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Formamido-Diterpenes from the South China Sea Sponge *Acanthella cavernosa*

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Received: 15 May 2012; in revised form: 1 June 2012 / Accepted: 16 June 2012 /

Published: 2 July 2012

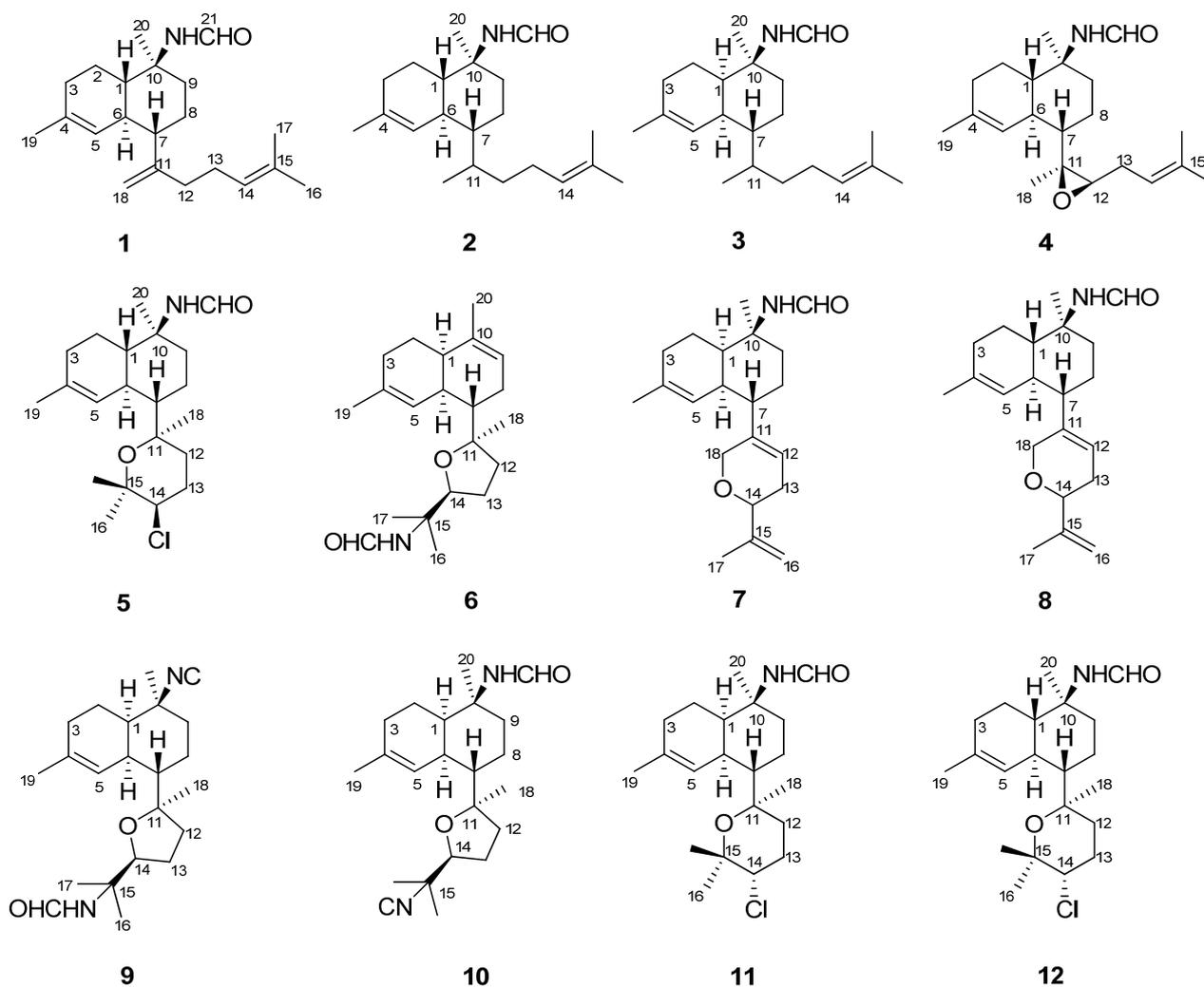
Abstract: Seven new formamido-diterpenes, cavernenes A–D (1–4), kalihinenes E and F (5–6), and kalihipyran C (7), together with five known compounds (8–12), were isolated from the South China Sea sponge *Acanthella cavernosa*. Structures were established using IR, HRESIMS, 1D and 2D NMR, and single X-ray diffraction techniques. The isolated compounds were assessed for their cytotoxicity against a small panel of human cancer cell lines (HCT-116, A549, HeLa, QGY-7701, and MDA-MB-231) with IC₅₀ values in the range of 6–18 μM. In addition, compound 9 showed weak antifungal activity against *Trichophyton rubrum* and *Microsporium gypseum* with MIC values of 8 and 32 μg/mL, respectively, compound 10 displayed weak antifungal activity against fungi *Candida albicans*, *Cryptococcus neoformans*, *T. rubrum*, and *M. gypseum* with MIC values of 8, 8, 4, and 8 μg/mL, respectively.

Keywords: *Acanthella cavernosa*; formamido-diterpenes; cytotoxicity; antifungal activity

1. Introduction

Marine sponges of the genus *Acanthella* have proven to be a rich source of new diterpenes and sesquiterpenes containing nitrogenous functional groups, including isonitrile ($-\text{NC}$), isothiocyanate ($-\text{NCS}$), isocyanate ($-\text{NCO}$), and formamide ($-\text{NHCHO}$) functionalities, which show various promising biological activities [1–7]. Diterpenes isolated from this genus have demonstrated cytotoxic [8], anthelmintic [9,10], antimalarial [11], antimicrobial [12], antifungal [1,13,14], and antifouling activities [15–17]. In general, these diterpenes can be divided into two types, defined by having a *trans*- or *cis*-decalin moiety. The diterpene precursor has been supposed to be geranylgeraniol, which cyclizes to form a *cis*- or *trans*-biflorane skeleton [6]. In our previous study, we had reported the isolation of eight new diterpenes, kalihinols M–T, together with seven known compounds from the CH_2Cl_2 extract of the sponge *A. cavernosa* [18]. As part of our interest on bioactive secondary metabolites from the marine sponges of the genus *Acanthella*, a petroleum ether extract of *A. cavernosa* was investigated and found to be cytotoxic. Further bioassay-guided fractionation of the extract led to the isolation of 12 formamido-diterpenes (**1–12**), including seven new ones (**1–7**) (Figure 1). Details of the isolation, structure elucidation, cytotoxic and antifungal activities of these compounds are described herein.

