CHARACTERIZING THE MACROCYCLIZATION ACTIVITY OF FUNGAL POLYKETIDE SYNTHASE THIOESTERASES

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(…) Later when I started doing chemistry, I did it the way I fished – for the excitement, the discovery, the adventure, for going after the most elusive catch imaginable in uncharted seas.
--K. Barry Sharpless

Like all sciences, chemistry is marked by magic moments. For someone fortunate enough to live such a moment, it is an instant of intense emotion: an immense field of investigation suddenly opens up before you.
--Yves Chauvin

The one thing that matters is the effort.
--Antoine de Saint-Exupery

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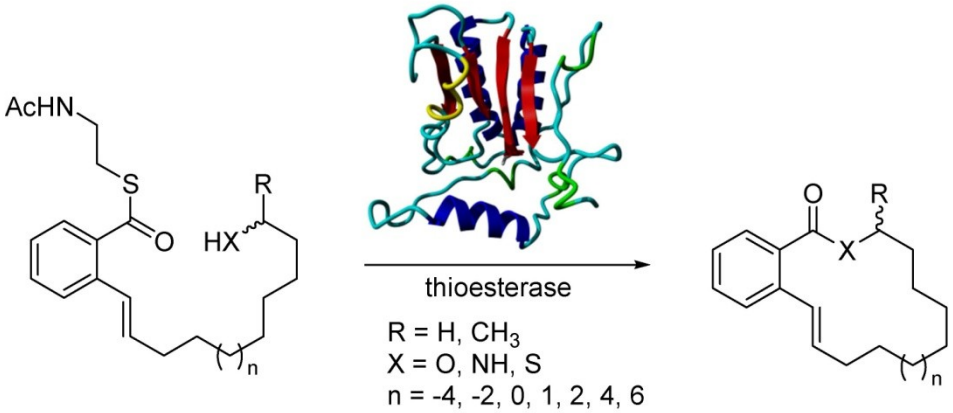
Abstract

Fungal polyketides are a diverse class of natural products that possess many pharmacological properties, including anticancer properties. These properties are evident in the resorcylic acid lactones, a family of polyketides, including zearalenone and radicicol, which shows potent inhibition of tumour cell growth. The key step in the biosynthesis of these lactones is macrocyclization of a linear carboxylic acid into the macrolactone. This reaction is catalyzed by a polyketide synthase (PKS) thioesterase enzyme. Bacterial PKS thioesterases (TEs) have been extensively studied and their substrate specificity has been characterized in vitro. They are highly substrate selective for the macrocyclization reaction. Since Fungal PKS TEs show little sequence homology to bacterial TEs, we have begun investigating their substrate specificity. In particular we are examining the ability of fungal TEs to macrocyclize compounds with varying ring sizes, stereogenic configuration, and nucleophiles. Herein we present the synthesis of a number of diverse TE substrates and the *in vitro* macrocyclization results for the TEs from zearalenone and radicicol biosynthetic pathway with these substrates.



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ACP	Acyl carrier protein
Asp	Aspartic acid
AT	Acyltransferase
ATP	Adenosine triphophate
bm	Broad multiplet
Boc	Tert-butyl carbonate
Boc_2O	Di-tert-butyl dicarbonate
br	Broad
bs	Broad singlet
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{o}C	Degree Celsius
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
COSY	Correlation Spectrroscopy or homonuclear Correlation
d	Doublet
Da	Dalton(s)
dd	Doublet of doublet
6-dEB	6-deoxyerythronolide B
DEBS	6-deoxyerythronlide B synthase
DH	Dehydratase
DIAD	Diisopropyl azodicarboxylate
DMAP	4-N, N-dimethylaminopyridine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dt	Doublet of triplet
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide
Epo	Epothilone
Equiv	Equivalent(s)
ER	Enoylreductase
ESI-MS	Electrospray ionization mass spectrometry

EtOAc	Ethyl Acetate
FAS	Fatty acid synthase
g	Gram(s)
GrsB	Gramicidin S
H	Hydrogen
HCl	Hydrochloric acid
His	Histidine
HMBC	Heteronuclear Multiple Bond Correlation
HMQC (HSQC)	Heteronuclear Multiple Quantum Coherence
^{1}H NMR	Proton Nuclear Magnetic Resonance
H_2O	Water
HPLC	High Pressure Liquid Chromatography
HR	Highly reducing
HRMS	High Resolution Mass Spectrometry
HSP	Heat shock protein
HSV	Herpes simplex virus
Hz	Hertz
IR	Infrared
J	Coupling Constant
KAS	β-ketoacyl synthase
KR	β-Ketoreductase
KS	β-Ketoacyl synthase
LC	Liquid chromatography
LC/MS/MS	Liquid chromatography coupled to tandem mass spectrometry
M	Molar
m	Multiplet
MAP	Mitogen-activated protein
MeCN	Acetonitrile
MEK	Mitogen-activated protein kinase kinase
mg	Milligram(s)
$MgSO_4$	Magnesium sulfate

MHz	Megahertz
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar(s)
mmol	Millimole(s)
mol	Mole(s)
MS	Mass Spectrometry
MSAS	6-methyl salicylic acid synthase
MS/MS	Tandem mass spectrometry
MT	Methyltransferase
MW	Molecular Weight
N	Normal
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
nm	Nanometer(s)
NMR	Nuclear Magnetic Resonance
NR	Nonreducing
NRPS	Non-ribosomal peptide synthase
Pik	Pikromycin
Pim	Pimaricin
PKS	Polyketide synthase
ppm	Parts per million
PR	Partially reducing
PT	Product templating
PTLC	Preparative thin Layer Chromatography
q	Quartet
Rad	Radicicol

RAL	Resorcylic acid lactone
R_f	Retention factor
rt	Room Temperature (~22 ^oC)
s	Singlet
SAM	S-adenosyl methionine
SAT	Starter unit acyltransferase
Ser	Serine
SM	Starting material
SNAC	N-acetylcyteamine
t	Triplet
TBAF	Tetrabutyl ammonium fluoride
TCEP	Tris(2-carboxyethyl)phosphine
TE	Thioesterase
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
Tyc	Tyrocidine
µL	Microliter(s)
µM	Micromolar
UV	Ultraviolet
v/v	Volume per volume
Zea	Zearalenone

Chapter 1 Introduction

Drugs have been shown to improve the quality of life for billions worldwide by treating a variety of diseases and disorders. As such, tremendous efforts have been placed into drug discovery in many fields, including medicine, biology, and chemistry.[1, 2] Over the years, there has been an increasing demand for rapid and novel drug development. Drug costs, from discovery to market, exceed 400 million dollars and takes over a decade.[3] While the interest and investments placed into drug research and development has increased, the number of newly approved drugs has fallen. This has amplified the necessity to discover active compounds as leads in drug development.

Natural products comprise over 60% of drugs on the market today, an amount even greater in the antiinfective and anticancer fields. They have been isolated from microorganisms, plants and animals to be used as hormones, toxins, flavours, fragrances, pigments, drugs and other materials of commercial value.[4] Their prevalence is attributed to their privileged role in nature, as their size, stereochemistry and composition have been selected by evolution to bind to biologically relevant targets.[5, 6] Just as importantly, natural products also display increased stability, solubility and cell permeability.

Even when natural products have all the essential criteria to be developed as drugs, they are often used as lead compounds for the discovery of more potent analogues. These natural product derived molecules also perturb biological systems and are thus an appealing approach to investigate the biological mechanism of different molecules.

However, there are still major scientific questions pertaining to why some natural products are synthesized in the first place. Especially in light of the observation that many do not act, for example, as antibiotics or anticancer agents in their native environments. Furthermore, many natural products and their analogues,

even those undergoing clinical evaluation or approved as drugs, still have cellular activities and mode of action that remain a mystery.[7] This applies to many drugs, such as antidepressants, including citalopram, fluoxetine, amitriptiline and sertraline, the anti Parkinson's disease drug pramipexol, thalidomide, the analeptic drug modafinil and analgesics, including lamotrigene and even morphine; whose precise mechanism of action are still unknown and remain mainly speculative.[8-13] This lack of knowledge on cellular targets inhibits the rational design and development of more potent compounds.

The goal of rational drug design is to discover and optimize novel drugs by the chemical and structural information of a specific protein target.[14] This will provide new insights into the function and mechanism of enzymes and proteins involved in the biosynthesis of different natural products. Subsequently, it will allow the simplification of the design and development of drugs. In addition, the timeline, costs of drug development and the role serendipity plays in discovering novel drugs will decrease.

Most biological and biochemical drug development research is based on rational drug design, as nature excels in the combinatorial biosynthesis of multifunctional organic molecules. Some of these are polyketides, which arise from multiple two-carbon condensation reactions to yield structurally diverse natural products.[15] Numerous decades were spent on the discovery and analysis of the enzymes that catalyze the biosynthesis of those polyketides to a specific compound, and work still continues on unravelling details of polyketide biosynthesis.

1.1 Polyketides

Polyketides are a significant group of natural products found in a variety of plants, bacteria, fungi and marine organisms.[16, 17] They encompass a high degree of functional and structural diversity, possess many stereocentres and typically have low molecular weight (200-700 g/mol).[18] Their specific functions in nature are numerous,

with polyketides acting as infochemicals, virulence factors, pigments, and for defense.[19] Drugs derived from polyketides are in the top 20% of top-selling drugs, including the cholesterol-lowering blockbuster drug lovastatin.[18] Furthermore, while the usual hit rate average from a typical high-throughput screen is expected to be around 0.001%, polyketides have a 0.3% success rate for making it to the market as commercial drugs, which is obviously an enormous improvement. Polyketides have been shown to possess a wide array of pharmaceutically relevant activity, including antibiotic, antifungal, antiparasitic, insecticidal, anticancer, immunosuppressant, and cholesterol-lowering activity (Figure 1.1).[16, 18, 20-24]

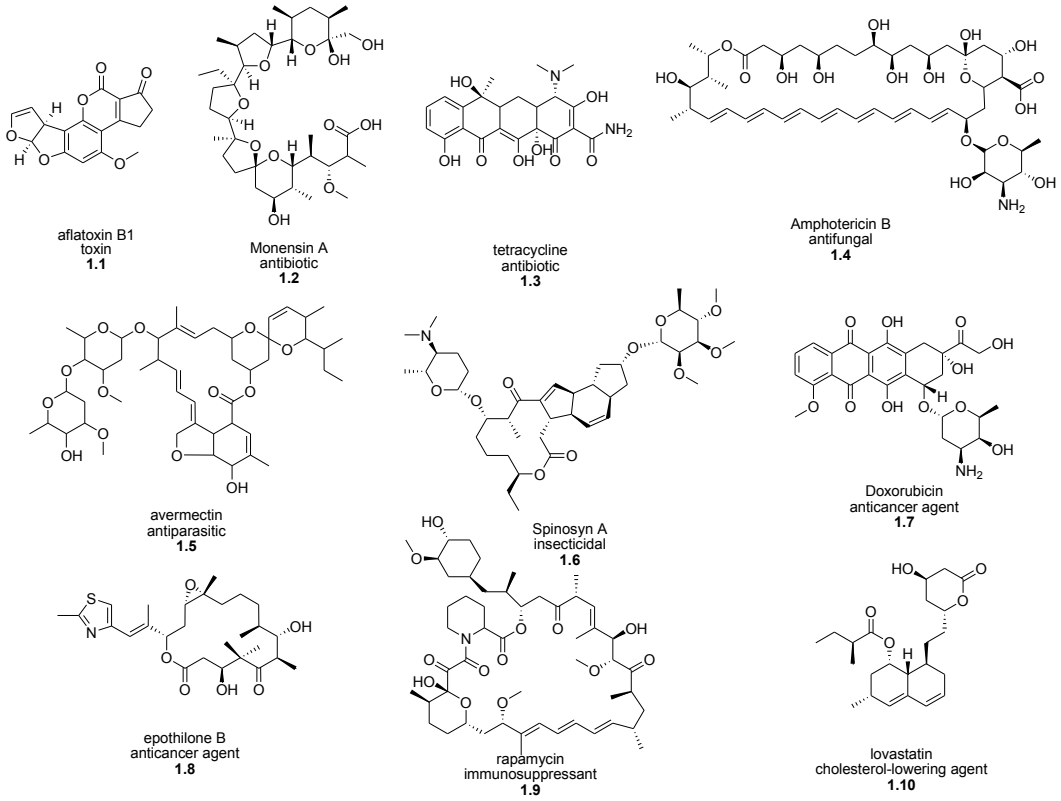


Figure 1.1 A select array of polyketides that displays a vast variety of biological activities.

1.1.1 Biosynthetic steps and reactions of polyketide synthases

Polyketides are biosynthesized in an analogous way to fatty acid biosynthesis. However, polyketides vary from fatty acids as they display much greater variations in structure. Despite the fact that polyketides yield a wide variety of structures, many contain a common structural feature, a macrolactone, which is thought to be crucial for their bioactivity.[25, 26]

Polyketides are all biosynthesized from small carboxylic acids, such as acetate, propionate and butyrate.[27] These carboxylic acids are assembled by polyketide synthases (PKSs), found in the genomes of polyketide producing organisms. PKSs use diverse thioester activated acyl groups during the biosynthesis.[16, 28, 29] The starting unit can vary from acetyl-, propionyl, benzoyl-, isobutyryl-, and phenylacetyl-CoAs bound to a thioester, while the chain elongation step comprises mostly of added malonyl- or methylmalonyl-CoA units.[15, 30, 31] These CoA activated substrates are added via a catalytic cycle that utilises a decarboxylative Claisen condensation as its key step; followed by other reactions such as a ketoreduction, dehydration and an enoyl reduction in both polyketide and fatty acid biosynthesis (Figure 1.2).[21, 30, 32-34]

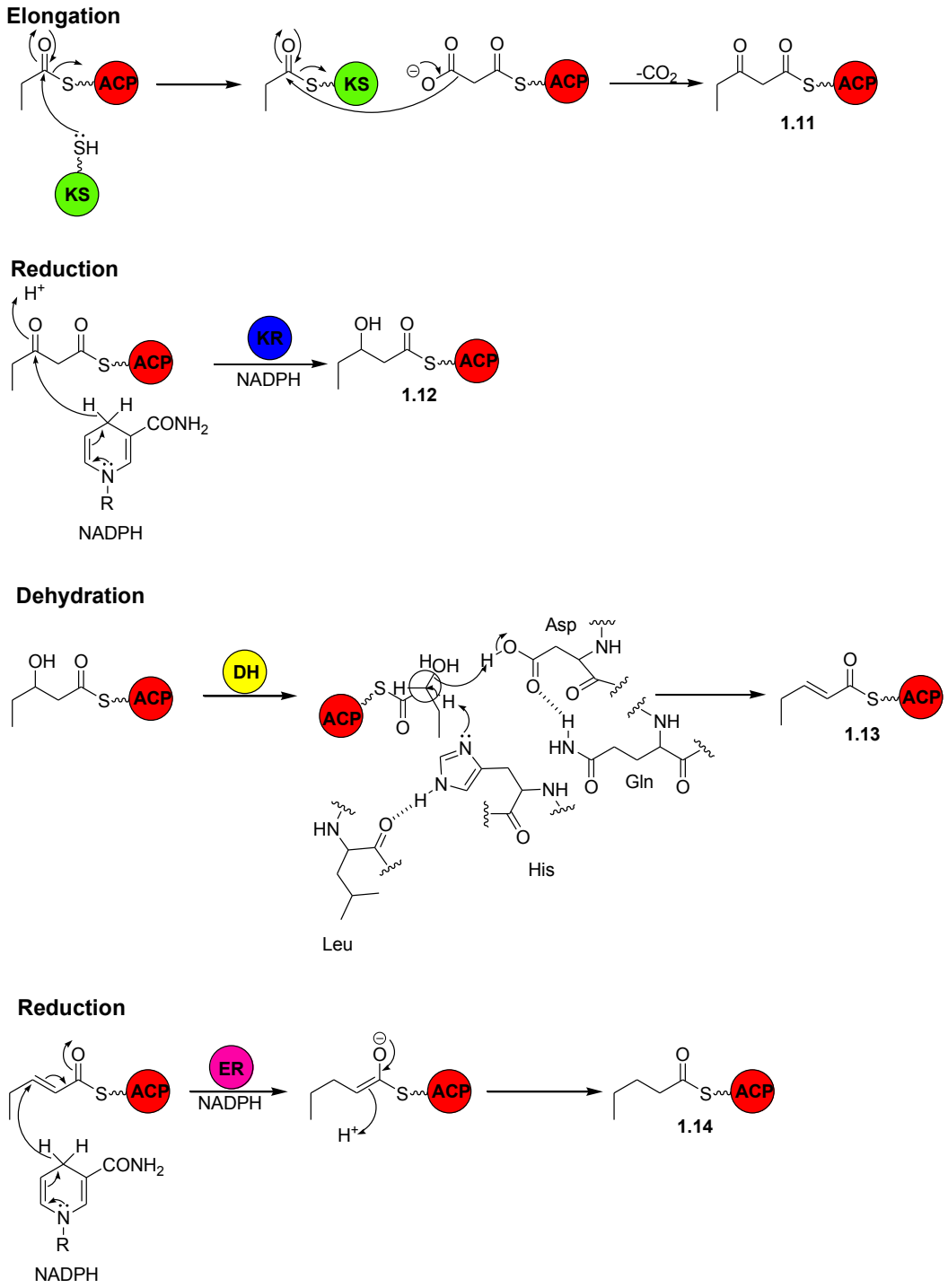


Figure 1.2 Reactions and mechanisms of the different enzymatic domains of a polyketide synthase. Note: not all intermediates and proton transfers are shown.

One cycle of chain elongation contains three core chemical reactions. First, there is recognition of the extender unit, which is followed by covalent binding to a carrier protein, and finally condensation with the acyl chain attached to the enzyme complex.[35] Finally, there is a reduction reaction, which converts the ketone moiety into an alcohol, olefin, or alkane. These reactions occur by reduction of the ketone to the alcohol. Following this, there is dehydration of the alcohol into an olefin and finally reduction of the olefin into an alkane. These elongation and reduction cycles occur until the substrate is released from the enzyme complex to give a specific chain length or macrocycle. The released product may then undergo additional modifications to yield molecules that vary in complexity. Furthermore, in polyketide synthesis, the reductive steps are highly stereochemically controlled and are not essential, as in fatty acid synthesis, as they can be completely or partially omitted, yielding a wide diversity of oxidation states compounds.[19]

This thiotemplate biosynthesis is common to the non-ribosomal peptide synthases (NRPSs), which generate a family of compounds closely resembling polyketides. NRPSs differ from PKSs by incorporating amino acids into the growing linear acyl chain length via amide bond formation rather than two carbon units via decarboxylative Claisen condensations.[29] For both polyketides and NRPSs, the final step is often macrocyclization to the active compound, which they can interact with a specific biological target.[18]

1.1.2 PKS classification

Polyketide synthases can be classified as three different types based on their mechanisms of biosynthesis. Type I PKS are typically large multifunctional polypeptides containing discrete functional catalytic domains.[18, 36] Type I can be further broken down as to whether the enzyme act in a modular or iterative function and are found in bacteria and fungi. Modular type I PKS have been extensively studied and typical examples of type I PKS products include 6-deoxyerythronolide B (DEBS) and pikromycin (Pik).[16]

Type II PKS typically produce aromatic compounds, are non-reducing during the biosynthesis, and each catalytic domain is found as a standalone protein that functions iteratively.[27] They are only found in bacteria. Typical examples of type II PKS products include tetracenomycin and actinorhodin.

Type III PKS do not correspond with any known fatty acid synthases and are found in plants, bacteria and fungi. They are very simple β-ketoacyl synthase (KAS) proteins which do not require an ACP domain or other typical functionalities found in type I and type II PKSs.[27] These cover chalcone and stillbene synthases and common examples of type III PKS products include resveratrol and naringenin chalcone.

1.2 Modular type I PKS

Modular type I PKS are large multifunctional enzymes responsible for the biosynthesis of polyketides, such as macrolides. These multifunctional enzymes possess many catalytically active domains grouped together into modules. Each module catalyzes the addition of one "ketide" unit, a two carbon building block before passing the growing polyketide chain downstream to the subsequent module.[37] Typically, a modular PKS pathway consists of multiple proteins each containing two or three modules, although single proteins with up to nine modules are known.[18] The number of modules generally correlates with the number of "ketide" units present in the final polyketide product. Along with the mandatory domains that all modular polyketide possess in each module, there are three optional reductive domains that can be present. As a result the length, functionalities and stereochemistries of the polyketide product can be predicted by studying the modules in a PKS pathway and is used for rational design and programming of complex polyketides.[19, 30, 35, 38] However, not all modular type I PKS correlate to this, and the final structure sometimes does not resemble what is predicted by the PKS genes, as some modules are used more than once or can be skipped completely.

Bacterial PKSs, especially from strains of the order actinomycetales have been extensively studied. Each enzyme is used for a specific role, such as β-ketoacyl synthase (KS) for the decarboxylative condensation of the extender unit; acyltransferase (AT) for the selection of the extender unit and its transfer; and an acyl carrier protein (ACP) for the attachment of the extender unit. Other enzymes used in the biosynthesis include β-ketoreductase (KR); dehydratase (DH); enoyl reductase (ER); and thioesterase (TE).[16, 35] These enzymes catalyze many steps, including the initiation, elongation, reduction and ultimately, release of the compound. Once the compound is released, tailoring enzymes are used to add further regiospecific and stereospecific functionalities to the molecules.

The thioesterase functionality is important for the release of the compound from the enzyme. These compounds are frequently released by either hydrolysis; attack from another nucleophile, or more commonly, by macrocycle formation.[38] Macrocyclization is crucial for bioactivity and is crucial in many pharmaceutical compounds, including erythromycin, epothilone and daptomycin.[24] Macrocyclization is catalyzed by a TE domain, where 12-, 14- and 16-member lactones such as 10-deoxymethynolide, 6-deoxyerythronolide B and tylactone are common (Figure 1.3).[29] Macrocyclization is generated by way of a two step mechanism. The first step is rate limiting, in in vitro assays, and constitutes the addition of the linear polyketide chain to the active site serine leading to an acyl-enzyme intermediate. The subsequent step is intramolecular nucleophilic attack that leads to the macrocyclic product.[20, 25] Hydrolysis occurs when there are no intramolecular nucleophiles in the binding pocket of the enzyme.

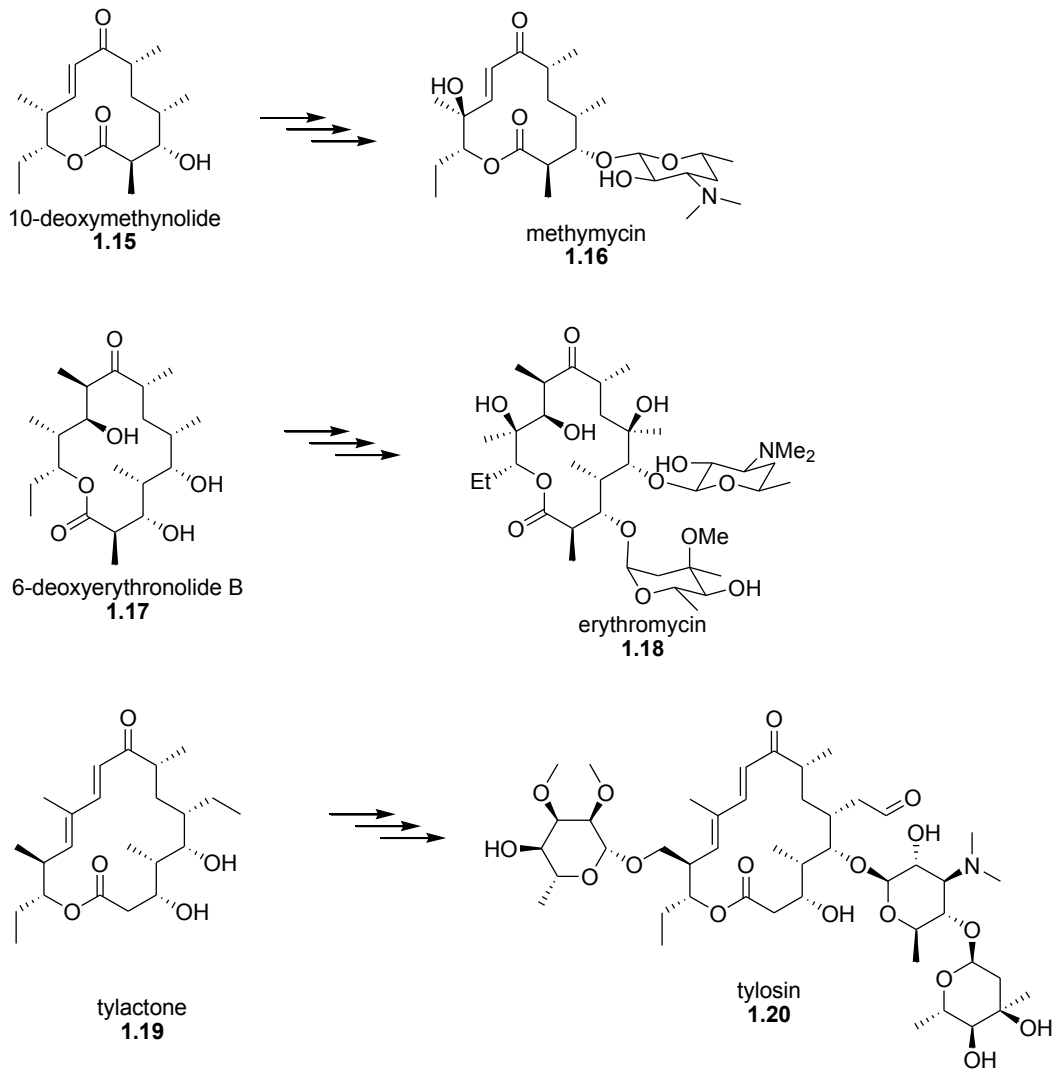


Figure 1.3 Different type I polyketides varying in ring sizes from the thioesterase release product to the final biologically active compound.

Erythromycin is the modular polyketide that has garnered the most attention since its discovery. Understanding of its biosynthesis pathway, via in vitro characterization, has paved the way for understanding other PKSs over the past two decades.[21] Erythromycin is biosynthesized by 6-deoxyerythronolide synthase (DEBS) which is comprised of 6 different modules on three polypetides (DEBS 1, 2 and 3) (Figure 1.4).[15, 36, 39, 40] DEBS 1 starts with a loading didomain containing an AT and ACP, which load the starter unit propionate onto the ACP from propionyl-CoA. After the loading domain, DEBS 1 contains two modules, module 1 and 2.

These both possess the minimal required KS, AT and ACP, which carry out a Claisen condensation to form a new carbon-carbon bond as a β-keto ester intermediate. In addition module 1 and 2 each contain a KR domain that reduces the ketone to the hydroxyl functionality found in the natural product. The growing polyketide chain is then passed on the second protein, DEBS 2, which includes module 3 and 4. Module 3 contains an inactive KR group, and so the ketone remains intact throughout the biosynthesis. Module 4 contains all three DH, ER and KR enzymes and the ketone is fully reduced to a methylene. The chain is then transferred onto DEBS 3, which contains modules 5 and 6, as well as the release domain. Module 5 and 6 only contains a KR group and thus the ketone is only reduced up to a hydroxyl group. DEBS 3 also contains a TE which undergoes macrocyclization to produce 6-dEB. Further tailoring enzymes add the missing functionalities to yield erythromycin.

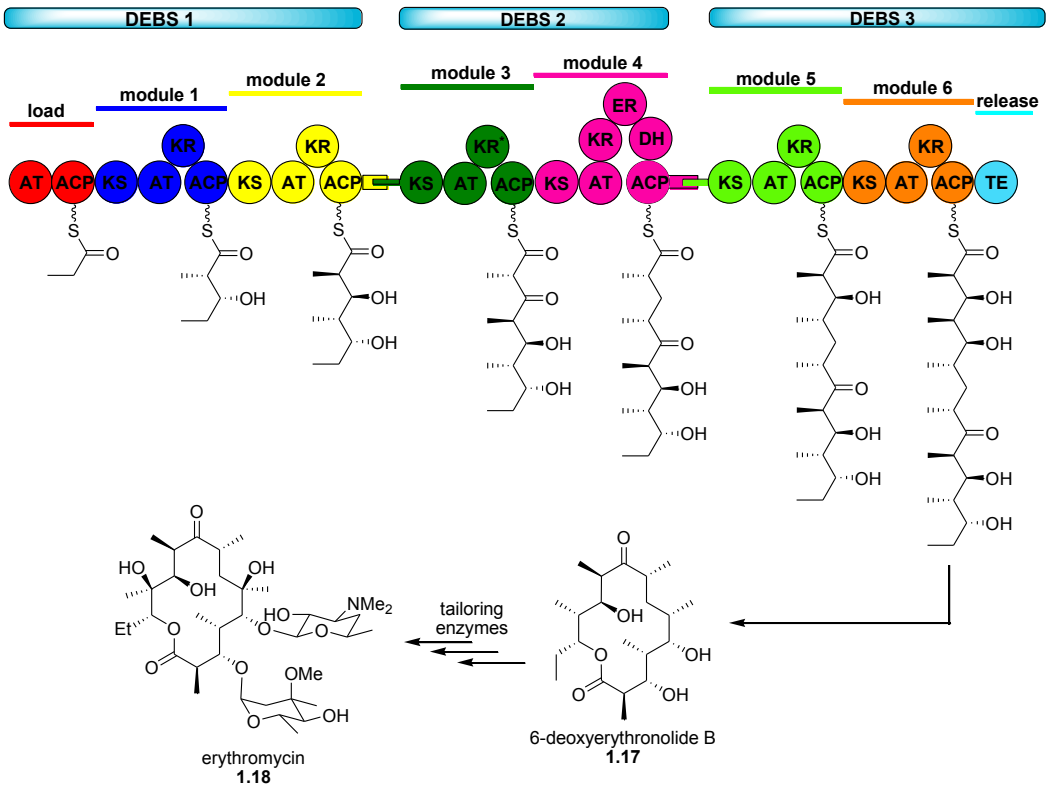


Figure 1.4 Biosynthesis of erythromycin, a modular type I bacterial polyketide

The other modular PKS that has been extensively studied is the pikromycin biosynthetic pathway. This is a unique pathway as it naturally forms two compounds of varying chain length; the 12-membered methymycin and the 14-membered pikromycin (Figure 1.5).[41, 42] The inherent substrate specificity suggested from this observation is very appealing, as poor substrate specificity might make it easier to genetically engineer analogues of varying lengths and stereochemistries.

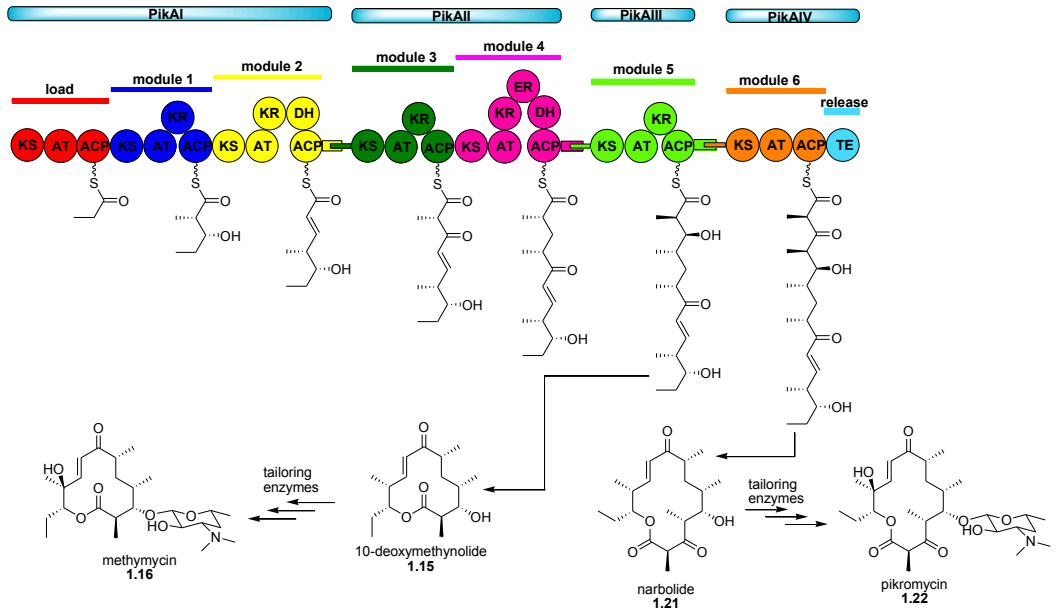


Figure 1.5 Biosynthesis of pikromycin, a modular type I bacterial polyketide

Both 6-dEB and Pik are generated similarly, with comparable enzymes, modules and domains. As with DEBS, Pik contains 6 modules; however it is organised into 4 domains instead of 3. Analogous to DEBS, the KS, AT and ACP units are key for the generation and elongation of the polyketide chain. Modules 1 and 5 contain a KR enzyme that reduces the resulting ketone to the hydroxyl group. Module 2 contains a KR and a DH moiety, which reduces the ketone to the hydroxyl groups, but then undergoes dehydroxylation to form the double bond. Module 3 contains an inactive KR, which leaves the ketone unreduced on the growing polyketide chain. Module 4 contains a KR and DH, but also an additional ER which

fully reduces the ketone to a methylene. Module 6 contains a TE that undergoes macrolactonization to give narbolide. Narbolide is further functionalised by tailoring enzymes to produce pikromycin. However, it is possible that the sixth module is skipped completely and that the TE macrocyclizes the polyketide formed at the end of the fifth module to give 10-deoxymethynolide which is further functionalised to form methymycin.

1.3 Type II PKS

Type II PKS systems consist of mono- or bi-functional multienzymes that generate unreduced aromatic compounds.[16, 19] They are commonly found in prokaryotes, especially actinomycetales that produce aromatic compounds like doxorubicin, tetracenomycin and actinorhodin. Elongation of the growing polyketide occurs in an iterative fashion. It is unclear whether the growing acyl chain stays attached to the same carrier protein, or if it gets transferred. A typical type II PKS is thought to consist of two KS and an ACP unit, with acyl monomers on distinct subunits.[29] Additional catalytic domains, such as KR, cyclases and aromatases control the level of reduction and regiochemistry of aromatisation of the polyketide intermediate. The archetypical type II PKS can be found in the actinorhodin biosynthetic pathway (Figure 1.6).[15, 28] The entire chain is synthesized using one distinct enzyme complex, often called the minimal PKS. Each additions and reactions are repeated in an iterative manner until the backbone reaches the required length. The chain then undergoes a controlled regiospecific aromatisation, followed by a cyclization to give compound **1.27**. Once cyclized, other modifications and cyclizations are performed, to give the final natural product.

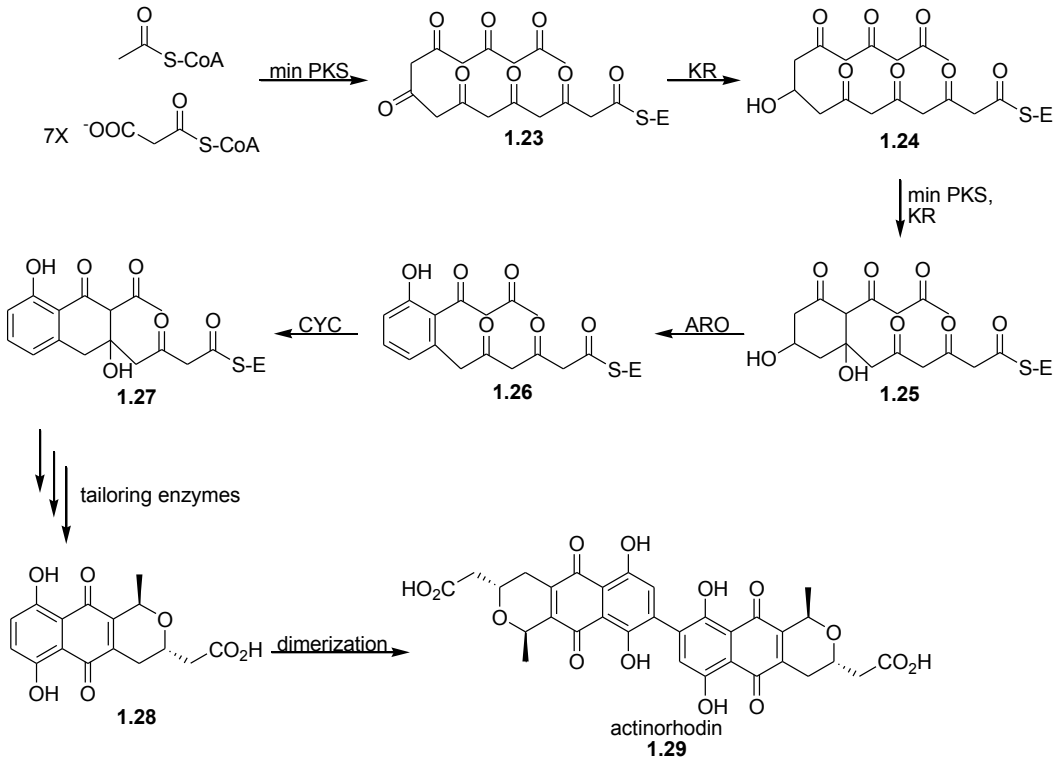


Figure 1.6 Biosynthesis of actinorhodin, an archetypical bacterial type II PKS.

1.4 Type III PKS

Type III PKSs, such as chalcone and stillbene synthases, are a class closely related to known fatty acid synthases. They are simple β-ketoacyl synthase (KAS) proteins that lack typical PKS functionalities, such as AT, ACP, KR, DH, ER or TE domains and are found in plants and bacteria.[27, 43] Chalcone synthases are still considered PKSs, since they catalyze the linking of acyl CoA subunits by repetitive decarboxylative condensation, which is the definition of polyketide synthesis. These condensation reactions are used to build molecules that are similar to those in the biosynthesis of fatty acids and polyketides, however type III PKS use CoA bound intermediates directly rather than the ACP bound intermediates to deliver the nucleophile for the subsequent condensing reactions. Some plant chalcone compounds require a specific NADPH-dependent reductase, named polyketide reductase, that acts together with the chalcone synthase to reduce a specific carbonyl

group after the second or third condensation reaction, but prior to the aromatic ring cyclization.[44] In chalcone and stillbene syntheses, a phenylpropanoid CoA-ester substrate interacts with their synthase, a small homodimeric protein of 40-45 Da that acts in an iterative function to undergo three specific condensation reactions with malonyl-CoA to form a tetraketide intermediate. This intermediate then cyclizes into an aromatic ring to give in the case of the chalcone synthase, a naringenin chalcone via a Claisen condensation, and in the case of stillbene synthase, resveratrol via an aldol condensation (Figure 1.7).[23, 26, 43, 45, 46]

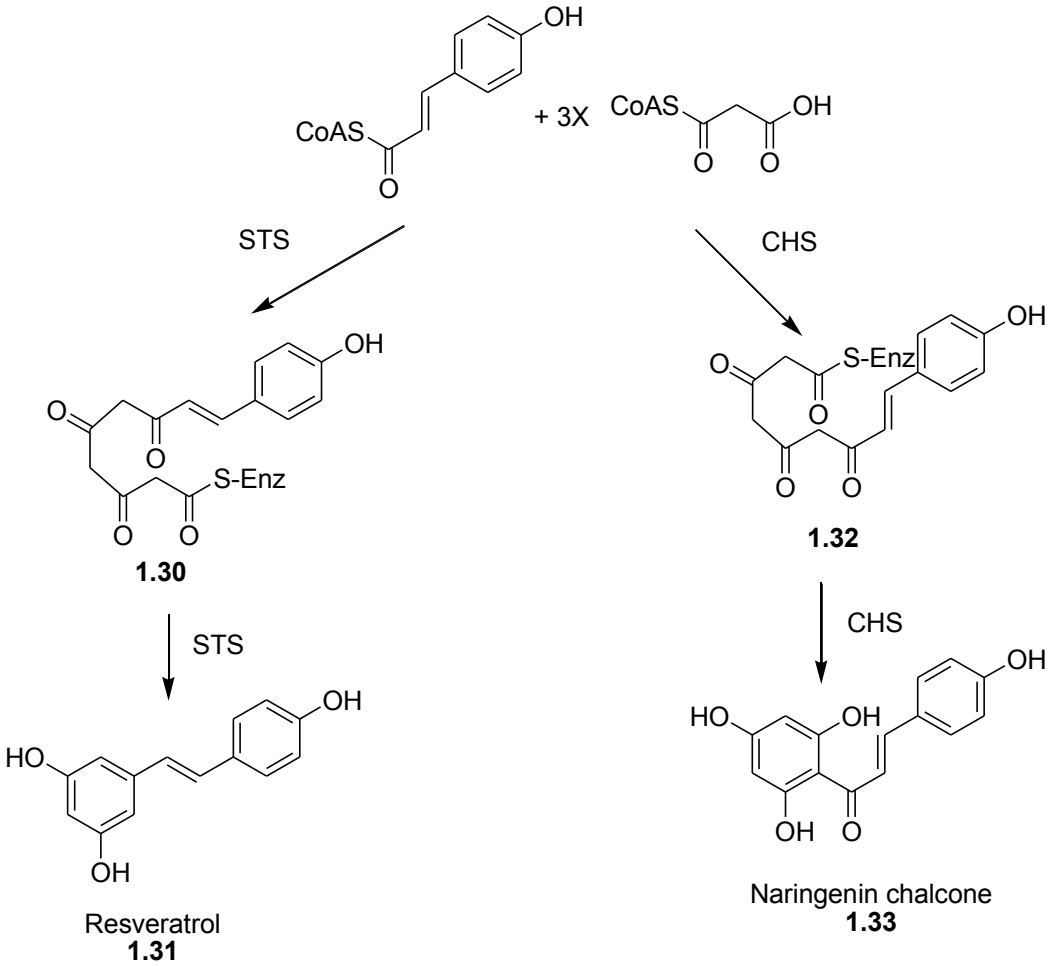


Figure 1.7 Biosynthesis of resveratrol and naringenin chalcone, well-known type III PKSs.

These products are the precursors to many pigments and other flavonoids in plants. Since these compounds all contain a polyphenol, as it is the starting material, it has the inherent ability to act as a free radical scavenger.

A new family of PKS compounds, called enediynes, have recently been discovered. Enediynes are unique in their structure and mechanism. They all possess a 9- or 10-member ring, along with two acetylene groups conjugated to a double bond.[47] These features make enediynes extremely potent anticarcenogenics by cleaving DNA.[48] They are unlike any polyketide product known to date, and as such, have generated a lot of interest in elucidating their biosynthesis.[49-51] While this research is still going on today, it has been found that it is a mixed iterative type 1/type 3 PKS that biosynthesize these unique compounds.[52]

1.5 Iterative type I PKS

Fungi produce many polyketides. The first fungal PKS gene to be cloned was 6-methyl-salycilic acid (MSAS) from *Penicilium patulum* by Lynen and coworkers.[16] Since then, many fungal PKS have been cloned, including those responsible for lovastatin, zearalenone, radicicol and orsellinic acid biosynthesis.[16, 53-55] Fungal PKSs are for the most part multidomain type 1 PKSs that can act either iteratively or non-iteratively. Fungal PKSs are considered iterative when more than one chain elongation cycle is catalyzed by the same KS domain.[19, 40, 56] Despite being used in an iterative function, the substitution pattern is controlled by different uses of KR, DH, ER, and methyltransferase (MT) domains in each elongation cycle. However, the factors controlling the substitution pattern in each cycle is more cryptic and virtually unknown.[4, 27]

The biosynthesis of fungal PKS depends on their reduction patterns. These can either be nonreducing (NR), partially reducing (PR) or highly reducing (HR) PKS. These reduction patterns are governed by the presence or absence of β-keto processing domains.[19] The biosynthesis of fungal PKS starts with malonyl-CoA as a

precursor and is then loaded as acyl thioesters by the AT domain on the ACP domain. Just like in other types of PKSs, it is the KS domain that undergoes the decarboxylative condensation of the growing chain. Fungal nrPKS, which produces unreduced polyketide chains also include an N-terminal starter unit acyltransferase (SAT) domain, a centrally located product templating (PT) domain, and a C-terminal TE domain.[57] The TE domain in this case is believed to play a role in controlling the chain-length. Fungal prPKS do not contain SAT, PT or TE domains, but possesses β-keto processing domains, such as a KR and a DH which are selectively used during the biosynthesis to produce partially reduced compounds.[58] The factors controlling these partial reduction at each chain length remain virtually unkown.[19, 27]

Fungal hrPKSs produce non-aromatic compounds. Their domain architecture closely resembles modular bacterial type 1 PKSs, as they do not contain SAT, PT or TE domains, but contain KS, AT, ACP, KR, DH and ER domains. Moreover, the chain can be methylated by a MT domain, using a methyl group from SAM. This is unique to hrPKS.[27] The regiospecific cyclization of the final product remains nearly unknown at the present. Of the three fungal PKSs, hrPKS remain the biggest mystery, as the factors that control the number of chain elongation and reduction of the carbon chain are still unknown. Furthermore, hrPKS, with nearly identical domain architecture, have the ability to synthesize compounds with extreme variations in their chain-lengths and reduction levels. Additionally, the chain termination and the offloading of the linear polyketide chain in hrPKS are still unknown.

Some unique fungal PKSs have been discovered to make use of two different PKSs (for example, in the case of resorcylic lactones, an nrPKS and a hrPKS) in their biosynthesis. A typical example of this is the biosynthesis of zearalenone, which is typical of most resorcylic lactone compounds (Figure 1.8).[59, 60] The first PKS, a hrPKS, named PKS4, contains KS, AT and ACP, the minimal PKS condensation domains, as well as DH, ER and KR domains. The product from PKS4 is then transferred to PKS13, a nrPKS. PKS13 contains, in addition to the minimal domains,

a putative SAT and PT, and a C-terminal TE domain. Its role is to incorporate three malonyl-CoA units without reduction to give the complete acyl chain, and catalyze formation of the resorcylate moiety via an aldol condensation and tautomerization. Subsequently, the TE domain forms the 14-membered macrolactone via intramolecular macrolactonization. An FAD-dependent oxidation of the secondary alcohol found on the macrocycle yields zearalenone.

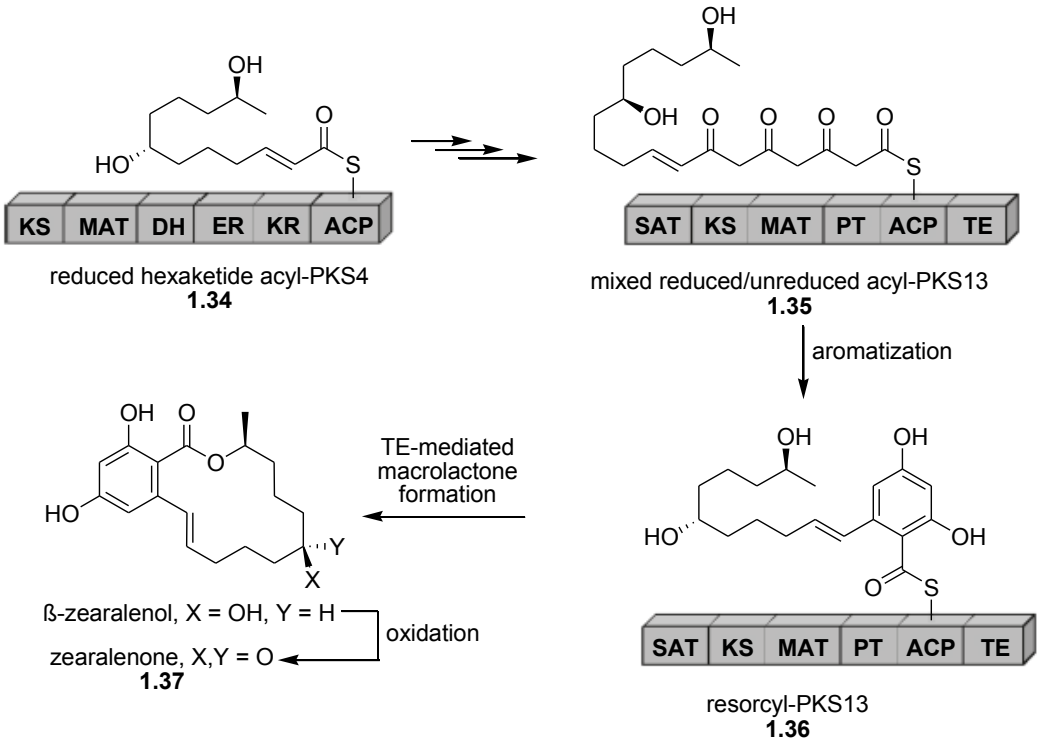


Figure 1.8 Biosynthesis of the fungal agent zearalenone, an archetypical resorcylic acid lactone.

The other resorcylic acid lactones are biosynthesized in a similar fashion. hrPKS reduces the growing chain uniquely for each resorcylic acid lactone to yield different polyketides. This explains the variety of oxidation states, functionalities and stereochemistries in the final compounds of that family. The use of two PKSs during the biosynthesis is unique, and extremely remarkable. It suggests that PKS13, on top of its other roles mention above, interacts with PKS4 so that the growing polyketide chain can transfer to the PKS13 and the SAT unit can then initiate its biosynthesis.[61, 62] Studies have shown that PKS13 has a broad substrate tolerance toward different

start-units and acyl carrier, however, substrate tolerance on its TE domain is still practically unknown.

Despite the fact that the stereochemistry and the whole sequence of the compound can be predicted by the different modules present, the loading and offloading steps, as well as the chain length and the reductive steps at specific sites are still not well understood by the fungal PKS.[21] On top of that, while the functions of individual PKS genes have been established, products from individual PKSs have seldom been identified.

1.6 Resorcylic acid lactones (RALs)

Resorcylic acid lactones are from a unique family of macrolides that possess a resorcylate (2,4-dihydroxybenzoate) and a 14-membered macrolactone, produced by a mixed reduced/unreduced PKS. This family of natural products are all mycotoxins produced by many different fungi and they possess a wide array of potent pharmaceutical properties, including HSP90 inhibition, MAP kinase inhibition, estrogen receptor agonist, MEK kinase inhibition, antimalarial and HSV inhibition (Figure 1.9).[57, 59, 60, 63, 64] The majority of RALs possess a 14-membered lactone ring; however there is one example of a 12-member RAL, called lasiodiplodin. Two important RALs are zearalenone, an estrogen receptor agonist, and radicicol, an anticancer agent.

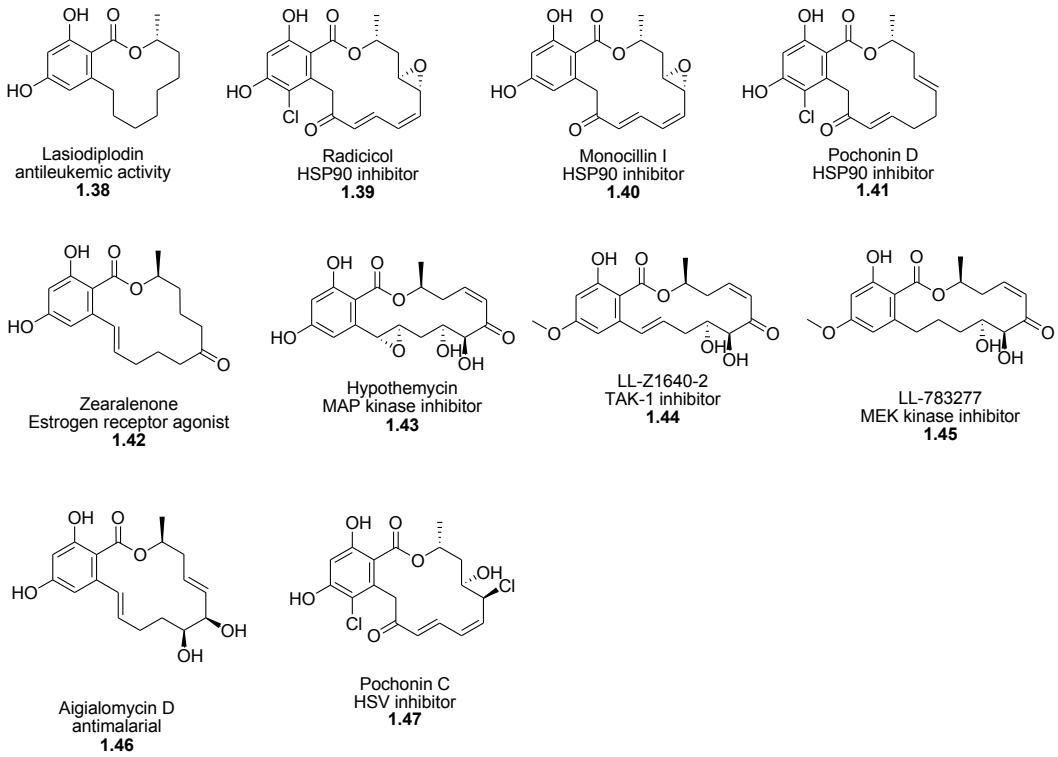


Figure 1.9 Different resorcylic acid lactones with differing biological activities.

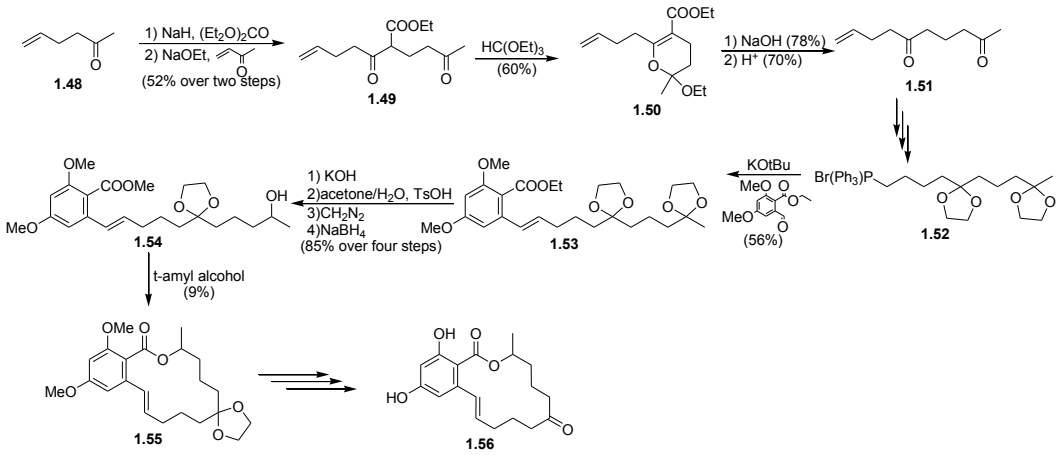
1.6.1 Zearalenone

Zearalenone is an estrogen receptor agonist isolated from different *Fusarium* species, including *F. graminearum, F. culmorum*, *F. sporotrichioides*, *F. equiseti* and *F. smistectum* and was first isolated in 1962.[57, 61, 62, 64] Zearalenone is sometimes used for growth promotion in cattle, yet is more identified with *Gibberella zeae* infections found in maize, barley, wheat, sorghum and oats. *G. Zeae* infections dramatically reduce the yield and quality of these grains. Zearalenone is found in food supplies worldwide, including cereal crops, cornmeal, corn flakes, corn porridge, and beer. Even though its toxicity and carcinogenicity levels are low, chronic exposure to its estrogenic properties has been shown to cause estrogenic disorders. In swine, rats and mice, chronic zearalenone exposure can lead to vaginal eversion in females and promotes the growth of mammary glands in both males and females. Zearalenone was found to act on the estrogen receptor by adopting a

conformation analogous to estradiol and thus competing with the steroid for the estrogen receptor.[5, 64]

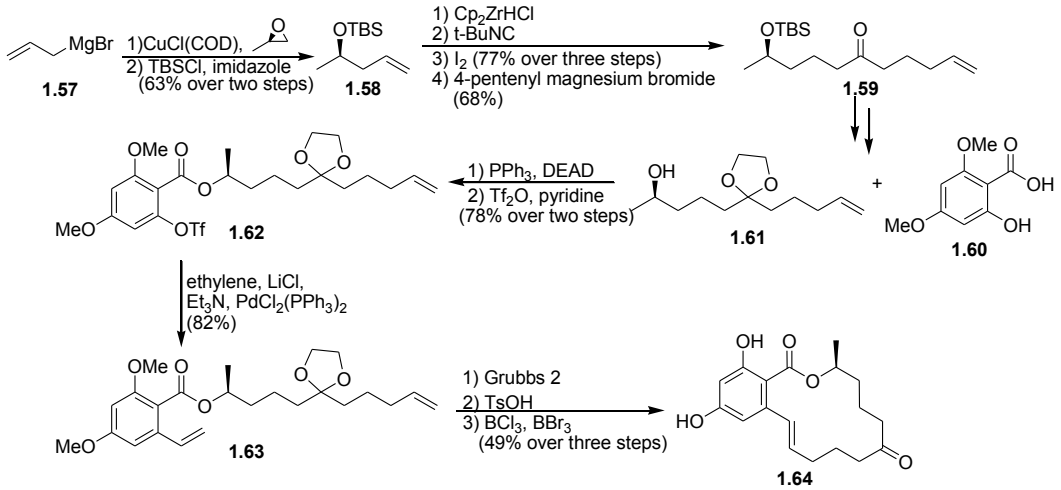
Since the discovery of zearalenone, many total and formal syntheses have been completed. Through extensive research, it has been shown that macrocyclization of zearalenone is extremely challenging under Corey-Nicolaou and Yamaguchi conditions.[53] Because of this, many syntheses have relied on alternative macrocyclization approaches, such as ring closing metathesis and Stille macrocyclization. Representative of the various approaches to zearalenone are the syntheses by Vlattas, Fürstner, Nicolaou and Barrett, which will be discussed below.

Vlattas and his group was one of the first to publish a synthesis of racemic zearalenone in 1968 (Scheme 1.1).[65] This synthesis relied on a macrolactonization strategy, which was ultimately very low yielding. First compound **1.49** was generated using a Michael addition. Then, after a series of functional transformations, the phosphonium salt **1.52** was generated, and a Wittig reaction was performed. Finally, compound **1.55** underwent macrolactonization via base-catalyzed esterification in only 8% yield, following another series of deprotection and protection reactions.



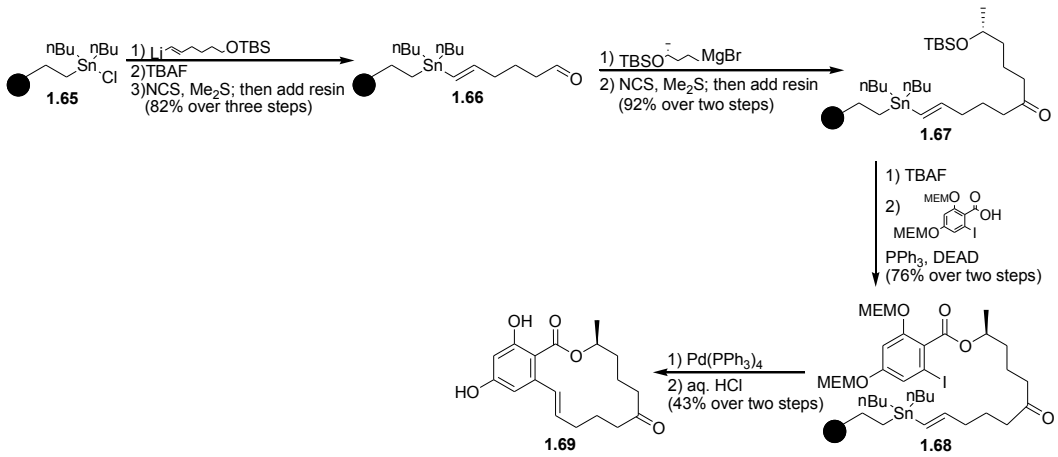
Scheme 1.1 Synthesis of zearalenone by Vlattas and his group.

Fürstner and his group published an elegant racemic and enantiopure synthesis of zearalenone(Scheme 1.2).[66] This study relied on a very successful ring closing metathesis strategy to access the macrocycle. **1.61** was synthesized via two different routes, one relying on the Jacobsen's hydrolytic kinetic resolution, and the other on a coupling reaction with copper. Coupling of **1.61** to **1.60** via a Mitsunobu esterification generated the ring closing metathesis precursor, which was macrocyclized in 91% yield using Grubbs second generation catalyst.



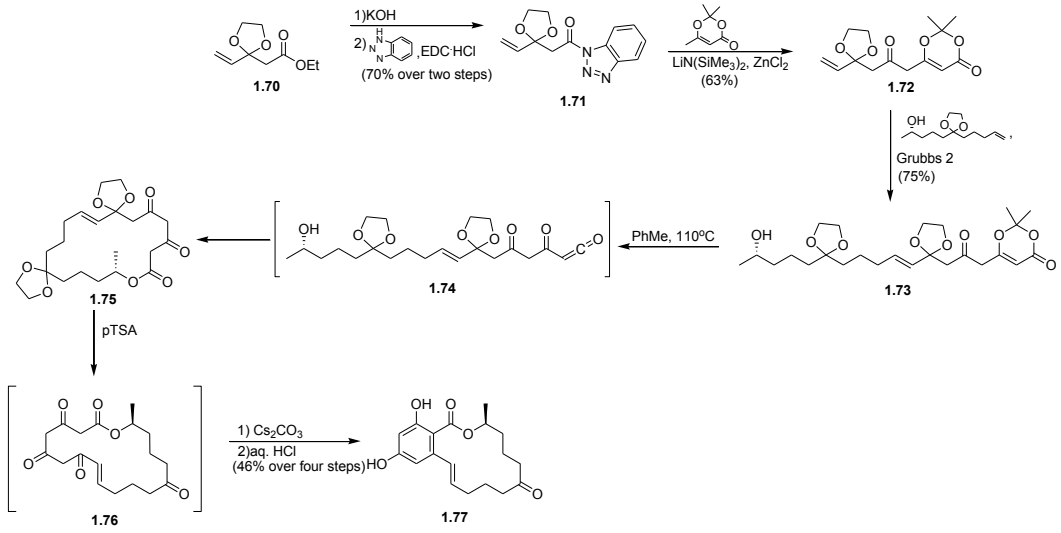
Scheme 1.2 Synthesis of zearalenone by Fürstner and his group.

Nicolaou and his group was the first to synthesise zearalenone and various analogues by solid phase (Scheme 1.3).[67] Their strategy relied on a Stille macrocyclization to construct the 14-member ring. After its synthesis, **1.66** underwent a Grignard reaction before the Mitsunobu esterification. In the last step, the 14-membered ring was formed by a Stille coupling in 54% yield, with concomitant release of the final product from the solid phase bead.



Scheme 1.3 Synthesis of zearalenone by Nicolaou and his group.

Barrett and his group recently published a concise and elegant synthesis of zearalenone, using a unique route to form the aromatic ring (Scheme 1.4).[68] Compounds **1.59** and **1.60** were prepared and underwent a cross metathesis with Grubbs second generation catalyst. Following this, compound **1.57** could be synthesized by intramolecular ketene trapping. Finally, zearalenone was achieved by a late-stage aromatization of compound **1.57**.



Scheme 1.4 **S**ynthesis of zearalenone by Barrett and his group.

Zearalenone is a resorcylic acid lactone found in food supplies worldwide with estrogen receptor agonist properties. While a simple molecule, zearalenone has

proven to be quite challenging synthetically, mostly due to the 14-member ring macrocyclization formation. Common Corey-Nicolaou and Yamaguchi conditions do not yield macrocycles. Over the years, several groups have synthesized zearalenone, with varying success. The most common route was via Stille coupling or Mitsunobu macrocyclization, which gave low to moderate yields, until 2000 when Fürstner and his group published a synthesis where macrocyclization was performed via Grubbs II ring closing metathesis. Since then, most groups have used this approach as it is high yielding.

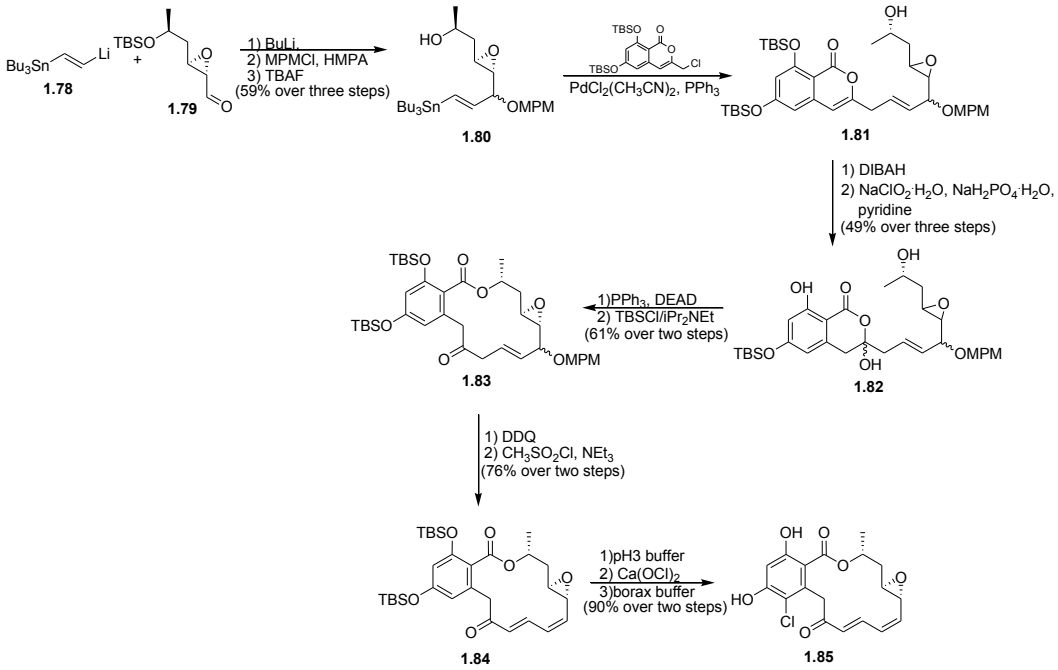
1.6.2 Radicicol

Radicicol was first isolated in 1952, and at first was discovered to have mild sedative properties, as well as moderated antibiotic properties.[64] It has since been found to be a potent antitumor inhibitor of the stress chaperone Hsp90. Hsp90 is one of the most abundant proteins in cells, comprising of 1 to 2% of proteomic mass and is responsible for folding, maturing, stabilizing and activating proteins. Radicicol binds to the Bergerat-fold ATP-binding pocket of Hsp90, inhibiting its activity.[5, 57] By inhibiting Hsp90, radicicol promotes the degradation of oncogenic proteins, blocking many cancer-causing pathways.[69, 70] Inhibition of Hsp90 was also found to reduce protein aggregation for many neurodegenerative diseases, including Huntington, spinal and bulbar muscular atrophy, Parkinson, and many other Tau protein-related neurodegenerative diseases.[5, 69, 70] Hsp90 is thought to be in an active form in cancer cells and is responsible for the formation of proteins involved in angiogenesis, metastasis and apoptosis. Since it is responsible for the production of so many proteins involved in tumour growth, inhibition of Hsp90 has the potential to be a broader, more potent target for anticancer treatment than drugs targeting a single oncogenic protein.[64] These could work synergistically with already commercially available anticancer drugs as effective anticancer agents.

However, even though radicicol is the most potent RAL inhibitor of Hsp90 in vitro, its metabolic instability, due to the reactivity of the strained allylic epoxide

group, as well as the reactivity of its conjugated dienone to Michael additions causes it to be inactive in vivo. This led to the search of more stable analogs by combinatorial chemistry, including the works by Winssinger and his group.[5, 71] Some total syntheses of radicicol have also been published, among them syntheses by Lett and his group as well as Danishefsky and his group.[72-77]

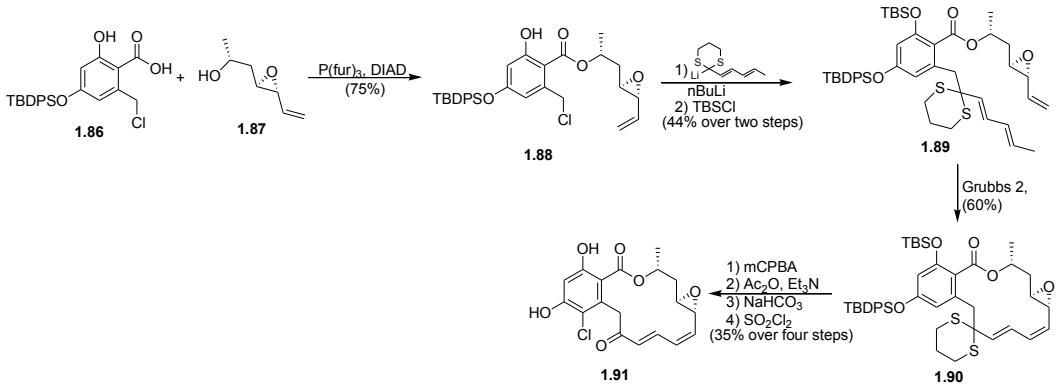
Lett and his group published the first total synthesis of radicicol in 1992, and 10 years later, published a second generation synthesis (Scheme 1.5).[72-75] Their synthesis relied on a Mitsunobu macrocyclization to form the 14-member ring. Compounds **1.78** and **1.79** were built and underwent a vinyl addition to form compound **1.80**. A palladium-catalyzed cross-coupling generated product **1.81**. Finally, the 14-membered ring was formed in 66% yield via a Mitsunobu macrocyclization and the dienone was formed by elimination.



Scheme 1.5 Synthesis of radicicol by Lett and his group.

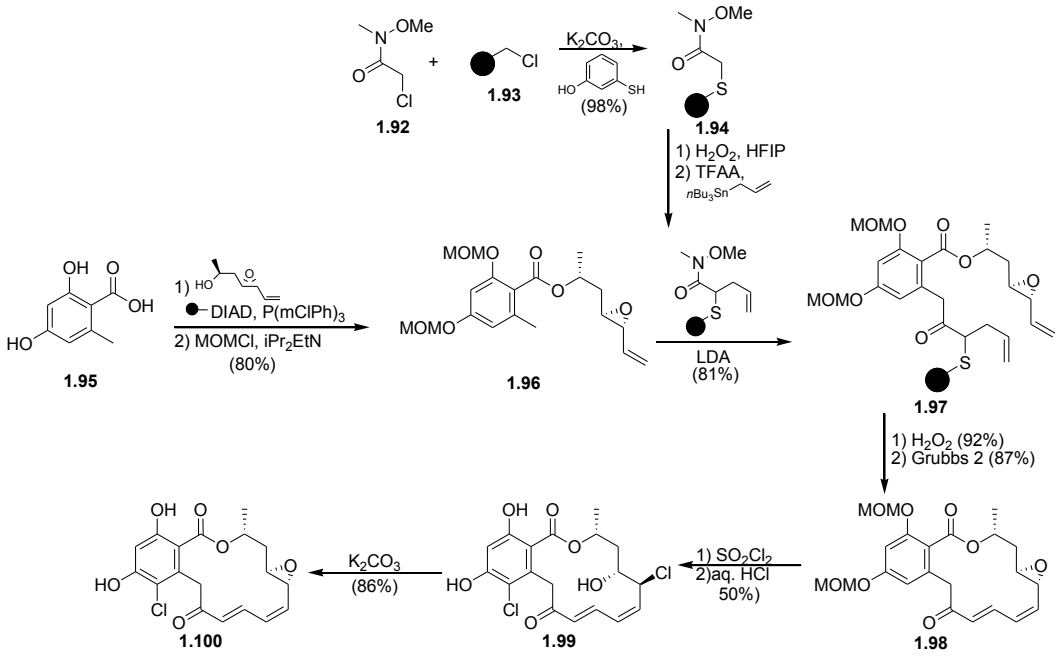
Danishefsky and his group synthesized radicicol in an extremely concise and elegant manner (Scheme 1.6).[76, 77] Their strategy to form the macrocycle involved a

ring closing metathesis. The main steps to form the 14-membered macrocycle included a Mitsunobu esterification in 75% yield, followed by a dithiane addition and finally a ring closing metathesis, achieved in 60% yield, using Grubbs second generation catalyst.



Scheme 1.6 Synthesis of radicicol by Danishefsky and his group.

Finally, Winssinger and his group published a polymer-assisted synthesis of radicicol, in the hopes of using this to combinatorially build synthetic analogs of the molecule (Scheme 1.7).[5, 71] Their strategy also involved a ring closing metathesis. Firstly, the solid phase side chain was built using an allylation reaction. Subsequently, the 14-membered ring was built in an analogous fashion to the Danishefsky synthesis, first by undergoing a Mitsunobu esterification, then an acylation and finally a ring closing metathesis, in 94% yield, using Grubbs second generation catalyst.



Scheme 1.7 Synthesis of radicicol by Winssinger and his group.

Radicicol is a resorcylic acid lactone that has potent anticarcinogenic properties by binding to Hsp90. Its compound is a bit more complex than zearalenone, with an epoxide and a conjugated dienone. These functional groups are chemically unstable and must be masked during synthesis, thereby adding complexity to the total syntheses. Similarly to zearalenone, Mitsunobu esterification is a popular way to generate the macrolactone, despite often being low to moderate yielding. Another popular strategy, just like for zearalenone, is using a ring closing metathesis to introduce the 14-member ring. Because of the added complexity and functionalities, this method is often lower yielding for radicicol. Despite having widespread uses, macrocycle formation, especially via macrolactonization, is often challenging and selective.[78, 79] Often, a molecule that was macrolactonized has dropped completely or partially in efficiency by adding a functionality or changing the ring sizes.[29] This makes it harder to predict and build synthetic routes. In nature, this is achieved in RALs by a thioesterase, leading exclusively to the product. If the thioesterase proves to have broad substrate tolerance, this would be an excellent tool to use in organic chemistry to build macrolactones.

1.7 PKS thioesterases

The PKS thioesterases (TE) domain is structurally and functionally homologous to NRPS TEs.[20] These TEs display an α/β hydrolase fold, thus making them a member of the α/β hydrolase superfamily of enzymes.[29] This fold consist of seven-stranded β-sheets in the center, with the second strand, called β2, antiparallel to the remaining strands.[36] In solution, some TEs, including DEBS and Pik TEs, are homodimers and contain a hydrophobic substrate channel that completely traverses the TE domain.[19, 80, 81] PKS TEs vary between 28 to 35 kDa and generally possess a Ser-His-Asp catalytic triad in their active site.

The function of TEs is to cleave the polyketide product, via either inter- or intramolecular nucleophilic attack. In type I PKSs, the TE domains are found at the C-terminus of the last PKS protein. In type II PKS, the TEs are separate proteins.[30, 80] Their physiological function is to cleave the PKS product after it reaches its final length by intra- or intermolecular attack of a nucleophile to form either a cyclized or linear product. When the attack is intramolecular, an amine or alcohol moiety on the compound will attack, regiospecifically, to cleave the acyl-enzyme intermediate, yielding a macrocycle product. However, when an intermolecular attack prevails, a water molecule attacks the substrate to form the hydrolysis product. Rarely, a separate amine or alcohol molecule is used to cleave the TE and form the amide or ester product. It is proposed that defined hydrophobic interactions in the substrate channel and binding cavity influence the ring size of the product by dictating the substrate specificity.

The TE domain in DEBS TE is linked covalently to the ACP of the sixth module. This ACP transfers the final linear polyketide intermediate to the TE domain. In vitro this TE substrate loading step is rate limiting, however, in vivo it is not known what step is rate determining in polyketide synthase catalyzed chemistry.[25]

DEBS TE is the first PKS TE for which a crystal structure was obtained (Figure 1.10).[81] DEBS TE is a homodimer in solution with a molecular mass of 66 kDa. Unlike all other α/β hydrolases, the DEBS TE possesses a hydrophobic substrate channel which passes through the center of the fold. It contains, as mentioned above, seven-stranded β-sheets at the center, with the second strand, β2, antiparallel to the other strands, the β7 strand is also shorter than β8. Part of the substrate channel is formed by β7, as it is twisted in a 90^{o} angle. The α-helices are present on both side of the β-sheets, four being on one side, and two on the other. There is also presence of two other α-helices at the N-terminal of the TE that forms the interface of the dimer. The expected catalytic triad is easily identifiable at residues Ser-142-His-249-Asp-169.

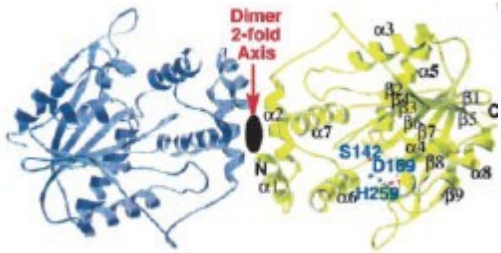


Figure 1.10 Structure of the DEBS TE dimer. The catalytic triad is found in the middle of the substrate channel.

