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ACULEATUSQUINONES A–D, NOVEL METABOLITES FROM THE MARINE-DERIVED FUNGUS *ASPERGILLUS ACULEATUS*

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Abstract – Four new aculeatusquinones A–D (**1**–**4**) and five known compounds, (5a*S*,6*S*,7*S*)-3,7-dihydroxy-6-methoxy-1,4,6,9-tetramethyl-6,7-dihydro-5a*H*-dibenzo[*b*,*e*][1,4]dioxepine-8,11-dione (**5**), 3,8-dihydroxy-1,4,6,9-tetramethyl-dibenzo[*b*,*e*][1,4]dioxepin-11-one (**6**), 4-*O*-demethylbarbatic acid (**7**), atraric acid (**8**), and 2,5-dimethyl-1,3-benzenediol (**9**), were isolated from the marine-derived fungus *Aspergillus aculeatus*. The structures of the new compounds were elucidated by spectroscopic methods, including one- and two-dimensional NMR and high-resolution mass spectrometric analyses. Two new compounds (**2** and **4**) showed cytotoxic effects on the HL-60, K562, and A-549 cell lines, with IC_{50} values ranging from 5.4 μM to 76.1 μM.

Numerous natural products with novel structures and distinct biological activities have been discovered as the secondary metabolites of marine-derived microbes,[1] and some have been used as drugs, for example, echinocandins (antifungal drugs), ergot alkaloids (for the treatment of migraine), cyclosporine (an immunosuppressive drug), and lovastatin (a cholesterol-lowering drug).[2] To search for new anticancer compounds, more than 300 microbial strains isolated from sediment samples collected from the Min River estuary in China were screened for cytotoxicity against HL-60 cell.[3] Among these strains, a fungal strain identified as *Aspergillus aculeatus* showed significant cytotoxic activity. The broth extract of *A. aculeatus* was separated by chromatography on Si gel and Sephadex LH-20 columns and then purified by

reversed-phase HPLC to yield four new benzoquinone derivatives, aculeatusquinones A–D (**1**–**4**) and five known compounds, (5a*S*,6*S*,7*S*)-3,7-dihydroxy-6-methoxy-1,4,6,9-tetramethyl-6,7-dihydro-5a*H*-dibenzo-[*b*,*e*][1,4]dioxepine-8,11-dione (**5**),[4] 3,8-dihydroxy-1,4,6,9-tetramethyl-dibenzo[*b*,*e*][1,4]dioxepin-11-one (**6**),[5] 4-*O*-demethylbarbatic acid (**7**),[6] atraric acid (**8**),[7] and 2,5-dimethyl-1,3-benzenediol (**9**).[8]

Fungi of the genus *Aspergillus* are sources of many bioactive compounds.[9] Previous chemical investigations on *A. aculeatus* have afforded various bioactive secondary metabolites, such as asperaculin A,[9] aculeacins A–G,[10] CJ-15,183,[11] aspergillusol A,[12] secalonic acids D and F,[13] and okaramines H and I,[14] which exhibit antifungal,[10,11] enzymatic inhibitory,[11,12] and antimicrobial activities.[13] However, benzoquinone derivatives isolated from *A. aculeatus* are reported for the first time. Herein, we report the isolation, structural elucidation and bioactivities of compounds **1**–**9**.