Figure 1. Structures **1–12** isolated from *Acanthella cavernosa*.



2. Results and Discussion

The sponge *A. cavernosa* was exhaustively extracted with acetone, and after being subjected to extensive column chromatography on silica gel, Sephadex LH-20, ODS, and semipreparative HPLC, compounds **1–12** were obtained. The known compounds, kalihipyran A (**8**) [16], 15-formamido-kalihinine (**9**) [6], 10-formamido-kalihinine (**10**) [6], and kalihinenes X (**11**) and Y (**12**) [15], were identified by comparison of their spectroscopic data with literature values.

In the ^1H and ^{13}C NMR spectra of compounds **1–7**, most signals were doubled in the ratio of $\sim 1.8:1$, $2.2:1$, $1.1:1$, $1.4:1$, $2.1:1$, $1.9:1$, and $1.1:1$, respectively. This suggested that **1–7** existed as equilibrium mixtures derived from the *s-trans* and *s-cis* forms of formamide groups, as in the cases of **8–12** [6,15,16], which was supported by IR absorptions (**1**, 1670; **2**, 1681; **3**, 1668; **4**, 1681; **5**, 1682; **6**, 1686; and **7**, 1668 cm^{-1}) [16]. The assignments of the formamide groups (NH and CHO) in **1–7** for *s-trans* isomers and *s-cis* isomers were confirmed by HSQC and COSY data (Tables 1 and 2).

Table 1. ^1H NMR data of compounds **1–7** (CDCl_3 , J in Hz).

Position	1 ^{a,c}	2 ^{b,c}	3 ^{a,c}	4 ^{a,c}	5 ^{b,c}	6 ^{a,c}	7 ^{a,d}
1	1.24 m	1.26 m ^e	1.65 m ^e	1.30 m ^e	1.30 m	1.96 m	2.40 m
2a	1.84 m	1.34 m	1.73 m	1.85 m	1.84 m ^e	1.87 m	1.48 m ^e
2b	1.30 m	1.26 m ^e	1.65 m ^e	1.30 m ^e	1.28 m	1.48 m	1.48 m ^e
3a	1.99 m ^e	1.97 m ^e	1.99 m ^e	2.00 m ^e	1.93 m ^e	2.01 m	2.00 m ^e
3b	1.99 m ^e	1.97 m ^e	1.99 m ^e	2.00 m ^e	1.93 m ^e	1.89 m	2.00 m ^e
5	5.24 br s	5.47 br s	5.49 br d (4.0)	5.69 br s	6.37 br s	5.70 br s	5.39 br s
6	2.05 m	1.91 m	2.15 m	2.18 m	2.11 m	2.20 m	2.20 m
7	1.73 m	1.14 m	1.43 m ^e	1.17 m	1.58 m	1.62 m	1.78 m ^e
8a	1.66 m	1.53 m	1.43 m ^e	1.43 m	1.10 m	1.74 m	1.55 m ^e
8b	1.50 m	1.26 m	1.26 m	1.57 m	1.75 m	1.94 m	1.55 m ^e
9a	1.89 m	1.88 m	1.65 m ^e	1.90 dt (9.5, 3.5)	1.89 m		1.55 m ^e
9b	1.62 m ^e	1.56 m	1.55 m ^e	1.60 m	1.62 m	5.32 br s	1.55 m ^e
11		1.97 m ^e	1.75 m				
12a	1.99 m ^e	1.26 m ^e	1.24 m ^e		1.28 m ^e	1.78 m	
12b	1.99 m ^e	1.26 m ^e	1.24 m ^e	2.62 t (6.5)	1.28 m ^e	1.63 m	5.61 m ^e
13a	2.13 m	1.97 m ^e	1.99 m ^e	2.23 m ^e	2.27 m	1.83 m	2.18 m
13b	1.99 m ^e	1.97 m ^e	1.90 m	2.23 m ^e	1.97 m	1.56 m	2.13 m
14	5.13 t (5.5)	5.11 t (6.0)	5.08 br s	5.19 t (6.5)	3.93 m	3.75 t (7.0)	3.89 br s
16	1.69 s	1.69 s	1.67 s	1.72 s	1.39 s	1.31 s	4.88 s
17	1.62 s ^e	1.60 s	1.59 s	1.62 s	1.31 s	1.20 s	5.01 s
18	4.89 br s						1.78 s ^e
	4.80 br s	0.78 d (6.9)	0.83 d (6.5)	1.26 s	1.22 s ^e	1.18 s	4.16 br s ^e
19	1.62 s ^e	1.67 s	1.65 s ^e	1.68 s	1.65 s	1.68 s	4.16 br s ^e
20	1.28 s	1.22 s	1.43 s ^e	1.27 s	1.22 s ^e	1.68 s	1.62 s
NH	5.89 d (12.5)	6.10 d (11.8)	5.62 d (12.0)	5.92 d (12.0)	5.66 d (12.5)	5.86 m	5.61 m ^e
CHO	8.30 d (12.0)	8.27 d (12.3)	8.24 d (12.5)	8.29 d (12.5)	8.28 d (12.5)	8.19 d (12.5)	8.08 d (2.0)

^a Measured at 500 MHz; ^b Measured at 400 MHz; ^c Data for major *s-trans* isomer; ^d Data for major *s-cis* isomer; ^e Overlapped.

