UNIVERSITY OF CALIFORNIA

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EXPLORATION AND DEVELOPMENT OF TOOLS TO ISOLATE AND CHARACTERIZE BORON NATURAL PRODUCTS EXHIBITING SELECTIVE MOSQUITOCIDAL ACTIVITY

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by

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Abstract

Exploration and Development of Tools to Isolate and Characterize Boron Natural Products Exhibiting Selective Mosquitocidal Activity

by Jocelyn Marie Macho

Each year, mosquito-borne diseases such as Malaria, Dengue, and Zika continue to affect millions of people worldwide. New approaches for mosquito vector control are imperative as current insecticides are not only losing efficacy towards mosquitoes due to rising resistance but also bioaccumulate, resulting in adverse ecological effects due to their non-selectivity amongst target and non-target organisms. The major challenges to developing new control agents are ensuring human safety, limiting ecological off-target effects, and confronting resistance. To address these, novel and efficient high throughput screening platforms are being used to identify selective mosquitocidal agents that exhibit novel mechanisms of action.

Our library of marine microbial-derived natural products was screened in a novel mosquito cell-based assay to identify metabolites lethal to mosquito cells but inactive against other insect cell lines such as *Drosophila melanogaster* (Kc and S2R+) and *Spodoptera frugiperda* (SF9). One compound, a novel boron-containing macrolide, “NP-34,” was identified as a selective mosquito toxin as it led to approximately 80% *Anopheles gambiae* cell death at a concentration of 50 nM whereas toxicity to *D. melanogaster* and *S. frugiperda* cell lines was negligible with less than 10% cell death at the same concentration. This selectivity was further observed when tested against larval and adult stage mosquitoes at concentrations like that of permethrin treatment. Additionally, the compound has impressive activity against cells, larvae, and mosquitoes from permethrin-resistant strains of *Aedes aegypti*. Preliminary data with RNAseq and genomic data of *Culex quinquefasciatus* resistant mutants has revealed potential markers of resistance within the vector, which can likewise shed light onto NP-34’s mechanism of action.

Based on the cellular and subsequent mosquito and mouse studies, we believe this unique macrolide is a promising candidate for the development of an eco-friendly mosquitocide. As we sought to isolate more NP-34 and analogs to continue biological testing to find the best *in vivo* potency, there were extensive challenges including validating the presence of boron in analogs and issues in fermentation leading to diminishing yields of material. To aid in screening and validation, a pulse sequence was applied to ^{11}B NMR to increase the sensitivity of the experiment by eliminating external probe noise that was hindering data acquisition. This allowed for detection of low levels of boron rapidly from crude microbial extracts. The feasibility of this experiment was confirmed with the biologically relevant boronic compounds Aplasmomycin and autoinducer-2. Additional methods for identification explored included LC- and ICP-MS.

Once the ^{11}B NMR was optimized as a screening method, the experiment was used to determine the best conditions for fermentation and isolation of NP-34 and other co-eluting analogs. Improvements including shortened fermentation time, swapping of chromatographic techniques, and stripping of all acid buffer, resulted in a 12 time increase in NP-34 yield compared to when yields started diminishing. Along the course of optimization, various analogs have been isolated and characterized for biological testing, while other analogs have been generated with late-stage functionalization. Some of these efforts included installing clickable handles for affinity chromatography that were unsuccessful. To help start understanding the biology and reactivity of NP-34 and similar analogs, material has been crystalized to get confirmational information. This is all in effort to understand and confirm NP-34's selective mosquitocidal activity, to be able to carry it forward for development as an eco-friendly insecticide.

Acknowledgements

First and foremost, thank you Prof. John MacMillan without whom this work would not be possible. Thank you for letting me be a part of the lab, starting at UT Southwestern. It has been a most challenging five years, but I am always going to value how much I have grown as a scientist and as a person, and all the friendships I have made in the process. You never stopped from doing anything I wanted to do, project or career-development related, or thwarted my creativity, and I really appreciate that. Thank you for letting me be a part of this awesome project and thank you for an amazing five years in your lab.

To my committee: Prof. Scott Lokey, thank you for always being so kind and always challenging me to think about chemistry in new ways. Thank you to Prof. Bakthan Singaram, for always helping me with synthesis and my ^{11}B NMR. You are one of the kindest people I know and UCSC truly wouldn't have been the same without you.

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School programs: I am a first-generation American and college student. My family urged me to go to college but were unable to guide me or financially support me. In high school I was a part of amazing TRIO programs AVID and the Hillsborough Education Foundation to equip first generation, minority students with the resources needed to go to college. If it wasn't for them, I would not have applied to the University of Florida or found the Florida Opportunity Scholars Program that, as long as I kept a 3.5 GPA, paid my way through UF. Thank you to Mrs. Alexa Lutz, Mrs. Darlene-Sigle-Lam, and Ms. Mary Alice Kelley for guiding me and supporting me all those years and thank you to Ms. Roohi Abidi for nudging me to pursue chemistry. At UF, I had the privilege of being a Ronald McNair Scholar which provided the resources and skills on how to apply to and succeed in graduate school. Thank you to Dr. Samesha Barnes, Dr. Alexander Grenning, and Dr. Tadeusz Molinski for guiding me all those years. These days in our current political and cultural climate, more and more the validity of diversity-oriented programs similar to these are being questioned as issues of race, gender, and inequality are becoming more everyday conversations. These programs are absolutely worth our time and effort because students will look to us for guidance, and whether we find ourselves in academia, industry, the government etc. we will have to make decisions on how to allocate time and funding and resources, and programs like these make huge impacts and are worth our resources.

Family: Thank to parents and grandparents. You are the hardest working people I know, working my whole life odd jobs throughout the nights and weekends on top of full 40- hour work weeks and through surgeries to put food on the table. You always made time to be present in everything I did at school. You've been so supportive of everything I've wanted to do, trusted me when I said I was making the best decisions for my school and my career, and have been patient with me. Thank you for all your love throughout the years. To my partner Dr. Ryan Quinones, thank you for supporting of all my choices, even when that meant going to Texas for 5 years to work for John instead of San Diego with you. Thank you for your love and encouraging me to always pursue what will make me happy.

This thesis is dedicated to my mom and dad, Annelisse and Jose Macho, and my *Yeya* and *Yeyo*, Helga and Angel Trueba.

List of Experimental Abbreviations

4Å MS – 4Å molecular sieves

AChE - Acetylcholinesterases

ACN – Acetonitrile

BGC – Biosynthetic Gene Cluster

$B(OH)_3$ – Boric Acid

^{11}B – 11-Boron NMR

^{13}C – 13-Carbon NMR

°C – Celsius

$CDCl_3$ – Chloroform, deuterated

CoA – Coenzyme A

COSY – Homonuclear Correlation Spectroscopy

d – doublet

DCM – Dichloromethane

DIW – De-ionized Water

DMSO – Dimethyl Sulfoxide

EDG – Electron Donating Group

EtOAc – Ethyl Acetate

eq – Equivalent(s)

EWG – Electron Withdrawing Group

FA – Formic Acid

(g) – Gas (gaseous state)

Glu – Glutamate

^{1}H – Proton NMR

His - Histidine

HMBC – Heteronuclear Multiple-bond Correlation Spectroscopy

HPLC – High Pressure Liquid Chromatography

hr – Hour(s)

HRMS – High Resolution Mass Spectroscopy

HTS – High Throughput Screen

ICP-MS – Inductively Coupled Plasma Mass Spectroscopy

ICP-OES – Inductively Coupled Plasma - Optical Emission Spectroscopy

ig – Inverse Gated, indicative of proton decoupling with NMR

IGRs – Insect Growth Regulators

IPA – Isopropanol

L – Liter(s)

(l) – Liquid (liquid state)

LC-MS – Liquid Chromatography Mass Spectroscopy

LG – Leaving Group

MeOH- Methanol

MeOD- Methanol, deuterated

min – Minute(s)

mM – mili-Molar

mmol – mili-mole(s)

MoA – Mechanism of Action

nAChR – Nicotinic Acetylcholine Receptor

$NaHCO_3$ – Sodium Bicarbonate

nM – nano-Molar

m – multiplet

nmol – nano-mole(s)

NMR – Nuclear Magnetic Resonance

NP(s) – Natural Product(s)

p – pentet

ppb – Parts per Billion

ppm – Parts per Million

OP – Organophosphate

rt – Room Temperature

s – singlet

SAR – Structure Activity Relationship

Ser – Serine

SNAC – N-acteylcysteamine

t – Time (when in body of text)

t – triplet (when in NMR charts)

µM – micro-Molar

µmol – micro-mole(s)

wk – Week(s)

zg – Standard Bruker NMR Pulse Sequence

zgbs – Bruker NMR Composite Pulse Sequence

CHAPTER ONE

THE NEED FOR NOVEL MOSQUITO VECTOR CONTROL

1.1 Introduction

Mosquitoes are the deadliest animal threat to humans,[1] transmitting infectious diseases and killing more than any other creature. Approximately 700 million[2] people contract a mosquito-borne disease annually and approximately 1 million people die as a result.[3] *Aedes* is the most dangerous of the mosquito families, as members of transmit yellow fever, dengue fever, and chikungunya, with *Ae. aegypti* also responsible for transmitting Zika.[4] The *Anopheles* mosquitoes are vectors for malaria, brugian and bancroftian filariasis and the *Culex* family are vectors for Rift Valley fever, West Nile Virus, and various strains of encephalitis. Since these diseases severely hinder quality of life, curtailing the spread and impact of mosquito-borne diseases is a global public health goal, but it is not without its challenges. [5-7]

Climate change is also allowing spread of disease-carrying mosquitos to areas where, under normal conditions, they would not survive, and are threatening a larger percentage of the world population.[8,9] It is estimated, for example, that currently 40% of the world's population is at risk for dengue, as it's one of the fastest spreading diseases.[10] West Nile virus carrying *Aedes* mosquitos, which are typically found in warmer climates, such as the American States of Florida and Texas, have been spreading to historically colder states, such as Connecticut.[8]

Female mosquitoes feed on human hosts to extract protein from blood to develop their eggs. In the process, they transmit the pathogens responsible for diseases through their saliva. Controlling the mosquito vector to avoid disease transmission is a desired goal as therapeutics for the range of diseases are limited, prone to resistance, or in many cases non-existent. Unfortunately, in cases where drug therapies do exist, access to therapies can be challenging, costly, and often ineffective. In 2017, approximately $3.1 billion was invested by malaria endemic countries to expand malaria prevention, diagnostic testing, and treatment programs. One major area of investment is the use of artemisinin-based combination therapies (ACT) which drastically reduces most malaria parasites with efficacy rates over 95%. However, poorer communities continue to choose less costly and other unapproved drugs. As a result, treatment

failure from sub-optimal dosing, poor adherence, or bodily rejection of the medicines is frequently experienced. The lack of adherence to dose and completion of therapies leads to recrudescence, drug resistance, and further disease transmission.[11]

Therapeutic development is challenging due to the abundance of pathogens responsible for these diseases. Treatments for malaria (transmitted by *Plasmodium* viruses) will not be effective against dengue or yellow fever (transmitted by flavivirus), for example. Additionally, diseases will be transmitted more than one strain of microorganism, such as dengue with 4 different serotypes thus requiring 4 distinct treatments.[12] Rise of malaria pathogens resistant to all current treatments, including artemisinin, ivermectin, and chloroquine[13] make even combination therapies, such as Sulfadoxine-pyrimethamine, and triple ACTs increasingly ineffective in parts of the world like Southeast Asia due to genetic mutations in the *Plasmodium* strains.[14] So, at present, we cannot rely on drug therapies and vaccines alone to combat mosquito-borne diseases.

To curtail transmission of mosquito-borne diseases, historically there has been a heavy reliance on chemical insecticides to control the spread of the vector, as it is more effective to wipe out the disease carrier and avoid transmission than it is to treat the disease once it has manifested. An estimated 2.5 million tons of pesticides are used annually for pest management – from agricultural protection to combatting mosquito outbreaks.[15] While this can be effective, heavy usage has led to the emergence of resistance, resulting in a loss of vector control efficacy,[16] and continuation of disease spread. Although the efficacy is decreasing, the use of insecticides will not cease anytime soon as they are currently the most viable control option.

Perhaps more concerning than resistance are chemical insecticides' lack of selectivity for mosquitoes, resulting in harm to other insects and organisms in the ecosystems. Recent studies have shown that approximately 0.3% of pesticides interact with their target while the other 99.7% goes into the environment as runoff,[17] and this runoff affects non-target organisms.

Pollinator species including bees and butterflies are responsible for over 75% of crop production worldwide,[18] but the non-selectivity of insecticides are causing vast population declines of beneficial insects. Honey bees have been dying off in the past years, with over 40% colony loss in 2019 alone.[19] This is largely due to insecticide exposure as they not only elicit direct toxicity to worker bees, but also impact many downstream factors contributing to mortality including queen failure, susceptibility to mites and pathogens, and reduced resistance and immunity to infection, etc.[20-22] Traces of over 150 insecticides had been found in bee hives, wax, honey, nectar and pollen due bee exposure by drift droplets, dust, and inhalation.[23] As a result of heavy pesticide use, particularly the neonicotinoids, the agricultural landscape of America is considered 48 times more toxic to bees and other insects than it was a quarter of a century ago.[24] In addition to beneficial insects, heavy pesticide use is harmful to aquatic and terrestrial organisms, both altering the food chain and leading to serious acute and chronic health issues such as neurological damage and cancer. To limit off target consequences, it is imperative before their use, to develop more selective agents that limit this devastating impacts.

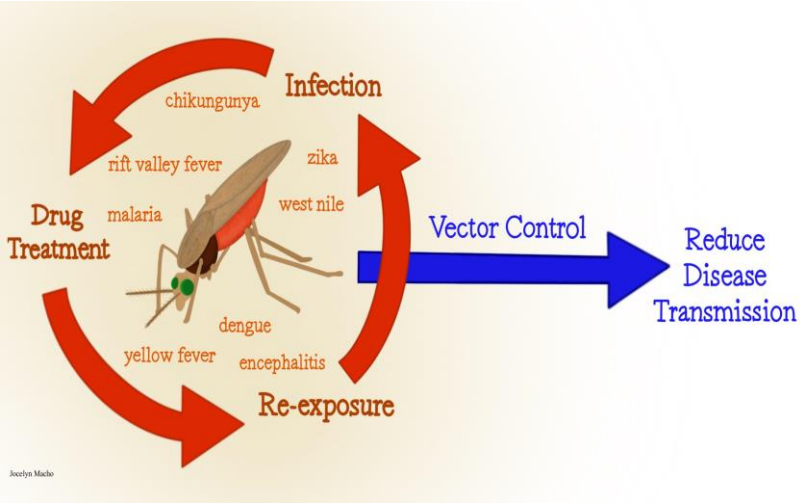


Figure 1.1 The perpetual cycle of infection, drug-treatment, and re-exposure to mosquito-borne diseases affects millions of people each year. Therapeutic development for many of these diseases are slow and challenging. For other diseases, many treatments are becoming increasing ineffective, making mosquito-borne diseases a global issue. Effective vector control strategies are currently the best option to reduce the rate and burden of disease transmission.

1.2 Currently Used Insecticides

There are six insecticide classes recommended to control adult mosquitoes. The most commonly used belong to the family of pyrethroids and organophosphates (OPs), while others in use include carbamates, organochlorines, pyrroles, and phenyl pyrazoles.[7] Additional insecticides used for agricultural purposes, such as neonicotinoids have shown mosquitocidal activity even though that is not their primary target. Pan-insecticidal insect growth regulators (IGRs), such as temephos (Section 1.2.7), are commonly used in mosquito-breeding areas. These pesticides are generally characterized via their chemical composition, target organism, and application type (Table 1.1). The major classes will be discussed below.

Overall Mechanistic Function	Insecticide Class	Application Type
Sodium Channel Modulators	Pyrethrins/Pyrethroids, Organochlorines	Agriculture livestock and crop protection; residential pest control; pet-parasite control; mosquito control
Acetlycholinesterase Inhibitors	Organophosphates, Carbamates	
Chlorine Channel Antagonists	Organochlorine cyclodienes, Phenylpyrazoles	

Table 1.1 Summarized mechanistic function and application type of the six most used insecticide classes used to treat adult mosquitoes.

1.2.1 Pyrethroids

Pyrethroids are the main class of insecticides used to combat mosquitoes. Due to their mechanism of action (MoA) in modulating sodium channels, they are fast acting and efficient against wild-type mosquitos, but have broad-spectrum insecticidal activity due to the high degree of similarity of sodium channels across insect species.[25] As such, pyrethroids are used against various mosquito species through aerosol spraying, indoor residual spraying (IRS) within homes, and are considered the only insecticide safe enough to use on bed nets.[4] Even though they make up approximately a fourth of the entire insecticide market overall,[26] pyrethroids are still expensive and beyond the financial resources of some developing countries to be used on scale.

Chemistry

The naturally occurring pyrethrins were discovered from the achenes of *Chrysanthemum cinerariifolium*, a plant known to have insecticidal properties for centuries. Isolated in the 1920s, pyrethrin I and II along with four other analogs (Figure 1.2) were identified as the source of the plant's insecticidal properties.[27] These six naturally occurring pyrethrins all contain a cyclopropane-carboxylic acid "chryanthemic acid," an ester linkage, and a cyclopentenolone.[28]

Of the naturally occurring analogs, the best insecticidal potency is seen with *cis*-penta-2',4'-dienyl sidechains, as in pyrethrins I and II. Insecticidal toxicity is reduced when dihydroesters are present or if unsaturation is two or more methylene groups away from the cyclopentenolone ring, but the most toxicity is exhibited with unsaturated sidechains. The natural occurring pyrethrins have 16 possible isomers, most of which have been fully explored, with the (+)-*trans*- and (+)-*cis*-chrysanthemic acid analogs showing greater insecticidal toxicity.[29] These compounds show broad-spectrum toxicities towards multiple insect species, like mustard beetles and houseflies. The pyrethrins, for example, have an LC_{50} of 2.861 mg/L against wingless aphids. No other naturally occurring compounds in *C. cinerariifolium* act synergistically, but the potency of pyrethrin I and II increases fourfold with the common insecticidal synergist piperonyl butoxide.[29,30]

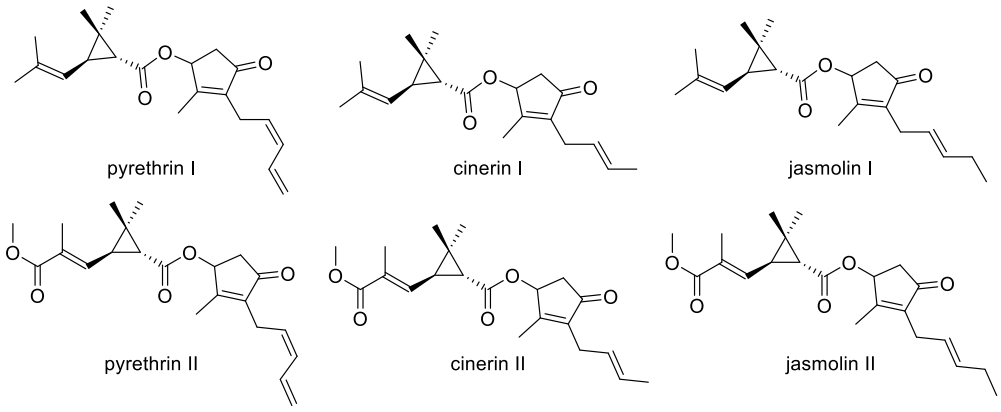


Figure 1.2 Natural pyrethrins from *Chrysanthemum cinerariifolium.*

Since their initial discovery, one aspect of optimization has been modification of the cyclopropane carboxylic ester core, due to the susceptibility to light induced degradation. Additionally, the isobutenyl group and furan rings are modified as they are easily epoxidized and degrade through unstable peroxide intermediates, respectively.[30] These modifications not only strengthen these compounds structurally, but also diversifies insecticidal activity, allowing for use against mosquito, household, and agricultural pests. More than 30 synthetic derivatives have been commercialized since the 1960s for their remarkable potency, economy, and stability compared to the original natural pyrethrins (Figure 1.3).

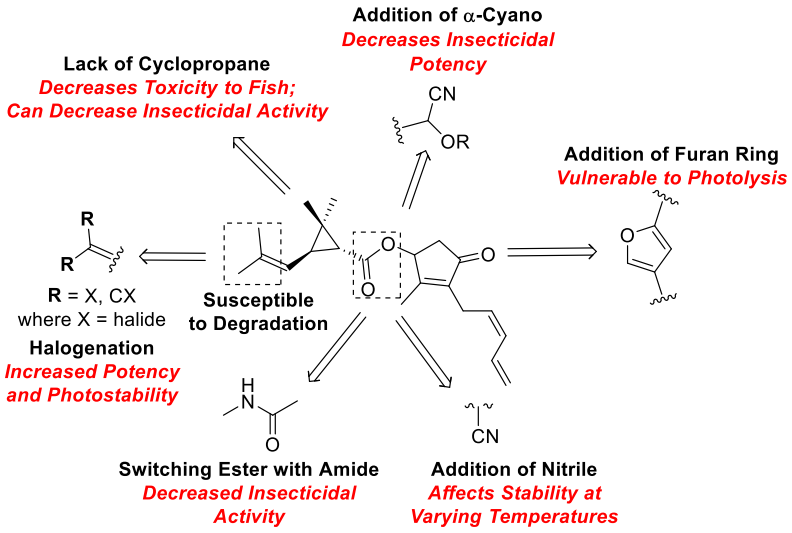


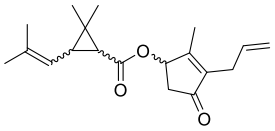
Figure 1.3 Select SAR strategies taken to affect biological activity and physiochemical properties of pyrethroids based of the natural pyrethrins.

Since discovery, there have been 4 generations of pyrethroid analogs. The 1st and 2nd generation were still light-sensitive due to the acid's isobutenyl side chain and the furan ring's vulnerability to photodecomposition. Analogs lacking the furan ring and analogs with changes to the isobutenyl chain, such as halogenation, resulted in the 3rd and 4th generation pyrethroids which are not susceptible to UV photolysis.[31] One example is Permethrin, the first photostable pyrethroid designed, which contains a 3-phenoxybenzyl group in place of the furan ring and a

chlorinated isobutenyl chain.[31] These late generation compounds are reported to have efficacy lasting between 4-10 days after application due to stability, compared to pyrethrin I, for example, which exhibits half-lives of 11.8 hours in water and 12.9 hours on soil when in the presence of light.[32,33]

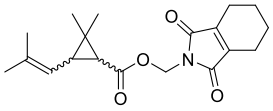
The keto-alcoholic ester and overall lipophilicity of the molecules are responsible for their insecticidal properties; the sidechains determine potency, environmental sensitivity, and how they are metabolized in organisms.[34] Addition of halogen groups at the gem-dimethyls also greatly increases potency and photostability.[35] The greatest larvicidal activity with halogens are with bromo< choloro< flouro substitutions, and vice versa with adulticidal activity in various mosquito strains.[36] Addition of cyano groups affect their physiological manifestations. Type I pyrethroids (T-syndrome) which lack a cyano group, work better at warmer temperatures, and produce aggressive sparring and tremors in targets. Type II (CS-syndrome) which have a cyano group, work better at lower temperatures, and produce choreoathetosis and salivation in their targets. Type II *cis*-isomers have the greatest toxicity of both type I and II.[26,37] Of the pyrethroids on the market, deltamethrin is currently the most active.[28]

1st Generation

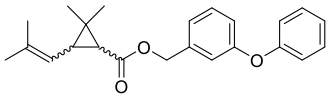


Allethrin

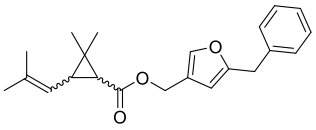
2nd Generation



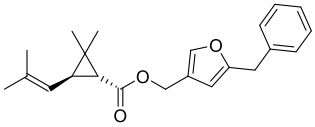
tetramethrin



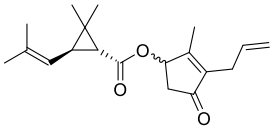
Phenothrin



Resmethrin

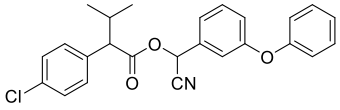


Bioresmethrin

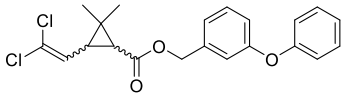


Bioallethrin

3rd Generation

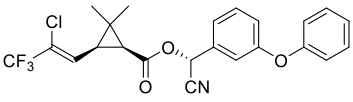


Fenvalerate

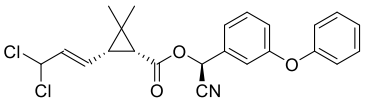


Permethrin

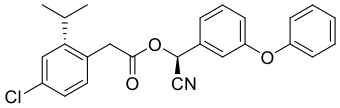
4th Generation



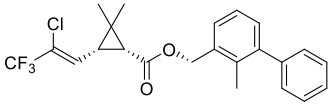
lambda-Cyhalothrin



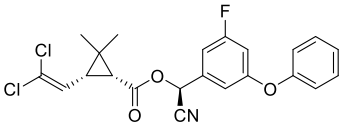
Cypermethrin



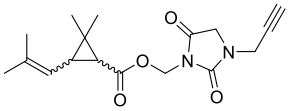
Esfenvalerate



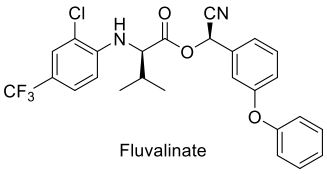
Bifenthrin



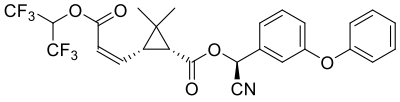
Cyfluthrin



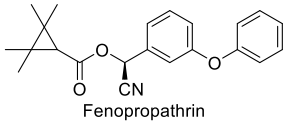
Imiprothrin



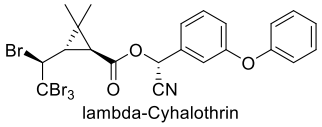
Fluvalinate



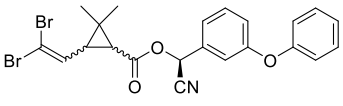
Acrinathrin



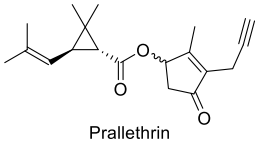
Fenopropathrin



lambda-Cyhalothrin



Deltamethrin



Prallethrin

Figure 1.4 Representative compounds from the pyrethroid class of insecticides.

Usage

Permethrin (Figure 1.4), registered in 1979, is the most widely used mosquitocidal agent to control outbreaks. It is employed for both residential and public indoor and outdoor insect sprays and foggers, flea treatments for pets, termite treatment, head lice and scabies treatments, and is used on agriculture and livestock. Phenothrin (Sumithrin®), registered in 1976, is likewise used for mosquito control (indoor and outdoor residential sprays), and for flea and tick eradication in pets.[38,39] Even though most pyrethroids are considered safe because they show low toxicity to mammals and birds, they can be extremely dangerous to fish and other insects.[38] 0.07 µg of Sumithrin, for example, has been found toxic enough to kill bees.[40] Some pyrethroids such as Remethrin, registered since 1967, are still used for mosquito control but only by public health officials as it is a "restricted use pesticide" due to its high toxicity to fish.[38]

Mechanism of Action and Resistance

The pyrethroids modify the gating-properties of sodium channels via delaying closures, causing drawn-out influx of Na^+. The organisms' central nervous system (CNS) discharges repetitively and nerve membranes depolarize while these lipophilic compounds distribute themselves throughout the liver, stomach, intestines, nervous systems, and kidneys. They generally have been thought to have low toxicity in mammals due to their poor absorption (by human skin) and quick metabolism in liver.[7,26]

Though pyrethroids are generally considered the safest class of insecticides used with mosquito-control, toxic human manifestations have been reported due to exposure. Facial paranesthesia, skin itching and burning, dizziness, nausea, and muscle fasciculations have been reported after acute exposure, and hemorrhage, pulmonary edema, seizures,

hyperglycemia, and coma upon greater exposure. Type I pyrethroids specifically have shown to contribute to reflex hyperexcitability and tremors, with type II specifically leading to choreoathetosis, salivation, seizures, and effects to skeletal and cardiac muscles.[26] Cypermethrin is suspected to be an endocrine-disrupting compound and decamethrin to have mutagenic and teratogenic effects.[41]

Environmental Impact

Since these synthetic pyrethroids are hydrophobic, they are notorious for high residue formation and time in the environment.[41] Bifenthrin, for example, has the longest soil-residual time of any other insecticide. It has the tendency to hold onto biosolids and organic matter, and frequently contaminates to runoff water. It is the most prevalent pyrethroid found in influent, effluent, and biosolids in Publicly Owned Treatment Works (POTWs) such as wastewater and sewage plants.[39,42] Bifenthrin is highly toxic to fish and is lethal to bees at 17 mg/L and at sublethal doses, it diminishes bees' fecundity.[43] The pyrethroids' hydrophobicity make them highly toxic by contact exposure and can persist in the environment yielded prolonged toxicity.[23] Doses at 0.02 µg have been reported fatal to bees, and application is recommended at night in liquid form, rather than dust, in order to avoid active pollinators.[44]

Resistance

Additionally, since they are used repeatedly for vector control and persist in the environment, resistance has risen. Resistance was first detected in 1993 in the Ivory Coast and has been developing at alarming rates since. However, there is little in the pipeline to safely replace them.[4] Resistance arises via point mutations at the *VGSC* gene (knockdown resistance, *kdr*) that alters both the binding affinity of the insecticide and the gating properties of the channel.[45] As a form of combatting resistance, new forms of pyrethroids have been created via combination with either OPs or piperonyl butoxide. The combination with OPs and

piperonyl butoxide increases insect sensitivity to the pyrethroid core by inhibiting detoxification via ester cleavage, but also increases toxicity to humans.[26]

1.2.2 Organophosphates

Organophosphorus compounds (OPs) are highly toxic, broad-spectrum insecticides used in agricultural and residential pest control. Use of OPs peaked in the 1970s after organochlorines (OCs) began phasing out due to their bioaccumulation and prolonged toxicity in the environment. In 2000, OPs accounted for 70% of all insecticides used in the USA, but high toxicity to humans and animals labels them as a societal and environmental concern and are being phased out as a result. OPs are still highly used for agricultural purposes and mosquito control, especially in developing countries due to their low costs.[46-48]

Chemistry

The organophosphates are phosphoric acid derivatives, composed of a pentavalent phosphorous containing either a thion (S=P) or an oxon (O=P) bond. The three other bonds are most commonly esters or thiols with alkyl or aromatic substituents. A key structural feature of the OPs is the presence of one leaving group, which is displaced upon phosphorylation of acetylcholinesterase, the biological target. (Figure 1.5)[47]

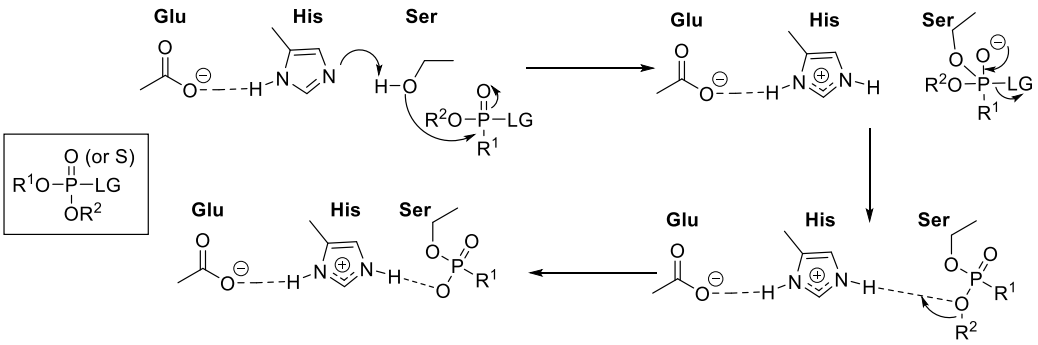


Figure 1.5 Phosphorylation mechanism by which acetylcholinesterase is inhibited by an organophosphate insecticide. The organophosphate first binds first to the serine and then binds

the histidine residue after transformation to a phosphate-containing molecule. This occupation of the esteratic site inhibits the acetylcholinesterase to be able to engage in cleavage activities.[49]

Oxons have very favorable insecticidal activity, but are very unstable.[46] As a result, most OP insecticides are thions, but end up requiring bioactivation via oxidative desulfuration, mediated by cytochrome P450 chromosomes (CYP 450).[47] CYP 450 activates transformation of thions to oxons, improving their ability to inhibit acetylcholinesterases (AChE).[50] Both oxons and thions undergo hydrolysis at the ester linkage, resulting in alkyl phosphates that are either further broken down and/or excreted by the organism.

The search for new OP analogs has been fueled not only by the desire of achieving effective insecticides, but also for the design of environmentally benign compounds. Various substituents on OPs fine tune their overall toxicities and physiochemical properties, resulting in analogs that are highly hydrophobic and hydrophilic. Most lipophilic groups applied to OPs yield very effective insecticides. Thiocyanate is commonly used as a leaving group on OPs and is present with aryl or alkyl amino groups. The hydrophobic nature of diazin, fenthion, and methyl parathion (Figure 1.6) for example, allow for significant lipid solubility and thus long-term toxicity.[46] Halogenated OPs are less prone to photolysis tallowing for bioaccumulation like the organochlorines (Section 1.2.4), so caution is needed with halogens.[51]

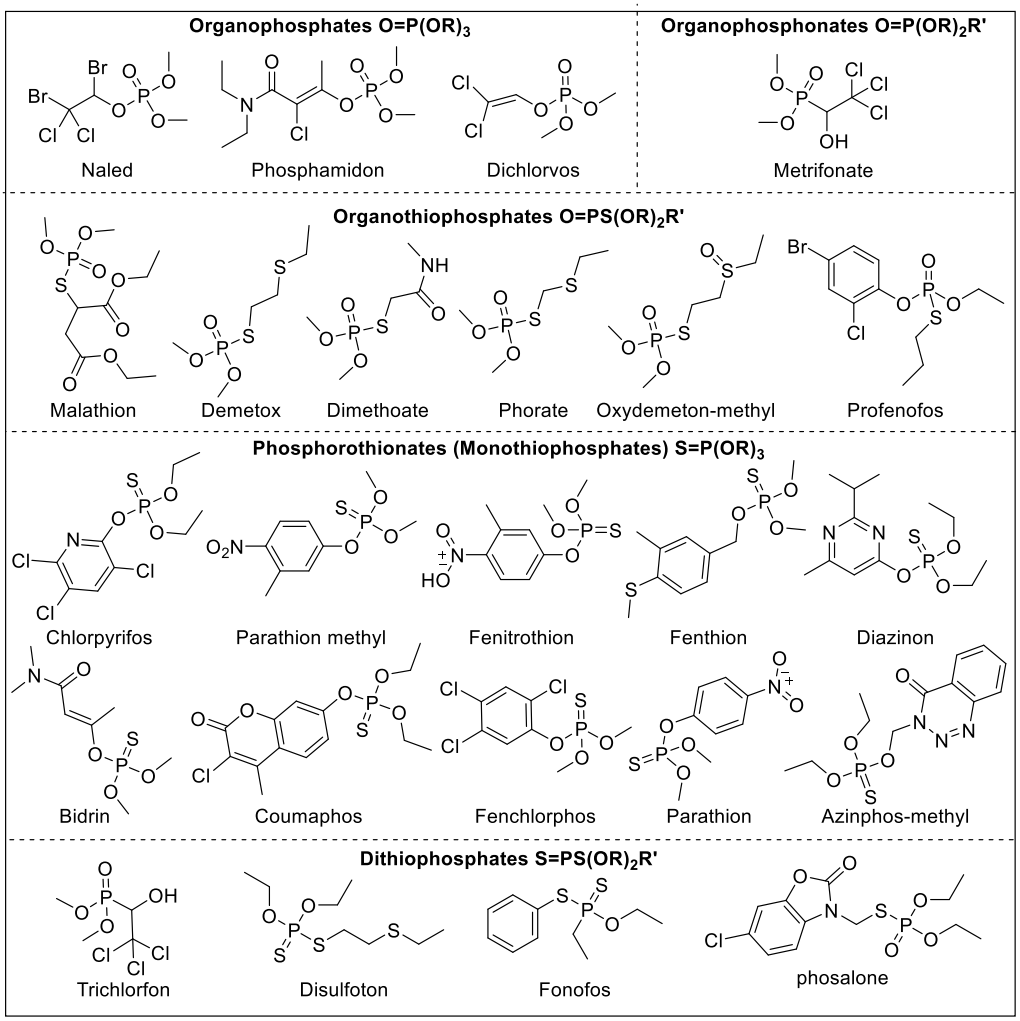


Figure 1.6 Representative compounds from the organophosphate class of insecticides.

The optimization of OP insecticides has been greatly enhanced via *in silico* methods such as Quantitative Structure Activity Relationship (QSAR), a computational method of determining physiochemical properties of compounds.[52,53] QSAR calculations have validated that lipophilic groups, commonly C_2H_5, and aryl groups with an electron withdrawing group (EWG) at the (meta) *m*- or (para) *p*- position are best for activity. One calculation indicated that a compound 4-(diethoxy phosphoryloxy) benzene sulfonic acid would be a very effective OP with an LD_{50} of -7.293.[54]

Usage

Used as insecticides since the 1930s, OPs are mainly used as agricultural agents but also used for mosquito control. In the US, Malathion and Naled (Figure 1.6) are the most utilized OPs for adult mosquito control. Malathion has been used since 1956 and is mostly applied as a ULV (ultra-low volume spray) via ground application (foggers on trucks). It is also used in agricultural crop protection, in private residences for garden protection, and it has been used in Cotton Boll Weevil Eradication and Fruit Fly (Medfly) control programs.[55,56] Likewise, Naled (Dibrom) is also a pan-insecticidal that is also used on food crops and in greenhouses. Used in the US since 1959, it's mostly applied aerially as a ULV at 1-2 tbspn/acres, and yearly about 16 million acres of mainland US are treated.[55,57] OPs were so common that in the early 2000s, Diazinion (Figure 1.6) was the most widely used OP ingredient in lawn and garden sprays: for almost 50 years, Diazinion sold approximately 13 million pounds in the USA.[58]

Mechanism of Action

OPs are neuromuscular transmission inhibitors that work via inactivating AChE by phosphorylating hydroxyl groups on the catalytic serine enzyme, similarly to how AChE would be acetylated. The resulting bond is highly stable, and depending on the groups off the phosphorous, are generally irreversible. Once the serine hydroxyl is blocked, it can no longer hydrolyze acetylcholine (ACh) to the inactive choline and acetic acid, resulting in ACh accumulation at the synaptic and neuromuscular junctions. OP compounds thus act by covalently modifying the serine residue causing AChE to terminate cholinergic transmission via rapid hydrolysis of AChE, resulting in excessive stimulation of nerve and muscle fibers which lead to exhaustion, tetany, and death of the insects.[59] Mutations to AChE1 have shown to decrease sensitivity to OP insecticides.[7]

OPs pose great human health threats, especially to those in developing countries and agricultural workers, and is a frequent cause of hospitalization. Binding to AChE at cholinergic

synapses in plasma and red blood cells, it results in asthma, confusion, convolutions, tremors, ataxia, muscle weakness, decreased respiration and circulation, respiratory distress, hypertension, tachycardia, and coma.[60,61] Farmworkers with prolonged exposure have reported deficits in respiratory health, visual memory, visuomotor skills, confusion, lower vibrotactile sensitivity, and OPIDN.[61] Family members of agricultural workers are also highly at risk. Children, especially under the age of 6, have shown the highest exposure to OPs due to oral exploration and increased proximity to surfaces from dust in homes tracked by household members or by pesticide drift. OP presence in breastmilk, and prenatal exposure has led to increased occurrences of brain tumors, leukemia, lymphoma, and various cancers in children. Unfortunately, children do suffer the most toxicity due to decrease activity in enzymes to detoxify active OP metabolites and have higher fatality rates than the adults suffering from OP-poisoning.[61]

The OPs' significant health effects have caused numerous countries to reevaluate their use. For example, a class of OPs known as “chlorpyrifos” have known adverse toxicity yet were still the top-selling organophosphate insecticide in the 2010s.[62] They are neurotoxic insecticides that cause developmental delays in children and have been in numerous proposals for suspension of use. In 1997, the US Center for Disease Control (CDC) let out a public health statement of chlorpyrifos causing “dizziness, fatigue, runny nose or eyes, salivation, nausea, intestinal discomfort, sweating and changes in heart rate,” while higher levels could result in “paralysis, seizures, loss of consciousness and death in humans.[63] Studies have shown that even prenatal exposure to chlorpyrifos leads to developmental defects in babies and children including poor neurological functions and a decrease in muscarinic receptors in fetal brains.[61] California, which accounts for 20% for the US’s approximate 5 million agricultural workers, issued a ban against chlorpyrifos, beginning in early 2020.[61,63]

Environmental Impact

The variety in structure of OPs results in various ecological consequences. Their nonpolar nature causes them to bioaccumulate and persist in the environment, deteriorating soil via acidification, nitrate leaching, causing loss of fertility and biodiversity.[64] OPs can be water soluble and rural and urban water sampling has indicated that non-chlorinated and chlorinated OPs respectively are commonly present, resulting in exposure and toxicity to non-target insects, fish, birds, reptiles, and mammals.[17,65]

Chlorpyrifos is one of the most notorious OPs. It is a cheap synthetic insecticide used since 1965 in the USA. It is very dangerous and recently more and more countries have been restricting and banning their use altogether.[66,67] Bees are a high risk for chlorpyrifos as they are frequently applied, are found in approximately 14% of pollen samples worldwide, and persist in honey and wax. Chlorpyrifos present 0.24 ng bee^{-1} oral toxicity and contact LD50 of 70 ng/bee.[23,68] Under laboratory conditions, 60% of bee larvae died within a week when exposed to chlorpyrifos as opposed to 15% with controls.[69] Their persistence is not only affecting bees, but also humans – 75% of Americans show bodily traces of chlorpyrifos from food as in 2014 alone, the US used between 3 – 5 million kg of chlorpyrifos.[67,70]

Resistance

Like the pyrethroids, repeated use of OPs has led to resistance by VGSC point mutations that alters its binding and gating potentials. Reduced or total loss of sensitivity to organophosphates has been reported with mutations at codon 1014 of the *VGSC* gene[71] and at G119S and F455W on AChE1 by the *ace-1* gene.[7] Cross resistance amongst different OPs, carbamates, and pyrethroids is felt after only a few (approximately six) generations.[72]

1.2.3 Carbamates

Carbamates are derivatives of carbamic acid (NH_2COOH) typically with small alkyl substitutions on the nitrogen and larger aryl or alkyl substituents on the oxygen which inhibit

neural acetylcholinesterase enzymes like the OPs. The first insecticidal carbamate was physostigmine (Figure 1.7), a metabolite from the plant *Physostigma venenosum*, which has been used against insects since the 19th century. The structure was fully elucidated in 1925, leading to its first synthetic derivatives in 1926.[73] The carbamates, as a class, are used for agricultural pest control, and function via oral, dermal, and respiratory exposure.

Chemistry

Carbamates are hydrophobic insecticides with the core structure $R^1R2NCOOR^3$, with the carbamate ester being essential for activity. The R^1 and R^2 functional groups affect binding to AChE which in turn affects insecticidal activity and selectivity, while the R3 group is commonly an unsaturated polycyclic or an oxime group.[74] R^1 and R^2 are typically short chained alkyl groups, between 1 and 3 carbons long, as any longer tends to decrease anticholinesterase activity. Optimizing substituents for high lipophilicity facilitates their crossing of insect cuticles.[75,76]

Phenyl carbamates, such as Promecarb or Bufencarb (Figure 1.7), commonly have alkyl groups at the *meta* position which yield better AChE inhibition. Additionally, sterically bulkier substituents on the phenyl rings, like branched chains or rigid rings like dioxolane and benzofuran, block access to AChE catalytic sites, decreasing reactivity and selectivity of the molecules.[73-75] Electronically, electron donating groups (EDGs) on the phenyl carbamates increases activity. Adding additional aromatic rings facilitate the insects' ability to detoxify itself of these compounds.[73]

Halogenation with chlorine or bromine at the *ortho* or *meta* position enhances insecticidal activity. This is thought to be due to restricted rotation of the carbamoyl core by the bulky substituent, yielding to a better fit within AChE. When halogens are present at the *para* position though, little difference is seen from the *p*-methyl substitution. Unfortunately, halogen

substituents have been shown to contribute hydrolytic instability of the carbamate, making them ineffective larvicidal agents. The best insecticidal activity is seen with uncharged, lipophilic materials, which most easily penetrate the cuticle.[73]

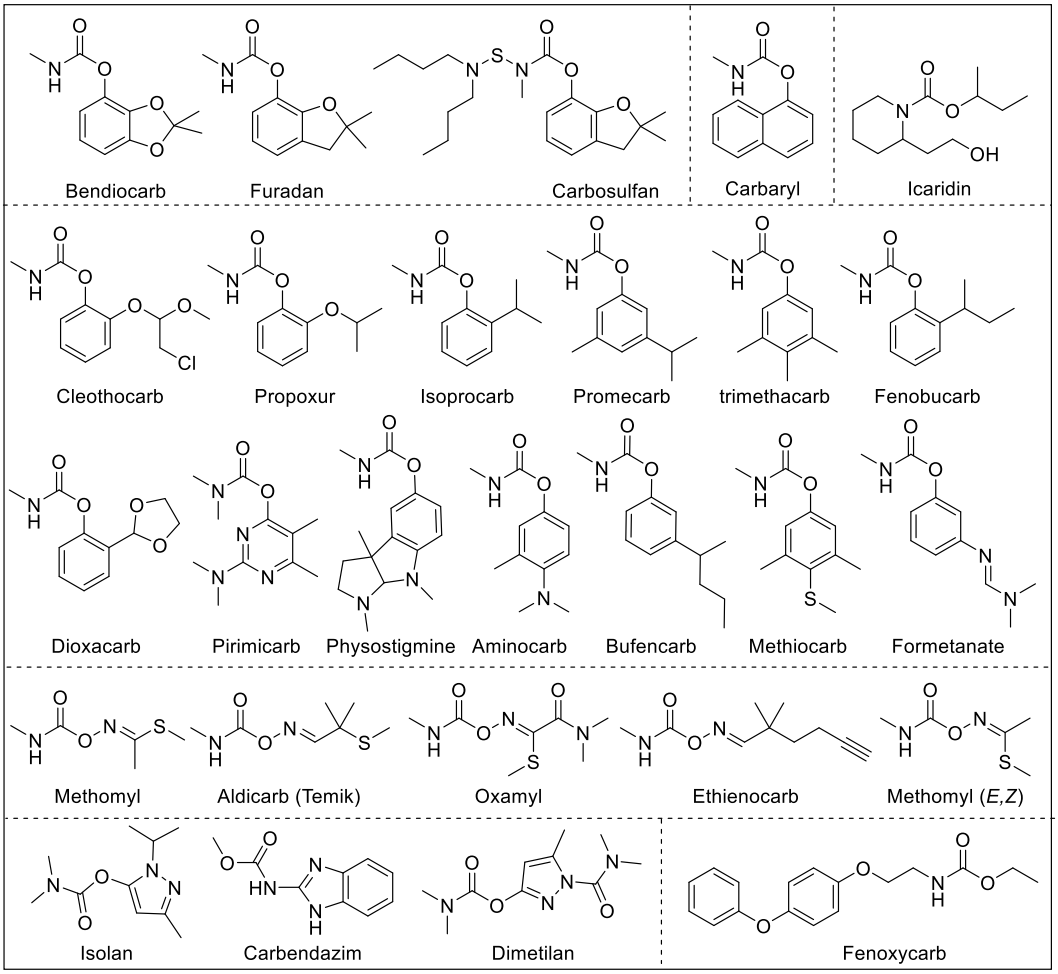


Figure 1.7 Representative compounds from the carbamate class of insecticides.

Usage

Carbamates were widely popular from the 1950s – 1980s and used as soil-applied insecticides (and fungicides). They exhibit broad-spectrum activity against various insects, mites, and nematodes making them valuable chemicals in protecting large commodity crops

(fruit trees, vegetables, cotton, row crops, etc.). Generally, carbamates like aldicarb are applied below the soil surface where they are rapidly absorbed systemically by roots.[77]

Mechanism of Action

Carbamates activate AChE via a covalent reaction on serine hydroxyl groups in the active site. Unlike the OPs, the carbamates interaction with the serine hydroxyl group is reversible.[59]

Carbamates are absorbed via respiratory, oral, and/or contact exposure, the latter being the least-toxic route.[78] Overall, carbamate poisoning is very similar to OP poisoning, with ill-effects to the central nervous, cardiovascular, endocrine, and reproductive systems.[74] Human symptoms of poisoning include malaise, headache, diarrhea, incoordination, and muscle twitching amongst others. The most severe manifestations of carbamate poisoning occur in the respiratory and CNS including coma, hypertension, seizures, and cardiorespiratory or respiratory depression, with children more likely to experience symptoms of the CNS.[78]

Environmental Impact

Carbamates easily degrade in the environment within weeks or months. Their few oxidative metabolites, sulfones and sulfoxides, have been found in various water sources as some are water soluble while others adhere to soil sediment in aqueous banks. This presence in ground and well water sources has caused concerned over human toxicity since carbamates can exhibit high toxicity to mammals and other vertebrates.[76,77] Carbamates show higher toxicity to mammals than OPs as they inactivate AchE without the need of metabolic activation first.[59] Carbamates are rapidly excreted from human systems and have shown to possess no carcinogenic effects or long-term health effects, though acute toxicity has been documented in lab animals - rats, rabbits, and Guinea pigs.[77]

Carbaryl has been found in honey hives at concentrations as high as 1.4 ppm which is threatening for honeybee longevity.[79] Like toxic neonicotinoids (see below), carbaryl has shown to diminish honeybee immunity and antioxidant mechanisms, when exposed to at sublethal doses. Overall, exposed bees have shorter lifespans due to changes in genome and their gut microbiota, which can leave bees susceptible to disease and attack by pathogenic microorganisms.[79,80]

1.2.4 Organochlorines

Organochlorines (OC) are broad-use pesticides functioning as insecticides, fungicides, herbicides, and rodenticides. They were greatly used in the 1940s – 1970s until their ban in the USA and Europe. Dichlorodiphenyltrichloroethane (DDT), is the most notorious organochlorine, used to control malaria and typhus amongst troops and in residential homes during WWII.[81] The use of this deadly adulticide lead to the eradication of malaria in the United States in 1949.[82]

Chemistry

OCs are synthetic, aliphatic, and aromatic rings that have at least one covalently bonded chlorine. Critical for their insecticidal activity are 2 electronegative/polar centers, one being a chlorine and the other either an olefin or an epoxide. The removal of chlorines from OCs most often significantly increases their toxicity.[83] With the DDT-like compounds, substitution at the *para* position is critical for activity; any change in substitution to the aromatic rings with greatly diminish activity. These substitutions are all alkyls, alkyloxys, alkylthios, or halogens, as any other group would be too polar and affect the compounds' ability to penetrate insect nerve sheaths. The structures of the chlorinated alicyclic OCs differ much more than DDT-like compounds and their requirements for an effective insecticidal aren't as well-defined.

These compounds vary greatly in both position and number of chloro-substituents. Overall, compounds with epoxides and cyclopentadiene rings have greater bioactivity.[84]

The goal with varying the structures of OCs was to create compounds that had effective insecticidal toxicity but would readily degrade, which proved difficult. With DDT, for example, replacing a hydrogen with a hydroxyl group on dicofol's central carbon (Figure 1.8), makes it more environmentally-friendly by making it more water-soluble, but decreases its stability.[83] For these compounds to remain biologically active, they characteristically tend to stay as bulky, stable molecules, with poor water solubility and low soil degradability.

The orientation of a compound's structural motifs can also elicit differences in biological properties. Structurally similar Dieldrin and Endrin (Figure 1.8) differ in the orientation of the methano bridge and chlorinated bridges, with Endrin having a decreased toxicity as a result. When the methano and chlorinated bridges are syn to each other, as is the case with Endrin and Isodrin (Figure 1.8), the toxicity decreases due to blockage of interaction with target proteins.

OCs are lipophilic compounds, which has its pros and cons. Dieldrin's high lipophilicity is what makes it an effective insecticide, allowing easy absorption into insect cuticles. Their high lipophilicity, though, leads to persistence in the environment and for its ease of CNS absorption, which leads to OC poisoning in humans.[83] *Para*-position substitutions of the DDT-like derivatives also prevents easy detoxification of these compounds.[84]

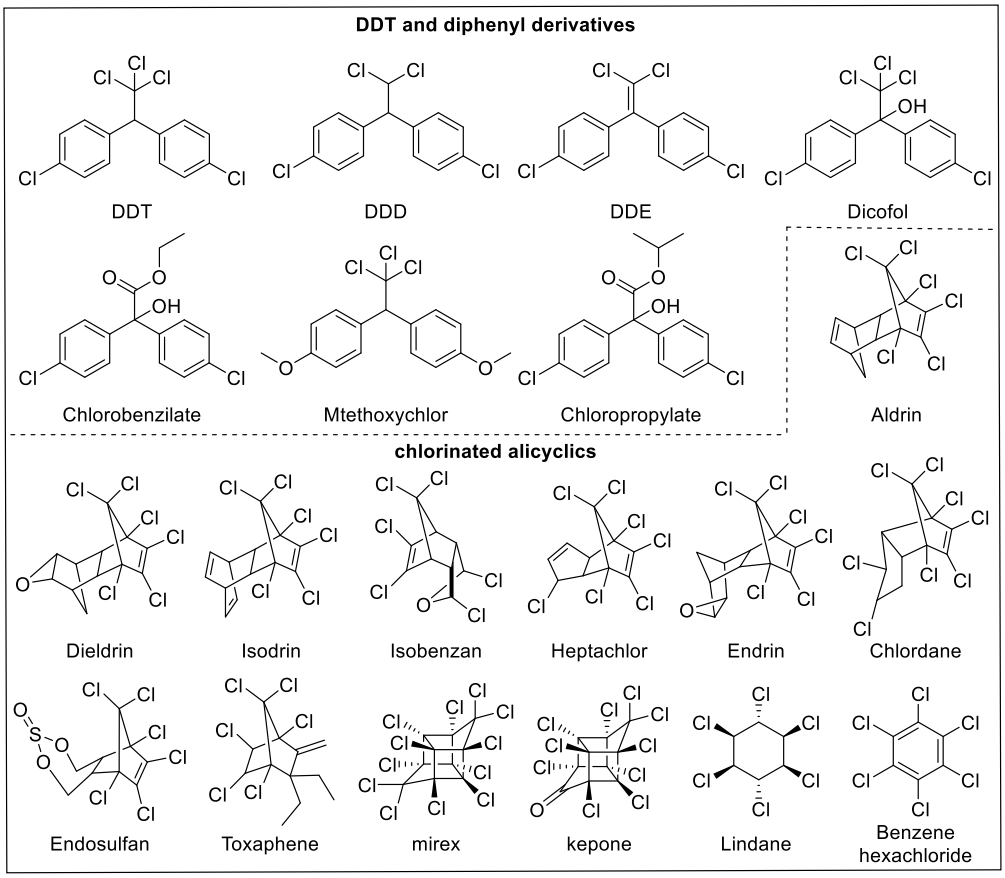


Figure 1.8 Representative compounds from the organochlorine class of insecticides.

Usage

Many OCs were heavily used for agricultural pests and mosquito control, without knowing the disastrous ecological effects they were to elicit, leading to eventual ban of the insecticidal class. Dieldrin (Figure 1.8), for example, used until its ban in 1970, is listed as one of the 12 most dangerous pesticides due to its environmental persistence and toxicity.[85]

Conservationist Rachel Carson's 1962 novel *Silent Spring* addressed the issues in lack of regulation, discriminated insecticide use, and illuminated the ecological concerns of DDT use and its threat to human and wildlife health. This helped lead to its ban in the United States in 1972.[86] Use of DDT was later prohibited under the Stockholm Convention on Persistent

Organic Pollutants (2004) but is still allowed by the UN for malaria control in Africa and for disease outbreak as a last-case scenario. Other OCs banned in 2004 include Aldrin, Chlordane, Chlordecone, heptachlor, and mirex due to their bioaccumulation and toxicity.[15]

Unfortunately, other countries have yet to ban DDT[87] and its use globally has not changed significantly. For example, India continues to heavily rely on DDT-use and from 2000 – 2009 accounted for 82% of global-DDT use. After India, the heaviest use of DDT is in Africa, who in 2008 began incorporating its use to indoor residual spraying (IRS) to combat malaria.[88] Overall, developing countries continue to use OCs due to low cost; in Asia alone, the among the most used insecticides are aldrin, dieldrin, and DDT.[17]

Mechanism of Action

OCs bind to GABA receptors, inhibiting the closure of ion channels, causing hyperexcitation of the CNS and poisoning like OPs and carbamates. The ion channel influenced with OC binding is determined by the structural features of the molecule. Their poisoning can result in neurological, endocrine, mutagenic, CNS, or peripheral disorders.[15]

The DDT-like compounds prevent closure of Na^+ channels after membrane depolarization. The influx of Na^+ ions cause destabilizing negative potentials, leading to hyperexcitation and repetitive neuronal discharges. This primarily affects the peripheral nervous system. Whereas the chlorinated alicyclics bind at the picrotoxinin site on the GABA Cl^- ion receptor. This leads to an influx of Cl^- ions, resulting in hyperexcitation as well but with GABA-ergic inhibitory neurons impaired.[84]

The lipophilic nature or organochlorines causes them to bioaccumulate, and so even though OCs were banned or are at “reduced” use in some areas, there effects are still felt. Exposure to OCs disrupt the endocrine channels and functions, increasing risk to breast, lung, stomach, and prostate cancer. Additionally, issues of high blood pressure, disruption of thyroid

hormones, and type II diabetes can present themselves. For women with prenatal exposure to OCs, children have been born with decreased birthweights, and have had predispositions to obesity and ADHD.[17] Intake of OCs are common for people living in regions where OCs are used through inhalation, direct contact, and consumption of contaminated water. Additionally, OCs can accumulate in fat-rich foods including meats and milk.

Environmental Impact

As mentioned above, OCs are highly volatile, lipophilic, and lack ease of biodegradation, allowing them to easily bioaccumulation thru their adherence to soil, water, and air. Their potential for long range transport and long half-lives, greater than 2 months in water and greater than 6 months in soil, has led to their classification as persistent organic pollutants (POPs). For example, Dieldrn has a half of life of 25 years, and DDT 2-15 years in the natural environment.[17]

In the mid-20th century, uptake of DDT by earthworms caused widespread reproductive failure of many bird populations feeding off them. Bird embryos experienced skeletal abnormalities, impaired reproductive and nervous systems, wasting syndrome, and reduced hatchability. Adult avium experienced reduced fertility, impaired reproductive behavior and egg thinning, and acute mortality. Ill effects are also noticed in amphibians and farm animals; stress to honeybees and contamination of honey, for example, is noted in areas where OCs are still employed.[17]

1.2.5 Pyrroles

Pyrroles are 5-membered, heterocyclic rings with the core structure C_4H_4NH. They are a new class of insecticides, “pro-insecticides,” developed to combat the rising resistance to pyrethroids. Currently, chlorfenapyr (Figure 1.9) is the only commercially available pyrrole and

is mainly used with agricultural pests and termites. Ongoing work is trying to incorporate it into Long lasting Insecticidal Nets (LLINs) and for IRS.

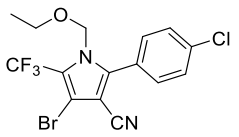


Figure 1.9 Structure of chlorfenapyr, the only commercially available pyrrole insecticide.

Being pro-insecticides, pyyroles are dependent on activation by other chemicals or oxidases to elicit biological activities.[89] Oxidative cleavage of N-ethoxymethyl on chlorfenapyr by Cytochrome P450 results in the toxic metabolite known as “CL 303268,” which uncouples oxidative phosphorylation in mitochondria, disrupting respiratory pathways and proton gradients. This inhibits insects’ mitochondria ability to convert ADP to ATP, leading to cellular death.[89-91] This novel and unique MoA within insecticides shows no cross resistance with Pyrethroids, OPs, and carbamates.[92] As a result, *An. gambiae*, *An. funestus*, and *C. quinquefasciatus* have shown no cross resistance to chlorfenapyr.[90]

Chlofenapyr elicits activity through oral and contact exposure and is considered a low toxicity insecticide when compared to ivermectin, a microbial derived insecticide used against mites and arthropods, and fipronil (Figure 1.10).[93] A limitation of chlorfenapyr is that it is a broad-spectrum insecticide. Since the 1990s, it has been used as an agricultural pesticide for treatment against mites, spiders, worms, flies, etc.[92] So even though it is effective towards mosquito species, it’s not the solution for mass mosquito vector control as it still is harmful towards beneficial insects.

1.2.6 Phenylpyrazoles

Phenylpyrazoles are broad-spectrum insecticides composed of a central pyrazole ring with decorated phenyl groups attached to N1 of the pyrazole core. Fipronil, the first and most

utilized phenylpyrazole, was discovered in 1987 by Rhode-Poulene Agro. It was developed for agricultural and residential pest use against sucking insects like fleas and ticks.[94]

Chemistry

Changes to the substituents on the phenyl rings alters the lipophilicity and electronic properties of the molecules. Changing the electron withdrawing group (EWG) CF_3 on fipronil to EDG C_2H_5, resulting in ethiprole (Figure 1.10), for example, decreases the lipophilicity of ethiprole, decreasing its rate of absorption through insect cuticles and resulting in a compound that is more stable in the environment.[95]

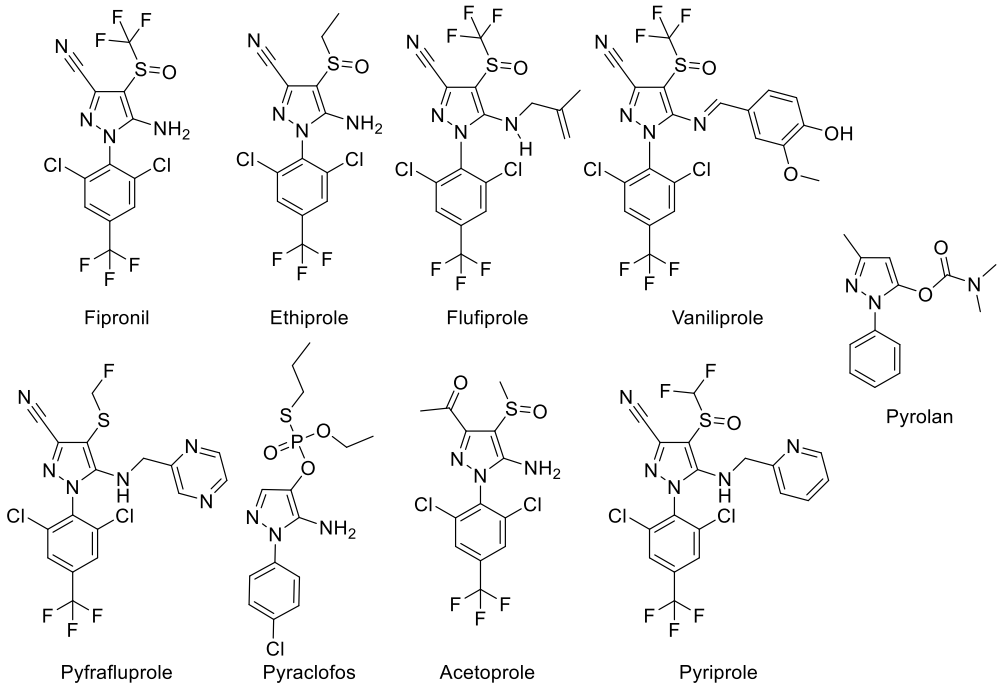


Figure 1.10 Representative compounds from the phenylpyrazole class of insecticides.

Mechanism of Action

Phenylpyrazoles, target GABA receptors, causing hyperexcitation of insect CNS and eventual death. The MoA is unique in that they block glutamine-activated, GABA chlorine

channels. These channels are lacking in mammalian systems, which is thought to be why they exhibit low mammalian toxicity. They are also thought to cause cellular death by targeting mitochondria and decreasing ATP levels.[94,96] When compared to dieldrin in roaches, there was little evidence of cross-resistance.[97]

Usage

Phenylpyrazoles were primarily developed and used for agricultural pests on corns and grains and for residential control of ticks and fleas, but their broad-spectrum activity made them appealing to use for vector control. In controlled studies, fipronil was active towards various species of mosquitoes, as efficiently as the best IGRs.[94]

Environmental Toxicity

Fipronil is considered a highly toxic insecticide. Termite populations experience severe and long-lasting population effects when treated with fipronil, which calls into question its risk to beneficial insects.[93,98] Contact exposure to honeybees is highly toxic (LD_{50} 0.007 ug/bee) even at low residual loads (1.6–29 ppb).[23] Similarly high (relative) toxicities are seen for lizards and birds, and there is evidence of carcinogenic action in rats at 300 ppm.[98]

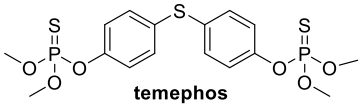
These compounds decompose slowly in water and soil, mainly via photolytic cleavage of the pyrazole. The main metabolites (sulfides, sulfoxides, sulfones, and desulfinyls) are in themselves highly toxic. This is problematic as they are generally immobile in soil and thus readily bioaccumulate. There is evidence of high concentrations of fipronil and metabolites in food products, especially in fish.[95,98]

1.2.7 Larvicidal Agents

As only adult mosquitoes carry disease-inducing parasites, larvicides are often developed and used to attack breeding sites before populations can reach adulthood and pose

a threat to communities. They are administered directly in aquatic breeding sites mainly in the USA, EU, and other countries economically able to do so. They are favorable due to their ease of administration and are generally associated with low risk to other species. Mosquito larvae are mainly targeted with OPs and IGRs. Several reports argue that vector control should focus on controlling larvae as it is more effective than controlling free-flying adults due to limited habitat and weakness of freshly laid eggs.[99]

OP larvicides work through the CNS just like adulticides but are distributed in mosquito breeding sites to cut them off at the source. Temephos (Figure 1.11) is one such OP, which has been the most utilized control strategy for dengue virus since 1965 and is applied in aqueous mosquito, midge, and black fly breeding sites at 0.1–0.5 kg/ha. The ease of application and selectivity towards mosquito larvae make it an ideal control strategy, but environmental factors such as sunlight, temperature, water type, etc. all control its effectiveness.[100] As a means of reducing breeding and larval development site, granules of temephos are applied to urban and domestic water sources to control breeding sites. [101] IGRs, like temephos, are usually analogs of juvenile hormones (JH). These chemicals that do not kill insects upon contact but rather inhibit maturity into adulthood by interfering with the molting process.[102]



temephos

Figure 1.11 Temephos is an organophosphate insect growth regulator that is used to control dengue at mosquito breeding sites.

Overall, larvicides are an effective, localized way to curtail the spread of mosquitoes and the diseases they disperse without running the risk of developing resistance. The problems are the needs of reformulation for specificity and their high costs pose challenges to the

countries that could use them most. Additionally, we cannot rely on larvicides alone and still need adulticides to deal with times of outbreak.

1.2.8 Other Common Insecticides: Neonicotinoids

The most widely used class of insecticides overall are a family of synthetic neuroactive compounds introduced in the 1990s, the neonicotinoids.[103-105] They include acetamiprid, Dinotefuran, Clothianidin, Thiamethoxam, Sulfoxaflor, and Imidacloprid (but). They are primarily applied as seed coating for use in agriculture and integrate into the plant tissue and, ideally, would only affect pests that feeding on those plants. Neonicotinoids have not been necessarily employed in widescale mosquito-control efforts.

