Antimicrobial Alkaloids from the Tubers of *Stephania succifera*

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Chemical investigation of the EtOH extract from the tubers of *Stephania succifera* collected in Hainan Province of China resulted in the isolation of the two new alkaloids Cepharanone D (**1**) and *N*-formyl-asimilobine (**2**), and a known alkaloid, *N*-formyl-annonain (**3**). Their structures were elucidated by spectroscopic techniques (UV, IR, 1D and 2D NMR). The antimicrobial activities of the three compounds were also investigated.

Key words: *Stephania succifera*, Alkaloid, Cepharanone D, *N*-Formyl-asimilobine, Antimicrobial Activity

Introduction

Stephania succifera H. S. Lo et Tsoong (Menispermaceae) is a traditional Li folk medicine hosted in Hainan Province of China, the tuber of which is used in the treatment of sedation, relieving pain, heat-clearing and detoxification [1]. The genus *Stephania* is comprised of more than sixty species in the world, among which thirty-nine species are distributed in China, and most of them are folk medicine. Domestic and international phytochemical studies on the plants of this genus indicated that the main chemical constituents were isoquinoline alkaloids [2, 3], which show several pharmacological activities such as antitumor, anti-malarial, analgesia, and antihypertensive properties [4 – 6]. The main chemical constituents of *S. succifera* are aporphine alkaloids and berberine alkaloids [7, 8]. In our screening for antimicrobial agents from Li folk medicine in Hainan Province, the ethanol extract from the tubers of *S. succifera* showed antimicrobial activities. The solvent-solvent partition of the ethanol extract combined with a bioassay revealed that the petroleum ether extract, the ethyl acetate extract, and the water extract from *S. succifera* exhibited a strong inhibitory effect on *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Further bioassay-guided fractionation of the ethyl acetate extract led to the isolation of two new alkaloids, an aristolactam derivative, Cepharanone-D (**1**), an *N*-formyl-aporphine, *N*-formyl-asimilobine (**2**), and a known alkaloid, *N*-formyl-annonain (**3**) (Fig. 1). The present paper discusses their structure elucidation and antimicrobial activities.

* Equally contributed

Results and Discussion

Compound **1** was obtained as a yellowish powder and gave a positive reaction to Dragendorff's reagent. The high-resolution electrospray ionization mass spectrum (HR-ESI-MS) of **1** showed a molecular ion peak at *m/z* = 324.0862 (calcd. 324.0871 for $C_{18}H_{14}NO_5$, $[M+H]^+$), indicating a molecular formula of $C_{18}H_{13}NO_5$, which was supported by ^{13}C NMR and DEPT data (Table 1). The UV absorptions of **1** at 216, 240, 262 (sh), 288, 340 (sh), 376, and 396 nm were consistent with a typical aristolactam derivative [9]. The ^{13}C NMR (DEPT) spectra of **1** showed the presence of fourteen aromatic carbons (δ_C = 99.5 – 151.0), a carbonyl (δ_C = 170.1), a methylenedioxy (δ_C = 102.7), and two methoxyl (δ_C = 60.9 and 56.0) groups. The ^1H NMR spectrum of **1** exhibited signals attributable to a methylenedioxy, and two methoxyl groups at δ_H = 6.25 (s, 2H), 3.92 (s, 3H) and 3.88 (s, 3H), respectively. A set of *ortho* coupled aromatic protons appeared at δ_H = 8.24 and 7.12 (d, J = 8.9 Hz, each 1H). The lower field signal is the characteristic

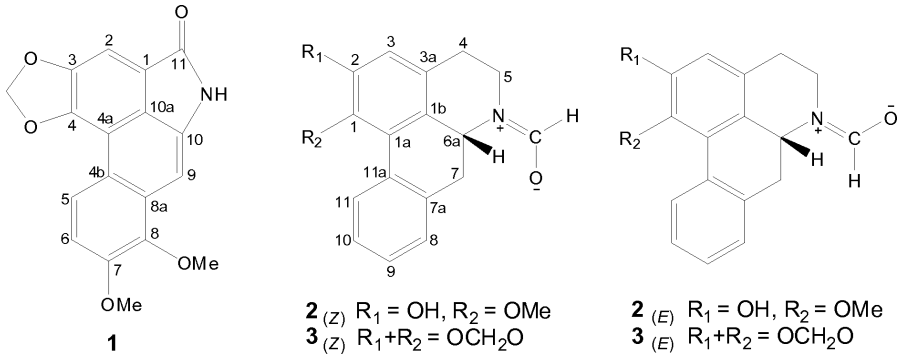

Fig. 1. Structures of compounds **1**–**3**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compound **1** (in $CDCl_3$ + CD_3OD).

