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Eunicellin-based diterpenoids from the cultured soft coral *Klyxum simplex*

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ABSTRACT

Eight new eunicellin-base diterpenoids, klysimplexins A–H (**1**–**8**), were isolated from a cultured soft coral *Klyxum simplex*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The structure of **1** was further confirmed by a single-crystal X-ray diffraction analysis and the application of modified Mosher's method. Metabolites **2** and **8** were found to be cytotoxic toward a limited panel of cancer cell lines.

1. Introduction

Worldwide marine secondary metabolites are of considerable interest due to the highly diversified structures and wide range of biological activites.[1] The eunicellin-based diterpenoids are secondary metabolites which possess a cladiellane skeleton with a C-2, C-9 ether bridge. Previously reported eunicellin-based diterpenoids have been mostly isolated from octocorals (Alcyonaceae) belonging to the genera *Acalycigorgia*,[2] *Alcyonium*,[3] *Astrogorgia*,[4] *Briareum*,[5] *Cladiella*,[6] *Eleutherobia*,[7] *Eunicella*,[8] *Klyxum*,[9] *Litophyton*,[10] *Muricella*,[11] *Pachyclavularia*,[12,13] *Sclerophytum*,[14] *Sinularia*[15] and *Solenopodium*.[16] Some of these metabolites have been shown to exhibit cytotoxic activity against the growth of various cancer cell lines.[5–7,12–14] In continuation of our investigation on the bioactive substances from marine invertebrates,[17–25] the chemical content of a cultured soft coral *Klyxum simplex* has been studied. In this paper, we report the isolation, structure determination and biological activity of eight new eunicellin-based metabolites, klysimplexins A–H (**1**–**8**), from this soft coral. The structures of **1**–**8** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopy. Cytotoxicity of metabolites **1**–**8** against a limited panel of human tumor cell lines including human liver carcinoma (Hep G2 and Hep G3B), human breast carcinoma (MDA-MB-231 and MCF-7), human lung carcinoma (A-549), and human oral cancer cells (Ca9-22) was also evaluated. Klysimplexin B (**2**) and H (**8**) have been shown to exhibit moderate cytotoxicity against the growth of the above six cancer cell lines.

2. Results and discussion

The octocoral (1.5 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (3×10 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between CH_2Cl_2 and water. The combined CH_2Cl_2-soluble fraction was concentrated under reduced pressure and the residue was repeatedly purified by chromatography to yield metabolites **1**–**8**.

Klysimplexin A (**1**) was isolated as colorless crystals following recrystallization from acetone. The HRESIMS of **1** exhibited a $[M+Na]^+$ peak at *m*/*z* 489.2832 and established a molecular formula $C_{26}H_{42}O_7$, implying six degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxy and carbonyl functionalities from absorptions of 3442 and 1728 and 1712 cm^{-1}. The ^{13}C NMR spectroscopic data of **1** exhibited 26 carbon signals (Table 1), which were assigned by the assistance of DEPT spectrum

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Table 1
^{13}C NMR data for compounds **1**–**8**

Position	**1**[a]	**2**[b]	**3**[c]	**4**[c]	**5**[d]	**6**[c]	**7**[e]	**8**[d]
1	45.5 (CH)[f]	45.0 (CH)	43.0 (CH)	43.1 (CH)	43.0 (CH)	44.2 (CH)	42.3 (CH)	42.5 (CH)
2	93.0 (CH)	91.5 (CH)	91.6 (CH)	91.5 (CH)	91.5 (CH)	93.6 (CH)	92.0 (CH)	93.7 (CH)
3	84.9 (qC)	83.9 (qC)	84.5 (qC)	84.4 (qC)	84.8 (qC)	85.9 (qC)	86.2 (qC)	84.3 (qC)
4	34.2 (CH_2)	34.2 (CH_2)	29.9 (CH_2)	29.9 (CH_2)	29.7 (CH_2)	36.6 (CH_2)	36.2 (CH_2)	29.6 (CH_2)
5	35.5 (CH_2)	36.1 (CH_2)	35.4 (CH_2)	29.7 (CH_2)	29.7 (CH_2)	30.5 (CH_2)	30.5 (CH_2)	30.8 (CH_2)
6	211.4 (qC)	200.3 (qC)	73.5 (CH)	87.2 (CH)	73.7 (CH)	80.6 (CH)	80.6 (CH)	71.0 (CH)
7	45.0 (CH)	147.5 (qC)	150.0 (qC)	145.5 (qC)	150.1 (qC)	77.2 (qC)	77.2 (qC)	136.0 (qC)
8	42.2 (CH_2)	42.1 (CH_2)	41.2 (CH_2)	41.9 (CH_2)	41.3 (CH_2)	47.5 (CH_2)	47.6 (CH_2)	124.9 (CH)
9	76.8 (CH)	78.6 (CH)	79.2 (CH)	79.0 (CH)	79.2 (CH)	76.1 (CH)	75.8 (CH)	79.4 (CH)
10	52.0 (CH)	49.7 (CH)	45.5 (CH)	45.3 (CH)	45.3 (CH)	52.5 (CH)	52.4 (CH)	56.9 (CH)
11	84.1 (qC)	82.6 (qC)	83.5 (qC)	83.4 (qC)	83.4 (qC)	83.7 (qC)	82.2 (qC)	73.0 (qC)
12	43.3 (CH_2)	43.3 (CH_2)	42.2 (CH_2)	42.4 (CH_2)	42.5 (CH_2)	41.9 (CH_2)	32.4 (CH_2)	76.4 (CH)
13	66.1 (CH)	66.5 (CH)	66.8 (CH)	66.8 (CH)	66.8 (CH)	66.4 (CH)	17.6 (CH_2)	70.4 (CH)
14	50.2 (CH)	49.7 (CH)	50.3 (CH)	49.9 (CH)	50.2 (CH)	50.4 (CH)	42.5 (CH)	48.0 (CH)
15	22.8 (CH_3)	23.6 (CH_3)	22.7 (CH_3)	22.9 (CH_3)	22.7 (CH_3)	23.5 (CH_3)	23.1 (CH_3)	24.5 (CH_3)
16	14.4 (CH_3)	117.9 (CH_2)	117.0 (CH_2)	118.2 (CH_2)	116.9 (CH_2)	22.8 (CH_3)	22.8 (CH_3)	19.6 (CH_3)
17	25.5 (CH_3)	25.9 (CH_3)	25.2 (CH_3)	25.3 (CH_3)	25.2 (CH_3)	24.6 (CH_3)	24.7 (CH_3)	26.6 (CH_3)
18	31.1 (CH)	30.7 (CH)	28.4 (CH)	28.5 (CH)	28.4 (CH)	30.5 (CH)	29.0 (CH)	30.7 (CH)
19	25.5 (CH_3)	25.8 (CH_3)	24.7 (CH_3)	24.8 (CH_3)	24.7 (CH_3)	24.7 (CH_3)	21.8 (CH_3)	24.3 (CH_3)
20	16.6 (CH_3)	16.8 (CH_3)	15.8 (CH_3)	15.7 (CH_3)	15.8 (CH_3)	16.3 (CH_3)	15.4 (CH_3)	17.4 (CH_3)
3-*n*-Butyrate	14.3 (CH_3)	14.8 (CH_3)	13.6 (CH_3)	13.6 (CH_3)		13.7 (CH_3)		14.8 (CH_3)
	19.6 (CH_2)	20.0 (CH_2)	18.6 (CH_2)	18.5 (CH_2)		18.7 (CH_2)		19.2 (CH_2)
	37.9 (CH_2)	37.9 (CH_2)	37.4 (CH_2)	37.3 (CH_2)		37.3 (CH_2)		37.4 (CH_2)
	173.4 (qC)	170.4 (qC)	172.6 (qC)	172.6 (qC)		172.6 (qC)		170.8 (qC)
3-OAc					22.4 (CH_3)		22.3 (CH_3)	
					169.8 (qC)		169.8 (qC)	
11-OAc	22.8 (CH_3)	22.8 (CH_3)	22.4 (CH_3)	22.4 (CH_3)	22.4 (CH_3)	22.5 (CH_3)	22.6 (CH_3)	
	170.6 (qC)	167.6 (qC)	170.0 (qC)	169.9 (qC)	169.9 (qC)	170.1 (qC)	170.3 (qC)	
12-OAc								21.8 (CH_3)
								168.4 (qC)
13-*n*-Butyrate								15.0 (CH_3)
								19.3 (CH_2)
								37.6 (CH_2)
								170.8 (qC)

