Biosynthetic Study of CcsA and a Polyketide Synthase with Homology to Carnitine Acyltransferase

by

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Abstract

Polyketides are a class of natural products with large structural and biochemical diversity. Polyketides are assembled by the polymerization of short-chain fatty acids by enzymes named polyketide synthases. Fungal iterative polyketide synthases are notable in that they contain multiple active sites that are used in a cyclical fashion to construct their natural product. There is potential for competing reactions on the growing substrate, and how these enzymes are programmed to synthesize only one final product is not well understood.

Cytochalasin E is a fungal polyketide that is currently utilized as an angiogenesis inhibitor in cellular assays. The enzyme responsible for production of cytochalasin E is named CcsA. CcsA is a polyketide synthase that is fused to a non-ribosomal peptide synthetase domain, and both work in tandem to create cytochalasin E. The biosynthetic pathway in the production of cytochalasin E has been proposed, but the pathway has only been partially elucidated.

This thesis will first outline the synthetic effort undertaken toward the synthesis of compounds that will be used to study the biosynthesis of cytochalasin E. Progress made toward the synthesis of the proposed octaketide late-stage product of the polyketide synthase will be described.

This thesis will then illustrate the discovery of a novel polyketide system that may help to answer pressing questions the polyketide community has concerning enzymatic control of methylation and reduction events. The product of this polyketide system contains an interesting pattern of methylation and reduction, and the enzyme responsible contains a domain with homology to carnitine acyltransferase instead of the standard

thioesterase domain for off-loading of the polyketide product. It is hoped that by understanding this fungal polyketide system, the results can be translated to other polyketides assembled by fungal iterative polyketide synthases. The carnitine acyltransferase domain also represents a new mode of polyketide off-loading by polyketide synthases, and adds to the already broad diversity of polyketide natural products.

Preface

The experiments presented in this thesis were done to supply substrates for our collaborators, the lab of Dr. Yi Tang at UCLA. The members of Dr. Tang's lab are to utilize these substrates for enzymatic assays, and are responsible for the required molecular biology and *in vitro* testing. I was responsible for performing synthetic experiments and collecting data for the substrates outlined in the thesis. Dr. Christian Foerster was responsible for synthesis of the enantiomeric series of the compounds outlined in chapter 3. The work presented in this thesis is unpublished at the time of writing.

Acknowledgments

I want to thank my research supervisor, Dr. John Vederas, for his role. You were an extremely knowledgeable source of intellectual input, and a role model for enthusiasm and dedication to science. Without the opportunity to work in your lab and the resources you were able to provide, none of this would have been possible. I would like to also acknowledge the members of my supervisory committee, Dr. Derrick Clive and Dr. Liang Li. Thank you for the time that you have put into reading progress reports, attending meetings, and discussing research.

I want to thank Randy Sanichar for his support in the lab and for his reading of this thesis. Randy was a readily accessible source of synthetic chemistry knowledge who was always willing to provide input to problems I was having. He is passionate about chemistry and is always eager to learn about the work that other people are doing, and to offer helpful suggestions for improvement.

Last but not least, I want to thank Dr. Christian Foerster for his support in the lab, the reading of this thesis, and for his role in the synthesis of selected compounds outlined in the thesis. Christian has a unique sense of humour that makes working with him very enjoyable. He was exceptional at motivating me to push forward when things were working well, and bringing me out of the gutter when things would inevitably fail. Without his encouragement and support, this work would not have been accomplished.

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Abbreviation list

[α]	specific rotation
A	adenylation domain
Å	angstrom
ACP	acyl carrier protein
AT	acyl transferase
n-BuLi	*n*-butyllithium
BVMO	Baeyer-Villiger monooxygenase
c	concentration in g/mL (optical rotation)
C	condensation domain
CAT	carnitine acyltransferase
CoA	coenzyme A
CPT I	carnitine palmitoyltransferase I
CPT II	carnitine palmitoyltransferase II
δ	chemical shift in ppm
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DEBS	6-deoxyerthronolide B synthase
DH	dehydratase
DIPEA	diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DML	dihydromonocolin L
DMP	Dess-Martin periodinane

DMSO	dimethyl sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EI	electron impact ionization
eq	equivalents
ER	enoyl reductase
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FAS	fatty acid synthase
FDA	Food and Drug Administration (USA)
HRMS	high resolution mass spectrometry
HWE	Horner-Wadsworth-Emmons
IR	infrared
J	coupling constant (in hertz)
kDa	kilodalton
KR	ketoreductase
KS	ketosynthase
KHMDS	potassium hexamethyldisilazide
LC-MS	liquid chromatography-mass spectrometry
m	multiplet
MAT	malonyl-CoA:acyl transferase
MT	methyltransferase
NADPH	nicotinamide adenine dinucleotide phosphate

NaHMDS	sodium hexamethyldisilazide
NOESY	nuclear Overhauser effect spectroscopy
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
PBS	phosphate-buffered saline
PE	pentaerythritol
PKS	polyketide synthase
PKS-NRPS	polyketide synthase-nonribosomal peptide synthetase
Ppant	phosphopantetheine
ppm	parts per million
q	quartet
quant.	quantitative yield
R	reductase domain
rt	room temperature
satd	saturated
SAM	*S*-adenosyl methionine
SNAC	*N*-acetylcysteamine
soln	solution
T	thiolation domain
TBAF	tetrabutylammonium fluoride
TBS	*tert*-butyldimethylsilyl
TE	thioesterase
TEA	triethylamine

THME	1,1,1-tris(hydroxymethyl)ethane
TLC	thin-layer chromatography
uv	ultraviolet

1 Introduction

Natural products can be simply defined as secondary metabolites produced by a biological source.[1] Natural products are of interest to chemists for many reasons. They present challenging opportunities for total synthesis to synthetic organic chemists, and these efforts are one of the driving forces for the development of new synthetic methodologies.[2] For more biologically oriented chemists, how nature makes these compounds is an intriguing puzzle to be solved. Medicinal chemists harness natural products as drugs and work to create derivatives with improved pharmaceutical properties.

Polyketides are a class of natural products that contain complex chemical structures and diverse bioactivities, despite being constructed from the oligomerization of simple fatty acids such as acetic acid and malonic acid through their activated coenzyme A (CoA) derivatives. Polyketides form the basis for a vast number of pharmaceutically active drugs, either in their original form or through synthetic modification.[2,3] A few representative examples are anti-infectives such as erythromycin A, anti-cancer agents such as epothilone B, and statin drugs including lovastatin – the first FDA approved drug for lowering high blood cholesterol levels[3,4] (Figure 1-1).

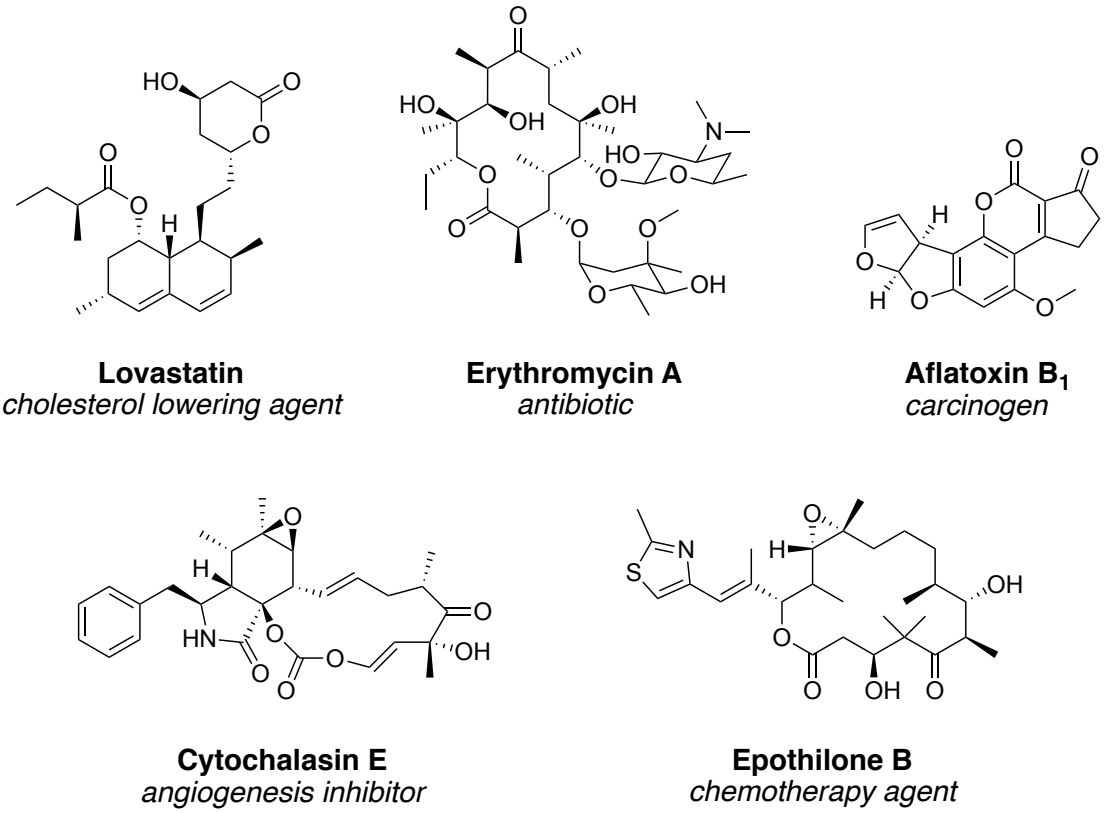


Figure 1-1. Selected examples of polyketide natural products, highlighting their diversity of structure and bioactivity

Microorganisms are rich sources of natural products for human applications. During the early years of natural product discovery, compounds with interesting bioactivities were easily found by low-throughput fermentation and whole cell screening procedures. With the development of molecular biology and medicinal chemistry, efforts began on improving pharmacologically active scaffolds (pharmacophores) within known compounds. In the 1990s, the pharmaceutical industry moved largely toward high-throughput screening of large synthetic libraries to discover compounds with improved activity against a desired biological target.[5] With this trend toward high-throughput screening and the modification of existing natural products, the discovery rate of new compounds from biological sources has decreased. However, microorganisms are still valuable in the discovery of new drugs. Certain actinomycetes possess the ability to

produce 30-50 secondary metabolites with different backbones – much more than previously proposed. Even though the field of genome mining is still relatively new, it has already contributed to the advancement of genetic sequencing, gene expression, and metabolomics. Genome mining represents an important part of the future of natural product discovery and development. It will accelerate the discovery of pharmacologically active compounds, and may also be a useful way of obtaining synthetically complex molecules.[6]

A potential application of understanding biosynthetic pathways is the combinatorial biosynthesis of molecules. Only after revealing how enzymes facilitate the biosynthesis of natural products will it be possible to rationally manipulate these enzymes. This potentially would pave the way for the biosynthetic production of novel compounds with improved and/or new pharmacological activities. The most well understood polyketide system is that of a type I modular PKS, 6-deoxyerythronolide B synthase (DEBS).[7] DEBS is the enzyme responsible for the biosynthesis of the polyketide precursor to erythromycin A, an antibiotic listed on the World Health Organization's list of essential medicines.[8] It is noteworthy that there are successful instances of using chemical biology to modify this system to make novel compounds. By modifying enzymatic domains to accept new substrates, researchers were able to incorporate amides, benzyl groups, alkenes, and remove methyl groups to create new erythronolide analogues.[3,7] However there are still many challenges to harnessing enzymes to do truly combinatorial synthesis. The main obstacle is overcoming the inherent selectivity that most enzymes exhibit. In the near future it is believed that PKS synthetic biology will become complementary to synthetic chemistry to create derivatives of known structures.[9]

1.1 Biosynthesis of fatty acids and polyketides

Fatty acid and polyketide biosynthetic pathways share a large degree of homology. Indeed, phylogenetic analysis suggests that both eukaryotic fatty acid synthases (FASs) and polyketide synthases (PKSs) evolved from bacterial FAS.[10] Mechanistically both classes of compounds are built through a number of 'chain elongation' and 'tailoring' steps. Before discussing polyketide biosynthesis in detail, it is useful to understand how classical fatty acid biosynthesis is accomplished. Fatty acids and polyketides are assembled in an analogous fashion, and fatty acids can be seen as simplified polyketides.

Human FAS is a complex (552 kDa) homodimeric enzyme that contains seven catalytic domains.[11] The domains are arranged head to tail, and are used in an iterative fashion. FAS is primarily responsible for the construction of the 16 carbon palmitic acid (hexadecanoic acid).[2,11]

The order of domain reactivity in the assembly of fatty acids is shown in Figure 1-2. There are three domains involved in elongation of the carbon backbone. The acyl carrier protein (ACP) is a non-catalytic domain of the enzyme. The *apo* form of the ACP must first be converted to the *holo* form by the attachment of a phosphopantetheine (Ppant) group derived from CoA (Figure 1-2, step 1). The Ppant moiety is linked to the ACP through a serine residue to create an 18Å-long prosthetic group with a free thiol at its distal end.[2] The *holo* ACP acts as a tether that covalently attaches to the growing acyl chain at different points in its elaboration and shuttles it from one domain to another.

The malonyl-CoA:acyltransferase (MAT) domain is responsible for loading the 'starter unit' (acetyl-CoA) and 'extender unit' (malonyl-CoA) onto the enzyme.[2] As a consequence of the affinity of MAT for both acetyl-CoA and malonyl-CoA, the two substrates are competitive inhibitors of each other. This is not rate limiting because both are in fast equilibrium between their CoA and protein-bound forms[12] (Figure 1-2, step 2).

Once the cysteine residue in the ketosynthase (KS) domain's active site is loaded with a starter unit (Figure 1-2, step 2a), and the ACP is loaded with the extender unit (Figure 1-2, step 2b), the enzyme is ready for chain extension. The net result of a chain elongation event is a decarboxylative Claisen condensation that extends the backbone of the growing carbon chain by one ketide unit [$C(O)CH_2$]. The KS catalyzes decarboxylation of the malonyl extender unit, creating a nucleophilic enolate. The enolate attacks the acyl carbon of the starter unit to form a new C-C bond and yield a β-ketothioester species that is bound to the ACP[2] (Figure 1-2, step 3).

After chain elongation the β-carbonyl may be reduced all the way down to the aliphatic methylene in a series of reactions referred to as tailoring steps. The tailoring steps all take place while the acyl chain is covalently attached to the ACP domain. First the β-keto functionality is reduced by the NADPH-dependent ketoreductase (KR)[13] (Figure 1-2, step 4a). The dehydratase (DH) domain effects a *syn*-elimination of the β-hydroxy group to create a *trans*-alkene that is in conjugation with the thioester (Figure 1-2, step 4b). This enone is then reduced by the NADPH-dependent enoyl reductase (ER), yielding a saturated carbon chain. It is interesting to note that in mammalian and prokaryotic FAS this is a *syn*-addition of hydride from NADPH and a proton from water, while in yeast FAS this is an *anti* addition.[2,13]

This concludes the chain tailoring events for fatty acid biosynthesis. The reduced substrate will then go down one of two paths. The acyl chain will either become the starter unit in another round of chain extension and tailoring (Figure 1-2, step 5), or be off-loaded by a thioesterase (TE) domain as a carboxylic acid[2] (Figure 1-2, step 6).

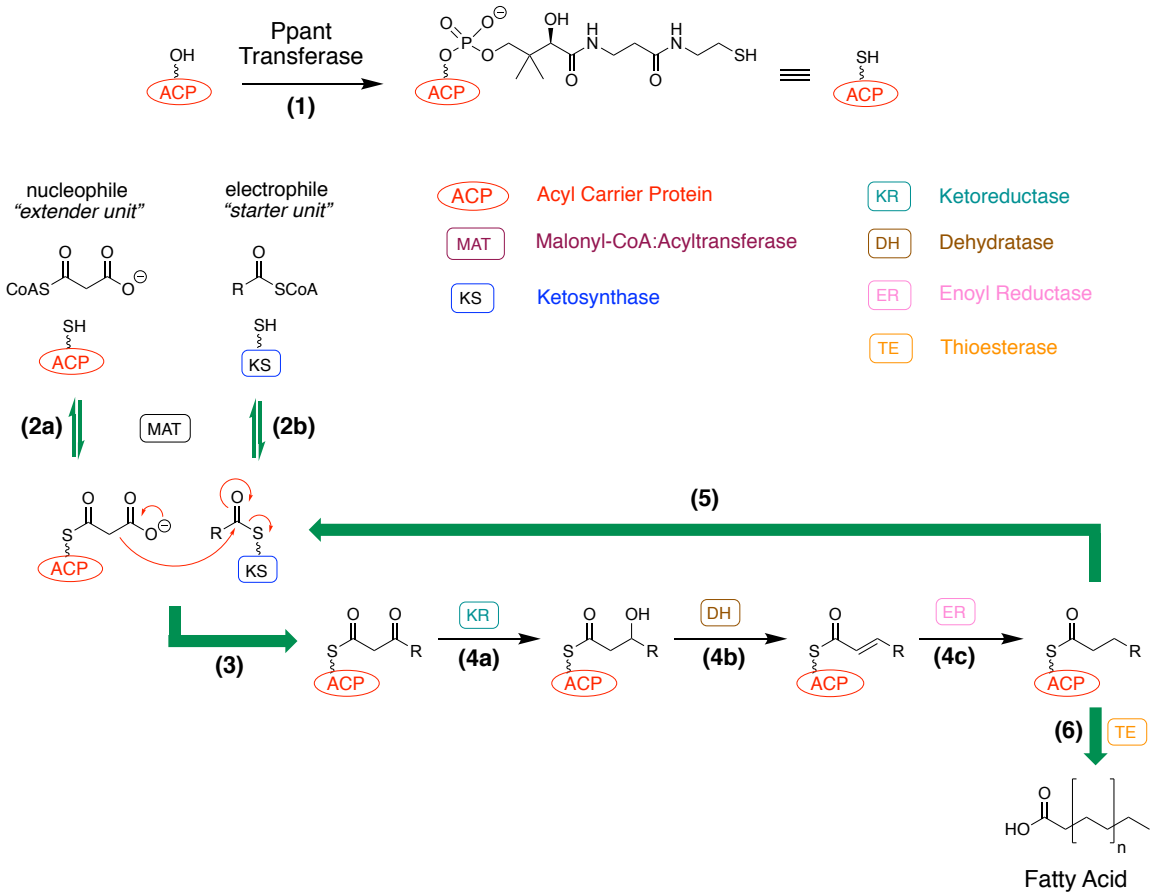


Figure 1-2. General mechanism of fatty acid biosynthesis. ACP = acyl carrier protein, MAT = malonyl-CoA:acyltransferase, KS = ketosynthase, KR = ketoreductase, DH = dehydratase, ER = enoyl reductase, TE = thioesterase. Step numbers are bolded and in parentheses.

Polyketides are assembled in an analogous fashion to that of fatty acids with a few key differences (Figure 1-3). Importantly, the post-translational modification of the ACP domain to attach a Ppant arm, the loading of the starter and extender units, and the mechanism of chain elongation are all similar (Figure 1-3, steps 1-3). Polyketides are

different from fatty acids in that tailoring events can be interrupted at any stage by another chain extension (Figure 1-3, steps 5a-d), resulting in ketone, alcohol, or alkene moieties being incorporated into the final product (Figure 1-3, steps 6a-c). In addition a methyltransferase (MT) may be present that can introduce a methyl group from *S*-adenosyl methionine (SAM) to the growing polyketide chain, and the TE domain can catalyze the off-loading of the polyketide using nucleophiles other than water. After release of the polyketide from the enzyme, post-translational modifications including glycosylation, oxidation, and cyclization can also take place.[3] These unique aspects of polyketides add elements of structural diversity and increase the opportunity for producing compounds with biological activity.

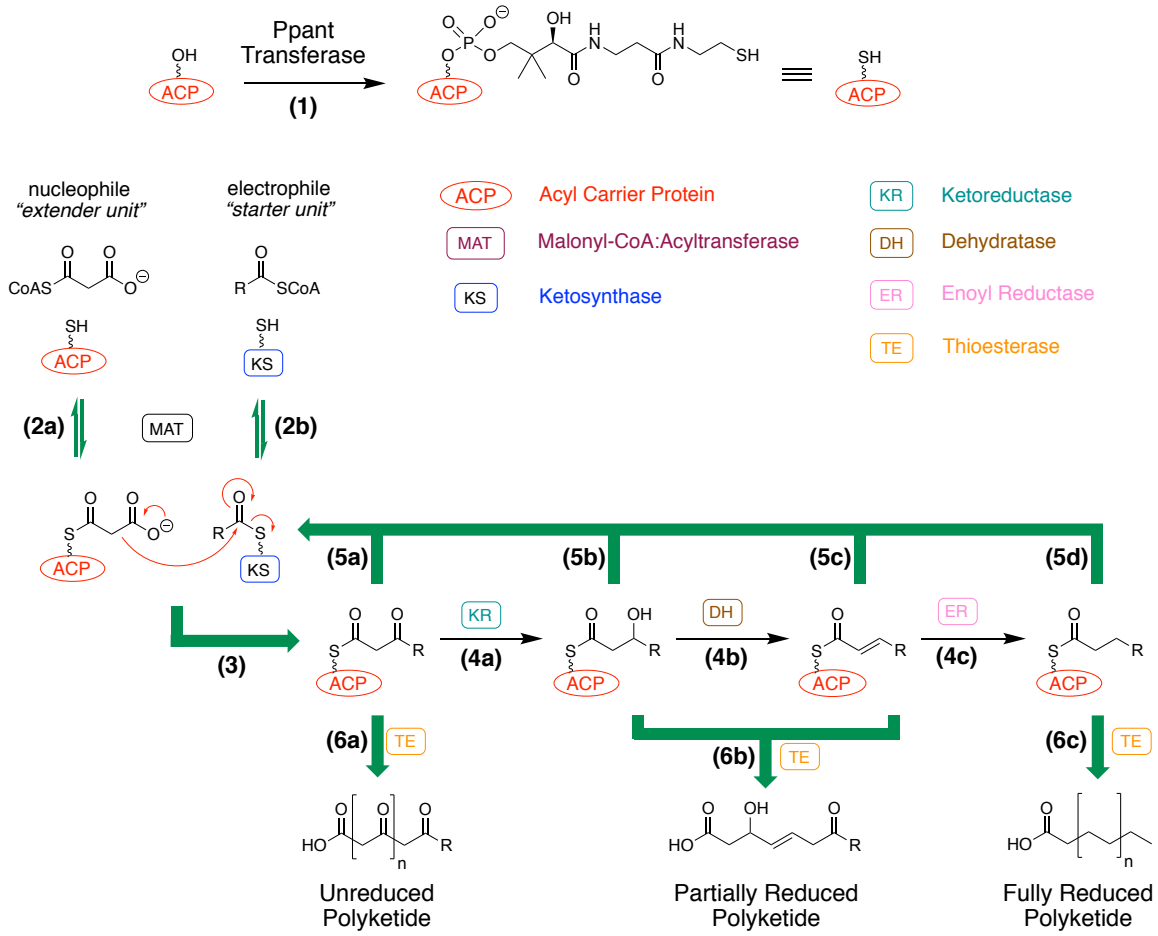


Figure 1-3. General mechanism of polyketide biosynthesis. ACP = acyl carrier protein, MAT = malonyl-CoA:acyltransferase, KS = ketosynthase, KR = ketoreductase, DH = dehydratase, ER = enoyl reductase, TE = thioesterase. Step numbers are bolded and in parentheses.

1.2 Classification and features of polyketide synthases

PKSs are classified into several different groups depending on the primary structure of the enzyme, the organization of the active site, and the structure of the product (Table 1-1). The PKS domains are either used once (modular) or repeatedly (iterative) during the construction of the polyketide. Modular PKSs contain many copies of the same set of domains. After a growing substrate is transformed by one domain it is transferred to the next domain where it is further modified, like an assembly line. Modular PKS systems obey the rule of "co-linearity", where the organization of the domains as well as their presence or absence allows us to form a hypothesis for how the polyketide product is made. Iterative PKSs are much more mysterious. Iterative PKSs utilize their domains in a cyclical fashion for the construction of a polyketide. This involves some inherent programming of the enzyme that is not well understood. In fact, iterative PKSs are sometimes referred to as a 'black box'.[14] The PKS types are also classified based on how the domains are arranged (Figure 1-4). Type I enzymes have all their domains constructed from one large polypeptide chain. The domains are said to act *in cis* since they are part of the same protein. Type II PKSs are a group of smaller individual proteins that are associated with each other and are said to act *in trans*. Type III PKSs act *in trans* in the same manner as type II PKS, but do not require an ACP. Type III PKSs utilize acyl-CoA building blocks directly and use a single active site for the iterative processing of polyketides. Lastly there is a type of PKS that is fused to a nonribosomal peptide synthetase (NRPS) domain. This type of enzyme facilitates the construction of a polyketide chain, followed by the incorporation of an amino acid residue into the final off-loaded product.[2]

PKS Type	Subtype	Organism
I	Modular	Bacteria, some protists
	Iterative	Fungi, some bacteria
II	Iterative	Bacteria only
III	Iterative	Mostly plants, some bacteria and fungi
PKS-NRPS	Modular	Bacteria
	Iterative	Fungi and bacteria

Table 1-1. PKS types and subtypes. Modified from a review by C. Hertweck[14]

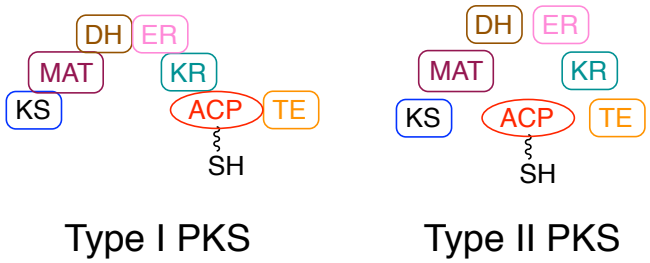


Figure 1-4. Structural organization of type I (*cis*) and type II (*trans*) PKS

1.3 Conclusion

Although much is known about type I modular PKSs, iterative PKSs remain puzzling. The dynamic nature of the domains and the large size of the proteins make them very difficult to crystallize. The absence of co-linearity means that we are not able to make the same degree of educated inferences as to how iterative PKSs work based on their structure.[15]

The molecular basis of how iterative PKS domains are programmed to control the length of the polyketide chain (number of iterations) and the chemical functionality of the polyketide (tailoring steps) is not well understood. During the tailoring steps there are a number of plausible chemical outcomes that can occur with each substrate. Recent breakthroughs have provided us with tools to help elucidate the complex nature by which iterative PKSs function to produce natural products.[16] The first efforts focused on feeding isotopically labeled small building blocks into PKS systems in order to gain knowledge about the biosynthetic pathway. Newer efforts have involved using chemical synthesis to create labeled proposed biosynthetic intermediates that are subsequently fed into the biological system. If a synthetic compound is recognized and transformed by the PKS, it provides evidence that the compound is a natural substrate for the enzyme.

