# AN ABSTRACT OF THE THESIS OF

Yan Sheng for the degree of Doctor of Philosophy in Pharmacy presented on June 19, 2015

Title: Bioactive Microbial Natural Products from Unique Indonesian Black Water Ecosystems.

Abstract approved:

_____

Taifo Mahmud

The discovery of new antibiotics reached its peak during the golden era of antibiotics in the mid 20$^{th}$ century and continued to thrive until the 1980s. However, after more than 50 years of fruitful exploration and exploitation of microbial natural products the discovery rate of new antibiotics has dropped drastically in recent decades. On the other hand, known compounds were re-discovered again and again, possibly from related producing organisms, making natural products discovery an expensive and time-consuming endeavor. Therefore, in recent years, efforts for discovering new natural products have been redirected towards organisms from unique and untapped areas of the globe with remarkable biodiversity potential and/or extreme environment, such as deep-sea vents, caves, and other unusual ecosystems. This dissertation describes our efforts to discover new bioactive natural products from microorganisms isolated from a unique black water ecosystem in Indonesia.

Indonesia is recognized as a major center in the world for biodiversity and is one of the countries with the highest level of endemic species and microorganisms

that live in its various ecosystems. Among them are the “black-water” rivers, which flow in the steamy jungles of islands along the Indonesian archipelago, providing unique environments for its flora and fauna. In collaboration with scientists at the Indonesian Center for Biodiversity and Biotechnology (ICBB), we investigated bioactive secondary metabolites of microorganisms from the soil samples of a black water ecosystem on Borneo Island, Indonesia. 16S rRNA sequence analysis was used for the identification of the bacteria, and from 141 bacterial strains investigated, 77% were *Streptomyces*, 6% were non-*Streptomyces* actinomycetes, and 17% were non-actinomycete strains. About one third of the strains were screened for their bioactive secondary metabolites production. The EtOAc and *n*-BuOH extracts of the culture broths of the bacteria were subjected to antibacterial and antifungal assays, as well as mass spectrometry analysis. More than half of the screened *Streptomyces* (18 out of 24) showed activities against one or more pathogenic microorganisms, whereas only 2 out of 14 of the non-actinomycete strains showed antibacterial activities. Furthermore, chemical screening of 9 non-*Streptomyces* actinomycetes revealed that 3 strains, all of which are *Amycolatopsis* spp., produce the anticancer agents the apoptolidins.

Chemical investigation of the strain *Streptomyces albiflaviniger* ICBB 9297 delivered four new elaiophylin-like macrolides and five known elaiophylins. The new compounds have macrocyclic skeletons distinct from those of the known elaiophylins, in which one or both of the dimeric polyketide chains contain(s) an additional pendant methyl group. However, they showed comparable antibacterial activity to elaiophylin against *Staphylococcus aureus*. Interestingly, those with the additional

pendant methyl group on only one of the polyketide chains showed significantly increased activity against *Mycobacterium smegmatis*, whereas the one with two additional methyl groups and the known elaiophylin analogues showed no activity. The production of the new analogues suggests that the last acyltrasferase (AT) domain of the elaiophylin polyketide synthases has relaxed substrate specificity. Bioinfomatic analysis of the conserved active site residues within the AT domains revealed an unusual amino acid sequence of the active site motif in the AT7 domain.

Investigations of the apoptolidin producer *Amycolatopsis* sp. ICBB 8242 led to the discovery of two succinylated apoptolidin analogues and linear apoptolidin A. The two succinylated compounds can inhibit the proliferation and viability of human H292 and HeLa cells. However, in contrast to apoptolidin A, they do not inhibit cellular respiration in H292 cells. This seemingly contradictory results may be due to an alternative biological mechanism of action or due to degradation of succinyl-apoptolidin A to apoptolidin A in the cell viability assay. This hydrolysis may not occur during the real time analysis of cellular respiration, which was performed in a relatively short experimental time. The production of succinylated apoptolidins by *Amycolatopsis* sp. ICBB 8242 suggests that succinylation may play a role in self-resistance and/or as an export mechanism.

# Bioactive Microbial Natural Products from Unique Indonesian Black Water Ecosystems

by
Yan Sheng

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented June 19, 2015
Commencement June 2016

Doctor of Philosophy thesis of Yan Sheng presented on June 19, 2015

APPROVED:

Major Professor, representing Pharmacy

Dean of the College of Pharmacy

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yan Sheng, Author

# ACKNOWLEDGEMENTS

First and foremost, I would like to convey my sincere gratitude to Dr. Taifo Mahmud for giving me the opportunity to join his research group and for introducing me to the world of natural product discovery. His supervision, enthusiasm, encouragement, support and dedication were and are still invaluable to me.

I am so thankful to my advisory committee members Dr. Mark Zabriskie, Dr. Philip Proteau, Dr. Jane Ishmael and Dr. Adrian Gombart for their generous time, support, advice and constructive criticism throughout my graduate career.

Many thanks are also extended to many scholars, postdocs and students who have contributed to the ICBB project including but not limited to Dr. Dwi Andreas Santosa and Salmah Shahab from Indonesian Center for Biodiversity and Biotechnology, and Dr. Serge Fotso, Dr. Dahai Zhang, Jeffrey Serrill, Nick Lowery and Phillip Lam from College of Pharmacy at Oregon State University.

I am also thankful to all former and current members of the Mahmud group for their friendship, encouragement, and for generous sharing their expertise with me and creating a positive atmosphere for learning and working. I would like to acknowledge the friendships of the members of the McPhail, Zabriskie, Proteau, Philmus, and Sikora groups. I also would like to thank the faculty and staff of the Department of Pharmaceutical Sciences for their generous help. I would like to specially thank the NMR facility personnel Rodger Kohnert and Stephen Huhn.

I am greatly indebted to my family, especially my parents for their love and unconditional support throughout the years. Many thanks for my husband for his encouragement and patience throughout the entire process.

# CONTRIBUTION OF AUTHORS

Drs. Taifo Mahmud, Philip Proteau and Mark Zabriskie designed the study. The microorganisms isolated from soil samples collected on the island of Borneo (aka Kalimantan) were kindly provided by Dr. Dwi Andreas Santosa at the Indonesian Center for Biodiversity and Biotechnology (ICBB). Glycerol stock cultures were prepared by Dr. Dahai Zhang. Nick Lowery performed the 16S rRNA sequencing and analysis described in Chapter 2. Phillip Lam assisted with the isolation of elaiophylin analogues described in Chapter 3 and contributed to isolation and characterization of actinomycin analogues. Dr. Serge Fotso contributed the initial discovery of the new apoptolidin analogues described in Chapter 4. Jeffrey D. Serrill performed the biological activity tests for cancer cell viability and cellular respiration under the guidance of Dr. Jane Ishmael. Dr. Jane Ishmael and Jeffrey D. Serrill contributed to writing and editing the paragraphs for biological activity described in Chapter 4. Yan Sheng performed all other experiments. Yan Sheng and Dr. Taifo Mahmud analyzed the data and wrote the manuscripts for Chapters 3 and 4. Drs. Philip Proteau and Mark Zabriskie edited the manuscripts.

# TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

# LIST OF FIGURES

Figure Page

# LIST OF FIGURES (Continued)

Figure Page

# LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES (Continued)

Figure Page

# LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

Table Page

LIST OF TABLES (Continued)

Table Page

# CHAPTER ONE

## General Introduction

Yan Sheng

### 1.1. Overview of Natural Products

For thousands of years, humans have been taking advantage of natural products for their health benefits and used them as medicines to treat diseases and promote health. There are tremendous amounts of written evidence found in many regions of the world describing the use of natural products as traditional medicines in the forms of herbal remedies, potions, oils, etc. The earliest known records of natural products was written on a 4000 year old Sumerian clay tablet, which described the use of remedies for various illness.[1] Clearly Nature was the major source for ancient civilization to cure and prevent diseases. For instance, the oils from *Cupressus sempervirens* (cypress) and *Commiphora* species (myrrh) were used to treat coughs, colds and inflammation.[2] The ancient Chinese people believed that dried ginseng roots were beneficial for patients that were recovering from their illnesses. In fact, nowadays dried ginseng roots are still being used for various purposes, such as boosting the immune system, improving memory and increasing work efficiency. In addition, the extracts of ginseng roots have been frequently used as ingredients for skin and health care products.

Traditionally, natural products, such as herbs, mineral, and materials from animals, were used in the form of extracts or consumed directly with most of the active components unidentified. It was not until the nineteenth century, when pure components from various medicinal plants started to be isolated. Morphine, which is generally believed to be the first active ingredient associated with a medicinal plant, was isolated from *Papaver somniferum* by Friedrich Sertürner in 1806.[2] The isolation and usage of pure ingredients, starting with morphine, laid the basis of modern natural product

discovery. Since then, numerous important compounds have been isolated from natural sources and developed as medicines with various pharmacological effects. Ziconotide (Figure 1.1), derived from a toxin of the cone snail *Conus magus*, is an atypical analgesic agent for the amelioration of severe and chronic pain.[3] Taxol (Figure 1.1), isolated from the bark of the Pacific yew tree, is used to treat a number of types of cancers.[4] Artemisinin (Figure 1.1), obtained from the leaves of *Artemisia annua*, is well known for its remarkable antimalarial activity.[5]

In summary, natural products discovery has played a critical role throughout the drug discovery and development history. Over the time period of 1940 and 2010, 85 out of 175 small molecules (~48.6%) approved for cancer treatments are either natural products or directly derived from them. From 1981 to 2010, 65% of the new chemical entities (NCEs) indicated with antibacterial activities were natural products or related to natural products. For all of the small-molecule approved drugs, natural products or their derivatives constitute about 39% of the total number.[6]

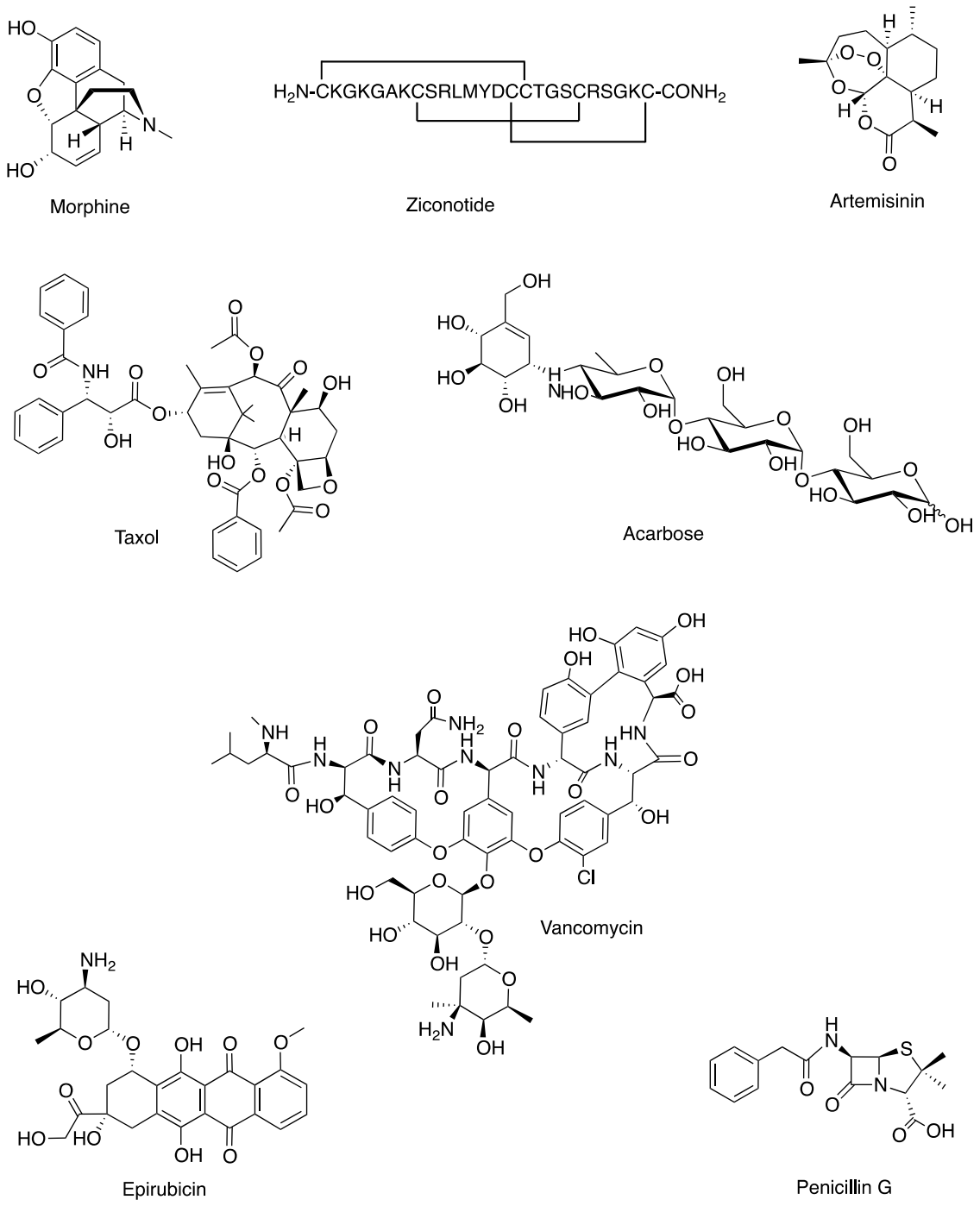


Figure 1.1 Structures of historically-important natural products.

### 1.2. Secondary Metabolites

The term of secondary metabolites is commonly referred to biologically-derived small molecules or natural products (<3000 Daltons), which are not directly involved in the normal growth, development and reproduction of the producing organism. Although the lack of secondary metabolites may not cause immediate death of the organisms (microbes, plants and animals), they play important roles in increasing the likelihood of their survival. Secondary metabolites may be used as weapons against other organisms living in the same environment to compete for limited nutrient source or to protect themselves from enemies. They may also be used as signaling molecules in symbiosis (between microbes and microbes, microbes and plants, insects, or higher animals), biofilm formation, and pathogenicity. Furthermore, secondary metabolites may also serve as sexual hormones, differentiation effectors, and many of them including some antibiotics have been found to be related to spore formation and germination.[7]

### 1.3. Microbial Secondary Metabolites

Microbes, particularly soil bacteria and fungi, are a prolific source of bioactive natural products. About half of structurally characterized natural products (~160,000) were isolated from microbes, whereas most of the rest were from plants.[8] Secondary metabolites produced by microbes have been used clinically to treat various diseases, such as antibacterial (e.g., penicillin, vancomycin), anti-diabetics (e.g., acarbose), and anti-cancers (e.g., epirubicin) (Figure 1.1). Unlike plants, microorganisms had only been extensively explored after the discovery of penicillin (Figure 1.1) in 1929.[8] Since then new bioactive natural products from microbes have been discovered at an amazing

pace, at least until the 1980s. By the end of 2014, more than 23,000 active compounds produced by microbes were reported in scientific and patent literature, 32% are generated by filamentous bacteria, the Actinomyces, while 42% are produced by fungi.[8] In spite of the great number of discovered natural products, the need for new compounds with novel chemical scaffolds and molecular targets remains high, especially for treating infectious diseases, as drug resistant microorganisms have become a major threat to public health.

### 1.4. Biosynthesis of Microbial Natural Products

With the rapid development of molecular biology and bioinformatic techniques, as well as subsequent dramatic growth in biological knowledge, genetics has played a more and more important role in natural product discovery. The first whole genome sequencing for a free-living organism (bacterium, *Haemophilus influenzae*, 1.8 Mbp) was completed by Fleischmann *et al.* in 1995.[9] Back then, the cost for DNA sequencing was about $0.80 per base pair.[10] Nowadays, it is possible to sequence a complete bacterial genome in a few hours with affordable price ($3 × $10^{-7}$ per bp, in 2011).[10] As of 2014, more than 30,000 sequenced bacterial genomes are publically available.[11] The advanced DNA sequencing technology provides new opportunity to explore microbial natural products production at the genetic level, which not only help us understand more about natural products biosynthetic machineries and novel enzyme-catalyzed transformations, but also facilitate the discovery of novel secondary metabolites by “Genome Mining” process. Major chemical classes, such as terpenoids, non-ribosomal/ribosomal peptides and polyketides, are well represented in microbial natural products and have been

extensively studied by the biosynthetic community.

Terpenoids, also known as isoprenoids, constitute the largest group of natural products. More than 35,000 members with diverse structures have been discovered. Biosynthetically they are derived from five-carbon isoprene units joined in a head-to-tail fashion, and thus terpenoid natural products usually contain a multiple of 5C-atoms. Most of the terpenoids were obtained from terrestrial and marine plants as well as fungi. However in the past 5-10 years, as more and more bacteria genome sequence were available, it has become evident that genes encoding terpene synthases are widely distributed in bacteria, especially the soil-dwelling Gram-positive bacteria the actinomycetes.

Peptide natural products are defined as short chains of amino acids linked by peptide bonds. They can be divided into two groups, non-ribosomal and ribosomal peptides, depending on whether they are synthesized by the ribosomes or by the multi-modular proteins called non-ribosomal peptide synthetases (NRPSs). Non-ribosomal peptides (NRP) are usually produced by microorganisms like bacteria and fungi. Ribosomal peptide natural products have been discovered from various sources including bacteria, fungi, plants, eukaryotes (*eg*. cone snails) and archaea. Although non-ribosomal peptide synthetases (NRPSs) had been thought to be the most common biosynthetic machineries to the production of peptide natural products, in recent years, genetic studies and genome sequence data showed that some peptide natural products are ribosomally synthesized and post-translationally modified.

Polyketides are a highly diverse group of natural products with structurally intriguing carbon skeletons. Among different chemical classes of polyketide-derived compounds are polyphenols, macrolides, polyenes, enediynes, and ansamycins. Many important therapeutic drugs belong to this class of compounds, such as the antibiotics erythromycin and tetracycline, the immunosuppressants rapamycin and FK 506, the cholesterol-lowering drug lovastatin, and the anticancer agents epothilone and geldanamycin (Figure 1.2). To date, more than 10,000 polyketides have been identified and most of them are produced by bacteria or fungi.[8]

**1.5. Polyketide Biosynthesis**

Polyketide natural products are generally derived from simple building blocks, such as malonyl- and/or methylmalonyl-CoA, through repetitive decarboxylative Claisen thioester condensations, involving β-ketoacyl synthase (KS), acyltransferase (AT), and phosphopantetheinylated acyl carrier protein (ACP) proteins (Figure 1.3 B). In most cases, additional proteins or domains, such as ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER), are also involved, leading to full or partial reduction of the polyketide chains. Although polyketide synthases (PKSs) and fatty acid synthases (FASs) (Figure 1.3 A) have much in common in terms of their catalytic mechanism and their use of simple precursors as building blocks,[12] PKSs provide much more structurally diverse natural products. This is due to the fact that PKSs can incorporate more diverse building blocks than FASs. Moreover, unlike FASs, which typically produce fully reduced fatty acid chains, PKSs produce more complex patterns of

functionalization. Once the polyketide backbone is synthesized, the chain is released from the protein through different types of reactions, such as lactonization, hydrolysis, esterification/amidation, or reductive release. Subsequent modifications of the resulting carbon skeleton, such as cyclization, C-C bond cleavage, and rearrangement reactions, may generate novel carba- and heterocycles. A recent study also found that a non-canonical polyketide synthase (PKS) module from the endofungal bacterium *Burkholderia rhizoxinica* can generate a branch in the carbon chain through a Michael-type acetyl addition.[13] Finally, the polyketide structure may be decorated through a broad range of tailoring reactions, which include glycosylation, acylation, alkylation, hydroxylation, and epoxidation.

Based on the architecture of the protein(s) and the way the polyketide is assembled, PKS can be classified into type I, type II and type III. In addition, PKSs are also classified as iterative or non-iterative, depending on whether or not each KS domain catalyzes more than one elongation cycle.[14]

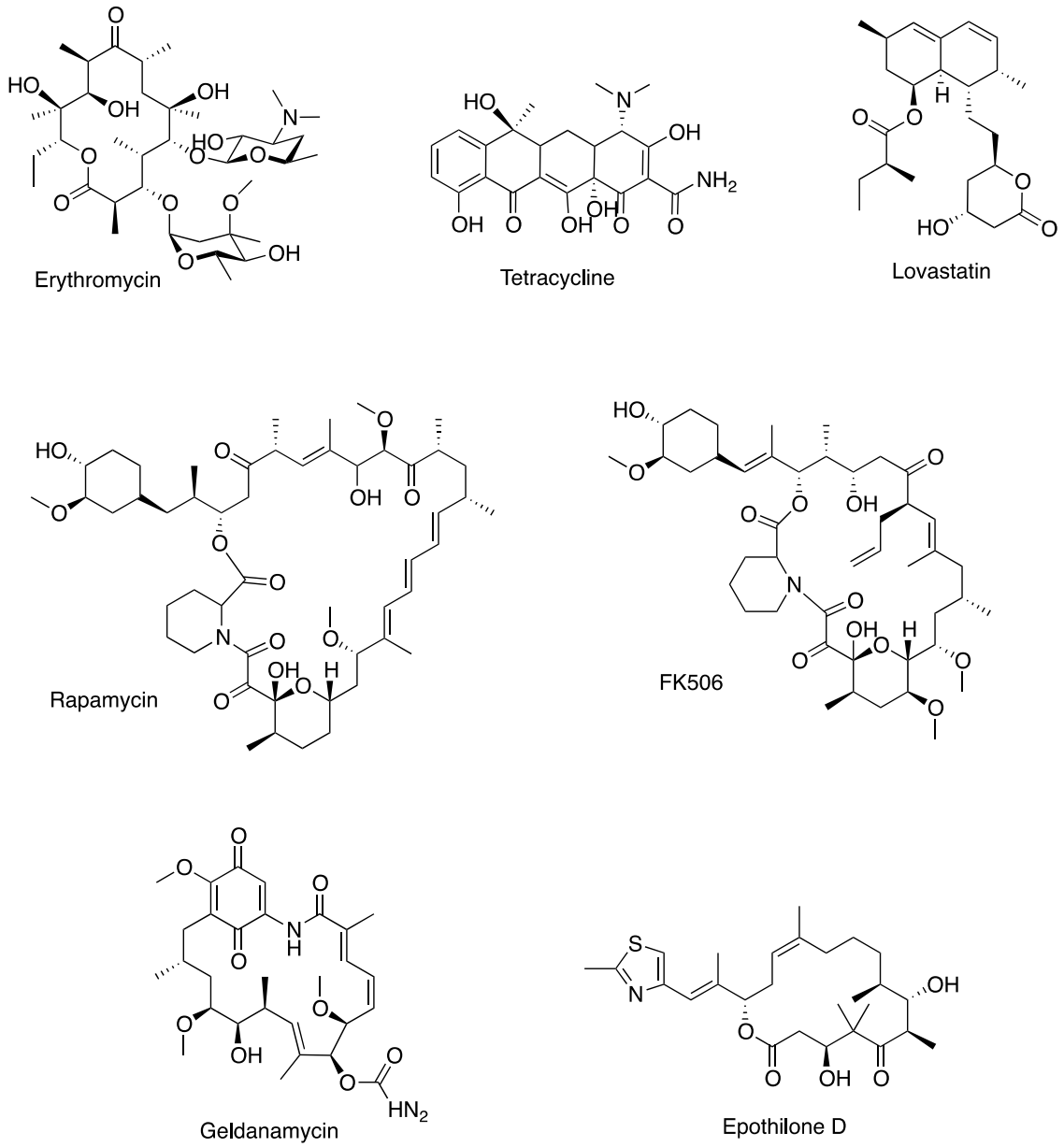


Figure 1.2 Chemical structures of polyketide natural products.

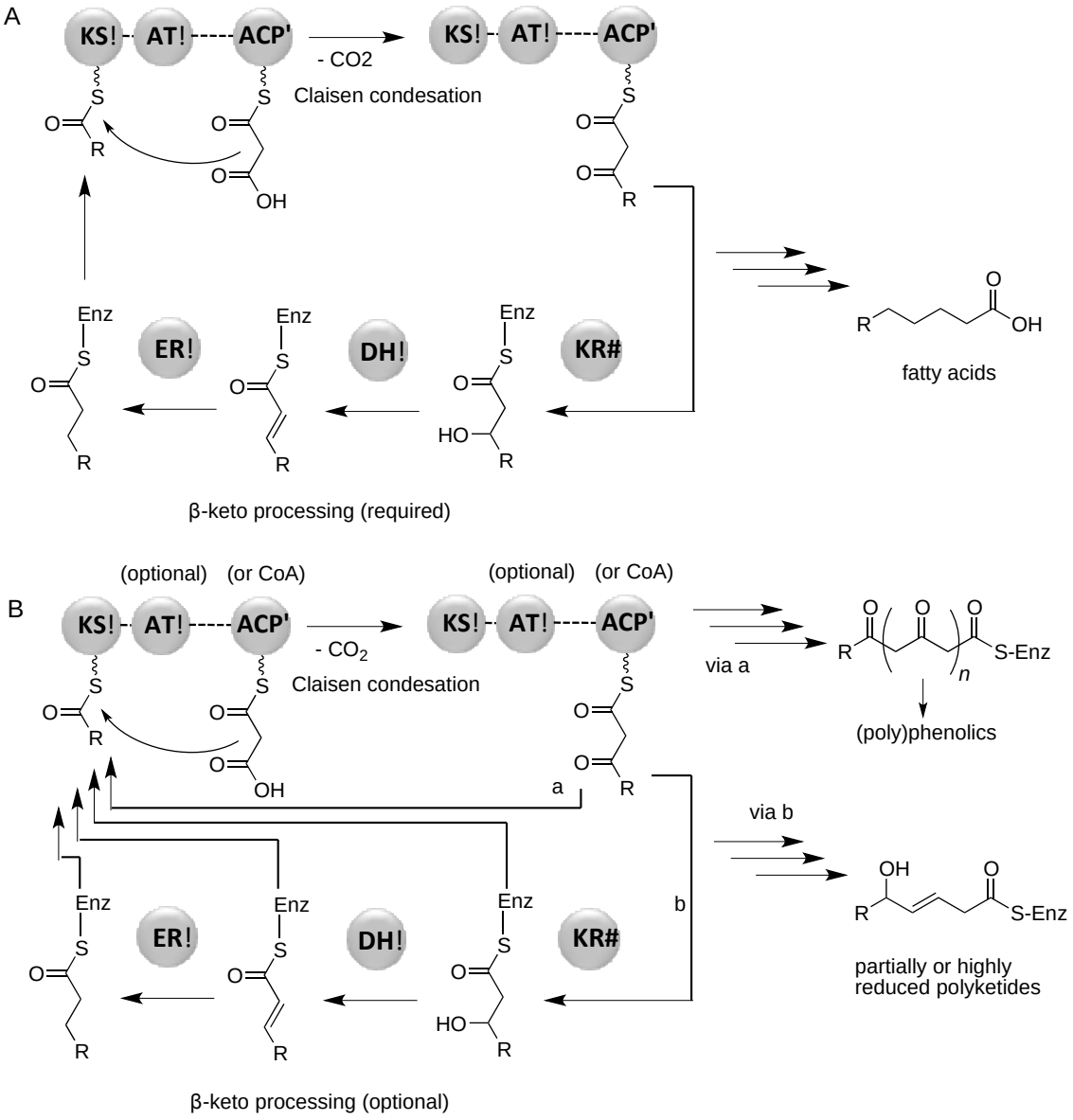


Figure 1.3 Basic mechanisms involved in fatty acid (A) and polyketide (B) biosynthesis. Enz = enzyme.[15]

### 1.5.1. Noniterative (modular) type I PKS

Noniterative type I PKSs (modular PKSs) such as the archetypal 6-deoxyerythronolide synthase (DEBS) (Figure 1.4)[16] are large multifunctional enzymes mainly found in prokaryotes. Modular polyketide biosynthetic pathways usually consist of several large

polyketide synthase proteins, each containing linearly arranged and covalently fused catalytic domains. A set of KS, AT, and ACP domains, as well as optional beta-keto processing domains, required for accomplishing one single elongation cycle, are grouped together to form a module. To make a polyketide chain, the starter unit or the growing acyl chain from the previous module needs be loaded onto the KS domain. An extender unit is selected and loaded to the ACP via the acyltransferase and incorporated to the polyketide chain through a ketosynthase catalyzed Claisen condensation. In each module, optional ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains can further modify the β-keto group resulting partially reduced or fully reduced product. Finally, the growing polyketide chain reaches the last module containing a thioesterase domain (TE), which catalyzes the chain release from the covalent attachment to the assembly line by hydrolysis or macrocyclization.

As the consequence of the co-linearity principle of modular PKSs, the chain length and the degree of reduction of their products can usually be predicted based on the order and architecture of their modules. This principle has been utilized in the "genome mining" strategy to discover novel polyketides,[17,18] as well as for rational engineering of type I PKS to generate novel non-natural products.[19,20] Besides the canonical model of modular PKS, a distinct multimodular PKS type, the trans-AT PKS, was also found present in bacteria. Unlike the textbook modular PKS (cis-AT PKS), the knowledge of which was mainly derived from the studies of PKS present in soil dwelling filamentous actinomycetes, most of the trans-AT PKS were discovered through the investigation of secondary metabolism of bacteria from more unusual

habitats or taxa (*eg*. *Bacillus subtilis*, *Pseudomonas fluorescens* and myxobacteria). Modules in these enzymes lack individual AT domains and the ACPs are loaded by freestanding ATs. Each gene cluster contains only one to three ATs, either present as individual genes fused as tandem ATs or fused with a trans-acting enoyl reductase domain (ER)[21, 22]. Standalone ATs are used iteratively for each module and usually malonyl-CoA was incorporated as building blocks.

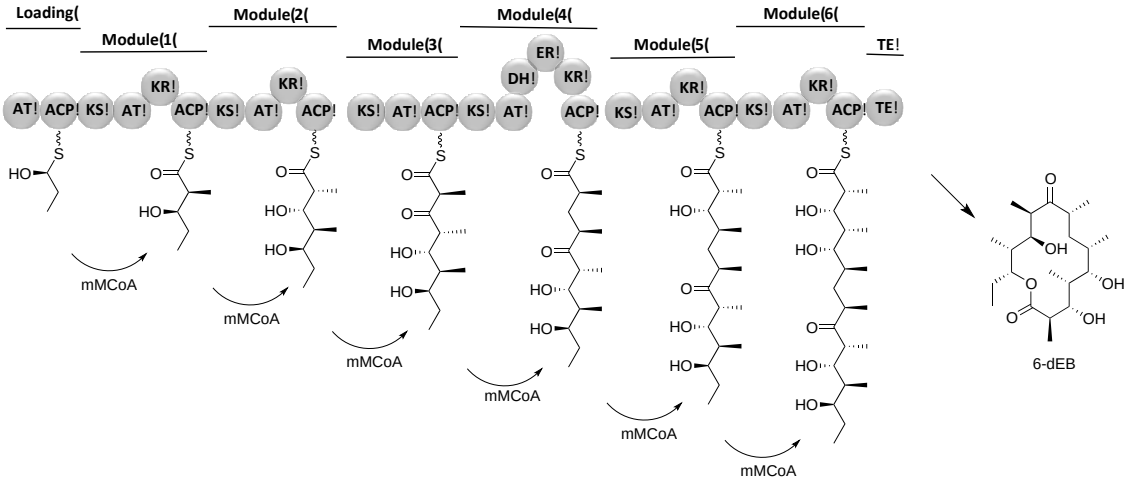


Figure 1.4 The PKS assembly line for 6-deoxyerythronolide (6-dEB).[15]

### 1.5.2. Prediction of Substrate Specificity of Modular PKS

A series of programmed events occur before, during, and after chain assembly lead to the impressive diversity of polyketide structures. In all cases, the determinant factors of the structure diversity are the type and number of biosynthetic building blocks employed. Various extender units can be used to make a polyketide backbone, but malonyl-CoA and methylmalonyl CoA are the most common substrates. For modular PKS, the selection and incorporation of a thioester derivative is controlled by the acyltransferase domain, the specificity of which correlates well with its conserved

amino acid residues.

Bioinformatic analysis of 187 AT domains in PKS and FAS proteins indicated that AT domains with the same substrate specificity usually fell into the same cluster.[23] By aligning the amino acid sequences of AT domains from PKSs with those from *E. coli* FAS, 13 putative active site residues in PKS AT domains were identified. Nine of those active site residues were completely conserved in most of the analyzed AT domains (181 out of 187) (Table 1.1, 1.2).[23] Eleven additional conserved positions, which were identified as specificity determining residues, were also compared and the results indicated that four residues (93, 197, 198, 200) showed more correlation with substrate specificity and they were only conserved in AT domains from the same group (Table 1.1, 1.2).[23] Among them, amino acid residue 200 has been proposed to be crucial for substrate selection as it is highly conserved within malonate or methylmalonate specific AT domains and the amino acid residue in each group is significantly different (F in malonate specific ATs, S in methylmalonate specific ATs).

In addition, molecular modeling of the active site of the proteins and the substrates suggests the crucial role of the amino acid residue 200 in substrate selection.[23] This has also been confirmed by site directed mutagenesis studies. In a study performed by Reeves *et al.*, three regions in or near the active site of a methylmalonate specific domain (AT4) from DEBS were mutated to the malonyl-CoA specific motif.[24] The results showed that mutations of all three identified motif did affect substrate specificity of the domain, though to varying extents. Of these three

regions, the S200F and Y198H mutants displayed the highest altered selectivity. Consequently, the conserved four consecutive amino acid residues (198-201) motif was proposed to play an important role in substrate specificity. Methylmalonyl-CoA specific ATs usually contain a YASH motif, whereas malonyl-CoA specific ATs have a HAFH motif (Table 1.3). Mutation of the YASH motif in AT1[25], AT4[24] and AT6[26] of DEBS to the HAFH successfully generated promiscuous ATs capable of taking both extender units. In a relaxed AT (AT3) from the epothilone PKSs, a hybrid motif (HASH) was observed.[27]

The current state of knowledge of PKS enzymes indicates that it is possible to predict the products of uncharacterized PKS clusters based on their genetic organizations and specific sequences (particularly of the AT domains), and vice versa. However, it must be noted that although the substrate specificity of AT domains can be well correlated with one or several active site residues, they are not the only determinants of AT substrate specificity, as previously performed site directed mutagenesis never completely blocks the incorporation of the original substrate and usually led to a mixture of products with decreased total titers. In addition, previous study showed that a region at the C terminus of AT domains, located at a considerable distance from the active site was also related to substrate specificity.[28]

Table 1.1 Variation of the 13 active site residues and 11 additional conserved residues for the malonate specific AT domains.[23]

| Domain | Specificity | 11 | 63 | 90 | 91 | 92 | 93 | 94 | 117 | 200 | 201 | 231 | 250 | 255 | 15 | 58 | 59 | 60 | 61 | 62 | 70 | 72 | 197 | 198 | 199 | Freq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| averm_02 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V | R | Q | T | R | Y | A | Q | A | N | H | A | 2 |
| averm_05 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V | R | Q | T | P | Y | A | Q | A | N | H | A | 2 |
| averm_04 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V | R | Q | T | P | Y | A | Q | A | K | N | A | 1 |
| myxot_04 | Malonate | Q | Q | G | H | S | L | G | R | F | H | N | H | V | H | Q | S | G | V | S | E | A | T | H | A | 2 |
| myxal_06 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | Y | D | T | E | Y | T | E | A | S | H | A | 1 |
| rapam_11 | Malonate | Q | Q | G | H | S | V | G | R | F | H | . | Q | V | R | E | T | G | Y | A | Q | A | S | H | A | 6 |
| ascom_03 | Malonate | Q | Q | G | H | S | I | G | R | F | H | . | Q | V | R | D | T | G | Y | A | Q | A | S | H | A | 1 |
| nidda_03 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | H | R | T | E | Y | T | Q | A | S | H | A | 4 |
| ascom_10 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | H | V | R | D | T | L | Y | A | E | A | G | H | A | 1 |
| rapam_14 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V | R | D | T | L | Y | A | E | A | R | H | A | 1 |
| spino_01 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V | W | R | T | L | W | A | Q | G | S | H | A | 4 |
| spino_06 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V | W | Q | T | L | W | A | Q | G | S | H | A | 2 |
| spino_05 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V | W | R | T | V | W | A | Q | G | S | H | A | 1 |
| spino_07 | Malonate | Q | Q | G | H | S | V | G | R | F | H | A | Q | V | W | R | T | L | W | A | Q | G | S | H | A | 1 |
| epoth_02 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | T | Q | T | A | F | T | E | A | S | H | A | 2 |
| epoth_08 | Malonate | Q | Q | G | H | S | L | G | R | F | H | N | H | V | V | Q | T | A | F | T | E | A | S | H | A | 1 |
| epoth_04 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | M | Q | T | A | Y | A | E | A | S | H | A | 1 |
| ampho_09 | Malonate | Q | Q | G | H | S | V | G | R | F | H | D | H | V | R | R | T | E | Y | A | E | A | S | H | A | 1 |
| nysta_09 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | R | T | A | Y | A | E | A | S | H | A | 1 |
| ampho_12 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | R | T | G | Y | A | E | A | S | H | A | 2 |
| pimar_05 | Malonate | Q | Q | G | H | S | I | G | R | F | H | G | H | V | R | Q | T | G | Y | A | E | A | S | H | A | 1 |
| ampho_13 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | H | V | R | E | T | G | W | T | E | A | S | H | A | 1 |
| nysta_13 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | H | V | H | Q | T | G | F | T | E | A | S | H | A | 1 |
| ampho_18 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | H | V | R | D | T | G | H | T | E | A | S | H | A | 1 |
| nysta_18 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | D | T | R | H | T | E | A | S | H | A | 1 |
| ampho_LD | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | R | T | G | W | T | E | A | S | H | A | 2 |
| nysta_16 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | R | T | R | F | T | E | A | S | H | A | 2 |
| pimar_09 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | E | T | G | F | T | E | A | S | H | A | 5 |
| pimar_10 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | E | T | G | Y | T | E | A | S | H | A | 1 |
| ampho_14 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | Q | E | T | G | H | T | E | A | S | H | A | 1 |
| nysta_14 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | A | T | G | N | T | E | A | S | H | A | 1 |
| ampho_16 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | A | T | A | F | T | E | A | S | H | A | 1 |
| ampho_17 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | S | T | A | F | T | E | A | S | H | A | 1 |
| pimar_03 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | Q | T | A | Y | A | E | A | S | H | A | 1 |
| pimar_04 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | R | T | G | Y | A | E | A | S | H | A | 1 |
| pimar_02 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | R | T | V | Y | A | E | A | S | H | A | 1 |
| ampho_03 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | E | T | G | W | T | E | A | S | H | A | 10 |
| nysta_03 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | D | T | G | W | A | E | A | S | H | A | 1 |
| nysta_04 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | R | T | G | Y | T | E | A | S | H | A | 1 |
| ampho_10 | Malonate | Q | Q | G | H | S | I | G | R | F | H | D | H | V | R | R | T | G | W | T | E | A | S | H | A | 1 |
| nysta_10 | Malonate | Q | Q | G | H | S | I | G | R | F | H | D | H | V | R | E | T | G | W | T | E | A | S | H | A | 1 |
| pimar_01 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V | R | Q | T | A | Y | T | E | A | S | H | A | 1 |
| pimar_08 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V | R | D | T | G | Y | T | E | A | S | H | A | 1 |
| rifam_02 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V | W | Q | T | M | Y | T | E | A | S | H | A | 1 |
| pimar_06 | Malonate | Q | Q | G | H | S | I | G | R | F | H | G | H | V | R | R | T | E | W | T | G | A | S | H | A | 1 |
| tylac_07 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V | R | R | T | E | Y | T | E | A | A | H | A | 1 |
| nidda_07 | Malonate | Q | Q | G | H | S | I | G | R | F | H | E | Q | V | R | R | T | A | Y | A | E | A | S | H | A | 1 |
| tylac_03 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V | R | R | T | E | F | T | Q | A | S | H | A | 1 |
| sorap_02 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | Q | T | A | F | T | E | A | S | H | A | 3 |
| averm_10 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | D | T | G | F | A | E | A | S | H | A | 1 |
| pikro_02 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V | R | E | T | R | Y | T | E | A | S | H | A | 1 |
| averm_12 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V | R | R | T | R | Y | A | Q | A | G | H | A | 1 |
| pimar_00 | Malonate | Q | Q | G | H | S | V | G | R | P | H | T | H | V | R | D | T | T | Y | T | Q | A | S | T | A | 1 |
| rifam_09 | Malonate | Q | Q | G | H | S | I | G | R | F | H | N | H | V | R | Q | T | V | F | T | E | A | S | H | A | 1 |
| pyolu_03 | Malonate | Q | Q | G | H | S | F | G | R | F | H | S | H | V | Y | Q | T | R | Y | A | E | A | S | H | A | 1 |
| myxal_04 | Malonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | R | E | I | D | V | N | E | A | D | V | P | 1 |
| myxal_05 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | N | V | W | E | T | E | V | A | Q | A | N | Y | A | 1 |
| myxot_02 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | N | V | P | R | T | E | I | E | Q | A | N | Y | A | 1 |
| pyolu_01 | Malonate | Q | Q | G | H | S | M | G | R | F | H | T | N | V | W | L | T | E | F | A | Q | A | E | Y | A | 1 |
| olean_LD | Mal/acet | Q | Q | G | H | S | V | G | R | F | H | N | H | V | O | E | T | H | Y | T | E | A | S | H | A | 1 |

Table 1.2 Variation of the 13 active site residues and 11 additional conserved residues for AT domains specific for methylmalonate and other unusual substrates.[23]

| Domain name | Specificity | 11 | 63 | 90 | 91 | 92 | 93 | 94 | 117 | 200 | 201 | 231 | 250 | 255 | 15 | 58 | 59 | 60 | 61 | 62 | 70 | 72 | 197 | 198 | 199 | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| eryth_01 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | S | D | Y | A | 39 |
| ascom_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | E | V | V | A | S | D | Y | A | 4 |
| ampho_02 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | A | D | Y | A | 2 |
| nysta_02 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | G | D | Y | A | 4 |
| spino_03 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | L | M | S | D | Y | A | 1 |
| spino_LD | Methylmal/Prop | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | H | D | D | V | V | M | S | E | Y | A | 1 |
| averm_01 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | A | D | V | V | M | S | D | Y | A | 2 |
| averm_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | Q | A | D | V | V | M | S | D | Y | A | 2 |
| rifam_05 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | L | M | G | D | Y | A | 3 |
| rifam_07 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | S | V | E | V | L | M | G | D | Y | A | 1 |
| rifam_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | G | D | Y | G | 1 |
| ampho_01 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | S | V | W | R | V | D | V | V | M | S | D | Y | A | 2 |
| nidda_04 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | V | A | D | Y | A | 1 |
| eryth_04 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | L | M | S | R | Y | A | 2 |
| megal_05 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | P | V | D | V | V | M | S | D | Y | A | 1 |
| averm_11 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | S | D | W | A | 1 |
| spino_08 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | D | D | V | I | M | S | D | Y | A | 1 |
| myxal_01 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | E | I | N | V | V | Q | A | N | V | A | 1 |
| myxot_05 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | D | I | D | V | L | S | S | D | V | A | 1 |
| epoth_06 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | R | I | D | V | V | E | A | D | V | A | 3 |
| epoth_01 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | R | I | D | V | V | A | A | D | V | A | 1 |
| myxal_02 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | E | I | D | V | V | A | A | D | V | A | 1 |
| myxal_03 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | E | I | D | V | I | E | A | D | V | A | 1 |
| myxal_07 | Methylmalonate | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | D | I | D | V | I | Q | A | D | V | A | 1 |
| myxot_03 | Methylmalonate | Q | Q | G | S | S | I | G | R | S | H | T | N | V | W | Q | A | A | I | S | Q | A | D | Y | A | 1 |
| **Unusual substrates** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| eryth_LD | Propionate | Q | Q | G | H | S | I | G | W | A | H | S | S | V | W | R | V | E | V | V | Q | S | S | M | A | 1 |
| megal_LD | Propionate | Q | Q | G | H | S | I | G | W | A | H | G | S | V | W | R | V | E | V | V | Q | S | S | M | A | 1 |
| ascom_04 | Ethylmalonate | Q | H | G | H | S | Q | G | R | T | H | T | N | V | W | R | V | D | V | V | M | S | D | C | P | 1 |
| nidda_05 | Ethylmalonate | Q | Q | G | H | S | Q | G | R | G | H | T | N | V | W | R | V | D | V | V | M | S | D | T | A | 2 |
| sorap_07 | Glycerate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | V | D | V | V | M | S | D | F | A | 1 |
| sorap_03 | Glycerate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V | W | R | I | E | V | V | M | S | D | V | A | 1 |
| averm_LD | 2 me butyrate | Q | Q | G | H | S | L | G | W | A | H | S | N | V | W | R | V | D | V | V | M | S | D | V | P | 1 |
| myxal_LD | 2 me butyrate | H | L | G | H | S | I | G | Q | V | H | T | N | T | W | R | I | D | V | T | E | S | N | V | A | 1 |
| sorap_LD | Benzoate | Q | L | G | H | S | T | G | Y | P | H | E | N | A | W | E | I | D | V | S | E | A | D | V | A | 1 |
| myxot_01 | 3 me butyrate | A | L | G | Y | S | V | G | H | A | H | T | N | V | W | Q | G | E | R | Q | Q | S | D | F | Y | 1 |
| ascom_07 | Methoxymalonate | Q | P | G | H | S | L | G | R | G | H | . | Q | V | H | D | P | T | Q | G | Q | A | R | H | A | 1 |
| ascom_08 | Methoxymalonate | Q | Q | G | H | S | L | G | R | G | H | . | Q | V | H | D | P | T | H | S | Q | A | P | H | A | 1 |
| nidda_06 | hydroxymalonate | Q | Q | G | H | S | Q | G | R | G | H | T | N | V | W | R | A | D | V | V | M | S | D | F | A | 1 |
| epoth_03 | Mal / Memal | Q | Q | G | H | S | A | G | R | S | H | N | H | V | T | Q | T | A | F | T | E | A | S | H | A | 1 |

Table 1.3 Variation of the conserved motifs for AT domains with different specificities.

| Domain name | Specificity | 198 | 199 | 200 | 201 |
|---|---|---|---|---|---|
| averm_05 | Malonate | H | A | F | H |
| nidda_03 | Malonate | H | A | F | H |
| epoth_02 | Malonate | H | A | F | H |
| ampho_18 | Malonate | H | A | F | H |
| rifam_02 | Malonate | H | A | F | H |
| | | | | | |
| ampho_02 | Methylmalonate | Y | A | S | H |
| averm_01 | Methylmalonate | Y | A | S | H |
| rifam_07 | Methylmalonate | Y | A | S | H |
| nidda_04 | Methylmalonate | Y | A | S | H |
| eryth_04 | Methylmalonate | Y | A | S | H |
| | | | | | |
| nidda_05 | Ethylmalonate | T | A | G | H |
| tylac_05 | Ethylmalonate | T | A | G | H |
| | | | | | |
| epoth_03 | relax | H | A | S | H |

### 1.5.3. Iterative type I PKS

Iterative type I PKSs, mainly present in fungi, comprise only a single module that is used iteratively during cycles of chain elongation. Different from modular type I PKS, only malonyl-CoA can be used as extender unit for iterative type I PKS. A typical example is lovastatin synthase (Figure 1.5). In addition to KR, DH, ER domains, a methyltransferase (MT) domain could also be involved in the reductive tailoring process of polyketide chain.[15] Although the same multidomain enzyme is used repeatedly for every elongation cycle, the degree of reduction can vary in each extender unit. It is still unclear what factors govern this variability.

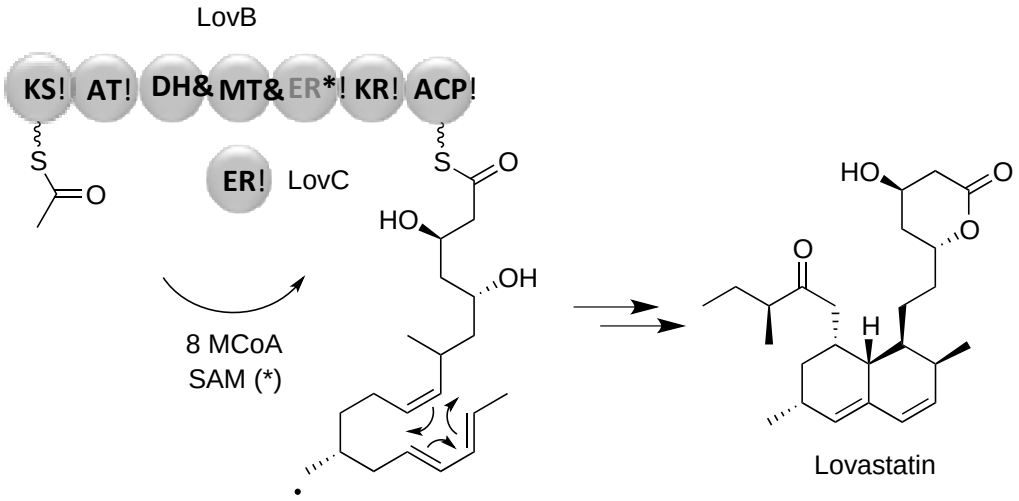


Figure 1.5 Iterative type I PKS involved in the biosynthesis of lovastatin.[15] *This ER domain is inactive. A separate ER enzyme (LovC), is required for lovastatin biosynthesis.

### 1.5.4. Iterative type II PKS

Type II PKSs are comprised of several individual enzymes and usually each enzyme is of single function. Type II PKS gene clusters are a hallmark for the biosynthesis of aromatic polyketides such as tetracycline (antibiotic), doxorubicin (anti cancer drug) and chromomycin (anti cancer drug) (Figure 1.6). A minimal set of individual proteins, the so-called 'minimal PKS', including two ketosynthase units [$KS_\alpha$ and $KS_\beta$ or chain length factor (CLF)] and an acyl carrier protein (ACP) is used iteratively to assemble the polyketide chain (Figure 1.7). The ketosynthase ($KS_\alpha$) subunit catalyzes decarboxylative condensation of malonyl-CoA extender units with an acyl starter unit and the growing polyketide chain is tethered to the acyl carrier protein (ACP). The $KS_\beta$ subunit, which is lacking the active site cysteine, plays an important role for determination of the carbon chain length, thus it has also been called the 'chain length factor'.[29] Moreover, in previous studies of the actinorhodin (*act*) and tetracenomycin

(*tcm*) PKSs, it was found that the active site cysteine in $KS_\beta$ subunit was mutated into a glutamine (Q). The mutated $KS_\beta$ subunit also served as a chain initiation factor responsible for loading malonyl-CoA and catalyzing the decarboxylation of malonyl-ACP to give an acetyl starter unit.[30] Interestingly, the $KS_Q$ domain, usually found in the loading module of modular type I PKSs, contains the same mutation and also provides an acetate starter unit by decarboxylation of malonyl-CoA.[30] Type II PKSs shows a typical $KS_\alpha/KS_\beta$/ACP architecture responsible for making a polyketide chain. The cyclization pattern of the nascent poly-β-keto intermediate is controlled and catalyzed by additional PKS subunits including ketoreductases, cyclases and aromatases, which define the folding pattern of the nascent poly-β-keto intermediate.

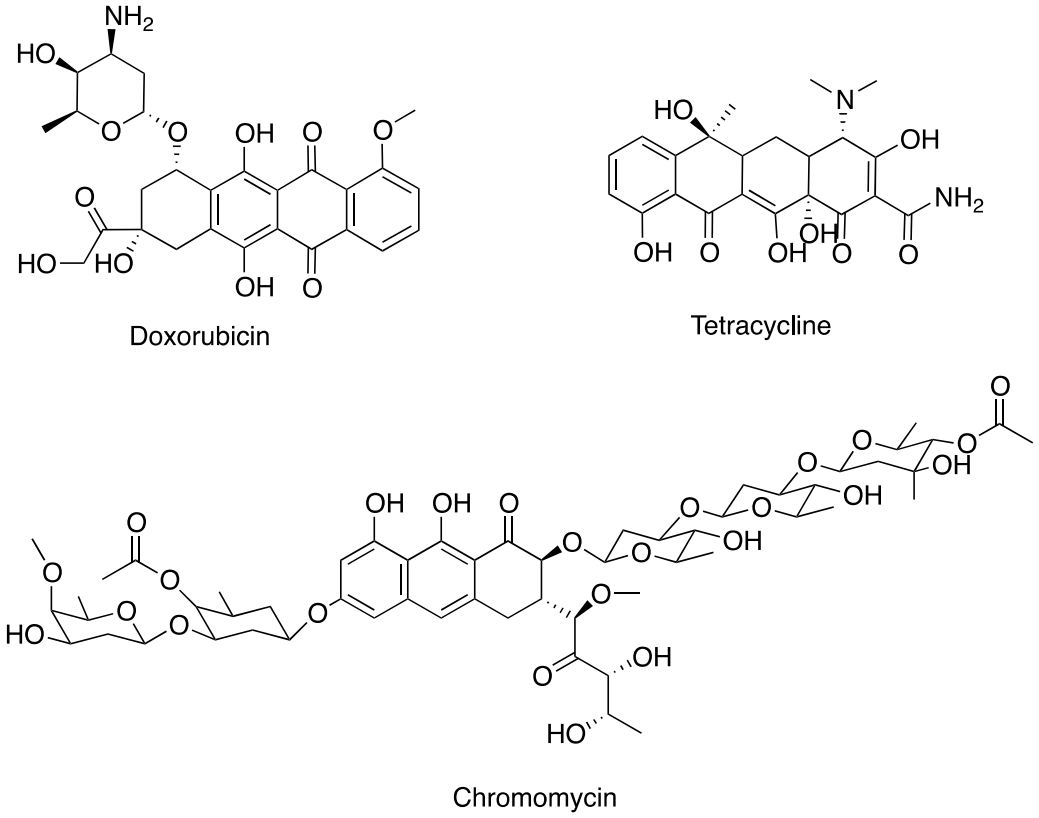


Figure 1.6 Chemical structures of polyketides made by type II PKS.

