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Antibacterial monoterpenoid indole alkaloids from *Alstonia scholaris* cultivated in temperate zone

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ABSTRACT

Three new monoterpenoid indole alkaloids, named normavacurine-21-one (**1**), 5-hydroxy-19, 20-*E*-alschomine (**2**), and 5-hydroxy-19, 20-*Z*-alschomine (**3**), together with thirteen known indole alkaloids (**4**–**16**) were isolated from the leaves of *Alstonia scholaris* cultivated in Kunming. Their structures were elucidated on the basis of extensive spectroscopic analysis, as well as by comparison with the reported spectroscopic data. The leaves of *A. scholaris* cultivated in Kunming, contained picrinine-type alkaloids, scholaricin-type alkaloids and nareline as major alkaloids. New compounds **1**–**3** might be derived from a common biogenetic precursor **(5)**. Compounds **1**, **5** and **10** exhibited significant antibacterial activity against *Enterococcus faecalis*, and **3**, **9** and **14** against *Pseudomonas aeruginosa* with an MIC value of 0.781 μg/mL, while **14** showed moderate activity against *Klepsiella pneumonia* with an MIC value of 1.56 μg/mL.

1. Introduction

Alstonia scholaris (Apocynaceae), is widely distributed in the tropical regions of Africa and Asia [1]. It has also been planted as shade tree in Pu'er, Nanning, Guangzhou, Shenzhen and some other tropical and subtropical regions, P. R. China. Kunming located at temperate zone, is known as the "City of Eternal Spring", the monthly 24-hour average temperature ranges from 8.1 °C (46.6 °F) in January to 19.9 °C (67.8 °F) in June, and temperature has seldom exceeded 30 °C (86 °F) [2]. Interestingly, *A. scholaris* has been cultivated successfully in Kunming Botanic Garden in recent years. The different parts of the plant exhibited anticancer [3] and antibacterial [4] activities. In addition, the leaves of *A. scholaris* induced pronounced bronchodilatory activity in aesthetized rats [5] and the crude extract of its leaves has also been prescribed in hospitals and sold over the counter in drug stores [6]. In our previous phytochemical studies, a series of monoterpenoid indole alkaloids were isolated from different parts of plant [7–9]. Besides, alkaloids from leaf of *A. scholaris* cultivated in Pu'er, indicated anti-tussive, anti-asthmatic, anti-inflammatory, analgesic and expectorant activities both in vitro and in vivo [10–13]. The remarkable difference of the alkaloidal patterns in *A. scholaris* in different ecological environments has been reported in 1990 [14]. Moreover, we found that plant secondary metabolites would be influenced significantly by the ecological environment [15]. Then, we investigated HPLC fingerprints of alkaloids of *A. scholaris* from Pu'er and Kunming, and the result exhibited visible difference, which encouraged us to search for structurally unique and biologically active indole alkaloids of *A. scholaris* from different climate zones. As a result, three new monoterpenoid indole alkaloids, normavacurine-21-one (**1**), 5-hydroxy-19, 20-*E*-alschomine (**2**), and 5-hydroxy-19, 20-*Z*-alschomine (**3**) (See Fig. 1), together with thirteen known monoterpenoid indole alkaloids (**4**–**16**), were isolated. All the compounds except **8** were evaluated for their antibacterial activities against five bacterial strains. In this paper, we report the isolation, structure elucidation and antibacterial activity of the compounds from *A. scholaris* cultivated in Kunming.

2. Experiment part

2.1. General experimental procedures

Optical rotations were measured with a JASCO P-1020 digital polarimeter. UV spectra were obtained using a Shimadzu UV-2401 PC spectraphotometer. IR spectra were recorded on a Bruker Tensor-27 infrared spectrophotometer using KBr pellets. 1D and 2D NMR spectra were performed on Bruker AM-400 and DRX-600 spectrometers (Bruker BioSpin GmBH, Rheinstetten, Germany) with TMS as the internal standard. ESIMS spectra were recorded on a Bruker HTC/Esquire spectrometer. HREIMS spectra was recorded on a Waters AutoSpec Premier P776 spectrometer. Semi-HPLC was performed on an Agilent 1100 HPLC with a ZORBAX SB-C_{18} (9.4 × 250 mm). Column Chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), RP-18 (40–63 μm, Merk). Fractions were monitored by TLC (GF254, Qingdao Marine Chemical Ltd., Qingdao, China), and spots were visualized by Dragendorff's reagent and 10%

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Table 1
^{1}H NMR data for compounds **1–3** (600 MHz, δ in ppm, J in Hz).

