·H₂O (5 mL) was added and evaporated *in vacuo*. The residue was partitioned between H₂O (10 mL) and CHCl₃ (3 × 10 mL). The CHCl₃ layer was concentrated and purified by Sephadex LH-20 column chromatography (MeOH) to afford **1a** (7.9 mg): pink amorphous powder (MeOH); [α]_D²³ – 112.1 (c 0.19, MeOH); CD (c 0.0020, MeOH) Δε – 0.60 (240), – 1.52 (207); ¹H and ¹³C NMR, see ● Table 1; ESIMS *m/z* 460 [M]⁺.

(+)-4-Nitroisoapocavidine (**2**): yellow powder (CHCl₃); m.p. 290–293 °C; [α]_D²⁵ + 82.7 (c 0.20, CHCl₃); UV (CHCl₃) λ_{max} (log ε) 424 (3.05), 343 (3.49), 279 (3.78), 240 (3.93) nm; CD Δε (c 0.0021, MeOH) + 0.23 (207); IR (KBr) ν_{max} 3430, 1628, 1534, 1510, 1467, 1358, 1292 cm⁻¹; ¹H and ¹³C NMR, see ● Table 1; ESIMS *m/z* 385 [M + H]⁺; EIMS *m/z* 384 [M]⁺ (45), 369 (8), 354 (7), 223 (5), 162 (100); HREIMS *m/z* 384.1322 [M]⁺ (calcd. for C₂₀H₂₀N₂O₆, 384.1321).

General procedure for preparation of hydrochlorides

A drop of concentrated hydrochloric acid was added to a solution of the alkaloids (1.0 mg) in MeOH (0.5 mL). The mixture was evaporated under reduced pressure to yield the hydrochlorides of compounds **1**–**7**.

Bioassays

Microplate assay for AChE and BuChE: The AChE inhibitory activities of samples were measured quantitatively by the improved Ellman's method with tacrine (Sigma, purity 98%) as the positive control (IC₅₀ = 0.19 μM) [13, 14]. Inhibitory activity of compounds against BuChE was performed by the improved Ellman's method using tetraisopropylpyrophosphoramidate (Sigma, purity 98%) as the positive control (IC₅₀ = 1.35 μM) [13, 15].

Cell culture and cytotoxicity assays: The embryonic feline kidney F81 cell line (Cell Bank of the Chinese Academy of Sciences, Shanghai) was used for growing canine parvovirus, which was isolated by the authors in Yunnan Agricultural University. The cells were grown in monolayer in a 5% carbon dioxide and 95% atmosphere at 37 °C, with minimal essential medium (MEM; HyClone) containing 10% fetal bovine serum (HyClone). Cytotoxicity assays were performed by the WST-8 method [16, 17], using Cell Counting Kit-8 (CCK-8; Dojindo) according to the supplier recommendations. Briefly, cells were incubated in a 96-well microculture plate (Corning) in the absence or presence of twofold serial dilutions of compounds **1**–**12** and ribavirin (Sigma). After 4 days of culture, 10 μL of CCK-8 solution was added, and the cells were incubated for 1 hour. The number of surviving cells was measured with a Bio-Tek ELx 800.

ELISA microplate reader could show the detection wavelength of 450 nm (*L*₁) and the reference wavelength of 650 nm (*L*₂). The 50% cytotoxic concentration (CC₅₀) was obtained by nonlinear

Table 1 ¹HNMR data for compounds **1**, **1a**, and **2** (δ in ppm, J in Hz).