They are derivatives of 3-pyridylmethyl amine and bind with the nicotinic acetylcholine receptor (nAChR) in insects. Interestingly, they have low hydrophobicity, which is sets them apart from a lot of other insecticides. With structure activity relationship (SAR), introduction of chlorine, which is common in a lot of insecticides, increased the hydrophobicity of analogs and their translocation into CNS, increasing insecticidal activity. It is also thought that a full or partial positive charge on the 3-pyridylmethylamine nitrogen atom helps with selectivity of insecticidal nAChR over vertebrates.[106]

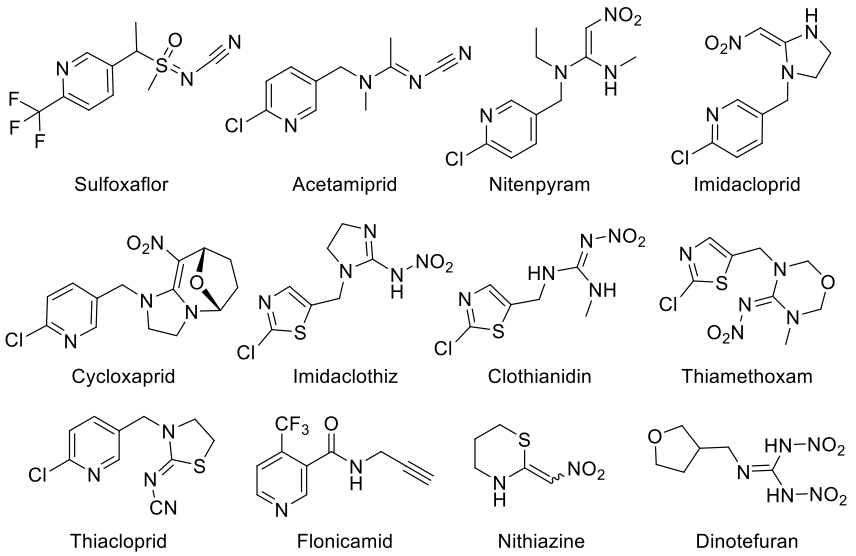


Figure 1.12 Representative compounds from the neonicotinoid class of insecticides.

Development of neonicotinoids for mosquito control has been considered, as they are known to be effective to all insects, show low mammalian toxicity, and show little cross resistance with pyrethroids, OPs, and carbamates.[107] Hence, they have been tested and some reports show promising efficacy towards mosquitoes[108-111] while other reports show lesser toxicity compared to the currently used insecticides.[107,112] As these compounds show promise, there is interest in optimizing their potential for mosquito control, but their off target effects to pollinators make them ill-suited for widescale vector control.

Neonicotinoids are known to be lethal to honeybees, (topical LD_{50} values 0.02 – 0.09 µg/bee).[23] They are known to cause including colony collapse disorder (CCD) with honeybee hives, and other species have experienced defects to their cognitive, motor, immune, and survival functions with sub-lethal exposures.[80,104,113] Imidacloprid and lamba-cyhalothrin are known to decrease production of queens and worker bees, increase the mortality of worker bees in the nest, and cause less forager bees to return to the nest.[114] Their severe physiological affects to bees caused several European countries, in 2013, to restrict the use of clothianidin, thiamethoxam, and imidacloprid to further avoid hive damage, and were completely banned for

outdoor use in 2018 by the EU.[104,115-117] Their harmful side effects not only exclude them as a potential source for aerial mosquito-spray but also for personal protection instances like for IRS and bed net use. So even though there is consideration over neonicotinoid-use for mosquito control, they are broad-spectrum and pose too many off target effects to ethically consider for use in dealing with mosquitoes.

1.2.9 Briefly, Natural Compounds for Mosquito Vector Control

Natural compounds have been increasingly sought after for developing insecticides. Plants especially have classically been sources of folk insecticides[118] and are thought to be environmentally and toxicologically safer than synthetic molecules. As current synthetic molecules are associated with development of resistance and off-target, ecological consequences, there is a renewed fervor to discover plant compounds with mosquitocidal activity.[119]

For centuries, the vast biological diversity of plants has led to the exploitation of their secondary metabolite natural products (NPs) and essential oils (EOs) to be used as active ingredients for vector control.[120] EOs are the volatile, aromatic substances present in the flower, leaves, stems, and roots, etc. of plants. Typically, about 20-60 lipophilic compounds, with two or three major compounds accounting for 20–70% of the total, comprise an EO.[121] Even though botanical EOs have already been heavily employed, these compounds are continuously explored for their insecticidal properties.

There are also the secondary metabolites produced by plants, which offer more complexity in structure, and are found distributed amongst the EOs. The most well-known inseciticdal NPs include the aforementioned pyrethrins, Rotenone, isolated from *Lonchocarpus nicou* and *Derris elliptica,*[122] and Ryanodine, from *Ryania speciosa* (Figure 1.13), amongst many others.[123] They are widely used against agricultural and residential pests including beetles, worms, lice, mites, as well as against mosquitoes.[124]

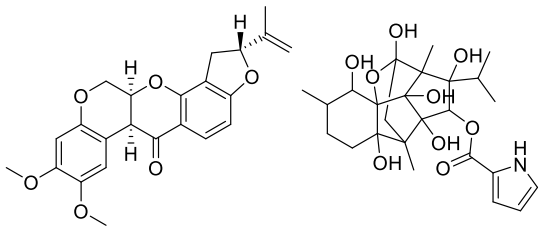


Figure 1.13 Plant natural products Rotenone and Ryanodine which exhibit broad-spectrum insecticidal activity.

Compounds from natural sources though can elicit toxic off target effects just like synthetic ones.[125,126] Exposure to Rotenone is known to affect mitochondrial respiration in animals[127] and lead to development of Parkinson's disease.[128,129] Ryanodine blocks calcium channels, leading to CNS issues, and can cause hormonal disruption, and miotic poisoning.[127,130]

Overall, the biggest problem seen in reports on natural compounds continues to be their broadness of scope. Most natural compounds are tested for activity over a wide variety of organisms just like synthetic compounds, to serve as residential and agricultural pest control and not just for mosquito control. Broad-spectrum insecticides are commercially favorable for their efficiency in wiping out various pests, so very few studies cite a control organism to ensure selectivity to only mosquitoes. However, it is ill-advised to continue producing broad-spectrum insecticides based on the issues in resistance and adverse effects to off-target organisms.

Diversity in natural sources will lead to various compounds produced, with a wide range in toxicities. Thus, the assumption cannot be made that just because something is natural that it is safe for use as it depends on the context in which it is employed. A compound can be mammalian safe but should not be developed as an insecticide if it is harmful to pollinator species, for example. No matter the source, it is crucial for compounds of interest to be evaluated with an empirically-based risk assessment, instead of assuming elicited effects.[131] Thorough experimentation should be done against various controls early in the screening

processes, such as Swale *et al*. who not only surveyed 5 different mosquito species for activity, but also 6 different insect control species to ensure selectivity.[132]

The challenge lies in the lack of selective screening approaches. Current methods of screening for active insecticidal agents involve application of candidate compounds on the thoraxes of insect bodies, through feeding solutions, or applying solutions to where eggs and larvae will rest. After a few days, death in the animals is quantified to qualify the compounds, which is tedious and laborious. Additionally, controls for environmental safety early in the discovery pipeline need to be prioritized, but these traditional animal screens limit testing scope and capacity. If there was a way to optimize testing in lieu of application to individual animals, the number of testable compounds and controls would greatly increase, potentially helping find more optimal candidates.

1.3 HTS for Screening Insecticidal Compounds

One way of ensuring selectivity towards mosquitoes is applying the use of high throughput screening (HTS) platforms that can test compound libraries for potency against various cell lines. HTS are automated systems that can rapidly identify active compounds, genes, or antibodies, etc. against specific biological pathways. They are heavily relied on in drug discovery and their use has led to rapid, mass data generation since their integration.[133] The ability to create or tailor HTS with insect cell lines would help economize insecticidal screens as thousands of compounds could be screened in a matter of days against both target and control cell lines. Having methods of HTS would ease the amount of time, labor, and material needed for testing compound libraries.

1.3.1 Mosquito and Other Insect Cell Line Assays

There have been many cell-based, phenotypic platforms established that are highly appealing for ensuring target-specific effects. Since the 1960s, cell lines have been established for over 10 different species of disease-carrying mosquitoes that could be incorporated into assays for screening compound libraries.[134] Ideally a screening strategy would screen

compounds against mosquito cell lines, and those compounds which show the desirable potency would be tested against other insect lines to qualify selectivity.

One such cell-based platform was created to rapidly screen compounds for toxicity against various mosquito and beneficial-insect cell-lines.[135] The platform tests compounds against cultured *Anopheles gambiae* (cell line 4A3A) and control lines of *Drosophila melanogaster* (S2R+ and Kc), *Spodoptera frugiperda* (SF9), and 30 human cell lines to confirm mosquito selectivity and safety to non-target organisms. Selective compounds are then tested against additional *Anopheles gambiae* cell line (MRA-921), *Anopheles stephensi* (MRA-858), and *Aedes aegypti* (Aag2) cell lines.[135] All compounds that successfully passed through all 3 cell screens are deemed potential mosquitocidal agents and in larval and adult mosquitoes to confirm that activity translates *in vivo*.

HTS composed of various cells lines offer versatility. They can be created using not only mosquito cell lines, but also any other insects who have cultured lines established. As over 1000 insect cell lines of various species have been developed,[136,137] HTS could be developed to test for off-target effects by mosquitocidal-compounds towards relatively any insect of interest. Additionally, cell lines created from various tissue types can be incorporated to create a complete picture of how compounds will behave mechanistically in a system.

Most insect cultures are derived from *Diptera* and *Lepidoptera* species, with few from *Hymenoptera* species. As the honeybee is one of the most important pollinators, designing insecticides with low to no potency towards *Apis mellifera*, is crucial moving forward. Facile testing of compounds against bees is necessary so having cell lines in a HTS would be greatly beneficial. While the culturing of bee cell-lines has proved challenging, there have been a handful of successful cultures established. Cell lines from *A. mellifera* have been successfully cultured from their antennae flagella and lobes, pupal brains, mushroom body, motor and projection neurons, Kenyon cells, embryo, gut, midgut, and eggs.[136,138,139] This wide range of

honeybee and other insect cell lines gives a variety of possibilities to test against when screening compounds to prioritize safe insecticide design.

1.4 Discussion

Mosquitoes are the deadliest animal in the world, affecting hundreds of millions per year by spreading of viral infections including Zika, malaria, yellow fever, West Nile virus, dengue, rift valley fever, chikungunya, and encephalitis. As current drug treatments for these diseases are either losing efficacy or are non-existent, there is a heavy reliance worldwide on chemical insecticides to control mosquito populations to curtail the spread of these diseases.

As previously discussed, the 6 classes of chemical insecticides used to deal with mosquito outbreaks are nonselective as, even though their MoA varies slightly amongst the classes, their target receptors are conserved among organisms. As a result, non-target insects, fish and other aquatic species, birds, and mammals including humans experience their toxic effects. In 2009 it was reported that over 5.5 billion pounds of insecticides were being sprayed worldwide each year, but less than 20% actually interact with their target;[140] the rest bioaccumulates, seeping into soil, are absorbed by plants, and leeched into water ways. This results in toxic effects felt long after initial spraying. Additionally, these insecticides are used against agricultural pests, so there is an increasing resistance amongst mosquito species to these chemicals. With all this, there is a global, dire need of new chemical control agents that can be used specifically against mosquitoes without ecological consequences.

Ensuring mosquito selectivity is one of the best ways to provide limited or no off-target effects in the ecosystem. Unfortunately, it's costly time- and money-wise to screen various controls with animal-based assays. The time constraint with adult or larval assays can limit the scope of compounds tested. Insect cell based HTS are time and labor efficient and can be designed to be biased for compounds with novel MoA when created with cultures derived from nonneuronal tissue(s). Though cellular toxicity does not necessarily indicate *in vivo* activity, it is easier to survey large fragment or compound libraries initially and then check for translation

with the animal. HTS can additionally be designed with cell lines from pollinator insects, such as bees and butterflies, to ensure compounds selected for mosquitocidal development are environmentally benign.

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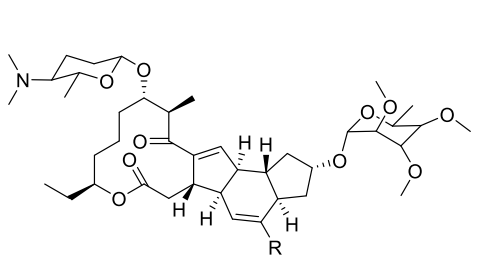
CHAPTER TWO

IDENTIFICATION OF A BORON-CONTAINING NATURAL PRODUCT WITH MOSQUITOCIDAL ACTIVITY

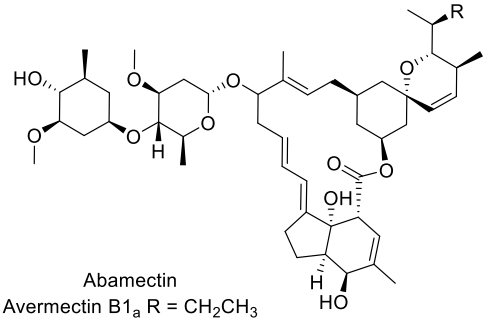
2.1 Microbial Natural Products as Insecticidal Leads

As previously mentioned, insecticides utilizing synthetic molecules as active ingredients are associated with harmful, ecological effects. Resulting in a renewed fervor for discovery of mosquitocidal agents from natural sources.[1] NPs are compounds produced by secondary metabolism in organisms including plants, fungi, algae, and bacteria for protection in their environments. They are diverse classes of molecules made up of alkaloids, polyketides, sterols, unsaturated fatty acids, etc. that have vast biological applications. They are commonly used as active ingredients in pharmaceuticals, additives in food industry, or in agriculture including as active ingredients in fertilizers or pesticides.

Little work though has been to identify insecticidal leads from microbial sources. The spinosyns are one example (Figure 2.1). They are glycosylated polyketides produced by *Saccharopolyspora spinosa* that are used as a broad spectrum pesticide against species of *Lepidoptera*, *Coleoptera*, *Diptera, Thysanoptera*, *Hymenoptera*, and *Orthoptera* since 1997.[2] Abamectin, is an acaricide (miticide) and nematicide from *Streptomyces avermitilis* (Figure 2.1) which is likewise active as a mixture of two naturally occurring compounds avermectin $B1_a$ (~80%) and avermectin $B1_b$ (~20%). It elicits activity via blockage of GABA receptors and is classified as moderately hazardous by the WHO.[3]



Spinosad
Spinosyn A: R = H
Spinosyn D: R = CH_3



Abamectin
Avermectin $B1_a$ R = CH_2CH_3
Avermectin $B1_b$ R = CH_3

Figure 2.1 Microbial natural products Spinosyn A and Spinosyn D, which make up “Spinosad,” and Avermectin $B1_a$ and Avermectin $B1_b$, which make up “Abamectin,” exhibit broad-spectrum insecticidal activity.

The commercial product Spinosad, the combination of both Spinosyn A and D, while readily degradable,[4] is a broad-spectrum pesticide that is very harmful to off-target organisms, including pollinators, through hyperexcitation of the CNS.[5,6] Birds and mammals experience neurotoxic symptoms or acute mortality when exposed to 1-30 mg/kg of abamectin, and CNS symptoms with chronic exposure at 2 mg/kg per day. Overall though, when used as dilute solutions, it poses little threat to mammals, and it degrades readily, so it does not bioaccumulate[7]

Additionally, there have been reports of *Pseudomonas* and *Bacillus* species exhibiting toxicity towards mosquito larvae.[8-12] While there are some reports of interesting compounds, the microbial world still remains a vastly untapped realm of biodiversity[13] that has not been profiled to the extension that plant NPs have been. As these NPs offer diverse and complex structural classes, there is the potential for toxicities elicited through novel MoAs. There is potential for safe mosquitocidal agents if they are tailored to ensure selectivity, instead of as broad-spectrum insecticides. If a compound from a natural source could be identified as mosquito-specific, or synthetically modified to enhance selectivity, it would be a safer and more effective control agent.

Since microbes have not been extensively sourced for pesticides, we aimed to screen our NP fraction library to identify lead compounds capable of selective mosquito toxicity that would help meet the challenges of insuring human safety, limiting off-target effects in the ecosystem, and confronting resistance in insecticide development.

2.2 High-throughput Screen of Natural Products Library

As issues of non-selectivity amongst target and beneficial insects are a main concern, a screening platform was needed that would identify compounds that discern between the two. We sought phenotype-based screens for a discovery-first approach as unique targets within the mosquito to screen against were initially unknown to us. The goal was to identify a compound with the desired phenotype of mosquito-specific toxicity, that could then be used to extrapolate out target proteins. Given the impractical amount of compound necessary (milligrams) for animal screens, subsequent SAR studies would also be hindered. We needed a much more robust and high-throughput method of screening our crude and semi-crude fraction library, which would quickly identify target compounds.

As a result, a novel mosquito-cell based assay, developed at UT Southwestern that could quickly identify selective and toxic candidates, was highly appealing.[14] This assay employs 4 mosquito cell lines whose mortality is quantified via CellTiterGlo, a measurement of cell viability through luminescence indicating the number of metabolically active cells proportional to adenosine triphosphate (ATP) present. Additionally, it is front-loaded with fly, moth, and human cell lines that would serve as a selectivity control early in the discovery pipeline. The platform would identify compounds lethal to various non-neuronal mosquito cell lines.[14] This would confront both the challenges of non-selectivity and rising resistance as most insecticides are neurotoxins, and target-based analyses screen for potency against conserved acetylcholine receptors and cytochrome P450s. This was a quicker and more viable method for iterative qualification of compounds as much less material would be needed (micrograms) to test in shorter windows of time.

2.3 Identification of A Lead Compound

Our library of approximately six thousand crude and semi-crude natural product fractions at the time was screened by a post doc in the lab, identifying three hits. Bioassay guided fractionation with iterative tests through the mosquito-cell assay, eliminated two

fractions that exhibited a loss of activity at the semi-pure and pure stages. This is unsurprising as it is difficult to distill activity to solely one compound, and often greater activity is seen form a synergistic relationship caused by a mixture of compounds.[15] One NP fraction though from strain "SNC-034," derived from marine *Streptomyces malachitospinus* (See E.2.1), retained potent and selective toxicity to mosquito cell lines throughout the entire isolation sequence. Bioassay guided purification using the selective cytotoxicity led to the pure compound "**NP-34**," (**2.1**) a boron-containing macrolide of the aplasmomycin[16] (**2.2**) family of compounds (Figure 2.4 and 2.5).

When pure **2.1** was tested against *Culex quinquefasciatus* cells, approximately 80% *Culex* cell death was observed at 50 nM selectively over *Drosophila melanogaster* (Kc and S2R+) and *Spodoptera frugiperda* (SF9), control cell lines which experienced less than 10% cell death (Figure 2.2). Furthermore, the molecule exerted no toxicity against a panel of 30 human cell lines of either epithelial or neuroendocrine origin at concentrations up to 30 μM (Figure 2.3).

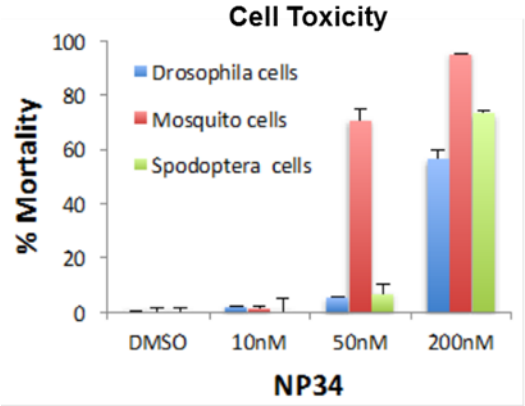


Figure 2.2 NP-34 showed ~80% cell death in mosquito cells but negligible (<10%) cell death in lines derived from *Drosophila* and *Spodoptera* species as measured by CellTiter Glo after 4 days.

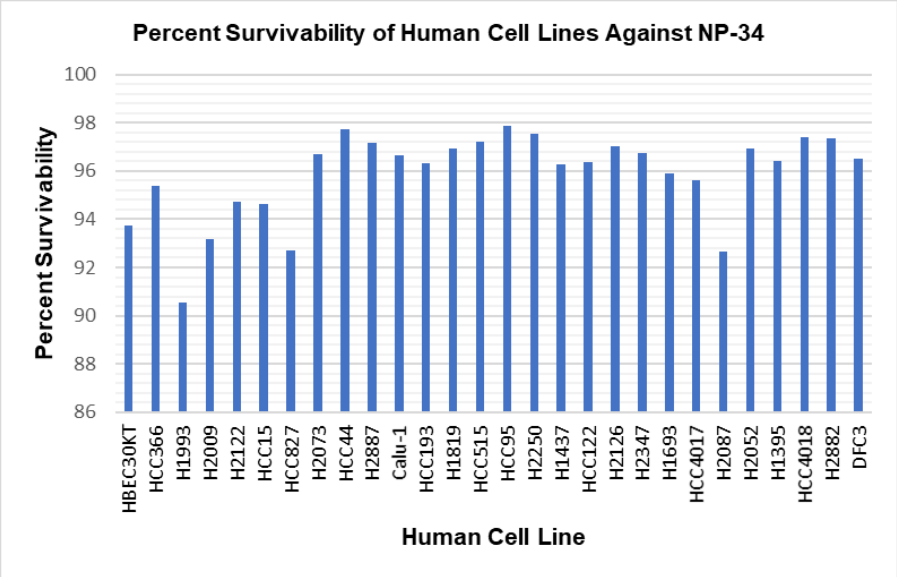


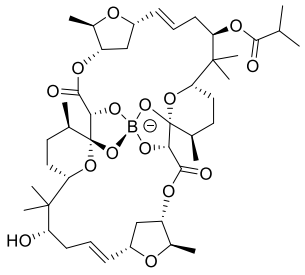
Figure 2.3 Percent survivability of 28 human cell lines against NP-34 at 20 µM, normalized to DMSO control. The lowest survivability, with 90.5% survival, was experienced by lung cell line H1993, indicating that NP-34 is safe against humans.

2.4 Characterization of the Lead Compound

With **2.1**'s promising biological activity, the isolate was characterized via ^{1}H and ^{13}C NMR. ^{1}H-^{13}C HSQC spectra revealed the existence of 10 methyl, 8 methylene, 4 olefinic methine, and 15 aliphatic methine groups, leaving 1 exchangeable proton. The carbon spectrum resolved 7 additional carbon atoms: 3 carbonyls and 4 oxygenated sp3 hybridized quaternary carbons. ^{1}H-^{13}C HMBC correlations formed the 34-membered symmetrical core characteristic of a metabolite like **2.2**. Two six-membered rings from C-3 to C-7 (and C'-3 to C-7') and two tetrahydrofurans from C-13 to C-16 (C-13' to C-16') were confirmed in the core with TOCSY. The point of asymmetry arises at side chains off C-9 and C-9' established by ^{1}H-^{13}C HMBC and confirmed by TOCSY. The side chain at C-9 was determined to be an isobutyrate with ^{1}H-^{13}C HMBC correlations from ^{1}H 2.54 ppm to C-1'', C-3'' and C-4'', and from C-9 to C-1''. TOCSY with ^{1}H 2.54 ppm irradiated showed only a doublet at 1.16 for the two methyl groups at C-3'' and C-4". The side chain at C-9' was determined to be an alcohol when it's proton at ^{1}H 5.51 ppm had ^{1}H-^{13}C HMBC correlations to C-8', C-9', and C-10', but not an ^{1}H-^{13}C HSQC

correlation between ^{1}H 5.51 ppm and C-9'. Additionally, TOCSY showed no other protons in the spin system with ^{1}H 5.51 ppm.

The quaternary carbons off the boron core, C3 and C3', were confirmed with ^{1}H-^{13}C HMBC with ^{1}H 3.73 and ^{1}H 4.49 ppm correlating to C-3 and with ^{1}H 3.79 and ^{1}H 4.55 ppm corelating to C-3'. There was no ^{1}H or ^{13}C signal indicating that a boron was present, but with the established core by HMBC and LC-MS, it was speculated to be a boron-binder like previously known **2.2**. ^{11}B NMR with an optimized pulse sequence, utilized in our lab to search for novel boron-containing natural products (unpublished work from the MacMillan lab Macho *et al*), confirmed the presence of the boron in a BO_4^- configuration known as the Böeseken complex at 10.5 ppm. It has been known that boric acid ($B(OH)_3$) readily acts as a monobasic acid in aqueous solutions, a conjugate base to the tetrahedral $B(OH)_4^-$.[17]



NP34 (**2.1**)

Figure 2.4 NP-34 is a novel boron-containing macrolide from the Aplasmomycin family of compounds.

While a plethora of aromatic and lipophilic boronic acids and boranes are known from plant sources,[18,19] only a handful of macrocycles with boron binding its core structure are known. **2.1** is one of few of this small group of microbially-derived macrolides which includes Boromycin (**2.3**), (Figure 2.5) isolated in 1967 from *Streptomyces antibioticus,* from an African soil sample. It is a D-valine ester macrolide whose Böeseken complex was established via x-ray analysis.[20] The compound has antibiotic activity against gram positive bacteria, some fungi and protozoae, and against antihuman immunodeficiency virus (HIV).[20,21]

In 1976, **2.2** was isolated from *Streptomyces griseus* from shallow sea sediment in Sagami Bay. Initial characterization lacked the boron and it was thought to contain a $C(O)_4$ central moiety instead, which was later remedied with crystal structure.[22,23] Of all the known boronic NPs, it is the most similar to **2.1** as they both have the same symmetrical, dimeric ring core. The difference between the two is the presence of one isobutyrate sidechain in **2.1** off C9. **2.2** was shown to have antibiotic activity against gram-positive bacteria, including various strains of *Staphylococcus, Bacillus, and Mycobacterium.*[22] Two naturally occurring analogs of **2.2** are known - Aplasmomycin B (**2.4**) and C (**2.5**) (Figure 2.5) – that are mono-substituted and bis-substituted, respectively, at C9 and C9 and C9' with acetate groups.[24]

Other analogs include borophycin (**2.6**), (Figure 2.5), a cytotoxin isolated from cyanobacterium *Nostoc spongiaeforme* var. *tenue* and *Nostoc lincka,* the antibiotic tartrolons B (**2.7**), C (**2.8**), and E (**2.9**), (Figure 2.4) from *Sorangium cellulosum,* and the inflammasome-stimulating Hyaboron (**2.10**) from *Hyalangium minutum* (Figure 2.5).[25-27] All these macrolides are dimeric polyketides with similar biosynthesis, and are centered around a non-enzymatically incorporated Böeseken complex. **2.10** and the tartrolons are bigger in ring size with higher degrees of unsaturation than the Aplasmomycin family, and all lack the tetrahydrofuran moiety. Interestingly, **2.10** has an additional tetrahydropyran and an epoxide ring. Of these, **2.8** was tested and had insecticidal activity against beet army worm,[28] but there were no studies reported on its toxicity towards mosquitoes or its insecticidal MoA to the best of our knowledge.

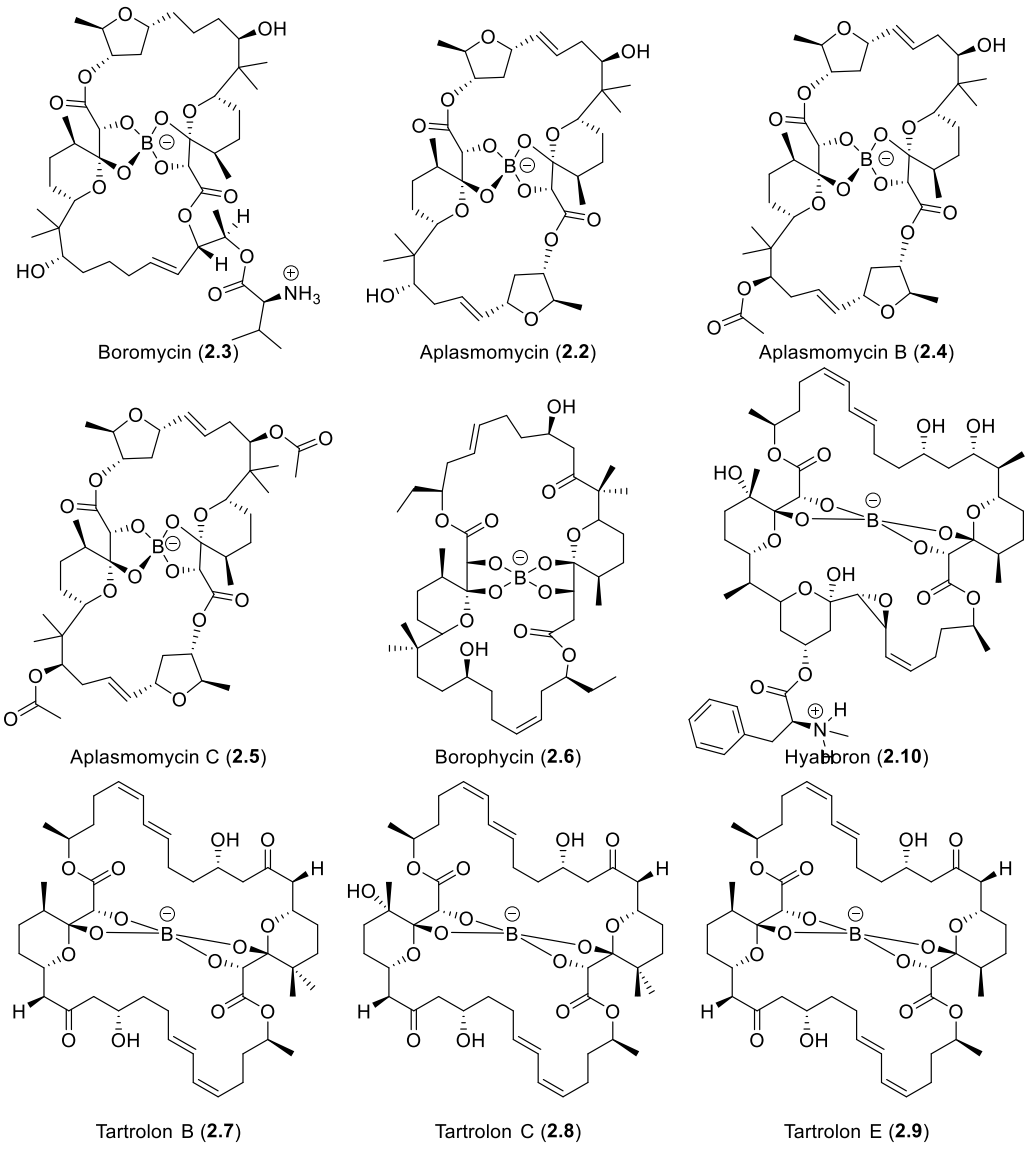


Figure 2.5 Previously known boron-containing macrolides from microbial sources.

Subsequent fermentations of SNC-034 continued to yield **2.1** whose isolation was guided therein out using ^{11}B NMR. Every fermentation produced 1 mg **2.1**/L of SNC-034. As mosquitocidal activity of **2.1** was retained after iterative tests for confirmation, we decided to carryout additional biological studies to determine its potential for insecticidal use.

2.5 Biological Activity

2.5.1 Analogs for Structure Activity Relationship

Along with **2.1**, two additional co-occurring compounds with different acylation patterns were isolated from SNC-034 – **2.2** and NP-34-915 (**2.11**), a bis-substituted isobutyrate analog of **2.1** (Figure 2.6). Comparison to **2.1** would allow us to explore how the steric and electronic effects of acylation on the macrocyclic core affect toxicity towards the mosquito cell.

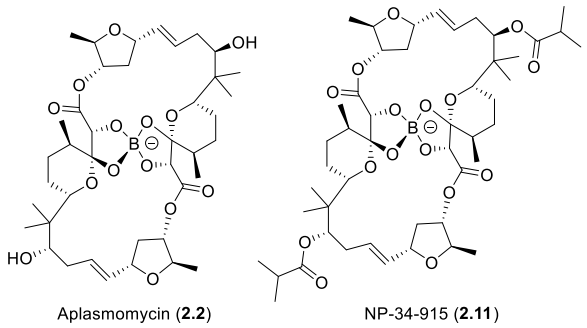


Figure 2.6 Aplasmomycin and NP-34-915 are two-occurring analogs of NP-34 whose different acylation patterns affect mosquitocidal activity.

Vast differences in activity were seen between all three analogs when subjected through the mosquito-cell assay. Both the non- and bis-substituted analogs experienced a decrease in efficacy compared to **2.1**. With its lack of acyl sidechains, **2.2** elicited 10% *Aedes aegypti* cell death at 10 µM whereas **2.1** showed 100% cell death at the same concentration (Figure 2.7). Likewise, the bis-substituted analog, **2.11**, had diminished activity of 40% cell death at 200 nM against *Ae aegypti* whereas **2.1** exerted approximately 90% cell death at the same concentration.

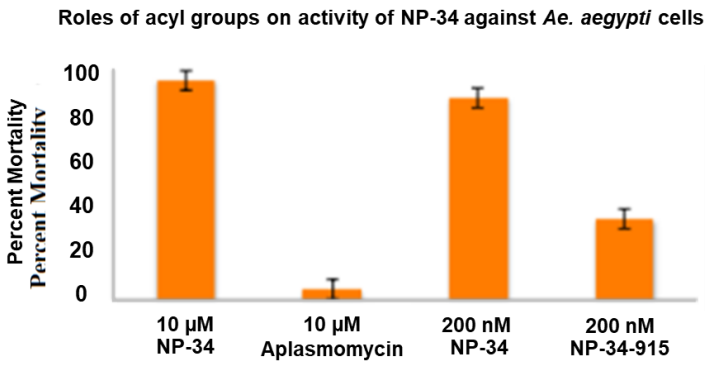
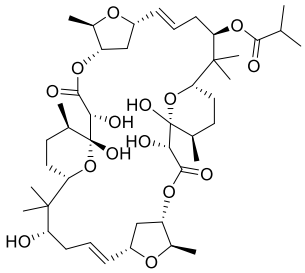


Figure 2.7 Aplasmomycin showed 10% cell death at 10 µM and bis-substituted analog NP-34-915 showed 40% mortality at 200 nM against *Ae. aegypti* cells – both were lesser toxicity than elicited by NP-34 at the same concentrations.

Thus far, the selectivity and potency of **2.1** made it a very promising candidate. This interesting and rather unique family of compounds has received relatively little attention from the synthetic chemistry community, with the only a few reported syntheses and relatively no access to analogs for SAR studies. Though, we were especially interested the role the boron played in the molecule's toxicity.

The deboronated analog of **2.1** was very easily obtained under acidic conditions, yielding "NP-34-DB," **2.12**, in full yield (Figure 2.8, See E.8.1). Biological evaluation of **2.12** at both 50 and 200 nM in the cell-based assay showed no activity against *Ae. aegypti* cells, comparable to vehicle control, whereas **2.1** showed approximately 70% and 95% mosquito cell death, respectively (Figure 2.9). With this study, we believe that **2.1**'s activity is dependent on the boron. Additionally, we see a decrease in selectivity at high concentrations of **2.1** as at 200 nM it begins to affect *Drosphila* cells with approximately 55% cell death.



De-boro NP-34 (2.12)

Figure 2.8 NP-34-DB, the boron-less analog of NP-34 generated under acidic conditions.

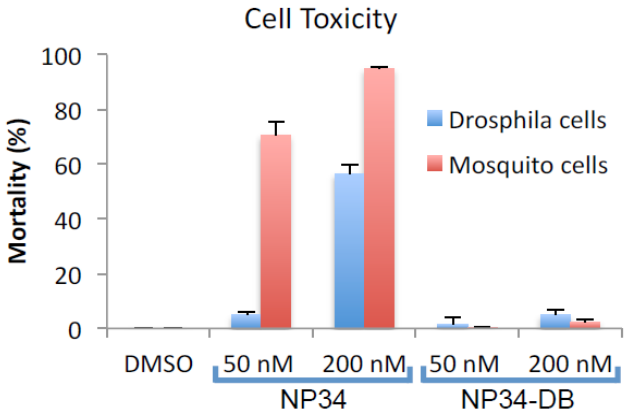


Figure 2.9 NP-34 and NP-34-DB are applied to cultured *Ae. aegypti* cells to show that boron is necessary for mosquitocidal activity.

2.5.2 Activity Against Adult and Mosquito Larvae

While the cellular activity of **2.1** showed great promise, confirmation that the phenotypes would translate to *in vivo* systems was needed. As now milligram quantities of a reasonable selection of compounds were available, it was feasible to do follow-up studies with adult and larvae mosquitoes. First, 0.5 µL of **2.1** was topically applied to the thoraxes of (3-5 days old) adult *Ae. aegypti* and *Musca domestica* as control at 1, 2, and 3 mM in acetone (Figure 2.10). Additionally, the pyrethroid insecticide Permethrin was applied at 26 mM, typical treatment conditions, as a positive control, and vehicle acetone as a negative control. Three trials were done with 20 adults each, and mortality was quantified after 24 hrs. **2.1** showed the

most activity at 5 nM with over 80% mosquito death, comparable to Permethrin, and houseflies were not susceptible to **2.1** at all concentrations. House flies only experienced 40% death with the non-selective Permethrin, which also resulted in over 90% mosquito death. This shows **2.1**'s competitiveness to currently used insecticides while retaining desired selectivity.

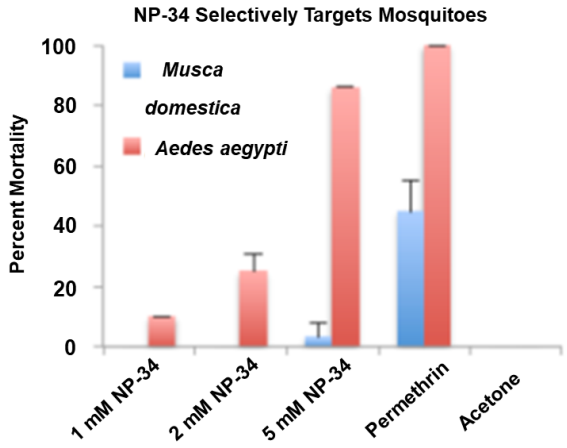


Figure 2.10 NP-34 tested at various concentrations against *Ae. aeygpti* and *Musca domestica*. Permethrin (26mM) was included as a positive control and the vehicle, acetone, was tested as a negative control.

The adulticidal activity being consistent with cellular assays was very promising. Next, confirmation that cellular activity would translate to *in vivo* toxicity amongst different mosquito species was needed. Again, 1- and 5-mM concentrations of **2.1** in acetone were applied to the thoraxes of adult *Cx. quinquefasciatus* and *Anopheles quadrimaculatus* mosquitoes (Figure 2.11). Likewise, triplicate trials were done on different days with 20 adults each (3-5 days old), and mortality was quantified after 24 hrs. Acetone and lack of **2.1** application were both used as negative controls. After 24 hrs, 5 mM of **2.1** had the best activity with over 80% cell death of both mosquito species, *Cx. quinquefasciatus* being the most susceptible of the two.

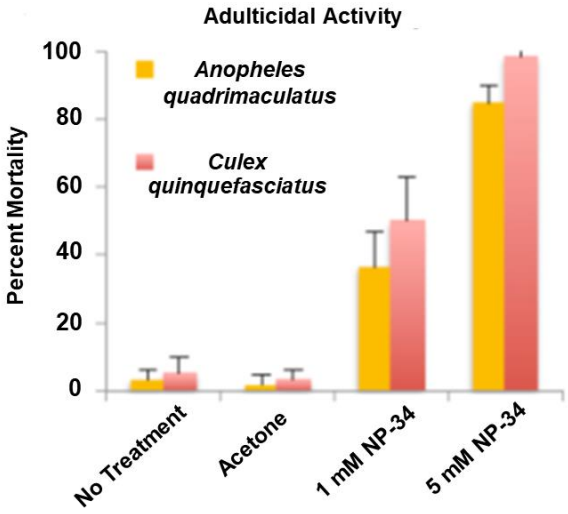


Figure 2.11 NP-34 tested at 1- and 5 mM against adult *Cx. quinquefasciatus* and *Anopheles quadrimaculatus*. No treatment and vehicle acetone were tested as negative controls.

Further evaluation was done to test **2.1**'s efficacy against pyrethroid-resistant and -susceptible strains. As pyrethroids are the most used pesticide – accounting for one fourth of the entire market[29] - and being the only class acceptable for use on bed nets,[30] pyrethroid susceptibility is crucial to test as mosquitoes quickly develop resistance to this class. 1- and 5 mM concentrations of **2.1** in acetone were applied to thoraxes of Permethrin-resistant and -susceptible strains of *Ae. aegypti,* (3-5 days old) adult mosquitoes (Figure 2.12). Permethrin was applied as positive control at 26 mM and acetone as a negative control. Analyses were done in triplicate on 20 mosquitoes on different days. After 24 hrs, approximately 90% adult death was observed in susceptible strains, and approximately 80% mortality was observed in resistant adults. This demonstrates that **2.1** retains activity against permethrin resistant *Ae. aegypti* mosquitoes which is significant, as permethrin resistance is common in regions where mosquito-borne diseases are prevalent. Additionally, the susceptibility of resistant strains to **2.1** indicates that it is potentially acting through a different MoA.

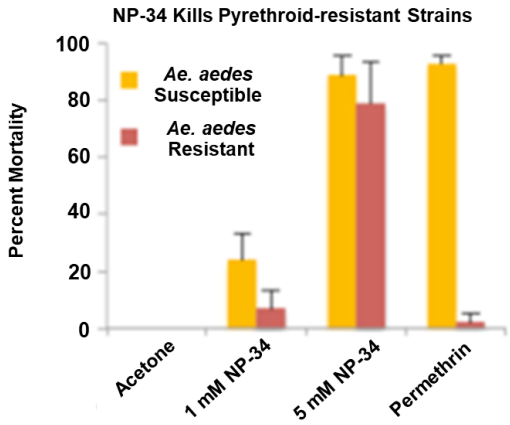


Figure 2.12 Both *Ae. aegypti*-susceptible and -resistant strains experience mortality at 5 nM NP-34 compared to Permethrin control which is ineffective towards resistant strains.

As only adult mosquitoes spread disease-inducing parasites, larvicides are used to thwart populations before they can reach adulthood. They are favored due to their ease of administration directly into aquatic breeding sites and are therefore “localized” and associated as “low risk to other species.” Several reports argue that vector control should prioritize targeting the larval stage since the limited habitat range and weakness of freshly laid eggs would make them easier to control than free-flying adults.[31] As a result, we tested **2.1** for its efficacy against larval stage mosquitoes (Figure 2.13). Triplicate trials of 10 instar-I *Cx. quinquefasciatus*, *Ae. aegypti*-susceptible, and *Ae. aegypti*-resistant larvae were exposed to 85 µM **2.1** in DMSO. Larvae were also exposed to DMSO as negative control. Over 75% mortality to all three larval strains were observed after 24 hrs, showing that **2.1** is also active as a larvicidal agent.

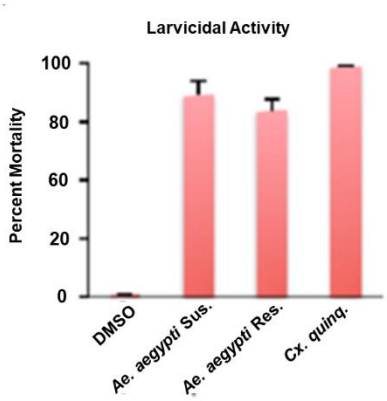


Figure 2.13 NP-34 is an active larvicide agent. *Cx. quinquefasciatus*, *Ae. aegypti*-susceptible, and *Ae. aegypti*-resistant 1st instars were challenged at 85 µM and assessed for mortality 24hr later, whom all experienced greater than 80% cell death.

As **2.12** experienced lesser toxicity against mosquito cells, we sought to confirm if the lack of boron translated *in vivo.* Triplicate studies were done on various days towards instar I larvae of *Ae. aegypti*-susceptible and -resistant strains (Figure 2.14). 50- and 75 µM of **2.1** and of **2.12** in DMSO solutions were applied, and mortality was quantified after 24 hrs. DMSO and no treatment were both used as negative controls. Approximately 95% death was quantified for *Ae. aegypti*-susceptible at both 50 µM and 75 µM of **2.1**, and approximately 50% and approximately 90% death was quantified for *Ae. aegypti*-resistant at 50 µM and 75 µM of **2.1**, respectively. While activity is seen amongst both *aegypti* strains with **2.1**, no activity is seen with **2.12**, indicating that the boron is necessary for activity in animals as it was necessary for cellular activity.

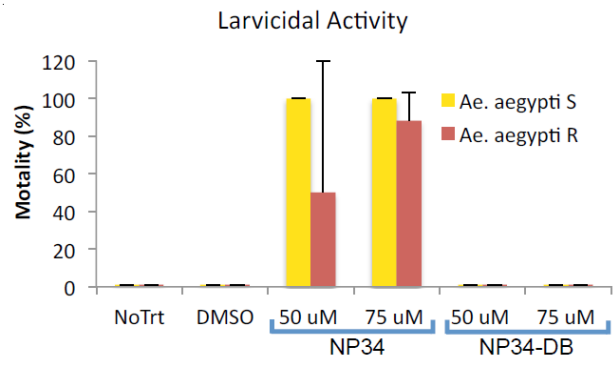


Figure 2.14 NP-34-DB is clearly inactive when tested against *Ae. aegypti*-susceptible and -resistant strains at both 50 µM and 75 µM compared to NP-34, thus indicating that the boron is necessary for NP34 activity in both cells and whole animals.

2.5.3 Activity Against Other Organisms

Acute oral, dermal, and inhalation toxicity, acute eye and dermal irritation, and skin sensitization tests are required by the U.S. Environmental Protection Agency for any chemical seeking EPA registration as an insecticide. These are done to minimize the acute or chronic effects possible to mammals and other organisms exposed to these chemicals. Preliminary experiments with **2.1** were evaluated for acute oral toxicity towards mammals (Figure 2.15). Six-week-old female mice were monitored for 30 days after given oral doses of 0.25 mL **2.1** at 5.5, 11.6, 55, 175 or 500 mg/kg. The appearance and body weight of the mice that were challenged with **2.1** were indistinguishable from mice treated with vehicle only, indicating that **2.1** shows no acute toxicity in this model. This indicated that **2.1** has the potential to be a mammalian-safe insecticidal agent.

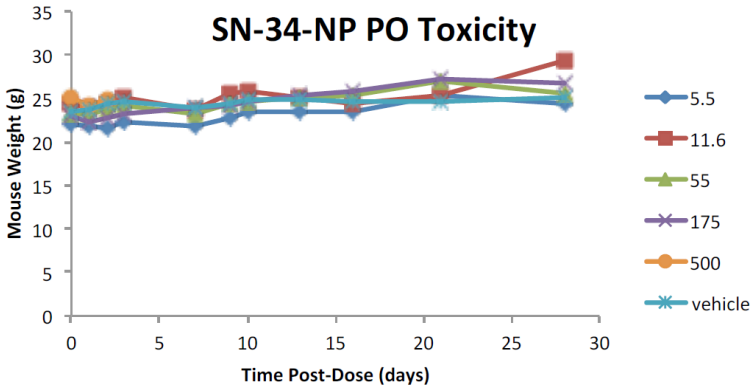


Figure 2.15 Females (age 6 weeks) were given 0.25 ml NP-34 orally at doses of 5.5, 11.6, 55, 175 or 500 mg/kg and monitored for 30 days. The appearance and body weight of animals challenged with NP-34 were indistinguishable from mice treated with vehicle only.

2.6 Discussion

Analysis of our NP-fraction library with a high throughput cell-based assay, yielded a novel boron-macrolide **2.1,** based on its mosquitocidal activity. Since various pyrethroid-susceptible and -resistant strains experienced mortality by **2.1** in both cellular and whole animal

studies, this macrolide is a promising compound for the development of an eco-friendly insecticide. Activity at both larval and adult stages amongst various mosquito species indicates that **2.1** is targeting something conserved within the vector regardless of species or developmental stage. This gives **2.1** a wide range of possibilities for use including as a larvicide to limit communities at breeding sites, as an aerosol strictly in times of disease outbreak, or a combination of the two. Likewise, if **2.1** continues to show inactivity towards humans, it can be used as a human-safe and effective bednet additive.

While further studies are needed to elucidate its MoA, activity to non-neuronal cells in the assay suggest that **2.1** is acting differently than commonly used neurotoxic insecticides. Compounds screened for mosquitocidal activity should not exhibit CNS activity to avoid development of broad-spectrum insecticides that will perpetuate issues of non-selectivity amongst organisms. Identification of novel targets with **2.1** can also elucidate unique vulnerabilities within the mosquito-vector to help inform insecticide development and reveal potential mutations that can lead to resistance.

Work in target ID is needed to establish how **2.1** interacts in the vector, though it is perhaps the most difficult challenge associated with phenotypic-based discovery. Due to the selectivity profile of **2.1**, a two-pronged approach is planned to understand its MoA against mosquitoes. First, is with affinity purification utilizing a tagged version of **2.1** to pull down target proteins. For this approach, medicinal chemistry efforts have been carried out to identify regions of the molecule appropriate for attaching the affinity tags (Section 4.6). Second, will be with the development of resistant mutants combined with RNAseq to reveal susceptibilities to **2.1** at the genomic level.

For the former, MoA studies can be conducted with unbiased biochemical strategies accessed through functionalized analogs. Installation of clickable handles will allow for addition of biotin for subsequent pull-down and purification if the reactivity at **2.1**'s side chains or points of vulnerability within its macrocyclic core can be exploited. Alkylated analogs can be

biotinylated using azides and studied within the lysates from cells treated with **2.1** can identify target protein(s) against *D. melanogaster* Kc and S2R+ and *Spodoptera frugiperda* SF9 cells as control. This will allow identification of potential non-specific binding of probe compounds as well as reveal whether insensitive cell lines have homologs of the target of **2.1**.

For the latter, when cultured cells are challenged with an antagonistic compound, surviving populations evolve a selective resistance through diverse mechanisms, such as gene amplification.[32,33] This resistance is brought about by producing elevated levels of the protein directly antagonized by the introduced compound. For example, selective resistance was produced in *Caenorhabditis elegans* with exposure to the pesticides Levamisole and Ivermectin, both hyperpolarizers of muscle and neuronal cells.[34] This allowed for genetic evaluation of the surviving generations, giving insight into their resistance mechanisms. Since gene amplification is often unstable, in the absence of challenge, it is rapidly lost because the relevant amplicons frequently reside on extrachromosomal fragmentes,[14,32,33] allowing identification of involved genes that can be prioritized for analyses.

Cultures of *Cx. quinquefasciatus* cells will be serially passaged with sub-lethal, elevating doses of **2.1** to generate resistant lines that can grow in concentrations at least 10-fold higher than the LD_{50} of the parental line. These populations will be analyzed for resistance consistent with gene amplification mechanisms, where relaxation of amplified genes would be seen in absence of **2.1**. RNA-seq experiments will profile RNAs present in the resistant lines and subsequent populations released from selection, to compare to the parental cells. Genes which are highly over-expressed in both resistant strains and are substantially reduced or restored to normal levels in both relaxed strains, will be prioritized for analysis.

This should lead to identification of genes interacting with **2.1**, hopefully one that is not conserved, but rather something unique to insects and, more specifically, something uniquely designed to mosquitoes. A potential target, for example, would be Olfactory receptors (*Or*). ORs are diverse receptors exclusive to insects that translate odor molecules into electrical

signals in peripheral neurons.[35] Their uniqueness to insects could explain **2.1**'s lack of toxicity towards the human cell lines in the assay and the mice in the study. Sensillar lymphs on the antennae and maxillary palps contain odorant-binding proteins (OBPs) in conjunction with the Ors. These proteins help solubilize odorants, as they tend to be hydrophobic compounds,[35] which could explain the ready uptake of **2.1**. Additionally, *Or* genes are extremely divergent which yields the possibility of unique receptor(s) to the mosquito not found in other insects that can be selectively targeted.

With the range of activity this macrocycle elicits with different acylation patterns, it is interesting to see how further functional decoration will affect both its potency and selectivity. Further SAR can be achieved by generating a library consisting of analogs naturally occurring and co-isolated, biosynthetically manipulated, or semi-synthetically altered. Manipulation of the alcohol side chains and skeletal olefins to carbonyls, epoxides, and diols, etc. (Section 4.5) can create analogs whose steric and electronic effects can be probed for differences in activity, while also providing handles for affinity chromatography. Care should be taken though with SAR to not change the overall polarity of **2.1**, as insecticidal agents are typically lipophilic compounds for ease of access into insect cuticles. A systematic survey of fatty acid length and branching with generated analogs could potentially yield a compound with better toxicity. Analogs that could possess greater potency towards mosquitoes or elicit improved *in vivo* efficacy are welcomed as the best mosquito antagonist is desired.

While changes to the macrocyclic core are thought to not affect the activity of deboronated analogs, such analogs are needed for confirmation. Deboronated analogs should remain ineffective towards mosquito species as the boron is crucial for activity in both cellular and animal assays. Since it is tetravalent with a full octet in **2.1**, it is unlikely that the boron itself is eliciting toxicity as it does not have the electronic freedom to interact with any substrate. Thus, in the macrolide itself, the boron should not participate in any target-site coordination that would lead to mosquitocidal effects. As physiological systems are pH neutral, which is crucial

to retaining the integrity of the Böeseken complex (Section 4.2), there should not be conditions acidic enough in the organism to cause the boron to dissociate from the macrolide and interact with the active site itself as a $B(OH)_3$ derivative. Thus, it is logical to consider the boron to be an overall unreactive entity and rather its role is to keep the macrolides's confirmation favorable for target site interaction. Analogs lacking the boron moiety would hence be unable to access its coordination site because of the molecule's flexibility regardless of side chain or skeletal decoration.

Follow-up studies with **2.1** and all analogs of interest against not only live flies and moths, but also live bees will be very important as *in vivo* controls. If **2.1** is indeed only mosquito-toxic, the lack of cellular toxicity towards flies and moths should translate *in vivo*, but most importantly to confirm this lack of activity against bee species as those are currently the most affected populations by insecticides. Fortunately, pesticides that are being linked to bee toxicity are being increasingly regulated or discontinued,[36,37,38,39,40] and requirements for approving new insecticides should and will become increasingly more stringent, limiting the number of new broad-spectrum insecticides. Thus, developing a compound that will contribute to pollinator decline, or ultimately be denied by the EPA for lack of selectivity, should be avoided. Confirmation of **2.1**'s inactivity towards bees can be checked initially in cultured bee cell lines (Section 1.3), to validate its selectivity with minimal risk to the population, and then move to live bees for *in vivo* confirmation. With this data, **2.1** or another candidate with better promise, can confidently be brought forward for development as a mosquitocide.

Currently the biggest hurdle to working with **2.1** is its miniscule yield biosynthetically, which will limit SAR efforts and the scope of biological testing. Being a secondary metabolite, it is typical to only receive a few milligrams with standard fermentation conditions. As our *Streptomyces* strain has only produced a maximum of 1 mg/L of bacteria, the generation of new analogs and further biological studies, including the necessary animal and controlled aerosol field studies, has been bottlenecked. Down the road for commercial development, **2.1**

would also need to be acquired in gram and kilogram quantities, further fueling the need for upscaling its production.

The bulk of this project has been dedicated to optimizing the detection, fermentation, and isolation conditions to optimize the yield of **2.1** and its co-occurring analogs. In parallel, semi-synthetic efforts have been undergone to generate a functionally rich and diverse library of analogs. Supplemented by the optimization of detection, fermentation, and isolation methods in this work will be the synthetic biology efforts to heterologously express the biosynthetic gene cluster (BGC) responsible for producing **2.1** (unpublished work from the MacMillan Lab). It is expected that **2.1** and other lead compounds emerging from this initiative will advance vector control by delivering a powerful new class of insecticides for field-testing programs and expose new vulnerabilities that are specific to mosquitoes. These should illuminate biochemical opportunities inspiring novel vector control strategies through rational design, that are more effective in targeting vectors while being safe for the environment.

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CHAPTER THREE

OPTIMIZATION OF ^{11}B DETECTION METHODS

3.1 The Need for NP-34 Detection Methods

Over the course of scale-up to isolate more **2.1**, there were extensive challenges in validating the presence of boron in analogs and issues in fermentation leading to diminished yields. With subsequent regrows of SNC-034, yields dropped from 1 mg/L to approximately 0.1 mg/L. This issue needed addressing to be able to move forward with SAR and planned biological testing.

To aid in screening and validation of **2.1**'s presence in crude, purse, or pure fractions, a method was needed that could rapidly identify it. The first option was to use ^{1}H NMR as NMR has served as an important tool for screening, whether in fragment-based approaches for drug discovery or NP discovery efforts, and proton is the most used method due to its sensitivity and richness of information. But identification of **2.1** with ^{1}H NMR during fermentation and early stages of isolation is unhelpful as characteristic proton peaks at 2.54, 3.92, and especially 6.45 ppm are completely overshadowed by other metabolites present (Figure 3.1). Thus, using ^{1}H NMR to validate optimization methods would be tedious and inefficient since presence of **2.1** would be unknown until very late into the isolation process.

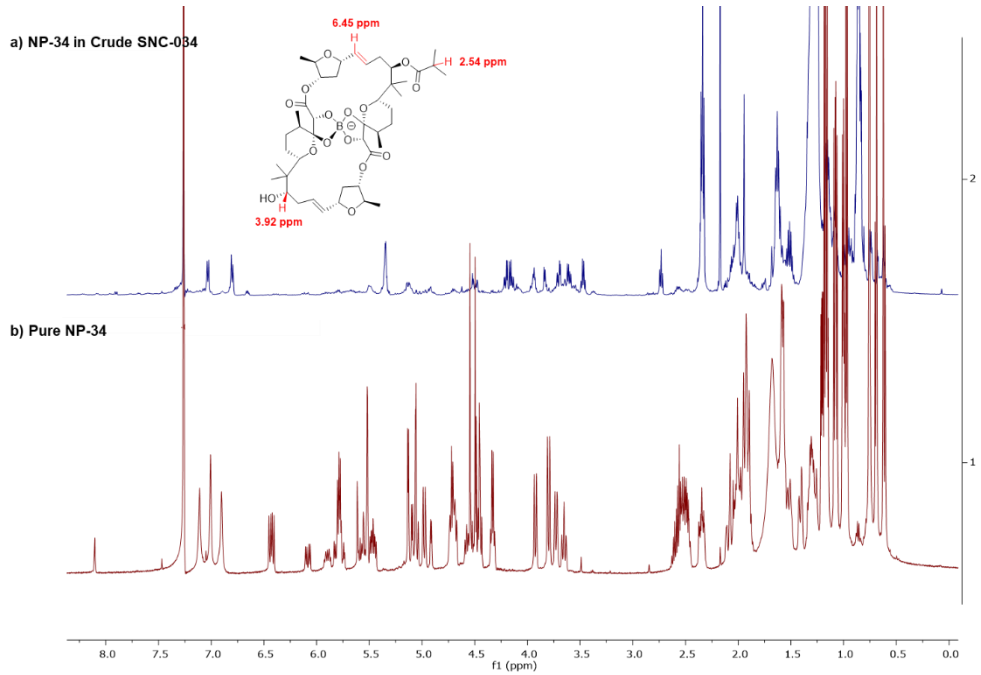


Figure 3.1 ^{1}H NMR (500 MHz, $CDCl_3$) of an aliquot of a) 41.4 mM NP-34 in SNC-034 after resin extraction from aqueous media vs. b) 41.1 mM pure NP-34. Characteristic peaks of NP-34 at 2.54, 3.92, and 6.45 ppm are completely overshadowed by other metabolites present in crude samples, making confirmation of NP-34's presence impossible with ^{1}H NMR at this stage.

Our lab has previously taken advantage of other NMR-active nuclei, such as ^{15}N[1,2] and ^{19}F (unpublished work from the MacMillan lab), to study the mechanism of formation and discovery of biologically-interesting compounds. Based on this success, we sought to use ^{11}B NMR as an approach to detect NP-34 rapidly from crude extracts, enriched fractions, and purse compounds.

Boron has two naturally occurring, stable isotopes: both quadrupolar, ^{10}B (19.9% abundant) with a spin of 3 and ^{11}B (80.1%) with a spin of 3/2.^{11}B being the more sensitive of the two, has been successfully used in NMR analysis of synthetic and naturally occurring boronic compounds.[3] As boron is relatively rare in microbial NPs, there should not be interference from other compounds, and we can be certain that any ^{11}B NMR signals in spectrum from SNC-034 arise from **2.1** and its co-occurring analogs. Likewise, fermentation nutrients should not interfere, like any excess $B(OH)_3$ unincorporated into **2.1** should stay behind in in aqueous media after resin extraction, for example (See E.5).

Initial studies to detect boron signals with 3.93 mM pentaphenylfluoroboronic acid (**3.1**) gave large protrusions of asymmetrical noise arising with the ^{11}B NMR experiment. This noise is due to broadening of resonance peaks from ^{11}B having a nuclear spin of 3/2[3] coupled with internal boron probe components in the instrument itself yielding stronger signals than those coming from the sample. These extraneous signals result in the asymmetrical boron NMR noise referred to as the "boron hump," (Figure 3.3) and typically covers a range of -30 to 30 of ppm.[4] Attempts to rectify this by screening in quartz instead of borosilicate NMR tubes did not silence the extraneous noise. We determined that this experiment would not be sensitive enough to detect boron in NP samples as we would be expecting low milligram amounts of material incapable of yielding a stronger signal than those from the probe components themselves.

3.2 Methods of Optimizing ^{11}B NMR and Proof of Concept

The hypothesis was that optimizing the sensitivity of ^{11}B NMR by silencing the extraneous probe signals and reducing the boron hump would provide the means to detect lower levels of boron material. Based on work by Cory and Ritchey, DEPTH pulse sequences can be applied to NMR to select homogeneous regions of radiofrequency.[5,6] They were able to successfully apply a three-pulse DEPTH experiment to Si NMR to suppress glass resonance from siloxysilanes. We believed this method could likewise suppress extraneous boron resonances from the NMR probe itself. Figure 3.2 shows the composite pulse series, which is the basic 90° pulse with two subsequent 180° pulses with phase cycling.[5,7]

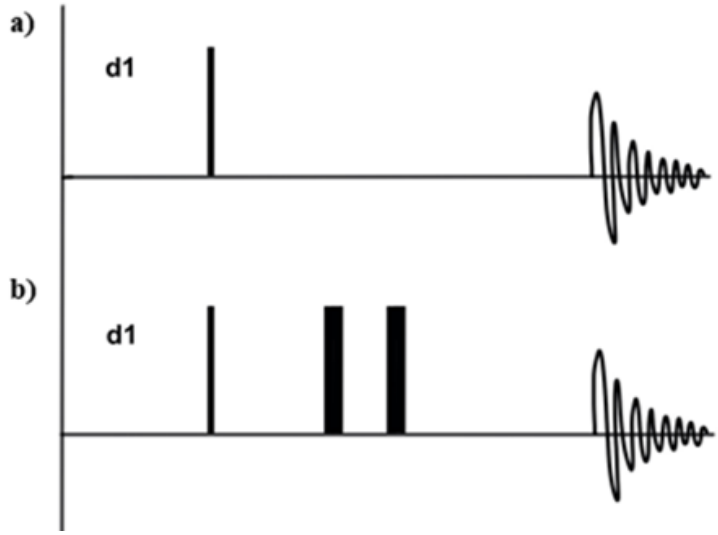


Figure 3.2 a) The typical 90° pulse on basic NMR acquisitions versus b) the composite pulse consists of two additional 180° pulses in sequence after the 90° pulse.

This phase cycled composite pulse is application of a 90° pulse for the spins inside the coil and 0° for the outside, cancelling all extraneous noise from outside the coil.[7,8] The excess noise is neglected, and resultant NMR signals arise only from the boron sample within the coil. As this pulse sequence was successfully applied to Si NMR, yielding more ample signals,[5] we sought to achieve the same with boron. The pulse sequence was available on Bruker's pulse program library as "zgbs." We applied it to the standard ^{11}B proton decoupled experiment on

the Bruker. The nucleus was changed for boron detection, and neither the phase cycling nor delay parameters were modified. It was named “11Bzgbsig” to indicate “inverse gated” as this was the proton decoupled experiment. To further reduce noise, we continued conducting all experiments in quartz NMR to ensure signal would not be compromised by borosilicate in regular tubes. Proof of concept was tested with a solution of 3.93 mM of **3.1** in MeOD-*d* (Figure 3.3) and total reduction of the boron hump was seen with the applied zgbs pulse sequence.

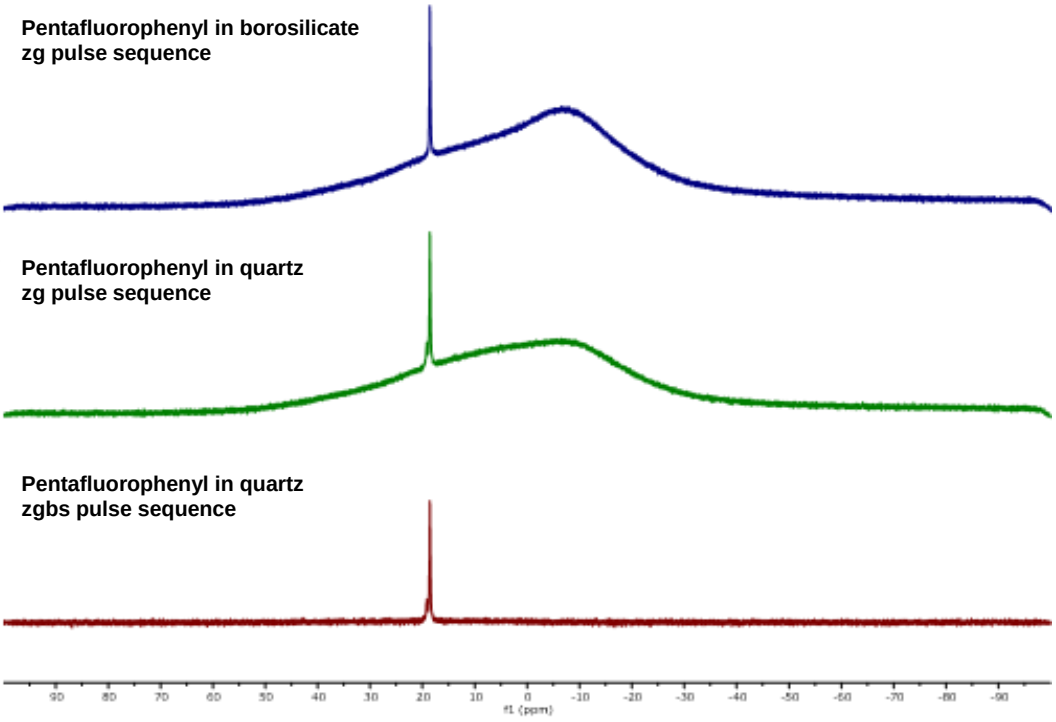


Figure 3.3 ^{11}B NMR (500 MHz, MeOD) of 3.93 mM pentafluorophenylboronic acid in a) borosilicate NMR tube with zg pulse sequence, b) quartz NMR tube with zg pulse sequence, and c) quartz NMR tube with zgbs pulse sequence, all with ns = 128. The “boron hump” is seen with ^{11}B NMR experiments taken in borosilicate NMR tubes or with the zg pulse sequence. Significant reduction of extraneous signals is observed with the zgbs pulse sequence applied to samples in quartz tubes, as the baseline noise is leveled, resulting in clearly distinguishable boron peaks.

