

Article

Klyflaccicembranols A–I, New Cembranoids from the Soft Coral *Klyxum flaccidum*

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Abstract: New cembranoids klyflaccicembranols A–I (1–9), along with gibberosene D (10), have been isolated from the organic extract of a Formosan soft coral *Klyxum flaccidum*. Their structures were established by extensive spectroscopic analyses, including 2D NMR spectroscopy, and spectral data comparison with related structures. The cytotoxicity of the isolated metabolites, as well as their nitric oxide (NO) inhibitory activity, were evaluated and reported. Metabolites 2, 4, 6, 8 and 9 were found to exhibit variable activities against a limited panel of cancer cell lines in a range of IC₅₀ 16.5–49.4 μM. Among the tested cembranoids, compounds 4, 5, 6, and 9 significantly inhibited NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages at a dose of 50 μg/mL.

Keywords: soft coral; *Klyxum flaccidum*; cembranoid; NO inhibition; cytotoxicity

1. Introduction

The marine environment has long been recognized as an exceptional source of new natural products with a diverse range of chemical structures and bioactivities, including anti-cancer, antiviral, immunosuppressive and anti-inflammatory activities [1–3]. This structural diversity has supplied unique chemicals for the pharmaceutical, cosmetics, and even agrochemicals industry [4]. Soft corals belonging to the genus *Klyxum* (Alcyoniidae), including *Klyxum flaccidum*, are considered rich sources of eunicellin-based diterpenoids species [2,5–10], of which many compounds have been found to exert anti-inflammatory [7–10] and cytotoxic [8,11] effects. A series of cytotoxic and anti-inflammatory steroids was also discovered from *K. flaccidum* in our previous investigations [12,13]. Cembranoid-based diterpenoids have not hitherto been isolated from soft corals of the genus *Klyxum*. However, our continuing investigation into the chemical constituents of a Formosan soft coral *K. flaccidum* has led to the discovery of a series of new polyoxygenated cembranoids. Extensive spectroscopic analyses, including 2D NMR spectroscopy and spectral comparison, were applied

to establish the structures of these new metabolites. The cytotoxicity of the isolated metabolites was further assessed against a limited panel of cancer cell lines via Alamar Blue assay [14,15], and anti-inflammatory activity was evaluated in terms of their potential to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages.

2. Results and Discussion

Sliced bodies of the soft coral *K. flaccidum* were extracted exhaustively with ethyl acetate (EtOAc). The solvent-free extract was then fractionated over a silica gel 60 column to yield 26 fractions. Fractions that displayed terpenoidal methyl, olfeinic and oxymethine proton signals in the ^1H NMR spectrum were selected and further purified using a successive series of silica gel 60 and RP-18 gel columns to yield compounds 1–10 (Figure 1).

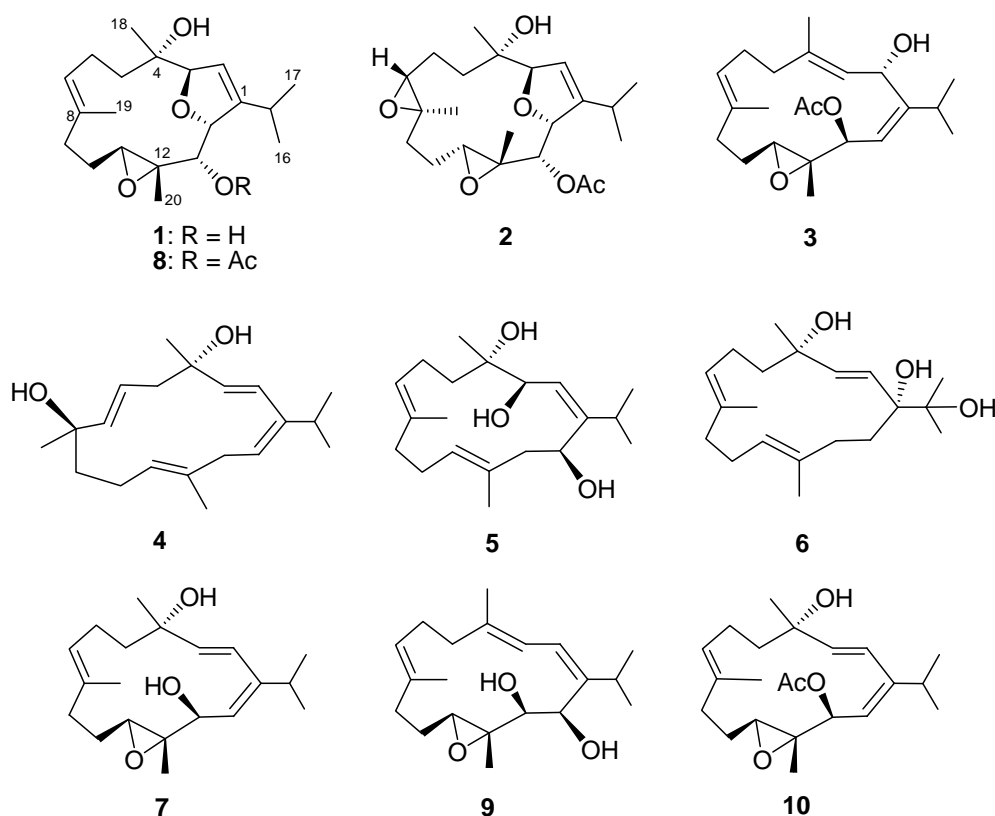


Figure 1. Structures of cembranoids isolated from *Klyxum flaccidum*.

Klyflaccicembranol A (**1**) was isolated as a colorless oil. Its molecular formula $\text{C}_{20}\text{H}_{32}\text{O}_4$ was deduced from the sodium adduct ion peak (m/z 359.2196 $[\text{M} + \text{Na}]^+$) in the high-resolution electrospray ionization mass spectrometry (HRESIMS) and NMR data measured in C_6D_6 (Tables 1 and 2). Thus, five degrees of unsaturation were indicated. The infrared (IR) absorption band at ν_{max} 3445 cm^{-1} revealed the presence of hydroxy functionality. The ^{13}C NMR spectrum displayed 20 carbon signals (Table 1), which were characterized by distortionless enhancement by polarization transfer (DEPT) and heteronuclear single-quantum correlation (HSQC) spectra as five methyls, four methylenes, seven methines (including two olfeinic and four sp^3 oxymethines), and four nonprotonated carbons (including two olfeinic and two sp^3 oxycarbons). The NMR signals at δ_{C} 147.8, C; 132.1, C; 127.0, CH and 121.0, CH; δ_{H} 5.59, 1H, br s and 5.38, 1H, dd, $J = 5.2, 5.2$ Hz indicated the presence of two trisubstituted double bonds. Thus, the remaining three unsaturations suggested **1** to be a tricyclic diterpenoid. However, it was found that the NMR data of **1** differed from those of eunicellins discovered from *Klyxum* species [2,5–10] and resembled those of cembranoids isolated from *Sinularia* and *Sarcophyton*

species [16–18], as no correlation spectroscopy (COSY) correlated ring-juncture sp^3 methine protons were detected in **1**. One trisubstituted epoxide (δ_C 61.9, C and 59.7, CH; δ_H 3.15, 1H, dd, $J = 6.4$, 2.0 Hz) and an ether bridge (δ_C 89.7, CH; δ_H 4.61, 1H, d, $J = 4.8$ Hz and δ_C 85.8, CH; δ_H 4.78, 1H, br d, $J = 4.8$ Hz) of a 2,3,5-trisubstituted dihydrofuran moiety [18] were also deduced. Thus, the remaining two oxygen atoms of the molecular formula were ascribed to two hydroxy groups linked to the carbon atoms resonating at 72.8 (CH) and 74.2 (C). Analysis of the COSY correlations of **1** indicated the presence of four proton-correlated partial structures, including those of an isopropyl group (Figure 2). By analysis of heteronuclear multiple bond correlation (HMBC) correlations observed from the methyl, olefinic, and oxymethine protons, it was possible to connect the four partial structures and to assign the positions of the two hydroxy groups, one trisubstituted double bond, and a trisubstituted epoxide at C-4, C-13, C-7/C-8, and C-11/C-12, respectively (Figure 2). The ether linkage of the dihydrofuran was confirmed by the HMBC correlations found from H-2 (δ_H 5.59, br s) to C-1 (δ_C 147.8, C), C-3 (δ_C 89.7, CH) and C-14 (δ_C 85.8, CH) and from H-14 (δ_H 4.78, br d, $J = 4.8$ Hz) to C-3, respectively. The planar structure of compound **1** was thus established as 4,13-dihydroxy-3,14:11,12-bisepoxy-cembra-1,7-diene.

Table 1. ^{13}C NMR spectral data of compounds 1–9.