Cavernene A (**1**) was isolated as a colorless oil. A molecular formula of $C_{21}H_{33}ON$ was established by the $[M + Na]^+$ ion peak at m/z 338.2461 in the HRESIMS and supported by NMR data (Tables 1 and 2), indicating six degrees of unsaturation. The 1H NMR spectrum showed the presence of four tertiary methyl [δ_H 1.28 (3H, s), 1.62 (6H, s), and 1.69 (3H, s)] and four olefinic proton signals resonated at δ_H 4.80 (1H, br s), 4.89 (1H, br s), 5.13 (1H, t, $J = 5.5$ Hz), and 5.24 (1H, br s). The ^{13}C NMR and DEPT spectra of **1** displayed 21 carbon resonances for four methyls, seven methylenes (one olefinic), six methines (two olefinic and one formamido), and four quaternary carbons (three olefinic). The above moieties accounted for four of six degrees of unsaturation, indicating a bicyclic structure for **1**. The COSY correlations of H-9/H-8/H-7/H-6/H-1/H-2/H-3/H-5/H-19, together with the HMBC correlations of H₃-19/C-3, C-4, and C-5, H₃-20/C-1, C-9, and C-10, H-5/C-1, C-3, C-6, and C-7, and CHO/C-10 indicated the presence of a decalin moiety (Figure 2). The HMBC correlations from the geminal methyls (H₃-16 and H₃-17) to C-14 and C-15, from terminal olefinic protons (H₂-18) to C-7, C-11, and C-12, and from H-13 to C-11, C-12, C-14, and C-15 and COSY correlation between H-13 and H-14 revealed the presence of an isoprenoid unit homolog in **1** and connectivity of the two moieties between C-12 and C-7 through the quaternary carbon C-11.

Table 2. ^{13}C NMR data ($CDCl_3$, δ in ppm) of compounds **1**–**7**.

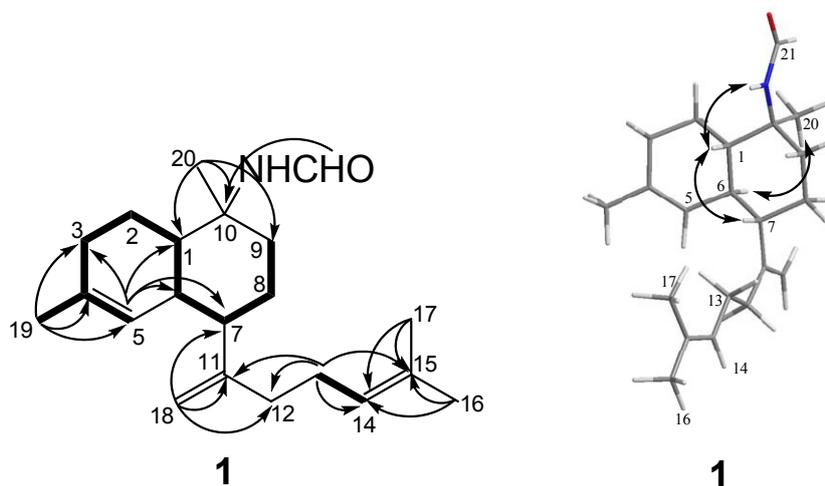
C	1 ^a		2 ^b		3 ^a		4 ^a		5 ^b		6 ^a		7 ^a	
	<i>s-trans</i>	<i>s-cis</i>	<i>s-cis</i>	<i>s-trans</i>										
1	48.8	45.3	49.0	45.7	45.6	41.1	48.4	45.0	48.8	46.1	40.1	40.1	40.9	45.4
2	22.8	23.1	22.9	23.4	19.1	19.5	22.7	23.1	23.3	23.7	24.6	24.7	18.8	19.1
3	30.96	31.04	30.8	30.9	31.3	31.4	30.8	30.9	30.5	30.6	30.5	30.6	31.1	31.2
4	134.4	134.1	134.9	134.6	134.3	134.2	134.8	134.3	132.4	131.9	131.1	130.8	134.5	134.4
5	123.3	123.8	121.8	122.3	124.3	124.0	122.3	122.7	125.7	126.2	127.1	127.3	123.9	124.2
6	39.5	39.4	38.4	38.2	35.2	34.8	38.6	38.4	39.5	39.2	36.5	36.5	37.0	36.5
7	50.3	50.3	44.6	44.4	42.3	42.0	45.2	45.2	51.2	50.4	44.9	45.2	43.3	43.6
8	29.7	29.9	20.9	20.9	20.1	19.9	25.7	25.8	24.5	24.7	29.48	29.51	28.4	28.4
9	42.0	37.6	41.8	37.4	33.0	33.7	41.7	37.3	42.1	37.8	120.2	120.5	33.5	32.8
10	55.5	57.2	55.6	57.3	55.5	57.1	55.3	56.8	55.4	56.9	136.9	136.8	56.8	55.4
11	151.3	151.9	30.8	30.8	31.3	31.1	62.33	62.7	76.9	77.2	86.4	86.3	139.4	139.0
12	34.2	34.2	35.65	35.69	35.7	35.8	62.27	62.33	31.0	31.7	37.2	37.1	118.6	118.9
13	26.3	26.3	26.2	26.2	26.2	26.3	27.0	27.0	25.9	26.1	25.6	25.8	29.6	29.6
14	124.2	124.3	124.6	124.8	124.7	124.9	119.3	119.5	64.2	64.5	84.2	84.5	77.2	76.8
15	131.7	131.6	131.3	131.1	131.2	131.1	134.2	134.1	74.2	74.4	54.5	55.9	145.4	145.3
16	25.7	25.7	25.7	25.7	25.7	25.7	25.7	25.7	29.7	29.7	27.4	25.1	110.5	110.6
17	17.8	17.8	17.7	17.7	17.7	17.7	18.0	18.0	28.9	28.4	23.8	21.7	18.9	18.9
18	110.0	109.6	13.3	13.3	13.3	13.3	18.5	18.5	21.9	21.2	20.3	21.1	67.63	67.58
19	23.3	23.4	23.6	23.7	23.5	23.4	23.55	23.60	23.8	23.8	23.92	23.87	23.3	23.4
20	19.0	18.9	18.8	18.7	27.2	23.7	18.9	18.7	19.0	18.5	21.53	21.47	23.6	29.7
21	162.8	160.4	163.1	160.5	162.8	160.0	162.8	160.4	162.7	160.4	163.1	160.9	160.1	162.8

^a Measured at 125 MHz; ^b Measured at 100 MHz.

