ENGINEERING NOVEL TANDEM REACTIONS USING ORGANOMETALLIC CATALYSTS AND METALLOENZYMES

BY

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THESIS
Submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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Abstract

Organometallic chemistry and bioinorganic chemistry are two prominent sub-disciplines of chemistry. Complexes of the transition metals are prominent components of both disciplines. On the one hand, considerable efforts in transition-metal catalysis have culminated in practical and efficient transformations such as isomerization, olefin metathesis, hydroxylation, epoxidation and others. On the other hand, metalloenzymes and enzymes with bound metal centers have been investigated and engineered to catalyze reactions with organic compounds that are synthetically and industrially important.

While these two fields of transition metal catalysis have grown and matured, they have often learned from each other, but not worked cooperatively. Enzymes work mostly in buffer, at mild conditions, and in air. Organometallic catalysts often require very different conditions, such as organic solvents, inert atmosphere, and high temperatures. Research interests over the last several years have grown in attempting to combine these two sub-disciplines for asymmetric synthesis to achieve atom-economical transformations, a process called aptly tandem catalysis. However, until now, in no case has a metalloenzyme and an organometallic complex been shown to react cooperatively.

Our project aims at combining organometallic catalysts and metalloenzymes for cooperative reactions to access products and selectivities that would not occur with either catalyst alone and would occur in lower yield if conducted as two sequential reactions. In particular we present our work on tandem reactions between ruthenium catalysts and P450s for enantioselective and regioselective enzymatic asymmetric epoxidations, a reaction which still poses a few issues for
organic chemists with respect to scope and enantioselectivity. In Chapter 2, we combined a ruthenium isomerization catalyst and P450 BM3 mutants for the oxidation of olefins from an equilibrium established by the ruthenium-catalyzed isomerization. We found that the two catalysts can effectively work together in a biphasic system, for a successive isomerization and epoxidation of alkenes and short olefinic acids. However, we observed that under the reaction conditions, the \textit{trans- to cis-} isomerization rate of olefins is too slow. Lastly, we also found that the enzymatic reaction is not selective enough, and that yields are mass transfer limited. Therefore, the enzymatic epoxidation cannot exert a driving force powerful enough to drive the reaction towards the epoxidation of one alkene of interest. In Chapter 3, we engineered a cross-metathesis-epoxidation reaction using a ruthenium olefin metathesis catalyst and the wild type P450 from \textit{Bacillus megaterium}. We show that 90\% yield of a single epoxide can be obtained selectively from the cross metathesis of two alkenes, a reaction that would yield a maximum 64\% yield if ran sequentially. This study suggests that these two classes of catalyst can be combined in new ways as the metal catalysts become more tolerant of functional groups and the enzymes become more tolerant of organic media.
To God

To my family
Acknowledgments

I first wish to thank my advisors, Huimin Zhao and John Hartwig, for giving me the opportunity to work on these projects, for their patience and for the constant guidance and support they have provided me through this journey. I also thank the Zhao, Hartwig, and Denmark research groups for their invaluable advice and useful discussions, in particular Dr. Haige Lu, Dr. Martin Jarenmark, Dr. Ramesh Giri, Dr. Levi Stanley, for their useful discussions. This would not have been possible without the support of my significant other and of my family.
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CHAPTER 1. INTRODUCTION

1.1 Introduction

Organometallic chemistry and bioinorganic chemistry are two prominent sub-disciplines of chemistry. Complexes of the transition metals are prominent components of both disciplines. In the last several decades, considerable efforts in transition-metal catalysis have culminated in practical and efficient transformations such as isomerization, olefin metathesis, hydroxylation, epoxidation, hydroformylation and other asymmetric syntheses. On the other hand, metalloenzymes and enzymes with bound metal centers have been investigated and engineered to catalyze reactions with organic compounds that are synthetically and industrially important. For example, several metalloenzymes catalyze the oxidation of alkanes and alkenes with high enantio- and regioselectivity in most cases not accessible by man-made catalysts. Such transformations are highly desirable, since alkanes are ubiquitous in nature, a rich source of energy, and abundant as chemical feedstocks derived from petroleum cracking. Enantiopure epoxides and alcohols serve as important starting material for the pharmaceutical and chemical industries.

While these two fields of transition metal catalysis have grown and matured, they have often learned from each other, but not worked cooperatively. Enzymes work mostly in buffer, at mild conditions, and in air. Organometallic catalysts often require very different conditions, such as organic solvents, inert atmosphere, and high temperatures. Research interests over the last several years have grown in attempting to streamline these two sub-disciplines for asymmetric synthesis to achieve atom-economical transformations, a process called aptly tandem catalysis.
However, in no case has a metalloenzyme and an organometallic complex been shown to react cooperatively.

Our project aims at combining organometallic catalysts and metalloenzymes in tandem for enantioselective and regioselective enzymatic asymmetric epoxidations, a reaction which still poses a few issues for organic chemists with respect to scope and enantioselectivity. In the next several sections, we will first give an overview of tandem catalysis, with an emphasis on metal catalysts and metalloenzymes. Secondly, we will present the state-of-the-art technological advances in the enantioselective epoxidation of double bonds using metal catalysts, enzymes and artificial enzymes. Examples of exploiting hydrolytic kinetic resolutions for the preparation of enantiopure epoxides are also included. Lastly, we present two tandem catalysis approaches, which are expanded on in Chapters 2 and 3.

1.2 Asymmetric tandem catalysis

In nature, multi-enzymatic systems cooperate to accomplish extremely efficient one-pot tandem catalysis. As in an assembly line, tens of enzymes are well organized to transform simple materials to complex molecules with perfect control of selectivity by a series of coupled reactions in the cell. In attempts to emulate multi-enzymatic systems, one goal of synthetic chemistry is to streamline chemical syntheses by employing strategies of modular combination of catalytic reactions into one synthetic operation. As a powerful strategy to improve synthetic efficiency, tandem catalysis offers several advantages over sequential reactions including cooperative effect between two or more catalysts, stereochemical control, suppression of side reactions, and minimum workup and change in conditions [1]. From simple achiral starting
materials, complex molecules with one or more stereogenic centers can be synthesized, which could not be accessed through one catalytic center alone.

As promising as it sounds, asymmetric tandem catalytic reactions generally suffer from three major shortcomings: catalyst compatibility, reaction selectivity, and reaction condition compatibility. For example, putting two or more metal catalysts in one system may scramble the coordination of each metal with its chiral ligand, which makes the selectivity of the reaction unpredictable. To promote reaction selectivity, every step of a tandem reaction should strictly follow the designed sequence to avoid the generation of side products or termination of the tandem reaction. Lastly, each reaction in its optimized condition may not be compatible with others; therefore changes in reaction conditions may not suit every reaction in the tandem process. Nonetheless, there are many examples in the literature of tandem reactions for asymmetric synthesis (reviewed in [2-4]). Chemists engineered several strategies to overcome the obstacles mentioned above, firstly through the use of compatible catalysts, or catalyst encapsulation to avoid catalyst interference. Secondly, phase separation techniques can be used. Thirdly, catalysts and substrates can be added sequentially, and these additions can be fine-tuned to improve reaction selectivity.

Based on these approaches, one can classify several kinds of tandem catalytic reactions (Scheme 1.1). For example, two catalytic centers can work in tandem in which catalyst I acts upon A to produce the intermediate B, which becomes a substrate for catalyst II to produce the final product P (Scheme 1.1-I). In other cases, one catalyst can effect two transformations, either through the addition of an additional substrate C (Scheme 1.1-III), or by using both the starting
material A and C as substrates towards the final step (Scheme 1.1-V). Similar systems as III and IV can be envisioned as well, except that two catalysts operate instead of just one (Scheme 1.1-II and Scheme 1.1-IV). The polymerization of ethylene to branched polyethylene by two different catalysts serves as an example of Scheme 1.1-IV. Catalyst I dimerizes ethylene exclusively into 1-butene, whereas catalyst II copolymerizes 1-butene and ethylene to high molecular weight poly(ethylene-co-butene) materials. Systems with three catalysts are rare, but have been reported as well (insert citation) (Scheme 1.1-VI).

Depending on the nature of the catalysts (metal catalysts, organocatalysts, biocatalysts), several combinations can be envisioned between catalysts from the same discipline or across disciplines. One can appreciate therefore, how tandem catalysis approaches expand the scope of asymmetric catalysis, assuming that many of these combinations are made possible. In the next sections, a few examples of tandem catalytic reaction using multiple metal catalysts and multiple enzymes will be presented. Lastly, tandem reactions involving metal catalysts and enzymes will also be presented. For full review of tandem reactions involving multiple organocatalysts, metal catalysts and organocatalysts, the readers should consult more thorough reviews [1-5].