[a] Spectra recorded at 125 MHz in C_5D_5N at 25 °C.
[b] Spectra recorded at 100 MHz in C_6D_6 at 25 °C.
[c] Spectra recorded at 75 MHz in $CDCl_3$ at 25 °C.
[d] Spectra recorded at 100 MHz in $CDCl_3$ at 25 °C.
[e] Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C.
[f] Multiplicities deduced by DEPT.

to seven methyls, six sp^3 methylenes, eight sp^3 methines (including three oxymethines), three sp^2 carbonyls and two sp^3 oxygenated quaternary carbons. The ^{13}C NMR spectrum of **1** showed the presence of a ketone (δ_C 211.4). Two ester carbonyls (δ_C 170.6 and 173.4) were also assigned from the ^{13}C NMR spectrum and were HMBC correlated with an acetate methyl (δ_H 2.06 s) and methylenes (δ_H 1.78 m and 2.58 dd, J=14.5 and 7.0 Hz, each 2H) of an *n*-butyrate, respectively. Therefore, the remaining three degrees of unsaturation identified compound **1** as a tricyclic compound. In the ^{1}H NMR of **1** (Table 2), a doublet at δ_H 1.31 (3H, d, J=7.0 Hz) was attributed to H_3-16 and two doublet at δ_H 1.21 and 1.49 (each 3H, d, J=7.0 Hz) were arised from two methyls of an isopropyl group. Furthermore, two singlets of the tertiary methyls bonded to oxygenated carbons at δ_H 1.53 and 1.62 (each, 3H, s) were due to the resonances of H_3-15 and H_3-17. Signals resonating at δ_H 2.51 (1H, dd, J=12.0, 7.0 Hz), 3.54 (1H, br t, J=7.5 Hz), 3.78 s and 4.20 (1H, ddd, 13.0, 7.0, 4.0 Hz), and at δ_C 45.5, 52.0, 93.0 and 76.8, indicated the presence of a tetrahydrofuran structural unit.[6] The gross structure of metabolite **1** was elucidated by analysis of ^{1}H–^{1}H COSY and HMBC correlations (Fig. 2). From the ^{1}H–^{1}H COSY spectrum of **1**, it was possible to identify three structural units, which were further assembled by HMBC correlations (Fig. 1). Key HMBC correlations from H-2 to C-1, C-9 and C-10; H_2-4 to C-5 and C-6; H_3-15 to C-2, C-3 and C-4; H_3-16 to C-6, C-7 and C-8; H_3-17 to C-10, C-11 and C-12; and both H_3-19 and H_3-20 to C-14 and C-18 permitted the connection of the carbon skeleton. Furthermore, the detailed structure and relative configuration of **1** were established unambiguously from a single-crystal X-ray diffraction analysis (Fig. 3). Finally, in order to resolve the absolute structure of **1**, we determined the configuration at C-13 using a modified Mosher's method.[26,27] The *S*- and *R*-α-methoxy-α-trifluoromethylphenylacetic (MPTA) esters of **1** (**1a** and **1b**, respectively) were prepared by using the corresponding *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chlorides, respectively. The values of $\Delta\delta$ [δ(*S*-MPTA ester)−δ(*R*-MPTA ester)] for H-9, H-10, H-12 and H_3-17 were positive, while the values of $\Delta\delta$ for H-18, H_3-19, H_3-20 were negative, revealing the *R*-configuration at C-13 (Fig. 4).

