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FOUR NEW CANTHARIDIN DERIVATIVES FROM THE CHINESE BLISTER BEETLES, *MYLABRIS PHALERATA*

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Abstract – Four new cantharidin derivatives, cantharacidines A-D (**1-4**) were isolated from the Chinese blister beetles *Mylabris phalerata*. Their structures with absolute configurations were elucidated by means of NMR spectroscopy, single-crystal X-ray diffraction and electronic circular dichroism (ECD) spectral analyses. Compounds **1-3** are the first examples of natural cantharimide-carboxylic acid adducts. The inhibitory effects of compounds **1-4** on the viability of three cancer cell lines (HepG2, MDA-MB-231 and A549 cells) were evaluated by the MTT assay.

The Chinese blister beetles *Mylabis phalerata* Palla ('Ban-mao' in Chinese) is widely distributed in southern China. The dried body of *M. phalerata* had been used as a traditional Chinese medicine to treat tumor, carbuncle, and scrofula.[1] Previously chemical and pharmacological investigation of *M. phalerata* had led to the discovery of a number of cantharidin derivatives, some of which exhibited significant antitumor activities.[2-7] Norcantharidin, a typical cantharidin derivative, had been used in clinical cancer therapy in China for a long time.[8,9] As a part of our ongoing efforts to search for structurally diverse natural

products for antitumor agents,[10,11] four new cantharidin derivatives, cantharacidines A-D (**1-4**) were isolated from the whole body of *Mylabis phalerata*. Among them, compounds **1-3** were found to be the first natural adduct that is constructed through cantharimide and carboxylic acid units. In addition, the cytotoxic effects of the isolated compounds against HepG2, MDA-MB-231 and A-549 cell lines were evaluated.

