



CEMBRANOIDS CONTENT OF SOFT CORAL SARCOPHYTON FROM ACIDIFIED ENVIRONMENT AT VOLCANO ISLAND, INDONESIA

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Abstract

Cembranoid content in soft coral is known as a chemotype that relate with genotype and environment. This research aimed to characterize the cembranoid Sarcophyton soft coral from the reef that acidified by CO₂ volcanic vents (pH_T 7.8) at Volcano Islands waters, Banda-Neira (Indonesia), as a means of predicting the future impact of ocean acidification to the genetic diversity of Sarcophyton soft coral. 30 random colonies were taken, combined, and extracted with ethanol. Cembranoid isolation and identification had been done by high performance liquid chromatography and spectroscopic techniques. Results of the study found sarcophytol derivatives (sarcophytol A, 11,12-epoxy sarcophytol A, sarcophytol B, and sarcophytol M) as the only chemotype in the sample. This may suggest low genetic diversity in the observed Sarcophyton sample. Therefore, it may suggest that even soft coral is known to be resilient to future acidification pressures, the genetic diversity or the production of diverse cytotoxic metabolite may be hampered due to ocean acidification in future climate change adaptation.

Keywords: cembranoid; chemotype; ocean acidification; soft coral; *Sarcophyton*

1. Introduction

Soft corals are reef organisms that expected to survive in the increasing ocean acidification pressure on the future climate change. Observation into coral community structure at natural acidified environment at CO₂ volcanic vents in Iwotorishima Island waters (Japan) detected an increase in the cover of soft coral Sarcophyton (Inoue, Kayanne, Yamamoto, & Kurihara, 2013). The increasing of soft coral cover was also shown in pH 8 waters located close to volcanic CO₂ at Volcano Island (Maluku), Mahengetang Island (North Sulawesi), and seashore of Northern Minahasa (Januar, Zamani, Soedharma, & Chasanah, 2016a; 2017a). Soft corals are known to be able to mitigate the effects of acidic environment because the protection of external soft bodies to their endoskeleton (Gabay, Benayahu & Fine, 2013; Gabay, Fine, & Barkay, 2014). Moreover, the competitiveness in living space competition is due to their ability to produce the allelochemical cembranoid compounds.

Cembranoid is a compound that generally composed by 20 carbon atoms, with 14 carbon atoms main ring and several functional groups. The combination of functional groups such as hydroxyl, carbonyl, epoxy, and lactone, becomes a side chain that attaches to various carbon atom positions on the main ring (Januar, Zamani, Soedharma, & Chasanah, 2016b). Cembranoid is the main soft coral allelochemical, with cytotoxic biological properties that may serve as a chemical weapon for predator or benthic living space competitors, such as hard coral (Lages, Fleury, Ferreira, & Pereira, 2006). The content of cembranoid is highly dependent upon environmental factors, as the ecological relationship with the number of predators and spatial competitors (Slattery, Starmer, & Paul, 2001). Soft coral that was taken from natural coral reefs was detected to contain diverse cembranoid compounds than cultivated aquariums (Frag et al., 2016). Our previous finding also showed cembranoid was produced in higher concentration at healthier coral reef environment (Januar, Hendarto,

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Chasanah, & Wright, 2011; Januar, Marraskuranto, Patantis, & Chasanah, 2012; Januar et al., 2015). Meanwhile, cembranoid content also depends on the genetic diversity of soft coral. Previous studies on *Sarcophyton glaucum* found that each genetic clade (Clade A to F) contains a combination of 8 cembranoid chemotypes (Aratake et al., 2012; Farag et al., 2016). Furthermore, multivariate statistics for cembranoid quantity was successfully validated to be a profiling taxonomic approach between soft coral *Lobophytum*, *Sinularia*, and *Sarcophyton* (Farag et al., 2017).

Therefore, cembranoid identification from soft coral may serve as an indicator of genetic diversity in a particular location. This research aimed to characterize the cembranoid content in soft coral genus (*Sarcophyton*) on the reef that acidified by CO₂ volcanic vents (Volcano Islands waters, Banda-Neira region, Indonesia), as a means of predicting the future impact of ocean acidification to the genetic diversity of *Sarcophyton* soft coral. Even through the resistance of soft corals in acidic condition is already well-known, the impact of ocean acidification into their genetic and allelochemical metabolite diversity is still limited.