Position	δ_C	δ_H (*J* in Hz)	HMBC (H to C)
1	119.0 (s)		
2	104.7 (d)	7.42 (s)	C-3, C-4, C-10a, C-11
3	148.9 (s)		
4	146.9 (s)		
4a	111.9 (s)		
4b	119.6 (s)		
5	111.2 (d)	8.24 (d, 8.9)	C-4a, C-7, C-8a
6	123.3 (d)	7.12 (d, 8.9)	C-4b, C-7, C-8
7	151.0 (s)		
8	144.4 (s)		
8a	129.2 (s)		
9	99.5 (d)	7.31 (s)	C-4b, C-8, C-10, C-10a
10	134.8 (s)		
10a	125.3 (s)		
11	170.1 (s)		
OCH_2O	102.7 (t)	6.25 (s)	C-3, C-4
7-OMe	56.0 (q)	3.92 (s)	C-7
8-OMe	60.9 (q)	3.88 (s)	C-8

signal of H-5 in an aristolactam derivative. Two singlet signals at δ_H = 7.42 (s, 1H) and δ_H = 7.31 (s, 1H) were assigned to H-2 and H-9, respectively. By comparison of the ^{1}H NMR data of **1** with those of cepharanone C [9], the presence of an additional methoxyl group at δ_H = 3.88 (s, 3H) at higher field indicated that C-8 was substituted by a methoxyl group in compound **1**. This substitution was confirmed by the HMBC experiments (Fig. 2), which showed the correlations of 7-OMe (δ_H = 3.92) to C-7 (δ_C = 151.0), and of 8-OMe (δ_H = 3.88) to C-8 (δ_C = 144.4) (Fig. 2). On the basis of the above results, the structure of compound **1** was elucidated and named cepharanone D.

Compound **2** was obtained as colorless crystals and showed a positive Dragendorff test. Its molecular formula was determined to be $C_{18}H_{17}NO_3$ based on the HR-ESI-MS data (*m/z* = 296.1280, calcd. 296.1287 for $C_{18}H_{18}NO_3$, $[M+H]^+$). The UV absorptions at 232

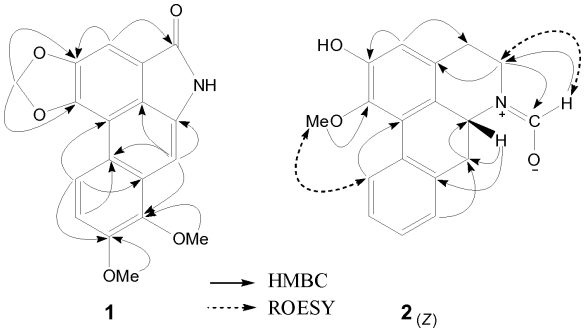

Fig. 2. Key correlations for compounds **1** and **2** $_{(Z)}$.

(sh), 272, and 308 nm were characteristic of a 1,2-disubstituted aporphine [10]. A peak at 1654 cm^{-1} in the IR spectrum was suggestive of a carbonyl group. The ^{1}H NMR spectrum of **2** was complex, arising from the resonances of two rotational isomers that occur due to restricted rotation about the *N*-formyl group. Amidic aporphines, either *N*-formyl or *N*-acetyl, always exist as a mixture of enolates, as clearly demonstrated by NMR studies [11]. Two separate sets of signals in the ratio of 2 : 1 indicated slow exchange on the NMR time scale between the two rotational isomers in solution. The ^{1}H NMR data of the major component showed a typical resonance of an *N*-formyl proton at δ_H = 8.26 (s, 1H), an AA′BB′ aromatic system at δ_H = 7.27 (td, *J* = 8.0, 1.2 Hz, 1H, H-9), 7.30 (d, *J* = 7.3 Hz, 1H, H-8), and 7.33 (td, *J* = 8.0, 1.2 Hz, 1H, H-10) and a typical downfield-shifted proton at δ_H = 8.32 (d, *J* = 8.2 Hz, 1H, H-11) in ring D, a methoxyl group at δ_H = 3.57 (s, 3H), and a singlet signal at δ_H = 6.74 (s, 1H, H-3). A signal at δ_H = 4.89, assigned to H-6a of the major isomer, was coupled to signals at δ_H = 3.15 (dd, *J* = 4.1 Hz, 1H) and 2.78 (d, *J* = 13.8 Hz, 1H); these coupling constants were compatible with axial-equatorial and axial-axial relationships, respectively. Therefore,

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compound **2** (in $CDCl_3$).

