Screening of New Bioactive Compounds from Marine Bacteria Associated with Stony Corals

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June 2020

Contents

List of Abbreviations

1-BuOH	1-butanol
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography-ultraviolet
HR-ESI-TOFMS	High resolution-electrospray ionization-time of flight- mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
MeCN	Acetonitrile
MeOH	Methanol
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance
ODS	Octadecyl-silica
TFA	Trifluoroacetic acid
XTT	Sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

CHAPTER 1

Introduction

Natural products are small organic molecules produced by living organisms. They are usually called secondary metabolites because these molecules are not essential for the survival of organisms. Secondary metabolites are produced from key intermediates of the primary metabolism and relatively limited in occurrence and are often unique to particular groups of organisms or even species [1]. Secondary metabolites normally represent a chemically extremely diverse group of organic molecules and include products as a consequence of nutrient limitation, compounds for defense, regulatory molecules, or signaling molecules [1]. Secondary metabolites can be classified on the basis of the primary metabolic pathway from which they are derived or in terms of their structural similarity [1]. In contrast to secondary metabolites, primary metabolites are essential for life, and include proteins, carbohydrates, lipids, nucleic acids, and their precursors and these molecules are produced by metabolic pathways common to most of organisms and include all of the modes of synthesis, interconversion, or degradation involved with the production of primary metabolites [1,2].

Historically, higher plants are considered as the most important source of drugs and medicinal plants are well documented throughout human history. In the history of natural product drug discovery, synthesis of acetylsalicylic acid (aspirin), an anti-inflammatory agent derived from the salicin, the natural product originally isolated from the bark of willow trees is one of the most noted examples [3] (Figure 1-1). Investigation of *Papver somniferous* (opium poppy) resulted in the isolation of morphine including several alkaloids, commercially available as analgesic agent, first reported in 1800s [4]. The bark of *Cinchona succirubra* had been used for the centuries for the treatment of fever and malaria [5], and the active component of bark of *Cinchona succirubra* was identified as quinine, and commonly used as anti-malaria drug and approved by the Food and Drug Administration (FDA) in 2004 [2]. Pilocarpine, L-histidine-derived alkaloid isolated from *Pilocarpus jaborandi*, which has been used as a drug for the treatment of glaucoma for over centuries and an oral formulation of pilocarpine was approved by the FDA in 1998 for the treatment of dry mouth (xerostomia) [2].

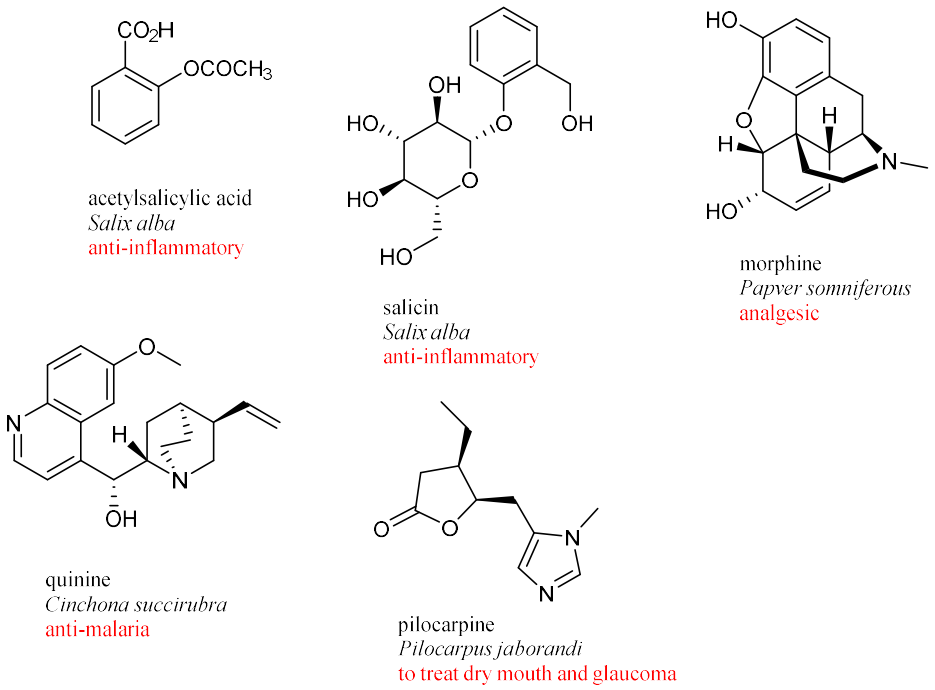


Figure 1-1. Examples of plant-derived drugs.

Traditionally, microbial natural product drug discovery program includes the isolation of targeted microorganisms, fermentation of individual isolated strains, and extraction of fermentation broth with desired organic solvent and if the extract showed any activity in bioassays, then each compound from extract was isolated and the structure was elucidated, and biological activity of pure compound was evaluated [6]. The modern era of antibiotic chemotherapy was started with introduction of penicillin in 1940s in clinical trials for the treatment of bacterial infections. Penicillin was first discovered on September 3, 1928 with the somewhat serendipitous event by Sir Alexander Fleming as a secondary metabolite derived from fungus *Penicillum notatum* [7]. After the first clinical use of penicillin in 1940s, there was a worldwide search for new antibiotics from microorganisms and bioactive natural products began [2]. Another important milestone in the history of antibiotics was the discovery of streptomycin from *Streptomyces griseus*, the first aminoglycoside antibiotic, in 1943 by Selman Waksman [8]. Chloramphenicol is a broad-spectrum antibiotic originally isolated from *Streptomyces venezuelae* in 1947. It was the first antibiotic synthesized by chemical means soon afterwards its structure was determined [9]. The first tetracycline antibiotic discovered, aureomycin, was isolated in 1948 from soil derived *Streptomyces aureofaciens* [10]. Erythromycin is the first macrolide antibiotic introduced into clinincal practice since 1952 was isolated from *Streptomyces erythraeus* [11]. Vancomycin is a glycopeptide antibiotic used for the treatment of methicillin-resistant *Staphylococcus aureus*. It was originally discovered from

Streptomyces orientalis isolated from soil sample in 1956 [12]. Doxorubicin is one of anthracycline family of antibiotic originally isolated from *Streptomyces peucetius* from soil of sourthern Italy in 1957, is a potent chemotherapeutic agent to treat various types of liquid and solid tumors [13,14]. Avermectins are series of antihelmentic drugs originally discovered from fermentation broth of *Streptomyces avermitilis* isolated from a soil sample in Japan [15]. Most of antibiotic classes in use today were identified in the 1940-1960s, a period referred to as the antibiotic golden age [16] (Figure 1-2).

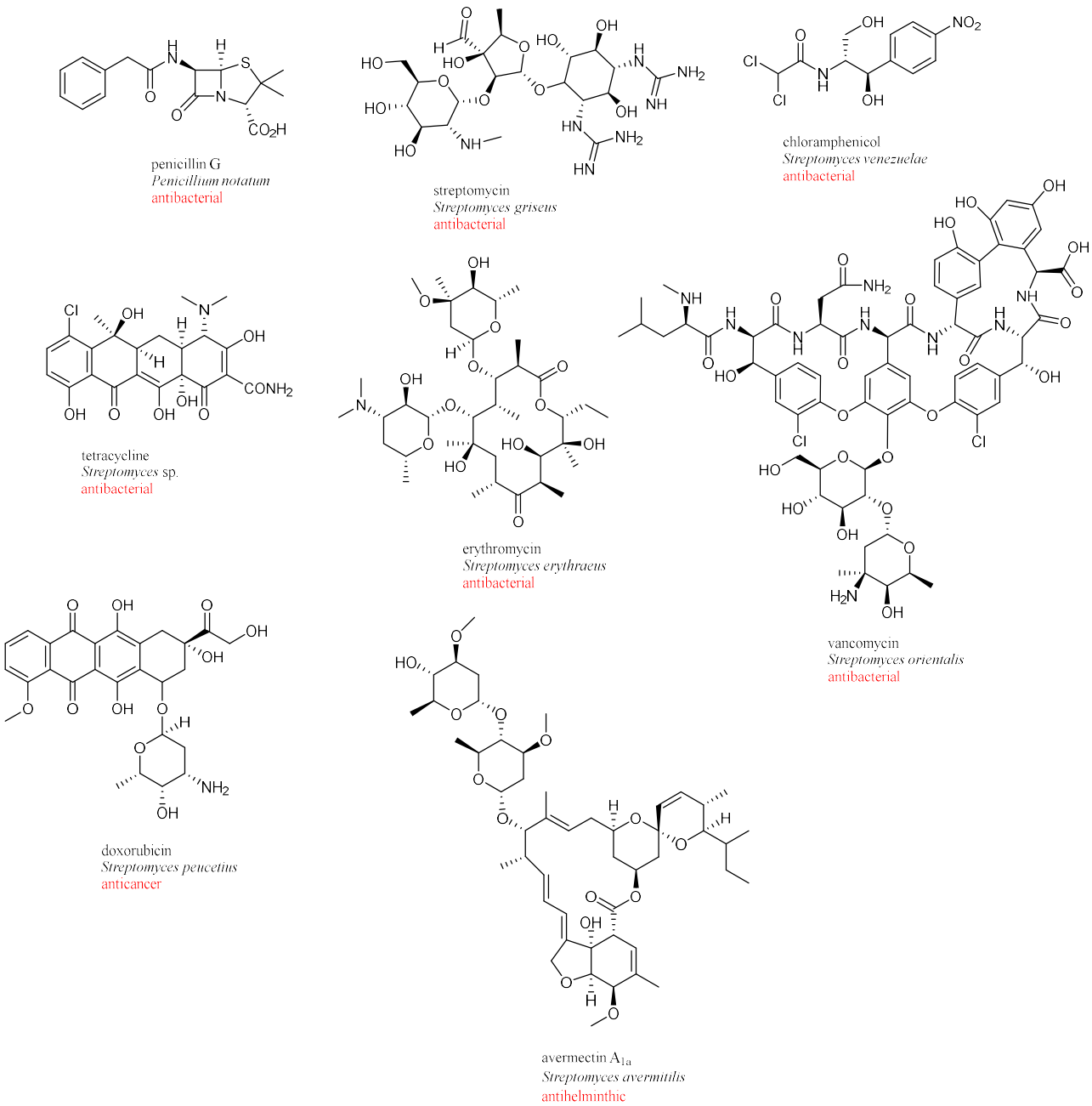


Figure 1-2. Examples of representative drugs discovered from microorganisms.

Recently, the rate of discovery of novel bioactive compounds from well-established traditional terrestrial sources have fallen dramatically [17], which in some extent preclude researchers to use common source for the quest of new bioactive compounds and lead researchers to explore new, unexplored, unusual and extreme habitat such as the marine environment, for potentially new biosynthetic diversity [18]. Principally, unexplored organisms represent the best way of discovery new metabolites because of divergence of biosynthetic pathways [19]. Two points should be considered when researchers seeking for new bioactive compounds such as (i) geographically untapped sources, and (ii) overlooked group of organisms [20].

1-1 Marine Natural Products as a Source of Pharmaceuticals

Natural products are organic molecules derived from living organisms such as plant, animals and microbes. They have been exploited by people for various purposes such as fragrances, food, medicine, pigments, and insecticides [21]. They often traditionally called secondary metabolites and it is restricted to small molecules less than 3,000 Daltons [22]. These molecules are not essential to the normal growth, development or reproduction of an organism [23], but they are important for survival, defending (e.g. against predators or prey organisms) or attracting (e.g. odorant fragrances of plants attracting insects attract animals for pollination and seed dispersal) other organisms [22]. These compounds are mostly genetically encoded and produced by secondary metabolic pathways [24,25]. About 50-70% of all therapeutical agents in clinical use today are natural products insipired by such small molecules [26].

Oceans cover more than 70% of the Earth's surface and host a vast species diversity, and there are still immense untapped resources available for the drug discovery [27]. Some marine ecosystems such as coral reefs are thought to surpass even tropical rain forest in terms of species diversity [28]. This species diversity provided by the marine environment can be anticipated to translate into structural diversity at the level of secondary metabolite production [29,30]. Marine organisms represnt the most recent source of novel bioactive natural products as compared terresterial plants and non-marine microorganisms [31]. Marine natural products show a great varitey of biological activities, which play a pivotal role in the discovery of drugs for the treatment of human diseases [32,33].

Extreme enviroments are unsual, yet promising sources of new bioactive molecules [34]. Organisms residing unexplored harsh environment such as marine enviroments featured by high salinity, low oxygen concentration, extreme temperature (low and high), high pressure

and limited light availabity favored the production of therapeutically invaluable molecules. To live under such harsh conditions, organisms must physioloigally and biologically adapted for survival. Such adaptations usually involve modification of metabolic pathways of organisms resulting in production of a great variety of molecules bearing unique structures in terms of structural diversity and functional features [31].

Marine invertebrates, in particular, have proven to be major sources of marine natural products (MNPs) in clinical trials. Sponges, corals, tunicates, and molluscs are the major source of marine natural produts retrieved from invertebrates. It was not evident that the organism itself produced those natural products or symbiotic or assoictead microbes did. About 28,500 MNPs had been identified by the end of 2016 with cytotoxic and anticancer properties [31]. The great majority of antibiotics currently used have been obtained from terrestrial sources, accounting for more than 75% of all currently used antibiotics, however antibiotics from marine sources have not yet been developed into any clinical trial phases. So most of research is devoted to the devolpment in anti-cancer drugs.

When we trace the history of marine natural products with a clear impact on the development of drugs for human use, we should not forget two arabino-nucleosides spongothymidine and spongouridine. At first they were isolated in the early 1950s from the extract of sponge *Tethya crypta* (currently *Tectitethya crypta*) [35] (Figure 1-3). Two nucleosides, the anticancer cytarabine (ara-C) and antiviral vidarabine (ara-A), are derivatives of sponge dervived arabino-nucleosides were the first FDA approved marine drugs in 1969 and 1976, respectively [31]. Untill now, nine marine-derived compounds are the FDA pharmaceutical drugs, and six compounds are undergoing phase III clinical trials. Fourteen other compounds are in phase II clinical trials, and 10 compounds are in phase I and several drugs in preclinical trails. Targets of these drugs include a wide range of diseases such as cancer, viral, Alzheimer's, chronic pain, and hypertriglyceridemia [36] (Table 1-1).

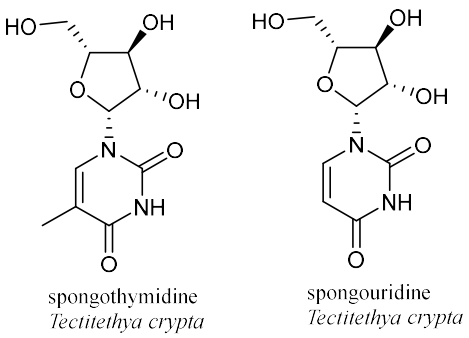


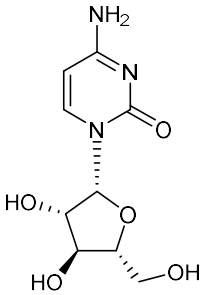
Figure 1-3. Structures of arabino-nucleosides.

Marizomib (NPI-0052; salinosporamide A), a proteasome inhibitor discovered from a marine bacteria *Salinospora tropica* and *Salinispora arenicola*. It is undergoing phase I clinical trail for the treatment of solid tumors and refractory lymphoma [37]. Bryostatin, macrocyclic polyketide, was first isolated from the bryozoan *Bugula neritina* in 1968 and characterized chemically in 1982 and the main pharmacological mechanism of action is modulation of protein kinase C (PKC) activity. Bryostatin is currently in phase I clinical trial for the Alzheimer's disease [38]. Plinabulin is a synthetic analogue of halimide, originally isolated from *Aspergillus* sp. Plinabulin is now undergoing phase III clinical trail against solid tumors and lymphomas [39]. Plocabulin, new polyketide isolated from the Madagascan sponge *Lithoplocamia lithistoides* [40]. Plocabulin currently in phase II trial in patients with advanced malignancies. Omega-3 acids are essential, because human do not synthesize them and three main types of omega-3 fatty acids are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic (DHA). EPA and DHA are found in all fish and are effective strategy for reducing hypertriglyceride levels in patients with hypertriglyceridemia [41]. Monomethyl auristatin E (MMAE) is a synthetic analog of dolastatin 10, a cytotoxic compound originally isolated from the the sea hare *Dolabella auricularia*. MMAE, as a highly potent microtubule inhibitor, and demonstrated wide spectrum of anticancer activity against a multitude of lymphomas, leukemia, and solid tumors [42] (Figure 1-4).

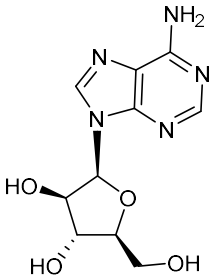
Table 1-1. Pipeline of marine pharmaceuticals until 2019 (according to https://www.midwestern.edu/departments/marinepharmacology/clinical-pipeline.xml, accessed on 27 October 2019) [36].

Compounds	Chemical class	Source organism	Therapeutical use	Status in 2019
Cytarabine	Nucleoside	Sponge	Cancer	FDA-approved
Vidarabine	Nucleoside	Sponge	Antiviral	FDA-approved
Ziconotide	Peptide	Cone snail	Chronic pain	FDA-approved
Omega-3-acid ethyl esters	Omega-3-fatty acid	Fish	Hypertriglyceridemia	FDA-approved
Eribulin mesylate	Macrolide	Sponge	Cancer	FDA-approved
Brentuximab vedotin	ADC (MMAE)*	Mollusc	Cancer	FDA-approved
Trabectedin	Alkaloid	Tunicate	Cancer	FDA-approved
Plitidepsin	Depsipetide	Tunicate	Cancer	FDA-approved
Polatuzuman ventodin	ADC (MMAE)	Mollusc	Cancer	FDA-approved
Plinabulin	Diketopiperazine	Fungus	Cancer	Phase III
Tetrodotoxin	Guanidinium alkaloid	Puffer fish	Chronic pain	Phase III
Lurbinectedin	Alkaloide	Tunicate	Cancer	Phase III
Marizomib	Beta-lactone-γ-lactam	Bacterium	Cancer	Phase III
Enfortumab	ADC (MMAE)	Mollusc	Cancer	Phase III
Plocabulin	Polyketide	Sponge	Cancer	Phase II
Tisotumab Vedotin	ADC (MMAE)	Mollusc	Cancer	Phase II
Bryostatin	Macrolide lactone	Bryozoan	Alzheimer's	Phase I

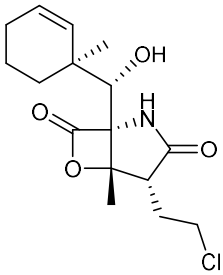
*Antibody Drug Conjugate (Monomethylauristatin E)



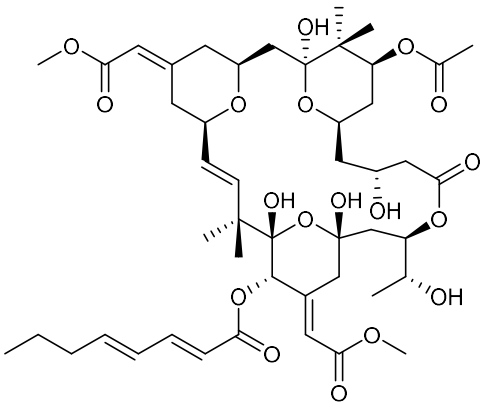
cytarabine
Tectitethya crypta (sponge)
anticancer



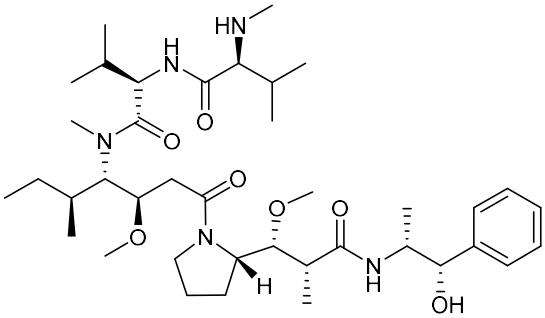
vidarabine
Tectitethya crypta (sponge)
antiviral



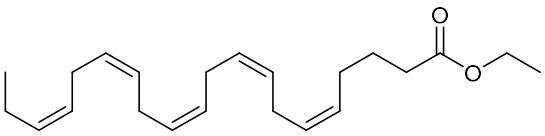
marizomib
Salinospora sp.
anticancer



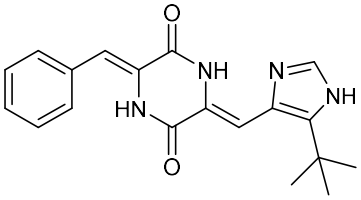
bryostatin
Bugula neritina (Bryozoan)
anti-Alzheimer's



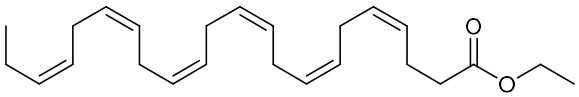
monomethyl auristatin E
moullsc
anticancer



EPA ethyl ester

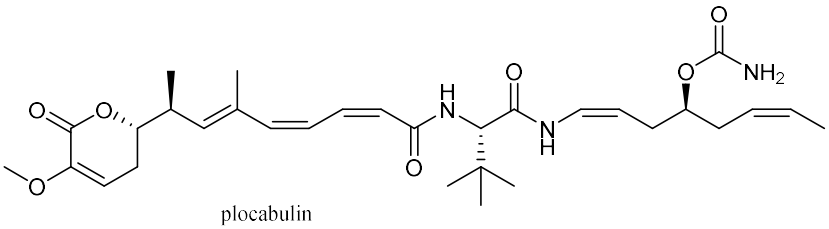


plinabulin
Aspergillus sp.
anticancer



DHA ethyl ester

omega-3-acid ethyl esters
fish
decrease triglyceride



plocabulin
Lithoplocamia lithistoides (sponge)
anticancer

Figure 1-4. Examples of marine natural products in clinical trials.

Chemoinformatics insight into the difference between MNPs and terrestrial natural products (TNPs) revealed that MNPs have lower solubility and are often larger than TNPs. MNPs possess longer chains and larger rings, usually the 8- to 10- membered rings containing ester bonds, which facilitate marine organisms to adapt to the water habitat, while the scaffolds of TNPs often have more stable ring systems and bond types. MNPs contain more halogens, because halogens are prevalent in seawater, and nitrogen and less oxygen atom compare to TNPs, which is indicative of MNPs may be synthesized by more diverse biosynthetic pathways than TNPs [43].

The main drawback of MNPs for drug discovery is the supply problem because active MNPs obtained from natural sources are in very minute amounts [31]. Total chemical synthesis or semi synthesis is the most useful technique to solve this problem [31]. Biotechnological techniques such as large scale fermentation of producing microorganisms, aquaculture of invertebrates, change in nutrient regimes of media, change in physical parameter of cultivation media, co-cultivation such as prokaryote-prokaryote co-cultivation, prokaryote-eukaryote co-cultivaton, addition of chemical elicitors, addition of adsorptive polymeric resin in fermentation media and high throughput methods to streamline cultivation could be helpful for mass production of natural products from marine organisms [31,44].

1-2 Natural Products from Corals

Corals are large, diverse group of sessile marine invertebrates belonging to the phylum Cnidaria include over 7,500 species [45]. Corals are the one of the most prolific sources of marine natural products ranking next to sponges in marine invertebrates [46]. Corals are basically classified into stony and soft corals. Stony corals are reef-building scleractinian corals and contain skeleton made of calcium carbonate and soft corals lacking calcium carbonate skeleton and includes a range of species, like gorgonians, sea fans, sea whips, sea pens, sea feathers, and blue corals. Soft corals are not responsible for reef building but may live in them [47-50]. To date, there have been more than 5,800 compounds reported from corals all over the world, attributing almost 20% of the total marine natural products. These natural products are mostly representative as terpenoids and steroids [45].

1-2-1 Natural Products from Soft Corals

Soft corals are promising potential source of marine natural products among marine organisms and received significant attention. Many bioactive compounds with diverse structures include diterpenes, sesquiterpenes, steroids and other chemical types have been

isolated from soft corals. The natural products retrieved from soft corals showed wealth of biological activities such as antimicrobial, antiviral, anticancer, antifouling and anti-inflammatory (Figure 1-5). Some of those compounds isolated from soft corals have provided a great opportunity for the development of new pharmaceuticals and antifoulants [51-59].

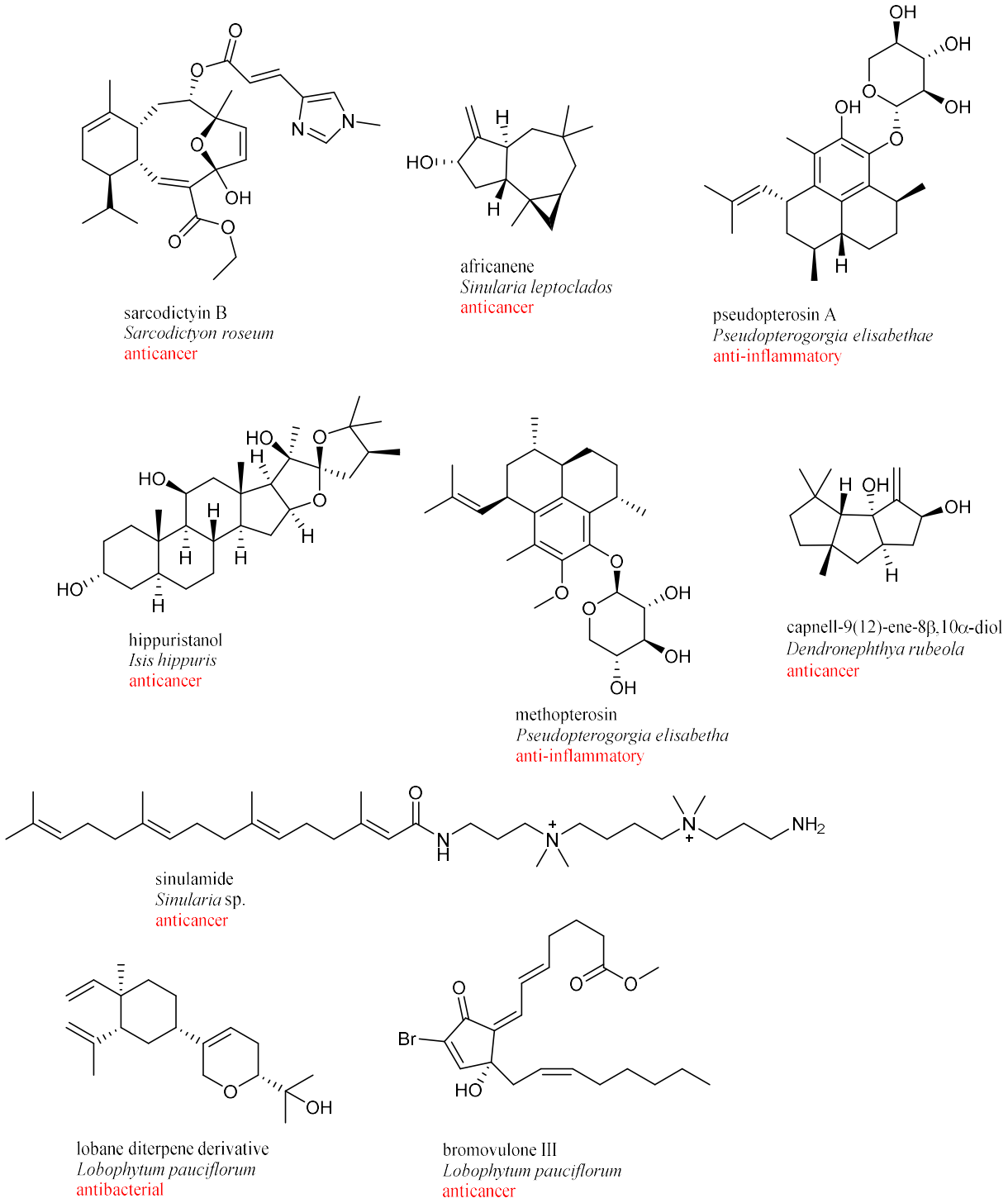


Figure 1-5. Chemical structures of new bioactive compounds from soft corals.

1-2-2 Natural Products from Stony Corals

Stony corals (or Scleractinian, hard coral, hexacoral, reef-building coral, hermatypic coral) has been rarely investigated for their ability to produce bioactive natural products, different from soft corals. The reason behind it is that researchers consider stony corals would hardly produce secondary metabolites as they consist mostly calcareous skeleton which is responsible for defensive role and thus the organic material is relatively few to the body weight. However, in soft corals secondary metabolites are produced to protect soft corals from predators because of lacking physical means of defense. New natural products obtained from stony corals usually belong to chemical classes such as alkaloids, sesterterpenes, anthraquinoids, phenols, macrolides, and acetylenic compounds. Many of them showed intriguing biological activities, such as antiviral, antibacterial, and cytotoxic activity (Figure 1-6).

Tubastrine, a new guanidinostyrene derivative was isolated from the acetone extract of stony cooral *Tubastrea aurea*. Tubastrine exhibited antiviral activity [60]. Brine shrimp lethality assay guided fractionation of stony coral *Montipora* sp. led to the discovery of *N*-alkyl pyridinium alkaloid, montipyridine [61]. This compound showed cytotoxic activity against panel of human cancer cell lines, A549 (human lung cancer), SK-OV-3 (human ovarian cancer), SK-MEL-2 (human skin cancer), XF498 (human CNS cancer), and HCT15 (human colon cancer) with ED_{50} values higer than 30 μg/mL. Aplysinopsin derivative was isolated from ethanol extract of stony coral *Tubastrea aurea* by solvent/solvent fractionation and silica gel column chromatography. This inhibits the first cleavage of fertilized sea urchin eggs at 2.5 μg/ml [62]. Ethanol extract of air dried sample of stony coral *Tubastraea micrantha* followed by defatting with hexane and extracted with $CHCl_3$ and LH-20 purification and finally purified by RP-8 column led to the discovery of alkaloid designated tubastraine [63]. Two new macrolide derivatives, mycalolides D and E, were isolated from organic extract of stony coral *Tubastraea faulkneri* [64]. Mycalodie D showed modest cytotoxicity against NCI-60 cancer cell line panel with LC_{50} value of 0.6 μM. Two new polyacetylene carboxylic acids, montiporic acids A and B, were isolated from the stony coral *Montipora digitate* [65]. Montiporic acid A showed antimicrobial activity against *Escherichia coli* but, also cytotoxicity against P-388 murine leukemia cells with IC_{50} value of 5 μg/mL. Six new acetylenic compounds including montiporyne A with cytotoxic activities against human solid tumor cell lines (SKOV-3, SK-MEL-2, XF498, and HCT15) were isolated from the stony coral *Montipora* sp [66].

Montiporyne A exhibited cytotoxicity against human cell lines SK-MEL-2 with ED_{50} value of 1.4 µg/mL.

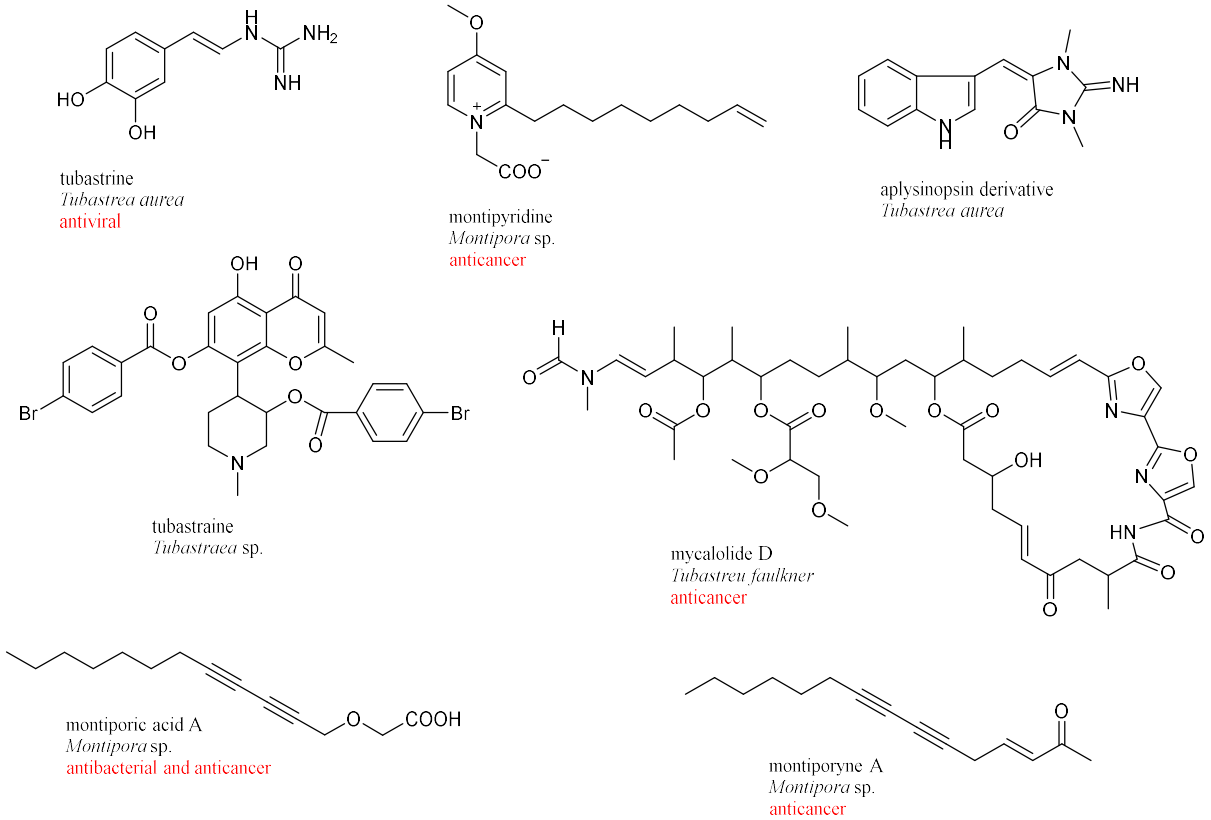


Figure 1-6. Chemical structures of natural products from stony corals.

Over the last decade, number of publications and number of new compounds from corals has been slightly decreased, whereas number of publications of new compounds from coral-associated microorganisms is slightly increasing [67]. Although corals and other marine invertebrates are rich source of bioactive natural products with a wide range of bioactivities, their pharmaceutical potential to drug discovery is often hindered by a supply problem. These marine invertebrates produce bioactive natural products in very minute amounts and it is need to collect a large amount of animal samples for extraction and ultimately drug discovery. Thus, the exploitation of bioactive natural products from corals for drug developments is still restricted by a supply problem and the need of sustainable conservation of coral reefs. So it is rational to use host-associated microorganisms for the bioactive natural products because associated microorganisms also produce a wide range of bioactive compounds.

1-3 Natural Products from Coral-Associated Microorganisms

1-3-1 Microbial Community in Corals

Corals are colonized by bacteria and other microorganisms, and the relation between corals and their colonized microbes vary from commensal to mutualistic or pathogenic or having no effect [68,69]. Corals harbor diverse and abundant microbial community, mainly consisting of eukaryota, bacteria, and archaea, as well as numerous viruses [70]. Corals live in symbiosis with unicellular dinoflagellate algae, commonly called zooxanthellae. In this association, dinoflagellate algae of genus *Symbiodinium* (zooxanthellae) lives within the gastrodermal cells of the coral host [70]. In this relationship, both corals and *Symbiodinium* are benefited. Corals provide protection of environment and compounds needed for the photosynthesis, whereas *Symbiodinium* furnish corals with glucose, glycerol and amino acids, which are products of photosynthesis [72]. *Symbiodinium* cells also provide pigmentation to corals [73] (Figure 1-7). Microorganisms are usually found in the coral surface mucus, coral tissue, and calcium carbonate skeleton and some particularly associated with *Symbiodinium* and each of which harbor distinct bacterial population [74]. It is assumed that coral-associated microorganisms are playing critical role in the chemical defense of their hosts by production of secondary metabolites [75]. In addition, microorganisms-associated with corals contribute to carbon cycling, sulfur cycling, nitrogen fixation, and phosphorous fixation [76]. However, some bacteria associated with corals also have negative effect on corals, including pathogenic bacteria such as *Vibrio* species, *Aspergillus sydowii*, *Serratia marcescens*, *Thalassomonas loyan* and *Aurantimonas coralicida*. These are the major group of bacteria responsible for the diseases of corals [77]. Study on bacteria associated with corals has been mainly focused on stony corals. Studies on the genetic diversity of bacteria associated with corals have disclosed extreme species richeness and abundance [78,79]. Diverse groups of bacteria are found from the different parts of corals [80,81]. Proteobacteria are the most abundant class of bacteria associated with corals [82].

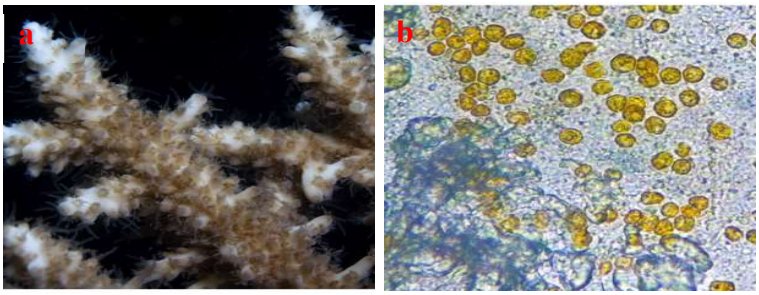


Figure 1-7. *Symbiodinium* cells in the host tissues [73]. a) Stony coral with brown coloration due to the presence of *Symbiodinium*, b) *Symbiodinium* cells in the gastrovascular cavity of larva (under a microscope) of the stony coral *Mussismilia hispida*.

Some metabolites isolated from invertebrate-associated microbes are closely identical to compounds isolated from their hosts and sometimes associated-microbes are considered as the true producers of some marine bioactive compounds. For a long time, natural products of microbes associated with corals are largely neglected. Recently, secondary metabolites of associated microbes are attracting attention of researchers, mainly focused on associated fungi.

Marine invertebrates (e.g., corals, sponges, tunicates) contain secondary metabolites in minute quantities, so it is inevitable to consume large amount of such organisms to get adequate amount of compounds for detail chemical and biological study and ultimately drug development. The concentrations of many highly active compounds in marine invertebrates are very low accounting for less than 10^{-6} % of their wet weight. To get approximately 1 g of ecteinascidin-743 (ET-743), an anticancer agent isolated from tunicate, about one metric ton of wet weight of tunicate *Ecteinascidia turbinate* must be used for extraction [83] (Figure 1-8). Therefore, exploration of new bioactive compounds for drug developments has been gradually shifted from hosts to their associated microbes. Here, bioactive new compounds from coral-associated microorganisms are categorized into two points.

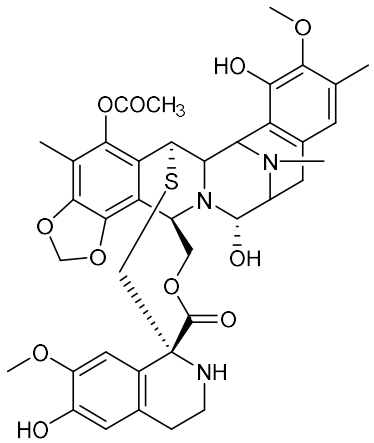
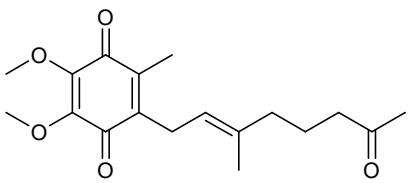


Figure 1-8. Chemical structure of ecteinascidin-743 (ET-743).

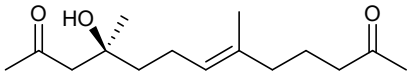
1-3-2 Bioactive Compounds from Soft Coral-Associated Microorganisms

A handful of bioactive compounds were discovered from soft coral-associated bacteria, actinomycetes and fungi with a wide range of bioactivity like antimicrobial, antifungal, anticancer, antibiofilm or anti-inflammatory activity. Comparing to bacteria and actinomycetes, substantial amount of new natural products were isolated from soft coral-associated fungi, mostly belonging to genus *Aspergillus* followed by *Penicillium*.

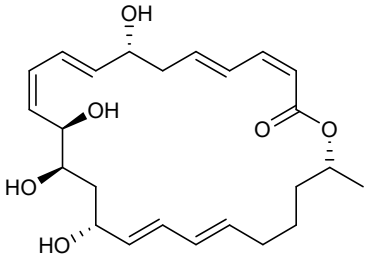
Pseudoalteromones A and B were isolated from *Pseudoalteromonas* collected from the cultured octocoral *Lobophytum crassum* [84,85]. Pseudoalteromone A, a novel ubiquinone derivative displayed cytotoxicity toward the human acute lymphoblastic leukemia cells (MOLT-4) with IC_{50} value of 3.7 μg/mL. Pseudoalteromone B showed moderate inhibitory effect on the release of elastase by human neutrophils. A new 24-membered ring lactone, macrolactin V, was isolated from culture broth of *Bacillus amyloliquefaciens* collected from the gorgonian coral *Junceella juncea* [86]. Macrolactin V exerted strong antibacterial activity against *E. coli*, *Bacillus subtilis*, and *S. aureus*, with MIC value of 0.1 μg/mL. Seven novel maleimide derivatives, aqabamycins A-G, were isolated from culture broth of *Vibrio* obtained from soft coral *Sinularia polydactyla* [87]. Aqabamycins A-G showed antimicrobial activity against *Micrococcus luteus*, *Bacillus subtilis*, *Proteus vulgaris*, and *Escherichia coli* and the fungus *Nematospora coryli* (Figure 1-9).



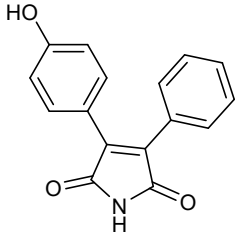
pseudoalteromone A
Pseudoalteromonas sp.
anticancer



pseudoalteromone B
Pseudoalteromonas sp.
anti-inflammatory



macrolactin V
Bacillus amyloliquefaciens
antibacterial



aquabamycin A
Vibrio sp.
antibacterial, antifungal, anticancer

Figure 1-9. Chemical structures of bioactive natural compounds from bacteria associated with soft corals.

Five new spirocyclic polyketides, pteridic acids C-G, were isolated from *Streptomyces* sp. collected from gorgonian coral *Melitodes squamata* [88]. Pteridic acids C, D and E showed weak antibacterial activity against *Bacillus subtilis* with inhibition zones of 8, 7 and 7 mm, respectively, at the equal concentration of 50 µg per disc. Chemical investigation of culture broth of *Streptomyces* sp. isolated from gorgonian coral *Melitodes squamata* led to the discovery of five novel polyhydroxy polyketides nahuoic acids B-E [89]. These compounds showed weak antibiofilm activity against *Shewanella onedensis* MR-1 biofilm. Unidentified soft coral-associated *Streptomyces* sp. produces two chlorinated polyketides, strepchloritides A and B that possess cytotoxicity against breast cancer (MCF-7) cells with IC_{50} values of 9.9 and 20.2 µM, respectively [90]. Chemical analysis of *Streptomyces* sp. collected from the surface of the gorgonian octocoral *Pacifigorgia* sp. led to the obtainment of two octatomic ring lactones, octalactins A and B [90]. Octalactin A exhibited strong cytotoxic activity toward human melanoma B-16-F10 and colon carcinoma HCT-116 cell lines with IC_{50} values of 7.2 x 10^{-3} and 0.5 µM, respectively (Figure 1-10).

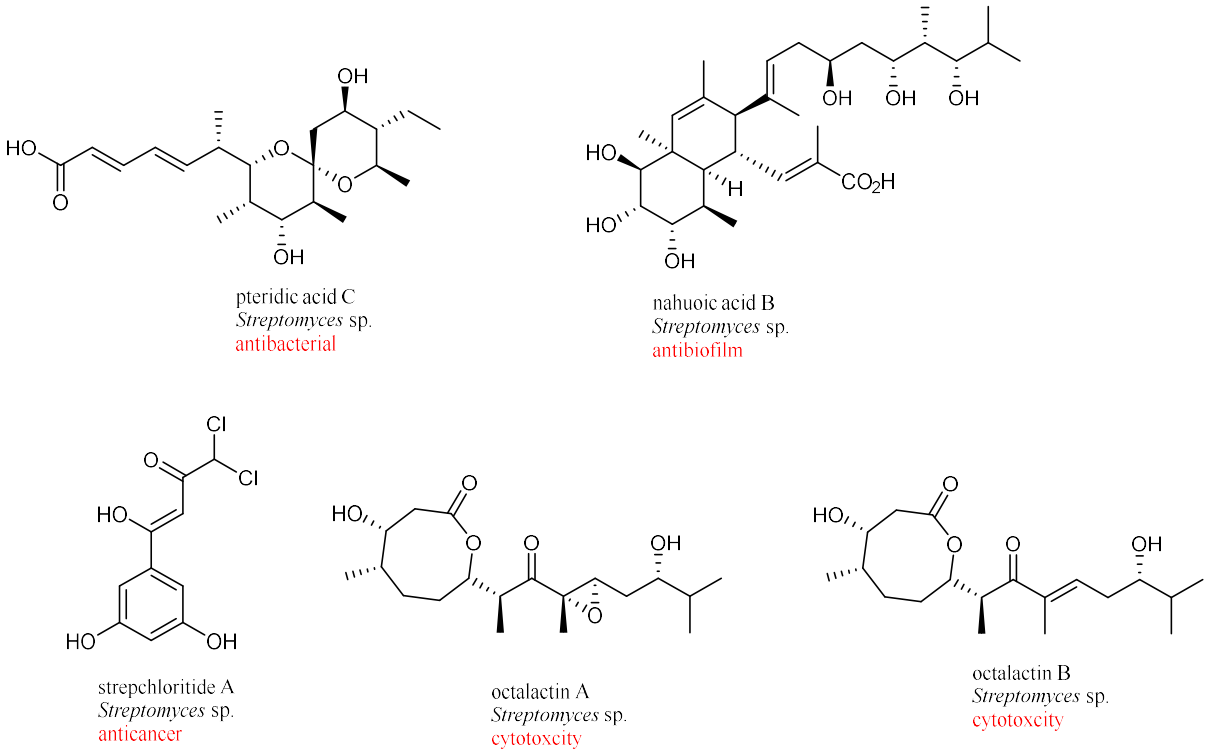


Figure 1-10. Chemical structures of bioactive compounds isolated from actinomycetes associated with soft corals.

Chemical investigation of culture broth of *Aspergillus* sp. obtained from a gorgonian coral *Dichotella gemmacea* led to the discovery of two new benzylazaphilone derivatives with an unprecedented carbon skeleton, aspergilone A and its symmetrical dimer with a unique methylene bridge, aspergilone B [92]. Aspergilone A displayed cytotoxicity toward HL-60 human promyelocytic leukemia, MCF-7 human breast adenocarcinoma and A-549 human lung carcinoma cell lines with IC_{50} values of 3.2, 25, and 37 µg/mL, respectively. Aspergilone A also showed potential antifouling activity against the larval settlement of barnacle *Balanus amphitrite* at nontoxic concentration with EC_{50} value of 7.68 µg/mL. Three phenolic bisabolane-type sesquiterpenoids (+)-methyl sydowate, 7-deoxy-7,14-didehydrosydonic acid, and 7-deoxy-7,8-didehydrosydonic acid, were obtained from the culture broth of *Aspergillus* sp. isolated from gorgonian coral *Dichotella gemmacea* [93]. (+)-Methyl sydowate showed weak antibacterial activity against *Staphylococcus aureus*. Investigation of *Aspergillus sydowii* associated with the sea fan (gorgonian) coral *Annella* sp. led to the isolation of 15 compounds, including three new sesquiterpenes, aspergillusenes A and B and (+)-(7*S*)-7-*O*-methylsydonic acid [94]. These compounds did not show antioxidant activity in antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay. Three cyclic tetrapeptides, aspergillipeptides A-C, and asteltoxin B were isolated from a fermentation broth of *Aspergillus*

sp. associated with gorgonian coral *Melitodes squamata* [95]. Aspergillipeptide C exhibited strong antifouling activity against *Bugula neritina* larvae settlement with EC_{50} value of 11 µg/mL. Four mycotoxins aluminiumneoaspergillin, zirconiumneoaspergillin, aspergilliamide, and aochratoxin A *n*-butyl ester were isolated from the culture broth of *Aspergillus* sp. associated with gorgonian coral *Melitodes squamata* [96]. All these compounds displayed strong or medium brine shrimp toxicity. Two prenylated indole alkaloids, 17-epinotoamides Q and M, and two new phenyl ether derivatives, cordyols D and E were obtained from static culture of *Aspergillus* sp. isolated from gorgonian coral *Dichotella gemmacea* [97]. Anthraquinone derivative, named 8-*O*-methylnidurufin was isolated from culture broth of *Aspergillus* sp. associated with gorgonian coral *Dichotella gemmacea* [98]. 8-*O*-Methylnidurufin exhibited strong antibacterial activity against *Micrococcus luteus* with the MIC value of 6.25 µM (Figure 1-11).

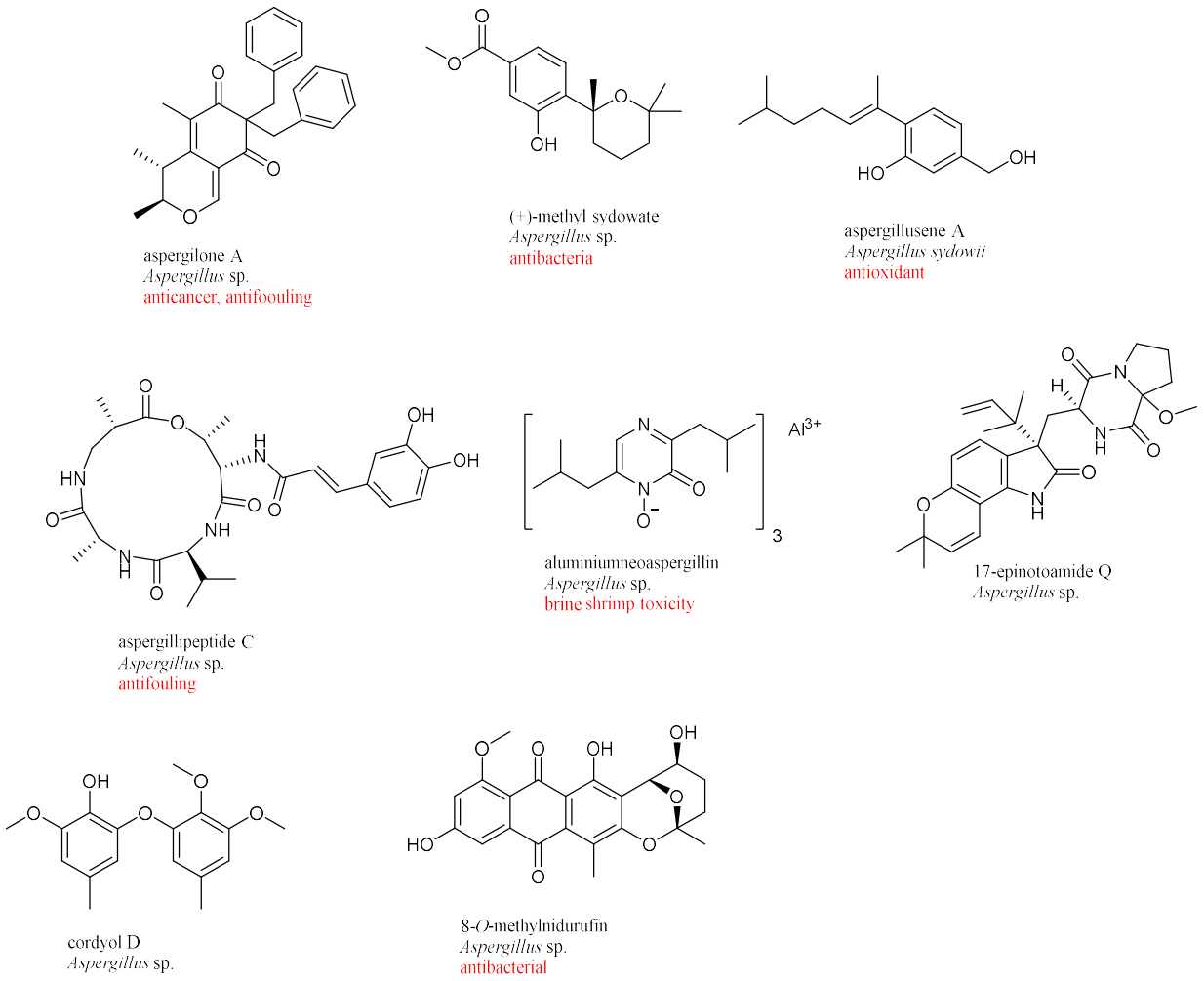


Figure 1-11. Chemical structures of new compounds isolated from *Aspergillus* sp. associated with soft corals.