1.2.1 Multiple metal catalysts
Asymmetric tandem reactions with multiple metal catalysts still remain challenging. In general, these one-pot reactions are catalyzed by one metal center (Scheme 1.1-III and Scheme 1.1-V), or two very similar metal catalysts (two ruthenium catalysts for example). Such is the case for the tandem metathesis-isomerization reported by Fustero and coworkers for the preparation of a series of unsaturated lactams with up to 93% yield [6]. In one example in which two similar
catalysts are used, Arisawa et al. used a ruthenium carbene in tandem with a ruthenium hydride for the synthesis of several heterocycles with yields up to 74% (insert citation) (Scheme 1.2).

In 2004, B. L. Feringa reported the catalytic asymmetric synthesis of (-)-pumiliotoxin C, the key step of which is a Cu/Pd catalyzed tandem asymmetric conjugate addition–allylic substitution reaction (Scheme 1.3) [7]. To prevent catalyst contamination, the palladium catalyst was added after the first reaction was completed and the reaction conditions were changed. Other reports in which one of the catalysts or substrates is added separately, or the reaction conditions are changed to afford the second step of the reaction are quite common with metal catalysts in tandem. In 2006, Hartwig and coworkers developed a sequential reaction for the conversion of easily synthesized branched aromatic esters to branched allylic products in good yield and excellent regio- and enantioselectivity (Scheme 1.4) (insert citation). At the end of the first reaction, the reaction mixture was passed through a plug of silica to remove the bulk of the palladium catalyst. Next, the ligand, iridium catalyst and the amine nucleophile were added [8].

More recently, Nibayashi and coworkers reported the deracemization of secondary benzylic alcohols by a two-step process by using two different chiral catalysts. The first ruthenium catalyst catalyzes the S-selective oxidation of the secondary alcohol to the ketone, while the second catalyst reduces the ketone to the R-alcohol. This DKR (specify the acronym when it is first used)-type reaction yielded quantitative R-benzylic alcohols with high ee of >90% [9] (Scheme 1.5).

1.2.2 Multiple enzymes

Tandem biocatalysis is a powerful tool for enantioselective synthesis owing to its high selectivity and non-toxicity. Multiple enzyme catalysts can perform under the same mild conditions. With
protein engineering, enzymes have been endowed with activity on non-natural substrates. However, to date, many synthetically and industrially relevant substrates are not catalyzed by enzymes with acceptable activity. As a result, tandem biocatalysis for synthetic chemistry is still not a very popular and examples are limited. In 2009, Kroutil and coworkers developed the first biological deracemization of secondary alcohols, a reaction analogous to the deracemization reported by Niyibashi but with enzymes. In their system, *Alcaligenes faecalis* used molecular oxygen for an *R*-selective oxidation of the secondary alcohol to the respective ketone, leaving the *S*-alcohol intact. An alcohol dehydrogenase from *Rhodococcus ruber* 44541 then performs an *S*-selective reduction, which effectively transforms the racemic alcohol to an *S*-alcohol with 99% ee and 99% yield [10]. Their substrate scope included aliphatic, aromatic sec- alcohols, as well as ethyl esters.

A whole-cell biphasic tandem reaction for the asymmetric dihydorxylation of styrene oxide derivatives was reported by Xu and coworkers. Whole cells of *E. coli* expressing *styA* epoxidized styrene derivatives to *S*- epoxides (with >99 ee), while the lyophilized whole cells of *Sphingomonas sp.* HXN-200 selectively ring opened the formed epoxides to afford highly enantiopure diols [11] (Scheme 1.6). By adjusting the reaction rates, this work also highlighted that the addition of epoxide hydrolase accelerated the epoxidation reaction. In another report from the Kroutil lab, terminal epoxides were synthesized starting from prochiral *α*-halo ketones, which were asymmetrically reduced to the corresponding halohydrins by an alcohol dehydrogenase. The latter was transformed further into the corresponding terminal epoxides by a non-selective halohydrin dehalogenase. The stereoselectivity of the reaction was however, only
controlled by the dehydrogenase reaction [12], thus this reaction cannot be used as a kinetic resolution.

1.2.3 Metal catalysts and enzymes
1.2.3.1 Kinetic dynamic resolutions using chemical catalysts and enzymes
A powerful strategy for the synthesis of important chiral compounds is the DKR, a way to transform a racemic mixture exclusively into one enantiomer. Kinetic resolutions of racemates using enzymes are very efficient in terms of selectivity; however, only a maximum of 50% yield for the desired enantiomer can be achieved. Therefore, adding a racemization catalyst to continuously replenish the pool of reacted enantiomer can ensure that the reaction goes to 100% completion. The first DKR using enzymes and metal catalysts were reported in the late 90s [13, 14]. These DKR typically required long reaction times and a lower yield. More recently, Bäckvall and coworkers have been in the forefront of developing DKR, combining a lipase-catalyzed trans-esterification with an in situ racemization catalyzed by a metal complex. The groups of Williams and Bäckvall first reported the combination of enzymes and transition metals for DKR of sec-alcohols. They used the immobilized Pseudomonas cepacia lipase B (CALB) as the KR enzyme, different Shvo’s ruthenium complexes for the racemization and p-chlorophenyl as the acyl donor [15] (Scheme 1.7). In their best case, a yield of 92% and ee of >99% was obtained for the DKR of phenyl-1-ethanol in under 3h. As the first practical route to prepare enantiomerically pure phenyl-1-ethanol, this approach has been licensed to DSM.

Naturally occurring lipases are (R)-specific, which means only (R)-acetate can be formed, enriching the (S)-alcohol. Serine proteases, on the other hand, are known to catalyze trans-
esterifications similar to those catalyzed by lipases but, interestingly, with reversed enantioselectivity. Bäckvall and coworkers used this enzyme to engineer a DKR to produce (S)-phenyl-1-ethanol [16]. Serine proteases, such as subtilisin, are not as thermostable as lipases, thus require a lower operating temperature, which slows down the racemization. As a result, the highest yield obtained for their process was 52%.

Racemization of amines is difficult to achieve and usually requires harsh reaction conditions. Using a newly designed Shlov ruthenium complex, Bäckvall and coworkers reported a DKR of a variety of primary phenyl amines [17]. A few limitations with this reaction include very long reaction times (3 days), high enzyme loading and a high operating temperature (90°C). More recently, the DKR of primary amines has been used in the synthesis of the antidepressant norsertraline [18].

1.2.3.2 Other chemoenzymatic reactions

The lipase-catalyzed kinetic dynamic resolution has a compatibility advantage because lipases are particularly solvent resistant and are active in pure organic solvents or at the aqueous:organic solvent interphase. Pairing a racemization metal catalyst in an organic solvent with a lipase is more straightforward than using other enzymes that have lower organic solvent resistance. As a result, besides the kinetic dynamic resolution using metal-catalyzed racemization and the Candida lipase B (CALB), there are very few reports on the successful combination of chemical catalysts and enzymes in one pot. Typically, the enzymatic reaction products are separated and partially purified prior to starting the other reactions involving chemical catalysts, or vice versa. For example, Kohler and coworkers recently reported a stereoselective synthesis of
prolyl peptides using a monoamine oxidase which does a desymmetrization of substituted pyrrolidines followed by a Ugi reaction [19, 20]. Huang and coworkers also recently integrated a glucose isomerase and an acid catalyst to convert glucose to hydroxymethylfurfural [21]. The two-step chemoenzymatic fluorination of unactivated carbons using P450 BM3 variants and diethylaminosulfur trifluoride as the fluorine donor is another such example of sequential enzymatic/chemical catalytic reactions [22]. Kroutil and coworkers recently published a tandem chemoenzymatic route for the production of chiral biaryl alcohols [23]. They first performed a Suzuki coupling reaction to form biaryl ketones, which were selectively reduced to enantioselective alcohols using an alcohol dehydrogenase. A biphasic ionic liquid: buffer system was designed which housed the Suzuki coupling and the lyophilized cells of E. coli expressing the alcohol dehydrogenase, respectively. Since the Suzuki coupling has to occur at 110°C and the enzyme reaction at 30°C, this tandem reaction was performed as a cascade.