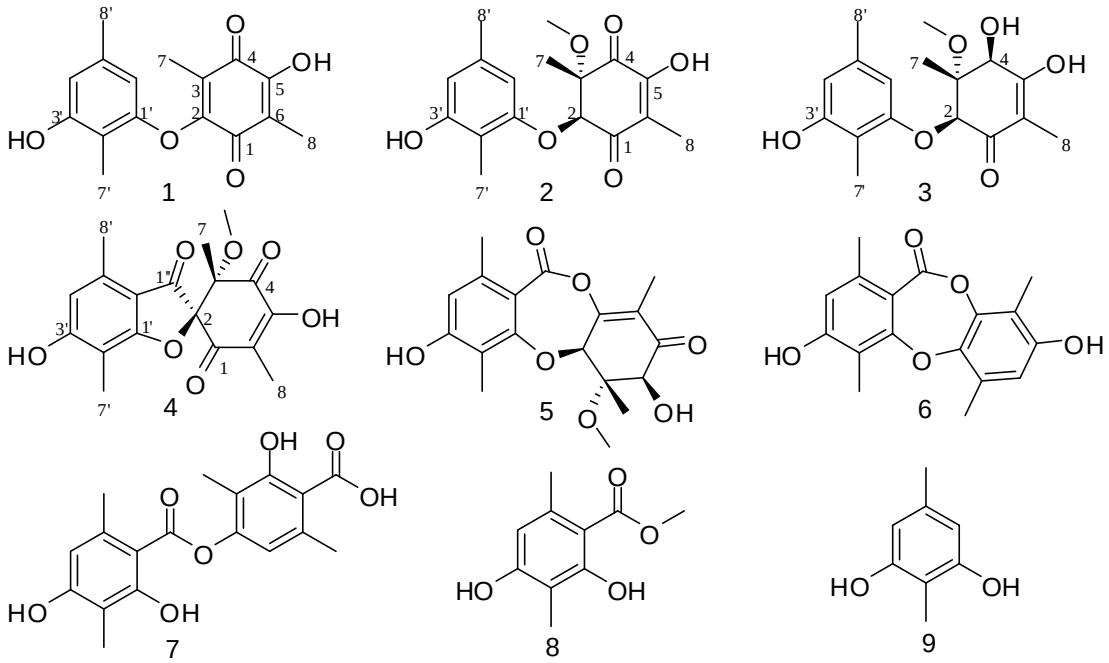

Figure 1. Structures of Compounds **1**–**9**

Table 1. ^{1}H NMR Data (500 MHz, δ in ppm) of Compounds **1**–**4** in DMSO-d_6

No	**1**	**2**	**3**	**4**
2		5.42 (H, s)	5.04 (H, s)	
4			4.68 (H, s)	
7	1.83 (3H, s)	1.38 (3H, s)	1.09 (3H, s)	1.10 (3H, s)
8	1.73 (3H, s)	1.82 (3H, s)	1.61 (3H, s)	1.78 (3H, s)
4'	6.34 (H, brs)	6.28 (H, brs)	6.23 (H, brs)	6.45 (H, s)
6'	6.02 (H, brs)	6.28 (H, brs)	6.19 (H, brs)	
7'	2.05 (3H, s)	1.93 (3H, s)	1.97 (3H, s)	2.07 (3H, s)
8'	2.06 (3H, s)	2.11 (3H, s)	2.11 (3H, s)	2.28 (3H, s)
OMe		3.08 (3H, s)	3.12 (3H, s)	3.09 (3H, s)
5-OH	10.79 (H, s)	10.93 (H, s)	10.06 (H, s)	11.47 (H, s)
3'-OH	9.37 (H, s)	9.22 (H, s)	9.06 (H, s)	11.16 (H, s)

