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

(マレーシアサバ州で採集された海洋藍藻 *Moorea bouillonii* から  
得られた新規二次代謝産物に関する研究)

**Jakia Jerin Mehjabin**

**Ph.D Dissertation**

**2020**

**Division of Environmental Science Development**

**Graduate School of Environmental Science**

**Hokkaido University, Japan**

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# 学 位 論 文 内 容 の 要 旨

博士 (環境科学)

氏 名 Jakia Jerin Mehjabin

## 学 位 論 文 題 名

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

(マレーシアサバ州で採集された海洋藍藻 *Moorea bouillonii* から  
得られた新規二次代謝産物に関する研究)

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 nonribosomal 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.

For this study, a total twenty one samples of marine cyanobacteria were collected by SCUBA from different locations of Northern Bornean coast, Sabah, Malaysia. Among them nine samples were successfully identified by 16S rRNA gene sequence analysis using cyanobacteria specific primers. Based on 16S rRNA gene sequencing and phylogenetic tree analysis M1621 was identified as *Symploca* or *Caldora* sp., M1623 was identified as *Okeania* sp., and other collected samples M1620, M1622, M1629, M1701, M1704 and M1705 were identified as *Moorea* sp. The cyanobacterial samples were extracted with MeOH and then partitioned with ethyl acetate (EtOAc), *n*-butanol (BuOH) and water (H<sub>2</sub>O). The chemical profiling and dereplication of EtOAc fractions of *Moorea* sp. by LC-MS revealed the richness of secondary metabolites including known compounds apratoxins, wewakazole, lyngbyabellins, laingolides, madangolide, kanamienamide and unknown halogenated compounds. The cyanobacterial extracts were subjected to oil displacement assay for examining the surfactant properties of secondary metabolites. The EtOAc fractions of cyanobacterial extracts showed higher activity in terms of oil displacement area compared to other fractions and subjected to MTT assay against human MCF7 breast cancer cell lines for cytotoxicity. Among the extracts two *Moorea bouillonii* samples M1629 and M1705 were chosen for the isolation of novel secondary metabolites because of their higher amount, rich chemical profile and activity.

Biosurfactant assay guided isolation of *M. bouillonii* (M1705) afforded three new acyl amides, columbamide F-H along with known cytotoxic apratoxin A and C, kanamienamide and columbamide D. The planar structure of columbamide F-H was established by mass spectrometric and NMR spectroscopic analyses. An *E* configuration of the double bond for columbamides was obtained by measuring the coupling constant ( $^3J_{H,H} = 16-17$  Hz) from the  $^{13}C$  satellites observed by non-decoupled HSQC analysis. The absolute configuration of the *N,O*-dimethylserinol of columbamide F-H were determined by synthesis and Marfey's analysis of the synthetic (*R*)- and (*S*)-*N,O*-dimethylserinol and the hydrolysates of the columbamides.

The absolute configuration of chloromethine on the long alkyl chain of columbamide F 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. Since, columbamide D and F have similar alkyl chain structure, columbamide D standards (10*S*,20*R*) and (10*R*,20*R*) were used as standards in the Ohrui reaction for columbamide F. Synthesized diastereomers of columbamide D and natural columbamide F were converted to diol by dihydroxylation and then cleaved oxidatively with sodium periodate (NaIO<sub>4</sub>) to form two

aldehydes. After separation, aldehyde that contained chloromethine was reduced with NaBH<sub>4</sub> to furnish linear alcohol which was esterified with (S,S)-2-(anthracene-2,3-dicarboximido)cyclohexanecarboxylic acid in the presence of EDCI and DMAP to afford Ohrui ester of synthetic standards and natural columbamide F. Chiral-phase HPLC analysis of synthetic 10S and 10R standards revealed that a coinjection experiment can separate both standards. Then, by a coelution experiment, columbamide F gave a single peak with the synthetic 10R isomer while two peaks were observed with the synthetic 10S isomer confirming the *R*-configuration for the chloromethine.

The biosurfactant properties of columbamide F was measured using oil displacement assay which is an indirect measurement of surface activity. The diameter of the clear zone was measured as ~ 90 mm for 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. Due to the limited amount of sample it was not possible to determine the biosurfactant properties of columbamide G and H, however, the related fatty acid amides columbamide D and serinolamide C were checked for their biosurfactant properties. We measured the critical micelle concentration of these two fatty acid amides that showed biosurfactant properties in their initial screening results by oil displacement assay. The critical micelle concentrations (CMC) of columbamide D and serinolamide C were 0.34 mM, and 0.78 mM, respectively, that are lower than the CMC value of the synthetic surfactant SDS (8.2 mM) and comparable to pluronic F-68 (0.07-0.35) mM and triton X-100 (0.33 mM). Serinolamide C isolated from *Okeania* sp. showed potent antifouling activity (0.88 µg/mL) against *Amphibalanus amphitrite* larvae. The biosurfactant properties of serinolamide C could reduce the surface tension of seawater in the microplate well, and therefore the larvae cannot adhere easily. Furthermore, columbamides F-H were tested for the cytotoxicity against MCF-7 breast cancer cell line but were inactive (IC<sub>50</sub> >10 µg/mL).

LC-MS guided separation of organic extract from *M. bouillonii* (M1629) collected in Udar Island, Sabah, Malaysia led to the isolation of new madangolide along with known compounds madangolide, laingolide, laingolide A and (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*,11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one which was originally isolated from the sponge *Dysidea* sp. The planar structure of new madangolide was established by combination of 1D and 2D NMR spectroscopic and mass analyses. The <sup>13</sup>C NMR peak revealed the presence of two carbonyls (δ 176.7, 175.5), four olefinic methines (δ 134.9, 122.7, 121.7, and 129.7), four methines; one is connected to oxygen atom (δ 76.2), one *N*-methyl (δ 35.4) which showed similarities of this compound with known madangolide except the replacement of isopropyl group instead of *tert*butyl group. The anti-allergic activity of crude extract and pure compounds laingolide A, madangolide and (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*,11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one were checked by β-Hexosaminidase release in rat basophil leukemia (RBL-2H3) cells. An allergic condition described a hypersensitivity disorder in which immune system response to a normally harmless environmental substance. The preliminary screening of the anti-allergic activity of crude extract as well as pure compound laingolide A and madangolide exhibited the reduction of β-Hexosaminidase, chemical mediator responsible for the allergic reactions. The details analysis of absolute configuration of new madangolide and the anti-allergic activities will be investigated in the future.

This study demonstrated the capabilities of marine cyanobacteria from Sabah, Malaysia to produce novel secondary metabolites. To date, only one paper reported the secondary metabolites from *M. bouillonii* collected in this region and very few reports revealed the biosurfactant and anti-allergic properties of secondary metabolites from marine cyanobacteria. The unique structure of new acyl amides columbamide F-H and new madangolide would be leading to the further future research about their bioactivities and isolation of novel compounds from the unexplored marine cyanobacteria from Sabah, Malaysia.

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## Introduction

Cyanobacteria, also known as blue-green algae, are prokaryotic gram-negative organisms that have the ability to perform photosynthesis. Cyanobacteria have a long evolutionary history and have the ability to produce two types of specialized cells: (1) heterocysts, which can fix nitrogen gas and thereby counteract nitrogen demand under conditions of nitrogen deficiency, and (2) akinetes, which are resting cells that allow the species to survive in unfavorable growth conditions.<sup>1</sup> Cyanobacteria produce a wide range of bioactive compounds includes 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides, 9% amides and others.<sup>2</sup> This large spectrum of secondary metabolites can be explained on the basis of their ubiquitous occurrence and long evolutionary history. They are the ancient group of organisms that can survive in diverse habitats including marine, terrestrial, freshwater and furthermore in extreme conditions because of their physiological capacities and adaptations.<sup>3-5</sup>

Early studies of natural products are mainly focused on terrestrial plants and microorganisms and proved to be potential source of useful compounds like aspirin, penicillin and taxol.<sup>2</sup> Researcher focused on ocean as a source of novel compounds in the beginning of 1960s and to date more than 35000 compounds with intriguing bioactivities have been isolated from marine organisms.<sup>6,7</sup> Marine cyanobacteria have gained attention from natural product chemists because of their magnificent biosynthetic capacity in producing novel secondary metabolites.<sup>8</sup> 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.<sup>9-13</sup> 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.<sup>14</sup> Many of these nitrogen containing metabolites are produced through the non-ribosomal peptide (NRP) or the hybrid polyketide-NRP biosynthetic pathways and discovered at the laboratories of Professor Richard Moore (University of Hawaii) and William Gerwick (University of California, San Diego).<sup>15,16</sup>

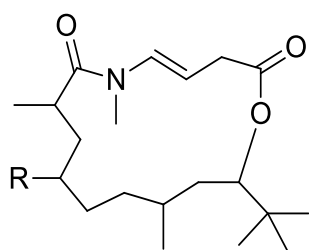
Besides the broad biological activities of secondary metabolites from cyanobacteria, 40% of species are supposed to be toxigenic and produce neurotoxins, hepatotoxins, and cytotoxins.<sup>17</sup> Cyanobacteria can synthesize the allelopathic compounds which tend to target the other

competitive species and cause disease, and mortality.<sup>18,19</sup> However, a number of mechanical, physical, chemical and biological methods are recently used to prevent the harmful blooms of cyanobacteria.<sup>20</sup> 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 *Moorea 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.<sup>21</sup> 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.

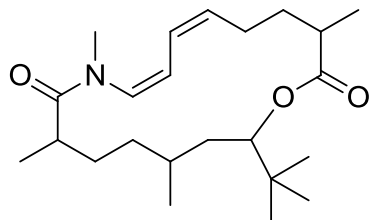
### **Compounds from *Moorea bouillonii***

Filamentous marine cyanobacterium *Lyngbya bouillonii* was collected from Hansa Bay, Laing Island, Papua New Guinea and morphologically described in 1991.<sup>22</sup> 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*.<sup>23</sup> According to MarinLit database total 42 compounds were reported from *M. bouillonii* and gaining attention as a prolific producer of novel secondary metabolites.<sup>24</sup>

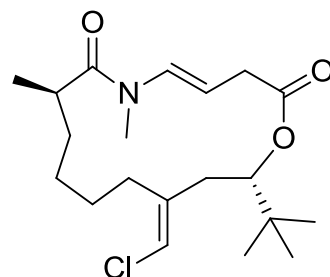
A 15-membered macrolides laingolide (**1**), laingolide A (**2**) and 17-membered macrolide madangolide (**3**) were isolated from *Lyngbya bouillonii* collected Laing island and vicinity of Madang, north coast of Papua New Guinea.<sup>25,26</sup> These macrolides are the first reported compounds from *M. bouillonii*. Unfortunately, the absolute configuration and biological activity couldn't be determined because of the degradation of these compounds. Later in 2010, laingolide B (**4**) with unique *tert*-butyl carbinol moiety and the chlorinated exo-methylene group was isolated from Guam.<sup>27</sup> The absolute configuration of laingolide B was established as (2*S*,9*R*) by total synthesis.<sup>28</sup>



**1** Laingolide R=CH<sub>3</sub>  
**2** Laingolide A R=H



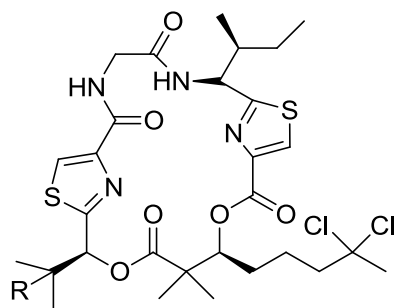
**3** Madangolide



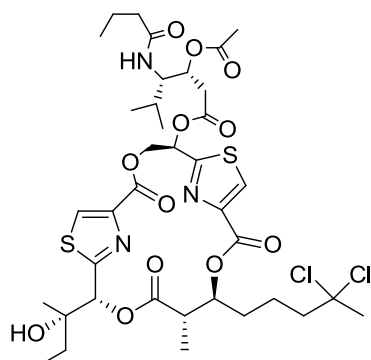
**4** Laingolide B

Two analogues of lyngbyabellin A (**5**), 27-deoxylyngbyabellin A (**6**) and lyngbyabellin J (**7**), have been isolated from *M. bouillonii* collected in Guam along with lyngbyabellin A (**5**), B (**8**) and showed strong cytotoxicity against HT29 colon cancer cells and HeLa cervical cancer cells ( $IC_{50}=0.012-1.1 \mu M$ ).<sup>27</sup> Four novel cyclic peptides, lyngbyabellins K (**9**) and L (**10**), lyngbyabellin N (**11**) and 7-*epi*-lyngbyabellin L (**12**) and one thiazole containing linear peptides lyngbyabellin M (**13**) have been isolated from *M. bouillonii* collected in Palmyra Atoll in the Central Pacific Ocean.<sup>29</sup>

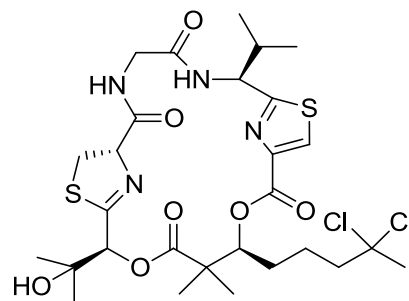
Among them lyngbyabellin N (**11**) showed potent cytotoxicity against HCT116 colon cancer cell line with  $IC_{50}=40.9\pm3.3 \text{ nM}$ .<sup>29</sup>



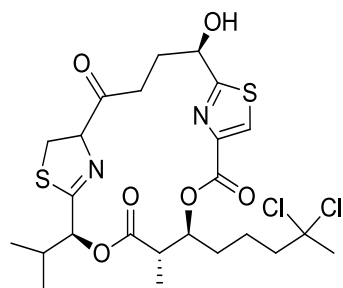
**5** Lyngbyabellin A, R=OH  
**6** 27-Deoxylyngbyabellin A, R=H



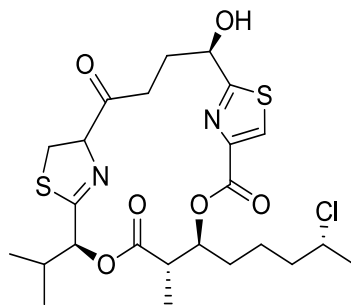
**7** Lyngbyabellin J



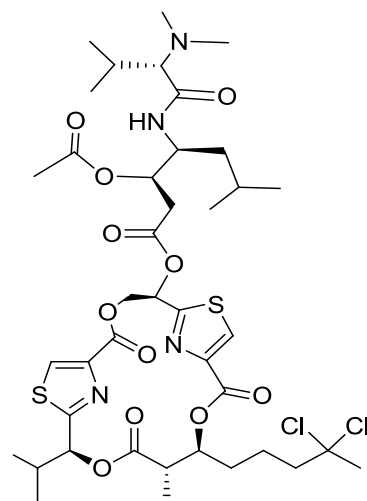
**8** Lyngbyabellin B



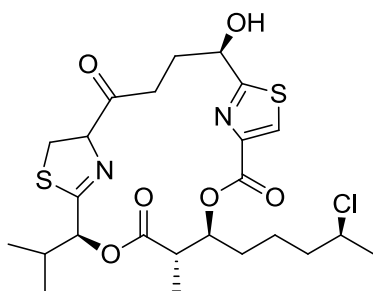
**9** Lyngbyabellin K



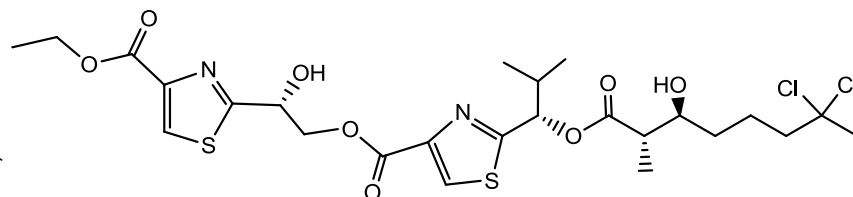
**10** Lyngbyabellin L



**11** Lyngbyabellin N

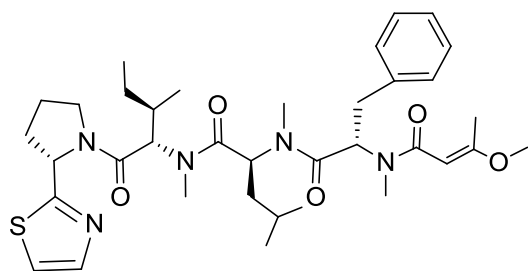


**12** 7-*epi*-Lyngbyabellin L

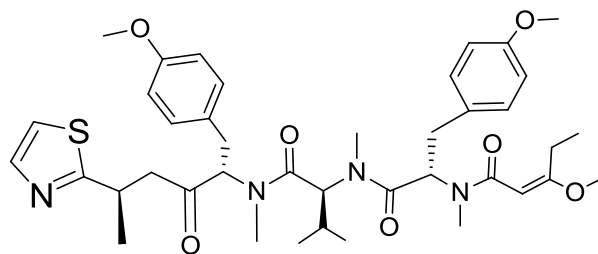


**13** Lyngbyabellin M

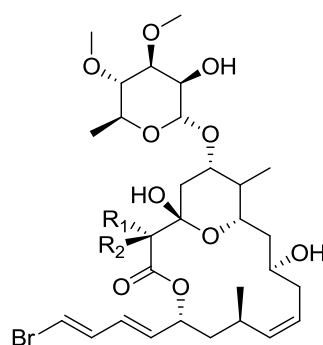
Linear peptide lyngbyapeptin A (**14**) and 16-membered macrolide lyngbyaloside (**15**, **17**) were isolated from different collection of *M. bouillonii* collected in Papua New Guinea.<sup>30,31</sup> Later in 2010 lyngbyapeptin D (**16**) along with lyngbyapeptin A (**14**) and lyngbyalosides (**18-20**), were isolated from cyanobacterium collected from Apra Harbor, Guam<sup>27</sup> Lyngbyalosides showed weak cytotoxicity to HT29 colon cancer and HeLa cervical cancer cell lines.<sup>27</sup> On the other hand glycosidic macrolide, lyngbouilloside (**21**) were collected from Papua New Guinea showed moderate cytotoxicity (IC<sub>50</sub>=17  $\mu$ M) against neuroblastoma cells.<sup>32</sup>



**14** Lyngbyapeptin A

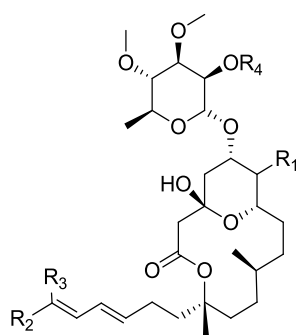


**16** Lyngbyapeptin D



**15** Lyngbyaloside,

**17** 2-*epi*-Lyngbyaloside

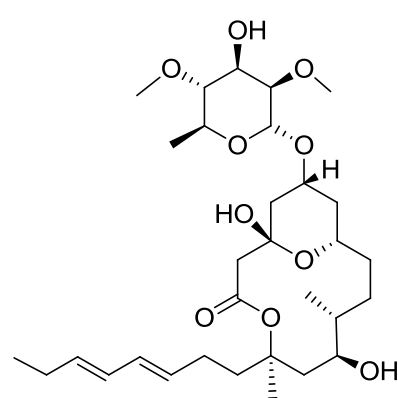


**18** Lyngbyaloside B,

**19** 18*E*-Lyngbyaloside C

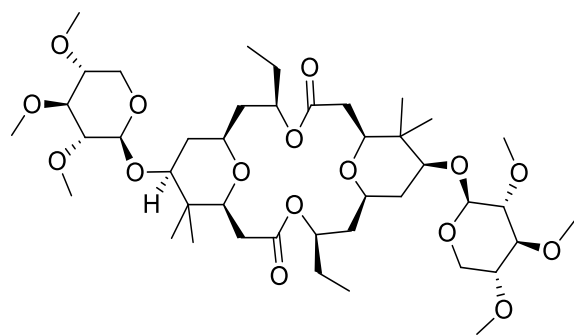
**20** 18*Z*-Lyngbyaloside C

| R <sub>1</sub>  | R <sub>2</sub> | R <sub>3</sub> | R <sub>4</sub>  |
|-----------------|----------------|----------------|-----------------|
| CH <sub>3</sub> | Br             | H              | H               |
| H               | Br             | H              | CH <sub>3</sub> |
| H               | H              | Br             | CH <sub>3</sub> |

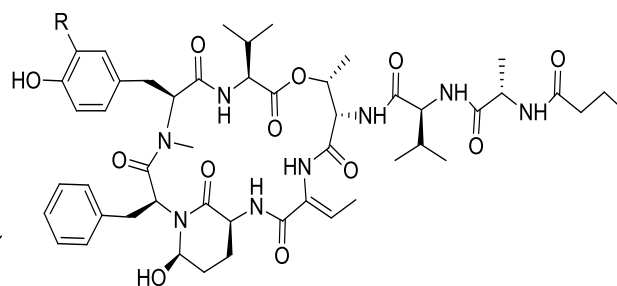


**21** Lyngbouilloside

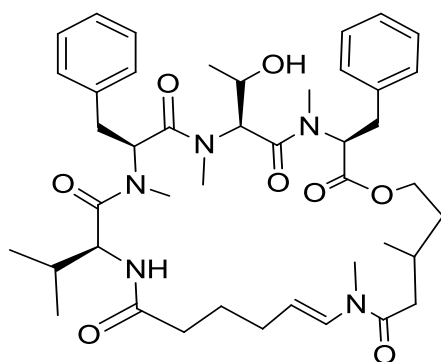
Cyanolide A (**22**), a glycosidic macrolide isolated from extracts of a Papua New Guinea collection that showed potent molluscicidal agent against the snail vector *Biomphalaria glabrata* (LC<sub>50</sub>= 1.2 μM).<sup>33</sup> Two cyclic depsipeptide bouillomides A (**23**) and B (**24**), isolated from a Guamanian sample of *L. bouillonii*.<sup>34</sup> These two analogues of dolastatin 13 inhibited serine proteases elastase (IC<sub>50</sub>=1.9 μM for both) and chymotrypsin (IC<sub>50</sub>=0.17 and 9.3 μM, respectively).<sup>34</sup> Other cyclic depsipeptides, bouillonamide (**25**) and alotamide A (**26**) were reported from different collection of *M. bouillonii* collected in Papua New Guinea.<sup>35,36</sup>



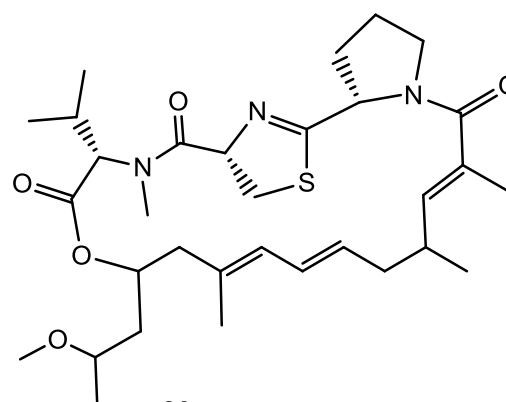
**22** Cyanolide A



**23** Bouillomide A R=H  
**24** Bouillomide B R=Br

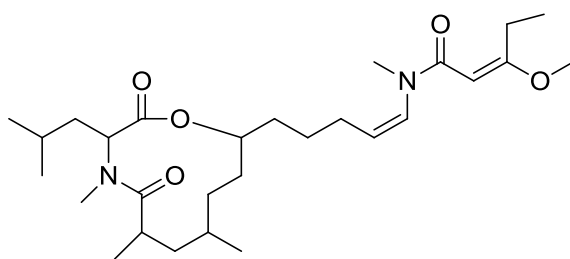


**25** Bouillonamide

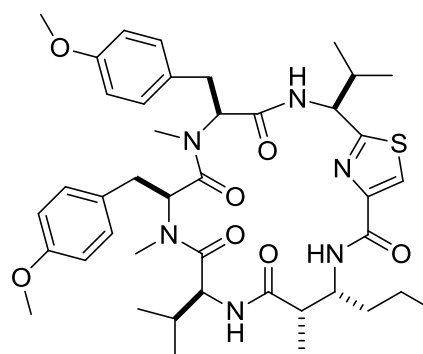


**26** Alotamide A

A cytotoxic kanamienamide (**27**) containing *N*-Me-enamide moiety was isolated from *M. bouillonii* collected in Kagoshima, Japan which showed cytotoxicity against HeLa human cervical cancer cell lines with an  $IC_{50}$  value 2.5  $\mu$ M.<sup>37</sup> Kakeromamide (**28**), a cyclic pentapeptide containing a thiazole ring moiety was reported from another collection of *M. bouillonii* from Kakeroma Island in Kagoshima Prefecture, Japan which induce differentiation of neural stem cells into astrocytes without cytotoxicity.<sup>38</sup>

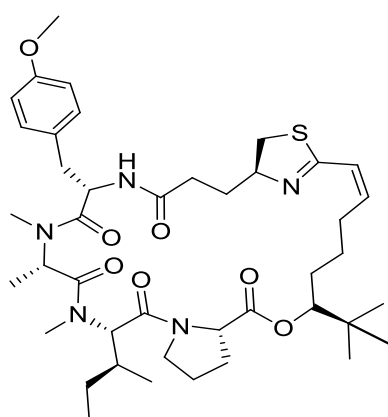


**27** Kanamienamide

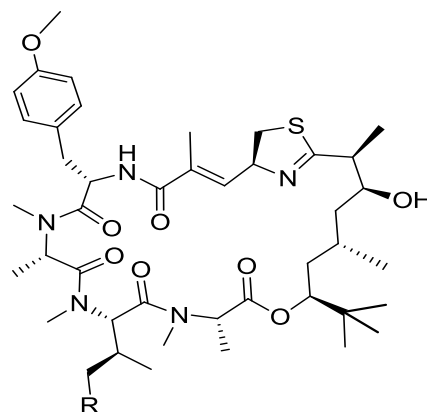


**28** Kakeromamide

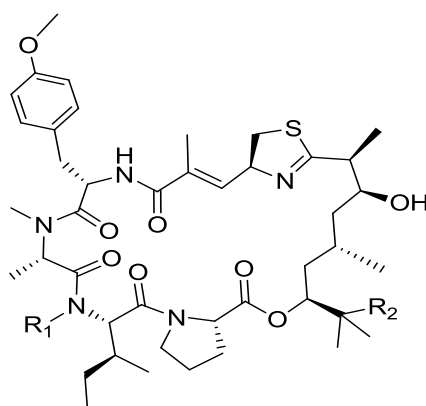
A cytotoxic peptolide, apratoxin E (**29**) was isolated from *M. bouillonii* collected in Apra Harbor, Guam at 12-14 m depth, and at the same time apratoxin A (**30**) was isolated from shallow water *M. bouillonii* at a depth 1-3 m.<sup>39</sup> Apratoxin E (**29**) exhibited strong cytotoxicity against colon, cervix, and bone cancer cell lines with IC<sub>50</sub> value ranging from 21 to 72 nM.<sup>39</sup> *M. bouillonii* collection from Palmyra Atoll in the Central Pacific resulted in the isolation two new cytotoxins apratoxin F (**31**) and G (**32**) showed strong cytotoxicity against H460 lung cancer and HCT-116 colon cancer cell lines with IC<sub>50</sub> values in nanomolar concentration.<sup>40</sup> Although apratoxins have been isolated from *Lyngbya* species collected in Guam and Palau, later apratoxins A-C (**30**, **33-34**) were isolated from different collections of *M. bouillonii* collected in Papua New Guinea and Sabah, Malaysia.<sup>41,42</sup>



**29** Apratoxin E



**31** Apratoxin F R=CH<sub>3</sub>  
**32** Apratoxin G R=H



**30** Apratoxin A R<sub>1</sub>=CH<sub>3</sub> R<sub>2</sub>=CH<sub>3</sub>  
**33** Apratoxin B R<sub>1</sub>=H R<sub>2</sub>=CH<sub>3</sub>  
**34** Apratoxin C R<sub>1</sub>=CH<sub>3</sub> R<sub>2</sub>=H

The acyl amide, mooreamide A (**35**) was isolated from *Moorea bouillonii* collected in Papua New Guinea and exhibited selective ligand binding activity to cannabinoid receptors, CB<sub>1</sub> ( $K_i = 0.47 \mu\text{M}$ ) versus CB<sub>2</sub> ( $K_i > 25 \mu\text{M}$ ).<sup>43</sup> Another group of fatty acid amides columbamides A-C (**36-38**) were isolated from laboratory culture of *M. bouillonii* and showed the highest affinity for both CB<sub>1</sub> and CB<sub>2</sub> ( $K_i$  0.59 and 1.03  $\mu\text{M}$  for columbamide A;  $K_i$  0.41 and 0.86  $\mu\text{M}$  for columbamide B.<sup>44</sup> Columbamides D (**39**) and E (**40**) were the first reported compounds from the *M. bouillonii* sample collected in Mantanani island, Sabah, Malaysia.<sup>42</sup>

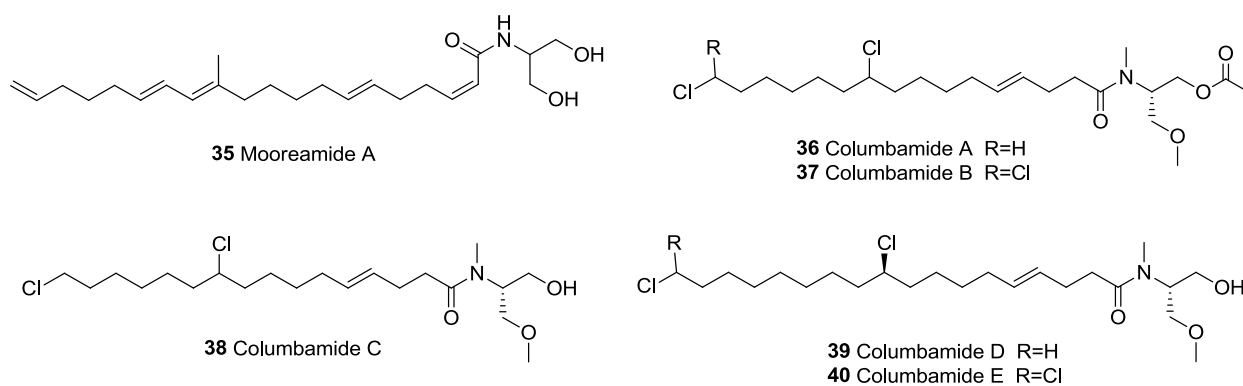


**Table 1. Natural products with bioactivity from marine cyanobacteria *Moorea bouillonii***

| Secondary Metabolites       | Sources          | Biological Activities                                                                                                                                                            | Ref |
|-----------------------------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Lyngbyabellin A (5)         | Guam             | Cytotoxic against colorectal adenocarcinoma HT29 cancer cells (IC <sub>50</sub> =0.047 µM)                                                                                       | 27  |
| 27-Deoxylyngbyabellin A (6) | Guam             | Cytotoxic against colorectal adenocarcinoma HT29 cancer cells (IC <sub>50</sub> =0.01 µM)                                                                                        | 27  |
| Lynbyabellin J (7)          | Guam             | Cytotoxic against colorectal adenocarcinoma HT29 cancer cells (IC <sub>50</sub> =0.054 µM)                                                                                       | 27  |
| Lyngbyabellin N (11)        | Palmyra Atoll    | Cytotoxic against HCT116 colon cancer cells (IC <sub>50</sub> =40.9±3.3 nM)                                                                                                      | 29  |
| Lyngboulloside (21)         | Papua New Guinea | Moderate cytotoxicity against neuroblastoma cells (IC <sub>50</sub> =17 µM)                                                                                                      | 32  |
| Cyanolide A (22)            | Papua New Guinea | Molluscicidal activity against the snail vector <i>Biomphalaria glabrata</i> (LC <sub>50</sub> =1.2 µM)                                                                          | 33  |
| Bouillomide A (23)          | Guam             | inhibited serine proteases elastase (IC <sub>50</sub> =1.9 µM) and chymotrypsin (IC <sub>50</sub> =0.17 µM)                                                                      | 34  |
| Bouillomide B (24)          | Guam             | inhibited serine proteases elastase (IC <sub>50</sub> =1.9 µM) and chymotrypsin (IC <sub>50</sub> =9.3 µM)                                                                       | 34  |
| Bouillonamide (25)          | Papua New Guinea | Mildly cytotoxicity against neuro 2a mouse neuroblastoma cells (IC <sub>50</sub> =6 µM)                                                                                          | 35  |
| Alotamide A (26)            | Papua New Guinea | Cytotoxic against brine shrimp <i>Artemia salina</i> (IC <sub>50</sub> =9 µM)<br>Calcium influx activation profile in murine cerebrocortical neurons (EC <sub>50</sub> =4.18 µM) | 36  |
| Kanamienamide (27)          | Kagoshima, Japan | Cytotoxic against HeLa cervical cancer cells (IC <sub>50</sub> =2.5 µM)                                                                                                          | 37  |
| Kakeromamide (28)           | Kagoshima, Japan | Cytotoxic against HeLa cervical cancer cells (IC <sub>50</sub> =10 µM)                                                                                                           | 38  |
| Apratoxin E (29)            | Guam             | Cytotoxic against HT29, HeLa, and U2OS cells (IC <sub>50</sub> =21-72 nM)                                                                                                        | 39  |
| Apratoxin A (30)            | Papua New Guinea | Cytotoxic against neuro 2a mouse neuroblastoma cells (IC <sub>50</sub> =1 µM)                                                                                                    | 39  |
| Apratoxin F (31)            | Palmyra Atoll    | Cytotoxic against H460 lung cancer cells                                                                                                                                         | 40  |

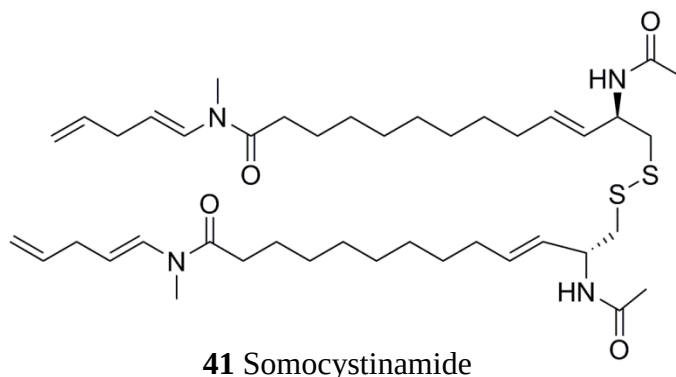
|                    |                    |                                                                                             |    |
|--------------------|--------------------|---------------------------------------------------------------------------------------------|----|
|                    |                    | (IC <sub>50</sub> =2 nM)                                                                    |    |
| Apratoxin G (32)   | Palmyra Atoll      | Cytotoxic against H460 lung cancer cells (IC <sub>50</sub> =14 nM)                          | 40 |
| Mooreamide A (35)  | Papua New Guinea   | CB <sub>1</sub> cannabinoid receptor (K <sub>i</sub> =0.47 μM)                              | 43 |
| Columbamide A (36) | Laboratory Culture | CB <sub>1</sub> and CB <sub>2</sub> cannabinoid receptor (K <sub>i</sub> =0.59 and 1.03 μM) | 44 |
| Columbamide B (37) | Laboratory Culture | CB <sub>1</sub> and CB <sub>2</sub> cannabinoid receptor (K <sub>i</sub> =0.41 and 0.86 μM) | 44 |

### Biosurfactants from Cyanobacteria

Biosurfactants have recently emerged as diverse group of surface active amphipathic compounds with distinct chemical structure produced by a wide variety of microorganisms are potentially useful for many therapeutic applications.<sup>45,46</sup> Biosurfactants are mainly formed as secondary metabolites generally extracellularly produced or localized on microbial cell surfaces, play important roles in the survival of their producing microorganisms by facilitating nutrient transport, interfering in microbe-host interactions and quorum sensing mechanisms, or by acting as biocide agents.<sup>47,48</sup> Biosurfactants are usually classified as glycolipids, lipopeptides, fatty acid and polymers.<sup>45,49,50</sup> Biosurfactants have attracted much attention as potential replacement for synthetic surfactants in industrial and environmental applications and also for the discovery of several therapeutic agents.<sup>51-53</sup>

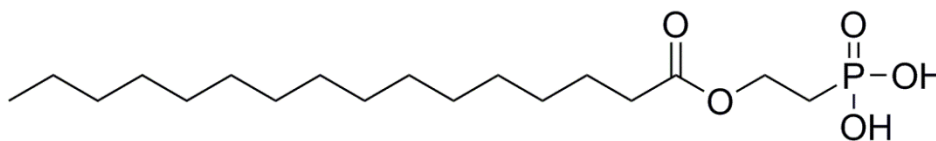
Biosurfactants produced by cyanobacteria have not been reported so far, however, it is expected that amphipathic metabolites frequently found in cyanobacteria as lipoproteins, lipopeptides, and lipopolysaccharides will exhibit surfactant properties.<sup>54-56</sup> Some new biosurfactant structures have been reported namely, somocystinamide A (41), apratoxin A (30) from marine cyanobacteria and 2-aceoxyethylphosphonate (42) from the freshwater cyanobacterium strain *Aphanizomenon flos-aquae*.

Lipopeptide biosurfactant somocystinamide (**41**) from cyanobacteria *Lyngbya majuscula* exhibited cytotoxicity against a number of cancer cell lines including leukaemia, lung, breast and prostate cancer cells with IC<sub>50</sub> values ranging from 1.3  $\mu$ M to 970 nM depending on the cancer model.<sup>57</sup>



Apratoxins are cyclic lipopeptides which exhibit a significant cytotoxicity against a number of cancer cell lines. Apratoxin A (**30**) and its derivatives were isolated from marine cyanobacteria and has been found to induce G1-phase cell cycle arrest and apoptosis.<sup>58</sup>

A novel biosurfactant 2-Aceloxyethylphosphonate (**42**) was isolated from freshwater cyanobacterium *Apahanizomenon flos-aquae* having critical micelle concentration about 22 mM. This biosurfactant have been tested for brine shrimp (*Artemia salina*) assay and found to be non toxic at concentration upto 50  $\mu$ g/mL.<sup>59</sup>



The majority of the studies on biosurfactant mainly focused on the known compounds produced by the microbes of terrestrial origin. Therefore the marine ecosystem can be great

opportunity to identify new biosurfactants with significant bioactivities. Because of the high diversity of chemical compounds and bioactivities of the secondary metabolites from cyanobacteria can be a potential source of biosurfactants with significant biological activities.

### **Anti-allergic properties of secondary metabolites from cyanobacteria**

Allergic diseases are a number of conditions caused by hypersensitivity of the immune system to typically harmless substances in the environment.<sup>60</sup> Food allergy are one of the most important health problems now a day. Food allergy, associated with nausea, vomiting, diarrhoea, peptic ulcers, asthma, allergic dermatitis, allergic rhinitis, allergic shock and even death, commonly triggered by immunoglobulin (Ig)E has seriously affected people in developing countries.<sup>61</sup> Although some medicines are now available to treat the allergic symptoms such as anti-histamine drugs (diphenhydramine, chlorpheniramine maleate, terfenadine, etc.), mast cell stabilizers (disodium cromoglycate, sodium hydroxypropylcromate, ketotifen, etc.) and immune suppressors (adrenal cortical hormones, dexamethasone, hydrocortisone, etc.) however, these drugs have side effects.<sup>62</sup>

The anti-allergic compounds from cyanobacteria has not reported so far, however few studies reported the anti-allergic properties of *Spirulina* based dietary supplements. *Spirulina Platensis* is one of the oldest organisms with long evolutionary history and have been used as human food. The powder of *Spirulina platensis* was first assessed in the *in vivo* model of systemic anaphylaxis using compound 48/80 as a systemic fatal anaphylaxis inducer and showed inhibitory effect by reducing the histamine release.<sup>63</sup> Also the clinical efficacy of tablets prepared from *Spirulina platensis* was proved by applying into 150 allergic rhinitis patients.<sup>64</sup>

Anti-allergic properties were found in the extract of marine cyanobacteria that was attempted in our continuous study to identify bioactive compounds. In 2018, Ryosuke Takahashi first checked the anti-allergic properties from the extracts of *M. bouillonii* and found potent anti-allergic properties in silica gel hexane/EtOAc 80:20 (v/v): 12±3%, EtOAc/MeOH 50:50 (v/v): 33±3%, and 100% MeOH: 22±3% fractions. These results revealed the possibility of having the anti-allergic properties in the compounds derived from *M. bouillonii*.

