Cytotoxic Amides and Quinolones from *Clausena lansium*

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Two new amides, claulansamides A and B (**1** and **2**, resp.), together with five known amides, **3**–**7** and six known quinolones, **8**–**13**, were isolated from the stems and roots of *Clausena lansium*. Their structures were elucidated on the basis of extensive spectroscopic methods. Their absolute configurations were determined by single-crystal X-ray diffraction technique, CD, and optical rotation. HPLC Chiral separation of **1** afforded two enantiomers, (+)- and (−)-claulansamide A, respectively. Compounds **9**, **12**, and **13** were isolated from the genus *Clausena* for the first time. All compounds were evaluated for their cytotoxic activities against A549, BGC-823, and HeLa cancer cell lines. However, only **9** showed cytotoxic activity against A549 cell line with an IC_{50} value of 46.3 μM, and **11** against BGC-823 and HeLa cell lines with the IC_{50} values of 55.0 and 14.7 μM, respectively.

Introduction. – *Clausena lansium* (LOUR.) SKEELS (Rutaceae) is a plant widely distributed in South China. Its fruits have been used as a folk medicine to treat indigestion, cold, cough, and stomach pain [1]; its leaves and roots for the treatment of cough, asthma, dermatological diseases, viral hepatitis, and gastro-intestinal disease; and its seeds for treating acute and chronic gastrointestinal inflammation, and ulcer [2]. Previous studies revealed that the genus *Clausena* contains mainly coumarins, cabazole alkaloids, amides, and terpenoids [3–7]. *Huang* and co-workers isolated the first amide, named clausenamide, featuring reduction of SGPT (serum glutamic-pyruvic transaminase) activity from the leaves of *C. lansium* [8–10]. So far, *ca.* 30 amides have been isolated from this plant [11–17]. Some of them showed cytotoxic and hepatoprotective activities [13][14]. There are also about ten quinolones isolated from this plant [4][18–22]. Our efforts to obtain bioactive constituents from *C. lansium* has led to the isolation of two new amides, claulansamides A and B (**1** and **2**, resp.), together with five known amides, **3**–**7**, and six known quinolones, **8**–**13**. Herein, we report the isolation, structure elucidation, and cytotoxicity of these compounds.

Results and Discussion. – Claulansamide A (**1**) was obtained as colorless crystals. The HR-EI-MS showed a molecular-ion peak at *m*/*z* 297.1364 (M^+, $C_{18}H_{19}NO_3^+$; calc. 297.1365), suggesting ten degrees of unsaturation. The UV spectrum showed typical maximum absorptions of a conjugated benzene chromophore at λ_{max} 208 and 256 nm. The IR spectrum displayed absorption bands of OH (3423 cm^{-1}) and amide CO (1640 cm^{-1}) groups. The ^{1}H-NMR spectrum (*Table*) revealed the presence of two benzene rings (δ(H) 7.20–7.37 (10 H, overlapped)), three O-bearing CH groups (δ(H)

5.14 (*dd*, $J = 8.8, 3.2$), 4.98 (*d*, $J = 9.4$), and 4.15 (*d*, $J = 9.4$)), one CH_2 group (δ(H) 3.29 (*dd*, $J = 14.4, 8.8$) and 3.13 (*dd*, $J = 14.4, 3.2$), and one MeN group (δ(H) 3.02 (*s*)). The ^{13}C-NMR spectrum (*Table*) exhibited 18 signals attributable to one CO group (δ(C) 170.0), twelve benzene C-atoms (δ(C) 137.8 – 126.6), three O-bearing CH groups (δ(C) 88.7, 73.7, and 70.2), one CH_2 group (δ(C) 37.8), and one MeN C-atom (δ(C) 31.3). These data evidenced the presence of two benzene rings and a pyran ring bearing a CO group. The latter was supported by the HMBCs from H–C(7) to C(9) and C(11), from H–C(8) to C(9), from MeN to C(9) and C(11), and from H–C(11) to C(9), and by the ^{1}H,^{1}H-COSY correlations between H–C(7) and H–C(8), and between H–C(11) and H–C(12). Positions of the CH_2 group and two benzene rings were established by the HMBC cross-peaks from H–C(8) and H–C(7) to C(1), and from H–C(12) to C(11) and C(13). These analyses suggested that **1** was an amide, and its constitution was established (*Fig. 1*).