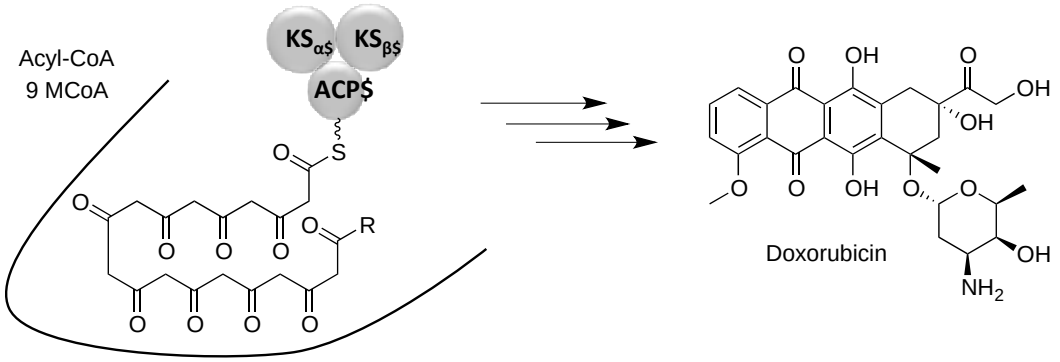


Figure 1.7 Iterative type II PKS involved in the biosynthesis of doxorubicin.[15]

### 1.5.5. Iterative type III PKS

Type III PKSs are simple homodimeric enzymes with multifunctions including the starter unit selection, chain assembly and cyclization. Unlike type I and type II PKSs, which are discovered only in microorganisms, type III PKSs are widely distributed both in plants and bacteria.[31] The most well-known examples of type III PKSs are the well-studied family of plant chalcone/stilbene synthases (CHS/STS), which produce aromatic products such as naringenin chalcone (Figure 1.8). A variety of molecules activated as CoA thioesters can be used as starter units by the type III PKSs, for example, hydroxyl-substituted and nonsubstituted coumaroyl in CHS/THS, benzoyl units in biphenyl synthases[32] and fatty acids in alkylresorcinol synthases.[33]

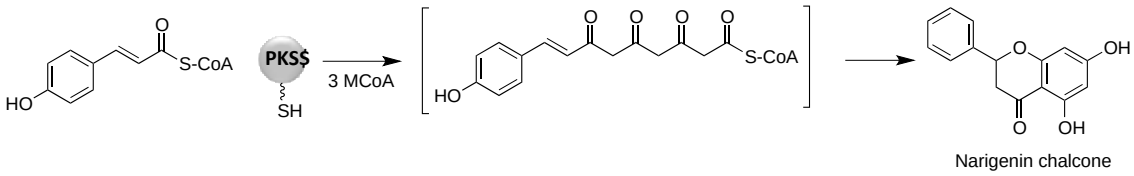


Figure 1.8 Iterative type III PKS involved in the biosynthesis of naringenin chalcone.[15]

Table 1.4 Overview of different types of PKSs.[15]

| PKS type | Minimal PKS | Building blocks | Organisms |
|---|---|---|---|
| Modular type I (cis-AT, trans-AT) | KS, AT*, ACP | various extender units | Bacteria, (protists) |
| Iterative type I | KS, AT, ACP | malonyl-CoA extenders | Mainly fungi, some bacteria |
| Iterative type II | $KS_{\alpha}$ , $KS_{\beta}$ , ACP | malonyl-CoA extenders | Excusively bacteria |
| Iterative type III | Multifunctional homodimeric enzyme | malonyl-CoA extenders | Mainly plants, some bacteria and fungi |

* Stand alone AT for trans-AT PKS.

## 1.6. Challenge of natural products discovery and Indonesian Black Water Ecosystems

The investigation of microorganisms as a source of bioactive natural products was inspired by the serendipitous discovery of penicillin by Alexander Fleming in the late 1920s and the pioneering work of Selman Waksman soon after.[8] The discovery of new antibiotics reached its peak during the golden era of antibiotics in the mid 20$^{th}$ century and continued to thrive until the 1980s. However, after more than 50 years of fruitful exploration and exploitation of microbial natural products the discovery rate of new antibiotics has dropped drastically in recent decades. On the other hand, the same compounds, possibly from related producing organisms, were isolated repeatedly. Therefore, in recent years, efforts for discovering new natural products have been redirected toward organisms from unique and untapped areas of the globe with remarkable biodiversity potential and/or extreme environment, such as deep sea, caves, and unusual ecosystems. The focus of this research is to study the secondary metabolite

production from microorganisms living in a unique ecosystem in Indonesia, the black water rivers.

### 1.7. Research objectives and thesis overview

The objective of this research is to discover novel bioactive natural products from microorganisms isolated from unique black water ecosystems in Indonesia. The bacteria, which were obtained from our collaborators in Indonesia, were subjected to 16S rRNA analysis and screened for their potential for producing bioactive natural products (Chapter 2). This preliminary chemical/biological screening resulted in a number of promising candidates, one of which, *Streptomyces albiflaviniger* ICBB 9297, was selected for further chemical investigation. Details of the investigation of bioactive compounds from *S. albiflaviniger* ICBB 9297 are described in Chapter 3. Furthermore, preliminary chemical screening of a group of non-*Streptomyces* actinomycete strains revealed that several of these strains produce the anticancer agents the apoptolidins. This group of compounds has previously been isolated from *Amycolatopsis* sp. ICBB 8242. Chemical investigation of this strain led to the discovery of two new succinylated apoptolidin analogues and linear apoptolin A. Details of the isolation, characterization, and biological activity of the succinylated apoptolidin analogues and the linear apoptolin A are described in Chapter 4.

## 1.8 Reference


1. Kong, J. M.; Goh, N. K.; Chia, L. S.; Chia, T. F. *Acta Pharmacol Sin* **2003,** *24*, 7-21.
2. Cragg, G. M.; Newman, D. J. *Pure Appl. Chem.* **2005**, *77*, 7-24.
3. McIntosh, M.; Cruz, L. J.; Hunkapiller, M. W.; Gray, W. R.; Olivera, B. M. *Arch Biochem Biophys* **1982,** *218*, 329-334.
4. Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J Am Chem Soc* **1971,** *93*, 2325-2327.
5. Miller, L. H.; Su, X. *Cell* **2011,** *146*, 855-858.
6. Newman, D. J.; Cragg, G. M. *J Nat Prod* **2012,** *75*, 311-335.
7. Demain, A. L.; Fang, A. *Adv Biochem Eng Biotechnol* **2000,** *69*, 1-39.
8. Demain, A. L. *J Ind Microbiol Biotechnol* **2014,** *41*, 185-201.
9. Fleischmann, R. D.; Adams, M. D.; White, O.; Clayton, R. A.; Kirkness, E. F.; Kerlavage, A. R.; Bult, C. J.; Tomb, J. F.; Dougherty, B. A.; Merrick, J. M. *Science* **1995,** *269*, 496-512.
10. Carlson R. February 12, 2014, http://www.synthesis.cc.
11. Land, M.; Hauser, L.; Jun, S. R.; Nookaew, I.; Leuze, M. R.; Ahn, T. H.; Karpinets, T.; Lund, O.; Kora, G.; Wassenaar, T.; Poudel, S.; Ussery, D. W. *Funct Integr Genomics* **2015,** *15*, 141-161.
12. Smith, S.; Tsai, S. C. *Nat Prod Rep* **2007,** *24*, 1041-1072.
13. Bretschneider, T.; Heim, J. B.; Heine, D.; Winkler, R.; Busch, B.; Kusebauch, B.; Stehle, T.; Zocher, G.; Hertweck, C. *Nature* **2013,** *502*, 124-128.
14. Hopwood, D. A. *Chem Rev* **1997,** *97*, 2465-2498.
15. Hertweck, C. *Angew Chem Int Ed Engl* **2009,** *48*, 4688-4716.
16. Rawlings, B. J. *Nat Prod Rep* **2001,** *18*, 190-227.
17. Challis, G. L. *J Med Chem* **2008,** *51*, 2618-2628.
18. Gross, H. *Appl Microbiol Biotechnol* **2007,** *75*, 267-277.
19. Zhang, W.; Tang, Y. *J Med Chem* **2008,** *51*, 2629-2633.
20. Reeves, C. D. *Crit Rev Biotechnol* **2003,** *23*, 95-147.
21. Bumpus, S. B.; Magarvey, N. A.; Kelleher, N. L.; Walsh, C. T.; Calderone, C. T. *J Am Chem Soc* **2008,** *130*, 11614-11616.
22. Piel, J. *Nat Prod Rep* **2010,** *27*, 996-1047.
23. Yadav, G.; Gokhale, R. S.; Mohanty, D. *J Mol Biol* **2003,** *328*, 335-363.
24. Reeves, C. D.; Murli, S.; Ashley, G. W.; Piagentini, M.; Hutchinson, C. R.; McDaniel, R. *Biochemistry* **2001,** *40*, 15464-15470.
25. Del Vecchio, F.; Petkovic, H.; Kendrew, S. G.; Low, L.; Wilkinson, B.; Lill, R.; Cortés, J.; Rudd, B. A.; Staunton, J.; Leadlay, P. F. *J Ind Microbiol Biotechnol* **2003,** *30*, 489-494.
26. Sundermann, U.; Bravo-Rodriguez, K.; Klopries, S.; Kushnir, S.; Gomez, H.; Sanchez-Garcia, E.; Schulz, F. *ACS Chem Biol* **2013,** *8*, 443-450.
27. Petković, H.; Sandmann, A.; Challis, I. R.; Hecht, H. J.; Silakowski, B.; Low, L.; Beeston, N.; Kuscer, E.; Garcia-Bernardo, J.; Leadlay, P. F.; Kendrew, S. G.; Wilkinson, B.; Müller, R. *Org Biomol Chem* **2008,** *6*, 500-506.

28. Lau, J.; Fu, H.; Cane, D. E.; Khosla, C. *Biochemistry* **1999,** *38*, 1643-1651.
29. Hertweck, C.; Luzhetskyy, A.; Rebets, Y.; Bechthold, A. *Nat Prod Rep* **2007,** *24*, 162-190.
30. Bisang, C.; Long, P. F.; Cortés, J.; Westcott, J.; Crosby, J.; Matharu, A. L.; Cox, R. J.; Simpson, T. J.; Staunton, J.; Leadlay, P. F. *Nature* **1999,** *401*, 502-505.
31. Yu, D.; Xu, F.; Zeng, J.; Zhan, J. *IUBMB Life* **2012,** *64*, 285-295.
32. Liu, B.; Raeth, T.; Beuerle, T.; Beerhues, L. *Planta* **2007,** *225*, 1495-1503.
33. Baerson, S. R.; Schröder, J.; Cook, D.; Rimando, A. M.; Pan, Z.; Dayan, F. E.; Noonan, B. P.; Duke, S. O. *Plant Signal Behav* **2010,** *5*, 1286-1289.
34. Lewis, K. *Nat Rev Drug Discov* **2013,** *12*, 371-387.
35. Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K. *Nature* **2015,** *517*, 455-459.

# CHAPTER TWO

## Identification and Biological Screening of Soil Bacteria from Indonesian Black Water Ecosystems

Yan Sheng, Nicholas C. Lowery, Phillip Lam, Dahai Zhang, Dwi A. Santosa, Philip J. Proteau, T. Mark Zabriskie, and Taifo Mahmud*

## 2.1 Abstract

The diverse and untapped microorganisms from Indonesian Black Water Ecosystems have great potential as a source of bioactive natural products. Bacterial strains isolated from the peaty soils of the black water rivers on Borneo Island, Indonesia, were subjected to 16S rRNA gene analysis and biological screening. The 16S rRNA gene sequences of 141 bacterial strains were obtained and about one third of the strains were screened for bioactive secondary metabolites production. The results showed that *Streptomyces* were the most abundant species (77%) in this collection. More than half of the screened *Streptomyces* (18 out of 24) showed activities against one or more pathogenic microorganisms, whereas only 2 out of 14 of those that are not actinomycete showed antibacterial activities.

## 2.2 Introduction

Indonesia, an archipelago of more than 18,000 islands, is known as one of the major centers in the world for biodiversity. It is home to 11% of all flowering plant species, 12% of mammalian species, 16% of reptile and amphibian species and 17% of bird species. Also, it is estimated that Indonesia has more than 25% of the world's microorganisms, living in the various ecosystems around the country. Among them are "Black water" rivers in Indonesia, unusual ecosystems that harbor diverse microorganisms with great potential as a source of novel bioactive natural products. "Black water" rivers lie in the steamy jungles of several large islands like Borneo, Sumatra, Papua, as well as of hundreds of the nation's smaller islands. They are typically being described as deep, slow-moving channels flowing through wetlands and forested swamps. Black water

does not mean muddy water. As vegetation decays in the rainforest, tannins from peaty soils leach into the water. Years after years, the rivers became tea-like tinted but transparent. The low levels of pH (4.0-5.5) and dissolved minerals, almost unmeasurable water hardness, as well as the nutrient poor water condition, all result in a unique black water ecosystem with a composition of flora and fauna differs significantly from that found in other water ecosystems.[2, 3] Microorganisms living in such untapped unique environments hold promise for novel bioactive natural products discovery.

As part of our efforts to identify valuable microorganisms that can produce new bioactive compounds, we investigated microorganisms isolated from the soil samples collected from the BWEs in Indonesia. Here, we report the identification of 141 strains of the BWE bacteria and antimicrobial screening of EtOAc and *n*-BuOH extracts from a selection of those strains against a set of pathogenic bacteria and a fungus.

## 2.3 Results and Discussion

### 2.3.1 The 16S rRNA analysis of ICBB strains (performed by Nicholas C. Lowery)

16S rRNA gene is a commonly used tool for identifying bacteria and creating phylogenetic trees, as it is highly conserved between different species of bacteria. To identify the bacterial strains in the library, 16S rRNA genes from 141 strains, which constitute about 75% of a total of 200 strains we received from ICBB, were PCR amplified using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') or 1522R (5'-

AAGGAGGTGATCCANCCRCA -3') primers and sequenced.[4] The 16S rRNA sequences were obtained and compared to those in the NCBI database by BLAST. The results showed that the majority of the strains, 108 out of 141 (77%), belong to various *Streptomyces* spp. Nine strains (6%) were identified as non-*Streptomyces* actinomycetes, such as *Amycolatopsis*, *Kitasatospora*, and *Kocuria* (Table 2.1, 2.2). The remaining 24 strains (17%) belong to non-actinomyces genus, such as *Bacillus*, *Pandoraea*, and *Staphylococcus warneri*.

Table 2.1 Summary of 16S rRNA analysis for strains from ICBB library.

| **Species** | **Total number** |
|---|---|
| *Streptomyces* | 108 |
| Non-*Streptomyces* actinomycetes | 9 |
| Non-actinomycetes | 24 |
| Total | 141 |

Table 2.2 Non-*Streptomyces* actinomycetes isolated from the ICBB library.

| **Strain ID** | **Species** |
|---|---|
| 9301 | *Amycolatopsis rubida** |
| 9343 | *Amycolatopsis rubida* |
| 9389 | *Amycolatopsis rubida** |
| 9355B | *Amycolatopsis rubida* |
| 9212 | *Amycolatopsis rubida* |
| 9385 | *Amycolatopsis echigonensis** |
| 9421 | *Kitasatospora sampliensis* |
| 9448 | *Kitasatospora sampliensis* |
| 9379A | *Kocuria rhizophila* |

* These strains were identified as apoptolidin producers.

### 2.3.2 Biological screening of secondary metabolites produced by ICBB strains

From a total of 141 strains that have been identified, 45 strains, including 31 *Streptomyces* and 14 non-actinomycetes, were cultured in a number of media (e.g., Modified Bennett's, M6, Kings, MB, YPGM). In addition, tannic acid (1mM) and aluminum chloride (100 μg/mL) were also added to a batch of cultures and the pH was adjusted to around 5 to resemble the natural condition of the black water ecosystems. The culture broths were then extracted with EtOAc and *n*-BuOH and the resulting extracts were screened for their antibacterial and antifungal activities by agar diffusion assay. Three Gram-positive pathogenic bacteria (*S. aureus*, *M. smegmatis* and *B. subtilis*), two Gram-negative bacteria (*E. coli*, *P. aeruginosa*), and a fungus (*P. sasaki*) were used for preliminary screening. The results were summarized in Table 2.3, 2.4.

Overall, the biological screening results indicate that *Streptomyces* strains remain the best producers of bioactive compounds. Fifteen out of 24 *Streptomyces* (strains that produced the same set of secondary metabolites were excluded) produced compounds that are active against Gram-positive bacteria and two of them are also active against the Gram-negative bacteria *P. aeruginosa* and/or *E. coli*. Furthermore, nine *Streptomyces* strains produced compounds that are active against the fungus *P. sasakii.* On the other hand, only 2 out of 14 non-actinomycete strains tested showed good activities against Gram-positive bacteria. The results also showed that there is no significant differences between extracts obtained from modified media and those of normal media in terms of their bioactivities. Screening of those non-*Streptomyces* actinomycetes were performed by other members of the laboratory. Three out of six

strains belonging to *Amycolatopsis sp*. were identified by mass spectrometry analysis as apoptolidin producers (Figure 2.1 and Table 2.2). Investigations of other members of this group did not lead to any new bioactive natural products.

### 2.3.3 Isolation and identification of the *Streptomyces* active metabolites

Six *Streptomyces* strains, *S. albiflaviniger* ICBB 9297, *S. asiaticus* ICBB 9276, *S. herbaricolor* ICBB 9409, *S. hiroshimensis* ICBB 9371 A, *S. hiroshimensis* ICBB 9261, and *S. minutiscleroticus* ICBB 9275 were cultured in large scales (≥4 L) and the culture broths were extracted with EtOAc and *n*-BuOH. The resulting active extracts were fractionated by column chromatography and the active compounds were purified by HPLC and analyzed by mass spectrometry and NMR spectroscopy.

The EtOAc extract of *S. herbaricolor* ICBB 9409 showed potent activity against the Gram-positive bacteria *S. aureus* and *M. smegmatis.* Bioassay-guided isolation and purification led to the isolation of one red crystalline compound. High-resolution mass spectrometry (HR-MS) analysis of the compound revealed a molecular formula of $C_{64}H_{90}N_{12}O_{16}$ (*m/z* 1283.6652, $[M+H]^+$; calcd for $C_{64}H_{90}N_{12}O_{16}$ 1283.6676), which is, based on ANTIBASE® and SciFinder® searches, identical to those of 20 published actinomycin analogs (one example is shown in Figure 2.1). Actinomycins are a class of polypeptide antitumor antibiotics isolated from several *Streptomyces* spp. and they are in fact distinctive in their red color. Structurally, they consist of an actinoyl chromophore ring (2-amino-4,6-dimethylphenoxazine-3-one-1,9-dicarboxylic acid) and two cyclic pentapeptidolactones. NMR analysis of the ICBB 9409 product indicated the

presence of the same actinoyl chromophore ring, however, the cyclic pentapeptidolactone moieties in this compound appear to be slightly different from those reported in the literature. Therefore, full characterization of the amino acid residues is needed and is still in progress.

The EtOAc extract of *S. hiroshimensis* ICBB 9261 exhibited relatively good activity against *S. aureus*. However, the activity was lost during a bioassay guided isolation process, preventing full characterization of the active compound. This loss of activity may be due to decomposition of the active compound or irreversible binding to silica gel. No further attempt was made to pursue this presumably highly unstable compound.

The EtOAc and *n*-BuOH extracts of *S. hiroshimensis* ICBB 9371A inhibited the growth of several tested pathogenic bacteria. However, the active compound(s) was not identified due to low yields. On the other hand, the EtOAc extract of *S. minutiscleroticus* ICBB 9275 was active against *S. aureus* and *M. smegmatis*. However, we were not able to identify the active compounds, as further fractionation of this red-colored extract resulted in broad distribution of antibacterial activity and red color through out all fractions.

The EtOAc extracts of *S. albiflaviniger* ICBB 9297 and *S. asiaticus* ICBB 9276 showed good activities against the Gram-positive bacteria *S. aureus* and *M. smegmatis*. Isolation and detailed investigation of the active compounds by MS and NMR revealed

that both strains produce elaiophylin analogues, albeit the ICBB 9297 strain is a better producer. Elaiophylins, a group of polyketide antibiotics, have been reported to have antibacterial, cytotoxic and immunosuppressive activities. Further studies of the ICBB 9297 strain led to the isolation of four new elaiophylin analogues, together with 5 known elaiophylins. The structure of elaiophylin is shown in Figure 2.1. Detailed isolation, structure characterization, and biological activity of these compounds are described in Chapter 3.

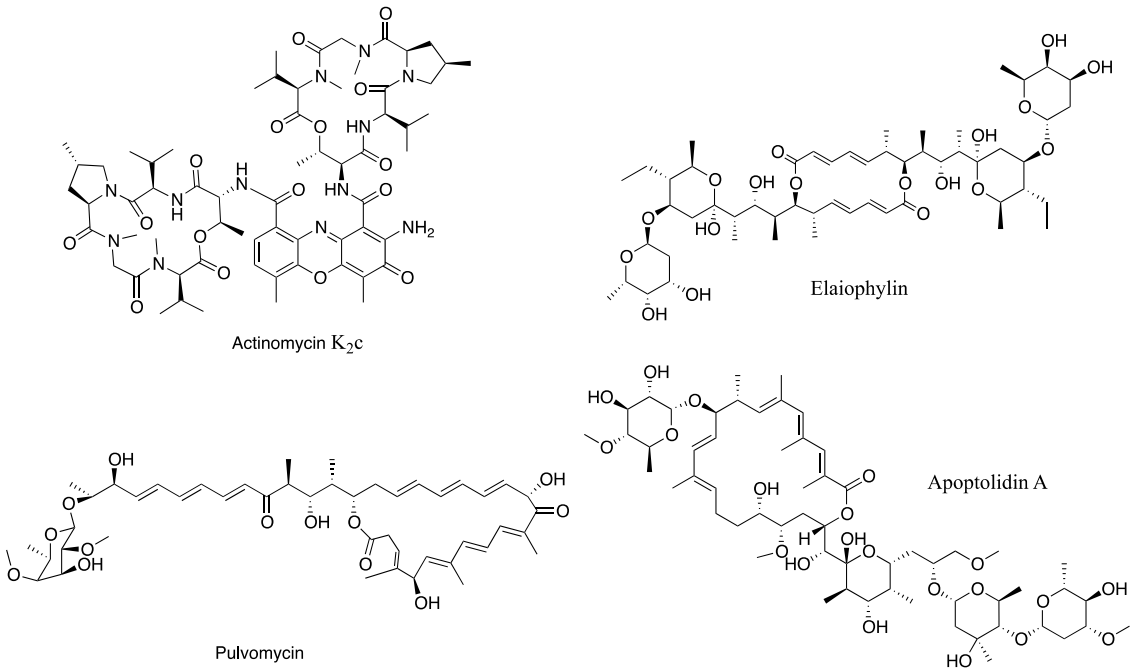


Figure 2.1 Chemical structures of bioactive compounds from ICBB strains.

Besides *S. asiaticus* ICBB 9276 and *S. albiflaviniger* ICBB 9297, *S. asiaticus* 9282 and 9299B were also found to produce elaiophylin analogues. Similarly, the strain *S. lilacinus* ICBB 9173 showed the same activity and secondary metabolite profile as *S. lilacinus* ICBB 9374, from which a known polyketide compound, pulvomycin (Figure

2.1) was isolated by another member of the laboratory, Khaled Almabruk. It is not surprising that closely related bacterial strains collected from the same place could produce similar secondary metabolites. It is also possible that the same strain was repeatedly isolated from the soil samples, but was cataloged with a different number in the library.

Table 2.3 Preliminary activity screening of *Streptomyces* strains.

| Strain No. | | 9297 | | | | 9276 | | | | 9173 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *S. albiflaviniger* | | | | *S. asiaticus* | | | | *S. lilacinus* | | | |
| Medium | | MB | | M6 | | MB | | M6 | | MB | | A-3M | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | + | - | + | - | + | - | + | - | + | + | + | + |
| | M.S. | + | - | + | - | + | - | + | - | + | + | + | +* |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | + | + | + | + |
| Large scale | | ✓ | | - | | ✓ | | - | | - | | - | |
| Natural products | | Ela analogs | | - | | Ela analogs | | - | | Act analogs | | - | |
| Strain NO. | | 9409 | | | | 9371A | | | | 9261 | | | |
| Species | | *S. herbaricolor* | | | | *S. hiroshimensis* | | | | *S. hiroshimensis* | | | |
| Medium | | MB | | M6 | | MB | | YPGM | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | + | - | + | - | + | + | + | - | + | - | + | - |
| | M.S. | + | - | + | - | + | + | - | - | - | - | - | - |
| | B.S. | - | - | - | - | +* | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | +* | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | + | + | +* | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | + | - | - | - | - | - |
| Large scale | | ✓ | | - | | ✓ | | - | | ✓ | | - | |
| Natural products | | Act analogs | | - | | unident | | - | | unident | | - | |
| Strain NO. | | 9275 | | | | 9451 | | | | 9453 | | | |
| Species | | *S. minutiscleroticus* | | | | *S. longwoodensis* | | | | *S. longwoodensis* | | | |
| Medium | | MB | | M6 | | MB | | M6 | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | + | - | - | - | + | - | - | - | +* | - | - | - |
| | M.S. | + | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | NA | NA | NA | NA | - | - | - | - |
| Large scale | | ✓ | | - | | - | | - | | - | | - | |
| Natural products | | unident | | - | | - | | - | | - | | - | |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower pH (~5).

Table 2.3 Preliminary activity screening of *Streptomyces* strains. (Continued)

| Strain NO. | | 9308 | | | | | | 9153A | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *S. minutiscleroticus* | | | | | | *S. mobaraensis* | | | | | |
| Medium | | MB | | A-3M | | M6 [M] | | MB[M] | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | + | + | + | + | - | - | + | - | +* | - | - | - |
| | M.S. | + | + | + | - | - | - | - | - | + | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | - | + | - | - |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |
| Strain NO. | | 9331 | | | | | | 9188 | | | | | |
| Species | | *S. hiroshimensis* | | | | | | *S. hiroshimensis* | | | | | |
| Medium | | MB | | A-3M | | M6 | | M6 [M] | | FR23 | | Kings | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | +* | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | +* | +* |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | + | - | - | - | - | - | - | - | + |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |
| Strain NO. | | 9352 | | | | | | 9329 | | | | | |
| Species | | *S. griseocarneus* | | | | | | *S. luteireticuli* | | | | | |
| Medium | | MB | | A-3M | | M6 | | M6 [M] | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | +* | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | +* | - | - | - |
| | P. S. | + | + | - | - | - | - | - | - | - | + | | |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower pH (~5)

Table 2.3 Preliminary activity screening of *Streptomyces* strains. (Continued)

| Strain NO. | | 9278 | | | | | | 9204 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *S. aurantiogriseus* | | | | | | *S. polychromogenes* | | | | |
| Medium | | M6 | | M6 [M] | | MB [M] | | MB | | M6 | | A-3M |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E |
| Activities | S.A. | +* | - | - | - | +* | +* | - | - | - | - | - |
| | M.S. | +* | - | - | - | +* | +* | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | + | - | - | NA |
| Large scale | | - | | - | | - | | - | | - | | - |
| Natural products | | - | | - | | - | | - | | - | | - |

| Strain NO. | | 9156 | | | | 9390 | | | | 9347 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *S. luteireticuli* | | | | *S. seoulensis* | | | | *S. griseoluteus* | | | |
| Medium | | M6 | | MB [M] | | MB | | M6 | | FR23 | | Kings | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | + | - | - | - | - | - | - | - | - |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |

| Strain NO. | | 9334 | | | | 9367 | | | | 9345 | | | | 9382 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *S. misionensis* | | | | *S. misionensis* | | | | *S. misionensis* | | | | *S. misionensis* | | | |
| Medium | | M6 | | M6[M] | | MB | | M6 | | M6 | | M6 [M] | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Large scale | | - | | - | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | | - | | - | |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower pH (~5)

Table 2.4 Preliminary activity screening of non-actinomycete strains.

| Strain NO. | | 9419 | | | | 9317 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species | | *Pandoraea/Burkholderia* | | | | *Erwinia amylovora* | | | |
| Medium | | MB | | M6 | | MB | | M6 | |
| Extracts | | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - |
| | M.S. | - | - | + | - | + | - | + | - |
| | B.S. | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - |
| | P. S. | + | + | + | + | NA | NA | NA | NA |
| Large scale | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | |

| Strain NO. | | 9387B | | | | 9309 | | | | 9280 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *Bacillus thuringiensis* | | | | *Balneimonas flocculans* | | | | *Ochrobactrum intermedium* | | | |
| Medium | | MB | | YPGM | | MB [M] | | M6 | | FR23 | | King | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | - | - | - | - |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |

| Strain NO. | | 9322 | | | | | | 9257A | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *Pandoraea pnomenusa* | | | | | | *Bacillus thuringiensis* | | | | | |
| Medium | | M6 [M] | | MB [M] | | MB | | YPGM | | MB | | YPGM | |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | - | - | - | - |
| Large scale | | - | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | | - | |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower PH (~5)

Table 2.4 Preliminary activity screening of non-actinomycete strains. (Continued)

| Strain NO. | | 9195 | | | | | 9344 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *Pandoraea pnomenusa* | | | | | *Bacillus thuringiensis* | | | | |
| Medium | | MB | | YPGM | | Kings | A-3M | | MB | | YPGM |
| Extracts | | E | B | E | B | E | E | B | E | B | E |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | NA | NA | - | - | - | - |
| Large scale | | - | | - | | - | - | | - | | - |
| Natural products | | - | | - | | - | - | | - | | - |

| Strain NO. | | 9447 | | | | | | 9443 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *Pandoraea pnomenusa* | | | | | | *Mycobacterium llatzerense* | | | |
| Medium | | MB | | YPGM | | Kings | A-3M | MB | | YPGM | |
| Extracts | | E | B | E | B | E | E | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | NA | NA | - | - | - | - |
| Large scale | | - | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | | - | |

| Strain NO. | | 9320B | | | | | | 9319 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | | *Lysinibacillus fusiformus* | | | | | | *Brevibacillus centrosporus* | | | | |
| Medium | | MB | | A-3M | | M6 | | MB | | M6 | | A-3M |
| Extracts | | E | B | E | B | E | B | E | B | E | B | E |
| Activities | S.A. | - | - | - | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - | - | - | NA |
| Large scale | | - | | - | | - | | - | | - | | - |
| Natural products | | - | | - | | - | | - | | - | | - |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower PH (~5)

Table 2.4 Preliminary activity screening of non-actinomycete strains. (Continued)

| Strain No. | | 9399 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species | | *Staphylococcus warneri* | | | | | | | |
| Medium | | M6 | | M6 [M] | | FR23 | | Kings | |
| Extracts | | E | B | E | B | E | B | E | B |
| Activities | S.A. | - | - | - | - | - | - | - | - |
| | M.S. | - | - | - | - | - | - | - | - |
| | B.S. | - | - | - | - | - | - | - | - |
| | P.A. | - | - | - | - | - | - | - | - |
| | E. C. | - | - | - | - | - | - | - | - |
| | P. S. | - | - | - | - | - | - | - | - |
| Large scale | | - | | - | | - | | - | |
| Natural products | | - | | - | | - | | - | |

* Weak activity; E, EtOAc extract; B, *n*-BuOH extract. S.A., *Staphylococcus aureus*; M.S., *Mycobacterium smegmatis*; B.S., *Bacillus subtilis*; P.A., *Pseudomonas aeruginosa*; E.C., *Escherichia coli*; P.S., *Pellicularia sasaki*. Act, actinomycin; Ela, elaiophylin; unident, unidentified. [M] Modified media with tannic acid (1mM), aluminum chloride (100 μg/mL) and lower PH (~5)

### 2.3.4 Isolation and Identification of the non-*Streptomyces* actinomycete active metabolites

Based on the TLC and MS analyses of their products, strains *Amycolatopsis rubida* ICBB 9389, *A. rubida* ICBB 9301, *A. echigonensis* ICBB 9385 were found to produce a set of compounds called apoptolidins. Apoptolidins are a group of macrolide antibiotics originally isolated from a soil bacterium, *Nocardiopsis* sp. Several members of this family of natural products have been reported as potent apoptosis inducers of certain cancer cells. Biosynthetically, they are produced by modular type I polyketide synthases, followed by a series of tailoring processes including glycosylation, methylation and oxidation.[5] Apoptolidin A (Figure 2.1) and other known apoptolidin analogues have been isolated from another ICBB strain, *Amycolatopsis* sp. ICBB 8242. Previous studies by our laboratories revealed that this strain produced apoptolidin A as a major product in higher yield (~300 mg/mL) than *Nocardiopsis* sp. (150 mg/mL).[6, 7]

Further investigations of the ICBB 8242 strain has led to the discovery of two new succinylated apoptolidins, along with a linear apoptolidin A. Isolation, structure characterization and bioactivity of the new apoptolidin analogues are described in Chapter 4.

## 2.4 Experimental section

### 2.4.1 Sample Collection

The soil samples were collected from the beds of the Pangkoh Lima and Sungai Kala black water rivers on Kalimantan Island (the Indonesian part of Borneo). These rivers represent the typical Black Water ecosystems in Kalimantan, which lies in the remote area, about 150 km from the coast of South Kalimantan. A detailed isolation protocol of microorganisms from the soil samples is described in our previous publication.[8] Bacterial isolates were provided by Dr. Dwi Andreas Santosa from the Indonesian Center for Biodiversity and Biotechnology (ICBB). Glycerol stocks were prepared by Dr. Dahai Zhang and stored in -80 °C for further investigations.

### 2.4.2 Fermentation and extraction

The bacterium was streaked on Modified Bennett's or YPGM agar plates and placed in 30 °C incubator for 3 days. To prepare the liquid cultures, the bacterium was then added into 250 mL Erlenmeyer flasks containing 50 mL of different liquid media. After 7 days incubation in a rotary shaker set to 200 rpm at 30 °C, the cultures were centrifuged and the supernatants were collected and extracted three times with an equal volume of EtOAc. The aqueous layers were then extracted two times with an equal

volume of *n*-BuOH. The EtOAc and BuOH extracts were then dried on rotary evaporator.

Modified Bennett's medium (MB): glucose 10 g, yeast extract 1 g, beef extract 1 g soytone 2 g, metal ion solution 1 mL, in 1 L $ddH_2O$.

YPGM: yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g, in 1 L $ddH_2O$.

M6: glycerol 20 mL, molasses 10 g, casein 5 g, $CaCO_3$ 4 g, peptone 1 g, in 1 L $ddH_2O$.

3-AM: Glucose 5 g, glycerol 20 mL, soluble starch 20 g, pharmamedia 15 g, yeast extract 3 g, in 1 L $ddH_2O$.

FR-23: molasses 10 g, pharmamedia 20 g, glucose 5 g, soluble starch 30 g, in 1 L $ddH_2O$.

Kings: protease peptone B 20 g, glycerol 10 g, $K_2HPO_4$ 1.5 g, $MgSO_4.7H_2O$ 1.5 g in 1 L $ddH_2O$.

The modified media were prepared by adding tannic acid (1 mM), aluminum chloride (100 μg/mL) and adjusting the pH to about 5 by HCl.

Agar plates were prepared by adding 15% of agar to the liquid medium.

### 2.4.3 Agar diffusion assay

For bioassay, bacteria were first grown on agar plates overnight and then added to a liquid medium. After shaken at 200 rpm overnight, 0.5 mL of the culture was mixed with 10 mL of a warm agar medium. The mixture was poured on top of agar plates and let stand until solidified. Each sterilized Whitman disc was loaded with 10 μL of MeOH or DMSO solution of each extract and placed on the agar plates. The plates were placed in the incubator for overnight and then stained with MTT solution (1

mg/mL). A growth inhibition zone is expected if the extract has activity. Except for *E. coli*, where LB medium was used and the cultures were incubated at 37 °C, other pathogenic bacteria were grown on YMG (yeast extract 4 g, malt extract 4 g, glucose 10 g, agar 15 g, in 1 L dd$H_2O$, pH 7.3) plates and the cultures were incubated at 30 °C.

For antifungal activity tests, the fungus *P. sasakii* spores were placed at the center of an YMG agar plate and Whitman discs impregnated with the samples were placed on the agar plate around the test organism. The plate was then placed in the incubator at 30 °C. After 2 to 3 days, the plate was observed for zones of growth inhibition.

## 2.5 References

1. Prepared for the United Nations Environment Programme (UNEP). Jakarta, **1993**.
2. Janzen, D. H. *Biotropica* 6, 69–103.
3. Harald S. *Tropical Ecological Systems/Trends in Terrestrial and Aquatic Research* (New York City: Springer-Verlag), 1075, 275–288.
4. Suzuki, M. T.; Giovannoni, S. J. *Appl Environ Microbiol* **1996,** *62*, 625-630.
5. Du, Y.; Derewacz, D. K.; Deguire, S. M.; Teske, J.; Ravel, J.; Sulikowski, G. A.; Bachmann, B. O. *Tetrahedron* **2011,** *67*, 6568-6575.
6. Kim, J. W.; Adachi, H.; Shin-ya, K.; Hayakawa, Y.; Seto, H. *J Antibiot (Tokyo)* **1997,** *50*, 628-630.
7. Wender, P. A.; Sukopp, M.; Longcore, K. *Org Lett* **2005,** *7*, 3025-3028.
8. Fotso, S.; Mahmud, T.; Zabriskie, T. M.; Santosa, D. A.; Sulastri; Proteau, P. J. *J Nat Prod* **2008,** *71*, 61-65

# CHAPTER THREE

## Elaiophylin-like Macrolides from a Soil Bacterium of an Indonesian Black Water Ecosystem

Yan Sheng, Phillip Lam, Dwi Andreas Santosa, Philip J. Proteau, T. Mark Zabriskie,

Taifo Mahmud*

On July 2, 2015, we were informed by our collaborators in Indonesia that strain ICBB 9297 was not isolated from a black water ecosystem in Kalimantan, but from a soil sample of an agricultural field in Jatiroto, East Java, Indonesia.

## 3.1 Abstract

Four new elaiophylin-like macrolides (**1**-**4**), together with five known elaiophylins (**5**-**9**), have been isolated from an Indonesian soil bacterium *Streptomyces albiflaviniger* ICBB 9297. The new compounds have macrocyclic skeletons distinct from those of the known elaiophylins in that one or both of the dimeric polyketide chains contain(s) an additional pendant methyl group. However, they showed comparable antibacterial activity to elaiophylin against *Staphylococcus aureus*. Interestingly, those with the additional pendant methyl group only on one of the polyketide chains (**1** and **3**) showed significantly increased activity against *Mycobacterium smegmatis*, whereas the one with two additional methyl groups and all the known elaiophylin analogues (**5**-**7**) showed no activity. The production of the new compounds in *S. albiflaviniger* ICBB 9297 indicates that one of the acyltransferase (AT) domains in the elaiophylin polyketide synthases (PKSs) has relaxed substrate specificity, utilizing both malonyl-CoA and methylmalonyl-CoA as substrates. Bioinformatic analysis of the AT domains of the elaiophylin PKSs revealed that the AT-7 domain contains unusual active site amino acid residues, distinct from those conserved for malonyl-CoA or methylmalonyl-CoA ATs.

## 3.2 Introduction

Indonesia is recognized as one of biodiversity hotspots in the world with a high level of endemic species and microorganisms.[1] The biodiversity and species richness of the various ecosystems in this archipelagic country represents a wealth of untapped resources for pharmaceutical, agricultural and environmental applications. However, many of those biologically diverse ecosystems are threatened by rapid economic growth

and human activities. Several of the most interesting and most endangered ones are found on the island of Borneo (aka Kalimantan), known as the black water ecosystems, which consist of a wetland of interconnected lakes surrounded by peaty swamp forests.

In continuation of our efforts to discover new bioactive natural products from microorganisms from the Indonesian black water ecosystems, we investigated the secondary metabolites of the soil bacterium *Streptomyces albiflaviniger* ICBB 9297, which was isolated from a soil sample of the Borneo Island. Four new elaiophylin analogues (**1**-**4**), together with five known elaiophylins, elaiophylin (**5**),[2, 3, 4] 11-*O*-monomethylelaiophylin (**6**),[5] 11, 11´-*O*-dimethylelaiophylin (**7**),[5] efomycin G (**8**),[6] 11,11´-*O*-dimethyl-14´-deethyl-14´-methylelaiophylin (**9**),[7] were isolated from the culture broths of the ICBB 9297 strain. Elaiophylin (**5**), which is also called azalomycin and gopalamycin, has previously been isolated from several *Streptomyces* strains and reported to have antibacterial,[4, 5, 8] cytotoxic[4, 5] and immunosuppressive activities.[9] Similar to many other macrolide antibiotics, this group of compounds exhibits good activity against Gram-positive bacteria but have no effect on Gram-negative bacteria.

Herein, we report the isolation, structural elucidation, and bioactivity of the new elaiophylin analogues (**1**-**4**), as well as bioinformatic analysis of acyltransferase domains in the elaiophylin PKSs.

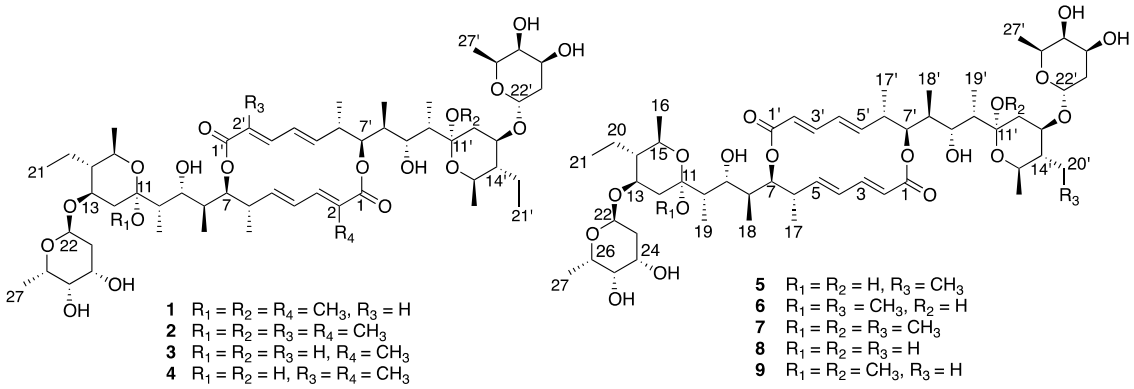


Figure 3.1. Chemical structures of elaiophylin analogues.

### 3.3 Results and discussion

The EtOAc extract of strain ICBB 9297, which was based on analysis of its 16S rRNA sequence identified as *Streptomyces albiflaviniger*, exhibited good activities against the Gram-positive bacterium *Staphylococcus aureus*. The EtOAc extract was then subjected to SPE $C_{18}$ fractionation followed by silica gel and Sephadex LH-20 column chromatography to yield an active fraction. HPLC and (+)-ESI-MS analyses of this fraction showed that it contains a mixture of macrolide natural products related to the elaiophylins (Figure 3.1 and Table 3.1, Peaks A-L). Further separation of the compounds using HPLC resulted in nine pure metabolites (**1**-**9**). Comparison of the $^1$H NMR spectra of the products with those of previously reported for elaiophylin analogues revealed that the two major peaks (F and J) are 11-*O*-methylelaiophylin (**6**) and 11,11´-*O*-dimethylelaiophylin (**7**). On the other hand, inspections of the (+)-ESI-MS data for the other products revealed that peak K (**1**) has an *m*/*z* value of 1089 ($[M+Na]^+$), which is different from the molecular masses of any known elaiophylins, suggesting that this compound is a new elaiophylin analogue.

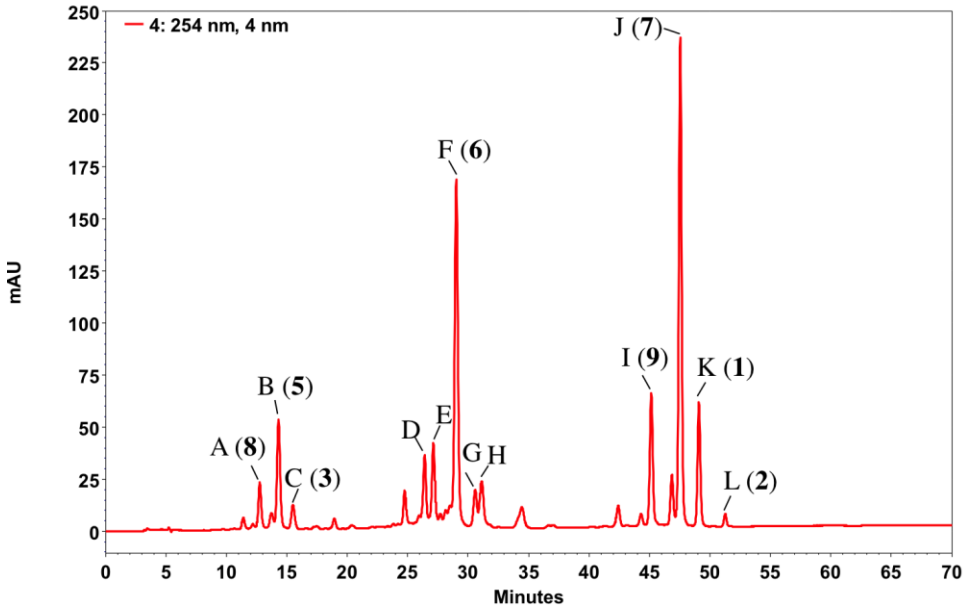


Figure 3.2. HPLC profile of elaiophylin mixture. Compound **4** cannot be observed in this figure due to low yield.

Table 3.1. (+)-ESI-MS profile of elaiophylin mixture

| **Peak No.** | ***m/z* [M+Na]$^+$** | **Peak No.** | ***m/z* [M+Na]$^+$** |
|---|---|---|---|
| A | 1033 | G | 1075 |
| B | 1047 | H | 1075 |
| C | 1061 | I | 1061 |
| D | 1047 | J | 1075 |
| E | 1047 | K | 1089 |
| F | 1061 | L | 1103 |

Compound **1** was isolated as white powder and the molecular formula was determined as $C_{57}H_{94}O_{18}$ (*m/z* 1089.6377 [M+Na]$^+$; calcd for $C_{57}H_{94}O_{18}Na$ 1089.6338) by HR-TOF-MS analysis. The $^1$H NMR spectrum of **1** showed close similarity to that of **7** except that an additional singlet for one methyl group was observed at 1.78 ppm. Moreover, proton signals in the olefinic region of **1** is more complex than that of **7**, indicating the lost of symmetry in the structure, presumably is related to the presence of the additional pendant methyl group. $^{13}$C and DEPT-135 NMR spectra of **1** showed the

presence of three quaternary carbons including two overlapping hemiketal carbon signals at 102.8 ppm and one olefinic carbon signal at 126.6 ppm. These are different from compound **7**, which has two quaternary carbons from the hemiketal moieties. The observed differences in the 1D NMR spectra of **1** and **7** suggested that one of the olefinic proton in **7** has been replaced with a methyl group in **1**, resulting in a quaternary olefinic carbon.

Detailed analysis of 2D NMR experiments (COSY, HSQC, HMBC, and TOCSY) revealed that the additional methyl group attaches to the C-2 olefinic carbon. The proton signal of this pendant methyl group shows HMBC correlations with the C-2 quaternary olefinic carbon ($\delta_C$ 126.6), the C-1 carbonyl carbon ($\delta_C$ 168.0) and C-3 ($\delta_C$ 138.6). Due to the absence of proton at C-2, the signal for H-3 appears as a doublet in **1** ($\delta_H$ 6.66), instead of a doublet of doublet seen in **7**. Compound **1** was elucidated as a new elaiophylin like macrolide with one additional methyl group attached to one of the olefinic carbons in the macrocyclic core structure of **7**. The substitution of the methyl group mainly affects the chemical environment of the macrodiolide ring, resulting in different chemical shifts between the upper and the lower parts of the ring structure. On the other hand, signals for the side polyketide chains, the hemiketal rings and the sugar moieties from both sides of the molecule are almost identical. The coupling constants of the protons and the ROESY correlations are all consistent with the published data for known elaiophylins, indicating they all share the same relative stereoconfiguration.

The core structures of all known elaiophylins are constructed from two linear polyketide chains via $C_2$-symmetric dimerization. Although there are some variations in the C-20 and -20´, which can be a methyl or an ethyl group, as well as C-11 and C-11´ hydroxyl groups, which may be methylated, the polyketide backbones that form the macrodiolide ring of the known elaiophylins are all identical. Therefore, the identification of **1**, in which one of the polyketide backbones has an additional pendant methyl group at the C-2 position, is rather unusual. This may indicate that the polyketide synthase (PKS) proteins that are responsible for elaiophylin biosynthesis have relaxed substrate specificity or there is a post-PKS tailoring enzyme that catalyze the C-methylation of the polyketide backbone. In either case, there is a high likelihood that another analogue with pendant methyl groups on both sides of the polyketide chains present in the mixture. This has prompted us to examine the HPLC and mass spectrometry profiles of the elaiophylin mixture more carefully, and in fact we discovered a peak (peak L, Figure 3.1) with an *m/z* of 1103 $[M+Na]^+$ (Table 3.1), 14 dalton higher than **1**. As the compound appeared to be produced in a significantly low yield, we grew the strain in 20 liters culture and purified the compound (**2**, 1.7 mg) through successive chromatography experiments.

Compound **2** was isolated as white powder and the molecular formula was determined to be $C_{58}H_{96}O_{18}$ (*m/z*, 1103.6531 $[M+Na]^+$; calcd for $C_{58}H_{96}O_{18}Na$ 1103.6494). Compared to the $^1H$ NMR spectrum of **1**, fewer signals were observed in that of **2**, particularly those of the olefinic protons. The rest of the spectrum is similar to that of **1,** including the presence of a methyl singlet at 1.75 ppm. On the basis of the MS

data for **2** and the number of protons and carbons in its $^{1}H$, $^{13}C$, and DEPT-135 spectra, it was postulated that **2** has a $C_2$-symmetric structure. Further analysis of the 2D NMR (COSY, HSQC, HMBC) of **2** confirmed the presence of additional pendant methyl groups at C-2 and C-2´. The HMBC correlation between H-7 (or H-7´) ($\delta_H$ 5.13) and C-1 (or C-1´) ($\delta_C$ 168.2) signifies the connection between the two linear polyketide chains. The characteristic doublet for H-3 (and H-3´) ($\delta_H$ 6.66 ppm) and the quaternary olefinic carbon for C-2 (and C-2´) ($\delta_C$ 126.7 ppm) were both observed. The coupling constants and the ROESY correlations of compound **2** are consistent with those of compound **1**, indicating that compound **2** has the same relative stereoconfiguration as compound **1**.

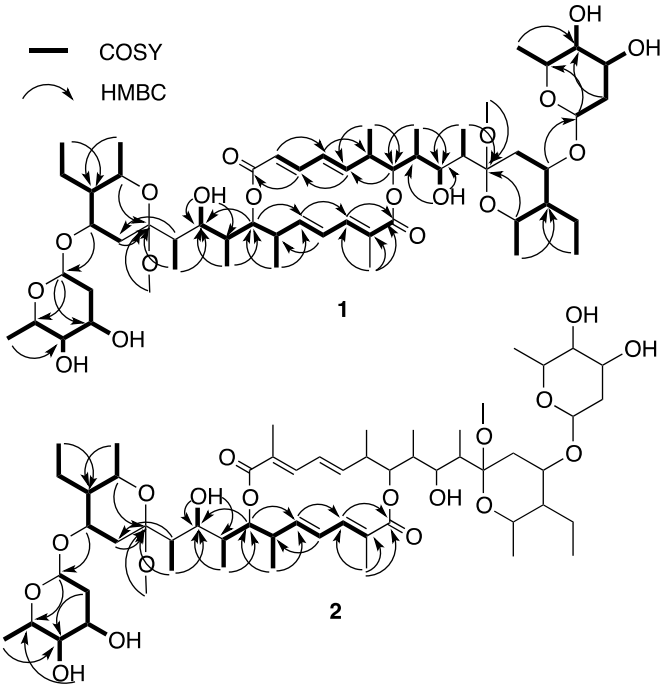


Figure 3.3. Selected HMBC and COSY correlations of **1** and **2**.