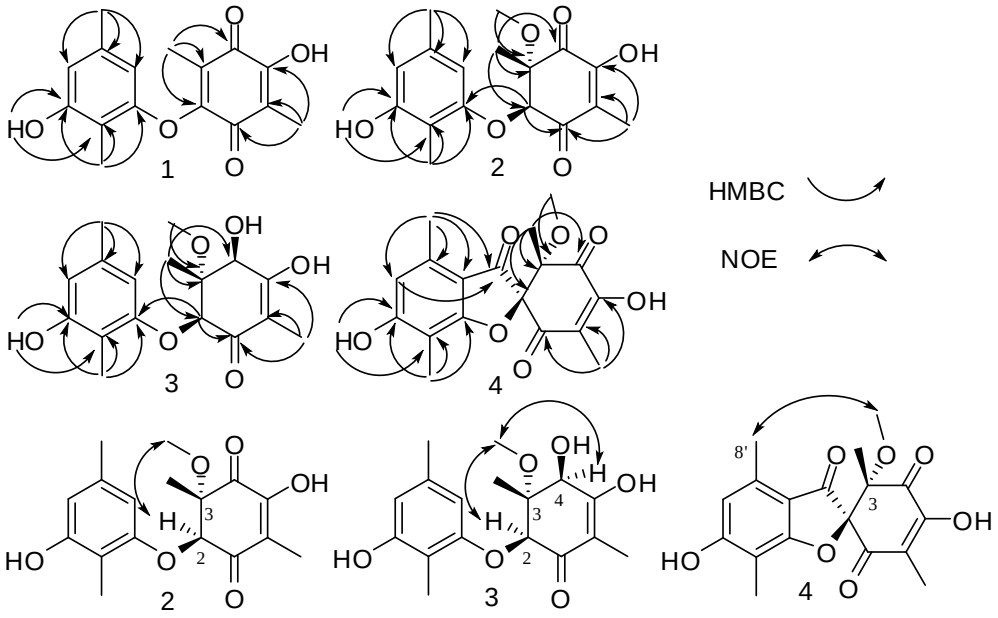

Figure 2. Key HMBC and NOE Correlations of Compounds **1**–**4**

The molecular formula of aculeatusquinone A (**1**) was determined to be $C_{16}H_{16}O_5$ on the basis of HRESIMS (*m/z*: 287.0920 $[M - H]^-$, calcd for $C_{16}H_{15}O_5$, 287.0919). The ^{13}C NMR and DEPT spectra of **1** (Table 2) displayed 16 carbons assignable to one benzene ring, one quinone ring and four methyl groups. The HMBC correlations from 3'-OH to C-2' and C-3', from CH_3-7' to C-1', C-2', and C-3', and from CH_3-8' to C-4', C-5', and C-6' (Figure 2) suggested the presence of a tetrasubstituted benzene ring. Moreover, the HMBC from CH_3-7 to C-2, C-3, and C-4, as well as the HMBC from CH_3-8 to C-1, C-5, and C-6 revealed the presence of a symmetric quinone ring. FT-IR microscopy data was useful in determining that **1** was the 1,4-*p*-quinone rather than the 4,5-*o*-quinone, since *p*-quinones typically exhibit strong IR absorptions near 1645 and 1585 cm^{-1}.[15] Furthermore, an absorption at 3383 cm^{-1} indicated the presence of an intramolecularly H-bonded hydroxyl group, which also proved that **1** was the 1,4-*p*-quinone. The two moieties were further connected via an oxygen atom after full consideration of the molecular formula and the 1D and 2D NMR spectral data. Thus, the structure of aculeatusquinone A was established to be **1**, as shown in Figure 1.

Aculeatusquinone B (**2**) was obtained as yellow oil. The molecular formula was established by negative HRESIMS (*m/z*: 319.1192 $[M - H]^-$, calcd for $C_{17}H_{19}O_6$, 319.1187). The structure of **2** was found to be similar to that of **1** through the ^1H and ^{13}C NMR data comparison, except for the appearance of a methoxy group (δ_H 3.08 and δ_C 52.7), a methine (δ_H 5.42 and δ_C 81.0), and a quaternary carbon (δ_C 83.3), and for the disappearance of two aromatic carbons (δ_C 128.6 and 152.1). In addition, the chemical shifts of the two carbonyl carbons (δ_C 184.1 and 182.4) were mobiled to lower field (δ_C 194.5 and 193.9). After the 1D and 2D NMR spectral data analyses of **1** and **2**, the benzene ring was reserved in **2**. The differences in

the quinone ring were determined by the HMBC of Me-7 with C-2, C-3, and C-4, as well as the HMBC of OMe with C-3 (Figure 2). The two rings were further connected via an oxygen atom, which was determined by its molecular formula and the HMBC correlations between H-2 and C-1', C-1, C-3, and C-7. In the NOE experiment for **2**, enhancement of the signal of the methoxy protons (OMe) occurred on irradiation of the signal of the methine proton (H-2). Therefore, the structure of aculeatusquinone B (**2**) was elucidated, as shown in Figure 1.