2. Material and Methods

2.1. General Methods

Flash vacuum chromatography was performed using Bulk Phenomenex C₁₈. Preparative HPLC was performed using a Shimadzu C₁₈ column (250 × 21 mm) attached to a Shimadzu Preparative HPLC system with a fraction collector. 1D and 2D-NMR spectra were recorded on Jeol ECS 400 MHz NMR spectrometer, with spectra referenced to ¹H and ¹³C resonances in the deuterated solvents. Accurate mass spectrometric data were obtained from Shimadzu IT-TOF mass spectrometer.

2.2. Study Site

The samples were taken from the reef (4-6 m, 4°30.371'S, 129°53.067'E) that acidified by CO₂ from a vent of an active volcano near Gunung Api Island, Banda-Neira region, in Indonesian - Maluku Province. Specific site with pH_T (pH in total scale) 7.8 was selected based on our previous study (Januar, Zamani, Soedharma, & Chasanah, 2017a). Toxic H₂S may come from the hydrothermal vents. Therefore, the site was also selected according to Anthony, Kline, Diaz-Pulido, Dove, & Hoegh-Guldberg (2008) where the sulfide level was below 2 ppm. The site with pH_T 7.8 was chosen to enable predictions of pH condition that might be seen in the coral reef region for next 100 years (Lemke, Ren, & Alley, 2007).

2.3. Animal Materials

30 random *Sarcophyton* genus (contained with several random species) colonies (20 g) from three replications of 30 m line intercept transect (4-6 m) were taken from observed site. Soft coral taxonomy was identified by morphological inspection according to Fabricius & Alderslade (2001). All colonies were combined (600 g in total) and extracted with 600 mL ethanol. Samples were placed in a cool box containing ice packs for preservation to the laboratory. Samples were then exhaustively re-extracted with ethanol, to yield the extracts (1.23 g and 0.205% w/w from wet sample) that were used for isolation study. A voucher of samples has been lodged and preserved in Indonesia Research Center for Marine and Fisheries Product Competitiveness and Biotechnology, Jakarta, Indonesia.

2.4. Extraction, Isolation, and Elucidation Study

Crude ethanol extract was filtered through a column chromatography of reversed phase C₁₈ silica using a mixture (1:1) of methanol and dichloromethane as eluent. The filtrate solvent was evaporated to 50 mL and then fractionated with ethyl acetate (50 mL) with three replications. The ethyl acetate solvent was then removed with rotary evaporator and freeze dryer. The resultant dry extract was subjected to a preparative reversed-phase HPLC (15 mL/min, gradient elution from 20% Acetonitrile:H₂O to 100% Acetonitrile; column 250 × 20 mm Shimadzu RP C₁₈, over 60 mins). Fractions were collected every 7.5 mL or 30s. Proton NMR analysis was conducted to find all cembranoid in the fractions, by finding the chemical shift in olefinic and oxymethine regions as according to Faraq et al. (2016; 2017). Identification of cembranoid was conducted according to Januar, Zamani, Soedharma, & Chasanah (2016b), with spectroscopic techniques (mass and nuclear magnetic resonance analyses) and LSD-CASE (Logic Structure Determination – Computer Assisted Structure Elucidation) software.

3. Results and Discussion

Isolation and elucidation from *Sarcophyton* sample detected four cembranoid compounds. NMR and MS analysis revealed the yellow color fractions number 24, 26, 30, and 32 are 1 (*Sarcophytol B*, 9 mg, 0.002% w/w wet weight sample, 8% w/w ethyl acetate fraction), 2 (11,12-epoxy *sarcophytol A*, 11 mg, 0.002%, 10%), 3 (*Sarcophytol A*, 47 mg, 0.008%, 42%), and 4 (*Sarcophytol M*, 7 mg, 0.001%, 6%). Computer assisted structure elucidation into the spectroscopic

