Klysimplexins I–T, eunicellin-based diterpenoids from the cultured soft coral *Klyxum simplex*†

Bo-Wei Chen,[a] Chih-Hua Chao,[a] Jui-Hsin Su,[b] Chung-Wei Tsai,[c] Wei-Hsien Wang,[a,b] Zhi-Hong Wen,[a] Chiung-Yao Huang,[a] Ping-Jyun Sung,[a,b] Yang-Chang Wu[c] and Jyh-Horng Sheu*[a,d]

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New eunicellin-base diterpenoids, klysimplexins I–T (**1–12**), were isolated from a cultured soft coral *Klyxum simplex*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The absolute stereochemistry of **4** was determined by Mosher's method. Compounds **9** and **12** have been shown to exhibit cytotoxicity toward a limited panel of cancer cell lines. Compounds **2–6**, **10** and **11** were found to display significant *in vitro* anti-inflammatory activity in LPS-stimulated RAW264.7 macrophage cells by inhibiting the expression of the iNOS protein. Compounds **10** and **11** also could effectively reduce the level of COX-2 protein.

Introduction

During the course of our investigation on new natural substances from the cultured and wild-type soft corals *K. simplex*, new metabolites klysimplexins A–H[1] and klysimplexin sulfoxides A–C[2] were isolated from the cultured soft coral, and simplexins A–I were obtained from the wild-type soft coral.[3] Previously reported eunicellin-based diterpenoids were isolated mostly from octocorals (Alcyonaceae) belonging to the genera *Acalycigorgia*,[4] *Alcyonium*,[5] *Astrogorgia*,[6] *Briareum*,[7] *Cladiella*,[8] *Eleutherobia*,[9] *Eunicella*,[10] *Klyxum*,[11] *Litophyton*,[12] *Muricella*,[13] *Pachyclavularia*,[14,15] *Sclerophytum*,[16] *Sinularia*,[17] and *Solenopodium*.[18] Some of these metabolites have been shown to exhibit cytotoxic activity against the growth of various cancer cell lines.[1,2,7–9,14–16] In continuation of our recent effort on discovering novel and bioactive substances from marine invertebrates,[19–24] the chemical constituents of the cultured soft coral *Klyxum simplex* were further studied. In this paper, we report the isolation, structure determination, and biological activity of twelve new eunicellin-based metabolites, klysimplexins I–T (**1–12**, Scheme 1), from *K. simplex*. The relative structures of **1–12** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopy, and the absolute structure of **4** was determined by Mosher's method. Besides the normal THF-containing eunicellins **1–7** which are similar to those isolated previously from this soft coral,[1–19] this investigation also led to the isolation of eunicellins containing a long-chain ester substitution at C-6 (compounds **1–3**), a 6,7-secoeunicellin **8**, two 2,9-deoxygenated derivatives **9** and **10**, and a tetradecahydrophenanthrene-type diterpene **12** for the first time from the genus *Klyxum*. Cytotoxicity of metabolites **1–12** 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) are also discussed, and the ability of **1–12** to inhibit up-regulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was also evaluated.

Results and discussion

The soft coral (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 **1–12**.

Klysimplexin I (**1**) was obtained as a colorless oil. The HRESIMS of **1** exhibited a $[M + Na]^+$ peak at m/z 701.4974 and established a molecular formula $C_{40}H_{70}O_8$, implying six degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxy and carbonyl functionalities from absorptions at 3460 and 1738 cm^{-1}. The ^{13}C NMR data of **1** was made up 40 carbon signals in total (Table 1), which were assigned by the DEPT spectrum to eight methyls, nineteen sp^3 methylenes, seven sp^3 methines (including three oxymethines), three sp^2 carbonyls and three sp^3 oxygenated quaternary carbons. Three ester carbonyl carbons (δ_C 174.7, 172.6 and 170.1) were HMBC correlated by the methylene protons (δ_H 2.31 m, 2H and 1.61, m, 2H) of a long-chain ester unit, methylene protons (δ_H 2.38 m, 2H and 1.67 m, 2H) of an *n*-butyrate and protons of an acetate (δ_H 1.98, 3H, s), respectively. The long-chain ester was found to be myristate as the negative ESIMS of **1** exhibited a peak at m/z 227.2, consistent with the molecular formula $C_{14}H_{27}O_2$. Therefore, the remaining three degrees of unsaturation identified metabolite **1** as a tricyclic compound. In the ^{1}H NMR spectrum of **1** (Table 3), two doublets at δ_H 0.94 and 0.80 (each 3H, d, J = 7.2 Hz) arose from two methyls of an isopropyl group. Signals resonating at δ_H 2.15 (1H, dd, J = 11.6, 7.2 Hz), 3.13 (1H, br t, J = 7.2), 3.54 (1H, s) and 4.08 (1H,

[a]*Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan*
[b]*Taiwan Coral Research Center, National Museum of Marine Biology & Aquarium, Checheng, Pingtung 944, Taiwan*
[c]*Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[d]*Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 804, Taiwan*

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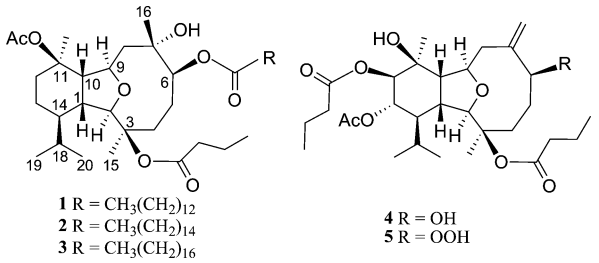
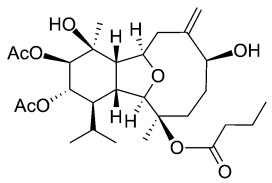
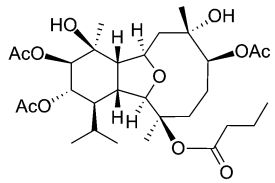
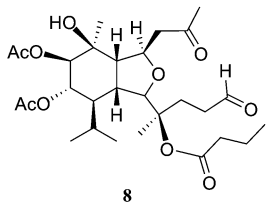
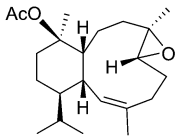
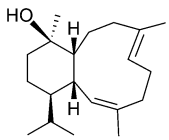


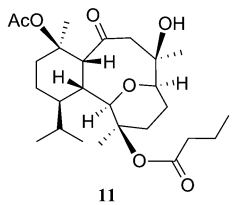


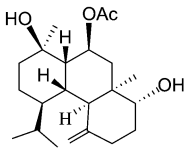




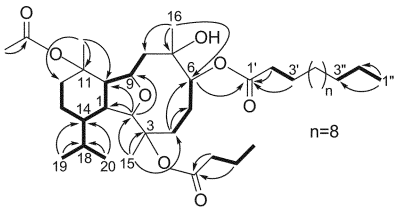


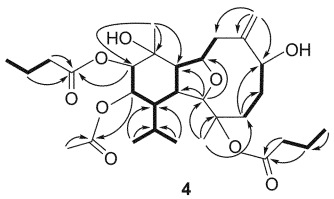


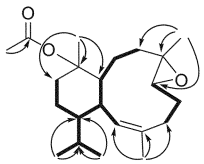


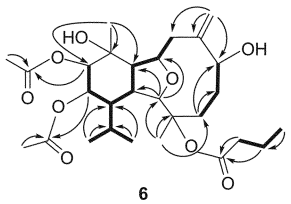


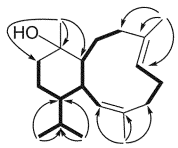
Scheme 1

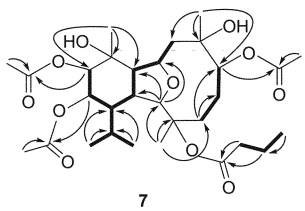


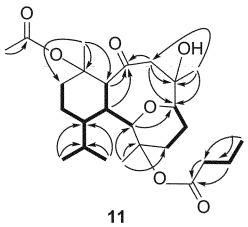


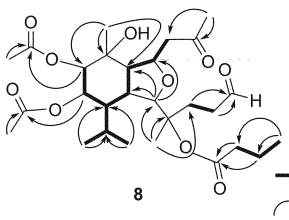


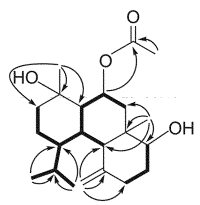












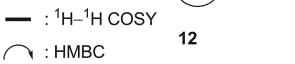
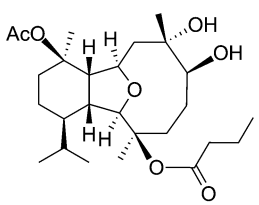
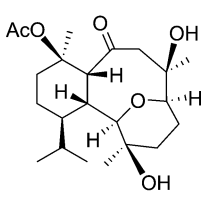


