# Isolation and structure elucidation of novel cyanobacterial secondary metabolites using OSMAC approach

# OSMAC 法を用いた藍藻由来新規二次代謝産物の単離と構造決定

Nandani Balloo

## Introduction

Cyanobacteria are photosynthetic gram-negative prokaryotes that have colonized diverse habitats. They have been successfully mined for natural product derived drug candidates. One such drug currently on the market and used in cancer treatments is Brentuximab vedotin which was developed from dolastatin 10 isolated from the cyanobacterium *Symploca hydnoides*. In the past decades, the issue of redundancy in natural product research has become a concern with the repeated isolation and characterization of natural products that have already been reported in the literature. Moreover, with the advent of genome sequencing, a huge inconsistency has been found between the number of secondary metabolites biosynthetic gene clusters encoded in a particular strain and the number of metabolites produced by that strain. This indicates that in typical laboratory conditions, a significant number of gene clusters are not transcribed, suggesting a considerable amount of untapped chemical potential. One reason for this is that the optimal conditions for the production of secondary metabolites are not essentially identical to those for growth. In their natural environment, microorganisms thrive in ecosystems together with other species and ever-changing physical parameters. Hence, the need to protect itself against the changing conditions, predation and competition for the same resources giving rise to signaling and cross-feeding between organisms that will induce the biosynthesis of novel metabolites.

New strategies have been devised to identify and characterize the chemical diversity of these microorganisms. One such strategy is the OSMAC (One Strain Many Compounds) approach, which comes from the theory that a single strain is able to produce many compounds in different growth conditions to access the cryptic metabolic pathways that are otherwise silent. Culture conditions can significantly impact the production of natural products resulting in either upregulation or downregulation of certain compounds or even the production of new metabolites through the activation of cryptic biosynthetic pathways. Many studies have reported the impact of cyanobacterial culture conditions with various objectives: to increase biomass, lipid content,

improve production of bioactive compounds or to investigate the ecology and physiology of strains related to harmful algal blooms. OSMAC approaches have effectively demonstrated that cyanobacteria have the capacity to modulate their metabolite profiles based on environmental cues. However, despite the success of these methods in exploring the adaptability of cyanobacteria, there remains a scarcity of published literature focused specifically on the discovery of novel metabolites using the OSMAC approach in cyanobacterial research. The overall aim of the study is to isolate and elucidate the structure of new metabolites from cyanobacterial species from the NIES collection and to induce the production of new metabolites using the OSMAC approach.

## Chapter 1: Screening for new metabolites using the OSMAC approach

The objective of this first chapter is the chemical profiling of cyanobacterial strains grown in different conditions using the OSMAC approach and genome mining.

In order to induce the production of new metabolites from cyanobacteria, a selected number of strains were cultured in different conditions by changing one parameter at a time while keeping the other parameters identical (except when different media was used). From a 200 mL 7-day old pre-culture of the strain, 1.0-1.2 x $10^5$ cells/mL were transferred to 500 mL of medium in the appropriate condition in triplicates. The control group was maintained in BG-11 medium at a pH of 7.5 and a temperature of 25 °C. Growth of the cyanobacterial strains was monitored every 2 days by counting the cells using a hemocytometer. After 7-10 days, the culture medium was centrifuged, and intracellular metabolites extracted for chemical profiling by LC-MS. The 500 mL culture was centrifuged, and the resulting pellet was extracted with 80% MeOH. The crude extract was evaporated to dryness and dissolved in 2 mL of 20% methanol solution. Solid phase extraction (SPE) was used to prepare the crude extract for chemical profiling. The sample was dissolved in 50 % MecN and was analyzed by LC-MS. LC-MS was conducted on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer equipped with a Cadenza CD-C18 column (2 mm × 150 mm, flow rate 0.2 mL/min at 25 °C). The mobile phase conditions were: 0−30 min 20−80% (*v*/*v*) acetonitrile with 0.1% (*v*/*v*) formic acid in Milli-Q water; 30−45 min, isocratic 80% (*v*/*v*) MeCN in Milli-Q water with 0.1% (*v*/*v*) formic acid. Injection volume was 5 µL and MS analysis was in wide mode in the range of 50 – 3,000 *m/z*. All *m/z* obtained in chemical profiles were cross referenced with online databases such as the 'Natural Products Atlas (npatlas) and 'MarinLit' for identification.