Our lab has a history of collaboration with the molecular biologists in the Yi Tang lab at UCLA. The chemical synthesis of hypothesized biosynthetic intermediates coupled with screening by enzyme assays is a useful method to gain knowledge of how iterative PKS systems operate. Chemical synthesis also affords the opportunity to create compounds with unnatural stereochemistry and functionality to tease out the essential properties for recognition by the PKS domains. Enzyme assays allow us to quantify how

selective the domains are for one compound versus another, and together these two fields can help us to understand how nature so effectively carries out chemistry that currently eludes us.

This thesis will describe the work that has been conducted to answer questions posed by two polyketide systems: CcsA, the PKS responsible for producing cytochalasin E, and a novel PKS system identified through genome mining by the Yi Tang lab at UCLA. The following chapters will describe the synthetic efforts directed at making analogues of proposed biosynthetic intermediates in each pathway.

2 Investigating Cytochalasin E Biosynthesis

2.1 Introduction to cytochalasin E: discovery, structure, and biological activity

Cytochalasin E was originally discovered in 1972 by Aldridge and co-workers while studying the fungus *Rosellinia necatrix*, a plant pathogen.[17] The Büchi group independently discovered cytochalasin E in 1973 as a mycotoxin produced by *Aspergillus clavatus* and is credited with being the first to unambiguously determine its structure using NMR, IR, and X-ray crystallography.[18] *A. clavatus* was first isolated from mold-infected rice in a Thai home after the related death of a young boy. Since these original findings cytochalasin E has been isolated from a number of sources, including infected grains (*A. clavatus*), soil (*Mycotypha sp.*), and the marine environment (*Spicaria elegans*).[19] Though cytochalasin E is produced by many organisms, *A. clavatus* is considered the model organism for studying its biosynthesis.

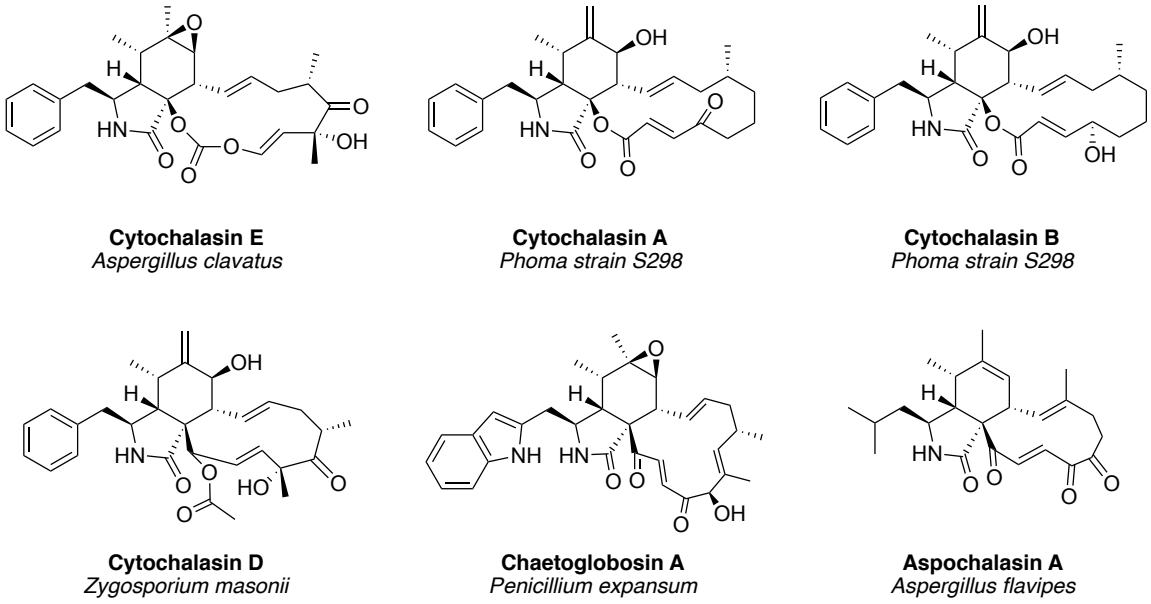


Figure 2-1. Structures of selected members of the cytochalasin family, and their producing organism

The cytochalasin family of natural products contains over 100 members with a diverse arrangement of functionality and bioactivity.[20] Several representative examples of cytochalasins are shown in Figure 2-1. Cytochalasins are typically characterized by the presence of an 11- to 14-membered macrocycle fused to a highly substituted perhydroisoindolone core derived from an amino acid. Cytochalasin E, as well as cytochalasins A, B, D, and F inhibit cell proliferation through their prevention of actin polymerization. Anti-proliferative activity, combined with low cytotoxicity, is why cytochalasin E is widely used as an angiogenesis inhibitor in cellular assays.[21]

Despite being studied for more than 40 years and the topic of hundreds of publications, little is known about how cytochalasin E is made in nature. There are many questions to be answered regarding how this complex natural product is biosynthesized.

2.1.1 Proposed biosynthesis of cytochalasin E

The gene cluster responsible for the biosynthesis of cytochalasin E in *A. clavatus* was published in 2011 by Tang and coworkers.[22] Using this knowledge, the authors proposed a biosynthetic sequence with intermediates for the production of cytochalasin E (Figure 2-2). With the previously proposed chaetoglobosin biosynthesis as a guide, the authors assigned roles to the genes found in the *A. clavatus* cluster. Bioinformatic analysis showed the gene CcsA contains all of the domains expected for a PKS-NRPS hybrid, including an inactivated ER domain that can be compensated for by a *trans-acting* ER in the same gene cluster, CcsC. It is hypothesized that the PKS-NRPS enzyme with the same name (CcsA) is responsible for the production of an octaketide chain *via* the polymerization of eight C2 units. The NRPS C domain is proposed to catalyze the formation of an amide bond between the amino group of a phenylalanine loaded on the T

domain, and the polyketide acyl chain. The NRPS R domain contains an NADPH-binding motif and is hypothesized to mediate a reductive off-loading of the ACP-bound thioester to the corresponding aldehyde. There is previous evidence for this type of off-loading in other fungal natural products, such as in thiopyrazine biosynthesis.[23] After being released from the enzyme, the β-keto amide is speculated to undergo a Knoevenagel-type condensation to create a deoxytetramic acid. This species is then positioned to undergo an intramolecular Diels-Alder reaction to construct the isoindolone core. It is plausible that the condensation and Diels-Alder may be either spontaneous or enzyme catalyzed. The authors proposed that the next steps are installation of the ketone, alcohol, and epoxide moieties by a pair of P450 monooxygenases, CcsG and CcsD. This is plausible, as there is precedent for multiple oxidations being performed by a single oxygenase.[24] The final series of reactions in cytochalasin E biosynthesis were thought to be installation of the labile carbonate moiety by a flavin-dependent Baeyer-Villiger monooxygenase, CcsB.

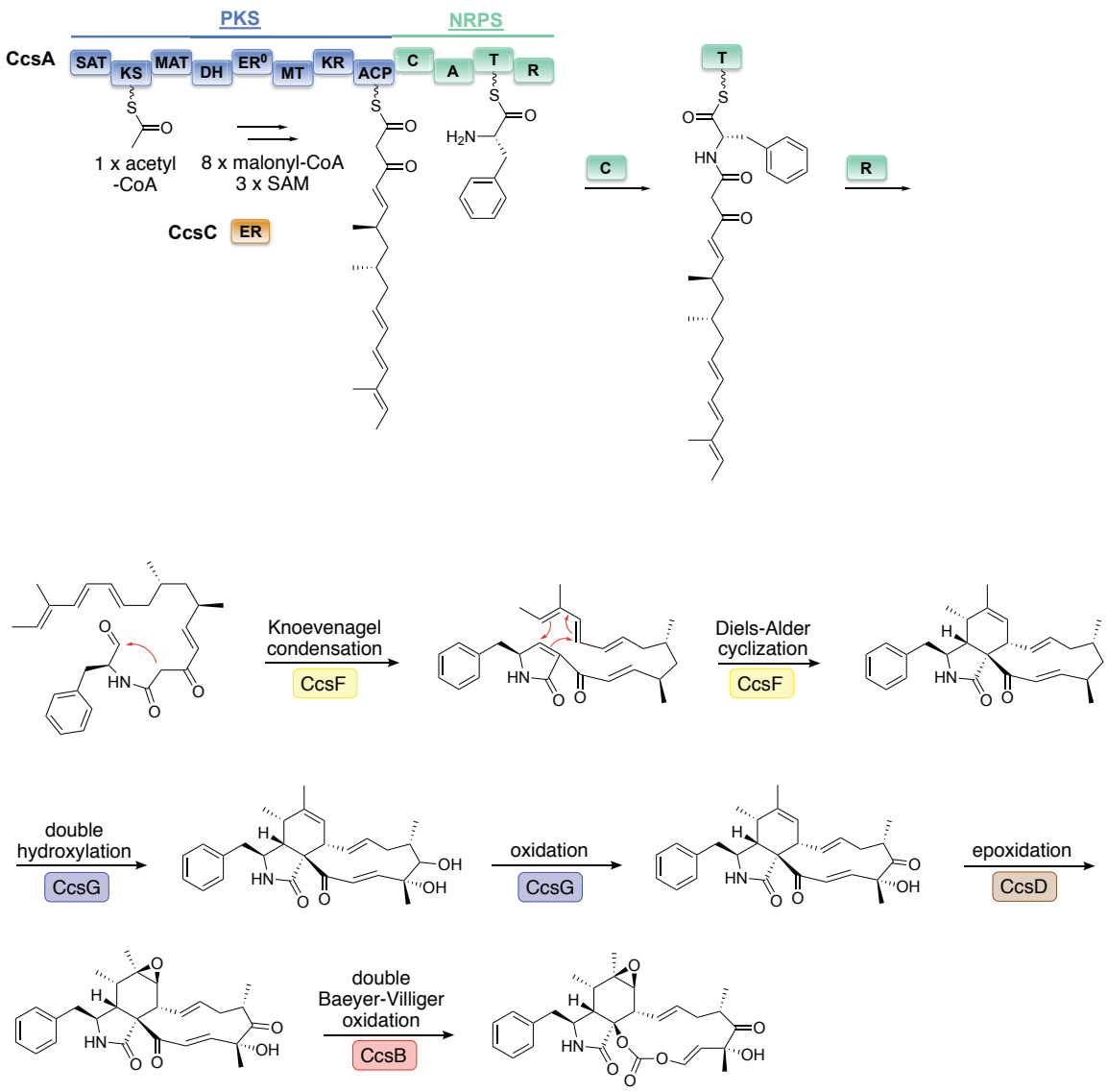


Figure 2-2. Proposed biosynthetic pathway of cytochalasin E production by Qiao *et al.* in 2011[22]

Subsequent experiments have shown that the cytochalasin E biosynthetic pathway does not follow that of chaetoglobosin as closely as first proposed. Work by the Oikawa group in 2013 reported the heterologous expression of CcsA in *Aspergillus oryzae*.[25] The authors were able to isolate the released product of CcsA. The reduction of the thioester carbonyl appeared to not stop at the expected aldehyde, and continued to the alcohol. It is not clear if this is a true intermediate or a shunt product. If the rest of the proposed

pathway is correct, this would require oxidation back to the aldehyde before cyclization can occur. Work by the Vederas and Tang labs has shown that CcsB is a flavin-dependent monooxygenase that catalyzes a double Baeyer-Villiger oxidation to transform a ketone into a carbonate – a reaction that is unprecedented in natural product biosynthesis.[26] The proposed mechanism requires that carbonate formation takes place after the Diels-Alder, but before the epoxidation and installation of the 3° hydroxyl group – which is not in agreement with the original proposed biosynthetic pathway.

A biosynthetic proposal in agreement with the information that has already been obtained from biosynthetic studies of chaetoglobosin and cytochalasin E is presented in Figure 2-3. However, it is still conjecture at this point and a number of questions remain to be answered:

- Is the released product of CcsA an alcohol or an aldehyde?
- If the off-loaded product is an alcohol, does CcsG oxidize it to the aldehyde?
- Does CcsF catalyze a Knoevenagel-like condensation followed by a Diels-Alder reaction?
- Additionally, an alternative mechanism of off-loading from CcsA *via* a Dieckmann cyclization is plausible (Figure 2-4). How is the polyketide product off-loaded from CcsA?
- After off-loading and cyclization, does CcsG subsequently effect hydroxylation and oxidation to create the allylic ketone before creation of the carbonate?
- Does CcsD mediate consecutive hydroxylation and epoxidation in the final step of cytochalasin E biosynthesis?

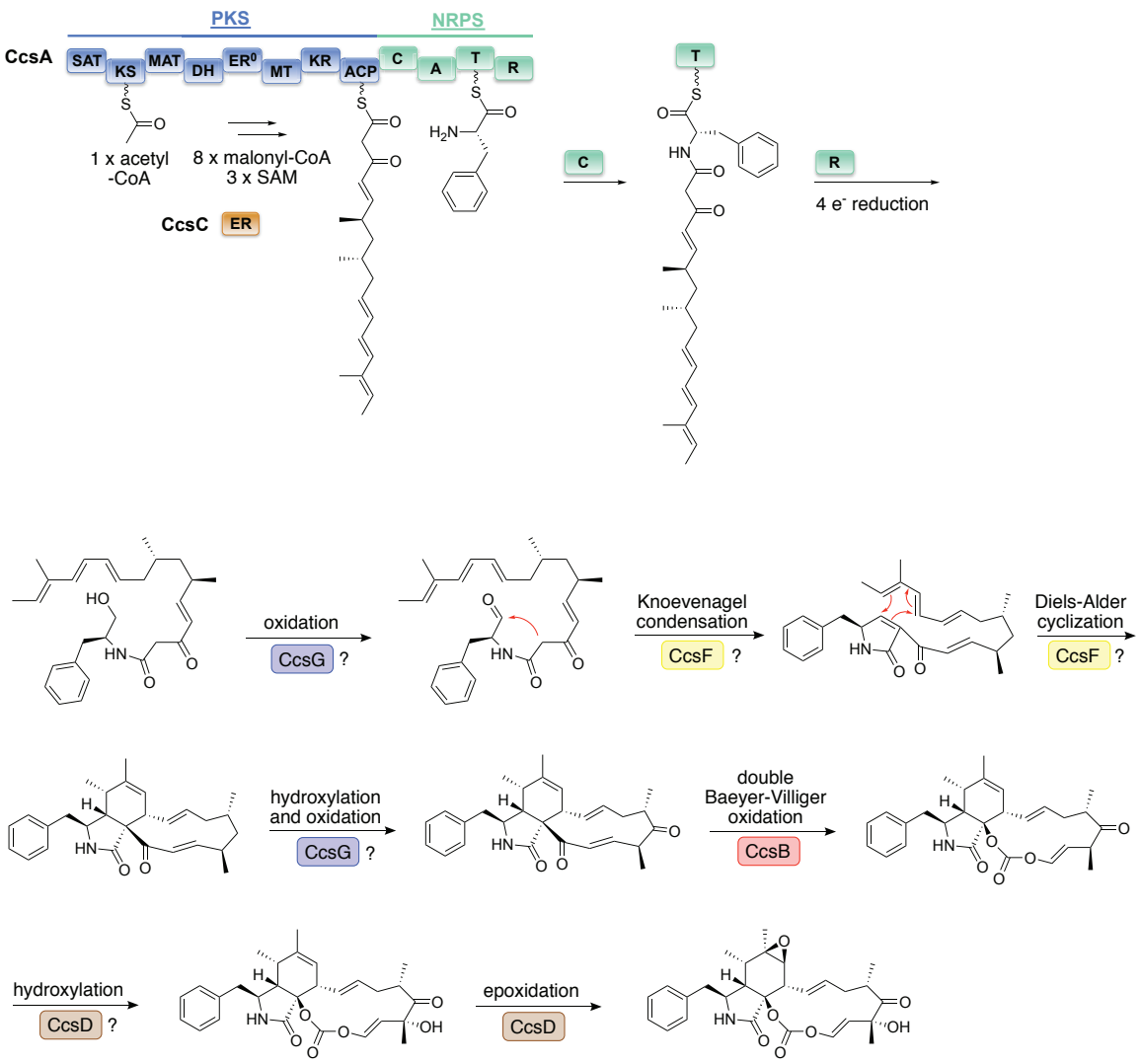


Figure 2-3. Updated proposed biosynthetic pathway of cytochalasin E. The functions of CcsD, CcsF, and CcsG still remain to be confirmed.

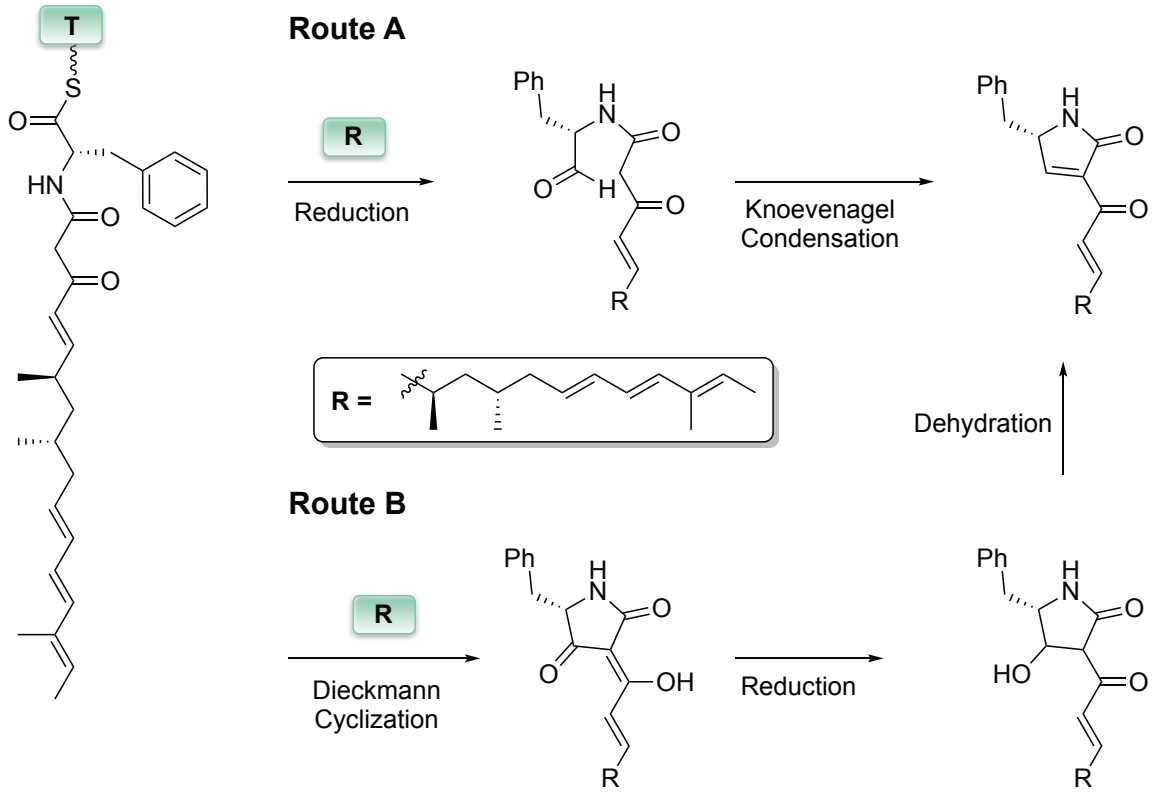
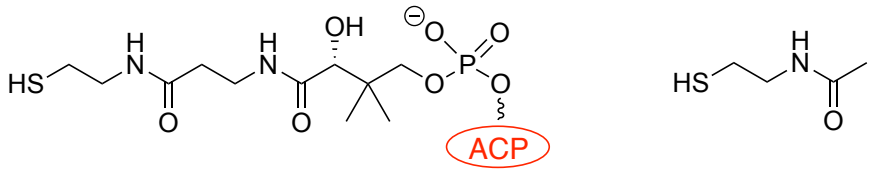


Figure 2-4. Two plausible methods of off-loading and cyclization of the polyketide product of CcsA

2.1.2 Project goals

A combination of organic chemistry and molecular biology techniques will be used to probe the biosynthetic pathway for cytochalasin E. Efforts toward the chemical synthesis of the hypothesized final octaketide product of CcsA will be described. The goal of this project is to synthesis the *N*-acetylcysteamine (SNAC) thioester of the proposed advanced polyketide product. SNAC is a sufficient mimic of the Ppant prosthetic group that is attached to the *holo* ACP (Figure 2-5). SNAC thioesters of fatty acid and polyketide biosynthetic intermediates are recognized and transformed in the same manner as if they were ACP bound.[16,27]



holo ACP *N*-acetylcysteamine

Figure 2-5. *N*-Acetylcysteamine is a mimic of the distal end of the *holo* ACP

To study the final polyketide product of CcsA and whether it is off-loaded as the aldehyde or the alcohol, synthesis of the proposed CcsA-bound product before off-loading was planned. Traditionally, a ^{13}C label is incorporated into the proposed substrate before feeding into the enzymatic system. Isotopic enrichment in the isolated final product can provide additional evidence of incorporation of the chemically synthesized proposed substrate. This technique is not always foolproof, as it can be impeded by low incorporation rates of the ^{13}C label being overwhelmed by the concentration of unlabeled material synthesized by the enzymatic machinery. Additional limiting factors are the cost of incorporation of a ^{13}C label into a chemical synthesis, the synthetic plan may be more difficult than it would be otherwise, and it can be stymied by low yields of small-scale reactions. Synthesis of a late-stage intermediate would circumvent these problems and would allow us to simply add the substrate, phenylalanine, and NADPH to the purified enzyme. Previous work with hypothemycin using ^{13}C labelled intermediates showed that more advanced intermediates are incorporated more readily than more primitive intermediates.[16] Furthermore, studies of the PKS-NRPS systems responsible for the production of tenellin[28] and aspyridone[29] have shown that the PKS and NRPS sections of a PKS-NRPS hybrid function independently. These studies suggest that there is a high likelihood the NRPS portion will recognize the octaketide SNAC thioester as a substrate. If this is the case, it will provide strong evidence for the identity of the polyketide

intermediate, and would allow us to confirm the structure of the final polyketide product of CcsA. We would also potentially see if the product is reductively off-loaded as the aldehyde or alcohol, or if it is off-loaded by undergoing a Dieckmann cyclization.

In collaboration with Dr. Yi Tang's lab at UCLA, we plan to illuminate what the final polyketide product is of the PKS portion of CcsA, and how this advanced intermediate is off-loaded from the enzyme. Our lab is responsible for the chemical synthesis of the proposed biosynthetic product of CcsA. The Tang lab will clone, express, and purify the enzymes CcsA (PKS-NRPS) and CcsC (*trans-acting* ER) (Figure 2-6). Enzyme assays will be carried out with the synthetic intermediate and the resulting product analyzed by LC-MS and NMR. If the proposed octaketide thioester intermediate is correct and it is recognized by the NRPS, only phenylalanine and NADPH will be required to transform it to the final released product.

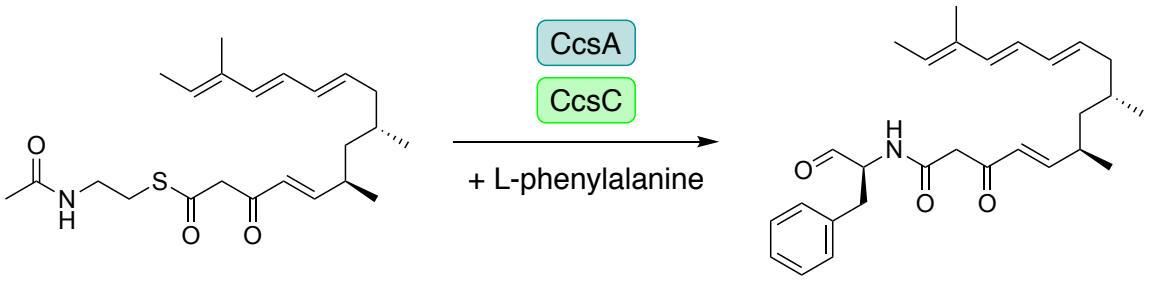
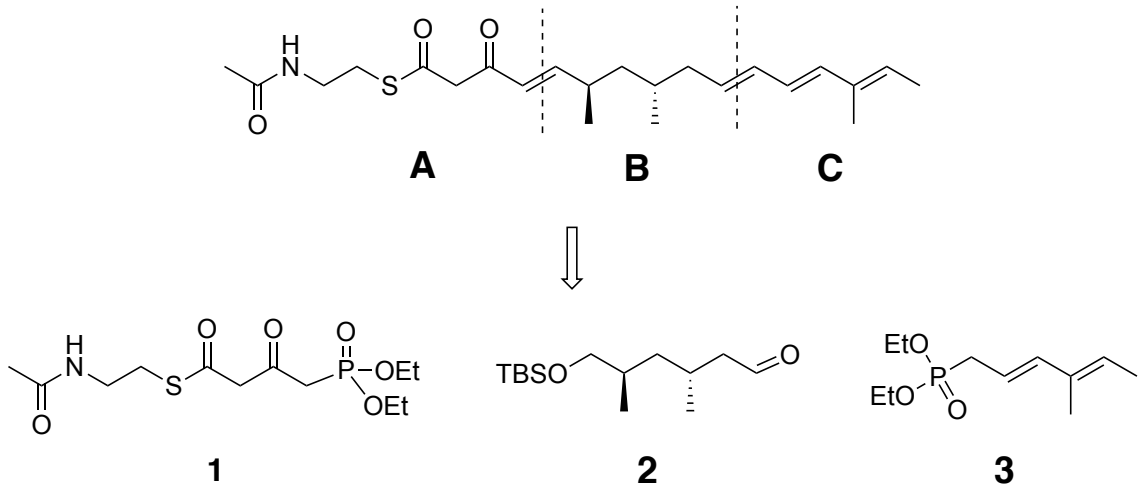


Figure 2-6. Proposed enzymatic assay and substrate for identifying the product of CcsA

2.1.3 Synthetic plan for the proposed late stage octaketide SNAC thioester

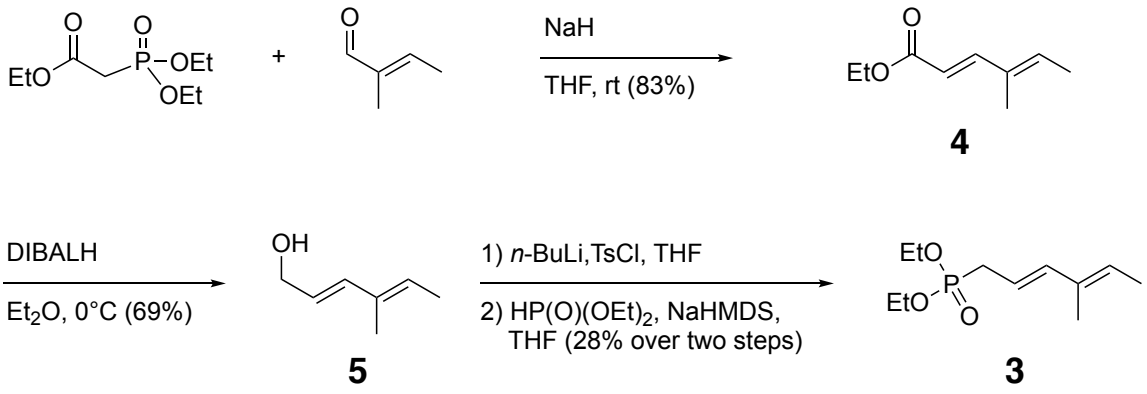
A retrosynthetic strategy for the synthesis of the proposed octaketide product of CcsA is shown in Scheme 2-1. The octaketide SNAC thioester will be assembled as three fragments (head, middle, tail) that will then be linked together through *E*-selective Horner-Wadsworth-Emmons (HWE) reactions.