Fig. 1 Key ^{1}H-^{1}H COSY and HMBC correlations of **1**, **4**, and **6–12**.

ddd, J = 11.7, 8.0 and 4.0), and at δ_C 42.3, 52.8, 92.0 and 75.5, indicated the presence of a tetrahydrofuran structural unit.[1,3] The molecular framework was established mainly by ^{1}H–^{1}H COSY and HMBC experiments (Fig. 1). The placement of a myristate and an *n*-butyrate at C-6 and C-3, respectively, was proven from the HMBC correlations from H-6 (δ 5.58) and H-2 (δ 3.54) to the carbonyl carbons resonating at δ 174.7 (qC) and δ 172.6 (qC). The proton resonances for H_3-17 (δ 1.48) and H_3-16 (δ 1.16) also determined the positions of the acetate and hydroxy groups at C-11 and C-7, respectively. Thus, the molecular framework of **1** was established unambiguously, and was found to be similar to that of a known compound **13** (Scheme 2).[3] The relative structure of **1** also was found to be the same as that of **13** by comparison of the chemical shifts and coupling constants for protons of both compounds, and by analysis of NOE correlations. In order to unambiguously confirm the structure, a base-catalyzed hydrolysis of **1** was performed and the reaction was found to afford **13**. The structure of **1** was thus fully established.





Scheme 2

Klysimplexins J (**2**) and K (**3**) were also isolated as colorless oils. The molecular formulae of $C_{42}H_{74}O_8$ and $C_{44}H_{78}O_8$, 28 and 56 mass units higher than that of **1**, were determined by HRESIMS, respectively. The negative mode ionization of **2** and **3** by LC-ESI MS/MS fragmentation exhibited $[M - H]^-$ peaks at m/z 255.6 and 283.5, consistent with the molecular formulae $C_{16}H_{31}O_2$ and $C_{18}H_{35}O_2$, and indicated the presence of palmitate and stearate in **2** and **3**, respectively. The ^{1}H and ^{13}C NMR spectroscopic data of

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

Position	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[b]	**6**[b]	**7**[c]	**8**[b]
1	42.3 (CH)[d]	42.4 (CH)	42.9 (CH)	41.6 (CH)	42.0 (CH)	41.6 (CH)	43.0 (CH)	42.2 (CH)
2	92.0 (CH)	92.0 (CH)	91.8 (CH)	91.2 (CH)	91.3 (CH)	91.1 (CH)	93.0 (CH)	88.5 (CH)
3	86.0 (qC)	86.1 (qC)	85.9 (qC)	84.2 (qC)	84.4 (qC)	84.3 (qC)	85.9 (qC)	84.4 (qC)
4	35.9 (CH_2)	36.0 (CH_2)	35.4 (CH_2)	29.7 (CH_2)	28.3 (CH_2)	29.7 (CH_2)	35.9 (CH_2)	27.8 (CH_2)
5	29.1 (CH_2)	29.1 (CH_2)	29.8 (CH_2)	35.2 (CH_2)	29.9 (CH_2)	35.2 (CH_2)	29.2 (CH_2)	39.4 (CH_2)
6	84.9 (CH)	84.9 (CH)	84.8 (CH)	72.7 (CH)	86.7 (CH)	72.6 (CH)	84.8 (CH)	201.4 (CH)
7	75.9 (qC)	75.9 (qC)	75.5 (qC)	150.1 (qC)	146.1 (qC)	150.0 (qC)	75.8 (qC)	206.3 (qC)
8	47.8 (CH_2)	47.8 (CH_2)	48.3 (CH_2)	40.9 (CH_2)	41.8 (CH_2)	40.9 (CH_2)	47.6 (CH_2)	50.7 (CH_2)
9	75.5 (CH)	75.5 (CH)	75.5 (CH)	78.7 (CH)	78.8 (CH)	78.7 (CH)	75.6 (CH)	75.4 (CH)
10	52.8 (CH)	52.8 (CH)	53.2 (CH)	49.2 (CH)	49.5 (CH)	49.2 (CH)	56.6 (CH)	53.9 (CH)
11	82.2 (qC)	82.2 (qC)	82.1 (qC)	72.8 (qC)	72.8 (qC)	72.8 (qC)	72.7 (qC)	72.0 (qC)
12	32.0 (CH_2)	32.0 (CH_2)	32.7 (CH_2)	76.4 (CH)	76.7 (CH)	75.7 (CH)	76.7 (CH)	77.0 (CH)
13	17.5 (CH_2)	17.5 (CH_2)	18.4 (CH_2)	70.9 (CH)	71.2 (CH)	71.0 (CH)	70.7 (CH)	70.9 (CH)
14	42.4 (CH)	42.4 (CH)	43.0 (CH)	47.7 (CH)	47.7 (CH)	47.6 (CH)	47.3 (CH)	46.1 (CH)
15	22.9 (CH_3)	23.0 (CH_3)	23.8 (CH_3)	22.2 (CH_3)	22.6 (CH_3)	22.2 (CH_3)	23.2 (CH_3)	21.4 (CH_3)
16	23.9 (CH_3)	23.9 (CH_3)	24.7 (CH_3)	117.0 (CH_2)	118.3 (CH_2)	117.0 (CH_2)	23.8 (CH_3)	30.6 (CH_3)
17	24.8 (CH_3)	24.9 (CH_3)	25.7 (CH_3)	26.2 (CH_3)	26.6 (CH_3)	26.1 (CH_3)	25.8 (CH_3)	26.1 (CH_3)
18	28.9 (CH)	28.9 (CH)	29.7 (CH)	28.0 (CH)	28.3 (CH)	28.0 (CH)	30.2 (CH)	28.7 (CH)
19	21.7 (CH_3)	21.7 (CH_3)	22.6 (CH_3)	23.6 (CH_3)	23.8 (CH_3)	23.6 (CH_3)	23.4 (CH_3)	23.6 (CH_3)
20	15.2 (CH_3)	15.2 (CH_3)	16.1 (CH_3)	15.7 (CH_3)	15.9 (CH_3)	15.7 (CH_3)	16.1 (CH_3)	15.4 (CH_3)
3-*n*-butyrate	13.6 (CH_3)	13.6 (CH_3)	14.6 (CH_3)	13.7 (CH_3)	14.0 (CH_3)	13.6 (CH_3)	13.7 (CH_3)	13.7 (CH_3)
	18.7 (CH_2)	18.7 (CH_2)	19.6 (CH_2)	18.4 (CH_2)	18.6 (CH_2)	18.5 (CH_2)	18.4 (CH_2)	18.4 (CH_2)
	37.3 (CH_2)	37.3 (CH_2)	37.9 (CH_2)	35.9 (CH_2)	36.2 (CH_2)	37.3 (CH_2)	37.4 (CH_2)	37.4 (CH_2)
	172.6 (qC)	172.6 (qC)	171.2 (qC)	172.8 (qC)	172.7 (qC)	172.4 (qC)	172.3 (qC)	172.6 (qC)
6-OAc							21.5 (CH_3)	
							172.1 (qC)	
11-OAc	22.5 (CH_3)	22.5 (CH_3)	23.3 (CH_3)					
	170.1 (qC)	170.1 (qC)	168.7 (qC)					
12-OAc						20.7 (CH_3)	20.7 (CH_3)	20.6 (CH_3)
						170.2 (qC)	170.0 (qC)	170.0 (qC)
13-OAc				21.5 (CH_3)	21.7 (CH_3)	21.4 (CH_3)	21.4 (CH_3)	21.1 (CH_3)
				170.1 (qC)	170.4 (qC)	170.2 (qC)	170.4 (qC)	170.1 (qC)
12-*n*-butyrate				13.6 (CH_3)	13.8 (CH_3)			
				18.5 (CH_2)	18.7 (CH_2)			
				37.4 (CH_2)	37.6 (CH_2)			
				172.4 (qC)	173.1 (qC)			
3′	25.1 (CH_2)	25.1 (CH_2)	25.9 (CH_2)					
2′	34.7 (CH_2)	34.8 (CH_2)	35.4 (CH_2)					
1′	174.7 (qC)	174.7 (qC)	173.3 (qC)					
$(CH_2)_n$	29.1–29.6 (CH_2)	29.1–29.7 (CH_2)	29.8–30.4 (CH_2)					
3″	32.0 (CH_2)	31.9 (CH_2)	32.6 (CH_2)					
2″	22.7 (CH_2)	22.7 (CH_2)	23.5 (CH_2)					
1″	14.1 (CH_3)	14.1 (CH_3)	15.1 (CH_3)					

[a] 100 MHz in $CDCl_3$. [b] 125 MHz in $CDCl_3$. [c] 100 MHz in $CDCl_3$. [d] Multiplicities deduced by DEPT.

