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ORIGINAL ARTICLE

Monoterpenoid Indole Alkaloids from Inadequately Dried Leaves of *Alstonia scholaris*

Xu-Jie Qin · Yun-Li Zhao · Chang-Wei Song ·
Bei Wang · Ying-Ying Chen · Lu Liu ·
Qiong Li · Dan Li · Ya-Ping Liu · Xiao-Dong Luo

Abstract Six new indole alkaloids, named alstoniascholarines L–Q (**1–6**), together with nineteen known analogues were isolated from the inadequately dried leaves of *Alstonia scholaris*. Their structures were elucidated on the basis of extensive analysis of spectroscopic data and by comparison of their physical and spectroscopic data with the literature values. In addition, the new alkaloids were tested for their cytotoxic and neurite outgrowth-promoting activities.

1 Introduction

Plants of the genus *Alstonia* (Apocynaceae), which are usually shrubs or trees, grow mainly in the tropical regions of Africa and Asia. *A. scholaris* has been historically used to treat chronic respiratory diseases in 'dai' ethnopharmacy in Yunnan province, People's Republic of China. Previous phytochemical and phar-

X.-J. Qin · Y.-L. Zhao · C.-W. Song · B. Wang · Y.-Y. Chen ·
L. Liu · Q. Li · D. Li · Y.-P. Liu (✉) · X.-D. Luo (✉)
State Key Laboratory of Phytochemistry and Plant Resources in
West China, Kunming Institute of Botany, Chinese Academy of
Sciences, Kunming 650201, People's Republic of China
e-mail: liuyaping@mail.kib.ac.cn

X.-D. Luo
e-mail: xdluo@mail.kib.ac.cn

X.-J. Qin · B. Wang · Y.-Y. Chen · L. Liu · Q. Li · D. Li
University of Chinese Academy of Sciences, Beijing 100049,
People's Republic of China

macological studies on this species afforded a number of structurally diverse indole alkaloids [1–5] with various bioactivities, such as antineoplastic [6], antibacterial [7], anti-inflammatory and analgesic effects [8], and broncho-vasodilatory [9] activities. The leaves of *A. scholaris* are usually collected and dried by exposed to sunshine in an open yard. However, some leaves of *A. scholaris* might not be dried in time because of rainy days in Pu'er city. Then, the green color of these leaves might fade in couples of day even they were died finally. Whether these leaves can be used for medical raw material is still unknown, which encouraged us to carried out HPLC analysis of total alkaloids from the inadequately dried leaves of *A. scholaris*. The results indicated that picrinine, the major bioactive compound was decreased remarkably and more peaks were appeared in the HPLC fingerprint profile of inadequately dried leaves (see Supplementary data). Correspondingly, the anti-tussive efficacy reduced significantly (Table 1). In our continuing efforts to search for structurally interesting and bioactive indoles of this plant [10–15], the inadequately dried leaves of *A. scholaris* were investigated. As a result, six new indole alkaloids, alstoniascholarines L–Q (**1–6**), along with nineteen known analogues were isolated. We report herein the isolation, structural elucidation and bioactivities of alkaloids.

Table 1 Effect of the different alkaloid extracts on ammonia-induced cough in mice

Group	Dose (mg/kg)	Frequency of cough	Inhibition (%)
Control		31.3 ± 6.5	–
Codeine phosphate	30	$7.0 \pm 2.5^{**}$	77.6
Alkaloids from the dried leaves	20	$13.1 \pm 4.8^{**}$	58.0
	10	$17.0 \pm 5.6^{**}$	45.6
Alkaloids from the inadequately dried leaves	20	$19.4 \pm 3.9^{**}$	38.0
	10	$22.8 \pm 7.1^{*}$	27.2

Values expressed as mean $\pm$ SEM ($n = 10$), $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison of treated groups with control

Table 2 ^{1}H and ^{13}C NMR spectroscopic data for alkaloids 1–3 in CD$_3$OD

Position	1[a] δ_C	1[a] δ_H (J, Hz)	2[a] δ_C	2[a] δ_H (J, Hz)	3[b] δ_C	3[b] δ_H (J, Hz)
2	109.2		109.0		84.5	
3	51.2	3.63 br d (5.7)	51.4	3.64 br d (5.8)	68.5	3.66 br d (3.1)
5	178.1		178.1		150.7	8.81 d (4.8)
6a	44.2	3.73 d (18.0)	44.0	3.78 d (18.0)	118.4	7.49 d (4.8)
6b		2.77 d (18.0)		2.75 d (18.0)		
7	51.2		51.2		nd[c]	
8	136.7		136.6		128.1	
9	124.3	7.20 d (7.5)	124.2	7.20 d (7.5)	129.2	9.19 d (8.6)
10	121.1	6.79 t (7.6)	121.0	6.80 t (7.5)	128.0	7.69 t (8.4)
11	130.2	7.08 t (7.5)	130.2	7.08 t (7.7)	131.0	7.79 t (8.2)
12	110.3	6.62 d (7.8)	110.3	6.62 d (7.8)	129.7	8.06 d (8.3)
13	148.7		148.7		150.1	
14a	20.7	1.92 ddd (8.0, 6.0, 2.0)	21.5	2.00 ddd (8.1, 6.1, 2.0)	26.0	2.22 d (13.8)
14b		1.77 dd (14.6, 4.3)		1.77 dd (14.5, 4.3)		1.98 dt (13.8)
15	32.3	2.83 m	34.3	2.62 m	42.5	3.11 m
16	49.1	3.23 d (11.5)	48.6	3.28 d (11.5)	55.7	3.36 d (7.9)
17	178.8		178.7		176.5	
18	17.5	1.20 d (6.5)	17.4	1.20 d (6.5)	17.5	1.26 d (6.5)
19	68.9	3.72 q (6.5)	71.3	3.78 q (6.5)	70.4	3.81 q (6.5)
20	92.8		92.1		92.0	
21a	45.7	3.06 d (13.3)	44.6	3.24 d (13.4)	54.2	2.94 d (12.8)
21b		2.68 d (13.3)		2.73 d (13.4)		2.79 d (12.8)
N-Me					46.2	2.65 s