## Objective

Marine cyanobacteria are known as the prolific producer of structurally diverse bioactive compounds. Many bioactive compounds were reported from *Moorea* sp. especially from *M. producens* previously known as *Lyngbya* sp., whereas, compounds isolated from *M. bouillonii* are limited. Recent studies about the bioactive compounds with unique structure from this species made them as potential source of unknown secondary metabolites. The main objective of this study is to isolate and elucidate the structure of novel bioactive compounds from marine cyanobacteria collected from different locations of Sabah, Malaysia. The details of the sample collection, identification, chemical profiling and bioactivity like cytotoxicity and biosurfactant activity are reported in chapter 1. Chapter 2 deals with isolation and structure elucidations of chlorinated fatty acid acyl amides Columbamide F-H including the absolute configuration assignment by Marfey's analysis and Ohruai method. Then chapter 3 deals with the isolation and structure elucidations of new 17-membered macrolide, 20-desmethylmadangolide.

## CHAPTER 1

### LC-MS Profiling and Screening of Marine Cyanobacteria from Sabah, Malaysia

A total 21 samples were collected from different locations of Sabah, Malaysia. Among them 14 samples were preserved in *RNAlater*® for 16S rRNA gene sequencing. All samples were subjected to solvent extraction by using MeOH. The MeOH extractions of all samples were partitioned using EtOAc, *n*-BuOH, and H<sub>2</sub>O. The chemical profiling of extracts were analyzed by LC-MS and checked for cytotoxicity and oil spreading assay.

#### 1.1 16S rRNA Gene Sequencing Analysis

##### 1.1.1 Sampling

The marine cyanobacterial samples (Table I-1) were collected by scuba at different locations in Kota Kinabalu, Sabah, Malaysia in September 2016 and November 2017. The collections were conducted in collaboration with Professor Charles S. Vairappan of the Universiti Malaysia Sabah (UMS). Seawater was squeezed out from samples and preserved in the MeOH-filled I-boy bottle after collection. A small amount of fresh cyanobacteria was preserved in the 8 mL RNA stabilization solution *RNAlater*® (Ambion, USA) in the 15 mL Falcon tube for identification. To preserve the sample in *RNAlater*® sea water and corals were removed carefully. Collected samples were stored in sea water BG-11 medium for culturing. A total of 16 samples were collected in 2016 and five samples were collected in 2017 from Kota Kinabalu, Sabah, Malaysia. Samples were transported to the laboratory of UMS and evaporated to remove MeOH. The MeOH and *RNAlater*® preserved sample were stored in freezer before transporting to Japan. The underwater and microscopic pictures of the collected samples are shown in Fig. I-1 and I-2.

**Table I-1.** Collected samples from Sabah, Malaysia

| Sample Code | Collection Date | Area                    | Location, Depth              | GPS Coordinates          | RNA <sup>later</sup> Preserved |
|-------------|-----------------|-------------------------|------------------------------|--------------------------|--------------------------------|
| M1620       | Sep 2016        | Island near UMS         | Sepangar Island, 7-8 m       | N 6°4'26"; E 116°4'11"   | ✓                              |
| M1621       |                 |                         |                              |                          | ✓                              |
| M1622       |                 |                         |                              |                          | ✓                              |
| M1623       |                 |                         |                              |                          | ✓                              |
| M1624       |                 |                         |                              | N 6°4'30"; E 116°4'14"   | ✓                              |
| M1625       |                 |                         |                              |                          | ×                              |
| M1626       |                 |                         |                              |                          | ×                              |
| M1627       |                 |                         |                              |                          | ×                              |
| M1628       |                 |                         |                              |                          | ×                              |
| M1629       |                 |                         | Udar Island, 5-10 m          | N 6°4'53"; E 116°5'16"   | ✓                              |
| M1630       |                 |                         |                              |                          | ×                              |
| M1631       |                 |                         | Main Island (Sepangar) 7-8 m | N 6°5'44"; E 116°5'23"   | ✓                              |
| M1632       |                 |                         |                              |                          | ✓                              |
| M1633       |                 |                         |                              |                          | ✓                              |
| M1634       |                 |                         | Udar Island, 5-10 m          | N 6°4'53"; E 116°5'16"   | ✓                              |
| M1635       | Nov 2017        | Tunku Abdul Rahman Park | Sulug Island 5-10 m          | -                        | ✓                              |
| M1701       |                 | Mantanani Island        | Sandy bar point              | N 6°42'30"; E 116°21'0"  | ✓                              |
| M1702       |                 |                         | Jack sparrow reef            | N 6°42'0"; E 116°20'26"  | ×                              |
| M1703       |                 |                         | Jack sparrow reef            | N 6°42'0"; E 116°20'26"  | ×                              |
| M1704       |                 |                         | Rocky point                  | N 6°42'17"; E 116°19'13" | ✓                              |
| M1705       |                 |                         | Rocky point                  | N 6°42'16"; E 116°19'23" | ✓                              |



**M1620**



**M1621**



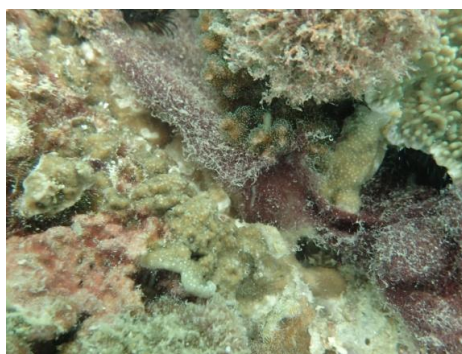
**M1623**



**M1624**



**M1625**



**M1629**



**M1631**



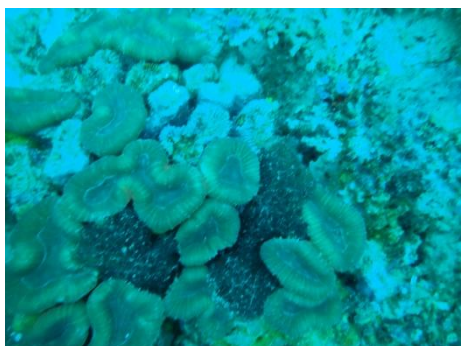
**M1632**



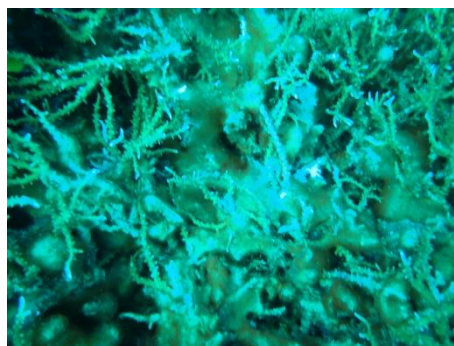
**M1634**



**M1635**



**M1701**



**M1702**



**M1703**



**M1704**



**M1705**

**Fig. I-1** Underwater picture of collected marine samples.



**M1622 (*M. bouillonii*)**



**M1629 (*M. bouillonii*)**



**M1701 (*M. bouillonii*)**



**M1702**



**M1703**



**M1703**



**M1704 (*M. bouillonii*)**



**M1705 (*M. bouillonii*)**

**Fig. I-2** Images taken under a microscope. Scale =100  $\mu$ m.

### 1.1.2 Experimental Section

**Genomic DNA Extraction.** The marine samples were identified by molecular phylogenetic analysis based on 16S rRNA gene sequence data.<sup>65</sup>

At first DNA was extracted from the samples preserved in *RNAlater*® by the following solution (Table I-2).

**Table I-2.** Ingredients of DNA extraction solution

| <b>A - Extraction buffer (50 mL)</b> | <b>B - Urea solution (50 mL)</b> | <b>C (100 mL)</b> | <b>D (100 mL)</b>    |
|--------------------------------------|----------------------------------|-------------------|----------------------|
| 0.6 M NaCl                           | 10 M urea                        | Mixture of        | phenol:chloroform:   |
| 0.1 M Tris-HCl,<br>pH 7.5            | 20 mM 2 mecaptoethanol           | solutions A       | isoamyl alcohol =    |
| 40 mM EDTA, pH 8.0                   | 10% (v/v) phenol                 | and B             | volume ratio 25:24:1 |
| 1% SDS                               |                                  |                   |                      |

For preparing the extraction solution, 25 mL C and 25 mL D were put into Erlenmeyer flasks. The cells of marine samples preserved in *RNAlater*® were disrupted using liquid nitrogen and dismembrator II for 90 sec per run and put into the extraction solution. Then the flasks were put in a shaker for 15 min at 25 °C. After shaking, the solutions were centrifuged at 7,500 rpm for 15 min at 4 °C. The top layer of the solution were collected and put into the new tube. Ethanol 99.5% (-30 °C) (Wako, Japan) were added (volume ratio 1:2, DNA solution: EtOH) to give ethanol precipitation and centrifuged again at 7,500 rpm for 15 min at room temperature. The supernatants were discarded and 70% EtOH was added to sample to and put into the ice for precipitation and centrifuged at 7500 rpm for 5 min. The supernatant were discarded and the precipitated DNA was dried completely in vacuum. The DNA was dissolved in 150 µL TE buffer (10 mM Tris-HCl, pH 8.0 + 1 mM EDTA) and transferred to eppendorf tubes to add 6 µL RNase quickly. Then the samples were put in ice for 5 min. The amount of DNA were calculated using nanodrop lite (Thermo Scientific, USA).

For the repurification of DNA, 20  $\mu\text{L}$  of DNA sample was taken if the concentration was greater than 100 ng/ $\mu\text{L}$  and 50  $\mu\text{L}$  if the concentration was less than 100 ng/ $\mu\text{L}$ . TE buffer was added to the sample to get 100  $\mu\text{L}$  and 100  $\mu\text{L}$  of phenol solution (from  $\text{CHCl}_3$ : phenol: isoamylalcohol) was added. The solution were mixed using Tomy microtube at mixer speed 4.5 for 2-3 min and centrifuged at 14,800 rpm for 5 min. The upper part was collected, and put into new tubes, and ethyl ether (saturated by  $\text{H}_2\text{O}$ ) was added to remove the excess phenol. Then the solutions were centrifuged at 14,800 rpm for 3 min. The upper parts of the ether layer were discarded and NaOAc (10% of DNA volume) and 99% ethanol (2.5 times of solution) was added to recover DNA as precipitants. The solutions were centrifuged at 14,500 rpm for 10 min to remove ethanol. After that 300  $\mu\text{L}$  of 70% EtOH was added and centrifuged at 14500 rpm for 3 min. The supernatants were removed and dried completely to check the amount of DNA.

### PCR and DNA Sequence Analysis

The 16S rRNA genes were amplified by PCR by using Takara PCR thermal cycler (Takara, Japan) and the 16S rRNA genes were amplified using the 106F and 1509R primer.<sup>65</sup> Each PCR tube should contain the following solution (Table I-3).

**Table I-3.** Solution for PCR

| <b>Solution</b>                      | <b>Volume, <math>\mu\text{L}</math></b> |
|--------------------------------------|-----------------------------------------|
| 10X reaction buffer                  | 2.5                                     |
| dNTP mix                             | 4.0                                     |
| ExTaq polymerase                     | 0.25                                    |
| forward primer (4 $\mu\text{M}$ )    | 1                                       |
| reverse primer (4 $\mu\text{M}$ )    | 1                                       |
| template DNA (20 ng/ $\mu\text{L}$ ) | 1                                       |
| $\text{H}_2\text{O}$                 | 15.25                                   |

The PCR conditions were used in this study as follows, initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and

extension at 72 °C for 60 sec and a final extension step at 72 °C for 3 min. All PCR reactions were prepared in a volume of 25.0 µL containing 2.5 µL of 10×PCR buffer, 4 µL of each deoxynucleotide triphosphate, 1 µL of each of 4 µM primer, 0.25 µL of *ExTaq* DNA polymerase and 20 ng of template DNA. PCR was done to amplify the DNA fragment followed by gel electrophoresis to check the PCR products.

The PCR product was analyzed on a 1.2% (w/v) agarose gel made by 0.6 g agarose and 50 mL TBE buffer (Tris-boric acid-EDTA). For sample preparation, 2 µL of sample, 7 µL TE buffer and 1.5 µL of 6X dye were mixed and for markers, 4 µL of marker ( $\lambda$ -Hind III and  $\phi$ X174 Hae III), 5 µL of TE buffer and 1.5 µL of 6X dye were mixed. Samples and markers were loaded at 100 V for 30 min. After running the gel was soaked in ethidium bromide for 10 min and checked under UV light. In the present study, the Mupid® – 2 plus Submarine electrophoresis system was used.

PCR products were purified using QIAquick PCR purification Kit (Diagen, Dusseldorf, Germany). Purified PCR products were used as templates in sequencing reactions using 3130xl Genetic Analyzer ABI PRISM 3100 (Hitachi, Japan). The forward primers CYA106F and CYA359F were used in this study and reverse primer CYA1509R and CYA781R were used as an internal primer (Table I-4).

**Table I-4.** Primer sequences and target sites\*

| <b>Primer</b>  | <b>Sequence (5' to 3')</b>        | <b>Target site</b> |
|----------------|-----------------------------------|--------------------|
| <b>106F</b>    | CGG ACG GGT GAG TAA CGC GTG A     | 106-127            |
| <b>359F</b>    | GGG GAA TTT TCC GCA ATG GG        | 359-378            |
| <b>1509R</b>   | GGT TAC CTT GTT ACG ACT T         | 1491-1509          |
| <b>781R(a)</b> | GAC TAC TGG GGT ATC TAA TCC CAT T | 781-805            |
| <b>781R(b)</b> | GAC TAC AGG GGT ATC TAA TCC CTT T | 781-805            |

\*CYA781R primer is used as an equimolar mixture of CYA781R (a) and CYA718R (b)

The full gene sequences of the samples were compared using the BLAST (Basic Local Alignment Search Tool) against the sequences of bacteria available in GenBank for identification of species.

### **Phylogenetic Analysis**

The Phylogenetic analysis of all nucleotide sequence of 16s rRNA gene was conducted using Mega 7 software.<sup>66</sup> The 16S rRNA sequences were aligned using MUSCLE. Briefly, pairwise distances were calculated by the K2+G+I method, and a tree was constructed with the maximum likelihood optimally criteria. Bootstrap analyses were performed with 1000 replicates.

#### **1.1.3 Results**

Among the 13 RNAlater<sup>®</sup> preserved samples, the 16S rRNA gene sequence of nine samples were successfully acquired (Table I-5). These sequence data were used to construct the phylogenetic tree (Fig. I-3) 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.

**Table I-5. Results of 16S rRNA gene sequence analysis**

| Sampling Site<br>(depth)       | Sample<br>Code | Morphology               | RNA<br>Later | Identification by 16S<br>rRNA gene                         | Identity (%)                                                    |
|--------------------------------|----------------|--------------------------|--------------|------------------------------------------------------------|-----------------------------------------------------------------|
| Pulau Sepangar<br>depth 7~8 m  | M1620          | Tuft forming             | ✓            | <i>Moorea producens</i>                                    | <i>Moorea producens</i> PNG5-192 (98.8%)                        |
|                                | M1621          | Tuft forming             | ✓            | <i>Symploca</i> or <i>Caldora</i> sp.                      | <i>Caldora</i> sp. Ch4 (85%)<br><i>Symploca</i> sp. VP377 (85%) |
|                                | M1622          | Mat forming              | ✓            | <i>Moorea bouillonii</i>                                   | <i>Moorea bouillonii</i> PNG6-42 (94%)                          |
|                                | M1623          | Small patch<br>purple    | ✓            | <i>Okeania</i> sp.                                         | <i>Okeania</i> sp. PAB (95.5%)                                  |
|                                | M1624          | Tuft forming<br>(Orange) | ✓            | Uncultured Bacterium<br>clone                              | -                                                               |
|                                | M1625          | Tuft forming             | ✓            | No DNA based on PCR                                        | -                                                               |
| Udar Island depth<br>5~10 m    | M1629          | Mat forming              | ✓            | <i>Moorea bouillonii</i>                                   | <i>Moorea bouillonii</i><br>PAL08-16 (95%)                      |
|                                | M1630          | Mat forming              | -            | <i>Moorea bouillonii</i> *<br>(supported by<br>morphology) | -                                                               |
| Sepangar Island<br>depth 7~8 m | M1631          | Tuft forming             | ✓            | No DNA based on PCR                                        | -                                                               |
|                                | M1632          | Small tuft<br>forming    | ✓            | No DNA based on PCR                                        | -                                                               |
| Sulug<br>Island<br>(5~10 m)    | M1635          | -                        | ✓            | No DNA based on PCR                                        | -                                                               |
| Mantanani Island               | M1701          | Mat<br>forming           | ✓            | <i>Moorea bouillonii</i>                                   | <i>Moorea</i> sp. RS05 (93%)                                    |
|                                | M1704          | Mat<br>forming           | ✓            | <i>Moorea bouillonii</i>                                   | <i>Moorea bouillonii</i> Kjy 9<br>(96.2%)                       |
|                                | M1705          | Mat<br>forming           | ✓            | <i>Moorea bouillonii</i>                                   | <i>Moorea bouillonii</i> png7-34 (99.7 %)                       |

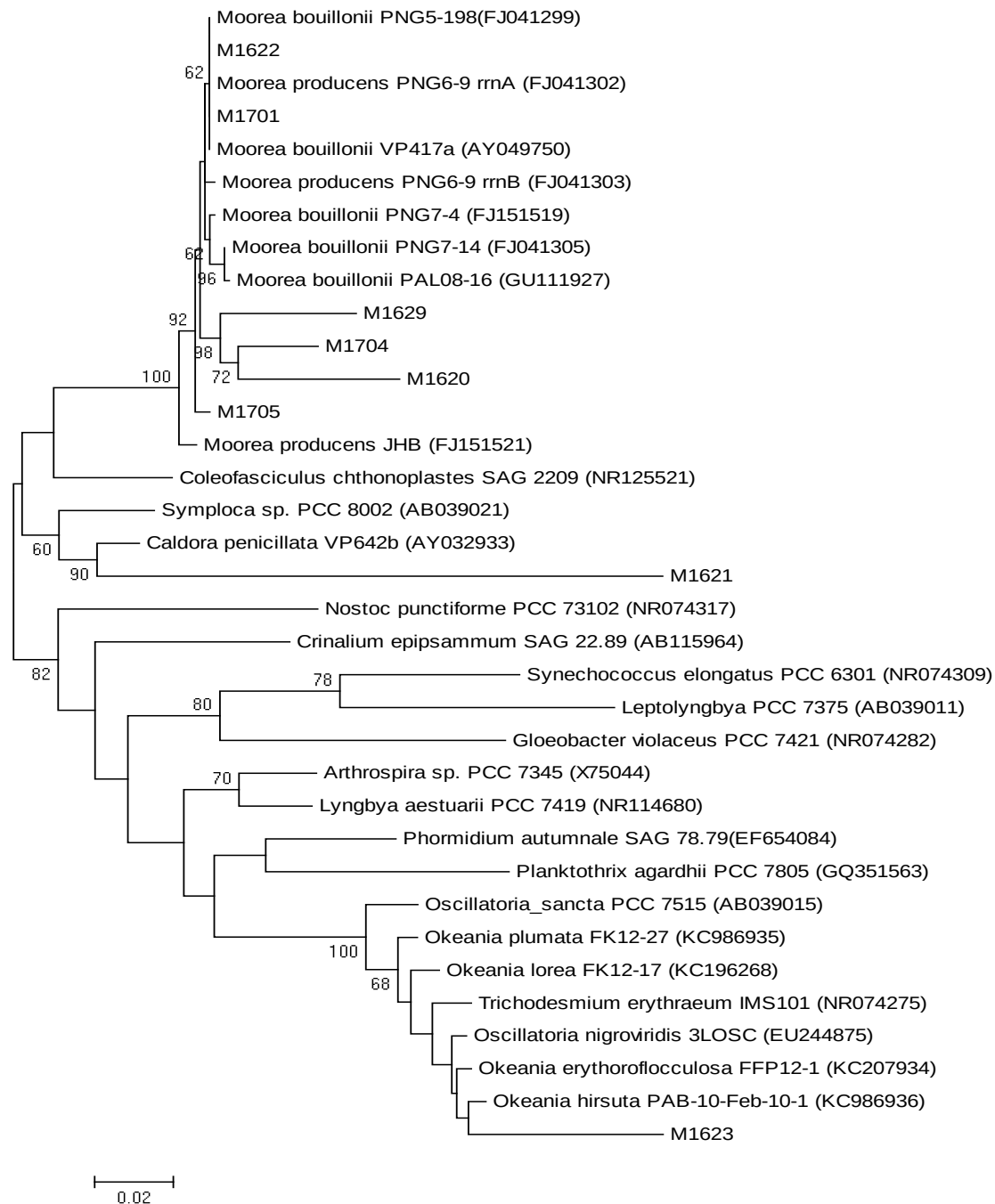


**Figure I-3.** The evolutionary history was inferred by using the Maximum Likelihood method (MEGA 7) based on 16S rRNA gene sequences. Bootstrap support are indicated at the nodes (values < 60% are not shown). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. The final dataset contained a total of 819 positions.

## 1.2 Chemical Profiling of Crude Extracts by LC-MS

### 1.2.1 Experimental Section

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<sub>2</sub>O). The chemical profile of each ethyl acetate (EtOAc), *n*-butanol (BuOH), and water (H<sub>2</sub>O) 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<sub>2</sub>O; 15 - 30 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in Milli-Q water.

### 1.2.2 Results

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. Figure I-4 shows the chemical profiles of *M. bouillonii* samples collected from different locations. All known compounds detected in these fractions were listed in Table I-6. Although the chemical profiles of these three samples (M1622, M1629, and M1705) showed the similar patterns, halogenated compounds were abundant in sample M1629 and M1705 collected from Udar and Mantanani island, respectively compared to the sample M1622 collected in Sepangar island. 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. The chemical profiles of BuOH fractions of these samples contained the same compound like apratoxins, kanamienamide, laingolide and cyanolide though lacking of halogenated compounds. Inconsistent results with high background noise obtained from H<sub>2</sub>O fractions may be because of the presence of large amount of sea salt in the sample.

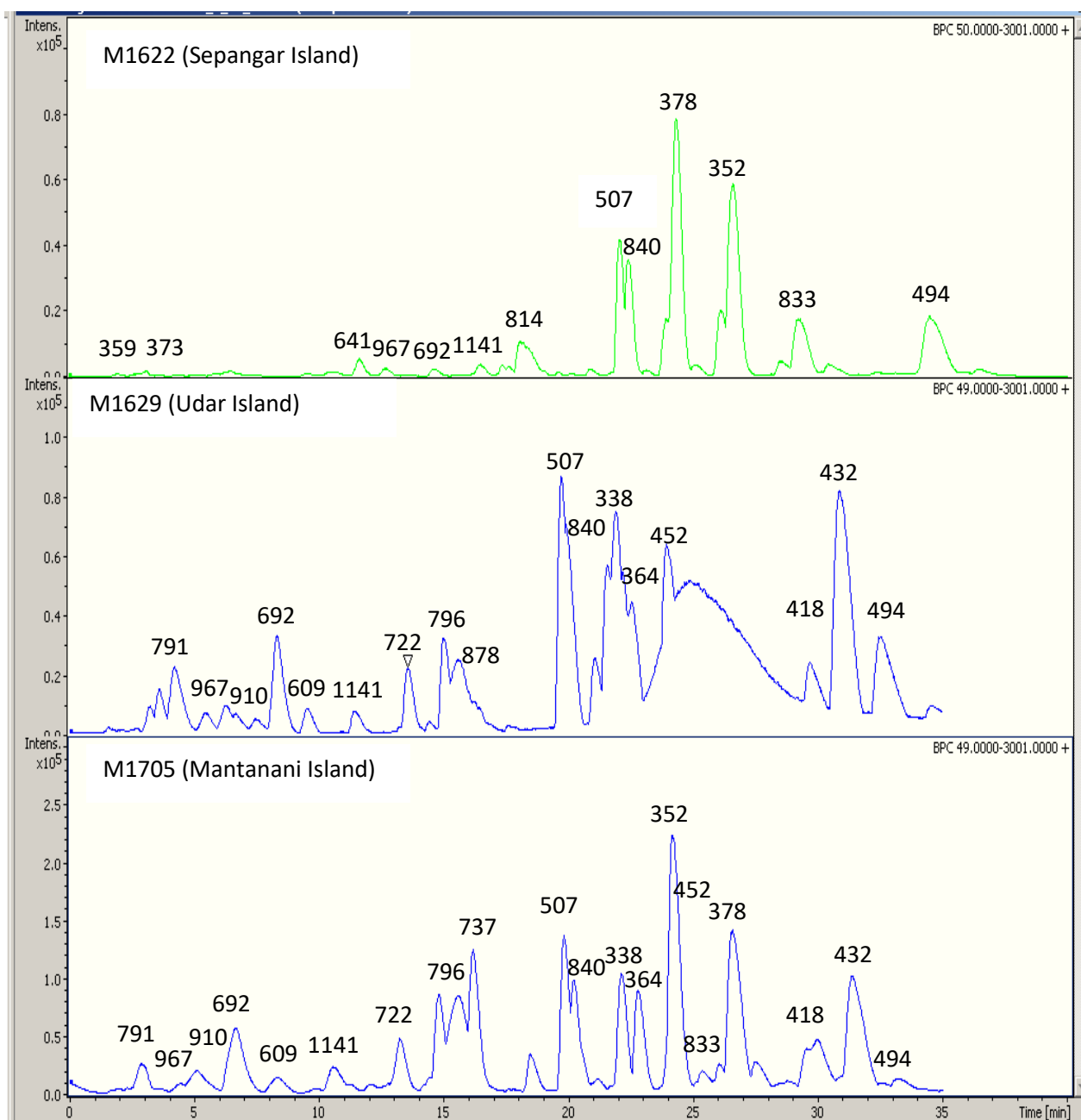


**Figure I-4** Chemical profiles of *M. bouillonii* samples collected from different locations.

**Table I-6.** List of known compounds detected in LC-MS

| <b>Compound</b>             | <b>Retention Time (min)</b> | <b>Calculated Mass (m/z) [M+H]<sup>+</sup></b> | <b>Observed Mass (m/z) [M+H]<sup>+</sup></b> | <b><math>\Delta</math>, ppm</b> | <b>Molecular Formula</b>                                                                      |
|-----------------------------|-----------------------------|------------------------------------------------|----------------------------------------------|---------------------------------|-----------------------------------------------------------------------------------------------|
| <b>laingolide A</b>         | 17.4                        | 338.2695                                       | 338.2649                                     | -13.59                          | C <sub>20</sub> H <sub>35</sub> NO <sub>3</sub>                                               |
| <b>laingolide</b>           | 19.9                        | 352.2851                                       | 352.2886                                     | 9.93                            | C <sub>21</sub> H <sub>37</sub> NO <sub>3</sub>                                               |
| <b>laingolide B</b>         | 15.7                        | 370.2149                                       | 370.2180                                     | 8.37                            | C <sub>20</sub> H <sub>32</sub> ClNO <sub>3</sub>                                             |
| <b>madangolide</b>          | 20.9                        | 378.3082                                       | 378.3083                                     | 0.26                            | C <sub>23</sub> H <sub>39</sub> NO <sub>3</sub>                                               |
| <b>columbamide D</b>        | 25.0                        | 452.2697                                       | 452.2659                                     | -8.40                           | C <sub>23</sub> H <sub>43</sub> Cl <sub>2</sub> NO <sub>3</sub>                               |
| <b>kanamienamide</b>        | 20.7                        | 493.3641                                       | 493.3678                                     | 7.49                            | C <sub>28</sub> H <sub>48</sub> N <sub>2</sub> O <sub>5</sub>                                 |
| <b>lyngbyabellin C</b>      | 10.7                        | 609.0898                                       | 609.0953                                     | 9.02                            | C <sub>24</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>2</sub> O <sub>8</sub> S <sub>2</sub>  |
| <b>ulongamide C</b>         | 9.7                         | 692.3117                                       | 692.3150                                     | 4.76                            | C <sub>36</sub> H <sub>45</sub> N <sub>5</sub> O <sub>7</sub> S                               |
| <b>lyngbyapeptin B</b>      | 14.5                        | 722.3587                                       | 722.3606                                     | 2.63                            | C <sub>38</sub> H <sub>51</sub> N <sub>5</sub> O <sub>7</sub> S                               |
| <b>apratoxin B/C</b>        | 19.2                        | 826.4788                                       | 826.4819                                     | 3.75                            | C <sub>44</sub> H <sub>68</sub> N <sub>5</sub> O <sub>8</sub> S                               |
| <b>cyanolide A</b>          | 26.5                        | 833.4898                                       | 833.4826                                     | -8.63                           | C <sub>42</sub> H <sub>73</sub> O <sub>16</sub>                                               |
| <b>apratoxin A</b>          | 20.4                        | 840.4945                                       | 840.4981                                     | 4.28                            | C <sub>45</sub> H <sub>70</sub> N <sub>5</sub> O <sub>8</sub> S                               |
| <b>lyngbyabellin E or J</b> | 15.9                        | 864.2369                                       | 864.2433                                     | 7.40                            | C <sub>37</sub> H <sub>52</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>12</sub> S <sub>2</sub> |
| <b>lyngbyabellin D or F</b> | 15.2                        | 896.2631                                       | 896.2669                                     | 4.23                            | C <sub>38</sub> H <sub>56</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>13</sub> S <sub>2</sub> |
| <b>wewakazole</b>           | 14.6                        | 1141.5470                                      | 1141. 5326                                   | -12.61                          | C <sub>59</sub> H <sub>73</sub> N <sub>12</sub> O <sub>12</sub>                               |

### 1.3 Biosurfactant and Cytotoxicity Assay Screening

#### 1.3.1 Experimental Section (Cytotoxicity Assay)

Standard MTT assay was performed to check the cytotoxicity against human MCF 7 breast cancer cell line.<sup>67</sup> Cancer cells were cultured in RPMI-1640 (Wako) with L glutamine supplemented by 10% (v/v) fetal bovine serum (FBS, BioWest). Cells were incubated in a humidified atmosphere with 5% of CO<sub>2</sub>, at 37 °C. 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.<sup>68</sup> The

reduction of MTT to formazan is directly proportional to the mitochondrial activity and consequently cell viability. Cells were seeded in 96-well culture plates (10,000 cells per well) in 90  $\mu$ L of RPMI medium supplemented by fetal bovine serum (FBS) 10% for 24 h before treatment at 37°C and with 5% CO<sub>2</sub>. Cisplatin was used as positive control in this study. The test samples were prepared approximately 3 h before treatment. The crude extracts were diluted in medium without FBS to make desired concentrations and 10  $\mu$ L samples were added in the well and incubated for 72 h.

After the incubation time, the medium (100  $\mu$ L) was carefully aspirated from the well because the healthy cells should be attached to the wells/plate. Then, 100  $\mu$ L of MTT solution (0.5 mg/mL) was added. After 3 h incubation, the MTT solution was removed and 100  $\mu$ L of DMSO was added to the well. The residual formazan was dissolved in DMSO and the absorbance was measured at 570 nm in a microplate reader (Multiskan, Japan). The plate was shaken for 10 sec prior to the absorbance measurement. The solution would give a purple color in the existence of the live cells. All of the incubations were done in the incubator at 37 °C and with 5% CO<sub>2</sub>.

### 1.3.2 Results

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  $\mu$ g/ mL.

**Table I-7.** Cytotoxicity vs. human MCF breast cancer cells

| Sample code | Cytotoxicity<br>(%, EtOAc) |          |         | Identity based on 16S rRNA gene sequencing |
|-------------|----------------------------|----------|---------|--------------------------------------------|
|             | 100 µg/mL                  | 10 µg/mL | 1 µg/mL |                                            |
| M1620       | 100                        | 92       | 91      | <i>Moorea producens</i>                    |
| M1622       | 100                        | 90       | 4       | <i>Moorea bouillonii</i>                   |
| M1623       | 83                         | 48       | 41      | <i>Okeania</i> sp.                         |
| M1624       | 68                         | 44       | 46      | Uncultured bacterium clone                 |
| M1625       | 46                         | -8       | 10      | -                                          |
| M1629       | 100                        | 96       | 8       | <i>Moorea bouillonii</i>                   |
| M1630       | 100                        | 98       | 90      | <i>Moorea bouillonii</i>                   |
| M1631       | 100                        | 86       | 54      | -                                          |
| M1632       | 86                         | 17       | -14     | -                                          |
| M1634       | 98                         | 58       | 13      | -                                          |
| M1635       | 74                         | 26       | 10      | -                                          |
| M1701       | 97                         | 96       | 88      | <i>Moorea bouillonii</i>                   |
| M1704       | 89                         | 97       | 88      | <i>Moorea bouillonii</i>                   |
| M1705       | 96                         | 92       | 47      | <i>Moorea bouillonii</i>                   |

### 1.3.3 Experimental Section (Biosurfactant Activity)

**Oil Displacement Assay.** The oil displacement test was performed by determining the diameter of the oil displacement area.<sup>69</sup> For this assay, 10 µL of crude oil were added to the surface of 40 mL of distilled H<sub>2</sub>O in a 15 cm glass petri dish to form a thin oil layer. The crude oil used in this experiment was manufactured by Tokyo Chemical Industry Co., LTD (S0432). Extracted sample were dissolved in H<sub>2</sub>O for hydrophilic fractions and EtOH for lipophilic fractions to make final concentration 10 mg/mL. Then, 10 µL of extracted samples were gently placed on the center of the oil layer. If biosurfactant was present in the sample, the oil was displaced and a clearing zone was formed. The clearing zone was observed by light and the diameter of this zone was measured with a vernier Caliper. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity.

### 1.3.4 Results

The preliminary screening of marine samples was done by oil displacement assay. In oil displacement assay the clearing zone is an indirect measurement of the activity of the tested sample against crude oil, the higher clearing zone represents the higher activity.<sup>70</sup> Each fraction (Ethyl acetate, butanol and water) from solvent partition results were tested. The oil displacement results showed that the ethyl acetate fraction had the potent activity to remove the crude oil by oil displacement assay.

**Table I-8.** Oil spreading assay results of EtOAc, BuOH and H<sub>2</sub>O fractions

| Species name                      | Oil displacement area (mm) |          |                      |
|-----------------------------------|----------------------------|----------|----------------------|
|                                   | EtOAc Fr.                  | BuOH Fr. | H <sub>2</sub> O Fr. |
| M1620( <i>Moorea producens</i> )  | 139                        | 88       | 1                    |
| M1622 ( <i>Moorea bouilloni</i> ) | 102                        | 0        | 5                    |
| M1623 ( <i>Okeania sp</i> )       | 79                         | 101      | 12                   |
| M1624                             | 54                         | 101      | 0                    |
| M1625                             | 99                         | 0        | 12                   |
| M1626                             | 101                        | 0        | 8                    |
| M1627                             | 139                        | 49       | 0                    |
| M1628                             | 104                        | 30       | 0                    |
| M1629 ( <i>Moorea bouilloni</i> ) | 91                         | 0        | 5                    |
| M1630                             | 129                        | 30       | 0                    |
| M1631                             | 120                        | 44       | 1                    |
| M1632                             | 109                        | 71       | 0                    |
| M1634                             | 79                         | 4        | 12                   |
| M1635                             | 109                        | 51       | 44                   |
| M1701 ( <i>Moorea bouilloni</i> ) | 100                        | 40       | 25                   |
| M1705 ( <i>Moorea bouilloni</i> ) | 110                        | 60       | 35                   |
| M1704 ( <i>Moorea bouilloni</i> ) | 98                         | 55       | 40                   |

## 1.4 Discussion

Marine cyanobacteria were identified successfully by 16S rRNA gene sequencing (Table I-5). Some samples did not show good results because of the quality of DNA or lack of the amount of DNA or some species are not cyanobacteria. On the basis of 16S rRNA gene sequencing the species M1620 was identified as *M. producents* while M1622, and M1629 are close to *M. bouillonii*. The morphology of M1629 and M1630 were similar and collected from the same location and depth. The LC-MS analyses of these two samples revealed the same chemical profile. On the basis of these results we supposed that this two species are *M. bouillonii*. The species labeled as M1621 and M1623 are *Symploca* sp. and *Okeania* sp. respectively. The samples collected from Mantanani island in November 2017 labeled as M1701, M1704 and M705 were identified as *M. bouillonii*. These results concluded that most of the cyanobacteria samples collected from different locations of Sabah, Malaysia were identified as *M. bouillonii*.

16S rRNA gene sequencing is a powerful approach to identify the cyanobacteria having similar morphology. However, sometimes it is difficult to differentiate between *M. bouillonii* and *M. producents*. In 2012, Eugene and colleagues proposed that this *M. producents* and *M. bouillonii* had distinct colony morphology and *M. bouillonii* always attached to surrounding substrate and each colony associated with snapping shrimp *Alpheus frontalis*.<sup>23</sup> In our collected samples we also observed the similar features for *M. bouillonii* samples labeled as M1622, M1629, M1701, M1704 and M1705.

The LC-MS profiles of *M. bouillonii* samples showed the richness of secondary metabolites including known cytotoxic compound apratoxins. Although all *M. bouillonii* samples showed the similar profile of known compounds, samples labeled as M1629, M1704 and M1705 exhibited the presence of additional unknown halogenated compounds ( $m/z$  935, 967, 910, 878, 434).

As the EtOAc fractions exhibited the presence of known compounds with a number of unknown metabolites based on MarinLit database, these fractions were subjected to cytotoxicity assay against human MCF7 breast cancer cells. Most of the *M. bouillonii* samples showed

cytotoxicity. These toxicities may results from the apratoxins, lyngbyabellins or unknown compounds.

Besides the cytotoxicity assay we also checked the fractions from marine cyanobacteria for their surface activities using oil displacement assay. 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. On the basis of screening results and chemical profile, sample M1705and M1629 were selected to isolate new metabolites.

### **1.5 Summary**

Nine samples were successfully identified based on their morphology and 16S rRNA gene sequence analysis. 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 Three New Columbamides F-H from *Moorea bouillonii*

In chapter 1, a sample (M1705) collected from Mantanani Island, Sabah, Malaysia was selected for the isolation of new compounds based on the biosurfactant activity and the amount of sample. Chemical profiling of organic extracts by LC-MS detected the presence of halogenated compounds and three new halogenated acyl amides, columbamide F-H. The structure elucidations of new compounds were done by using mass spectrometric and NMR spectroscopic analyses. The absolute configuration was done by Marfey's analysis and Ohruai-Akasaka method followed by chiral-phase HPLC analysis.

#### 2.1 Isolation of Apratoxin A and C, Columbamides F-H

##### 2.1.1 Experimental Section

**General Procedures.** MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer. The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 µm, flow rate 0.2 mL/min at 25 °C) and operated under the following conditions: 0-15 min 50-80% MeCN with 0.1% (v/v) formic acid (FA) in Milli-Q H<sub>2</sub>O; 15-30 min, isocratic 80% MeCN with 0.1% (v/v) FA. Preparative HPLC systems used a Cosmosil Cholesterol column (10 × 250 mm, 5 µm) at a flow rate of 3 mL/min, with UV detection at 210 nm.

**Extraction and Isolation.** The cyanobacterial sample (M1705) was collected from Rocky Point, Mantanani Island, Sabah, Malaysia (06°42'16" N; 116°19'23" E) in November, 2017 by using scuba diving at a depth of 5-10 m. The sample was cleaned by removing foreign particles and the seawater was squeezed out by hand before storing in MeOH for transportation. The details of isolation are illustrated in schematic diagram (Figure II-I). The *M. bouillonii* cyanobacterium sample was homogenized at 10,000 rpm for 10 min with MeOH and extracted three times. The extract was filtered and concentrated *in vacuo*. The residue of the MeOH extract was partitioned

between EtOAc and H<sub>2</sub>O, and then BuOH. These fractions were dried and subjected to an oil displacement assay for biosurfactant activity and a cytotoxicity assay against MCF7 breast cancer cell line. The chemical profiling of EtOAc and BuOH fractions revealed the presence of known cytotoxic compounds including apratoxins, wewakazole and possible new halogenated compounds according to the MarinLit database. 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 solvents were removed *in vacuo* using a rotary evaporator and the sample was moved to a clean small vial to be weighed. The 50:50 (v/v) hexane-EtOAc fraction was then subjected to semi-preparative reversed-phase HPLC (gradient 0-30 min 50-80% MeCN; 30-40 min 80-100% MeCN, Cosmosil cholesterol, 10 × 250 mm, 3 mL/min and UV detection at 210 nm) using HPLC grade MeCN (Wako) and MilliQ H<sub>2</sub>O to obtain columbamides F (**43**) (5 mg, *t<sub>R</sub>*=43.3 min) and G (**44**) (2 mg, *t<sub>R</sub>*=44.7 min). The silica gel fraction that eluted with 100% EtOAc was subjected to RP-HPLC with similar condition to obtain columbamide H (**45**) (3.5 mg, *t<sub>R</sub>*=39.1 min). Then compounds **43** and **45** were further purified using RP-HPLC (gradient 0-30 min, 80-100% MeCN, Cosmosil cholesterol, 4.6 × 250 mm, 1 mL/min and UV detection at 210 nm ) to yield **43** (3.2 mg, *t<sub>R</sub>*=13.9 min) and **45** (2 mg, *t<sub>R</sub>*= 12 min).

