Novel Alkaloids from *Alstonia scholaris*

Xiang-Hai Cai[a], Jian-Hua Shang[b], Tao Feng[a], and Xiao-Dong Luo[a]

[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, Yunana 650204, China
[b] Yunnan Institute of Medical Material, Kunming 650111, China

Reprint requests to Prof. Dr. Xiao-Dong Luo. Fax: +86-871-5150227. E-mail: xdluo@mail.kib.ac.cn

Z. Naturforsch. **2010**, *65b,* 1164 – 1168; received April 6, 2010

The alkaloidal fraction of *Alstonia scholaris* leaves shows anti-inflammatory and analgesic bioactivity *in vivo*. A phytochemical study on this fraction led to the isolation of two novel alkaloids, scholarisine I and (±)-scholarisine II. Their structures were elucidated on the basis of spectroscopic methods, and a possible biogenesis is proposed. (±)-Scholarisine II selectively inhibited the inducible COX-2 rather than COX-1, and also markedly inhibited 5-LOX, comparable to positive controls.

Key words: Alstonia scholaris, Scholarisines I and II, COX, 5-LOX

Introduction

The leaves of *Alstonia scholaris*, named dengtaiye by local people, have been historically used in "dai" ethnopharmacy to treat chronic respiratory diseases in the Yunnan province of the People's Republic of China [1]. The aqueous extract of the leaves, developed as a commercially available traditional Chinese medicine used to release tracheitis and cold symptoms, has also been prescribed in hospitals and sold over the counter in drug stores [2]. This established clinical efficiency stimulated us to investigate the bioactive compounds from this plant. The traditional and clinical uses are related to anti-inflammatory and analgesic actions. *In vivo* anti-inflammatory and analgesic assays of a fractionated extract indicated that the alkaloid fraction contained the bioactive component. At doses of 10, 20, 40 and 80 mg per kg, the alkaloid fraction reduced the writhing reflex in mice by 48.4, 40.1, 36.6 and 49.5 %, respectively, comparable to aspirin at 200 mg/kg (57.2 %). At doses of 50 and 100 mg/kg, the alkaloid fraction decreased xylene-induced ear edema in mice by 46.0 and 41.2 %, comparable to aspirin at 200 mg/kg (45.7 %). This promising bioactivity led us to initiate a phytochemical and pharmacological investigation on the *A. scholaris* extract. As part of this endeavor, more than 40 alkaloids, including three monoterpenoid indole alkaloids with rearranged skeletons and further elaboration, were isolated [3, 4] and subjected to anti-inflammatory evaluation against COX-1, COX-2 and 5-LOX *in vitro*. In this paper, we describe the isolation, structure elucidation and anti-inflammatory effect of two novel quinoline alkaloids from *A. scholaris* (Fig. 1).

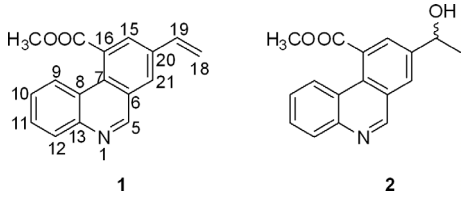

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Fig. 1. Structures of scholarisines I (**1**) and II (**2**).

Results and Discussion

Compound **1** was deduced to have a molecular formula of $C_{17}H_{13}NO_2$ as evidenced by ^{1}H and ^{13}C NMR data and confirmed by HRESIMS at m/z = 264.1025, $[M+H]^+$. Its UV spectrum indicated the presence of a conjugated system based on absorption maxima at 222 and 267 nm. The ^{13}C NMR and DEPT spectra displayed thirteen aromatic carbon signals at δ(C) = 153.4 (d), 146.0 (s), 136.1 (s), 130.4 (s), 130.2 (s), 129.2 (d), 129.0 (d), 128.9 (s), 128.7 (d), 127.5 (s), 126.8 (d), 124.9 (d), and 122.5 (s), a carboxylic methyl ester (δ(C) = 171.4 (s), 52.9 (q)), and a terminal double bond (δ(C) = 134.8 (d), 116.9 (t)). The above data, together with the ^{1}H NMR spectrum δ(H) = 9.25 (1H, s, H-C(5)), 8.20 (1H, d, J = 7.5 Hz, H-C(9)), 8.11 (1H, d, J = 7.5 Hz, H-C(12)), 7.76 (1H, t, J = 7.5, H-C(10)), 7.61 (1H, t, J = 7.5, H-C(11)) suggested that **1** was a unsubstituted A-ring quinoline-type alkaloid [5].