The ability of this pulse sequence to suppress asymmetrical peak noise in ^{11}B NMR seemed very promising for us to screen and detect low level of boron-containing molecules. As proof of concept, we sought to detect boron molecules from crude extracts, enriched

fractions, and pure compounds using only their boron signals with this optimized experiment. Three compounds **2.2**, bortezomib (**3.2**), and autoinducer-2 (**3.3**), (Figure 3.4) were used to illustrate the different applications of this optimized NMR experiment.

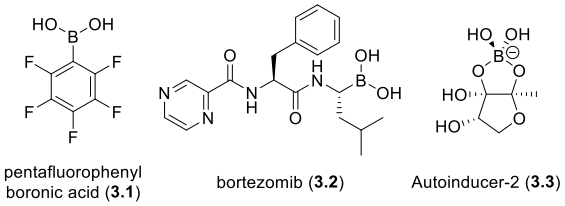


Figure 3.4 Structures of some of the boron-containing compounds used to illustrate effectiveness of ^{11}B NMR detection with the applied pulse sequence.

As previously stated, **2.2** is a microbially-derived macrolide with a Böeseken complex[9] produced by SNC-034 in our library. Over the course of isolation its structural analog **2.1**, we found there to be extensive challenges in the analytical chemistry, including validating the presence of boron. With the standard Bruker ^{11}B decoupled pulse sequence (zg) on crude, semi-crude fraction, or pure **2.2**, only the asymmetrical noise was seen (Figure 3.5). This broad background signal dominated the spectrum, making it difficult to see **2.2**'s desired signal at 10.5 ppm. This confirmed our suspicion that the standard ^{11}B NMR experiment, without the pulse sequence, would be useless as a screening method for detecting **2.1** or discovering other NPs. Running the same samples with the composite pulse sequence, ^{11}Bzgbisg experiment, in quartz NMR tubes gave the boron peak against a flattened baseline, confirming the experiment's ability to detect low quantities of boron in crude and pure fractions. Enrichment of **2.2**'s boron NMR is seen with subsequent steps of purification (Figure 3.6).

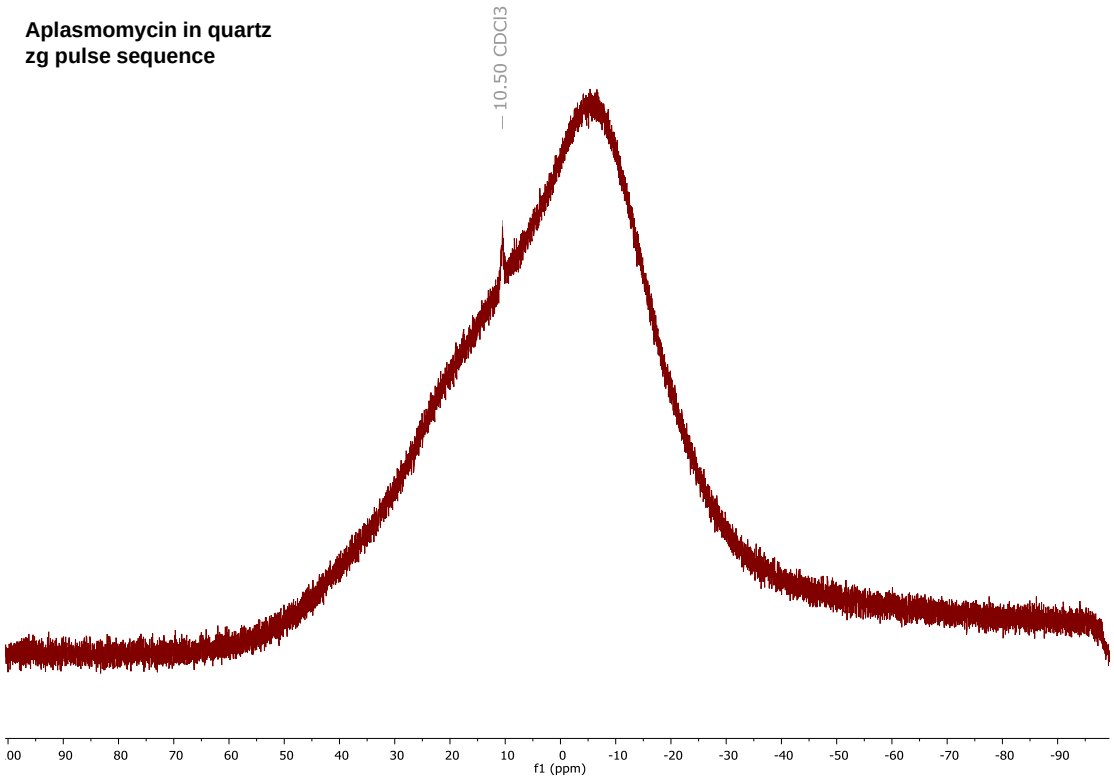


Figure 3.5 3.23 mM of Aplasmomycin (500 MHz, $CDCl_3$) via standard ^{11}B decoupling NMR experiment in quartz NMR tubes, ns = 128, yields the boron hump, overshadowing distinctive chemical signals.

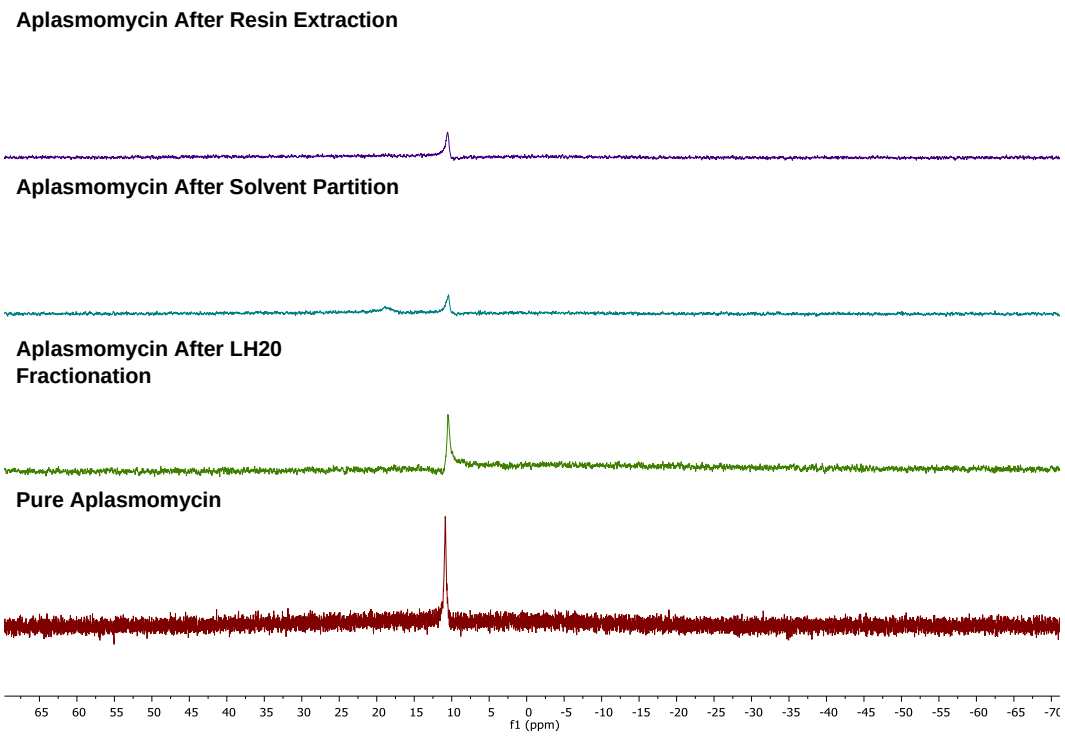


Figure 3.6 Standard ^{11}B NMR proton decoupled experiment (500 MHz, MeOD) in quartz with zgbs pulse sequence of 3.23 mM aplasmomycin, ns = 128, throughout various stages of purification. Significant enhancement of boron peak is seen upon iterative purification steps.

For further confirmation that a boron compound could be seen at low quantities in crude and semi-crude fractions, we wanted to introduce a boron compound into a microbial fraction free of boron-molecules, at quantities comparable to NP-yield. We chose the synthetic compound, **3.2**, a N-protected dipeptide $B(OH)_3$ used for treatment of multiple myeloma and mantle cell lymphoma. Its boron reversibly binds to the catalytic site of the 26S proteasome in mammalian cells, eliciting chymotrypsin-like activity.[10,11] It also elicits apoptosis via endoplasmic reticulum (ER)-induced stress affecting NF-κB activity.[12] The boron in a 1 mg sample of pure **3.2** was easily detectable with ^{11}Bzgbsig with 512 scans. The sample was then introduced into a crude fraction of metabolites produced by a strain of bacteria (SNC-117) that we knew via ^{11}BNMR did not produce boron-NPs. The ^{11}Bzgbsig experiment was likewise able to detect the boron signal with ns = 1024 scans (Figure 3.7). Lower quality peaks were observed in this instance due to **3.2**'s instability at ambient temperature.

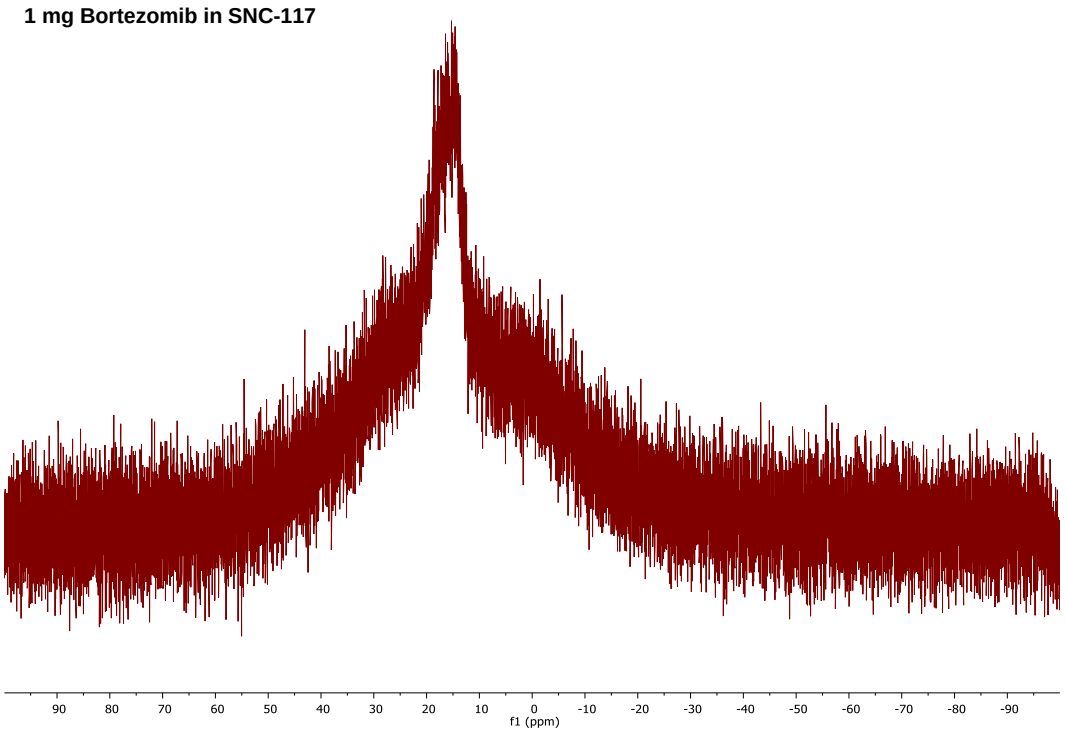


Figure 3.7 11Bzgbsig (500 MHz, MeOD) of 1 mg of Bortezomib (4.34 mM) added to SNC-117 in quartz NMR tube, ns = 1024. The boron peak is distinctive from the baseline, but with lower resolution due to compound degradation.

Lastly, the sensitivity of this experiment was probed with its ability to detect **3.3** *in situ*. **3.3** is a naturally occurring, boron-containing furan. It is produced in both Gram-positive and -negative bacteria serving as an extracellular signaling molecule for inter- and intraspecies communication.[13] It is highly studied in the reporter bacterial strain *Vibrio harveyi* which uses **3.3** for quorum sensing-mediated bioluminescence.[14,15]

In *V. harveyi,* the concentration of **3.3** is proportional to the luminescent expression of the bacteria. It is typically detected via the engineered strain,[16] but the bioassay used for detection is not quantitative and can be thwarted at high concentrations of culture medium. **3.3** is also relatively unstable at lower concentrations and is sensitive to growth conditions. For example, low pH or the addition of glucose to media inhibits luminescence, and the presence of borate can shift chemical equilibrium between stereoisomers[17] thus making it very challenging to standardize quantification of **3.3** in culture.

Various methodologies have been developed throughout the years to facilitate detection and quantification of **3.3** including Fe^{3+} supplementation to growth medium;[17] LuxP-based fusion proteins;[18] 1,2-phenylenediamine reactions forming HPLC measurable quinoxaline derivatives;[19] environmentally sensitive protein receptors with fluorescent dyes;[20] GC-MS;[21] and chemical probes such as d-desthiobiotin-AI-2.[22] But these methods have their shortcomings including sensitivity to environmental inference, invasiveness, and dependence on concentration. NMR being quick, sensitive, and noninvasive would be an efficient and facile way to detect **3.3**. More so, exploiting the boron with ^{11}B NMR would eliminate the need of purification for other naturally occurring metabolites in culture, making it an efficient detection method. ^{11}B NMR has been reported once for **3.3**, but with low resolution after 80,000 scans.[13] If ^{11}Bzgbsig could improve data acquisition, it would be a better experiment to employ for detecting **3.3**.

After a 24 hr incubation of a 50 mL culture of *V. harveyi*, **3.3** was detectable with the ^{11}Bzgbsig NMR experiment from aqueous media without the need of purification (Figure 3.8).

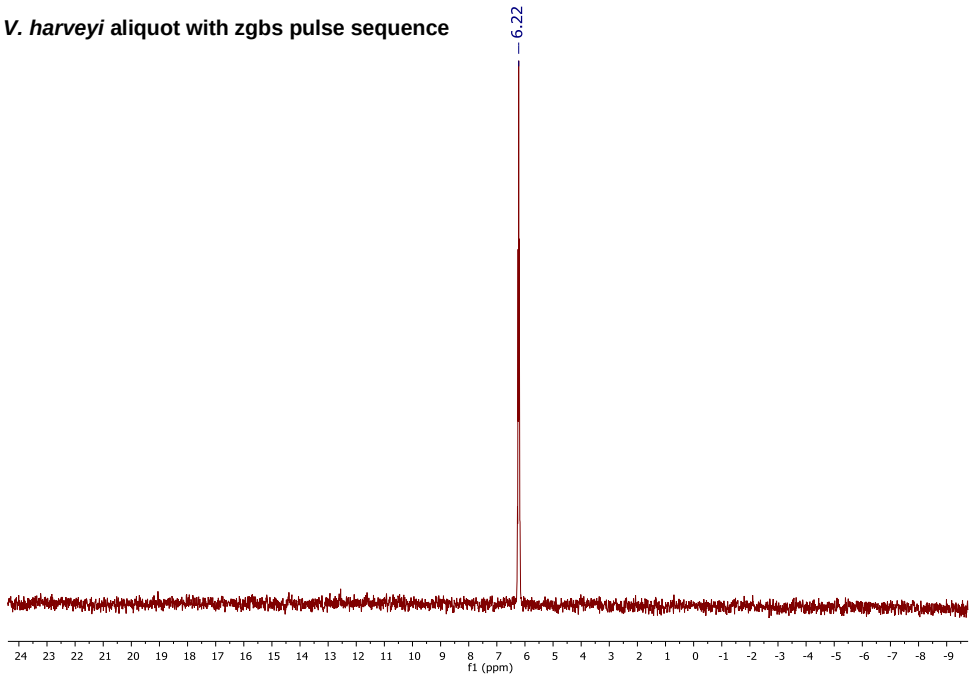


Figure 3.8 ^{11}Bzgbsig (500 MHz, MeOD) decoupling NMR spectra of *V. harveyi*, ns = 512.

When screening solvents for the NMR analysis, differences in **3.3**'s ^{11}B NMR were observed. A broad singlet at 6.2 ppm in DMSO-*d* and a sharp singlet at 6.4 ppm in MeOD are indicative of the one boron atom in **3.3**. When taken in D_2O though, a clear pentet with splitting $J_{B\text{-}D}$ = 3.50 Hz is observed (Figure 3.9). The splitting is indicative of deuterium exchange, further confirming that we are observing **3.3** via ^{11}B NMR. Observing **3.3** with ^{11}B NMR exemplifies the experiment's ability to detect quantitative amounts of extracellular metabolites. Additionally, as NMR can quantify material, this method could be an appealing method for quantification of **3.3**, which is an ongoing challenge.

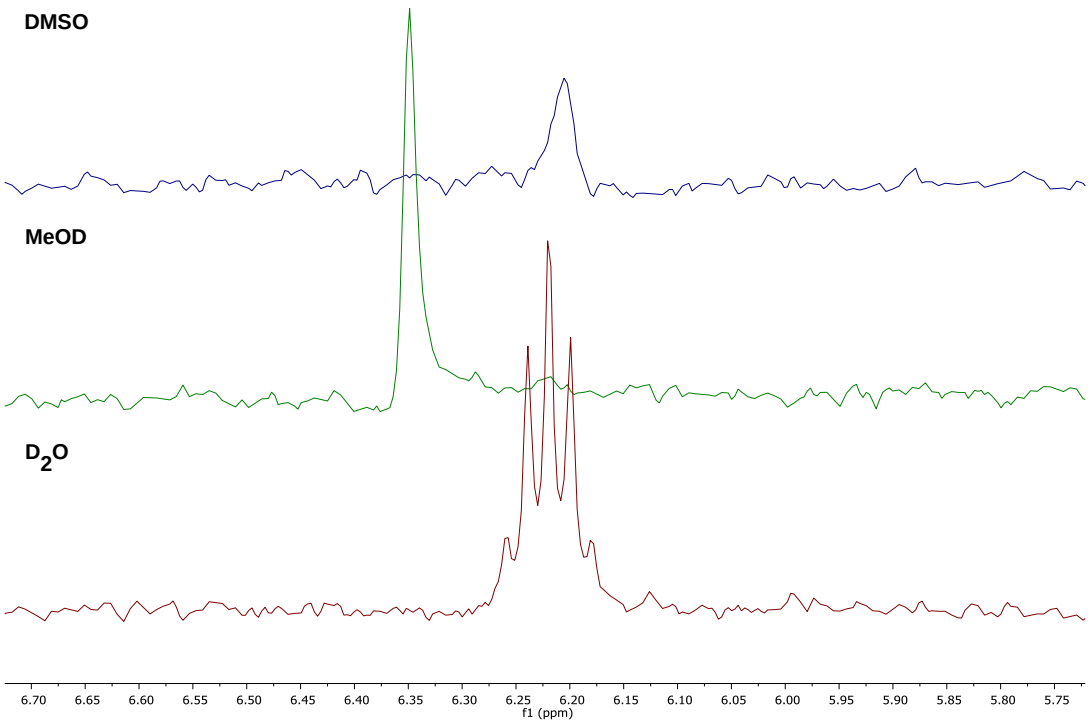


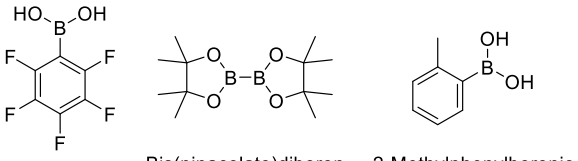
Figure 3.9 ^{11}B NMR (500 MHz) of autoinducer-II from an aliquot of *V. harveyi* in various NMR solvents: DMSO-*d* (ϱ 6.2 ppm), MeOD (ϱ 6.4 ppm), and D_2O (ϱ 6.2 ppm). Closer inspection of the boron NMR reveals a pentet splitting (J = 3.50 Hz), due to deuterium exchange, further proving detection of the molecule.

3.2.1 Quantifying Boron Metabolites with an Internal Standard

Quantitative NMR is a method of deducing how much analyte is in a sample proportional to an analytical standard.[23] Since we planned on using ^{11}B NMR to determine

which isolation and fermentation conditions yield the most **2.1** (Chapter 4), a way to quickly deduce how much relative **2.1** was being produced was needed. Since internal standards are used for quantification of compounds in ^{1}H NMR, it was thought that a compound could be found to do the same with ^{11}B NMR.

As **2.1** and analogs have a characteristic 10.5 ppm shift, a boronic compound was needed with a shift more downfield that would not interfere. Additionally, a compound was needed that was unreactive towards the macrolide as to not react in the NMR sample and give a false indication of the conditions' actual yields. At first, boric acid was sought as the reference, but due to its ability to coordinate with *cis*-1,2 diols to form the Böeseken complex,[24] this could react with potential deboronated macrolide in a sample. Thus, boronic acids with more steric bulk were analyzed as potential standards. Additional characteristics sought after were 1) solubility in $CDCl_3$, as **2.1** was characterized in $CDCl_3$; 2) solubility in MeOD, for reaction monitoring if needed; 3) a chemical shift starkly different from 10.5 ppm. Out of 16 boranes and boronic acids found in our lab, 3 were found to meet these characteristics: **3.1**, Bis(pinacolato)diboron (B_2Pin_2), and 2-Methylphenylboronic acid (Figure 3.10).



3.1 Bis(pinacolato)diboron (B_2Pin_2) 2-Methylphenylboronic acid

Figure 3.10 Three boronic compounds soluble in both $CDCl_3$ and MeOD that would be suitable for use as internal standards with ^{11}B NMR.

These compounds could thus be added into fractions of SNC-034 at known concentrations to generate a relative ratio of boron macrolide present in an NMR sample. Figure 3.11 shows the ^{11}B NMR of 1.0:0.5, 1.0:1.0, and 1.0:2.0 solutions of 2-Methylphenylboronic acid to **3.1** as proof of concept where 2-Methylphenylboronic acid is acting as an internal standard. If the relative ^{11}B intensity of **3.1** reflected the relative amount

of **3.1** compared to 2-Methylphenylboronic acid, then this would be a successful way of quantifying boronic compounds.

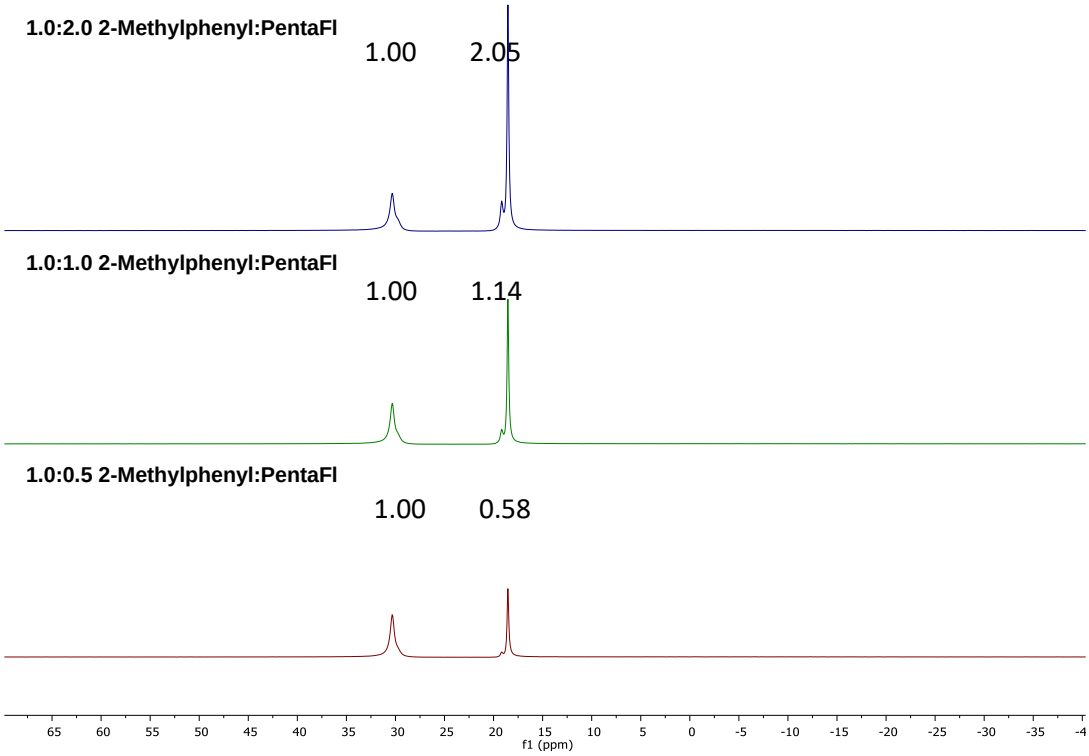


Figure 3.11 ^{11}B NMR (500 MHz, MeOD) of varying ratios between pentaflurorophenyl boronic acid (18.5 ppm) and 2-Methylphenylboronic acid (30.3 ppm) as proof of concept to test the feasibility of using an internal standard with ^{11}B NMR.

As the ^{11}B NMR intensity of **3.1** showed a relative increase to 2-Methylphenylboronic acid as its concentration in sample increased, this method could work at helping deduce relative amounts of boron macrolides from samples. This technique was greatly used in determining if fermentation and isolation conditions were viable at yielding **2.1** and analogs (Chapter 4). Something to consider going forward with this method is use of a coaxial NMR insert which would allow for the same quantification without having to mix our fractions with **3.1**. This could eliminate any potential interactions occurring that are unseen and will allow for easier recovery of material when measuring purer samples of material.

3.3 Detecting Boron Macrolides with Mass Spectroscopy

While NMR was great for the detection and quantification of **2.1** and analogs, mass spectrometry (MS) was also needed to determine the molecular weight of compounds. It is a versatile, analytical tool that measures the mass of molecules, used in conjunction with NMR to unravel NP complexity, elucidate new structures, and dereplicate known ones. It can help identify NPs without the need of isolation[25] and also be used to monitor reaction progress, so it offers a complimentary screening method to determine if **2.1** is present in a fraction.

3.3.1 LC-MS

As secondary metabolites are mostly non-volatile, we sought the use of LC-MS, which uses various types of ionization, in our case electro spray ionization (ESI), and can offer rapid determination of molecules of interest. Upon initial screening of our fractions, our samples were tested with a 20 – 100% ACN gradient with 0.1% FA, over 12 minutes, (standard in the MacMillan lab) on an analytical C18 Kinetex column, at 0.3 mL/min method. In fractions with **2.1** present, confirmed by ^{11}B NMR, the corresponding mass was absent, and instead the mass of **2.12**, M^- = [873], was seen. As I will discuss in Section 4.2, boron dissociates from the macrolide skeleton in acidified aqueous conditions, so the mass of **2.1**, M^- = [845], would not be present or detectable under these mass spectrometry conditions.

To accurately observe the masses of these macrolides, a method that bypassed the acidic buffer was needed. Thus, samples would need to be injected directly into the mass spectrometer, avoiding the column and solvent lines altogether. Samples were prepped at 0.5 mg/mL in MeOH and monitored with negative ion mode as molecules were better ionized and gave mass peaks with superior resolution than in positive ion mode.

Commonly seen in mass spectrometry, due to in-source fragmentation, are adduct formations between the compounds of interest and an ion that shifts observed molecular weights, complicating the process of structure elucidation. For example, in positive mode, compounds will generally form a sodium cluster – a [M + Na]+ ion – which will exhibit a mass 22 Daltons higher than what the molecule actually weighs.[25] Working in negative mode,

positively charged sodium adducts would not be formed, but adducts with anions such as acetate or chlorine were possible. For the macrolides with boron present, this was never an issue, as the molecule was already negatively charged, and only the $[M]^-$ ion was seen. For analogs lacking the boron though, there was always an adduct observed with a mass shifted by 35 Daltons in addition to the $[M-H]^-$ ion. These ions were chlorine adducts, which explains why for **2.12**, the masses observed were always M^- = [873] instead of just M^- = [838]. Figure 3.12 shows a curious isotope distribution pattern common amongst the analogs containing boron. The patten has been reported in other boron-binding siderophores and is what indicates that there is an element present other than the expected C, H, N, or O.[26] In fact, the observed isotope distribution pattern is what lead us to considering boron and the **2.2** family of compounds upon initial identification and isolation of **2.1**.

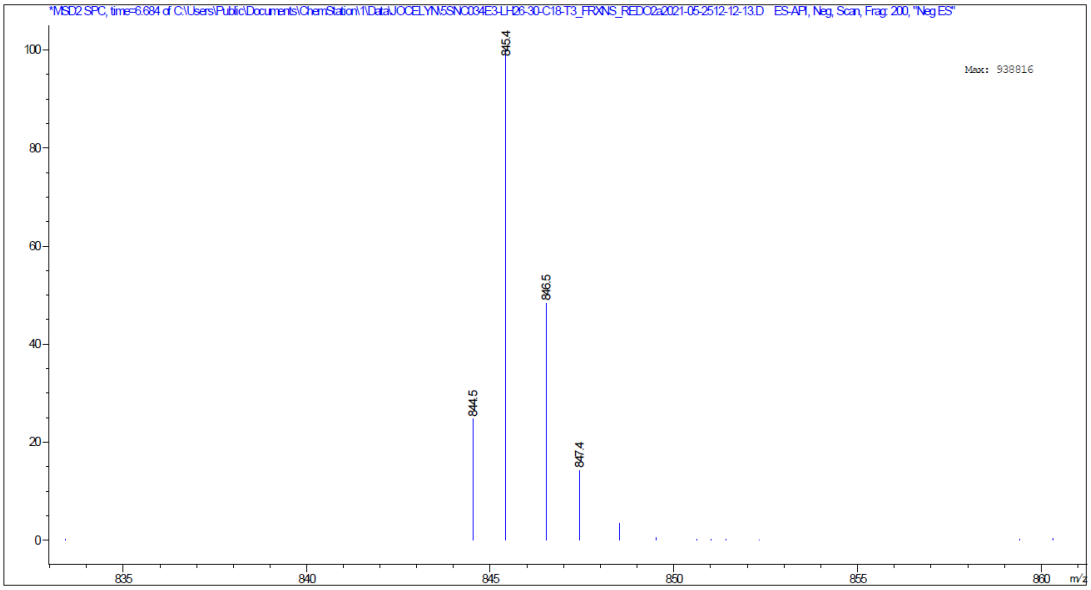


Figure 3.12 A curious isotope distribution pattern was observed amongst all macrolides containing boron.

3.3.2 ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) was also attempted as it's regarded as a facile method with the ability to detect low concentrations of metals and

nonmetals from samples. A sister method, inductively coupled plasma optical emission spectroscopy's (ICP-OES), has reported success in detecting boron in samples.[27]

There was much troubleshooting in sample preparation due to boron's prevalence in the environment to limit artificial noise in the ICP-MS matrix that would interfere with samples. Various grades of water were analyzed for their boron content to determine the best for sample prep (Table 3.1). Samples were additionally tested with 1 – 3% EtOH as **2.1** was not soluble in total aqueous conditions, and EtOH stored in plastic would not introduce leeched boron like MeOH from borosilicate glass bottles potentially could. LC-MS-grade water was determined to be the cleanest of boron at 0.12 ppb boron per 1 mL sample.

Sample	[B] ppb
MiliQ Water	0.15
MiliQ Water, 1% EtOH	0.16
MiliQ Water, 2% EtOH	0.16
MiliQ Water, 3% EtOH	0.16
HPLC Water	0.42
LCMS Water	0.12
25% Seawater in LCMS Water	1.00

Table 3.1 Analysis of different grades of water purity for sample prep to limit artificial boron content for ICP-MS analysis.

As other sources of boron contaminants would be sample vials, sample prep was done in plastic to avoid borosilicate leeching. Seawater preparation was also analyzed in glass versus plastic flasks. No significant difference was observed: 1.79 ppb B glass to 1.76 ppb B plastic, suggesting that there would not be significant differences in boron counts between the two and thus our seawater prep could continue with glass flasks.

2.12 was run in triplicate and gave 0.19 ppb boron count compared to blank 25% seawater in LCMS H_2O with 2% EtOH which gave 0.24 ppb B – consistent for samples not having boron in them. The samples containing boron though gave inconsistent readings between each other, with high relative standard deviations, making quantification inconclusive.

While sample prep was minimally invasive, data acquisition became challenging in weeks following due to high levels of boron in the ICP-MS instrument matrix and inconsistency with readings. Samples were run in triplicate and yielded wide ranges of values, and much background boron was detected even though the samples were prepped in LCMS H_2O. The problem was boron retention and carryover in the ICP-MS by samples ran before our macrolides shifted boron counts to high levels – hundreds of thousands of counts per second that dwarfed our macroldie's signals. "Flushing" the system to lower the boron baseline would require much time, making ICP-MS unviable for quick sample analysis. So, while ICP-MS could have been a viable method to extrapolate quantitative data of **2.1** growth conditions, efforts were focused on ^{11}B NMR and LC-MS methods as they were much more rapid experiments with consistent results.

3.4 Discussion

Boron is a fascinating element with remarkable chemical properties and biological activities due to its unique electronic structure. It plays an ever-increasing role in drug development, making up the active agents in antibacterial, anticancer, antifungal, and antiviral therapies, amongst others.[28,29] The ability to find structurally new boronic acids, boronic esters, and boronates from natural sources has the potential to discover equally novel biologies elicited through mechanisms not yet known.

As NPs are chemically diverse metabolites with complex structures, innovation in the field is driven by the need for techniques to identify novel structures. HTS for identifying boron NPs has not been executed before due to lack of boron's presence in NP space from sources other than plants.[14,30] Only a fraction of the known boron-containing NPs are found from microbial sources, for example. This, we believe, can be remedied by identifying boron's presence in libraries, with ^{11}B NMR, before isolation as opposed to elucidating it within a pure compound.

Boranes and $B(OH)_3$'s can be sensitive to acidic environmental conditions. It is easy to lose the boron moiety in a molecule with traditional HTS and isolation methods, e.g., a chelated boron five-membered ring like that of NP-34's can be removed with exposure to acidic buffers in assays or chromatography (See Section 4.2), for example. To look for boron after isolation can be fruitless due to loss before structural elucidation. That is why the non-invasiveness of NMR is an appealing screening method. NMR gives an indication of the classes of molecules present in libraries, better informing isolation schemes, to protect the structural integrity of desired molecules.

Thus, the composite phase-cycling pulse sequences to silence extraneous background noise from NMR probe's components was a great application to ^{11}B NMR. With its newfound sensitivity, quantitative amounts of boron compounds are now detectable from crude, semi-crude, and pure fractions. Not only does this give a novel screening handle, but it is also an exciting opportunity for furthering the biology of boron NPs. Isolated NPs frequently exhibit suboptimal properties in their natural state, and functionalization is needed to yield analogs with superior properties. This sensitive NMR experiment will be a quick and easy confirmation that synthetic efforts have not altered the boron's presence or confirmation – especially when the boron is crucial for activity, which would otherwise be difficult on such miniscule scales.

Additionally, this sensitive experiment will allow ease of elucidation of novel boron-containing structures. Boron being diamagnetic, can cover large ranges of about 250 ppm within its various configurations.[3] Having its NMR shift can easily determine electronic structure and geometrical configuration to assist in structural determination. Additionally, this pulse program was also successfully applied to the standard ^{11}B no proton decoupling experiment (zg standard experiment). In the case that naturally occurring boranes are identified, the no proton decoupling experiment can show splitting from a B-H bond with the same sensitivity as the decoupling experiment. As most natural boron species are expected are to be $B(OH)_3$ or esters, the decoupled experiment is suggested to screen libraries via identification of the

number and the species of boron, followed by the no proton decoupling for structural elucidation once a pure compound is obtained. As the boron in NP-34 is covalently bonded to 4 oxygens, no splitting is observed when using ^{11}Bzgbs and so ^{11}Bzgbsig is used for screening for NP-34 as we need to look only for its characteristic BO_4^- shift at 10.5 ppm.

Aside from using to guide NP-34 and analog isolation and characterization, future work with this experiment consists of moving forward to screen and identify microbially-produced boron compounds within microbial libraries to diversify the chemical diversity of NP space. We are optimistic at the prospects of using this to identify and isolate novel boron-containing structures of biological interest.

MS data provide an excellent complement to NMR data for solving unknown structures and are useful for rapidly searching a database to determine whether a compound was previously identified. Those benefits notwithstanding, NMR is the far more effective technique for solving unknown structures, provided enough purified material are available. MS can enable identification without isolation, so it offers the potential to resolve many of these issues. Still, limitations loom, and they must be addressed before we can consider MS an optimal technique for structure elucidation.[25]

3.5 Chapter Three References

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CHAPTER FOUR

MEDIA AND ISOLATION OPTIMIZATION, CRYSTALIZATION, AND GENERATING A LIBRARY OF BORON-CONTAINING MACROLIDES

4.1 Problems with NP yield

One of the biggest hurdles to working with NPs, no matter the producing organism, is low yields.[1] As these compounds are products of secondary metabolism, they are not required for growth or basic metabolic functions. Rather they mediate ecological interactions, increasing the survivability or fecundity of the host organism, and are only produced when needed. Extractions often yield low amounts of material due to slow biomass accumulation and low molecular produced by the host.[2] The limited material restrains the scope of SAR and biological testing available on the molecules of interest. Additionally, NPs are very structurally diverse and complex molecules, making total chemical synthesis difficult with similarly diminished yields, so it is an unviable option for scale-up unless greatly optimized. Hence, it is challenging to get enough of these compounds for industrial-scale purposes.[2]

As a result, it is very common to explore methods to alter microbial biosynthesis that result in higher production of these desired metabolites. Optimization of SNC-034's growth conditions, including media components and culture conditions, could affect the growth of **2.1**. But, producing large quantities of **2.1** and analogs would be fruitless if suboptimal methods are used for isolation the metabolites. As will be discussed, the isolation of **2.1** is challenging due to the instability of the boron and the challenges of detection. Optimization of the isolation is needed to be able to extract as much of the metabolite from biomass to compliment optimized biosynthetic processes.

A thorough analysis of **2.1**'s isolation methods were conducted to validate if there was cause for suboptimal yields of **2.1** during purification. This was followed by analysis of media conditions for optimal SNC-034 growth. Success in identifying methods to create and isolate **2.1** were promising as there was an increase from 0.1 mg/L isolated to 12.3 mg/L isolated based on the work explored here. In the process, various analogs were isolated and or semi-synthetically created to generate a library for biological testing. Crystallization methods for these analogs were established to study the structural and thus biological activity of these

molecules. Additional work revolved around trying to perform click chemistry for MoA studies but were unsuccessful.

4.2 Optimization of NP-34 Isolation

Initial isolation of **2.1** was achieved with an XAD-7 and acetone extraction of organic material from aqueous bacteria culture, which are typical extraction conditions in the MacMillan Lab. This was followed by a solvent partition with 2:1 EtOAc:Deionized water (DIW), and then by a series of chromatography to separate individual compounds. The chromatographic series involved fractionation by silica column with 0%, 1%, 2%, 3%, 5%, and 10% MeOH in DCM elution. **2.1** and other boron-macrolides would coelute at 3% MeOH and would then be separated on a series of iterative C5 HPLC columns with 100% ACN in 0.1% FA (Figure 4.1).

Figure 4.1 The initial isolation procedure for NP-34

This process was lengthy, and overtime began yielding less macrolide. Efforts to streamline this process and address the loss of material with aqueous conditions during HPLC resulted in loss of the Böeseken complex within **2.1**. This was surprising as the other known macrolides like **2.5**, **2.3**, and **2.10**, etc. were isolated previously with boron covalently bound.

Typical HPLC conditions would include the use of acid buffer in aqueous solvents to not only help ionize material for better separation, but also keep column matrices intact and free of harmful bacterial growth. Our lab employs 0.1% formic acid (FA) in solvent solutions to achieve this, but unaware to us at the time, acidified aqueous conditions readily cause dissociation of boron from *cis*-diol systems as such (Figure 4.2).[3]

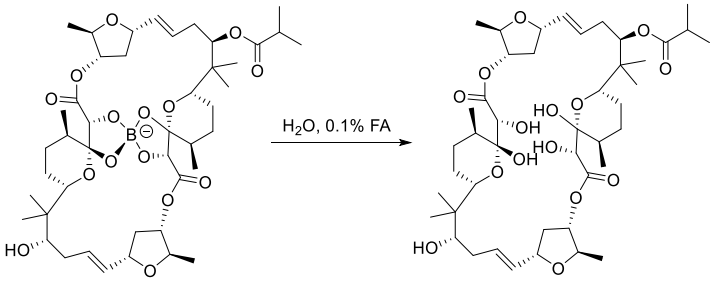


Figure 4.2 Dissociation of boron from NP-34 was observed upon attempting to streamline its isolation with mobile phases employing H_2O with 0.1% formic acid.

For further validation, two samples of 2.5 mg semi-crude SNC-034 containing **2.1**, ready for HPLC separation, were dried in separate 1 mL scintillation vials. One sample was dissolved with 1 mL HPLC H_2O without 0.1% FA and the other with 1 mL HPLC H_2O with 0.1% FA. Both samples prior to the experiment gave the 10.5 ppm Böeseken complex shift via ^{11}B NMR. After stirring in their respective solutions for approximately 10 minutes, both samples were dried and resuspended in chloroform-*d.* The neutral sample (pH 7.0) showed boron signal at 10.5 ppm whereas the acidified sample (pH 3.0) did not, indicating that boron had dissociated from the complex (Figure 4.3). The acidified sample vial contained a white solid insoluble in $CDCl_3$; it dissolved in MeOH-*d* and gave a ^{11}B NMR shift at 18.3 ppm, indicative of $B(OH)_3$, further confirming dissociation.

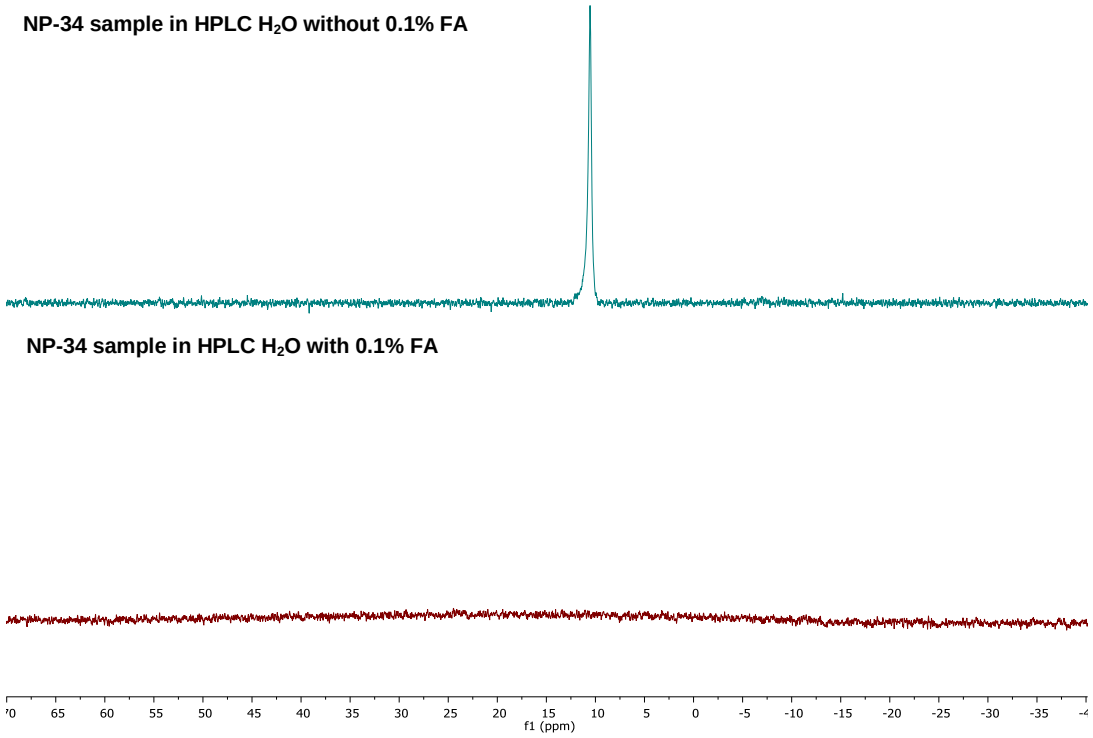
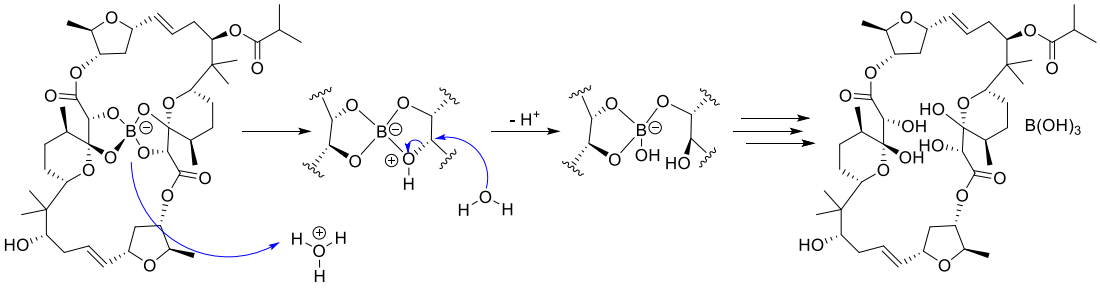


Figure 4.3 ^{11}B NMR on 500 MHz in $CDCl_3$, shows that NP-34 in water without acid buffer retains its boron (top) whereas NP-34 in acidified water loses its boron (bottom). No peak is seen for $B(OH)_3$ as it is insoluble in $CDCl_3$.

As the mosquitocidal activity of **2.1** is dependent on the boron, this was problematic. Retention of the boron would be more efficient and better yielding than synthetically reintroducing it after isolation. This would require optimization either without the use of acid or without the use of aqueous conditions. Using **2.10**'s isolation as a guide,[4] numerous gradient and isocratic-based methods were attempted on a Phenomenex Kinetex C18 column with varying mobile phase mixtures of neutral ACN, MeOH, and H_2O to test for separation and boron retention. With each trial, **2.1** was isolated without dissociation of the boron, confirmed by ^{1}H, ^{11}B NMR, and LC-MS.

Since dissociation only occurred when acidified aqueous solution was introduced and not when 100% organic solvent with FA was used, I believe H_3O^+ causes the dissociation of boron (Scheme 4.1). I propose dissociation occurs through iterative nucleophilic attacks of H_2O

at the diol carbons of the boron complex: C2, C2', C3, and C3'. This reaction would be catalyzed by deprotonation of H_3O^+ by the diol's oxygens, causing a subsequent break in the covalent bonds between boron and oxygen.



Scheme 4.1 Proposed mechanism for dissociation of boron from macrolides with H_3O^+

With the issue of acidity affecting the macrolide resolved, further analysis into the isolation of **2.1** and other analogs could be conducted. Further efforts to streamline the isolation process would require validation that there was no further macrolide decomposition.

4.2.1 Effects of Chromatography

One aspect to optimize was the chromatographic series used to isolate **2.1**. Chromatography is the separation of compounds in a mixture by use of solvent (mobile phase) carrying material through a solid matrix (stationary phase) which is mounted on a surface such as a column or a plate, etc. The chemical composition of both the mobile and stationary phases affects the compounds' interactions with the stationary phase. The better affinity a compound has for the stationary phase, the longer it'll retain on the matrix, allowing those with less affinity to elute out with the mobile phase, resulting in separation. Subtle differences in both phases can result in differential retention, thus affecting level of purification. The extent of these interactions to separate boron macrolides or to, hopefully not, disrupt boron bonds can be tested by varying both phases.

Silica Flash and LH20 Chromatography

Early in the isolation pipeline, there's the need to separate molecules of interest from other smaller metabolites before HPLC separation. HPLC separation is typically the final round of purification as it offers highest resolution and very precise separation of compounds. Too many coeluting metabolites will hinder it as it takes longer than flash chromatography and is higher pressure, requiring work at smaller concentrations.

Early isolations used silica gel for this initial fractionation step. Silica chromatography is "normal phase" where the silica stationary phase is polar and interacts strongly with polar compounds, and nonpolar compounds, like **2.1**, elute out first with much less polar mobile phase gradients. As it is a slightly acidic matrix, there was no certainty that no other decomposition was occurring due to use of this column. Since this method doesn't offer vast separation, other methods were explored to offer more effective fractionation without the presence of acid.

Sephadex® LH-20 was considered a suitable alternative. It is a gel column composed of a beaded, hydroxypropylated cross-linked dextran that offers separation of hydrophilic and lipophilic compounds including steroids, fatty acids, lipids, and vitamins, etc.[5] Various solvents will cause the beads to swell in size, giving access to channels that offer an added element of separation by molecular size: smaller compounds retain on the column by swimming through the channels, while larger molecules elute faster between the beads. **2.1** being a large, lipophilic molecule could achieve great separation due to its hydrophobicity and its large size would help separate it from smaller co-eluting metabolites. Additionally, since it is a non-acidic matrix, these less harsh conditions could potentially lead to better yield of compound.

To test its viability, 10L of SNC-034 were grown: 5L to be dedicated to fractionation with silica gel (13B) and 5L for LH20 (13A), to compare the yield of **2.1** between the two. There was no significant difference between semi-crude yields of organic material after extraction and subsequent EtOAc extraction. After silica chromatography, 101.8 mg of material were collected that needed further separation whereas 48.0 mg of material were collected from LH20. ^{11}B

NMR with **3.1** as internal standard showed a higher abundance of boron macrolides from semi-pure material after LH20 than from silica fractionation (Figure 4.4). Both sets of material were purified with isocratic 95% ACN on C18 under acid-less conditions (See E.5.2).

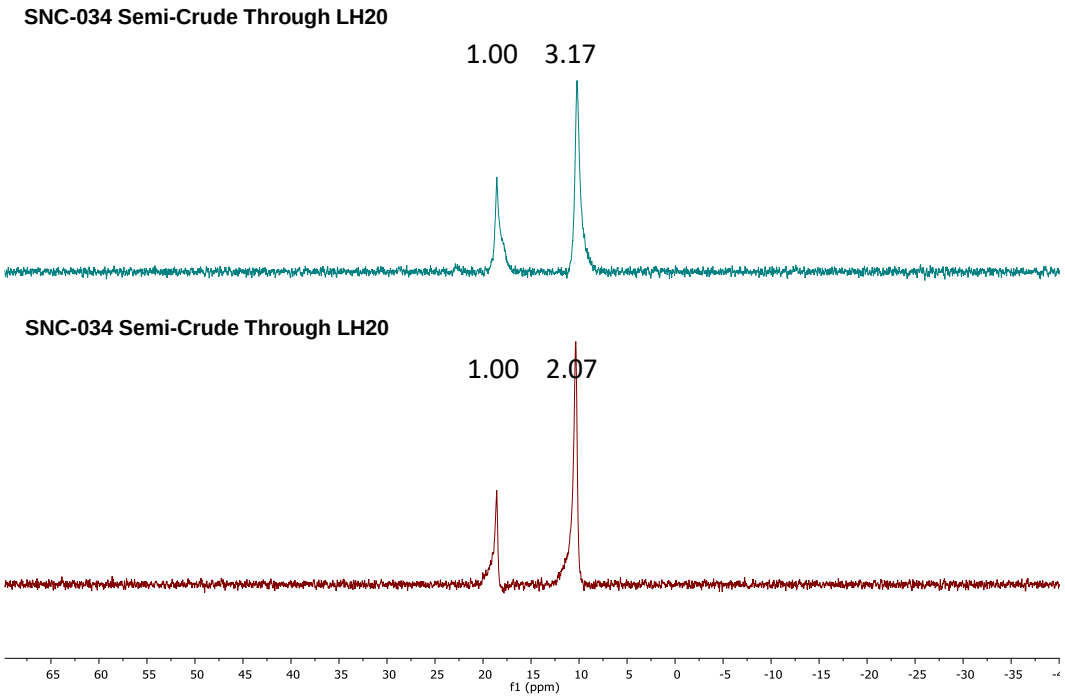


Figure 4.4 ^{11}B NMR on 500 MHz in MeOD of semi-crude SNC-034 samples after LH20 (top) vs. silica (bottom) chromatography. Comparison to internal standard pentafluorophenylboronic acid shows higher macrolide intensity after LH20 chromatography than with silica.

Relatively no difference was seen in the final yield of **2.1**: 0.6 mg were collected from the silica batch and 0.9 mg from the LH20 batch. This indicates that there should not be any considerable degradation of **2.1** and co-occurring analogs between either column. A higher volume of macrolides overall were isolated from the LH20 route, in accordance with the slightly larger ^{11}B NMR intensity of 13A sample. The LH20 column though did separate macrolides better from smaller co-eluting metabolites, decreasing the amount of HPLC purification required, and overall hastening isolation. For that reason, silica gel flash chromatography was replaced with LH20 chromatography.

Reverse Phase HPLC Chromatography

C18 is the most used reverse phase column. Its matrix is composed of 18 carbon-long alkyl chains bonded to silica dioxide and is regarded as an excellent separator of long fatty acids as compared to smaller molecules. As previously mentioned, C18 chromatography was chosen for HPLC purification of **2.1** based on its success at isolating **2.10**. The solvent system however needed tweaking as **2.10**'s didn't fully purify **2.1**. Isocratic methods at 100% and 90% ACN and MeOH on C18 showed purification of **2.1** comparable to 95% isocratic ACN, but 95% yielded the most amount of pure **2.1** without the need of further purification. Step gradients and gradual inclines from 20 – 100% or 80 – 100% organic solvent in water were tried on C18 and likewise, 95% isocratic ACN showed the best separation.

Since iterative C5 with only ACN with 0.1% FA was originally used for **2.1**'s separation, it was tested to see if addition of the aqueous component would better separate the compounds without the need of subsequent fractionation. With all efforts, 95% ACN on C18 remained the best method. This is because of C18's greater retention of more lipophilic compounds compared to C5. C18 having a longer carbon chain length than C5 (5 carbon-long alky chains) creates a denser column matrix due to the increased surface area. The increased density causes an increase in retention time on the column, resulting in better separation of compounds.

The Phenomenex Synergi Fusion column, which is a polar embedded C18 column, offers improved selectivity of mixtures with both polar and non-polar characteristics. Before discovery that acid was harmful to the structural integrity of **2.1**, purification was attempted with Synergi Fusion with an acidified ACN and AQ gradient. Separation of compounds was achieved, but they were boron-less analogs including **2.12**. With the retention of boron, these compounds remain highly nonpolar and thus C18 should be kept for their separation. But for natural or synthetic analogs where there is an increased amount of alcohol and diol groups present, the Synergi Fusion without an acidified matrix should be a good column for separation.

Normal phase silica HPLC was not attempted due to the acidity of the silica matrix.

Chiral HPLC Chromatography

Chiral chromatography was attempted for final stage purification, after C18 chromatography, to purify potential enantiomers within fractions. At the onset of **2.1** discovery and isolation, purification was attempted on a chiral OD-H column with 1% isopropanol (IPA) in hexanes without FA. The matrix of Chiralcel OD-H column is cellulose tris(3,5-dimethylphenyl carbamate) (Figure 4.5) on silica gel, which was problematic because polysaccharides like cellulose and alginate are known to be boron sorbents.[6,7]

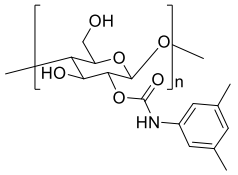


Figure 4.5 The structure of the Chiralcel OD-H matrix is cellulose tris(3,5-dimethylphenyl carbamate) on silica gel, a sugar that can interact with the boron in these macrolides.

Semi-pure samples of SNC-034 were injected through the chiral column with an isocratic run of 1% IPA in hexanes. When the sample was collected and dried, ^{1}H and ^{11}B NMR showed decomposition of macrolide skeleton and loss of BO_4^- moiety, respectively (Figure 4.6). This indicates that the cellulose matrix of the column caused boron dissociation and subsequent breakdown occurred, eluting out metabolic chunks of the macrolide. Based on this, chiral chromatography is unviable for purification of these molecules.

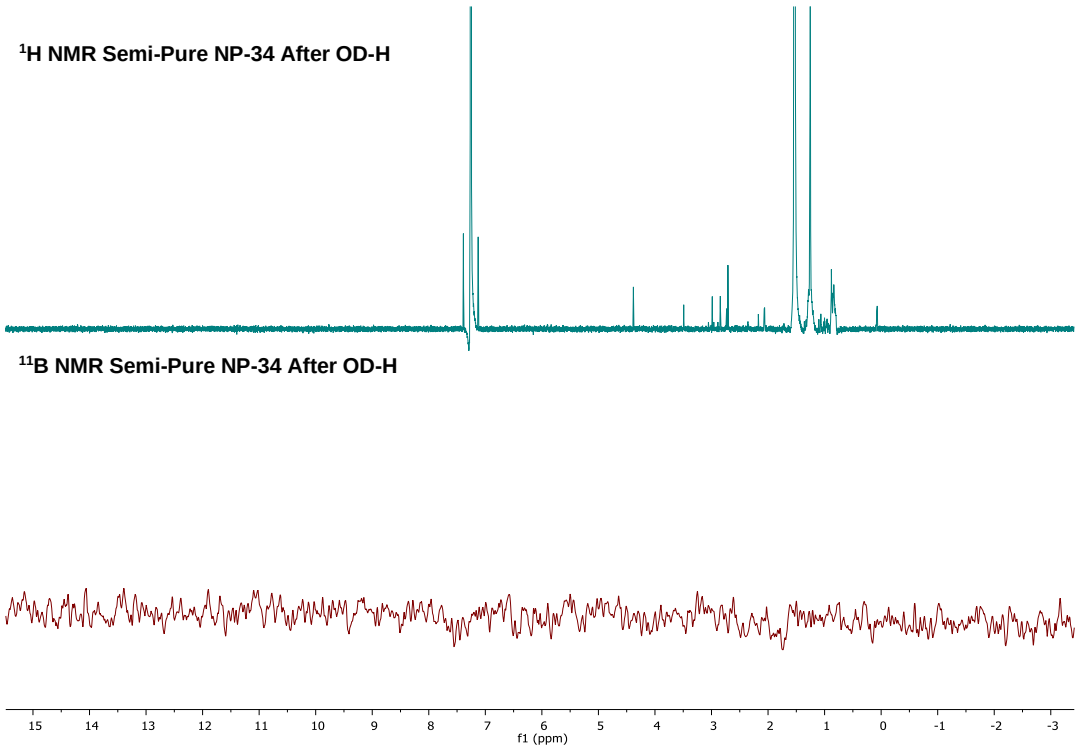


Figure 4.6 ^{1}H (top) and ^{11}B (bottom) NMR (500 MHz, $CDCl_3$) of 2.5 mg semi-pure SNC-034 fraction containing NP-34 put through chiral OD-H HPLC column for purification. The ^{1}H NMR shows decomposition of the metabolites and the ^{11}B NMR shows a loss of boron, indicating that this column is not good for purification of these macrolides.

4.2.2 Resin Efficiency

Resin vs. Organic Solvent Extraction

Before separation of compounds occurs, organic metabolites need to be extracted from their host organisms. Solvent partition is the classical and most used method of extraction.[8] The MacMillan lab instead uses non-ionic resins to extract NPs from aqueous media. These resins are commercially available and have gained popularity in the field due to their avoidance of hazardous organic solvents; their ease of handling with large volumes of aqueous material; and their suitability for processing large volumes of samples at a time.[9]

One limitation with resin, though, is that it will not necessarily extract all organic material from seawater, as the hydrophobicity, polarity, and size of a molecule will affect how

it interacts and is absorbed by the resin. Care must be taken when choosing an adequate resin to extract desired compounds, so the efficiency of our chosen resin for extraction of boron macrolides was questioned. For some compounds, like potentially the boron macrolides, perhaps the more traditional solvent partition would be more efficient than resin.

First, the issue of resin versus solvent extraction was addressed. Could a traditional EtOAc partition extract more bulk macrolide than resin, increasing yields of **2.1**? Twenty liters of SNC-034 were grown: 10L to be dedicated to an EtOAc extraction and 10L to be dedicated to resin, to compare the yield of **2.1** between the two. Partitioning with EtOAc was very tedious and after extracting the 1st liter 3 times with EtOAc (2:1 EtOAc:aqueous media), no material was observed via ^{1}H (not shown) and ^{11}B NMR (Figure 4.7). To validate that it wasn't a bad batch of SNC-034, before extracting the remaining 9L, 1L was extracted with resin for comparison. The 1L resin extraction showed the BO_4^- NMR peak at 10.5 ppm indicative of boron macrolides. Since **2.1** was seen in 1L after resin extraction, it was concluded that EtOAc did not have the capability to effectively extract these metabolites from aqueous media.

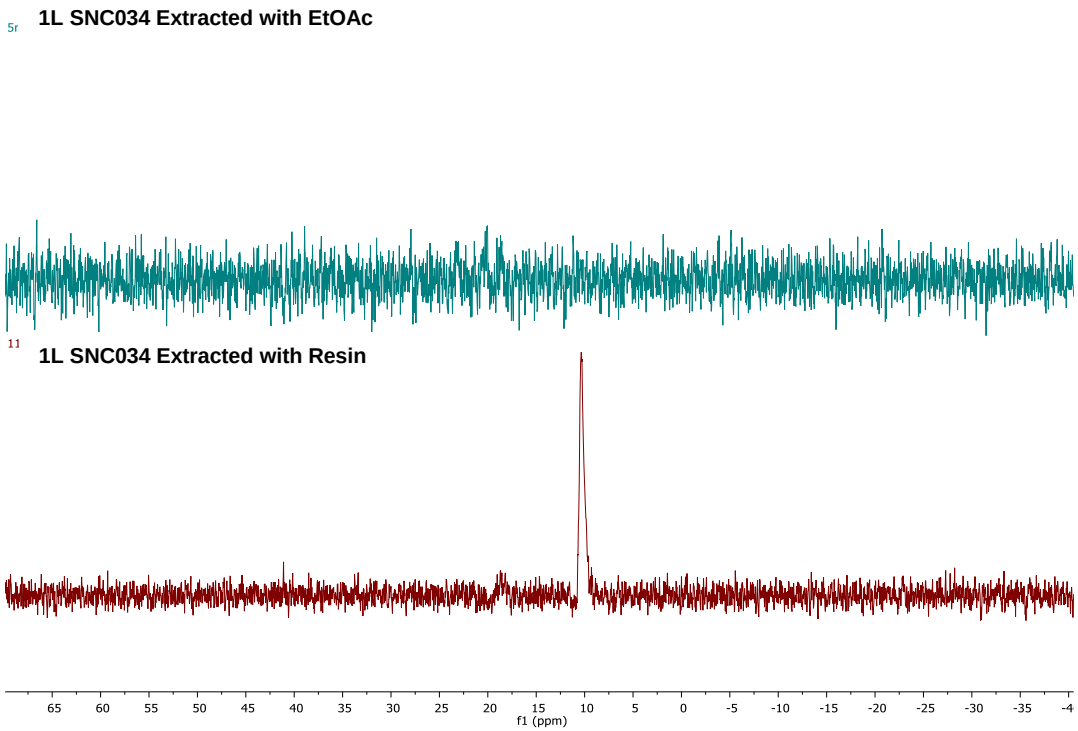


Figure 4.7 ^{11}B NMR (500 MHz, $CDCl_3$) of one liter of SNC-034 extracted with EtOAc (top) vs. one liter of SNC-034 extracted with resin (bottom). Ethyl acetate is not an effective method for extracting boron macrolides from fermentation media.

Factors including diffusivity, polarity (thus solubility), temperature, solvent-to-solid ratio, etc. can affect extraction efficiency.[8] To test if it was not an issue of solvent partition but rather of solvent choice, partitions were attempted with hexanes and DCM, but likewise were poor at extracting organic material from the fermentation media. Heating of the fermentation media or extraction solvent was not attempted due to (1) the volatility of the proposed organic solvents, (2) the scale of fermentation was too large to adequately heat with the lab's instruments at hand, and (3) NPs can decompose at elevated temperatures which was undesirable. Based on the ineffectiveness of solvent partition and lack of options for optimization, resin was deemed best for extracting these macrolides.

Note: This is separate from the 2:1 EtOAc:DIW partition done after resin extraction. EtOAc seems to not have the capability to extract from extracellular media that is very dense. Though, the 2:1 EtOAc:DIW partition is successful when used on organic residue after resin

extraction as a method to remove all water soluble components, like salts, that might have carried over accidentally and are undesirable to load onto columns.

Resin Type

Based on the work by Surup *et al.*, their isolation of **2.10** involves the use of nonionic macroreticular resin XAD-16.[4] While it captures organic material from aqueous media through hydrophobic and polar interactions like XAD-7 does, XAD-16 is specifically tailored for hydrophobic compounds whereas XAD-7 is for moderately polar compounds.[10] Since **2.1** is nonpolar and very hydrophobic, changing to the more nonpolar XAD-16 could lead to greater capture of **2.1** from fermentation media. If XAD-7 did not have the proper affinity for **2.1**, product could have been lost over the course of this project due to inadequate capture from media. Additionally, the time of resin introduction could be making a difference. Based on personal communications, there was reason to believe that resin added at the onset of fermentation could capture NPs as they were produced. Potentially this would be more efficient as the media would not get as saturated, allowing more product to form.

Eight liters of SNC-034 were grown for this experiment: 4L for XAD-7 and 4L for XAD-16 extraction that would be used to compare the yield of **2.1** between them. Of each of the 4L, 2 liters would have resin added at the onset of fermentation and be extracted at day 3 and day 7 of fermentation. The remaining 2L would have resin added 2 hours before extraction, which is the typical procedure in our lab (See E.2.2). Addition of XAD-7 on day 7, 2 hr before extraction would serve as the control liter. Day 3 was chosen due to being the highest **2.1** yielding condition in an experiment testing for extraction date of SNC-034 (See section on addition of boric acid). The resin to be added on the onset of fermentation was autoclaved in DIW prior to the experiment to avoid contamination that could kill SNC-034 upon addition. Filtration through cheesecloth and acetone soak was done as normal (See E.2.2). The 4L that had resin added at the onset of fermentation were removed from the shaker and filtered through cheesecloth on their respective days without any further processing. The other 4L had resin added on their

respective extraction day and allowed to shake for an additional 2 hr before filtering through cheesecloth (Figure 4.8).

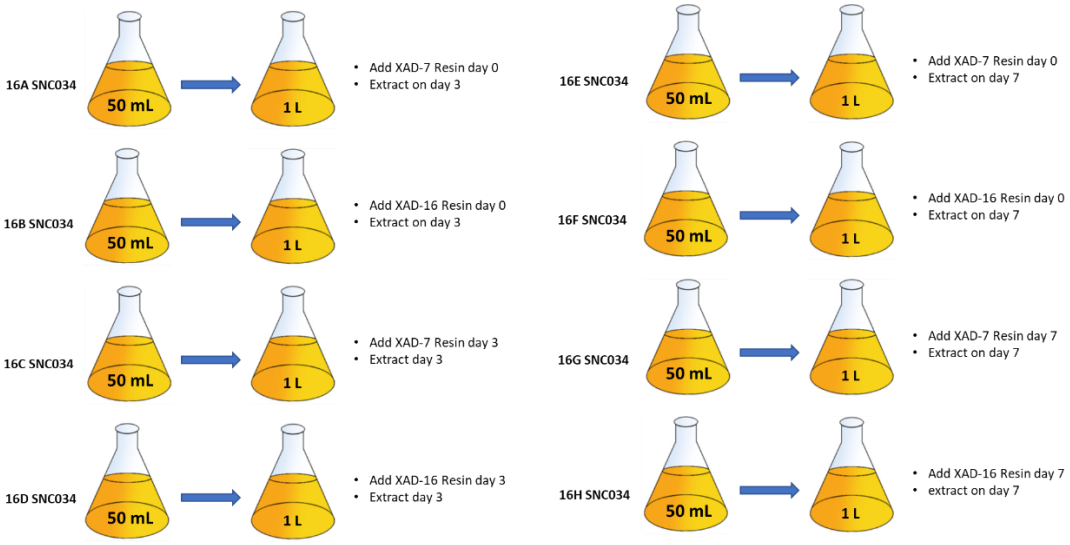


Figure 4.8 Experimental setup for analysis of resin efficiency including what day of resin addition, which type of resin, and which day it would be extracted.