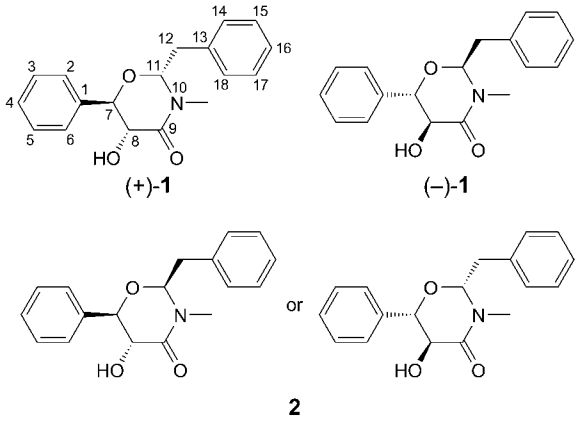

Fig. 1. *Structures of compounds* **1** *and* **2**

The relative configuration of **1** was determined by the ^{1}H-NMR coupling constants and ROESY spectrum. In the ^{1}H-NMR spectrum, the coupling J(7,8) constant is 9.4 Hz, and there is a correlation between H–C(7) and H–C(12) in the ROESY spectrum. These data indicated that H–C(7) and H–C(12) are in the same orientation, but H–C(8) and H–(11) are in another orientation (*Fig. 2*). To establish the configuration, **1** was crystallized from acetone to afford a crystal of the monoclinic space group *P*21/*c*, which was analyzed by X-ray crystallography with CuK_α ($\lambda =$ 1.54178 Å) radiation (*Fig. 3*). Finally, **1** was determined as a racemate by single-crystal X-ray diffraction analysis. Subsequent HPLC separation of **1** on chiral column gave two individual enantiomers (−)-**1** and (+)-**1**, which displayed opposite *Cotton* effects in their electronic CD (ECD) spectra and opposite optical rotations. The HPLC integral area of the two enantiomers, (−)-**1** and (+)-**1**), were in a ratio of *ca.* 1:2. Furthermore, the geometry was optimized using DFT at the B3LYP/CC-pVDZ level on the basis of the crystal structure. The harmonic vibrational frequency was then calculated to confirm its stability at the same level. The ECD spectrum and the optical rotation

Table. *^1H- and ^{13}C-NMR Data* (400 and 100 MHz, resp.; $CDCl_3$) *of Compounds* **1** *and* **2**. δ in ppm, J in Hz.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1		137.8		137.6
2,6	7.37 (overlapped)	126.6	7.40 (overlapped)	126.6
3,5	7.34 (overlapped)	128.5	7.40 (overlapped)	128.5
4	7.34 (overlapped)	128.5	7.36 (overlapped)	128.5
7	4.98 (d, J = 9.4)	73.7	4.51 (d, J = 9.6)	78.0
8	4.15 (d, J = 9.4)	70.2	3.77 (d, J = 9.6)	70.2
9		170.0		171.1
11	5.14 (dd, J = 8.8, 3.2)	88.7	5.17 (d, J = 2.6)	89.0
12	3.29 (dd, J = 14.4, 8.8), 3.13 (dd, J = 14.4, 3.2)	37.8	3.23 (dd, J = 14.4, 2.6), 3.07 (overlapped)	40.0
13		135.5		134.5
14,18	7.20 (overlapped)	129.3	7.30 (overlapped)	130.1
15, 17	7.29 (overlapped)	128.7	7.31 (overlapped)	128.5
16	7.26 (overlapped)	127.1	7.27 (overlapped)	127.1
MeN	3.02 (s)	31.3	3.03 (s)	29.7

(589.3 nm) of the optimized geometry were calculated at the B3LYP/Aug-CC-pVDZ level in MeCN [23]. All the calculations were carried out with the Gaussian 03 program. The computed and experimental ECD spectra are shown together in *Fig. 4*. The calculated optical rotation value of (+)-**1** was +28.39 (experimental value: +24.0) and of (−)-**1** was −28.39 (experimental value: −36.3). The experimental optical rotation value of **1**, +24.8, indicated that **1** was a mixture (−)-**1**/(+)-**1** with a ratio of *ca.* 1:2. Accordingly, the absolute configurations of (+)-**1** and (−)-**1** were established.