#	1 ^a	2 ^b	3 ^c	4 ^a	5 ^c	6 ^b	7 ^c	8 ^c	9 ^a
1	147.8 (C)	147.7 (C)	155.9 (C)	146.3 (C)	154.6 (C)	80.9 (C)	146.0 (C)	147.4 (C)	146.9 (C)
2	121.0 (CH) ^d	121.2 (CH)	67.2 (CH)	124.3 (CH)	122.7 (CH)	129.2 (CH)	123.7 (CH)	121.2 (CH)	120.8 (CH)
3	89.7 (CH)	91.1 (CH)	126.3 (CH)	136.2 (CH)	70.7 (CH)	138.0 (CH)	138.3 (CH)	89.1 (CH)	121.0 (CH)
4	74.2 (C)	74.6 (C)	137.6 (C)	72.9 (C)	75.2 (C)	71.9 (C)	73.0 (C)	74.6 (C)	136.6 (C)
5	41.6 (CH ₂)	40.5 (CH ₂)	39.0 (CH ₂)	45.5 (CH ₂)	38.6 (CH ₂)	43.8 (CH ₂)	39.0 (CH ₂)	41.3 (CH ₂)	39.1 (CH ₂)
6	22.1 (CH ₂)	25.2 (CH ₂)	24.2 (CH ₂)	121.3 (CH)	22.3 (CH ₂)	22.3 (CH ₂)	24.2 (CH ₂)	21.8 (CH ₂)	25.6 (CH ₂)
7	127.0 (CH)	64.6 (CH)	125.2 (CH)	141.4 (CH)	127.0 (CH)	128.6 (CH)	127.6 (CH)	126.3 (CH)	126.5 (CH)
8	132.1 (C)	59.9 (C)	133.2 (C)	72.5 (C)	133.9 (C)	132.7 (C)	131.9 (C)	132.6 (C)	133.8 (C)
9	36.8 (CH ₂)	36.7 (CH ₂)	36.9 (CH ₂)	43.6 (CH ₂)	39.0 (CH ₂)	39.0 (CH ₂)	36.7 (CH ₂)	36.8 (CH ₂)	36.6 (CH ₂)
10	25.1 (CH ₂)	23.6 (CH ₂)	24.3 (CH ₂)	23.5 (CH ₂)	24.2 (CH ₂)	23.8 (CH ₂)	24.3 (CH ₂)	24.5 (CH ₂)	24.8 (CH ₂)
11	59.7 (CH)	59.0 (CH)	59.7 (CH)	126.7 (CH)	125.6 (CH)	126.9 (CH)	61.3 (CH)	59.2 (CH)	59.9 (CH)
12	61.9 (C)	60.3 (C)	61.3 (C)	132.8 (C)	131.9 (C)	136.1 (C)	64.7 (C)	60.4 (C)	62.9 (C)
13	72.8 (CH)	71.9 (CH)	71.6 (CH)	37.3 (CH ₂)	44.1 (CH ₂)	36.1 (CH ₂)	71.6 (CH)	72.7 (CH)	75.2 (CH)
14	85.8 (CH)	85.0 (CH)	115.6 (CH)	122.0 (CH)	70.6 (CH)	29.9 (CH ₂)	122.9 (CH)	84.9 (CH)	68.1 (CH)
15	26.6 (CH)	25.7 (CH)	27.6 (CH)	31.6 (CH)	27.8 (CH)	75.1 (C)	32.1 (CH)	25.7 (CH)	28.2 (CH)
16	22.2 (CH ₃)	20.9 (CH ₃)	23.7 (CH ₃)	22.6 (CH ₃)	22.8 (CH ₃)	24.5 (CH ₃)	22.1 (CH ₃)	22.2 (CH ₃)	24.0 (CH ₃)
17	21.2 (CH ₃)	20.6 (CH ₃)	24.5 (CH ₃)	22.6 (CH ₃)	23.5 (CH ₃)	24.7 (CH ₃)	22.3 (CH ₃)	20.9 (CH ₃)	25.4 (CH ₃)
18	23.1 (CH ₃)	22.2 (CH ₃)	15.7 (CH ₃)	29.7 (CH ₃)	25.1 (CH ₃)	27.8 (CH ₃)	30.0 (CH ₃)	22.6 (CH ₃)	16.7 (CH ₃)
19	16.7 (CH ₃)	16.8 (CH ₃)	15.2 (CH ₃)	29.6 (CH ₃)	15.1 (CH ₃)	14.7 (CH ₃)	15.1 (CH ₃)	16.7 (CH ₃)	15.2 (CH ₃)
20	15.5 (CH ₃)	16.0 (CH ₃)	15.2 (CH ₃)	17.6 (CH ₃)	17.1 (CH ₃)	14.8 (CH ₃)	15.5 (CH ₃)	16.0 (CH ₃)	16.1 (CH ₃)
OAc		169.9 (C)	170.7 (C)					169.9 (C)	
		21.0 (CH ₃)	21.1 (CH ₃)					20.6 (CH ₃)	

Spectra recorded in ^a C₆D₆ at 100 MHz; ^b CDCl₃ at 125 MHz; and ^c CDCl₃ at 100 MHz at 25 °C; ^d Attached protons were determined by distortionless enhancement by polarization transfer (DEPT) experiments. Values are presented as ppm downfield from tetramethylsilane (TMS).

Table 2. ^1H NMR spectral data for compounds 1–5.

#	1 ^a	2 ^b	3 ^c	4 ^a	5 ^c
2	5.59 br s	5.65 br s	5.70 d (10.0)	6.19 d (16.4)	5.41 d (7.6)
3	4.61 d (4.8) ^d	4.78 d (5.0)	5.25 d (10.0)	5.83 d (16.4)	4.34 d (7.6)
5	1.48 m; 1.85, m	1.82 m; 1.92 m	2.10 m; 2.24 m	2.31 2H, d (6.8)	1.55 m; 1.86 m
6	2.02 m; 2.15, m	1.59 m; 1.86 m	2.10 m; 2.27 m	5.56 dd (15.6, 6.8)	2.11 m; 2.34 m
7	5.38 dd (5.2, 5.2)	3.07 dd (6.0, 2.5)	4.83 br d (6.0)	5.52 d (15.6)	4.99 dd (6.0, 6.0)
9	2.04 m; 2.09 m	2.10 m; 1.39 m	2.16 m; 2.20 m	1.58 m; 1.67 m	1.98 m; 2.15 m
10	1.76 m; 1.83 m	1.54 m; 1.95 m	1.61 m; 1.86 m	2.01 m; 2.37 m	2.14 m; 2.18 m
11	3.15 dd (6.4, 2.0)	2.98 d (7.5)	2.47 dd (7.2, 2.0)	5.11 dd (7.2, 7.2)	4.93 dd (6.8, 6.0)
13	3.58 br s	5.21 s	5.52 d (10.4)	2.71 2H, d (8.0)	2.27 m; 2.37 m
14	4.78 br d (4.8)	5.05 d (5.0)	5.03 d (10.4)	5.48 dd (8.0, 5.6)	4.78 dd (5.6, 5.6)
15	2.25 sept (6.8)	2.17 m	2.78 sept (6.8)	2.53 sept (6.8)	2.48 m
16	0.94 3H, d (6.8)	1.05 3H, d (6.5)	1.05 3H, d (6.8)	1.09 3H, d (6.8)	1.06 3H, d (6.8)
17	1.12 3H, d (6.8)	1.10 3H, d (6.5)	1.09 3H, d (6.8)	1.10 3H, d (6.8)	1.12 3H, d (6.8)
18	0.98 3H, s	1.05 3H, s	1.80 3H, s	1.27 3H, s	1.14 3H, s
19	1.48 3H, s	1.24 3H, s	1.55 3H, s	1.16 3H, s	1.55 3H, s
20	1.18 3H, s	1.42 3H, s	1.22 3H, s	1.60 3H, s	1.68 3H, s
OAc		1.91 3H, s	2.10 3H, s		

Spectra recorded in ^a C₆D₆, at 400 MHz; ^b CDCl₃ at 500 MHz; and ^c CDCl₃ at 400 MHz at 25 °C; ^d J values (Hz) in parentheses.

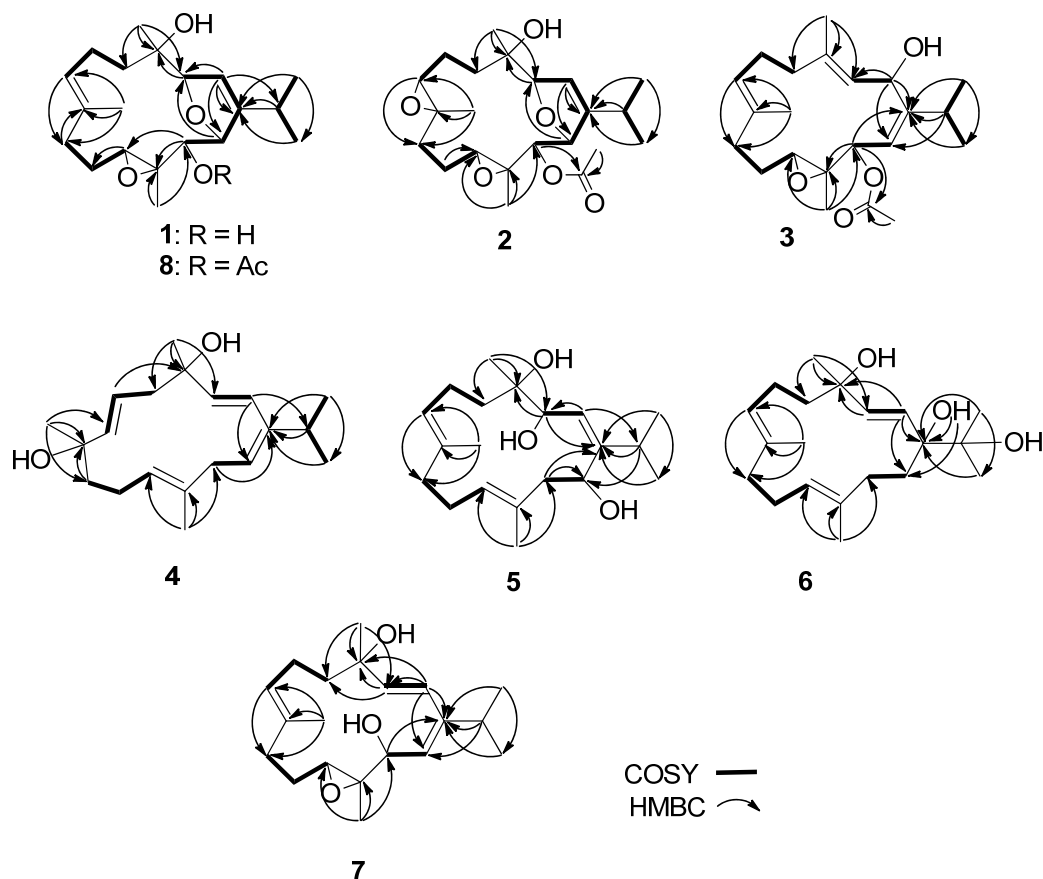


Figure 2. Correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) correlations in 1–8.

