



# HOKKAIDO UNIVERSITY

Title	Studies on Natural Products from Untapped Bacterial Resources [an abstract of entire text]
Author(s)	Haedar, Jabal Rahmat
Description	この博士論文全文の閲覧方法については、以下のサイトをご参照ください。【担当：薬学部図書室】 <a href="https://www.lib.hokudai.ac.jp/dissertations/copy-guides/">https://www.lib.hokudai.ac.jp/dissertations/copy-guides/</a>
Degree Grantor	北海道大学
Degree Name	博士(薬科学)
Dissertation Number	甲第14839号
Issue Date	2022-03-24
Doc URL	<a href="https://hdl.handle.net/2115/85734">https://hdl.handle.net/2115/85734</a>
Type	doctoral thesis
File Information	Jabal_Rahmat_Haedar_summary.pdf



## Summary of Doctoral Dissertation

Degree requested Doctor of Pharmaceutical Science Applicant's name Jabal Rahmat Haedar

Title of Doctoral Dissertation

Studies on Natural Products from Untapped Bacterial Resources  
(未開拓細菌類を起源とする天然物に関する研究)

### Introduction

Natural product (NP) and its derivatives have made a major contribution to drug discovery and development. Thousands of NPs have been clinically approved to treat various human diseases. However, new therapeutic agents are still required to treat cancer and infectious diseases. In addition, recent exploration of novel NPs is challenged by high-rate rediscovery of previously reported molecules. On the other hand, metagenomic analysis of many marine invertebrates and soil ecosystems revealed enormous of biosynthetic gene clusters (BGCs) encoded for new bioactive small molecules. Furthermore, these potential BGCs are associated with diverse and rare bacteria that have never been investigated before. Thus, targeting these untapped microbial strains from marine and soil ecosystems would lead to discovery of novel natural products. This study covered explorations of novel NPs from untapped and rare bacterial strains living in marine invertebrates and soil ecosystem.

### Natural Product from Marine Sponge *Theonella swinhoei*

Marine sponges *Theonella swinhoei* have been well-known as prolific sources of many biologically active compounds. Lately, it has been revealed that the true producers of these *Theonella*-derived potent compounds are as-yet uncultured bacterial symbiont *Candidatus Entotheonella* sp. However, up to date, the cultivation of these promising symbionts remains elusive. Therefore, to access its secondary metabolite, we analyzed the chemical constituent of the marine sponge *Theonella swinhoei* and purified several new analogs of theonellapeptolide type compounds.

To purify these compounds, the crude extract of marine sponges was separated between water and ethyl acetate, followed by gel filtration chromatography and semipreparative HPLC to yield new theonellapeptolides namely IIa (**1**), IIb (**2**), and IIc (**3**) and known compounds II d (**4**), II e (**5**), and II d (**6**) (Figure 1). The planar structures of these molecules have been elucidated by means of NMR and tandem MS analysis. In addition, their absolute configurations have been determined by employing Marfey's method to the hydrolyzed compounds **1-6**. Furthermore, comparing Marfey's method data of some targeted fragments from each new compound allowed us to unambiguously determine the absolute configuration of repeated amino acid residues.

To examine anti-pancreatic cancer activity of theonellapeptolides, all isolated compounds were tested for their preferential cytotoxic activity against the human pancreatic cancer cell line MIA-PaCa-2 in standard nutrient-rich medium (Dulbecco's Modified Eagle Medium, DMEM) and nutrient-deprived medium using the antiausterity strategy. The data are presented as preferential cytotoxicity (PC<sub>50</sub>) values, representing the concentration that kills 50% of cancer cells in NDM without any toxicity in DMEM. Among the tested compounds (**1-6**), theonellapeptolides (**5**, **4**, **6**, and **3**) displayed the most potent preferential cytotoxicity, with PC<sub>50</sub> 3.5, 7.8, 8.2, and 10.0 μM, respectively and the moderate activity was observed for theonellapeptolides **1** and **2**. The structure-activity relationship of the tested compounds revealed that the combination of methyl β-alanine and methyl isoleucine at the positions 4 and 6 of **5** increased the activity (PC<sub>50</sub> 3.5 μM). In contrast,