When *M. aeruginosa* NIES-88 was grown in higher temperatures of 30 °C and 37 °C, an increased production of a previously unreported metabolite having *m/z* 922 was observed (Figure 1) with the most pronounced effect being at 37 °C. While carefully analyzing the chemical profile of *M. aeruginosa*, two more unknown compounds with *m/z* 1058 and *m/z* 990 were found. When *M. aeruginosa* NIES-88 was grown in iron limited BG-11 medium, the chemical profile showed the production of a new compound with *m/z* 1074 (Figure 2).

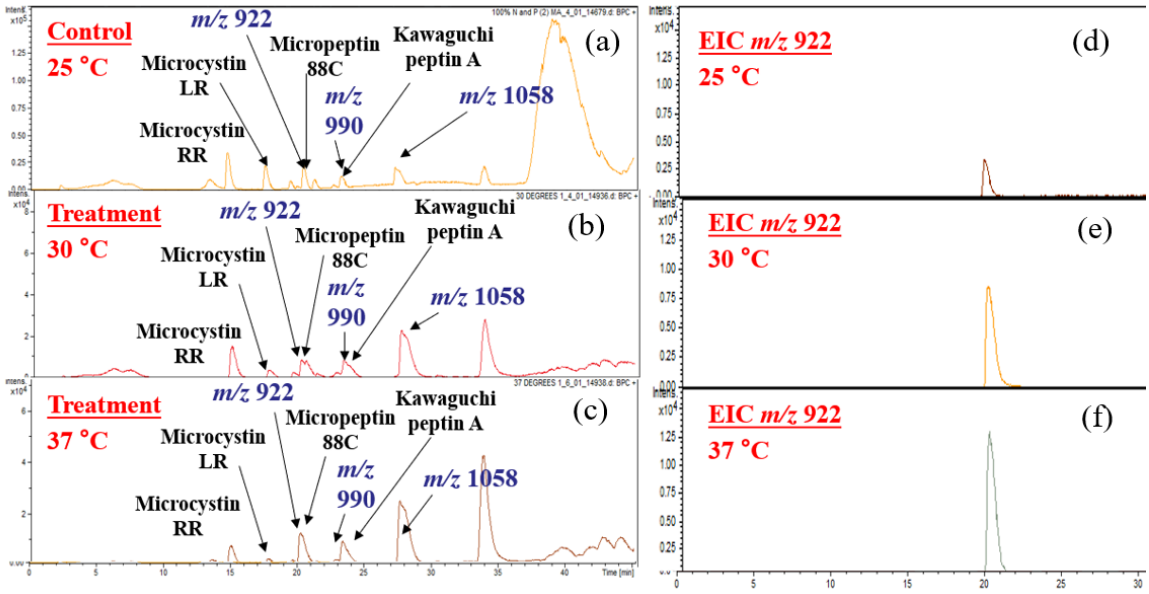


Figure 1: LC-MS chromatograms of *M. aeruginosa* NIES-88 grown in BG-11 medium at (a) 25 °C, (b) 30 °C and (c) 37 °C. Extracted ion chromatogram (EIC) of *m/z* 922 of LC-MS chromatograms of *M. aeruginosa* grown in BG-11 medium at (d) 25 °C, (e) 30 °C and (f) 37 °C.

Based on the screening results, the study was directed towards exploring the impact of high temperature on a large-scale culture of *M. aeruginosa* NIES-88. Subsequently, specific metabolites were selected for isolation and structure elucidation. These included the *m/z* 922 (compound C), *m/z* 990 (compound B), and *m/z* 1058 (compound C) detected from the culture of *M. aeruginosa* NIES-88, as well as the *m/z* 1074 which was produced only under limited iron conditions. Lastly, the isolation, structure elucidation and configuration of a new compound produced by the cyanobacterium *T. bourrellyi* was also targeted.

