A New Cytotoxic Carbazole Alkaloid and Two New Other Alkaloids from *Clausena excavata*

by **Wen-Wen Peng**[a])[b]), **Guang-Zhi Zeng***[a]), **Wei-Wu Song**[a])[b]), and **Ning-Hua Tan***[a])

[a]) State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China (phone: +86-871-65223800; fax: +86-871-65223800; email: nhtan@mail.kib.ac.cn, gzh_zeng@mail.kib.ac.cn)

[b]) University of Chinese Academy of Sciences, Beijing 100049, P. R. China

One new carbazole alkaloid, excavatine A (**1**), and two additional new alkaloids, excavatine B (**2**) and excavatine C (**3**), were isolated from the stems and leaves of *Clausena excavata* BURM.f. (Rutaceae). Their structures were determined on the basis of detailed spectroscopic analyses, especially 2D-NMR and HR-EI-MS data. Compounds **1**–**3** were tested for their cytotoxic activities against A549, HeLa, and BGC-823 cancer cell lines, and for their antimicrobial activities against *Candida albicans* and *Staphylococcus aureus*. Only **1** exhibited cytotoxicity against A549 and HeLa cell lines with the IC_{50} values of 5.25 and 1.91 μg/ml, respectively.

Introduction. – *Clausena excavata* BURM.f. (Rutaceae), mainly growing in Xishuangbanna, Yunnan Province, P. R. China, has been used as a folk medicine for the treatment of dysentery, enteritis, and urethra infection in China [1][2]. Carbazole alkaloids [3–5] and coumarins [6–12] are known as the main chemical constituents of this plant. Being interested in alkaloids, the constituents of the stems and leaves of *C. excavata* collected in Xishuangbanna have been studied. Here, we report the isolation and structure elucidation of three new alkaloids **1**–**3** (*Fig. 1*), together with their cytotoxic and antimicrobial activities.

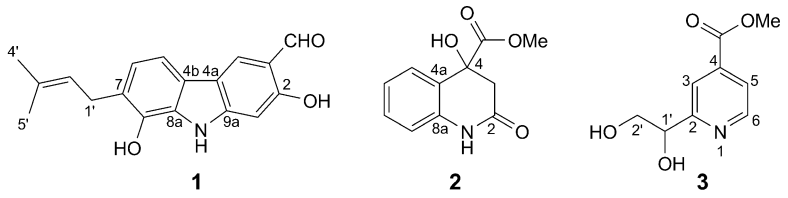


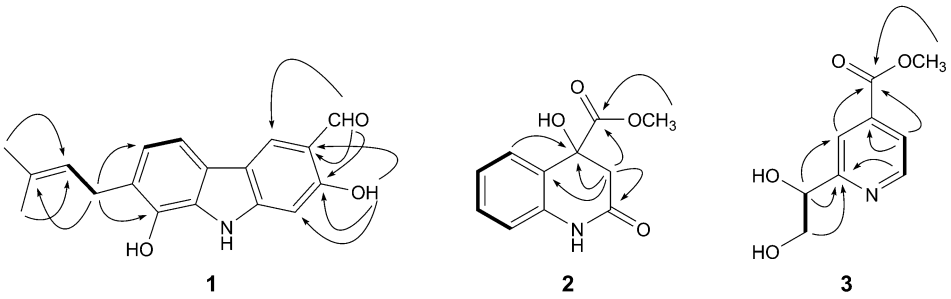
Fig. 1. *Structures of compounds* **1**–**3**

Results and Discussion. – Excavatine A (**1**) was obtained as pale-yellow powder. Its molecular formula $C_{18}H_{17}NO_3$ was determined on the basis of the HR-EI-MS (*m*/*z* 295.1213; calc. 295.1208), implying eleven degrees of unsaturation. The IR spectrum indicated the presence of the OH and NH groups (3421 and 3398 cm^{-1}), and of a conjugated C=O group (1631 cm^{-1}). The ^{1}H-NMR spectrum (*Table 1*) of **1** exhibited signals of one OH H-atom with the H-bond (δ(H) 11.44), one CHO H-atom (δ(H)