No.	1	2	3
3	5.03, (t, 6.8)	4.39, (t, 3.1)	4.57, (br, s)
5	4.70, (m), 3.60, (m)	5.28, (t, 5.7)	5.30, (br, s)
6	3.17, (m); 3.01, (m)	3.18, (m); 2.22, (m)	3.23, (m); 1.99, (m)
9	7.38, (d, 7.8)	7.08, (d, 7.4)	7.24, (d, 7.4)
10	7.00, (t, 7.8)	6.83, (t, 7.4)	6.79, (t, 7.4)
11	7.06, (t, 8.2)	7.14, (t, 7.8)	7.11, (t, 7.8)
12	7.25, (d, 8.2)	6.76, (d, 7.8)	6.73, (d, 7.8)
14	2.26, (m)	2.23, (m); 2.44, (m)	2.25, (m); 3.31, (m)
15	3.81, (m)	3.35, (m)	3.14, (br, s)
16	4.01, (m)	2.64, (d, 3.8)	2.62, (br, s)
17	4.72 (m), 4.03 (m)		
18	1.67, (d, 6.8)	1.79, (d, 7.5)	1.87, (d, 7.0)
19	5.43, (q, 6.8)	6.03, (q, 7.5)	5.72, (q, 7.0)
21		7.60 (s)	7.93 (s)
OCH_3		3.70 (s)	3.74 (s)

H_2SO_4 in ethanol. OD values were measured by Multiskan GO (Thermo Fisher Scientific, USA).

2.2. Plant material

Air-dried leaves of *A. scholaris* were collected in January 2014 from Kunming Botanic Garden, Yunnan province, PR China. The plant materials were identified by Dr. Ya-Ping Liu, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 20140125) has been deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried leaves of *A. scholaris* (2.5 kg) were powdered and extracted with MeOH (6 L × 3, 24 h each) under reflux conditions, and the solvent was evaporated in vacuum. The residue dissolved in 0.37% HCl, and the solution was subsequently basified using 10% ammonia to pH 9–10. The basic solution was partitioned with EtOAc, affording a two-phase mixture including the aqueous phase and the EtOAc/organic phase. The organic fraction (23 g) was collected and then dissolved in MeOH, and the resulting solution was subjected to column chromatography over silica gel eluting with $CHCl_3$/MeOH (from 1:0–0:1) to afford five fractions (Fr. A–D). Fr. A (7.2 g) was subjected to RP-18 column chromatography (MeOH/H_2O 35:1, 50:1, 70:1, 90:1, 0:1) and silica gel column chromatography ($CHCl_3$/MeOH 95:5–10:1) to yield compounds **4** (950 mg), **6** (11 mg) and subfraction A1. Subfraction A1 was chromatographed over silica gel column chromatography (CHCl3/MeOH, 50:1–10:1) and then chromatographed over Sephadex LH-20 column chromatography (MeOH) to afford **5** (5 mg), **7** (9 mg), **8** (1 mg) and **9** (12 mg). Compounds **3** (58 mg), **11** (18 mg), **14** (117 mg), and **15** (7 mg) were obtained from Fr. B (3.4 g) by using silica gel column chromatography eluted with $CHCl_3$/MeOH (from 39:1 to 10:1). Fr. C (2.5 g) was subjected to RP-18 column chromatography (MeOH/H_2O 50:1, 70:1, 0:1) and further resolved by HPLC to yield compounds **2** (20 mg), **10** (5 mg), **12** (40 mg), and **13** (12 mg). Fr. D (5.3 g) was subjected to RP-18 column chromatography (MeOH/H_2O 10:1, 40:1, 0:1) and further resolved by HPLC to yield compounds **1** (15 mg) and **16** (9 mg).

Normavacurine-21-one (**1**): white powder; [α]21 *D* −137.9 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 219.5 (4.39), 271.0 (3.68); IR (KBr) ν_{max} 3441 cm^{-1}, 1632 cm^{-1}, 1562 cm^{-1}, 1452 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; positive ESIMS *m/z* 309 $[M + H]^+$; positive HRESIMS *m/z* 309.1596 (calcd for $C_{19}H_{21}N_2O_2$ $[M + H]^+$, 309.1603).