Position	1	1a	2
	$\delta_{\text{H}}^{\text{a}}$	$\delta_{\text{C}}^{\text{b}}$	$\delta_{\text{H}}^{\text{c}}$
1		140.0 (qC)	144.9 (qC)
2		139.7 (qC)	140.7 (qC)
3		149.0 (qC)	154.8 (qC)
4	6.93 (1H, s)	114.4 (CH)	7.45 (1H, s)
4a		127.2 (qC)	127.8 (qC)
5	3.01 (1H, m) 2.88 (1H, ddd, 16.3, 5.3, 5.3)	29.8 (CH ₂)	3.60 (2H, m)
6	3.13 (1H, m) 2.69 (1H, ddd, 11.4, 7.5, 5.3)	48.2 (CH ₂)	4.10 (2H, m)
8	4.16 (1H, d, 16.5) 3.78 (1H, d, 16.5)	53.8 (CH ₂)	5.42 (1H, d, 16.4) 5.15 (1H, d, 16.4)
8a		122.9 (qC)	119.8 (qC)
9		150.3 (qC)	150.9 (qC)
10		140.4 (qC)	143.2 (qC)
11		146.2 (qC)	151.6 (qC)
12	7.67 (1H, s)	108.1 (CH)	7.90 (1H, s)
12a		126.2 (qC)	122.9 (qC)
13	3.19 (1H, dd, 18.0, 3.6) 2.98 (1H, dd, 18.0, 11.4)	32.2 (CH ₂)	4.00 (1H, m) 3.56 (1H, m)
14	3.90 (1H, dd, 11.4, 3.6)	55.4 (CH)	5.01 (1H, dd, 11.3, 5.6)
14a		122.3 (qC)	116.0 (qC)
2-OMe			3.94 (3H, s)
3-OMe	3.92 (3H, s)	56.8 (CH ₃) ^g	4.01 (3H, s)
7-NMe			3.52 (3H, s)
9-OMe			4.04 (3H, s)
11-OMe	3.93 (3H, s)	57.0 (CH ₃) ^g	4.02 (3H, s)
13-Me			
OCH ₂ O			
			62.5 (CH ₃) 57.2 (CH ₃) 50.9 (CH ₃) 61.7 (CH ₃) 56.9 (CH ₃)
			3.89 (1H, m) 2.85 (1H, m)
			3.95 (1H, m) 3.14 (1H, m)
			4.90 (1H, d, 15.5) 4.02 (1H, d, 15.5)
			108.4 (qC) 143.7 (qC) 146.4 (qC) 109.0 (CH) 121.6 (CH) 130.2 (qC) 36.1 (CH) 63.6 (CH) 120.9 (qC) 56.8 (CH ₃)
			6.76 (1H, d, 8.0) 6.70 (1H, d, 8.0)
			111.1 (CH) 122.9 (qC)
			3.64 (1H, m)
			4.66 (1H, br s)
			1.28 (3H, d, 6.8) 5.97 (1H, d, 1.5) 5.93 (1H, d, 1.5)

^a Measured in CD₃OD-DMSO-*d*₆ (6:1) at 500 MHz. ^b In CD₃OD-DMSO-*d*₆ (6:1) at 100 MHz. ^c In acetone-*d*₆ at 500 MHz. ^d In acetone-*d*₆ at 100 MHz. ^e In CDCl₃-CD₃OD (6:1) at 400 MHz. ^f In CDCl₃-CD₃OD (6:1) at 100 MHz. ^g Data under the same entry are interchangeable

regression analysis of logistic curves (the value of $L_1 - L_2$ to different concentrations of compounds).

Protection assay for CPV-infected F81 cells: By the determination of cell viability, the ability of the compounds to protect CPV-infected F81 cells from death was evaluated [18,19] using the WST-8 method as described above. Tissue culture medium infective dose (TCID₅₀) of 300 viral particles with twofold serial dilutions of the compounds were added to each test well, and the plates were reincubated for 4 days to allow development of a cytopathologic effect (CPE) if any. The number of surviving cells was measured. The 50% effective concentration (EC₅₀) and therapeutic index (TI) were calculated. Ribavirin (Sigma, purity 99.5%) was used as a positive control (EC₅₀ = 121.72 μ M; TI = 10.5) (● Fig. 1).

Supporting information

The optical rotation values and CD data of compounds **1–7** as well as 1D NMR and 2D NMR spectra of compounds **1**, **1a**, and **2** are available as Supporting Information.

Results and Discussion

The molecular formula C₁₉H₁₉N₃O₈ of **1** was determined from the HRESIMS ([M + H]⁺ 418.1251, calcd. 418.1250). The IR spectrum of **1** indicated the presence of hydroxy (3432 cm⁻¹), aromatic (1629 cm⁻¹), and nitro (1327 and 1517 cm⁻¹) functional groups. NMR data of **1** (● Table 1) showed the presence of two pentasub-

stituted phenyl rings [δ_{H} 7.67 (1H, s, H-12), 6.93 (1H, s, H-4); δ_{C} 150.3 (qC), 149.0 (qC), 146.2 (qC), 140.4 (qC), 140.0 (qC), 139.7 (qC), 127.2 (qC), 126.2 (qC), 122.9 (qC), 122.3 (qC), 114.4 (CH), 108.1 (CH)]. The ¹H NMR spectrum also showed signals for two aryl methoxy groups, three aliphatic protons (H₂-13 and H-14) as an AMX system, and two aliphatic protons of the same methylene (H₂-8) as an AX system, together indicating that **1** may be a tetrahydroprotoberberine [20].

Using the ¹H-¹H COSY and HMBC results (● Fig. 2), the carbon skeleton of tetrahydroprotoberberine **1** was established. The ROESY spectrum showed the correlations of H-4/3-OMe and H-12/11-OMe, helping to establish that the two methoxy groups were located at C-3 and C-11, respectively.

The remaining four substituent groups in the two benzene rings were deduced as two hydroxy groups and two nitro groups according to the molecular formula of **1**. In order to determine their locations, a derivative (**1a**) of **1** was prepared.