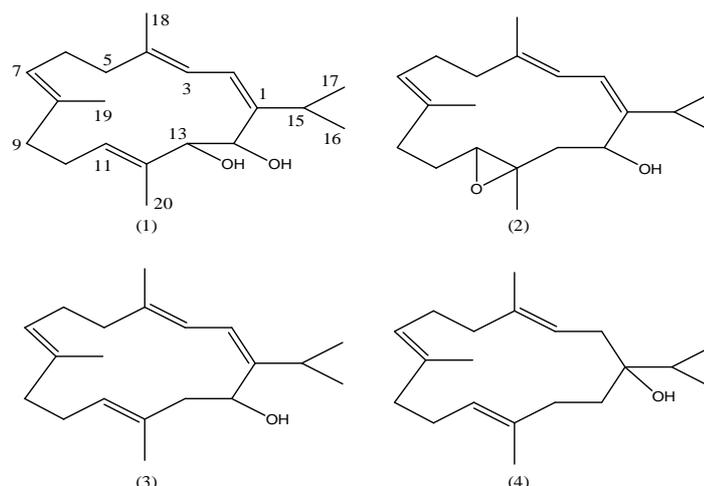


Figure 1. Structures of sarcophytol B (1) 11,12-epoxy sarcophytol A (2), sarcophytol A (3), and sarcophytol M, isolated from the ethanol extracts of *Sarcophyton* genus from acidified reef (up to pH_T 7.8) by CO₂ vents at Volcano Island waters, Banda – Neira Region (Maluku Province), Indonesia.

(Table 1) and 2D-NMR correlation (Heteronuclear Single Quantum Correlation/HSQC, Heteronuclear Multiple Quantum Coherence/HMQC, Homonuclear Correlation Spectroscopy/COSY, Heteronuclear Multiple Bond Correlation/HMBC, and Heteronuclear 2 Bond Correlation/H2BC) data revealed the structure of all compounds (Figure 1).

The backbone of 14 carbon ring of all isolated cembranoid was identified by NMR analysis. 8 double bond carbons indicated 4 double bonds at (1) and (3), while 6 double bond carbons suggested 3 double bonds at (2) and (4). All compounds showed ¹H-¹H COSY continuous chains from H-5 to H-7, H-9 to H-11, and H-14 or H-13 to H-2. Long-range ¹H-¹³C couplings (HMBC) were observed between H₃-18 to C-3 and C-5; H₃-19 to C-7 and C-9; and H₃-20 to C-11 and C-13. Further two bonds ¹H-¹³C couplings (H2BC) confirm the bond of C-4 to H₃-18, C-8 to H₃-19, C-12 to H₃-20, and C-15 to H₃-16 and H₃-17. These data may link all of the main carbons in cembranoid ring of all isolated compounds. The specific configuration of each compound was found with direct H2BC couplings of carbon with C-13 hydroxyl to H-14 (the same couplings with on the other way around) for sarcophytol B (1), H2BC couplings of epoxide between C-12 to H-11 for 11,12-epoxy sarcophytol A (2), tertiary C-14 with hydroxyl for Sarcophytol A, and quaternary C-1 with hydroxyl for Sarcophytol M. All of the isolated compounds in this study was already characterized previously by Kobayashi, Nakagawa, & Mitsuhashi, (1979) and Kobayashi & Osabe (1989). The spectroscopic data (MS, ¹H-NMR, and ¹³C-NMR) for all samples were as follows;

- Sarcophytol B (1), m/z 305.2319 for [M+H]⁺ (calculated for C₂₀H₃₃O₂⁺); ¹H (CDCl₃) δ 5.96 (1H, *d*, *J* = 10.83 Hz, H-2), 5.79 (1H, *d*, *J* = 10.79 Hz, H-3), 2.09 (1H, *overlapped*, H-5 α), 2.15 (1H, *overlapped*, H-5 β), 2.17 (1H, *overlapped*, H-6 α), 2.19 (1H, *overlapped*, H-6 β), 5.01 (1H, *overlapped*, H-7), 2.07 (1H, *overlapped*, H-9 α), 2.13 (1H, *overlapped*, H-9 β), 2.15 (1H, *overlapped*, H-10 α), 2.19 (1H, *overlapped*, H-10 β), 4.96 (1H, *overlapped*, H-11), 4.02 (1H, *d*, *J* = 7.19 Hz, H-13), 5.13 (1H, *overlapped*, H-14), 2.65 (1H, *m*, *J* = 26.97 Hz, H-15), 1.16 (3H, *d*, *J* = 7.13 Hz, Me-16), 1.05 (3H, *d*, *J* = 6.99 Hz, Me-17), 1.52 (3H, *s*, Me-18), 1.73 (3H, *s*, Me-19), 1.38 (3H, *s*, Me-20); and ¹³C (CDCl₃) δ 146.33 (C-1), 122.14 (C-2), 123.16 (C-3), 137.89 (C-4), 39.31 (C-5), 24.49 (C-6), 125.18 (C-7), 136.72 (C-8), 39.44 (C-9), 24.92 (C-10), 126.62 (C-11), 132.46 (C-12), 73.42 (C-13), 76.15 (C-14), 27.16 (C-15), 25.32 (C-16), 24.92 (C-17), 19.20 (Me-18), 15.14 (Me-19), 14.82 (Me-20).