Table 2. ^{13}C NMR Data (125 MHz, δ in ppm) of Compounds **1**–**4** in DMSO-d_6

No	1	2	3	4
1	182.4 s	194.5 s	193.9 s	186.6 s
2	152.1 s	81.0 d	80.9 d	99.6 s
3	128.6 s	83.3 s	82.7 s	82.5 s
4	184.1 s	193.9 s	69.3 d	189.1 s
5	154.1 s	156.8 s	169.9 s	159.9 s
6	115.0 s	122.7 s	107.5 s	120.9 s
7	9.1 q	19.9 q	13.4 q	10.7 q
8	8.2 q	9.5 q	8.2 q	9.4 q
1'	156.7 s	157.5 s	158.4 s	174.1 s
2'	109.9 s	110.2 s	109.9 s	105.1 s
3'	156.7 s	156.3 s	156.0 s	165.6 s
4'	110.9 d	109.9 d	109.0 d	113.1 d
5'	136.0 s	135.8 s	135.2 s	138.4 s
6'	106.4 d	105.9 d	105.6 d	108.7 s
7'	8.9 q	8.9 q	8.9 q	7.7 q
8'	21.3 q	21.8 q	21.7 q	17.6 q
1"				191.1 s
OMe		52.7 q	50.6 q	52.1 q

The structure of aculeatusquinone C (**3**) was found by comparing its NMR and MS data with those of **2**. The molecular formula of **3** was determined to be $C_{17}H_{22}O_6$ based on HRESIMS (*m/z*: 321.1349 $[M - H]^-$, calcd for $C_{17}H_{21}O_6$, 321.1344). Its degree of unsaturation is seven, whereas that of **2** is eight. After comparing the ^{1}H, ^{13}C NMR, and DEPT data of **3** and **2** (Tables 1 and 2), an oxygenic methine (δ_H 4.68 and δ_C 69.3) in **3** was found to replace the carbonyl (δ_C 193.9) in **2**. This finding was confirmed by the HMBC and HMQC data. In the NOE experiments for **3**, the signals of H-2 (δ_H 5.04) and H-4 (δ_H 4.68) were enhanced upon irradiation of the signal of OMe (δ_H 3.12), indacating that H-2, H-4, and the methoxy group were on the same side of the plane. Thus, aculeatusquinone C (**3**) was established to be a reduction product of aculeatusquinone B (**2**), as shown in Figure 1.

Aculeatusquinone D (**4**) exhibited an anion peak at *m/z* 345.0981 in HRESIMS (calcd for $C_{18}H_{17}O_7$, 345.0974). The ^{13}C NMR and DEPT spectral data showed four methyl groups, a methoxy group, a

methine, and 12 quaternary carbons. The HMBC from Me-7' to C-1', C-2', and C-3', from Me-8' to C-4', C-5', and C-6', and from 3'-OH to C-2' and C-3' indicated the presence of a pentasubstituted phenyl ring. The HMBC of Me-7 with C-2, C-3, and C-4, Me-8 with C-1, C-5, and C-6, and OMe with C-3 indicated the presence of a quinone ring similar to that of **2**, except for a loss of H-2. In addition, the 1,4-*p*-quinone structure of **4** was determined by its IR data. Considering the weak HMBC from H-4' and Me-8' to C-1", as well as the lower field of C-2 and C-1' than those of **2**, the two moieties were finally connected via an oxygen atom and a carbonyl (C-1"), which is consistent with its molecular formula. In the NOE experiment for **4**, enhancement of the signal of Me-8' occurred on irradiation of the signal of OMe, revealing the relative configurations of C-2 and C-3. Thus, the structure of aculeatusquinone D was established to be **4**, as shown in Figure 1.