The ESI-MS afforded the positive ion of **1a** at m/z 460 [M]⁺, which implied that three more *O*- or *N*-methyl groups were linked to **1** through the reaction. The ¹H-NMR spectrum (● Table 1) of **1a** indicated four *O*-methyl groups (δ_{H} 3.94, 4.01, 4.02, and 4.04) and one *N*-methyl group (δ_{H} 3.52). The 3-OMe and 11-OMe groups were located on **1a** by the ROESY correlations (● Fig. 2) of H-4/3-OMe and H-12/11-OMe, respectively, while 2-OMe and 9-OMe groups were determined by the HMBC correlations (● Fig. 2) from H-4 and 2-OMe to C-2, and H₂-8 and 9-OMe to C-9, respectively. Thus, the remaining two nitro groups must be

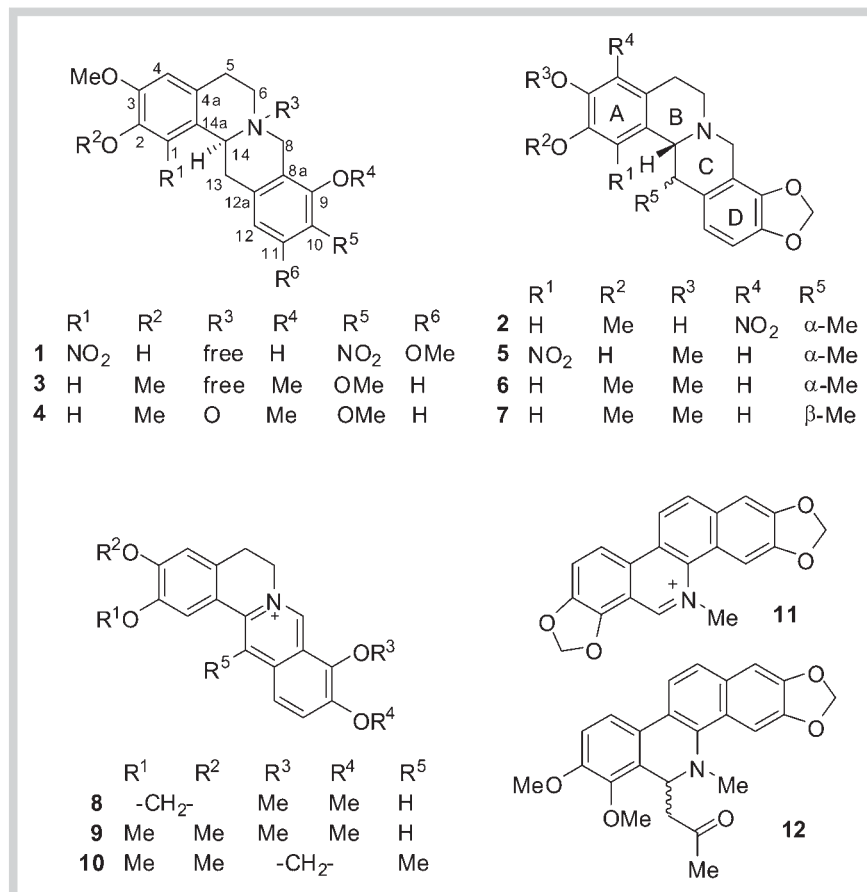


Fig. 1 Structures of compounds 1–7.

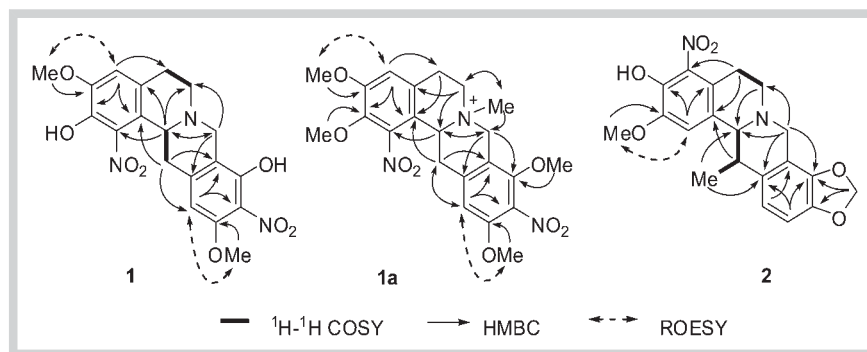


Fig. 2 Key 2D NMR correlations of 1, 1a, and 2.

linked to C-1 and C-10, respectively. Therefore, the planar structure of **1** was elucidated as 2,9-dihydroxy-3,11-dimethoxy-1,10-dinitrotetrahydroprotoberberine.