Scheme 2-1. Retrosynthetic analysis of the octaketide SNAC thioester target. Fragment A = head group, fragment B = middle group, fragment C = tail group.

2.2 Results

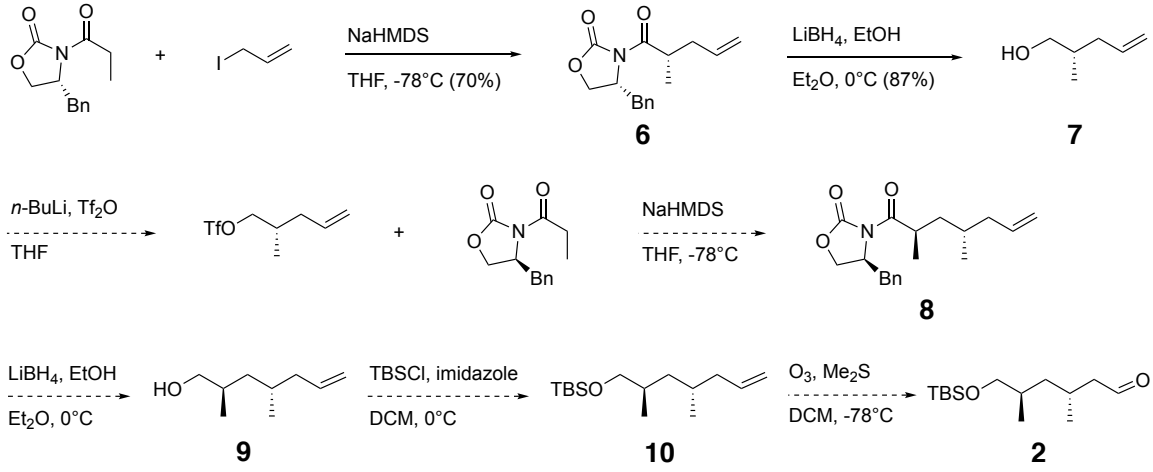
2.2.1 Synthesis of the octaketide tail fragment



Scheme 2-2. Synthesis of the octaketide tail fragment **3**

The strategy used for accessing the tail fragment is outlined in Scheme 2-2. The first step in assembling the allylic phosphonate was an HWE reaction using triethyl phosphonoacetate and tiglic aldehyde to yield conjugated ester **4**.[30] The ester was reduced to allylic alcohol **5** using diisobutylaluminum hydride (DIBAL-H) in moderate yield.[31] The alcohol was converted to phosphonate **6** by converting the hydroxyl to a good leaving group, then displacing it with freshly prepared sodium diethyl phosphite.[32]

2.2.2 Synthesis of the octaketide middle fragment

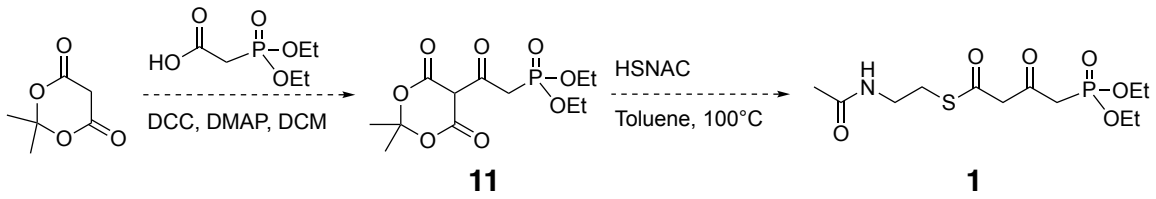


Scheme 2-3. Enantioselective synthesis of the octaketide middle fragment **12**

The approach for synthesizing the middle fragment of the proposed octaketide intermediate is outlined in Scheme 2-3. Evans' chiral oxazolidinone chemistry was used because it works well with a variety of substrates and is considered by some to be the standard for stereochemical induction of this type. The first steps in the synthesis of fragment B were the alkylation of commercially available (*R*)-4-benzyl-3-propionyl-2-oxazolidinone, followed by reductive cleavage to the unsaturated alcohol.[33] The *Z*-enolate of (*R*)-4-benzyl-3-propionyl-2-oxazolidinone was created with NaHMDS before adding allyl iodide to obtain the desired product **6**. The next step, a reductive cleavage of the chiral auxiliary using lithium monoethoxy borohydride, yielded the unsaturated alcohol **7**. The subsequent plan is to activate the alcohol with triflic anhydride and displace with (*S*)-4-benzyl-3-propionyl-2-oxazolidinone to install the second stereocenter and elongate the chain, creating Evans adduct **8**. Reductive cleavage of the chiral auxiliary using lithium monoethoxy borohydride will yield primary alcohol **9**. The alcohol will then be protected as a silyl ether to access compound **10**. Ozonolysis of the terminal alkene will then provide aldehyde **2** that will be reacted soon after in the series of reaction to stitch the fragments together.

2.2.3 Synthesis of the octaketide head fragment

Synthesis of the head fragment is outlined in Scheme 2-4. The synthesis will begin by coupling of diethyl phosphonoacetic acid to Meldrum's acid using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP) to create acylated Meldrum's acid derivative **11**. Heating the resultant compound at 100°C in the presence of *N*-acetylcysteamine (HSNAC) will then provide compound **1**.

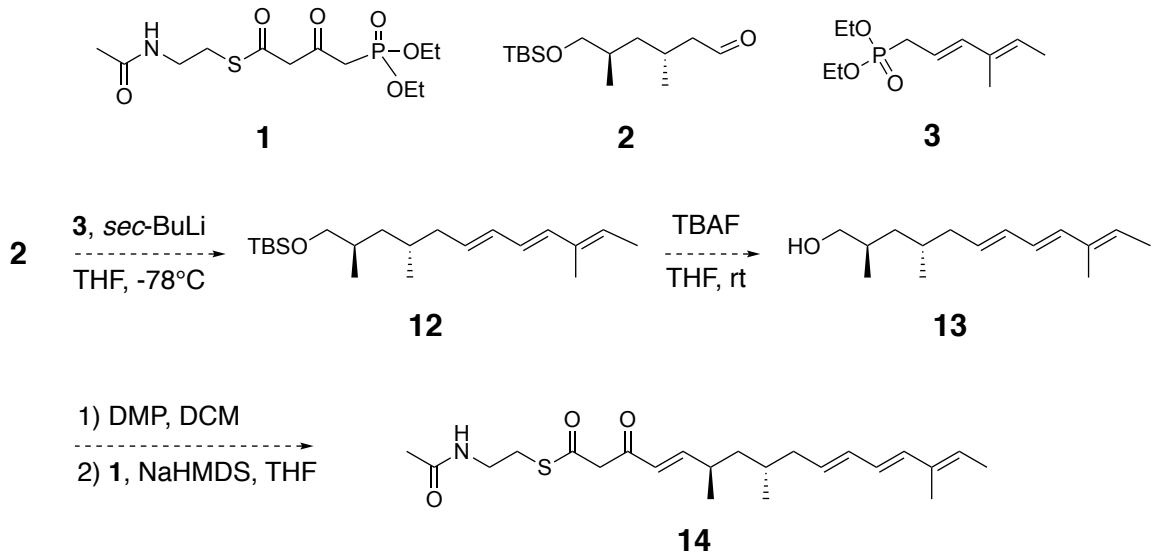


Scheme 2-4. Synthesis of the octaketide head fragment **1**

2.2.4 Linking of the three octaketide fragments to obtain the proposed CcsA polyketide product

The proposed scheme to connect the head, middle, and tail fragments together is outlined in Scheme 2-5. An HWE reaction will combine the middle and tail fragment to yield the conjugated triene containing all three bonds of *E* configuration. The product is expected to be sensitive to light and acid, so special handling is required to avoid isomerization and degradation. Exposure to light will be minimized and purification will be done using base-washed silica.

The first step will be to synthesize the ylide of phosphonate **3**, and then react with aldehyde **2** to create compound **12**. Due to the acid-sensitivity of the substrate, the reaction will need to be quenched with pH 7 phosphate buffer and purified on base-washed silica. Deprotection of the silyl ether with tetrabutylammonium fluoride (TBAF) will then give alcohol **13**. This compound will be oxidized to the corresponding aldehyde using Dess-Martin Periodinane (DMP) and then undergo the final HWE reaction to connect it to the head fragment and complete the synthesis of octaketide SNAC thioester **14**. For the final HWE reaction, three equivalents of base will be required to abstract all acidic protons on substrate **1**. The ylide is expected to react at the most nucleophilic carbon to create the desired oxaphosphetane which will eliminate to form the last (*E*)-alkene.



Scheme 2-5. Strategy for connecting the synthetic fragments of the proposed CcsA polyketide product

2.3 Conclusion and future work

The octaketide tail fragment was successfully synthesized, as well as intermediates in the production of the middle fragment. The proposed synthetic routes remain to be carried out. The synthesis will be challenging after the generation of the conjugated triene, which will need to be treated delicately in order to avoid degradation of the compound.

3 A Novel Polymethylated Polyketide

3.1 Introduction to methyltransferase, ketoreductase, and thioesterase domains, carnitine acyltransferases, and a novel polyketide system

3.1.1 Introduction to polyketide methyltransferases

In type I modular PKSs methyl groups can be incorporated into polyketides through the use of methylmalonyl-CoA derived extender units during chain elongation events, or the action of a C-methyltransferase (MT).[34] For all other PKSs, with only a handful of exceptions, a MT is responsible for the incorporation of a methyl group at the α-position during the elaboration of a polyketide intermediate.[14] In fungal PKS this is accepted to occur on β-ketoacyl-ACP species after chain elongation and before β-keto reduction.[35,36] PKS MTs belong to the class I MT superfamily that is characterized by a conserved SAM binding motif. There is low homology among the active sites residues as a consequence of the wide substrate variety that MT domains encounter. As a result, MT domains are harder to characterize than other domains – aside from recognizing a SAM binding motif as evidence for a MT.[37]

The lovastatin PKS system contains an in-line MT that acts *in cis* at the tetraketide stage. It is proposed that there are two possible mechanisms that can account for the selectivity of LovB MT (Figure 3-1). The first is that the enzyme may adopt an assembly line-like model in which the substrate is passed to each of the tailoring domains in the order of MT → KR → DH → ER and a binary decision is made (yes/no). In this model a domain will recognize and act upon the substrate, or it will not. The second hypothesis is that the transformations are kinetically controlled. The ACP-bound

substrate can sample all of the domains in the enzyme, and the relative rate of reaction is what determines whether methylation, reduction, or any other step occurs.

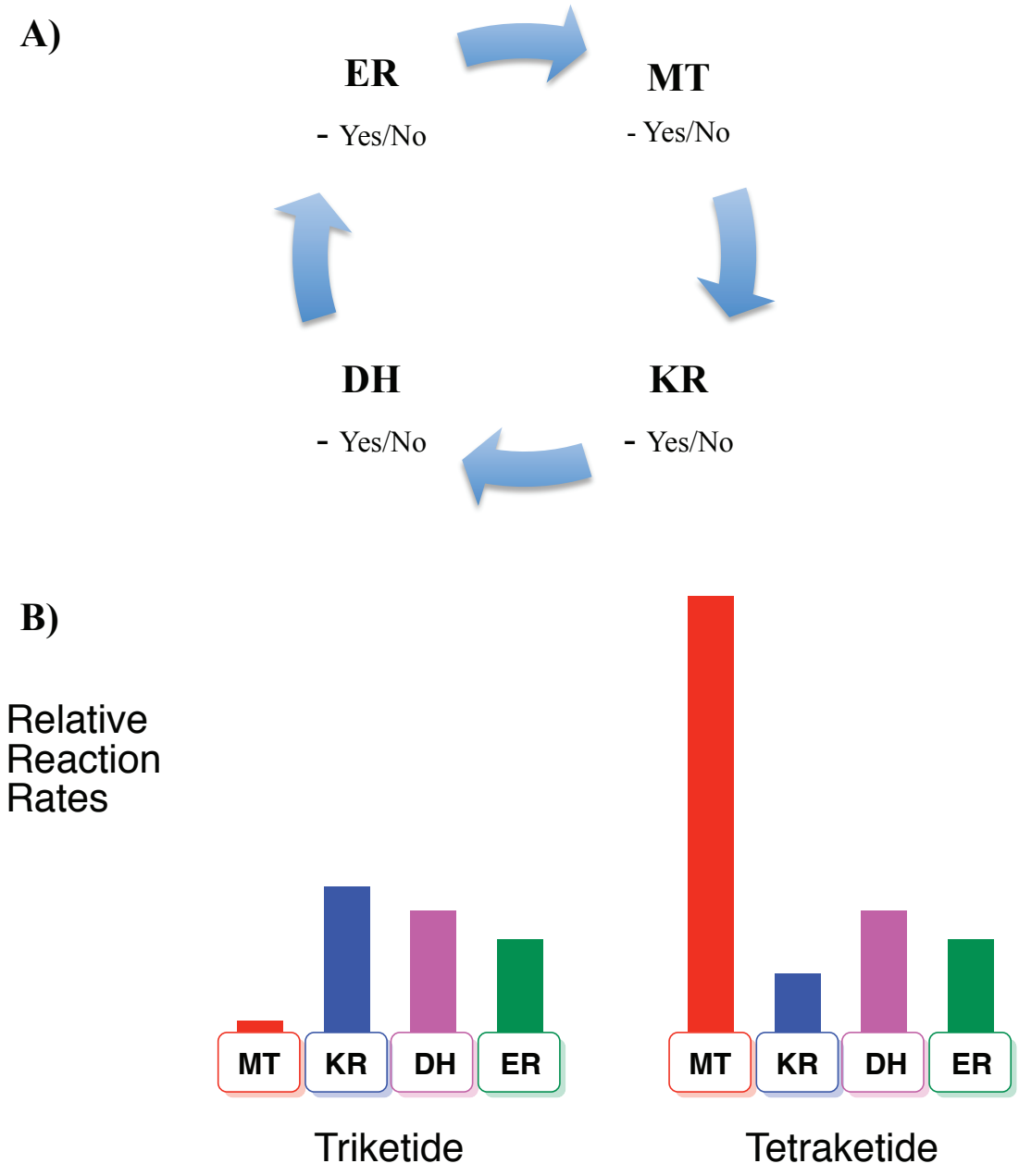


Figure 3-1. Two hypothesized methods of PKS tailoring domain selectivity in the lovastatin system. A) Assembly line or binary (yes/no). B) Kinetically-controlled.

Cacho *et al.* measured the rates of transformation of the MT and KR domains toward natural and model β-keto-SNAC thioester substrates.[36] These kinetic studies

showed that LovB MT is much more selective toward its natural substrate versus the KR domain. The selectivity shown by the MT is achieved predominantly by the high kinetic efficiency at which it transforms its natural substrate relative to that of the KR. The results of kinetic assays are shown in Figure 3-2. LovB MT was determined to have a 2500-fold higher catalytic efficiency for the natural tetraketide substrate than for the triketide, arising from an approximately 50-fold difference in both the k_{cat} and K_M values. The other natural β-keto substrates of the LovB system were not methylated in detectable amounts. There was some activity measured with unnatural tetraketide substrate, however it was significantly less than the natural tetraketide.

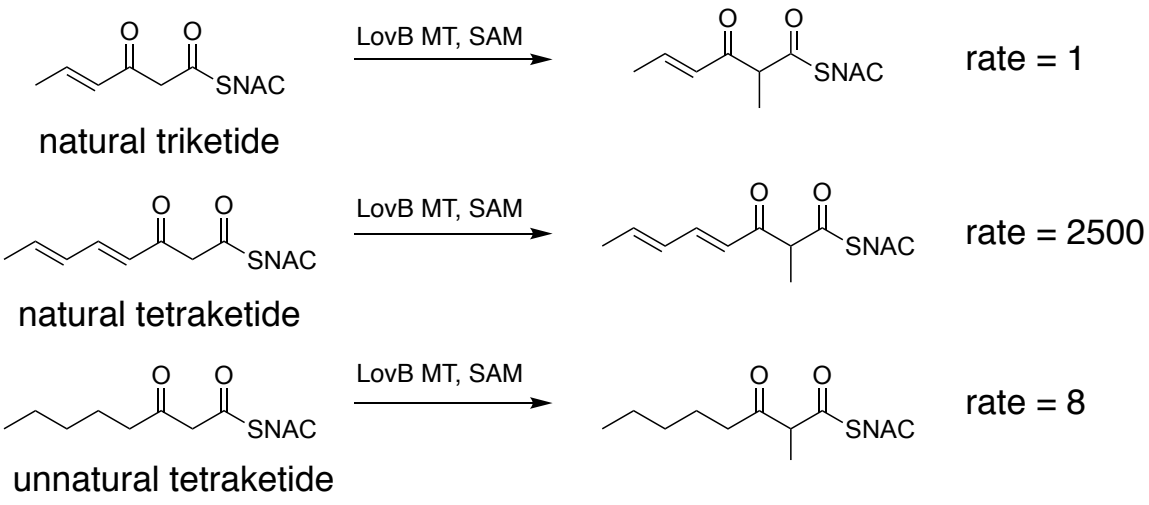


Figure 3-2. Rates of transformation of natural and unnatural substrates by LovB methyltransferase

3.1.2 Introduction to ketoreductases

KR domains are responsible for reduction of the β-keto functionality of ACP-bound β-ketothioester species. Ketoreductases belong to the short-chain dehydrogenase/reductase family and are known to be NAD(P)H-dependent oxidoreductases.[38] KRs may be present as part of the polypeptide chain that is responsible for the construction of the polyketide (*cis*) or may be a separate protein that associates

with the PKS for reduction events (*trans*). In systems that produce reduced polyketides, *trans-acting* KRs replace a nonfunctioning *cis-acting* KR if it lacks an NADPH binding motif or the conserved catalytic Ser-Tyr-Lys triad. KRs are classified based on the stereochemistry of their hydroxyl product, with Type-A KRs producing an L-configuration and Type-B KRs producing a D-configuration. KRs are also involved in setting the stereochemistry of methyl groups added by MTs, and this is another way to further classify KRs.[39] It is accepted that methyl groups are incorporated at the α-carbon during the β-ketoacyl stage, and it is relatively easy to epimerize this stereocenter due to the acidic α-proton. Once the β-keto group is reduced to a β-hydroxy group, epimerization is no longer possible (without the action of a dedicated epimerase), and the methyl stereochemistry will be set at this point. As such, KRs can be subclassified depending on how they discriminate against β-keto substrates. Some KR will only accept one epimer as a substrate, and may or may not work in tandem with a KS domain that catalyzes epimerization of the substrate. Other KR domains directly catalyze epimerization of the substrate before reducing the desired epimer, or cryptically epimerize through the reduction of the enol tautomer that is then protonated on the correct face to yield the desired stereochemistry at the α-centre.

The active site of a KR contains a conserved Ser-Tyr-Lys triad that is responsible for activation of the β-ketothioester and stabilization of the oxyanion intermediate (Figure 3-3). The stereochemical outcome of the reduction is dependent on the path by which the substrate enters the active site, since this determines which prochiral face of the β-ketothioester is attacked by the hydride from NADPH.[38] The domain is structured in a way that restricts the entrance of the ACP-bound β-ketothioester. Type A KR contain

a tryptophan residue positioned between the catalytic serine and tyrosine residues that prevents the ACP-bound β-ketothioester from entering on that side. Type B KR do not have this tryptophan residue present and the substrate will enter from this side of the active site. They also have an additional "LDD" motif present on a loop near the other side that prevents access. Based on this model of β-keto reduction, it is expected that all hydroxyl groups that are introduced by a single PKS domain and remain in the product will have the same relative stereochemistry. This is generally the case, with a notable exception being that of hypothemycin[40] (Figure 3-4).

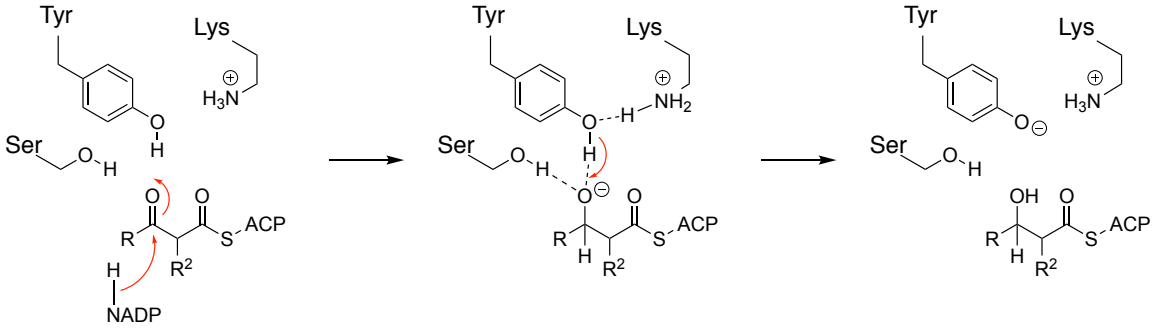


Figure 3-3. General mechanism of a ketoreductase domain catalyzed reduction. Modified from Xu *et al.*[37]

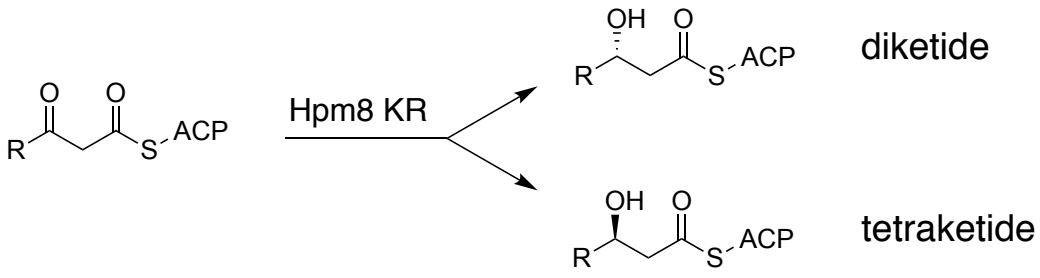


Figure 3-4. Differential stereoselectivity in hypothemycin ketoreductase. Modified from Xu *et al.*[38]

In the first part of lovastatin biosynthesis, LovB in tandem with LovC, a *trans-acting* ER, is responsible for the construction of dihydromonocolin L (DML). DML is a cyclized nonaketide and the first enzyme-free intermediate in lovastatin biosynthesis.[41]

LovB KR is utilized in every iteration during the production of DML and is predicted to have a relaxed substrate tolerance as a consequence. Cacho *et al* found this to be the case, with the turnover rate of the isolated KR domain on the tested aliphatic triketide and tetraketide substrates agreeing with each other within an order of magnitude[36] (Figure 3-5). Due to problems with the LC-MS assay, the authors were not able to test all of the desired substrates – including the natural substrate. However the results that were obtained do suggest that the KR domain has relaxed selectivity and its activity is not significantly affected by the presence of an α-methyl group.

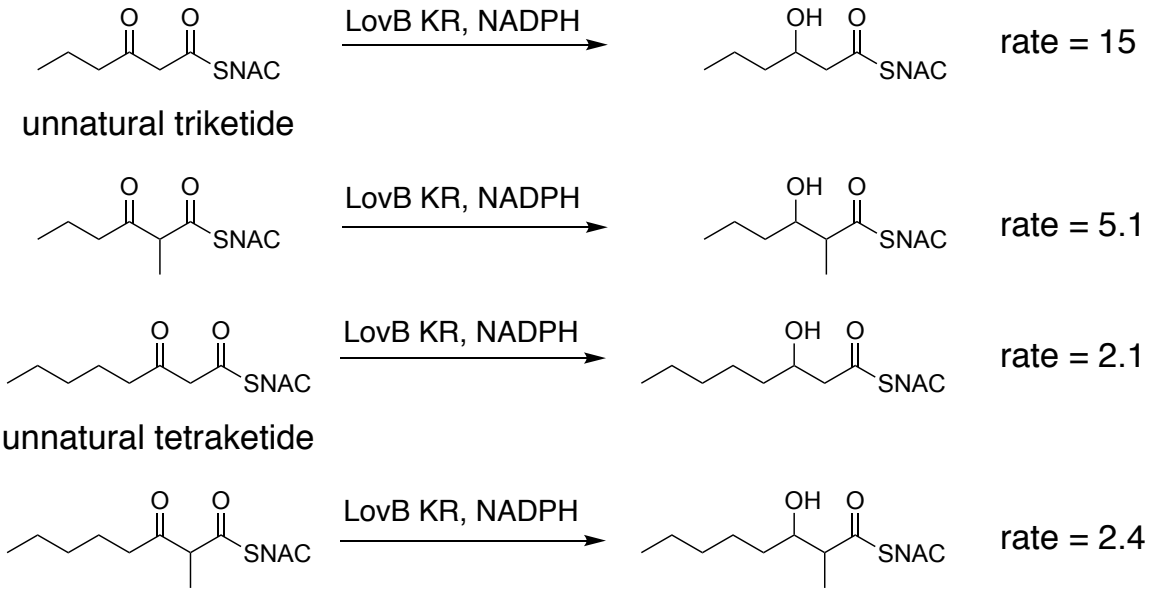


Figure 3-5. Relative reduction rates by LovB ketoreductase on β-keto-SNAC thioester compounds

3.1.3 Competition between methyltransferase and ketoreductase domains

In lovastatin biosynthesis the function of the KR domain was studied simultaneously with that of the MT domain.[36] To obtain a more accurate picture of the domain specificity toward different acyl groups, a combined MT/KR assay was performed. A LovB mutant with an inactivated DH domain (LovB DH°) was incubated

with substrate and equimolar amounts of both SAM and NADPH. The results are summarized in Figure 3-6 and are consistent with the individually determined kinetic parameters and the natural programming rules of LovB. The KR domain is more active than the MT domain on the aliphatic triketide substrate by a 4:1 margin. The product ratio is inverted with the aliphatic tetraketide substrate. The selectivity is even higher on the natural triketide with a 10:1 ratio of reduced product relative to methylated product. As expected, when testing the natural tetraketide only methylated product was detected. This suggests that the MT domain acts extremely fast on its natural tetraketide substrate and outcompetes the KR domain before it has an opportunity to reduce the β-keto functionality.

