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New secondary metabolites from marine cyanobacteria of the genus *Moorea* collected in Malaysia and Saudi Arabia

(マレーシアおよびサウジアラビア由来海洋ラン藻*Moorea*属から得られた新規2次代謝産物)

by Julius Adam Velasco Lopez

Marine cyanobacteria have been a consistent source of significant bioactive compounds with novel structures. For example, over a hundred compounds have been discovered from *Moorea producens* and *Moorea bouillonii* (formerly *Lyngbya majuscula* and *Lyngbya bouillonii*, respectively). Nonetheless, there are still areas, such as the Red Sea in Saudi Arabia and the northern Bornean coast of Sabah Malaysia, where information about marine cyanobacteria and their chemistry is limited. Moreover, it is also recognized that environmental factors may influence the secondary metabolite production of marine cyanobacteria. Therefore, it is hypothesized that there is a huge potential of finding novel compounds from collections done in these underexplored areas. Thus, the aim of this study is to isolate and elucidate the structure of novel bioactive compounds from marine cyanobacteria collected from Malaysia and Saudi Arabia. In addition, by doing marine natural products research, we gain insight into the chemistry of the subject organisms and their communities, as well as their ecological significance.

A total of 44 marine cyanobacterial samples from Malaysia and Saudi Arabia were collected by scuba at a depth of 4-20 m. A small portion of the sample was preserved in RNAlater[®] for DNA extraction. Identification was carried out by 16S rRNA gene sequencing, morphological, and phylogenetic analysis. Cyanobacteria-specific primers were used for polymerase chain reaction (PCR) experiments on a Takara PCR thermal cycler. Sequencing experiments were performed on a Hitachi 3130xl Genetic Analyzer. Phylogenetic analyses were conducted using the MEGA6 software. Nine samples from Saudi Arabia were identified as *Moorea producens* (2), *Moorea bouillonii* (1),

Symploca sp. (1), *Trichodesmium erythraeum* (1), and *Okeania* sp. (1) or related to *Okeania* sp. (3), while 11 samples from Malaysia were found to be *Caldora penicillata* (1) and *M. bouillonii* (10).

Bulk of the samples were soaked in MeOH and transported to the laboratory. A small portion of the samples were also immersed in seawater BG-11 medium for culturing. The cyanobacteria were homogenized and further extracted with MeOH. The MeOH extracts were combined, evaporated *in vacuo*, and partitioned between EtOAc, H₂O, and BuOH. These extracts were dried and screened in a cytotoxicity assay and subjected to chemical profiling by using liquid chromatography-mass spectrometry (LC/MS).

The cytotoxicity assay was performed by exposing human MCF7 breast cancer cells to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and measuring the absorbance of the formazan product at 570 nm with a Thermo Labsystems Multiskan JX instrument. Eleven of the identified samples showed potent cytotoxicity at 1 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 x 150 mm). The mass data were tabulated and the dereplication of known compounds was performed by using the MarinLit database. Known compounds such as apratoxins, curacin D, dolastatins, and lyngbyabellins were identified by high resolution mass spectrometry (HRMS) data. Accordingly, samples with unique mass data were marked as potential sources of new compounds. Summarizing the results of the 16S rRNA gene sequencing, cytotoxicity screening, and chemical profiling, two samples were selected for the isolation of new compounds. These two samples were M1407 (*M. bouillonii*) from Malaysia and S1301 (*M. producens*) from Saudi Arabia.

The lipophilic fraction of M1407 exhibited di- and trichlorinated compounds in its mass spectrum. This was subjected to silica gel column chromatography eluting with a stepwise gradient composed of hexane and EtOAc, followed by EtOAc and MeOH to obtain ten fractions. The 100% EtOAc fraction was further subjected to ODS (YMC Gel ODS-A) column chromatography eluting with a stepwise gradient of MeCN and H₂O, followed by MeOH and DCM to obtain seven fractions. The 60% MeCN fraction was purified using semi-preparative reversed-phase (RP) HPLC (Cosmosil Cholesterol, 10 × 250 mm) to obtain the known cyclic peptide, wewakazole. The 80% MeCN fraction was also purified using RP HPLC to yield the highly cytotoxic apratoxins, apratoxin A and C, and two new acyl amides, columbamides D and E, which are di- and trichlorinated, respectively.