The relative configuration of **1** was established on the basis of NOESY data (Figure 2). The NOESY correlations of H-1/H-7 and NH/H-1 indicated that these protons were on the same face of the

decalin ring and arbitrarily assigned β -orientations. The α -orientation of H-6 was determined by the NOESY correlation between H₃-20 and H-6. In addition, the carbon resonances at δ_C 48.8 (C-1), 39.5 (C-6), 42.0 (C-9), and 19.0 (C-20) in **1** further confirmed the *trans* fusion of the decalin ring [6,8,15]. Cavernene A (**1**) can be envisaged as a decomposable intermediate product of isocyanobifloradiene epoxides in the plausible biogenetic pathway of kalihiprans [3,19].

Figure 2. COSY (—), key HMBC (→), and key NOESY correlations of **1**.



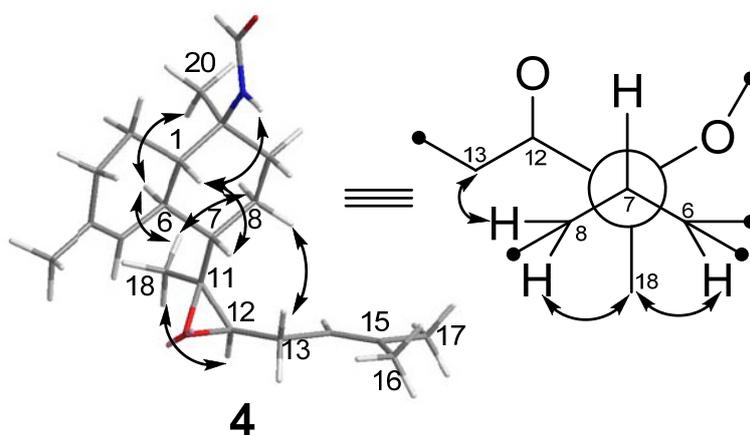
Cavernene B (**2**) was obtained as a colorless oil. Its molecular formula of C₂₁H₃₅ON was deduced from the HRESIMS (m/z 340.2617 [M + Na]⁺) combined with its NMR data (Tables 1 and 2), indicating five degrees of unsaturation. The ¹H and ¹³C NMR spectra of **1** and **2** were comparable except for a marked difference in the isoprenoid unit. Signals for a C-11–C-18 double bond were absent in **2** and replaced by saturated carbons resonated at δ_C 30.8 and 13.3, respectively. The HMBC correlations of H₃-18/C-7, C-11, and C-12 and the COSY correlations of H₃-18/H-11/H-12 and H-11/H-7 supported the assignment of cavernene B as **2**. The relative configuration of the decalin ring in **2** was the same as in **1**, with the observation of the NOESY correlations of NH/H-1, H₃-20/H-6, NH/H-9b, H-7/H-9b, and H₃-20/H-9a (Supporting Information). The relative configuration at C-11, however, could not be conclusively determined due to conformational flexibility between C-7 and C-11.

Cavernene C (**3**), obtained as white needles, showed the same molecular formula of C₂₁H₃₅ON as **2** determined by pseudomolecular [M + Na]⁺ ion peak at m/z 340.2614 in HRESIMS. Its carbon skeleton was readily assignable as the same as **2** by HSQC, HMBC, and COSY spectra. In particular, the carbon resonances of the isoprenoid unit in **3** were almost superimposable on those of **2** (Table 2), suggesting the same stereostructure of the isoprenoid unit for both **2** and **3**. On the other hand, differences were observed for the signals of the decalin ring. Specifically, the carbon resonances at δ_C 49.0 (C-1), 38.4 (C-6), 41.8 (C-9), and 18.8 (C-20) in **2** were replaced by resonances at δ_C 45.6, 35.2, 33.0, and 27.2 in **3**, respectively, indicating a *cis* fusion of the decalin ring [6,8,15]. The relative configuration at C-11, however, could not be conclusively determined due to the free rotation around the C-7–C-11 bond.

Cavernene D (**4**) displayed a HRESIMS [M + Na]⁺ peak at m/z 354.2407 corresponding to a molecular formula of C₂₁H₃₃O₂N, implying six degrees of unsaturation. Many similarities of the ¹H and ¹³C NMR data between **2** and **4** (Tables 1 and 2) suggested they were structural analogs, with the main differences due to the presence of a trisubstituted epoxide (δ_C 62.33 and 62.27) in **4** and the

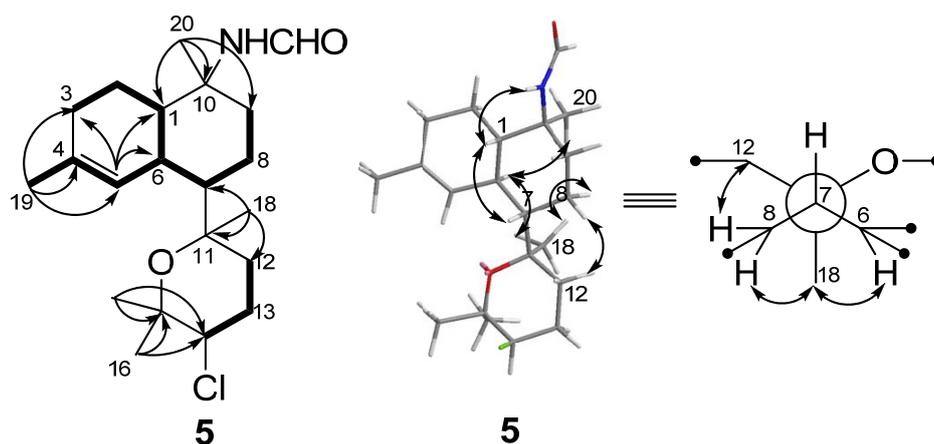
absence of two saturated carbons (δ_C 30.8 and 35.65) in **2**. The C-11/C-12 position of the epoxy group was determined by a COSY correlation of H-12/H-13 and HMBC correlations of H₃-18/C-7, C-11, and C-12 and H-13/C-11 and C-12. The relative configuration of the decalin ring of **4**, found to agree with **2**, was established by observation of NOESY correlations of NH/H-1, H-1/H-7, and H₃-20/H-6. The relative configurations of C-7 and C-11 between the conjoined bicyclic ring systems in **4** were assigned as 7*S** and 11*S**, respectively, based on NOESY correlations of H₃-18/H-6, H₃-18/H-8b, and H-8a/H-13, as shown in the Newman projection (Figure 3). Thus, the epoxy group was determined as in β -orientation.