[a] Recorded at 400 and 100 MHz

[b] Recorded at 600 and 150 MHz

[c] Not detected

2 Results and Discussion

Alkaloid **1** was obtained as a white amorphous powder. The HREIMS spectrum showed a quasi-molecular ion peak at m/z 356.1366 [M]$^+$ (calcd for $C_{19}H_{20}N_2O_5$, 356.1372) from which, in conjunction with the ^{13}C NMR data, the molecular formula was determined to be $C_{19}H_{20}N_2O_5$, requiring 11 indices of hydrogen deficiency. The UV absorption maxima 204, 240, and 298 nm suggested the presence of an indole chromophore, while the IR spectrum of **1** indicated the presence of indolic amino (3440 cm^{-1}), ester carbonyl (1748 cm^{-1}), and aromatic ring (1612 and 1485 cm^{-1}) functionalities, respectively. The ^{13}C NMR and DEPT spectra (Table 2) for **1** revealed 19 carbon

signals, including characteristic signals due to an indole ring [δ_C 109.2 (s, C-2), 51.2 (s, C-7), 136.7 (s, C-8), 124.3 (d, C-9), 121.1 (d, C-10), 130.2 (d, C-11), 110.3 (d, C-12), and 148.7 (s, C-13)], two ester carbonyls (δ_C 178.1 and 178.8), one oxygen-bearing quaternary carbon (δ_C 92.8), four methines (δ_C 68.9, 51.2, 49.1, and 32.3), three methylenes (δ_C 45.7, 44.2, and 20.7), and one methyl group (δ_C 17.5).

The ^{1}H–^{1}H COSY spectrum of **1** disclosed the presence of three structural fragments, **a** (C-9–C-12), **b** (C-3–C-14–C-15–C-16), and **c** (C-18–C-19), as shown in Fig. 2. The position of functional groups and the skeleton of alkaloid **1** were assigned by its HMBC data. In the H MBC spectrum, the correlations of δ_H 3.73 and 2.77 (both d, J = 18.0 Hz, H$_2$-6) with δ_C 109.2 (s, C-2), 178.1 (s, C-5) and 51.2 (s, C-7), indicated the presence of a five-membered lactone ring E which was formed by the connection of C-5 and C-2 via an oxygen atom. Besides, the HMBC correlations of H-16 with C-7 and of δ_H 3.63 (1H, br d, J = 5.7 Hz, H-3) with C-2 suggested the formation of a six-membered ring C. The relative downfield shifts of δ_C 51.2 (d, C-3) and 45.7 (t, C-21) required that they both be connected to a nitrogen atom. Likewise, the HMBC correlations of δ_H 2.68 (1H, d, J = 13.3 Hz, H-21b) with δ_C 32.3 (d, C-15), 92.8 (s, C-20) and C-3, and of δ_H 2.83 (1H, m, H-15) with C-20 established the occurrence of another six-membered ring. Moreover, considering the last one degree of unsaturation in **1**, another ring should be constructed. The key HMBC correlations of H-16 with C-17 and C-20 revealed that another five-membered lactone ring was present between C-17 and C-20. Finally, a linkage of C-18/19/20 was deduced from HMBC correlations of Me-18 (δ_H 1.20, d, J = 6.5 Hz) with δ_C 68.9 (d, C-19) and C-20. On the basis of the aforementioned information, the planar structure of **1** was elucidated as an monoterpenoid indole alkaloid with a rare 6/5/6/6/5/5 fused ring system. The stereochemistry was then considered. In the ROESY spectrum, the correlations of H-3/H$_2$-14, H$_2$-14/H-15, and H-15/H-16 were observed, indicating that they should be placed on the same side. However, it still could not determine the configuration for such complicated ring system. According to the similarities between the NMR data of **1** and the recently reported bio-relationship (Scheme 1) of alstolactines A–C [14], the configuration of **1** was assigned as 2*R*, 3*S*, 7*R*, 15*R*, 16*R*, 19*R*, 20*S*. This was further confirmed by the comparison of its CD spectrum with that of alstolactine A (Fig. 3). It is worthy to note that alstolactines A–C were also isolated from the leaves of *A. scholaris* and their absolute configurations were determined by the X-ray diffraction. On the basis of the above results, the structure of alkaloid **1** was established to be as shown in Fig. 1 and named alstoniascholarine L.

Alkaloid **2** was shown to have the same molecular formula of $C_{19}H_{20}N_2O_5$ as that of **1** based on its HREIMS

spectrum (m/z 356.1358 [M]$^+$, calcd for $C_{19}H_{20}N_2O_5$, 356.1372). The ^{1}H and ^{13}C NMR spectral data (Table 2) of **2** were almost identical with those of **1**, except for the carbon signals of C-15, C-19, and C-21, which indicated that **2** could be a 19-epimer of **1**. Following a similar analysis of the ^{1}H, ^{13}C and 2D NMR spectroscopic data as described before, the gross structure of alkaloid **2** was the same as that of **1**. The well matched CD curves of alkaloids **1** and **2** (Fig. 3) proposed the absolute configurations of **1** and **2**. The absolute configurations of 2*R*, 3*S*, 7*R*, 15*R*, 16*R*, 20*S* were those commonly accepted from biogenetic point of view. However, the configuration of C-19 was assigned as 19*R* rather than 19*S* based on its similar NMR data with that of alstolactine B [14]. Therefore, the structure of **2** was characterized as shown in Fig. 1 and named alstoniascholarine M.