5-hydroxy-19,20-*E*-alschomine (**2**): white powder; [α]21 D + 108.3 (*c* 0.1, $CDCl_3$); UV ($CDCl_3$) λ_{max} (log ε) 239 (3.85), 289 (4.28) nm; IR (KBr) ν_{max} 3438, 1729, 1593, 1467, 1248 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; positive ESIMS *m/z* 393 $[M + Na]^+$; positive HRESIMS *m/z* 393.1423 (calcd for $C_{20}H_{22}N_2O_5Na$ $[M + Na]^+$, 393.1426).

5-hydroxy-19,20-*Z*-alschomine (**3**): white powder; [α]25 D + 27.9 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.42), 232 (4.01), 290 (4.11) nm; IR (KBr) ν_{max} 3442, 1738, 1632, 1469, 1384 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; positive ESIMS *m/z* 393 $[M + Na]^+$; positive HRESIMS *m/z* 393.1423 (calcd for $C_{20}H_{22}N_2O_5Na$ $[M + Na]^+$, 393.1426).

Table 2
^{13}C NMR data for compounds **1–3** (150 MHz, δ in ppm).

No.	1	2	3
2	131.0 (s)	102.5 (s)	102.6 (s)
3	70.5 (d)	67.8 (d)	68.6 (d)
5	52.3 (t)	100.3 (s)	100.1 (s)
6	18.9 (t)	43.1 (t)	42.6 (t)
7	105.5 (s)	54.8 (s)	54.3 (s)
8	126.9 (s)	135.6 (s)	135.1 (s)
9	119.4 (d)	123.0 (d)	123.8 (d)
10	120.8 (d)	120.7 (d)	120.2 (d)
11	123.6 (d)	128.6 (d)	128.8 (d)
12	112.4 (d)	111.2 (d)	111.1 (d)
13	138.9 (s)	145.1 (s)	145.5 (s)
14	35.3 (t)	29.5 (t)	29.4 (t)
15	43.5 (d)	27.2 (d)	32.6 (d)
16	72.9 (d)	52.9 (d)	54.5 (d)
17	66.7 (t)	171.1 (s)	170.7 (s)
18	14.4 (q)	14.1 (q)	13.6 (q)
19	119.3 (d)	134.8 (d)	131.9 (d)
20	134.2 (s)	129.8 (s)	128.5 (s)
21	169.3 (s)	142.5 (d)	135.9 (d)
OCH_3		51.6 (q)	51.7 (q)

2.4. Antibacterial assays

The antibacterial assay of compounds **1–16** except **8** was evaluated against *Staphylococcus aureus* ATCC 25922, *Klepsiella pneumoniae* ATCC 13883, *Enterococcus faecalis* ATCC 10541, *Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 27853. All the bacteria were obtained from the American Type Culture Collection (Rockville, USA). The antibacterial assay was carried out as described in the literature elsewhere [16]. The preparation of bacterial inocula was done by using 18 h old overnight bacterial cultures prepared in Nutrient Agar. A few colonies of bacteria were collected aseptically with a sterile loop and introduced into 10 ml of sterile 0.90% saline solution. The concentration of the suspension was then standardized by adjusting the optical density to 0.10 at 630 nm, corresponding to bacterial cell suspension of 10^8 colony-forming units/mL (CFU/mL) [17]. This cell suspension was diluted 100 times to obtain 10^6 CFU/mL before use. The compounds were dissolved in DMSO and then added to bacteria suspension to obtain the final concentration of 5% (v/v) DMSO or less. Serial twofold dilutions from 200 μg/mL of the compounds were performed in 96-well microtiter plates. Each well contained 100 μL of sample of each concentration. Into each well was then introduced 100 μL of the bacterial suspension. The final concentration range of the test compounds was 100–0.781 μg/mL, and the plates were incubated at 37 °C for 24 h. After incubation, the wells were examined for growth of microorganisms by measuring optical density (OD) value of the wells. Each experiment was repeated three times and Norfloxacin, bacteria suspension of 5% (v/v) DMSO were used as a positive control and a blank control, respectively. By comparing to OD values, we can point out MIC values of these compounds among the selected concentration range and MIC > 100 μg/mL was considered to be inactive.

3. Results and discussion

Compound **1** was obtained as white powder. Its molecular formula of $C_{19}H_{20}N_2O_2$ was established by ^{13}C NMR and HR-ESI-MS data (*m/z* 309.1596 $[M + H]^+$, calcd. for 309.1603), indicating 11 indices of hydrogen deficiency. The UV spectrum showed the characteristic maximal absorptions of indole alkaloid at 219 and 271 nm [18]. The ^{1}H and