Klysimplexin B (**2**) was isolated as a colorless oil. Its molecular formula $C_{26}H_{40}O_7$ was established by HRESIMS (*m*/*z* 487.2669, $[M+Na]^+$). Thus, **2** has seven degrees of unsaturation. Its IR spectrum exhibited strong absorptions at 3442, 1738 and 1696 cm^{-1}, indicative of hydroxy, ester carbonyl and conjugated carbonyl groups. The ^{13}C NMR signals of **2** were found to be very closely related to those of compound **1**, suggesting the very similar eunicellin-based skeleton for both compounds, except that the single bond between C-7 and C-16 in **1** was oxidized to a double bond in **2**, as evidenced by two exocyclic methylene protons resonating at δ 4.96 (s) and 5.61 (s). The structure of **2** was unambiguously determined by the extensive analysis of ^{1}H–^{1}H COSY and HMBC (Fig. 2), and NOESY correlations (Fig. 5).

Klysimplexin C (**3**) was obtained as a colorless oil that gave a pseudomolecular ion peak at *m*/*z* 489.2825 $[M+Na]^+$ in the HRESIMS, consistent with the molecular formula $C_{26}H_{42}O_7$ and implying six degrees of unsaturation. The IR absorptions at ν_{max} 3431 (br) and 1733 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The assignments of ^{1}H and ^{13}C NMR spectroscopic

Table 2
^{1}H NMR data for compounds **1**–**6**

Position	**1**[a]	**2**[b]	**3**[c]	**4**[c]	**5**[d]	**6**[c]
1	2.51 dd (12.0, 7.0)[e]	2.13 t (7.5)	2.24 dd (11.6, 7.3)	2.23 dd (11.8, 7.2)	2.25 dd (11.2, 7.6)	2.12 dd(11.0, 7.1)
2	3.78 s	3.40 s	3.59 s	3.59 s	3.60 s	3.49 s
4	β 2.90 dd (14.0, 8.5)	β 2.70 dd (14.0, 9.2)	β 2.19 m	1.95 m	1.60 m	β 2.66 dd (14.5, 8.4)
	α 2.36 br t (12.5)	α 2.16 m	α 1.86 m			α 1.81 m
5	α 2.84 m	α 2.64 m	α 2.12 m	α 2.12 m	α 2.12 m	α 1.61 m
	β 2.20 m	β 2.24 m	β 1.77 m	β 1.51 m	β 1.77 m	β 1.42 m
6			4.30 dd (10.6, 2.8)	4.63 dd (11.3, 2.8)	4.33 dd (10.8, 3.6)	4.55 d (6.0)
7	2.56 m					
8	β 2.60 m	α 2.80 dd (13.2, 4.8)	α 2.83 dd (13.8, 4.7)	α 2.83 dd (13.6, 4.9)	α 2.86 dd (13.6, 4.8)	β 2.00 m
	α 2.18 m	β 2.65 d (13.2)	β 2.54 d (14.0)	β 2.54 d (13.8)	β 2.50 d (13.6)	α 1.86
9	4.20 m	3.61 m	4.13 dd (10.7, 4.3)	4.12 dd (10.7, 4.9)	4.14 dd (10.4, 4.8)	4.08 td (11.1, 3.8)
10	3.54 br t (7.5)	3.32 br t (8.4)	3.08 dd (9.6, 7.8)	3.11 dd (10.4, 7.4)	3.15 dd (10.4, 7.4)	3.11 t (7.2)
12	β 2.78 dd (14.0, 2.5)	β 2.00 dd (13.2, 4.4)	β 2.39 dd (13.6, 4.0)	β 2.39 dd (13.5, 3.7)	β 2.40 m	β 2.36 m
	α 1.90 dd (14.0,11.5)	α 1.04 dd (13.2, 11.6)	α 1.53 m	α 1.53 m	α 1.56 m	α 1.48 m
13	4.27 m	3.76 td (10.8, 4.0)	3.90 td (10.7, 4.0)	3.91 td (11.0, 4.0)	3.93 td (11.2, 4.0)	3.86 td (10.7, 3.6)
14	1.58 t (11.0)	1.06 m	1.29 m	1.24 m	1.23 m	1.17 m
15	1.53 s	1.25 s	1.56 m	1.54 s	1.58 s	1.40 s
16	1.31 d (7.0)	5.61 s	5.47 s	5.45 s	5.49 s	1.16 s
		4.96 s	5.21 s	5.33 s	5.24 s	
17	1.62 s	1.27 s	1.57 s	1.58 s	1.61 s	1.51 s
18	1.92 m	1.64 m	1.92 m	1.90 m	1.93 m	1.72 m
19	1.49 d (7.0)	1.29 d (7.2)	1.18 d (7.2)	1.19 d (7.0)	1.22 d (7.0)	1.19 d (7.3)
20	1.21 d (7.0)	0.94 d (7.2)	0.95 d (7.2)	0.96 d (7.0)	1.00 d (7.0)	1.01 d (7.3)
3-*n*-Butyrate	1.03 t (7.0)	0.89 t (7.2)	0.94 t (7.6)	0.94 t (7.4)		0.99 t (7.3)
	1.78 m	1.60 m	1.63 m	1.60 m		1.67 m
	2.58 dd (14.5, 7.0)	2.22 m	2.17 dd (14.4, 7.4)	2.16 dd (14.9, 7.3)		2.31 dd (15.8, 7.2)
3-OAc					1.98 s	
11-OAc	2.06 s	1.62 s	2.00 s	2.00 s	2.04 s	1.98 s
13-OH	5.94 d (6.0)					
6-OOH				7.88 s		