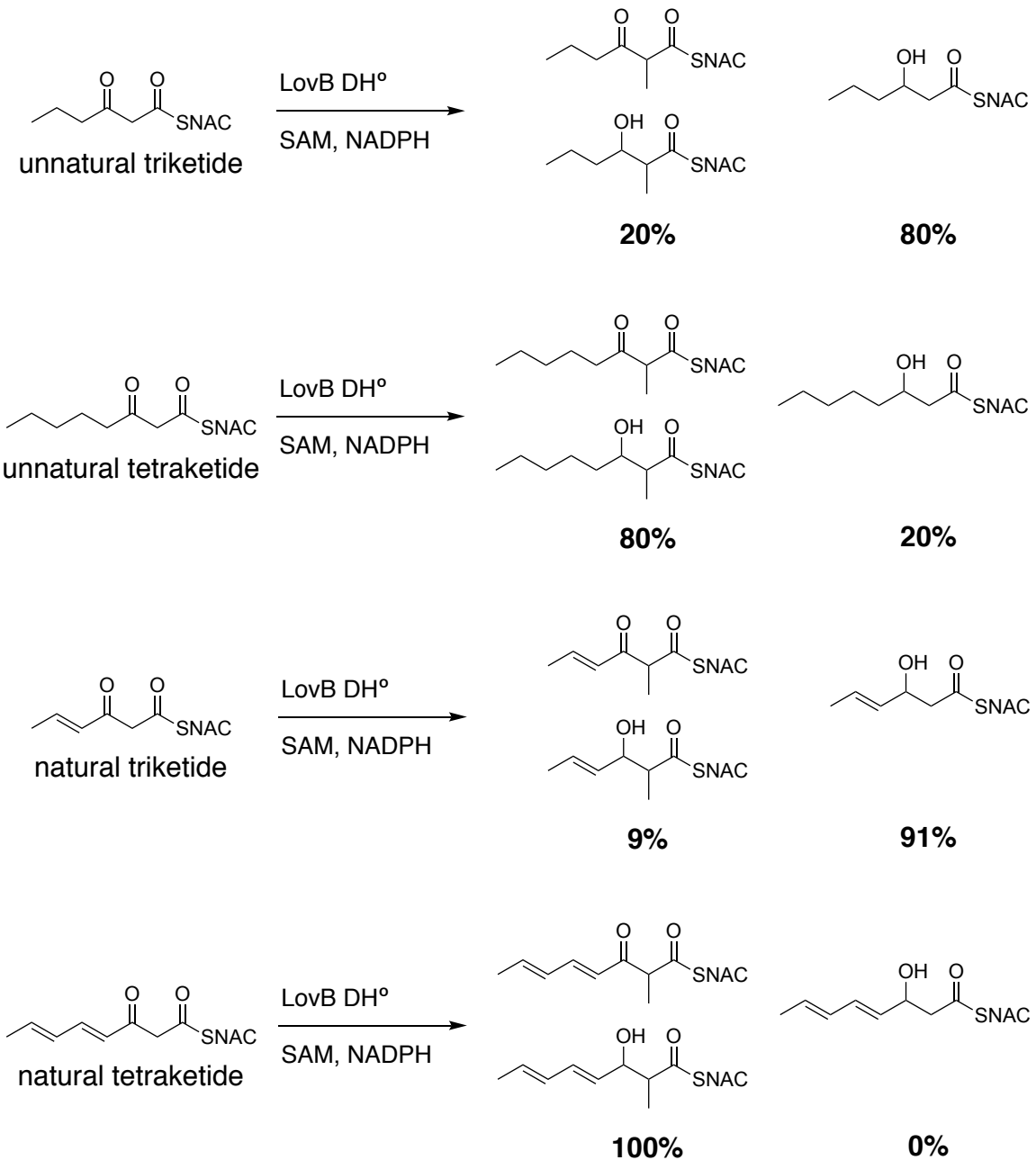


Figure 3-6. Relative reaction rates by LovB DH° on β-keto-SNAC thioester compounds. Numbers indicate relative amounts of products measured for each substrate. Left number refers to the methylated product, right number to the reduced product.

In summary, chain length is important for discrimination of the MT domain against the substrate, but functionality is even more important. When the natural substrate with the correct functionality and chain length is presented to the MT domain, it is

processed with an exceptionally higher efficiency than any other potential substrates. It outcompetes the KR domain for the first reaction after chain elongation to create the tetraketide. Notably, these experiments suggest that the transformations are kinetically-controlled by the relative rates that the domains act upon the maturing substrate, and not in an all-or-nothing fashion.

3.1.4 Introduction to thioesterases

Thioesterase (TE) domains are essential for the production of polyketide, fatty acid, and NRPS-derived natural products. A TE domain catalyzes the cleavage of the thioester bond that attaches the polyketide to the enzyme.[14] This prevents stalling of the enzyme and allows for timely turnover. TE domains also play a role in adding diversity to natural products. TEs may catalyze off-loading of a polyketide substrate through an external nucleophile, for example water or an alcohol. The result in these cases will be a carboxylic acid or ester product. It is also possible for macrolides or Claisen-condensation products to be obtained through an intramolecular *O*-, *N*-, or *C*-nucleophile.

As of 2016 there are nine high-resolution crystal structures of TE domains.[7] Crystal structures show two helices in the TE domain that form a lid that is proposed to be the basis for substrate selectivity. The lid and the TE domain core form a channel that limits the approach of the ACP-bound substrate and potential nucleophiles. In some TE domains this appears to be a restrictive channel that limits what can enter it, for example DEBS TE. In other TE domains the channel is more open - like a trough – as is the case in the curacin biosynthetic pathway. Crystal structures obtained with diphenylphosphonate-based inhibitors have shown that the primary substrate-enzyme interactions are hydrophobic packing, rather than dipole-dipole or hydrogen bonding. TE

domains typically display substrate selectivity – particularly those associated with iterative systems - though some TE domains show more promiscuity than others.

There are two structural classifications of TEs: α/β hydrolase-fold TEs and "hot-dog" fold TEs.[38] α/β hydrolase-fold TEs can act *in cis* as part of the same PKS responsible for the production of the polyketide (type I TEs) or can be distinct proteins that act *in trans* (type II TEs) to hydrolyze stalled chains from a PKS. With α/β hydrolase-fold TEs off-loading is accomplished through a two-step mechanism[38] (Figure 3-7). First the ACP-bound polyketide chain is loaded onto a serine residue on the TE domain, consistent with known serine hydrolase mechanisms. The serine residue is activated by a His-Asp dyad to increase its nucleophilicity. The serine attacks the carbonyl of the ACP-bound thioester, the resulting charged tetrahedral intermediate is stabilized by an oxyanion hole, and then the ACP thiolate is lost to create the acyl-TE intermediate. The carbonyl of the acyl-TE is then attacked by a nucleophile, the charged tetrahedral intermediate is stabilized by the oxyanion hole, then the intermediate collapses to release the off-loaded product as well as the serine alkoxide with concurrent protonation. "Hot-dog" fold TEs are characterized by a five-strand anti-parallel β-sheet wrapping around a long α-helix, along with several smaller helices present. "Hot-dog" fold TEs use a different catalytic mechanism than α/β hydrolase-fold TEs that does not create any covalent substrate-enzyme intermediates (Figure 3-8).

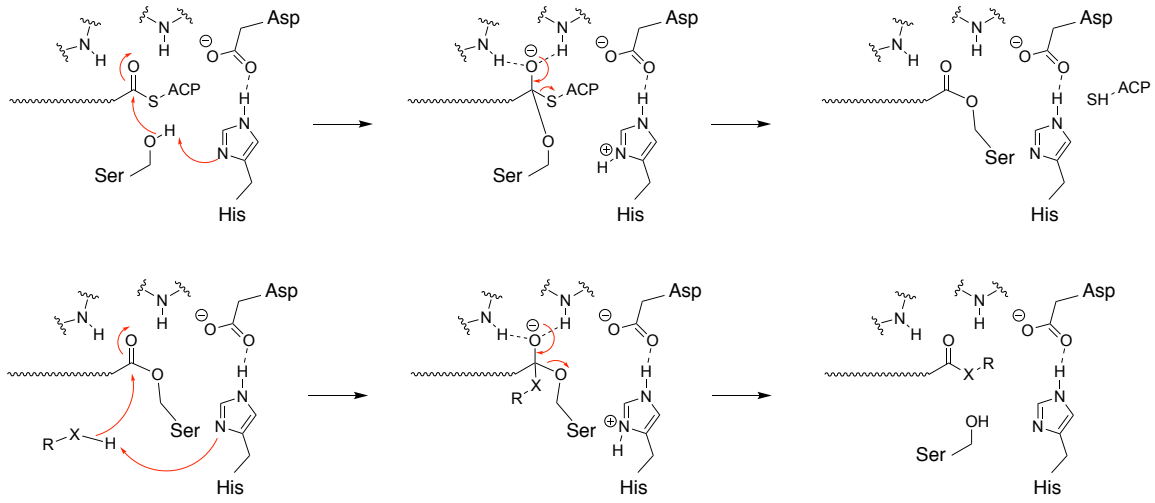


Figure 3-7. Generalized mechanism of loading and off-loading in α/β hydrolase-fold TEs. "X" can be an intramolecular nucleophile or an external nucleophile. Modified from Xu *et al.*[38]

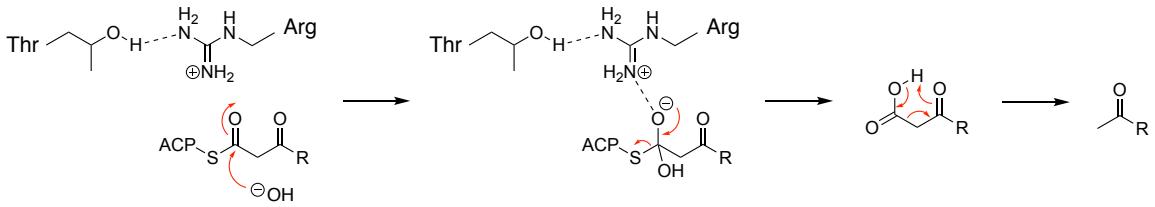


Figure 3-8. Generalized mechanism of decarboxylative hydrolysis in "hot-dog" fold TEs. Modified from Xu *et al.*[38]

3.1.5 Introduction to carnitine acyltransferases

Fatty acids are often activated in metabolic processes by an ATP-driven conversion to their respective CoA thioester.[42] The simplest relevant example of this is the conversion of malonic acid to malonyl-CoA – the extender unit for most PKSs. While this is not a problem for the synthesis of limited quantities of metabolites, the downside to doing so is that it sequesters the limited CoA resources within the cell, and the resulting esters are not membrane permeable. Cells have a method for controlling the concentration of CoA thioesters without the energy-intensive and time-consuming process of resynthesizing them from the free acid. This method also enables the acyl

moiety to permeate membranes without the expenditure of additional energy.[43] The cell achieves this through the formation of carnitine esters from CoA esters and L-carnitine.[42] By catalyzing this reaction, carnitine acyltransferases (CATs) regenerate non-esterified CoA while creating activated esters that can move more easily through cell membranes.

CATs catalyze the reversible transesterification between long chain acyl-CoA esters and carnitine (Figure 3-9). There are several different types of carnitine acyltransferases, of which many are named carnitine palmitoyltransferases (CPTs) for historical reasons due to their role in the metabolism of palmitic acid.[43] CATs are found in the mitochondria, peroxisomes, and the endoplasmic reticulum. CATs are divided into two classes depending on whether or not they are inhibited by malonyl-CoA. All CATs that face the cytosol are integral membrane proteins that are inhibited by binding of malonyl-CoA to an allosteric site on the protein.[42] This class of CATs is also inhibited by CoA esters of substrate analogues that contain a 2-oxirane ring moiety. CATs that are not sensitive to inhibition by malonyl-CoA occur as soluble proteins that are spread throughout the lumen of the organelle they are present in, or may be loosely associated with the inner membrane.[43] Each organelle contains both types of CATs and together they form a transferase system which allows the shuttling of acyl moieties across membranes that would be impermeable to the analogous CoA thioester.

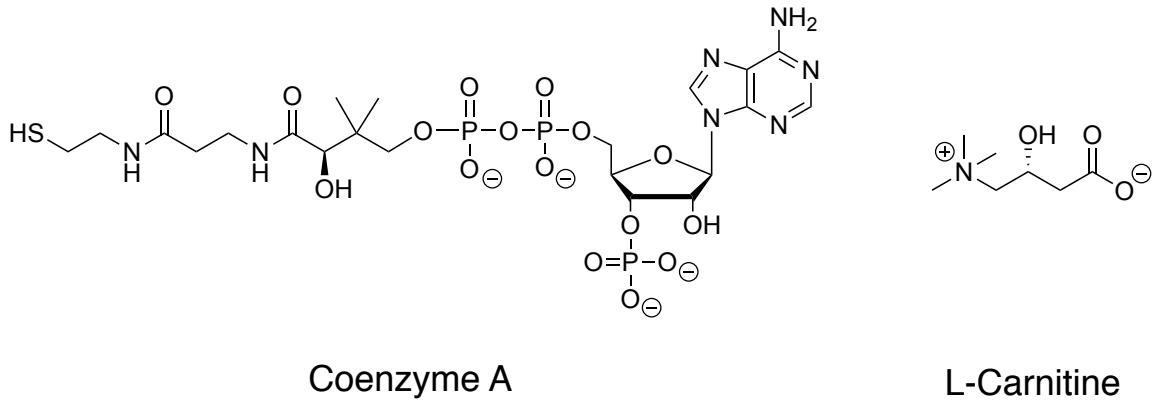


Figure 3-9. Structures of CoA and carnitine, and the transesterification catalyzed by carnitine acyltransferase

CATs were first studied in the 1950s in the context of energy homeostasis in the mitochondrion.[43] Carnitine palmitoyltransferase I (CPT I) and II (CPT II) are the most well characterized members of the CAT family. CPT I and CPT II are essential for the transportation of fatty acids across the mitochondrial membranes and eventual utilization as fuel in the Krebs cycle.[44] CPT I and CPT II work in concert with a translocase on the mitochondrial inner membrane to transport fatty acids across the outer and inner membrane, then prepare the activated fatty acid ester for degradation in the β-oxidation pathway[43] (Figure 3-10). The β-oxidation pathway is used to generate acetyl-CoA, which is ultimately utilized to generate ATP through the Krebs cycle.[42]

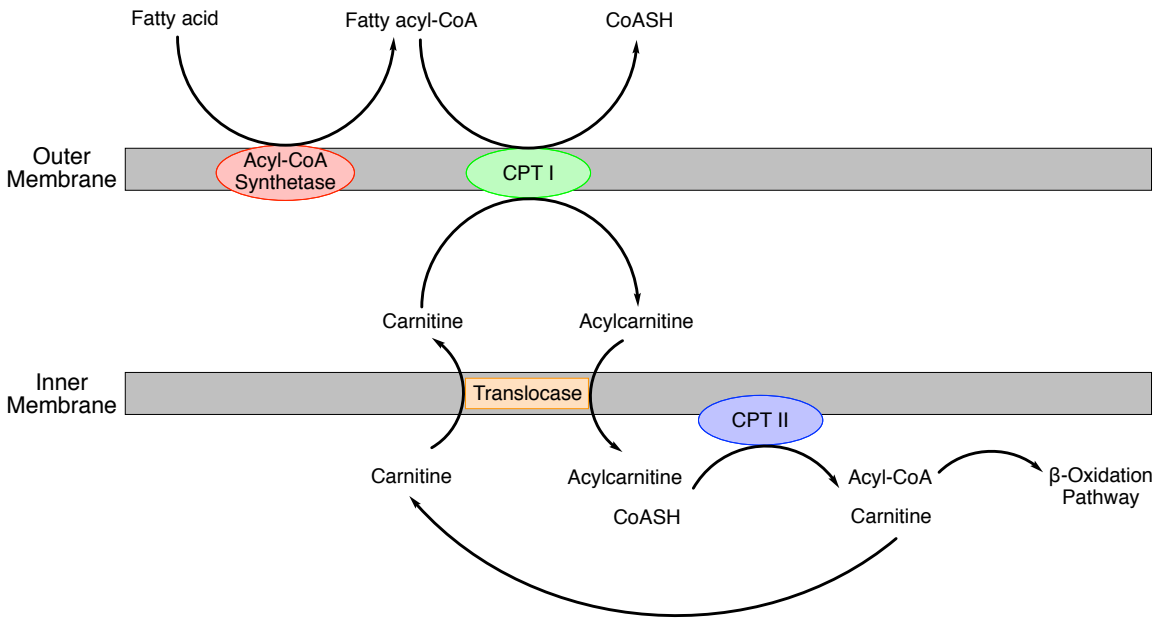


Figure 3-10. The mitochondrial carnitine palmitoyltransferase system. Adapted from McGarry and Brown.[43]

CATs contain a long tunnel that runs through the center of the enzyme. The two substrates enter from opposite ends of the tunnel and have access to the centrally positioned histidine residue in the active site.[44] The acyl chain is stabilized by a hydrophobic pocket, and the carboxyl group of carnitine is stabilized by a number of hydrogen bonding interactions. There appears to be no specific interactions with the trimethylammonium group of carnitine, although it is necessary for catalysis and may be involved in stabilizing the oxyanion intermediate formed during the reaction. There is also a conserved serine that helps stabilize the oxyanion intermediate in the same fashion as the oxyanion hole in serine hydrolases. Even though the mechanism by which CATs work is not completely understood, it is accepted that it involves a catalytic histidine residue. In the 1980s experiments showed that lowering the pH from 8 to 6 significantly decreases the activity of carnitine acyltransferases.[43] It has since been demonstrated that the histidine modifying reagent diethyl pyrocarbonate quickly inactivates the enzyme.

The enzyme is also inactivated by the presence of bromoacetyl-CoA and L-carnitine through the alkylation of the active site histidine. Studies of the related choline acetyltransferase suggest that the catalytic mechanism involves a His-Asp-Ser triad similar to serine proteases. In serine proteases, and in α/β hydrolase-fold TEs, a serine is deprotonated by a histidine residue and an aspartic acid residue acts as a charge relay system. By analogy, in carnitine acyltransferases it may be a catalytic dyad that is present, with the hydroxyl group of carnitine in place of the typical serine residue. A crystal structure of human peroxisomal carnitine acyltransferase shows the histidine residue is in the correct position to act as a general base to deprotonate either the carnitine hydroxyl group or the CoA thiol group, depending on the direction of the reaction[45] (Figure 3-11). Alternatively the production of acylcarnitine may proceed through an acyl-*S*-enzyme intermediate,[46] though there is not yet consensus on this proposal.

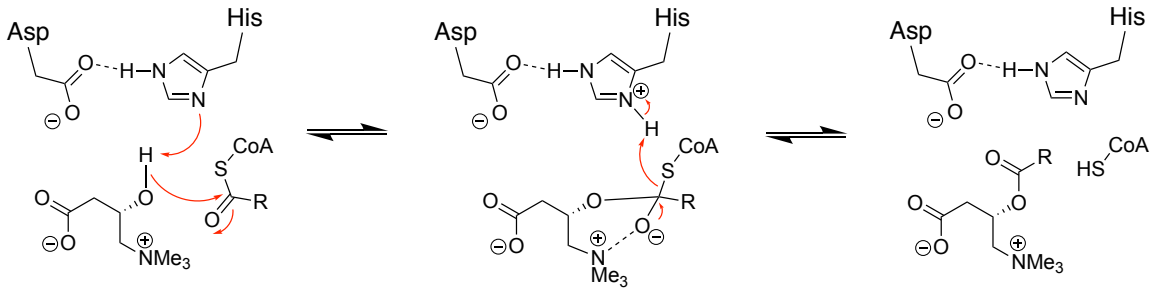


Figure 3-11. Putative catalytic mechanism of carnitine acyltransferases.

3.2 A novel polyketide system

3.2.1 An unexpected PKS product

Our collaborators, the Yi Tang group at UCLA, undertook the genome mining of 581 sequenced ascomycetes and basidiomycetes genomes. Within these genomes they discovered an uncharacterized clade of approximately 100 members that contain a PKS cluster with a C-terminal domain that has a strong sequence homology to the CAT family.[47] One member of the identified PKS clusters was within the genome of *Trichoderma virens* strain Gv29-8. *T. virens* is a fungus associated with the root systems of plants that is known to be beneficial for growth and development.[48] *T. virens* is also being studied for its ability to produce secondary metabolites, and for bioremediation. The gene cluster and its PKS are both named Tv6-931.

Expression and isolation of Tv6-931 yielded enough protein for enzymatic assays. The chain extending and tailoring domains were determined to be functional, but the addition of carnitine and related compounds did not yield esterified off-loaded products. Serendipitously, when the assay was repeated using different buffer conditions, esterified products were detected (Figure 3-12). When using Tris buffer a polyketide product was observed to have been off-loaded onto one of the hydroxyl groups of Tris, and when using PBS buffer containing glycerol the polyketide fragment was off-loaded onto a primary hydroxyl of glycerol. NMR, MS, and further incubation experiments with [2-^{13}C]-malonate confirmed the tetraketide structures shown in Figure 3-12.

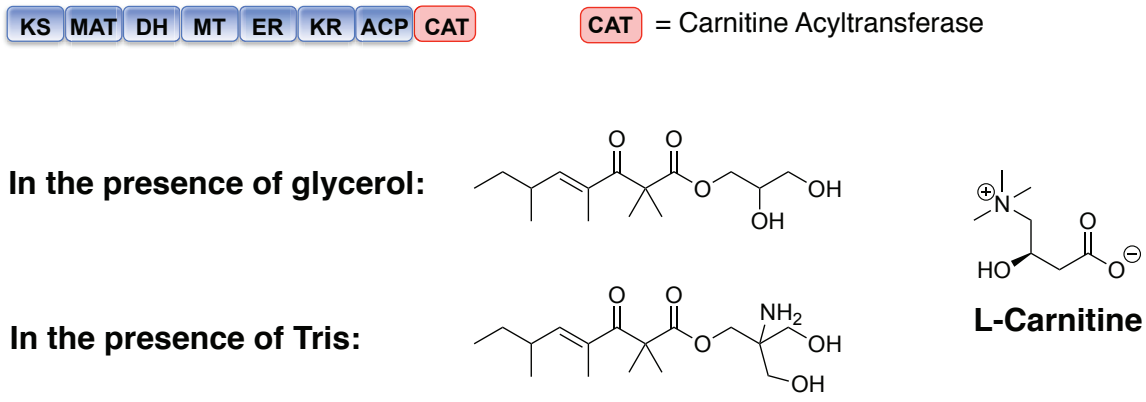


Figure 3-12. Unexpected product from expression of a PKS gene cluster from *Trichoderma virens* Gv29-8

Heterologous expression was done in *Saccharomyces cerevisiae* and *Aspergillus nidulans* with 1% of various off-loading nucleophiles. It was revealed that 1,1,1-tris(hydroxymethyl)ethane (THME) and pentaerythritol (PE) were equally effective at off-loading the substrate from the enzyme, and were even better substrates than glycerol or Tris.[47] Experiments with simplified polyketide substrates and an inactivated CAT domain demonstrated that methylation takes place independently of the CAT domain and the MT domain distinguishes between triketide and tetraketide substrates. It was confirmed that the CAT domain was responsible for off-loading and will do so with a range of polyol substrates. Comparison of the Tv6-931 CAT domain with other CATs revealed that the catalytic active site residues are conserved, though the residues specifically responsible for recognizing carnitine are absent. This partially explains the domain's relative promiscuity in alcohol nucleophiles utilized for off-loading.

An important aspect of CATs is the reversibility of the transesterification of acyl-CoA thioesters and carnitine that the enzymes catalyze. This allows the transfer of acyl moieties across membranes, then their conversion back to the corresponding activated CoA thioester. This could be advantageous to Tv6-931 with respect to obtaining

the desired gem-dimethyl moiety, since it was observed that the second methylation is very slow (catalytic efficiency of 1 turnover per hour with the unnatural tetraketide substrate).[47]

The reversibility of the CAT domain was tested by mixing equimolar amounts of THME tetraketide esters with PE in the presence of Tv6-931.[47] A roughly 1:1 mixture of THME and PE esters was observed (Figure 3-13). The transesterification was not able to be replicated with a stand-alone CAT domain, or with a Tv6-931 variant lacking a functional ACP. This suggests that the α-monomethyl product may be reloaded onto the ACP as a thioester and then eventually undergoes a second α-methylation before being off-loaded as an ester. The reloading and off-loading seems unproductive, though this quirk is an effective way of recycling prematurely off-loaded α-monomethyl polyketide. It allows the substrate to reenter the PKS for a second α-methylation, which appears to be a kinetically slow step. By off-loading as an ester it also allows the product to be easily reloaded onto the enzyme without going through the energetically costly steps of activating a free acid, which would be the product if the substrate sat on the enzyme long enough to be released hydrolytically.

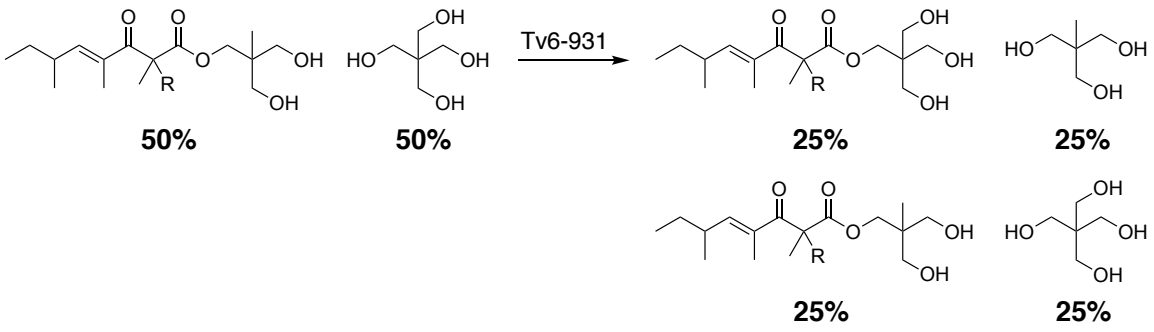


Figure 3-13. Transesterification of off-loaded polyketide product catalyzed by the CAT domain in Tv6-931. Bolded numbers represent the relative amounts of starting materials and products. R = H or CH_3.

These polyketide products are peculiar in that they are *gem*-dimethylated, which is relatively rare in natural products. It is even less common in fungal polyketides than in bacterial systems, with no MT responsible for α-dimethylation identified as of yet.[47] The products of this PKS system display an interesting and varied methylation pattern at the di-, tri-, and tetraketide stages, as well as varied degrees of reduction carried out at each stage. This system is well suited for studying the interplay of the enzyme domains involved in methylation and reduction events. Additionally, the fusion of a PKS with a CAT domain represents a potentially new mode of product release. This system is relatively promiscuous in its substrates and adds to the already large diversity of polyketide natural products.

3.2.2 Proposed biosynthesis of the isolated polyketides

The proposed biosynthetic pathway for the creation of the isolated polyketide products is shown in Figure 3-14. As already established, methylation is thought to take place at the β-keto stage following chain elongation by the KS domain. Methylation is followed by varying degrees of tailoring before another round of chain extension. It is not known at this time what the natural substrate is for off-loading of the polyketide product.