As shown in the HPLC chromatogram (Fig. 2), the elaiophylin mixture was composed of three clusters of compounds. The major peaks from each cluster, peaks B (**5**), F (**6**) and J (**7**), have been identified as known elaiophylin analogues. Upon

examinations of their chemical structures, it is postulated that the first cluster consists of compounds that have free hemiketal hydroxy groups (11-OH and 11´-OH). The second cluster consists of elaiophylins with one of the hemiketal hydroxy groups has been converted to a methoxy, whereas the third cluster consists of compounds that have two methoxy groups at C-11 and C-11´. Examinations of the minor components of the clusters also showed these correlations, where similar compounds differing only in the number of methoxy groups at the C-11/11´ positions were found in every cluster. For example, peak I, which was identified as 11,11´-*O*-dimethyl-14´-deethyl-14´-methylelaiophylin (**9**), is a dimethoxy analogue of peak A (efomycin G, **8**). This observation suggested that peak C (**3**), with a molecular mass of 14 dalton higher than peak B (**5**), is a new elaiophylin analogue. Thus, isolation and chemical characterization of this compound was pursued.

$^{1}$H NMR spectrum of **3** is almost identical as that of **1,** except that it lacks signals for oxygenated methyl groups shown in **1** ($\delta_H$ 2.94, 2.96 ppm). Additionally, it showed an extra signal for a hydroxy group at $\delta_H$ 5.43 ppm. Detailed analysis of HR-ESI-MS and 1D and 2D NMR spectra of **3** confirmed that **3** has the same macrolide ring as **1** but has free hemiketal hydroxy groups at C-11 and C-11´. In addition, we have also isolated a small amount of compound **4** (Figures. 3.1 and S 3.22), which, based on its mass and $^{1}$H NMR spectra, is postulated to be a symmetric version of **3**.

The absolute configurations of the new elaiophylins were determined on the basis of their $[\alpha]_D$ values. Elaiophylin (**5**), isolated from strain ICBB 9297, showed

$[\alpha]^{23}_{D}$ values of -51.7 (***c*** 1.0, $CHCl_3$) and -51.4 (***c*** 1.0, MeOH), which are comparable to those reported in the literature, $[\alpha]^{25}_{D}$ -46.9 (***c*** 0.83, $CHCl_3$)[20] and $[\alpha]^{25}_{D}$ -42.4 (***c*** 1.0, MeOH).[10] A closely related compound efomycin G (**8**) has also been reported to have a similar $[\alpha]^{25}_{D}$ value of -43.2 (***c*** 1.0, MeOH).[10] Interestingly, 11,11´-*O*-dimethyl-14´-deethyl-14´-methylelaiophylin (**9**), which bears methoxy groups at C-11 and C-11´, showed an opposite sign $[\alpha]^{25}_{D}$ value of +21.6 (***c*** 0.59, MeOH).[7] Consistent with this observation, compound **1** and **2**, which both bear methoxy groups at C-11 and C-11´, showed positive $[\alpha]^{22}_{D}$ values of +39.0 (c 0.10, MeOH) and +35.7 (c 0.06, MeOH), respectively, whereas compound **3**, which contain hydroxy groups at C-11 and C-11´, showed a negative $[\alpha]^{22}_{D}$ value of -29.4 (***c*** 0.33, MeOH). Combined together the results indicate that the elaiophylin analogues from strain ICBB 9297 adopt the same absolute stereoconfigurations as those of the reported compounds.

Recently, it has been reported that methylation of the C-11 and C-11´ hemiketals in elaiophylins is a non-enzymatic event that occurs during purification and isolation processes.[7] When let stand for several days in MeOH, compound **5** was found to convert gradually to **6** and **7**. This may also be the case with compounds **1** and **2**, as MeOH was used in their purification steps. Therefore, it is likely that compounds **1** and **2** are derived from compounds **3** and **4**, respectively. To investigate if this modification can be prevented we repeated the experiments using a freshly prepared culture. The EtOAc extract was prepared and then fractionated by silica gel column, Sephadex LH-20, and HPLC without the use of MeOH during the entire purification process. As shown in Fig. 3.4, the fresh mixture, which has no contact with MeOH, only contained a

cluster of "hydroxy" elaiophylins, whereas those that were exposed to MeOH for 3 h or overnight showed the presence of a "monomethoxy" elaiophylin cluster (Fig. 3.4 b) and a mono/dimethoxy elaiophylin cluster (Fig. 3.4 c), respectively, confirming that compounds **1** and **2** are experimental artifacts of compounds **3** and **4**.

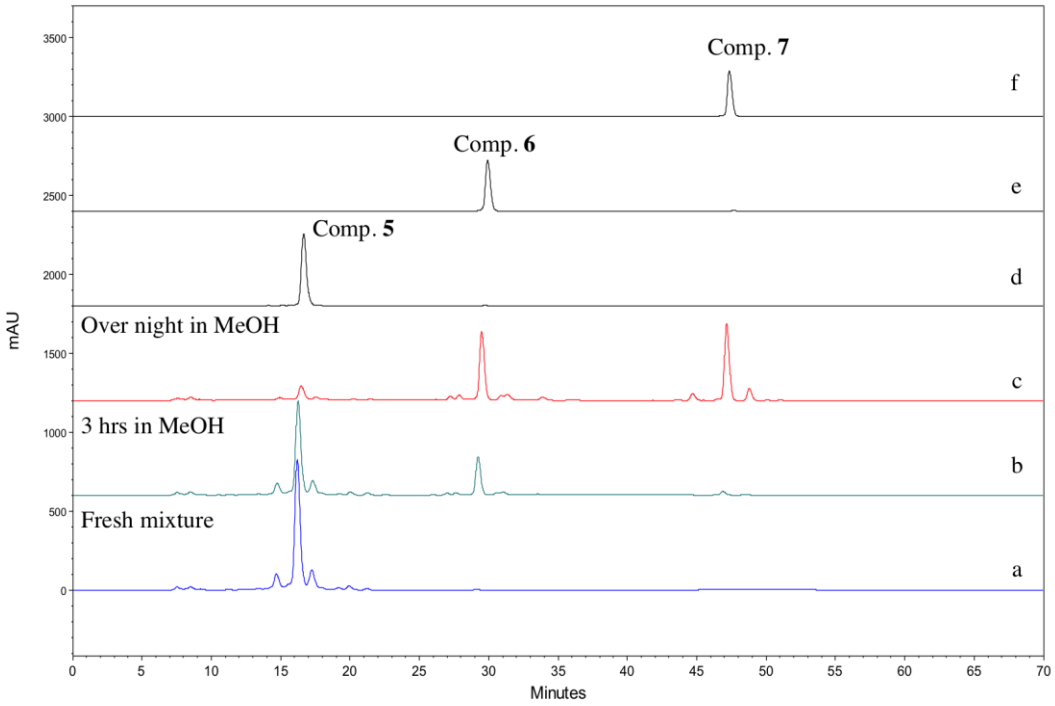


Figure 3.4. HPLC analysis of elaiophylin mixtures. **a**, Fresh elaiophylin mixture which has no contact with MeOH; **b**, Elaiophylin mixture in MeOH after 3 h at room temperature; **c**, Elaiophylin mixture in MeOH after an overnight stand at room temperature; **d**, pure compound **5**; **e**, pure compound **6**; **f**, pure compound **7.**

As described above, the identification of compound **1**-**4**, in which one or both of the polyketide backbones has an additional pendant methyl group at the C-2/C-2´ position(s), is rather unusual. This may be due to relaxed substrate specificity of the PKSs responsible for elaiophylin biosynthesis. The elaiophylin biosynthetic gene clusters have been identified in *Streptomyces* sp. DSM4137[21] and NRRL 30748.[11] The

proposed 63.2 kb gene cluster contains 24 individual open reading frames (ORFs) including five type I polyketide synthase genes (orf 7-11) responsible for the biosynthesis of the polyketide backbone (Figure 3.5). In modular type I PKS systems, extension of the polyketide chains occurs through repeated condensation of acyl-CoA thioesters. Generally, each module is responsible for one chain elongation cycle. It is known that the acyltransferase (AT) domains, which catalyze the transfer of an acyl group from a CoA thioester to the phosphopantetheine arm of the acyl carrier protein (ACP), is responsible for the selection of the extender units. Based on the sequence of the active site residues in AT domains, the substrate specificity could be predicted with a high confidence level.[12,13]

In elaiophylin biosynthesis, the polyketide backbone starts with an acetate unit, which is derived from $KS_Q$ domain-catalyzed decarboxylation of a malonyl thioester.[14] Then the growing polyketide backbone is passed through next seven extension modules which specifically recruit one ethylmalonate (but sometimes methylmalonate) (module 1), three malonates (module 2, 6, 7) and three methylmalonates (modules 3, 4, 5) to reach the full length of elaiophylin polyketide backbone. However, the discovery of compounds (**1**-**4**) with additional pendant methyl group(s) in the macrocyclic ring structure indicates that the last module in elaiophylin PKS (module 7), could use not only malonate but also methylmalonate as extender units, although based on the production yields of the products, malonate appears to be the preferred substrate. Careful analysis of the conserved active site residues of the AT domains within the elaiophylin PKS revealed that except for AT7, all other ATs have a conserved motif and

active side residues that match perfectly with those in other AT domains with known specificity (Table S3.3, Table 3.2).[12] Interestingly, instead of having the typical conserved 'HAFT' motif (residues 198-201) for malonate specific ATs, the AT7 domain has an unusual 'IAAH' motif (Table 3.2).[12,13,15,16] On the basis of computational and site-specific mutation analyses, amino acid residue 200 has been identified to play a crucial role in controlling substrate selection by AT domains.[13] However, instead of a phenylalanine commonly found in malonate-specific ATs, the elaiophylin AT7 contains an alanine at this position. This may explain its relaxed substrate specificity, but it does not explain why it still prefers malonyl-CoA as substrate. Also, the motif does not match the previously reported hybrid motif (HASH) in the epothilone PKS, epo_AT3 (Table 3.2)[17] Nevertheless, the Ala200 residue of AT7 is consistent with that in the loading module ATs of the erythromycin and megalomicin PKSs,[12] which recognize propionyl-CoA as substrate.

In order to confirm the AT sequence of *Streptomyces albiflaviniger* ICBB 9297, the AT7 domain was PCR amplified and sequenced. The primers were designed based on the published sequence of the elaiophylin PKSs from *Streptomyces* sp. NRRL 30748. The genomic DNA of strain *Streptomyces albiflaviniger* ICBB 9297 was used as template and the obtained PCR product was sequenced. The result showed that the ICBB 9297 AT7 domain shares 96% identity to that of *Streptomyces* sp. NRRL 30748 (Figure S3.23). Multiple amino acid sequence alignments of the AT domains showed that the conserved residues including those proposed to be important for substrate specificity were identical.

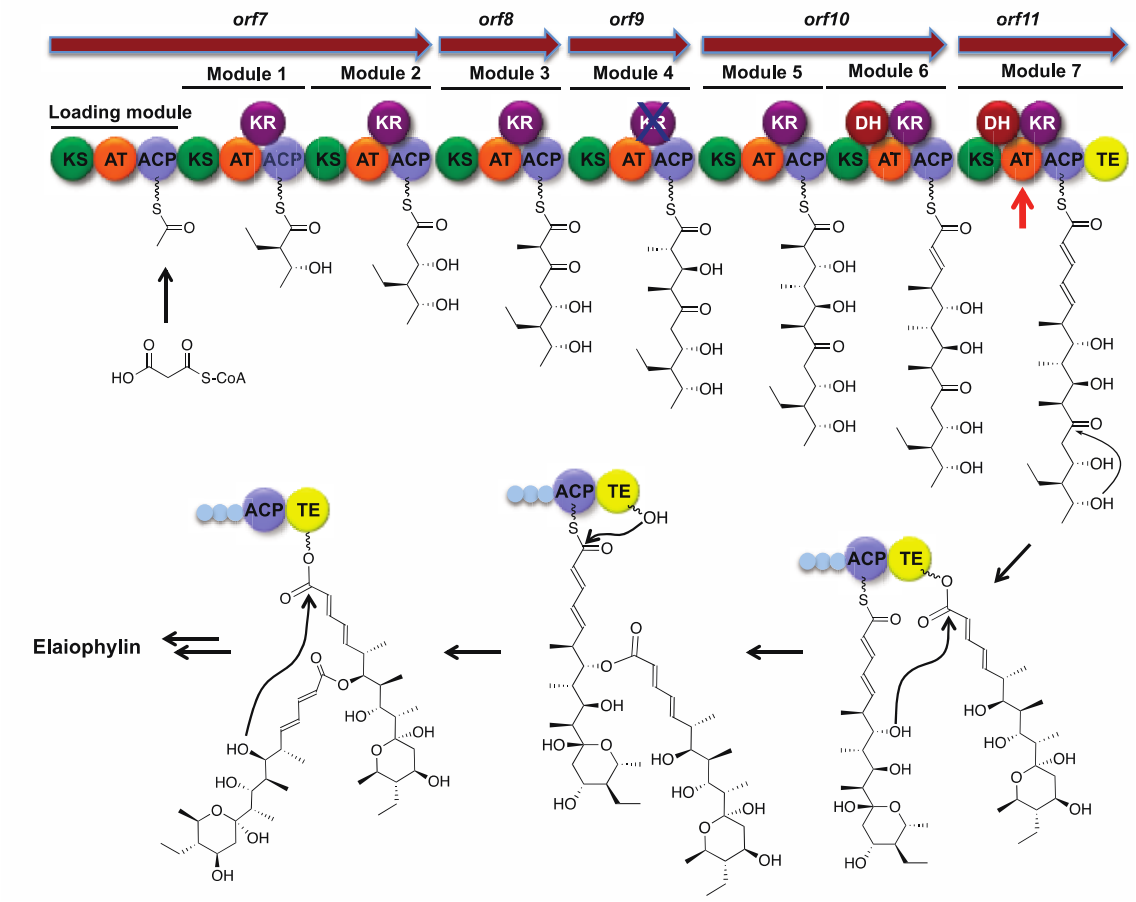


Figure 3.5. A proposed elaiophylin PKS assembly line. The PKS gene cluster organization was adapted from Haltli[11] with some modifications and the dimerization mechanism was adapted from Zhou et al.[22] However, the relative stereoconfiguration of the intermediates is different from those depicted in both reports, [11, 22] but is consistent with the stereoconfiguration reported for elaiophylin.

Table 3.2. Alignment of conserved motifs in the active site of AT domains.

| Domain name | Specificity | 198 | 199 | 200 | 201 |
|---|---|---|---|---|---|
| ave_05 | Malonate | H | A | F | H |
| nid_03 | Malonate | H | A | F | H |
| epo_02 | Malonate | H | A | F | H |
| amp_18 | Malonate | H | A | F | H |
| rif_02 | Malonate | H | A | F | H |
| **ela_LD** | **Malonate** | **H** | **A** | **F** | **H** |
| **ela_02** | **Malonate** | **H** | **A** | **F** | **H** |
| **ela_06** | **Malonate** | **H** | **A** | **F** | **H** |
| amp_02 | Methylmalonate | Y | A | S | H |
| ave_01 | Methylmalonate | Y | A | S | H |
| rif_07 | Methylmalonate | Y | A | S | H |
| nid_04 | Methylmalonate | Y | A | S | H |
| ery_04 | Methylmalonate | Y | A | S | H |
| **ela_03** | **Methylmalonate** | **Y** | **A** | **S** | **H** |
| **ela_04** | **Methylmalonate** | **Y** | **A** | **S** | **H** |
| **ela_05** | **Methylmalonate** | **Y** | **A** | **S** | **H** |
| nid_05 | Ethylmalonate | T | A | G | H |
| tyl_05 | Ethylmalonate | T | A | G | H |
| **ela_01** | **Ethylmalonate** | **T** | **A** | **G** | **H** |
| ery_LD | Propionate | M | A | A | H |
| meg_LD | Propionate | M | A | A | H |
| epo_03 | Flexible | H | A | S | H |
| **ela_07** | **Malonate** | **I** | **A** | **A** | **H** |

Abbreviations: ave, avermectin; nid, niddamycin; epo, epothilone; amp, amphotericin; rif, rifamycin; ery, erythromycin; tyl, tylactone; meg, megalomicin; ela, elaiophylin.

Elaiophylin and its derivatives were reported to exhibit antimicrobial activities against Gram-positive bacteria including several drug-resistant strains.[4,5,7,8] Similar to many other macrolide antibiotics, this group of compounds is not active against Gram-

negative bacteria or fungi.[4,5,18,19] The antimicrobial activities of the isolated new elaiophylin-like macrolides (**1-3**) together with some of the known elaiophylin analogues (**5-7**) were evaluated against *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Compound **4** was not included due to the lack of a sufficient amount. The minimum inhibitory concentrations (MIC) were determined by microdilution assay at final concentrations ranging from 0.05 μg/ml to 100 μg/ml (Table 3.3). All compounds (**1**-**3**, **5**-**7**) displayed good activities against *Staphylococcus aureus* with MIC values in the range of 0.78-3.13 μg/ml but did not inhibit the growth of *Bacillus subtilis* with a final concentration up to 100 μg/ml. As previously reported, none of the tested compounds exhibited activity against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Table 3.3). Remarkably, the new compounds **1** and **3**, which have only one additional pendant methyl group in the macrocyclic core structure, showed good activities against the Gram-positive bacterium *Mycobacterium smegmatis* with an MIC value of 6.25 μg/ml, whereas compound **2**, which contains two additional pendant methyl groups, and the known elaiophylins **5-7** showed no significant growth inhibition (Table 3.3, Figure S3.24).

Table 3.3. Antimicrobial activities (MIC, μg/ml) of elaiophylin analogues.

| Microorganism | **1** | **2** | **3** | **5** | **6** | **7** | Amp | Apra |
|---|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | 1.56 | 3.13 | 0.78 | 0.78 | 1.56 | 1.56 | 0.78 | NT |
| *Mycobacterium smegmatis* | 6.25 | >100 | 6.25 | >100 | >100 | >100 | >100 | 0.39 |
| *Bacillus subtilis* | >100 | >100 | >100 | >100 | >100 | >100 | 0.78 | NT |
| *Pseudomonas aeruginosa* | >100 | >100 | >100 | >100 | >100 | >100 | 1.56 | NT |
| *E. coli* | >100 | >100 | >100 | >100 | >100 | >100 | 6.25 | NT |

NT, not tested.

## 3.4 Experimental section

### 3.4.1 General Experimental Procedures

Optical rotations were determined on a Jasco P1010 polarimeter. UV spectra were measured on an Eppendorf biospectrometer kinetic. NMR spectra were recorded in $(CD_3)_2SO$ referenced to residual solvent signals ($\delta_H$ 2.50 ppm, $\delta_C$ 39.52 ppm) on a Bruker Avance III 700 MHz spectrometer equipped with a 5 mm $^{13}C$ cryogenic probe. ESI-MS data were recorded on a ThermoFinnigan LCQ Advantage system with a quaternary Rheos 4000 pump (Flux Instrument). High-resolution mass spectrometry was performed in a positive ion mode on an AB SCIEX Triple TOF 5600 mass spectrometer. HPLC was performed using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector.

### 3.4.2 Organism Collection and Identification

The strain ICBB 9297 was isolated from the Black Water Ecosystems (BWE) in Kalimantan, Indonesia by the ICBB (Indonesian Center for Biotechnology and

Biodiversity). The isolate was stored in 20% glycerol at -80 °C. For amplification of 16S rDNA, primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were selected. The genomic DNA was isolated and used as template. The 16S rRNA gene sequence of strain ICBB 9297 was found to have 99% identity over the sequenced region to several *Streptomyces* strains in the database, with *Streptomyces albiflaviniger* being the best hit.

### 3.4.3 Fermentation and Isolation

*Streptomyces albiflaviniger* ICBB 9297 glycerol stock was streaked on Modified Bennett's agar plate [glucose (10 g), yeast extract (1 g), beef extract (1 g), soytone (2 g), metal ion solution (1 mL), agar (15 g) in dd$H_2O$ (1L)] and placed in 30 °C incubator for 3 days. Then the bacteria were inoculated into 500 mL Erlenmeyer flasks containing 100 mL of Modified Bennett's liquid medium. After 3 days, shaken at 200 rpm at 30 °C, the resulted seed culture (7%) was transferred to 10 replicate 2 L Erlenmeyer flasks each containing 400 ml production medium (Modified Bennett's) and cultivated on rotary shaker (200 rpm) at 30 °C for 7 days. The culture was centrifuged and the resulted supernatant was collected and extracted three times with EtOAc. 20 liters culture were grown and extracted by the same process. The resulted EtOAc extract (4.88 g) was fractionated by reversed-phase RP $C_{18}$ solid phase extracton (SPE) using a stepped solvent gradient (10%, 30%, 60%, 80%, 90%, 100% MeOH-$H_2O$). The fractions eluted by 60%, 80%, 90% MeOH-$H_2O$ were pooled and further chromatographed by silica flash column eluting with a stepwise gradient solvent system from 100% $CHCl_3$ to 100% MeOH (2%, 4%, 6%, 8%, 16%, 100%). The fractions

eluting with, 6% - 8% $CHCl_3$-MeOH were combined and further separated by Sephadex LH-20 (100% MeOH) followed by semi preparative reverse phase HPLC purification to yield compounds **1**-**4**. For RPHPLC purification and analysis (column: YMC-Pack ODS-A, 250×10 mm I.D.), MeOH-$H_2O$ were used as mobile phase (2 ml/min) and the time program is described as below: the column was equilibrated with 85% MeOH for 10 min, then developed with a gradient of 85% to 90% MeOH for 10 min. Then the column was eluted by 90% MeOH for 10 min, followed by a linear gradient of 90% to 100% MeOH for 25 min. Finally the column was eluted by 100% MeOH for 15 min. Retention time are 51.7, 53.8, 19.6, 21.5 minutes for compound **1** (11.7 mg), **2** (1.7 mg), **3** (4.9 mg), **4**.

2-methyl-11,11´-*O*-dimethylelaiophylin (**1**): white powder; $[\alpha]^{22}_D$ +39.0 (c 0.10, MeOH); UV (MeOH) λmax (log ε) 254 (4.64) nm; $^1H$ and $^{13}C$ NMR data, see Table S3.1 and S3.2; HRTOFMS (ES+) m/z 1089.6377 $[M + Na]^+$ (calcd for $C_{57}H_{94}O_{18}Na$, 1089.6338 ).

2,2´-methyl-11,11´-*O*-dimethylelaiophylin (**2**): white powder; $[\alpha]^{22}_D$ +35.7 (c 0.06, MeOH); UV (MeOH) λmax (log ε) 258 (4.56) nm; $^1H$ and $^{13}C$ NMR data, see Table S3.1 and S3.2; HRTOFMS (ES+) m/z 1103.6531 $[M + Na]^+$ (calcd for $C_{58}H_{96}O_{18}Na$, 1103.6494).

2-methylelaiophylin (**3**): white powder; $[\alpha]^{22}_{D}$ -29.4 (c 0.33, MeOH); UV (MeOH) λmax (log ε) 254 (4.84) nm; $^{1}H$ and $^{13}C$ NMR data, see Table S3.1 and S3.2; HRTOFMS (ES+) m/z 1061.6002 $[M + Na]^{+}$ (calcd for $C_{55}H_{90}O_{18}Na$, 1061.6025).

2,2´-dimethylelaiophylin (**4**): HRTOFMS (ES+) m/z 1075.6156 $[M + Na]^{+}$ (calcd for $C_{56}H_{92}O_{18}Na$, 1075.6181).

### 3.4.4 Evaluation of antibacterial activity

Bacteria were grown on agar plate (YMG/LB) for 1-2 days at 30/37 °C and then inoculated to liquid medium (YMG for *S. aureus, M. smegmatis, B. subtilis* and *P. aeruginosa*, LB for *E. coli*). After shaken at 200 rpm 5-20 h at 30 °C (*S. aureus, M. smegmatis, B. subtilis* and *P. aeruginosa*) or at 37 °C (*E. coli*), the culture was diluted with YMG/LB medium to obtain an optical absorbance value around 0.1 at the wavelength of 600 nm. 100 μl of the bacterial culture with desired optical density value was added to 50 ml medium and then mixed gently. 98 μl of the bacteria-containing medium was added to each well of 96-well plate containing 2 μl compound solution with different concentrations. The solution was prepared by first dissolving the pure compound in DMSO followed by serial two fold dilutions to obtain the desired concentrations. DMSO was used as the negative control, ampicillin or apramycin were used as positive controls. The 96 well plates were placed in incubator for 17 to 48 h. Then 50 μl of MTT solution (1 mg/ml) was added to each well and left stand for 30 min. All experiments were performed in triplicates. The minimum inhibitory values

(MIC) were determined as the lowest concentration required for inhibiting bacterial growth.

## 3.5 Supporting Information

Table S3.1. $^{1}$H NMR Data for Compound **1-3** in DMSO-$d_6$

| | **1** | **2** | **3** | **4** | | **1** | **3** |
|---|---|---|---|---|---|---|---|
| no. | $\delta_H$, mult. ($J$ in Hz) | $\delta_H$, mult. ($J$ in Hz) | $\delta_H$, mult. ($J$ in Hz) | $\delta_H$, mult. ($J$ in Hz) | no. | $\delta_H$, mult. ($J$ in Hz) | $\delta_H$, mult. ($J$ in Hz) |
| 1 | | | | | 1' | | |
| 2 | | | | | 2' | 5.62, d (15.3) | 5.63, d (15.4) |
| 3 | 6.66, d (11.6) | 6.66, d (11.5) | 6.64, d (11.6) | 6.63, d (11.1) | 3' | 6.85, dd (15.1, 11.3) | 6.83, dd (15.0, 11.3) |
| 4 | 6.20 dd (14.6, 12.0) | 6.23, dd (14.6, 11.9) | 6.19, dd (14.5, 11.8) | 6.21, dd (14.8, 11.2) | 4' | 6.07, dd (14.8, 11.3) | 6.05, dd (14.7, 11.3) |
| 5 | 5.56, dd (14.6, 9.9) | 5.58, dd (14.6, 9.9) | 5.55, dd (14.8, 10.1) | 5.56, dd (15.1, 9.7) | 5' | 5.67, dd (14.9, 10.0) | 5.65, dd (14.8, 9.9) |
| 6 | 2.54, m | 2.56, m | 2.54, b | 2.55, b | 6' | 2.47, m | 2.47, m |
| 7 | 5.15, d (10.5) | 5.13, d (10.5) | 5.10, d (10.5) | 5.10, d (10.2) | 7' | 5.11, d (10.4) | 5.08, d (10.4) |
| 8 | 1.70, (m) | 1.72, m | 1.77, b | 1.77, b | 8' | 1.73, m | 1.8, b |
| 9 | 3.32, b | 3.33b | 3.79, b | 3.79, b | 9' | 3.32, b | 3.79, b |
| 10 | 1.89, q (6.9) | 1.90, q (6.7) | 1.58, b | 1.58, b | 10' | 1.89, q (6.9) | 1.58, b |
| 11 | | | | | 11' | | |
| 12ax | 1.08, b | 1.08b | 0.97, b | 0.96, b | 12'ax | 1.08, b | 0.97, b |
| 12eq | 2.39, dd (13.3, 3.9) | 2.39, dd (13.0, 3.9) | 2.26, brd | 2.26, dd (11.9, 3.8) | 12'eq | 2.39, dd (13.3, 3.9) | 2.26, brd (8.5) |
| 13 | 3.70, b | 3.70, td (10.7, 4.7) | 3.80, b | 3.81, b | 13' | 3.70, b | 3.8, b |
| 14 | 1.05, b | 1.04, b | 1.06, b | 1.06, b | 14' | 1.05, b | 1.06, b |
| 15 | 3.40, m | 3.40, dq (10.3, 5.9) | 3.77, b | 3.78, b | 15' | 3.40, m | 3.77, b |
| 16 | 1.11, d (5.7) | 1.11, d (6.1) | 1.04, d (5.7) | 1.04, d | 16' | 1.11, d (5.7) | 1.04, d (5.7) |
| 17 | 0.97, d (6.1) | 0.97, d (6.4) | 0.98, d (4.7) | 0.97, d (6.4) | 17' | 0.96, d (6.2) | 0.98, d (4.7) |
| 18 | 0.85, d (6.8) | 0.89, d (6.9) | 0.80, b | 0.83, d (7.0) | 18' | 0.87, d (6.8) | 0.81, (6.7) |
| 19 | 0.82, d (6.4) | 0.83, d (6.9) | 0.86, d (6.7) | 0.86, d (7.0) | 19' | 0.82, d (6.4) | 0.86, d (6.7) |
| 20a | 1.60, m | 1.59, m | 1.61, b | 1.60, b | 20'a | 1.60, (m) | 1.61, b |
| 20b | 1.36, b | 1.35, b | 1.37, b | 1.37, b | 20'b | 1.36, b | 1.37, b |
| 21 | 0.77, t (7.4) | 0.78, t (7.5) | 0.79, t (7.4) | 0.79, t (7.2) | 21' | 0.77, t (7.4) | 0.79, t (7.4) |
| 22 | 4.9, brs | 4.9, d (3.0) | 4.92, d (2.9) | 4.92, d (2.5) | 22' | 4.9 brs | 4.92, d (2.9) |
| 23a | 1.8, b | 1.79, b | 1.81, b | 1.81, b | 23'a | 1.8, b | 1.81, b |
| 23b | 1.38, b | 1.38, b | 1.40, b | 1.40, b | 23b | 1.38, b | 1.40, b |
| 24 | 3.72, b | 3.72, b | 3.74, b | 3.74, b | 24' | 3.72, b | 3.74, b |
| 25 | 3.37, b | 3.37, b | 3.38, b | 3.38, b | 25' | 3.37, b | 3.38, b |
| 26 | 3.73, b | 3.73, b | 3.75, b | 3.75, b | 26' | 3.73, b | 3.75, b |
| 27 | 1.06, d (6.3) | 1.05, d (6.4) | 1.07, d (6.3) | 1.07, d (6.3) | 27' | 1.06, d (6.3) | 1.07, d (6.3) |
| 11-OMe | 2.96, s | 2.95, s | | | 11'-OMe | 2.94, s | |
| 11-OH | | | 5.42, brs | no | 11'-OH | | 5.42, brs |
| 9-OH | 4.43, d (8.0) | 4.43, d (7.4) | 4.47, d (8.4)* | no | 9'-OH | 4.44, d (8.8) | 4.48, d (7.4)* |
| 24-OH | 4.56, brs | 4.54 brs | 4.54, brs | 4.50, brs | 24'-OH | 4.56, brs | 4.54 |
| 25-OH | 4.32, brs | 4.28, d (3.8) | 4.3, brs | 4.27, brs | 25'-OH | 4.32, brs | 4.3 |
| 2-Me | 1.78, s | 1.75, s | 1.78, s | 1.74, s | 2'-Me | | |

*These signals are exchangeable. b, Multiplicity patterns were unclear due to signal overlap. no, signal not observed.

Table S3.2. $^{13}C$ NMR Data for Compound **1**-**3** in DMSO-$d_6$

| | **1** | **2** | **3** | | **1** | **3** |
|---|---|---|---|---|---|---|
| no. | $\delta_c$, type | $\delta_c$, type | $\delta_c$, type | no. | $\delta_c$, type | $\delta_c$, type |
| 1 | 168.0, C | 168.2, C | 168.0, C | 1' | 167.4, C | 167.4, C |
| 2 | 126.6, C | 126.7, C | 126.6, C | 2' | 120.2, CH | 120.2, CH |
| 3 | 138.6, CH | 138.6, CH | 138.4, CH | 3' | 145.1, CH | 145.0, CH |
| 4 | 127.7, CH | 126.7, CH | 127.7, CH | 4' | 129.6, CH | 129.6, CH |
| 5 | 143.8, CH | 144.5, CH | 143.6, CH | 5' | 145.8, CH | 145.6, CH |
| 6 | 41.6, CH | 41.5, CH | 41.5, CH | 6' | 41.2, CH | 41.2, CH |
| 7 | 76.2, CH | 76.2, CH | 76.0, CH | 7' | 76.0, CH | 75.7, CH |
| 8 | 36.9, CH | 37.2, CH | 36.4, CH | 8' | 37.0, CH | 36.4, CH |
| 9 | 68.1, CH | 68.2, CH | 69.5, CH | 9' | 68.1, CH | 69.5, CH |
| 10 | 37.6, CH | 37.6, CH | 43.0, CH | 10' | 37.6, CH | 43.0, CH |
| 11 | 102.8, C | 102.8, C | 99.2*, C | 11' | 102.8, C | 99.3*, C |
| 12 | 33.7, $CH_2$ | 33.7, $CH_2$ | 36.6, $CH_2$ | 12' | 33.7, $CH_2$ | 36.6, $CH_2$ |
| 13 | 68.2, CH | 68.2, CH | 68.3, CH | 13' | 68.2, CH | 68.3, CH |
| 14 | 47.2, CH | 47.2, CH | 48.0, CH | 14' | 47.2, CH | 48.0, CH |
| 15 | 66.9, CH | 66.9, CH | 65.8, CH | 15' | 66.9, CH | 65.8, CH |
| 16 | 19.0, $CH_3$ | 19.0, $CH_3$ | 19.1*, $CH_3$ | 16' | 19.0, $CH_3$ | 19.2*, $CH_3$ |
| 17 | 15.9, $CH_3$ | 15.8, $CH_3$ | 15.8, $CH_3$ | 17' | 15.5, $CH_3$ | 15.5, $CH_3$ |
| 18 | 10.2, $CH_3$ | 10.0, $CH_3$ | 9.8, $CH_3$ | 18' | 10.0, $CH_3$ | 9.7, $CH_3$ |
| 19 | 7.3, $CH_3$ | 7.2, $CH_3$ | 7.0, $CH_3$ | 19' | 7.3, $CH_3$ | 7.0, $CH_3$ |
| 20 | 18.8, $CH_2$ | 18.8, $CH_2$ | 18.8, $CH_2$ | 20' | 18.8, $CH_2$ | 18.8, $CH_2$ |
| 21 | 8.8 $CH_3$ | 8.8 $CH_3$ | 8.8 $CH_3$ | 21' | 8.8 $CH_3$ | 8.8 $CH_3$ |
| 22 | 92.5, CH | 92.5, CH | 92.5, CH | 22' | 92.5, CH | 92.5, CH |
| 23 | 32.7, $CH_2$ | 32.7, $CH_2$ | 32.7, $CH_2$ | 23' | 32.7, $CH_2$ | 32.7, $CH_2$ |
| 24 | 66.4, CH | 64.9, CH | 65.0, CH | 24' | 66.4, CH | 65.0, CH |
| 25 | 70.3, CH | 70.3, CH | 70.3, CH | 25' | 70.3, CH | 70.3, CH |
| 26 | 65.0, CH | 66.4, CH | 66.4, CH | 26' | 65.0, CH | 66.4, CH |
| 27 | 17.2, $CH_3$ | 17.1, $CH_3$ | 17.2, $CH_3$ | 27' | 17.2, $CH_3$ | 17.2, $CH_3$ |
| 11-Ome | 45.6*, $CH_3$ | 45.6, $CH_3$ | | 11'-Ome | 45.7*, $CH_3$ | |
| 2-Me | 12.26, $CH_3$ | 11.9, $CH_3$ | 12.2, $CH_3$ | 2'-Me | | |

* These signals are exchangeable.

Table S3.3. Alignment of active site residues in AT domains with different substrate specificity.

| Domain name | Specificity | 11 | 63 | 90 | 91 | 92 | 93 | 94 | 117 | 200 | 201 | 231 | 250 | 255 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| eryth_01 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| ascom_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| ampho_02 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| spino_03 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| spino_LD | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| averm_01 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| averm_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| rifam_07 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| rifam_06 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| elaio_03 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | H | A | V |
| elaio_04 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| elaio_05 | Methylmalonate | Q | Q | G | H | S | Q | G | R | S | H | T | N | V |
| averm_02 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V |
| averm_05 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V |
| averm_04 | Malonate | Q | Q | G | H | S | L | G | R | F | H | A | Q | V |
| myxot_04 | Malonate | Q | Q | G | H | S | L | G | R | F | H | N | H | V |
| myxal_06 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V |
| rapam_11 | Malonate | Q | Q | G | H | S | V | G | R | F | H | - | Q | V |
| ascom_03 | Malonate | Q | Q | G | H | S | I | G | R | F | H | - | Q | V |
| nidda_03 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V |
| ascom_10 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | H | V |
| rapam_14 | Malonate | Q | Q | G | H | S | I | G | R | F | H | T | Q | V |
| spino_01 | Malonate | Q | Q | G | H | S | V | G | R | F | H | T | Q | V |
| elaio_ LD | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V |
| elaio_02 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V |
| elaio_06 | Malonate | Q | Q | G | H | S | V | G | R | F | H | N | H | V |
| ascom_04 | Ethylmalonate | Q | H | G | H | S | Q | G | R | T | H | T | N | V |
| nidda_05 | Ethylmalonate | Q | Q | G | H | S | Q | G | R | G | H | T | N | V |
| elaio_01 | Ethylmalonate | Q | Q | G | H | S | Q | G | R | G | H | T | N | V |
| eryth_LD | Propionate | Q | Q | G | H | S | I | G | W | A | H | S | S | V |
| megal_LD | Propionate | Q | Q | G | H | S | I | G | W | A | H | G | S | V |
| elaio_07 | flexible | Q | Q | G | H | S | V | G | R | A | H | N | H | V |

Abbreviations: eryth, erythromycin; acsom, ascomycin; ampho, amphotericin; spino, spinosad; averm, avermectin; rifam, rifamycin; myxot, myxothiazol; myxal, myxalamid; rapam, rapamycin; nidda, niddamycin; megal, megalomicin; elaio, elaiophylin.

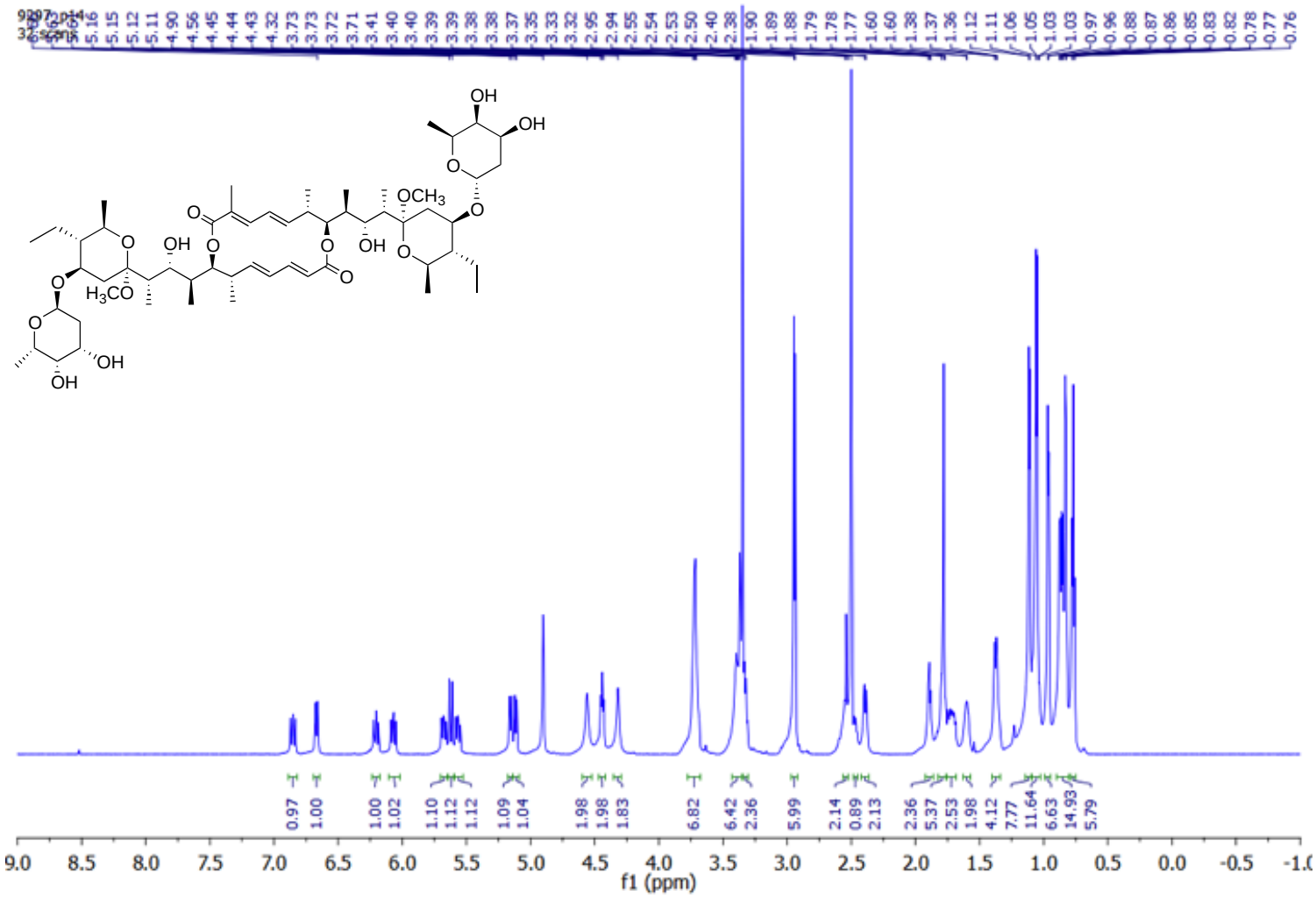


Figure S3.1. $^{1}$H NMR spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

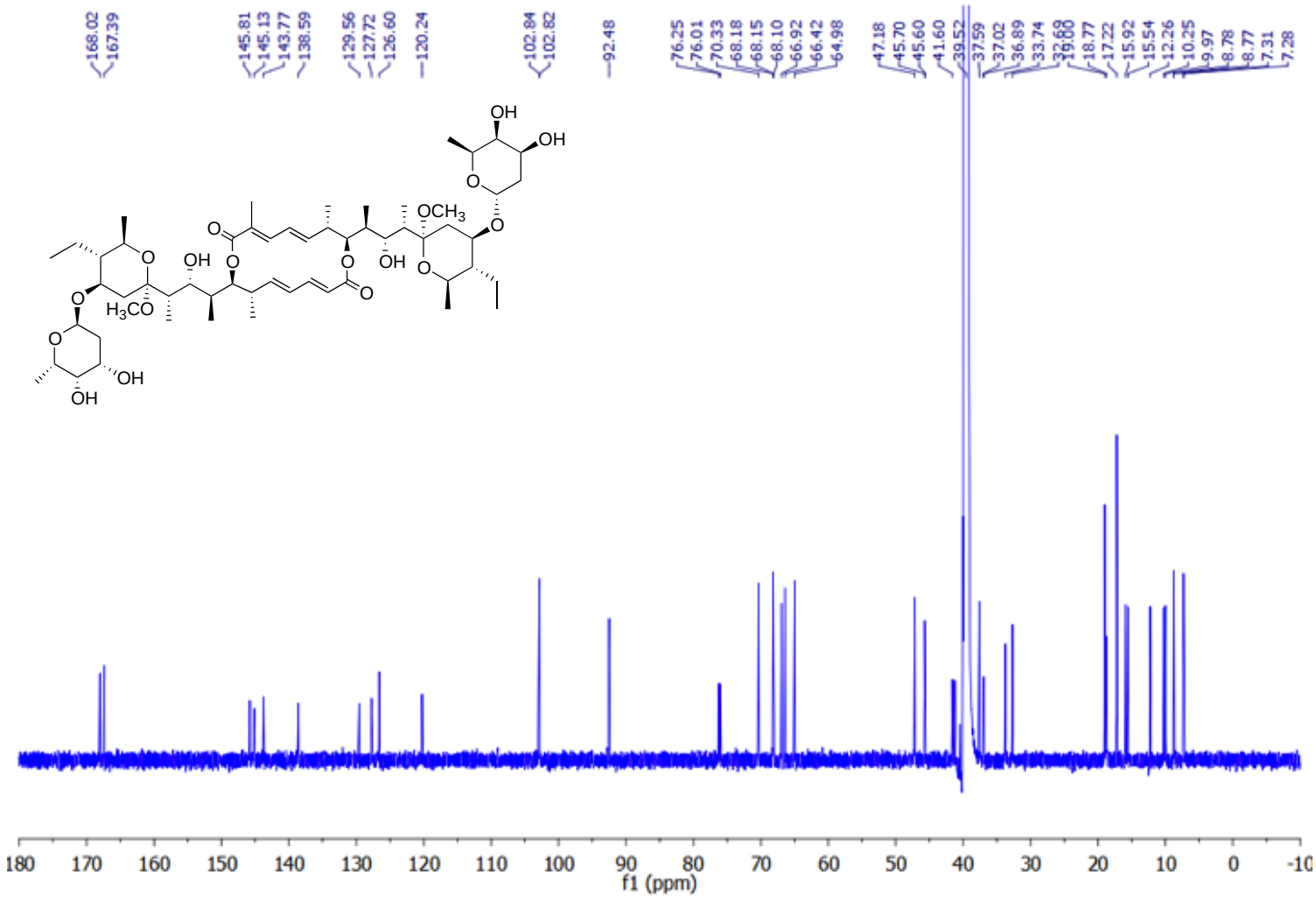


Figure S3.2. $^{13}$C NMR spectrum of compound **1** in DMSO-$d_6$ (175 MHz).