Figure 3. Key NOESY correlations of **4**.



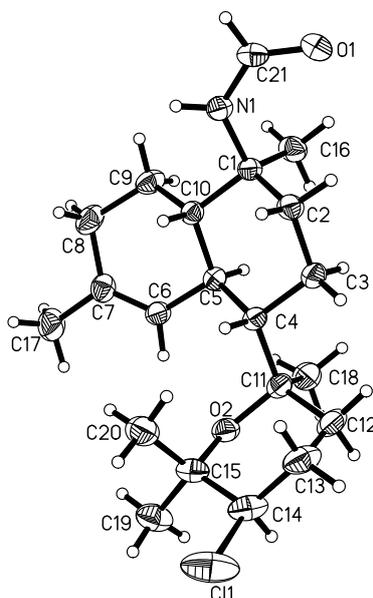
Kalihinene E (**5**) was isolated as colorless needles (MeOH), and given a C₂₁H₃₄NO₂Cl molecular formula with five degrees of unsaturation, based on the HRESIMS (m/z 368.2354 [M + H]⁺) and NMR spectra. The ESIMS of **5** showed a cluster of isotopic [M + H]⁺ ion peaks at m/z 368/370 in a ratio of ~3:1, indicating the presence of a chlorine atom in the molecule. The NMR spectra of **5** revealed the presence of five methyls [δ_H 1.22 (6H, s), 1.31 (3H, s), 1.39 (3H, s), and 1.65 (3H, s)], an olefinic methine [δ_H 6.37 (1H, br s), δ_C 125.7 (CH), and δ_C 132.4 (qC)], a chlorine-bearing methine [δ_H 3.93 (1H, m)/ δ_C 64.2], and two oxygenated quaternary carbons (δ_C 74.2 and 76.9) for the major *s-trans* isomer (Tables 1 and 2). Analysis of the 2D NMR (HSQC, HMBC, and COSY) data (Figure 4) revealed that **5** possessed the same carbon skeleton as kalihinene X (**11**) and Y (**12**) [15].

Figure 4. COSY (—), key HMBC (→), and key NOESY correlations of **5**.



The relative configuration of the decalin ring in **5** was the same as **12**, inferred from the chemical shifts of C-1 to C-10 and NOESY correlations of H-1/H-7, H₃-20/H-6, and NH/H-1. A significant difference between **5** and **12** was found in the chemical shift of C-12 (δ_C 31.0 in **5** instead of δ_C 38.2 in **12**), which was caused by the γ -*gauche* effect [20–24], indicating an *axial* orientation of Cl-14. The relative configurations of C-7 and C-11 in **5** were determined as *S** and *R**, respectively, from NOESY correlations of H₃-18/H-6, H₃-18/H-8b, and H-8a/H-12, as shown in the Newman projection (Figure 4). Finally, the absolute configuration of **5** was unambiguously determined as 1*S*, 6*S*, 7*S*, 10*S*, 11*R*, and 14*R* by single crystal X-ray diffraction using Cu K α radiation (Figure 5).

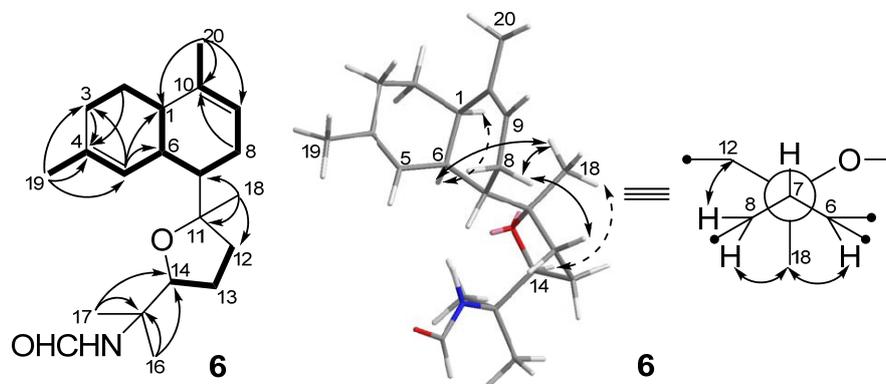
Figure 5. ORTEP drawing of compound **5**.



Kalihinene F (**6**) was isolated as a colorless oil and given a C₂₁H₃₃NO₂ molecular formula based on HRESIMS measurements (m/z 354.2406 [M + Na]⁺) in combination with extensive NMR analysis. The NMR spectra of **6** (Tables 1 and 2) revealed the presence of five methyls [δ_H 1.18 (3H, s), 1.20 (3H, s), 1.31 (3H, s), and 1.68 (6H, s)], two trisubstituted double bonds [δ_H 5.70 (br s), δ_C 127.1 (CH), and δ_C 131.1 (qC); δ_H 5.32 (br s), δ_C 120.2 (CH), and δ_C 136.9 (qC)], an oxymethine [δ_H 3.75 (t, $J = 7.0$ Hz)/ δ_C 84.2], and an oxygenated quaternary carbons (δ_C 86.4) for the major *s-trans* isomer. The COSY correlations of H₃-20/H-9/H-8/H-7/H-6/H-1/H-2/H-3 and H-6/H-5/H₃-19, together with the HMBC correlations of H₃-19/C-3, C-4, and C-5, H₃-20/C-1, C-10, and C-9, H-5/C-1, C-3, and C-6, H-2 and H-3/C-4, and H-8/C-10 indicated the presence of a decalin moiety (Figure 6). A tetrahydrofuran ring, attached to the decalin ring at C-7, was established by carbon resonances at δ_C 84.2 (C-14) and 86.4 (C-11), COSY correlations of H-12/H-13/H-14, HMBC correlations of H₃-18/C-7, C-11, and C-12 and geminal methyls (H₃-16 and H₃-17)/C-14 and C-15, and NOESY correlation between H₃-18 and H-14. The location of the formamide functionality was assigned to be at C-15 by the observation of doubled singlets for H₃-16 [δ_H 1.31 (s) and 1.38 (s) for *s-trans* and *s-cis* isomers, respectively] and H₃-17 [δ_H 1.20 (s) and 1.34 (s) for *s-trans* and *s-cis* isomers, respectively] and doubled triplets for H-14 [δ_H 3.75 (t, 7.0) and 3.80 (t, 7.0) for *s-trans* and *s-cis* isomers, respectively] [6]. The relative configuration of **6** was determined by NOESY correlation of H-1/H-6 and carbon resonances at δ_C 40.1

(C-1), 36.5 (C-6), and 44.9 (C-7) [6]. NOESY correlations of H₃-18/H-6, H₃-18/H-8b, and H-8/H-12b defined the relative configurations of 7*S**, 11*R**, as shown in the Newman projection (Figure 6).