Alkaloid **3** was determined to have the molecular formula of $C_{20}H_{22}N_2O_4$ from an HREIMS ion peak at m/z 354.1577 ([M]$^+$). The ^{1}H and ^{13}C NMR spectroscopic data (Table 2) of **3** showed its structure resembling that of alstolactine A. The main difference between them was the presence of two olefinic carbon signals (δ_C 118.4 and 150.7) in alkaloid **3** and the absence of the lactone (δ_C 177.2) and methylene (δ_C 43.8) groups in **2**. The HMBC correlation of H-5 (δ_H 7.49, d, J = 4.8 Hz) with C-7 (δ_C 84.5) and a ^{1}H-^{1}H COSY correlation between H-5 (δ_H 7.49) and H-6 (δ_H 8.81) allowed the location of the double bond was at C-5 and C-6. Other parts of **3** were identical to those of alstolactine A, as supported by its HSQC, HMBC, and COSY experimental data. The configuration of C-19 was determined to be *R* based on the chemical shifts of C-19 (δ_C 70.4) and C-20 (δ_C 92.0), which were in agreement to those of alstolactine A (δ_C 69.2 and 92.1) rather than those of alstolactine B (δ_C 71.7 and 91.4). The other configurations of **3** were assigned to be the same as those of **1** based on their same biogenetic pathway (Scheme 1) and similar NMR data. Accordingly, the structure of **3** was elucidated as shown in Fig. 1 and named alstoniascholarine N.

Biosynthetically, the three related alkaloids might be derived from the precursor picrinine, which was also isolated as a main chemical constituent in this experiment. A plausible biogenetic pathway for **1**–**3** suggested that a Hoffmann degradation led to the formation of a dihydrofuran intermediate **A**. Further oxidation would yield a lactone derivative **B**. Then, epoxidation at C-20–C-19 double bond, followed by ring opening could produce another lactonic F-ring. Subsequently, alkaloids **1** and **2** would finally be obtained via demethylation of the methyl N(4)-Me of alstolactines **A** and **B**. Coincidentally, alkaloid **3** might also derive from intermediate **A** without oxidation and demethylation.

Alkaloid **4**, isolated as a white amorphous powder, had the molecular formula $C_{20}H_{24}N_2O_4$, as established by
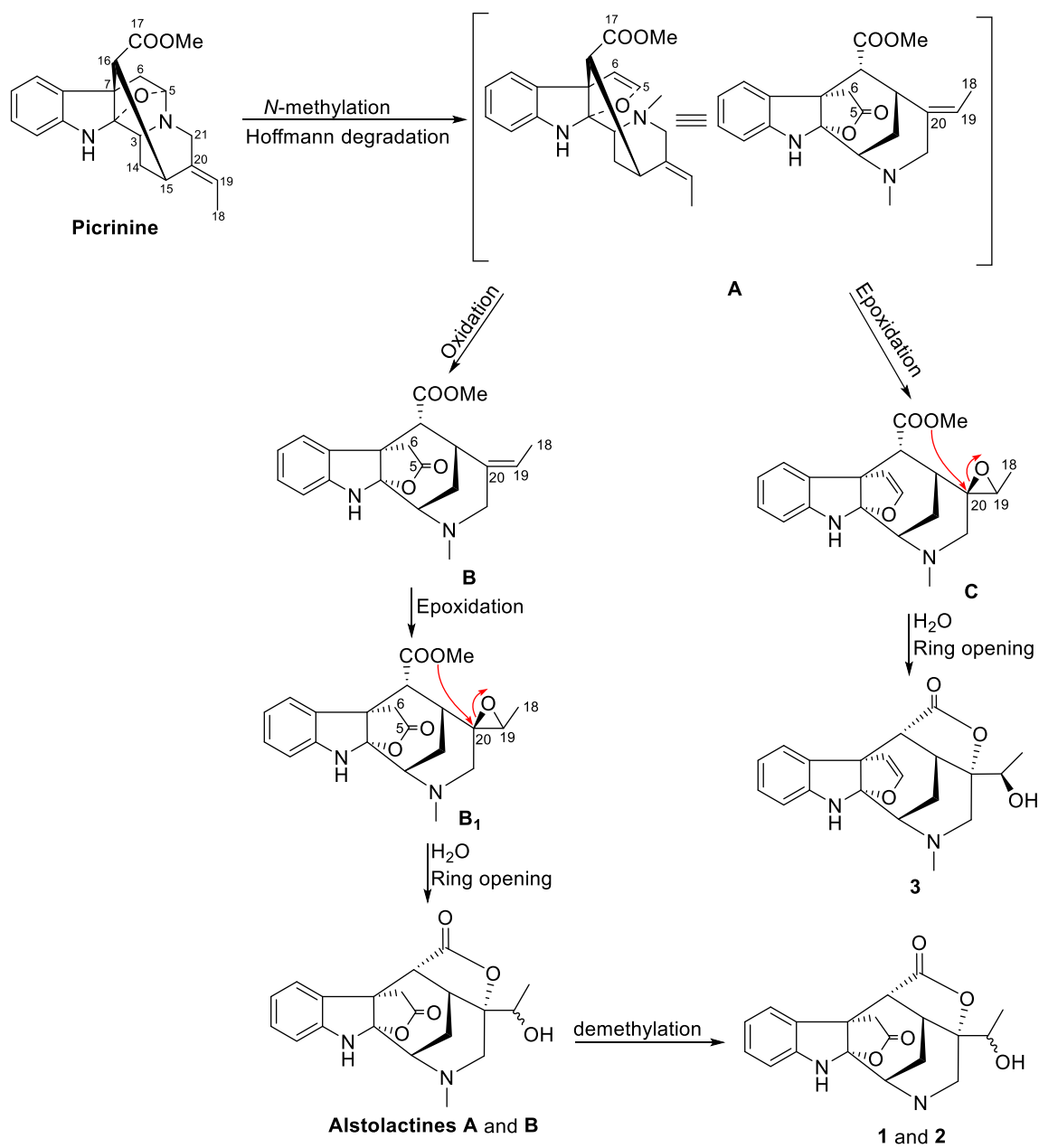
Scheme 1 Putative biosynthetic pathway of alkaloids **1–3** from picrinine
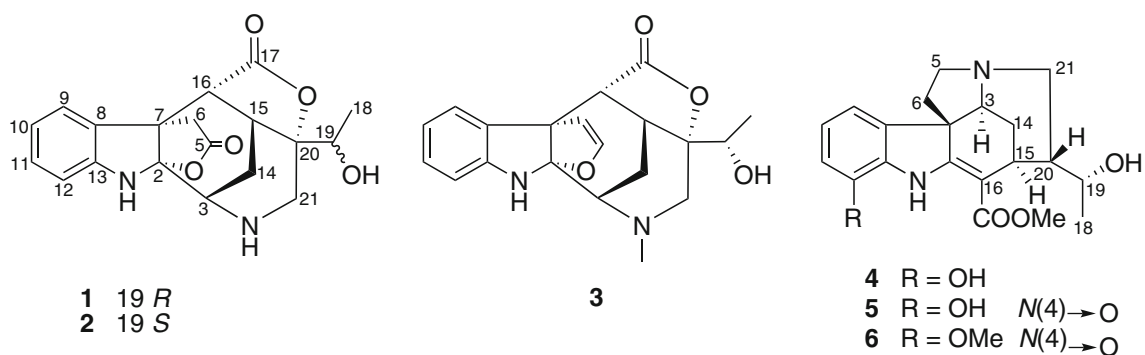
**Fig. 1** Structures of alkaloids **1–6**