One pot tandem reactions combining chemical catalysts and enzymes are scarce. In one of only a few examples, Feringa and coworkers reported the first one pot “click” chemoenzymatic reaction, combining namely a tandem enantioselective biocatalytic epoxide ring opening and a [3+2] azide alkyne cycloaddition [17]. The epoxide ring opening is catalyzed by the halohydrin dehalogenase HheC which catalyzes the enantioselective azidolysis of substituted styrene oxides with over 99% ee. Concurrently, a copper catalyzed 1,3-dipolar cycloaddition of azides and alkynes occurs to afford the click product in high yields and high ee. One limitation to this system is that a maximum of 50% yield can be obtained, since one enantiomer of the epoxide remains unreacted by HheC. The reaction is rendered easier because all the chemical components (ligands, copper salt) are soluble in buffer; however the one pot reaction required a very high
enzyme loading (25U) while the substrate loading was only 32 µmol. Although enzyme stability is not addressed in the report, one cannot exclude significant enzyme deactivation by chemical components of the reaction.

1.3 Chemical synthesis of enantiopure epoxides

Enantiopure epoxides are very important building blocks for the synthesis of enantiomerically pure complex molecules, in particular, of biologically active compounds [5]. Many active natural products, drugs, pharmaceutical intermediates and metabolites contain epoxides with high enantiopurity, which are often required to confer their biological and medicinal properties [24] (Figure 1). As a result, discovery of practical methods to prepare enantiopure epoxides by asymmetric epoxidation of olefins has long been an area of active research. Asymmetric olefin epoxidation methods fall into four general categories: directed epoxidations, metal-oxo catalyzed epoxidations, dioxane-catalyzed epoxidations and nucleophilic epoxidations. Other miscellaneous methods have surfaced in the last ten years as well. This section will quickly summarize the most prominent epoxidation methods in each category, as well as their limitations. For more in-depth mechanistic insights into each reaction and catalyst, readers are encouraged to consult recent reviews [5, 25]

1.3.1 Directed epoxidations

The Sharpless epoxidation is one of the most powerful epoxidation reactions available for the preparation of 2,3-epoxyalcohols [26]. This well-known reaction comprises the enantioselective epoxidation of an allyl alcohol using tert-butyl hydroperoxide as oxidant and titanium-tartrate complex Ti(OiPr)₄ as the catalyst. The enantioselectivity of the reaction can be predicted from a
simple mnemonic [27]. The reactivity and regioselectivity of the epoxidation is directed exclusively to the double bond allylic to the alcohol, even in the presence of other double or triple bonds. Although this selectivity is remarkable, the Sharpless epoxidation is quiet ineffective in the absence of the alcohol directing group, in the epoxidation of terminal olefins, and does not tolerate functional groups such as carboxylic acids and most aryl groups. Very low reaction temperatures (-40°C-0°C) also present a limitation in terms of the compatibility of this reaction in tandem. As a slight modification of the Sharpless method, Yamamoto and coworkers recently reported the epoxidation of homoallylic alcohols using a vanadium triisopropoxide oxide to achieve 90% ee and yields up to 78% for simple homoallylic alcohols [28, 29].

1.3.2 Metal-oxo catalyzed epoxidations

1.3.2.1 Salen catalysts

As opposed to the Sharpless epoxidation, metal-oxo catalyzed epoxidation do not require bonding interactions between the substrate and the catalyst. For this reason, this epoxidation does not require an alcohol as a directing group. Various metal-salen catalysts have been reported over the last 25 years, including chromium and manganese-salens. The first breakthrough manganese-salen complex as catalysts for enantioselective epoxidation was reported independently by Jacobsen and Katsuki (Scheme 1.8) [30, 31]. This epoxidation allowed the enantioselective formation of epoxides from various cis-substituted olefins by using a chiral Mn-salen catalyst and a stoichiometric oxidant such as bleach or iodomesitylene. Compared to the Sharpless epoxidation, the Jacobsen epoxidation allows a broader substrate scope for the transformation, such as conjugated cis-olefins (R: Ar, alkenyl, alkynyl; R': Me, alkyl) or alkyl-
substituted cis-olefins bearing one bulky alkyl group. However, common limitations to the early Mn-salen catalysts developed include the requirement of an unsaturated conjugated group such as an arene, alkene, or alkyne for acceptable reactivity. Terminal epoxidation can be achieved at very low temperatures (−40°C) with high yields and acceptable enantioselectivity as well (up to 85% ee) [32, 33]. However, Mn-salen catalysts often perform badly with trans-alkenes, aliphatic linear alkenes, and create trans-epoxides from cis-alkenes: thus, Cr-salen catalysts are preferred for some specific trans-alkene to trans-epoxide transformations [25].

1.3.2.2 Metal porphyrins
In many proteins, porphyrins complete several functions, including O₂ storage (myoglobin Mb), and transport (hemoglobin Hb), oxidation of inactivated carbon-hydrogen bonds (cytochrome P450), and oxygen reduction (cytochrome c oxidase). Since John T. Groves reported that man-made porphyrins catalyze enantioselective epoxidation via a highly reactive oxo-metal (M=O) intermediate similar to cytochrome P450 in 1979 [34], transition metal porphyrins-catalyzed enantioselective epoxidations has been of utmost importance and widely studied. Their rigid macrocyclic core and alterable periphery of porphyrins make them attractive templates for building asymmetric catalysts. Chiral groups can be attached to porphyrins in various geometries, aiming at systems that might give high enantioselectivities and turnover numbers. Most studies on metalloporphyrin catalysts are confined to the porphyrin complexes of iron, manganese, ruthenium, and molybdenum.

While many iterations of iron-porphyrin catalysts were designed, only a few could achieve very high enantioselectivities (>over 80%), led mainly by the seminal works of Eric Rose. In 1999,
Collman et al. presented a highly efficient catalyst based on a novel chiral iron porphyrin (Scheme 1.9). This system gave very high enantioselectivities and turnover numbers for the epoxidation of styrene derivatives (83% ee for styrene, 88% ee for pentafluorostyrene, and 82% ee for m-chlorostyrene) and some non-conjugated branched terminal alkenes, ee reaching over 90% for trimethylbutene [35], exceeding the highest values obtained from any catalytic systems, including Salen-Mn derivatives. Later in 2004, they synthesized a new chiral binaphthyl-strapped iron porphyrin, which exhibited unprecedented catalytic activity toward the enantioselective epoxidation of terminal olefins. For the epoxidation of styrene, typical ee values were measured to be 90-97%, whereas the turnover numbers (TON) averaged 16 000 [36].

1.3.3 Dioxiranes
A few organocatalyzed epoxidations have been published in recent years. These include epoxidations with BINOL systems, dioxiranes [37], chiral ketones [38, 39] and chiral aldehydes [40] and chiral iminium salts [41, 42]). Many of these systems are efficient for various epoxidations, especially for the epoxidation of trans-di- and trisubstituted olefins. Shi and coworkers, who have been leading this effort of synthesizing chiral ketone catalysts, also reported a diooxirane catalyst capable of doing terminal epoxidation of styrene derivatives with high ee, comparable with the iron porphyrin catalysts developed by Rose, with ee for styrene reaching 90% [43, 44].

In the last several years, as one can appreciate, many catalysts have been designed for the enantioselective epoxidation of olefins. Nonetheless, man-made catalysts for asymmetric epoxidations suffer from several shortcomings. Firstly, the epoxidation of terminal aliphatic
alkenes with high enantioselectivity still remains a synthetic challenge. A conjugated ring (phenyl) is usually present to help increase the activity and enantioselectivity of the reaction. Secondly, asymmetric epoxidations, and enatioselective oxidations in general, are not streamlined in tandem reactions, as are other important reactions such as metathesis, isomerization, hydroformylation and polymerization.

1.4 Enzymes for asymmetric oxidation

Numerous enzymes are capable of epoxidizing double bonds with high enantio- and regioselectivity, including styrene monooxygenases, ω-hydroxylases, peroxidases, and cytochrome P450s. However, many of these enzymes have low activity and total turnovers, are multicomponent, have little to no organic solvent tolerance and catalyze only their native biological substrates. These obstacles, in many cases, diminish the importance and usage of these enzymes from a synthetic chemistry and industrial biocatalysis point of view. This section will only highlight the enzymes which have been engineered or naturally catalyze the epoxidation of double bonds of synthetically relevant organic compounds with high ee, as well as enzymes which catalyze the hydroxylation of terminal carbons.

1.4.1 Styrene monooxygenases

Of all the monooxygenases that have been investigated and engineered, styrene monooxygenases hold the most promise as an industrial biocatalyst for the production of enantiopure styrene oxide and styrene oxide derivatives. The two-component styrene monooxygenase (oxygenase StyA) and a NADH-flavin oxidoreductase (StyB) [45, 46]) from Pseudomonas sp. strain VLB120 exhibits a specific activity on styrene of 2.1 U mg\(^{-1}\) cdw, the highest activities known for styrene
monooxygenases. By expressing the two-component system recombinantly in an engineered *E. coli*, Schmid and coworkers demonstrated the feasibility of recombinant whole-cell biotransformations in two-liquid phase systems on a pilot scale [47]. They produced 388 g of styrene oxide (>99% ee) from a 30 L fermentation, with an average overall volumetric activity was 170 U per liter.