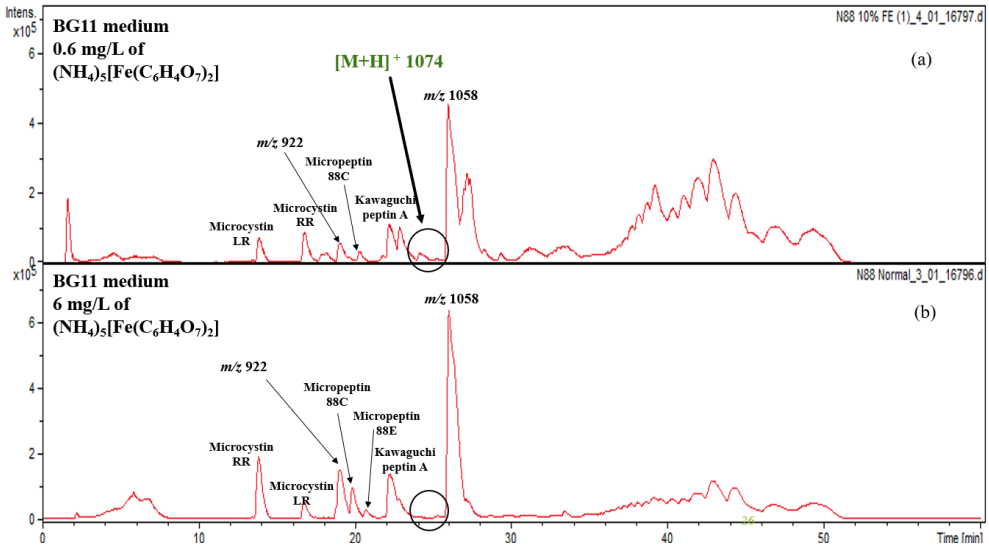


Figure 2: Chromatogram of crude extracts of *M. aeruginosa* grown in (a) BG-11 medium with 0.6 mg/L of $(NH_4)_5[Fe(C_6H_4O_7)_2]$ (b) BG-11 medium with 6 mg/L of $(NH_4)_5[Fe(C_6H_4O_7)_2]$.

## Chapter 2: Effect of high temperature on the metabolic profile of *Microcystis aeruginosa* NIES-88

The objective of this chapter is to determine how the production of secondary metabolites produced by *M. aeruginosa* changes in function of time when exposed to a temperature of 37 °C.

A temperature of 37 °C was chosen for further upscaling experiments as it showed the most pronounced effect based on screening results. 1.0 – 1.2 x $10^5$ cells/mL from 500 mL culture was inoculated in 10L glass bottles. The control conditions were BG-11 medium supplemented with continuous aeration with filtered air (0.3 L/min) at 25 °C under illumination of 80 µE/$m^2$s on a 12L:12D. The heat shock treatment consisted of *M. aeruginosa* NIES-88 (hereafter, *M. aeruginosa*) grown in BG-11 medium aerated with filtered air at 0.3 L/min at 37 °C under illumination of 80 µE/$m^2$s on a 12L:12D cycle. All experiments were conducted in triplicates and lasted for 38 days. Every 3-7 days, 40 mL of the cyanobacterial culture was collected aseptically, cells were counted, and the sample centrifuged at 10,000 rpm at 4°C. The supernatant was discarded, and the pellet stored at -30°C until further analysis. Metabolites were extracted with methanol by sonication for 20 min. The extract was filtered with a 0.45 µm syringe filter, evaporated to dryness and SPE was used for LC-MS sample preparation. The dried extract from the SPE step was then reconstituted with 1 mL of 50% MeCN (*v*/*v*) in milli-Q water prior to LC-MS analysis.

At higher temperatures, production of microcystin-LR, microcystin-RR and micropeptins 88C-E are declined by at least 6-fold. The lowest concentration of microcystins and micropeptins were found at the end of the stationary phase. Production of compound A (*m/z* 1058), B (*m/z* 990) and kawaguchipeptin A seems to vary throughout the experiment with compound B showing the least change. Day 14 and 30 seem to favor the production of compound A at 37 °C while on the other days there is a slight reduction in their production at 37°C. A 2-fold increase in kawaguchipeptin A was observed on day 14 and a subsequent decrease in its production at the end of the growth cycle. On the other hand, the production of compound C (*m/z* 922) seems to be significantly favored at 37 °C. Production of compound C during the exponential phase of *M. aeruginosa* seems to be the highest. The highest production showed a 10-fold increase on day 14. This provided the first indication that the metabolic signature of *M. aeruginosa* was highly dependent on incubation temperatures.