Figure 6. COSY (—), key HMBC (→), and key NOESY (↔) correlations of **6**.



Kalihypran C (**7**), a colorless oil, had a molecular formula of C₂₁H₃₁NO₂ established by HRESIMS at *m/z* 352.2255 [M + Na]⁺, indicating seven degrees of unsaturation. Its ¹H and ¹³C NMR spectra (Tables 1 and 2) showed the presence of three methyls [δ_{H} 1.55 (3H, s), 1.62 (3H, s), 1.78 (3H, s)], two trisubstituted double bonds [δ_{H} 5.39 (br s), δ_{C} 123.9 (CH), and δ_{C} 134.5 (qC); δ_{H} 5.61 (br s), δ_{C} 118.6 (CH), and δ_{C} 139.0 (qC)], a disubstituted double bond [δ_{H} 4.88 (br s), 5.01 (br s), δ_{C} 110.5 (CH₂), and δ_{C} 145.4 (qC)], an oxymethylene [δ_{H} 4.16 (s, 2H)/ δ_{C} 67.63], and an oxymethine δ_{H} [3.89 (br s)/ δ_{C} 77.2] for *s-cis* isomer. Many similarities of the ¹H and ¹³C NMR data between **7** and **8** suggested they were structural analogs [16], the COSY correlations of H₃-19/H-5/H-6, H₃-17/H-16, and H-18/H-12/H-13/H-14 and the HMBC correlations of H₃-19/C-3, C-4, and C-5, H₃-20/C-1, C-9, and C-10, H₃-17/C-14, C-15, and C-16, and H₂-16/C-14, C-15, and C-17 further confirmed **7** possessed the same carbon skeleton as **8**. The chemical shifts of C-1, C-6, and C-20 for *s-trans* isomers in **7** (δ_{C} 45.4, 36.6, and 29.7, respectively) were different from those in **8** (δ_{C} 48.9, 39.4, and 18.9, respectively), indicated *cis* fusion of the decalin ring in **7** [16]. The NOESY correlations of H-1/H-6, H-1/H₃-20, and H-6/H₃-20 confirmed the relative configurations as **7**. However, the relative configuration of H-14 was not determined.

The isolated compounds were assessed for their cytotoxicity against a small panel of human cancer cell lines (human colon cancer cell line HCT-116, human lung epithelial cell line A549, human cervical carcinoma cell line HeLa, human hepatocellular carcinoma cell line QGY-7701, and human mammary cancer cell line MDA-MB-231) using a MTT method, and camptothecin (Shanghai Diba Chemical Co., Shanghai, China; purity $\geq 98\%$) was used as positive control. Compounds **1** and **2** showed moderate cytotoxic activities against HCT-116 with IC₅₀ values of 6.31 and 8.99 μM , respectively. Compounds **5** showed cytotoxic activity against HCT-116, HeLa, QGY-7701 and MDA-MB-231 with IC₅₀ values of 14.36, 13.36, 17.78 and 12.84 μM , respectively (Table 3). In addition, compounds **1–12** were tested for antifungal activity against fungi *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Microsporum gypseum*, and *Aspergillus fumigatus*. Compound **9** showed weak antifungal activity against *T. rubrum* and *M. gypseum* with MIC values of 8 and 32 $\mu\text{g/mL}$, respectively. Compound **10** displayed weak antifungal activity against fungi *C. albicans*, *C. neoformans*, *T. rubrum*, and *M. gypseum* with MIC values of 8, 8, 4, and 8 $\mu\text{g/mL}$, respectively. Ketoconazole (Shanghai Aiyuan

Chemical Co., Shanghai, China; purity $\geq 98\%$) was used as positive control with MIC value ≤ 0.25 $\mu\text{g/mL}$. It is worth noting that the isonitrile functionalities in the diterpenes play an important role in their antifungal activity.

Table 3. Cytotoxicities of compounds 1–12 in five cancer cell lines.

	Cytotoxicity IC ₅₀ (μM)				
	HCT-116	A549	HeLa	QGY-7701	MDA-MB-231
1	6.31	>50	>50	>50	>50
2	8.99	>50	>50	>50	>50
3	>50	>50	>50	>50	>50
4	>50	>50	>50	>50	>50
5	14.36	>50	13.36	17.78	12.84
6	>50	>50	>50	>50	>50
7	>50	>50	>50	>50	>50
8	>50	13.09	11.19	13.53	>50
9	>50	17.53	14.74	16.39	>50
10	>50	6.98	13.30	14.53	6.84
11	12.25	8.55	10.59	13.02	7.46
12	>50	17.12	10.05	14.41	15.23
camptothecin	9.25	2.32	6.98	4.05	0.50

3. Experimental Section

3.1. General Experimental Procedures

Optical rotation data were determined with a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Inc., Waltham, MA, USA) with a 1 dm cell. UV spectra were collected using a Shimadzu UV 240 spectrophotometer (Shimadzu Corp., Kyoto, Japan). IR spectra were recorded on a Bruker vector 22 spectrometer (Bruker Optics, Inc., Billerica, MA, USA) with KBr pellets. NMR experiments were conducted on Bruker Avance-500 and AMX-400 spectrometers (Bruker Biospin Corp., Billerica, MA, USA). The HRESIMS spectra were acquired with a Waters Q-ToF micro YA019 mass spectrometer (Waters Corp., Milford, MA, USA). X-ray structure analysis was performed on a Bruker SMART APEX-II CCD diffractometer (Bruker Optics, Inc.). Melting points were obtained on an SGW X-4 melting point apparatus (Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China). Reversed-phase HPLC was carried out on a YMC-Pack ODS-A column (250 \times 10 mm, 5 μm ; YMC Co., Ltd., Kyoto, Japan) using a Waters 1525 HPLC instrument with Waters 2998 UV detector and monitored at 210 nm. Silica gel (200–300 mesh, Qingdao Chengyang Ocean Chemical Co., Jinan, China), Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, USA), and YMC ODS-A (50 μm , YMC Co., Ltd, Kyoto, Japan) were used as column packing materials. Fractions were monitored by TLC (HSGF 254, Yantai Huiyou Co., Yantai, China), and spots were visualized by heating silica gel plates sprayed with 12% H_2SO_4 in EtOH.