The engineering of styrene monooxygenases has also been reported recently for the epoxidation of α- and β-substituents, as well as halogenated styrenes [48]. A newly characterized styrene monooxygenase StyAB2 was engineered by site-directed mutagenesis for enhanced activity against many styrene derivatives with α-substituents (2-fold improvement for α-ethylstyrene) through alanine scanning of four active site residues [49]. In addition, the wild-type StyAB2 protein showed high activities for β-methylstyrene, butenylbenzene, as well as a few diaryl compounds with whole-cell biphasic conversions ranging from 35 – 100% [50]. The longer the α-substituent alkyl chain, the lower the resulting conversion which was obtained.

1.4.2 ω-Hydroxylases

Many bacterial species, such as *Mycobacterium sp.* and a few marine bacteria, have the ability to grow on alkanes as their only carbon source. The first and key step in alkane metabolism is the terminal hydroxylation of alkanes to 1-alkanols, a reaction catalyzed by a diverse group of methane, propane, and butane monooxygenases and by some membrane-bound cytochrome P450s. Genome mining revealed that cytochrome P450 alkane diiron hydroxylases of the CYP153 family, are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases [51, 52]. A few of these alkane hydroxylases have been characterized to different
extents. The CYP153A6 from *Mycobacterium sp.* HXN-1500 was shown to hydroxylate medium-chain-length alkanes (C6 to C11) to 1-alkanols with a maximal turnover number of 70 min\(^{-1}\) and has a regiospecificity of over 95% for the terminal carbon atom position [53]. This enzyme was expressed in *Pseudomonas putida* as a polycistronic construct of the CYP153A6 P450 enzyme, ferredoxin, and ferredoxin reductase from *Mycobacterium* sp. HXN-1500 [54]. Through fusion with the reductase domain from *Rhodobacter sp.* NCIMW 9784, various CYP153 were reconstituted [55]. The CYP153A13a from *Alcanivorax burkomensis* SK2 was found to catalyze the terminal oxidation of alkanes (C6-C10) and produce α-ω-diols [56]. This chimeric enzyme was also shown to mono-hydroxylate various aromatics and biaryl compounds with high regioselectivity, although with fairly low activities [57].

Alkane hydroxylases exhibit a tight carbon length preference, and efforts at expanding their substrate scope is of interest. However, directed evolution of CYP153 oxygenases has not been reported as frequently. Arnold and coworkers used an *in vivo* selection method to engineer AlkB and CYP153A6 for butane oxidation, two enzymes which have C8 carbon chain length preference [58]. *Pseudomonas* cells were transformed with error prone libraries of AlkB and Cyp153A6, respectively. Only cells containing the improved variants were able of growing on butane. Although they found a few mutants of each enzyme that could oxidize butane to produce 1-butanol, the mutation found in the Cyp153A6 gene was not responsible for the cell growth on butane, which suggested that genome adaptation occurred to afford the improvement.

1.4.3 Cytochrome P450s
Cytochrome P450 monooxygenases are versatile biocatalysts that introduce oxygen into a vast range of molecules at allylic positions, across double bonds, and into non-activated C–H bonds in a regio- and stereo- selective manner [59-61]. Epoxide rings found in metabolic pathways are generally formed through the epoxidation activity of cytochrome P450s. Early research done in the area of microbial epoxidation showed that rat liver microsomes were capable of epoxidation. More recently, several cytochrome P450s have been expressed and purified in heterologous hosts, most often *E. coli*. However, many P450 enzymes, especially the mammalian ones are not very stable, and their native reductase partners are not known, as they are not found on the same polypeptide chain. This hinderance reduces their synthetic applicability, especially on the preparative scale. Previously characterized reductase partners, such as ferredoxin reductase and flavin reductase domains from known P450s are usually co-expressed in the heterologous hosts; however, in many cases the reconstituted active enzymes does not match the redox coupling of the native activities. The CYP102 family of P450s has been fruitfully blessed with a few single chain bacterial P450s, in which the heme and reductase domains are found on a single polypeptide chain. Among these, the cytochrome P450 BM-3 from *Bacillus megaterium* catalyzes the sub-terminal hydroxylation of long-chain (C12–C20) fatty acids as well as the epoxidation of mono and polyunsaturated fatty acids with high regio- and enantioselectivity. Due to its high activity and catalytic self-sufficiency [59, 60], the P450 BM-3 represents an excellent engineering platform for oxidation.

Arnold and coworkers successfully re-specialized the P450 BM-3 enzyme, through successive rounds of laboratory evolution, to catalyze the selective hydroxylation of short alkanes, including propane with native-like activity and coupling efficiency [62], a reaction for which no practical
catalysts are available [63]. An initial round of mutagenesis afforded the P450 BM-3 variant 139-3 that showed low activity towards propane [64], but very good activity for C6-C8. A domain-based protein engineering strategy was used, in which the heme, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) domains were evolved separately in the context of the holoenzyme, and beneficial mutations were recombined in a final step. Mutant libraries were created by random mutagenesis and site-directed mutagenesis. Two mutants, P450PMO R1 and R2, containing mutations in both the reductase and heme domains, could catalyze propane oxidation with high total turnovers (35,600 and 45,800 for R1 and R2, respectively), while maintaining close to 98% coupling efficiency [65]. In P450PMO, little trace of the original P450 BM-3 activity remained (Figure 2A).

During the course of the evolution, through saturation mutagenesis, the Arnold group altered the chemoselectivity of P450 BM-3 by engineering of the heme domain to create two mutants, RH-47 and SH-44, for the selective epoxidation of simple terminal alkenes, versus hydroxylation at the allylic position (C-3) (Figure 2B) [19]. The two mutants show inverted enantioselectivity (toward an enantiomer of product), with the RH-47 variant forming the (R)-epoxide, while the (S)-epoxide is formed by the SH-44 variant. Up to 83% ee, catalytic turnovers up to 1,370 and high epoxidation selectivities (up to 95%) were obtained. However, these two variants suffered from heme alkylation that limited their catalytic turnovers [66]. Because the active site of the P450 BM3 can accommodate different sizes and shapes of substrates, simple rational design or directed evolution libraries have afforded activities on other non-native substrates such as aromatics (α- substituted styrene derivatives, steroids, macromolecules, disaccharides) and have been reviewed and reported thoroughly elsewhere [60, 67-73].
Besides P450 BM3 as a model enzyme for alkane hydroxylation, the Auclair group has engineered another P450, namely the CYP 3A4 for the regioselective oxidation of short alkanes. This is important because, although P450s have an unprecedented ability to oxidize C-H bonds, it is still challenging to predict and control the regioselectivity in the presence of multiple hydroxylation or epoxidation sites without using some sort of enzyme evolution. Regioselectivity in systems in which non-bonding interactions exist between substrate and catalytic center is also generally difficult to achieve. Strukul and coworkers demonstrated with a Pt catalyst, that one could direct double bond epoxidation, even in the presence of other double bonds [74, 75]. Using the chemical auxiliary theobromine, Auclair and coworkers directed the epoxidation and hydroxylation of small chain alkanes and alkenes attached to theobromine, towards C4 carbon hydroxylation exclusively [76]. The theobromine cleavage is straightforward and can generate an amine or another functional group. Enantiomeric excess varied for each substrate, and could not be predicted, but epoxidation of 3,7-Dimethyl-1-(pent-4-enyl)-1H-purine-2,6(3H,7H)-dione (1-pentene attached to theobromine) yielded only the R-epoxide with over 99% ee. Unfortunately, with many other substrates (C2-C6 alkanes and alkenes), the ees were not as favorable.

1.5 Hydrolytic kinetic resolution of terminal epoxides

Although a lot of research has been directed towards the enantioselective epoxidation of olefins, it is safe to say that the asymmetric epoxidation of simple linear terminal alkenes remains challenging to afford with high enantiopurity (>90% ee), with both enzymes and metal catalysts. One way enantiopure terminal epoxides can be prepared is through kinetic resolution. In 2002, the hydrolytic kinetic resolution of several terminal epoxides was accomplished with a Co(II)
salen catalyst developed by Jacobsen and coworkers [77, 78]. Using a catalytic amount of water, a low loading of this commercially available catalyst, the kinetic hydrolysis of a very wide range of terminal epoxides, ranging from aliphatic, halogenated, epoxides bearing ethers, aryl and vinyl, could be resolved, with yields close to the theoretical 50%. Reaction time varied from 8 to 24h, but the unreacted enantiopure epoxide starting material could be recovered completely. In this way, many terminal epoxides could be obtained with over 99% ee.