Table 3 ^{1}H and ^{13}C NMR spectroscopic data for alkaloids **4–6** in CD$_3$OD[a]

| Position	4		5		6	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	165.6		164.8		166.2	
3	61.8	4.58 br s	78.0	4.83 br s	78.4	4.33 br s
5a	53.0	3.70 m	66.8	4.08 2H m	69.1	3.75 m
5b		3.43 m				3.68 m
6a	43.0	2.56 m	41.0	2.59 m	41.0	2.50 m
6b		2.19 m		2.38 m		2.24 m
7	57.9		57.1		57.0	
8	134.5		134.1		135.2	
9	113.0	6.89 d (7.5)	113.0	7.00 d (7.4)	114.0	7.10 d (7.4)
10	123.7	6.82 t (7.8)	123.8	6.85 t (7.8)	123.8	6.97 t (8.0)
11	117.4	6.74 d (8.1)	117.6	6.76 d (8.1)	112.5	6.92 d (8.2)
12	143.3		143.4		146.2	
13	132.7		132.8		133.6	
14a	26.5	2.37 dt (14.6, 3.4)	25.9	2.62 m	25.8	2.65 br d (14.5)
14b		1.35 dt (14.6, 2.5)		1.47 br d (14.8)		1.29 br d (14.5)
15	25.8	3.32 m	25.1	3.43 br s	25.7	3.36 br s
16	105.3		105.0		105.6	
18	20.5	1.32 d (6.6)	20.8	1.37 d (6.4)	20.8	1.33 d (6.4)
19	69.0	3.82 m	68.4	3.91 m	69.0	3.95 m
20	42.0	2.10 m	44.4	2.13 m	44.8	1.97 m
21a	48.5	3.48 dd (13.5, 11.7)	64.1	4.00 dd (13.4, 6.1)	66.3	3.71 dd (13.6, 6.1)
21b		3.22 dd (13.5, 6.2)		3.81 dd (13.0, 10.6)		3.47 dd (13.4, 9.3)
12-OMe					56.3	3.88 s
<u>C</u>O$_2$Me	168.4		168.1		168.4	
CO$_2$<u>Me</u>	51.7	3.79, s	51.8	3.79 s	51.8	3.78 s

[a] Recorded at 600 and 150 MHz
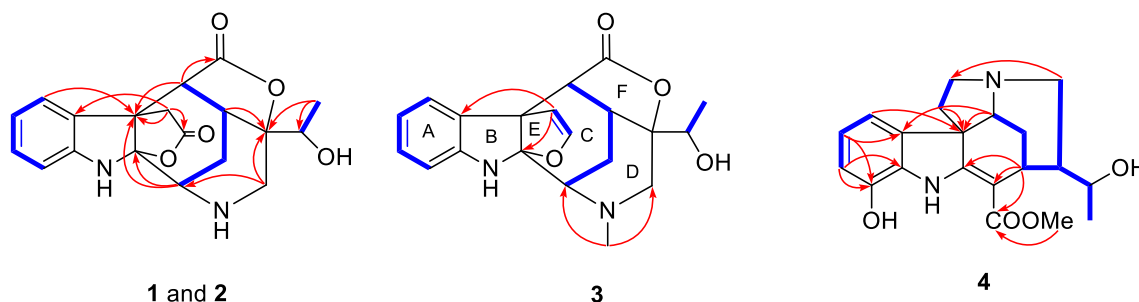
Fig. 2 Key ^{1}H-^{1}H COSY (———) and HMBC (——→) correlations for **1–4**