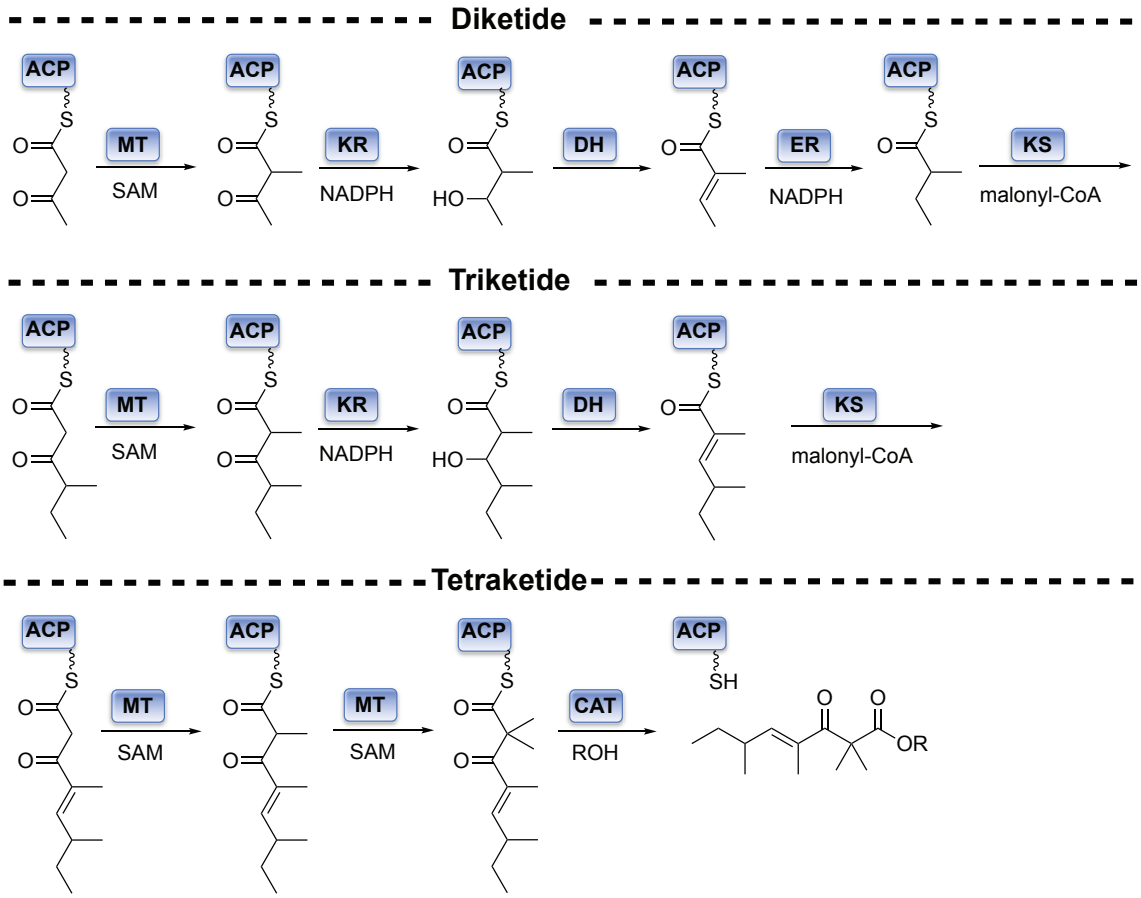


Figure 3-14. Proposed biosynthesis of isolated polyketide products from Tv6-931.

3.2.3 Project goals

The synthesis of SNAC thioesters of ten proposed biosynthetic intermediates was undertaken with the goal of using them to probe a number of questions raised by this polyketide system (Figure 3-15). It is hoped that the following questions can be addressed by these experiments:

- Will the results already obtained with simplified polyketide substrates be replicated by the proposed biosynthetic intermediates?
- What is the configuration of the methyl stereocenter and does it influence subsequent transformations?
- Why does methylation occur only once on the diketide and triketide, but twice at the tetraketide stage?
- How are the activities of the methyltransferase and ketoreductase domains coordinated to achieve this methylation pattern?
- What is the natural product of this PKS system?

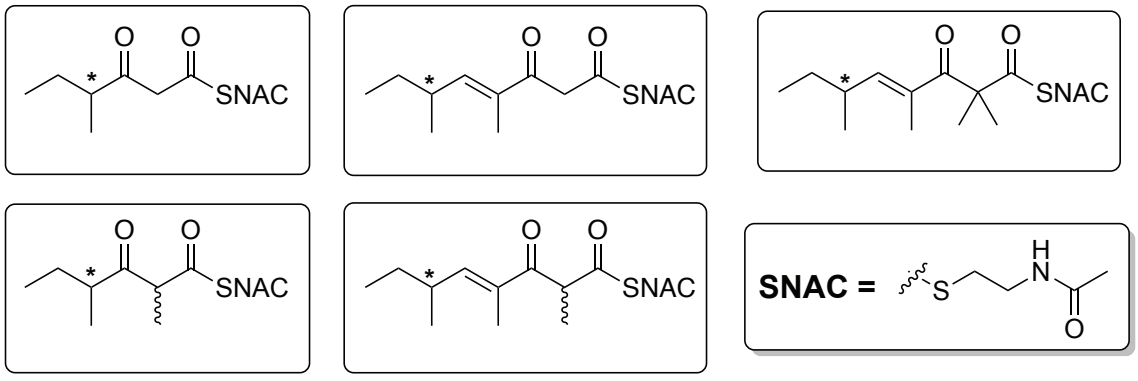


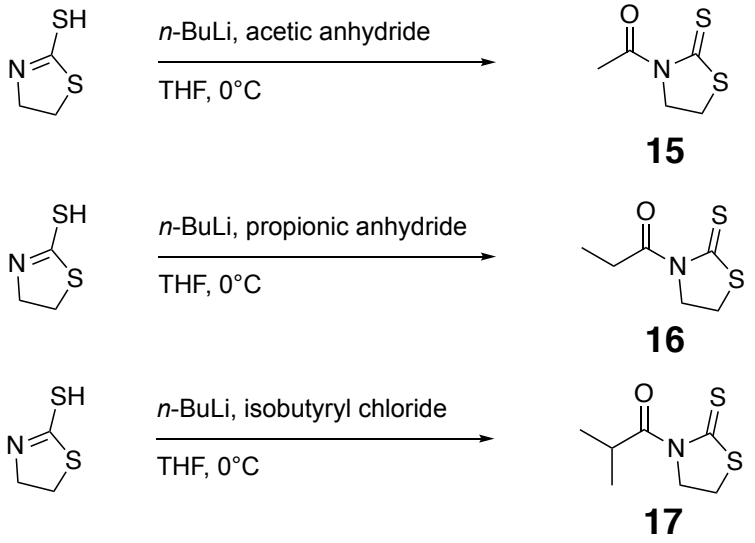
Figure 3-15. Targeted synthetic compounds. Both stereoisomers at the (*)-labeled carbon were targeted.

Substrates were synthesized with both stereochemical configurations at the distal methyl group. One example is shown to illustrate the process for asymmetric synthesis. The work for the shown stereoisomer in the synthetic schemes was performed by myself, and the enantiomeric series was synthesized by Dr. Christian Foerster.

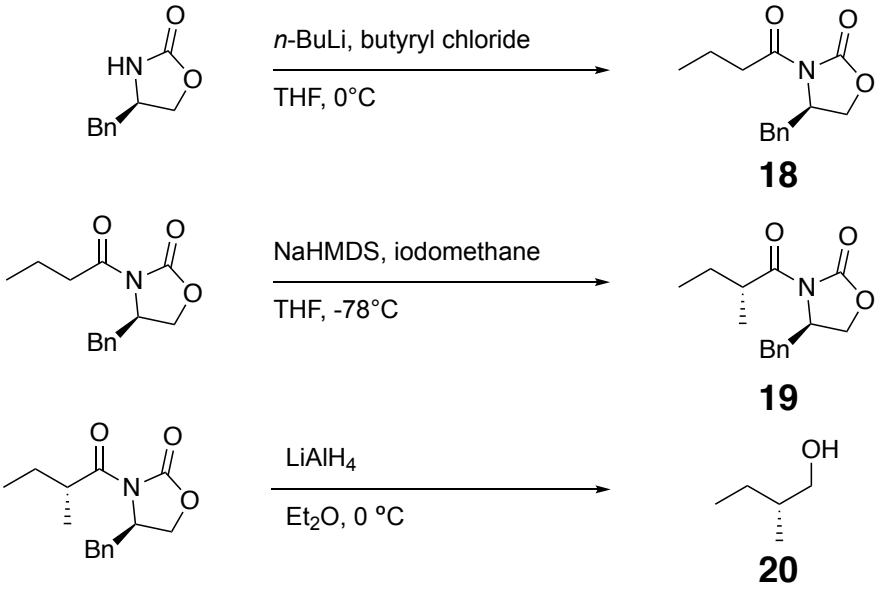
3.2.4 Synthetic plan for SNAC thioesters of proposed biosynthetic intermediates

The original synthetic scheme is presented in Scheme 3-1, Scheme 3-2, and Scheme 3-3. The compounds will be accessed through a divergent methodology that will allow material to be redirected at different stages of elaboration to access all of the desired compounds. The first reactions undertaken were to synthesis the *N*-acyl-thiazolidine-2-thiones **15**, **16**, and **17** that will be utilized for Crimmins' aldol reactions. Crimmins' auxiliary (thiazolinethione, originally known as the Fujita-Nagao auxiliary)[49] is preferable to Evans' auxiliary (oxazolidinone) in this synthetic scheme because the resultant aldol product can be easily converted to the SNAC thioester by displacing the auxiliary with HSNAC and K_2CO_3. A secondary benefit is that the aldol adducts are easily purified – aided by the bright yellow colour of *N*-acyl-thiazolinethiones. It is also noteworthy that the stereoselectivity of aldol reactions using Crimmins' auxiliaries are typically lower than those obtained with Evans' auxiliaries, though this is not a concern with the planned synthetic route. The alcohol stereocenters generated during the aldol will be removed by subsequent reactions, so there is no need for any stereochemical induction in the aldol reactions. To control the configuration of the methyl stereocenter in the synthetic products, the appropriate Evans' auxiliary will be used. One example of the chemistry used to obtain the enantiomerically pure alcohol will be shown, though both routes were undertaken in the same manner. The diketide backbone of the first set of

targeted compounds will be accessed by acylation of (*R*)-4-benzyl-3-((*R*)-2-methylbutanoyl)oxazolidin-2-one to obtain **18**. Deprotonation with a strong base will preferentially create the *Z*-enolate and addition of iodomethane will provide **19**. The enantiopure 2-methylbutan-1-ol **20** will then be obtained by reductive cleavage of the Evans' auxiliary.

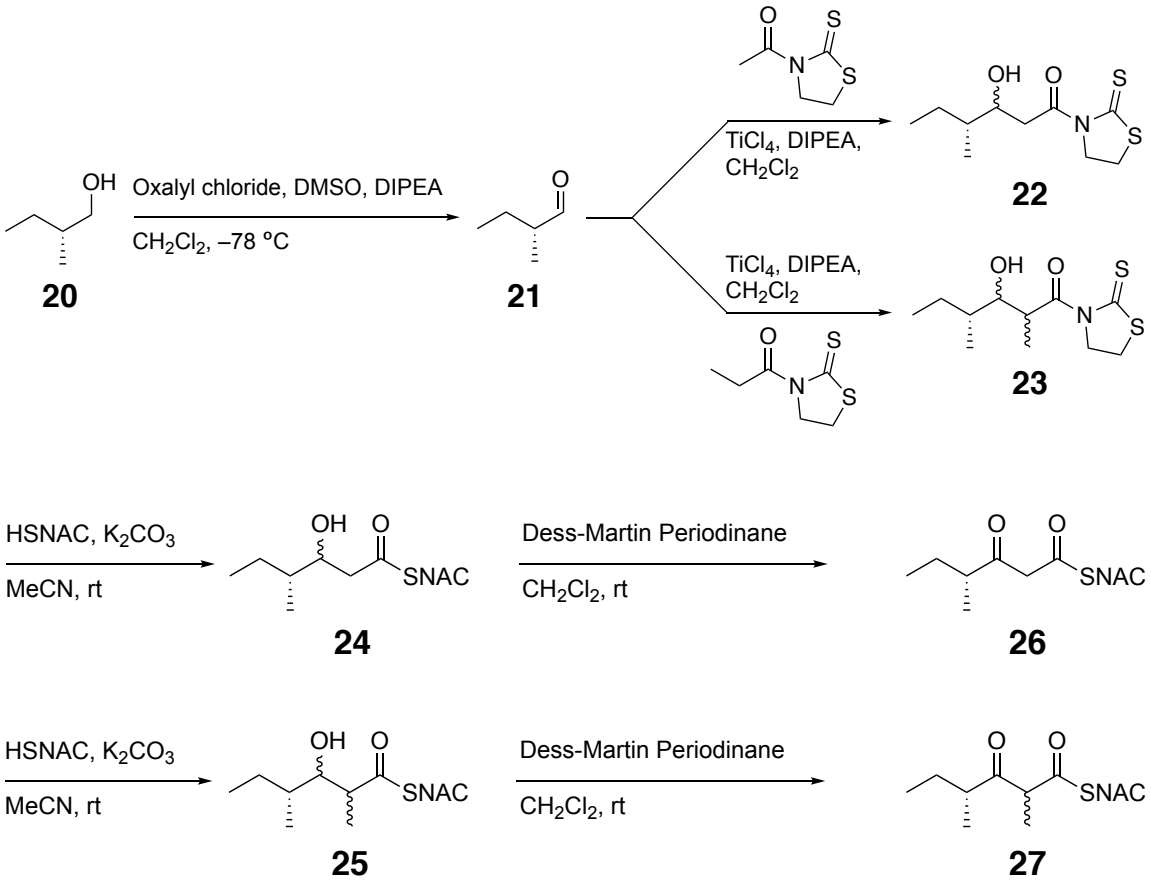


Scheme 3-1. Synthetic plan for the synthesis of Crimmins' auxiliaries



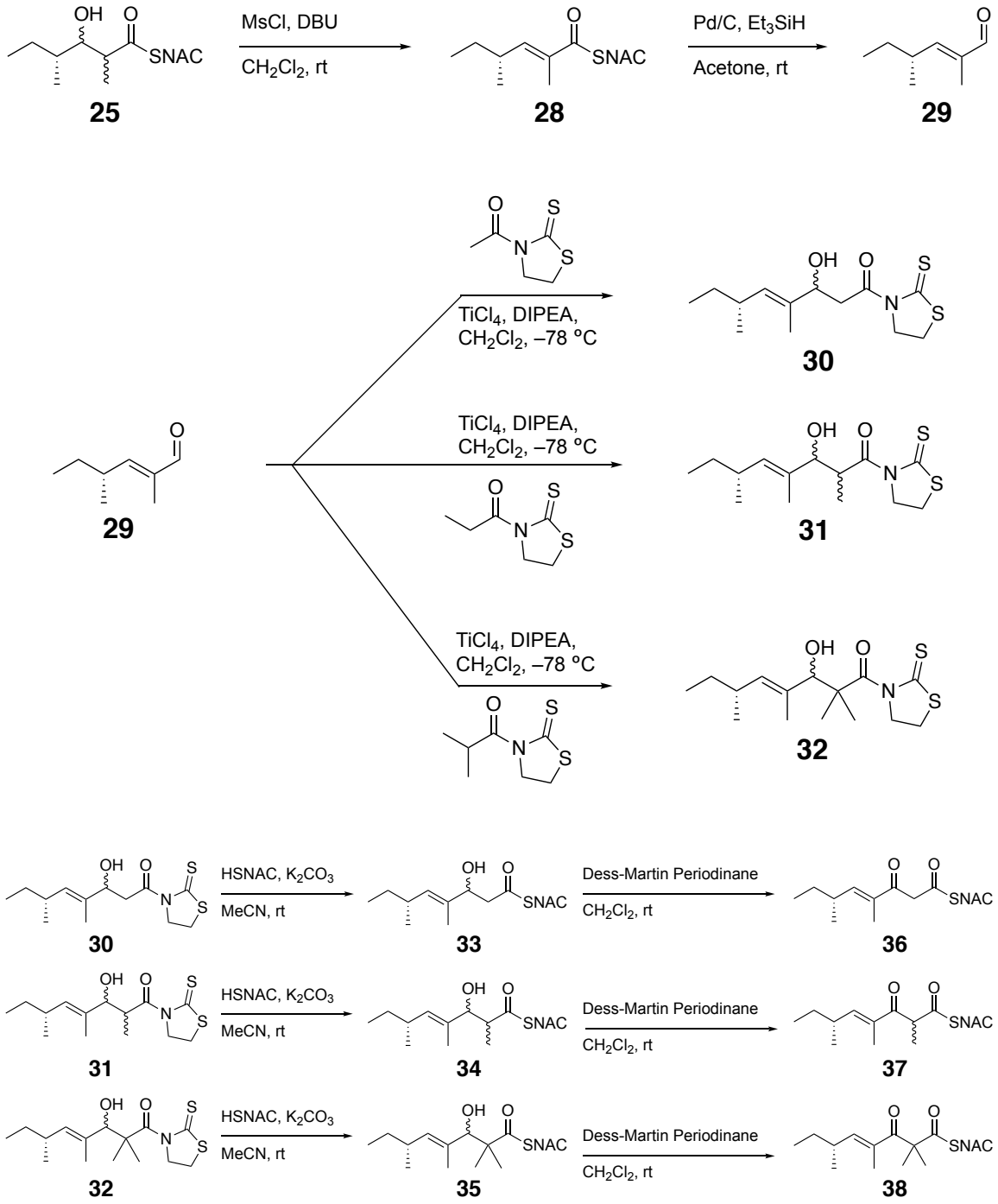
Scheme 3-2. Synthetic plan for the asymmetric synthesis of 2-methylbutan-1-ol

Scheme 3-3 outlines the plan to obtain the first two targeted compounds. The enantiomerically pure 2-methylbutan-1-ol will undergo a Swern oxidation to create aldehyde **21**, which will be split into two directions for aldol reactions with *N*-acetyl and *N*-propionyl Crimmins' auxiliaries to create **22** and **23** respectively. These two compounds will then undergo SNAC exchanges to create **24** and **25**. Compound **25** is a splitting point for the synthesis of the remaining compounds. Compounds **24** and **25** will then be oxidized to the targeted β-keto compounds **26** and **27**.



Scheme 3-3. Synthetic plan for accessing the first two β-keto compounds

The synthetic plan for the three remaining β-keto compounds is shown in Scheme 3-4. Compound **25** will be converted to α,β-unsaturated enone **28** by mesylation of the β-hydroxy functionality, followed by elimination with a non-nucleophilic base. A NOESY experiment performed on the isolated polyketide product from the biological system confirmed that the alkene is in the *E*-configuration and it was anticipated that performing the elimination at ambient temperature would produce the thermodynamically favoured alkene. Enone **28** will then undergo a Fukuyama reduction to furnish the unsaturated aldehyde **29** while scavenging the produced thiolate *in situ*, preventing a Michael fashion addition of the thiolate to the α,β-unsaturated starting material and products. Aldehyde **29** will be split into three separate Crimmins' aldol reactions to obtain products **30**, **31**, and **32**. The aldol products will then undergo SNAC exchanges to yield products **33**, **34**, and **35**. The final reaction will be an oxidation to obtain the final β-keto compounds **36**, **37**, and **38**.

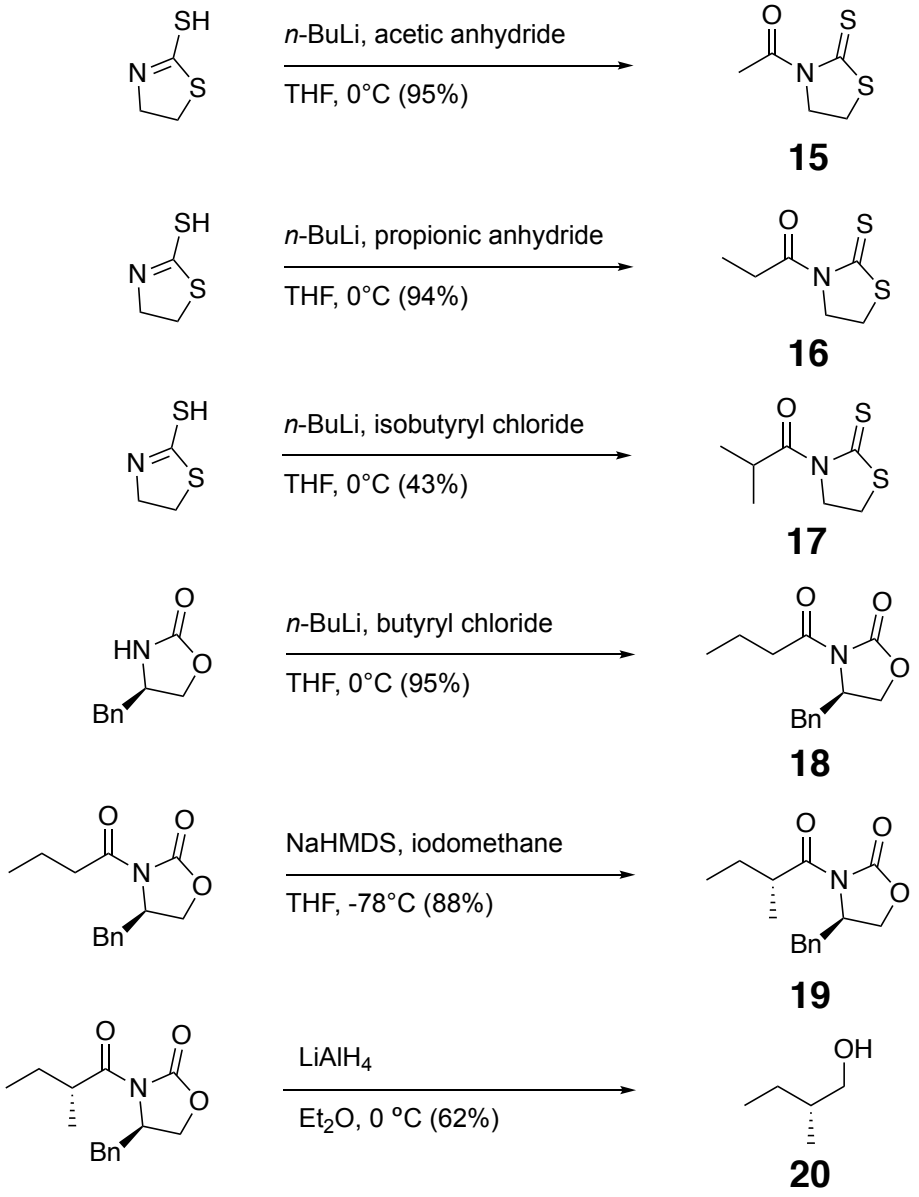


Scheme 3-4. Planned synthesis of the three remaining β-keto compounds

3.3 Results

3.3.1 Synthesis of aldol reagents and enantioselective synthesis of 2-methylbutanol

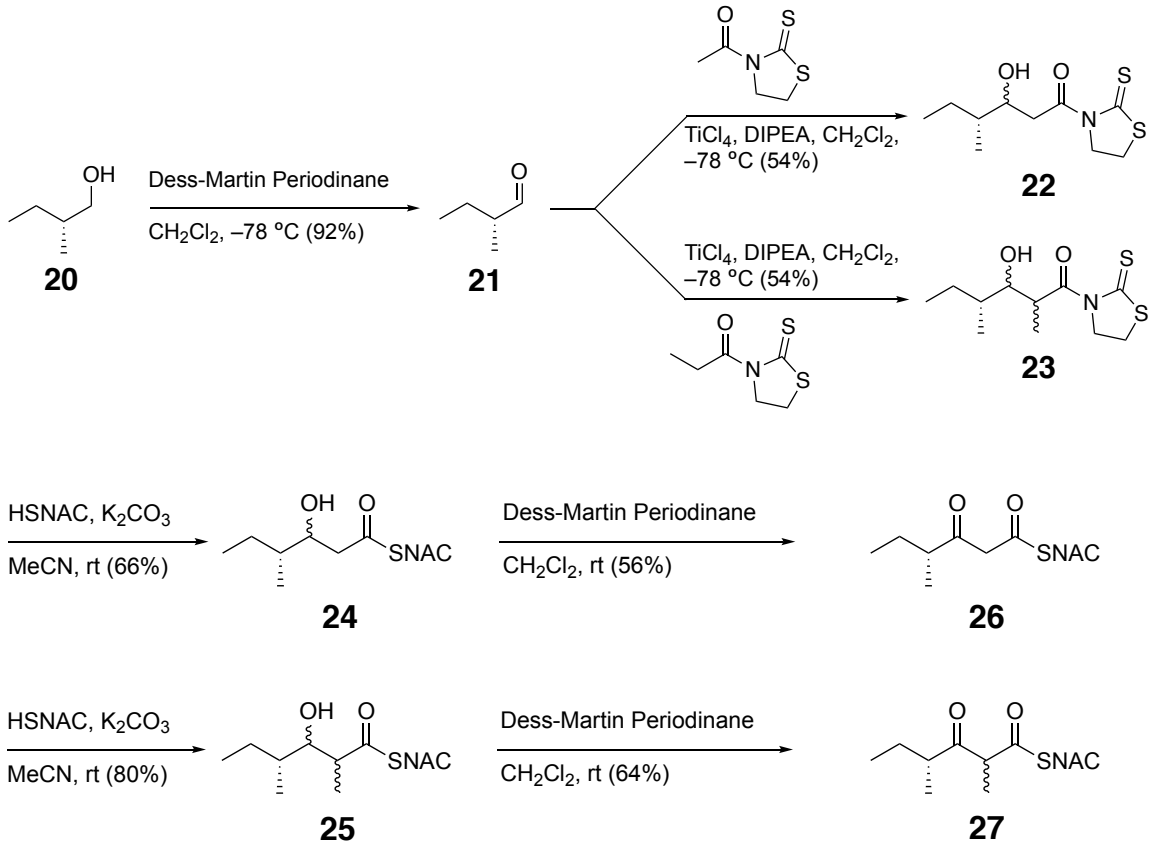
Synthesis of Crimmins' auxiliaries was done according to established methods[36] and proceeded smoothly in excellent yields, except for the isobutyryl auxiliary **17** (Scheme 3-5). The appending of a butyryl group to Evans' auxiliary to create **18** also proceeded smoothly, as did the following alkylation reaction to produce compound **19**.[49] Compound **19** was reductively cleaved to **20** using lithium aluminum hydride in a lower than expected yield. This was originally attributed to difficulty with emulsions, which was improved slightly in subsequent repeats by filtering the reaction mixture through Celite. An alternative method of preparation through the use of lithium monoethoxy borohydride did not improve yields. Characterization data matched what is reported in the literature for these known compounds, and the enantiomeric series also fit with what was expected.