The relative configuration of **1** was determined by the analysis of nuclear Overhauser effect (NOE) correlations in a nuclear Overhauser enhancement spectroscopy (NOESY) experiment and with the assistance of $^5J_{H,H}$ coupling constants, in addition to molecular modeling using molecular mechanical parameters (MM2 force field) calculations (Figure 3). The high magnitude of the long-range coupling constant of H-3 and H-14 ($J = 4.8$ Hz) of the 2,5-dihydrofuran ring in **1** suggested the *trans* orientation of protons at C-3/C-14 [19]. Accordingly, no NOE correlation was detected between H-3 and H-14. From the NOESY spectrum of **1**, it was found that H₃-18 showed NOE interaction with H-14, and H-14 with H-13 and H₃-20; therefore, due to the β -orientation of H₃-18, H-13 and H-14 should also be positioned on the β -face. Furthermore, H-3 exhibited NOE correlation with the olefinic H-7, as did H-7 with H-11, while H-11 did not show NOE interaction with H₃-20, revealing that H-3 and H-11 should be positioned on the α -face. Moreover, the NOE interactions exhibited for the olefinic H-2 with H₃-17 and for H₃-19 with H-9, but not with H-7, enabled the assignment of *Z* and *E* geometries of the double bonds at C-1/C-2 and C-7/C-8, respectively. As a result, the (3*R**,4*S**,11*S**,12*R**,13*S**,14*R**)-configuration of **1** was established.

After isolation and chemical identification of **1**, related compound klyflaccembranol H (**8**) was isolated from the less polar fraction of the same extract of the organism. Compound **8** possessed the molecular formula C₂₂H₃₄O₅, as indicated by the HRESIMS (m/z 378.2404 [M]⁺) and NMR data (Tables 1 and 3). IR absorptions at ν_{\max} 3310 and 1730 cm⁻¹ and electron ionization mass spectroscopy EIMS ion peaks at m/z 318 [M – AcOH]⁺ and m/z 300 [M – AcOH – H₂O]⁺ revealed the presence of hydroxy and acetoxy functionalities in the molecule of **8**. On comparison of spectroscopic data, the NMR spectrum of **8** was found to be very similar to that of **1** (Tables 1–3), with the exception of the presence of signals of an additional methyl and an ester carbonyl carbon of an acetoxy group

at δ_C 169.9, C and 20.6, CH₃; and δ_H 2.06, 3H, s. These were associated with a downfield shift displayed by H-13 in **8** (δ_H 5.16, d, $J = 2.0$ Hz) relative to that of **1** (δ_H 3.58, br s). Therefore, **8** was suggested to be the 13-acetylated derivative of **1**, as proven by complete 2D NMR correlations analyses (Figure 2). Moreover, compound **8**, measured in CDCl₃, showed the same NOE correlations that had been observed in the NOESY spectrum of **1**, indicating the same relative configuration. Furthermore, hydrolysis of **8** yielded **1**. To clarify the absolute configuration at C-13 in **1** (and hence also in **8**), a modified Mosher's method [20,21] was employed to prepare (*S*)- and (*R*)- α -methoxy- α -(trifluoromethyl)phenylacetic (MTPA) esters of **1** (**1a** and **1b**, respectively) using the corresponding (–)- and (+)-MTPA-chloride, respectively. Calculation of $\Delta\delta$ values ($\delta_H S - \delta_H R$) for the protons adjacent to C-13 led to the assignment of the *S* configuration at C-13 in **1**, and consequently therefore in **8** (Figure 4). On the basis of the above findings and detailed NOESY correlations analyses (Figure 3), compound **1** was identified as (3*R*,4*S*,11*S*,12*R*,13*S*,14*R*,1*Z*,7*E*)-4,13-dihydroxy-3,14:11,12-bisepoxy-cembra-1,7-diene. Compound **8** (klyflaccembranol H) was thus subsequently characterized as the C-13 acetyl derivative of **1**.

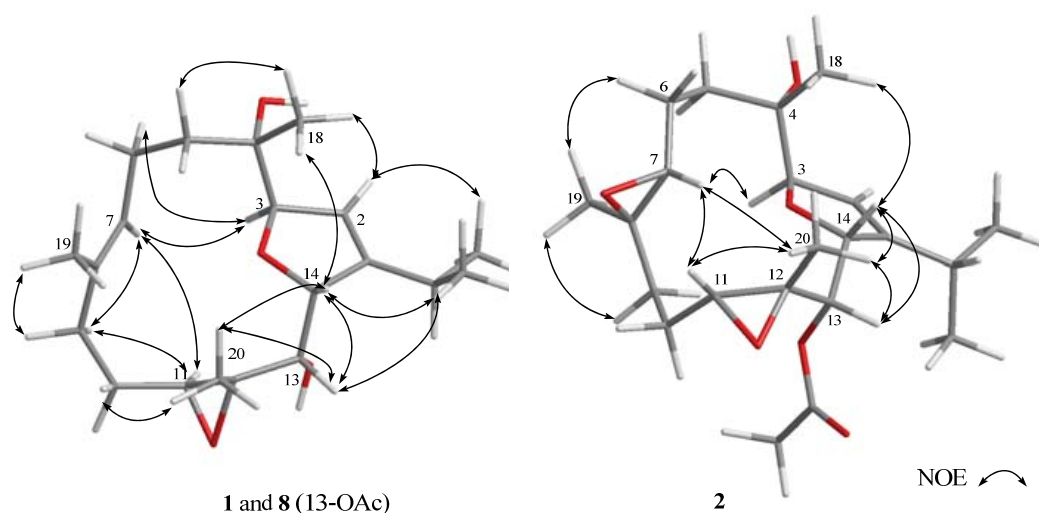


Figure 3. Key nuclear Overhauser effect (NOE) correlations of **1**, **8** and **2**.

Table 3. ^1H NMR spectral data for compounds 6–9.

#	6 ^a	7 ^b	8 ^b	9 ^c
2	5.61 d (16.0) ^d	6.24 d (16.0)	5.60 br s	6.25 d (11.2)
3	6.10 d (16.0)	5.75 d (16.0)	4.57 d (5.2)	5.85 d (11.2)
5	1.51 m; 2.01 m	2.10 m; 2.24 m	1.63 m; 1.89 m	2.00 2H, m
6	2.22 m; 2.39 m	2.10 m; 2.27 m	2.16 m; 2.18 m	1.97 m, 2.06 m
7	5.34 dd (7.5, 7.5)	5.03 dd (6.0, 6.0)	5.36 dd (5.5, 5.5)	5.03 dd (6.0, 6.0)
9	1.95 m; 2.20 m	2.16 m; 2.20 m	2.04 m; 2.09 m	1.95 m, 2.11 m
10	2.07 m; 2.24 m	1.61 m; 1.86 m	1.76 m; 1.83 m	1.43 2H, m
11	5.19 br d (9.0)	2.67 m	2.89 dd (6.0, 3.6)	3.08 dd (6.0, 6.0)
13	2.13 m; 2.19 m	4.59 dd (8.0, 8.0)	5.16 d (2.0)	3.72 d (6.0)
14	1.63 m; 2.11 m	5.10 d (8.0)	5.00 dd (5.2, 2.0)	4.60 d (6.0)
15		2.52 m	2.13 m	2.76 m
16	1.21 3H, s	1.04 3H, d (6.8)	1.03 3H, d (6.8)	1.06 d (6.8)
17	1.13 3H, s	1.07 3H, d (6.8)	1.08 3H, d (6.8)	1.28 d (6.8)
18	1.40 3H, s	1.35 3H, s	1.00 3H, s	1.59 3H, s
19	1.62 3H, s	1.60 3H, s	1.59 3H, s	1.32 3H, s
20	1.67 3H, s	1.42 3H, s	1.39 3H, s	1.34 3H, s
15-OH	2.60 s			
13-OH		1.74 d (8.0)		
OAc			2.06 3H, s	

Spectra recorded in ^a CDCl₃ at 500 MHz; ^b CDCl₃ at 400 MHz; and ^c C₆D₆ at 400 MHz at 25 °C; ^d J values (Hz) in parentheses.

