Research Article

Isopropyl(ene)-type cembrene diterpene an important secondary metabolite in soft coral *Sinularia flexibilis* of Tun Sakaran Marine Park, Malaysia

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**ABSTRACT.** Two cembrene diterpenes; (3S, 4S, 11S, 12S, 1E, 7E)-3,4:11,12-bisepoxyembre-1,7-diene (1) and (1E,3E,7E)-11,12-epoxyembre-1,3,7-triene (2) were isolated from a population of *Sinularia flexibilis* collected from Kapikan Reef, Semporna, Sabah. The chemical structures were elucidated based on ¹H-NMR and ¹³C-NMR spectroscopic data. This is the first record of cembranoid diterpenes isolated from the Malaysian soft coral genus *Sinularia*. These cembrenes could be used as chemotaxonomical markers and have also exhibited potent anti-bacterial activity against pathogenic *Escherichia coli* and *Staphylococcus aureus*.

**Keywords:** Soft coral, *Sinularia*, Kapikan Reef, cembranoid diterpenes, chemotaxonomic, antibacterial activity.

**INTRODUCTION**

The soft coral genus *Sinularia* (Aleyonacea, Aleyoniidae) is known as a prolific producer of secondary metabolites among marine organisms (Yu et al., 2006). A total of 30 of the 96 known species of *Sinularia* have been chemically examined and contain diterpenoids, sesquiterpenoids and steroids as their secondary metabolites (Goud et al., 2002; Jin et al., 2005; Amira et al., 2006; Liang et al., 2010). Due to their soft bodies and sedentary life, soft corals are known to biosynthesize secondary metabolites to chemically protect themselves from predators and colonizers (Li et al., 2006; Bonnard et al., 2010). In the last 30 years, there have been more than 15,000 novel secondary metabolites discovered from these organisms (Li et al., 2006).

Soft corals are known to synthesize and accumulate terpenes, particularly sesquiterpenes and diterpenes of eudesmane and cembranoid types (Matthee et al., 1998). It has been suggested that some of these terpenes are used as chemical defence compounds with ichthyotoxicity activity to avoid predatory fishes (Iwagawa et al., 1999). In light of some recent findings, these compounds have also shown anti-fungal, anti-bacterial and anti-bleaching potentials in nature, and for this reason have been suggested as ecological chemicals of importance for these organisms (Chao et al., 2006; Ishii et al., 2010a). In addition, these compounds are also actively evaluated for biological activities of pharmacological significance (Lin et al., 2009).

To date, biomedical and pharmaceutical studies have shown interesting activities such as in anti-cancer, anti-microbial, anti-fungal, anti-inflammatory, anti-coagulant, anti-platelet and anti-viral (Kamel et al., 2005; Chao et al., 2006; Arepalli et al., 2009; Chen et al., 2010). In addition to their importance as test metabolites for pharmacological assay,
information pertaining to the distribution of secondary metabolites based on their chemical structures could be used to assist the systematics of soft corals. There has been some success in the application of cembrane-type diterpenes in the chemotaxonomy of major soft coral genus such as Sinularia, Lobophytum and Nepthea (Longeon et al., 2002; Lin et al., 2009; Cheng et al., 2010).

Despite these advances in the chemistry of soft corals, there is shortage of information pertaining to the chemistry of soft corals in Malaysia. The only available to date are on the chemistry of the soft coral genus Nepthea collected from the coastal waters of Kota Kinabalu, Sabah. This genus was found to synthesis compounds such as sterols, norsesquiterpenoids and cembranoid diterpenes (Ishii et al., 2009a, b; 2010a, b). Therefore, this paper reports the discovery of cembranoid diterpenes from the population of soft coral genus Sinularia at the Kapikan Reef, Semporna, Sabah.

METHODOLOGY

Collection

The Sinularia flexibilis population was collected from a coral reef at 10 m depth in the waters of Kapikan Reef (Semporna, Sabah) (N 04°06' 53.35" E 118°37' 41.60") . The samples were photographed underwater, collected by SCUBA divers and a representative specimen (SC022011SI) is deposited at the Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. The samples were weighed and cut into pieces. Then the samples were brought back to the laboratory under cool conditions (4°C) and processed according to the procedures described by Ishii et al. (2009a).