Epoxide hydrolases from microbial sources, yeast and fungi are very popular for the kinetic resolution of terminal and internal epoxides or the preparation of their enantiopure diols. Significant kinetic resolutions of terminal epoxides are listed in table 1 [79-81]. In general, these epoxide hydrolases are S-selective.

1.6 Artificial metalloenzymes for epoxidation

Over the last decades, the design of many metal porphyrin catalysts has been inspired by the active site configurations of natural porphyrins. Recently, with the advance of computational tools, many artificial enzymes have been designed. In particular, researchers have sought to use enzyme scaffolds as natural ligands, and fitted in different catalytic metals. Chemical reactions such as Diels-Alder, olefin metathesis [82], reactions which are not performed by any enzyme, as well as C-H activation (hydroxylation) have been achieved using streptavidin or biotin scaffolds [83]. So far, only two examples exist as far as artificial metalloenzyme for epoxidations. The first one was reported by Kazlauskas, in which they created a manganese-substituted carbonic anhydrase (CA[Mn]), which showed peroxidase activity with a bicarbonate-dependent mechanism. In the presence of bicarbonate and hydrogen peroxide, CA[Mn] catalyzed the
epoxidation of chlorostyrene, styrene, β-methyl styrene with moderate enantioselectivities. A maximum conversion of 12.5% was obtained with p-chlorostyrene, a reaction not catalyzed by the native zinc enzyme [84]. More recently, Ricoux and coworkers incorporated manganese complexes into a xylanase to create new artificial metalloenzymes for enantioselective epoxidation [85]. In particular, they inserted a Mn(III)-meso-tetrakis(p-carboxyphenyl)porphyrin [Mn(TpCPP), 1-Mn] into a host protein, xylanase 10A from *Streptomyces lividans* (Xln10A). The artificial metalloenzyme was able to catalyze the oxidation of p-methoxystyrene by KHSO$_5$ with a 16% yield and the best enantioselectivity (80% in favor of the R isomer) ever reported for an artificial metalloenzyme.

### 1.7 Conclusions

From the sections expanded upon above, one can be convinced of several facts. Firstly, asymmetric tandem catalysis even in the realm of organic chemistry is still in its infancy. Most asymmetric tandem reactions between chemical entities are sequential, or cascade reactions, as in most cases, the reaction conditions and reaction selectivity cannot be matched. Truly concurrent tandem reactions are more difficult to engineer. Secondly, finding ways to combine chemical catalysis and biocatalysis would greatly expand the pool of tandem reactions possible. In particular, tandem reactions between the two metal catalysts of the respective fields of organometallic chemistry and metalloenzyme chemistry would open the door to many combinations of reactions. Dynamic kinetic resolutions using racemization catalysts and lipases/proteases represent the only example of metal catalyst-enzyme combination. This technology is only restricted to lipases and other enzymes with very high solvent tolerance. In addition, the end product of a dynamic kinetic resolution may be accessible through one catalytic
route alone, such as an enantioselective chemical reduction of alcohols. To that effect, groundbreaking tandem reactions would be one in which metalloenzymes and organometallic catalysts are combined to achieve a final product not accessible by one catalyst alone. In addition, the two catalysts in the reaction must work cooperatively, which means that the final yield obtained from the tandem reaction should be higher than doing the two reactions separately.

In Chapter 2 of this thesis, we report on the engineering of our first tandem reaction involving a transition metal isomerization catalyst and a cytochrome P450 for the successive isomerization/epoxidation of olefins. In Chapter 3, we extend the approach to encompass an olefin metathesis/epoxidation tandem reaction. We show that the action of the enzyme reaction on the metathesis mixture drives it forward, thus creating a dynamic concurrent system.
1.8 References


1.9 Schemes

Scheme 1.1 Tandem reaction configurations
Scheme 1.2 Combination of Grubbs-2 and [Ru]-H catalysts for lactone synthesis
Scheme 1.3 Combination of copper and palladium catalysts
Scheme 1.4 Combination of palladium and iridium catalysts

83% yield, 94% ee
linear/branched 2:98
Scheme 1.5 Deracemization of sec-alcohols through a tandem ruthenium oxidation and reduction
Scheme 1.6 Asymmetric dihydroxylation of styrene derivatives

1a: R=H  1b: 4-Cl  1c: R=3-Cl  1d: R=2-Cl

up to >99% ee
up to 95% yield
Scheme 1.7 DKR of secondary alcohols
Scheme 1.8 Katsuki epoxidation
Scheme 1.9 Chiral iron metal porphyrin
1.10 Figures

![Chemical structures]

Beloranib
Carbamazepine 10,11-oxide
Oleandomycin
Anticapsin

Figure 1.1 Epoxide-containing bioactive macromolecules
Figure 1.2 A) Major P450 reactions B) Examples of evolved P450 BM-3 mutants obtained from the wild-type fatty acid hydroxylase by directed evolution (Adapted from [86])
### 1.11 Tables

Table 1.1 Selected microbial kinetic resolution of terminal epoxides

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**Adapted from [81]**
CHAPTER 2. TANDEM ISOMERIZATION-EPOXIDATION OF ALKENES INVOLVING RUTHENIUM TRIPHENYLPHOSPHINE CATALYSTS AND P450 BM3 MUTANTS

2.1 Introduction

As part of the Center for Enabling New Technologies through Catalysis (CENTC), we are interested in the selective functionalization of carbon bonds. One such transformation, namely the enantio- and regioselective epoxidation of olefins, is of great synthetic value. Enantiopure epoxides, as well as their corresponding vicinal diols, are valuable intermediates in fine organic synthesis, in particular for the preparation of biologically active compounds [1]. Effectively, the very reactive oxirane ring can be attacked by a wide variety of nucleophiles to produce chiral intermediates (Figure 2.1). In recent years, the preparation of enantiopure epoxides has been pursued using both pure chemical and enzymatic approaches [2]. The first methodologies of Katsuki and Sharpless using titanium-tartrate complexes allowed for the epoxidation allylic alcohols [3]. Since then, many more catalysts allowing for the epoxidation of non-functionalized olefins have been developed, leading to high stereoselectivities when applied to the epoxidation of cis-alkenes [4, 5]. However, these complexes preferably oxidize the internal double bond over the terminal double bond. In addition, the selectivity of these complexes suffers with trans-olefins and terminal olefins [6]. On the other hand, as mentioned in Chapter 1, several enzymes can catalyze the epoxidation of olefins with high enantioselectivity, naturally or through enzyme engineering [7-10].
The recent initiative for green and sustainable chemistry has increased the interest of chemists in improving synthetic efficiency of chemical transformations. Synthetic chemists thrive to develop novel catalysts that work in mild conditions, require less separation costs, to achieve transformations with high purity, selectivity, and yield through minimal synthetic steps. To that effect, concurrent tandem catalytic processes, aptly named multicatalyst promoted asymmetric tandem reactions have emerged as a powerful strategy to improve said synthetic efficiency. Although the complexity of engineering these reactions still remain a synthetic challenge in terms of fine-tuning substrate and product selectivities, as well as reaction condition compatibility, there are several successful examples of concurrent asymmetric tandem reactions in the literature involving two or more catalysts from the same or different disciplines of chemistry [11, 12].

Although tandem reactions involving multiple catalysts have found success in synthetic chemistry, only a few reports of a tandem catalytic system incorporating both enzyme and organometallic catalysts have previously been published [13, 14]. These have used an organometallic catalyst that reversibly and dynamically racemize a mixture of enantiomers and a lipase enzyme that selectively transforms only one of the isomers into a product that is enantiomerically enriched or pure with a yield higher than 50%. Similarly one can envision a dynamic kinetic resolution where regioisomers are resolved instead. An interesting example of such a dynamic kinetic resolution would be the tandem isomerization and epoxidation of olefins. This process would allow for a mixture of aliphatic olefins (as obtained from alkane dehydrogenation for example) which usually are quite difficult to separate, to be reacted only to yield a single epoxide regio-isomer, in the hardest case the terminal olefin (Figure 2.2).
Isomerization is a mature field, as several transition metal isomerization catalysts are commercially available. Using an enantioselective enzyme would make it possible, from a mixture of various pro-chiral olefins, to synthesize enantiomerically and regio-isomerically pure epoxides. One can appreciate how difficult this tandem reaction would be to monitor, considering that only 5% terminal olefin is formed from the thermodynamically uphill isomerization from internal to terminal olefins. Firstly, the thermodynamic barrier for the isomerization of internal olefins to terminal olefins would make it difficult to follow the progress of isomerization reaction. The success of the epoxidation reaction would also be difficult to establish at low concentrations of the terminal epoxide. To make it easier to set up the proof of concept, one could start from the terminal olefin and isomerize to the internal olefin mixture. The enzyme would in turn selectively epoxidize one of the internal olefins (Figure 2.3). This approach alleviates the thermodynamic problem while addressing cis- trans- selectivity issues and opens up new directions for development of tandem catalysis.