Scheme 3-5. Preparation of Crimmins' aldol reagents and 2*R*-methylbutan-1-ol

The remaining steps to obtain the first two targeted products are shown in Scheme 3-6. Alcohol **20** was oxidized to aldehyde **21**. The original attempts involved a Swern oxidation using a large excess of DIPEA. After quenching with ammonium chloride solution and extracting with CH_2Cl_2, the aldehyde was intended to be concentrated carefully and used immediately in the ensuing aldol reaction. After obtaining very little aldol product, further investigation revealed that DIPEA was

carrying over from the extraction – presumably due to its lipophilicity. This would not be a problem for the following aldol reaction, except it was the DIPEA hydrochloride salt that was being carried over and quenching the pre-developed enolate upon addition of aldehyde solution in CH_2Cl_2. Reducing the excess of DIPEA used and extending the Swern reaction time still resulted in poor yields of aldol product. Substituting the less lipophilic TEA for DIPEA improved the yield, though not drastically. Quenching with aqueous citric acid solution instead of aqueous ammonium chloride solution to avoid the possibility of imine formation slightly improved yields. In the end, aldehyde **21** was obtained in excellent yield by oxidizing with DMP,[51] concentrating carefully after workup, then drying with $MgSO_4$ before splitting into two fractions and adding to the subsequent aldol reactions[36] to obtain compounds **22** and **23**. The two aldol products were then readily converted to their SNAC thioesters,[36] yielding compounds **24** and **25**. The β-hydroxy SNAC compounds were then oxidized to the targeted β-keto compounds **26** and **27** using DMP.[36] NMR analysis of compound **26** in $CDCl_3$ revealed the compound to be present as a 2:1 mixture of keto-enol tautomers. The yields for the DMP oxidations were less than expected due to side reactions. It is known that these substrates are prone to DMP-mediated over-oxidation at the α-position.[52] This can be controlled by lowering the temperature, avoiding the use of excess DMP reagent, and limiting reaction time.



Scheme 3-6. Synthetic scheme for accessing the first two β-keto compounds

Compound **25** was a splitting point for the remaining synthesis (Scheme 3-7). Compound **25** was converted to the mesylate and eliminated with DBU to afford **28**.[53] Compound **28** produced one peak by LC-MS, and no unassigned peaks in the NMR spectrum. The geometry of the alkene double bond was assigned as being in the *E*-configuration, which was confirmed by a NOESY experiment done on final β-keto compound **37**.

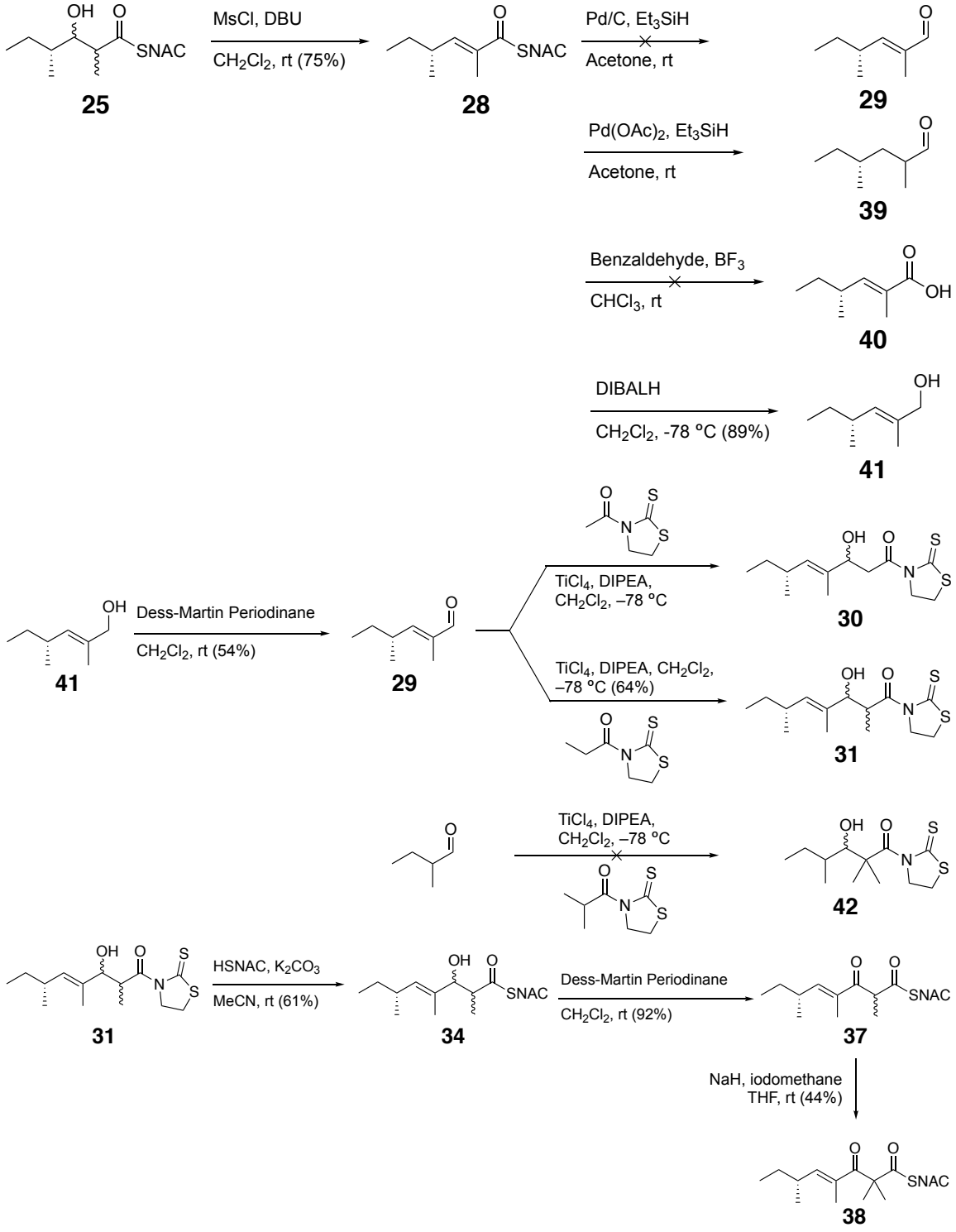
A Fukuyama reduction of **28** was attempted, but was not successful in producing aldehyde **29**. It was thought the thioester may be adopting a conformation or aggregated state that was preventing it from accessing the palladium inside the pores of the charcoal, so palladium (II) acetate was tried instead. This resulted in the conversion of the thioester

to the aldehyde, but with the undesired reduction of the alkene moiety to obtain saturated aldehyde **39**. The next approach was to convert the thioester to carboxylic acid **40**, then to reduce the acid to the alcohol and oxidize back up to the aldehyde. This method would have scavenged the freed SNAC as a benzyl thioether, though the reaction did not proceed. Finally it was decided to reduce the SNAC thioester to allylic alcohol **41** using DIBAL-H.[54] Gratifyingly, the reaction proceeded in excellent yield.

Alcohol **41** was oxidized to the α,β-unsaturated aldehyde **29** using DMP,[36] then split into three fractions for the following aldol reactions. The reaction to make compound **30** proceeded in low yield. More material was required to complete the synthesis of the final β-keto compound through this route, so work was continued to access the other compounds while bringing up more material. Aldol product **31** was successfully synthesized in moderate yield.[36] Since there was no literature precedent for an aldol reaction using isobutyryl Crimmins' auxiliary **17**, a pilot reaction was done with racemic 2-methylbutanal. Upon adding DIPEA to the titanium-complexed auxiliary, the expected deep-purple enolate colour was not observed. A stronger non-nucleophilic base (DBU) was added and the reaction mixture turned cherry-red in colour. Still, the expected product **42** was not detected by TLC or by NMR after quenching and workup. Another route would need to be taken to obtain the final product with the *gem*-dimethyl moiety. It was decided to introduce the final methyl group by α-methylation of compound **37**.

Compound **31** was converted to SNAC thioester **34** in moderate yield.[36] Compound **34** was then converted to targeted β-keto compound **37** in excellent yield.[36] The final α-methyl group was installed on compound **37** using NaH and iodomethane to obtain *gem*-dimethyl β-keto compound **38**. The yield could likely be improved due to the

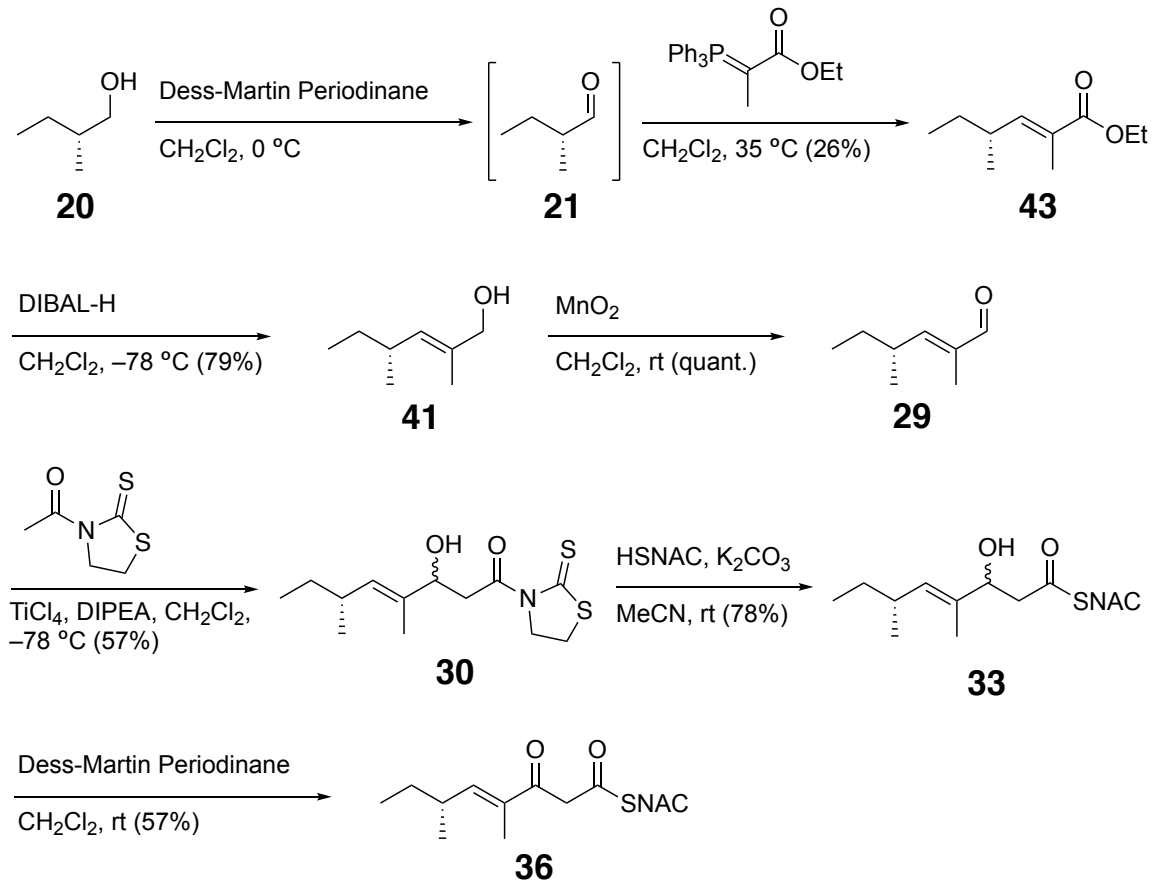
milligram scale of the reaction and the difficulty of measuring sub-milligram quantities of reagents.



Scheme 3-7. Synthetic scheme for accessing the third and fourth β-keto compounds

3.3.2 Alternative route to access the final β-keto product

The necessity of bringing more material through the synthesis to access the final β-keto compound presented an opportunity to develop a shortcut to accessing the final compounds (Scheme 3-8). After synthesizing alcohol **20**, it was oxidized to aldehyde **21** before proceeding with an HWE reaction to create unsaturated ester **43** in low yield.[54] The ester was reduced to allylic alcohol **41** before oxidizing to aldehyde **29** with freshly prepared manganese dioxide.[54] This was a very clean and easy way to oxidize the allylic alcohol and it produced the conjugated aldehyde quantitatively. The aldehyde was used in a Crimmins' aldol reaction to obtain compound **30** in moderate yield. A facile SNAC exchange to produce **33** was then followed by a DMP oxidation to yield the final β-keto compound **36**. NMR analysis in $CDCl_3$ revealed the compound to be present as a 3:1 mixture of keto-enol tautomers.



Scheme 3-8. Synthetic scheme for accessing the final β-keto compound

Our collaborators are currently using the substrates in enzymatic assays to probe the functioning of the biological system. Products of the *in vitro* assays will be quantified and characterized by LC-MS and NMR in order to make conclusions about how the system operates.

3.4 Conclusion and future work

Tailoring domains have well-defined substrate preferences that ensure the correct product is made, with few exceptions. The off-loading step, by a TE or CAT domain, is also coordinated with the tailoring domains to ensure a product is not off-loaded prematurely. This is probably kinetically controlled, which means the rate of reaction for the different domains determines which reaction occurs next on the substrate.

The CAT domain that is part of Tv6-931 appears to catalyze a reversible off-loading of α-monomethyl product before an irreversible final α-methylation. This is potentially a new method of polyketide off-loading and augments what we know about the diversity of polyketide natural products and how they are made.

Our collaborators are currently using the synthesized proposed biosynthetic intermediates in enzymatic assays to probe the functioning of the biological system. Products of the *in vitro* assays will be quantified and characterized. This will be done by using LC-MS with standard curves obtained from the synthetic compounds, as well as NMR.

It is not known what the natural nucleophile is in the Tv6-931 system for off-loading of the polyketide product. This is a problem that is inherent to many genome mining efforts – when a substrate utilized by the natural host is not present in a heterologous organism. It is hoped that further metabolomics work on *T. virens* Gv29-8 will be able to identify the natural nucleophile used by the CAT domain to off-load the polyketide product.

4 Experimental Procedures

4.1 General experimental

4.1.1 Solvents, reagents, and solutions

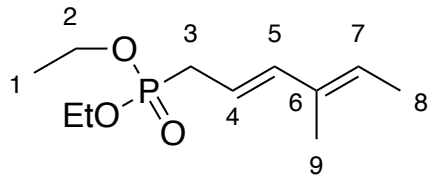
All reactions involving air or moisture sensitive reactants were conducted under a positive pressure of dry argon. All solvents, reagents, and chemicals were reagent grade and used as supplied unless otherwise stated. For anhydrous reactions, solvents were dried according to the procedures detailed in Armarego and Chai.[55] Tetrahydrofuran and diethyl ether were distilled over sodium and benzophenone under an atmosphere of dry argon. Acetonitrile and dichloromethane were distilled over calcium hydride. DMSO was dried over 4Å molecular sieves. Solvent was removed under reduced pressure using a diaphragm pump attached to a Büchi rotary evaporator and finished with a high-powered vacuum, unless the compound was considered volatile. Reagents were purchased from Sigma-Aldrich. Solutions of NH_4Cl, $NaHCO_3$, NaOH, and $Na_2S_2O_3$ refer to aqueous solutions. Brine refers to a saturated solution of NaCl. All reactions and fractions from column chromatography were monitored by thin-layer chromatography. Compounds were visualized by exposure to uv light and by dipping the plate in a potassium permanganate staining solution followed by heating with a heat gun. Flash chromatography was performed according to the method of Still *et al.*[56] on silica gel (EM Science, 60Å, 230-400 mesh).

4.1.2 Characterization and instrumentation

Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at 25 °C. Nuclear magnetic resonance spectra were obtained on a Varian 500 equipped with a cryo-probe. ^{1}H NMR chemical shifts are reported in parts per million (ppm) using the residual proton resonance of solvents as reference: $CDCl_3$ δ 7.26, or CD_2Cl_2 δ 5.32. ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ δ 77.1, or CD_2Cl_2 δ 53.8. Infrared spectra were recorded on a Nicolet Magna 750 or a 20SX FT-IR spectrometer. Cast film refers to the evaporation of a solution on a NaCl plate. Mass spectra were recorded on a Kratos IMS-50 (high-resolution, electron impact ionization), and an Agilent Technologies 6220 oaTOF (high-resolution, electrospray ionization).

4.2 Synthesis and characterization

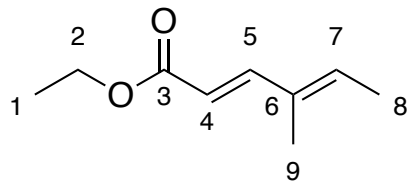
(2*E*,4*E*)-4-Methylhexa-2,4-dienylphosphonic acid diethylester (3):



This known compound was synthesized using an established procedure.[32] To a stirred solution of (2*E*,4*E*)-4-methyl-2,4-hexadien-1-ol (0.456 g, 4.15 mmol) in THF (8 mL) at −78 °C was added *n*-BuLi soln (2.5 M, 1.82 mL, 4.56 mmol) dropwise *via* syringe. The yellow solution was stirred at −78 °C for 10 min. A solution of *p*-toluenesulfonylchloride (0.870 g, 4.56 mmol) in THF (4 mL) was then added dropwise *via* cannula to the reaction mixture. The reaction mixture was stirred for 1 h at −78 °C. Meanwhile, a soln of KHMDS in THF (1 M, 6.84 mL, 6.84 mmol) was added slowly *via* syringe to a stirred solution of diethyl phosphite (0.945 g, 6.84 mmol) in 8 mL THF at 0 °C. After 30 min, this solution was added to the −78 °C solution of the preformed tosylate *via* cannula. The reaction mixture was stirred at −78 °C for 10 min then slowly warmed to rt overnight. The reaction mixture was partitioned between aqueous pH 7 buffer (15 mL) and diethyl ether (20 mL). The organic phase was washed sequentially with satd $NaHCO_3$ (2 x 10 mL), water (2 x 10 mL), and brine (10 mL). The organic phase was dried with $MgSO_4$, concentrated *in vacuo*, then purified by flash chromatography (Al_2O_3, ether then EtOAc) to yield a colourless oil, R_f = 0.46 in EtOAc, (0.266 g, 28%). IR (CH_2Cl_2, cast) 2983, 2912, 1444, 1029 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.15 (dd, *J* = 15.5, 5.0 Hz, 1H, H5), 5.51 – 5.46 (m, 1H, H4), 5.43 (q, *J* = 7.5 Hz, 1H, H7), 4.11 – 4.04 (m, 4H; H2), 2.61 (dd, *J* = 22.0, 7.6 Hz, 2H, H3), 1.70 (s, 3H, H9), 1.69

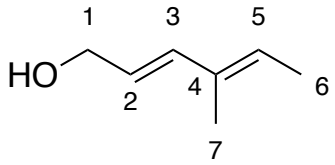
(d, J = 7.5 Hz, 3H, H8), 1.29 (t, J = 7.5 Hz, 6H, H1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 139.7 (d, J = 14.6 Hz), 134.0 (d, J = 4.2 Hz), 126.6 (d, J = 4.1 Hz), 114.6 (d, J = 12.0 Hz), 63.6 (d, J = 5.9 Hz), 61.9 (d, J = 6.7 Hz), 30.7 (d, J = 139.4 Hz), 16.5 (d, J = 5.8 Hz), 13.8 (d, J = 1.4 Hz), 12.0. HRMS (ESI): Calcd for $C_{11}H_{21}O_3P$ $[M+H]^+$ 233.1301, found 233.1301.

Ethyl (2*E*,4*E*)-4-methyl-2,4-hexadienoate (4):



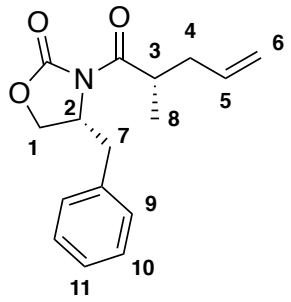
This known compound was synthesized using an established procedure.[30] Sodium hydride (0.520 g, 13.0 mmol, 60% suspension in oil) was suspended in 120 mL of dry THF at rt. Triethyl phophonoacetate (2.77 mL, 13.8 mmol) was added dropwise and stirred for 1 h. Tiglic aldehyde (1.15 mL, 11.9 mmol) was added dropwise and the reaction was stirred overnight. After 16 h the reaction was quenched with 30 g of ice. THF was removed *in vacuo*. The aqueous phase was extracted with ether (3 x 40 mL). The organic layers were pooled and concentrated *in vacuo*. The yellow oil was purified by flash chromatography (SiO_2, 25% EtOAc in hexanes) to provide the title compound as a light-yellow oil, R_f = 0.59 in 25% EtOAc in hexanes, (1.52 g, 83%). IR (neat) 2982, 2923, 2857, 1714, 1633, 1622 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.31 (d, *J* = 15.8 Hz, 1H, H5), 5.99 (q, *J* = 7.0 Hz, 1H, H7), 5.78 (d, *J* = 15.6 Hz, 1H, H4), 4.21 (q, *J* = 7.2 Hz, 2H, H2), 1.81 (d, *J* = 7.0 Hz, 3H, H8), 1.77 (s, 3H, H9), 1.30 (t, *J* = 7.2 Hz, 3H, H1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.7, 149.5, 136.3, 133.8, 115.3, 60.1, 14.6, 14.4, 11.8. HRMS (ESI): Calcd for $C_9H_{14}O_2Na$ $[M+Na]^+$ 177.0886, found 177.0887.

(2*E*,4*E*)-4-Methyl-2,4-hexadien-1-ol (5):



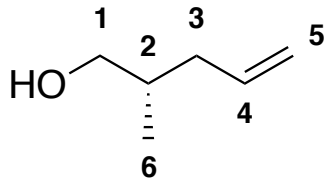
This known compound was synthesized using an established procedure.[31] To a stirred solution of ethyl (2*E*,4*E*)-4-methyl-2,4-hexadienoate (1.38 g, 8.95 mmol) in dry CH_2Cl_2 (90 mL) at –40 °C was added DIBAL-H solution (1 M in CH_2Cl_2, 22.5 mmol) dropwise. The reaction mixture was then brought to −20 °C and stirred for 1 h. The reaction was quenched at 0 °C by addition of satd Rochelle salt soln (5 mL) and the suspension was stirred vigorously for 1 h. The phases were separated and the aqueous phase extracted with CH_2Cl_2 (3 x 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, 50% EtOAc in hexanes) to provide a colorless oil, R_f = 0.35 in 50% EtOAc in hexanes, (0.693 g, 69 %). IR (CH_2Cl_2, cast) 3327, 3034, 2991, 2919, 2860, 1653, 1443, 1109 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.26 (d, *J* = 15.6 Hz, 1H, H3), 5.71 (dt, *J* = 15.6, 6.1 Hz, 1H, H2), 5.57 (q, *J* = 6.9 Hz, 1H, H5), 4.19 (d, *J* = 6.6 Hz, 2H, H1), 1.74 (s, 3H, H7), 1.73 (d, *J* = 7.5 Hz, 3H, H6). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 136.8, 133.9, 127.6, 124.8, 64.1, 13.9, 12.1. HRMS (EI): Calcd for $C_7H_{12}O$ $[M]^+$ 112.0888, found 112.0890.

(*R*)-4-Benzyl-3-((*S*)-2-methylpent-4-enoyl)oxazolidin-2-one (6):



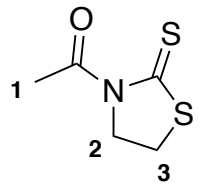
This known compound was synthesized using an established procedure.[33] To a stirred solution of (*R*)-(-)-4-benzyl-3-propionyl-2-oxazolidinone (1.50 g, 6.43 mmol) in THF (24 mL) was added dropwise NaHMDS (1.0 M in THF, 6.5 mL, 6.5 mmol) at −78 °C. The reaction mixture was allowed to stir for 30 min at −78 °C, then allyl iodide (0.88 mL, 9.7 mmol) was added *via* syringe. The mixture was stirred at −78 °C for 4 h, then quenched at −78 °C with satd NH_4Cl soln (18 mL). The aqueous layer was extracted with ether (3 x 15 mL) and the combined organic layers were dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, 10% EtOAc in hexanes) to yield the title compound as a yellow oil (0.70 g, 39%). $[\alpha]_D^{25} = -36.3$ (*c* 1.0, $CHCl_3$); ref $[\alpha]_D^{25} = -39.0$ (*c* 1.0, $CHCl_3$)[33]; IR ($CHCl_3$, cast) 3076, 3029, 2979, 2934, 1781, 1699, 1641, 1605, 1498 cm^{-1}; 1 H NMR (500 MHz, $CDCl_3$) δ 7.36 – 7.30 (m, 2H, H10), 7.30 – 7.24 (m, 1H, H11), 7.24 – 7.19 (m, 2H, H9), 5.84 (ddt, *J* = 17.0, 10.0, 7.1 Hz, 1H, H5), 5.14 – 5.03 (m, 2H, H6), 4.69 (ddt, *J* = 9.8, 7.6, 3.2 Hz, 1H, H2), 4.23 – 4.09 (m, 2H, H1), 3.88 (dq, *J* = 6.8, 6.8 Hz, 1H, H3), 3.29 (dd, *J* = 13.4, 3.4 Hz, 1H, H7), 2.72 (dd, *J* = 13.4, 9.8 Hz, 1H, H7'), 2.54 (dt, *J* = 13.7, 6.8 Hz, 1H, H4), 2.25 (dt, *J* = 14.0, 7.1 Hz, 1H, H4'), 1.19 (d, *J* = 6.7 Hz, 3H, H8). ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.5, 153.1, 135.4, 135.3, 129.4, 129.0, 127.4, 117.2, 66.1, 55.4, 38.2, 38.0, 37.2, 16.5. HRMS (ESI) Calcd for $C_{16}H_{20}NO_3$ $[M+H]^+$ 274.1438, found 274.1439.

(*S*)-2-Methylpent-4-en-1-ol (7):



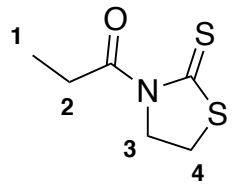
This known compound was synthesized using a modified procedure.[33] A solution of (*R*)-4-benzyl-3-((*S*)-2-methylpent-4-enoyl)oxazolidin-2-one (1.50 g, 5.49 mmol) in dry ether (35 mL) was stirred while cooling to 0 °C under argon. Absolute EtOH (0.38 mL, 6.6 mmol) was added, followed by $LiBH_4$ soln (2 M in THF, 3.3 mL, 6.6 mmol). After stirring for 3.5 h, the reaction was quenched by addition of aqueous NaOH soln (1 M, 35 mL) and stirred until the mixture became clear. Layers were separated and the aqueous extracted with ether (3 x 20 mL). The pooled organic layers were washed with brine (20 mL), dried with $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, 25% ether in pentanes) to provide the title compound as a pale-yellow oil (0.478 g, 87%). ^{1}H NMR (500 MHz, CD_2Cl_2) δ 5.82 (ddt, J = 17.3, 10.2, 7.2 Hz, 1H, H4), 5.06 – 4.98 (m, 2H, H5), 3.47 (dd, J = 10.6, 6.1 Hz, 1H, H1), 3.41 (dd, J = 10.6, 6.2 Hz, 1H, H1'), 2.20 – 2.13 (m, 1H, H3), 1.95 – 1.88 (m, 1H, H3'), 1.75 – 1.65 (m, 1H, H2), 0.91 (d, J = 6.8 Hz, 3H, H6); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 137.6, 116.0, 68.0, 38.1, 36.1, 16.5. HRMS (EI) Calcd for $C_6H_{12}O$ $[M]^+$ 100.0888, found 100.0889.