its HRESIMS data (*m/z* 357.1810, [M + H]$^+$, calcd for C$_{20}$H$_{25}$N$_2$O$_4$ 357.1810), which indicated 10 degrees of unsaturation. Its IR spectrum displayed characteristic absorptions attributing to amino/hydroxyl (3417 cm^{-1}), double bond (1678 cm^{-1}), and aromatic ring (1620, 1440 cm^{-1}) functionalities. The ^{1}H NMR spectrum (Table 3) showed the presence of a 1, 2, 3-trisubstituted aromatic moiety due to the signals of three contiguous aromatic hydrogens (δ_H 6.89, d, *J* = 7.5 Hz, H-9; 7.14, t, *J* = 7.8 Hz, H-10; 6.82, d, *J* = 8.1 Hz, H-11) and two methyls (δ_H 3.79 and 1.32). The ^{13}C NMR spectrum (Table 3) displayed a total of 20 carbon signals, which were classified as two methyls, four methylenes, seven methines, and seven quaternary carbons, respectively. The
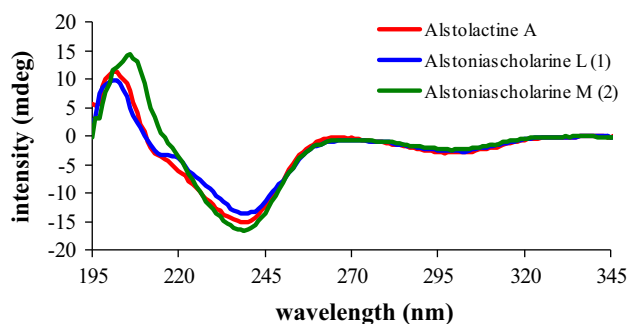
Fig. 3 Experimental CD spectra of alstolactine A (*red*), **1** (*blue*) and **2** (*green*). (Color figure online)
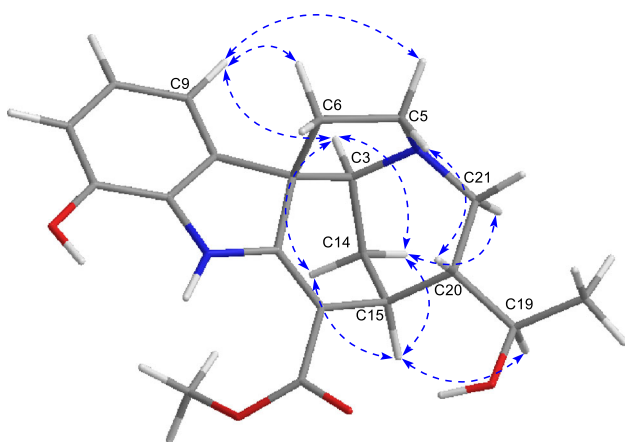
Fig. 4 Key ROESY (←- - →) correlations for alkaloids **4–6**

presence of conjugated ester functionality was supported by the observed carbon signals at δ_C 168.4 and 51.7, while the signals due to the two olefinic quaternary carbons at δ_C 165.6 (C-2) and 105.3 (C-16) are consistent with the presence of a β-anilinoacrylate moiety [8]. One downfield signal at δ_C 69.0 was associated with the presence of oxymethine. The COSY and HMQC data (Figs. 2, 3) disclosed the following partial structures, viz., NCH_2CH_2, $NCHCH_2CHCHCH_2$, and $CHCHCH_3$, corresponding to the C-5–C-6, C-3–C-14–C-15–C-20-C–21, and C-20–C-19–C-18 fragments, respectively. The above NMR data and the partial structures from the COSY spectrum indicated a similarity $N(4)$-demethylalstogustine (**25**) except for the observed difference in the aromatic region. The replacement of H by OH at C-12 was verified by the HMBC correlations of δ_H 6.82 (H-10) and 6.74 (H-11) with δ_C 143.3 (C-12). The relative configurations at the various stereogenic centers were established from ROEs and by comparison of the ^{13}C NMR spectra with the reported values. The ROESY spectrum (Fig. 4) displayed cross peaks of H-3/H-9, H-3/H-5b, H-3/H-6b, H-3/H$_2$-14, H$_2$-14/H-15 indicating the relative configurations of C-7 and C-3, which also in turn allowed the orientation of H-15 to be

assigned as α. The preferred boat conformation adopted by the piperidine ring D could be deduced from the observed H-14a/H-21a ROE correlation [16]. Likewise, the key ROE correlations of H-9/H-6b and H-6a/H-20 suggested a β orientation for H-20. Characteristic chemical shifts of C-16 (δ 105.6) and C-19 (69.0) suggested that the configuration of C-19 in **4** should be *R*, which was consistent with that of alstogustine (δ_C 105.3, 68.8) [17], rather than that of 19-*epi*-alstogustine (δ_C 104.8, 70.6) [17]. On the basis of the above evidences, the structure of **4** was deduced to be as shown in Fig. 1 and named as alstoniascharine O.

Alkaloid **5** exhibited a molecular ion peak at *m/z* 372.1676 in its HREIMS spectrum, indicating the molecular formula of $C_{20}H_{24}N_2O_5$ with sixteen mass units higher than that of **4**. Examination of the ^1H NMR spectrum revealed four downfield shifts for the protons H-3 (δ_H 4.83), H-5 (δ_H 4.08), and H-21 (δ_H 4.00 and 3.81), while the ^{13}C NMR data exhibited noticeable downfield shifts involving C-3 (δ_C 78.0), C-5 (δ_C 66.8), and C-21 (δ_C 64.1), when compared to those of **4**. These features are characteristic of $N(4)$-oxides, and this conclusion was further confirmed by the HMBC correlations of δ_H 4.08 (H$_2$-5), 2.62 (H-14a), and 4.00 (H-21a) with δ_C 78.0 (d, C-3). The ROESY correlations (Fig. 4) indicated that the relative configuration of **5** was the same as that of **4**. Other parts of **5** were identical to those of **4** as secured by detailed analysis of extensive 2D NMR spectral data of **5**. Thus, the structure of alkaloid **5** was determined to be as shown in Fig. 1 and named alstoniascharine P.

Alkaloid **6** was deduced to have the molecular formula $C_{21}H_{26}N_2O_5$, as indicated by the observed ion peak at *m/z* 386.1847 [M]$^+$ in its HREIMS data. The IR, UV and 1D NMR spectra of **6** were similar to those of **5**, which suggested that **6** was also a strychnan type indole alkaloid. The ^1H and ^{13}C NMR data (Table 3) of **6** exhibited high similarities with those of N^b-demethylalstogustine *N*-oxide (**25**), except for the loss of one aromatic proton and the existence of an additional methoxy group (δ_C 56.3, δ_H 3.88). By comparing the ^1H and ^{13}C NMR spectral data of **6** with those of **25**, the C-12 carbon signal was significantly deshielded, while the C-11 and C-13 carbon signals were relatively shielded, suggesting that the methoxy was attached to C-12. This conclusion was further confirmed by the correlation of methoxy protons at δ_H 3.88 with C-12 (δ_C 146.2) in the HMBC spectrum. Complete analysis of 2D NMR spectral data of **6** suggested that its other parts were the same to those of **4** and **5**. Hence, the structure of alkaloid **6** was assigned as shown in Fig. 1 and named as alstoniascharine Q.