Pik TE is highly similar to DEBS TE and it contains the arrangements of β-sheets and α-helices as is seen in the DEBS TE (Figure 1.11).[82] Furthermore, it is also a homodimer in solution and it contains an open hydrophobic substrate channel with the same Ser-His-Asp catalytic triad.[83] Due to these similarities, DEBS TE and Pik TE can be superimposed on each other. As with other TEs, DEBS TE and Pik TE catalyzes the macrocyclization of a highly functionalize product by lactonization, where substrate specificity is most likely a result of the hydrophobic substrate channel.

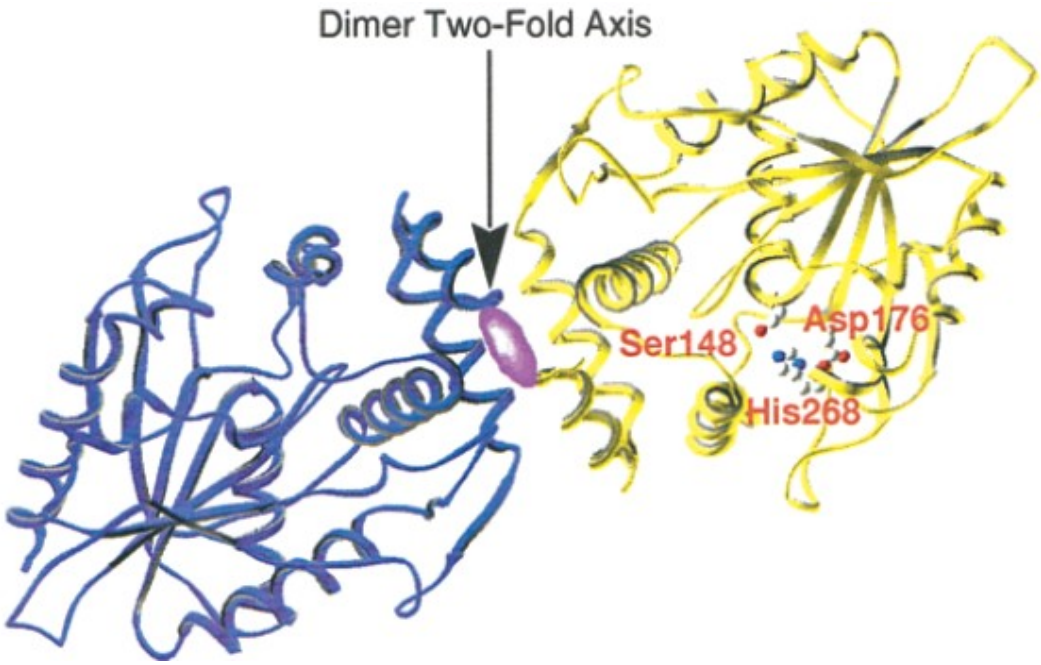


Figure 1.11 Structure of the Pik TE dimer. The catalytic triad is found in the middle of the substrate channel.

Many other PKS TEs have been identified and their primary structures are highly homologous to the DEBS TE, with up to 75% sequence identity. The regions around the catalytic triad and substrate channel are especially highly conserved among many TEs. The residues that line and stabilize the hydrophobic channel are also highly conserved, suggesting that the open substrate channel is a conserved element among the various TEs in modular PKSs.

While fungal iterative PKSs contain many differences from modular type I PKSs, their product release is also catalyzed by a TE domains.[53, 60] Just like modular type I PKS, the zearalenone (zea) TE contains a catalytic triad of Ser-His-Asp where serine is the active-site nucleophile. Based on sequence analysis, the Zea TE is expected to be part of the α/β hydrolase superfamily. Alignment with both the DEBS and Pik TE show low sequence homology to both, with about 30% homology and 10% identity (Figure 1.12). This suggests that zea TE may be unique in both tertiary structure and function.

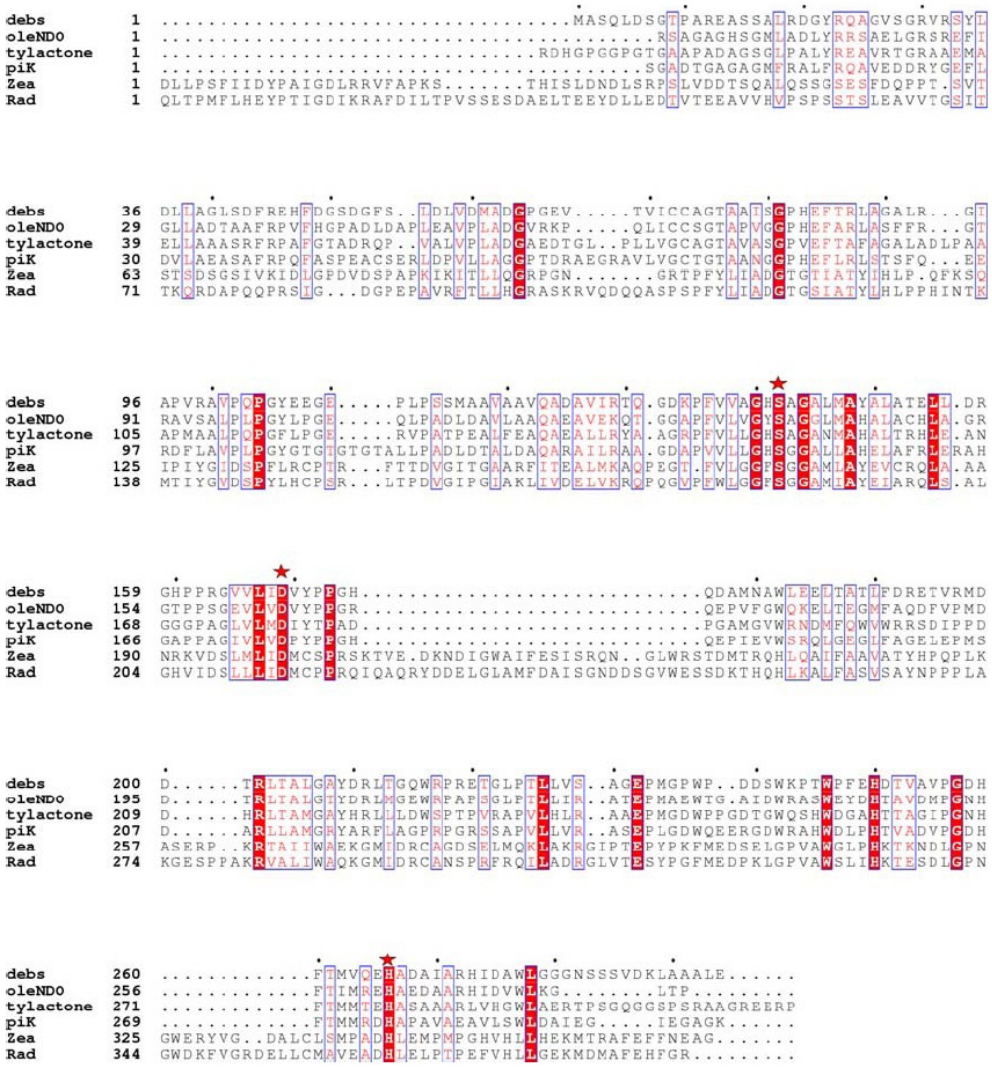


Figure 1.12 Sequence analysis of zea TE with other TEs, including DEBS and Pik, known to undergo macrocyclization as product release.

1.8 Substrate specificity of thioesterases

Understanding TE substrate specificity is an extremely important goal in characterizing and utilizing polyketide biosynthesis. Macrocyclization by TEs is highly biologically relevant since many polyketide and non-ribosomal peptide natural

products are only active in their macrocyclized form.[80] In the context of metabolic engineering, it would be extremely ineffective to engineer a PKS to produce a new compound or libraries of compounds, if macrocyclization and product release from the final ACP is not possible due to limited TE substrate specificity. Because TE substrate specificity plays a key role in the activity of the resulting natural product, turn-over of the native biosynthetic enzymes, and efforts to engineer PKS systems, these enzymes have been extensively studied over the past ten years.[84, 85]

Using PKS TEs as chemoenzymatic tools for macrocyclization may be highly advantageous. The advantages of chemoenzymatic synthesis include high regio- and stereoselectivity, rapid reaction rates, and limited by-product formation afforded by the use of the enzyme. Using TEs in chemoenzymatic synthesis overcomes some of the limitations of enzymatic transformations in organic chemistry, namely the limited scale, and low solubility of many organic reagents in the compatible enzyme buffer. As macrocyclization occur late in syntheses, small scale is not problematic and as macrocyclizations are generally synthesizing natural product-like molecules, they are often densely functionalised with polarisable groups, increasing their solubility in organic buffer.[86, 87] Unfortunately, there is still a lack of understanding of the substrate specificity of these TEs, which makes their use in chemoenzymatic transformations difficult.

The assembly-line mechanisms of modular PKSs systems make them particularly attractive and easily amenable to protein engineering. So far however substrate specificity, kinetics, and rate limiting steps that dictate product formation are still unknown. Without this information it is extremely challenging to rationally engineer PKS genes to produce libraries of complex natural product-like compounds.[88] While additional understanding of the structure of PKS enzymes, by both X-ray crystallography and NMR methods will increase the successes in metabolic engineering, a thorough understanding of substrate specificity in the key off-loading enzyme, the TE, is the key for successful pathway engineering.

Substrate specificity of PKS, NRPS, and fatty acid synthase (FAS) TEs have been examined and in some cases the molecular interactions between substrate and enzyme governing substrate specificity have been characterized. For example in NRPS and FAS TEs, the substrate-enzyme interaction is driven by hydrophobic interactions between the substrate alkyl side chains and the hydrophobic residues in the binding cavity.[89] While a clear understanding of PKS TE substrate-enzyme interactions, especially those involved in controlling macrocyclization, is yet to emerge, it appears that hydrophobic interactions also play a major role in PKS TE substrate specificity. Characterizing the different molecular interactions that lead to substrate specificity will render rational substrate specificity engineering possible, leading to the formation of non-native compounds.

TE-catalyzed macrocyclization occurs in a two step process: the first step involves the formation of the acyl-enzyme intermediate, which is followed by cleavage of the acyl-enzyme bond. Macrocyclization occurs when the nucleophilic heteroatom is appropriately positioned to attack the acyl-enzyme intermediate in the active site cavity. When no intramolecular nucleophile is positioned to attack the acyl-enzyme intermediate, due either to substrate specificity limitations to the lack of an intramolecular nucleophile in the substrate, the most likely outcome is water-mediated hydrolysis.[30] In some cases, such as some NRPS TEs, the acyl-enzyme is stable and remains attached to the megasynthase, inhibiting product formation.[89]

Key studies investigating the substrate specificity of both acyl-enzyme intermediate forming step and macrocyclization/hydrolysis step are presented below. These examples review the current breath of what is known regarding TE substrate specificity.

In probing the specificity of acyl-enzyme intermediate formation for different PKS TEs, including pimaricin (Pim TE), epothilone (Epo TE) and DEBS TE, it was found that these TEs are able to accept peptide-like substrates with much better pseudofirst order rate constants than ahs been seen for other PKS TEs. In fact, this

study showed that in many cases, the PKS TEs had less difficulty accepting these NRPS-like peptidyl substrates than PKS-like substrates. Examination of the specificity constants showed that the different TEs have different functional group and stereochemistry specificity, thus making it challenging to develop a broad model describing substrate specificity.[82]

During the in vitro TE-catalyzed macrocyclization, hydrolysis has been shown to compete, significantly in some cases, with cyclization.[56, 83] This property appears to be unique to in vitro TE activity and may be related to the proteins being excised from their native megasynthases. However this property may ultimately hinder the use of these enzymes as chemoenzymatic catalysts for complex molecule formation and library generation. It was found, however, that adding non-ionic detergents helped shift the yield towards macrocyclization and decrease hydrolysis formation.[90]

While many bacterial PKSs have been probed for substrate specificity, DEBS and Pik TE have been studied the most extensively. In general, the cyclorelease step for DEBS TE appears to be too highly substrate specific to be amenable to protein engineering to generate a broadly functional macrocyclization catalyst. For example, DEBS TE was tested with different analogs resembling **1.101** (Figure 1.13).[84, 91] It was found that substrates corresponding to the 9-, 10- and 11-membered macrocycle product were not macrocyclized. The major products observed were from hydrolysis; however trace glycerol ester could be detected. These were likely formed via coupling with the glycerol found in the enzyme preparation. This study was one of the first to indicate that the TE had varying mechanisms for product release, including hydrolysis and ester formation. These results demonstrate that the DEBS TE is substrate specific for that macrolactone formation.

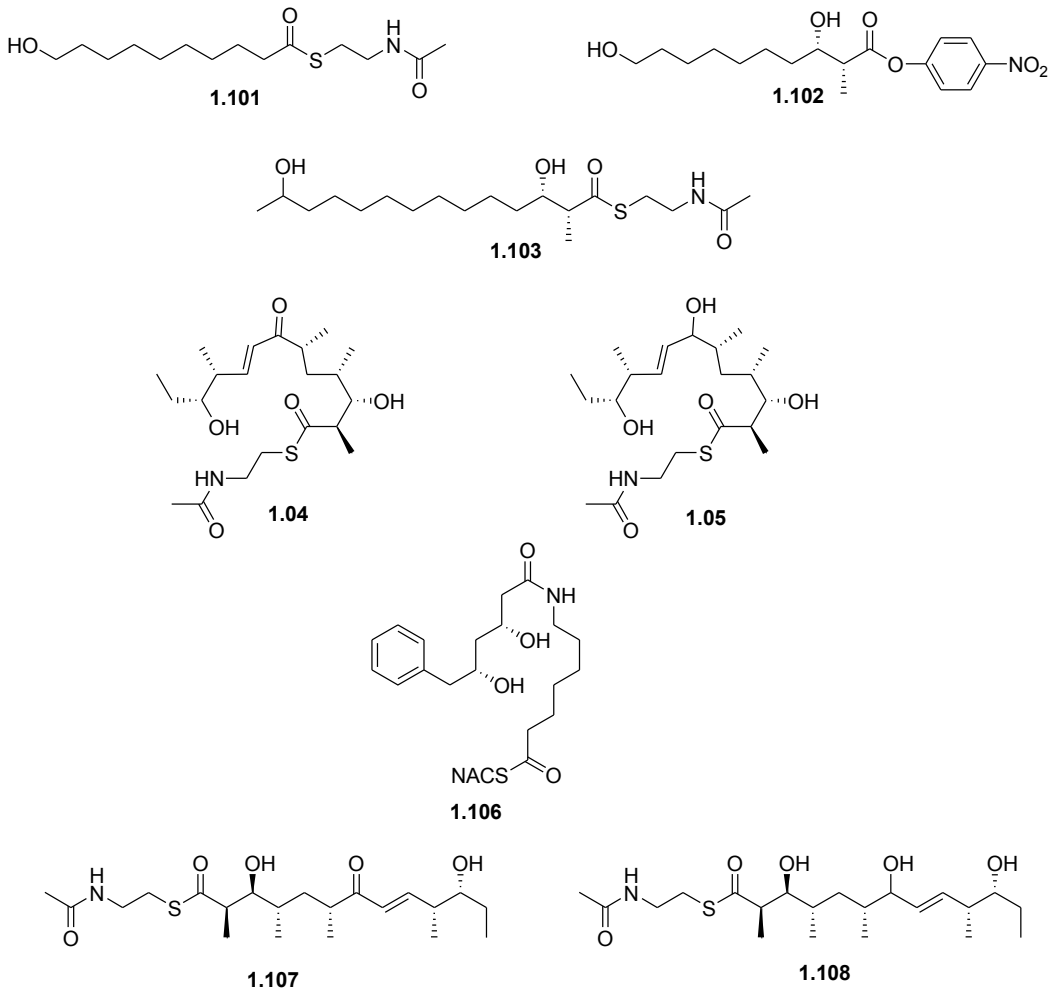


Figure 1.13 A few substrates that were built to probe the substrate specificity of DEBS and Pik TE

Even when more native-like substrates are examined, the DEBS TE is highly substrate specific for macrolactonization. Analogues of the native DEBS TE substrate, which possessed the native functionality at C2 and C3, were examined by Khosla and coworkers. These analogues of different ring sizes and different stereochemistries resembling **1.102** were built (Figure 1.13).[41, 42, 92] Analogous to previous experiments, the substrate specificity of DEBS TE and genetically modified DEBS TE showed preference for hydrolysis over macrolactonization of these analogues, even when replacing water with acetonitrile as a solvent. These results suggest that other structural features on the chain are needed to ensure macrolactonization. However, given that no stereochemical conformation of the 2-

methyl and 3-hydroxyl moiety favoured macrolactonization over hydrolysis, this might imply that as long as hydrolysis is properly suppressed, any stereochemical configuration might macrocyclize without preference over another, suggesting poor stereochemical substrate specificity.

The ability of secondary alcohols versus more native-like primary alcohols to macrocyclize using DEBS was probed by Khosla and his group. These analogues of different ring sizes and stereochemical pattern similar to **1.103** were built (Figure 1.13).[93, 94] Again, these analogues were unable to macrolactonize, but instead underwent hydrolysis. This result suggests that the substitution pattern and stereochemistry may be more important for the macrocyclization than previous studies implied. Hydrolytic rates were also measured in this study. DEBS TE showed significant preference for longer chain length substrates, as well as for similar substitution and stereochemistry to the native substrate. Therefore, hydrolysis, and most likely macrocyclization, might be dependent on the natural stereochemistry and substitution pattern of the native substrate and might require additional conformational constraints analogous to the native substrate.[40]

DEBS TE is able to macrocyclize varying ring sizes when the natural substrate substitution pattern is followed. This was demonstrated by Khosla, in an in vivo study of DEBS. Surprisingly, as long as the substitution pattern for DEBS is adhered to, DEBS TE show remarkable tolerance to macrocycles varying in ring sizes. DEBS TE has proven to be able to cyclized 6-, 8-, 12-, 14- and 16-membered rings, with no 10-membered ring cyclized so far.[81, 95] These results suggest that DEBS TE does have broad tolerance for varying ring sizes, and is not too substrate specific.

Lack of macrocyclization from DEBS TE might in part be due to lack of hydrogen bonding. A study on DEBS TE by Boddy and Wang showed that hydrogen bonds in the binding pocket between the substrate and the enzyme do not direct substrate specificity in the loading step of DEBS TE.[25] However, hydrogen bonding

may be important for cyclization as it may direct the linear 6-deoxyerythronolide precursor into a cyclic conformation in the active site, while at the same time shielding the binding pocket from external water molecules to favour macrocyclization.[36] This hypothesis could explain why the previously investigated analogs did not macrocyclize, but instead underwent hydrolysis, as they lacked the required hydrogen bond donor/acceptor in the substrate.

Observations from a number of in vivo experiments also suggest that DEBS TE appears to require a carbonyl group four carbons from the nucleophilic alcohol for lactonization to occur.[25] The most definitive of these studies is an in vitro study examining macrocyclization of 12-member ring 10-deoxymethynolide derivatives, **1.104** and **1.105** (Figure 1.13).[83] Indeed, when the carbonyl group was present, compound **1.104**, the TE could easily catalyze macrocyclization without any competing hydrolysis. However, when that same carbonyl group was reduced to a hydroxyl (compound **1.105**), no macrolactonization was detectable and only hydrolysis occurred. The results strongly suggest that the carbonyl functionality is required for macrocyclization. However, 6- and 8-member analogues lacking the carbonyl functional group have also been macrocyclized in vivo by DEBS TE.[81, 95] These results run contrary to the above in vitro requirement for the carbonyl group and further suggest a complex substrate-enzyme interaction dictating partitioning between macrocyclization and hydrolysis.

Recently, our group has systematically investigated the influence of stereochemistry on DEBS TE catalyzed macrocyclization, regioselectivity and hydrolysis. Analogues with the four different possible stereoisomers of **1.106** were synthesized (Figure 1.13). The (*R*,*R*)-substrate was the only one to macrocyclize. The (*R*,*S*)-substrate mimicked the native diastereochemistry and did not macrocyclize. It is still unclear, however, if the (*R*,*S*)-substrate does macrocyclize and a subsequent ring opening reaction occurred to form the macrocycle, as is seen with the TE catalyzed macrocyclization of epothilone D. Additionally, the (*S*,*R*) substrate did not form either the 14- or the possible 12-member ring, rather only

hydrolysis was observed. This indicates that the last possible nucleophile is used preferentially by DEBS TE in these substrates. So far, our result indicates that absolute *R* configuration is required for macrocyclization by DEBS TE, making it extremely substrate selective in regards to stereoselectivity.

It was also shown that DEBS can macrocyclize the substrates for Pik TE, although hydrolysis was a side product.[42] All these studies show that while DEBS TE has some substrate tolerance, it still displays significant substrate selectivity and hydrolysis is often a problem that hinders macrocyclization.

Pik TE has also been extensively studied, due to its natural ability to macrocyclize two products, 10-deoxymethynolide, a 12-member ring, and narbolonide, a 14-member ring.[17, 19, 38, 96] Since both products are catalyzed by a TE domain, this suggests that Pik TE has inherent substrate tolerance for varying chain lengths. This is confirmed when looking at the substrate channel of the crystal structure of Pik TE, which indicates that the channel provides a hydrophobic environment for the hydrophobic acyl substrates. However, the size, the hydrophobic surface of the channel and especially the deficiency of hydrogen bonding groups suggests that it can accept acyl chains containing a wide variety of lengths and conformations, thus being poorly substrate specific. What does seem to drive substrate recognition and thus specificity however, is the nonpolar interactions between the enzyme and the substrate.[25]

While analysis of the active site binding cavity supports broader substrate specificity for the Pik TE, no biochemical evidence has yet been obtained indicating that this TE has broader substrate scope than the DEBS TE. For example both modules 5 and 6 from the pikromycin biosynthetic pathway were expressed in *E. coli*, purified and incubated with natural and modified pentaketide and hexaketide thioester substrates.[97] In these experiments both modules showed extreme preference for their natural substrates, suggesting that the Pik TE may also be highly substrate specific.[97]

Many of the studies performed on the DEBS TE were also performed on the Pik TE. Similar to the DEBS TE, Pik TE will hydrolyse a variety of simple diketide thioesters, with a small preference to the natural stereochemistry of narbolide. Like DEBS TE, Pik TE will macrocyclize 6-, 8-, 12-, 14- and 16-member rings, but as of yet, no 10-membered rings have been successfully cyclized.[83] Furthermore, as in DEBS TE, Pik TE macrocyclizes, with little hydrolysis substrate **1.107** when in presence of a carbonyl group at the fifth carbon from the nucleophilic hydroxyl group (Figure 1.13).[98] However, when that carbonyl is reduced to a hydroxyl (compound **1.108**), only hydrolysis occurs. This seems to suggest that Pik TE is also highly specific; and that while Pik TE has good substrate tolerance for different chain lengths, minute changes to functional groups, even those farther from where the reaction occurs, significantly affects the substrate specificity of the TE. Based on these in vitro biochemical results, the Pik TE does not appear to tolerate even small changes to the natural substrates.

Despite being similar in substrate specificity, Pik and DEBS TE vary greatly kinetically. Compared to DEBS TE, Pik TE catalyzes the same analogues at a much higher rate than DEBS TE, especially for 12-membered rings, where in DEBS TE macrocyclization of 12-member macrolactone competes significantly with hydrolysis.[1] Therefore, Pik TE is more appealing for protein engineering experiments to produce a PKS TE with expanded substrate scope.

Other bacterial TEs have also been probed for substrate tolerance. Tyc TE, from the modular biosynthetic pathway that produces tyrocidine, was probed for its efficiency to catalyze different ring sizes.[99] However, only hydrolysis of the substrates was detectable, most likely due to the fact that the substrate channel of Tyc TE does not contain a hydrophobic environment. Addition of non-ionic detergents significantly improved the macrocyclization versus hydrolysis ratio and Tyc TE was found to be able to macrolactonize ring sizes varying from 6- to 14-amino acid residues. Furthermore the Tyc TE appears to be tolerant to changes in amino acid

composition within the peptide chain to be cyclized.[100] For example, a linear peptide containing the amino acid sequence of tyrocidine was built, with a library of amino acids included at the fourth amino acid position. The study compared the macrocyclization to hydrolysis ratio of each analogue. The results show that Tyc TE can macrocyclize many amino acid analogues, with a preference on the D-amino acid over the L-amino acid.

The TE responsible for the formation of the polyketide vicenistatin was also probed with analogues varying in ring sizes.[101] While the TE was able to efficiently macrocyclize the substrate to form vicenistatin, the other analogues had a 1:1 ratio of macrolactam formation and hydrolysis.

The epothilone TE (Epo TE) was also studied for substrate specificity.[83] Epo TE could only catalyze its native substrate, and even then hydrolysis versus macrocyclization was a major issue and it proved to be too substrate specific to be of any use to combinatorial biochemistry.

Our group also studied the substrate specificity for the pimaricin TE (pim TE).[102] The substrates built did not contain an accessible intramolecular electrophile and thus could not undergo macrocyclization, only hydrolysis. The analogues built had variation at the α and β positions of both stereochemistry and oxidation states. Most analogues underwent rapid hydrolysis, suggesting relaxed substrate specificity by Pim TE, at least for acyl-enzyme intermediate formation.

In contrast to bacterial TEs, there is very limited information on the substrate specificity of iterative fungal TEs. Since these TEs show little sequence homology to bacterial TEs, their substrate specificity may be significantly different from the bacterial TEs (Figure 1.12). To date, there has been no biochemical characterization of these TEs nor the corresponding substrate specificity for macrocyclization. However, it is believed that the cyclization electrophile and nucleophile seems to be of importance and making changes to them seem to decrease the ability of the TEs to

macrocyclize.[56] Iterative fungal PKSs in vivo have the ability to macrocyclize different chain lengths, just like modular bacterial TEs.[101]

The work presented in this thesis is the first in vitro characterization of macrocyclization by fungal TEs and shows without ambiguity that zearalenone (zea TE) is responsible for macrocyclization, as in other TEs.[53] Furthermore, the k_{cat}/K_M (specificity constant or pseudofirst-order rate constant) is 10 to 100 times greater than those seen for macrocyclization by the Pik TE, and 1000 times higher than those of DEBS and Epo TE. Since macrocyclization of zearalenone and related RALs are challenging to do chemically, chemoenzymatic synthesis of zearalenone and its analogs via TE-mediated macrocyclization may be a promising route to these compounds. Biochemically evaluating these RAL TEs and the substrate scope is thus a first step in this long-term goal. Herein we also show that the RAL TEs have broad substrate scope being able to macrocyclize ring sizes from 10- to 18-member rings. We show that these TEs are not sensitive to the absolute stereochemistry of the nucleophilic alcohols during macrocyclization and that the alcohol can be replaced by an amine to generate macrolactams. This work solidifies that the RAL TEs are one of the most broadly tolerant TEs yet characterized and opens the door to the use of TEs as catalysts in organic chemistry.

1.9 Conclusions

There is tremendous interest in discovering polyketide natural product analogues, especially given their current clinical successes and the improved or new pharmacological properties seen in semi-synthetic derivatives.[18, 28] Since polyketides share similar mechanisms in their biosynthesis there is great interest in rational design of combinatorial libraries through metabolic engineering of PKS pathways. Library generation, however, relies on systems with broad substrate tolerance and has not as yet been realized in PKS systems due to the lack of knowledge on the substrate specificity of many PKS catalytic domains, including the TE.[97,102] While bacterial PKS TEs have been extensively studied and have shown to be highly substrate

selective for the macrocyclization reaction in vitro, the low sequence homology of fungal TEs to bacterial TEs might indicate broader substrate tolerance. To this end, we have investigated the macrocyclization ability of zea TE and radicicol TE (rad TE) for varying chain lengths, stereochemistries and various nucleophiles. If proven to have broad substrate tolerance, these fungal TEs could be of great use in chemoenzymatic reactions as well as for the rational design of engineered polyketide biosynthetic pathways to produce a diverse set of compounds in a library.

Macrocyclization is important to render compounds bioactive.[100] Unfortunately, macrocyclization, including macrolactonization, is often challenging.[78, 79] Just by changing the functionality or ring size, a molecule that could previously macrolactonize is now unable to undergo macrocyclization in an efficient manner under the same chemical conditions[29]. This makes it harder to predict and build synthetic routes. If the zea or rad TE proves to have high substrate tolerance for different ring sizes, nucleophiles and stereochemistries, they could be used chemoenzymatically to effectively yield macrocycles.

1.10 References

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Chapter 2 Resorcylic acid lactone TEs can rapidly and exclusively macrocyclize simple 14-member ring thioesterase substrates

2.1 Introduction

2.1.1 Resorcylic acid lactones (RALs)

Resorcylic acid lactones (RALs) are a unique family of macrolides that possess a wide array of potent pharmaceutical properties, including HSP90 inhibition, MAP kinase inhibition, estrogen receptor agonist, MEK kinase inhibition, antimalarial and HSV inhibition (Figure 2.1).[1-5] Structurally, all RALs contain a resorcylate group (2,4-dihydroxybenzoate) and a 14-membered macrolactone. While all of the lactones are formed from secondary alcohols, the stereochemistry of the alcohols vary from compound to compound. This variable stereochemistry at the lactone alcohol is unique among polyketides and has major implications for the biosynthesis of the macrolactone.

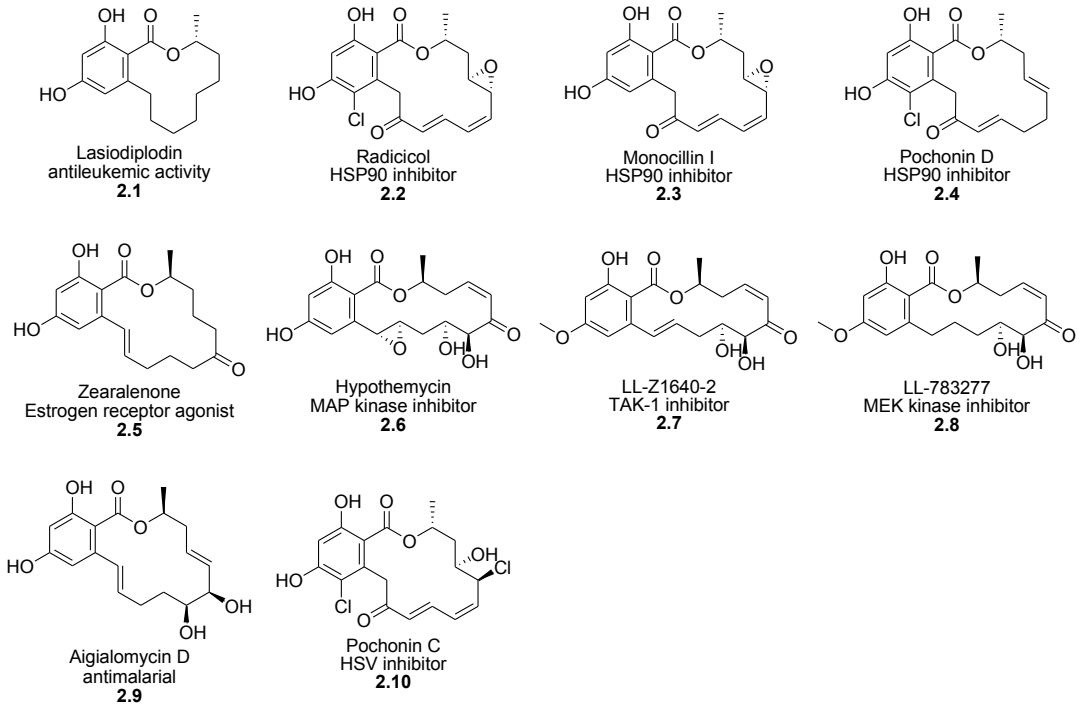


Figure 2.1 Different resorcylic acid lactones with differing biological activity.

The majority of RALs possess 14-membered lactone rings. The exception to this is lasiodiplodin, which possesses a 12-member macrolactone. This study focuses on two important RALs, zearalenone, an estrogen receptor agonist, and radicicol, an anticancer agent. These two compounds have the opposite stereogenic configurations at their respective lactone alcohols and thus can provide insight into how the enzymes that catalyze macrocyclization are influenced by absolute stereochemical configuration.

RAL biosynthetic pathways typically consist of two PKSs, a nonreducing PKS (nrPKS) and a highly reducing PKS (hrPKS), in their biosynthesis. The biosynthesis of zearalenone is archetypical of most resorcylic lactone compounds (Figure 2.2).[1, 3] The first PKS, an hrPKS, named PKS4, contains KS, AT and ACP, the minimal PKS condensation domains, as well as DH, ER and KR domains. The product from PKS4 is then transferred to PKS13, an nrPKS. PKS13 contains, in addition to the minimal domains, a putative SAT and PT, and a C-terminal TE

domain. Its role is to incorporate three malonyl-CoA units without reduction to give the complete acyl chain, and catalyze formation of the resorcylate moiety via an aldol condensation and tautomerization. Subsequently, the TE domain forms the 14-membered macrolactone via intramolecular macrolactonization. An FAD-dependent oxidation of the secondary alcohol found on the macrocycle yields zearalenone.

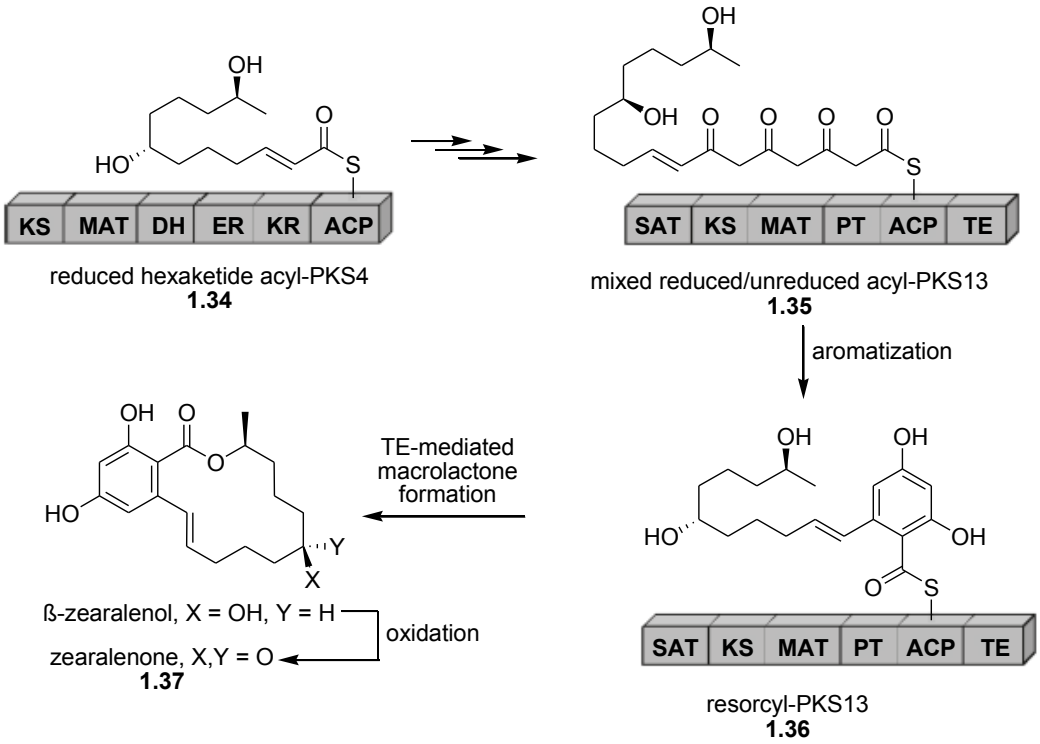


Figure 2.2 Biosynthesis of the fungal agent zearalenone, an archetypical resorcylic acid lactone.

2.1.2 PKS thioesterases

Macrocyclization in PKS biosynthesis is generally catalyzed by thioesterase domains (TEs). PKS TEs are structurally and functionally homologous to NRPS TEs. PKS TEs were the first catalytic domain for which crystal structures were obtained.[6] These TEs display an α/β hydrolase fold, making them a member of the α/β hydrolase superfamily of enzymes.[7] This fold consist of seven-stranded β-sheets in the center, with the second strand, called β2, antiparallel to the remaining strands.[8] In solution, some TEs, including DEBS and Pik TEs, are homodimers and contain a

hydrophobic substrate channel that completely traverses the TE domain.[9-11] TEs vary between 28-35 kDa and possess a Ser-His-Asp catalytic triad in their active site.

The primary function of TEs is to release the polyketide products and incorrectly processed intermediates from the PKS enzyme to which they are covalently linked. Release can occur by either inter- or intramolecular nucleophilic attack. In bacterial type I PKSs, the TE domains are found at the downstream C-terminus of the last PKS module and typically catalyze release of the final product, generating either a linear or cyclic product. When the attack is intramolecular, an amine or alcohol moiety on the polyketide itself will attack regiospecifically to cleave the acyl-enzyme intermediate, yielding a macrocycle. When intermolecular nucleophilic attack prevails, a water molecule attacks the acyl-enzyme intermediate, forming the free carboxylic acid. In some rare cases, an intermolecular amine or alcohol molecule is used to cleave the acyl-enzyme intermediate to form the amide or ester product. It is proposed that defined hydrophobic interactions in the substrate channel and binding cavity play a major role in influencing the regio- and stereospecificity of these reactions.

Similar to the bacterial type I PKS TEs, fungal iterative PKSs also catalyze product release via TE domains.[3, 12] Based on sequence alignment, the TE identified in the zearalenone biosynthetic pathway (zea TE) is likely a α/β hydrolase with a Ser-His-Asp catalytic triad. The regions around the catalytic triad and substrate channel are especially highly conserved among bacterial and fungal TEs. The residues that line and stabilize the hydrophobic channel are also highly conserved, suggesting that the open substrate channel is a conserved element among the various TEs.

While sequence alignment of fungal PKS TEs shows they are clearly members of the α/β hydrolase family, the fungal TEs do differ substantially from bacterial TEs. Alignment with both the DEBS TE and PICS TE show low sequence homology, with about 30% homology and 10% identity (Figure 2.3). This analysis

suggests that fungal TEs, such as zea TE may be unique in both some aspect of their tertiary structure and their function, such as specificity and selectivity.

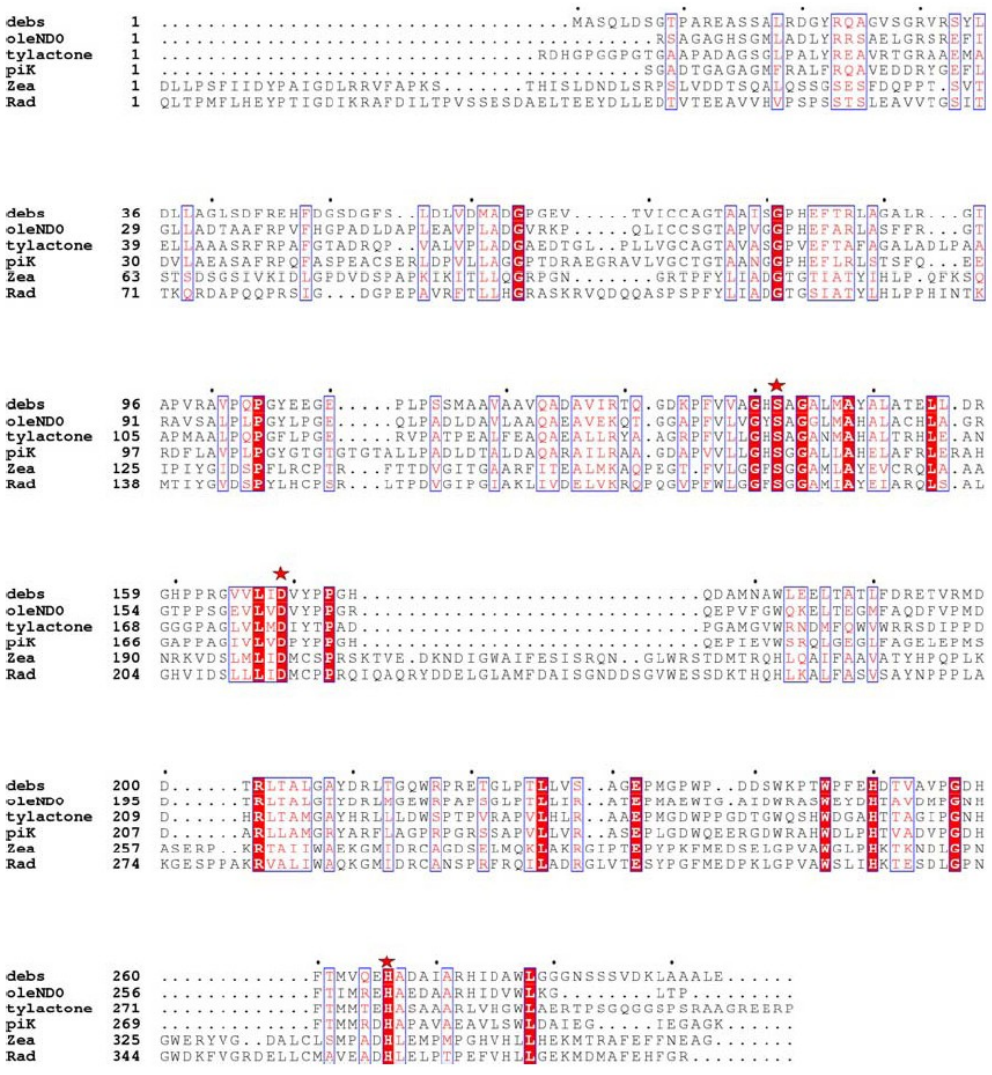


Figure 2.3 Sequence analysis of zea TE with other TEs, including DEBS and Pik, known to undergo macrocyclization as product release.

Herein we develop the reagents and assays required to evaluate the macrocyclization activity of fungal PKS TE. This chapter describes the synthesis of an analogue of the linear polyketide intermediate from both the zearalenone and radicicol biosynthetic pathways. We also develop a LC/MS/MS based assay to evaluate the efficiency of TE-mediated macrocyclization of this analogue by both zea

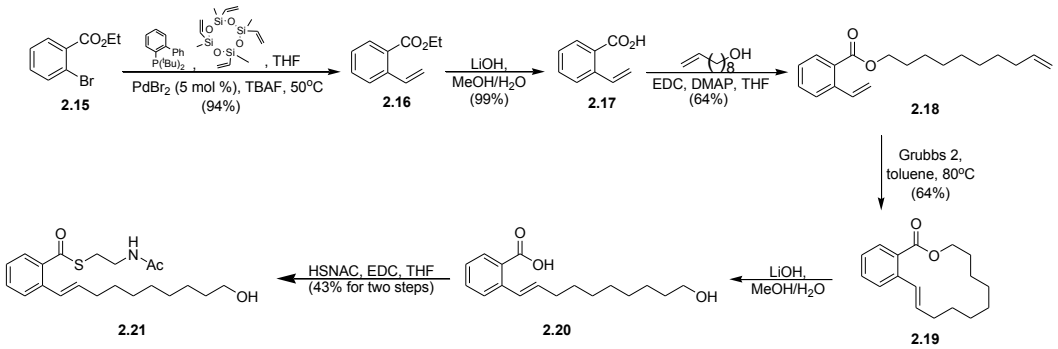
and rad TE. These results demonstrate that fungal TEs exhibit substantially different reaction rates and substrate specificities than bacterial macrocyclizing PKS TEs. This work sets the stage for further evaluation of the substrate specificity and tolerance of this class of TEs.

2.2 Results and discussion

Our first goal was to synthesize an analogue of the PKS intermediate which undergoes TE-catalyzed macrocyclization in the zearalenone and radicicol biosynthetic pathways. This analogue resembles zearalenone precursor, but is lacking the ketone moiety, the methyl group next to the alcohol and both phenols. These simplifications make the analogue highly synthetically tractable.

2.2.1 Synthetic route to 14-member analogue

To be able to probe the cyclization ability of zea and rad TE, an analogue resembling the acyl-enzyme intermediate of the biosynthesis was synthesized (Scheme 2.1). An N-acetylcysteamine (SNAC) side chain is used to mimic the substrate-enzyme thioester bond that is found at that step by the thioesterase.



Scheme 2.1 Reaction sequence to build the 14-member SNAC analogue.

The first step of the synthesis was to introduce the vinyl group to the aromatic ethyl ester core. While this transformation can be done by a Stille coupling, we chose to use the vinylation published by Denmark.[13] Not only is this reaction high yielding,

but it makes use of an inexpensive, non-toxic siloxane, in contrast to the highly toxic stannane require for the Stille coupling.[14] The desired styrene product is generated in 94% yield.

To complete the formation of the carbon backbone of the substrate, the ethyl ester was hydrolyzed to the corresponding carboxylic acid, using lithium hydroxide in a 3:1 mixture of methanol and water with quantitative yield. Following the hydrolysis, the ester derived from 9-decenol was generated following standard EDC and DMAP coupling conditions providing the ring closing metathesis precursor in 64%. Using the Grubbs II catalyst and heating in toluene at 80°C this linear diene was macrocyclized in 64% yield. These macrocyclization conditions are similar to those used by Fürstner and his group in their synthesis of zearalenone.[15]

To complete the synthesis of the substrate the lactone was ring opened using lithium hydroxide. The free carboxylic acid was then coupled to N-acetylcysteamine (SNAC) using EDC and DMAP. This gave the desired thioester activated enzyme substrate in a yield of 43% for the final two steps.

The reaction sequence was achieved in 6 steps with and overall yield of 16%. This concise synthesis makes use of an efficient ring closing metathesis reaction to form the macrocycle and a siloxane-mediated vinylation reaction to replace the commonly used Stille vinylation.

2.2.2 Enzymatic cyclization by Zea and Rad TE

With the 14-member thioester activated analogue in hand, it was possible to evaluate the ability of the zea TE and the rad TE to catalyze macrocyclization. The thioester substrate was incubated for 30 minutes with either the zea TE or the rad TE and LC/MS/MS analysis was performed to determine if macrocyclization had occurred (Figure 2.4 and 2.5 respectively). Incubation with both enzymes led to the

14-member macrocyclization product, with no detectable carboxylic acid **2.20** as a by-product.

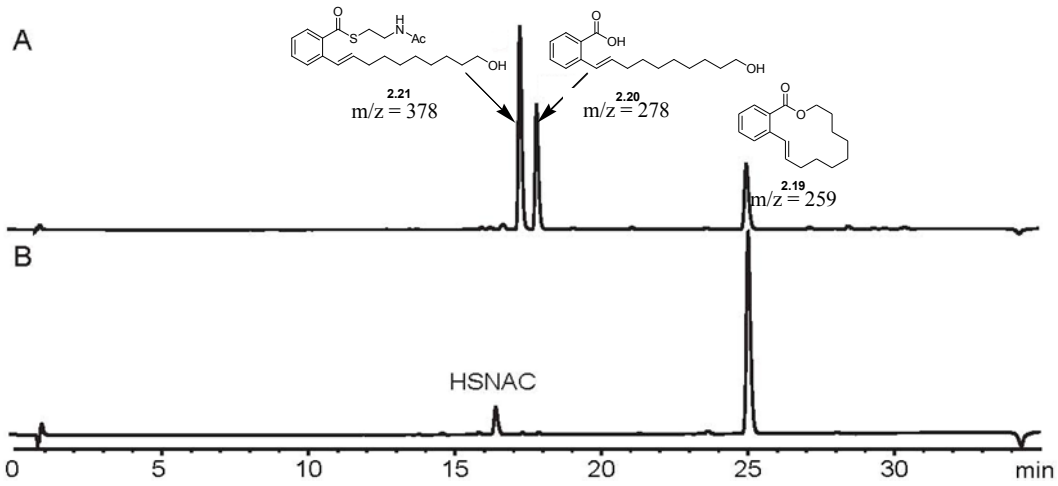


Figure 2.4 LC/MS/MS analysis of zea TE incubation to macrocyclize 14-member rings run on the API2000. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN) **A**: LC/MS/MS analysis of authentic standards. **B**: LC/MS/MS analysis of a 30 min incubation of 15 µM zea TE with 5 mM **2.21** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

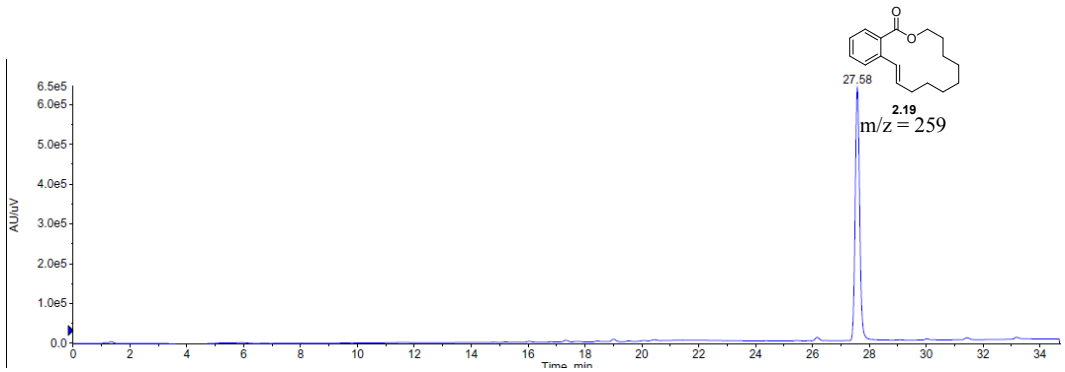


Figure 2.6 LC/MS/MS analysis of rad TE to macrocyclize 14-member rings. Chromatograms are absorbance at 210 nm. LC/MS/MS analysis of a 30 min incubation of 15 µM Rad TE with 5 mM

2.21 at pH 7.4, 23°C. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN). Identity of by-products were determined by ESI-MS analysis.

These results clearly indicate that both zea TE and rad TE can catalyze macrocyclization of this 14-member ring substrate. These results suggest that neither the ketone functionality, the aromatic alcohols, nor the methyl group α to the lactone are necessary for substrate recognition by these TEs. These results are in contrast to those seen for bacterial macrocyclizing TEs such as the DEBS TE. In the case of the DEBS TE, it has been shown that simple substrates, lacking functional groups found in the native substrate are not macrocyclized.[16-21] Thus in comparison to bacterial macrocyclizing TEs, the fungal TEs investigated herein appear to have much broader substrate tolerance.[22-24]

In addition to their enhanced substrate tolerance, the fungal TEs investigated do not catalyze non-productive hydrolysis of the activated thioester substrate to the free carboxylic acid. Work with the Epothilone (Epo) TE, and TEs from many NRPS and PKS pathways, have shown that the carboxylic acid is frequently a significant side product.[25, 26] Hydrolysis presumably occurs when water can access the active site and can out-compete the native nucleophile for attack on the acyl-enzyme intermediate. The ability of both zea and rad TE to exclusively macrocyclize the acyl-enzyme intermediate, with no detectable hydrolysis, is fairly unique and suggests that these TEs may ultimately be useful tools in chemoenzymatic macrocyclization.

The kinetic parameters were determined for the zea TE mediated macrocyclization. Kinetics were determined using a continuous spectrophotometric assay monitoring for the release of free thiol via reactivity with Ellman's reagent.[23] The results obtained showed that zea TE catalyzes macrocyclization with a specificity constant of $k_{cat}/K_M = 2.92 \pm 0.16 \times 10^3\ M^{-1}s^{-1}$. This data was collected by Meng Wang[12]. Due to the limited solubility of the reagent, k_{cat} and K_M could not be

determined independently. Similar to the TE from the pikromycin biosynthetic pathways, the rad TE is inactivated by Ellman's reagent, and thus it could not be kinetically characterized using this assay. Examination by HPLC analysis of the initial velocities at a single substrate concentration for both zea TE and rad TE catalyzed macrocyclization, shows that the 14-membered ring macrocycle is produced approximately 3 times faster than rad TE to the zea TE. In comparison to bacterial macrocyclizing TEs, these fungal TEs are kinetically more competent with specificity constant that are ten to one hundred times greater than those seen for Pik TE (k_{cat}/K_M = 1.67 ± 0.027 $mM^{-1}min^{-1}$), and one thousand times greater than those seen for DEBS (k_{cat}/K_M = 1.5 ± 0.8 $M^{-1}s^{-1}$) and Epo TE (k_{cat}/K_M = 0.41 ± 0.03 $mM^{-1}min^{-1}$).[16, 25, 27]

2.3 Conclusions

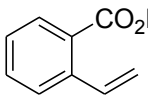
In conclusion, both rad TE and zea TE efficiently macrocyclize a simplified model of the native substrate. These results show that limited structural complexity is required for substrate recognition and processing by the TEs. Furthermore, enzymatic hydrolysis is not observed by HPLC. Instead, only macrocyclization occurs, with specificity constant much faster than bacterial macrocyclizing TEs studied to date. The start contrast between the specificity and reaction rates of the fungal TE studied to date, suggest that these two classes of TEs will likely behave very differently and thus fungal TEs should be further studied. Lastly our results indicate that RAL TEs have the potential to be useful in chemoenzymatic macrocyclization reactions, as well as used in building combinatorial macrocycle libraries.

2.4 Experimental section

2.4.1 General methods

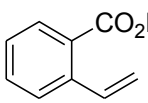
All reactions were carried out under nitrogen or argon atmosphere with dry solvents under anhydrous conditions in oven-dried glassware equipped with a magnetic stir bar and rubber spectrum. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reagents were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light (254 nm) as a visualizing agent and cerium molybdate stain and heat as developing agent. E. Merck silica gel 60 (230-400 mesh) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254). IR spectra were collected with neat thin films on sodium chloride disk using a Bomem Michaelson 100 Fourier transform infrared (FTIR) spectrometer. ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, NOSEY, and COSY spectra were recorded on a Brucker AVANCE 300 MHz or 400 MHz spectrometer. Deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) were used as NMR solvents unless otherwise noted. All chemical shifts are reported in parts per million (δ), integration and coupling constant(s) in Hz downfield from TMS and corrected using the solvent residual peak as internal standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, br = broad, bs = broad singlet. Mass spectra were recorded on an Applied Biosystem API2000 triple quadrupole mass spectrometer using electrospray ionization. High resolution mass spectra (HRMS) were collected by positive ion electrospray on Kratos Analytical Concept-11A mass spectrometer with an electron beam of 70ev (Ottawa-Carleton Mass Spectrometry Centre). LC/MS/MS was collected with an Agilent 1100 quadrupole ion trap mass spectrometer or an Applied Biosystems API 2000 triple quadrupole ion trap mass spectrometer using electrospray ionization interfaced with a Shimadzu UFLC. A Hypersil C_{18} 100 × 2.1 mm, 3 μM particle size column was used with both instruments.

2.4.2 Experimental procedures



2.16

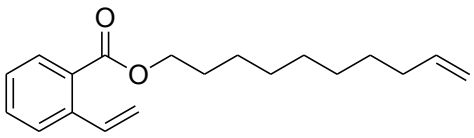
2-vinyl-benzoic acid ethyl ester (2.16). 2.16 was prepared following the procedure described by Denmark et al.[13] (2-biphenyl)di-tert-butylphosphine (94 mg, 0.31 mmol, 10 mol %) and palladium (II) bromide (42 mg, 0.16 mmol, 5 mol %) were added to a 50 mL round-bottom flask with a stir bar under argon atmosphere. The flask was evacuated and refilled with argon three times. 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane (544 µL, 1.57 mmol, 0.5 equiv.) was then added, followed by tetrabutylammonium fluoride (6.4 mL, 1 M in THF, 2 equiv.) and THF (0.16 mL) and the resulting mixture was stirred for 10 minutes. Ethyl 2-bromobenzoate (500µL, 3.15 mmol, 1 equiv.) was then added and the reaction was warmed to 50°C in an oil bath for 7 hours. Once the reaction was judged complete by TLC, the reaction was put to room temperature and 15 mL of ether was added. The resulting mixture was stirred for 10 minutes before being filtered through silica and washed with ether (50 mL). The reaction mixture was evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 4% ethyl acetate in hexanes) to afford a light orange oil (523.5 mg, 2.97 mmol, 94%). Observed ^{1}H agrees with literature values.[13] R_f = 0.35 (silica gel, 9:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (ddd, *J* = 7.8, 1.4, 0.3 Hz, 1 H), 7.56 (ddd, *J* = 8.0, 0.7, 0.6 Hz, 1 H), 7.46 (t, *J* = 7.9 Hz, 1 H), 7.44 (dd, *J* = 17.6, 11.0 Hz, 1 H), 7.30 (td, *J* = 7.7, 1.3 Hz, 1 H), 5.63 (dd, *J* = 17.4, 1.3 Hz, 1 H), 5.33 (dd, *J* = 11.0, 1.3 Hz, 1 H), 4.35 (q, *J* = 7.2 Hz, 2 H), 1.38 (t, *J* = 7.1 Hz, 3 H).



2.17

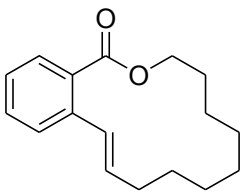
2-Vinyl benzoic acid (2.17). To **2.16** (469 mg, 2.66 mmol, 1 equiv.) in methanol (30 mL) at room temperature was added lithium hydroxide mohohydrate (1.06 g, 25.27 mmol, 9.5 equiv) that was first dissolved in water (10 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached a pH of 2 and extracted with EtOAc (3 × 20 mL). The organic layer was washed with brine (70 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford yellow crystals (388.9 mg, 2.63 mmol, 99%). Observed ^{1}H agrees with literature values.[28] R_f = 0.18 (silica gel, 1:1

hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.99 (bs, 1 H); 8.09 (dd, *J* = 7.8, 1.4 Hz, 1 H), 7.63-7.51 (m, 3 H); 7.35 (ddd, *J* = 7.9, 7.3, 1.0 Hz, 1 H), 5.66 (dd, *J* = 17.4, 1.3 Hz, 1 H), 5.38 (dd, *J* = 11.1, 1.3 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 140.6, 136.1, 133.1, 131.3, 127.5, 127.5, 127.2, 116.7.



2.18

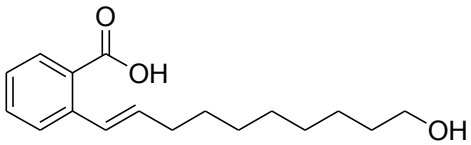
2-Vinyl benzoic acid dec-9-enyl ester (2.18) To **2.17** (147 mg, 0.99 mmol, 1 equiv) in THF (5 mL) under argon atmosphere was added 9-decen-1-ol (224 μL, 1.26 mmol, 1.5 equiv.) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (225 mg, 1.17 mmol, 1.4 equiv.) before addition of 4-(dimethylamino)pyridine (20 mg, 0.17 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with 10% HCl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a colorless oil (173 mg, 0.64 mmol, 64%). Observed ^{1}H agrees with literature values.[15] R_f = 0.74 (silica gel, 9:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.56 (d, *J* = 7.9 Hz, 1 H), 7.50-7.38 (m, 2 H), 7.30 (ddd, *J* = 7.7, 7.4, 0.9 Hz, 1 H), 5.79 (ddt, *J* = 17.1, 10.3, 6.5 Hz, 1 H), 5.63 (dd, *J* = 17.4, 1.3 Hz, 1 H), 5.33 (dd, *J* = 10.9, 1.3 Hz, 1 H), 4.98 (ddt, *J* = 17.1, 2.0, 1.7 Hz, 1 H), 4.92 (ddt, *J* = 10.3, 1.1, 1.1 Hz, 1 H), 4.29 (t, *J* = 6.8 Hz, 2 H), 2.08-1.97 (m, 2 H), 1.80-1.62 (m, 2 H), 1.48-1.21 (m, 10 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 139.5, 139.1, 136.0, 132.0, 130.2, 129.1, 127.4, 127.2, 116.3, 114.2, 65.2, 33.8, 29.4, 29.2, 29.0, 28.9, 28.7, 26.1.



2.19

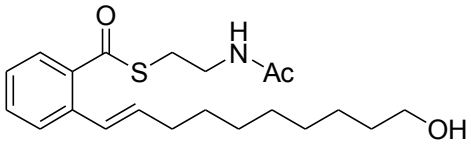
7,8,9,10,11,12,13,14-octahydro-6-oxa-benzocyclotetradecen-5-one (2.19) 2.19 was prepared following the procedure described by Fürstner et al.[15] To **2.18** (173 mg, 0.64 mmol, 1

equiv.) in toluene (151 mL) was added Grubbs catalyst 2nd generation (27 mg, 0.03 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 30:1 hexanes/ethyl acetate) to afford **2.19** (105 mg, 0.41 mmol, 64 %) as a colourless oil. R_f = 0.78 (silica gel, 9:1 hexanes/EtOAc); IR (thin film) ν_{max} = 2930, 2853, 1705, 1200, 1254 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 7.8, 1.3 Hz, 1 H), 7.48 (ddd, *J* = 7.8, 1.3, 0.6 Hz, 1 H), 7.41 (dddd, *J* = 7.8, 7.4, 1.4, 0.5 Hz, 1 H), 7.26 (ddd, *J* = 7.7, 7.6, 1.2 Hz, 1 H), 6.91 (d, *J* = 15.7 Hz, 1 H), 5.93 (dt, *J* = 15.7, 7.2 Hz, 1 H), 4.34 (t, *J* = 5.2 Hz, 2 H), 2.34-2.24 (m, 2 H), 1.80-1.68 (m, 2 H), 1.59-1.46 (m, 4 H), 1.45-1.28 (m, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 138.4, 133.2, 131.7, 130.6, 130.1, 129.4, 127.3, 126.7, 65.5, 30.7, 27.2, 26.7, 26.6, 24.2, 23.9, 23.2; HRMS (+EI) : calcd for $C_{17}H_{22}O_2$ (M) 258.1620, obsd 258.1618.



2.20

2-(10-Hydroxy-dec-1-enyl)-benzoic acid (2.20). To **2.19** (105 mg, 0.41 mmol, 1 equiv.) in methanol (9 mL) at room temperature was added lithium hydroxide mohohydrate (162 mg, 3.86 mmol, 9.5 equiv) that was first dissolved in water (3 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached a pH of 2 and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and carried over to the next step without further purification.



2.21

2-(10-Hydroxy-dec-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (2.21) To **2.20** (112 mg, 0.41 mmol, 1 equiv) in THF (30 mL) at 0°C under argon atmosphere was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (203 mg, 1.06 mmol, 2.6 equiv.) before the addition of N-acetylcysteamine (99 µL, 0.94 mmol, 2.3 equiv.).

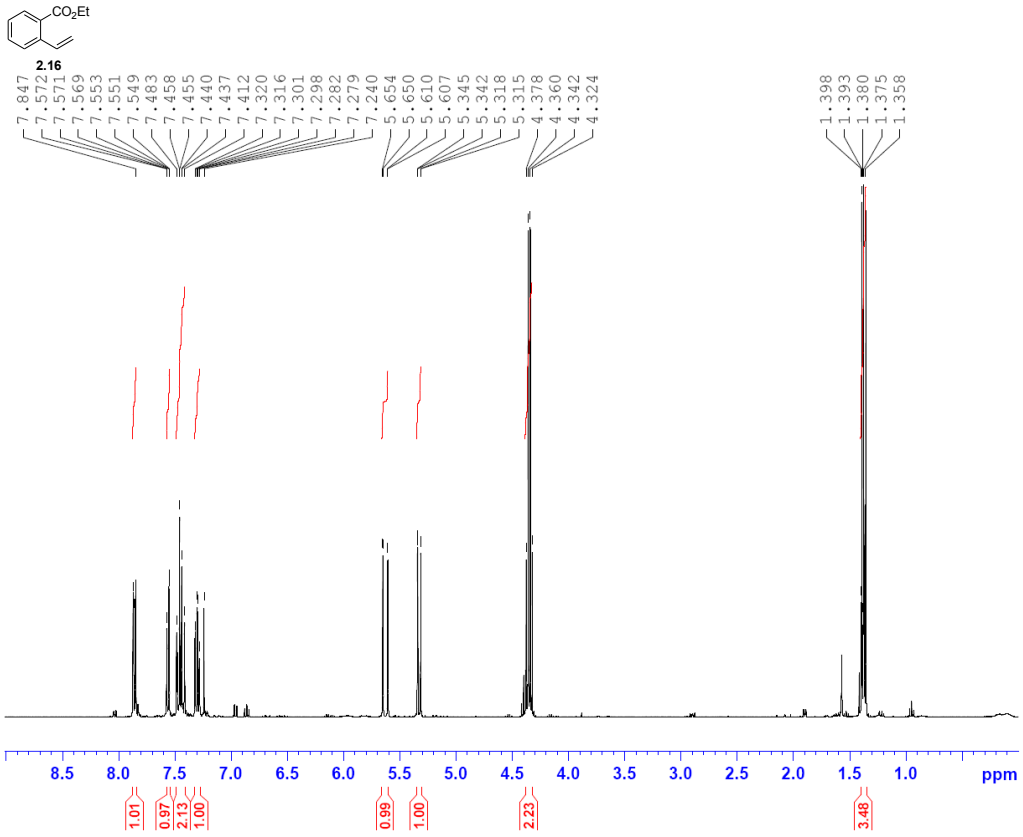
The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated ammonium chloride solution (30 mL) and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 60% acetone in hexanes) to afford a colourless oil (66.5 mg, 0.18 mmol, yield for two steps 43%) R_f = 0.23 (silica gel, 1:1 hexanes/acetone); IR (thin film) ν_{max} = 2930, 2853, 1705, 1200, 1254 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 7.8, 1.1 Hz, 1 H), 7.53 (d, *J* = 7.8 Hz, 1 H), 7.42 (ddd, *J* = 7.7, 7.5, 0.7 Hz, 1 H), 7.24 (ddd, *J* = 7.7, 7.4, 1.2 Hz, 1 H), 6.76 (d, *J* = 15.7 Hz, 1 H), 6.16 (dt, *J* = 15.7, 6.9 Hz, 1 H), 6.00 (bs, 1 H), 3.62 (t, *J* = 6.6 Hz, 2 H), 3.52 (dt, *J* = 6.3, 6.1 Hz, 2 H), 3.18 (t, *J* = 6.3 Hz, 2 H), 2.24-2.16 (m, 2 H), 1.96 (s, 3 H), 1.59-1.41 (m, 6 H), 1.38-1.21 (m, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.8 170.3, 136.7, 135.8, 134.8, 132.0, 128.4, 127.1, 127.0, 126.6, 63.0, 39.8, 33.1, 32.8, 30.9, 29.3, 29.1, 29.0, 25.7, 23.3, 14.1; LRMS (+APCI) : calcd for $C_{21}H_{31}NO_3S$ (M+H) 378, obsd 378

2.4.3 Enzymatic protocol

Assay of enzymatic catalyzed macrocyclization reaction

For a typical assay, a 200 µL solution containing 50 mM sodium phosphate pH 7.43, 5 mM thioester substrate **2.21**, 15 µM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1:4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

2.4.4 Spectra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7.301
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7.279
7.240
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5.650
5.610
5.607
5.345
5.342
5.318
5.315
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4.360
4.342
4.324
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1.393
1.380
1.375
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7.0
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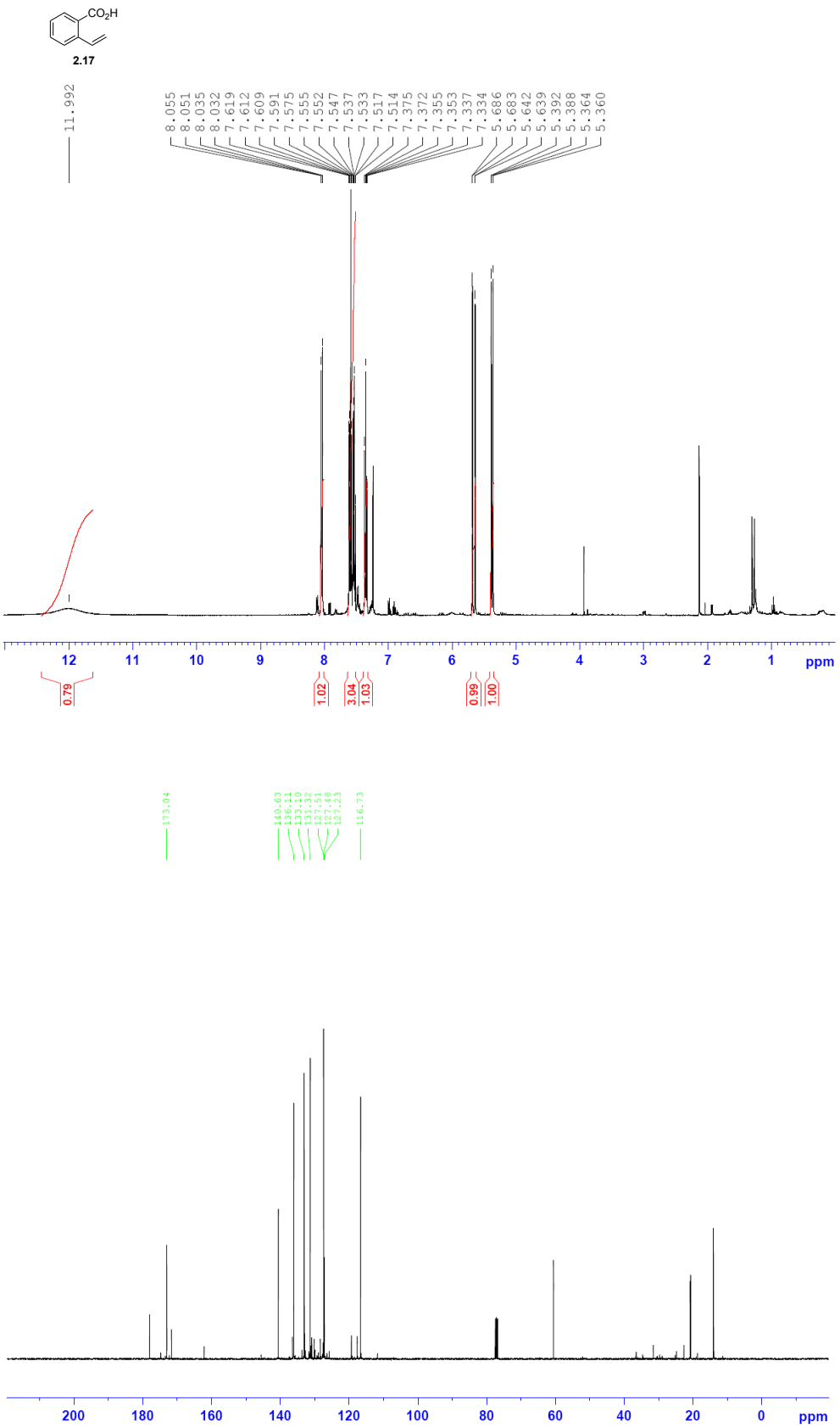
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7.552
7.547
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7.353
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5.642
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1.00
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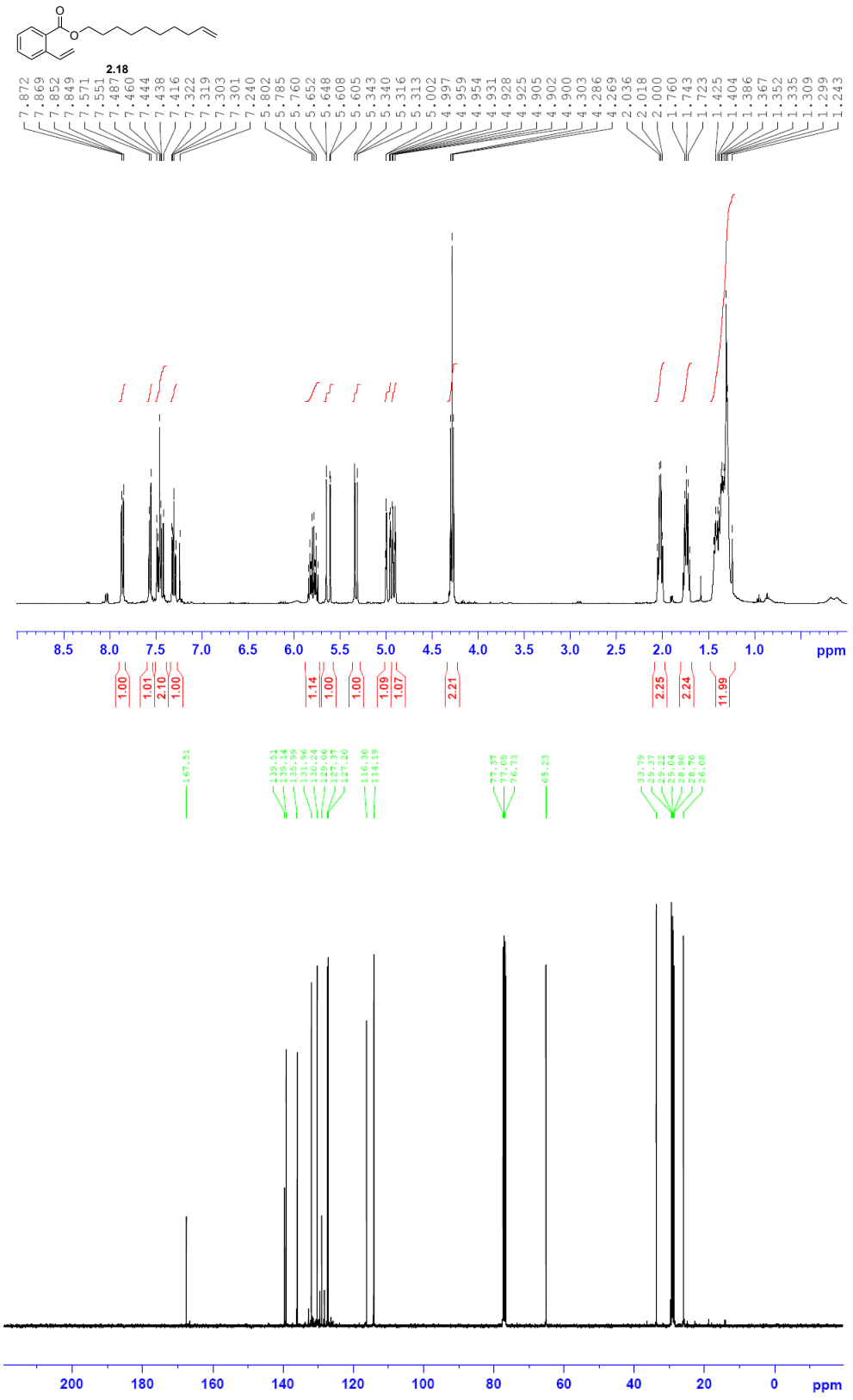
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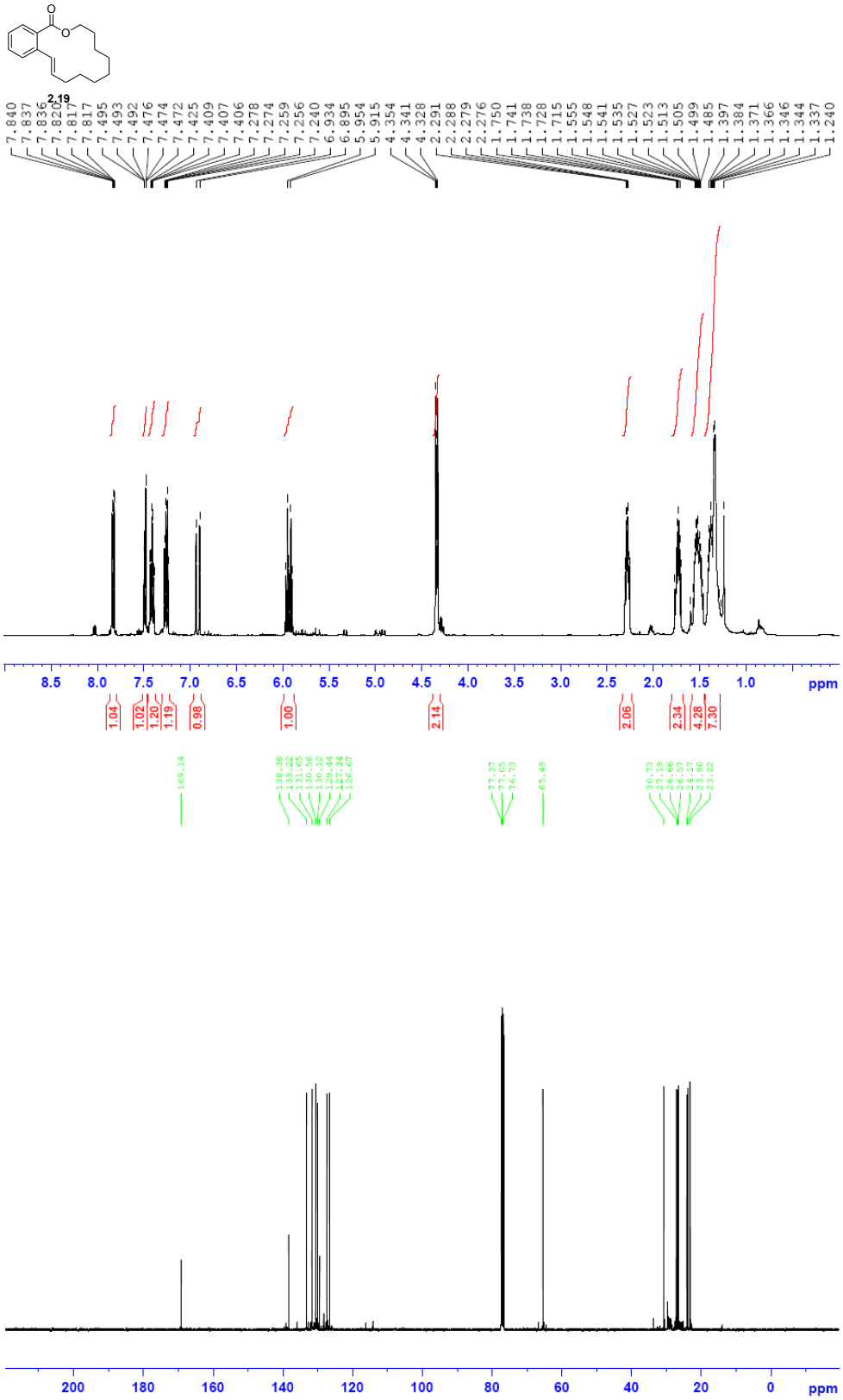
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7.407
7.406
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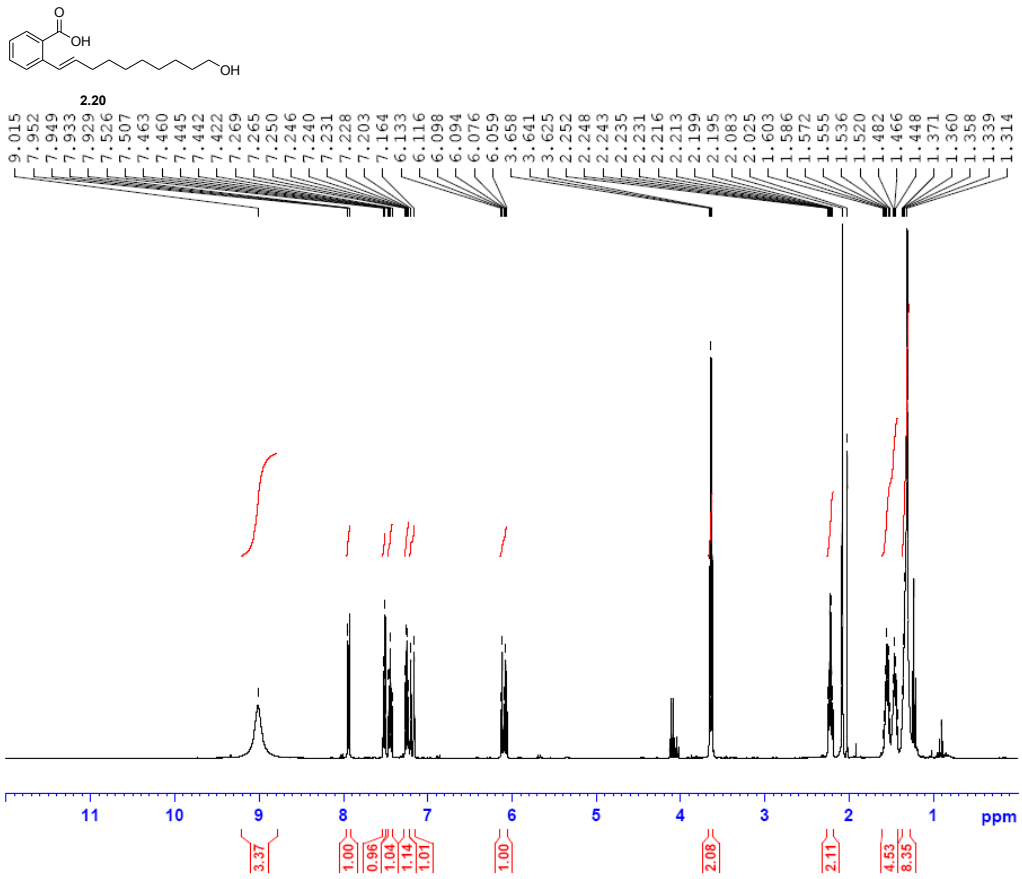
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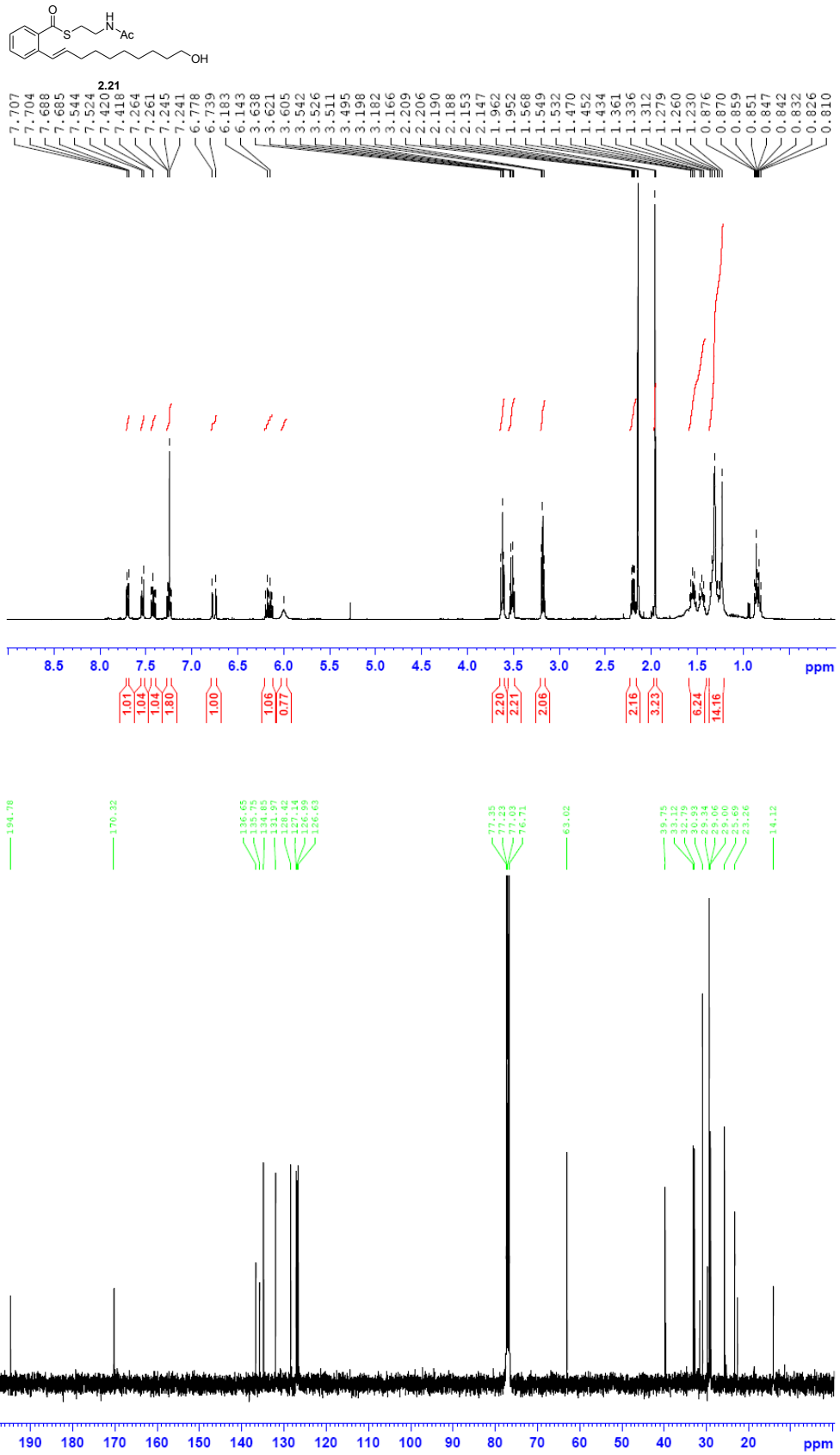
8.35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6.183
6.143
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3.621
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3.526
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3.495
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1.230
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0.870
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0.832
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0.810
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170.32
136.65
135.75
134.85
131.97
128.42
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63.02
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32.79
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23.26
14.12
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2.5 References

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Chapter 3 Resorcylic acid lactones TEs can macrocyclize substrates of varying ring sizes

3.1 Introduction

Understanding TE substrate specificity is an extremely important goal in characterizing and utilizing polyketide biosynthesis. Macrocyclization by TEs is highly biologically relevant since many polyketide and non-ribosomal peptide natural products are only active in their macrocyclized form.[1, 2] In the context of polyketide biosynthetic engineering, it is a waste of time, money and effort to engineer a PKS to produce a new compound or libraries of compounds, if macrocyclization and product release step from the final ACP is not possible due to limited TE substrate specificity. Because TE substrate specificity plays a key role in the activity of the resulting natural product, turn-over of the native biosynthetic enzymes, and efforts to engineer PKS systems, these enzymes have been extensively studied over the past ten years.[3, 4]

3.1.1 TEs can catalyze macrocyclization and hydrolysis

Substrate specificity of PKS, NRPS, and fatty acid synthase (FAS) TEs have been examined and in some cases the molecular interactions between substrate and enzyme governing substrate specificity have been characterized. For example in NRPS and FAS TEs, the substrate enzyme interaction is driven by hydrophobic interactions between the substrate alkyl side chains and the hydrophobic residues in the binding cavity.[5, 6] While a clear understanding of PKS TE substrate-enzyme interactions, especially those involved in controlling macrocyclization, is yet to emerge, it appears that hydrophobic interactions also play a major role in PKS TE substrate specificity. Characterizing the different molecular interactions that lead to substrate specificity will render rational substrate specificity engineering possible, leading to the formation of non-native compounds.