2 and **3** were found to be very close to those of **1** (Tables 1 and 3), indicating the very similar structures for these three metabolites. The relative stereochemistries of **2** and **3** were suggested to be the same as that of **1** due to the biogenetic consideration, NMR spectroscopic data, as well as the same sign of specific optical rotations.

Klysimplexin L (**4**) was obtained as a colorless oil that gave a pseudomolecular ion peak at *m/z* 575.3193 $[M + Na]^+$ in the HRESIMS, consistent with the molecular formula $C_{30}H_{48}O_9$ and implying seven degrees of unsaturation. The NMR spectra data of **4** (Tables 1 and 3) showed the appearance of an 1,1-disubstituted carbon–carbon double bond (δ_C 150.1, qC and 117.0, CH_2; δ_H 5.46 s and 5.12 s). Three ester carbonyls (δ_C 172.8, 172.4 and 170.1) were also assigned from the ^{13}C NMR spectrum and were HMBC correlated with the methylenes (δ_H 2.32 m, 2H and 1.66 m, 2H; 2.13 m, 2H and 1.58 m, 2H) of two *n*-butyrate units and an acetate methyl (δ_H 2.00 s, 3H), respectively. Therefore, the remaining three degrees of unsaturation identified compound **4** as a tricyclic compound. In the ^{1}H NMR spectrum of **4** (Table 3), two doublets at δ_H 0.99 and 0.92 (each 3H, d, $J = 7.2$ Hz) arose from two methyls of an isopropyl group. The molecular framework was established by ^{1}H–^{1}H COSY and HMBC experiments (Fig. 1). The placement of the acetate at C-13 was confirmed from the HMBC correlations of acetate methyl (δ_H 2.00 s, 3H) and H-13 (δ 5.49) with the carbonyl carbon resonating at δ_C 170.1 (qC). Also, the location of an *n*-butyryloxy group at C-12 was proven from the HMBC correlations of H-12 (δ 5.04) to the carbonyl carbon resonating at δ_C 172.4 (qC). The downfield chemical shifts for H_3-15 (δ 1.61) and C-3 (δ 84.2), and the upfield chemical shifts of H_3-17 (δ 1.17) and C-11 (δ 72.8), determined the positions of the other *n*-butyrate and hydroxy group at C-3 and C-11, respectively. From the above results, the structure of compound **4** was shown to be very similar to that of a known compound, klysimplexin sulfoxide C.[2] Therefore, the molecular framework

Table 2 ^{13}C NMR data for compounds **9–12**

Position	**9**[a]	**10**[b]	**11**[a]	**12**[a]
1	36.7 (CH)[c]	36.8 (CH)	50.1 (CH)	31.4 (CH)
2	129.6 (CH)	130.6 (CH)	78.0 (CH)	51.7 (CH)
3	133.8 (qC)	133.4 (qC)	81.0 (qC)	144.9 (qC)
4	29.0 (CH_2)	32.3 (CH_2)	28.5 (CH_2)	30.4 (CH_2)
5	26.1 (CH_2)	25.8 (CH_2)	21.5 (CH_2)	32.2 (CH_2)
6	64.9 (CH)	124.6 (CH)	80.3 (CH)	69.8 (CH)
7	60.9 (qC)	138.3 (qC)	85.3 (qC)	38.9 (qC)
8	39.4 (CH_2)	39.9 (CH_2)	50.0 (CH_2)	36.2 (CH_2)
9	23.5 (CH_2)	24.0 (CH_2)	209.0 (qC)	70.3 (CH)
10	42.5 (CH)	47.0 (CH)	56.2 (CH)	46.6 (CH)
11	85.8 (qC)	73.5 (qC)	83.3 (qC)	71.4 (qC)
12	32.3 (CH_2)	36.1 (CH_2)	31.3 (CH_2)	38.6 (CH_2)
13	19.9 (CH_2)	20.4 (CH_2)	20.3 (CH_2)	21.1 (CH_2)
14	42.9 (CH)	45.6 (CH)	37.2 (CH)	40.9 (CH)
15	25.8 (CH_3)	25.0 (CH_3)	24.5 (CH_3)	111.9 (CH_2)
16	19.2 (CH_3)	17.0 (CH_3)	23.9 (CH_3)	23.2 (CH_3)
17	23.8 (CH_3)	26.1 (CH_3)	25.2 (CH_3)	28.5 (CH_3)
18	27.5 (CH)	26.5 (CH)	28.5 (CH)	26.8 (CH)
19	22.7 (CH_3)	22.1 (CH_3)	22.6 (CH_3)	22.8 (CH_3)
20	16.6 (CH_3)	18.5 (CH_3)	14.9 (CH_3)	21..8 (CH_3)
3-*n*-butyrate			15.6 (CH_3) 19.6 (CH_2) 37.9 (CH_2) 171.1 (qC)	
9-OAc				22.7 (CH_3) 169.0 (qC)
11-OAc	23.5 (CH_3) 168.8 (qC)		23.4 (CH_3) 168.0 (qC)	

[a] 100 MHz in $CDCl_3$. [b] 125 MHz in $CDCl_3$. [c] Multiplicities deduced by DEPT.

of **4** was established. In the NOESY spectrum of **4** (Fig. 2), observation of the NOE correlations between H-10 and both H-8β (δ 2.86) and H-1; and H-1 and H-13 suggested that H-1, H-10 and H-13 are β-oriented. Also, correlations between H-2 and both H_3-15 and H-14; H-9 and H-12, H-14 and H_3-17; and H-6 and both H-8α (δ 2.44) and H_3-15 suggested that all of H-2, H-6, H-9, H-12, H-14, H_3-15 and H_3-17 are α-oriented. Thus, the relative

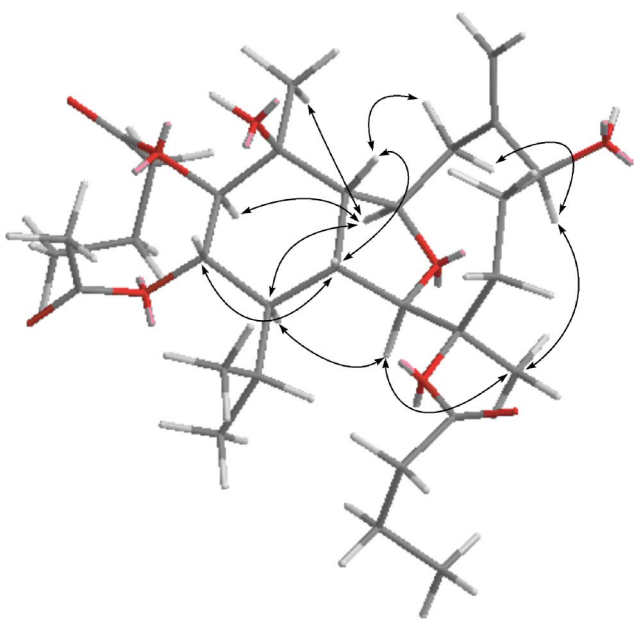
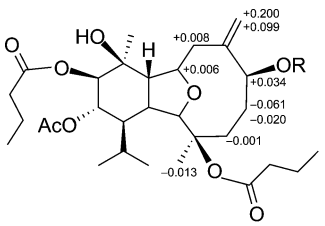


Fig. 2 Key NOESY correlations of **4**.

structure of diterpenoid **4** was established. In order to resolve the absolute structure of **4**, we determined the absolute configuration at C-6 using Mosher's method.[25,26] The (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl) phenylacetic (MTPA) esters of **4** (**4a** and **4b**, respectively) were prepared by using the corresponding *R*-(–)- and *S*-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chlorides, respectively. The values of $\Delta\delta$ [δ(*S*-MTPA ester) – δ(*R*-MTPA ester)] for H-8, H-9 and H_2-16 were positive, while the values of $\Delta\delta$ for H-4, H_2-5 and H_3-15 were negative, revealing the *S*-configuration at C-6 (Fig. 3).