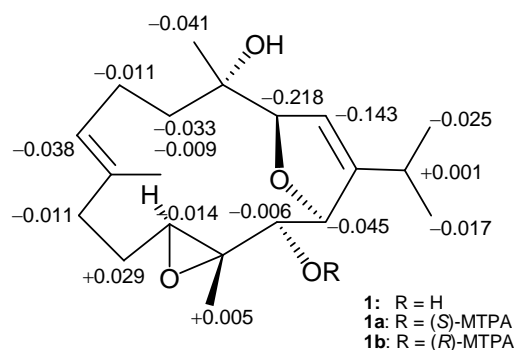


Figure 4. ^1H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the α -methoxy- α -(trifluoromethyl) phenylacetic (MTPA) esters of **1**.

Klyflaccicembranol B (**2**) was also isolated as a colorless oil. The sodium adduct ion peak $[\text{M} + \text{Na}]^+$ appearing at m/z 417.2250 in the HRESIMS was appropriate for the molecular formula $\text{C}_{22}\text{H}_{34}\text{O}_6$ with six unsaturations. The IR absorption bands at ν_{max} 3443 and 1746 cm^{-1} indicated hydroxy and ester functionalities in the molecule. The NMR signals (δ_{C} 169.9, C and 21.0, CH₃; δ_{H} 1.91, 3H, s) showed the ester functionality to be an acetoxy group. A trisubstituted-2,5-dihydrofuran moiety (δ_{C} 147.7, C; 121.2, CH; 91.1, CH and 85.0, CH; δ_{H} 5.65, br s; 5.05, d, $J = 5.0$ Hz and 4.78, d, $J = 5.0$ Hz) was also deduced, as in case of **1** and **8**. However, the ^{13}C NMR spectral comparison of compound **2** with **8** in the regions of δ_{C} 125–140 and 58–65 ppm indicated that **2** had an additional trisubstituted epoxide, instead of the 7,8-trisubstituted double bond in **8**. The gross structure of **2** was thus established by analysis of COSY and HMBC correlations (Figure 2). The relative configuration of **2** was determined by analysis of NOE correlations, as shown in Figure 3. The NOE interactions observed for H₃-19 (δ_{H} 1.24, s) with H-6 (δ_{H} 1.59, m), H-7 (δ_{H} 3.07, dd, $J = 6.0, 2.5$ Hz) with H-3, and H-11 (δ_{H} 2.98, d, $J = 7.5$ Hz) with H₃-20 (δ_{H} 1.42, s), indicated the *trans* and *cis* geometries of the trisubstituted 7,8- and 11,12-epoxides, respectively. As H-3 exhibited a NOE correlation with H-7, H-7 and H₃-19 were thus α - and β -oriented, respectively. Moreover, the NOE correlations observed for H-14 with both H₃-20 and H-13 disclosed the α -orientation of the acetyl group at C-13 and the β -orientation of the

methyl group at C-12, and hence the β -oriented epoxide proton at C-11. On the basis of the above findings and the absolute configuration of biogenetically-related **1**, compound **2** was thus determined as (3*R*,4*S*,7*R*,8*R*,11*R*,12*R*,13*S*,14*R*,1*Z*)-7,8:11,12:3,14-triepoxy-4-hydroxy-13-acetoxycembra-1-ene.

New metabolite **3** was found to have the molecular formula $C_{22}H_{34}O_4$, as deduced from the HRESIMS (m/z 385.2354 $[M + Na]^+$) and NMR data (Tables 1 and 2), implying six degrees of unsaturation. As in the cases of **2** and **8**, the IR absorptions at 1731 and 3450 cm^{-1} further indicated the presence of an ester moiety and a hydroxy group. The NMR data (Tables 1 and 2) of an acetoxy group (δ_C 170.7, C; 21.1, CH_3 ; δ_H 2.10, 3H, s), a trisubstituted epoxide (δ_C 61.3, C and 59.7, CH) and three trisubstituted olefins (δ_C 155.9, 137.6, 133.2, each C, and 126.3, 125.2, 115.6, each CH) established five degrees of unsaturation in the molecule. By comparison of NMR spectroscopic data with those of gibberosene D (**10**) isolated from the soft coral *S. gibberosa* [16], compound **3** was suggested to be a regioisomer of **10**. Interpretation of 2D NMR correlations (Figure 2) indicated a secondary hydroxy group at C-2 and a trisubstituted double bond at C-3/C-4 in **3** instead of the disubstituted double bond and the tertiary hydroxy group at C-2/C-3 and C-4, respectively, in **10**. Detailed COSY and HMBC correlations (Figure 2) further determined the C-7/C-8, C-1/C-14, C-11/C-12, and C-13 positions for the two other olefins, the epoxide, and the acetoxy group in the structure of **3**, respectively, as were identified in **10** (Figure 1). Thus, the gross structure of **3** was established as 13-acetoxy-11,12-epoxy-2-hydroxycembra-3,7,14-triene. The NOE interaction of H-13 with H-2 enabled assignment of their *syn* positions, and hence the α -orientation of the hydroxy group at C-2, based on the previously defined *S* configuration at C-13 (as in **1** and **8**). The NOE interactions of H-14 with H₃-20 (δ_H 1.22, 3H, s) and H-13 with H-11 (δ_H 2.47, dd, $J = 7.2, 2.0$ Hz) indicated the *trans* geometry of the 11,12-epoxide, and, consequently, the 11*S* and 12*R* configurations. Furthermore, other NOE interactions of H-2 with H₃-18 (δ_H 1.80, 3H, s), H-3 with H-7 (δ_H 4.83, br d, $J = 6.0$ Hz), and H₃-19 (δ_H 1.55, 3H, s) with H-6 (δ_H 2.27, m) established the *E* geometries of the C-3/C-4 and C-7/C-8 double bonds. The *Z* geometry of the double bond at C-14/C-1 in **3** was proven by the NOE correlation (Figure 5) of H-14 with the isopropyl methyls at C-15 (δ_H 1.05 and 1.09, each 3H, d, $J = 6.8$ Hz). The above observations thus established the structure of klyflaccicembranol C (**3**) as (2*S*,11*S*,12*R*,13*S*,3*E*,7*E*,14*Z*)-13-acetoxy-11,12-epoxy-2-hydroxycembra-3,7,14-triene.

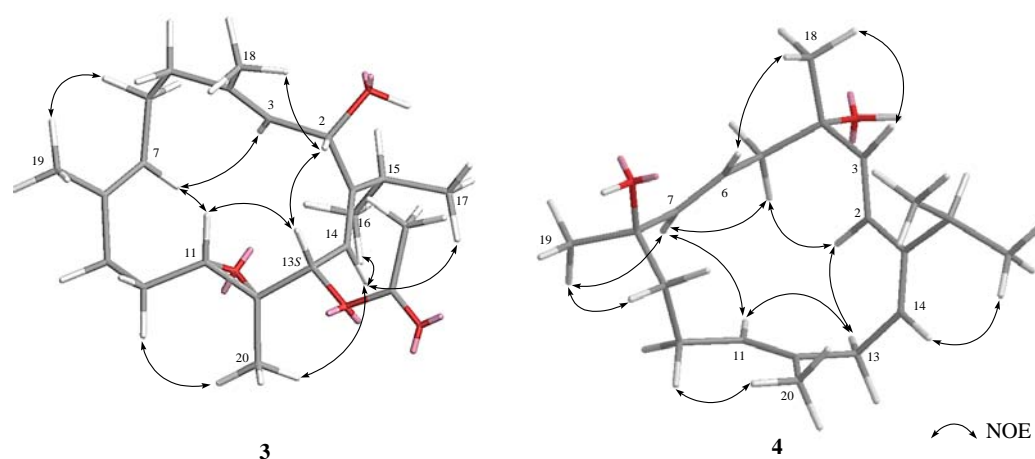


Figure 5. Key NOE correlations of **3** and **4**.

New metabolite klyflaccicembranol D (**4**) was isolated as a pale oil. A pseudomolecular ion peak $[M + Na]^+$ at m/z 327.2200 in the HRESIMS data was observed, corresponding to the molecular formula $C_{20}H_{32}O_2$ and five degrees of unsaturation. The IR absorption at ν_{max} 3419 cm^{-1} and two sp^3 oxycarbon signals appearing at δ_C 72.9 and 72.5 indicated the hydroxyl-bearing characteristic of the compound. Two trisubstituted double bonds were also identified from the NMR signals at δ_C 146.3, C; 132.8, C; 126.7, CH; 122.0, CH; δ_H 5.48 (1H, dd, $J = 8.0, 5.6$ Hz), and 5.11 (1H, dd,

$J = 7.2, 7.2$ Hz). Analysis of COSY correlations of **4** established five consecutive proton sequences, including those of the isopropyl group (Figure 2). Careful study of the HMBC spectrum of **4** further established the connectivities of the five proton sequences with the diagnostic nonprotonated carbons (C-1, C-4, C-8, and C-12), as illustrated in Figure 2. Thus, the two 1,2-disubstituted and the two trisubstituted double bonds were localized at C-2/C-3, C-6/C-7, C-11/C-12, and C-14/C-1, respectively, whereas the hydroxy groups were positioned at C-4 and C-8. Thus, the planar structure of **4** was established as 4,8-dihydroxycembra-2,6,11,14-tetraene. The large coupling constants of $J_{2,3}$ (16.4 Hz) and $J_{6,7}$ (15.6 Hz) reflected the *E* geometries of the two disubstituted double bonds at C-2/C-3 and C-6/C-7. Moreover, the NOE correlations (Figure 5) found for H₃-20 (1.60, 3H, s) with one proton of H₂-10 (δ_{H} 2.37, m), but not with H-11, and for H-14 with the protons of the two methyls at C-15, indicated the *E* and *Z* geometries of the double bonds at C-11/C-12 and C-1/C-14, respectively. On the basis of the previously defined α -orientation of 4-OH in biogenetically-related compounds **1**, **2**, and **8**, and the NOE interactions of the β -oriented H₃-18 (δ_{H} 1.27, 3H, s) with the olefinic H-6 and the *trans* downward-oriented H-7 with H₃-19 (δ_{H} 1.16, 3H, s), the β -orientation of the hydroxy group at C-8 was proven. These findings, together with detailed analysis of other NOE correlations (Figure 4), indicated the structure of klyflaccicembranol D (**4**) to be (4*S*,8*R*,2*E*,6*E*,11*E*,14*Z*)-4,8-dihydroxycembra-2,6,11,14-tetraene.