The first two necessary parts of this tandem system are the two catalysts: an organometallic catalyst that efficiently isomerizes olefins and a regio- and stereo- selective epoxidation enzyme. Secondly, suitable reaction conditions must be engineered to house both reactions and have them operate compatibly. The success of this tandem reaction relies on several additional requirements as listed in Table 2.1. In this chapter, we present our work on engineering a reaction for the tandem isomerization and epoxidation of short olefins.

2.2 Results and discussion

2.2.1 Choice of enzyme, selectivity and activity
2.2.1.1 T4MO versus P450 BM3

As pointed out previously, there are several enzymes that are capable of doing epoxidation of aliphatic olefins. However, not many of them are capable of selectively oxidizing linear aliphatic olefins. After surveying the literature, two enzymes were selected for the epoxidation of short alkenes: a toluene-4-monooxygenase (T4MO) [15] and two cytochrome P450 BM3 mutants obtained from the Arnold lab [7]. The variant T4MO I100G was previously shown to increase the wild-type activity for epoxidation [16]. Regioselectivity and activity experiments on hexenes were conducted (Table 2.2). P450 BM3 mutants was 30-fold higher selective for the terminal alkene in a 1:1 mixture of terminal and trans-2-hexene, while no regioselectivity was observed in the case of T4MO and T4MO I100G. In addition, the P450 mutants exhibited higher turnovers than T4MO. It is noteworthy that although the RH47 and SH44 mutants react with trans-2-hexene when supplied as the only substrate, terminal selectivity predominates in a 1:1 mixture of terminal and trans-2-olefins. This may be due to the faster binding of the terminal olefin in the enzyme active site of the enzyme through having a more exposed double bond than the trans-internal alkenes.

2.2.1.2 P450 BM3 RH47 and SH44 alkene regioselectivity and activity on branched and straight chain short alkenes

The isomerization of terminal olefin produces a thermodynamic mixture of terminal, cis- and trans- internal olefins, although the thermodynamic ratio favors the trans- isomers. When testing the regioselectivity of P450 BM3 mutants in mixtures of terminal and cis-alkenes, we found that the selectivity drops dramatically. In the case of 1-hexene and cis-2-hexene for example, the regioselectivity dropped to 1.3-1.6 fold for the RH47. For the SH44 mutant, the enzyme
preferred the cis- alkenes in most cases. The activity of the P450 RH47 and SH44 was further tested against C₅-C₇ aliphatic straight or branched alkenes, as well as the enzyme regioselectivity in mixtures of these alkene isomers, as shown in Table 2.3 in accordance with the envisioned substrate scope of the tandem reaction. The SH44 mutant does not show any terminal selectivity except slightly in the case of pentene isomers. However, one can observe terminal selectivity for RH47, especially with 4-methyl-pentenes and t-butyl-propenes, affording 8.3 and 4.2-fold terminal selectivity, respectively. This terminal selectivity of RH47 can be explained by the presence of a phenylalanine at residue 87, contrary to SH44 which has a valine at this position. Residue 87 in P450 BM3 has been studied in depth in several works and has been shown to be one of the main residues that determine regioselectivity in hydroxylation [17-19]. In addition, the bulky residue 87 provides increased protection to the heme, alleviating the devastating effects of heme alkylating agents such as aldehydes,[20] acetylenic substrates and terminal double bonds [21].

P450 BM3 RH47 and SH44 were able to react with all the olefin substrates fed with reasonable activity (TTN of 200-1000). Lower TTNs (~200) were observed for the branched alkenes, especially the 4-methyl-pentenes. When NADPH depletion was monitored for this substrate, it was observed that rapid enzyme deactivation occurred, caused most likely by heme alkylation. Addition of enzyme partly restarted the reaction.
2.2.2 Reaction optimization

2.2.2.1 Screening of organic solvents

While enzymes are naturally active in aqueous buffers, aqueous environments (air, water, room temperature) are generally not suitable for chemically-catalyzed synthetic transformations. Firstly, the large isoelectric constant of water is not favorable for many chemical catalysts; secondly, many organic compounds of commercial interest afford minute water solubility. Therefore, it is not surprising that various organometallic catalysts were not preferably synthesized to be soluble in aqueous solutions. In our tandem reaction, it became quickly evident that a biphasic aqueous/nonpolar two phase reaction system should be employed. Separation of the two catalysts by having them in different phases would minimize cross-talk and potentially afford higher catalyst compatibility. For example, the aqueous buffer phase would include the biocatalyst (cells, lysate or purified enzyme), and the organic solvent as a second phase would contain the soluble organometallic catalyst, serve as a substrate reservoir and product extraction pool, exhibiting high product recovery capacity and biocompatibility [22] (Figure 2.4). The advantages of this aqueous/nonpolar medium two-phase systems would include not only the production of sparingly water-soluble compounds (aliphatic olefins), but also the in situ product recovery, which reduces end-product inhibition and favors the bioconversion by shifting the thermodynamic equilibrium (Table 2.1) [23].

While the other criteria are relative, biocompatibility and NADPH uncoupling remain very restrictive criteria. Biocompatibility of an organic solvent is closely related to the log of its octanol/water partition coefficient (logP), which is a measure of its hydrophobicity [24]. In general, organic solvents with a logP of 4 or higher are regarded as being biocompatible with
both cells and enzymes [23]. One of our reaction designs used *E. coli* whole-cells as the epoxidation catalyst. Since P450 BM3 substrate oxidation is accompanied by the reduction of stoichiometric amount of the expensive cofactor NADPH, we hypothesized that using the *E. coli* cell metabolism to regenerate NADPH would be economical. In addition, a regeneration enzyme, such as glucose dehydrogenase, could be expressed in the cells simultaneously to compensate the natural regeneration [25]. The biocompatibility of many organic solvents with respect to cell growth was screened. Expectedly, we found that the growth of *E. coli* cells harboring the plasmids P450-bm3-RH47 was not significantly affected (no organic solvent) under up to 50% v/v decane, dodecane, and hexadecane which have logPs of 5.6, 6.8 and 8.8, respectively (Figure 2.6). On the other hand, when pure olefins were chosen as the organic solvents markedly high toxicities were observed (not shown). Even at a concentration of 1 M 1-hexene in decane or dodecane, cell growth was decreased about 100%. In addition, hexadecane proved to be the solvent that gave the highest yield of 1,2-epoxyhexane after 6h.

We further optimized the substrate concentrations in hexadecane to be 1.0 M, as well as the organic phase: buffer ratio to be 40% v/v. At this concentration of substrate in hexadecane, *E. coli* cells did not show adverse growth effects and the epoxidation rate was the highest reached (Figure 2.6). Whole cells concentration was adjusted to 20 g/L in phosphate buffer supplemented with 2% glucose, based on a calculated *E. coli* glucose consumption rate of 0.63 g glucose. g cdw⁻¹. h⁻¹.

In addition, we studied the effect of these solvents on their NADPH uncoupling properties. As pointed out by Schrader and others [26], an organic solvent may uncouple NADPH from
substrate oxidation, albeit not oxidized itself, thus driving NADPH away from the reaction. Studying the NADPH uncoupling effects of several solvents showed us that in fact, dodecane caused NADPH uncoupling of P450 BM3 RH47. This solvent has been used in previous reports as an organic phase, but with a different P450 mutant [27]. Hexadecane and diisononyl phthalate caused no NADPH uncoupling of the P450 BM3 mutants (Figure 2.7). Keeping in mind that the isomerization catalyst was being fitted to achieve solubility in higher alkanes, hexadecane was chosen as the organic solvent for the tandem reactions in our further experiments.