3.2. Animal Material

Samples of *A. cavernosa* were collected by hand using scuba around Xisha Islets in the South China Sea in March 2009 and identified by Professor Jin-He Li (Institute of Oceanology, Chinese Academy of Sciences, China). A voucher sample (JHQ-0901) was deposited in the Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, China.

3.3. Extraction and Isolation

The sponge (5.5 kg, wet weight) was extracted with acetone at room temperature five times (5×5 L) and the extract was concentrated to a brown oil, which was redissolved in H₂O (2 L). The aqueous solution was extracted with CH₂Cl₂ (4×2 L) to afford a CH₂Cl₂-soluble extract (89 g). The resulting extract was partitioned between petroleum ether (4×1 L) and 90% aqueous MeOH (4×1 L) to yield a brownish red oil (49 g), which was subjected to column chromatography (80×7.0 cm) on silica gel (1000.5 g) eluting with petroleum ether-acetone gradient (stepwise, 0:1, 50:1, 30:1, 15:1, 8:1, 3:1 to 0:1, 40 L), and finally MeOH, to give eight fractions (Fractions 1–8). Fraction 5 (5.4 g) was fractionated over Sephadex LH-20 (40×2000 mm, eluted with CH₂Cl₂/MeOH 1:1, 1.5 L), and further fractionated by CC on ODS (RP-18, 30×500 mm), eluted with 70%–100% MeOH/H₂O, to give four subfractions (Fractions 5a–d). Fraction 5a (203.4 mg) was purified by HPLC (YMC-Pack ODS-A, 5 μ m, 10×250 mm, 2.0 mL/min, UV detection at 210 nm), using MeOH/H₂O (88:12) as eluent, to yield **7** (2.1 mg, $t_R = 40.5$ min) and **8** (4.2 mg, $t_R = 42.5$ min). Fraction 5b (659 mg) was subjected to CC (15×300 mm) on silica gel (15 g), using CH₂Cl₂/MeOH with increasing polarity (1:0, 100:1, 50:1) as mobile phase, to yield compound **4** (13.3 mg). Fraction 5c (368.0 mg) was purified by HPLC (YMC-Pack ODS-A, 5 μ m, 10×250 mm, 2.0 mL/min, UV detection at 210 nm), using MeOH/H₂O (92:8) as eluent, to afford **1** (48.9 mg, $t_R = 36.6$ min), **3** (4.2 mg, $t_R = 40.8$ min), and **2** (50.7 mg, $t_R = 43.8$ min). Fraction 5d (267.5 mg) was chromatographed (15×300 mm) on silica gel (15 g, eluted with CH₂Cl₂/MeOH 50:1), to obtain **10** (160 mg). Fraction 6 (7.4 g) was applied to Sephadex LH-20 (40×2000 mm) eluted with CH₂Cl₂/MeOH (1:1) to furnish three subfractions (Fractions 6a and c). Fraction 6b (1.3 g) was subjected to CC on ODS (RP-18, 30×500 mm), eluting with 80%–100% MeOH, to give five subfractions (Fractions 6b1–5). Fraction 6b2 (123 mg) was purified by HPLC (YMC-Pack ODS-A, 5 μ m, 10×250 mm, 2.0 mL/min, UV detection at 210 nm) using MeOH/H₂O (92:8) as eluent to **9** (1.0 mg, $t_R = 37.5$ min). Fraction 6b3 (31.5 mg) was fractionated by a Sephadex LH-20 column (15×2000 mm) eluting with hexane/CH₂Cl₂/MeOH (5:4:1) to afford **6** (4.5 mg). Compound **5** (50.9 mg) was obtained from fraction 6b4 (478.5 mg) by a CC (15×300 mm) on silica gel (15 g, eluted with CH₂Cl₂/MeOH 30:1). Fraction 7 (8.3 g) was subjected to CC (40×2000 mm) on Sephadex LH-20 eluting with MeOH followed by HPLC purification (YMC-Pack ODS-A, 5 μ m, 10×250 mm, 2.0 mL/min, UV detection at 210 nm) using MeOH/H₂O (90:10) as eluent to yield **11** (201.5 mg, $t_R = 50.4$ min) and **12** (197.6 mg, $t_R = 56.8$ min).

Cavernene A (**1**): colorless oil; $[\alpha]_D^{20} +25.0$ (c 0.06, MeOH); UV (CH₃CN) λ_{\max} ($\log \epsilon$) < 200 (2.45) nm; IR (KBr) ν_{\max} 3323, 3051, 2927, 2856, 1670, 1541, 1457, 1386 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z 338.2461 [$M + Na$]⁺ (calcd for C₂₁H₃₃NONa, 338.2460).

Cavernene B (**2**): colorless oil; $[\alpha]_D^{20} +46.2$ (*c* 0.07, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.79), 234 (sh, 2.25) nm; IR (KBr) ν_{\max} 3295, 3053, 2960, 2926, 2869, 1681, 1537, 1453, 1382 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 340.2617 [M + Na]⁺ (calcd for C₂₁H₃₅NONa, 340.2616).

Cavernene C (**3**): white needles (MeOH); m.p. 98.0–102.0 °C; $[\alpha]_D^{20} +20.0$ (*c* 0.03, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.95) nm; IR (KBr) ν_{\max} 3304, 3062, 2958, 2926, 2855, 1668, 1538, 1455, 1381 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 340.2614 [M + Na]⁺ (calcd for C₂₁H₃₅NONa, 340.2616).

