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Two unprecedented cembrene-type terpenes from an indonesian soft coral *sarcophyton* sp.

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ABSTRACT

Two unusual cembranoids, sarcofuranocembrenolides A (**1**) and B (**2**), were isolated from a soft coral *Sarcophyton* sp. together with five known cembranoids (**3**–**7**). Compound **1** had a unique carbon skeleton of 8,19-bisnorfuranocembrenolide. Compound **2** was a furanocembrenolide, but a C_1 unit (C-20) was attached to C-10 instead of C-12 of the ordinary cembrenolides. These rearrangements are unique in the biosynthesis of cembranoid diterpenes. Lobohedleolide (**5**), (7*Z*)-lobohedleolide (**6**), and denticulatolide (**7**) inhibited the colony formation of V79 cells at ED_{50} values of 4.6 (abt. 1.52), 3.7 (1.22), and 3.6 (1.40) μM (μg/mL), respectively, and reduced TNF-α production from LPS-stimulated RAW264.7 cells at 3.0–10.0 μM.

1. Introduction

Soft corals tend to produce terpenes, and cembranoid diterpenes have been reported from many species of soft corals.[1] During our studies on bioactive metabolites from marine organisms, we found that the methanol extract of an Indonesian soft coral, *Sarcophyton* sp., showed inhibitory activity against the colony formation of Chinese hamster V79 cells. Bioassay-guided separation of the extract gave two new cembrene-type terpenes, named sarcofuranocembrenolides A (**1**) and B (**2**), together with five known cembranoids, 5-*epi*-sinuleptolide (**3**),[2–6] **4**,[6] lobohedleolide (**5**),[7,8] (7*Z*)-lobohedleolide (**6**),[7,8] and denticulatolide (**7**).[9,10] Compounds 5–7 inhibited the colony formation of V79 cells at ED_{50} values of 4.6, 3.7, and 3.6 μM, respectively, and reduced TNF-α production from lipopolysaccharide (LPS)-stimulated mouse macrophage RAW264.7 cells at 3.0–10.0 μM. We describe herein the isolation, structure assignment, and bioactivity of these compounds.

2. Results and discussion

The methanol extract of *Sarcophyton* sp., collected in North Sulawesi, Indonesia, was separated by SiO_2, LH-20, and ODS column chromatographies followed by HPLC to afford **1**–**7**. Compounds **3**–**7** were assigned the structures on the basis of their spectroscopic data and comparison with those of the reported values.[2–10]

2.1. Structure of sarcofuranocembrenolide A (1)

Sarcofuranocembrenolide A (**1**) had a molecular formula of $C_{21}H_{24}O_7$, which was determined by HR-FABMS and NMR spectra. ^{13}C and ^1H NMR data (Table 1) revealed the presence of three carbonyl carbons, two oxygenated nonprotonated sp^2 carbons, one *exo*-methylene, two protonated and two nonprotonated sp^2 carbons, one methoxyl, one ethoxyl, two oxymethines, one methine, three methylenes, and one methyl. ^1H–^1H COSY spectrum of **1** showed partial structures, as indicated in Figure 1. The

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presence of a 3-carboxymethylfuran ring was deduced from the analysis of ^{13}C, ^{1}H, and 2D NMR spectra of **1** and comparison of the data with those of the reported values for furanocembrenes isolated from soft corals. An α,β-unsaturated γ-lactone moiety was assigned by comparison of NMR data for **1** with those for **4** and related compounds.[3,6] The skeletal structure of **1** was elucidated by the analysis of HMBC spectra measured in $CDCl_3$ and in C_6D_6, and key HMBC correlations are shown in Figure 1. The proton signals due to H_2-8 and H_2-19 were observed at the same position in $CDCl_3$, but in C_6D_6, four protons showed different chemical shifts, while, the proton signals of H-9/H-12 and H-1/H-2 (δ 2.68) were well separated in $CDCl_3$ but resonated close to each other in C_6D_6. The position of an ethoxy group was elucidated at C-12 by HMBC correlation from H_2-19 to C-12. HMBC correlations from H-1 to C-14 and -15, H_2-13 to C-14, H_2-15 to C-1, and from H_3-16 to C-1 and -14 established a connection between C-1 and -14. HMBC correlations from H_2-2 to C-3, H-12 to C-10, H_2-13 to C-11, and from H_2-8 to C-7 revealed connections between the furan ring and C-2, γ-lactone and C-12, and C-7 and -8. C-6 and -7 were connected to satisfy the molecular formula of **1**.