TE-catalyzed macrocyclization occurs in a two step process, the first step involves the formation of the acyl-enzyme intermediate, which is followed by cleavage of the acyl-enzyme bond. Macrocyclization occurs when the nucleophilic heteroatom is appropriately positioned to attack the acyl enzyme intermediate in the active site cavity. When no intramolecular nucleophile is positioned to attack the acyl-enzyme intermediate, due either to substrate specificity limitations to lack of an intramolecular nucleophile in the substrate, the most likely outcome is water-mediated hydrolysis.[7] In some cases, such as some NRPS TEs, the acyl-enzyme intermediate is stable and remains attached to the megasynthase, inhibiting product formation.[5, 8]

TE-catalyzed hydrolysis can often compete with macrocyclization activity. For example, the epothilone TE (epo TE) was also shown to macrocyclize the *N*-acetylcysteamine thioester of its native substrate in vitro and subsequently hydrolyze the macrocyclization product (Figure 3.1).[9] As high levels of concomitant hydrolysis render these PKS TEs unsuitable for chemoenzymatic synthesis of macrocycles, a number of approaches have been investigated to reduce hydrolysis and promote macrocyclization.[9, 10] Most promising has been the observation that non-ionic detergents can shift the outcome in favour of macrocyclization formation.[10, 11]

3.1.2 Limited data on the ability of PKS TEs to catalyze macrocyclization of varying ring sizes in vitro

A number of studies have focused on the ability of NRPS TEs to produce macrolactams of varying ring size. One of the best studied of these NRPS TE systems is the GrsB TE, which produces gramicidin S (Figure 3.1).[12, 13] The GrsB TE was found to be able to macrolactonize ring sizes varying from 6 amino acid residues to 15 amino acid residues (18- to 45-member rings) in vitro. Another NRPS study with Tyc TE, which produces tyrocidine, was probed for its efficiency to catalyze different ring sizes(Figure 3.1).[12] However, only hydrolysis of the substrates was detectable, most likely due to the fact that the substrate channel of Tyc TE does not

contain a hydrophobic environment. Addition of non-ionic detergents significantly improved the macrocyclization versus hydrolysis ratio and Tyc TE was found to be able to macrolactonize ring sizes varying from 6- to 14-member rings. Another study on Tyc TE studied the ability to macrocyclize different amino acids analogues.[14] A linear peptide containing the amino acid sequence of tyrocidine was built, with differences at the fourth amino acid position. The study compared the macrocyclization to hydrolysis ratio of each analogue. The results show that Tyc TE can macrocyclize many amino acid analogues, with a preference on the D-amino acid over the L-amino acid.

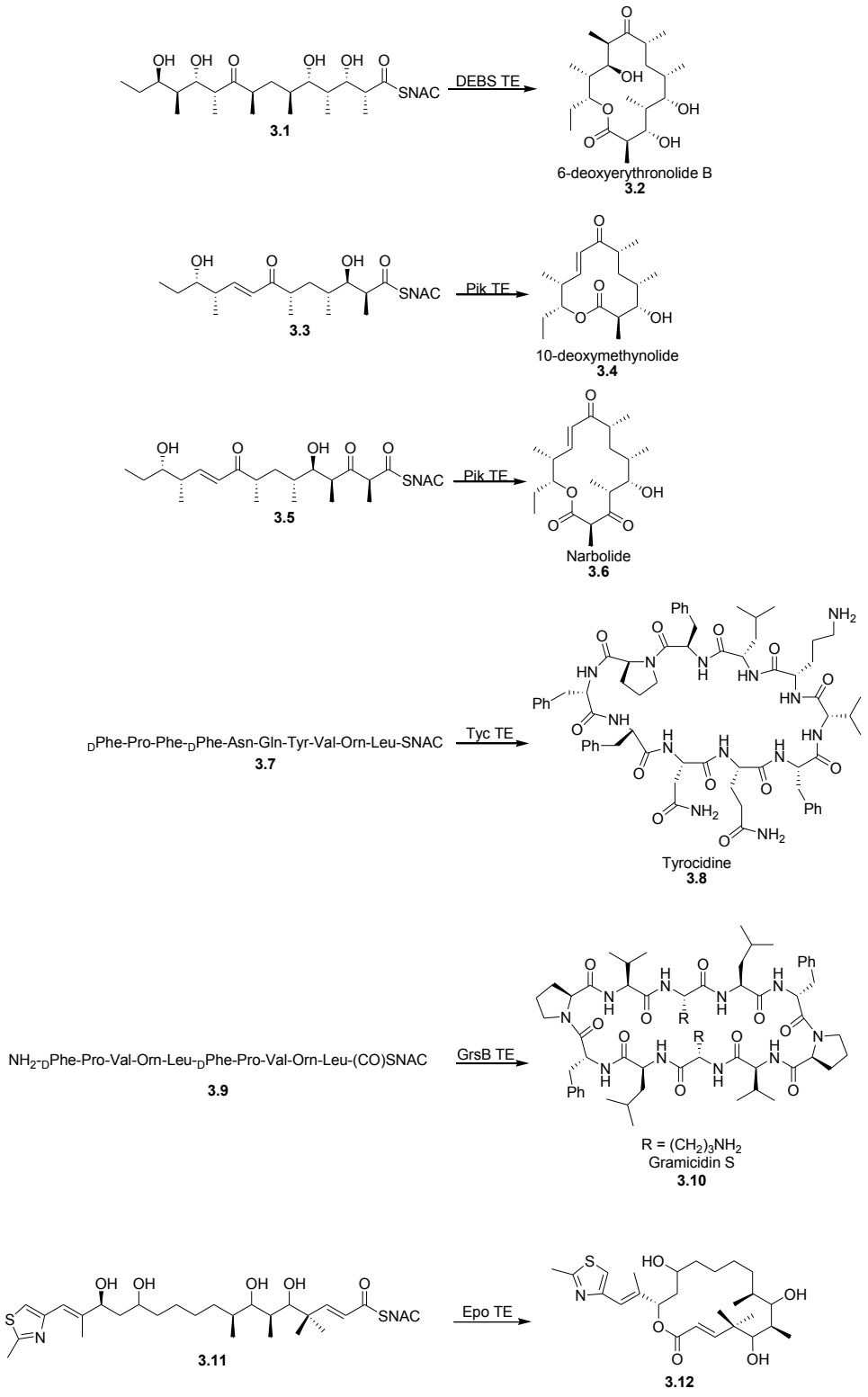


Figure 3.1 Different PKS TEs that were probed for their substrate specificity of varying ring sizes.

Less is known about the ability of PKS TEs to catalyze macrolactonization of varying ring sizes. Khosla was able to demonstrate in vivo that the DEBS TE can macrocyclize 6-, 8-, 12-, 14- and 16-membered rings (Figure 3.1).[2, 15] These results suggest that in vivo DEBS TE has tolerance for varying ring sizes, as long as the native substitution pattern for 6-deoxyerythronolide is adhered to. The pikromycin biosynthetic pathway naturally generates two macrocyclic products, 10-deoxymethynolide, a 12-member ring, and narbolide, a 14-member ring (Figure 3.1).[16-19] Since both products are generated via the same TE domain, this suggests that Pik TE has inherent substrate tolerance for varying chain lengths.

This is confirmed when looking at the substrate channel of the crystal structure of Pik TE, which indicates that the channel provides a hydrophobic environment for the hydrophobic acyl substrates. However, the size, the hydrophobic surface of the channel and especially the deficiency of hydrogen bonding groups suggests that it can accept acyl chains containing a wide variety of lengths and conformations, thus being poorly substrate specific. Like DEBS TE, Pik TE will macrocyclize 6-, 8-, 12-, 14- and 16-member rings, but as of yet, no 10-membered rings have been successfully cyclized.[9]

In vitro, there has been limited characterization of the ability of PKS TEs to catalyze formation of macrocycles of varying sizes. Both DEBS and Pik TEs can catalyze formation of the 12-member macrocycle 10-deoxymethynolide.[20] However, the vast majority of non-native substrates are not macrocyclized by these PKS TEs.

3.1.3 Ability of fungal TE to catalyze macrocyclization of varying ring sizes is an unaddressed question in the field

In contrast to modular bacterial PKS TEs, iterative fungal PKS TEs have barely been studied for substrate specificity. Since they show little sequence homology to bacterial TEs, their substrate specificity may be significantly different

from the bacterial TEs. So far, however, very little is known about what controls macrocyclization activity and substrate specificity. Iterative fungal PKSs have the ability to macrocyclize different chain lengths, just like modular bacterial PKS TEs.[21]

Macrocyclization seem to be important to render compounds bioactive.[14] Unfortunately, macrocyclization, including macrolactonization, is often challenging and selective. Often, just by changing the functionality or ring size, a molecule that could previously macrolactonize is now unable to undergo macrocyclization in an efficient manner.[22-24] This makes it harder to predict and build synthetic routes. If the fungal PKS TEs, zea TE or rad TE, prove to have high substrate tolerance for different ring sizes, they could be used chemoenzymatically to effectively yield macrocycles.

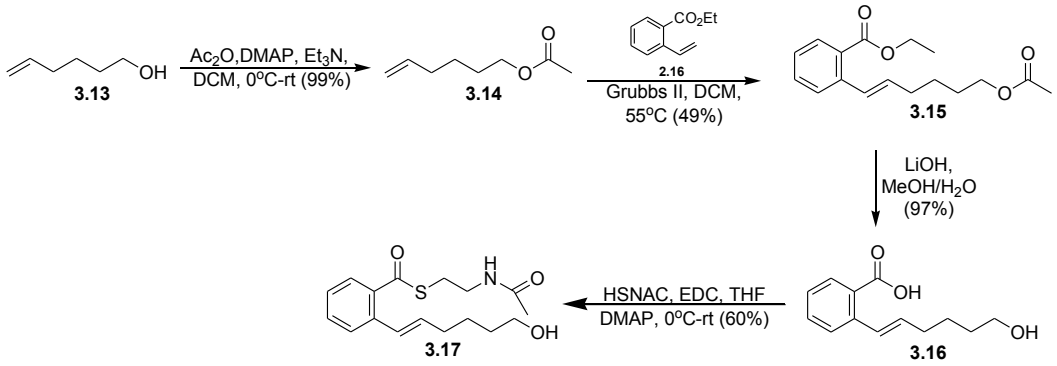
To investigate the ability of fungal PKS TEs to macrocyclize substrates with differing ring size, analogues of varying chain lengths were synthesized and probed for in vitro cyclization by both rad TE and zea TE. If the TEs display broad substrate tolerance, then cyclization should occur. However, if the reaction is slow, or if it shows high substrate specificity to the native substrate, then either hydrolysis or glycerolysis should be the main products observed. If broad substrate tolerance for these substrates is found, these TEs could be a tool to aid in macrocyclization of molecules that have been shown to undergo difficult macrolactonization in the past. This is especially true of 10-member rings, which are known to often be low yielding in chemical synthesis.

3.2 Results and discussion

3.2.1 Enzymatic cyclization of 10-member ring

The synthesis of the linear 10-member ring SNAC substrate followed the route shown in Scheme 3.1. This method is different to the synthesis of the 14-member analogue, since the ring-closing metathesis reaction to form the 10-member

ring did not occur. Instead, we used a route that utilized a cross-metathesis reaction to avoid having to form a 10-member ring as it proved exceedingly difficult to achieve synthetically.



Scheme 3.1 Reaction sequence to build the 10-member SNAC analogue.

The first step of the synthesis was to protect the alcohol so that the cross-metathesis could be achieved in good yield. This was done by acetylating the alcohol by using acetic anhydride and triethylamine. This was achieved in good yield, and gave the protected alcohol quantitatively.

The next step was a cross-metathesis reaction using Grubbs II.[25] The reagents were dissolved in methylene chloride and the reaction was heated at 55°C for 24 hours. The reaction was only moderate yielding, at 49%. However, considering that ring closing metathesis did not provide any product, this moderate yield was a huge improvement.

The final two steps of the synthesis follow that of the 14-member ring analogue. Hydrolysis was performed using lithium hydroxide in methanol and water followed by SNAC coupling with EDC and DMAP.

The analogue was generated in 4 steps, with an overall yield of 28%. With the SNAC analogue material in hand we investigated the ability of zea TE and rad TE to macrocyclize this substrate. The SNAC substrate was mixed with recombinant

purified zea and rad TEs for 24 hours. The reaction mixtures were then analyzed by LC/MS/MS (Figure 3.2). The reaction with zea TE showed trace cyclized product and hydrolysis by-product. There was still significant amount of starting material, but the main by-product was the glycerolysis product. This comes from the small amount of glycerol present from the enzyme preparation.

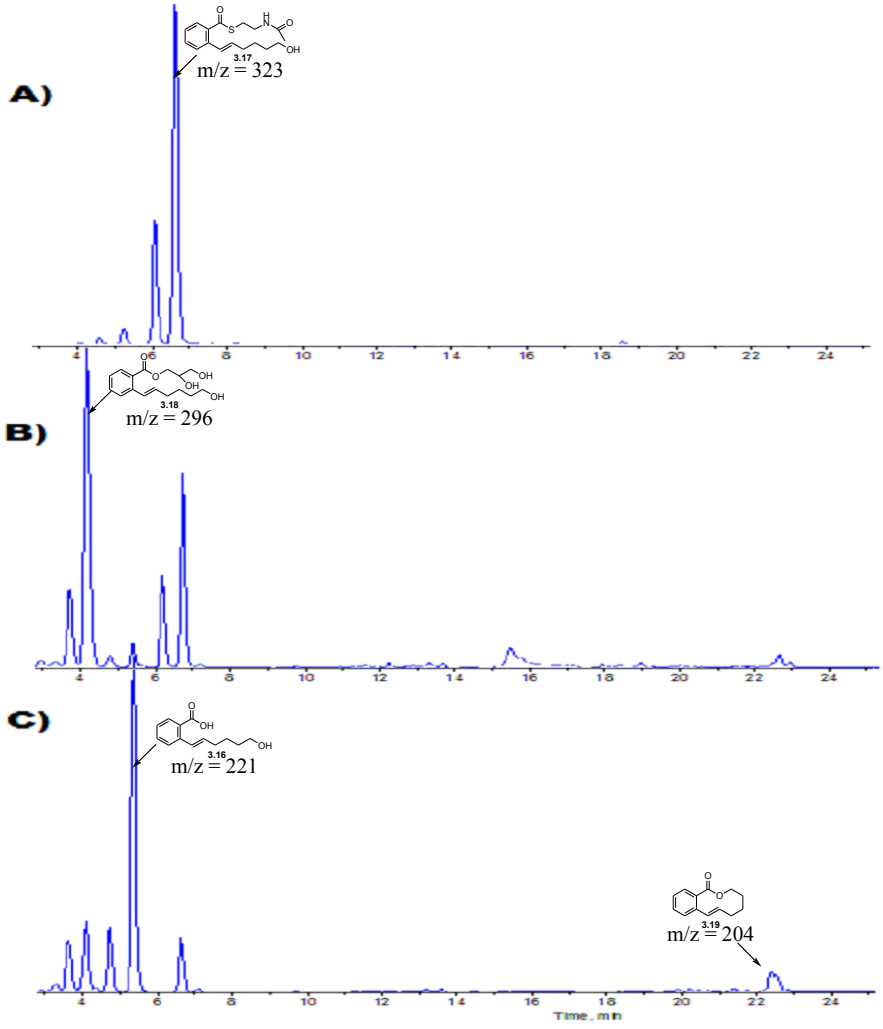


Figure 3.2 LC/MS/MS analysis of macrocyclization of substrate **3.17**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to

100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.17**. B) LC-MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **3.17** at pH 7.4, 23^oC. C) LC-MS/MS analysis of a 24 hours incubation of 2 μM rad TE with 5 mM **3.17** at pH 7.4, 23^oC. Identity of by-products were determined by ESI-MS analysis.

With radicicol TE, however, hydrolysis was the main by-product, followed by glycerolysis. Compared to zea TE, there was less unreacted substrate present. Zea TE showed very small amounts of cyclized product. Rad TE showed more cyclized product than zea TE, even though hydrolysis was still the main product obtained by this reaction. Since there was still a significant amount of starting material in these reactions, it is unclear if the TEs became inactive during the 24 hours incubation, or if the concentration of TE in the reaction was too low for sufficient reaction to occur. To probe the effects on increase of concentrations of the TEs in relation to cyclized product formation, the 10-member SNAC substrate was incubated with 15 μM of enzyme instead of 2 μM (Figure 3.3).

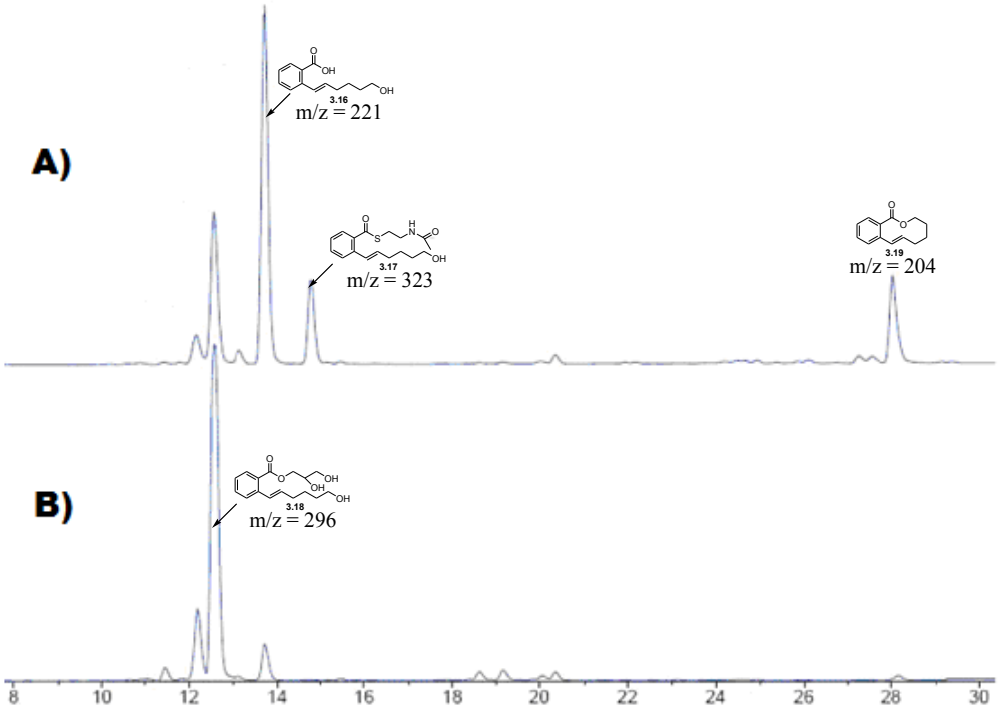
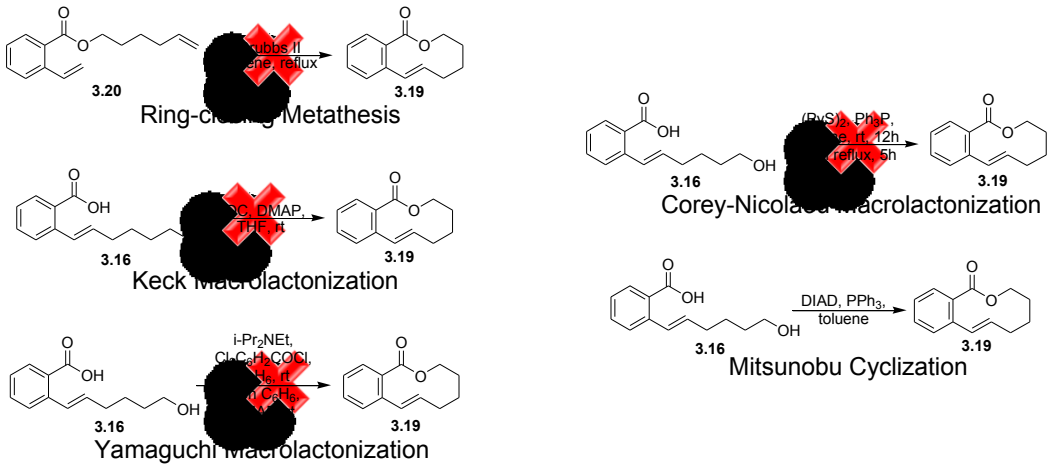


Figure 3.3 LC/MS/MS analysis of macrocyclization of substrate **3.17**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid /H_2O; B: 0.05% formic acid/ MeCN). A) LC/MS/MS analysis of a 24 hours incubation of 15 μM rad TE with 5 mM **3.17** at pH 7.4, 23^oC. C) LC/MS/MS analysis of a 24 hours incubation of 15 μM zea TE with 5 mM **3.17** at pH 7.4, 23^oC. Identity of by-products were determined by ESI-MS analysis.

When the concentration of zea TE increased, the starting material was entirely consumed during the reaction. However, only a trace amount of the cyclized product was observed, with some hydrolysis by-product and significant amount of the glycerolysis by-product. Compared to zea TE, rad TE still had the SNAC substrate present. This indicates that rad TE reacts slower than zea TE, or that it becomes inactive before 20 hours, thus leaving unreacted starting material present in the reaction mixture. Furthermore, rad TE shows the ability to macrocyclize 10-member rings, while zea TE does not. Zea TE, however seem to possess a greater affinity for

glycerol than rad TE, as that is the main by-product, while rad TE has the hydrolysis product as the main by-product.

The fact that rad TE can catalyze 10-member ring formation is very surprising. First, zea TE was unable to perform this reaction, even though both TEs are highly homologous in sequence and product composition. Additionally, bacterial TEs that have been probed for substrate specificity regarding ring sizes have all been unable to macrocyclize 10-member ring formation.[2, 9, 12, 14, 15] Lastly, it is extremely challenging to chemically macrocyclize 10-member rings.[26] Out of all the methods possible for macrolactonization, the Corey-Nicolaou and Yamaguchi reactions are some of the only ones that has shown success in cyclization of 10-member rings, and even then it is often low yielding.[26] To probe the ability of well known chemical methods to access this strained 10-member ring, we have attempted the Yamaguchi, Corey-Nicolaou, and Mitsunobu cyclizations (Scheme 3.2).[27-30] Out of all the methods attempted, only the Mitsunobu reaction yielded the desired 10-member ring product, albeit in very low yields. The failure of the 10-member product to be cyclized chemically makes rad TE even more attractive for this reaction.



Scheme 3.2 Different cyclization reactions attempted to form the 10-member ring.

It has been shown that adding detergents to the chemoenzymatic TE cyclizations suppresses hydrolysis and favours cyclization.[10-12] To determine if

adding non-ionic detergents to rad TE or zea TE could improve macrocyclization yields, we incubated both zea TE and rad TE with **3.5** and Brij-58, a non-ionic detergent (Figure 3.4).

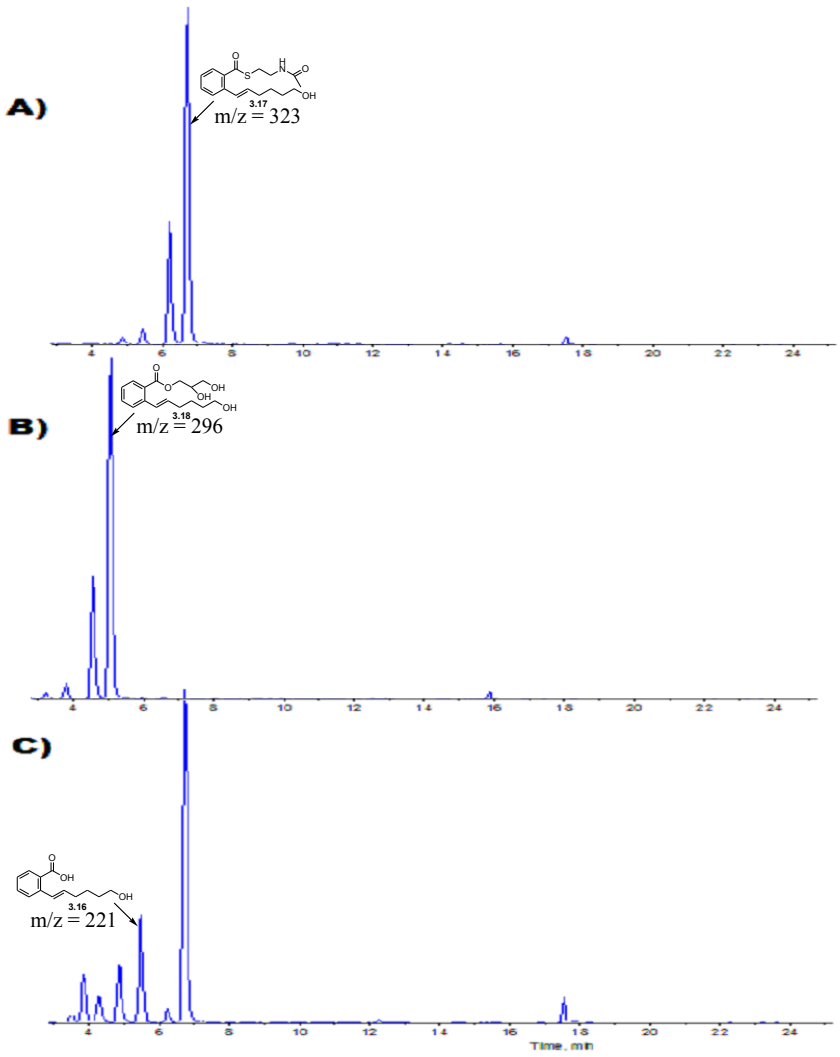


Figure 3.4 LC/MS/MS analysis of macrocyclization of substrate **3.17**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.17**. B) LC/MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **3.17** and Brij-58 at pH 7.4, 23°C. C) LC/MS/MS analysis of a 24 hours incubation of

2 μM rad TE with 5 mM **3.17** and Brij-58 at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

When Brij-58 was added, zea TE showed no starting material or cyclized product, and exclusively glycerolysis by-product. This could indicate that while Brij-58 suppressed hydrolysis, it did not suppress glycerolysis. Since glycerol seems to have better affinity with zea TE than the free alcohol on **3.17**, and hydrolysis is suppressed, only the glycerolysis product is formed.

The rad TE also showed no cyclized product. However, it did not react significantly with glycerol. It does have a bit of hydrolysis by-product, as Brij-58 does not seem to suppress it entirely. However, the main compound in this reaction is the SNAC starting material. This shows that rad TE has very poor affinity for glycerol. If glycerolysis is slower than hydrolysis, it also shows that Brij-58 may inactivate the enzyme before glycerolysis occurs.

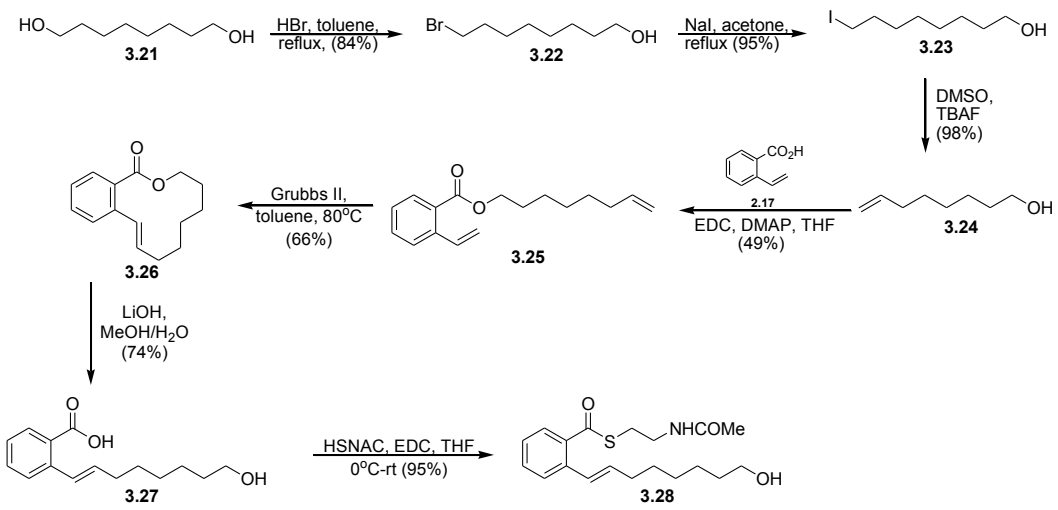
Addition of a non-ionic detergent did not increase the ability of zea TE or rad TE to perform cyclizations. In fact, we observed a suppression of cyclization activity for rad TE. This is contrary to what has been published for bacterial TEs.[10-12] This difference in reactivity between bacterial and fungal TEs could be due to kinetics. 10-member rings are already strained molecules, but this 10-member macrocycle is even more strained due to the trans double bond and the attached aromatic ring. Because of this, the SNAC analogue will react very slowly as it needs to be present in the right conformation. It is possible that Brij-58 reacts with the enzyme and inactivates it faster than the macrocyclization rate. For zea TE, since Brij-58 does not prevent glycerol from the enzymatic pocket, only water, it is possible that glycerolysis occurs faster than Brij-58 enzyme inactivation. This could explain glycerolysis as the only product for the zea TE incubation.

Overall, while zea TE didn't cyclize 10-member rings, rad TE has shown the ability to do so. Considering the difficulties in chemically synthesizing 10-member

rings in general, and this once especially, rad TE could become a useful tool in organic synthesis for these cyclizations. However, rad TE would have to be metabolically engineered to increase the macrocyclization rate and affinity to give higher yields before being use chemoenzymatically.

3.2.2 Enzymatic cyclization of 12-member ring

To probe the ability of zea TE and rad TE to cyclize 12-member rings, the corresponding SNAC analogue was synthesized (Scheme 3.3). Analogous to the 14-member ring formation, the ring-closing metathesis reaction scheme was followed. However, the 7-octenol is not commercially available, so a route to generate that reagent had to be developed.



Scheme 3.3 Reaction sequence to build the 12-member SNAC analogue.

The synthesis of 7-octenol started with 1,8-octanediol. Since it is a symmetrical molecule, it is hard to activate one alcohol over the other and generate product in high yield. After trying a wide variety of reactions, the most satisfactory route in terms of overall yield was by monobromination of the diol.[31] This was done by treatment with bromic acid in refluxing toluene for three days. The yield achieved was 84%, which is extremely high considering the theoretical yield based on statistical likelihood of monobromination is 50%.

The next step in the synthesis was a Finkelstein reaction to exchange the bromine atom to an iodine.[32] This was achieved in good yields by adding sodium iodide in refluxing acetone overnight. Many methods were attempted to eliminate the iodine to form the terminal olefin. The most satisfactory one was elimination with TBAF in DMSO for 4 hours.[33] These mild conditions led to terminal olefin in 98% yield.

Once the olefinic alcohol was synthesized, the subsequent steps were the same as those to form the 14-member ring. The alcohol was coupled to the carboxylic acid **2.17** with EDC and catalytic DMAP in 49% yield. Following this was the ring closing metathesis using Grubbs II in toluene gave the macrocycle in 66% yield. Hydrolysis with lithium hydroxide in methanol and water followed yielding the seco acid in 74% yield. Finally SNAC coupling was performed with EDC as a coupling agent and catalytic DMAP. This was achieved in a very satisfying 95% yield.

Once the analogue was obtained, incubation with both zea TE and rad TE was performed, to probe the enzymes' ability to catalyze the macrolactonization of a 12-member ring. The SNAC substrate was mixed with recombinant purified zea and rad TEs for 24 hours. The reaction mixtures were then analyzed by LC/MS/MS (Figure 3.5).

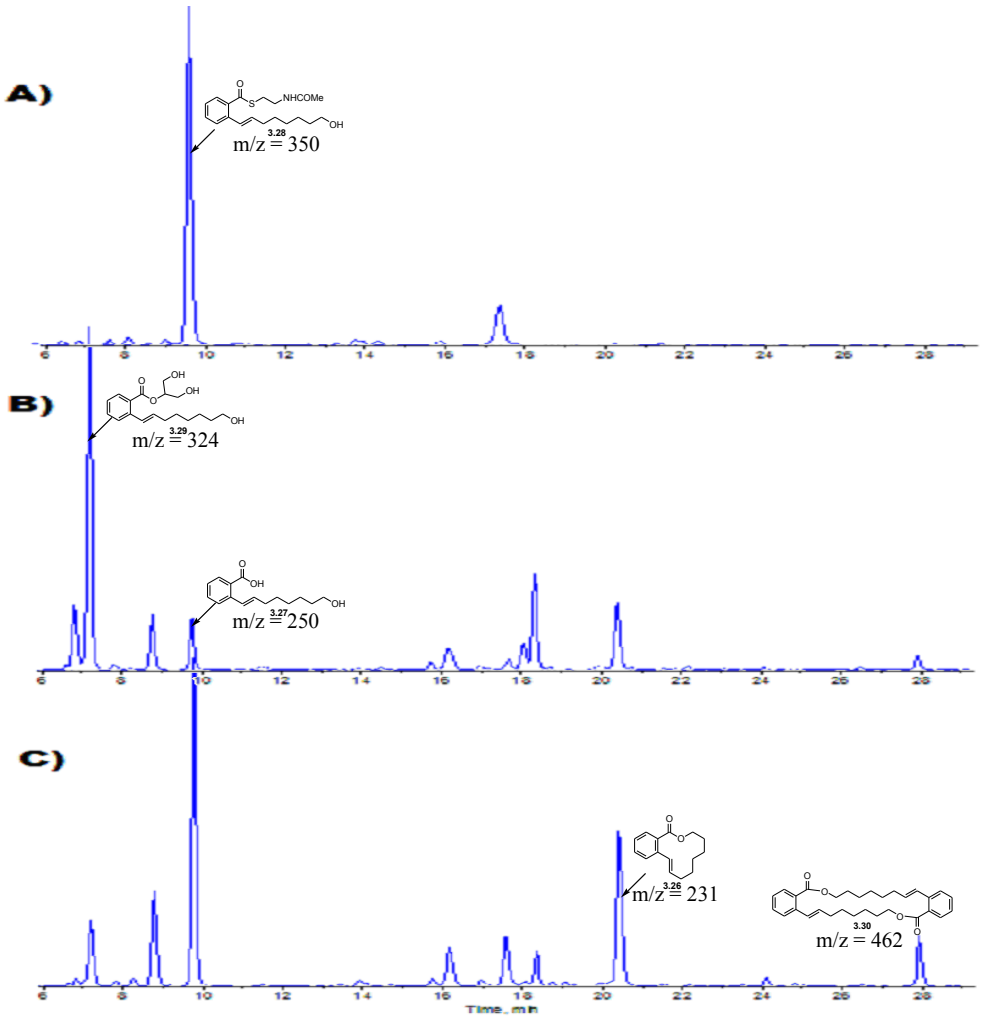


Figure 3.5 LC/MS/MS analysis of macrocyclization of substrate **3.28**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.28**. B) LC/MS/MS analysis of a 1 hour incubation of 2 µM zea TE with 5 mM **3.28** at pH 7.4, 23°C. C) LC/MS/MS analysis of a 1 hour incubation of 2 µM rad TE with 5 mM **3.28** at pH 7.4, 23°C. By-product identities were proposed based on ESI-MS data.

After a 1 hour incubation with zea TE, the main product observed was glycerolysis. There was also some hydrolysis product, starting material and cyclized

product. For rad TE, there was a significant amount of starting material left in the reaction. The main product formed was cyclized product, although there was also presence of dimerization, hydrolysis and glycerolysis products. After 1 hour, zea TE and rad TE are capable of cyclizing 12-member rings. This is analogous to previous studies done with other TEs.[2, 9, 12, 15, 21] This enzymatic cyclization is qualitatively slower than the one to form the 14-member ring, and it is possible that if left longer, starting material would not be present. Presence of hydrolysis is not entirely surprising, as it was shown by other TEs that macrolactonization reactions by TEs often compete significantly with hydrolysis. It is possible that adding non-ionic detergents would hinder hydrolysis and push the formation of the macrolactone. Presence of both hydrolysis and glycerolysis suggests that the substrate might not fit into the active site as well as the 14-member ring. This allows competition of both water and glycerol with the nucleophile, and increase by-products. It is also possible, since the molecule is smaller, there is more room in the enzyme pocket for those molecules, thus favouring both hydrolysis and glycerolysis compared to the 14-member ring.

To probe if starting material would be entirely consumed if given more reaction time, rad TE and zea TE were incubated for 24 hours (Figure 3.6). As expected, no starting material remained with either the zea TE or rad TE incubation. Surprisingly, however, no cyclized product was present with zea TE, and very little was found in the rad TE reaction; however dimerization of the cyclized product was present with rad TE. Incubation with zea TE showed little hydrolysis product, and glycerolysis as the major product. Incubation with rad TE showed little glycerolysis product, and hydrolysis as the main by-product.

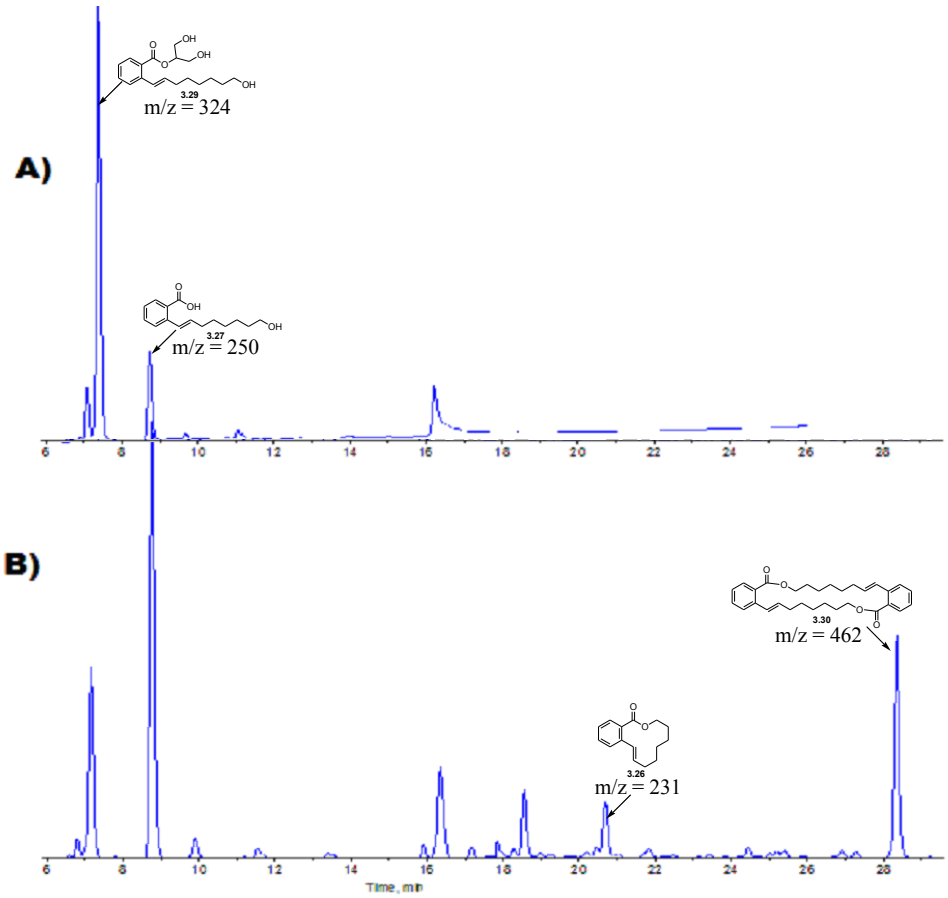


Figure 3.6 LC/MS/MS analysis of macrocyclization of substrate **3.28**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) LC/MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **3.28** at pH 7.4, 23°C. B) LC/MS/MS analysis of a 24 hours incubation of 2 μM rad TE with 5 mM **3.28** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

These results indicate that both zea TE and rad TE have the ability to form 12-member rings. However, 12-member rings do not seem to be stable in the presence of these TEs and over time the 12-member macrocycle likely reacts with the TEs and either water or glycerol to form the hydrolysis or glycerolysis by-products, respectively, as has been previously seen with epo TE. With rad TE, the cyclized product can also react to form the 24-member ring dimer product. This indicates that

rad TE has the ability to form 24-member rings, and that this product is more stable than its 12-member counterpart.

To probe if hydrolysis could be suppressed, and the cyclized product favoured, Brij-58 was added to the enzymatic reaction (Figure 3.7). Contrary to the literature, but analogous to the 10-member ring incubation, no cyclization or dimerization product was present with Brij-58 incubation. For zea TE, glycerolysis was the main by-product, with a bit of hydrolysis. Rad TE on the other hand, showed equal amounts of both the glycerolysis and hydrolysis by-products with little starting material left in the reaction.

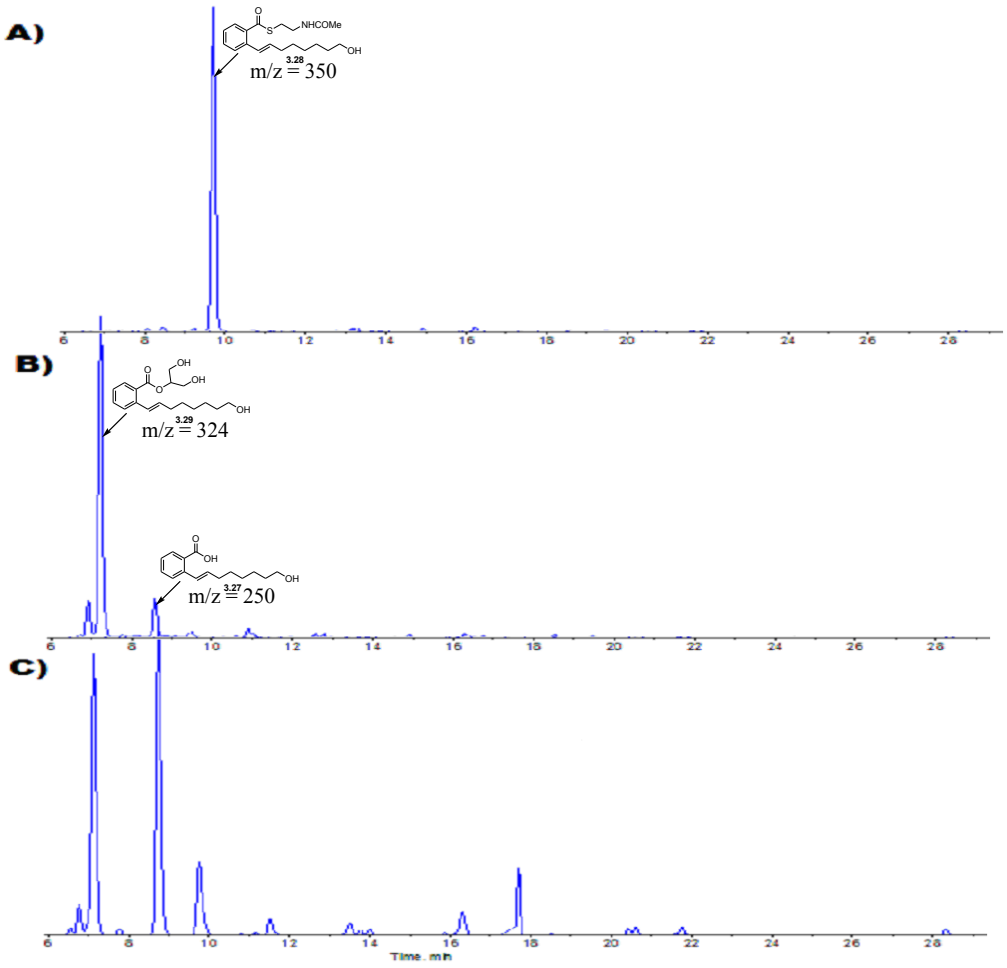


Figure 3.7 LC/MS/MS analysis of macrocyclization of substrate **3.28**. Chromatograms are absorbance at 210 nm. LC/MS/MS

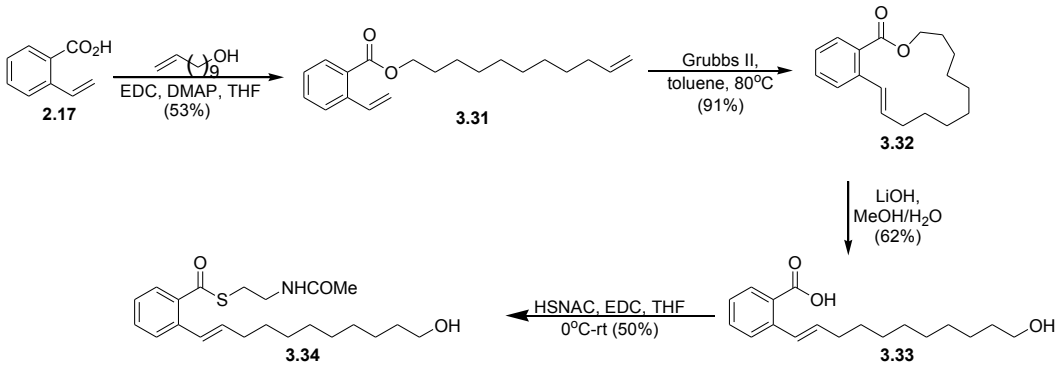
conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.28**. B) LC/MS/MS analysis of a 24 hours incubation of 2 μM Zea TE with 5 mM **3.28** and Brij-58 at pH 7.4, 23^oC. C) LC/MS/MS analysis of a 24 hours incubation of 2 μM Rad TE with 5 mM **3.28** and Brij-58 at pH 7.4, 23^oC. Identity of by-products were determined by ESI-MS analysis.

Similar to the experiments to form the 10-member rings, Brij-58 seem to interact with the enzyme and inactivate the fungal TEs. This results in no cyclized or dimerized product. However, rad and zea TE produce both hydrolysis and glycerolysis products. This might again indicate the increased reactivity of external nucleophiles over the intramolecular reaction. The linear substrate might have difficulty fitting in the right conformation in the active site, thus suppressing intramolecular attack. The non-ionic detergent might be able to stabilize the hydrophobic substrate in water, leading to increase hydrolysis or glycerolysis products. Similar to the 10-member ring formation reaction, zea TE has greater affinity for glycerol than rad TE does. This results in a higher amount of glycerolysis product with zea TE.

In conclusion both zea TE and rad TE have the ability to cyclize 12-member rings. However, this reaction is not as efficient as the 14-member ring formation and in both cases there is presence of hydrolysis and glycerolysis by-products. Over time, the cyclized product gets dimerized by the rad TE. The cyclized product can also react with water or glycerol by the TEs over time. Adding non-ionic detergent does not increase the presence of the wanted cyclized product, rather a greater amount of glycerolysis product is found in the reaction mixture.

3.2.3 Enzymatic cyclization of 15-member ring

Both zea TE and rad TE have shown the ability to cyclize smaller ring sizes. However, it is still unclear whether they are able to catalyze the macrolactonization of larger ring sizes. It could be possible that the enzymatic pocket is too small to accommodate larger molecules. To probe the macrolactonization ability of both zea and rad TEs for 15-member rings, the SNAC analogue was synthesized (Scheme 3.4).



Scheme 3.4 Reaction sequence to build the 15-member SNAC analogue.

The reaction sequence followed is identical to the synthesis of the 14-member SNAC analogue. First, there was an esterification reaction between **2.17** and the alcohol using EDC and DMAP at 57% yield. A ring-closing metathesis using Grubbs II catalyst followed. Stirring overnight at 80°C gave the wanted 15-member ring in very satisfying yield of 91%. A hydrolysis reaction using lithium hydroxide in methanol and water followed by SNAC coupling using EDC and DMAP generated the desired substrate. The overall yield of this synthesis was 15% after 4 steps.

With the SNAC analogue in hand, it was possible to incubate it with zea TE and rad TE and test their macrolactonization capabilities. The SNAC analogue was incubated with rad TE and zea TE for 1 hour before the analysis of the reaction by LC/MS/MS (Figure 3.8). After 1 hour, only the 15-member macrolactone was present in the reaction mixture. Trace starting material remained with zea TE, and no detectable starting material remained with the rad TE incubation. This shows the

unique affinity for both zea TE and rad TE to macrocyclize 15-member ring without competition of hydrolysis. This is unique to both rad TE and zea TE, as most other TEs have a mixture of both the macrolactone and hydrolysis products after incubation.[2, 14] These results are similar to those obtained for the 14-member ring. Since they are similar in size and reactivity, this was expected.

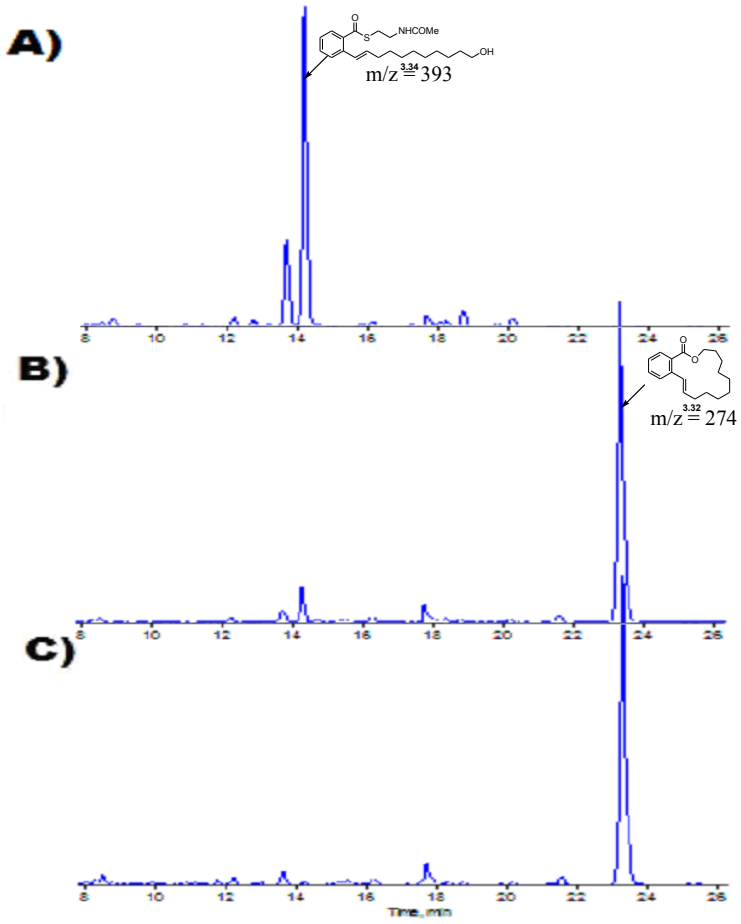


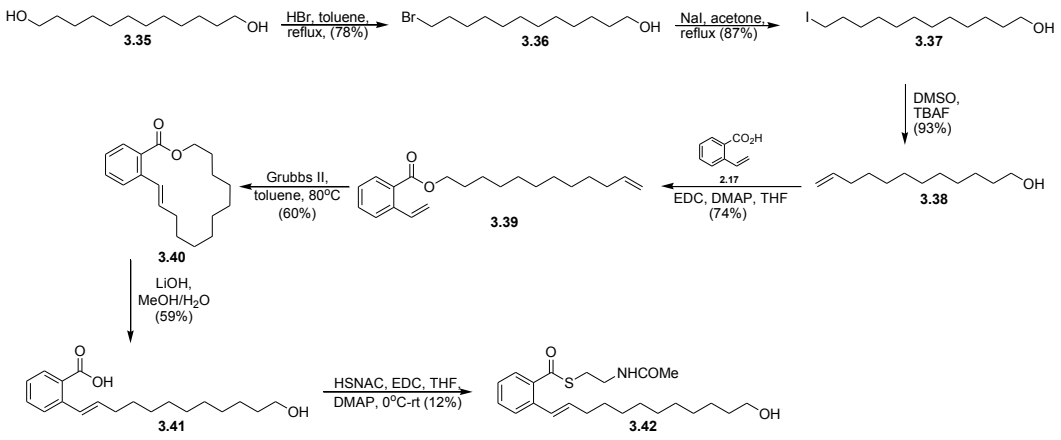
Figure 3.8 LC/MS/MS analysis of macrocyclization of substrate **3.34**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.34**. B) LC/MS/MS analysis of a 1 hour incubation of 2 µM zea TE with 5 mM **3.34** at pH 7.4, 23°C. C) LC/MS/MS analysis of a 1 hour incubation of 2 µM rad TE

with 5 mM **3.34** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

To conclude, both zea TE and rad TE can cyclize exclusively the macrolactone product in very low enzyme concentration, making those TEs extremely efficient for this type of cyclization reactions.

3.2.4 Enzymatic cyclization of 16-member ring

To probe the ability of both zea TE and rad TE to build larger ring sizes, and have an idea of their limitations, the 16 member SNAC analogue was built (Scheme 3.5). As the alcohol was not commercially available it needed to first be synthesized. To this end, the reaction scheme followed the same scheme as the one to build the 12 member ring.



Scheme 3.5 Reaction sequence to build the 16-member SNAC analogue.

The first step of the synthesis was a monobromination reaction on the symmetric diol achieved in 78% yield. The next step was a Finkelstein reaction, followed by TBAF elimination to generate the double bond. Both reactions were high yielding, in 87% and 93%, respectively.

The rest of the reaction sequence was similar to the ones used to build the other analogues. Esterification with EDC as a coupling agent and ring-closing metathesis using Grubbs II were the next steps. However, the longer acyl chain rendered the molecule substantially more hydrophobic than previous substrates. Different purification methods, including column chromatography with different solvent systems and even silver nitrate were tried, to purify this compound but to no avail. Finally, PTLC was used to purify the ring-closing metathesis compound. Those multiple purification steps lowered the overall yield of the reaction.

Finally, hydrolysis with lithium hydroxide and SNAC coupling with EDC were performed. Again, due to the greasy nature of the compound, purification proved to be very challenging. After several purification attempts, the molecule was purified by HPLC, leading to the low yield of 12%.

Once the SNAC analogue was in hand, it was incubated with zea TE and rad TE to probe the enzymes' selectivity for larger ring sizes (Figure 3.9). After 1 hour, zea TE showed some cyclized product, but still had significant amounts of starting material. After 24 hours however, the starting material was fully converted to the cyclized product. After 1 hour, rad TE fully converted the starting material to the 16-member ring. Analogous to the results with the 14- and 15-membered rings, no detectable hydrolysis or glycerolysis products were observed with either rad TE or zea TE.

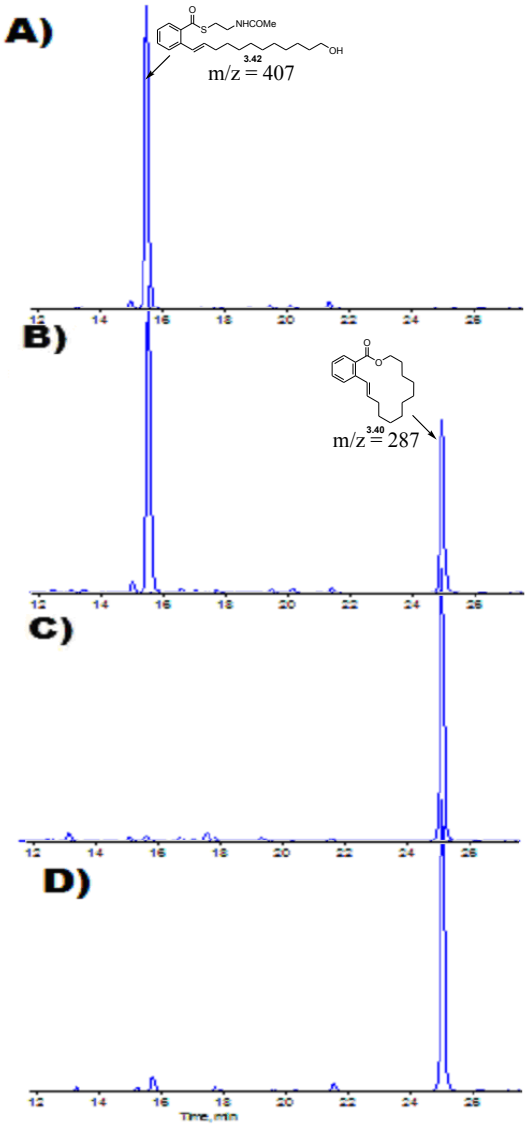


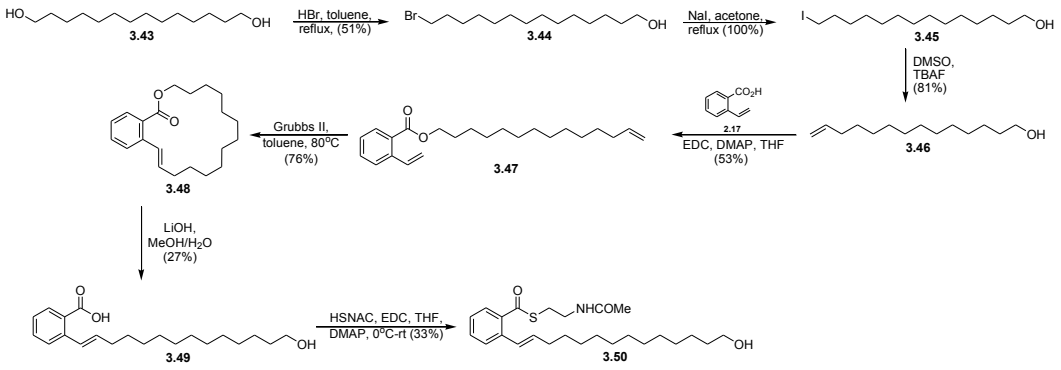
Figure 3.9 LC/MS/MS analysis of macrocyclization of substrate **3.42**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.42**. B) LC/MS/MS analysis of a 1 hour incubation of 2 μM zea TE with 5 mM **3.42** at pH 7.4, 23°C. C) LC/MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **3.42** at pH 7.4, 23°C. D) LC/MS/MS analysis of a 1 hour incubation of 2 μM rad TE with 5 mM **3.42** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

These results are different than those observed from other bacterial TEs, where, while the enzyme was able to convert the substrate to the 16-member macrolactone, there was significant competing hydrolysis.[2, 9, 12, 15, 21] This suggests that the rad and zea TE have a greater affinity for the substrate and for the intramolecular reaction. It also suggests that once the substrate is present in the active-site cavity, there is no additional room for water or glycerol.

Overall, the RALs fungal TEs studied seem to be better enzymes for chemoenzymatic reactions than previous bacterial TEs as they exclusively generate the cyclized product. Rad TE also seems to qualitatively have greater rates of conversion than the zea TE, as within 1 hour, at only 2 μM enzyme concentration, the 16-membered ring is fully produced. This makes both TEs, but especially rad TE, useful potential tools in chemoenzymatic chemistry for macrolactonization reactions.

3.2.5 Enzymatic cyclization of 18-member ring

To probe if zea and rad TE could macrolactonize even larger ring sizes, the 18-member SNAC analogue was synthesized (Scheme 3.6). Similar to the 16- and 12-member analogues, the alcohol was not commercially available and had to first be synthesized.



Scheme 3.6 Reaction sequence to build the 18-member SNAC analogue.

The first step was the monobromination using bromic acid. The yield in this case was a bit lower than previously, at 51%. This is closer to the theoretical yield of 50% and could be because the acyl chain is longer and the bromine and alcohol moieties are farther away. This could have less of an impact when both bromines are added to the molecule instead of just one. The next step was a Finkelstein reaction, and the product was obtained in quantitative yield. It was followed by TBAF elimination, generating the olefin in 81% yield.

The next steps were esterification with EDC as a coupling agent and ring-closing metathesis using Grubbs II. Similar to the 16-member ring, the acyl chain on the molecule is very long and this renders the whole molecule highly hydrophobic. This makes the purification much more difficult, as both the starting materials and the product have similar retention factors on normal phase column chromatography. After trying different solvent systems and adding silver nitrate and still not seeing much difference in co-elution and separation, we decided to purify the molecule at the hydrolysis step. This is because the hydrolysis reaction renders the molecule significantly more polar than the macrocyclic product and is thus easier to separate and purify. However, this modification accounts for the low yield of 27% at that step. The SNAC coupled product was also difficult to purify, and since there was only a small amount of compound in the reaction, we decided to purify using HPLC methods. This method gave better yields than with the 16-member SNAC analogue, giving 33% yield of the desired final product.

Substrate **3.50** was incubated with zea TE and rad TE for 1 hour before LC/MS/MS analysis (Figure 3.10). After that time, cyclized product is observed by LC/MS/MS for both zea and rad TEs. However, there is much more starting material present after that time than there is cyclized product for zea TE. There are also no hydrolysis or glycerolysis products detectable by LC/MS/MS. With the rad TE incubation, there is no detectable glycerolysis product. However, the major peak corresponds to the hydrolysis product, with some starting material present.

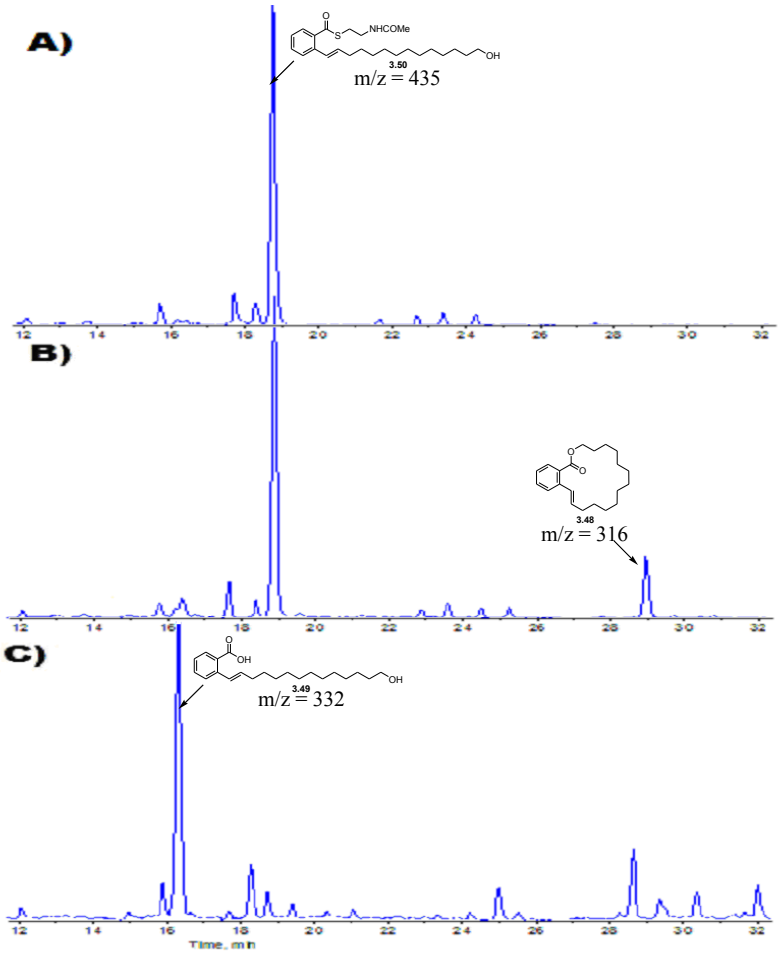


Figure 3.10 LC/MS/MS analysis of macrocyclization of substrate **3.50**. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **3.50**. B) LC/MS/MS analysis of a 1 hour incubation of 2 µM zea TE with 5 mM **3.50** at pH 7.4, 23°C. C) LC/MS/MS analysis of a 1 hour incubation of 2 µM rad TE with 5 mM **3.50** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

The hydrolysis product is consistent with previous literature results on bacterial TEs.[2, 9, 12, 15, 21] The lack of hydrolysis product in the zea TE incubation might indicate better affinity by zea TE for the intramolecular reaction and the SNAC substrate. It could also indicate the there is not sufficient room in the enzymatic pocket to accommodate both the substrate and water or glycerol molecules, thus inhibiting hydrolysis and glycerolysis and favouring macrolactonization.

The large amount of starting material could indicate that zea TE is a slow reaction and that given more time, there would be an increase in the cyclized product. However, after 24 hours, the ratio of starting material and cyclized product remained similar (Figure 3.11). At that time, there was still very little hydrolysis present in the reaction mixture. This indicates that the hydrolysis reaction is not favoured with the 18-member ring substrate with zea TE. This could be due to lack of space in the enzymatic pocket. It is possible that increasing enzyme concentration would increase the amount of cyclized product formed within 24 hours during the reaction. However, no such experiments have been performed to date.

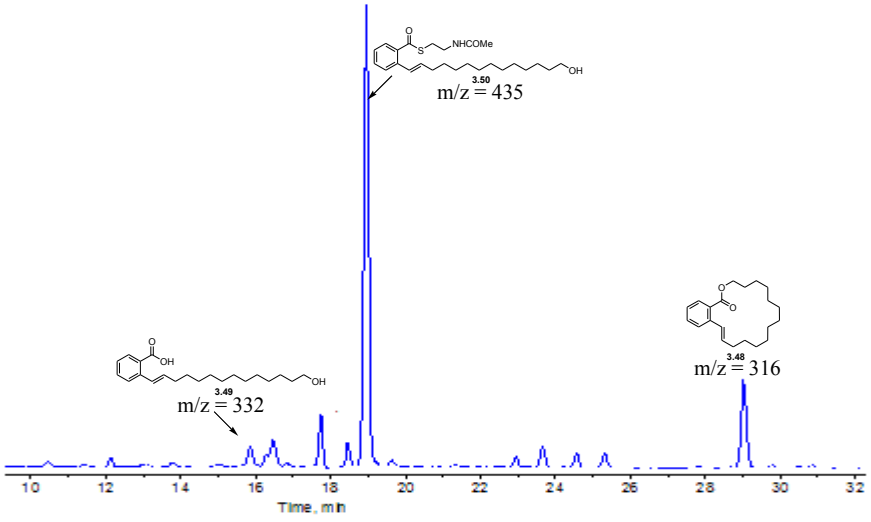


Figure 3.11 LC/MS/MS analysis of a 24 hours incubation of 2 µM zea TE with 5 mM **3.50** at pH 7.4, 23°C. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min.

Overall, both rad TE and zea TE have the ability to macrocyclize 18-member rings. However, hydrolysis is the main by-product with rad TE, and with zea TE there is a significant amount of starting material that remains unreacted even after 24 hours. Unless they are metabolically engineered to suppress hydrolysis and to render them more reactive, they would not be useful tools in chemoenzymatic chemistry to form 18-member rings.