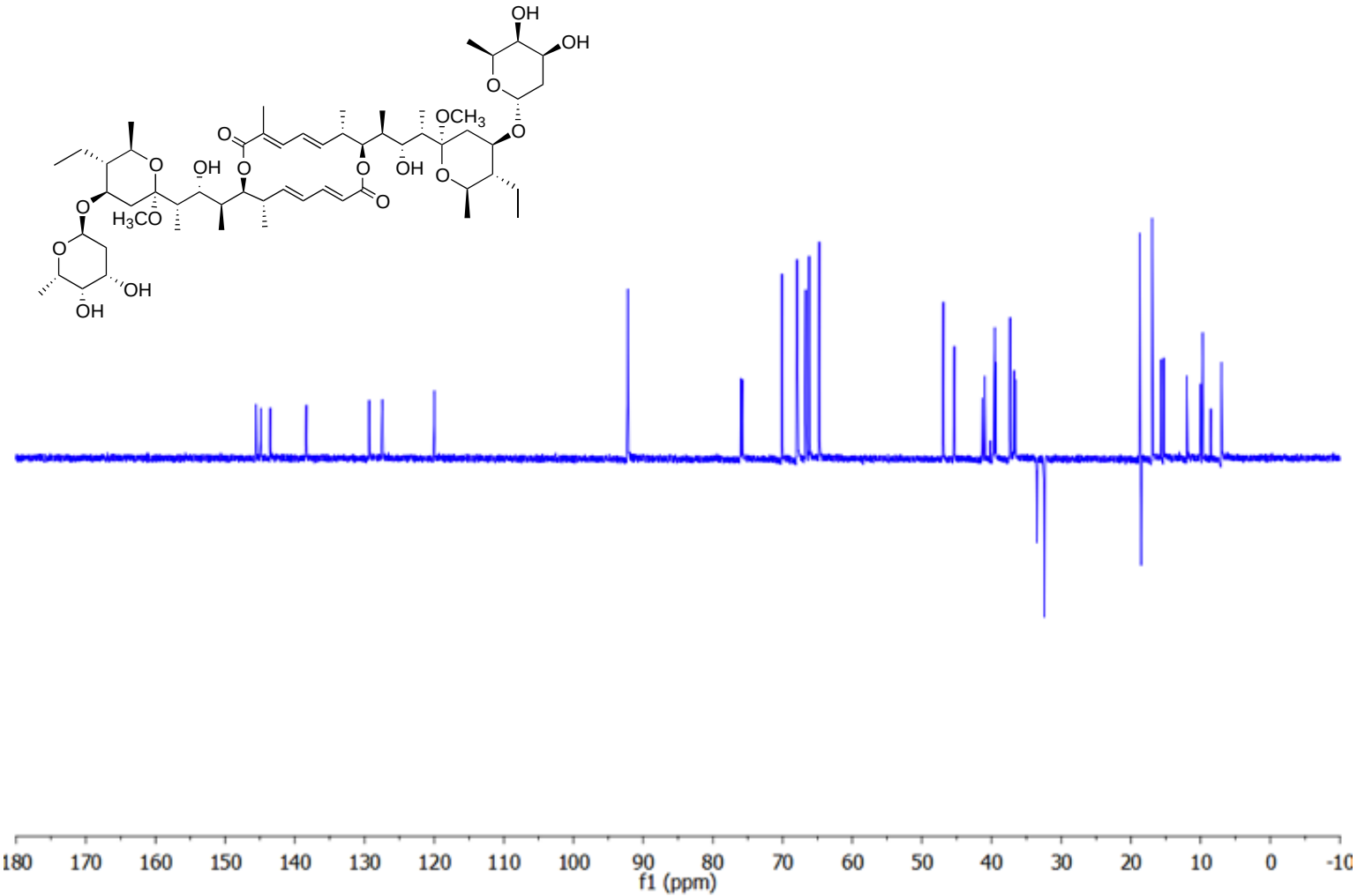


Figure S3.3. DEPT-135 spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

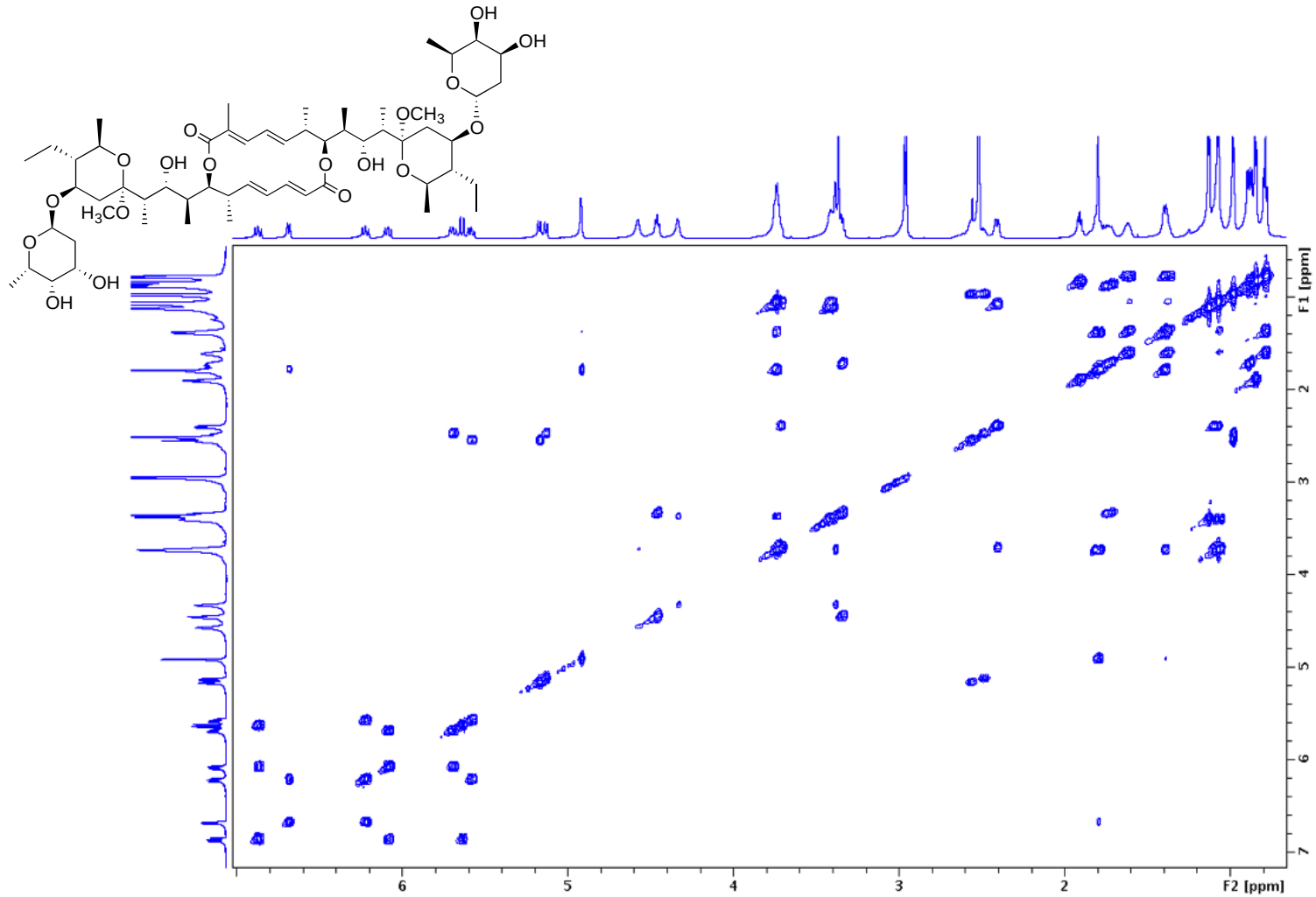


Figure S3.4. COSY spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

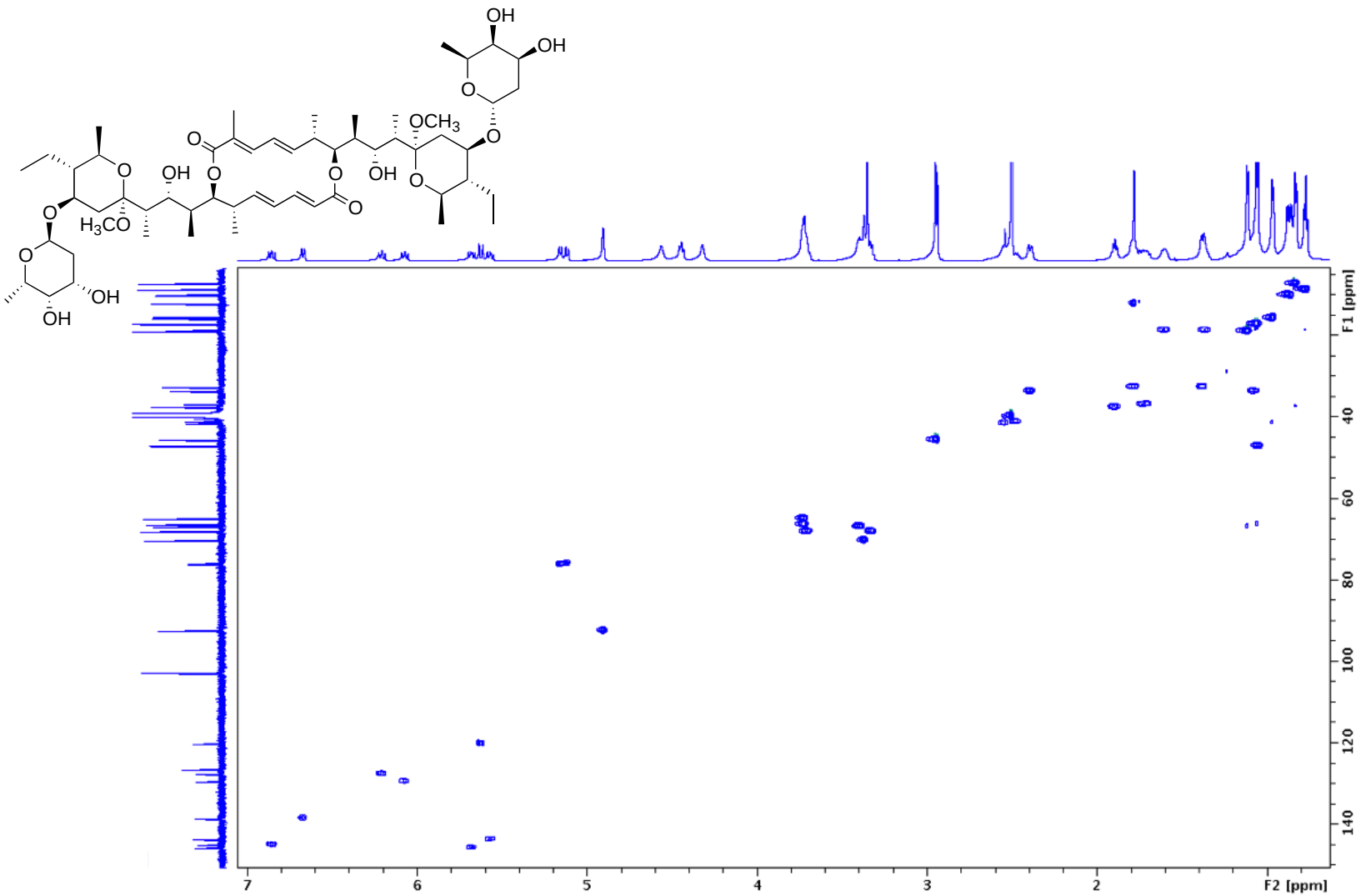


Figure S3.5. HSQC spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

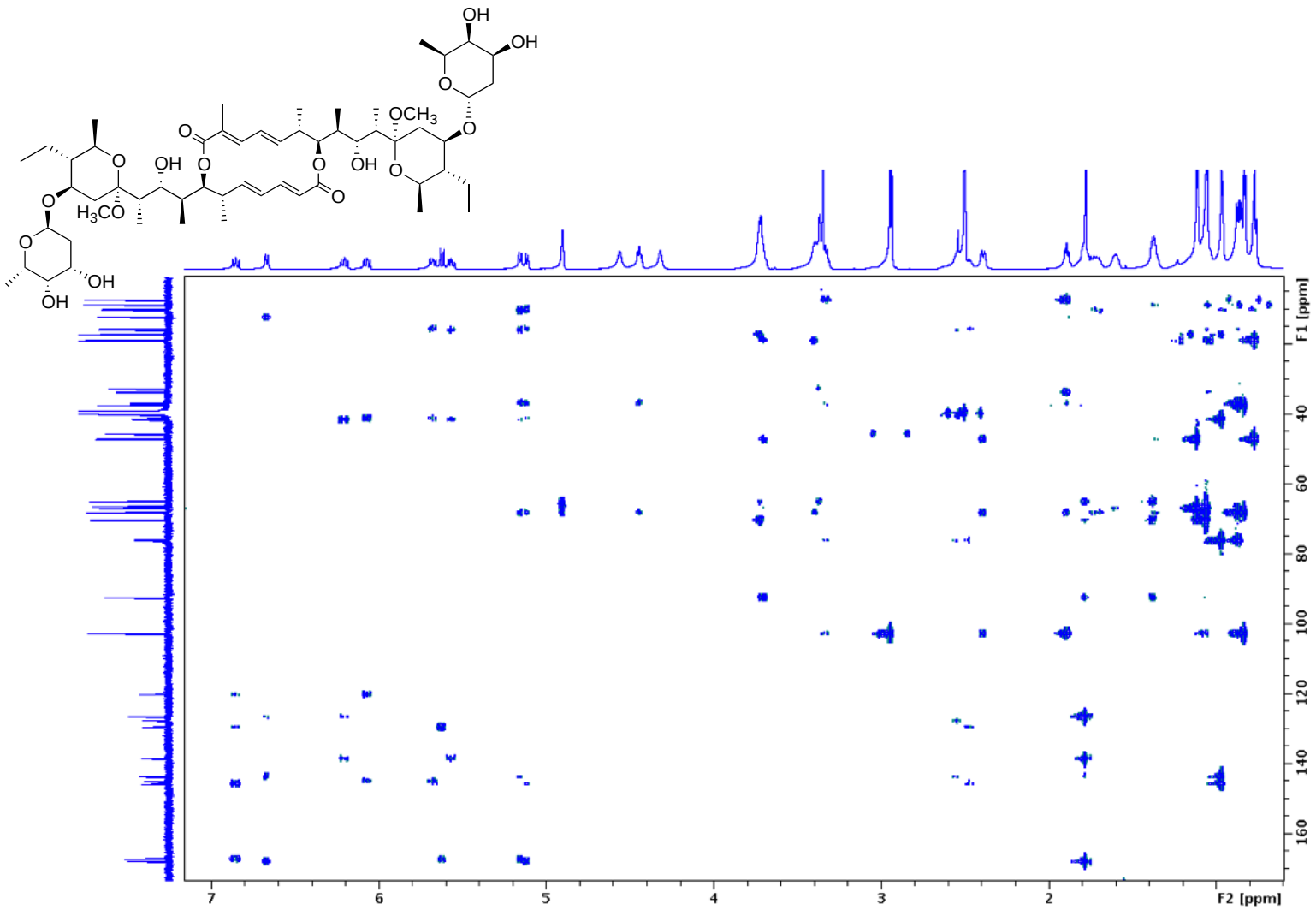


Figure S3.6. HMBC spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

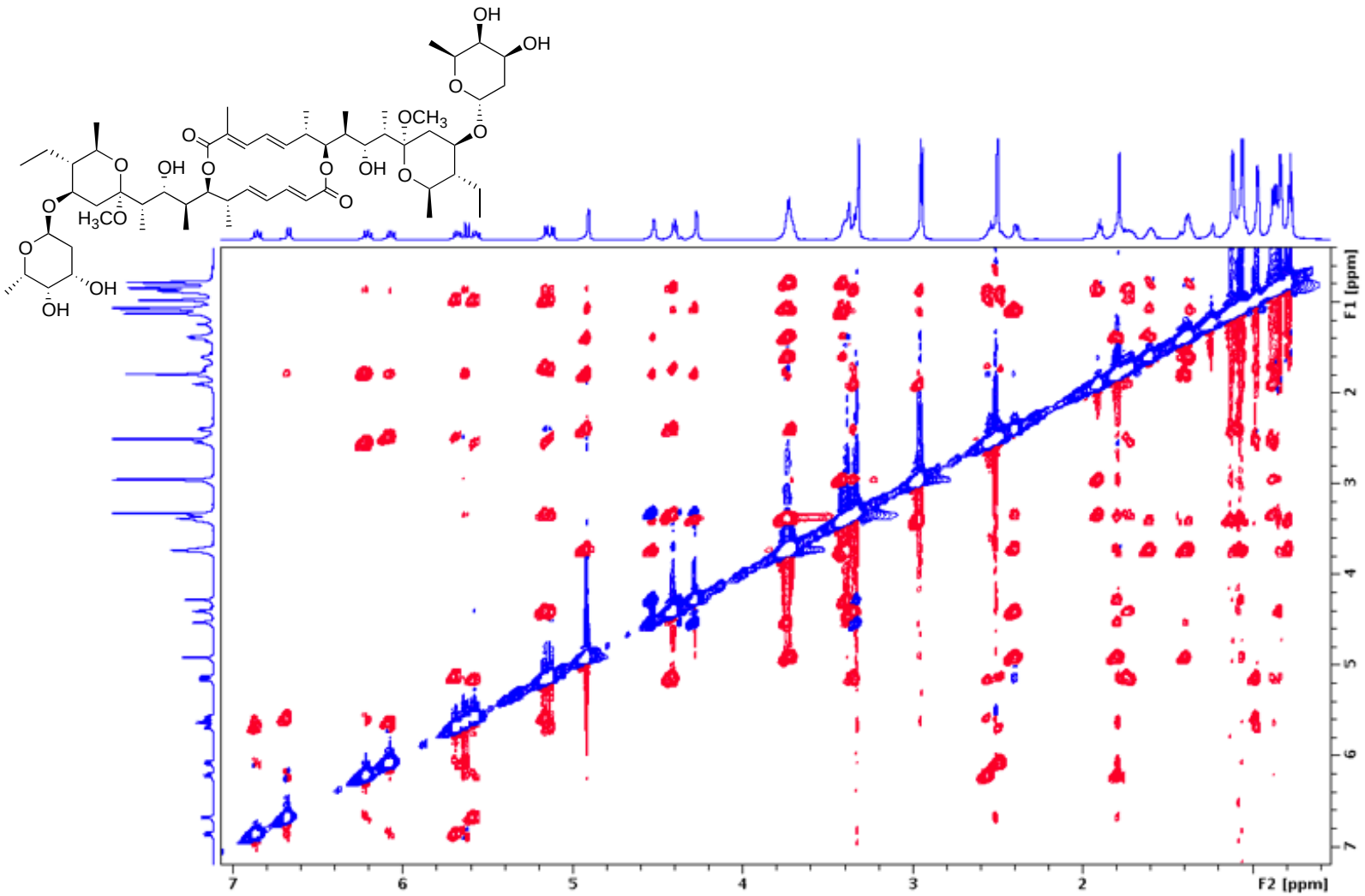


Figure S3.7. ROESY spectrum of compound **1** in DMSO-$d_6$ (700 MHz).

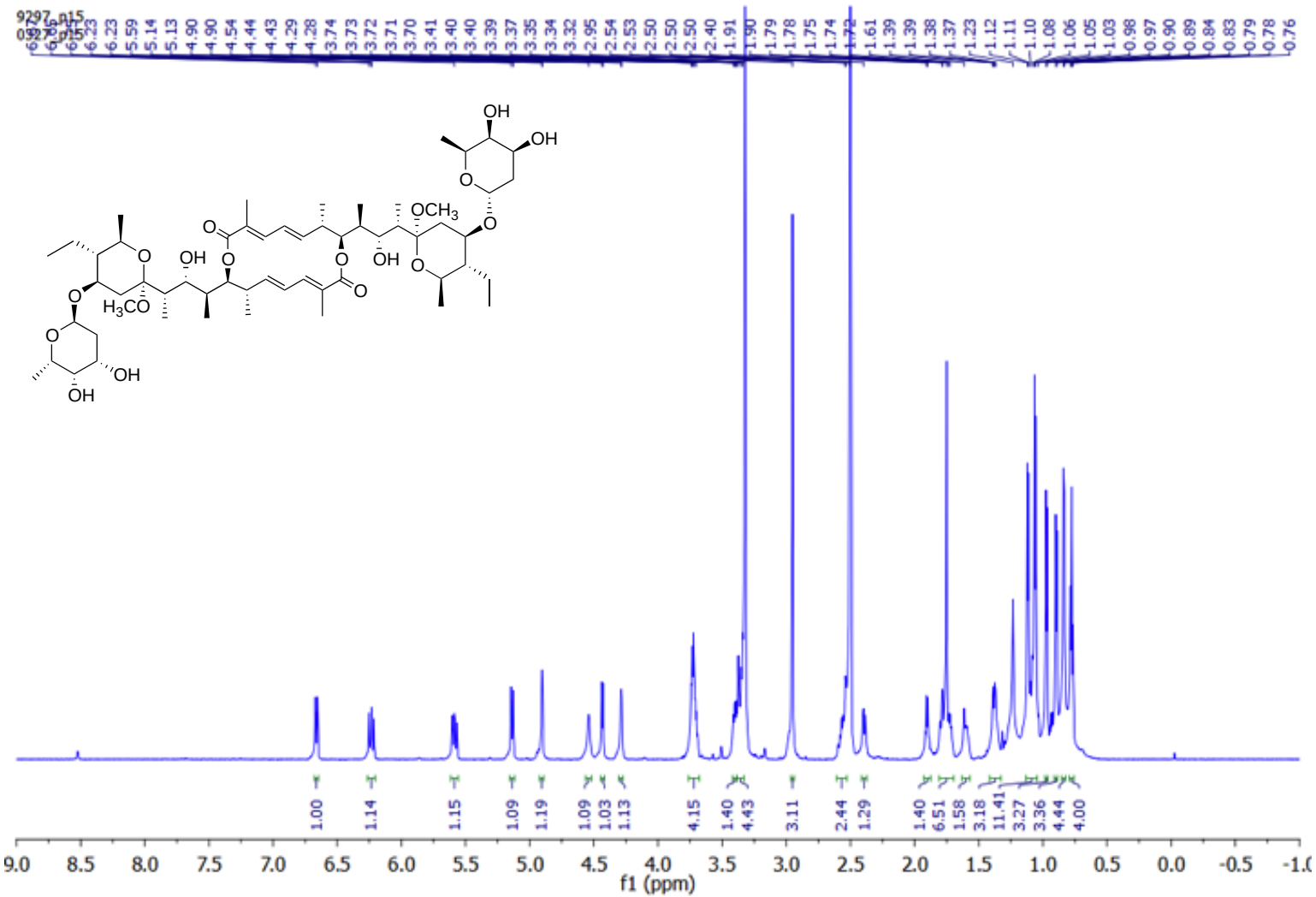


Figure S3.8. $^{1}$H NMR spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

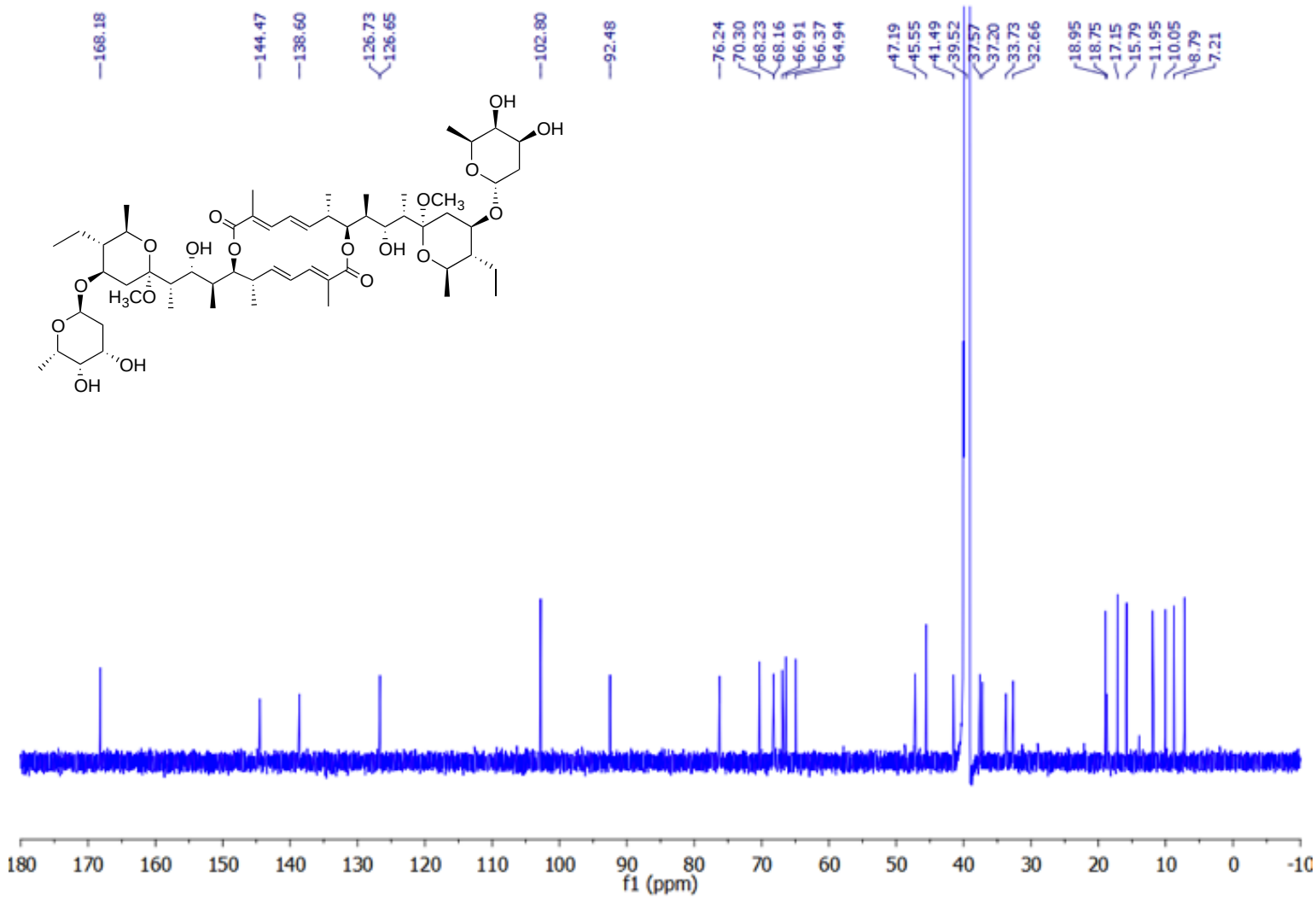


Figure S3.9. $^{13}$C NMR spectrum of compound **2** in DMSO-$d_6$ (175 MHz).

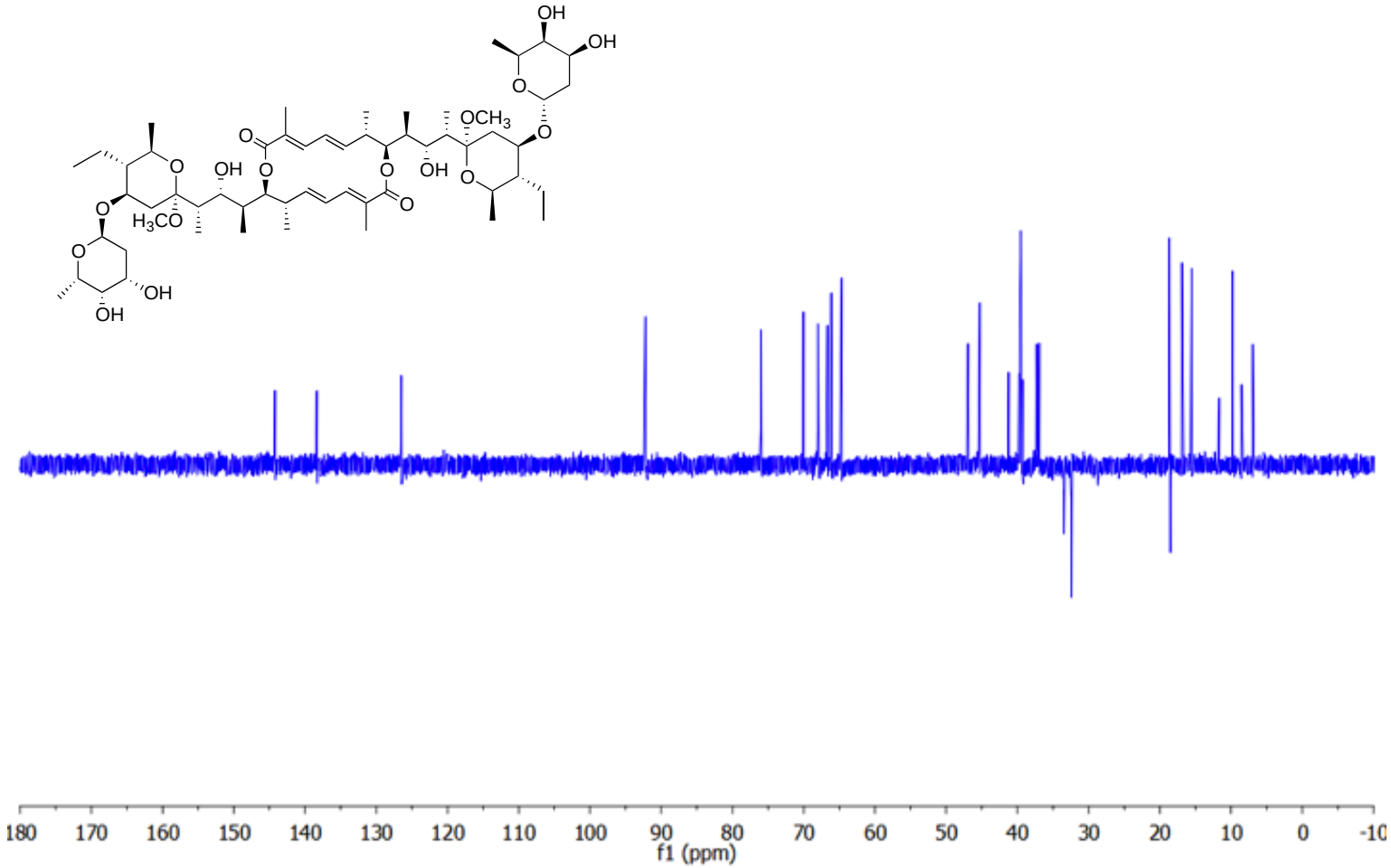


Figure S3.10. DEPT-135 spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

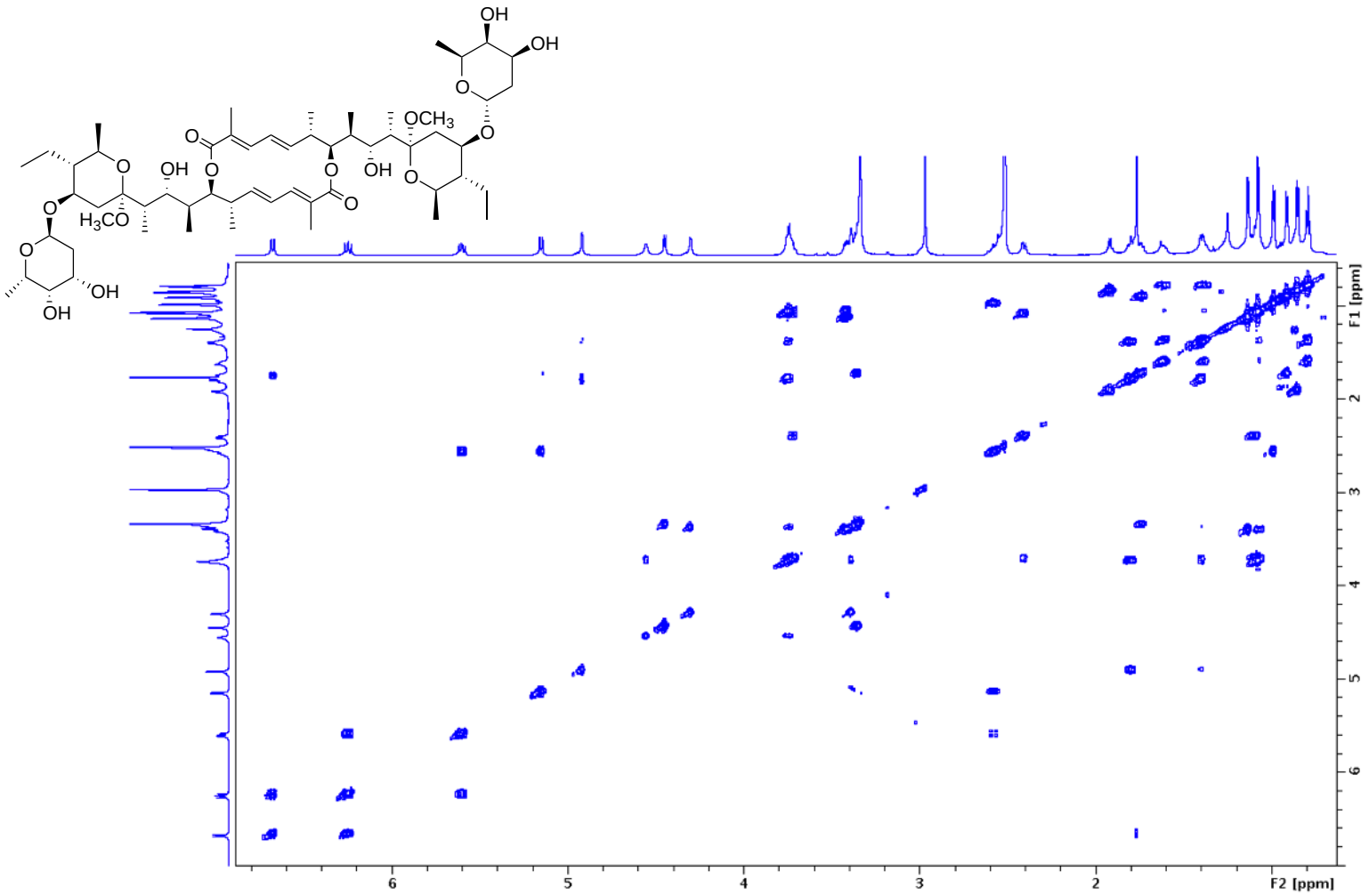


Figure S3.11. COSY spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

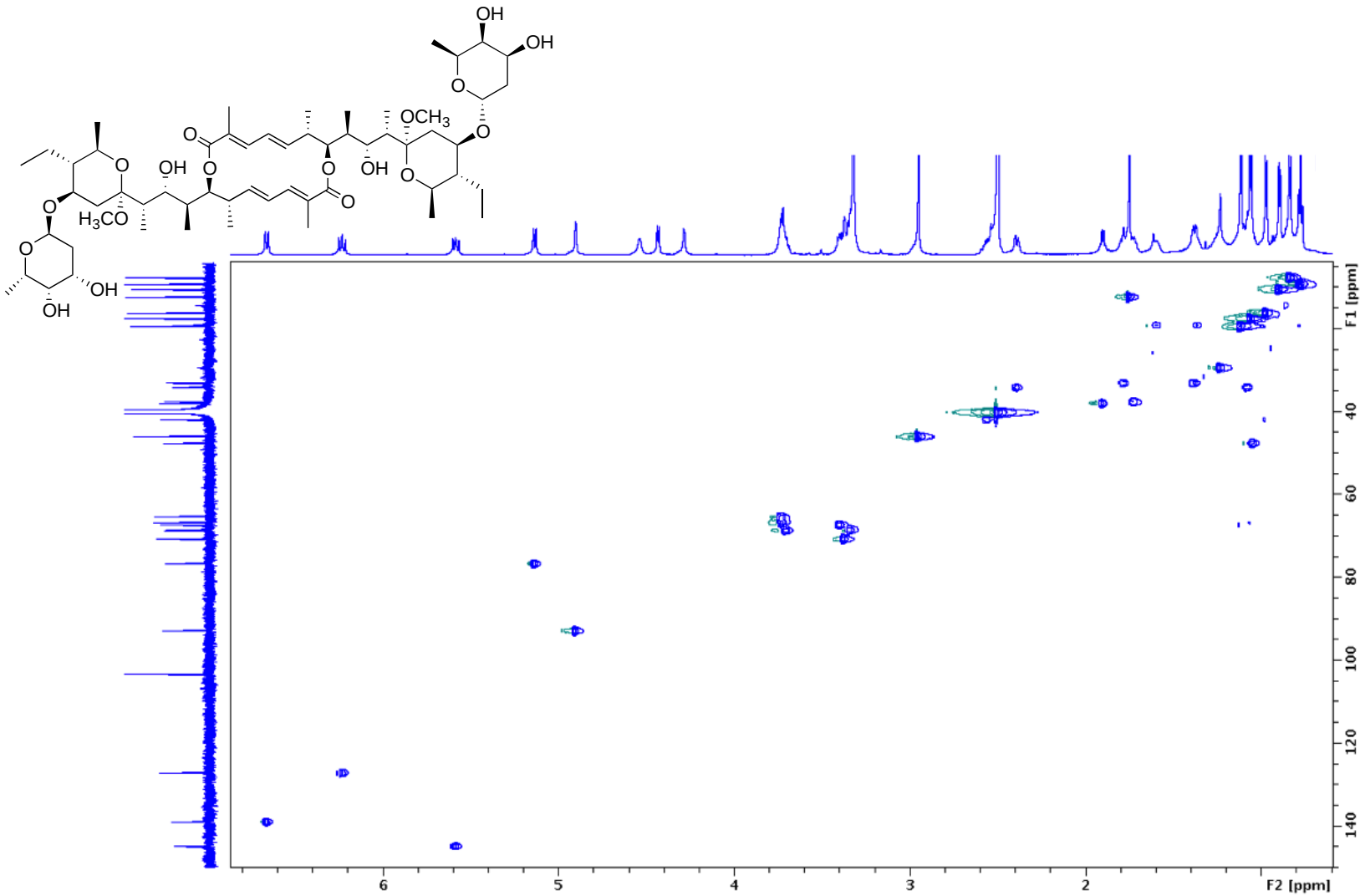


Figure S3.12. HSQC spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

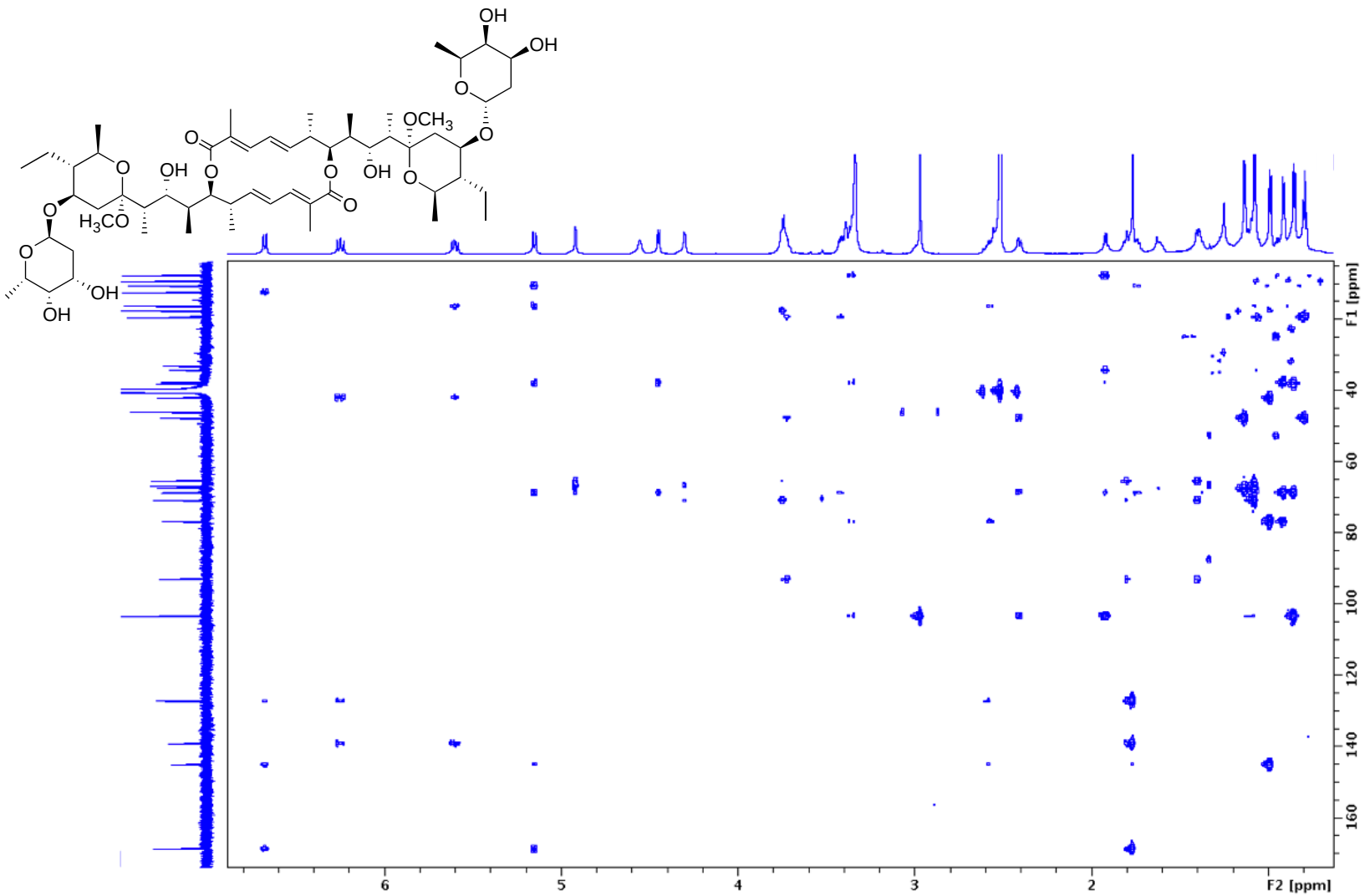


Figure S3.13. HMBC spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

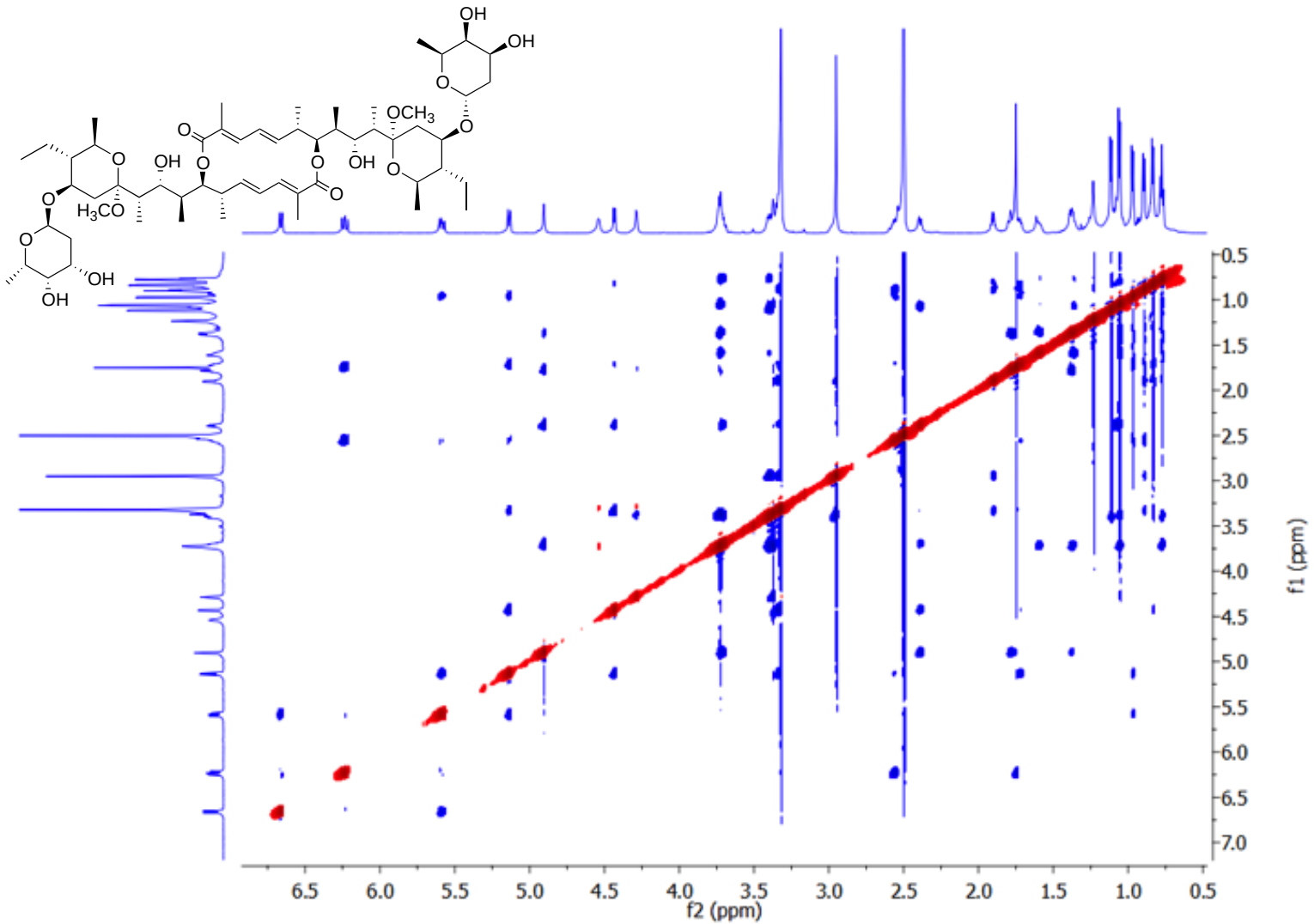


Figure S3.14. ROESY spectrum of compound **2** in DMSO-$d_6$ (700 MHz).

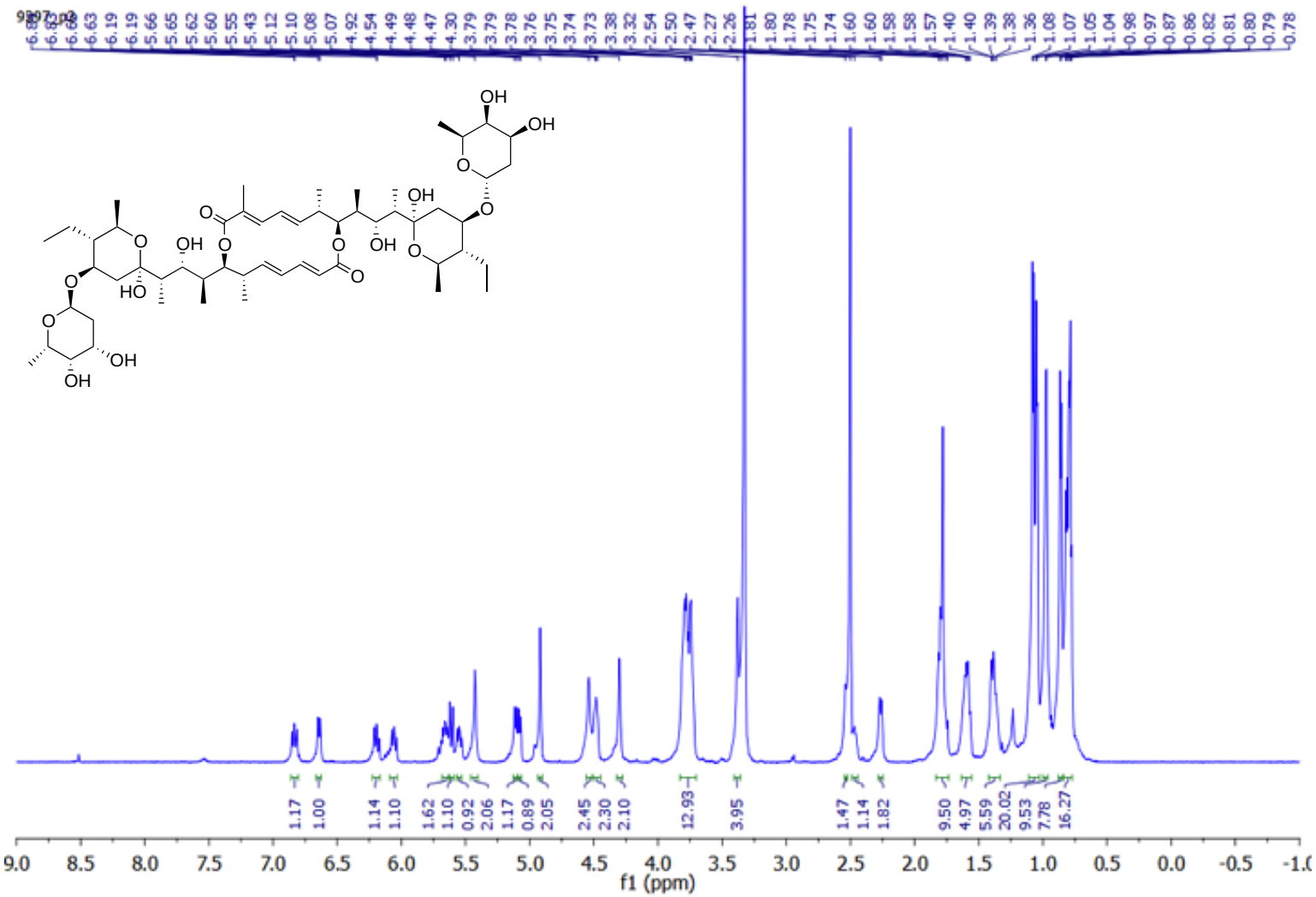


Figure S3.15. $^{1}$H NMR spectrum for compound **3** in DMSO-$d_6$ (700 MHz).

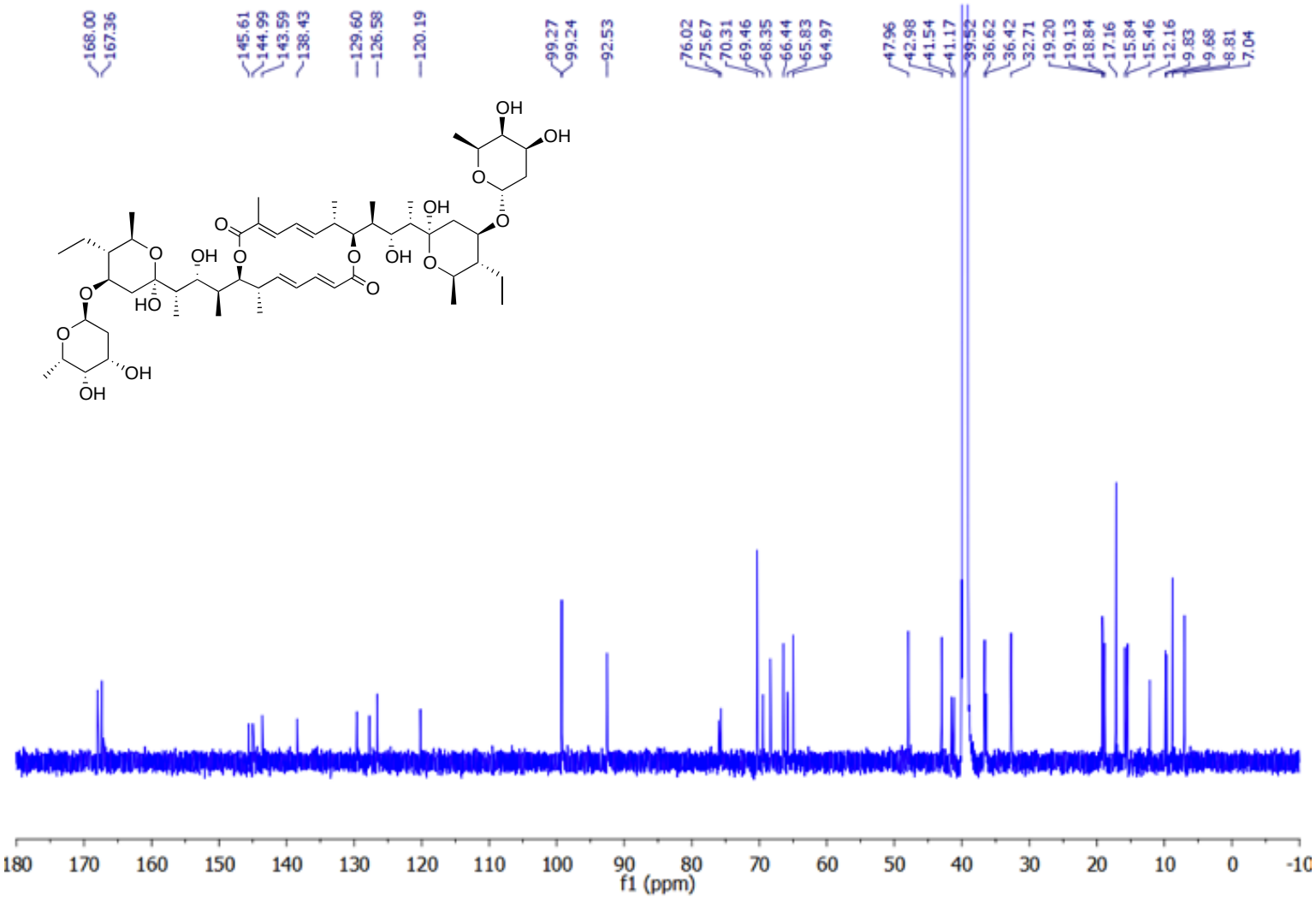


Figure S3.16. $^{13}$C NMR spectrum of compound **3** in DMSO-$d_6$ (175 MHz).

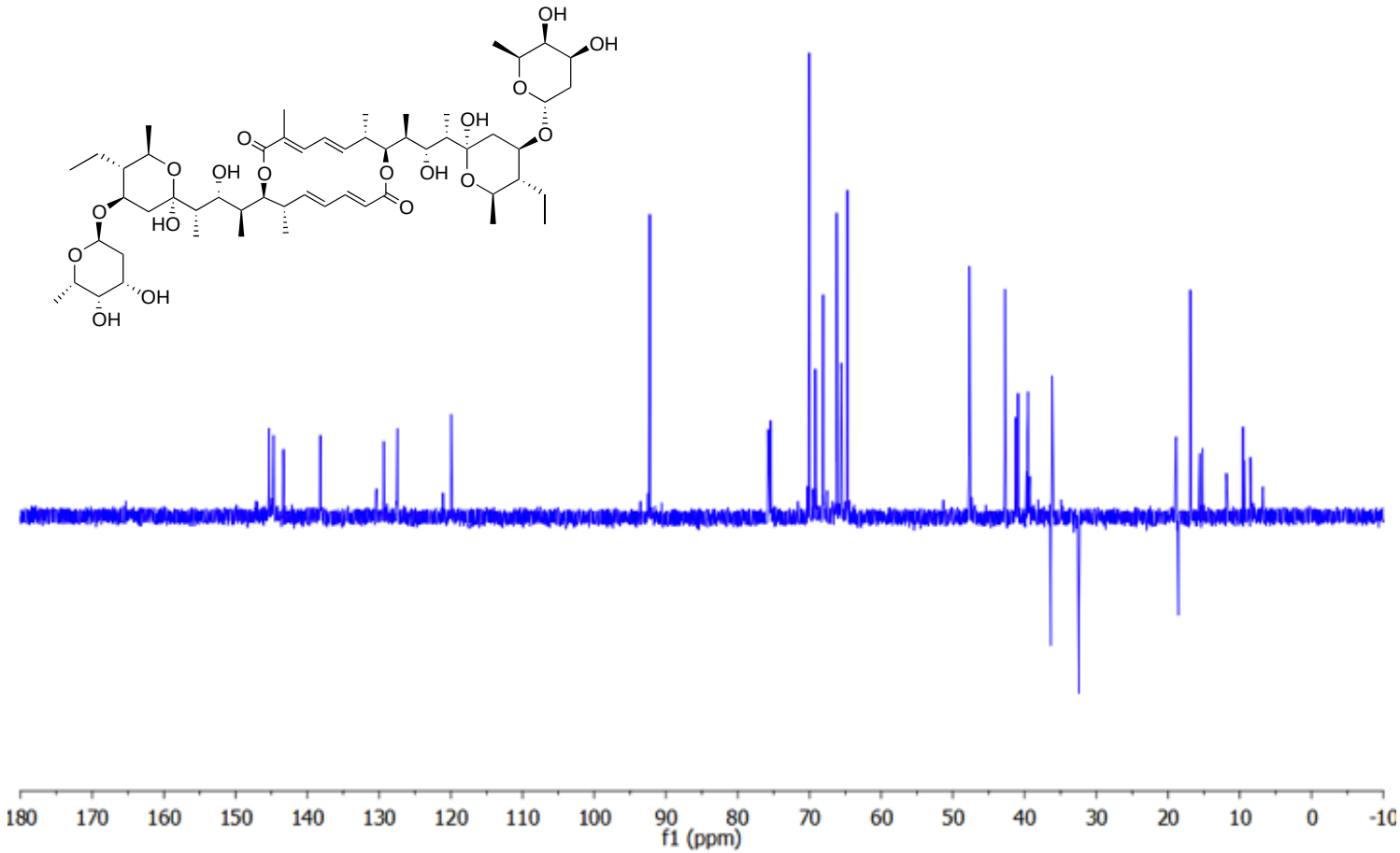


Figure S3.17. DEPT-135 spectrum of compound **3** in DMSO-$d_6$ (700 MHz).

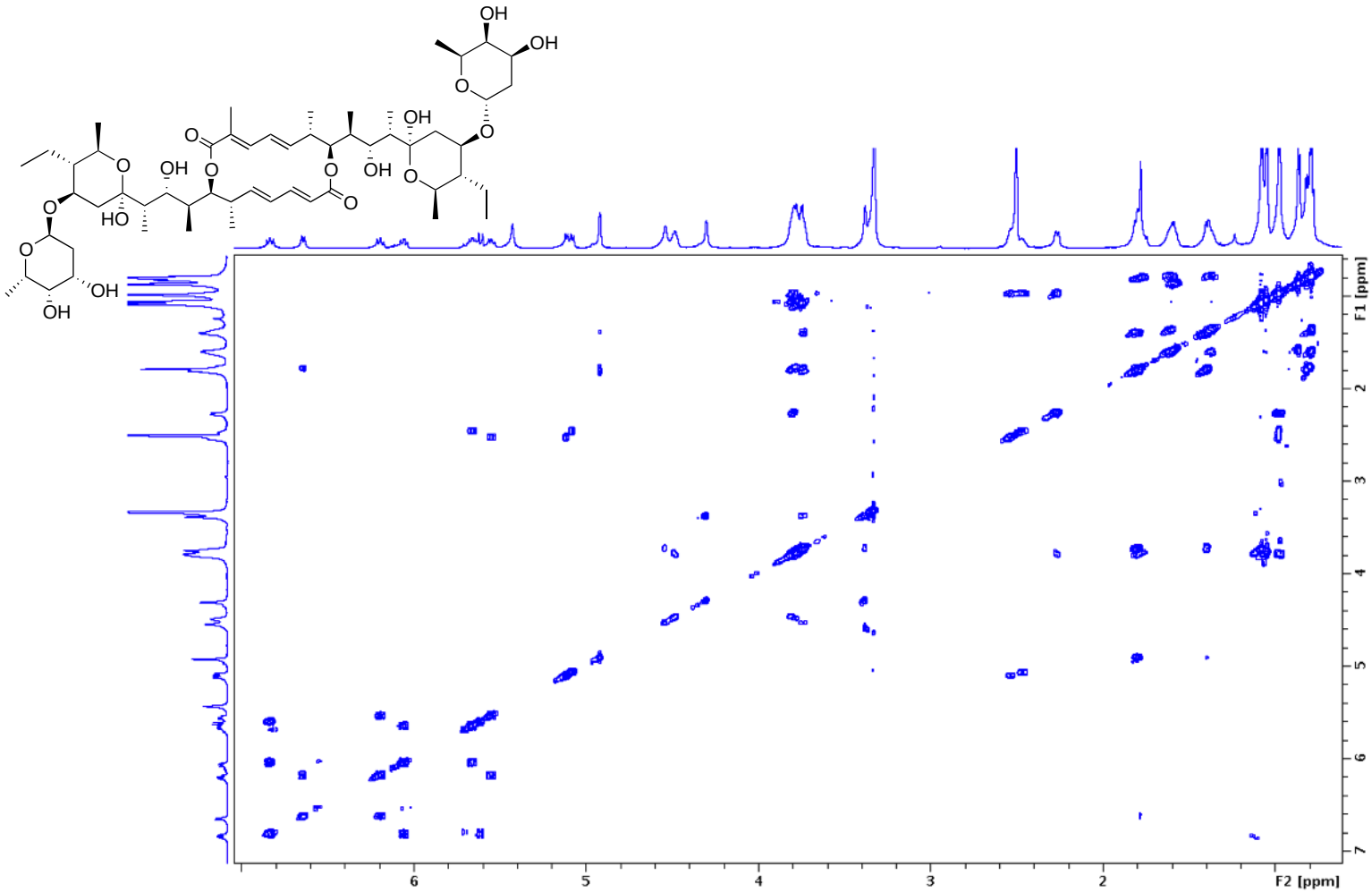


Figure S3.18. COSY spectrum of compound **3** in DMSO-$d_6$ (700 MHz).