*Columbamide F (43)*: colorless oil; [ $\alpha$ ]<sub>D</sub><sup>22</sup> +6.2 (c 0.29, CHCl<sub>3</sub>); IR (neat)  $\lambda_{\text{max}}$  2929, 2856, 1743, 1648, 1456, 1109, 1042 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table II-1; HRESIMS *m/z* 494.2816 [M+H]<sup>+</sup> (calcd for C<sub>25</sub>H<sub>46</sub>Cl<sub>2</sub>NO<sub>4</sub>, 494.2804)

*Columbamide G (44)*: colorless oil; [ $\alpha$ ]<sub>D</sub><sup>23</sup> +9 (c 0.07, CHCl<sub>3</sub>); IR (neat)  $\lambda_{\text{max}}$  2923, 2853, 1741, 1651, 1456, 1106 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table II-1; HRESIMS *m/z* 528.2451 [M+H]<sup>+</sup> (calcd for C<sub>25</sub>H<sub>45</sub>Cl<sub>3</sub>NO<sub>4</sub>, 528.2414)

*Columbamide H (45)*: colorless oil; [ $\alpha$ ]<sub>D</sub><sup>23</sup> +23.1 (c 0.06, CHCl<sub>3</sub>); IR (neat)  $\lambda_{\text{max}}$  3394, 2925, 2855, 1624, 1456, 1121 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table II-1; HRESIMS *m/z* 418.3042 [M+H]<sup>+</sup> (calcd for C<sub>23</sub>H<sub>45</sub>ClNO<sub>3</sub> 418.3087)

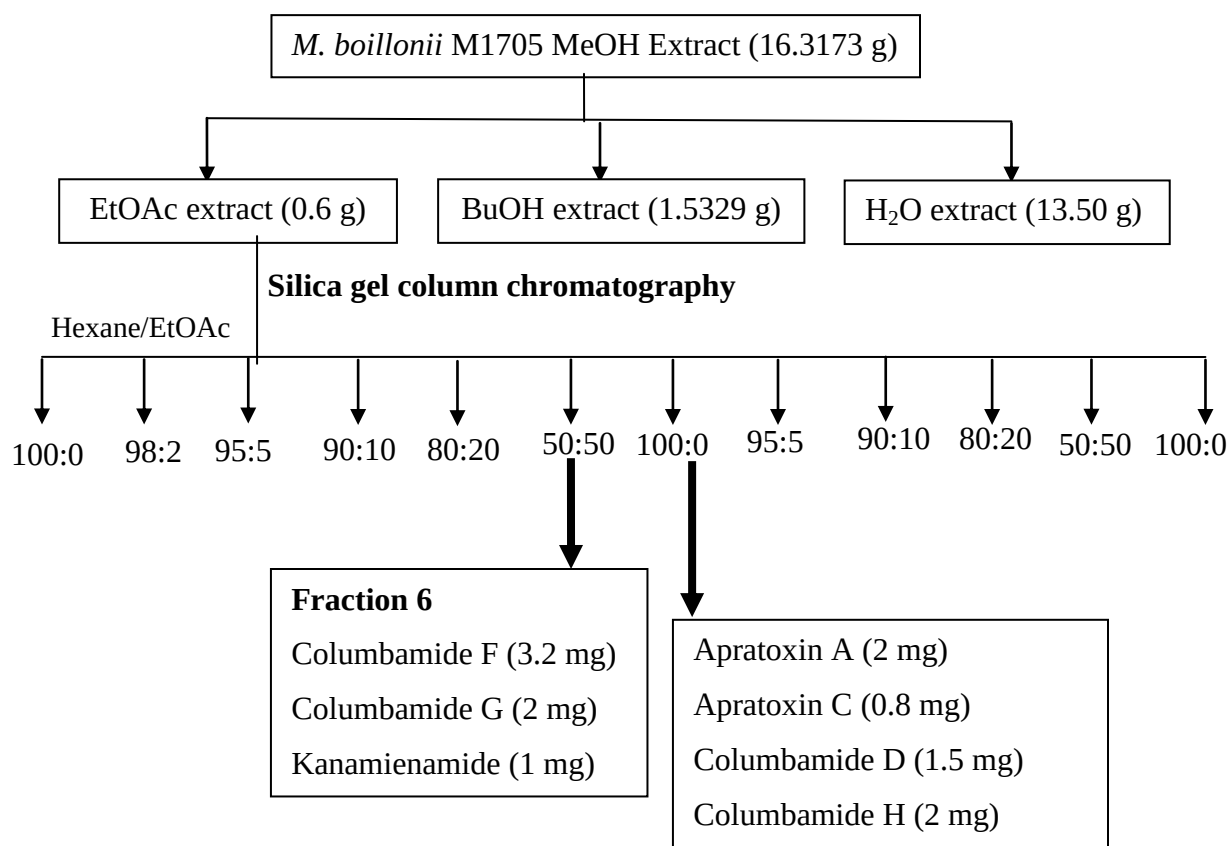


**Fig. II-1.** Isolation scheme of *M. bouillonii* (M1705).

### 2.1.2 Results

The chemical profiling of the EtOAc fraction by LC-MS exhibited the presence of a number of known compounds including apratoxins A and C, wewakazole, lyngbyabellin A, laingolides, kanamienamide and additional possibly new chlorinated compounds. The EtOAc fraction was subjected to normal phase silica gel column chromatography and divided into 12 fractions. The silica gel fractions of hexane/EtOAc (50:50-v/v), 100% EtOAc and EtOAc/MeOH (90:10-v/v) exhibited higher value for the diameter of clear zone (~140 mm) compared to other fractions in an oil displacement assay. On the basis of their bioactivity and chemical profile these fractions were chosen for the isolation of new halogenated compounds. The LC-MS analysis of hexane/EtOAc (50:50-v/v) fraction revealed the presence of cytotoxic kanamienamide as well as di- and tri- chlorinated compounds with  $m/z$  494 and 528, respectively (Figure II-2). These chlorinated columbamides were isolated and purified along with other known compound kanamienamide and unknown compounds with  $m/z$  507 and  $m/z$  434. Unfortunately, the isolated amounts of these unknown compounds were not enough for NMR analysis. Another new columbamide with monochlorinated pattern was purified from the 100% EtOAc fraction along with known compounds columbamide D, apratoxins A and C and confirmed by comparing their  $^1\text{H}$  NMR chemical shift data. The HPLC chromatogram for hexane/EtOAc (50:50-v/v) and 100% EtOAc fraction is shown in Figure II-3. The structure elucidations of three new columbamides are discussed in the next section. The  $^1\text{H}$  NMR spectra for known compounds kanamienamide, apratoxin A and C, columbamide D are shown in Figures II-4 to II-7.

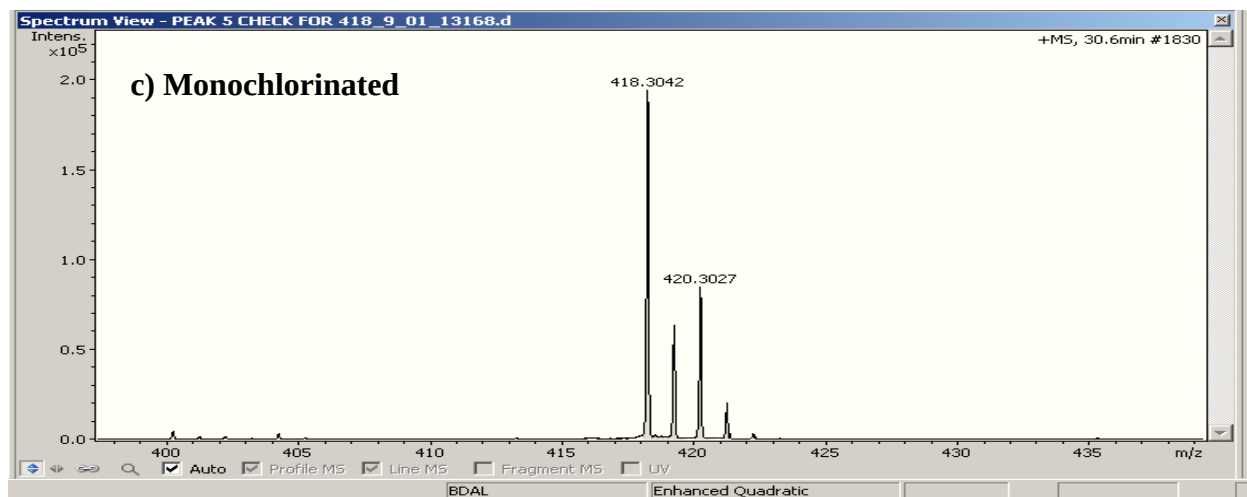
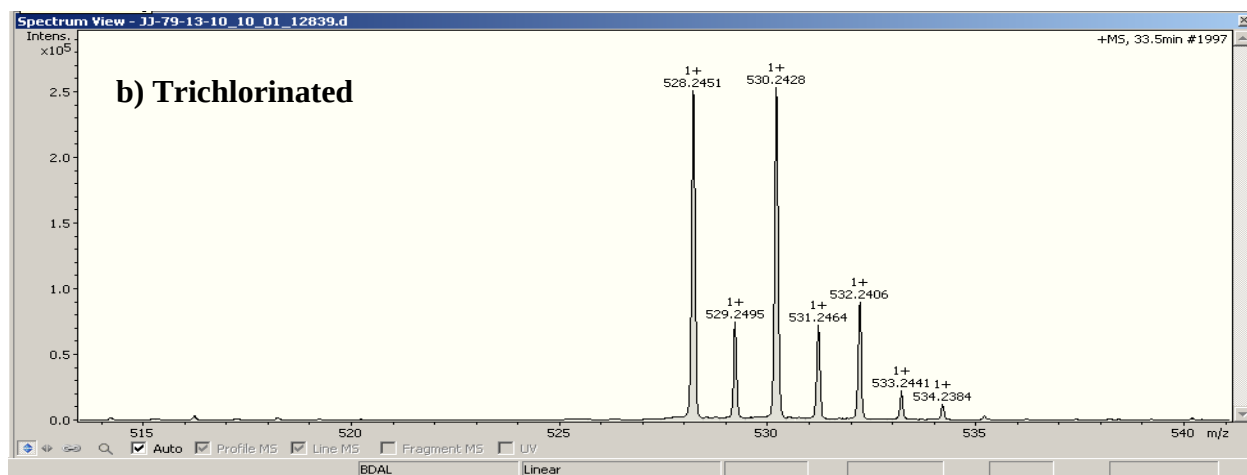
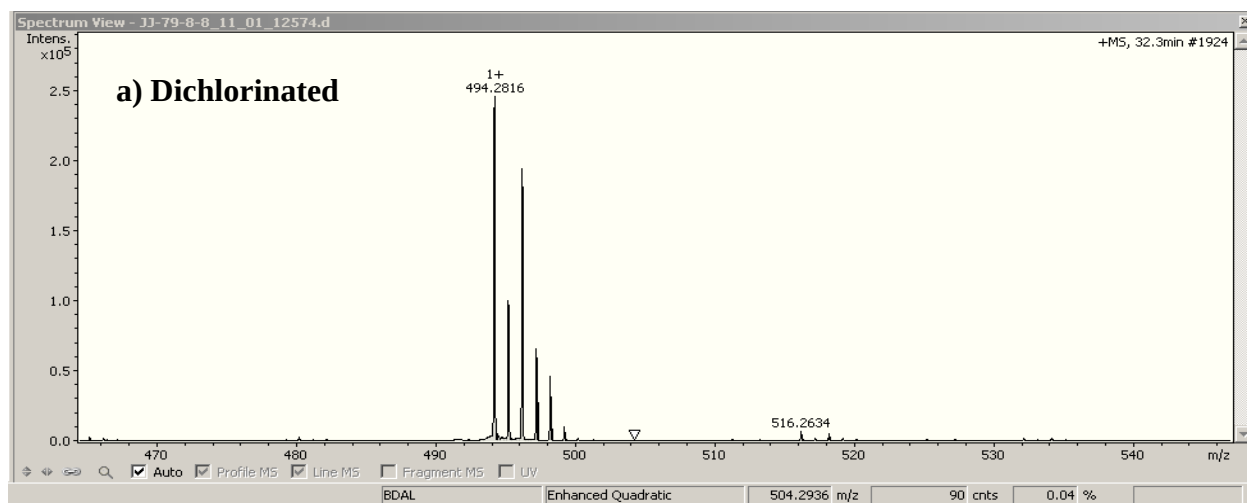


**Figure II-2.** a) dichlorinated, b) trichlorinated and c) monochlorinated mass pattern of **43**, **44** and **45**, respectively.

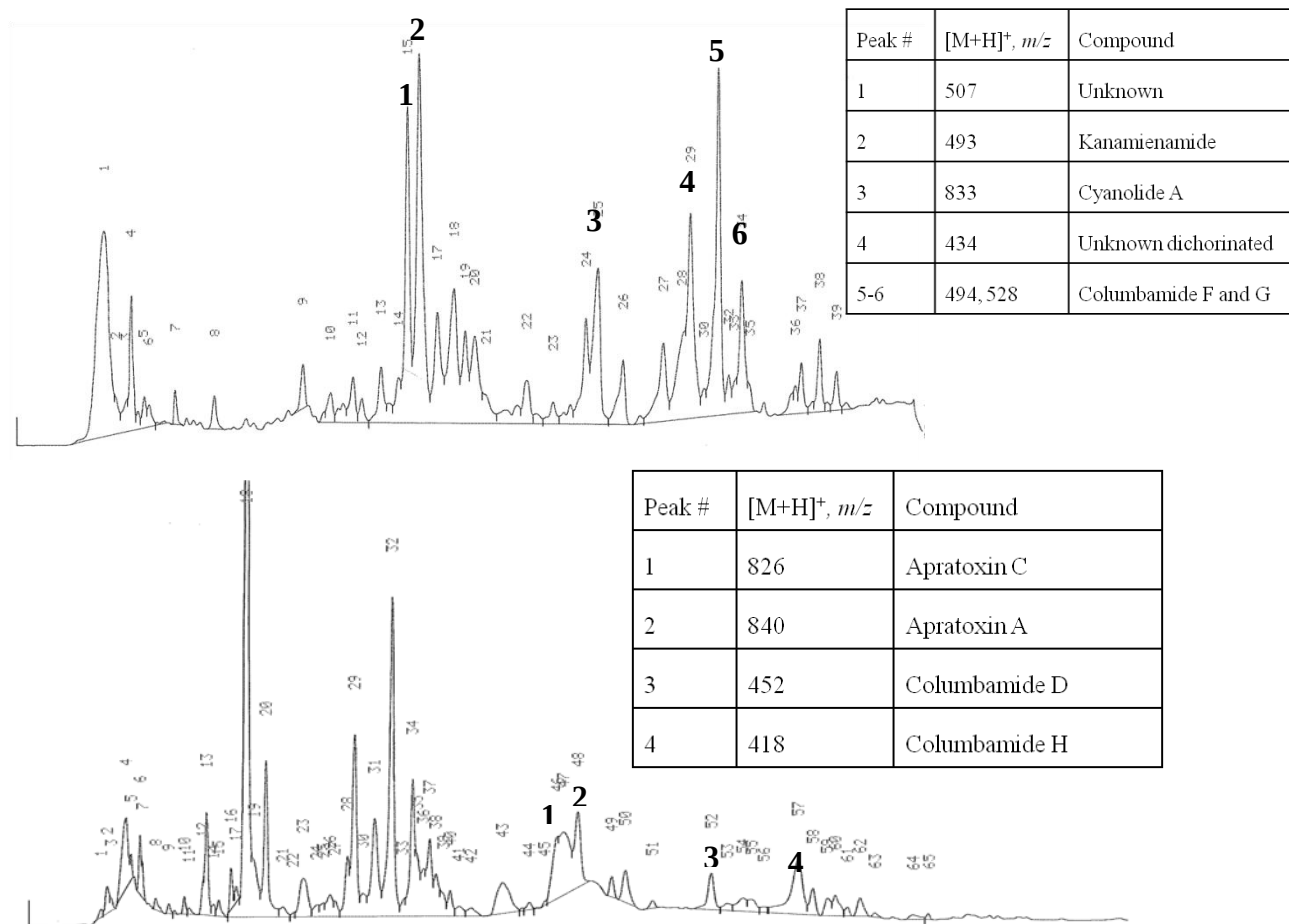


**Figure II-3.** HPLC chromatogram of the hexane/EtOAc (50:50-v/v) 100% EtOAc fraction. The mass data reported in the tables was obtained by LC-MS.

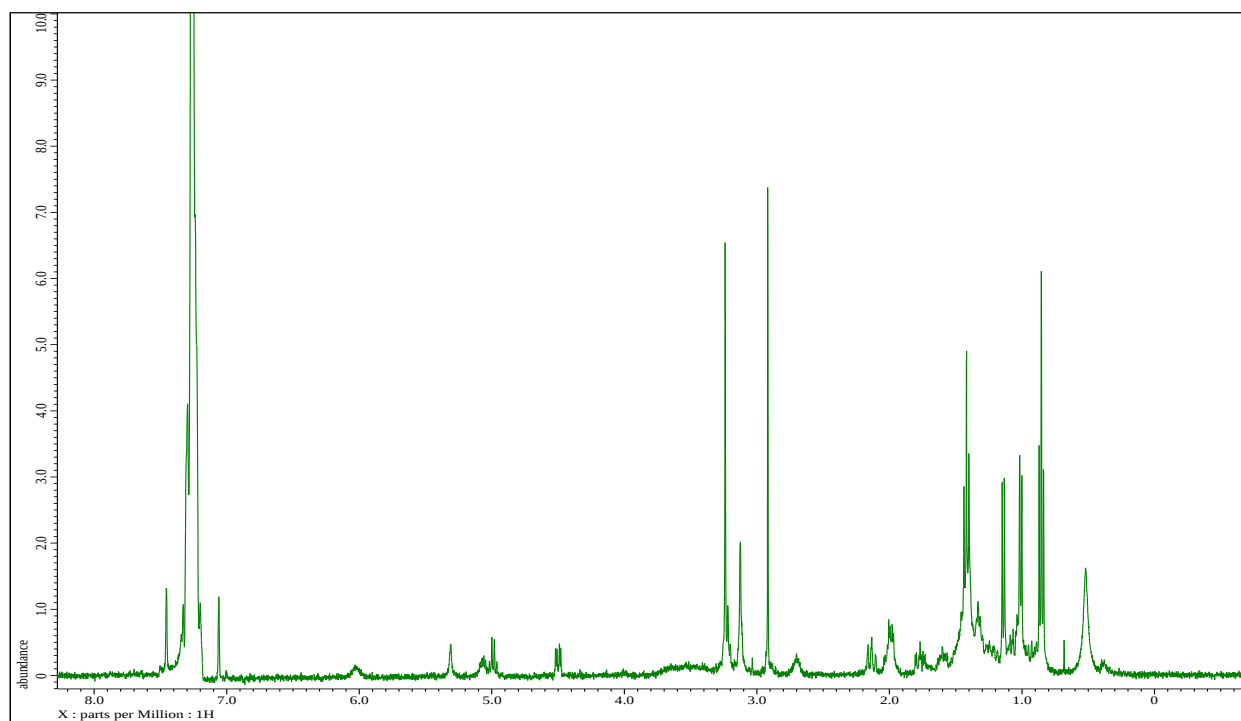


**Figure II-4.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) of kanamienamide (**27**).

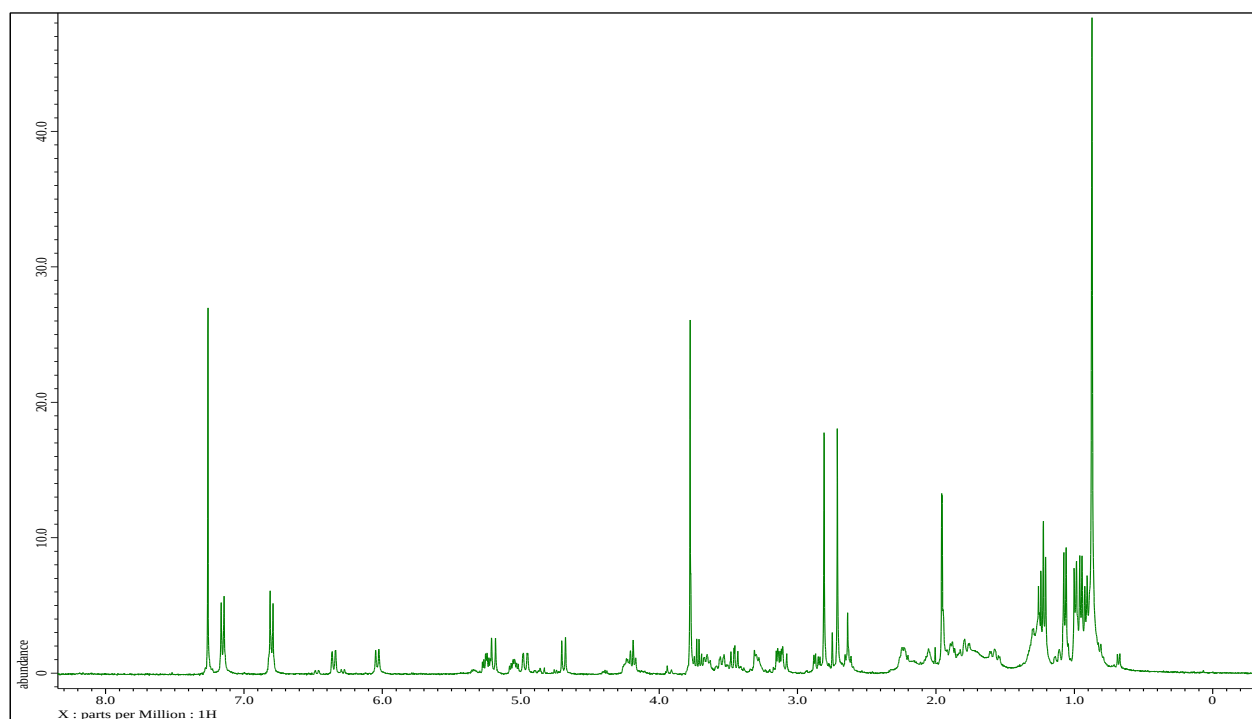


**Figure II-5.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) of apratoxin A (**30**).

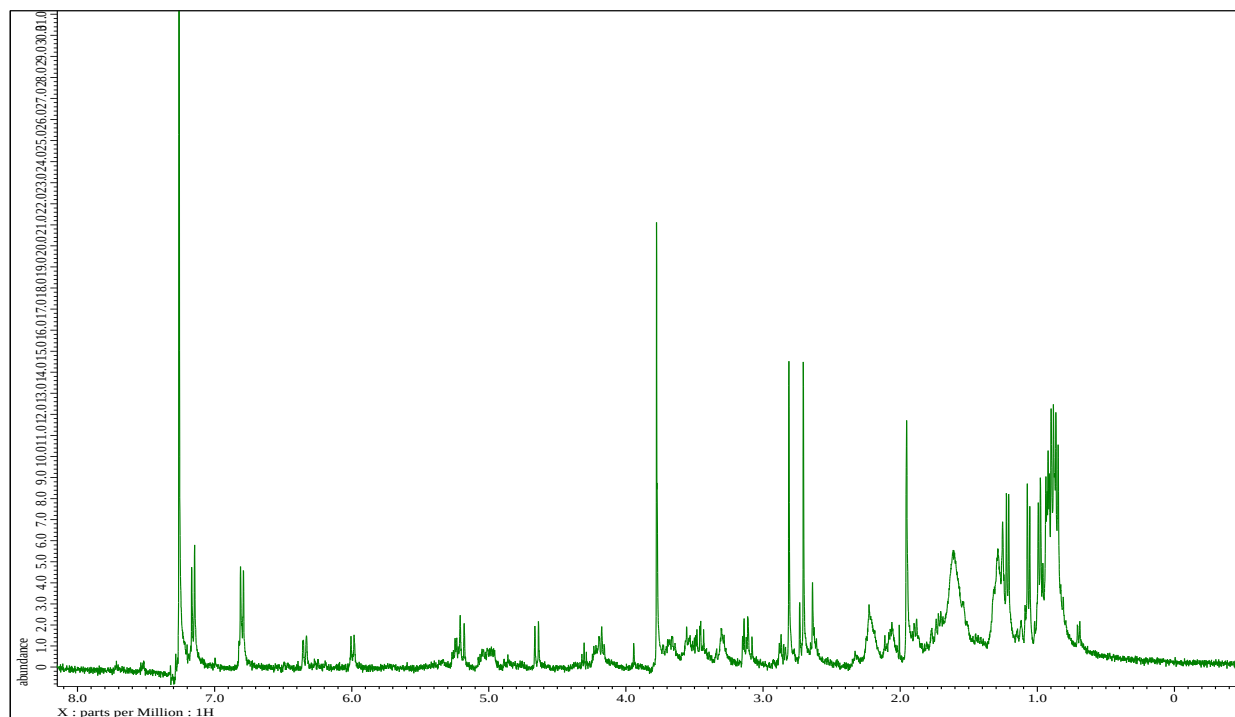


**Figure II-6.**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) of apratoxin C (**34**).

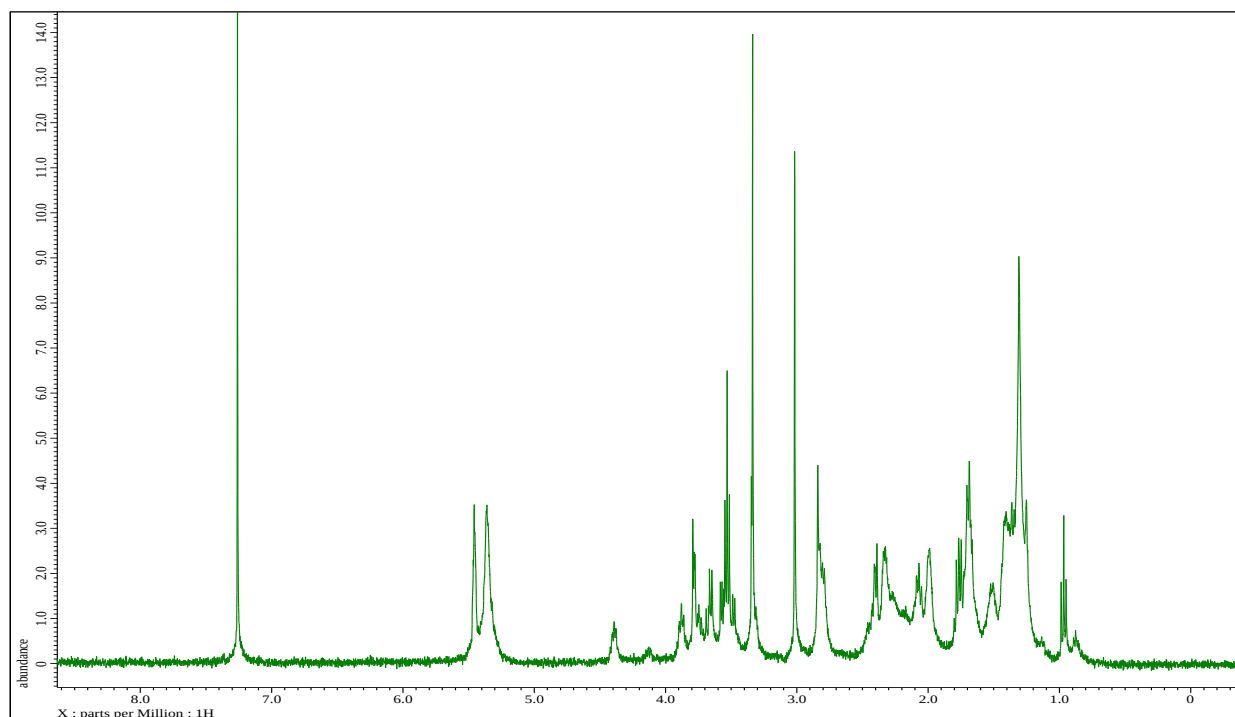


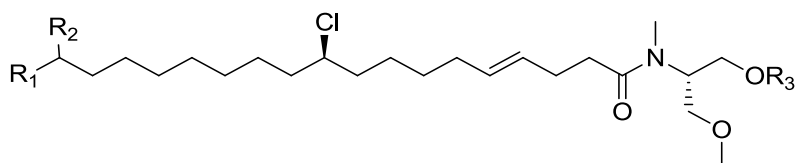
**Figure II-7.**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) of columbamide D (**39**).

## 2.2 Structure Elucidations of Columbamide F-H

### 2.2.1 Experimental Procedure

Optical rotation data were measured on a HORIBA SEPA-300 polarimeter using chloroform as a solvent. IR spectra were measured on a JASCO FT/IR-4100 type A. The  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and 2D NMR spectra for compounds **43**, **44** and **45** were acquired using a JEOL 600 MHz spectrometer. For all NMR experiments,  $\text{CDCl}_3$  (Cambridge Isotope Laboratories, Inc.) was used as solvent as an internal standard ( $\delta_{\text{H}}$  7.26 and  $\delta_{\text{C}}$  77.0). LC-MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer.

### 2.2.2 Results



Columbamide F (**43**),  $\text{R}_1=\text{Cl}$   $\text{R}_2=\text{H}$   $\text{R}_3=\text{Ac}$   
Columbamide G (**44**),  $\text{R}_1=\text{Cl}$   $\text{R}_2=\text{Cl}$   $\text{R}_3=\text{Ac}$   
Columbamide H (**45**),  $\text{R}_1=\text{H}$   $\text{R}_2=\text{H}$   $\text{R}_3=\text{H}$

The planar structure of **43** was established by analyzing NMR and MS data (Figure II-8 and Table II-1). The molecular formula of **43** was determined to be  $\text{C}_{25}\text{H}_{45}\text{Cl}_2\text{NO}_4$  by ESITOFMS, suggesting three degrees of unsaturation. The presence of carbonyls ( $\delta_{\text{C}}$  173.3, 170.7), two olefinic methines ( $\delta_{\text{C}}$  129.1, 130.9) with identical protons ( $\delta_{\text{H}}$  5.46), a chlorinated methine ( $\delta_{\text{C}}$  64.2,  $\delta_{\text{H}}$  3.88), an *N*-methyl ( $\delta_{\text{C}}$  31.7,  $\delta_{\text{H}}$  2.95), an *N*-methine ( $\delta_{\text{C}}$  51.9,  $\delta_{\text{H}}$  4.90), a chlorinated methylene ( $\delta_{\text{C}}$  45.1,  $\delta_{\text{H}}$  3.53), a methoxy ( $\delta_{\text{C}}$  58.9,  $\delta_{\text{H}}$  3.34), two oxo-methylenes ( $\delta_{\text{C}}$  70.9,  $\delta_{\text{H}}$  3.57 and  $\delta_{\text{C}}$  62.0,  $\delta_{\text{H}}$  4.26), and other methylenes ( $\delta_{\text{C}}$  26.4-38.4,  $\delta_{\text{H}}$  1.31-2.42) revealed the similarities of this compound with the columbamides.<sup>42,44</sup> The mass difference between columbamide D (**39**) and **43** is 42, indicating the possibility of the acetylation of columbamide D for **39**. Columbamide A has an acetyl function as well. An acetylated *N,O*-dimethylserinol moiety was elucidated by HMBC correlations from both the methyl singlet  $\text{H}_3\text{-24}$  and the deshielded methylene protons  $\text{H}_2\text{-22}$  to an acetoxycarbonyl at  $\delta_{\text{C}}$  170.7,  $\text{H}_2\text{-20}$  to  $\text{H}_2\text{-21}$  and  $\text{H}_2\text{-22}$

by COSY, H-20 to C-19 and H<sub>3</sub>-25 to C-21 by HMBC, and H-20 to C-21 and C-22 by H2BC. The connection between C-1 and H-19 was also made by HMBC correlations. COSY and H2BC correlations allowed the assignment from H-3 to H-4. The double bond between C-4 and C-5 was established by H2BC signals from H-4/5 to C-3 and C-6 and corresponding HMBC signal from H-5 to C-4. COSY signals from H-5 to H-6, H-6 to H-7, and H-8 to H-9 allowed the connections of these protons to the adjacent methylenes. The position of the chlorine atom at position C-10 was established by the following signals: COSY correlations between H-10 to H-9 and 11, H2BC correlations between H-10 to C-9 and 11 as well as HMBC correlations between H-10 to C-8, 9, 11 and 12. The assignment of methylenes from C-11 to C-17 was confirmed by COSY, HMBC and H2BC correlations. The position of the second chlorine at the terminal C-18 was supported by COSY signals from H-17 to H-18 and vice versa. The corresponding H2BC and HMBC signals completed the planar structure of columbamide F (**43**) (Figure II-8). The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectrum and 2D NMR data (HSQC, COSY, TOCSY, HMBC, and H2BC) for **43** are shown in Figure II-9 to II-15.