After all 8L were extracted with acetone and dried down, the organic residue was extracted with 2:1 EtOAc:DIW (ph 7.0). Seventy milligrams of each semi-crude organic layer were resuspended in a solution of 1 mg **3.1**/mL MeOD for relative comparison of macrolide yields (Table 4.1). All were then purified through LH-20 chromatography, and the fractions containing ^{11}B-macrolides were purified with 95% ACN on C18 HPLC for yields of **2.1**.

Sample	Resin	Addition Day	Extraction Day	Organic Semi-Crude	^{11}B NMR Ratio	NP-34 Yield
16A	XAD-7	0	3	424.1 mg	1.95	--
16B	XAD-16	0	3	106.9 mg	4.23	--
16C	XAD-7	3	3	134.7 mg	11.09	12.3 mg
16D	XAD-16	3	3	161.3 mg	7.17	3.0 mg
16E	XAD-7	0	7	95.3 mg	7.79	1.6 mg
16F	XAD-16	0	7	81.2 mg	6.30	--
16G	XAD-7	7	7	185.2 mg	5.45	6.3 mg
16H	XAD-16	7	7	115.3 mg	4.97	--

Table 4.1 Data from resin extraction test summarizing the yields of semi-crude metabolites, their relative ^{11}B NMR ratios compared to internal standard pentafluorophenylboronic acid, and final NP-34 yield for the 4 conditions that yielded quantifiable amounts of NP-34. Based on the results, XAD-7 addition on day 3, 2 hr before extraction yields the most NP-34.

Only conditions 16C, 16D, 16E, and 16G gave a quantifiable amount of **2.1** based on presence of 6.45 ppm proton after LH20 chromatography. These 4 fractions were carried forward to isolate **2.1** with isocratic 95% acid-less ACN on C18. Comparison of ^{1}H NMRs (not shown) after HPLC purification shows greatest intensity of **2.1** under condition 16C: XAD-7 addition 2 hours before extraction on day 3 of fermentation. This condition yielded 12.3 mg **2.1**, the most yield per liter seen yet. The second-best condition with 6.3 mg yield was how **2.1** had been extracted to date: XAD-7 added 2 hours before extraction on day 7 of fermentation. So, it had been best to add XAD-7 2 hr before extraction, but with extraction on day 3 instead of day 7.

Chronologically, this was the last experiment performed of all the optimization studies. It combined of all the conclusions gathered with the only factors tested being resin and time of addition. This experiment gave the highest yield of **2.1** than any other experiment.

4.3 Optimization of Fermentation Media

Biosynthesis of NPs can be stimulated with altering growth parameters including media composition and cultivation time, etc. - even the most subtle adjustments can result in drastic changes.[11] Some sort of shock to the organism by environmental stimulation, or lack of or supplementing a nutrient could promote secondary metabolism. For example, limiting alanine in culture combined with a presence of low pH can induce production of methylenomycin in *Streptomyces coelicolor*.[11,12] Likewise, optimization of SNC-034 media and growth conditions to mimic environmental conditions naturally found could trigger secondary metabolism into producing **2.1** in large quantities.

4.3.1 Boric Acid

Boron is prevalent everywhere, including in rocks, soil, and water, rarely found in elemental form, but rather as borate esters in biological systems and as inorganic oxides in minerals.[13] It has a solubility in water of 57 g/L and is abundant in seawater at 4 x $1O^{-4}$ M,

existing as $B(OH)_3$ and $B(OH)^{4-}$.[14,15] While it is an essential micronutrient for a plethora of organisms including terrestrial plants and marine algae, it can be toxic at high environmental concentrations.[16] Toxicity in plants starts at concentrations exceeding 100 ppm, and at concentrations ranging from 10 – 300 mg/L , it begins to be toxic for different fish species.[15]

Possibly, large concentrations of $B(OH)_3$ could also be toxic to microbes such as *Streptomyces malachitospinus,* the bacterium making up our SNC-034 strain. As boron is highly concentrated in the ocean, marine organisms can develop homeostatic control mechanisms to aid in detoxification of external toxins as such.[16] Potentially then, the production of **2.1** and other analogs could be a way of shuttling out excess boric acid to avoid toxicity.

Shuttling of boron has been seen in marine bacteria with Fe^{3+} chelating siderophores that compete with boron for binding. One such example is vibrioferrin, when isolated from strains of *Marinobacter*. When 0.4 mM $B(OH)_3$ was added to media, boronylated vibrioferrin was isolated, confirmed with LCMS and ^{11}B NMR shift at 8.5 ppm. This yields the possibility that vibrioferrin could be acting as a boron-transporter for these marine species. It would not be a surprising mechanism of defense as plants are known to have membrane-associated boron transporters.[16,17] Thus, affecting boron content during fermentation can likely induce a change in the biosynthesis of **2.1** and analogs as a response to needing to transport boron extracellularly.

Amount of Boric Acid

The first experiment was to test the feasibility that varying the concentration of $B(OH)_3$ could stress the bacterium into producing more macrolide. Additionally, assurance was needed that adding more $B(OH)_3$ wouldn't pose excess toxicity and kill the bacterium. The lab's seawater recipe is made to mimic conditions in natural seawater. The recipe requires 460 mg/20L of fermentation, so per liter of media, one equivalent of $B(OH)_3$ is equal to 23 mg/L.

Two 20L batches of SNC-034 were grown with no alteration to fermentation media except that one batch (series 9BA) had two eq of $B(OH)_3$ whereas the second batch (series 9) had only one eq $B(OH)_3$ and served as control. The resin extraction and chromatographic isolation was the same between both batches. ^{11}B NMR of semi-crude material with **3.1** internal standard showed a 12-time difference between the two batches – two eq of $B(OH)_3$ yielded a higher intensity of macrolide peaks compared to control (Figure 4.9).

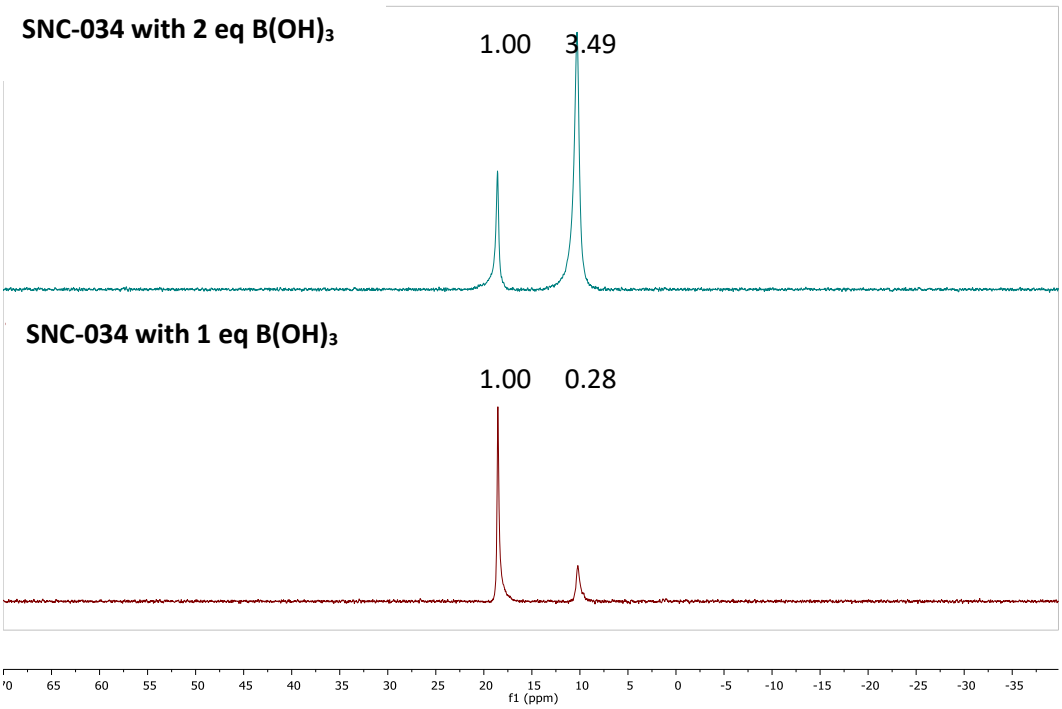


Figure 4.9 ^{11}B NMR (500 MHz, $CDCl_3$) of SNC-034 with 2 eq of $B(OH)_3$ during fermentation (top) vs. 1 eq (bottom). Comparison with internal standard pentafluorophenylboronic acid shows approximately 12 times more boron macrolide present in $B(OH)_3$-supplemented media.

The supplemented $B(OH)_3$ yielded 4.1 mg pure **2.1** and 2.2 mg needing further purification, whereas the control with only 1 eq $B(OH)_3$ yielded 2.0 mg pure **2.1**. Thus, the doubling of $B(OH)_3$ in seawater resulted in approximately a 2-3 time increase in **2.1** yield compared to control. This is promising for efforts in inducing macrolide production, and it seemed like there was no harmful consequences to the bacterium.

Following the success of this experiment was to test the maximum eq of $B(OH)_3$ that would positively influence macrolide production. Eight 1L cultures of SNC-034 were prepared, each containing a different amount of $B(OH)_3$, between 0 -7 eq, to test which condition yielded the highest amount of **2.1** (Table 4.2). Liter 10B contained 1 eq $B(OH)_3$ and served as the control group. All cultures were extracted after 7 days of fermentation with XAD-7 resin and isolated with the same chromatographic steps.

Sample	Equivalents of Boric Acid	^{11}B NMR intensity	Semi-crude SNC-034 (mg)
10A	0	0.69	80.0
10B	1	4.14	154.3
10C	2	9.58	92.1
10D	3	11.05	225.5
10E	4	8.72	99.3
10F	5	4.97	92.1
10G	6	3.85	78.0
10H	7	4.79	90.6

Table 4.2 Results from varying boric acid equivalents in SNC-034 fermentation.

Figure 4.10 shows the ^{11}B NMR of all 8L, after EtOAc partition, with **3.1** internal standard. Based on ^{11}B NMR, samples 10D with 3 eq $B(OH)_3$ during fermentation was the best condition as a gradual increase in macrolide intensity is seen from 0 – 3 eq and a gradual decrease from 4 – 7 eq $B(OH)_3$. Two and 4 eq would have also been viable options at inducing production of macrolides, but not to the extent that 3 eq did. As 1 eq was the control group, it was not surprising that it had a lower ^{11}B intensity.

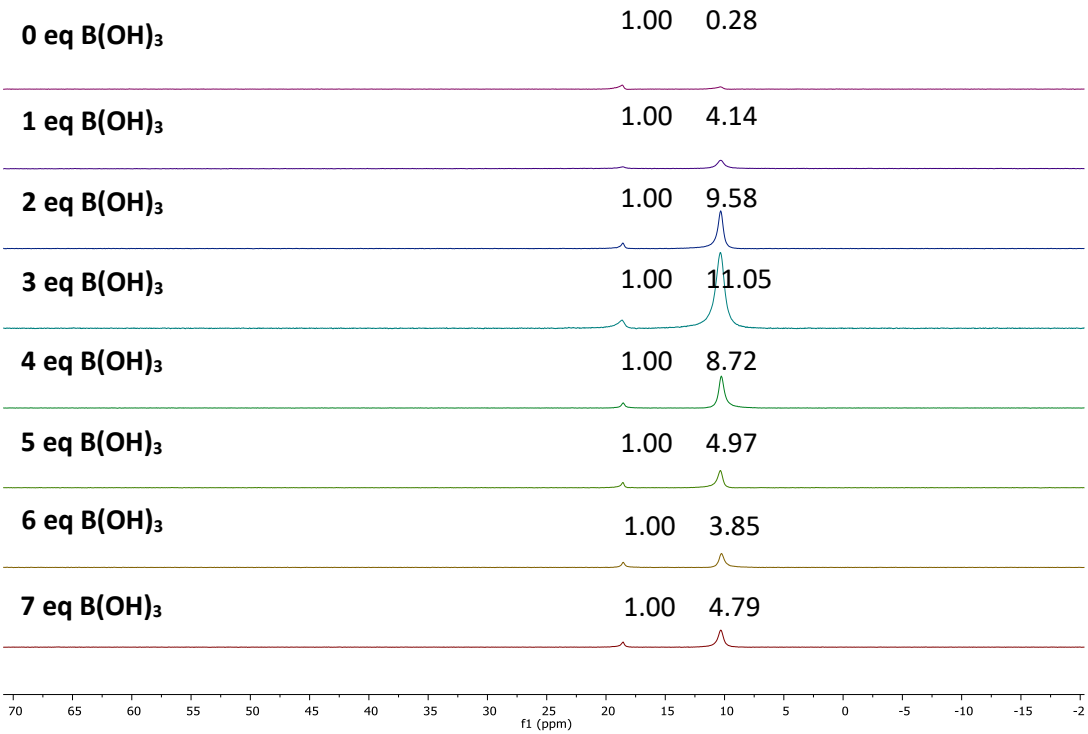


Figure 4.10 ^{11}B NMR (500 MHz, $CDCl_3$) of 10A – 10H, the 8 different SNC-034 liters with varying equivalents of $B(OH)_3$ compared to pentafluorophenylboronic acid internal standard.

Fraction 10D was purified through C18 HPLC chromatography, isocratic 95% acid-less ACN to yield 5.9 mg pure **2.1**. The remaining conditions B – H were combined for purification, and between the 6L gave a total yield of 10.7 mg **2.1**. This illustrates that 3 eq $B(OH)_3$ gives maximum production of these macrolides. Higher than 3 eq of $B(OH)_3$ could potentially be eliciting a toxic effect towards the bacteria where they are unable to respond to the external stimuli with NP production.

Addition of Boric Acid

We also wanted to consider whether the timing of $B(OH)_3$ addition affected production. The hypothesis that SNC-034 responds to $B(OH)_3$ with creation of the macrolide skeleton to shuttle out boron would support that the BGC is activated upon the external stimuli. But the question was posed if the bacterium needed to grow first before it can start producing macrolide and the presence of $B(OH)_3$ is hindering that? Could production be thwarted at the onset of

fermentation due to presence of $B(OH)_3$? While the former seemed the more probable explanation for macrolide production, late-stage addition of $B(OH)_3$ was still attempted to validate if it would or would not result in higher yields of. **2.1**.

Seven liters of SNC-034 were prepared as normal but without $B(OH)_3$ in the seawater. Each day, 1 eq of $B(OH)_3$ was added to one liter and on day 7, all 7L were extracted with XAD-7 resin. The first liter (12A) was the same as control (12G) as both had $B(OH)_3$ added at the onset of fermentation. Potentially, more **2.1** could be seen over time if the late-stage addition would stress a grown and mature bacterium into producing these secondary metabolites to protect itself from external stimulus. Figure 4.11 shows the ^{11}B NMR of all 7L after resin extraction. 12A and 12B, which had addition of $B(OH)_3$ at the onset and after 24 hr of fermentation, respectively, yielded macrolide whereas addition any time after 24 hr fermentation did not.

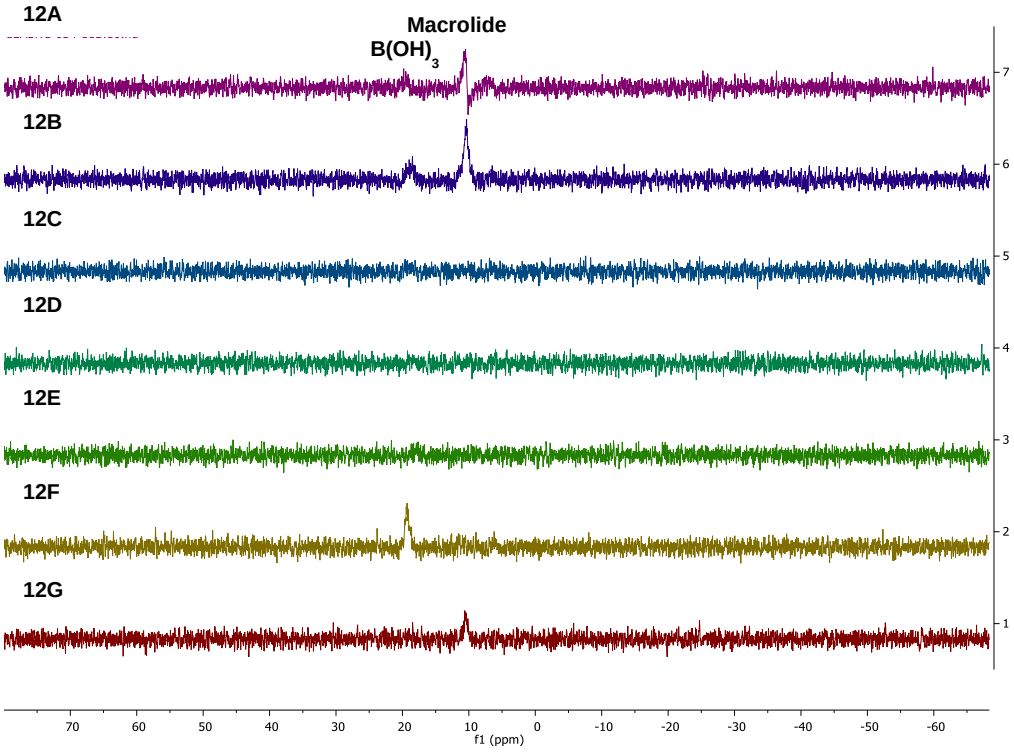


Figure 4.11 ^{11}B NMR (500 MHz, $CDCl_3$) of the 7 liters involved in late-stage addition of $B(OH)_3$ experiment. Macrolide peaks are seen at 10.5 ppm in conditions 12A, 12B, and 12G – addition of $B(OH)_3$ at 0 and 24 hr of fermentation; no other conditions produced quantifiable amounts of macrolide. Unincorporated $B(OH)_3$ that carried over from aqueous media is seen at 18.3 ppm.

The results of this experiment are summarized in Table 4.3. Peak production was seen at day 1 addition. Potentially, the nutrients of fermentation are spent after 48 hours of fermentation, causing metabolic production by the bacteria to stop, which could explain why the bacteria did not produce macrolide upon the boric acid stimuli after the first couple of days. Both liters with addition of $B(OH)_3$ at hour 0 had the most **2.1** production. Boron macrolides are present by mass in 12A and 12B via LCMS analysis. Conditions 12C and 12D show very low quantities of **2.12** – indicative by baseline molecular ions – and is not present by mass in 12E or 12F. This leads to the conclusion that boric acid is the stressor causing production of **2.12** that will uptake $B(OH)_3$ nonenzymatically, rather than the bacterium produces the macrolide just because and that it just happens to take up boric acid. Thus, $B(OH)_3$ is needed at the beginning of bacterial growth to induce the production of the macrolide. Additionally, the crude NMR showing specs of $B(OH)_3$ at 18.3 ppm suggests that premixed seawater with $B(OH)_3$ is more homogenized solution and as a result is better incorporated by the macrolide.

Sample	Day of Boric Acid Addition	Semi-crude SNC-034	NP-34 yield
12A	0	52.9 mg	< 0.5 mg
12B	1	59.0 mg	< 0.5 mg
12C	2	44.4 mg	0.0
12D	3	45.7 mg	0.0
12E	4	54.6 mg	0.0
12F	5	46.1 mg	0.0
12G	0	62.9 mg	< 0.5 mg

Table 4.3 Results of late-stage boric addition. The time of boric acid addition and semi-crude yield after EtOAc partition are listed, with the semi-crude's corresponding ^{11}B intensity as compared to pentafluorophenylboronic acid. The final yields of NP-34 indicate that addition of $B(OH)_3$ is only viable between 0 – 24 hr fermentation.

Something left to try is spiking in $B(OH)_3$ over the course of fermentation. For example, have SNC-034 start growing in presence of $B(OH)_3$ to activate the BGC into producing macrolide, then after a few hours, spike in more B(OH)3 to see if that further promotes

production. If the carbon source for polyketide production is indeed spent, there shouldn't be a spike in production in production.

4.3.2 pH and Other Supplements

All cellular processes function best at specific pHs, and changes in those values lead to conformational changes in enzymes that impede their function. Changes in the pH of culture medium can affect cell membrane function, the cell structure, and the uptake nutrients, thus affecting the biosynthesis of metabolites.[18] Hence, a drastic shift in the media's pH, could affect the production of **2.1**. We sought to analyze the change of SNC-034 pH over the course of fermentation to validate if it influenced macrolide production. This would be achieved by daily pH tests and extraction to isolate and compare the yields of **2.1** at each time point.

Additionally, the use of the iron-supplemented media at the onset of fermentation was questioned. To grow SNC-034, 50 mL cultures of bacteria are grown in our "A1+C" media then moved to liter batches of it supplemented with iron, "A1FeB+C" for scale up (See E.2.1). If the scale up requires iron supplementation, wouldn't growth of the initial colonies also require it? This could easily be tested by setting up parallel experiments in which one batch of SNC-034 is grown in A1+C and then transferred to A1FeB+C for scale up, whereas a second batch is grown in A1FeB+C initially and during scale up, to compare **2.1** yields between the two. That experiment could easily be combined with a pH study.

Sixteen liters of SNC-034 were grown for this experiment: 8L would be derived from SNC-034 in 50 mL A1+C (14A series) and the remaining 8L would be derived from SNC-034 in 50 mL A1FeB+C (14B series) starter culture. Two liters of each of those batches would be extracted at day 7, acting as control. All 16L would commence fermentation on the same day, but each day, 1L from each batch would have its pH checked and resin extracted (Figure 4.12). The yield of **2.1** between all 16L would be compared to see at which pH and if iron-supplement was affecting **2.1** production.

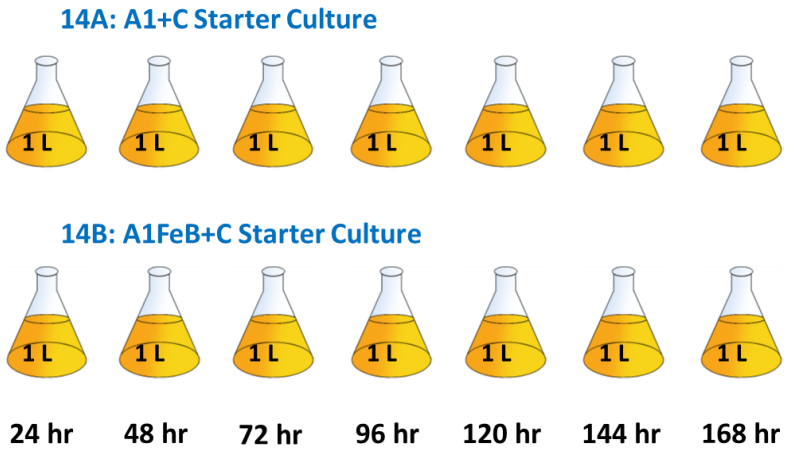


Figure 4.12 Experimental setup for pH analysis. Series 14A is derived from A1+C starter culture and series 14B is derived from A1FeB+C starter culture. Both series would be grown and extracted in parallel. Times listed indicated after how many hours of fermentation a liter would have its pH tested and be extracted. Control flask is not shown.

The pH was seen to gradually increase over the course of fermentation from 6.0 to 8.0. Boron signals were not seen with day 1 extraction (after 24 hr fermentation) or at day 7 extraction (after 168 hr fermentation) with either condition. This was reasonable for day 1 as the bacterium probably hadn't enough time to fully process metabolites. What was peculiar was that Day 7 and Control (both extracted after 168 hr fermentation) were with the same conditions, but Day 7 did not produce boron macrolides whereas the control did, but the yields of **2.1** should have been the same. But this phenomenon was seen across both series. LCMS showed M^- = $[845]^-$ for control flasks but not day 7 flasks. Thus, only days 2-5 and control groups were kept for analysis.

Figure 4.13 shows the ^{11}B NMR of 14A series compared to **3.1** internal standard and Figure 4.14 shows the ^{11}B NMR of 14B series compared to **3.1**. Series 14B shows 14A-D2 having the highest intensity of boron macrolides with a gradual decrease until the control peak spikes up again. Series 14A however didn't have a general trend in ^{11}B macrolide intensity. Both series overall had higher ^{11}B NMR intensity of macrolides with fermentations lasting 2 and

3 days compared to fermentations lasting 4 and 5 days. Based on NMR, the control group had the best intensity of boron macrolides, and these results are summarized in Table 4.4.

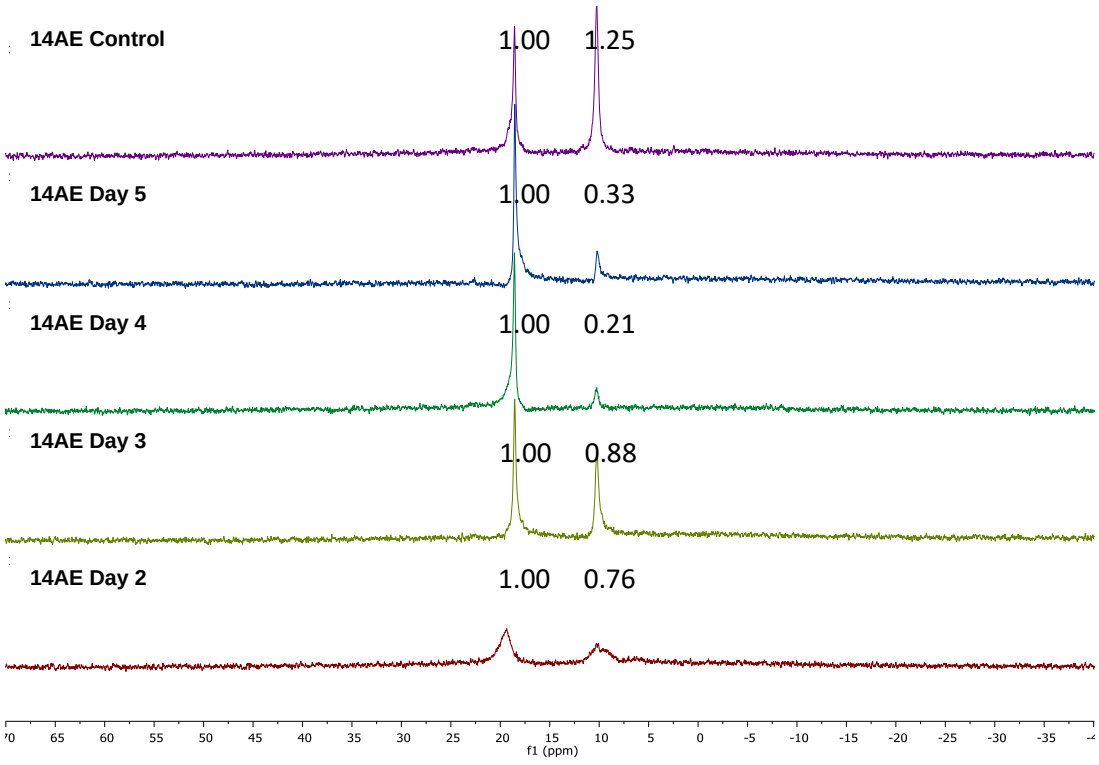


Figure 4.13 ^{11}B NMR (500 MHz, $CDCl_3$) of 14ASNC34E Days 2-5 and Control with 1 mg/mL pentafluoroboronic acid as internal standard at 18.3 ppm.

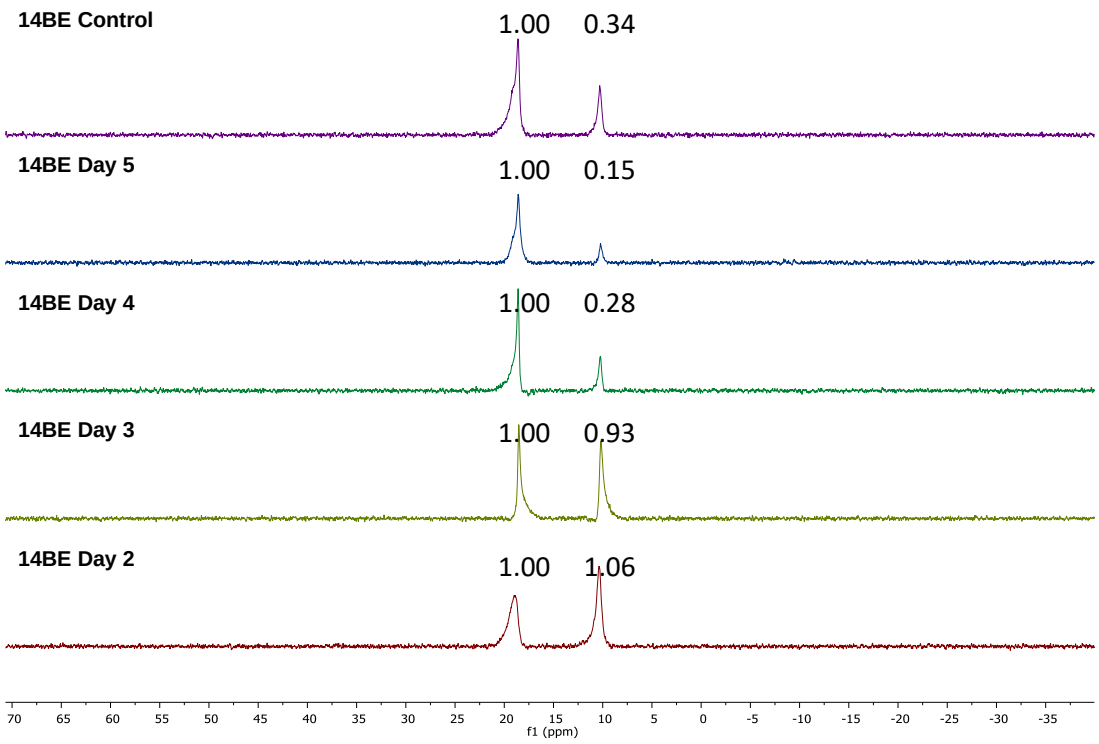


Figure 4.14. ^{11}B NMR (500 MHz, $CDCl_3$) of 14BSNC34E Days 2-5 and Control with 1 mg/mL pentafluoroboronic acid as internal standard at 18.3 ppm.

Sample	Starter Culture Media	Extraction Day	pH	Organic Semi-crude Yield (mg)	^{11}B NMR Intensity
14A-d1	A1BFe+C	1	6.0	396.4	--
14A-d2	A1Bfe+C	2	6.0	630.1	0.76
14A-d3	A1Bfe+C	3	7.0	533.0	0.88
14A-d4	A1Bfe+C	4	8.0	455.3	0.21
14A-d5	A1Bfe+C	5	8.0	327.3	0.33
14A-d7	A1Bfe+C	7	8.5	173.0	--
14A Control	A1Bfe+C	7	8.5	300.2	1.25
14B-d1	A1+C	1	6.0	358.6	--
14B-d2	A1+C	2	6.0	535.2	1.06
14B-d3	A1+C	3	7.0	588.5	0.93
14B-d4	A1+C	4	8.0	365.1	0.28
14B-d5	A1+C	5	8.0	346.8	0.15
14B-d7	A1+C	7	8.5	138.3	--
14B Control	A1+C	7	8.5	297.3	0.34

Table 4.4 Results from the 14A and 14B series of SNC-034 growth analyzing the effect of pH and iron-supplements in media for NP-34 production.

Figure 4.15 shows that both fermentation series experienced a gradual increase in pH (blue) over the course of fermentation from pH 6 to pH 8.5. Both series correspondingly produced a maximum amount of organic metabolites between day 2 and day 3 of fermentation (green and orange), and yields of material tapered off as time and pH went on.

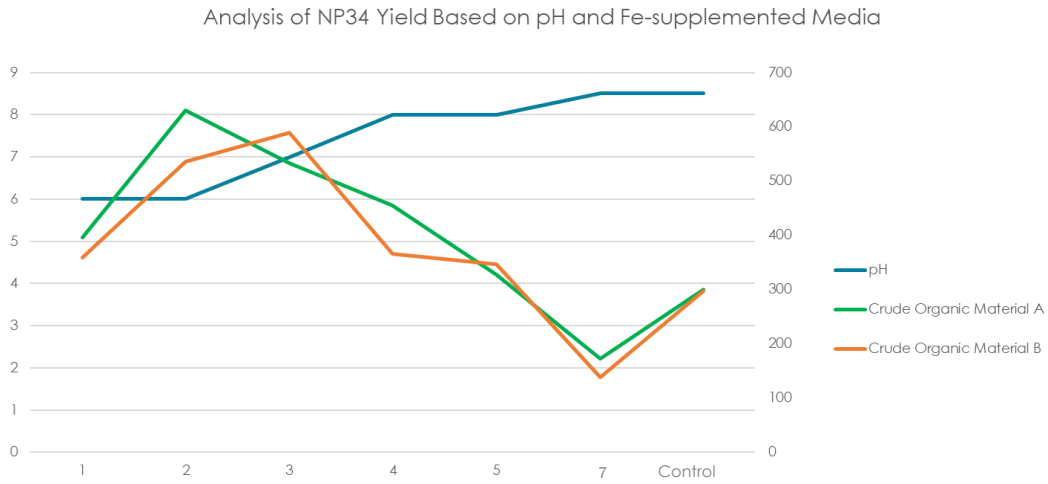


Figure 4.15 Production of NP-34 is decreasing as basicity of fermentation cultures increased over time. The amounts of NP-34 produced by both iron-lacking (14A) and iron-supplemented (14B) cultures were similar, except for day 3 extraction which 14A yielded 0.3 mg and 14B yielded 1.1 mg of NP-34, the best yield of molecule in this experiment, and that is what determined time point for harvesting cultures.

All samples were purified to determine their **2.1 yield**. The most **2.1** were isolated from 14A-D2, 14B-D2, and 14A-D3 with 0.2 mg, 0.3 mg, and 0.2 mg yields of **2.1**, respectively. Interestingly, while 14B-D3 had the second highest ^{11}B intensity, it yielded the maximum amount of **2.1** with 1.1 mg isolated. All other conditions gave unquantifiable amounts of **2.1**. Based on these results, SNC-034 with both starter and fermentation cultures supplemented with iron, extracted after 3 days of fermentation were the best conditions for **2.1** yield as opposed to our typical 7-day fermentation. Something of concern was the difference in **2.1** yields between day 7 extraction and control as both time points are essentially the same (both extracted after 7 days of fermentation). The difference between the two yields (amongst both series) illustrates a variability in production that requires further investigation.

Based on the day 3 extraction having the best yield of **2.1**, we sought to test if increasing $B(OH)_3$ to 3 eq within that 3-day fermentation window would successfully induce macrolide production. 15L of SNC-034 were grown: 5L with 1 eq $B(OH)_3$ extracted after 3 days serving as a pseudo-control; 5L with 3 eq $B(OH)_3$ extracted after 3 days; and 5L with 1 eq $B(OH)_3$ extracted after 7 days serving as the control. Each would be purified to isolate **2.1** to compare yields and determine which combination of extraction and $B(OH)_3$ eqs would be best for **2.1** production.

^{11}B NMR showed relatively the same numbers between both 3-day extractions with the $B(OH)_3$-supplemented having slightly higher intensity, but day 7 extraction had the overall highest intensity (Figure 4.16). Upon isolation though, 15A (Day 3 extraction, 1 eq $B(OH)_3$) yielded the most **2.1** with 2.2 mg and 15B yielded roughly the same with 2.0 mg. The higher intensity of ^{11}B with the 7-day fermentation could be because of the larger distribution of macrolides within the fraction: while each analog was overall less yielding, there were more differences in macrolides being formed as indicated by the variety of masses in LCMS analysis compared to Day 3 extractions, which would contribute to the same macrolide boron peak. The results of this experiment are summarized in Table 4.5.

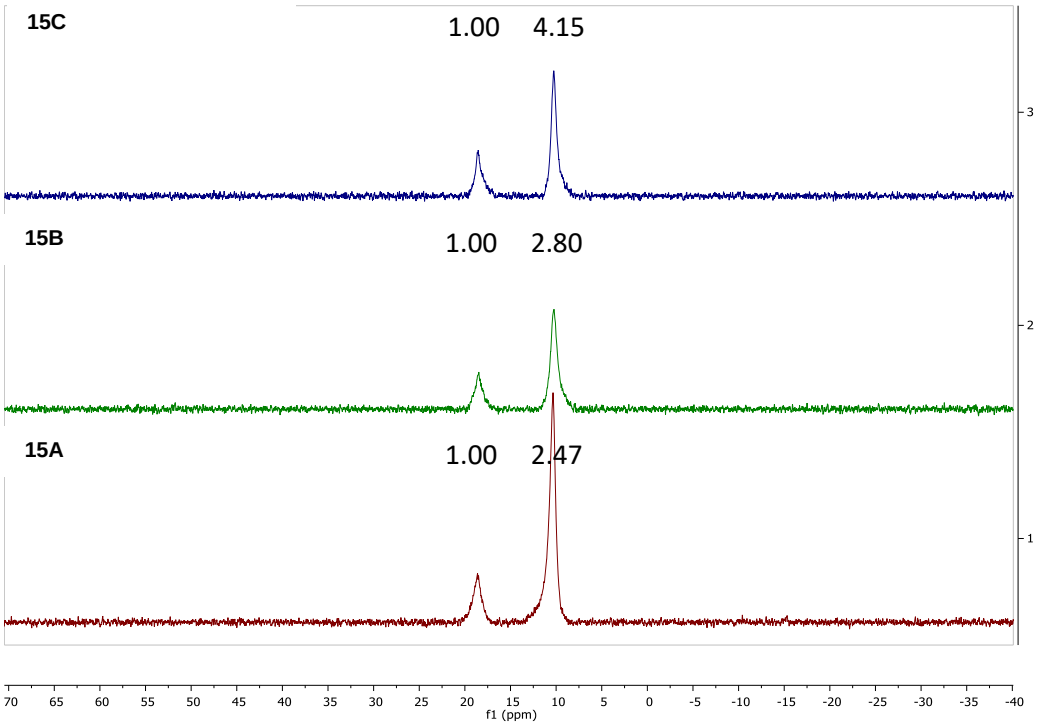


Figure 4.16 ^{11}B NMR (500 MHz, $CDCl_3$) of samples 15A, B, and C SNC34E testing the difference in $B(OH)_3$ eq paired with extraction dates, with 1 mg/mL pentafluoroboronic acid as internal standard at 18.3 ppm.

Sample	Amount of Boric Acid	Extraction Day	pH	Organic Semi-crude	^{11}B NMR Intensity	NP-34 Yield
15A	1 eq	3	7.0	420.9 mg	2.47	2.2 mg
15B	3 eq	3	7.0	296.5 mg	2.80	2.0 mg
15C	1 eq	7	8.0	205.7 mg	4.15	1.5 mg

Table 4.5 Summarized results of 15A, B, and C SNC34E testing the difference in B(OH)3 eq paired with different extraction dates.

4.4 Additional Isolated Analogs

Nature is amazing at synthetizing complex compounds with rich, chemical diversity. In living organisms, primary metabolism constructs fatty acids including carbohydrates, amino acids, and nucleic acids necessary for basic metabolic function like growth, nutrient assimilation, and energy production Additionally, there are the aforementioned NPs, composed of alkaloids, polyketides, steroids, etc. produced by secondary metabolism which are evolutionary advantages, not necessary for basic function and survival. These molecules serve

a wide range of purposes including signaling (pheromones), communication (quorum sensing), protection (venom and toxin), and nutrient transport (siderophores).[19]

BGCs are bins of non-homologous genes within bacteria and some fungi that are responsible for the production of secondary metabolites. These clusters can code for several structural analogs that vary in biological activity. SAR is studying the connection between the chemical structure and activity of a molecule to determine which motifs lead to optimal biological and physiochemical characteristics in specific contexts. To elaborate, BGCs will code for various metabolites that improve the fitness of their producing organisms, but when tested against our biological assays, subtle differences in chemical structure can lead to drastic differences between analogs. Analyzing these differences help determine the best molecular design for the specific function it is intended for. Additionally, not all possible analogs will be produced under a certain set of environmental conditions as there are many "silent" BGC under laboratory conditions. Subtle changes in fermentation though can lead to activation of more analogs being produced (see future directions).

Over the course of isolating **2.1**, other naturally occurring analogs were collected to test their mosquitocidal activity and selectivity compared to **2.1**. All macrolides exhibited the same 10.5 ppm BO_4^- shift via ^{11}B NMR and were identified as separate compounds based on their ^1H NMR and molecular ions observed via LCMS. Figure 4.17 shows the structures of the highest yielding analogs that were able to be characterized. Note: many more potential analogs were identified in semi-crude and semi-pure fractions, but either not enough material was available to isolate them, or they require further optimization of purification methods. For example, some of these analogs, such as **4.1**, were purified with a subsequent 80-100% MeOH in H_2O gradient after purification with HPLC 95% ACN on C18, but that additional round of purification was not suitable for purifying other analogs.

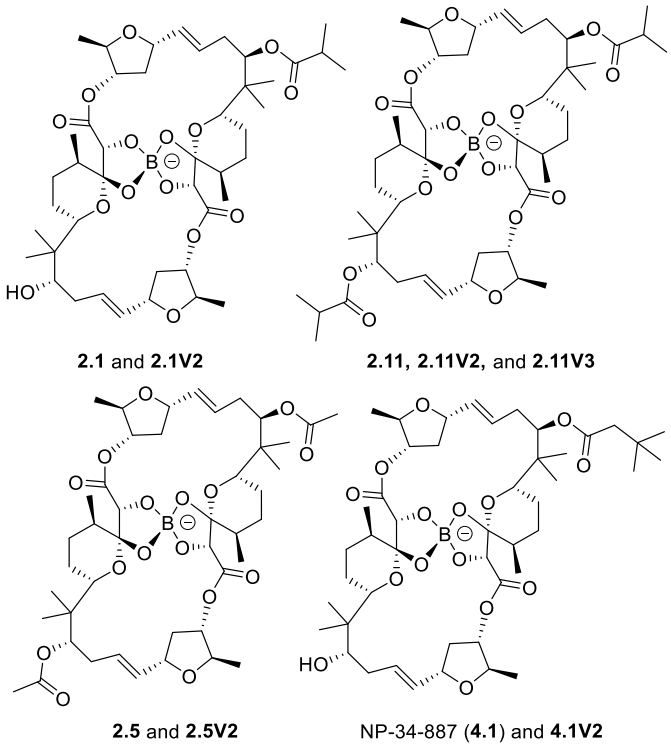


Figure 4.17 Structures of some additional naturally occurring analogs of NP-34.

The highest yielding analogs in virtually every condition were **2.5**, **2.11**, and NP-34-887 (**4.1**), with **4.1** being the most yielding fraction overall, as they were collected in high yield with every fermentation. Additional uncharacterized family members are present in large-scale yields; however, they have not been pursued at this time. Some of these masses were only observed upon subsequent changes in fermentation conditions, supporting the claim that changes in stimuli can induce production of different analogs.

Interestingly, throughout the conditions tried, very little **2.2** was isolated. While **2.2** was originally isolated from *Streptomyces griseus,*[20] being the precursor of **2.1**, I thought there would be much more of it present. Only about number 3 mg of **2.2** was isolated overall. In actuality, the highest yielding acylation pattern of **2.1** was the bis-substituted **2.11**, followed by **2.1**, and **2.2** had the lowest yields (Figure 4.18). This has nothing to do with mosquito toxicity, as these molecules aren't producing these molecules to specifically combat mosquitoes, rather there's an environmental stimulus that causes the bacteria to preferentially produce this bis-

substituted analog for some sort of advantage. It'd be beneficial to know the reason because that could elucidate why this BGC is produces these molecules and thus help tailor conditions to favor **2.1** instead. Furthermore, this should be something culturally because, based on the BGC, it is believed that uptake of the sidechains happens nonenzymatically/outside of the BGC (unpublished work by the MacMillan Lab).

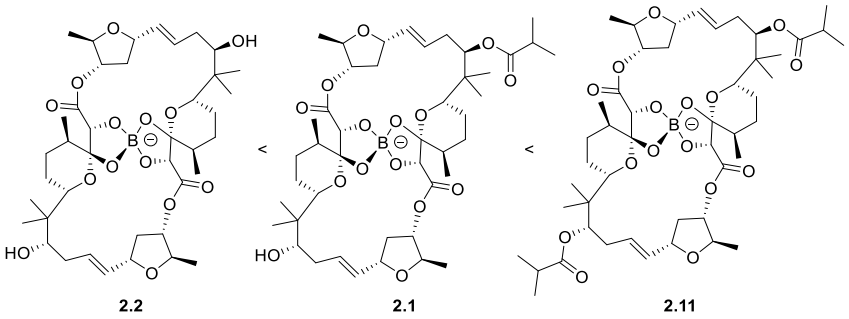


Figure 4.18 SNC-034 preferentially produces the bis-substituted isobutyrate version of NP-34, NP-34-915, in highest yields compared to NP-34 and Aplasmomycin. The preference in distribution of these acylation patterns could help inform culture conditions to favor production of NP-34.

Another analog of **2.1** was also a symmetrical bis-substituted macrolide like **2.11**, but acetate groups at C9 and C9'. It is the previously known **2.5**. By mass, the mono-substituted acetate **2.4** was also observed with subsequent fermentation conditions but in low yields that it could not be isolated for biological testing at the moment (x ~ 500 ug in need of further purification). Having identified both analogs is great as they would be direct comparisons to **2.1** and **2.11**, giving insight into how the sterics of acyl side chain affect mosquitocidal activity. Both **2.1** and **2.4** with mono-substitution and **2.11** and **2.5** with bis-substitution. It would be interesting to see if this smaller acyl group improves or weakens the activity, since **2.4** is hypothesized to have activity since its monosubstituted. Likewise, it will be interesting to see if the smaller side chain unit in **2.5** gives improved mosquitocidal activity compared to **2.11**, but it is likewise expected to have diminished activity compared to **2.1**. Important to note would be if the subtle difference causes any change in selectivity between organisms. I predict that **2.4** will have better mosquitocidal activity than **2.5** based on the relationship between **2.2** and **2.11**, but

without information on the binding site, no hypothesis can be made atm if **2.2** will elicit better activity than **2.4**.

Like **2.2** relative to **2.11**, **2.4** was produced in much smaller yields than **2.5**, just like **2.1** was produced in smaller yields than **2.11**. This pattern further suggests that formation of the bis-substituted analog is favored over the mono-substituted analog. During fermentation studies, conditions 14A-D3, 14D, and 16G yielded 817 mw, which was the first time I had observed the mass via LCMS. If needed, more SNC-034 can be grown up under those specific conditions to prioritize isolation of **2.4** for biological testing.

The ananlog NP-34-887 (4.1) was highly prevalent, isolated in almost every condition tested, but was very difficult to fully characterize. ^{1}H-^{13}C HMBC analysis showed 3 different carbonyls, indicating an asymmetrical structure – a monosubstituted macrolide like **2.1**. TOCSY confirmed the side chain off C9 to be 3,3-Dimethylbutyrate, but by mass, there was still 14 mass units to be elucidated – a CH3 group – thought to be distributed elsewhere in the core ring structure. With ^{1}H-^{13}C 2D analysis, all protons and carbons were accounted for C1-C20 and C1'-C20', giving the same core structure as **2.1**. TOCSY of the tetrahydropyran ring by irradiating the protons at 3.66 and 3.68 ppm (C4' and C4, respectively), determined that there was no extra methyl substitution in rings besides those typical at C4 and C4' (C18 and C18', respectively). In the proton spectrum, there was one methyl group unaccounted for: a singlet at 2.07 ppm. TOCSY irradiating the signal at 2.07 ppm revealed no other protons in its spin system. While its proton shift is too upfield to be thought of as a methoxy group, our tentative structure currently includes it as a methoxy group at C9', and validation is needed with crystal structure.

Other analogs that were isolated and are currently under characterization include macrolides with the molecular weights 831, 841, 901, and 929 g/mol. The analogs are likewise expected to exhibit differences in the side chains at C9 or C9', but potentially can exhibit additional differences at the tetrahydrofuran or tetrahydropyran rings.

As mentioned previously, different culture conditions can provide different stimuli that prompt SNC-034 into producing different structural analogs of **2.1**. Depending on what needs to be prioritized for isolation for biological testing, or what analog proves to have the best physiochemical properties and mosquitocidal activity, fermentation conditions can be tailored to yield the specific analog. Different analogs would be produced in intensity dependent on the fermentation conditions – for example, the molecular weight analog 817 wasn't seen in all fermentations. Figure 4.19 is a chart summarizing various analogs by mass seen in various fermentation conditions. This can be used as a guide for SNC-034 growth conditions if the growth of a particular analog need be prioritized. Care must be taken though when following strictly mass data, though. Mass spectroscopy is very sensitive data, so analogs of interest should be confirmed by differences observed in ^{1}H NMR as ^{1}H NMR is tangible data.

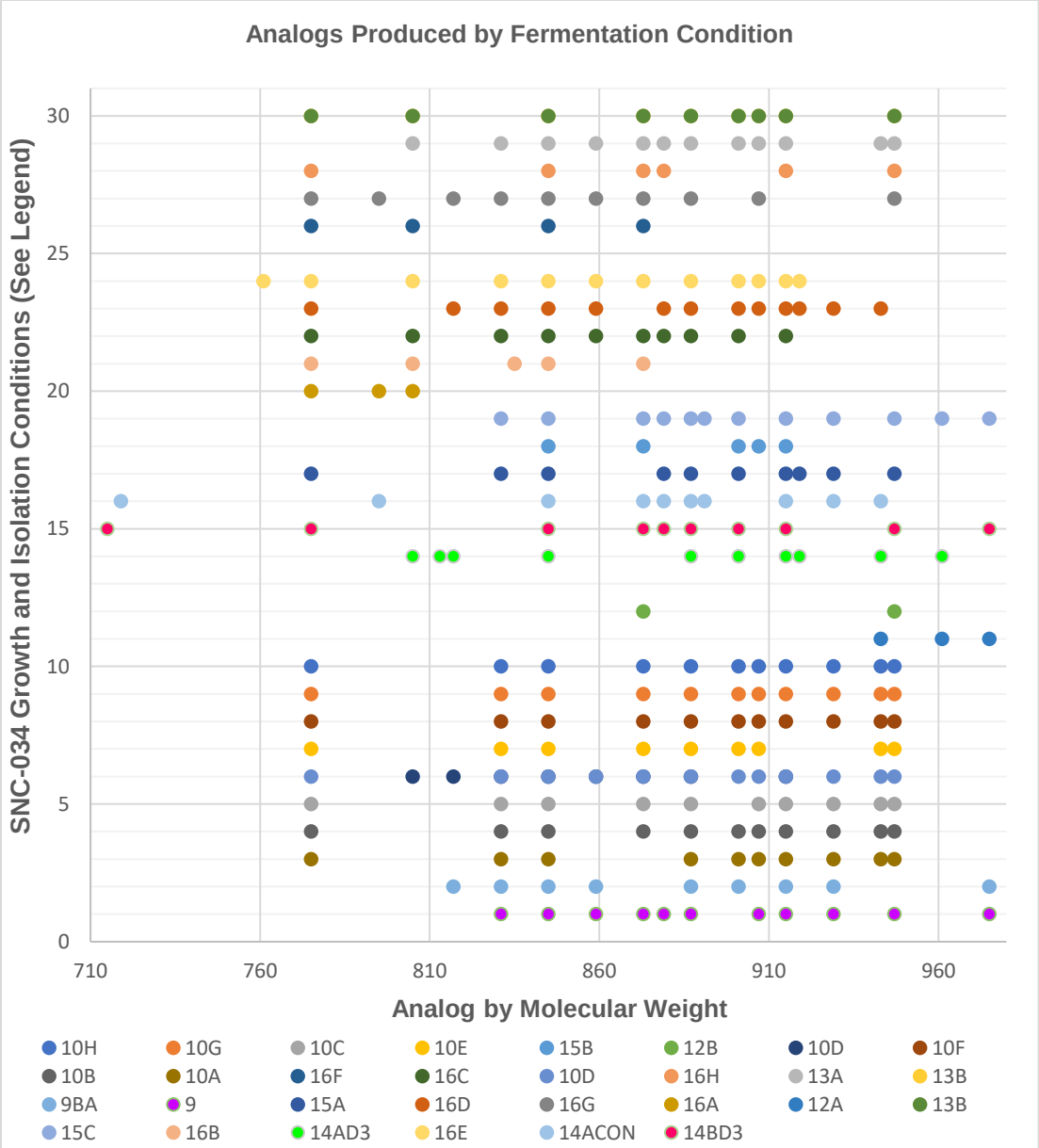


Figure 4.19 Analogs of NP-34 by molecular weight produced in various fermentation conditions. This data can be used for SNC-034 growth dependent on analog prioritized for isolation. This can be used as a guide for SNC-034 growth, if one analog, even if still a minor analog, to prioritize for isolation and biological testing.

In addition to different structural analogs, we have isolated peaks from the column that have the same molecular weight, but with observed differences in ^{1}H NMR shifts. For example, 3 “versions” of **2.1** were isolated: **2.1, 2.1V2, 2.1V3** (Figure 4.16) and they were all constitutionally the same macrolide based on 2D NMR assignments, but there were key

differences in their assignments. For example, a key vinyl proton was shifted in each sample: δ 6.45 ppm (**2.1**), 6.36 ppm (**2.1V2**), 6.11 ppm (**2.1V3**). This was observed for analogs **2.11** (**2.11V2**, **2.11V3**), **2.5** (**2.5V2**), and **NP-34-887** (**NP-34-887V2**). This was not observed though for the boron-less version of **212, 2.12**. With some fermentations, the stark difference in analogs was notable - moving to 3-day fermentation yielded approximately 50:50 **2.1** and **2.1V2** (reported as **2.1** in its totality though) with very little yields of V3. The isolations of these species were guided by vinyl protons 6.45 (**2.1**) vs 6.36 (**2.1V2**) vs. 6.11 (**2.1V3**) into separate vials to assure there was no difference in biological activity.

My hypothesis is that a different cation is coordinating to the boron in **2.1, 2.1V2,** and **2.1V3** which would explain why the differences are not seen in the boron-less analogs. Since these differences arose with differences in fermentation, it raises the questions, could the affinity for a certain cation change with different environmental conditions (Section 4.6)?

4.5 Synthetically Manipulated Analogs

While a wide variety of structural analogs were desired for testing, the scope of biosynthetically occurring analogs by any one species is limited. Synthetic manipulation of **2.1** and co-occurring analogs would allow exploration of chemical space that would not naturally occur with these analogs. While NPs are excellent sources for biologically active compounds, it is very common that they have suboptimal physicochemical properties, which can also be improved with synthetic alteration.[21]

Figure 4.20 shows a summarized conceptualization of different functional groups that can be installed on **2.1**. Longer fatty-acid chains, isocyanates, allyl or propargyl substitutions at the C9' position can be attempted with S_N2 acylation and alkylations that could also serve as handles for further diversification. Other manipulations include olefin reductions that could provide a degree of flexibility to the macrolide, and epoxidations which could lend itself to installation of more alcohol moieties. Amines in place of the alcohol at C9' can be converted to phenylureas, N-phenylcarbamates, or phenylsulfonates, and ester linkages could also be

converted to thioamides. Use of a double-inversion Mitsonobu, to convert to the amine, would allow conversion with retention of the molecule's stereochemistry. This would allow analysis of mosquitocidal activity with a less nucleophilic group at C9' position and allow for further functionalization with amination reactions.

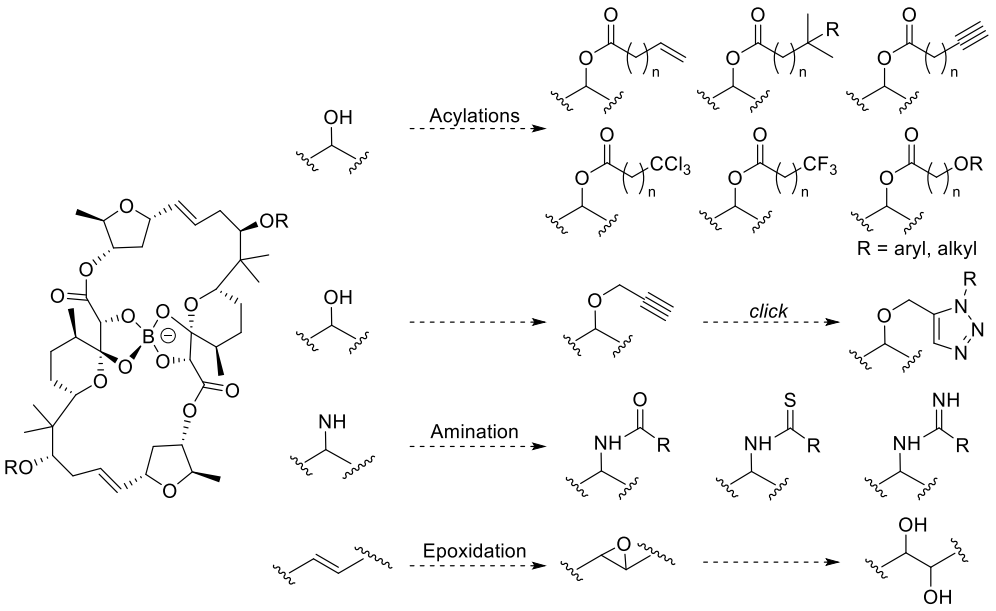
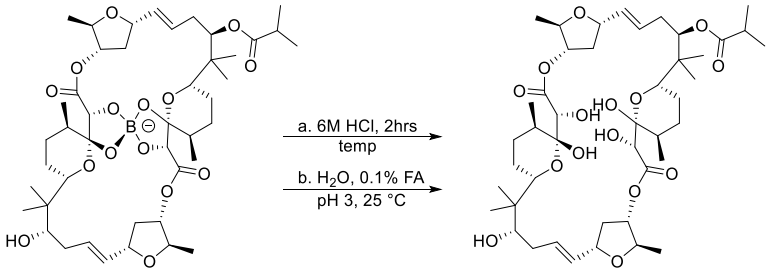


Figure 4.20 Proposed semi-synthetic SAR studies to modify the alcohol and olefin substituents on NP-34 and other analogs.

4.5.1 Boron Removal

As previously stated, acidic conditions cause the boron to dissociate from the macrolide. This was illustrated under two conditions which, caused us to move to pH > 6 conditions whenever possible. The first instance was experimentally planned (Scheme 4.2 a) where 6M HCl was added to a solution of **2.1** in THF. After 2 hrs stirring, the **2.12** was observed. With this observation, it was assumed that only harsh conditions, such as 6M HCl would deboronate **2.1** and analogs (especially since purification occurred with 100% ACN with 0.1% FA), but as previously mentioned, **2.1** in the presence of H_2O, 0.1% FA will readily cause deboronation (Scheme 4.2 b). Upon addition of the a few drops of acidified water, white solid $B(OH)_3$ is

observed to crash out into solution. This was the reason behind use of strictly acid-less conditions in all chromatographic steps.



Scheme 4.2 Removal of boron from macrolides containing *cis*-1,2 diols is seen with a) addition of 6M HCl in organic solutions or b) addition of 0.1% formic acid in aqueous solutions.

This provided us the easiest handle to create analogs. 5 drops of HPLC grade H_2O with 0.1% FA were added to 1 mL solutions of **2.5**, **2.11**, and **4.1** stirring in EtOAc at 25 °C, to create the boron-less analogs **4.2**, **4.3**, **4.4**, respectively (Figure 4.21, See E.8.1).

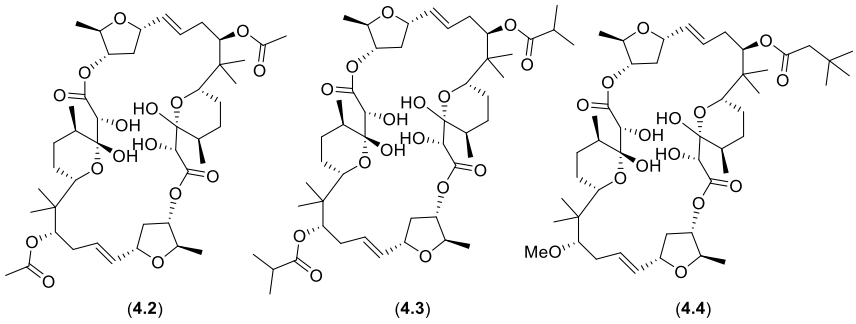
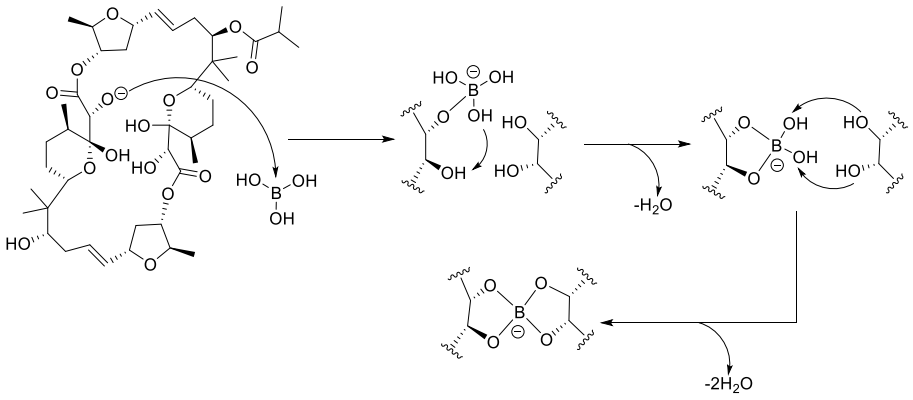


Figure 4.21 Structures of three additional boron-less analogs created from Aplasmomycin C, NP-34-915, and NP-34-887 analogs.

4.5.2 Boron Reintroduction

Since there was a significant loss in **2.1** due to boron-loss in early isolation efforts, reintroduction of the boron moiety into the macrolide skeleton was attempted to recover material and restore mosquitocidal properties. Formation of the Böeseken complex is believed to be non-enzymatic since it is known for $B(OH)_3$ to form stable tetrahedral anionic complexes with diols present on biomolecules like carbohydrates and nucleotides.[22] These cyclic macrocycles readily incorporate boron naturally due to the orientation of the alcohols allowing

a tetrahedrally coordinated boron.[23] Since that orientation is conserved with the loss of boron, it was thought it could be reintroduced with $B(OH)_3$. Trials in water and in MeOH-*d* with 2.0 eq of $B(OH)_3$ resulted in approximately 75% yield incorporation observed via NMR (a 4:1 ratio BO_4^- NMR shift to BO_3 was seen). So, formation of the boron complex can be achieved in the lab, which is good in case unique structural analogs are isolated lacking the boron, or if synthetic conditions unintentionally cause boron dissociation, the boron can be reintroduced. Scheme 4.3 shows the agreed upon proposed mechanism for formation of the Böeseken complex, which occurs by iterative nucleophilic attacks at the diol carbons by boric acid.[24]

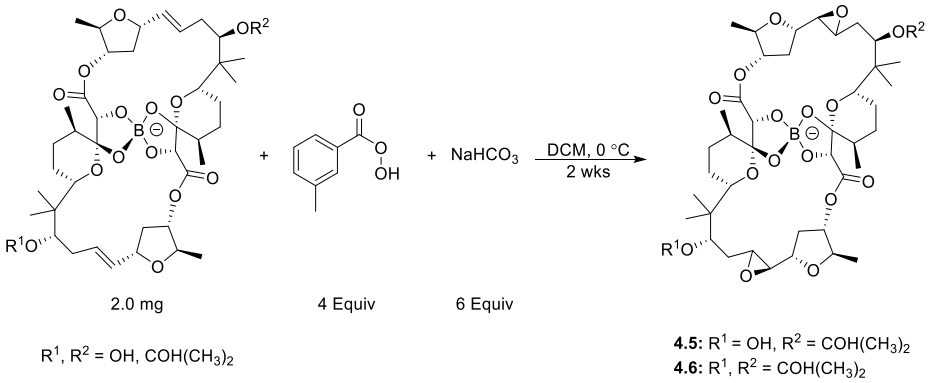


Scheme 4.3 The proposed mechanism for complex formation between NP-34 and analogs with boric acid.

4.5.3 Epoxidation of Alkenes

The other reaction that readily went was epoxidation of olefins with *meta*-Chloroperoxybenzoic acid (*m*CPBA).[25] Epoxidations are concerted reactions creating a 3-membered heteronuclear rings with an oxygen, retaining the *cis* or *trans* geometry of the alkene starting material. It would be interesting to observe the resultant flexibility of these molecules with the replacement of the olefin group, and epoxides are good “intermediates” for nucleophilic attack to add on alcohol and other groups to a molecule.

The reactions went to full conversion after two weeks stirring in the hood with 4 eq *m*CPBA and 6 eq $NaHCO_3$ in DCM (Scheme 4.4). A 1:1 ratio of **2.1 (**or **2.11**) with one epoxide to **2.1** (or **2.11**) with epoxides at both olefins was seen after one week of stirring via LC-MS. On one attempt, the reaction was quenched to attempt isolation one from the other with C18 HPLC chromatography, but separation was unsuccessful. This reaction was expected to give a complex mixture of products, as seen at the halfway point of the reaction (1 week) due to the symmetrical nature of the macrolide, however the reaction seemed to go to completion via LCMS.



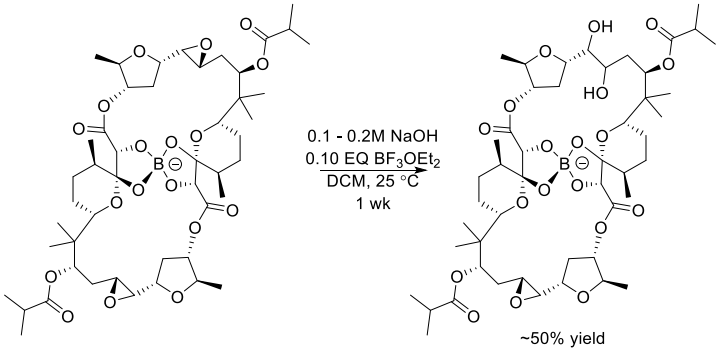
Scheme 4.4 Methods of epoxidation for the macrolides NP-34 and NP-34-915.

2.1 under these conditions yielded "NP-34-EPOX" (**4.5**) and **2.11** yielded "NP-34-915-EPOX" (**4.6**), both in over 90% yields by ^{1}H NMR and LCMS analysis. The mass corresponding to only one epoxide formed (Figure 4.22) on each molecule was seen via LCMS after one week stirring.