4a: R=(*S*)–MTPA
4b: R=(*R*)–MTPA

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

Klysimplexin M (**5**) was isolated as a colorless oil and exhibited a pseudomolecular ion peak at *m/z* 591.3146 [M + Na]$^+$ by HRESIMS, appropriate for a molecular formula of $C_{30}H_{48}O_{10}$, with one more oxygen atom than that of **4**. The NMR spectra data of **5** were found to be very similar to those of **4** (Tables 1 and 3), except for those of CH-6, which were downfield shifted (δ_C 86.7 and δ_H 4.66) relative to these of **4** (δ_C 72.7 and δ_H 4.33). Therefore, the hydroxy group attached at C-6 in **4** was assumed to be replaced by a hydroperoxy group in **5**. The NOE correlations of **5** also showed that the stereochemistry of this metabolite is similar to that of **4**. A structure related metabolite, klysimplexin N (**6**), was also isolated as a colorless oil with a molecular formula of $C_{28}H_{44}O_9$, implying seven degrees of unsaturation. NMR spectroscopic data of **6** (Tables 1 and 4) showed the presence of an *n*-butyryloxy group (δ_C 172.4, qC; 37.3, CH_2; 18.5, CH_2; 13.6, CH_3; δ_H 2.12 m, 2H, 1.63 m, 2H, and 0.93 t, *J* = 7.0, 3H) and two acetoxy groups (δ_C 170.2, qC; 21.4, CH_3; δ_C 170.2, qC and 20.7, CH_3; δ_H 2.10, s and 2.01, s). Comparison of the 1D and 2D NMR data of **6** with those of **4** revealed that the only difference between both compounds arose from the replacement of the *n*-butyryloxy moiety at C-12 in **4** by an acetoxy group in **6**, as confirmed by HMBC correlations of both acetate methyl (δ 2.10) and H-12 (δ 5.01) with the carbonyl carbon resonating at δ 170.2 (qC).

The HRESIMS spectrum of **7** exhibited a pseudomolecular ion peak at *m/z* 607.3095 [M + Na]$^+$, consistent with a molecular formula $C_{30}H_{48}O_{11}$ and implying seven degrees of unsaturation. By comparison of the NMR data of **7** with those of **6** (Tables 1 and 4), it was found that a C-7/C-16 double bond in **6** was replaced by a quaternary carbon bearing a methyl and a hydroxy group in **7**. Moreover, the hydroxy group attached at C-6 of **6** was replaced by an acetoxy group in **7**. This was further evidenced by the HMBC correlations observed from H_3-16 (δ 1.19, 3H, s) to C-6 (δ 84.8, CH), C-7 (δ 75.8, qC), and C-8 (δ 47.6, CH_2); and from H-6 (δ 5.61) to the carbonyl carbon resonating at δ_C 172.1 (qC). The more detailed analysis on the ^{1}H and ^{13}C NMR spectroscopic data

Table 3 ^{1}H data for compounds **1–5**

Position	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[b]
1	2.15 (dd, 11.6, 7.2)[c]	2.16 (dd, 11.2, 6.8)	2.18 (dd, 11.2, 6.8)	2.55 (dd, 11.6, 7.6)	2.55 (dd, 11.5, 7.5)
2	3.54 (s)	3.55 (s)	3.57 (s)	3.59 (s)	3.60 (s)
4	2.63 (dd, 15.2, 7.6)	2.64 (dd, 15.2, 8.0)	2.66 (dd, 15.2, 7.2)	2.25 (m)	2.26 (m)
	1.98 (m)	2.00 (m)	2.00 (m)	1.71 (m)	1.88 (m)
5	1.46 (m)	1.46 (m)	1.49 (m)	α 2.12 (m)	α 2.15 (m)
				β 1.70 (m)	β 1.52 (m)
6	5.58 (d, 6.4)	5.58 (br s)	5.59 (d, 5.6)	4.33 (m)	4.66 (dd, 11.5, 3.5)
8α	1.87 (dd, 14.4, 3.6)	1.89 (d, 14.8)	1.91 (dd, 14.4, 3.6)	2.44 (d, 14.0)	2.51 (d, 14.5)
8β	1.96 (m)	1.96 (m)	1.98 (m)	2.86 (dd, 14.0, 4.0)	2.85 (dd, 14.0, 4.5)
9	4.08 (ddd, 11.7, 8.0, 4.0)	4.09 (m)	4.11 (ddd, 11.2, 7.2, 3.6)	4.30 (m)	4.29 (dd, 11.0, 4.5)
10	3.13 (br t, 7.2)	3.14 (br t, 6.8)	3.16 (br t, 6.8)	2.66 (dd, 10.8, 7.6)	2.66 (dd, 11.0, 7.5)
12	β 2.21 (m)	β 2.18 (m)	β 2.21 (m)	5.04 (d, 9.6)	5.04 (d, 9.5)
	α 1.38 (m)	α 1.39 (m)	α 1.42 (m)		
13	1.39 (m)	1.41 (m)	1.44 (m)	5.49 (dd, 10.4, 10.4)	5.49 (dd, 11.0, 10.0)
14	1.16 (m)	1.17 (m)	1.20 (m)	1.75 (t, 11.6)	1.76 (t, 11.5)
15	1.36 (s)	1.37 (s)	1.40 (s)	1.61 (s)	1.60 (s)
16	1.16 (s)	1.18 (s)	1.22 (s)	5.46 (s); 5.12 (s)	5.44 (s); 5.22 (s)
17	1.48 (s)	1.49 (s)	1.52 (s)	1.17 (s)	1.18 (s)
18	1.73 (m)	1.72 (m)	1.73 (m)	1.97 (m)	1.96 (m)
19	0.94 (d, 7.2)	0.95 (d, 6.8)	0.99 (d, 6.8)	0.99 (d, 7.2)	1.00 (d, 7.5)
20	0.80 (d, 7.2)	0.81 (d, 6.8)	0.85 (d, 6.8)	0.92 (d, 7.2)	0.92 (d, 7.5)
3-*n*-butyrate	0.99 (t, 7.2)	1.00 (t, 7.2)	1.03 (t, 7.6)	0.97 (t, 7.5)	0.97 (t, 7.5)
	1.67 (m)	1.68 (m)	1.71 (m)	1.66 (m)	1.67 (m)
	2.38 (m)	2.35 (m)	2.36 (m)	2.32 (m)	2.31 (m)
3′	1.61 (m)	1.63 (m)	1.65 (m)		
2′	2.31 (m)	2.32 (m)	2.32 (m)		
$(CH_2)_n$	1.25 (br s)	1.26 (br s)	1.28 (br s)		
3″	1.26 (m)	1.26 (m)	1.29 (m)		
2″	1.28 (m)	1.28 (m)	1.31 (m)		
1″	0.87 (t, 7.2)	0.88 (t, 6.4)	0.92 (t, 7.2)		
11-OAc	1.98 (s)	1.99 (s)	2.02 (s)		
12-*n*-butyrate				0.91 (t, 7.2)	0.91 (t, 7.0)
				1.58 (m)	1.60 (m)
				2.13 (m)	2.13 (m)
13-OAc				2.00 (s)	2.00 (s)

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

and the detected 2D correlations in ^{1}H–^{1}H COSY and HMBC spectra led to the establishment of the molecular framework of **7** (Fig. 1). The relative configurations of all chiral centers except that of C-7 in **7** were confirmed to be mostly the same as those of **4** by analysis of NOE correlations. H_3-16 was found to exhibit an NOE correlation with H-5β but not with H-6, revealing the β-orientation of the acetoxy group at C-6 and α-orientation of the hydroxy group at C-7. Thus, the structure of diterpenoid **7** was established.