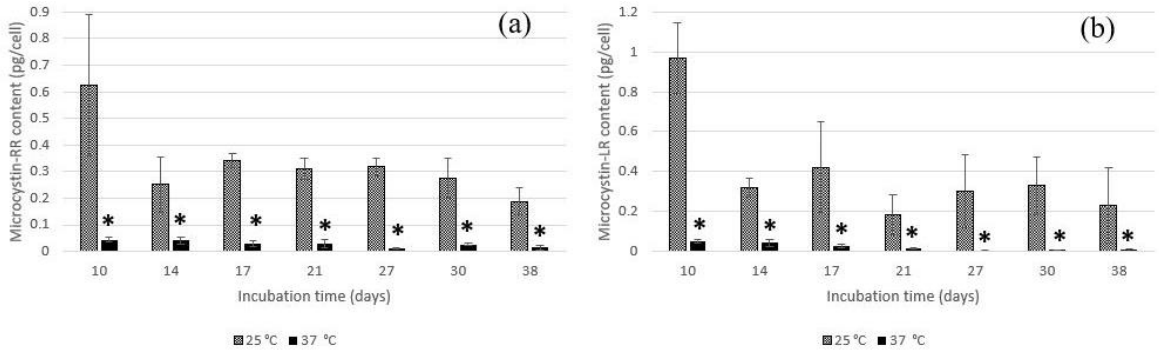


Figure 3: Production of (a) microcystin-LR and (b) microcystin-RR by *M. aeruginosa* at 25 °C and 37 °C. Asterisk on each plot denotes a significant difference between groups ($p < 0.05$).

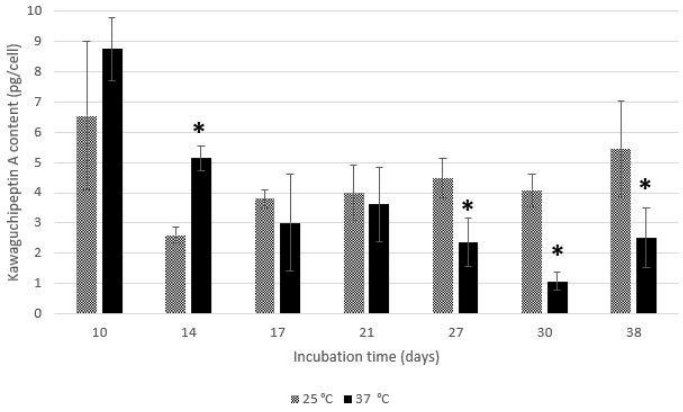


Figure 4: Production of kawaguchipeptin A by *M. aeruginosa* at 25 °C and 37 °C. Asterisk on each plot denotes a significant difference between groups ($p < 0.05$).