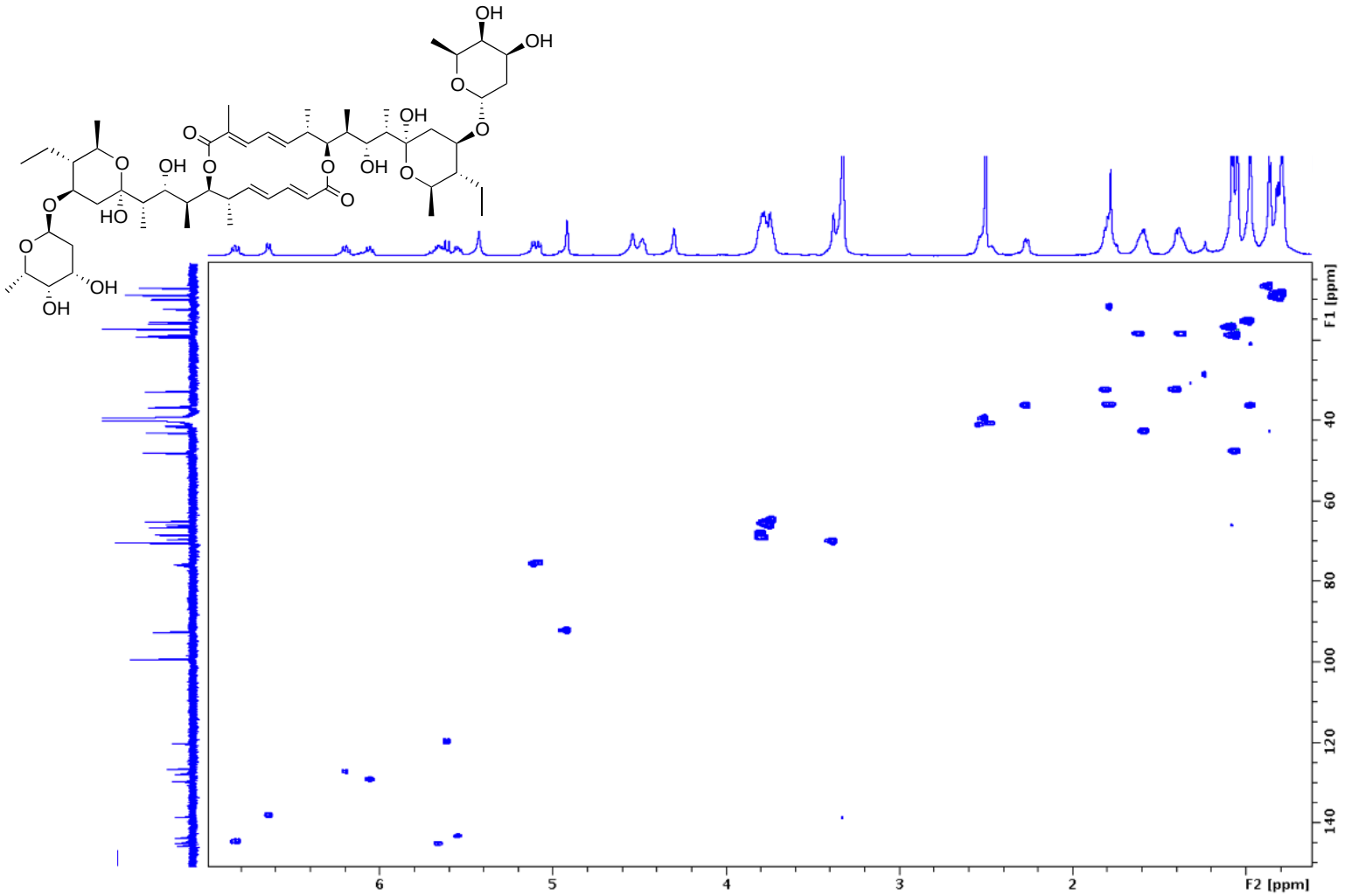


Figure S3.19. HSQC spectrum of compound **3** in DMSO-$d_6$ (700 MHz).

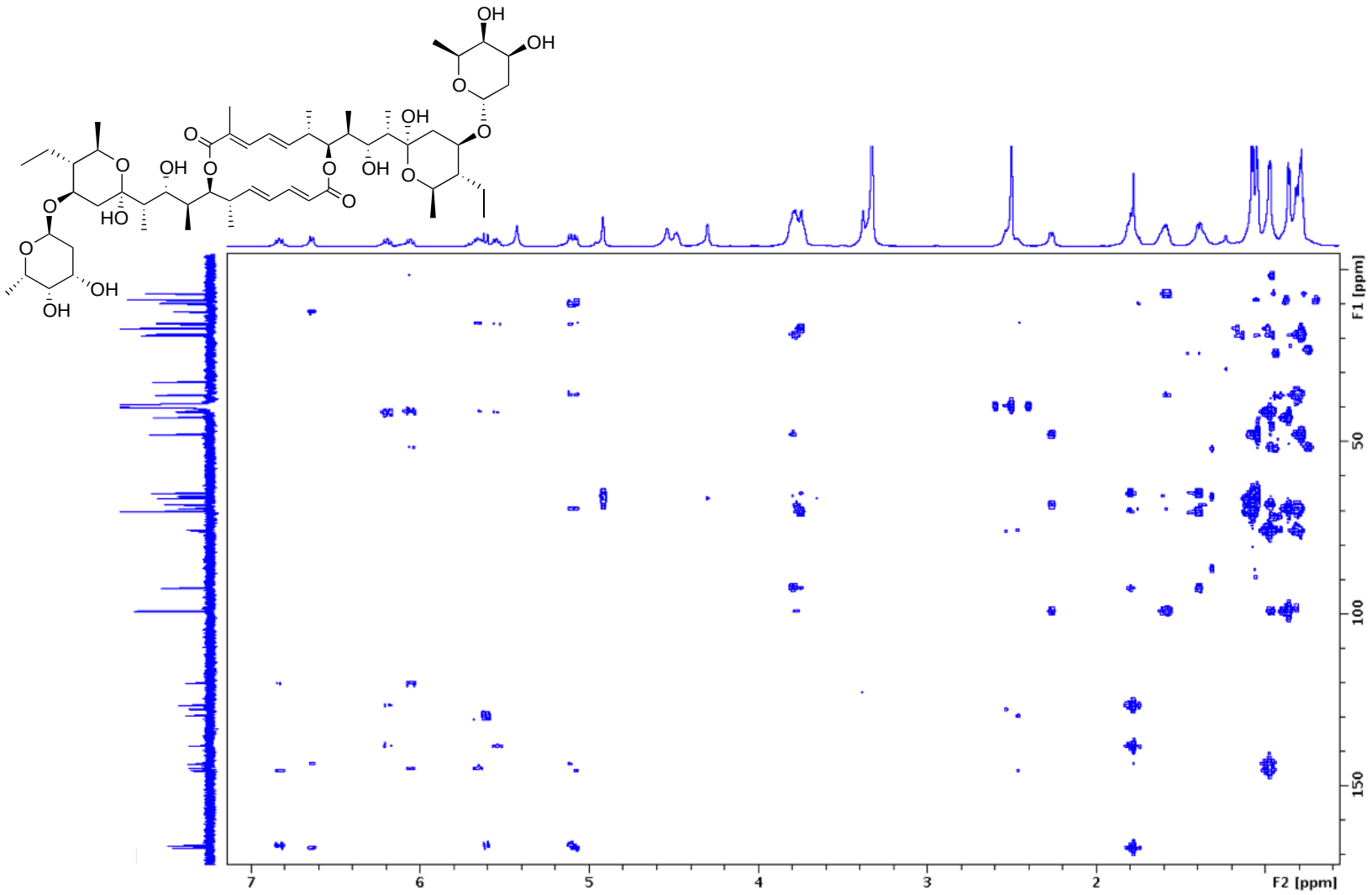


Figure S3.20. HMBC spectrum of compound **3** in DMSO-$d_6$ (700 MHz).

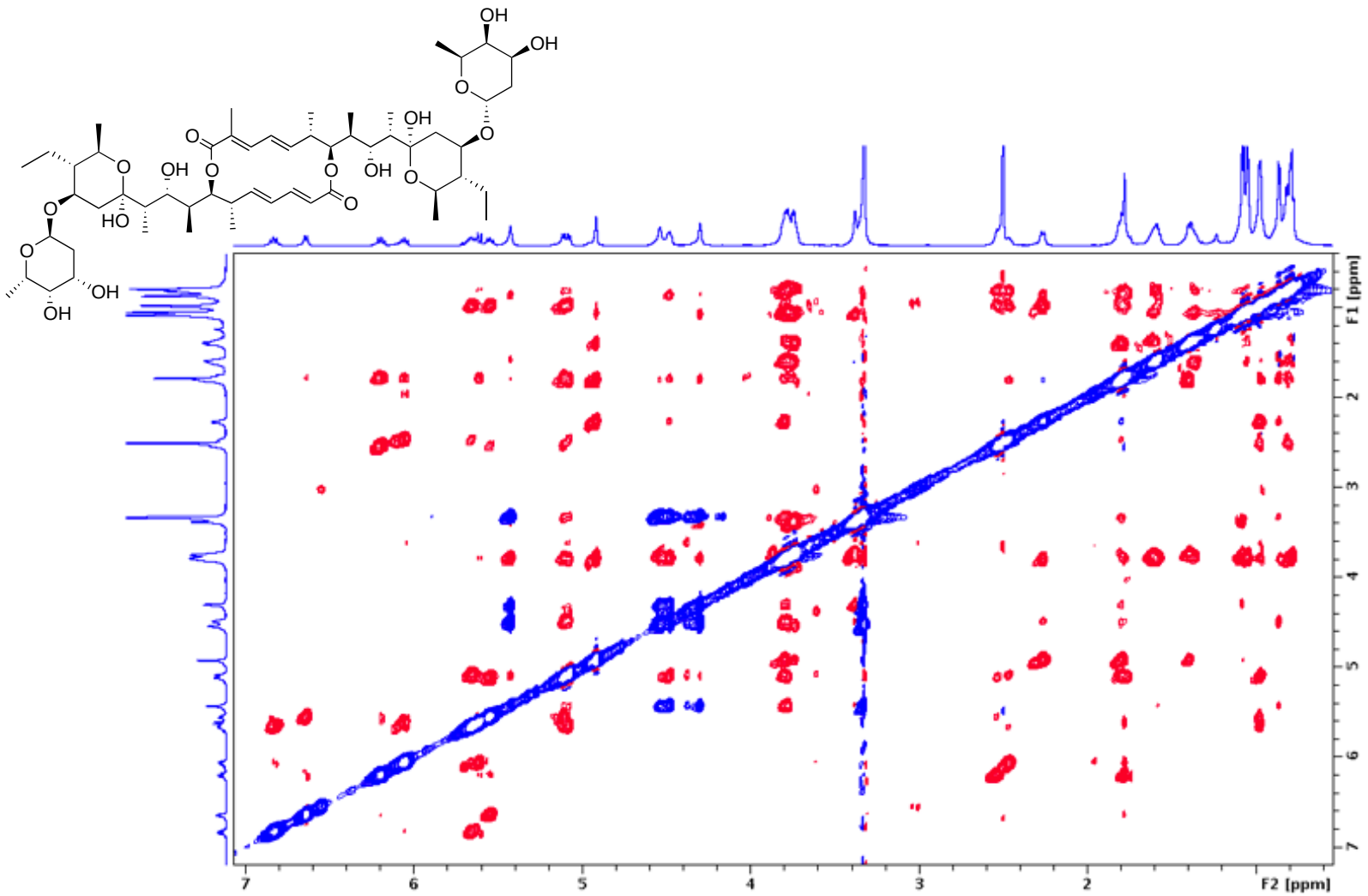


Figure S3.21. ROESY spectrum of compound **3** in DMSO-$d_6$ (700 MHz).

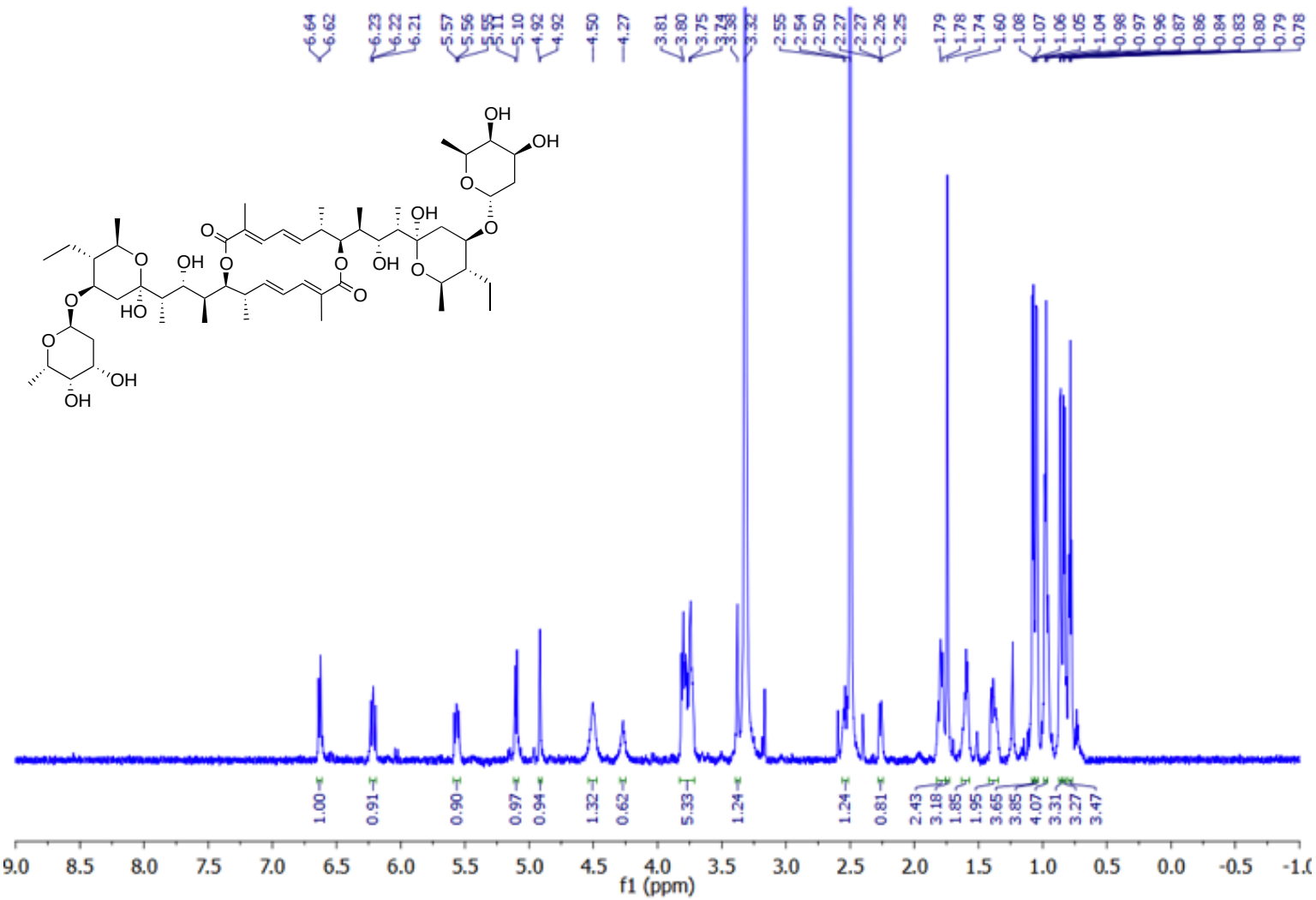


Figure S3.22. $^{1}$H NMR spectrum for compound **4** in DMSO-$d_6$ (700 MHz).

```
S. albiflaviniger ICBB 9297 ----------------------------------------------------ARSRLLDR
S. sp NRRL 30748            LFTGQGAQRLGMGRALHTAFPVFAAAFDAVCAELDRHLDGHVGHAVRDVVFGADAEPLDR
                                                                              *    ***
                                 63                                 90  94          117
S. albiflaviniger ICBB 9297 TLYTQAGLFATEVALYRLVESWGVTADFLVGHSVGELAAAHVAGVFSLEDACALVAARGR
S. sp NRRL 30748            TLYTQTGLFAVEVALYRLLESWGVTADFLVGHSVGELAAAHVAGVFSLEDACALVAARGR
                            ***** **** ******* ****************************************

S. albiflaviniger ICBB 9297 LMDALPAGGAMVSLQTGEAEVLPHLAGEEGQVSLGALNGPAATVISGEEKAVLRIADAVG
S. sp NRRL 30748            LMDALPAGGAMVSLQTGEAEVLPHLEGEEGQVSLGAVNGPAATVISGEEKAVLRIADAVG
                            ************************ ********** ************************
                                                200                             231
S. albiflaviniger ICBB 9297 VKSKRLRIGIAAHSPLMDPMLEEFAKVAGELTYHTPRIAVVSNVTGEAVAEELRSPEYWV
S. sp NRRL 30748            VKSKRLRIGIAAHSPLVDPMLEEFAKVAGELTYATPRIAVVSNVTGEAVAEELCSPDYWV
                            **************** **************** ****************** ** ***
                             250  255
S. albiflaviniger ICBB 9297 RHVRQPVRFQDGVRFLEDQGVTRYVXGGPVRCAVRAGAGVRRRH------------
S. sp NRRL 30748            RHVRQPVRFQDGVRFLEDQGVTRYVEVGPSGVLSVMGQECVADPDAAAFVPLLRKD
                            ************************  **       *
```

Figure S3.23. Alignment of AT domain in module 7 of elaiophylin PKS from *S. albiflaviniger* ICBB 9297 and *Streptomyces* sp. NRRL 30748. Conserved active site residues and motif are indicated by yellow and red color.

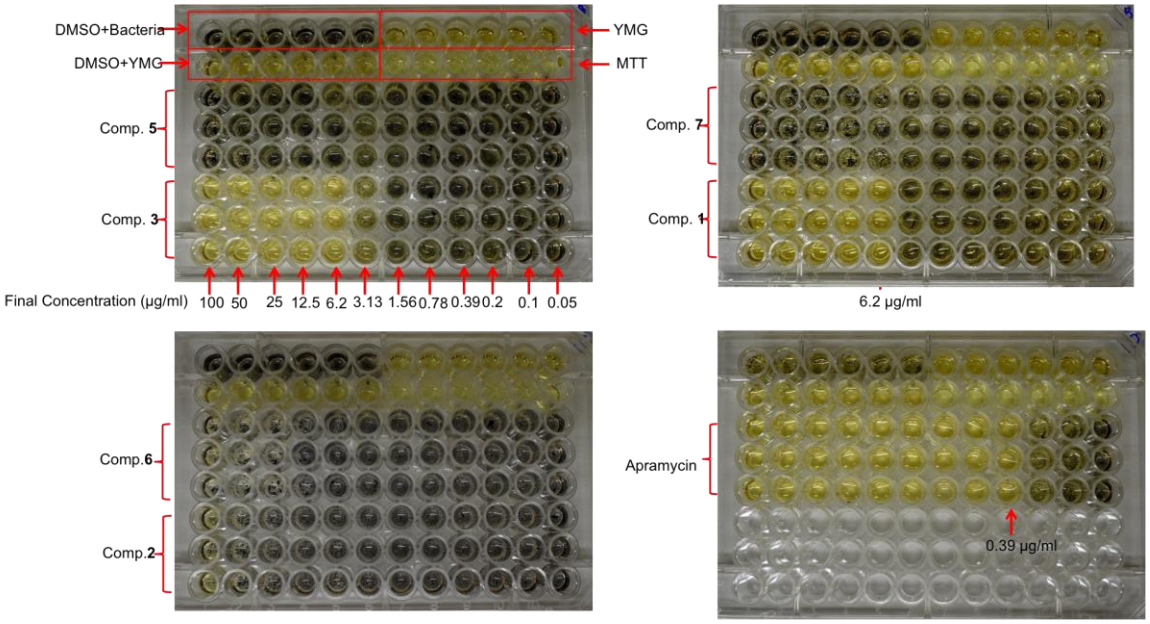


Figure S3.24. Microdilution assay for *Mycobacterium smegmatis.*

## 3.6 Reference


1. Myers, N.; Mittermeier, R. A.; Mittermeier, C. G.; da Fonseca, G. A.; Kent, J. *Nature* **2000**, *403*, 853-858.
2. Nair, M. G.; Chandra, A.; Thorogood, D. L.; Ammermann, E.; Walker, N.; Kiehs, K. *J. Agric. Food Chem.* **1994**, *42*, 2308-2310.
3. Kaiser, H.; Keller-Schierlein, W. *Helv. Chim. Acta* **1981**, *64*, 402-424.
4. Lee, S. Y.; Kim, M. S.; Kim, H. S.; Kim, Y. H.; Hong, S. D.; Lee, J. J. *J. Microbiol. Biotechnol.* **1996**, *6*, 245-249.
5. Ritzau, M.; Heinze, S.; Fleck, W. F.; Dahse, H. M.; Gräfe, U. *J Nat Prod* **1998,** *61*, 1337-1339.
6. Frobel, K.; Muller, H.; Bischoff, E.; Salcher, O.; De Jong, A.; Berschauer, F.; Scheer, M., U.S. Patent 4,927,810. 1990.
7. Wu, C.; Tan, Y.; Gan, M.; Wang, Y.; Guan, Y.; Hu, X.; Zhou, H.; Shang, X.; You, X.; Yang, Z.; Xiao, C. *J Nat Prod* **2013,** *76*, 2153-2157.
8. Arcamone, F. M.; Bertazolli, C.; Ghione, M.; Scotti, T. *G. Microbiol.* **1959**, *7*, 207-216.
9. Lee, S. Y.; Kim, H. S.; Kim, Y. H.; Han, S. B.; Kim, H. M.; Hong, S. D.; Lee, J. J. *J. Microbiol. Biotechnol.* 1997, *7*, 272-277.
10. Nakakoshi, M.; Kimura, K.; Nakajima, N.; Yoshihama, M.; Uramoto, M. *J Antibiot (Tokyo)* **1999,** *52*, 175-177.
11. Haltli, B. A., U.S. Patent 2006/0141583 A1.
12. Yadav, G.; Gokhale, R. S.; Mohanty, D. *J Mol Biol* **2003,** *328*, 335-363.
13. Reeves, C. D.; Murli, S.; Ashley, G. W.; Piagentini, M.; Hutchinson, C. R.; McDaniel, R. *Biochemistry* **2001,** *40*, 15464-15470.
14. Bisang, C.; Long, P. F.; Cortés, J.; Westcott, J.; Crosby, J.; Matharu, A. L.; Cox, R. J.; Simpson, T. J.; Staunton, J.; Leadlay, P. F. *Nature* **1999,** *401*, 502-505.
15. Del Vecchio, F.; Petkovic, H.; Kendrew, S. G.; Low, L.; Wilkinson, B.; Lill, R.; Cortés, J.; Rudd, B. A.; Staunton, J.; Leadlay, P. F., Active-site residue, domain and module swaps in modular polyketide synthases. *J Ind Microbiol Biotechnol* **2003,** *30*, 489-94.
16. Sundermann, U.; Bravo-Rodriguez, K.; Klopries, S.; Kushnir, S.; Gomez, H.; Sanchez-Garcia, E.; Schulz, F. *ACS Chem Biol* **2013,** *8*, 443-450.
17. Petković, H.; Sandmann, A.; Challis, I. R.; Hecht, H. J.; Silakowski, B.; Low, L.; Beeston, N.; Kuscer, E.; Garcia-Bernardo, J.; Leadlay, P. F.; Kendrew, S. G.; Wilkinson, B.; Müller, R. *Org Biomol Chem* **2008,** *6*, 500-506.
18. Arai, M., 12b. *J. Antibiot.* **1960**, *13*, 51-56.
19. Hammann, P.; Kretzschmar, G.; Seibert, G. *J Antibiot (Tokyo)* **1990,** *43*, 1431-1440.
20. Fiedler H.P., Wörner W., Zähner H., Kaiser H.P., Keller-Schierlein W., Müller A. *J Antibiot* (Tokyo) **1981**, *34*, 1107-1118.
21. Haydock, S. F.; Mironenko, T.; Ghoorahoo, H. I.; Leadlay, P. F. *J Biotechnol* **2004**, *113*, 55-68.
22. Zhou, Y.; Prediger, P.; Dias, L. C.; Murphy, A. C.; Leadlay, P. F. *Angew Chem Int Ed Engl* **2015**, *54*, 5232-5235.

# CHAPTER FOUR

## Succinylated apoptolidins from *Amycolatopsis sp*. ICBB 8242

Yan Sheng, Serge Fotso, Jeffrey D. Serrill, Salmah Shahab, Dwi Andreas Santosa, Jane E. Ishmael, Philip J. Proteau, T. Mark Zabriskie, Taifo Mahmud

## 4.1 Abstract

Two new apoptolidins, 2´-O-succinyl-apoptolidin A (**11**) and 3´-O-succinyl-apoptolidin A (**12**), were isolated from the culture broth of an Indonesian Amycolatopsis sp. ICBB 8242. These compounds inhibit the proliferation and viability of human H292 and HeLa cells. However, in contrast to apoptolidin A (**1**), they do not inhibit cellular respiration in H292 cells. It is proposed that apoptolidins are produced and secreted in their succinylated forms and 1 is the hydrolysis product of **11** and **12**.

## 4.2 Introduction

Apoptolidin A (**1**, Figure 4.1), the first member of the apoptolidin family, was isolated from the cultures of a *Nocardiopsis* sp. by Seto and co-workers in 1997. Remarkably, this macrolide antibiotic induced apoptosis in rat glia cells transformed with adenovirus E1A oncogene ($IC_{50}$ =11 ng/mL), but did not show cytotoxicity in normal glia cells ($IC_{50}$ >100 μg/mL).[1,2] Studies by the National Cancer Institute involving 37,000 substances against the 60 human cancer cell lines concluded that **1** was among the top 0.1% of the most selective cytotoxic agents known at that time.[3] Further mechanistic studies revealed that **1** is an inhibitor of the eukaryotic mitochondrial $F_0F_1$-ATP synthase, which is associated with its apoptotic activity.[3,4] However, subsequent investigations suggested that the inhibition of mitochondrial $F_0F_1$-ATP synthase was not the only determinant of the potent anti-proliferative activity of **1** and its analogues.[5] We have recently determined that although apoptolidins A and C are potent inhibitors of mitochondrial function they differ from the commonly known ATP synthase inhibitor oligomycin A in the way they trigger acute metabolic stress in several cancer cells.[6]

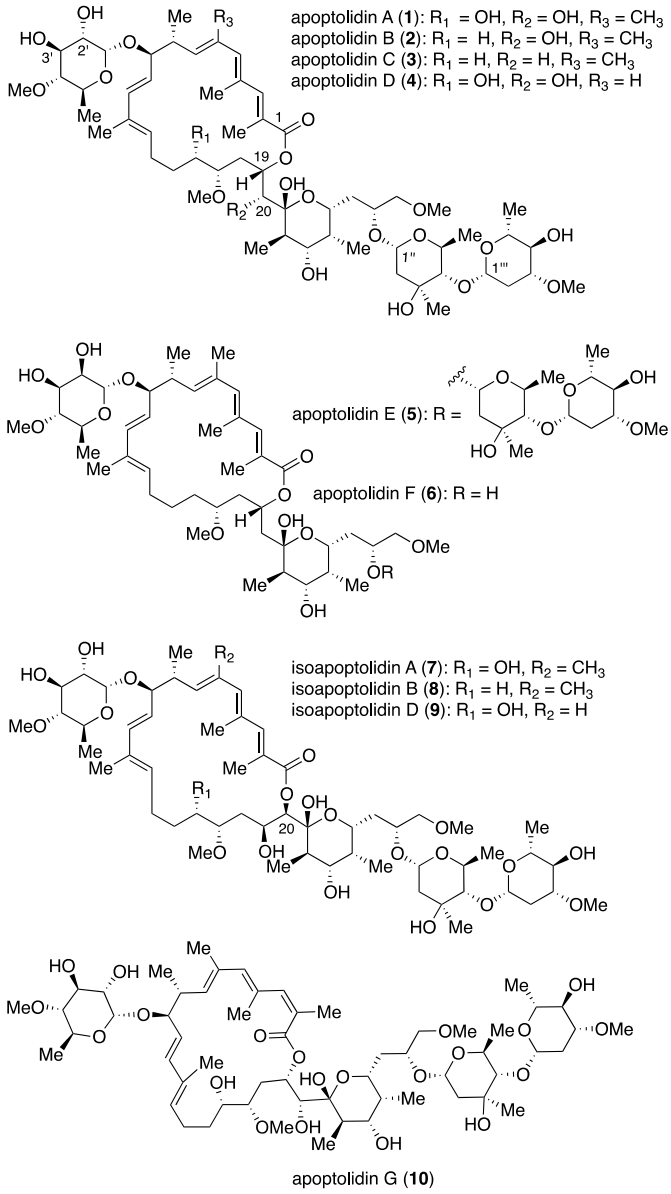


**Figure 4.1.** Chemical structures of apoptolidins isolated from *Nocardiopsis* sp. and *Amycolatopsis* sp. ICBB 8242.

Following the discovery of **1**, which was isolated as the major compound from culture broths of a *Nocardiopsis* sp. (150 mg/L), various minor apoptolidin analogues (2-7 mg/L), namely apoptolidin B-F (**2-6,** Figure 4.1), have also been identified.[7-9] In addition, during isolation and purification steps, apoptolidins may also undergo various

isomerization events. Isoapoptolidin A (**7**, Figure 4.1), isolated as the second major compound, is believed to be derived from **1** through an acyl migration from C19 to the C20 hydroxy group.[10] On the other hand, apoptolidin G (**10,** Figure 4.1), which was isolated from the same cultures, may be derived from **1** by a light induced olefin isomerization.[11] In addition to the above natural products and their artifacts, a number of synthetic or semisynthetic derivatives of apoptolidins have also been generated, opening up opportunities for exploring the structure activity relationships and mechanism of action of this group of natural products.[12-14]

As part of our on-going drug discovery efforts, we investigated bioactive natural products from microorganisms isolated from a unique black water ecosystem on the island of Borneo in Indonesia. Based on preliminary screenings using mass spectrometry (MS) and bioactivity assay of extracts, we identified a number of strains of *Amycolatopsis* that produce apoptolidins. Among them is *Amycolatopsis* sp. ICBB 8242, which produces the apoptolidins in high yields. Similar to the original producer *Nocardiopsis* sp., this strain produced apoptolidin A (**1**) and isoapoptolidin A (**7**) as major products, along with apoptolidins B (**2**), C (**3**), and D (**4**) as minor products.[6] Interestingly, MS analysis of the *n*-BuOH extract of this strain showed signals with significant intensity at *m*/*z* 1169 $[M+Na]^+$ and 1251 $[M+Na]^+$, indicating the presence of apoptolidins having molecular masses higher than any known naturally occuring apoptolidin analogues. The *n*-BuOH extract of the culture broth was consecutively subjected to solid phase extraction (SPE) $C_{18}$ column and Sephadex LH-20 column chromatography, followed by HPLC to give two new apoptolidin analogues

(compounds **11** and **12**) with an identical *m/z* value of 1251 ($[M+Na]^+$) (Figure 4.2), which is 100 atom mass units (amu) higher than that of **1** (*m/z* 1151, $[M+Na]^+$). Moreover, another pure compound (**13**) with an *m/z* value of 1169 ($[M+Na]^+$), 18 amu higher than **1**, was also isolated (Figure 4.2). We discovered that compounds **11** and **12** are relatively sensitive to pH, as they may be hydrolyzed to **1** under HPLC conditions with trifluoroacetic acid-containing solvent systems.

### 4.3 Results and discussion

The molecular formula of compound **11** ($[\alpha]^{21}_D$ -72 (***c*** 0.3, MeOH), UV (MeOH) $\lambda_{max}$ (log ε) 234 (4.46), 319 (4.22) nm) was determined by HR-ESI-MS to be $C_{62}H_{100}O_{24}$ (*m/z* 1251.6539, $[M+Na]^+$; calcd for $C_{62}H_{100}O_{24}Na$ 1251.6497). The $^1H$ NMR spectrum of **11** is very similar to that of **1** in terms of chemical shifts and coupling constants except that extra resonances were observed around 2.6 ppm. Also similar to the spectrum of **1**, six methyl doublets and five methyl singlets in the aliphatic region, four methoxy singlets, as well as sugar and $sp^2$ proton signals were observed. The $^{13}C$ and DEPT-135 spectra of **11** also showed extensive similarity to those of **1**. However, there are three carbonyl carbons present in **11** ($\delta_C$ 176.2, 173.9 and 172.8) instead of one carbonyl carbon in **1** ($\delta_C$ 172.8) and in other known apoptolidin analogues. Moreover, in addition to seven methylene carbons found in **1**, compound **11** possesses two additional methylene carbons ($\delta_C$ 30.2 and 30.1 ppm). These confirmed the presence of 62 carbons in **11**, instead of 58 in **1**. Based on the molecular formula and detailed analysis of two-dimensional (2D) NMR experiments (COSY, HSQC, HMBC, TOCSY, ROESY) compound **11** was determined to be a succinylated apoptolidin A. The two additional

carbonyl carbons and two methylene carbons in **11** were assigned to the succinyl moiety. The methylene protons [$\delta_H$ 2.69, 2.62, 2.58 (2H) ppm] showed HMBC correlations to both carbonyl carbons ($\delta_C$ 176.2, 173.9). The shift of the H-2´ signal from 3.40 ppm in **1** to 4.55 ppm in **11** and the HMBC correlation between H-2´ ($\delta_H$ 4.55 ppm) and one of the succinyl carbonyl carbons ($\delta_C$ 173.9) indicate that the succinyl moiety is attached to the C-2´ hydroxy group of **1**. This was confirmed by comparisons of the MS/MS fragments of **11** and **1** (Figures S4.31 and S4.33 in Supporting Information).

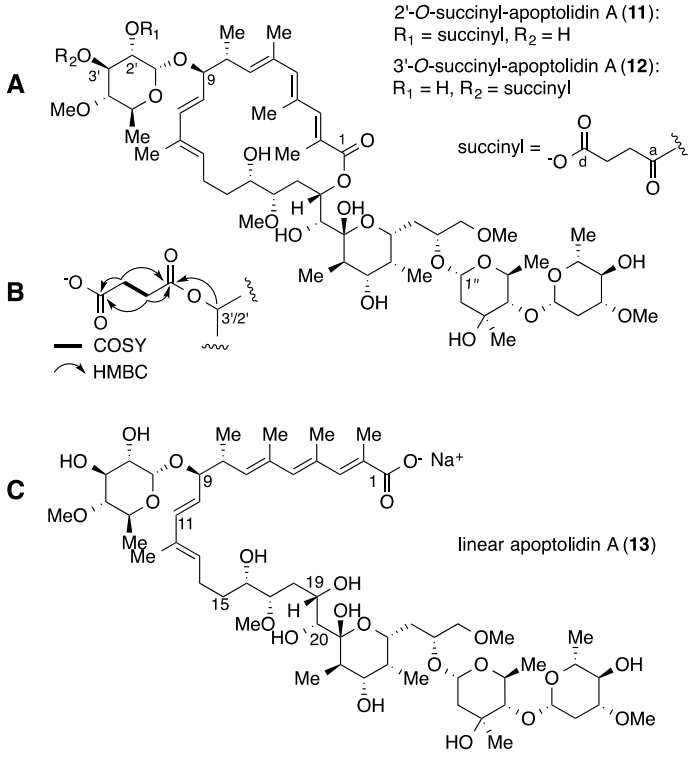


Figure 4.2. Chemical structures of the new apoptolidin analogues isolated from *Amycolatopsis* sp ICBB 8242. A. Structures of succinylated apoptolidins. B. COSY and HMBC correlations of the succinyl moiety in **11** and **12**. C. A linear analogue of apoptolidin A (**13**).

Compound **12** ($[\alpha]^{21}_D$ +17 (***c*** 0.3, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 235 (4.38), 319 (4.18) nm) has a molecular formula of $C_{62}H_{100}O_{24}$ (*m/z* 1227.6586, $[M-H]^-$; calcd for $C_{62}H_{99}O_{24}$ 1227.6532). The $^1H$, $^{13}C$ and DEPT-135 spectra of **12** are highly similar to those of **11**, including the presence of resonances for three carbonyls ($\delta_C$ 177.2, 174.1, 172.8) and nine methylenes. Based on extensive 2D NMR studies, **12** was also identified as a succinylated apoptolidin A. Interestingly, unlike **11**, in which the succinyl moiety is attached to the C-2´ hydroxy group, **12** has the succinyl moiety linked to the C-3´ hydroxy group, as indicated by an HMBC correlation between H-3´ and one of the succinyl carbonyl carbons ($\delta_C$ 174.1). Consequently, the resonance for H-3´ appears at 5.26 ppm, deshielded 1.54 ppm from that in **1**. Interestingly, compound **12** has a significantly different specific rotation (+17) than compound **11** (-72). Although the reason for this significant divergence is unclear, it may be due to conformational/rotational differences of the succinylated sugar moieties between the two compounds.

Compound **13** ($[\alpha]^{21}_D$ -180 (***c*** 0.4, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 236 (4.41), 288 (4.09) nm) has a molecular formula of $C_{58}H_{98}O_{22}$ (*m/z* 1169.6447, $[M+Na]^+$; calcd for $C_{58}H_{98}O_{22}Na$ 1169.6442). The molecular mass of **13** is 18 amu higher than **1**, indicating the formal addition of one water molecule. Based on its $^{13}C$ and DEPT-135 data, compound **13** has, among others, one carbonyl carbon, 10 olefinic carbons, including four quaternary ones, suggesting that the 18 amu difference was not a result of water addition to a double bond. Detailed analysis of the 2D NMR spectra of **13** revealed significant differences in the proton and carbon resonances at and around the

C-19 position. The $^{1}$H NMR signal for H-19 in **13** is 4.32 ppm, shifted from 5.29 ppm in **1**, suggesting that compound **13** lacks an ester functionality at this position. In addition, no HMBC correlation between H-19 and C-1 was observed. Considered together the above data suggest that compound **13** is the ester hydrolysis product of **1,** bearing a hydroxy group at C-19 and a free carboxylic acid moiety at C-1 (isolated as the sodium salt). To confirm the presence of a free carboxylic acid in **13**, the compound was subjected to methylation with TMS diazomethane. ESI-MS analysis of the reaction mixture showed a product with an *m*/*z* value of 1183 ($[M+Na]^{+}$), which is 14 amu higher than **13** ($[M+Na]^{+}$, 1169), consistent with the addition of a methyl group (Figure S4.28a, S4.28b). Compound **1** was also treated with trimethylsilyldiazomethane under the same conditions. However, no product was observed in this reaction, indicating that no other functionalities in **13** can be methylated under the reaction conditions used (Figure S4.28c). The $^{1}$H NMR spectrum of the purified methylated product of **13** is almost identical to that of **13**, except for the presence of an additional methyl group resonance at 3.76 ppm (Figure S4.29). Moreover, a significant hypsochromic shift of $\lambda_{max}$ from 320 nm in **1** to 288 nm in **13** confirmed the distinctive structural backbone of **1**, which has a conjugated ester chromophore, and **13**, which possesses a conjugated acid.

To investigate the biological activities of the new apoptolidins, we tested the ability of the compounds to inhibit the viability of human NCI-H292 (H292) lung and HeLa cervical cancer cells. The viability of H292 and HeLa cells was assessed after continuous exposure to increasing concentrations of the compounds for 6 days, using a

3 day + 3 day strategy to avoid nutrient deprivation.[6] The results show that **11** and **12** can inhibit the viability of both cancer cell lines tested with similar activity; **11** and **12** were 4- to 6-fold less potent than **1**, whereas **13** did not show any activity up to 1 μM (Table 4.1, Figure S4.34), suggesting that an intact macrocyclic ring is important for biological activity. We next used a target-based assay to examine the effects of the compounds on mitochondrial function in intact cells. Real-time analysis of cellular respiration in H292 cells (Seahorse XF 24 Analyzer, Seahorse Bioscience, Billerica, MA) showed that only **1** was affecting the oxygen consumption rate (OCR) of living cells following short-term exposure, whereas **11**, **12**, and **13** produced no change in cellular respiration (Figure 4.3). Although the result for the linear analogue **13** was consistent with that of the cell viability assays, the lack of activity of **11** and **12** was seemingly contradictory to their activity at nanomolar concentrations in standard end point assays and potentially indicative of an alternate biological mechanism of action.

In light of the relatively unstable nature of compounds **11** and **12**, we hypothesized that hydrolysis of **11** and **12** to the more active product **1** may have occurred during the time course of the cell viability assay, and the observed activities were not for **11** and **12** but for the hydrolytic product **1**. To test this hypothesis, we incubated compounds **11** and **12** individually in RPMI-1640 mammalian cell culture medium in a humidified cell culture incubator at 37 °C with 5% $CO_2$, and analyzed their potential conversion to **1** by ESI-MS after 2 h and 72 h. We observed a significant conversion (up to 30%) of the compounds to **1** after 72 h incubation but not at 2 h (Figures S4.35). Taken together our findings indicate that **11** and **12** are not acute

inhibitors of mitochondrial function. However, as hydrolysis is anticipated to be higher inside the cells due to non-specific cellular esterase enzymes,[15] it is likely that **1** contributes to the anti-proliferative activities of **11** and **12**, and that **11** and **12** may act as prodrugs of **1**.

Table 4.1. Effect of apoptolidin A (1) and the new analogues (11-13) on the viability of human cancer cell lines.

| compound | H292 cell $IC_{50}$ | HeLa cell $IC_{50}$ |
|---|---|---|
| apoptolidin A (**1**) | 22 nM | 39 nM |
| 2´-*O*-succinyl-apoptolidin A (**11**) | 91 nM | 240 nM |
| 3´-*O*-succinyl-apoptolidin A (**12**) | 82 nM | 260 nM |
| linear apoptolidin A (**13**) | NA | NA |

Compound **13** was not active up to 1 μM.

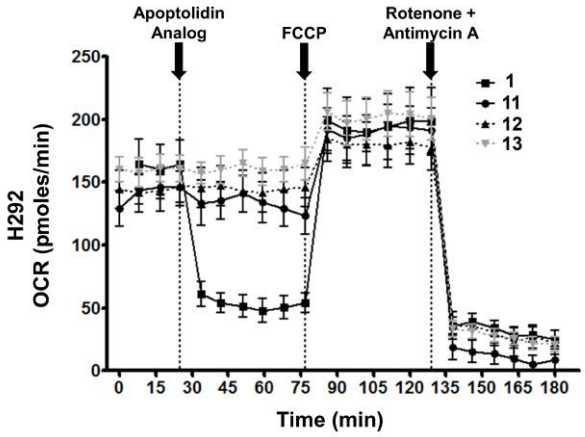


Figure 4.3. Real-time analysis of cellular respiration in human H292 lung cancer cells in response to apoptolidins plus target-specific inhibitors of the electron transport chain. OCR, oxygen consumption rate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone.

The production of succinylated apoptolidins by *Amycolatopsis* sp. ICBB 8242 suggests that succinylation may play a role in self-resistance and/or as an export mechanism. In addition to compounds **11** and **12** (*m/z* 1251, $[M+Na]^+$), we observed

other minor metabolites with *m/z* values consistent with those for succinylated apoptolidin analogues, such as succinylated apoptolidin B (*m/z* 1235 $[M+Na]^+$) and a related succinylated apoptolidin (*m/z* 1221 $[M+Na]^+$) in the ESI-MS spectrum of the EtOAc extract of the culture broths (Figure S4.36). This observation further suggests that all known natural apoptolidins may be produced and secreted in their succinylated forms and then hydrolyzed to the apoptolidins during fermentation and/or the isolation process. To test this hypothesis, we produced cell-free extracts of *Amycolatopsis* sp. ICBB 8242 by treating the cells (from 5 day cultures) with lysozyme followed by sonication and centrifugation to remove the cell debris. The cell-free extracts were lyophilized and extracted with MeOH. ESI-MS analysis of the MeOH extracts revealed the presence of **11** and/or **12** as predominant products (Figure S4.37), supporting the notion that natural apoptolidins are produced and secreted in their succinylated forms by *Amycolatopsis* sp. ICBB 8242. Inspection of the biosynthetic gene cluster of the apoptolidins in *Nocardiopsis* sp. FU40 did not provide clear indication of the presence of genes that may encode proteins for the succinylation reaction.[16] Therefore, the question of whether or not succinylation occurs in the *Nocardiopsis* producer, and if this modification is important in self-resistance and/or secretion remains unclear and warrants further investigation.

## 4.4 Experimental section

### 4.4.1 General Experimental Procedures.

Optical rotations were measured on a Jasco P1010 polarimeter. UV spectra were measured on an Eppendorf Biospectrometer kinetic. NMR spectra were recorded in

$CD_3OD$ referenced to residual solvent signals ($\delta_H$ 3.31 ppm, $\delta_C$ 49.00 ppm) on a Bruker 500 MHz Avance III 500 MHz spectrometer equipped with a 5 mm TXI probe or a Bruker 700 MHz Avance III 700 MHz spectrometer equipped with a 5 mm $^{13}C$ cryogenic probe. ESI-MS and MS/MS data were recorded on a ThermoFinnigan LCQ Advantage system with a quaternary Rheos 4000 pump (Flux Instrument) and an AB SCIEX 3200 Q TRAP mass spectrometer. High-resolution mass spectrometry was performed in positive/negative ion mode on an AB SCIEX Triple TOF 5600 mass spectrometer. HPLC was performed using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector.

**4.4.2 Organism Collection and Identification.**

The strain ICBB 8242 was isolated from the Black Water Ecosystems (BWE) in Kalimantan, Indonesia by the ICBB (Indonesian Center for Biotechnology and Biodiversity). The isolate was stored in 20% glycerol at -80 °C. For amplification of 16S-rDNA, primers F243 (GGATGAGCCCGCGGCCTA) and R1378 (CGGTGTACAAGGCCCGGGAACG) were selected. The genomic DNA was isolated and used as template. The 16S rRNA gene sequence (GenBank KJ888154) of strain ICBB 8242 was found to have 99% identity over the sequenced region to several *Amycolatopsis* strains in the database.[6]

**4.4.3 Fermentation and Isolation**.

*Amycolatopsis* sp. ICBB 8242 glycerol stock was streaked on Modified Bennett's agar plates [glucose (10 g), yeast extract (1 g), beef extract (1 g), soytone (2 g), metal ion

solution (1 mL), agar (15 g) in dd$H_2O$ (1L)] and placed in a 30 °C incubator for 3 days. To prepare the seed culture, bacteria were then inoculated into 500 mL Erlenmeyer flasks containing 100 mL of Modified Bennett's liquid medium. After 3 days, shaken at 200 rpm at 30 °C, the seed culture (10%) was transferred to the production medium (Modified Bennett's). The strain was cultivated in 25 Erlenmeyer flasks (500 mL) containing Modified Bennett's liquid medium (100 mL each) at 30 °C, 200 rpm, for 7 days. The culture centrifuged and the resulted supernatant was collected and extracted three times with EtOAc to give EtOAc extract (0.483 g). The aqueous layer was then extracted with *n*-BuOH to yield an *n*-BuOH extract (1.145 g). The *n*-BuOH extract was fractionated using an SPE $C_{18}$ cartridge with MeOH/$H_2O$ as the solvent system (stepped MeOH gradient: 10%, 20%, 30%, 50%, 60%, 70%, 80%, 100%). The desired compounds eluted together in the 60%, 70%, 80% MeOH/$H_2O$ fractions. The fractions were pooled and further separated by Sephadex LH-20 chromatography (100% MeOH) followed by semi-preparative reversed-phase HPLC purification to give pure 2´-*O*-succinyl-apoptolidin A (**11**) and 3´-*O*-succinyl-apoptolidin A (**12**) and linear apoptolidin A (**13**). For RPHPLC purification (column: YMC-Pack ODS-A, 250×10 mm I.D.), $CH_3CN$-sodium phosphate buffer (pH 7.0) was used as the mobile phase with the following time program: the column was equilibrated with 30% $CH_3CN$ for 5 min, then developed with a gradient of 30% to 50% $CH_3CN$ in phosphate buffer for 30 min and stay at 50% $CH_3CN$ for 30 min (2 mL/min). The retention times for 2´-*O*-succinyl-apoptolidin A (**11**), 3´-*O*-succinyl-apoptolidin A (**12**), and linear apoptolidin A (**13**) were 26, 25, and 19 min, respectively. Finally, the samples were desalted by passing them through a Sephadex LH-20 column (100% MeOH) to give **11** (2.7 mg), **12** (8.7

mg), and **13** (14 mg).

### 4.4.4 Methylation Reaction and Product Purification.

TMS diazomethane (40 μL, 2 M in diethyl ether, Aldrich) was added to a MeOH solution (250 μL) of **13** (0.5 mg) in an ice bath. The reaction mixture was stirred for 1 h and dried by nitrogen blow down. The product was dissolved in MeOH and purified by reversed-phase HPLC (YMC-Pack ODS-A, 250 × 4.6 mm I.D.) using a gradient of 5% to 100% $CH_3CN/H_2O$ over 45 min.

### 4.4.5 Mammalian Cell Culture.

Human NCI-H292 (H292) lung and HeLa cervical cancer cells were from the American Type Culture Collection (ATCC, Manassas, VA). H292 cells were cultured in RPMI-1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (1.5 g/L), 1% penicillin/streptomycin and 10% FBS. HeLa cells were grown in Minimal Essential Medium Eagle (MEM) formulation (Mediatech Inc., Manassas, VA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT), L-glutamine (2 mM), and 1% penicillin/streptomycin. All cells were maintained in a humidified chamber at 37 °C with 5% $CO_2$.

### 4.4.6 Cell Proliferation/Viability Assays.

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously with the viability of vehicle-treated cells defined as 100%.[6] Concentration-response relationships were analyzed using Graphpad

Prism Software (Graphpad Software Inc., San Diego, CA), and IC50 values derived using nonlinear regression analysis fit to a logistic equation.

### 4.4.7 Analysis of Mitochondrial Function.

Mitochondrial function was assessed in intact H292 cells with a Seahorse XF 24 Analyzer (Seahorse Bioscience, Billerica, MA). Cells were seeded in XF cell culture microplates (Seahorse Bioscience) at $5\times10^4$ cells/well in complete medium and maintained in a humidified chamber at 37 °C with 5% $CO_2$. Prior to measurements, the standard culture medium was replaced with unbuffered XF Assay Media (Seahorse Biosciences) supplemented with 1 mM sodium pyruvate and 25 mM D-glucose, pH 7.4, and allowed to equilibrate for 1 h at 37 °C in a non-$CO_2$ incubator. Cell culture plates were inserted into the instrument and the basal cellular oxygen consumption rate (OCR) was acquired before exposing cells to: (1) apoptolidins (1 μM) at 25 min, (2) FCCP (1 μM) at 77 min and, (3) rotenone plus antimycin A (0.5 μM) at 129 min by serial injection. OCR was monitored continuously over time to profile the mitochondrial stress response.