Klysimplexin P (**8**) was obtained as a colorless oil. The HRESIMS (*m*/*z* 563.2833 $[M + Na]^+$) of **8** established a molecular formula of $C_{28}H_{44}O_{10}$, appropriate for seven degrees of unsaturation. Inspection of the NMR spectroscopic data of **8** by the assistance of DEPT spectrum revealed the presence of eight methyls, five methylenes, eight methines (including four oxymethines), two sp^3 oxygenated carbons, and five carbonyl carbons (including one ketone, one aldehyde, and three ester carbonyls). Three ester carbonyls (δ_C 172.6, 170.1 and 170.0) were also assigned from the ^{13}C NMR spectrum and were HMBC correlated with the methylenes (δ_H 2.26 m, 2H and 1.63 m, 2H) of an *n*-butyrate unit and two acetate methyls (δ_H 2.01 s, 3H; 2.09 s, 3H), respectively. Therefore, the remaining two degrees of unsaturation identified metabolite **8** as a bicyclic compound. The ^{1}H NMR data of **8** (Table 4) showed a methyl (δ 2.23, 3H, s) attached to a carbonyl carbon, two tertiary methyls attached to oxygenated carbons (δ 1.42 and 1.14, each 3H, s), and two secondary methyls (δ 1.01 and 0.88, each 3H, d, J = 7.5 Hz) of an isopropyl moiety. Two oxymethines observed at δ_C 77.0 (CH) and 70.9 (CH) and δ_H 5.04 (1H, d, J = 9.5 Hz) and 5.45 (1H, dd, J = 11.0, 10.0 Hz) indicated the presence of two acetoxy substituents in the six-membered ring, as those of compounds **6** and **7**. By comparison of the ^{1}H NMR and ^{13}C NMR spectroscopic data of **8** with those of **1–7**, signals resonating at δ_H 2.47 (1H, m), 2.48 (1H, m), 3.73 s and 4.46 (1H, br t, J = 8.5), and at δ_C 42.2, 53.9, 88.5 and 75.4 also indicated the presence of a tetrahydrofuran structural unit in **8**. The 1D NMR and HSQC data showed signals at δ_C 206.3 (qC) and 201.4 (CH); δ_H 9.70 (1H, br s), and further supported the presence of a ketone and an aldehyde. The above findings together with careful analysis of ^{1}H–^{1}H COSY and HMBC correlations (Fig. 1), led to the establishment of the 6,7-secoeunicellin skeleton of **8**, as confirmed by key HMBC correlations from H_3-16 to C-7 (δ 206.3) and C-8 (δ 50.7), and one proton of H_2-4 (δ 2.44) and H_2-5 (δ 2.50) to aldehyde carbon (δ 201.4). Therefore, the molecular framework of **8** was established. In the NOESY spectrum of **8** (Fig. 4), the NOE correlations of H-10 with both H_2-8 and H-1; and H-1 with H-13 suggested that H-1, H-10 and H-13 are

Table 4 ^{1}H data for compounds **6–10**

Position	**6**[a]	**7**[b]	**8**[a]	**9**[c]	**10**[a]
1	2.55 (dd, 11.5, 7.5)[d]	2.41 (m)	2.47 (m)	2.38 (m)	2.82 (m)
2	3.59 (s)	3.55 (s)	3.73 (s)	5.17 (d, 6.4)	5.29 (d, 9.0)
4	2.24 (m)	2.66 (m)	2.44 (m)	2.34 (m)	2.10 (m)
	1.71 (m)	1.98 (m)	2.19 (m)	1.95 (m)	1.83 (m)
5	α 2.18 (m)	α 1.57 (m)	2.50 (m)	β 2.19 (m)	2.25 (m)
	β 1.70 (m)	β 1.47 (m)		α 1.40 (m)	2.03 (m)
6	4.33 (dd, 11.0, 4.0)	5.61 (d, 5.4)	9.70 (br s)	3.28 (dd, 11.2, 4.0)	5.24 (dd, 11.0, 4.5)
8α	2.44 (d, 14.5)	1.81 (m)	2.73 (m)	1.99 (m)	2.19 (m)
8β	2.86 (dd, 14.0, 5.0)	1.93 (m)		1.03 (m)	1.85 (m)
9	4.28 (dd, 11.5, 3.5)	4.29 (m)	4.46 (br t, 8.5)	1.33 (m)	1.57 (m)
					1.29 (m)
10	2.67 (dd, 11.0, 7.0)	2.63 (br t, 8.4)	2.48 (m)	2.96 (m)	1.94 (m)
12	5.01 (d, 10.0)	5.02 (d, 9.6)	5.04 (d, 9.5)	1.90 (m)	1.48 (m)
				1.56 (m)	
13	5.49 (dd, 11.0, 10.0)	5.48 (dd, 10.9, 9.9)	5.45 (dd, 11.0, 10.0)	1.37 (m)	1.53 (m)
					1.42 (m)
14	1.73 (m)	1.74 (m)	1.83 (t, 11.0)	1.09 (m)	0.94 (m)
15	1.60 (s)	1.39 (s)	1.42 (s)	1.70 (s)	1.72 (s)
16	5.46 (s); 5.12 (s)	1.19 (s)	2.23 (s)	1.17 (s)	1.53 (s)
17	1.18 (s)	1.12 (s)	1.14 (s)	1.44 (s)	1.25 (s)
18	1.98 (m)	1.72 (m)	1.73 (m)	1.92 (m)	1.88 (m)
19	0.99 (d, 7.5)	1.01 (d, 7.0)	1.01 (d, 7.5)	0.92 (d, 6.8)	0.98 (d, 7.0)
20	0.92 (d, (7.5)	0.96 (d, 7.0)	0.88 (d, 7.5)	0.69 (d, 6.8)	0.77 (d, 7.0)
3-*n*-butyrate	0.93 (t, 7.0)	0.99 (t, 7.1)	0.99 (t, 7.5)		
	1.63 (m)	1.69 (m)	1.63 (m)		
	2.12 (m)	2.37 (m); 2.28 (m)	2.26 (m)		
6-OAc		2.09 (s)			
11-OAc				2.01 (s)	
12-OAc	2.10 (s)	2.08 (s)	2.09 (s)		
13-OAc	2.01 (s)	1.99 (s)	2.01 (s)		

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

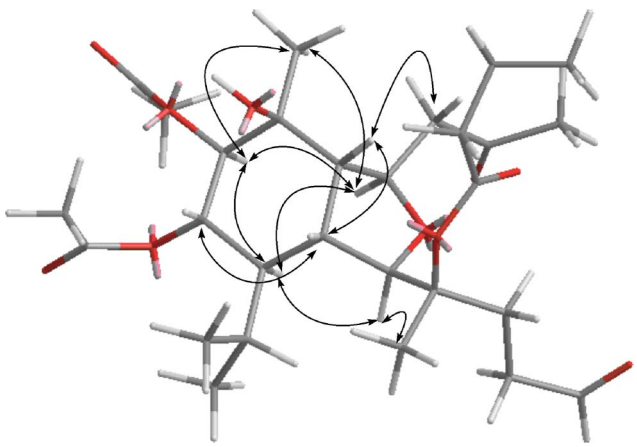


Fig. 4 Key NOESY correlations of **8**.

β-oriented. Also, correlations between H-2 and both H_3-15 and H-14; H-9 and H-12, H-14 and H_3-17; and H-12 and both H-14 and H_3-17, suggested that all of H-2, H-9, H-12, H-14, H_3-15 and H_3-17 are α-oriented. Thus, the structure of diterpenoid **8** was unambiguously established.

Klysimplexin Q (**9**) was found to possess a molecular formula of $C_{22}H_{36}O_3$, as revealed from its HRESIMS (m/z 371.2560 $[M + Na]^+$). Thus, the compound possesses five degrees of unsaturation. The 3H singlet appearing at δ 2.01 in the ^{1}H NMR spectrum and the carbonyl signal at δ 168.8 in the ^{13}C NMR spectrum were ascribable to an acetate. The twenty-two carbon signals appearing in the ^{13}C NMR spectrum of **9** (Table 2) were identified by DEPT spectrum to six methyls, six methylenes, six methines (including one vinylic CH and one epoxide CH), two sp^3 oxygenated quaternary carbons, one sp^2 quaternary carbon and one ester carbonyl carbon. Moreover, the ^{13}C signals at δ 133.8 (qC), 129.6 (CH), 64.9 (CH), and 60.9 (qC) assigned one trisubstituted double bond and one epoxide in the molecule. Three 3H singlets appearing in the ^{1}H NMR spectrum at δ 1.70, 1.44, and 1.17 were assigned to an olefinic methyl, one methyl attached to a quaternary oxycarbon, and one methyl of a trisubstituted epoxide in the molecule, respectively. Also, two doublets at δ_H 0.92 and 0.69 (each 3H, d, J = 6.8 Hz) arose from two methyls of an isopropyl group. The above functionalities revealed that compound **9** is a bicyclic compound. 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 molecular framework of **9** (Fig. 1). In the NOESY spectrum of compound **9** (Fig. 5), the NOE correlations between H_3-15 and H-2 revealed the Z geometry of the double bond at C-2 and C-3. In addition, H-6 showed NOE interactions with H-10, H-5β (δ 2.19), and H-4β (δ 2.34), but not with H_3-16; and H-1 showed NOE responses with both H-10 and H-4β (δ 2.34), but not with H-14, indicating the β-orientation for all of H-10, H-6, and H-1. The above observations and correlations between H-14 and both H-12α (δ 1.56) and H-2; H-12α (δ 1.56) and H_3-17; H-2 and both H_3-16 and H_3-15; and H_3-16 and H_3-15 suggested that all of H-14,

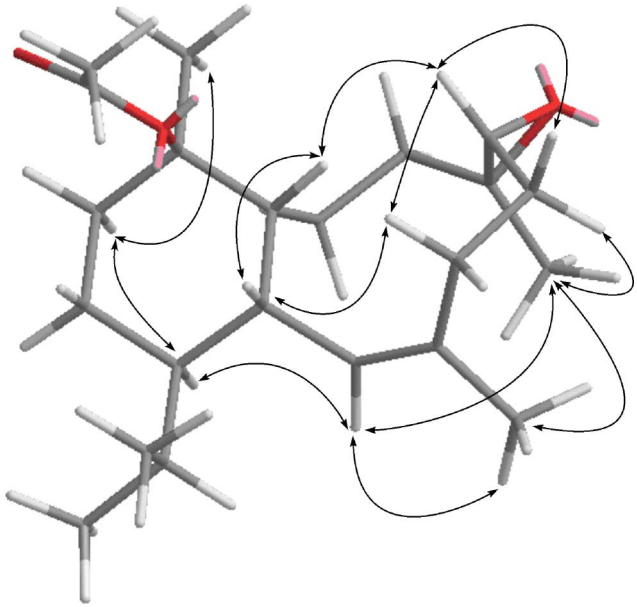


Fig. 5 Key NOESY correlations of **9**.