[a] Spectra recorded at 500 MHz in C_5D_5N at 25 °C.
[b] Spectra recorded at 400 MHz in C_6D_6 at 25 °C.
[c] Spectra recorded at 300 MHz in $CDCl_3$ at 25 °C.
[d] Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C.
[e] *J* values (in Hz) in parentheses.

data of **3** were assisted by a series of 2D NMR (^{1}H–^{1}H COSY, HMQC and HMBC) experiments (Fig. 2). In addition, comparison of the ^{13}C NMR spectroscopic data of **3** with those of **2** revealed that the signal of ketone functionality (δ_C 200.3, C, C-6) in **2** was replaced by that of a secondary alcohol (δ_C 73.5, CH, C-6) in **3**. The relative configurations of all chiral centers of **3**, except C-6, were confirmed to be identical with those of **2** by the NOE correlations (Fig. 5). H-6 was found to exhibit an NOE correlation with H_3-15, revealing the β-orientation of C-6 hydroxy group. The absolute configuration of **3** was also determined by the use of a modified Mosher's method. The (*S*)- and (*R*)-MTPA esters of **3** (**3a** and **3b**, respectively) were also prepared using the corresponding *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chlorides, respectively. The determination of the chemical shift differences ($\delta_S-\delta_R$) for the protons neighboring C-6 and C-13 led to the assignment of *S* and *R* configurations at both C-6 and C-13 of **3** (Fig. 4), respectively. Thus, the structure of diterpenoid **3** was fully established.

Klysimplexin D (**4**) was isolated as a colorless oil and exhibited a pseudomolecular ion peak at *m*/*z* 505.2773 $[M+Na]^+$ by HRESIMS, appropriate for a molecular formula of $C_{26}H_{42}O_8$, with one more oxygen atom than that of **3**. The NMR spectroscopic data of **4** were found to be very similar to those of **3** (Tables 1 and 2), but not for those of CH-6, which were more down-field shifted (δ_C 87.2 and δ_H 4.63), relative to these of **3** (δ_C 73.5 and δ_H 4.30). Therefore, the hydroxy group attached at C-6 in **3** was assumed to be replaced by a hydroperoxy group in **4**. The correlations from a NOESY experiment of **4** also showed that the relative configuration of this metabolite is similar to that of **3**. A structure-related metabolite, klysimplexin E (**5**), was also isolated as a colorless oil with a molecular formula of $C_{24}H_{38}O_7$, implying six degree of unsaturation. Similar to compound **3**, the IR spectrum of **5** indicated the presence of hydroxy (3457 cm^{-1}) and ester (1733 cm^{-1}) groups. NMR spectroscopic data of **5** (Tables 1 and 2) showed the presence of two acetoxy groups (δ_C 169.8, qC; 169.9, qC; 22.4, CH_3; and 22.4, CH_3; δ_H 1.98, 3H, s; and 2.04, 3H, s). Comparison of the NMR data of **5** with those of **3** revealed that the only difference between both compounds arised from the replacement of the *n*-butyryloxy moiety at C-3 in **3** by an acetoxy group in **5**.

The HRESIMS spectrum of **6** exhibited a pseudomolecular ion peak at *m*/*z* 507.2933 $[M+Na]^+$, consisting with a molecular formula $C_{26}H_{44}O_8$ and implying five degrees of unsaturation. The IR spectrum of **6** showed the presence of hydroxy (3416 cm^{-1}) and ester carbonyl (1733 cm^{-1}) groups. By comparison of the NMR data of **6** with those of **3** (Tables 1 and 2), it was found that a C-7/C-16 double bond in **3** was replaced by an oxymethine bearing a methyl and a hydroxy group in **3**, as confirmed by HMBC correlations observed from H_3-16 (δ 1.16, 3H, s) to C-6 (δ 80.6, CH), C-7 (δ 77.2, qC) and C-8 (δ 47.5, CH_2). The more detailed analysis on the ^{1}H and ^{13}C NMR spectroscopic data and the detected 2D correlations in ^{1}H–^{1}H COSY and HMBC spectra led to the establishment of the gross structure of **6** (Fig. 2). The relative configurations of all chiral centers except that of C-7 in **6** were confirmed to be mostly the same as those of **1** by comparison of the proton shifts, coupling constants, and NOE correlations (Fig. 5). H_3-16 was found to exhibit an NOE correlation with H-5β but not with H-6, revealing the β-orientation of hydroxy group at C-6 and α-orientation of hydroxy group at C-7. Thus, the structure of diterpenoid **6** was established.

Klysimplexin G (**7**) was obtained as a colorless oil with a molecular formula of $C_{24}H_{40}O_7$, implying five degrees of unsaturation, as established by the HRESIMS *m*/*z* 463.2670 $[M+Na]^+$. The IR absorptions at 3411 and 1738 cm^{-1} suggested the presence of hydroxy and ester carbonyl groups. The ^{1}H and ^{13}C NMR spectra also revealed

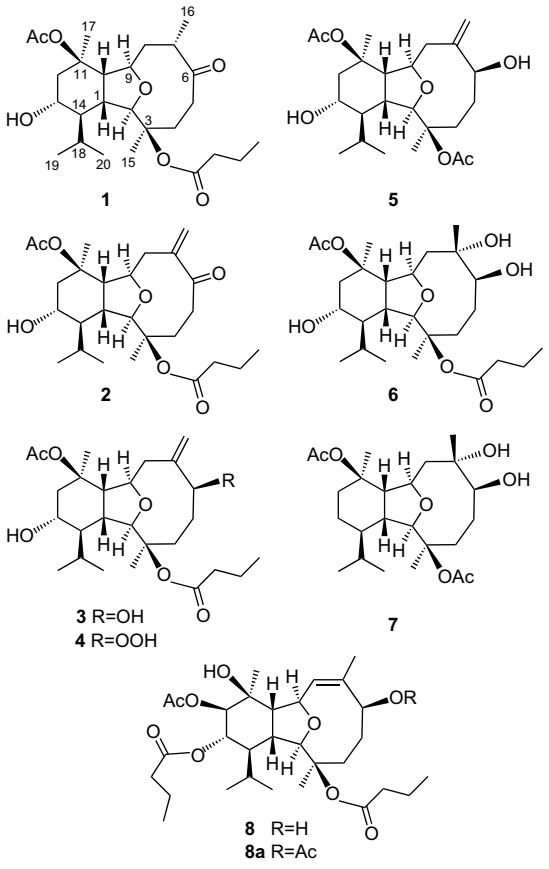

Figure 1. Structures of metabolites **1**–**8**.