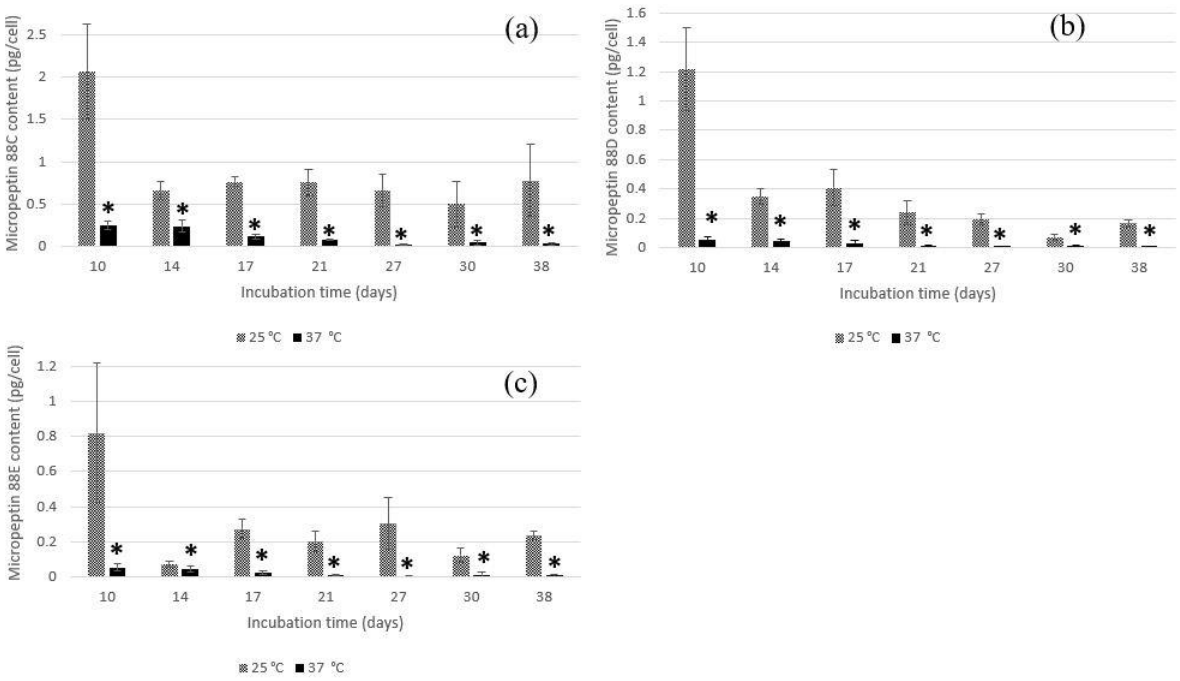


Figure 5: Production of (a) micropeptin 88C, (b) micropeptin 88D and (c) micropeptin 88E by *M. aeruginosa* at 25 °C and 37 °C. Asterisk on each plot denotes a significant difference between groups ($p < 0.05$).

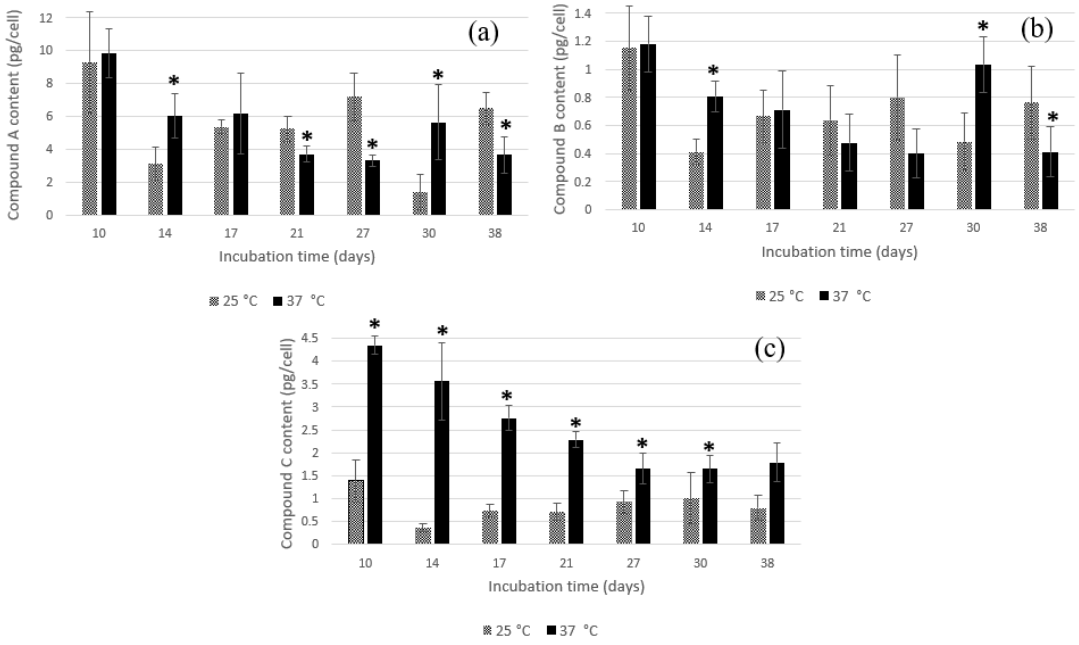


Figure 6: Production of (a) compound A, (b) compound B and (c) compound C by *M. aeruginosa* at 25 °C and 37 °C. Asterisk on each plot denotes a significant difference between groups ($p < 0.05$).