3.3 Conclusions

In conclusion, both zea and rad TEs have the ability to macrolactonize larger and smaller ring sizes than the native 14-member ring. Contrary to previous literature results by different bacterial TEs, the larger ring sizes do not compete with hydrolysis but rather exclusively form the macrolactone. The only exception however, is the 18-member ring formation with rad TE. The 10- and 12- member rings however, do compete with hydrolysis, glycerolysis and macrolactonization products. Larger substrates might completely occupy the enzymatic cavity, leaving no room for water or glycerol molecule. This would favour macrolactonization. Substrates smaller than the native substrate, however, might not fully occupy the enzymatic cavity, leaving room for glycerol and water to react intermolecularly.

Rad TE seems more reactive toward smaller ring sizes. With zea TE, no 10-member ring was formed, and very little 12-member cyclized product was present. However, when it came to the 18-member ring formation, rad TE was almost exclusively hydrolysed while zea TE had a mixture of starting material and cyclized product.

Adding a non-ionic detergent to the enzymatic reaction for the 10- and 12-member ring formation did not increase macrolactone formation, contrary to what has been previously published in the literature on bacterial TEs. Instead, glycerolysis was favoured and macrocyclization seems to have been suppressed completely.

Since fungal and bacterial TEs display low sequence homology, it is possible that the detergent inactivates the enzyme, thus decreasing macrocyclic reactivity.

The 10-member ring was successfully macrocyclized by rad TE. This is the first example in the literature where a PKS TE has been able to macrolactonize a 10-member ring. Given the difficulty in forming 10-member rings both enzymatically and in chemical syntheses, rad TE might be a useful tool in forming these rings.

Overall, the TEs are not substrate specific regarding ring sizes. They could therefore be used in both combinatorial library formation for different PKS products, or as potential tools for chemoenzymatic macrolactone reactions.

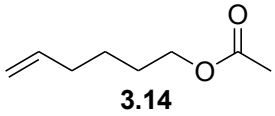
3.4 Experimental section

3.4.1 General methods

All reactions were carried out under nitrogen or argon atmosphere with dry solvents under anhydrous conditions in oven-dried glassware equipped with a magnetic stir bar and rubber spectrum. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reagents were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light (254 nm) as a visualizing agent and cerium molybdate stain and heat as developing agent. E. Merck silica gel 60 (230-400 mesh) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254). IR spectra were collected with neat thin films on sodium chloride disk using a Bomem Michaelson 100 Fourrier transform infrared (FTIR) spectrometer. ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, NOSEY, and COSY spectra were recorded on a Brucker AVANCE 300 MHz or 400 MHz spectrometer. Deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) were used as NMR solvents

unless otherwise noted. All chemical shifts are reported in parts per million (δ), integration and coupling constant(s) in Hz downfield from TMS and corrected using the solvent residual peak as internal standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, b = broad, br s = broad singlet. High resolution mass spectra (HRMS) were collected by positive ion electrospray on Kratos Analytical Concept-11A mass spectrometer with an electron beam of 70ev (Ottawa-Carleton Mass Spectrometry Centre). LC/MS/MS was conducted with an Applied Biosystems API2000 triple quadrupole mass spectrometer using electrospray ionization and a Hypersil C_{18} 100 × 2.1 mm, 3 μM particle size column. LC/MS/MS was conducted with an Applied Biosystems API2000 triple quadrupole ion trap mass spectrometer using electrospray ionization and a Hypersil C_{18} 150 × 4.6 mm, 5 μM particle size column.

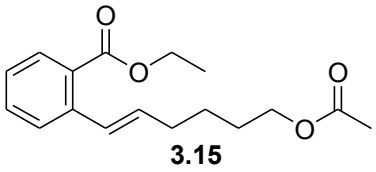
3.4.2 Experimental procedures



3.14

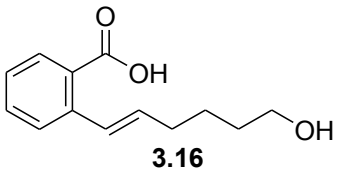
Hex-5-enyl acetate (3.14). 5-hexenol (600 μL, 5.07 mmol, 1 equiv.) and 4-(dimethylamino)pyridine (31 mg, 0.25 mmol, 5 %) were dissolved in methylene chloride (6 mL). Triethylamine (777 μL, 5.58 mmol, 1.1 equiv.) was added and the resulting mixture was stirred for 10 minutes at 0°C. Acetic anhydride (527 μL, 5.58 mmol, 1.1 equiv.) was added dropwise and the reaction mixture was slowly warmed to room temperature for 5 hours. The reaction was quenched with a saturated ammonium chloride solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo to afford a colourless oil (713.4 mg, 5.01 mmol, 99%). Observed ^{1}H NMR agrees with literature values.[34] Rf = 0.33 (silica gel, 9:1 hexanes/EtOAc) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 (ddt, *J* = 16.9, 10.2, 6.8 Hz, 1 H), 4.90 (ddt, *J* = 17.1, 3.5, 1.7 Hz, 1 H), 4.84 (ddt, *J* = 10.2,

1.9, 0.9 Hz, 1 H), 3.94 (t, *J* = 6.4 Hz, 2 H), 2.00-1.95 (m, 2 H), 1.92 (s, 3 H), 1.57-1.48 (m, 2 H), 1.39-1.29 (m, 2 H).



3.15

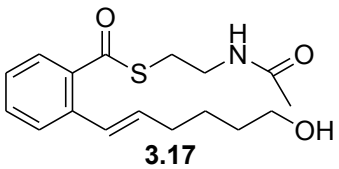
2-(6-acetoxy-hex-1-enyl)-benzoic acid ethyl ester (3.15). To **2.16** (100 mg, 0.57 mmol, 1 equiv.) in methylene chloride (3 mL) was added hex-5-enyl acetate (713 mg, 5.01 mmoles, 8.8 equiv.) and the reaction mixture was stirred at room temperature for 10 minutes. Grubbs catalyst 2nd generation (24 mg, 0.03 mmol, 5 mol%) was added and the reaction mixture was stirred at reflux for 48 hours. The reaction mixture was cooled to room temperature and was then filtered through silica with methylene chloride (50 mL) and the solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 15 % ethyl acetate in hexanes) to afford **3.15** (81.4 mg, 0.28 mmol, 49 %) as a yellow oil. **3.15**: Rf = 0.21 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2947, 2862, 1737, 1238 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (dd, *J* = 7.8, 1.4 Hz, 1 H), 7.50 (d, *J* = 7.9 Hz, 1 H), 7.41 (ddd, *J* = 7.5, 7.4, 1.4 Hz, 1 H), 7.26-7.21 (m, 1 H), 7.12 (d, *J* = 15.8 Hz, 1 H), 6.08 (dt, *J* = 15.8, 7.1 Hz, 1 H), 4.34 (q, *J* = 7.2 Hz, 2 H), 4.07 (t, *J* = 6.6 Hz, 2 H), 2.30-2.23 (m, 2 H), (2.03 (s, 3 H), 2.01-1.96 (m, 3 H), 1.72-1.64 (m, 2 H), 1.64-1.50 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.2, 167.8, 139.4, 132.9, 131.8, 130.2, 129.7, 129.1, 127.2, 126.6, 64.4, 60.4, 32.1, 28.1, 25.8, 21.0, 14.3; HRMS (+EI) : calcd for $C_{13}H_{14}O_2$ (M-$C_2H_3O_2$-C_2H_5) 202.0994, obsd 202.1004.



3.16

2-(6-hydroxy-hex-1-enyl)-benzoic acid (3.16). To **3.15** (79 mg, 0.27 mmol, 1 equiv.) in methanol (12 mL) at room temperature was added lithium hydroxide monohydrate (108 mg, 2.56 mmol, 9.5 equiv.) that was first dissolved in water (4 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached a pH of 2 and extracted with EtOAc (3 × 15

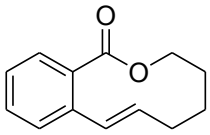
mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 50% ethyl acetate in hexanes) to afford (57.9 mg, 0.26 mmol, 97 %) as a yellow oil. **3.16**: Rf = 0.10 (silica gel, 1:1 hexanes/EtOAc); IR (NaCl) νmax = 3340 (br), 2933, 2856, 1700, 1254, 1007 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.92 (dd, *J* = 7.8, 1.1 Hz, 1 H), 7.49 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.43 (ddd, *J* = 7.6, 7.4, 1.4 Hz, 1 H), 7.24 (ddd, *J* = 7.8, 7.2, 1.4 Hz, 1 H), 7.17 (d, *J* = 15.8 Hz, 1 H), 6.06 (dt, *J* = 15.7, 6.8 Hz, 1 H), 3.66 (t, *J* = 6.5 Hz, 2 H), 2.29-2.22 (m, 2 H), 2.00-1.95 (m, 2 H), 1.68-1.60 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.3, 140.5, 133.5, 132.7, 131.1, 130.4, 129.3, 127.6, 126.7, 62.9, 32.2, 32.1, 25.6; HRMS (+EI) : calcd for $C_{13}H_{14}O$ (M-H_2O) 202.0994, obsd 202.1007.



3.17

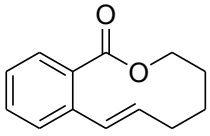
2-(6-Hydroxy-hex-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (3.17). To **3.16** (53 mg, 0.24 mmol, 1 equiv.) in THF (18 mL) at 0°C under argon atmosphere was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (120 mg, 0.62 mmol, 2.6 equiv.) before the addition of *N*-acetylcysteamine (59 μL, 0.55 mmol, 2.3 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated NH_4Cl solution (30 mL) and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 60% acetone in hexanes) to afford a colorless oil (45 mg, 0.14 mmol, 58 %). **3.17**: Rf = 0.17 (silica gel, 1:1 hexanes/acetone); IR (NaCl) νmax = 3745, 3286(br), 2920, 2854, 1655, 1195, 1090 cm^{-1}; ^1H NMR (75 MHz, $CDCl_3$) δ 7.69 (dd, *J* = 7.8, 1.0 Hz, 1 H), 7.52 (d, *J* = 7.3 Hz, 1 H), 7.43 (ddd, *J* = 7.7, 7.4, 1.3 Hz, 1 H), 7.27 (dd, *J* = 7.8, 1.4 Hz, 1 H), 6.78 (d, *J* = 15.9 Hz, 1 H), 6.15 (dt, *J* = 15.8, 6.8 Hz, 1 H), 5.91 (bs, 1 H), 3.67 (t, *J* = 6.3 Hz, 2 H), 3.53 (dt, *J* = 6.3, 6.1 Hz, 2 H), 3.19 (t, *J* = 6.8 Hz, 2 H), 2.29-2.22 (m, 2 H), 1.97 (s, 3 H), 1.66-1.55 (m, 4 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.8, 170.3, 136.5,

135.8, 134.2, 132.0, 128.3, 127.6, 127.0, 126.7, 62.7, 39.7, 32.7, 32.1, 29.3, 25.1, 23.2; HRMS (+EI): calcd for $C_{17}H_{23}NO_3S$ (M-$SC_2H_4NHCOCH_3$) 202.0993, obsd 202.0978.



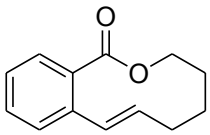
3.19

3,4,5,6-tetrahydrobenzooxecin-1-one (3.19). To 2-Vinyl benzoic acid hex-5-enyl ester (138 mg, 0.56 mmol, 1 equiv) in toluene (133 mL) was added Grubbs catalyst 2nd generation (24 mg, 0.05 mmol, 5 mol%). The reaction mixture was stirred at 80^{o}C for 48 hours. The solvent was then evaporated to dryness in vacuo. Crude NMR did not show **3.19** product.



3.19

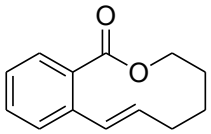
3,4,5,6-tetrahydrobenzooxecin-1-one (3.19). To **3.16** (42 mg, 0.19 mmol, 1 equiv) in THF (6 mL) under argon atmosphere was added N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (51 mg, 0.26 mmol, 1.4 equiv.) before addition of 4-(dimethylamino)pyridine (5 mg, 0.04 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 48 hours. The reaction was quenched with a saturated ammonium chloride solution (20 mL) and extracted with EtOAc (3 × 10 mL). A saturated $NaHCO_3$ solution (20 mL) was then added to the organic layer and extracted with EtOAc (3 × 10 mL). The resulting organic layers were combined and washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated to dryness in vacuo. Crude NMR did not show **3.19** product.



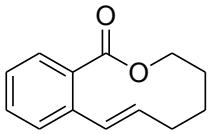
3.19

3,4,5,6-tetrahydrobenzooxecin-1-one (3.19). To **3.16** (73 mg, 0.33 mmol, 1 equiv) in toluene (6.6 mL) under argon atmosphere was added 2,2'-dithiodipyridine (109 mg, 0.50 mmol, 1.5 equiv.) and triphenylphosphine (130 mg, 0.50 mmol, 1.5 equiv.). The resulting mixture was stirred at room temperature for 18 hours. Toluene (50 mL) and triethylamine (1 mL, 0.066 mmol, 0.2 equiv.) were then added and the reaction mixture was heated at reflux for 24 hours. The solvent was evaporated and EtOAc

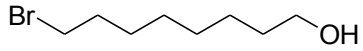
(20 mL) was added. The reaction was extracted with 1M HCl (3 × 15 mL) and the organic layer was dried over $MgSO_4$, filtered, concentrated to dryness in vacuo. Crude NMR did not show **3.19** product.


3.19

3,4,5,6-tetrahydrobenzooxecin-1-one (3.19). 3.16 (35 mg, 0.16 mmol, 1 equiv) was azeotropically dried before the addition of benzene (1 mL). The reaction was stirred for 15 minutes before the addition of *N*,*N*-diisopropylethylamine (835 µL, 4.80 mmol, 30 equiv.) and 2,4,6-trichlorobenzoyl chloride (500 µL, 3.20 mmol, 20 equiv.). The resulting mixture was stirred at room temperature overnight. The reaction mixture was diluted with benzene (8 mL) and 4-(dimethylamino)pyridine (782 mg, 6.40 mmol, 40 equiv.) was added. The resulting mixture was stirred at room temperature for 4 hours. The reaction was quenched with a saturated ammonium chloride solution (30 mL) and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and dried over $MgSO_4$, filtered and concentrated to dryness in vacuo. Crude NMR did not show **3.19** product.

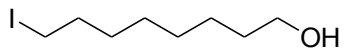

3.19

3,4,5,6-tetrahydrobenzooxecin-1-one (3.19). 3.16 (73 mg, 0.33 mmol, 1 equiv) was diluted with toluene (45 mL) and was heated to 45°C under argon atmosphere. Diisopropyl azodicarboxylate (390 µL, 1.98 mmol, 6 equiv.) and triphenylphosphine (519 mg, 1.98 mmol, 6 equiv.) were mixed together in toluene (180 mL) at 0°C. Half of the DIAD solution was added dropwise to the **3.16** solution over a period of 2 hours and the reaction mixture was stirred at 45°C for 3 hours. The remaining of the DIAD solution was added dropwise over a period of 2 hours and the resulting mixture was stirred at 45°C for 18 hours. The solvent was evaporated, and EtOAc (10 mL) was added. The mixture was filtered through silica and was concentrated to dryness in vacuo to afford a yellow oil. Crude NMR showed trace amounts of product. **3.19**: HRMS (+EI): calcd for $C_{13}H_{14}O_2$ (M) 202.0994, obsd 202.0999.



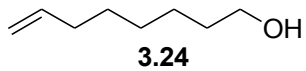
3.22

8-bromooctanol (3.22). To 1,8-octanediol (150 mg, 1.03 mmoles, 1 equiv.) in toluene (2.5 mL) was added bromic acid 9M (48%) (183 μL, 1.65 mmoles, 1.6 equiv.) under argon atmosphere. The resulting mixture was refluxed for 72 hours. The reaction was then put to room temperature and the aqueous and organic phases were separated. The organic phase was diluted with ether (20 mL), washed with 1M NaOH (50 mL) and brine (50 mL). The resulting organic layer was dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford a colorless oil (181.2 mg, 0.87 mmoles, 84 % yield). Observed ^{1}H NMR data agrees with literature values.[35] Rf = 0.28 (silica gel, 9:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.61 (t, *J* = 6.7 Hz, 2 H), 3.38 (t, *J* = 6.9 Hz, 2 H), 1.87-1.78 (m, 2 H), 1.57 (bs, 1 H), 1.56-1.49 (m, 2 H), 1.45-1.36 (m, 2 H) 1.35-1.27 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.0, 34.0, 32.8, 32.7, 29.2, 28.7, 28.1, 25.6.



3.23

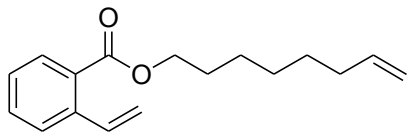
8-iodooctanol (3.23). To 8-bromoctanol (176 mg, 0.84 mmoles, 1 equiv.) in acetone (2 mL) was added sodium iodide (441 mg, 2.94 mmoles, 3.5 equiv.) under argon atmosphere. The resulting mixture was refluxed for 18 hours. The reaction was then put to room temperature and the mixture was washed with brine (20 mL) and extracted with EtOAc (3 × 15 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford a yellow oil (205.0 mg, 0.80 mmoles, 95% yield). Observed ^{1}H NMR data agrees with literature values.[36] Rf = 0.32 (silica gel, 9:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (t, *J* = 6.5 Hz, 2 H), 3.16 (t, *J* = 7.1 Hz, 2 H), 1.84-1.75 (m, 2 H), 1.59-1.50 (m, 4 H), 1.41-1.27 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.0, 33.5, 32.7, 30.4, 29.2, 28.5, 25.7, 7.3.



3.24

7-octenol (3.24). To 8-iodooctanol (201 mg, 0.78 mmoles, 1 equiv.) in DMSO (86 mL) was added TBAF hydrate (1230 mg, 3.9 mmoles, 5 equiv.) under argon atmosphere. The resulting mixture was

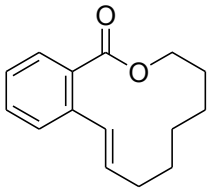
stirred at room temperature for 5 hours. The reaction was quenched with H_2O (100 mL) and the aqueous phase was extracted with EtOAc (3 × 150 mL). The resulting organic layers were combined and extracted with H_2O (3 × 75 mL) and brine (75 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford a yellow oil (98.5 mg, 0.77 mmoles, 98% yield). Observed ^{1}H NMR data agrees with literature values.[37] Rf = 0.34 (silica gel, 9:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1 H), 4.97 (ddt, *J* = 17.0, 1.9, 1.7 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 2.1, 1.0 Hz, 1 H), 3.61 (t, *J* = 6.5 Hz, 2 H), 2.07-1.99 (m, 2 H), 1.62-1.50 (m, 4 H), 1.42-1.27 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.1, 114.3, 63.0, 33.7, 32.7, 29.9, 28.9, 25.6.



3.25

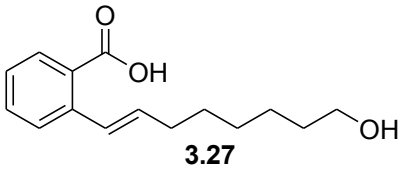
2-Vinyl benzoic acid dec-9-enyl ester(3.25). To 2-vinyl benzoic acid (128 mg, 0.86 mmol, 1.2 equiv.) in THF (3 mL) under argon atmosphere was added 7-octenol (92 mg, 0.72 mmol, 1 equiv.) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (414 mg, 2.16 mmol, 3 equiv.) before addition of 4-(dimethylamino)pyridine (18 mg, 0.14 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with NH_4Cl (50 mL) solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a colorless oil (91.3 mg, 0.35 mmol, 49%). **3.25**: Rf = 0.63 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2945, 2871, 1718, 1286 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (dd, *J* = 7.9, 1.4 Hz, 1 H), 7.56 (ddd, *J* = 7.9, 0.8, 0.7 Hz, 1 H), 7.48-7.40 (m, 2 H), 7.30 (ddd, *J* = 7.7, 7.5, 1.3 Hz, 1 H), 5.79 (ddt, *J* = 17.1, 10.2, 6.7 Hz, 1 H), 5.62 (dd, *J* = 17.5, 1.9 Hz, 1 H), 5.33 (dd, *J* = 11.0, 1.9 Hz, 1 H), 4.98 (ddt, *J* = 17.2, 2.1, 1.6 Hz, 1 H), 4.92 (ddt, *J* = 10.2, 1.1, 1.1 Hz, 1 H), 4.28 (t, *J* = 6.6 Hz, 2 H), 2.08-2.00 (m, 2 H), 1.79-1.70 (m, 2 H), 1.48-1.30 (m, 6 H); ^{13}C NMR (75

MHz, $CDCl_3$) δ 167.5, 139.5, 139.0, 136.0, 132.0, 130.2, 129.0, 127.4, 127.2, 116.3, 114.4, 65.2, 33.7, 28.8, 28.7, 28.7, 26.0 ; HRMS (+EI) : calcd for $C_{17}H_{22}O_2$ (M) 258.1620, obsd 258.1610.



3.26

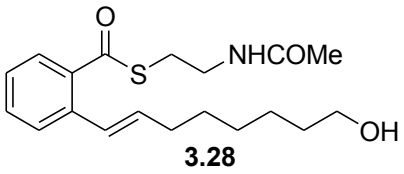
7,8,9,10,11,12-hexahydro-6-oxa-benzocyclododecen-5-one (3.26). To 3.25 (91 mg, 0.35 mmol, 1 equiv.) in toluene (84 mL) was added Grubbs catalyst 2nd generation (15 mg, 0.02 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 2% EtOAc in hexanes) to afford **3.26** (53.7 mg, 0.23 mmol, 66 %) as a colourless oil. **3.26**: Rf = 0.47 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2924, 2867, 1720, 1288, 1249 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.73 (dd, *J* = 7.7, 1.3 Hz, 1 H), 7.41 (ddd, *J* = 7.6, 7.4, 1.4 Hz, 1 H), 7.31-7.24 (m, 2 H), 6.69 (d, *J* = 15.9 Hz, 1 H), 5.69 (dt, *J* = 15.8, 7.1 Hz, 1 H), 4.31 (t, *J* = 5.6 Hz, 2 H), 2.31-2.24 (m, 2 H), 1.76-1.68 (m, 2 H), 1.59-1.45 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ169.5, 139.3, 132.3, 131.5, 131.5, 130.5, 129.8 127.8, 126.6, 63.1, 30.9, 26.7, 26.2, 25.5, 23.8; HRMS (+EI) : calcd for $C_{15}H_{18}O_2$ (M) 230.1307, obsd 230.1302



3.27

2-(8-Hydroxy-oct-1-enyl)-benzoic acid (3.27). To **3.26** (45 mg, 0.19 mmol, 1 equiv.) in methanol (6 mL) at room temperature was added lithium hydroxide monohydrate (76 mg, 1.81 mmol, 9.5 equiv.) and water (2 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched to pH 2 with a 10% HCl solution and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 1:1 EtOAc:hexanes) to afford **3.27** (35.7 mg, 0.14 mmol, 74 %) as a colorless oil. **3.27**: Rf = 0.19 (silica gel, 1:1

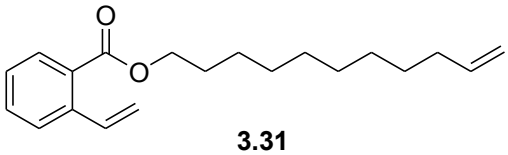
EtOAc in hexanes); IR (NaCl) νmax = 3859, 2936, 2863, 1692, 1260 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.95 (dd, *J* = 8.0, 1.2 Hz, 1 H), 7.51 (dd, *J* = 7.9, 1.4 Hz, 1 H), 7.45 (ddd, *J* = 7.6, 7.5, 1.2 Hz, 1 H), 7.26 (ddd, *J* = 7.6, 7.5, 1.2 Hz, 1 H), 7.19 (d, *J* = 15.7 Hz, 1 H), 6.07 (dt, *J* = 15.7, 6.8 Hz, 1 H), 5.66 (bs, 1 H), 3.66 (t, *J* = 6.3 Hz, 2 H), 2.29-2..21 (m, 2 H), 1.63-1.54 (m, 2 H) 1.54-1.47 (m, 2 H), 1.44-1.36 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.9, 140.6, 133.7, 132.6, 131.0, 129.1, 127.5, 127.4, 126.6, 62.7, 32.6, 32.6, 28.7, 28.4, 25.3; HRMS (+EI) : calcd for $C_{15}H_{20}O_3$ (M) 248.1412, obsd 248.1405.



3.28

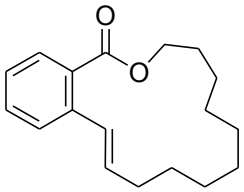
2-(8-Hydroxy-oct-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (3.28) To **3.27** (35 mg, 0.14 mmol, 1 equiv.) in THF (3 mL) at 0°C under argon atmosphere was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (81 mg, 0.42 mmol, 3 equiv.) before the addition of DMAP (3 mg, 0.03 mmol, 0.2 equiv.) and *N*-acetylcysteamine (37 μL, 0.35 mmol, 2.5 equiv.). The resulting mixture was slowly put to room temperature and stirred for 48 hours. The reaction was quenched with 10% HCl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 60% acetone in hexanes) to afford a colourless oil (46.4 mg, 0.13mmol, 95%). **3.28**: Rf = 0.44 (silica gel, 1:1 hexanes/acetone); IR (NaCl) νmax = 3282(br), 3066, 2931, 2858, 1653, 1191 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 7.8, 1.0 Hz, 1 H), 7.52 (d, *J* = 8.0 Hz, 1 H), 7.42 (ddd, *J* = 8.0, 7.5, 1.1 Hz, 1 H), 7.24 (ddd, *J* = 7.8, 7.4, 1.3 Hz, 1 H), 6.75 (d, *J* = 15.7 Hz, 1 H), 6.13 (dt, *J* = 15.7, 7.0 Hz, 1 H), 6.17 (bs, 1 H), 3.63 (t, *J* = 6.7 Hz, 2 H), 3.50 (dt, *J* = 6.6, 6.2 Hz, 2 H), 3.17 (t, *J* = 6.6 Hz, 2 H), 2.24-2.17 (m, 2 H), 1.95 (s, 3 H), 1.84 (bs, 1 H),1.61-1.52 (m, 2 H), 1.52-1.43 (m, 2 H), 1.41-1.34 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ1.94.8, 170.5, 136.7, 135.7, 134.6, 132.0, 128.4, 127.5, 127.1, 126.7, 62.9,

39.7, 32.9, 32.8, 29.3, 28.9, 28.7, 25.5, 23.2; HRMS (+EI) : calcd for $C_{15}H_{19}O_2$ (M-$SC_2H_4NHCOCH_3$) 231.1385, obsd 231.1365.



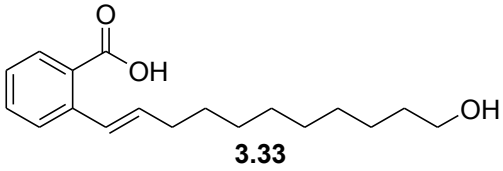
3.31

2-Vinyl benzoic acid undec-10-enyl ester (3.31). To 2-vinyl benzoic acid (50 mg, 0.34 mmol, 1 equiv.) in THF (2 mL) under argon atmosphere was added 10-undecen-1-ol (103 μL, 0.51 mmol, 1.5 equiv.) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (196 mg, 1.02 mmol, 3 equiv.) before addition of 4-(dimethylamino)pyridine (8 mg, 0.07 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with a saturated NH_4Cl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a yellow oil (54.4 mg, 0.18 mmol, 53 %). **3.31**: Rf = 0.40 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2930, 2862, 1718 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 7.2, 1.2 Hz, 1 H), 7.56 (dd, *J* = 7.5, 1.4 Hz, 1 H), 7.49-7.40 (m, 2 H), 7.30 (ddd, *J* = 7.7, 7.0, 1.7 Hz, 1 H), 5.79 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1 H), 5.62 (dd, *J* = 17.5, 1.4 Hz, 1 H), 5.32 (dd, *J* = 11.1, 1.3 Hz, 1 H), 4.97 (ddt, *J* = 17.3, 3.0, 1.4 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 2.1, 1.2 Hz, 1 H), 4.28 (t, *J* = 7.1 Hz, 2 H), 2.06-1.98 (m, 2 H), 1.78-1.69 (m, 2 H), 1.64-1.55 (m, 2 H), 1.46-1.39 (m, 2 H), 1.39-1.31 (m, 6 H), 1.31-1.23 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.5, 139.5, 139.2, 136.0, 132.0, 130.2, 129.1, 127.4, 127.2, 116.3, 114.1, 65.3, 33.8, 29.5, 29.4, 29.3, 29.1, 28.9, 28.7, 25.9; HRMS (+EI) : calcd for $C_{20}H_{28}O_2$ (M) 300.2089, obsd 300.2094.



3.32

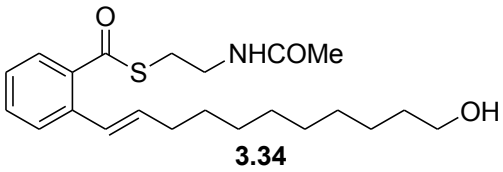
7,8,9,10,11,12,13,14,15-nonahydro-6-oxa-benzocyclopentdecen-5-one (3.32). To **3.31** (45 mg, 0.15 mmol, 1 equiv.) in toluene (36 mL) was added Grubbs catalyst 2nd generation (6 mg, 0.01 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 2 % ethyl acetate/hexanes) to afford **3.32** (37.0 mg, 0.14 mmol, 91 %) as a colourless oil. **3.32**: Rf = 0.55 (silica gel, 9:1 hexanes/EtOAc IR (NaCl) νmax = 2930, 2862, 1717 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (dd, *J* = 7.6, 1.0 Hz, 1 H), 7.47 (d, *J* = 7.7 Hz, 1 H), 7.39 (ddd, *J* = 8.0, 7.5, 1.5, 1 H), 7.24 (ddd, *J* = 7.7, 7.5, 1.2, 1 H), 6.94 (d, *J* = 15.7 Hz, 1 H), 5.96 (dt, *J* = 15.7, 7.4 Hz, 1 H), 4.38 (t, *J* = 5.4 Hz, 2 H), 2.34-2.20 (m, 2 H), 1.77-1.67 (m, 2 H), 1.58-1.19 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ169.4, 138.1, 134.6, 131.3, 129.9, 129.9, 129.4, 127.4, 126.6, 65.1, 31.3, 28.9, 27.7, 27.1, 26.1, 25.3, 24.8, 24.1; HRMS (+EI) : calcd for $C_{18}H_{24}O_2$ (M) 272.1776, obsd 272.1794.



3.33

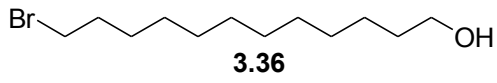
2-(11-Hydroxy-undec-1-enyl)-benzoic acid (3.33). To **3.32** (31 mg, 0.11 mmol, 1 equiv.) in methanol (9 mL) at room temperature was added lithium hydroxide monohydrate (44 mg, 1.05 mmol, 9.5 equiv.) and water (3 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched to pH 2 with a 10% HCl solution and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 1:1 hexanes:ethyl acetate) to afford **3.33** (20.5 mg, 0.07 mmol, 62 %) as a colourless oil. **3.33**: Rf = 0.31 (silica gel, 1:1 EtOAc in hexanes); IR (NaCl) νmax = 3402 (br), 2928, 2851, 1694, 1294, 1238 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.54 (dd, *J* = 7.9, 0.7 Hz, 1 H), 7.45 (ddd, *J* = 7.9, 7.5, 1.1 Hz, 1 H), 7.28-7.19 (m, 2 H), 6.12 (dt, *J* = 15.7, 6.8 Hz, 1 H), 3.64 (t, *J* = 6.6 Hz, 2 H), 2.28-2.20 (m, 2 H), 1.60-1.51 (m, 2 H), 1.51-1.43 (m, 2 H),

1.41-1.22 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 140.5, 134.3, 132.6, 131.1, 128.7, 127.4, 127.3, 126.5, 63.0, 33.0, 32.5, 29.4, 29.2, 29.2, 29.0, 29.0, 25.6; HRMS (+EI) : calcd for $C_{18}H_{26}O_3$ (M) 290.1882, obsd 290.1901.



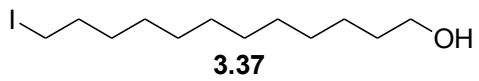
3.34

2-(11-Hydroxy-undec-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (3.34) To **3.33** (15 mg, 0.05 mmol, 1 equiv.) in THF (1 mL) at 0°C under argon atmosphere was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (29 mg, 0.15 mmol, 3 equiv.) and 4-(dimethylamino)pyridine (1 mg, 0.01 mmol, 0.2 equiv.) before the addition of *N*-acetylcysteamine (13 μL, 0.12 mmol, 2.5 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated NH_4Cl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 50% acetone in hexanes) to afford a colorless oil (9.8 mg, 0.03mmol, 50%). **3.34**: Rf = 0.21 (silica gel, 1:1 hexanes/acetone); IR (NaCl) νmax = 3280 (br), 3071, 2927, 2847, 1653, 1193 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 7.9, 1.4 Hz, 1 H), 7.54 (d, *J* = 8.1 Hz, 1 H), 7.42 (ddd, *J* = 7.6, 7.5, 1.2 Hz, 1 H), 7.25 (ddd, *J* = 7.6, 7.5, 1.4 Hz, 1 H), 6.76 (d, *J* = 15.8 Hz, 1 H), 6.17 (dt, *J* = 15.7, 6.9 Hz, 1 H), 5.93 (bs, 1 H), 3.62 (t, *J* = 6.6 Hz, 2 H), 3.53 (dt, *J* = 6.2 Hz, 2 H), 3.19 (t, *J* = 6.4 Hz, 2 H), 2.24-2.16 (m, 2 H), 1.97 (s, 3 H), 1.60-1.50 (m, 2 H), 1.50-1.41 (m, 2 H), 1.39-1.27 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.8 170.3, 136.7, 135.7, 134.9, 132.0, 128.4, 127.1, 127.0, 126.6, 63.7, 39.8, 33.2, 32.8, 29.5, 29.4, 29.4, 29.3, 29.1, 29.1, 25.7, 23.3; HRMS (+EI) : calcd for $C_{18}H_{24}O$ (M-SNAC-OH) 256.1827, obsd 256.1843.



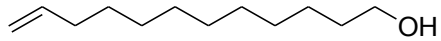
3.36

12-bromododecan-1-ol (3.36). To 1,12-dodecanediol (200 mg, 0.98 mmoles, 1 equiv.) in toluene (4 mL) was added bromic acid 9 M (48%) (175 μL, 1.57 mmoles, 1.6 equiv.) under argon atmosphere. The resulting mixture was refluxed for 72 hours. The reaction was then put to room temperature and the aqueous and organic phases were separated. The organic phase was diluted with ether (20 mL), washed with 1M NaOH (50 mL) and brine (50 mL). The resulting organic layer was dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 15% EtOAc in hexanes) to afford a clear oil (204.5 mg, 0.77 mmoles, 78% yield) and was carried over to the next step without further purification. Observed ^{1}H NMR data agrees with literature values.[38] Rf = 0.31 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.61 (t, *J* = 6.8 Hz, 2 H), 3.38 (t, *J* = 6.9 Hz, 2 H), 1.86-1.78 (m, 2 H), 1.59-1.49 (m, 3 H), 1.43-1.35 (m, 2 H) 1.34-1.21 (m, 14 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.0, 34.7, 32.8, 32.8, 29.6, 29.5, 29.5, 29.5, 29.4, 28.8, 28.2, 25.7.



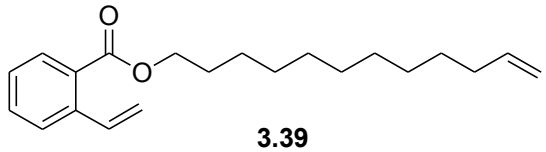
3.37

12-iododododecanol (3.37). To 12-bromododecanol (204 mg, 0.77 mmoles, 1 equiv.) in acetone (2 mL) was added sodium iodide (404 mg, 2.70 mmoles, 3.5 equiv.) under argon atmosphere. The resulting mixture was refluxed for 18 hours. The reaction was then put to room temperature and the mixture was washed with brine (20 mL) and extracted with EtOAc (3 × 15 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 20% EtOAc in hexanes) to afford an orange solid (209.4 mg, 0.67 mmoles, 87% yield). Observed ^{1}H NMR data agrees with literature values.[39] Rf = 0.26 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (t, *J* = 6.6 Hz, 2 H), 3.17 (t, *J* = 7.0 Hz, 2 H), 1.84-1.76 (m, 2 H), 1.59-1.50 (m, 2 H), 1.39-1.22 (m, 16 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.0, 33.6, 32.8, 30.5, 29.6, 29.5, 29.5, 29.4, 29.4, 28.5, 25.8, 7.4.



3.38

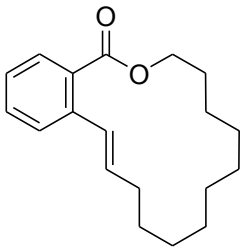
11-dodecen-1-ol (3.38). To 12-iodododecanol (201 mg, 0.64 mmoles, 1 equiv.) in DMSO (70 mL) was added TBAF hydrate (1.011 g, 3.20 mmoles, 5 equiv.) under argon atmosphere. The resulting mixture was stirred at room temperature for 5 hours. The reaction was quenched with H_2O (100 mL) and the aqueous phase was extracted with EtOAc (3 × 150mL). The resulting organic layers were combined and extracted with H_2O (3X75mL) and brine (75mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo to afford a colourless oil (110.5 mg, 0.60 mmoles, 93% yield) and was carried over to the next step without further purification. Observed ^{1}H NMR data agrees with literature values.[40] Rf = 0.32 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (ddt, *J* = 16.1, 10.0, 6.7 Hz, 1 H), 5.01-4.88 (m, 2 H), 3.62 (t, *J* = 6.7 Hz, 2 H), 2.06-1.97 (m, 2 H), 1.59-1.50 (m, 2 H), 1.42-1.19 (m, 14 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.3, 114.1, 63.1, 33.8, 32.8, 29.6, 29.5, 29.5, 29.4, 29.1, 28.9, 25.7.



3.39

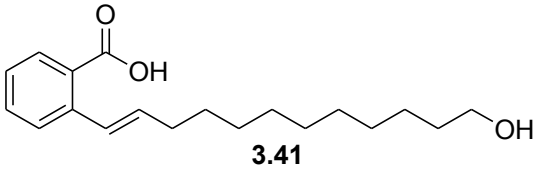
2-Vinyl benzoic acid dodec-11-enyl ester (3.39). To 2-vinyl benzoic acid (100 mg, 0.67 mmol, 1.2 equiv.) in THF (4 mL) under argon atmosphere was added 11-dodecen-1-ol (103 mg, 0.56 mmol, 1 equiv.) and *N*-(3- Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (322 mg, 1.68 mmol, 3 equiv.) before addition of 4-(dimethylamino)pyridine (14 mg, 0.11 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with NH_4Cl (50 mL) solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a colorless oil (130.7 mg, 0.42 mmol, 74%). **3.39**: Rf = 0.55 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2925, 2853, 1712,

1260 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 7.8, 1.3 Hz, 1 H), 7.56 (d, *J* = 7.8 Hz, 1 H), 7.49-7.40 (m, 2 H), 7.30 (ddd, *J* = 7.8, 7.4, 0.8 Hz, 1 H), 5.79 (ddt, *J* = 17.1, 10.3, 6.7 Hz, 1 H), 5.63 (dd, *J* = 17.5, 1.3 Hz, 1 H), 5.33 (dd, *J* = 11.0, 1.3 Hz, 1 H), 4.97 (ddt, *J* = 17.2, 1.9, 1.6 Hz, 1 H), 4.91 (ddt, *J* = 10.3, 1.1, 1.0 Hz, 1 H), 4.28 (t, *J* = 6.8 Hz, 2 H), 2.06-1.99 (m, 2 H), 1.78-1.70 (m, 2 H), 1.46-1.23 (m, 14 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 139.5, 139.2, 136.0, 132.0, 130.2, 129.1, 127.4, 127.2, 116.3, 114.1, 64.7, 33.8, 29.5, 29.5, 29.3, 29.1, 28.9, 28.6, 26.1, 25.9; HRMS (+EI) : calcd for $C_{21}H_{30}O_2$ (M) 314.2246, obsd 314.2248.



3.40

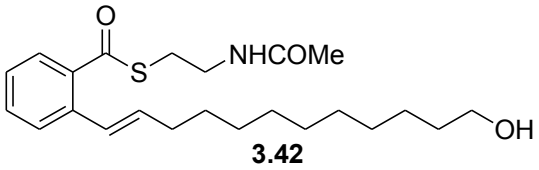
7,8,9,10,11,12,13,14,15,16-decahydro-6-oxa-benzocyclohexadecen-5-one (3.40). To **3.39** (122 mg, 0.39 mmol, 1 equiv.) in toluene (95 mL) was added Grubbs catalyst 2nd generation (17 mg, 0.02 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by PTLC (silica gel, 5% EtOAc/hexanes, Rf = 0.54) to afford **3.40** (66.4 mg, 0.23 mmol, 60 %) as a colourless oil. **3.40**: Rf = 0.64 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 1703 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.72 (dd, *J* = 7.8, 1.2 Hz, 1 H), 7.57 (d, *J* = 7.9 Hz, 1 H), 7.39 (dd, *J* = 7.5 Hz, 1 H), 7.23 (dd, *J* = 7.5 Hz, 1 H), 7.03 (d, *J* = 15.7 Hz, 1 H), 6.13 (dt, *J* = 15.7, 7.0 Hz, 1 H), 4.41 (t, *J* = 5.4 Hz, 2 H), 2.29-2.21 (m, 2 H), 1.79-1.71 (m, 2 H), 1.58-1.49 (m, 2 H), 1.47-1.21 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.2, 137.5, 133.7, 131.3, 130.1, 129.9, 128.1, 126.5, 126.1, 65.0, 31.2, 28.8, 27.1, 26.9, 26.5, 26.4, 26.2, 25.4, 24.6; HRMS (+EI) : calcd for $C_{19}H_{26}O_2$ (M) 286.1933, obsd 286.1921.



3.41

2-(12-Hydroxy-dodec-1-enyl)-benzoic acid (3.41). To **3.40** (63 mg, 0.22 mmol, 1 equiv.) in methanol (9 mL) at room temperature was added lithium hydroxide monohydrate (88 mg, 2.09 mmol, 9.5 equiv.) and water (3 mL). The resulting

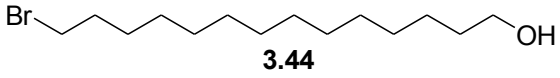
mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched to pH 2 with a 10% HCl solution and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 1:1 EtOAC:hexanes) to afford **3.41** (39.8 mg, 0.13 mmol, 59 %) as a white crystals. **3.41**: Rf = 0.41 (silica gel, 50 % EtOAc in hexanes); IR (NaCl) νmax = 3861 2925, 2853, 1717 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.96 (dd, *J* = 7.9, 1.2 Hz, 1 H), 7.54 (d, *J* = 8.0 Hz, 1 H), 7.46 (dd, *J* = 7.5, 1.2 Hz, 1 H), 7.26 (ddd, *J* = 7.8, 7.5, 1.2 Hz, 1 H), 7.22 (d, *J* = 15.7 Hz, 1 H), 6.13 (dt, *J* = 15.7, 7.0 Hz, 1 H), 3.64 (t, *J* = 6.7 Hz, 2 H), 2.27-2.21 (m, 2 H), 1.59-1.52 (m, 2 H), 1.51-1.44 (m, 2 H), 1.38-1.25 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.2, 140.6, 134.4, 132.7, 131.2, 128.6, 127.4, 127.1, 126.5, 63.1, 33.1, 32.6, 29.4, 29.3, 39.3, 29.3, 29.1, 29.0, 25.6; HRMS (+EI) : calcd for $C_{19}H_{28}O_3$ (M) 304.2038, obsd 304.2048.



3.42

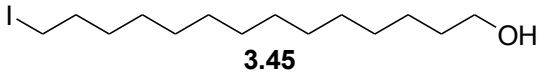
2-(12-Hydroxy-dodec-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (3.42). To **3.41** (34 mg, 0.11 mmol, 1 equiv.) in THF (1 mL) at 0°C under argon atmosphere was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (62 mg, 0.33 mmol, 2.6 equiv.) and 4-(dimethylamino)pyridine (3 mg, 0.02 mmoles, 0.2 equiv) before the addition of *N*-acetylcysteamine (23 μL, 0.22 mmol, 2.3 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturate NH_4Cl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by HPLC/MS (Flow rate 2.00 mL/min, gradient solvent of 0 to 20% B over 6 min and 20 to 100% B over 26 minutes. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).). **3.42**: Rf = 0.48 (silica gel, 1:1 hexanes/acetone); IR (NaCl)

νmax = 3755, 3285 (br), 2928, 2856, 1662, 1189, 1098 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (ddd, *J* = 7.8, 1.4, 0.6 Hz, 1 H), 7.54 (ddd, *J* = 7.9, 0.7, 0.5 Hz, 1 H), 7.42 (dddd, *J* = 7.8, 7.4, 1.4, 0.6 Hz, 1 H), 7.25 (ddd, *J* = 7.7, 7.5, 1.3 Hz, 1 H), 6.77 (d, *J* = 15.7 Hz, 1 H), 6.17 (dt, *J* = 15.7, 7.0 Hz, 1 H), 5.92 (bs, 1 H), 3.62 (t, *J* = 6.6 Hz, 2 H), 3.53 (dt, *J* = 6.5, 6.2 Hz, 2 H), 3.19 (t, *J* = 6.2 Hz, 2 H), 2.25-2.15 (m, 2 H), 1.97 (s, 3 H), 1.64-1.49 (m, 4 H), 1.49-1.39 (m, 2 H), 1.38-1.24 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.8 170.3, 136.7, 135.7, 135.0, 132.0, 128.4, 127.0, 127.0, 126.6, 63.1, 39.8, 33.2, 32.8, 31.6, 29.5, 29.5, 29.4, 29.4, 29.2, 25.7, 22.7, 14.1; HRMS (+EI) : calcd for $C_{23}H_{35}NO_3S$ (M) 405.2338, obsd 405.2356.



3.44

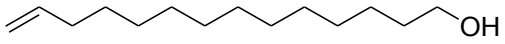
14-bromotetradecan-1-ol (3.44). To 1,14-tetradecanediol (300 mg, 1.30 mmoles, 1 equiv.) in toluene (2.6 mL) was added bromic acid 9 M (48%) (231 μL, 2.08 mmoles, 1.6 equiv.) under argon atmosphere. The resulting mixture was refluxed for 72 hours. The reaction was then put to room temperature and the aqueous and organic phases were separated. The organic phase was diluted with ether (20 mL), washed with 1M NaOH (50 mL) and brine (50 mL). The resulting organic layer was dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) to afford a white solid (194.1 mg, 0.66 mmoles, 51% yield). Observed ^1H NMR data agrees with literature values.[41] Rf = 0.34 (silica gel, 20% EtOAc in hexanes); ^1H NMR (400 MHz, $CDCl_3$) δ 3.61 (t, *J* = 6.6 Hz, 2 H), 3.38 (t, *J* = 6.8 Hz, 2 H), 1.87-1.78 (m, 2 H), 1.58-1.50 (m, 2 H), 1.44-1.35 (m, 2 H), 1.34-1.22 (m, 18 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.1, 34.1, 32.9, 32.8, 31.6, 29.6, 29.6, 29.5, 29.4, 28.8, 28.2, 25.8, 22.7, 14.1.



3.45

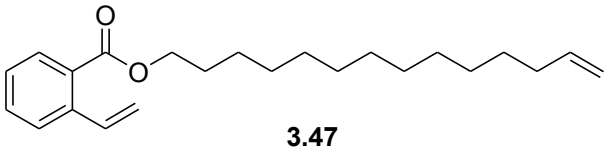
14-iodotetradecan-1-ol (3.45). To 14-tetradecane-1-ol (194 mg, 0.66 mmoles, 1 equiv.) in acetone (1.7 mL) was added sodium iodide (347 mg, 2.32 mmoles, 3.5

equiv.) under argon atmosphere and the resulting mixture was refluxed for 18 hours. The reaction was then put to room temperature and the mixture was washed with brine (20 mL) and extracted with EtOAc (3X15 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) to afford light pink crystals (224.7 mg, 0.66 mmoles, 100% yield). **3.45**: Rf = 0.47 (silica gel, 20% EtOAc in hexanes); IR (NaCl) νmax = 3300(br), 2932, 2849 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.61 (t, *J* = 6.7 Hz, 2 H), 3.16 (t, *J* = 7.0 Hz, 2 H), (1.84-1.75 (m, 2 H), 1.58-1.50 (m, 2 H), 1.42-1.20 (m, 20 H); ^{13}C NMR (100 Hz, $CDCl_3$) δ 63.1, 33.6, 32.8, 30.5, 29.6, 29.6, 29.6, 29.5, 29.4, 29.4, 29.3, 28.6, 25.8, 7.4; HRMS (+EI) : calcd for $C_{14}H_{29}IO$ (M) 213.2218, obsd 213.2209.



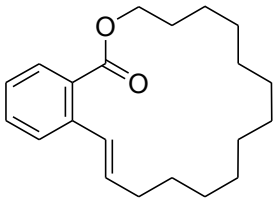
3.46

14-tetradecen-1-ol (3.46). To 14-iodotetradecanol (225 mg, 0.66 mmoles, 1 equiv.) in DMSO (72.3 mL) was added TBAF hydrate (1034 mg, 3.3 mmoles, 5 equiv.) under argon atmosphere. The resulting mixture was stirred at room temperature for 5 hours. The reaction was quenched with H_2O (100 mL) and the aqueous phase was extracted with EtOAc (3 × 150mL). The resulting organic layers were combined and extracted with H_2O (3 × 75mL) and brine (75mL), dried over $MgSO_4$, filtered, and concentrated to dryness and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) in vacuo to afford a yellow oil (113.6 mg, 0.53 mmoles, 81% yield) and was carried over to the next step without further purification. Observed ^1H NMR data agrees with literature values.[42] Rf = 0.37 (silica gel, 1:1 hexanes/EtOAc); ^1H NMR (400 MHz, $CDCl_3$) δ 5.79 (ddt, *J* = 17.1, 10.3, 6.8 Hz, 1 H), 4.97 (ddt, *J* = 17.1, 2.1, 1.4 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 1.4, 1.0 Hz, 1 H), 3.62 (t, *J* = 6.7 Hz, 2 H), 2.05-1.98 (m, 2 H), 1.59-1.50 (m, 2 H), 1.40-1.22 (m, 18 H); ^{13}C NMR (75 Hz, $CDCl_3$) δ 139.3, 114.1, 63.1, 33.8, 32.8, 29.6, 29.6, 29.6, 29.6, 29.5, 29.4, 29.2, 29.0, 25.7.



3.47

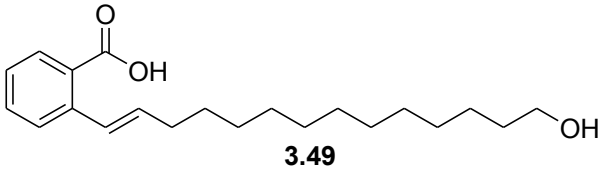
2-Vinyl benzoic acid tetradec-13-enyl ester (3.47). To 2-vinyl benzoic acid (100 mg, 0.67 mmol, 1.3 equiv.) in THF (3 mL) under argon atmosphere was added 14-tetradecen-1-ol (113 mg, 0.53 mmol, 1 equiv.) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (385 mg, 2.01 mmol, 3.8 equiv.) before addition of 4-(dimethylamino)pyridine (16 mg, 0.13 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with a saturated NH_4Cl (50 mL) solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford a colorless oil (96.5 mg, 0.28 mmol, 53%). **3.47**: Rf = 0.63 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2926, 2855, 1743, 1245 cm^{-1}; ^1H NMR (400 MHz, CDCl3) δ 7.85 (dd, *J* = 7.8, 1.3 Hz, 1 H), 7.56 (d, *J* = 7.5 Hz, 1 H), 7.48-7.40 (m, 2 H), 7.30 (ddd, *J* = 7.5, 7.5 1.3 Hz, 1 H), 5.79 (ddt, *J* = 17.2, 10.4, 6.7 Hz, 1 H), 5.62 (dd, *J* = 17.4, 1.4 Hz, 1 H), 5.32 (dd, *J* = 11.1, 1.3 Hz, 1 H), 4.97 (ddt, *J* = 17.1, 1.5, 0.3 Hz, 1 H), 4.90 (ddt, *J* = 10.0, 1.3, 0.5 Hz, 1 H), 4.28 (t, *J* = 6.8 Hz, 2 H), 2.05-1.98 (m, 2 H), 1.78-1.67 (m, 2 H), 1.65-1.55 (m, 2 H),1.44-1.22 (m, 16 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 139.5, 139.3, 136.0, 132.0, 130.2, 129.0, 127.4, 127.2, 116.3, 114.1, 64.7, 33.8, 29.6, 29.6, 29.5, 29.5, 29.3, 29.2, 29.0, 28.7, 28.6, 25.9. HRMS (+EI) : calcd for $C_{23}H_{34}O_2$ (M) 342.2559, obsd 342.2542.



3.48

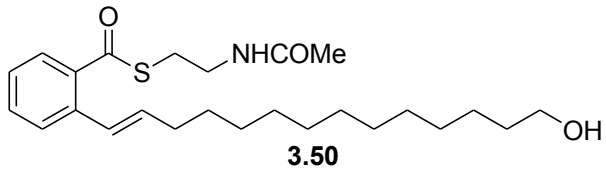
7,8,9,10,11,12,13,14,15,16,17,18-dodecahydro-6-oxa-benzocyclooxadecen-5-one (3.48). To **3.47** (96 mg, 0.28 mmol, 1 equiv.) in toluene (67 mL) was added Grubbs catalyst 2nd generation (12 mg, 0.01 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column

chromatography (silica gel, 100% hexanes) to afford **3.48** (67.3 mg, 0.21 mmol, 76%) as a colorless oil. Due to the non-polar properties of the molecules, there was a 3:2 mixture of product:SM. The mixture was carried over to the next step without further purification. **3.48**: Rf = 0.70 (silica gel, 9:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, *J* = 7.8, 1.3 Hz, 1 H), 7.54 (dd, *J* = 8.0, 1.3 Hz, 1 H), 7.49-7.35 (m, 1 H), 7.23 (ddd, *J* = 7.6, 7.5, 1.2 Hz, 1 H), 7.03 (d, *J* = 15.8 Hz, 1 H), 6.14 (dt, *J* = 15.7, 6.8 Hz, 1 H), 4.36 (t, *J* = 6.5 Hz, 2 H), 2.27-2.17 (m, 2H), 1.78-1.20 (m, 22 H).



3.49

2-(14-Hydroxy-tetradec-1-enyl)-benzoic acid (3.49). To **3.48** (67 mg, 0.21 mmol, 1 equiv.) in methanol (6 mL) at room temperature was added lithium hydroxide monohydrate (85 mg, 2.00 mmol, 9.5 equiv.) and water (2 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached pH 2 and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 1:1 hexanes:EtOAc) to afford **3.49** (19.2 mg, 0.06 mmol, 27 %) as a yellow oil. **3.49**: Rf = 0.43 (silica gel, 50 % EtOAc in hexanes); IR (NaCl) νmax = 3460(br), 2930, 2857, 1694, 1261 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (dd, *J* = 7.9, 1.2 Hz, 1 H), 7.54 (d, *J* = 7.8 Hz, 1 H), 7.46 (ddd, *J* = 8.0, 7.5, 1.2 Hz, 1 H), 7.29-7.20 (m, 2 H), 6.14 (dt, *J* = 15.6, 6.9 Hz, 1 H), 3.63 (t, *J* = 6.7 Hz, 2 H), 2.28-2.20 (m, 2 H), 1.59-1.52 (m, 2 H), 1.51-1.43 (m, 2 H), 1.38-1.21 (m, 16 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 140.6, 134.4, 132.7, 131.2, 128.6, 127.4, 127.0, 126.5, 63.1, 33.2, 32.7, 29.5, 29.5, 29.5, 29.5 29.4, 29.4, 29.2, 29.1, 25.7; HRMS (+EI) : calcd for $C_{21}H_{32}O_3$ (M) 332.2351, obsd 332.2343.



3.50

2-(14-Hydroxy-tetradec-1-enyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (3.50) To **3.49** (19 mg, 0.058 mmol, 1 equiv.) in THF (1 mL) at 0°C under argon atmosphere was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (33 mg, 0.17 mmol, 3.0 equiv.) before the addition of *N*-acetylcysteamine (16 μL, 0.15 mmol, 2.6 equiv.) and 4-dimethylaminopyridine (1 mg, 0.012 mmol, 0.2 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated NH_4Cl solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by HPLC-MS (Flow rate 2.00 mL/min, gradient solvent of 0 to 20% B over 6 min and 20 to 100% B over 26 minutes. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).) to afford a colourless oil (8.3 mg, 0.019 mmol, 33%). **3.50**: Rf = 0.51 (silica gel, 1:1 hexanes/acetone); IR (NaCl) νmax = 3287 (br), 3072, 2926, 2853, 1655, 1187 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 7.7, 1.2 Hz, 1 H), 7.54 (d, *J* = 7.9 Hz, 1 H), 7.42 (ddd, *J* = 7.9, 7.7, 1.4 Hz, 1 H), 7.25 (ddd, *J* = 7.7, 7.5, 1.3 Hz, 1 H), 6.77 (d, *J* = 15.7 Hz, 1 H), 6.18 (dt, *J* = 15.7, 7.0 Hz, 1 H), 5.92 (bs, 1 H), 3.62 (t, *J* = 6.5 Hz, 2 H), 3.53 (dt, *J* = 6.2 Hz, 2 H), 3.19 (t, *J* = 6.6 Hz, 2 H), 2.25-2.15 (m, 2 H), 1.97 (s, 3 H), 1.64-1.49 (m, 2 H), 1.49-1.41 (m, 2 H), 1.39-1.21 (m, 16 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.8 170.3, 135.7, 134.1, 133.0, 130.9, 129.5, 128.0, 126.0, 125.5, 63.0, 39.7, 32.8, 31.2, 29.6, 29.3, 29.5, 29.4, 29.2, 27.9, 27.8, 25.8, 24.1, 22.4, 20.7; HRMS (+EI) : calcd for $C_{25}H_{39}NO_3S$ (M-$SC_2H_4NHCOCH_3$) 314.2246, obsd 314.2247.

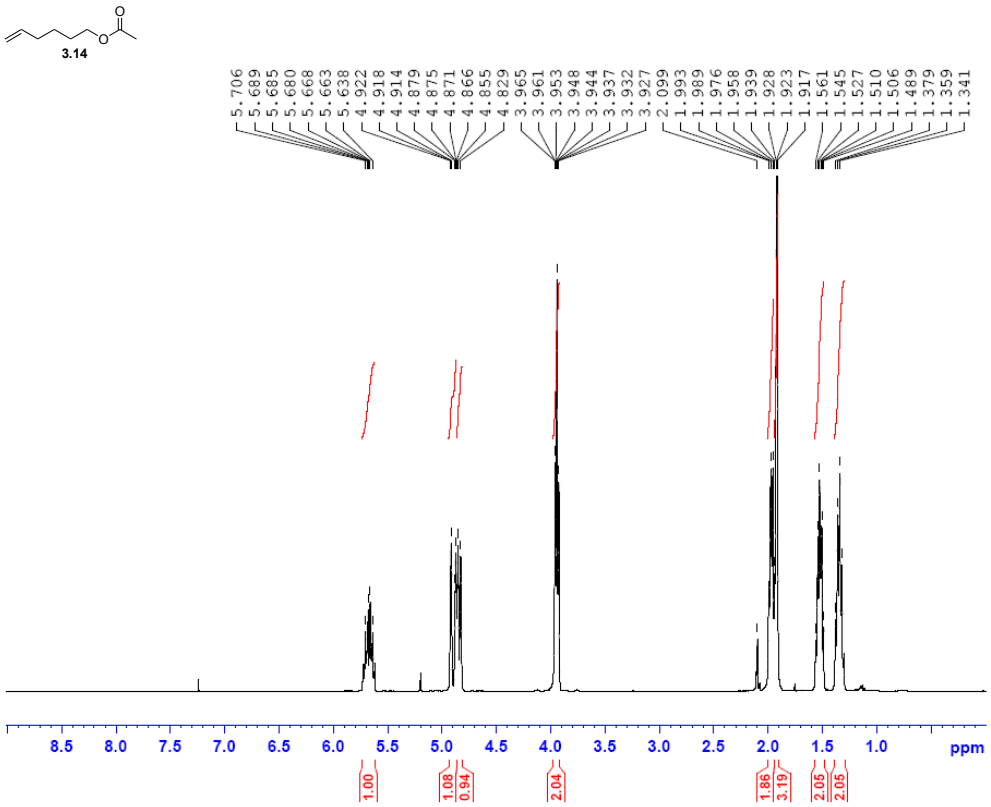
3.4.3 Enzymatic protocol

Assay of enzymatic catalyzed macrocyclization reaction

For a typical assay, a 20 μL solution containing 50 mM sodium phosphate pH 7.43, 5 mM thioester substrate **2.21**, 2 μM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1 : 4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

For a typical assay with Brij-58, a 20 μL solution containing 50 mM sodium phosphate pH 7.43, 2 mg Brij-58, 5 mM thioester substrate **2.21**, 2 μM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1 : 4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

3.4.4 Spectra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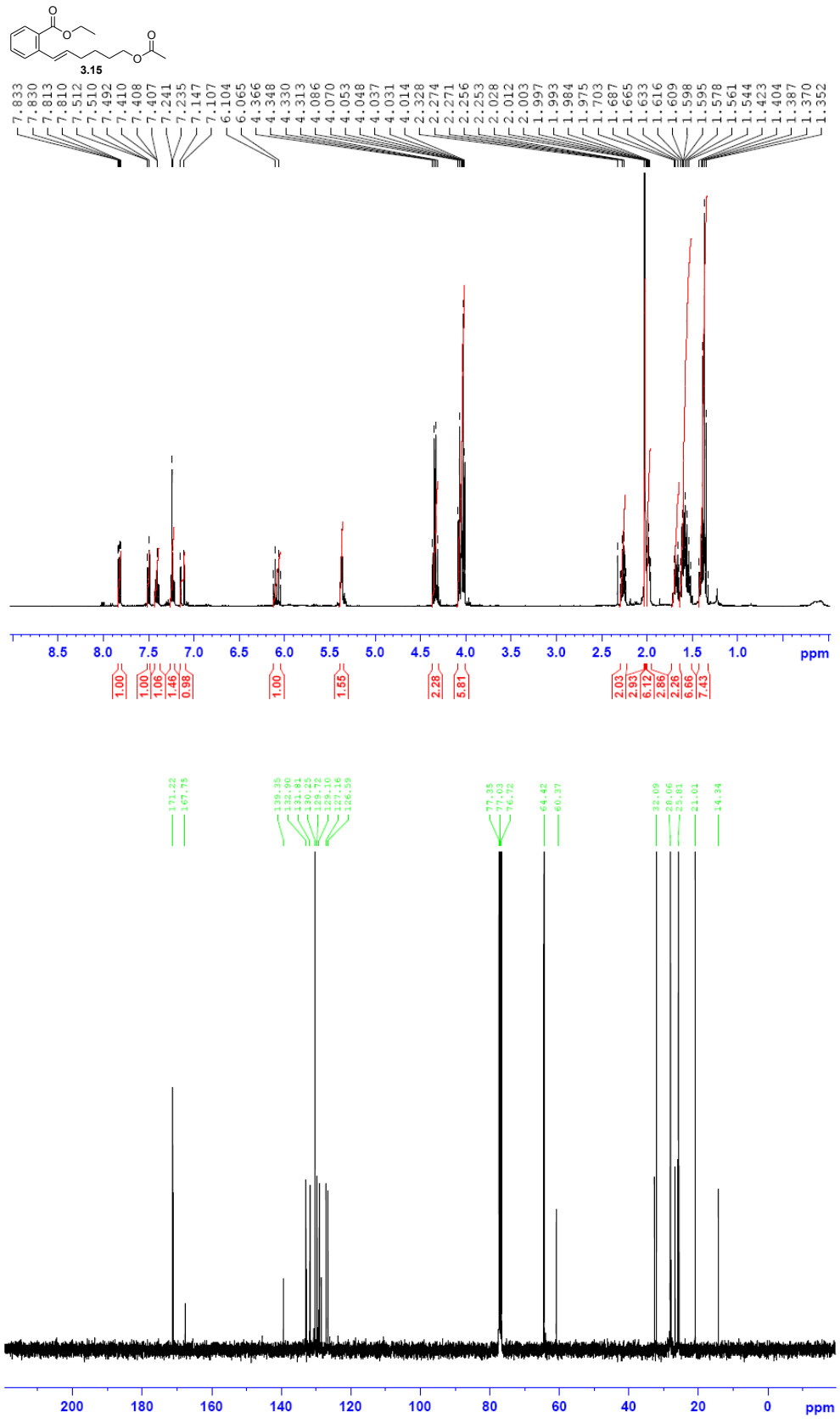
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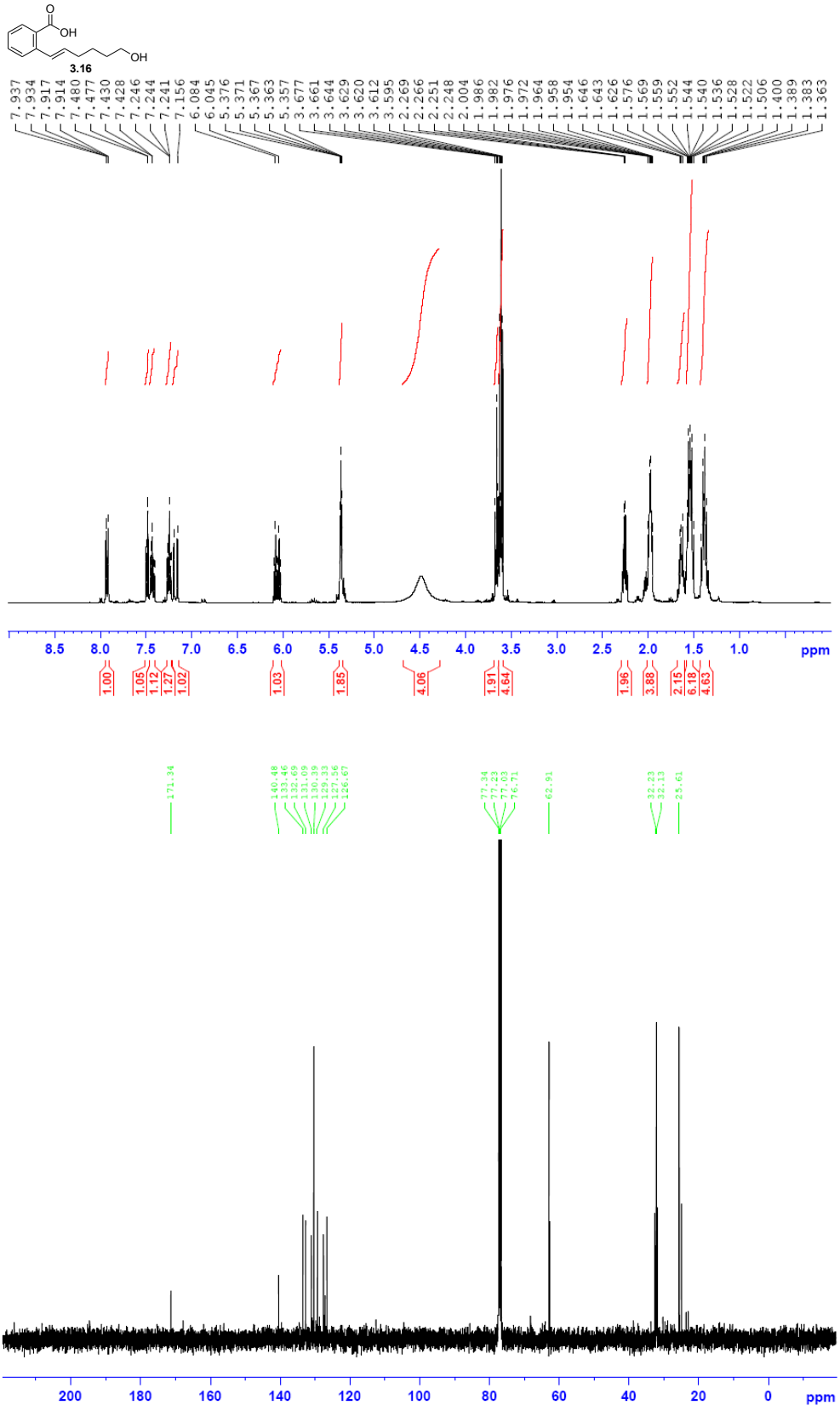
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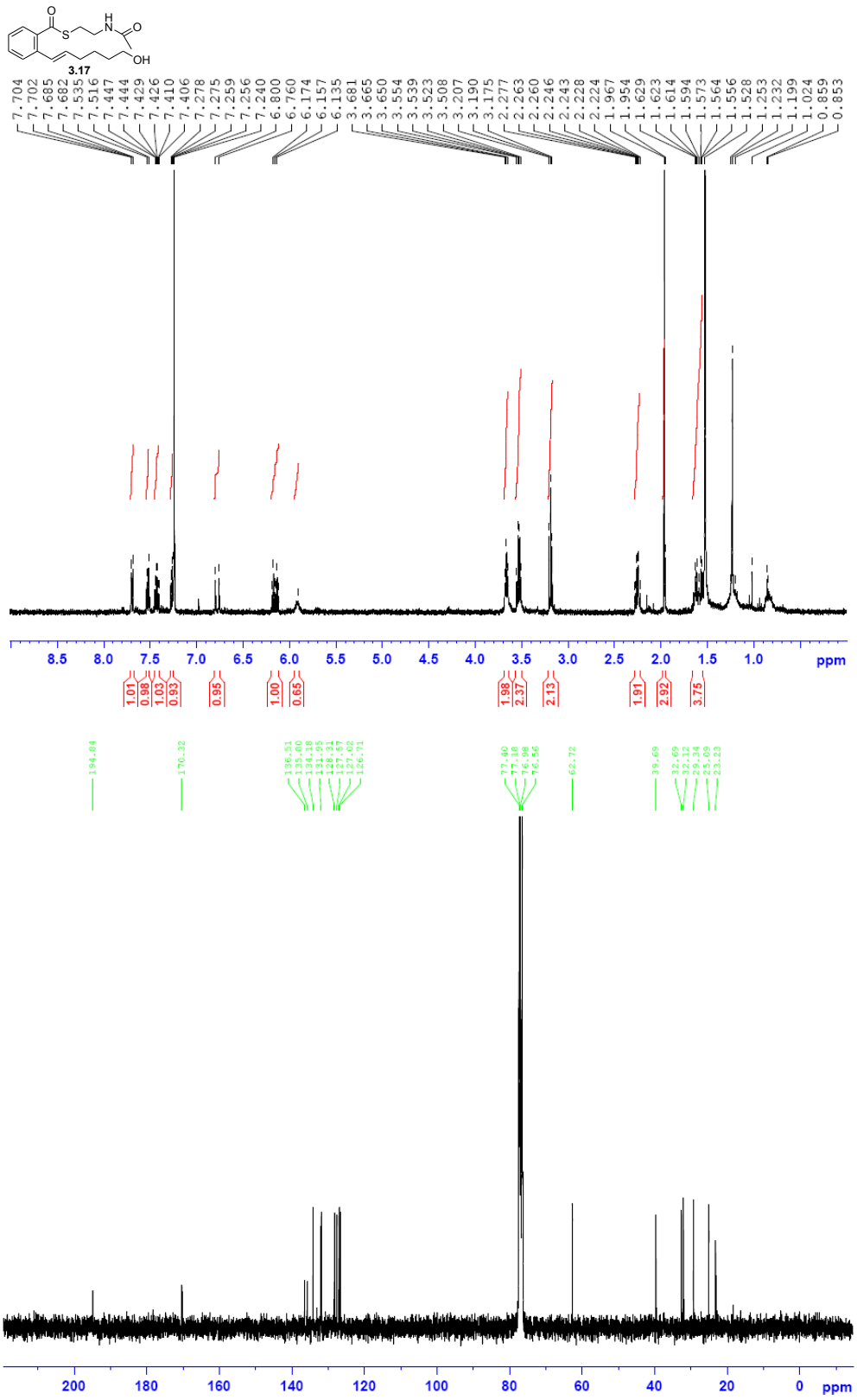
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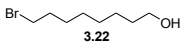
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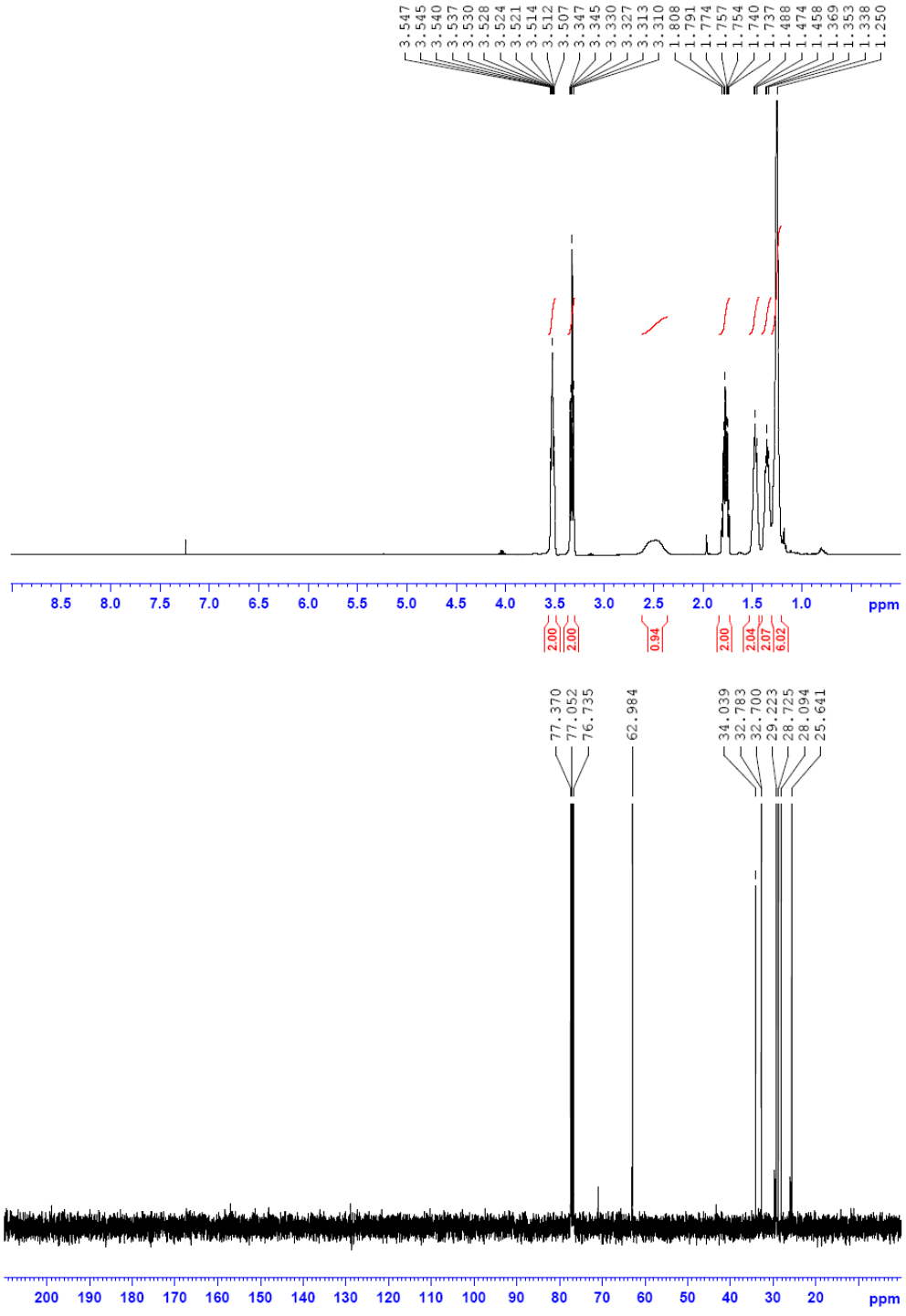
3.22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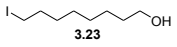
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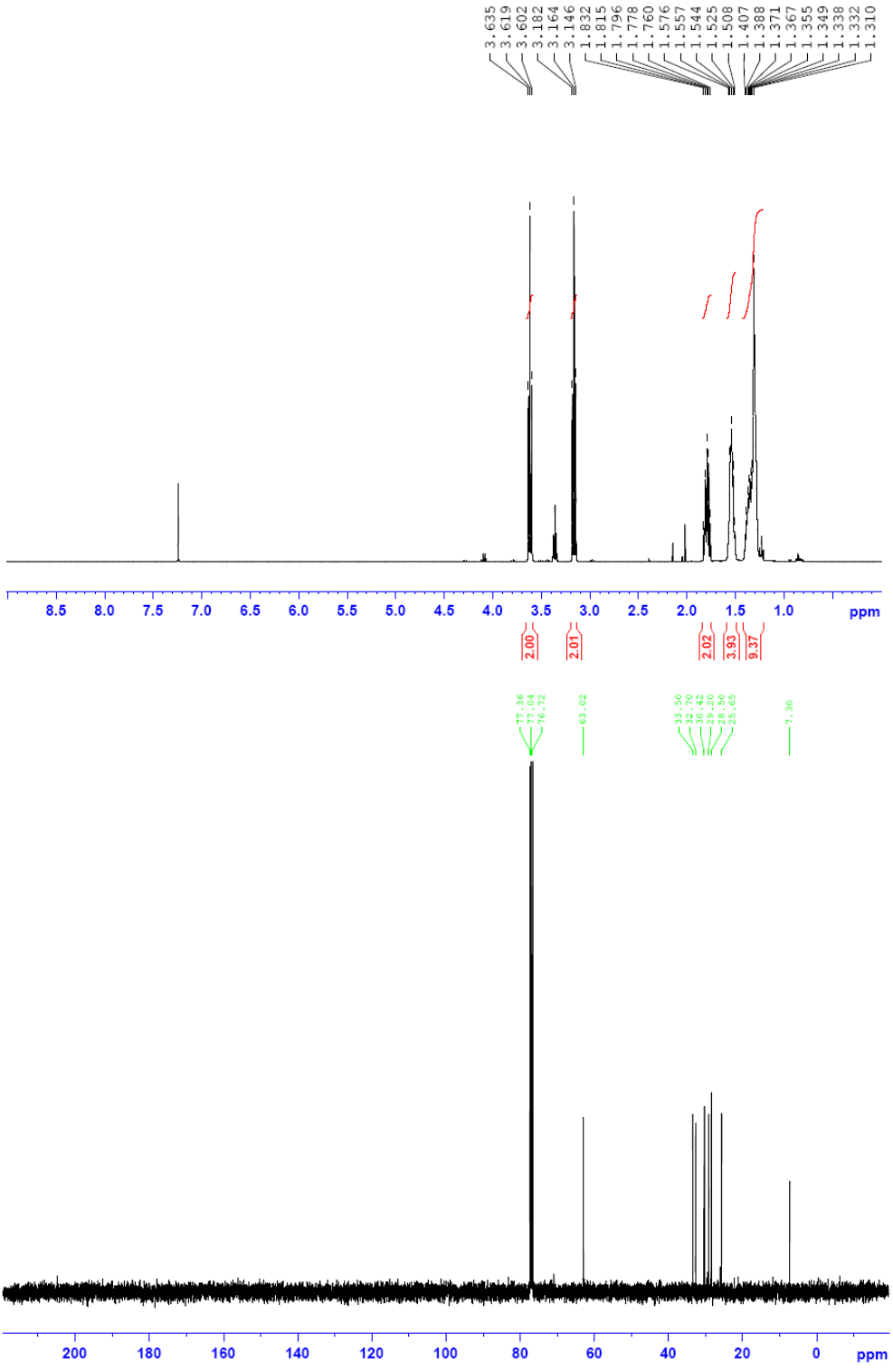
3.23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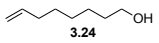
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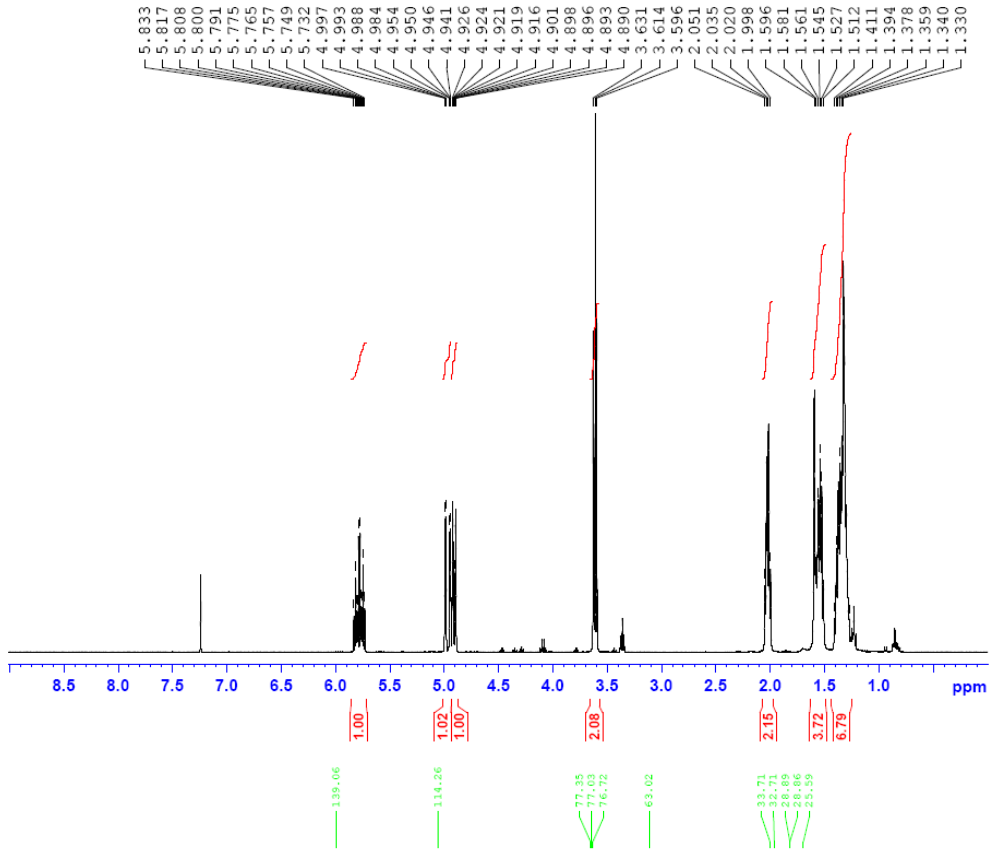
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1.00
2.08
2.15
3.72
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76.72
63.02
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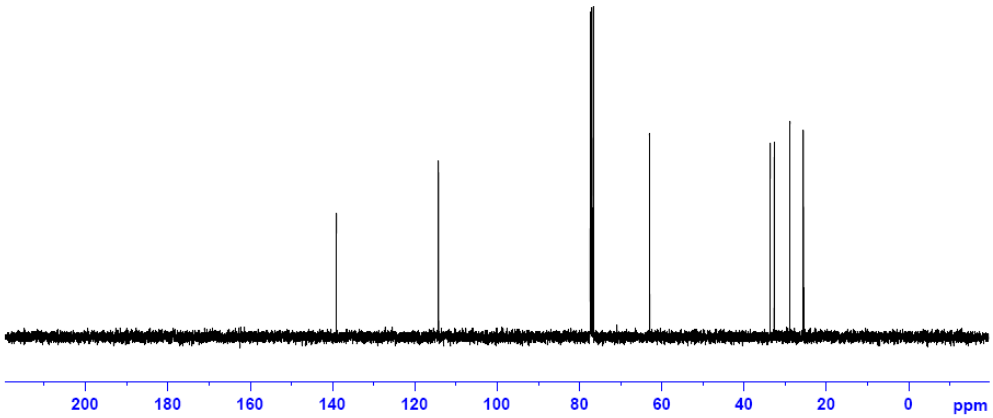
25.59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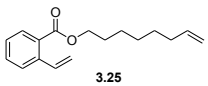