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** *at 400 and 100 MHz, Respectively, in (D_6)Acetone.* δ in ppm, J in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
1	6.91 (*s*, 1 H)	97.5 (*d*)	8a		132.2 (*s*)
2		161.5 (*s*)	9a		146.9 (*s*)
3		116.0 (*s*)	1′	3.50 (*d*, J=7.2, 2 H)	28.9 (*t*)
4	8.29 (*s*, 1 H)	128.1 (*d*)	2′	5.37 (*t*, J=7.2, 1 H)	124.0 (*d*)
4a		119.2 (*s*)	3′		132.5 (*s*)
4b		126.2 (*s*)	4′	1.74 (*s*, 3 H)	25.9 (*q*)
5	7.50 (*d*, J=7.9, 1 H)	112.4 (*d*)	5′	1.71 (*s*, 3 H)	17.9 (*q*)
6	6.97 (*d*, J=7.9, 1 H)	123.1 (*d*)	CHO	9.93 (*s*, 1 H)	196.5 (*d*)
7		123.9 (*s*)	NH	10.60 (*s*, 1 H)	
8		140.7 (*s*)	2-OH	11.44 (*s*, 1 H)	

9.93), and one NH H-atom (δ(H) 10.60). In the same spectrum, two *singlet*s at δ(H) 8.29 and 6.91 were attributed to H–C(4) and H–C(1), respectively and the *doublet*s at δ(H) 7.50 (J=7.9) and 6.97 (J=7.9) were assigned to H–C(5) and H–C(6), respectively. The ^{13}C-NMR spectrum (*Table 1*) of **1** showed 18 C-atom signals, namely those of one CHO group (δ(C) 196.5), two benzene rings (δ(C) 161.5, 146.9, 140.7, 132.2, 128.1, 126.2, 123.9, 123.1, 119.2, 116.0, 112.4, 97.5), and one prenyl group (δ(C) 132.5, 124.0, 28.9, 25.9, 17.9). The above evidence indicated the presence of a 2,3,7,8-tetrasubstituted carbazole skeleton [13][14]. The ^{1}H,^{1}H-COSY (*Fig. 2*) correlation H–(5)/H–C(6) further confirmed the presence of a 7,8-disubstituted carbazole skeleton. In addition, the ^{1}H,^{1}H-COSY correlation H–C(1′)/H–C(2′), and the HMBCs (*Fig. 2*) H–C(1′)/C(3′) and H–C(4′), H–C(5′)/C(2′) evidenced that **1** had a Me_2C=$CHCH_2$– unit. The HMBCs H–C(1′)/C(6), C(7), and C(8) suggested that the Me_2C=$CHCH_2$– moiety was attached to C(7). The HMBCs CHO/C(2), C(3), and C(4), and H–(4)/CHO revealed the location of the CHO group at C(3). The HMBCs 2-OH/C(1), C(2), and C(3) indicated one OH group at C(2). Thus, the structure of **1** was deduced as shown in *Fig. 1*.



1 **2** **3**

Fig. 2. *Key ^{1}H,^{1}H-COSY* (—) *and HMB* (H→C) *correlations of compounds* **1**–**3**

Excavatine B (**2**) was obtained as pale-yellow oil and had a molecular formula $C_{11}H_{11}NO_4$ as determined by HR-EI-MS (*m*/*z* 221.0690; calc. 221.0688), suggesting seven degrees of unsaturation. The IR spectrum displayed absorptions at 3419 and 1729 cm^{-1}, indicating the presence of OH and C=O groups, respectively. Analyses of

