Constituents from *Clausena excavata*

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Three new coumarins containing a C_{10} terpenoid side chain, clauslactones R—T (1—3), together with 14 known coumarins (4—17) and 11 known carbazole alkaloids (18—28), were isolated from the leaves and stems of *Clausena excavata*. Their structures were established by detailed spectroscopic analyses. Furthermore, the stereochemistry of 1 was confirmed by single-crystal X-ray diffraction analysis, which was the first example among coumarins with a C_{10} terpenoid side chain. Additionally, compounds 22 and 27 were found to show moderate topoisomerase II inhibitory effects at 50 μM.

Key words clauslactone R; clauslactone S; clauslactone T; *Clausena excavate*; coumarin; C_{10} terpenoid side chain

Clausena excavata (Rutaceae) has been claimed to be a useful folk medicine in the treatment of dysentery, enteritis, and urethra infection.[1] Previous studies revealed that chemical constituents of this plant included carbazole alkaloids,[2—4] coumarins[5—7] and tetranortriterpenoids.[8] The coumarins featured with a C_{10} terpenoid side chain in their structures have attracted considerable attentions due to their potent inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells.[5] In a recent bioassay guided study, a carbazole alkaloid was reported to have cyclin-dependent kinase 5 (CDK5) inhibitory activity.[9] In our investigation on *C. excavata*, three new coumarins together with 25 known compounds (14 coumarins and 11 carbazole alkaloids) were isolated. We described herein the isolation and structural elucidation of compounds **1**—**3**, as well as the *in vitro* topoisomerase II inhibition of some isolated compounds.

Clauslactone R (**1**) was obtained as optically active colorless needles, whose molecular formula was deduced as $C_{20}H_{20}O_7$ by HR-EI-MS at *m*/*z* 372.1216 (M^+). The presence of a 7,8-dioxygenated coumarin nucleus was demonstrated by two pairs of doublets at δ_H 6.25 and 7.88 (each 1H, J=9.6 Hz), and at 7.36 and 7.14 (each 1H, J=8.7 Hz).[5] The IR band at 1762 cm^{-1} suggested the presence of a lactone moiety. Its ^{1}H- and ^{13}C-NMR signal patterns [(δ_H 4.76—4.83 (m, H-6′), 3.18 (ddt, J=17.2, 7.6, 2.5 Hz, Ha-7′), 2.66—2.72 (m, Hb-7′), 6.08 (t, J=2.5 Hz, Ha-10′), 5.66 (t, J=2.5 Hz, Hb-10′); δ_C 75.4 (C-6′), 34.2 (C-7′), 136.0 (C-8′), 170.4 (C-9′), 121.7 (C-10′)] were in good agreement with those of a 5-substituted α-methylene-γ-lactone ring.[5] The ^{1}H-chemical shifts of Me-4′ at δ_H 1.50 (s), H-2′ at 3.25 (dd) and the ^{13}C-chemical shifts of C-2′ at δ_C 61.6 and C-3′ at 58.8 suggested a trisubstituted oxirane ring in the molecule. The COSY correlation between H-5′ and H-6′ and HMBC correlations of H-5′/C-2′ and H-5′/C-4′ suggested that the 5-substituted α-methylene-γ-lactone ring and the trisubstituted oxirane ring were connected by the methylene at C-5′. The linkage of H-1′ to the trisubstituted oxriane ring was deduced from the COSY interactions between H-1′ and H-2′. Cross peaks of Ha-1′, Hb-1′/C-7 and MeO-8/C-8 in HMBC spectrum suggested the attachment of the side chain to C-7 while the methoxy group to C-8. Such allocation of the functional groups was further supported by the significant mass fragment at *m*/*z* 192 resulting from the cleavage at O-7/C-1′ with a hydrogen transfer. Thus the planar structure of **1** was established.

The relative configuration of **1** was determined by a ROESY experiment. Interactions of Me-4′/H-1′, Me-4′/H-6′ and H-2′/H-5′ suggested that H-6′, Me-4′ and H-1′ were on the same face while H-2′ was on the other face. Thus the relative configuration of C-2′, C-3′ and C-6′ was deduced as *R**, *R** and *S**. The X-ray crystallographic diffraction analysis (Fig. 2) of **1** further confirmed the elucidated structure as shown in Fig. 1.