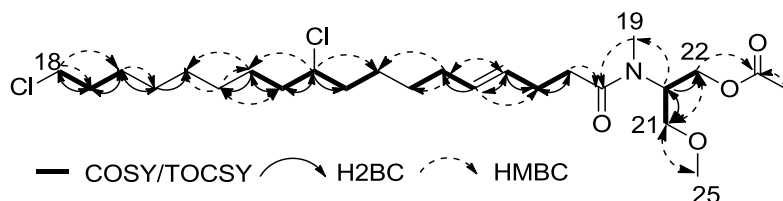


**Figure II-8.** 2D NMR correlations for **43**.

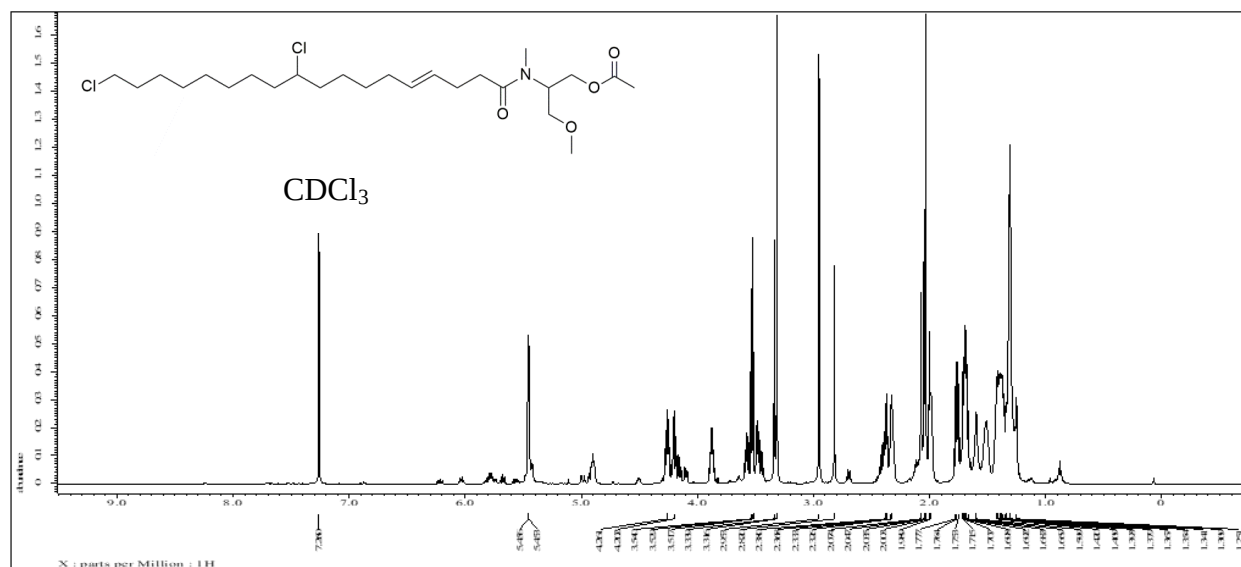


**Figure II-9.**  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide F (43).

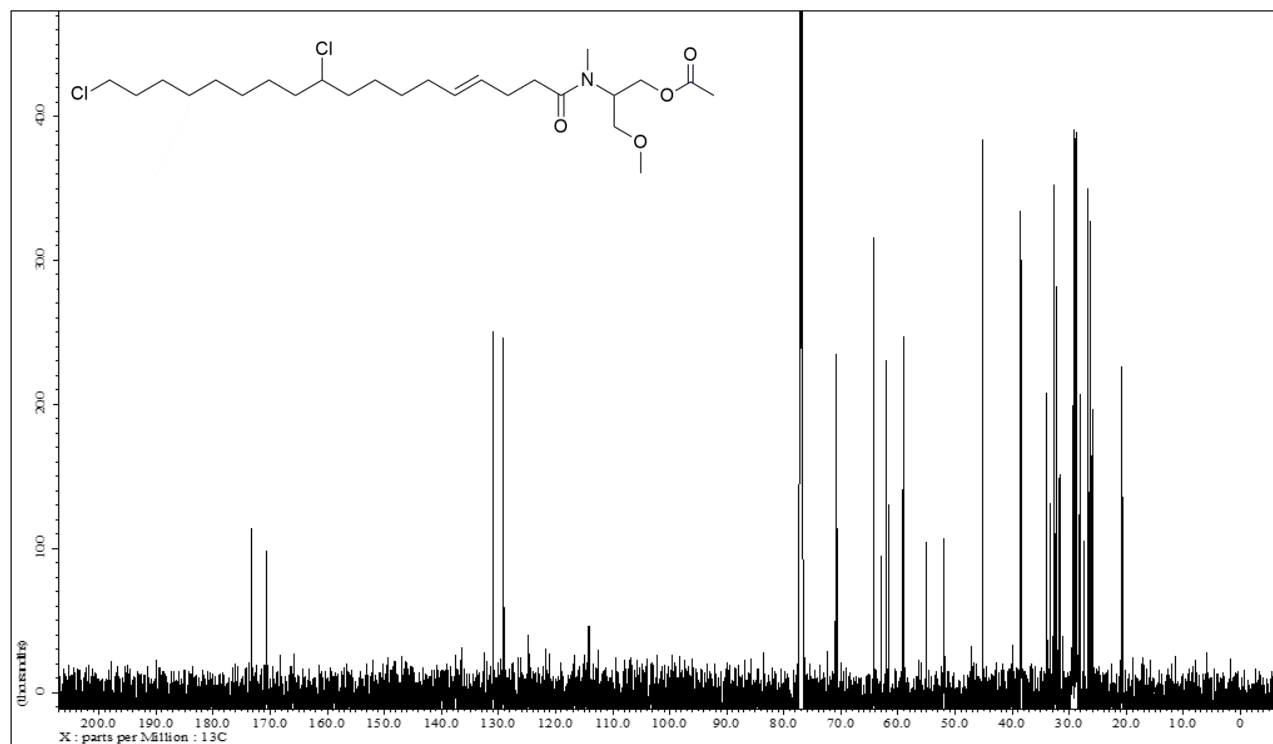


**Figure II-10.**  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide F (43).

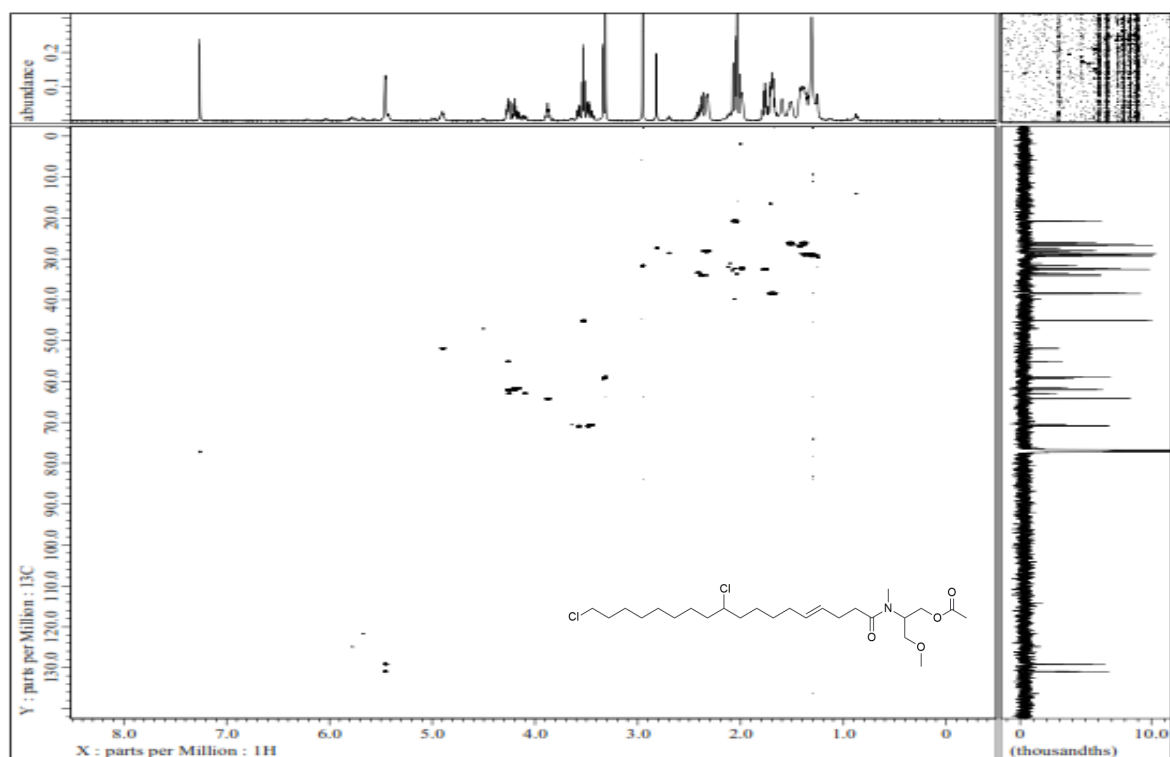


**Figure II-11.** HSQC data (600 MHz, CDCl<sub>3</sub>) of columbamide F (43).

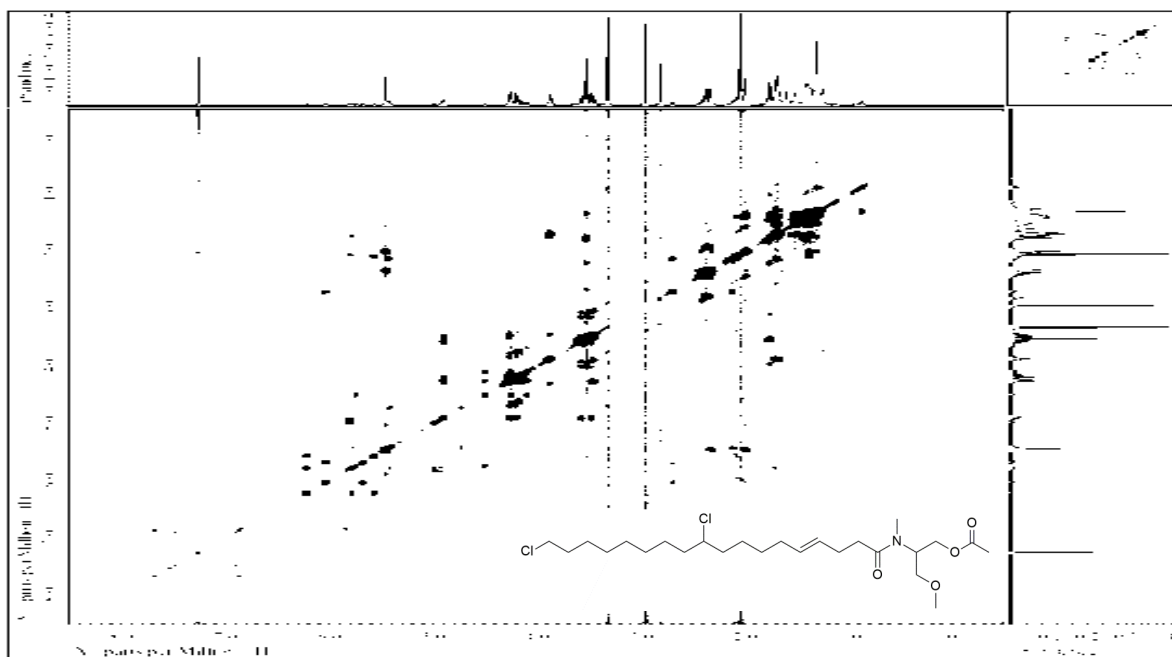


**Figure II-12.** COSY data (600 MHz, CDCl<sub>3</sub>) of columbamide F (43).

**Table II-1. NMR Spectroscopic Data for Columbamides F (43), G (44) and H (45) in CDCl<sub>3</sub>**

| Columbamide F (43) |                              |                                              |                             |                        | Columbamide G (44)           |                                          | Columbamide H (45)     |                                   |
|--------------------|------------------------------|----------------------------------------------|-----------------------------|------------------------|------------------------------|------------------------------------------|------------------------|-----------------------------------|
| Position           | $\delta_C^a$ , Type          | $\delta_H$ (J in Hz) <sup>b</sup>            | COSY/<br>TOCSY <sup>b</sup> | HMBC/H2BC <sup>b</sup> | $\delta_C^c$ , Type          | $\delta_H$ (J in Hz) <sup>b</sup>        | $\delta_C^c$ , Type    | $\delta_H$ (J in Hz) <sup>b</sup> |
| 1                  | 173.3, C                     |                                              |                             |                        | 173.2, C                     |                                          | 174.3, C               |                                   |
| 2                  | 34.02, CH <sub>2</sub>       | 2.38, m                                      | 3                           | 1, 3'                  | 34.02, CH <sub>2</sub>       | 2.38, m                                  | 33.9, CH <sub>2</sub>  | 2.40, m                           |
| 3                  | 28.0, CH <sub>2</sub>        | 2.33, m                                      | 4                           | 2', 4'                 | 28.0, CH <sub>2</sub>        | 2.33, m                                  | 28.0, CH <sub>2</sub>  | 2.34, m                           |
| 4                  | 129.1, CH                    | 5.46, m                                      | 3, 3'                       | 3', 5', 6              | 129.1, CH                    | 5.45, m                                  | 128.9, CH              | 5.46, m                           |
| 5                  | 130.9, CH                    | 5.46, m                                      | 6                           | 3, 4, 4', 6'           | 130.9, CH                    | 5.45, m                                  | 131.1, CH              | 5.46, m                           |
| 6                  | 32.6, CH <sub>2</sub>        | 2.00, m                                      | 5, 7                        | 4, 5, 7, 8             | 32.3, CH <sub>2</sub>        | 2.00, m                                  | 32.3, CH <sub>2</sub>  | 2.0, m                            |
| 7                  | 28.8, CH <sub>2</sub>        | 1.31, m*                                     | 6                           |                        | 28.9, CH <sub>2</sub>        | 1.33, m                                  | 28.04, CH <sub>2</sub> | 1.32, m                           |
| 8                  | 25.9, CH <sub>2</sub>        | 1.51, 1.39, m                                | 9                           |                        | 25.8, CH <sub>2</sub>        | 1.39, m                                  | 25.9, CH <sub>2</sub>  | 1.38, m                           |
| 9                  | 38.5, CH <sub>2</sub>        | 1.69, m                                      | 8, 10'                      |                        | 38.5, CH <sub>2</sub>        | 1.70, m                                  | 38.3, CH <sub>2</sub>  | 1.70, m                           |
| 10                 | 64.2, CH                     | 3.88, m                                      | 9, 9', 11                   | 8, 9, 9', 11', 11, 12  | 64.2, CH                     | 3.88, m                                  | 64.3, CH               | 3.88, m                           |
| 11                 | 38.4, CH <sub>2</sub>        | 1.69, m                                      | 10, 12                      | 10, 10', 12', 12, 13   | 38.5, CH <sub>2</sub>        | 1.70, m                                  | 38.3, CH <sub>2</sub>  | 1.70, m                           |
| 12                 | 26.4, CH <sub>2</sub>        | 1.51, 1.39, m                                | 11                          | 11, 11', 13'           | 26.4, CH <sub>2</sub>        | 1.54, m 1.38, m                          | 26.5, CH <sub>2</sub>  | 1.51, m 1.38, m                   |
| 13                 | 28.8, CH <sub>2</sub>        | 1.31, m*                                     |                             | 11, 12, 14             | 28.4, CH <sub>2</sub>        | 1.32, m                                  | 28.0, CH <sub>2</sub>  | 1.28, m                           |
| 14                 | 29.0, CH <sub>2</sub>        | 1.31, m*                                     |                             |                        | 29.2, CH <sub>2</sub>        | 1.29, m                                  | 29.2, CH <sub>2</sub>  | 1.29, m                           |
| 15                 | 28.7, CH <sub>2</sub>        | 1.31, m*                                     |                             | 14', 16'               | 28.7, CH <sub>2</sub>        | 1.32, m                                  | 28.0, CH <sub>2</sub>  | 1.29, m                           |
| 16                 | 26.8, CH <sub>2</sub>        | 1.40, m                                      | 17                          | 15, 17, 15', 17'       | 26.8, CH <sub>2</sub>        | 1.42, m                                  | 26.5, CH <sub>2</sub>  | 1.38, m                           |
| 17                 | 32.5, CH <sub>2</sub>        | 1.76, m                                      | 16, 18, 18'                 | 16, 16', 18, 18'       | 43.6, CH <sub>2</sub>        | 2.2, m                                   | 22.6, CH <sub>2</sub>  | 1.27, m                           |
| 18                 | 45.1, CH <sub>2</sub>        | 3.53, t (6.8)                                | 17                          | 16, 17, 17'            | 73.6, CH                     | 5.75, t (6.1)                            | 14.0, CH <sub>3</sub>  | 0.88, t (6.8)                     |
| 19                 | 31.7 (27.3), CH <sub>3</sub> | 2.95, s (2.82, s)                            |                             | 1                      | 31.5 (27.2), CH <sub>3</sub> | 2.95, s (2.82, s)                        | 31.8, CH <sub>3</sub>  | 3.02, s (2.84, s)                 |
| 20                 | 51.9, CH                     | 4.90, m                                      | 21, 22                      | 19, 21', 22'           | 51.9, CH                     | 4.90, m                                  | 57.9, CH               | 4.39, m                           |
| 21                 | 70.9, CH <sub>2</sub>        | 3.57, dd (10.3, 6.8)<br>3.48, dd (10.3, 5.5) | 20                          | 20', 20, 22, 25        | 70.9, CH <sub>2</sub>        | 3.59, dd (10.3, 6.8) 3.48, dd (9.6, 4.8) | 71.0, CH <sub>2</sub>  | 3.57, dd (10.3, 4.8)              |
| 22                 | 62.0, CH <sub>2</sub>        | 4.26, dd (11.0, 7.5)<br>4.20, dd (12.3, 4.8) | 20                          | 20, 21, 23             | 62.0, CH <sub>2</sub>        | 4.26, dd (11.6, 7.5) 4.20, dd            | 62.3, CH <sub>2</sub>  | 3.79, m                           |
| 22-OH              |                              |                                              |                             |                        |                              |                                          |                        | 3.02, bs                          |
| 23                 | 170.7, C                     |                                              |                             |                        | 170.7, C                     |                                          | 58.9, CH <sub>3</sub>  | 3.34, s                           |
| 24                 | 20.8, CH <sub>3</sub>        | 2.04, s (2.05, s)                            |                             | 23                     | 20.8 CH <sub>3</sub>         | 2.04, s (2.05, s)                        |                        |                                   |
| 25                 | 58.9, CH <sub>3</sub>        | 3.32, s 3.34, s                              |                             | 21                     | 59.0, CH <sub>3</sub>        | 3.32, s 3.34, s                          |                        |                                   |

<sup>a</sup>Measured at 150 MHz. <sup>b</sup>Measured at 600 MHz. Values in (') are from TOCSY and H2BC experiments. <sup>c</sup>Derived from HSQC, HMBC and H2BC data. \*Overlapping signals

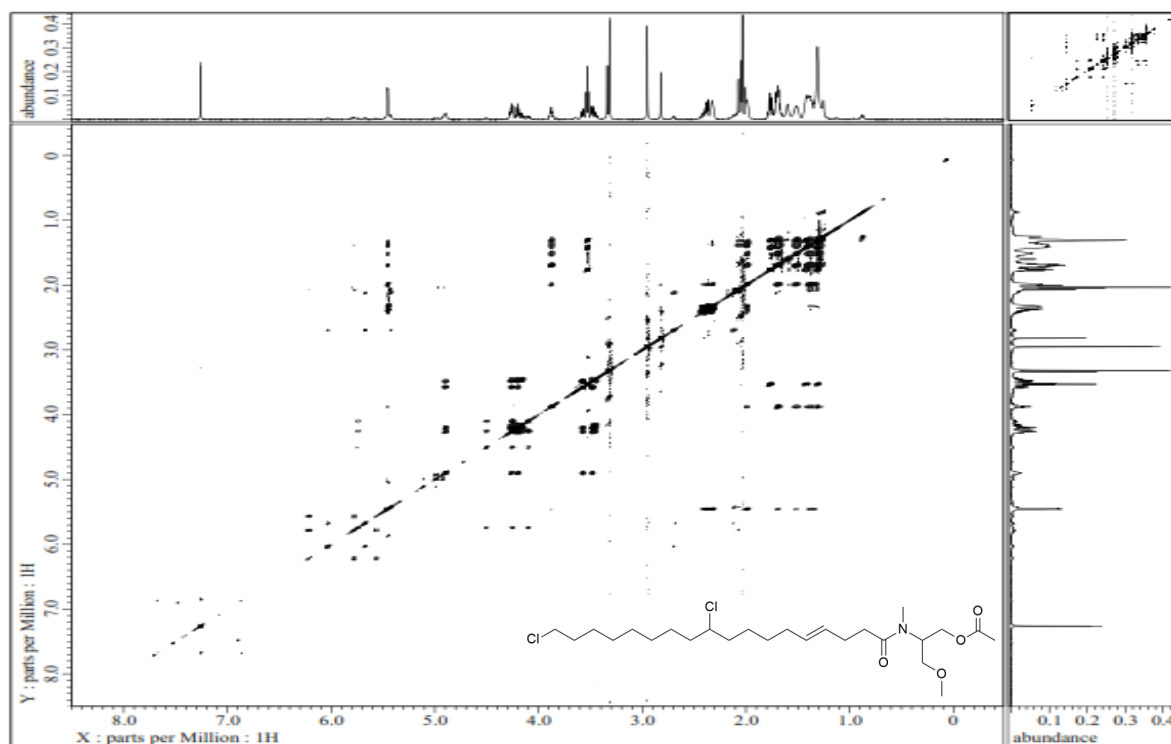


**Figure II- 13.** TOCSY data (600 MHz, CDCl<sub>3</sub>) of columbamide F (43).

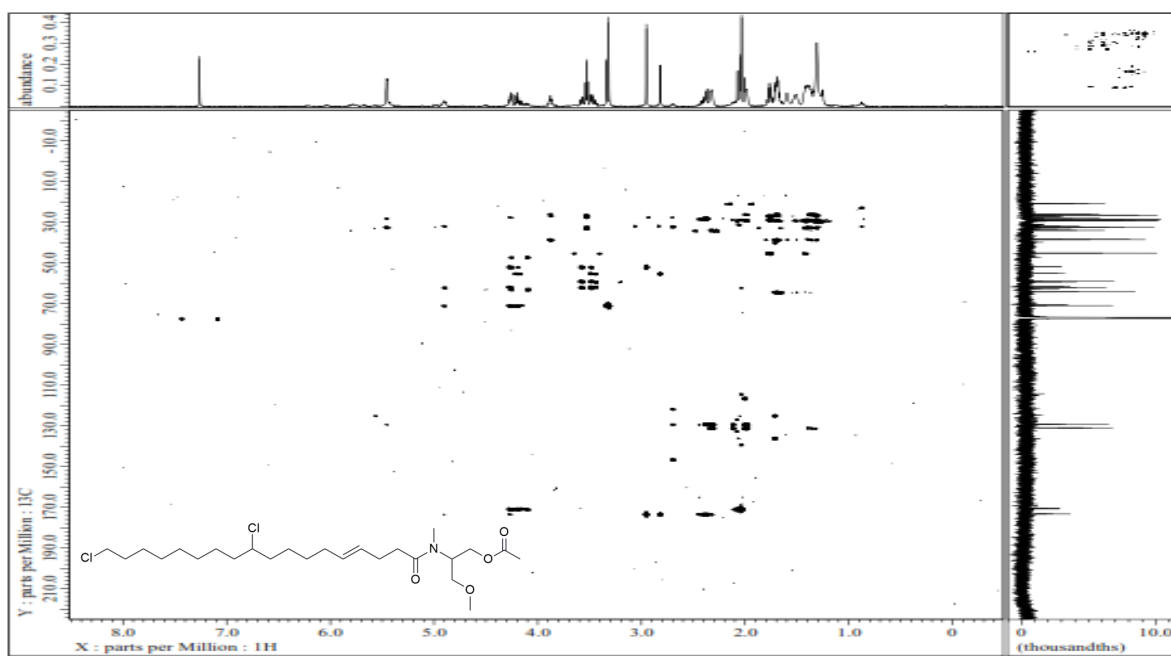


**Figure II-14.** HMBC data (600 MHz, CDCl<sub>3</sub>) of columbamide F (43).

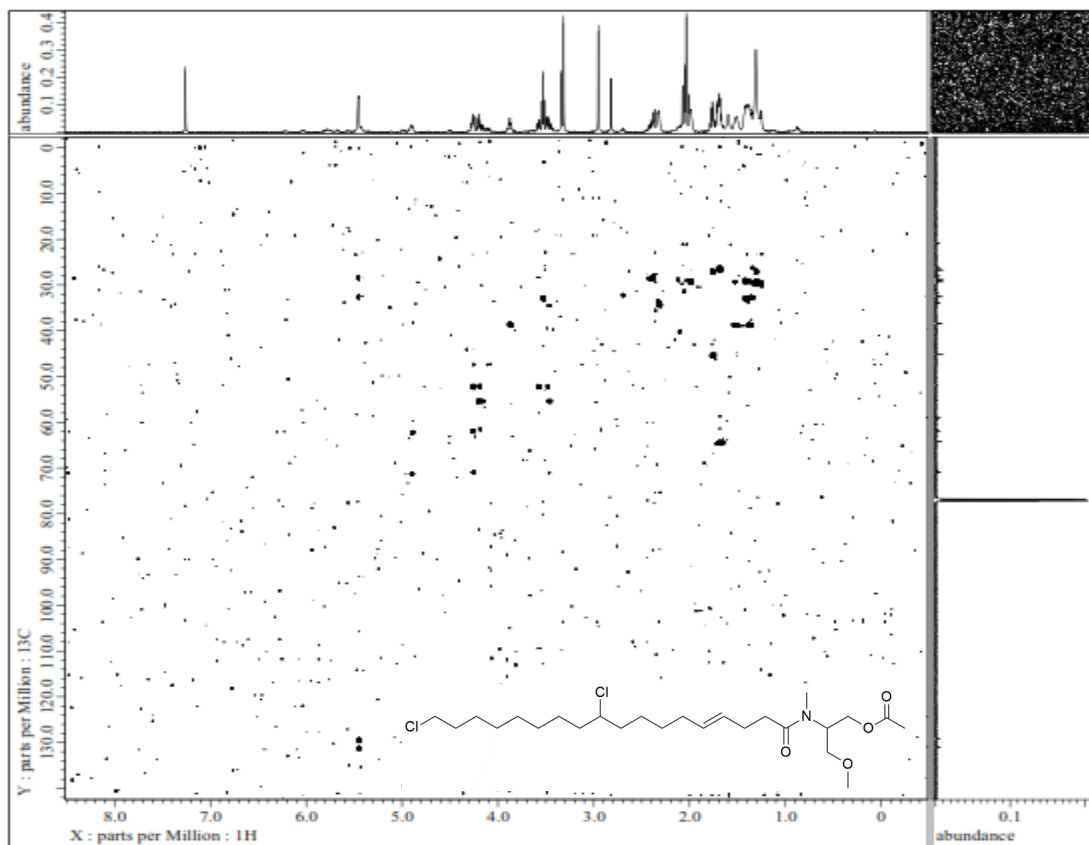


**Figure II-15.** H2BC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide F (**43**).

ESITOFMS analysis of **44** showed the  $[\text{M}+\text{H}]^+$  ion at  $m/z$  528.2451, corresponding with the molecular formula  $\text{C}_{25}\text{H}_{44}\text{Cl}_3\text{NO}_4$ , indicating three degrees of unsaturation. The mass difference between columbamide G (**44**) and columbamide F (**43**) was 34 dalton suggesting the presence of an additional chlorine. The position of the additional chlorine was confirmed by the triplet proton signal at  $\delta_{\text{H}}$  5.75 and associated carbon signal at  $\delta_{\text{C}}$  73.6 ppm which indicated a terminal dichloromethine at C-18, similar to columbamide E (**40**). The 1D and 2D NMR data for columbamide G (**44**) are shown in Figure II-16 to II-22.

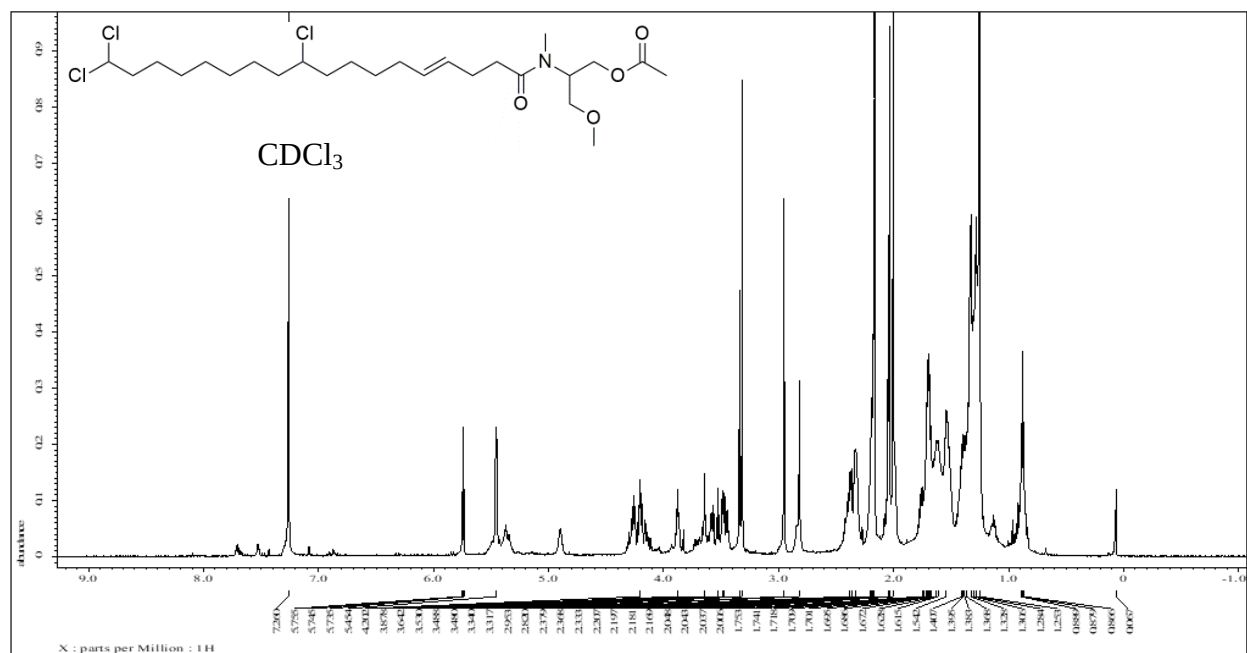


**Figure II-16.**  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide G (44).

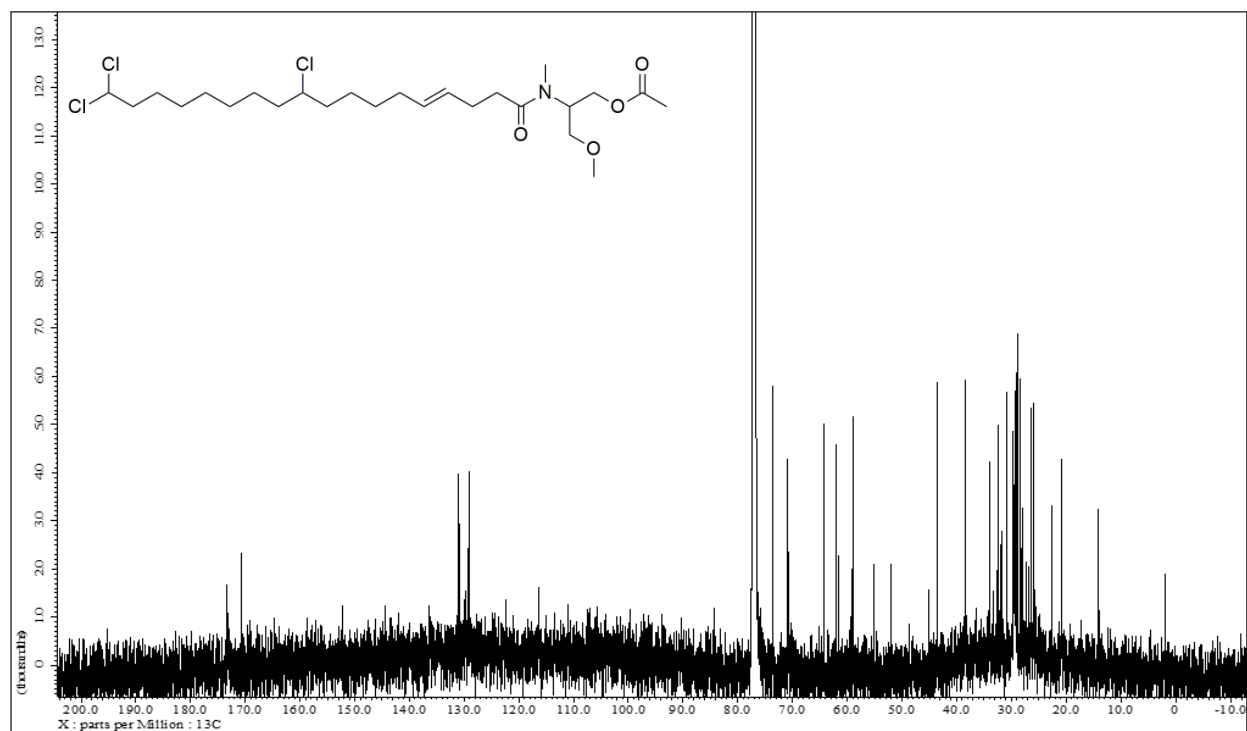


**Figure II-17.**  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide G (44).

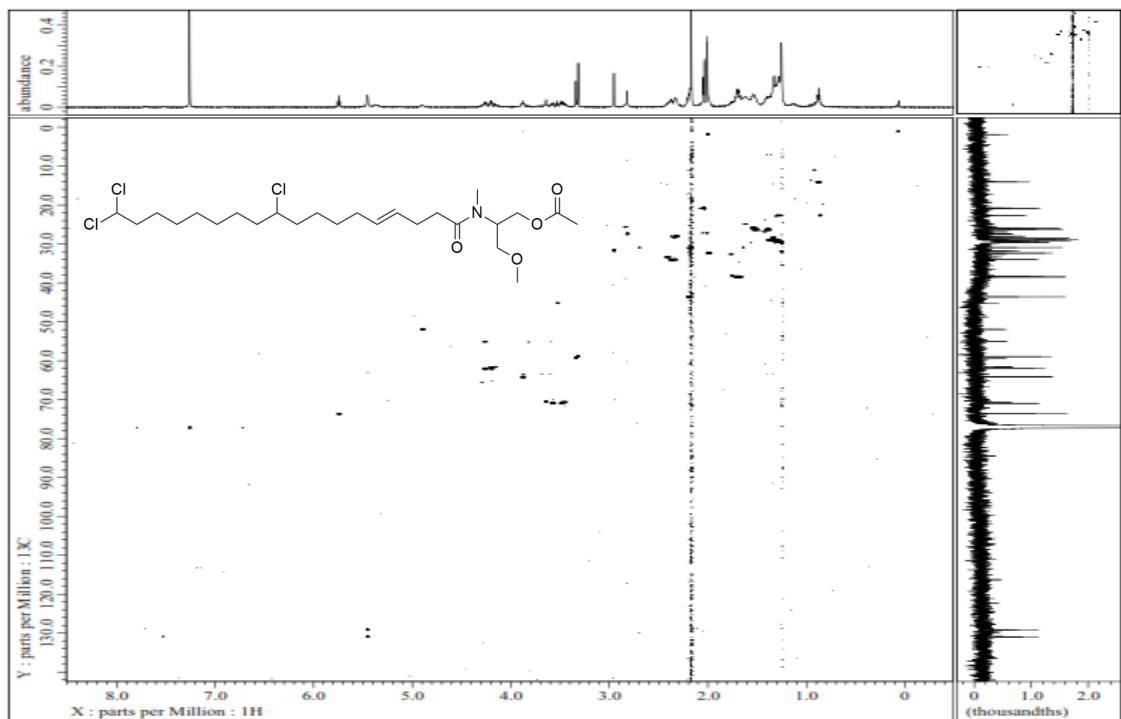


**Figure II-18.** HSQC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide G (44).

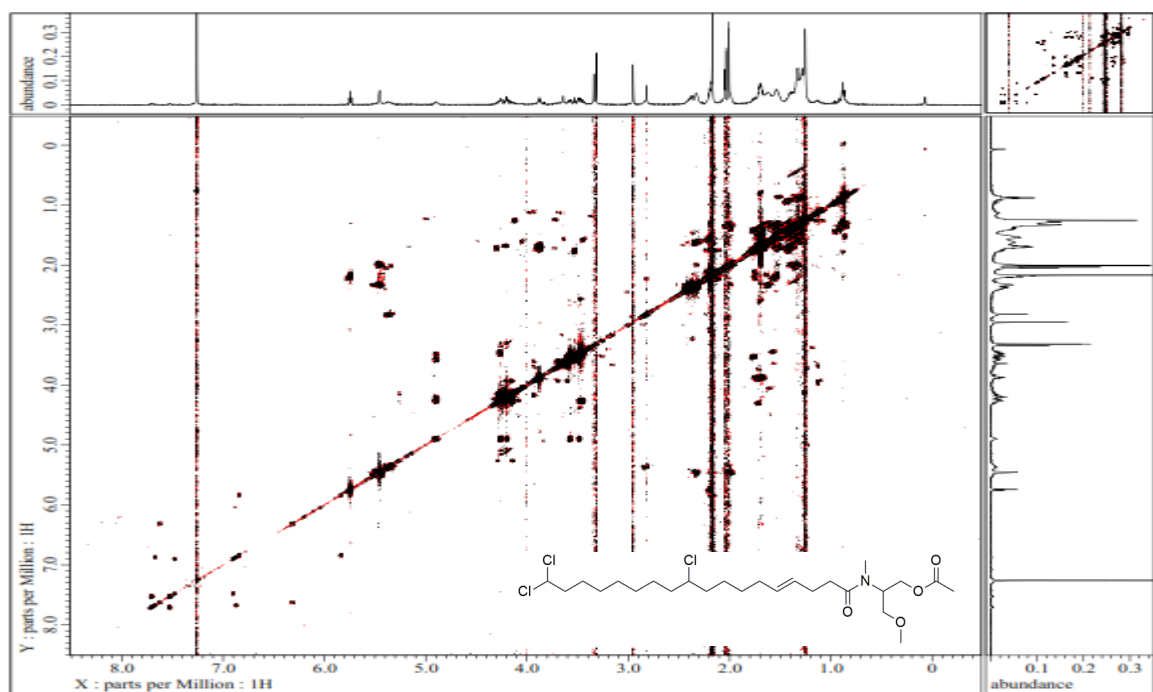


**Figure II-19.** COSY data (600 MHz,  $\text{CDCl}_3$ ) of columbamide G (44).

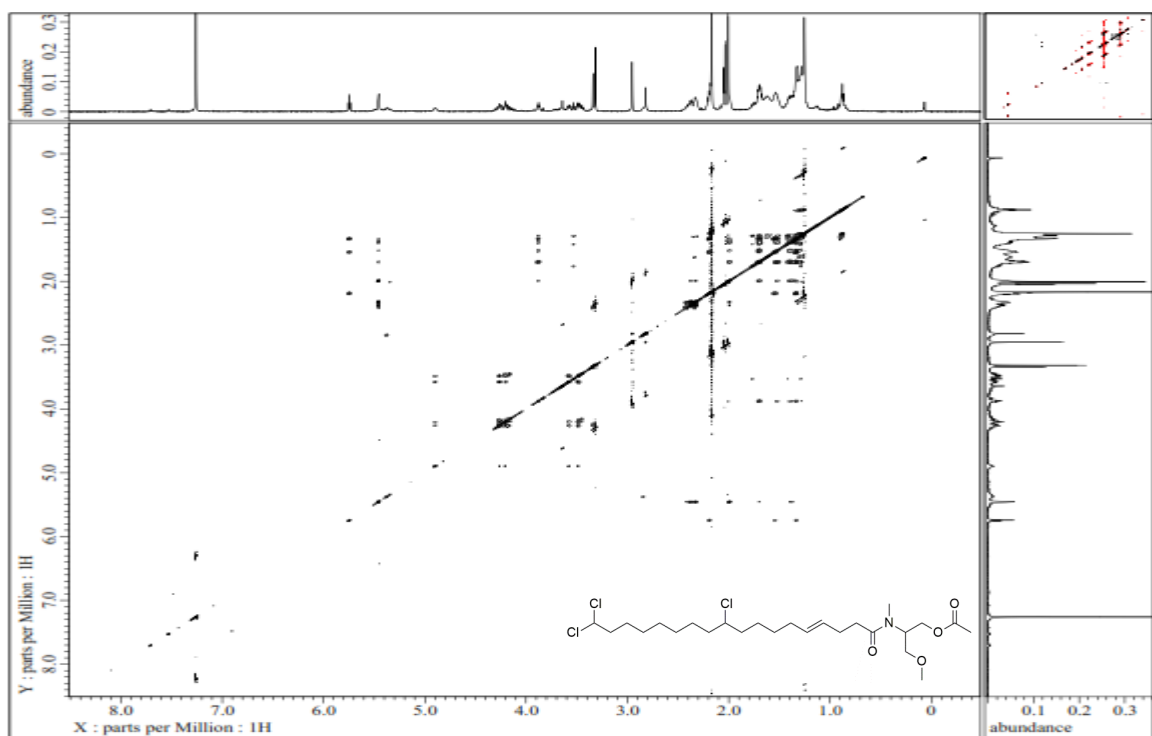


**Figure II-20.** TOCSY data (600 MHz, CDCl<sub>3</sub>) of columbamide G (44).

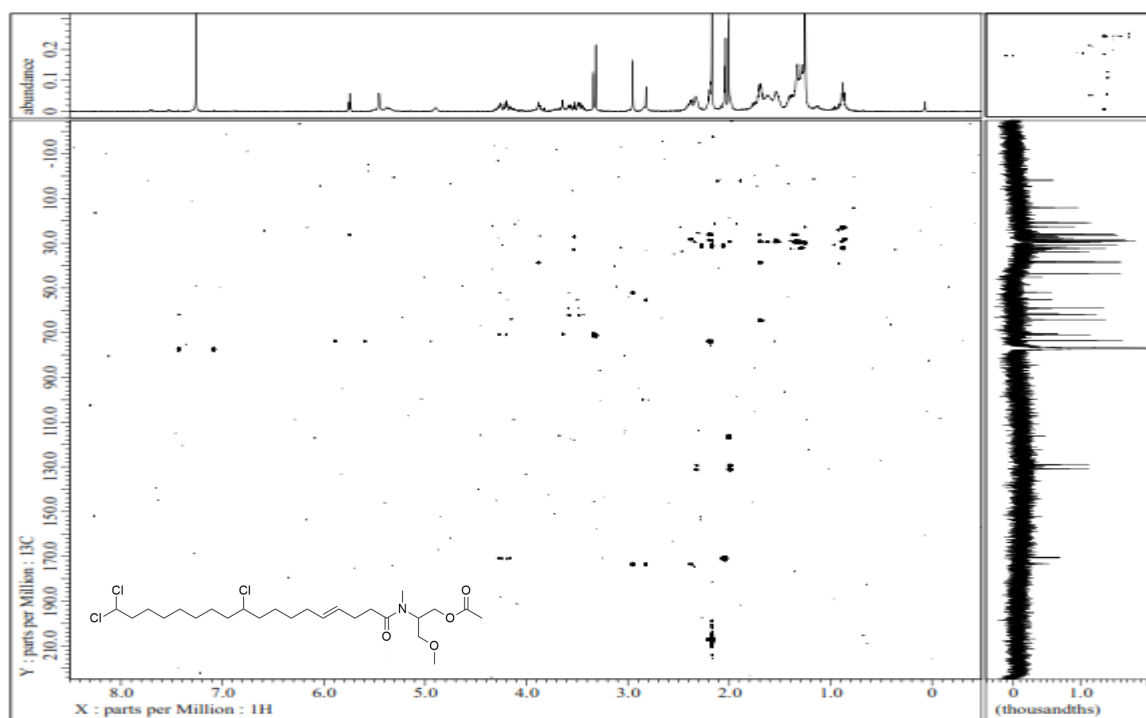


**Figure II-21.** HMBC data (600 MHz, CDCl<sub>3</sub>) of columbamide G (44).

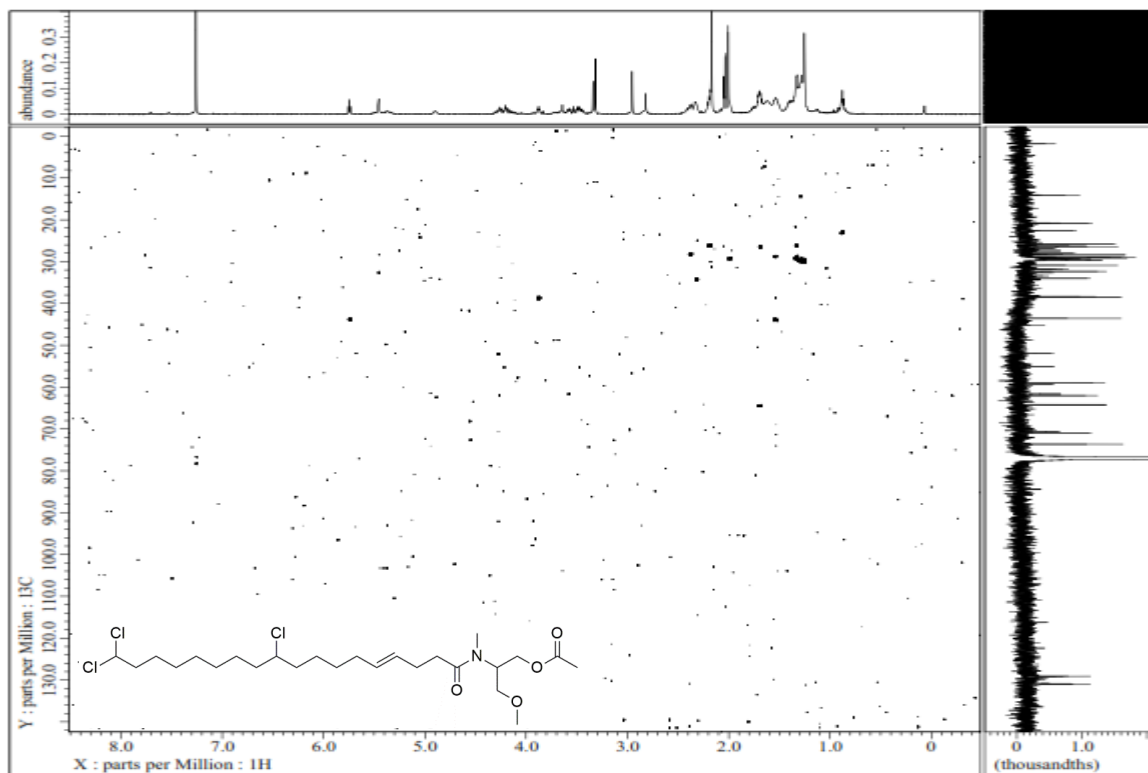


**Figure II-22.** H2BC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide G (**44**).

The molecular formula of columbamide H (**45**) was determined as  $\text{C}_{23}\text{H}_{44}\text{ClNO}_3$  by ESITOFMS indicating two degrees of unsaturation. The NMR signals for columbamide H (**45**) are similar to columbamide F (**43**) except for the replacement of the acetoxy group at C-22 by a hydroxy group ( $\delta_{\text{H}}$  3.02) resulting in the shielded shift of the adjacent methylene group to H-22 ( $\delta_{\text{H}}$  3.79) and the lack of a terminal chlorine at position C-18 confirmed by the triplet signal for methyl group at H-18 ( $\delta_{\text{H}}$  0.88 and  $\delta_{\text{C}}$  14) (Table II-1). The 1D and 2D NMR data for columbamide H (**45**) are shown in Figure II-23 to II-29.