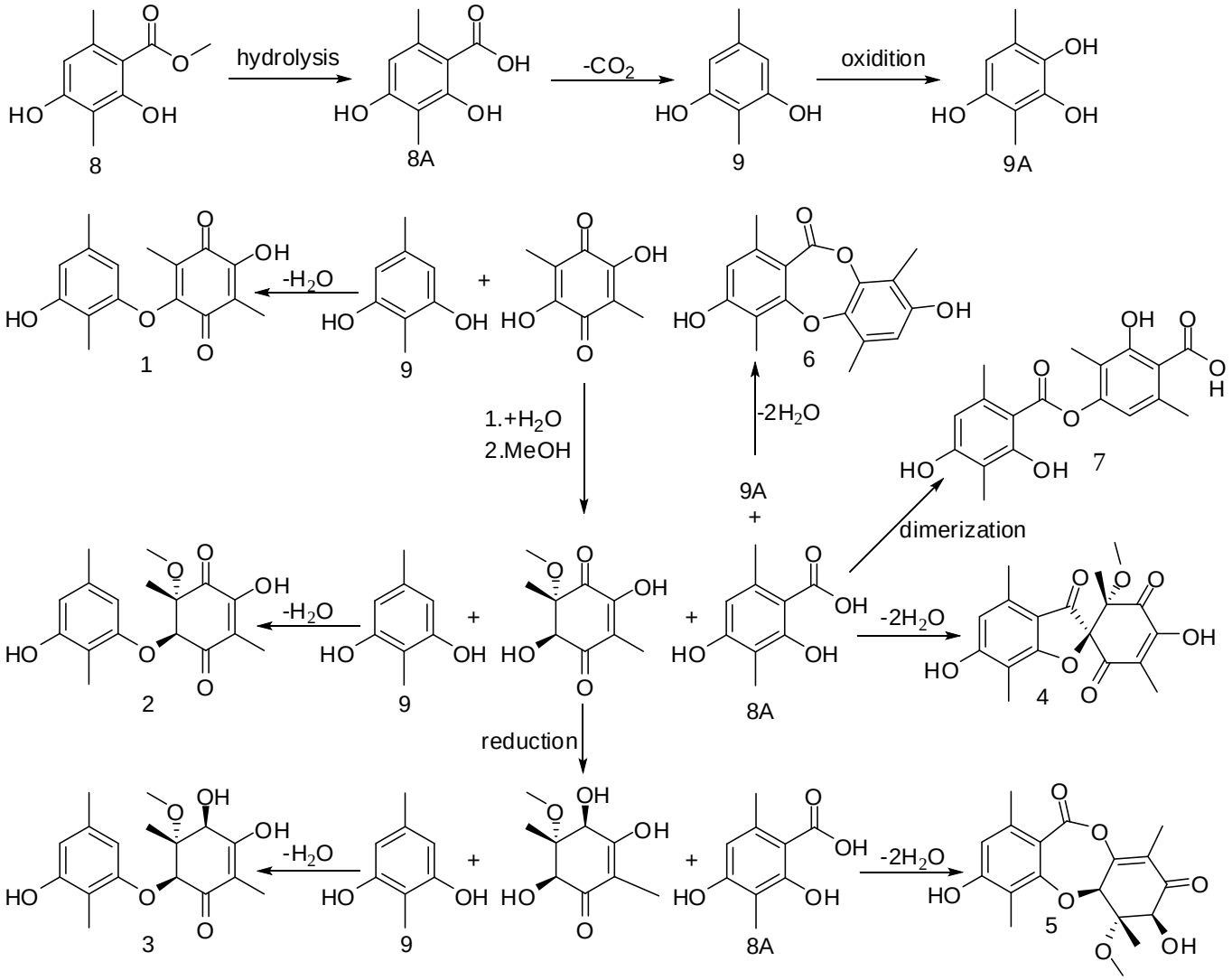

Figure 3. Plausible Biosynthetic Pathway of Compounds **1**–**9**

Structurally, compounds **1**–**7** are composed of two moieties, either a benzene ring and a quinone ring or two benzene rings. The benzene ring can be divided into two types, depending on the presence or absence of the carbonyl group, whereas the quinone ring may be regarded as a derivative product of multi-substituted benzoquinone. Ultimately, these two moieties are connected by dehydration reaction. For a detailed explanation of the interrelationship of these metabolites, a plausible biosynthetic pathway is proposed in Figure 3.

The new compounds (**1**–**4**) were tested for cytotoxic effects on the HL-60 and K562 cell lines using the MTT method and on the A-549 cell line using the SRB method.[16] Compounds **2** and **4** showed moderate cytotoxicity against HL-60 cell, with IC_{50} values of 7.3 and 5.4 μM, and weak activity against A-549 and K562 cells, with IC_{50} values of 42.3, 76.1, 37.2, and 29.7 μM. By contrast, compounds **1** and **3** were inactive (Table 3).