The structures were elucidated by using a combination of MS and nuclear magnetic resonance (NMR) techniques. NMR data were recorded on JEOL ECA600 and JEOL EX400 spectrometers. The molecular formulas were deduced based on HRMS data. The 2D NMR data were used to assemble the structure. In particular, the heteronuclear 2-bond correlation (H2BC) technique was useful in positioning the chloromethine in the long chain alkyl group. Meanwhile, nondecoupled heteronuclear single quantum correlation (HSQC) data revealed the *E* geometry of the olefin (³*J*_{H,H} = 16 Hz). The partial synthesis of the *N,O*-dimethylserinol was carried out and the synthetic products were used in Marfey's analysis to conclude an *R* configuration. Subsequently, total synthesis of two diastereomers (10*S*,20*R* and 10*S*,20*S*) of columbamide D were achieved. By comparison of the ¹H and ¹³C NMR chemical shifts and results of the Marfey's analysis of the synthetic versus the natural compounds, columbamides D and E were concluded to have the 10*S*,20*R* configuration.

The second sample, S1301, was processed accordingly. A possible new compound with [M+H]⁺ *m/z* 1127 was found in the EtOAc fraction and was targeted for purification. The sample was

subjected to silica gel column chromatography eluting with a stepwise gradient composed of hexane and EtOAc, followed by EtOAc and MeOH to obtain ten fractions. The 75:25 EtOAc:MeOH fraction was subjected to semi-preparative RP HPLC (Cosmosil C18-AR, 10 × 250 mm). The fraction that eluted at 18 min was further purified by RP HPLC (Cosmosil C18-MS, 4.6 × 250 mm) to yield pure wewakazole B (0.4 mg). Four other samples (SH92, SH95, SH97-98) having similar chemical profiles were combined and subjected to the same isolation scheme to yield an additional 0.2 mg of wewakazole B. This process also resulted in the successful isolation of the known cytotoxic compound, curacin D.

The structure elucidation was carried out by a combination of NMR, MS, and MS/MS techniques. MS/MS fragmentation data were acquired using Thermo Scientific LTQ Orbitrap XL (ESI) and AB Sciex TOF/TOF 5800 (MALDI) instruments. The molecular formula was deduced based on HRMS data. Nine common and three modified amino acid residues were assembled from the COSY, TOCSY, HSQC, and HMBC data. In addition, using the same 2D NMR data plus ROESY data, five fragments were constructed. However, connections across these five fragments could not be established using the existing data. Nonetheless, all possible combinations were considered and a cyclic structure was realized. The cyclic structure was supported by the number of unsaturations and MS/MS data.

After ozonolysis and acid hydrolysis, the absolute configurations of the amino acid residues were determined by chiral-phase LC/MS and HPLC analyses. Chiral-phase (Supelco Chirobitotic TAG, 2.1 x 250 mm) LC/MS revealed that the Ala, Phe and Pro residues were in the L-configuration. However, this technique failed to distinguish between L-Ile and L-*allo*-Ile; therefore, a different chiral-phase column (Sumichiral OA-5000, 3.6 x 150 mm) was required to separate them in conjunction with a co-elution experiment. This technique clearly identified that wewakazole B

contained L-Ile. Wewakazole B was cytotoxic to MCF7 cancer cells ($IC_{50} = 0.58 \mu M$) and to human H460 lung cancer cells ($IC_{50} = 1.0 \mu M$). It was also found to be inactive in a siderophore assay in contrast to a previous computational study that showed its analogue, wewakazole, as a potential iron-binding compound.

By using an LC/MS-based approach, the chemical profiles of different marine cyanobacterial samples from Malaysia and Saudi Arabia were analyzed against the MarinLit database. This strategy efficiently dereplicated several known compounds such as apratoxins, dolastatins, and lyngbyabellins. Thus, the investigator was able to save time and resources by having clear targets and focusing on them through MS. This led to the successful isolation of three new compounds. The results also show the potential of marine cyanobacteria as a source of cytotoxic compounds. The wewakazoles showed moderate cytotoxicity, while the synthesized columbamide D stereoisomers were inactive. The high cytotoxicity observed in the Malaysian *M. bouillonii* was most likely due to the apratoxins, which are cytotoxic at nanomolar levels. This study was also able to confirm that the wewakazoles are not siderophores as previously predicted.

Furthermore, the 16S rRNA gene and MS data showed the difference in cyanobacteria biodiversity between the Red Sea and South China Sea. It is noteworthy that even without 16S rRNA gene data, the samples could be differentiated based on their chemical profiles. The compounds found in a certain species may serve as chemotaxonomic markers for easy identification. Finally, it is concluded that the genus *Moorea* remains to be a promising source of new bioactive compounds and an attractive subject for drug discovery.