Clauslactone S (**2**), isolated as a colorless powder, had the molecular formula $C_{20}H_{22}O_8$ according to HR-EI-MS at *m*/*z*

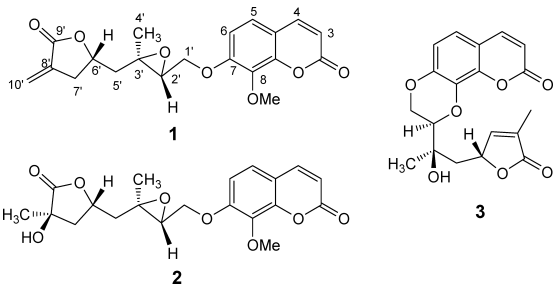

Fig. 1. Structures of Compounds **1**—**3**

Figure 1 depicts **1**—**3** with the relative configuration at the chiral centers.

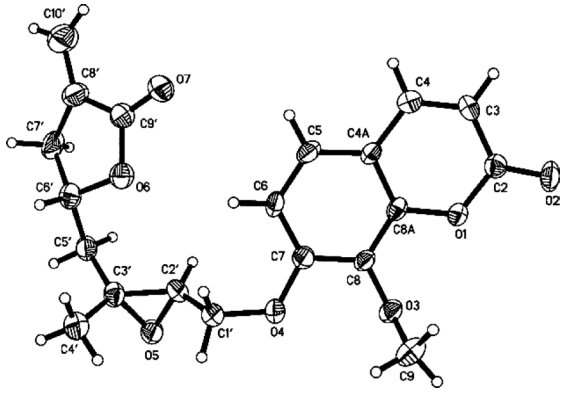

Fig. 2. X-Ray Crystallographic Structure of **1**

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Table 1. ^{1}H-NMR Data of Compounds **1**—**3**[a)]

No.	**1**[b)]	**2**[b)]	**3**[c)]
3	6.25 (d, 9.6)	6.25 (d, 9.6)	6.23 (d, 9.6)
4	7.88 (d, 9.6)	7.88 (d, 9.6)	7.82 (d, 9.6)
5	7.36 (d, 8.7)	7.36 (d, 8.7)	7.07 (d, 8.8)
6	7.14 (d, 8.7)	7.14 (d, 8.7)	6.84 (d, 8.8)
1′a	4.54 (dd, 11.5, 3.4)	4.52 (dd, 11.4, 3.5)	4.64 (dd, 11.2, 2.0)
1′b	4.20 (dd, 11.5, 6.6)	4.24 (dd, 11.4, 6.5)	4.13 (dd, 11.2, 9.0)
2′	3.25 (dd, 6.6, 3.4)	3.26 (dd, 6.5, 3.5)	4.03 (dd, 9.0, 2.0)
4′	1.50 (s)	1.46 (s)	1.44 (s)
5′a	1.88—2.00 (m)	1.97—2.03 (m)	2.09 (dd, 14.9, 3.9)
5′b			1.92 (dd, 14.9, 9.5)
6′	4.76—4.83 (m)	4.79—4.85 (m)	5.29—5.31 (m)
7′a	3.18 (ddt, 17.2, 7.6, 2.5)	2.45 (dd, 13.4, 5.5)	7.31—7.32 (m)
7′b	2.66—2.72 (m)	1.86 (dd, 13.4, 9.5)	
10′-Me		1.40 (s)	1.86 (d, 1.6)
10′a	6.08 (t, 2.5)		
10′b	5.66 (t, 2.5)		
MeO	3.92 (s)	3.94 (s)	

a) δ in ppm and *J* in Hz, *b*) in acetone-d_6 at 400 MHz, *c*) in MeOH-d_4 at 600 MHz.

