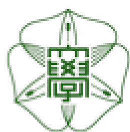
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Studies on new secondary metabolites from the marine cyanobacterium *Moorea bouillonii* collected in Sabah, Malaysia

Jakia Jerin Mehjabin

Introduction

Marine cyanobacteria are prolific producer of diverse secondary metabolites with intriguing bioactivities including cytotoxic, antiviral, antifungal, antifeedant, anti-inflammatory, antimicrobial, and antiproliferative activities. A large number of these nitrogen-containing metabolites with an enormously broad spectrum of pharmacological properties are mainly biosynthesized by non ribosomal peptide (NRP) or mixed polyketide-NRP enzymatic system. Having an increasing number of new secondary metabolites in recent decade, the marine cyanobacteria have become an attractive source for the isolation of novel compounds.

Marine cyanobacteria have gained attention from natural product chemists because of their magnificent biosynthetic capacity in producing novel secondary metabolites. The adaptation of marine cyanobacteria to extreme conditions could be attributed to the ecological significance of these compounds including nitrogen fixation, UV radiation protection, feeding deterrence, allelopathy and signaling. More than 550 secondary metabolites have been reported from different species of marine cyanobacteria including *Lyngbya*, *Moorea*, *Symploca* and *Oscillatoria* and many of them were mainly collected from Papua New Guinea, Guam, Palau and Palmyra Atoll. Filamentous marine cyanobacterium *Lyngbya bouillonii* was collected from Hansa Bay, Laing Island, Papua New Guinea and morphologically described in 1991. Later based on phylogenetic data, its taxonomy has been reclassified under the *Moorea* genus and named as *Moorea bouillonii* which had different colony morphologies with *Moorea producens* formally known as *Lyngbya majuscula/sordid*. According to MarinLit database total 42 compounds were reported from *M. bouillonii* and gaining attention as a prolific producer of novel secondary metabolites.

Marine ecosystem contains a broad scale of under discoverable biodiversity in terms of novel organisms as well as novel compounds which have made this system as an attractive bioresource. In particular, we are interested in marine cyanobacterium *M. bouillonii* because of the structurally diverse secondary metabolites with intriguing bioactivities isolated from this species. As the environmental factors influence the chemical profile of *Moorea* sp. and the probability of the presence of novel secondary metabolites in the unexplored localities of Asian region, our research interest is mainly focused on the isolation of new secondary metabolites with unique bioactivities from underexplored areas of Bornean coast in northern Sabah, Malaysia.

CHAPTER 1: LC-MS Profiling and Screening of Marine Cyanobacteria from Sabah, Malaysia

1.1. Material and Methods

1.1.1. 16S rRNA Gene Sequencing Analysis

A total 21 samples were collected from different location of Northern Bornean Coast, Sabah, Malaysia by SCUBA. A small amount of fresh cyanobacteria samples were preserved in the 8 mL RNA stabilization solution RNAlater® (Ambion, USA) in the 15 mL Falcon tube for identification. The marine samples were identified by molecular phylogenetic analysis based on 16S rRNA gene sequence data using cyanobacteria specific primers. Sequencing experiments were conducted using 3130xl Genetic Analyzer ABI PRISM 3100 (Hitachi, Japan). The Phylogenetic analysis of all nucleotide sequence of 16s rRNA gene was conducted using Mega 7 software.

1.1.2. Chemical Profiling of Crude Extracts by LC-MS

Samples preserved in MeOH were homogenized again with MeOH after arriving in Japan and solution was vacuum filtered through a filter paper. The combined MeOH extracts were then partitioned with ethyl acetate (EtOAc), *n*-butanol (BuOH), and water (H_2O). The chemical profile of each ethyl acetate (EtOAc), *n*-butanol (BuOH), and water (H_2O) fraction were analyzed by LC-MS at concentration of 100 µg/mL. LC-MS analyses were conducted on an Agilent 1100 Series HPLC system coupled with a Bruker Daltonics micrOTOF-HS mass spectrometer (ESI). The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 µm, 25 °C, 0.2 mL/min) under the following conditions: 0 - 15 min, gradient elution of 50 - 80% MeCN with 0.1% (v/v) formic acid in Milli-Q H_2O; 15 - 30 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in Milli-Q water.