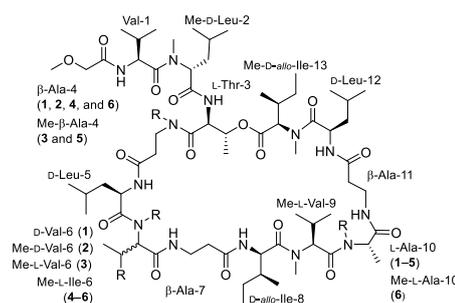


Figure 1. Isolated compounds from marine sponge *T. swinhoei*

the absence of both residues, as in **1** and **2**, resulted in moderate activity (PC<sub>50</sub> 33.4 and 42.8 μM) in MIA-PaCa-2 cells. Furthermore, in the presence of one of them, the activity of **3**, **4**, and **6** increased (PC<sub>50</sub> 10.0, 7.8 and 8.2 μM, respectively) but was lower than that of **5**. These data suggested that the presence of methyl β-alanine-4 and/or methyl isoleucine-6 is important for the activity of the theonella-peptolide type compounds as anti-cancer candidate under nutrient starvation conditions.

### Natural Product from Ichip-domesticated Bacteria

Many factors support the growth of bacteria in their natural habitats such as unique essential nutrients and environmental factors that are difficult to mimic under traditional isolation methods. Alternatively, the application of *in situ* cultivation using a diffusion chamber-like device called isolation chip (ichip) allows bacteria to acquire those critical factors from their natural habitat and eventually promote their growth, thereby increasing the diversity of cultivated strains. The ichip-based cultivation in principle involves (i) plating the environmental bacterial cells into multiple through-holes containing agar and (ii) subsequent covering it using a 0.03 μm membrane filter to prevent cells mobility but allow diffusion of surrounding organic matters. Upon incubation in their natural habitats, successfully domesticated bacterial strains were identified by the homologous search of 16S DNA amplicon sequences obtained from Illumina MiSeq® sequencing against NCBI non-redundant database.

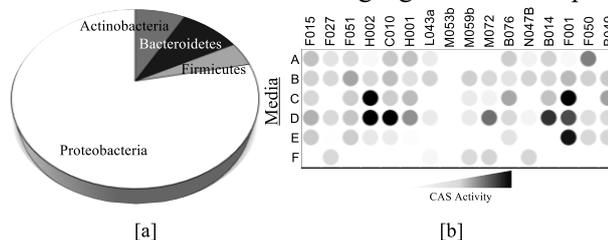


Figure 2. [a] Pie chart showed phyla distributions of ichip domesticated strains, [b] CAS activity assay-based screening.

Employing *in situ* cultivation approach using a small device called ichip enabled to domesticate 1046 bacterial strains. These successfully domesticated bacterial strains were screened based on their antibacterial activity after being cultivated under rich (LB) and define (M9) media. As the result, we selected strain *Variovorax* sp. H002 that showed antibacterial activity exclusively after cultivation under M9 minimal media. This media is widely used for productions of small molecules having metal chelator activity from diverse microorganisms. Therefore, we further screened all the *Variovorax* strains in our collection using chrome azurol S (CAS) assay for detection of metal chelator (particularly iron) producer. Accordingly, *Variovorax* sp. H002 and *Variovorax* sp. B014 were selected for investigation of novel NPs.

To isolate small bioactive molecules from *Variovorax* sp. H002, the crude extract obtained from the strain cultivated in an iron-deprived medium was subjected to gel filtration chromatography followed by purification using semi-preparative HPLC to yield three new analogs (**7-9**) and two known compounds (**10** and **11**). The planar structures of the isolated compounds were elucidated by NMR combined with tandem MS analyses. Based on 2D NMR correlations and tandem MS analysis, we deduced the structure of the major compound **10** as variochelin A previously reported by Kurth. Furthermore, based on HR-ESI-MS data combined with tandem MS and GC/MS analysis, we established that the other purified compounds were different from **10** in fatty acid moiety, which are identified as decanoic acid in **7**, 9-dodecenoic acid in **8**, 7-tetradodecenoic acid in **9**, and tetradecanoic acid in **11** (Figure 3).