The known alkaloids picrinine (**7**) [18], strictamine (**8**) [19], tubotawine (**9**) [20], alstolucine D (**10**) [8], nareline (**11**) [21], picralinal (**12**) [18], isoalschomine (**13**) [18], polyneuridine (**14**) [22], burnamine (**15**) [23], echitamidine

(**16**) [24], scholarisine I (**17**) [25], 19,20-*Z*-vallesamine (**18**) [26], 19,20-*E*-vallesamine (**19**) [26], scholaricine (**20**) [27], 19-*epi*-scholaricine (**21**) [16], pseudoakuammigine N^b-oxide (**22**) [28], tubotawine N^b-oxide (**23**) [29], vallesamine N^b-oxide (**24**) [16], and N^b-demethylalstogustine *N*-oxide (**25**) [30], were identified by comparison of their spectroscopic data with those reported in the literature.

Considering the various bioactivities of the chemical constituents from *A. scholaris* reported previously [5], six new alkaloids (**1–6**) were evaluated for their cytotoxic and neurite outgrowth-promoting activities. Unfortunately, the alkaloids **1–6** neither showed cytotoxic activity against five human cancer cell lines (SW-480, SMMC-7721, HL-60, MCF-7, and A-549), nor exhibited neurite outgrowth-promoting activity.

3 Experimental Section

3.1 General Experimental Procedures

Optical rotations were recorded on a JASCO P-1020 polarimeter. UV spectra were measured on a Shimadzu UV-2401PC spectrophotometer. IR spectra were performed on a Bruker FT-IR Tensor-27 infrared spectrophotometer with KBr discs. NMR spectroscopic data were obtained on Bruker AM-400 and DRX-500 spectrometers using TMS as an internal standard. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. ESIMS spectra were performed on Waters Xevo TQ-S and Bruker HCT/Esquire mass spectrometers; HREIMS and HRESIMS analyses were carried out on Waters AutoSpec Premier P776 and Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS mass spectrometers, respectively. Column chromatography (CC) was conducted on silica gel (200–300 mesh, Qingdao Marine Chemical Co., Ltd., Qingdao, People's Republic of China), RP-18 gel (20–45 μm, Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (GE Healthcare Bio-sciences AB, Sala, Sweden). Fractions were monitored by TLC (GF 254, Qingdao Marine Chemical Co., Ltd., Qingdao), and spots were visualized by Dragendorff's reagent.

3.2 Plant Material

The inadequately dried leaves of *A. scholaris* were collected in June 2013 in Pu'er city of Yunnan Province, P. R. China and identified by Dr. Xiao-Dong Luo, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. Luo20130720) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3 Extraction and Isolation

The inadequately dried leaves (40 kg) of *A. scholaris* were extracted with MeOH at room temperature, and the solvent was evaporated *in vacuo*. The extract was dissolved in 0.3 % HCl solution and then partitioned with EtOAc three times. The acidic water-soluble material, subsequently basified with 5 % ammonia solution to pH 9–10, was repeatedly partitioned with EtOAc three times to give the crude alkaloid extract (155 g). The alkaloid extract was subjected to a silica gel column and eluted with CHCl$_3$–MeOH (30:1, 15:1, 10:1, 5:1, 1:1) to afford fractions I–V. Fraction I (40.0 g) was separated by RP-18 CC, eluted with MeOH-H$_2$O (55:1 → 100:1) to give five subfractions (Fr. I–I to Fr. I–V). Fraction I–II (3.8 g) was further separated by silica gel CC, eluted with CHCl$_3$-acetone (10:1 → 5:1) to yield alkaloids **8** (25 mg), **9** (18 mg), **10** (38 mg), and **11** (123 mg). Fraction I–III (16 g) was subjected to RP-18 CC, eluted with MeOH-H$_2$O (75:25 → 95:5) to obtain alkaloids **1** (18 mg), **2** (20 mg), and **7** (9.8 g). Fraction II (10 g) was separated by RP-18 CC, eluted with MeOH-H$_2$O (50:50–80:20) and subsequently purified by Sephadex LH-20 CC to yield alkaloids **12** (17 mg), **13** (12 mg), **14** (10 mg), **15** (15 mg) and **16** (32 mg). Fraction III (35 g) was subjected to RP-18 CC and eluted with MeOH-H$_2$O (40:60 → 65:35) to obtain five fractions (Fr. III–I to Fr. III–V). Fraction III–II (200 mg) was chromatographed repeatedly over silica gel CC, eluted with CHCl$_3$–MeOH (12:1 → 10:1) to give alkaloids **3** (12 mg) and **17** (8 mg). In the same way, alkaloid **20** (6.5 g) was obtained from fraction III–III (8.2 g) by silica gel CC and eluted with CHCl$_3$–MeOH (12:1 → 10:1). Fraction III–IV (1 g) was separated by RP-18 CC, eluted with MeOH-H$_2$O (35:65 → 60:40) to afford alkaloids **4** (7 mg), **18** (45 mg), **19** (18 mg), and **21** (85 mg). Fraction IV (14 g) was separated over RP-18 CC, eluted with MeOH-H$_2$O (25:75 → 60:40) to afford five fractions (Fr. IV–I to Fr. IV–V). Fraction IV–II (120 mg) was subjected to silica gel CC, eluted with CHCl$_3$–MeOH (8:1 → 5:1) to yield alkaloid **22** (15 mg). Fraction IV–III (200 mg) was purified by RP-18 CC, eluted with MeOH-H$_2$O (30:70 → 50:50) to give alkaloids **23** (4 mg) and **24** (6 mg). Fraction IV–IV (300 mg) was subjected to RP-18 CC eluted with MeOH-H$_2$O (25:75 → 50:50) and further purified by silica gel CC eluted with CHCl$_3$–MeOH (6:1 → 4:1) to yield alkaloids **5** (6 mg), **6** (8 mg) and **25** (6 mg).