the ^{1}H-NMR spectrum (*Table 2*) of **2** revealed the presence of a 1,2-disubstituted benzene ring (δ(H) 7.35 (*d*, $J=7.5$, H–C(5)); 7.02, (*t*-like, $J=7.5$, H–C(6)); 7.25 (*t*-like, $J=7.5$, H–C(7)); 6.88 (*d*, $J=7.5$, H–C(8))), and one MeO (δ(H) 3.47 (*s*)) and one CH_2 group (δ(H) 3.06 (*d*, $J=1.5$, CH_2(3))). Its ^{13}C-NMR spectrum (*Table 2*) exhibited characteristic signals of two C=O C-atoms (δ(C) 179.6, 169.8), of one benzene ring (δ(C) 142.3, 130.4, 129.7, 123.9, 122.3, 110.0), of one quaternary C-atom (δ(C) 73.5), and of one MeO (δ(C) 50.8), and one CH_2 group (δ(C) 41.3). The ^{1}H,^{1}H-COSY (*Fig. 2*) correlations H–C(5)/H–C(6), H–C(6)/H–C(7), and H–C(7)/H–C(8) were indicative of the presence of a 1,2-disubstituted benzene ring. Pertinent cross-peaks observed in the HMBC spectrum (*Fig. 2*), MeO/COO, and CH_2(3)/C(2), C(4), and COO, allowed the identification of the –C(COOMe)(OH)CH_2CONH– moiety. In addition, the HMBC interactions H–C(5)/C(4) and CH_2(3)/C(4a) confirmed that the –C(COOMe)(OH)CH_2CONH– moiety was attached to C(4a). The remaining unsaturation indicated that NH was attached to C(8a). In contrast to the known compound (–)-(*S*)-4-ethyl-3,4-dihydro-4-methylcarbostyril ($[\alpha]_D^{21.0}=-7.1$) [15], **2** could be racemic ($[\alpha]_D^{26.0}=+0.3$). Therefore, the structure of **2** was established as depicted in *Fig. 1*.

Table 2. *^{1}H- and ^{13}C-NMR Data of* **2** *at 400 and 100 MHz, Respectively, in CD_3OD.* δ in ppm, J in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
2		179.6 (*s*)	7	7.25 (*t*-like, $J=7.5$, 1 H)	129.7 (*d*)
3	3.06 (*d*, $J=1.5$, 2 H)	41.3 (*t*)	8	6.88 (*d*, $J=7.5$, 1 H)	110.0 (*d*)
4		73.5 (*s*)	8a		142.3 (*s*)
4a		130.4 (*s*)	COO		169.8 (*s*)
5	7.35 (*d*, $J=7.5$, 1 H)	123.9 (*d*)	MeO	3.47 (*s*, 3 H)	50.8 (*q*)
6	7.02 (*t*-like, $J=7.5$, 1 H)	122.3 (*d*)			

Excavatine C (**3**) was obtained as pale-yellow oil. It had a molecular formula $C_9H_{11}NO_4$ as deduced from the HR-EI-MS (*m*/*z* 197.0729; calc. 197.0688), implying five degrees of unsaturation. The IR spectrum indicated the presence of OH (3414 cm^{-1}) and C=O (1731 cm^{-1}) groups. The ^{1}H-NMR spectrum (*Table 3*) of **3** exhibited in the low-field three chemical shifts characteristic of one pyridine ring (δ(H) (7.20, *d*, $J=5.1$, H–C(6)); 6.65 (*s*, H–C(3)); 6.34 (*dd*, $J=5.1$, 1.4, H–C(5))), and the resonance of one MeO group (δ(H) 2.49(*s*)). The ^{13}C-NMR spectrum (*Table 3*) of **3** confirmed the presence of the pyridine ring (δ(C) 162.5, 148.7, 138.0, 121.0, 119.9). This spectrum also exhibited signals of one C=O (δ(C) 165.2), one MeO (δ(C) 51.5), one CH_2O (δ(C) 65.7) and one CHOH group (δ(C) 74.2). The ^{1}H,^{1}H-COSY (*Fig. 2*) correlation H–C(1′)/CH_2(2′) revealed the presence of a CH_2(OH)CH(OH)– moiety. The HMBC (*Fig. 2*) interactions of H–C(1′)/C(2) and C(3), and CH_2(2′)/C(2) indicated that the CH_2(OH)CH(OH)– group was attached to C(2). In addition, the HMBCs H–C(3), H–C(5)/COO, H–C(6)/C(4), and MeO/COO revealed that the COOMe group was at C(4). The optical rotation of **3** was determined as –9.3, opposite to the known (*S*)-2-(1,2-dihydroxyethyl)pyridine ($[\alpha]_D^{25}=+80.6$) [16]. However, the measured absolute value is only *ca.*10% of the reference compound. Therefore, we can assume that **3** is racemic (as **2**). So, the structure of **3** was established as shown in *Fig. 1*.