## Chapter 3: Isolation and structure elucidation of argicyclamides A-D from *Microcystis aeruginosa* NIES-88

The objectives of this chapter are the isolation, structure elucidation and configuration of (i) argicyclamides A-C from *M. aeruginosa* NIES-88 grown in BG-11 medium and (ii) argicyclamide D from *M. aeruginosa* culture in modified iron limited BG-11 medium.

80 L of *M. aeruginosa* was cultured in BG-11 medium with aeration (filtered air, 0.3 L/min) at 25 °C under illumination of 80 μE/$m^2$s on a 12L:12D cycle. After 1 month the culture was centrifuged at 10,000 rpm to separate the cells from the medium. 7.8 g of freeze-dried cells was homogenized three times with methanol for metabolites extraction. The crude extract (2.2 g) was evaporated to dryness and then fractionated with water and ethyl acetate. The water fraction (0.98 g) was further fractionated by ODS open column chromatography and eluted with a stepwise gradient from 20% to 100% MeOH. After each fractionation step, the sample was analyzed with LC-MS. The 100% MeOH fraction (124 mg) was subjected to reverse phase HPLC using an isocratic condition of 60% MeCN supplemented with 0.05% trifluoroacetic acid with Wakosil AR 5C18 (250mm x 20mm) column, a flow rate of 4 mL/min and UV detection 215 nm to yield 3.1 mg of argicyclamide A, 1.5 mg of argicyclamide B and 2.2 mg of argicyclamide C.

For the iron limitation experiments, *M. aeruginosa* was first transferred to 500 mL of $(NH_4)_5[Fe(C_6H_4O_7)_2]$ free BG-11 medium for 3 days and then inoculated at a concentration of 1.0 – 1.2 x $10^5$ cells/mL to 400 L (40 × 10 L) of iron limited BG-11 medium containing only 0.6 mg/mL $(NH_4)_5[Fe(C_6H_4O_7)_2]$ supplemented with aeration with filtered air (0.3 L/min) at 25 °C under illumination of 80 μE/$m^2$s on a 12L:12D cycle. Cells were counted every 3-7 days using a hemocytometer to monitor growth rate. After 5 weeks of fermentation time, the cells were separated from the medium by centrifugation at 10,000 rpm. *M. aeruginosa* freeze-dried cells (15.5 g) from 400 L of culture was homogenized at 10,000 rpm for 15 min and extracted with 80 % MeOH (250 mL × 3). The extract was then dried in vacuo and partitioned between EtOAc and $H_2O$. The $H_2O$ fraction (1.5 g) was subjected to open column chromatography with a stepwise gradient elution starting with aqueous 20% MeOH to 100% MeOH. The 100% MeOH (104 mg) fraction was further purified by reverse phase HPLC (Wakosil II 5C18 AR, 20 x 250 mm, UV detection 215 nm, flow rate 4.0 mL/min) and running condition of 60% MeCN with 0.1% trifluoracetic acid to yield 0.9 mg of argicyclamide D.

Structure elucidation of the purified compounds was done with a combination of spectroscopic methods. Compounds A-C, named argicyclamides A-C, respectively, were octapeptides comprised of only L-amino acids with varying degrees of prenylation on the guanidine moiety. Argicyclamide A, the bis-prenylated compound, had good antibacterial activity against Gram-positive with MIC of 6.25 µM including the MRSA strain which is responsible for the healthcare-associated infections worldwide and livestock infections.

When *M. aeruginosa* was grown in iron limited BG-11 medium, a new argicyclamide analog was induced which was not found in any other culture conditions. *M. aeruginosa* culture was then upscaled to target its isolation and 0.9 mg of argicyclamide D was isolated. $^{1}$H NMR and tandem mass spectrometry data showed much similarity to previously isolated argicyclamides. Advanced Marfey's analysis revealed the presence of only L-amino acids similar to argicyclamides A-C. Argicyclamide D was proposed to comprise of the same macrocyclic structure as argicyclamide A with bis-prenylation on the arginine residue with an $N^{\delta}$ hydroxy group. Argicyclamide D showed negligible antibacterial or siderophore activity.