Klyflaccicembranol E (**5**) was obtained as a colorless oil. It possessed the molecular formula C₂₀H₃₄O₃ and four unsaturations, as concluded from the pseudomolecular ion peak [M + Na]⁺ at m/z 345.2404 in the HRESIMS. The presence of hydroxy groups in **5** was demonstrated by an IR absorption band at ν_{max} 3408 cm⁻¹ and ¹³C NMR signals at δ_{C} 75.2 (C), 70.7 (CH), and 70.6 (CH). The NMR spectroscopic data comparison of **5** with semisynthetic product **11** (Figure 6), obtained by chromic acid oxidation of sarcophytol A [22], suggested that **5** (C₂₀H₃₄O₃) was the hydrated derivative of **11** (C₂₀H₃₂O₂). Therefore, the 3,14-dihydroxy compound **5**, relative to the 3,14-epoxy compound **11**, exhibited significant upfield ($\Delta\delta_{\text{C}} - 8.5, -6.5, -13.9$ ppm) and downfield ($\Delta\delta_{\text{C}} + 3.6$ ppm) shifts at C-2, C-3, C-14, and C-1, respectively. Furthermore, extensive interpretation of 2D NMR correlations further established the gross structure of compound **5** to be 3,4,14-trihydroxycembra-1(2),7,11-triene (Figure 2). The geometries of the double bonds and stereochemistries at C-3, C-4, and C-14 were determined by careful investigation of NOE correlations exhibited in the NOESY spectrum (Figure 7) in combination with molecular modelling. The NOE correlations of the olefinic H-2 with protons of the isopropyl methyls (H₃-16 and H₃-17) revealed the *Z* geometry of the double bond at C-1/C-2. The chemical shift values of C-19 (15.1) and C-20 (17.1) reflected the *E* geometries of the trisubstituted double bonds ($\delta_{\text{C}} < 20$ ppm) at C-7/C-8 and C-11/C-12 in the molecule of **5**. Assuming an *S*-configuration at C-4 (as found in biogenetically-related metabolites **1**, **2**, and **8**), the NOE interaction of H-2 with H₃-18, but not with H-3, reflected the *R*-configuration at C-3. Consequently, the *S*-configuration was allocated for C-14, as H-14 exhibited strong NOE correlation with H-3. The above-mentioned findings, along with detailed analysis of other NOE correlations (Figure 7), identified klyflaccicembranol E (**5**) as (3*R*,4*S*,14*S*,1*Z*,7*E*,11*E*)-3,4,14-trihydroxycembra-1,7,11-triene.

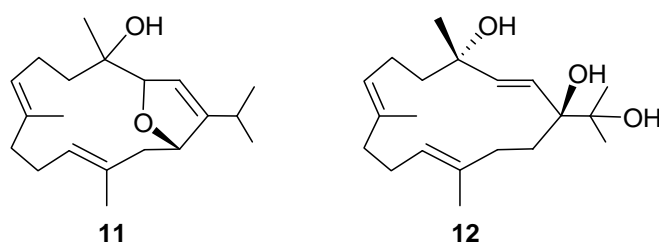


Figure 6. Structures of semisynthetic cembranoid (**11**) and crassumol A (**12**).

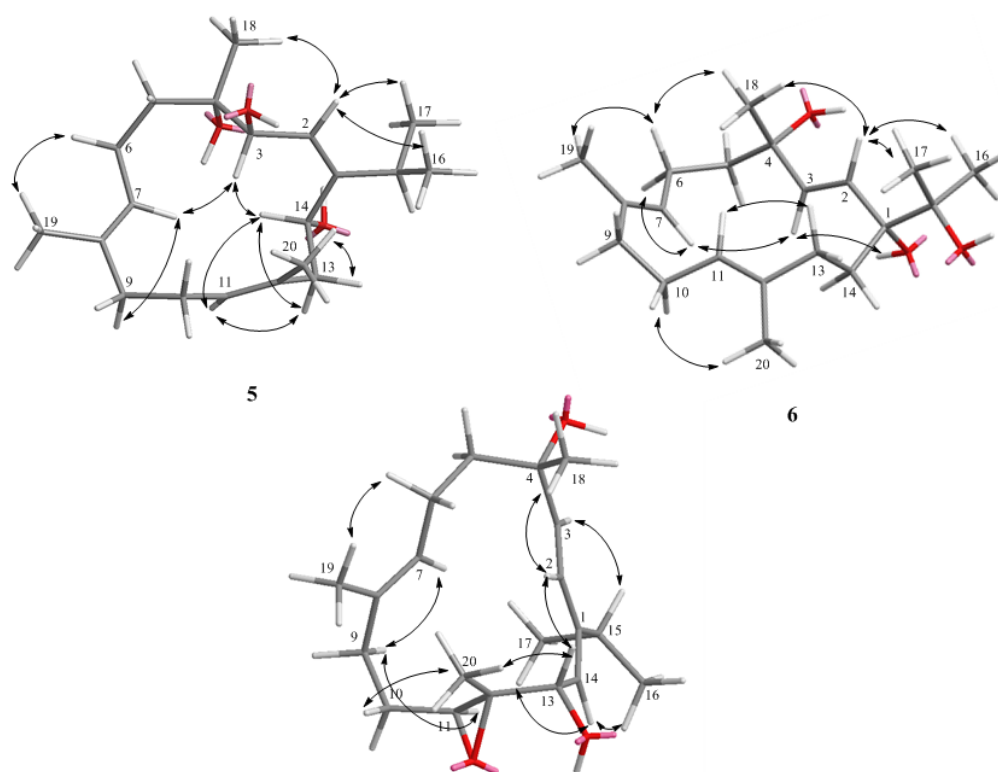


Figure 7. Key NOE correlations of 5–7.

Klyflaccicembranol F (**6**) displayed a pseudomolecular ion peak at m/z , 345.2405 ($[M + Na]^+$) in the HRESIMS, consistent with a molecular formula of $C_{20}H_{34}O_3$ and four degrees of unsaturation. Its IR spectrum also showed a broad absorption band at ν_{max} 3392 cm^{-1} , representing a hydroxy group. This was further supported by NMR signals resonating at δ_C 80.9, 75.1, and 71.9 (each C) of tertiary hydroxyl-bearing carbons and a hydroxy proton at δ_H 2.60 (1H, s). Moreover, carbon signals appearing at δ_C 129.2 (C-2, CH), 138.0 (C-3, CH), 128.6 (C-7, CH), 132.7 (C-8, C), 126.9 (C-11, CH), and 136.1 (C-12, C) indicated the presence of one disubstituted double bond and two trisubstituted olefins in **6**, respectively. The analysis of COSY correlations (Figure 2) of **6** indicated four consecutive proton sequences. The connection of four partial structures was subsequently resolved by the HMBC. Furthermore, long-range correlations observed from both H₃-16 and H₃-17 (δ_H 1.13 and 1.21, each 3H, s) to C-15 (δ_C 75.1, C) and C-1 (δ_C 80.9, C), 1-OH (δ_H 2.60, 1H, s) to C-1 and C-14, and H-2 (δ_H 5.61, 1H, d, $J = 16.0$ Hz) to C-1 and C-4 (δ_C 71.9, C), enabled localization of the hydroxy groups at C-15, C-1, and C-4, respectively. The planar structure of compound **6** was thus described as 1,4,15-trihydroxycembra-2,7,11-triene (Figure 2). Comparison of NMR data of **6** with the known cembranoid crassumol A (**12**) [23] revealed that **6** had similar 1H and ^{13}C chemical shifts, except for the significant downfield shifts noticed at C-3, C-4, C-5 ($\Delta\delta_C + 1.1$, $+1.8$, and $+1.5$, respectively) and H-2 ($\Delta\delta_H + 0.18$), and upfield shifts at C-2 ($\Delta\delta_C - 0.9$ ppm) and H-3 ($\Delta\delta_H - 0.18$ ppm), which suggested klyflaccicembranol F (**6**) to be the 1-epimer of **12** (Figure 6). Careful investigation of key NOE correlations (Figure 7) enabled us to prove the α -orientation of 1-OH. In the NOESY spectrum of **6**, H₃-18 was found to exhibit correlation with H-2, and H-2 with both H₃-16 and H₃-17; therefore, assuming a β -orientation of H₃-18, H-2 and the isopropyl methyls should also be positioned on the β -face. Therefore, 1-OH and 4-OH should be α -oriented. The chemical shift values of C-19 (δ_C 14.7) and C-20 (δ_C 14.8) reflected the *E* geometries of the trisubstituted double bonds at C-7/C-8 and C-11/C-12 in the molecule of **6**. Moreover, it should be noted that the large J_{HH} values for H-2 and H-3 (δ_C 16.0 Hz) and the null NOE response reflected the *trans* positions of these two protons. On the basis of the above findings, metabolite **6** was determined to be (1*R*,4*S*,2*E*,7*E*,11*E*)-1,4,15-trihydroxycembra-2,7,11-triene.