390.1332 (M^+), an increase of H_2O compared with **1**. Its IR band at 3430 cm^{-1} revealed the presence of a hydroxyl group. Its ^{1}H- and ^{13}C-NMR spectra were very similar to those of **1**, except for a quaternary methyl [Me-10′ at δ_H 1.40 (s), δ_C 23.6] attached to an oxygenated carbon (C-8′ at δ_C 73.5) in **2** instead of an *exo*-methylene of a γ-lactone ring in **1**. All proton and carbon signals were assigned by a detailed analysis of 1D- and 2D-NMR data (Tables 1, 2), suggesting that it was a hydrated analogue of **1**. The NOE correlations H-6′/Ha-7′ and Me-10′/Hb-7′ revealed the relative configuration of C-8′ as *R**, while the relative configuration of C-2′, C-3′ and C-6′ remained the same as those in **1** (Fig. 3). Thus, the entire structure of **2** was determined as in Fig. 1.

Clauslactone T (**3**) was obtained as a colorless powder and its molecular formula was determined as $C_{19}H_{18}O_7$ by HR-EI-MS at *m*/*z* 358.1057 (M^+). A thorough analysis of MS, IR, 1D-, and 2D-NMR data of **3** revealed the presence of a 7,8-dioxygenated coumarin nucleus. The absence of the methoxy group in **3** indicated that the side chain was attached to C-8 in the coumarin moiety *via* an ether bond. The presence of an α-methyl-α,β-unsaturated-γ-lactone moiety was evident from the ^{1}H- and ^{13}C-NMR data [δ_H 5.29—5.31 (m, H-6′), 7.31—7.32 (m, H-7′), 1.86 (d, *J*= 1.6 Hz, Me-10′); δ_C 79.9 (C-6′), 153.3 (C-7′), 133.3 (C-8′), 176.9 (C-9′) and 10.9 (C-10′)]. The AMX-type signals at δ_H 4.64 (dd, *J*=11.2, 2.0 Hz), 4.13 (dd, *J*=11.2, 9.0 Hz) and 4.03 (dd, *J*=9.0, 2.0 Hz) were assignable to an oxygenated methylene (H-1′) and an adjacent hydroxymethine group (H-2′). The correlation between H-5′ and H-6′ in the COSY spectrum revealed the linkage of C-5′ to the α-methyl-α,β-unsaturated-γ-lactone ring. The linkage of these partial structural units as shown in Fig. 1 was further elucidated from the HMBC long-range correlations of H-2′, H-5′/C-4′, as well as Me-4′/C-2′. In fact, **3** was a 7′,8′-bisdehydro analogue of murrayacoumarin C, whose relative configuration at C-2′, C-3′, C-6′ and C-8′ remains unspecified.[10)] The ROESY experiment of **3** showed correlations between Me-4′ and H-2′ and H-6′, suggesting that Me-4′, H-2′, H-6′ shared the same face. Thus the relative stereochemistry of **3** was elucidated in Fig. 1. The stable stereochemical consequences of configurations at C-2′ and C-3′ might be explained by a possible biogenesis that an epoxide was opened intramolecularly by the free hydroxyl at C-8, although no direct evidence was provided to fully support the entire relative configuration assignment of **3**.

Table 2. ^{13}C-NMR Data of Compounds **1**—**3**[a)]

No.	**1**[b)]	**2**[b)]	**3**[c)]
2	160.8 (s)	160.9 (s)	163.3 (s)
3	114.1 (d)	114.0 (d)	114.1 (d)
4	144.9 (d)	145.0 (d)	146.7 (d)
4a	115.0 (s)	115.0 (s)	115.3 (s)
5	124.1 (d)	124.2 (d)	121.6 (d)
6	114.4 (d)	111.5 (d)	115.3 (d)
7	155.5 (s)	155.6 (s)	148.7 (s)
8	137.6 (s)	137.1 (s)	130.4 (s)
8a	148.8 (s)	149.0 (s)	145.4 (s)
1′	69.3 (t)	69.4 (t)	66.8 (t)
2′	61.6 (d)	61.7 (d)	80.8 (d)
3′	58.8 (s)	59.0 (s)	72.9 (s)
4′	17.1 (q)	17.3 (q)	22.7 (q)
5′	45.3 (t)	44.7 (t)	43.7 (t)
6′	75.4 (d)	75.5 (d)	79.9 (d)
7′	34.2 (t)	44.6 (t)	153.3 (d)
8′	136.0 (s)	73.5 (s)	133.3 (s)
9′	170.4 (s)	177.9 (s)	176.9 (s)
10′	121.7 (t)	23.6 (q)	10.9 (q)
MeO	61.4 (q)	61.5 (q)	

a) δ in ppm, *b*) in acetone-d_6 at 400 MHz, *c*) in MeOH-d_4 at 600 MHz.