Three new lumazine peptides, penilumamides B-D and cyclic pentapeptide, asperpeptide A were isolated from the gorgonian coral-associated *Aspergillus* sp. [99]. Steroid derivative, (22*E*)-ergosta-4,6,8(14),22,24(28)-pentaen-3-one was obtained from the culture broth of *Aspergillus* sp. isolated from a gorgonian coral *Muricella abnormalis* [100]. This compound inhibited the larval settlement of barnacle *Balanus amphitrite* with EC_{50} value of 18.40 µg/mL. Phenylalanine derivative, 4'-*O*-methyl-asperphenamate and two cytochalasins, aspochalasin A1 and cytochalasin Z24 were isolated from the culture broth of *Aspergillus elegans* obtained from a soft coral *Sarcophyton* sp. [101]. 4'-*O*-Methyl-asperphenamate showed selective antibacterial activity against *Staphylococcus epidermidis* with the MIC value of 10 µM. Nucleoside aroyl uridine derivative was isolated from fermentation broth of *Aspergillus versicolor* collected from the gorgonian coral *Dichotella gemmacea* [102]. This compound showed antibacterial activity against *Staphylococcus epidermidis* with MIC value of 12.5 µM. Spirocyclic diketopiperazine alkaloid, spirotryprostatin F was isolated from *Aspergillus fumigatus* collected from soft coral *Sinularia* sp. [103]. Spirotryprostatin F in low and ultralow doses (10^{-6}-10^{-17} M) exhibited stimulating action on the growth of sprout roots of soy (*Glycine max*) buckwheat (*Fagopyrum esculentum*), and corn (*Zea mays*). Three new cyclopentapeptides, versicoloritides A-C, a new orcinol tetramer, tetraorcinol A, and two new lactones, versicolactones A and B were isolated from the fermentation broth of *Aspergillus versicolor* associated with the coral *Cladiella* sp. [104]. Tetraorcinol A exhibited weak radical-scavenging activity against the DPPH radical with an IC_{50} value of 67 µM. Two aromatic butenolides, aspernolides A and B were isolated from the fermentation broth of *Aspergillus terreus* isolated from soft coral *Sinularia kavarattiensis*. Aspernolide A showed mild cytotoxicity against cancer cell lines [105]. 3-Methoxy-4-methyl-2,4-dien-pentanoic acid, was isolated from EtOAc extract of *Aspergillus* sp. collected from soft coral *Sarcophyton tortuosum* [106]. Chemical investigation on a coral-associated fungus *Aspergillus terreus*, led to the discovery three new compounds including a prenylated tryptophan derivative, luteoride E, a butenolide derivative, versicolactone G, and a linear aliphatic alcohol, (3*E*,7*E*)-4,8-dimethyl-undecane-3,7-diene-1,11-diol [107]. Versicolactone G showed potent α-glucosidase inhibitory activity (Figure 1-12).

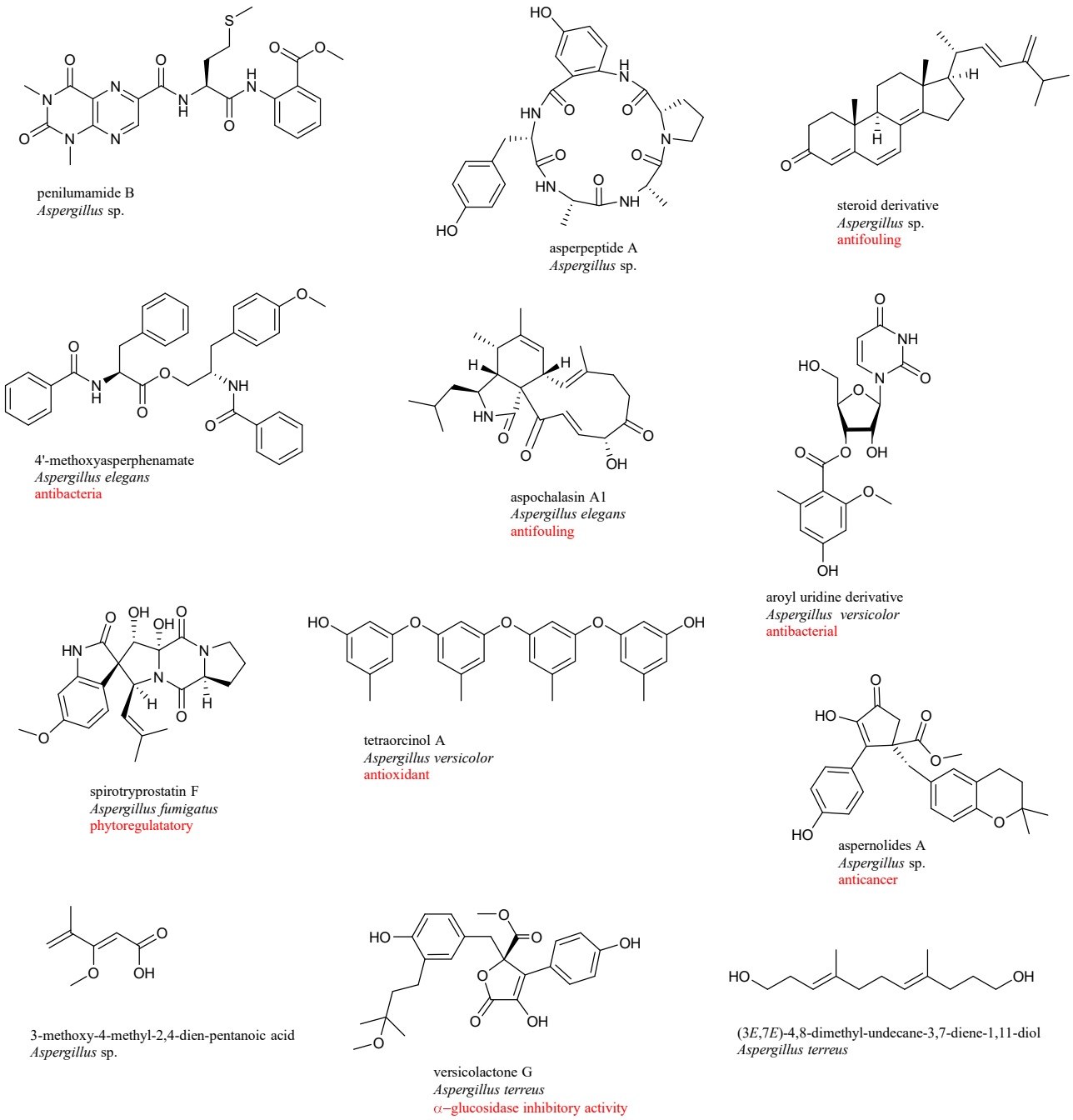


Figure 1-12. Chemical structures of new compounds isolated from *Aspergillus* sp. associated with soft corals.

Seven aromatic polyketides, communols A-G, were isolated from the fermentation broth of *Penicillium commune* associated with the gorgonian coral *Muricella abnormalis* [108]. Communol A showed weak antimicrobial activities against *Escherichia coli* and *Enterobacter aerogenes* with MIC values of 4.1 and 16.4 μM, respectively. Two polyketides, 6,8,5',6'-tetrahydroxy-3'-methylflavone and paecilin C were obtained from a fermentation broth of *Penicillium* sp. associated with gorgonian coral [109]. 6,8,5',6'-Tetrahydroxy-3'-

methylflavone showed significant antifouling activity against *Balanus amphitrite* larvae settlement with EC_{50} value of 6.7 µg/mL. Dihydrothiophene-condensed chromones, oxalicumones A and B and a new natural chromone, oxalicumone C were obtained from a fermentation broth of *Penicillium oxalicum* associated with gorgonian coral *Muricella flexuosa* [110]. Oxalicumones A and B showed moderate cytotoxicity against A375 and SW-620 cell lines. Three azaphilone derivatives, pinophilins D-F and one diphenyl ether derivative, hydroxypenicillide were isolated from *Penicillium pinophilum* associated with gorgonian coral [111]. Five polyketides including two benzopyranones, one isochroman and two anthraquinone-citrinin derivatives were isolated from the culture broth of *Penicillium citrinum* associated with gorgonian coral [112]. Penicillanthranin A showed displayed moderate antibacterial activity against both *Staphylococcus aureus* and methicillin-resistant *S. aureus* with equal MIC value of 16 µg/mL. Two 2,5-piperazinedione derivatives, janthinolides A and B were isolated from the fermentation broth of *Penicillium janthinellum* collected from soft coral *Dendronephthya* sp. [113] (Figure 1-13).

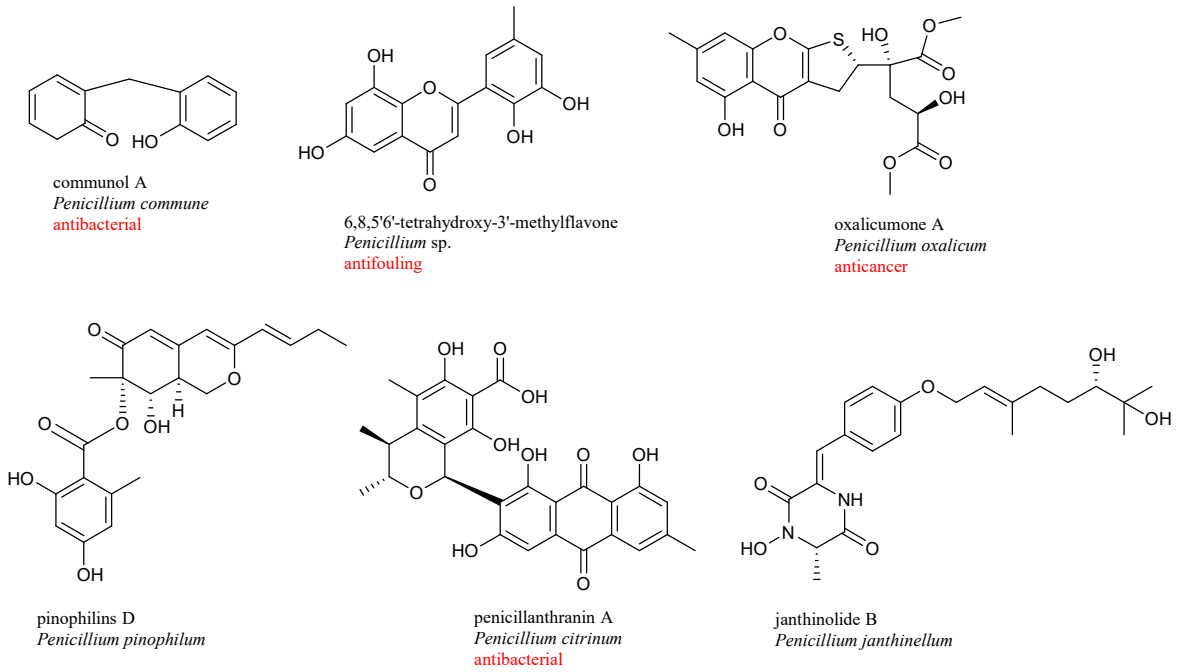


Figure 1-13. Chemical structures of new compounds isolated from *Penicillium* sp. associated with soft corals.

Chlorinated benzophenone derivative, (±)-pestalachloride D was isolated from *Pestalotiopsis* sp. isolated from a soft coral *Sarcophyton* sp. [114]. (±)-Pestalachloride D exhibited moderate antibacterial activity against *Escherichia coli*, *Vibrio anguillarum* and

Vibrio parahaemolyticus with MIC values of 5.0, 10.0 and 20.0 μM, respectively. Chemical investigation of culture broth of *Cladosproium* sp. collected from a gorgonian coral *Anthogorgia ochracea* led to the discovery of a bicyclic lactam, cladosporilactam A with promising cytotoxic activity against cervical cancer HeLa cell line with an IC_{50} value of 0.76 μM [115]. Five triquinane-type sesquiterpenoids, chondrosterins A-E and the known sesquiterpenoid hirsutanol C were isolated from fermentation broth of *Chondrostereum* sp. collected from soft coral *Sarcophyton tortuosum* [116]. Chondrosterin A displayed significant cytotoxic activities against cancer lines A549, CNE2, and LoVo with IC_{50} values of 2.45, 4.95, and 5.47 μM, respectively. Chemical investigation of *Eurotium rubrum* associated with soft coral *Sarcophyton* sp. led to the discovery of two new sulfur-containing benzofuran derivatives, eurothiocins A and B. Both compounds showed potent inhibitory effects against α-glucosidase activity [117]. Anthraquinone alterporriol Q and the hydroanthraquinone tetrahydroaltersolanol C were isolated from a static culture broth of *Alternaria* sp. retrieved from a soft coral *Sarcophyton* sp., showed antiviral activity against the porcine reproductive and respiratory syndrome virus (PRRSV) [118]. Two C12 polyketides, cladospolides E and F were isolated from *Cladosporium* sp. associated with a soft coral [119]. Cladospolide E showed potent lipid-lowering activity in HepG2 hepatocytes. Nigrospoxydons A-C and nigrosporapyrone, were obtained from *Nigrospora* sp. associated with a sea fan coral *Annella* sp [120]. Nigrospoxydon A displayed antibacterial activity against *S. aureus* and MRSA with MIC values of 64 and 128 μM, respectively. Four pyrone derivatives, nigrosporapyrones A-D, were isolated from the culture broth of *Nigrospora* sp. associated with sea fan coral *Annella* sp. [121]. Nigrosporapyrone A showed antibacterial activity against *Staphylococcus aureus* with MIC value of 128 μM. A cytochalasin derivative, xylarisin was isolated from *Xylaria* sp. associated with the gorgonian coral *Annella* sp. [122]. Xylarisin showed weak antibacterial activity. Hirsutane sesquiterpenoid, hirsutanol E was isolated from EtOAc extract of *Chondrostereum* sp. collected from soft coral *Sarcophyton tortuosum*. Hirsutanol E displayed potent cytotoxicity toward several cell lines [100] (Figure 1-14).

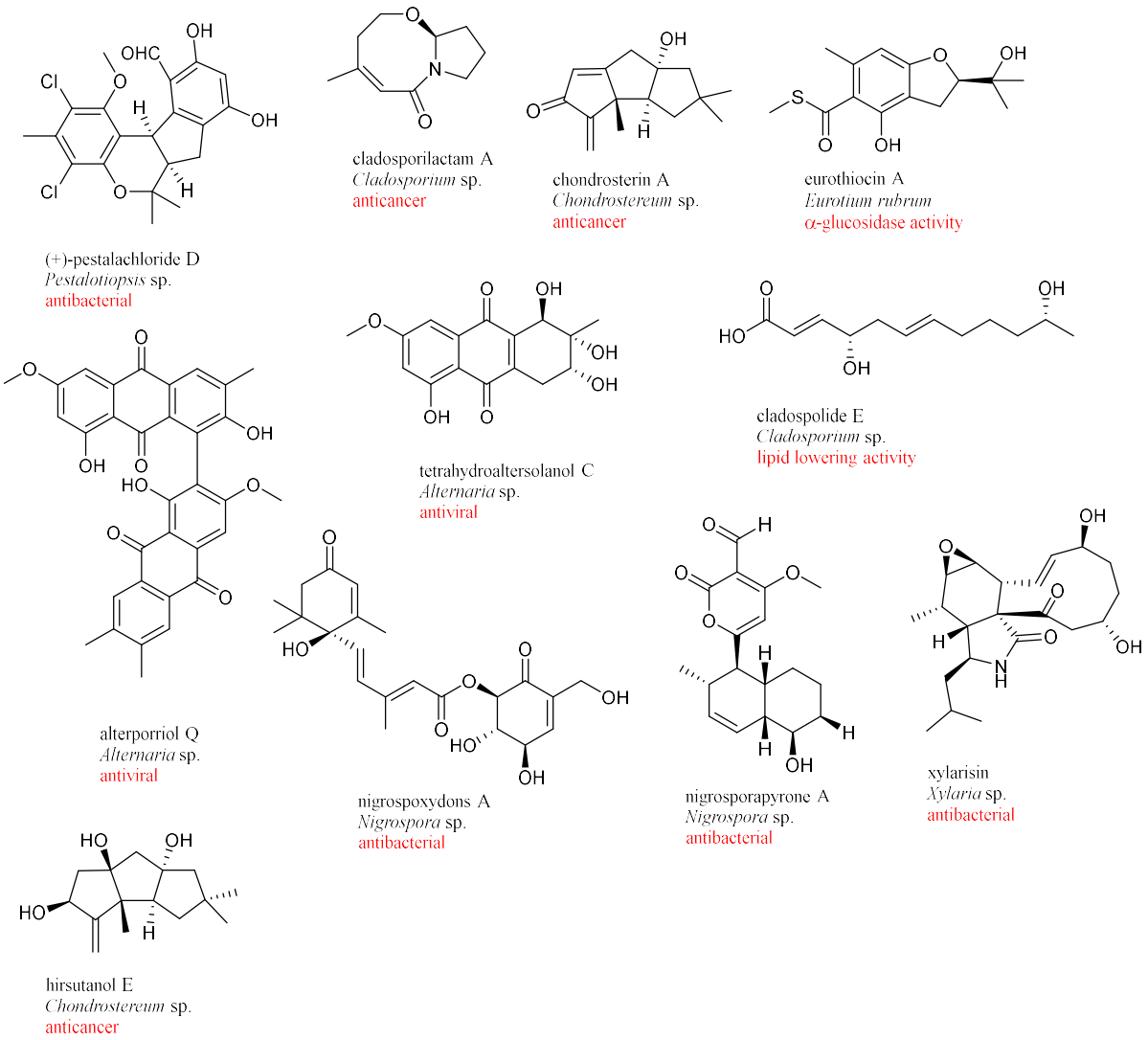


Figure 1-14. Chemical structures of new compounds isolated from other fungi associated with soft corals.

1-3-3 Bioactive Compounds from Stony Coral-Associated Microorganisms

Soft-coral associated microorganisms are attracting attention of researchers for discovering novel bioactive compounds. Meanwhile, stony coral-associated microorganisms are not exploited for screening of new bioactive compounds. Although stony coral-associated bacteria showed higher bioactivity than soft coral-associated bacteria, their secondary metabolites are not studied extensively [124]. This is maybe because only limited compounds were reported from stony corals and researchers speculate that the presence of calcium carbonate skeleton and very few amount of organic compounds hinders to get novel bioactive compounds from them. In addition, sustainable conservation of coral reefs, stony corals (reef

building corals) cannot be taken for the drug discovery. However, several studies revealed the presence of diverse and abundant microbial community in stony corals [125-130].

Stony coral-associated bacteria were selected for the screening of new bioactive compounds in this study because stony corals and its associated microorganisms are largely neglected for drug discovery and using new source, which has not been exploited for isolation of microorganism, ensure the isolation of new microbes with genome containing different biosynthetic pathway. To the best of our knowledge, no studies have been published with respect to new compounds from microorganisms residing stony corals before this work. So this study was conducted to estimate the ability of bacteria associated with stony corals to produce new bioactive compounds.

Literature reviews divulged that coral-associated microorganisms are the source of structurally diverse metabolites with various bioactivities. These compounds include polyketones, pyrones, cyclic peptides, lactones, alkaloids and so on, and show promising activities such as cytotoxicity, antibacterial, antifouling, phytoregulatory, and antiviral etc., which suggested that coral-associated microorganisms are promising source for finding new compounds with diverse activities. Most of the research focused on fungi associated with soft corals and, but only limited research has done with bacteria associated with soft corals. Many cultivable associated microorganisms have not been thoroughly exploited in terms of natural product biosynthesis abilities. Natural products derived from coral-associated microorganisms are still in their infancy.

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CHAPTER 2

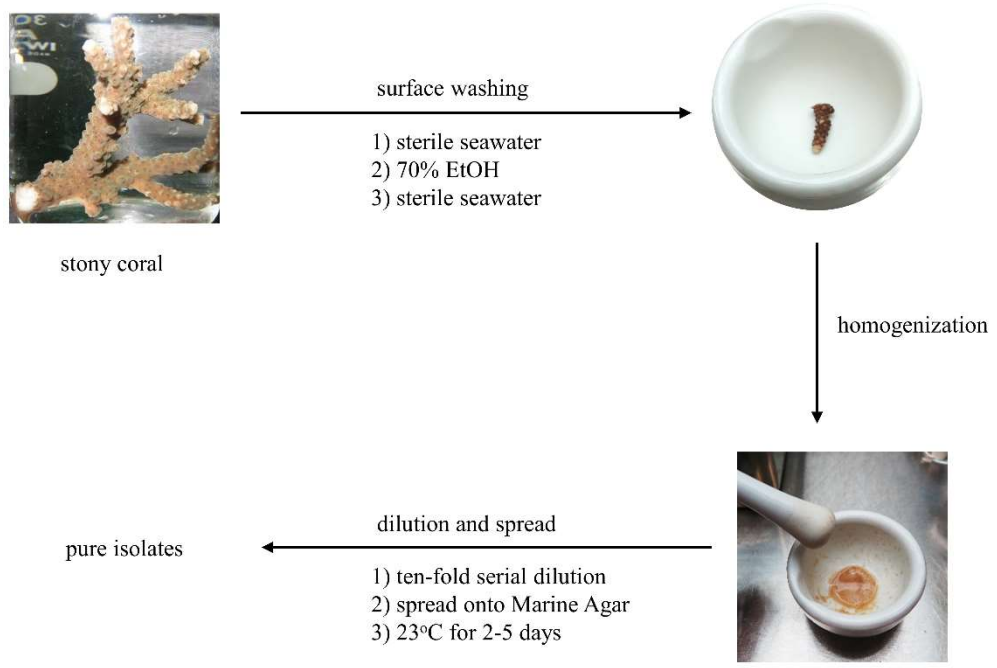
General Methodology

2-1 Collection of Stony Corals

Stony corals were obtained from aquarium vendors in Nagasaki and Osaka, Japan. Upon arrival of corals in our laboratory, they were kept alive in an aquarium tank until use for the isolation of bacteria.

2-2 Isolation and Identification of Coral-Associated Bacteria

In this study, aquacultured, healthy stony corals were used for isolation of bacteria. Bacteria were isolated by serial dilution and spread plate technique as follows: a small fragment of coral was taken and washed with sterile natural seawater to remove dirty particles and transiently attached microorganisms, then the surface was sterilized by using 70% ethanol to kill transiently associated microorganisms, and then washed with sterile natural seawater to remove the toxic effect of ethanol. A fragment of coral (*ca*. 1 g) was homogenized with mortar and pestle with an equal volume of natural seawater (1 mL), then 10-fold serial dilution was made up to 10^{-5}, and 0.1 ml of each dilution liquid was plated onto Marine Agar 2216 (Difco). The plates were incubated at room temperature for 2 to 5 days and after two days of incubation at ambient temperature, single colonies were transferred onto Marine Agar and cultures were further purified using the same agar until to get axenic cultures (Scheme 2-1). Pure bacterial cultures were preserved by cryopreservation at -85°C in 20% glycerol in Marine Broth.



Scheme 2-1. Isolation of bacteria from stony corals.

Bacterial strains were identified by 16S rRNA gene sequencing. The 16S rRNA gene of strains were amplified from a single colony by PCR with GoTaq Green Master Mix (Promega) with two universal primers (27F and 1492R). The PCR reaction was started with denaturation 98ºC for 2 min followed by total 40 cycles that consisted of denaturation at 98ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 68ºC for 10 sec. PCR products were assessed by 1% agarose gel electrophoresis (1X TAE, 1% agarose w/v). Purification of the PCR products were carried out according to the protocol of the Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co. Ltd.). The amplified 16S rRNA gene products were sequenced by Eurofins Genomics K. K., Tokyo, Japan.

2-3 Fermentation, Extraction, Isolation and Purification of Natural Products from Bacteria Associated with Stony Corals

Bacterial strains were cultured on Marine Agar was inoculated into 500 mL K-1 flask containing 100 mL of Marine Broth (Difco) or V-22 as seed medium. The flask was placed on a rotary shaker (200 rpm) at 30ºC for 2 days. The seed culture (3 mL) was transferred into 500 mL K-1 flasks each containing 100 mL of A3M, A11M and A16 production medium (Table 2-1). All fermentation media except Marine Broth were prepared in natural seawater collected in Toyama Bay, Toyama, Japan. In order to increase the productivity of bacterial strains, HP-20 was added to fermentation broth, which adsorbs metabolites and minimize the toxic effects of produced metabolites [1]. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 rpm) at 30ºC for 5 days. *n*-butanol was selected as a solvent for the extraction, because *n*-butanol has a wide solubility range from hydrophilic to hydrophobic compounds, but remove high molecular compounds such as polysaccharides and proteins in order to separate into organic and water layer [2]. After fermentation, 100 mL of 1-butanol was added to each flask, and the flasks were allowed to shake for 1 h. The mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer. Purification of crude extract was carried out by mainly three steps. Firstly, crude extract was purified by silica gel column chromatography or solvent/solvent extraction, followed by ODS column chromatography and final purification was achieved by semi-preparative HPLC.

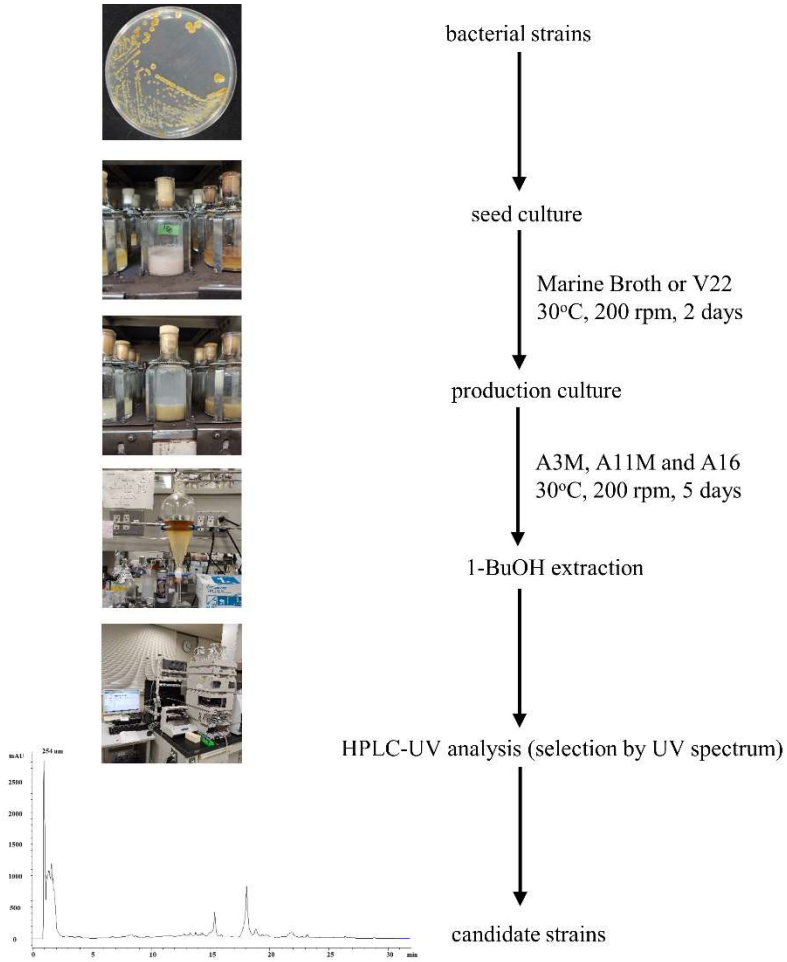
Table 2-1. Composition of seed culture and production culture media.

Media	Composition
Marine Broth	peptone 0.5%, yeast extract 0.1%, and major consitutents of inorganic salts present in seawater
V22	soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3%
A3M	glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical, Japan) 1%
A11M	glucose 0.2%, soluble starch 2.5%, yeast extract 0.5%, polypeptone (Wako Pure Chemical Industries, Ltd.) 0.5%, NZ-amine (Wako Pure Chemical Industries, Ltd.) 0.5%, $CaCO_3$ 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1%
A16	glucose 2%, Pharmamedia (Traders Protein) 1%, $CaCO_3$ 0.5%, and Diaion HP-20 (Mitsubishi Chemical, Japan) 1%

2-4 HPLC-UV Screening of Metabolites of Bacteria Associated with Stony Corals

Chemical screening was employed to discover new compounds from bacteria associated with stony corals. HPLC-UV was used for chemical screening of compounds. Two milliliter of 1-butanol extract was put into vial and dried by vacuum centrifugation. Dried extract was resupeneded in 200 µL DMSO and filtered using 0.45 µm membrane filter before HPLC-DAD analysis. The extract metabolites were separated using reversed-phase HPLC and photodiode array detector (200-600 nm) was used to record their characteristic UV spectra. The conditions for the HPLC analysis were as follows: Varian Microsorb C18 column (4.6 x 100 mm) on an Agilent HP1100 system with a binary pump equipped with DAD for 200 to 600 nm. The mobile phase used the following stepwise gradient of acetonitrile (MeCN) and 0.1% formic acid at pH 2.7: 15% MeCN for 0-3 min, 15-85% MeCN for 3-25 min, 85% MeCN for 25-29 min and 85-15% MeCN for 29-32 min, with a flow-rate of 1.2 mL/min (Scheme 2-2).

Normally peaks with similar UV absorbance with in house-UV database were not considered for further study. However, this was a preliminary study conducted to assess the biosynthetic ability of associated bacteria to produce new compounds and our in-house database mainly contain UV of actinomycetes and fungi and lacking UV data for bacteria. UV only represent the chromophore present in the molecules but not the complete structure of molecules. So I considered to work with UV have hit from in-house database. Peaks with selected UV absorbance were considered for further purification and structure determination.



Scheme 2-2. Screening of metabolites of bacteria isolated from stony corals.

2-5 Structure Determination

Structure of pure compounds were determined by high resolution-mass spectrometer (HR-MS) and one dimensional and two dimensional NMR techniques and corroborated with optical rotation, UV spectrometry and IR spectrometry. Bioassays for new compounds were performed.

2-6 Dereplication of Natural Products Using HPLC-UV

Screening of natural products for novel bioactive compounds becomes increasingly a challenge. The chance of finding new bioactive compounds have become more arduous due to astronomical number of known compounds already reported in literatures. A major problem of finding new bioactive compounds from natural products is reisolation of known compounds. Reisolation of known compounds wastes time, money and resources, and easily distracts natural product chemists for more promising leads for drug discovery, and to obviate such reisolation and recharacterization of known compounds, dereplication process should be

considered before isolation of target compounds. The rapid characterization of known compounds, a process known as dereplication, has become strategically important for natural product chemists involved in screening program [3]. Nowadays, several commercial databases have been developed that can assist the natural products chemists by avoiding unnecessary structure elucidation of known compounds. Several databases are available such as CAS Registry [4-7], CSLS [8], ChemSpider [9], PubChem [10], Dictionary of Natural Products (DNP) [11], Dictionary of Marine Natural Products (DMNP) [12], AntiBase [13], MarinLit [14], Reaxys [15], and NAPRALERT [16].

Several combinations of LC hyphenated techniques such as LC-UV, LC-MS, LC-MS/MS, LC-ELSD-MS, LC-UVSPE-NMR, LC-NMR, and LC-MS/MS-NMR have been developed over the last three decades to obtain structural information of natural products [17]. UV is the simplest and most widely used detector among HPLC. Most of natural products absorb UV light in the range of 200-600 nm, including all substances having one or more double bonds and all substances that have unshared electron. Thus, even compounds with weak chromophores, such as triterpene glycosides, can be efficiently detected by UV at short wavelengths (203 nm) [18]. The major drawback of this detector is that not all natural products possess UV chromophores. UV spectra are easily acquired using a HPLC-UV examination of a crude extract or fraction. The UV profile and the maxima (λ_{max}) of the natural products provide information on the chromophores and these distinctive spectral properties can be used for database search. For UV spectra data including, λ_{max} and ϵ, are available in DNP and DMNP. However, partial UV spectral data available database are NAPRALERT, AntiMarin and AntiBase [19]. The UV spectra profile, including the λ_{max} are suggestive of a chromophore present in a structure, which does not necessarily give the structure of the entire molecule. However, it offers clues for the potential substructures that can be searched for during dereplication process [19]. The UV spectrum is more informative other than just the λ_{max} data. Matching spectra is a superior and more effective approach of UV spectra in the dereplication approach for natural products [19]. Such approaches are carried out by matching UV spectra with in-house UV spectra database [20]. Most of HPLC have the essential software for the capturing and comparing acquired UV spectral data. However, UV spectral data acquired from other HPLC cannot be compared [19]. Unfortunately, there is no UV spectral database available which contain other information required for the dereplication process [19]. UV data with mass data is a powerful and efficient tool for dereplication.

Fixed wavelength, multiple wavelength, and diode array detector (DAD) are the three main types of UV detectors which are currently available. The fixed wavelength detector has a higher instrinsic sensitivity and low cost because the light emitted at specific wavelengths with given lamp. The multiple wavelength detector is more versatile, but lower sensitivity compared to the fixed wavelength detector. The diode array detector (DAD) also known as photodiode array and provides several advantages such as peak measurement at all wavelengths, determination of the correct wavelengths in one run, detection of multiple wavelengths, peak purity analysis, scan spectrum very quickly and so on.

In this study, HPLC-UV screening of natural products from bacteria associated with stony corals was used. Having some severe drawbacks, UV is still widely used in natural products screening because of easy to use, cheap, and adapted to a relatively large numbers of natural products with detection at low wavelengths. In addition, UV spectrum is only indicative of a chromophore present in a molecule, but not the entire structure. So there is still a chance of getting new molecules even after matching UV spectra profile with an in-house database if we are using unexplored sources or organisms from different taxonomic group for screening (Figure 2-1).

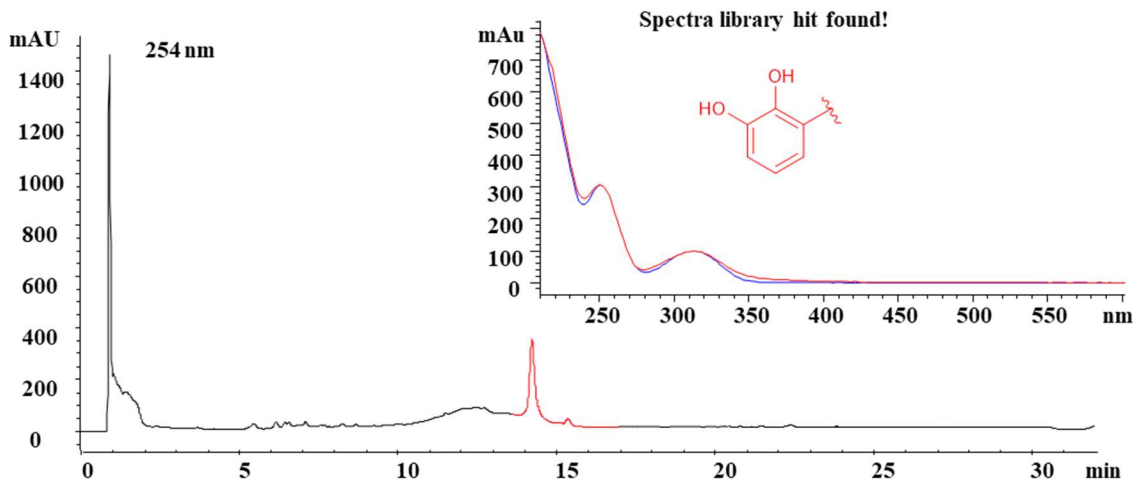


Figure 2-1. Characteristic UV profile of a 1,2,3-trisubstituted aromatic system.

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CHAPTER 3

Labrenzbactin from a Coral-Associated Bacterium *Labrenzia* sp. C1-1

3-1 Background

This study was conducted to assess the biosynthetic ability of coral-associated bacteria to produce new bioactive metabolites. Corals are marine invertebrates and represent one of the most exploited marine invertebrate for natural product discovery after sponges [1]. A wealth of new bioactive compounds was reported from soft corals. However, only handful of compounds were reported from stony corals. The reason behind it may be that researchers thought that stony coral rarely produces bioactive metabolites as a chemical defense as its outer skeleton made of calcium carbonate [2]. Recently, the rate of finding new compounds from common terrestrial conventional sources decrease whereas the chance of finding already know compounds is dramatically increases. Because there is myriad of new compounds already reported from common sources. So it is wise to use unexplored sources or unexploited organisms for the natural product discovery. The reason behind it is that there is a high chance of finding new organisms with diverse metabolic pathway which can lead to the discovery of novel bioactive compounds. Stony corals and its associated bacteria are unexplored sources and largely neglected for natural products screening program. In this study, stony coral-associated bacteria were used to find new bioactive compounds because there is no any published report on bioactive compounds from stony coal-associated bacteria and they are highly ignored by scientists for screening of new bioactive compounds.

HPLC-UV screening was employed to check the secondary metabolites produced by coral-associated bacterial strain C1-1. The producing strain was isolated from aquacultured stony coral of genus *Montipora* sp. Three types of fermentation media were used to compare the metabolite production. However, this strain produced metabolites only in A3M seawater fermentation broth. In the solvent extract of culture broth of C1-1 in A3M showed one major peak with retention time 14.2 min. The UV spectrum of peak showed absorption maxima at 208, 250 and 314 nm (Figure 3-1). This type of UV spectrum usually represents the compound containing dihydroxy benzene chromophore. When I searched this UV spectrum with our in-house database, there were two hits for known compounds such as catechoserine (211, 248, 319 nm) [3] and ulbactin F (204, 252, 317 nm) [4]. However, our in-house database only contains UV spectrum for compounds retrieved from mainly actinomycetes and fungi. UV spectrum only gave hint of the chromophore present in the molecule. However, it could not give full information of the structure. This was the first time I worked with coral-associated bacteria and producing strain is not commonly used for natural products screening in my laboratory. So I decided to work with this strain although similar UV spectrum was present in

our in-house database. I thought that there is a chance of finding new compounds if I work with new bacterial strains or unexploited group of organisms obtained from unexplored sources.

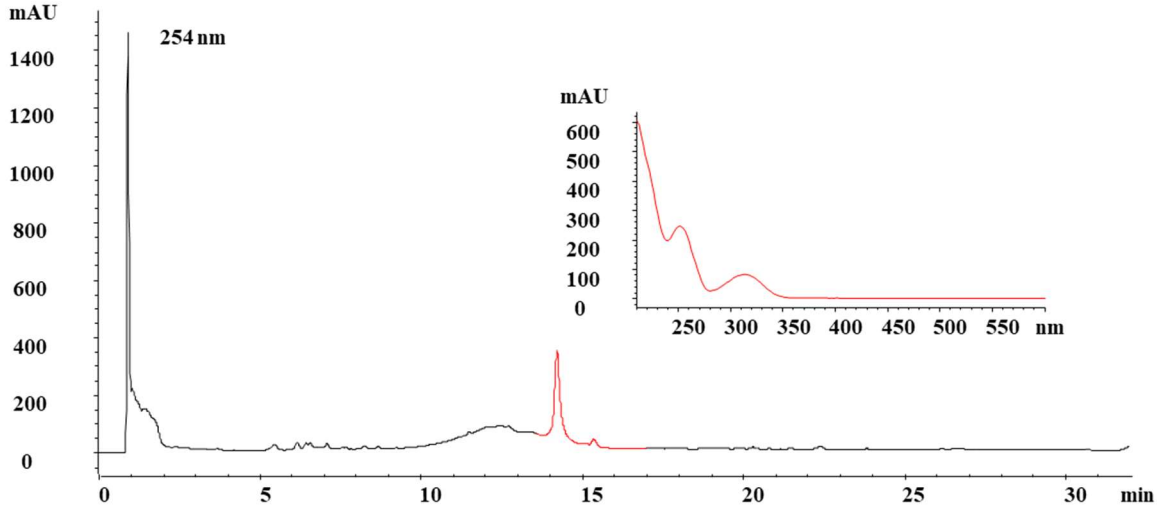


Figure 3-1. HPLC analysis of 1-butanol extract of producing strain *Labrenzia* sp. C1-1.

The producing strain C1-1 was identified as a member of genus *Labrenzia* on the basis of 99.9% similarity in the 16S rRNA gene sequence (1395 nucleotides; DDBJ accession number LC456786) to *Labrenzia aggregata* IAM 12614^{T} (accession number AAUW01000037). The genus *Labrenzia* falls within the class Alphaproteobacteria and encompasses Gram-negative, motile, strictly aerobic, and rod-shaped bacteria. The *Labrenzia* strains require a sea salt-based medium for growth and they are therefore marine obligate bacteria (Figure 3-2). *Labrenzia* are usually isolated from marine habitats, in many reported cases, in association with marine invertebrates such as corals [5-7]. Although the species of *Labrenzia* possess biosynthetic genes for the production of polyketide, non-ribosomal peptide, and terpenoid, very little is known about their secondary metabolites [7]. To date, a polyketide-derived pederin-analogue and two cyclopropane-containing fatty acids have been reported from *Labrenzia* sp. (Figure 3-3). Polyketide-derived new pederin-analogue was isolated from culture broth of *Labrenzia* sp. isolated from marine sediment. This compound showed strong cytotoxicity toward the panel of cell lines [8]. Similarly, chemical investigation of fermentation broth of marine sediment-derived *Labrenzia* sp. led to the discovery of two cyclopropane-containing medium-chain fatty acids [9]. These compounds displayed strong inhibition against wide range of bacteria and fungi.

In our HPLC-UV chemical screening of secondary metabolite production by coral-associated bacteria, *Labrenzia* sp. C1-1 was found to produce a new catecholate-class

siderohpore designated labrenzbactin (**1**). In this chapter, the isolation, characterization, and bioactivity of **1** are described.

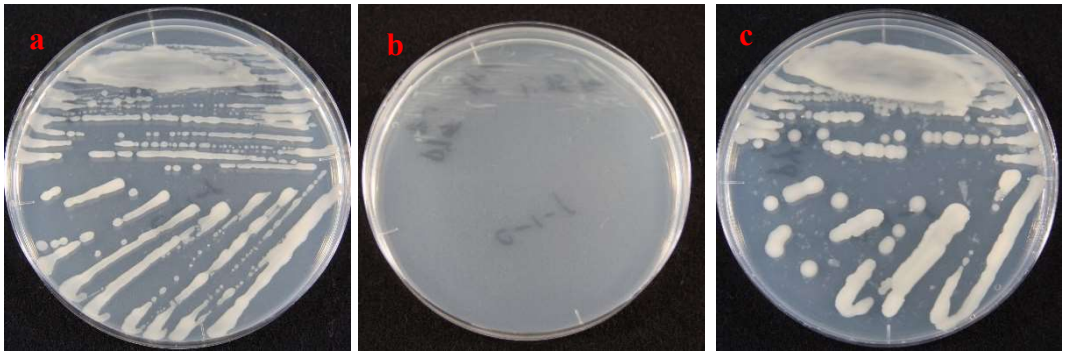


Figure 3-2. Colony morphology of *Labrenzia* sp. C1-1 on a) Marine Agar, b) YP medium (yeast extract 0.1% and peptone 0.5%) in distilled water, c) YP medium in natural seawater.

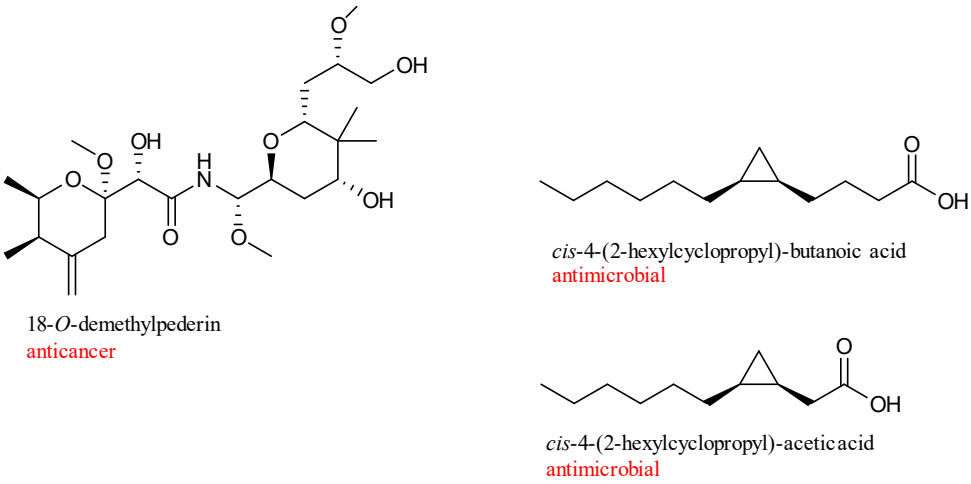
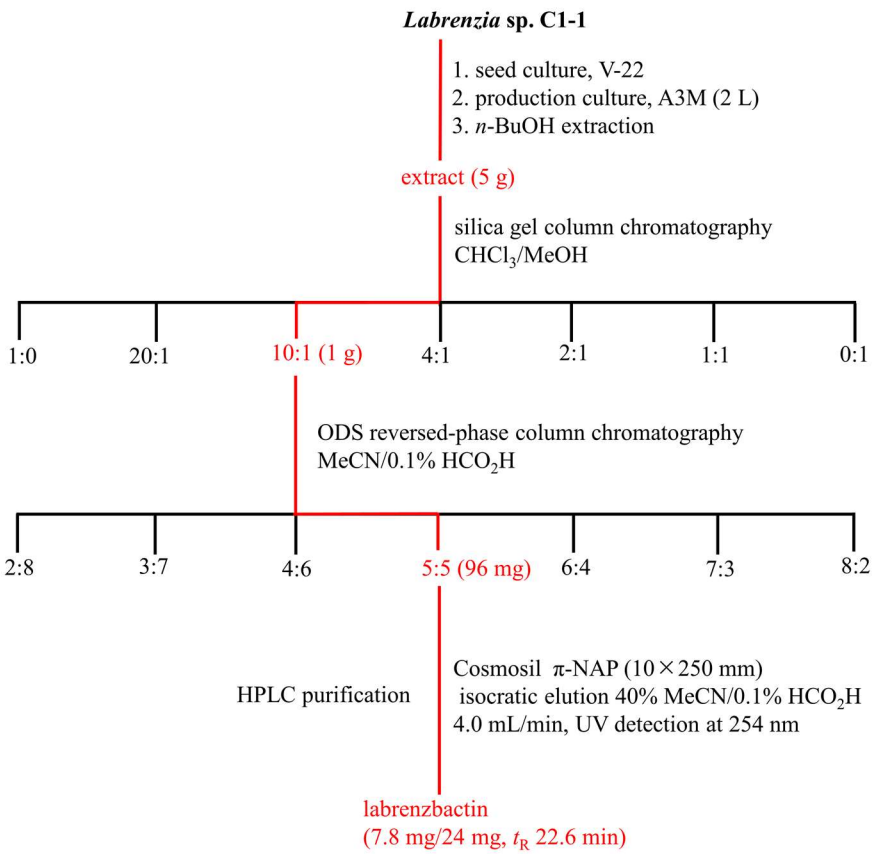


Figure 3-3. Chemical structures of compounds from *Labrenzia* sp.

3-2 Results and Discussion

3-2-1 Fermentation and Isolation

The producing strain C1-1 was isolated from a cultured Scleractinian (stony) coral *Montipora* sp., and identified as a member of the genus *Labrenzia* by 16S rRNA gene sequence analysis. The fermentation broth of strain C1-1 cultured in A3M seawater medium was extracted with 1-butanol. After evaporation, the solvent extract was fractionated by a sequence of chromatographies, and final purification was achieved by reversed-phase HPLC to render labrenzbactin (**1**) (Scheme 3-1).



Scheme 3-1. Isolation scheme of labrenzbactin (**1**).

3-2-2 Structure Determination

Labrenzbactin (**1**) was obtained as a brown amorphous solid. Its molecular formula was assigned as $C_{32}H_{36}N_4O_{10}$ with 17 degrees of unsaturation by HR-ESI-TOFMS that gave a molecular ion $[M+Na]^+$ at *m/z* 659.2324 (calcd for $C_{32}H_{36}N_4O_{10}Na$, 659.2329) and NMR spectroscopic data (Table 3-1). The IR spectrum of **1** indicated the presence of OH and/or NH (3313 cm^{-1}) and carbonyl (1634 cm^{-1}) groups. Our in-house UV database indicated that the UV spectrum with the absorption bands at λ_{max} 208, 250 and 314 nm was similar to those for catechoserine (211, 248, 319 nm) [3] or ulbactin F (204, 252, 317 nm) [4], suggestive of the presence of a hydroxylated benzene ring conjugated with a double bond like a carbonyl group.

NMR spectroscopic data of **1** revealed the presence of 32 carbons attributable to four carbonyl-like downfield sp^2 carbons, eighteen sp^2 carbons (nine are proton-bearing), eight methylenes, one methine, and one methoxy group (Table 3-1). COSY analysis identified proton resonances for three sets of 1,2,3-trisubstituted benzene rings, H8/H9/H10, H9'/H10'/H11', and H8"/H9"/H10" (Figure 3-4). HMBC correlations from H8 and H10 to C6 and H9 to C5 and C7 established the 2,3-dihydroxyphenyl substructure. An oxazoline ring was deduced from

the HMBC correlations from H2 and H3 to C4 and chemical shift consideration for H2, H3, and C4. The linkage between C4 and C5 and the attachment of C1 to C2 were determined by the observed HMBC correlations. Another 2,3-dihydroxyphenyl moiety consisting of the carbons from C5" to C10" and a 2-hydroxy-3-methoxyphenyl moiety comprising C6' to C11' were also established by analyzing HMBC correlation data. The position of the methoxy group was confirmed by NOE detected between H12' and H9'. COSY spectrum also provided two short methylene chains from H1' to H4' and from H1" to H3". Mutual HMBC correlations between H1' and H1" linked these carbon chains through a nitrogen atom which was in turn connected with the carbonyl carbon C1 by HMBC correlations from H1' and H1" to C1. Finally, two aromatic parts were connected to the methylene chains via an amide bond on the basis of HMBC correlations from H4' and H11' to C5' and from H3" and H10" to C4" to complete the planar structure of **1** (Figure 3-4). This structure was further validated by analyzing the NMR spectral data obtained in DMSO-d_6 that gave higher peak resolution for ^{1}H NMR spectrum (Supplementary Information). Some of the ^{1}H and ^{13}C NMR signals were observed as duplicated peaks presumably due to the tautomeric isomerization of amide bonds but all the signals could be assigned to its structure (Table 3-1).

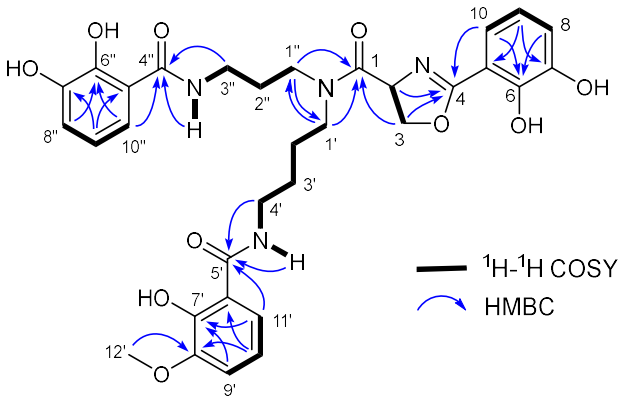


Figure 3-4. COSY and key HMBC correlations for labrenzbactin (**1**).

3-2-3 Absolute Configuration

In order to determine the absolute configuration of oxazoline moiety derived from the serine residue in **1**, advanced Marfey's analysis was utilized [10] (Scheme 3-2). Following acid-catalyzed hydrolysis, **1** was derivatized using 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and retention time was compared with L-serine standard that was similarly derivatized with L-FDLA and 1-fluoro-2,4-dinitrophenly-5-D-leucinamide (D-FDLA) using LC-MS. The derivatized L-serine standard with L-FDLA and D-FDLA eluted at 9.5 and 10.7 min, respectively, while the L-FDLA derivative of the acid hydrolysate of **1** eluted at 9.5 min. The serine residue was determined to have the L-configuration and thus the configuration of C2 was established to be *S* (Figure 3-5 and 3-6).

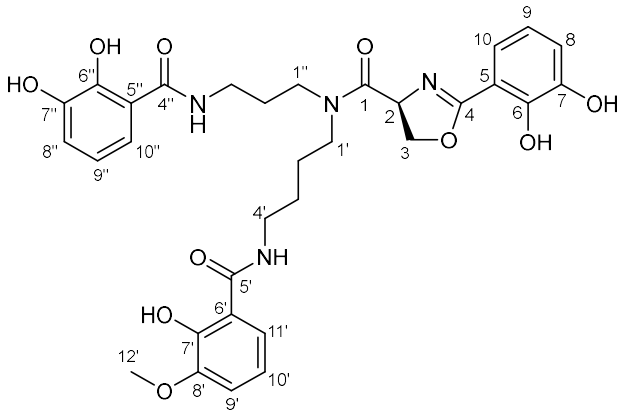


Figure 3-5. Structure of labrenzbactin (**1**).