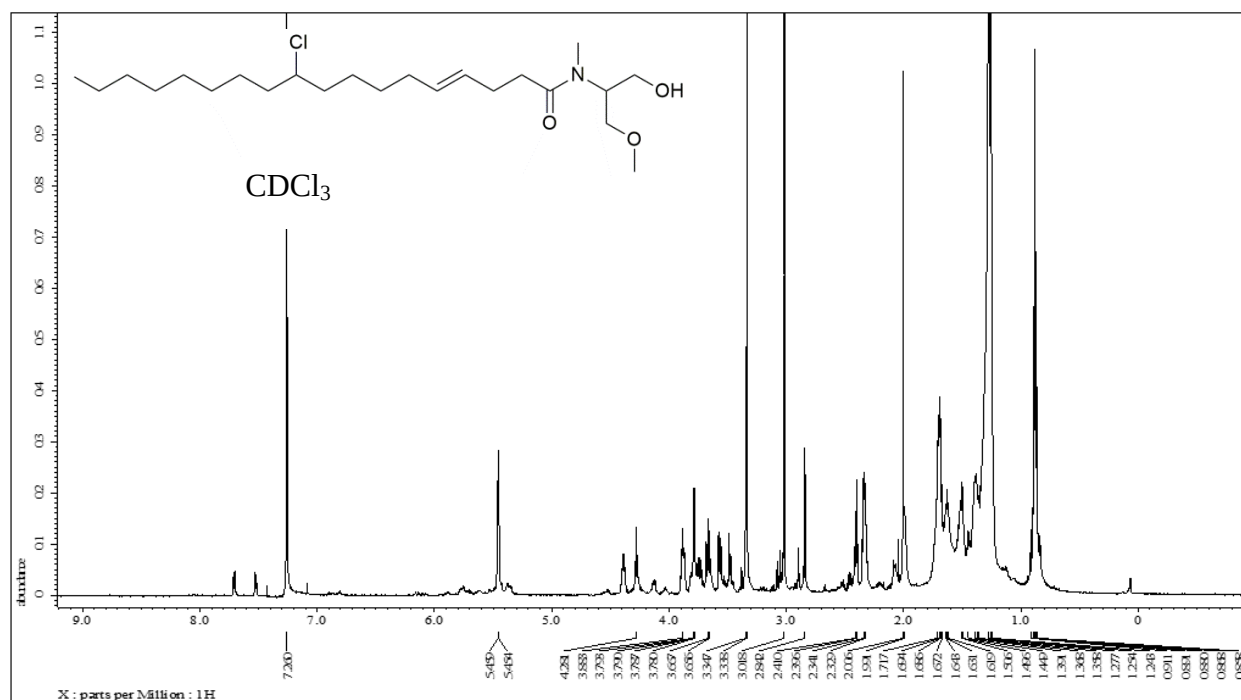


**Figure II-23.**  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide H (45).

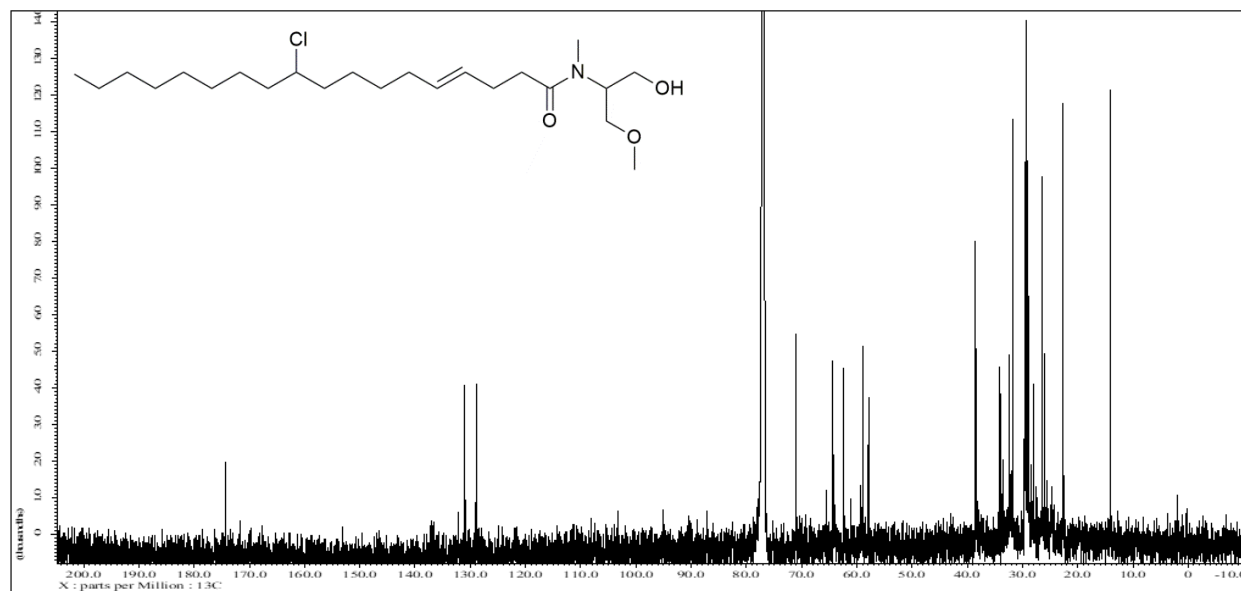


**Figure II-24.**  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ) spectrum of columbamide H (45).

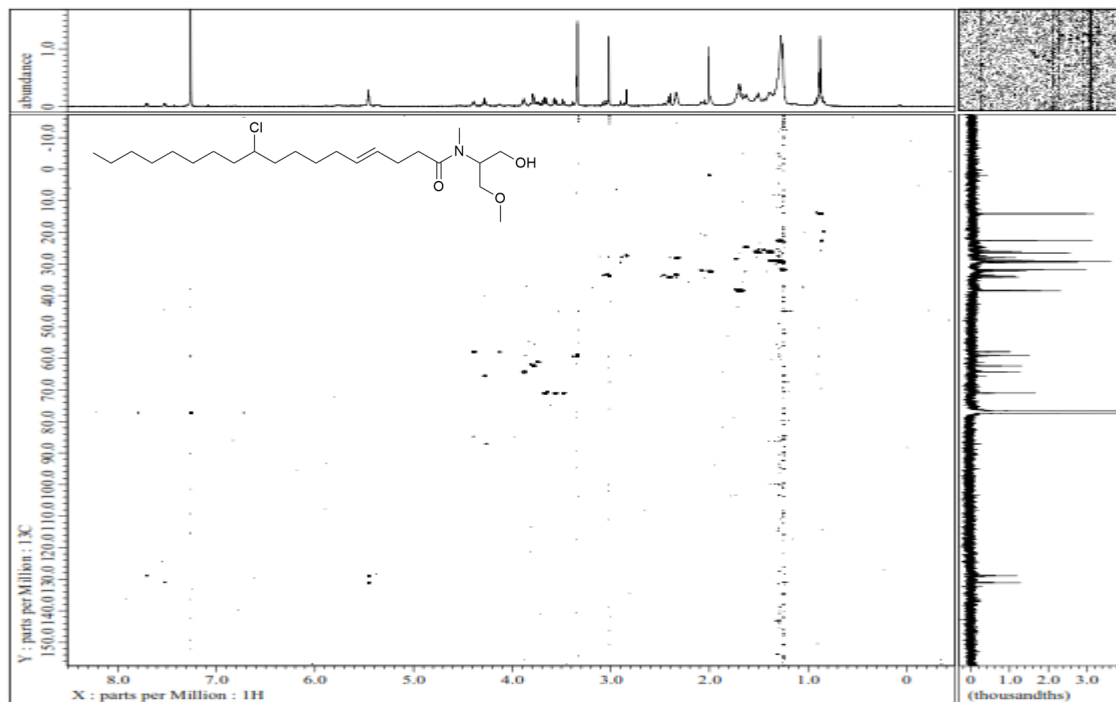


**Figure II-25.** HSQC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide H (45).

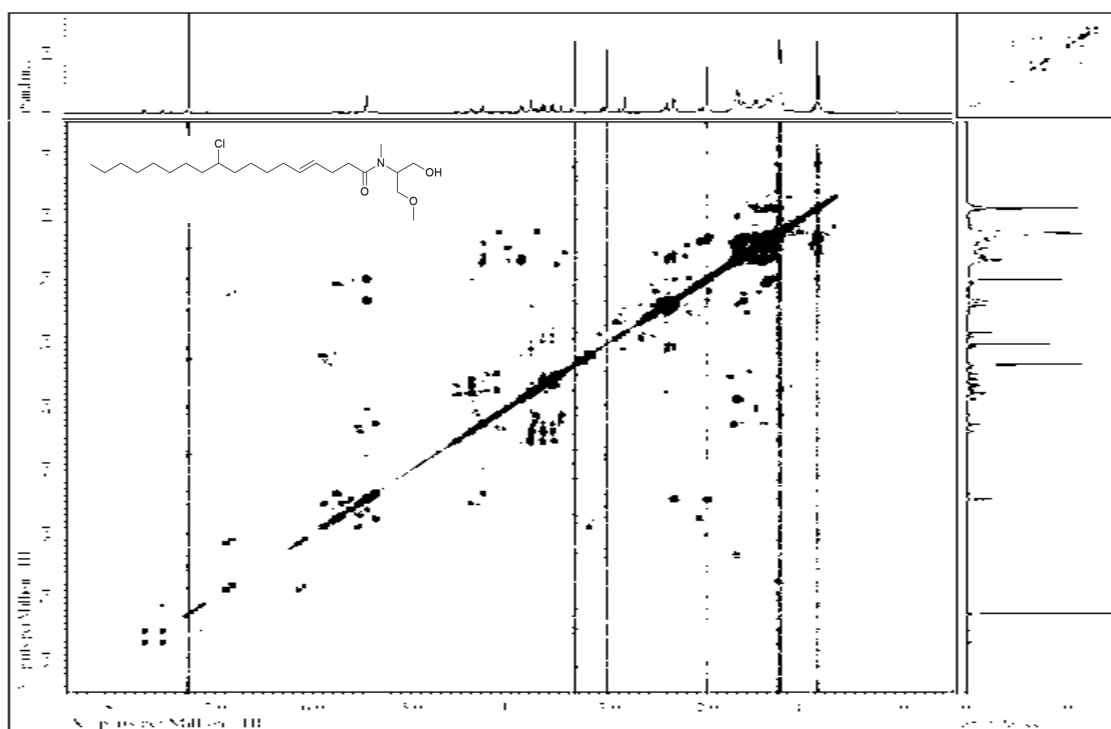


**Figure II-26.** COSY data (600 MHz,  $\text{CDCl}_3$ ) of columbamide H (45).

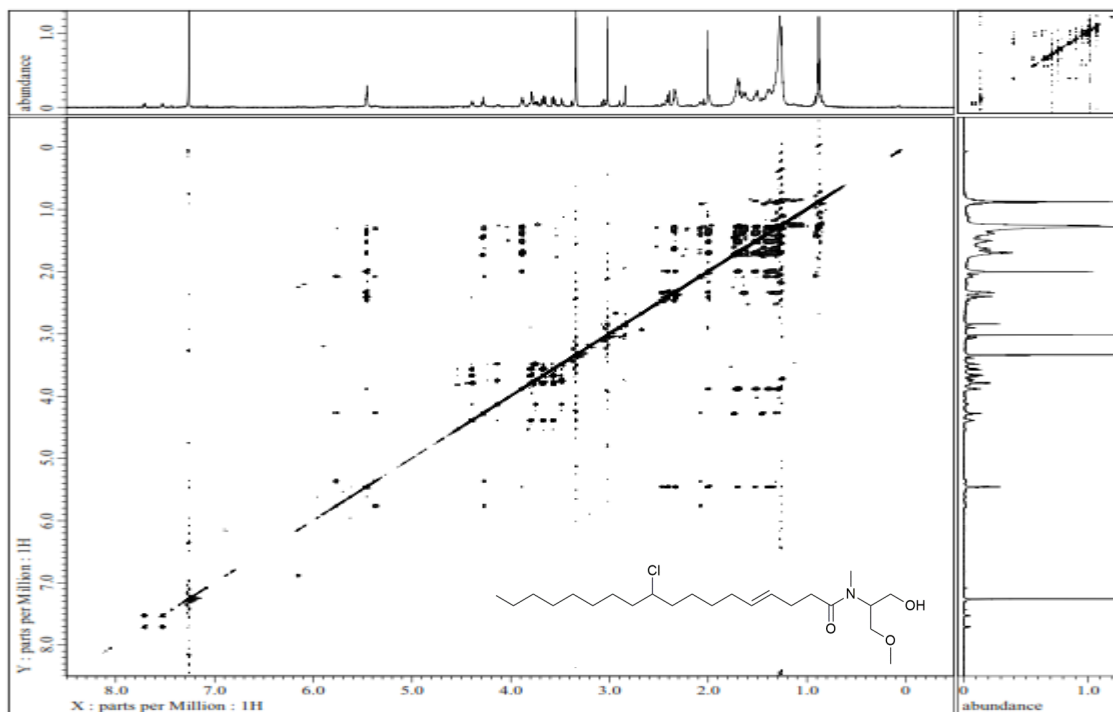


**Figure II-27.** TOCSY data (600 MHz,  $\text{CDCl}_3$ ) of columbamide H (45).

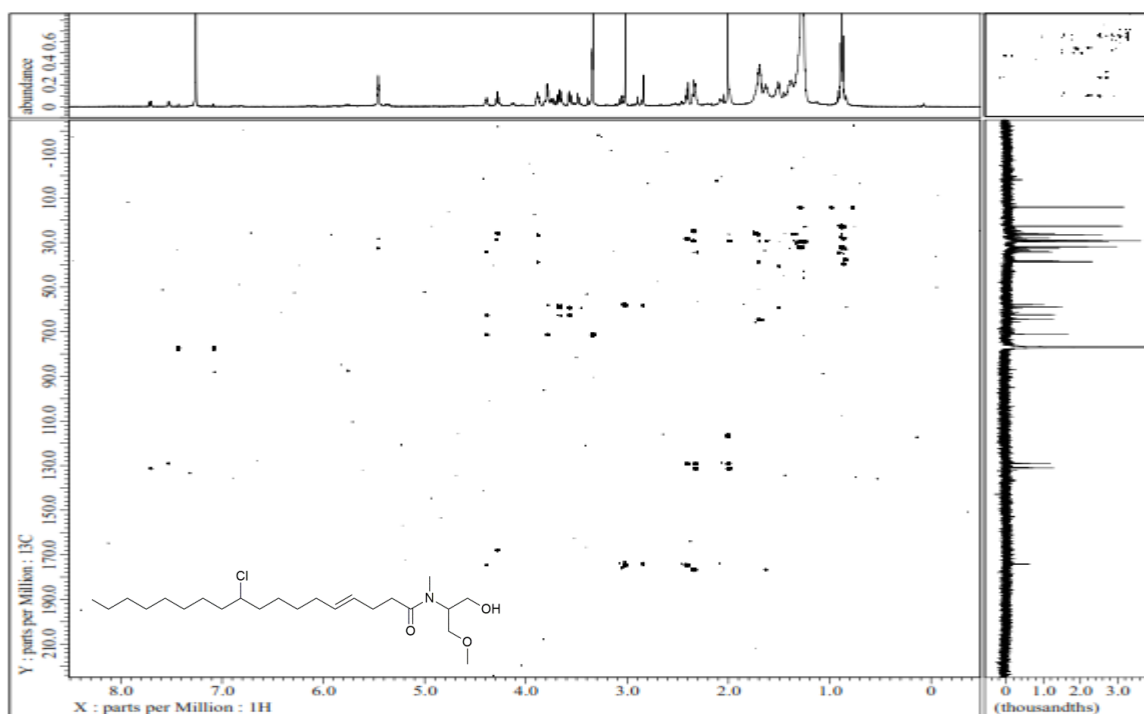


**Figure II-28.** HMBC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide H (45).

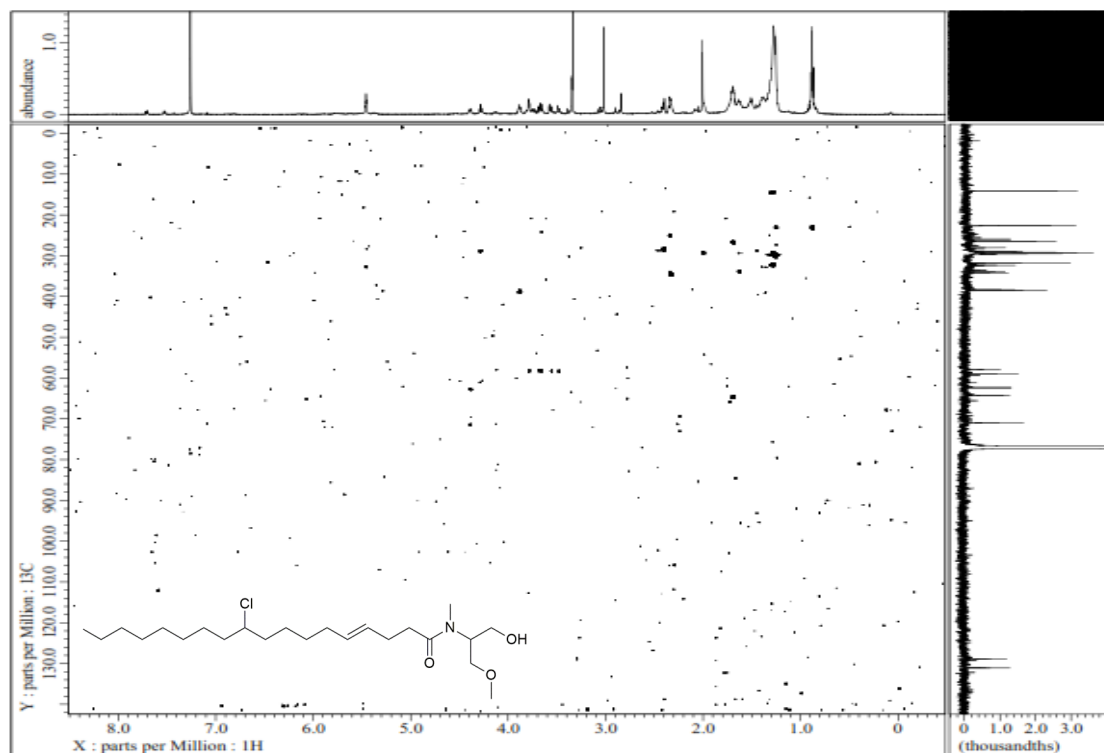


**Figure II-29.** H2BC data (600 MHz,  $\text{CDCl}_3$ ) of columbamide H (**45**).

## 2.3 Configuration of the Double Bond

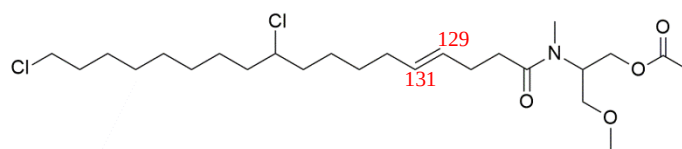
### 2.3.1 Experimental Section

Non-decoupled HSQC spectra were recorded to determine the configuration of double bond for columbamides F-H (**43-45**) using JEOL ECA 600 spectrometer at 600 MHz.

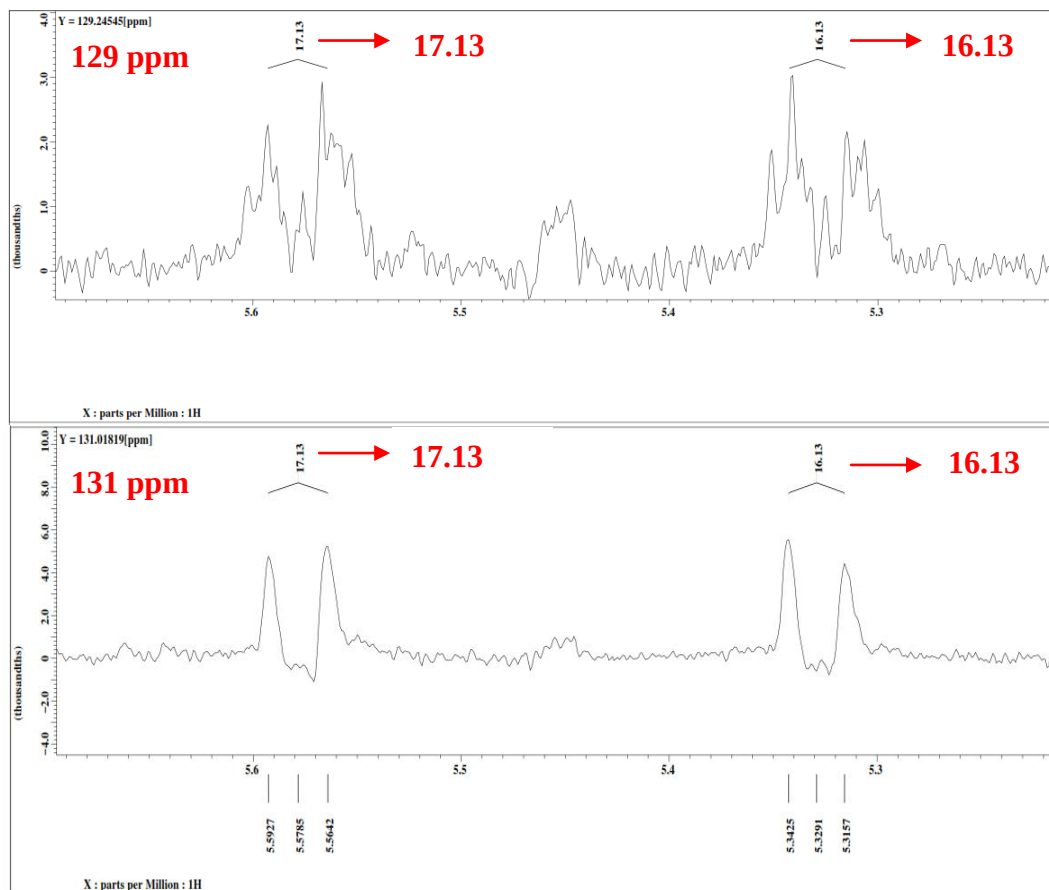
### 2.3.2 Results

An *E* configuration of the double bond for compounds **43-45** was obtained by measuring the coupling constant ( $^3J_{\text{H,H}} = 16\text{-}17\text{ Hz}$ ) from the  $^{13}\text{C}$  satellites observed by non-decoupled HSQC analysis.<sup>71</sup> (Figure II-30).

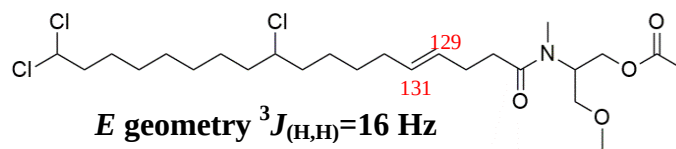
a)



*E* geometry  $^3J_{(H,H)}=16-17$  Hz

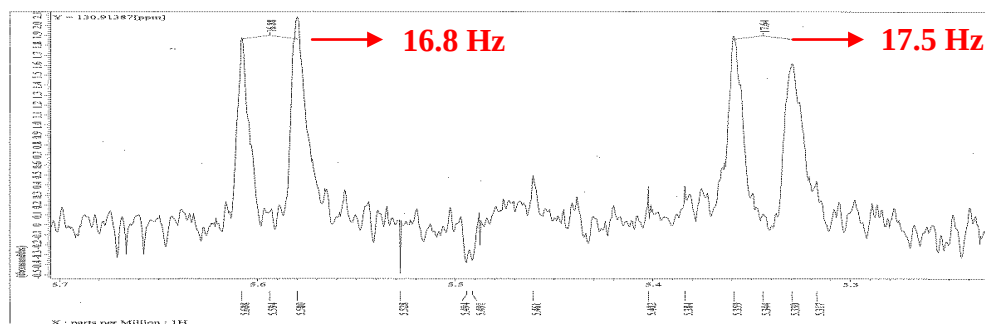


b)



*E* geometry  $^3J_{(H,H)}=16$  Hz

131 ppm



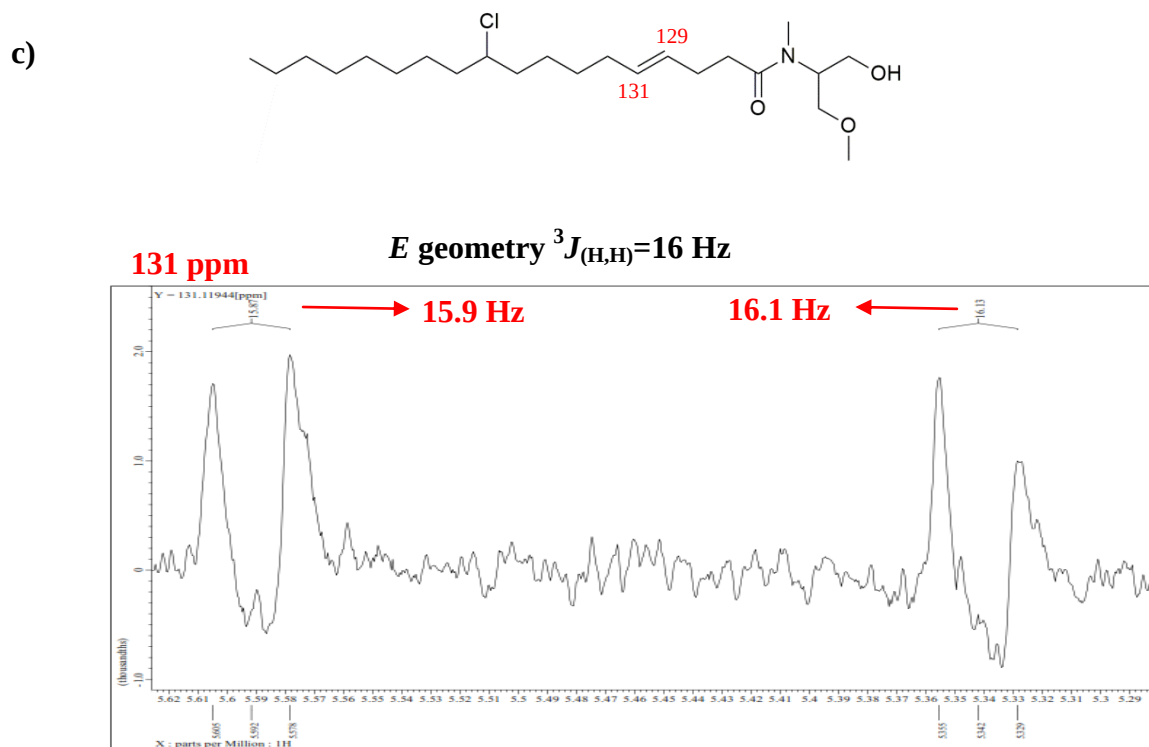


**Figure II-30.** Non-decoupled HSQC (600 MHz,  $\text{CDCl}_3$ ) data showing the  $^{13}\text{C}$  satellite signals for a) columbamide F (**43**), b) columbamide G (**44**) and c) columbamide H (**45**).

## 2.4 Absolute Configuration of the *N,O*-dimethylserinol Residues

### 2.4.1 Experimental Section

The synthesis of (*S*)-*N,O*-dimethylserinol was carried out following the method reported by Gao and colleagues<sup>72</sup> (Figure II-31). The (*R*)-*N,O*-dimethylserinol residue was given by Umezawa Sensei.

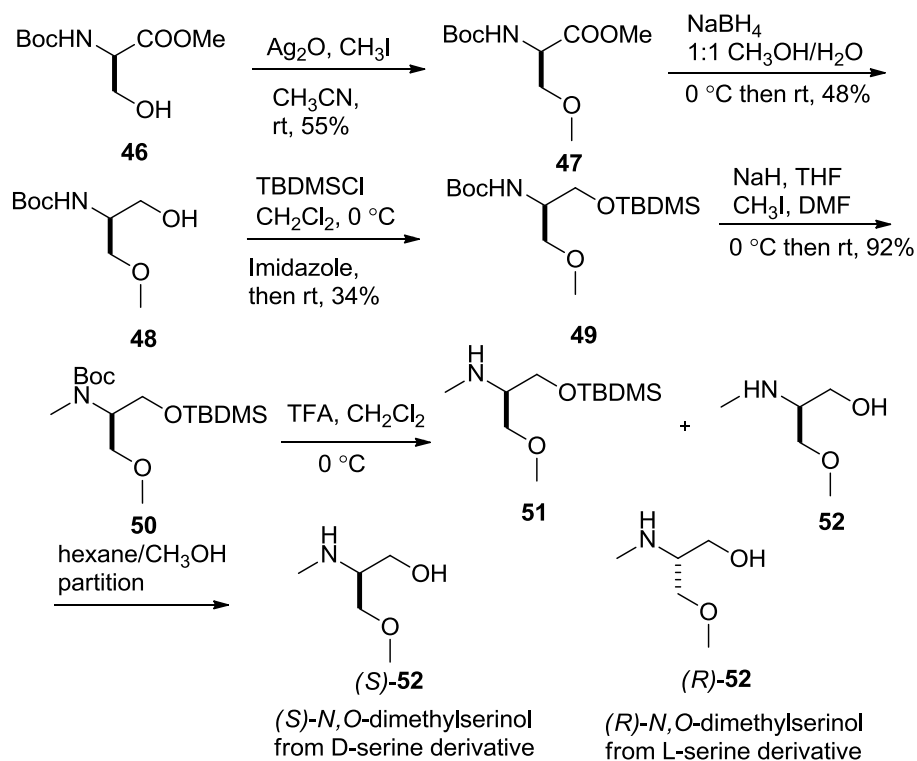


**Figure II-31.** Synthesis of *N,O*-dimethylserinol (**50**) moiety.

**(R)-methyl-2-(*tert*-butoxycarbonylamino)-3-methoxypropanoate (**47**):** To a solution of **46** (1.5 g, 6.84 mmol) in MeCN (34.2 mL), Ag<sub>2</sub>O (2.4 g) and MeI (1.7 mL) were added successively. The flask was protected from light and refluxed overnight. The mixture was filtered through a pad of Celite and the solvent was evaporated. The residue was purified by flash chromatography (EtOAc/hexane 1:5) to afford **47** as a pale yellow oil (0.89 g).

**(S)-*tert*-butyl-1-hydroxy-3-methoxypropane-2-ylcarbamate (**48**):** To a solution of **47** (0.89 mg, 3.82 mmol) in MeOH/H<sub>2</sub>O (1:1, 7.64 mL), NaBH<sub>4</sub> (0.29 g) was added in several portions at 0 °C. The mixture was allowed to warm to room temperature and stirred overnight. Then, it was extracted with EtOAc (3 × 5 mL) and the organic layer was washed with brine (5 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue **48** (0.44 g) was a colorless oil and was pure by TLC and NMR inspection.

**(R)-tert-butyl-1-(tert-butyldimethylsilyloxy)-3-methoxypropan-2-ylcarbamate(49):**

Compound **48** (0.44 g, 1.84 mmol) and *tert*-butyldimethylsilyl chloride (0.56 g) were dissolved in dichloromethane at 0 °C. Then, imidazole (0.25 g) was added in portions over 5 min. The mixture was allowed to warm to room temperature and stirred overnight. Then, H<sub>2</sub>O was added to quench the reaction. The organic layer was separated and washed with an aqueous solution of HCl (2 M, 3 × 3 mL). The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Flash chromatography of the residue over silica gel (EtOAc:hexane 1:5) afforded **49** (193.2 mg) as a colorless oil.

**(R)-tert-butyl-1-(tert-butyldimethylsilyloxy)-3-methoxypropan-2-ylmethylcarbamate (50):**

To a solution of **49** (193.2 mg, 0.638 mmol) in THF (2 mL), NaH (60% dispersion in mineral oil, 19 mg) was added at 0 °C in one portion. After stirring for 10 min, MeI (0.16 mL) and DMF (0.1 mL) were added. The mixture was stirred for further 2 h at 0 °C and allowed warm to room temperature for 15 h. Then the reaction was quenched with H<sub>2</sub>O (3 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (3 × 3 mL). The combined organic layer was dried and concentrated to afford pure **50** (188 mg).

**(S)-3-methoxy-2-(methylamino)propan-1-ol or (S)-N,O-dimethylserinol (52):** To compound **50** (188 mg, 0.59 mmol), CH<sub>2</sub>Cl<sub>2</sub> (1.18 mL) was added at 0 °C. After 3h, the mixture was evaporated. Excess TFA was removed by repeated addition of CH<sub>2</sub>Cl<sub>2</sub> and evaporation. Two layers were observed corresponding to products, **51** and **52**. These were separated by solvent partition using hexane/MeOH (1:1) to obtain pure **52** (40 mg).

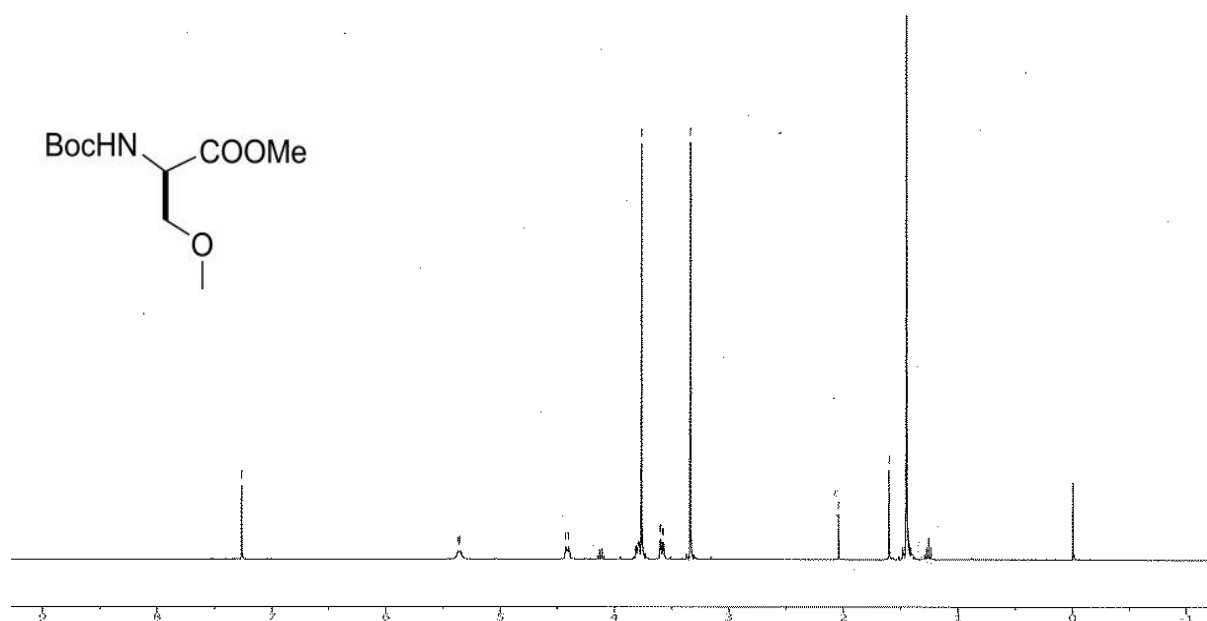


**Fig II-32.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **47**.

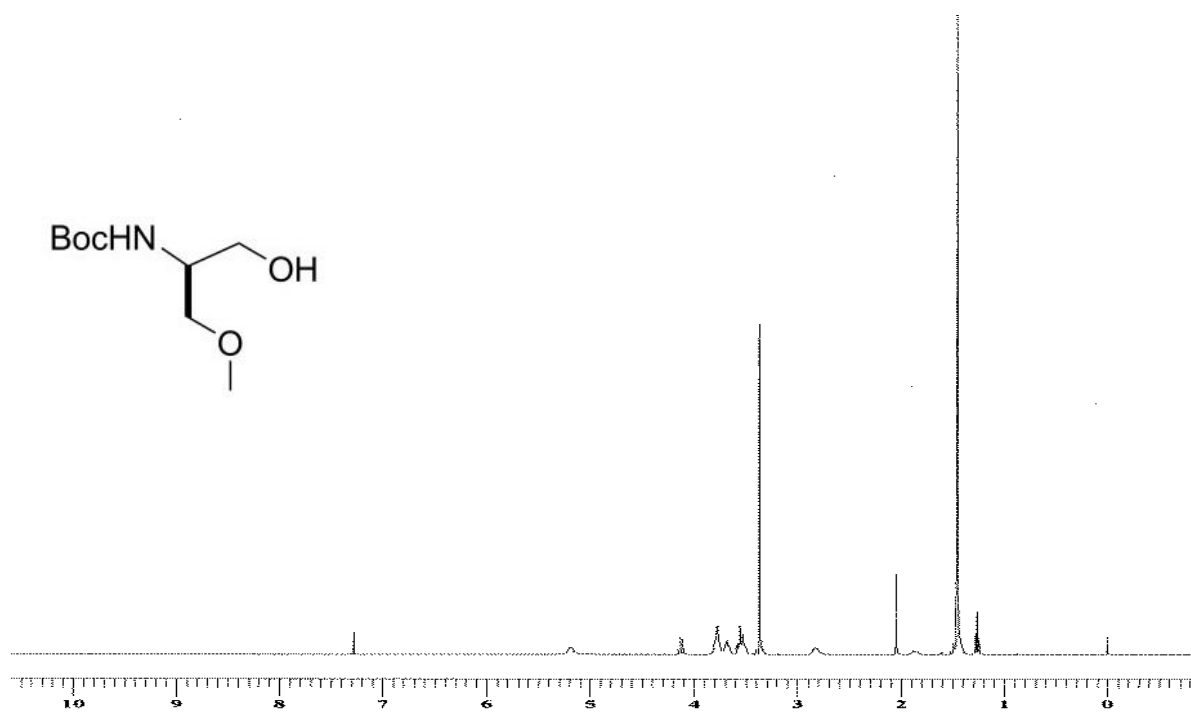


**Fig II-33.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **48**.



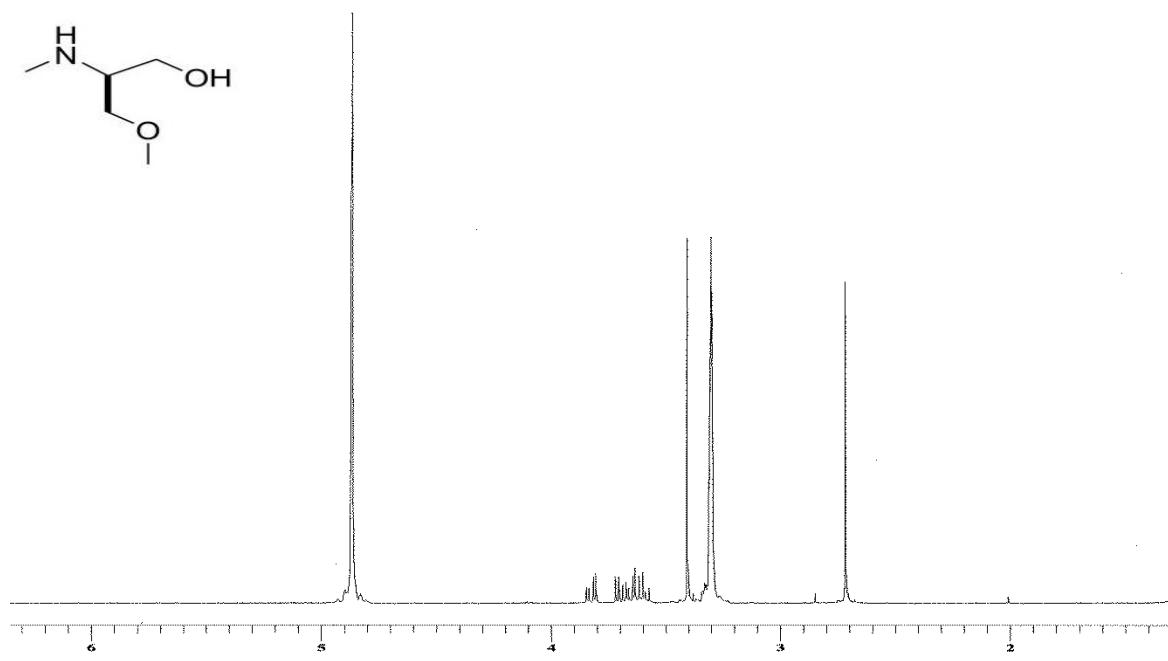


**Fig II-36.**  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ) spectrum of **52**.

**Acid Hydrolysis and Marfey's Analysis.** The absolute configurations of the *N,O*-dimethylserinol moiety were determined by comparing with synthetic *N,O*-dimethylserinol standards. Columbamides **F** (**43**) and **G** (**44**) (0.2 mg) were hydrolyzed using 12 M HCl at 120 °C for 24 h to cleave both the amide and acetate groups. Columbamide **H** (**45**) (0.2 mg) was hydrolyzed using 6 M HCl at 120 °C for 16 h. The reaction was quenched with  $\text{NaHCO}_3$  and evaporated to dryness. The hydrolysates from the columbamides and the synthesized *N,O*-dimethylserinol were subjected to Marfey's analysis.<sup>73</sup> The hydrolysates and standards were first dissolved in 100  $\mu\text{L}$  of  $\text{H}_2\text{O}$  and then 200  $\mu\text{L}$  of FDAA (5-fluoro-2,4-dinitrophenyl-L-alaninamide) solution (in 1% acetone) was added, followed by addition of 40  $\mu\text{L}$  1 M  $\text{NaHCO}_3$ . The solutions were heated in an oven at 40 °C for 1 h, and then the reaction was quenched with 20  $\mu\text{L}$  of 2 M HCl. 200  $\mu\text{L}$  of MeCN were added and this mixture was directly analyzed by LC-MS (gradient 0-80 min 10-50% MeCN with 0.1% FA in Milli-Q  $\text{H}_2\text{O}$ , Cosmosil 5C18-AR II column,  $4.6 \times 250$  mm, flow rate: 0.2 mL/min at 25 °C, UV Detection at 340 nm).