1.1.3. Biosurfactant and Cytotoxicity Assay Screening

The biosurfactant properties of crude extracts were measured by determining the diameter of the oil displacement area. The crude oil used in this experiment was manufactured by Tokyo Chemical Industry Co., LTD (S0432).

Standard MTT assay was performed to check the cytotoxicity against human MCF7 breast cancer cell line. The cellular viability was evaluated by the reduction of the 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) a yellow tetrazole soluble in water, to purple formazan crystals that are insoluble in water.

1.2. Results

1.2.1. Identification of Marine Cyanobacteria

Among the 13 RNA*later*® preserved samples, the 16S rRNA gene sequence of nine samples were successfully acquired. These sequence data were used to construct the phylogenetic tree along with type and reference strain sequences from the NCBI Nucleotide database. Samples labeled as M1620 was identified as *Moorea producens* while two samples labeled as M1622 and M1629 were identified as *Moorea bouillonii*. The tuft forming cyanobacteria M1621 was identified as *Symploca* or *Caldora* sp. and M1623 was identified as *Okeania* sp. The sample labeled as M1624 was identified as uncultured bacterium clone. Unfortunately, DNA couldn't be retrieved from M1625. The samples (M1701, M1704, and M1705) collected near Mantanani Island in November 2017 were successfully identified as *Moorea* sp.

1.2.2. Chemical Profiling and Bioactivity of Crude Extracts

The chemical profiles of the EtOAc fractions from *M. bouillonii* samples showed the richness of known secondary metabolites like the apratoxins, wewakazoles, lyngbyabellins, laingolides, madangolide, kanamienamide with a number of unknown halogenated compounds based on MarinLit database search results. Halogenated compounds were abundant in sample M1629 and M1705 collected from Udar and Mantanani island, respectively compared to other *M. bouillonii* samples. The possible new halogenated compounds with *m/z* 878, 935, 967, 910, 418, 432, 434, 494, 528 were detected with other unknown compounds with *m/z* 359, 406, 472, 364, 507, 737 in *M. bouillonii* samples.

Each fraction (Ethyl acetate, butanol and water) from solvent partition results were tested for the biosurfactant activity. The oil displacement results showed that the ethyl acetate fraction had the potent activity to remove the crude oil by oil displacement assay. The EtOAc fractions of all collected samples were checked for the cytotoxicity against MCF-7 cancer cell lines. Highest numbers of collected samples were identified as *M. bouillonii* and showed potent cytotoxicity at concentration 10 and 1 μg/ mL.

1.3. Discussion

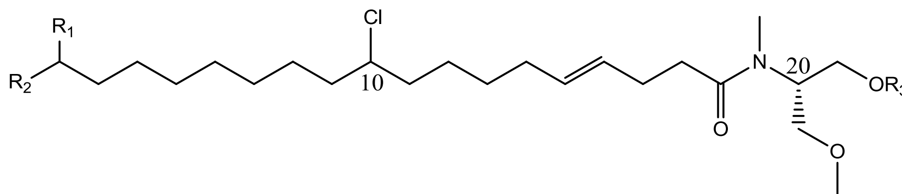
Nine samples were successfully identified based on their morphology and 16S rRNA gene sequence analysis. The LC-MS profiles of *M. bouillonii* samples showed the richness of secondary metabolites. The screening results of the EtOAc fractions showed the higher oil displacement area compared to other fractions because of the presence of surface active compounds in these fractions. Most of the *M. bouillonii* samples showed cytotoxicities. These toxicities may results from the apratoxins, lyngbyabellins or unknown compounds. Among them M1705 and M1629 were selected for the isolation of new compounds based on the screening results and amount of sample.