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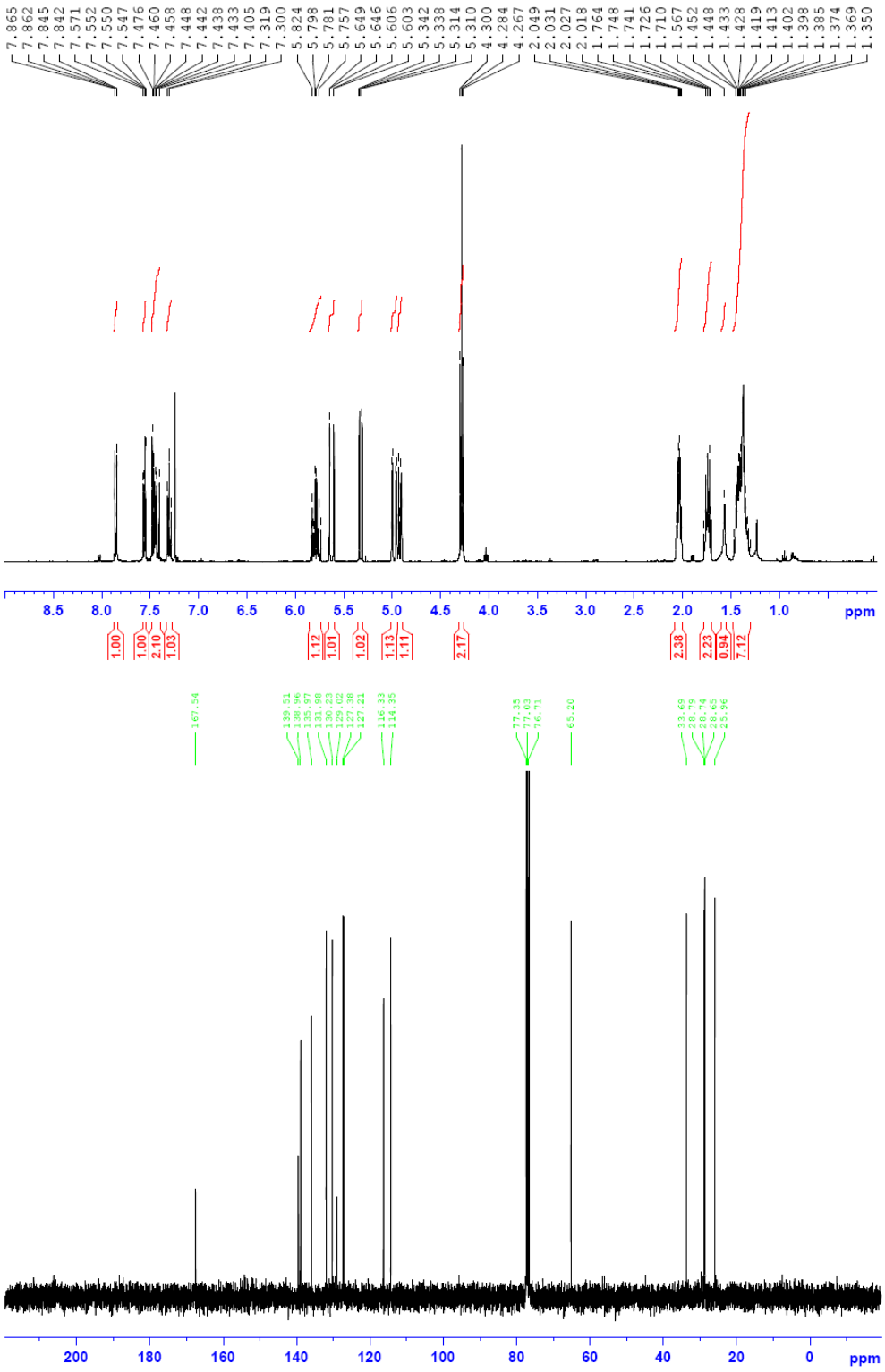
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7.433
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2.027
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1.726
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2.5
2.0
1.5
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ppm
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1.03
1.12
1.01
1.02
1.13
1.11
2.17
2.38
2.23
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7.12
167.54
139.51
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135.97
131.98
130.23
129.02
127.38
127.21
116.33
114.35
77.35
77.03
76.71
65.20
33.69
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28.63
25.96
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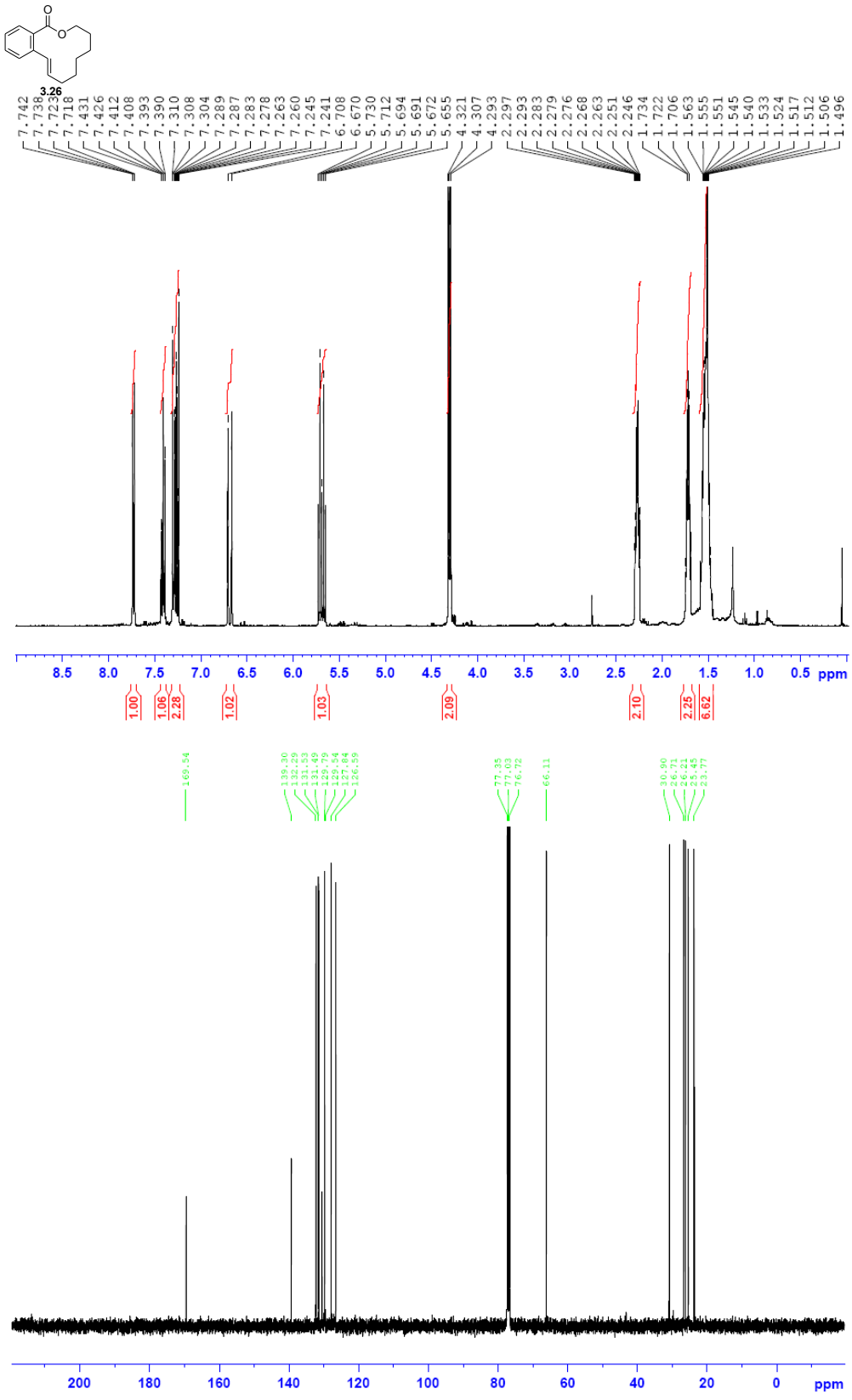
180
160
140
120
100
80
60
40
20
0
ppm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7.304
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7.287
7.283
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7.263
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7.241
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5.730
5.712
5.694
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5.672
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2.297
2.293
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2.279
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2.268
2.263
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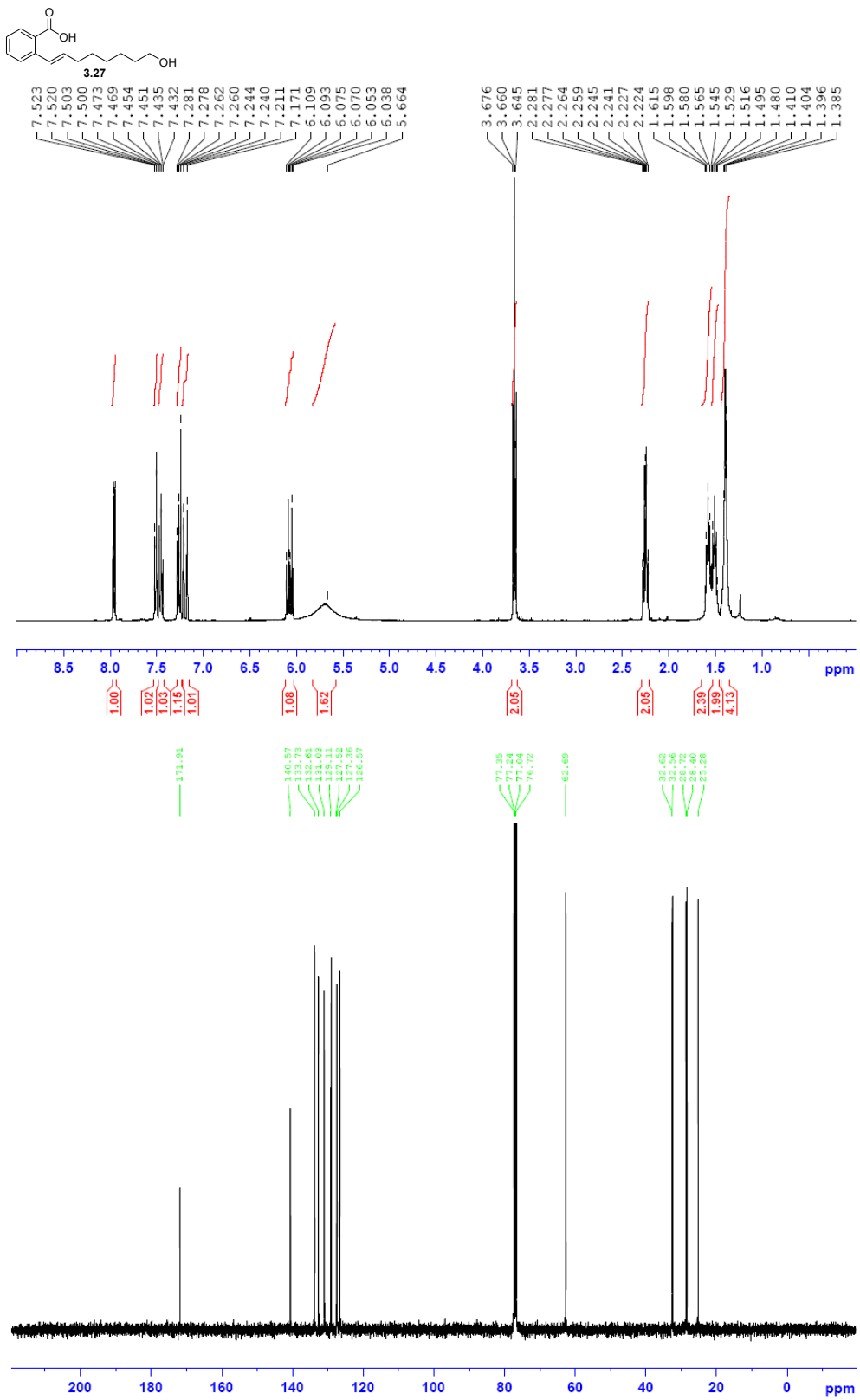
1.551
1.545
1.540
1.533
1.524
1.517
1.512
1.506
1.496
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
1.06
2.28
1.02
1.03
2.09
2.10
2.25
6.62
169.54
139.90
132.29
131.53
131.49
129.79
129.54
127.84
126.59
77.35
77.00
76.72
66.11
30.90
26.71
26.21
25.45
23.77
200 180 160 140 120 100 80 60 40 20 0 ppm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7.451
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7.432
7.281
7.278
7.262
7.260
7.244
7.240
7.211
7.171
6.109
6.093
6.075
6.070
6.053
6.038
5.664
3.676
3.660
3.645
2.281
2.277
2.264
2.259
2.245
2.241
2.227
2.224
1.615
1.598
1.580
1.565
1.545
1.529
1.516
1.495
1.480
1.410
1.404
1.396
1.385
8.5
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7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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1.02
1.03
1.15
1.01
1.08
1.62
2.05
2.05
2.39
1.99
4.13
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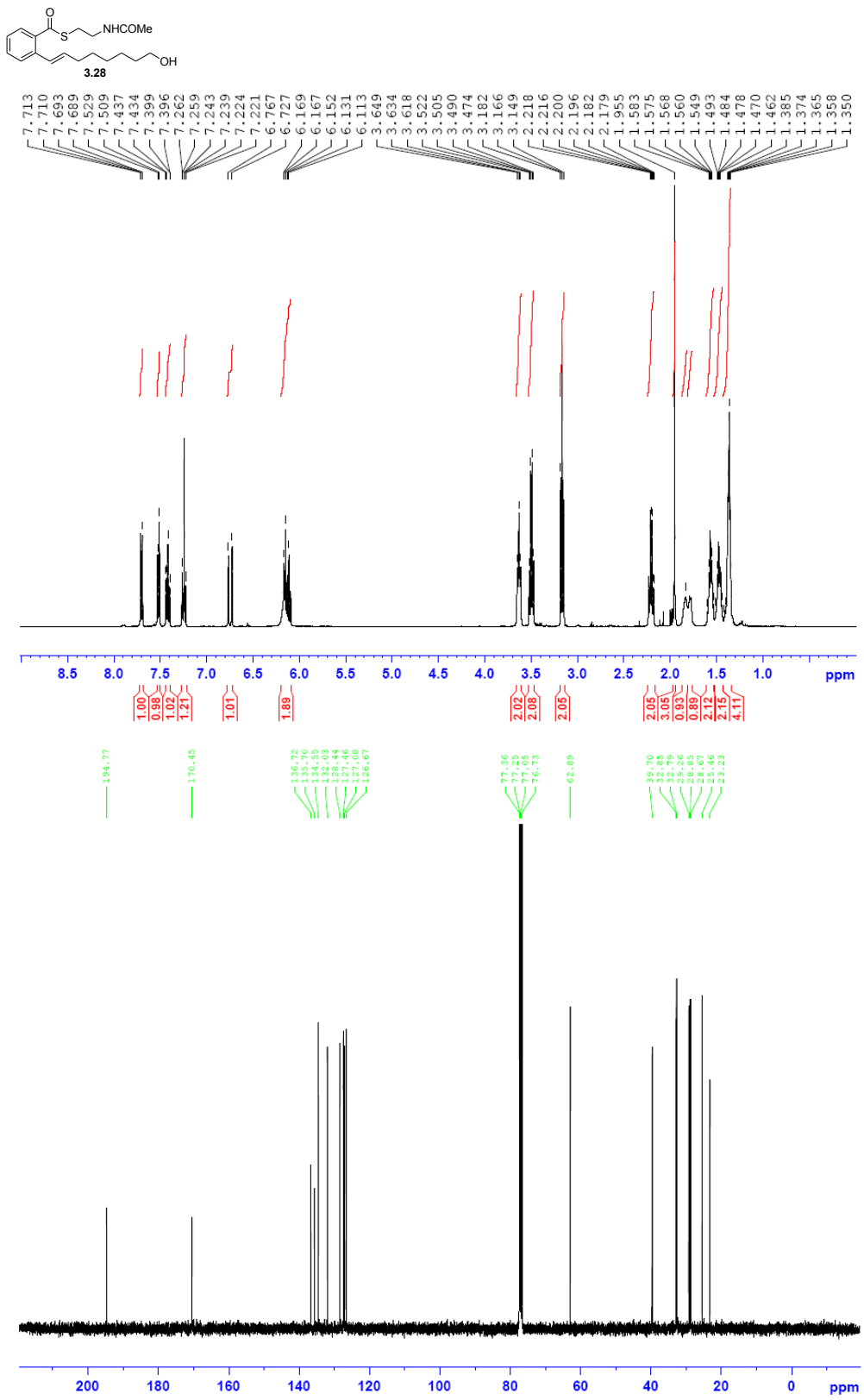
131.03
129.11
127.52
127.36
126.57
77.35
77.24
77.04
76.72
62.69
32.62
32.56
28.76
28.72
28.40
25.28
200
180
160
140
120
100
80
60
40
20
0
ppm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S
NHCOMe
OH
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7.437
7.434
7.399
7.396
7.262
7.259
7.243
7.239
7.224
7.221
6.767
6.727
6.169
6.167
6.152
6.131
6.113
3.649
3.634
3.618
3.522
3.505
3.490
3.474
3.182
3.166
3.149
2.218
2.216
2.200
2.196
2.182
2.179
1.955
1.583
1.575
1.568
1.560
1.549
1.493
1.484
1.478
1.470
1.462
1.385
1.374
1.365
1.358
1.350
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8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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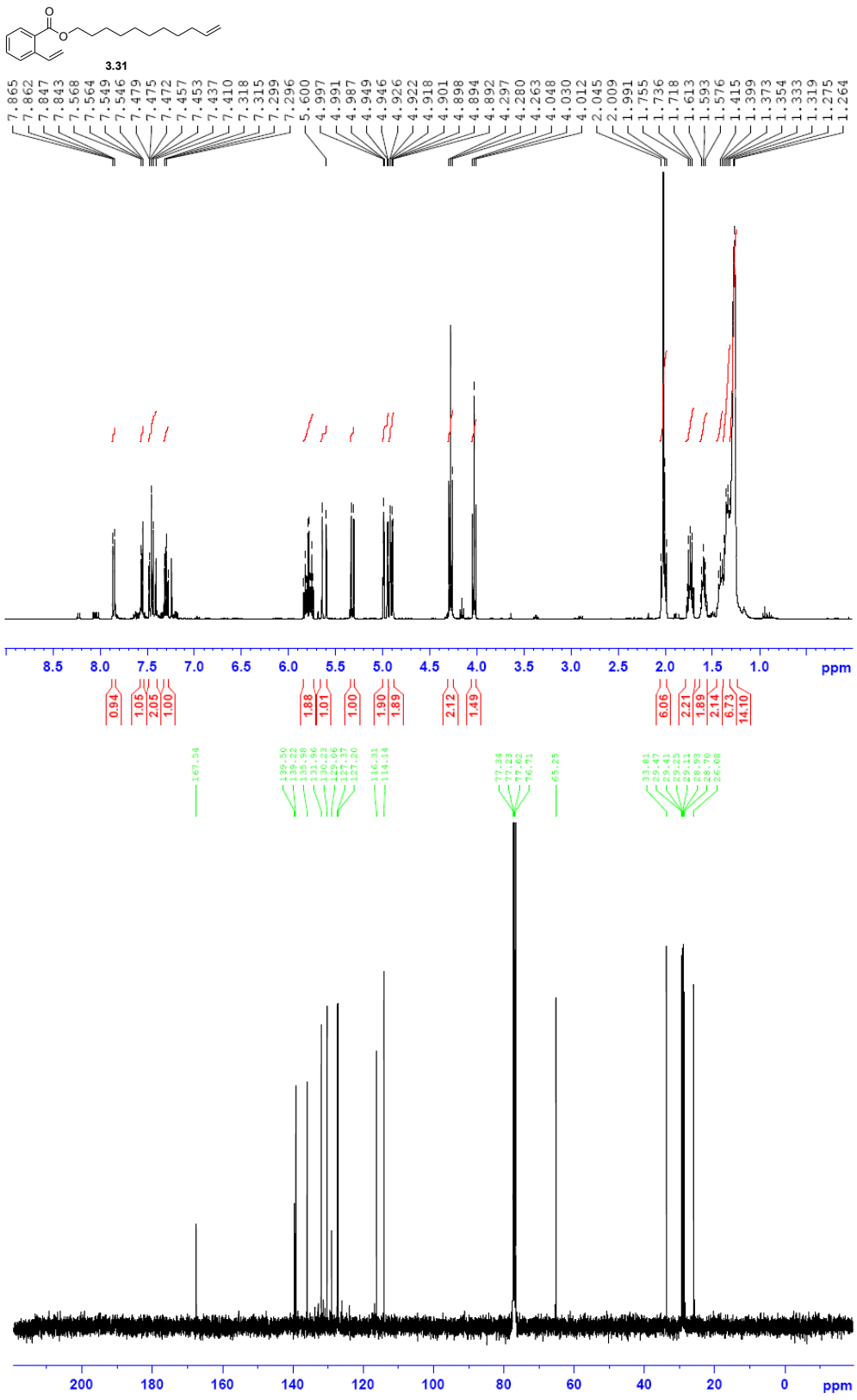
1.21
1.01
1.89
2.02
2.08
2.05
2.05
3.05
0.93
0.89
2.12
2.15
4.11
194.77
170.45
136.72
135.70
134.55
130.08
128.44
127.46
127.08
126.67
77.36
77.25
77.05
76.73
62.89
39.70
32.85
32.79
29.26
28.85
28.67
25.46
23.23
200
180
160
140
120
100
80
60
40
20
0
ppm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7.549
7.546
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7.475
7.472
7.457
7.453
7.437
7.410
7.318
7.315
7.299
7.296
5.600
4.997
4.991
4.987
4.949
4.946
4.926
4.922
4.918
4.901
4.898
4.894
4.892
4.297
4.280
4.263
4.048
4.030
4.012
2.045
2.009
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1.736
1.718
1.613
1.593
1.576
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1.399
1.373
1.354
1.333
1.319
1.275
1.264
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7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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2.05
1.00
1.88
1.01
1.00
1.90
1.89
2.12
1.49
6.06
2.21
1.89
2.14
6.73
14.10
167.54
139.50
138.21
135.98
131.96
130.23
129.06
127.37
127.20
116.31
114.14
77.34
77.23
77.02
76.71
65.25
33.61
29.47
29.41
29.25
29.11
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28.70
26.08
200
180
160
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120
100
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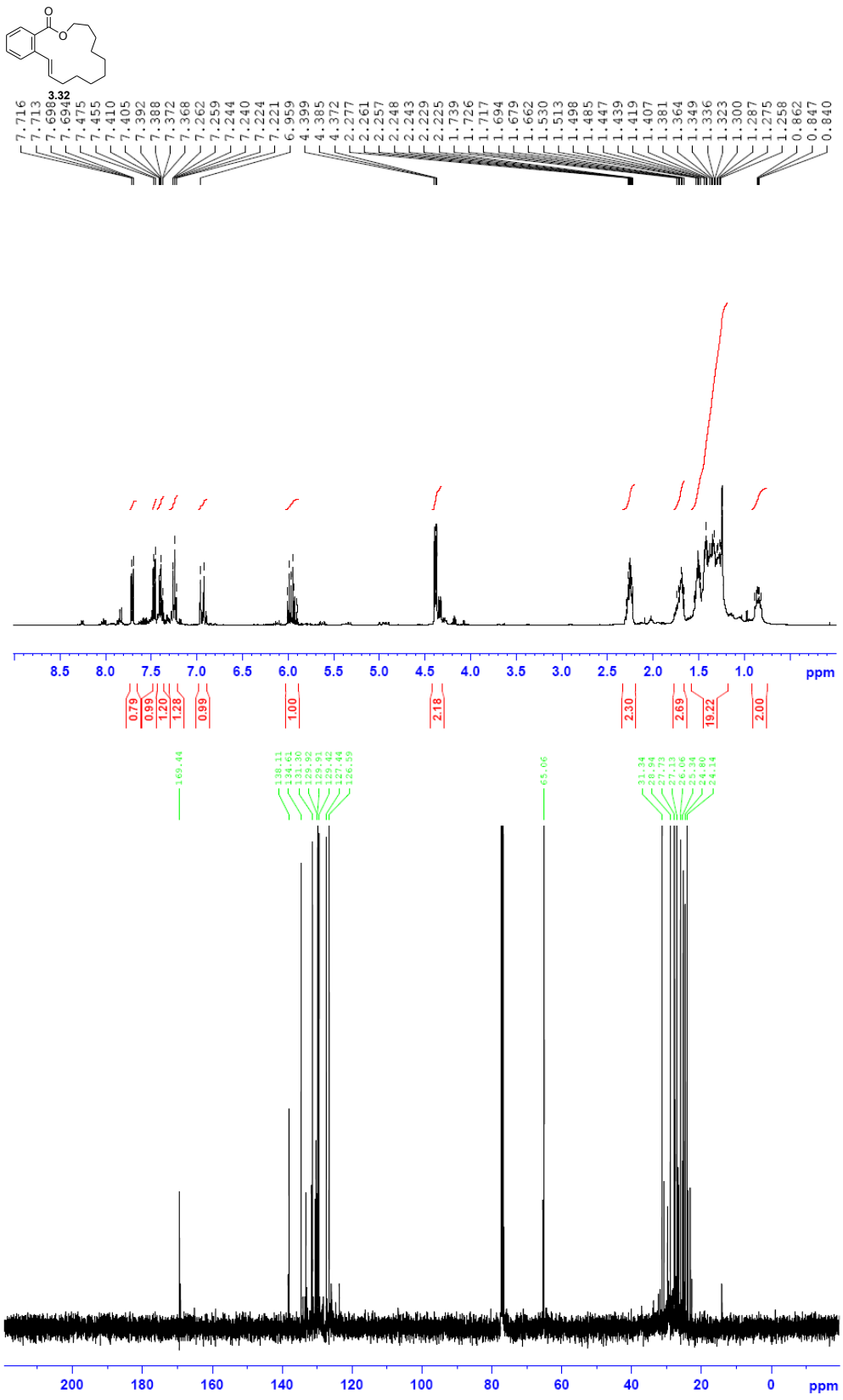
60
40
20
0
ppm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7.368
7.262
7.259
7.244
7.240
7.224
7.221
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2.261
2.257
2.248
2.243
2.229
2.225
1.739
1.726
1.717
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1.530
1.513
1.498
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1.439
1.419
1.407
1.381
1.364
1.349
1.336
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1.258
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0.847
0.840
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6.0
5.5
5.0
4.5
4.0
3.5
3.0
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2.0
1.5
1.0
ppm
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0.99
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1.28
0.99
1.00
2.18
2.30
2.69
19.22
2.00
169.44
138.11
134.61
131.30
131.32
129.91
129.42
127.44
126.59
65.06
31.34
28.94
27.73
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24.80
24.44
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120
100
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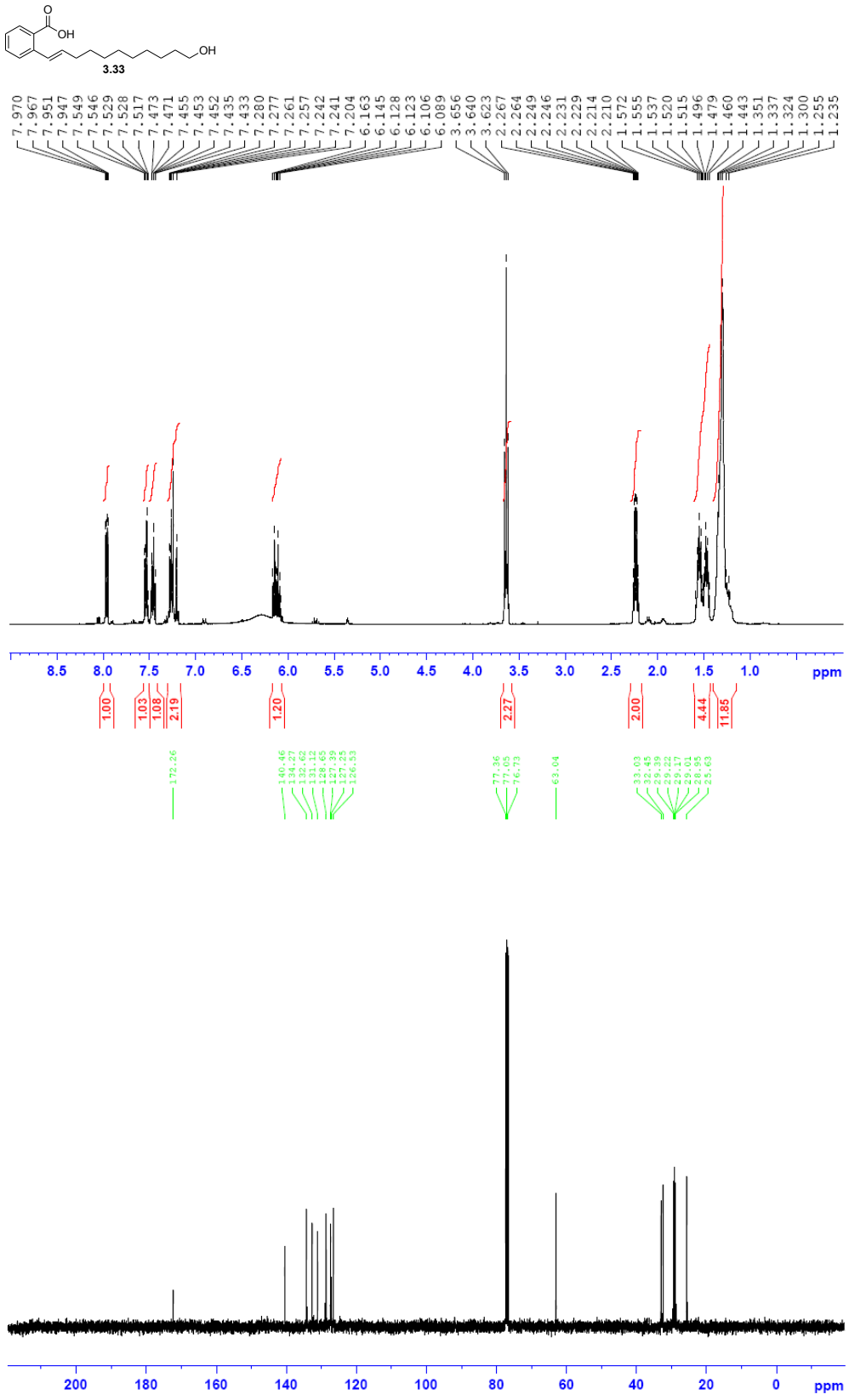
60
40
20
0
ppm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7.471
7.455
7.453
7.452
7.435
7.433
7.280
7.277
7.261
7.257
7.242
7.241
7.204
6.163
6.145
6.128
6.123
6.106
6.089
3.656
3.640
3.623
2.267
2.264
2.249
2.246
2.231
2.229
2.214
2.210
1.572
1.555
1.537
1.520
1.515
1.496
1.479
1.460
1.443
1.351
1.337
1.324
1.300
1.255
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7.0
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6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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1.03
1.08
2.19
1.20
2.27
2.00
4.44
11.85
172.26
140.46
134.27
132.62
131.12
128.45
127.39
127.25
126.53
77.36
77.05
76.73
63.04
33.03
32.45
30.39
29.29
29.22
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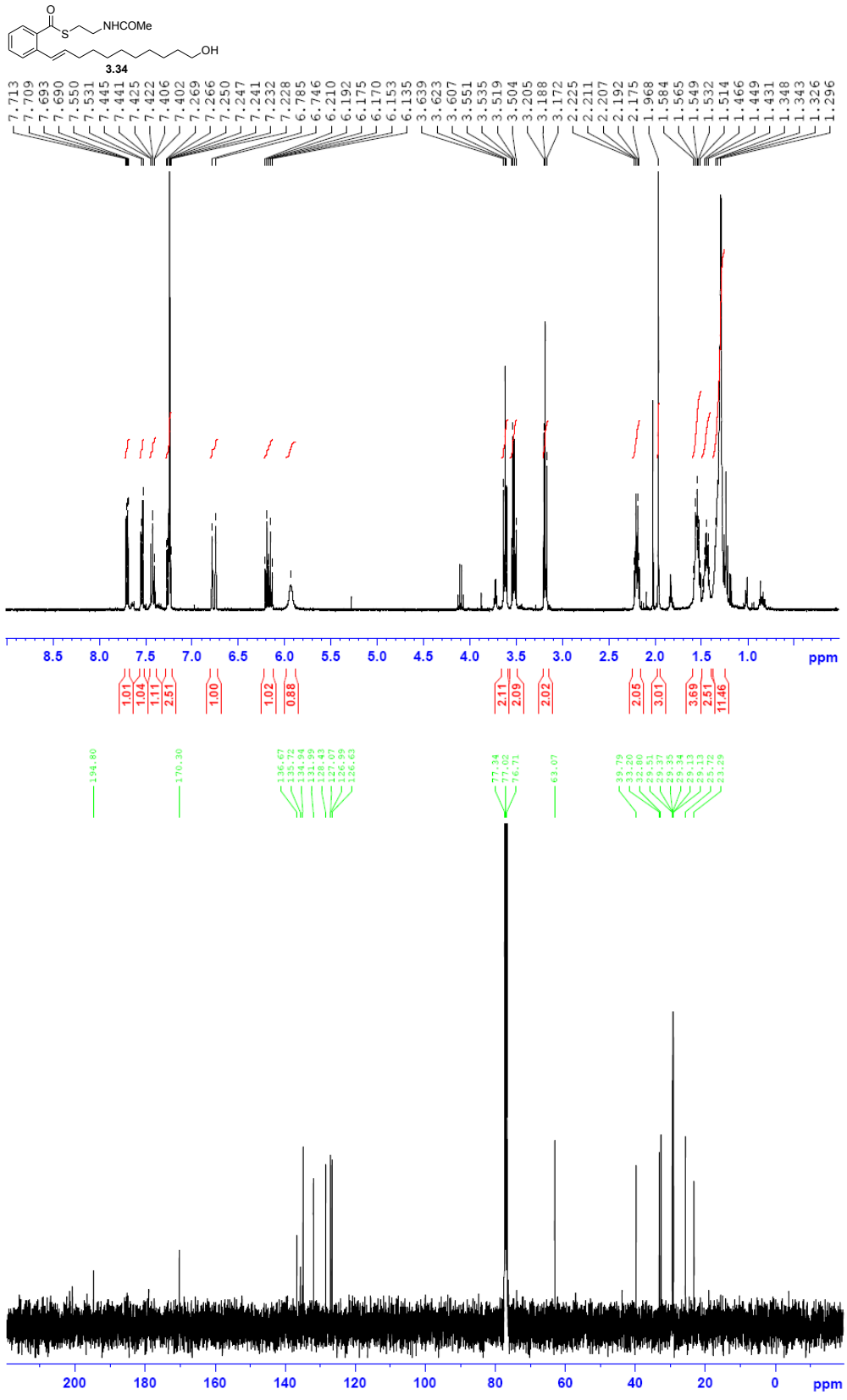
29.01
28.95
25.63
200
180
160
140
120
100
80
60
40
20
0
ppm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S
NHCOMe
OH
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7.531
7.445
7.441
7.425
7.422
7.406
7.402
7.269
7.266
7.250
7.247
7.241
7.232
7.228
6.785
6.746
6.210
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6.175
6.170
6.153
6.135
3.639
3.623
3.607
3.551
3.535
3.519
3.504
3.205
3.188
3.172
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2.207
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2.175
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1.565
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1.431
1.348
1.343
1.326
1.296
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7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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1.11
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1.00
1.02
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2.11
2.09
2.02
2.05
3.01
3.69
2.51
11.46
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170.30
136.67
135.72
134.94
131.99
128.43
127.07
126.99
126.63
77.34
77.02
76.71
63.07
39.79
33.20
32.60
29.51
29.37
29.35
29.34
29.13
29.13
25.72
23.29
200
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160
140
120
100
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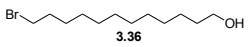
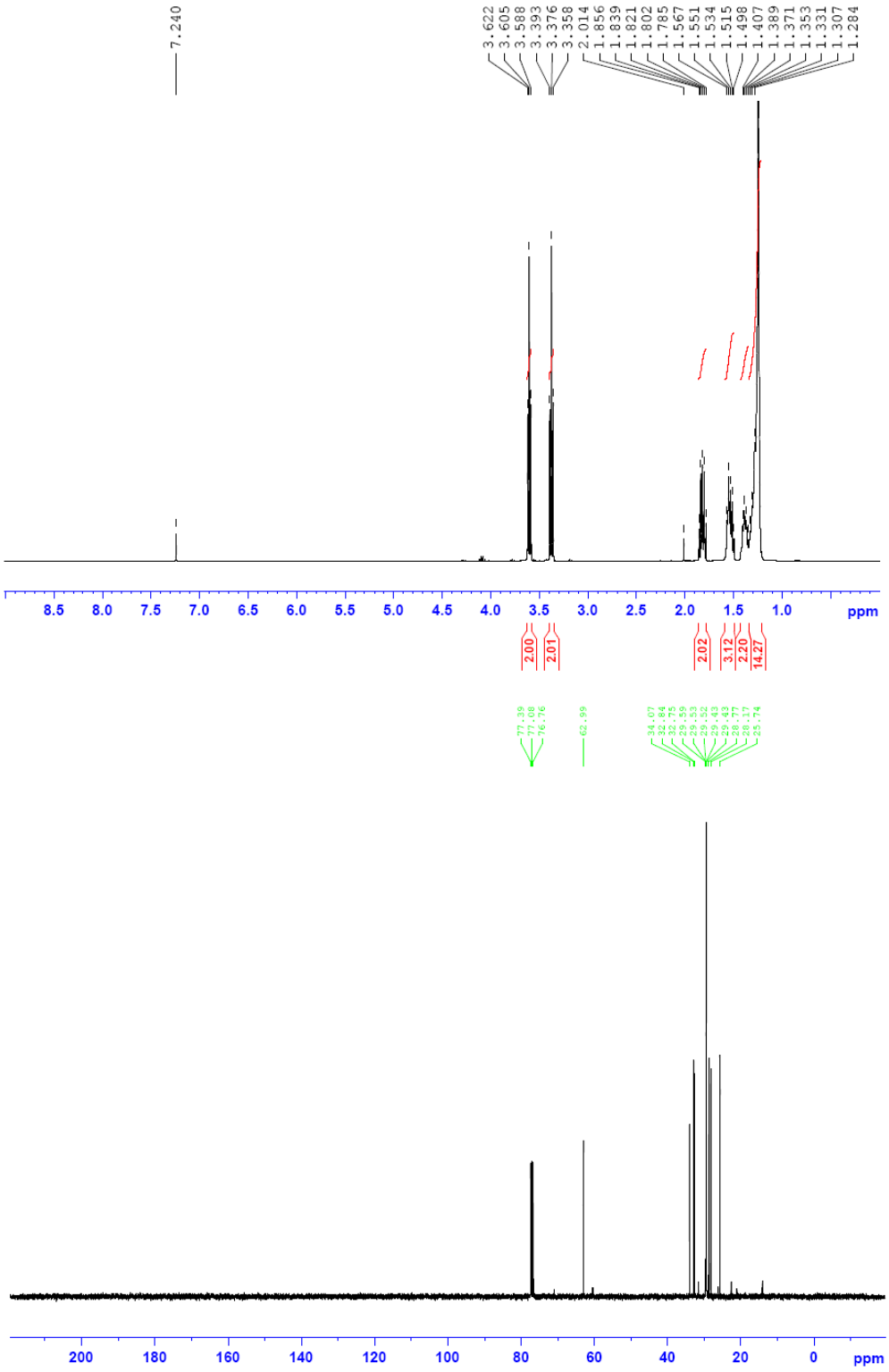
60
40
20
0
ppm


Br
OH

3.36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1.802
1.785
1.567
1.551
1.534
1.515
1.498
1.407
1.389
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1.353
1.331
1.307
1.284
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5.0
4.5
4.0
3.5
3.0
2.5
2.0
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1.0
ppm
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2.01
2.02
3.12
2.20
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77.39
77.08
76.76
62.99
34.07
32.84
32.75
29.59
29.53
29.52
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120
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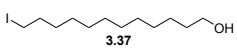
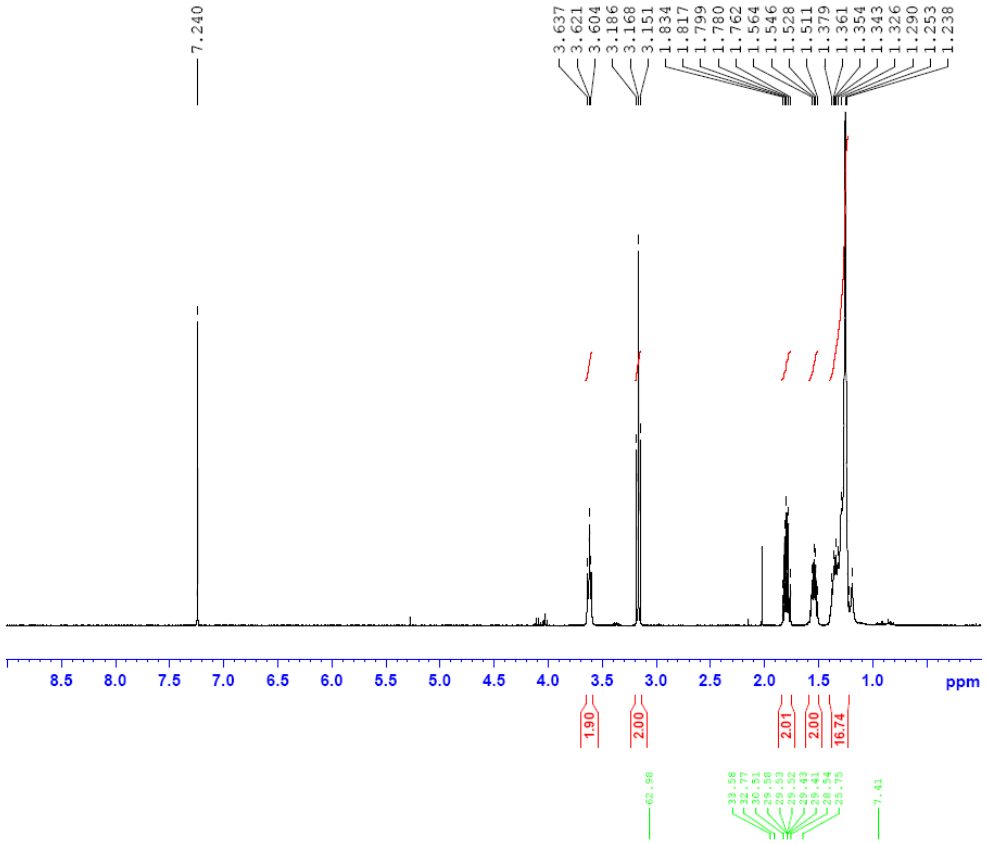
60
40
20
0
ppm


I
OH

3.37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1.546
1.528
1.511
1.379
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1.354
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1.290
1.253
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7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
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2.01
2.00
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29.43
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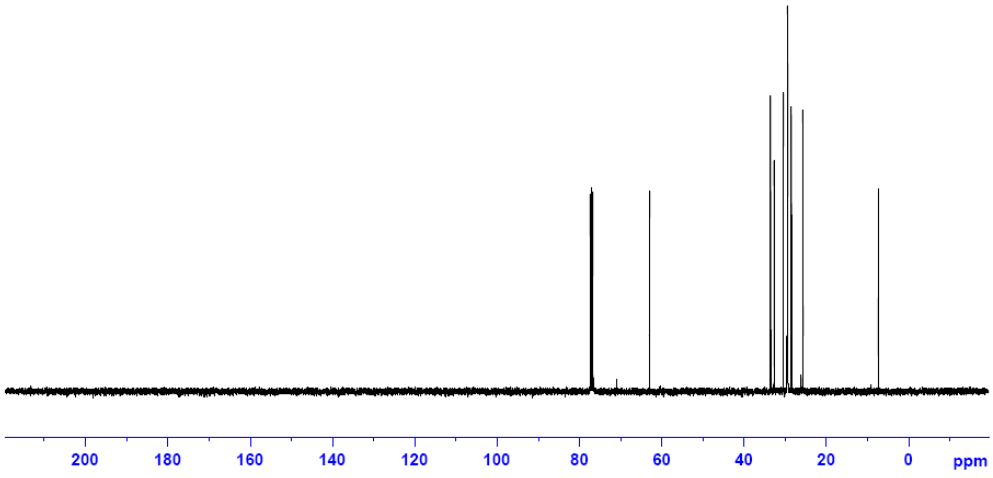
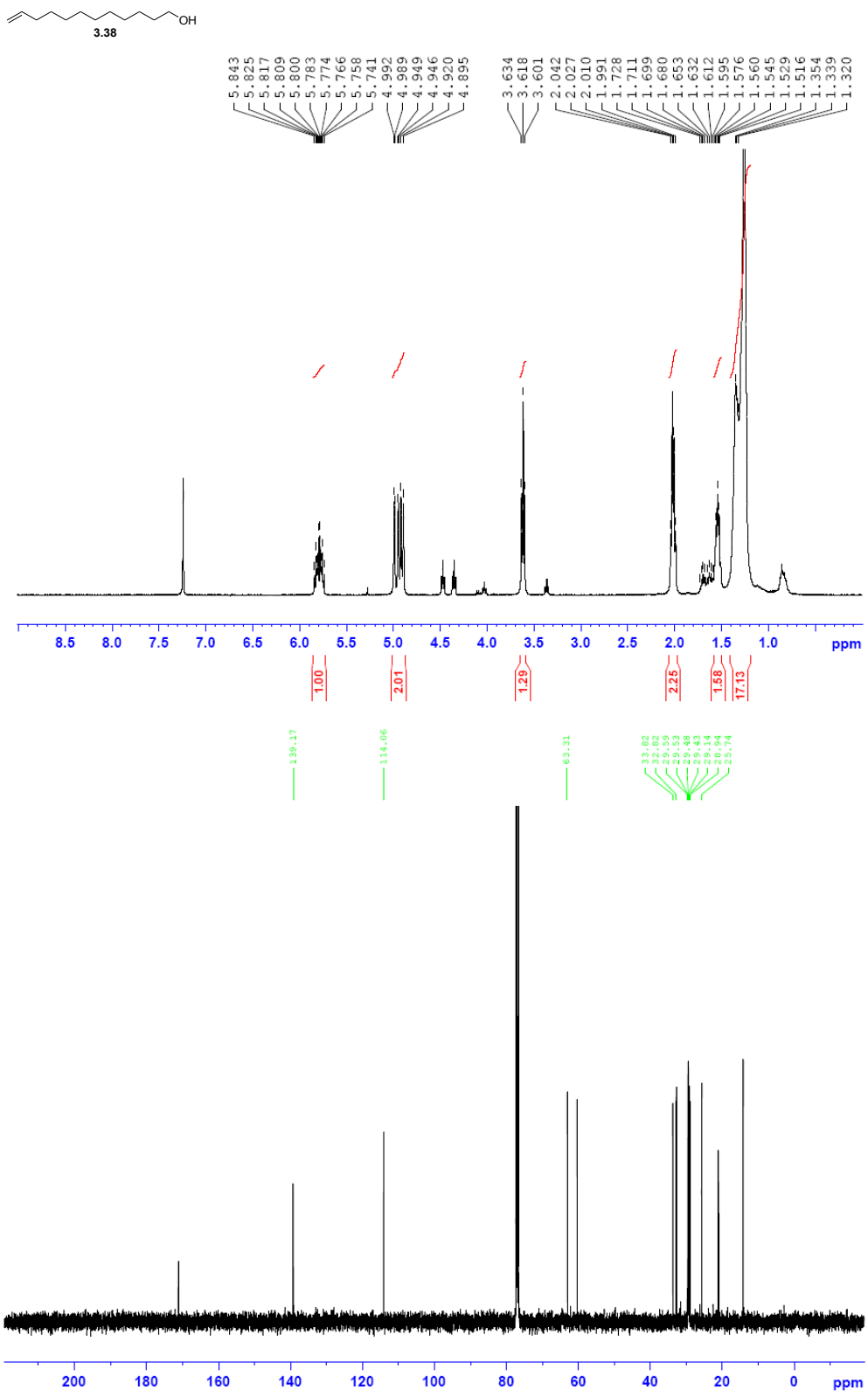
28.54
25.75
7.41