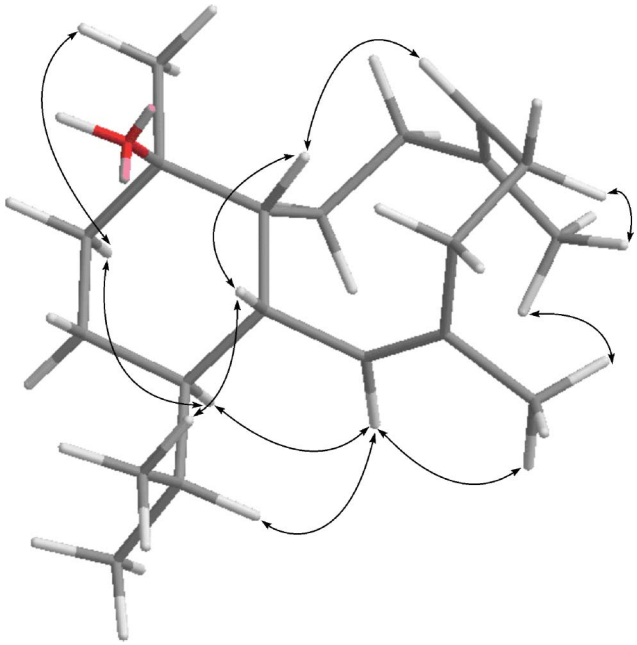


Fig. 6 Key NOESY correlations of **10**.

H_3-16, and H_3-17 are α-oriented. The structure of diterpenoid **9** was thus fully established.

Klysimplexin R (**10**) was isolated as a colorless oil and exhibited a pseudomolecular ion peak at *m/z* 290.2607 $[M]^+$ by HREIMS, appropriate for a molecular formula of $C_{20}H_{34}O$ and implying four degrees of unsaturation. The IR spectrum of **10** revealed the presence of hydroxy group (3398 cm^{-1}). The ^{13}C NMR spectroscopic data of **10** were found to be very similar to those of **9**, except that the 6,7-epoxide (δ 64.9, CH and 60.9, qC) in **9** was converted to an olefinic group (δ 138.3, qC and 124.6, CH) in **10**, as also confirmed by HMBC correlations (H_3-16 to C-6, C-7, and C-8), and the acetoxy group at C-11 in **9** was replaced by a hydroxy group in **10**. It was further observed that the NOE correlations of **10** (Fig. 6) are very similar to those of **9** and the *E* geometry 6,7-double bond in **10** was established by the NOE correlation between H_3-16 and one proton of H_2-5 (δ 2.25) and the upfield chemical shift of C-16 (δ 17.0) in **10**. Thus, the structure of **10** was determined unambiguously.

Klysimplexin S (**11**) was obtained as a colorless oil. The HRESIMS of **11** established the molecular formula $C_{26}H_{42}O_7$, implying 6 degrees of unsaturation. The IR absorption bands at 3347, 1731, and 1716 cm^{-1} revealed the presence of hydroxy and carbonyl functionalities. The ^{13}C NMR spectrum of **11** showed the presence of a ketone (δ_C 209). Two ester carbonyl carbons (δ_C 171.1 and 167.9) were HMBC correlated with the methylenes (δ_H 2.45 m, 2H and 1.75 m, 2H) of an *n*-butyrate and an acetate methyl (δ_H 2.02 s, 3H), respectively. Therefore, **11** is a tricyclic diterpenoid. The molecular framework was also confirmed by ^1H–^1H COSY and HMBC experiments (Fig. 1). It was shown that the NMR data of **11** (Tables 2 and 5) were almost identical to those of australin A (**14**),[8] except that the hydroxy group at C-3 in **14** (Scheme 2) was replaced by an *n*-butyryloxy in **11**, as confirmed by the downfield shifted δ value of H_3-15 (δ 1.55) of **11**, relative to that of **14** (δ 1.23), and the HMBC correlation from H-2 (δ 3.90) to the carbonyl carbon resonating at δ 171.1 (qC).

Table 5 ^1H data for compounds **11–12**

Position	**11**[a]	**12**[a]
1	2.56 (dd, 12.0, 4.4)[b]	2.34 (dd, 12.8, 6.4)
2	3.90 (s)	2.22 (d, 13.2)
4	2.98 (dd, 13.6, 4.0)	2.15 (m)
	1.41 (dd, 13.6, 7.6)	
5	1.70 (m)	α 1.86 (m)
		β 1.51 (m)
6	3.85 (dd, 11.2, 6.0)	4.35 (dd, 12.0, 5.2)
8	α 2.02 (d, 12.0)	α 2.30 (m)
	β 2.79 (d, 12.0)	β 1.42 (m)
9		5.31 (m)
10	4.06 (d, 4.4)	1.94 (d, 5.6)
12	2.26 (dd, 9.6, 3.6)	1.59 (m)
		1.54 (m)
13	1.65 (m)	1.57 (m)
	1.23 (m)	1.37 (m)
14	1.98 (m)	1.15 (m)
15	1.55 (s)	4.84 (s)
		4.66 (s)
16	1.16 (s)	0.86 (s)
17	1.49 (s)	1.32 (s)
18	1.92 (m)	1.80 (m)
19	1.01 (d, 6.8)	0.90 (d, 6.8)
20	0.76 (d, 6.8)	0.87 (d, 6.8)
3-*n*-butyrate	1.05 (t, 7.2)	
	1.75 (m)	
	2.45 (m)	
9-OAc		2.08 (s)
11-OAc	2.02 (s)	
7-OH	4.88 (s)	

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

The HRESIMS (*m/z* 387.2509, $[M + Na]^+$) of klysimplexin T (**12**) established the molecular formula $C_{22}H_{36}O_4Na$, consistent with five degrees of unsaturation. The IR absorptions of **12** indicated the presence of hydroxy (3641 cm^{-1}) and carbonyl

(1735 cm^{-1}) functionalities. The NMR spectra of **12** (Tables 2 and 5) showed signals of an 1,1-disubstituted carbon–carbon double bond (δ_C 144.9, qC and 111.9, CH_2; δ_H 4.84 s and 4.66 s). The presence of an acetoxy group was indicated by the ^1H NMR signal at δ 2.08 (s, 3H) and ^{13}C NMR signals at δ 22.7 (CH_3) and 169.0 (qC). ^1H NMR data of **12** (Table 5) showed the presence of a methyl (δ_H 1.32) attached to an oxygenated carbon, and a methyl (δ_H 0.86) bound to a quaternary carbon, respectively. Also, two doublets at δ_H 0.90 and 0.87 (each 3H, d, J = 6.8 Hz) arose from two methyls of an isopropyl group. The above functionalities revealed that **12** is a tricyclic terpenoidal compound. The molecular framework of **12** was further established by 2D NMR studies, in particular ^1H–^1H COSY and HMBC correlations (Fig. 1). In the NOESY spectrum of **12** (Fig. 7), NOE correlations of H-1 and all of H-6, H-10, H-18 and H_3-19 were observed, suggesting that H-1, H-6, H-10 and the C-14 isopropyl group are β-oriented, and H-14 should be placed α-oriented. Also, correlations of H-2 and H-14, H_3-16 and H_3-17; and H-9 and H_3-17 suggested that of all of H-2, H-9, H-14, H_3-16 and H_3-17 are α-oriented. Thus, the relatively structure of diterpenoid **12** could be established.