The structure of **1** was assigned as a unique bisnorcembrenolide, which may be biosynthesized via an acyloin- or pinacol-like rearrangement followed by deacetoxylation, as observed in the biogenesis of vitamin B_{12} (Fig. 2).

The relative stereochemistry of **1** was assigned by the analysis of NOESY spectra measured in $CDCl_3$ and in C_6D_6. NOESY correlations between H-15 (δ 4.95 in $CDCl_3$, 5.13 in C_6D_6)/H-1, H-2β (2.68 in $CDCl_3$, 2.41 in C_6D_6), and H-13β (2.05 in $CDCl_3$, 2.08 in C_6D_6), H-2β/H-13β, and H-1/H-12 (detected in $CDCl_3$) suggested that the isopropenyl and ethoxy groups were on the same side of the ring (Fig. 3). On the other hand, NOESY correlations were observed between H-2α (δ 3.61 in $CDCl_3$, 3.66 in C_6D_6)/H-13α (1.17 in $CDCl_3$, 1.15 in C_6D_6) and H_3-16, H-13α/H-10, and H-10/H-9. Considering these NOE data, Monte Carlo conformational analysis was performed with MMFF94 force field utilizing Spartan'04.[11] Consequently, the structure of **1** was assigned as shown in the Scheme 1 and Figure 3.

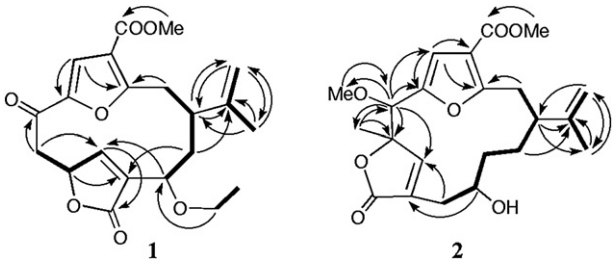


Figure 1. ^{1}H–^{1}H COSY and key HMBC correlations of **1** and **2**.

2.2. Structure of sarcofuranocembrenolide B (2)

Sarcofuranocembrenolide B (**2**) was determined as the molecular formula of $C_{22}H_{28}O_7$ from HR-FABMS and NMR data. The analysis of ^{1}H and ^{13}C NMR data (Table 1) revealed the presence of two carbonyl carbons, two oxygenated nonprotonated sp^2 carbons, one *exo*-methylene, two protonated and two nonprotonated sp^2 carbons, two methoxyls, two oxymethines, one methine, four methylenes, two methyls, and one quaternary carbon. ^{1}H-^{1}H COSY data for **2** showed two partial structures (Fig. 1). A 3-carboxymethylfuran ring was elucidated by comparing NMR data for **2** with those for **1**. The presence of an α,β-unsaturated γ-lactone moiety was assigned by comparing NMR data for **2** with those for **1**, **4**, and related cembranolides.[3,6] HMBC correlations from H-1 to C-15 and -17, H_2-14 to C-15, H_2-16 to C-1, and from H_2-2 to C-3 revealed connections between C-1/C-15 and C-2/C-3 (Fig. 1). The partial structure and connection of the γ-lactone moiety were elucidated on the basis of HMBC correlations from H-7 to C-5, -6,