200
180
160
140
120
100
80
60
40
20
0
ppm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3.618
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2.042
2.027
2.010
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1.632
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6.5
6.0
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5.0
4.5
4.0
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3.0
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2.0
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1.0
ppm
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2.25
1.58
17.13
139.17
114.06
63.31
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29.59
29.53
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200
180
160
140
120
100
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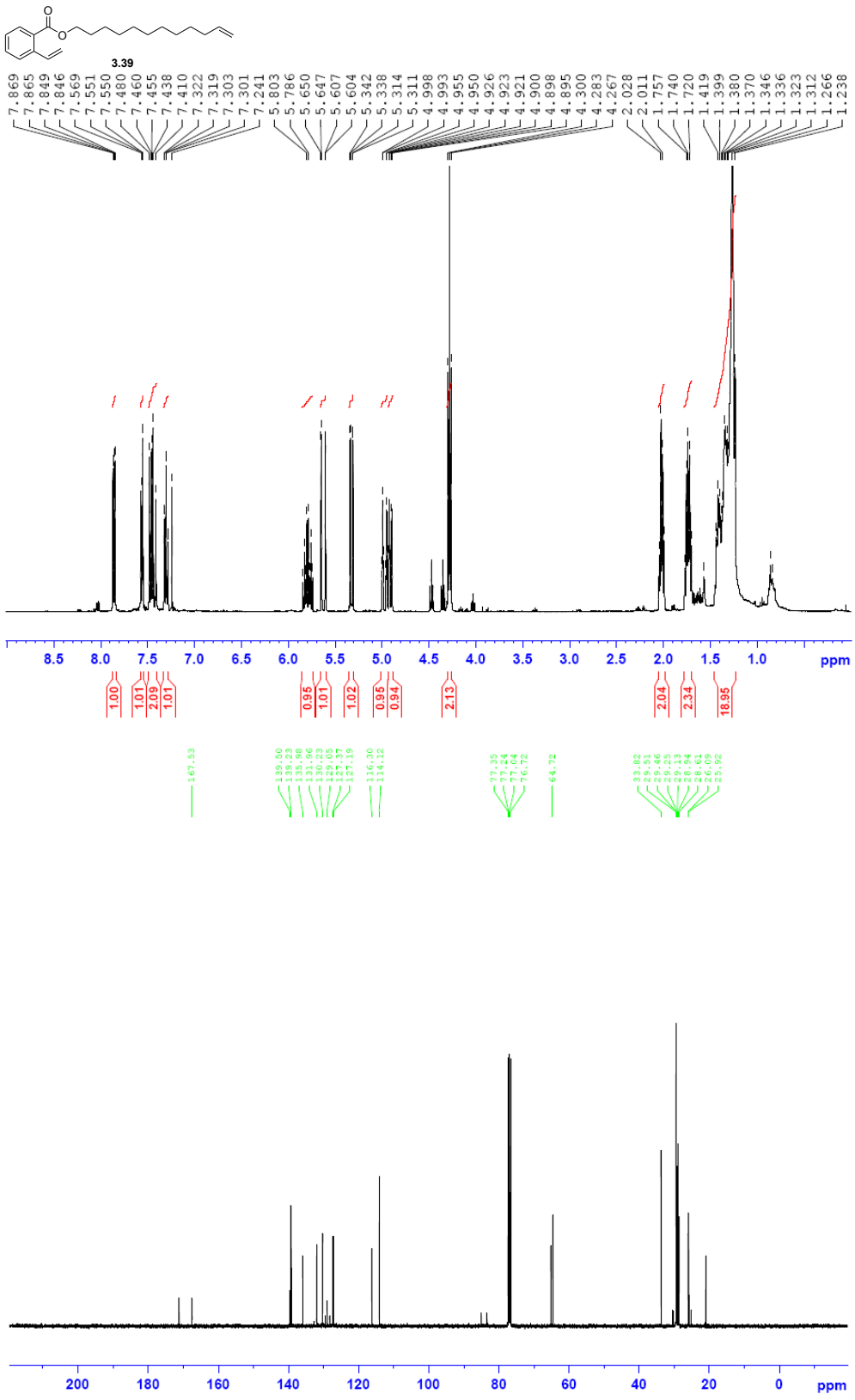
ppm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5.338
5.314
5.311
4.998
4.993
4.955
4.950
4.926
4.923
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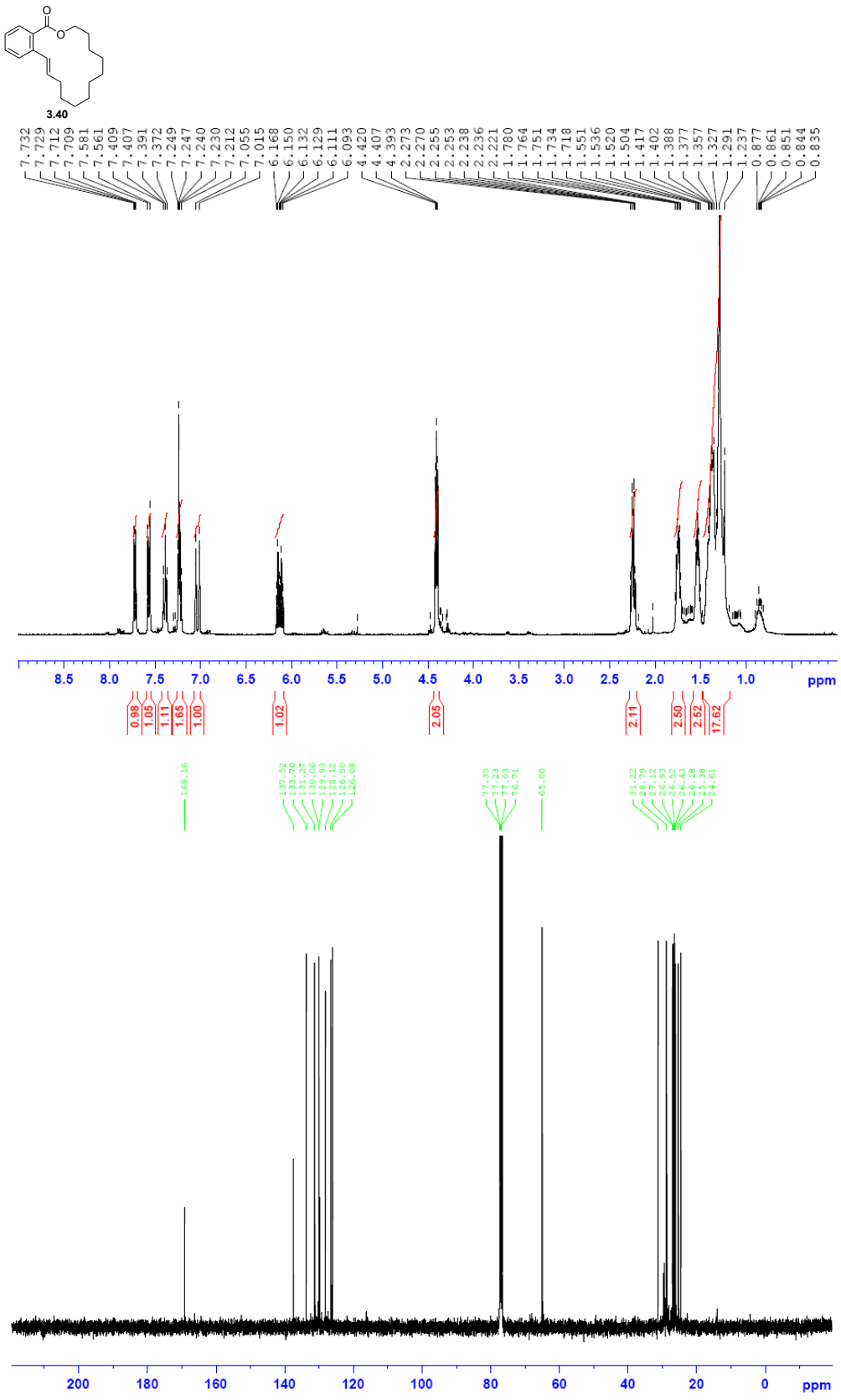
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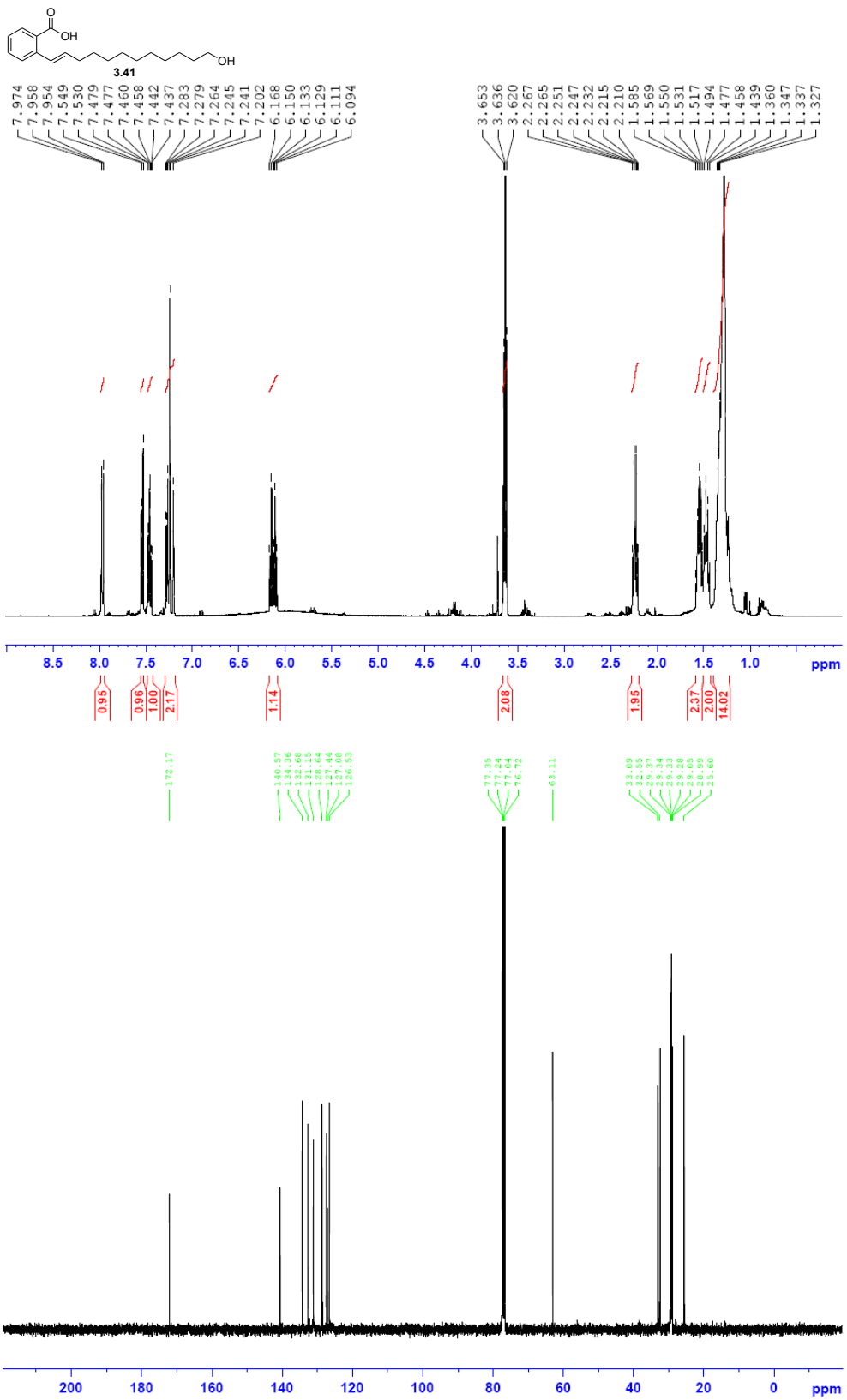
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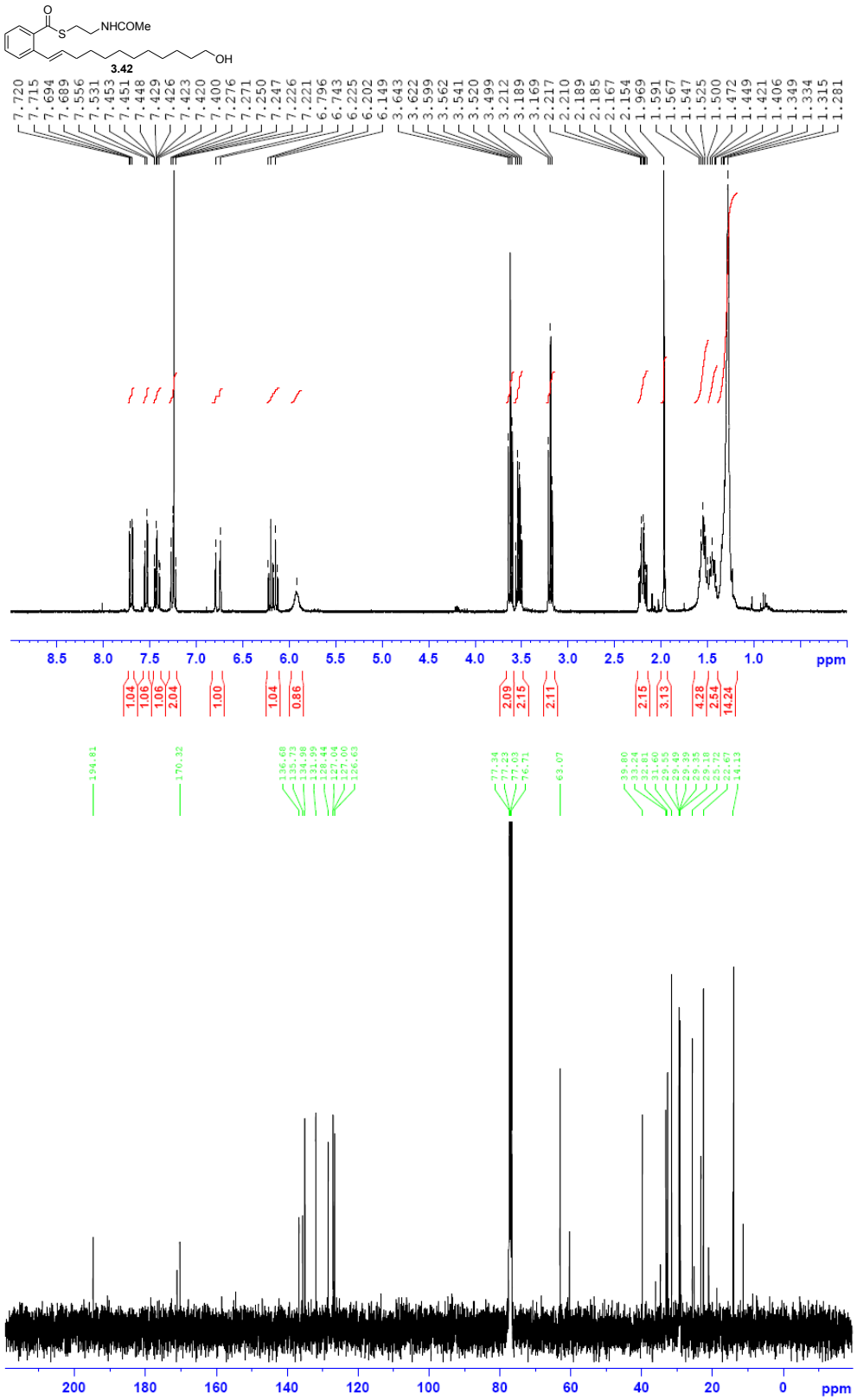
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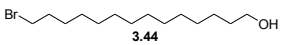
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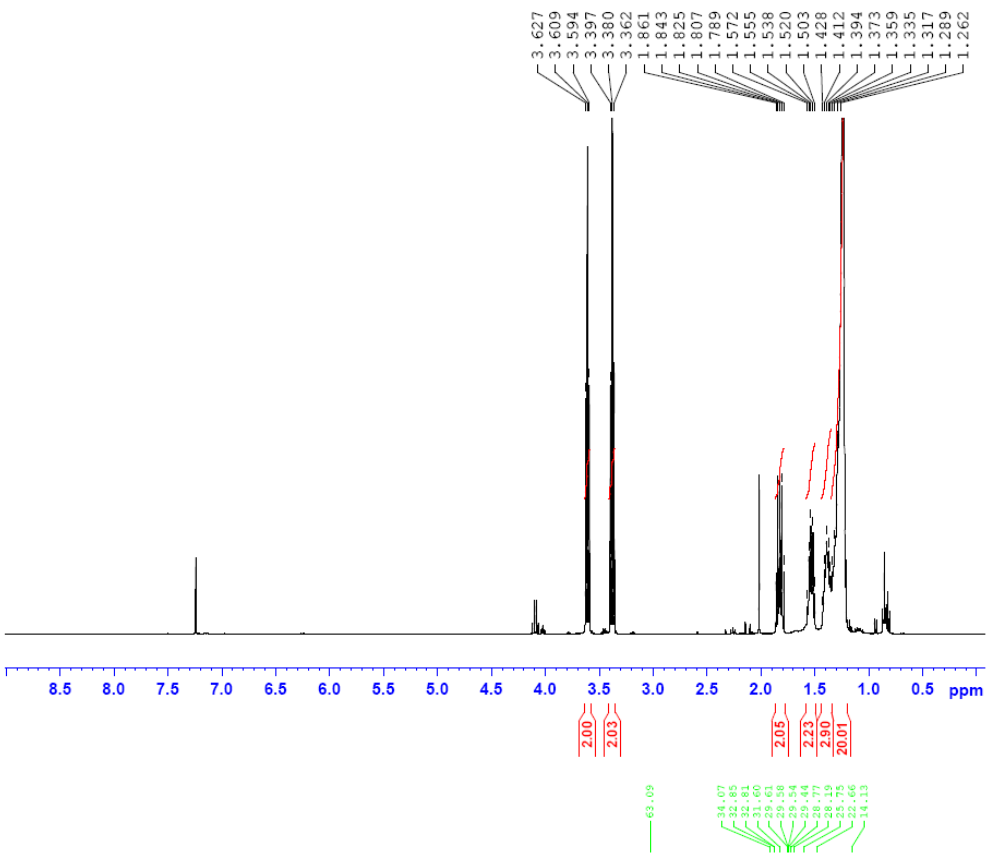
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1.373
1.359
1.335
1.317
1.289
1.262
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
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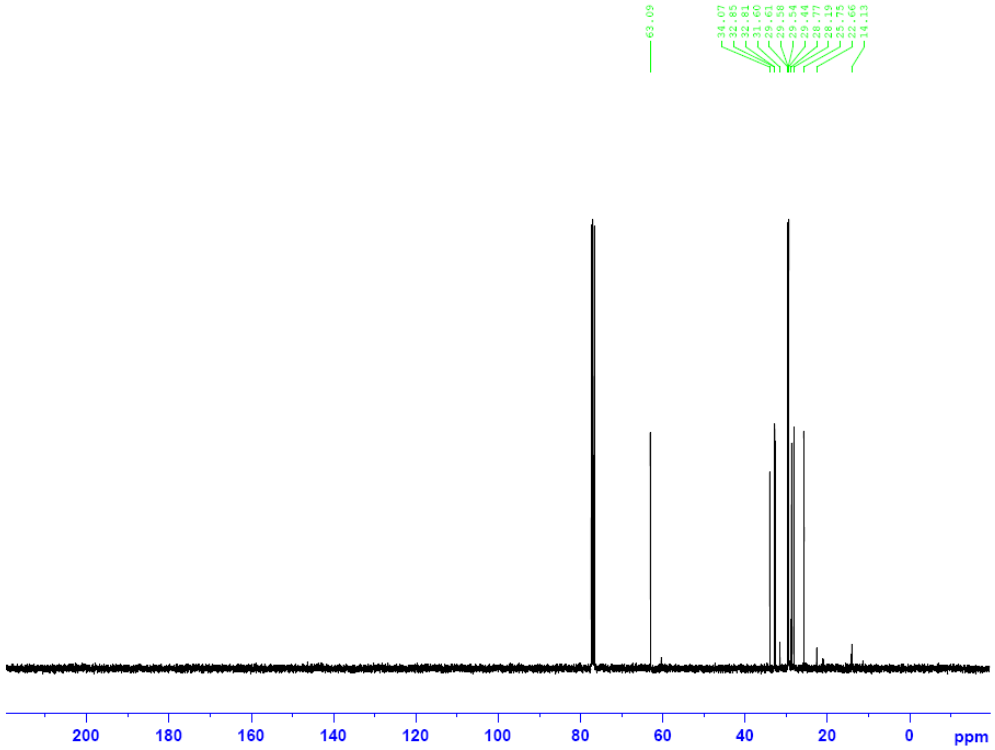
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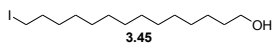
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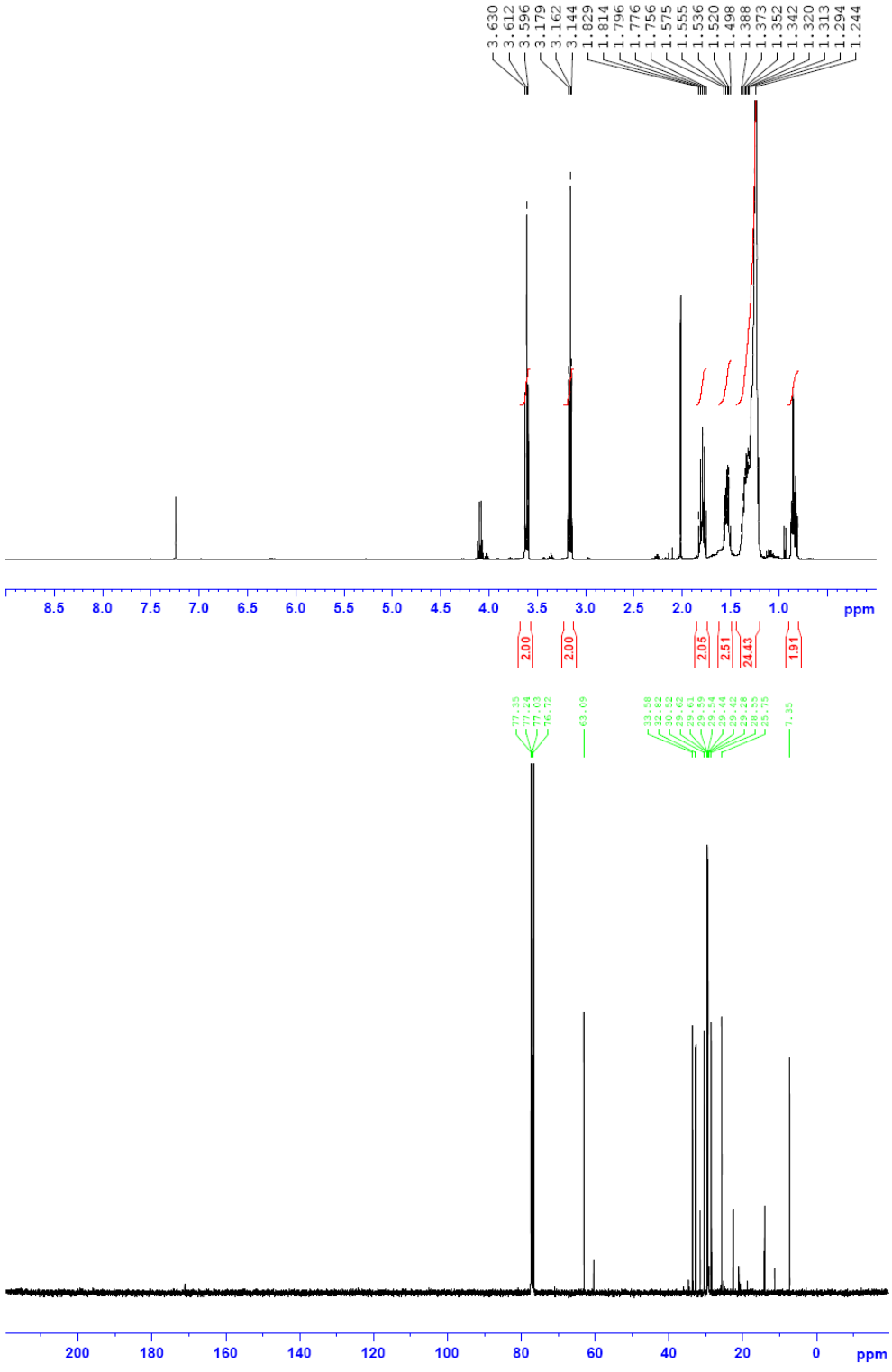
3.45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1.536
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1.244
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2.00
2.05
2.51
24.43
1.91
77.35
77.24
77.03
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63.09
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32.82
30.52
29.62
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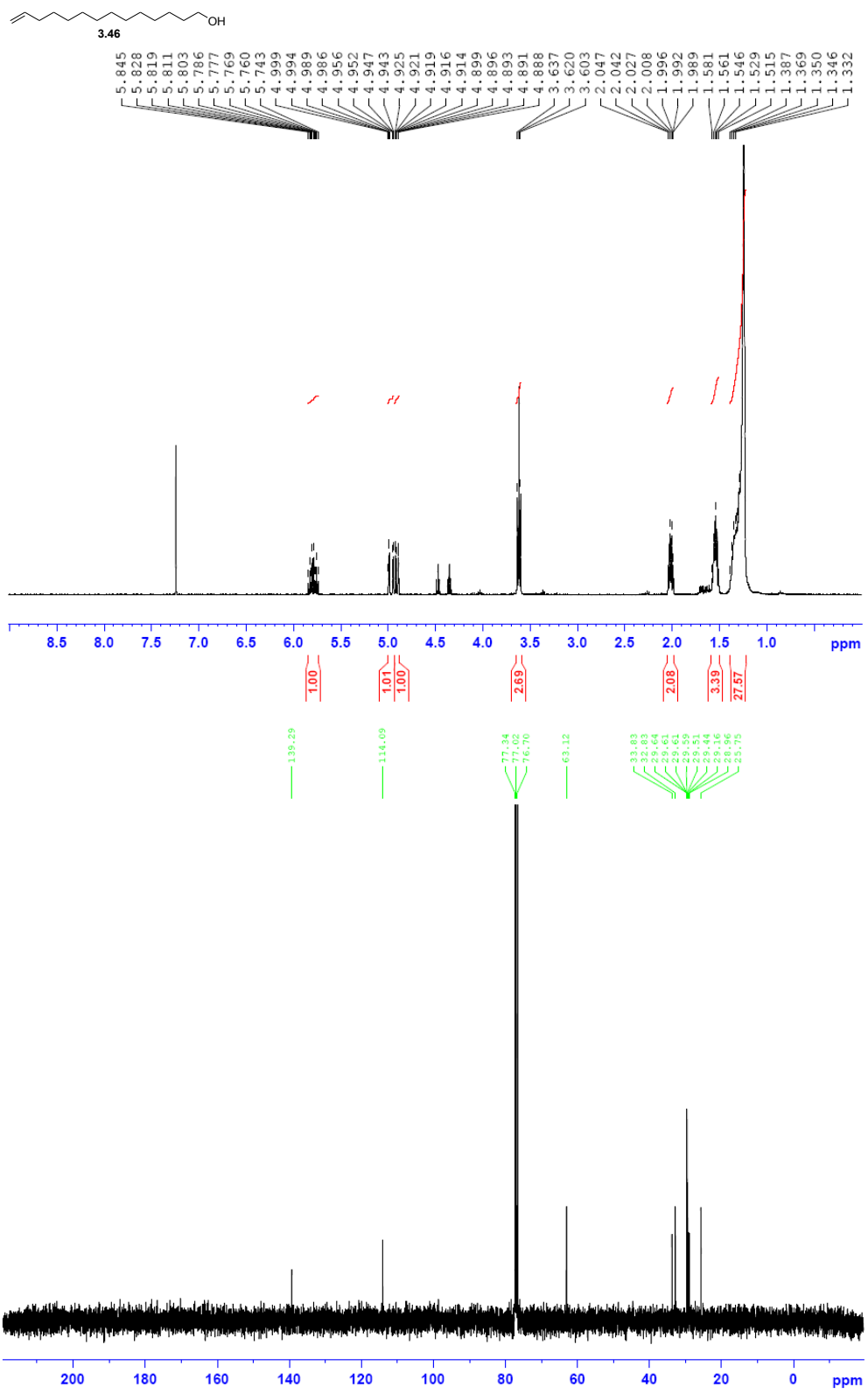
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1.01
1.00
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3.39
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77.02
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32.83
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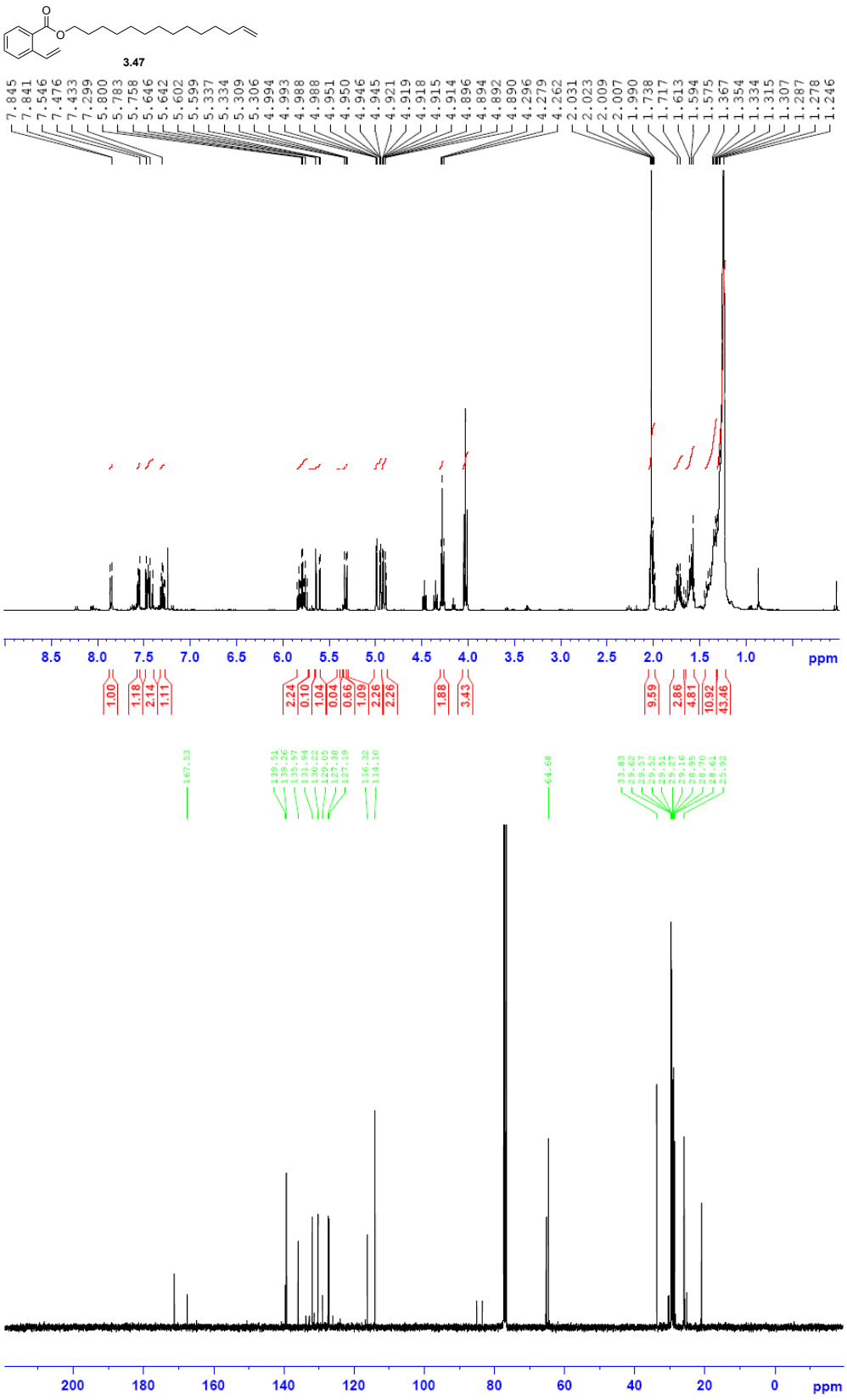
200 180 160 140 120 100 80 60 40 20 0 ppm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ppm
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0.04
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130.00
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29.57
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29.51
29.27
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120
100
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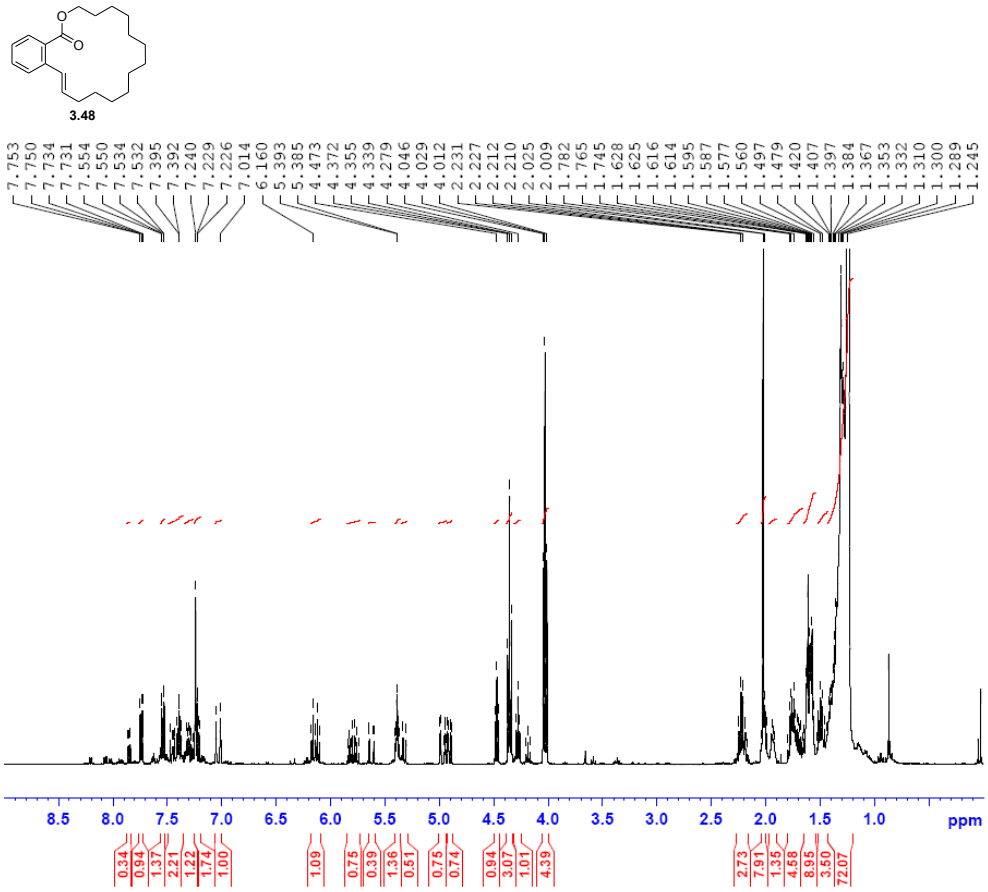
0
ppm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1.5
1.0
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0.51
0.75
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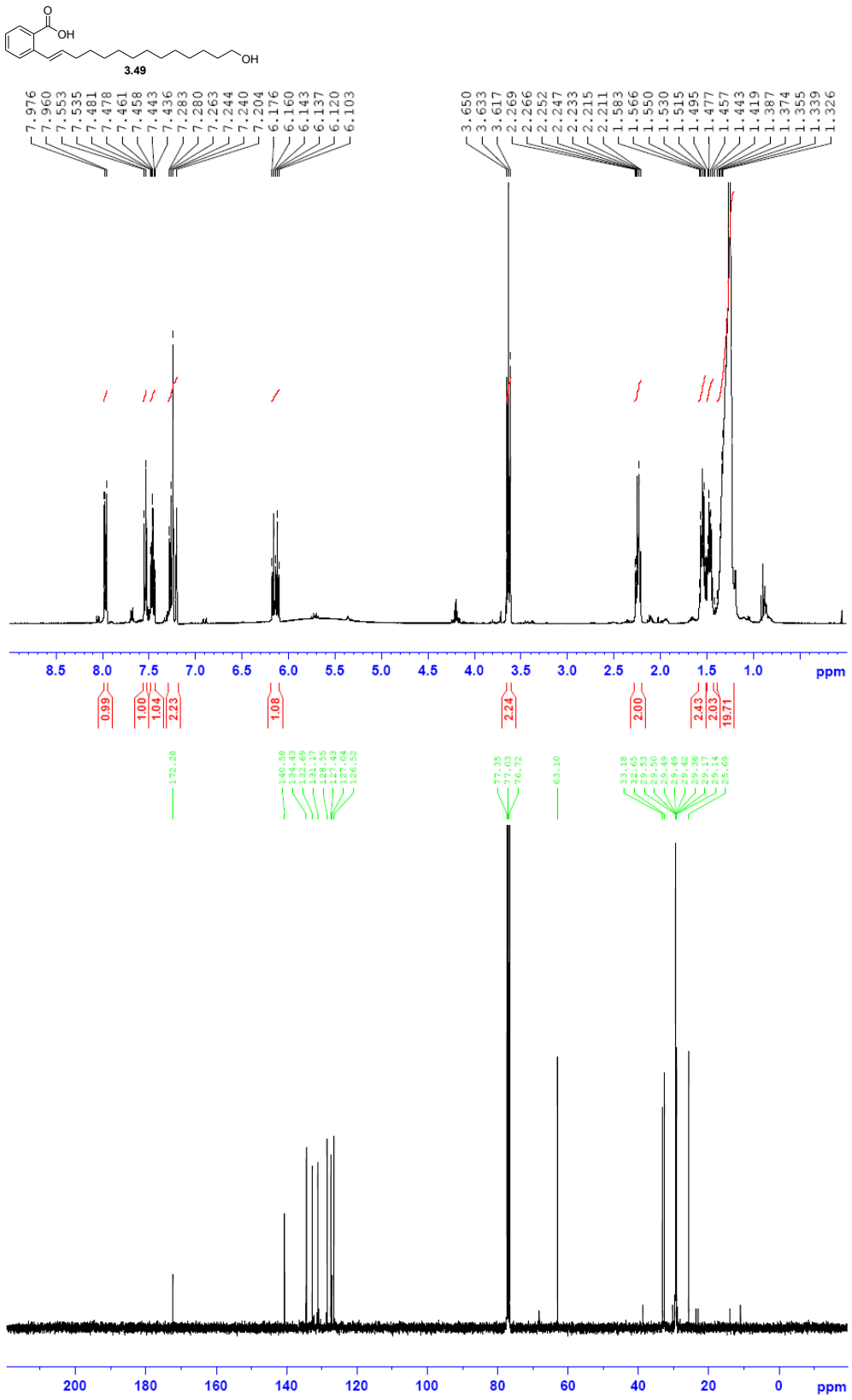
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2.03
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76.72
63.10
33.18
32.65
29.53
29.50
29.49
29.49
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140
120
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60
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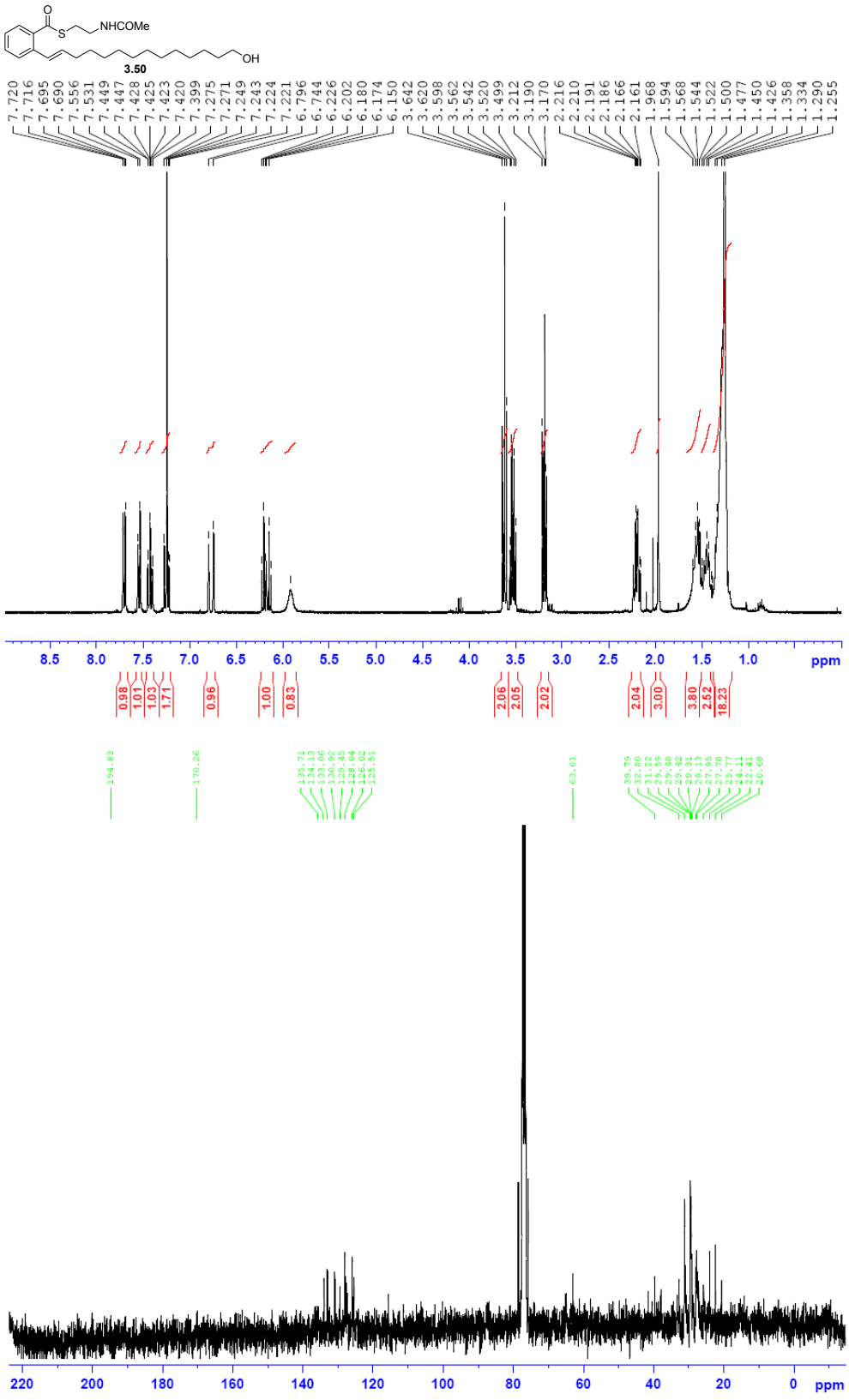
0
ppm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0.83
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2.05
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29.31
29.13
27.95
27.79
27.77
24.11
22.41
20.68
220
200
180
160
140
120
100
80
60
40
20
0
ppm

3.5 References

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Chapter 4 Probing the effect of substrate absolute stereochemistry on fungal TE macrocyclization activity

4.1 Introduction

4.1.1 Stereochemical substrate specificity of known TEs

To date, despite the importance and utility of PKS TEs, there is little known about the substrate specificity of these catalysts. In the context of metabolic engineering, understanding the substrate specificity of TE is crucial. For example, it would be extremely ineffective to engineer a PKS to produce a new "natural-like" product or libraries of compounds, if the macrocyclization and product release step is not possible due to limited TE substrate specificity. Since many polyketide and non-ribosomal peptide natural products are only biologically active in their cyclized form, TE-mediated macrocyclization is of additional importance for generating biologically active compounds.[1] While the substrate tolerance of bacterial PKS TEs have been studied, much less is known about the corresponding fungal PKS TEs. Ultimately, understanding the substrate specificity of TEs and the enzyme-substrate interactions which control specificity will enable engineered TEs to be constructed for use in the synthesis of natural product-like libraries and combinatorial libraries through chemoenzymatic synthesis and metabolic engineering.[2, 3]

Bacterial PKS TEs, such as the 6-deoxyerythronolide B synthase (DEBS) TE, have been biochemically characterized in vitro. In general it has been observed that PKS TEs are highly substrate specific in catalyzing macrocyclization chemistry. In the absence of macrocyclization activity, TE catalyze highly substrate tolerant hydrolysis of activated thioester substrates, generating linear carboxylic acids.[4]

Initial attempts to characterize the macrocyclization activity of the DEBS TE focused on very simple primary alcohol containing substrates. Simple analogues of the native DEBS TE substrate, possessing the native substitution functionality at C2

and C3, were examined by Khosla and coworkers. Analogues corresponding to macrocycles of different ring sizes with various stereochemistries of **4.1** were synthesized; however none of these compounds were successfully macrocyclized by the DEBS TE (Figure 4.1).[5-7] In all cases hydrolysis of the activated thioester generating the linear carboxylic acid was the only product observed. These results suggest that additional substrate structural features are needed to promote substrate recognition and macrolactonization. Given that the stereochemical configurations of the C2 and C3 substituents did not substantially impact activity, it is possible that if hydrolysis is properly suppressed, macrocyclization may be broadly stereochemically tolerant.

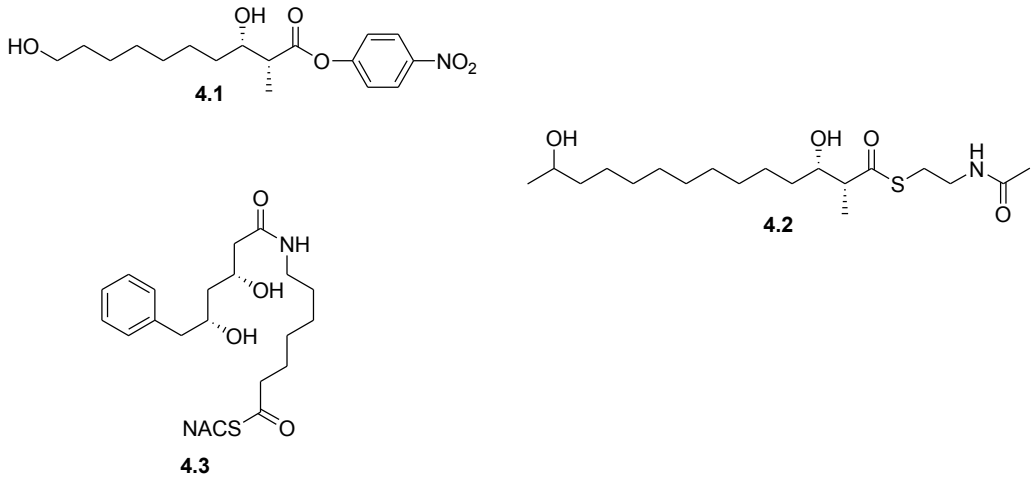


Figure 4.1 A few substrates that were built to probe the substrate specificity of DEBS TE in regards to stereochemistry.

The Khosla group also investigated the ability of secondary alcohols to undergo macrocyclization.[8, 9] Various stereochemical analogues of **4.2** were synthesised (Figure 4.1). These analogues were also not macrolactonized by the DEBS TE. Again in all cases the substrate underwent DEBS TE catalyzed hydrolysis. Taken together these results indicate that bacterial PKS TEs are highly substrate specific for macrocyclization. With no examples of synthetically tractable substrates that are effectively macrocyclized by these TEs, characterization of the elements responsible for substrate recognition in TE-catalyzed macrocyclization has not been possible.[10]

Recently, our group has identified the first analogues of 6-deoxyerythrnolide that can be macrocyclized by the DEBS TE, enabling us to probe the substrate specificity of DEBS TE with different stereoisomers. Four stereoisomeric analogues of **4.3** of the native DEBS TE substrate were synthesized (Figure 4.1). The analogue with the 11*R*, 13*R* stereochemistry was successfully macrocyclized by DEBS TE, where the other analogues underwent hydrolysis exclusively. These results are the first glimpse into the stereoselectivity of bacterial PKS TEs and they clearly indicate that these TEs are highly stereoselective.

Many of the studies carried out with the DEBS TE have also been performed by the pikromycin (Pik) TE. Similar to DEBS TE, Pik TE hydrolyzes a simple thioesters effectively.[11] So far, Pik TE does not tolerate all small changes to the natural substrates, and it is still unknown what mechanistic and structural modification leads to macrocyclization and which leads to hydrolysis.

While little is known about modular bacterial TEs, even less is known about the substrate tolerance and specificity of iterative fungal TEs. Fungal PKS TEs show limited sequence homology to bacterial TEs. This suggests that their substrate specificity may differ significantly from the bacterial TEs. In this chapter we will examine in vitro the absolute stereoselectivity of the fungal PKS TEs responsible for the biosynthesis of zearalenone (zea TE) and radicicol (rad TE). The results presented herein demonstrate that unlike bacterial PKS TEs, fungal PKS TE are much less stereoselective.

4.2 Results and discussion

The resorcylic acid lactone family, of which zearalenone and radicicol are members, is unique in that different members of the same family have different stereochemical configurations for the alcohol involved in macrolactone formation. For example, in zearalenone the secondary alcohol involved in formation of the

lactone is in the *S* configuration. In radicicol, the same group is in the *R* configuration (Figure 4.2). The biosynthetic pathway for these two compounds thus provide the ideal situation to examine the absolute stereochemical substrate specificity for fungal PKS TEs.

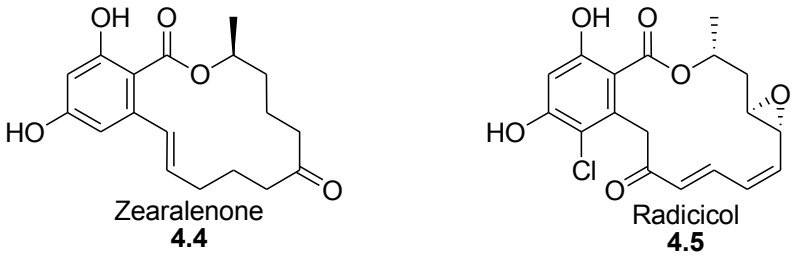
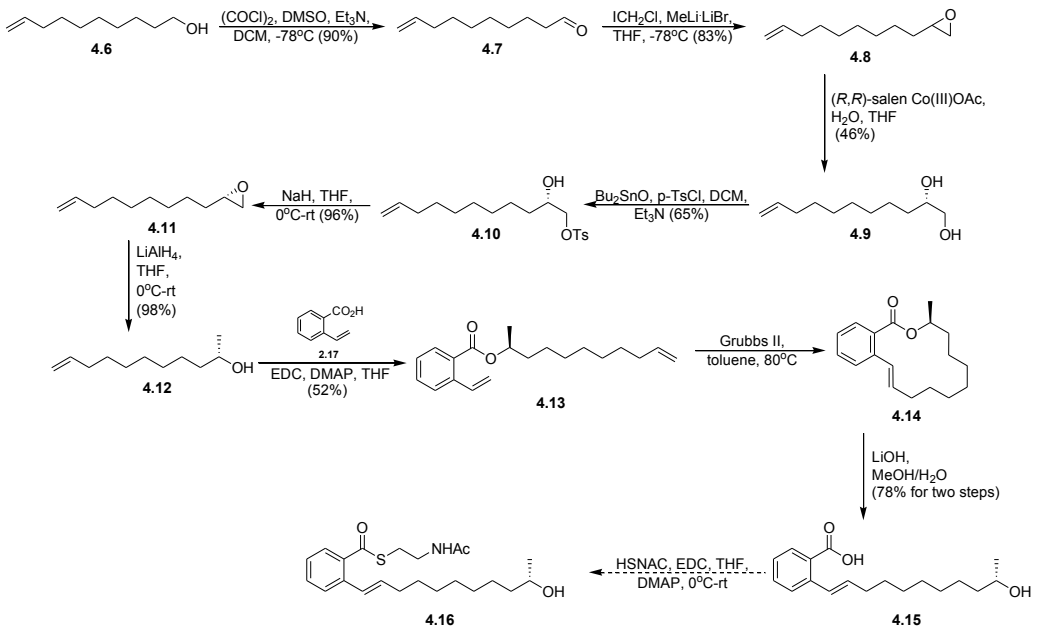


Figure 4.2 Zearalenone and radicicol, two resorcylic acid lactones with different enantiomeric configurations.

Examining in vitro the absolute stereochemical substrate specificity of the macrocyclizing TEs from the zearalenone and radicicol biosynthetic pathways will determine if these TE show stringent or relaxed absolute stereoselectivity. In the case of stringent substrate specificity, the zea TE is expected to macrocyclize substrates with alcohols in the *S* configuration and hydrolyze substrates with the *R* configuration. The rad TE is expected to provide the converse, macrocyclization of the *R* substrates and hydrolysis of the *S* substrates. If the fungal PKS TEs are tolerant of absolute stereochemistry, both TEs are expected to macrocyclize the *R* and *S* configured substrates. To test these hypotheses, synthetic enantiopure and racemic substrates will be incubated with recombinant purified zea TE and rad TE and the formation of macrocyclic products will be monitored by LC/MS/MS.

4.2.1 Synthetic route to the *S* stereoisomer

The synthesis of the *S* isomer followed the route shown in Scheme 4.1. A variety of different methods and reaction conditions were investigated for the introduction of stereochemistry. Ultimately we relied upon the Jacobsen hydrolytic kinetic resolution to provide an enantiopure terminal epoxide.



Scheme 4.1 Reaction sequence to build the *S* stereoisomer SNAC analogue.

The synthesis started using the inexpensive and commercially available 9-decenol, which was also used to synthesize the 14-member ring. The alcohol underwent a Swern oxidation, generating the aldehyde. Using standard Swern oxidation conditions with oxalyl chloride and DMSO, the aldehyde was produced in high yield (90%).[12]

The next step was methylenation to form an epoxide.[13] A number of reagents were investigated for this transformation, including trimethylsulfonium iodide and propylene oxide. Chloromethyllithium generated from chloroiodomethane and methyl lithium complexed with lithium bromide at -78°C, proved to be the most effective method generating the epoxide in 83% yield.

Once the epoxide was constructed, the Jacobsen hydrolytic kinetic resolution could be used to set the stereocenter.[14-16] The (*R*,*R*)-salenCo(III) catalyst was used to form the (*S*,*S*) diol and leaving the *R* epoxide unreacted. While this approach generates enantiopure diol, as the initial mixture is racemic, the highest possible yield

is 50%. To improve the yield, the reaction was stirred for two days before purification and the next step. This yielded the diol in 46% yield.

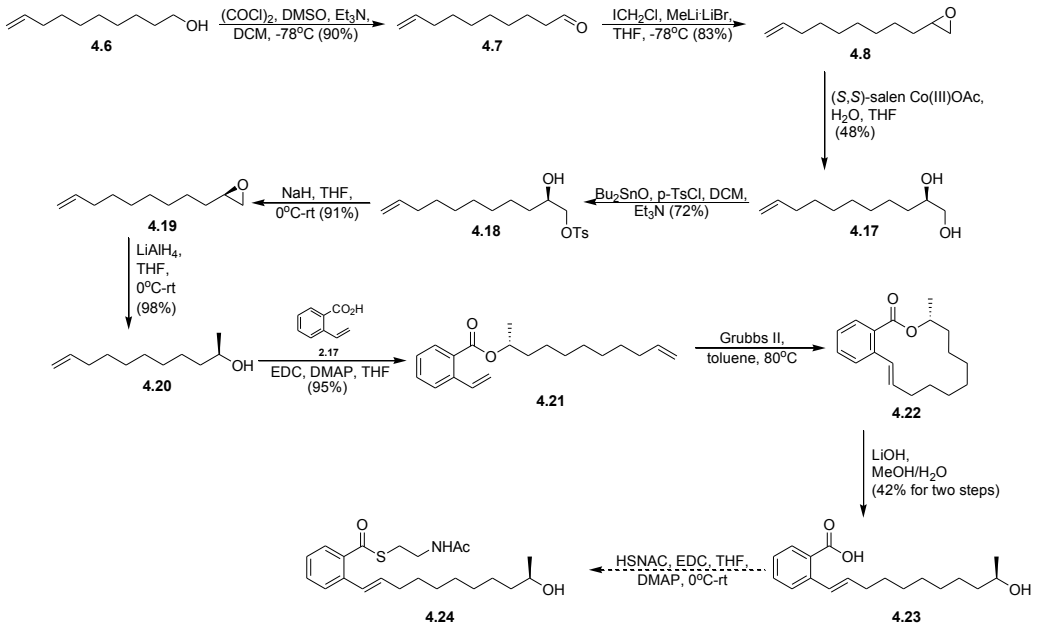
With the *S*-diol in hand, we focused on generating the enantiopure secondary alcohol. To do this we envisioned monotosylation oh the primary alcohol using dibutyltin oxide, triethylamine and p-toluenesulfonyl chloride.[17] Despite dibutyltin being used to selectively tosylate the primary alcohol, there was still a mixture of mono- and ditosylated product in the reaction mixture. Separating them in the purification by column chromatography led to the lower yield of 65%. The monotosylate was then converted into the epoxide via treatment with sodium hydride at 0°C in 96% yield. The final step in generating the desired secondary alcohol was the regioselective opening of the epoxide using lithium aluminium hydride at 0°C which gave near quantitative yield.

Once the alcohol was obtained, the remaining steps were similar to those used in the synthesis of the 14-membered ring (Scheme 2.1). Esterification with the carboxylic acid **2.17** and EDC gave 52% of the ester. The lower yield than seen in the primary alcohol analog may be in part due to the added steric hindrance from the methyl group.

The ring closing metathesis using Grubbs II gave an inseparable mixture of starting material and product. Since we had limited amount of material, and the next product from the next step is much more polar compared to the lactone and its coeluting impurities, we decided to purify after the hydrolysis step. Hydrolysis with lithium hydroxide was easily achieved and yielded 78% for two steps. Unfortunately due to the limited amount of material obtained, we were unable to carry out the final SNAC coupling.

4.2.2 Synthetic route to the *R* stereoisomer

The synthesis strategy for the *R* SNAC analogue is identical to the strategy used for the *S* enantiomer (Scheme 4.2). To generate the *R* isomer, the (*S*,*S*)-salenCo(III)OAc was used in the Jacobsen hydrolytic kinetic resolution instead of the (*R*,*R*)-reagent. This gives the expected *R*-diol. Yields obtained for all transformations are, as is expected, very similar to the yield for the *S*-steroisomer.



Scheme 4.4 Reaction sequence to build the *R* stereoisomer SNAC analogue.

4.2.3 Enzymatic cyclization of the racemic SNAC analogue by rad and zea TEs

The racemic SNAC analogue was built using a strategy similar to those used for the *R* or *S* enantiomers. In this case however the racemic epoxide **4.8** was opened directly using lithium aluminium hydride.

With the racemic material in hand we investigated the ability of zea TE and rad TE to macrocyclize this substrate. The racemic substrates was mixed with recombinant purified zea and rad TEs for 20 hours. The reaction mixtures were then analyzed by LC/MS/MS (Figure 4.3). LC/MS/MS analysis showed that both the zea

TE and rad TE could affect conversion of the racemic mixture into the macrocycle. In both cases the racemic starting material was completely consumed, indicating that both substrates could be loaded as acyl-enzyme intermediates by the TEs. Analysis of the product peaks by MS-MS showed the formation of the macrocycle as the major product with trace formation of the free acid and glycerol ester.

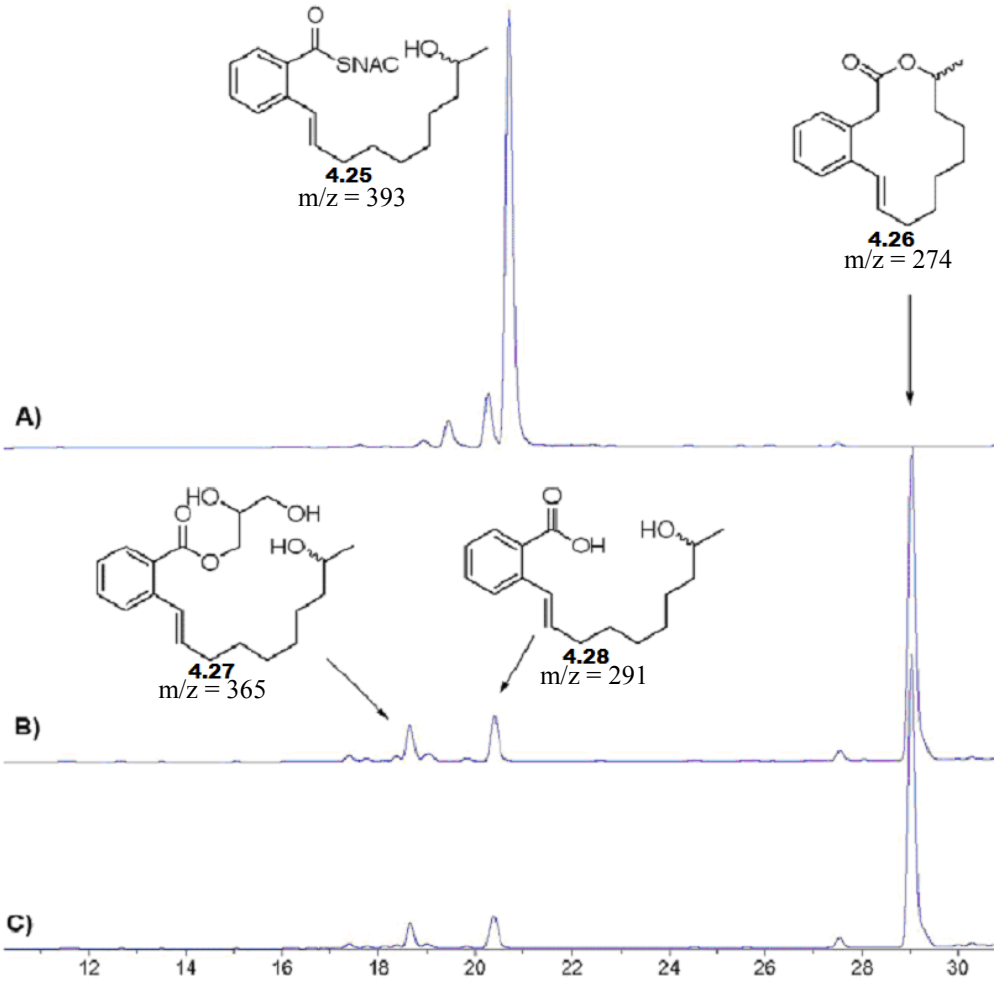


Figure 4.3 LC/MS/MS analysis of macrocyclization of racemic substrate. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN). A) Standard of racemic SNAC substrate **4.23**. B) LC/MS/MS analysis of a 20 hours incubation of 15 μM rad TE with 5 mM **4.23** at pH 7.4, 23°C. C) LC/MS/MS

analysis of a 20 hours incubation of 15 μM zea TE with 5 mM **4.23** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

These results are consistent with fungal macrocyclizing TEs being able to catalyze macrocyclization regardless of the nucleophile absolute stereochemistry. The LC traces show the vast majority of the racemic starting material is converted into macrocyclic products by both TEs. Based on these results the zea TE must be able to process some of the non-native *S* configured substrate as well as the native *R* configured material. Similarly the rad TE must be able to macrocyclize the non-native *R* configured material as well as the native *S* configured substrate

These results differ from previous observations with bacterial TEs.[8-10, 18] Bacterial TEs appear to be very specific to the stereochemistry of their substrates. Often, only hydrolysis occurs instead of macrolactonization when there is change in stereochemistry. Since this does not appear to be the case with both zea and rad TEs, they could be good enzymes to build different PKS combinatorial libraries. They could also be great tools in chemoenzymatic synthesis.

With these results, it would be extremely interesting and informative to have the kinetics of each enantiomer, as it is possible that zea TE can catalyse the macrolactonization reaction of the *S*-enantiomer faster than the *R*-enantiomer. From this data set it is not possible to determine a kinetic preference for either enantiomers by the two TEs. Use of the enantiopure thioester substrates also described in this chapter will facilitate collection of this data. This would give us further insights on these very appealing enzymes.

4.3 Conclusions

In conclusion, both zea and rad TE can macrocyclize the racemic SNAC analogue, with very little glycerolysis and hydrolysis by-products. This means that they can catalyze the macrolactonization of both enantiomers. Further experiments,

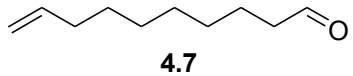
especially kinetic experiments, are needed to gain more information and better understand these enzymes. So far, they are more appealing than previous PKS TEs due to their broad substrate tolerance for the stereochemistry of the molecules.

4.4 Experimental section

4.4.1 General methods

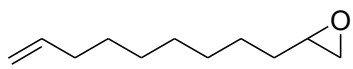
All reactions were carried out under nitrogen or argon atmosphere with dry solvents under anhydrous conditions in oven-dried glassware equipped with a magnetic stir bar and rubber spectrum. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reagents were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light (254 nm) as a visualizing agent and cerium molybdate stain and heat as developing agent. E. Merck silica gel 60 (230-400 mesh) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254). IR spectra were collected with neat thin films on sodium chloride disk using a Bomem Michaelson 100 Fourrier transform infrared (FTIR) spectrometer. ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, NOSEY, and COSY spectra were recorded on a Brucker AVANCE 300 MHz or 400 MHz spectrometer. Deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) were used as NMR solvents unless otherwise noted. All chemical shifts are reported in parts per million (δ), integration and coupling constant(s) in Hz downfield from TMS and corrected using the solvent residual peak as internal standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, b = broad, br s = broad singlet. High resolution mass spectra (HRMS) were collected by positive ion electrospray on Kratos Analytical Concept-11A mass spectrometer with an electron beam of 70ev (Ottawa-Carleton Mass Spectrometry Centre). LC/MS/MS was conducted with an Applied Biosystems API2000 triple quadrupole mass spectrometer using electrospray ionization and a Hypersil C_{18} 100 × 2.1 mm, 3 μM particle size column.

4.4.2 Experimental procedures



4.7

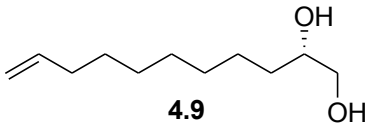
9-Decenal (4.2). Oxalyl chloride (1.5 mL, 17.7 mmol, 3.5 equiv.) was dissolved in methylene chloride (25 mL) and the reaction was cooled to -78°C. DMSO (1.8 mL, 25.2 mmol, 5 equiv.) was added dropwise and the resulting mixture was stirred for 30 minutes at -78°C. 9-decenol (900 μL, 5.04 mmol, 1 equiv.) was dissolved in methylene chloride (25 mL) and cooled to -78°C before being cannulated into the above reaction mixture. The resulting solution is then stirred for 1 hour at -78°C. Triethylamine (7.0 mL, 50.4 mmol, 10 equiv.) is added dropwise and the reaction is put slowly to room temperature. A brine solution (50 mL) is added and the solution is extracted with methylene chloride (3 × 20 mL),dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 100% hexanes) to afford a dark yellow oil (702.5 mg, 4.55 mmol, 90%). Observed ^1H and ^{13}C NMR data agrees with literature values.[19] **4.2**: Rf = 0.47 (silica gel, 9:1 hexanes/EtOAc). ^1H NMR (400 MHz, $CDCl_3$) δ 9.74 (t, *J* = 1.4 Hz, 1 H), 5.78 (ddt, *J* = 17.0, 10.3, 6.3 Hz, 1 H), 4.96 (ddt, *J* = 17.1, 2.0, 1.5 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 1.0, 0.9 Hz, 1 H), 2.39 (dt, *J* = 7.4, 1.7 Hz, 2 H), 2.05-1.97 (m, 2 H), 1.66-1.55 (m, 2 H), 1.39-1.21 (m, 8 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.7, 139.0, 114.2, 43.8, 33.7, 29.2, 29.1, 28.8, 28.8, 22.0.



4.8

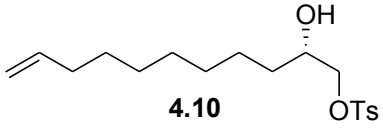
2-(8-noneyl)oxirane (4.3). 9-decenal (698 mg, 4.52 mmol, 1 equiv.) and chloroiodomethane (396 μL, 5.42 mmol, 1.2 equiv.) were dissolved in THF (11 mL) and cooled to -78°C. Methyl lithium complexed with lithium bromide 1.5 M solution in diethyl ether (3.31 mL, 4.97 mmol, 1.1 equiv.) was added dropwise to the solution at

-78°C and the resulting mixture was stirred at -78°C for 45 minutes before being warmed up to room temperature. The reaction was quenched with ammonium chloride (15 mL) and extracted with EtOAc (3 × 10 mL). The resulting organic layer was washed with brine (20 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a yellow oil (631.6 mg, 3.75 mmol, 83%). Observed ^{1}H NMR data agrees with literature values.[20] **4.3**: Rf = 0.61 (silica gel, 9:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (ddt, *J* = 17.0, 10.3, 6.6 Hz, 1 H), 4.97 (ddt, *J* = 17.3, 2.1, 1.6 Hz, 1 H), 4.90 (ddt, *J* = 10.3, 2.3, 1.3 Hz, 1 H), 2.92-2.85 (m, 1 H), 2.72 (dd, *J* = 5.1, 4.1 Hz, 1 H), 2.44 (dd, *J* = 5.1, 2.8 Hz, 1 H), 2.06-1.97 (m, 2 H), 1.40-1.17 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.0, 114.1, 52.3, 47.0, 33.8, 32.5, 29.4, 29.4, 29.0, 28.9, 25.9.



4.9

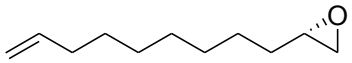
(*S*)-10-undecene-1,2-diol (4.4). To 2-(8-noneyl)oxirane (392 mg, 2.33 mmol, 1 equiv.) was dissolved in THF (100 µL) and the Jacobsen's (*S,S*)-salenCo(III)OAc catalyst (28 mg, 0.05 mmol, 0.02 equiv.) was added and the solution was cooled to 0°C. H_2O (23 µL, 1.28 mmol, 0.55 equiv.) was added to the solution and the resulting mixture was warmed up to room temperature and stirred for 16 hours. The reaction was purified by column chromatography (silica gel, 30% ethyl acetate in hexanes) to afford a light brown oil (199.7 mg, 1.07 mmol, 46%). Observed ^{1}H NMR data agrees with literature values.[21] **4.4**: Rf = 0.21 (silica gel, 1:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.59 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1 H), 4.97 (ddt, *J* = 17.2, 2.1, 1.7 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 2.2, 1.2 Hz, 1 H), 3.72-3.66 (m, 1 H), 3.64 (dd, *J* = 10.9, 3.1 Hz, 1 H), 3.42 (dd, *J* = 10.9, 7.6 Hz, 1 H), 2.05-1.96 (m, 2 H), 1.45-1.21 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 114.2, 72.3, 66.9, 33.8, 33.2, 29.6, 29.4, 29.0, 28.9, 25.5.



4.10

(*S*)-2-hydroxyundec-10-enyl 4-methylbenzenesulfonate (4.5). 4.5 was prepared

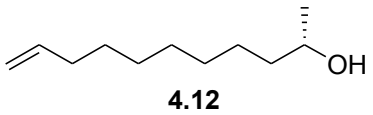
following the procedure described by Martinelli et al.[17] (*S*)-undece-10-ene-1,2-diol (196 mg, 1.05 mmol, 1 equiv.) was dissolved in methylene chloride (3 mL). Dibutyltin oxide (5 mg, 0.02 mmol, 0.02 equiv.) and p-Toluenesulfonyl chloride (200 mg, 1.05 mmol, 1 equiv) was added before the addition of triethylamine (146 µL, 1.05 mmol, 1 equiv) and the resulting mixture was stirred for 4 hours. The reaction was quenched with ammonium chloride (15 mL) and extracted with EtOAc (3 × 10 mL). The resulting organic layer was washed with brine (20 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford a yellow oil (232.5 mg, 0.68 mmol, 65%). Observed ^{1}H NMR data agrees with literature values.[22] **4.5**: Rf = 0.61 (silica gel, 1:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73-7.64 (m, 2 H), 7.36-7.25 (m, 2 H), 5.76 (ddt, *J* = 17.1, 10.3, 6.6 Hz, 1 H), 4.98-4.92 (m, 1 H), 4.92-4.87 (m, 1 H), 4.58-4.46 (m, 1 H), 3.99 (dd, *J* = 4.4, 0.8 Hz, 2 H), 2.40 (s, 3 H), 2.03-1.90 (m, 2 H), 1.62-1.49 (m, 2 H), 1.39-0.93 (m, 10 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.0, 139.0, 129.9, 128.0, 127.9, 114.3, 78.9, 69.5, 33.7, 31.0, 29.1, 28.9, 28.9, 28.8, 24.4, 21.7.



4.11

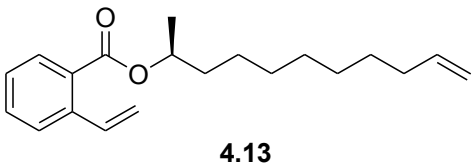
(*S*)- 2-(8-noneyl)oxirane (4.6). 4.5(230 mg, 0.67 mmol, 1 equiv.) was dissolved in THF (7 mL) and the solution was cooled to 0°C. Sodium hydride in a 60% oil dispersion (54 mg, 2.23 mmol, 2 equiv.) was added and the resulting mixture was slowly put to room temperature and was stirred for 18 hours. The reaction was quenched with ammonium chloride (15 mL) and extracted with EtOAc (3 × 10 mL). The resulting organic layer was washed with brine (20 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford a yellow oil (108.9 mg, 0.65 mmol, 96%). Observed ^{1}H NMR data agrees with literature values.[20] **4.6**: Rf = 0.42 (silica gel, 9:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1 H), 4.96 (ddt, *J* = 17.2, 2.2, 1.7 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 2.3, 1.2 Hz, 1 H), 2.91-2.85 (m, 1 H), 2.72 (dd, *J* = 5.1, 4.1 Hz, 1 H), 2.44 (dd, *J* =

5.1, 2.7 Hz, 1 H), 2.05-1.98 (m, 2 H), 1.54-1.19 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 114.2, 52.4, 47.1, 33.8, 32.5, 29.4, 29.4, 29.0, 28.9, 26.0.



4.12

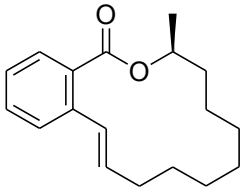
***S*-Undec-10-en-2-ol (4.7).** **4.6** (100.9 mg, 0.60 mmol, 1 equiv.) was dissolved in THF (5 mL) and cooled to 0°C. Lithium aluminium hydride (23 mg, 0.60 mmol, 1 equiv.) was added to the solution at 0°C in three portions over 30 minutes and the resulting mixture was warmed to room temperature and stirred for 4 hours. The reaction was cooled again to 0°C and H_2O (20 μL) was added dropwise, before addition of a 15% sodium hydroxide solution (20 μL) and H_2O (60 μL). The mixture was filtered and the filtrate was extracted with brine (15 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) to afford a light yellow oil (100.0 mg, 0.59 mmol, 98%). Observed ^{1}H NMR data agrees with literature values.[23] **4.7**: Rf = 0.35 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 (ddt, *J* = 16.6, 10.4, 6.6 Hz, 1 H), 4.95 (ddt, *J* = 17.0, 1.8, 1.7 Hz, 1 H), 4.89 (ddt, *J* = 10.0, 1.4, 0.9 Hz, 1 H), 3.79-3.69 (m, 1 H), 2.03-1.96 (m, 2 H), 1.46-1.18 (m, 14 H), 1.14 (d, *J* = 6.3 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 114.1, 68.2, 39.4, 33.8, 29.9, 29.6, 29.5, 29.1, 25.8, 23.5.



4.13

(*S*)-2-Vinyl benzoic acid undec-10-en-2-yl ester (4.8). To 2-vinyl benzoic acid (98 mg, 0.66 mmol, 1.2 equiv.) in THF (5 mL) under argon atmosphere was added (*S*)-undec-10-en-2-ol (93 mg, 0.55 mmol, 1 equiv.) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (316 mg, 1.65

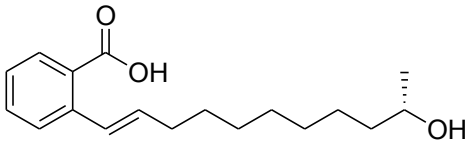
mmol, 3.0 equiv.) before addition of 4-(dimethylamino)pyridine (13 mg, 0.11 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with a saturated ammonium chloride solution and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford a yellow oil (85.3 mg, 0.28 mmol, 52%). **4.8:** Rf = 0.54 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2976, 2928, 2856, 1718, 1254, 1126 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 7.8, 1.3 Hz, 1 H), 7.56 (ddd, *J* = 7.8, 0.6, 0.6 Hz, 1 H), 7.49-7.39 (m, 2 H), 7.30 (ddd, *J* = 7.7, 7.5, 1.3 Hz, 1 H), 5.78 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1 H), 5.62 (dd, *J* = 17.4, 1.3 Hz, 1 H), 5.32 (dd, *J* = 11.0, 1.3 Hz, 1 H), 5.18-5.08 (m, 1 H), 4.97 (ddt, *J* = 17.2, 2.1, 1.6 Hz, 1 H), 4.91 (ddt, *J* = 10.1, 2.3, 1.3 Hz, 1 H), 2.06-1.97 (m, 2 H), 1.79-1.21 (m, 15 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 139.4, 139.2, 136.0, 131.8, 130.1, 129.4, 127.4, 127.1, 116.2, 114.2, 71.9, 36.0, 33.8, 29.4, 29.4, 29.0, 28.9, 25.5, 20.1; HRMS (+EI): calcd for $C_{20}H_{28}O_2$ (M) 300.2089, obsd 300.1958.



4.14

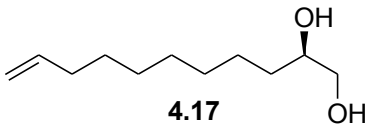
(*S*,E)-3-methyl-7,8,9,10,11,12,13,14-octahydro-6-oxa-benzocyclotetradecen-5-one (4.9). To **4.8** (80 mg, 0.27 mmol, 1 equiv.) in toluene (64 mL) was added Grubbs catalyst 2nd generation (11 mg, 0.01 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford **4.9** (62.6 mg) as a yellow oil. . Due to the non-polar properties of the molecules, there was a 1:1 mixture of product:SM. The mixture was carried over to the next step without further purification. **4.9**: Rf = 0.43 (silica gel, 9:1 hexanes/EtOAc); ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (dd, *J* = 7.7, 1.3 Hz, 1 H), 7.49-7.37 (m, 2 H), 7.25 (ddd, *J* = 7.7, 7.3, 1.1 Hz, 1 H), 6.98 (d, *J* = 15.7

Hz, 1 H), 5.90 (dt, *J* = 15.7, 7.2 Hz, 1 H), 5.26-5.17 (m, 1 H), 2.31-2.25 (m, 2 H), 1.70-1.19 (m, 15 H).



4.15

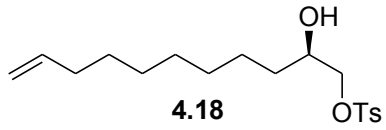
(*S*,E)-2-(10-Hydroxy-undecenyl)-benzoic acid (4.10). To **4.9** (60 mg, 0.22 mmol, 1 equiv.) in methanol (9 mL) at room temperature was added lithium hydroxide monohydrate (86 mg, 2.04 mmol, 9.5 equiv.) that was first dissolved in water (3 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached a pH of 2 and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 30% ethyl acetate: hexanes) to afford **4.10** (62 mg, 0.21 mmol, yield for two steps: 78 %) as a light yellow oil. **4.10**: Rf = 0.54 (silica gel, 1:1 hexanes/EtOAc); IR (NaCl) νmax = 3859 (br), 2936, 2853, 1710, 1260, 1089 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 7.9 Hz, 1 H), 7.51 (d, *J* = 7.9 Hz, 1 H), 7.44 (dd, *J* = 7.6 Hz, 1 H), 7.26 (dd, *J* = 7.4 Hz, 1 H), 7.18 (d, *J* = 15.7 Hz, 1 H), 6.07 (dt, *J* = 15.6, 7.0 Hz, 1 H), 3.87-3.77 (m, 1 H), 2.28-2.19 (m, 2 H), 1.52-1.21 (m, 12 H), 1.18 (d, *J* = 6.2 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.2, 140.3, 134.1, 132.4, 131.0, 130.9, 129.5, 129.1, 126.5, 68.5, 39.1, 32.7, 29.4, 29.1, 28.7, 28.4, 25.4, 23.5; HRMS (+EI): calcd for $C_{18}H_{25}O_2$ (M-OH) 273.1855, obsd 273.1890.



4.17

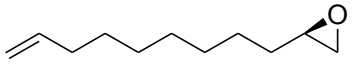
(*R*)-10-undecene-1,2-diol (4.12). 2-(8-noneyl)oxirane (237 mg, 1.41 mmol, 1 equiv.) was dissolved in THF (100 μL) and the Jacobsen's (*R,R*) catalyst (17 mg, 0.03 mmol, 0.02 equiv.) was added and the solution was cooled to 0°C. H_2O (14 μL,

0.78 mmol, 0.55 equiv.) was added to the solution and the resulting mixture was warmed up to room temperature and stirred for 16 hours. The reaction was purified by column chromatography (silica gel, 30% ethyl acetate in hexanes) to afford a light brown oil (125.7 mg, 0.67 mmol, 48%). Observed ^{1}H NMR data agrees with literature values[21]. **4.12**: Rf = 0.21 (silica gel, 1:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (ddt, *J* = 17.1, 10.2, 6.7 Hz, 1 H), 4.97 (ddt, *J* = 17.2, 2.1, 1.7 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 2.2, 1.2 Hz, 1 H), 3.73-3.66 (m, 1 H), 3.64 (dd, *J* = 11.0, 3.0 Hz, 1 H), 3.41 (dd, *J* = 11.1, 7.6 Hz, 1 H), 2.05-1.97 (m, 2 H), 1.47-1.24 (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 114.2, 72.3, 68.9, 33.8, 33.2, 29.6, 29.4, 29.0, 28.9, 25.5



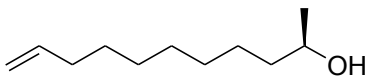
4.18

(*R*)-2-hydroxyundec-10-enyl 4-methylbenzenesulfonate (4.13). (*S*)-undece-10-ene-1,2-diol (126 mg, 0.68 mmol, 1 equiv.) was dissolved in methylene chloride (2 mL). Dibutyltin oxide (3 mg, 0.013 mmol, 0.02 equiv.) and p-Toluenesulfonyl chloride (130 mg, 0.68 mmol, 1 equiv) was added before the addition of triethylamine (95 μL, 0.68 mmol, 1 equiv) and the resulting mixture was stirred for 4 hours. The reaction was quenched with a saturate ammonium chloride solution (15 mL) and extracted with EtOAc (3 × 10 mL). The resulting organic layer was washed with brine (20 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 20% ethyl acetate in hexanes) to afford a yellow oil (165.4 mg, 0.49 mmol, 72%). Observed ^{1}H NMR data agrees with literature values[22]. **4.13**: Rf = 0.31 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73-7.64 (m, 2 H), 7.36-7.25 (m, 2H), 5.76 (ddt, *J* = 17.1, 10.3, 6.6 Hz, 1 H), 4.98-4.92 (m, 1 H), 4.92-4.87 (m, 1 H), 4.58-4.46 (m, 1 H), 3.99 (dd, *J* = 4.4, 0.8 Hz, 2 H), 2.40 (s, 3 H), 2.03-1.90 (m, 2 H), 1.62-1.49 (m, 2 H), 1.39-0.93 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.0, 139.0, 129.9, 128.0, 127.9, 114.3, 78.9, 69.5, 33.7, 31.0, 29.1, 28.9, 28.9, 28.8, 24.4, 21.7.



4.19

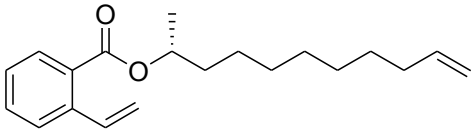
(*R*)- 2-(8-noneyl)oxirane (4.14). 4.13 (163 mg, 0.48 mmol, 1 equiv.) was dissolved in THF (5 mL) and the solution was cooled to 0°C. Sodium hydride in a 60% oil dispersion (38 mg, 1.60 mmol, 2 equiv.) was added and the resulting mixture was slowly put to room temperature and was stirred for 18 hours. The reaction was quenched with ammonium chloride (15 mL) and extracted with EtOAc (3 × 10 mL). The resulting organic layer was washed with brine (20 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford a yellow oil (73.6 mg, 0.44 mmol, 91%). Observed ^{1}H NMR data agrees with literature values.[24] **4.14**: Rf = 0.49 (silica gel, 9:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, CDCl3) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.77 (ddt, *J* = 17.1, 10.3, 6.6 Hz, 1 H), 4.95 (ddt, *J* = 17.2, 2.1, 1.5 Hz, 1 H), 4.89 (ddt, *J* = 10.2, 2.3, 1.2 Hz, 1 H), 2.90-2.84 (m, 1 H), 2.71 (dd, *J* = 5.1, 4.1 Hz, 1 H), 2.43 (dd, *J* = 5.1, 2.8 Hz, 1 H), 2.04-1.97 (m, 2 H), 1.53-1.19 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.1, 114.2, 52.4, 47.2, 33.8, 32.5, 29.4, 29.4, 29.0, 28.9, 25.9.



4.20

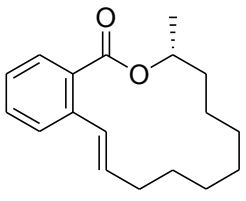
***R*-Undec-10-en-2-ol (4.15). 4.14** (73 mg, 0.43 mmol, 1 equiv.) was dissolved in THF (5 mL) and cooled to 0°C. Lithium aluminium hydride (16 mg, 0.43 mmol, 1 equiv.) was added to the solution at 0°C in three portions over 30 minutes and the resulting mixture was warmed to room temperature and stirred for 4 hours. The reaction was cooled again to 0°C and H_2O (20 μL) was added dropwise, before addition of a 15% sodium hydroxide solution (20 μL) and H_2O (60 μL). The mixture was filtered and the filtrate was extracted with brine (15 mL) and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, evaporated to near dryness in vacuo and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) to afford a light yellow oil (72.0 mg, 0.42 mmol, 98%). Observed ^{1}H NMR data agrees with literature values.[25] **4.15**: Rf = 0.31 (silica gel, 4:1 hexanes/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (ddt, *J* = 17.0, 10.3, 6.6 Hz, 1 H), 4.97 (ddt, *J* = 17.1, 1.7,

1.6 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 1.2, 1.1 Hz, 1 H), 3.81-3.73 (m, 1 H), 2.05-1.98 (m, 2 H), 1.49-1.22 (m, 14 H), 1.16 (d, *J* = 6.2 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.2, 114.1, 68.1, 39.2, 33.8, 29.6, 29.4, 29.0, 28.9, 25.7, 23.3.



4.21

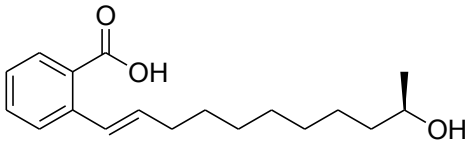
(*R*)-2-Vinyl benzoic acid undec-10-en-2-yl ester (4.16). To 2-vinyl benzoic acid (93 mg, 0.63 mmol, 1.5 equiv.) in THF (3.5 mL) under argon atmosphere was added (*S*)-undec-10-en-2-ol (71 mg, 0.42 mmol, 1 equiv.) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (242 mg, 1.26 mmol, 3.0 equiv.) before addition of 4-(dimethylamino)pyridine (10 mg, 0.08 mmol, 0.2 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was quenched with ammonium chloride and extracted with EtOAc (3 × 15 mL). A saturated $NaHCO_3$ solution (50 mL) was then added to the organic layer and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford a yellow oil (119.1 mg, 0.40 mmol, 95%). **4.16**: Rf = 0.57 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2926, 2855, 1726, 1245, 1126 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 7.7, 1.2 Hz, 1 H), 7.56 (ddd, *J* = 7.6, 0.6, 0.6 Hz, 1 H), 7.48-7.38 (m, 2 H), 7.30 (ddd, *J* = 7.7, 7.6, 1.3 Hz, 1 H), 5.78 (ddt, *J* = 17.1, 10.7, 6.9 Hz, 1 H), 5.62 (dd, *J* = 17.5, 1.4 Hz, 1 H), 5.32 (dd, *J* = 11.0, 1.4 Hz, 1 H), 5.17-5.07 (m, 1 H), 4.97 (ddt, *J* = 17.1, 2.2, 1.0 Hz, 1 H), 4.91 (ddt, *J* = 10.2, 1.3, 1.2 Hz, 1 H), 2.06-1.97 (m, 2 H), 1.77-1.64 (m, 2 H), 1.64-1.51 (m, 2 H), 1.43-1.15 (m, 11 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 139.4, 139.2, 136.0, 131.8, 130.1, 129.5, 127.3, 127.1, 116.2, 114.2, 71.9, 36.0, 33.8, 29.7, 29.4, 29.0, 28.9, 25.5, 20.1; HRMS (+EI): calcd for $C_{20}H_{28}O_2$ (M) 300.2089, obsd 300.2120



4.22

(*R*,E)- 3-methyl-7,8,9,10,11,12,13,14-octahydro-6-oxa-

benzocyclotetradecen-5-one (4.17). To **4.16** (109 mg, 0.36 mmol, 1 equiv.) in toluene (86 mL) was added Grubbs catalyst 2nd generation (15 mg, 0.02 mmol, 5 mol%). The reaction mixture was stirred at 80°C for 23 hours. The solvent was then evaporated to dryness in vacuo and purified by column chromatography (silica gel, 2% ethyl acetate in hexanes) to afford **4.17** (54.3 mg) as a yellow oil. . Due to the non-polar properties of the molecules, there was a 1.5:1 mixture of product:starting material. The mixture was carried over to the next step without further purification. **4.16**: Rf = 0.33 (silica gel, 9:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (dd, *J* = 7.8, 1.1 Hz, 1 H), 7.47 (ddd, *J* = 7.8, 0.7, 0.5 Hz, 1 H), 7.39 (dddd, *J* = 7.9, 7.3, 0.9, 0.3 Hz, 1 H), 7.25 (ddd, *J* = 7.6, 7.4, 1.2 Hz, 1 H), 6.98 (d, *J* = 15.7 Hz, 1 H), 5.89 (dt, *J* = 15.6, 7.1 Hz, 1 H), 5.25-5.18 (m, 1 H), 2.31-2.24 (m, 2 H), 1.70-1.18 (m, 15 H).



4.23

(*R*,E)- 2-(10-Hydroxy-undecenyl)-benzoic acid (4.18). To **4.17** (45 mg, 0.17 mmol, 1 equiv.) in methanol (9 mL) at room temperature was added lithium hydroxide monohydrate (66 mg, 1.57 mmol, 9.5 equiv.) that was first dissolved in water (3 mL). The resulting mixture was stirred for 13 hours at room temperature under argon atmosphere. The reaction was quenched with a 10% HCl solution until the mixture reached a pH of 2 and extracted with EtOAc (3 × 15 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and purified by column chromatography (silica gel, 30% ethyl acetate: hexanes) to afford **4.18** (45 mg, 0.15 mmol, yield for two steps: 42 %) as a light yellow oil. **4.18**: Rf = 0.55 (silica gel, 1:1 hexanes/EtOAc); IR (NaCl) νmax = 3372 (br), 2920, 2855, 1707, 1249, 1090 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (dd, *J* = 7.8, 1.1 Hz, 1 H), 7.53-7.43 (m, 2 H), 7.29-7.22 (m, 1 H), 7.19 (d, *J* = 15.7 Hz, 1 H), 6.08 (dt, *J* = 15.7, 7.0 Hz, 1 H), 3.87-3.78 (m, 1 H), 2.89-2.29 (m, 2 H), 1.52-1.26 (m, 12 H), 1.18 (d, *J* = 6.2 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.2, 140.3, 134.1,

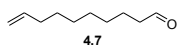
132.4, 131.0, 130.9, 129.5, 129.1, 126.5, 68.5, 39.1, 32.7, 29.4, 29.1, 28.7, 28.4, 25.4, 23.5; HRMS (+EI): calcd for $C_{18}H_{24}O_2$ (M-H_2O) 272.1776, obsd 272.1752.