- 11,12-epoxy sarcophytol A (2), m/z 305.2315 for [M+H]⁺ (calculated for C₂₀H₃₃O₂⁺ 305.2317), ¹H (CDCl₃): δ 5.99 (1H, *d*, *J* = 10.99 Hz, H-2), 5.76 (1H, *d*, *J* = 10.98 Hz, H-3), 1.93 (1H, *overlapped*, H-5 α), 2.06 (1H, *overlapped*, H-5 β), 2.12 (1H, *overlapped*, H-6 α), 2.15 (1H, *overlapped*, H-6 β), 5.12 (1H, *t*, *J* = 11.07 Hz, H-7), 2.07 (1H, *overlapped*, H-9 α), 2.14 (1H, *overlapped*, H-9 β), 2.15 (1H, *overlapped*, H-10 α), 2.17 (1H, *overlapped*, H-10 β), 4.76 (1H, *t*, *J* = 11.91 Hz, H-11), 1.98 (1H, *overlapped*, H-13 α), 2.12 (1H, *overlapped*, H-13 β), 4.73 (1H, *t*, *J* = 13.33 Hz, H-14), 2.60 (1H, *m*, *J* = 27.02 Hz, H-15), 1.10 (3H, *d*, *J* = 6.92 Hz, Me-16), 1.06 (3H, *d*, *J* = 7.39 Hz, Me-17), 1.58 (3H, *s*, Me-18), 1.71 (3H, *s*, Me-19), 1.32 (3H, *s*,

Me-20); ^{13}C (CDCl_3) δ 146.61 (C-1), 119.71 (C-2), 118.92 (C-3), 138.41 (C-4), 40.32 (C-5), 24.95 (C-6), 126.32 (C-7), 134.84 (C-8), 38.43 (C-9), 22.45 (C-10), 67.82 (C-11), 62.16 (C-12), 38.84 (C-13), 59.12 (C-14), 27.45 (C-15), 25.42 (C-16), 24.49 (C-17), 19.02 (Me-18), 17.11 (Me-19), 15.92 (Me-20).

- Sarcophytol A (3), m/z 289.2126 for $[\text{M}+\text{H}]^+$ (calculated for $\text{C}_{20}\text{H}_{33}\text{O}^+$ 289.2123) ^1H (CDCl_3): δ 6.15 (1H, d, $J = 11.91$ Hz, H-2), 6.01 (1H, d, $J = 11.45$ Hz, H-3), 1.96 (1H, overlapped, H-5 α), 2.01 (1H, overlapped, H-5 β), 2.14 (1H, overlapped, H-6 α), 2.21 (1H, overlapped, H-6 β), 4.97 (1H, overlapped, H-7), 2.02 (1H, overlapped, H-9 α), 2.21 (1H, overlapped, H-9 β), 1.83 (1H, overlapped, H-10 α), 1.93 (1H, overlapped, H-10 β), 5.01 (1H, overlapped, H-11), 2.25 (1H, overlapped, H-13 α), 2.41 (1H, overlapped, H-13 β), 5.02 (1H, t, $J = 13.51$ Hz, H-14), 2.58 (1H, m, $J = 27.05$ Hz, H-15), 1.11 (3H, d, $J = 6.87$ Hz, Me-16), 1.05 (3H, d, $J = 7.33$ Hz, Me-17), 1.60 (3H, s, Me-18), 1.74 (3H, s, Me-19), 1.45 (3H, s, Me-20); ^{13}C (CDCl_3) δ 146.91 (C-1), 120.63 (C-2), 121.21 (C-3), 136.16 (C-4), 38.84 (C-5), 24.56 (C-6), 124.58 (C-7), 134.78 (C-8), 39.73 (C-9), 25.62 (C-10), 125.34 (C-11), 131.34 (C-12), 44.44 (C-13), 69.93 (C-14), 27.11 (C-15), 25.50 (C-16), 24.50 (C-17), 18.34 (Me-18), 16.46 (Me-19), 15.01 (Me-20).