Table 1
^{13}C and ^{1}H NMR Data for Compounds **1** and **2**

C#	**1** in $CDCl_3$[a]		**1** in C_6D_6[b]		**2** in $CDCl_3$[a]	
	^{13}C	^{1}H mult. (*J* in Hz)	^{13}C	^{1}H mult. (*J* in Hz)	^{13}C	^{1}H mult. (*J* in Hz)
1	40.4	2.40 m	40.5	2.44 m	44.1	2.15 m
2	32.1	2.68 dd (16.3, 3.5) 3.61 dd (16.3, 13.2)	32.1	2.41 m 3.66 dd (16.5, 14.0)	31.9	2.66 dd (14.4, 2.4), 3.44 dd (14.4, 12.4)
3	164.0		163.4		161.2	
4	119.1		118.8		116.3	
5	117.8	7.50 s	116.9	7.39 s	109.3	6.65 s
6	149.6		144.9		150.3	
7	184.4		183.6		85.1	3.99 s
8	45.8	3.34 m (2H)	45.7	2.74 dd (11.4, 8.8) 2.89 dd (11.4, 8.8)	73.0	
9	77.2	5.30 ddd (8.5, 8.5, 1.7)	76.4	4.50 dd (8.4, 8.4)	148.4	5.67 br s
10	151.9	6.73 d (1.7)	151.7	6.01 s	133.4	
11	135.9		135.6		42.3	1.76 dd (14.9, 11.7) 2.56 dd (14.9, 4.1)
12	69.5	4.15 dd (12.0, 3.5)	69.9	4.39 dd (12.1, 3.3)	78.6	4.89 m
13	38.4	1.17 m 2.05 m	38.9	1.15 m 2.08 m	21.6	2.08 m 2.30 m
14	144.7		145.3		27.7	1.49 m 1.80 m
15	113.8	4.92 s 4.95 s	113.7	4.87 s 5.13 s	146.2	
16	19.0	1.81 s	18.8	1.60 s	112.6	4.78 s 4.82 t (1.4)
17	162.7		162.6		19.0	1.75 s
18	172.5		172.3		163.9	
19	64.7	3.34 m (2H)	64.4	3.13 dq (7.0, 6.0) 3.21 dq (7.0, 6.0)	20.1	
20	15.2	1.06 t (7.0)	15.4	1.00 t (7.0)	173.4	1.35 s
21	52.0	3.85 s	51.3	3.40 s	51.6	3.85 s
22					57.6	3.31 s

[a] ^{1}H, 400 MHz; ^{13}C, 100 MHz.
[b] ^{1}H, 600 MHz; ^{13}C, 150 MHz.

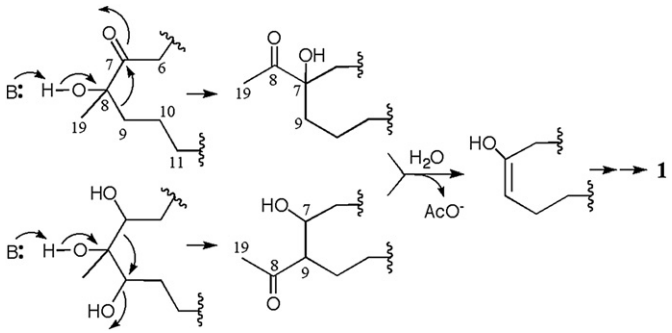
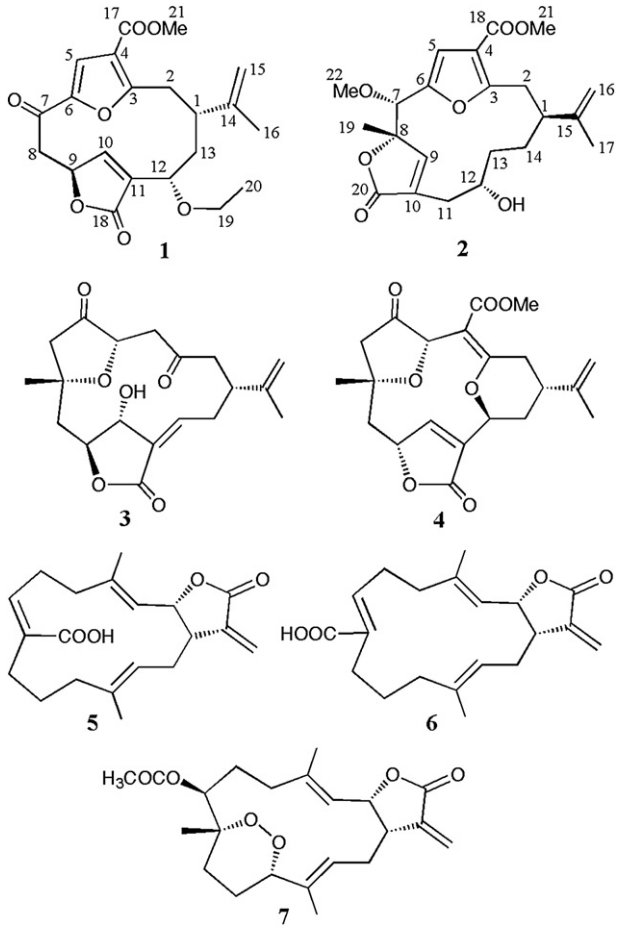