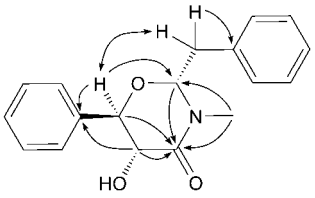

Fig. 2. *Key HMBC* (H → C) *and ROESY* (H ↔ H) *correlations of compound* **1**

Claulansamide B (**2**) has the same molecular formula as **1** determined by HR-EI-MS (m/z 297.1362 (M^+, $C_{18}H_{19}NO_3^+$, calc. 297.1365)). Comparison of the NMR data of **2** with those of **1** revealed that they have the same planar structure, and the only difference was the configuration at C(11). In the ROESY spectrum of **2**, the correlation between H–C(7) and H–C(11) indicated that H–C(7) and H–C(11) are in the same orientation. Comparison of the experimental optical rotation ($[\alpha]_D^{24.0} = +1.9$ (c = 0.35, MeOH)) with the calculated value of ±54.9 indicated that **2** was a mixture with a ratio of *ca.* to 1:1.

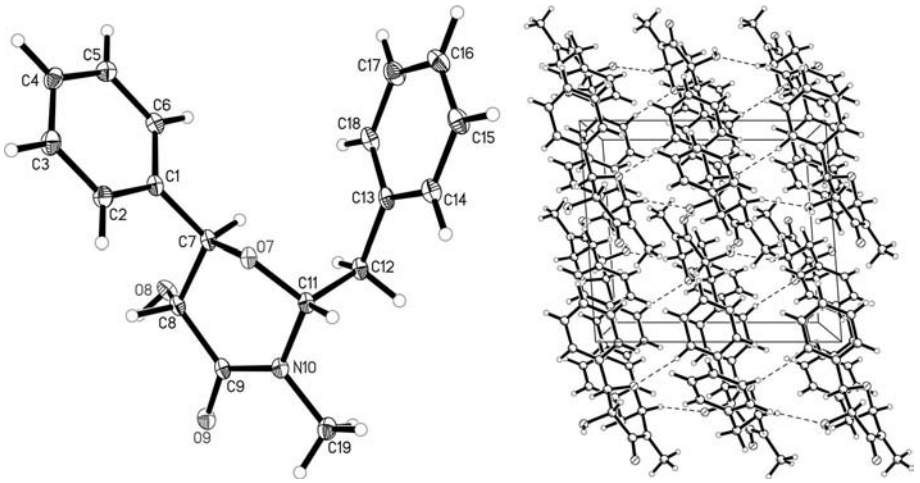

Fig. 3. *X-Ray crystal structures of compound* **1**

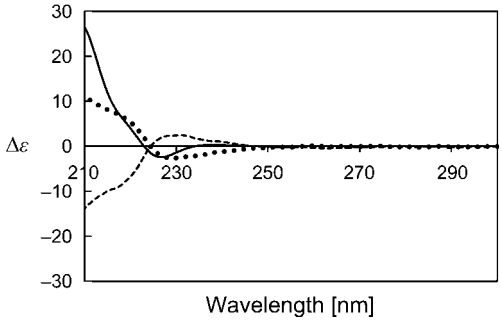

Fig. 4. *Calculated ECD spectrum of* (+)-**1** *in MeCN* (—)*; experimental ECD spectrum of* (+)-**1** *in MeCN* (····)*; and experimental ECD spectrum of* (−)-**1** *in MeCN* (– – –)

The known compounds (*Fig. 5*) were identified as clausenalansamide A (**3**) [13], clausenalansamide B (**4**) [13][15], secodemethylclausenamide (**5**) [16], (−)-(*R*)-tembamide (**6**) [17], (−)-clausenamide (**7**) [9][10], 4-methoxy-1-methyl-2-quinolone (**8**) [20], 6-hydroxy-4-methoxy-1-methyl-2-quinolone (**9**) [24], 4-methoxy-1*H*-2-quinolone (**10**) [21], dictamine (**11**) [22], atanine (**12**) [25], and (+)-(*S*)-platydesmine (**13**) [26] by comparing the NMR and MS data with those in the literature. The absolute configurations of **3** and **5** were determined by induced CD experiments [27], and of **4**, **6**, **7**, and **13** were determined by comparing the optical rotation data with those in the literature.