3.3.1 Alstoniascholarine L (*1*)

White amorphous powder; $[\alpha]_D^{24}$ –121.1 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.45), 240 (3.87), 298 (3.49) nm; IR (KBr) ν_{max} 3440, 2924, 1748, 1630, 1612, 1473,

1090 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 202 (+8.1), 239 (–10.8), 302 (–2.1) nm; ^{1}H (400 MHz, CD$_3$OD) and ^{13}C NMR (100 MHz, CD$_3$OD) data, see Table 2; ESIMS m/z 379 [M + Na]$^+$; HREIMS m/z 356.1366 ([M]$^+$ calcd for C$_{19}$H$_{20}$N$_2$O$_5$, 356.1372).

3.3.2 Alstoniascholarine M (2)

White amorphous powder; $[\alpha]_D^{24}$ –117.1 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.48), 240 (3.90), 298 (3.52) nm; IR (KBr) ν_{max} 3441, 2923, 1748, 1631, 1474, 1241, 1085 cm^{-1}; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 202 (+8.3), 239 (–11.6), 302 (–2.4) nm; ^{1}H (400 MHz, CD$_3$OD) and ^{13}C NMR (100 MHz, CD$_3$OD) data, see Table 2; ESIMS m/z 379 [M + Na]$^+$; HREIMS m/z 356.1358 ([M]$^+$ calcd for C$_{19}$H$_{20}$N$_2$O$_5$, 356.1372).

3.3.3 Alstoniascholarine N (3)

White amorphous powder; $[\alpha]_D^{23}$ +2.7 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.38), 229 (4.35), 291 (3.63) nm; IR (KBr) ν_{max} 3426, 2976, 1764, 1640, 1511, 1244, 1104 cm^{-1}; ^{1}H (600 MHz, CD$_3$OD) and ^{13}C NMR (150 MHz, CD$_3$OD) data, see Table 2; ESIMS m/z 377 [M + Na]$^+$; HREIMS m/z 354.1577 ([M]$^+$ calcd for C$_{20}$H$_{22}$N$_2$O$_4$, 354.1580).

3.3.4 Alstoniascholarine O (4)

White amorphous powder; $[\alpha]_D^{23}$ –348.7 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.09), 230 (4.00), 289 (3.61), 337 (4.06) nm; IR (KBr) ν_{max} 3417, 2977, 1678, 1620, 1440, 1260, 1074 cm^{-1}; ^{1}H (600 MHz, CD$_3$OD) and ^{13}C NMR (150 MHz, CD$_3$OD) data, see Table 3; ESIMS m/z 357 [M + H]$^+$; HRESIMS m/z 357.1810 ([M + H]$^+$ calcd for C$_{20}$H$_{25}$N$_2$O$_4$, 357.1809).

3.3.5 Alstoniascholarine P (5)

White amorphous powder; $[\alpha]_D^{23}$ –329.3 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.12), 287 (3.60), 336 (3.98) nm; IR (KBr) ν_{max} 3431, 2977, 1680, 1621, 1439, 1202, 1059 cm^{-1}; ^{1}H (600 MHz, CD$_3$OD) and ^{13}C NMR (150 MHz, CD$_3$OD) data, see Table 3; ESIMS m/z 373 [M + H]$^+$; HREIMS m/z 372.1676 ([M]$^+$ calcd for C$_{20}$H$_{24}$N$_2$O$_5$, 372.1685).

3.3.6 Alstoniascholarine Q (6)

White amorphous powder; $[\alpha]_D^{24}$ –298.7 (c 0.12 MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.21), 287 (3.76), 334 (4.06) nm; IR (KBr) ν_{max} 3438, 2965, 1681, 1615, 1461, 1266,

1060 cm^{-1}; ^{1}H (600 MHz, CD$_3$OD) and ^{13}C NMR (150 MHz, CD$_3$OD) data, see Table 3; ESIMS m/z 387 [M + H]$^+$; HREIMS m/z 386.1847 ([M]$^+$ calcd for C$_{21}$H$_{26}$N$_2$O$_5$, 386.1842).

3.4 Antitussive Effects Against Ammonia-Induced Cough

The antitussive assay was performed using the technique as described in the literature [31]. ICR (Institute of Cancer Research) mice of either sex (19–24 g) were purchased from Kunming Medical College (license number SYXK 2005-0001). All mice were housed at room temperature (20–25 °C) and constant humidity (40–70 %) under a 12 h light–dark cycle in SPF (Specific Pathogen Free) grade laboratory. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care. The mice were divided randomly, of which 12 mice per group. The negative control group of animals was treated with distilled water orally, and the positive control was treated with codeine phosphate, the remaining groups were treated with test samples. Antitussive activity was investigated on a classical mouse cough model induced by ammonia liquor. Briefly, each mouse was placed in a 300 mL special glass chamber and exposed to 40 μL 25 % ammonia solution. The cough frequency produced during 2 min exposure period was counted. In the second assay for alkaloids, cough frequency and latent period of cough were recorded.

3.5 Cytotoxic Activity Assay

The following human cancer cell lines were used: SW-480, SMMC-7721, HL-60, MCF-7, and A-549. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT), supplemented with 10 % fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5 % CO$_2$. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) [32]. Briefly, 100 μL of adherent cells was seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with an initial density of 1×10^5 cells/mL in 100 μL of medium. Each cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with cisplatin and paclitaxel (Sigma) as positive controls. After the incubation, MTT (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20 % SDS-50 % DMF

after removal of 100 µL of medium. The optical density of the lysate was measured at 595 nm in a 96-well Microtiter plate reader (Bio-Rad 680).

3.6 Neurite Outgrowth-Promoting Activity Assay

The neurotrophic activities of the test compounds were examined according to an assay using PC12 cells as reported elsewhere [33]. Briefly, PC12 cells were maintained in F12 medium supplemented with 12.5 % horse serum (HS), and 2.5 % fetal bovine serum (FBS), and incubated at 5 % CO_2 and 37 °C. Test compounds were dissolved in DMSO. For the neurite outgrowth-promoting activity bioassay, PC12 cells were seeded at a density of 5×10^4 cells/mL in 48-well plate coated with poly-L-lysine. After 24 h, the medium was changed to that containing 10 µM of each test compounds plus 5 ng/mL NGF, or various concentrations of NGF (50 ng/mL for the positive control, 5 ng/mL for the negative control). The final concentration of DMSO was 0.05 %, and the same concentration of DMSO was added into the negative control. After 72 h incubation, the neurite outgrowth was assessed under a phase contrast microscope. Neurite processes with a length equal to or greater than the diameter of the neuron cell body were scored as neurite bearing cells. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/view area; 5 viewing area/well) was determined and expressed as a percentage.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest in this work.