Table 3. Cytotoxicities of Compounds **1**–**4** in Three Cancer Cell Lines

	cytotoxicity (IC_{50}, μM)		
compounds	A-549 cell	HL-60 cell	K562 cell
1	>100	>100	>100
2	42.3	7.3	76.1
3	>100	>100	>100
4	37.2	5.4	29.7

EXPERIMENTAL

General Experimental Procedures. Optical rotations were obtained from a Shenguang SGW-1 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. IR spectra were recorded on a Nicolet Avatar 670 spectrophotometer. ^{1}H NMR, ^{13}C NMR, DEPT spectra and 2D NMR were recorded on a BRUKER BIOSPIN AVANCE III spectrometer using TMS as the internal standard. HRESIMS were obtained by an Agilent Q-TOF 6520 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (ODS-A, 10×250 mm, 5 μm) at 5 mL/min.

Fungal Material. The fungus *A. aculeatus* was isolated from marine sediments collected from Langqi Island, Fujian, China. It was identified according to its morphological characteristics and ITS by Beijing Sunbiotech Co. Ltd, and preserved in our laboratory at −80 °C. The producing strain was prepared on Martin medium and stored at 4 °C.

Fermentation and Extraction. The fungus *A. aculeatus* was cultured under static conditions at 28 °C for 30 days in 1000 mL conical flasks containing the liquid medium (400 mL/flask), composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L),

$MgSO_4 \cdot 7H_2O$ (0.3 g/L), yeast extract (3 g/L), and seawater. The fermented whole broth (60 L) was filtered through cheese cloth to separate supernatant from mycelia. The former was extracted two times with EtOAc to yield an EtOAc solution that was concentrated under reduced pressure to give a broth extract (48.9 g).

Purification. The crude extract (48.9 g) of the fungus *A. aculeatus* was separated into five fractions on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction 3 (5.6 g) was further purified on a Sephadex LH-20 column ($CHCl_3$: MeOH, 1 : 2) to afford four subfractions. Subfraction 3-2 (1.3 g) was subjected on a Si gel column using a step gradient elution of CH_2Cl_2 and MeOH, followed by semipreparative HPLC (40% MeCN, 0.1% TFA) to yield compounds **1** (8.7 mg, t_R 21.2 min), **2** (10.3 mg, t_R 15.1 min), **3** (9.4 mg, t_R 11.6 min), **4** (6.5 mg, t_R 13.4 min), and **5** (6.1 mg, t_R 18.5 min). Subfraction 3-3 (850 mg) was purified by semipreparative HPLC (50% MeCN, 0.1% TFA), yielding compounds **6** (5.5 mg, t_R 9.3 min), **7** (4.2 mg, t_R 11.4 min), **8** (7.4 mg, t_R 7.1 min), and **9** (9.4 mg, t_R 5.2 min).

Aculeatusquinone A (**1**): yellow oil (MeOH); UV (MeOH) λ_{max} 277 nm; IR (KBr) ν_{max} 3383, 2989, 1645, 1585, 1296, 1108 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 287.0920 $[M - H]^-$, calcd for $C_{16}H_{15}O_5$, 287.0919).

Aculeatusquinone B (**2**): yellow oil (MeOH); $[\alpha]^{25}_D$ +213.7 (*c* 0.11, MeOH); UV (MeOH) λ_{max} 278 nm; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 319.1192 $[M - H]^-$, calcd for $C_{17}H_{19}O_6$, 319.1187).

Aculeatusquinone C (**3**): yellow oil (MeOH); $[\alpha]^{25}_D$ +245.4 (*c* 0.17, MeOH); UV (MeOH) λ_{max} 263 nm; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 321.1349 $[M - H]^-$, calcd for $C_{17}H_{21}O_6$, 321.1344).

Aculeatusquinone D (**4**): colorless oil (MeOH); $[\alpha]^{25}_D$ +379.6 (*c* 0.15, MeOH); UV (MeOH) λ_{max} 292 nm; IR (KBr) ν_{max} 3409, 2937, 1699, 1658, 1590, 1381, 1092 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 345.0981 $[M - H]^-$, calcd for $C_{18}H_{17}O_7$, 345.0974).

Biological Assays. The cytotoxic activity for the HL-60 and K-562 cancer cell lines was evaluated by the MTT method, whereas that for the A-549 cell line was evaluated by the SRB method.[16] Doxorubicin was used as the reference drug.

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