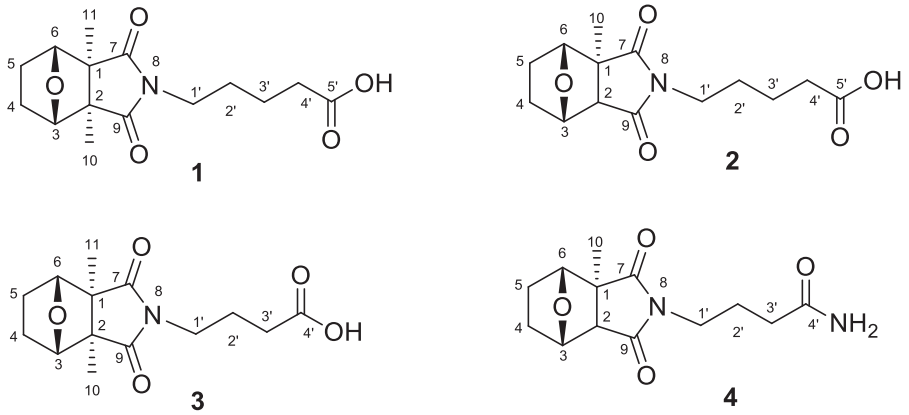

Figure 1. Chemical structures of compounds **1-4**

Cantharacidine A (**1**) was obtained as colorless blocks. The molecular formula of **1** was determined as $C_{15}H_{21}NO_5$ by its HRESIMS at *m/z* 296.1490 $[M+H]^+$ (calcd for $C_{15}H_{22}NO_5$, 296.1492). The UV spectrum of **1** showed absorption maximum at 206 nm. The IR spectrum exhibited the characteristic absorptions for hydroxyl (3438 cm^{-1}) and carbonyl (1702 cm^{-1}) groups. The analysis of ^1H and ^{13}C NMR spectra revealed **1** possessed two methyls [δ_H 1.15 (6H, s); δ_C 12.6], six methylenes [δ_H 3.50 (2H, t, J = 5.6 Hz), 2.30 (2H, t, J = 6.4 Hz), 1.90 (2H, m), 1.63 (2H, m), and 1.57 (4H, m); δ_C 39.6, 34.2, 28.0, 24.6×2, and 22.9], two methines [δ_H 4.47 (2H, overlapped), δ_C 85.1×2], two quaternary carbons (δ_C 55.2×2), and three carbonyl groups (δ_C 183.8×2, 177.0). With the aid of ^1H-^1H COSY, HSQC, and HMBC experiments, all the ^1H and ^{13}C NMR signals of **1** were assigned as shown in Table 1. The ^1H-^1H COSY spectrum of **1** revealed the presence of two spin systems (H-3 to H-6 and H-1′ to H-4′, Figure 2). In the HMBC spectrum, correlations between H-6 and C-2/C-4, between H-3 and C-1, between H-10 and C-3/C-9, as well as between H-11 and C-6/C-7 allowed the assignment of the planar structure of cantharimide moiety (**1a**). The spin system from H-1′ to H-4′ in the ^1H-^1H COSY spectrum, the HMBC correlations between H-3′ and C-5′, as well as the carbonyl group signal δ_C 171.5 (C-5′) indicated the presence of a butyric acid unit (**1b**). Furthermore, the linkage between **1a** and **1b** through the N_8-C_1' bond was deduced by the HMBC correlations between H-1′ and C-7/C-9. Thus, the planar structure of **1** was defined (Figure 2). The relative configuration of **1** was determined by a single-crystal X-ray diffraction experiment (Figure 3) (See Supporting Information). To determine the absolute configuration of **1**, a circular dichroism measurement was applied. The CD spectrum of **1** showed positive Cotton effect at

201.4 nm ($\Delta\varepsilon$ + 18.1) and negative one at 210.7 nm ($\Delta\varepsilon$ − 6.4), which was accordance with the known compound cantharimide (Figure 4). Thus, the absolute configuration of **1** was determined as 1*S*, 2*R*, 3*S*, 6*R*. Based on the above evidences, the structure of **1** was elucidated. This is the first report of natural cantharidin derivatives which is constructed through cantharimide and carboxylic acid.