***N*-Acetyl-thiazolidine-2-thione (15):**



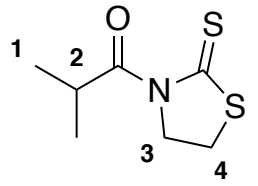
This known compound was synthesized according to an established method.[36] 2-Thiazoline-2-thiol (5.00 g, 41.9 mmol) was dissolved in dry THF (100 mL) and cooled to 0 °C under argon while stirring. *n*-BuLi (2.5 M in hexanes, 18.5 mL, 46.1 mmol) was added over 15 min and the reaction was stirred for an additional 15 min. Acetic anhydride (4.8 mL, 50 mmol) was added slowly *via* syringe and the reaction stirred for 2 h at rt. Satd NH_4Cl soln (100 mL) was added and the phases were separated, followed by extraction of the aqueous layer with EtOAc (2 x 100 mL). The combined organic phases were washed with brine (100 mL), dried with Na_2SO_4, concentrated *in vacuo,* and purified by column chromatography (SiO_2, 20% EtOAc in hexanes), yielding the product as a bright-yellow oil, R_f = 0.20 in 20% EtOAc in hexanes, (5.87 g, 87%). IR ($CHCl_3$, cast) 3004, 2941,1694 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.57 (t, *J* = 7.5 Hz, 2H, H2), 3.29 (t, *J* = 7.5 Hz, 2H, H3), 2.77 (s, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.0, 171.5, 55.7, 28.2, 27.0. HRMS (EI) Calcd for $C_5H_7NOS_2$ $[M]^+$ 160.9969, found 160.9968.

***N*-Propionyl-thiazolidine-2-thione (16):**



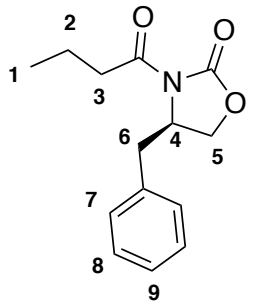
This known compound was synthesized according to an established method.[36] 2-Thiazoline-2-thiol (10.0 g, 83.9 mmol) was dissolved in dry THF (200 mL) and cooled to 0 °C under argon while stirring. *n*-BuLi (2.5 M in hexanes, 37 mL, 92 mmol) was added slowly and the reaction stirred for an additional 30 min. Propionic anhydride (11.8 mL, 92.3 mmol) was added slowly and the reaction stirred for 2 h at rt. Satd NH_4Cl soln (100 mL) was added and the phases were separated, followed by extraction of the aqueous layer with EtOAc (2 x 100 mL). The combined organic phases were washed with brine (200 mL), dried with Na_2SO_4, concentrated *in vacuo,* and purified by column chromatography (SiO_2, 20% EtOAc in hexanes), yielding the product as a bright-yellow oil, R_f = 0.33 in 20% EtOAc in hexanes, (14.2 g, 97%). IR ($CHCl_3$, cast) 2980, 2939, 1698 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.58 (t, *J* = 7.5 Hz, 2H, H3), 3.28 (t, *J* = 7.5 Hz, 2H, H4), 3.25 (q, *J* = 7.2 Hz, 2H, H2), 1.17 (t, *J* = 7.2 Hz, 2H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 201.5, 175.6, 56.1, 32.3, 28.3, 8.8. HRMS (EI) Calcd for $C_5H_7NOS_2$ $[M]^+$ 175.0126, found 175.0124.

***N*-Methylpropionyl-thiazolidine-2-thione (17):**



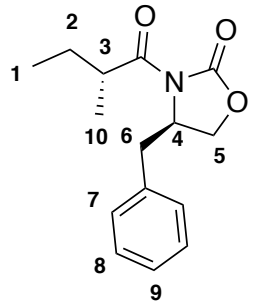
This compound was synthesized according to a modified procedure.[50] 2-Thiazoline-2-thiol (1.00 g, 8.39 mmol) was dissolved in dry THF (20 mL) and cooled to 0 °C under argon while stirring. *n*-BuLi (2.5 M, 3.7 mL, 9.2 mmol) was added *via* syringe and the reaction was stirred for 30 min. Isobutyryl chloride (0.97 mL, 9.2 mmol) was added dropwise and the reaction was stirred for 5 min at 0 °C before removing the cooling bath. After 2 h at rt, the reaction was quenched with satd NH_4Cl soln (10 mL) and the THF was removed *in vacuo.* The aqueous was extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with satd aqueous $NaHCO_3$ (10 mL), brine (10 mL), dried over Na_2SO_4, and concentrated *in vacuo.* The residue was purified by flash chromatography (SiO_2, 20% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.33 in 20% EtOAc in hexanes, (0.755 g, 43%). IR ($CHCl_3$, cast) 2975, 2934, 1701 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.54 (t, *J* = 7.5 Hz, 2H, H3), 4.47 (heptet, *J* = 7.0 Hz, 1H, H2), 3.27 (t, *J* = 7.5 Hz, 2H, H4), 1.21 (d, *J* = 7.0 Hz, 6H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 201.5, 179.8, 56.7, 33.9, 28.3, 19.3. HRMS (EI) Calcd for $C_7H_{11}NOS_2$ $[M]^+$ 189.0282, found 189.0286.

(*R*)-4-Benzyl-3-butyryloxazolidin-2-one (18):



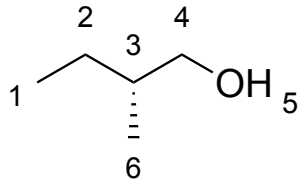
This known compound was synthesized according to an established procedure.[50] (*R*)-4-benzyloxazolidin-2-one (10.0 g, 56.4 mmol) was dissolved in dry THF (80 mL) and cooled to –78 °C under argon while stirring. *n*-BuLi (2.5 M, 24.8 mL, 62.1 mmol) was added *via* syringe and the reaction was stirred for 30 min. Butyryl chloride (6.45 mL, 62.1 mmol) was added dropwise and the reaction was stirred for 1 h at –78 °C before removing the cooling bath. After 15 min, the reaction was quenched with satd NH_4Cl soln (40 mL) and the THF was removed *in vacuo.* The aqueous was extracted with EtOAc (3 × 40 mL). The combined organic extracts were washed with satd $NaHCO_3$ soln (40 mL), brine (40 mL), dried over Na_2SO_4, and concentrated *in vacuo.* The residue was purified by flash chromatography (SiO_2, 20% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.30 in 20% EtOAc in hexanes, (14.0 g, quant.). $[\alpha]_D^{25}$ = –54.4 (*c* 1.0, $CHCl_3$); ref $[\alpha]_D^{25}$ = –56.4 (*c* 1.0, $CHCl_3$)[50]; IR ($CHCl_3$, cast) 3029, 2965, 2934, 2876, 1781, 1700 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.38 – 7.32 (m, 2H, H8), 7.31 – 7.23 (m, 1H, H9), 7.20 – 7.19 (m, 2H, H7), 4.69 – 4.65 (m, 1H, H4), 4.25 – 4.09 (m, 2H, H5), 3.31 (dd, *J* = 13.4, 3.4 Hz, 1H, H6), 3.02 – 2.84 (m, 2H, H3), 2.77 (dd, *J* = 13.4, 9.6 Hz, 1H, H6'), 1.82 – 1.69 (m, 2H, H2), 1.03 (t, *J* = 7.4 Hz, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.3, 153.5, 135.4, 129.5, 129.0, 127.4, 66.2, 55.2, 38.0, 37.4, 17.8, 13.7. HRMS (ESI) Calcd for $C_{14}H_{17}NO_3Na$ $[M+Na]^+$ 270.1106, found 270.1104.

(*R*)-4-Benzyl-3-((*R*)-2-methylbutanoyl)oxazolidin-2-one (19):



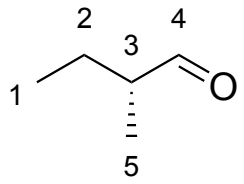
This known compound was synthesized using an established procedure.[50] (*R*)-4-Benzyl-3-butyryloxazolidin-2-one (14.0 g, 56.6 mmol) was dissolved in dry THF (200 mL) and cooled to –78 °C under argon while stirring. NaHMDS (1 M, 62.3 mL, 62.3 mmol) was added slowly *via* syringe and the reaction was stirred for 30 min. Methyl iodide (8.81 mL, 142 mmol) was added slowly and the reaction stirred for 2.5 h. The cooling bath was removed and 10 min later the reaction was quenched with brine (100 mL). The THF was removed *in vacuo* and the aqueous extracted with EtOAc (3 x 100 mL). The combined organic phases were dried with Na_2SO_4, concentrated *in vacuo*, and purified by column chromatography (SiO_2, 10% EtOAc in hexanes), yielding the product as a pale-yellow oil, R_f = 0.28 in 15% EtOAc in hexanes, (12.7 g, 86%). $[\alpha]_D^{25}$ = –72.9 (*c* 1.0, $CHCl_3$); ref $[\alpha]_D^{25}$ = –69.1 (*c* 1.0, $CHCl_3$)[50]; IR ($CHCl_3$, cast) 3029, 2969, 2933, 2877, 1779, 1697 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.35 – 7.31 (m, 2H, H8), 7.29 – 7.25 (m, 1H, H9), 7.23 – 7.20 (m, 2H, H7), 4.70 – 4.65 (m, 1H, H4), 4.22 – 4.15 (m, 2H, H5), 3.66 – 3.60 (m, 1H, H3), 3.27 (dd, *J* = 13.4, 3.3 Hz, 1H, H6), 2.77 (dd, *J* = 13.4, 9.6 Hz, 1H, H6'), 1.82 – 1.73 (m, 1H, H2), 1.52 – 1.43 (m, 1H, H2'), 1.22 (d, *J* = 6.9 Hz, 3H, H10), 0.93 (t, *J* = 7.4 Hz, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.2, 153.2, 135.4, 129.5, 129.0, 127.4, 66.1, 55.4, 39.2, 38.0, 26.5, 16.9, 11.7. HRMS (ESI) Calcd for $C_{15}H_{19}NO_3Na$ $[M+Na]^+$ 284.1257, found 284.1260.

(*R*)-2-Methylbutan-1-ol (20):



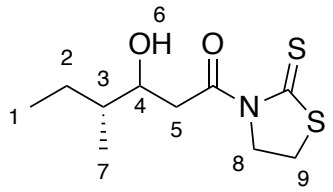
This known compound was synthesized using an established procedure.[50] (*R*)-4-Benzyl-3-((*S*)-2-methylbutanoyl)oxazolidin-2-one (9.13 g, 34.9 mmol) was dissolved in dry ether (70 mL) and cooled to –20 °C under argon while stirring. $LiAlH_4$ (3.17 g, 83.3 mmol) was added in three portions and the reaction was stirred at 0 °C for 3 h. Water (10 mL) was carefully added and the reaction was stirred at rt for 15 min. The mixture was filtered through Celite, washing with ether (4 x 100 mL). The organic extracts were washed with brine (100 mL), dried with Na_2SO_4, and concentrated *in vacuo* using an ice bath. The residue was purified by column chromatography (SiO_2, 30% ether in pentanes), yielding the product as a colourless oil, R_f = 0.46 in 50% EtOAc in hexanes, (1.90 g, 62%). $[\alpha]_D^{25}$ = 6.1 (*c* 0.95, CH_2Cl_2); IR (CH_2Cl_2, cast) 3442, 2960, 2928, 2857, 1274 cm^{-}1; ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 3.43 (dd, *J* = 10.5, 5.8 Hz, 1H, H4), 3.35 (dd, *J* = 10.5, 6.4 Hz, 1H, H4'), 1.51 – 1.44 (m, 1H, H3), 1.43 – 1.36 (m, 1H, H2), 1.31 (br s, 1H, H5), 1.14 – 1.08 (m, 1H, H2'), 0.87 (t, *J* = 7.5 Hz, 3H, H1), 0.86 (d, *J* = 6.5 Hz, 3H, H3). ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 68.2, 37.8, 26.1, 16.2, 11.5. HRMS (EI) Calcd for $C_5H_{12}O$ $[M]^+$ 88.0888, found 88.0878.

(*R*)-2-Methylbutanal (21):



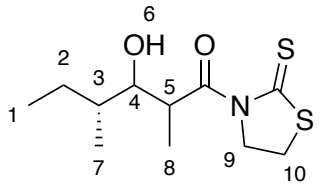
This known compound was synthesized using a modified procedure.[51] To a solution of (*R*)-2-methylbutan-1-ol (1.90 g, 21.6 mmol) in dry CH_2Cl_2 (40 mL) at 0 °C was added Na_2CO_3 (2.17 g, 25.9 mmol) followed by Dess-Martin periodinane (11.0 g, 25.9 mmol). The suspension was stirred for 2 h and became yellow in colour. The reaction was quenched with a 1:1 mixture of satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln (80 mL) and stirred until the bubbling stopped. The aqueous was extracted with CH_2Cl_2 (2 × 15 mL). The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo* using an ice bath. R_f = 0.70 in CH_2Cl_2 (1.71 g, 92%). ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 9.60 (d, *J* = 1.9 Hz, 1H, H4), 2.31 – 2.19 (m, 1H, H3), 1.73 (dqd, *J* = 13.9, 7.5, 6.4 Hz, 1H, H2), 1.46 – 1.37 (m, 1H, H2'), 1.06 (d, *J* = 7.0 Hz, 3H, H5), 0.93 (t, *J* = 6.5 Hz, 3H, H1). ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 205.5, 48.1, 23.9, 13.0, 11.5.

3-Hydroxy-4*R*-methyl-1-(2-thioxothiazolidin-3-yl)hexan-1-one (22):



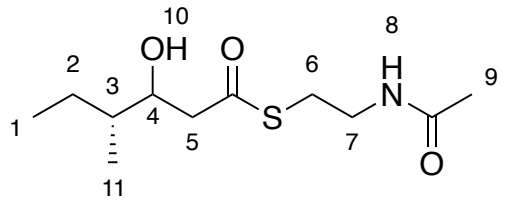
This new compound was synthesized using an established procedure.[36] To a stirred solution of *N*-acetyl-thiazolidine-2-thione (0.23 g, 1.4 mmol) in dry CH_2Cl_2 (25 mL) was added $TiCl_4$ (1.0 M soln in CH_2Cl_2, 1.4 mL, 1.4 mmol) at 0 °C under argon. The reaction mixture was stirred for 5 min to dissolve, then cooled to –78 °C. Diisopropylethylamine (0.29 mL, 1.7 mmol) was added and the reaction stirred for 1 h. A solution of (*R*)-2-methylbutanal (0.11 g, 1.3 mmol) in CH_2Cl_2 was added dropwise and the reaction stirred for 15 min at –78 °C, then 2 h at rt. The reaction was quenched by addition of half-satd NH_4Cl soln (25 mL). Layers were separated and the aqueous extracted with CH_2Cl_2 (3 x 25 mL). The combined organic phases were washed with brine (100 mL), dried with $MgSO_4$, concentrated *in vacuo*, and purified by column chromatography (SiO_2, 30% EtOAc in hexanes), yielding the product as a yellow oil, a 60:40 mixture of diastereomers, R_f = 0.11 in 30% EtOAc in hexanes, (0.19 g, 54%). $[\alpha]_D^{25}$ = 3.0 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, cast) 3499, 2961, 2932, 2875, 1697, 1368, 1281, 1157, 1053 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.65 – 4.55 (m, 2H, H8), 4.07 – 3.97 (m, 1H, H4), 3.50 – 3.34 (m, 2H, H5), 3.31 – 3.26 (m, 2H, H9), 2.86 (br, 1H, H6), 2.82 (br, 0.4H, H6), 2.76 (br, 0.6H, H6'), 1.61 – 1.44 (m, 2H, H2+H3), 1.22 – 1.17 (m, 1H, H2'), 0.94 – 0.90 (m, 6H, H7+H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.0, 174.7, 71.1, 55.8, 43.4, 39.9, 28.4, 25.6, 14.1, 11.8. HRMS (ESI) Calcd for $C_{10}H_{17}NO_2S_2Na$ $[M+Na]^+$ 270.0593, found 270.0599.

3-Hydroxy-2,4*R*-dimethyl-1-(2-thioxothiazolidin-3-yl)hexan-1-one (23):



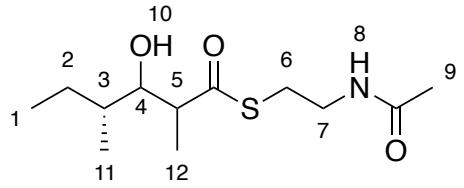
This new compound was synthesized using an established procedure.[36] To a stirred solution of *N*-propionyl-thiazolidine-2-thione (0.25 g, 1.4 mmol) in dry CH_2Cl_2 (25 mL) was added $TiCl_4$ (1.0 M soln in CH_2Cl_2, 1.4 mL, 1.4 mmol) at 0 °C under argon. The reaction was stirred for 5 min to dissolve, then cooled to –78 °C. Diisopropylethylamine (0.29 mL, 1.7 mmol) was added and the reaction stirred for 1 h. A solution of (*R*)-2-methylbutanal (0.11 g, 1.3 mmol) in CH_2Cl_2 was added dropwise and the reaction stirred for 15 min at –78 °C, then 2 h at rt. The reaction was quenched by addition of half-satd NH_4Cl soln (25 mL). Layers were separated and the aqueous extracted with CH_2Cl_2 (3 x 25 mL). The combined organic phases were washed with brine (100 mL), dried with $MgSO_4$, concentrated *in vacuo*, and purified by column chromatography (SiO_2, 30% EtOAc in hexanes), yielding the product as a yellow oil, a 70:30 mixture of diastereomers, R_f = 0.20 in 30% EtOAc in hexanes, (0.21 g, 57%). $[\alpha]_D^{25}$ = -2.9 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, cast) 3490, 2963, 2933, 2875, 1697, 1366, 1279, 1155, 1060 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.93 – 4.80 (m, 1H, H4), 4.59 – 4.52 (m, 2H, H9), 3.80 – 3.66 (m, 1H, H5), 3.33 – 3.27 (m, 2H, H10), 2.74 (br, 0.7H, H6), 2.32 (br, 0.3H, H6'), 1.81 – 1.75 (m, 1H, H2), 1.53 – 1.38 (m, 1H, H2'+H3), 1.19 (d, *J* = 7.0 Hz, 3H, H8), 0.99 – 0.83 (m, 6H, H1+H7). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 201.8, 179.8, 75.1, 56.5, 41.1, 37.2, 28.2, 25.3, 14.8, 11.1, 9.8. HRMS (ESI) Calcd for $C_{11}H_{19}NO_2S_2Na$ $[M+Na]^+$ 284.0749, found 284.0753.

***S*-2-Acetamidoethyl 3-hydroxy-4*R*-methylhexanethioate (24):**



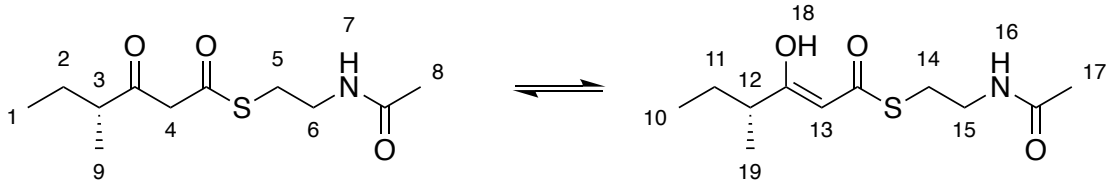
This new compound was synthesized using an established procedure.[36] To a stirred solution of 3-hydroxy-4*R*-methyl-1-(2-thioxothiazolidin-3-yl)hexan-1-one (0.135 g, 0.55 mmol) in 5 mL of dry acetonitrile was added K_2CO_3 (0.226 g, 1.64 mmol) followed by *N*-acetylcysteamine (0.070 mL, 0.65 mmol). The reaction was stirred for 30 min before removing the solvent *in vacuo*. The residue was suspended in CH_2Cl_2, washed with brine, and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.18 in EtOAc, (0.089 g, 66%). $[\alpha]_D^{25}$ = 1.70 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, cast) 3309, 3089, 2962, 2933, 1688, 1658, 1552 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.84 (br, 1H, H8), 4.03 – 3.98 (m, 0.7H, H4), 3.96 – 3.91 (m, 0.3H, H4'), 3.50 – 3.39 (m, 2H, H7), 3.10 – 3.00 (m, 2H, H6), 2.76 – 2.66 (m, 2H, H5), 2.62 (br, 0.3H, H10), 2.49 (br, 0.7H, H10'), 1.97 (s, 3H, H9), 1.56 – 1.48 (m, 1H, H2), 1.48 – 1.40 (m, 1H, H3), 1.22 – 1.12 (m, 1H, H2'), 0.94 – 0.88 (m, 6H, H1+H11). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 200.2, 200.0, 170.4, 72.4, 71.8, 48.7, 47.7, 40.1, 40.0, 39.3, 28.9, 25.6, 24.9, 23.3, 14.5, 13.8, 11.8, 11.5. HRMS (ESI) Calcd for $C_{11}H_{21}NO_3SNa$ $[M+Na]^+$ 270.1134, found 270.1135.

***S*-2-Acetamidoethyl 3-hydroxy-2,4*R*-dimethylhexanethioate (25):**



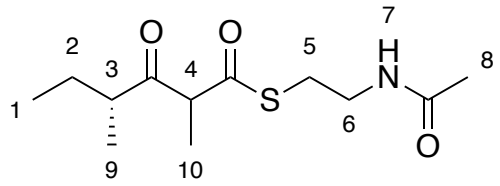
This new compound was synthesized using an established procedure.[32] To a stirred solution of 3-hydroxy-2,4*R*-dimethyl-1-(2-thioxothiazolidin-3-yl)hexan-1-one (0.127 g, 0.49 mmol) in 5 mL of dry acetonitrile was added K_2CO_3 (0.201 g, 1.46 mmol) followed by *N*-acetylcysteamine (0.062 mL, 0.58 mmol). The reaction was stirred for 30 min before removing the solvent *in vacuo*. The residue was suspended in CH_2Cl_2, washed with brine, and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.25 in EtOAc, (0.101 g, 80%). $[\alpha]_D^{25}$ = 0.89 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$, cast) 3308, 3087, 2965, 2935, 1660, 1552 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.83 (br, 1H, H8), 3.76 – 3.66 (m, 1H, H4), 3.51 – 3.39 (m, 2H, H7), 3.09 – 2.98 (m, 2H, H6), 2.89 – 2.84 (m, 1H, H5), 2.45 (br, 0.7H, H10), 2.14 (br, 0.3H, H10'), 1.96 (s, 3H, H9), 1.80 – 1.28 (m, 3H, H2+H2'+H3), 1.24 – 1.19 (m, 3H, H11), 0.92 – 0.88 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 204.8, 170.4, 75.7, 50.6, 39.4, 37.2, 28.7, 24.9, 23.3, 15.1, 11.0, 10.3. HRMS (ESI) Calcd for $C_{12}H_{23}NO_3SNa$ $[M+Na]^+$ 284.1291, found 284.1287.

***S*-2-Acetamidoethyl 4*R*-methyl-3-oxohexanethioate (26):**



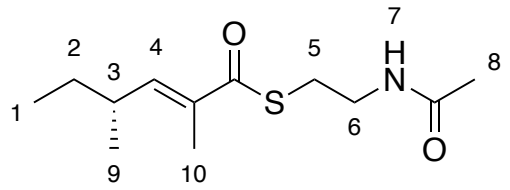
This new compound was synthesized using an established procedure.[36] To a stirred solution of *S*-2-acetamidoethyl 3-hydroxy-4*R*-methylhexanethioate (0.076 g, 0.31 mmol) in dry CH_2Cl_2 (5 mL) was added a suspension of Dess-Martin periodinane (0.047 g, 0.34 mmol) in dry CH_2Cl_2. The reaction was stirred for 2.5 h before quenching with 1:1 satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln (10 mL). The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 10 mL). Organic phases were combined, washed with brine (40 mL), and dried with Na_2SO_4 before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 70-100% EtOAc in hexanes), yielding the product as a colourless oil, a 2:1 mixture of keto-enol tautomers, R_f = 0.25 in EtOAc, (0.042 g, 56%). $[\alpha]_D^{25} = -8.0$ (*c* 0.14, CH_2Cl_2); IR (CH_2Cl_2, cast) 3296, 3078, 2968, 2933, 1720, 1657, 1613, 1551 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.98 (br, 1H, H7+H16), 4.45 (s, 0.3H, H13) 3.73 (s, 1.4H, H4), 3.49 – 3.44 (m, 2H, H6+H15), 3.10 – 3.07 (m, 2H, H5+H14), 2.59 – 2.53 (m, 0.7H, H3), 2.14 – 2.07 (m, 0.3H, H12), 1.97 (s, 3H, H8+H17), 1.75 – 1.59 (m, 2H, H2+H11+H3+H12), 1.49 – 1.39 (m, 1H, H2'+H11'), 1.14 – 1.10 (m, 3H, H9), 0.91 – 0.88 (m, 3H, H1+H10). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 206.0, 194.5, 192.6, 181.4, 170.5, 170.3, 98.3, 55.5, 48.6, 41.1, 40.0, 39.4, 29.3, 27.9, 27.2, 25.6, 23.2, 23.1, 17.6, 15.4, 11.8, 11.5. HRMS (ESI) Calcd for $C_{11}H_{19}NO_3SNa$ $[M+Na]^+$ 268.0978, found 268.0977.

***S*-2-Acetamidoethyl 2,4*R*-dimethyl-3-oxohexanethioate (27):**



This new compound was synthesized using an established procedure.[36] To a stirred solution of *S*-2-acetamidoethyl 3-hydroxy-2,4*R*-dimethylhexanethioate (0.083 g, 0.34 mmol) in dry CH_2Cl_2 (10 mL) was added Dess-Martin periodinane (0.051 g, 0.37 mmol). The reaction was stirred for 2.5 h before quenching with 10 mL of 1:1 satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln. The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 10 mL). Organic phases were combined, washed with brine (40 mL), and dried with Na_2SO_4 before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 70-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.30 in EtOAc, (0.053 g, 64%). $[\alpha]_D^{25} = -5.6$ (*c* 0.11, CH_2Cl_2); IR ($CHCl_3$, cast) 3297, 3077, 2968, 2922, 1722, 1660, 1600, 1551 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.85 (br, 1H, H7), 3.97 – 3.91 (m, 1H, H4), 3.50 – 3.35 (m, 2H, H6), 3.12 – 3.00 (m, 2H, H5), 2.72 – 2.64 (m, 1H, H3), 1.96 (s, 3H, H8), 1.76 – 1.64 (m, 1H, H2), 1.41 – 1.35 (m, 4H, H2'+H10), 1.09 (d, *J* = 7.0 Hz, 1.5H, H9), 1.08 (d, *J* = 7.0 Hz, 1.5H, H9'), 0.87 (t, *J* = 7.0 Hz, 1.5H, H1), 0.86 (t, *J* = 7.0 Hz, 1.5H, H1'). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 208.6, 208.5, 196.8, 196.6, 170.4, 60.1, 59.9, 47.4, 47.3, 39.3, 28.9, 26.1, 25.6, 23.2, 16.4, 16.0, 13.9, 13.8, 11.6. HRMS (ESI) Calcd for $C_{12}H_{21}NO_3SNa$ $[M+Na]^+$ 282.1134, found 282.1133.