Figure 2. Plausible biosynthetic pathways for **1**.

-8–9, -19 and -22, H_2-11 to C-9, H-12 to C-10, H_3-19 to C-7 and -8, and from H_3-22 to C-7. Thus, the skeletal structure of **2** was assigned as shown in Figure 1.

The relative configurations at C-7, -8, and -12 of **2** were elucidated on the basis of NOESY correlations observed between H-7/H_3-19, H_3-19/H-9, H_3-19/H-11, and H-9/H-12 (Fig. 3). NOESY correlations detected for H-1, H_2-2, H_2-16, and H_3-17 were very similar to those of **1**, but no relation with the 7, 8, and 12 positions could be identified. Therefore, Monte Carlo conformational analysis was performed on H-1α and H-1β isomers with MMFF94 force field using Spartan'04. The H-1α isomer had lower energy minimum value and the H-1β isomer showed relatively higher energy value [+11.0 kJ/mol (+2.6 kcal/mol)]. Thus, we have selected the H-1α isomer as the most probable structure of **2** (Scheme 1 and Fig. 3). However, the H-1β isomer cannot be excluded because biogenetic enzymes may construct a thermodynamically more unfavorable structure.

The C_1 unit (C-20) in **2** was attached to the C-10-position instead of C-12 in ordinary cembrene-type diterpenes. One possibility of this rearrangement may be formation of a four-membered ring.



Scheme 1.

2.3. Bioactivity of compounds 1–7

Compounds **1**–**7** were tested for their inhibitory activity against the colony formation of Chinese hamster V79. This bioassay reflects the direct action of the compounds on the cells and is used to select active compounds for the bioassay of TNF-α production. Three known compounds, **5**, **6**, and **7**, inhibited the colony formation at ED_{50} values of 4.6 (abt. 1.52), 3.7 (1.22), and 3.6 (1.40) μM (μg/mL), respectively, but **1**–**4** were not active at 10 μM (3.88, 4.04, 3.48, and 3.88 μg/mL, respectively). Compounds **5**–**7** were then examined for their inhibitory activity against the production of TNF-α from LPS-stimulated RAW264.7 cells. These compounds inhibited TNF-α production at 3.0–10.0 μM (Fig. 4), and **5** showed the most potent activity among three compounds. The cell proliferation of LPS-stimulated RAW264.7 cells was not affected by **5**–**7** at 10.0 μM: therefore, the dose-dependent inhibition of TNF-α production will be induced by these compounds. Two epimers of **3** at C-5 (sinuleptolide) and at C-10 were reported to inhibit TNF-α production from LPS-stimulated RAW264.7 cells at rather higher concentrations (IC_{50}'s=15 (abt. 43.1) and 20 (57.4) μg/mL (μM), respectively).[12] Compound **3** slightly reduced the production of TNF-α at 10.0 μM (3.48 μg/mL). Sesquiterpenes possessing an α-methylene-γ-lactone and an α,β- or α,β,γ,δ-unsaturated carbonyl group showed *anti*-inflammatory activity and inhibited TNF-α production in LPS-stimulated mouse lymphocytes through inhibition of the transcription factor nuclear factor κB (NF-κB).[13,14] Compounds **5**–**7** have an α-methylene-γ-lactone moiety and, therefore, this partial structure will be important for their activity and may inhibit the activity of NF-κB. Investigation of the mode of action of **5**–**7** is now in progress.