4.5.4 Epoxide Ring Opening

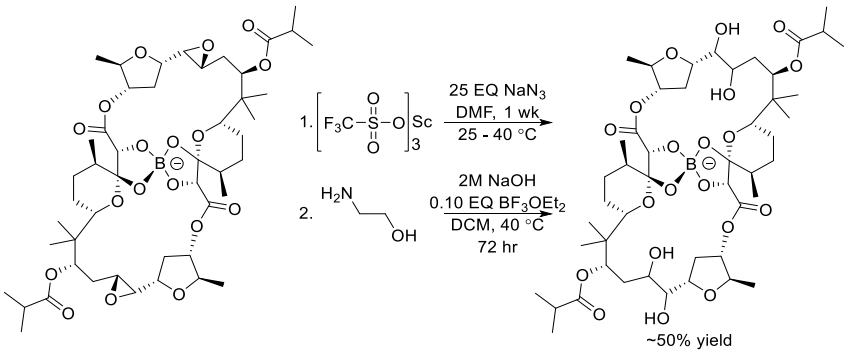
As exciting as it was to have the epoxide analogs for biological testing, opening of the epoxides to introduce additional alcohol handles was very appealing for the physiochemical potential of the molecules and as handles for click chemistry. Many conditions were attempted to open the epoxides to the corresponding diols including NaOH with $KMnO_4$ in DCM at 0 °C[26] and with 0.1 – 0.2 M NaOH alone in anhydrous DCM at 25 °C. With those attempts, only starting

material was observed via LCMS. With the latter condition, a 1:1 ratio of starting epoxide to singly opened epoxide (Scheme 4.5) was observed via LCMS when 0.1 eq BF_3OEt_2 was added as catalyst. Increasing the equivalents of NaOH did not drive the reaction: one mg of **4.5** was stirred with 0.1 eq of BF_3OEt_2 and 2M NaOH, and via LCMS, the masses of **4.5**, **2.11**, and of degradation fragments were seen via LCMS, and none of desired product.



Scheme 4.5 A 1:1 ratio of starting epoxide to singly opened epoxide was observed via LCMS when 0.1 eq BF_3OEt_2 was added to reaction with 0.1 M NaOH in anhydrous DCM at 25 °C.

Interesting, another condition that worked wasn't intended to open the epoxide with a diol, but rather with an N_3 group. Placzek *et al.*[27] and Coca *et al.*[28] reported aminolysis of epoxides with scandium triflate. Their reaction conditions were used but with but DMF solvent instead due to **2.1**'s low solubility in water. The reaction was set up at 25 °C and after one week, starting material remained unaltered via LCMS analysis. The reaction was heated to 40 °C, and likewise after 48 hr, no change to the starting material was observed. Assuming that the NaN_3 was spent, 2.5 eq of ethanolamine were added to reaction matrix while still at 40 °C and left stirring (Scheme 4.6).



Scheme 4.6 Methods of Ring Opening of Epoxides on the NP-34 and NP-34-915 Macrolides.

After one week, $NaHCO_3$ was added to the reaction, and macrolide was extracted with anhydrous DCM, and dried over MgSO4. Via LCMS, not only was epoxide starting material present, but also the singly (**4.7**) and doubly opened (**4.8**) epoxides (Figure 4.22). The mixture of products was later resuspended in DCM with 2.5 equivalents of ethanolamine. After 1 week, no reaction progress. Another 2.5 equivalents of ethanolamine were added did not drive full conversion, potentially suggesting that NaN3 and/or scandium triflate were needed to assist in the epoxide ring opening on **4.6**.

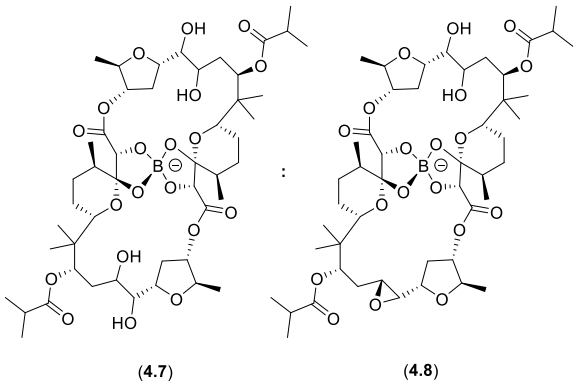


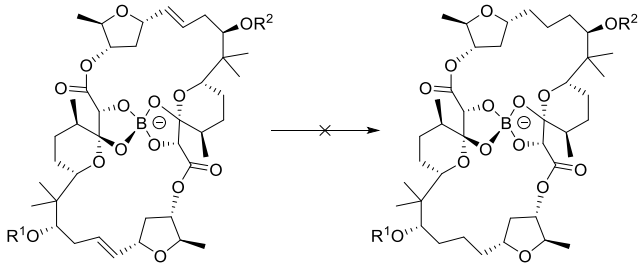
Figure 4.22 Structures of the singly (**4.7**) and doubly opened (**4.8**) epoxides. Under various chromatographic efforts, the two analogs were unable to be separated from one another and require more chromatographic troubleshooting.

Having both versions of this analog would be beneficial as there would be more analogs for biological testing and having one ring still closed meant more controlled click

chemistry as there was only 2 possible alcohols to react with instead of 4. Various conditions with HPLC C18 and C5 were attempted to separate product from starting material to no avail. Follow up with TLC to see if silica column would be a good option, but in silica, as seen before, these macrolides co-elute together like with HPLC. As mentioned previously, these analogs would be good candidates to try for separation with the Synergy Fusion HPLC column.

4.5.5 Attempts at Hydrogenation

Multiple hydrogenation conditions at the olefins between C11 and C12 and C11' and C12' (Scheme 4.7) were attempted on both **2.1** and **2.11** to compare consequential biology after a small loss of skeletal rigidity of the molecule. Conditions tried with **2.1** include H_2 on Pd/C;[29] manganese dipivaloyl-methane with phenylsilane and *tert*-butyl hydroperoxide;[30] and Snelling Salt.[31] Possibly, these reductions could not be working due to inactivation of the reagents. For example, the reason that the Snelling Salt reaction could not be working is not because it won't reduce **2.1**, but rather at the 2 mg reaction scale, it was challenging to add 1 µL to the reaction without exposing it to air and thus the reagent is quenched upon addition.



Scheme 4.7 Desired Hydrogenation of NP-34 and NP-34-915 were unsuccessful under various conditions.

4.5.6 Attempts at Modification of Alcohol Side Chain

Creation of mono- or bis-substituted acyl analogs were attempted with **2.1** and **2.2**, respectively. Multiple attempts at derivatization of the secondary alcohol at C9 (and C9') position included acid chlorides with EDCI, DMAP, and TEA to attempt addition of not only fatty acid-like side chains, but also propargyl substitutions for click chemistry (Section 4.5). Further

attempts to alkylate included reactions of propargyl bromide using either KH, NaH, n-BuLi, and/or imidazole in tetrahydrofuran or MeOH (0 – 40 *C).[32,33] Additional experiments included attempts to mesylate the compounds with mesyl chloride, DMPA, and pyridine,[34] and attempts to add on methyl isothiocyanate with trifluoroacetic acid in DCM,[35] but to no avail.

All attempts were unsuccessful (no yields of desired products), due to the unsurprising, low reactivity of this group. This neopentyl alcohol (pinacolyl) is known to have low reactivity due to steric hinderance imparted by the adjacent gem-dimethyl group. Additionally, the low yields of macrolide biosynthetically in the lab limited the scope of reaction optimization possible. Conversions of the alcohol at C9 to an amine were not carried out due to these same reasons. When larger quantities of **2.1** and **2.2** are obtained, further optimization of the reactivity of this secondary alcohol can be investigated.

4.6 Attempted Analogs for Affinity Chromatography

Having a larger supply of **2.1** to create functional analogs allowed for exploration into clickable analogs. Figure 4.23 shows the workflow for using alkylated NPs to determine MoA using affinity chromatography. Bioactive compounds with insignificant toxicity towards beneficial insects, like **2.1**, can be treated with affinity probes (alkynes, benzophenones, alkyl halides) and introduced into mosquito cells. Replacement of the isobutyrate moiety with other acetyl groups should retain mosquitocidal activity, providing a handle for introduction of the biotin probe. Biontinylation using click chemistry approaches[36] and subsequent affinity purification with streptavidin chromatography would yield protein lysates that would be investigated with LCMS analysis to elucidate mosquito targets of interest. Furthermore, these experiments would be carried out in *D. melanogaster* and *Drosophila* cells for parallel comparison to mosquito targets, to reveal potential non-specific binding.

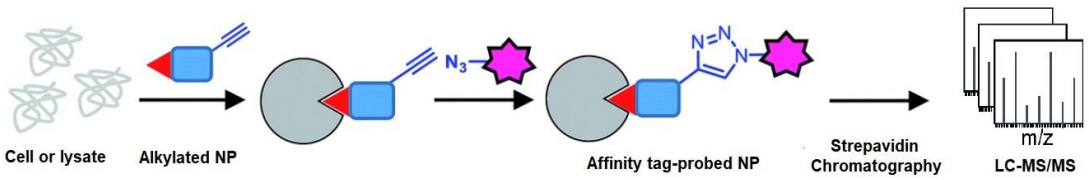
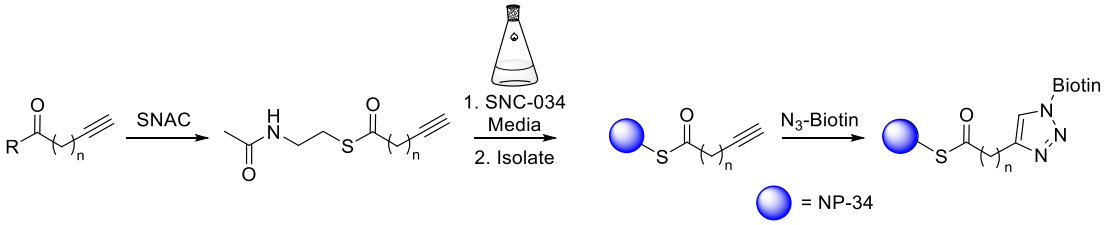


Figure 4.23 Workflow for use of alkylated NPs for protein pulldown using streptavidin affinity chromatography.

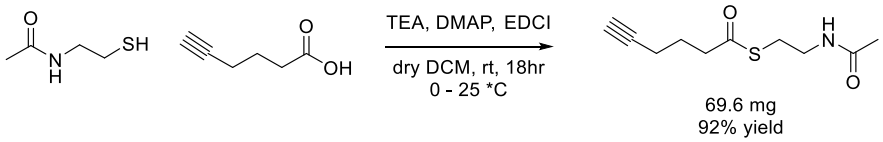
Limited reactivity at the pinacolyl alcohol as previously mentioned made installation of an alkyne group at C9' position unsuccessful. Efforts were then focused on the creation of *N*-acetylcysteamine (SNAC) derivatives that could be incorporated biosynthetically into **2.1**'s macrolide skeleton during fermentation. SNAC is an acetyl-CoA mimic, which is a NP elongation unit used in the biosynthesis of polyketides like **2.1**. Previous work by Klopries *et al.* have performed feeding studies using SNAC derivatives for successful biosynthetic incorporation into erythromycin and rapamycin.[37]

Scheme 4.8 shows the proposed workflow to prepare the SNAC derivatives. Alkylated carboxylic acids or acid chlorides can react with SNAC to create alkylated-SNAC derivatives that will be added to SNC-034 in A1FeB+C media. As SNAC mimics malonyl-CoA, it can compete with malonyl-CoA for biosynthetic incorporation into macrolides produced during fermentation, producing analogs with alkylated handles. These analogs can be isolated, which with the extra alkyl handle at C9', should be relatively easy to separate from **2.1,** for click chemistry to attach biotin for subsequent affinity chromatography. Biosynthetic incorporation would achieve a new biosynthetic analog for 1) biological testing and 2) click chemistry that would simplify the process by removing one synthetic step.



Scheme 4.8 Proposed workflow for preparation of alkylated-SNAC derivatives to add to SNC-034 media. During fermentation, the analogs should compete with malonyl-CoA for biosynthetic incorporation into the macrolides, producing analogs with alkylated handles. These can be isolated for click chemistry to attach biotin for subsequent affinity chromatography.

Attempts with acid chlorides resulted in low yields of desired products, but when 0.95 eq of SNAC was added to a solution of 5-hexynoic acid in DMC with EDCI, TEA, and catalytic DMAP[38] and the desired product was observed after 18 hrs (Scheme 4.9) when stirred in an ice bath that was allowed to warm to room temperature.



Scheme 4.9 Reaction of SNAC with 5-hexynoic acid in the presence of DMAP, EDCI, and TEA yielded the desired material after 18 hrs stirring in an ice bath that was allowed to warm to room temperature.

A desired 4 mM worth of material was needed for 50 mL cultures of SNC-034, so all 69.6 mg (approximately 4.7 mM) were added to the culture without further media alteration. The culture was allowed to ferment for 7 days and then was extracted with XAD-7 resin as usual. ^{11}B NMR of the organic residue showed no BO_4^- peak, indicating that potentially there were some acidic side-effects to incorporation of this SNAC derivative to the macrolides. Thus, with LCMS analysis, molecular ions $[M-1]^-$ = 861 and $[M-1]^-$ = 969 were sought after, indicative of a mono-substituted and bis-substituted alkyl group, respectively (Figure 4.24), but neither were observed. This indicates that there was not incorporation of the SNAC derivative into the macrocyclic ring.

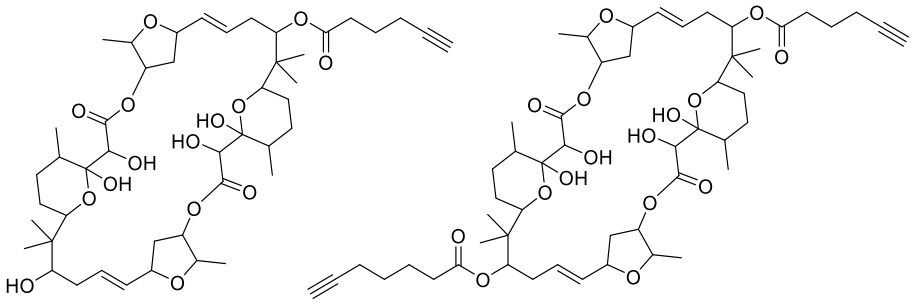


Figure 4.24 Structures of the potential alkylated SNAC-derivatives of NP-34

Since there was no incorporation with just simple addition of compound to fermentation media, it is thought that an enzyme is needed to catalyze the reaction. Use **2.2** with an acyl transferase from the gene cluster to catalyze the reaction as acyltransferases tightly control the carboxyacyl-CoA substrates that are added onto polyketides.[39]

4.7 Crystal Structures

As differences in ^{1}H NMR for the same analog were thought to arise from differences in the cationic species coordinating to the boron, a method of determining their identities was needed. Crystal structure would be a good way of determining what cations were present as crystallography is an excellent tool for analyzing inorganic material. Not only would achieving crystal structures be good for determining the present cationic species, but it would also give much insight into the 3D shape of these macrolides. When material in solution crystalizes, they take on an ordered confirmation that reveal much about a molecule including absolute stereochemistry and 3D confirmation which is important when thinking about biological properties.

The biggest challenge was finding the proper solvent system to crystalize these macrolides as all analogs showed excellent solubility in organic solvents. No issues in solubility were seen with IPA, hexanes, MeOH, ACN, chloroform, DCM, EtOAc, DMSO, isooctane, or acetone. Combinations of all these solvents were unsuccessfully employed with the three most common methods of growing crystals: slow evaporation, slow cooling of solvent, and gas-phase diffusion of precipitant into solution.[40] Attempts included placing a heated hexane solution in -20 °C freezer; slow vapor diffusion between $CDCl_3$ and iso-octane;[41] and slow evaporation of $CDCl_3$ in a NMR tube,[41] etc. What could be contributing to these challenges was the limited material to work with. As only a few milligrams of each analog were available, only microgram amount of material were used per crystallization condition, thus parameters that could've been successful potentially weren't because good crystals are formed with slow evaporation from super saturated solutions.

Crystalline material was unintentionally observed in vials that were placed under high pressure, N_2 (l) cooled vacuum (Figure 4.25). Unfortunately, x-ray analysis with XtaLab Synergy Rigaku Oxford Diffractor showed misaligned reflections indicating that crystals were not optimally packed. This implied that high pressure vacuum crystalized material too quickly and the resulting misalignment that would yield misinformed and inconclusive structures.

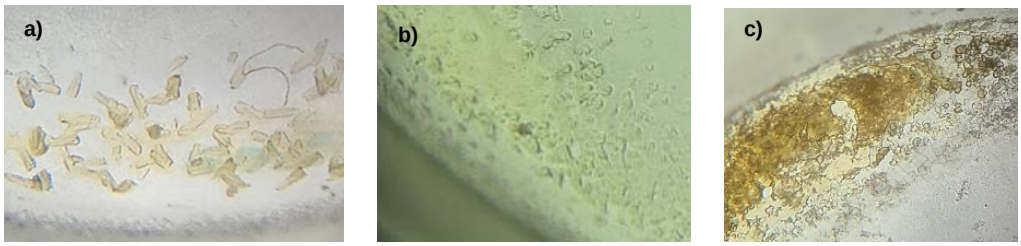


Figure 4.25 Examples of malformed crystals produced after material was dried in 20 mL scintillation vials in vacuum chamber. a) NP-34-915 b) NP-34V2 c) NP-34

The only method that yielded proper crystals was suspending material in benzene in a 1 mL glass insert, placed in a capped 20 mL scintillation vial, left on the benchtop at ambient temperature. Slow evaporation of the benzene left behind clear, colorless, planar crystals in a bed of yellow oily material after approximately one week (Figure 4.26).

Figure 4.26 Crystals of NP-34-915 after slow benzene evaporation.

Figures 4.25.a and Figure 4.26 show the stark differences between the malformed and properly formed crystals of the same NP, respectively. Figure 4.25.a shows a clear yellow, rod-like material, which are unevenly distributed in thickness, whereas the proper crystals in 4.26

are planar, thin, colorless crystals. Such physical differences in the molecular stacking rendered data acquisition meaningless in the first instance – incredible! Diffraction of the proper crystal (Figure 4.25) gave reflections with an atomic resolution at 0.8Å and revealed the crystal to have C2 symmetry, with a tetrahedral boron coordinating to Na^+ (Figures 4.27 and 4.28).

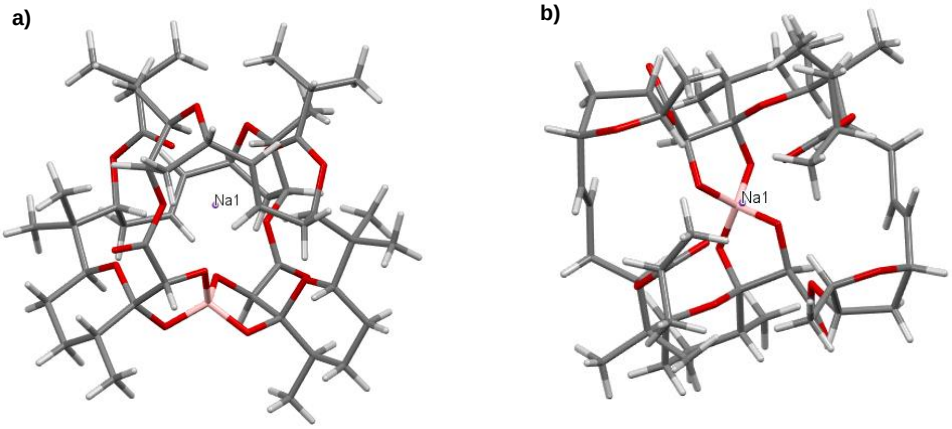


Figure 4.27 a) Side view of the crystal structure of NP-34-915 with all hydrogens shown. b) Top view of the crystal structure of NP-34-915 with all hydrogens shown. Na^+ is seen clearly coordinating to tetravalent boron.

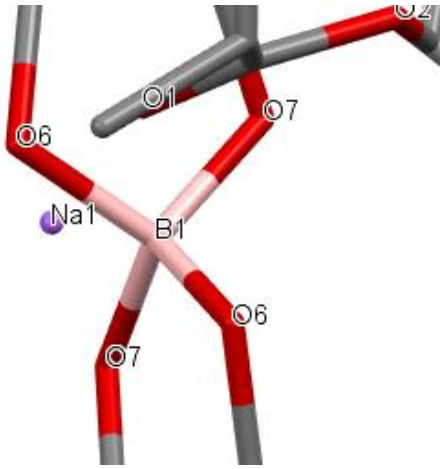


Figure 4.28 Crystal structure of NP-34-915 with non-hydrogens and carbons annotated and hydrogens omitted, zoomed into the Böeseken complex to highlight the tetrahedral orientation of boron coordinated to sodium.

The macrolide sits in a concave, “cupped” confirmation with the isobutyrate sidechains pointing towards each other due to the Boron’s tetrahedral character. I hypothesize that the

concaved confirmation is a result of the sodium coordinating to the boron. I propose there's an electronic interaction going on between the side chains and skeletal oxygens that are near the cation holding the molecule in this tight shape, potentially giving it access to the active site within the mosquito.

As aforementioned, various analogs were slightly different versions of themselves via ^{1}H NMR. For example, **2.11**, **2.11V2**, and **2.11V3** all had the same molecular weight M^{-} = $[915]^{-}$ via LCMS and the same molecular structure via ^{1}H and ^{13}C NMR, but the ^{1}H NMRs were slightly different. It was thought that the cationic binding partner of boron would lead to these slight differences.

Interestingly, **2.11** vs **2.11V3** were the first samples to crystalize, and upon inspection of crystal structures, both showed coordination to Na^{+}. This was very surprising as a difference in cation was expected to account for the difference in ^{1}H NMR. That led to the idea that it could indeed be a different in stereochemistry, but when both structures were superimposed, they stacked perfectly indicating that both structures were identical (Figure 4.29). Forces that hold crystals together, such as bond lengths and angles, will conserve between solution and crystalline state, whereas other factors such as dihedral angles and folding can change between the two. Potentially the coordinating cation can change in solution as well, if there is more than one present in solution, and molecules will crystalize only under certain conditions. That would explain why both analogs showed sodium coordination. It would also explain why there's crystal evenly distributed in a bed of oil – the oil can be material that that is coordinating to another cation and isn't able to crystalize under these conditions.

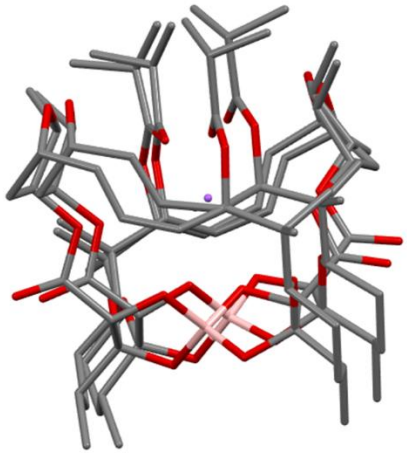


Figure 4.29 When structures of "two versions" of the bis-substituted isobutyrate molecule, NP-34-915 V1 and NP-34-915 V3, were taken and stacked. Both molecules were identical which could mean there is an exchange of cations possible when in solution, but material is crystalized when in presence of sodium cation.

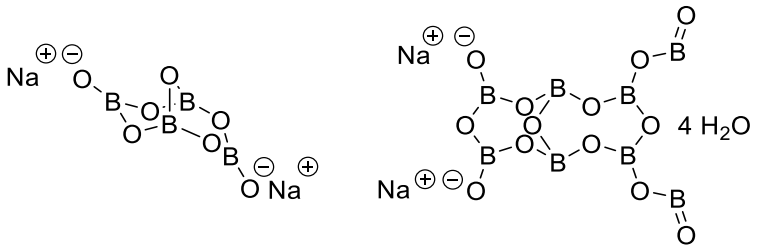
Very important will be the biological results between these same molecular species with different ^{1}H spectrum like **2.11**, **2.11V2**, and **2.11V3**. Ultimately, if there is no difference in the mosquitocidal selectivity or toxicity, then this will not be a problem. If there are substantial differences in activity, then we can assume the cation is making the difference within the molecule, and further work can be done into testing the affinity for and exchanging cations.

4.8 Discussion

Boron

Macrocycles, like **2.1**, are 12-membered or larger cyclic structures, that are biologically relevant. They are regarded as platforms for inaccessible targets because of their optimal properties compared to smaller, linear molecules. These appealing characteristics include that constrained cycles can be preorganized for target binding and high molecular weights are more suitable for complexation with a protein's surface area.[21] This could explain why this class of molecule has such great activity and selectivity – it's well suited for binding with a very specific mosquito protein.

Boron-containing macrolides, to the best of our knowledge, have not previously been exploited as insecticides, but their potential due to their selective mosquitocidal activity, is very exciting. Non-organic elements like metallic iron, lead, tin, and arsenic, etc. are prevalent in insecticides, but not as much as the metalloid boron. It is commonly found in insecticides such as boric acid, borax, and disodium octaborate tetrahydrate (DOT) (Figure 4.30). Borax and boric acid are readily absorbed by insects, eliciting toxic effects in the gut, and abrading their exoskeletons, and DOT stops enzyme functions.



Borax **Disodium octaborate tetrahydrate**

Figure 4.30 Structures of two commonly used insecticides containing boron.

While **2.1** is likewise a boron compound, the boron itself should not be eliciting any of the observed mosquitocidal activity. Rather, it should only be retaining the optimal confirmation required to access the mosquito target site. In these macrolides, boron does not have the electronic freedom to participate in interactions that can result in binding, proton exchange, etc. that would affect the active site, resulting in toxicity. Had the boron in **2.1** not been tetravalent, potentially it could've acted as a receptor. Boron is an electrophilic atom and readily undergoes reactions with different alcohol and amine species, so had it been trigonal planar, it could've formed a bond at the active site that inactivated the target, and lead to toxic effects.

Np-34 Analogs and Mosquitocidal Activity

The extent of mosquitocidal activity is yet to be seen as various analogs of **2.1** are available for testing after isolation and synthetic derivatized. These analogs are to be tested in the same mosquito-cell based assay used to identify **2.1** and against the same controls of *D.*

melanogaster and *S. frugiperda.* Follow up studies will likewise be conducted in adult and larvae mosquito to check for translation into *in vivo* activity. Differences in chemical structure are desired to understand more about the binding site within the mosquito and what about their structures cause such selective and potent activity.

Based on what has been observed with **2.1**, **2.2**, **2.11**, and **2.12**, there are a few hypotheses that can be drawn before testing. For one, the boron-less analogs **4.2**, **4.3**, and **4.4** should exhibit less potent mosquitocidal activity like **2.12** did. Reduced activity is also hypothesized for the epoxide analogs **4.5** and **4.6** of **2.1** and **2.11**, respectively, as epoxides will increase the flexibility in the molecule as compared to the olefin. It would be interesting to observe though how the opening of the epoxides to the corresponding diols will affect the activity. Analogs lacking the acyl substituents at C9 and C9' exhibited loss of activity, but this analog contains an acyl group at the C9 position, and typically, alcohols tend to be good for physiochemical conditions. Addition of the alcohol groups throughout the core of the molecule could potentially help with cell permeability, solubility in water (which will be good for insecticidal development since right now **2.1** is insoluble in pure water), etc.

Interestingly, bis-substituted analogs seemed to be more favored than mono-substituted analogs, and it would be interesting to understand why, because even though the side chains are believed to be non-enzymatically incorporated, that structure of the NPs produced says more about the bacterium than the mosquito. Yes, **2.1** is produced by marine bacteria, but these molecules weren't produced with the intent of being mosquito toxins. These molecules are produced to provide their producing bacterium with an environmental fitness, and mosquito species are not a threat to *Streptomyces malachitospinus* residing in marine sediment. These were possibly produced as a self-defense mechanism to detoxify itself of excess boric acid in its system. The fact that it is mosquito-toxic only illustrates the wide range of possibilities NPs have for biological applications due to their structural complexity. It is very fortunate that **2.1** happens to fit perfectly in an active site within that mosquito vector that elicits

death. NPs have a wide variety of potential, and **2.1** being from a known class of NPs, shows that even though there is a decrease in the number of structurally novel NPs being identified,[42] there is still biological activity yet to be discovered in because the full potential of a single molecule hasn't been fully explored.

Optimized Growth and Purification Pipeline

At the start of this project, SNC-034 was yielding 1 mg/L of media grown. Then issues with fermentation and isolation arose that rendered yields to 0.1 mg/L, leading to reexamination of the entire procedure. Optimization of both culturing conditions to better mimic environmental conditions better suited for SNC-034 growth and isolation steps that retain the structural integrity of **2.1** resulted in a 12.3 mg yield from 1L of SNC-034 when all the positive "results" of experimentation were incorporated. This is very promising for carrying the project forward as there are now methods to isolate higher yields of **2.1** (Figure 4.31) in shorter windows of time than before. These higher yields also allow for more flexibility in terms of derivatization for both SAR and MoA studies.

Figure 4.31 Optimized isolation procedure for NP-34.

The more optimal yields with day 3 extractions were determined after noticing a decrease in amount of macrolide as pH of media increased over the course of fermentation. What could also be occurring is the bacteria is redigesting produced metabolites because of spent media being unable to provide nutrients and carbon sources needed to continue forming NPs. In redigesting the macrolides, the overall yield of **2.1** and other macrolides would decrease, so fermentations should be kept to only 3 days at maximum.

Additionally, the best yields of material were seen in extractions where resin was acetone soaked in individual batches as opposed to combining multiple liters. This is potentially because the acetone solution becomes highly saturated and is unable to pull more material

from resin. Moving forward, resin extractions should be treated as such – one acetone soak per liter grown, no matter the scale up size, to maximize amount of macrolide collected.

Crystallography

Now that there are methods to crystalize these molecules, substantial confirmational data can be achieved. For one, relatively no analysis of stereochemistry has been done on these molecules and the crystal structures would give absolute configurations. These macrolides could be identical constitutionally but differ in 3D space, which is important as the slightest change in orientation, whether in an isolated isomer or by inversion caused by a reaction, can drastically hinder the activity and/or selectivity of the molecule. Knowing the orientation of the molecule also gives further insight into the binding pocket, which informs MoA and further mosquito-control strategies.

One of the biggest limitations to x-ray crystallography will be the relative low yield of these macrolides. We're working with relatively such small scales of material that challenge crystal growth. Furthermore, good crystal conditions do not guarantee good quality crystals as they can pack poorly, be misaligned, etc. The more volume of NP available, the better chance at growing good quality crystals. Additionally, more amounts of crystals are desired due to the lower power limits of diffraction.[43]

Microcrystal electron diffraction (MicroEd), a 3D application of single-particle cryo-electron microscopy (CryoEm) for small molecules like NPs, can offer complimentary data to crystallography,[44] but is also good alternative for dealing with small(er) crystals. Since it uses electrons, which can interact more strongly than x-rays, it can help achieve data acquisition in cases with lesser yielding natural analogs or synthetic analogs because there will be relatively less of those crystals. It can even yield data for crystals with a moderate solvent content present, allowing minimal amounts of crystal to give data.[43] Crystals created under the same conditions for crystallography can be used with MicroED, and it is an up-and-coming technique in the field of NPs. In terms of complimentary data, Crystallography is well equipped to provide

functional data including temperature, pressure, and other perturbations, whereas cryo-EM-based methods can offer more insight into conformation and energy landscapes.[45] Together, they could answer virtually any question regarding the structure of **2.1** and analogs and how they behave in 3D space.

Additionally, crystallography can help visualize these molecules' binding within mosquito vectors, giving insight into target sites, greatly informing further SAR and analog design. After successful affinity chromatography, isolated target protein(s) can be co-crystalized with **2.1** to form protein-ligand complexes, to visualize the antagonists' fitting in the binding pockets. This method of co-crystallization allows antagonist to bind to protein freely without the constraint of a crystal lattice,[46] allowing qualification of fragment-fitting based on conformation. This can help suggest subsequent design of stronger binders to improve mosquitocidal activity.

Future Directions

While a great increase in **2.1** production was seen from altering both the fermentation and isolation methods of SNC-034, it is still not the increase that is needed. Milligram amounts of material will be needed of **2.1** and analogs for the required animal and human toxicity studies to get approval as an insecticide. Similarly, 1 g will be needed per planned controlled field study, which is necessary to understand **2.1**'s behavior as an aerosol. Also, if it were to get EPA approval, it would have to be produced on the kilogram scale. Thus, much work is needed in inducing production of these macrolides as what has been done here is good for laboratory studies and complimentary with synthetic biology efforts, but not for mass production.

Synthesis of the molecule would be feasible had it been a smaller petroleum-based product. The synthesis of Aplasmomycin was first reported by in the early 1980s, constructed by coupling precursors made from the inexpensive, commercially available (+)-pulegone (C3-C10) and D-mannose (C11-C17).[47,48] This was followed by a synthesis based on the (+)-dithiane (C3-C11) key intermediate of that synthesis with controlled 1,3- and 1,5-asymmetric

reductions on (+)-aldehyde, starting from (-)-(S)-2-hydroxy-4-butanolide.[49,50] And most recently by macrocyclizations from subunits derived from (+)-pulegone (C3-10) and (2S, 3R)-1,2-epoxy-3-butanol with propargyl alcohol (C11-C17), who likewise reported Na^+ coordination to boron.[51] These total syntheses, while are amazingly designed chemical feats that achieved the desired product in its totality, suffered from the notorious trait of NP total synthesis being multiple steps with low final yields. The 1,2-epoxy-3-butanol derived product, for example, reported an final yield of 8.8 mg.[51] The construction of these macrolides currently are not feasible for industrial processes by chemical means.

Only so much can be done in the lab to mimic the environmental cues that promote secondary metabolism, so further work will need to focus on the genomic level. This can include altering regulatory genes necessary for NP production. For example, regulatory genes within BGCs, which are those dictated by environmental stimuli, repress or activate the biosynthesis of NPs. Lack of said genes could lead to production without the necessary environmental stresses.[11] These synthetic biology methods would be more feasible for production of the molecule as it's a NP. Synthetic biology efforts involve the redesign and engineering of organisms for specific purposes, such as producing a substance. Manipulations to the BGC responsible for coding NPs within an organism could result in an enhancement in production. This includes activating promoters or positive regulatory genes; deletion or inactivation of repressor and regulatory genes; exchange of promoters; and/or ribosome engineering.[52] Additionally, these BGC can be cloned and expressed in heterologous hosts, such as yeast, *E. coli,* and *Saccharomyces cerevisiae,* workhorses that could potentially yield gram quantities of NPs.

Thus, these optimized fermentation conditions of SNC-034 coupled with synthetic biology tools to yield higher titers can be the key to producing these macrolides on an industrial scale. This can be used to prioritize production of **2.1** or an analog with improved efficacy to develop an ecofriendly mosquitocide. We are optimistic that **2.1** or a slightly modified analog

possess the efficacy and selectivity to be used in times of mosquito outbreak in developed areas or to thwart mosquito populations in areas where diseases are still endemic.

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EXPERIMENTAL

E.1 General Information

Commercial chemicals (Sigma Aldrich) and deuterated solvents (Cambridge) were used without further drying or purification. Compounds from natural sources were grown from culture and purified. The ^{1}H and ^{11}B NMR spectra were recorded on Bruker Avance III HD 800 MHz, Varian Inova 600 MHz, or Bruker Avance III HD 500 MHz spectrometers. ^{11}B NMR taken on Bruker 500 MHz BBFO SmartProbe spectrometer. Chemical shifts were referenced to TMS (^{1}H: δ 0.0 ppm) and BF_3OEt_2 (^{11}B: δ 0.0 ppm), with downfield positive shifts. Chemical shifts (δ) are given in ppm relative to residual solvent chloroform (CDCl3: 1 H, δ = 7.26 ppm, 13C, δ = 77.16 ppm) and coupling constants (J) in Hz. Multiplicity is tabulated as s for singlet, d for doublet, t for triplet, q for quadruplet, m for multiplet, and br when the signal in question is broadened. For ^{11}B NMR, 128 – 2048 scans were collected depending on sample over a spectral width of (-100 – 100 ppm). NMR samples were prepared in 5 mm Wilmad® Quartz NMR tubes, 535-PP-7QTZ, and lock was achieved with deuterated solvents. Data was collected at ambient temperature. LC-MS measurements were performed via direct injection to the MS Agilent 6130 Quadrupole LC/MS with Electrospray ionization mass spectra (ESI-MS), bypassing acidic solvents and column matrix. Mass spectra were recorded on LTQ-Orbitrap Velos Pro MS. 100 µL injections of 0.5 mg/mL solutions in MeOH were tested. Semipreparative and analytical HPLC purifications were carried out on Agilent 1200 HPLC consisting of a UV−vis detector (G1315D), dual pumps (G1312A), and a dynamic mixer (G1312A). Reactions were performed under an atmosphere of nitrogen with magnetic stirring unless noted otherwise. Thin layer chromatography was performed using precoated plates purchased from E. Merck (silica gel 60 PF254, 0.25 mm).

E.2 General procedure for Fermentation of Biological Material

E.2.1 Collection of biological material. Microbial strains SNC-034 and SNC-117 from the MacMillan lab were isolated from dry stamping marine sediments collected in Vava'u,

Tonga at depths of 5 and 9 meters, respectively. The collection coordinates for the sediment for SNC-034 are 18º 39'04" S, 173º 59'21" W and SNC-117 are 18º 39'01" S, 173º 59'37" W. The sediment was desiccated and pasted onto agar plates using gauze 1 acidic media (10 g starch, 1 g NaNO3, 0.5 g K2HPO4, 0.5 g MgSO4, 0.5 g NaCl, 0.01 g FeSO4, 1 L seawater, 15 g agar, adjust pH to 5.3 with phosphate buffer). Bacterial colonies were selected and streaked to purity using the same agar media. Analysis of the strain SNC-034 by 16S rRNA revealed 99.9% identity to *Streptomyces malachitospinus.* The sequence is deposited in GenBank under accession no. MF159575. Analysis of the strain SNC-117 by 16S rRNA revealed 97.0% identity to *Streptomyces intermedius.* The sequence SNC-117 the GenBank accession number is pending. *Vibrio harveyi,* strain BB152 that produces AI-2, was cultured, and supplied by the Karen Ottemann lab at UC Santa Cruz.

E.2.2 Cultivation and Extraction of SNC-034 and SNC-117 cultures. Bacterium SNC-034 and SNC-0117 were cultured in 20 x 2.8 L and 5 × 2.8 L Fernbach flasks, respectively. 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO3, 40mg Fe2(SO4)3·4H2O, 100 mg KBr) was added to each liter and and shaken at 200 rpm at 27 °C. After seven days, sterilized XAD-7- HP resin (20 g/L) was added to each flask for 2 hr to adsorb the organic products. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone soluble fraction was dried *in vacuo.*

E.2.3. Fermentation of *Vibrio harveyi.* Supplied by Prof. Karen M. Ottemann and Ms. Frida Salgado. Fermentation and subsequent liquid culture provided by the Karen Ottemann lab at UC Santa Cruz. *V. harveyi* was grown overnight on LB plates, without antibiotics, in Autoinducer Biossay (AB) media (17.53g NaCl, 6.02g $MgSO_4$, Casamino acids, 2.0g vitamin-free) in 960 mL distilled water. pH was adjusted to 7.0 KOH medium and sterilized (15-minute autoclave at 121°C). When cooled, filter sterilized 1 M Potassium phosphate, pH 7.0 (10.0 mL), 0.1 M L-Arginine (10.0 mL), and 50% Glycerol (20.0 mL) are added to the media. 10 µL of BB (sterile) with the *V. harveyi* are used as control. 90 µL of *V. harveyi* for every 10 µL of cell free

culture were added to produce a 50 mL culture. The culture grew at 27 °C for 24 hr. After 24 hr, the 50 mL culture was dried *in vacuo* to yield testable crude material.

E.3 Identification of Mosquitocidal Agents HTS with Cell Line. Crude and semi-crude NP samples from the MacMillan fraction library were screen against cultured cell lines of *Anopheles gambiae* 4A3A, with follow up against *Anopheles gambiae* (MRA-921), *Anopheles stephensi* (MRA-858), and *Aedes aegypti* (Aag2). All compounds were tested against control cell lines of *Drosophila melanogaster* (Kc and $S2R^{+}$), *Spodoptera frugiperda* (SF9), and 30 human cell lines of epithelial and neuroendocrine origin to confirm selectivity in parallel. Additional controls included in the assay were DMSO and blasticidin. A all compounds were injected at 5 uM in 1% DMSO to cells incubated for 96 hours in 384 well plates,[1] and the toxins that showed no activity towards control cell lines would then move forward for *in vivo* efficacy in whole animal studies which were conducted by external collaborators. All cell death in the assay was quantified via CellTiterGlo.

E.4 Optimization of ^{11}B NMR The pulse sequence was available on the 500 MHz Bruker instrument in pulse program library called "zgbs." To further reduce noise, all experiments were conducted in quartz NMR to ensure signal is not compromised by borosilicate NMR tubes as previously specified. Proof of concept was tested with 600 uL pentafluorophenylboronic acid, 3.93 mM in MeOD.

E.4.1 Test of alignment compared to standard ^{11}B NMR. To ensure that the pulse sequence did not alter acquisition parameters or interfere with referencing, synthetic compounds were tested with ^{11}Bzgbsig vs standard ^{11}B experiment to ensure the correct chemical shifts were observed. 600 µL of the boron reference standard boron trifluoride etherate (BF_3OEt_2, 0.675 M); a commercial borane reagent used for hydroborations and reductions, BMS (3M); and an aromatic boric acid, Pyrene-1-boronic Acid ($C_{16}H_{11}BO_2$, 0.169 M) were acquired with ns = 128 scans in MeOD. With pyrene-1-boronic acid, even with 25 mg

of a pure, synthetic compound of small molecular weight (x<300 g/mol), a small hump of asymmetrical noise is seen ranging from 30 to 15 ppm in standard ^{11}B decoupling experiment.

E.4.2 ^{11}B NMR Limit of Detection To probe the feasibility of NP-detection, a series of boric acid concentrations were created to test for detection limits. 4.72 mM (1 mg), 1.0 mM, 0.5 mM, 0.1 mM, 0.05 mM, and 0.01 mM boric acid in MeOD were prepared in quartz NMR tubes. Spectra acquired with ns = 128 and 512 scans with the zgbs pulse sequence applied to boron NMR experiment. Likewise, the limits of detection were probed with the proton no decoupling experiment under the same parameters, to observe ^{1}H-^{11}B splitting patterns. With ns = 128 scans, a boron compound as low as 0.5 mM can be distinguished by its 11B peak with the 11Bzgbsig experiment. With ns = 512 scans, a boron compound as low as 0.1 mM can be distinguished by its 11B peak with the 11Bzgbsig experiment.

E.5 Isolation of Boron-containing Macrolides

E.5.1 Initial Isolation Sequence of NP-34 and Other Analogs from SNC-034. Crude material was dissolved in 2:1 EtOAc:DIW and extracted three times. The organic layer was dried with $MgSO_4$ to give an oily orange-brown residue. The fraction was resuspended in DCM and separated by flash chromatography on silica (flash chromatography performed using E. Merck silica gel 60, 240–400mesh), eluting with a step gradient of DCM and MeOH (0%, 1%, 2%, 3%, 5%, 10% MeOH in DCM). Boron-containing macrolides would elute at 3% MeOH in DCM, R_f = 0.69, visualized with TLC, heated in 10:1 DCM:MeOH with vanillin stain. The dried residue would be further separated by HPLC on C5 column (250 x 10 mm), eluting with 100% ACN (0.l% FA), flow rate 2.5 mL/min, λ = 210 nm, at t_R = 12.9 min (NP-34). The sub-fraction containing **2.1** would be attempted for further purification on HPLC with OD-H chiral column (250 x 4.6 mm) eluting with 1% IPA in HEX at 0.7 mL/min.

E.5.2 Optimized Isolation Sequence of NP-34 and Other Analogs from SNC-034. The crude material was dissolved in 2:1 EtOAc:DIW and extracted three times. The organic

layer was dried with $MgSO_4$ and dried *in vacuo* to give an orange-brown oily residue. The residue was resuspended in MeOH and fractionated with Sephadex® LH-20 with Chromaflex Column, 2172 mL, 120 cm L x 4.8 cm ID, 1 drop per second rate with Gilson FC 203B Fraction Colelctor to yielded semi-crude, yellow-orange oil. The material was resuspended im MeOH and further purified by semi-prep HPLC on Kinetex 5 µm EVO C18 100 Å, 250 x 10.0 mm (Phenomenex). Solvent A: H2O, no acidic or basic buffer, pH 7.0. Solvent B: Acetonitrile, no acidic or basic buffer. Using an isocratic elution of 95% B over 90 minutes at flow rate 1 mL/min to yield white solid material. Boron macrolides would elute out after between 30 and 60 minutes, with the mono-substituted analogs eluting sooner and the bis-substituted analogs eluting later. For example, **2.1** would elute out at approximately t = 36 min and **2.11** at t = 48 min.

E.6 LCMS Analysis Samples for LCMS analysis are prepped at 100 µL per compound at a concentration of 1 mg/mL concentration in MeOH and centrifuged to separate out solid precipitant. 50 µL are added to a vial with 50 µL MeOH to yield a final sample concentration of 0.5 mg/mL. Using a Model 9013 Needle Port (IDEX RheFlex PEEK fitting), the sample is injected directly into mass spectrometer using a blunt tip 100 µL syringe, while method of 0.3 mL/min 100% ACN is flowing to waster (a method needs to be running to acquire mass spec data) with mass window 200 – 1500 m/z. Negative mode MSD2 is analyzed as these macrolides do not ionize well in positive mode.

E.7 Crystallography

E.7.1 Crystallization of Macrolides Approximately 1.5 mg of corresponding macrolide would be dissolved in 300 µL of benzene in a 1 mL glass insert and placed in a capped, 20 mL scintillation vial. The solution was left on the benchtop at 25 °C to allow for slow evaporation of the benzene over a period of approximately 1 week.

E.7.2 x-ray Crystallography Crystals were selected under a polarizing microscope, loaded onto a nylon fiber loop using Paratone-n, and mounted onto a Rigaku XtaLAB Synergy-

S single-crystal diffractometer. Each crystal was cooled to 100 K under a stream of nitrogen. Diffraction from a PhotonJet-S microfocus source was detected using a HyPix-6000HE hybrid photon counting detector. Screening, indexing, data collection, and data processing were performed with CrysAlisPro.[2] The structures were solved using SHELXT and refined using SHELXL.[3,4] All non-H atoms were refined anisotropically. H atoms were placed at geometrically calculated positions and refined with a riding model.

E.8 Synthetic Reaction Conditions

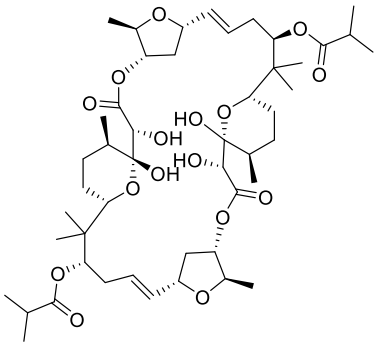
E.8.1 Removal of Boron

Procedure for Boron Removal of NP-34

One milligram of **2.12** (1.19 µmol) was synthesized by adding 5 drops of HPLC-grade H_2O with 0.1%FA dropwise to a solution of **2.1** (1.0 mg, 1.18 µmol) dissolved in 1 mL EtOAc with rapid stirring at 25 °C for 2 hrs in a capped scintillation vial. Reaction conversion was monitored via LC-MS and confirmed with total loss of boron signal in ^{11}B NMR. The solution was dried under vacuum to yield pure boron-less material. ^{1}H is the same as isolated **2.12**.

Procedure for Boron Removal of NP-34-915

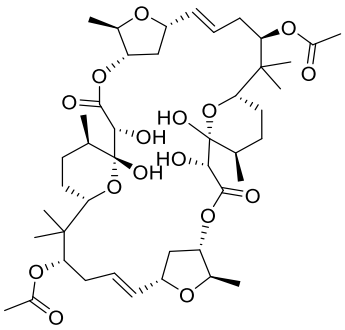
One milligram of **4.3** (1.10 µmol) was synthesized by adding 5 drops of HPLC-grade H_2O with 0.1%FA dropwise to a solution of **2.11** (1.0 mg, 1.09 µmol) dissolved in 1 mL EtOAc with rapid stirring at 25 °C for 2 hrs in a capped scintillation vial. Reaction conversion was monitored via LC-MS and confirmed with total loss of boron signal in ^{11}B NMR. The solution was dried under vacuum to yield pure boron-less material.



*NP-34-915-DB (**4.3**).* ^{1}H (600 MHz, Chloroform-*d*): δ 5.75 (2H, m), 5.35 (2H, m, 17.6, 11.3), 5.16 (2H, dd, 12.4), 4.90 (2H, d, 3.9), 4.58 (2H, d, 4.3), 4.49 (2H, d, 6.6), 4.13 (2H, s), 3.47 (2H, t, 6.6), 2.54 (2H, m), 2.35 (2H, m, 14.8, 7.6), 2.03 (2H, m), 2.01 (2H, m), 1.98 (2H, m), 1.56 (2H, m), 1.54 (2H, m), 1.44 (2H, m), 1.36 (2H, m), 1.17 (12H, m), 1.08 (6H, m), 0.88 (6H, d, 6.0), 0.75 (6H, s), 0.62 ppm (6H, s). ^{13}C (600 MHz, Chloroform-*d*): δ 180.7, 176.7, 132.5, 130.9, 101.4, 82.7, 81.0, 80.7, 78.1, 73.8, 67.7, 43.2, 36.8, 36.4, 29.9, 29.8, 27.3, 21.8, 21.4, 20.7, 20.3, 16.9 ppm. $[M]^-$ = 908 g/mol.

Procedure for Boron Removal of Aplasmomycin C

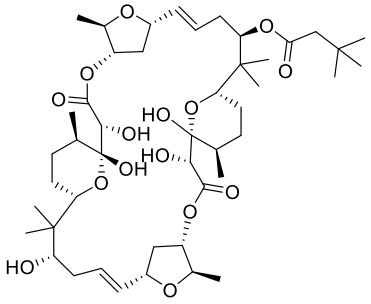
One milligram of **4.2** (1.17 µmol) was synthesized by adding 5 drops of HPLC-grade H_2O with 0.1%FA dropwise to a solution of Aplasmomycin C (1.0 mg, 1.29 µmol) dissolved in 1 mL EtOAc with rapid stirring at 25 °C for 2 hrs in a capped scintillation vial. Reaction conversion was monitored via LC-MS and confirmed with total loss of boron signal in ^{11}B NMR. The solution was dried under vacuum to yield pure boron-less material.



*Aplasmomycin-C-DB (**4.2**).* ^{1}H (800 MHz, Chloroform-*d*): δ 5.72 (2H, m, 7.2, 6.7), 5.50 (2H, dd, 15.6, 3.0), 5.24 (12.1, 1.6), 4.89 (2H, d, 5.4), 4.67 (2H, d, 7.2), 4.51 (2H, q, 6.8), 4.23 (2H, d, 11.8), 3.65 (2H, m), 2.50 (2H, m), 2.34 (2H, m, 14.5, 7.2), 2.15 (2H, m), 2.11 (6H, s), 2.05 (2H, m), 1.91 (2H, d, 14.5), 1.59 (2H, m), 1.45 (2H, dq, 6.1, 4.5), 1.30 (2H, m), 1.28 (2H, m), 1.11 (6H, d, 6.7), 0.98 (6H, d, 6.6), 0.78 (6H, s), 0.67 (6H, s) ppm. ^{13}C (800 MHz, Chloroform-*d*): δ 171.8, 168.7, 133.5, 126.3, 99.8, 80.8, 78.9, 76.8, 76.3, 75.3, 73.0, 40.5, 37.0, 32.8, 31.3, 27.9, 22.8, 21.1, 19.9, 18.4, 18.2, 16.7 ppm. $[M]^-$ = 852 g/mol.

Procedure for Boron Removal of NP-34-887

One milligram of **4.4** (1.14 µmol) was synthesized by adding 5 drops of HPLC-grade H_2O with 0.1%FA dropwise to a solution of **4.1** (1.0 mg, 1.13 µmol) dissolved in 1 mL EtOAc with rapid stirring at 25 °C for 2 hrs in a capped scintillation vial. Reaction conversion was monitored via LC-MS and confirmed with total loss of boron signal in ^{11}B NMR. The solution was dried under vacuum to yield pure boron-less material.



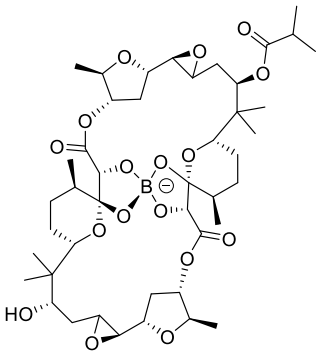
NP-34-887-DB. ^1H (500 MHz, Chloroform-*d*): δ 5.56 (1H, dd, 15.5, 4.1), 5.74 (1H, m), 5.68 (1H, m, 7.6), 5.44 (1H, dd, 16.2, 2.5), 5.30 (1H, dd, 11.6, 2.6), 5.27 (1H, dd, 11.5, 2.9), 5.22 (1H, dd, 12.3, 2.3), 4.90 (1H, t, 4.1), 4.70 (1H, m), 4.69 (1H, m), 4.56 (1H, q, 5.8), 4.45 (1H, q, 6.3), 4.23 (qs, 11.1), 3.50 (3H, s), 2.61 (1H, s), 2.54 (2H, m, 7.5), 2.41 (1H, m), 2.40 (1H, m), 2.36 (1H, m), 2.21 (1H, m), 2.17 (1H, s), 2.04 (1H, m), 2.00 (1H, m), 1.50 (2H, t, 13.9), 1.48 (2H, t, 13.9), 1.31 (2H, m), 1.28 (2H, m), 1.15 (9H, m, 7.5), 1.09 (3H, d, 6.6), 1.00 (3H, m, 3.3), 0.98 (3H, m), 0.89 (3H, m), 0.88 (3H, m), 0.79 (3H, d, 9.7), 0.69 (3H, d, 9.7) ppm. ^{13}C (500 MHz, Chloroform-*d*): δ 179.6, 168.9, 168.6, 133.6, 133.0, 126.4, 98.9, 9.7, 80.9, 80.3, 79.0, 76.9, 75.4, 40.6, 40.4, 34.3, 31.9, 31.6, 31.0, 51.0, 33.8, 32.9, 27.7, 25.3, 22.7 19.9, 19.6, 19.0, 18.1, 16.7, 14.5 ppm. $[M]^-$ = 880 g/mol.

E.8.2 Epoxidation of Alkenes

Procedure for Epoxidation of NP-34

Two mg of **2.1** (2.37 µmol) in 1 mL anhydrous DCM in a 20 mL glass, scintillation vial, and placed in an ice bath to set reaction temperature to 0 °C. Six eq of solid $NaHCO_3$ was added to the reaction, followed by 4 eq of *m*CPBA. The reaction was capped and kept stirring for two weeks until conversion of the corresponding starting material was seen via both LC-MS and ^1H NMR. To quench the reactions, solid $NaHCO_3$ was added to both maintain a high pH and to quench the *m*CPBA-carboxylic acid byproduct, and additional anhydrous DCM was added as needed. When pH 7 was observed, the product, a white powder, was extracted using saturated $NaHCO_3$ solution. No difference in yield was noted between refilling the ice bath to

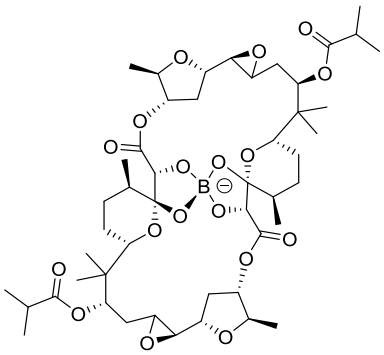
maintain the reaction at 0°C versus setting up at 0 °C and letting it warm to room temperature. Less than 10% COOH byproduct impurity was seen via ^{1}H NMR.



*NP-34-EPOX (**4.5**).* ^{1}H (800 MHz, Chloroform-*d*): δ 5.47 (1H, m), 5.28 (2H, d, 2.9), 5.15 (1H, d, 4.9), 4.91 (1H, d, 3.8), 4.64 (1H, m), 4.63 (1H, m), 4.51 (1H, s), 4.49 (1H, s), 4.15 (1H, q, 6.5), 3.89 (1H, dd, 11.7, 1.4), 3.65 (1H, d, 2.0), 3.56 (1H, dd, 11.5, 2.5), 3.32 (1H, m, 5.2), 3.02 (1H, dd, 6.1), 2.95 (1H, m, 6.5), 2.84 (1H, 12.3, 6.1), 2.69 (1H, m), 2.62 (1H, m), 2.40 (1H, m), 2.14 (1H, d, 4.0), 2.02 (1H, m), 2.01 (2H, m), 1.95 (1H, m), 1.93 (1H, m), 1.83 (1H, m), 1.65 (1H, m), 1.64 (1H, m), 1.54 (1H, m), 1.53 (1H, m), 1.51 (1H, m), 1.50 (1H, m), 1.42 (1H, m), 1.32 (1H, m), 1.30 (1H, m), 1.25 (6H, m, 7.2, 5.9), 1.13 (6H, d, 6.7), 1.00 (3H, 8.9), 0.98 (3H, d, 6.5), 0.74 (3H, d, 8.8), 0.70 (3H, s), 0.66 (3H, s), 0.62 (3H, d, 8.9) ppm. ^{13}C (800 MHz, Chloroform-*d*): δ 178.5, 176.7, 170.7, 105.8, 104.9, 79.6, 79.4, 79.0, 78.7, 78.4, 78.0, 76.6, 75.9, 75.7, 75.6, 71.3, 60.4, 55.7, 54.6, 40.7, 39.0, 35.8, 34.5, 33.6, 32.0, 31.7, 30.6, 28.0, 27.9, 25.4, 25.1, 22.2, 21.6, 19.2, 18.8, 18.5, 17.6, 16.8 ppm. ^{11}B (500 MHz, Chloroform-*d*): δ 10.5 ppm. $[M]^{-}$ = 877 g/mol.

Procedure for Epoxidation of NP-34-915

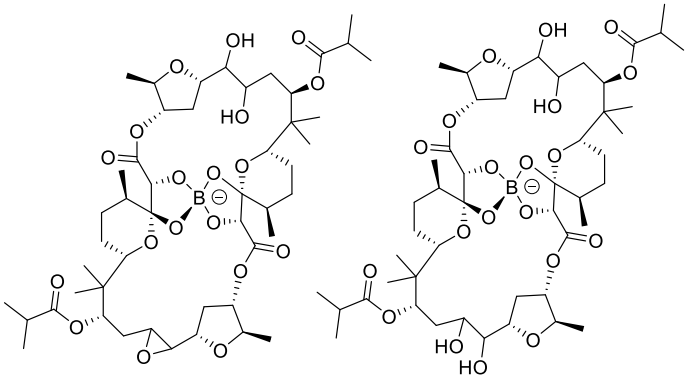
Two mg of **2.11** (2.19 µmol) were resuspended in 1 mL anhydrous DCM in a 20 mL glass, scintillation vial, and placed in an ice bath to set reaction temperature to 0 °C. Six eq of solid $NaHCO_3$ was added to the reaction, followed by 4 eq of *m*CPBA. The reaction was capped and kept stirring for two weeks until conversion of the corresponding starting material was seen via both LC-MS and ^{1}H NMR. To quench the reactions, solid $NaHCO_3$ was added to both maintain a high pH and to quench the mCPBA-carboxylic acid byproduct, and additional anhydrous DCM was added as needed. When pH 7 was observed, the product, a white powder, was extracted using saturated $NaHCO_3$ solution. No difference in yield was noted between refilling the ice bath to maintain the reaction at 0°C versus setting up at 0 °C and letting it warm to room temperature. Less than 10% COOH byproduct impurity was seen via ^{1}H NMR.



*NP-34-915-EPOX (**4.6**).* ^{1}H (600 MHz, Chloroform-*d*): δ 5.43 (2H, dt, 12.4, 3.5), 4.76 (2H, d, 5.7), 4.63 (2H, m), 4.46 (2H, s), 3.67 (2H, t, 7.5), 3.54 (2H, m, 11.3), 2.95 (2H, dd, 7.5, 1.7), 2.79 (2H, t, 5.0), 2.63 (2H, m, 6.7, 5.4), 2.34 (2H, m), 2.25 (2H, dt, 12.0, 5.0), 2.17 (2H, m), 2.02 (2H, m), 1.99 (2H, m), 1.68 (2H, m), 1.60 (2H, m), 1.53 (2H, m), 1.28 (2H, m), 1.20 (12H, d, 5.4), 1.08 (6H, m), 0.97 (6H, d, 6.8), 0.70 (6H, d, 3.5), 0.60 (6H, d, 3.5) ppm. ^{13}C (600 MHz, Chloroform-*d*): δ 181.5, 173.8, 107.5, 82.6, 81.3, 81.2, 78.4, 75.8, 73.2, 57.2, 54.0, 43.6, 36.8, 36.0, 35.6, 33.4, 30.1, 25.2, 22.4, 21.4, 21.3, 19.9,19.6 ppm. ^{11}B (500 MHz, Chloroform-*d*): δ 10.5 ppm. $[M]^-$ = 947 g/mol.

E.8.3 Epoxide Ring Opening

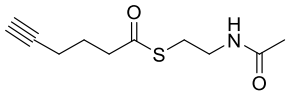
Seven hundred µg of **4.6** (0.739 µmol) were resuspended in 1 mL anhydrous DMF, due to **2.1**'s low solubility in water, in a 20 mL glass scintillation vial with 0.05 eq scandium triflate, and 25 eq sodium azide at 25 °C. After stirring for one week, starting material remained unaltered via LCMS. The reaction was heated to 40 °C, and likewise after 48 hr, no change in the starting material was observed. Assuming that the sodium azide was spent, 2.5 eq of ethanolamine were added at 40 °C. After one week of stirring, $NaHCO_3$ was added to the reaction, and macrolide was extracted with anhydrous DCM, and dried over MgSO4. Via LCMS, the epoxide starting material and doubly opened epoxide were present at a 1:1 ratio; the mass for a singly-opened epoxide was also observed. The epoxide and diol materials were unable to be separated with C18 HPLC and were left as a mixture of products.



(**4.7**) (**4.8**)

E.8.4 Creation of SNAC-derivative

5-hexynoic acid (0.100 g, 0.89 mmol) was added to 2 mL anhydrous DCM in a round bottom cooled to 0 °C in an ice bath under N_2 (g) with 0.5 equivalents of DMAP (0.055 g, 0.45 mmol), and 1.5 equivalents of EDCI (0.257 mg, 0.139 mL) and TEA (0.135 g, 1.338 mmol). After stirring for 10 minutes, 0.95 eq SNAC (0.090 mL, 0.847 mmol) were added dropwise to the solution. The solution was warmed to room temperature and allowed to stir for 16 hr. Additional DCM, H_2O, and 1M HCl were used to extract the product 5-hexynoyl-N-acetylcysteamine and dried over $MgSO_4$ without the need of further purification (0.069 g, 38.9% yield).

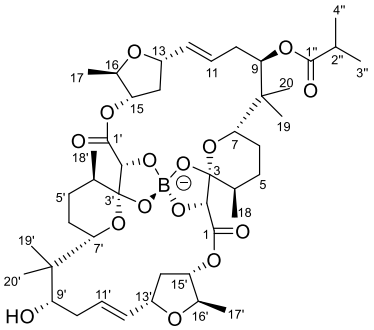


^{1}H NMR (500 MHz, $CDCl_3$): δ 3.35 (q, 2H, *J* = 5.8), 2.96 (t, 2H, *J* = 7.3), 2.65 (t, 2H, *J* = 7.8), 2.19 (dt, 2H, *J* = 6.1, 2.5), 1.95 (t, 1H, *J* = 2.5), 1.90 (s, 3H), 1.81 ppm (m, 2H). ^{13}C NMR (500 MHz, $CDCl_3$): 199.1, 170.7, 82.9, 69.5, 42.5, 39.4, 28.4, 24.1, 23.0, 17.7 ppm.