that **7** is a eunicellin-based compound (Table 3). By means of extensive 2D NMR experiments (COSY, HMQC and HMBC), the structure of **7** was found to be close to that of **6** except that the *n*-butyryloxy group at C-3 and the hydroxy group at C-13 in **6** were replaced by an acetoxy group and a hydrogen atom, respectively, in **7**. Furthermore, the analysis of NOE correlations of **7** revealed the same relative configurations at C-1, C-2, C-3, C-6, C-7, C-9, C-10 and C-11 as those of **6**, and the same β-orientation of the isopropyl substituent at C-14.

Klysimplexin H (**8**) was obtained as a colorless oil. The HRESIMS of **8** exhibited a $[M+Na]^+$ peak at *m*/*z* 575.3191 and established a molecular formula $C_{30}H_{48}O_9$, implying seven degrees of unsaturation. The IR spectrum of **8** revealed the presence of hydroxy and ester carbonyl groups from absorpions at 3460 and 1738 cm^{-1}. The NMR spectroscopic data of **8** (Table 3) showed the presence of a trisubstituted double bond (δ_H 5.19, s, 1H; δ_C 124.9 and 136.0). Three ester carbonyls at δ_C 168.4 (one carbon signal) and 170.8 (two carbon signals) were assigned from the ^{13}C NMR spectrum and were HMBC correlated with the acetate methyl (δ_H 2.09, s) and methylenes (δ_H 1.63 m and 2.22 m, 2H; 1.63 m and 2.23 m, 2H) of two *n*-butyrate units, respectively. Furthermore, the remaining three degrees of unsaturation identified compound **8** as a tricyclic diterpenoid. The molecular framework was established by ^1H–^1H COSY and HMBC experiments (Fig. 2). The placement of an acetate at C-12 was confirmed from the HMBC connectivities of an acetate methyl (δ_H 2.09, s) and H-12 (δ 5.03) with the carbonyl carbon resonating at δ_C 168.4 (qC). Also, the location of an *n*-butyryloxy group at C-13 was proven from the HMBC connectivity from H-13 (δ 5.51) to the carbonyl carbon resonating at δ_C 170.8 (qC). The proton resonances for H_3-15 (δ 1.47) and H_3-17 (δ 1.20) determined the positions of the other *n*-butyryloxy group and a hydroxyl group at C-3 and C-11, respectively. The chemical shift of H-6 was

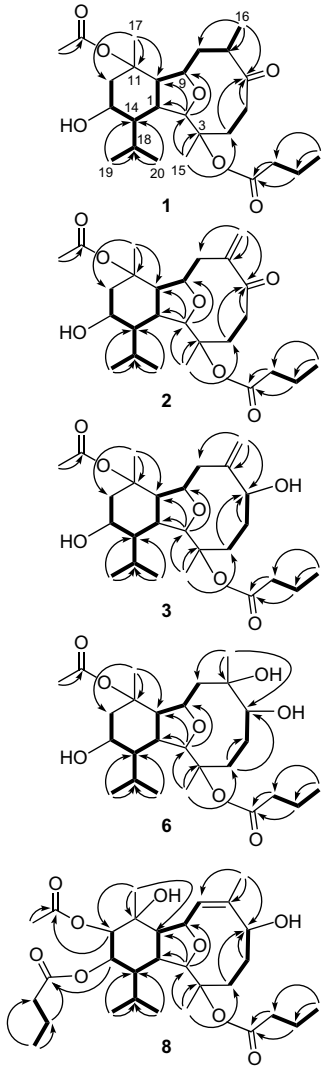

Figure 2. Key ^1H–^1H COSY and HMBC correlations for **1**–**3**, **6** and **8**.

apparently downfield-shifted, indicating that C-6 might bear an allylic hydroxy group. Consequently, compound **8** was acetylated to yield the corresponding ester **8a**, in which the chemical sift of H-6 (δ 5.45) in **8** was downfield shifted to δ 6.41 in **8a**, confirming the presence of a hydroxy group at C-6. The C-7/C-8 double bond was confirmed by HMBC correlations from H_3-16 to C-6 (δ 71.0)

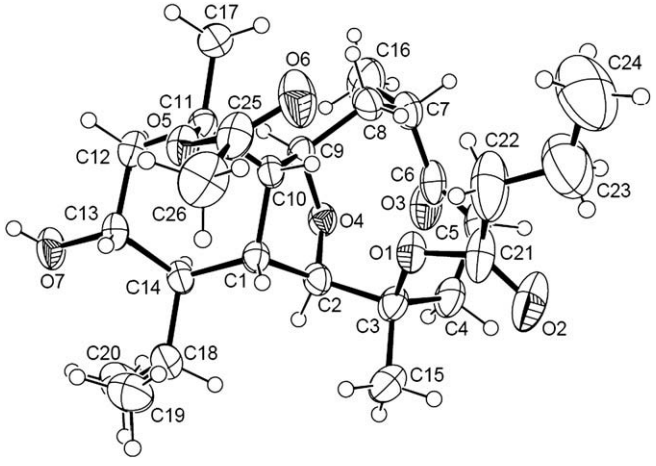

Figure 3. Molecular structure of **1** based on X-ray analysis.