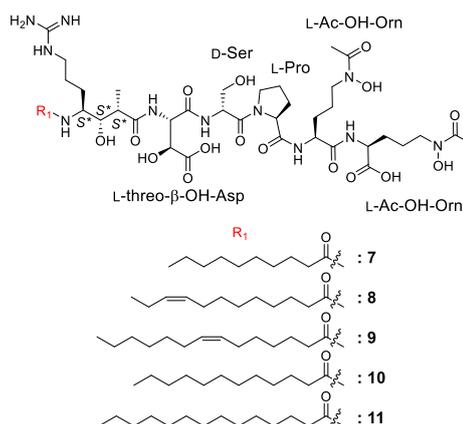


Figure 3. Isolated compounds from *Variovorax* sp. H002

Isolation of small molecules from *Variovorax* sp. B014 was initiated by its cultivation in modified M9 media. The cell-free supernatant of the culture was fractionated through an ODS column followed by purification with semipreparative HPLC to yield five new compounds **12-16**. The planar structures of the isolated compounds were elucidated by the combined NMR and tandem MS analyses. However due to broad NMR signals derived from intact compounds, we elucidated the planar structures of **13** in the form of complexes with metal gallium (III). Based on 2D NMR correlations, we observed seven spin systems that are typical to peptide compounds. Further comparison of the tandem MS analysis of intact compound **13** and its *seco*-methyl ester product (**13a**) allowed us to deduce the amino acid sequence of **13** (from N-C termini) as shown in (Figure 4). In addition, application of Marfey's method to the hydrolyzed **13** revealed its absolute configuration as L-serine, D-ornithine, L-threonine, and L-ornithine.

Compounds **7-16** shared common features with several group hydroxamates installed on each of their ornithine residues that might be responsible for iron coordination. Expectedly, the productions of **10** and **11** are significantly decreased after cultivation of *Variovorax* sp. H002 under M9 minimal medium fortified 0.5  $\mu$ M ferric ions and completely abolished in the presence of 5  $\mu$ M of ferric ions, suggesting that these compounds are siderophores, produced to acquire ferric ion from environment. Therefore, their ferric ion chelating properties were investigated by using CAS assay. As a result, isolated compounds from *Variovorax* sp. H002 exhibited chelating activity similar to EDTA as a positive control at 7.81  $\mu$ M. Additionally, isolated compounds from *Variovorax* sp. B014 showed chelating activity at minimal concentration of 15.6  $\mu$ M, a slightly lower compared to the EDTA. Moreover, antibacterial activity against gram negative *Escherichia coli* and *Burkholderia multivorans* were exhibited by compounds **7-16**. Compounds **12** and **13** showed antibacterial activity against *Burkholderia multivorans* while compound **14-16** were inactive, suggesting antibacterial property preferred for cyclic compounds.

## Conclusion

In this study, we have identified several new natural products from marine sponge *Theonella swinhoei* inhabited by promising uncultured symbiont bacteria *Ca. Entotheonella* spp. as well as from two different strains of ichip domesticated *Variovorax* sp. H002 and B014. The isolated theonellapeptolide type compounds **1-6** showed activity against human pancreatic MIA-PaCa-2 cancer cells in nutrient-deprived medium, where the most potent activity was observed in compound **5**. On the other hand, the purified siderophores from *Variovorax* sp. H002 and B014 showed strong ferric ion chelating activity. Additionally, antibacterial activities against tested gram-negative bacteria were observed from compounds **7-13**. Finally, this study suggests that untapped bacterial strains are potential sources of biologically active small molecules that could contribute to drug discovery and development.

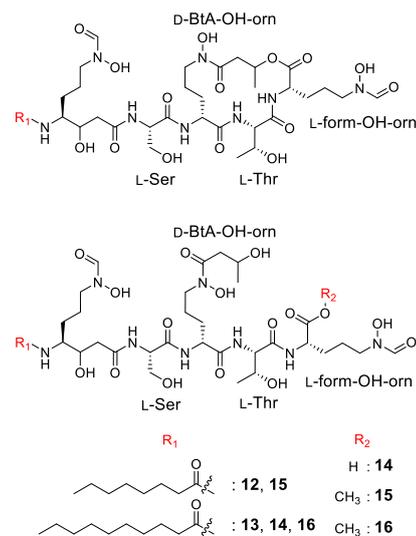


Figure 4. Isolated compounds from *Variovorax* sp. B014