Cytotoxic activities of all compounds were evaluated *in vitro* against A549 (human lung adenocarcinoma), BGC-823 (human gastric cancer), and HeLa (cervical cancer) cancer cell lines. Only **9** showed cytotoxic activitiy against A549 cancer cell line with an IC_{50} value of 46.3 μM, and **11** against BGC-823 and HeLa cancer cell lines with the IC_{50} values of 55.0 and 14.7 μM, respectively.

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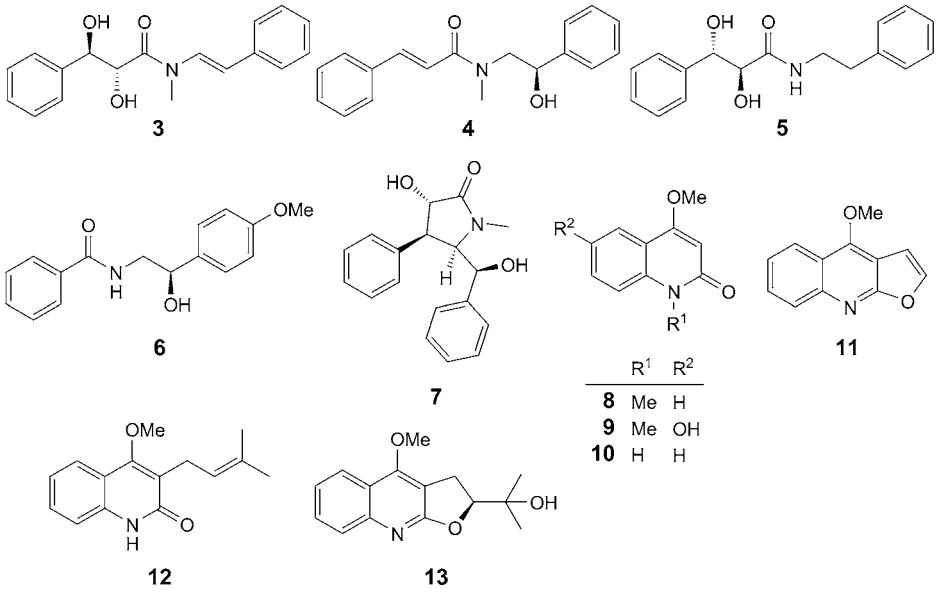

Fig. 5. *Structures of compounds* **3**–**13**

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Experimental Part

General. TLC: Silica gel GF_{254} plates (*Qingdao Yuminyuan Silica Reagent Factory*, Qingdao, P. R. China); visualization with UV light (254 and 365 nm), and by spraying with 5% aq. H_2SO_4 soln., followed by heating. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Yuminyuan Silica Reagent Factory*, Qingdao, P. R. China); *Sephadex LH-20* (*Pharmacia*, *Amersham Biosciences*, SE-Uppsala); *RP-18* silical gel (40–60 μm, *Merck*, DE-Darmstadt); and *MCI* gel (*CHP-20P*, 70–150 μm, *Mitsubishi Chemical Corporation*, Japan). MPLC: *EZ Purifier 100/200* equipped with a UV detector (*Lisure Science (Suzhou) Co., Ltd.*, P. R. China) and a *RP-18* column (3.5 × 25 or 4.5 × 40 cm, 40–60 μm) or a *MCI* column (4.5 × 40 cm); flow rate, 25 ml/min. Semiprep. HPLC: *Agilent 1100* apparatus equipped with a UV detector and *CHIRALCEL® OD-H* (10 × 250 mm, 5 μm, *Daicel Chiral Technologies (China) Co., Ltd.*); flow rate, 2 ml/min. Prep. reversed-phase (RP) HPLC: *Agilent 1100* apparatus equipped with a UV detector and a *SunFire OBD* (1.9 × 25 cm, 5 μm, *Waters*) column, flow rate, 10 ml/min. M.p.: *Gongyi Yuhua X-4* digital melting-point apparatus. Optical rotations: *Jasco P-1020 Polarimeter*. UV Spectra: *Shimadzu UV-2401PC* spectrophotometer; in nm. CD Spectra: *Agilent Applied Photophysics* spectrometer; in nm. IR Spectra: *Bruker Tensor 27* FT-IR spectrometer; KBr pellets; in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker AM-400* (at 400 and 100 MHz, resp.), *DRX-500* (at 500 and 125 MHz, resp.), or *Bruker AV-600* (at 600 and 150 MHz), *Spectrometer* in (D_6)acetone, $CDCl_3$, or (D_5)pyridine at r.t.; δ in ppm rel. to TMS, J in Hz. ESI-MS: *Waters Xevo TQ-S or Bruker HCT/Esquire*;

in *m*/*z*. EI-MS and HR-EI-MS: *Waters AutoSpec Premier P776*; in *m*/*z*. X-Ray crystallography: *Bruker APEX DUO* diffractometer.