2.2.2.2 In vitro epoxidation with cofactor regeneration

In parallel, we also studied the epoxidation reaction in vitro with cofactor regeneration. Several reasons prompted this investigation. First, the whole cell biocatalysis gave a low yield of epoxides. Secondly, the epoxide products would at times be further metabolized and not be recovered in the extraction. To efficiently regenerate NADPH, we used the alcohol dehydrogenase from Thermoanaerobium brockii. The NADP+ alcohol dehydrogenase from T. brockii has been shown previously to efficiently regenerate NADPH in previous reports and has high organic solvent resistance [7, 28, 29]. There are several commercially available NAD+ dependent enzymes used for regeneration, but the same cannot be said for NADP+ counterparts. A mutant of formate dehydrogenase from Pseudomonas sp. capable of using NADP+ has been reported, however, the specific mutations to obtain this mutant were not revealed. NADP+ glucose dehydrogenases and isocitrate dehydrogenases have also been reported; however, the organic solvent resistance of many of these enzymes has not been well studied, nor has their specific activity outcompeted the T. brockii ADH. Lastly, a thermostable NADP+-dependent phosphite dehydrogenase, engineered in our lab, could have been used for regeneration [30].
However, in our initial studies, we found that the activity of PTDH may not be high enough in phosphate buffer, the buffer of choice for P450 activity. In the future, switching to a different buffer for the P450 will enable us to use this enzyme.

Using the NADP+-dependent *T. brockii*, we found that a minimum of 1 U/ml had to be used for the regeneration reaction when 10 µM P450 BM3 RH47 reacted with 1-hexene. As a rule of thumb, 1 U/ml of ADH was used in the subsequent tandem reactions.

2.2.3 Isomerization catalyst optimization (with Martin Jarenmark)

2.2.3.1 Achieving solubility in higher alkanes

As mentioned before, the first and foremost requirement for the isomerization catalyst is to be functional and stable at room temperature, in air, and in the presence of enzymatic entities (e.g. cells, buffer, enzymes). The second requirement is that it should be soluble and active in a biocompatible solvent, in our case higher alkanes such as hexadecane. While the commercially available RuHCl(CO)(P(Ph₃)₃ and RhHCO(Ph₃P)₃ catalysts were found to be stable in air and at biological conditions, they afforded no solubility or activity in alkanes (Table 2.5). To increase solubility in alkanes, alkyl chains were incorporated into the *para*-position of the phenyl groups of the triaryl phosphine ligands. Incorporation of t-butyl groups increased the solubility and activity in pentane for the ruthenium complexes, however, in hexadecane, the solubility was still low (<1 mg/ml estimated). Therefore, in hexadecane: buffer, the conversion was only 50% after 24h (Table 2.6). At last, simply switching from t-butyl groups to n-butyl groups afforded solubility in hexadecane. Unfortunately, even in the presence of 20 mol% catalyst, the
isomerization rate of 1-hexene was still too slow compared to the epoxidation rate of olefins by P450 (60% in 6 hours at room temperature).

2.2.3.2 Optimizing activity in hexadecane (sulfur activation) and activation at biological conditions

The isomerization of olefins by metal-hydride catalysts generally requires coordination of the olefin to the metal center. Therefore, one of the ligands would have to dissociate for the substrate to bind to the metal center. It was found that activation of the Ru(n-butyl) complex with 1.5 eq. elemental sulfur (after optimization) increased the rate 7-fold (from 10% at 30 min to 68% with sulfur activation) (Figure 2.8). The activation time was also optimized (See experimental procedures for details) to 5 minutes for optimal activity. Longer activation times deactivated the ruthenium catalyst by possibly withdrawing additional ligands from the metal center. Although activation on sulfur made the catalyst much faster, the trade-off was that it steadily decreased the catalyst lifetime. With 20 mol% catalyst, after about 6h, the catalyst activity had dramatically decreased to about 5-10%. Finally, we also found that the isomerization was as effective in hexadecane: buffer (with lysate containing no P450) as in hexadecane alone (Figure 2.9). In hexadecane, we noted that there was very minimal formation of 3-hexenes.

2.2.3.3 Isomerization of various alkenes in hexadecane: buffer

With these optimized isomerization conditions, isomerization of various alkenes were attempted. The results are summarized in Figure 2.10. Interestingly, when starting from cis-2 isomers, the amount of terminal isomer formed in the isomerization increase with the degree of substitution of the olefin (b, e and h). This is due to the decreased stability of the cis-2 isomer caused by the
larger steric interaction between the 1, 2- substituents of the ethylene moiety. While the cis-2 isomers seems to work decently in the isomerization reaction, the trans-2 isomers are generally poor substrates, due to the thermodynamic stability of the trans- double bond compared to the cis- bond (c, f and i).

2.2.4 Tandem isomerization-epoxidation in hexadecane: buffer biphasic systems

2.2.4.1 Whole cell tandem reactions

In our quest to achieve solubility in hexadecane, we made several pit stops. In one of them, we used an isomerization catalyst with t-buty1 groups at the para- position of the phenyl ring. Although this catalyst has very low solubility in hexadecane, it could be dissolved to up to 1 mol% in pure 1-hexene (used as substrate and organic phase). In the tandem reaction, we observed for the first time the formation of the internal cis-2,3-epoxyhexane. The overall yield of the reaction was 150 µM of total products, with 1.6 fold higher terminal epoxide.

In the previous sections, we optimized the catalyst loading and activity in hexadecane, the substrate concentration in the organic phase, as well as the cell density. Tandem reactions were set up with hexadecane as the organic phase in which 0.1 M 1-hexene and 20 mol% Ru-n-butyl catalyst were dissolved. The aqueous phase contained 4.8 g/L BL21 cells harboring pET28aRH47 in a phosphate buffer pH 7.5. Catalyst activation on sulfur prior to the tandem reaction fastened the isomerization, therefore increasing the amount of cis-2-hexene in the tandem reaction at early times. Without sulfur activation, the yield of internal epoxide was only 15 µmol/g cdw, compared to 33 µmol/g cdw, when activated sulfur was used. In this system,
however, the reaction yields were very low and stopped after 5h, and as mentioned previously, the epoxides were further metabolized or reacted by the cells (Figure 2.11 A and B).

2.2.4.2 Cell lysate based tandem reactions

Using cell lysate or purified P450 BM3 RH47 or SH44, we set up several tandem reactions with the optimized conditions. Starting with 1-hexene for the isomerization, we observed the formation of cis-2,3-epoxyhexane at 2.5-fold higher concentration than the internal epoxide. Interestingly, starting with trans-2-hexene, the terminal epoxide was also observed, although at a much lower concentration of 6-fold less than both the internal epoxides (cis- and trans-). We found earlier that in a mixture of terminal and trans- internal alkenes, we observed very little activity against the trans-alkenes. With a high starting concentration of trans-2-hexene, and very slow isomerization, at most 5% of terminal hexene formed, it was interesting to observe that the terminal epoxide was formed in the tandem reaction using the RH47 mutant. Using the SH44 mutant, the terminal epoxide could not be observed (Figure 2.12).

The tandem reaction was expanded to a few straight and branched aliphatic alkenes (Table 2.7). As a general observation, in most of the reactions starting with the terminal alkenes, the cis-internal epoxides are formed with higher yields than the starting terminal alkene product (entries 1, 2, 5, 6). One particular exception (entries 3 and 7) involves 4-methyl-1-pentene. As far as the RH47 mutant, the terminal selectivity of the RH47 in mixtures of 4-methyl-pentenes explains why the tandem reaction does show formation of the epoxides with particular preference. Conversely, entries 10 and 11 represented our best results. Starting with either the trans- or cis-4-methyl-2-pentenes, all three epoxides were formed in equimolar concentrations with the RH47
mutant. In entries 4, 8, 9 and 12, the enzymes showed not high enough terminal selectivity, therefore, low amounts of the terminal epoxide was formed. A theme of these reactions is the low overall epoxide conversion. With such low conversions, we did not expect the epoxidation reaction to have any impact on the isomerization reaction, i.e., to exert a selective and “dynamic” pull of the substrates from the organic phase. In order to double check entries 10 and 11, control reactions were run starting with 4-methyl-2-pentenes without the addition of isomerization catalyst. The 4-methyl-1,2-epoxypentane peak did not appear unless the isomerization catalyst was added (Figure 2.13). This peak was also confirmed with spiking with authentic standard, prepared chemically. Although the isomerization that only produces 5% terminal alkene at most, we could observe a 1:1:1 mixture of epoxides in the tandem reaction. To further understand this, we performed a mutual solubility test. While in the organic phase, the ratio of internal to terminal alkenes could be 10:1, in the aqueous phase, the ratio was determined to be lower (Table 2.8). This is due to the cis-2-hexene going beyond the threshold for solubility-dependent partition, highlighting that the ratio of isomers obtained in the organic phase is not the same seen by the P450 enzyme in the aqueous phase.