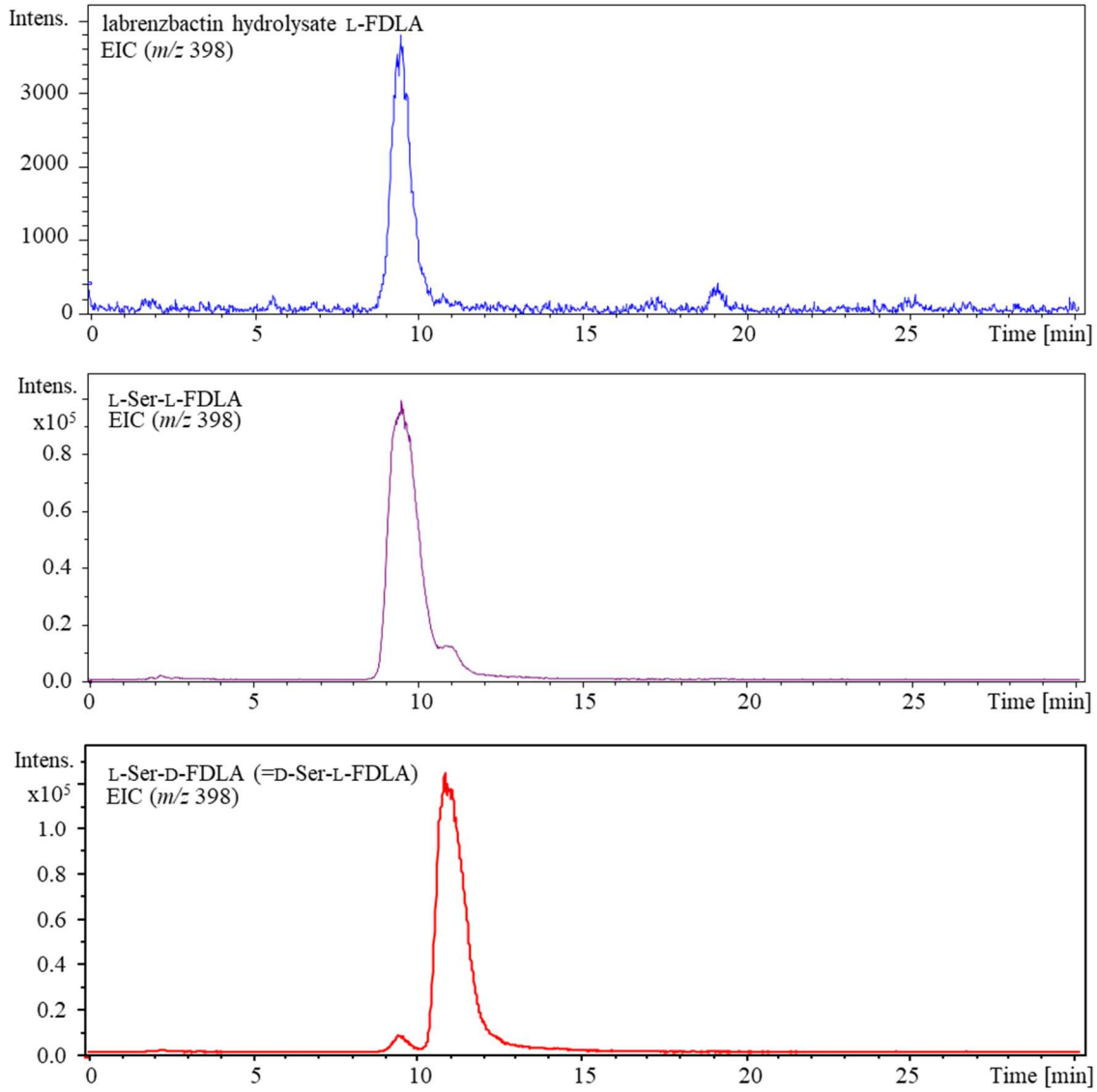


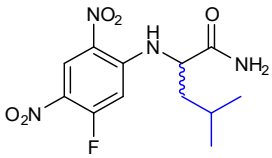
Figure 3-6. HPLC chromatogram of hydrolysate from labrenzbactin (**1**) and comparison to standard amino acid, derivatized with Marfey's reagent (FDLA).

Column: Cosmosil AR-II (Nacalai Tesque, 2×150 mm)
Solvent: Gradient MeCN (%): 0-30 min: 25-65% in 0.1% HCO_2H
Flow rate: 0.2 mL/min
Mass detection: negative ion mode

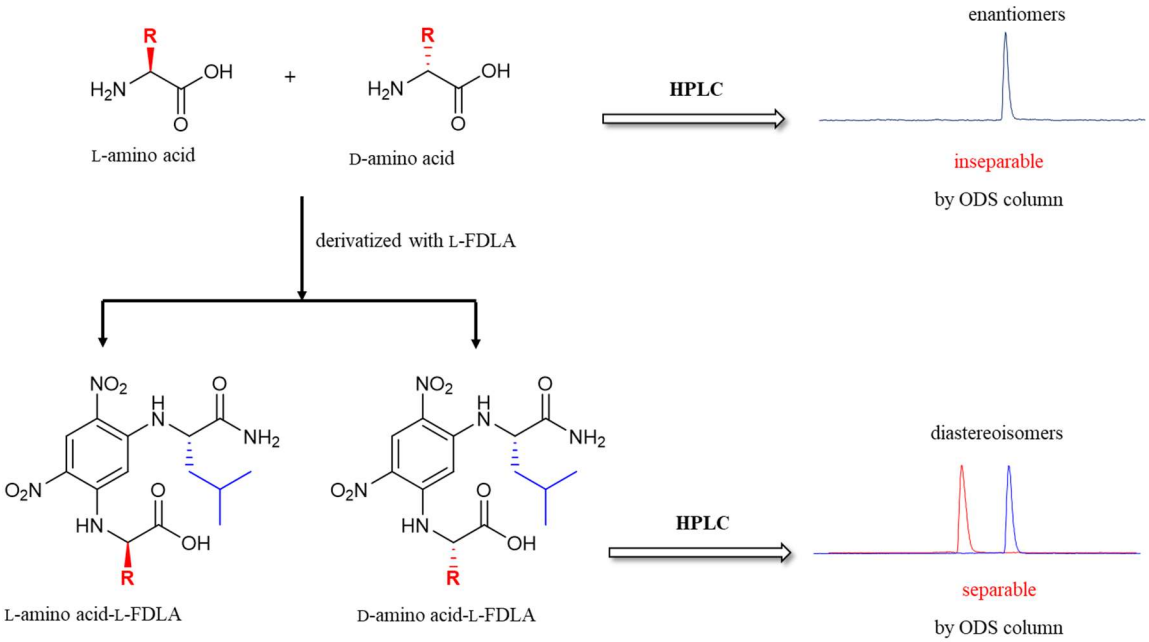
Marfey's method is a procedure for determining the absolute configurations of hydrolytically accessible amino acid residues present in natural products (typically but not exclusively peptides) [11]. This method consists four steps: (i) the natural product is subjected to acid hydrolysis (6 N HCl) to release amino acid residues, (ii) the hydrolysate is derivatized using chiral Marfey's reagents such as D- or L-1-fluoro-2-4-dinitrophenyl-5-alanine amide (D- or L-FDAA) or D- or L-1-fluoro-2-4-dinitrophenyl-5-leucinamide (FDLA) under alkaline

condition (1 N $NaHCO_3$), (iii) the neutralized derivatized analyte is subjected to reversed-phase HPLC-UV (340 nm) analysis or HPLC-MS, using generally C18 columns, and (iv) HPLC retention times of derivatized amino acid residues in the analyte is compared with L and D-amino acid standards derivatized with Marfey's reagent.

Based on Marfey's method, the relationship between the L-FDLA derivative of D-amino acid (D-L type) and that of L-amino acid (L-L type) is diastereomeric, and this pair of diastereoisomers has different retention times on chromatogram so readily separated by C18 reveresd-phase HPLC. On the contrary, the L-D and D-L type derivatives, as well as the L-L and D-D type derivatives, are enantiomers, and each pair of enantiomers has the same retention time and cannot be separated by C18 reversed-phase HPLC (Scheme 3-2).



D- or L-FDLA (1-fluoro-2,4- dinitrophenyl-5-leucinamide)



Scheme 3-2. Principle of Marfey's method.

Table 3-1. ^{1}H and ^{13}C NMR data for labrenzbactin (**1**) in $CDCl_3$-CD_3OH (9:1) or in DMSO-d_6.

	$CDCl_3$-CD_3OH (9:1)			DMSO-d_6	
Position	δ_C[a]	δ_H mult (*J* in Hz)[b]	HMBC[b,c]	δ_C[a]	δ_H mult (*J* in Hz)[b]
1	169.8, C			168.5/168.6	
2	65.4, CH	5.16 t (8.4)	1, 3, 4	64.3	5.36 t (8.5)/5.37 t (8.5)
3	69.0, CH_2	4.48 t (8.9)	1, 2, 4	69.3	4.53 t (9.0)
		5.08 t (7.5)	1, 2, 4		4.76 t (7.3)
4	167.4, C			166.0	
5	110.2, C			110.1	
6	147.5, C			148.1	
7	145.0, C			145.7	
8	119.1, CH	6.99 d (7.2)	6, 10	119.5	6.96 d (7.6)
9	119.3, CH	6.75 t (7.3)	5, 7	118.8	6.74 t (8.0)
10	119.4, CH	7.15 d (7.9)	4, 6, 8	117.87	7.07 d (7.9)
1’	47.6, CH_2	3.62 m	1, 2’, 1”	45.1/46.8	3.32, 3.36 m/3.54, 3.63 m
2’	26.3, CH_2	1.82[d]	1’, 3’	26.1	1.63, 1.72 m
3’	27.0, CH_2	1.72 m	1’, 2’, 4’	24.5/26.2	1.51 m/1.50 m
4’	38.9, CH_2	3.52[e]	3’, 5’	38.5/38.6	3.36 m/3.28 m
5’	169.5, C			169.49/169.53	
6’	111.5, C			114.75/114.83	
7’	150.0, C			150.9/151.0	
8’	148.6, C			148.4	
9’	114.7, CH	6.87 d (7.9)	7’, 11’	115.3	7.06[f]
10’	118.7, CH	6.72 t (7.9)	6’, 8’	117.7	6.78 t (8.1)
11’	118.6, CH	7.20 d (7.7)	5’, 7’, 9’	118.45/118.50	7.39 d (8.6)/7.41 d (8.6)
12’	56.2, CH_3	3.82 s	10’	55.7	3.74 s/3.75 s
4’-NH		7.50 br.s			8.82 t (5.4)/8.86 t (5.5)
1”	43.3, CH_2	3.46[e]	1, 1’, 2”, 3”	43.4/44.8	3.38 m/3.53, 3.69 m
2”	27.2, CH_2	1.82[d]	1”, 3”	27.1/28.5	1.77 m/1.95 m
3”	36.1, CH_2	3.29 m	1”, 2”, 4”	36.7	3.26 m, 3.39 m
4”	170.3, C			169.7/169.9	
5”	114.5, C			114.9/115.1	
6”	149.3, C			149.6/149.7	
7”	145.8, C			146.2	
8”	118.3, CH	6.95 d (7.5)	6”, 10”	118.7	6.89 d (7.8)
9”	118.8, CH	6.71 t (7.9)	5”, 7”	117.86	6.66 t (7.9)
10”	116.9, CH	7.10 d (7.9)	4”, 6”, 8”	117.0/117.2	7.24 d (8.0), 7.27 d (7.9)
3”-NH		8.03 br.s	4”		8.76 t (5.4)/8.85[g]
OH					9.23 br.s
OH					11.78 br.s
OH					12.77 br.s

[a] Recorded at 125 MHz (reference δ_C 77.2 for $CDCl_3$-CD_3OH; δ_C for 39.5 for DMSO-d_6).

[b] Recorded at 500 MHz (reference δ_H 7.26 for $CDCl_3$-CD_3OH; δ_H for 2.50 for DMSO-d_6).

[c] HMBC correlations are from proton(s) stated to the indicated carbon.

[d-g] Overlapping signals.

3-2-4 Bioactivity

Biological activity of labrenzbactin (**1**) was evaluated by cytotoxicity and antimicrobial assays. Labrenzbactin showed moderate cytotoxicity against P388 murine leukemia cells at IC_{50} 13 μM (IC_{50} of a reference drug doxorubicin: 0.018 μM) and no appreciable activity in antimicrobial assay against *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* FDA209P JC-1, *Rhizobium radiobacter* NBRC14554, and *Escherichia coli* NIHJ JC-2. However, it showed weak antibacterial activity against *Ralstonia solanacearum* SUPP1541 and *Micrococcus luteus* ATCC9341 with MIC values of 25 and 50 μg/mL, respectively.

3-3 Conclusion

In summary, HPLC-UV chemical investigation of a marine bacterium *Labrenzia* sp. isolated from stony coral *Porites* sp. led to the discovery of labrenzbactin (**1**). This compound displayed weak antibacterial and cytotoxic activity. The antibacterial activity is likely due to the ability of **1** to bind Fe^{3+}, inhibiting microbial proliferation via cellular iron depletion [12]. This is a new member of catecholate-type bacterial siderophores in which a linear triamine, spermidine or norspermidine, is amidated with acyl groups containing 2,3-dihydroxyphenyl and/or 2-hydroxyphenyl units. Several related compounds are reported: parabactin (**2**) from *Paracoccus denitrificans* [13]; agrobactin (**3**) from a plant pathogen *Agrobacaterium tumefaciens* [14]; fluvibactin (**4**) and vibriobactin (**5**) from *Vibrio fluvialis* and *Vibrio cholerae*, respectively [15,16] (Figure 3-7). *Labrenzia* spp. have the same origin with *Agrobacaterium tumefaciens*, and they were initially called the marine *Agrobacterium* then it was transferred to new group *Stappia* sp. then finally transferred to new genus *Labrenzia*. This results showed that *Agrobacterium* sp. and *Labreniza* sp. are closely related. Maybe *Labrenzia* sp. by some means transferred to the marine environment and adapted to live there but still carry the biosynthetic gene cluster for the synthesis of siderophore, which can be expressed in iron-difficient environment for their survivial. The concentration of dissolved iron is extrememly low in marine environments [17]. Iron is required for the living organisms and paly important role in the oxygen metabolism, electron transfer, and DNA and RNA synthesis [17]. Under the normal physiological condition iron is present as ferric hydroxide, higly insoluble form, which cannot be utilized by microorganisms [18]. To get such limited iron, microorganisms produce siderophores and they bind Fe^{3+} and transport it into the cells where it is reduced to Fe^{2+} and released from siderophore and microorgansims get iron in iron-deficient environment [19] (Scheme 3-3). Marine organisms like *Labrenzia* sp. overcome the iron limitation probably by

producing siderophores like labrenzbactin. *Labrenzia* species contains biosynthetic gene clusters for the production of non-ribosomal peptides, polyketides and terpenoids, but only two bioactive compounds are reported from this genus before this study. So *Labrenzia* spp. are underexplored bacteria with potential ability to produce secondary metabolites and waiting for exploitation by researchers for secondary metabolites screening program. From this study, we can conclude that bacterial strain retrieved from unexplored sources such as a stony coral could be the source of new compounds and stony coral-associated bacteria have great potential in the discovery of bioactive molecules.

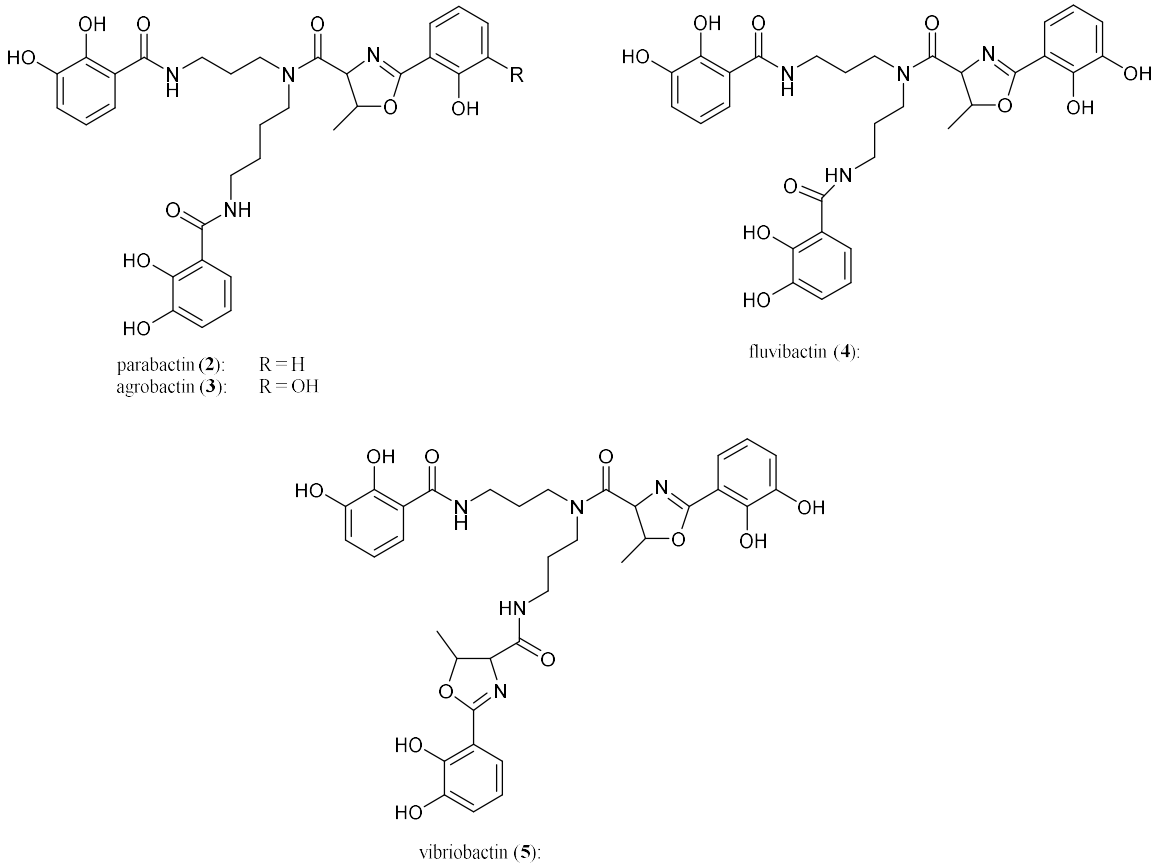
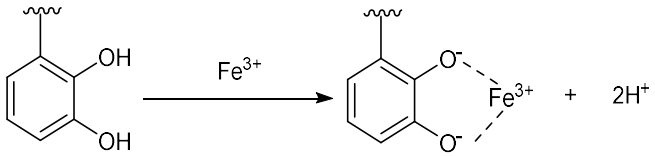


Figure 3-7. Related metabolites from bacteria, parabactin (**2**), agrobactin (**3**), fluvibactin (**4**), and vibriobactin (**5**).



Scheme 3-3. Iron acquisition by catecholate-type siderophores.

Until now, terrestrial microorganisms especially Gram-positive Actinobacteria and filamentous fungi, have furnished the majority of natural drugs. Proteobacteria is the major phylum of Gram-negative bacteria and includes many medically important pathogens such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, and many others. Although Proteobacteria is the most abundant phylum in marine ecosystems representing 55% of all bacteria [20], only limited bioactive compounds have been isolated from them [21-33] (Figure 3-8).

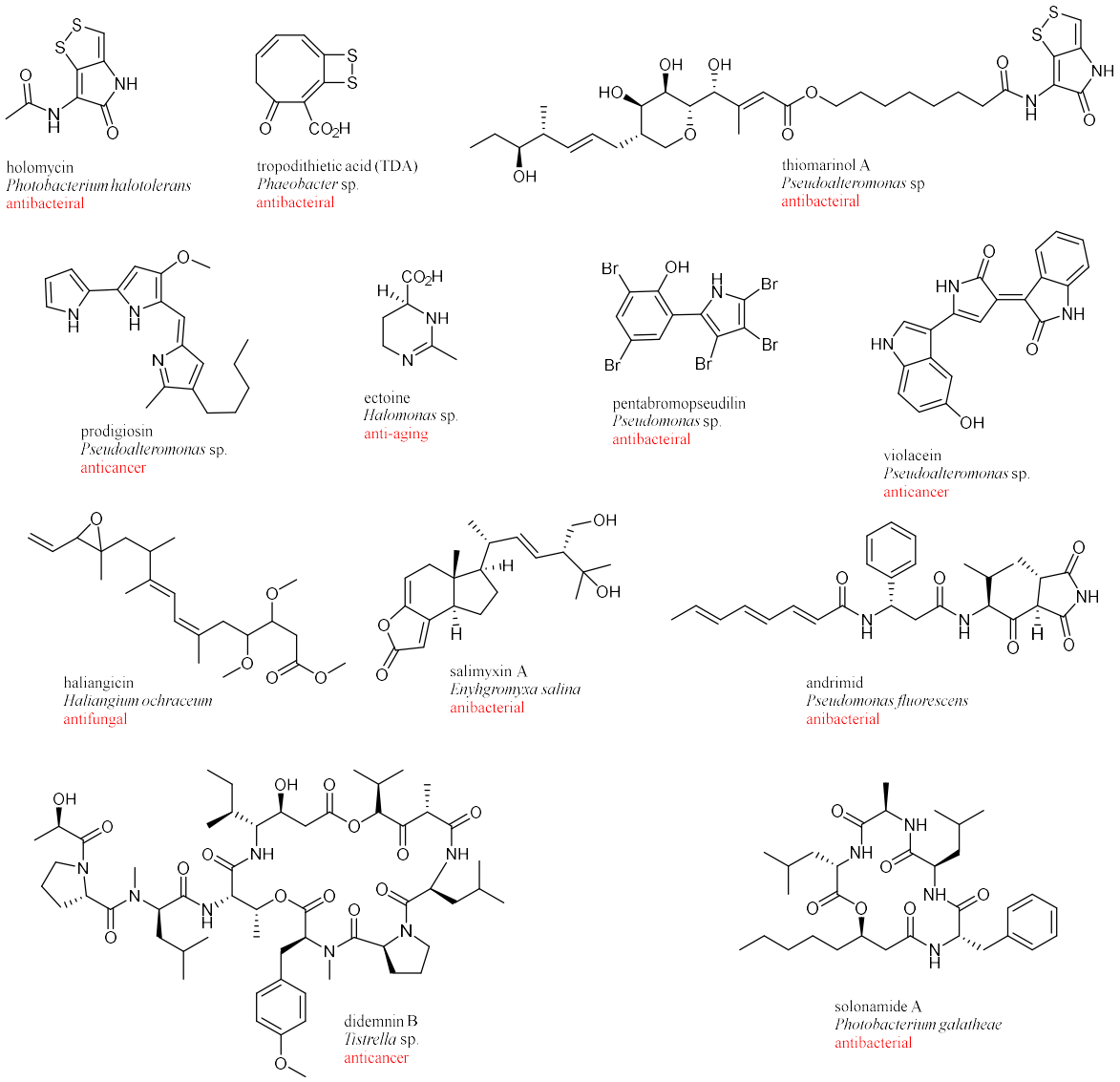


Figure 3-8. Selected examples of bioactive metabolites from marine Proteobacteria.

One of the most interesting example of such molecules is didemnin B, which was originally isolated from tunicate *Trididemnum solidum,* a potent anti-tumor agent, that was discovered to be a product of marine free living *Alphaproteobacteria* of genus *Tistrella* sp. by two independent research groups from Japan and the USA [31,32]. Most of compounds produced by Proteobacteria are not unique to marine environments, and are also produced by terrestrial microorganisms. This is likely due to the horizontal gene transfer-mediated spread of biosynthetic gene clusters. Bacteria of phylum Proteobacteria are dominant group in marine environment, because they evolved diverse metabolic and biosynthetic strategies in order to adapt to harsh marine ecosystems, which makes them attractive and reasonable candidate group of marine bacteria for large scale study for new bioactive compounds [20]. Marine Proteobacteria remain an intriguing source of new drug leads because up to 15% of genomes devoted to biosynthesis of secondary metabolites [20].

3-4 Experimental Section

General Experimental Procedures. Optical rotation was measured using a JASCO DIP-3000 polarimeter. The UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer. The IR spectrum was measured on a PerkinElmer Spectrum. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer in a mixture of $CDCl_3$ and CD_3OH (9:1) due to the low solubility of **1** in a single solvent or in DMSO-d_6. The residual solvent signals ($CDCl_3$: δ_H 7.26 and δ_C 77.24; DMSO-d_6: δ_H 2.50 and δ_C 39.5) were used as internal standards. HR-ESI-TOFMS were recorded on a Bruker micrOTOF focus mass spectrometer.

Microorganism. Strain C1-1 was isolated from a cultured stony coral (*Montipora* sp.) obtained from an aquarium vendor in Nagasaki, Japan. The coral specimen was washed with 70% ethanol and then washed with sterile natural seawater. A piece of the coral (*ca* 1 g) was homogenized by mortar and pestle with equal amount of sterile natural seawater (1 mL), and the suspension was diluted up to 10^{-5} and 0.1 ml of each dilution liquid was spread on plates of Marine Agar 2216 (Difco). After cultivation at 23°C for 2 days, a single colony was repeatedly transferred onto the same agar medium to obtain the pure isolate of strain C1-1. The isolated strain was identified as a member of genus *Labrenzia* on the basis of 99.9% similarity in the 16S rRNA gene sequence (1395 nucleotides; DDBJ accession number LC456786) to *Labrenzia aggregata* IAM 12614^T (accession number AAUW01000037).

Fermentation. Strain C1-1 cultured on Marine Agar 2216 (Difco) was inoculated into a 500 mL K-1 flask containing 100 mL of V-22 seed medium consisting of soluble starch 1%, glucose

0.5%, NZ-case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories, Sparks, MD, USA) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0) in natural seawater. The flasks were placed on a rotary shaker (200 rpm) at 30°C for 2 days. The seed culture (3 mL) was transferred into 500 mL K-1 flasks each containing 100 mL of A3M production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical, Kanagawa, Japan) 1% in natural seawater. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 rpm) at 30°C for 5 days.

Extraction and Isolation. At the end of the fermentation period, 100 mL of 1-butanol was added to each flask, and the flasks were allowed to shake for 1 h. The mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer. Evaporation of solvent gave 5.0 g of crude extract from 2.0 L of culture. The crude extract was subjected to silica gel column chromatography with a step gradient of $CHCl_3$-MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). Fraction 3 (10:1) was concentrated to provide a dark brown oil (1.0 g), which was then fractionated by reversed-phase ODS column chromatography with a gradient of MeCN-0.1% HCO_2H (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). Fraction 4 (5:5) was concentrated and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentered to give 96 mg of semi-pure material. Final purification was achieved by preparative HPLC (Cosmosil π-NAP, Nacalai Tesque Inc., 10 x 250 mm, 4 mL/min, UV detection at 254 nm) with 40% MeCN in 0.1% HCO_2H to give compound **1** (7.8 mg, t_R 22.6 min) from 24.0 mg of semi-pure compound.

Labrenazbactin (**1**): light brown amorphous solid; $[\alpha]^{24}_D$ +6.0 (*c* 0.10, 9:1 $CHCl_3$/MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.81), 250 (4.33), 314 (3.87); (MeOH – 0.01 M HCl (99:1)) 208 (4.74), 256 (4.23), 313 (3.76); (MeOH – 0.01 M NaOH (99:1)) 223 (4.78), 334 (4.03) nm; IR ν_{max} (ATR) 313, 1716 cm^{-1}; For ^1H and ^{13}C NMR data, see Table 3-1; HR-ESI-TOFMS $[M+Na]^+$ 659.2324 (calcd for $C_{32}H_{36}N_4O_{10}Na$, 659.2329).

Marfey's Analysis. A portion of **1** (0.1 mg) was hydrolyzed at 105°C with 5 M HCl (100 μL) for overnight in sand bath, and the reaction mixture was evaporated to dryness. A 0.1 M $NaHCO_3$ solution (100 μL) was added to the dried hydrolysate of **1**, as well as to standards of L-serine (Ser). A solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) in acetone (0.05 mg in 50 μl) was added to each reaction tube and a solution of 1-fluro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA) in acetone (0.05 mg in 50 μL) was added to L-serine reaction tube.

Each tube was sealed and heated at 50°C for 30 min. To quench reactions, 0.2 M HCl (50 μL) was added and then diluted with 50% MeCN/0.05% TFA (100 μL, 50:50). The Marfey's derivatives of the hydrolysate and standards were analyzed by LC-MS using a Cosmosil 5C18-AR-II column (Nacalai Tesque, 2×150 mm) eluted with MeCN-0.1% HCO_2H at a flow rate of 0.2 mL/min, monitoring at 340 nm in negative ion mode. The gradient elution was set as follows: 0-30 min (25-65% MeCN), 30-32 min (65-100% MeCN). Retention times for the amino acid standard were 9.5 min for L-Ser-L-FDLA and 10.7 min for L-Ser-D-FDLA, while the L-FDLA-hydrolysate of **1** gave a peak at 9.5 min.

Antimicrobial Assay. Antimicrobial activity was evaluated by the liquid microculture method using round-bottomed 96-well microtiter plates against six bacteria, *Bacillus subtilis* ATCC6633, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* FDA209P JC-1, *Ralstonia solanacearum* SUPP1541, *Rhizobium radiobacter* NBRC14554, and *Escherichia coli* NIHJ JC-2 and two yeasts *Candida albicans* NBRC0197 and *Saccharomyces cerevisiae* S100 as indication strains. Tryptic Soy Broth (Difco) and Potato Dextrose Broth (Difco) were used for bacteria and yeasts, respectively. Compound **1** and reference drugs, kanamycin sulfate for bacteria and amphotericin B for yeasts, were made in 2-fold dilution series along the longer side of the plates by sequential transfer of 100 μL aliquots between the adjacent wells, to which the same amount of medium was pre-dispensed. To each well was added a 100 μL suspension of the indication strains prepared at 0.5 McFarland (~10^8 cfu/mL) from a culture at the logarithmic growth phase. The solvent vehicle added to the top rows was set at the 0.5% of the final culture volume to avoid the effect on the growth of microbes. The plates were incubated for 48 h at 37°C for bacteria and at 32°C for yeasts. The tests were done in triplicate and the MIC values were read from the lowest drug concentrations at which no growth was observed.

Cytotoxicity Assay. P388 murine leukemia cells were maintained in RPMI-1640 medium containing phenol red, L-glutamine, and HEPES (product no. 189-02145) supplemented with 10% fetal bovine serum and 0.1 mg/mL gentamicin sulfate. Compound **1** and doxorubicin as a reference were serially diluted by a factor of 3.16 (half-logarithmic dilution) in a 96-well round bottom microtiter plate. To each well were seeded the cells at a final density of 1×10^4 cells/well, and 200-μL cultures thus made were incubated for 48 h at 37°C in an atmosphere of 5% CO_2 in air with 100% humidity. Viability of the cells was visualized by MTT, added to each well as a 50 μL solution in phosphate buffered saline without Ca^{2+} prepared at 1 mg/mL. After incubating for 4 h at 37°C, medium was carefully removed by a suction aspirator, and formazan dye formed by respiratory reduction by living cells was solubilized by 100 μL of DMSO. The

absorption at 540 nm was read by a microplate reader to calculate the rate of cell growth inhibition at each concentration, and the results of triplicate experiments were plotted on single-logarithmic charts to deduce IC_{50} values.

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3-5 Spectral Data

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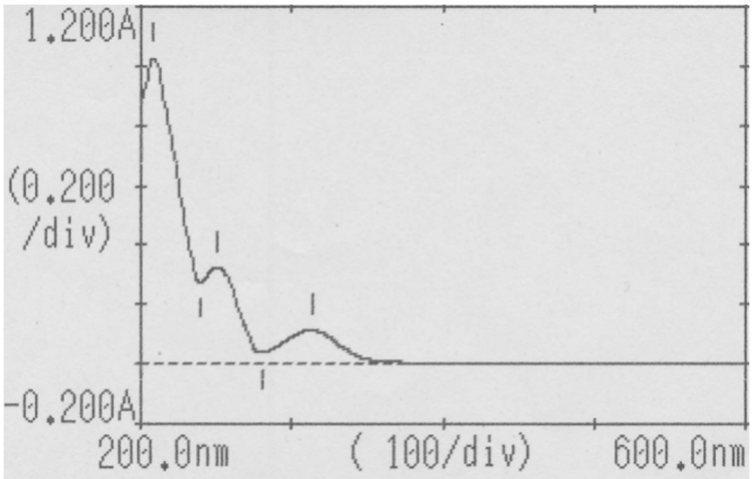
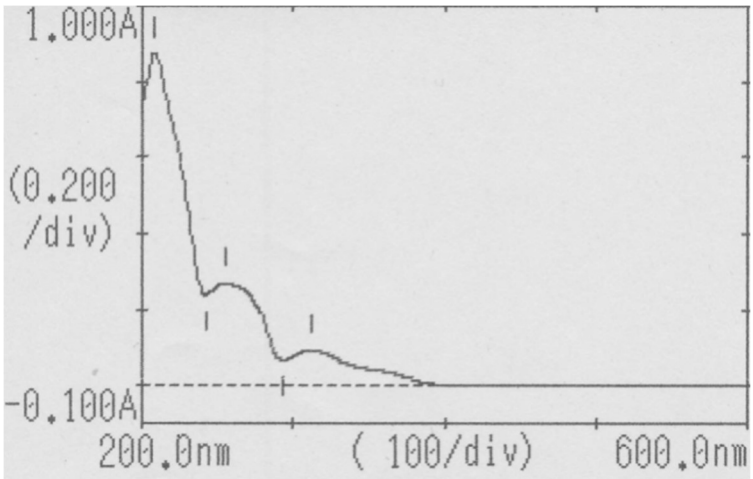
Figure S1. UV spectra of labrenzbactin (**1**)

(A) MeOH



(B) MeOH – 0.01 M HCl (99:1)



(C) MeOH – 0.01 M NaOH (99:1)

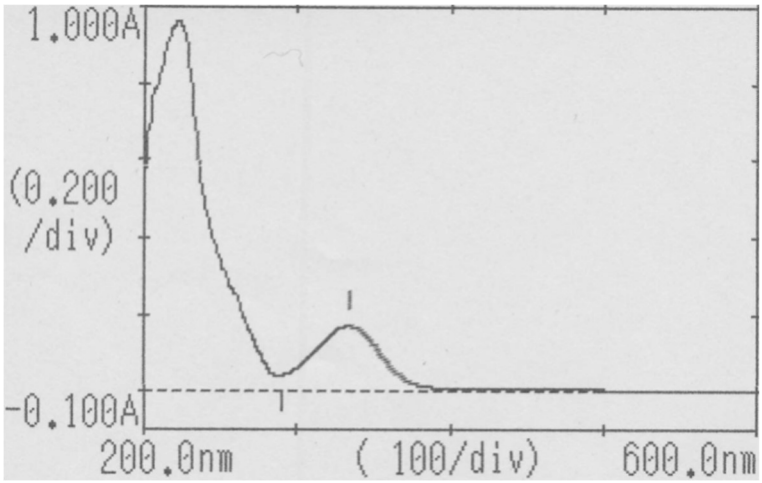


Figure S2. IR spectrum of **1**

(ATR)

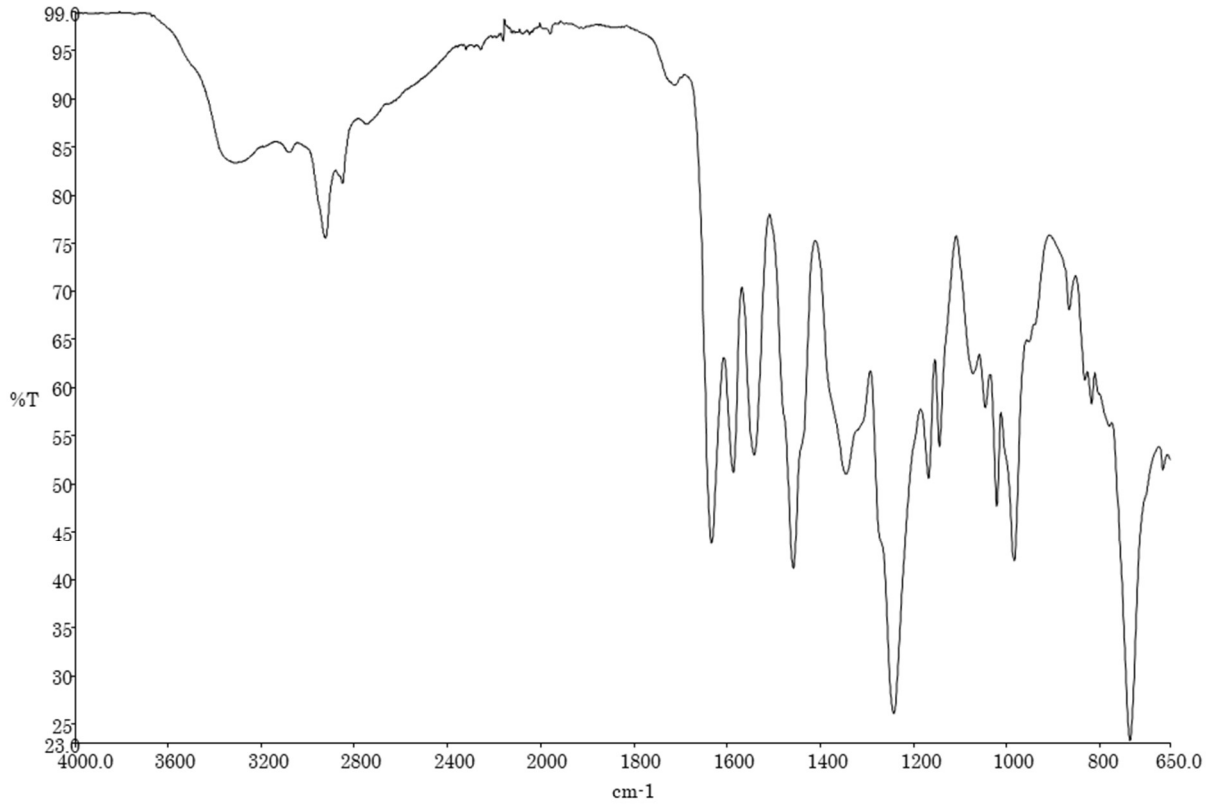


Figure S3. ^{1}H NMR spectrum of **1** (500 MHz, $CDCl_3$–CD_3OH (9:1))

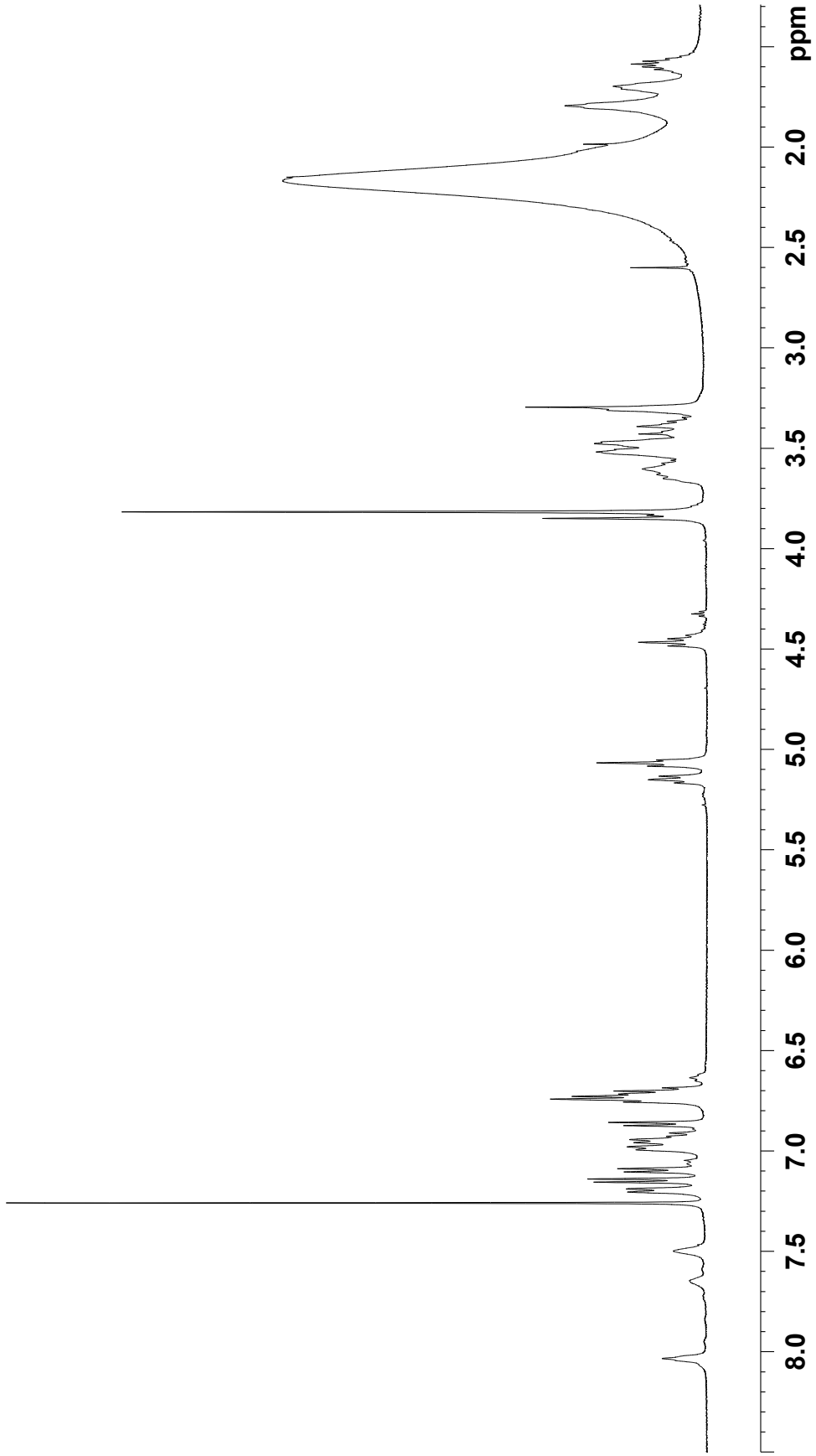


Figure S4. ^{13}C NMR spectrum of **1** (125 MHz, $CDCl_3$–CD_3OH (9:1))

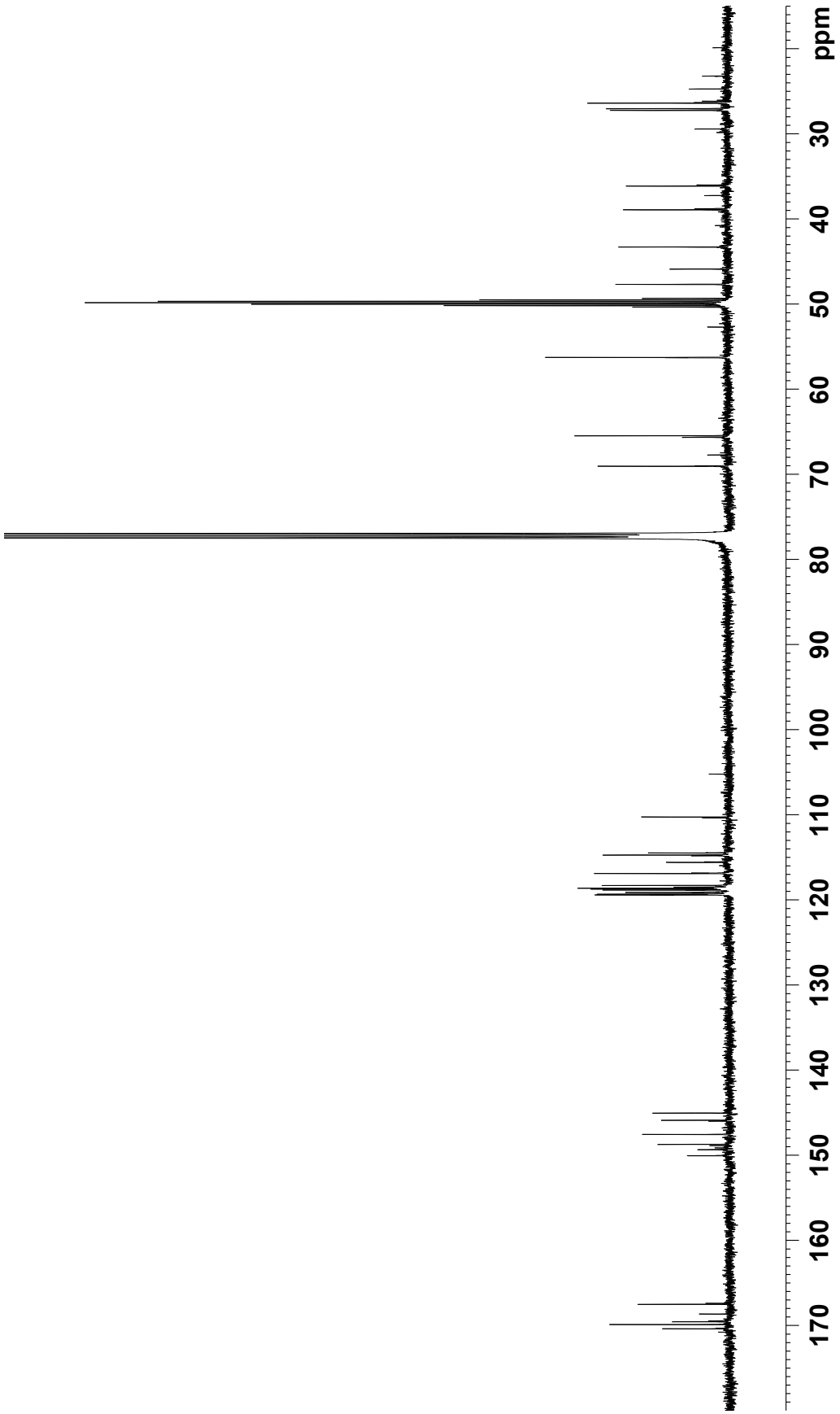


Figure S5. DEPT135 spectrum of **1** (125 MHz, $CDCl_3$–CD_3OH (9:1))

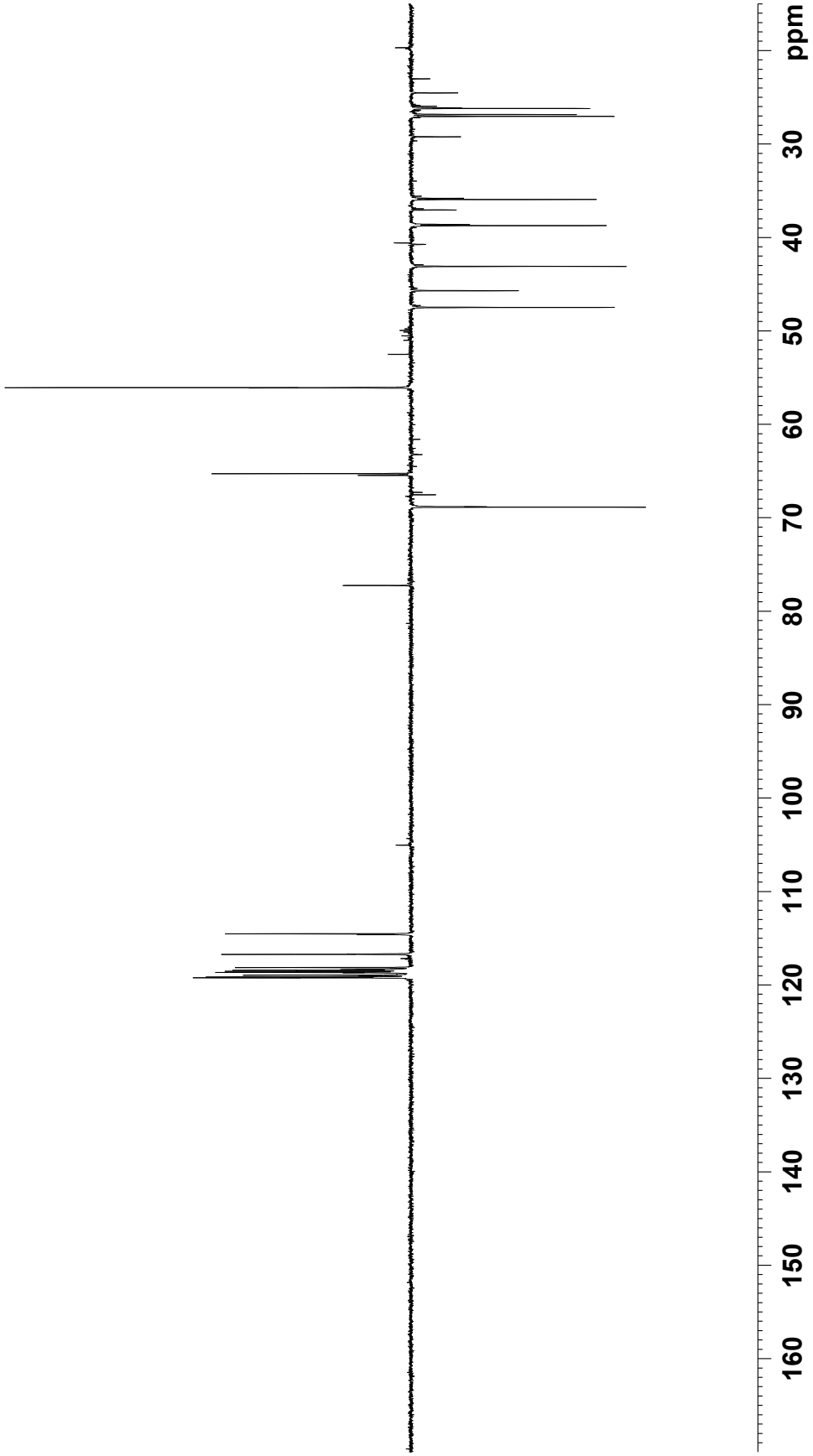


Figure S6. COSY spectrum of **1** (500 MHz, $CDCl_3$–CD_3OH (9:1))

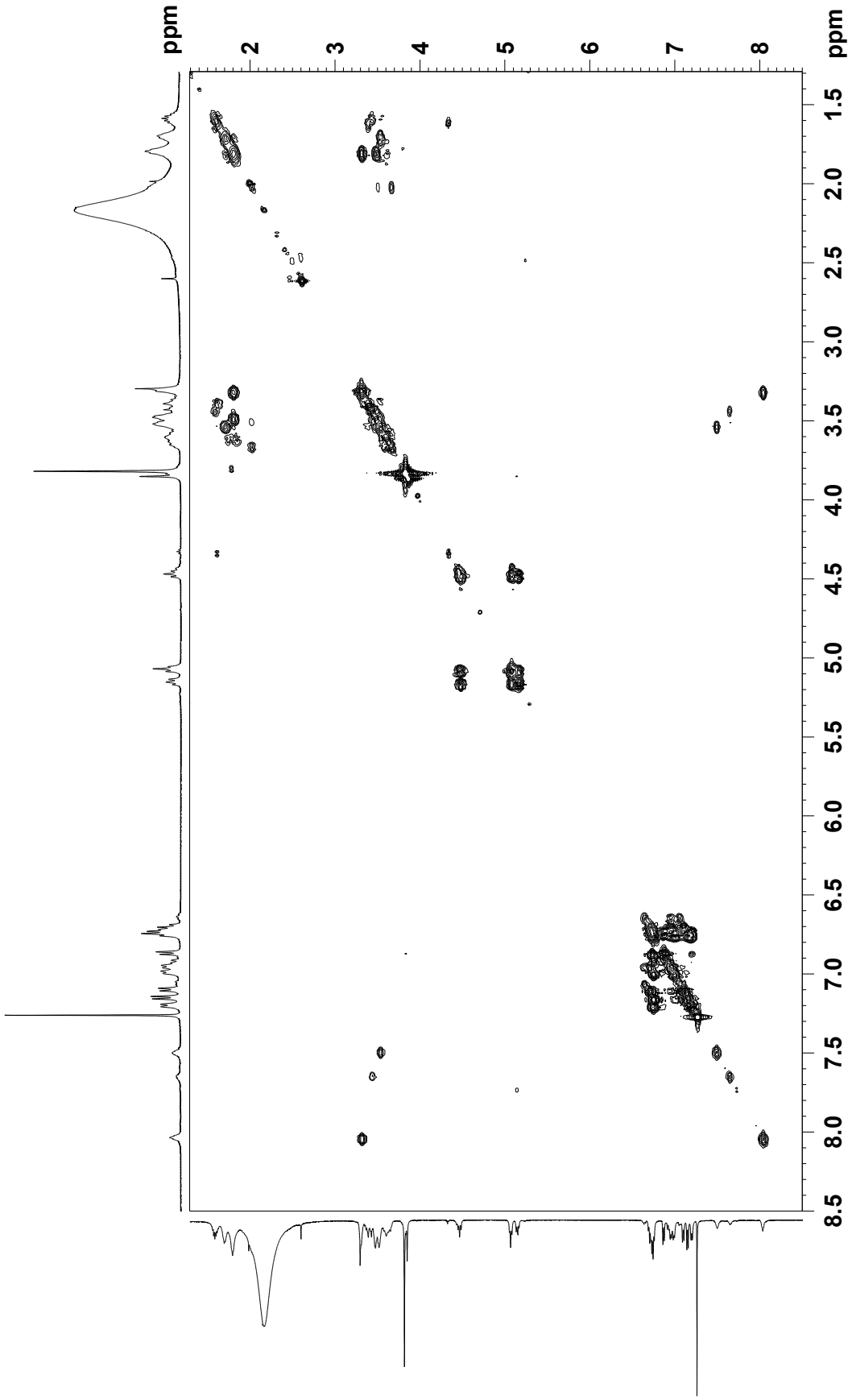


Figure S7. HSQC spectrum of **1** (500 MHz, $CDCl_3$–CD_3OH (9:1))