Appendix

NMR Tables and Spectra by Molecule

NP-34 (2.1)

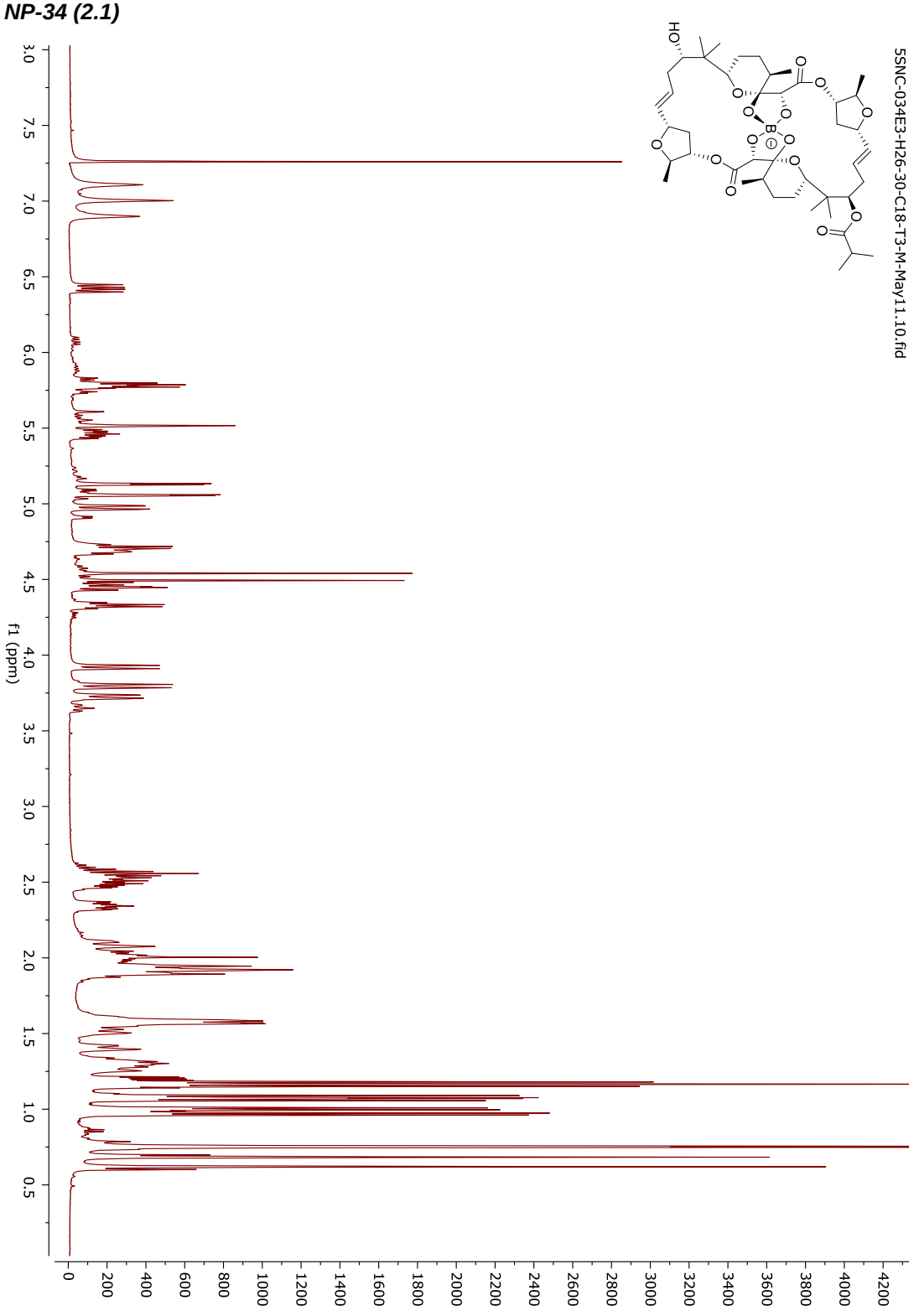


NP-34

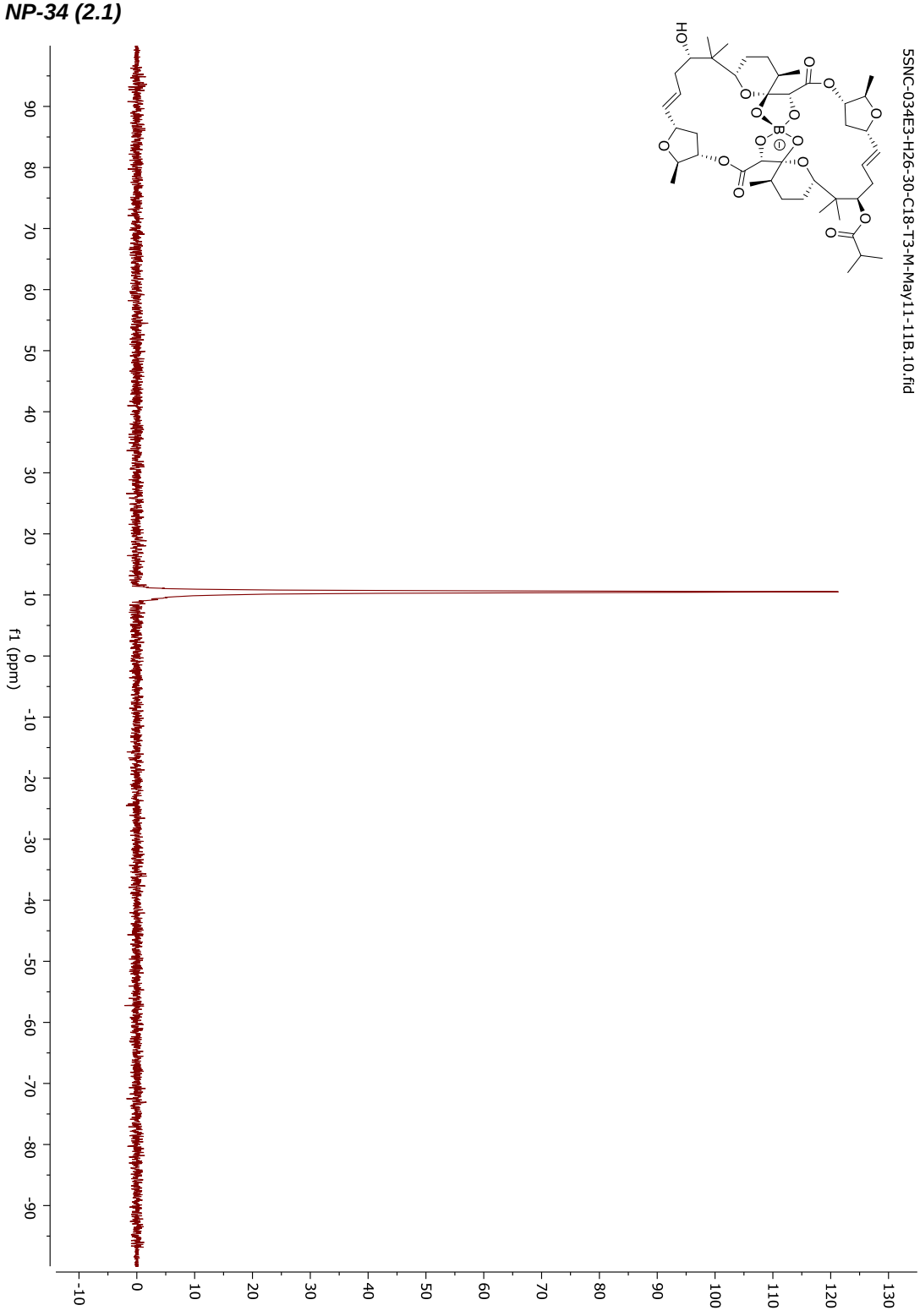
	NP-34	
#	δ_C	δ_H (*J* in Hz)
1	171.3	--
2	78.7	4.49 (1H, s)
3	104.5	--
4	33.5	1.92 (1H, m)
5	28.4	1.57 (1H, m) 1.56 (1H, m)
6	25.1	1.28 (1H, m) 1.40 (1H, m)
7	71.3	3.73 (XH, dd, 11.5, 2.1)
8	41.0	--
9	78.8	4.95 (1H, dd, 11.8, 1.9)
10	30.9	2.00 (1H, m) 2.52 (1H, m)
11	131.0	5.45 (1H, ddd, 15.3, 8.9, 5.2)
12	135.8	6.45 (1H, dd, 15.4, 8.9)
13	76.5	4.45 (1H, dd, 8.9, 7.1)
14	36.1	1.89 (1H, m) 2.34 (1H, m)
15	79.8	5.05 (1H, d, 3.2)
16	78.0	4.32 (1H, q, 6.8)
17	19.0	1.08 (1H, d, 6.7)
18	17.1	1.00 (1H, d, 6.5)
19	17.7	0.62 (3H, s)
20	18.0	0.75 (3H, s)
1’	171.7	--
2’	79.4	4.55 (1H, s)
3’	105.9	--
4’	33.7	1.89 (1H, m)
5’	29.0	1.56 (1H, m) 1.56 (1H, m)
6’	25.5	1.29 (1H, m) 1.50 (1H, m)

7’	80.1	3.79 (1H, dd, 11.5, 1.1)
8’	39.2	--
9’	78.7	3.92 (1H, d, 10.7)
10’	31.8	1.91 (1H, m) 2.08 (1H, m)
11’	129.9	5.75 (1H, ddd, 15.8, 4.2)
12’	131.1	5.80 (1H, dd, 15.8, 6.1)
13’	77.4	4.68 (1H, dd, 7.3, 6.2)
14’	36.7	1.92 (1H, m) 2.48 (1H, m)
15’	79.3	5.11 (1H, d, 4.0)
16’	78.4	4.71 (1H, q, 6.6)
17’	19.3	1.06 (3H, d, 6.6)
18’	17.0	0.97 (3H, d, 6.7)
19’	22.2	0.68 (3H, s)
20’	13.6	0.75 (3H, s)
1’’	177.5	--
2’’	34.5	2.54 (1H, m)
3’’	19.3	1.16 (3H, d, 6.5)
4’’	19.5	1.17 (3H, d, 6.5)
9’-OH	--	5.51 (1H, s)
B – 10.5 ppm		
$[M]^-$ = 845 g/mol		

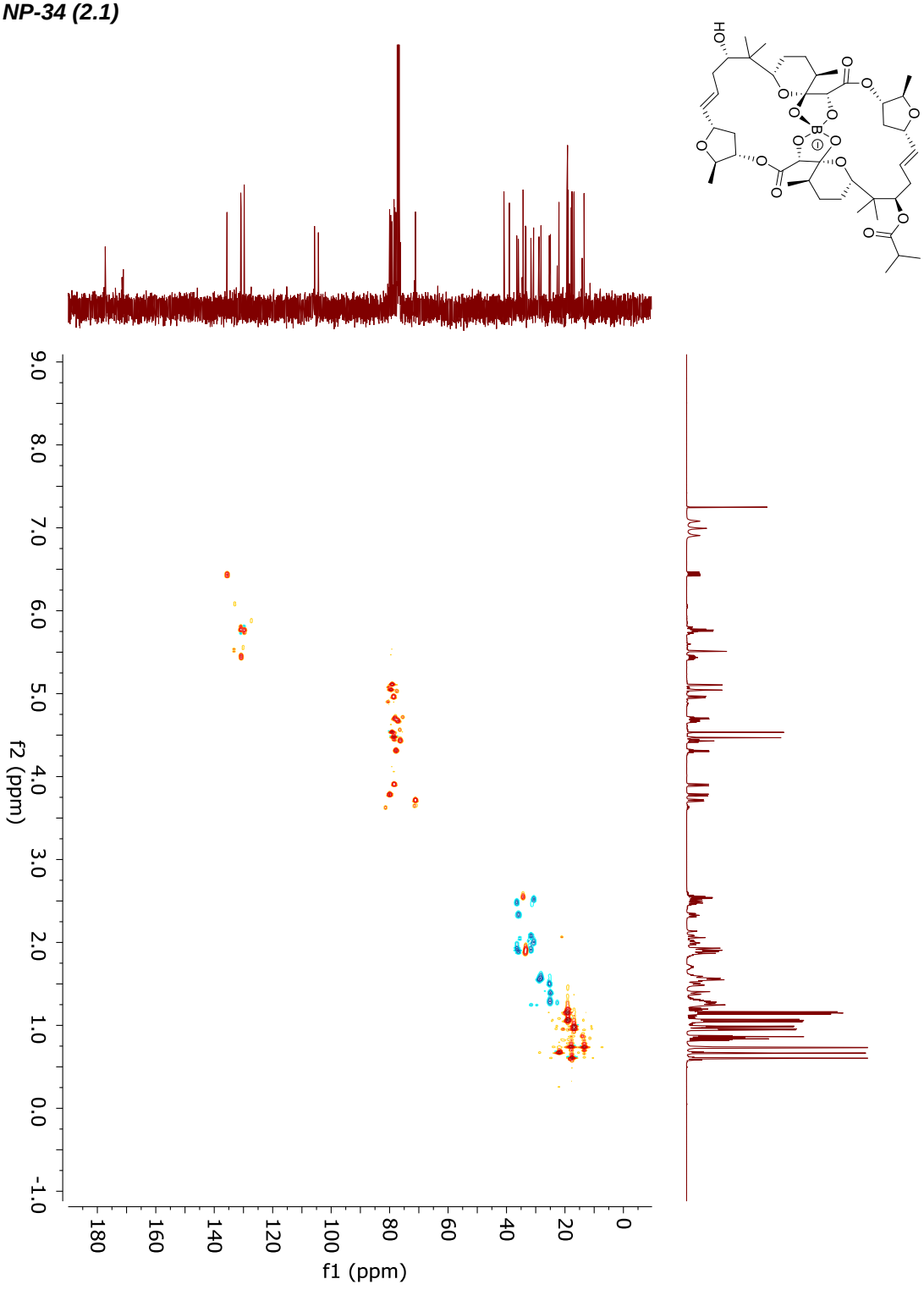
NP-34 (2.1)



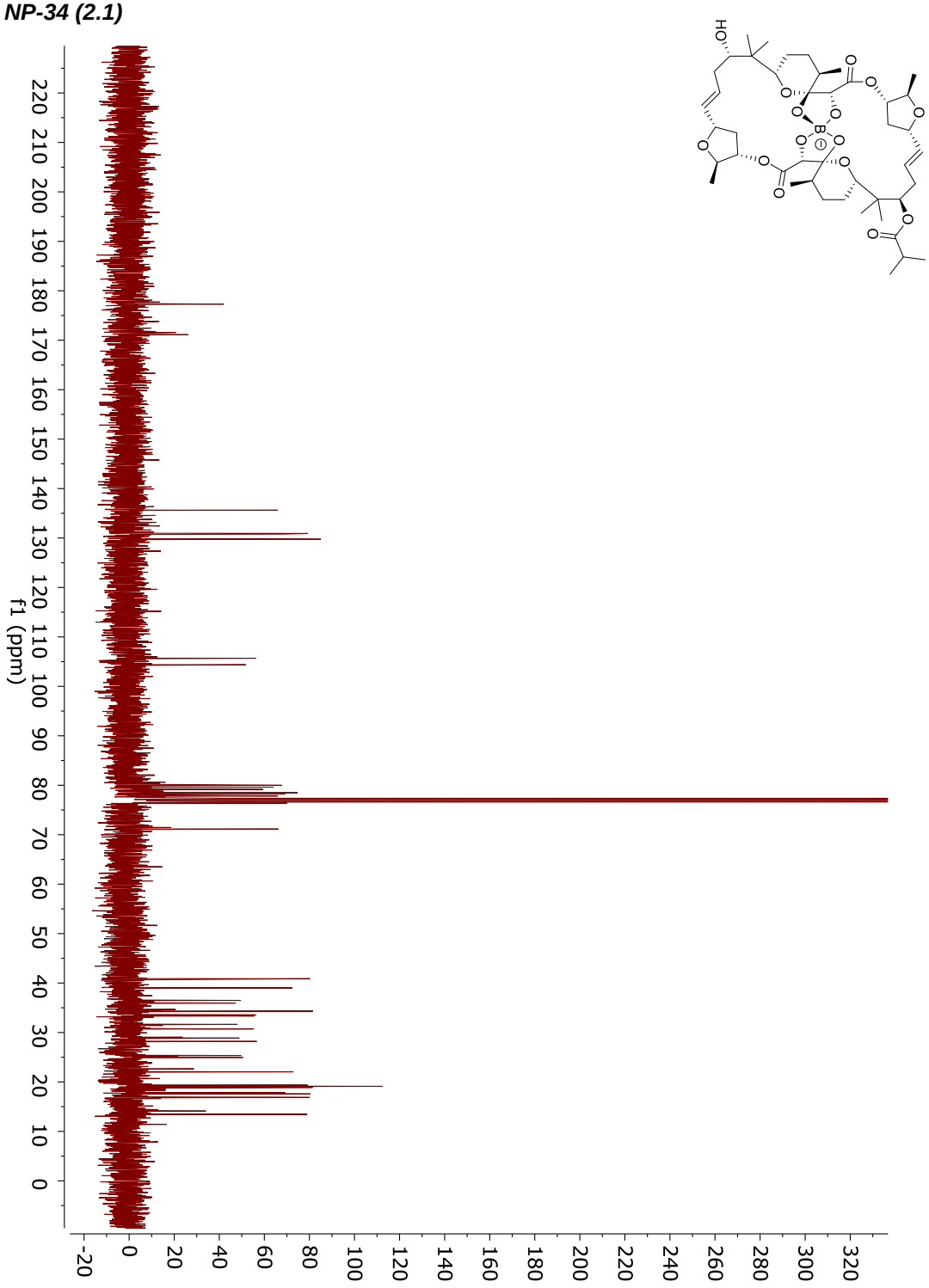
NP-34 (2.1)



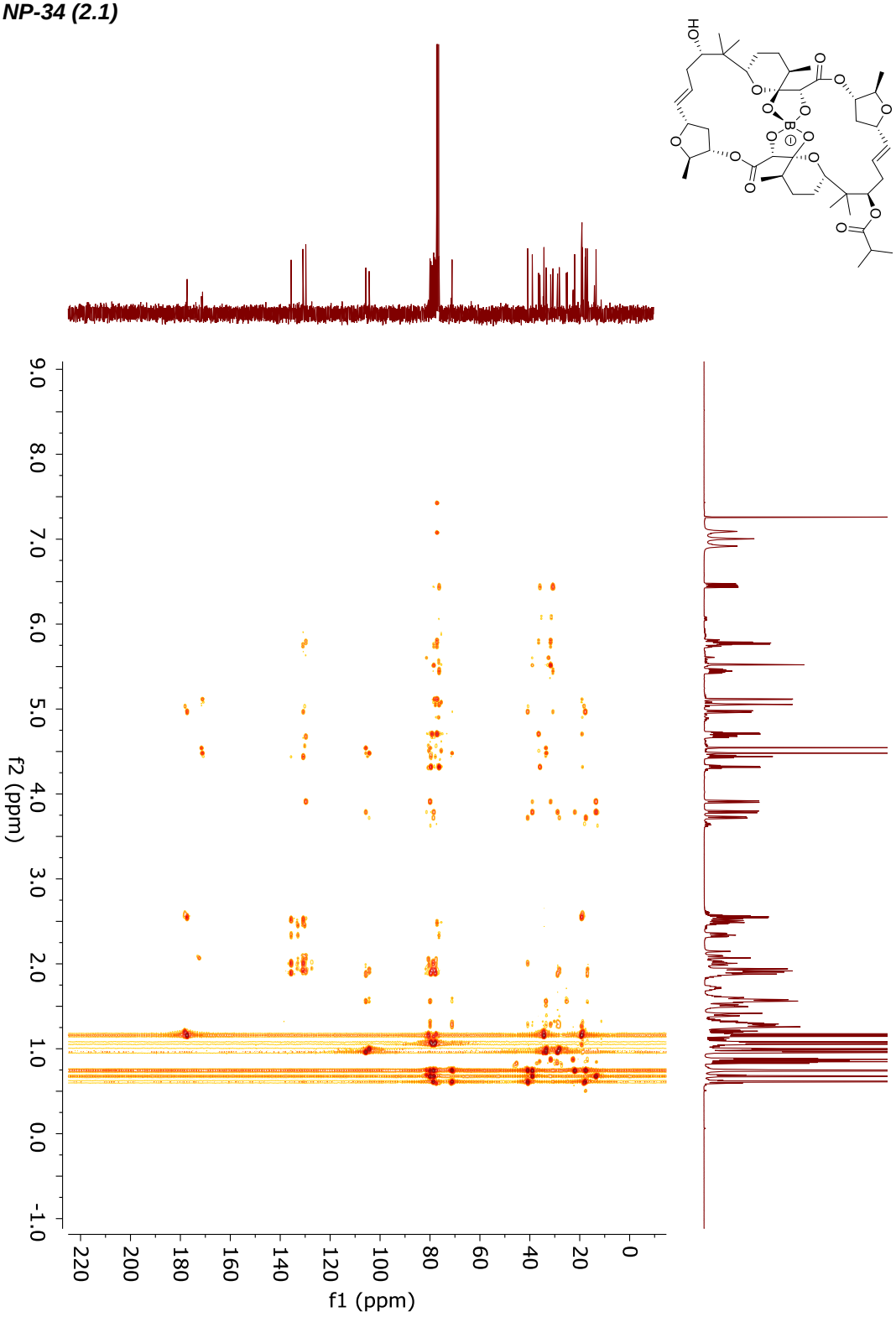
NP-34 (2.1)


HO
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
f2 (ppm)
180
160
140
120
100
80
60
40
20
0
f1 (ppm)

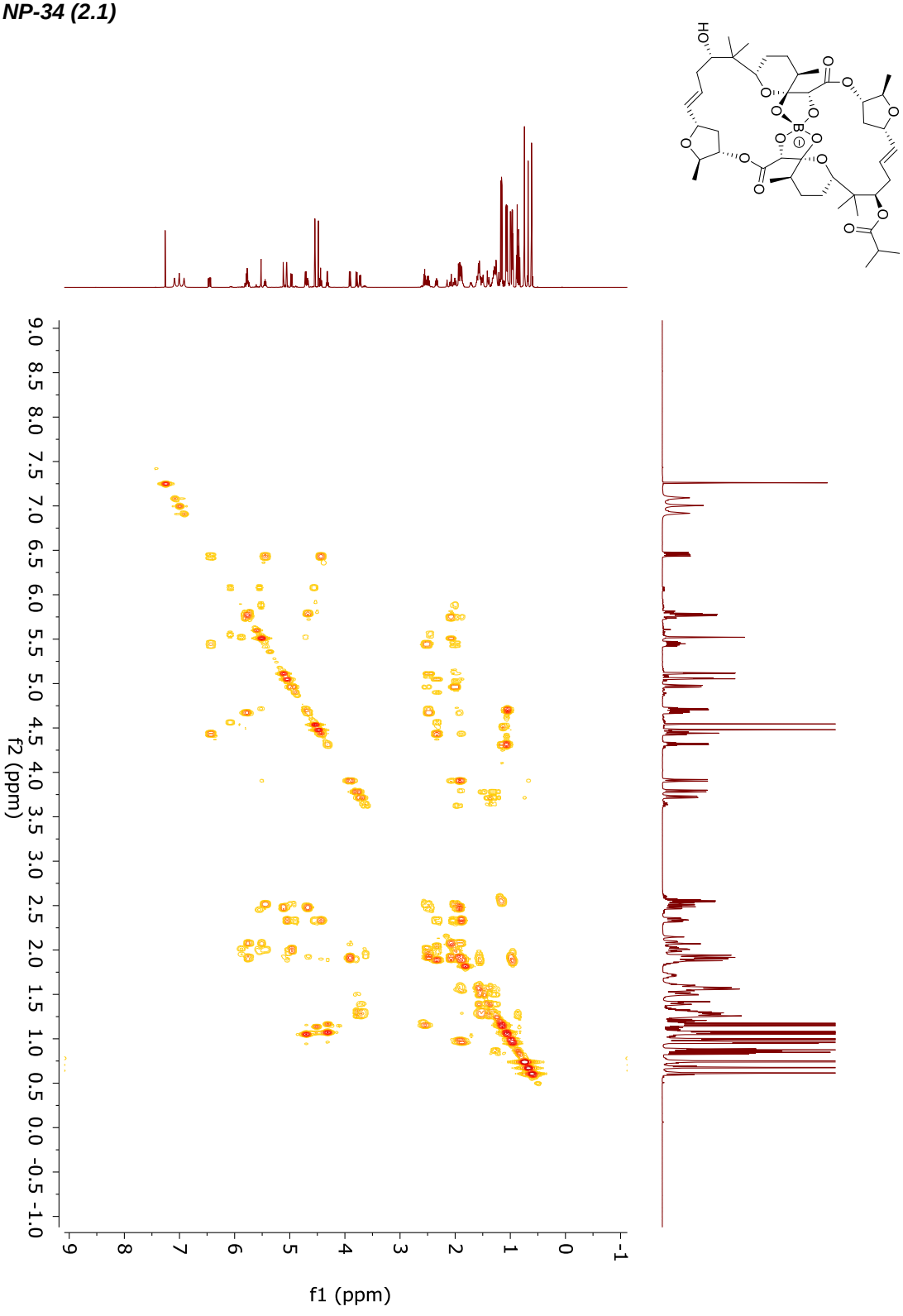
NP-34 (2.1)


HO
B
f1 (ppm)
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
-20 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320

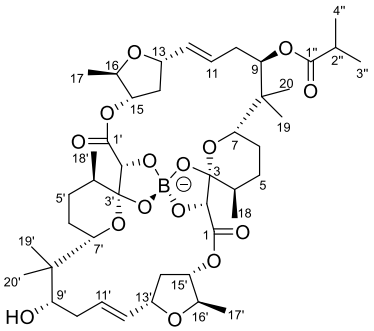
NP-34 (2.1)


HO
B
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
f2 (ppm)
220
200
180
160
140
120
100
80
60
40
20
0
f1 (ppm)

NP-34 (2.1)



NP-34-V2 (2.1V2)

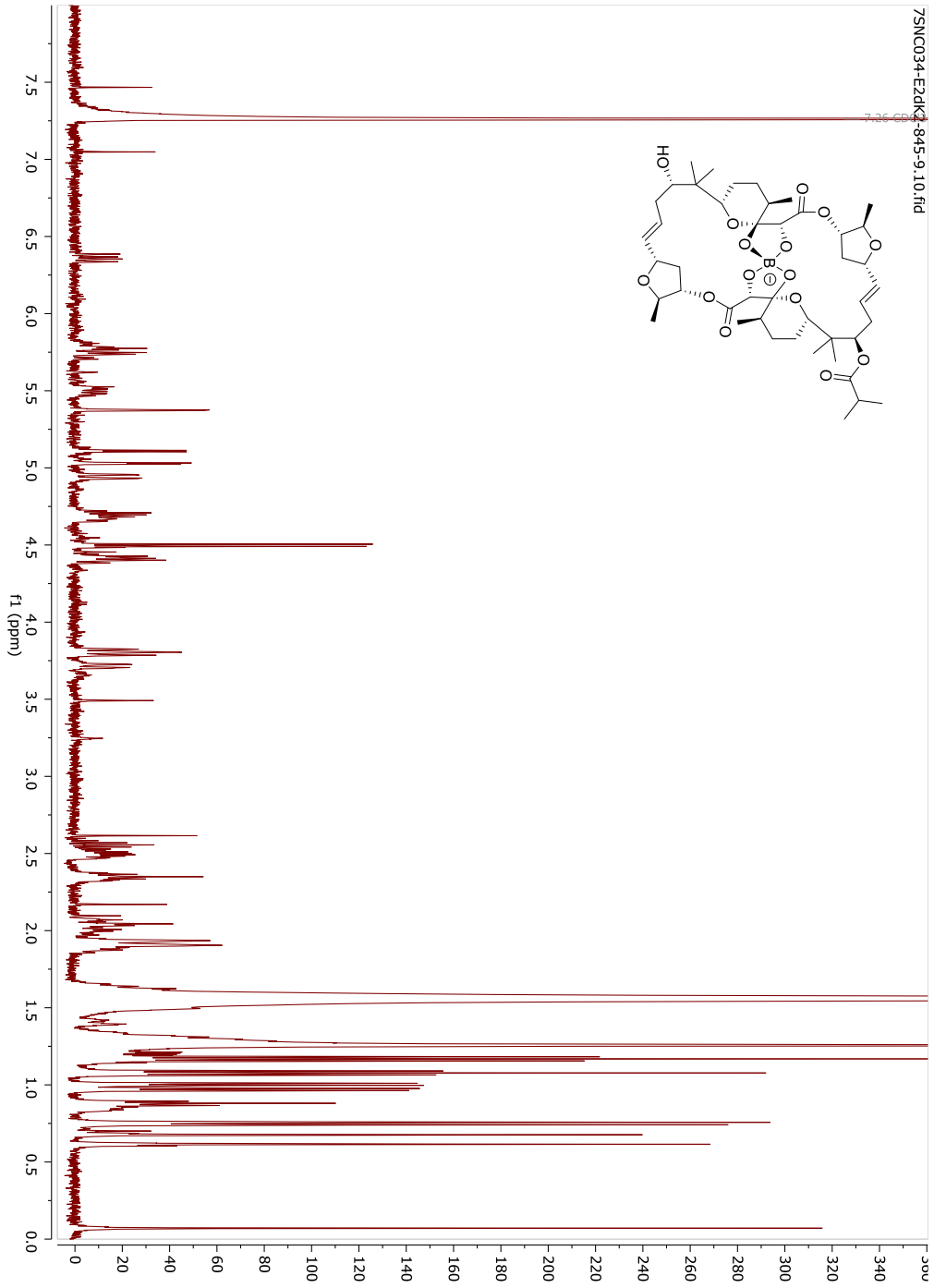


NP-34-V2

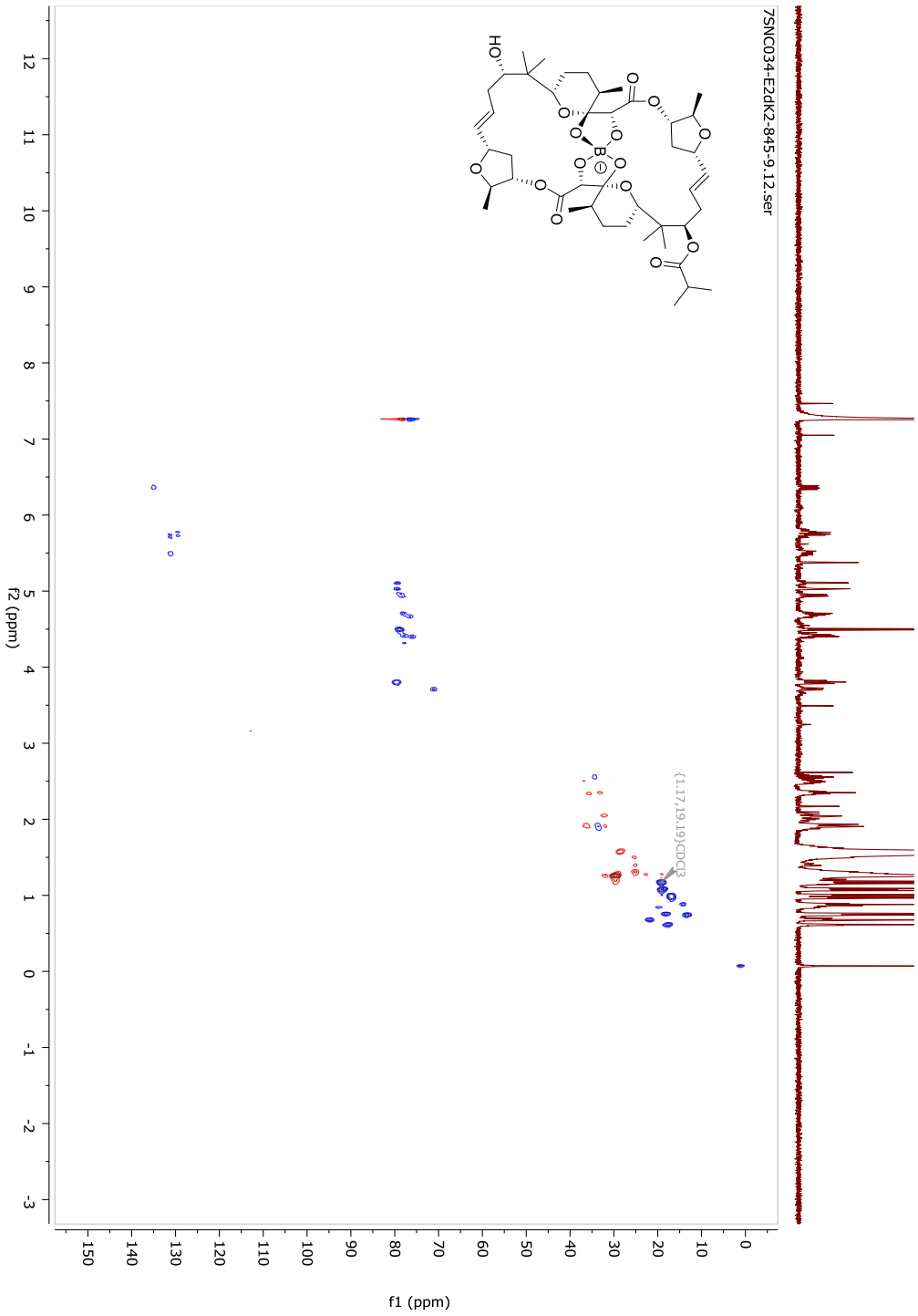
		NP-34-V2
#	δ_C	δ_H (*J* in Hz)
1	174.8	--
2	78.8	4.49 (1H, s)
3	104.7	--
4	33.4	1.91 (1H, m)
5	28.0	1.59 (1H, m) 1.20 (1H, m)
6	24.8	1.30 (1H, m) 1.21 (1H, m)
7	71.2	3.71 (1H, dd, 11.5, 2.3)
8	40.6	--
9	78.6	4.94 (1H, dd, 12.1, 2.1)
10	32.2	2.02 (1H, m) 2.50 (1H, m)
11	131.1	5.50 (1H, 14.6, 7.9, 5.9)
12	135.1	6.36 (1H, dd, 15.4, 9.5)
13	77.9	4.42 (1H, dd, 7.6, 6.9)
14	35.9	2.36 (1H, m) 2.50 (1H, m)
15	79.5	5.03 (1H, d, 3.5)
16	75.9	4.40 (1H, q, 8.6)
17	19.5	1.12 (3H, d, 6.5)
18	16.9	1.00 (1H, d, 6.6)
19	17.7	0.61 (3H, s)
20	17.9	0.75 (3H, s)
1’	171.7	--
2’	78.8	4.51 (1H, s)
3’	105.3	--
4’	33.3	1.91 (1H, m)
5’	28.8	1.60 (1H, m) 1.29 (1H, m)
6’	25.3	1.88 (1H, m) 1.30 (1H, m)
7’	79.5	3.80 (1H, t, 9.7)
8’	39.1	--

9’	79.2	4.68 (1H, m)
10’	32.1	2.06 (1H, m) 2.49 (1H, m)
11’	129.5	5.77 (1H, t, 4.8)
12’	131.1	5.74 (1H, d, 5.8)
13’	76.4	4.68 (1H, m)
14’	36.7	2.50 (1H. m) 1.94 (1H, m)
15’	79.5	5.11 (1H, d, 4.2)
16’	78.5	4.50 (1H, s)
17’	19.1	1.14 (3H, s)
18’	16.9	0.97 (3H, d, 6.8)
19’	13.4	0.74 (3H, s)
20’	21.8	0.67 (3H, s)
1’’	177.6	--
2’’	34.5	2.56 (1H, m)
3’’	19.2	1.17 (3H, d, 7.8)
4’’	19.2	1.17 (3H, d, 7.8)
9’-OH	--	5.37
^{11}B 10.5 ppm		
$[M]^-$ = 845 g/mol		

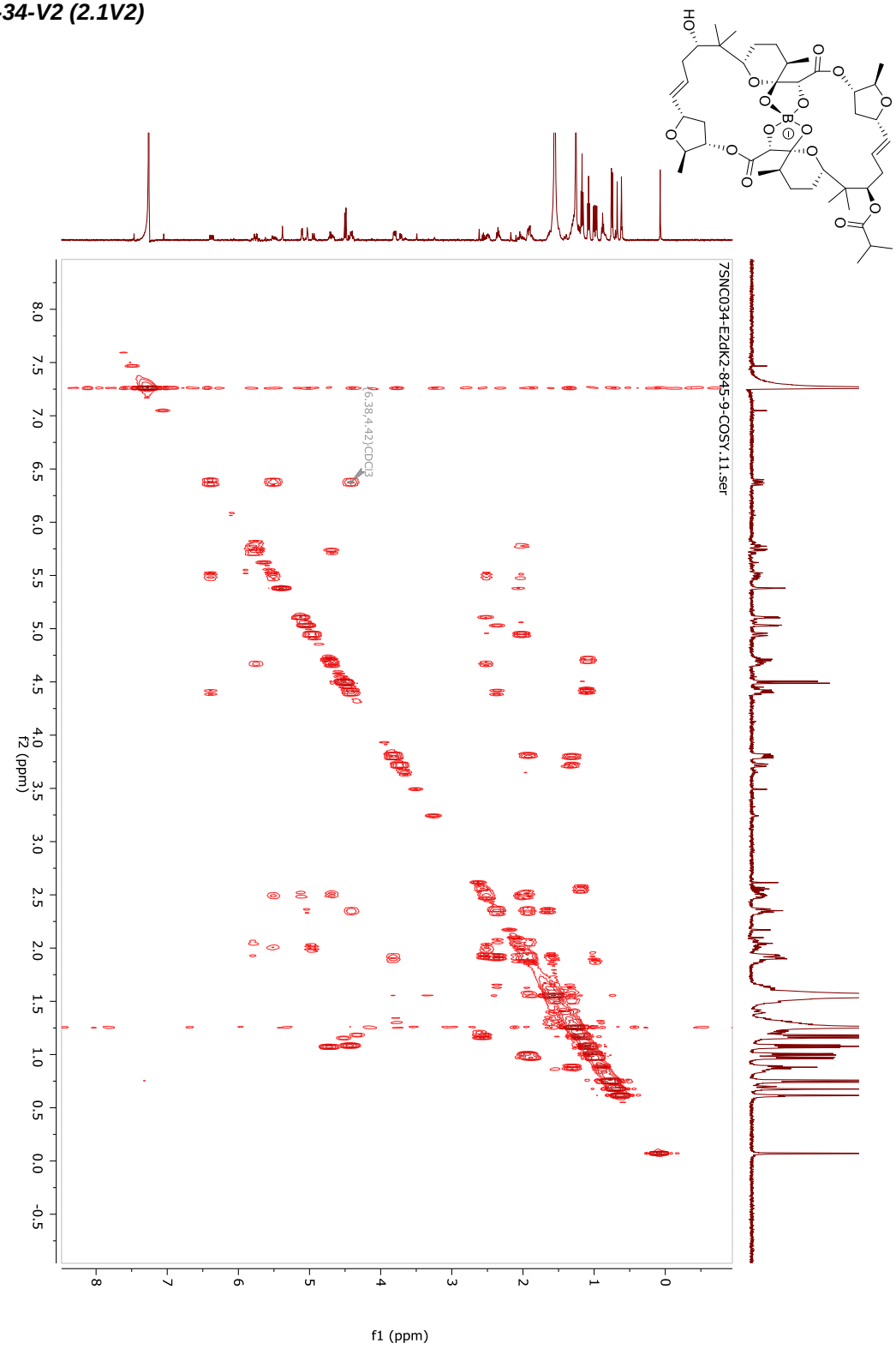
NP-34-V2 (2.1V2)



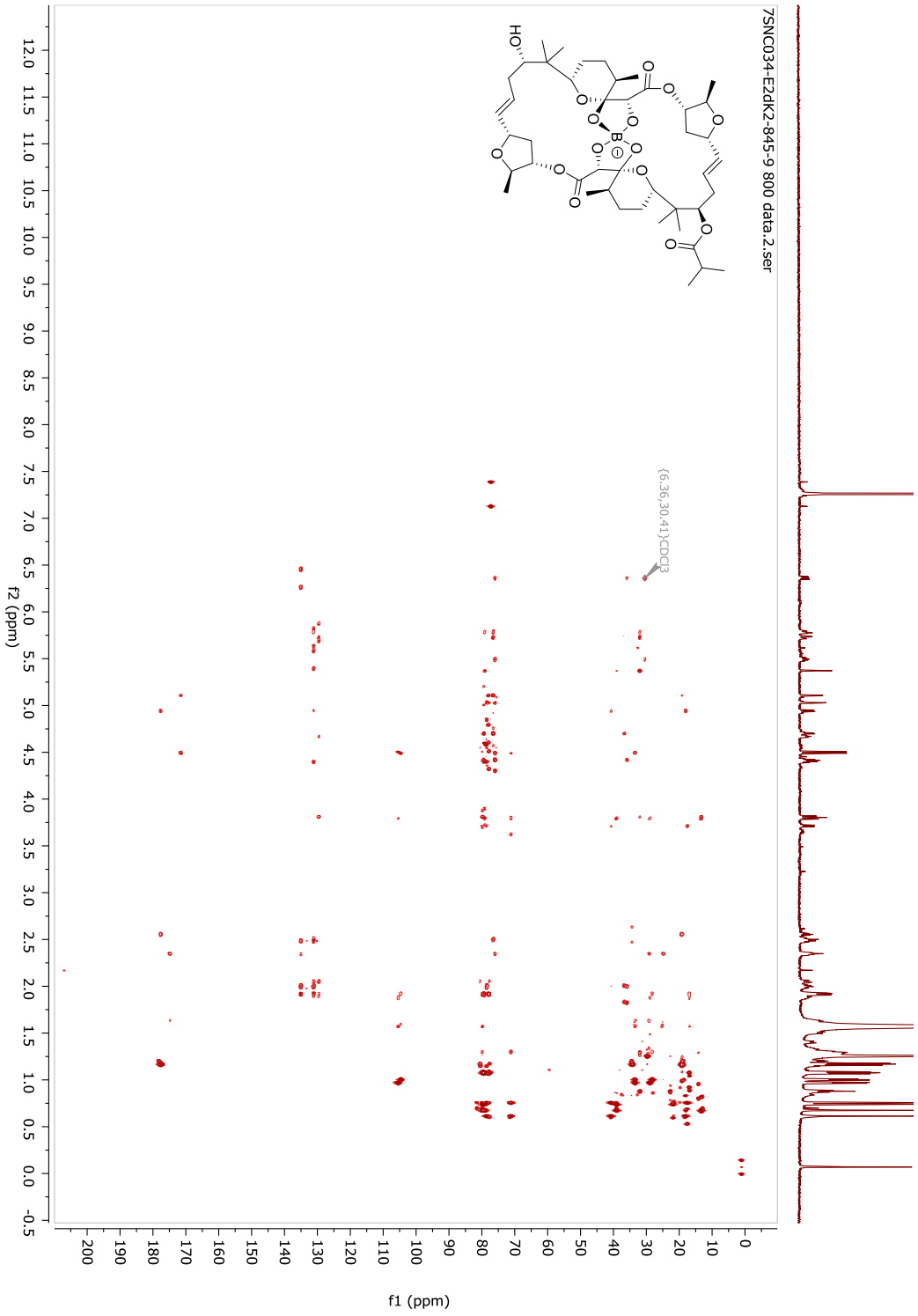
NP-34-V2 (2.1V2)



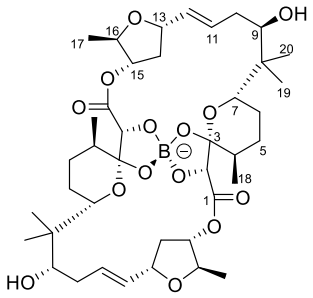
NP-34-V2 (2.1V2)



NP-34-V2 (2.1V2)

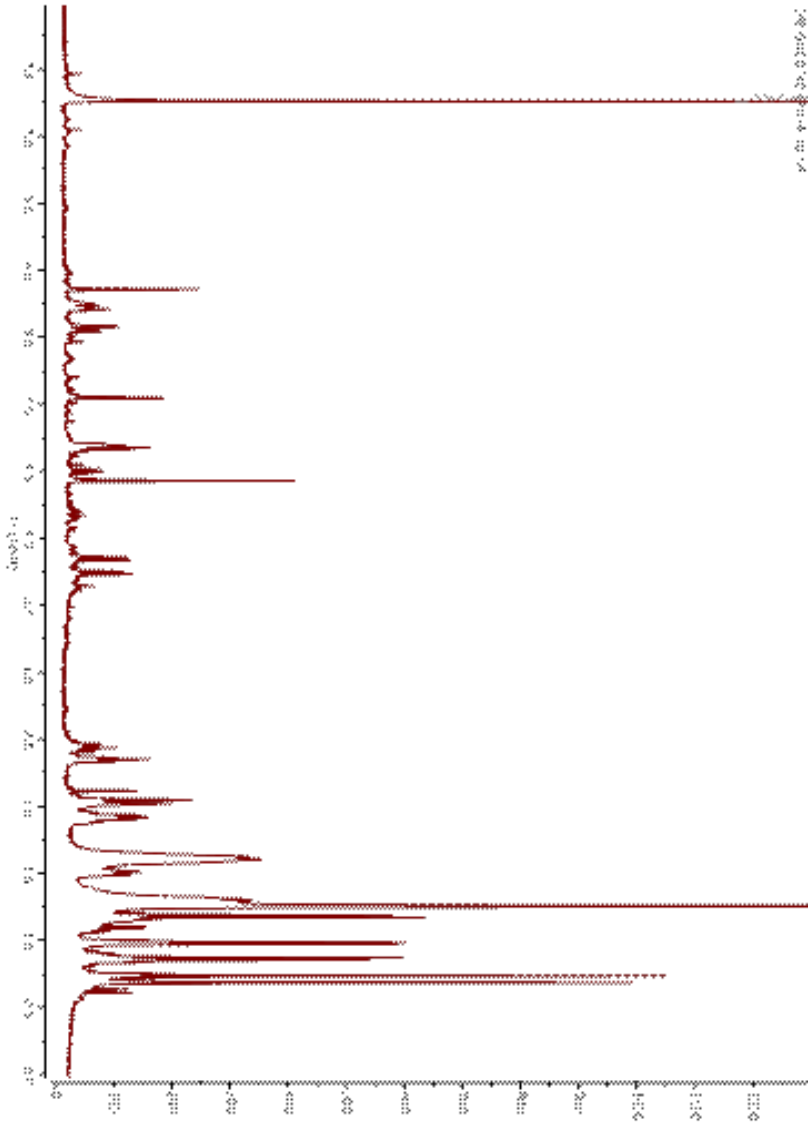


Aplasmomycin (2.2)

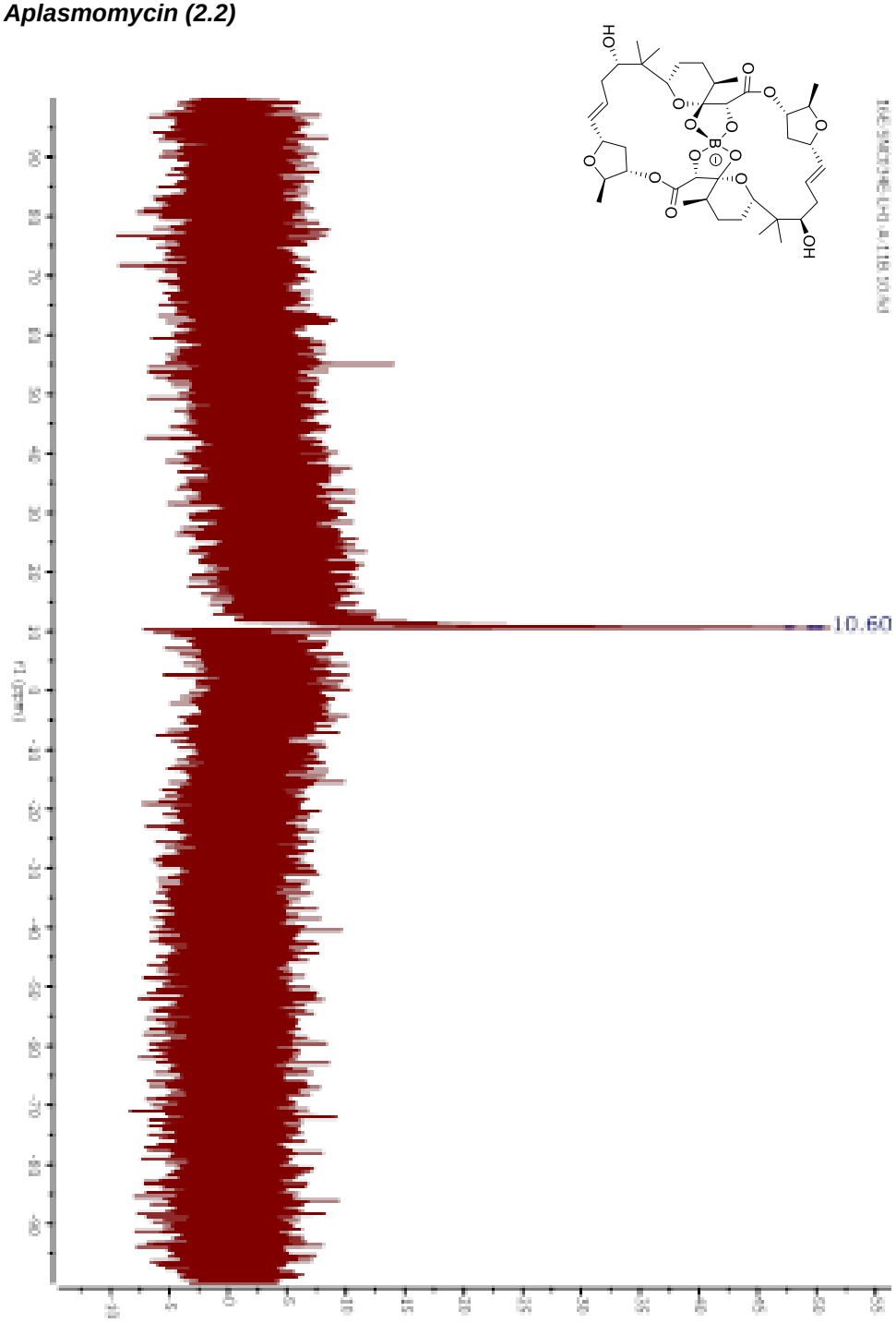


Aplasmomycin

Structure as published[5]

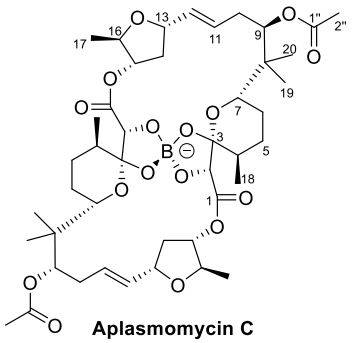


Aplasmomycin (2.2)

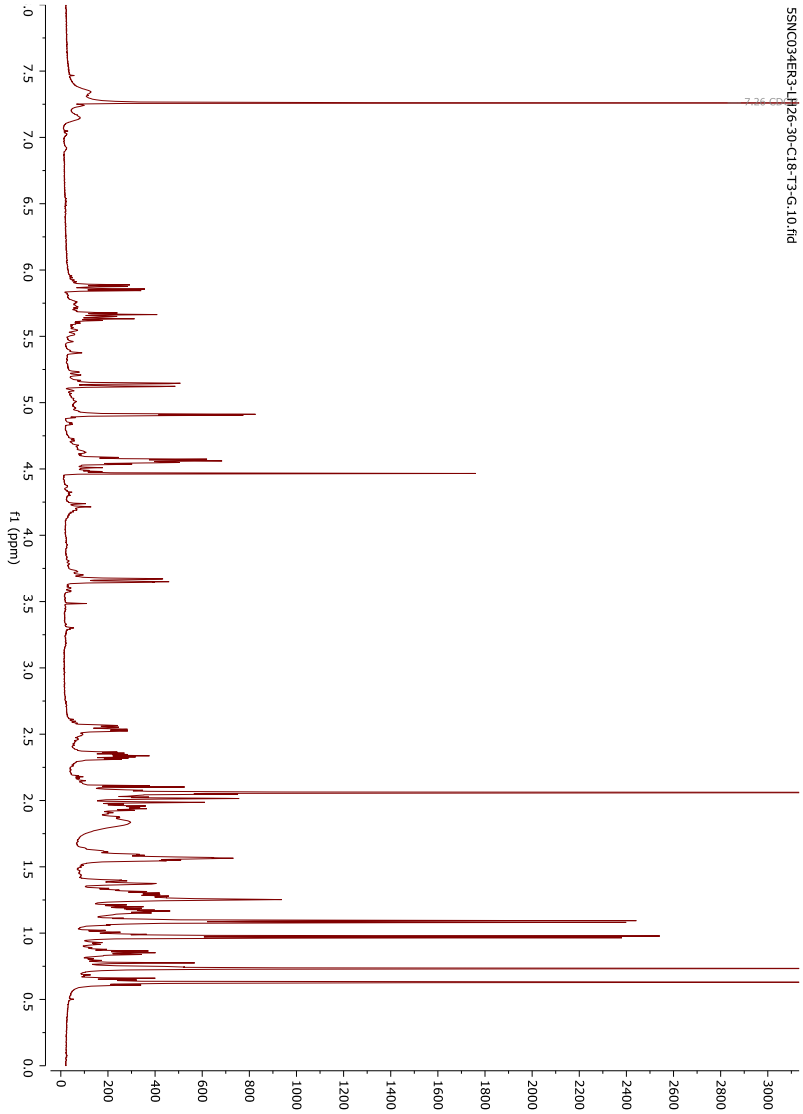


$[M]^-$ = 775 g/mol

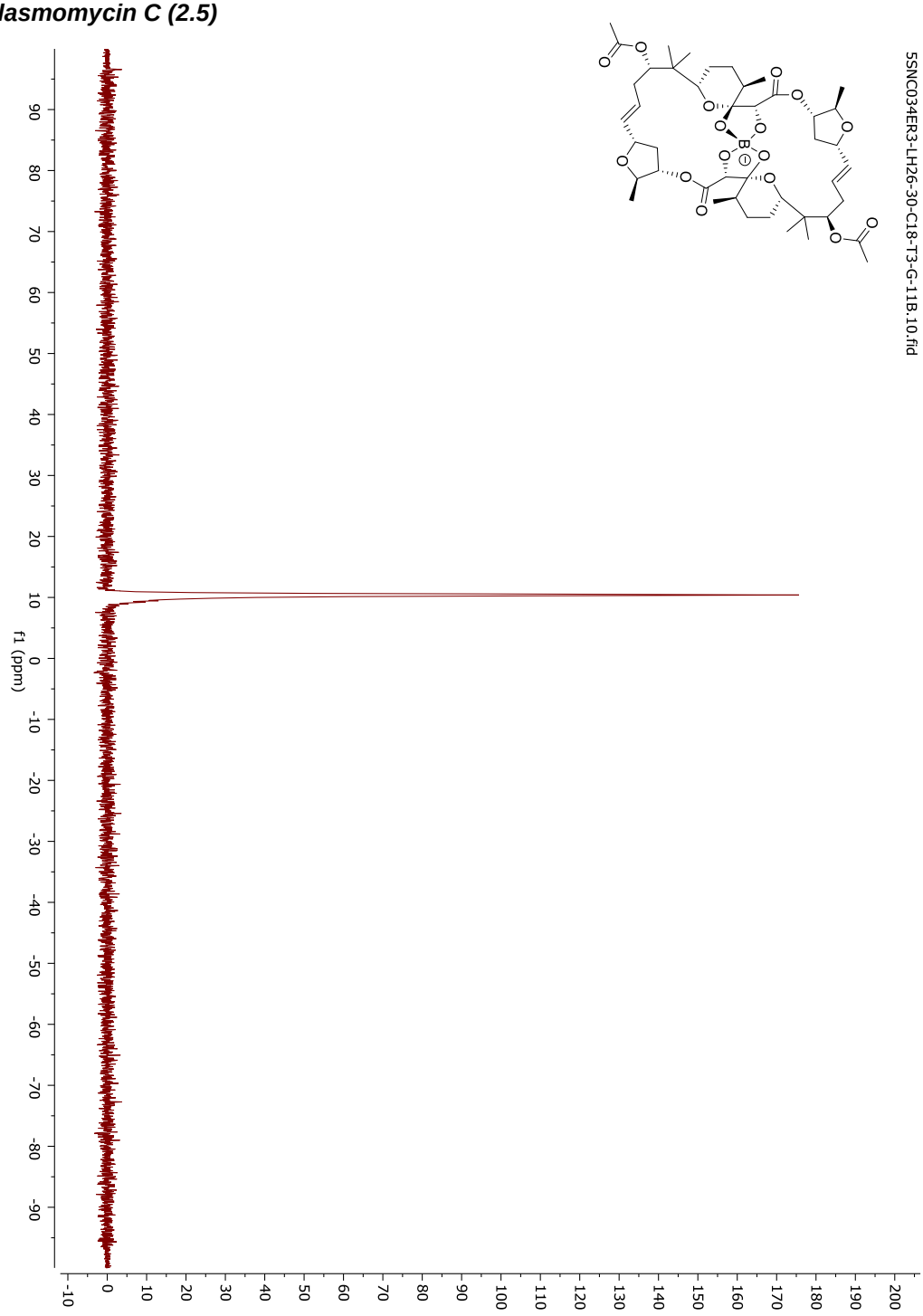
Aplasmomycin C (2.5)



Structure as published[6]

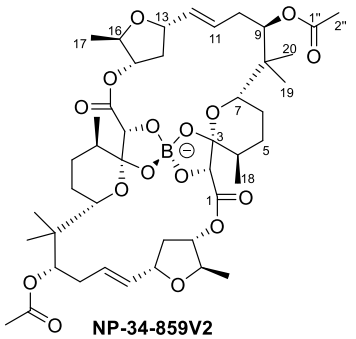


Aplasmomycin C (2.5)



$[M]^-$ = 859 g/mol

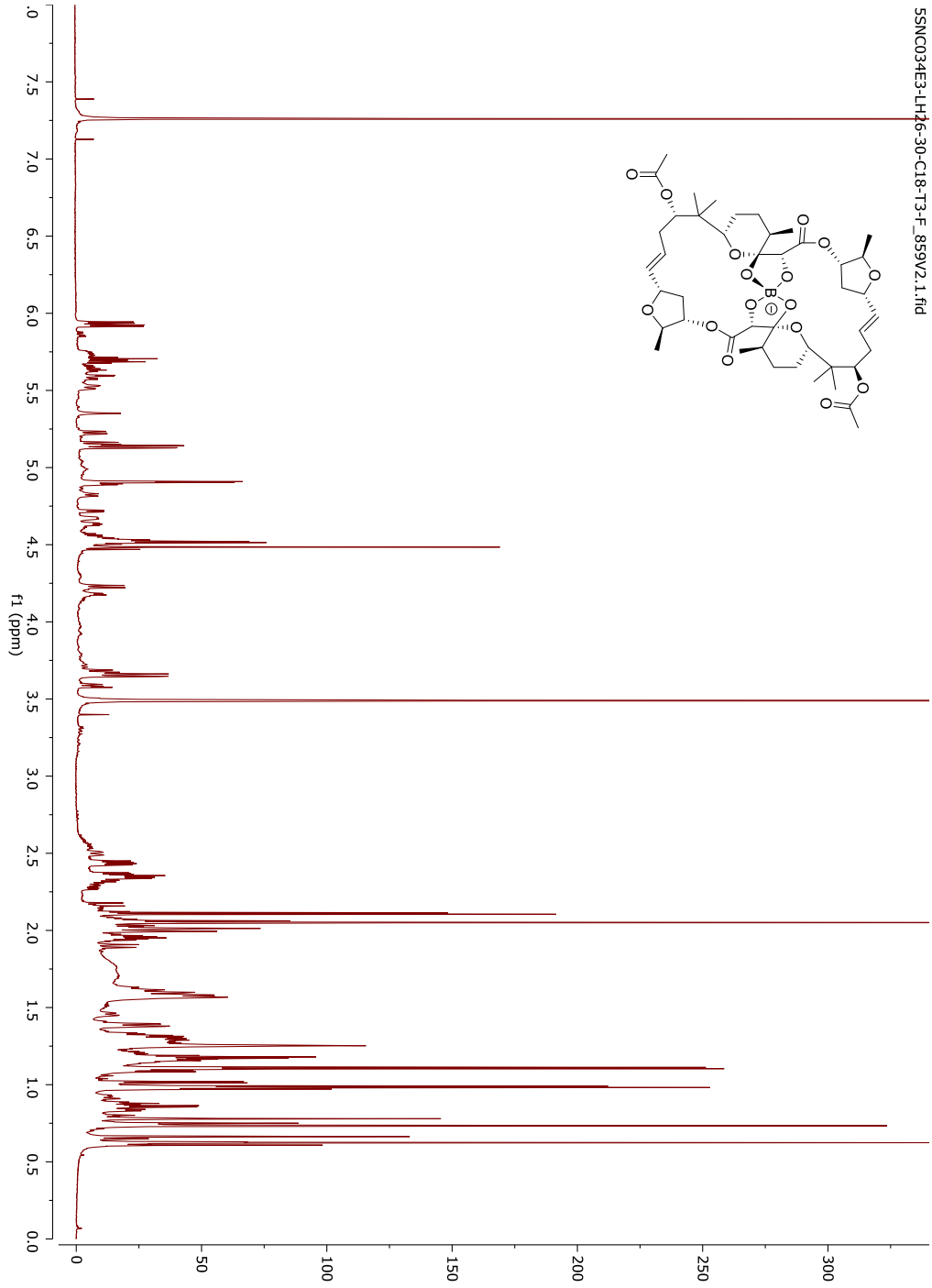
NP-34-859V2 (2.5V2)



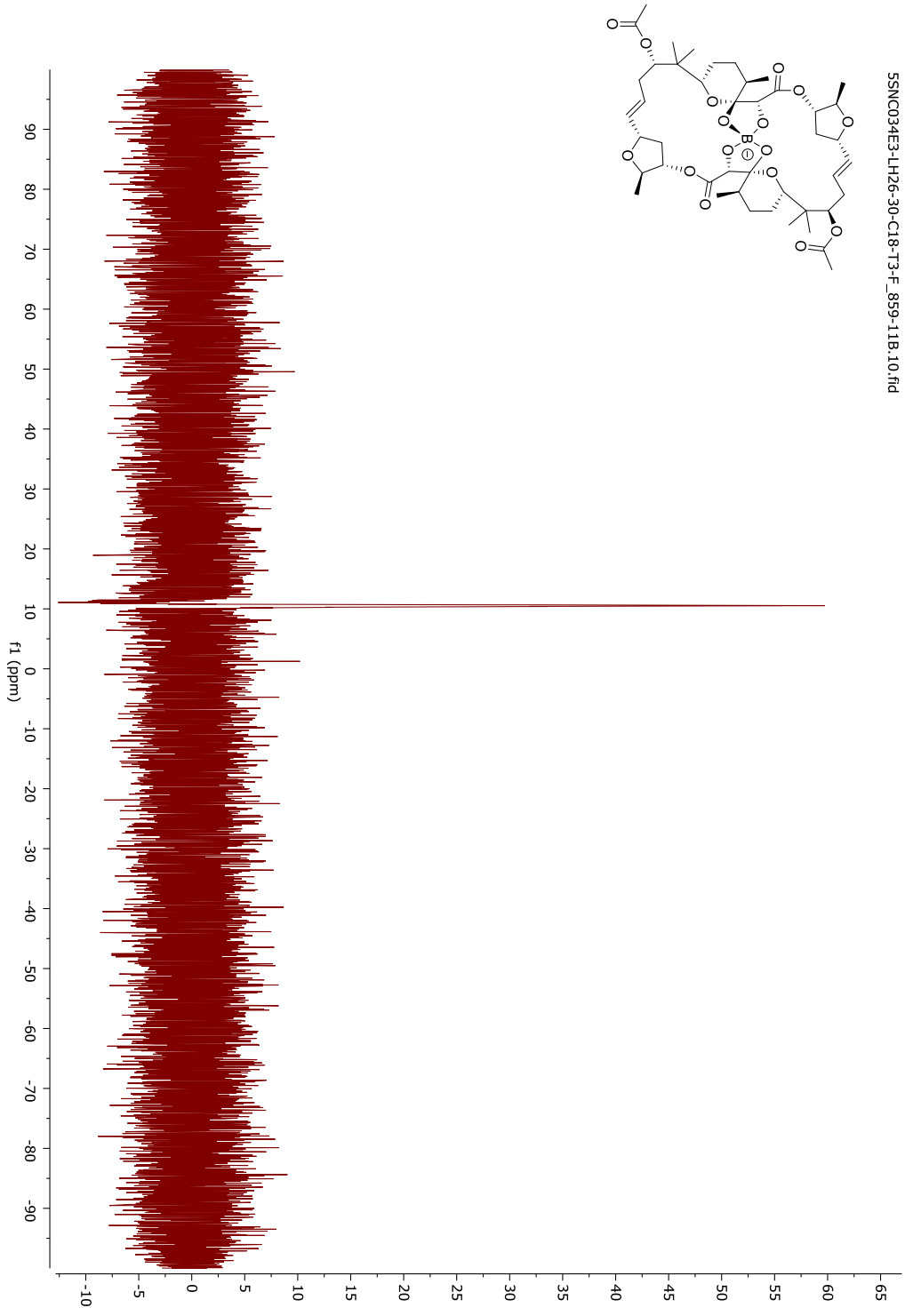
NP-34-859V2

		NP-34-859-V2
#	δ_C	δ_H (*J* in Hz)
1	171.9	--
2	79.1	4.48 (1H, s)
3	104.6	--
4	33.7	1.95 (1H, m)
5	28.0	1.55 (1H, m) 1.60 (1H, m)
6	24.9	1.38 (1H, m) 1.32 (1H, m)
7	70.8	3.65 (1H, dd, 11.5, 2.5)
8	40.8	--
9	78.3	5.13 (1H, dd, 11.3, 1.6)
10	30.7	2.43 (1H, qs, 7.3) 2.02 (1H, m)
11	130.8	5.69 (1H, m, 14.9, 6.9)
12	130.8	5.93 (1H, dd, 14.9, 6.8)
13	75.5	4.52 (1H, m)
14	36.4	2.35 (1H, qs, 4.5) 1.97 (1H, m, 17.9, 11.3, 6.9)
15	80.3	4.90 (1H, d, 4.5)
16	77.8	4.52 (1H, m)
17	19.0	1.11 (3H, dd, 7.3)
18	16.9	0.98 (3H, d, 7.3)
19	17.7	0.62 (3H, s)
20	18.4	0.73 (3H, s)
1''	172.3	--
2''	21.1	2.05 (3H, s)
^{11}B 10.5 ppm		
$[M]^-$ = 859 g/mol		

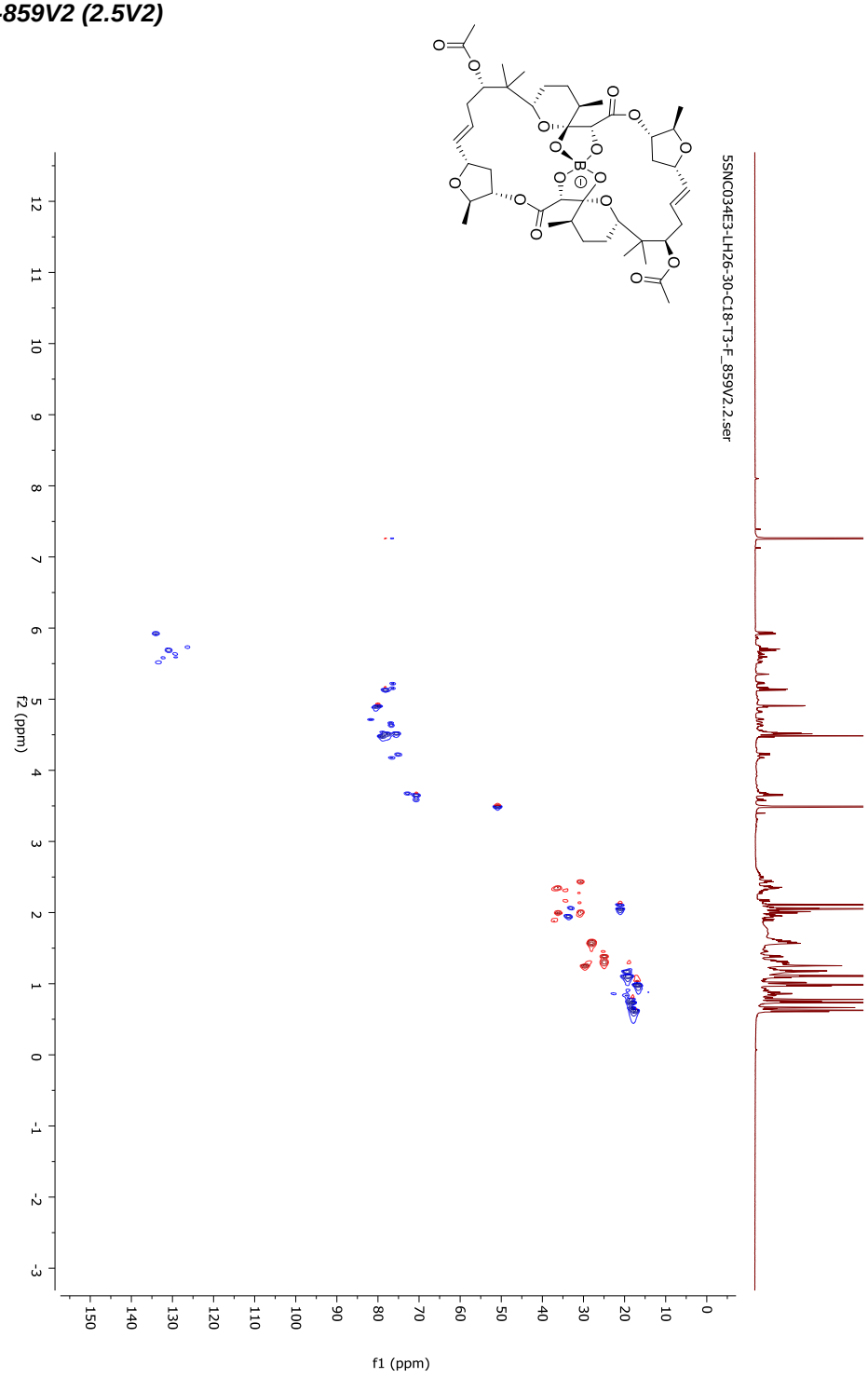
NP-34-859V2 (2.5V2)