Table 3. *^{1}H- and ^{13}C-NMR Data of* **3** *at 500 and 125 MHz, Respectively, in CD_3OD, δ* in ppm, *J* in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
2		162.5 (*s*)	COO		165.2 (*s*)
3	6.65 (*s*, 1 H)	119.9 (*d*)	1′	3.34–3.36 (*m*, 1 H)	74.2 (*d*)
4		138.0 (*s*)	2′	2.39 (*dd*, *J* = 11.3, 4.1, 1 H),	65.7 (*t*)
5	6.34 (*dd*, *J* = 5.1, 1.4, 1 H)	121.0 (*d*)		2.24 (*dd*, *J* = 11.3, 6.3, 1 H)	
6	7.20 (*d*, *J* = 5.1, 1 H)	148.7 (*d*)	MeO	2.49 (*s*, 3 H)	51.5 (*q*)

Compounds **1**–**3** were tested for their cytotoxic activities against A549, HeLa, and BGC-823 cancer cell lines, and for their antimicrobial activities against *Candida albicans* and *Staphylococcus aureus*. Only **1** exhibited cytotoxicity against A549 and HeLa cell lines with the IC_{50} values of 5.25 and 1.91 μg/ml, respectively.

Experimental Part

General. Column chromatography (CC): silica gel (100–200 and 200–300 mesh, and 10–40 μm, *Qingdao Marine Chemical, Inc.*, China). TLC: precoated silica-gel GF_{254} glass plates (*Qingdao Marine Chemical, Inc.*, China). Semiprep. reversed-phase (RP) HPLC: *Agilent 1100* apparatus equipped with a UV detector and a *YMC-Pack ODS-A* (*YMC*, 1 × 15 cm) column at a flow rate of 2 ml/min. Optical rotations: *Horiba SEPA-300* polarimeter. UV Spectra: *Shimadzu UV-2401 A* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Tensor 27* FT-IR spectrometer with KBr pellets; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra: *Bruker AV-400* (at 400 (^{1}H) and 100 MHz (^{13}C)) or *DRX-500* (at 500 (^{1}H) and 125 MHz (^{13}C)) spectrometers in (D_6)acetone or CD_3OD with Me_4Si as the internal standard ($\delta = 0$ ppm) at r.t. ESI-MS, EI-MS, and HR-EI-MS: *API QSTAR Pular-1* mass spectrometer; *m*/*z* (rel.).

Plant Material. The stems and leaves of *C. excavata* were collected in Xishuangbanna, Yunnan Province, P. R. China, in August 2010, which were identified by Prof. *Yu-Min Shui* of Kunming Institute of Botany. A voucher specimen (No. 2010813) has been deposited with the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried and powdered stems and leaves of *C. excavata* (32 kg) were extracted by refluxing with 95% MeOH (3 × 35 l). The MeOH extract was submitted to the liquid-liquid fractionation with petroleum ether (PE), AcOEt, and BuOH. The AcOEt- soluble fraction (1.1 kg) was subjected to CC (silica gel (100–200 mesh); PE/acetone (10 : 1–0 : 1) to yield five fractions, *Frs. 1–5*. Further separation of *Fr. 4* (34 g) by CC (PE/acetone 5 : 1–1 : 2) gave subfractions *Frs. 4.1–4.6. Fr. 4.4* was purified by CC (PE/acetone 3 : 1) and HPLC (MeOH/H_2O 6 : 4) to yield **2** (13 mg). *Fr. 4.5* was also purified by CC (PE/acetone 2 : 1) and HPLC (MeOH/H_2O 55 : 45) to yield **1** (24 mg) and **3** (4 mg).