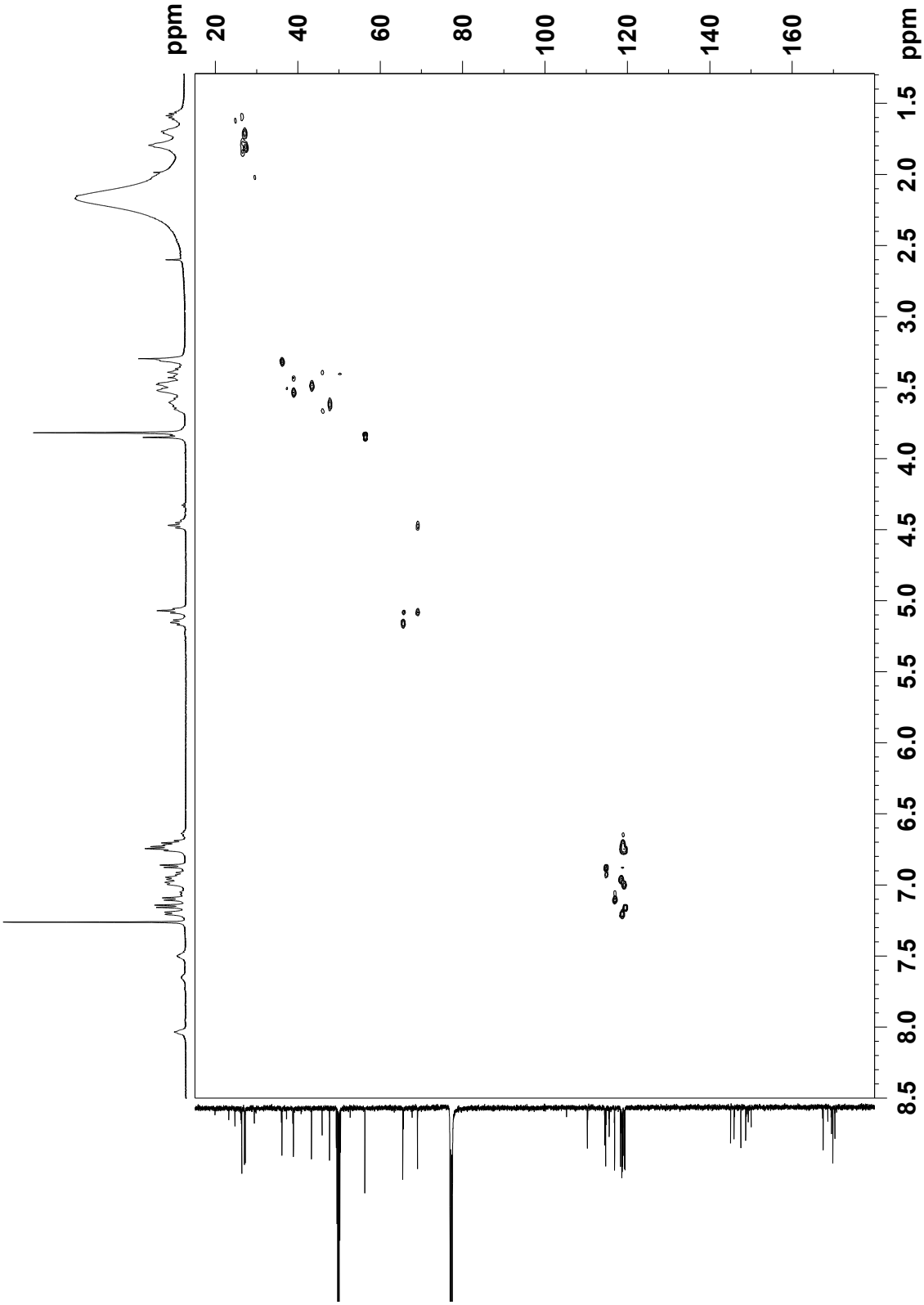


Figure S8. HMBC spectrum of **1** (500 MHz, $CDCl_3$–CD_3OH (9:1))

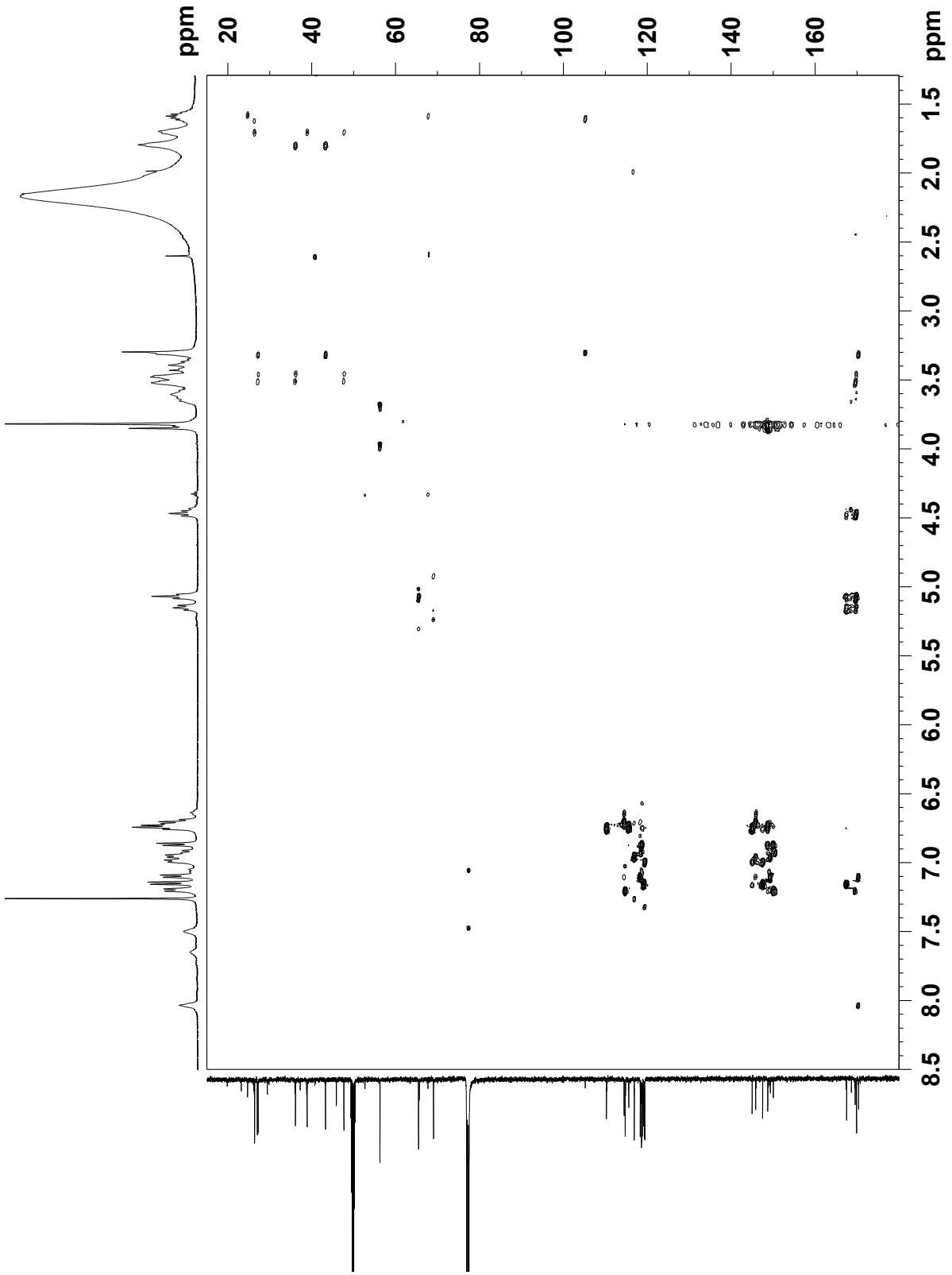


Figure S9. NOESY spectrum of **1** (500 MHz, $CDCl_3$–CD_3OH (9:1))

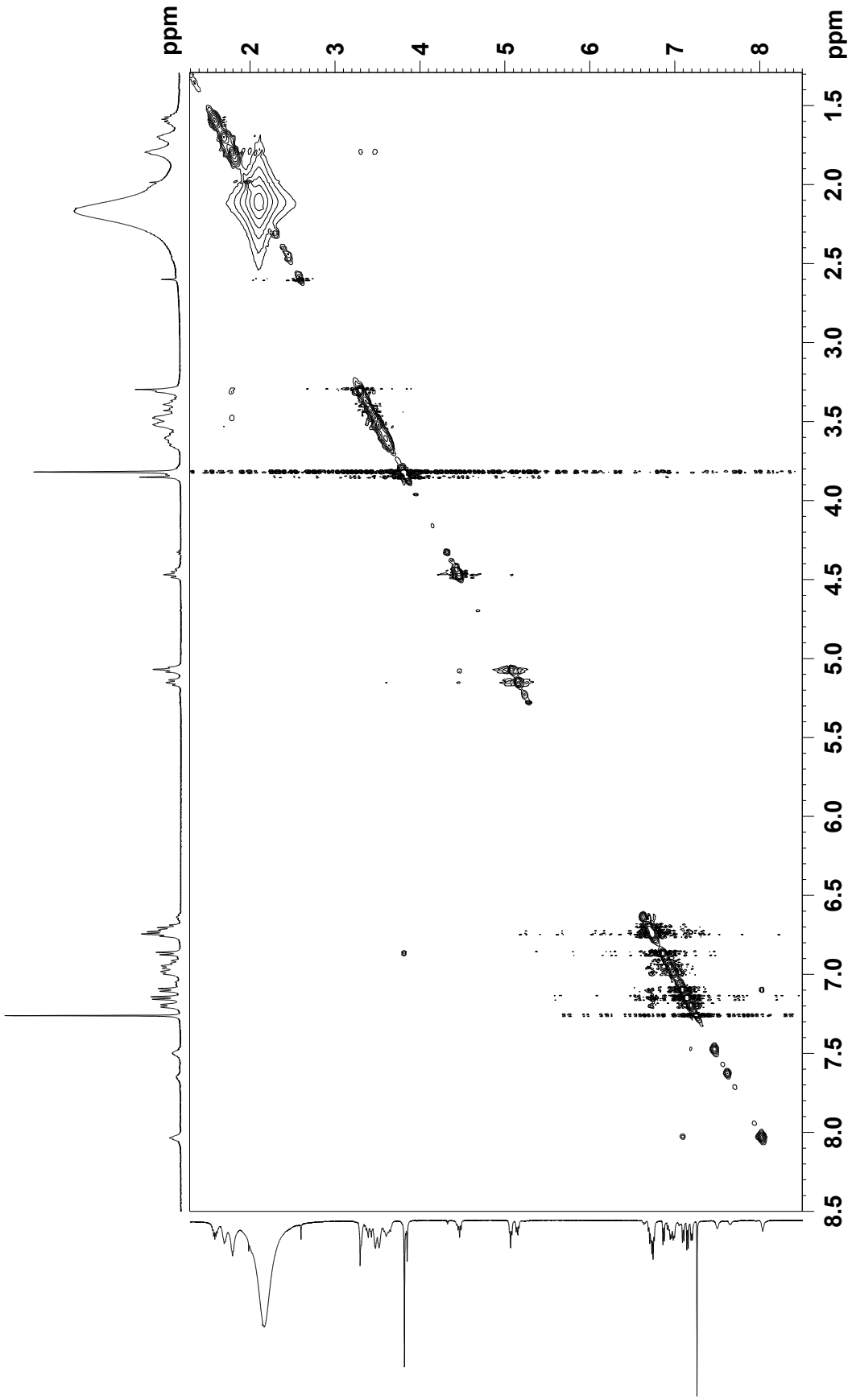


Figure S10. ^{1}H NMR spectrum of **1** (500 MHz, DMSO-d_6)

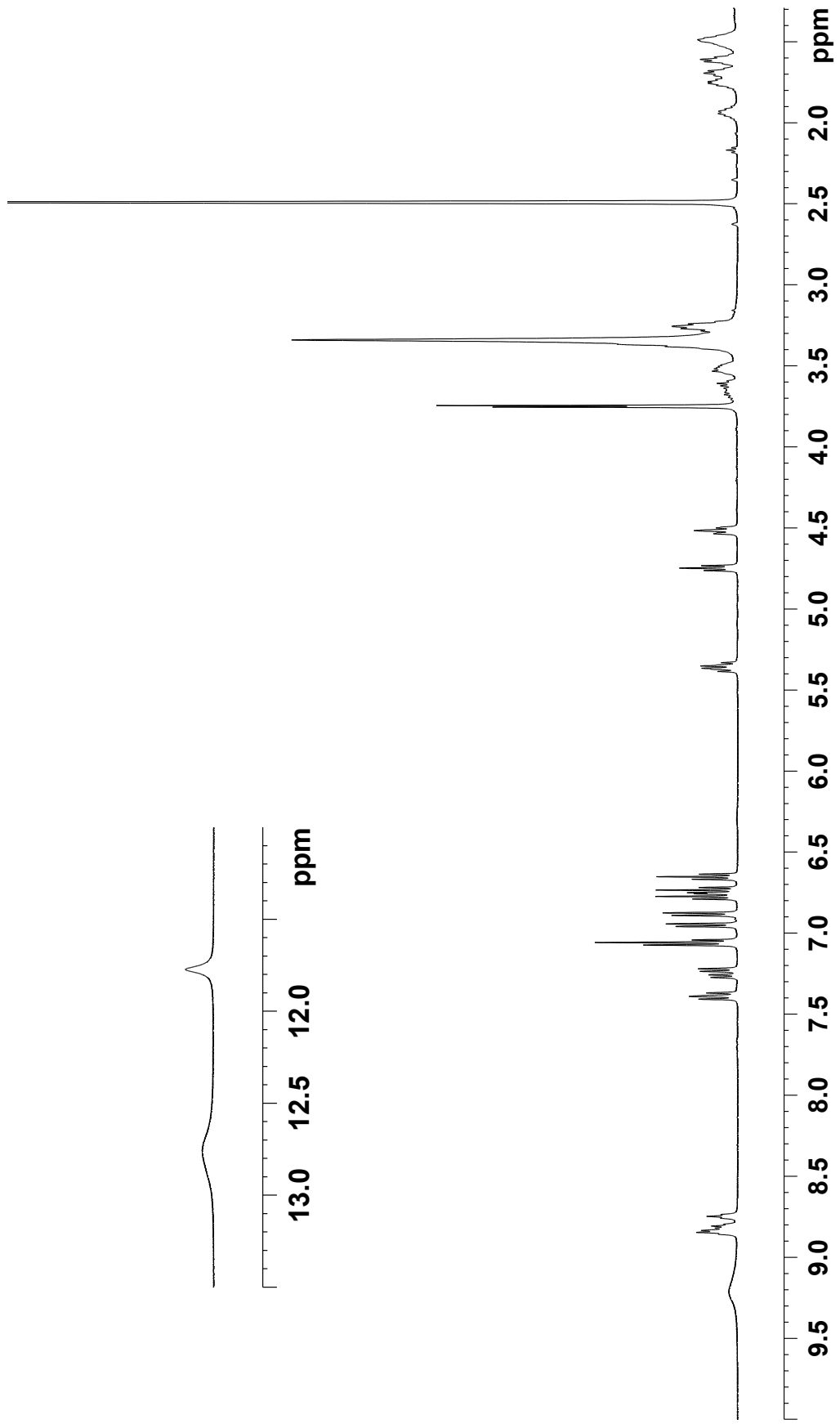


Figure S11. ^{13}C NMR spectrum of **1** (125 MHz, DMSO-d_6)

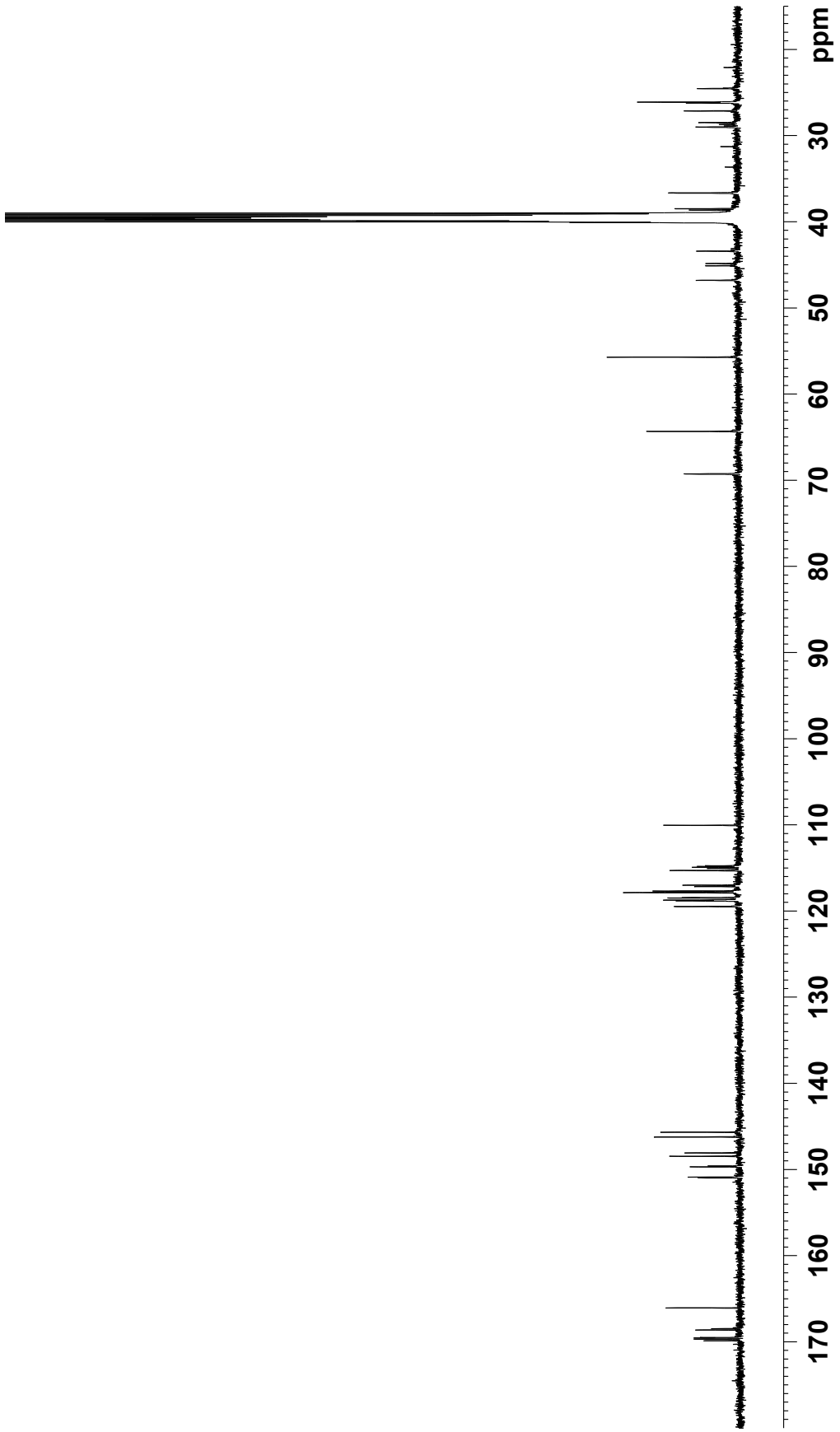


Figure S12. DEPT135 spectrum of **1** (125 MHz, DMSO-d_6)

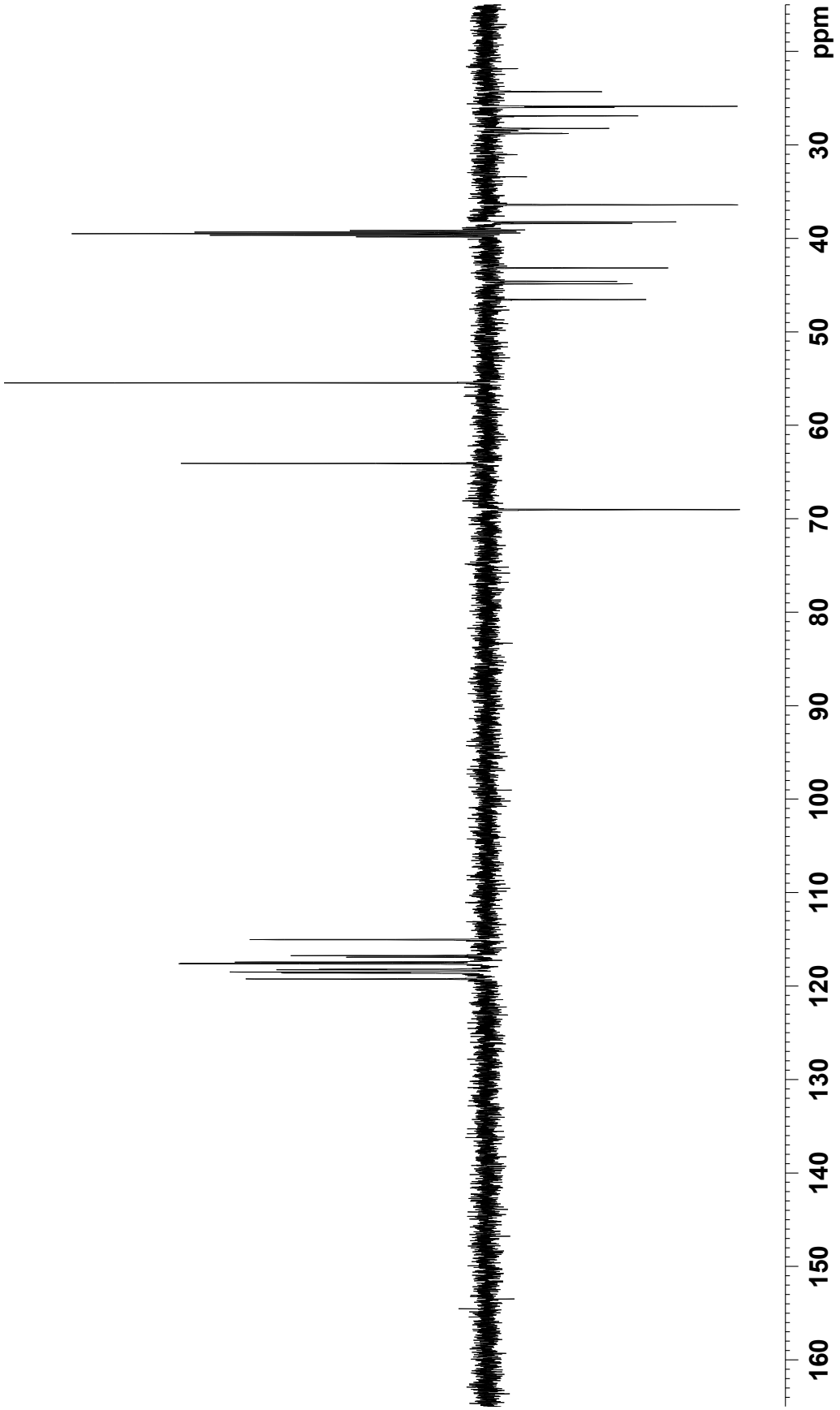


Figure S13. COSY spectrum of **1** (500 MHz, DMSO-d_6)

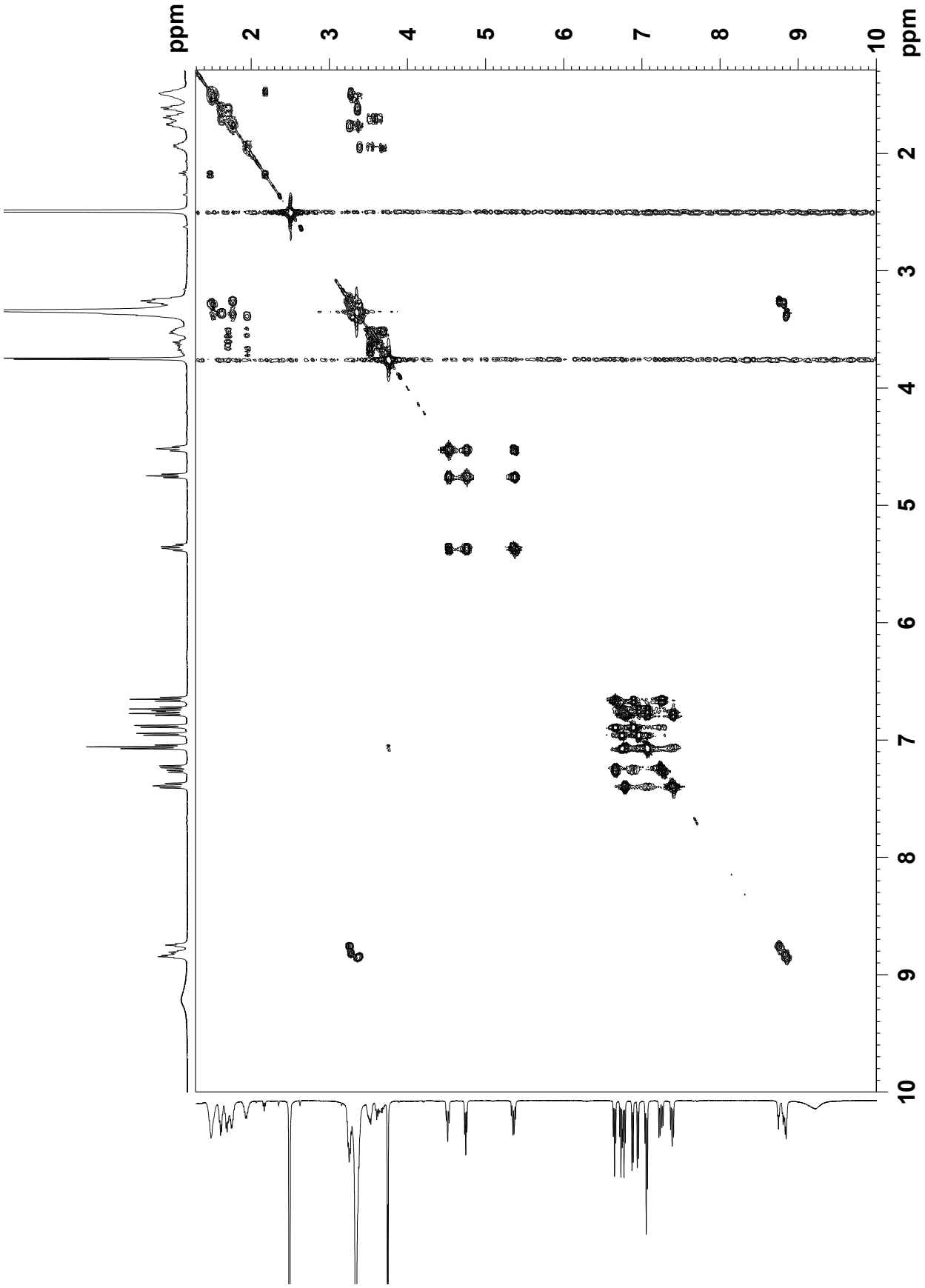


Figure S14. HSQC spectrum of **1** (500 MHz, DMSO-d_6)

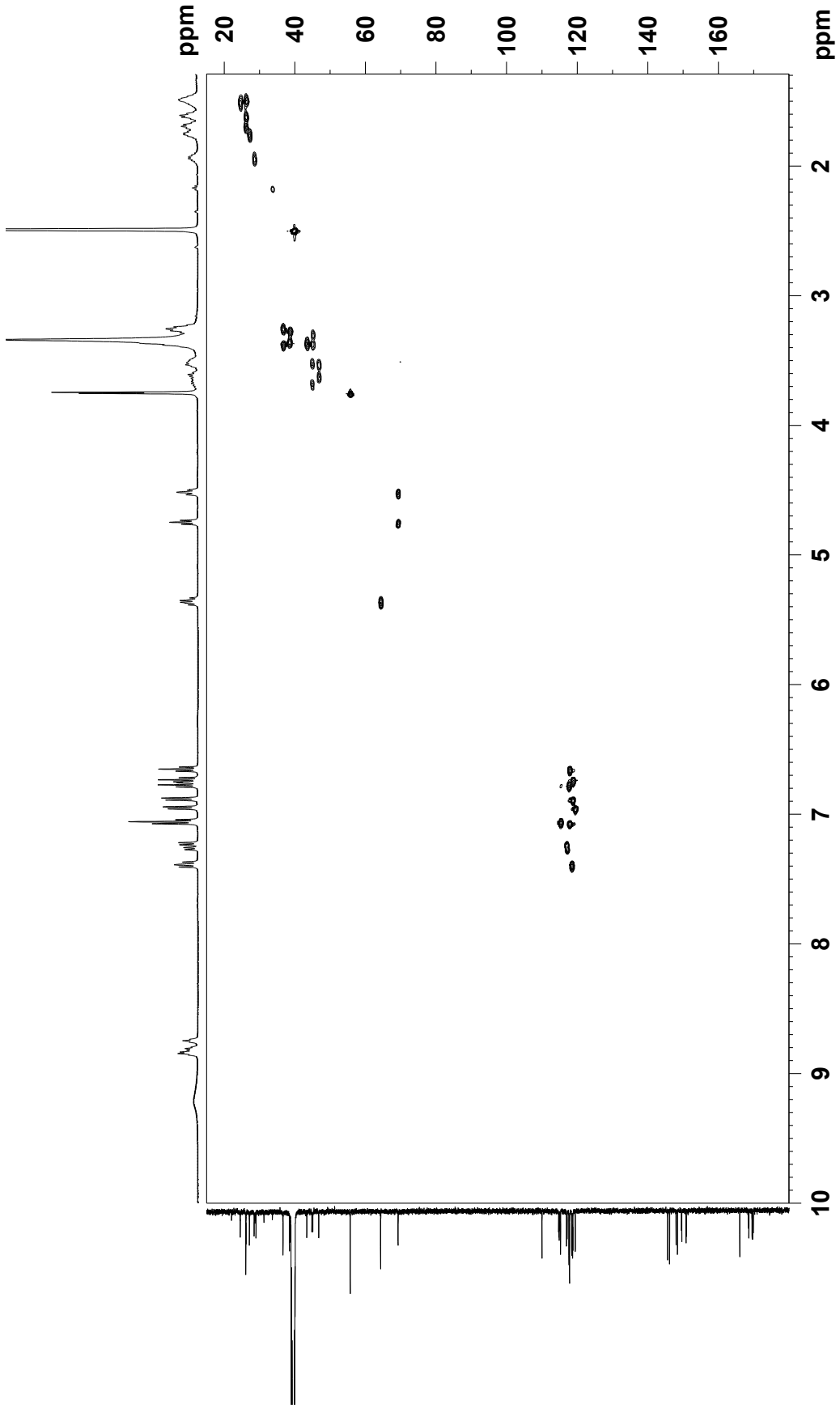
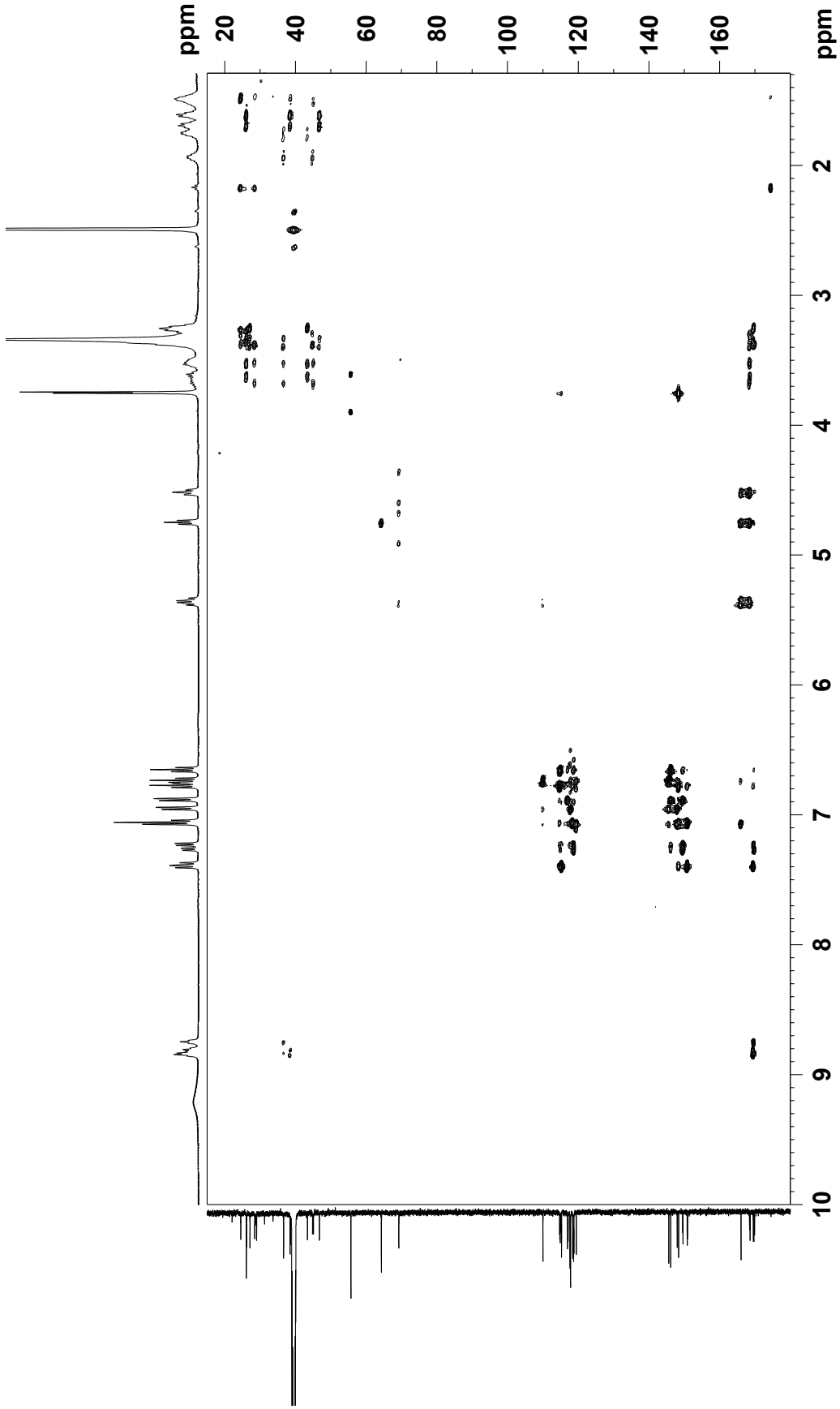


Figure S15. HMBC spectrum of **1** (500 MHz, DMSO-d_6)

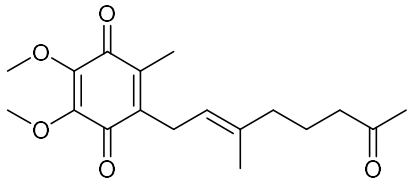


CHAPTER 4

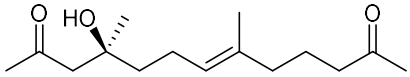
Isolation and Biosynthesis of an Unsaturated Fatty Acid with Unusual Methylation Pattern from a Coral-Associated Bacterium *Microbulbifer* sp. C4-6

4-1 Background

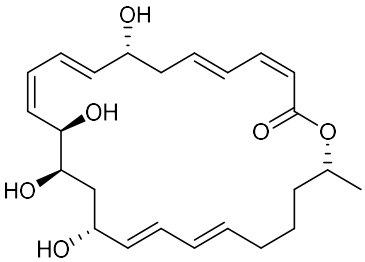
Stony corals and their associated bacteria are largely overlooked for natural product research. Although stony coral harbor diverse group of microbial community, coral-associated bacteria and their natural products have not received considerable attention [1]. To date, a handful of new natural products such as pseudoalteromones A and B [2,3], macrolactin V [4], and aqabamycins A-G [5] were discovered from soft coral-associated bacteria, but there is no report on the new compounds from stony coral-associated bacteria except our previously reported compound in chapter 3 [6] (Figure 4-1).



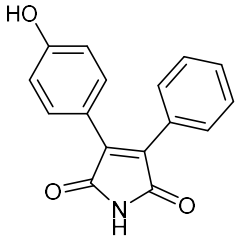
pseudoalteromone A
Pseudoalteromonas sp.
anticancer



pseudoalteromone B
Pseudoalteromonas sp.
anti-inflammatory



macrolactin V
Bacillus amyloliquefaciens
antibacterial



aquabamycin A
Vibrio sp.
antibacterial, antifungal, anticancer

Figure 4-1. Chemical structures of new bioactive compounds from bacteria associated with soft corals.

I decided to undertake HPLC-UV screening for more bacteria associated with stony corals after successful isolation and structure determination of labrenzbactin, which is the first natural product reported from stony coral-associated bacteria. As described in Chapter 3, in this study also I used HPLC-UV-guided fractionation of strain C4-6 which led to the discovery of new unsaturated fatty acid with unique methylation pattern.

HPLC-UV screening was utilized to examine the secondary metabolite production by strain C4-6. Strain C4-6 was collected from stony coral of genus *Porites* and identified as a member of genus *Microbulbifer*. Likewise, for strain C1-1, this strain also screened using three

types of seawater-based fermentation media (A3M, A11M and A16). Fermentation media were extracted with 1-butanol. Solvent extracts were analyzed by HPLC-UV, and results revealed that only A11M medium supported the metabolite production. In HPLC chromatogram, only one major peak was observed with retention time at 17.3 min and UV spectrum of the peak showed maximum absorbance at 262 nm (Figure 4-2).

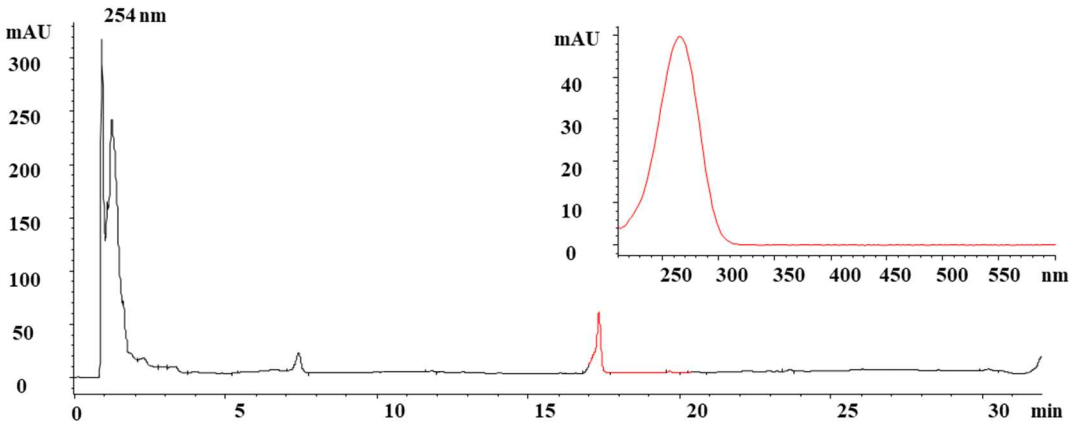


Figure 4-2. HPLC analysis of 1-butanol extract of strain *Microbulbifer* sp. C4-6.

Microbulbifer is a genus of the Gram-negative bacteria belonging to the class Gammaproteobacteria [7]. Members of this genus are frequently isolated from halophilic environments including marine solar saltern [8], marine sediments [9], and marine invertebrates [10]. The *Microbulbifer* species are considered to be marine obligate bacteria as they require sodium salt for growth [7]. The strain C4-6 was cultured on Marine Agar, YP medium in distilled agar and natural seawater. The strain C4-6 showed growth only on Marine Agar and YP medium in natural seawater, this result showed that strain C4-6 cannot grow without NaCl and thus marine obligate bacterium (Figure 4-3). To date, only two benzoate derivatives were isolated from tunicate derived *Microbulbifer* sp. [11] (Figure 4-4).

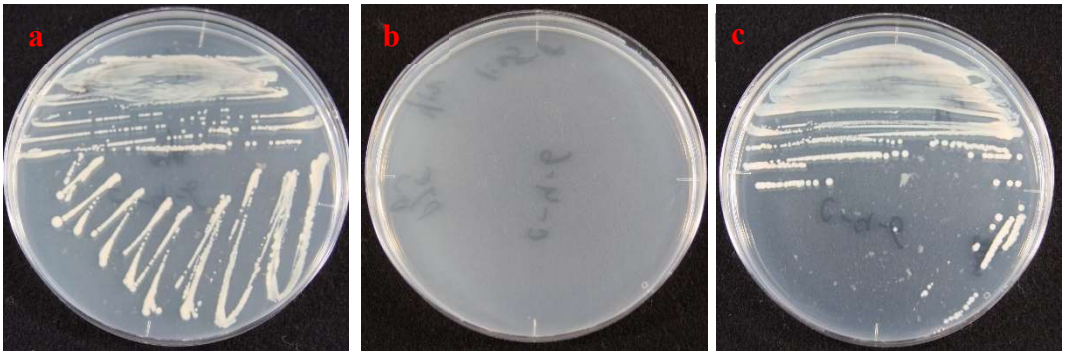


Figure 4-3. Colony morphology of *Microbulbifer* sp. C4-6 on a) Marine Agar, b) YP medium (yeast extract 0.1% and peptone 0.5%) in distilled water, c) YP medium in natural seawater.

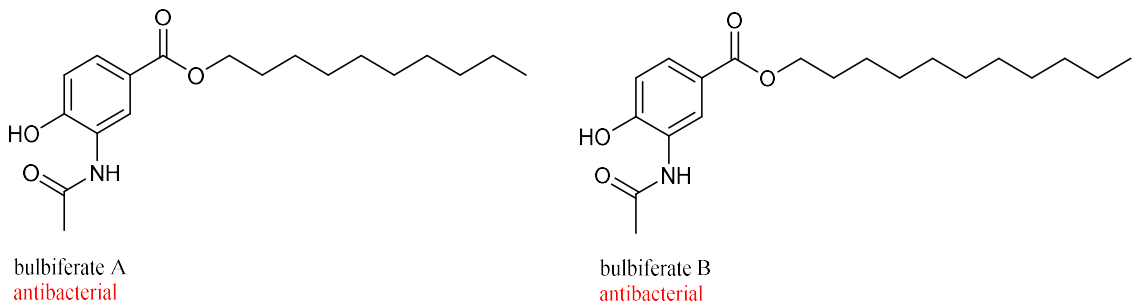


Figure 4-4. Chemical structure of bulbiferates A and B isolated from *Microbulbifer* sp.

In our continuing investigation of natural products from stony coral-associated bacteria, (2*Z*,4*E*)-3-methyl-2,4-decadienoic acid (**1**) was obtained from the culture extract of *Microbulbifer* sp. C4-6 (Figure 4-5). Compound **1** is known as a synthetic compound [12], and was used for the synthesis of a retinoid containing the alkylidene butenolide core. Alkylidene butenolides constitute the core of many natural and unnatural compounds which display a wide range of biological activities. But this is the first finding as a natural product. In addition, **1** is biosynthetically unique: it has an uncommon methylation pattern in its carbon chain, derived from the *C*-methylation with L-methionine at a carbon originated from a carbonyl carbon of acetate. In this chapter, the isolation and structure determination of **1** and its biosynthetic origin proven by the feeding experiments of ^{13}C-labeled precursors are described (Figure 4-5).

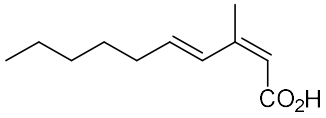
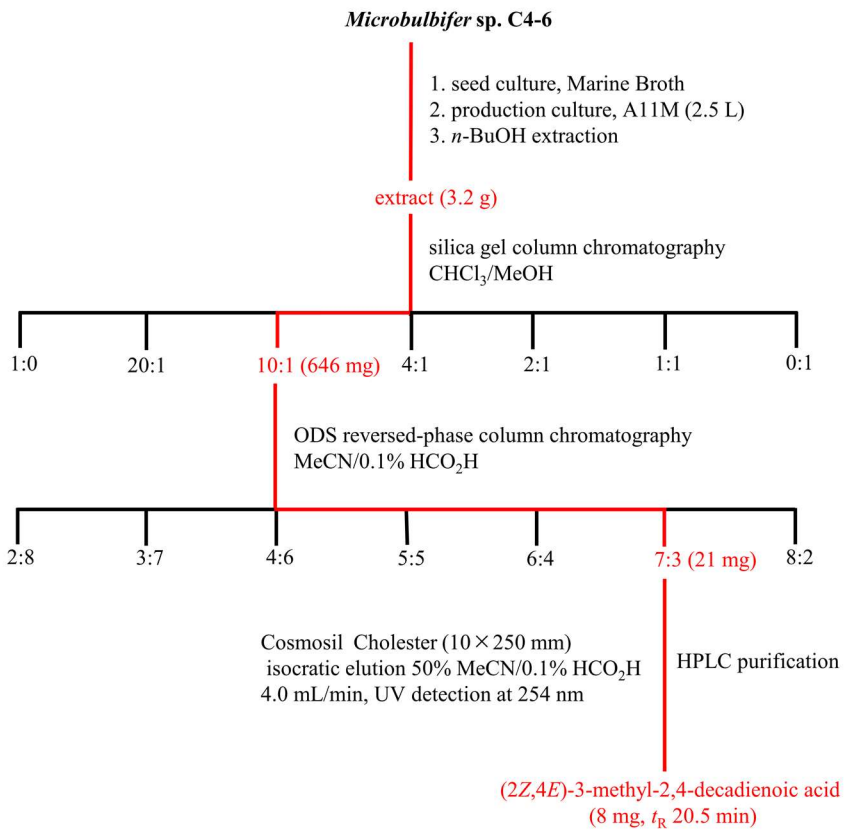


Figure 4-5. Structure of (2*Z*,4*E*)-3-methyl-2,4-decadienoic acid (**1**).

4-2 Results and Discussion

4-2-1 Fermentation and Isolation

The bacterial strain C4-6 was isolated from a Scleractinian (stony) coral *Porites* sp. On the basis of 16S rRNA gene sequence, this strain was identified as *Microbulbifer*. Strain C4-6 was cultured in A11M seawater medium at 30°C for five days, and the whole culture broth was extracted with 1-butanol. The extract was consecutively fractionated by normal- and reversed-phase column chromatographies, followed by HPLC purification to yield compound **1** (Scheme 4-1).



Scheme 4-1. Isolation scheme of (2*Z*,4*E*)-3-methyl-2,4-decadienoic acid (**1**).

4-2-2 Structure Determination

The molecular formula of **1** was determined to be $C_{11}H_{18}O_2$ with three degrees of unsaturation on the basis of its NMR and HR-ESI-TOFMS (m/z 181.1230 $[M - H]^-$; calcd for $C_{11}H_{17}O_2$, 181.1229) data. The UV spectrum of **1** in methanol exhibited an absorption maximum at 262 nm. The IR absorption bands at 1678 and 2800~3200 cm^{-1} suggested the presence of carboxyl group. The ^1H and ^{13}C NMR data of **1** (Table 4-1) showed the presence of 11 carbon signals, which were identified by the assistance of a DEPT135 spectrum as two methyls, four sp^3 methylenes, three sp^2 methines (δ_C 140.6, 127.7, 115.1), and two sp^2 non-protonated carbons including one carbonyl group (δ_C 171.3, 153.9). Besides, the ^1H NMR spectrum showed characteristic resonances, especially for two methyl groups at δ_H 2.02 (3H, s) and 0.90 (3H, t) in the highfield region and for multiple methylene signals, suggesting the presence of an alkyl chain. COSY analysis established two separate spin systems, H4/H5/H6/H7 and H8/H9/H10. These partial structures were joined by the mutual HMBC correlations between H7 and H9. Furthermore, long-range correlations from H11 to C2, C3, and C4 and from H2 to C1, C3, and C4 established the carbon connectivity among these

carbons to complete the structure of **1** (Figure 4-6). Double bond geometry was determined on the basis of NOESY analysis and $^{3}J_{HH}$ coupling constant. NOE was detected between H2 and H11 but not between H2 and H4, reflecting the *Z*-configuration for the C2-C3 double bond. Similarly, NOESY correlations for H4/H6 and H5/H11 reflected the *E*-configuration for the C4–C5 double bond, which was corroborated by $^{3}J_{HH}$ vicinal coupling constant ($J_{H4,H5}$ = 15.8 Hz).

Table 4-1. ^{1}H and ^{13}C NMR data for compound **1** in $CDCl_3$.

position	δ_C[a]	δ_H mult (*J* in Hz)[b]	HMBC[b,c]
1	171.3, C		
2	114.8, CH	5.63, s	1, 3, 4, 11
3	153.8, C		
4	127.5, CH	7.55, d (15.8)	2, 3, 6, 11
5	140.3, CH	6.22, dt (15.8, 7.2)	3, 6, 7
6	33.3, CH_2	2.24, dt (7.2, 7.2)	4, 5, 7, 8
7	28.7, CH_2	1.46, m	5, 8, 9
8	31.4, CH_2	1.32[d], m	6, 7, 10
9	22.4, CH_2	1.32[d], m	
10	13.9, CH_3	0.90, t (7.1)	8, 9
11	21.3, CH_3	2.02, s	2, 3, 4

[a]Recorded at 125 MHz (reference δ_C 77.0).

[b]Recorded at 500 MHz (reference δ_H 7.26).

[c]HMBC correlations are from proton(s) stated to the indicated carbon.

[d]Overlapping signals.

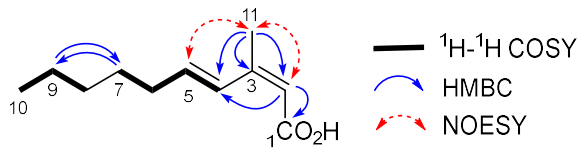


Figure 4-6. COSY and key HMBC correlations for **1**

4-2-3 Biosynthesis

In fungi and certain kinds of bacteria, methyl substituents in the fatty acid carbon chain or the polyketide chain are derived from the methyl group of *S*-adenosylmethionine (SAM) (Figure 4-7). For example, anthracimycin is a bioactive decalin-fused tricyclic macrolide isolated from *Streptomyces* species. In actinomycetes, the pendant methyl groups in polyketide chain usually derived from methylmalonyl CoA but in some cases like anthracimycin pendant methyls were dervived from L-methionine (via SAM) [13,14] (Figure 4-7A and 4-8). This

methylation reaction usually occurs at the nucleophilic carbons originated from the methyl carbon of acetate (C2) since SAM acts as an electrophilic methyl donor. In most of the bacteria including actinomycetes, methyl branching in polyketide chain is derived from methylmalonyl CoA, thereby the methylation position must be also the α-position (C2) of acetate unit [13]. For example, biosynthetic study of erythromycin, showed pendant methyl groups of 6-deoxyerythronolide B, which is a aglycone precursor of erythromycin were derived from propionates (the metabolic precursor of methylmalonate) [15] (Figure 4-7B and 4-9). In contrast, methylation at the carbons derived from the carbonyl carbon of acetate (C1) is quite unusual in fatty acids and polyketides. This unusual methylation pattern is reported for the polyketides of eukaryotic algae dinoflagellates. Some of the methyl groups in dinoflagellate compounds are positioned at the carbons derived from the carbonyl carbon of acetate (C1). Precursor labeling studies of amphidinolide B indicated that the origin of the methyl carbon is not SAM but one carbon fragment derived from the cleavage of another acetate unit [16] (Figure 4-7C and 4-10). The only single example of *C*-methylation with SAM at a carbon derived from the carbonyl carbon of acetate (C1; Figure 4-7D) is reported for sphingolipid biosynthesis in the yeast, *Pichia pastoris* [17]. SAM-dependent *C*-methylation takes place at the alkenyl carbon C9 of glucosylceramide, yielding a cationic intermediate, from which deprotonation occurs at C9 to give an internal olefin (Scheme 4-2).

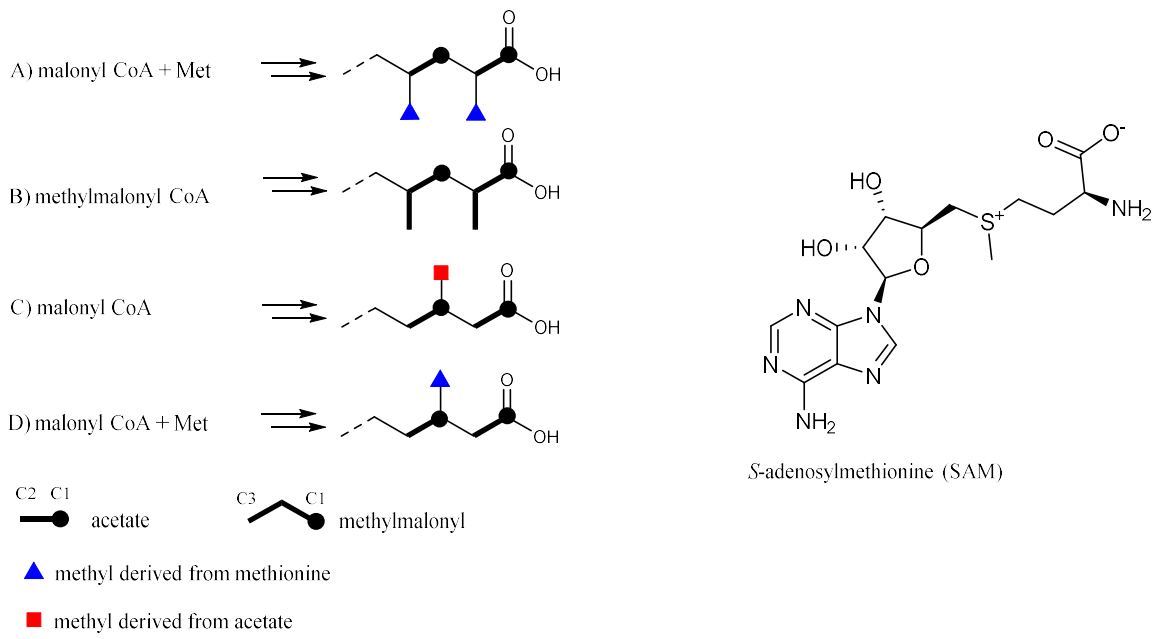


Figure 4-7. Methylation pattern that can occur in fatty acids and polyketides.