4.4.3 Enzymatic protocol

Assay of enzymatic catalyzed macrocyclization reaction

For a typical assay, a 200 μL solution containing 50 mM sodium phosphate pH 7.43, 5 mM thioester substrate **2.21**, 15 μM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1 : 4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.40 mL/min, gradient solvent of 5 to 95% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

4.4.4 Spectra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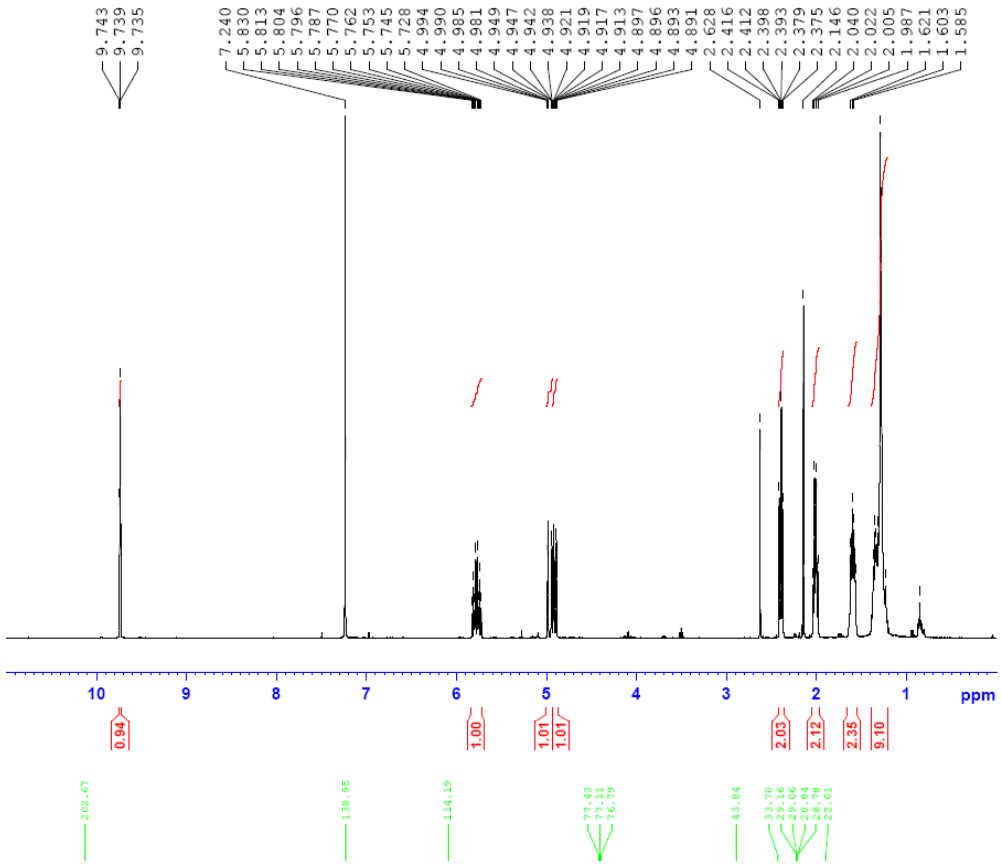
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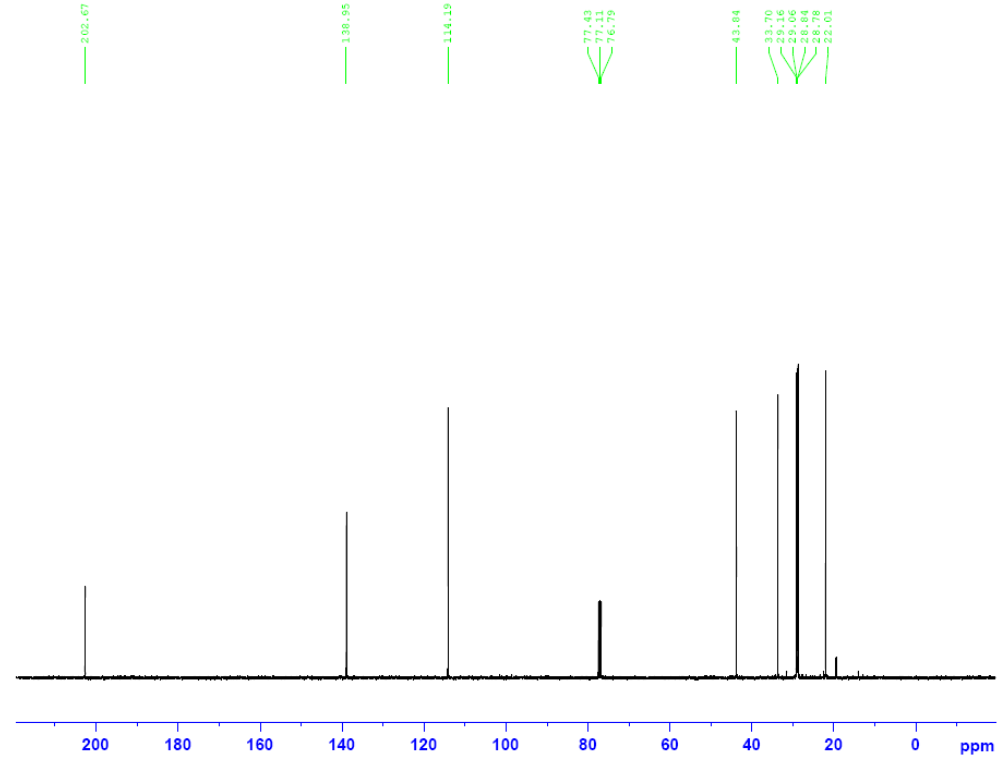
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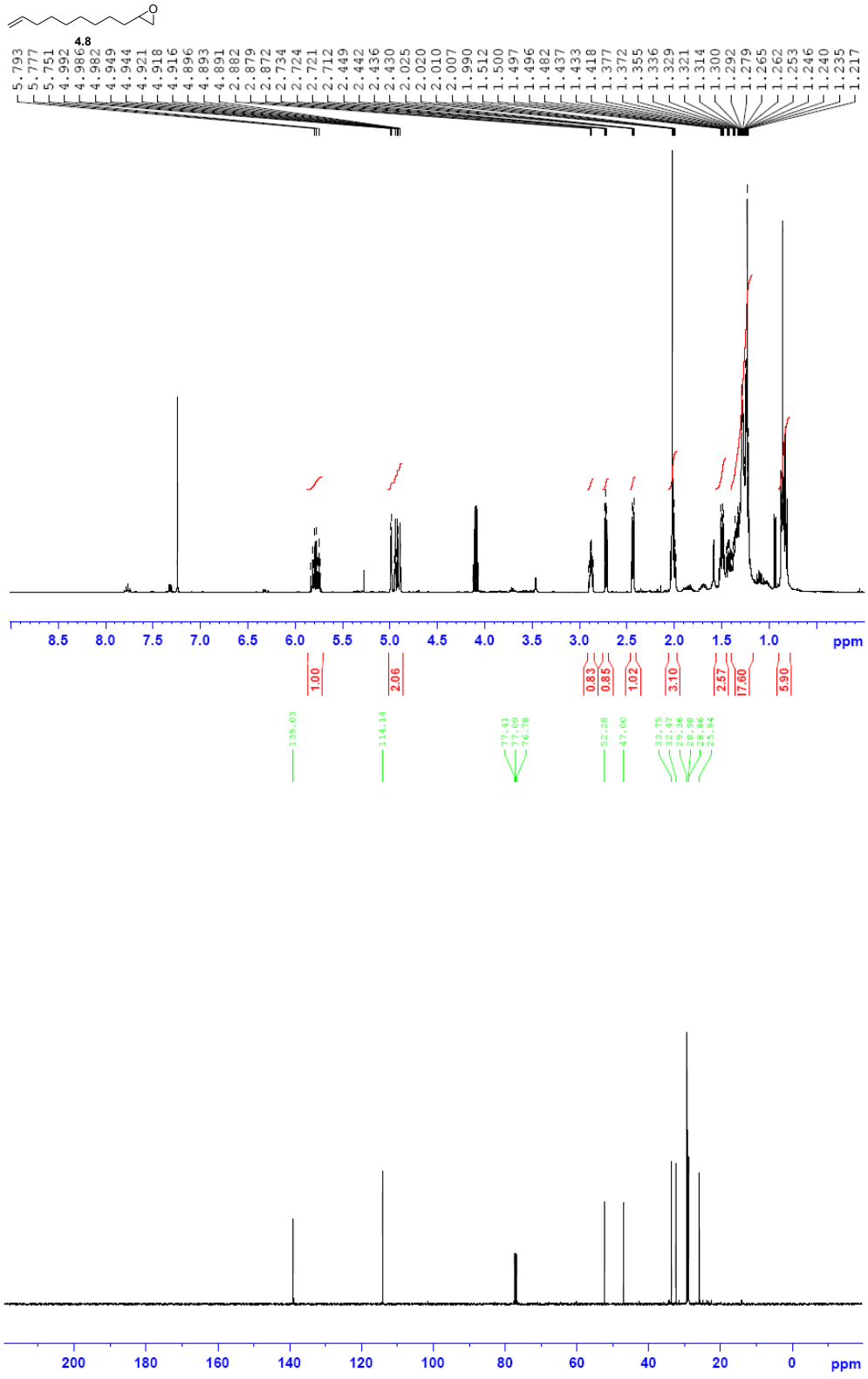
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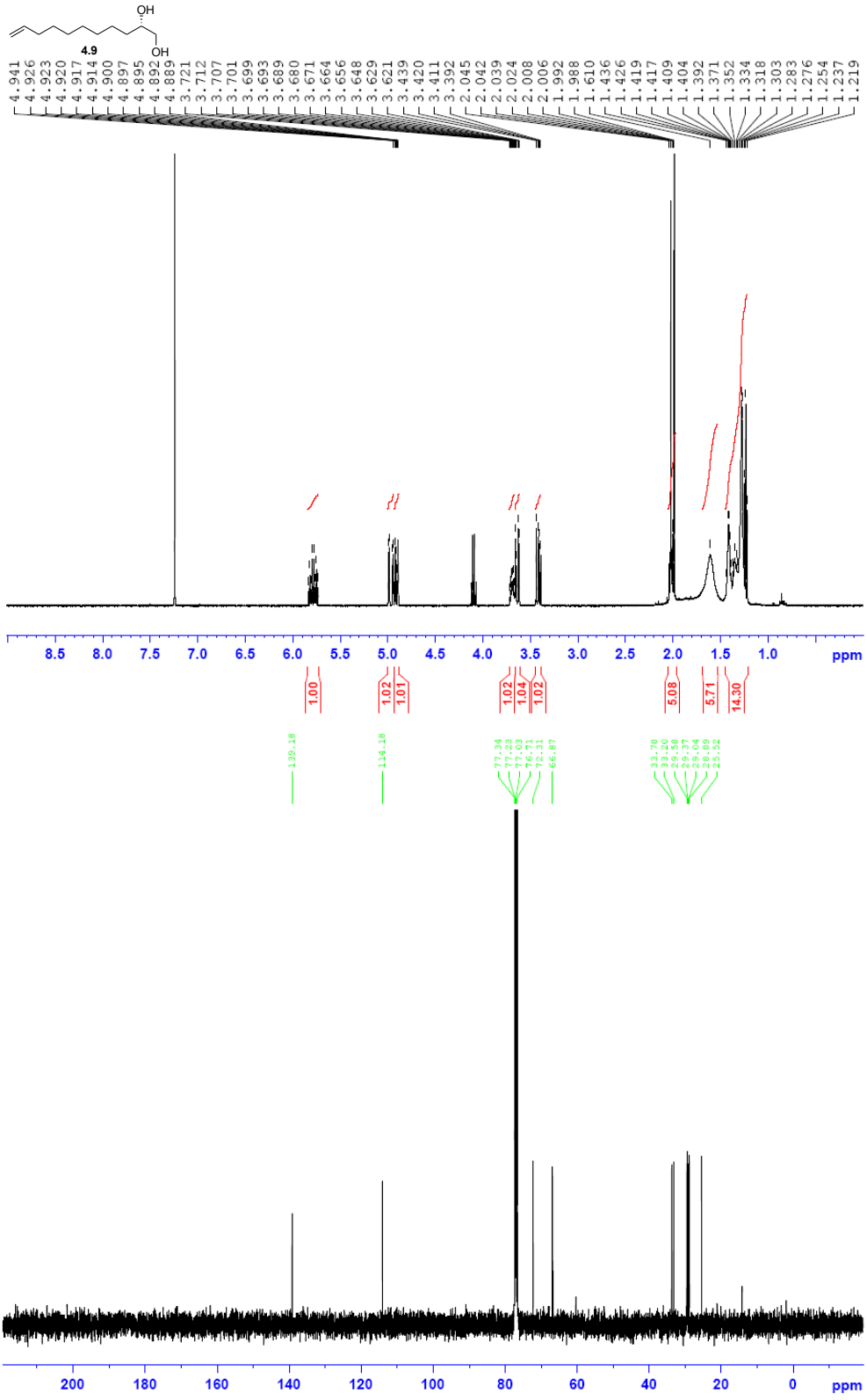
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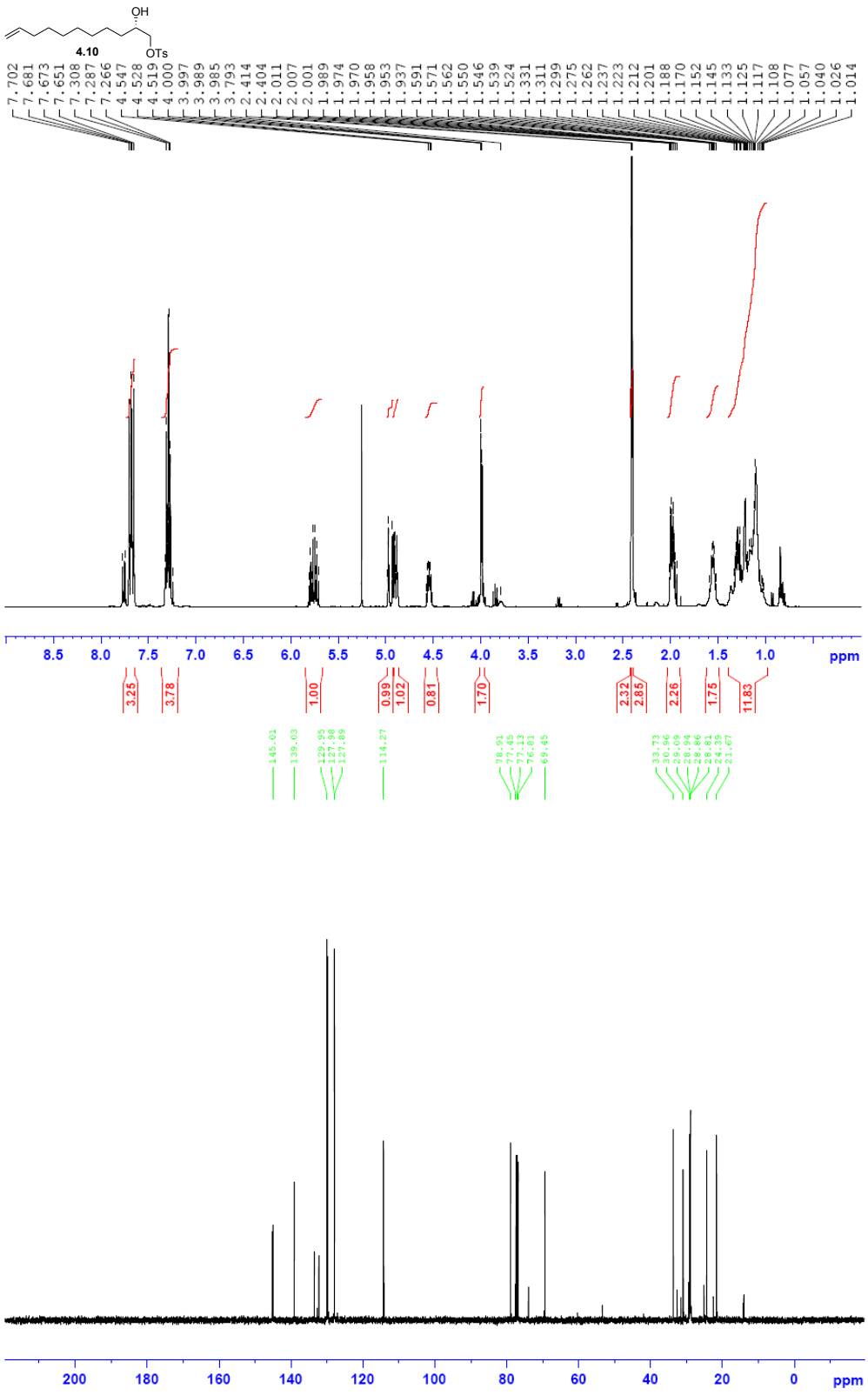
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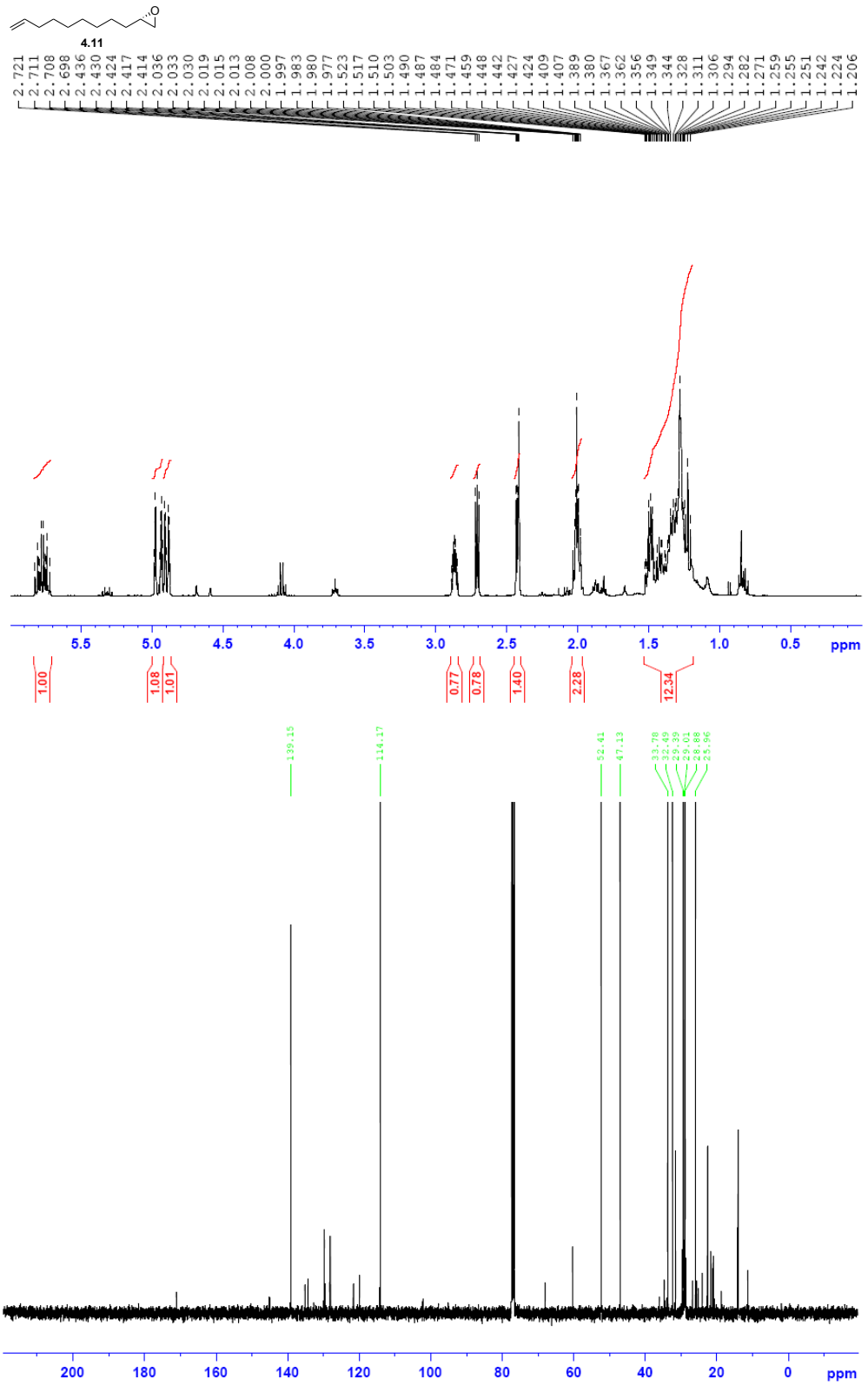
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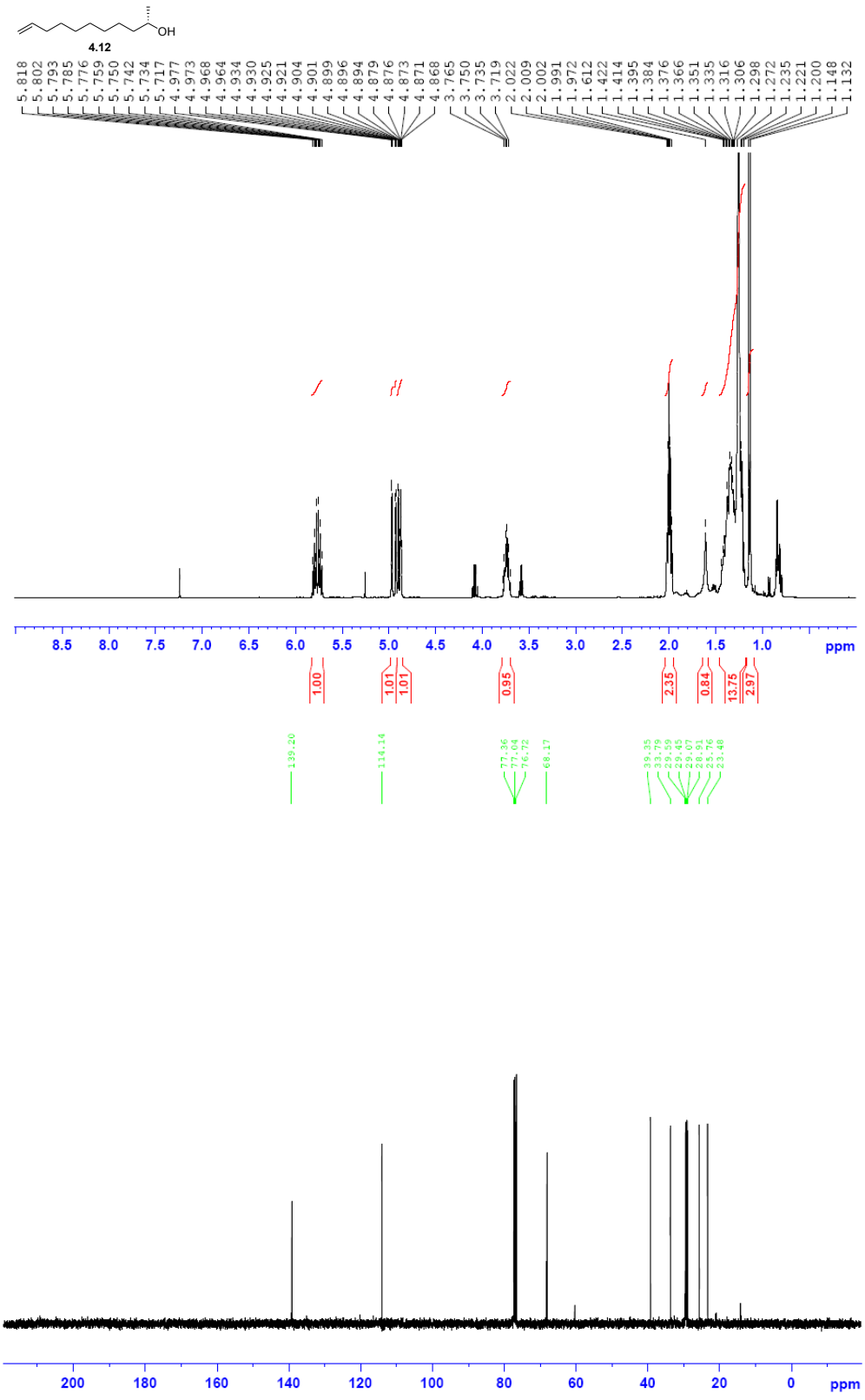
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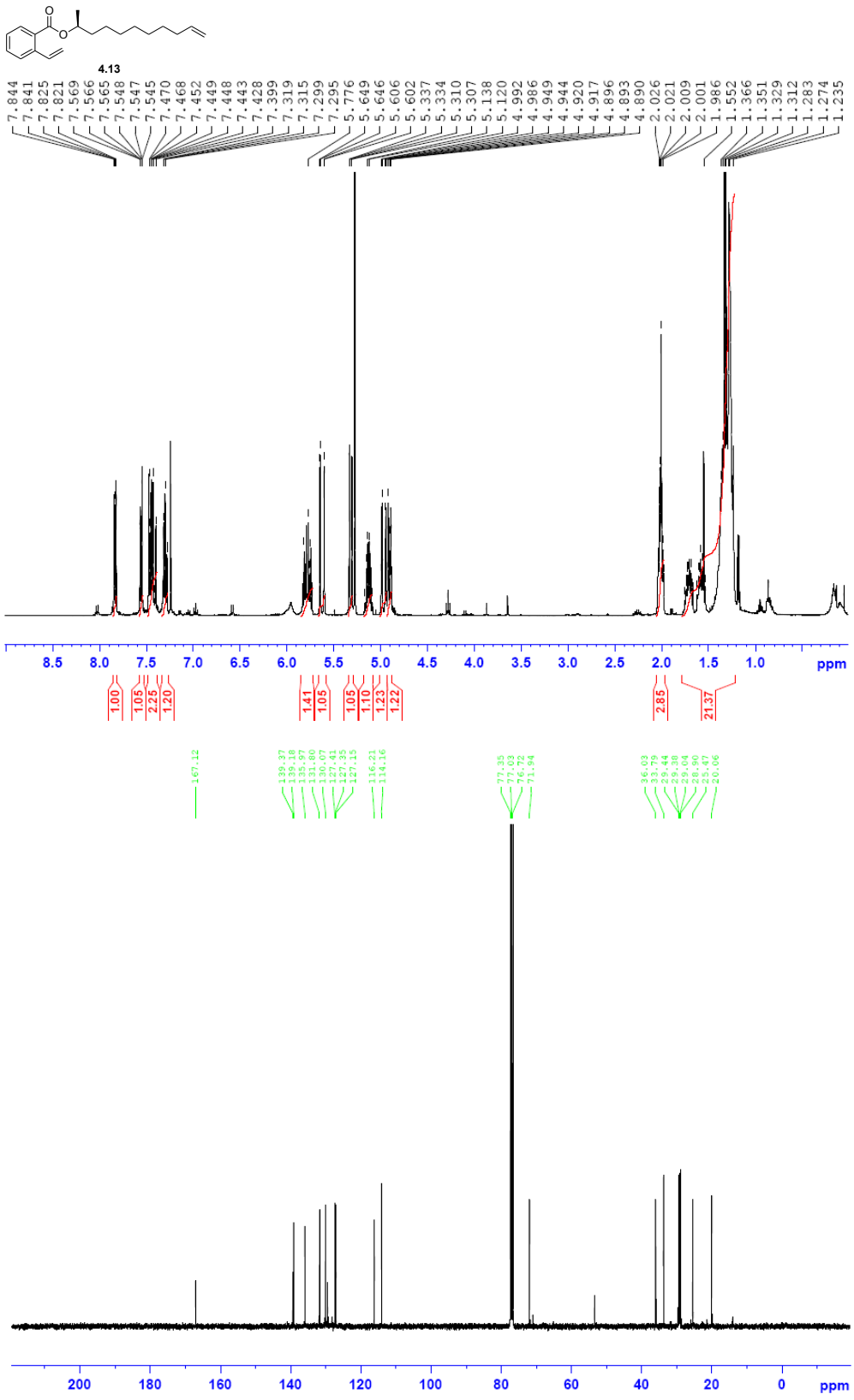
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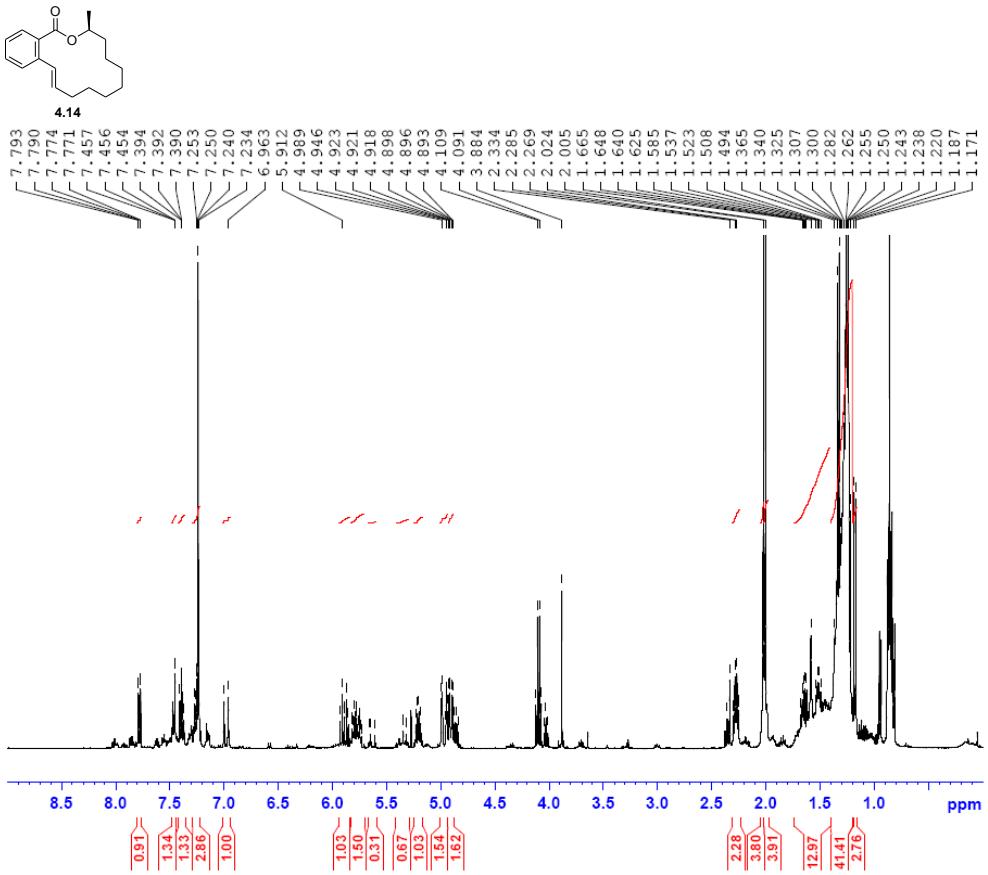
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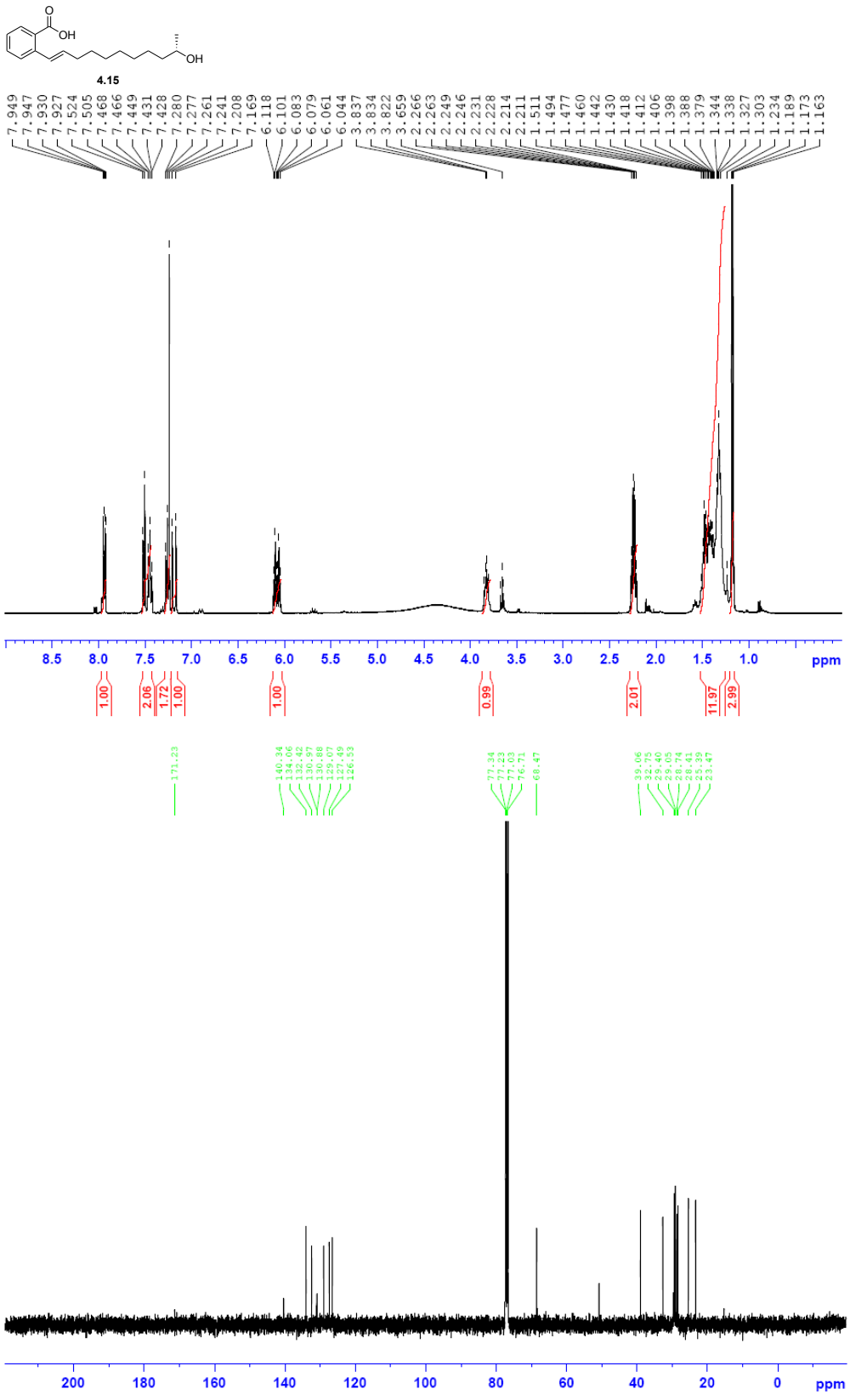
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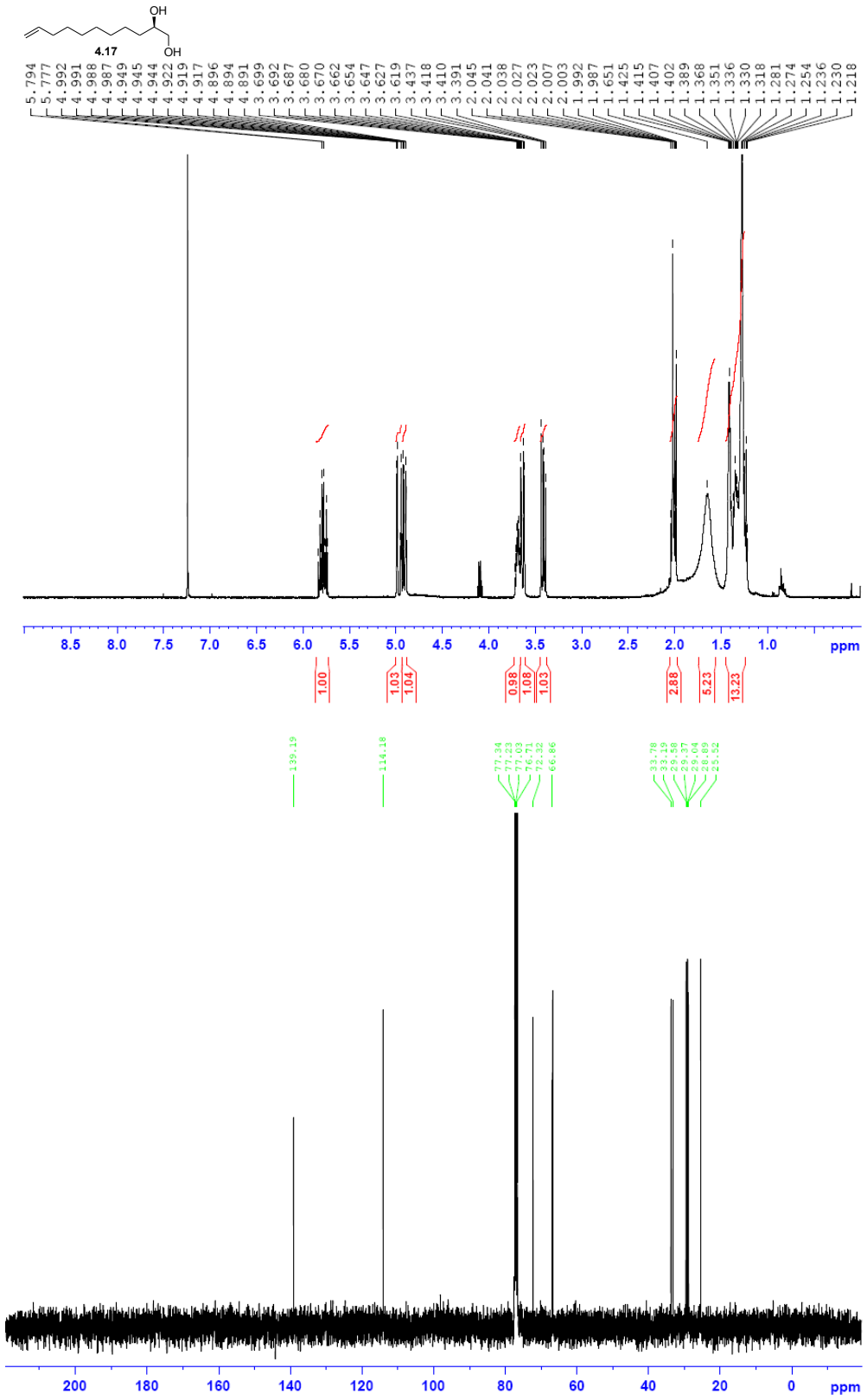
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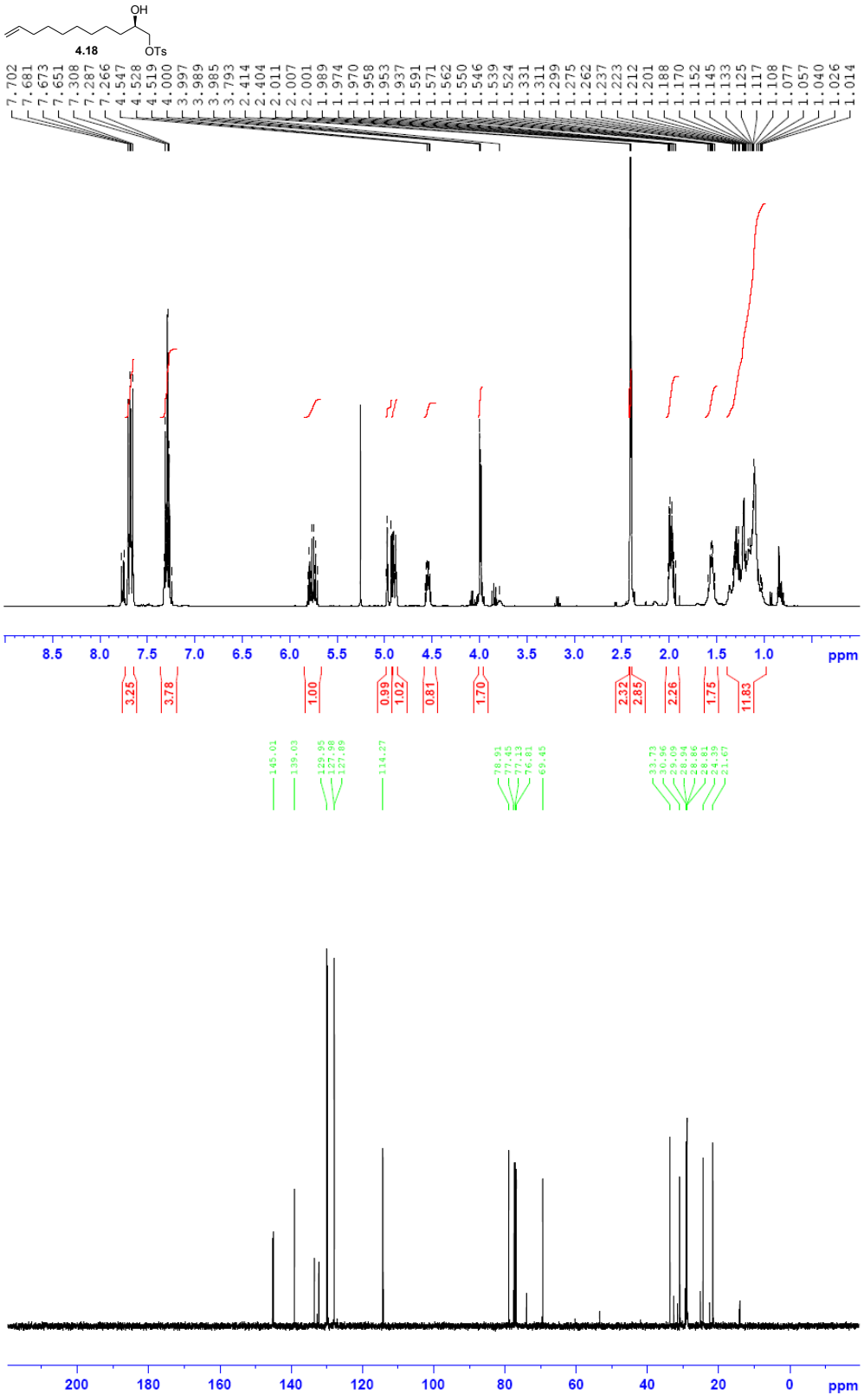
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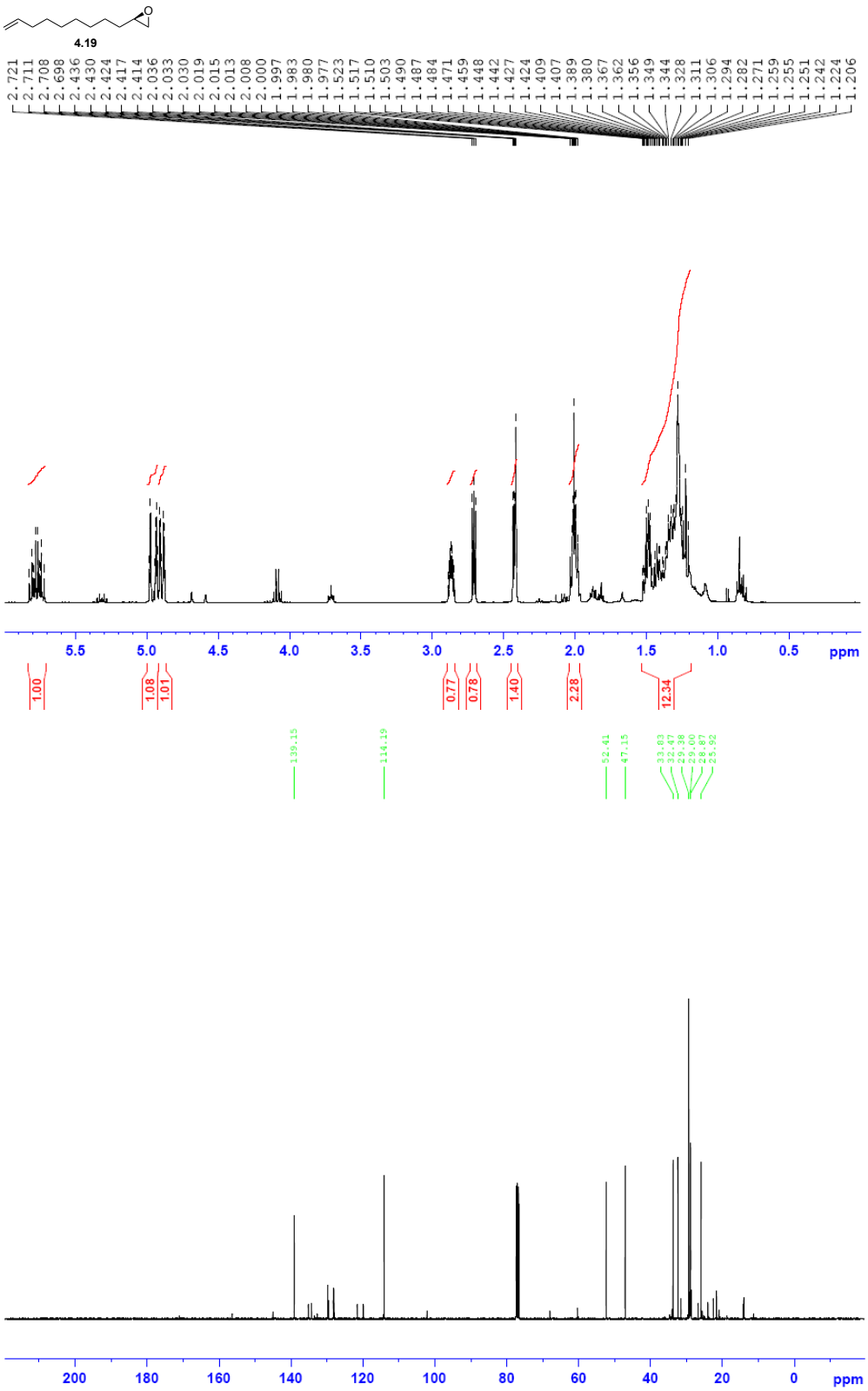
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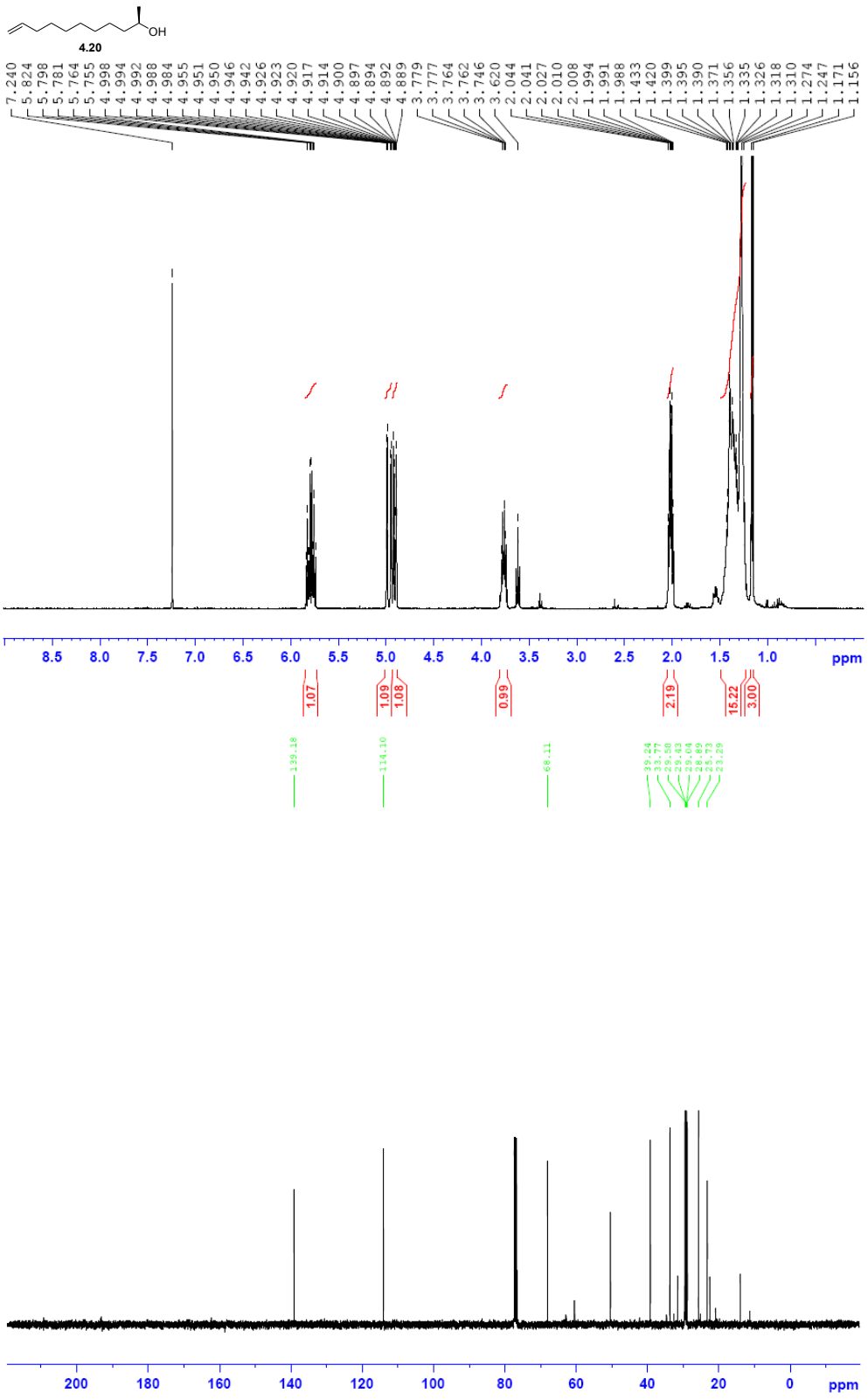
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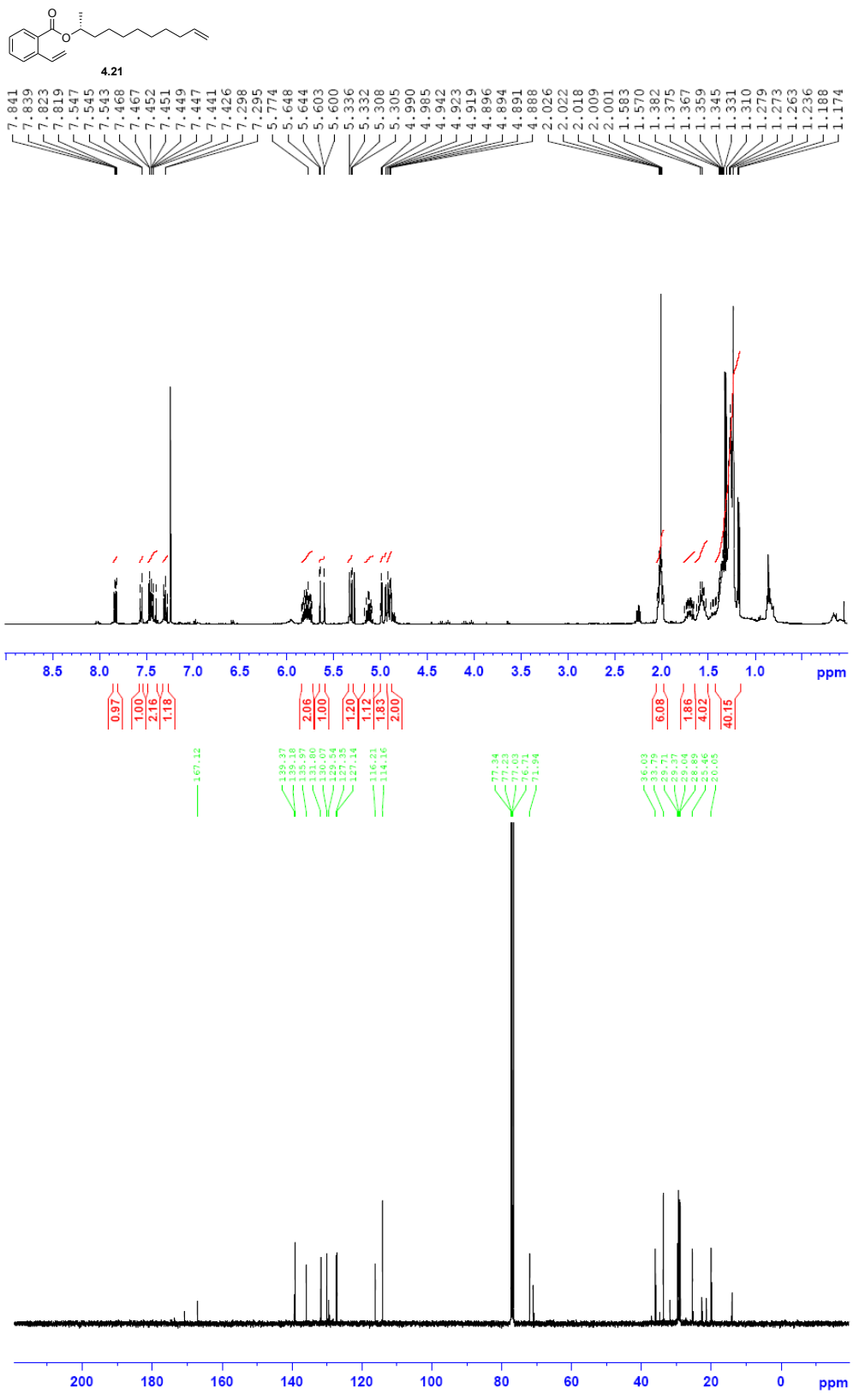
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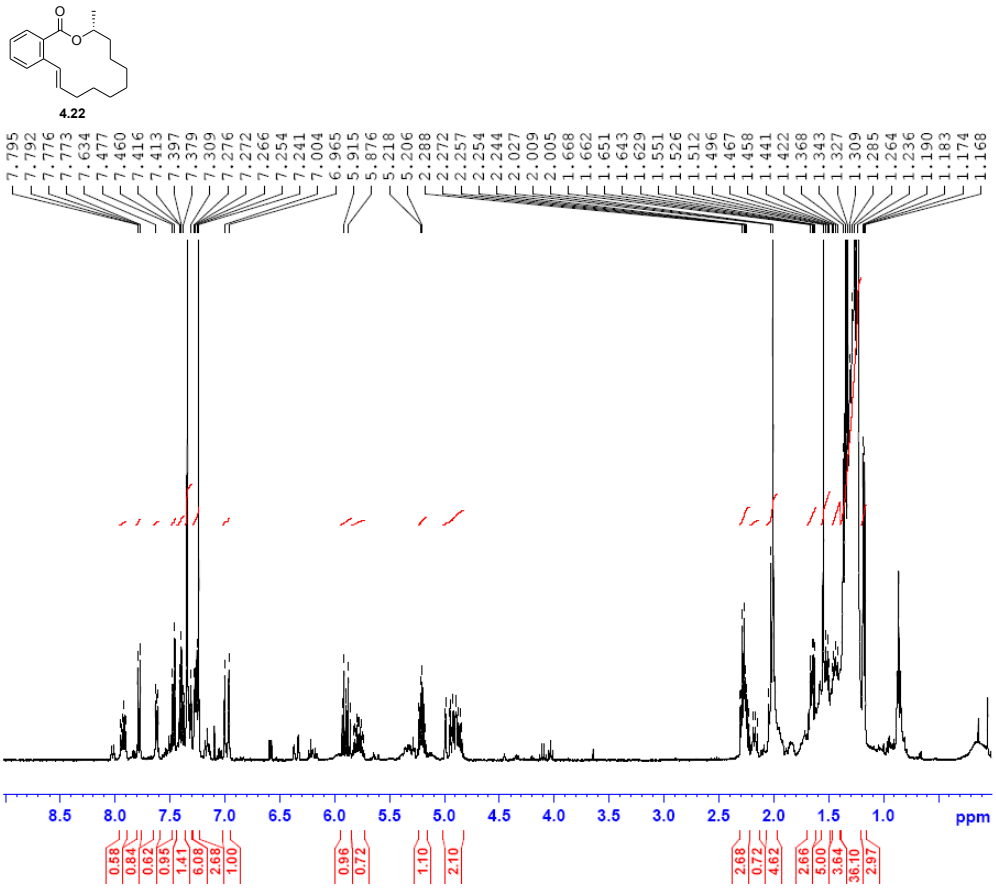
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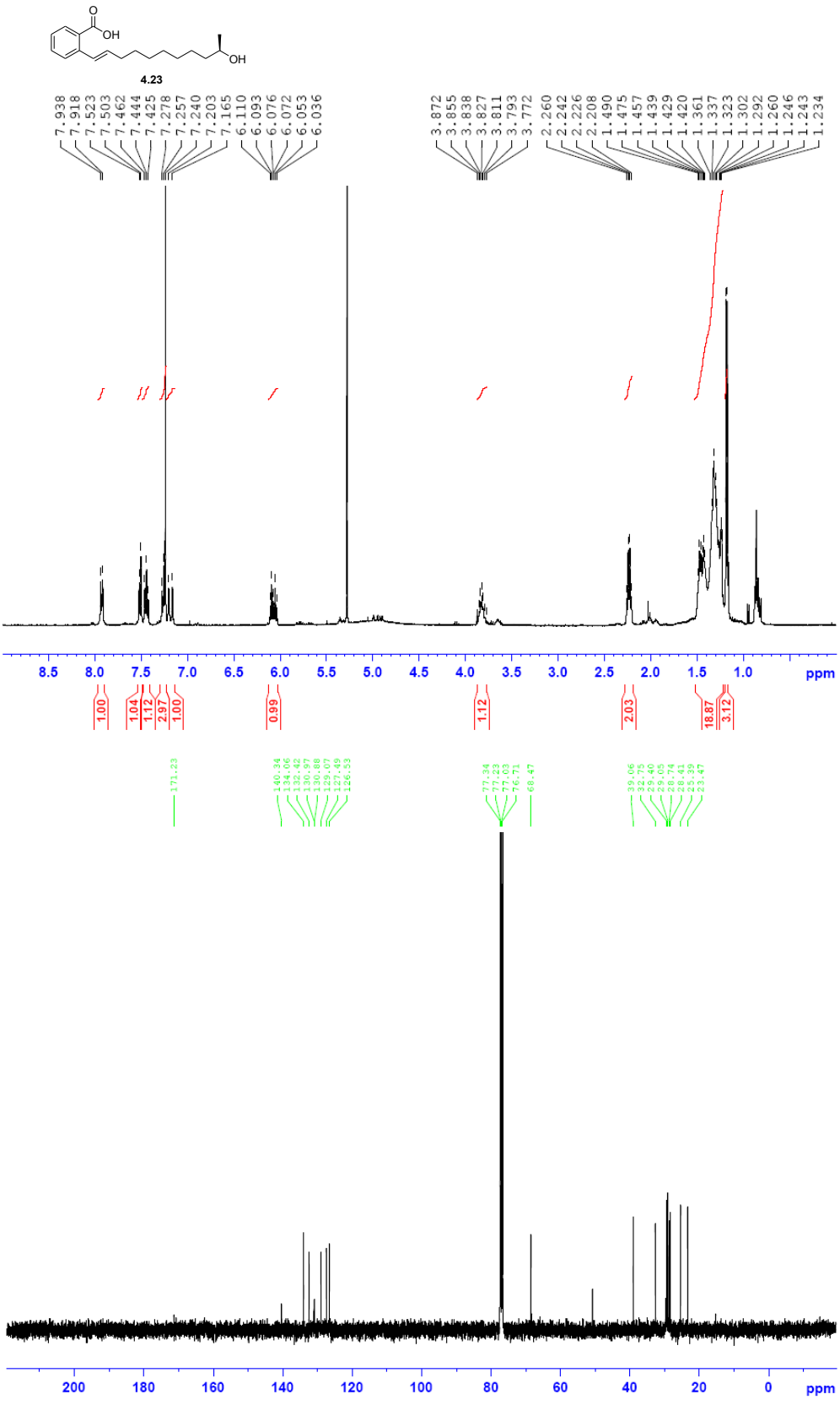
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Chapter 5 Resorcylic acid lactone TEs can macrocyclize substrates with alternative nucleophilic atoms

5.1 Introduction

Having demonstrated the versatility of the zea TE and rad TE in accessing macrolactones of varying ring size and with varying absolute configuration, we set out to determine if these broadly substrate tolerant enzymes could catalyze macrolactam or macrothiolactone formation.

5.1.1 Many amide containing polyketides are known suggesting PKS TEs can form amides

In nature, a great number of macrolactams, such as tyrocidine, are formed by NRPSs. However, there are a few PKS that also produces macrolactams. Some examples include ansamitocin, rifamycins, antibiotic TA, geldanamycin and vicenistatin (Figure 5.1).[1-3] These macrolactams possess many biological properties, including antimicrobial and anticancer properties. They are biosynthesized either by type 1 PKS, or a mixed PKS/NRPS system. Similarly to polyketides that contain macrolactone moieties, a TE is often responsible for the release of the compound from the enzyme.[4] TEs perform this chemistry via either an intermolecular or intramolecular reaction. When there is a free amine on the molecule, then the intramolecular reaction prevails. However, if there is no amine moiety far enough on the molecule to form a lactam, then the TE can also react with different amino acids via an intermolecular reaction.

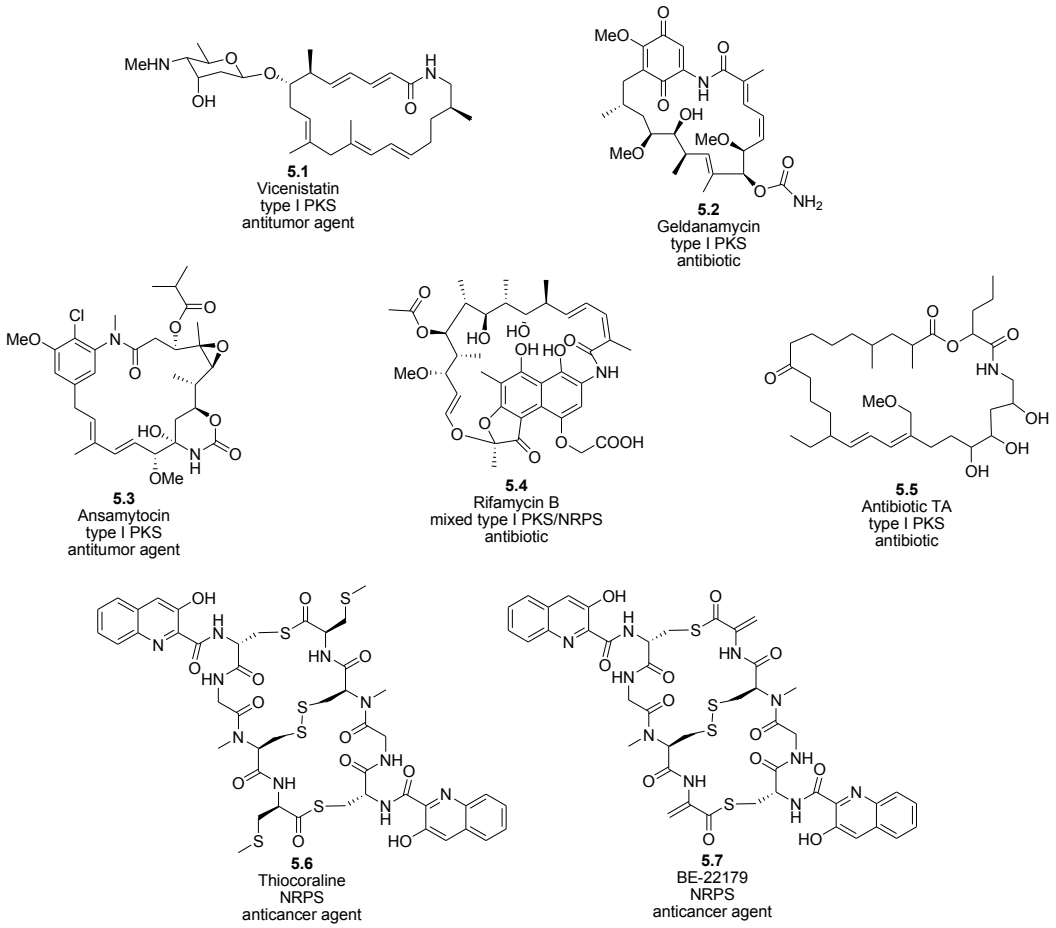


Figure 5.1 Different amides and thioesters from PKSs and NRPSs found in nature.

In contrast to macrolactam formation, very few PKSs or NRPSs produce thioesters. In fact, to our knowledge, there are only two thioester compounds, thiocoraline and BE-22179, that are formed by a NRPS, and none formed by PKSs (Figure 5.1).[5-8] Both these cyclic thiodepsipeptides possess potent antitumor properties. Since both these thioester and macrolactam compounds often possess such appealing biological properties, it would be a great tool if current macrolactone forming PKS TEs could also macrocyclize lactams and thioesters to further diversify combinatorial biosynthesis libraries. This could in turn lead to novel drug discovery.

Very little is known about the ability of TEs to utilize different nucleophiles, such as alcohols, amines and thiols. Preliminary studies by our group probed zea TE's ability to catalyze the cross coupling of different nucleophiles. We were able to demonstrate the successful cross coupling of alcohol and amines into their corresponding esters and amides, and unlike previous bacterial TEs, without any detectable level of the carboxylic acid product from hydrolysis. This observation suggested to us that it may be possible for the resorcylic acid lactones to generate macrolactams as well as macrolactones.

Toward this end, herein we investigate the ability of the zea TE and rad TE to catalyze macrolactamization and macrothiolactonization. If these TEs are shown to have sufficiently broad substrate tolerance that they can macrolactamize or macrothiolactonize substrates, these TEs will likely become useful tools in organic synthesis. Furthermore, they could potentially be used to engineer the epothilone biosynthetic pathway to access ixabepilone (Figure 5.2).[9-12] Ixabepilone is a recently FDA approved anticancer pharmaceutical and as the macrolactam analog of epothilone B, it is currently generated semi-synthetically from the natural product.

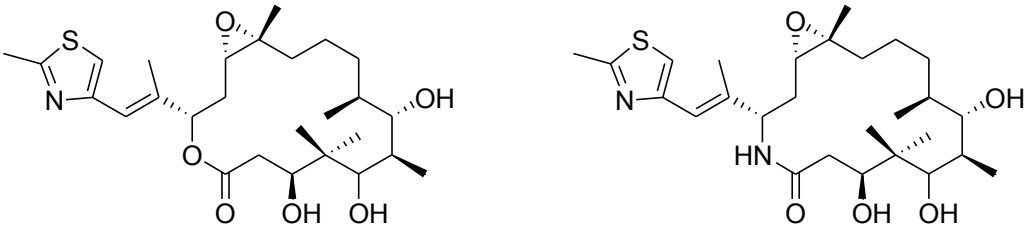


Figure 5.2 Epothilone B and ixabepilone, two anticancer agents.

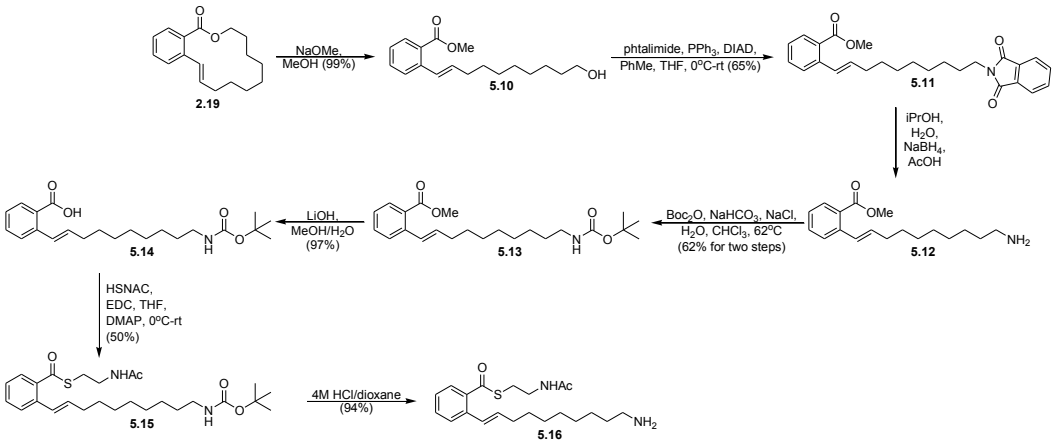
5.2 Results and discussion

Amide and thioester analogues of zearalenone were synthesized and probed for in vitro cyclization by both rad TE and zea TE. If the TEs display broad substrate tolerance, then cyclization should occur. However, if the reaction is slow, or if it shows high substrate specificity to the native substrate, then either no reaction will

occur or hydrolysis will be observed. To test these hypotheses, synthetic substrates of varying nucleophiles were incubated with recombinant purified zea TE and rad TE and the formation of macrocyclic products were monitored by LC/MS/MS.

5.2.1 Enzymatic cyclization to form lactams

The amine analogue was synthesized following the route shown in Scheme 5.1. First, the compound was constructed from the 14-member ring-closing metathesis product that we had previously synthesized. The 14-member ring was opened using sodium methoxide and methanol in quantitative yields.



Scheme 5.1 Reaction sequence to build the amine SNAC analogue.

Several methods were attempted to introduce a nitrogen group and reduce it to the amide. The most reliable method turned out to be by using phtalimide and mixing with DIAD, triphenylphosphine in toluene which gave the desired phtalimindo product in 65% yield.[13] The amine was then accessed using a mild modification of Gabriel synthesis conditions and protected as a Boc group without further purification in 65% for two steps.[14, 15] The desired product, **5.11**, and the Boc reagent had similar polarities during column chromatography which accounts for the lower yield.

The next steps were methyl ester hydrolysis with lithium hydroxide in methanol and water in 97% yield, followed by SNAC coupling with EDC and DMAP in 50% yield. The last step of the synthesis was removal of the Boc group, using hydrochloric acid in dioxane.[16] These conditions selectively hydrolyzed the Boc group without attacking the thioester. The desired product was obtained in 94% yield.

With the amine analogue in hand we investigated the ability of zea TE and rad TE to form amides. The SNAC substrate was mixed with recombinant purified zea and rad TEs for one hour. The reaction mixtures were then analyzed by LC/MS/MS (Figure 5.3). Zea TE showed lactam formation as the main product formed, with little hydrolysis product and trace starting material after just 1 hour. Zea TE also showed amide formation as the main product, but had equal amounts of starting material and hydrolysis product in the reaction mixture.

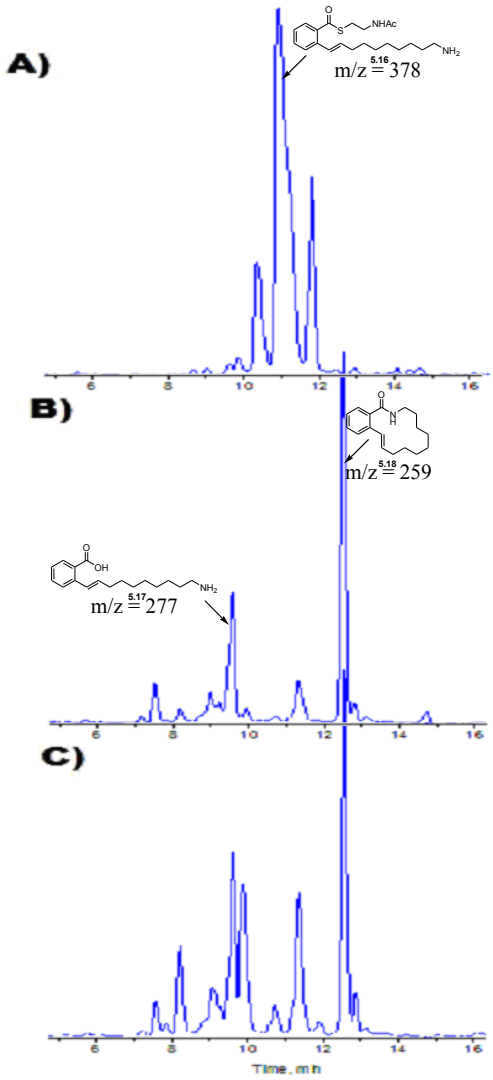


Figure 5.3 LC/MS/MS analysis of macrocyclization of the amine substrate. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **5.14**. B) LC/MS/MS analysis of a 1 hour incubation of 2 μM zea TE with 5 mM **5.14** at pH 7.4, 23°C. C) LC/MS/MS analysis of a 1 hour incubation of 2 μM rad TE with 5 mM **5.14** at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

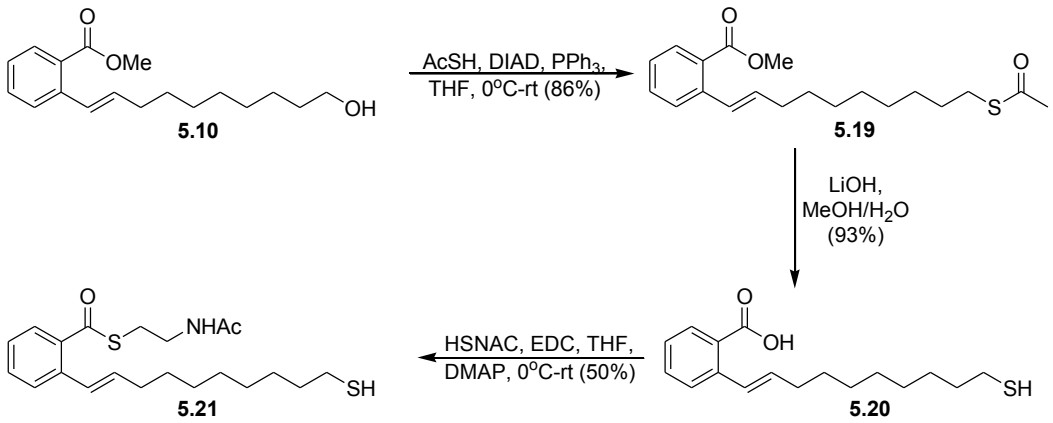
Both zea TE and rad TE have the ability to cyclize amides. The reaction seems to occur quickly since with low enzyme concentrations in just an hour almost

no starting material is present with incubation with zea TE. Since there is presence of hydrolysis, the reaction does not seem to be as selective as it is to form esters, the more native-like product.

The ability of both zea TE and rad TE to cyclize amides could be very useful in biosynthetic library generation. It would be useful to perform similar studies on other PKS TEs, both bacterial and fungal. These studies could determine if broad substrate tolerance for amines are selective only to RAL PKSs, or if they apply to other PKSs as well. If proven that only zea and rad TEs have broad substrate tolerance for differing nucleophiles, then they would be even more powerful tools in chemoenzymatic chemistry. However, metabolic engineering of the enzyme might be needed to suppress hydrolysis before they become useful tools as chemoenzymes.

5.2.2 Enzymatic cyclization to form thioesters

Since zea and rad TEs can form amide bonds, the ability of these TEs to form other nucleophilic bonds was investigated. To probe if zea TE and rad TE could form thioester bonds, the thiol analogue first needed to be synthesized (Scheme 5.2). Analogous to the amine synthesis, the route followed the 14-member SNAC synthesis up to the ring closing metathesis reaction. Once the 14-member ester was formed, the ring was opened using sodium methoxide in methanol to form the methyl ester product.



Scheme 5.2 Reaction sequence to build the thiol SNAC analogue.

Introduction of the sulphur moiety was achieved with thioacetic acid via a Mitsunobu reaction. Mixing the alcohol with thioacetic acid, DIAD and triphenylphosphine achieved the desired product in 86% yield. Hydrolysis with lithium hydroxide in methanol and water gave both the thiol and the carboxylic acid moieties. The last step of the synthesis was SNAC coupling with EDC and DMAP. To favour SNAC coupling, and reduce intramolecular thioester formation, excess SNAC was added in minimal amount of solvent. This gave 50% yield, with no detectable 14-member macrocycle.

With the thiol analogue in hand we investigated the ability of zea TE and rad TE to form amides. The SNAC substrate was mixed with recombinant purified zea and rad TEs for 24 hours. The reaction mixtures were then analyzed by LC/MS/MS. However, only starting material was present. Checking the substrate by LC/MS/MS, it was found that it had dimerized to the disulfide in solution. To break the disulfide bond, tris(2-carboxyethyl)phosphine (TCEP) was added to the enzymatic reaction and the substrate and TE were mixed for 24 hours before being analyzed by LC/MS/MS (Figure 5.4).

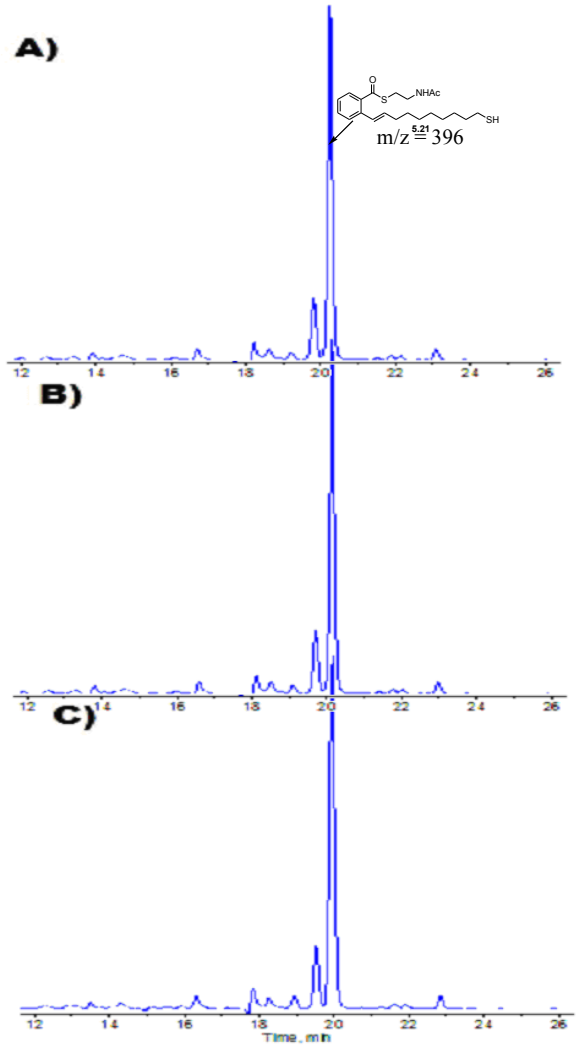


Figure 5.4 LC/MS/MS analysis of macrocyclization the thiol substrate. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). A) Standard of SNAC substrate **5.19**. B) LC/MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **5.19** and TCEP at pH 7.4, 23°C. C) LC/MS/MS analysis of a 24 hours incubation of 2 μM rad TE with 5 mM **5.19** and TCEP at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

With both zea TE and rad TE incubations, only starting material was present after 24 hours. By LC/MS/MS analysis, TCEP had successfully broken the disulfide

bond of the starting material. However, it was unclear if TCEP had also reacted and deactivated the enzyme, or if the thiol is not an active nucleophile for the TEs.

To test if TCEP affected the enzyme, a reaction with TCEP, zea TE and the 14-member SNAC alcohol analogue **2.21** was performed. 14-member SNAC alcohols have been shown previously to be an extremely fast reaction, where after only one hour 14-membered macrocycle is produced exclusively. If TCEP does not affect the enzymatic reaction, then we expect to see only cyclized product in the reaction mixture. If TCEP affect the enzymatic reaction however, only SNAC starting material should be present. After 24 hours of incubation with zea TE, TCEP and **2.21**, LC/MS/MS analysis was performed (Figure 5.5).

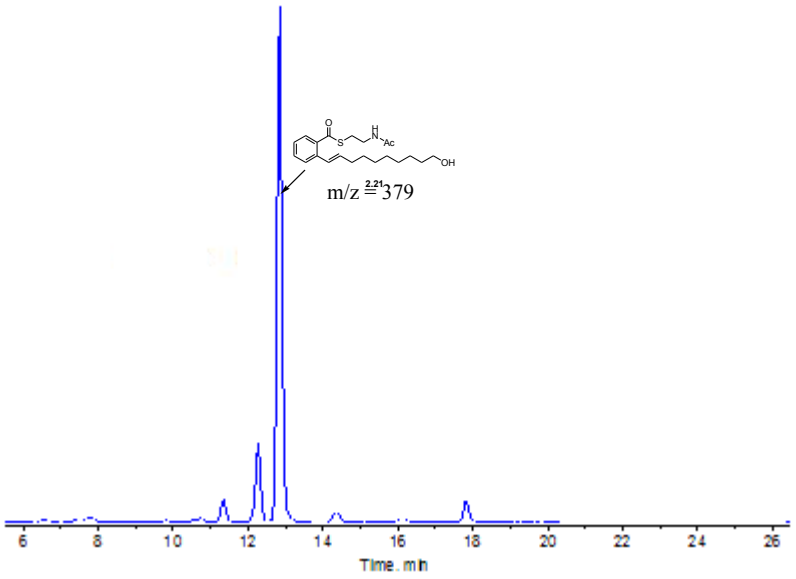


Figure 5.5 LC/MS/MS analysis of zea TE to catalyze macrocyclization of 14-member rings with TCEP present. Chromatograms are absorbance at 210 nm. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/5% MeCN/H_2O; B: 0.05% formic acid/95% MeCN/H_2O). LC/MS/MS analysis of a 24 hours incubation of 2 μM zea TE with 5 mM **2.21** and TCEP at pH 7.4, 23°C. Identity of by-products were determined by ESI-MS analysis.