- Sarcophytol M (4), m/z 291.2614 for $[\text{M}+\text{H}]^+$ (calculated for $\text{C}_{20}\text{H}_{35}\text{O}^+$ 291.2612) ^1H (CDCl_3): δ 2.03 (1H, overlapped, H-2 α), 2.07 (1H, overlapped, H-2 β), 6.03 (1H, t, $J = 7.52$ Hz, H-3), 2.02 (1H, overlapped, H-5 α), 2.05 (1H, overlapped, H-5 β), 2.14 (1H, overlapped, H-6 α), 2.16 (1H, overlapped, H-6 β), 5.02 (1H, overlapped, H-7), 2.18 (1H, overlapped, H-9 α), 2.21 (1H, overlapped, H-9 β), 1.88 (1H, overlapped, H-10 α), 1.96 (1H, overlapped, H-10 β), 4.96 (1H, overlapped, H-11), 2.08 (1H, overlapped, H-13 α), 2.11 (1H, overlapped, H-13 β), 2.09 (1H, overlapped, H-14 α), 2.13 (1H, overlapped, H-14 β), 4.81 (1H, t, $J = 27.05$ Hz, H-15), 1.13 (3H, d, $J = 6.89$ Hz, Me-16), 1.03 (3H, d, $J = 7.22$ Hz, Me-17), 1.49 (3H, s, Me-18), 1.53 (3H, s, Me-19), 1.32 (3H, s, Me-20); ^{13}C (CDCl_3) δ 78.16 (C-1), 32.16 (C-2), 121.52 (C-3), 134.82 (C-4), 38.92 (C-5), 24.14 (C-6), 124.56 (C-7), 136.82 (C-8), 39.84 (C-9), 24.86 (C-10), 125.92 (C-11), 131.36 (C-12), 34.22 (C-13), 36.12 (C-14), 33.16 (C-15), 22.14 (C-16), 21.16 (C-17), 16.32 (Me-18), 15.26 (Me-19), 14.92 (Me-20).

The finding of sarcophytol A as the major cembranoid compound in Sarcophyton sample was also detected by previous study on the same genus, which are *S. crassocaule*, *S. digitatum*, *S. infundibuliforme*, and *S. glaucum* (Koh et al., 2000; Tanaka, Yoshida, & Benayahu, 2005). Meanwhile, Sarcophytol M as a minor sarcophytol analogue was

also found from a previous study on *S. glaucum* (Kobayashi & Osabe, 1989). However commonly, sarcophytol A content was only up to 28% w/w from lipid fraction (Koh et al., 2000), while this study found sarcophytol A up to 42%. Moreover, studies on Indonesian tropical Sarcophyton species usually found other type cembranoid analogues, particularly sarcophytoxide/sarcophine (Iswani, Tohir, & Januar, 2014; Putra, M. Y., Saparhadi, Karim, Murniasih, & Swasono, 2017; Januar, Zamani, Soedharma, & Chasanah, 2017b). Other studies from the temperate region also commonly found more than one type of cembranoid analogue in Sarcophyton species (Pham, Butler, & Quinn, 2002; Cheng et al. 2010; Wang et al. 2011; Cao, Zhou, Xu, Zhang, & Wang, 2013). The result from this study, which only found sarcophytol analogue as the only cembranoid type in the sample, is rare. The same results were also found in *Sarcophyton* species from Okinawa Island (Japan) and Bornean Island (Indonesia), that only found a soft coral contained only with sarcophytol analogue cembranoid (Kobayashi, Nakagawa, & Mitsuhashi, 1979; Phan & Vairappan, 2015).