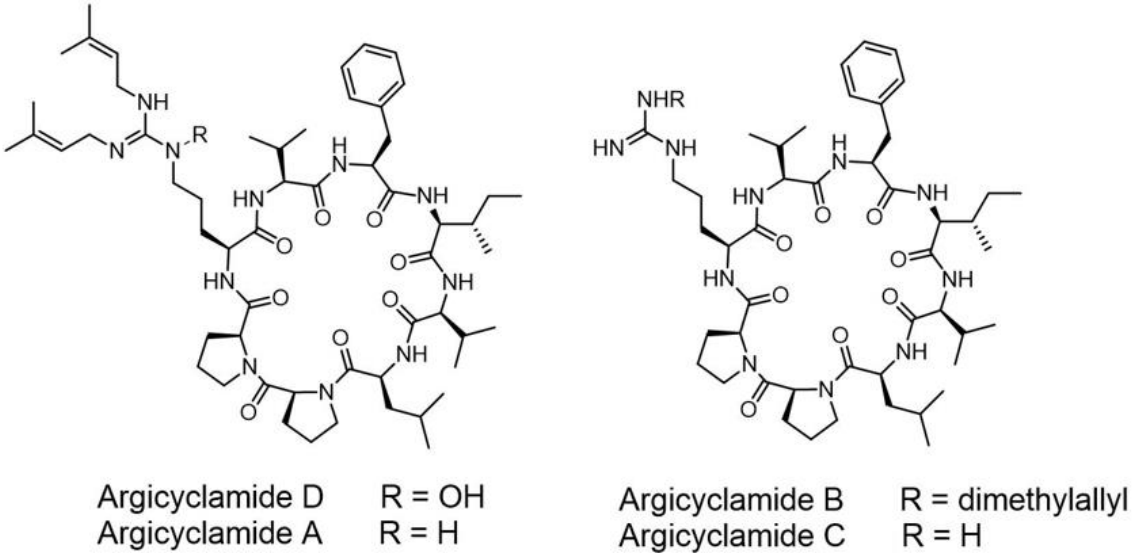


Figure 7: Structures of argicyclamides A-C and proposed structure of argicyclamide D

## Chapter 4: Isolation and structure elucidation of a new compound from the cyanobacterium *Tychonema bourrellyi*

*T. bourrellyi* was cultured in 100L of BG-11 medium supplemented with continuous aeration with filtered air (0.3 L/min) at 25 °C under illumination of 80 µE/$m^2$s on a 12L:12D photoperiod. After 4 weeks, the culture was centrifuged at 10000 rpm to separate the cells from the medium and freeze dried. The freeze-dried cells (22.4 g) were homogenized with methanol and the process repeated three times for maximum metabolite extraction. The crude extract (9.7 g) was evaporated to dryness and then fractionated with water and ethyl acetate and further purified to yield 15.3 mg of the new compound which was analyzed by NMR for structure elucidation. The compound demonstrated moderate activity with an $IC_{50}$ value of 43 µM and the positive control Trolox had an $IC_{50}$ of 24 µM.

## General discussion

Cyanobacteria have been recognized as a rich source of secondary metabolites with remarkable biological activities. In recent years, numerous studies have focused on the isolation and characterization of new compounds from cyanobacteria, particularly those with potential therapeutic applications. This study highlights the importance of using multiple screening strategies to identify new natural products. Three different strategies were employed to screen for new metabolites: standard laboratory cultures, OSMAC and genome mining. OSMAC can be a powerful tool that can be used to expand the chemical diversity of natural products and facilitate the discovery of new drugs. These strategies have allowed us to identify a diverse set of metabolites. Five new secondary metabolites with rare structural features were isolated and characterized from the culture of two cyanobacterial strains. The compounds were purified using a combination of chromatographic techniques and their structures were elucidated using spectroscopic methods such as NMR and mass spectrometry. Most importantly, this study marks the first report of isolation and characterization of a secondary metabolite specifically induced by the OSMAC approach in cyanobacteria.