## 4.5 Supporting information

**Table S4.1.** $^{1}H$ and $^{13}C$ NMR Spectroscopic Data for Compounds **11-13** (700 MHz, $CD_3OD$).

| No. | **1** | | **11** | | **12** | | **13** | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 1 | 172.8 | | 172.8 | | 172.8 | | 172.6 | |
| 2 | 123.7 | | 123.7 | | 123.7 | | 126.5 | |
| 3 | 149.3 | 7.39 (s) | 149.4 | 7.39 (s) | 149.4 | 7.39 (s) | 145.0 | 7.15 (s) |
| 4 | 133.1 | | 133.1 | | 133.1 | | 133.2 | |
| 5 | 147.1 | 6.20 (s) | 147.1 | 6.20 (s) | 147.2 | 6.20 (s) | 139.9 | 6.20 (s) |
| 6 | 133.5 | | 133.8 | | 133.6 | | 133.2 | |
| 7 | 143.1 | 5.24 (d, 9.3) | 142.9 | 5.22 (d, 10.0) | 143.0 | 5.24 (m) | 135.5 | 5.22 (d, 9.7) |
| 8 | 38.9 | 2.76 (m) | 39.0 | 2.70 (m) | 38.8 | 2.81 (m) | 39.6 | 2.80 (m) |
| 9 | 84.1 | 3.84 (t, 9.2) | 84.5 | 3.82 (t, 8.8) | 84.3 | 3.86 (t, 9.3) | 82.1 | 3.88 (t, 8.2) |
| 10 | 126.5 | 5.23 (dd, 9.2, 16.0) | 125.7 | 5.10 (dd, 8.9, 15.5) | 126.3 | 5.23 (m) | 125.8 | 5.40 (dd, 8.7, 15.7) |
| 11 | 141.2 | 6.18 (d, 16.0) | 141.3 | 6.13 (d, 15.5) | 141.3 | 6.19 (d, 15.8) | 140.9 | 6.24 (d, 15.7) |
| 12 | 134.8 | | 134.8 | | 134.9 | | 134.4 | |
| 13 | 133.3 | 5.68 (dd, 6.8, 8.3) | 133.6 | 5.65 (brt, 7.5) | 133.4 | 5.69 (dd, 6.7, 8.8) | 134.4 | 5.54 (brt, 7.4) |
| 14 | 24.6 | 2.47 (m), 2.06 (m) | 24.6 | 2.45 (m), 2.08 (m) | 24.6 | 2.46 (m), 2.06 (m) | 25.8 | 2.34 (m), 2.24 (m) |
| 15 | 36.5 | 1.50 (m), 1.41 (m) | 36.5 | 1.52 (m), 1.41 (m) | 36.5 | 1.52 (m), 1.41(m) | 33.4 | 1.60 (m), 1.51 (m) |
| 16 | 74.6 | 3.43 (m) | 74.6 | 3.41 (m) | 74.6 | 3.43 (m) | 73.7 | 3.55 (m) |
| 17 | 83.8 | 2.72 (m) | 83.9 | 2.72 (m) | 83.8 | 2.72 (m) | 82.4 | 3.40 (m) |
| 18 | 38.4 | 2.16 (m), 1.74 (m) | 38.5 | 2.15 (m), 1.74 (m) | 38.4 | 2.16 (m), 1.73 (m) | 36.8 | 1.84 (m), 1.54 (m) |
| 19 | 72.4 | 5.29 (brd, 11.4) | 72.4 | 5.28 (brd, 11.3) | 72.4 | 5.28 (m) | 67.6 | 4.32 (brd, 10.0) |
| 20 | 75.3 | 3.53 (d, 0.98) | 75.4 | 3.53 (brs) | 75.3 | 3.53 (d, 1.2) | 75.1 | 3.34 (m) |
| 21 | 101.3 | | 101.3 | | 101.3 | | 103.4 | |
| 22 | 36.4 | 2.05 (m) | 36.4 | 2.05 (m) | 36.4 | 2.06 (m) | 36.2 | 2.27 (m) |
| 23 | 73.9 | 3.72 (m) | 73.9 | 3.71 (dd, 4.6, 10.9) | 73.9 | 3.71 (dd, 4.6, 11.0) | 73.2 | 3.78 (dd, 4.8, 11.3) |
| 24 | 40.7 | 1.73 (m) | 40.7 | 1.71 (m) | 40.7 | 1.71 (m) | 40.7 | 1.78 (m) |
| 25 | 69.4 | 3.95 (ddd, 2.0, 3.8, 8.1) | 69.4 | 3.95 (ddd, 2.0, 4.0, 8.4) | 69.4 | 3.95 (ddd, 2.0, 3.8, 8.1) | 68.8 | 4.14 (m) |
| 26 | 37.3 | 1.58 (ddd, 2.5, 8.3, 14), 1.46 (m) | 37.1 | 1.58 (ddd, 3.4, 8.5, 14.8), 1.46 (m) | 37.3 | 1.58 (ddd, 2.6, 8.1, 14.1), 1.46 (m) | 36.8 | 1.77 (m), 1.49 (m) |
| 27 | 76.9 | 3.44 (m) | 76.9 | 3.32 (m) | 76.9 | 3.44 (m) | 75.5 | 3.75 (m) |
| 28 | 76.8 | 3.32 (m), 3.32 (m) | 76.8 | 3.44 (m), 3.44 (m) | 76.8 | 3.33 (m), 3.33 (m) | 76.9 | 3.44 (m), 3.39 (m) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2-Me | 14.1 | 2.11 (s) | 14.1 | 2.11 (s) | 14.1 | 2.11 (s) | 14.4 | 1.99 (s) |
| 4-Me | 18.0 | 2.18 (s) | 17.9 | 2.20 (s) | 17.9 | 2.19 (s) | 18.7 | 1.93 (s) |
| 6-Me | 16.5 | 1.94 (s) | 16.5 | 1.96 (s) | 16.5 | 1.95 (s) | 18.4 | 1.79 (s) |
| 8-Me | 18.3 | 1.14 (d, 6.6) | 18.3* | 1.13 (d, 6.7) | 18.4* | 1.17 (d, 6.6) | 17.6 | 1.11 (d, 6.6) |
| 12-Me | 12.0 | 1.68 (s) | 12.2 | 1.67 (s) | 12.1 | 1.68 (s) | 12.6 | 1.76 (s) |
| 22-Me | 12.2 | 1.03 (d, 6.6) | 12.2 | 1.02 (d, 6.6) | 12.3 | 1.02 (d, 6.7) | 11.7 | 1.00 (d, 6.7) |
| 24-Me | 5.2 | 0.89 (d, 6.9) | 5.2 | 0.89 (d, 6.9) | 5.2 | 0.89 (d, 6.9) | 5.4 | 0.92 (s, 6.9) |
| 17-OMe | 61.4 | 3.36 (s) | 61.4 | 3.36 (s) | 61.4 | 3.36 (s) | 60.12 | 3.50 (s) |
| 28-OMe | 59.5 | 3.27 (s) | 59.5 | 3.27 (s) | 59.5 | 3.27 (s) | 59.4 | 3.34 (s) |
| 1′ | 96.0 | 4.82 (d, 4.1) | 93.5 | 4.99 (d, 3.8) | 95.9 | 4.85 (d, 3.8) | 95.6 | 4.78 (d, 3.9) |
| 2′ | 73.6 | 3.40 (dd, 4.0, 9.7) | 75.3 | 4.55 (dd, 3.9, 10.2) | 71.8 | 3.58 (dd, 3.8, 10.0) | 73.6 | 3.38 (m) |
| 3′ | 74.9 | 3.72 (m) | 72.3 | 3.93 (dd, 9.7, 9.2) | 76.5 | 5.26 (m) | 74.9 | 3.71 (m) |
| 4′ | 87.5 | 2.72 (t, 9.2) | 87.3 | 2.82 (t, 9.2) | 85.0 | 2.94 (t, 9.4) | 87.5 | 2.71 (t, 9.2) |
| 5′ | 68.2 | 3.74 (m) | 68.2 | 3.77 (m) | 68.2 | 3.83 (m) | 68.2 | 3.74 (m) |
| 6′ | 18.3 | 1.26 (d, 6.2) | 18.1* | 1.27 (d, 6.2) | 18.1* | 1.27 (d, 6.1) | 17.8 | 1.24 (d, 6.3) |
| 4′-OMe | 61.0 | 3.58 (s) | 61.3 | 3.60 (s) | 60.7 | 3.47 (s) | 60.9 | 3.57 (s) |
| 1′′ | 99.6 | 4.95 (d, 4.27) | 99.5 | 4.94 (d, 4.4) | 99.5 | 4.94 (d, 4.1) | 99.5 | 5.04 (d, 4.3) |
| 2′′ | 45.5 | 1.92 (d, 13.7), 1.81 (dd, 4.3, 13.7) | 45.5 | 1.94 (m), 1.80 (dd, 4.2, 13.4) | 45.5 | 1.93 (m), 1.80 (dd, 4.3, 13.4) | 45.2 | 2.03 (d, 13.3), 1.80 (m) |
| 3′′ | 73.0 | | 73.0 | | 73.0 | | 73.1 | |
| 4′′ | 85.8 | 3.33 (d, 9.7) | 85.8 | 3.34 (m) | 85.8 | 3.34 (m) | 85.9 | 3.33 (m) |
| 5′′ | 67.4 | 3.67 (dq, 6.2, 10.1) | 67.4 | 3.67 (dq, 6.1, 9.8) | 67.4 | 3.67 (dq, 6.2, 9.8) | 67.4 | 3.72 (m) |
| 6′′ | 18.9 | 1.22 (d, 6.2) | 18.9 | 1.21 (d, 6.2) | 18.9 | 1.22 (d, 6.2) | 18.9 | 1.21 (d, 6.2) |
| 3′′-Me | 22.7 | 1.32 (s) | 22.7 | 1.33 (s) | 22.7 | 1.33 (s) | 22.7 | 1.35 (s) |
| 1′′′ | 101.9 | 4.83 (dd, 1.7, 9.8) | 101.9 | 4.83 (dd, 1.8, 9.7) | 101.9 | 4.83 (dd, 1.7, 9.7) | 101.9 | 4.83 (dd, 1.5, 9.7) |
| 2′′′ | 37.2 | 2.44 (ddd, 1.4, 4.8, 11.9) 1.29 (m) | 37.2 | 2.45 (m), 1.28 (m) | 37.1 | 2.44 (m), 1.29 (m) | 37.1 | 2.45 (ddd, 1.3, 4.9, 12.0), 1.30 (m) |
| 3′′′ | 82.0 | 3.17 (ddd, 5.0, 8.8, 11.6) | 81.9 | 3.17 (ddd, 5.0, 8.6, 11.5) | 81.9 | 3.17 (ddd, 5.0, 8.8, 11.6) | 81.9 | 3.17 (ddd, 5.0, 8.8, 11.7) |
| 4′′′ | 77.1 | 2.97 (t, 9.0) | 77.1 | 2.97 (t, 9.0) | 77.1 | 2.97 (t, 9.0) | 77.1 | 2.97 (t, 9.0) |
| 5′′′ | 73.2 | 3.21 (dq, | 73.2 | 3.21 (dq, 6.1, | 73.2 | 3.21 (dq, 6.1, | 73.2 | 3.21 (dq, |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 6.2, 9.5) | | 9.3) | | 9.5) | | 6.2, 9.3) |
| 6´´´ | 18.3 | 1.27 (d, 6.1) | 18.2* | 1.27 (d, 6.2) | 18.4* | 1.27 (d, 6.1) | 18.3 | 1.28 (d, 6.2) |
| 3´´´-OMe | 57.3 | 3.42 (s) | 57.3 | 3.43 (s) | 57.3 | 3.43 (s) | 57.3 | 3.43 (s) |
| a | | | 173.9 | | 174.1 | | | |
| b<br>c | | | 30.1<br>30.2 | 2.58 (m)*, 2.58 (m)*, 2.62 (m)*, 2.69 (m)* | 30.7<br>30.8 | 2.70 (m)*, 2.68 (m)*, 2.62 (m)*, 2.60 (m)* | | |
| d | | | 176.2 | | 177.2 | | | |

*These signals are exchangeable.

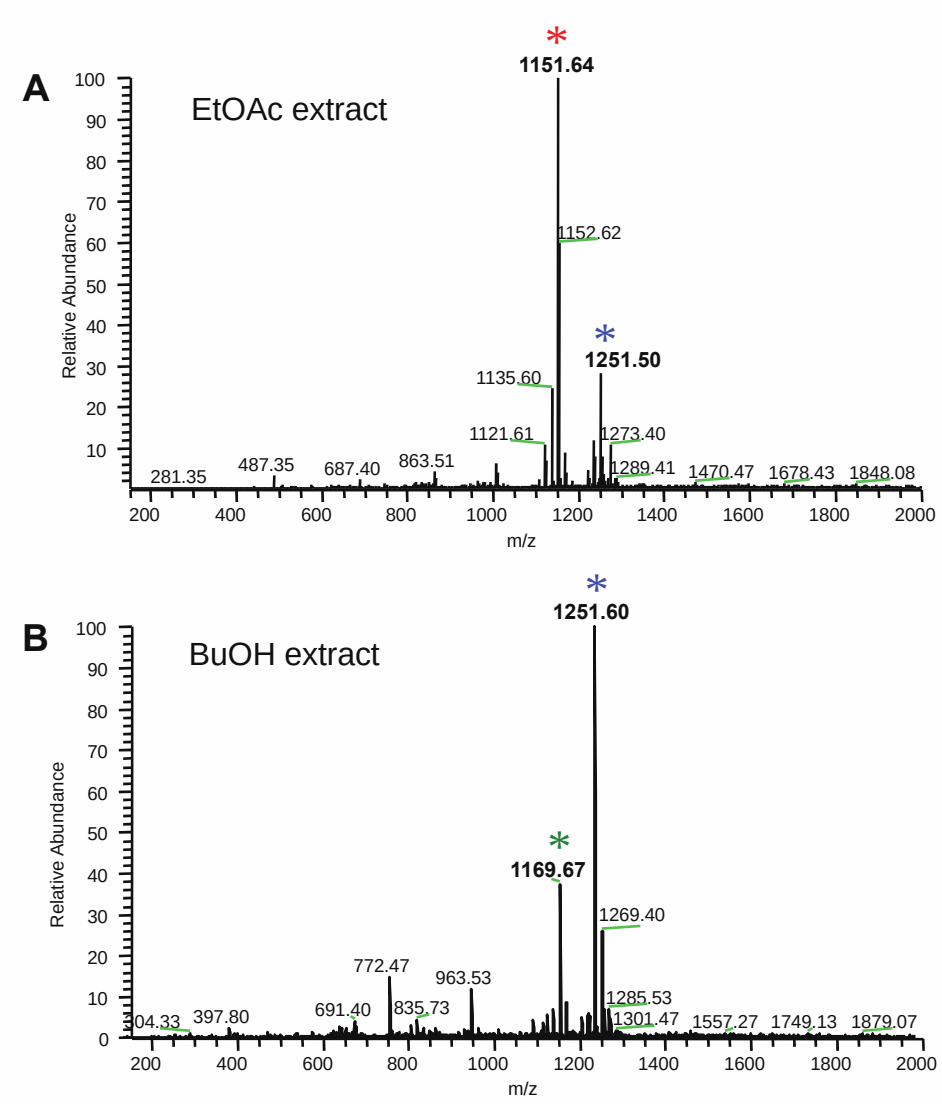


**Figure S4.1.** (+)-ESI-MS analysis of extracts of *Amycolatopsis* sp. ICBB 8242 culture broths. **A**, EtOAc extract; **B**, *n*-BuOH extract. Red asterisk indicates apoptolidin A and its isomers, blue asterisk indicates succinylated apoptolidins, and green asterisk indicates linear apoptolidin A.