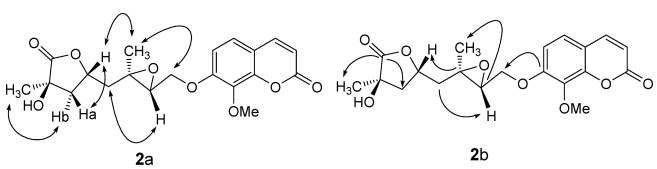

Fig. 3. a) Key ROESY Correlations of **2**; b) Selected HMBC Correlations for **2**

The structures of known compounds were confirmed by the comparison of their spectroscopic properties with published data, identified as 5-geranyloxy-7-hydroxycoumarin (**4**),[11)] clauslactone B (**5**),[5)] clauslactone M (**6**),[12)] anisocoumarin J (**7**),[13)] excavatin E (**8**),[6)] excavatin G (**9**),[6)]

clausenidin (**10**),[14,15] dentatin (**11**),[15,16] clausarin (**12**),[17] nordentatin (**13**),[18,19] xanthyletin (**14**),[20] xanthoxyletin (**15**),[20] murrayacoumarin C (**16**),[10] umbelliferone (**17**),[21] 3-formylcarbazole (**18**),[22] 2-methoxy-3-formylcarbazole (**19**),[23] 2-hydroxy-3-formyl-7-methoxycarbazole (**20**),[24] clausine C (**21**),[25] clausine E (**22**),[3] clausine K (**23**),[3] clausine L (**24**),[26] mukonal (**25**),[27] heptaphylline (**26**),[28] 2,7-dihydroxy-3-formyl-1-(3′-methyl-2′-butenyl)carbazole (**27**),[29] and heptazoline (**28**).[30]

Compounds **19**, **20**, **22**, **23**, **26** and **27** were applied in a topoisomerase II inhibition activity test, which was measured by using ATP-dependent decatenation of kDNA. Compounds **22** and **27** were found to exhibit moderate effects at the concentration of 50 μM.

Experimental

General Experiment Procedures Column chromatography (CC): silica gel (200—300 mesh). Fractions were monitored by TLC (silica gel). mp: Büchi 510 apparatus; uncorrected. Optical rotation: Perkin-Elmer 341 polarimeter. UV spectrum: Varian Cary 300 Bio spectrophotometer; λ_{max} (log ε) in nm. IR spectrum: Nicolet-Magna FT-IR 750 spectrometer, in cm^{-1}. EI-MS: Finnigan-MAT-95 mass instrument. ^{1}H- and ^{13}C-NMR spectra: Bruker AM-400 and Varian Unity Inova 600 MHz spectrometer.

Plant Material The leaves and stems of *C. excavata* were collected from Nanning, Guangxi Autonomous Region, P. R. China in 2001, and identified by Prof. Jin-Gui Shen of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. A Voucher specimen (2000133) was deposited in the Herbarium of the Shanghai Institute of Materia Medica.

Extraction and Isolation Air-dried leaves and stems (6.3 kg) of *C. excavata* were extracted with 95% EtOH for three times. After evaporation, the extract was partitioned into petroleum ether (134 g), Et_2O (80 g), AcOEt (70 g), and *n*-BuOH-soluble (13.7 g) parts respectively. The Et_2O and AcOEt fractions were combined due to their same TLC performance. The combined fractions were subjected to CC (silica gel, 200—300 mesh, 2.2 kg), eluting successively with petroleum ether/acetone (5 : 1—1 : 1), acetone and MeOH. Fractions A—H were afforded. Fractions E and F were further purified by CC (silica gel, petroleum ether/acetone), then Sephadex LH-20 (MeOH). Compounds **1** (>900 mg) and **2** (65 mg) were obtained. Compound **3** was also obtained with an impurity. A further purification by preparative HPLC (Merck RP-18 column, 10 μ, 220×25 mm), with a gradient from 20 to 30% acetonitrile (180 min, 15 ml/min) afforded compound **3** (6 mg).