Plant Material. The stems and roots of *C. lansium* were collected in Hekou, Yunnan Province, P. R. China, in September 2010, and identified by Prof. *Yu-Min Shui*, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 0599043) was deposited with the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. Air-dried and powdered stems and roots of *C. lansium* (27 kg) were extracted and refluxed with MeOH for three times each for 4 h (MeOH, 3 × 50 l). The extract was evaporated under reduced pressure to yield a dark brown residue (900 g). The residue was suspended in MeOH/H_2O 7:3 (3 l) and then partitioned with AcOEt (3 × 2 l). After removing solvent, the AcOEt-soluble part (406 g) was fractionated by CC (SiO_2 (200–300 mesh); $CHCl_3$/MeOH 30:1 to 4:1) to afford six fractions, *Frs. 1–6*, on the basis of TLC analysis. *Fr. 2* (38 g) was subjected successively to MPLC (*RP-18*; MeOH/H_2O 10:90 to 60:40), CC (SiO_2; PE/AcOEt 9:1 to 7:3), and prep. HPLC (MeCN/H_2O 60:40) to afford **11** (24 mg). *Fr. 4* (17.9 g) was separated by MPLC (*MCI*, MeOH/H_2O 10:90 to 40:60), repeated CC (SiO_2; PE/acetone 9:1), and prep. HPLC (MeCN/H_2O 60:40) to give **12** (6 mg). *Fr. 5* (6.9 g) was first submitted to MPLC (*MCI*; MeOH/H_2O 5:95 to 60:40) and then subjected to CC (SiO_2; PE/acetone 9:1). Finally, **6** (56 mg), **10** (55 mg), and **13** (152 mg) were obtained by prep. HPLC (MeCN/H_2O 50:50), (MeCN/H_2O 38:62), and (MeCN/H_2O 45:55), resp. *Fr. 6* (77 g) was purified by CC (SiO_2; $CHCl_3$/acetone 15:1 to 7:3) to afford *Fr. 6.1 – Fr. 6.4. Fr. 6.2* (14.3 g) was subjected successively to CC (SiO_2; PE/acetone 5:1), MPLC (*MCI*, MeOH/H_2O 10:90 to 60:40), and MPLC (*RP-18*; MeOH/H_2O 5:95 to 70:30) to remove pigments. Then, the residue was further purified by repeated CC (SiO_2; $CHCl_3$/acetone 20:1; SiO_2; $CHCl_3$/AcOEt 20:1; and SiO_2; PE/acetone 9:1). In the next step by prep. HPLC (MeCN/H_2O 35:65), six compounds were obtained: **8** (411 mg), **1** (14 mg), **2** (5 mg), **3** (146 mg), **4** (10 mg), and **5** (57 mg). Compound **1** was subjected to HPLC (hexane/^{i}PrOH 20:80) with a semi-prep. column on chiral stationary phase and afforded two enantiomers (−)-**1** (5.1 mg) and (+)-**1** (8.4 mg). *Fr. 6.4* (3.8 g) was subjected to CC for three times (*Sephadex LH-20*; $CHCl_3$/MeOH 1.5:1, 1:1 or 1:1.5) and then subjected to MPLC (*RP-18*; MeOH/H_2O 10:90 to 100:0). The residue was subjected to repeated CC (SiO_2; PE/acetone 9:1; SiO_2; PE/^{i}PrOH 20:1, resp.), followed by prep. HPLC (MeCN/H_2O 35:65) to afford **7** (25 mg) and **9** (6 mg).

Claulansamide A (=*2-Benzyl-5-hydroxy-3-methyl-6-phenyl-1,3-oxazinan-4-one*; **1**): Colorless crystals (acetone). $[\alpha]_D^{23.7} = +24.8$ ($c = 1.19$, MeCN). M.p. 178–180°. UV (MeOH): 208 (4.73), 256 (3.19). IR (KBr): 3423, 2923, 1640, 1453, 1409, 1116, 1080, 1029. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 298 ($[M+H]^+$), 320 ($[M+Na]^+$), 617 ($[2M+Na]^+$). HR-EI-MS: 297.1364 (M^+, $C_{18}H_{19}NO_3^+$; calc. 297.1365).