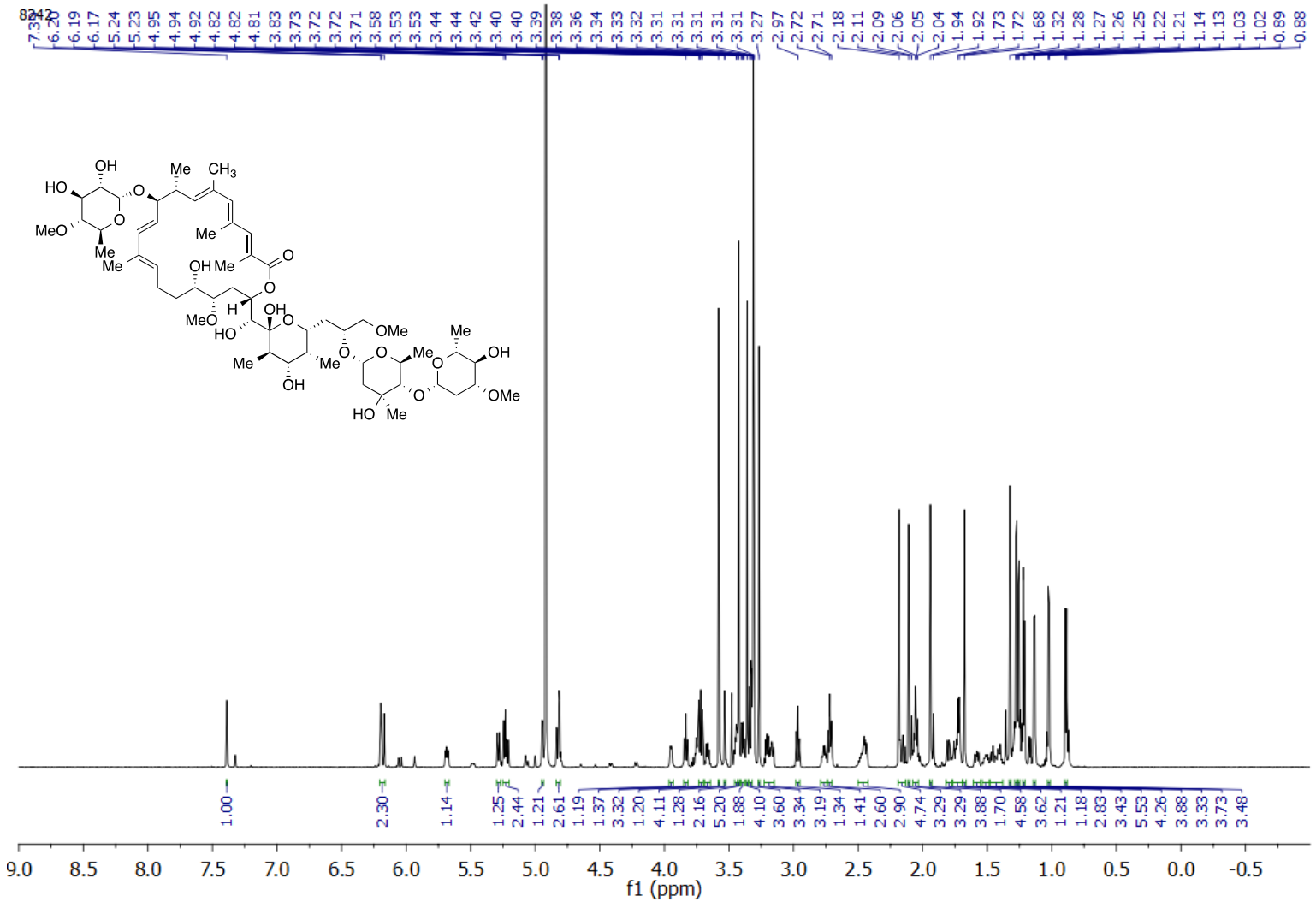


**Figure S4.2.** $^{1}$H NMR spectrum of apoptolidin A (**1**) (700 MHz, $CD_3OD$).

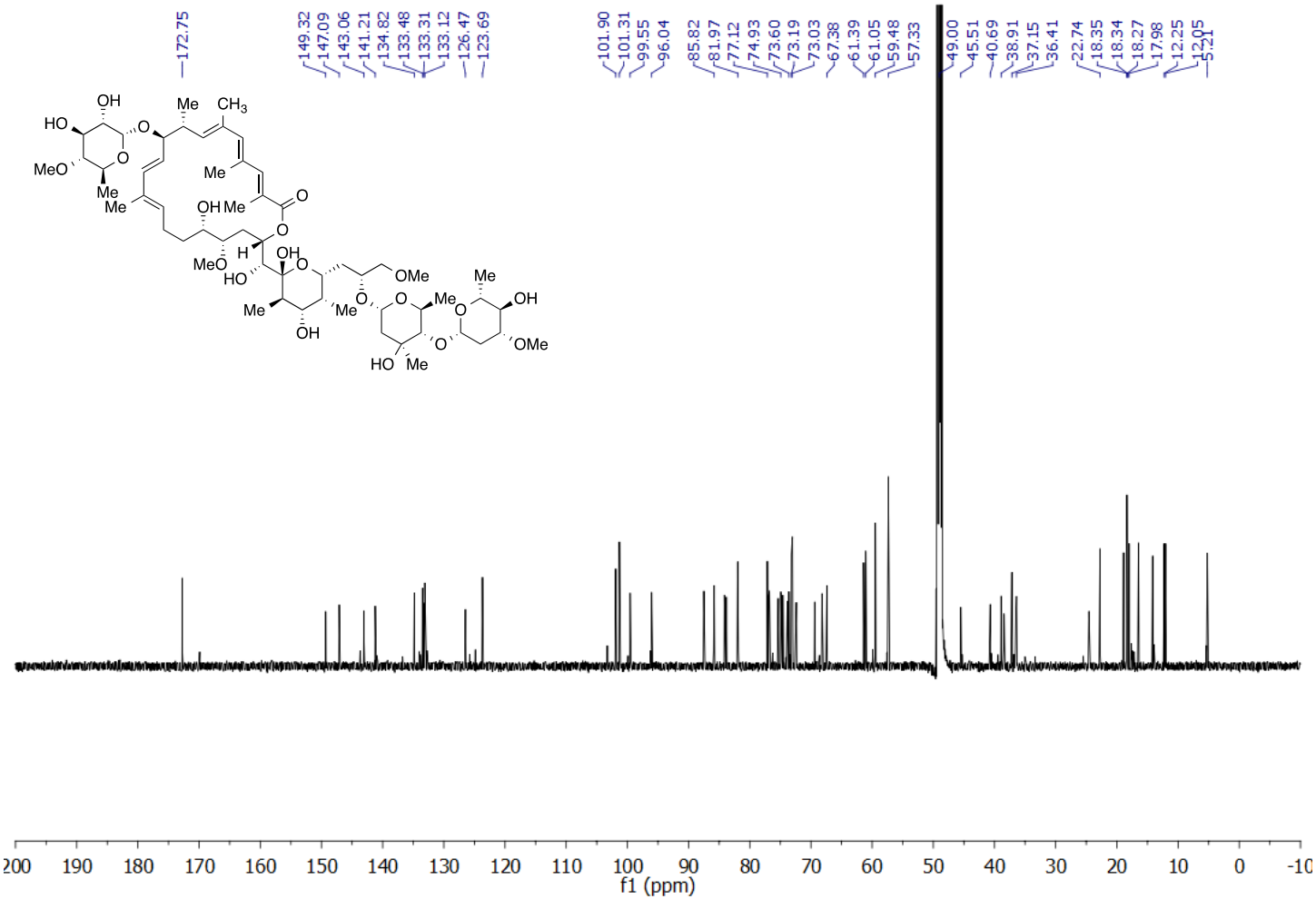


**Figure S4.3.** $^{13}C$ NMR spectrum of apoptolidin A (**1**) (175 MHz, $CD_3OD$).

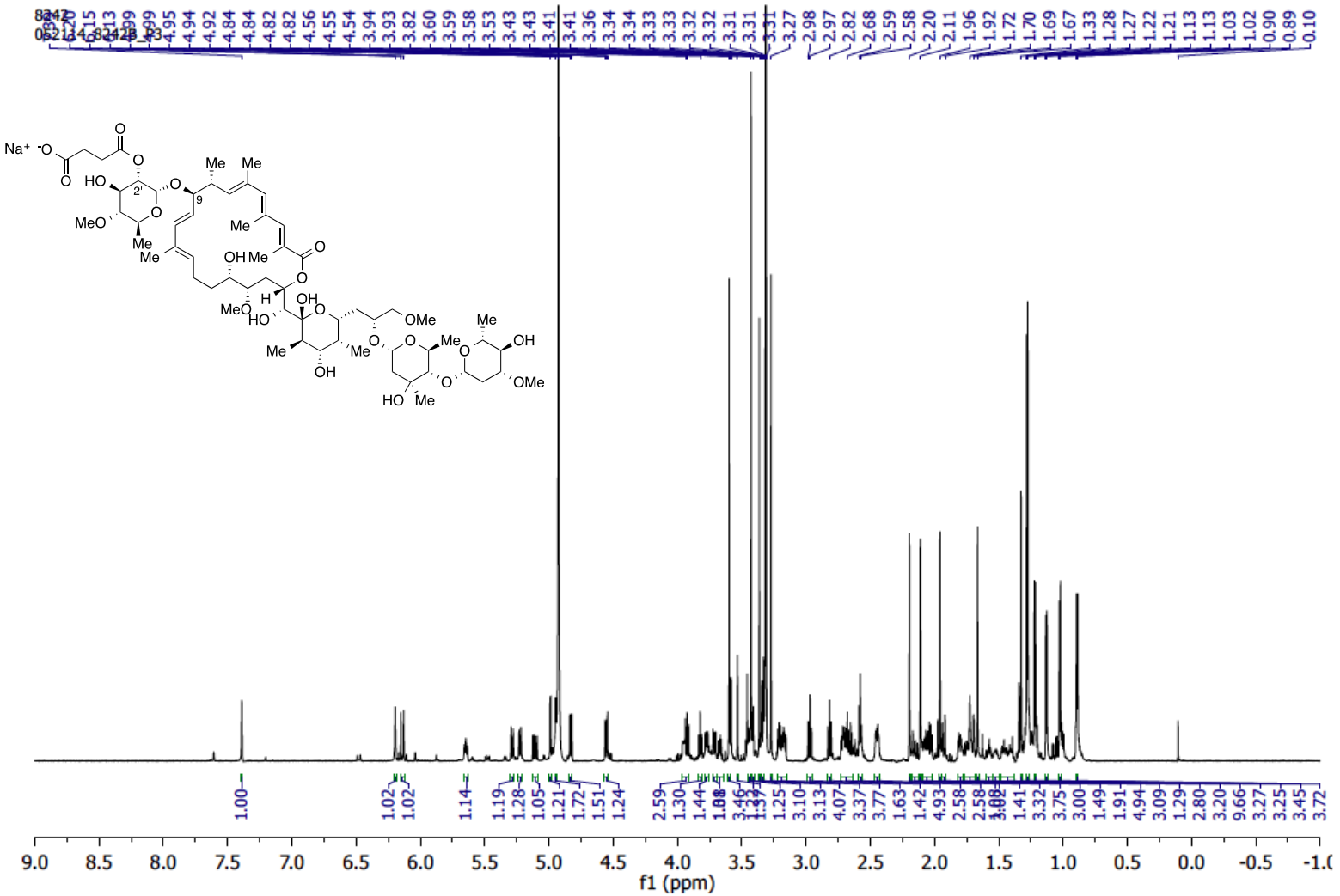


**Figure S4.4.** $^{1}$H NMR spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

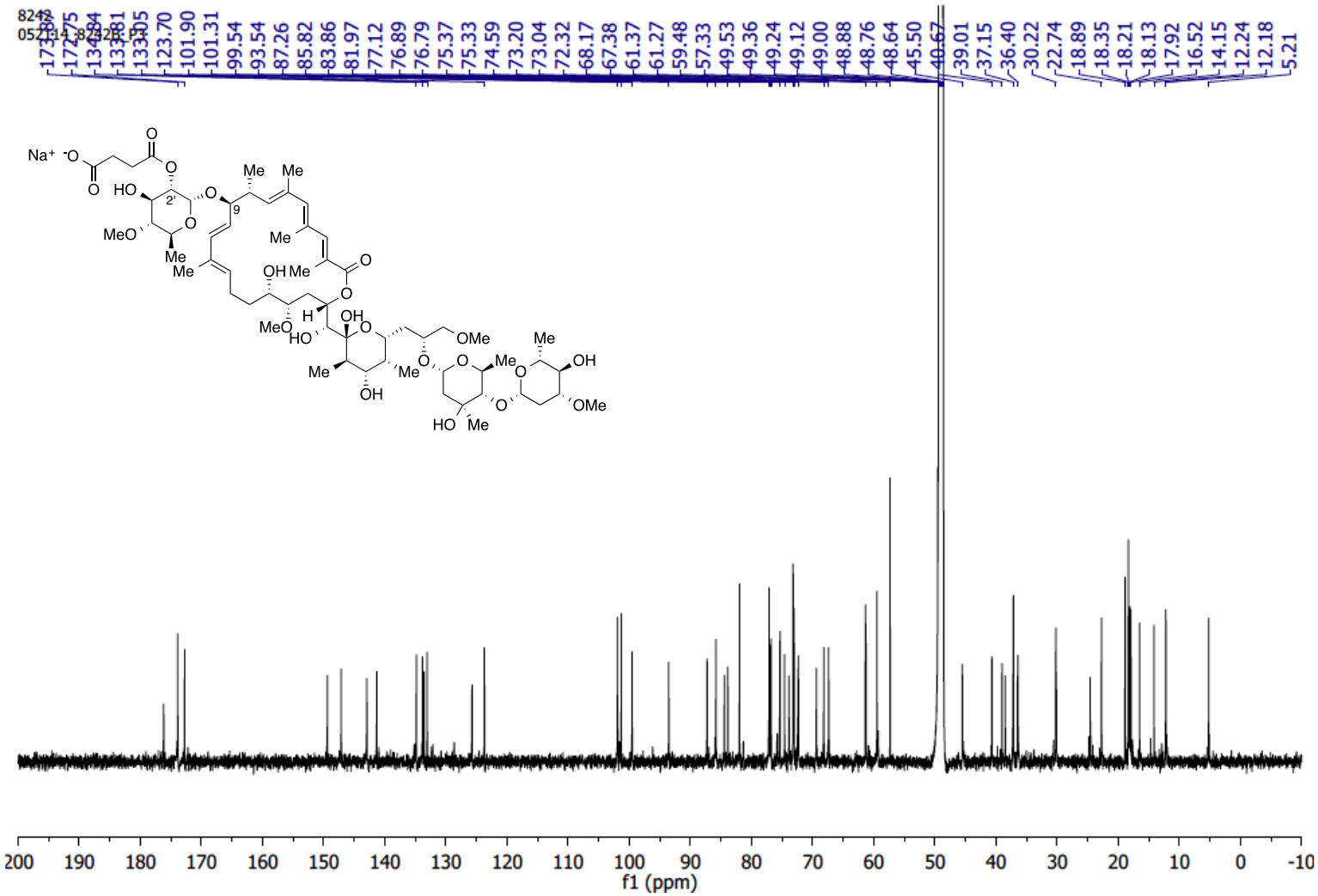


**Figure S4.5.** $^{13}$C NMR spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (175 MHz, $CD_3OD$).

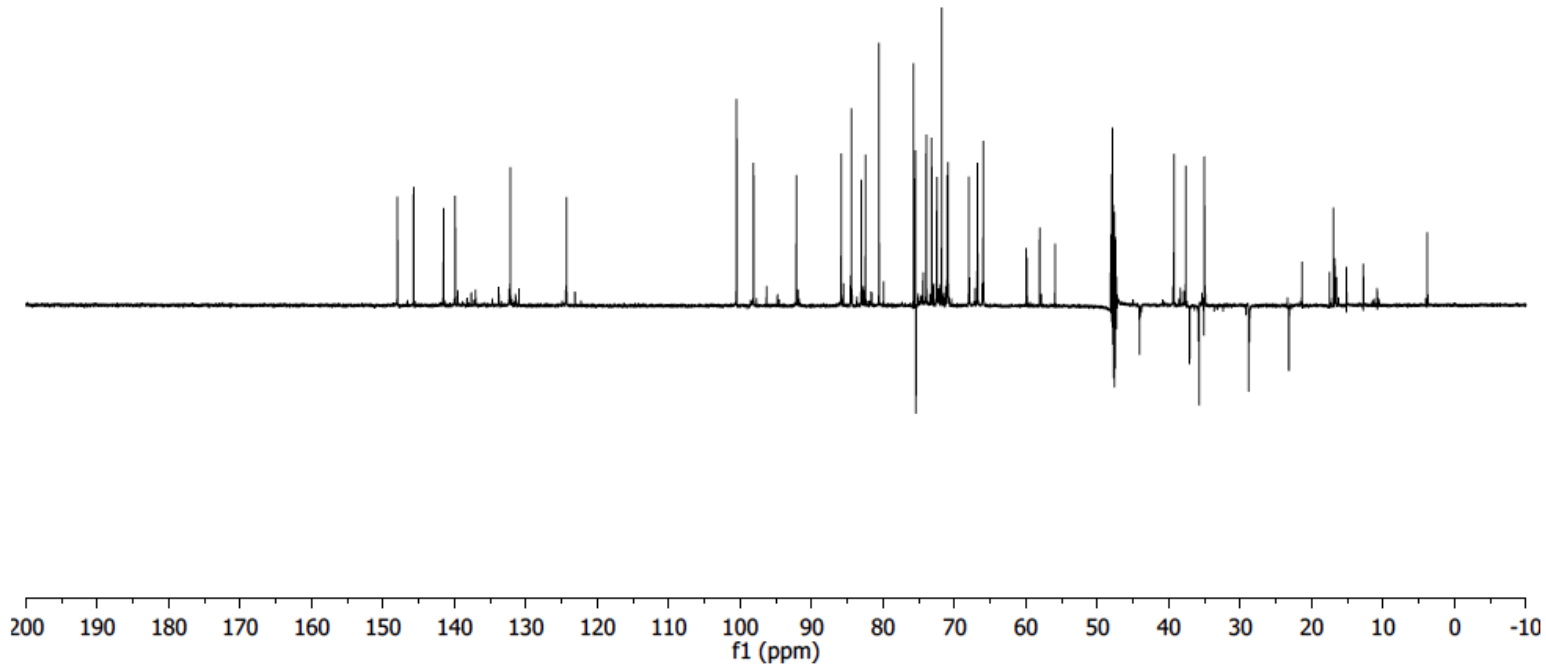


**Figure S4.6.** DEPT-135 spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

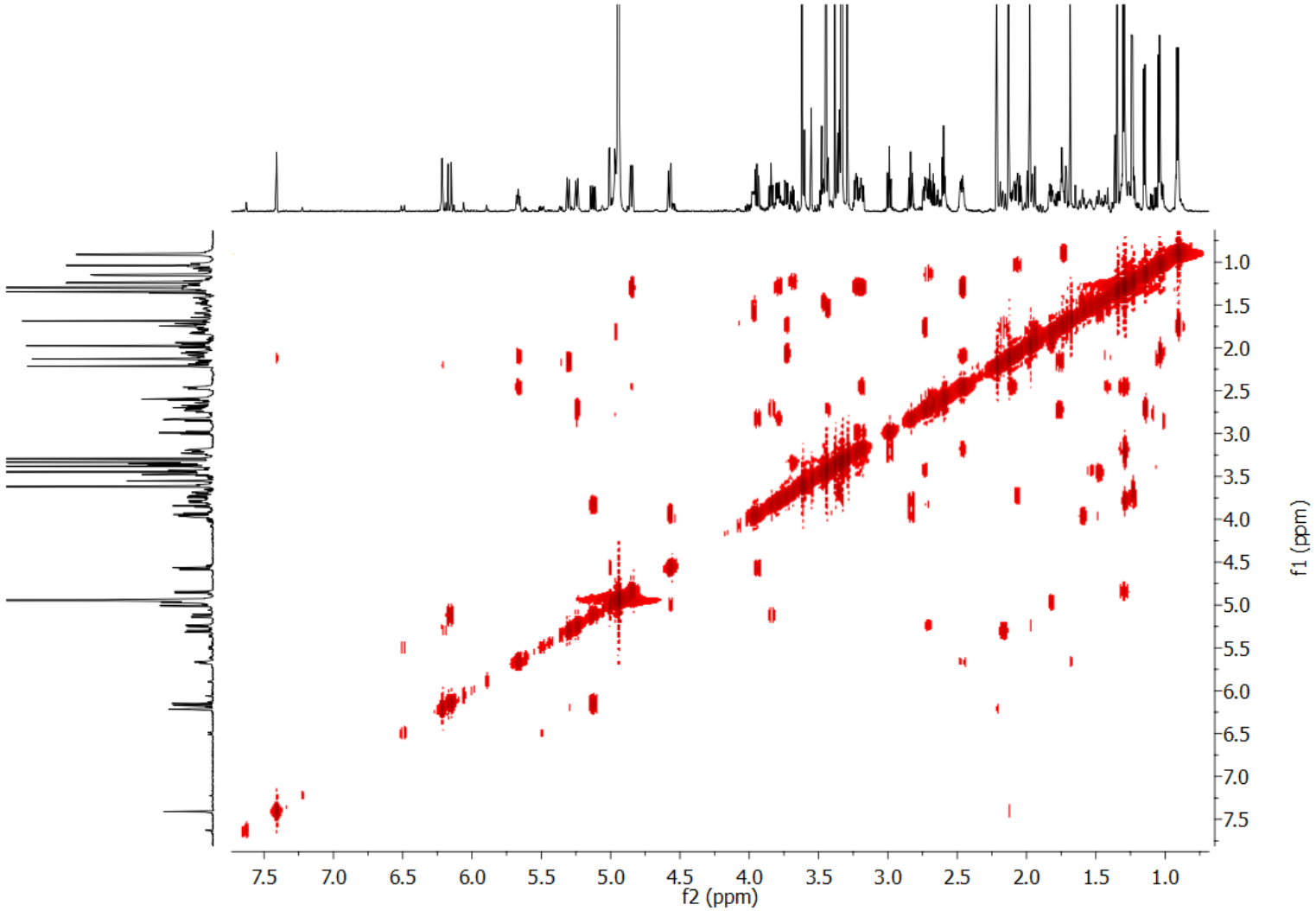


**Figure S4.7.** COSY spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

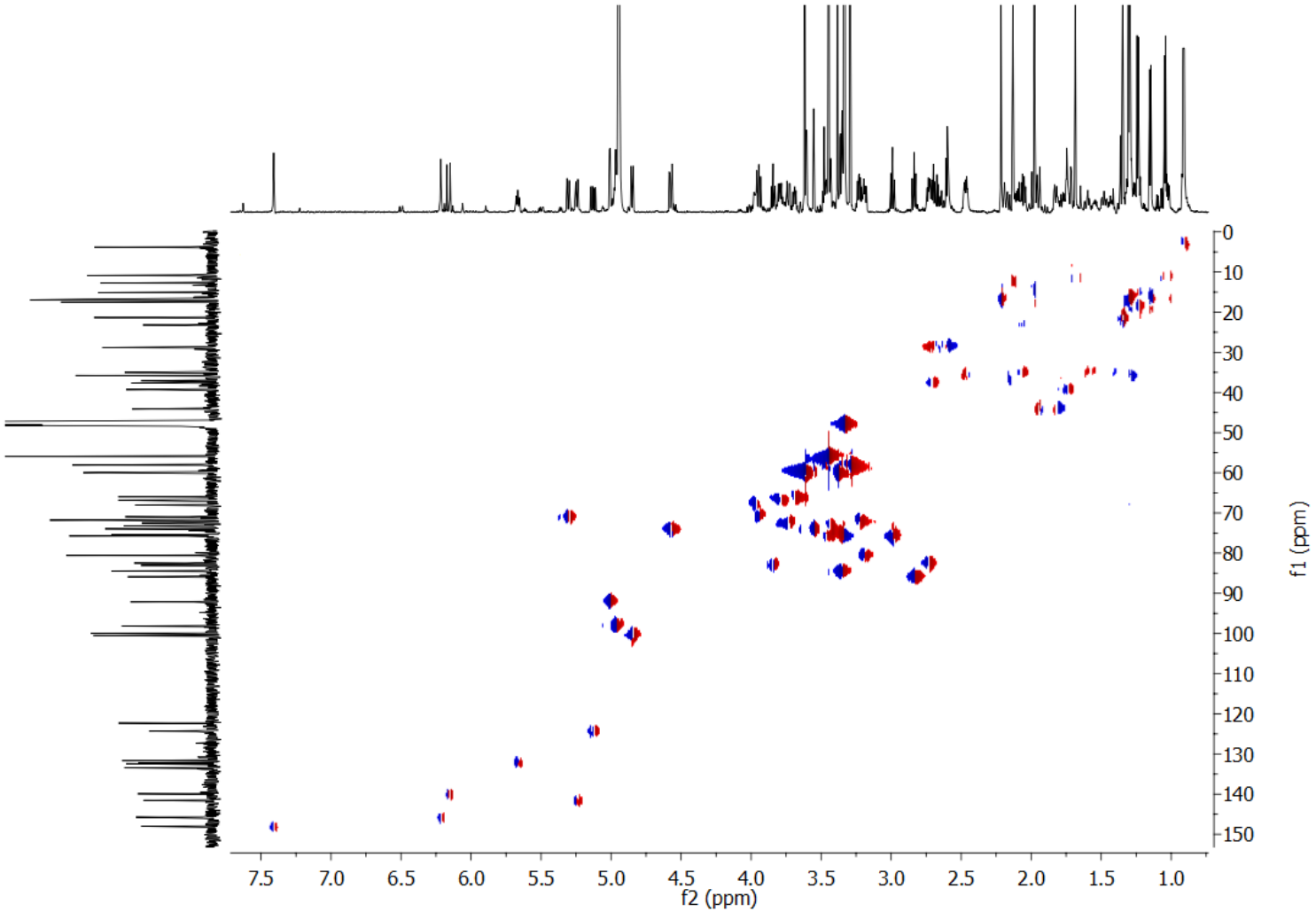


**Figure S4.8.** HSQC spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

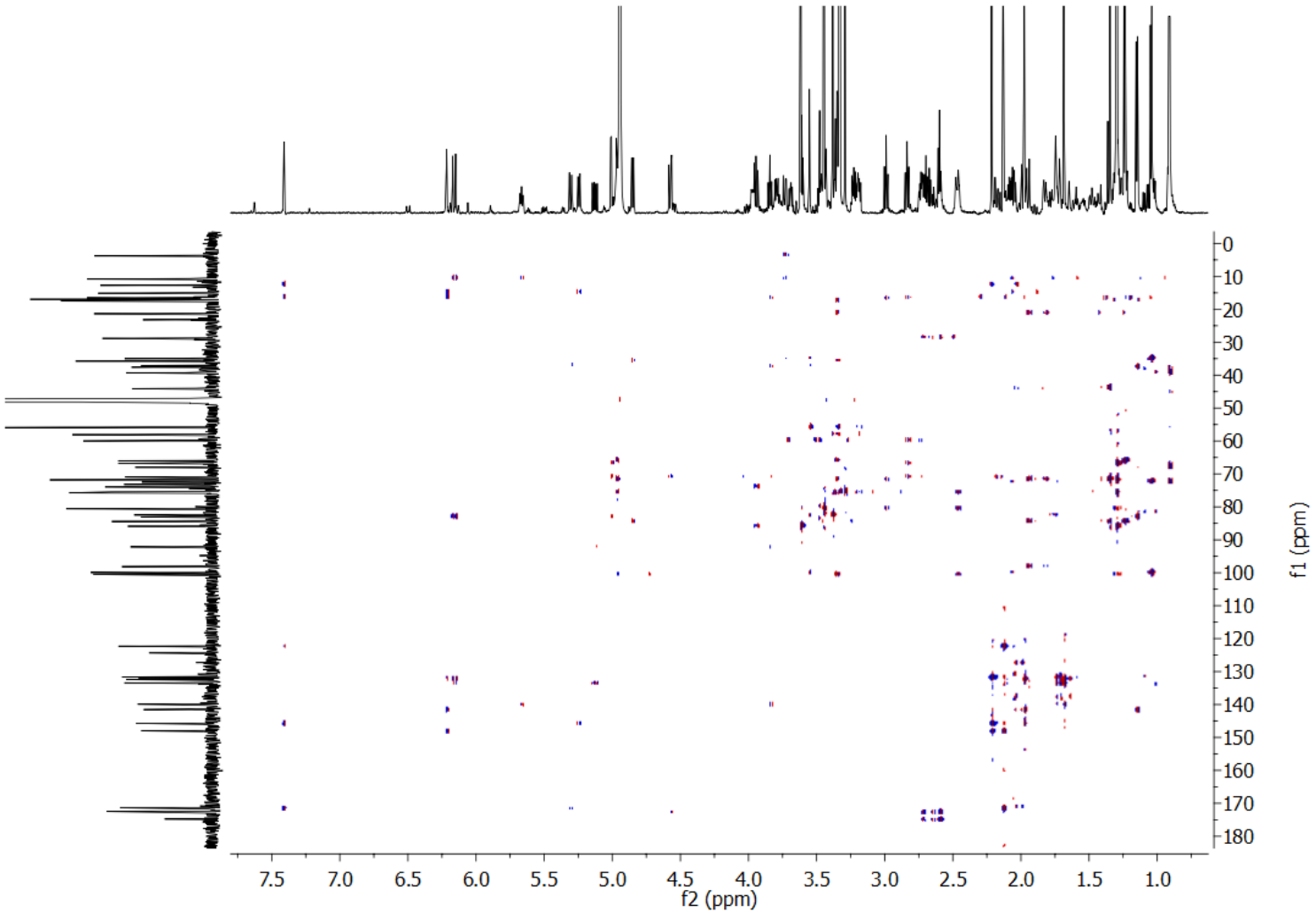


**Figure S4.9.** HMBC spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

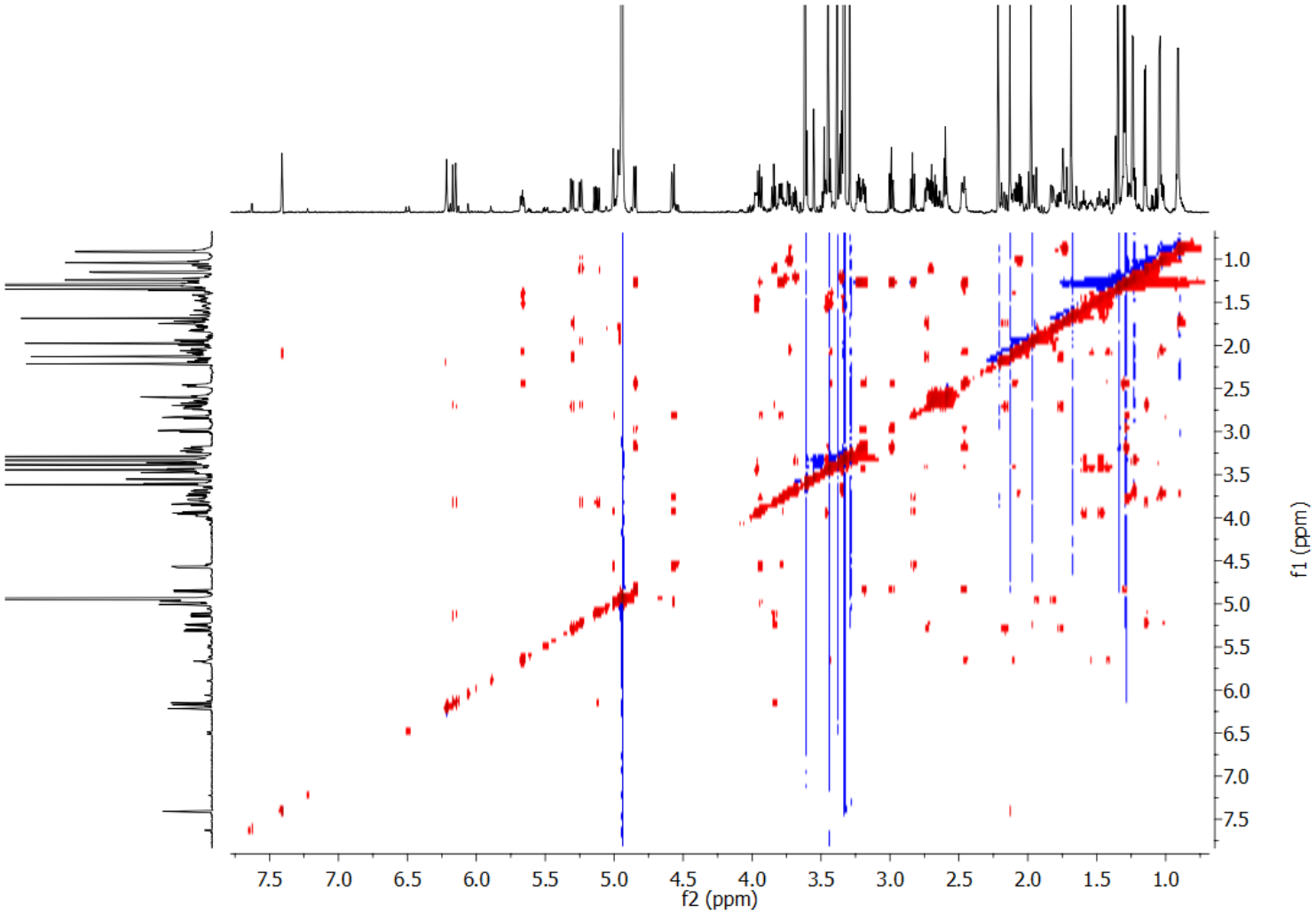


**Figure S4.10.** TOCSY spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (700 MHz, $CD_3OD$).

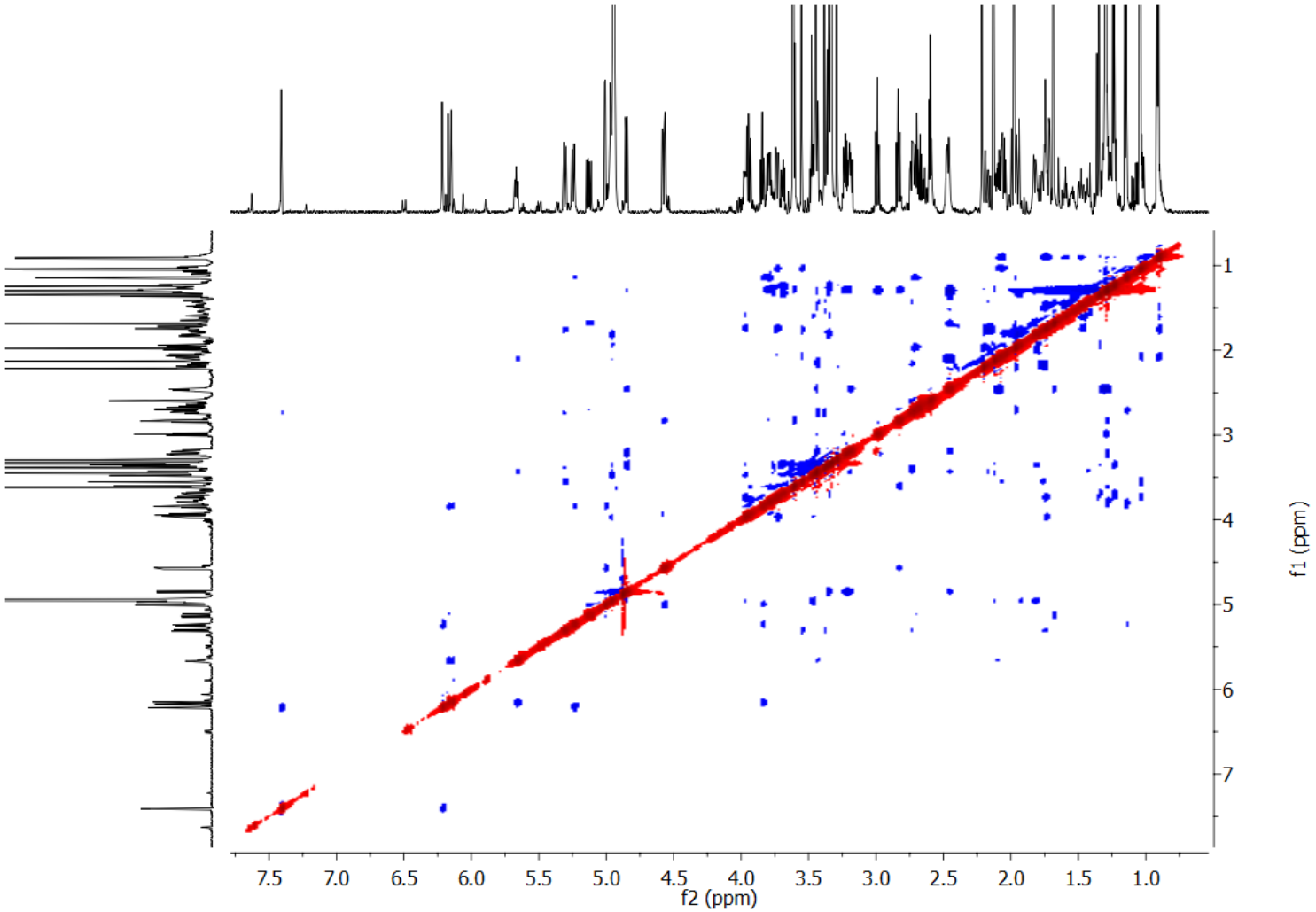


**Figure S4.11.** ROESY spectrum of 2´-*O*-succinyl-apoptolidin A (**11**) (500 MHz, $CD_3OD$).

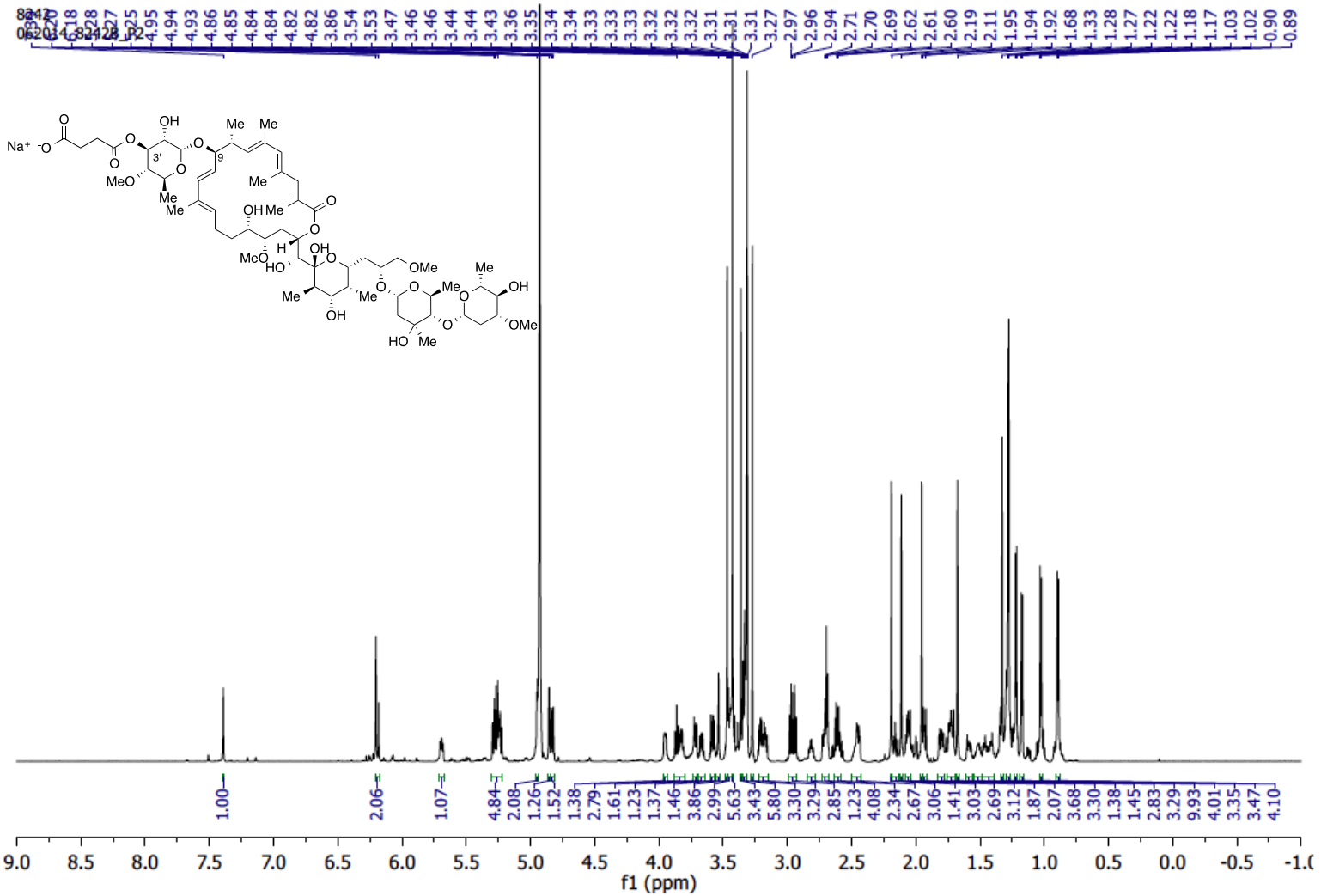


**Figure S4.12.** $^{1}$H NMR spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

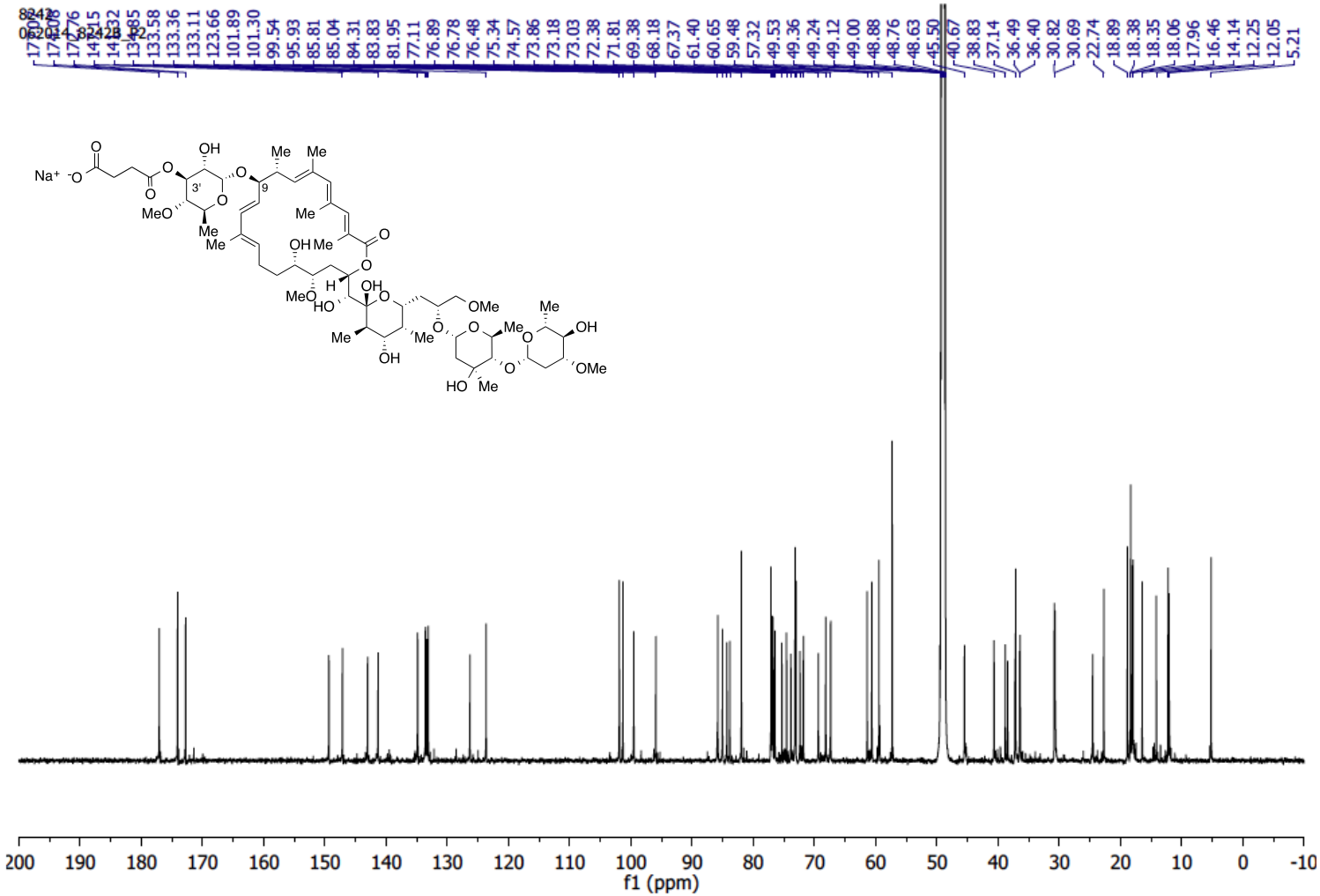


**Figure S4.13.** $^{13}$C NMR spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (175 MHz, $CD_3OD$).

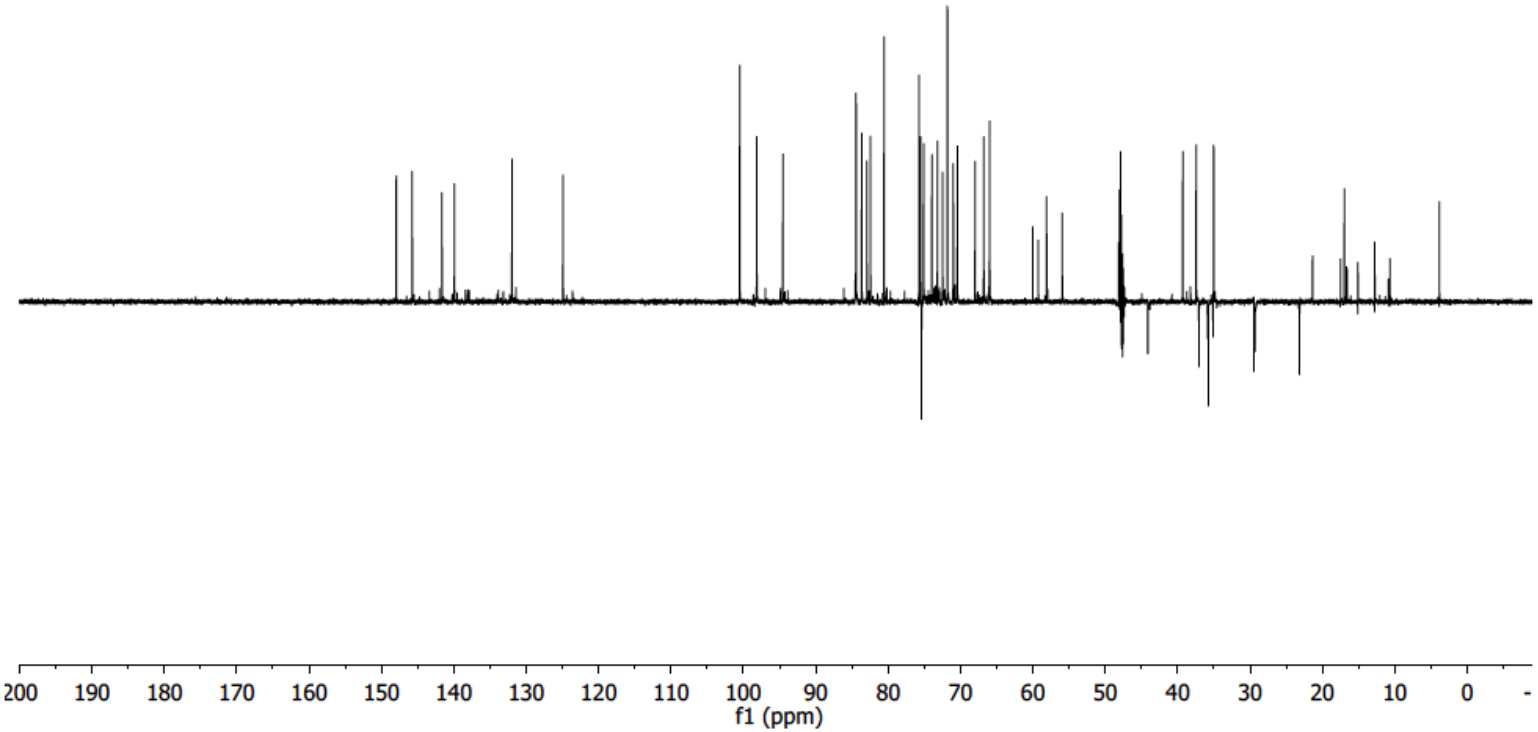


**Figure S4.14.** DEPT-135 spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

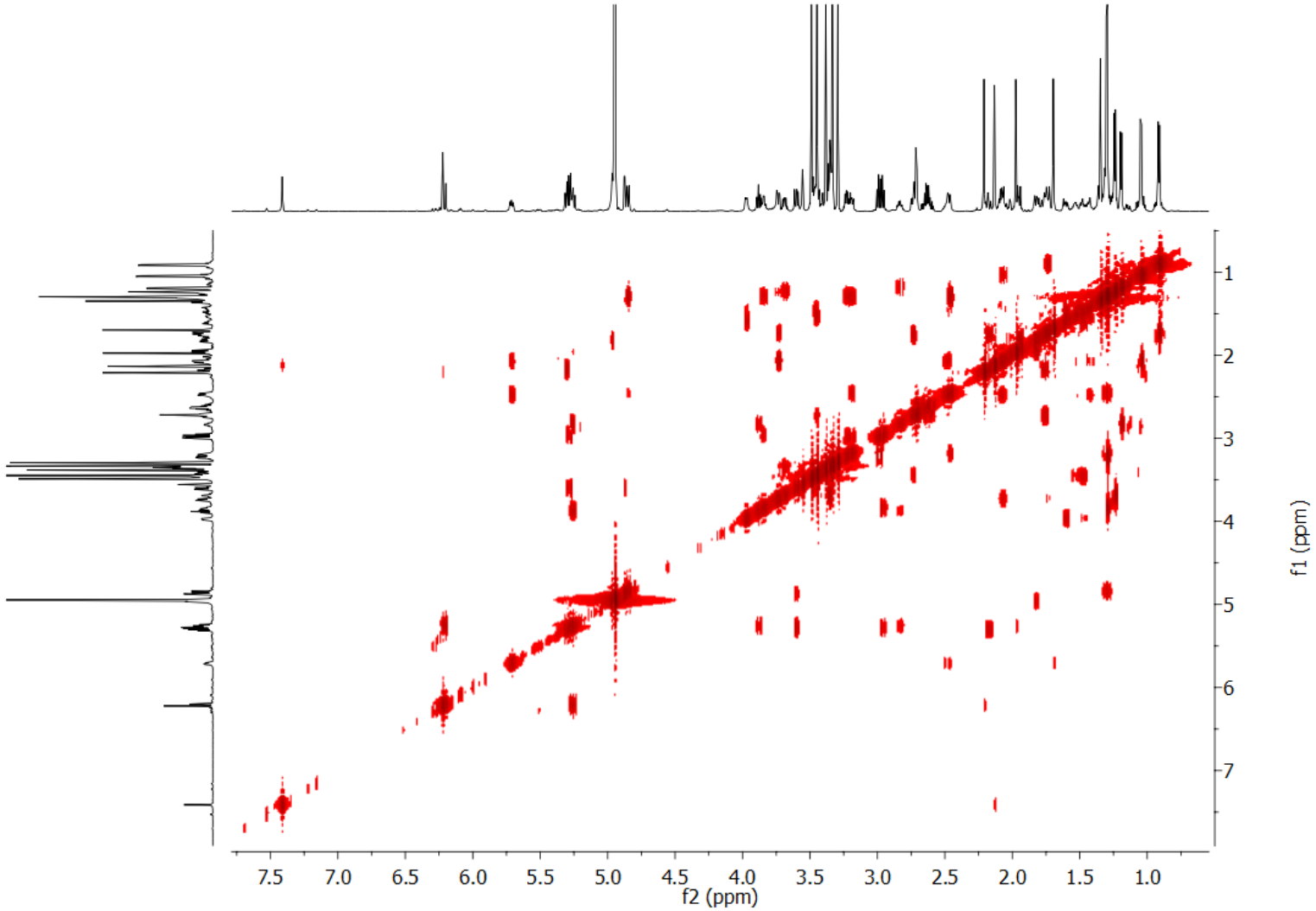


**Figure S4.15.** COSY spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

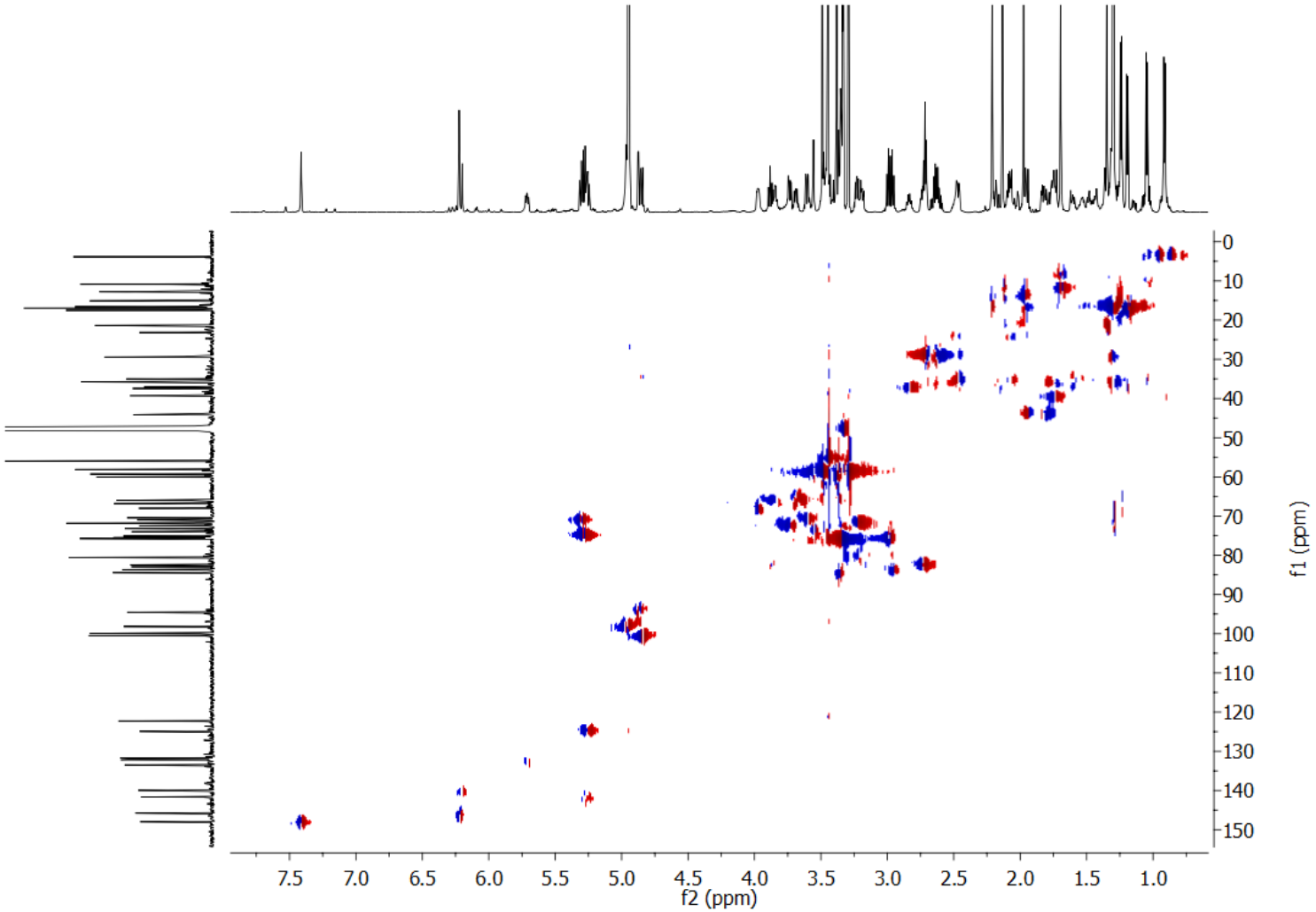


**Figure S4.16.** HSQC spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

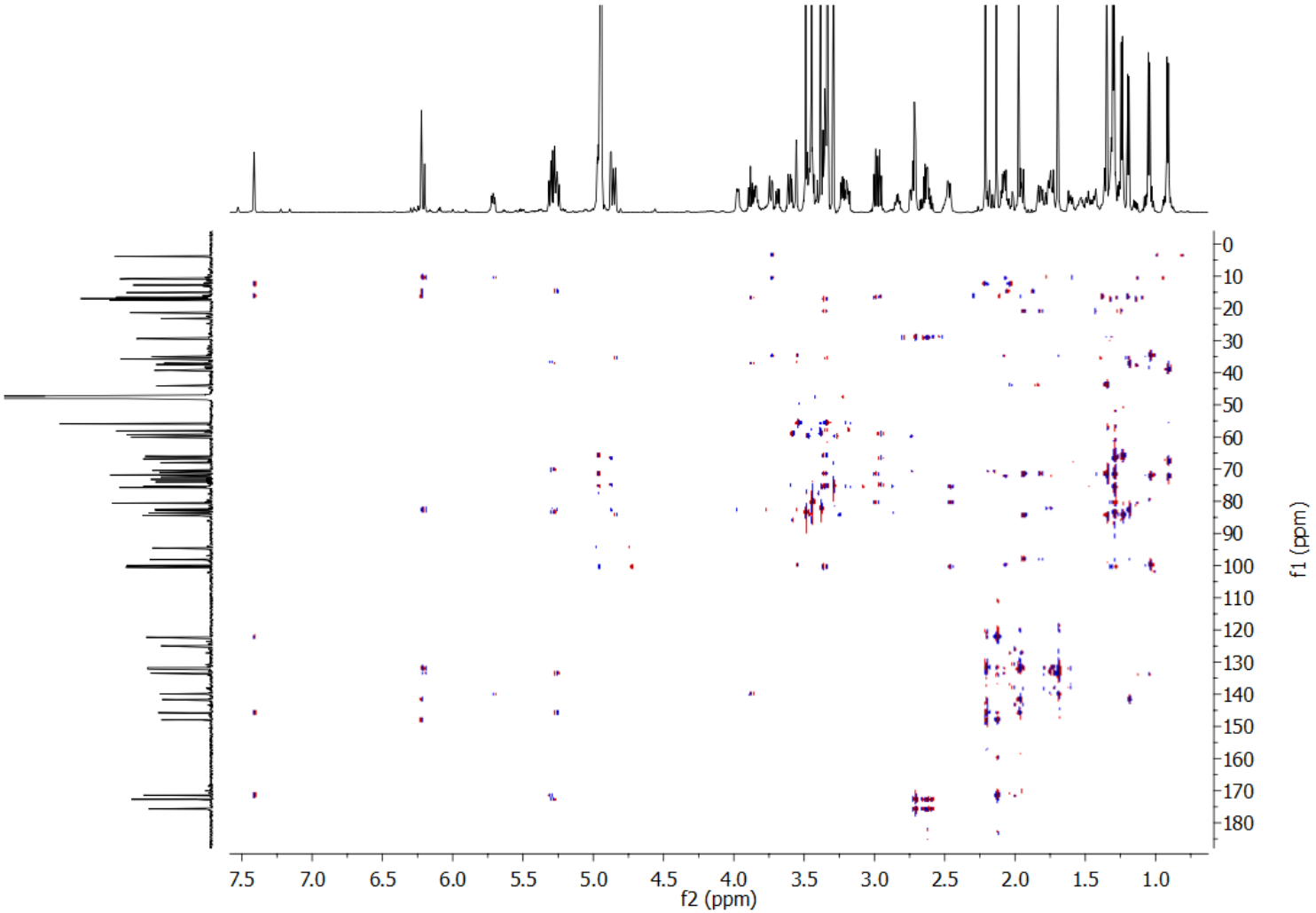


**Figure S4.17.** HMBC spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

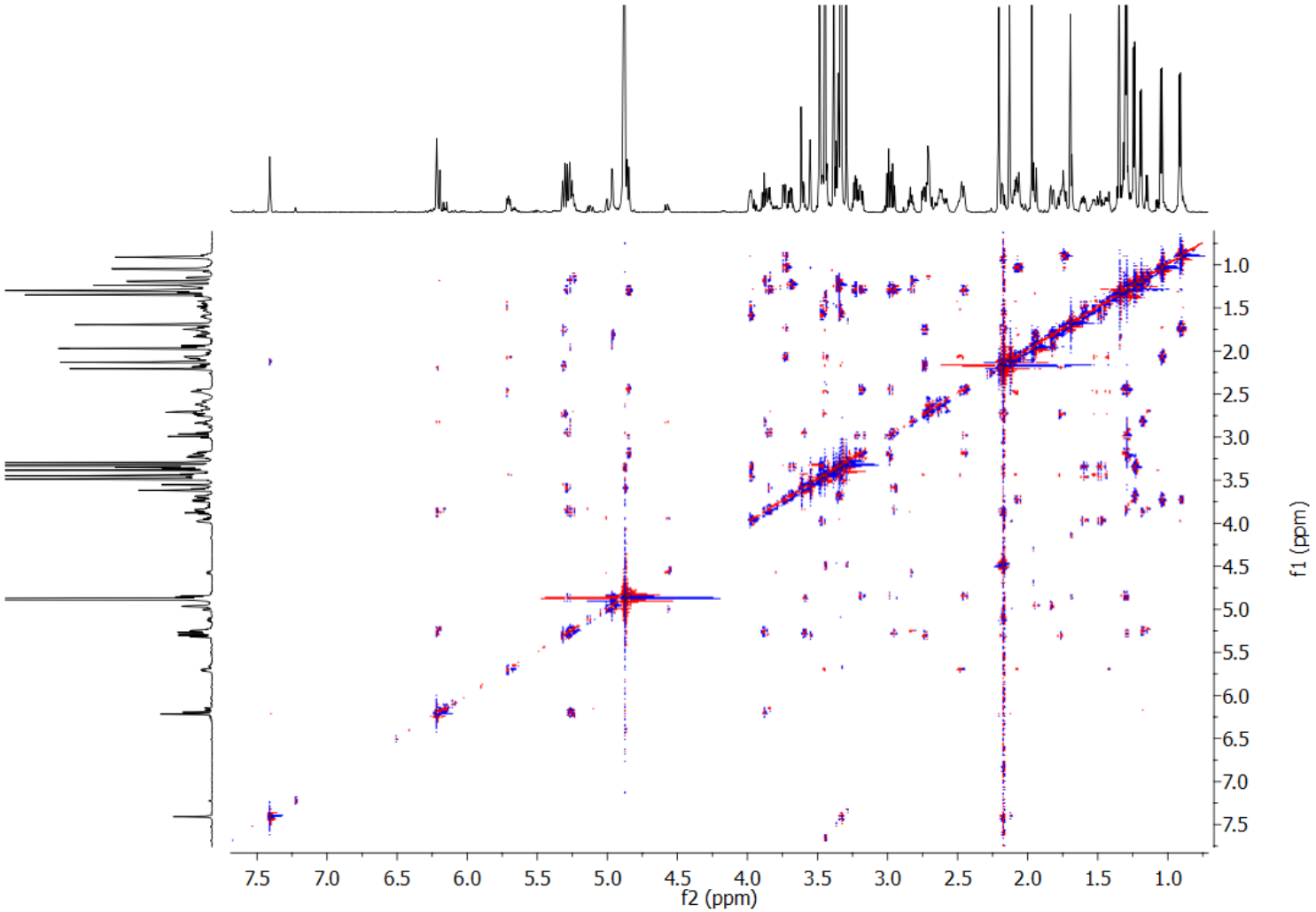


**Figure S4.18.** TOCSY spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

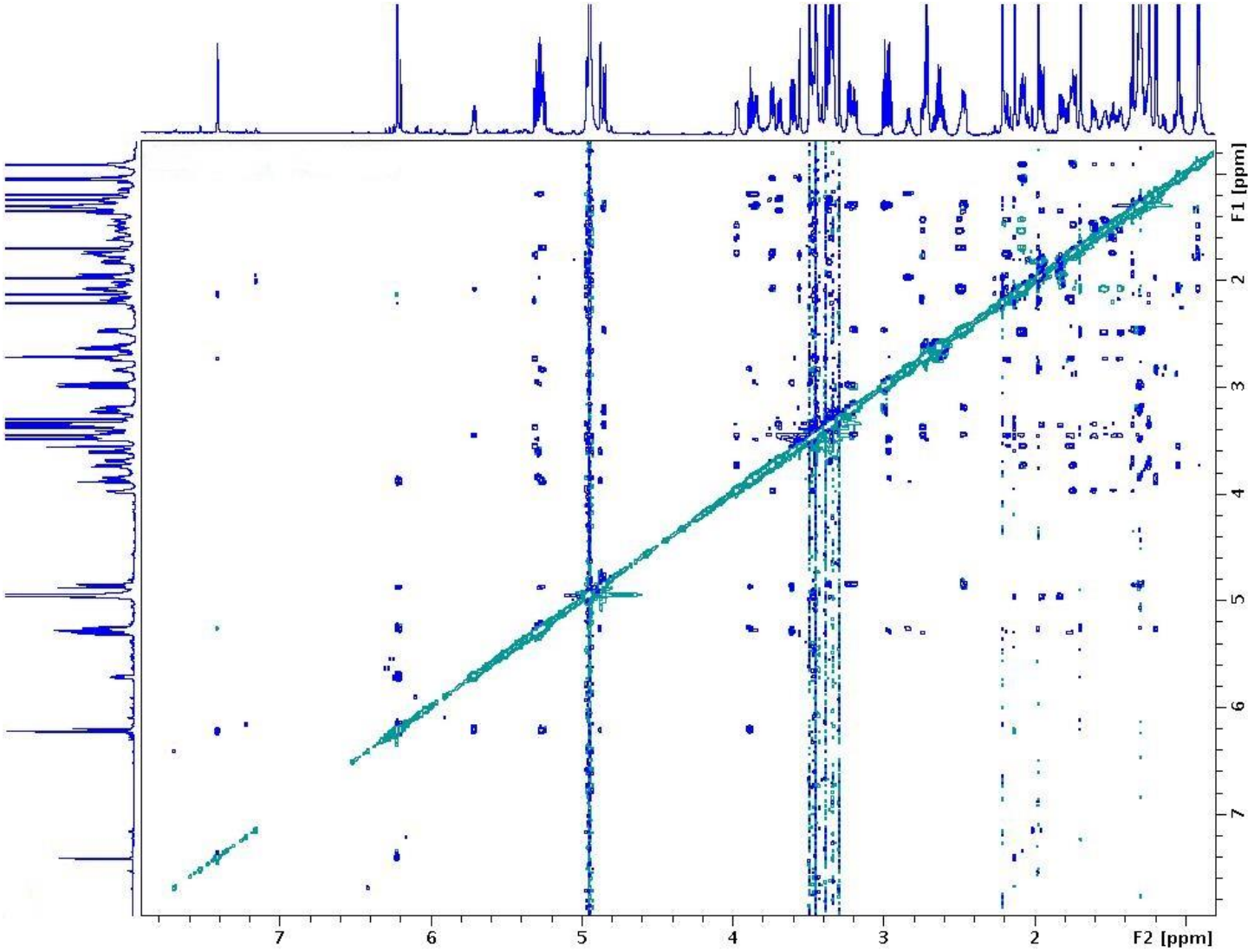


**Figure S4.19.** ROESY spectrum of 3´-*O*-succinyl-apoptolidin A (**12**) (700 MHz, $CD_3OD$).

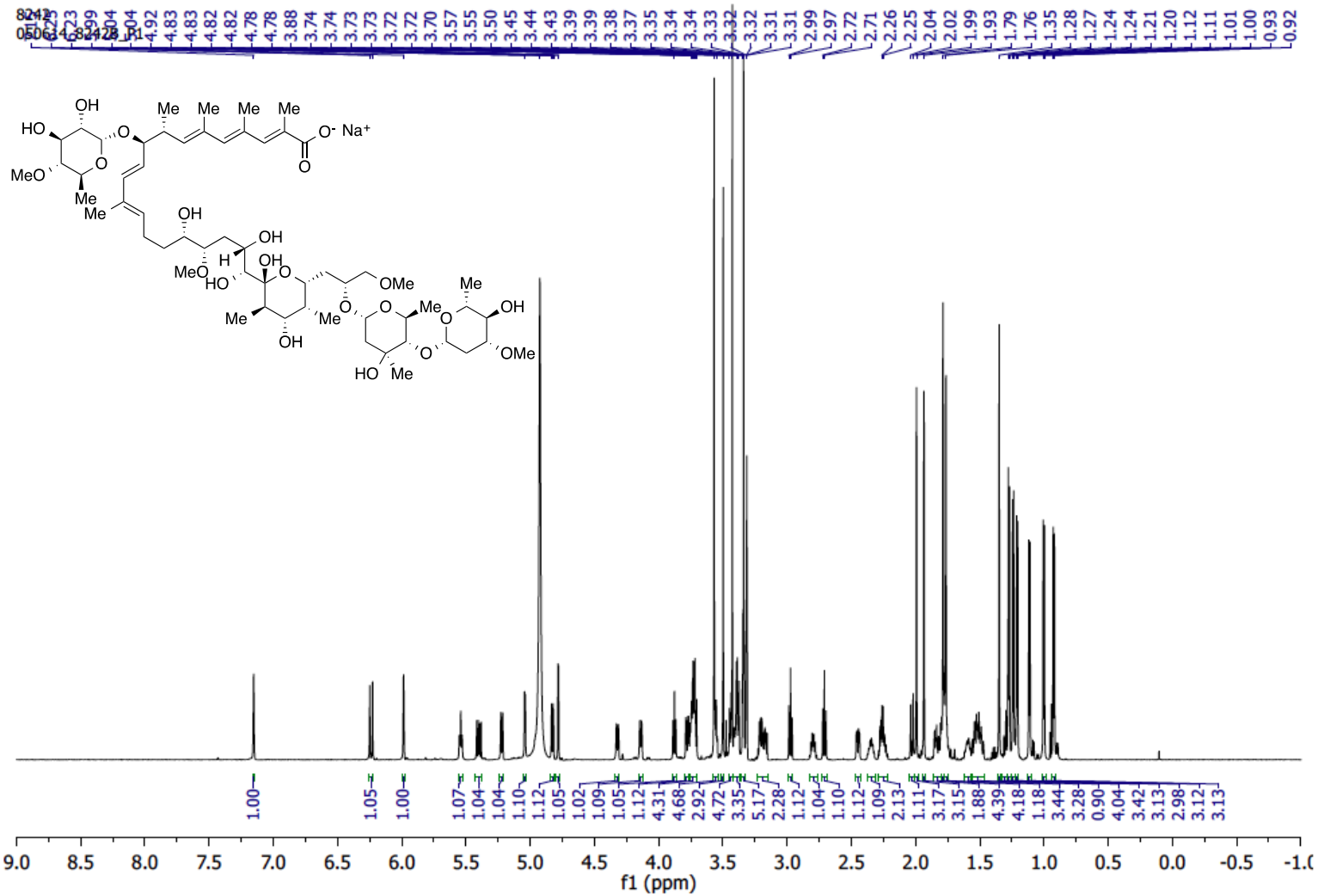


**Figure S4.20.** $^{1}$H NMR spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

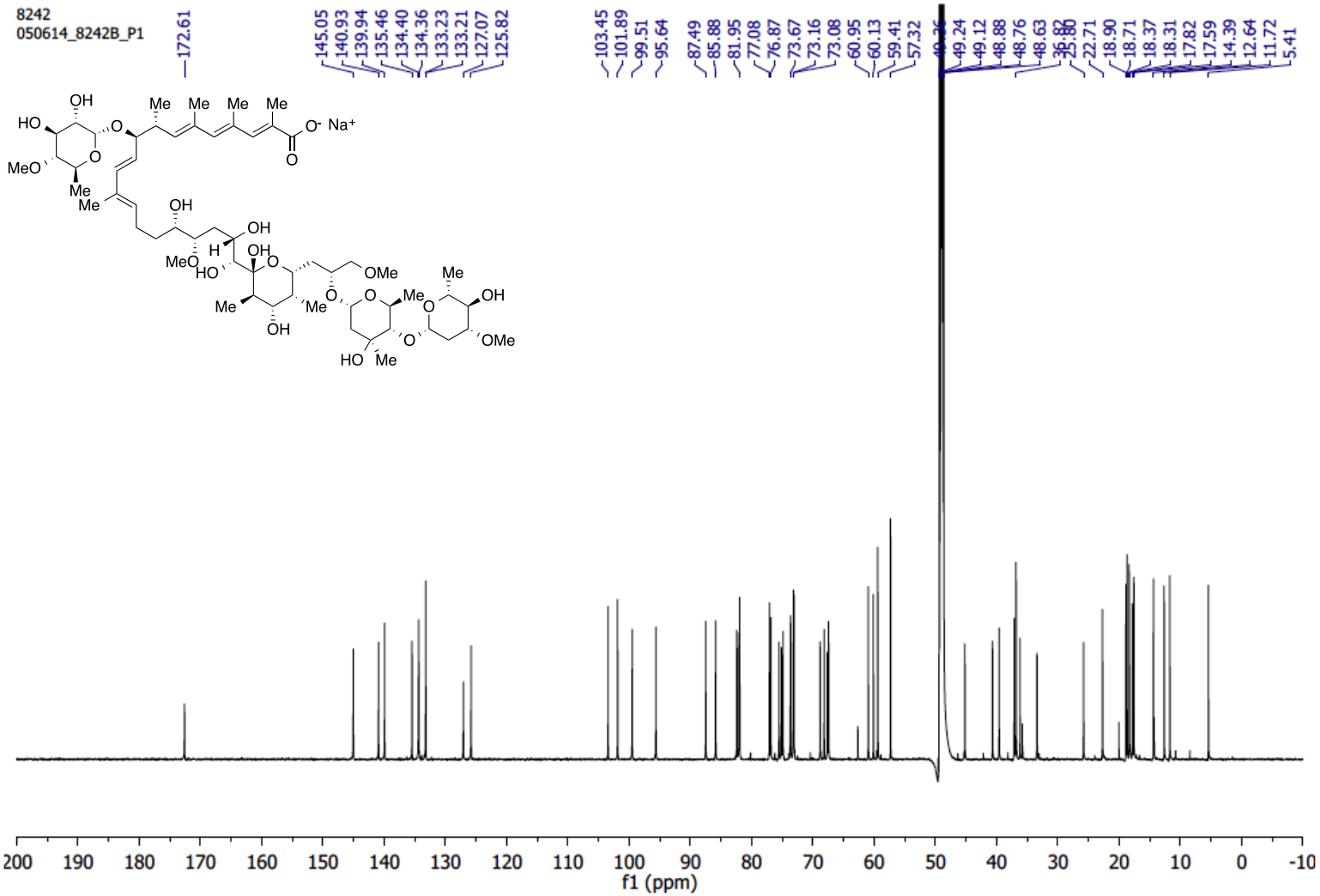


**Figure S4.21.** $^{13}$C NMR spectrum of linear apoptolidin A (**13**) (175 MHz, $CD_3OD$).

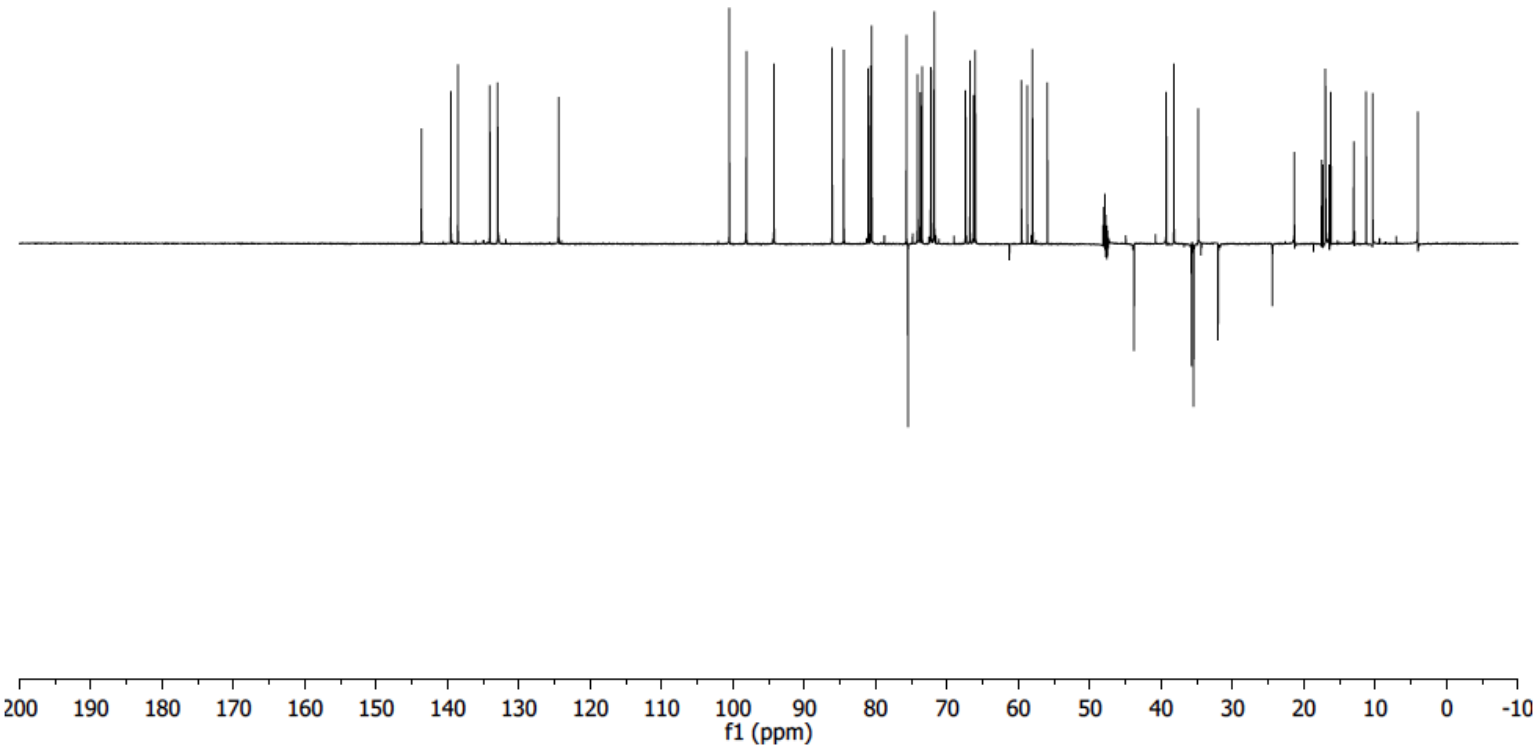


**Figure S4.22.** DEPT-135 spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

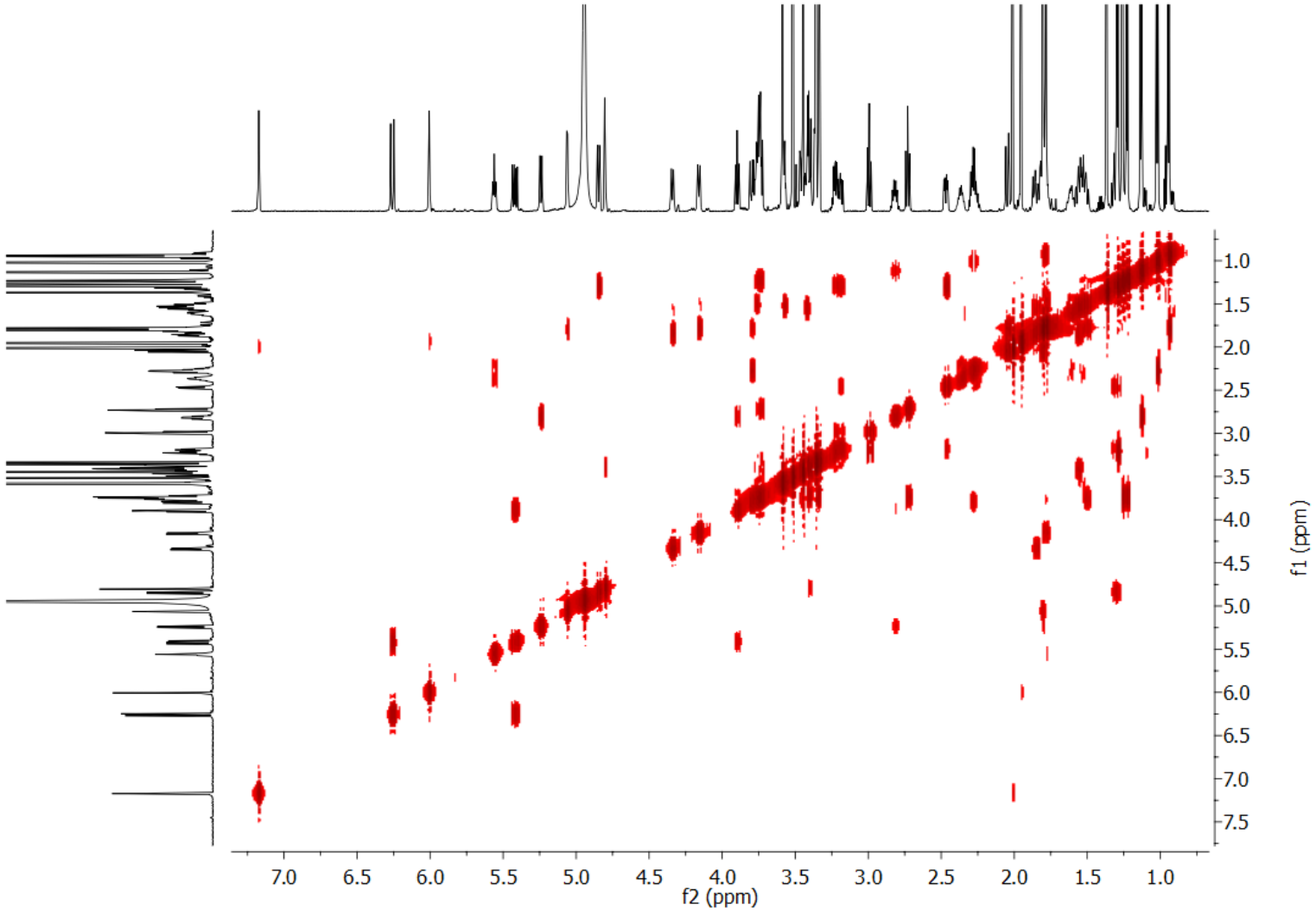


**Figure S4.23.** COSY spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

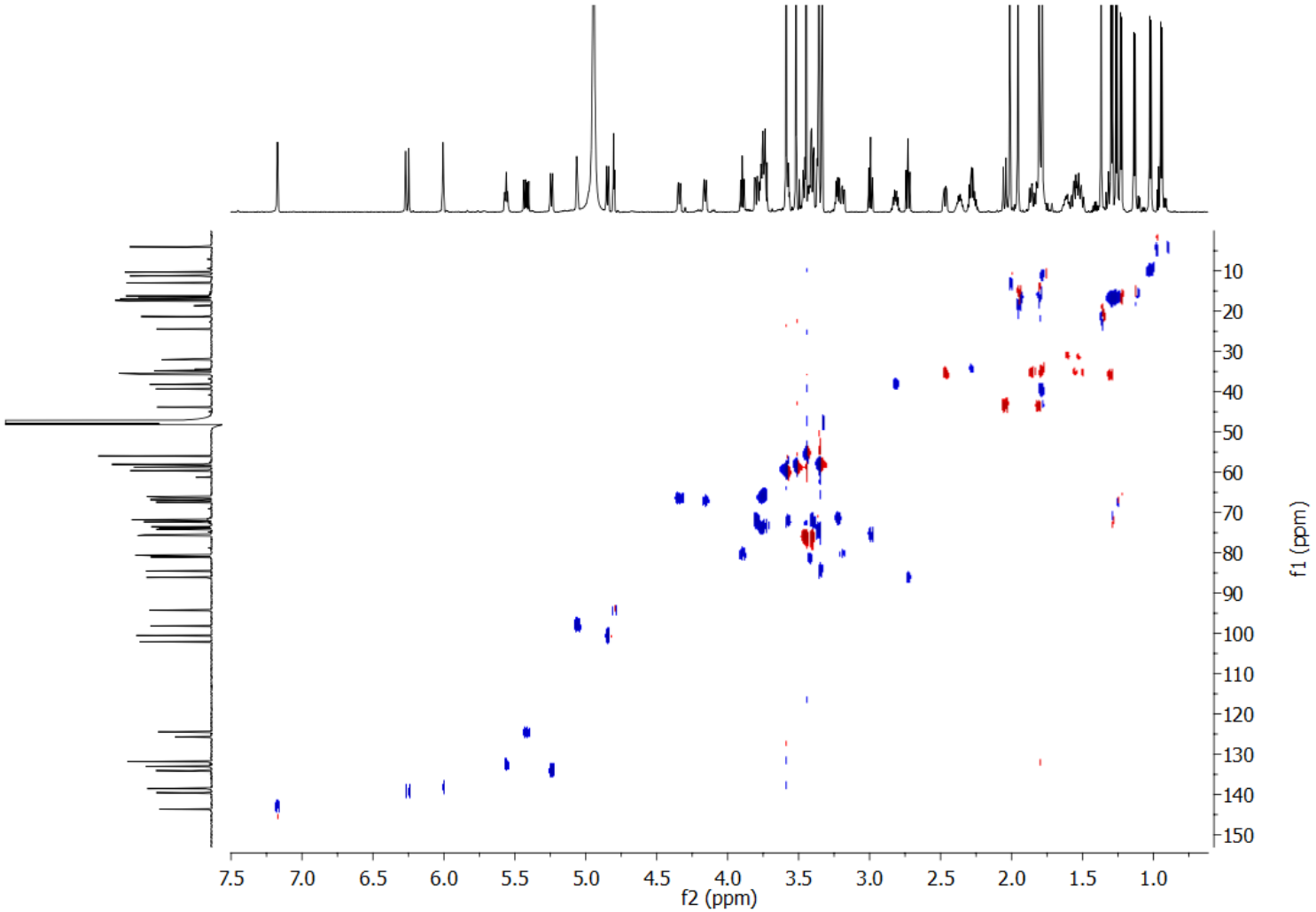


**Figure S4.24.** HSQC spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

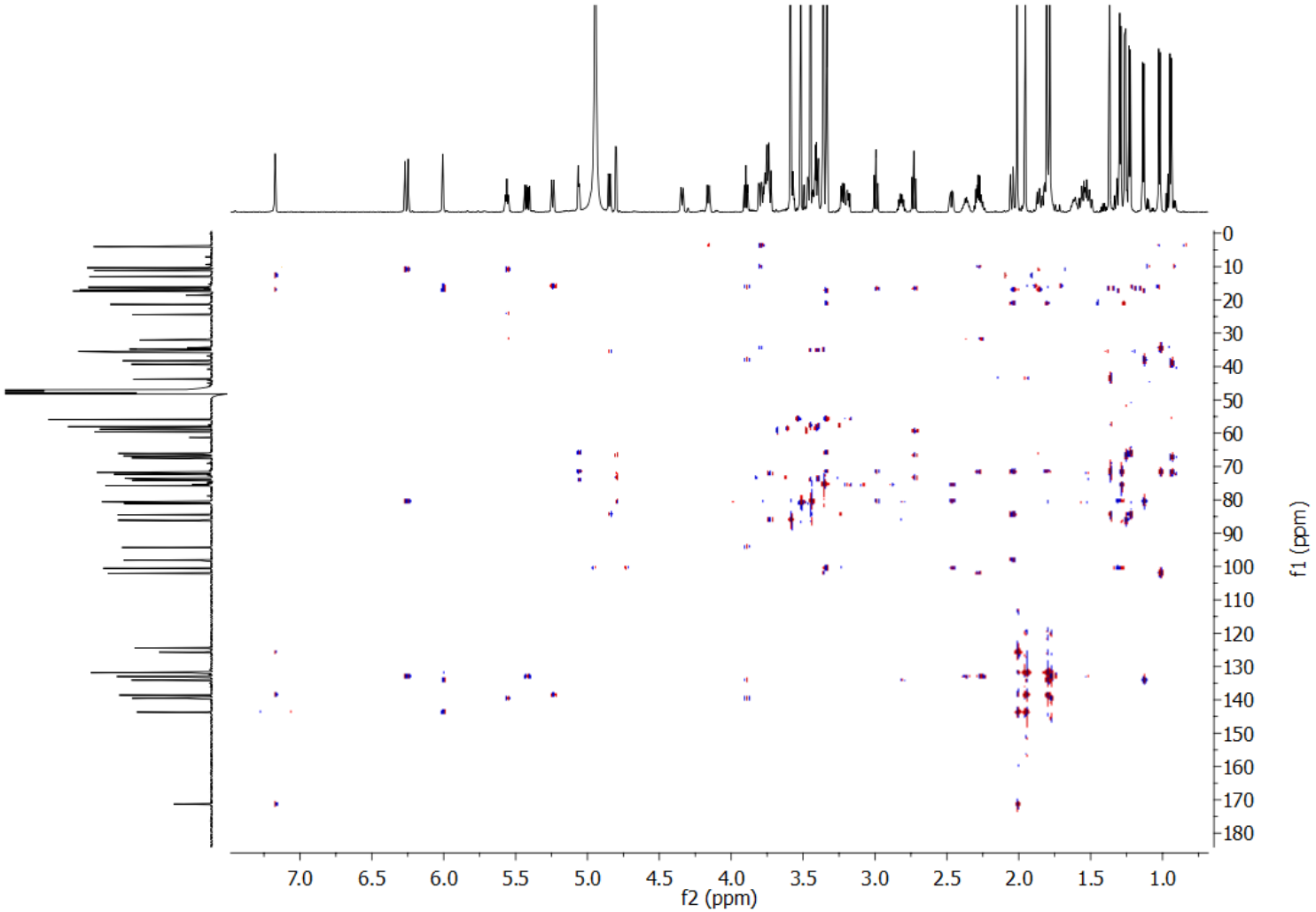


**Figure S4.25.** HMBC spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

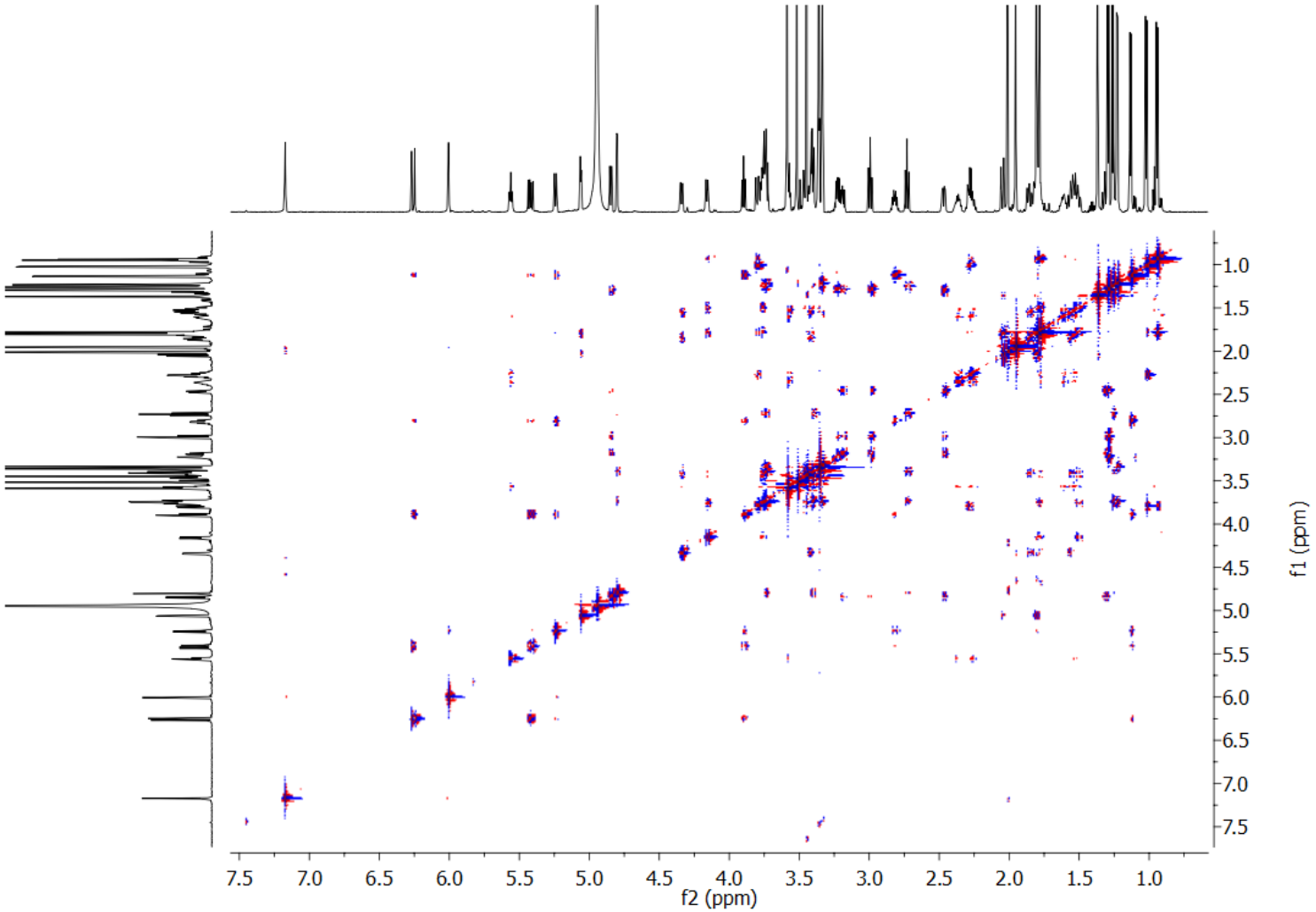


**Figure S4.26.** TOCSY spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

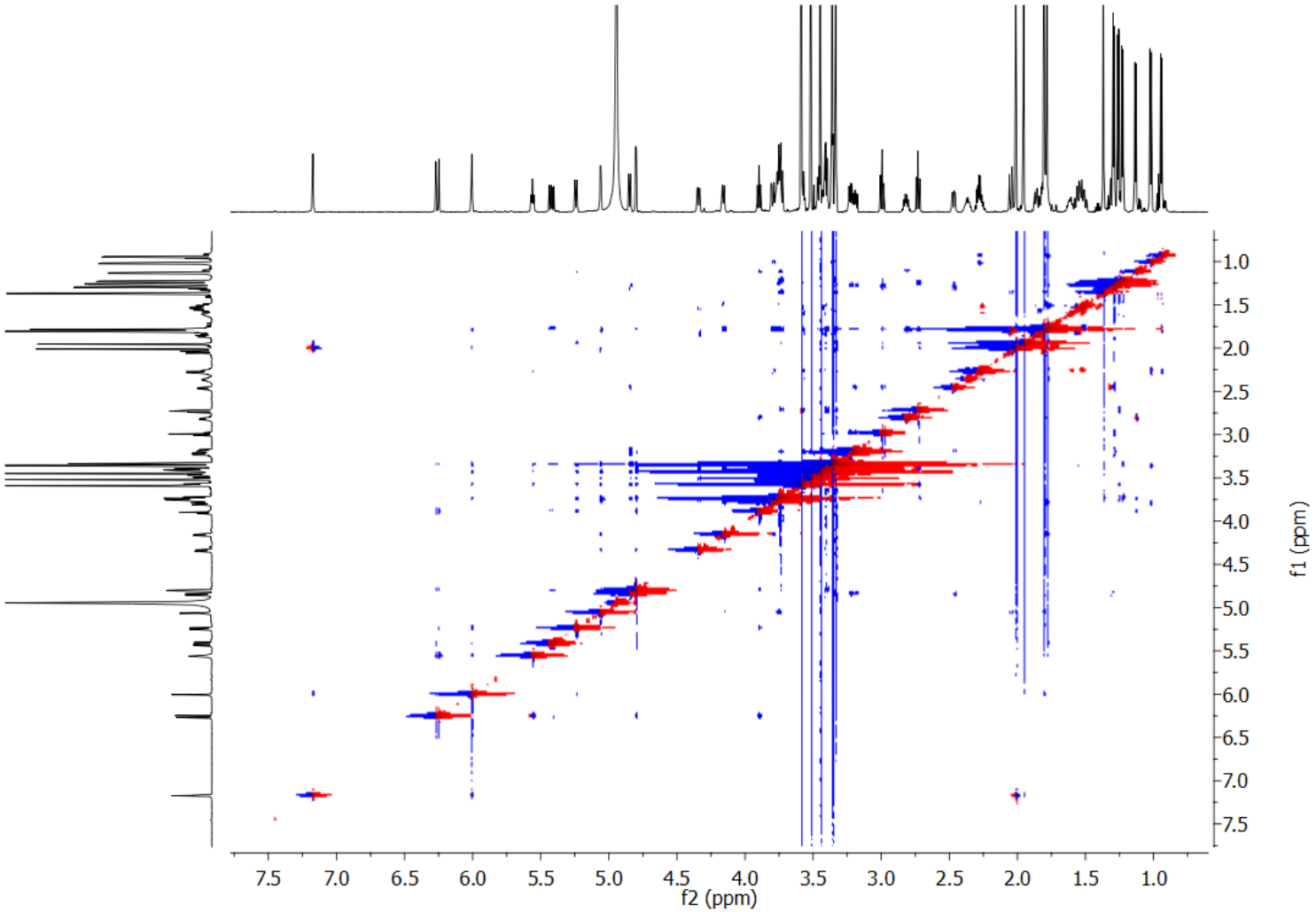


**Figure S4.27.** ROESY spectrum of linear apoptolidin A (**13**) (700 MHz, $CD_3OD$).

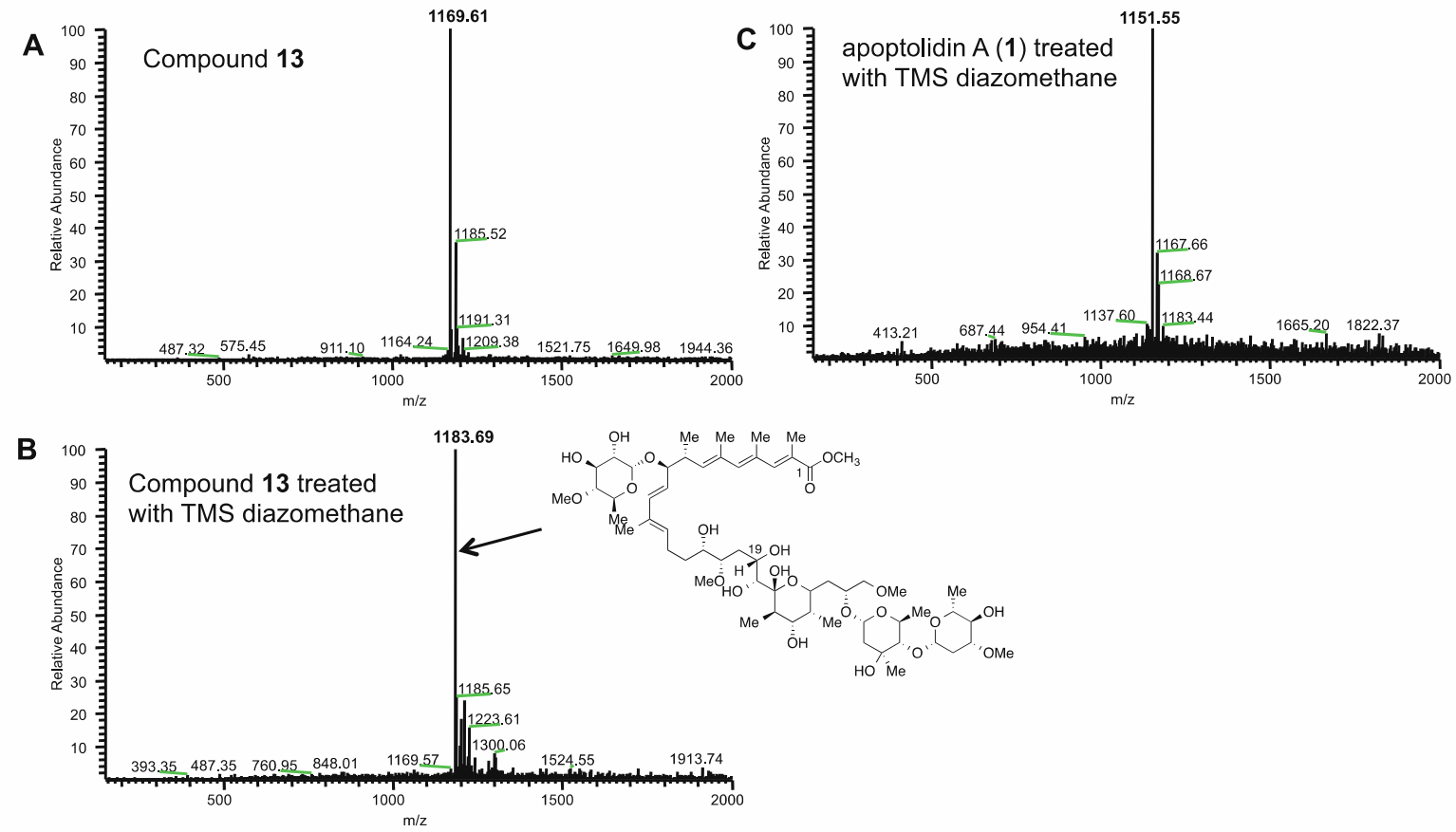


**Figure S4.28.** (+)-ESI-MS analysis of linear apoptolidin A (**13**) and its methylated product. **A**, linear apoptolidin A (**13**); **B**, product of linear apoptolidin A (**13**) treated with trymethylsilydiazomethane; **C**, product of apoptolidin A (**1**) treated with trymethylsilydiazomethane.

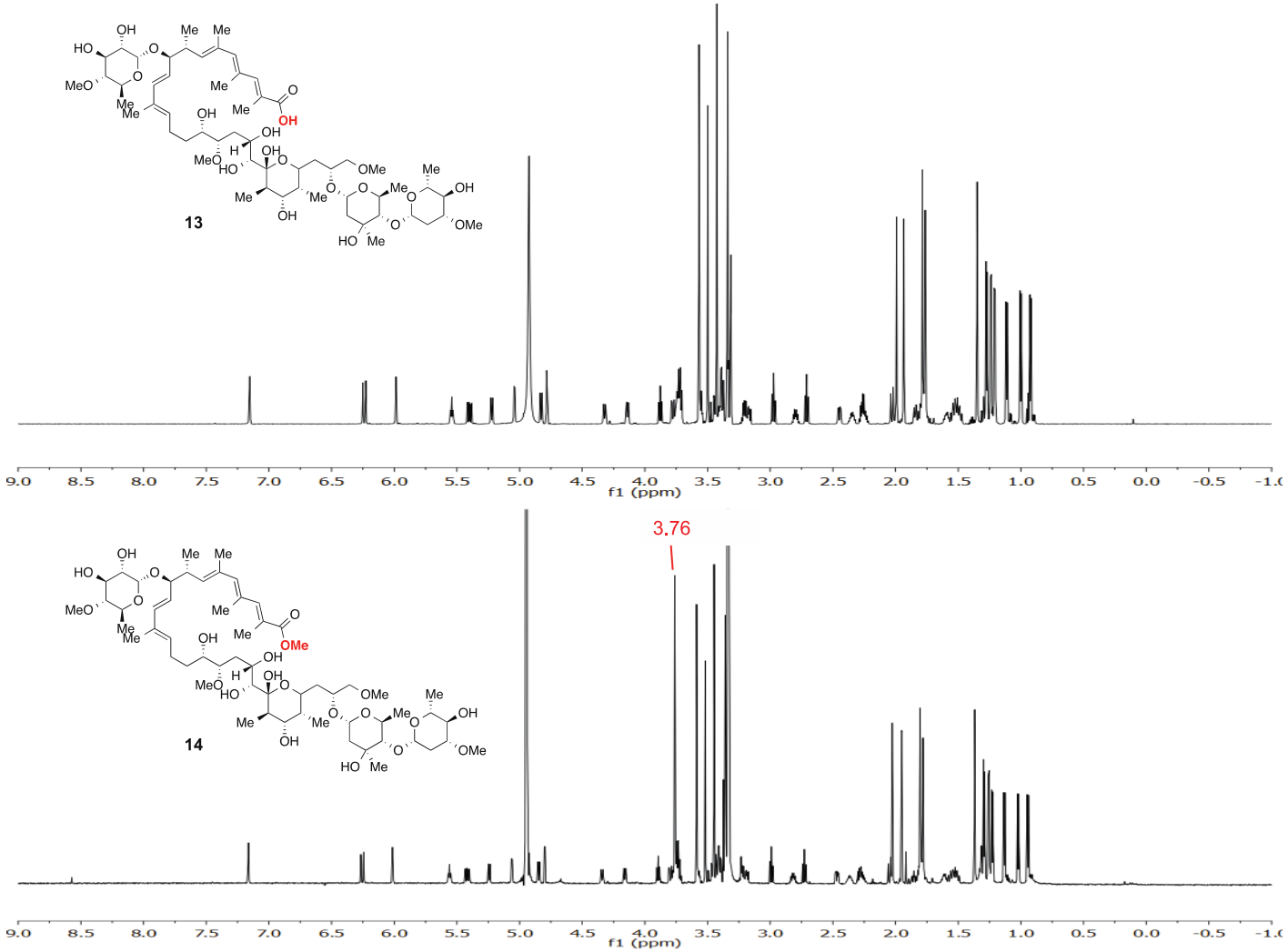


**Figure S4.29.** $^{1}$H NMR spectra of linear apoptolidin A (**13**) and its methylated product (**14**) (700 MHz, $CD_3OD$). The resonance at 3.76 ppm represents the newly formed methoxy group.

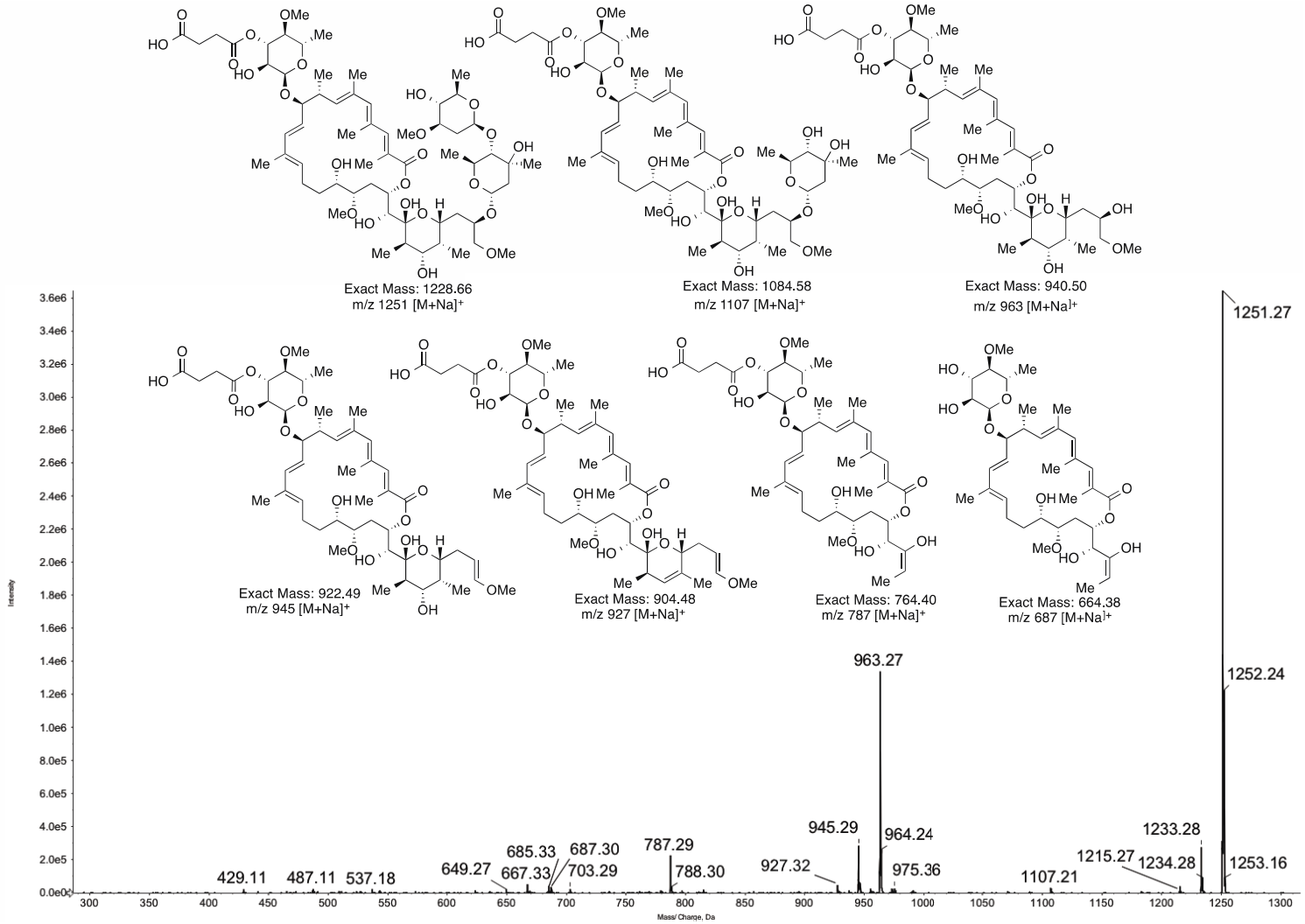


**Figure S4.30.** MS/MS analysis of 3´-*O*-succinyl-apoptolidin A (**12**).

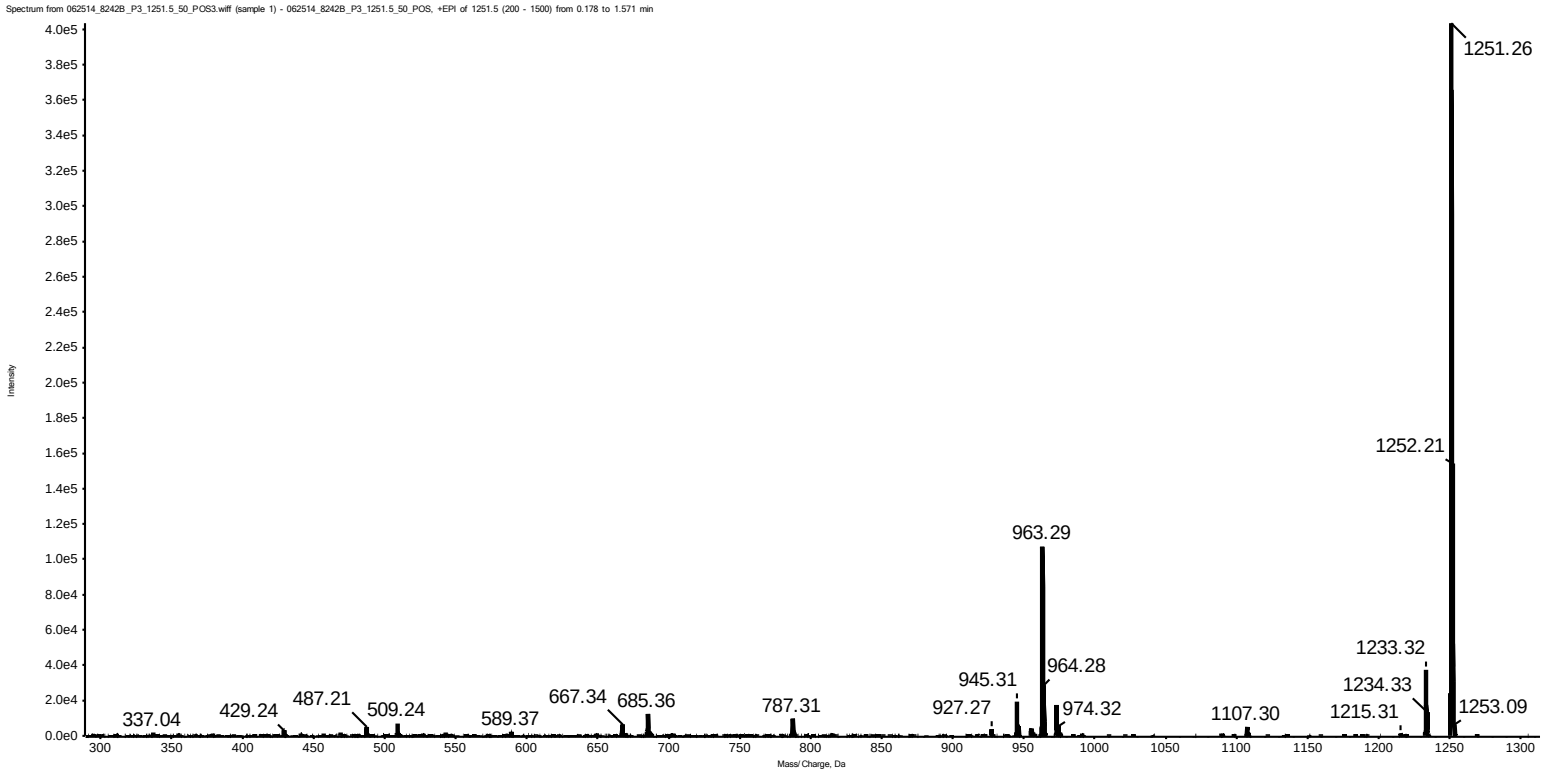


**Figure S4.31.** MS/MS analysis of 2´-*O*-succinyl-apoptolidin A (**11**).

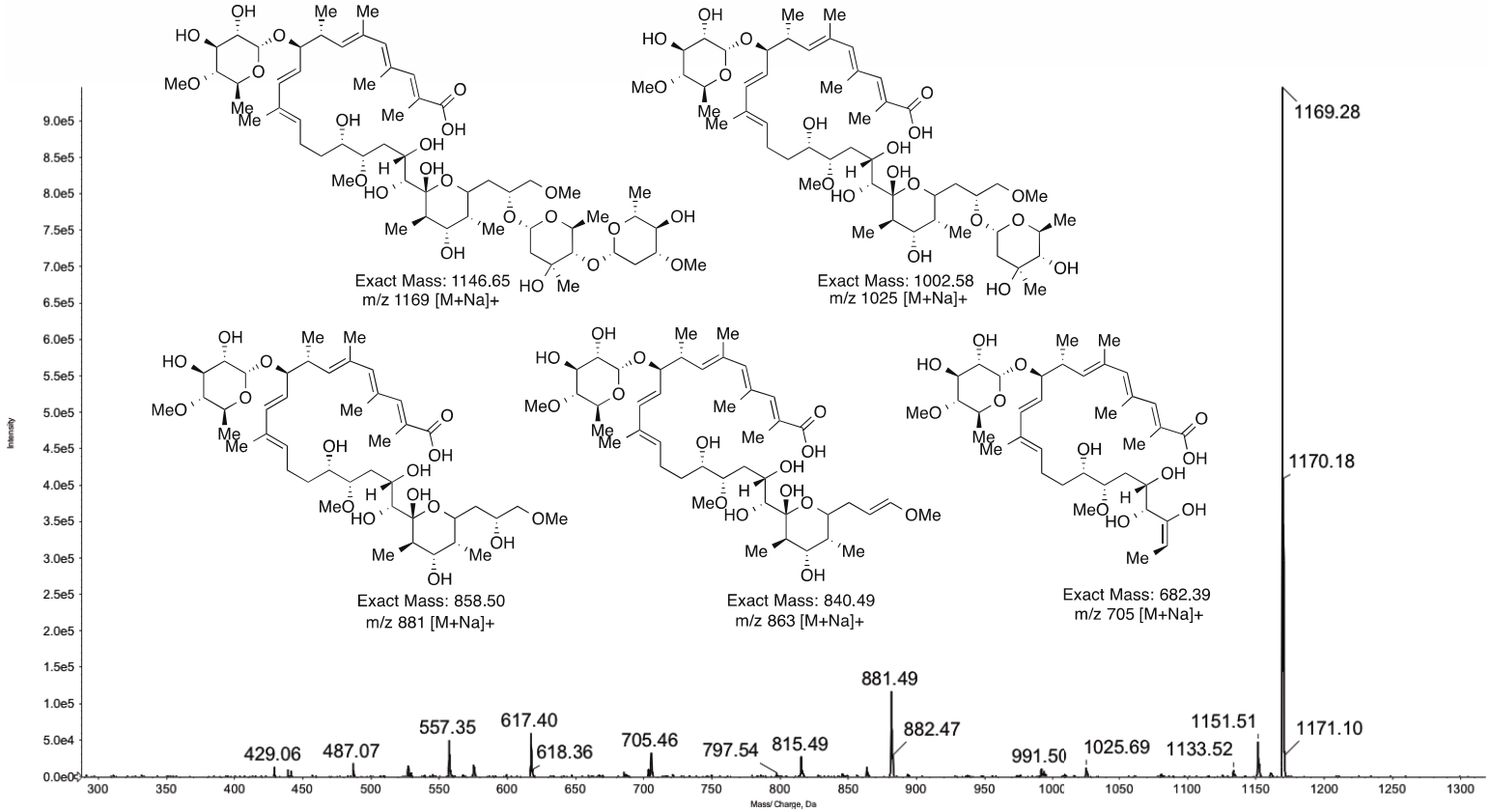


**Figure S4.32.** MS/MS analysis of linear apoptolidin A (**13**).

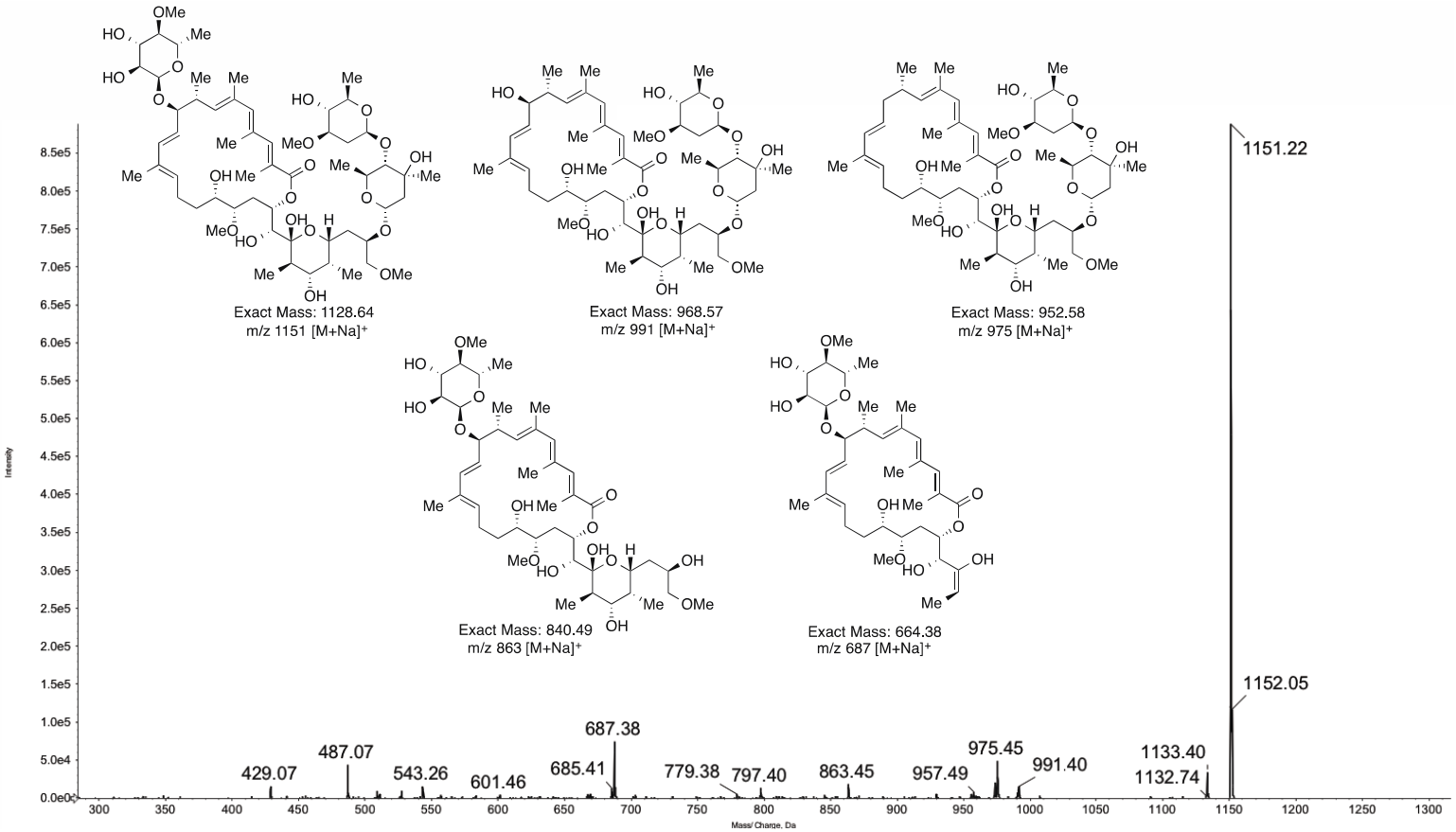


**Figure S4.33.** MS/MS analysis of apoptolidin A (**1**).

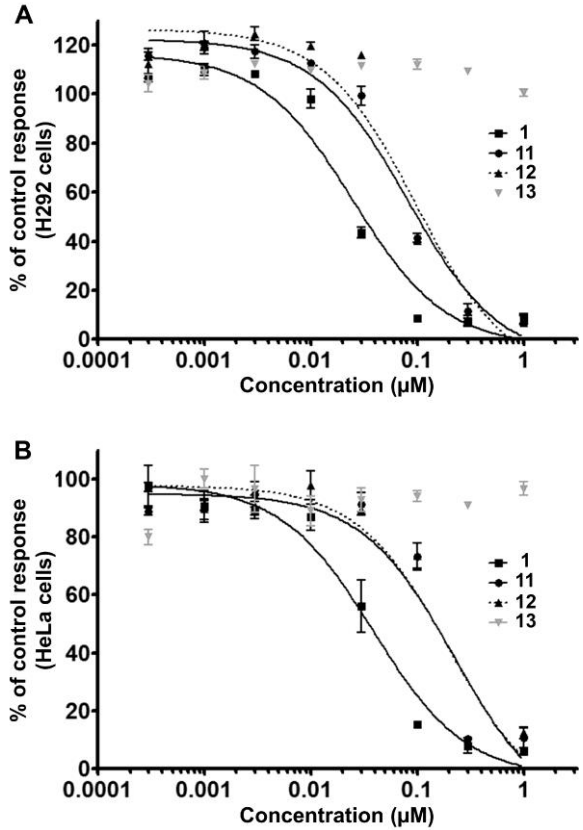


**Figure S4.34.** Concentration response profiles for apoptolidin A (**1**) and apoptolidin analogues (**11-13**) against human cancer cell lines. **A**, H292 lung cancer cells and **B**, HeLa cervical cancer cells.

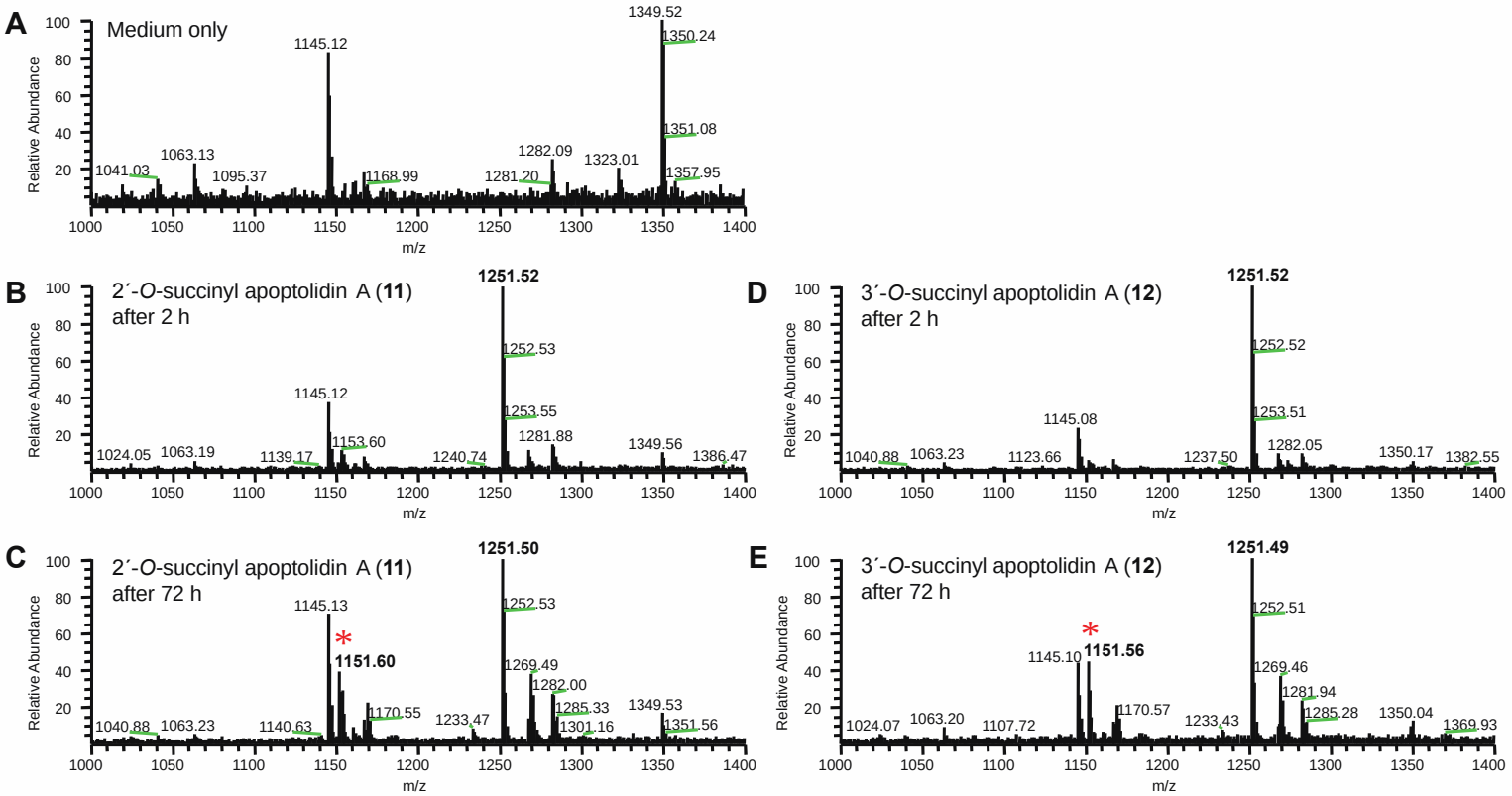


**Figure S4.35.** (+)-ESI-MS analysis of succinylated apoptolidins (**11** and **12**) and their hydrolysis products in RPMI-1640 cell culture medium after 2 h and 72 h incubations at 37 °C in a humidified cell culture incubator with 5% $CO_2$. **A**, medium only; **B**, 2´-*O*-succinyl-apoptolidin A (**11**) after 2 h; **C**, 2´-*O*-succinyl-apoptolidin A (**11**) after 72 h; **D**, 3´-*O*-succinyl-apoptolidin A (**12**) after 2 h; **E**, 3´-*O*-succinyl-apoptolidin A (**12**) after 72 h. Red asterisk indicates the newly formed apoptolidin A.

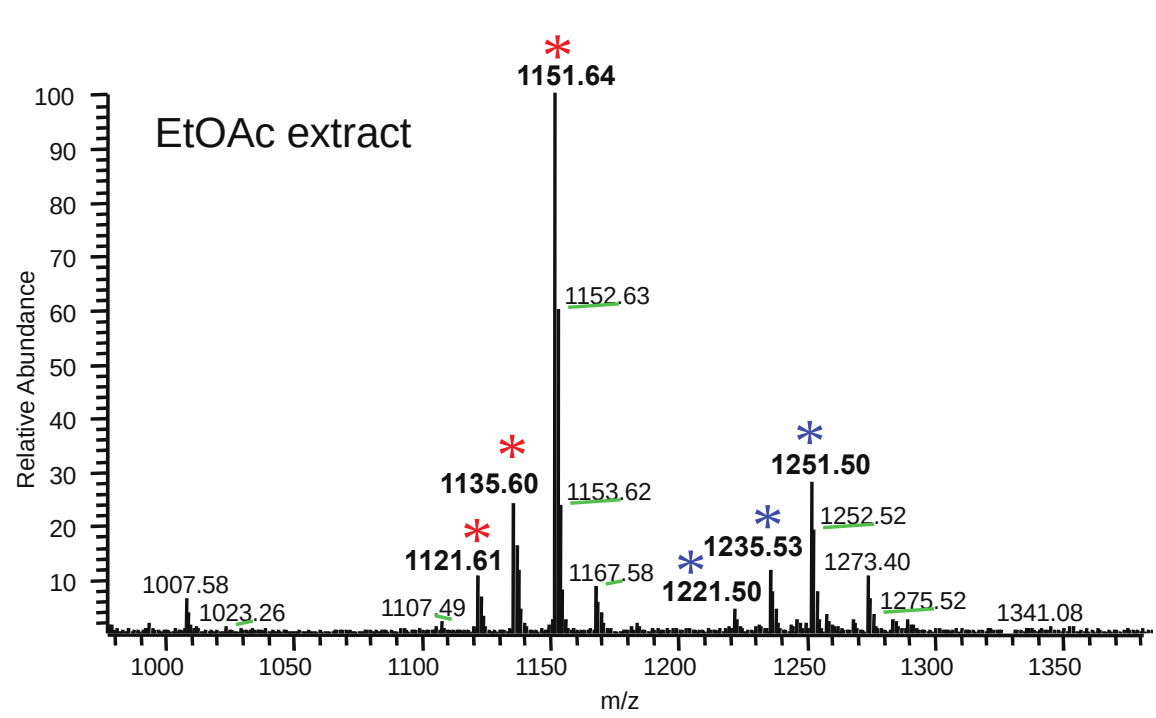


**Figure S4.36.** Evidence for the presence of succinylated apoptolidins B and C in the culture broth of *Amycolatopsis* sp. ICBB 8242. Red asterisks represent the apoptolidins and blue asterisks represent the corresponding succinylated apoptolidins.

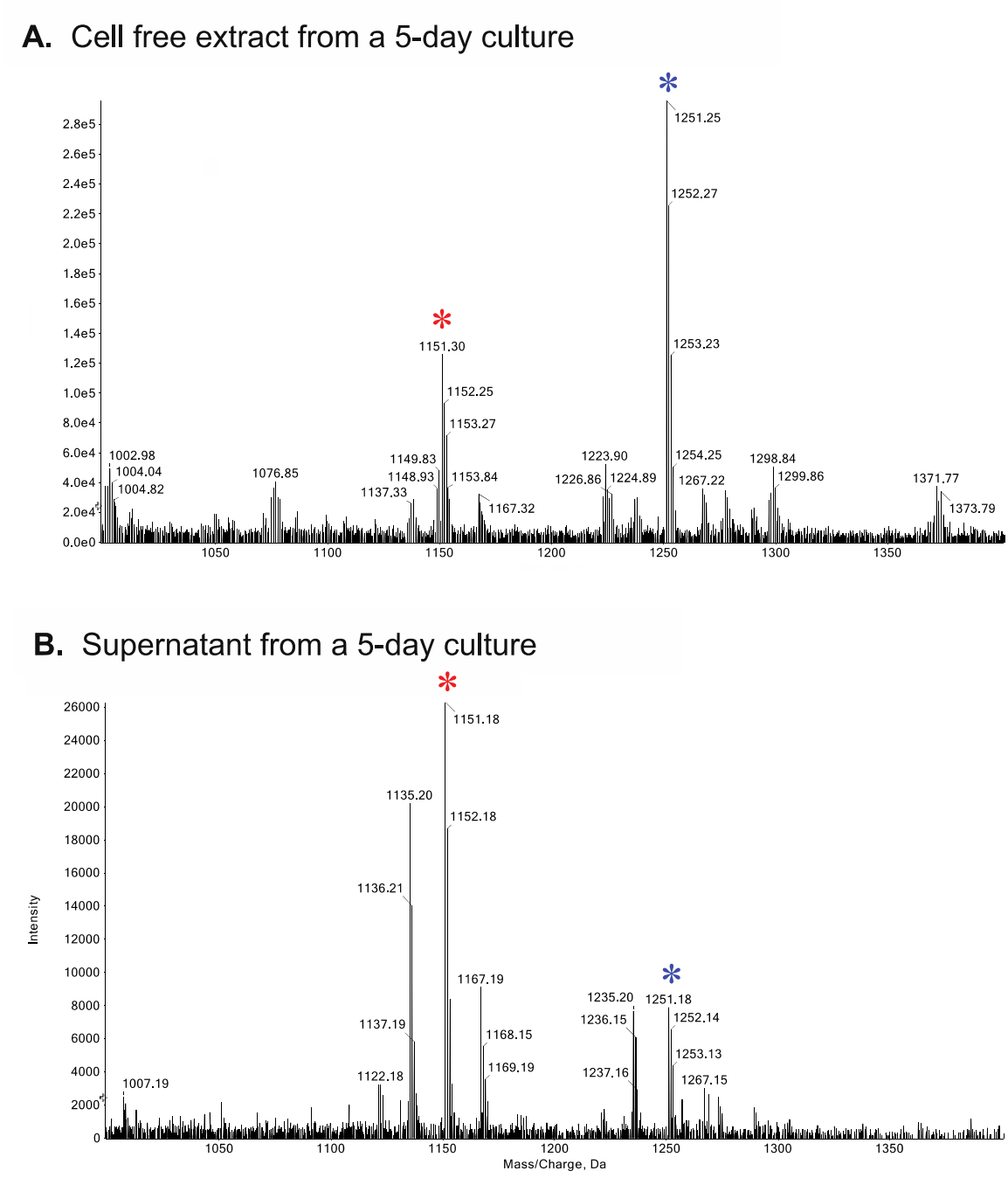


**Figure S4.37.** (+)-ESI-MS analysis of the cell-free extracts of *Amycolatopsis* sp. ICBB 8242 from 5-day cultures. **A**, cell-free extract from a 5-day culture; **B**, supernatant from a 5-day culture.

## 4.6 References


1. Kim, J. W.; Adachi, H.; Shin-ya, K.; Hayakawa, Y.; Seto, H. *J. Antibiot.* **1997**, *50*, 628-630.
2. Hayakawa, Y.; Kim, J. W.; Adachi, H.; Shin-ya, K.; Fujita, K.; Seto, H. *J. Am. Chem. Soc.* **1998**, *120*, 3524-3525.
3. Salomon, A. R.; Voehringer, D. W.; Herzenberg, L. A.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 14766-14771.
4. Salomon, A. R.; Voehringer, D. W.; Herzenberg, L. A.; Khosla, C. *Chem. Biol.* **2001**, *8*, 71-80.
5. Wender, P. A.; Jankowski, O. D.; Longcore, K.; Tabet, E. A.; Seto, H.; Tomikawa, T. *Org. Lett.* **2006**, *8*, 589-592.
6. Serrill, J. D.; Tan, M.; Fotso, S.; Sikorska, J.; Kasanah, N.; Hau, A. M.; McPhail, K. L.; Santosa, D. A.; Zabriskie, T. M.; Mahmud, T.; Viollet, B.; Proteau, P. J.; Ishmael, J. E. *Biochem. Pharmacol.* **2015**, *93*, 251-265.
7. Wender, P. A.; Sukopp, M.; Longcore, K. *Org. Lett.* **2005**, *7*, 3025-3028.
8. Wender, P. A.; Longcore, K. E. *Org. Lett.* **2007**, *9*, 691-694.
9. Wender, P. A.; Longcore, K. E. *Org. Lett.* **2009**, *11*, 5474-5477.
10. Wender, P. A.; Gulledge, A. V.; Jankowski, O. D.; Seto, H. *Org. Lett.* **2002**, *4*, 3819-3822.
11. Bachmann, B. O.; McNees, R.; Melancon, B. J.; Ghidu, V. P.; Clark, R.; Crews, B. C.; Deguire, S. M.; Marnett, L. J.; Sulikowski, G. A. *Org. Lett.* **2010**, *12*, 2944-2947.
12. Crimmins, M. T.; Christie, H. S.; Long, A.; Chaudhary, K. *Org. Lett.* **2009**, *11*, 831-834.
13. Salomon, A. R.; Zhang, Y.; Seto, H.; Khosla, C. *Org. Lett.* **2001**, *3*, 57-59.
14. Wender, P. A.; Jankowski, O. D.; Tabet, E. A.; Seto, H. *Org. Lett.* **2003**, *5*, 487-490.
15. Anderson, B. D.; Conradi, R. A.; Lambert, W. J. *J. Pharm. Sci.* **1984**, *73*, 604-611.