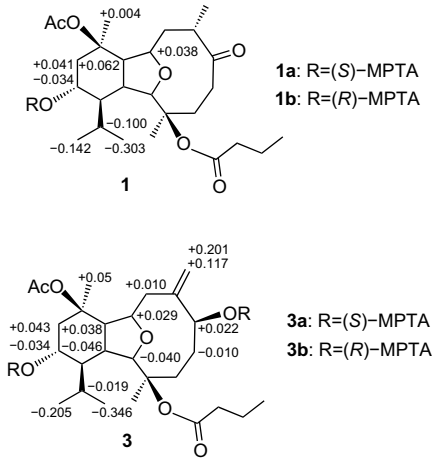

Figure 4. ^{1}H NMR chemical shift differences $\Delta\delta(\delta_S-\delta_R)$ in ppm for the MTPA esters of **1** and **3**.

and C-8 (δ 136.0). The *Z* geometry of the double bonds at C-7 and C-8 was evidenced by the presence of NOE correlations between H-8 and H_3-16. Therefore, the gross structure of **8** was established.

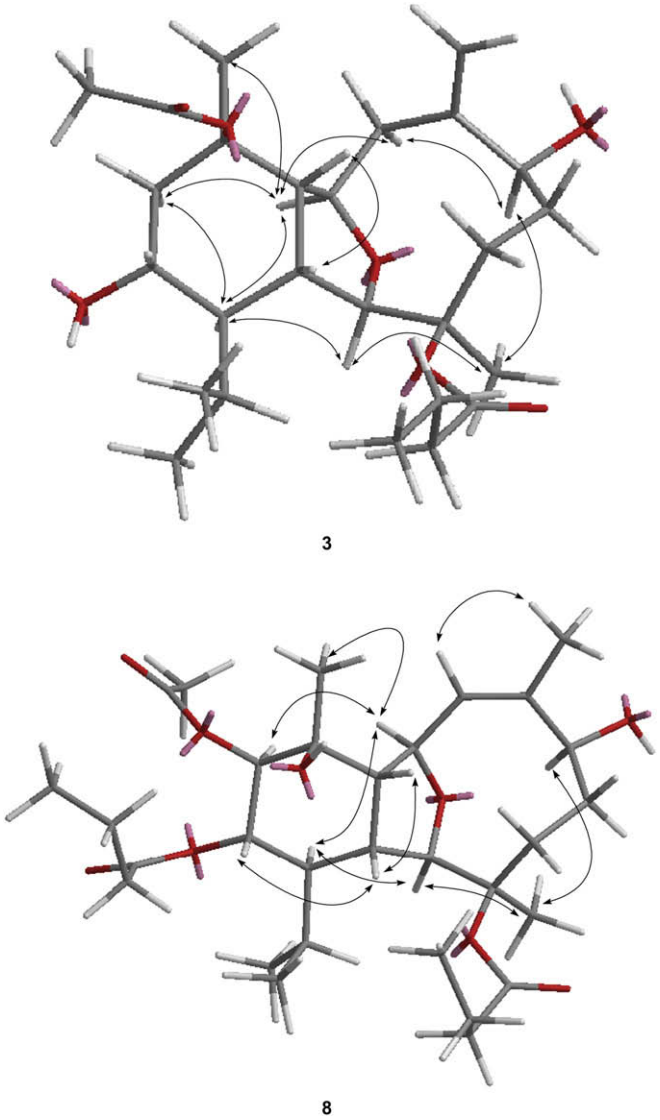

Figure 5. Key NOESY correlations of **3** and **8**.

Table 3
^{1}H NMR data for compounds **7** and **8**

Position	**7**[a]	**8**[b]
1	2.15 dd (11.5, 7.5)[c]	2.45 dd (10.4, 7.6)
2	3.49 s	3.60 s
4	β 2.62 dd (14.5, 9.0)	β 2.10 m
	α 1.80 dd (14.5, 11.0)	α 1.75 m
5	α 1.59 m	1.63 m
	β 1.43 m	
6	4.58 d (6.5)	5.45 d (10.0)
8	β 1.99 m	5.19 s
	α 1.87 m	
9	4.06 ddd	4.58 d (8.8)
10	3.18 t (6.5)	3.54 br t (7.5)
12	β 2.12 m	5.03 d (9.6)
	α 1.42 m	
13	1.42 m	5.51 t (9.6)
	1.38 m	
14	1.16 m	1.75 m
15	1.40 s	1.47 s
16	1.18 s	1.73 s
17	1.49 s	1.20 s
18	1.92 m	1.77 m
19	0.96 d (6.5)	0.95 d (7.2)
20	0.82 d (6.5)	0.98 d (7.2)
3-*n*-Butyrate		0.97 t (7.6)
		1.63 m
		2.23 m
3-OAc	2.10 s	
11-OAc	2.00 s	2.09 s
13-*n*-Butyrate		0.95 t (7.2)
		1.63 m
		2.22 m

[a] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C.
[c] *J* values (in Hz) in parentheses.

In the NOESY spectrum of **8** (Fig. 5), observation of the NOE correlations between H-1 with both H-10 and H-13 suggested that H-1, H-10 and H-13 are β-oriented. Also, correlations between H-2 with both H_3-15 and H-14; H-9 with H-12, H-14 and H_3-17; and H-6 with H_3-15 suggested that of all of H-2, H-6, H-9, H-12, H-14, H_3-15 and H_3-17 are α-oriented. Thus, the structure of diterpenoid **8** was established.

Cytotoxicity of metabolites **1**–**8** toward a limited panel of cancer cell lines was evaluated. The results showed that compound **2** exhibited moderate cytotoxicity toward Hep G2 and Hep 3B (human hepatocellular carcinoma), MDA-MB-231 and MCF-7 (human breast carcinoma), A549 (human lung carcinoma), and Ca9-22 (human gingival carcinoma) cell lines with IC_{50}'s of 3.0, 3.6, 6.9, 3.0, 2.0, and 1.8 μg/mL, respectively. Also, metabolite **8** showed cytotoxicity (IC_{50}'s 5.6, 6.9, 4.4, 5.6, 2.8 and 6.1 μg/mL) toward Hep G2, Hep 3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cell lines, respectively. Other metabolites were found to be inactive against the growth of the above six cancer cell lines. The above results revealed that the α,β-unsaturated ketone in **2** might be able to enhance the cytotoxicity.