Table 1. ^{1}H, ^{13}C NMR data and HMBC correlations of scholarisine I and II[a].

	— **1** —			— **2** —		
C	δ(C)	δ(H)	HMBC (^{1}H-^{13}C)	δ(H)	δ(C)	HMBC (^{1}H-^{13}C)
H-C(5)	153.4 d	9.25 (s)	C(6, 7, 8, 13, 21)	153.4 d	9.20 (s)	C(6, 7, 8, 13, 21)
C(6)	128.9 s			128.8 s		
C(7)	127.5 s			127.2 s		
C(8)	122.5 s			122.5 s		
H-C(9)	130.4 d	8.20 (d, 7.5)	C(7, 8, 10, 11, 13)	130.4 d	8.19 (d, 7.5)	C(7, 8, 10, 11, 13)
H-C(10)	126.8 d	7.76 (t, 7.5)	C(8, 9, 11, 12)	126.8 d	7.76 (t, 7.5)	C(8, 9, 11, 12)
H-C(11)	129.0 d	7.61 (t, 7.5)	C(9, 10, 12, 13)	129.0 d	7.61 (t, 7.5)	C(9, 10, 12, 13)
H-C(12)	124.9 d	8.11 (d, 7.5)	C(8, 10, 11, 13)	124.9 d	8.11 (d, 7.5)	C(8, 10, 11, 13)
C(13)	146.0 s			145.4 s		
H-C(15)	129.2 d	8.04 (s)	C(7, 19, 21, -CO-)	129.4 d	8.00 (s)	C(7, 19, 21, -CO-)
C(16)	130.2 s			130.1 s		
CH2(18)	116.9 t	6.01 (d, 17.6) 5.51 (d, 8.5)	C(19, 20)	25.4 q	1.61 (d, 6.0)	C(19, 20)
H-C(19)	134.8 d	6.92 (dd, 17.6, 8.5)	C(15, 20, 21)	69.4 d	5.16 (q, 6.0)	C(15, 18, 20, 21)
C(20)	136.1 s			144.7 s		
H-C(21)	128.7 d	8.07 (s)	C(5, 15, 19)	127.4 d	8.09 (s)	C(5, 7, 15, 19, 20)
CO	171.4 s			171.5 s		
OMe	52.9 q	4.06 (, s)	-CO-	53.0 q	4.05 (s)	-CO-

[a] 1D NMR (^{1}H, ^{13}C) spectroscopic data of **1** and **2** were recorded in $CDCl_3$ on Bruker AM-400 and DRX-500 instruments, respectively; δ in ppm, J in Hz.

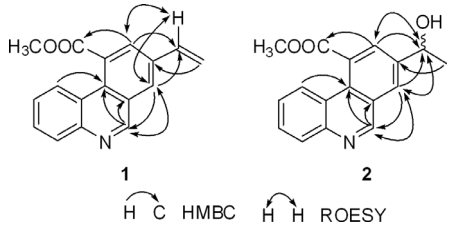

Fig. 2. Key HMBC and ROESY correlations of scholarisines I (**1**) and II (**2**).

Besides the carboxylic methyl ester and double bond, another 10 double bond equivalents required the presence of another benzene ring, which was fused to the quinoline ring at C-6 and C-7. The signal at δ(H) = 8.07 (s) showed HMBC cross peaks to both δ(C) = 153.4 (s, C(5)) and a terminal double bond carbon at δ(C) = 134.8 (d, C(19)), which placed a vinyl group at the *ortho*-position. This suggestion could be further supported by NOE correlations between H-C(21) and both H-C(5) and H-C(19). In the HMBC spectrum, correlations from the singlet signal at δ(H) = 8.04 to δ(C) = 171.4 (s) and 134.8 (d, C(19)) positioned the carboxylic methyl ester at C(16). Thus, **1** was elucidated as shown in Fig. 1, and named scholarisine I. Its numbering system is according to that of monoterpenoid indole alkaloids. All of the ^{1}H and ^{13}C NMR signals of **1** were assigned by HSQC, HMBC, and ROESY experiments (Table 1, Fig. 2).