Low cembranoid richness that was found throughout this study may be happened in several possibilities. First, it is probable that the moderate acidification environment at Volcano Island acted as a pressures that suppressed the richness of tropical Sarcophyton genetic biodiversity. The result that not found any trace of sarcophytoxide/sarcophine, was the key for this probability. Sarcophine is one the most common chemotype in Sarcophyton genus (Faraq et al., 2017). Sarcophine present almost in all Sarcophyton genres, which are *S. glaucum*, *S. trocheliophorum*, *S. ehrenbergi*, *S. elegans*, *S. infundibuliforme*, *S. pulchellum*, *S. cherbonnieri*, except in *S. crassocaule* and *S. digitatum* (Tanaka, Yoshida, & Benayahu, 2005). *S. glaucum* sub-species (clade A-F) can be also detected by specific cembranoid content that consists with sarcophine enantiomers (2S,7S,8S-sarcophine, 2S,7R,8R-sarcophine, or 2S,7R,8R-isosarcophine) and sarcophytolide cembranoid analogues (Aratake et al., 2012). Therefore, the lack of sarcophine and sarcophytolide analogues may indicate low genetic diversity within the Sarcophyton from Volcano Island. For example, *Sarcophyton glaucum* clade F may not present in this site. This species produces the sarcophytolide analogue that is not produced by any other *Sarcophyton glaucum* clade (Aratake et al., 2012).

The other probability is the moderate acidification environment affected the cembranoid chemotype diversity in the observed soft coral within direct or indirect ecological means. Our previous experiment showed a significant difference of cembranoid content

in *Sarcophyton glaucum* that were incubated in acidification environment over 3 days (Januar, Zamani, Soedhadma, & Chasanah, 2017c). Therefore, it may suggest that acidification environment directly disturbs several pathways of lactone ring cembranoid metabolism and result into low chemotype diversity in the organism. In another type of marine environmental stresses, eutrophication was detected as a probable cause for lower cembranoid diversity production in soft coral (Januar, Hendrarto, Chasanah, & Wright, 2011; Januar, Marraskuranto, Patantis, & Chasanah, 2012). Moreover, an indirect ecological term may also be applied, with the role of cembranoid in soft coral as a chemical defensive weapon to deterrent predator or benthic living place competition (Slattery, Starmer, & Paul, 2001; Lages, Fleury, Ferreira, & Pereira, 2006). Visual observation of studied soft coral site showed the benthic coral diversity was low. The reef was composed with a single dominant endemic coral species (up to 80%) *Acropora desalwii* (Januar, Zamani, Soedhadma, & Chasanah, 2017a). It is probable that *Sarcophyton* only produced specific type of cembranoid as their competitor was also only a specific type of acroporid coral.

Therefore, this study suggests that in general, the soft coral genetic diversity or the production of diverse cytotoxic metabolite may be hampered due to natural selection and adaptation to the ocean acidification in future climate change pressure. Even through soft coral could accommodate a wide range of environmental pressure conditions, particularly eutrophication, soft coral was suggested to struggle to grow in acidification environment (Koop et al., 2001; Fleury, Coll, Sammarco, Tentori, & Duquesne, 2004; Januar, Zamani, Soedhadma, & Chasanah, 2016a). This may happen because of living space competition with few benthic coral, e.g. acroporid, poritid, and helioporid, that is shown to grow well in acidic marine environment (Anthony, Kline, Diaz-Pulido, Dove, & Hoegh-Guldberg, 2008; Fabricius et al., 2011; Crook, Potts, Rebolledo-Vieyra, Hernandez, & Paytan, 2012; Dunn, Sammarco, & LaFleur, 2012; Januar, Zamani, Soedhadma, & Chasanah, 2017a). The struggling of soft coral to grow reflects a clear trade-off between energy for growth and the production of allelochemical for living space competition in coral reef organism (Turon, Martí, & Uriz, 2009).

4. Conclusion

The present work suggests that in general, the tropical *Sarcophyton* soft coral genetic diversity or their production of diverse cytotoxic metabolite may be compromised due to ocean acidification adaptation. A genetic profiling of soft coral from natural CO₂ vent

marine environment will be a prospective study, to comprehensively predict the resistant soft coral genotype and chemotype on the increasing ocean acidification pressure at the future climate change.

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