### 2.4.2 Results

The absolute configuration of the stereogenic center of the dimethylated and acetylated serinol moiety was determined by Marfey's analysis. The two standards, (*R*) and (*S*)-*N,O*-dimethylserinol (**52**) moiety were synthesized (Figure II-31) and derivatized with Marfey's reagent. In a similar way derivatization was done for the hydrolysates of compounds **43-45**. *R*-configurations for *N,O*-dimethylserinol residues were established (Figure II-37) by comparing the retention times.

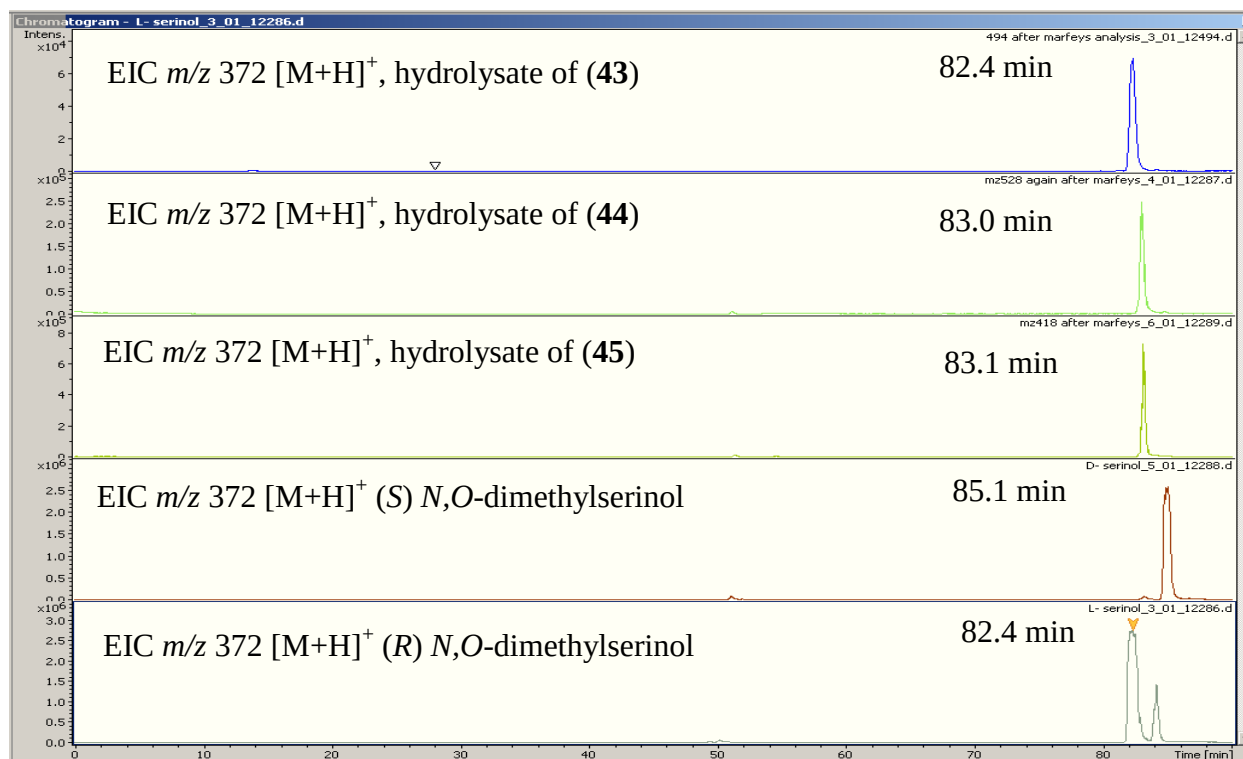


**Figure II-37.** Results of Marfey's analysis of the standards, and hydrolysates of columbamide F (**43**), columbamide G (**44**) and columbamide H (**45**).

## 2.5 Absolute Configuration of Chloromethine by Ohrui Method

### 2.5.1 Experimental Section

**Ohrui's Reaction and Chiral-Phase HPLC Analysis.** To a solution of synthetic **39**-(10*R*,20*R*) (4.9 mg, 0.0108 mmol) in THF (0.30 mL) were added NMO (50.0% in H<sub>2</sub>O, 3.3  $\mu$ L, 0.0162 mmol) and OsO<sub>4</sub> (0.020 M in H<sub>2</sub>O, 108  $\mu$ L, 0.00216 mmol) at room temperature. The mixture was stirred for 20 h, quenched with saturated aqueous Na<sub>2</sub>SO<sub>3</sub> and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. To a solution of crude diol **54** in CH<sub>2</sub>Cl<sub>2</sub> (0.50 mL) was added NaIO<sub>4</sub>-SiO<sub>2</sub> (50.0 mg), at rt.<sup>74</sup> The mixture was stirred for 30 min, then filtered through a pad of Celite (elution with EtOAc), and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc:Hexane = 2.5:97.5) to give aldehyde **55** (1.40 mg, 0.00499 mmol, 46% in 2 steps) as a colorless oil. To a solution of aldehyde **55** (1.40 mg, 0.00499 mmol) in MeOH (0.10 mL) was added NaBH<sub>4</sub> (0.50 mg, 0.0132 mmol) at 0°C. The mixture was stirred for 30 min, quenched with saturated NaHCO<sub>3</sub>, extracted with EtOAc, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The crude alcohol **57** was used for the next step. To a solution of the crude alcohol **57** in CH<sub>2</sub>Cl<sub>2</sub> (0.30 mL) were added (*S,S*)-Orui's acid (3.7 mg, 0.0114 mmol), EDCI (2.80 mg, 0.0146 mmol) and catalytic DMAP at rt under an Ar atmosphere. The mixture was stirred for 18 h, quenched with saturated NH<sub>4</sub>Cl, extracted with EtOAc, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc:hexane = 20:80) to give **58** (4.4 mg) which was used for HPLC analysis (HPLC conditions: 90:10 hexane/isopropanol, Chiralcel OJ-H, 4.6  $\times$  250 mm, 1 mL/min, UV 300 nm). The synthetic **39**-(10*S*,20*R*) (4.8 mg, 0.0106 mmol) and natural columbamide F (**43**) (3 mg, 0.006 mmol) were degraded and derivatized in a similar method as described previously to obtain (**53**)-ester of *S*-**57** (4.0 mg) and (**53**)-ester of Columbamide F (**43**) (2.0 mg), respectively. Using chiral-phase HPLC system, the coinjection experiments of the synthetic standards (**58**) were separated into two peaks at 22.2 min and 24.9 min. Again in coelution experiment of natural columbamide F (**43**) with synthetic standards, 10*R* gave a single peak at 25.2 min while 10*S* was separated from natural (**43**) and gave two peaks at 22.5 min and 25.1 min.

### 2.5.2 Results

In our previous study, the absolute configuration of the chloromethine of columbamide D (**39**) was determined by total synthesis of all four isomers followed by chiral-phase HPLC analysis. For this study we examined the potential of a chiral labeling reagent for determining the configuration of the chloromethine on the long alkyl chain of columbamide F (**43**). The absolute configuration of the chloromethine at C-10 was determined by chemical conversion of columbamide D (**39**) standards and natural compound (**39**) followed by esterification with Ohruí's acid (**53**).<sup>75</sup> The use of the highly potent fluorescent chiral labeling reagent, (*R,R*) and (*S,S*)-2-(anthracene-2,3-dicarboximido) cyclohexanecarboxylic acid, (Figure II-33) has been well reported for the discrimination of compounds having remote stereocenters.<sup>76</sup> Because our team previously synthesized columbamide D stereoisomers, the standards (10*S*,20*R*) and (10*R*,20*R*)-(**39**) were used as standards in the Ohruí reaction for columbamide F (**43**). Synthesized diastereomers of columbamide D and natural **43** were converted to diol (**54**) by dihydroxylation and then cleaved oxidatively with sodium periodate (NaIO<sub>4</sub>) to form two aldehydes (**55**, **56**). After separation, **55** was reduced with NaBH<sub>4</sub> to furnish linear alcohol (**57**) which was esterified with (*S,S*)-2-(anthracene-2,3-dicarboximido) cyclohexanecarboxylic acid (**53**) in the presence of EDCI and DMAP to afford **58**. Natural columbamide F (**43**) was converted and esterified with (**53**) in a similar way (Figure II-34). Chiral-phase HPLC analysis of synthetic 10*S* and 10*R* standards revealed that a coinjection experiment can separate both standards. Then by a coelution experiment, natural (**43**) gave a single peak with the synthetic 10*R* isomer while two peaks were observed with the synthetic 10*S* isomer confirming the *R*-configuration for the chloromethine of (**43**) (Figure II-36). Although in the Ohruí's original method, the HPLC separation of the diastereomers was done by using a C18 or C30 column and fluorescence detection using different temperatures ranging from 40 °C to -50 °C,<sup>76-78</sup> here we report the significance of chiral-phase HPLC for the separation of diastereomers using a UV detector at room temperature. Ohruí's acid was used to determine the absolute chloromethine using a small amount of sample and this can be a powerful approach for determining the absolute configuration of natural products having a remote stereogenic center.

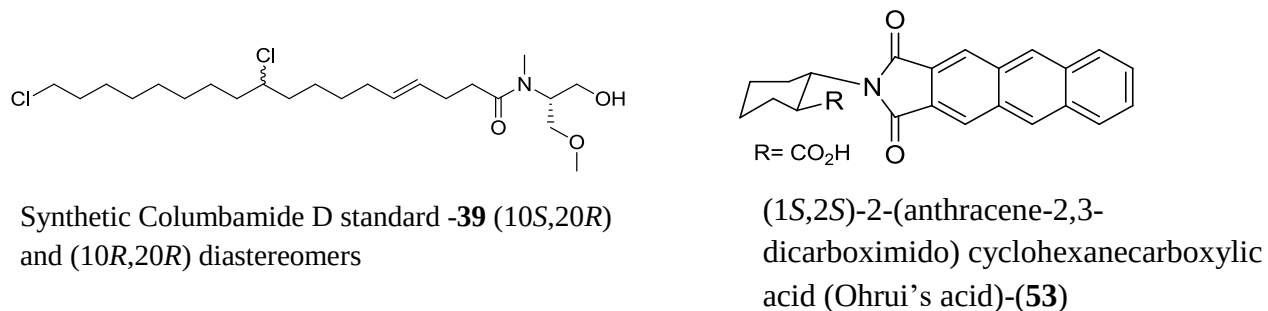


**Figure II-38.** Synthetic standards and Ohruí's acid for determining the configuration at a remote stereocenter.

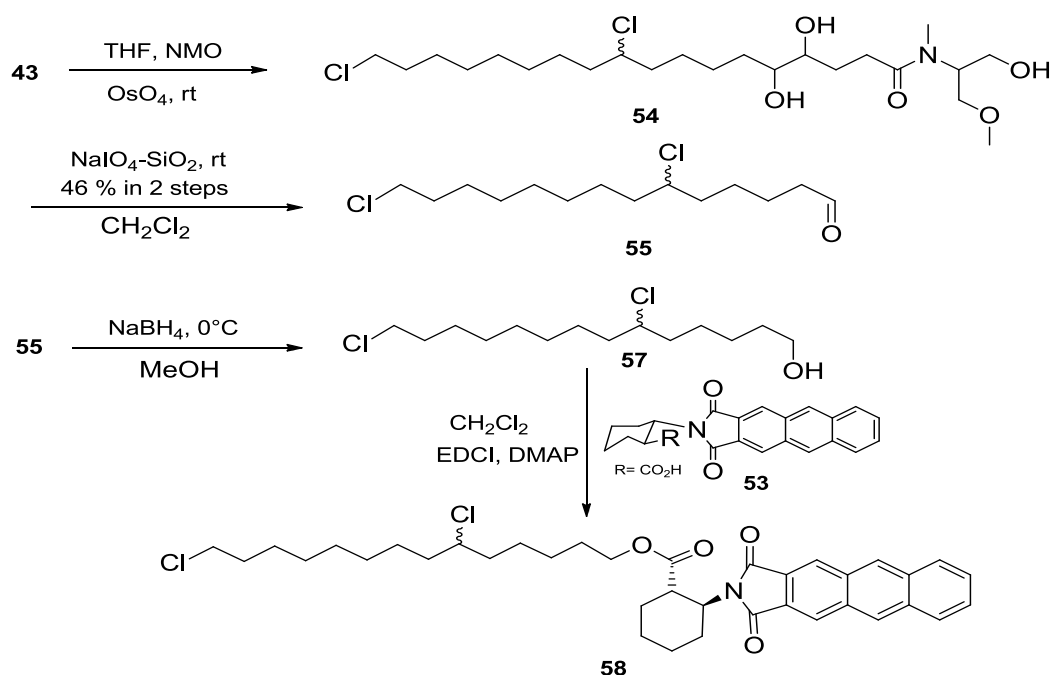


**Figure II-39.** Chemical conversion to apply Ohruí's method for determining the configuration of the chloromethine of columbamide F (**43**).

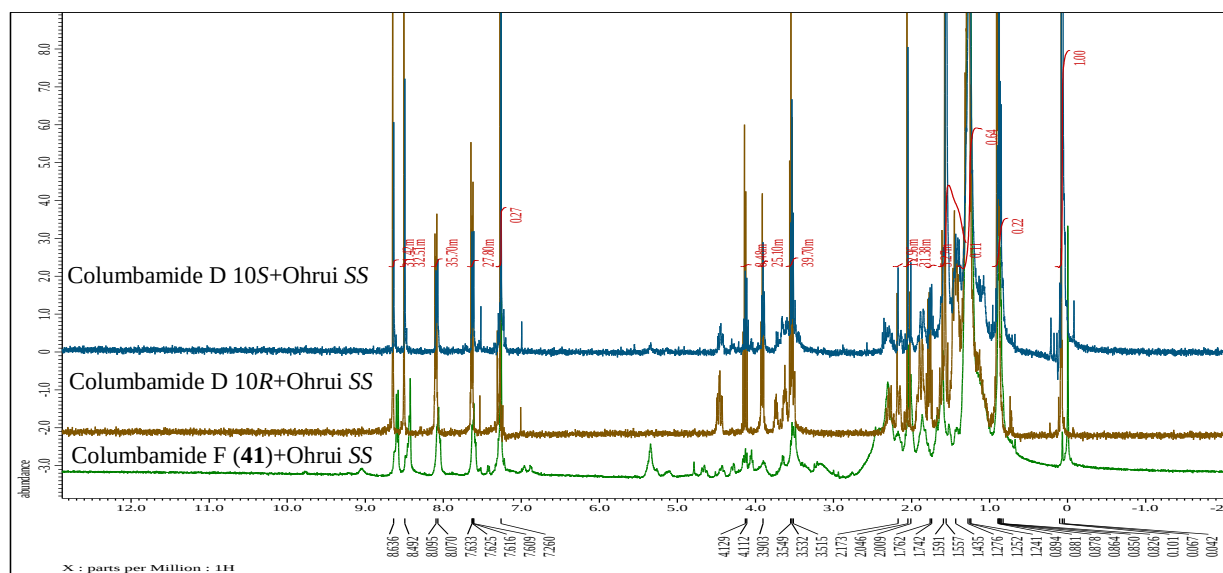


**Figure II-40.** Stacked spectra of  $^1\text{H}$  NMR of (53)-ester of *S*-57, (53)-ester of *R*-57 and (53) ester of columbamide F.

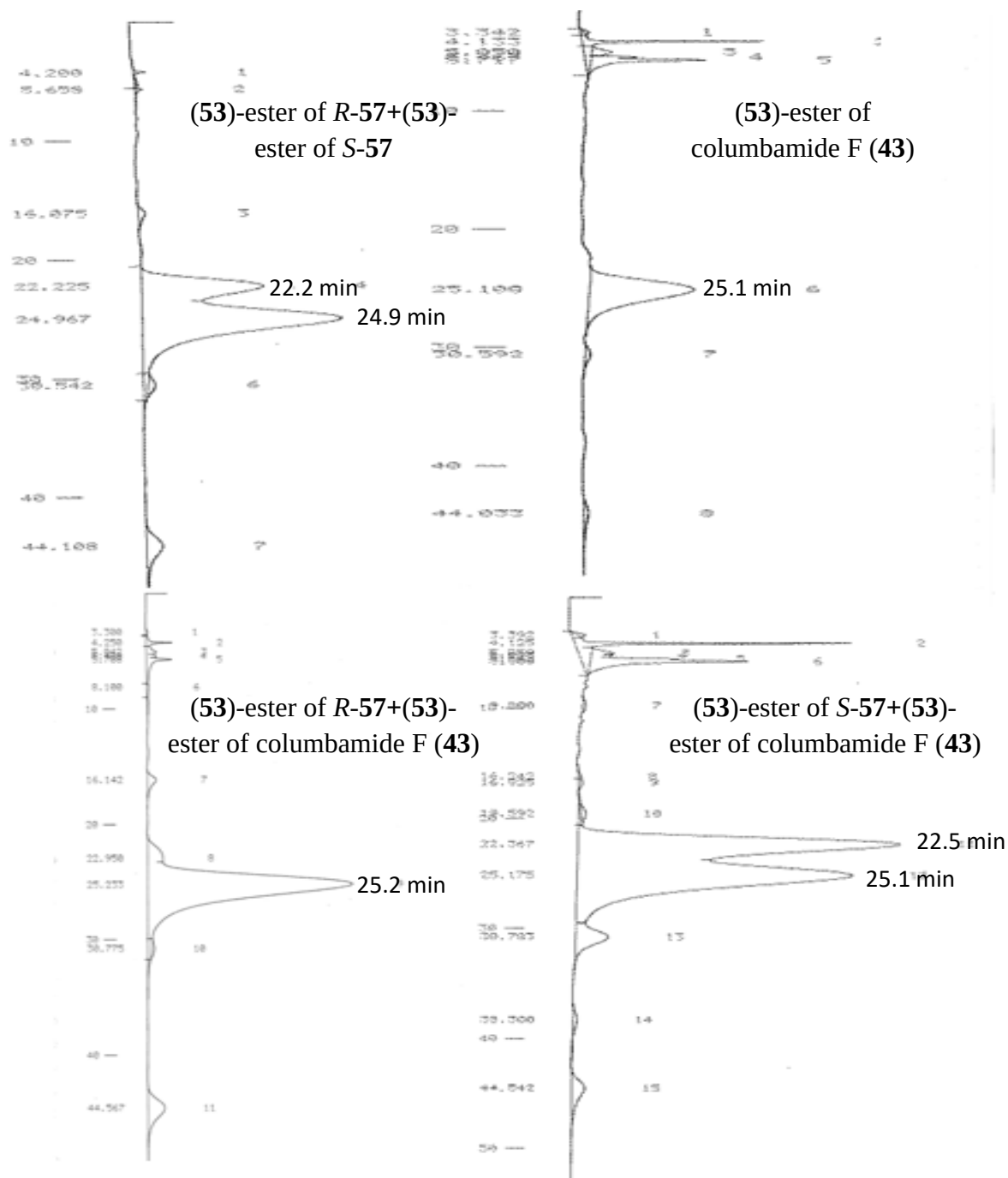


**Figure II-41.** Chiral-Phase HPLC results of Ohru ester with synthetic standards and natural **43**.

Chiral-phase HPLC conditions: Chiralcel OJ-H, 4.6 × 250 mm, 90:10 hexane/isopropanol,

1 mL/min, UV 300 nm

## 2.6 IR Data of Columbamides F-H (43-45)

The IR spectrum of **43**, **44**, and **45** (Figure II-42-II-44) showed the characteristic signals such as ester group ( $1743, 1741\text{ cm}^{-1}$ ) and amide group ( $1624\text{-}1651\text{ cm}^{-1}$ ). For compound **45** the ester group is replaced by a hydroxy group ( $3394\text{ cm}^{-1}$ ).

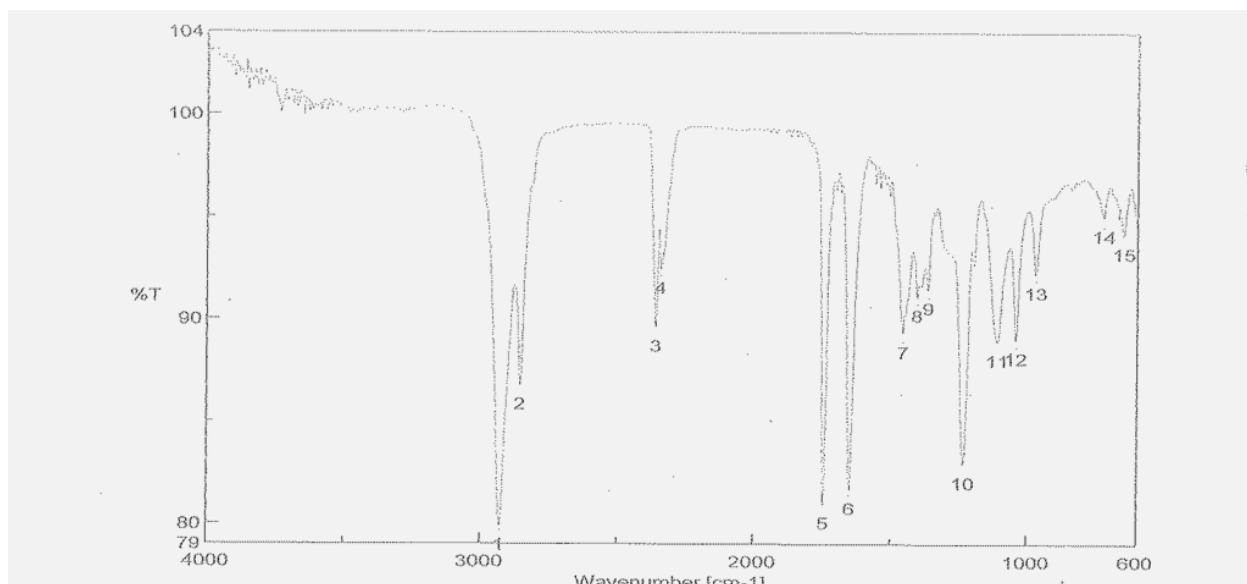


**Figure II-42.** IR data of Columbamide F (**43**).

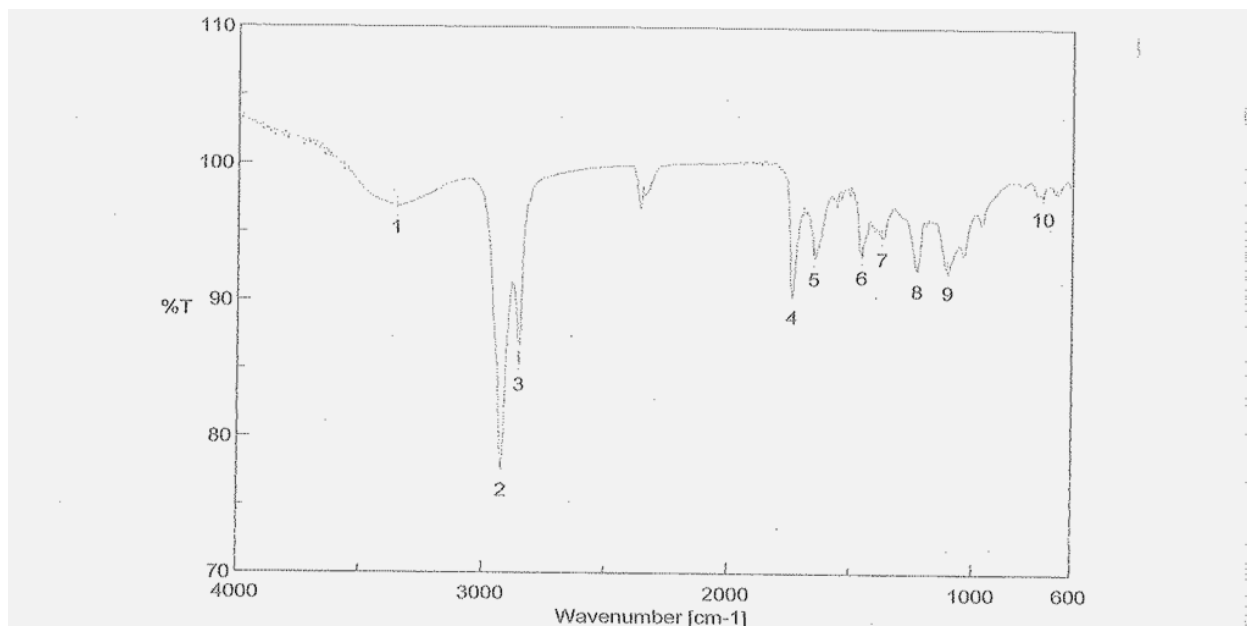


**Figure II-43.** IR data of Columbamide G (**44**).

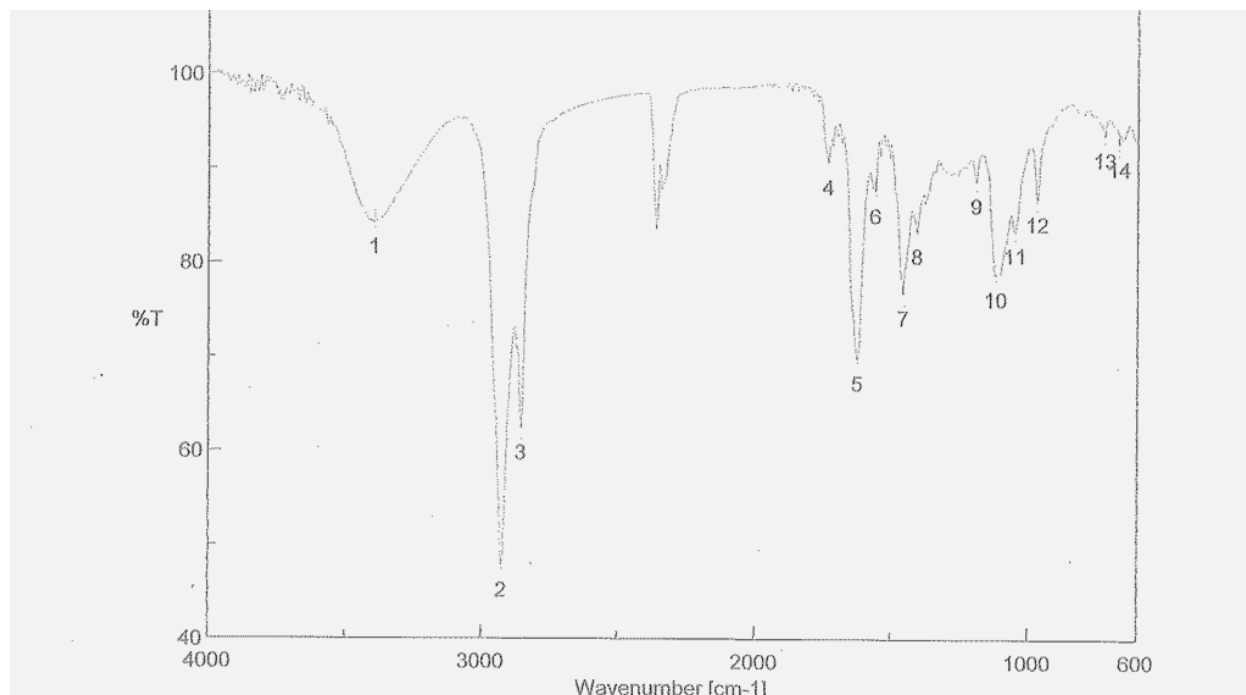


**Figure II-44.** IR data of Columbamide H (45).

## 2.7 Bioactivity

### 2.7.1 Experimental Section

**Surface Tension Measurement.** The surface tension of sample solution was determined by the ring method using a Du Noüy tensiometer (ITOH, No.4334) equipped with a platinum ring at 25 °C and measurements were done in triplicate. Calibration of the instrument was done by measuring the surface tension of the pure H<sub>2</sub>O before each set of experiments. The Critical Micelle Concentrations (CMC) for pure compounds were determined from the breakpoint of the surface tension versus the log of the bulk concentration curve.<sup>79</sup>

### 2.7.2 Results

Fatty acid amides columbamides A and B<sup>44</sup> isolated from cultured *Moorea bouillonii* showed cannabinomimetic activity similar to other fatty acid amides.<sup>41,80</sup> Here we report the

surface tension lowering activity of fatty acid amides columbamide D (**39**) and serinolamide C (**59**)<sup>81</sup> (isolated by our group). Because of the amphiphilic properties, these compounds reduced the surface tension of aqueous solutions at concentration 10-1200 µg/mL as determined by the ring method. The critical micelle concentrations (CMC) of **39** and **59** were 0.34 mM, and 0.78 mM, respectively, that are lower than the CMC value of the synthetic surfactant SDS (8.2 mM)<sup>82</sup> and comparable to pluronic F-68 (0.07-0.35) mM<sup>83</sup> and triton X-100 (0.33 mM).<sup>84</sup> Serinolamide C (**59**) isolated from *Okeania* sp. showed potent antifouling activity (0.88 µg/mL) against *Amphibalanus amphitrite* larvae. The biosurfactant properties of serinolamide C could reduce the surface tension of seawater in the microplate well, and therefore the larvae cannot adhere easily. Because of the small amount of sample, the biosurfactant activity of columbamide F (**43**) was checked by an oil displacement assay, which is an indirect measurement of surface activity. The diameter of the clear zone was measured as ~ 90 mm for 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. In addition columbamides F-H (**43-45**) were tested for the cytotoxicity against MCF-7 breast cancer cell line but were inactive (IC<sub>50</sub> >10 µg/mL).

**Table II-2.** Biosurfactant activity of pure compounds

| 201                          | Oil Displacement Area (mm) Concentration 10 mg/mL | Critical Micelle Concentration | Cytotoxicity                                                           | Ref        |
|------------------------------|---------------------------------------------------|--------------------------------|------------------------------------------------------------------------|------------|
| Columbamide D ( <b>39</b> )  | ~110 mm                                           | 0.33 mM                        | Couldn't be assessed because of the presence of cytotoxic apratoxins A | 42         |
| Columbamide F ( <b>43</b> )  | ~ 90 mm                                           | -                              | 84 % cytotoxicity at 100 µg/mL                                         | This study |
| Serinolamide C ( <b>59</b> ) | ~ 54 mm                                           | 0.78 mM                        | Couldn't assessed because of precipitation                             | 81         |
| SDS                          | ~ 84 mm                                           | 8.2 mM                         | IC <sub>50</sub> value 177.1 µg/mL against HeLa cells                  | 82, 85     |
| Pluronic F-68                | ~ 54 mm                                           | 0.07-0.35 mM                   | Slightly toxic to mammalian cells                                      | 83, 86     |
| Triton X-100                 | Not tested                                        | 0.33 mM                        | Toxic to PC-3 cells at concentration of 0.01% (w/v)                    | 84, 87     |

## 2.8 Discussion

The combination of mass spectrometric metabolic profiling and genomic analysis was resulted in the discovery of unique acyl amides columbamide (A-C, **36-38**) from laboratory culture of *M. bouillonii* sample and found to be potent ligands for the CB<sub>1</sub> and CB<sub>2</sub> receptor.<sup>44</sup> Later, related compounds columbamide D and E (**39, 40**) were isolated from the *M. bouillonii* sample collected from Mantanani Island and the absolute configuration was solved by Marfey's analysis and total synthesis of four diastereomers.<sup>42</sup> In this study, the biosurfactant-assay guided isolation led to the isolation of three new columbamides from *M. bouillonii* samples collected in Mantanani island, Sabah, Malaysia. The absolute configuration of chloromethine was determined by using highly potent chiral labeling reagent (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido) cyclohexanecarboxylic acid (Ohrui's acid) followed by chiral phase HPLC analysis. These results revealed the significance of using Ohrui reagent for determining the configuration of natural compounds having remote chiral center.

The molecular networking of chemical extracts from cultured *M. bouillonii* PNG revealed the presence of di- and tri-chlorinated compounds and these compounds were isolated and their biosynthetic gene cluster were described.<sup>44</sup> The biosynthetic pathway of columbamides starts with ColA by loading dodecanoic acid onto the acyl carrier protein ColC. Next ColD and ColE are responsible for the multiple chlorination of the acyl chain at the  $\omega$ -terminus and  $\omega$ -7 positions. The next gene ColF was found to contained two PKS module that extends the chain by four carbon and also responsible for the formation of double bonds. Afterward, ColG, the NRPS module extends the acyl ACP by incorporating *L*-serine in the molecule and performs *N*- and *O*-methylation via two methyltransferase domain. Lastly, the peptidyl carrier protein (PCP)-bound product was released as a primary alcohol, columbamide C and acetylated by ColI, a predicted acyltransferase to form columbamide A and B.

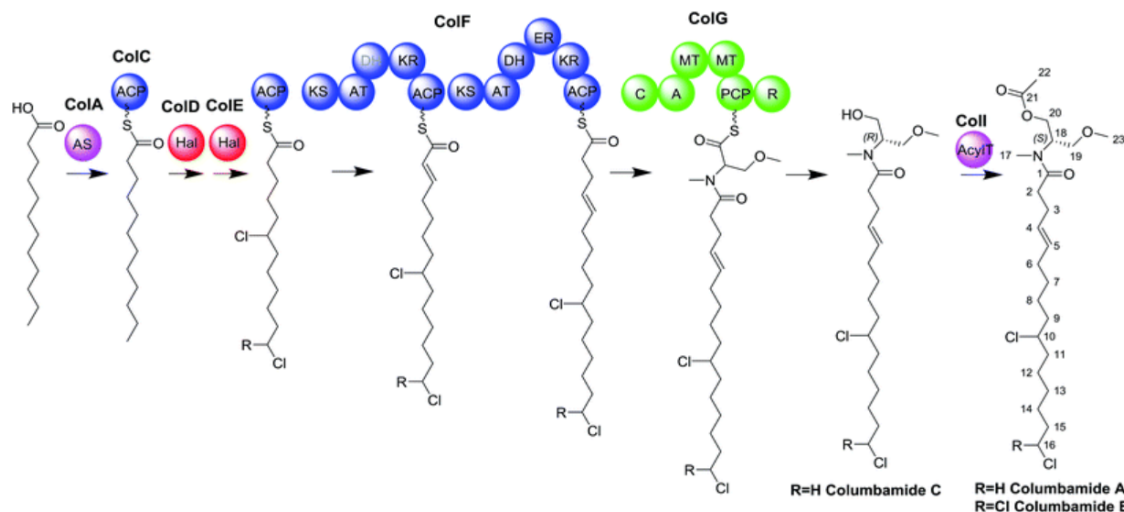
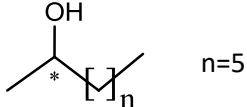
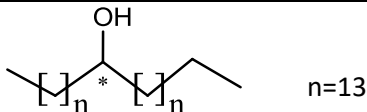
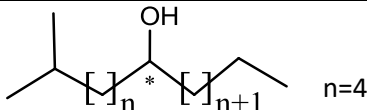
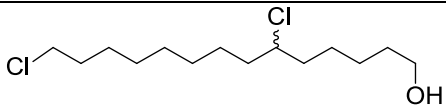


**Figure II-45.** Proposed biosynthetic pathway of the columbamides in *M. bouillonii* PNG. AS: acyl synthetase, ACP: acyl carrier protein, Hal: halogenase, KS:  $\beta$ -ketoacyl-ACP synthase; AT: acyl transferase, DH:  $\beta$ -hydroxy-acyl-ACP dehydratase, KR:  $\beta$ -ketoacyl-ACP reductase, ER: enoyl reductase, C: condensation domain, A: adenylation domain, MT: methyltransferase, PCP: peptidyl carrier protein, R: reductase, AcylT: acyltransferase.

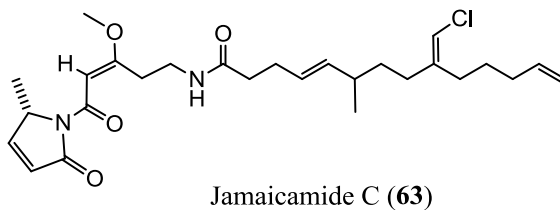
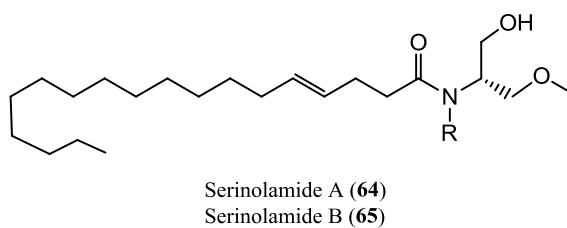
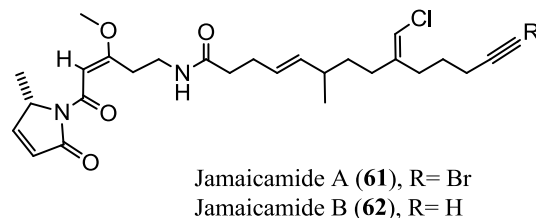
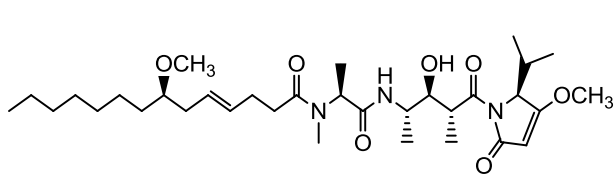
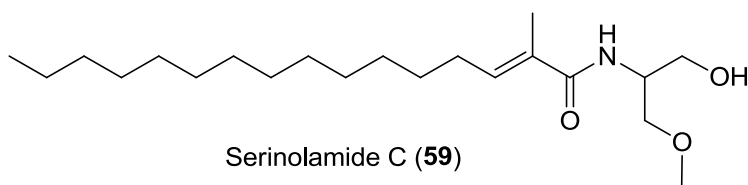
In this study the potential of a chiral labeling reagent from Ohruai-Akasaka method was examined for determining the configuration of the chloromethine of columbamide F (**43**). The widely used diastereomer method has the limited capability to discriminate optical isomers having chiral centers separated by more than four bonds. Previous report on the determination of absolute configuration of chloromethine of columbamide D (**39**) was done by total synthesis that requires laboratory facilities and extensive time relative to the other methods. Application of Ohruai-Akasaka method to determine the configuration of chloromethine of columbamide F (**43**) using UV detector in room temperature and chiral column revealed the implication of this method for the assignment of the absolute configuration of natural compounds having a remote stereogenic center. In the original method, the separation of diastereomers of chiral secondary alcohols with long methylene chains were carried out by using reversed phase HPLC system in low column temperature ( $-15\text{ }^{\circ}\text{C}$  to  $-50\text{ }^{\circ}\text{C}$ ) (Table II-3)<sup>88</sup>. However, we were able to separate the diastereomers in room temperature and UV detector that made this reagent suitable for using in minimum laboratory requirements.