New metabolite **7** was also isolated as a colorless oil, with the molecular formula $C_{20}H_{32}O_3$, as indicated by HRESIMS (m/z 343.2250 $[M + Na]^+$). Substitution of the hydroxy group of this compound was revealed by the IR absorption band at ν_{max} 3419 cm^{-1} and ^{13}C NMR signals at δ_C 73.0 (C) and 71.6 (CH). The two proton signals resonating at δ_H 6.24 and 5.75 (each 1H, d, $J = 16.0$ Hz) were found to represent two olefinic protons correlated in the HSQC spectrum with the carbon signals at δ_C 123.7 and 138.3 (each CH), respectively, attributable to a *trans* 1,2-disubstituted double bond. Moreover, carbon signals at δ_C 146.0 (C), 131.9 (C), 127.6 (CH), 122.9 (CH), 64.7 (C), and 61.3 (CH) indicated the presence of two trisubstituted double bonds and a trisubstituted epoxide in **7**. The remaining ten carbons were assigned to five methyls, one sp^3 methine and four methylene groups. By NMR spectroscopic data comparison, it was found that this compound was the 13-hydroxy derivative of **10**, isolated in this study and previously from *S. gibberosa* [16]. The hydroxy group at C-13 in **7** induced a significant upfield shift at H-13 ($\Delta\delta_H - 1.21$ ppm) and a downfield shift at C-14 ($\Delta\delta_C + 5.0$ ppm) relative to **10**. The structure of **7** was unambiguously determined by the extensive analysis of COSY and HMBC (Figure 2) and NOESY correlations (Figure 7). In addition, the appearance of the 1H double doublet signal of H-13 (δ_H 4.59, 1H, dd, $J = 8.0, 8.0$ Hz) was due to *vicinal* coupling with the olefinic H-14 (δ_H 5.10, 1H, d, $J = 8.0$ Hz) and the free proton of the hydroxy group at C-13 (δ_H 1.74, 1H, d, $J = 8.0$ Hz). Comparison of the splitting patterns of H-13 and H-14 of **7** (d, $J = 8.0$ Hz) with those of known cembranoid **10** (d, $J = 9.0$ Hz) suggested the same stereochemistry at C-13. The structure of klyflaccicembranol G (**7**) was thus established as (4*S*,11*S*,12*S*,13*S*,2*E*,7*E*,14*Z*)-4,13-dihydroxy-11,12-epoxy-cembra-2,7,14-triene.

Klyflaccicembranol I (**9**) possessed the molecular formula $C_{20}H_{32}O_3$, as revealed from the ESIMS (m/z 343 $[M + Na]^+$) and NMR data (Tables 1 and 3). The 1H and ^{13}C NMR data demonstrated the characteristic features of non-lactonized cembranoids (C_{20} signals, including those of five methyls) isolated previously from soft corals of the genus *Sinularia* and *Sarcophyton* [16–18,24,25]. By careful spectral comparison, it was found that the 1H and ^{13}C NMR data of **9** were identical to those of 11,12-epoxy-13,14-dihydroxycembrene obtained by hydrolysis of flaccidoxide [26], including the magnitude and sign of optical rotation $[\alpha]_D^{25} +124$.

Cytotoxicity of metabolites **1–6** and **8–10** against the growth of HT-29 (human colon adenocarcinoma), A549 (human lung adenocarcinoma), K562 (human erythromyeloblastoid leukemia), and P388 (mouse lymphatic leukemia) cell lines was evaluated. With the exception of inactive metabolites **1**, **3**, **5**, and **10**, all the other compounds exhibited variable potency against the tested cell lines (Table 4). Compounds **4** and **6** showed cytotoxicity against K562 and A549 (IC_{50} 44.9 and 21.4 μM , respectively). Compound **8** was capable of affecting the growth of three cancer cell lines (A549, K562, and P388) in the range of IC_{50} 34.6–49.4 μM ; however, it was found to be doubly-potent (IC_{50} 49.4 μM) relative to the positive control fluorouracil (IC_{50} 110 μM) against A549 cancer cells. Compound **2** was cytotoxic against two cell lines (A549 and K562), being 6.5-fold more potent than the positive control against the growth of A549. In addition, compound **9** was cytotoxic against another pair of cancer cells (HT-29 and P388), being very potent against P388.

Table 4. Cytotoxicities (IC₅₀ μM) of compounds 1–6 and 8–10.

Compound	HT-29	A549	K562	P388
1	– ^a	– ^a	– ^a	– ^a
2	– ^a	16.5	34.6	– ^a
3	– ^a	– ^a	– ^a	– ^a
4	– ^a	– ^a	44.9	– ^a
5	– ^a	– ^a	– ^a	– ^a
6	– ^a	21.4	– ^a	– ^a
8	– ^a	49.4	47.4	34.6
9	41.9	– ^a	– ^a	25.9
10	– ^a	– ^a	– ^a	– ^a
Fluorouracil	8.5	110	31.5	5.5

^a –: Compound was considered inactive when IC₅₀ > 50 μM.

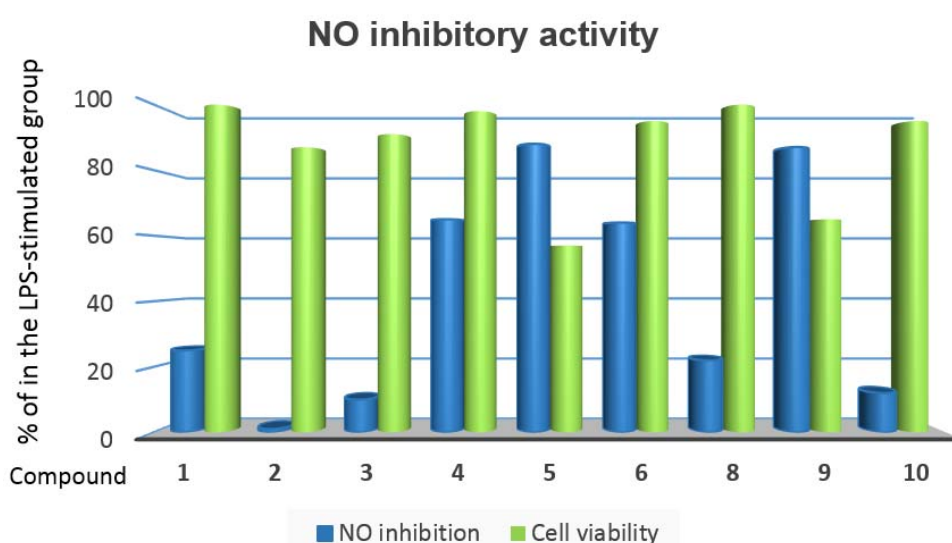


Figure 8. Inhibitory effects of compounds 1–6 and 8–10 at 50 μg/mL on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

The isolated compounds 1–6 and 8–10 also were evaluated in terms of their ability to suppress NO in LPS-activated RAW264.7 macrophages (Figure 8). The results showed that cembranoids 5 and 9 strongly inhibited 88% and 87% of NO production at 50 μg/mL, respectively. However, compounds 4 and 6 at the same dose possessed moderate potency (65% and 64% NO inhibition), with IC₅₀ values of 46.7 and 47.0 μg/mL, respectively. The higher cell viability indexes attained by 4 and 6 (98% and 95%, respectively) relative to 5 and 9 represented an advantageous characteristic in addition to the NO inhibitory effect over 5 or 9. A positive control, curcumin, at 10 μg/mL succeeded under the same experimental conditions in reducing the NO level by 92.5% (IC₅₀ 6.3 μg/mL), in association with 98% retention of cell viability. With the exception of the inactive metabolite 2, the rest of the tested compounds (1, 3, 8 and 10) showed weak NO inhibitory activity (12%–25%).

3. Materials and Methods

3.1. General Procedures

Optical rotations were measured on a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer (JASCO). Ultraviolet spectra were recorded on a JASCO V-650 spectrophotometer. ESIMS and HRESIMS spectral data were recorded on a BRUKER APEX II mass spectrometer (Bruker, Bremen, Germany). The NMR spectra were recorded

on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^1H and 125 MHz for ^{13}C or on a Varian 400 FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C or on a Bruker AMX-300 FT-NMR at 300 MHz for ^1H and 75 MHz for ^{13}C , in CDCl_3 or C_6D_6 using TMS as internal standard (δ in ppm, J in Hz). Silica gel 60 (Merck, 230–400 mesh), precoated silica gel plates (Merck, Darmstadt, Germany, Kieselgel 60 F254, 0.2 mm) were used for open CC and analytical TLC analysis, respectively. Isolation by HPLC was performed by a Hitachi L-2455 instrument equipped with a reversed-phase (RP-18) column (GL Sciences Inc., Tokyo, Japan ODS-3, 5 μm , 250 \times 20 mm).