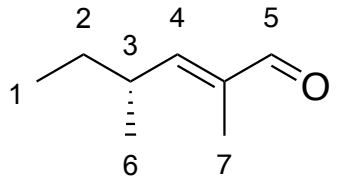
(*R*,*E*)-*S*-2-Acetamidoethyl 2,4-dimethylhex-2-enethioate (28):



This known compound[57] was synthesized using an established procedure.[53] To a stirred solution of *S*-2-acetamidoethyl 3-hydroxy-2,4*R*-dimethylhexanethioate (0.69 g, 2.6 mmol) in dry CH_2Cl_2 (20 mL) under argon at 0 °C was added diisopropylethylamine (0.91 mL, 5.2 mmol). After 5 min, methanesulfonyl chloride (0.62 mL, 7.8 mmol) was then added and the reaction stirred for 1 h. The cooling bath was removed and 1,8-diazabicyclo[5.4.0]undec-7-ene (1.55 mL, 10.4 mmol) was added slowly. Evolution of heat was noted. The reaction was stirred for 16 h at rt before cooling to 0 °C and quenching with 10% citric acid soln (20 mL). The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 20 mL). Organic phases were combined, washed with satd $NaHCO_3$ soln (80 mL), brine (80 mL), and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a yellow oil, R_f = 0.33 in EtOAc, (0.47 g, 74%). $[\alpha]_D^{25}$ = –29.7 (*c* 0.90, $CHCl_3$); ref $[\alpha]_D^{25}$ = –35.5 (*c* 1.0, $CHCl_3$)[57]; IR ($CHCl_3$, cast) 3289, 3080, 2962, 2929, 1658, 1598, 1551, 1456 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.53 (dq, *J* = 10.0, 1.4 Hz, 1H, H4), 5.90 (br, 1H, H7), 3.47 – 3.43 (m, 2H, H6), 3.07 – 3.05 (m, 2H, H5), 2.48 – 2.42 (m, 1H, H3), 1.96 (s, 3H, H8), 1.43 (d, *J* = 1.4 Hz, 3H, H10), 1.49 – 1.41 (m, 1H, H2), 1.40 – 1.31 (m, 1H, H2'), 1.02 (d, *J* = 6.5 Hz, 3H, H9), 0.86 (t, *J* = 7.5 Hz, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ

194.3, 170.2, 147.6, 135.4, 39.9, 35.1, 29.6, 28.5, 23.3, 19.6, 12.7, 11.9. HRMS (ESI) Calcd for $C_{12}H_{21}NO_2SNa$ $[M+Na]^+$ 266.1185, found 266.1181.

(*R,E*)-2,4-Dimethylhex-2-enal (29):

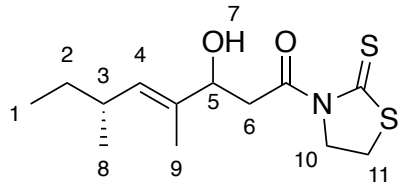


This compound was synthesized using a modified procedure.[36] (*R,E*)-2,4-dimethylhex-2-en-1-ol (0.76 g, 0.59 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and cooled to 0 °C under argon. Dess-Martin periodinane (0.090 g, 0.65 mmol) was added and the suspension was stirred for 5 min before warming to rt. After 3 h the reaction was cooled to 0 °C and quenched with 1:1 satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln (10 mL). The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 4 mL). Organic phases were combined, washed with 1:1 satd $NaHCO_3$ soln / brine (10 mL), and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, 10% EtOAc in hexanes), yielding the product as a yellow oil, R_f = 0.43 in 10% EtOAc in hexanes, (0.41 g, 54%). $[\alpha]_D^{25}$ = –18.4 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2, cast) 2964, 2930, 2876, 1689, 1644, 1388 cm^{-1}; ^1H NMR (CD_2Cl_2, 500 MHz) δ 9.38 (s, 1H, H5), 6.25 (dq, *J* = 10.0, 1.5 Hz, 1H, H4), 2.68 – 2.57 (m, 1H, H3), 1.72 (d, *J* = 1.5 Hz, 3H, H7), 1.54 – 1.46 (m, 1H, H2), 1.43 – 1.34 (m, 1H, H2'), 1.05 (d, *J* = 6.5 Hz, 3H, H6), 0.88 (t, *J* = 7.5 Hz, 3H, H1). ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 195.7, 160.6, 138.5, 35.5, 29.9, 19.6, 12.0, 9.5.

Alternative method of preparation

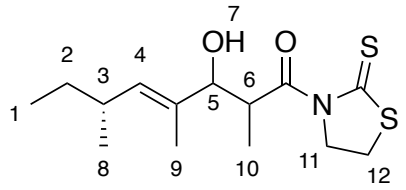
This compound was also prepared by an alternative method.[54] Activated MnO_2 was prepared according to an established procedure.[58] To a solution of (*R,E*)-2,4-dimethylhex-2-en-1-ol (0.016 g, 0.12 mmol) in dry CH_2Cl_2 (1 mL) was added activated MnO_2 (0.11 g, 1.2 mmol). The vessel was then sealed under argon and stirred for 12 h. The reaction mixture was filtered through Celite, washing with CH_2Cl_2. Reaction completion was confirmed by NMR, and the aldehyde was concentrated before running through a $MgSO_4$ plug and adding into the subsequent reaction (0.016 g, quant.).

(*6R,E*)-3-Hydroxy-4,6-dimethyl-1-(2-thioxothiazolidin-3-yl)oct-4-en-1-one (30):



This new compound was synthesized using a modified procedure.[36] To a stirred solution of *N*-acetyl-thiazolidine-2-thione (0.014 g, 0.09 mmol) in dry CH_2Cl_2 (5 mL) was added $TiCl_4$ (1.0 M soln in CH_2Cl_2, 0.10 mL, 0.10 mmol) at 0 °C under argon. The reaction mixture was stirred for 5 min to dissolve, then cooled to –78 °C. Diisopropylethylamine (0.018 mL, 0.10 mmol) was added and the reaction stirred for 30 min. A solution of (*R,E*)-2,4-dimethylhex-2-enal (0.010 g, 0.08 mmol) in CH_2Cl_2 was added dropwise and the reaction stirred for 15 min at –78 °C, then 1.5 h at rt. The reaction was quenched by addition of 10% citric acid soln (5 mL). Layers were separated and the aqueous extracted with CH_2Cl_2 (3 x 4 mL). The combined organic phases were dried with $MgSO_4$, concentrated *in vacuo*, and purified by column chromatography (SiO_2, 30% EtOAc in hexanes), yielding the product as a yellow oil, R_f = 0.16 in 30% EtOAc in hexanes, (0.013 g, 57%). $[\alpha]_D^{25}$ = –12.2 (*c* 0.27, $CHCl_3$); IR ($CHCl_3$, cast) 3467, 2957, 2924, 1700, 1460 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.27 – 5.23 (m, 1H, H4), 4.62 – 4.52 (m, 3H, H10+H5), 3.58 – 3.52 (m, 1H, H6), 3.48 – 3.44 (m, 3H, H6'), 3.34 – 3.26 (m, 2H, H11), 2.67 (br, 1H, H7), 2.32 – 2.24 (m, 1H, H3), 1.67 (s, 3H, H9), 1.39 – 1.29 (m, 1H, H2), 1.30 – 1.17 (m, 1H, H2'), 0.95 – 0.91 (m, 3H, H8), 0.86 – 0.81 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 201.9, 174.0, 134.1, 133.6, 55.8, 44.6, 33.7, 30.3, 28.6, 20.6, 12.5, 12.0. HRMS (ESI) Calcd for $C_{13}H_{21}NO_2S_2Na$ $[M+Na]^+$ 310.0906, found 310.0910.

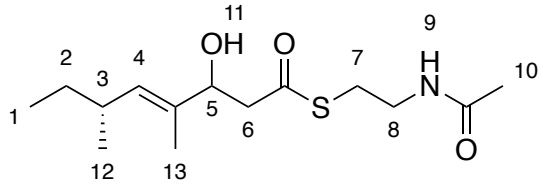
(*6R,E*)-3-Hydroxy-2,4,6-trimethyl-1-(2-thioxothiazolidin-3-yl)oct-4-en-1-one (31):



This new compound was synthesized using a modified procedure.[36] To a stirred solution of *N*-propionyl-thiazolidine-2-thione (0.015 g, 0.09 mmol) in dry CH_2Cl_2 (25 mL) was added $TiCl_4$ (1.0 M soln in CH_2Cl_2, 1.4 mL, 1.4 mmol) at 0 °C under argon. The reaction was stirred for 5 min to dissolve, then cooled to –78 °C. Diisopropylethylamine (0.018 mL, 0.10 mmol) was added and the reaction stirred for 30 min. A solution of (*R,E*)-2,4-dimethylhex-2-enal (0.010 g, 0.08 mmol) in CH_2Cl_2 was added dropwise and the reaction stirred for 15 min at –78 °C, then 1.5 h at rt. The reaction was quenched by addition of 10% citric acid soln (5 mL). Layers were separated and the aqueous extracted with CH_2Cl_2 (3 x 4 mL). The combined organic phases were dried with $MgSO_4$, concentrated *in vacuo*, and purified by column chromatography (SiO_2, 30% EtOAc in hexanes), yielding the product as a yellow oil and a 50:50 mixture of diastereomers, R_f = 0.19 in 30% EtOAc in hexanes, (0.015 g, 64%). $[\alpha]_D^{25}$ = –12.2 (*c* 0.26, $CHCl_3$); IR ($CHCl_3$, cast) 3472, 2958, 2924, 1702, 1456 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.30 – 5.28 (m, 1H, H4), 4.82 – 4.76 (m, 1H, H6), 4.62 – 4.46 (m, 2H, H11), 4.39 – 4.37 (m, 1H, H5), 3.34 – 3.20 (m, 2H, H12), 2.47 (br, 0.5H, H7'), 2.40 (br, 0.5H, H7'), 2.35 – 2.25 (m, 1H, H3), 1.60 (s, 3H, H9), 1.39 – 1.29 (m, 1H, H2), 1.31 – 1.22 (m, 1H, H2'), 1.17 (d, *J* = 7.0 Hz, 3H, H10), 0.94 – 0.92 (m, 3H, H8), 0.85 – 0.82 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 201.7, 178.6, 133.3, 133.0, 56.5, 42.3,

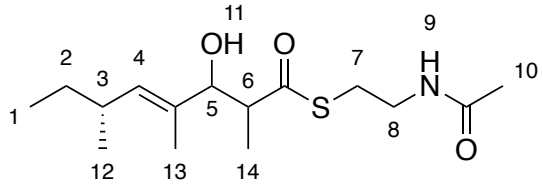
33.9, 30.4, 28.4, 20.7, 13.5, 12.1, 11.0. HRMS (ESI) Calcd for $C_{14}H_{23}NO_2S_2Na$ $[M+Na]^+$ 324.1062, found 324.1066.

(*6R,E*)-*S*-2-Acetamidoethyl 3-hydroxy-4,6-dimethyloct-4-enethioate (33):



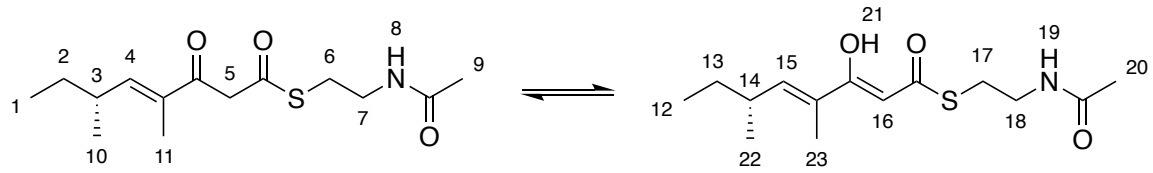
This new compound was synthesized using a modified procedure.[36] To a stirred solution of (*6R,E*)-3-hydroxy-4,6-dimethyl-1-(2-thioxothiazolidin-3-yl)oct-4-en-1-one (0.0034g, 0.012 mmol) in dry acetonitrile (1 mL) was added K_2CO_3 (0.049 g, 0.035 mmol) followed by *N*-acetylcysteamine (0.0015 mL, 0.014 mmol). The reaction was stirred for 30 min before removing the solvent *in vacuo*. The residue was suspended in CH_2Cl_2, washed with brine, and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.30 in EtOAc, (0.0022 g, 65%). $[\alpha]_D^{25} = -14.4$ (*c* 0.11, $CHCl_3$); IR ($CHCl_3$, cast) 3295, 3091, 2959, 2927, 2872, 1690, 1658, 1553 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.80 (br, 1H, H9), 5.26 – 5.22 (m, 1H, H4), 4.50 – 4.46 (m, 1H, H5), 3.50 – 3.40 (m, 2H, H8), 3.10 – 3.00 (m, 2H, H7), 2.88 – 2.81 (m, 1H, H6), 2.79 – 2.72 (m, 1H, H9'), 2.35 (br, 1H, H11), 2.32 – 2.22 (m, 1H, H3), 1.97 (s, 3H, H10), 1.64 (d, *J* = 1.0 Hz, 3H, H13), 1.38 – 1.29 (m, 1H, H2), 1.28 – 1.16 (m, 1H, H2'), 0.94 – 0.90 (m, 3H, H8), 0.85 – 0.79 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 199.0, 170.4, 133.9, 133.5, 77.3, 49.8, 39.5, 33.8, 30.2, 28.9, 23.3, 20.5, 12.2, 11.9. HRMS (ESI) Calcd for $C_{14}H_{25}NO_3SNa$ $[M+Na]^+$ 310.1447, found 310.1447.

(*6R,E*)-*S*-2-Acetamidoethyl 3-hydroxy-2,4,6-trimethyloct-4-enethioate (34):



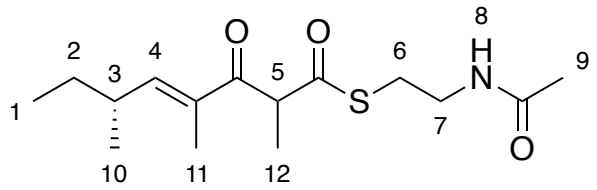
This new compound was synthesized using a modified procedure.[36] To a stirred solution of (*6R,E*)-3-hydroxy-2,4,6-trimethyl-1-(2-thioxothiazolidin-3-yl)oct-4-en-1-one (0.0092 g, 0.031 mmol) in dry acetonitrile (1 mL) at rt was added K_2CO_3 (0.013 g, 0.092 mmol) followed by *N*-acetylcysteamine (0.0039 mL, 0.037 mmol). The reaction was stirred for 20 min before removing the solvent *in vacuo*. The residue was suspended in CH_2Cl_2, washed with brine, and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.39 in EtOAc, (0.0056 g, 61%). $[\alpha]_D^{25} = -23.2$ (*c* 0.062, $CHCl_3$); IR ($CHCl_3$, cast) 3305, 3089, 2960, 2927, 1684, 1659, 1551, 1454 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.78 (br, 1H, H9), 5.27 – 5.20 (m, 1H, H4), 4.32 – 4.14 (m, 1H, H5), 3.52 – 3.38 (m, 2H, H8), 3.09 – 2.95 (m, 2H, H7), 2.91 – 2.84 (m, 1H, H6), 2.32 – 2.24 (m, 1H, H3), 2.20 (br, 0.5H, H11), 2.12 (br, 0.5H, H11'), 1.96 (s, 3H, H10), 1.61 (s, 3H, H13), 1.39 – 1.28 (m, 1H, H2), 1.28 – 1.19 (m, 1H, H2'), 1.20 – 1.17 (m, 3H, H14), 0.94 – 0.90 (m, 3H, H12), 0.85 – 0.79 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 203.3, 170.3, 134.3, 132.2, 76.8, 51.7, 39.6, 33.9, 30.2, 28.6, 23.3, 20.6, 13.2, 12.0, 11.5. HRMS (ESI) Calcd for $C_{15}H_{27}NO_3SNa$ $[M+Na]^+$ 324.1604, found 324.1607.

(*R*,*E*)-*S*-2-Acetamidoethyl 4,6-dimethyl-3-oxooct-4-enethioate (36):



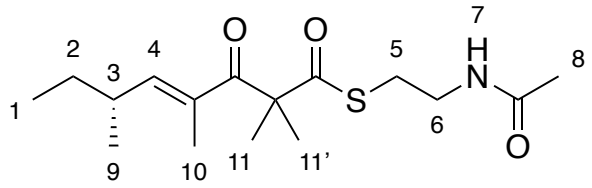
This new compound was synthesized using an established procedure.[36] To a stirred solution of (*6R*,*E*)-*S*-2-acetamidoethyl 3-hydroxy-4,6-dimethyloct-4-enethioate (0.0022 g, 0.0077 mmol) in dry CH_2Cl_2 (1 mL) at 0°C was added Dess-Martin periodinane (0.0036 g, 0.0085 mmol). The reaction was stirred for 16 h before quenching with 1:1 satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln (2 mL). The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 2 mL). Organic phases were combined and dried with $MgSO_4$ before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, 50% EtOAc in hexanes), yielding the product as a colourless oil, a 2:1 mixture of keto-enol tautomers, R_f = 0.41 in EtOAc, (0.0019 g, 87%). $[\alpha]_D^{25}$ = –23.2 (*c* 0.062, $CHCl_3$); IR ($CHCl_3$, cast) 3303, 3076, 2962, 2928, 1660, 1582, 1556 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 12.76 (br, 0.3H, H21), 6.44 – 6.42 (m, 0.3H, H15), 6.40 – 6.37 (m, 0.7H, H15), 5.96 (br, 1H, H10+H23), 5.64 (s, 0.3H, H16), 3.99, 3.96 (ABq, *J* = 15.0 Hz, 1.4H, H5), 3.50 – 3.44 (m, 2H, H7+H18), 3.12 – 3.08 (m, 2H, H6+H17), 2.58 – 2.42 (m, 1H, H3+H14), 1.98 (s, 2.1H, H9), 1.97 (s. 0.9H, H20), 1.80 (d, *J* = 1.4 Hz, 2.1H, H11), 1.78 (d, *J* = 1.4 Hz, 0.9H, H23), 1.51 – 1.42 (m, 1H, H2+H13), 1.28 – 1.19 (m, 1H, H2'+H13'), 1.04 (d, *J* = 7.0 Hz, 2.1H, H10), 1.01 (d, *J* = 7.0 Hz, 0.9H, H22), 0.89 – 0.84 (m, 3H, H1+H12). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.6, 193.7, 193.3, 170.9, 170.5, 170.3, 151.7, 144.8, 135.8, 126.9, 96.9), 52.6, 40.0, 39.3, 35.5, 35.0, 29.8, 29.6, 29.2, 28.0, 23.3, 19.9, 19.5, 12.2, 12.0, 11.9, 11.5, 11.0. HRMS (ESI) Calcd for $C_{14}H_{23}NO_3SNa$ $[M+Na]^+$ 308.1291, found 308.1289.

(*6R,E*)-*S*-2-Acetamidoethyl 2,4,6-trimethyl-3-oxooct-4-enethioate (37):



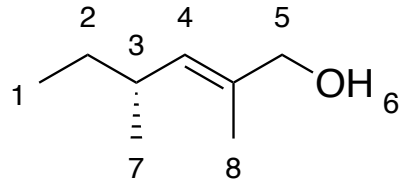
This new compound was synthesized using an established procedure.[36] To a stirred solution of (*6R,E*)-*S*-2-acetamidoethyl 3-hydroxy-2,4,6-trimethyloct-4-enethioate (0.0051 g, 0.017 mmol) in dry CH_2Cl_2 (1 mL) was added Dess-Martin periodinane (0.0079 g, 0.019 mmol). The reaction was stirred at rt for 3 h before quenching with 1:1 satd $NaHCO_3$ soln / satd $Na_2S_2O_3$ soln (2 mL). The phases were separated and the aqueous extracted with CH_2Cl_2 (3 x 2 mL). Organic phases were combined, washed with brine (5 mL), and dried with Na_2SO_4 before concentrating *in vacuo*. The residue was purified by column chromatography (SiO_2, gradient elution from 50-100% EtOAc in hexanes), yielding the product as a colourless oil, R_f = 0.41 in EtOAc, (0.0047 g, 92%). $[\alpha]_D^{25}$ = –19.2 (*c* 0.12, CH_3OH); IR (CH_3OH, cast) 3069, 2963, 2932, 1690, 1661, 1551 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.46 (d, *J* = 9.5 Hz 1H, H4), 5.81 (br, 1H, H8), 4.48 – 4.43 (m, 1H, H5), 3.51 – 3.34 (m, 2H, H7), 3.12 – 2.98 (m, 2H, H6), 2.57 – 2.48 (m, 1H, H3), 1.96 (s, 3H, H9), 1.80 (d, *J* = 1.0 Hz, 3H, H11), 1.53 – 1.43 (m, 1H, H2), 1.42 – 1.32 (m, 1H, H2'), 1.41 (d, *J* = 7.0 Hz, 3H, H12), 1.04 (d, *J* = 6.5 Hz, 3H, H10), 0.89 – 0.85 (m, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 197.4, 196.8, 170.7, 150.7, 135.4, 54.9, 39.5, 35.6, 29.7, 28.8, 23.2, 19.6, 15.0, 12.0, 11.9. HRMS (ESI) Calcd for $C_{15}H_{25}NO_3SNa$ $[M+Na]^+$ 322.1447, found 322.1444.

(*R,E*)-*S*-2-Acetamidoethyl 2,2,4,6-tetramethyl-3-oxooct-4-enethioate (38):



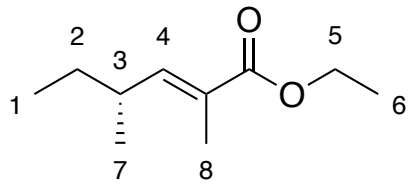
Sodium hydride (0.077 g, 0.19 mmol, 60% suspension in oil) was weighed into a conical-bottomed vial. A solution of (*6R,E*)-*S*-2-acetamidoethyl 2,4,6-trimethyl-3-oxooct-4-enethioate (0.0017 g, 0.0055 mmol) in dry THF (0.1 mL) was added to the vial. The contents were cooled to 0 °C and iodomethane (0.01 mL, 0.16 mmol) was added. The reaction was stirred under argon for 23 h before quenching with 10% citric acid soln (0.4 mL). The mixture was extracted with EtOAc. Organic phases were combined, dried with $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, 50% EtOAc in hexanes) to provide a pale-yellow oil, R_f = 0.53 in EtOAc, (0.00071 g, 42%). $[\alpha]_D^{25}$ = –37.3 (*c* 0.03, $CHCl_3$); IR ($CHCl_3$, cast) 3073, 2961, 2923, 1659, 1583, 1375, 1357 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.10 (dq, *J* = 7.0, 1.4 Hz, 1H, H4), 5.75 (br, 1H, H7), 3.45 – 3.40 (m, 2H, H6), 3.06 – 3.02 (m, 2H, H5), 2.48 – 2.42 (m, 1H, H3), 1.97 (s, 3H, H8), 1.79 (d, *J* = 1.4 Hz, 3H, H10), 1.47, 1.47 (ABd, 6H, H11+H11'), 1.44 – 1.22 (m, 1H, H2+H2'), 0.97 (d, *J* = 4.5 Hz, 3H, H9), 0.83 (t, *J* = 5.5 Hz, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 203.1, 199.6, 170.3, 149.5, 133.0, 61.2, 39.7, 35.5, 29.8, 28.7, 25.5, 24.5, 23.4, 19.6, 12.9, 12.0. HRMS (ESI) Calcd for $C_{16}H_{27}NO_3SNa$ $[M+Na]^+$ 336.1604, found 336.1602.

(*R,E*)-2,4-Dimethylhex-2-en-1-ol (41):



This compound was synthesized using a modified procedure.[59] (*R,E*)-*S*-2-acetamidoethyl 2,4-dimethylhex-2-enethioate (0.258 g, 1.06 mmol) was dissolved in dry CH_2Cl_2 (10 mL) under argon and cooled to –78 °C. DIBAL-H solution (1 M in CH_2Cl_2, 3.18 mmol) was added dropwise. After stirring for 2 h the reaction was cooled to 0 °C and quenched by addition of satd Rochelle salt soln (15 mL). The phases were separated and the aqueous phase extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, 20% EtOAc in hexanes) to provide a pale-yellow oil, R_f = 0.30 in 20% EtOAc in hexanes, (0.106 g, 78%). $[\alpha]_D^{25}$ = –25.6 (*c* 1.49, $CHCl_3$); IR (CH_2Cl_2, cast) 3315, 2960, 2923, 1456, 1012 cm^{-1}; ^1H NMR (CD_2Cl_2, 500 MHz) δ 5.17 – 5.14 (m, 1H, H4), 3.95 (d, *J* = 7.0 Hz, 2H, H5), 2.34 –2.25 (m, 1H, H3), 1.65 (d, *J* = 1.5 Hz, 3H, H8), 1.42 (t, *J* = 7.0 Hz, 1H, H6), 1.39 – 1.31 (m, 1H, H2), 1.27 – 1.18 (m, 1H, H2'), 0.93 (d, *J* = 7.0 Hz, 3H, H7), 0.84 (t, *J* = 7.5 Hz, 3H, H1). ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 134.1, 132.6, 69.1, 34.1, 30.6, 20.7, 13.9, 12.0. HRMS (EI) Calcd for $C_8H_{16}O$ $[M]^+$ 128.1201, found 128.1198.

(*R*,*E*)-Ethyl 2,4-dimethylhex-2-enoate (43):



This compound was synthesized using an established procedure.[54] To a solution of (*R*)-2-methylbutanal (0.617 g, 7.16 mmol) in dry CH_2Cl_2 (35 mL) was added (carbethoxyethylidene)triphenylphosphorane (3.89 g, 10.7 mmol). The reaction mixture was stirred under argon at 35 °C for 40 h before removing the solvent *in vacuo*. The residue was suspended in 2:1 pentanes/ether (40 mL) and filtered. The filtrate was concentrated *in vacuo* and purified by flash chromatography (SiO_2, 20:1 pentanes/ether) to provide a yellow oil, R_f = 0.31 in 20:1 pentanes/ether, (0.317 g, 26%). $[\alpha]_D^{25}$ = –33.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.53 (dq, *J* = 10.1, 1.3 Hz, 1H, H4), 4.18 (q, *J* = 7.2 Hz, 2H, H5), 2.46 – 2.34 (m, 1H, H3), 1.83 (d, *J* = 1.3 Hz, 3H, H8), 1.48 – 1.25 (m, 2H, H2), 1.29 (t, *J* = 7.1 Hz, 3H, H6), 0.99 (d, *J* = 6.7 Hz, 3H, H7), 0.85 (t, *J* = 7.4 Hz, 3H, H1). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.7, 148.0, 126.7, 60.5, 35.1, 29.8, 19.8, 14.4, 12.7, 12.1. HRMS (EI) Calcd for $C_{10}H_{19}O_2$ $[M+H]^+$ 171.1385, found 171.1379.

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