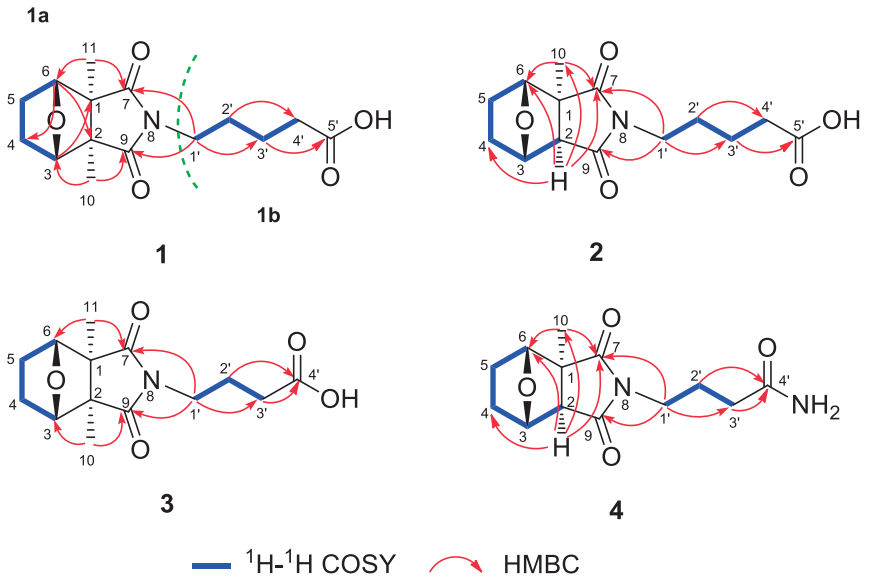

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of **1-4**

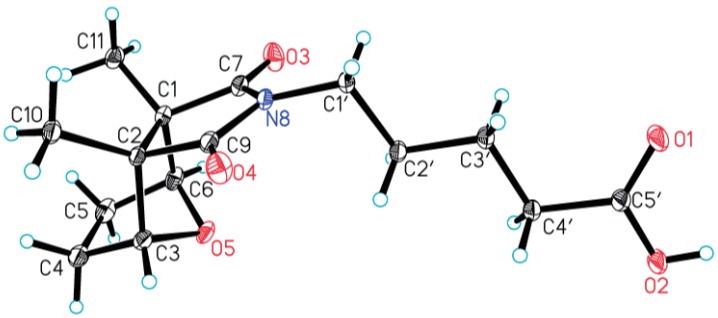

Figure 3. X-Ray ORTEP drawing of **1**

Cantharacidine B (**2**) was obtained as yellow oil. The molecular formula of **2** was determined as $C_{14}H_{19}NO_5$ by its quasi-molecular ion at *m/z* 282.1328 $[M+H]^+$ (calcd for $C_{14}H_{20}NO_5$, 282.1336). In the ^{1}H and ^{13}C NMR spectra of **2**, signals for one methyl [δ_H 1.30 (3H, s); δ_C 14.8], six methylenes [δ_H 3.47 (2H, t, J = 4.2 Hz), 2.28 (2H, t, J = 7.2 Hz), 1.96 (1H, m), 1.81 (1H, m), 1.65 (2H, m), and 1.55 (4H, m); δ_C 37.9, 33.0, 27.8, 26.6, 23.6, and 21.6], three methines [δ_H 4.64 (1H, d, J = 5.4 Hz), 4.52 (1H, d, J = 5.4 Hz), and 2.53 (1H, br s); δ_C 82.2, 80.4, 56.0], one quaternary carbon (δ_C 52.9), and three carbonyl groups (δ_C 182.0, 177.7, 176.0) were observed. Comparison of the NMR data of **2** with those of **1** revealed that their NMR signals were similar, except for the absence of a methyl and the presence of a methine (δ_C

56.0) at C-2 in **2**. This conclusion was confirmed by the spin system from H-2 to H-6 in ^{1}H-^{1}H COSY spectrum as well as the HMBC correlations between H-2 and C-4/C-6/C-7/C-10 (Figure 2). In the NOESY spectrum, correlations between H-6 and H_3-10, and between H-2 and H-3/H_3-10 suggested that the relative configurations of C-1, C-2, C-3 and C-6 as shown in Figure 5. The absolute configuration of **2** was established by comparison of experimental and TDDFT calculated ECD spectra (see Supporting Information). The calculated CD spectrum of 1*S*, 2*R*, 3*S*, 6*R*-**2** exhibited positive Cotton effects at λ_{max} 212.3 and 246.1 nm and negative Cotton effect at λ_{max} 203.9 nm, which were consistent with the measured curves (Figure 6). Thus, the structure of **2** was established.