CHAPTER 2: Isolation and Structure Elucidation of Columbamide F-H from *Moorea bouillonii*

2.1. Material and Methods

2.1.1. Isolation

The MeOH extract of *M. bouillonii* (M1705) was partitioned between EtOAc and H_2O, and then *n*BuOH. The EtOAc extract (600 mg) was then applied onto a silica gel (Merck) column with the size 0.063-0.200 mm and eluted with a stepwise gradient starting with 100% *n*-hexane to 100% EtOAc (98:2, 95:5, 90:10, 80:20, 50:50-v/v) followed by 100% EtOAc to 100% MeOH (95:5, 90:10, 80:20, 50:50) to yield 12 major fractions (F1 to F12). The 50:50 (v/v) hexane-EtOAc and 100% EtOAc fraction gave columbamide F (**1**) (3.2 mg), G (**2**) (2 mg) and columbamide H (**3**) (2 mg).



Columbamide F (**1**) $C_{25}H_{45}Cl_2NO_4$ (R_1=H, R_2=Cl, R_3=Ac)
Columbamide G (**2**) $C_{25}H_{44}Cl_3NO_4$ (R_1=Cl, R_2=Cl, R_3=Ac)
Columbamide H (**3**) $C_{23}H_{44}ClNO_3$ (R_1=H, R_2=H, R_3=H)

2.1.2 Structure Elucidation

The structure elucidations of columbamide F-H (**1-3**) was done by using combination of MS and NMR techniques. The ^{1}H NMR, ^{13}C NMR and 2D NMR spectra for compounds **1**, **2** and **3** were acquired using a JEOL 600 MHz spectrometer. Non-decoupled HSQC analysis was carried out to determine the configuration of double bond for columbamides. The synthesis of *N,O*-dimethylserinol was carried out and the absolute configurations were solved by using Marfey's analysis of synthetic standards and hydrolysates of **1-3**. The absolute configuration of chloromethine of columbamide F (**1**) was determined by chemical degradation and derivatization using chiral labeling reagent from Ohrui-Akasaka method.

2.1.3 Biosurfactant Properties

The biosurfactant properties of pure compounds were studied using oil displacement assay (Chapter 1) and ring method. The surface tension of compounds were determined to measure the critical micelle concentration (CMC) by using Du Noüy tensiometer (ITOH, No.4334) equipped with a platinum ring at 25 ºC .

2.2. Results

2.2.1 Structure Elucidation

The non-decoupled HSQC analysis revealed the *E*-geometry for the olefins (coupling constant, $^3J_{H,H=}$ 16-17 Hz). The absolute configuration of *N,O*-dimethylserinol moiety was determined by Marfey's nalysis. The LC-MS retention time of the hydrolysates of **1**-**3** were matched with synthetic (*R*)-*N,O*-dimethylserinol moiety indicating the *R*-configuration. The absolute configuration of chloromethine on the long alkyl chain of columbamide F (**1**) was determined by chiral-phase HPLC analysis after conversion and esterification with Ohrui's acid, (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanecarboxylic acid. The coelution experiment revealed that ester from synthetic 10*R* standard and natural **1** gave a single peak, while ester from synthetic 10*S* standard and natural **1** was divided into two peaks that indicated the *R*-configuration for the chloromethine at position C-10.

2.2.2 Biosurfactant Properties

The surfactant properties of columbamide F (**1**) was measured using oil displacement assay. The diameter of the clear zone was measured as ~ 90 mm for the concentration of 10 mg/mL which is a slightly higher displacement area compared to SDS (~ 84 mm) and pluronic F-68 (~54 mm) for the same concentration. The related fatty acid amides columbamide D and serinolamide C were also checked for the surfactant properties and showed surfactant properties in oil displacement assay (~110 mm and ~54 mm, respectively) and ring method by lowering the surface tension of water (CMC value=0.34 mM and 0.78 mM, respectively).

2.3 Discussion

New fatty acid amide columbamide F (**1**) along with related fatty acid amides columbamide D and serinolamide C showed biosurfactant properties. The absolute configuration of *N,O*-dimethylserinol moiety of columbamide F-H (**1**-**3**) were determined by acid hydrolysis and Marfey's analysis. The absolute configuration of chloromethine in the long alkyl chain of columbamide F (**1**) was determined by Ohrui-Akasaka method following chiral phase HPLC analysis at room temperature, which increases the potential of chiral labeling reagent to determine the configuration of natural compounds having a remote stereogenic center.