16. Du, Y.; Derewacz, D. K.; Deguire, S. M.; Teske, J.; Ravel, J.; Sulikowski, G. A.; Bachmann, B. O. *Tetrahedron* **2011**, *67*, 6568-6575.

# CHAPTER FIVE

## General Conclusion

Yan Sheng

Microorganisms are known as a major source of bioactive natural products with diverse chemical scaffolds. Recent research has shown that the bacterial biosynthetic diversity is closely related with two factors, geographic distance and biome-type.[1] Bacteria that are collected from more distant and unique locations are more likely to produce structurally distinct secondary metabolites. The focus of this research is to investigate secondary metabolite production in microorganisms living in a unique black water ecosystem in Indonesia.

Strains isolated from the peaty soils of the black water rivers were subjected to 16S rRNA analysis, as well as biological and chemical screening. Based on the 16S rRNA sequencing and alignment results, 108 out of 141 strains belong to 27 different species of *Streptomycetes*. Nine strains were identified as members of non-*Streptomyces* actinomycetes, e.g., *Amycolatopsis rubita*, *Amycolatopsis echigonensis*, *Kitasatospora sampliensis*, and *Kocuria rhizophila*. The remaining 24 strains belong to 17 different species of non-actinomyces genus, e.g., *Ochrobactrum intermedium*, *Erwinia amylovora*, and *Lysinibacillus fusiformus*. The 16S rRNA analysis showed the diversity of the bacterial collection, which holds promise for bioactive natural product discovery.

The biological screening results indicate that *Streptomyces* remain the best producers of bioactive compounds. Fifteen out of 24 *Streptomyces* produced compounds that are active against Gram-positive bacteria and two of them are also active against certain Gram-negative bacteria. Furthermore, nine *Streptomyces* strains produced compounds that are

active against the fungus *P. sasakii*. On the other hand, only 2 out of 14 non-actinomycete strains tested showed good activities against Gram-positive bacteria.

One of the "positive" strains, *Streptomyces albiflaviniger* ICBB 9297, was further investigated for its active secondary metabolites. From this strain, four new elaiophylin-like macrolides and five known elaiophylins were isolated. These new elaiophylin-like macrolides have macrocyclic skeletons distinct from those of the known elaiophylins, in which one or both of the dimeric polyketide chains contain(s) an additional pendant methyl group.

Similar to the known elaiophylin analogues, the new elaiophylins displayed good activity against *Staphylococcus aureus*, but did not inhibit the growth of *Bacillus subtilis*, as well as the Gram-negative bacteria *E. coli* and *P. aeruginosa*. Remarkably, two of the new compounds with only one additional pendant methyl group in the macrocyclic core showed good activity against the Gram-positive bacterium *Mycobacterium smegmatis* with an MIC value of 6.25 μg/ml. No significant inhibition was observed for the new compound that contains additional methyl groups in both sides of the macrocyclic core and for the known elaiophylins (MIC > 100 μg/ml). The reason for this disparity in activity toward *M. smegmatis* is currently unknown and will be a subject of our future investigations. However, the activity of against *M. smegmatis* may serve as a platform for development of new antibiotics against the human pathogenic strain *Mycobacterium tuberculosis*.

The biosynthesis of elaiophylins is catalyzed by modular type I polyketide synthases. Bioinfomatic analysis of the elaiophylin polyketide synthases revealed the presence of a flexible AT domain (AT7) capable of incorporating both malonyl CoA and methylmalonyl CoA as substrates, which explains the production of the new elaiophylin analogues by the ICBB 9297 strain. Instead of having the typical conserved 'HAFT' motif for malonate specific ATs or 'YASH' motif for methylmalonate specific ATs, the AT7 domain has an unusual 'IAAH' motif. The discovery of this non typical motif provide more information for identifying relaxed ATs and is useful for predicting the possible product of unidentified modular type I polyketide synthase. Additionally, residues in this region or the whole AT domain may be targeted for genetic manipulations to direct the production of elaiophylin analogues, for example, to increase the yield of new elaiophylin analogues, which shows better activities for *M. smegmatis*.

Chemical screening of non-*Streptomyces* actinomycetes revealed that strains *Amycolatopsis rubida* ICBB 9389, *A. rubida* ICBB 9301, and *A. echigonensis* ICBB 9385 produce apoptolidins, a group of glycosylated macrolide antibiotics with potent apoptosis inducing activity against certain cancer cells. Apoptolidins have also been isolated from another ICBB strain, *Amycolatopsis* sp. ICBB 8242. Previous studies revealed that this strain produced apoptolidin A as a major product in higher yield (~300 mg/mL) than that from the reported producing strain *Nocardiopsis* sp. (140 mg/mL). Further investigation of *Amycolatopsis sp* ICBB 8242 has led to the discovery of two new succinylated apoptolidin analogues and a linear apoptolidin A. The succinylated apoptolidins showed comparable

activities to apoptolidin A in human cancer viability assays, but in contrast to apoptolidin A, did not affect the cellular respiration. This seemingly contradictory results may be due to an alternative biological mechanism of action or due to degradation of succinyl-apoptolidin A to apoptolidin A in the cell viability assay. Two succinylated compounds were added in mammalian cell culture medium and left under the same condition used for biological activity tests. Significant conversion (up to 30%) of the succinyl apoptolidin As to apoptolidin A were observed after 72 h incubation but not at 2h. As hydrolysis is anticipated to be higher inside the cells due to non-specific cellular esterase enzymes, it is likely that apoptolidin A contributes to the anti-proliferative activities observed for two succinyl apoptolidin As. Since the addition of the succinyl moiety may help increase the solubility of the compound in water, it is likely that the succinyl apoptolidins act as prodrugs of apoptolidin A.

To explore the structure–activity relationship (SAR) and mode of action for this group of compounds, semisynthetic approaches[2] and peptide-based catalysts[3] have been utilized to modify the hydroxy groups in apoptolidin A. Our study is the first report on the isolation of naturally occurred succinylated apoptolidins. In addition, the production of succinylated apoptolidins by *Amycolatopsis* sp. ICBB 8242 suggests that succinylation may play a role in self-resistance and/or as an export mechanism, an interesting phenomenon that rarely reported in natural products biosynthesis.

The discovery of new bioactive natural products from the Indonesian microbes further highlights the great potential of the diverse and untapped microorganisms from the Indonesian black water ecosystems for natural products discovery. Since only a portion of the strains from the bacterial collection has been studied, further investigations may lead to the discovery of more novel bioactive compounds.

## 5.1 Reference

1. Charlop-Powers, Z.; Owen, J. G.; Reddy, B. V.; Ternei, M. A.; Guimarães, D. O.; de Frias, U. A.; Pupo, M. T.; Seepe, P.; Feng, Z.; Brady, S. F. *Elife* **2015,** *4*, e05048.
2. Wender P. A., Jankowski O. D., Tabet E. A., Seto H. *Org Lett* **2003**, *5*, 487-490.
3. Lewis C. A., Longcore K. E., Miller S. J., Wender P. A. *J Nat Prod* **2009**, *72*, 1864-1869.