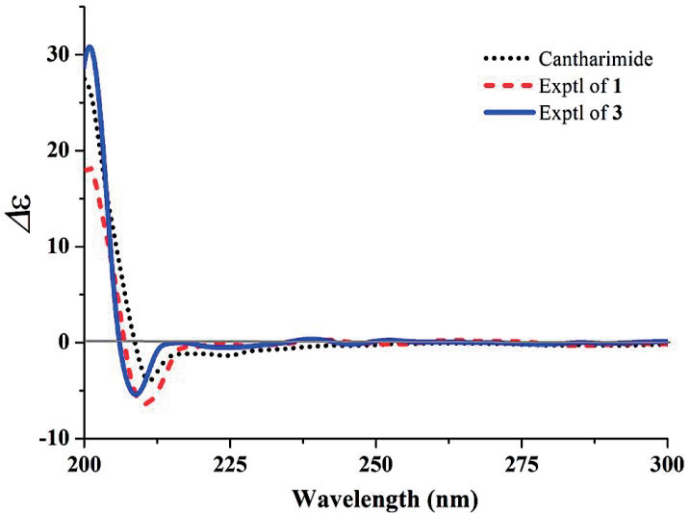

Figure 4. CD spectra of **1** and **3**

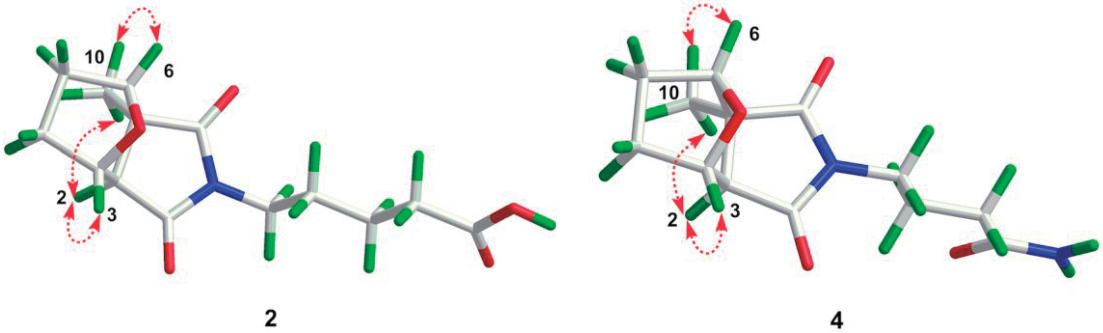

Figure 5. Key NOESY correlations of **2** and **4**

The molecular formula of cantharacidine C (**3**) was assigned as $C_{14}H_{19}NO_5$ from its HR-ESI-MS data (*m/z* 282.1340 $[M+H]^+$; calcd for $C_{14}H_{20}NO_5$, 282.1336). The UV and IR spectra of **3** were close to those of **1**, implying that they possessed similar functional groups. With the aid of ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY experiments, all the ^{1}H and ^{13}C NMR data were assigned (Table 1). Comparison of the NMR data of **3** with those of **1** revealed that their structures were similar except for the absence of a methylene in the side chain of **3**. This was confirmed by the spin system from H-1' to H-3' in ^{1}H-^{1}H

COSY spectrum, as well as the HMBC correlations between H-1' and C-3'/C-7/C-9, and between H-2'/H-3' and C-4' (Figure 2). The absolute configuration of **3** was identical to that of **1**, since they showed very similar Cotton effects in the CD measurement (Figure 4). Thus, the absolute configuration of **3** was assigned as 1*S*, 2*R*, 3*S*, 6*R*.