It is worthy to note here that we have recently isolated the same type compounds, simplexins A–I, from a wild-type soft coral of this species,[28] however, none of these metabolites was found in the cultured soft coral of the same species. Thus, it seems that farming soft corals could increase the structure diversity of natural products and also the possibility of discovering new bioactive substances.

3. Experimental

3.1. General experimental procedures

Melting points were determined using a Fisher-Johns melting point apparatus. Optical rotations were measured on a JASCO P-

1020 polarimeter. Ultraviolet spectra were recorded on a JASCO V-650 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS were obtained with a Bruker APEX II mass spectrometer. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C or on a Varian 400 MR FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C, or on a Bruker AVANCE-DPX 300 FT-NMR at 300 MHz for ^{1}H and 75 MHz for ^{13}C, respectively. Silica gel (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a C-18 column (250×21.2 mm, 5 μm).

3.2. Organism

Specimens of the cultured soft coral *K. simplex* were collected by hand in a 30 ton cultivating tank located in the National Museum of Marine Biology and Aquarium (NMMBA), Taiwan, in July 2005. A voucher sample (CSC-2) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and separation

The frozen bodies of *K. simplex* (1.5 kg, wet wt) were sliced and exhaustively extracted with EtOH (3×10 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4. After removal of solvent in vacuo, the residue (15.2 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0–100% of EtOAc, gradient) to yield 40 fractions. Fraction 20, eluted with *n*-hexane–EtOAc (10:1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (A1–A5). Subfraction A3 were separated by reverse-phase HPLC (CH_3CN–H_2O, 3:1) to afford **7** (2.3 mg). Fraction 29, eluted with *n*-hexane–EtOAc (1:3), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (B1–B5). Subfractions B3 and B4 were separated by reverse-phase HPLC (CH_3CN–H_2O, 3:1 to 1:3) to afford compounds **1** (4.3 mg), **2** (1.1 mg), **3** (2.2 mg), **4** (2.1 mg), **5** (4.4 mg) and **8** (1.0 mg), respectively. Fraction 36, eluted with EtOAc–MeOH (3:1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (C1–C4). Subfraction C3 were separated by reverse-phase HPLC (CH_3CN–H_2O, 1:1 to 1:3) to afford compound **6** (15.3 mg).

3.3.1. *Klysimplexin A* (***1***)

Colorless crystals; mp 165–172 °C; $[\alpha]_D^{22}$ −26 (*c* 0.43, $CHCl_3$); IR (neat) ν_{max} 3442, 1728 and 1712 cm^{-1}; ^{13}C and ^{1}H NMR data (500 MHz; C_5H_5N), see Tables 1 and 2; ESIMS *m*/*z* 489 $[M+Na]^+$; HRESIMS *m*/*z* 489.2832 $[M+Na]^+$ (calcd for $C_{26}H_{42}O_7Na$, 489.2828).

3.3.2. *Klysimplexin B* (***2***)

Colorless oil; $[\alpha]_D^{22}$ −42 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3442, 1738 and 1696 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; C_6D_6), see Tables 1 and 2; ESIMS *m*/*z* 487 $[M+Na]^+$; HRESIMS *m*/*z* 487.2669 $[M+Na]^+$ (calcd for $C_{26}H_{40}O_7Na$, 487.2672).

3.3.3. *Klysimplexin C* (***3***)

Colorless oil; $[\alpha]_D^{22}$ −34 (*c* 0.22, $CHCl_3$); IR (neat) ν_{max} 3431 and 1733 cm^{-1}; ^{13}C and ^{1}H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 489 $[M+Na]^+$; HRESIMS *m*/*z* 489.2825 $[M+Na]^+$ (calcd for $C_{26}H_{42}O_7Na$, 489.2828).

3.3.4. *Klysimplexin D* (***4***)

Colorless oil; $[\alpha]_D^{22}$ −44 (*c* 0.21, $CHCl_3$); IR (neat) ν_{max} 3452 and 1736 cm^{-1}; ^{13}C and ^{1}H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 505 $[M+Na]^+$; HRESIMS *m*/*z* 505.2773 $[M+Na]^+$ (calcd for $C_{26}H_{42}O_8Na$, 505.2777).

3.3.5. *Klysimplexin E* (***5***)

Colorless oil; $[\alpha]_D^{22}$ −63 (*c* 0.44, $CHCl_3$); IR (neat) ν_{max} 3457 and 1733 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 461 $[M+Na]^+$; HRESIMS *m*/*z* 461.2512 $[M+Na]^+$ (calcd for $C_{24}H_{38}O_7Na$, 461.2515).

3.3.6. *Klysimplexin F* (***6***)

Colorless oil; $[\alpha]_D^{22}$ −22 (*c* 1.53, $CHCl_3$); IR (neat) ν_{max} 3416 and 1733 cm^{-1}; ^{13}C and ^{1}H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 2 ; ESIMS *m*/*z* 507 $[M+Na]^+$; HRESIMS *m*/*z* 507.2933 $[M+Na]^+$ (calcd for $C_{26}H_{44}O_8Na$, 507.2934).

3.3.7. *Klysimplexin G* (***7***)

Colorless oil; $[\alpha]_D^{22}$ −54 (*c* 0.23, $CHCl_3$); IR (neat) ν_{max} 3411 and 1738 cm^{-1}; ^{13}C and ^{1}H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 463 $[M+Na]^+$; HRESIMS *m*/*z* 463.2670 $[M+Na]^+$ (calcd for $C_{24}H_{40}O_7Na$, 463.2672).