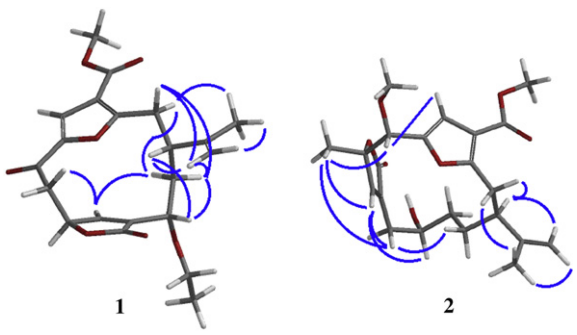


Figure 3. Key NOESY correlations of **1** and **2**.

3. Experimental section

3.1. General experimental procedures

NMR spectra were measured on a JEOL JNM-AL-400 or JNM-LA-600 spectrometer. Mass spectra were obtained by a JEOL JMS-MS 700 mass spectrometer (FAB mode, *m*-nitrobenzyl alcohol or glycerol as the matrix). UV and IR spectra were recorded on a Hitachi U-3310 spectrophotometer and a Perkin-Elmer Spectrum One FTIR spectrometer, respectively. Optical rotations were recorded with a JASCO DIP-370 digital polarimeter. Fetal bovine serum (FBS) was obtained from GIBCO after checking the lot, and all other reagents and chemicals for bioassay were of the highest grade available commercially.

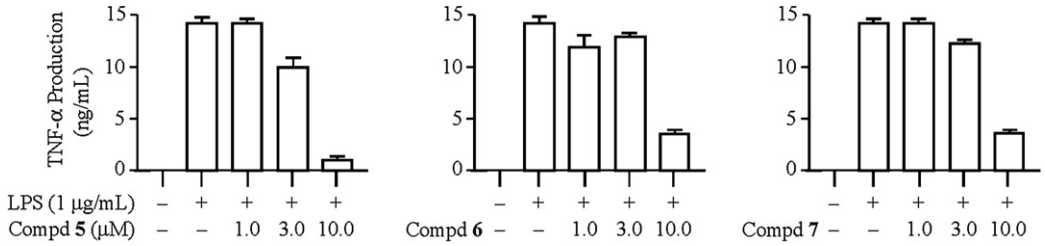


Figure 4. Inhibitory activity of compounds **5**–**7** on TNF-α production from LPS-stimulated RAW264.7 cells.

3.2. Cytotoxicity assay

Chinese hamster V79 cells were grown as a monolayer culture in Eagle's MEM with 10% heat-inactivated FBS. The relative plating efficiencies were determined as the ratio of the number of colonies in various concentrations of samples to that in the sample-free control, as described in previous papers.[15,16] Two hundred cells were seeded on a 60/15-mm plastic plate with 4 mL culture medium and incubated overnight at 37 °C. After each sample in DMSO (4 μL) was added to the culture medium, cells were further cultured for four days. The numbers of colonies in the sample plates were counted and compared with those in control cultures.

3.3. Quantitative Measurement of Murine TNF-α (mTNF-α)

RAW264.7 cells were treated with each test compound for 5 min and LPS (final concentration of 1 μg/mL) was added to the culture medium. The mixture was incubated at 37 °C for 24 h, and mTNF-α concentration in each culture supernatant was measured using the murine TNF-α ELISA development kit following the manufacturer's instructions. Each test sample was assayed in duplicate, and data are presented as the mean of three independent experiments.