Topoisomerase II Activity Inhibition Assay The inhibition of topoisomerase II activity was determined by ATP-dependent decatenation of kDNA.[33] The standard reaction mixture consisted of 50 mM Tris–HCl (pH 7.7), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, 50 μg/ml of bovine serum albumin (BSA) and 100 ng kDNA. The Reactions were initiated by the addition of hTopo II (TopoGEN, Columbus, OH, U.S.A.), incubated for 15 min at 37 °C, and stopped by addition of 2 μl 10% SDS. The samples were mixed with 2 μl of loading buffer (5% SDS, 50% glycerol, 0.05% bromophenol blue) and subjected to 1% agarose gel electrophoresis in TAE buffer (40 mM Tris acetate, pH 8.3, 2 mM EDTA) at 4 V/cm for 1.5 h. The gel was stained with ethidium bromide (EB) and photographed under a gel analysis system (Syngene, Cambridge, U.K.).

Clauslactone R (**1**): Colorless needles (acetone). mp 123—125 °C. $[\alpha]_D^{20}$ +51° (c=0.64, acetone). UV λ_{max} (MeOH) nm (log ε): 316 (4.16), 256 (3.67), 204 (4.69). IR (KBr) cm^{-1}: 2923, 1762, 1716, 1606. EI-MS *m/z*: 372 (14, M^+), 192 (29), 97 (100). HR-EI-MS *m/z*: 372.1216, M^+ (Calcd for $C_{20}H_{20}O_7$, 372.1209). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

X-Ray Crystallographic Data for **1** (Acetone): $C_{20}H_{20}O_7$, M=372.36, monoclinic, space group: $P2_1$, a=6.825(4) Å, b=13.239(7) Å, c=9.997(6) Å, V=903.0(9) Å^3, crystal size: 0.435×0.207×0.132 mm, Z=2, Dx=1.370 mg/m^3. The data were measured using a Bruker SMART CCD diffractometer, using Mo$K\alpha$ graphite-monochromated radiation (λ=0.71073 Å), T=293(2) K. 4581 reflections measured, 1751 were unique ($R_{(int)}$=0.1152) and used in all calculations. The final $\omega R(F^2)$ was 0.0578 (all data). The structures were solved by direct methods using the program SHELXS-97.[34] The refinement and all further calculations were carried out using SHELXL-97. The crystal structure had been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 603428. Copies of the data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge, CB21EZ, U.K.; E-mail: deposit@ccdc.cam.ac.uk; fax: +441223 336033).

Clauslactone S (**2**): Colorless powder. $[\alpha]_D^{20}$ +45° (c=0.3, acetone). UV λ_{max} (MeOH) nm (log ε): 318 (3.34), 261 (3.11), 204 (3.98). IR (KBr) cm^{-1}: 3430, 2979, 2937, 1778, 1728, 628. EI-MS *m/z*: 390 (M^+, 49), 192 (100), 87 (78), 85 (53). HR-EI-MS *m/z*: 390.1332, M^+ (Calcd for $C_{20}H_{22}O_8$, 390.1315). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Clauslactone T (**3**): Colorless powder. $[\alpha]_D^{20}$ +40° (c=0.13, MeOH). UV λ_{max} (MeOH) nm (log ε): 314 (3.52), 256 (3.02), 204 (3.99). IR (KBr) cm^{-1}: 3434, 2923, 1735, 1614. EI-MS *m/z*: 358 (M^+, 24), 204 (100), 189 (22), 175 (46), 95 (17). HR-EI-MS *m/z*: 358.1057, M^+ (Calcd for $C_{19}H_{18}O_7$, 358.1053). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

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