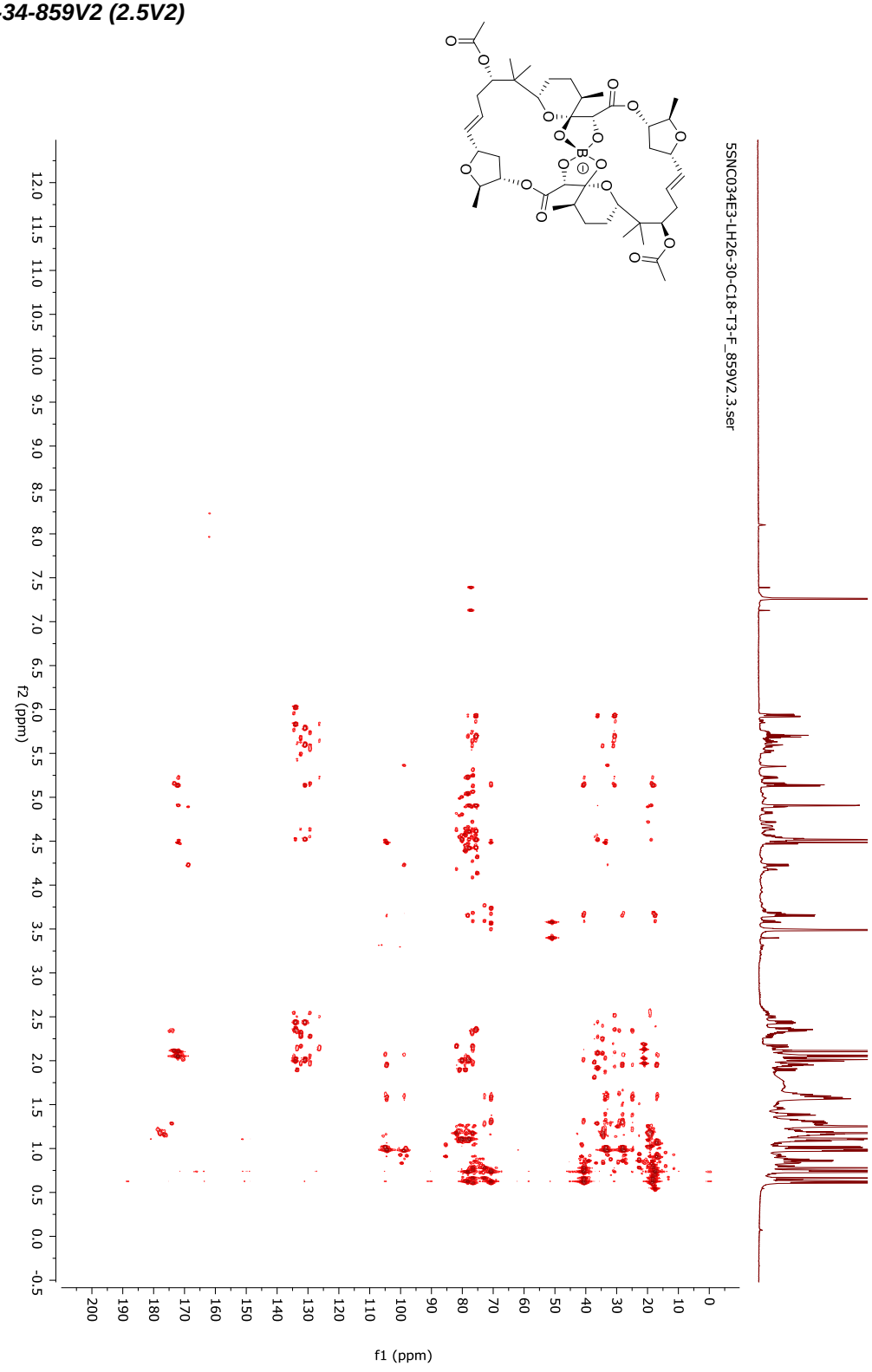
NP-34-859V2 (2.5V2)



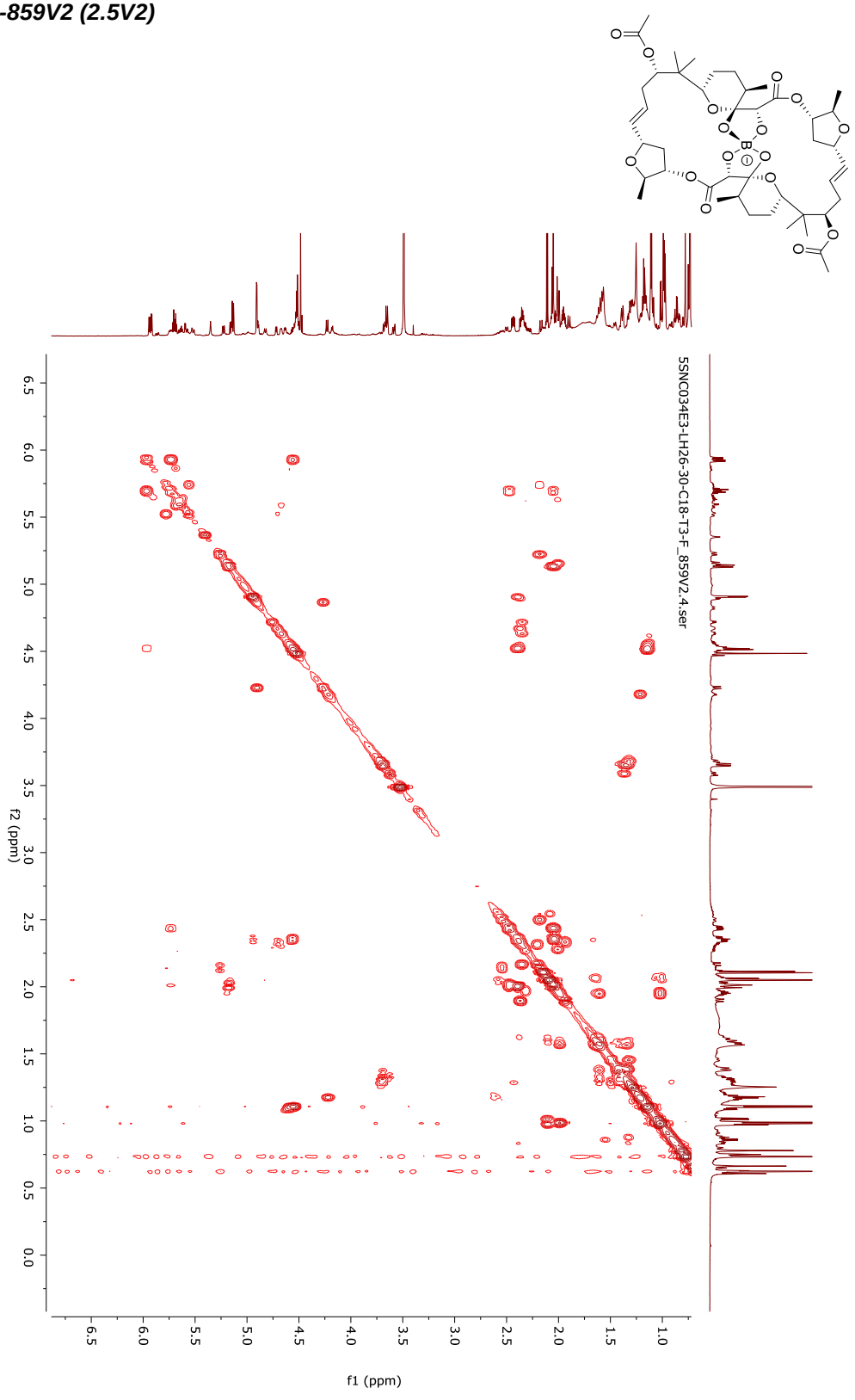
NP-34-859V2 (2.5V2)



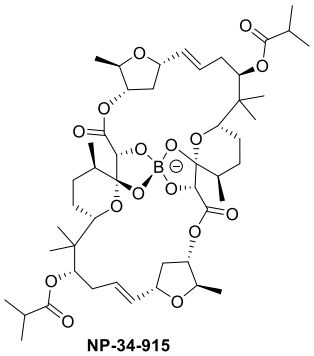
NP-34-859V2 (2.5V2)



NP-34-859V2 (2.5V2)



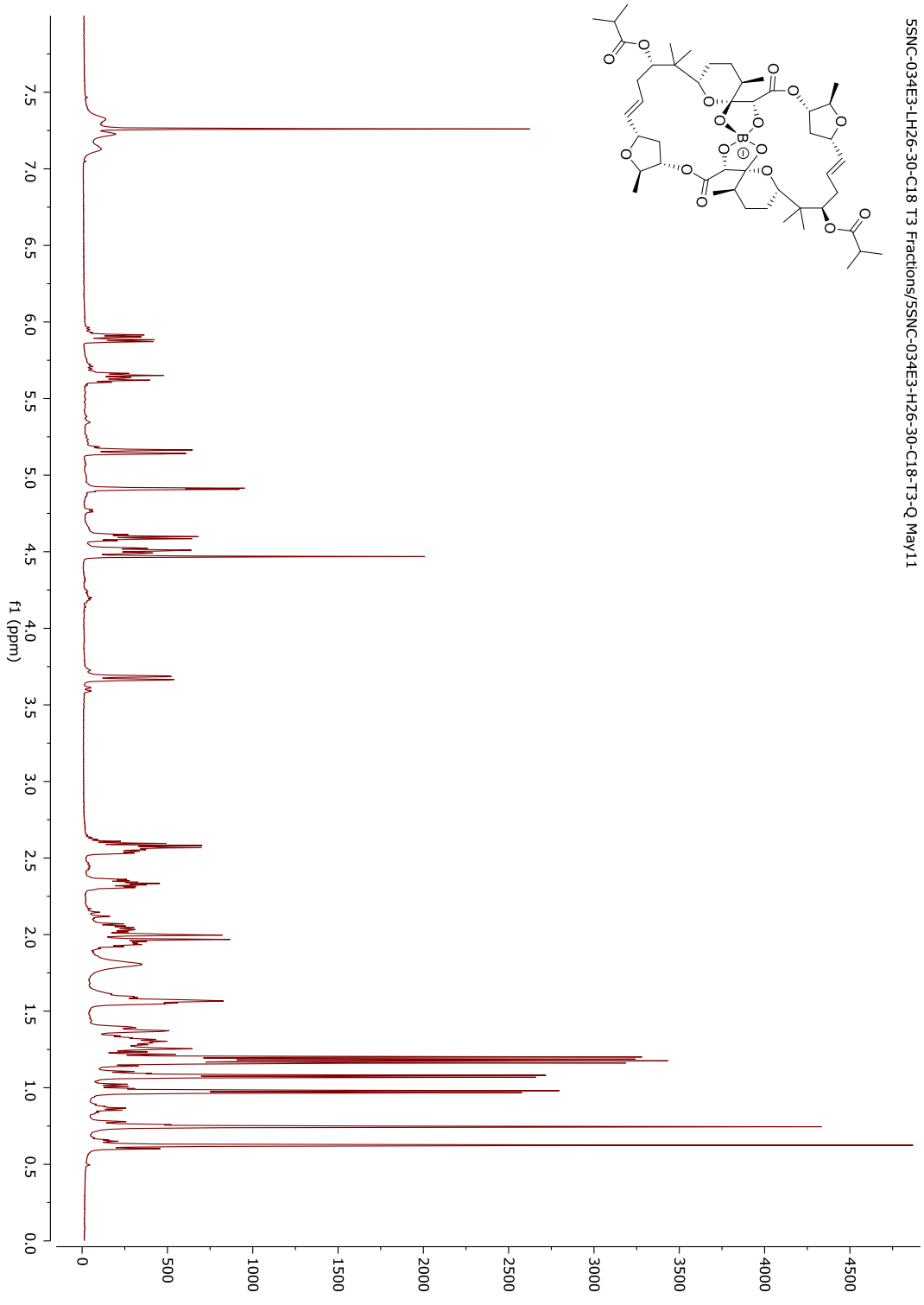
NP-34-915 (2.11)



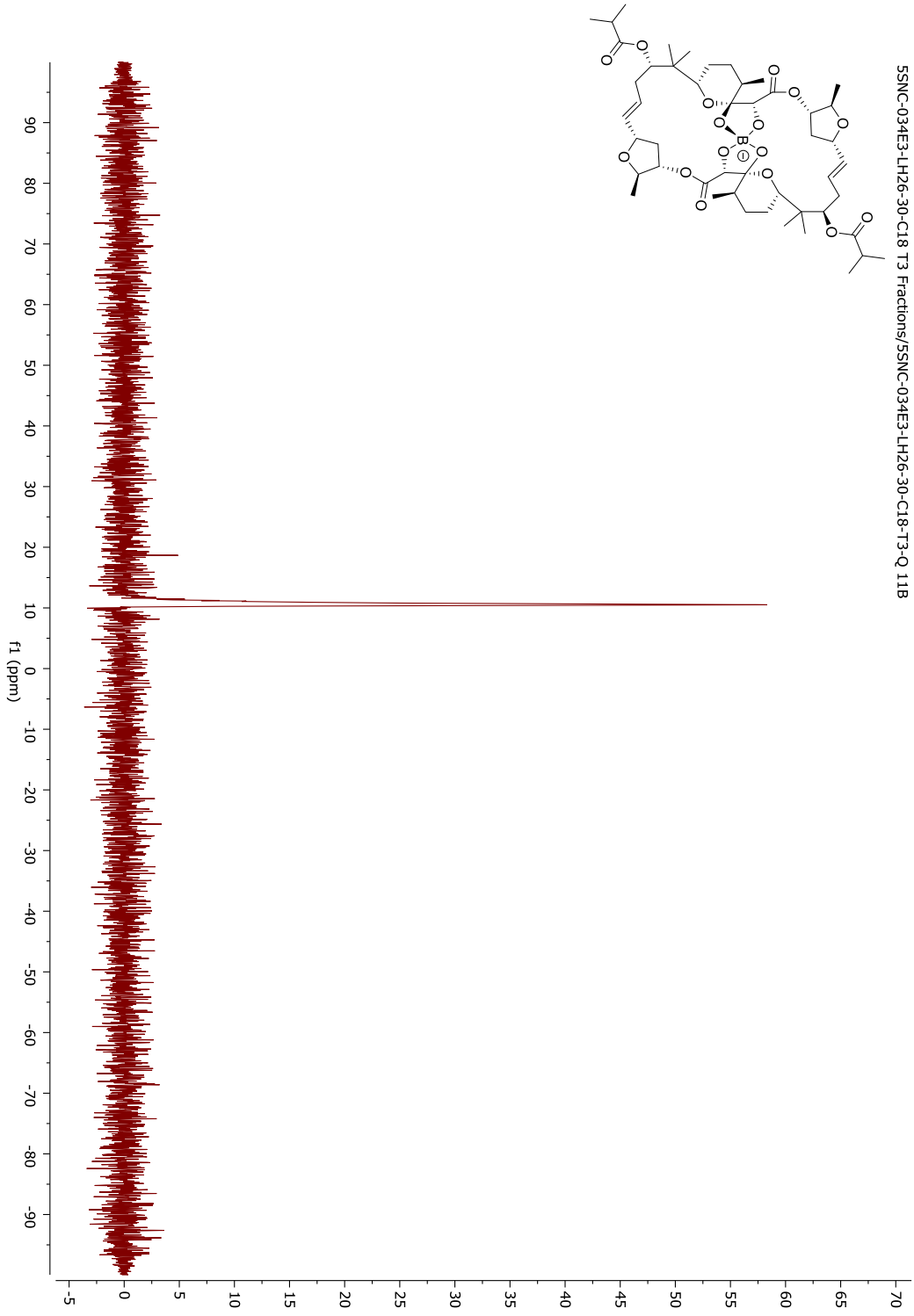
NP-34-915

	NP-34-915	
#	δ_C	δ_H (*J* in Hz)
1	171.6	--
2	78.9	4.47 (1H, s)
3	104.2	--
4	33.6	1.93 (1H, m)
5	28.2	1.56 (1H, m) 1.28 (1H, m)
6	24.9	1.38 (1H, dd, 12.1, 2.3) 1.32 (1H, m)
7	70.8	3.68 (1H, dd, 11.6, 1.5)
8	40.8	--
9	78.1	5.15 (1H, dd, 11.4)
10	30.7	2.03 (1H, m) 2.55 (1H, m)
11	129.6	5.64 (1H, m)
12	134.5	5.90 (1H, dd, 16.5, 6.5)
13	75.5	4.51 (1H, t, 6.5)
14	36.5	1.99 (1H, m) 2.34 (1H, m)
15	80.1	4.91 (1H, d, 3.70)
16	77.9	4.60 (1H, q, 9.8, 6.7)
17	19.1	1.07 (1H, d, 6.4)
18	16.9	0.97 (3H, d, 6.8)
19	17.9	0.62 (3H, s)
20	18.0	0.75 (3H, s)
1''	178.3	--
2''	34.6	2.56 (1H, m)
3''	19.2	1.18 (1H, q, 9.6, 7.0)
4''	19.2	1.18 (1H, q, 9.6, 7.0)
^{11}B 10.5 ppm		
$[M]^-$ = 915 g/mol		

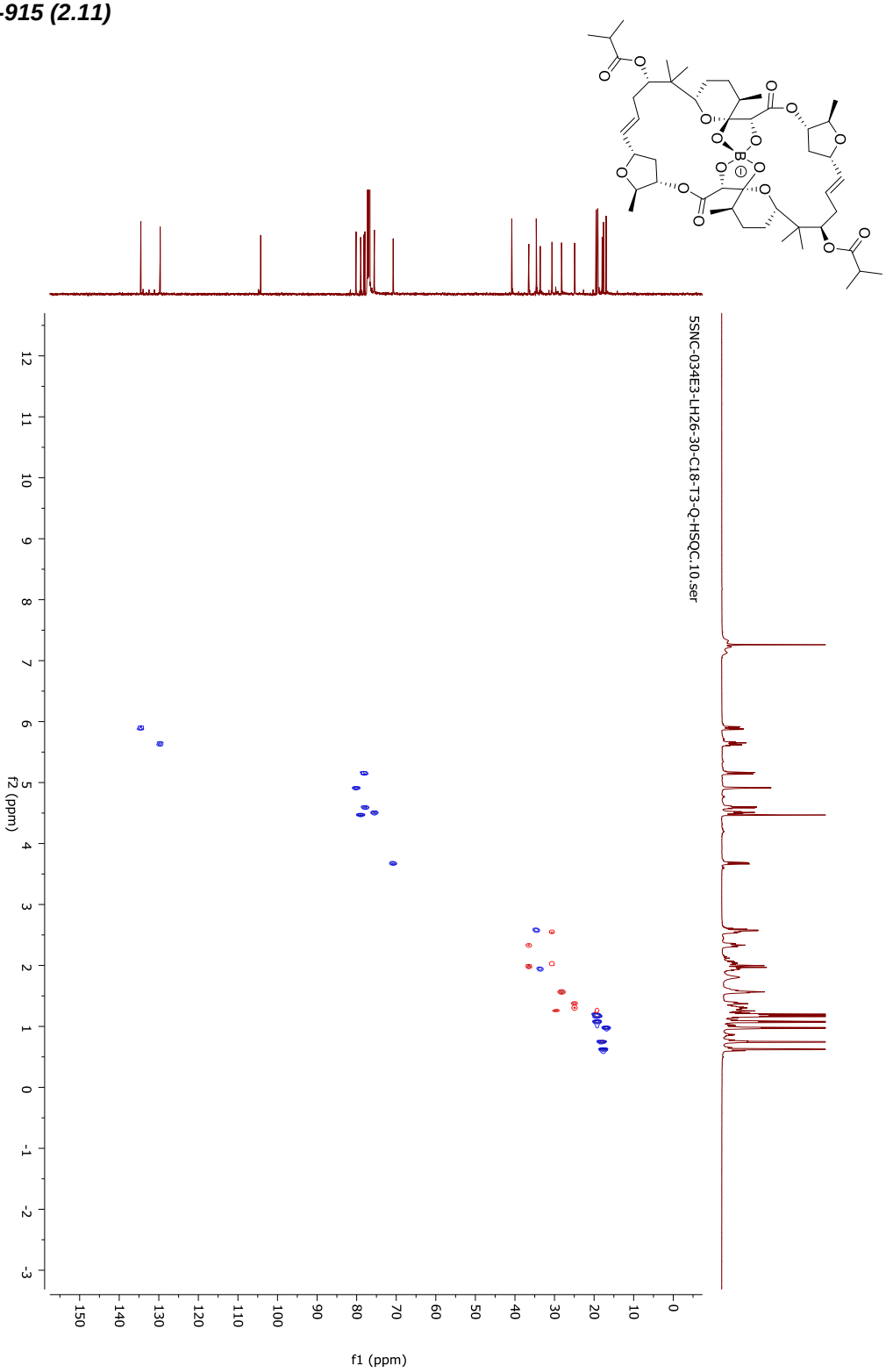
NP-34-915 (2.11)


5SNC-034E3-LH26-30-C18 T3 Fractions/5SNC-034E3-H26-30-C18-T3-Q May11
f1 (ppm)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
500
1000
1500
2000
2500
3000
3500
4000
4500

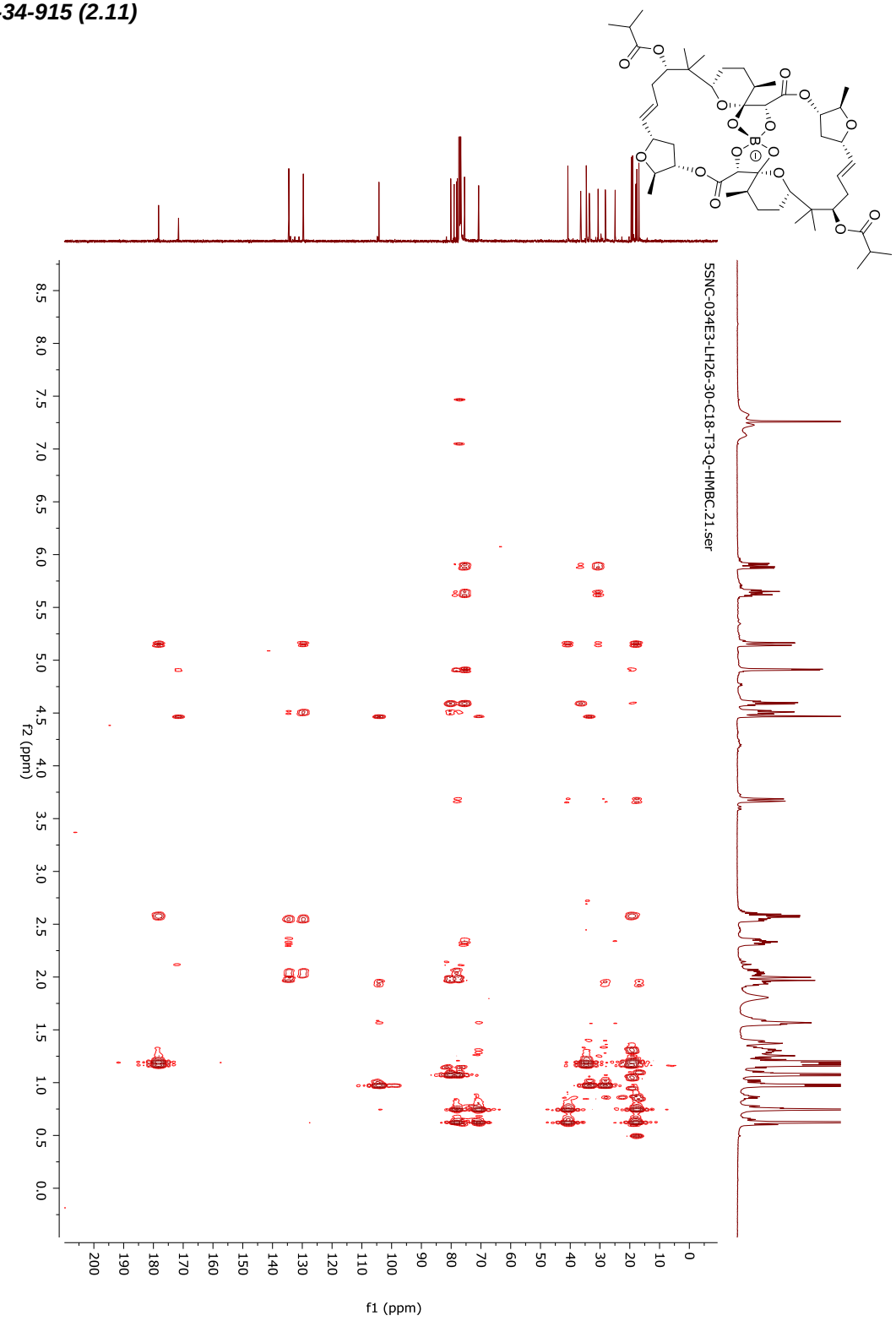
NP-34-915 (2.11)


5SNC-034E3-LH26-30-C18 T3 Fractions/5SNC-034E3-LH26-30-C18-T3-Q 11B
f1 (ppm)

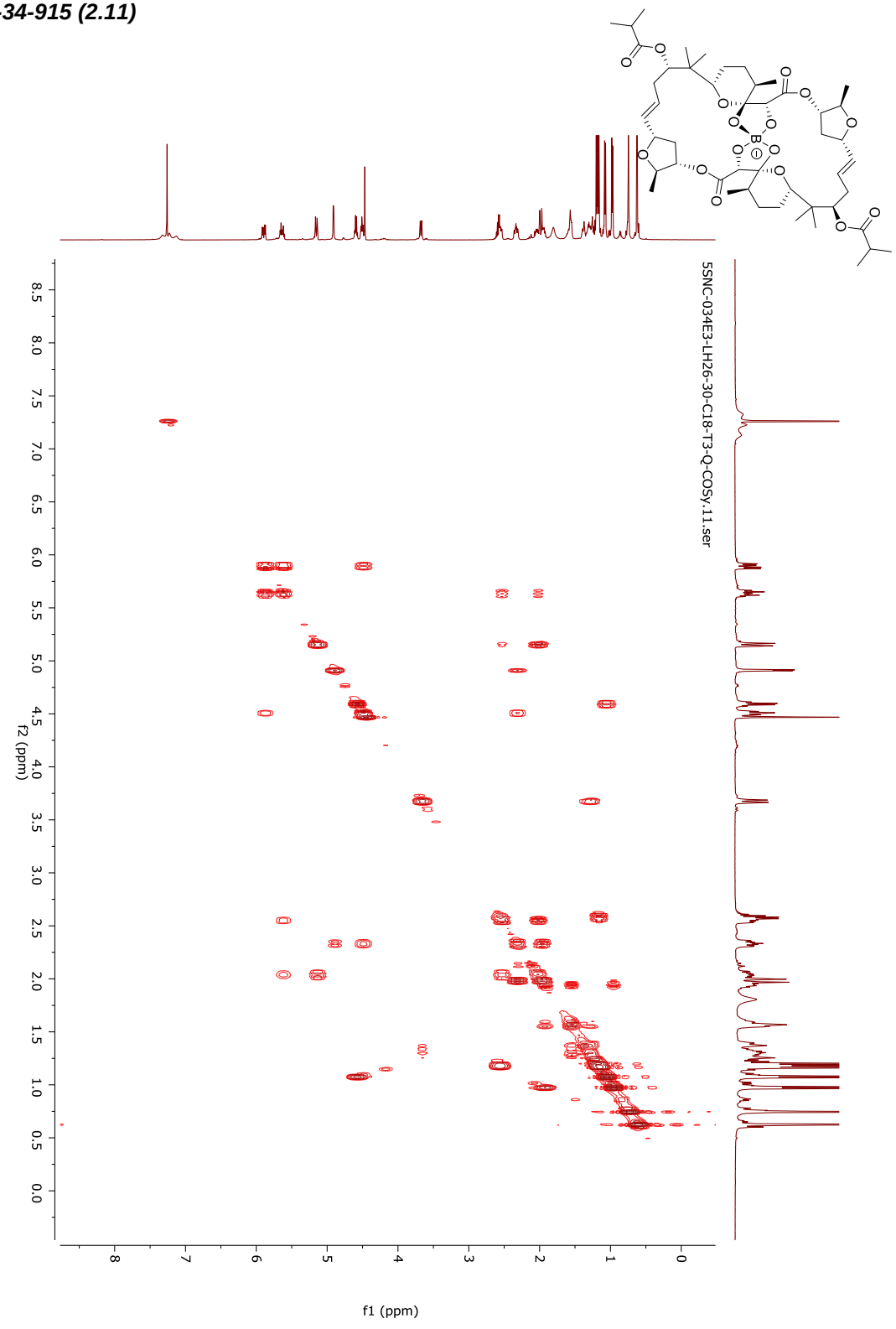
NP-34-915 (2.11)



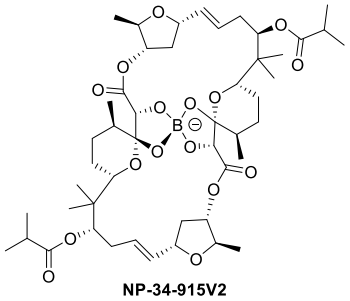
NP-34-915 (2.11)



NP-34-915 (2.11)



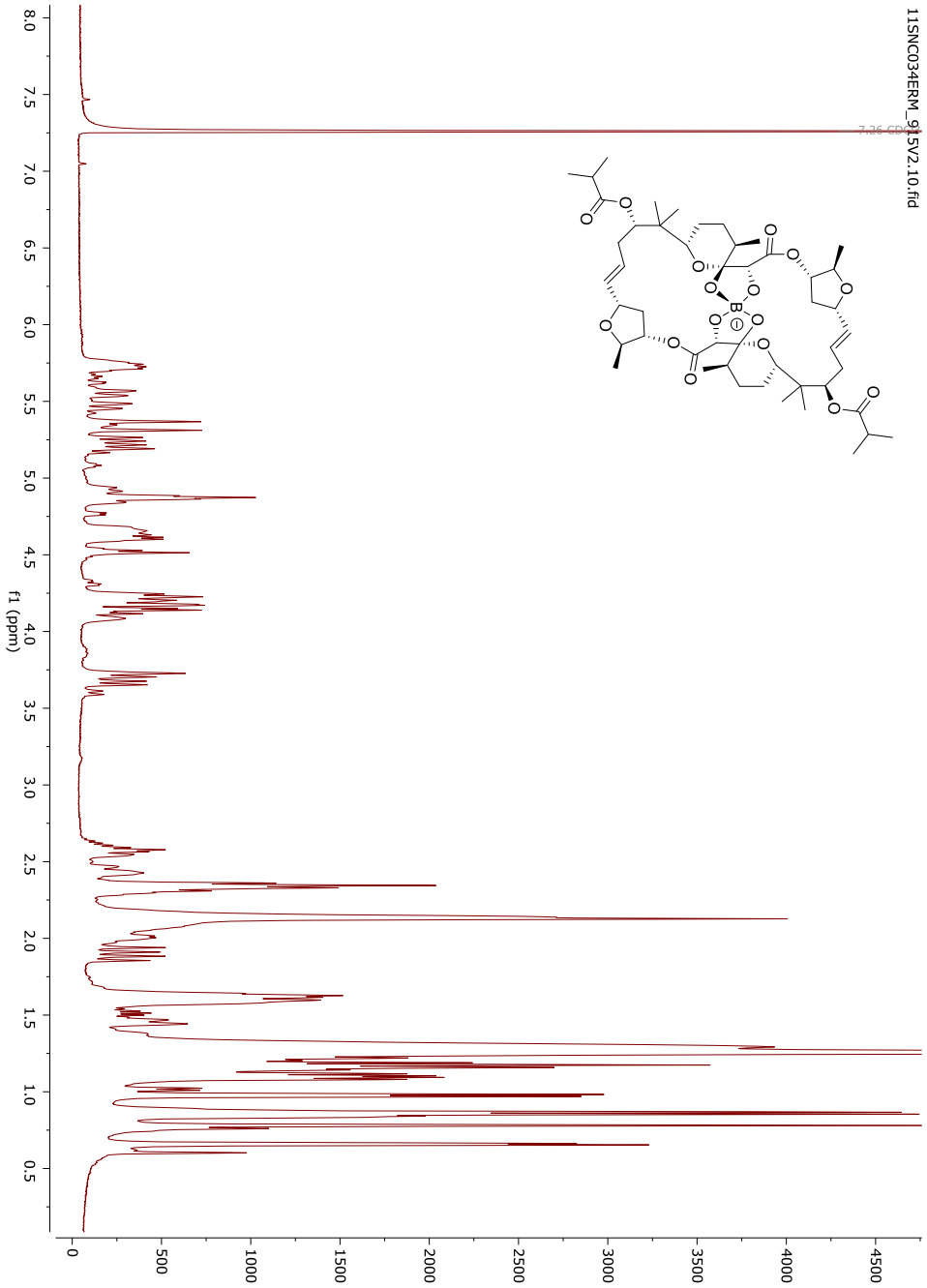
NP-34-915V2 (2.11V2)



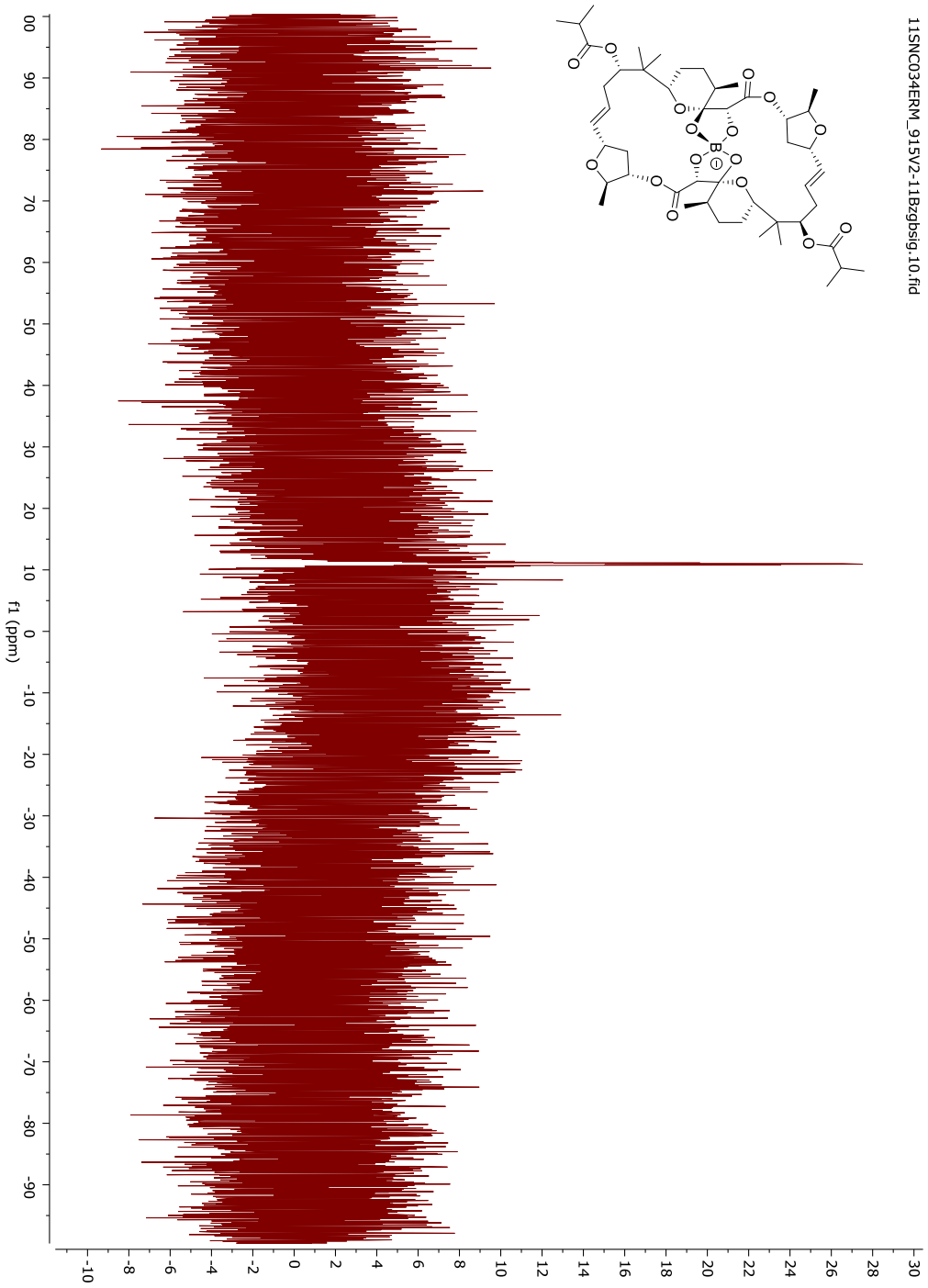
NP-34-915V2

		NP-34-915-V2
#	δ_C	δ_H (*J* in Hz)
1	172.0	--
2	75.1	4.24 (1H, s)
3	98.9	--
4	32.7	2.10 (1H, m)
5	27.5	1.60 (1H, m) 1.28 (1H, m)
6	29.5	1.24 (1H, m) 1.33 (1H, m)
7	72.9	3.71 (1H, dd, 17.8, 10.9)
8	40.6	--
9	76.2	5.25 (1H, qs, 12.9)
10	31.0	2.50 (1H, m) 2.17 (1H, m)
11	126.6	5.73 (1H, m)
12	133.4	5.50 (1H, d, 14.1)
13	77.0	4.69 (1H, m)
14	37.1	2.35 (1H, p, 15.6, 8.1) 1.95 (1H, m)
15	80.5	4.89 (1H, m)
16	78.2	4.63 (1H, q, 9.0, 6.4)
17	19.8	1.13 (3H, dt, 12.6, 6.8)
18	16.6	0.98 (3H, d, 6.6)
19	18.6	0.78 (3H, s)
20	17.9	0.68 (3H, ds, 4.7)
1''	177.3	--
2''	34.4	2.60 (1H, m)
3''	19.2	1.18 (3H, t, 8.3)
4''	19.2	1.18 (3H, t, 8.3)
^{11}B 10.5 ppm		
$[M]^-$ = 915 g/mol		

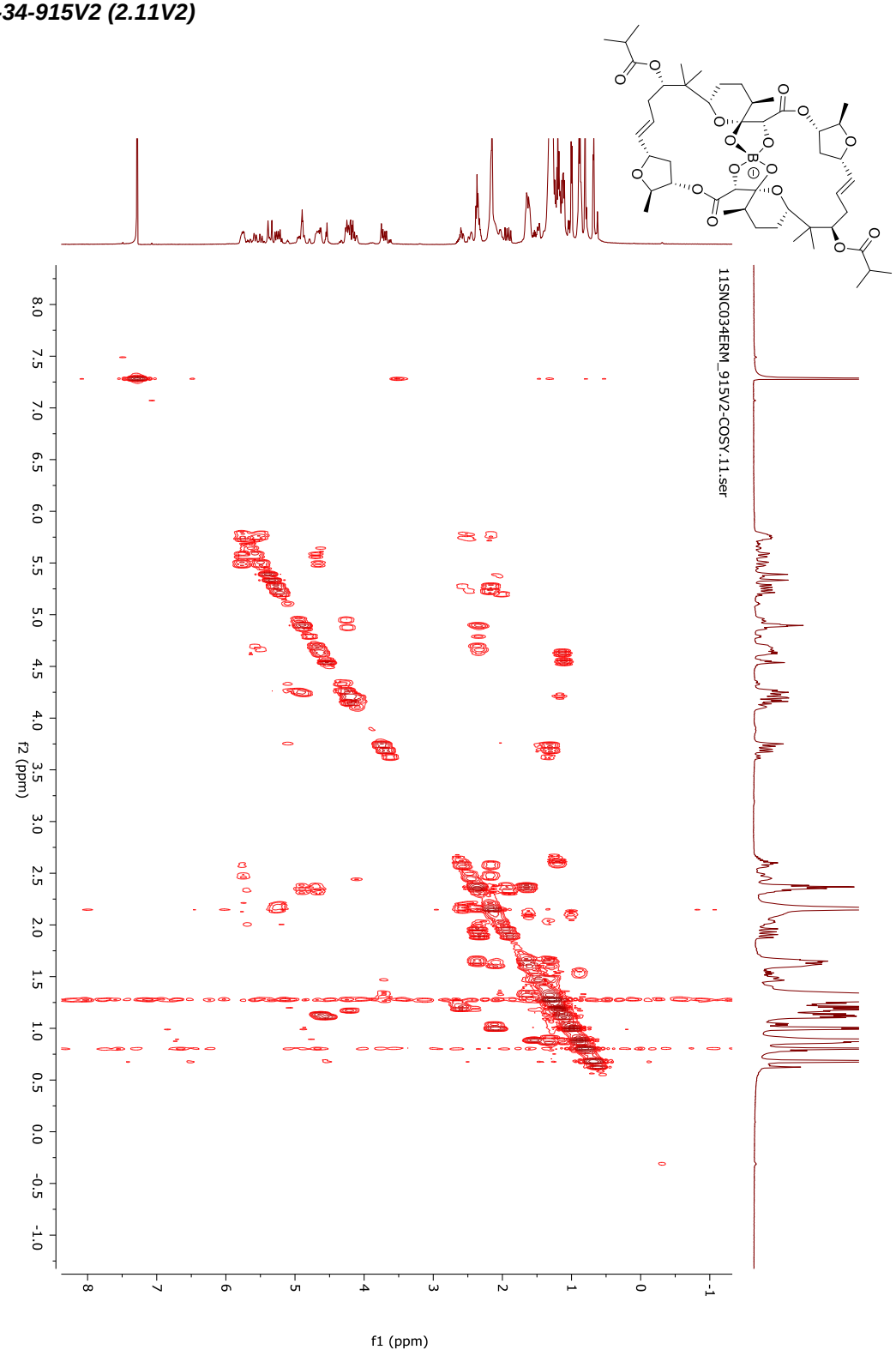
NP-34-915V2 (2.11V2)



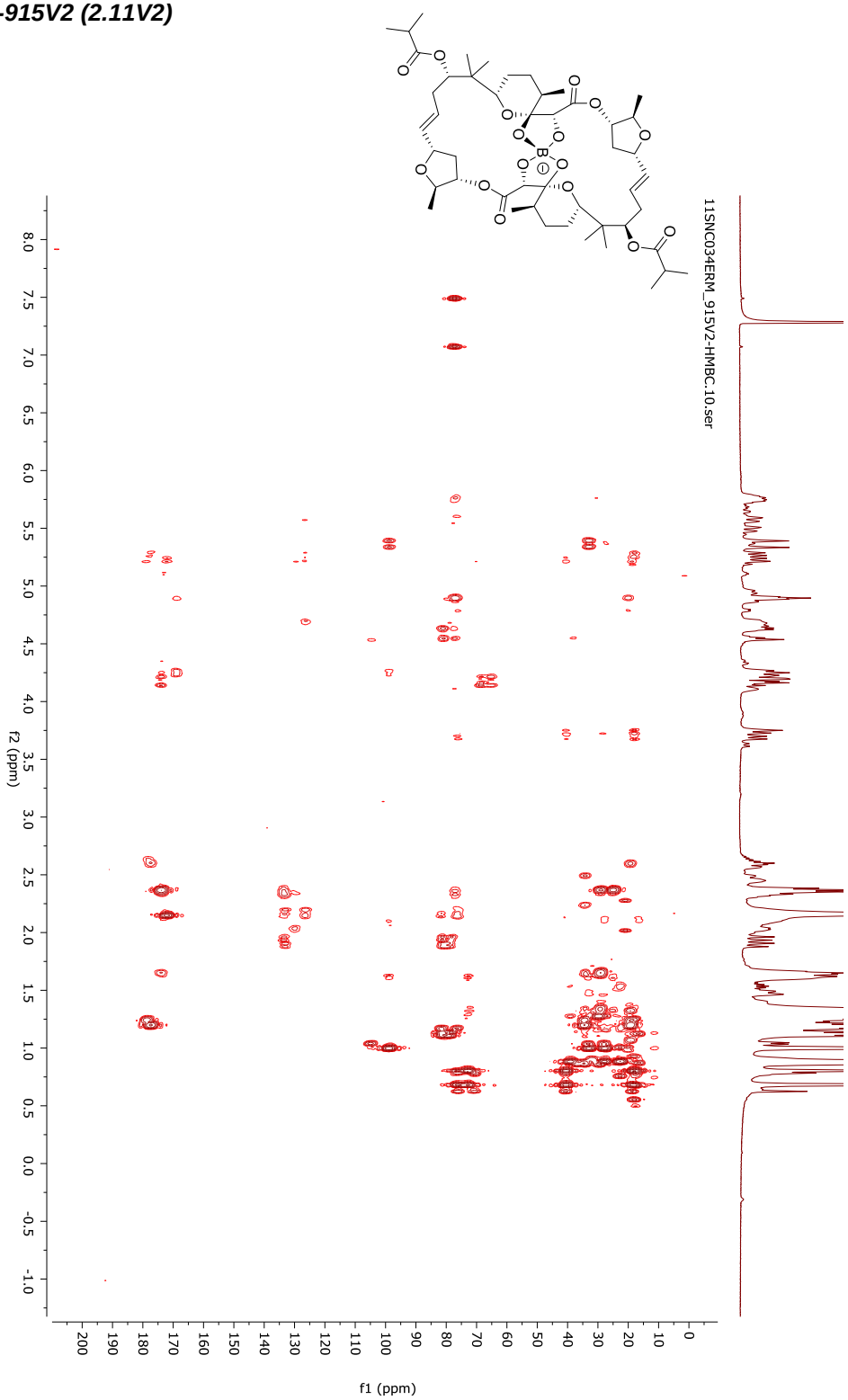
NP-34-915V2 (2.11V2)



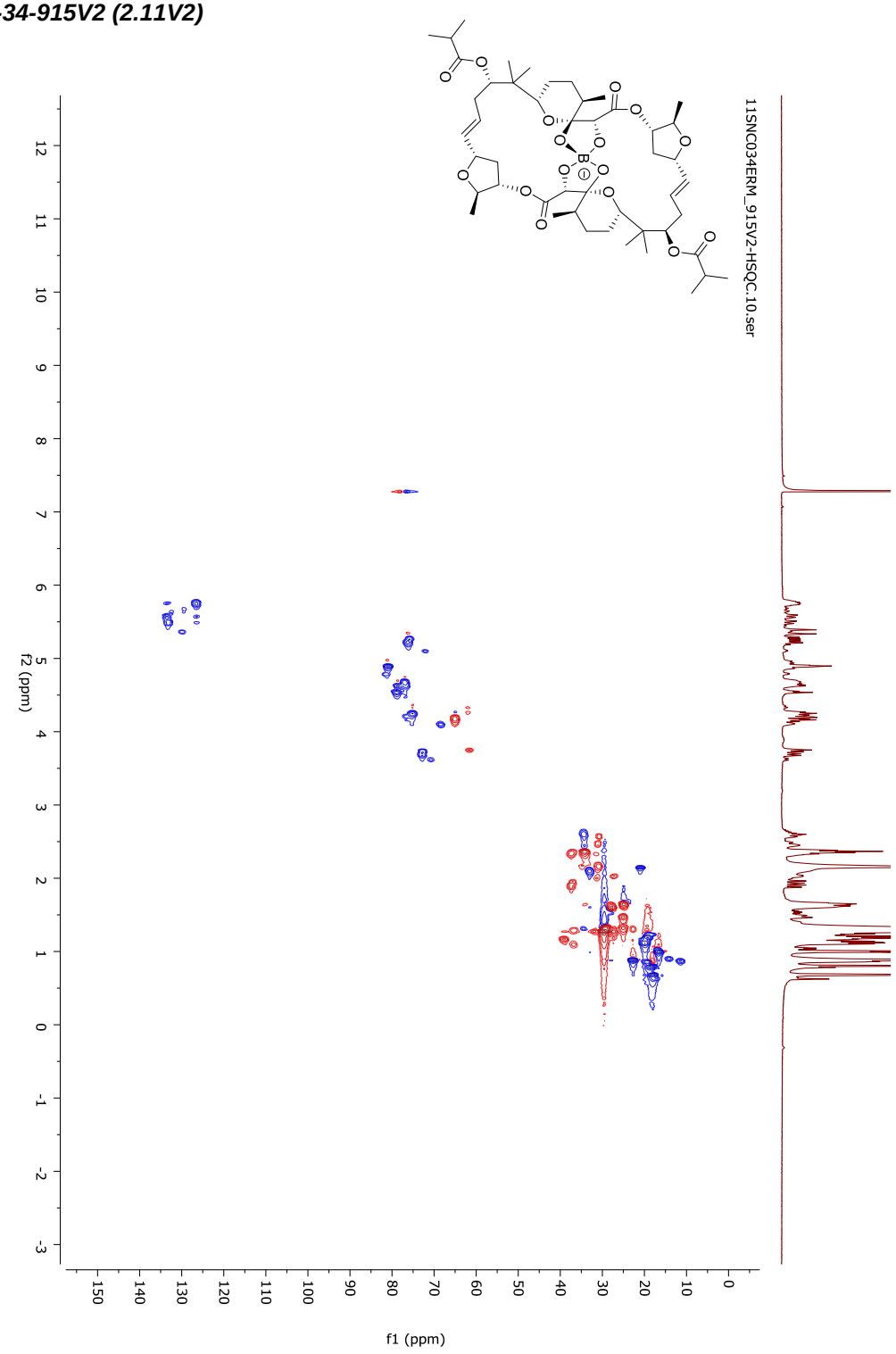
NP-34-915V2 (2.11V2)



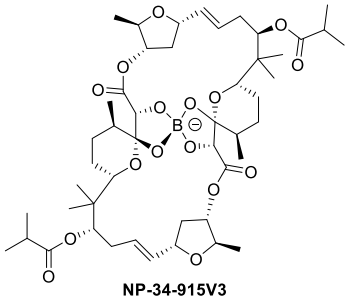
NP-34-915V2 (2.11V2)



NP-34-915V2 (2.11V2)



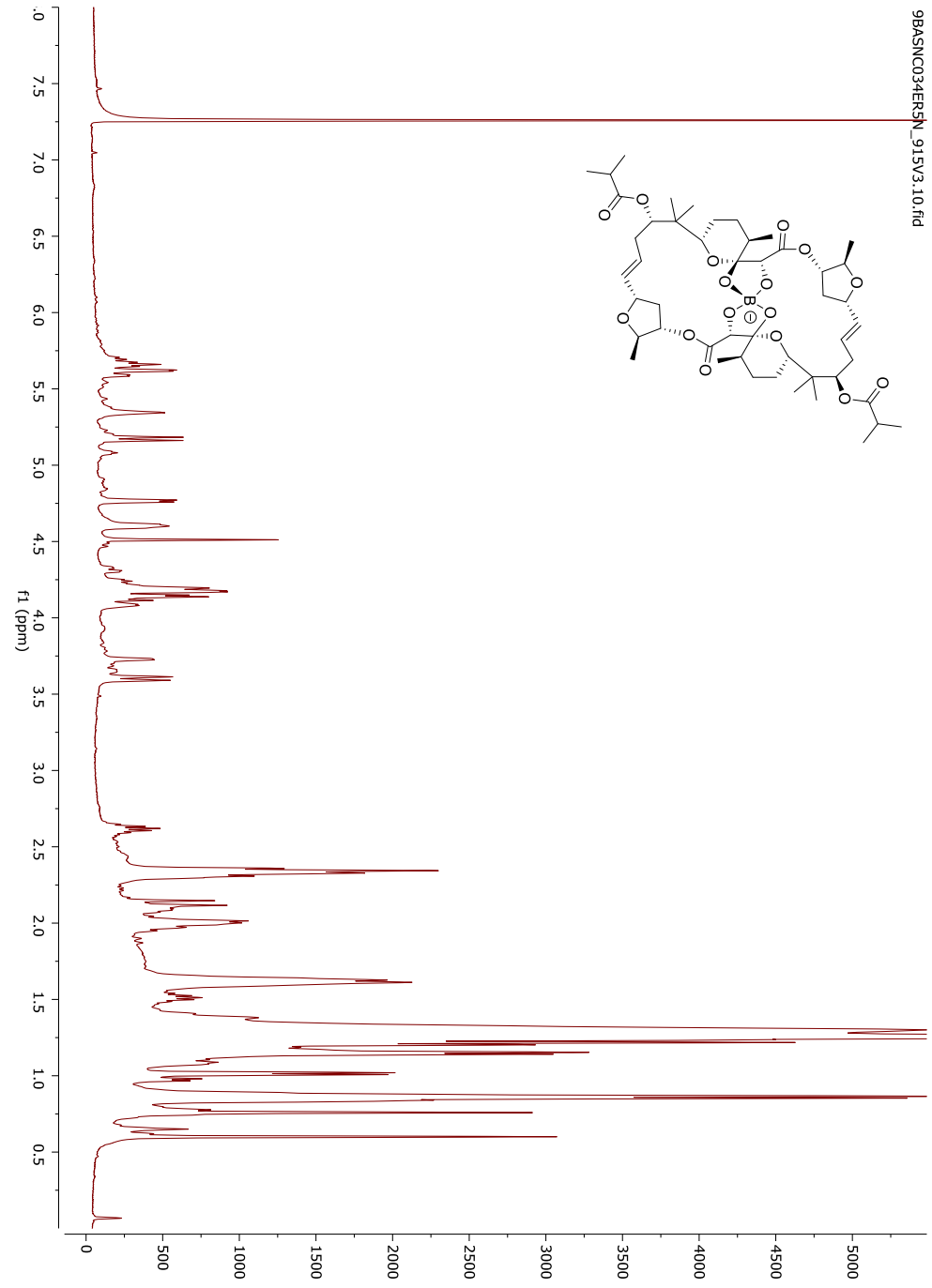
NP-34-915V3 (2.11V3)



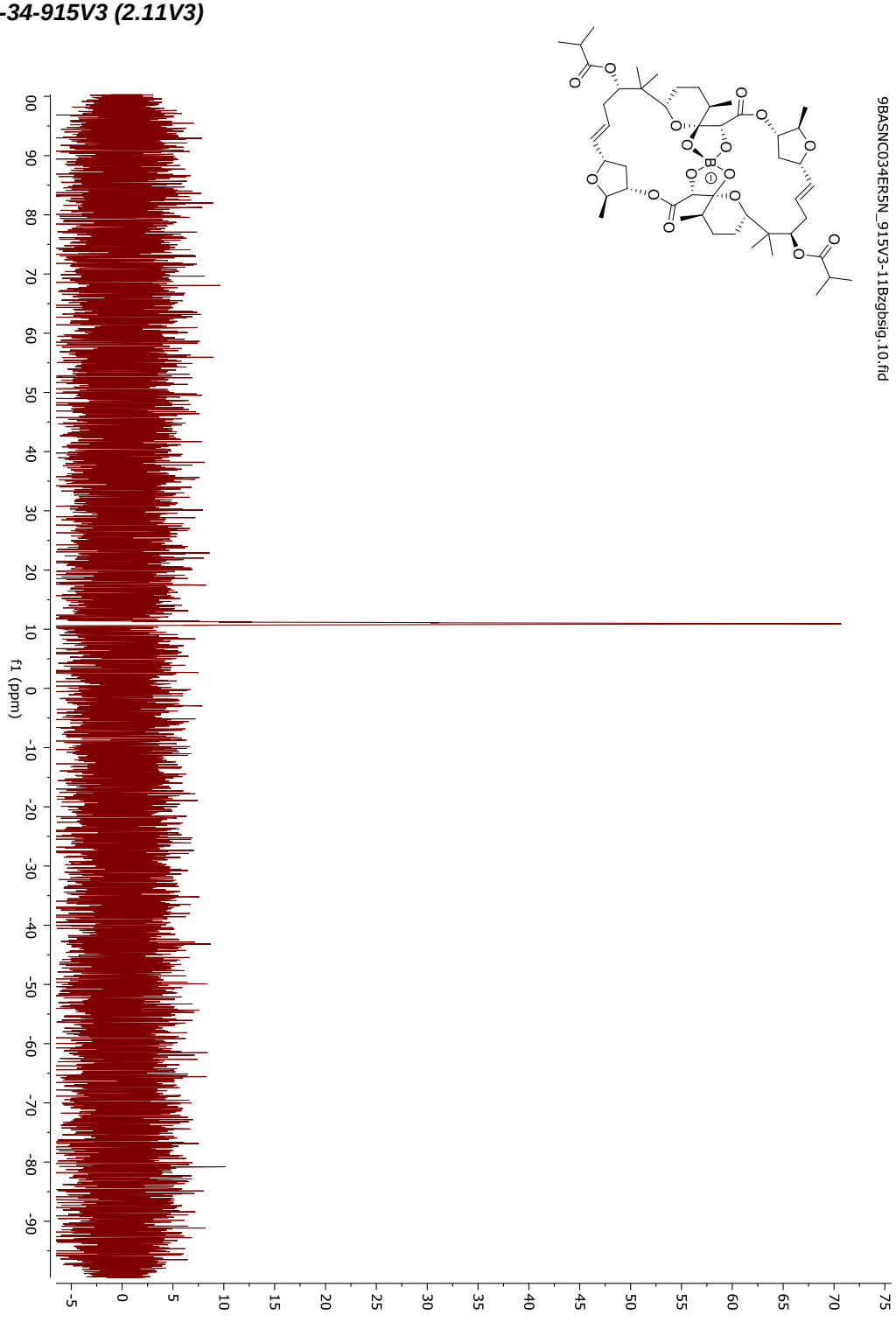
NP-34-915V3

		NP-34-915-V3
#	δ_C	δ_H (*J* in Hz)
1	174.1	--
2	78.7	4.52 (1H, s)
3	104.4	--
4	33.2	2.11 (1H, m)
5	28.3	1.62 (1H, m) 1.53 (1H, p, 13.0 6.8)
6	29.5	1.33 (1H, m) 1.23 (1H, m)
7	70.3	3.62 (1H, d, 12.0)
8	40.8	--
9	76.1	5.17 (1H, 10.7)
10	31.5	2.13 (1H, d, 15.2) 2.02 (1H, m)
11	129.6	5.36 (1H, bs)
12	132.4	5.64 (1H, m)
13	76.6	4.62 (1H, m)
14	34.2	2.36 (1H, dp, 15.3, 7.4) 1.95 (1H, m)
15	81.7	4.78 (1H, ds, 6.5)
16	76.3	4.20 (1H, m)
17	20.1	1.17 (3H, d, 5.9)
18	16.9	1.02 (3H, d, 5.3)
19	17.8	0.61 (3H, s)
20	18.6	0.76 (3H, s)
1''	178.5	--
2''	34.4	2.64 (1H, m, 20.0, 13.8, 7.5)
3''	18.9	1.23 (3H, d, 7.5)
4''	18.9	1.23 (3H, d, 7.5)

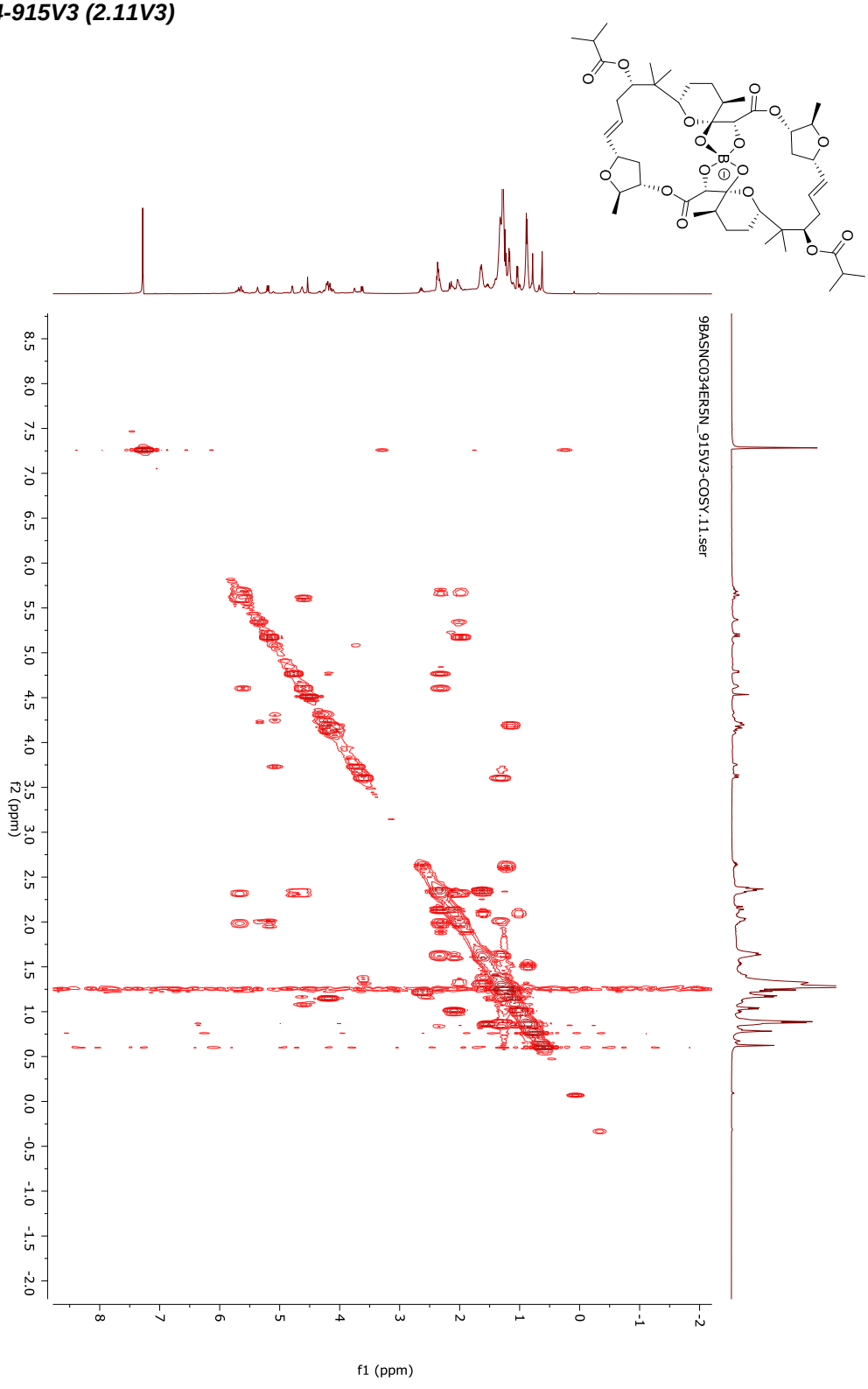
NP-34-915V3 (2.11V3)



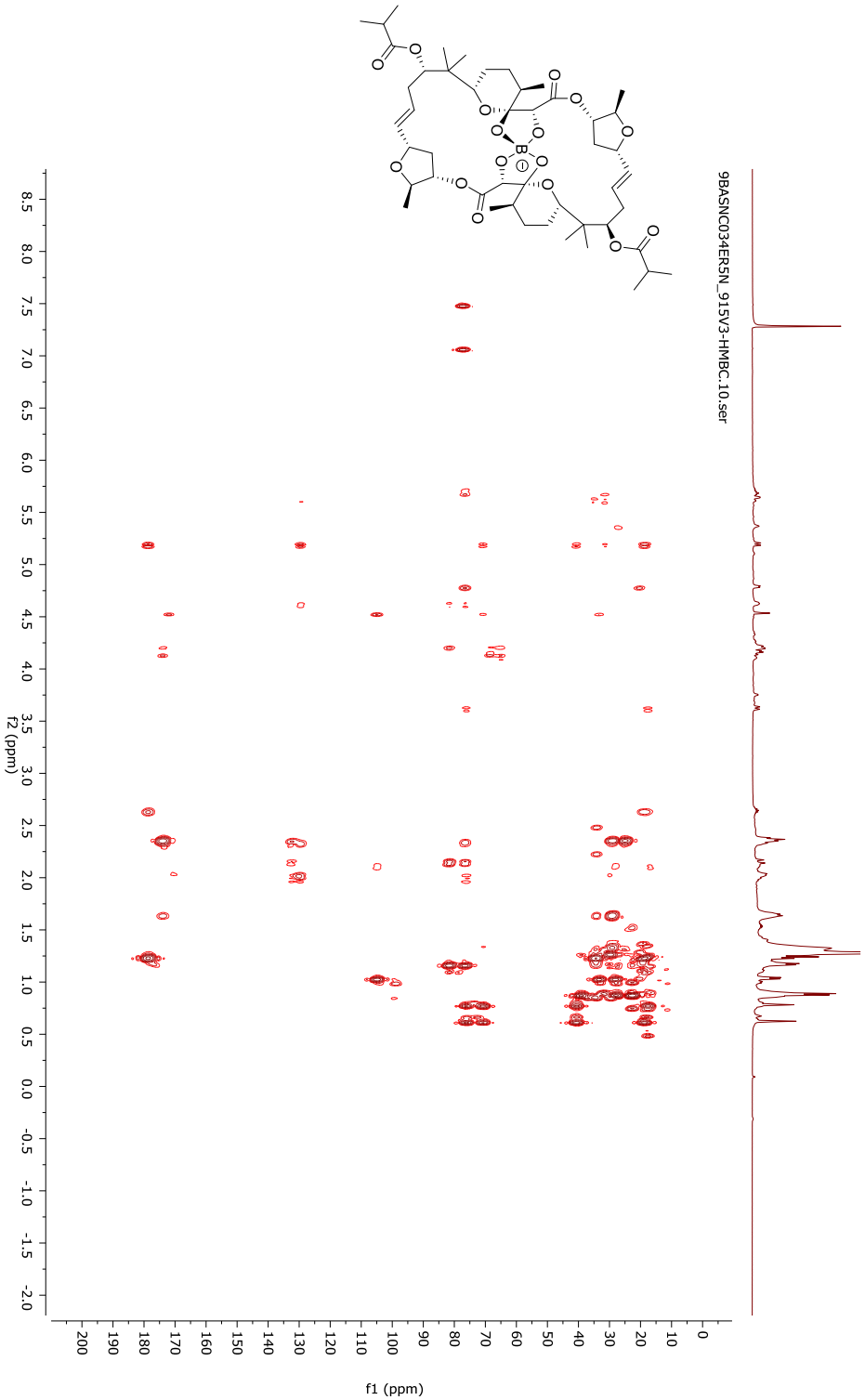
NP-34-915V3 (2.11V3)



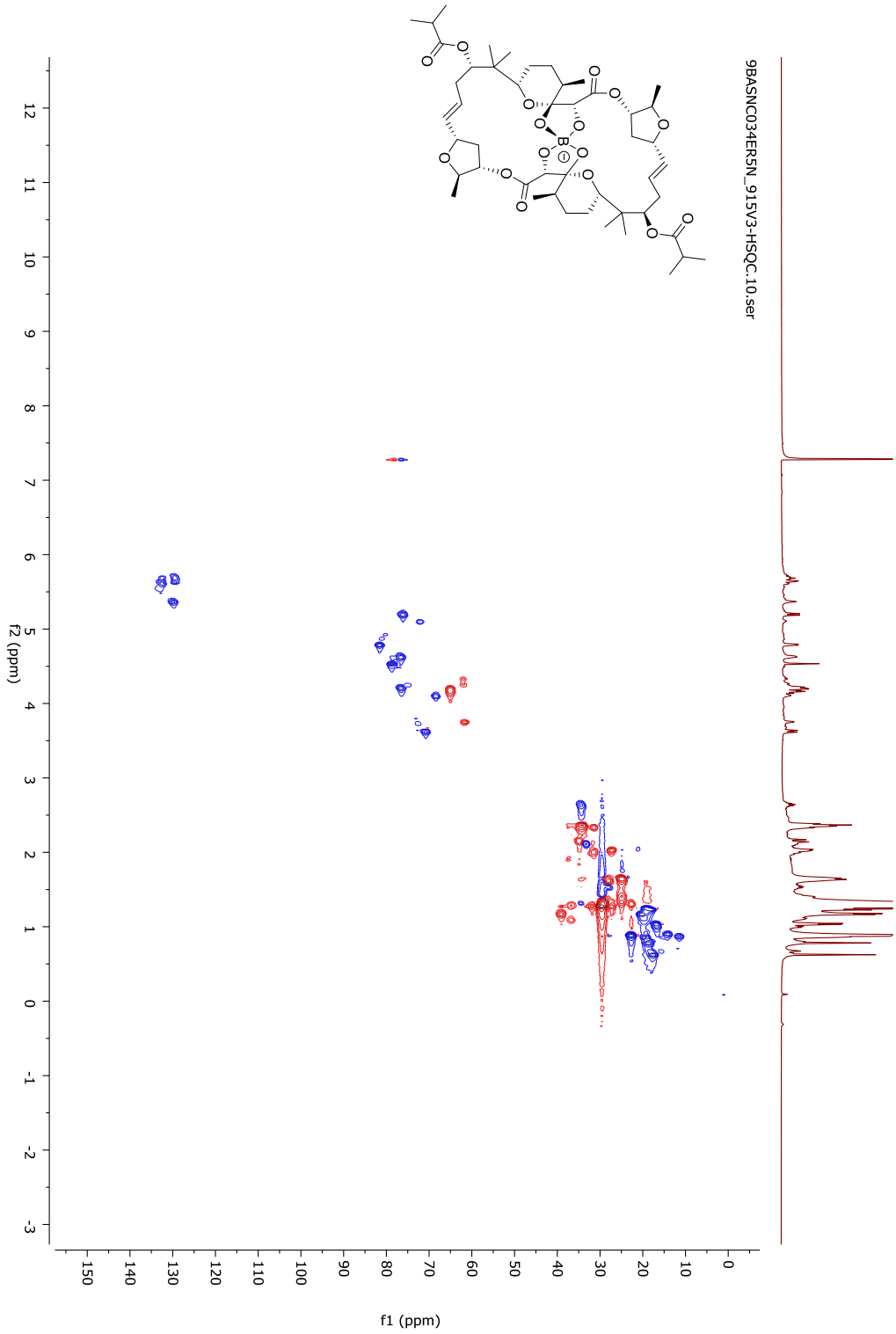
NP-34-915V3 (2.11V3)