Effects of compounds on cell proliferation were evaluated by enumerating viable cells using the MTT formazan production method.[17] RAW264.7 cells were incubated with various concentrations (0.1, 1, 3, and 10 μM) of test compounds for 24 h. The cells were treated with MTT solution and, after incubation for 3 h, formazan production was assessed by measuring the OD (570 nm).

3.4. Extraction and isolation

The soft coral was collected by scuba diving in Manado, North Sulawesi and identified as *Sarcophyton* sp. from shapes and appearances of the organism and sclerites (voucher specimen was deposited in the Faculty of Fisheries and Marine Science, Sam Ratulangi University as SC4). The wet sample was ground and extracted three times with MeOH. A portion of the concentrated extract (3.8 g) was dissolved in MeOH-water (9:1, 200 mL) and extracted with *n*-hexane. Water (250 mL) was added to the 90% MeOH layer and the solution (40% MeOH) was extracted successively with EtOAc and *n*-BuOH. The most active EtOAc fraction (1.35 g) against V79 cells (100% inhibition at 20 μg/mL) was separated by SiO_2 column (40 g) with $CHCl_3$–MeOH (gradient elution) into four fractions (E1–E4). Fraction E1 (165 mg) was subjected to HPLC (ODS, 70% MeOH containing 0.3% TFA) to give **1** (3.6 mg), **2** (1.8 mg), and crude **4**, which was further purified by HPLC (ODS, 20% to 70% MeOH (50 min) with 0.1% TFA) to afford 6.3 mg of **4**. Fraction E2 (646 mg) was separated by LH-20 (MeOH) followed by HPLC (ODS, 20% to 70% MeOH (50 min) with 0.1% TFA) to obtain **3** (11.3 mg). Compounds **1** and **2** were detected by HPLC eluted without a TFA buffer solution (ODS column, 5 mmφ x 250 mm; gradient elution with 50 to 100% MeOH in H_2O for 40 min) in the $CHCl_3$–MeOH (10:1) fraction obtained from the newly prepared MeOH extract of the soft coral followed by EtOAc extraction and SiO_2 column chromatography as above. Another portion (4.0 g) was similarly extracted with *n*-hexane, $CHCl_3$, and *n*-BuOH, and the most active $CHCl_3$ extract (1.60 g) was separated into six fractions (C1–C6) by SiO_2 column (40 g, gradient elution with $CHCl_3$–MeOH). Fraction C2 (1.15 g) was further separated by ODS column (60 g, gradient elution with MeOH-water) into six fractions (C21–C26). HPLC (ODS) separation of the fraction C23 (360 mg) with 70% MeOH gave **5** (100 mg) and **6** (33.1 mg), and of C24 (186 mg) by 80% MeOH yielded **7** (6.4 mg).

*3.4.1. Sarcofuranocembrenolide A (**1**).* $[\alpha]_D^{24}$ +61 (*c* 0.30, MeOH); UV (MeOH) λ_{max} nm (log ε) 223 (3.20), 282 (3.17); IR (KBr) ν_{max} cm^{-1} 3460, 1760, 1724, 1683, 1590, 1527, 1440; HR-FABMS *m*/*z* 389.1598 $(M+H)^+$, calcd for $C_{21}H_{25}O_7$, 389.1600; ^1H and ^{13}C NMR, see Table 1.

*3.4.2. Sarcofuranocembrenolide B (**2**).* $[\alpha]_D^{24}$ −12.7 (*c* 0.17, MeOH); UV (MeOH) λ_{max} nm (log ε) 222 (3.01), 246 (sh); IR (KBr) ν_{max} cm^{-1} 3460, 1765, 1725, 1683, 1589, 1527, 1439; HR-FABMS *m*/*z* 405.1921 $(M+H)^+$, calcd for $C_{22}H_{29}O_7$, 405.1914; ^1H and ^{13}C NMR, see Table 1.

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Supplementary data

NMR spectra of compounds **1** and **2**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.11.078.

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