Compound **2** was found to possess a molecular formula of $C_{17}H_{15}NO_3$ based on HRESIMS at m/z = 282.1135, $[M+H]^+$. The ^{13}C NMR and DEPT spectra displayed thirteen signals for aromatic carbons (δ(C) = 153.4 (d), 145.4 (s), 144.7 (s), 130.4 (d), 130.1 (s), 129.4 (d), 129.0 (d), 128.8 (s), 127.4 (d), 127.2 (s), 126.8 (d), 124.9 (d), and 122.5 (s)), a carboxylic methyl ester group (δ(C) = 171.5 (s), 53.0 (q)), an oxymethine (δ(C) = 69.4, d) and a methyl (δ(C) = 25.4, q) group. **2** was similar to **1** with the exception that the signals at δ(C) = 134.8 (d) and 116.9 (t) in **1** were replaced by δ(C) = 69.4 (d) and 25.4 (q) in **2**, in accordance with a hydroxyl group being positioned at C(19) in **2** instead of the double bond at C(18/19) in **1**. This assumption was confirmed by an HMBC correlation from δ(H) = 5.16 (1H, q, J = 6.0 Hz, H-C(19)) to δ(C) = 127.4 (d, C(21)) and 129.4 (d, C(15)). Thus, **2** was named scholarisine II. All ^{1}H and ^{13}C NMR signals of **2** were assigned by HSQC, HMBC, and ROESY experiments (Table 1, Fig. 2).

A small specific rotation value ($[\alpha]_D^{26} = -1.1$) was detected for compound **2**, caused by the only chiral carbon (C-19), which prompted us to determine its configuration by Mosher's method [6]. However, when (R/S)-Mosher's esters were obtained, ^{1}H NMR spectra for both esters exhibited the presence of a pair of isomers. The integrals of the peaks in the ^{1}H NMR spectrum suggested the presence of a pair of enantiomers with proportions of 10 : 9 for compound **2**. A chiral column, Chiralcel OD-H (5 μm, 10×250 mm^2), was used for further purification, eluting with petroleum ether-isopropanol (9 : 1). Finally,

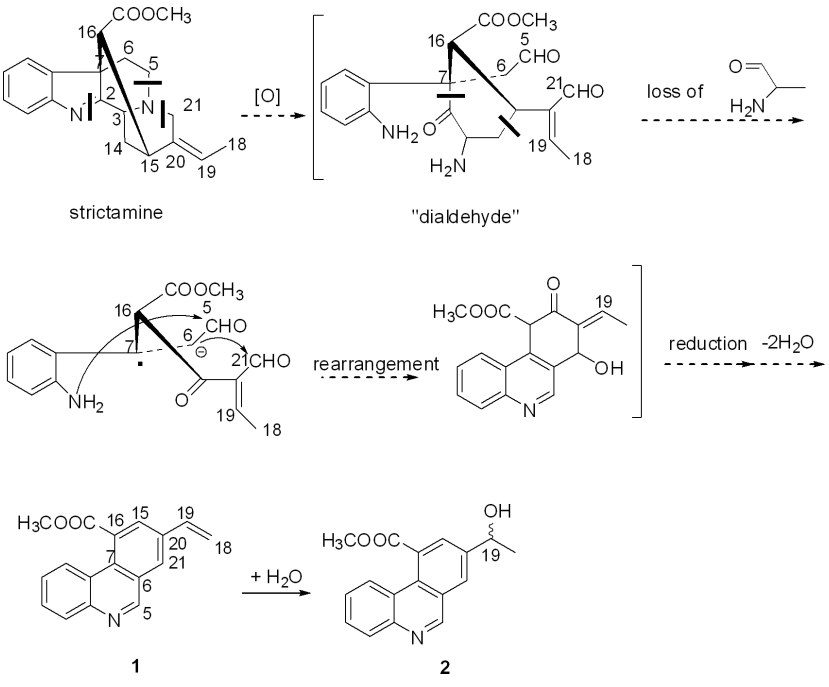

Fig. 3. Proposed biosynthesis of scholarisines I (**1**) and II (**2**).

1.1 mg of (–)-scholarisine II ($[\alpha]_D^{21} = -24°$) and 0.7 mg of (+)-scholarisine II ($[\alpha]_D^{21} = +24°$) were obtained. Due to the limited amounts obtained, the absolute configurations of the optical isomers of **2** could not be confirmed.