After 24 hours incubation with zea TE and TCEP, only starting material was detectable by LC/MS/MS. Since we know that 14-member macrocycle are exclusively produced by zea TE after only one hour, this shows that TCEP hinders TE activity. These results show that we are still unsure whether 14-member thioesters can be catalyzed by zea TE or rad TE.

It is possible, since sulphur groups are softer than oxygen, that the TE would not be able to catalyze their macrocyclization. However, since there is evidence of thioesters catalyzed by NRPS TEs in nature, at least some TEs have the ability to do so. Additionally, since both zea and rad TEs are able to cyclize 14-member amides, and react with a variety of amines, it is possible that they could also macrocyclize other nucleophiles.

To test if zea TE or rad TE has the ability to macrocyclize thioesters, additional experiments need to be performed. Other reducing agents that could break disulfide bonds could be tried out to see if they hinder TE activity. Additionally, TCEP could be added to the disulfide, isolated and purified, and the free thiol could be immediately incubated with the TEs to reduce the chance of disulfide bond formation.

5.3 Conclusions

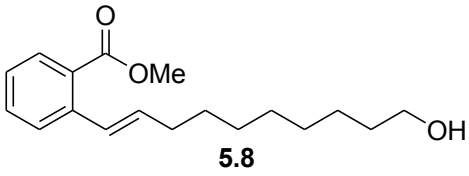
In conclusion, both zea TE and rad TE have the ability to form amide bonds, although hydrolysis is also a side product. More experiments are needed to probe the enzymes ability to form thioesters. Since these are the first experiments done on PKSs by varying nucleophiles, similar experiments done on bacterial and other fungal PKSs and NRPSs could prove very informative.

5.4 Experimental section

5.4.1 General methods

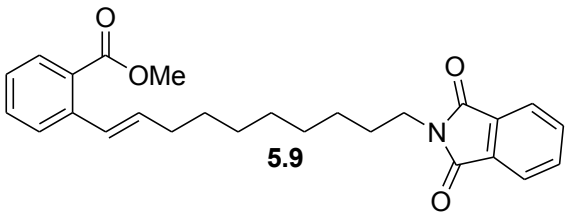
All reactions were carried out under nitrogen or argon atmosphere with dry solvents under anhydrous conditions in oven-dried glassware equipped with a magnetic stir bar and rubber spectrum. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reagents were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light (254 nm) as a visualizing agent and cerium molybdate stain and heat as developing agent. E. Merck silica gel 60 (230-400 mesh) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254). IR spectra were collected with neat thin films on sodium chloride disk using a Bomem Michaelson 100 Fourier transform infrared (FTIR) spectrometer. ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, NOSEY, and COSY spectra were recorded on a Brucker AVANCE 300 MHz or 400 MHz spectrometer. Deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) were used as NMR solvents unless otherwise noted. All chemical shifts are reported in parts per million (δ), integration and coupling constant(s) in Hz downfield from TMS and corrected using the solvent residual peak as internal standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, b = broad, br s = broad singlet. High resolution mass spectra (HRMS) were collected by positive ion electrospray on Kratos Analytical Concept-11A mass spectrometer with an electron beam of 70ev (Ottawa-Carleton Mass Spectrometry Centre). LC/MS/MS was conducted with an Applied Biosystems API2000 triple quadrupole mass spectrometer using electrospray ionization and a Hypersil C_{18} 100 × 2.1 mm, 3 μM particle size column.

5.4.2 Experimental procedures



5.8

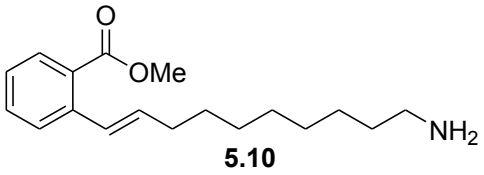
(E)-methyl-2-(10-hydroxydecenyl)benzoate (5.8). To **2.19** (101 mg, 0.39 mmol, 1 equiv.) in methanol (2 mL) under argon atmosphere was added sodium methoxide (32 mg, 0.59 mmol, 1.5 equiv.). The resulting mixture was stirred at room temperature for 18 hours. The reaction was acidified with 10% HCl solution to pH 3 and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 30% ethyl acetate in hexanes) to afford a colourless oil (112.6 mg, 0.39 mmol, 99%); **5.8**: Rf = 0.20 (silica gel, 4:1 hexanes/EtOAc); IR (NaCl) νmax = 3394 (br), 2923, 2854, 1712, 1257 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.81 (dd, *J* = 7.9, 1.2 Hz, 1 H), 7.51 (d, *J* = 7.8 Hz, 1 H), 7.41 (ddd, *J* = 7.7, 7.5, 0.8 Hz, 1 H), 7.22 (ddd, *J* = 7.8, 7.4, 0.9 Hz, 1 H), 7.10 (d, *J* = 15.7 Hz, 1 H), 6.11 (dt, *J* = 15.7, 6.9 Hz, 1 H), 3.87 (s, 3 H), 3.61 (t, *J* = 6.6 Hz, 2 H), 2.26-2.19 (m, 2 H), 1.58-1.52 (m, 2 H), 1.51-1.42 (m, 4 H), 1.38-1.28 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 139.7, 134.1, 131.9, 130.3, 128.4, 128.1, 127.2, 126.5, 63.0, 52.0, 33.2, 32.8, 29.4, 29.4, 29.2, 29.1, 25.7; HRMS (+EI) : calcd for $C_{18}H_{26}O_3$ (M) 290.1882, obsd 290.1867.



5.9

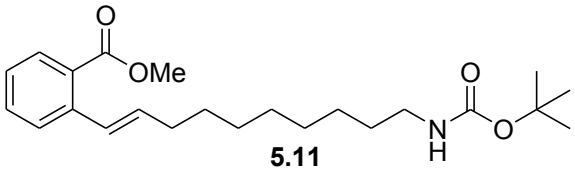
(E)-methyl-2-(10-phtalimidedecenyl)benzoate (5.9). To **5.8** (112 mg, 0.39 mmol, 1 equiv.) dissolved in THF (1.5 mL) was added phtalimide (57 mg, 0.39 mmol, 1 equiv.) and triphenylphosphine (153 mg, 0.59 mmol, 1.5 equiv.). The solution was cooled to 0°C. Diisopropyl azodicarboxylate (116 μL, 0.59 mmol, 1.5 equiv.) was dissolved in toluene (290 μL) and added to the

previous solution at 0°C. The resulting mixture was slowly put to room temperature and was stirred for 18 hours. The mixture was concentrated to dryness in vacuo and purified by column chromatography (silica gel, 5% ethyl acetate in hexanes) to afford a yellow oil (105.4 mg, 0.25 mmol, 65%). **5.9**: Rf = 0.38 (silica gel, 9:1 hexanes/EtOAc); IR (NaCl) νmax = 2926, 2855, 1768, 1717, 1445, 1386, 1248 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.81-7.75 (m, 3 H), 7.65 (dd, *J* = 5.6, 3.1 Hz, 2 H), 7.49 (d, *J* = 8.0 Hz, 1 H), 7.38 (ddd, *J* = 7.7, 7.6, 1.1 Hz, 1 H), 7.19 (ddd, *J* = 7.7, 7.6, 1.3 Hz, 1 H), 7.08 (d, *J* = 15.9 Hz, 1 H), 6.08 (dt, *J* = 15.6, 6.9 Hz, 1 H), 3.85 (s, 3 H), 3.63 (t, *J* = 7.3 Hz, 2 H), 2.23-2.15 (m, 2 H), 1.67-1.59 (m, 2 H), 1.47-1.39 (m, 2 H), 1.34-1.24 (m, 8 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 168.0, 139.6, 134.1, 134.0, 133.8, 132.1, 131.9, 130.2, 128.4, 127.1, 126.4, 123.1, 69.8, 51.9, 38.0, 33.1, 29.3, 29.1, 28.5, 26.8, 21.9; HRMS (+EI) : calcd for $C_{26}H_{29}NO_4$ (M) 419.2096, obsd 419.2066.



5.10

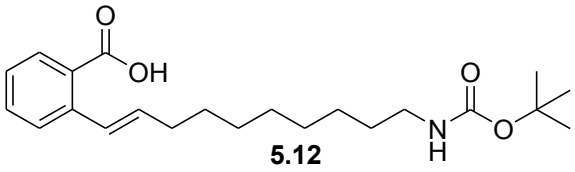
(E)-methyl-2-(10-aminodecenyl)benzoate (5.10). To **5.9** (101 mg, 0.24 mmol, 1 equiv.) in 2-propanol (2 mL) and H_2O (364 μL) at room temperature was added sodium borohydride (45 mg, 1.20 mmol, 5.0 equiv.). The resulting mixture was stirred for 24 hours at room temperature under argon atmosphere. Acetic acid (250 μL) was added and the solution was heated at 80°C for 2 hours. The reaction was neutralized with a saturated $NaHCO_3$ solution and extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$, filtered, and concentrated to dryness in vacuo and carried over to the next step without further purification.



5.11

(E)-methyl-2-(10-*tert*-butoxycarbonylamino decenyl)benzoate (5.11). To **5.10** (69 mg, 0.24 mmol, 1 equiv.) in H_2O (1 mL) was added sodium bicarbonate (22 mg, 0.26 mmol, 1.1 equiv.) and the reaction

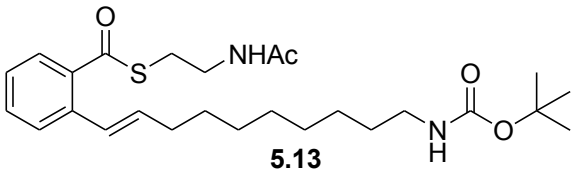
mixture was stirred for 5 minutes. Sodium chloride (46 mg, 0.79 mmol, 3.3 equiv.) and chloroform (1.1 mL) were added and the resulting mixture was stirred for an additional 5 minutes before the addition of Boc_2O (236 mg, 1.1 mmol, 2.0 equiv.). The resulting mixture was stirred at 62°C for 18 hours. The reaction was put to room temperature and extracted with EtOAc (3 × 15 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 15% ethyl acetate in hexanes) to afford a yellow oil (59.7 mg, 0.15 mmol, yield for two steps 62%). **5.11**: Rf = 0.49 (silica gel, 4:1 hexanes/EtOAc); IR (NaCl) νmax = 3376, 2926, 2855, 1739, 1707 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 (dd, *J* = 8.1, 1.5 Hz, 2 H), 7.51 (ddd, *J* = 7.5, 0.5, 0.5 Hz, 1 H), 7.40 (dddd, *J* = 8.0, 7.5, 1.5, 0.7 Hz, 1 H), 7.22 (ddd, *J* = 7.5, 7.5, 1.3 Hz, 1 H), 7.09 (d, *J* = 15.7 Hz, 1 H), 6.10 (dt, *J* = 15.7, 7.0 Hz, 1 H), 3.86 (s, 3 H), 3.07 (dt, *J* = 6.5, 6.2 Hz, 2 H), 2.26-2.17 (m, 2 H), 1.50-1.37 (m, 17 H), 1.36-1.17 (m, 4 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ168.1, 156.0, 139.7, 134.0, 131.9, 130.3, 128.4, 128.1, 127.2, 126.4, 52.0, 33.2, 30.9, 30.1, 29.4, 29.3, 29.3, 29.2, 29.1, 28.4, 26.8; HRMS (+EI) : calcd for $C_{18}H_{26}NO_2$ (M-$CO_2C_4H_9$) 288.1964, obsd 288.1935.



5.12

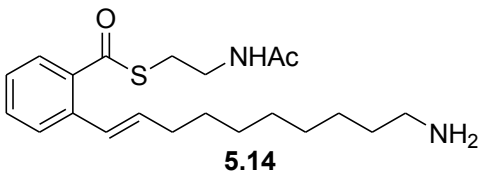
(E)-2-(10-(tert-butoxycarbonylamino) decenyl)benzoic acid (): 5.11 (58 mg, 0.14 mmol, 1 equiv.) was dissolved in methanol (6 mL) before the addition of lithium hydroxide monohydrate (56 mg, 1.33 mmol, 9.5 equiv.) and water (2 ml). The reaction mixture was stirred at room temperature for 16 hours. The reaction was brought to pH 2 with a 1M HCl solution and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (50 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 10% acetone in hexanes) to afford a yellow oil (52.1 mg, 0.14 mmol, 97%). **7**: Rf = 0.58 (silica gel, 3:2 hexanes/acetone); IR (NaCl) νmax = 3750, 3336 (br), 2927, 2850, 1699, 1253, 1169 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.6 Hz, 1 H), 7.49-7.38 (m, 2

H), (7.29-7.19 (m, 1 H), 7.10 (d, J = 15.8 Hz, 1 H), 6.86 (bs, 1 H), 5.99 (dt, J = 15.6, 6.8 Hz, 1 H), 3.18-3.04 (m, 2 H), 2.28-2.18 (m, 2H), 1.53-1.38 (m, 21 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 158.6, 140.9, 134.0, 132.5, 132.1, 130.8, 130.7, 127.8, 126.5, 41.1, 32.1, 30.0, 29.7, 29.2, 29.0, 28.8, 28.4, 28.0, 25.7; HRMS (+EI) : calcd for $C_{17}H_{24}NO_2$ (M) 274.1807, obsd 274.1812.



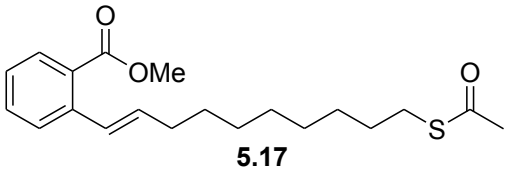
5.13

(E)-S-2-acetamidoethyl 2-(10-(tert-butoxycarbonylamino)dec-1-enyl)benzothioate (5.13): To **5.12** (52 mg, 0.14 mmol, 1 equiv) in THF (3 mL) at 0°C under argon atmosphere was added N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (81 mg, 0.42 mmol, 3.0 equiv.) and 4-(dimethylamino)pyridine (3 mg, 0.03 mmol, 0.2 equiv.) before the addition of N-acetylcysteamine (39 μL, 0.36 mmol, 2.6 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated NH_4Cl (10 mL) solution and extracted with EtOAc (3 × 10 mL). A saturated $NaHCO_3$ solution (15 mL) was then added to the organic layer and extracted with EtOAc (3 × 10 mL). The resulting organic layers were combined and washed with brine (20 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 40% acetone in hexanes) to afford a colourless oil (32.8 mg, 0.07 mmol, 50%). **5.13**: R_f = 0.57 (silica gel, 45% acetone in hexanes); IR (NaCl) ν_{max} = 3311 (br), 3074, 2925, 2854, 1699, 1667, 1167, 1096 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, J = 7.8, 1.2 Hz, 1 H), 7.54 (d, J = 7.8 Hz, 1 H), 7.42 (ddd, J = 8.0, 7.4, 1.2 Hz, 1 H), 7.25 (ddd, J = 7.5, 7.4, 1.2 Hz, 1 H), 6.76 (d, J = 15.4 Hz, 1 H), 6.17 (dt, J = 15.9, 6.4 Hz, 1 H), 5.97 (bs, 1 H), 4.49 (bs, 1 H), 3.53 (dt, J = 6.2 Hz, 2 H), 3.19 (t, J = 6.5 Hz, 2 H), 3.12-3.04 (m, 2 H), 2.26-2.16 (m, 2 H), 1.97 (s, 3 H), 1.65-1.54 (m, 2 H), 1.50-1.37 (m, 19 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.7, 170.3, 156.5, 144.5, 136.7, 134.8, 131.9, 128.4, 127.1, 127.0, 126.6, 39.7, 33.1, 30.0, 29.7, 29.6, 29.3, 29.2, 29.1, 29.0, 28.4, 26.7, 23.2; HRMS (+EI) : calcd for $C_{17}H_{23}NO$ (M-SNAC-$CO_2C_4H_9$) 257.1780, obsd 257.1789.



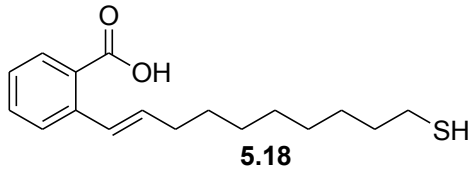
5.14

(E)-S-2-acetamidoethyl 2-(10-aminodecenyl)benzothioate (5.14): 5.13 (32 mg, 0.067 mmol, 1 equiv) was dissolved in a 4M HCl:dioxane solution (168 μL). The resulting mixture was stirred for 45 minutes. The solution was concentrated to dryness in vacuo to afford a colourless oil (23 mg, 0.063 mmol, 94%) **5.14**: R_f = 0.06 (silica gel, 45% acetone in hexanes); IR (NaCl) ν_{max} = 3474, 3413, 2921, 2855, 1640, 1443, 1199, 1083 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.67 (dd, *J* = 7.8, 1.1 Hz, 1 H), 7.58 (d, *J* = 7.7 Hz, 1 H), 7.45 (dd, *J* = 8.2, 8.1 Hz, 1 H), 7.28 (dd, *J* = 7.4, 7.2 Hz, 1 H), 6.73 (d, *J* = 15.6 Hz, 1 H), 6.20 (dt, *J* = 15.6, 7.0 Hz, 1 H), 3.72-3.63 (m, 2 H), 3.58-3.49 (m, 2 H), 3.42 (t, *J* = 6.6 Hz, 2 H), 3.16 (t, *J* = 6.5 Hz, 2 H), 2.92-2.83 (m, 2 H), 2.24-2.14 (m, 2 H), 1.93 (s, 3 H), 1.79-1.67 (m, 2 H), 1.67-1.43 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 188.2, 172.2, 136.3, 134.0, 133.7, 131.4, 129.4, 127.8, 126.9, 126.3, 61.7, 59.6, 39.6, 38.7, 32.7, 31.2, 29.7, 25.9, 22.2, 18.7, 13.1; HRMS (+EI) : calcd for $C_{17}H_{24}NO$ (M-$SC_4H_8NHCOCH_3$) 258.1858, obsd 258.1826.



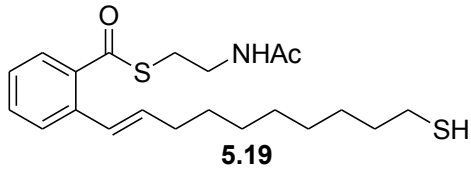
5.17

(E)-2-(10-(acetylthio)decenyl)benzoate (5.17): To triphenylphosphine (41 mg, 0.16 mmol, 1.2 equiv.) in THF (361 μL) was added diisopropyl azodicarboxylate (64 μL, 0.33 mmol, 2.5 equiv). The reaction mixture was stirred for 1 hour. Thioacetate (55 μL, 0.33 mmol, 2.5 equiv.) and **5.8** (38 mg, 0.13 mmol, 1.0 equiv.) was dissolved in THF (245 μL) and added to the previous mixture at 0°C. The resulting mixture was stirred for 18 hours. The solvent was then evaporated to dryness in vacuo. The mixture was dissolved in EtOAc and filtered through silica before being carried over to the next step without further purification. **4:** (38.8 mg, 0.11 mmol, 86 %) as a colourless oil. **7**: Rf = 0.55 (silica gel, 1:1 hexanes/EtOAc).



5.18

(E)-2-(10-mercaptodecenyl)benzoic acid (5.18): 5.17 (33 mg, 0.09 mmol, 1 equiv.) was dissolved in methanol (6 mL) before the addition of lithium hydroxide monohydrate (36 mg, 0.86 mmol, 9.5 equiv.) in H_2O (2 mL). The resulting mixture was stirred at room temperature for 18 hours. The reaction was acidified to pH 4 with 10% HCl solution and extracted with EtOAc (3 × 15 mL). The resulting organic layers were combined and washed with brine (20 mL), dried over $MgSO_4$, filtered, concentrated to dryness in vacuo and purified by column chromatography (silica gel, 30% ethyl acetate in hexanes) to afford a colourless oil (25.6 mg, 0.09 mmol, 93%). **5.18**: Rf = 0.53 (silica gel, 1:1 hexanes/EtOAc); IR (NaCl) νmax = 3861 (br), 3741, 2923, 2854, 1691, 1255, 1087 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.7 Hz, 1 H), 7.54 (d, *J* = 7.8 Hz, 1 H), 7.45 (dd, *J* = 7.5 Hz, 1 H), 7.31-7. 16 (m, 2 H), 6.14 (dt, *J* = 15.6, 6.9 Hz, 1 H), 2.66 (t, *J* = 7.2 Hz, 2 H), 2.29-2.18 (m, 2H), 1.70-1.58 (m, 2 H), 1.52-1.43 (m, 2H), 1.42-1.26 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 140.1, 134.2, 132.5, 131.1, 128.6, 128.4, 127.3, 126.5, 45.3, 39.6, 33.2, 29.7, 29.1, 29.0, 28.2, 8.5; HRMS (+EI) : calcd for $C_{17}H_{24}O_2S$ (M) 292.1497, obsd 292.1469.



5.19

2-(10-mercaptodecenyl)-thiobenzoic acid *S*-(2-acetylamino-ethyl) ester (5.19) To **5.18** (20 mg, 0.07 mmol, 1 equiv) in THF (1 mL) at 0°C under argon atmosphere was added N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (39 mg, 0.21 mmol, 3.0 equiv.) and 4-(dimethylamino)pyridine (2 mg, 0.014 mmol, 0.2 equiv.) before the addition of N-acetylcysteamine (37 μL, 0.35 mmol, 5.0 equiv.). The resulting mixture was slowly put to room temperature and stirred for 18 hours. The reaction was quenched with a saturated NH_4Cl (10 mL) solution and extracted with EtOAc (3 × 10 mL). A saturated $NaHCO_3$ solution (15 mL) was then added to the organic layer and extracted with EtOAc (3 × 10 mL). The resulting organic layers were combined and washed with brine (20 mL), dried over $MgSO_4$, filtered, concentrated to dryness in

vacuo and purified by column chromatography (silica gel, 40% acetone in hexanes) to afford a colourless oil (12.2 mg, 0.03 mmol, 44%). **5.19**: R_f = 0.53 (silica gel, 1:1 hexanes/acetone); IR (NaCl) ν_{max} = 3747, 3288 (br), 2919, 2853, 1655, 1286, 1087 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 7.9 Hz, 1 H), 7.54 (d, *J* = 7.9 Hz, 1 H), 7.42 (dd, *J* = 7.7, 7.4 Hz, 1 H), 7.30-7.21 (m, 1 H), 6.77 (d, *J* = 15.7 Hz, 1 H), 6.17 (dt, *J* = 15.7, 6.8 Hz, 1 H), 5.94 (bs, 1 H), 3.60-3.48 (m, 2 H), 3.19 (t, *J* = 6.4 Hz, 2 H), 2.77 (t, *J* = 6.2 Hz, 2 H), 2.72-2.62 (m, 2 H), 2.25-2.17 (m, 2 H), 1.97 (s, 3 H), 1.84-1.59 (m, 6 H), 1.58-1.41 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.7, 170.3, 136.6, 135.7, 134.8, 132.0, 128.4, 127.1, 127.0, 126.6, 39.8, 38.9, 38.0, 37.7, 33.1, 29.7, 29.3, 29.1, 29.0, 28.4, 23.3; HRMS (+EI) : calcd for $C_{16}H_{19}O$ (M-SNAC-SCH_4) 227.1436, obsd 227.1391.

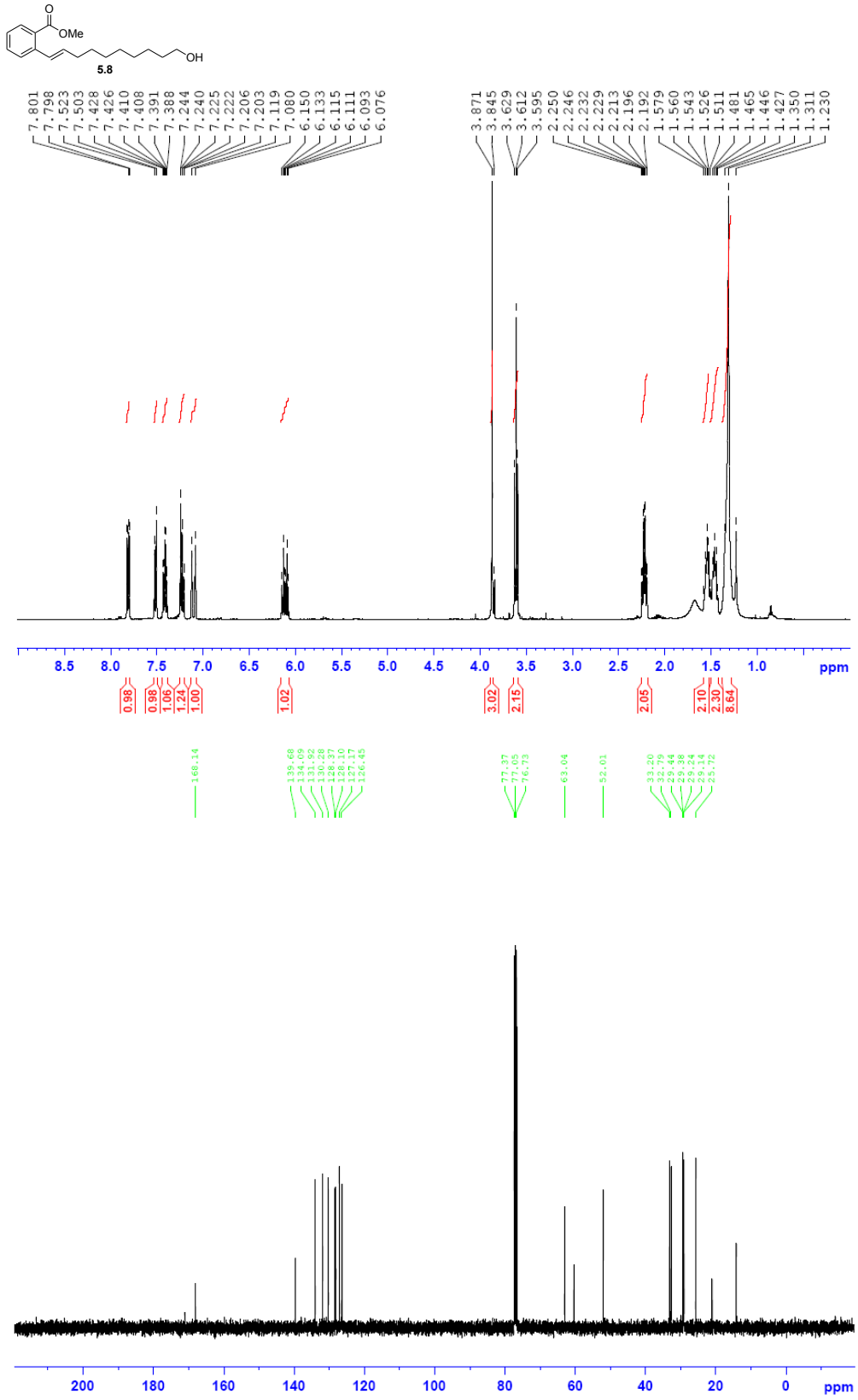
5.4.3 Enzymatic protocol

Assay of enzymatic catalyzed macrocyclization reaction

For a typical assay, a 20 μL solution containing 50 mM sodium phosphate pH 7.43, 5 mM thioester substrate, 2 μM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1 : 4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

For a typical assay with TCEP, a 20 μL solution containing 50 mM sodium phosphate pH 7.43, 4 mg TCEP, 5 mM thioester substrate **2.21**, 2 μM enzyme and 10% v/v DMSO. All reactions were quenched with MeCN (v/v = 1 : 4) prior to LC/MS/MS analysis. LC/MS/MS conditions: Flow rate 0.30 mL/min, gradient solvent of 20 to 100% B over 30 min. (A: 0.05% formic acid/H_2O; B: 0.05% formic acid/MeCN).

5.4.4 Spectra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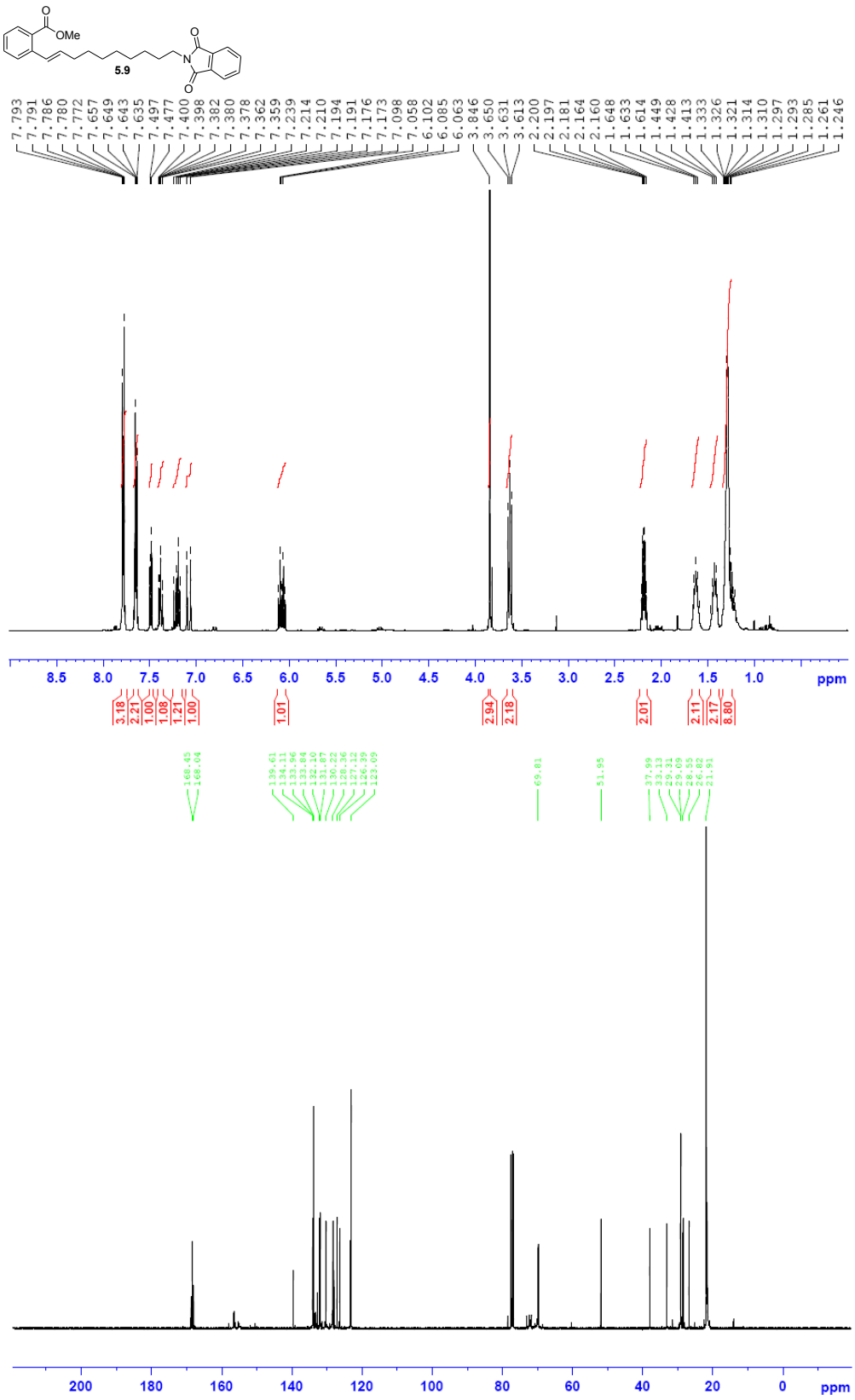
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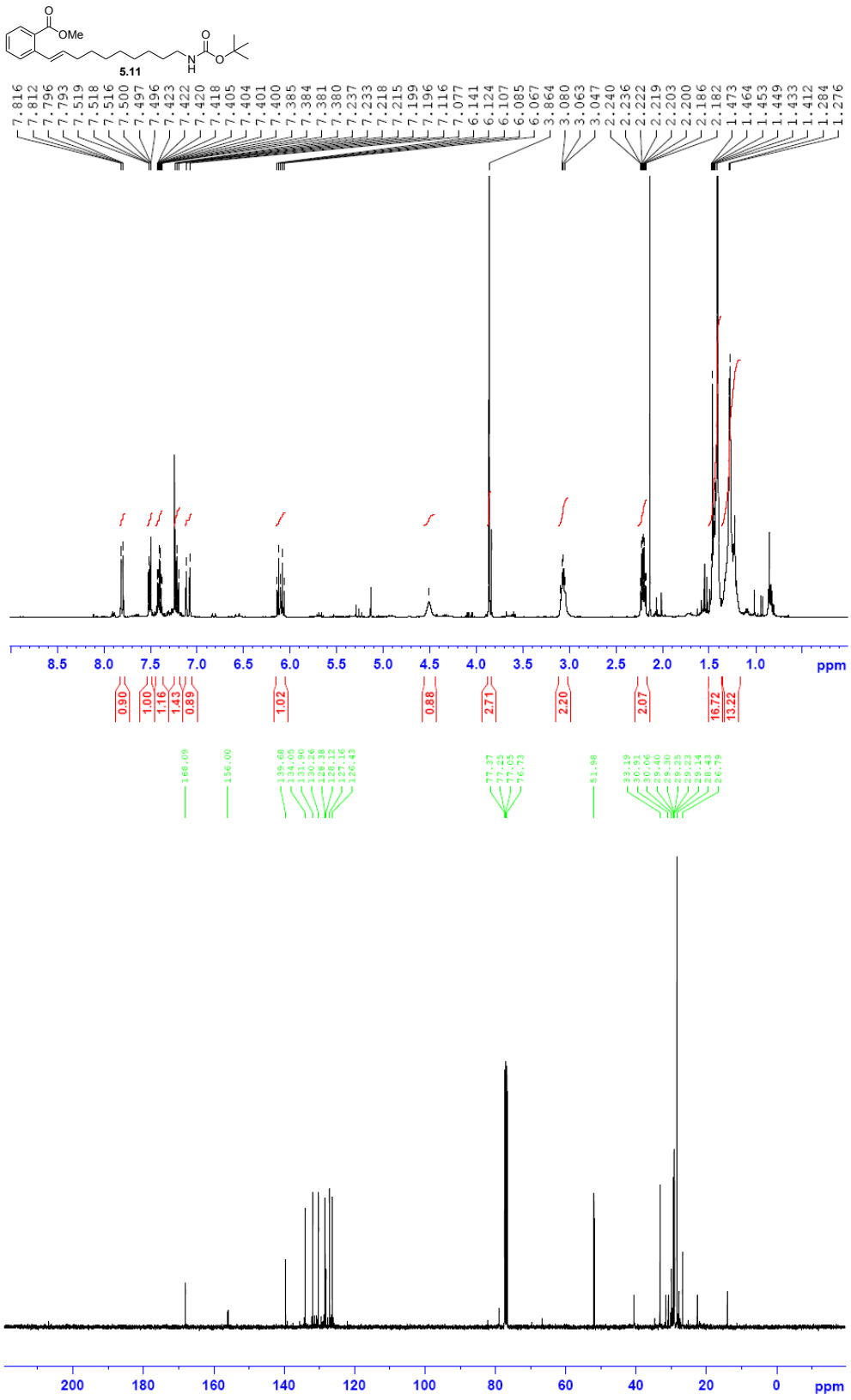
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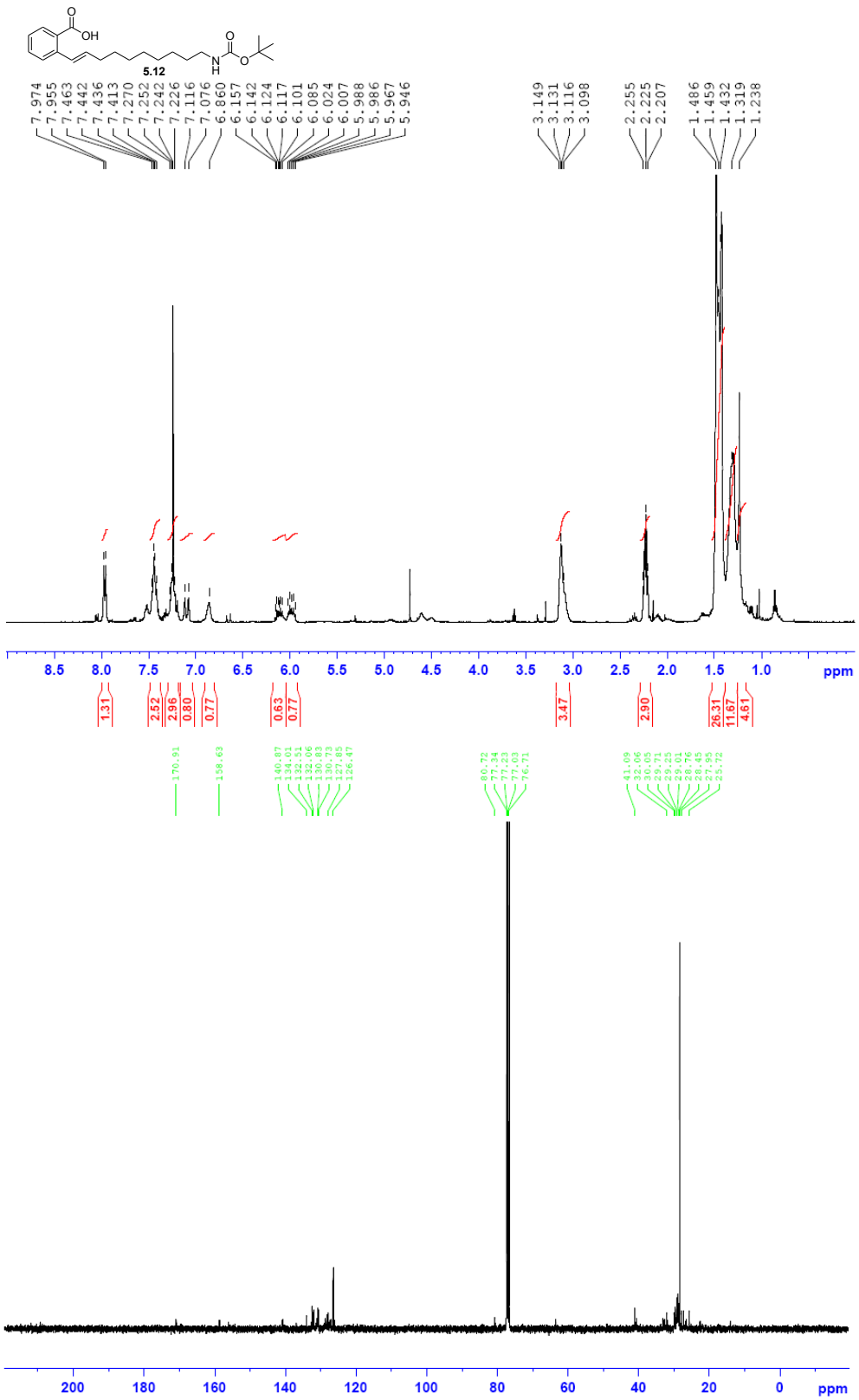
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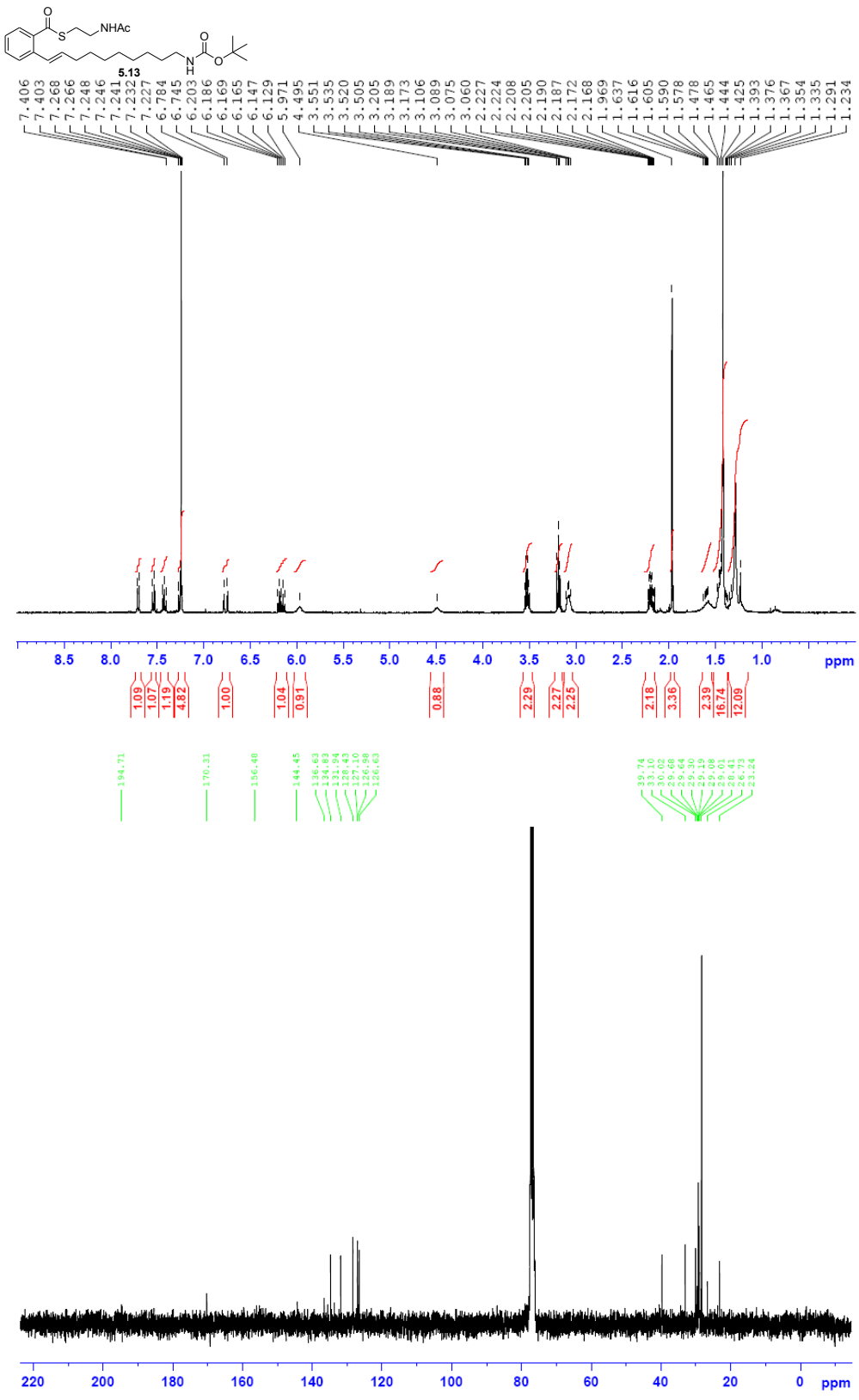
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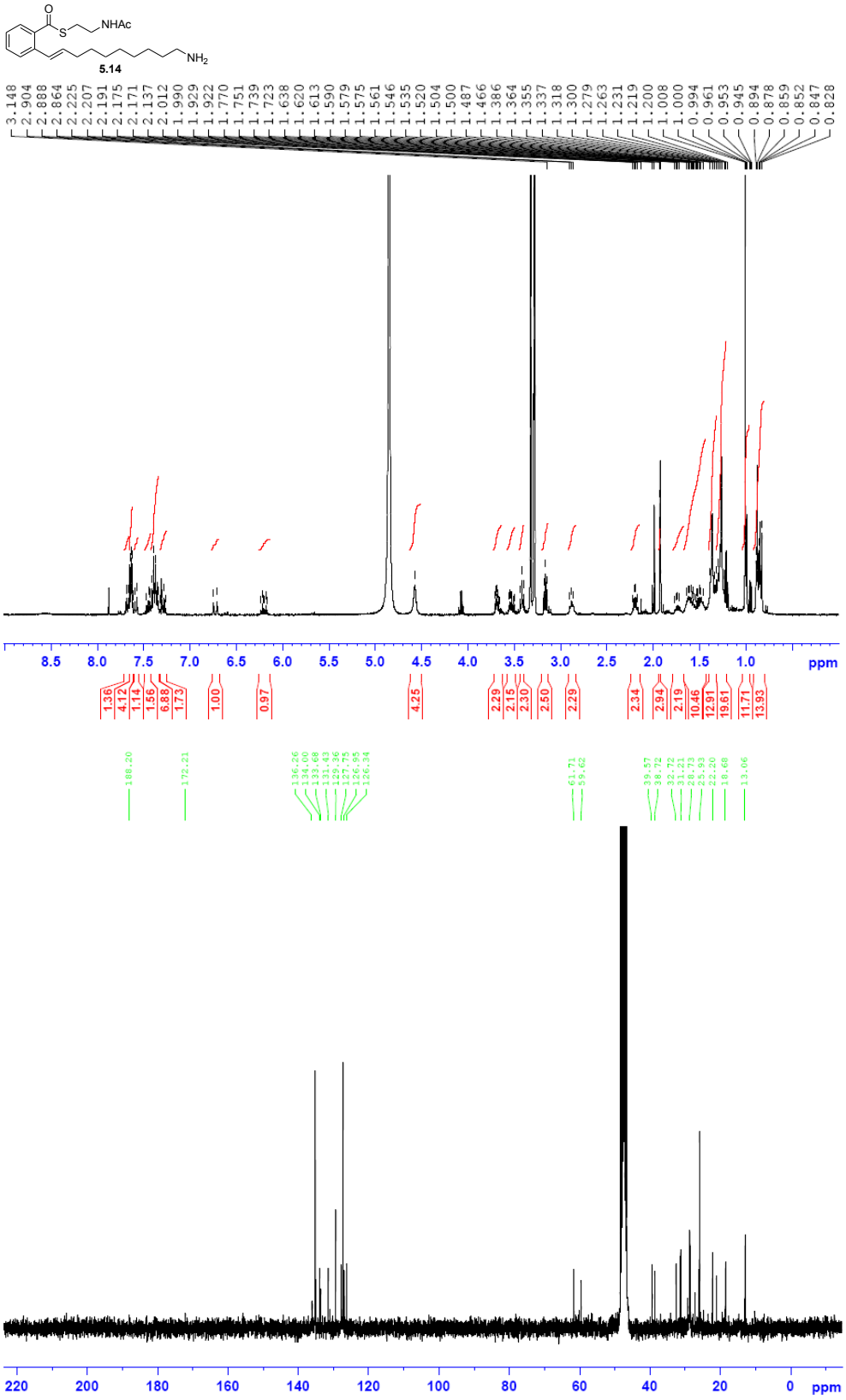
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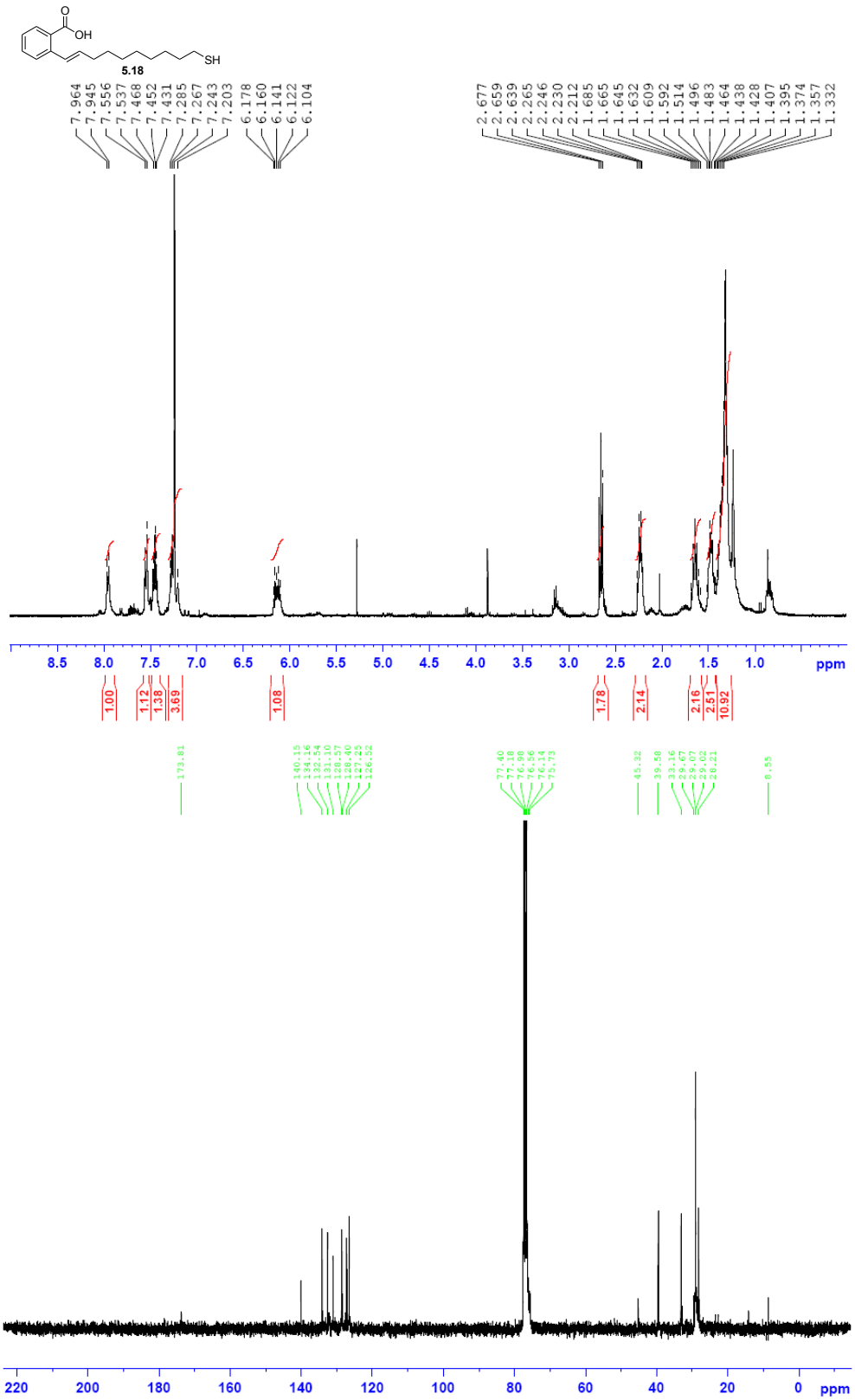
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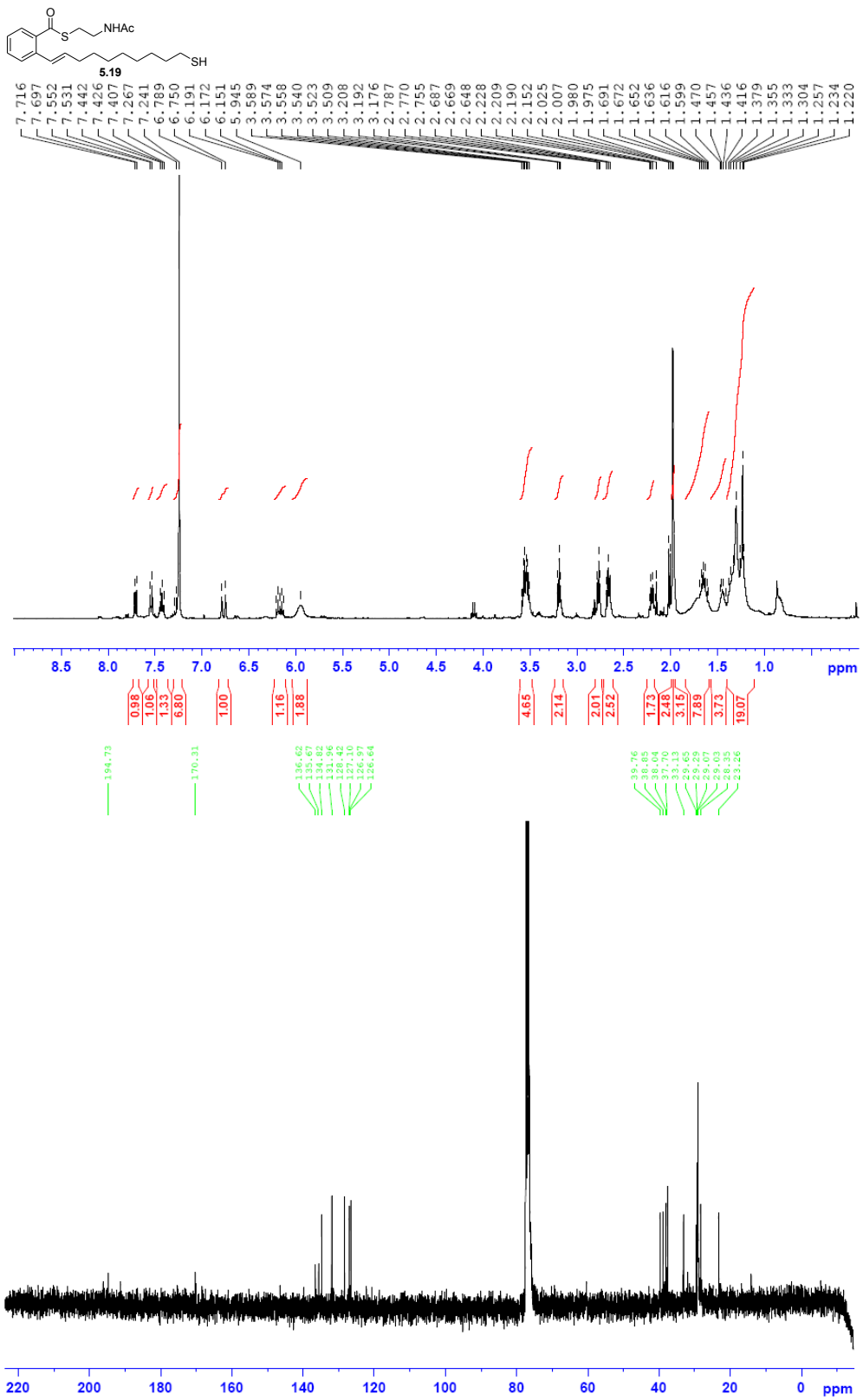
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220
200
180
160
140
120
100
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60
40
20
0
ppm

5.5 References

1. Hill, A. M., The biosynthesis, molecular genetics and enzymology of the polyketide-derived metabolites. *Natural Product Reports* **2006,** *23*, 256-320.
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Chapter 6 Conclusions and future work

6.1 Conclusions

In conclusion, rad TE and zea TE were found to be able to exclusively macrocyclize, without competing hydrolysis, a 14-member ring analogue of its natural product. In past experiments with other TEs, to counteract hydrolysis and favour esterification or macrocyclization, water molecules could not be present in the environment. The fact that these resorcylic acid lactone TEs can catalyze esterification in the presence of water suggests that they could be use chemoenzymatically for synthetic reactions.

Both zea and rad TEs were able to macrocyclize different ring sizes. The enzymes were able to exclusively macrocyclize 15- and 16-member rings. Rad TE seemed to macrocyclize smaller ring sizes more effectively than zea TE, where hydrolysis and macrocycle are the major products with very little glycerolysis product. With smaller ring sizes, incubation with zea TE led to glycerolysis as the major product with very little to no cyclization product. Adding non-ionic detergents did not increase macrocycle formation in either zea or rad TEs, but rather suppressed it. Zea TE on the other hand, macrocyclizes 18-member rings more effectively than rad TE. Zea TE macrocyclizes 18-member ring without competing hydrolysis, whereas rad TE catalyzes both hydrolysis and macrocycle products are present.

Both zea TE and rad TE are able to macrocyclize exclusively the racemic secondary alcohol substrate. However, macrocyclic rates seem to vary between enzymes and more work is needed to fully draw conclusions on their enantiomeric catalyzation capabilities.

Zea TE and rad TE are able to macrocyclize the formation of a 14-member amide, although hydrolysis is present as a by-product. More work is needed to understand the ability of both TEs to form 14-member thioesters. These are, to our

knowledge the first experiments on PKSs where different nucleophiles are studied for macrocyclization.

Overall, both zea and rad TEs display surprisingly broad substrate tolerance, far greater than bacterial TEs studied to date. This work solidifies that the RAL TEs are one of the most broadly tolerant TEs yet characterized and opens the door to the use of TEs as catalysts in organic chemistry. These results make the RAL TEs extremely intriguing as potential tools in chemoenzymatic chemistry as well as in combinatorial biosynthetic library formation and metabolic engineering.

6.2 Future work

Further studies could be performed on zea and rad TEs to better probe their substrate tolerance. First of all, bigger ring sizes could be built to see up to what size both TEs could macrocyclize, especially zea TE, before hydrolysis becomes a major by-product. Furthermore, rad TE could be metabolically engineered to reduce hydrolysis and increase their macrocyclic catalyzation, especially for 10-member ring formation.

For the stereochemical studies on the TEs, both the *R*- and *S*- enantiomers should be fully synthesized and incubated to fully understand the macrocyclic capabilities of zea TE and rad TE. Furthermore, kinetic studies should be undertaken to find out if one enzyme has a preference on one enantiomer over the other. Since both enzymes has shown that it can exclusively macrocyclize racemic methyl group α to the lactone, changing substation patterns at that area could prove very informative. For example, a methoxy or isopropyl group could be added at the α position of the lactone and then incubated with the enzymes to test their abilities to form macrocycles.

Since primary amines can be macrocycled to the amide, testing the ability of TEs to macrocyclize either methyl- or isopropyl- secondary amines could be

undertaken. Furthermore, experiments on the thiol, where no disulfide bonds or TCEP is present is needed to fully understand if the TEs can macrocyclize thiols to form thioesters. Other nucleophiles, such as phosphine, could also be tested.

Furthermore, different substitution patterns could be introduced to the 14-member ring, for example adding an amide where in nature there is a carboxylic group, to probe the substrate tolerance on different functional groups on the molecule.

Finally, adding the resorcylic moiety on the aromatic ring and testing the effect on macrocyclization rates for the 14-member ring could prove very informative. If it enhances rates, adding those to molecules that have a mixture of hydrolysis and macrocycle could potentially push the reaction to the macrocyclic compound.

Once these experiments are done, and we have a better understanding of the substrate tolerance of both zea TE and rad TE, they can be used as tools in chemoenzymatic chemistry.

Appendix A : Publications

- Wang, M.; Zhou, H.; Wirz, M.; Tang, Y.; Boddy, C. N., A Thioesterase from an Iterative Fungal Polyketide Synthase Shows Macrocyclization and Cross Coupling Activity and May Play a Role in Controlling Iterative Cycling Through Product Offloading. *Biochemistry* **2009,** *48 (27)*, 6288-6290.

6288 *Biochemistry* **2009,** *48,* 6288–6290
DOI: 10.1021/bi9009049

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

A Thioesterase from an Iterative Fungal Polyketide Synthase Shows Macrocyclization and Cross Coupling Activity and May Play a Role in Controlling Iterative Cycling through Product Offloading[†]

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ABSTRACT: Zearalenone, a fungal macrocyclic polyketide, is a member of the resorcylic acid lactone family. Herein, we characterize in vitro the thioesterase from PKS13 in zearalenone biosynthesis (Zea TE). The excised Zea TE catalyzes macrocyclization of a linear thioester-activated model of zearalenone. Zea TE also catalyzes the cross coupling of a benzoyl thioester with alcohols and amines. Kinetic characterization of the cross coupling is consistent with a ping-pong bi-bi mechanism, confirming an acyl−enzyme intermediate. Finally, the substrate specificity of the Zea TE indicates the TE may help control iterative cycling on PKS13 by rapidly offloading the final resorcylate-containing product.

Resorcylic acid lactones (RALs)[1] make up a large family of polyketide macrolactones with diverse biological activity (*1*). They include the estrogen receptor agonist zearalenone **5** and the HSP90 inhibitor, radicicol (*2*, *3*). The carbon framework of **5** is generated in the fungus *Gibberella zeae* by the actions of two iterative polyketide synthases (PKS) (Figure 1). PKS4 produces a highly reduced hexaketide intermediate **1**, which is transferred to the megasynthase PKS13. PKS13 functions iteratively, adding three unreduced ketide units to generate **2**, and, subsequently, catalyzes aromatization to produce **3** (*4*, *5*). The thioesterase (TE) domain of PKS13 is then postulated to generate the macrolactone **4**, releasing the macrocycle. FAD-dependent oxidation then gives **5** (*4*). In this study, we show that the TE of PKS13 (Zea TE) is responsible for macrocyclization of **3** and can catalyze rapid cross coupling to generate esters and amides. Characterization of the in vitro substrate specificity of the Zea TE suggests that the TE acts as a gatekeeper in vivo, ensuring cleavage of the growing polyketide from the synthase only after aromatization of the unreduced intermediate **2**. This model represents a new mechanism for controlling iterative processing in polyketide biosynthesis.

The fungal Zea TE is unique in both primary sequence and function. Alignment (SI) shows it to be substantially different (≤20% identical) from the well-characterized macrocyclizing bacterial PKS TEs (*6*−*10*) and the fungal TEs responsible for Claisen-like condensation activity (*11*, *12*). The proposed macrocyclization catalyzed by Zea TE has been shown to be chemically challenging under Corey-Nicolaou and Yamaguchi conditions (*13*). Furthermore, in vivo data suggest the Zea TE may be able to catalyze highly useful cross coupling with alcohol nucleophiles (*5*).

To demonstrate unambiguously that the Zea TE is responsible for macrocyclization of **4**, we synthesized a model macrocyclization precursor and evaluated the ability of the excised Zea TE to catalyze macrocyclization. *N*-Acetylcysteamine (NAC) thioester **6** and macrocyclic product **7** were synthesized following a short sequence (SI). Excised Zea TE was expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography (SI). Incubation of Zea TE with **6** led exclusively and rapidly to the formation of **7** as determined by HPLC (Figure 2) and ^{1}H NMR analysis (SI). To kinetically characterize Zea TE activity, release of NAC was monitored using Ellman's reagent and quantified spectrophotometrically. Kinetic analysis indicates that the TE catalyzes macrocyclization of **6** with a specificity constant (k_{cat}/K_M) of $(2.92 \pm 0.16) \times 10^3$ M^{-1} s^{-1}. Because of the limited solubility of **6**, k_{cat} and K_M could not be independently determined. This specificity constant is 10−100-fold greater than those reported for macrocyclization by the TE from the pikromicin biosynthetic pathway (*14*, *15*) and 1000-fold greater than those seen for the TEs from epothilone (Epo TE) and 6-deoxyerythronolide biosynthesis (*10*, *15*). Unlike Epo TE (*10*) and TEs from nonribosomal peptide synthetase pathways (*16*), the Zea TE does not catalyze detectable levels of hydrolysis to the linear carboxylic acid, making it an excellent potential tool for synthetic transformations involving macrolide macrocyclizations.

To confirm that the observed macrocyclization reaction is catalytic, the putative active site nucleophile S163 and the

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[1]Abbreviations: ACP, acyl carrier protein; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; KS, ketosynthase; MAT, malonyl-CoA:ACP acyltransferase; NAC, *N*-acetylcysteamine; PKS, polyketide synthase; PLP, pyridoxal 5′-phosphate; PT, product template; RAL, resorcylic acid lactone; SI, Supporting Information; SAT, starter unit: ACP transacylase; TE, thioesterase.

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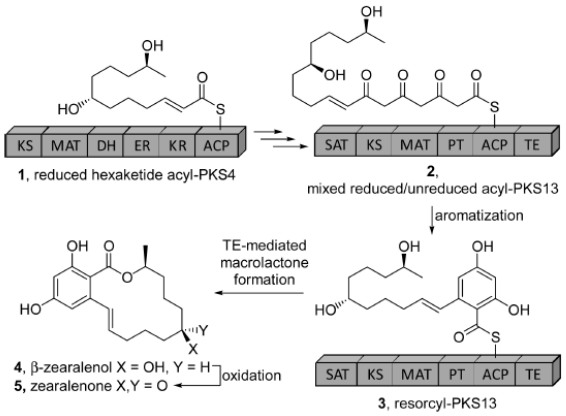

FIGURE 1: Biosynthesis of zearalenone, an archetypical RAL.

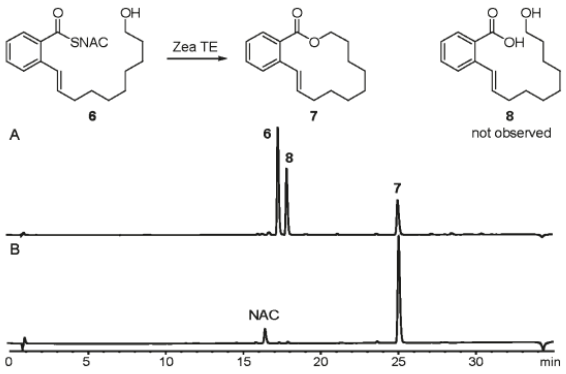

FIGURE 2: Zea TE catalyzes macrocyclization of 14-member rings. (A) HPLC analysis of authentic standards. (B) HPLC analysis of a 30 min incubation of 15 μM Zea TE with 5 mM **6** in 50 mM phosphate buffer at pH 7.4 and 23 °C. Negative controls lacking Zea TE showed no detectable macrocyclization activity.

putative catalytic base H332 were mutated and the mutants were kinetically characterized. The S163A and H332A mutants were generated by site-directed mutagenesis, expressed, and purified to homogeneity by affinity chromatography (SI). Neither the S163A mutant nor the H332A mutant exhibited detectable catalytic activity, indicating S163 and H332 play key roles in catalysis.

In vivo characterization of PKS13 had shown that ethanol and butanol could be coupled to acyl chains, produced by PKS13 to generate esters (*5*). To determine if Zea TE was responsible for this cross coupling activity, we investigated the consumption of **9** by Zea TE in the presence of exogenous nucleophiles. The Zea TE was incubated with **9** and varying concentrations of butanol. Liquid chromatography−mass spectroscopy (LC−MS) analysis of the reaction mixtures showed conversion exclusively to butyl benzoate without formation of benzoic acid even when stoichiometric butanol was present. Kinetic analysis performed at varying butanol concentrations showed Michaelis−Menten behavior, and parallel Lineweaver−Burk plots were obtained (SI). These results are consistent with a ping-pong bi-bi mechanism, fully supporting the postulated acyl−enzyme intermediate in the reaction mechanism.

A number of additional alcohol- and amine-based nucleophiles cross coupled with **9** to generate the corresponding

Table 1: Relative Velocities for Zea TE-Mediated Cross Coupling with Exogenous Nucleophiles[a]

nucleophile	v_{rel}	nucleophile	v_{rel}
butanol	1.00	2-propanol	0.38
benzyl alcohol	0.84	allylamine	0.38
aniline	0.58	glycerol	0.34
ethanol	0.50	water	0.20

[a] For 0.1 μM Zea TE with 1 mM **9** and 6 mM nucleophile (except for water at 55 M) at pH 7.4 and 23 °C. Negative controls lacking Zea TE showed no reactivity.

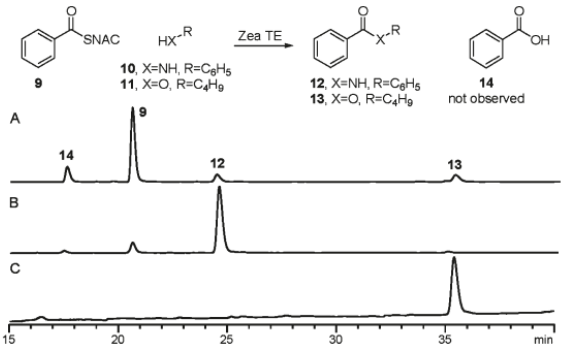

FIGURE 3: HPLC analysis of Zea TE-catalyzed cross coupling reactions. (A) Products and standards. (B) Cross coupling reaction with aniline. (C) Cross coupling reaction with butanol. Enzymatic reaction mixtures were incubated for 3 h with 5 μM Zea TE, 10 mM **9**, and 20 mM analine or butanol in 50 mM phosphate buffer at pH 7.4 and 23 °C. Negative controls lacking Zea TE showed no detectable cross coupling products.

benzoates and benzamides (Table 1 and Figure 3). Primary and secondary alcohols cross coupled with **9**, as did allyl amine and aniline. Tertiary alcohol (*tert*-butanol) and amino acids (glycine and methyl glycinate) were not cross coupled to **9**. In the absence of any exogenous nucleophile, the Zea TE used water to effect hydrolysis of **9**, generating benzoic acid. In general, α/β-hydrolases such as esterases, lipases, and proteases catalyze cross coupling (transesterification) without hydrolysis when the exogenous nucleophile is provided at a high concentration or water is excluded from the solvent system (*17*). The ability of the Zea TE to catalyze cross coupling in aqueous buffer with a low concentration of an exogenous nucleophile is thus particularly advantageous and complementary to existing α/β-hydrolases. Characterized aroyltransferases, which can catalyze cross coupling in aqueous buffer, require CoA thioesters as substrates (*18*−*20*), limiting their utility for in vitro chemistry. The use of the easy-to-prepare NAC thioesters by the Zea TE makes this an attractive enzyme for chemoenzymatic synthesis of commodity and fine chemicals.

Kinetic characterization of Zea TE with thioester substrates that model the intermediates in **2** and **3** (Figure 1) shows the enzyme to be highly specific for aromatic thioesters. **9**, a model of the resorcylate intermediate **3**, was consumed very rapidly in the presence of 6 mM butanol, with an apparent specificity constant (k_{cat}/K_M) of $(12.2 \pm 1.6) \times 10^3\ M^{-1}\ s^{-1}$ ($k_{cat} = 9.8 \pm 0.6\ s^{-1}$, and $K_M = 0.80 \pm 0.09$ mM). In the absence of butanol (and any other exogenous nucleophile, such as glycerol), Zea TE catalyzed the

hydrolysis of **9** to benzoic acid with a comparable specificity constant [k_{cat}/K_M = (16.0 ± 1.7) × 10^3 M^{-1} s^{-1} (k_{cat} = 1.18 ± 0.03 s^{-1}, and K_M = 78 ± 8 μM)]. This specificity constant is approximately 10000 times of that seen for thioester hydrolysis by bacterial PKS TEs (*6*, *7*, *21*−*24*). The NAC thioester of 3-ketopentanoate was originally selected as a representative model of intermediate **2**. However, the rapid rate of background hydrolysis precluded accurate kinetic characterization of the enzymatic reaction from this substrate. The NAC thioester of α-methyl-β-ketopentanoate was thus used as a surrogate of the poly-β-keto intermediate **2**. The pentanoate underwent hydrolysis to generate the corresponding carboxylic acid with a 100-fold lower specificity constant [k_{cat}/K_M = (0.088 ± 0.023) × 10^3 M^{-1} s^{-1} (k_{cat} = 0.39 ± 0.05 s^{-1}, and K_M = 4.4 ± 1.0 mM)], and the rate was not dependent on butanol concentration. The similarity of the α-methyl-β-ketopentanoate NAC thioester to the intermediate in **2** suggests that the Zea TE may not be able to process the growing polyketide until C2−C7 cyclization and the subsequent aromatization have occurred. The Zea TE may thus act as a gatekeeper ensuring rapid release of the aromatized intermediate during biosynthesis of **5**. Kinetic characterization of the substrate specificity of the Zea TE with more nativelike substrates is needed to rigorously test the hypothesis.

This gatekeeper activity may play a role in controlling iterative cycling on PKS13. Evidence supports iterative cycling in fungal PKSs (*25*), type II PKSs (*26*, *27*), and chalcone synthases (*28*) as being controlled by the overall chain length of the growing polyketide product. Iterative processing of the polyketide intermediate by the synthase is terminated when the growing polyketide fills the cavity available in the synthase. PKS13 has been shown not to use this well-characterized mechanism for controlling iterative cycling (*5*). Instead, the gatekeeper activity of the Zea TE (which is triggered by aromatization of the growing polyketide) may play a role in ensuring correct termination of iterative cycling and cleavage of the polyketide from PKS13. Recently, PLP-mediated offloading of a highly reduced polyketide has been shown to play a role in controlling iterative cycling of a fungal PKS (*29*, *30*). In conjunction with our results on the Zea TE, we propose that substrate selectivity in the offloading step may control iterative cycling in some iterative PKS systems.

In summary, we have characterized the first macrolactone forming fungal PKS TE in vitro. In addition to macrocyclization, the Zea TE catalyzes cross coupling to form esters and amides. We have also shown that the substrate specificity of the Zea TE suggests that it may play a role as a gatekeeper in the biosynthesis of zearalenone, ensuring that only appropriately functionalized intermediates can be released from the synthase. This may be a general mechanism seen in RAL biosynthesis.

SUPPORTING INFORMATION AVAILABLE

Experimental details, including synthesis of substrates and standards, and all kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Appendix B : Conference Presentations

- August 2010; Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases; 18th International Conference on Organic Synthesis (ICOS-18); Bergen, Norway – Poster presentation

- May 2010; Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases; Ottawa-Carleton Chemistry Institute (OCCI) day 2010; Ottawa, Ontario – Poster presentation

- May 2010; Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases; Keith Fagnou Organic Chemistry Symposium (KFOS); Ottawa, Ontario – Poster presentation

- May 2009; Caractérisation de polycétones synthase thioesterases fongiques et synthèse de substrats et évaluation de l'activitée de macrocyclisation; Association francophone pour le savoir (ACFAS); Ottawa, Ontario – Poster presentation

Appendix C : Curriculum vitae

Monica Wirz

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EDUCATION

MSc in chemistry ***2011 (Expected)***
University of Ottawa, Ottawa, Ontario – Undertook a Masters in Chemistry under the supervision of Professor Christopher N. Boddy.

B.S., biopharmaceutical sciences option medicinal chemistry ***May 2008***
University of Ottawa, Ottawa, Ontario – Graduated with honour while undertaking an Honour's Project at the National Research Council of Canada under the supervision of Dr. Prabhat Arya.

H.S. Diploma *June 2003*
Collège Louis-Riel, Winnipeg, Manitoba - Honour roll student. Participated on the green team and the awareness committee.

RELATED WORK EXPERIENCE

Research Assistant *September 2008 to Present*
University of Ottawa, Ottawa, Ontario – Built different analogues using organic chemistry methods in Dr. Christopher N. Boddy's laboratory. Tested the specificity and limitations of two enzymes.

Laboratory Teaching Assistant *September 2008 to December 2010*
University of Ottawa, Ottawa, Ontario – Responsible for training and monitoring 35 students per week, and preparing experiments for their first and second year organic chemistry laboratory courses. Graded laboratory reports, proctored exams, and provided office hours.

University Session Leader for High School Students *May 2010*
University of Ottawa, Ottawa, Ontario –Responsible for teaching basic chemistry and monitoring 60 high school students. Prepared theoretical classes and supervised laboratory experiments.

Teaching Assistant *September 2009 to December 2009*
University of Ottawa, Ottawa, Ontario - Responsible for teaching, training, and monitoring 60 students per week, and preparing discussion group for their third year spectroscopy courses. Graded homework, proctored exams, and provided office hours.

Research Assistant *June 2007 to August 2007*
National Research Council Canada, Ottawa, Ontario – Built different analogues using organic chemistry in Dr. Prabhat Arya's laboratory.

LABORATORY PROFICIENCY

- Previous laboratory experience while completing an Honour's Project and a MSc degree
- Successfully Completed WHMIS training (Workplace Hazardous Materials Information System)
- LC/MS/MS, HPLC, UV-VIS Spectroscopy, IR, GC-MS, NMR techniques

- Column chromatography, TLC, air and water sensitive organic synthesis techniques

CONFERENCE POSTER PRESENTATIONS

18th Internation Conference on Organic Synthesis (ICOS-18) *August 2010*
Bergen, Norway – Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases.

Ottawa-Carleton Chemistry Institute (OCCI) day *May 2010*
Ottawa, Ontario – Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases.

Keith Fagnou Organic Chemistry Symposium (KFOS) *May 2010*
Ottawa, Ontario – Characterizing the macrocyclization activity of fungal polyketide synthase thioesterases.

Association francophone pour le savoir (ACFAS) *May 2009*
Ottawa, Ontario – Caractérisation de polycétone synthase thioesterase fongique et synthèse de substrats et évaluation de l'activité de macrocyclisation.

PUBLICATIONS

- Wang, M.; Zhou, H.; Wirz, M.; Tang, Y.; Boddy, C. N., A Thioesterase from an Iterative Fungal Polyketide Synthase Shows Macrocyclization and Cross Coupling Activity and May Play a Role in Controlling Iterative Cycling Through Product Offloading. *Biochemistry* **2009,** *48 (27)*, 6288-6290.

PERSONAL PROFILE

- Fluent in English, French, and German, basic knowledge of Spanish, Portuguese, and Italian
- Reliable, open-minded, hard-worker, punctual and friendly
- Great problem solving, listening and interpersonal skills
- Participated in many sports, such as fencing, badminton, swimming, softball, and cycling

*References available upon request

*Ce C.V. est disponible en français sur demande

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