3.3.8. *Klysimplexin H* (***8***)

Colorless oil; $[\alpha]_D^{22}$ −74 (*c* 0.10, $CHCl_3$); IR (neat) ν_{max} 3460 and 1738 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 575 $[M+Na]^+$; HRESIMS *m*/*z* 575.3191 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_9Na$, 575.3196).

3.3.9. *Preparation of (S)-and (R)-MTPA esters of* ***1***

To a solution of **1** (0.5 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MPTA) chloride (25 μL), and the mixture was allowed to stand for 24 h at room temperature. The reaction was quenched by addition of 1.0 mL of water, and the mixture was subsequently extracted with EtOAc (3×1.0 mL). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was subjected to column chromatography over silica gel using *n*-hexane–EtOAc (13:2) to yield the (*S*)-MTPA ester, **1a** (0.6 mg, 86%). The same procedure was used to prepare the (*R*)-MTPA ester, **1b** (0.6 mg, 86%) from the reaction of (*S*)-MPTA chloride with **1** in pyridine. Selective ^{1}H NMR ($CDCl_3$, 400 MHz) of **1a**: δ 5.264 (1H, ddd, *J*=14.8, 11.2 and 4.0 Hz, H-13), 3.930 (1H, ddd, *J*=13.2, 9.6 and 4.0 Hz, H-9), 3.515 (1H, t, *J*=7.6 Hz, H-10), 2.481 (1H, m, H-12b), 1.530 (1H, m, H-18), 1.395 (1H, s, H-17), 0.721 (3H, d, *J*=7.0 Hz, H-19), 0.529 (3H, d, *J*=7.0 Hz, H-20). Selective ^{1}H NMR ($CDCl_3$, 400 MHz) of **1a**: δ5.298 (1H, ddd, *J*=15.2, 11.2 and 4.0 Hz, H-13), 3.892 (1H, ddd, *J*=13.2, 9.2 and 3.6 Hz, H-9), 3.453 (1H, t, *J*=7.6 Hz, H-10), 2.440 (1H, m, H-12b), 1.630 (1H, m, H-18), 1.391 (1H, s, H-17), 0.863 (3H, d, *J*=7.0 Hz, H-19), 0.832 (3H, d, *J*=7.0 Hz, H-20).

3.3.10. *Preparation of (S)-and (R)-MTPA esters of* ***3***

To a solution of **3** (0.5 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MPTA) chloride (25 μL), and the mixture was allowed to stand for 24 h at room temperature. The reaction was quenched by addition of 1.0 mL of water, and the mixture was subsequently extracted with EtOAc (3×1.0 mL). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was subjected to column chromatography over silica gel using *n*-hexane–EtOAc (6:1) to yield the (*S*)-MTPA ester, **3a** (0.8 mg, 87%). The same procedure was used to prepare the (*R*)-MTPA ester, **3b** (0.7 mg, 76%) from the reaction of (*S*)-MPTA chloride with **3** in pyridine. Selective ^{1}H NMR ($CDCl_3$, 400 MHz) of **3a**: 5.291 (1H, ddd, *J*=15.2, 11.2 and 4.0 Hz, H-13), 4.149 (1H, dd, *J*=10.4 and 5.2 Hz, H-9), 3.553 (1H, s, H-2), 3.296 (1H, dd,

J=10.4 and 7.2 Hz, H-10), 2.499 (1H, d, J=13.6 Hz, H-12b), 2.106 (1H, t, J=7.2 Hz, H-18), 0.674 (1H, d, J=7.0 Hz, H-19), 0.507 (1H, d, J=7.0 Hz, H-20). Selective ^{1}H NMR ($CDCl_3$, 400 MHz) of **3b**: δ 5.325 (1H, m, H-13), 4.120 (1H, m, H-9), 3.593 (1H, s, H-2), 3.258 (1H, dd, J=10.4 and 7.2 Hz, H-10), 2.456 (1H, d, J=14.0 Hz, H-12b), 2.125 (1H, t, J=7.2 Hz, H-18), 0.879 (1H, d, J=7.0 Hz, H-19), 0.853 (1H, d, J=7.0 Hz, H-20).

3.3.11. *Acetylation of* ***8***

A solution of **8** (0.5 mg) in pyridine (0.2 mL) was mixed with Ac_2O (0.1 mL), and the mixture was stirred at room temperature for 24 h. After evaporation of excess reagent, the residue was subjected to column chromatography over Si gel using *n*-hexane–acetone (6:1) to yield the acetyl derivative **8a** (0.6 mg, 90%).

3.4. X-ray diffraction analysis of klysimplexin A (1)[29]

A suitable colorless crystal ($0.8\times0.8\times0.3$ mm^3) of **1** was grown by slow evaporation of the acetone solution. Diffraction intensity data were acquired with a Rigaku AFC7S single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation (λ=0.71073 Å). Crystal data for **1**: $C_{26}H_{42}O_7$ (formula weight 466.60), approximate crystal size, $0.8\times0.8\times0.3$ mm^3, monoclinic, space group, $P2_1$ (#4), T=298(2) K, a=10.100(2) Å, b=10.467(2) Å, c=12.442(3) Å, β=95.11 (3)°, V=1310.1(5) Å^3, D_c=1.183 Mg/m^3, Z=2, F(000)=508, μ(Mo Kα)=0.084 mm^{-1}. A total of 5425 reflections were collected in the range $2.02°<\theta<26.01°$, with 5131 independent reflections [R(int)=0.0215], completeness to θ_{max} was 99.9%; psi-scan absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 5131/1/306; goodness-of-fit on F^2=1.011; final R indices [$I>2\sigma(I)$], R_1=0.0424, wR_2=0.1074; R indices (all data), R_1=0.0851, wR_2=0.1260, largest difference peak and hole, 0.178 and −0.224 e/Å^3.

3.5. Cytotoxicity testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[30,31]

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29. Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 699909). 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 or e-mail:deposit@ccdc.cam.ac.uk).
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