3.2. Animal Material

The soft coral *Klyxum flaccidum* Tixier-Durivault (Alcyoniidea) was collected by hand via SCUBA off the coast of Hsiao Liuchiu Island (22°19'48" N 120°21'55" E; Pingtung County), in October 2011, at a depth of 10–15 m along the coast of the island of Pratas, Taiwan. The species identification is based on three levels of morphological characters, i.e., colony shape, polyps' morphology and the morphology of sclerites in different parts of the coral colony, and then stored at $-20\text{ }^\circ\text{C}$ until extraction. A voucher sample (specimen no. LI6) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University (Kaohsiung, Taiwan). The organism was identified by Professor Chang-Feng Dai, Institute of Oceanography, National Taiwan University, Taipei 112, Taiwan.

3.3. Extraction and Separation

The frozen bodies of *K. flaccidum* (8.0 kg, wet weight) were sliced and exhaustively extracted with EtOAc and filtered off. The solvent-free residue (120 g) was fractionated by silica gel column chromatography, using EtOAc–*n*-hexane (0:100 to 100:0, gradient) and then MeOH–EtOAc (0:100 to 100:0, gradient) as eluting solvents, in order to yield 26 fractions (F1 to F26). F8 eluted with EtOAc–*n*-hexane (1:2) was further isolated on silica gel, using EtOAc–*n*-hexane (1:3 to 2:3, stepwise) to yield **3** (2.5 mg), **2** (1.5 mg), and **8** (40.5 mg), respectively. F10 eluted with EtOAc–*n*-hexane (1:1) was separated on silica gel, using EtOAc–*n*-hexane (1:2) to give four subfractions F101–F104. F101 was isolated on RP-HPLC using MeOH– H_2O (3:1) to give **4** (10.2 mg), **9** (9.7 mg), and **10** (11.3 mg), respectively. F12 eluted with EtOAc–*n*-hexane (2:1) was re-chromatographed on silica gel column, using EtOAc–*n*-hexane (1:2–2:1), in order to give four subfractions F121–F124. F122 was isolated on RP-HPLC, using MeOH– H_2O (15:1) as a mobile phase, to yield **1** (1.8 mg), **5** (3.4 mg), **6** (2.1 mg), and **7** (3.2 mg).

3.3.1. Klyflaccicembranol A (**1**)

Colorless oil; $[\alpha]_{\text{D}}^{25} +57.2$ (c 0.5, CHCl_3); IR (neat) ν_{max} 3445, 2959, 2924, 2870, 1456, 1381, 1118, 1087, 1064, 850 and 738 cm^{-1} ; ^{13}C (100 MHz, C_6D_6) and ^1H NMR (400 MHz, C_6D_6) data, see Tables 1 and 2, respectively; ESIMS m/z 359 $[\text{M} + \text{Na}]^+$; HRESIMS m/z 359.2196 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{20}\text{H}_{32}\text{O}_4\text{Na}$, 359.2198).

3.3.2. Klyflaccicembranol B (**2**)

Colorless oil; $[\alpha]_{\text{D}}^{25} +65.9$ (c 0.4, CHCl_3); IR (neat) ν_{max} 3443, 2924, 2856, 1746, 1457, 1375, and 1234 cm^{-1} ; ^{13}C (125 MHz, CDCl_3) and ^1H NMR (500 MHz, CDCl_3) data, see Tables 1 and 2, respectively; ESIMS m/z 417 $[\text{M} + \text{Na}]^+$; HRESIMS m/z 417.2250 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{22}\text{H}_{34}\text{O}_6\text{Na}$, 417.2253).

3.3.3. Klyflaccicembranol C (**3**)

Colorless oil; $[\alpha]_{\text{D}}^{25} +38.9$ (c 0.7, CHCl_3); IR (neat) ν_{max} 3450, 2927, 2858, 1731, 1455, 1373, 1240 and 1016 cm^{-1} ; ^{13}C (100 MHz, CDCl_3) and ^1H NMR (400 MHz, CDCl_3) data, see Tables 1 and 2, respectively; ESIMS m/z 385 $[\text{M} + \text{Na}]^+$; HRESIMS m/z 385.2354 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{22}\text{H}_{34}\text{O}_4\text{Na}$, 358.2355).

3.3.4. Klyflaccicembranol D (4)

Pale yellow oil; $[\alpha]_D^{25} -1.51$ (*c* 3.17, CHCl₃); IR (neat) ν_{\max} 3419, 2953, 2925, 2867, 1456, 1375, 977, and 757 cm⁻¹; ¹³C (100 MHz, C₆D₆) and ¹H NMR (400 MHz, C₆D₆) data, see Tables 1 and 2, respectively; ESIMS *m/z* 327 [M + Na]⁺; HRESIMS *m/z* 327.2200 [M + Na]⁺ (calcd. for C₂₀H₃₂O₂Na, 327.2198).

3.3.5. Klyflaccicembranol E (5)

Colorless oil; $[\alpha]_D^{25} -70.5$ (*c* 1.0, CHCl₃); IR (neat) ν_{\max} 3408, 2956, 2924, 2869, 1455, 1381, 1002, and 757 cm⁻¹; ¹³C (100 MHz, CDCl₃) and ¹H NMR (400 MHz, CDCl₃) data, see Tables 1 and 2, respectively; ESIMS *m/z* 345 [M + Na]⁺; HRESIMS *m/z* 345.2404 [M + Na]⁺ (calcd. for C₂₀H₃₄O₃Na, 345.2406).

3.3.6. Klyflaccicembranol F (6)

Colorless oil; $[\alpha]_D^{25} -32.0$ (*c* 0.6, CHCl₃); IR (neat) ν_{\max} 3392, 2924, 2855, 1455, and 1370 cm⁻¹; ¹³C (125 MHz, CDCl₃) and ¹H NMR (500 MHz, CDCl₃) data, see Tables 1 and 3; ESIMS *m/z* 345 [M + Na]⁺; HRESIMS *m/z* 345.2405 [M + Na]⁺ (calcd. for C₂₀H₃₄O₃Na, 345.2406).

3.3.7. Klyflaccicembranol G (7)

Colorless oil; $[\alpha]_D^{25} +18.0$ (*c* 1.0, CHCl₃); IR (neat) ν_{\max} 3419, 2953, 2925, 2867, 1460, 1375, 977 and 757 cm⁻¹; ¹³C (100 MHz, CDCl₃) and ¹H NMR (400 MHz, CDCl₃) data, see Tables 1 and 3, respectively; ESIMS *m/z* 343 [M + Na]⁺; HRESIMS *m/z* 343.2250 [M + Na]⁺ (calcd. for C₂₀H₃₂O₃Na, 343.2249).

3.3.8. Klyflaccicembranol H (8)

Colorless oil; $[\alpha]_D^{25} +83.0$ (*c* 1.4, CHCl₃); IR (neat) ν_{\max} 3310, 3070, 2964, 2958, 2931, 2867, 1730, 1644, 1460, 1441, 1371, 1251 and 1182 cm⁻¹; ¹³C (100 MHz, CDCl₃) and ¹H NMR (400 MHz, CDCl₃) data, see Tables 1 and 3, respectively; ¹H NMR (CD₃OD, 500 MHz) δ_H 5.65 (1H, d, *J* = 2.0 Hz, H-2), 5.37 (1H, dd, *J* = 5.5, 5.5 Hz, H-7), 5.15 (1H, d, *J* = 2.0 Hz, H-13), 5.07 (1H, ddd, *J* = 5.0, 2.0, 2.0 Hz, H-14), 4.57 (1H, d, *J* = 5.0 Hz, H-3), 2.95 (1H, dd, *J* = 7.5, 1.5 Hz, H-11), 2.21 (2H, m, H₂-9), 2.19 (1H, m, H-6), 2.13 (1H, m, H-15), 2.10 (1H, m, H-6), 1.93 (3H, s, Ac), 1.87 (1H, dd, *J* = 14.0, 9.5 Hz, H-5), 1.81 (1H, m, H-10), 1.70 (1H, m, H-10), 1.64 (1H, dd, *J* = 14.0, 8.0 Hz, H-5), 1.60 (3H, s, H₃-19), 1.39 (3H, s, H₃-20), 1.10 (3H, dd, *J* = 7.0 Hz, H₃-17), 1.06 (3H, dd, *J* = 7.0 Hz, H₃-16), 0.96 (3H, s, H₃-18); ¹³C NMR (CD₃OD, 125 MHz) δ_C 171.7 (C, 13-OAc), 148.4 (C, C-1), 133.2 (C, C-8), 128.2 (CH, C-7), 122.9 (CH, C-2), 90.6 (CH, C-3), 86.2 (CH, C-14), 75.2 (C, C-4), 74.2 (CH, C-13), 62.3 (C, C-12), 61.0 (CH, C-11), 42.1 (CH₂, C-5), 37.7 (CH₂, C-9), 25.6 (CH₂, C-10), 22.7 (CH₂, C-6), 25.7 (CH, C-15), 22.7 (CH₃, C-6), 22.7 (CH₃, C-18), 22.6 (CH₃, C-16), 21.3 (CH₃, C-17), 20.6 (CH₃, 13-OAc), 16.8 (CH₃, C-19), 16.2 (CH₃, C-20); ESIMS *m/z* 401 [M + Na]⁺, 385 [M - O + Na]⁺, 325 [M - AcOH - O + Na]⁺; EIMS *m/z* 379 [M + H]⁺, 361 [M - H₂O]⁺, 318 [M - AcOH]⁺, 300 [M - AcOH - H₂O]⁺; HRESIMS *m/z* 378.2404 [M]⁺ (calcd. for C₂₂H₃₄O₅, 378.2406).