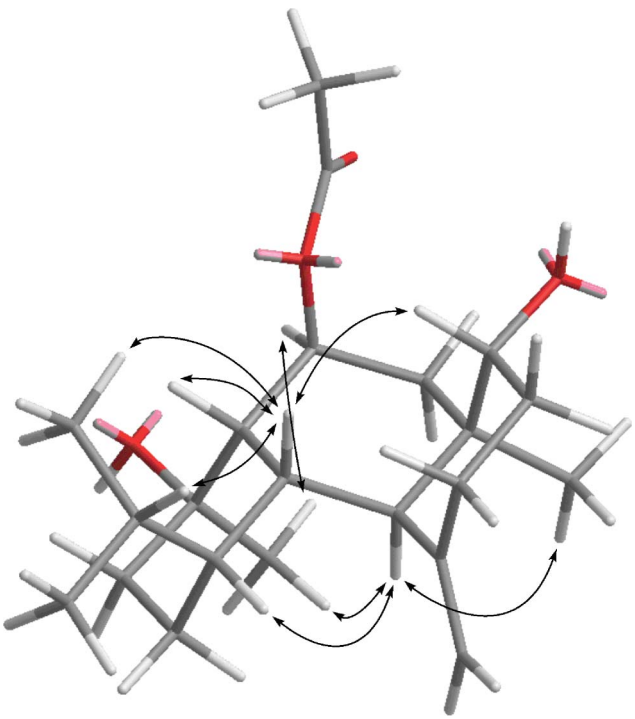
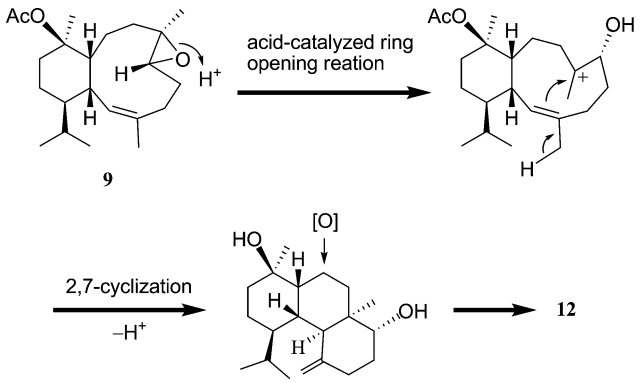


Fig. 7 Key NOESY correlations of **12**.

Compound **12** could be assumed to be biosynthesized *via* the acid-catalyzed ring opening of 6,7-epoxide to form a C-7 carbonium ion followed by carbon–carbon bond formation between C-2 and C-7 of a corresponding eunicellin, presumably the deacetyl derivative of **9**, and hydroxylation at C-9 and subsequent acetylation (Scheme 3). Alternatively, **1–3** and **7** might arise from the acid-catalyzed reaction of a 6,7-diastereomer of **9**, which was not found in this study, to form the cation at C-7 and the subsequent addition of water from α face in the next step.

Cytotoxicity of metabolites **1–12** toward a limited panel of cancer cell lines was evaluated. The results showed that compound



Scheme 3 Proposed biosynthetic pathway of **12**.

9 exhibited 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 53.2, 35.1, 44.0, 36.5, 40.5, and 40.5 μM, respectively. Also, metabolite **12** showed cytotoxicity (IC_{50}'s 34.3, 26.4, 44.0, 27.2, 42.0 and 37.4 μM) against the growth of Hep G2, Hep 3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cells, respectively. Other metabolites were found to be inactive against the growth of the above six cancer cells.

The *in vitro* anti-inflammatory effects of compounds **1–12** were also tested. In this assay, the inhibition of LPS-induced up-regulation of pro-inflammatory proteins, iNOS and COX-2 in RAW264.7 macrophage cells was measured by immunoblot analysis. At a concentration of 10 μM, compounds **2–6**, **10** and **11** were found to significantly reduce the expression of iNOS protein, relative to the control cells stimulated with LPS only. Furthermore, at the same concentration, metabolites **10** and **11** also could effectively reduce COX-2 expression in the same macrophage cells with LPS treatment. On the other hand, **7** could enhance the expression of both iNOS and COX-2 which might arise from the presence of acetoxy and hydroxy groups at C-6 and C-7, respectively. Thus, compounds **2–6**, **10** and **11** might be useful anti-inflammatory agents, while **11** is a promising anti-inflammatory lead compound as it showed potent inhibitory activity against the expression of both iNOS and COX-2 proteins (Fig. 8)

Conclusion

Our investigation demonstrated that the cultured soft coral, *K. simplex*, could be a good source of bioactive substances. Several of the isolated compounds, in particular **10** and **11**, are potential anti-inflammatory agents. Also, it is worthwhile to note here that **8** is a 6,7-secoeunicellin, while **12** is a tricarbocyclic compound which might be derived from the carbon–carbon bond formation between C-2 and C-7 of a corresponding 2,9-deoxygenated eunicellin.

Experimental

General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS spectra were obtained with a Bruker

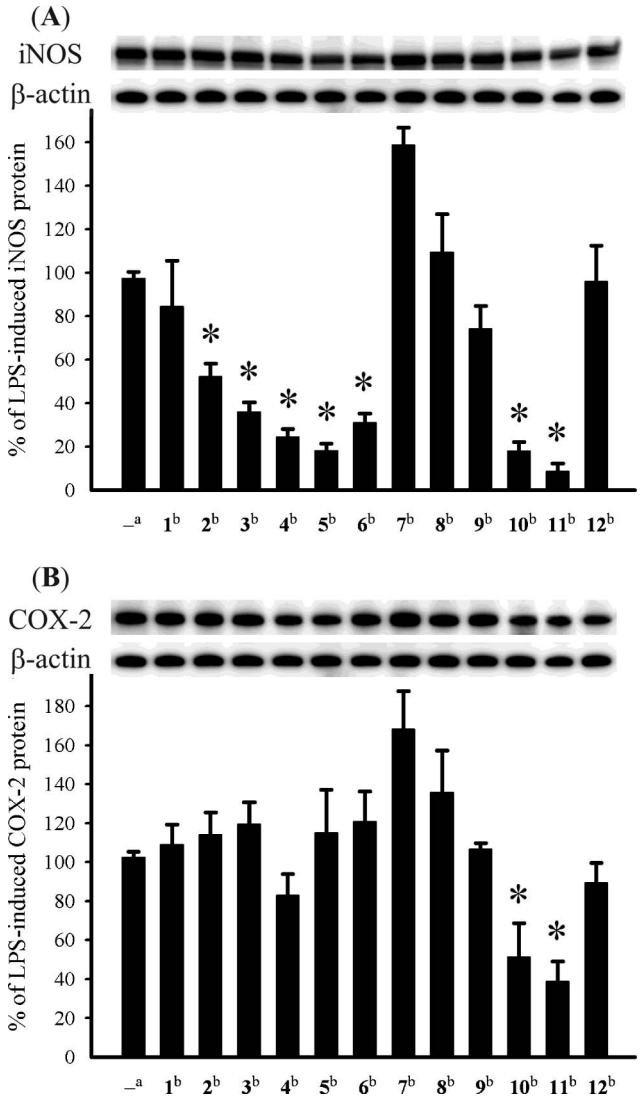


Fig. 8 Effect of compounds **1–12** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (**A**) Immunoblots of iNOS and β-actin; (**B**) Immunoblots of COX-2 and β-actin. The values are mean ± SEM. (n = 6). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS and COX-2 to 2.5 ± 3.7% and 67.2 ± 13.4%, respectively. *Significantly different from LPS alone stimulated group (*$P < 0.05$). [a]stimulated with LPS, [b]stimulated with LPS in the presence of **1–12** (10 μM).

APEX II mass spectrometer. LC-ESI MS/MS spectrometry analysis was carried out using an Applied Biosystem API 4000 tandem quadrupole mass spectrometer. 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 ODS column (250 × 21.2 mm, 5 μm).