Analysis of compound **1** indicated that the structure, consisting of a quinoline skeleton and a 4-methylhexenyl carboxylic methyl ester group (Fig. 3), most likely derives from degradation and rearrangement of a monoterpenoid indole alkaloid. Similar to the biosynthesis of quinine [7], the cleavage of the N(1)–C(2), N(4)–C(5) and N(4)–C(21) bonds in the indole heterocyclic ring of strictamine could generate a "dialdehyde" at C(5) and C(21) together with an amide group (Fig. 3). Subsequently, C(2/7) and C(14/15) might be cleaved, leading to the loss of the fragment -C(2)-C(3)-N(4)-C(14)-. Nucleophilic attack of C(6) on C(21) and of -NH_2 on C(5) would form a unique quinoline skeleton [8]. Finally, the intermediate might be reduced, and with elimination of two molecules of H_2O would yield **1**. Compound **2** could be formed by addition of H_2O across the C(18/19) double bond of **1**.

A direct route to the quinoline ring system, such as for dictamnine and skimmianine in *Dictamnus albus* and *Skimmia japonica* (Rutaceae), is possible through the combination of anthranilic acid and acetate/malonate, and an extension of this process also can account for the origin of the acridine ring system [9]. On the other hand, some remarkable quinoline alkaloids, such as quinine [10] and camptothecin [11], well known for their antimalarial and anticancer properties, have been established to arise by rearrangement of monoterpenoid indole alkaloids. Although few compounds of the latter type have been reported, they seem to show a high probability of having drug-like activity compared with other natural products.

The development of inflammatory diseases may be accompanied by increased production of leukotrienes and prostaglandins from arachidonic acid. COX-1 and COX-2 are responsible for the production of prostaglandins and LOX for leukotrienes. Inhibition of cyclooxygenase by non-steroidal anti-inflammatory drugs and selective COX-2 inhibitors reduces the levels of prostaglandins, resulting in a reduction in pain and inflammation. However, this inhibition can cause alternative processing of arachidonic acid *via* the 5-lipoxygenase (5-LOX) pathway, resulting in increased production of pro-inflammatory and gastrotoxic leukotrienes. The dual inhibitors of COX and 5-LOX decrease the production of both leukotrienes and prostaglandins, and as such, they should theoretically display enhanced anti-inflammatory effects and decreased cardiovascular side effects caused by some selective COX-2 inhibitors, such as rofecoxib

Table 2. COX-1 and -2, and 5-LOX inhibitory effects of compounds **1** and **2**[a].

Compound	COX-1 inhibition (%)	COX-2 inhibition (%)	5-LOX inhibition (%)
1	83.3	95.0	4.8
2	53.2	96.7	80.6
SC-560	61.3		
NS-398		97.1	
Zileuton			83.1

[a] Concentration of all compounds and reference drugs was 100 μM.

and valdecoxib. Thus, the development of new skeletons which show dual inhibition of COX and 5-LOX currently attracts much attention [12]. COX-1 and -2, and 5-LOX inhibitory effects for compounds **1** and **2** were assayed. Compound **1** showed inhibition to both COX-1 and -2 isoforms. To our interest, compound **2** selectively inhibited the inducible COX-2 to a much greater extent than COX-1, and also markedly inhibited 5-LOX, comparable to positive controls (Table 2). Because of the limited amount of the two alkaloids available, they were tested at a single concentration, 100 μM

Experimental Section

General

Optical rotations were measured with a Horiba SEAP-300 spectropolarimeter. UV spectra were recorded on a Shimadzu double-beam 210A spectrophotometer. IR (KBr) spectra were obtained on a Bio-Rac. FTS-135 infrared spectrophotometer. ^{1}H, ^{13}C and 2D NMR spectra were recorded on AM-400 and DRX-500 MHz NMR spectrometers with TMS as internal standard. MS data were obtained on API Qstar Pulsar I and Finnigan LCQ Advantage instruments. Silica gel (200 – 300 mesh) for column chromatography and GF_{254} for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, People's Republic of China, and sprayed with Dragdorff's reagent. C18 silica gel (20 – 45 μm) was bought from Fuji Chemical Ltd. HPLC was performed using Waters 600 pumps coupled with a Waters 2996 photodiode array detector and a Waters fraction collector II. All enzymes (COX-1 or COX-2 and 5-LOX) were purchased from Sigma-Aldrich Company Ltd.