CHAPTER 3: Isolation and Structure Elucidation of New Madangolide from *Moorea bouillonii*

3.1. Material and Methods

The LC-MS analysis of lipophilic fraction exhibited the possible new compound. The EtOAc fraction was subjected to silica gel column chromatography using the gradient solvent hexane, EtOAc and MeOH. The ^{1}H NMR, ^{13}C NMR and 2D NMR spectra were acquired using a JEOL 600 MHz spectrometer. For all NMR experiments, $CDCl_3$ (Cambridge Isotope Laboratories, Inc.) was used as solvent as an internal standard (δ_H 7.26 and δ_C 77.0). LC-MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer.

3.2 Results

The LC-MS analysis of hexane/EtOAc 80:20 fraction of *M. bouillonii* sample (M1629) revealed the presence of several known compounds like madangolide and laingolides with unknown compound based on MarinLit database. The compound was then isolated by RP-HPLC. Seventeen and fifteen membered macrolides derivatives, madangolide and laingolides were also purified and confirmed by comparing their ^{1}H NMR chemical shift data. The cyanobacterial macrolides hoiamide A, alotamide A, and palmyrolide A has proven for suppression or activation of spontaneous Ca^{2+} oscillations of murine cerebrocortical neurons.

3.3 Discussions

A small group of macrolides having trans-*N*-methyl enamide with *tert*-butyl carbinol moiety were found in marine cyanobacteria including laingolide, laingolide A, madangolide, and laingolide B. Madangolide was reported from *M. bouillonii* more than 10 years back. A new madangolide was isolated from hexane/EtOAc 80:20 fraction and the planar structure was established by combination of NMR spectroscopic and mass spectrometry analysis.

General Discussion

In this study we isolated three new columbamides F-H from *M. bouillonii* sample collected from Mantanani island, Sabah, Malaysia. The absolute configuration of chloromethine was not determined for columbamide A-C. In case of columbamide D, the absolute configuration of chloromethine was determined by total synthesis of all diastereomers and comparison of retention time by chiral HPLC column. In our study, the challenge of determining the absolute configuration of chloromethine for columbamide F was solved by using the potent chiral labeling reagent from Ohrui-Akasaka method. The chemical degradation and derivatization with Ohrui's acid was followed for both standards and natural columbamide F. Then by chiral phase HPLC analysis the configuration was determined. This result suggests that Ohrui method with chiral-phase HPLC analysis can be a powerful approach to determine the absolute configuration of natural compounds having remote chiral center.

Other fatty acid amides like serinolamide A, mooreamide and columbamide A-B showed cannabinomimetic properties. Columbamide D was checked for the cytotoxicity and was found inactive. For columbamide F, the biosurfactant properties of this compound was checked by oil displacement assay which is the indirect measurement of surface activities and found that columbamide F can displace the oil from the surface of water. Then it was decided to check the biosurfactant activity of columbamide D and serinolamide C by measuring the critical micelle concentration (CMC) value. Biosurfactants have gained attention in recent years and possess similar or enhanced performance compared to synthetic surfactant. For example, kaneka surfactin is widely used as cosmetic ingredient since 2001 showed low critical micelle concentration, easy biodegradation and less irritation than synthetic surfactant SDS. The CMC value of columbamide D and serinolamide C showed comparable value with synthetic surfactants. These results revealed the biosurfactant properties of fatty acid amides and increase their application in different fields.

A new madangolide was found in a few samples of *M. bouillonii* collected in Udar island and appeared as minor compound. The planar structure and preliminary screening of anti-allergic activity were done for related madangolide and laingolide.

The filamentous marine cyanobacteria *M. bouillonii* is frequently found in the Papua New Guinea, Guam, Palau, Palmyra atoll etc., French Polynesia, Malaysia and Japan. Although the excessive growth of this species due to the climate change and anthropogenic activities suppress the growth of coral and cause various diseases, careful steps should be taken to reduce the bloom from *M. bouillonii.* Finally, this study revealed the potentials of *M. bouillonii* samples for isolating novel secondary metabolites from Sabah, Malaysia.