References

1. Atta-ur-Rahman, K.A. Alvi, Phytochemistry **26**, 2139–2142 (1987)
2. T.S. Kam, K.T. Nyeoh, K.M. Sim, K. Yoganathan, Phytochemistry **45**, 1303–1305 (1997)
3. A.P.G. Macabeo, K. Krohn, D. Gehle, R.W. Read, J.J. Brophy, G.A. Cordell, S.G. Franzblau, A.M. Aguinaldo, Phytochemistry **66**, 1158–1162 (2005)
4. L. Zhang, C.J. Zhang, D.B. Zhang, J. Wen, X.W. Zhao, Y. Li, K. Gao, Tetrahedron Lett. **55**, 1815–1817 (2014)
5. M.S. Khyade, D.M. Kasote, N.P. Vaikos, J. Ethnopharmacol. **153**, 1–18 (2014)
6. G.C. Jagetia, M.S. Baliga, J. Ethnopharmacol. **96**, 37–42 (2005)
7. M.R. Khan, A.D. Omoloso, M. Kihara, Fitoterapia **74**, 736–740 (2003)
8. J.H. Shang, X.H. Cai, T. Feng, Y.L. Zhao, J.K. Wang, L.Y. Zhang, M. Yan, X.D. Luo, J. Ethnopharmacol. **129**, 174–181 (2010)
9. S. Channa, A. Dar, S. Ahmed, Atta-ur-Rahman, J. Ethnopharmacol. **97**, 469–476 (2005)
10. X.H. Cai, Z.Z. Du, X.D. Luo, Org. Lett. **9**, 1817–1820 (2007)
11. X.H. Cai, Q.G. Tan, Y.P. Liu, T. Feng, Z.Z. Du, W.Q. Li, X.D. Luo, Org. Lett. **10**, 577–580 (2008)
12. X.H. Cai, Y.P. Liu, T. Feng, X.D. Luo, Chin. J. Nat. Med. **6**, 20–22 (2008)
13. T. Feng, X.H. Cai, P.J. Zhao, Z.Z. Du, W.Q. Li, X.D. Luo, Planta Med. **75**, 1537–1541 (2009)
14. X.W. Yang, X.J. Qin, Y.L. Zhao, P.K. Lunga, X.N. Li, S.Z. Jiang, G.G. Cheng, Y.P. Liu, X.D. Luo, Tetrahedron Lett. **55**, 4593–4596 (2014)
15. X.J. Qin, Y.L. Zhao, P.K. Lunga, X.W. Yang, C.W. Song, G.G. Cheng, L. Liu, Y.Y. Chen, Y.P. Liu, X.D. Luo, Tetrahedron **71**, 4372–4378 (2015)
16. T. Yamauchi, F. Abe, R.F. Chen, G.I. Nonaka, T. Santisuk, W.G. Padolina, Phytochemistry **29**, 3547–3553 (1990)
17. M.E. Kuehne, F. Xu, C.S. Brook, J. Org. Chem. **59**, 7803–7806 (1994)
18. F. Abe, R.F. Chen, T. Yamauchi, N. Marubayashi, I. Ueda, Chem. Pharm. Bull. **37**, 887–890 (1989)
19. Habib-ur-Rehman Atta-ur-Rahman, Planta Med. **3**, 230–231 (1986)
20. T. Yamauchi, F. Abe, W.G. Padolina, F.M. Dayrit, Phytochemistry **29**, 3321–3325 (1990)
21. Y. Morita, M. Hesse, H. Schmid, A. Banerji, J. Banerji, A. Chatterjee, W.E. Oberhänsli, Helv. Chim. Acta **60**, 1419–1434 (1977)
22. L.D. Antonaccio, N.A. Pereira, B. Gilbert, H. Vorbrueggen, H. Budzikiewicz, J.M. Wilson, L.J. Durham, C. Djerassi, J. Am. Chem. Soc. **84**, 2161–2169 (1962)
23. R.H. Burnell, J.D. Medina, Phytochemistry **7**, 2045–2051 (1968)
24. D. Keawpradub, H. Takayama, N. Aimi, S.I. Sakai, Phytochemistry **37**, 1745–1749 (1994)
25. X.W. Yang, X.D. Luo, P.K. Lunga, Y.L. Zhao, X.J. Qin, Y.Y. Chen, L. Liu, X.N. Li, Y.P. Liu, Tetrahedron **71**, 3694–3698 (2015)
26. K.A. Atta-ur-Rahman, S.A. Alvi, W. Abbas, Voeter. Heterocycles **26**, 413–419 (1987)
27. Atta-ur-Rahman, M. Asif, M. Ghazala, J. Fatima, K.A. Alvi, Phytochemistry **24**, 2771–2773 (1985)
28. W.L. Hu, J.P. Zhu, M. Hesse, Planta Med. **55**, 463–466 (1989)
29. M. Pinar, U. Renner, M. Hesse, H. Schmid, Helv. Chim. Acta **55**, 2972–2974 (1972)
30. A.A. Salim, M.J. Garson, D.J. Craik, J. Nat. Prod. **67**, 1591–1594 (2004)
31. S.Y. Xu, R.L. Bian, X. Chen, *Pharmacological Experiment Methodology* (People's Medical Publishing House, Beijing, 1991), p. 1167
32. T. Mosmann, J. Immunol. Methods **65**, 55–63 (1983)
33. A. Pradines, M. Magazin, P. Schiltz, G. Le Fur, D. Caput, P. Ferrara, J. Neurochem. **64**, 1954–1964 (1995)