Cavernene D (**4**): colorless needles (MeOH); m.p. 112.0–115.0 °C; $[\alpha]_D^{20} +51.4$ (*c* 0.04, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.88) nm; IR (KBr) ν_{\max} 3296, 3055, 2928, 2857, 1681, 1538, 1453, 1383 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 354.2407 [M + Na]⁺ (calcd for C₂₁H₃₃NO₂Na, 354.2409).

Kalihine E (**5**): colorless needles (MeOH); m.p. 185.0–190.0 °C; $[\alpha]_D^{20} +25.0$ (*c* 0.04, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.94) nm; IR (KBr) ν_{\max} 3295, 3074, 2929, 2868, 1682, 1541, 1451, 1381 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 368.2354 [M + H]⁺ (calcd for C₂₁H₃₅NO₂Cl, 368.2356).

Kalihine F (**6**): colorless oil; $[\alpha]_D^{20} +2.5$ (*c* 0.08, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.63), 232 (sh, 2.12) nm; IR (KBr) ν_{\max} 3216, 3057, 2966, 2929, 1686, 1451, 1379, 1311 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 354.2406 [M + Na]⁺ (calcd for C₂₁H₃₃NO₂Na, 354.2409).

Kalihipyran C (**7**): colorless oil; $[\alpha]_D^{20} +24.6$ (*c* 0.07, MeOH); UV (CH₃CN) λ_{\max} (log ϵ) < 200 (2.87) nm; IR (KBr) ν_{\max} 3296, 3055, 2925, 2855, 1668, 1537, 1454, 1377 cm⁻¹; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z* 352.2255 [M + Na]⁺ (calcd for C₂₁H₃₁NO₂Na, 352.2252).

X-ray Crystallographic Analysis Data of Kalihine E (**5**): Colorless needles, C₂₁H₃₄NO₂Cl, *MW* = 367.94, monoclinic space group *P*2₁, *a* = 6.499 (2) Å, *b* = 7.952 (2) Å, *c* = 20.158 (4) Å, *V* = 1036.1 (4) Å³, *Z* = 2, *D*_{calcd} = 1.179 g/cm³, and *F* (000) = 400. A single crystal of dimensions 0.05 × 0.13 × 0.18 mm was used for X-ray measurements and data collected on a Bruker SMART APEX-II CCD diffractometer using Cu K α radiation and up to θ = 67.3 at 293 K. A total of 5789 reflections were collected, of which 3435 independent reflections were measured having an *R*_{int} of 0.0220, final *R* indices of *I* > 2 σ (*I*), *R*₁ = 0.0426, *wR*₂ = 0.1163, *R* indices for all data *R*₁ = 0.0445, and *wR*₂ = 0.1184. The crystal structure solution was achieved using direct methods, as implemented with the SHELX-97 software program. The refinement method was full-matrix least-square on *F*², goodness-of-fit on *F*² was 1.042, and the largest difference peak and hole were 0.237 and -0.196 e. Å⁻³. The absolute structure was determined giving a Flack parameter of 0.07 (2). The X-ray diffraction material has been deposited in the Cambridge Crystallographic Data Center (CCCD No. 847695).

3.4. Cytotoxicity Assay

Cytotoxic activity was evaluated by a MTT method as described previously [25]. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum in 5% CO₂ at 37 °C. An aliquot

(200 μL) of these cell suspensions at a density of 5×10^{-4} cell mL^{-1} was plated in 96-well microtiter plates and incubated for 24 h under the above conditions. 2 μL of the test compound in DMSO at different concentrations was added to each well for 48 h, and then incubated with 1 mg/mL MTT for 4 h. The formazan dye product was measured by the absorbance at 570 nm on a microplate reader. IC_{50} values were calculated by Reed and Muench's method.

3.5. Antifungal Activity Assay

Antifungal activity was determined by the broth macrodilution method following the National Center for Clinical Laboratory Standards (NCCLS) recommendations against the following strains: *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Microsporum gypseum*, and *Aspergillus fumigates* [26,27]. Briefly, bacterial strains were grown aerobically at 30 °C in SDA for 16–20 h in an orbital shaker. A set of tube with different concentrations of compounds 1–12 prepared in RPMI 1640 were next inoculated with the microorganisms and incubated 24 h for *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *A. fumigatus*, 72 h for *C. neoformans*, and 4–7 days for *T. rubrum* and *M. gypseum*. Broth tubes that appeared turbid were indicative of bacterial growth, while tubes that remained clear indicated no growth. The MIC, defined as the lowest concentration of inhibitory compound at which no growth was observed, was evaluated in triplicate for each compound (within the range 1.25–640 $\mu\text{g}/\text{mL}$). Cultures prepared under the same conditions but without compounds and cultures with the same proportions of DMSO (<1%) were used as controls. The growth of broth tubes without turbidity was further examined by counting the viable cells on the SDA plates.

4. Conclusions

A chemical investigation of the marine sponge of *Acanthella cavernosa* led to the isolation of seven new fomamido-diterpenes, cavernenes A–D (1–4), kalihinenes E and F (5,6), and kalihipyran C (7), and five known compounds, kalihipyran A (8), 15-formamido-kalihinene (9), 10-formamido-kalihinene (10), and kalihinenes X (11) and Y (12). The absolute configuration of 5 was determined by single X-ray diffraction. The isolated compounds showed modest cytotoxicity against a small panel of human cancer cell lines, HCT-116, A549, HeLa, QGY-7701 and MDA-MB-231. Compounds 9 and 10 displayed antifungal activity against *Trichophyton rubrum* and *Microsporum gypseum*, *Candida albicans*, and *Cryptococcus neoformans*.

Acknowledgments

We would like to thank Jin-He Li of the Institute of Oceanology, Chinese Academy of Sciences for his help in identifying the marine sponges. This work was funded by the National Natural Science Foundation of China (Nos. 81072573, 81172978, 41106127, and 81001394), the National Science & Technology Major Project of China (No. 2009ZX09103-427), and the Major Program of Modernization of Chinese Medicine (STCSM, 09dZ1975800). Financial support from the National High Technology Research and Development Program of China (863 Project, 2011AA09070107) is gratefully acknowledged.

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Samples Availability: Available from the authors.

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