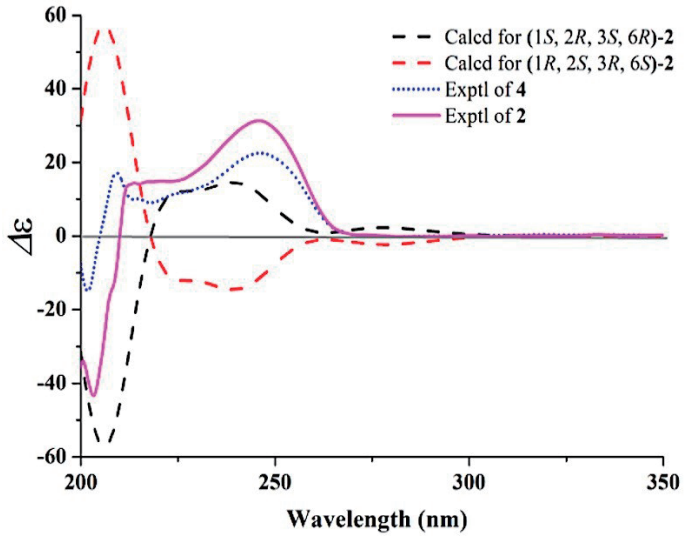

Figure 6. Calculated and experimental CD spectra of **2** and **4**

Cantharacidine D (**4**) was found to have the molecular formula $C_{13}H_{18}N_2O_4$ by its HR-ESI-MS signal at *m/z* 267.1336 $[M+H]^+$ (calcd for $C_{13}H_{19}N_2O_4$, 267.1339). In the ^{1}H NMR spectrum, signals for a methyl [δ_H 1.30 (3H, s)], five methylenes [δ_H 3.52 (2H, t, J = 4.8 Hz) 2.25 (2H, t, J = 7.8 Hz), 1.96 (1H, overlapped), 1.82 (3H, overlapped), 1.65 (2H, overlapped)], and three methines [δ_H 4.64 (1H, d, J = 5.4 Hz), 4.52 (1H, d, J = 4.8 Hz), 2.53 (1H, br s)] were observed. The ^{13}CNMR spectrum of **4** exhibited thirteen carbon signals including an imide group (δ_C 183.4, 179.1), two oxygenated methines (δ_C 83.7, 82.0), a methyl (δ_C 16.1), and a quaternary carbon (δ_C 54.3). Comparison the ^{1}H and ^{13}C NMR data of **4** with those of palasoninimide C[7] revealed that they possessed the same skeleton except for the presence of two more methylenes in the side chain at *N*-8 position in **4**. The molecular formula and characteristic IR peaks at 3575 (ν_{NH}), 3436 (ν_{NH}), 1646 (δ_{NH} and $\nu_{C=O}$) and 1442 (ν_{C-N}) cm^{-1} confirmed the presence of a carboxyamide moiety. The HMBC correlations between H-1' and C-7/C-9/C-3', between H-2'/H-3' and C-4', as well as the presence of a spin system from H-1' to H-3' in the ^{1}H-^{1}H COSY spectrum confirmed that the cantharimide connected with *N*-butyramide via N_8-$C_{1'}$ bond. In the NOESY spectrum, correlations between H-6 and H_3-10, and between H-2 and H-3/H_3-10 (Figure 5) indicated that the relative configurations of C-1, C-2, C-3 and C-6 in **4** are identical to those in palasoninimide C[7]. Furthermore, the Cotton effects for **4** were similar to those of **2** (Figure 6). Hence, the absolute configuration of **4** was determined as 1*S*, 2*R*, 3*S* and 6*R*.

The cytotoxicities of **1-4** against three cancer cell lines (HepG2, MDA-MB-231 and A549 cells) were evaluated by MTT assay. However, all the mentioned compounds were found to be devoid of significant cytotoxic activity at the concentration of 50 μM.

Table 1. NMR data of **1-4** (CD_3OD, J in Hz)[a]

No.	**1**		**2**		**3**		**4**	
	δ_C[b]	δ_H[c]	δ_C[d]	δ_H[e]	δ_C[b]	δ_H[c]	δ_C[d]	δ_H[e]
1	55.2	-	52.9	-	55.2	-	54.3	-
2	55.2	-	56.0	2.53, br s	55.2	-	57.4	2.53, br s
3	85.1	4.47	80.4	4.64, d (5.4)	85.1	4.48	82.0	4.64, d (5.4)
4	24.6	α 1.90	27.8	α 1.81	24.5	α 1.87	29.3	α 1.82
		β 1.63		β 1.65		β 1.65		β 1.65
5	24.6	α 1.90	23.6	α 1.96	24.5	α 1.87	24.9	α 1.96
		β 1.63		β 1.65		β 1.65		β 1.65
6	85.1	4.47	82.2	4.52, d (5.4)	85.1	4.48	83.7	4.52, d (4.8)
7	183.8	-	182.0	-	183.5	-	183.4	-
9	183.8	-	177.7	-	183.5	-	179.1	-
10	12.6	1.15	14.8	1.30 s	12.5	1.16	16.1	1.30 s
11	12.6	1.15			12.5	1.16		
1'	39.6	3.50, t (5.6)	37.9	3.47, t (4.2)	39.3	3.54, t (7.2)	39.2	3.52, t (4.8)
2'	28.0	1.57	26.6	1.55	23.8	1.87	24.0	1.82
3'	22.9	1.57	21.6	1.55	31.9	2.29, t (9.2)	32.1	2.25, t (7.8)
4'	34.2	2.30, t (6.4)	33.0	2.28, t (7.2)	176.4	-	176.8	-
5'	177.0	-	176.0	-				

[a]Overlapped signals are reported without designating multiplicity. [b]Measured at 100 MHz. [c]Measured at 400 MHz. [d]Measured at 150 MHz. [e]Measured at 600 MHz.