(+)-Claulansamide A (=*(2*R*,5*R*,6*R*)-2-Benzyl-5-hydroxy-3-methyl-6-phenyl-1,3-oxazinan-4-one*; (+)-**1**). Colorless oil. $[\alpha]_D^{24.6} = +24.0$ ($c = 0.14$, MeCN). CD (MeCN): 229 ($\Delta\varepsilon$ −2.79). ESI-MS: 298 ($[M+H]^+$), 320 ($[M+Na]^+$), 617 ($[2M+Na]^+$).

(−)-Claulansamide A (=*(2*S*,5*S*,6*S*)-2-Benzyl-5-hydroxy-3-methyl-6-phenyl-1,3-oxazinan-4-one*; (−)-**1**). Colorless oil. $[\alpha]_D^{24.6} = -36.3$ ($c = 0.30$, MeCN). CD (MeCN): 231 ($\Delta\varepsilon$ +2.45). ESI-MS: 298 ($[M+H]^+$), 320 ($[M+Na]^+$), 617 ($[2M+Na]^+$).

Claulansamide B (=*(2*S*,5*R*,6*R*)- or (2*R*,5*S*,6*S*)-2-Benzyl-5-hydroxy-3-methyl-6-phenyl-1,3-oxazinan-4-one*; **2**). Colorless oil. $[\alpha]_D^{24.0} = +1.9$ ($c = 0.35$, MeOH). UV (MeOH): 208 (4.32), 251 (2.90). IR (KBr): 3427, 2924, 1642, 1495, 1454, 1405, 1136, 1072, 1030. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 298 ($[M+H]^+$), 320 ($[M+Na]^+$), 617 ($[2M+Na]^+$). HR-EI-MS: 297.1362 (M^+, $C_{18}H_{19}NO_3^+$; calc. 297.1365).

X-Ray-Crystallographic Analysis of **1**[1]). Colorless crystals of **1** were obtained from acetone. Intensity data were collected at 100 K on a *Bruker APEX DUO* diffractometer equipped with an *APEX II CCD*, using CuK_α radiation. Cell refinement and data reduction were performed with *Bruker* SAINT. The structures were solved by direct methods using SHELXS-97. Refinements were performed with

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[1]) CCDC-944736 contains supplementary crystallographic data for this article. These data can be obtained free of charge from the *Cambridge Crystallographic Data Centre via* www.ccdc.cam.ac.uk/data_request/cif.

SHELXL-97, using full-matrix least-squares, with anisotropic displacement parameters for all the non-H-atoms. The H-atoms were placed in calculated positions and refined using a riding model. Molecular graphics were computed with SHELXP-97.

Crystallographic Data of Claulansamide A (**1**): $C_{18}H_{19}NO_3$, M_r 297.34, monoclinic, crystal size: 0.38 × 0.24 × 0.12 mm. $a = 9.7666(2)$ Å, $b = 14.3759(3)$ Å, $c = 10.9139(2)$ Å, $\alpha = 90.00°$, $\beta = 94.2170(10)°$, $\gamma = 90.00°$, $V = 1528.20(5)$ Å^3, $T = 100(2)$ K, space group $P21/c$, $Z = 4$, $\mu(CuK_\alpha) = 0.711$ mm^{-1}, 11833 reflections measured, 2691 independent reflections ($R_{int} = 0.0466$). The final R_1 values were 0.0465 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1232 ($I > 2\sigma(I)$). The final R_1 value was 0.0472 (all data). The final $wR(F^2)$ value was 0.1240 (all data). The goodness-of-fit on F^2 was 1.041.

Cytotoxicity Assay. The cytotoxic activities of all compounds were evaluated against A549, HeLa, and BGC-823 cancer cell lines using the SRB (sulforhodamine B) assay [28]. 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 the tested compounds with the maximum concentration of 20 µg/ml. Each compound was initially dissolved in DMSO and further diluted by 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% glacial AcOH, excessive dye was removed by washing with 1% glacial AcOH. After air-drying the plates, SRB was resuspended in 100 µl 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.

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