Position	**2** $_{(Z)}$ δ_C	δ_H (J in Hz)	**2** $_{(E)}$ δ_C	δ_H (J in Hz)
1	143.6 (s)		143.4 (s)	
1a	126.7 (s)		126.3 (s)	
1b	124.8 (s)		124.3 (s)	
2	148.6 (s)		148.8 (s)	
3	114.1 (d)	6.74 (s)	114.6 (d)	6.77 (s)
3a	129.8 (s)		130.8 (s)	
4ax	30.8 (t)	2.88 (td, 15.4, 4.6)	29.4 (t)	2.76 (overlapped)
4eq		2.74 (dt, 15.3, 2.6)		
5ax	42.2 (t)	3.40 (td, 12.6, 2.8)	36.3 (t)	3.20 (ddd, 12.6, 10.0, 4.0)
5eq		3.82 (ddd, 12.8, 4.6, 2.0)		4.38 (td, 12.6, 4.1)
6a	49.5 (d)	4.89 (dd, 13.8, 4.1)	53.4 (d)	4.48 (dd, 14.3, 4.1)
7ax	34.0 (t)	2.78 (d, 13.8)	37.8 (t)	3.14 (overlapped)
7eq		3.15 (dd, 13.6, 4.1)		2.82 (overlapped)
7a	136.1 (s)		135.4 (s)	
8	128.8 (d)	7.30 (d, 7.3)	128.4 (d)	7.30 (overlapped)
9	128.1 (d)	7.27 (td, 8.0, 1.2)	128.1 (d)	7.24 (td, 8.0, 1.3)
10	127.3 (d)	7.33 (td, 8.0, 1.2)	127.6 (d)	7.37 (m)
11	127.4 (d)	8.32 (d, 8.2)	127.8 (d)	8.34 (d, 8.1)
11a	131.1 (s)		131.3 (s)	
CHO	162.2 (d)	8.26 (s)	162.0 (d)	8.38 (s)
1-OMe	60.2 (q)	3.57 (s)	60.3 (q)	3.57 (s)

H-6a was axially disposed, and the H-7 axial proton was identified at δ_H = 2.78. The proton at δ_H = 2.74 for H-4eq was correlated with the proton at δ_H = 2.88 for H-4ax, and δ_H = 3.82 for H-5eq was correlated with the proton at δ_H = 3.40 for H-5ax. All the protons could be assigned clearly from examination of the data and comparison with the literature [11, 12], as well as by correlations from the HMQC and HMBC spectra (Fig. 2). The comparison of the NMR data of the major isomer in compound **2** with those of **3** [12] showed that compounds **2** and **3** were similar except for the presence of an additional methoxyl group at δ_H = 3.57 (s, 3H) in the ^{1}H NMR spectrum, and at δ_C = 60.3 in the ^{13}C NMR spectrum, and the absence of a methylenedioxy group at δ_C = 101.0, indicating that C-1 and C-2 were substituted by a methoxyl and a hydroxyl group in compound **2** instead of a methylenedioxy group in compound **3**. The relative substitution of methoxyl and hydroxyl groups in **2** were deduced from the ROESY experiment, which showed the correlation of 1-OMe (δ_H = 3.57) to H-11 (δ_H = 8.32) (Fig. 2). The correlation of H-5 (δ_H = 3.40) to the *N*-formyl proton (δ_H = 8.26, s) in the ROESY spectrum indicated a *Z*-form *N*-formyl-aporphine (Fig. 2). Thus, the structure of the major component of **2** was elucidated. In a similar way, the structure of the minor component was characterized as the *E*-form of *N*-formyl-aporphine. Complete assignments (Table 2) for the major isomer and the minor component were made through 2D NMR experiments. Therefore, compound **2**, a mixture of two isomers, was elucidated as a new compound and named *N*-formyl-asimilobine.

The known alkaloid, *N*-formyl-annonain (**3**), was characterized by comparison of the spectroscopic data (UV, IR, NMR and MS) with literature values [12].

The antimicrobial tests for compounds **1** – **3** demonstrated that compound **1** had inhibitory effects on a *Staphylococcus aureus* strain, with a diameter of the inhibition zone of 14.0 mm, and compound **3** possessed both inhibitory effects on *Staphylococcus aureus* and MRSA strains, with diameters of the inhibition zones of 18.0 mm and 16.0 mm, respectively, while compound **2** was inactive. The diameter of the inhibition zone of the positive control, kanamycin sulfate, was 34.0 mm.