EXPERIMENTAL

General

Melting points were obtained on an X-5 micro melting point apparatus without correction (Fukai Instrument, Beijing, China). Optical rotations were determined using a JASCO P-1020 digital polarimeter at 25 °C. UV spectra were recorded on a JASCO V-550 UV/vis spectrophotometer (JASCO, Tokyo, Japan). IR spectra were determined on a JASCO FT/IR-480 plus Fourier transform infrared spectrometer (JASCO, Tokyo, Japan) using KBr pellets. ECD spectra were obtained on a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature. NMR spectra were recorded on Bruker AV-400 and AV-600 spectrometers (Bruker, Fällanden, Switzerland). HRESIMS were carried out on an Agilent 6210 LC/MSD TOF mass spectrometer (Agilent Technologies, CA, USA). Column chromatography (CC) were performed on Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden),

silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Qingdao, P. R. China), and ODS (YMC, Kyoto, Japan). Preparative HPLC was carried out on an Agilent 1260 Chromatograph equipped with a G1311C pump and a G1315D photodiode array detector (Agilent Technologies, CA, USA) with a C18 reversed-phase column (Cosmosil, 10 mm × 250 mm, 5μm). All solvents used in HPLC were of chromatographic grade (Fisher Scientific, New Jersey, USA).

Insect Material

The dried bodies of *Mylabris phalerata* were collected from Kunming city, Yunnan province of P. R. of China, in November 2015. A voucher specimen (no. 2015112801) identified by Prof. Guang-Xiong Zhou (Jinan University) was deposited with the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. of China.

Extraction and isolation

The dried bodies of *M. phalerata* (30.0 kg) were pulverized and extracted three times with 95% EtOH at room temperature and concentrated at reduced pressure to obtain a crude extract. The extract (2.5 kg) was suspended in H_2O and partitioned with petroleum ether to remove the oil components. Then, the aqueous layer was partitioned with EtOAc. The EtOAc-soluble fraction was dried to yield a extract (290.0 g), which was subjected to silica gel column chromatography using $CHCl_3$-MeOH (99:1, 97:3, 95:5, 90:10, 85:15, 80:20, 70:30, 1:1, 0:100) as eluent to afford eight fractions (Fr. A-H). Fr. B (25.5 g) was subjected to silica gel column chromatography (petroleum ether-EtOAc, 95:5, 90:10, 85:15, 80:20, 70:30, 1:1, 0:100) to afford seven subfractions (Fr. B1-B7). Fr. B1 (1.8 g) was separated by reversed-phase preparative HPLC [MeOH-H_2O, (60:40, 5 mL/min)] to afford compound **4** (20.5 mg). Fr. B3 (2.2 g) was chromatographed on Sephadex LH-20 column using $CHCl_3$-MeOH (1:1) and purified by preparative HPLC using MeCN-H_2O (65:35, 5 mL/min) as the mobile phase to obtain compounds **2** (6.5 mg) and **3** (23.0 mg). Fr. B4 (2.3 g) was subsequently purified by reversed-phase preparative HPLC using MeCN-H_2O (75:25, 5 mL/min) as eluent to yield compound **1** (18.4 mg).

Cantharacidine A (1)

Colorless blocks; mp 147-148 ºC; $[\alpha]_D^{25}$ + 15.8 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.45) nm; IR (KBr) ν_{max}: 3438, 2947, 1770, 1702, 1409, 1355, 1265, 1209, 1083, 899, 548 cm^{-1}; ECD (MeCN, $\Delta\varepsilon$) λ_{max} 201.4 (+18.1), 210.7 (-6.4); ^1H NMR (CD_3OD, 600 MHz), ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; HR-ESI-MS *m/z* 296.1490 $[M + H]^+$ (calcd for $C_{15}H_{22}NO_5$, 296.1492).