**Table II-3. Chromatographic data of Ohrui esters of secondary alcohols**

| Alcohol                                                                                                                   | Mobile Phase<br>MeOH/MeCN/THF/n-<br>hexane/H <sub>2</sub> O<br>(v/v/v/v/v) | Flow rate<br>(mL/min) | Column<br>temperature<br>(°C)              | Retention<br>time<br>(min) |
|---------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------------|--------------------------------------------|----------------------------|
|  n=5                                     | 0:120:40:0:0                                                               | 0.6                   | -40<br>(Develosil<br>ODS-3<br>column)      | 58.3/61.3<br>(S/R)         |
|  n=13                                    | 0:80:120:120:0                                                             | 0.2                   | -20<br>(Develosil<br>C-30-UG-3<br>column)  | 76.5/80.5<br>(S/R)         |
|  n=4                                    | 0:50:100:0:0                                                               | 0.1                   | -40<br>(Develosil<br>ODS-3<br>column)      | 34.9/41.2<br>(R/S)         |
| <br>Alcohol from Columbamide F<br>(43) | Hexane/Isopropanol<br>(90:10)                                              | 1                     | Room<br>temperature<br>(Chiralcel<br>OJ-H) | 22.2/24.9<br>(S/R)         |

The long alkyl chain containing secondary metabolites from *Moorea* sp. exhibit the intriguing bioactivities including malyngamides and jamaicamides. Malyngamide X (**60**) with long chain acyl structure were reported to have a diverse bioactive profile including cancer cell cytotoxicity against oral human epidermoid carcinoma of the nasopharynx (KB), human small cell lung cancer (NCI-H187), and breast cancer (BC) cell lines with ED<sub>50</sub>=8.20, 4.12, and 7.03 μM, respectively, antimalarial (ED<sub>50</sub>=5.44 μM) and antitubercular activity (MIC=80 μM).<sup>89</sup> Jamaicamides (**61-63**) also showed cytotoxicity against H460 lung cancer cells and Neuro-2a neuro-blastoma cells with IC<sub>50</sub> value of 15 μM.<sup>90</sup>

Another fatty acid amides serinolamide A-B (**64-65**) and mooreamide (**35**) showed cannabinomimetic properties.<sup>41,80</sup> Because of the amphiphilic properties we checked the biosurfactant activities of columbamide D (**39**) and serinolamide C (**59**) and both of them showed comparable critical micelle concentration like synthetic surfactant SDS, pluronic f-68 and triton X-100 which revealed the potential use of this compounds in various fields.



## 2.9 Summary

Three new columbamides F (**43**), G (**44**) and H (**45**) were isolated from *Moorea bouillonii* collected in Sabah, Malaysia. In combination with NMR and MS data the planar structures of these three new columbamides were established. The absolute configuration of columbamide F (**43**) has been determined to be (10*R*,20*R*) by Marfey's analysis and chiral-phase HPLC analysis of synthetic standards and columbamide F (**43**) after derivatization with the Ohruí's acid. Ohruí's method combined with chiral-phase HPLC analysis at room temperature may increase its application to determine the absolute configuration of natural compounds having a remote stereogenic center. Because of having surface tension lowering properties with a low CMC value and weak cytotoxicity, the fatty acid amides columbamide D (**39**), serinolamide C (**59**), may have potential for applications in various fields such as healthcare, food or cosmetics. The current work reports the discovery of new potential biosurfactants, and has uncovered greater diversity in the columbamide family of compounds.

## CHAPTER 3

### Isolation and Structure Elucidation of 20-desmethylnadangolide from *Moorea bouillonii*

In chapter 1, the sample M1629 identified as *M. bouillonii* sample was chosen for the isolation of new compounds. An MS guided isolation led to the isolation of a 20-Desmethylnadangolide along with other known macrolids. The structure elucidations were done by combination of mass spectrometric and NMR spectroscopic analyses.

#### 3.1 Isolation of Nadangolides, Laingolides and (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*, 11*S*\*,12*R*\*)-10,18-diacetoxynadabella-2,7-dien-6-one

##### 3.1.1 Experimental Section

**General Procedures.** MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer. The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 μm, flow rate 0.2 mL/min at 25 °C) and operated under the following conditions: 0-15 min 50-80% MeCN with 0.1% (v/v) formic acid (FA) in Milli-Q H<sub>2</sub>O; 15-30 min, isocratic 80% MeCN with 0.1% (v/v) FA. Preparative HPLC systems equipped with a PU-980 pump and a UV-970 UV/vis detector were used to isolate the compounds.

**Extraction and Isolation.** The cyanobacterial sample (M1629) was collected from Rocky Point, Udar Island, Sabah, Malaysia (N 6°4'53"; E 116°5'16") in September, 2016 by using scuba diving at a depth of 5-10 m. The sample was preserved in MeOH for transportation. The cyanobacterial sample was extracted homogenized and extracted three times with MeOH. The MeOH extract (3.2 g) was partitioned between EtOAc and H<sub>2</sub>O. The EtOAc fraction (232 mg) was subjected to silica gel column chromatography 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 fractions (F1 to F12). The hexane-EtOAc 80:20

fraction was subjected to RP-HPLC (Cosmosil cholest column 10×250 mm, flow rate 3 mL/min, UV 210 nm, Gradient: 0-30 min 70-100% MeCN, 30-35 min 100% MeCN) to obtain 20-Desmethylnadanolide (0.5 mg,  $t_R$  17.7 min) along with known nadanolide (**3**) (0.4 mg,  $t_R$  21.3 min). To increase the amount of isolated compounds we subsequently combined other *Moorea boouillonii* samples (M1520, M1521, M1605 and M1630) that had a 20-Desmethylnadanolide with other known laingolides. This procedure resulted in the isolation of (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*, 11*S*\*,12*R*\*)-10,18-diacetoxynadabella-2,7-dien-6-one (5 mg,  $t_R$  13.1 min), 20-Desmethylnadanolide (5 mg,  $t_R$  17.5 min), known nadanolide (**3**) (5.5 mg,  $t_R$  22.1 min), laingolide A (**2**) (7 mg,  $t_R$  16.5 min) and laingolide (**1**) (5 mg,  $t_R$  18.7 min). These compounds were further purified by RP-HPLC (Cosmosil 5C<sub>18</sub> MS-II 4.6×250 mm, flow rate 1 mL/min, UV 210 nm, Gradient: 0-30 min 70-100% MeCN, 30-35 min 100% MeCN) to obtain 1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*, 11*S*\*,12*R*\*)-10,18-diacetoxynadabella-2,7-dien-6-one (2 mg,  $t_R$  8.5 min), 20-Desmethylnadanolide (3.2 mg,  $t_R$  13.1 min), known nadanolide (1.5 mg,  $t_R$  18.2 min), and laingolide A (1.3 mg,  $t_R$  12 min). The details of isolation procedure are presented in the schematic diagram (Figure III-1).

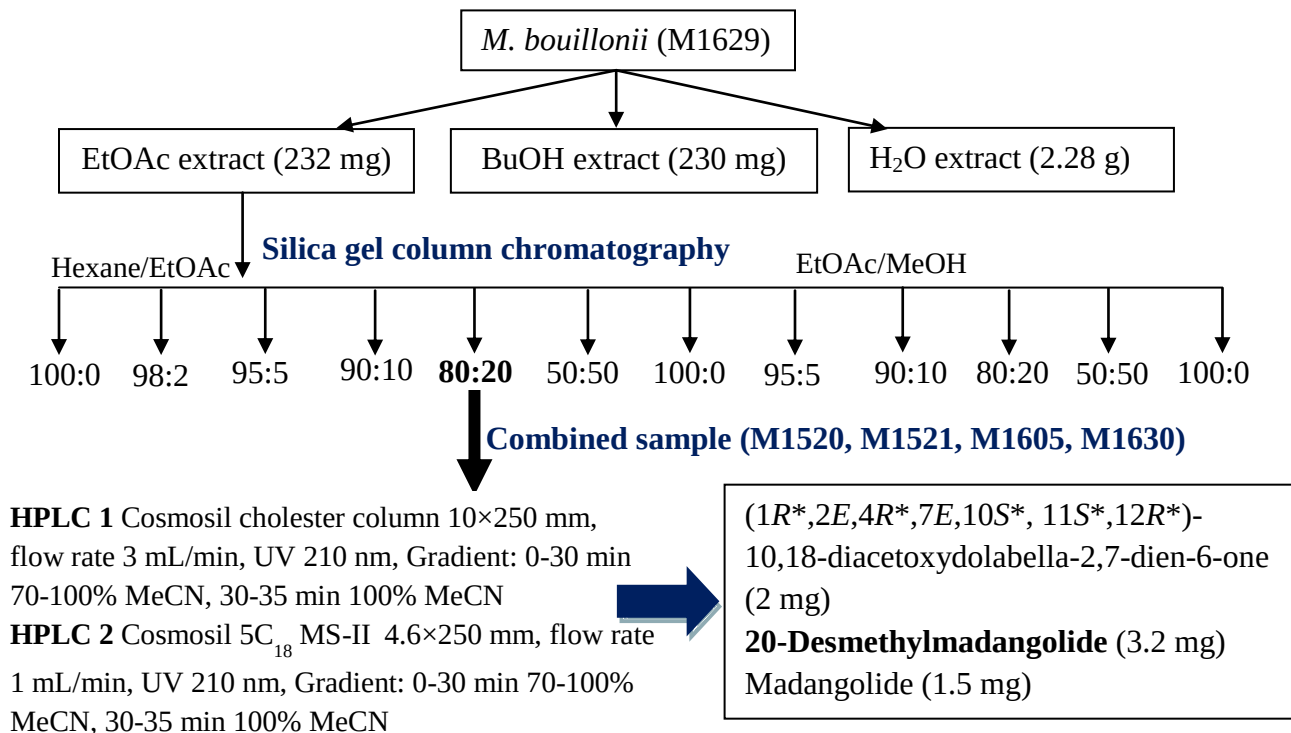


**Figure III-1.** A schematic diagram of the isolation of nadanolides and laingolides.

### 3.1.2 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 ( $m/z$  364) based on MarinLit database. The compound with  $m/z$  364 was then isolated by RP-HPLC. Although the amount ( $\sim 0.4$  mg) was not sufficient for NMR analysis and structure elucidations, a reisolation of this compound was done from other *M. bouillonii* samples and got 3.2 mg after purification along with other known compounds. The HPLC chromatogram of hexane/EtOAc 80:20 fraction of sample M1521 and M1605 are shown in Figure III-2.

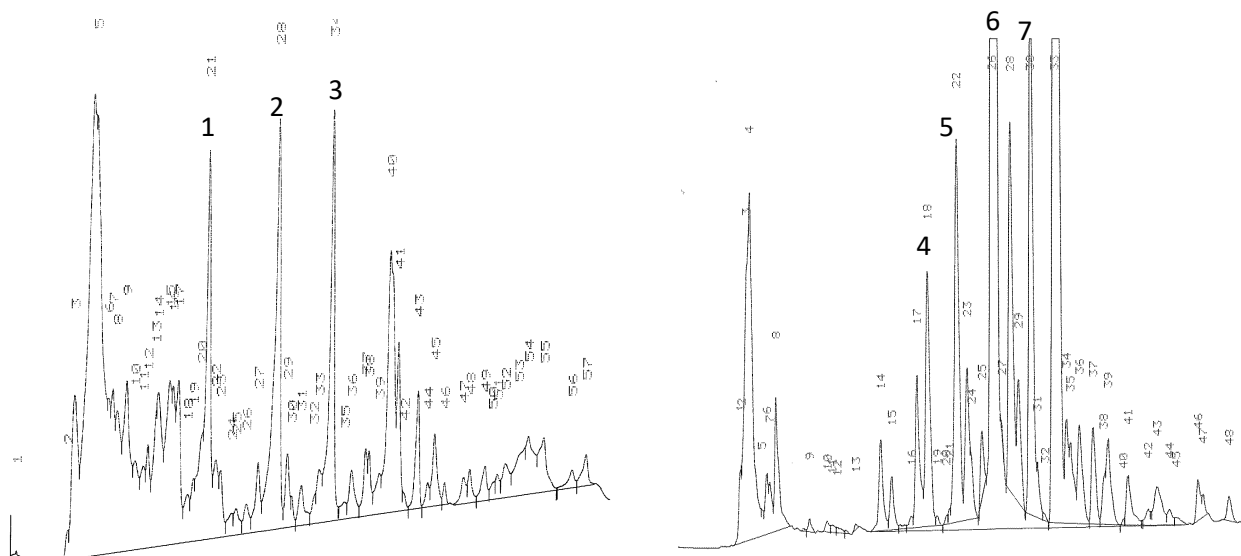


**Figure III-2.** HPLC chromatogram of the hexane/EtOAc (80:20-v/v) fraction of M1521 and M1605.

**Table III-1** list of compound isolated from hexane/EtOAc (80:20-v/v) fraction of M1521 and M1605

| Peak | $[M+H]^+ m/z$ | Compound                                                                |
|------|---------------|-------------------------------------------------------------------------|
| 1    | 345           | (1R*,2E,4R*,7E,10S*, 11S*,12R*)-10,18-diacetoxydolabella-2,7-dien-6-one |
| 2, 4 | 364           | 20-Desmethylmadangolide                                                 |
| 3, 6 | 378           | Madangolide                                                             |
| 5    | 338           | Laingolide A                                                            |
| 7    | 352           | Laingolide                                                              |

Seventeen and fifteen membered macrolides derivatives, madangolide and laingolides were purified and confirmed by comparing their  $^1\text{H}$  NMR chemical shift data shown in Figure III-3 to III-5.

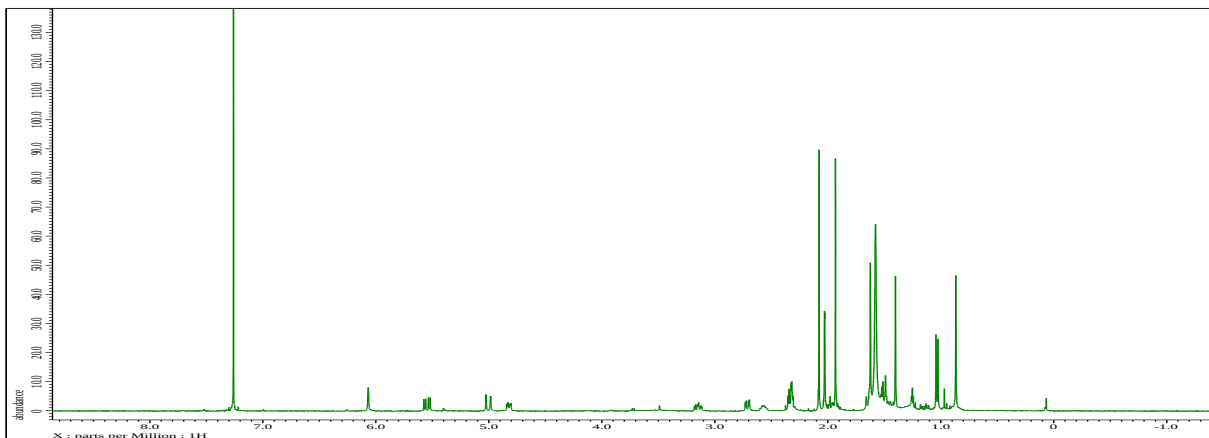


**Figure III-3.**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) of (1R\*,2E,4R\*,7E,10S\*, 11S\*,12R\*)-10,18-diacetoxydolabella-2,7-dien-6-one.

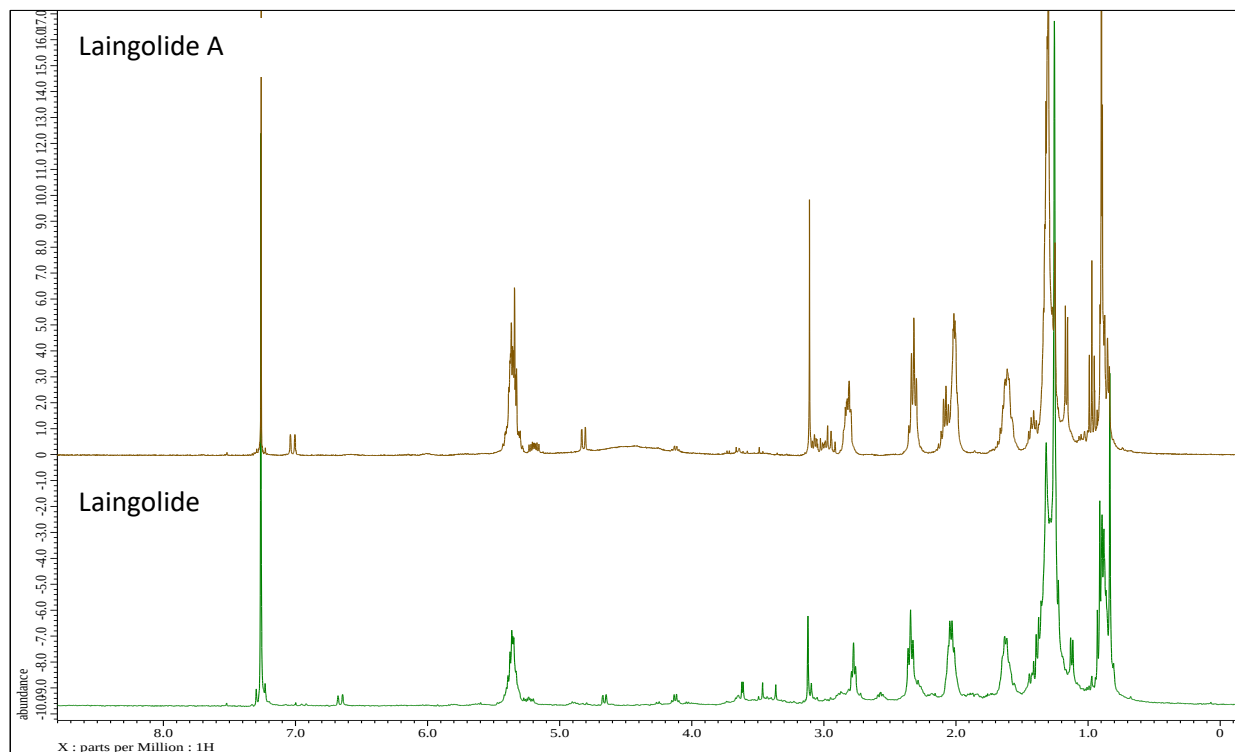


**Figure III-4.**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) of laingolide A and laingolide.

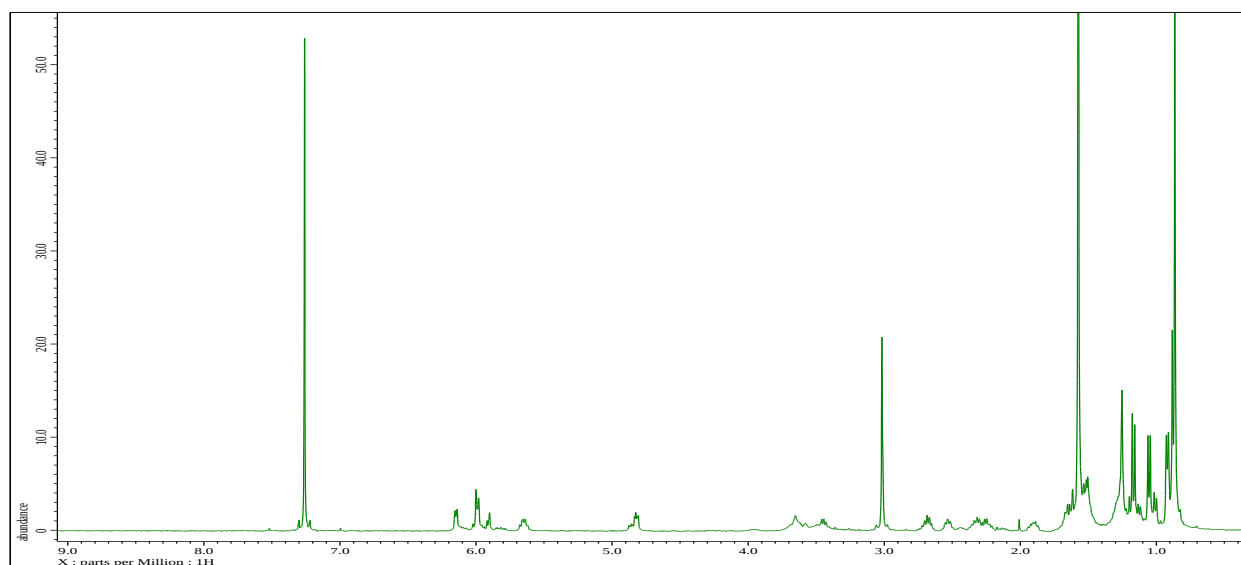


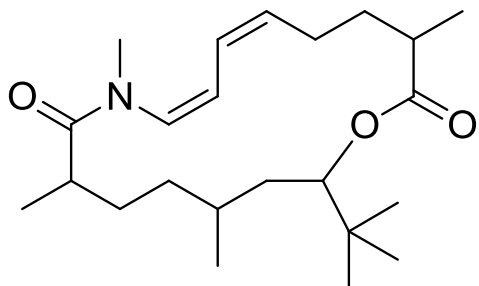
**Figure III-5.**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ) of madangolide.

### 3.2 Structure Elucidation of 20-desmethyldangolide

#### 3.2.1 Experimental Section

The  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and 2D NMR spectra were acquired using a JEOL 600 MHz spectrometer. For all NMR experiments,  $\text{CDCl}_3$  (Cambridge Isotope Laboratories, Inc.) was used as solvent as an internal standard ( $\delta_{\text{H}}$  7.26 and  $\delta_{\text{C}}$  77.0). LC-MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer.

#### 3.2.2 Results



20-desmethyldangolide (**66**)

The planer structure of **66** was established by combination of 1D and 2D NMR spectroscopic and mass analyses (Figure III-6-III-10, Table III-2). The LC-MS results of madangolide showed a molecular ion at  $m/z$  364.2901 corresponding to the molecular formula  $C_{22}H_{37}NO_3$ , indicating 5 degrees of unsaturation. The  $^{13}C$  NMR peak revealed the presence of two carbonyls ( $\delta$  176.7, 175.5), four olefinic methines ( $\delta_C$  134.9, 122.7, 121.7, and 129.7), four methines; one is connected to oxygen atom ( $\delta_C$  76.2), one *N*-methyl ( $\delta_C$  35.4) which showed similarities of this compound with madangolide. The mass difference of **66** with madangolide is 14 indicating lack of one  $CH_2$  group in new madangolide. The planar structure of **66** was established by using 2D NMR correlations. The connectivity from C-2 to C-18 and C-3 were established by corresponding COSY signals. Also the COSY correlations from C-5 to C-7 and C-10 to C-16 were observed. Corresponding HMBC correlations were observed for these signals. The connection between C-7 to C-20 by COSY revealed that the 20-desmethyldangolide contained the isopropyl group in the location of *tert*butyl group reported in madangolide. The connection of *N*- $CH_3$  at position C-23 showed HMBC correlations with carbonyl C-1 and C-16. The correlations observed by HSQC, HMBC, COSY and NOESY (Figure III-11) data led us to establish the planar structure of **66** (Figure III-12). The configuration of double bonds was assigned as *Z* on the basis of coupling constants (8 Hz and 11 Hz).

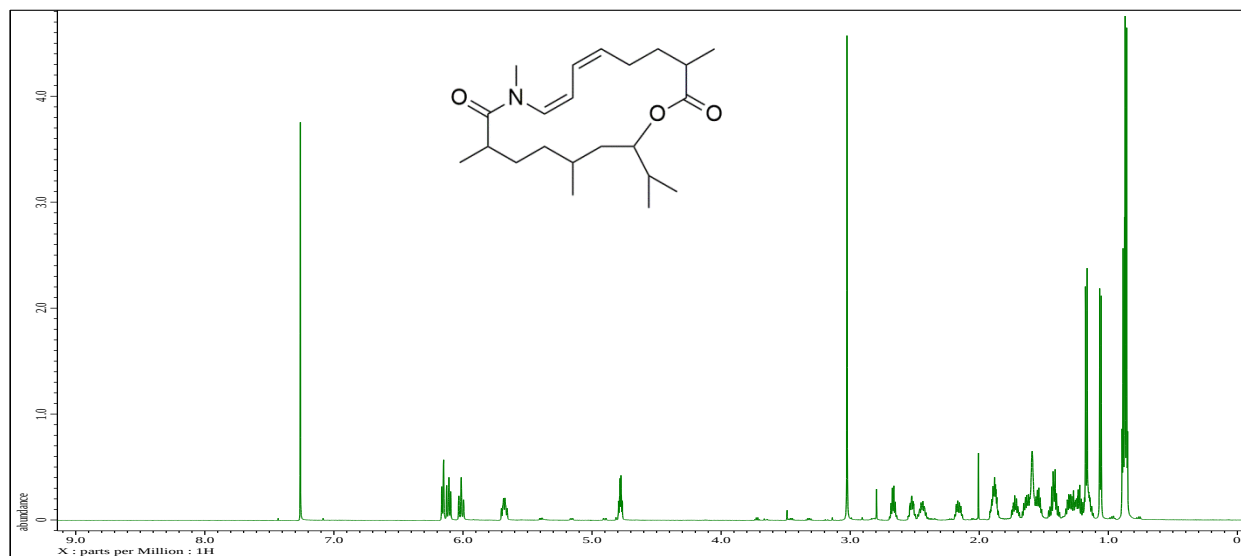


**Figure III-6.** <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) of 20-desmethylmadangolide (**66**).

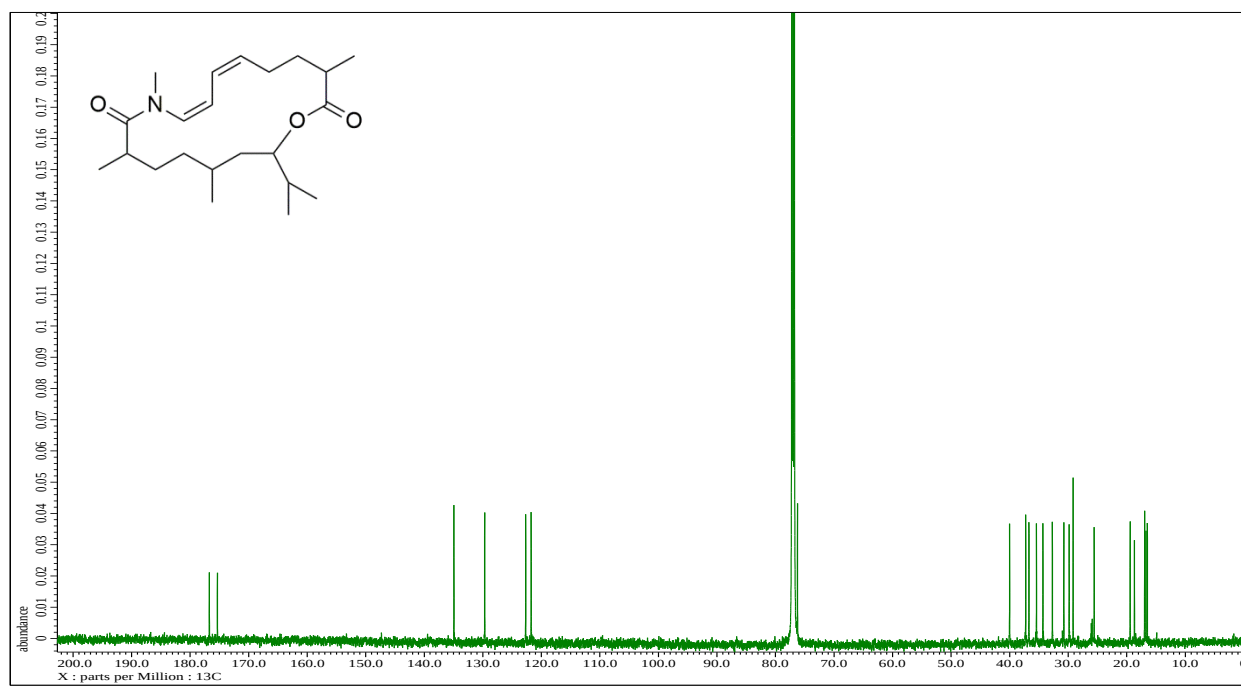


**Figure III-7.** <sup>13</sup>C NMR (600 MHz, CDCl<sub>3</sub>) of 20-desmethylmadangolide (**66**).

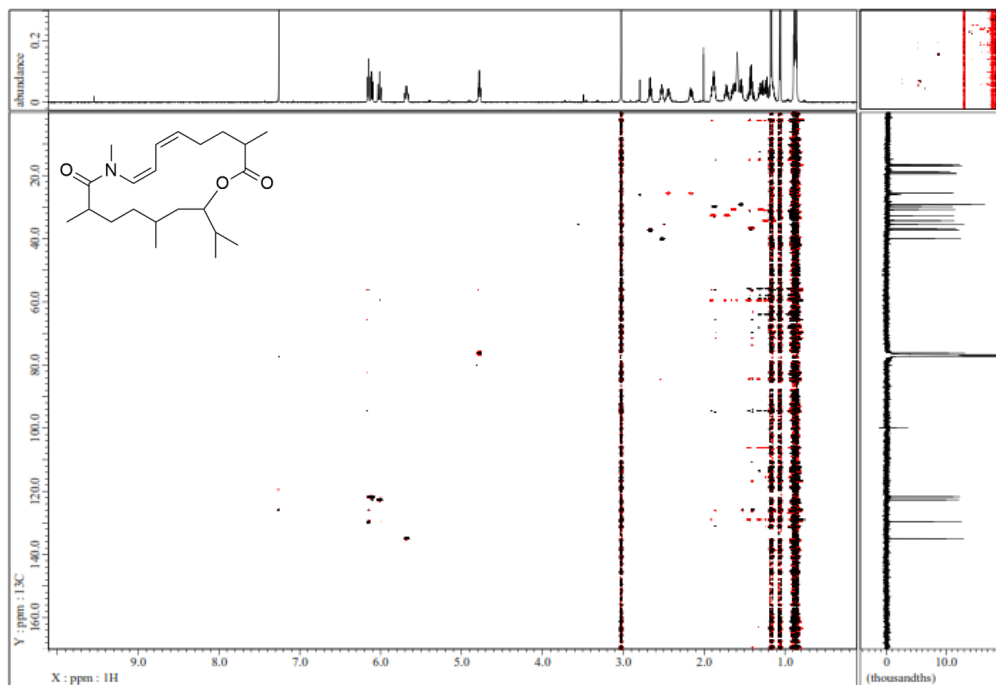


**Figure III-8.** HSQC data (600 MHz, CDCl<sub>3</sub>) of 20-desmethylnadanolide (**66**).

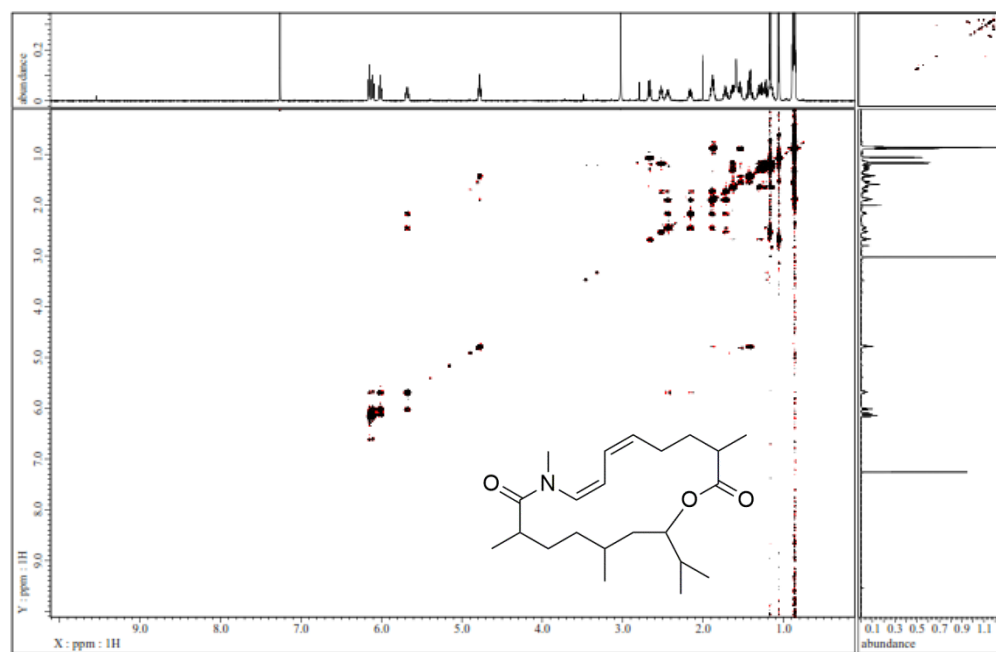


**Figure III-9.** COSY data (600 MHz, CDCl<sub>3</sub>) of 20-desmethylnadanolide (**66**).

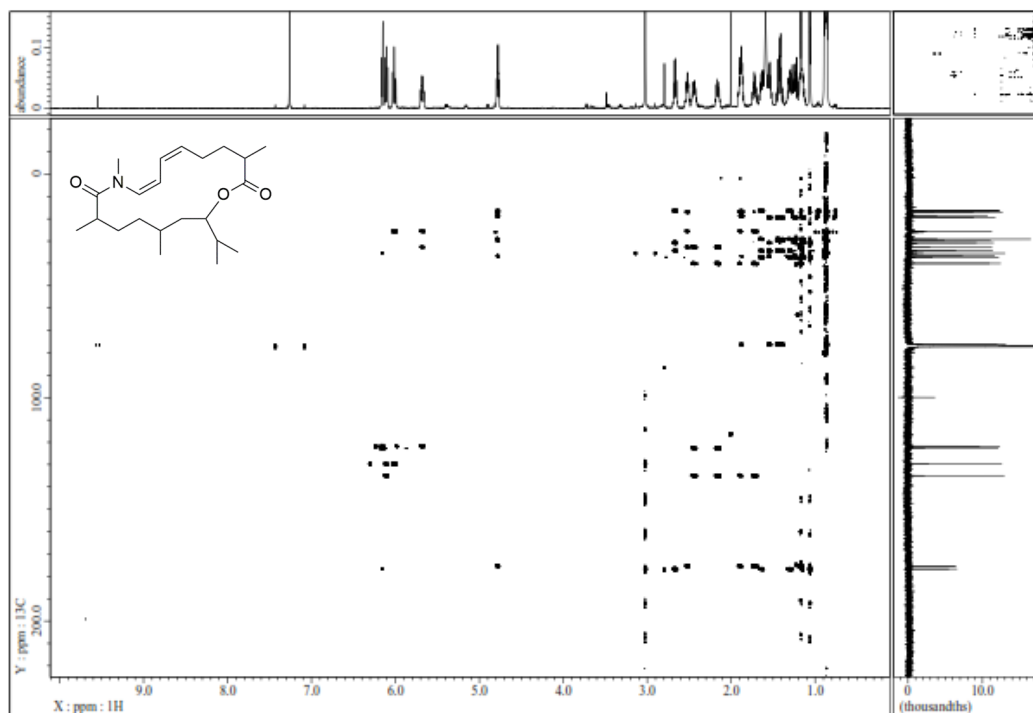


**Figure III-10.** HMBC data (600 MHz,  $\text{CDCl}_3$ ) of 20-desmethylmadangolide (66).

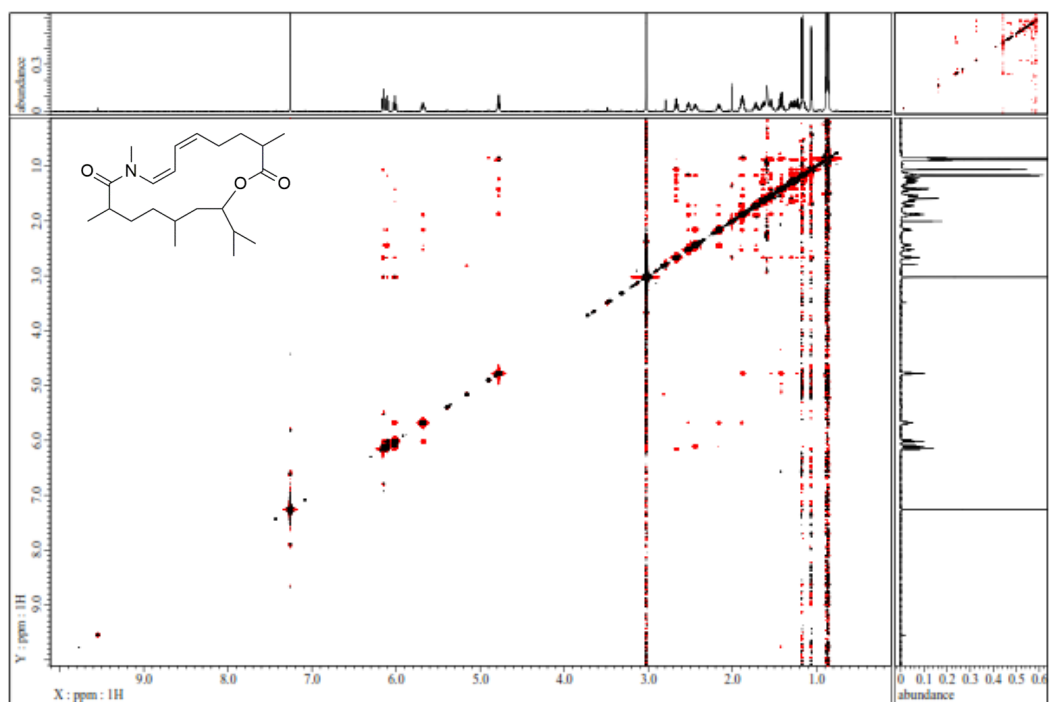


**Figure III-11.** NOESY data (600 MHz,  $\text{CDCl}_3$ ) of 20-desmethylmadangolide (66).

**Table III-2.** NMR data of 20-desmethylnadanolide (**66**) in CDCl<sub>3</sub>

| Position | <sup>1</sup> H | <sup>13</sup> C | COSY          | HMBC               |
|----------|----------------|-----------------|---------------|--------------------|
| 1        |                | 176.7           |               |                    |
| 2        | 2.66, sex      | 37.2            | 18            | 1, 3a, 4a, 18      |
| 3a       | 1.62, m        | 30.7            | 3b            | 1, 2, 4a, 5, 18    |
| 3b       | 1.32, m        |                 | 3a            | 2, 4a, 5, 18       |
| 4a       | 1.23, m        | 34.3            |               | 2, 3a, 5, 19       |
| 4b       | 1.14, m        |                 |               |                    |
| 5        | 1.53, m        | 29.1            | 6, 19         | 4a, 7, 19, 20      |
| 6a       | 1.42, m        | 36.6            | 7             | 4a, 5, 7, 19, 20   |
| 6b       | 1.42, m        |                 |               |                    |
| 7        | 4.78, m        | 76.2            | 6, 20         | 5, 6a, 9, 19       |
| 9        |                | 175.5           |               |                    |
| 10       | 2.52, m        | 39.9            | 22, 11b       | 9, 11a, 12a, 22    |
| 11a      | 1.90, m        | 32.7            | 11b, 12b      | 9, 10, 12a, 13     |
| 11b      | 1.73, m        |                 | 11a           | 9, 10, 12a, 13     |
| 12a      | 2.43, m        | 25.5            | 11a, 11b, 12b | 9, 10, 11a, 13, 14 |
| 12b      | 2.17, m        |                 | 11a, 12a      | 10, 11a, 13, 14    |
| 13       | 5.69, m        | 134.9           | 12            | 11a, 12a, 15       |
| 14       | 6.01, t        | 122.7           | 13, 15        | 112a, 16           |
| 15       | 6.11, t        | 121.7           | 14            | 13, 16             |
| 16       | 6.15, d        | 129.7           | 15            | 1, 14, 15, 23      |
| 18       | 1.07, d        | 16.4            | 2             | 1, 3a, 4a          |
| 19       | 0.88           | 19.4            | 5             | 6                  |
| 20       | 1.89, m        | 29.83           | 21            | 6a, 7, 19          |
| 21, 22   | 0.88           | 18.68           | 20            | 7, 20              |
| 23       | 1.17, d        | 16.9            | 10            | 9, 11a             |
| 24       | 3.03, s        | 35.4            |               | 1, 16              |

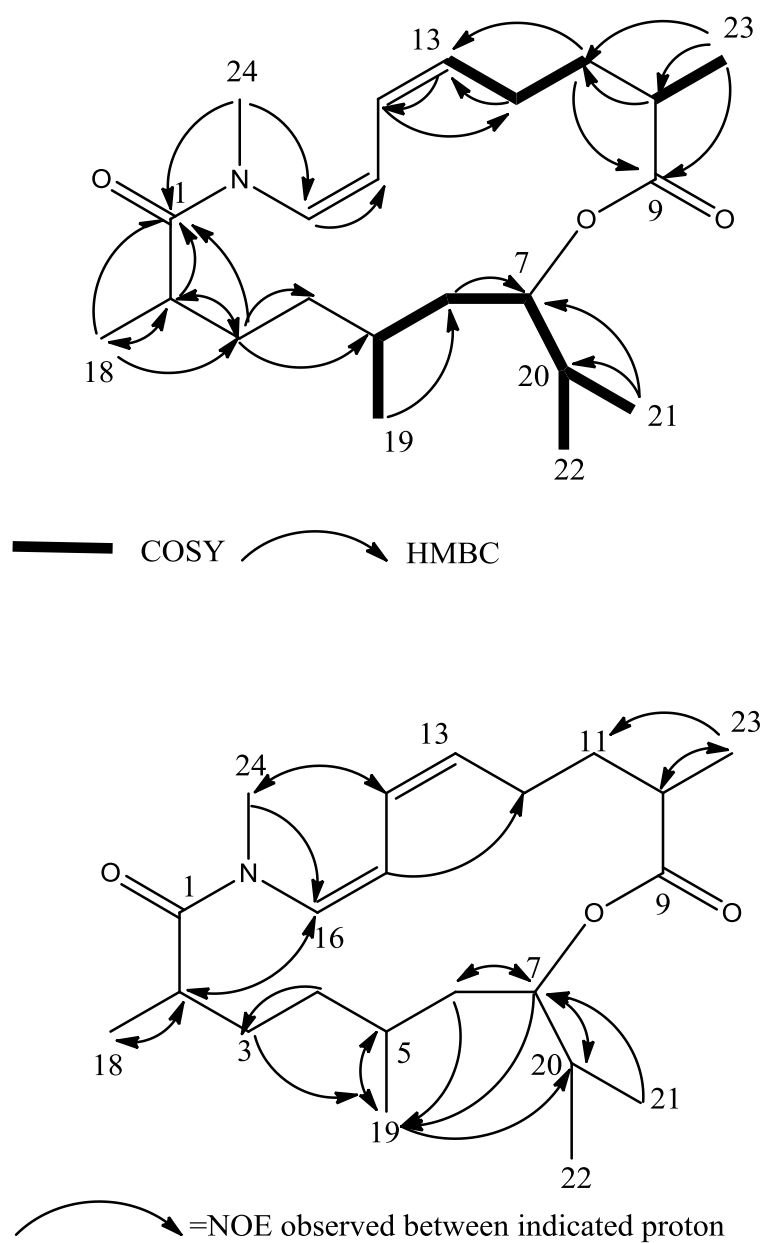


**Figure III-12.** 2D NMR correlations of 20-desmethylmadangolide (**66**).

### 3.3 Bioactivity

#### 3.3.1 Experimental Section

**Anti allergic assay.** The anti-allergic activity of crude extracts from silica gel fractions were checked by antigen induced  $\beta$ -hexosaminidase release assay.<sup>91</sup> RBL-2H3 cells were seeded into a 96-well microplate at  $5 \times 10^4$  cells/well and cultured at 37 °C in a 5% CO<sub>2</sub> incubator for 24 h. The medium was removed by suction, washed with 100  $\mu$ L/well PBS (-), and then 100  $\mu$ L /well Anti DNP-IgE solution (50 ng/mL) was added, and the cells were cultured at 37 °C in incubator for 2 h to sensitize cells. The anti DNP-IgE solution was removed, and the well was washed twice with 100  $\mu$ L/well MT buffer, and the MT buffer solution of the sample and control was placed in 50  $\mu$ L/ well. After 10 min incubation at 37 °C, 50  $\mu$ L /well DNP-HSA solution (100 ng /mL), and incubate at 37 °C in a 5% CO<sub>2</sub> incubator for 2 h. The cells were stimulated by adding the antigen and culturing. The microplate was taken out of the incubator and ice-cooled for 10 min to stop the reaction. The culture supernatant was completely transferred to the lower well to obtain a supernatant, and Triton-X was added as a cell lysis solution to the remaining cells at 100  $\mu$ L/well to lyse the cells. The lysate was cell lysate. After standing at room temperature for 10 minutes, preincubation was performed at 37 °C for 5 min. The substrate solution was added to the upper layer at 50  $\mu$ L / well, mixed well by pipetting, incubated at 37 °C for 25 min, and the reaction stop solution was added to the lower layer at 50  $\mu$ L/well. After 25 min, the reaction stop solution was added to the upper layer at 50  $\mu$ L/well, mixed well by pipetting, and the substrate solution was added to the lower layer at 50  $\mu$ L/well to stop the reaction. The absorbance at 405 nm was measured with a microplate reader.