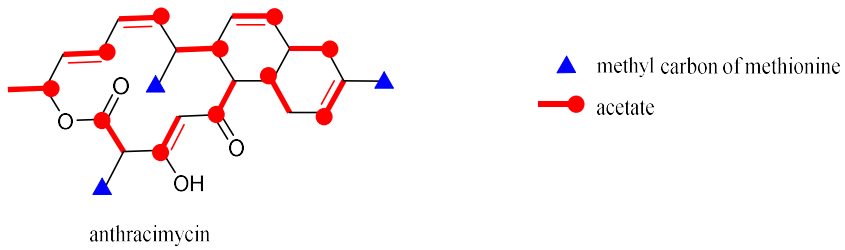


Figure 4-8. Biosynthesis of anthracimycin from *Streptomyces* sp.

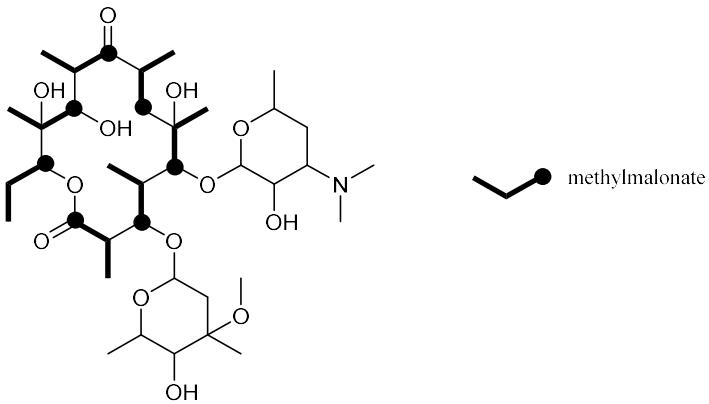


Figure 4-9. Biosynthesis of erythromycin from *Streptomyces erythraea*.

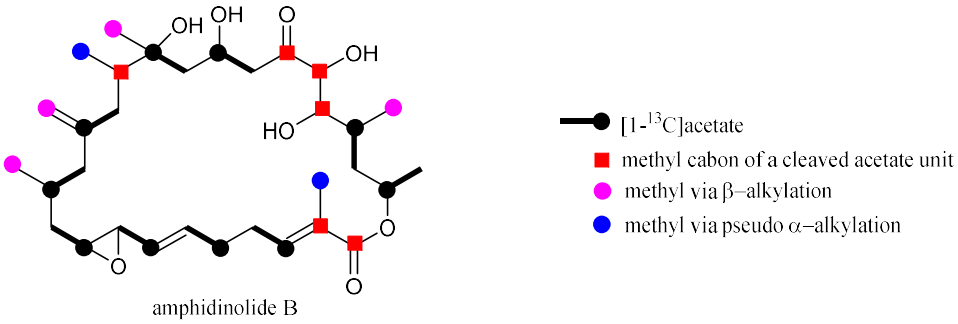


Figure 4-10. Biosynthesis of amphidinolide B from the dinoflagellate *Amphidinium* sp.

In the case of **1**, methylation at the C3 carbon is inconsistent with the regular methylation pattern that occurs in fatty acids synthesized by FAS (fatty acid synthase) or polyketides from PKS (polyketide synthase) system of bacterial group. Therefore, the biosynthetic origin of **1** was investigated by feeding experiments of ^{13}C-labeled precursors (Table 4-2). In order to elucidate the biosynthetic origin and incorporation pattern, producing strain C4-6 was cultured in the presence of plausible biosynthetic precursors labeled with carbon-13, namely [1-^{13}C]acetate, and L-[*methyl*-^{13}C]methionine. Firstly, in order to verify the origin of the carbon backbone, [1-^{13}C]acetate was fed to the culture which gave the high enrichment of the carbons at C1, C3, C5, C7, and C9 (Figure 4-11 and 12). Feeding experiment with L-[*methyl*-

^{13}C]methionine was then carried out. The high level of enrichment was observed only for C11 in the ^{13}C NMR spectrum, thereby confirming that the methyl group is derived from methionine via SAM (Figure 4-11 and 12). In addition to the fungal sphingolipids described above, methyl branches with SAM-origin are also found in some bacterial fatty acids like tuberculostearic acid, a major constituent of mycobacterial membrane phospholipids [18]. Methylation onto the olefinic double bond of oleic acid part in phospholipids is catalyzed by SAM-dependent methyltransferase, followed by 1,2-hydride shift and deprotonation, and a subsequent reduction of *exo*-methylene intermediate gives rise to a methyl group [19] (Scheme 4-3). The presence of the *exo*-methylene intermediate was experimentally proved but the enzyme responsible for the double bond reduction has not been identified [19]. Based on the *C*-methylation pattern found in the biosynthesis of sphingolipid and tuberculostearic acid, we propose a possible mechanism of methylation for compound **1** although further chemical/biochemical investigation is required (Scheme 4-4). First, 2,4-decadienoic acid is formed and methylation takes place at C3 to give a putative cationic intermediate. The following proton loss from C3 is likely probable to give directly **1** in a similar fashion to the glucosylceramide biosynthesis. Branched chain fatty acids are commonly present in cell membranes, and mono-methylation at the *iso* or *anteiso* position is the most common [20]. Branching at other positions is less common but represents a significant proportion of branched-chain fatty acids in some organisms [20]. Tuberculostearic acid (10-methyloctadecanoic acid (18:1me(10)), 10-methylhexadecanoic acid (16:1me(10)), and 10-methylnonadecanoic acid (19:1me(10)) are the major constituents of the cell wall of *Mycobacterium phlei* [21]. C9-Methylated glucosylceramides also possess a methyl-branching in the middle of the aliphatic carbon chain of the sphingosine part. C9-Methylated sphingolipids have not been found from plants and animals but are widely produced by many fungi and are thought to play an important role in the interaction between fungi and their host organisms. C9-Methylated sphingolipids are also found from some marine invertebrates such as sea anemone and starfish although their biological function is still unclear in these organisms. *C*-Methylation at C9 of fungal sphingosines is similarly catalyzed by SAM-dependent methyltransferases. The methylation takes place at the alkenyl carbon (C9) which is originally derived from the carbonyl carbon of an acetate unit: C1 and C2 of sphingosines are derived from L-serine and carbons from C3 to C18 are from palmitoyl-CoA. To the best of our knowledge, compound **1** is the first example of a simple fatty acid in which the carbonyl carbon of an acetate unit is methylated with SAM (Figure 4-7D).

Table 4-2. Incorporation of ^{13}C-labeled precursors into **1**.

		relative enrichment[a]	
position	δ_C[b]	[1-^{13}C]acetate	L-[*methyl*-^{13}C]-methionine
1	171.3	**8.99**	1.10
2	114.8	0.91	1.12
3	153.8	**10.13**	0.84
4	127.5	1.00	1.00
5	140.3	**11.19**	0.99
6	33.3	1.15	1.12
7	28.7	**11.35**	1.12
8	31.4	1.19	1.20
9	22.4	**12.01**	1.13
10	13.9	1.37	1.17
11	21.3	1.20	**17.74**

[a]The ^{13}C signal intensity of each peak in the labeled **1** divided by that of the corresponding signal in the unlabeled, normalized to the peak area of C4 to give an enrichment ratio for enriched peak. The numbers in bold type indicate ^{13}C-enriched atoms from ^{13}C-labeled precursors.

[b]Recorded at 125 MHz (reference δ_C 77.0).

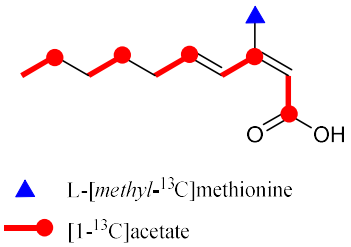


Figure 4-11. Incorporation of ^{13}C-labeled precursors into **1**.

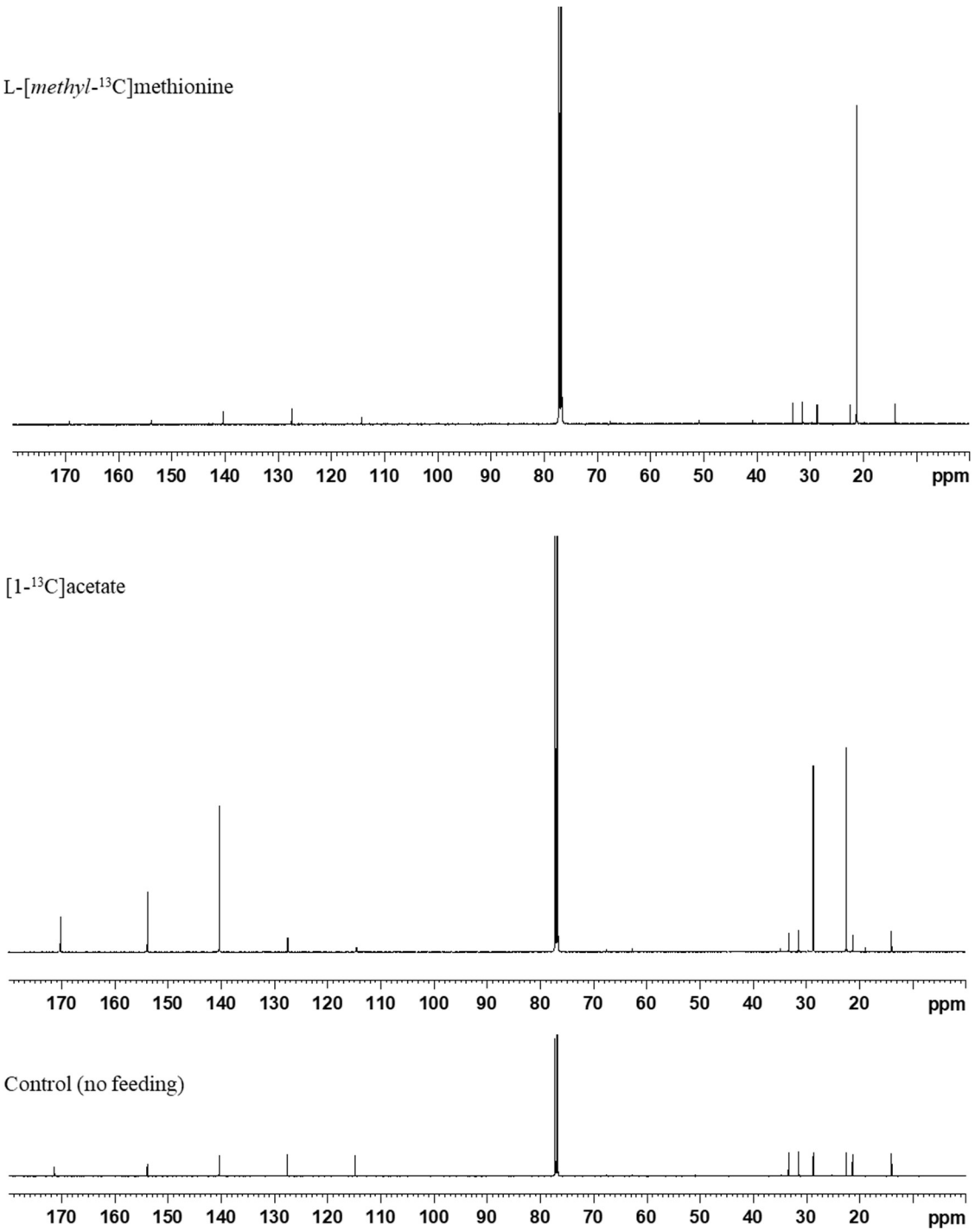
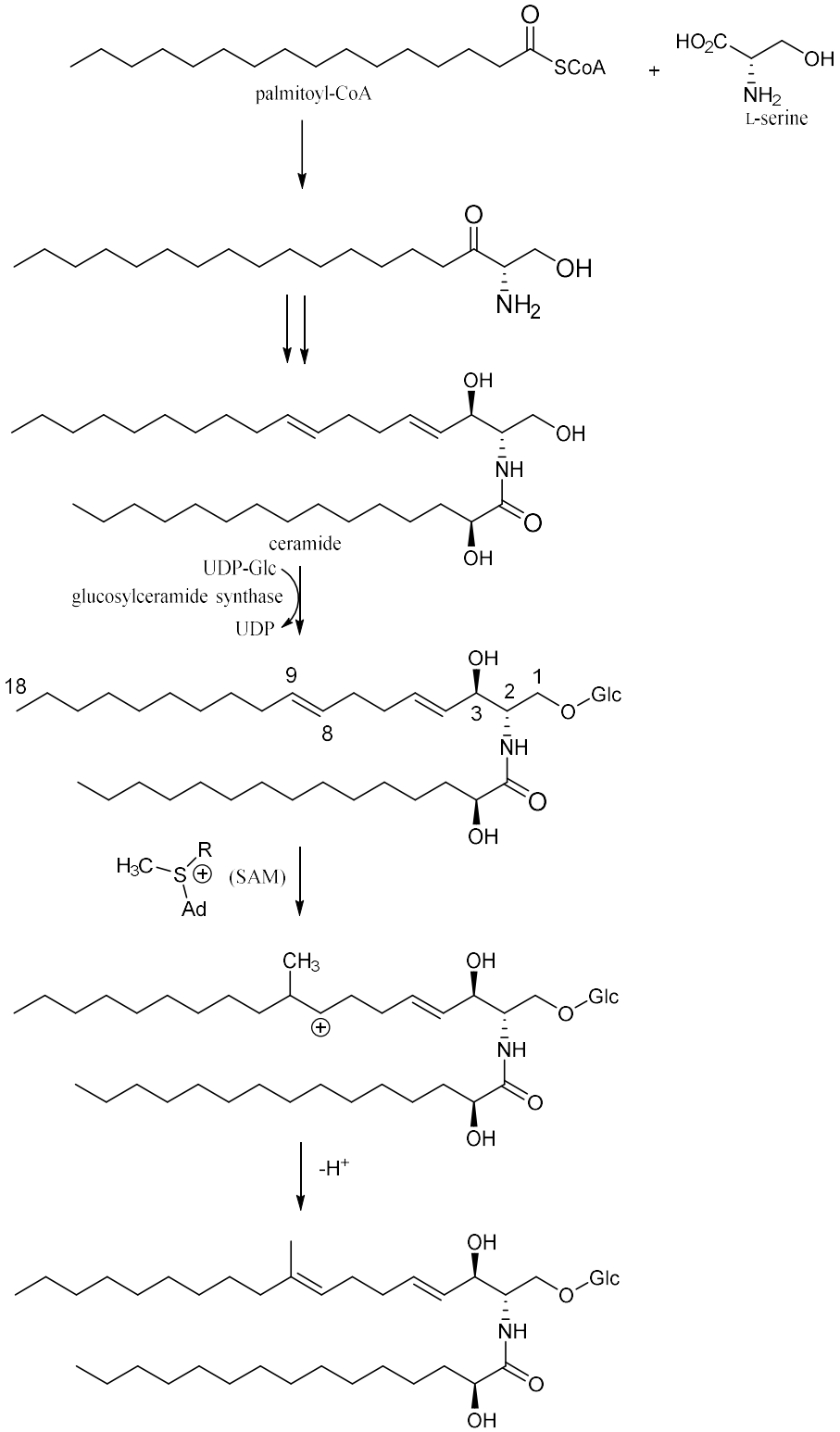
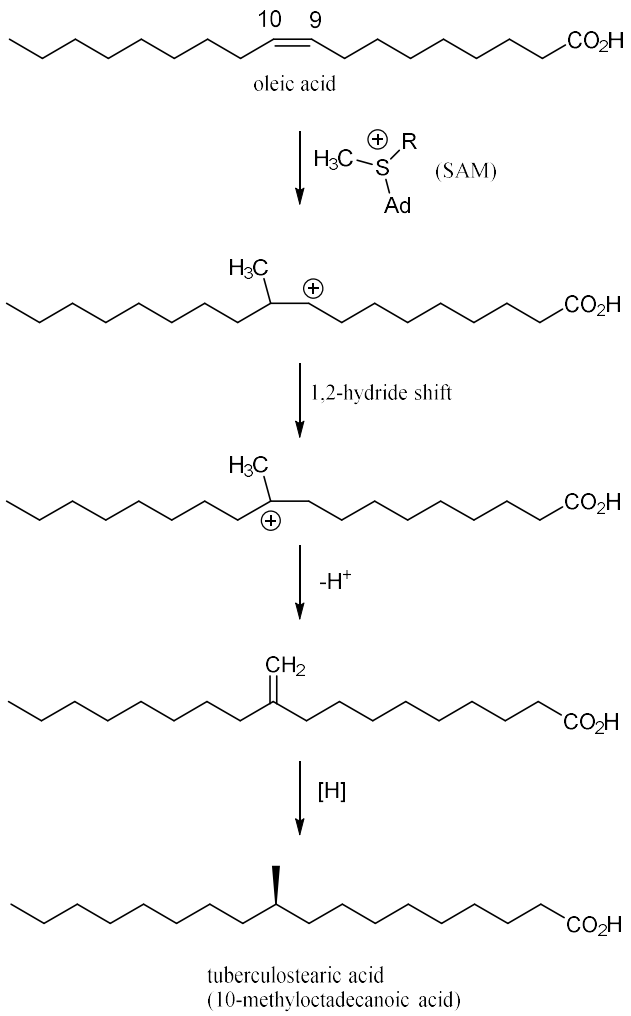


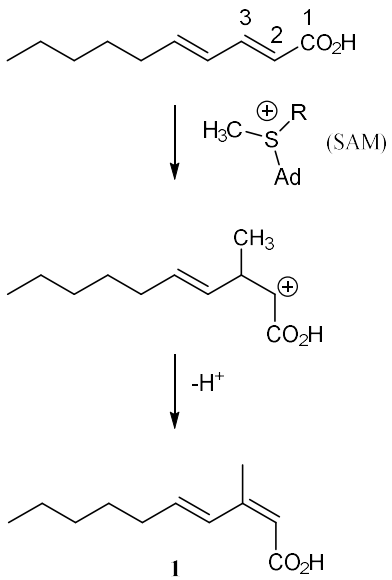
Figure 4-12. ^{13}C NMR Spectra of **1** labeled with [1-^{13}C]acetate and L-[*methyl*-^{13}C]methionine (125 MHz, $CDCl_3$).



Scheme 4-2. *C*-Methylation of alkenyl carbon in sphingolipid biosynthesis.



Scheme 4-3: Biosynthesis of tuberculostearic acid in *Mycobacterium* sp.



Scheme 4-4. Possible methylation mechanism for compound **1**.

4-2-4 Bioactivity

Compound **1** was not active against *Staphylococcus aureus* FDA209P JC-1, *Micrococcus luteus* ATCC9341, *Bacillus subtilis* ATCC6633, *Escherichia coli* NIHJ JC-2, *Ralstonia solanacearum* SUPP1541, *Rhizobium radiobacter* NBRC14554, and *Candida albicans* NBRC0197 (MIC >100 μg/mL) but weakly active against *Saccharomyces cerevisiae* S100 (MIC 100 μg/mL).

4-3 Conclusion

In summary, chemical investigation of *Microbulbifer* sp. associated with stony coral *Porites* sp. led to the discovery of a unique unsaturated fatty acid, (2Z,4*E*)-3-methyl-2,4-decadienoic acid (**1**) in which the carbon originated from the carbonyl carbon of an acetate unit is methylated, providing a quite rare case of *C*-methylation pattern in fatty acid/polyketide biosynthesis. Compound **1** and its (2*E*,4*E*)-isomer were reported previously as synthetic compounds but this is the first finding of **1** as a natural product [12,22]. In general, bacterial FAS catalyze the transfer of a methyl group from SAM to an inactivated double bond of a lipid chain, followed by deprotonation of the newly attached methyl group to form compound like **1**. Chemoinformatics insight into functional groups present in 186,000 molecules where natural products producing organisms were identified showed that carboxyl group conjugated with double bond type functional group like in compound **1** occurred only in 1.30% of molecules [23]. This results also showed that such functional group is not common in natural products. In addition, the presence of the pendant methyl group in odd number of carbon of fatty acid showed unique pattern of methylation occurred in compound **1**.

To date, benzoate derivatives are reported from a tunicate-derived *Microbulbifer* [11]. Therefore, this is the second report on the small molecule from this underexplored taxon. According to the genome sequence database, biosynthetic genes for NRPS and siderophore are present in *Microbulbifer* species, which offer researchers to utilize bacteria of this genus to quest for new bioactive compounds in the future.

The phylum Proteobacteria is a potential source of novel bioactive compounds amongst marine bacteria but have received considerably less attention than actinobacteria. Proteobacteria in marine environment usually found in the association with corals, sponges and mollusks. Within the phylum Proteobacteria, the classes of Alpha-, Gamma-, Beta-, Delta-, and Epsilonproteobacteria comprise 32, 13, 2, 2 and 0.4% of all bacteria in the water column,

respectively [24]. Gammaproteobacteria is one of the most diverse group of bacteria and a rich source of marine natural products with well-established genus *Pseudoalteromonas, Vibrio* and *Pseudomonas*. Although members of the Alphaproteobacteria seem to be the most abundant in marine environments, Gammaproteobacteria is the most successfully cultured [24]. But total number of natural products from this class of bacteria is relatively small [25-35] (Figure 4-13). Fewer number of bioactive compounds have been discovered from marine Proteobacteria than actinobacteria and fungi, possibly because these have not been studied extensively for their capability to produce secondary metabolites or they do not possess biosynthetic genes as commonly as Gram-positive bacteria.

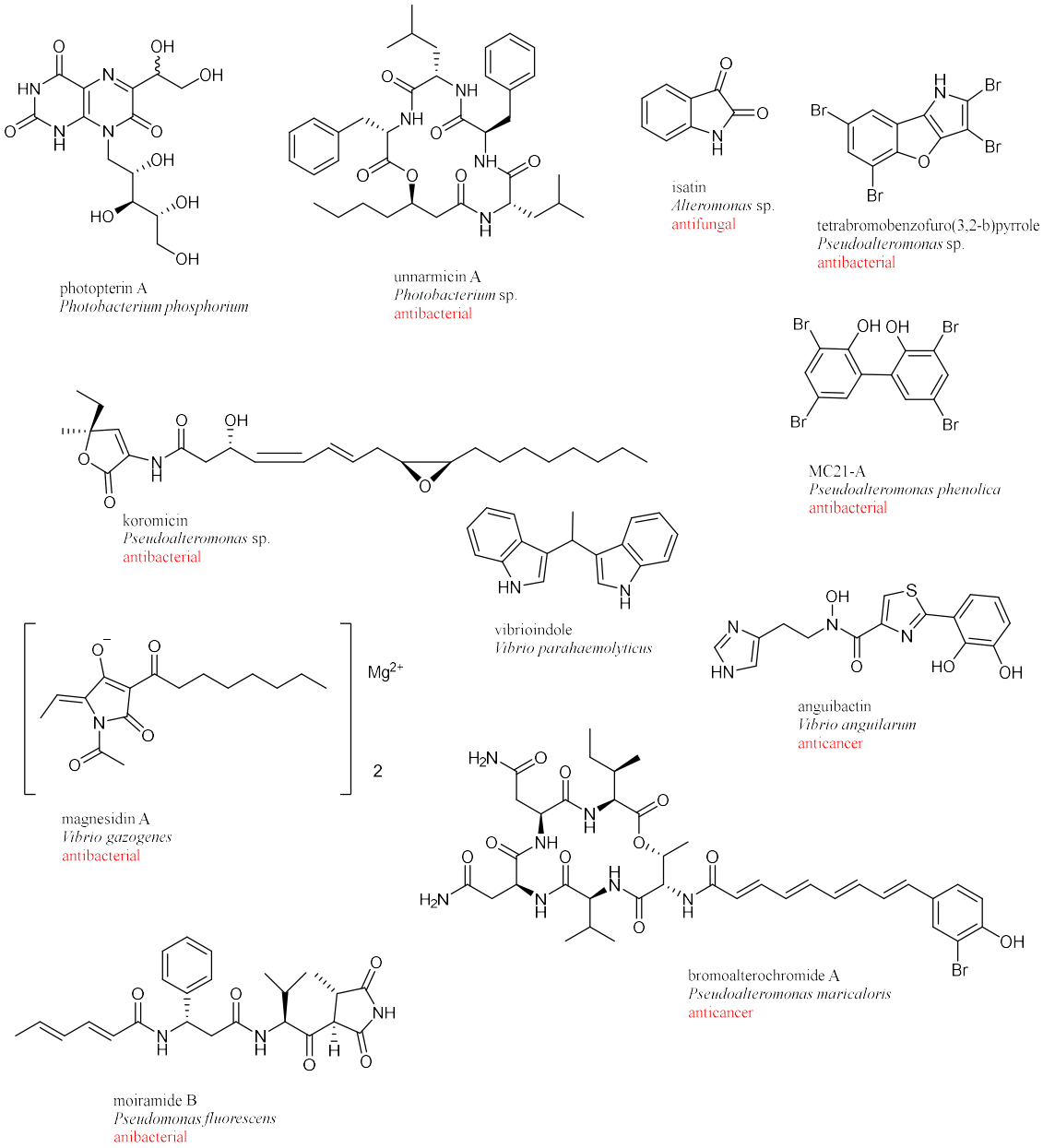


Figure 4-13. Structures of compounds from marine Gammaproteobacteria.

Fatty acids are carboxylic acids with a long aliphatic alkyl chains and they are ubiquitous in nature. Marine fatty acids are obtained from a variety of sources, including marine fishes, sponges, corals, sea cucumbers, limpets, and microorganisms. In past decades, marine derived unsaturated fatty acids have attracted considerable interest because of their structural diversity, potential biological activities and nutritional functions [36]. Although bacteria are one of the predominant microbiota of marine environments, only handful of fatty acids derivatives were reported from them [37-40] (Figure 4-14).

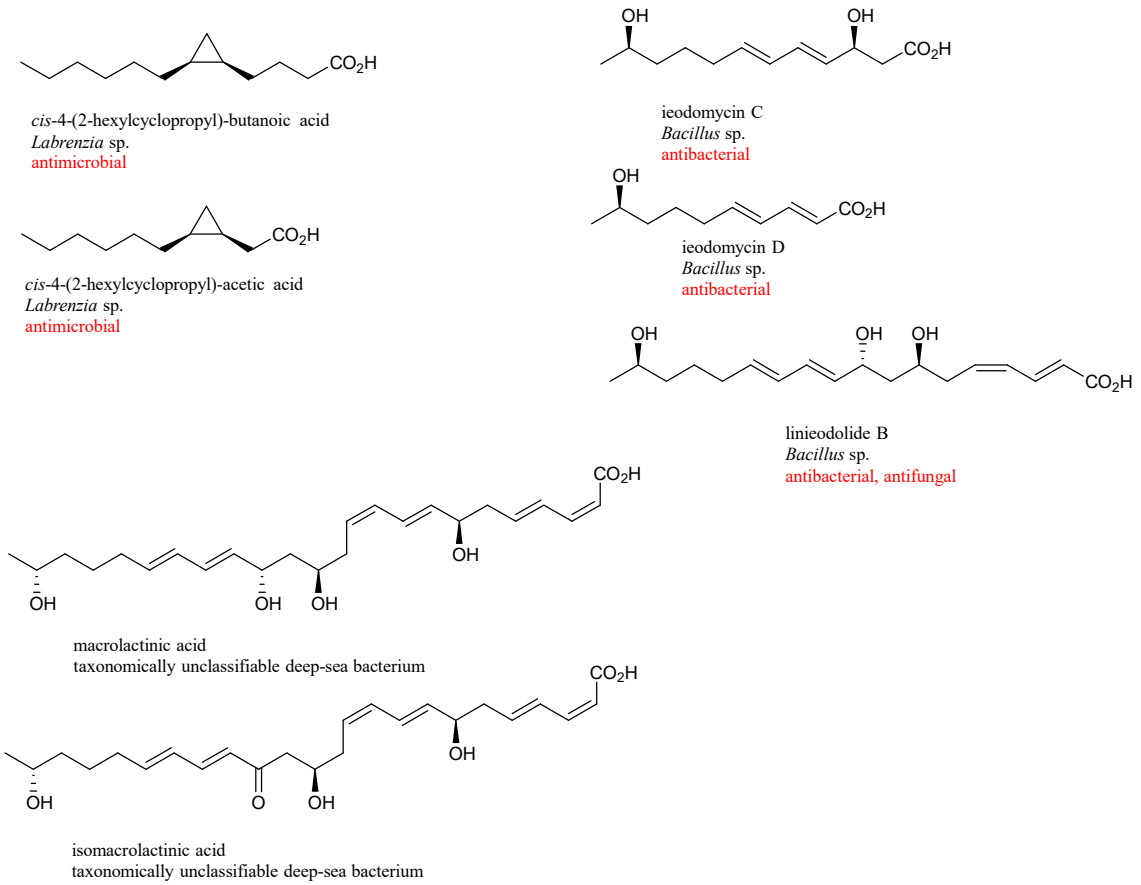


Figure 4-14. Structures of fatty acid derivatives from marine derived bacteria.

4-4 Experimental Section

General Experimental Procedures. The UV spectrum was recorded on a Shimadzu UV-1800 spectrophotometer. The IR spectrum was measured on a Perkin-Elmer Spectrum 100. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer in $CDCl_3$ using the signals of the residual solvent proton (δ_H 7.26) and carbon (δ_C 77.0) as internal standards. HR-ESI-TOFMS were recorded on a Bruker micrOTOF focus. Sodium [1-^{13}C]acetate was purchased from Cambridge Isotope Laboratories, Inc., and L-[*methyl*-^{13}C]methionine from Sigma-Aldrich Co. LLC.

Microorganism. Strain C4-6 was isolated from a cultured stony coral (*Porites* sp.) obtained from an aquarium vendor in Nagasaki, Japan. The coral specimen was washed with 70% ethanol and then washed with sterile natural seawater. A fragment of the coral (*ca* 1 g) was homogenized by mortar and pestle with an equal amount of sterile natural seawater (1 mL), and 10-fold serial dilution was carried out up to 10^{-5} and 0.1 mL of each dilution was spread onto Marine Agar 2216 (Difco). The plates were kept at 23°C, and a single colony was repeatedly transferred onto the same agar medium to obtain the pure isolate of strain C4-6. The strain was identified as a member of genus *Microbulbifer* on the basis of 99.0% similarity in the 16S rRNA gene sequence (1405 nucleotides; DDBJ accession number LC456787) to *Microbulbifer variabilis* Ni-2088T (accession number AB167354).

Fermentation. The producing strain C4-6 was maintained on Marine Agar 2216 (Difco). A single colony of strain C4-6 was inoculated into a 500 mL K-1 flask containing 100 mL of Marine Broth 2216 (Difco) as a seed culture. The seed culture was incubated at 30°C on a rotary shaker at 200 rpm for 2 days. Three mL of seed culture was inoculated into twenty-five 500 mL K-1 flasks each containing 100 mL of A11M production medium, which consists of glucose 0.2%, soluble starch 2.5%, yeast extract 0.5%, polypeptone (Wako Pure Chemical Industries, Ltd.) 0.5%, NZ-amine (Wako Pure Chemical Industries, Ltd.) 0.5%, CaCO3 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1% in natural seawater (collected from Toyama Bay, Toyama, Japan). The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated at 30°C on a rotary shaker at 200 rpm for 5 days.

Extraction and Isolation. After fermentation, 100 mL of 1-butanol was added to each production culture flask, and the flasks were allowed to shake for 1 h on a rotary shaker at 200 rpm. The mixture was centrifuged at 6000 rpm for 10 min in order to separate organic layer and aqueous layer. The organic layer was concentrated in vacuo to afford 3.2 g of extract from 2.5 L of production culture. The extract was fractionated by silica gel column chromatography with a step gradient of $CHCl_3$-MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). Fraction 3 (10:1) was concentrated to give a brown oil (646 mg), which was then fractionated by reversed-phase ODS column chromatography with a gradient of MeCN-0.1% HCO_2H (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). Fraction 6 (7:3) was concentrated and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentered to give a semi-pure material (21 mg). Final purification was achieved by preparative HPLC (Cosmosil Cholester 5 μm, 10 x 250 mm, 4 mL/min, UV detection at 254 nm) with an isocratic elution of MeCN-0.1%

HCO_2H solution (50:50) to yield (2*Z*,4*E*)-3-methyl-2,4-decadienoic acid (**1**, 8.0 mg, t_R 20.5 min).

(2Z,4*E*)-3-Methyl-2,4-decadienoic acid (**1**): colorless amorphous solid; UV (MeOH) λ_{max} (log ε) 262 (4.16) nm; IR (ATR) ν_{max} 2952, 2923, 2852, 2585, 1678, 1662 cm^{-1}; ^1H and ^{13}C NMR data, see Table 4-1; HR-ESI-TOFMS *m/z* 181.1230 $[M - H]^-$ (calcd for $C_{11}H_{17}O_2$, 181.1229).

Feeding Experiment. Feeding experiments were carried out using ^{13}C-labeled precursors, sodium [1-^{13}C]acetate and L-[*methyl*-^{13}C]methionine. The fermentation, extraction and purification of labeled compounds were performed in the same manner as describe for the unlabeled compound. After 24 h of the inoculation into the production medium from the seed culture, supplementation of ^{13}C-labeled precursors was commenced and carried out for four times at 24 h interval. After further 24 h incubation, the whole culture broth was extracted with 1-butanol.

1) Sodium [1-^{13}C]acetate: ^{13}C-Labeled **1** (1.6 mg) was obtained from 1 L of culture supplemented with sodium [1-^{13}C]acetate (total 800 mg; 20 mg × 10 flasks × 4 days). The ^{13}C NMR spectrum showed enriched signals at δ 170.2, 153.8, 140.3, 28.7, and 22.4.

2) L-[*Methyl*-^{13}C]methionine: ^{13}C-Labeled **1** (0.9 mg) was obtained from 1 L of culture supplemented with L-[*methyl*-^{13}C]methionine (total 80 mg; 2.0 mg × 10 flasks × 4 days). The ^{13}C NMR spectrum showed an enriched signal at δ 21.3.

Antimicrobial Assay. Antimicrobial activity was evaluated by the liquid microculture method using round-bottomed 96-well microtiter plates against six bacteria, *Bacillus subtilis* ATCC6633, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* FDA209P JC-1, *Ralstonia solanacearum* SUPP1541, *Rhizobium radiobacter* NBRC14554, *Escherichia coli* NIHJ JC-2, and two yeasts *Candida albicans* NBRC0197 and *Saccharomyces cerevisiae* S100 as indication strains. Tryptic Soy Broth (Difco) and Sabouraud Dextrose Broth (Difco) were used for bacteria and yeasts, respectively. Compound 1 and reference drugs, kanamycin sulfate for bacteria and amphotericin B for yeasts, were made in 2-fold dilution series along the longer side of the plates by sequential transfer of 100 μL aliquots between the adjacent wells, to which the same amount of medium was pre-dispensed. To each well was added a 100 μL suspension of the indication strains prepared at 0.5 McFarland (~10^8 cfu/mL) from a culture at the logarithmic growth phase. The solvent vehicle added to the top rows was set at the 0.5% of the final culture volume to avoid the effect on the growth of microbes. The plates were incubated

for 48 h at 37°C for bacteria and at 32°C for yeasts. The tests were done in triplicate and the MIC values were read from the lowest drug concentrations at which no growth was observed.

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4-5 Spectral Data

Table of Contents

Figure S1. UV spectrum of (2*Z*,4*E*)-3-methyl-2,4-decadienoic acid (**1**)

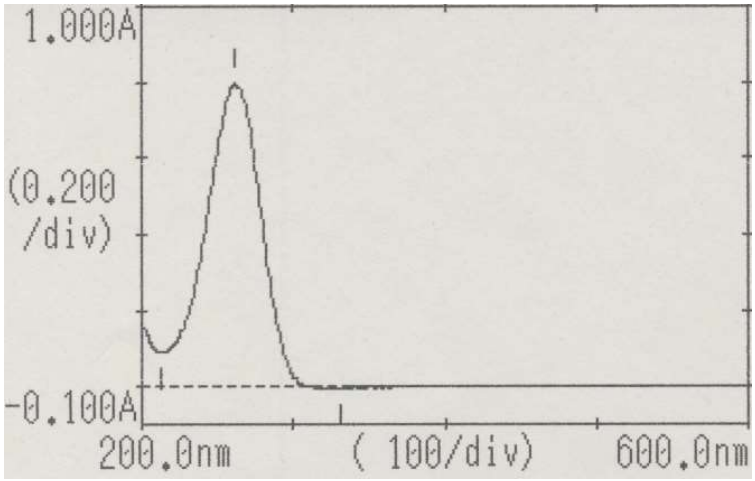


Figure S2. High resolution ESI-TOF mass spectrum of **1**

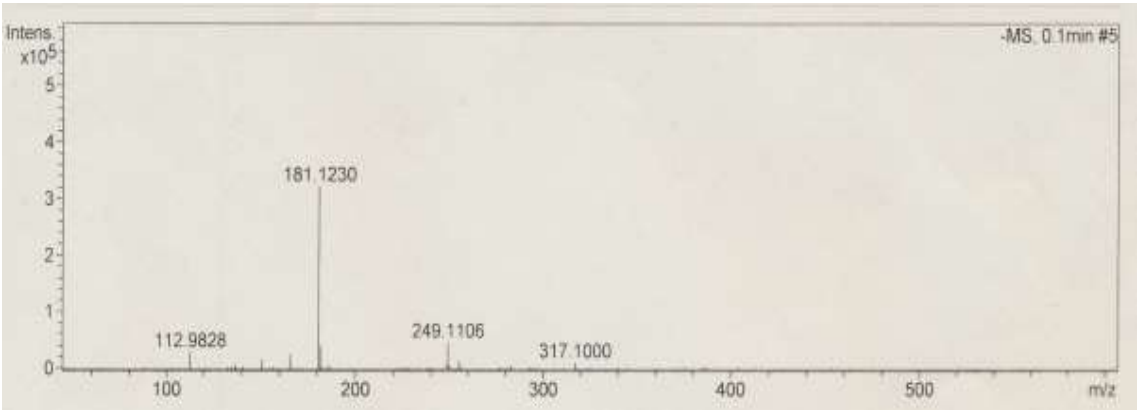


Figure S3. IR spectrum of **1**

(ATR)

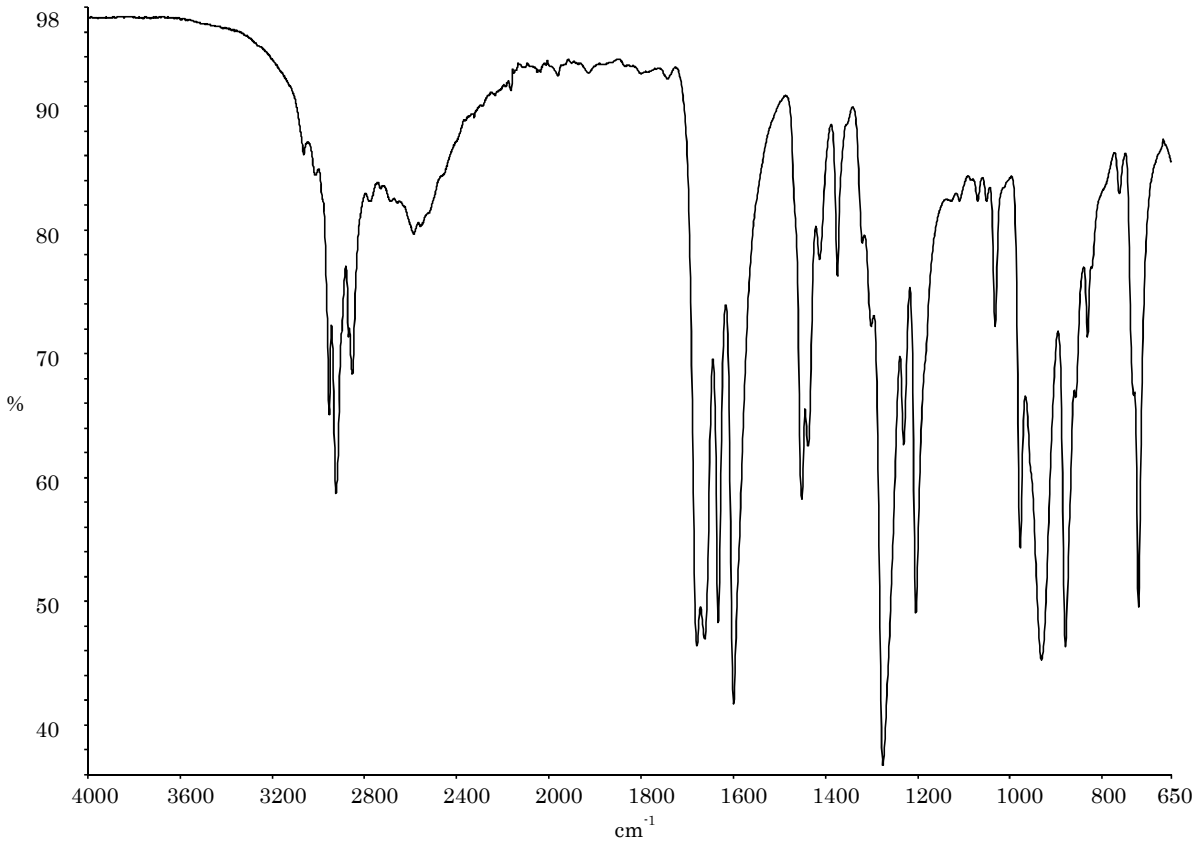


Figure S4. ^{1}H NMR spectrum of **1** (500 MHz, $CDCl_3$)

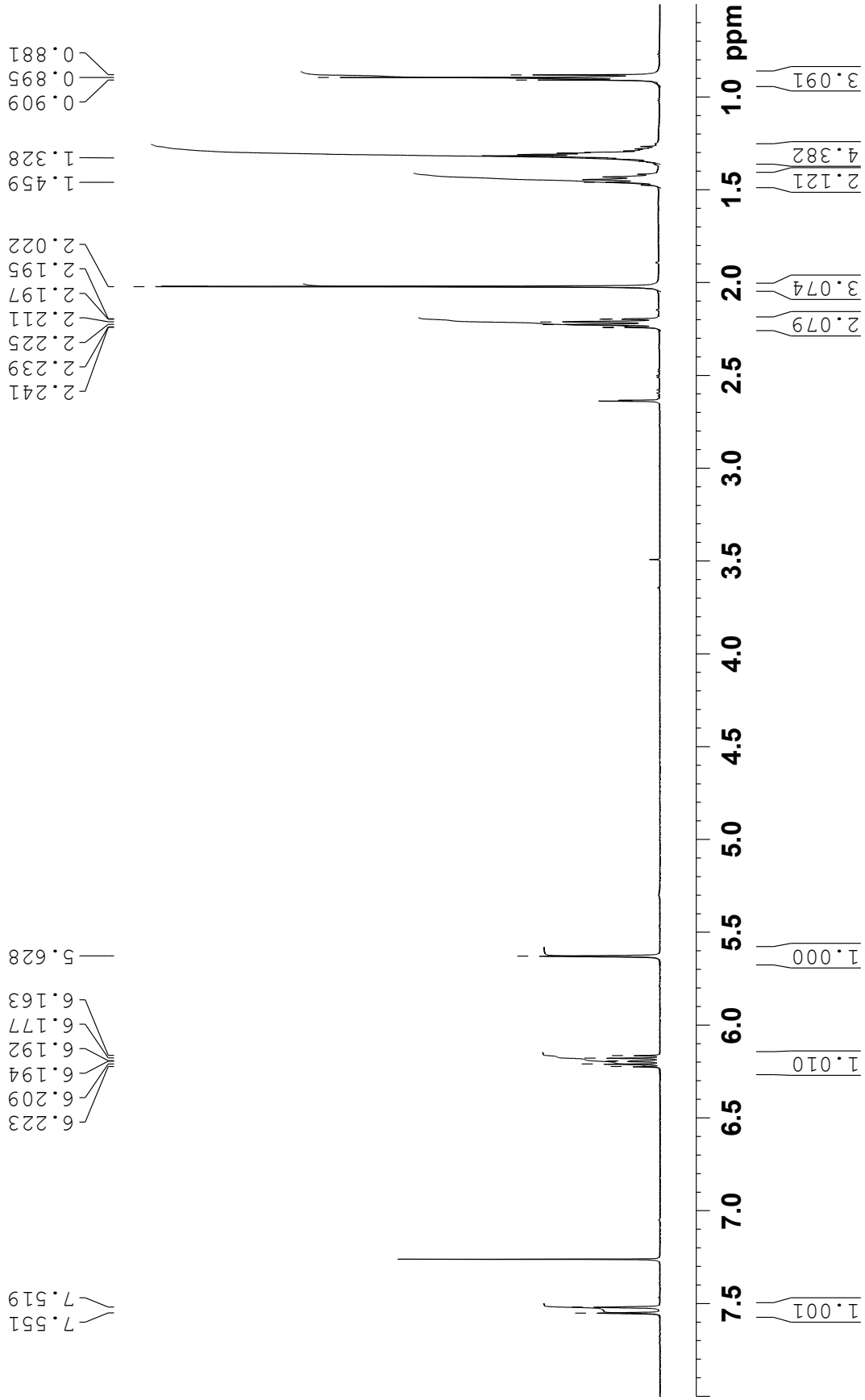


Figure S5. ^{13}C NMR spectrum of **1** (125 MHz, $CDCl_3$)

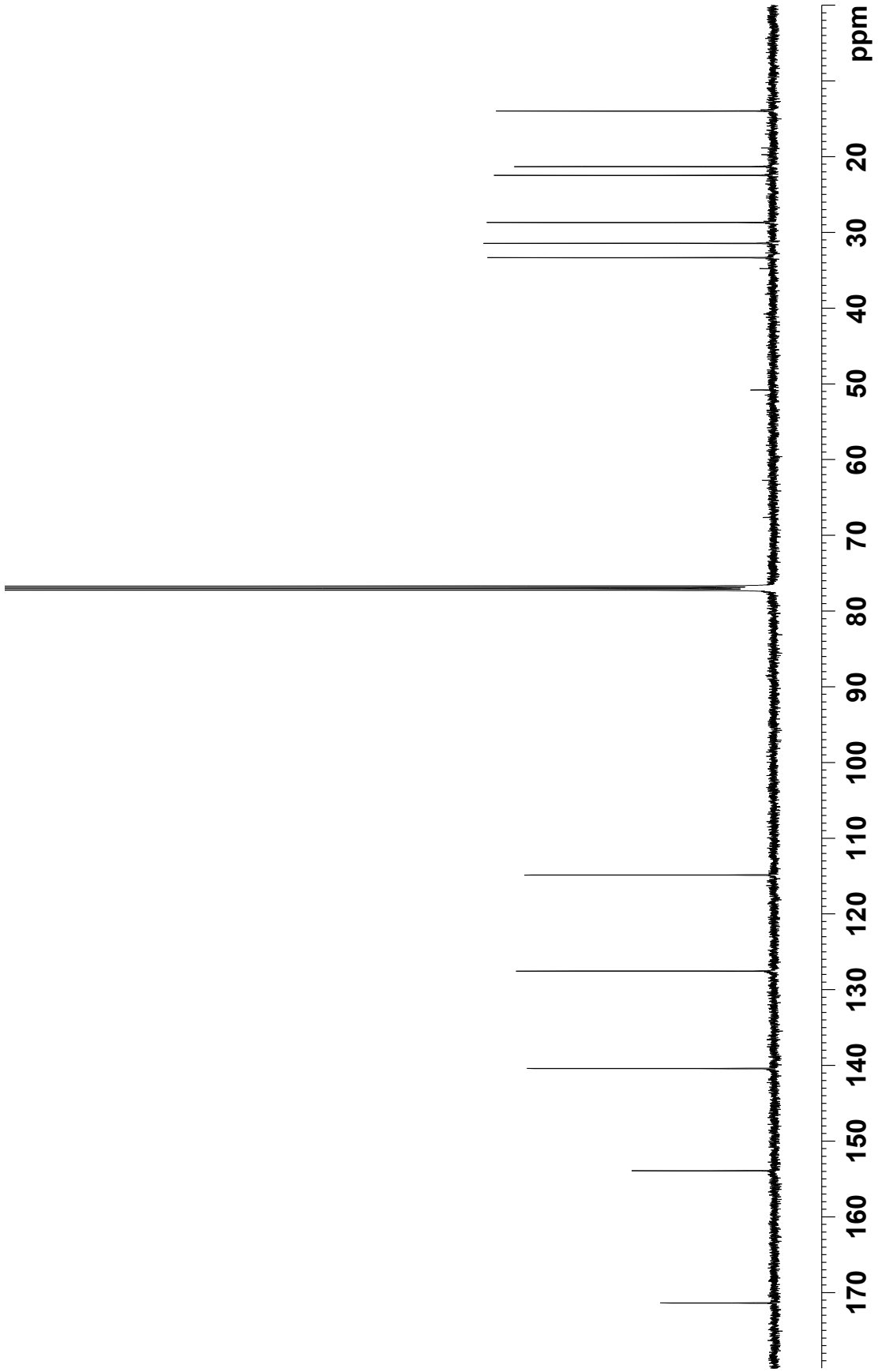


Figure S6. DEPT135 spectrum of **1** (125 MHz, $CDCl_3$)

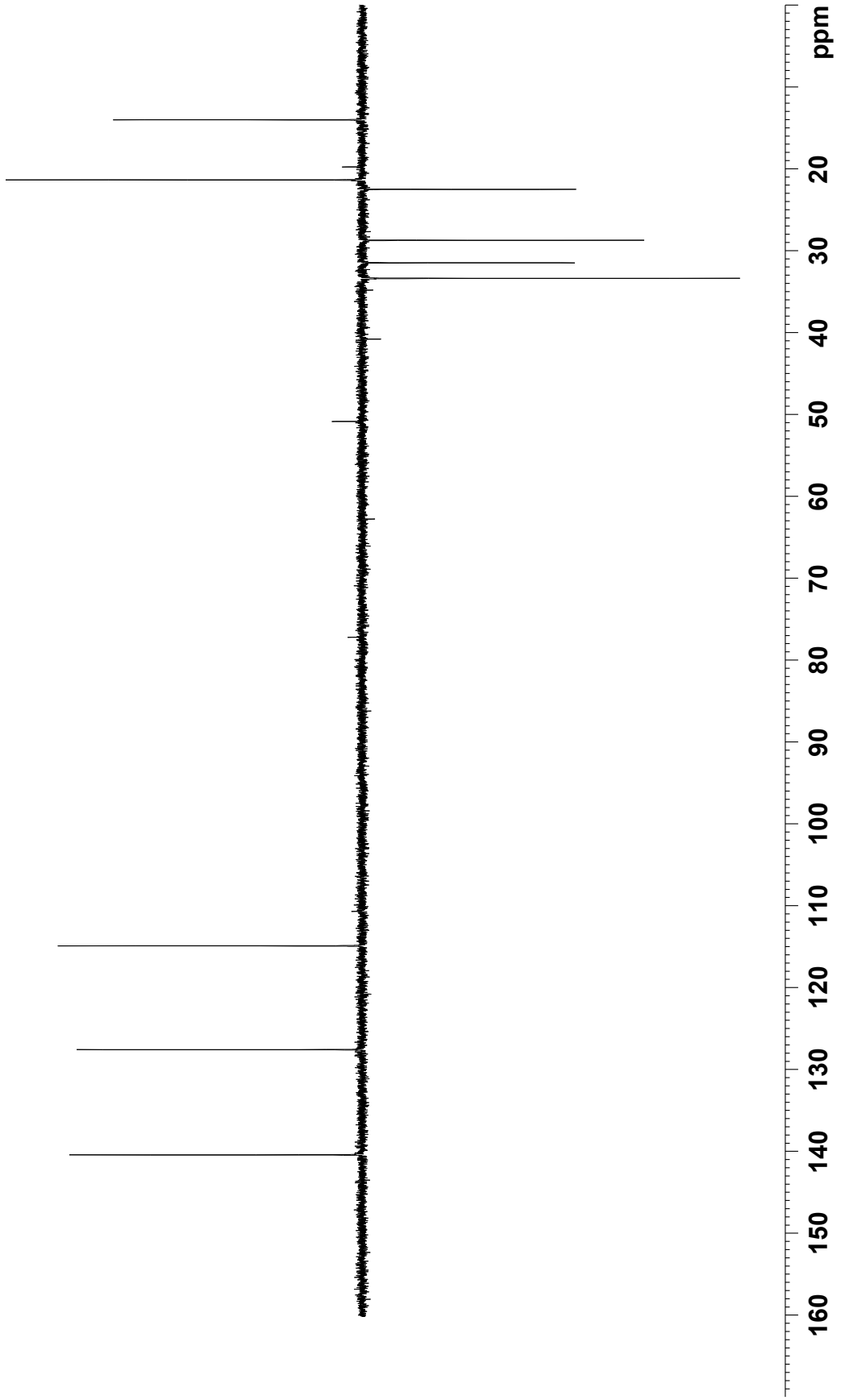


Figure S7. COSY spectrum of **1** (500 MHz, $CDCl_3$)