Extraction and isolation

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, Taiwan, in July 2005. A voucher sample (CSC-2) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University. 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 EtOH extract of the frozen organism was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2-soluble portion (15.2 g) was subjected to column chromatography on silica gel and eluted with EtOAc in n-hexane (0–100% of EtOAc, gradient) and then further with MeOH in EtOAc with increasing polarity to yield 40 fractions. Fraction 10, eluted with n-hexane–EtOAc (15 : 1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (A1–A4). Subfraction A3 was separated by reverse-phase HPLC (CH_3CN–H_2O, 6 : 1 to 3 : 1) to afford compounds **9** (6.0 mg) and **10** (2.2 mg). Fraction 21, eluted with n-hexane–EtOAc (9 : 1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (B1–B5). Subfraction B3 was separated by reverse-phase HPLC (CH_3CN, 100%) to afford compounds **1** (15.5 mg), **2** (4.2 mg), and **3** (1.1 mg), respectively. Fraction 23, eluted with n-hexane–EtOAc (5 : 1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (C1–C5). Subfractions C3 and C4 were separated by reverse-phase HPLC (CH_3CN–H_2O, 4 : 1 to 1 : 1) to afford compounds **4** (1.2 mg), **5** (1.1 mg), **6** (1.0 mg), and **12** (1.1 mg), respectively. Fraction 26, eluted with n-hexane–EtOAc (2 : 1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (D1–D4). Subfraction D3 was separated by reverse-phase HPLC (CH_3CN–H_2O, 3 : 1 to 1 : 2) to afford compounds **7** (15.3 mg), **8** (1.2 mg), and **11** (2.3 mg).

Klysimplexin I (1). Colorless oil; $[\alpha]_D^{25}$ −38 (c 1.55, $CHCl_3$); IR (neat) ν_{max} 3460, 1738 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CHCl_3$), see Tables 1 and 3; ESIMS m/z 701 $[M + Na]^+$; HRESIMS m/z 701.4974 $[M + Na]^+$ (calcd. 701.4968 for $C_{40}H_{70}O_8Na$).

Klysimplexin J (2). Colorless oil; $[\alpha]_D^{25}$ −40 (c 0.42, $CHCl_3$); IR (neat) ν_{max} 3463, 1723 cm^{-1};^{13}C and ^{1}H NMR data (400 MHz; $CHCl_3$), see Tables 1 and 3; ESIMS m/z 730 $[M + Na]^+$; HRESIMS m/z 729.5277 $[M + Na]^+$ (calcd. 729.5281 for $C_{42}H_{74}O_8Na$).

Klysimplexin K (3). Colorless oil; $[\alpha]_D^{25}$ −38 (c 0.11, $CHCl_3$); IR (neat) ν_{max} 3437, 1734 cm^{-1};^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS m/z 757.55 $[M + Na]^+$; HRESIMS m/z 757.5590 $[M + Na]^+$ (calcd. 757.5594 for $C_{44}H_{78}O_8Na$).

Klysimplexin L (4). Colorless oil; $[\alpha]_D^{25}$ −64 (c 0.12, $CHCl_3$); IR (neat) ν_{max} 3452, 1734 cm^{-1};^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS m/z 575 $[M + Na]^+$; HRESIMS m/z 575.3193 $[M + Na]^+$ (calcd. 575.3196 for $C_{30}H_{48}O_9Na$).

Klysimplexin M (5). Colorless oil; $[\alpha]_D^{25}$ −74 (c 0.11, $CHCl_3$); IR (neat) ν_{max} 3452, 1738 cm^{-1};^{13}C and ^{1}H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS m/z 591 $[M + Na]^+$; HRESIMS m/z 591.3146 $[M + Na]^+$ (calcd. 591.3145 for $C_{30}H_{48}O_{10}Na$).

Klysimplexin N (6). Colorless oil; $[\alpha]_D^{25}$ −53 (*c* 0.10, $CHCl_3$); IR (neat) ν_{max} 3467, 1738 cm^{-1};^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 4; ESIMS *m/z* 547 $[M + Na]^+$; HRESIMS *m/z* 547.2885 $[M + Na]^+$ (calcd. 547.2883 for $C_{28}H_{44}O_9Na$).

Klysimplexin O (7). Colorless oil; $[\alpha]_D^{25}$ −27 (*c* 1.53, $CHCl_3$); IR (neat) ν_{max} 3478, 1734 cm^{-1}; ^{13}C and ^1H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 4; ESIMS *m/z* 607 $[M + Na]^+$; HRESIMS *m/z* 607.3095 $[M + Na]^+$ (calcd. 607.3094 for $C_{30}H_{48}O_{11}Na$).

Klysimplexin P (8). Colorless oil; $[\alpha]_D^{25}$ −23 (*c* 0.12, $CHCl_3$); IR (neat) ν_{max} 3460, 1738 and 1711 cm^{-1}; ^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 4; ESIMS *m/z* 563 $[M + Na]^+$; HRESIMS *m/z* 563.2833 $[M + Na]^+$ (calcd. 563.2832 for $C_{28}H_{44}O_{10}Na$).

Klysimplexin Q (9). Colorless oil; $[\alpha]_D^{25}$ +56 (*c* 0.60, $CHCl_3$); IR (neat) ν_{max} 1734 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 2 and 4; ESIMS *m/z* 371 $[M + Na]^+$; HRESIMS *m/z* 371.2560 $[M + Na]^+$ (calcd. 371.2562 for $C_{22}H_{36}O_3Na$).

Klysimplexin R (10). Colorless oil; $[\alpha]_D^{25}$ +30 (*c* 0.22, $CHCl_3$); IR (neat) ν_{max} 3398 cm^{-1}; ^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 2 and 4; EIMS *m/z* 290 [(5.9) $M]^+$, 272 [(9.9) $M - H_2O]^+$, 257 [(5.9) $M - Me - H_2O]^+$; HREIMS *m/z* 290.2607 $[M]^+$ (calcd. 290.2610 for $C_{20}H_{34}O$).

Klysimplexin S (11). Colorless oil; $[\alpha]_D^{25}$ −43 (*c* 0.23, $CHCl_3$); IR (neat) ν_{max} 3347 1731 and 1716 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 2 and 5; ESIMS *m/z* 489 $[M + Na]^+$; HRESIMS *m/z* 489.2831 $[M + Na]^+$ (calcd. 489.2828 for $C_{26}H_{42}O_7Na$).

Klysimplexin T (12). Colorless oil; $[\alpha]_D^{25}$ −56 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3641 and 1735 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 2 and 5; ESIMS *m/z* 387 $[M + Na]^+$; HRESIMS *m/z* 387.2509 $[M + Na]^+$ (calcd. 387.2511 for $C_{22}H_{36}O_4Na$).

Base-catalyzed hydrolysis of 1

A solution of **1** (10.1 mg) was dissolved in 5% methanolic NaOH solution (2.7 mL), and the mixture was stirred at 0 °C for 12 h. The mixture was then neutralized with diluted HCl (0.1 N) and the resulted solution was evaporated. The afforded residue was extracted with $CHCl_3$ (2.0 mL × 3). The $CHCl_3$-soluble layers were combined, dried over anhydrous $NaSO_4$ and evaporated. The residue was subjected to column chromatograph over silica gel using EtOAc–n-hexane (1 : 1) to yield **13** (4 mg, 57.4%).

Preparation of (*S*)-and (*R*)-MTPA esters of 4

To a solution of **4** (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, **4a** (0.6 mg, 86%). The same procedure was used to prepare the (*R*)-MTPA ester, **4b** (0.6 mg, 86%) from the reaction of (*S*)-MTPA chloride with **4** in pyridine. Selective ^1H NMR ($CDCl_3$, 400 MHz) of **4a**: 5.425 (1H, m, H-6), 5.415 (1H, s, H-16a), 5.196 (1H, s, H-16b), 4.312 (1H, dd, *J* = 10.8 and 4.8, H-9), 2.467 (1H, d, *J* = 13.2 Hz, H-8a), 2.252 (1H, m, H-4a), 2.084 (1H, m, H-5a), 1.847 (1H, m, H-5b), 1.610 (3H, s, H_3-15). Selective ^1H NMR ($CDCl_3$, 400 MHz) of **4b**: *δ* 5.391 (1H, dd, *J* = 10.4 and 3.6 Hz, H-6), 5.215 (1H, s, H-16a), 5.097 (1H, s, H-16b), 4.306 (1H, dd, *J* = 10.8 and 4.8, H-9), 2.459 (1H, d, *J* = 13.6 Hz, H-8a), 2.253 (1H, m, H-4a), 2.145 (1H, m, H-5a), 1.867 (1H, m, H-5b), 1.623 (3H, s, H_3-15).

Cytotoxicity testing

Cell lines were purchased from the American Type Culture Collec-tion (ATCC). Cytotoxicity assays were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[27,28]

In vitro anti-inflammatory assay

Macrophage (RAW264.7) cell line was purchased from ATCC. *In vitro* anti-inflammatory activity of compounds **1–12** was measured by examining the inhibition of lipopolysaccharide (LPS)-induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophage cells using western blotting analysis.[29,30]

Acknowledgements

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