The principle of this operation is to act on  $\beta$ -hexosaminidase to release *p*-nitrophenol. By measuring the released *p*-nitrophenol by a colorimetric method (405 nm), the released amount of  $\beta$ -hexosaminidase from the cells at the time of untitled grains is determined. By comparing the amount of  $\beta$ -hexosaminidase released when the MT buffer was added instead of the sample, the rate of inhibition of the sample's reliable grain was determined. The  $\beta$ -hexosaminidase release inhibitory activity of the sample was calculated with the control “ $\beta$ -hexosaminidase release rate as 100%”.

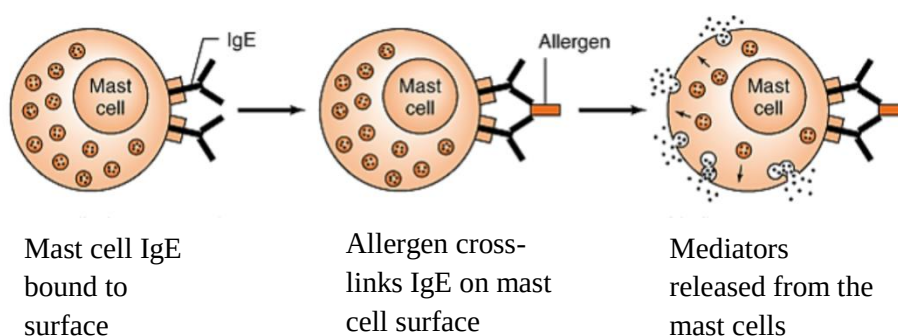


Fig III-13.  $\beta$ -hexosaminidase release assay.

$$\beta\text{-hexosaminidase release rate (\%)} = 100 * [(S - S_c) / \{(S - S_c) + (CL - CL_c)\}]$$

S : Absorbance of supernatant

S<sub>c</sub> : Absorbance of supernatant when stop solution added first then substrate

CL : Absorbance of cell lysate

CL<sub>c</sub> : Absorbance of cell lysate when stop solution add first then substrate solution

### 3.2.2 Results

The investigation of anti-allergic activity of silica gel fractions from *M. bouillonii* samples was done by assessing its impact on mast cell degranulation using RBL-2H3 cells.<sup>92</sup> Anti-DNP IgE-sensitized RBL-2H3 cells were treated with different fractions from silica gel column chromatography at concentration of 100  $\mu\text{g/mL}$  and the results suggested that the  $\beta$ -hexosaminidase release rate was significantly decreased (12%) in cells treated with hexane/EtOAc 80:20 fraction compared to control. The  $\beta$ -hexosaminidase release rate was also checked for the silica gel hexane/EtOAc 80:20 fraction of M1629 and showed reducing rate (21%) at concentration 100  $\mu\text{g/mL}$ . Then we decided to investigate the anti- allergic activity of pure compounds isolated from this fraction. Figure III-14 shows the  $\beta$ -hexosaminidase release rate of pure compounds madangolide (**3**) ( $\text{IC}_{50}$  value =20.5  $\mu\text{g/mL}$ ), (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*,11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one (**67**) and laingolide A (**2**) ( $\text{IC}_{50}$  value =33

μg/mL) along with wortmannin (**68**). Wortmannin is used as positive control in this study which is known to have RBL-2H3 grain suppressive activity (IC<sub>50</sub> value, 10-41 nM).

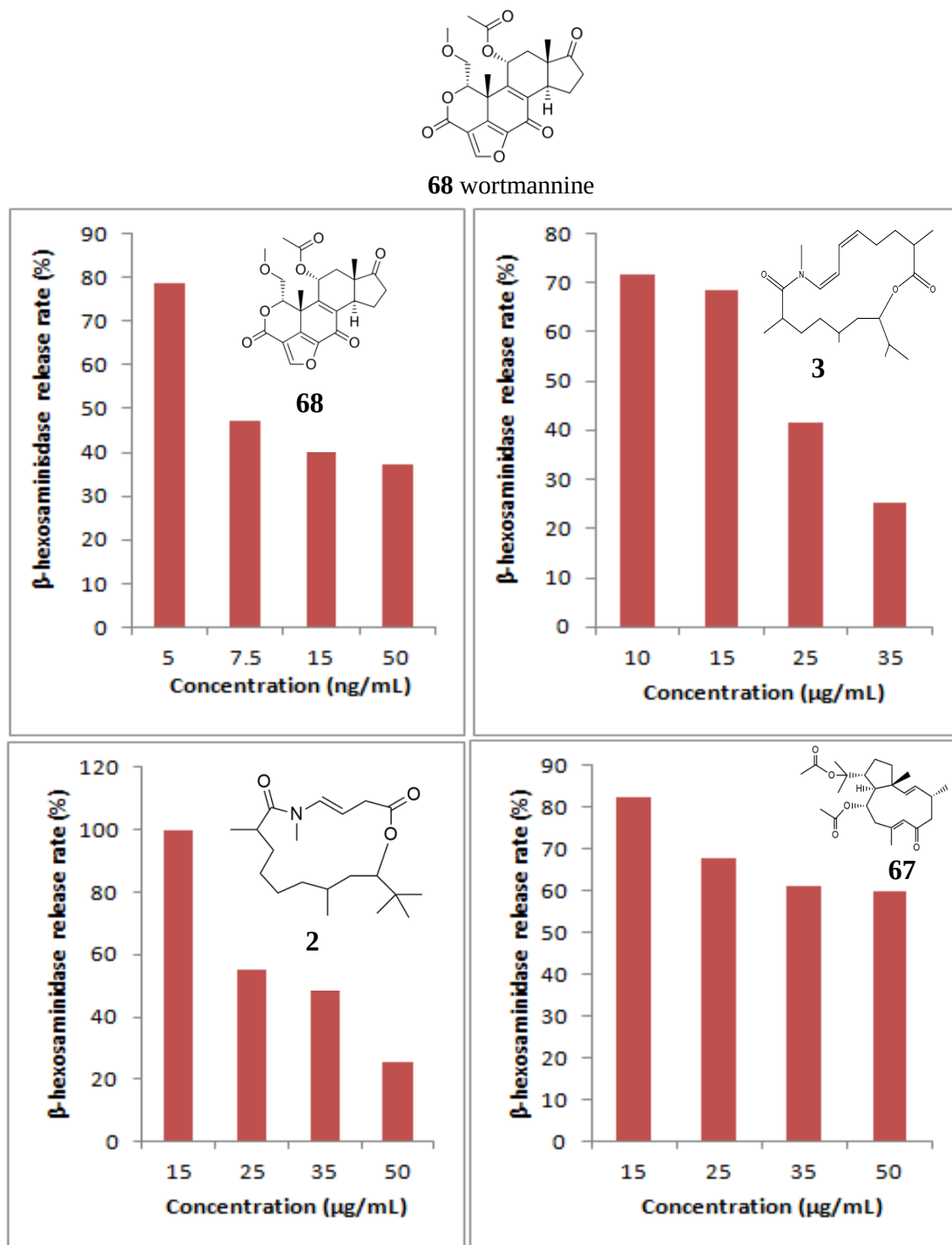
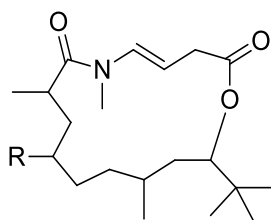


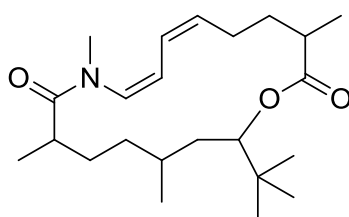
**Figure III-14.** β-hexosaminidase release rate of wortmannin (**68**), madangolide (**3**), laingolide A (**2**) and (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*,11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one (**67**).

### 3.4 Discussion

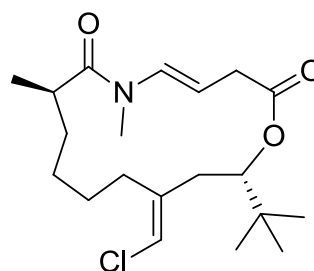
A small group of macrolides having trans-*N*-methyl enamide with *tert*-butyl carbinol moiety were found in marine cyanobacteria including laingolide (**1**), laingolide A (**2**), madangolide (**3**), and laingolide B (**4**).<sup>25,26,27</sup> Different collections of the marine cyanobacterium *Lyngbya bouillonii* produced laingolides and madangolides were collected from Laing island and Madang island in Papua New Guinea, whereas laingolide B was isolated from *M. bouillonii* collected at shallow patch reefs in Apra Harbor, Guam. Structurally similar palmyrolide A (**69**) was isolated from cyanobacterial assemblage of *Leptolyngbya* cf. and *Oscillatoria* spp.<sup>93</sup> Although the planar structure of laingolides and madangolides were proposed in their isolation paper, the absolute configuration has not been determined. The absolute configuration was determined by total synthesis for laingolide B (**4**).<sup>94</sup>



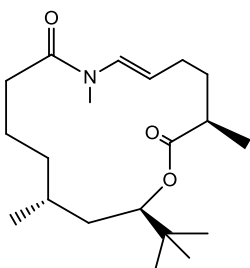
**1** Laingolide R=CH<sub>3</sub>  
**2** Laingolide A R=H



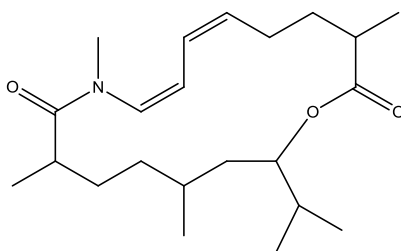
**3** Madangolide



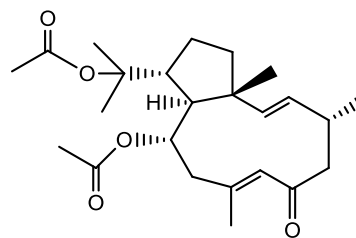
**4** Laingolide B



**69** Palmyrolide A



**66** New Madangolide

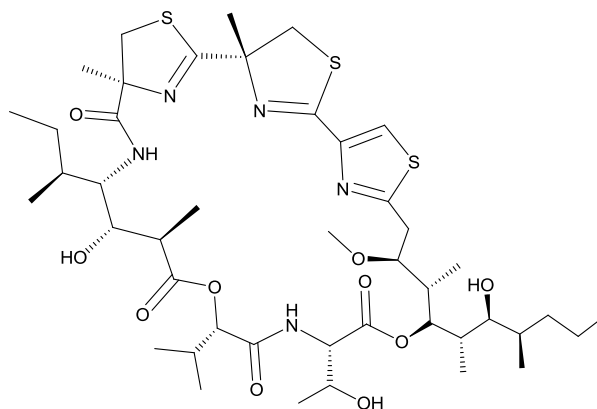
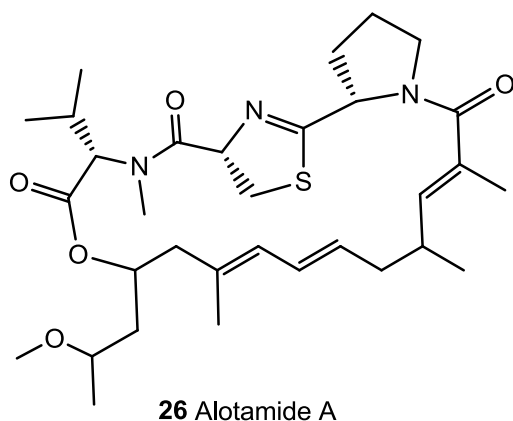


**67** 1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*,11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one

The chemical profile of hexane/EtOAc fraction of *M. bouillonii* (M1629) revealed the presence of laingolides, madangolide and 20-Desmethylmadangolidewith  $m/z$  364. In addition, the compound showed the molecular ion at  $m/z$  405 (**67**) was also isolated along with other known compounds. The NMR analysis of **67** showed similarity of this compound with (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*, 11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one previously isolated from sponge *Dysidea* sp.<sup>95</sup> from Palau. Diterpenes from dolabellane were isolated from sea hares of the genus *Dolabella* which are mainly produced by brown algae *Dictyota* sp.<sup>95,96</sup>

The absolute configuration of two chiral centers of laingolide B were determined by total synthesis and the synthesis of diastereomers of laingolide A are achieved in eleven to twelve steps.<sup>94,97</sup> The absolute configuration 15-membered palmyrolide A was determined by ozonolysis and oxidative workup followed by acid hydrolysis of the crude reaction product to afford 2-methylglutaric acid which was further derivatized with (S)-(+)-2-octanol. The commercial standard (R)-(-)- and (S)-(+)-2-methylglutaric acid are derivatized with (S)-(+)-2-octanol in a similar way. The comparison of the <sup>1</sup>H NMR spectrum and optical rotation data of standards and natural compound led to the assignment of configuration at position C-14 for palmyrolide A.<sup>93</sup> The relative configurations of the two chiral centers were solved by *J*-based configuration analysis.<sup>93</sup> Later, the absolute configuration at 1-3-methine system C5-C7 system was resolved by total synthesis.<sup>98,99</sup>

The cyanobacterial macrolides hoiamide A (**70**), alotamide A (**26**), and palmyrolide A (**69**) has proven for suppression or activation of spontaneous Ca<sup>2+</sup> oscillations of murine cerebrocortical neurons.<sup>36,93,100</sup> Here in this study the anti-allergic activity of macrolids laingolides and madangolides were investigated. Although the anti-allergic activity of madangolide, laingolide and compound (**67**) reduced the  $\beta$ -hexosaminidase release, the cytotoxicity assay will be done in the future investigation to determine that the suppressive effect of pure compounds on degranulation of RBL-2H3 cells might not be caused by cell death.



### 3.5 Summary

A 20-Desmethylmadangolide was isolated from hexane /EtOAc 80:20 fraction. The planar structure was established by combination of NMR spectroscopic and mass spectrometry analysis. The known compounds madangolide (**3**) and laingolide A (**2**) showed anti allergic activity with IC<sub>50</sub> value 20.5 and 33 µg/mL, respectively by reducing the β-hexosaminidase release from RBL-2H3 cells.

## General Discussion

In this study, the marine cyanobacterial samples collected from Bornean coast of Northern Sabah, Malaysia were identified by 16S rRNA gene sequencing, analyzed by LC-MS and screened for biosurfactant activity and cytotoxicity. Marine cyanobacteria are known to be a prolific producer of bioactive secondary metabolites. However, the records of secondary metabolites from marine cyanobacteria collected in Sabah, Malaysia are scarce. Acyl amides columbamide D and E were the first reported compounds isolated from *M. bouillonii* sample collected from Mantanani island, Sabah, Malaysia.

On the basis of 16S rRNA gene sequence analysis *Moorea* species was found to be the most abundant species among others. The chemical profile of *M. bouillonii* showed the richness of secondary metabolites with possible new halogenated compounds. The LCMS screening results suggested the possibility of finding novel compounds from marine cyanobacteria in this area. The presence of (1*R*\*,2*E*,4*R*\*,7*E*,10*S*\*, 11*S*\*,12*R*\*)-10,18-diacetoxydolabella-2,7-dien-6-one in *M. bouillonii* which is originally isolated from sponge *Dysidea* sp. might suggest that this cyanobacteria have the symbiotic relationship with associated sponge.

Novel acyl amides columbamide A-C were isolated from laboratory cultured *M. bouillonii*<sup>44</sup>, while columbamide D and E were isolated from *M. bouillonii* sample collected from Mantanani island, Sabah, Malaysia. In this study we isolated three new columbamides F-H from *M. bouillonii* sample collected from Mantanani island, Sabah, Malaysia. The presence of columbamide D and E were also detected in the same sample.

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 Ohruai-Akasaka method. The chemical degradation and derivatization with Ohruai'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 Ohruai 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 spreading 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 standards. These results revealed the biosurfactant properties of fatty acid amides and increase their application in different fields.

Madangolide was reported from *M. bouillonii* more than 10 years back. 20-Desmethylnadangolide 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 nadangolide and laingolide. The configuration analysis and details of bioactivity for 20-desmethylnadangolide and other related compounds are under investigation and will be reported in the future.

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<sup>101</sup>, 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.

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## APPENDIX

### List of 16S rRNA Gene sequences

>*Moorea producens* M1620 16S ribosomal RNA gene, partial sequence

GTGATTTTGTAGGGGAACCAACCGGAACAGTGGCTAATACCGGATGTGCCGAGAGGT  
GAAAGATTTATTGCAAAGGGGAGCTCGCGTCTGATTAGCTAGTTGGTAGGGYAAAT  
GCCTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGA  
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGG  
GCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTTTTGGGTGTAAAC  
CTCTTTTATCAGGGAAGAAGAAGTGAAGCCCTGAGGAAAAAGCATCGGCTAACTCC  
GTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGGCG  
TAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTAAAGACCAGGGCTTAAGTCTG  
GGCAGGCAGTGGAACTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGAATTCCTCG  
GTGTAGCGGTGAAATGCGTAGATTTTCGGGAAGAACATCGGTGGCGAAGGCGCTCTA  
CTGGACTGAACCTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATA  
CCCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTAGCTTGTATCGACCCAAG  
CTGTGCCGAAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTG  
AAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGTATGTGGTTTAATTC  
GATGCAACGCGAAGAACCTTACCAGGGCTTGACATGTCGCGAATCCCGGTGAAAGC  
TGGGAGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT  
CGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTCGTCTTTAGTTGCCAGC  
ACTTCAGTCAGCATGCCCTTACGCCCTGGGCGACACACGTACTACAATGGTCAGGA  
CAATGGGCAGCCAACTCGCAAGGGGGGAGCTAATCTCATCAAACCTGGCCTCAGTTC  
AGATTGCCGGCTGCAACTCGCCGGCATGAAGGAGGAATCGCTAGTAATCGCCGGTC  
AGATTACAGGGGTGAATCCGTTCCCGGGCCTTGTACACACCGTCCGCCCCCCCCATGG  
AAGCTGGCCACGCCCCGAAGTCGTTACCCTAACCTTTTTTGGAGGGGATGCCCATGAG  
CCCCTGGGACCCGCAACCCTCGTCTTTAGTTGCCAGCACTTCATGTGGGCACTCTAG  
AGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGCC  
CCTTACGCCCTGGGCGACACACGTACTACATGGTCAGGACATGGGCAGCCAACTCG  
CAGGGGGAGCTAATCTCATCAACCTGGGCCTCAGTTCAAATGCCGGCTGCA

>*Symploca* or *Caldora* sp. M1621 16S ribosomal RNA gene, partial sequence

CTTAGAGTTCCGCCCTGTACAGGAACACTGGACGTGCTATACCTTGGTTCCGGTGAA  
GTATTTTGAAGAGGAGGAGCTCGCGGCTGATTATCTAGTTGGGGGGGTAAAGCGT  
ACCAAGGCGGCGATCATTAGCTGGTGTGAGAGGAGGAGCATCCACCCTGGGGCTGA  
TACACCGCCACACTCCTACTGGAGGCGGCAGTGGGGAATTTTTTTCCTGGGGGCAA  
GCCTGATGGAGGATACCGCCTGAGGGAGGAAGGCTCTTGGGGCGTAAACCTCTTTT  
TTTGGAGGAAGTTCTGACGGTACCTGACGAATCAACATCGGCTAACTCCGTGCCAGC  
CACCGCGCTAATACAGAGGATGCAAGCGTTATCTTGAATTATTGGGCGTAAAGCAA

CCGCCGTGGGGTTTCAAGTCTGCTGTTAAAGACGGGGCTAAACTCCCGTAAAGCAGT  
GGAAGTGAAGATCTAGATAGAGGAGGGGTAGAGGAATTCCCTTTGTGCGGGGAATG  
CGTGATATCGGTAGAACATCCCCGGGGGAAGCACTCTACTGGACTGCACTTACCCTCA  
CGGACGAAACAAAGGGAGGAATTTATGACGGGGCCTGACAAATCAACATCGGCTAA  
CTCCCTGCCCCCACCCTCGGGAATACAGGGGGTGCAAGCGTTTTCCCGAATTATTGG  
GGGTAAAGCGTCCGCGGGGGGGATTTCAGTCTGCTGTTTAAAACCGGGGGCTAAAC  
TCCGGAAGGCAGTGGAAGTATAGCTAGAGTGCAGGAGGGGTAGAGGGGATTTT  
CGGTGTGGCGGGGAAATGCGTATATATCTGGAAAAAATCCGTGGGGAAAGGGCTC  
TTCTGGGCTGTATCTGACACTCACGGACCAAAGCTAGGGGAGCGAATGGGATTTGAT  
ACCCCCCTAGTCCTAGCCCTAAACAATGGATACTATGTGTGGCTTGTATCGACCCGA  
GCCGTGCCTTAGCTAACGCGTTTAATATCCCCCCCCGGGGAATACGCGCGCAAGCGTG  
AAACTCAAAAGAATTTACGGGGGGCCCCACAAACGGGGGAGTATGTGGTTTTATTTT  
ATGCAACCCGAAAAACCCTTCCAGGGGTTGACATGTCTCGAATMTTTTTTTAAACTA  
AAAATGCCTTTTGAACGCGAACACAGGTGGTGCATGGCTGTCCTCACCTCGTGTGCG  
TGAGAATGTTGGGTAAAGTCCCCCAACCAGCGCAACCCTTTTTTTTTATTTTGCCTTC  
ATTGAAATTGGGCACTCTATACAGACTGTAAGCGACAAAAGGGAGGAAGGTGGGGG  
ATGACTTCAAGTCCCCATGCCCTTGGGTTCGGGGCTTCTCTACATTCTACGAAGGA  
AAAGGAACAAAAGGTCTTCCAAACCCCAAAAGGAAAAATCAAATCCCATCAAACCT  
GGGCAATATGTTTCAAGATTCCTGCTGCGACTCAACCTTGCGAAAACCGCAAGAAAAA  
GGGAGGAATGGATTAATTCCCCCTAGTCTTCCCCTAAACAAAGATACAACCTTTTGGC  
TTGAACGACCCCCCGGGGCAAAACACCTAATAATTACCCCCGGGGGGAACCCCC  
ACCAGAAAAAATAAATGGGGCCCCCCCCGAGGGGTTTTTGGTTATTATTCA  
GCCACAAACACCCCCCTGTTTCCGAAATTTTTTATAAAAATCTTGGGGGAAACACCC  
GGGGTGCCTCC

>*Moorea bouillonii* M1622 16S ribosomal RNA gene, partial sequence

TCCAATCCGCCTTAGGCATCCCCTCCAAAGGGTTAGGGTAACGACTTCGGGGTGGCA  
GCTTCTGGAGGGACGGGSGGTGTGTACAAGGCCCGGAACGGATTCACCGCGTATTC  
GACCGGGATTACTAGGATTCCTCCTTCATGCCGGCAGTTGCACCGGCAATCTGAACT  
GAGGCCAGGTTTGATGAATTAGCTCCCCCTTGCGAGTTGGCTGCCCATTTGTCCTGAC  
CATTGTAGTACGTGTGTCGCCCAGGGCGAAGGGGCATGCTGACTTGACGTCATCCCC  
ACCTTCCTCCGGTTTGTACCGGCAGTCTCTCTAGAGTGCCCACCATACGTGCTGGC  
AACTAAAAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACACACGA  
GCTGACGACAGCCATGCACCACCTGTGTTTCGCGCTCCCGAAGGCACTCCCATTTTAC  
CGGGATTCGCGACATGCAAGCCCTGGTAAGGTTCTTCCGTTGCATCGAATTAAACCA  
CATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGTTTCACACTTGCGTGC  
GTACTCCCCAGGCGGGATACTTAACGCGTTGGCTTCGGCACAGCTTGGGTGATACA  
AGCTACACCTAGTATCCATCGTTTACRGCAGGACTACTGGGGTATCTAATCCCATT  
CTCCCCTAGTTTCGCCTCAGTGTACGGCAGCCAGTAGAGCGCCTTCGCCACCGATGT  
TCTTCCCGAAATCTACGCATTTCCGCTACACGGAATTCCTCTACCCCTACTGCCTCT

AGTCCCAGTTTCCATGCTGCCCAGAGTAAGCCCTGTTTTTTAACAGCCGACTTGAGG  
AC

>*Okeania* sp. M1623 16S ribosomal RNA gene, partial sequence

TCCGGGGGGGTGAGTACGGTGAGAATTGCCTTCAGGTTGGGGCACAAAAGAACTT  
TGCTAATCCCGGAGGGCGAATGGTAAAGATAATTGCCTGGAGATGAGCTCGCTTTG  
ATTAGCTAGTTGGTGGGGTAAAAGCTTACCAAGGCAACGATCAGTAACTGGTCTGA  
GAGGATGAGCAGCCCCACTGGGCTGAGACACGGCCCCAGACTCCTACGGGAGGCAG  
CAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAAGACCGCGTGGGGG  
AGGAAGGCTTTAGGGTTGTAAACCCCTTTTCTAGGGAAGAAGTAGGAGGGGCGAAA  
GGAATCAGCACCCCTTTTCTTTGGGAAGAAGTTCTGACGGTACCAAAGGAATCAGCC  
TCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGCGTTATCCGG  
AATGATTGGGCGTAAAGCGTCCGCAGGTGGCCATGTAAGTCTGCTGTCAAATCCCAG  
GGCTCAACTCTGGTCAGGCAGTGGAAGCTACAAAGCTAGAGTCTGGTAGAGGCAAA  
GGGAATTCCCGGCGTAGCGGTGAAATGCGTTTTTTCTGAAGAACATCGGTGGCGAA  
AGCGCTTTGCTAGACCTGATCTACAATAAGGAATGAAAGGTTCAAAGGG

>*Moorea bouillonii* M1629 16S ribosomal RNA gene, partial sequence

GCGGGCCTGCTTGGGGTACCACCGGAACAGTGGCTATACCGGATGTGCCGAGAGGT  
GAAAGATTTATTGCACAAAGGAGCTCGCGTCTGATTAGCTAGTTGGTAGGGTAAAG  
CCTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGAC  
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGG  
CGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTTTTGGGTTGTAAACC  
TCTTTTATCAGGGAAGAAGAAAGTGACGGTACCTGAGGAAAAAGCATCGGCTAACT  
CCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGG  
CGTAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTAAAGACCAGGGCTTAATCT  
TGGGCAGGCAGTGGAAACTGAGGAACTAGAGGGCAGTAGGGTAGAGGGAATTCGG  
TGTAGCGGTGAATGCGTAGATTTCCGGAAGAACATCGGTGGCGAAGSGCTCTACTG  
GACTGAACCTGACCTGAGGACGAAAGCTAGGGGAGCGAATGGGATTAATACCCCAT  
AGTCTAGCTGTAAACGATGGAATACTAGTAGCTTGTCGACCAGCTGTGCCGAAGCCA  
CCGCGTTAAGTTCCGCCTGGGGGAGTACGCCCCAGGTTGAAACTCAAGATTGACG  
GGGGCCGCCAAGCGGGGAAGGGTATCATGACCGAAAACCTACAGGCTGACATGTCCG  
ATCCTGTGAGTGGAAGCCTCGGGAGCGCGCAACAGGGTCATGCTGCTCACCTTGTCT  
GAAGTTGTAAATACCCAG

>*Moorea bouillonii* M1701 16S ribosomal RNA gene, partial sequence

GCCCGTTTCTTGGGGCACACCGGAACAGTGGCTATACCGGATGTGCCGAGAGGTGA  
AAGATTTATTGCATAAGAGGAGCTCGCGTCTGATTAGCTAGTTGGTAGGGTAAAGGC

CTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGACT  
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGC  
GAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTTTTGGGTTGTAAAACC  
TCTTTTATCAGGGAAGAAGAAAGTGAAAGAACCTGAGGAAAAAGCATCGGCTAACT  
CCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGG  
CGTAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTTAAAGACCAGGGCTTAACTC  
TGGGCAGGCAGTGGAAGCTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGAATTCC  
CGGTGTAGCGGTGAAATGCGTAGATTTTCGGGAAGAACATCGGTGGCGAAGGCGCTC  
TACTGGACTGAACCTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGA  
TACCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTAGCTTGTTTTTCGACCC  
AAGCTGTGCCGAAGCCAACGCGTTAGTATCCCGCCTGGGGAAGTACGCACGCATAG  
TGAACCTCAAAGGAATAGACSGGGGGCCCGCAATAGCAGTGGAGTTGTGGTTAACTT  
CGAACCCACCCGAAGAACCTTACCAGGCTTGGACTGGCCCGAATCCCCGGTGAAAG  
CTGGGAAGTGGCTTCCGGAACCGCCAACTCAGGTGGTGGCTGGCCTGCCCCCCCCCG  
GTCCTGAAATGTTGGTTAATTCCCGCACCATCGCCACCCCTGTTTTTTAGTTGGCCCC  
CCCTCAAGGTGGGGCCCTCTAAAGAGACCGGCGGTGCACAACAGGCTTTTGGGTTGT  
AAACCTCTTTTATCAGGGAAGAAGAAAGTGACGGTACCTGAGGAAAAAGCATCGGC  
TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTA  
TTGGGCGTAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTTAAAGACCAGGGCTT  
AACTCTGGGCAGGCAGTGGAAGCTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGA  
ATTCCCGGTGTAGCGGTGAACTGCTTATTTTCGGGAAGAACATCGGTGGCGAAGGCG  
CTCTACTGGACTGAACCTGACACCTGAAGTACGAAAGGGTAATTAGTTGTAAACCTC  
TTTTATCAGGGAAGAAGAAAGTGACGGTACCTGAGGAAAAAGCATCGGCTAACTCC  
GTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGGCG  
TAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTTAAAGACCAGGGCTTAACTCTG  
GGCAGGCAGTGGAAGCTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGAATTCCTCG  
GTGTAGCGGTGAAATGCGTAGATTTTCGGGAAGAACATCGGTGGCGAAGGCGCTCTA  
CTGGACTGAACCTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATA  
CCCCAGTAGTCCTAGCTGTAAACGATG

>*Moorea bouillonii* M1704 16S ribosomal RNA gene, partial sequence

GATGCCCCAACGCCGTCTGCCACGGACAGTGGCTATACCGGATGTGCCGAGAGGTGA  
AAGATTTATTGCCATAAGAGGAGCTCGCGTCTGATTAGCTAGTTGGTAGGGTAAAAG  
CCTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGGGAC  
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGG  
CGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTTTTGGGTTGTAAACC  
TCTTTTATCAGGGAAGAAGAAAGTGACGGTACCTGAGGAAAAAGCATCGGCTAACT  
CCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGG  
CGTAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTTAAAGACCAGGGCTTAACTCT

GGGCAGGCAGTGGAAGCTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGAATTCCC  
GGTGTAGCGGTGAAATGCGTAGATTTTCGGGAAGAACATCGGTGGCGAAGGCGCTCT  
ACTGGACTGAACCTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGAT  
ACCCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTAGCTTGTATCGACCCAA  
GCTGTGCCGAAGCCACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGT  
GAAACCTCAAGGAATTTGACGGGGGGCCCGCCAAGCGGTGGAGTATGTGGTTAATTC  
GATGCACCCGAAAAAACCTACCAGGGGCTTGACATGTCCCGAATCCCGGTGAAGCTG  
GGAATGCCTTTGGAAGCGGAAACACAGGTGGTGCAAGGTTTTAGTCAGCTCGGTTGT  
GAGATGTTGGGTTGAGTCCCGGAAGGGGGCAACGTTGTTTTTATTGCCAGCACGTAA  
TGGTGGGCACTCTAGAGAGACTCCGGTGCAAACGGGAGGAAGGTGGGGATGACGTC  
AAGTCAGCATGGCCTTACGCCCTGGGCTACACACGGACTACAATGGTCAGGCAATG  
GCAGCCAACTCGCAAGGGGGAGCTAATCTCATCAAACCTGGCCTCAGTTCAGTTGCC  
GGCTGCAACTCGCCGGCATGAAGGAGGAATCGCTAGTAATCGCCGGTCAGAATACG  
GCGGTGAATCCGTTCCCGGGCCTTGTACACAGCGCCTCGATCGGCAAACGCCAAAT  
GAGAACACAGGTGGGGGATGGCTGTCTCCTCCCGGGGCGGAAATGGTTGGGTTAA  
TTCCCGAACGAGCGAAACCCCTTGTTTTTAGTTGCCACCCCTGTAGGGGGGCCTCTC  
TTATAGAGAATTGCCGTGTACAAAGTCCGTAGGTGGTACCTCAGTCGGCTGTAAAGA  
ACAGGCTTAACTCTTGGGCAGGCAGTGGAAGCTGAGAACTAGAGGGCAGTAGGGTA  
GAGGGAATTCCCGTGTAGCGGTGAATGCGAGATTTCCGGAAGAACATCGTGGCGAA  
GGGCTCTACTGGACTGAACTGATCCTGAGGGACAAAGCTAAGGGAAGCGATGGGAT  
TAAAAACCCAGTGTCTACTGTAAACCATGGATAACAAGGGTAGCTGGATCCACC  
AGCTGTGCCAAAACAACGTTAGTTCCGCCTGGGAGTCCCCCAAGGGGAAATAAAAA  
AATATAGGGGCCCGCAACCGTGGAATTTTGTTTTATTTCCTTTGACGCGAAAACCTCC  
GCGTGGAATGTCGTATTCGGGTAACCGGGAGGCCTTAGGACGCGACCACTGTGGAC  
AAGTGTTTC

>*Moorea bouillonii* M1705 16S ribosomal RNA gene, partial sequence

GCCGATTTGGTTCGGGAACCAACCGGAAACAGTGGCTAATACCGGATGTGCCGAGAG  
GTGAAAGATTTATTGCCATAAGAGGAGCTCGCGTCTGATTAGCTAGTTGGTAGGGTA  
AAAGCCTACCAAGGCAACGATCAGTAGCTGGTCTGAGAGGATGAGCAGCCACACTG  
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAA  
TGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTTTTGGGTTGTA  
AACCTCTTTTATCAGGGAAGAAGAAAGTGACGGTACCTGAGGAAAAAGCATCGGCT  
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTAT  
TGGGCGTAAAGCGTCCGTAGGTGGTACCTCAAGTCGGCTGTTAAAGACCAGGGCTT  
AACTCTGGGCAGGCAGTGGAAGCTGAGGAACTAGAGGGCAGTAGGGGTAGAGGGA  
ATTCCCGGTGTAGCGGTGAAATGCGTAGATTTTCGGGAAGAACATCGGTGGCGAAGG  
CGCTCTACTGGACTGAACCTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGA  
TTAGATACCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTAGCTTGTATCG  
ACCCAAGCTGTGCCGAAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGC

AAGTGTGAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGTATGTGGT  
TTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTTGACATGTGCGAATCCCGGT  
GAAAGCTGGGAGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAG  
CTCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGT  
TGCCAGCACGTAATGGTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAA  
GGTGGGGATGACGTCAAGTCAGCATGCCCCCTTACGCCCTGGGCGACACACGTACTA  
CAATGGTCAGGACAATGGGCAGCCAACCTCGCAAGGGGGAGCTAATCTCATCAAACC  
TGGCCTCAGTTCAGATTGCCGGCTGCAACTCGCCGGCATGAAGGAGGAATCGCTAGT  
AATCGCCGGTCAGAATACGGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCG  
TCTCTCCATGGAAGCTGGCCACGCCC GAAGTCGTTACCCTAACCCAATTGGGAGGGG  
GATGCCTACGAAAGGAGAGC