Experimental Section

General

The NMR spectra were recorded on a Bruker AV-400 spectrometer, using TMS as an internal standard. The HR-ESI-MS spectra were measured with an API QSTAR Pulsar mass spectrometer. The IR spectra were obtained on a Nicolet 380 FT-IR instrument from KBr pellets. The UV spectra were measured on a Shimadzu UV-2550 spectrometer. Optical rotation was recorded using a Rudolph Autopol III polarimeter (USA). Melting points were obtained on a Beijing Taike X-5 stage apparatus and are uncorrected. Column chromatography was performed with silica gel (Marine

Chemical Industry Factory, Qingdao, China) and Sephadex LH-20 (Merck, Germany). TLC was preformed with silica gel GF254 (Marine Chemical Industry Factory, Qingdao, China).

Plant material

The tuber of *S. succifera* was collected in Wuzhishan county, Hainan Province, China (April, 2008). The specimen was identified by Associate Professor Zhu-nian Wang of the Tropical Crops Genetic and Resources Institute, Chinese Academy of Tropical Agricultural Sciences. A voucher specimen (No. 20080412) of *S. succifera* was deposited in the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences.

Extraction and isolation

The dried and crushed tubers (16.0 kg) of *S. succifera* were extracted three times with 95 % EtOH at r. t. The combined extracts were concentrated under reduced pressure to yield a dark-brown syrup, which was suspended in H_2O, and then partitioned successively with petroleum ether and EtOAc. The EtOAc fraction (90.3 g), which showed inhibitory activity against *S. aureus* and MRSA, was subjected to column chromatography on silica gel with a gradient solvent system of first petrol-acetone (0:1 – 5:1, v/v) and then $CHCl_3$-MeOH (0:1 – 0:1, v/v) to afford 10 fractions. Fraction 8 was submitted to repeated column chromatography on silica gel and Sephadex LH-20 and recrystallized finally to obtain compound **1** (25.5 mg). Fraction 6 was repeatedly chromatographed on a silica gel and a Sephadex LH-20 column and recrystallized and yielded compounds **2** (5.6 mg) and **3** (2.8 mg).

*Cepharanone-D (**1**)*

Yellowish needle-shaped crystals. – M. p. 234 – 237 °C. – $[\alpha]_D^{24} = 137°$ ($c = 1.0$, $CHCl_3$). – UV (MeOH): λ (log ε_{max}) = 216 (2.79), 240 (3.31), 262 (2.21), 288 (3.35), 340 (0.92), 376 (0.85), 396 (0.83) nm. – IR (KBr): ν = 3940, 3915, 3866, 3762, 3690, 3654, 2946, 2553, 2362, 1694, 1658, 1560, 1481, 1422, 1360, 1279, 1171, 1094, 1048, 940 (OCH_2O) cm^{-1}. – HRMS ((+)-ESI): *m/z* = 324.0862 (calcd. 324.0871 for $C_{18}H_{14}NO_5$, $[M+H]^+$). – ^1H and ^{13}C NMR: see Table 1.

*N-Formyl-asimilobine (**2**)*

Colorless crystals. – M. p. 233 – 236 °C. – $[\alpha]_D^{24} = -518°$ ($c = 0.5$, $CHCl_3$). – UV (MeOH): λ (log ε_{max}) = 216 (2.78), 232 (2.15), 272 (1.92), 308 (0.96) nm. – IR (KBr): ν = 3851, 3748, 3673, 3646, 3568, 3511, 3215, 2363, 1653, 1425, 406 cm^{-1}. – HRMS ((+)-ESI): *m/z* = 296.1280 (calcd. 296.1287 for $C_{18}H_{18}NO_3$, $[M+H]^+$). – ^1H and ^{13}C NMR: see Table 2.

*N-Formyl-annonain (**3**)*

Colorless crystals. – M. p. 244 – 245 °C. – $[\alpha]_D^{24} = -367°$ ($c = 0.1$, $CHCl_3$). – UV (EtOH): λ (log ε_{max}) = 238 (4.15), 274 (4.22), 292 (sh), 313 (3.60) nm. – IR (KBr): ν = 2870, 1655, 1565, 1490, 1425, 1395, 1280, 1255, 1220, 1200, 1180, 1145, 1075, 1040, 935, 910, 850, 780, 740, 730, 640 cm^{-1}.

Antimicrobial activity

These compounds were tested for *in vitro* antimicrobial activity against *Staphylococcus aureus* and MRSA strains (obtained from Professor Kui Hong of the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences) by the filter paper disc agar diffusion method [13]. The strains were cultured using Nutrient Agar (NA). 50 μL 10 mg mL^{-1} of compounds **1** – **3** were impregnated on sterile filter paper discs (6 mm diameter) and then applied aseptically to the surface of the agar plates. 10 μL 0.08 mg mL^{-1} kanamycin sulfate was used as positive control. As an expression of the antimicrobial activities, the diameters of the inhibition zones including the 6 mm disc diameter were measured after 24 h of incubation at r. t. Experiments were done in triplicate, and the results were presented as mean values.

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