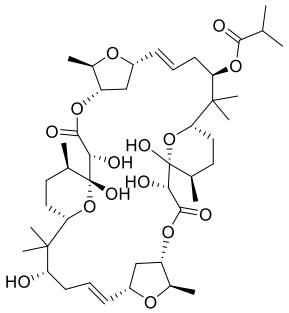
NP-34-915V3 (2.11V3)



NP-34-915V3 (2.11V3)



NP-34-DB (2.12)

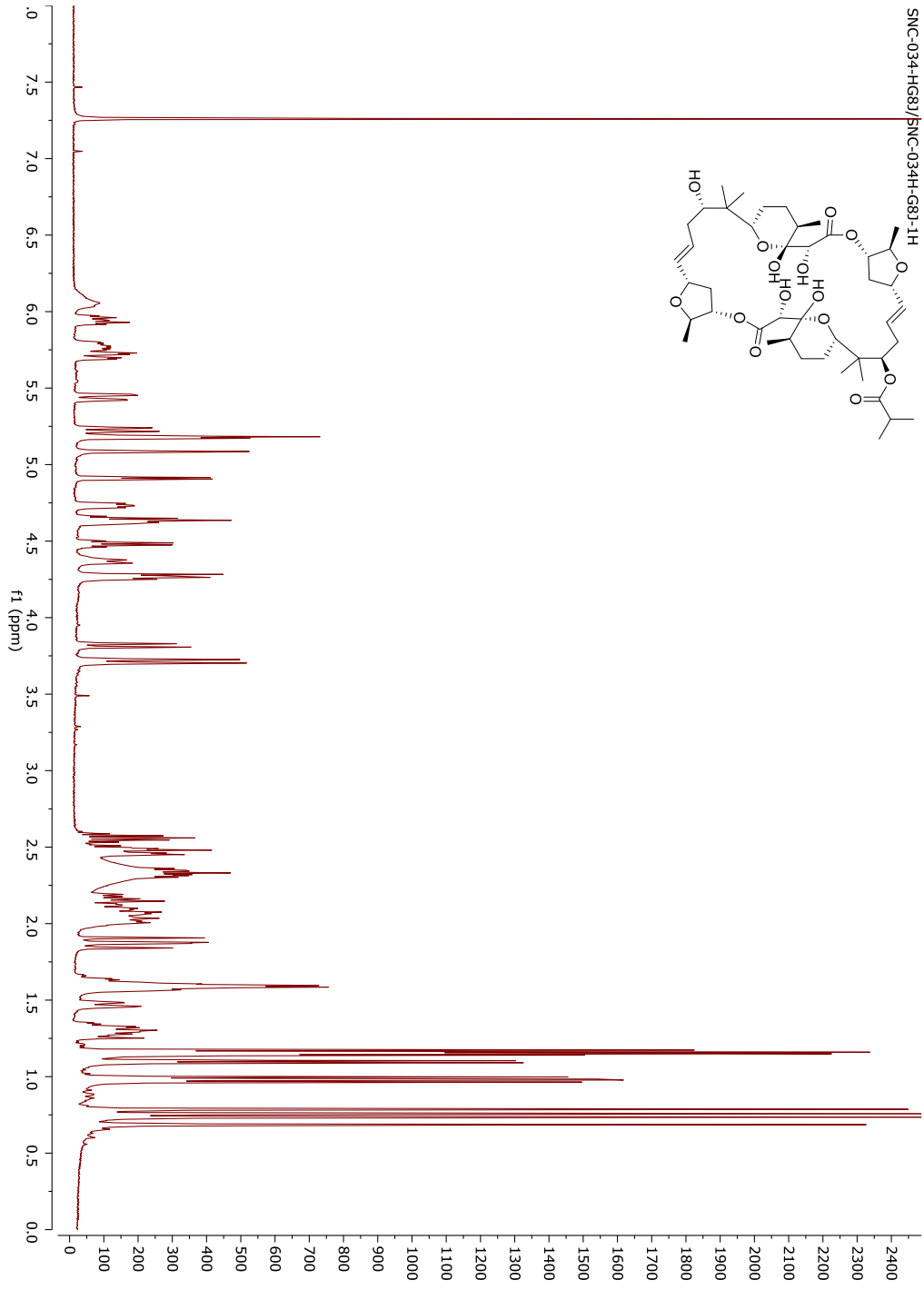


NP-34-DB

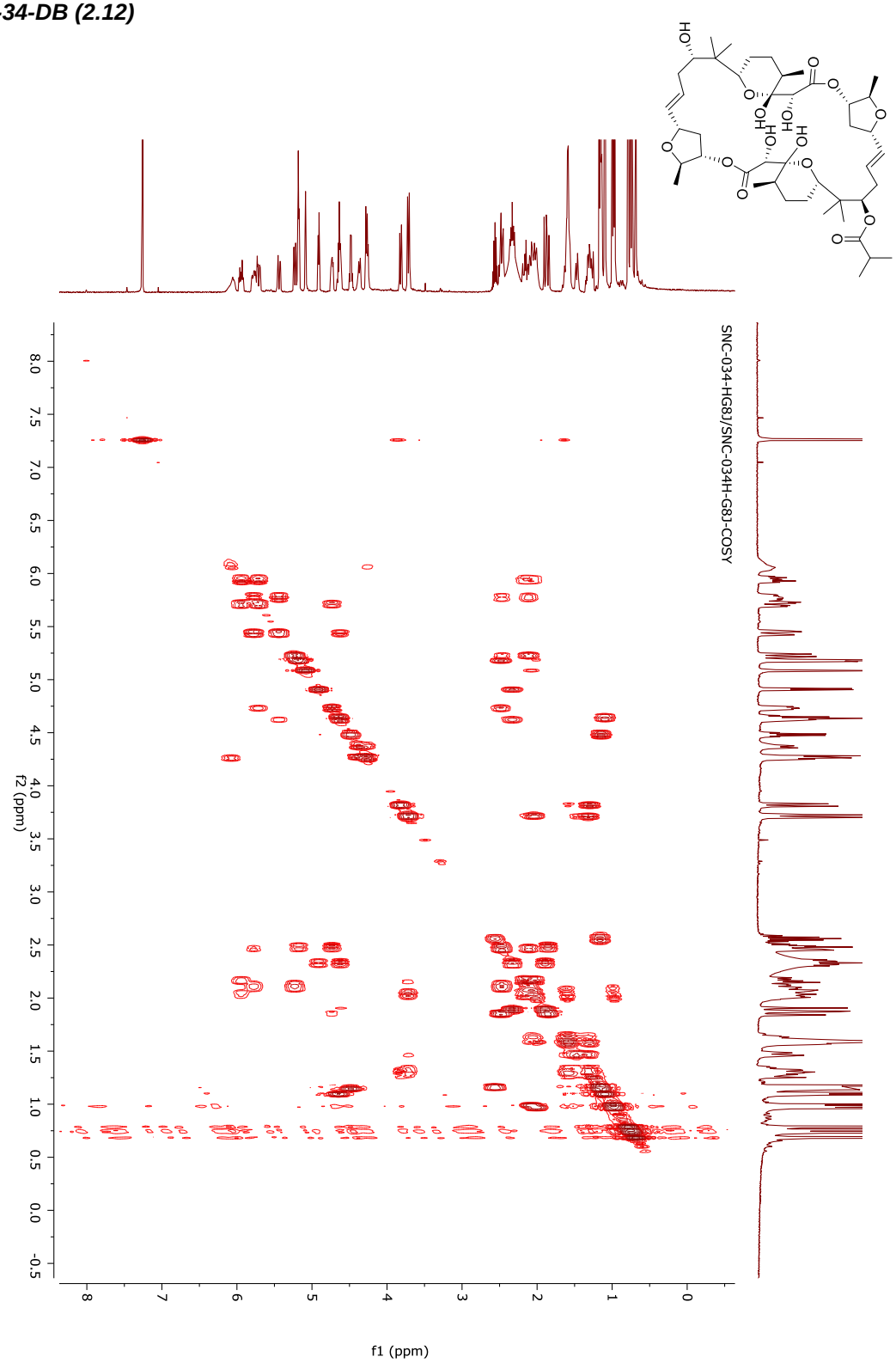
	NP-34-DB	
#	δ_C	δ_H (*J* in Hz)
1	168.0	--
2	75.0	4.27 (1H, m)
3	99.6	--
4	33.1	1.98 (1H, m)
5	27.9	1.56 (1H, m) 1.45 (1H, dq, 12.8, 3.9, 2.51)
6	25.3	2.02 (1H, m) 1.35 (1H, m)
7	72.8	3.70 (1H, s)
8	40.4	--
9	75.8	5.24 (1H, dd, 11.9, 2.1)
10	30.7	2.10 (1H, m) 2.49 (1H, m)
11	126.7	5.76 (1H, m)
12	133.1	5.43 (1H, dd, 15.6, 2.1)
13	79.6	4.65 (1H, m)
14	37.4	1.91 (1H, d, 14.6) 2.35 (1H, m)
15	81.7	4.91 (1H. d, 5.3)
16	76.8	4.61 (1H, q, 10.4, 6.4)
17	20.1	1.10 (3H, d, 6.4)
18	16.7	0.99 (3H, d, 6.4)
19	18.3	0.69 (3H, s)
20	18.1	0.79 (3H, s)
1’	169.2	--
2’	79.9	5.17 (1H, s)
3’	99.0	--
4’	33.1	2.02 (1H, m)
5’	32.2	1.60 (1H, m) 1.32 (1H, m)
6’	25.4	1.58 (1H, m) 1.33 (1H, m)
7’	80.3	3.82 (1H, d, 11.2)
8’	39.8	--
9’	79.6	3.72 (1H, dd, 11.1, 1.2)
10’	32.5	2.04 (1H, m)

		2.16 (1H, m)
11’	127.9	5.94 (1H, m)
12’	132.1	5.70 (1H, dd, 15.7, 4.5)
13’	77.9	4.73 (1H, dt, 6.7, 4.9)
14’	37.5	1.87 (1H, d, 4.6) 2.47 (1H, m)
15’	79.3	4.66 (1H, m)
16’	78.6	4.48 (1H, q, 9.9, 6.7)
17’	19.8	1.15 (3H, m)
18’	19.4	1.15 (1H, m)
19’	13.9	0.76 (3H, s)
20’	21.8	0.73 (3H, s)
1’’	177.5	--
2’’	34.6	2.56 (1H, p, 13.6, 6.2)
3’’	19.4	1.16 (3H, m)
4’’	19.4	1.16 (3H, m)
2-OH	--	5.08 (1H, bs)
9’-OH	--	6.06 (1H, bs)
$[M]^-$ = 838 g/mol, $[M+Cl^-]^-$ = 873 g/mol		

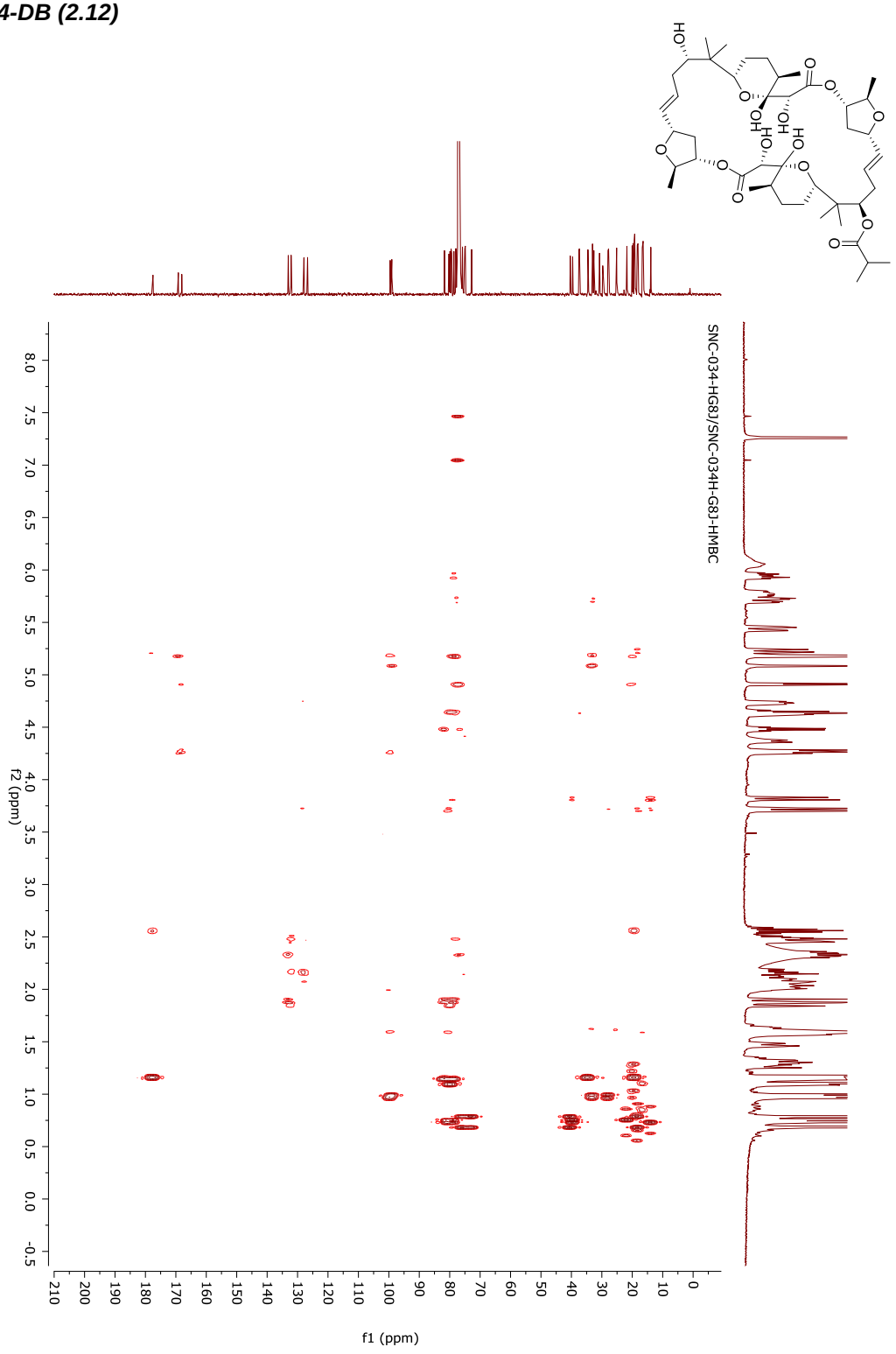
NP-34-DB (2.12)



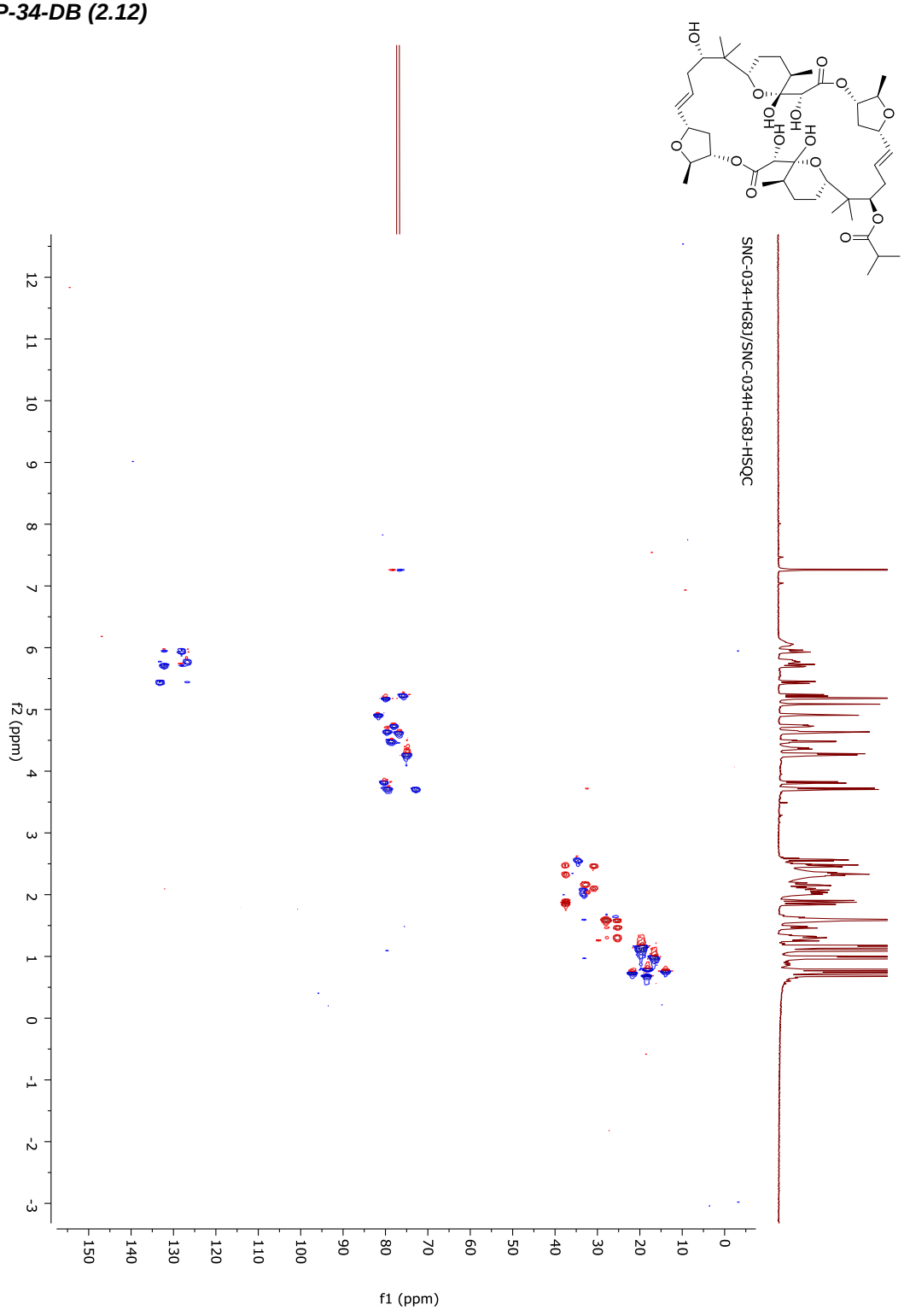
NP-34-DB (2.12)



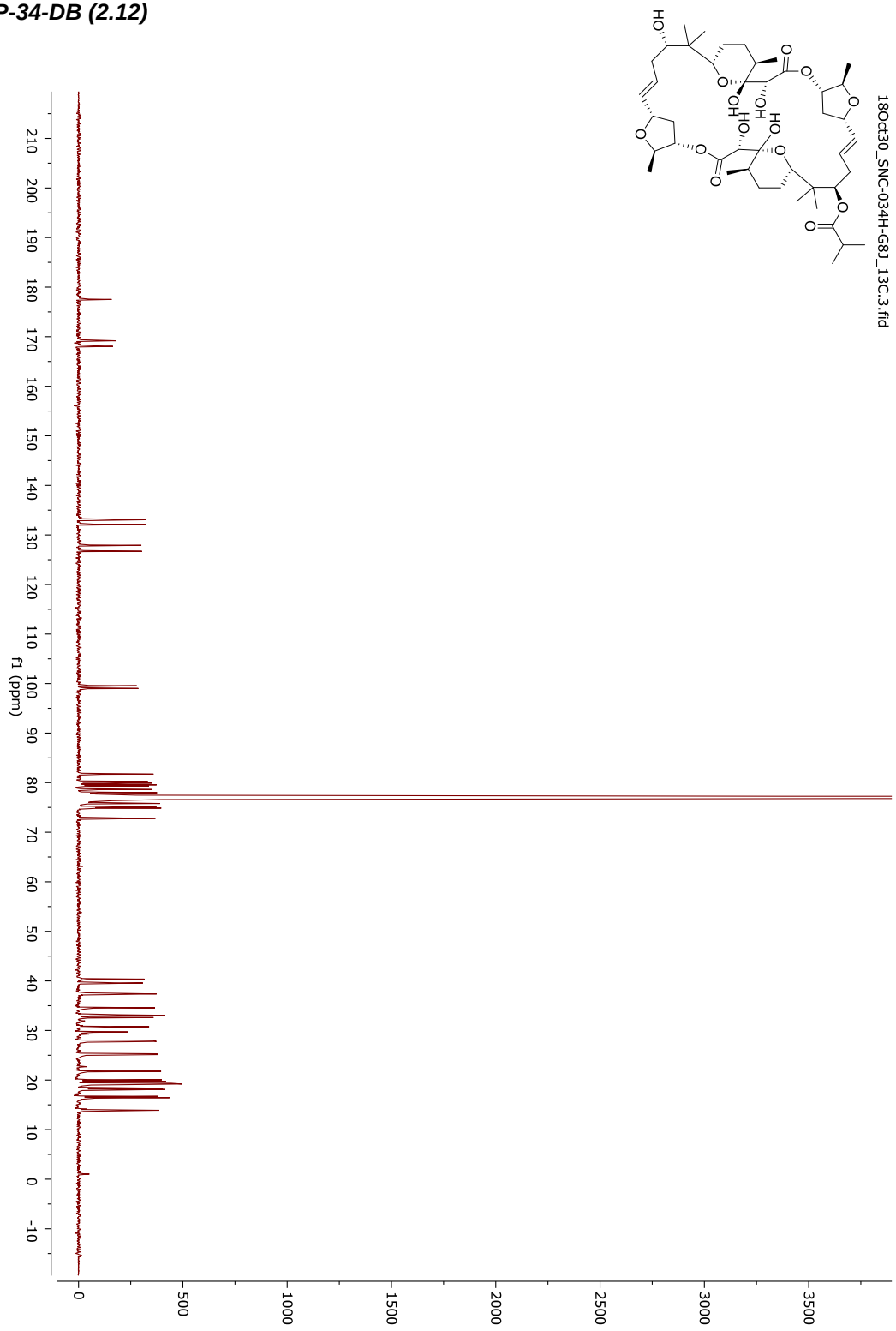
NP-34-DB (2.12)



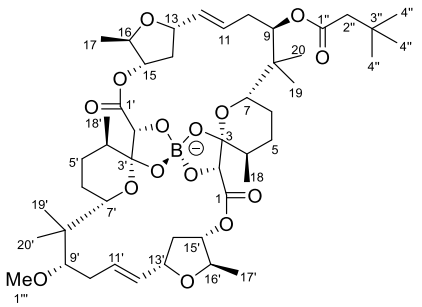
NP-34-DB (2.12)



NP-34-DB (2.12)


18Oct30_SNC-034H-G8J_13C.3.fid
f1 (ppm)
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
0
500
1000
1500
2000
2500
3000
3500

NP-34-887 (4.1)



NP-34-887

		NP-34-887
#	δ_C	δ_H (*J* in Hz)
1	172.5	--
2	78.7	4.48 (1H, s)
3	104.3	--
4	33.6	1.96 (1H, m)
5	25.2	1.40 (1H, m) 1.35 (1H, dq,12.3, 6.8)
6	26.3	1.60 (1H, m) 1.33 (1H, m)
7	70.8	3.68 (1H, dd, 6.9, 1.7)
8	40.7	--
9	78.2	5.17 (1H, p, 26.3, 14.3)
10	30.5	2.58 (1H, m) 2.06 (1H, m)
11	129.8	5.66 (1H, m)
12	134.8	5.93 (1H, dd, 15.4, 6.7)
13	75.6	4.54 (1H, m)
14	36.6	2.34 (1H, m) 2.00 (1H, t, 6.5)
15	80.4	4.90 (1H, d, 3.6)
16	78.2	4.55 (1H, m)
17	19.3	1.08 (1H, d, 6.7)
18	17.1	0.99 (3H, d, 6.2)
19	17.7	0.63 (3H, d, 5.5)
20	18.0	0.74 (3H, d, 5.5)
1’	171.4	--
2’	78.7	4.46 (1H, s)
3’	104.3	
4’	33.6	1.96 (1H, m)
5’	25.2	1.40 (1H, m) 1.35 (1H, m)
6’	26.3	1.60 (1H, m) 1.32 (1H, m)
7’	70.8	3.66 (1H, dd, 6.9, 1.7)
8’	40.8	--
9’	78.9	5.15 (1H, p, 26.3, 14.3)
10’	34.6	2.59 (1H, m)

		2.03 (1H, m)
11’	129.8	5.63 (1H, m)
12’	5.82	134.4 (1H, dd, 15.6, 6.1)
13’	75.6	4.52 (1H, m)
14’	36.4	2.34 (1H, m) 1.99 (1H, t, 6.5)
15’	80.0	4.97 (1H, d, 3.6)
16’	78.0	4.62 (1H, q, 10.1, 6.6)
17’	19.3	1.08 (1H, d, 6.7)
18’	17.1	0.99 (3H, d, 6.2)
19’	17.7	0.63 (3H, d, 5.5)
20’	18.0	0.74 (3H, d, 5.5)
1’’	178.5	--
2’’	34.5	2.57 (2H, m)
3’’	28.2	--
4’’	19.2	1.17 (9H, qs, 7.1)
1’’’	36.7	2.07 (3H, s)
^{11}B 10.5 ppm		
$[M]^-$ = 887 g/mol		

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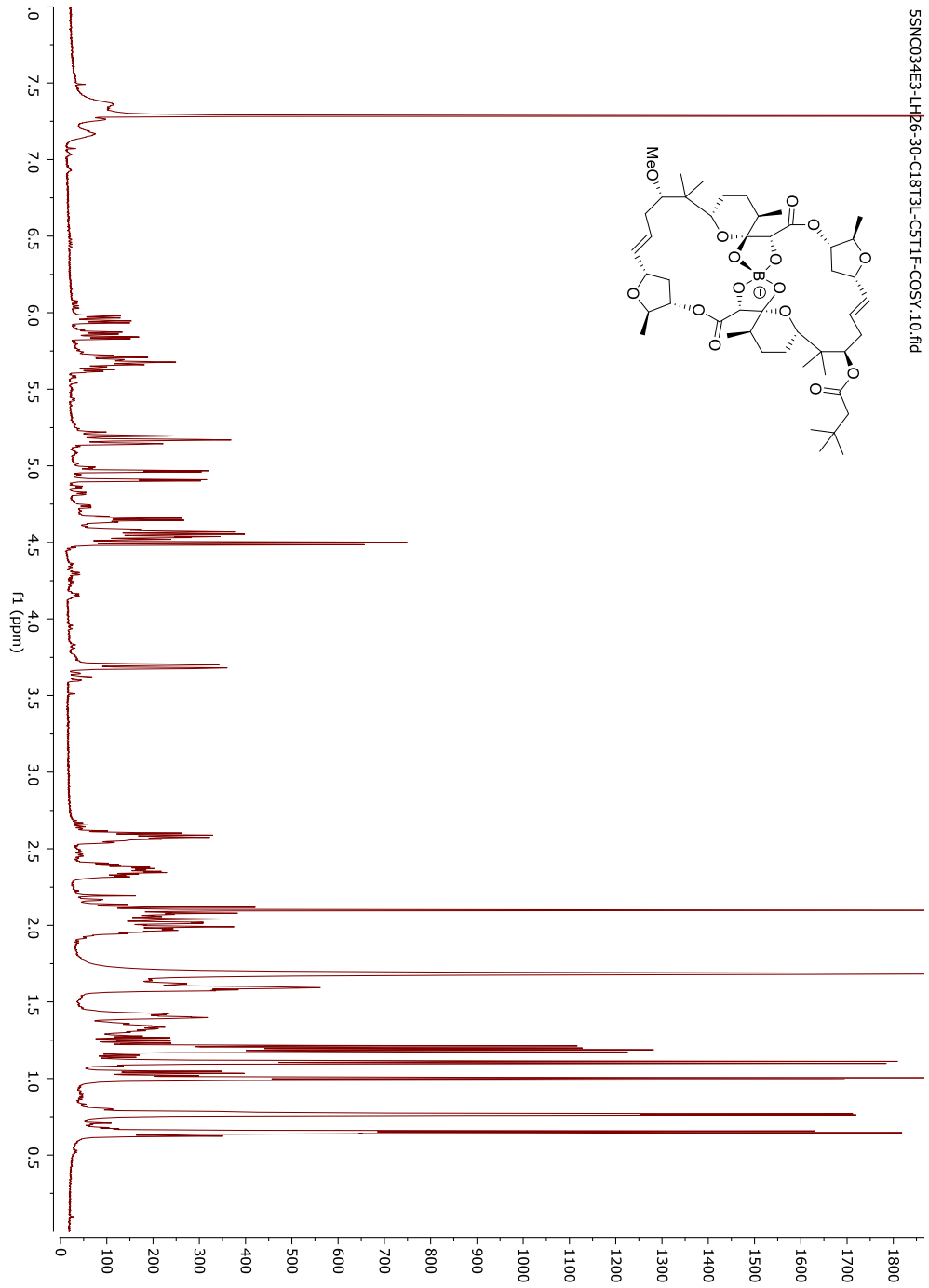

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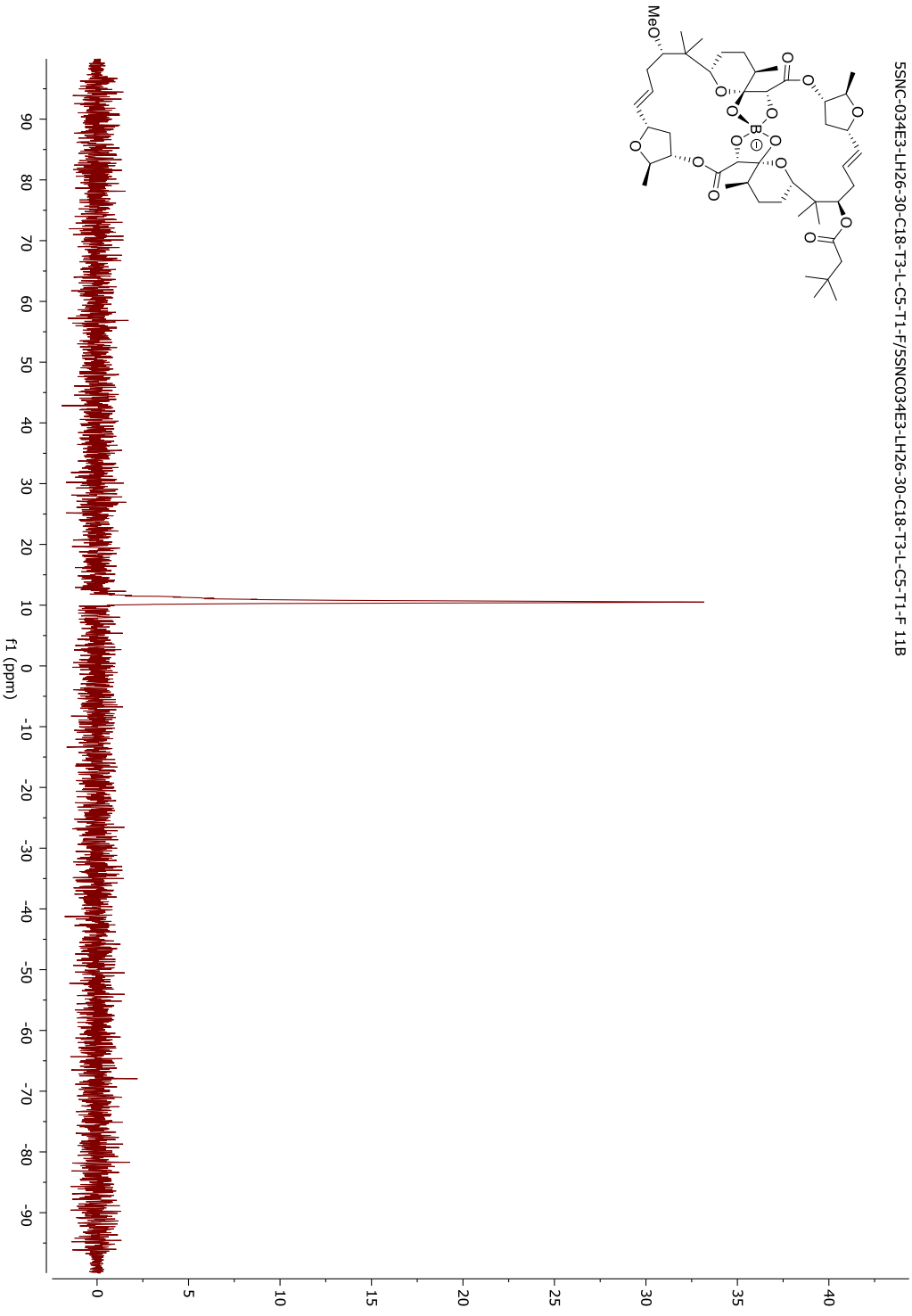
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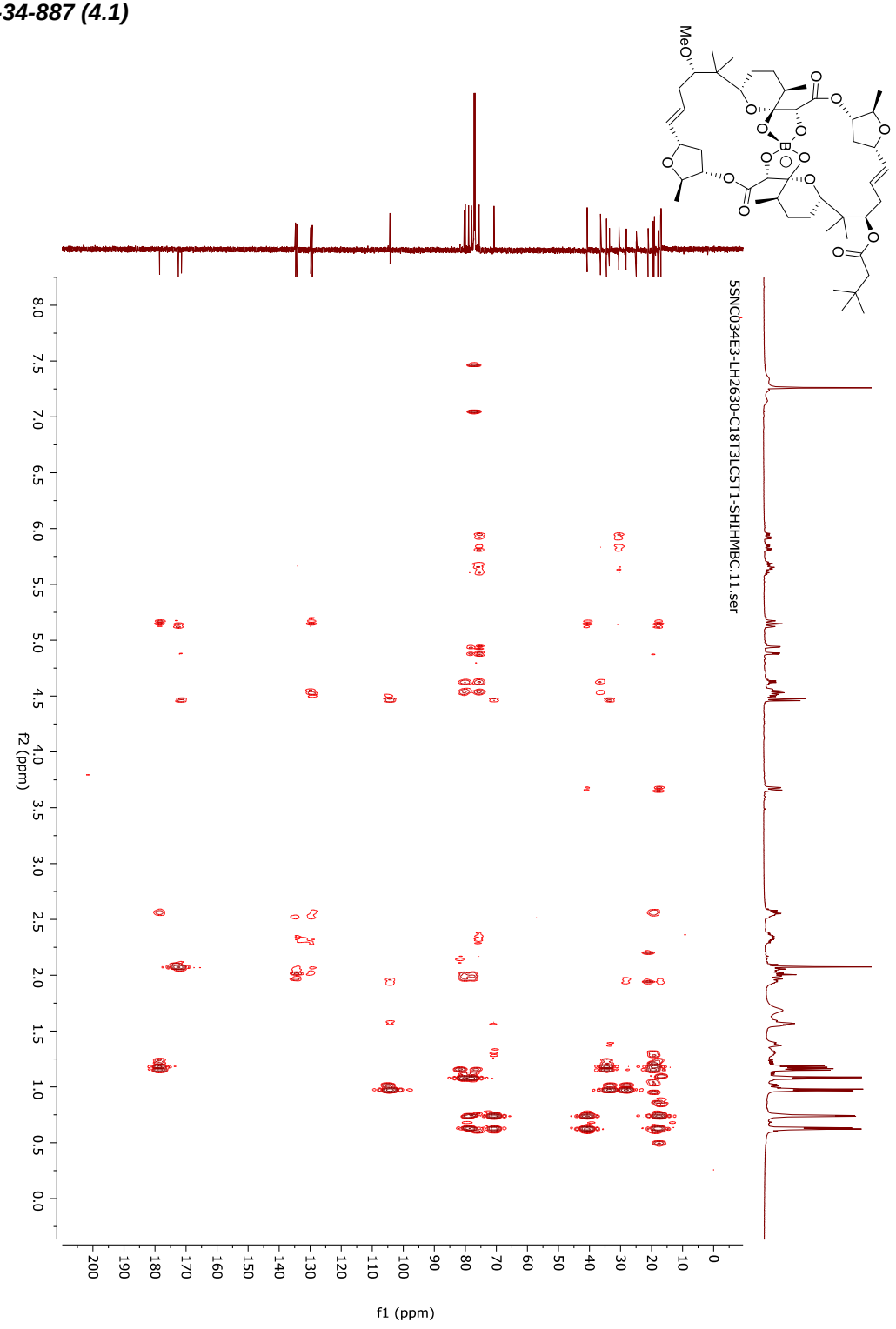
MeO
f1 (ppm)

NP-34-887 (4.1)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f1 (ppm)

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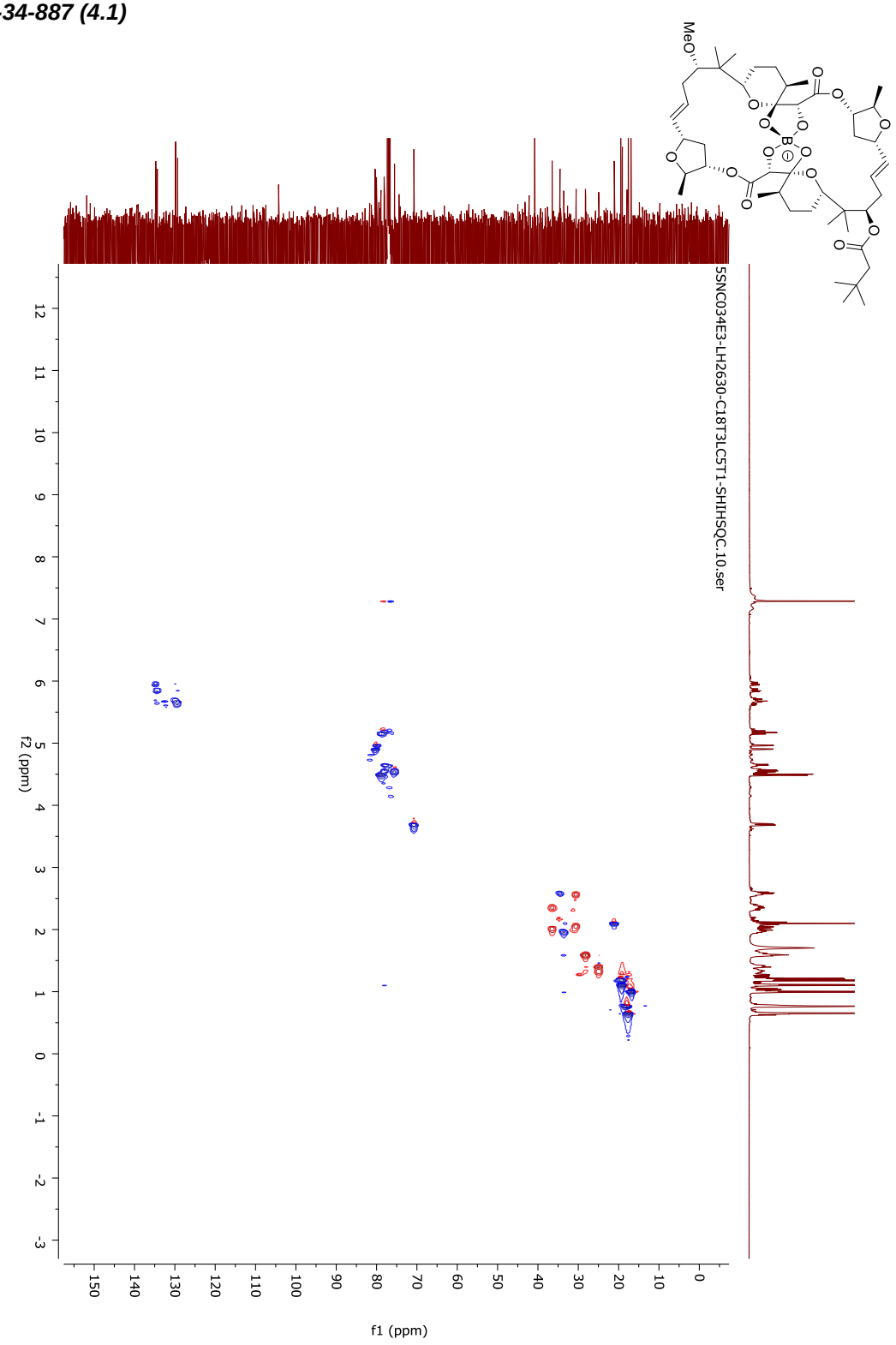
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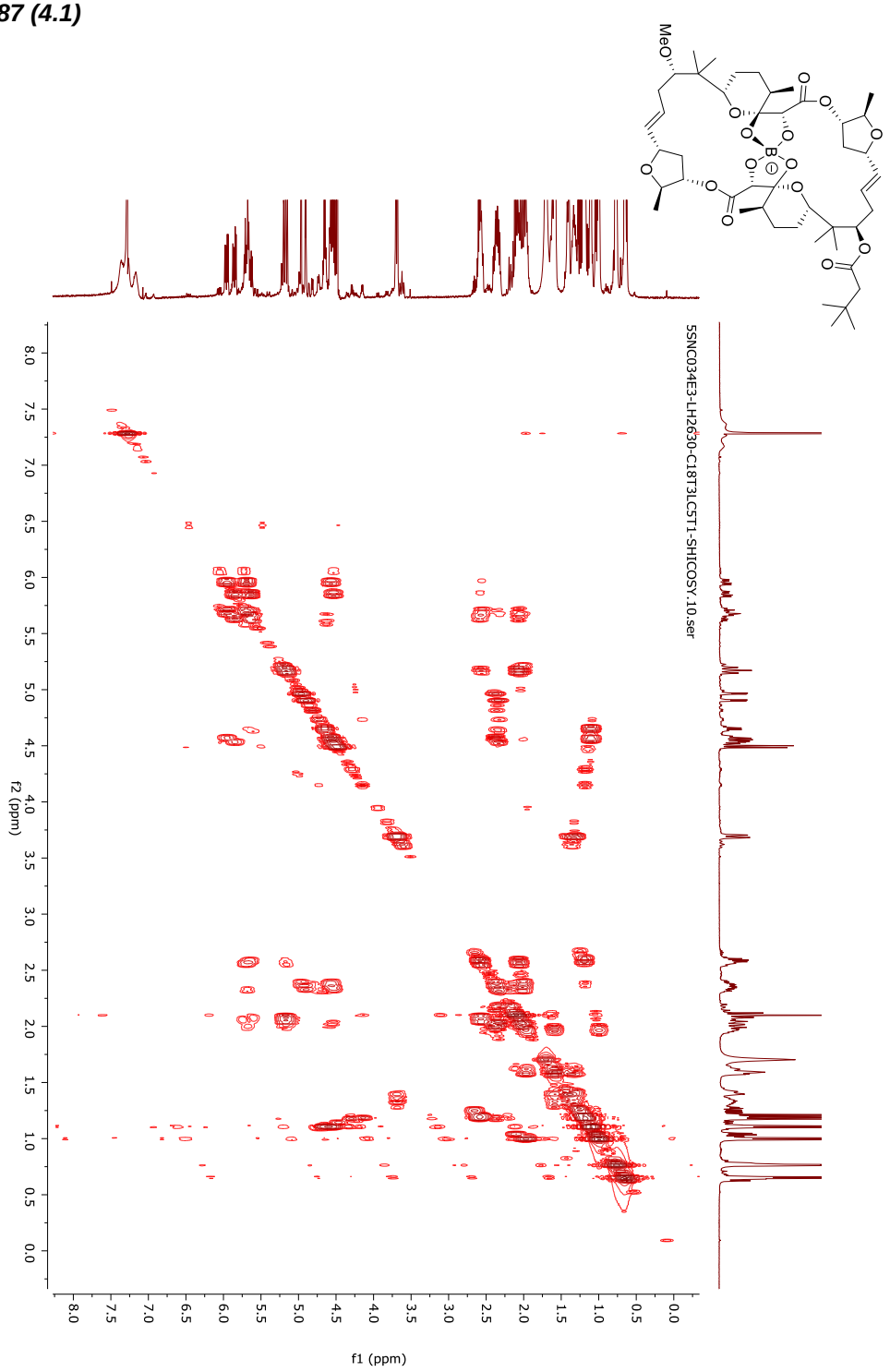
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f1 (ppm)

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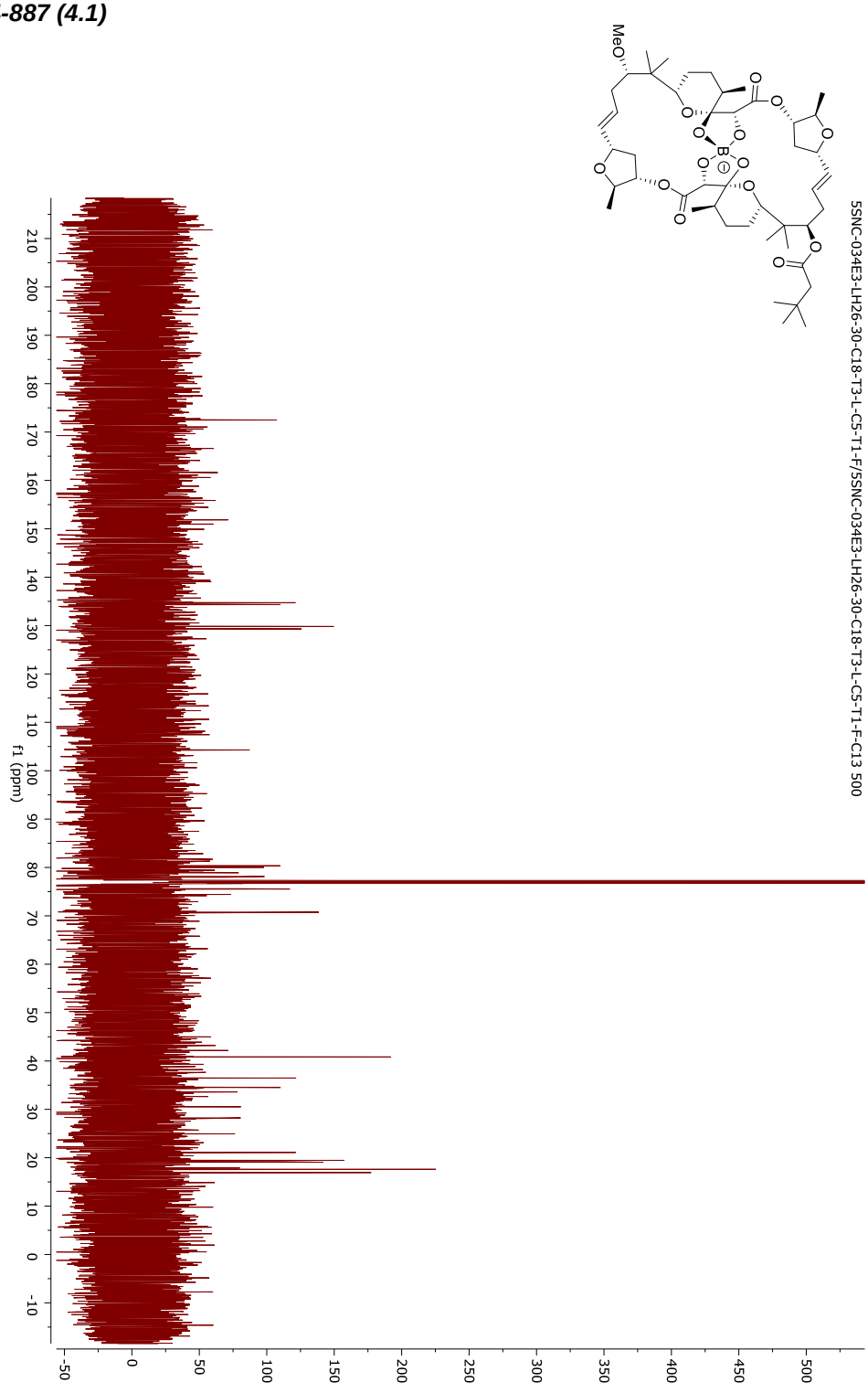
MeO
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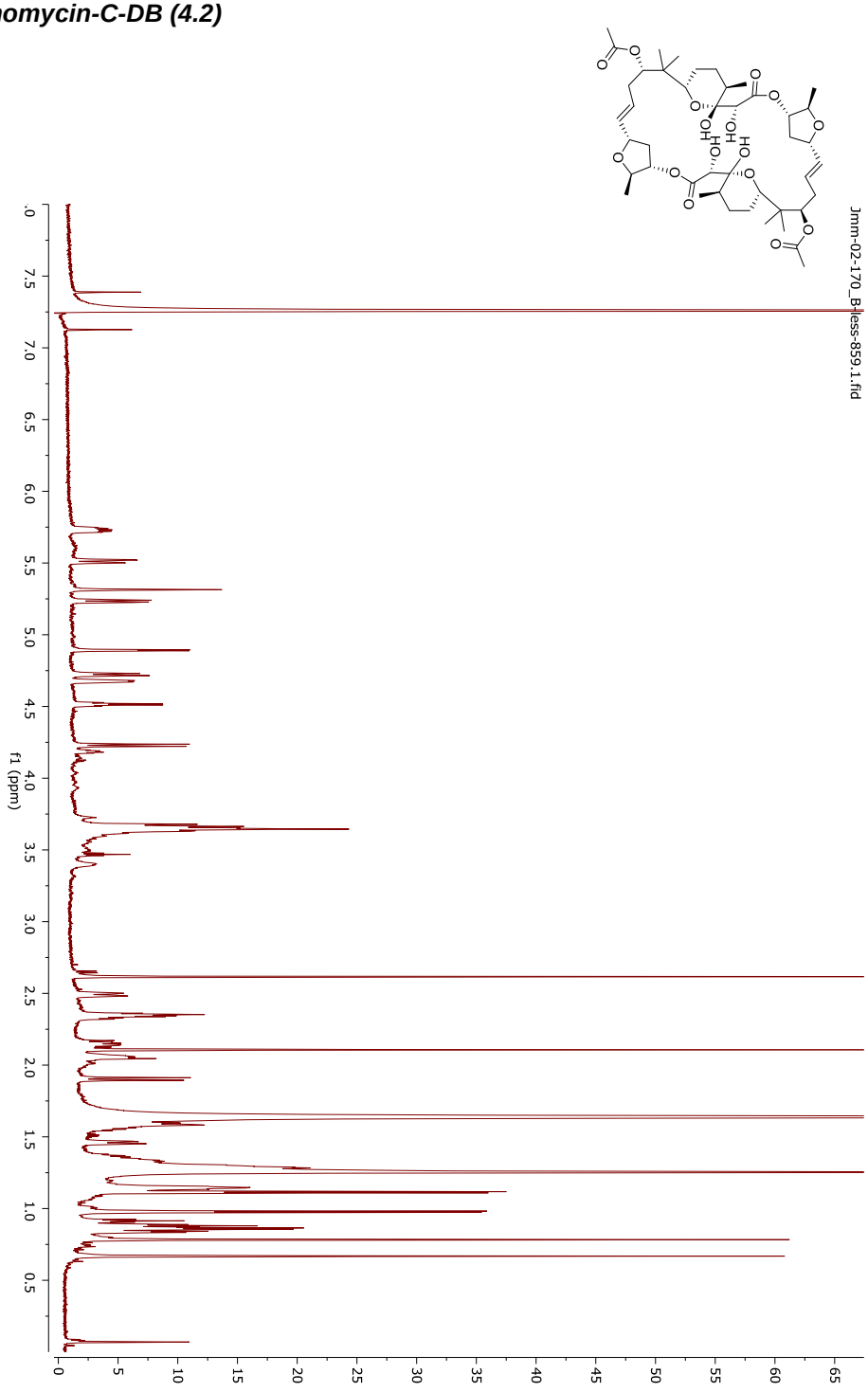
f1 (ppm)

Aplasmomycin-C-DB (4.2)



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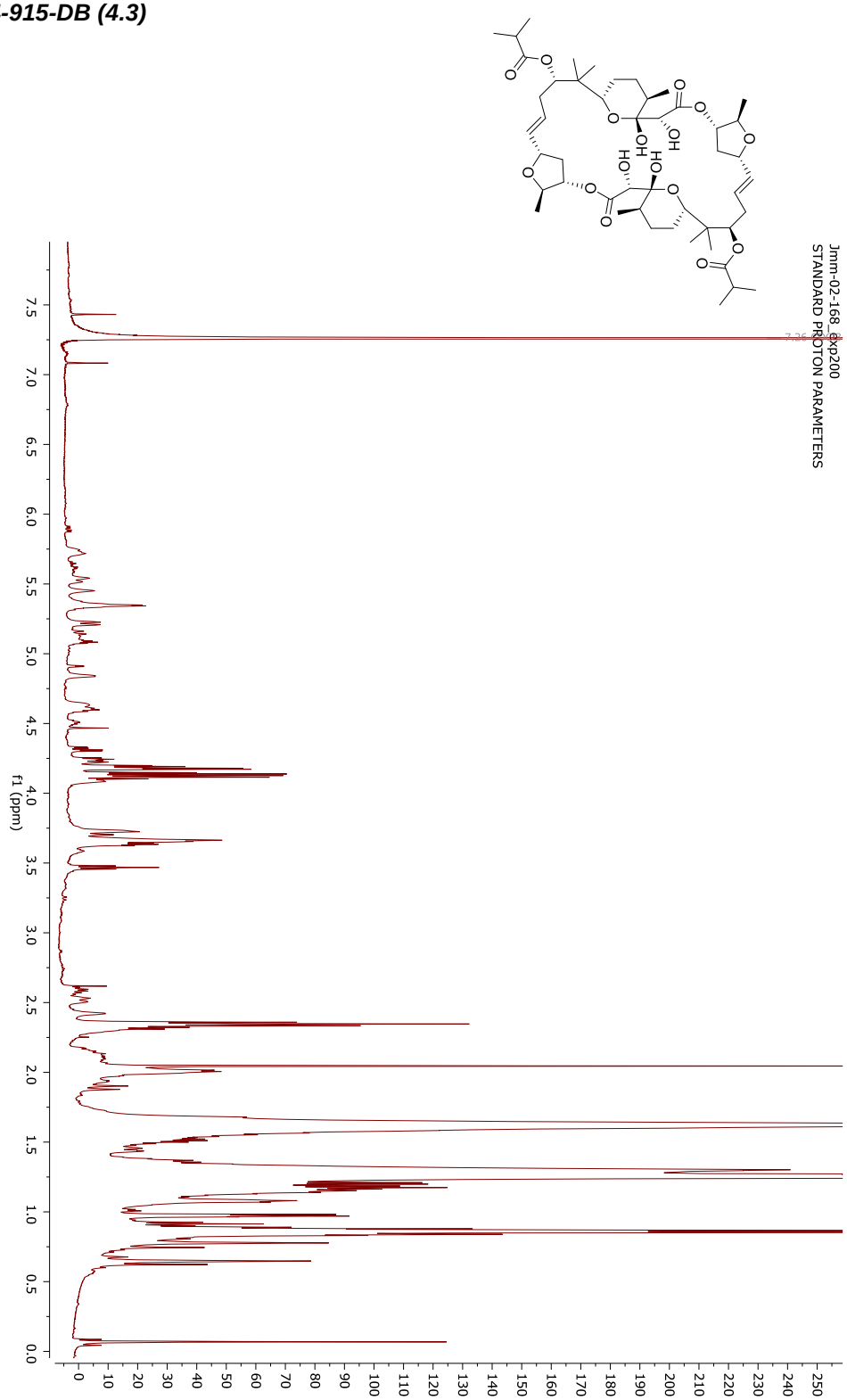

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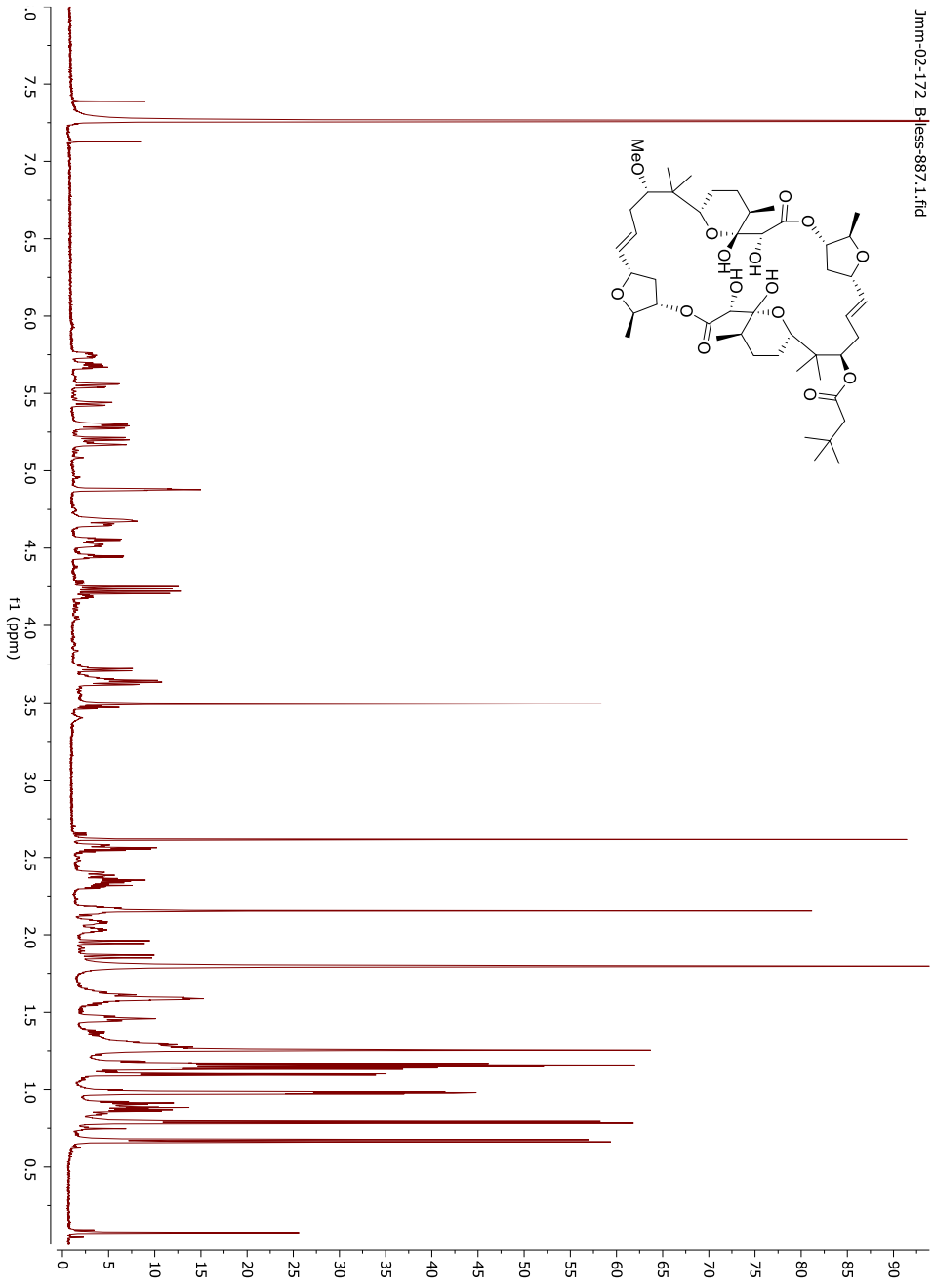

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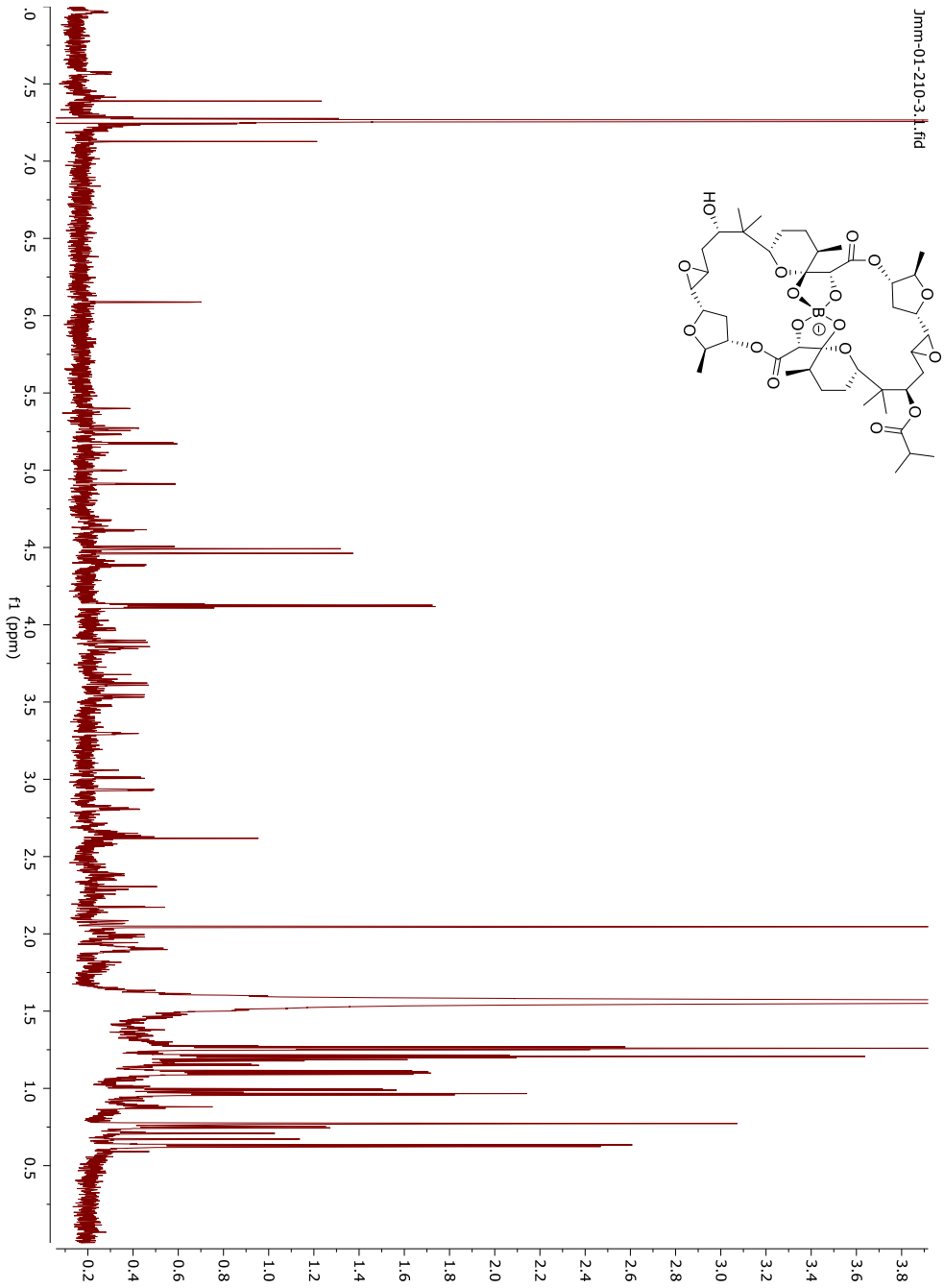

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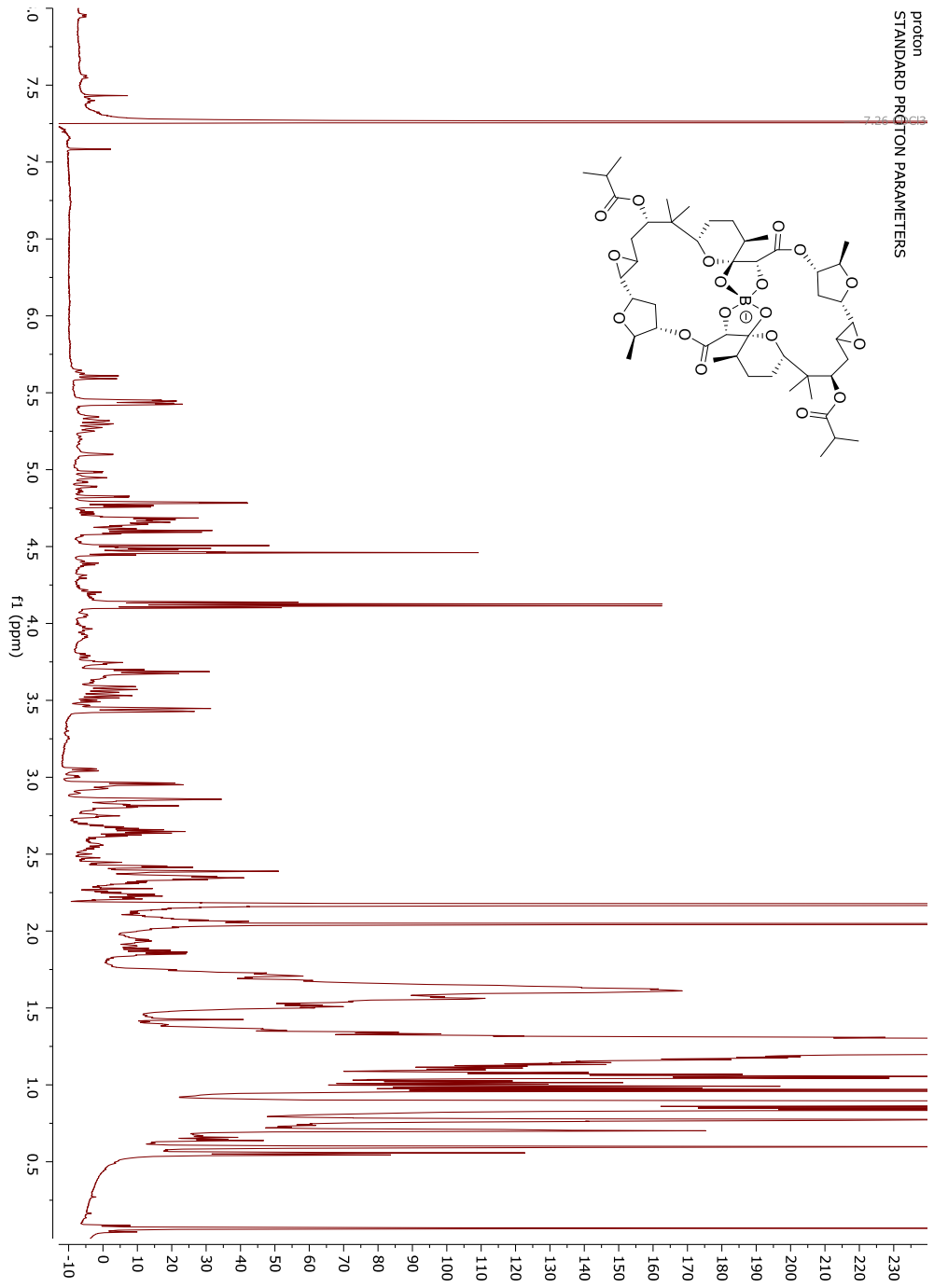

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