Plant material

The leaves of *A. scholaris* (L.) R. Br. were collected in April 2006 in Simao of Yunnan Province, People's Republic of China, and identified by Dr. C. X. Zeng, Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, The Chinese Academy of Sciences. A voucher specimen (Luo20060407) has been deposited in the herbarium of Kunming Institute of Botany, The Chinese Academy of Sciences (KUN).

Extraction and isolation

The dried and powdered leaves of *A. scholaris* (10 kg) were extracted with EtOH (60 L × 3) under reflux conditions, and the solvent evaporated *in vacuo*. The residue was dissolved in 1 % aqueous HCl, and the solution was subsequently basified using dilute aqueous ammonia to pH = 9 – 10. The basic solution was partitioned with EtOAc, affording an aqueous phase and an EtOAc/organic phase. The EtOAc fraction (105 g) was collected, redissolved in MeOH, and subjected to column chromatography on silica gel, eluting with $CHCl_3$-Me_2CO (from $CHCl_3$ to $CHCl_3$-Me_2CO (1 : 1)) to afford five fractions (I – V). Fraction II (4 g) was further chromatographed on RP-18 silica gel eluting with 70 % aqueous methanol to afford subfraction II-1. Subfraction II-1 was separated by silica gel once again using petroleum ether-Me_2CO (from 9 : 1 to 4 : 1) to give compounds **1** (6 mg) and **2** (4 mg).

Assay for COX-1 and -2, and 5-LOX inhibitory effects

The anti-inflammatory activity was tested according to the literature with minor modifications [13]. Briefly, the reaction system was incubated for 5 min at 25 °C before sequential addition of the buffer, heme, test compounds, and COX-1 or COX-2 into the system followed by mixing with TMPD and arachidonic acid, and soft agitation for several seconds. The absorbance value was recorded at a wavelength of 590 nm after another 15 min of incubation at 25 °C. The performance of the assay was checked using SC-560 and NS-398 as positive controls, which caused inhibition of COX-1 (61.3 %) and COX-2 (97.1 %), respectively. Different from the method described above, the reaction system was added to assay buffer with 5-LOX in the presence of the colorimetric substrate and test compounds and then incubated for a period of 5 min at 25 °C. After the completion of the reaction, the chromogen was added, and the plate was shaken softly for a few seconds. A further 5 min incubation was performed at 25 °C. The inhibitory effect against 5-LOX was determined by measuring the absorbance at a wavelength of 500 nm. The performance of the assay was checked using zileuton as a positive control, which inhibited 5-LOX activity by 83.1 %.

Scholarisine I (***1***): Colorless powder. – UV (MeOH): λ_{max} = 222, 267 nm. – IR (KBr): ν = 2928, 1766, 1600, 1575 cm^{-1}. – MS ((+)-ESI): m/z = 264 $[M+H]^+$. – HRMS ((+)ESI: m/z = 264.1025 (calcd. 264.1024 for $C_{17}H_{14}NO_2$, $[M+H]^+$). – ^{1}H NMR (400 MHz, $CDCl_3$), and ^{13}C NMR (100 MHz, $CDCl_3$) spectroscopic data: see Table 1.

Scholarisine II (***2***): Colorless powder. – $[\alpha]_D^{26}$ = −1.1 (c = 0.20, MeOH). – UV (MeOH): λ_{max} = 214, 255 nm. – IR (KBr): ν = 3432, 2931, 1765, 1623, 1572 cm^{-1}. – MS((+)-FAB): m/z = 282 $[M+H]^+$. – HRMS ((+)ESI: m/z = 282.1135 (calcd. 282.1130 for $C_{17}H_{16}NO_3$, $[M+H]^+$). –

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data: see Table 1.

Acknowledgement

This work was partial supported by the 973 Program of Ministry of Science and Technology of P. R. China (2009CB522300), the National Natural Science Foundation of China (30800091), the Natural Science Foundation of Yunnan Province (2007C085M), the Chinese Academy of Science (KZCX2-XB2-15, KSCX2-YW-R-202), and the Fund of State Key Laboratory of Phytochemistry and Plant Resources in West China (P2008-ZZ24).

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