3.3.9. Klyflaccicembranol I (9)

Colorless oil; $[\alpha]_D^{25} +124.0$ (*c* 2.7, CHCl₃); IR (neat) ν_{\max} 3487, 3275, 2955, 2933, 2868, 1732, 1644, 1453, 1370, 1247 and 1183 cm⁻¹; ¹³C (100 MHz, C₆D₆) and ¹H NMR (400 MHz, C₆D₆) data, see Tables 1 and 3, respectively; ESIMS *m/z* 343 [M + Na]⁺.

3.3.10. Hydrolysis of Klyflaccicembranol H (8)

A solution of **8** (3.2 mg, 8.12 μ mol) in MeOH (2.0 mL) was stirred with K₂CO₃ (30 mg) overnight at room temperature. The reaction mixture was diluted with distilled water (3.0 mL) and followed by extraction with CH₂Cl₂. The organic extract was then purified on short silica gel CC, using 25% EtOAc in *n*-hexane as an eluting solvent, to afford **1** (2.4 mg, 6.82 μ mol, 84% yield). **1**: colorless oil; ¹H NMR (CDCl₃, 300 MHz). δ_H 5.64 (1H, br s, H-2), 5.32 (1H, dd, *J* = 5.5, 5.5 Hz, H-7), 3.81 (1H, d, *J* = 1.8 Hz, H-13), 4.92 (1H, br d, *J* = 5.5 Hz, H-14), 4.57 (1H, d, *J* = 5.5 Hz, H-3), 3.12 (1H, dd, *J* = 9.5, 3.6 Hz, H-11),

2.20 (2H, m, H₂-9), 2.13 (2H, m, H₂-6), 2.37 (1H, septet, *J* = 6.9 Hz, H-15), 1.89 (1H, m, H-5a), 1.83 (2H, m, H₂-10), 1.65 (1H, m, H-5b), 1.59 (3H, s, H₃-19), 1.36 (3H, s, H₃-20), 1.10 (3H, dd, *J* = 6.9 Hz, H₃-17), 1.08 (3H, dd, *J* = 6.9 Hz, H₃-16), 1.01 (3H, s, H₃-18); ¹³C NMR (CDCl₃, 75 MHz) δ_C 148.4 (C, C-1), 132.8 (C, C-8), 126.8 (CH, C-7), 122.9 (CH, C-2), 89.6 (CH, C-3), 85.6 (CH, C-14), 74.9 (C, C-4), 72.6 (CH, C-13), 62.4 (C, C-12), 60.3 (CH, C-11), 41.6 (CH₂, C-5), 36.9 (CH₂, C-9), 24.8 (CH₂, C-10), 22.1 (CH₂, C-6), 26.6 (CH, C-15), 22.1 (CH₃, C-6), 22.7 (CH₃, C-18), 22.4 (CH₃, C-16), 21.4 (CH₃, C-17), 16.9 (CH₃, C-19), 15.7 (CH₃, C-20).

3.3.11. Preparation of (S)- and (R)-MTPA Esters of **1**

To a solution of **1** (1.1 mg, 3.1 μmol) in pyridine (50 μL), *R*-(-)-MTPA chloride (5 μL) was added and allowed to react overnight at room temperature. The reaction was terminated by the addition of 1.0 mL of water, and then processed as previously described [27] to yield the (*S*)-MTPA ester **1a** (1.2 mg, 2.1 μmol, 67.7%). Similarly, the correspondent (*R*)-MTPA ester **1b** was also obtained from the reaction of *S*-(+)-MTPA chloride with **1**. ¹H NMR (CDCl₃, 300 MHz) of **1a**: δ_H 5.496 (1H, br s, H-2), 5.341 (1H, br s, H-13), 5.185 (1H, dd, *J* = 4.5, 4.5 Hz, H-7), 5.030 (1H, br d, *J* = 4.5 Hz, H-14), 4.324 (1H, br d, *J* = 4.5 Hz, H-3), 2.609 (1H, m, H-11), 2.177 (1H, m, H-15), 2.100 (4H, m, H₂-6 and H₂-9), 1.985 (1H, m, H-5α), 1.825 (1H, m, H-5β), 1.666 (2H, m, H₂-10), 1.536 (3H, s, H₃-19), 1.387 (3H, s, H₃-20), 1.097 (3H, d, *J* = 6.9 Hz, H₃-17), 1.040 (3H, d, *J* = 6.9 Hz, H₃-16), 0.962 (3H, s, H₃-18). ¹H NMR (CDCl₃, 300 MHz) of **1b**: δ_H 5.639 (1H, br s, H-2), 5.347 (1H, br s, H-13), 5.223 (1H, dd, *J* = 4.5, 4.5 Hz, H-7), 5.075 (1H, br d, *J* = 4.5 Hz, H-14), 4.542 (1H, br d, *J* = 4.5 Hz, H-3), 2.623 (1H, m, H-11), 2.176 (1H, m, H-15), 2.111 (4H, m, H₂-6 and H₂-9), 1.994 (1H, m, H-5α), 1.858 (1H, m, H-5β), 1.637 (2H, m, H₂-10), 1.543 (3H, s, H₃-19), 1.382 (3H, s, H₃-20), 1.122 (3H, d, *J* = 6.9 Hz, H₃-17), 1.057 (3H, d, *J* = 6.9 Hz, H₃-16), 1.003 (3H, s, H₃-18).

3.4. Cytotoxicity Assay

Cancer cell (HT-29, A549, K562, and P388) lines were purchased from the American Type Culture Collection (ATCC). Evaluation of cytotoxicity for the isolated metabolites from *K. flaccidum* was performed according to Alamar Blue assay [14,15].

3.5. Nitric Oxide Inhibitory Assay

The inhibitory activity of isolated compounds on nitric oxide (NO) production by murine RAW 264.7 macrophage cells was assessed according to Griess reaction. Briefly, cells were cultured in 96-well plates for 1 h. The cells were challenged with LPS (5 μg/mL) and test samples for 48 h. The culture supernatant (100 μL) were reacted then with Griess reagent (1:1 mixture of 0.1% *N*-(1-naphthyl) ethylene-diamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid, 100 μL) in a 96-well plate, and absorbance was measured using the ELISA reader at 540 nm [28,29].

4. Conclusions

For the first time, cembranoid-based compounds were isolated and identified from the soft corals of genus *Klyxum* by this study. Five metabolites (klyflaccicembranols B, D, F, H, and I) exhibited variable activities against a limited panel of cancer cell lines while klyflaccicembranols D–F and I showed strong anti-inflammatory effect through inhibition of NO production in LPS-stimulated RAW264.7 macrophages.

Supplementary Materials: HRESIMS, ¹H, and ¹³C spectra of new compounds **1–9** are available online at www.mdpi.com/1660-3397/15/1/23/s1. Figure S1: HRESIMS spectrum of **1**, Figure S2: ¹H NMR spectrum of **1** in C₆D₆ at 400 MHz, Figure S3: ¹³C NMR spectrum of **1** in C₆D₆ at 100 MHz, Figure S4: HRESIMS spectrum of **2**, Figure S5: ¹H NMR spectrum of **2** in CDCl₃ at 500 MHz, Figure S6: ¹³C NMR spectrum of **2** in CDCl₃ at 125 MHz, Figure S7: HRESIMS spectrum of **3**, Figure S8: ¹H NMR spectrum of **3** in CDCl₃ at 400 MHz, Figure S9: ¹³C NMR spectrum of **3** in CDCl₃ at 100 MHz, Figure S10: HRESIMS spectrum of **4**, Figure S11: ¹H NMR spectrum of **4** in C₆D₆ at 400 MHz, Figure S12: ¹³C NMR spectrum of **4** in C₆D₆ at 100 MHz, Figure S13: HRESIMS spectrum

of **5**, Figure S14: ^1H NMR spectrum of **5** in CDCl_3 at 400 MHz, Figure S15: ^{13}C NMR spectrum of **5** in CDCl_3 at 100 MHz, Figure S16: HRESIMS spectrum of **6**, Figure S17: ^1H NMR spectrum of **6** in CDCl_3 at 500 MHz, Figure S18: ^{13}C NMR spectrum of **6** in CDCl_3 at 125 MHz, Figure S19: HRESIMS spectrum of **7**, Figure S20: ^1H NMR spectrum of **7** in CDCl_3 at 400 MHz, Figure S21: ^{13}C NMR spectrum of **7** in CDCl_3 at 100 MHz, Figure S22: HRESIMS spectrum of **8**, Figure S23: ^1H NMR spectrum of **8** in CDCl_3 at 400 MHz, Figure S24: ^{13}C NMR spectrum of **8** in CDCl_3 at 100 MHz, Figure S25: ESIMS spectrum of **9**, Figure S26: ^1H NMR spectrum of **9** in C_6D_6 at 400 MHz, Figure S27: ^{13}C NMR spectrum of **9** in C_6D_6 at 100 MHz.

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