Cantharacidine B (2)

Yellow oil; $[\alpha]_D^{25}$ + 33.0 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε): 205 (3.43) nm; IR (KBr) ν_{max}: 3449, 2971, 1767, 1701, 1697, 1404, 1358, 1203, 1140, 995, 819, 548 cm^{-1}; ECD (MeCN, $\Delta\varepsilon$) λ_{max} 203.9 (-43.5),

212.3 (+14.1), 246.1 (+31.4); ^{1}H NMR (CD_3OD, 400 MHz), ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; HR-ESI-MS *m/z* 282.1328 $[M+H]^+$ (calcd for $C_{14}H_{20}NO_5$, 282.1336).

Cantharacidine C (3)

Yellow oil; $[\alpha]_D^{25}$ + 11.0 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.26) nm; IR (KBr) ν_{max}: 3439, 2985, 1761, 1699, 1416, 1352, 1130, 1063, 998, 548 cm^{-1}; ECD (MeCN, $\Delta\varepsilon$) λ_{max} 201.0 (+30.2), 209.9 (-5.4); ^{1}H NMR (CD_3OD, 400 MHz), ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; HR-ESI-MS *m/z* 282.1340 $[M+H]^+$ (calcd for $C_{14}H_{20}NO_5$, 282.1336).

Cantharacidine D (4)

Yellow oil; $[\alpha]_D^{25}$ + 26.0 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.19) nm; IR (KBr) ν_{max}: 3575, 3436, 2981, 1766, 1697, 1646, 1442, 1407, 1361, 1191, 1141, 1049, 995, 925, 856, 817, 544 cm^{-1}; ECD (MeCN, $\Delta\varepsilon$) λ_{max} 201.8 (-15.1), 209.1 (+16.6), 246.9 (+21.6); ^{1}H NMR (CD_3OD, 600 MHz), ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; HR-ESI-MS *m/z* 267.1336 $[M+H]^+$ (calcd for $C_{13}H_{19}N_2O_4$, 267.1339).

Single-crystal X-ray crystallography of 1

Colorless blocks, $C_{15}H_{21}NO_5$, monoclinic, $P2_1/n$, a = 8.41720 (10) Å, b = 10.23860 (10) Å, c = 17.71080 (10) Å, β = 101.5320 (10), V = 1495.51 (3) Å^3, Z = 4, d_x = 1.312 g/cm^3, $F(000)$ = 632. Data collection was performed on a SMART CCD using graphite monochromated radiation (λ = 1.54178 Å) under low temperature (nitrogen gas); 3033 unique reflections were collected to θ_{max} = 74.15°, in which 2865 reflections were observed [$F^2 > 4\sigma(F^2)$]. The structures were solved by direct methods (SHELXL) and refined by full-matrix least-squares on F^2. Non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were located by different Fourier techniques and refined with isotropic thermal parameters. The final R_1 = 0.0376, wR_2 = 0.0984 and S = 1.034. Crystallographic data of **1** have been deposited with the Cambridge Crystallographic Data Centre (deposit No. CCDC 1539782). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2, 1EZ, UK (Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk).

Cytotoxic assay

HepG2, MDA-MB-231 and A549 cells were obtained from American Type Culture Collection (ATCC). All of the cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were cultured in 96-well plates for 24 h. Then the cells were treated with compounds **1-4** at various concentrations for 72 h. After incubated for another 4 h with 30 μL aliquot of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS), the medium was discarded, and 100 μL of DMSO was added to

dissolve the produced formazan. The absorbance was measured at 570 nm using a microplate Reader (Thermo scientific multiskan MK3, USA).

ACKNOWLEDGEMENTS

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