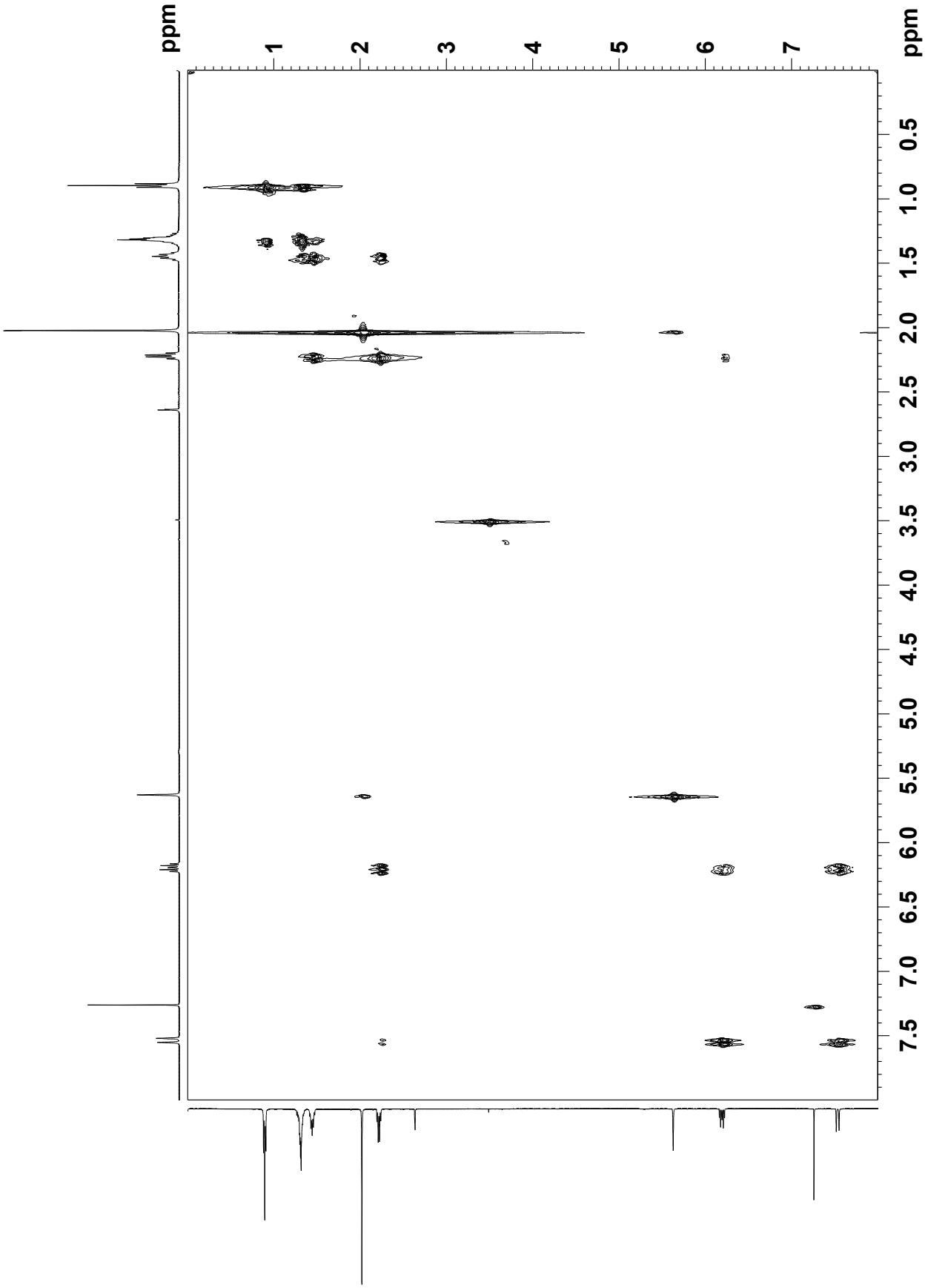


Figure S8. HSQC spectrum of **1** (500 MHz, $CDCl_3$)

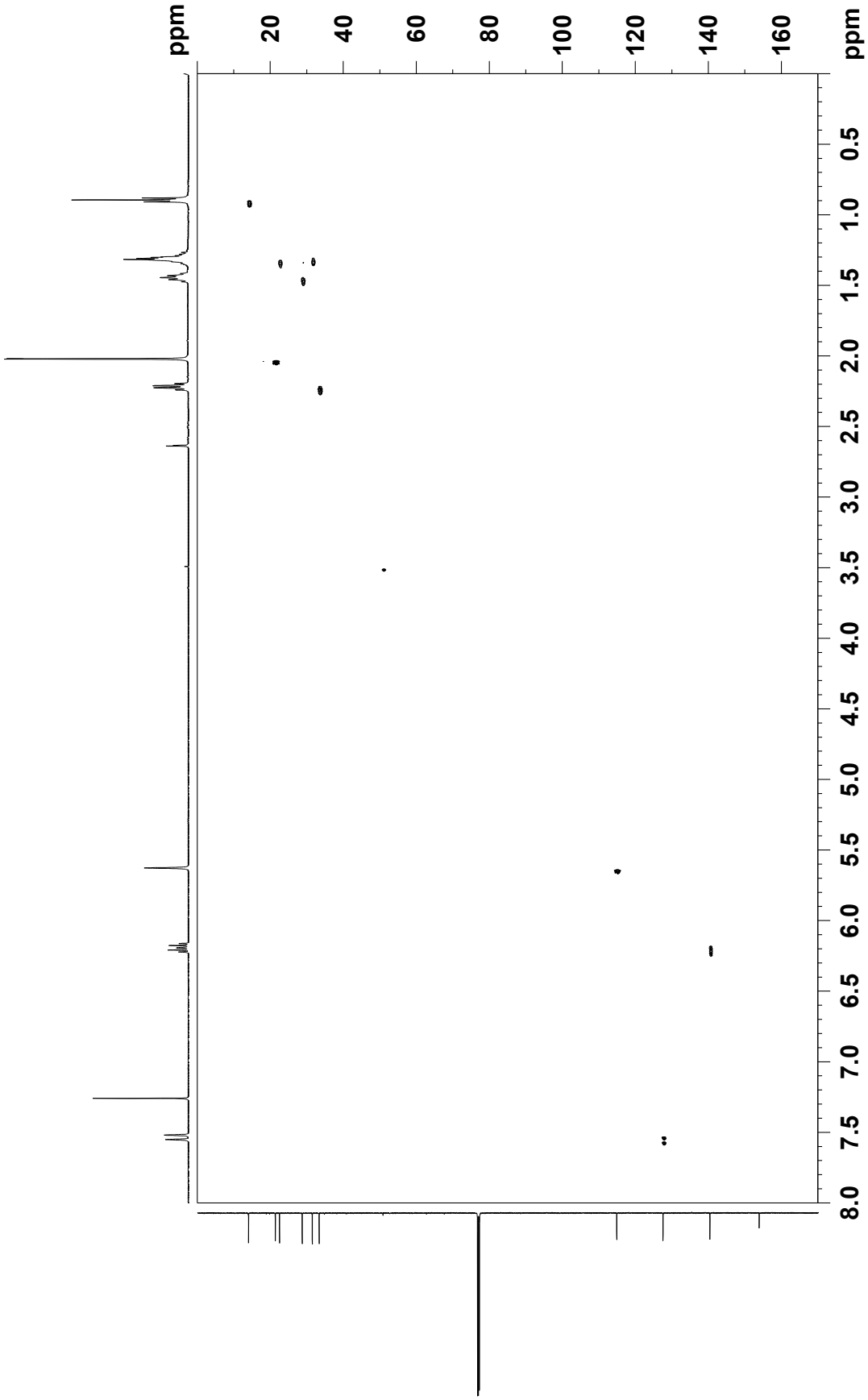


Figure S9. HMBC spectrum of **1** (500 MHz, $CDCl_3$)

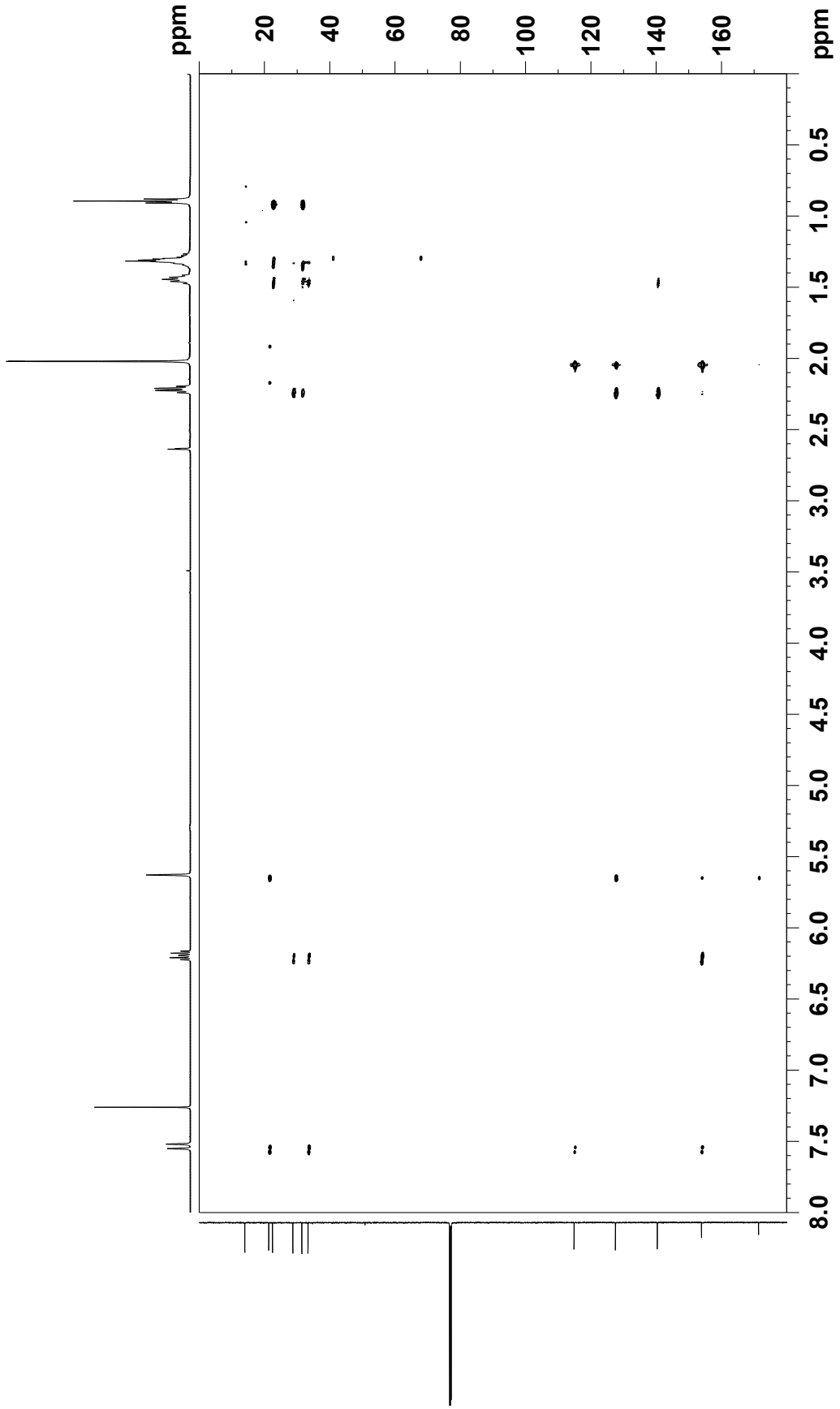
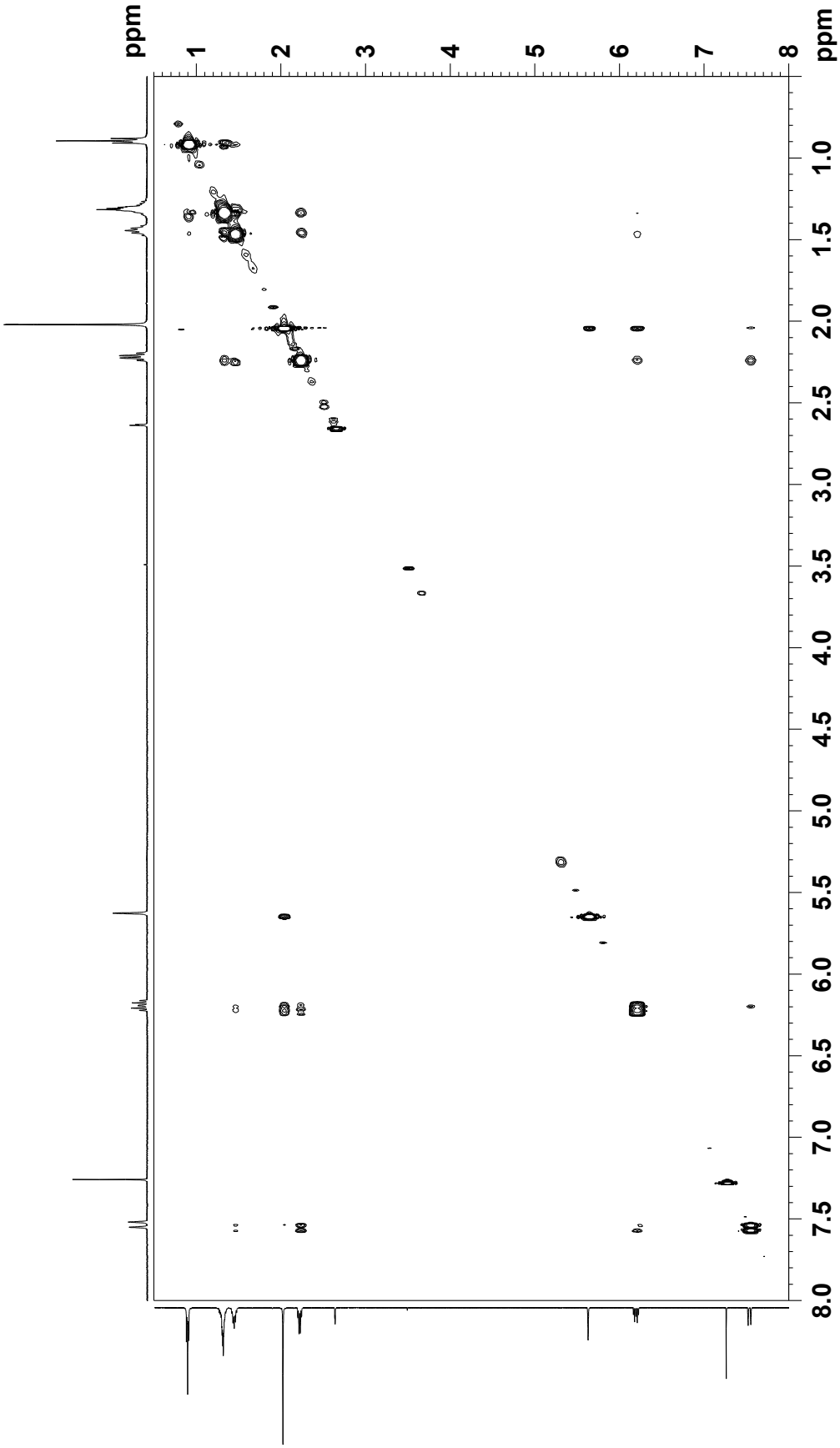


Figure S10. NOESY spectrum of **1** (500 MHz, $CDCl_3$)



CHAPTER 5

Two Antibacterial and PPARα/γ-Agonistic Unsaturated Keto Fatty Acids from a Coral-Associated Bacterium *Micrococcus* sp. C5-9

5-1 Background

After successful isolation and characterization of labrenzbactin and unsaturated fatty acid with a unique methylation pattern from *Labrenzia* and *Microbulbifer* as described in Chapter 3 and 4, respectively, I found marine obligate bacteria isolated from stony corals are the potential source of bioactive compounds. Previous studies in Chapter 3 and 4, substantiated that new types of bacteria are an intriguing source for the natural product screening program because they have not been exploited yet, even they contain biosynthetic gene clusters for some interesting group of molecules. So there is still a high chance of finding new bioactive molecules from such new group of bacteria. A new group of bacteria may carry genome for a new type of biosynthetic pathways resulting in the production of new bioactive molecules. However, in this chapter I worked with a common type of bacteria, *Micrococcus*, which can be readily isolated from marine as well as non-marine sources and NaCl is not essential for growth. Basically, if we work with very common type or overexploited group of bacteria, there is a high risk of finding already known compound. But I decided to undertake HPLC-UV screening of *Micrococcus* sp. C5-9 using different type of media varying in nutrient compositions because I think bacteria from the common taxonomic group could be interesting for the screening program if they are isolated from unexplored sources such as stony corals, which are not exploited for isolation of natural products as well as isolation of associated bacteria for the natural product screening program.

Marine actinobacteria are considered as a potential source for novel natural products with high structural diversity, unique biological activity, and molecular mode of action, beneficial to drug development [1-3]. Actinobacteria in marine environments are mostly found in association with higher organisms such as fish, sponges, corals, mollusks, ascidians, seaweeds, and mangroves, and have kept attracting attention due to their ability to produce various bioactive compounds [2,4]. Among the isolation sources for marine actinobacteria, substantial amounts of studies were devoted to sponges from which a wide range of actinobacterial species were found to produce intriguing natural products [5]. Corals, another large group of marine invertebrates, also harbor diverse symbiotic or associating microorganisms [6], however only a handful of natural products such as streptochloritides [7], nahuoic acids B-E [8], pteridic acids C-G [9], and octalactins A and B [10] were obtained from actinobacteria associated with soft corals (Figure 5-1). There is no report indeed on natural products from actinobacteria residing in stony corals.

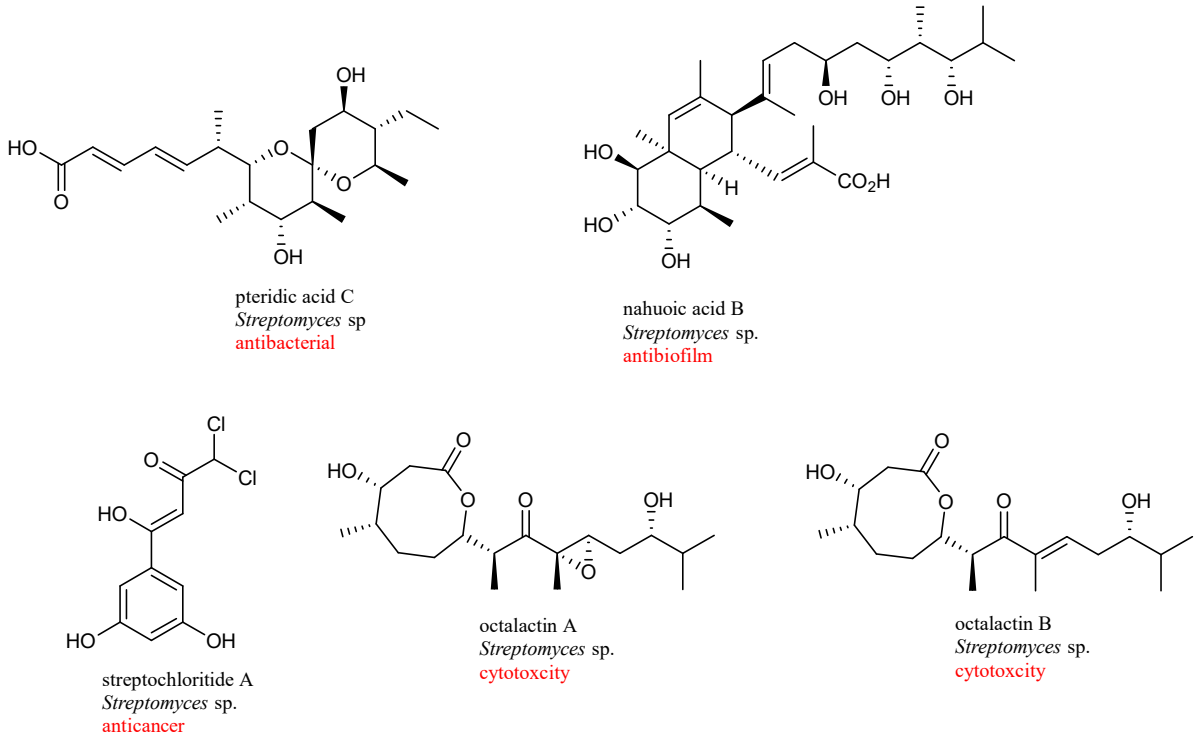
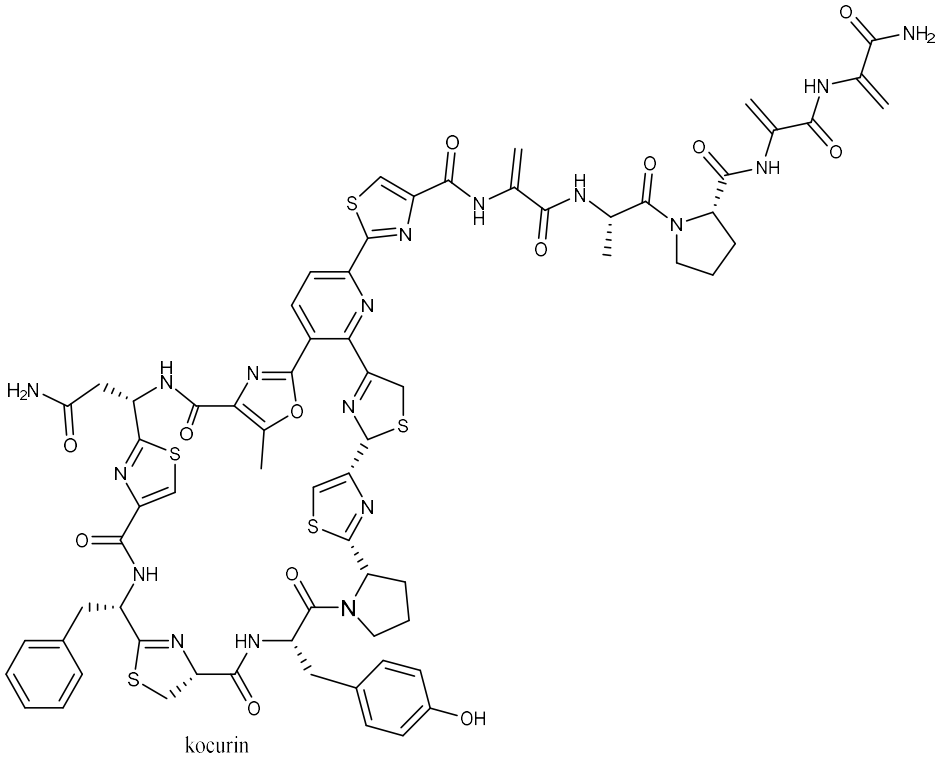
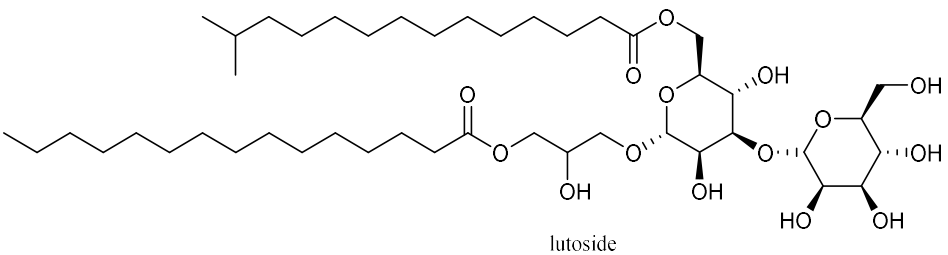


Figure 5-1. Bioactive compounds from actinobacteria associated with soft corals.

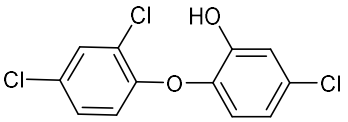
Actinomycetes of the genus *Micrococcus* are Gram-positive, aerobic, and non-motile cocci. Unlike the majority of actinomycetes, they typically form tetrad clusters but not hyphae [11]. *Micrococcus* is ubiquitous in distribution and, similar to other actinomycetes, marine *Micrococcus* are commonly associated with marine invertebrates such as sponges and corals [4,12,13]. Distinct classes of natural products have been isolated from sponge-associated *Micrococcus*, including glycosylated glycerolipid [14,15], cyclic peptide [16], xanthone glycoside [17], and halogenated diphenyl ether [15] (Figure 5-2). Until now, however, no natural products are known from coral-associated *Micrococcus*. Limazepines [18], micrococcin [19], neoberninamycin [20], and *iso*-branched octadecenoic acids [21] are the notable examples of natural products isolated from *Micrococcus* from various sources (Figure 5-3).



kocurin



lutoside



2,4,4'-trichloro-2'-hydroxydiphenylether

Figure 5-2. Natural products isolated from *Micrococcus* sp. collected from sponges.

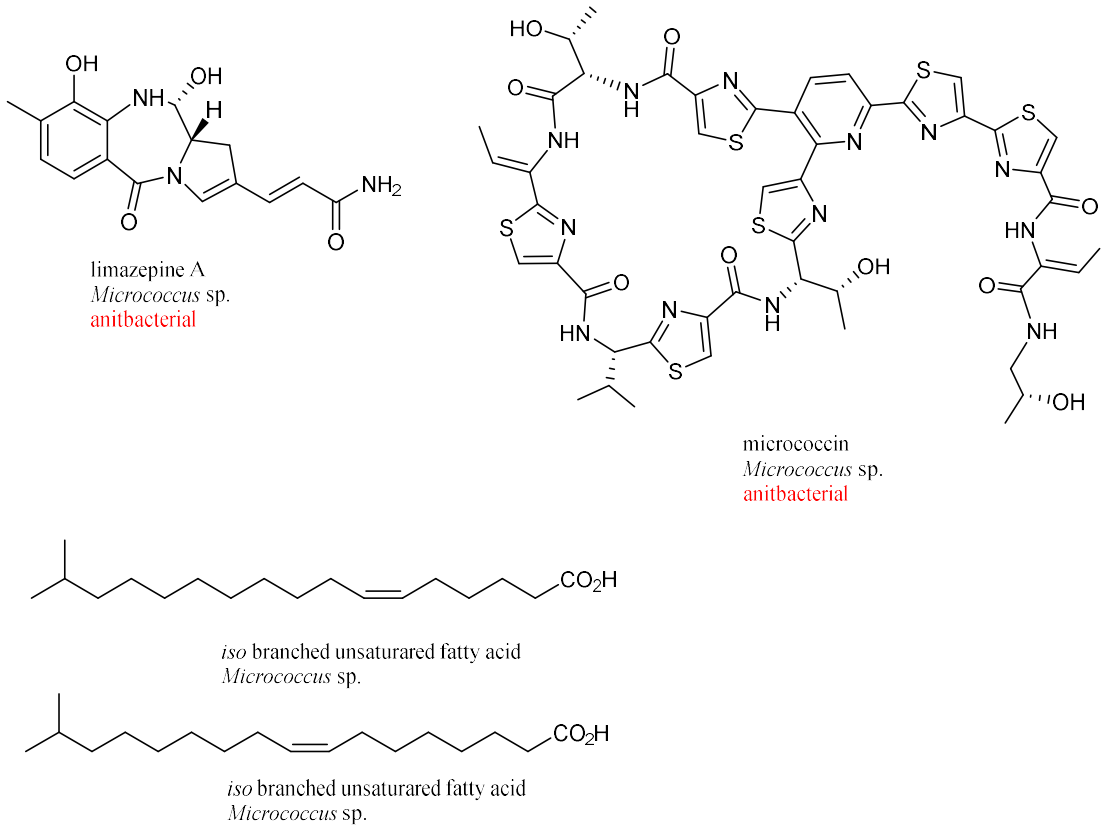


Figure 5-3. Natural products isolated from *Micrococcus* sp.

In this chapter, the fermentation, isolation, structure determination, and bioactivity of two new keto fatty acids, (6*E*,8*Z*)-5-oxo-6,8-tetradecadienoic acid (**1**) and its (6*E*,8*E*)-isomer (**2**) from a coral-associated actinomycete *Micrococcus* sp. C5-9 are described.

5-2 Results and Discussion

UV-chemical screening was employed to check the secondary metabolites production by *Micrococcus* sp. C5-9 associated with a stony coral. Three type of fermentation media (A3M, A11M and A16) were used for screening of metabolites. Strain C5-9 produced metabolites significantly only in medium A16, so this media was selected for further large scale fermentation and isolation of compound. This strain produced more than one peaks in A16 medium with similar UV profile but in this study I was able to obtain peaks A and B in sufficient amount for structure determination and bioactivity assays. Peaks A and B were eluted in 16.2 and 16.6 min, respectively (Figure 5-4). The UV spectra of peaks A and B showed absorption maxima at 277 and 275 nm, respectively.

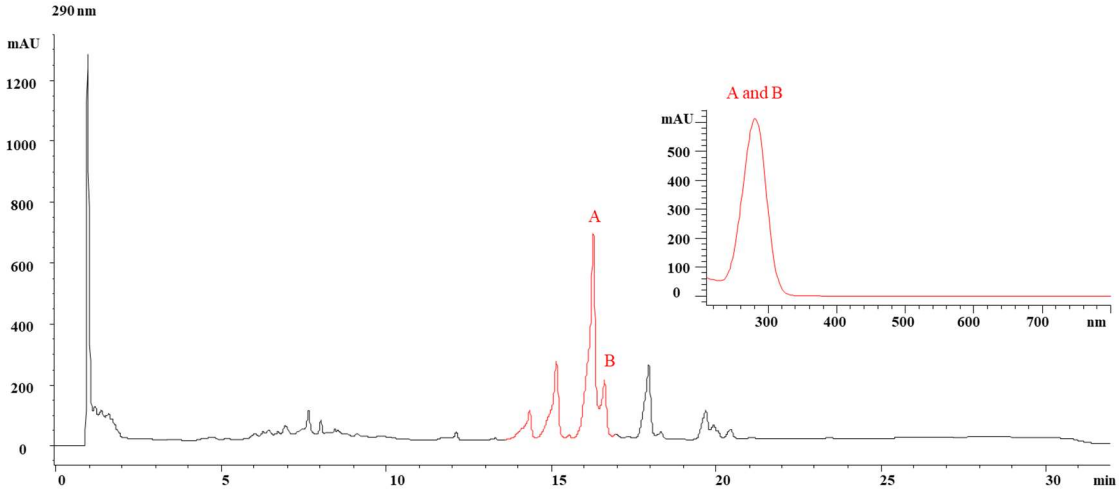


Figure 5-4. HPLC analysis of 1-butanol extract of producing strain *Micrococcus* sp. C5-9.

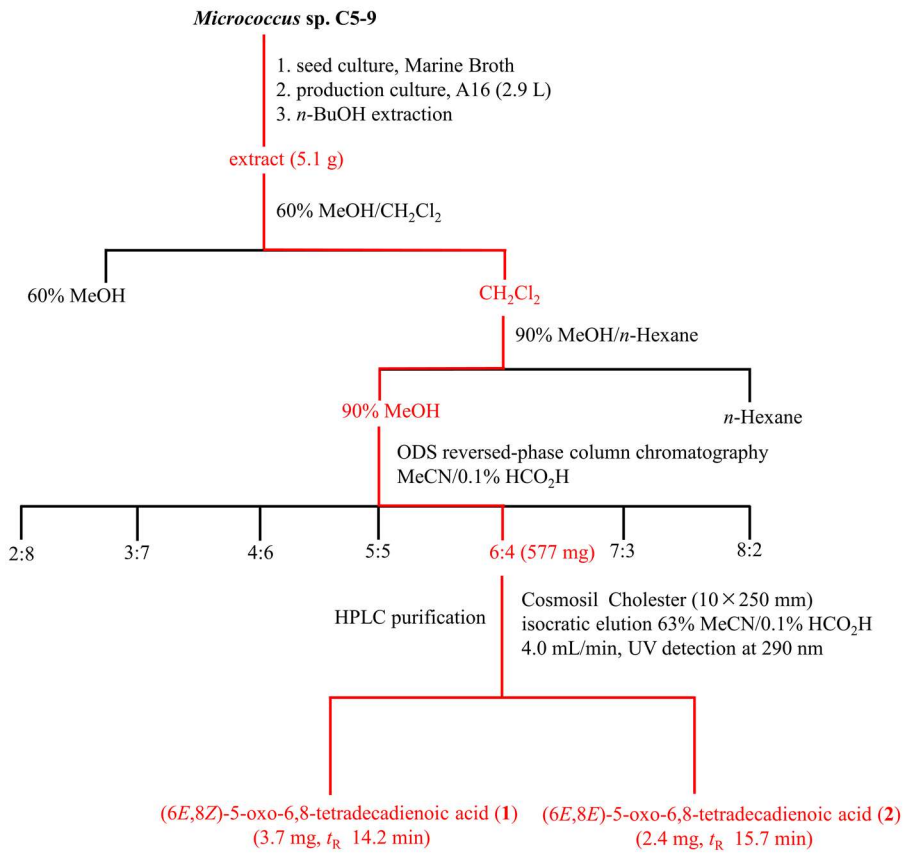
This strain readily grows on Marine Agar and other media without NaCl such as Muller-Hinton Agar prepared in distilled water. From this result, I concluded that this strain is halotolerant (Figure 5-5). *Micrococcus* sp. are commonly encountered bacteria in the nature but I selected this strain for my study because strain was collected from a stony coral, which is unexplored source for natural products discovery.

Figure 5-5. Colony morphology of *Micrococcus* sp. C5-9 on Marine Agar.

5-2-1 Fermentation and Isolation

The producing strain C5-9 was obtained from stony coral *Catalaphyllia* sp., and identified as a member of the genus *Micrococcus* by 16S rRNA gene sequence analysis. The HPLC-UV analysis of fermentation broth of strain C5-9 indicated the presence of metabolites showing UV absorption around 275 nm. Large-scale shaking culture (2.9 L) was carried out in A16 seawater medium at 30°C for five days to obtain adequate amounts of compounds for structure determination and bioassay. Fermentation broth was extracted with 1-butanol, and the extract

was fractionated by solvent/solvent partitioning. Following ODS column chromatography and isocratic reversed-phase preparative HPLC gave **1** (3.7 mg) and **2** (2.4 mg) (Scheme 5-1).



Scheme 5-1. Isolation of **1** and **2.**

5-2-2 Structure Determination

(6*E*,8*Z*)-5-Oxo-6,8-tetradecadienoic acid (**1**) was obtained as a pale yellow amorphous solid (Figure 5-6). The molecular formula was determined to be $C_{14}H_{22}O_3$ on the basis of its NMR and HR-ESITOFMS (*m/z* 261.1453 $[M+Na]^+$; calcd for $C_{14}H_{22}O_3Na$, 261.1461) data. The UV spectrum of **1** in methanol exhibited an absorption maximum at 277 nm. The IR absorption bands at 1708 and 2800~3400 cm^{-1} were suggestive of the carbonyl and hydroxy functionalities. The ^{13}C NMR and DEPT spectra of **1** (Table 5-1) displayed 14 carbon signals, including one methyl, seven sp^3 methylenes, four sp^2 methines (δ_C 143.1, 137.5, 129.1, and 126.8), one carboxyl carbon (δ_C 178.2), and one deshielded aldehyde or ketone carbon signal (δ_C 199.8). Four degrees of unsaturation indicated by ^{13}C signals were consistent with that calculated from the molecular formula, which indicated that **1** has a linear structure. The ^1H NMR spectrum showed characteristic resonances for a terminal methyl group at δ_H 0.89 (3H, t) in the shielded region and for multiple methylene signals, suggesting the presence of an alkyl

chain. COSY analysis established three spin systems, one from H2 to H4, a seven-carbon fragment from H6 to H12, and an ethyl fragment H13/H14. These partial structures were joined into one linear structure by HMBC correlations from H3, H4, H6, and H7 to C5, H14 to C12, and H11 to C13. Finally, a correlation from H2 and H3 to C1 connected the carboxyl group at C2 to complete the structure of **1** (Figure 5-7). The geometry of the double bonds was determined as *E* at C6 and *Z* at C8 based on the ^{1}H-^{1}H coupling constants $J_{H6,H7}$=15.3 Hz and $J_{H8,H9}$= 10.8 Hz, respectively.

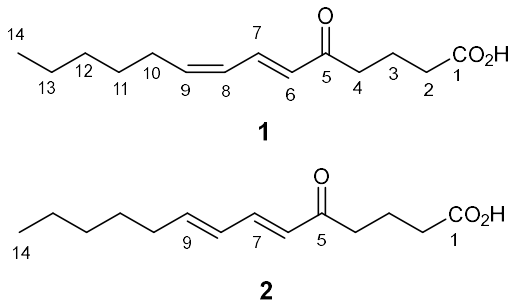


Figure 5-6. Structures of (6*E*,8*Z*)- and (6*E*,8*E*)-5-oxo-6,8-tetradecadienoic acids (**1** and **2**).

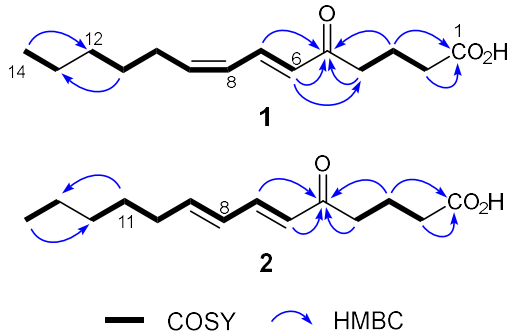


Figure 5-7. COSY and key HMBC correlations for **1** and **2**.

Table 5-1: ^{1}H and ^{13}C data for compounds **1** and **2** in $CDCl_3$.

	1			**2**		
position	δ_C[a]	δ_H mult (*J* in Hz)[b]	HMBC[b,c]	δ_C[a]	δ_H mult (*J* in Hz)[b]	HMBC[b,c]
1	178.2, C			177.7, C		
2	32.9, CH_2	2.44, m	1, 3, 4	32.9, CH_2	2.42, m	1, 3, 4
3	19.1, CH_2	1.97, m	1, 2, 4, 5	19.1, CH_2	1.96, quint (7.0)	1, 2, 4, 5
4	39.6, CH_2	2.66, t (6.6)	2, 3, 5	39.0, CH_2	2.65, t (7.1)	2, 3, 5
5	199.8, C			199.8, C		
6	129.1, CH	6.15, d (15.3)	4, 5, 8	127.6, CH	6.07, d (15.6)	4, 5, 8
7	137.5, CH	7.51, dd (15.3, 11.7)	5, 6, 8, 9	143.5, CH	7.15, dd (15.6, 9.5)	5, 8, 9
8	126.8, CH	6.10, dd (11.7, 10.8)	5, 6, 7, 10	128.7, CH	6.15, dd (15.6, 9.5)[g]	6, 7, 10
9	143.1, CH	5.92, dt (10.8, 7.9)	7, 8, 10, 11	146.2, CH	6.18, dt (15.6, 6.3)[g]	7, 10, 11
10	28.3[d], CH_2	2.31, q (7.5)	8, 9, 11, 12	33.1, CH_2	2.18, dt (6.3, 7.2)	8, 9, 11, 12
11	29.0[d], CH_2	1.43, quint (7.1)	9, 10, 12, 13	28.3, CH_2	1.43, quint (7.2)	10, 12, 13
12	31.4, CH_2	1.32[e], m	10, 11, 13	31.4, CH_2	1.29[f], m	11, 13
13	22.5, CH_2	1.30[e], m	12, 14	22.4, CH_2	1.31[f], m	12
14	14.0, CH_3	0.89, t (6.8)	12, 13	14.0, CH_3	0.89, t (6.9)	12, 13

[a]Recorded at 125 MHz (reference δ_C 77.0).

[b]Recorded at 500 MHz (reference δ_H 7.26).

[c]HMBC correlations are from proton(s) stated to the indicated carbon.

[d]Assignment interchangeable.

[e,f]Overlapping signals.

[g]Determined by NMR simulation.

The molecular formula of **2** was also determined to be $C_{14}H_{22}O_3$ on the basis of its NMR and HR-ESI-TOFMS (*m/z* 261.1458 $[M+Na]^+$; calcd for $C_{14}H_{22}O_3Na$, 261.1461) data. The ^{1}H and ^{13}C NMR spectra of **2** displayed similar feature to those for **1** except five $^{1}H/^{13}C$ resonances from C6 to C10, which indicated the structural difference between **1** and **2** to be in the double bond geometries. In fact, the composition of 14 carbon signals, the carbon connectivity, and the sites of functional groups in **2** proved to be completely the same as those in **1** by the interpretation of ^{13}C, DEPT, COSY, and HMBC correlations (Figure 5-7) except double bond configuration at C8.

5-2-3 Determination of Configuration for the C8-C9 Double Bond

While an *E*-configuration at C6 was evident from the coupling constant $J_{H6,H7}$ = 15.6 Hz, $J_{H8,H9}$ was unable to read from the multiplicity pattern of H8 and H9, due to the intense second order effects caused by a signal overlap of these resonances (δ_H 6.15 and 6.18, respectively). Although the *E*-geometry at C8 was circumstantially obvious and supported by the deshielded allylic carbon C10 (δ_C 33.1 for **2** *v.s.* 28.3 for **1**), a decisive evidence was acquired from spin-system simulations using a software 'nmrpeak' [22], which gave the best matching to the

experimentally obtained ^{1}H NMR spectrum with the setting of $^{3}J_{H8,H9}$ = 15.6 Hz and $^{3}J_{H7,H8}$ = 9.5 Hz (Figure 5-8). Thus, the C8 geometry was unambiguously determined to be *E*.

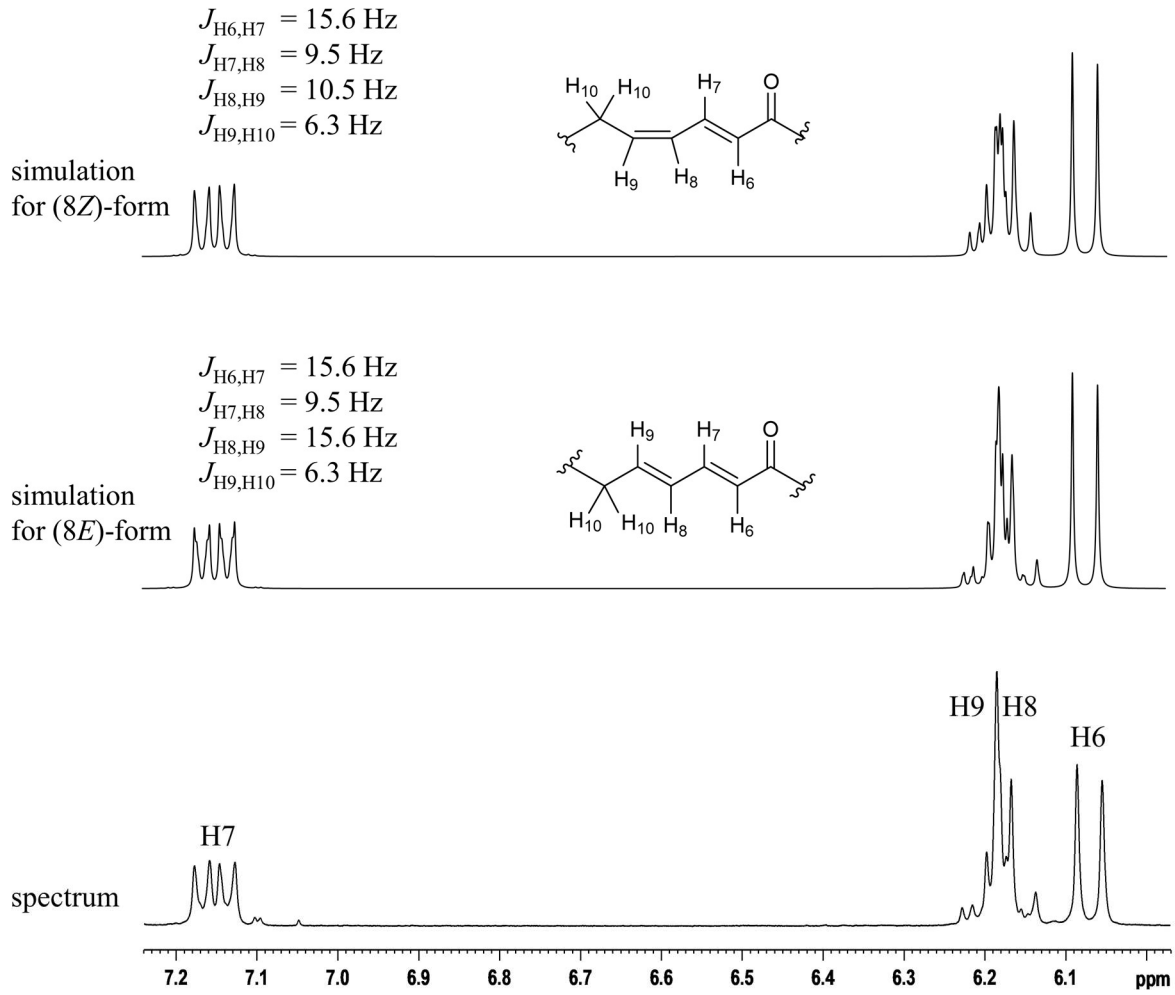


Figure 5-8 Spin-system simulation for the C8-C9 double bond of **2**.

Coupling constants (*J*) and chemical shifts (δ) can be directly acquired in first order spectra. However, second order spectra are more complex and their interpretation is much more complicated [23]. First-order spectra obey all general rules concerning the splitting, intensity distribution and integration of the peaks [23]. To acquire a first order spectrum, the chemical shift difference (Δv) between the coupled protons in hertz has to be much larger than the coupling constants (*J*) [24]. If Δv/*J* is lesser than 5 then second order effects appear. When Δv/*J* is less than 1 then second order effects become very noticeable and often preventing detailed manual interpretation of multiplets [24]. In this situation, quantum mechanical simulation and adjustment of NMR parameters (chemical shifts and *J* values) to match the observed pattern and intensities of NMR lines is the only option to analyze the data [25].

The effects of spectrometer field strength on the ability to resolve NMR coupling information is illustrated in the set of spectra of ethylbenzene, plotted at a constant Hz scale (Figure 5-9). The aromatic signals go from nearly a singlet at 60 MHz to a reasonably resolved set of peaks at 600 MHz. This increase in information content and greater ease of interpretation of NMR spectra at higher magnetic field strength is the main justification for the additional expense of more powerful magnets [24].

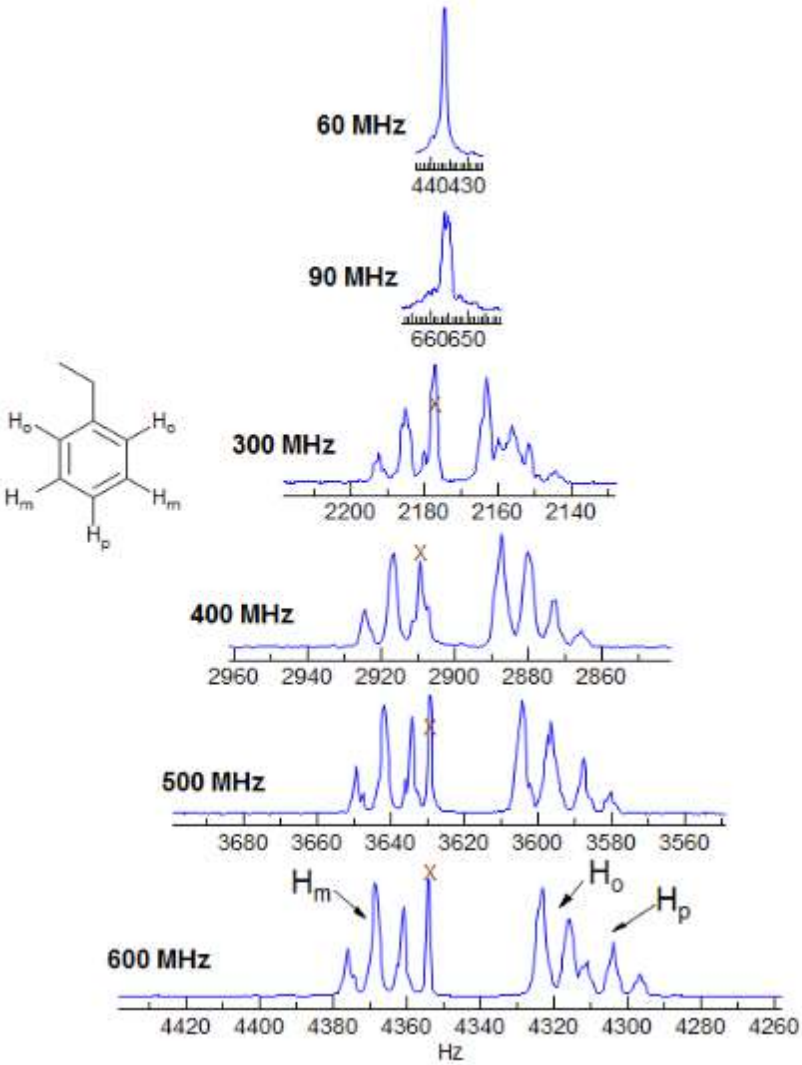


Figure 5-9. The effects of spectrometer field strength on the ability to resolve NMR coupling information [24].

A very good example of second order spectra is provided by the compound below (Figure 5-10). For the $BrCH_2CH_2O$ group, two methylenes at δ_H 3.48 and δ_H 3.81 (c and d) have a relatively large chemical shift separation, and they form noticeable triplets, although with a little leaning. For the $CH_3OCH_2CH_2O$ group (a and b) the chemical shift between the CH_2

groups is very small, and the signals are a complicated multiplet with only a vague resemblance to a triplet and resulted in second order spectra [24].

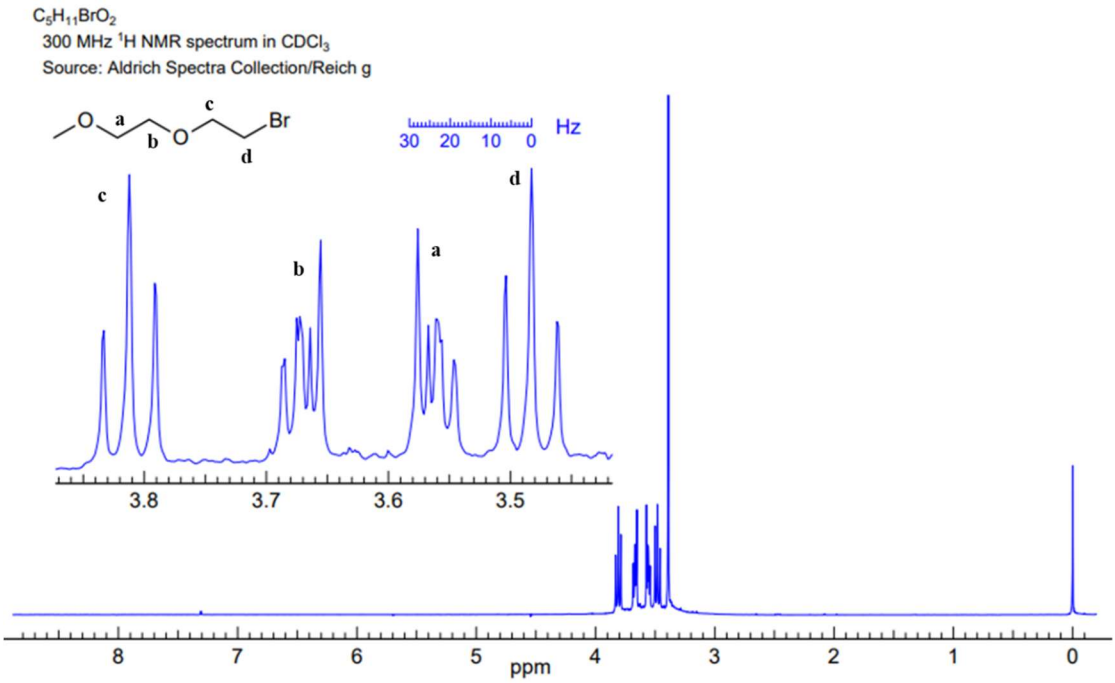


Figure 5-10. Example of second order spectrum [24].

5-2-4 Bioactivity

Compounds **1** and **2** inhibited the growth of *Tenacibaculum maritimum* NBRC16015, a causative agent of skin infection of marine fish, and *Rhizobium radiobacter* NBRC14554, a causative agent of crown gall disease of plant. The MIC values for **1** against *T. maritimum* and *R. radiobacter* were 50 and 6.2 μg/mL, respectively, while **2** was more potent against *T. maritimum* with MIC 12.5 μg/mL and less so against *R. radiobacter* with MIC 50 μg/mL, presenting an interesting contrast (Table 5-2). No appreciable antimicrobial activity was observed for both compounds against bacterial strains of *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* FDA209P JC-1, and *Escherichia coli* NIHJ JC-2, and yeast strains of *Candida albicans* NBRC0197 and *Saccharomyces cerevisiae* S100, nor cytotoxicity against murine leukemia P388 cells at 100 μM.