Extraction and isolation

The specimen was soaked in methanol (MeOH) at room temperature for approximately seven days. The resulting MeOH extract was concentrated in vacuo and the concentrate was partitioned between ethyl acetate (EtOAc) and H2O. The EtOAc layer was further concentrated and partitioned between hexane (Hex) and 90% MeOH. This procedure yielded Hex and 90% MeOH extracts. The hexane extract was then fractionated using Silica gel column chromatography with a step gradient of Hex and EtOAc with the ratio 9:1, 8:2, 7:3, 6:4, 1:1 and CHCl3:MeOH:H2O (65:25:4). The first fraction was then further isolated using Preparative Thin Layer Chromatography (PTLC) using toluene (Tol) and EtoAc with the ratio 98:2 to obtain 1 while fraction 2 was further isolated using Hex and EtoAC with the ratio 9:1 to obtain 2. Compounds 1 and 2 were subjected to 1H-NMR, 13C-NMR and 2D NMR measurements such as 1H-1H COSY, HSQC, HMBC and NOESY. High-resolution mass spectroscopy data was measured using Shimadzu LC-MS-IT-TOF. Other physical characteristics were obtained as described by Vairappan et al. (2001) and Ishii et al. (2009b).

Antibacterial assay

Six strains of bacteria, Clostridium sordelli, Clostridium novyi, Pseudomonas aurelis, Escherichia coli, Staphylococcus aureus and Vibrio parahaemolyticus, were used to test the anti-bacterial activity of compounds 1 and 2. One loopful of bacteria was inoculated in 10 ml of nutrient broth and incubated for 24 hours. The optical density of the inoculated bacteria was adjusted to 0.5 McFarland (0.5 McFarland = 0.105±0.0.05A@625 nm). Then, 100μl of the adjusted bacteria were seeded and spread evenly on pre-prepared nutrient agar plates using a cell spreader. Compounds 1 and 2 were then loaded onto paper discs (Whatman, 6 mm) and the impregnated discs were placed on the seeded agar plates. The diameters of the inhibitory zones were measured after the plates were incubated at 28°C for 24 hours. Minimum inhibitory concentration was determined upon serial dilution of the compound coupled with the observation of the inhibition zone disappearance (Vairappan et al., 2001).
RESULTS AND DISCUSSION

A total of 500 g fresh *Simularia flexibilis* was homogenized in 2 L of methanol and left to soak for seven days. Upon an extensive extraction and partition process as described above, 1.8 g of Hex extract and 4.6 g of 90% MeOH extract were obtained. Repetitive preparative thin layer chromatography of fraction 1 gave compound 1 in the amount of 3.7%, while fraction 2 gave compound 2 as 3.1% of the fresh soft coral biomass. The structure of 1 and 2 were elucidated independently and the resulting planar structure was compared to the data available in Marin Lit (version 2012) and were found to be (3S,4S,11S,12S,1E,7E)-3,4:11,12-bisepoxycebrina-1,7-diene (bisepxoxy) (1) and (1E,3E,7E)-11,12-epoxycebrina-1,3,7-triene (2) as reported by Bowden *et al.* (1983 & 1978), respectively.

Both compounds revealed a similar 14-member ring with terminal isopropyl functionality as shown in Figure 1. Compound 1 has four olifinic carbons (120.3, 127.1, 135.3 and 152.0 ppm) resulting in two pairs of double bond. $^{13}$C-DEPT experiments revealed the presence of five methyl carbons ($\delta$ 1.64 (s), 1.27 (s), 1.23 (s), 1.03 (d) and 1.03 (d) ppm), two epoxides (62.8 (d), 62.2 (s), 61.9 (s) and 60.2 (d)) and six methylene carbons. Presence of an allylic epoxide proton (3.33 (d)) and a nonallylic epoxide proton ($\delta$ 2.66 (dd)) further confirms the presence of two epoxide functionality.