Excavatine A (= *2,8-Dihydroxy-7-(3-methylbut-2-en-1-yl)-9*H-*carbazole-3-carbaldehyde*; **1**). Pale-yellow powder. UV (MeOH): 198 (4.07), 221 (4.07), 225 (4.22), 281 (4.24), 357 (3.79). IR: 3421, 3398, 2967, 2923, 2853, 1631, 1468, 1436, 1339, 1328, 1245, 1197, 1166, 1069, 801, 750, 725. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS (pos.): 318 ($[M+Na]^+$). HR-EI-MS: 295.1213 (M^+, $C_{18}H_{17}NO_3^+$; calc. 295.1208).

Excavatine B (= *Methyl 1,2,3,4-Tetrahydro-4-hydroxy-2-oxoquinoline-4-carboxylate*; **2**). Pale-yellow oil. $[\alpha]_D^{26.0} = +0.3$ (*c* = 0.31, MeOH). UV (MeOH): 208 (4.25), 253 (3.60), 289 (3.25). IR: 3419, 3030, 2955, 1729, 1622, 1473, 1439, 1384, 1352, 1337, 1206, 1181, 1113, 1084, 1060, 754. ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS (pos.): 244 ($[M+Na]^+$). HR-EI-MS: 221.0690 (M^+, $C_{11}H_{11}NO_4$; calc. 221.0688).

Excavatine C (= *Methyl 2-(1,2-Dihydroxyethyl)pyridine-4-carboxylate*; **3**). Pale-yellow oil. $[\alpha]_D^{25} = -9.3$ (*c* = 0.15, MeOH). UV (MeOH): 204 (3.90), 280 (3.31). IR: 3414, 2954, 2933, 2878, 1731, 1608, 1564, 1439, 1406, 1385, 1311, 1212, 1119, 1085, 1062, 764, 676. ^{1}H- and ^{13}C-NMR: see *Table 3*. EI-MS: 197 (M^+). HR-EI-MS: 197.0729 (M^+, $C_9H_{11}NO_4^+$; calc. 197.0688).

Cytotoxicity Assay. The cytotoxicities of compounds **1**–**3** against A549, HeLa and BGC-823 cancer cell lines were evaluated by the SRB (= sulforhodamine B) assay [17]. The cells were cultured in RPMI

1640 medium (*Sigma*). Aliquots of 90 µl were seeded in 96-well flat-bottomed microtiter plates for 24 h and then treated with serial dilutions of compounds **1**–**3** with the maximum concentration of 20 µg/ml. Each compound was initially dissolved in DMSO and further diluted with the medium to produce different concentrations. After incubation at 37° and 5% CO_2 for 48 h, cells were fixed with 25 µl of ice-cold 50% CCl_3COOH and incubated at 4° for 1 h. After washing, air-drying, and staining for 15 min with 100 µl 0.4% SRB in 1% AcOH, excessive dye was removed by washing with 1% AcOH. After airdrying of the plates, SRB was resuspended in 100 µl of 10 mM *Tris* buffer, and the absorbance was measured at 560 nm with a plate reader (*Molecular Devices*, *SPECTRA MAX 340*). All tests were performed in triplicate, and results are expressed as IC_{50} values.

Antimicrobial Assay. Compounds **1**–**3** were tested for their antibacterial activities against *Candida albicans* and *Staphylococcus aureus* in *vitro* by using the turbidimetric method [17]. *C. albicans* and *S. aureus* were inoculated in *Müller–Hinton* broth (*Oxiod*, CM0405, Hampshire, UK) to *McFarland 0.5* and diluted with the medium to 1×10^6 CFU/ml. Aliquots of 90 µl were filled in 96-well U-bottomed microplate, and then treated with serial dilutions of compounds **1**–**3** with the maximum concentration of 20 µg/ml. After being cultured at 37° for 24 h, the absorbance was measured at 620 nm with the microplate reader mentioned above. All tests were performed in triplicate, and results are expressed as IC_{50} values.

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