Table 5-2. Antimicrobial activity of **1** and **2**

	MIC (μg/mL)	
Microorganisms	**1**	**2**
Micrococcus luteus ATCC9341	>100	>100
Staphylococcus aureus FDA209P JC-1	>100	>100
Escherichia coli NIHJ JC-2	>100	>100
Rhizobium radiobacter NBRC14554	6.2	50
Tenacibaculum maritinum NBRC16015	50	12.5
Candida albicans NBRC0197	>100	>100
Saccharomyces cerevisiae S100	>100	>100

Additionally, compounds **1** and **2** were evaluated for agonist activity to peroxisome proliferator-activated receptors (PPARs), because similar oxo-fatty acids are known to act as PPAR agonist [26]. PPARs are ligand-activated transcription factors, playing key roles in lipid and carbohydrate metabolism [27,28]. PPARα upregulates lipid uptake and β-oxidation of fatty acids, whereas PPARγ promotes adipocyte differentiation and adipokine production in adipose tissues to improve insulin-sensitivity in diabetic patients [29-31]. Owing to these physiological functions in energy metabolism, PPARs are the molecular targets of metabolic disorders [32]. To assess the PPAR isoform specificity of **1** and **2**, three reporter cell lines that express luciferase gene in response to PPARα, PPARβ/δ, and PPARγ agonists were used. The agonist activity was determined as a relative potency to the positive controls, WY14643 for PPARα, GW0742 for PPARβ/δ, and troglitazone for PPARγ. Both **1** and **2** induced activations of PPARα and PPARγ transcription but were not effective against PPARβ/δ (Figure 5-11). Overall, **1** was lesser potent than **2**, indicating that the geometry at C8 may play an important role in the binding to PPARs.

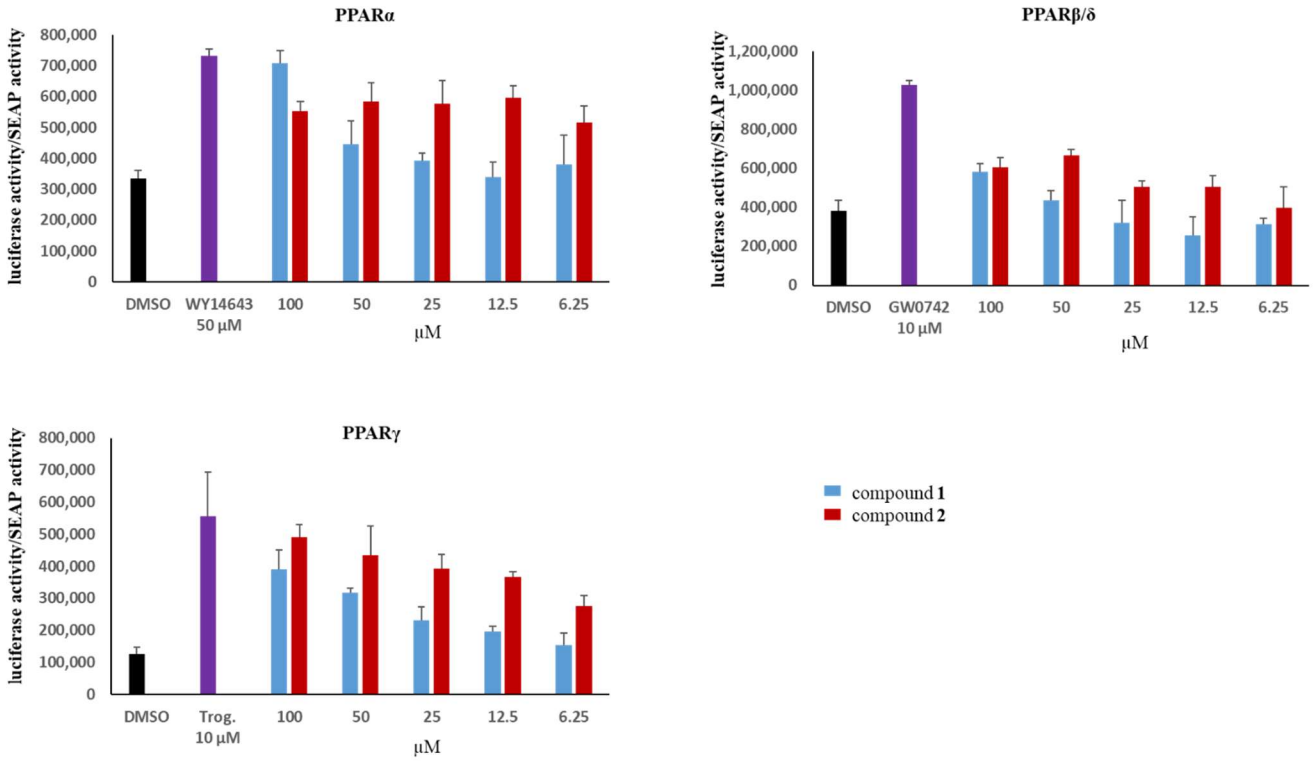


Figure 5-11. PPAR activation by compounds **1** and **2**.

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors, belongings to the superfamily of nuclear hormone receptors [33]. In mammals, three different isoforms of PPARs have been described yet: PPARα, PPARβ/δ, and PPARγ [33]. PPARα was the first identified member, whereas PPAR β/δ is the least described isoform [33]. PPARα mainly expressed in the high energy demanding tissues (liver, heart, kidney and muscles) and show high rates of β-oxidation of fatty acids [28]. Similarly, PPAR-β/δ is ubiquitously expressed in humans, whereas in mice it is expressed to a higher extent in the gastrointestinal duct [28]. PPAR-γ is expressed at high levels in the adipose tissue [28] (Figure 5-12).

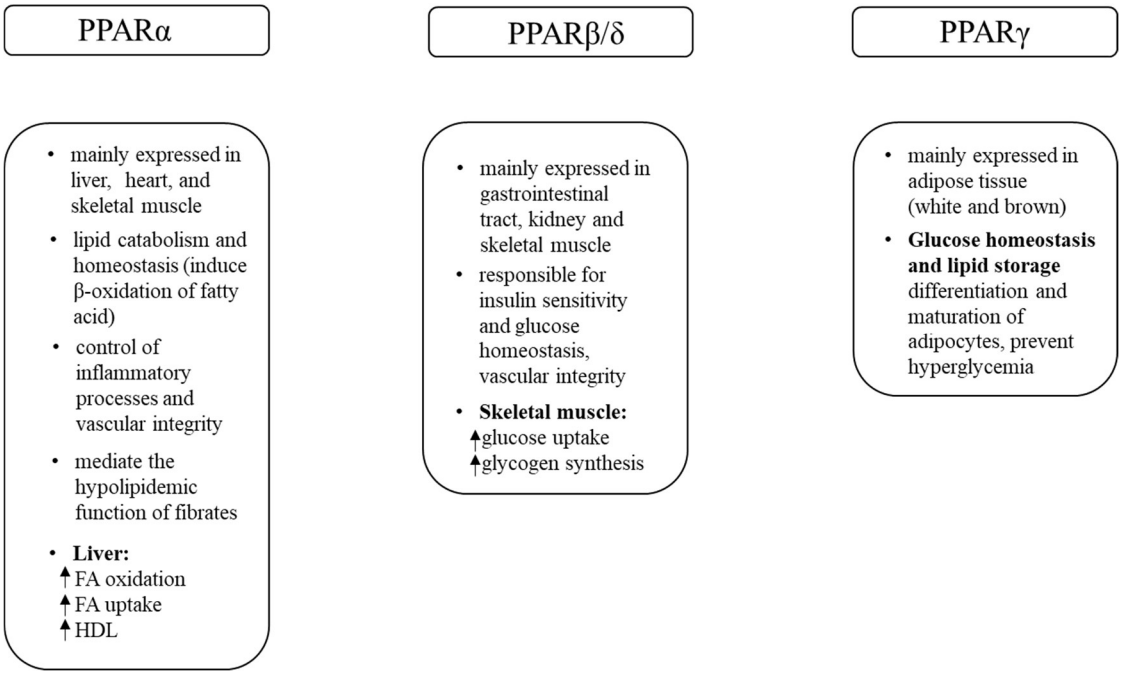


Figure 5-12. The physiological roles of PPARs.

PPAR agonists or activators, are drugs or chemicals that binds to the and activate peroxisome proliferator-activated receptor [34]. They are utilized for the treatment of metabolic syndroms, dyslipidemia and type II diabetes [35]. Fibrates and thiazolidinediones (TZDs) are used to treat these conditions by selectively activating PPARα and PPARγ, respectively [35]. Fibrates activate PPARα and reduce plasma triglycerides, and raise plasma HDL cholesterol (HDLc) levels [36]. Similarly, TZDs activate PPARγ and reduce glucose levels, improve insulin sensitivity, and can improve lipid homeostasis [37]. Many natural and synthetic fatty acid derivatives act as PPAR agonist and can play important role in the treatment of incidence of metabolic syndrome but they have severe side effects [34,38] (Figure 5-13, 14, 15, 16 and 17). So there is high demand of natural products which can acts as potent PPAR agonist without severe side effects.

PPARα

Natural activators:
- Unsaturated fatty acids
- Leukotriene B4
- 8-hydroxyl-eicosatetraenoic acid

Synthetic activators:
- Benzafibrate
- Ciprofibrate
- Clofibrate
- Gemfibrozil
- GW7647
- WY14643

PPARβ/δ

Natural activators:
- Unsaturated fatty acids
- carbaprostacyclin
- Components of VLDL

Synthetic activators:
- GW0742

PPARγ

Natural activators:
- Unsaturated fatty acids
- Prostaglandin PGJ2
- 15-hydroxyl-eicosatetraenoic acid

Synthetic activators:
- Ciglitazone
- GW1929
- Rosiglitazone
- Troglitazone

Figure 5-13. Natural and synthetic activators for PPARs.

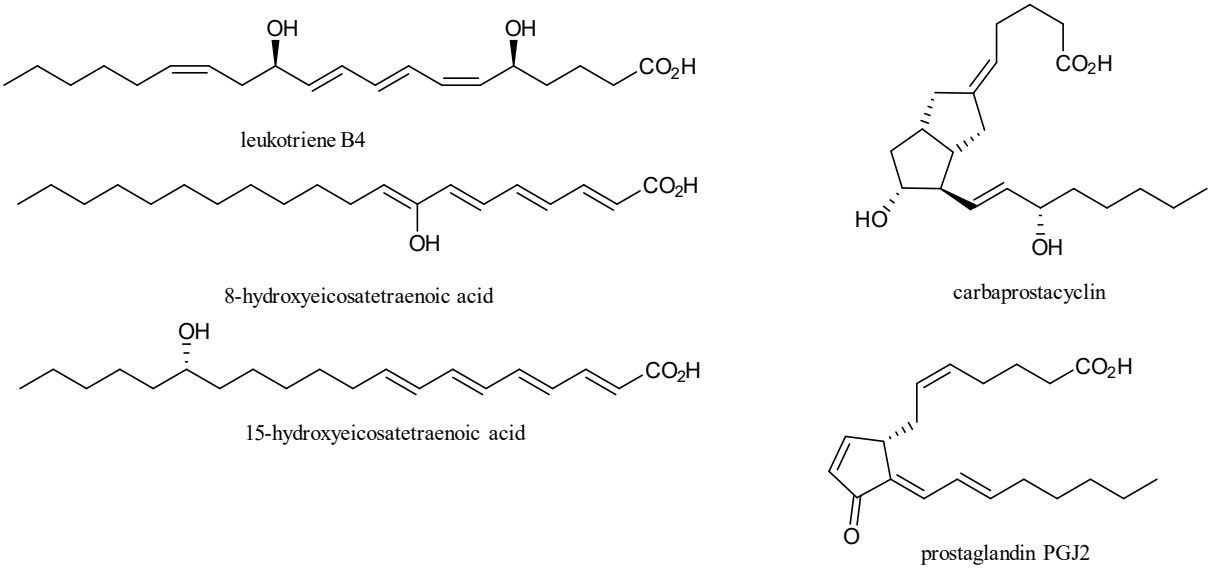


Figure 5-14. Natural activators for PPAR.

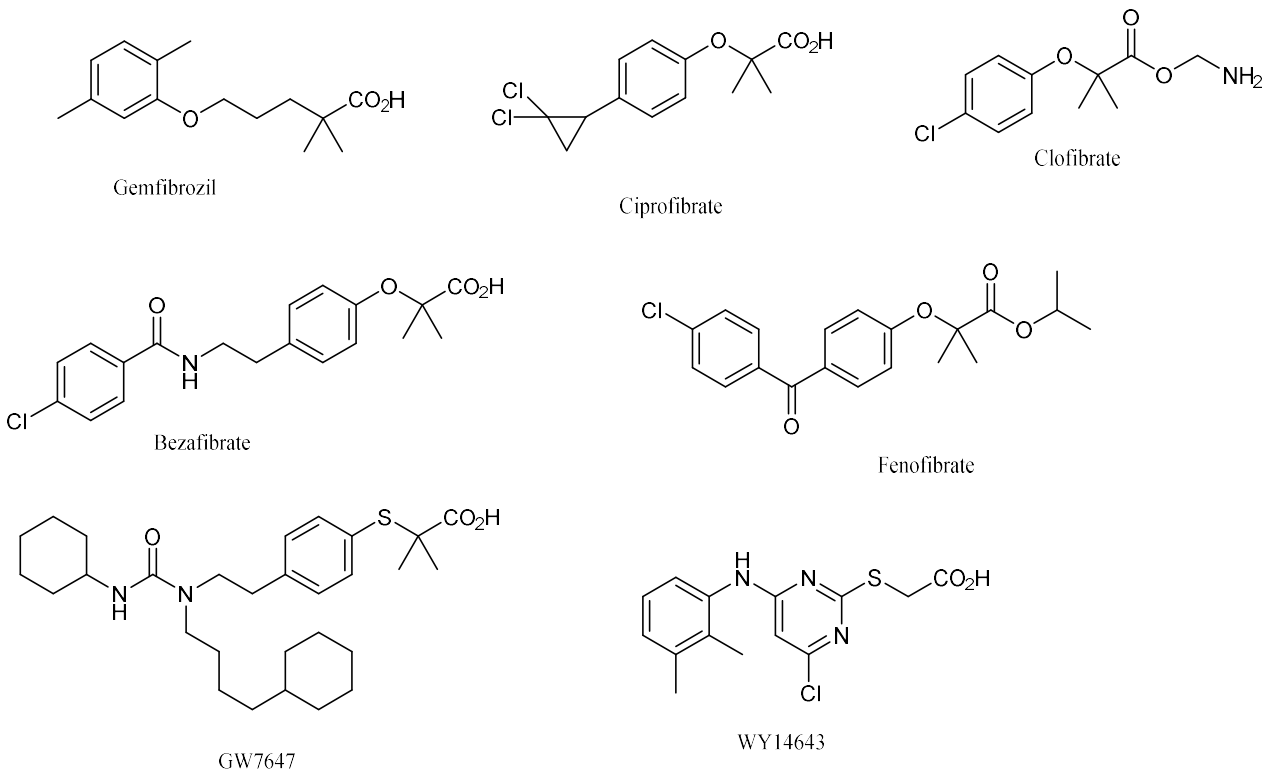


Figure 5-15. Synthetic PPARα activators.

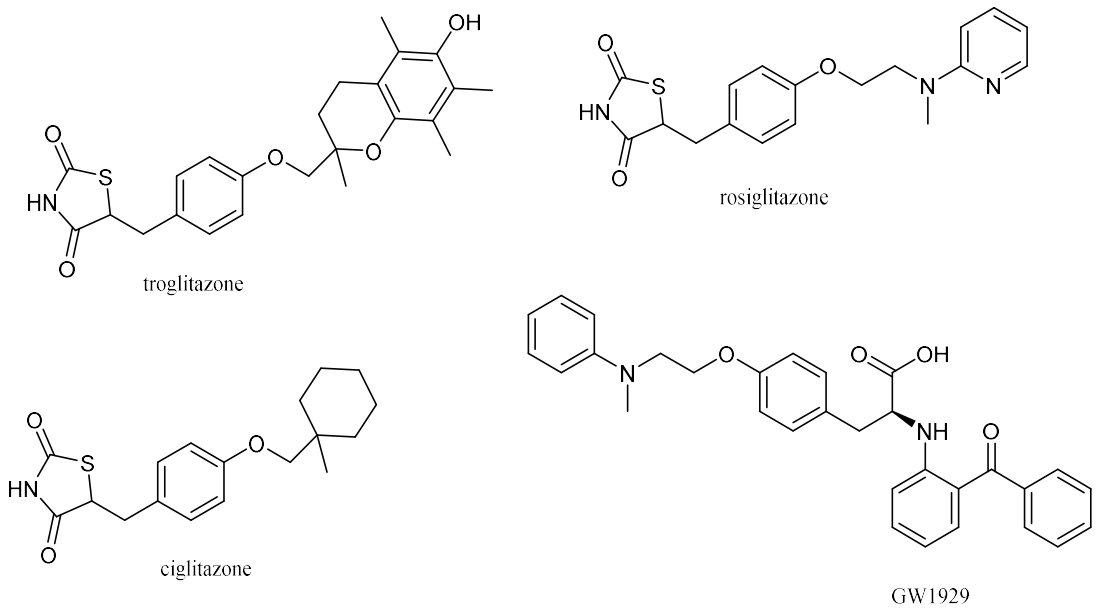


Figure 5-16. Synthetic PPARγ activators.

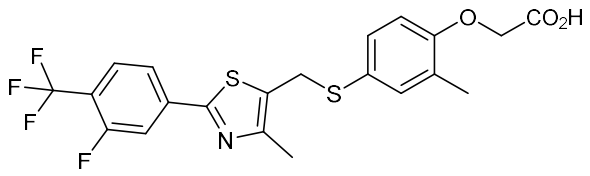


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Figure 5-17. Synthetic PPARβ/δ activator.

5-3 Conclusion

In summary, UV-chemical screening of coral-associated bacterium *Micrococcus* sp. C5-9 led to the discovery of two new unsaturated keto fatty acids, (6*E*,8*Z*)-5-oxo-6,8-tetradecadienoic acid (**1**) and its (6*E*,8*E*)-isomer (**2**). Compounds **1** and **2** showed selective antibacterial activity against a plant pathogen *R. radiobacter* and a fish pathogen *T. maritimum*, respectively. In addition, both **1** and **2** displayed agonistic activity against PPARα and PPARγ. Compounds similar to **1** and **2** showed antibacterial and antifungal activities. We do not know the exact role of compounds **1** and **2** in the nature but compounds **1** and **2** showed antibacterial activity against pathogenic bacteria in *in-vitro* assay so we can speculate that these compounds also may play important role to protect corals from pathogenic bacteria in nature.

Fatty acids are ubiquitous metabolites present in all organisms. They are organic compounds characterized by the presence of a carboxyl group (-COOH) at the one end and a methyl group at the other end. Some unusual fatty acids have two adjacent double bonds separated by more than one methylene group, and they are termed polymethylene-interrupted fatty acids [39]. The structures of fatty acids can be more complicated because they can have branched chains, an odd number of carbon atoms, or an array of other functional groups, containing acetylenic bonds, epoxy, hydroxy, or keto groups, and ring structures (cyclopropane, cyclopropene, cyclopentene, furan, and cyclohexyl) [39]. Recently, fatty acids have drawn significant attention because of their nutritional, cosmetic, and pharmaceutical importance [40].

Two isomeric keto fatty acids, (7*E*)-9-oxohexadecenoic acid and (10*E*)-9-oxohexadecenoic acid were isolated from the marine algae *Chaetoceros karianus*, and displyed dual PPAR α/γ agonist activity [26]. Majusculoic acid, a cyclopropane containing fatty acid isolated from an unidentified cyanobacterial mat assemblage, exhibited antifungal activity against *Candida albicans* with MIC value of 8 μM [41]. Acetylenic fatty acid, named scleropyric acid, was isolated from the twigs of *Scleropyrum wallichianum*, and exhibited antiplasmodial activity with an IC_{50} of 7.2 μg/mL against a K1 multidrug-resistant strain of

Plasmodium falciparum and showed antimycobacterial activity with an MIC value of 25 µg/mL [42]. *Pseudomonas aeruginosa* PR3 was found to convert linoleic acid to a new compound, (*E*)-9,12,13-trihydroxyoctadec-10-enoic acid (THOD). THOD displayed anti-plant pathogenic fungal activities [43]. Chemical investigation of of culture broth of *Pseudozyma focculosa* led to the discovery of two new fatty acids, (9*Z*)-9-heptadecenoic acid and (9*Z*)-6-methyl-9-heptadecenoic acid. These compounds showed antifungal activity [44]. A new furan containing fatty acid, plakorsin B was isolated from the Taiwanese marine sponge *Plakortis simplex*. Plakorsin B showed against COLO- 250 and KB-16 cells with IC_{50} values of 0.28 µg/mL and 3.43 µg/mL, respectively [45]. 12-methyltetradecanoic acid was isolated from endophytic Gram-negative bacterium *Stenotrophomonas maltophilia* has been shown to inhibit appressorium formation in the rice pathogen, *Magnaporthe oryzae* [46]. Polymethylene-interrupted fatty acid was isolated from gorgonian coral *Eunicea succinea*, (5*Z*,9*Z*)-14-methyl-5,9-pentadecadienoic acid. This compound was active against Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus faecalis* [47] (Figure 5-18).

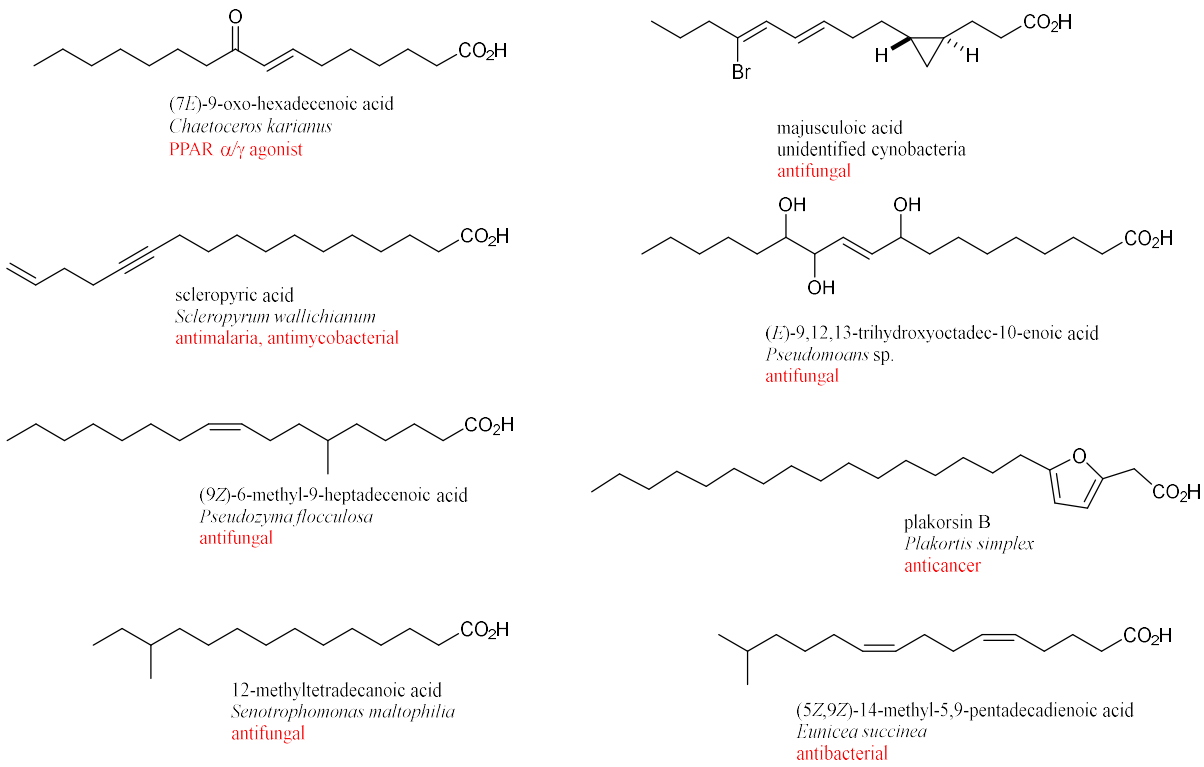

Figure 5-18. Examples of bioactive fatty acids from various sources.

α-Keto fatty acids are characterized by the presence of a ketone group at α-position of carboxylic acids. They are present in all living cells and play crucial roles in the biological system as they are involved in the Krebs cycle and glycolysis [48]. In contrast, keto fatty acids

bearing a ketone group in the middle of the carbon chain are relatively limited in their distribution in nature. Many of such natural keto fatty acids were found from plants, mainly as a constituent of seed oil [49-57]. Among them, rabdosia acids [58] and (10*E*,12*E*)-9-oxo-10,12-octadecadienoic acid [59] are the plant keto fatty acids containing the dienone moiety with *trans*-, *trans*-configuration, but congeners with *trans*-, *cis*-configuration have not been found from nature, until the present work (Figure 5-19). Some of the keto fatty acids of plant-origin are shown to exhibit pharmaceutically important activity. (9*E*,11*E*)-13-Oxo-octadecadienoic acid is a PPARα activator found in tomato juice. This keto fatty acid decreases plasma and hepatic triglyceride in obese diabetic mice by activating PPARα transcription [60]. (10*E*,12*E*)-9-Oxo-octadecadienoic acid isolated from eggplant calyx induces apoptosis in human ovarian cancer cells, leading to cell death [61]. One example of keto fatty acid from the animal kingdom is (*E*)-9-oxo-2-decenoic acid, a sex pheromone found in the royal jelly. Queen honey bees use this fatty acid to control the activity of worker bees [62]. (*E*)-7-Oxo-11,13-tetradecadienoic acid is another example of insect-origin, identified from hair pencils of male *Amauris* butterflies (*Amauris albimaculata*), which is supposed to be a precursor material for the butterfly pheromone [63]. Furthermore, 4-oxo-2-alkenoic fatty acids were characterized as antimicrobial metabolites from an actinomycete [64] and a Basidiomycete fungus [65]. In addition, long-chain saturated fatty acids possessing a keto group were detected in the solvent extract of *Legionella* by GC-MS analysis [66]. Fatty acid components in fresh water-derived *Micrococcus* species was comprehensively analyzed [67], but keto fatty acids like compounds **1** and **2**, bearing a dienone system, are unprecedented as microbial metabolites.

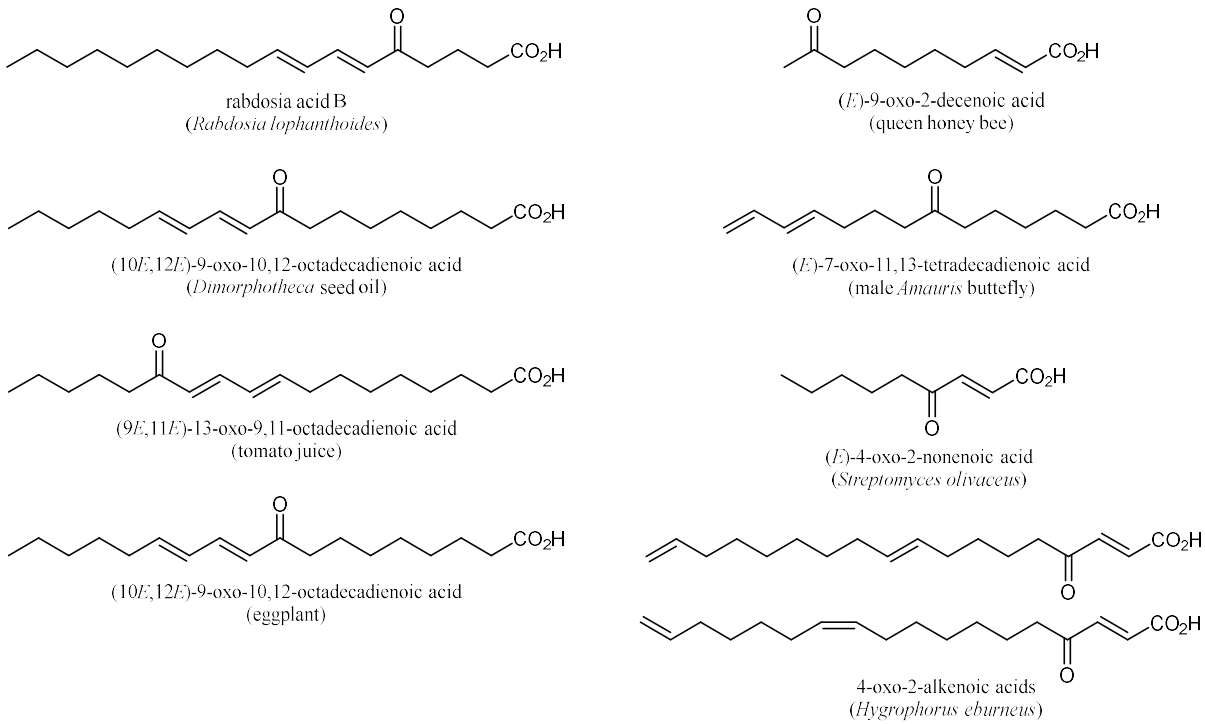

Figure 5-19. Natural keto fatty acids of various origins.

5-4 Experimental Section

General Experimental Procedures. UV and IR spectra were recorded on a Shimadzu UV-1800 and a Perkin-Elmer Spectrum 100 spectrophotometers, respectively. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer in $CDCl_3$ using the signals of the residual solvent protons (δ_H 7.26) and carbons (δ_C 77.0) as internal standards. HR-ESI-TOFMS were recorded on a Bruker micrOTOF focus.

Microorganism. Strain C5-9 was collected from a stony coral, *Catalaphyllia* sp., obtained from an aquarium vendor in Osaka, Japan. A piece of the coral specimen (*ca*. 1 g) was surface-sterilized by washing with 70% ethanol, followed by rinsing with sterile natural seawater. The coral piece was homogenized by mortar and pestle with an equal volume of sterile natural seawater (1 mL), and the resulting suspension was serially diluted by 10-fold up to 10^{-5}. One-hundred μL aliquots of each dilution were spread onto Marine Agar 2216 (Difco), and the agar plates were cultivated at 23°C for 2 days. Single colonies thus emerged on the plates were transferred onto a new agar medium to obtain pure isolates. One of these isolates, coded as C5-9, was identified as a member of genus *Micrococcus* on the basis of 99.9% similarity in the 16S rRNA gene sequence (1395 nucleotides; DDBJ accession number LC498624) to *Micrococcus yunnanensis* YIM 65004^T (accession number FJ214355).

Fermentation. Strain C5-9 was maintained on Marine Agar 2216 (Difco). A loopful of strain C5-9 was inoculated into a 500 mL K-1 flask containing 100 mL of Marine Broth 2216 (Difco) as a seed culture. The seed culture was incubated at 30°C on a rotary shaker at 200 rpm for 2 days. Three mL each of seed culture was inoculated into twenty-nine 500 mL K-1 flasks containing 100 mL of A16 production medium, which consists of glucose 2%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1%, $CaCO_3$ 0.5%, Diaion HP-20 (Mitsubishi Chemical, Kanagawa, Japan) 1%, and natural seawater collected in Toyama Bay, Toyama, Japan. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated at 30°C for 5 days with rotational shaking at 200 rpm.

Extraction and Isolation. After fermentation, 100 mL of 1-butanol was added to each flask, and the flasks were shaken for 1 h. The emulsified mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer. The organic layer was concentrated *in vacuo* to afford 5.1 g of crude extract from 2.9 L of production culture. The extract was successively partitioned between 60% aqueous MeOH (500 mL) and CH_2Cl_2 (500 mL × 3) and the latter between 90% aqueous MeOH (250 mL) and *n*-hexane (250 mL × 3). The 90% aqueous MeOH layer was evaporated to dryness (577 mg) and then fractionated by reversed-phase ODS column chromatography with a gradient of MeCN-0.1% HCO_2H (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). Fraction 5 (6:4) was concentrated *in vacuo* and the remaining aqueous layer was extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to give 27.5 mg of semi-pure material. Final purification was achieved by preparative HPLC (Cosmosil Cholester, Nacalai Tesque Inc., 10 x 250 mm, 4 mL/min, UV detection at 290 nm) with an isocratic elution of MeCN/0.1% HCO_2H (63:37) to afford **1** (3.7 mg, t_R 14.2 min) and **2** (2.4 mg, t_R 15.7 min).

(6*E*,8*Z*)-5-Oxo-6,8-tetradecadienoic acid (**1**): pale yellow amorphous solid; UV (MeOH) λ_{max} (log ε) 277 (4.25) nm; IR (ATR) ν_{max} 3050, 2955, 1708, 1689 cm^{-1}; HR-ESI-TOFMS *m/z* 261.1453 $[M + Na]^+$ (calcd for $C_{14}H_{22}O_3Na$, 261.1461).

(6*E*,8*E*)-5-Oxo-6,8-tetradecadienoic acid (**2**): pale yellow amorphous solid; UV (MeOH) λ_{max} (log ε) 275 (4.30) nm; IR (ATR) ν_{max} 3389, 2929, 1710, 1659 cm^{-1}; HR-ESI-TOFMS *m/z* 261.1458 $[M + Na]^+$ (calcd for $C_{14}H_{22}O_3Na$, 261.1461).

NMR Spin-System Simulation. In order to determine the multiplicity pattern and the coupling constants for the double bond system of **2**, spin-system simulations were performed using a freeware software, nmrpeak.exe [22].

Antimicrobial Assay. Antimicrobial activity was evaluated by the liquid microculture method using round-bottomed 96-well microtiter plates against five bacteria, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* FDA209P JC-1, *Rhizobium radiobacter* NBRC14554, *Escherichia coli* NIHJ JC-2, *Tenacibaculum maritimum* NBRC16015, and two yeasts *Candida albicans* NBRC0197 and *Saccharomyces cerevisiae* S100 as indication strains. Mueller-Hinton Broth (Difco), Sabouraud Dextrose Broth (Difco), and Marine Broth (Difco) were used for bacteria, yeasts and *Tenacibaculum maritimum* NBRC16015, respectively. Compounds **1** and **2**, reference drugs, kanamycin sulfate for bacteria, sulfamethoxazole for *R. radiobacter* NBRC14554 and *T. maritimum* NBRC16015 and amphotericin B for yeasts, were made in 2-fold dilution series along the longer side of the plates by sequential transfer of 100 μL aliquots between the adjacent wells, to which the same amount of medium was pre-dispensed. To each well was added a 100 μL suspension of the indication strains prepared at ~10^6 cfu/mL from a culture at the logarithmic growth phase. The solvent vehicle added to the top rows was set at the 0.5% of the final culture volume to avoid the effect on the growth of microbes. The plates were incubated at 37°C for 20 h for bacteria, at 24°C for *T. maritimum* NBRC16015, and at 32°C for yeasts. The tests were done in triplicate and the absorbance at 650 nm was measured using a microplate reader.

Cytotoxicity Assay. P388 murine leukemia cells were maintained in RPMI-1640 medium containing L-glutamine (product no. 186-02155) supplemented with 10% fetal bovine serum and 0.1 mg/mL gentamicin sulfate. Compounds **1** and **2** and doxorubicin as a reference were serially diluted by a factor of 3.16 (half-logarithmic dilution) in a 96-well round bottom microtiter plate. To each well were seeded the cells at a final density of 5×10^3 cells/well, and 200 μL cultures thus made were incubated for 96 h at 37°C in an atmosphere of 5% CO_2 in air with 100% humidity. Viability of the cells was visualized by addition of 50 μL of medium containing XTT (1 mg/mL) and PMS (40 μg/mL) to each well. After incubating for 4 h at 37°C, medium was carefully removed by a suction aspirator, and formazan dye formed by respiratory reduction by living cells was quantified by measurement of absorption at 450 nm was read by a microplate reader to calculate the rate of cell growth inhibition at each concentration, and the results of triplicate experiments were plotted on single-logarithmic charts to deduce IC_{50} values.

PPAR Activation Assay. Measurement of PPARα, -β/δ, and -γ ligand activity was evaluated by a luciferase reporter gene assay system. Briefly, COS-1 cells (5×10^5 cells) were transiently transfected with an expression plasmid containing the ligand-binding domain of human PPARα, -β/δ or -γ fused to the GAL4 DNA-binding domain (pPPARα-GAL4, pPPARδ-GAL4, or pPPARγ-GAL4, 0.25 μg), a luciferase reporter plasmid 17m2G TATA Luc (p17m2G, 1 μg), and the pSEAP-control vector (1 μg) (Clontech, CA, USA) by using the Effectene transfection reagent. Transfection was performed in 60-mm culture dishes, according to the manufacturer's instructions. After 16 h, the transfected cells were recovered and seeded into 96-well white multi-well plates, the indicated concentrations of the test compounds were added, and the plates were cultured for an additional 24 h at 37°C in a 5% CO_2 incubator. The luciferase activity and secreted alkaline phosphatase SEAP activity were measured in each well by using a Steady-Glo® luciferase assay (Promega, Madison, WI, USA) and Great ESCAPe SEAP Reporter System3 (Clontech), according to the manufacturer's instructions. The SEAP activity level was used to correct the luciferase activity in each well. Each data value is presented as the mean ± standard error of three experiments.

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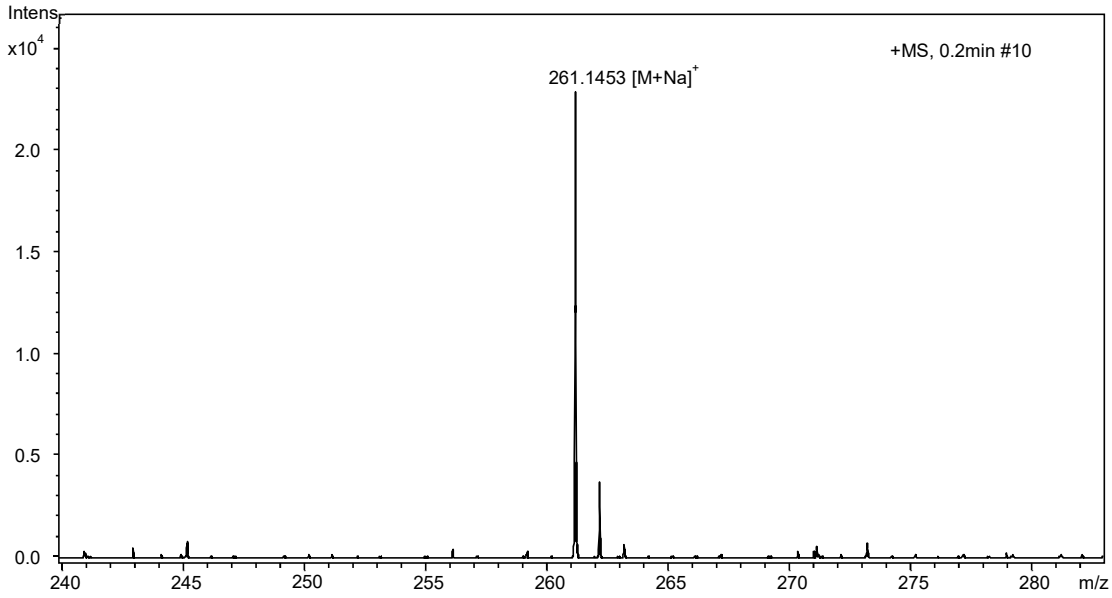
5-5 Spectral Data

Table of Contents

Figure S1. High resolution ESI-TOF mass spectra of (a) (6*E*,8*Z*)-5-oxo-6,8-tetradecadienoic acid (**1**) and (b) (6*E*,8*E*)-5-oxo-6,8-tetradecadienoic acid (**2**)

(a)



(b)

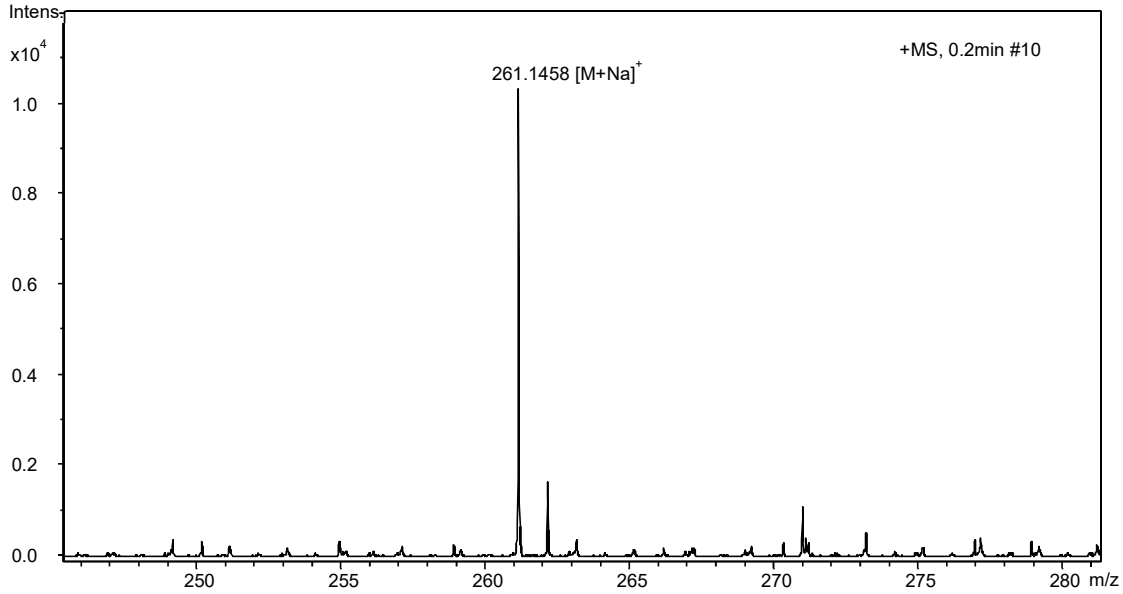
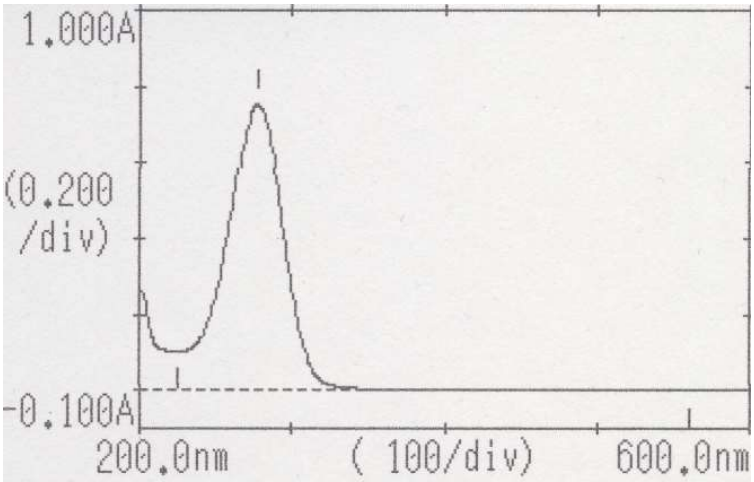


Figure S2. UV spectra of **1** and **2**

(a) (**1**) (MeOH)



(b) (**2**) MeOH

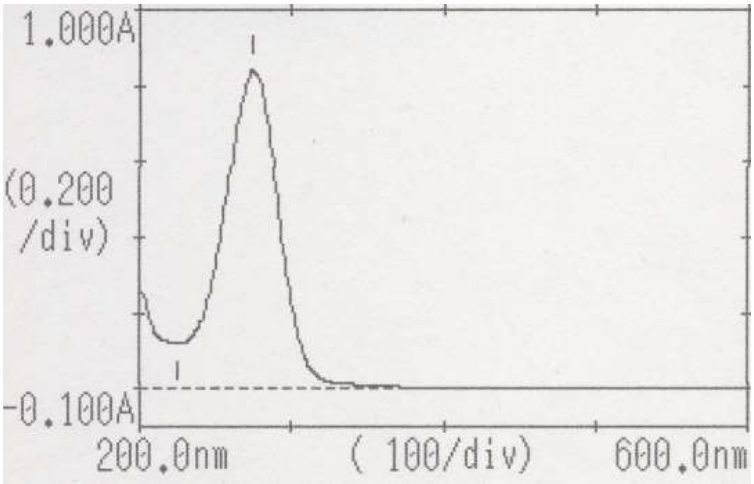
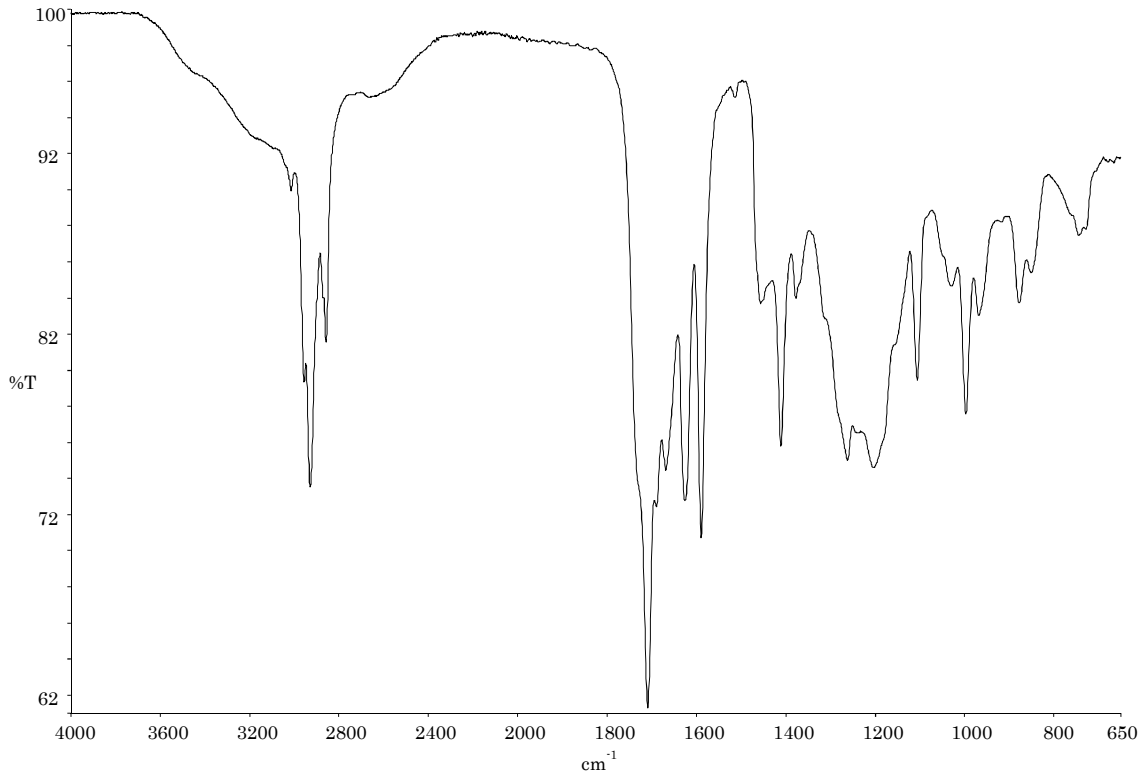


Figure S3. IR spectra of **1** and **2**.