On the other hand, detailed spectroscopic data analysis of compound 2 revealed the presence of six olifinic carbons (δ 147.8, 136.6, 134.1, 127.8, 121.6, 119.1) resulting in three pairs of double bond. $^{13}$C-DEPT experiments revealed the presence of five methyl carbons (δ 1.72 (s), 1.61 (s), 1.26 (s), 1.03 (d) and 1.03 (d)), and six methylene carbons. Presence of secondary methyl indicates the presence of isopropyl functionality and this was confirmed by HMBC correlations. An epoxy methane proton was observed at δ 2.70. In the earlier publication of these compounds, complete $^1$H and $^{13}$C assignments were lacking and these are given in this report. The detailed spectroscopy data are given below:

**Compound 1**, colourless oil, C_{28}H_{32}O_{2} : [$\alpha$]_{D}^{+} 48.0°, $^{13}$C NMR (CDCl$_3$, 150 MHz), 152.0 (C-1), 135.3 (C-8), 127.1 (C-7), 120.3 (C-2), 62.8 (C-11), 62.2 (C-4), 61.9 (C-12), 60.2 (C-3), 39.7 (C-13), 38.1 (C-5), 37.5 (C-9), 34.8 (C-15), 28.3 (C-14), 25.3 (C-10), 23.1 (C-16), 23.0 (C-6), 22.6 (C-17), 18.9 (C-18), 17.0 (C-20), 15.6 (C-19); $^1$H NMR (CDCl$_3$, 600 MHz), 5.29 (1H, t, H-7), 4.97 (1H, d, H-2), 3.33 (1H, d, H-3) 2.66 (1H, dd, H-11), 2.28 (1H, m, H-9), 2.23 (1H, m, H-15), 2.21 (1H, m, H-14), 1.17 (1H, m, H-6), 2.13 (1H, m, H-13), 2.11 (1H, m, H-10), 2.08 (1H, m, H-14), 2.06, (1H, m, H-9).

![Image](image.png)

**Figure 1.** Isopropyl cembrene diterpenes (1, 2) from Borneon *Simularia* sp.
cembranoid diterpenes are known secondary metabolites in soft corals, the presence of the 14-membered ring with an isopropyl terminal functionality is unique to soft corals genus *Sinularia* (Li et al., 2006; Lin et al., 2009). Similar enantiomers of isopropyl cembrane with alcohol functionality were isolated from another population of *Sinularia facile* by Bowden et al. (1981). These compounds were identified as (1R,4R,2E,7E,11E)-cembra-2,7,11-trien-4-ol (3) and (1E,4R,2E,7E,11E)-cembra-2,7,11-trien-4-ol or thunbergol (4) (Figure 2).

The soft coral genus *Sinularia* is known to be a prolific producer of diterpene secondary metabolites. Cyclization of a geranylgeraniol-derived precursor between one and 14 generates a 14 membered diterpenoid called cembrane. Members of this genus are known to mainly biosynthesize isopropyl(ene) type cembranoid diterpenes as reported in this paper. Therefore, the basic structure is characterized by terminal isopropyl functionality and three methyl-substituted 14-membered ring. Further structural changes in the position of double bonds, epoxidation, allylic and isopropyl oxidation, and carbon cyclization leads to another four rearranged cembranes such as -lactone-type cembrane, -lactone-type cembrane, -lactone-type and casbene-type.

![Figure 2](image_url)
Both these cembrenes were tested for their anti-bacterial activity against six species of bacteria: C. sordelli, C. novyi, P. aureus, E. coli, S. aureus and V. parahaemolyticus. Potent anti-bacterial activity was observed against E. coli and S. aureus with a minimum inhibitory concentration (MIC) value of 20 and 30 mg/disc, respectively. The activity observed with the inhibition against bacteria is not surprising as compounds of this skeleton are known to exhibit biological activities such as anti-fouling and an inhibition of pro-inflammatory proteins in RAW264.7 macrophage cells. They are also known to exhibit very low cytotoxic activities (Ahmed et al., 2008; Lai et al., 2011).

In conclusion, discovery of these two isopropyl cembrenes are an important milestone in the chemical investigation of the Bornean soft corals. These compounds could be regarded as the precursors for the biosynthesis of other cembrene diterpenes in this genus. The anti-bacterial activities exhibited by these compounds are indicators of the potency of these secondary metabolites in soft corals.

ACKNOWLEDGEMENT

The authors would like to acknowledge Sabah Parks for granting a permit for the collection of samples from Kapikan Reef, Tun Sakaran Marine Park, Semporna, Sabah. CSV would like to acknowledge MOSTI for research grant, MOSTI-NODE-Science-02-01-10-SF0131.

REFERENCES