The absolute configuration of **1** was determined as 14*S* according to the strong negative Cotton effect at 207 nm ($\Delta\epsilon$: -1.52) in the CD spectrum of **1** hydrochloride [21].

Compound **2** was obtained as yellow powder, which has the molecular formula of C₂₀H₂₀N₂O₆ according to its HREIMS at *m/z* 384.1322 [M]⁺ (calcd. 384.1321). The fragments with *m/z* 223 (5%) and 162 (100%) in the EIMS suggested the substitution pattern of the 13-methyl-9,10-methylenedioxy moiety at rings C and D [3]. The ¹H-NMR spectrum displayed one *O*-methyl group at δ 3.89 (s), one methyl group at δ 1.28 (d), and one methylenedioxy group at δ 5.97 (d) and 5.93 (d), respectively. Comparison of NMR data of compound **2** with those of known (+)-1-nitroapocavidine (**5**) [3] and isoapocavidine [22,23] revealed that com-

pound **2** is very similar to the two known alkaloids except for the substitution pattern in ring A. The presence of ROESY correlations (● Fig. 2) between H-1 and 2-OMe implied that the nitro group should be located at C-4 in **2** rather than at C-1 in **5**. In the 13-methyl tetrahydroprotoberberines with a *cis* configuration between H-13 and H-14, the carbon chemical shift of 13-Me is about 18 ppm rather than 22 ppm in those with a *trans* configuration [22,24,25]. Therefore, the relative configuration of **2** was confirmed as 4-nitroisoapocavidine. The absolute configuration of **2** was deduced as 13*S*, 14*R* by comparison of its CD spectrum with that of known (+)-cavidine (**6**) (see Supporting Information) [26,27].

Ringdahl et al. have studied the CD spectra of a series of 14*S*-tetrahydroprotoberberines and suggested that the intense 206–210 nm negative Cotton effects correlate directly with the absolute configuration of 14*S* [21]. However, 14*R*-tetrahydroprotober-

Table 2 AChE inhibitory activity of alkaloids from *Corydalis saxicola*.

Compound	IC ₅₀ (μM)
1	8.77 ± 0.20
1a	3.34 ± 0.69
2	53.30 ± 0.50
3	43.20 ± 1.20
4	64.40 ± 1.60
5	1.70 ± 0.31
6	> 200
7	14.50 ± 0.20
8	1.87 ± 0.48
9	2.20 ± 0.46
10	9.92 ± 0.23
11	1.93 ± 0.01
12	58.70 ± 1.34
Tacrine	0.19 ± 0.02

berines were not reported in the literature. We found that 14R compounds (**2** and **5–7**) showed positive Cotton effects at 206–210 nm, whose intensity was changed according to the configuration of C-13. In the case of the *cis* configuration between H-13 and H-14 (**2**, **5**, and **6**), the Cotton effects are much weaker than that in the *trans* one (**7**) (see Supporting Information).

To the best of our knowledge, compounds **1** and **2** are among the very few examples of the natural tetrahydroprotoberberine alkaloids with a nitro group.

The known alkaloids (–)-tetrahydropalmatine (**3**) [21,28], (–)-corynoxidine (**4**) [29], (+)-1-nitroapocavidine (**5**) [3], (+)-cavidine (**6**) [26,27], (+)-thalictrofoline (**7**) [26,27,30], berberine (**8**) [31,32], palmatine (**9**) [33], dehydrocavidine (**10**) [34], sanguinarine (**11**) [31], and 8-acetyldihydrochelerythrine (**12**) [35] were determined by comparison of their spectral data with those in the literature.

The inhibitory activity of all compounds against AChE, BuChE, and canine parvovirus were evaluated. The results showed that compounds **1** and **1a**, (+)-1-nitroapocavidine (**5**), berberine (**8**), palmatine (**9**), dehydrocavidine (**10**), and sanguinarine (**11**) owned potent inhibitory activity against acetylcholinesterase with IC₅₀ values less than 10 μM (● **Table 2**). Structure–activity relationships indicated that (i) all of tested berberines (**8–10**) were active (IC₅₀ < 10 μM). Other berberines such as jatrorrhizine, dehydrocorydaline, pseudocolumbamine, coptisine, and pseudo-palmatine possessed similar potency [20,36]. However, phenolic hydroxy groups (dehydrocorydalmine and dehydrocorytenchine) could reduce the activity [20]; (ii) nitro substitutions at ring A, especially at C-1, in the tetrahydroprotoberberines (**2**, **5**, and **6**) could increase the activity.

All of these compounds were inactive against BuChE (IC₅₀ > 200 μM), while compound **1** showed weak protection activity of CPV-infected F81 cells from death (EC₅₀ = 182.06 μM; SI = 2.2).

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Conflict of Interest

There are no conflicts of interest among the authors.

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