(a) **1** (ATR)



(b) **2** (ATR)

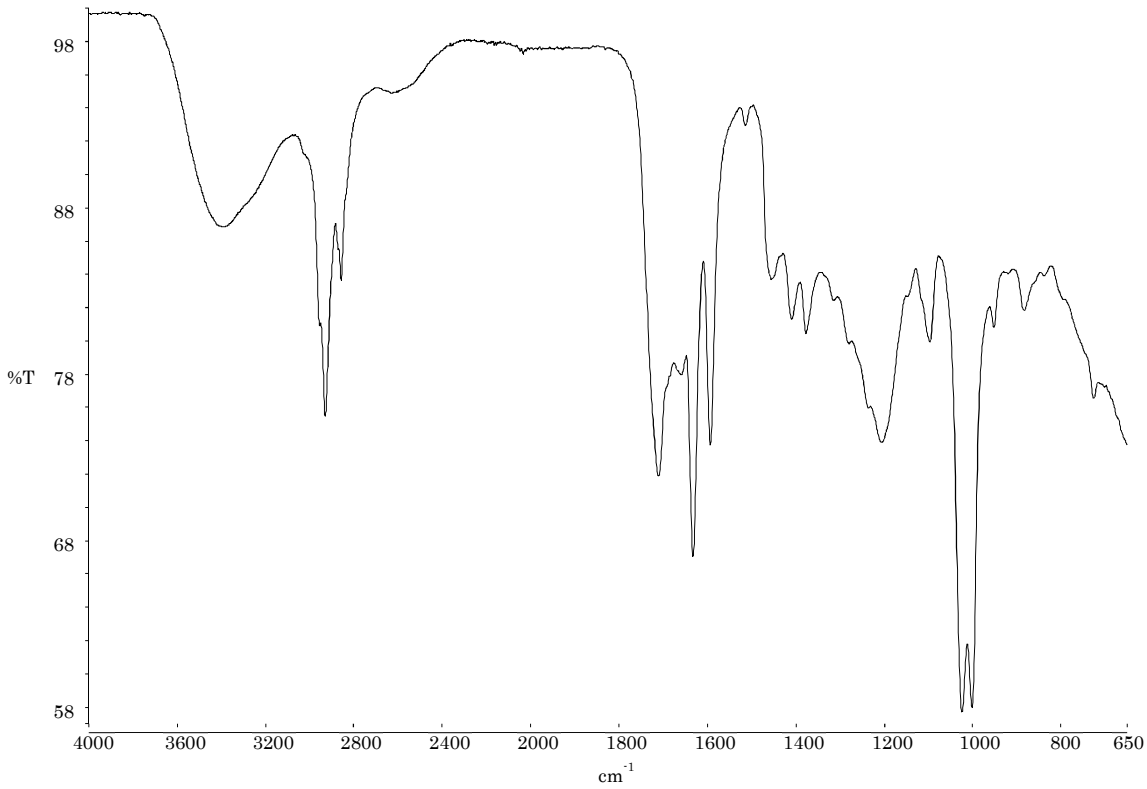


Figure S4. ^{1}H NMR spectrum of **1** (500 MHz, $CDCl_3$)

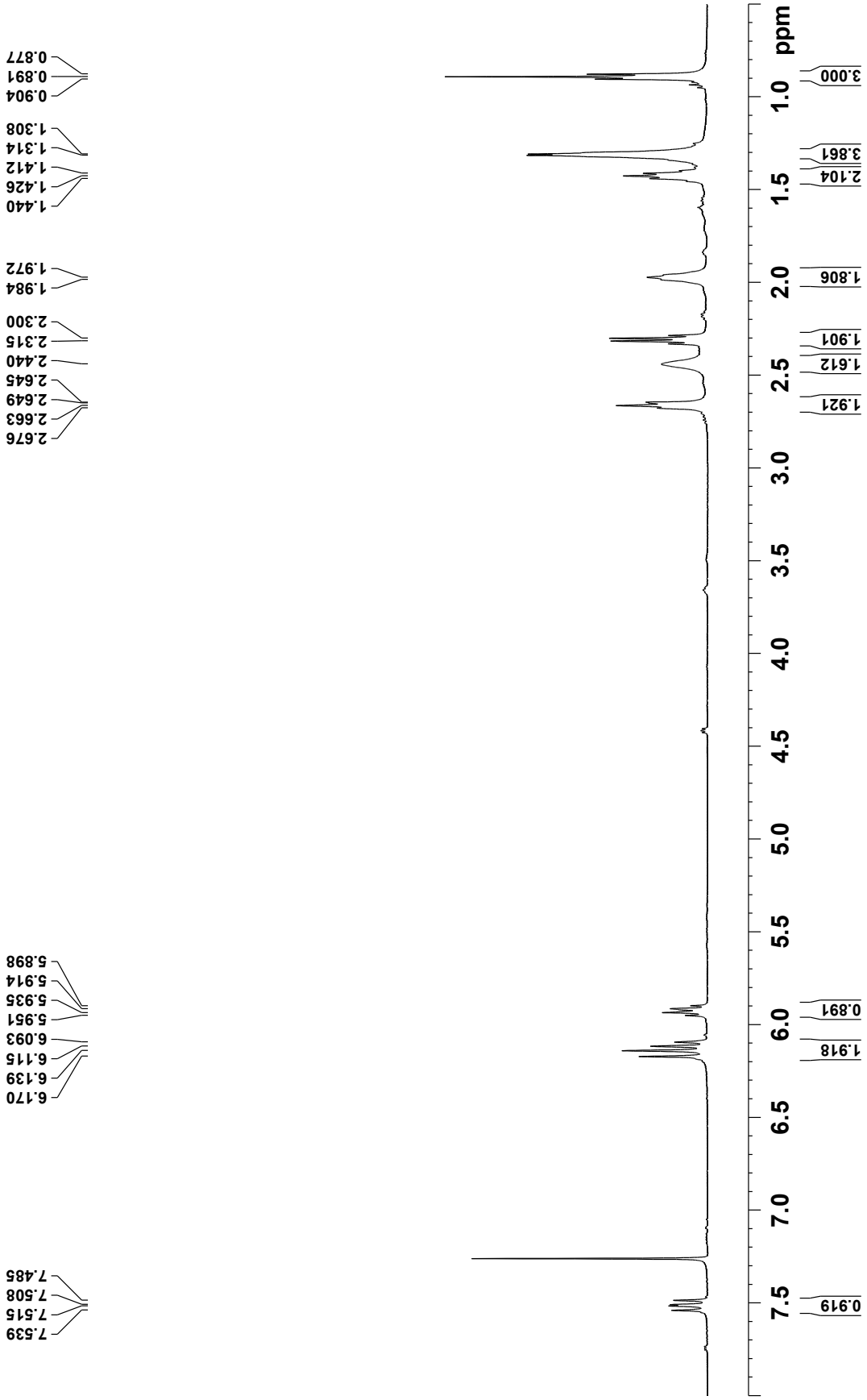


Figure S5. ^{13}C NMR spectrum of **1** (125 MHz, $CDCl_3$)

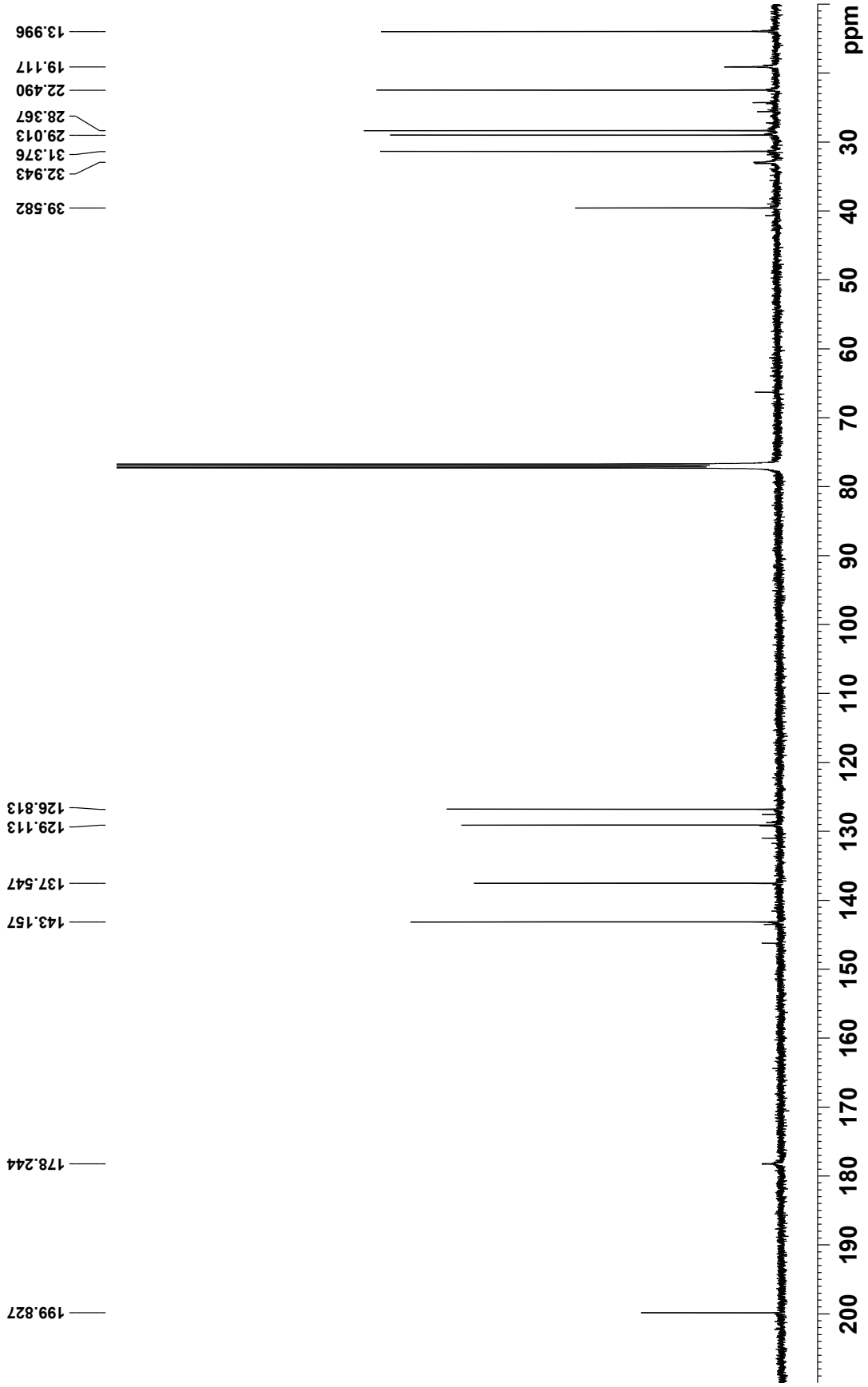


Figure S6. DEPT135 spectrum of **1** (125 MHz, $CDCl_3$)

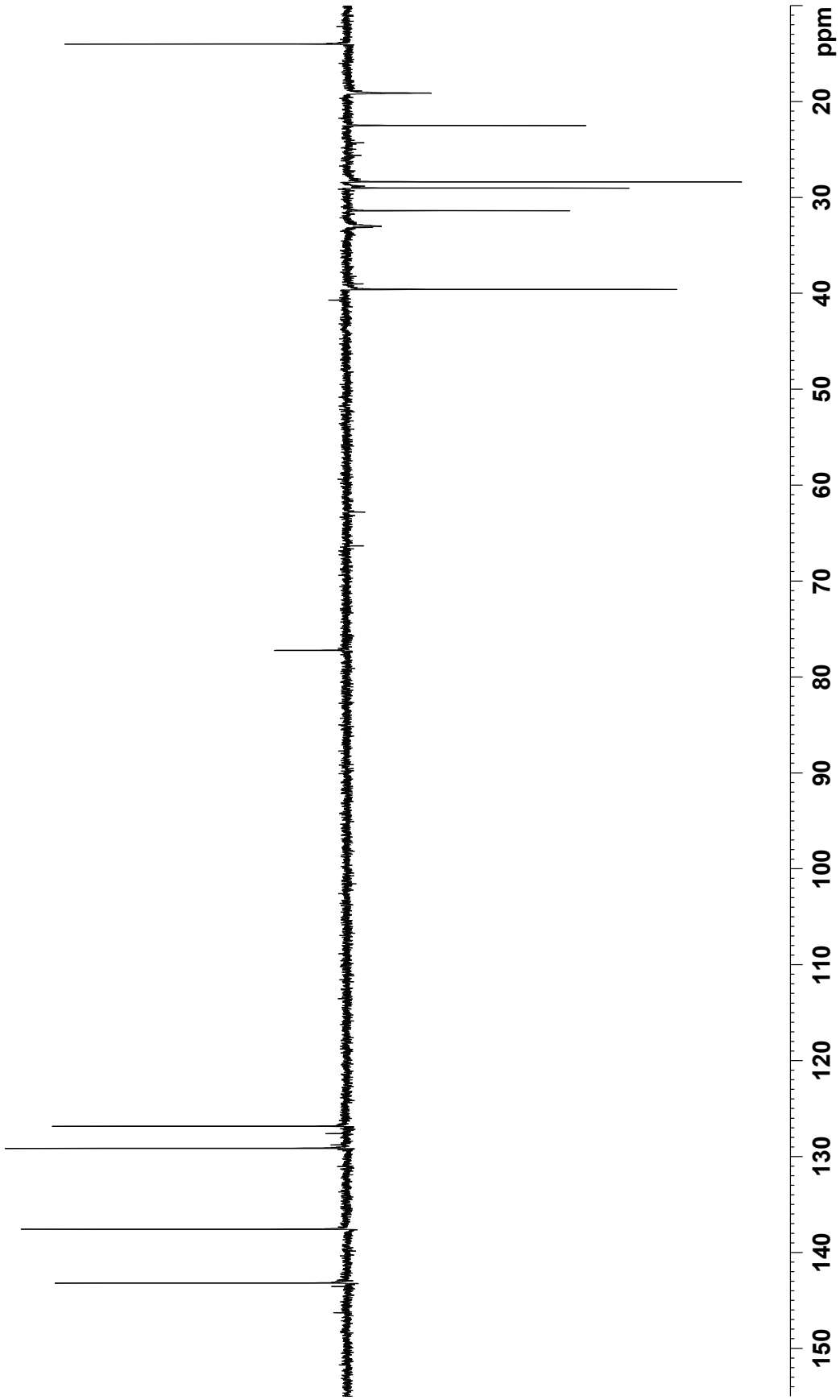


Figure S7. COSY spectrum of **1** (500 MHz, $CDCl_3$)

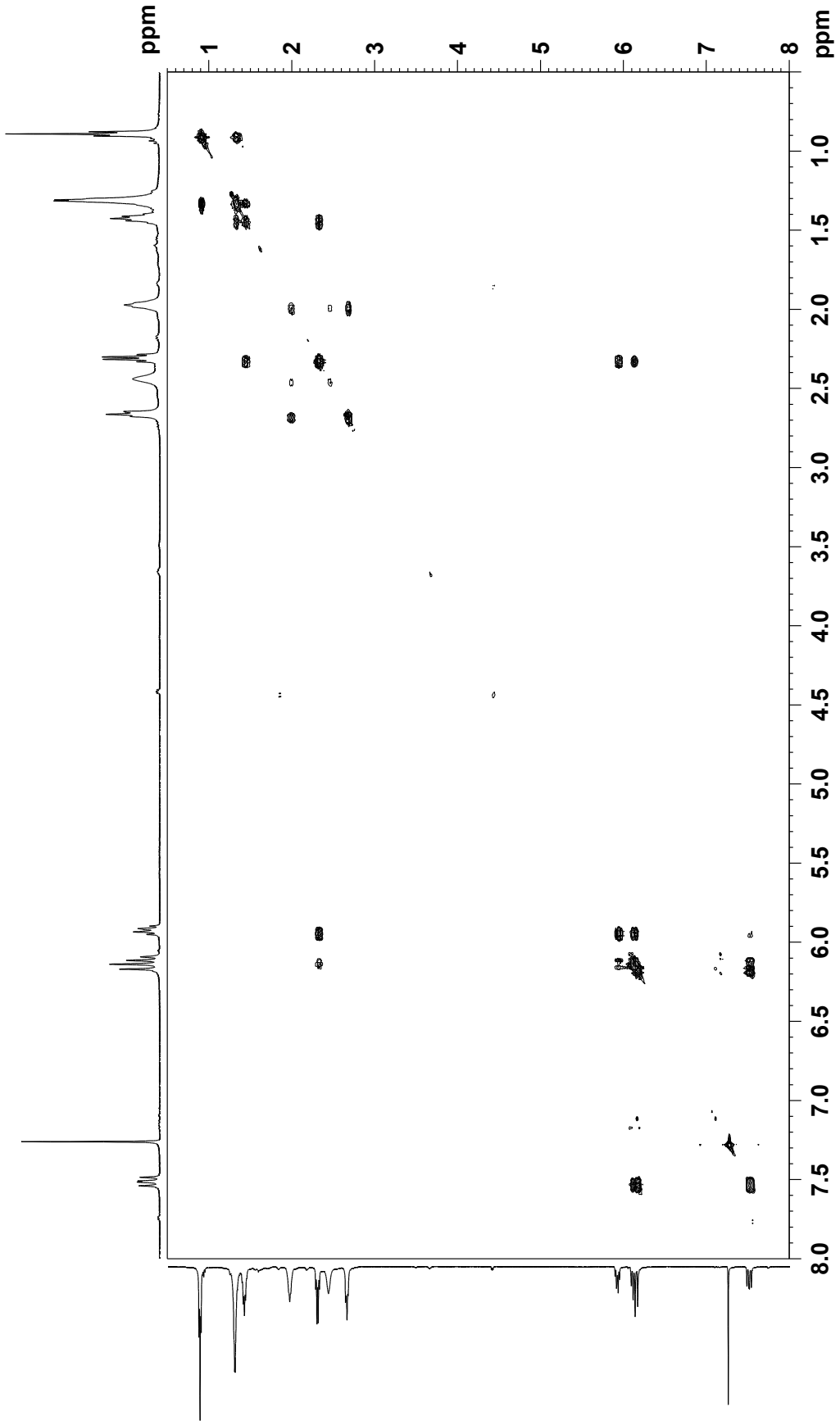


Figure S8. HSQC spectrum of **1** (500 MHz, $CDCl_3$)

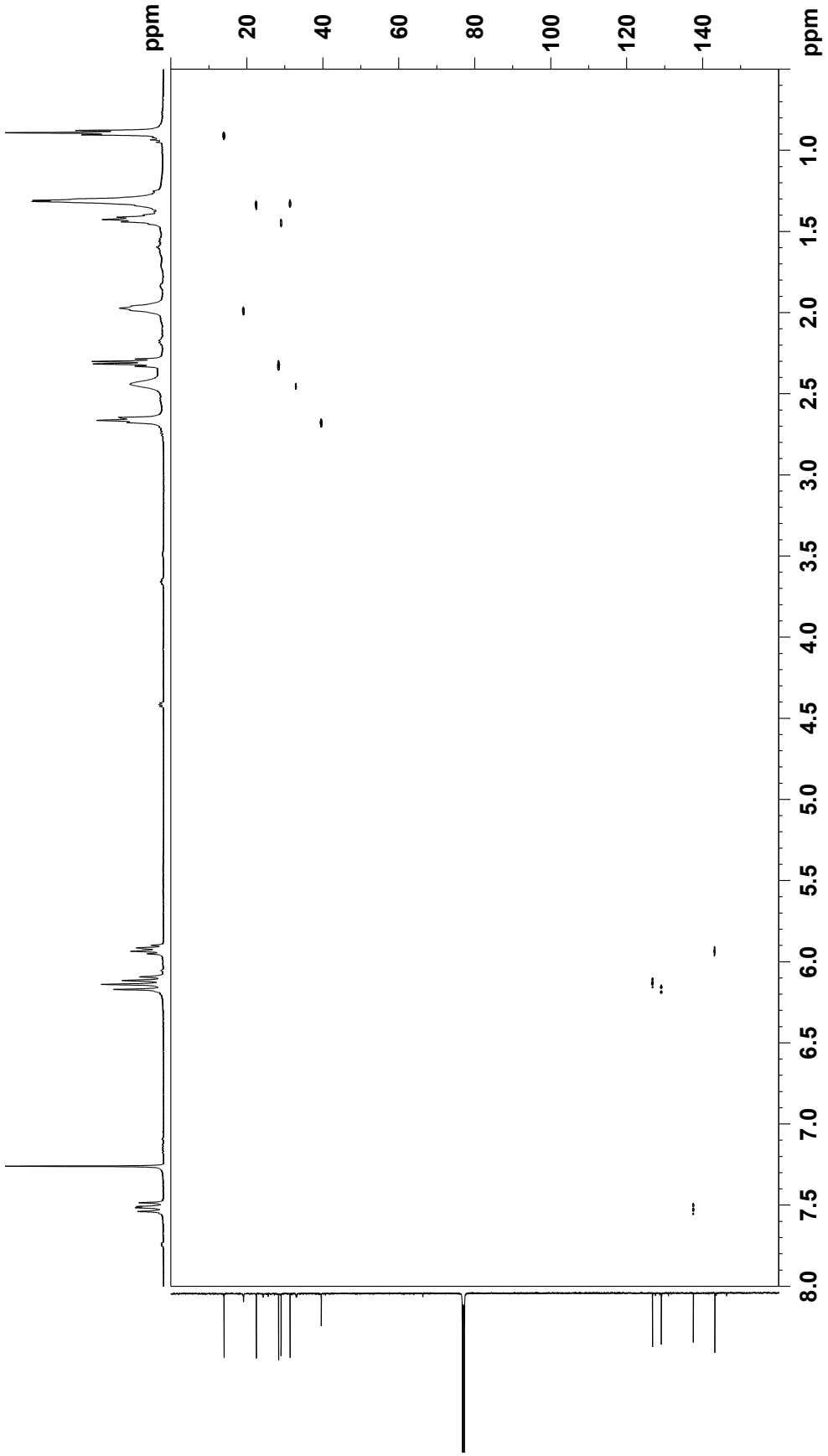


Figure S9. HMBC spectrum of **1** (500 MHz, $CDCl_3$)

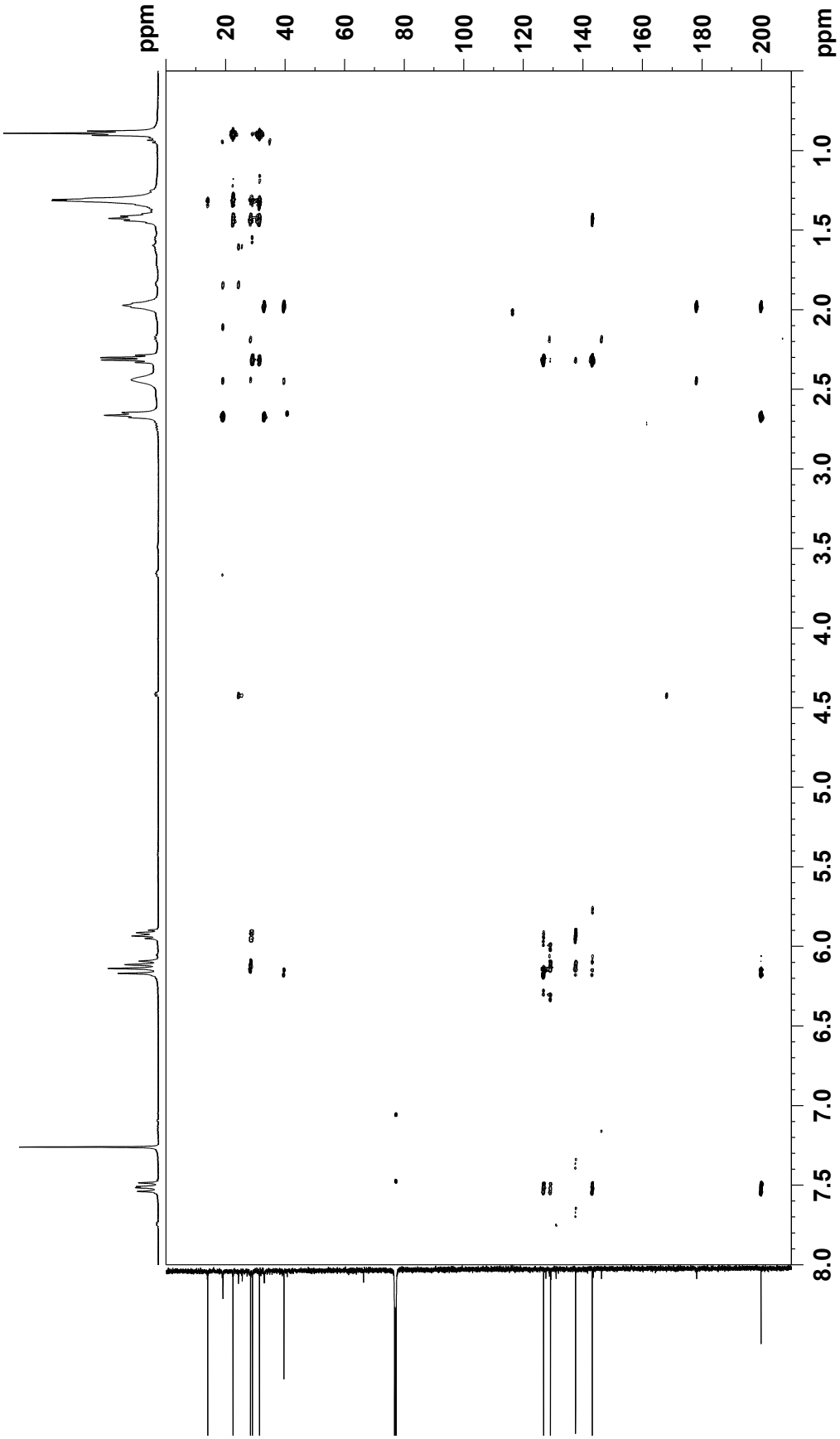


Figure S10. ^{1}H NMR spectrum of **2** (500 MHz, $CDCl_3$)

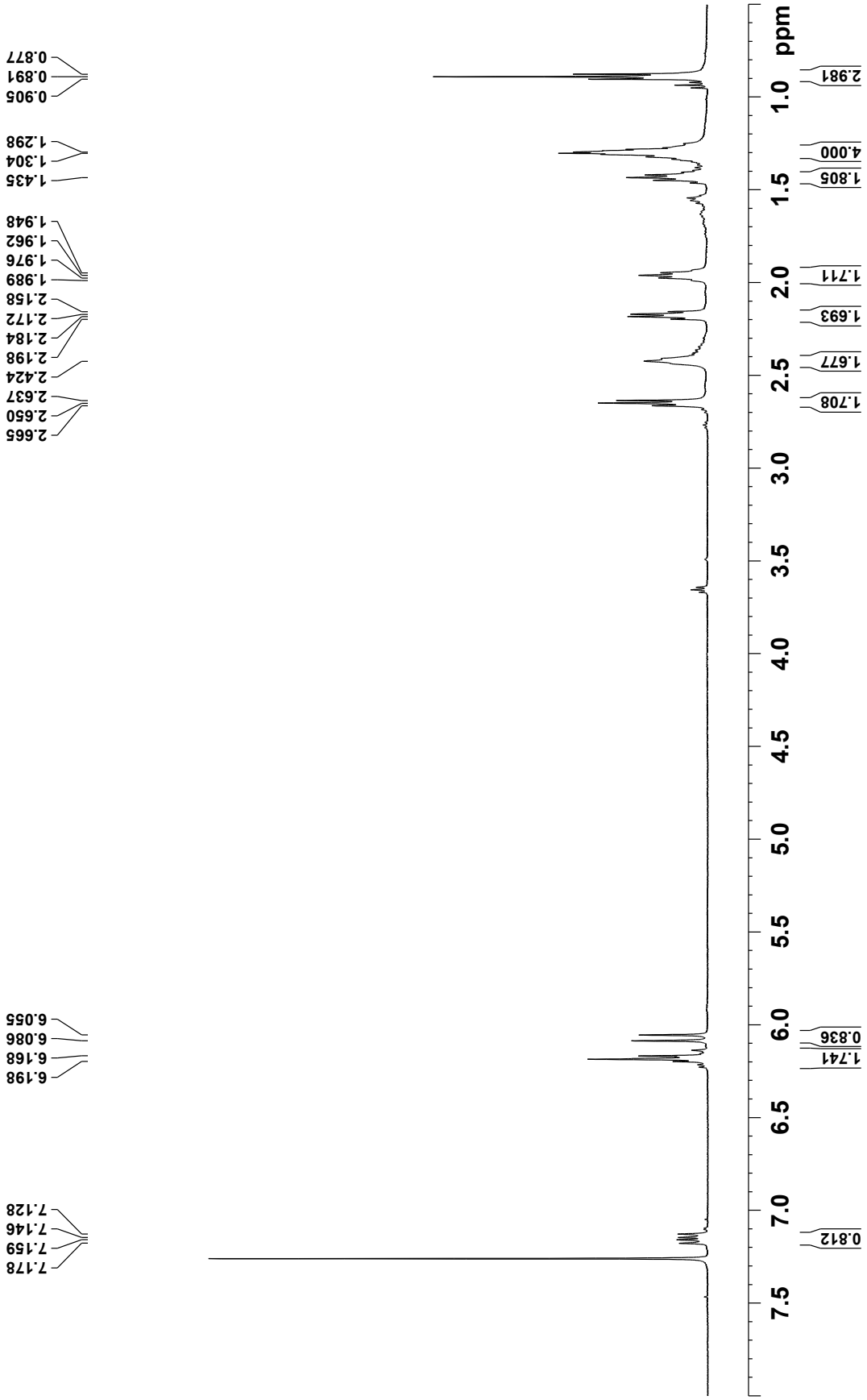


Figure S11. ^{13}C NMR spectrum of **2** (125 MHz, $CDCl_3$)

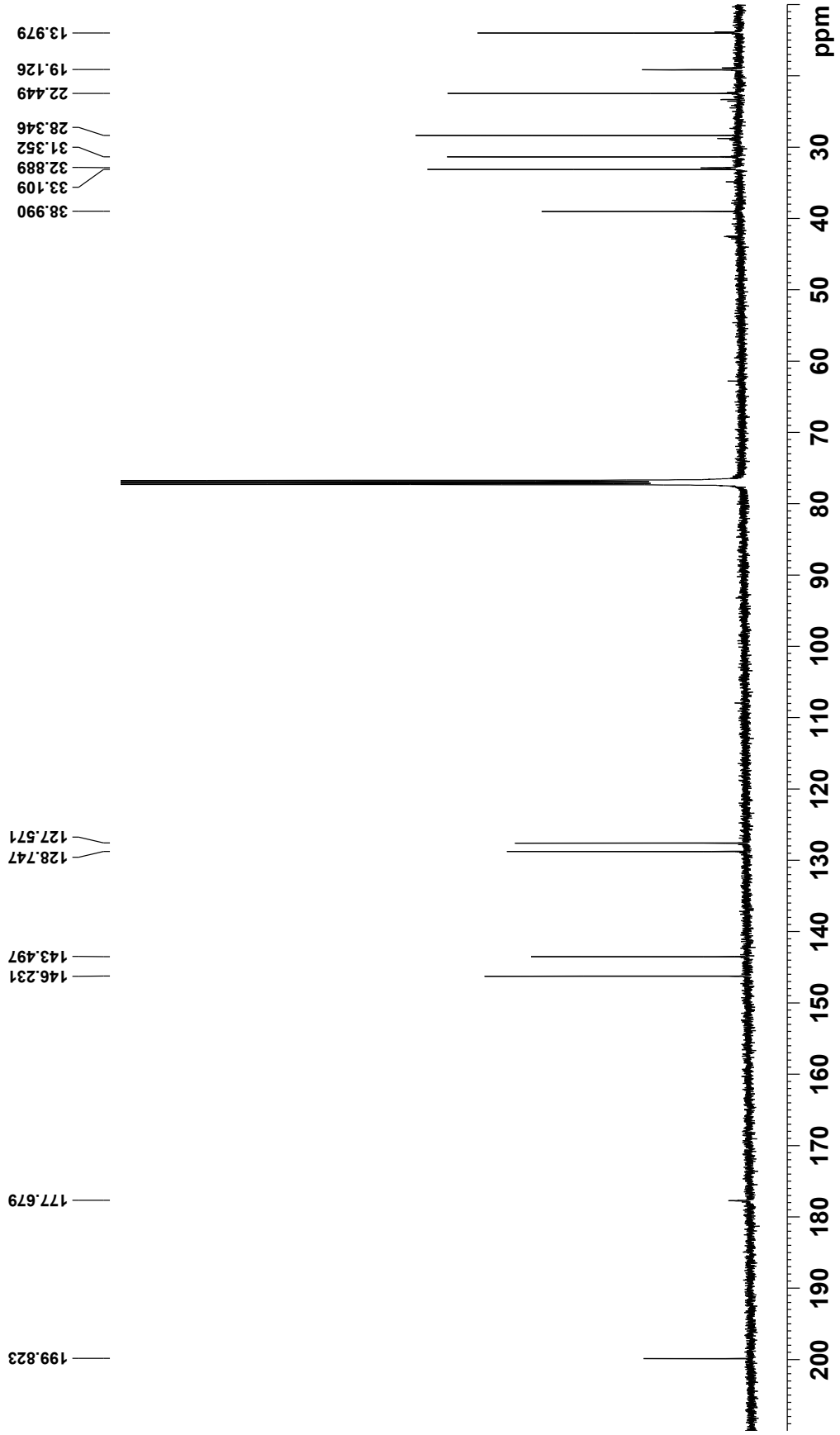


Figure S12. DEPT135 spectrum of **2** (125 MHz, $CDCl_3$)

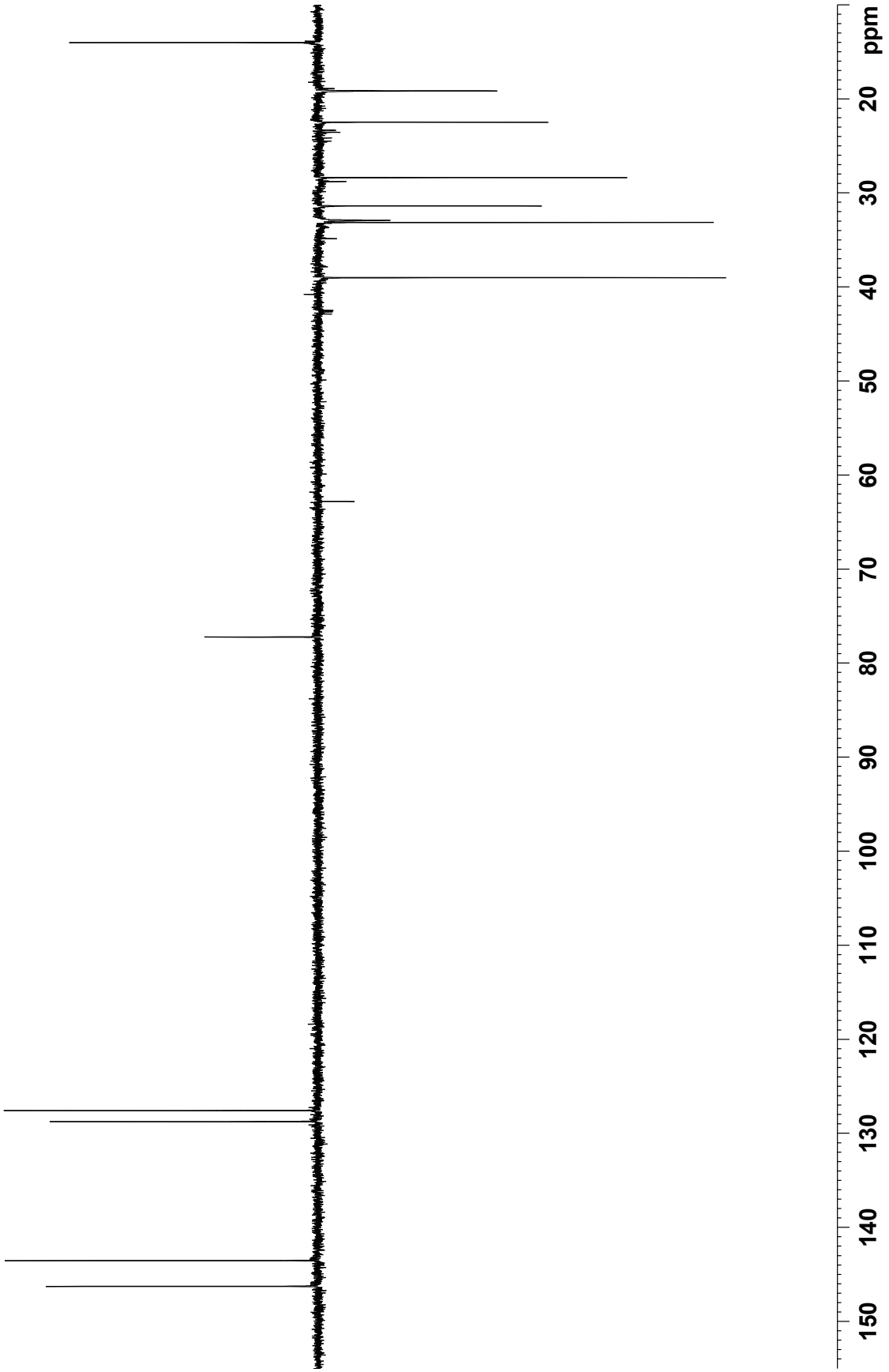


Figure S13. COSY spectrum of **2** (500 MHz, $CDCl_3$)

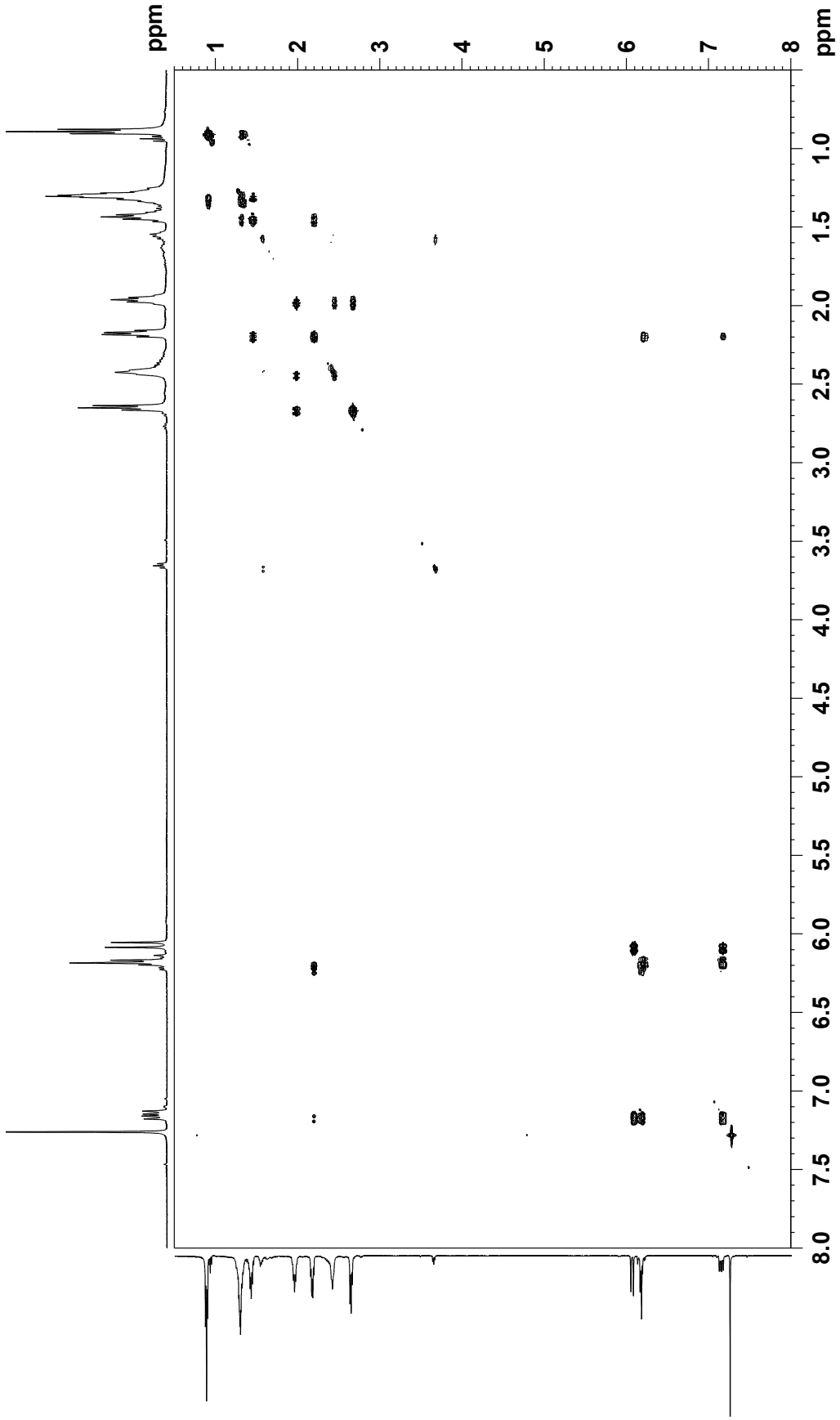


Figure S14. HSQC spectrum of **2** (500 MHz, $CDCl_3$)

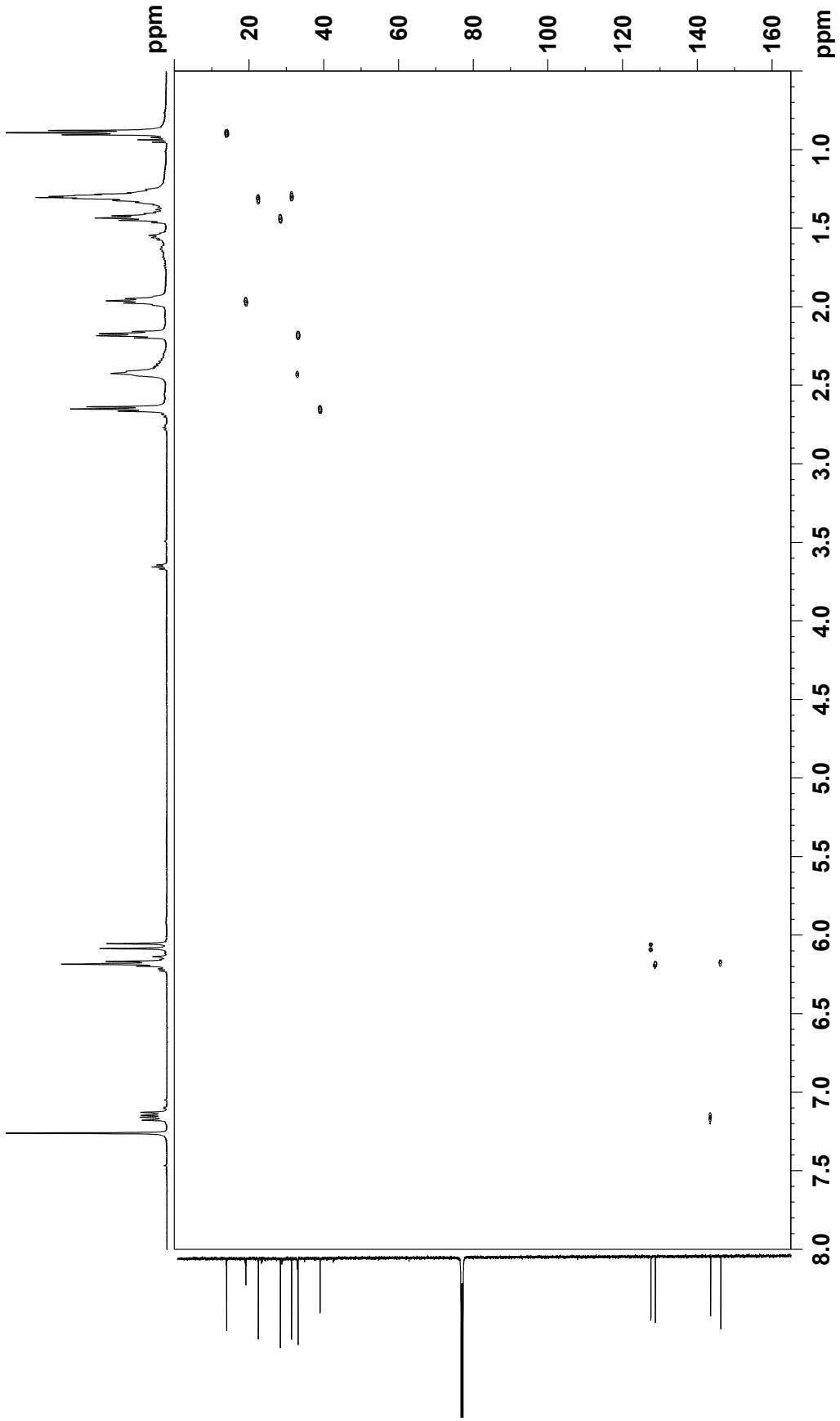
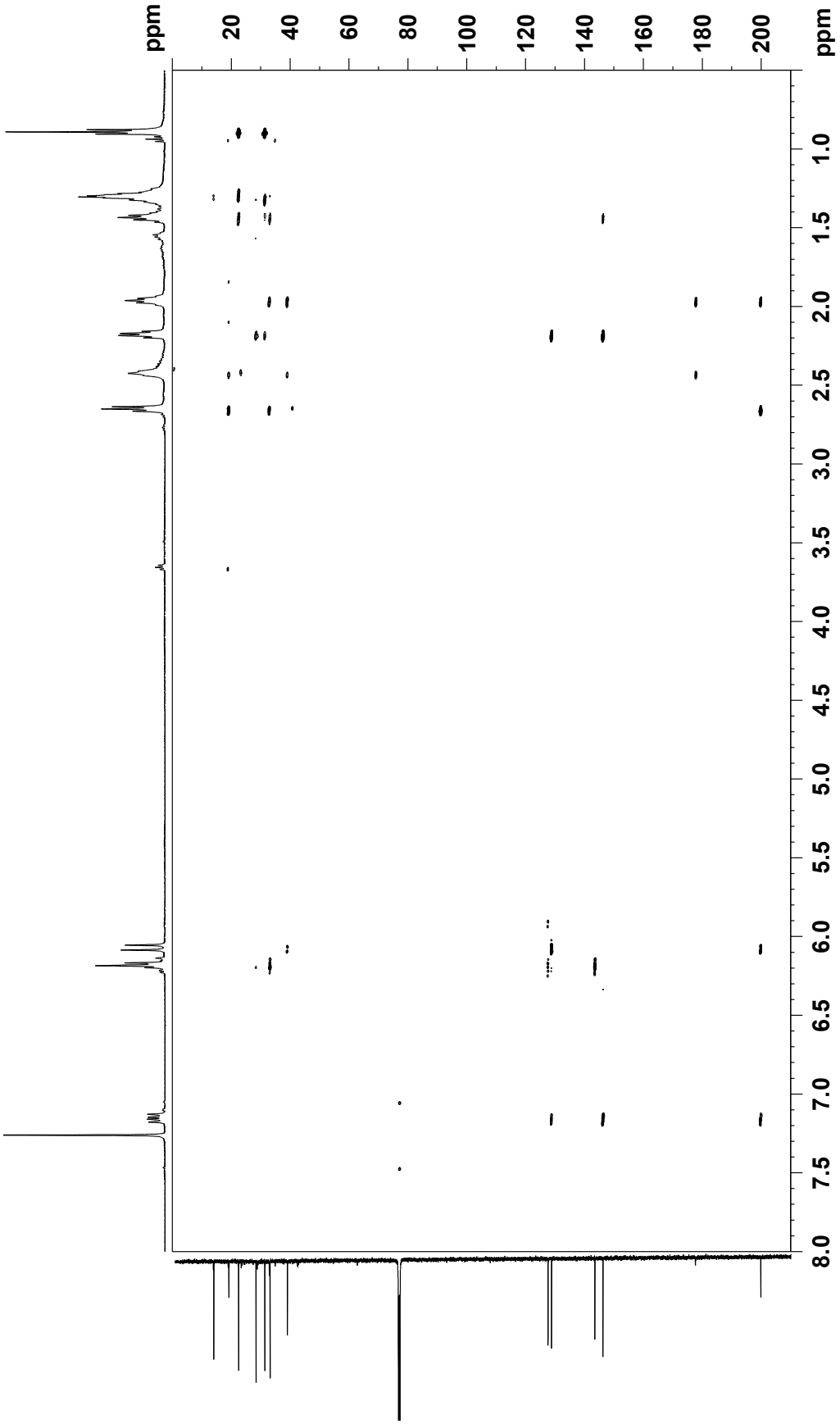


Figure S15. HMBC spectrum of **2** (500 MHz, $CDCl_3$)

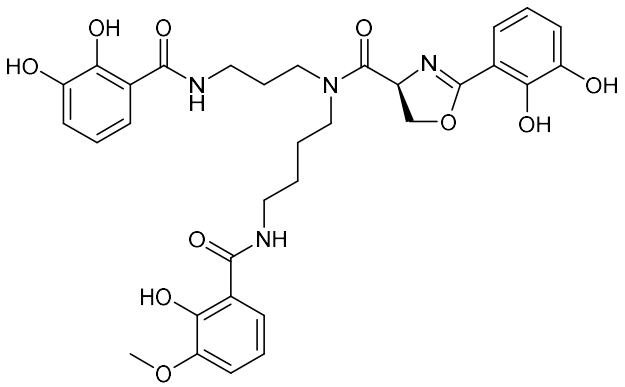


CHAPTER 6

Conclusion

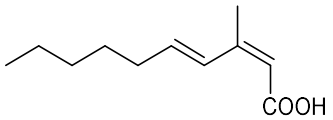
Stony corals and their associated microorganisms are largely neglected for natural product screening program. This study was conducted as our preliminary investigation to evaluate the coral-associated bacteria in terms of their ability to produce new bioactive metabolites. For the isolation purpose, eighteen aquacultured stony corals were used and 278 bacterial strains were isolated. Coral-associated bacteria were isolated by serial dilution and spread plate technique methods using Marine Agar and isolated pure strains were preserved at -85ºC in 20% glycerol in Marine Broth. HPLC-UV chemical screening was employed to find new compounds from bacteria associated with corals. Isolated bacterial strains were screened using three types of seawater based fermentation media (A3M, A11M and A16) supplemented with HP-20. The non-ionic polymeric adsorbent, HP-20 was added to all fermentation media in order to increase titres by sequestering metabolites, and preventing degradation or decrease the cytotoxic effects of final metabolites. In my screening program of coral-associated bacteria, 85 bacterial strains collected from different types of cultured stony corals were used. I selected three strains for further study on the basis of their HPLC chromatogram profile and UV spectrum profile.

In this study, I isolated and determined the structure of four new bioactive compounds from three stony coral-associated bacteria. First compound was designated as labrenzbactin (**1**). It is a catecholate-type of siderophore isolated from fermentation broth of *Labrenzia* sp. C1-1. *Labrenzia* is unexplored genus of marine bacteria and to date including **1** only three bioactive compounds were reported from this genus. *Labrenzia* is obligate marine bacteria and cannot grow in culture media without NaCl. According to biosynthetic gene cluster database, *Labrenzia* sp. possess biosynthetic genes for the production of polyketide, non-ribosomal peptide, and terpenoid. The structure of **1** was determined by spectroscopic analysis including NMR and MS, and absolute configuration was determined by advanced Marfey's method. Compound **1** showed antibacterial activities against plant pathogen *Ralstonia solanacearum* and *Micrococcus luteus* with MIC values of 25 and 50 μg/mL, respectively. Labrenzbactin showed weak cytotoxicity against P388 murine leukemia cells with an IC_{50} of 13 μM. Compound **1** is the first reported compound from stony coral-associated bacteria.



Labrenzbactin (**1**)

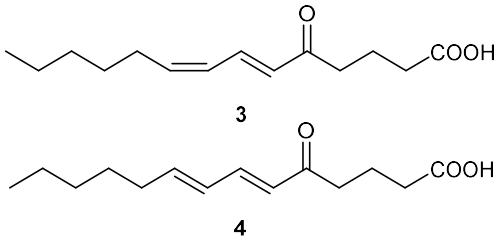
Similarly, HPLC-UV screening of *Microbulbifer* sp. C4-6 collected from stony coral *Porites* sp. led to discovery of a new unsaturated fatty acid, (2Z,4*E*)-3-methyl-2,4-decadienoic acid (**2**), with a unique methylation pattern. The structure of **2** was determined by NMR and MS data. The biosynthesis of **2** was elucidated by incorporation of ^{13}C isotope labeled precursors, [1-^{13}C]acetate and L-[*methyl*-^{13}C]-methionine. Supplementation of ^{13}C-labeled precursors was commenced after 24 h and carried out for four times at 24 h interval. The results revealed that origin of carbon backbone from acetate units, whereas methyl group from methionine. Compound **2** is biosynthetically unique because the carbon which is derived from carbonyl carbon of acetate unit is methylated by methionine via SAM. To the best of our knowledge, **2** is the first example of a simple fatty acid in which the carbonyl carbon of an acetate unit is methylated with SAM. In addition, compound **2** showed weak inhibition against *Saccharomyces cerevisiae* (MIC 100 μg/mL). *Microbulbifer* is marine obligate bacteria and cannot grow without NaCl. Like *Labrenzia*, *Microbulbifer* is also neglected for natural product drug discovery program. According to the genome sequence database, biosynthetic genes for NRPS and siderophore are present in *Microbulbifer* species which offer researchers to consider this genus for the investigation of natural product screening program.



(2*Z*,4*E*)-3-Methyl-2,4-decadienoic acid (**2**)

Chemical screening of fermentation broth of *Micrococcus* sp. C5-9 obtained from stony coral *Catalaphyllia* sp. led to the discovery of two new keto fatty acids, (6*E*,8*Z*)- and (6*E*,8*E*)-5-oxo-6,8-tetradecadienoic acids (**3** and **4**). Strain C5-9 readily grows on seawater-based

culture media as well as distilled water-based media, thus I considered this strain as halotolerant marine bacteria. Chemical structures of **3** and **4** were determined by spectroscopic analysis. Compounds **3** and **4** are unsaturated fatty acids containing ketone group at C5, conjugated with two carbon-carbon double bonds. Double bond configuration of C8 in **4** was determined by assistance of spin-system simulation studies. As microbial metabolites, compounds **3** and **4** are unprecedented, in terms of bearing a 2,4-dienone system. Both **3** and **4** displayed antibacterial activities against plant pathogen *Rhizobium radiobacter* and a fish pathogen *Tenacibaculum maritimum*. In addition, compounds **3** and **4** exhibited agonistic activity against peroxisome proliferator-activated receptors (PPARs) with an isoform specificity towards PPARα/γ. Previous studies revealed that *Micrococcus* associated with sponges are potential source for new bioactive compounds. *Micrococcus* species are ubiquitous in distribution and several compounds were reported from them. However, there is still chance to get new compounds from common bacterial taxa group if we isolated such bacteria from unexplored group of organisms such as stony corals. To date, there is no compounds reported from stony coral-associated *Micrococcus* except compounds **3** and **4**. Keto fatty are relatively rare in nature and usually reported from plant and animal sources.



(6*E*,8*Z*)- and (6*E*,8*E*)-5-Oxo-6,8-tetradecadienoic acids (**3** and **4**)

In this study, seawater-based fermentation media were used for the screening as well as large scale fermentation of bacteria associated with stony corals. Halophilic marine bacteria can thrive at high concentration of salts, which allow them to grow in low-cost seawater-based fermentation media instead of valuable fresh water-based fermentation media. In addition, contamination of fermentation broth can be reduced by using seawater because non-halophile cannot grow in high-salt containing media.

In summary, my research results demonstrate that screening of new natural products needs to be concentrated on unique, extreme, underexplored sources or uncommon microbial species or bacterial species isolated from such sources. However, tons of secondary metabolites were discovered from common type of bacteria recovered from conventional sources. And there is

a high risk of finding already discovered metabolites from common type of bacteria. Researchers should consider to work with common bacterial taxa if recovered from unexploited sources such as stony coral. The present study substantiated that bacteria isolated from unexplored source such as stony corals are a potential source of new bioactive compounds. In addition, bacteria from unexploited taxa could be the source of new compounds, but bacteria from common taxa collected from unexplored sources still can be a potential source of new compounds. Thus, coral-associated bacteria are the promising potential source for the screening of new bioactive metabolites.

Our current knowledge on stony coral-associated bacteria and their metabolites remains quite scarce and fragmented as it is limited to a few genus of bacteria, i.e., *Labrenzia* sp., *Microbulbifer* sp., and *Micrococcus* sp. Further detailed metabolite analysis of stony coral-associated bacteria of various genera needs to be carried out in larger range to estimate their biosynthetic ability to produce new bioactive metabolites and chemical diversity of the structures. In this study, Marine Agar was used as an isolation medium and this medium is very nutrient rich so it can prevent the isolation of slow growing bacteria because fast growing bacteria grow very fast and cover the medium. To address such problem in the future, we can use nutrient deficient media or some selective media to isolate specific group of bacteria such as *Labrenzia* species and *Microbulbifer* species, which already proved themselves as a poteintal source of new bioactive compounds. In addition to selective isolation of targeted bacteria, we can also use genetic approaches to assess their biosynethtic gene clusters to know the predicted types of molecules that can be produced by bacteria. Combination of selective isolation of bacterial strains, and analysis of their biosynthetic gene clusters along with HPLC-UV chemical screening can save time, cost and resources and can speed up the screening program.

Acknowledgements

I would like to express my deepest appreciation for my advisor Professor Yasuhiro Igarashi, Toyama Prefectural University, for his guidance, supervision, continuous encouragement and valuable advice in completing this work.

In addition, I would like to express my gratitude to Assoctiate Professor Naoya Oku, Assistant Professor Enjuro Harunari and Dr. Tao Zhou, Toyama Prefectural University, for their valuable advice and warm encouragement throughout this work.

Acknowledgements are also made to co-authors: Agus Trianto, Diponegoro University, Indonesia, and Nobuyasu Matsuura, Okayama University of Science, Japan.

I greatly appreciate all members of Microbial Engineering Laboratory, Biotechnology Center, Toyama Prefectural University.

I would like to thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for the Japanese government (Monbukagakusho) scholarship, which supported my stay at Toyama Prefectural University. Also I am grateful to Professor Yasuhiro Igarashi, and the former president Masaru Ishizuka, Toyama Prefectural University for giving me an opportunity to apply for the Monbukagakusho scholarship.

Finally, I extend my gratitude to my parents and family members for their prolonged inspiration, continue support, love and patience make this work possible and successful.

Publication List

1. Labrenzbactin from a coral-associated bacterium *Labrenzia* sp.

   Amit Raj Sharma, Tao Zhou, Enjuro Harunari, Naoya Oku, Agus Trianto and Yasuhiro Igarashi

   The Journal of Antibiotics 72: 634-639 (2019)

2. Isolation and biosynthesis of an unsaturated fatty acid with unusual methylation pattern from a coral-associated bacterium *Microbulbifer* sp.

   Amit Raj Sharma, Enjuro Harunari, Tao Zhou, Agus Trianto and Yasuhiro Igarashi

   Beilstein Journal of Organic Chemistry 15: 2327-2332 (2019)

3. Two antibacterial and PPARα/γ-agonistic unsaturated keto fatty acids from a coral-associated actinomycete of the genus *Micrococcus*

   Amit Raj Sharma, Enjuro Harunari, Naoya Oku, Nobuyasu Matsuura, Agus Trianto, and Yasuhiro Igarashi

   Beilstein Journal of Organic Chemistry 16: 297-304 (2020)