2.2.5 Attempts at increasing the yield of the P450 biphasic reaction

In all the above tandem reactions, rather low yields are obtained (1-2%). In order to increase the epoxidation yields, many strategies were employed. First, the P450 enzyme concentration was increased from 1-10 µM to 33 µM. Second, the highly soluble phase transfer catalyst (>200 g/L) methyl-β-cyclodextrin was added at different concentrations. The ability of cyclodextrins to form complexes with hydrophobic molecules has led to their usage in increasing the solubility of organic compounds in aqueous solutions and has been shown to increase biphasic reaction yields.
[31]. In addition, methyl-β-cyclodextrin was shown to increase the rate of styrene epoxidation by the styrene monooxygenase StyAB [32].

Hexene partitions were calculated as a function of methyl-β-cyclodextrin added. The results are shown in Figure 2.14. As expected, increasing the concentration of both phase transfer agent and hexene concentration in the organic phase increased the concentration in the aqueous phase. A concentration of 1 mM was eventually reached in the aqueous phase. Strangely, we did not reach the concentration-independent partition of 1-hexene. Increasing the partition of 1-hexene in the aqueous phase in accompanied by an increase in epoxidation yields, which suggests that the reaction is partly mass transfer limited (Figures 2.15C). Our best result of 7% yield was obtained when 40 µM of P450 was used with 30% methyl-β-cyclodextrin (Figure 2.16). Unfortunately, at these low yields, the tandem reaction would not show a dynamic pull as expected.

2.2.6 Tandem isomerization-epoxidation of terminal carboxylic acid methyl esters

Following our target to increase the epoxidation yields, we switched to olefinic methyl esters as substrates with higher solubility in the aqueous phase. These substrates should in principle give higher epoxidation yields. We chose 5-hexenoic acid methyl ester as the model substrate for this reaction because the P450 BM3 RH47 and SH44 mutants showed good turnovers on these substrates. A nitrobenzyl pyridine assay [33] was performed with these substrates and the TTN were estimated to be 500 and 700 for the RH47 and SH44 mutants, respectively. Although P450 BM3 has been engineered to catalyze the oxidation of short chain acids [34], it had not yet been shown to epoxidize short alkene methyl esters. We studied the epoxidation of 5-hexenoic acid methyl ester at 37°C in biphasic hexadecane: buffer. When the substrate concentration in the
organic phase was adjusted 5 mM (1 µmol), we observed 48% conversion of the starting material to the terminal epoxide product after 24h. A time course suggested that the reaction did finish in 6 to 7 hours.

2.2.6.1 Optimization and characterization of isomerization of carboxylic acid methyl esters in hexadecane

At room temperature, the isomerization 5-hexenoic acid methyl was significantly slower than with the alkenes (Figure 2.17). In the tandem reaction, we wanted to avoid catalyst activation (for easier handling and reproducibility). Therefore, in order to increase the isomerization rate, the concentration of 5-hexenoic acid methyl ester was optimized to 200 mM at 37°C. Optimizations of the volume of hexadecane used as the organic phase, catalyst loading were also performed as to increase the isomerization rate, while keeping the hexadecane organic phase volume low. Entry 2 in Figure 2.18 gave us the best result, and thus was used in the subsequent tandem reactions.

Proton NMR was used to determine the cis-trans isomeric ratio in the isomerization of 5-hexenoic acid methyl ester. ¹HNMR of the isomerization of 5-hexenoic acid methyl ester in hexadecane proved to be impractical because of the high boiling point of that solvent. In addition, the isomerization in THF yielded mostly the conjugated α-trans- olefin, of which we observe very little amount in hexadecane. Therefore, the cis -trans- isomeric ratio of the isomerization of methyl-2,2-dimethylhex-5-enoate (Figure 2.19), which only forms the cis- and trans-4 product, was studied and was found to be 60:40 trans: cis ratio (Figure 2.20)
2.2.6.2 Tandem isomerization-epoxidation of terminal carboxylic acid methyl esters

Tandem reactions were set up with several optimized conditions (Figure 2.21). Entries 7 and 8 in Table 2.9 represent our best results for this tandem reaction. For entries 7 and 8, yields of 36% and 32.2% were obtained, respectively, which were the highest were obtained with any of our previous tandem reactions with the alkenes. Unfortunately, the cis- versions of the internal olefin methyl esters are either not commercially available or very expensive. They are even harder to synthesize. Based on the mass spectrum and fragmentation of the products, we could determine that peak #4, #5 and #6 represented the 3,4-, 4,5-, and 5,6-(terminal) epoxides, respectively. In order to gather more insights into the tandem reaction such as selectivity, we performed reactions with only trans- internal alkene methyl esters. Surprisingly, we observe that there was no reaction when either trans-3-pentenoic acid methyl ester, trans-2-hexenoic acid methyl ester or trans-2-pentenoic acid methyl ester were used. This suggested that the tandem reaction was cis-selective (Figure 2.22).

With this cis-selectivity, we would expect the tandem reaction to selectively pull the cis-olefins. This is true based on some additional tests (data not shown); however, the trans-cis isomerization is way too slow to replenish the cis- olefin depleted. Therefore, we do not observe a dynamic effect. Trans-cis isomerization was attempted for various substrates in hexadecane, but all failed. The only successful trans-cis- isomerization occurred in THF with the more active RuHCl(CO)(PPh$_3$-tbutyl).

2.3 Conclusions and outlook
In this chapter, we have explored the possibility of combining a ruthenium catalyst with a metalloenzyme for the tandem isomerization-epoxidation of linear and branched olefins. P450 BM3 mutants RH47 and SH44 were found to be suitable biocatalysts for the epoxidation of alkenes and alkenoic methyl esters. To best of our knowledge, this represents the first example of successfully coupling a metalloenzyme and a metal catalyst in one system. Ruthenium catalysts were synthesized to achieve activity and solubility in hexadecane, and were active at biological conditions. We showed that two catalysts from very different disciplines, namely an organometallic catalyst and a metalloenzymes, could work in tandem in one-pot to achieve a transformation not accessible by a single catalyst alone. Using a biphasic reaction system (hexadecane: buffer), we showed that starting with the terminal alkene, the isomerization produced the internal olefin, which was subsequently epoxidized by the P450 BM3 mutants with at best 2-fold higher yields than the terminal olefin. We also tackled the thermodynamically challenging transformation starting with trans-2-hexene as the isomerization substrate. In our best case, starting with trans-4-methyl-pentene, one could observe the formation of the terminal 4-methyl-1,2-epoxypentane, at an equimolar concentration with the two internal epoxides. Meanwhile, there are several limitations to our tandem process. Firstly, the selectivity of the P450 mutants for terminal epoxidation over internal epoxidation is not high enough to truly drive the reaction. Secondly, the epoxidation yields were too low in the case of the alkenes to disturb the equilibrium of olefins established by the isomerization. Thirdly, the enzyme activity towards the alkene substrates is not high enough to overcome the mass transfer limitation imposed by the hexadecane: buffer system. In order to address the terminal selectivity, directed evolution was used to increase the selectivity of the P450 BM3 for epoxidation of terminal alkenes. A GC-MS based screening method was used to assess whether mutations at 10 active-site residues via
saturation mutagenesis would improve regioselectivity. Our most selective mutant, 87H9, reacted with only a 1.3-fold increase in selectivity for epoxidation of terminal over internal alkenes. Additional directed evolution was not conducted because the margin of increase was found to be small, and the screening method was too time-consuming. Difficulties faced when transforming the P450BM3 into a terminal hydroxylase has been described in the literature [35]. To increase the epoxidation yields, various strategies were employed. The most effective method proved to be the addition of the phase transfer catalyst methyl-β-cyclodextrin. Addition of 15% of the phase transfer catalyst effectively increases the partition of 1-hexene up to 0.01 and the epoxidation conversion up to 7%. Choosing more soluble substrates, such as unsaturated methyl esters, further increased the yield to 35% after reaction optimization. The epoxidation was found to be selective for the terminal alkene and cis-alkene (no trans-methyl ester tested reacted). However, we did not observe trans- to cis- isomerization; apparently, the isomerization between cis and trans alkenes was still slow. Trans- to cis- isomerization was only achieved in non-compatible solvents such as THF.

An additional key component of the tandem reaction that has not been tampered with has been the regeneration system. Although it has been shown to be effective, the alcohol dehydrogenase from T. brockii is not readily used in biocatalytic transformations. Inhibition of the activity of the ADH by acetone may be limiting the regeneration efficiency [36]. In addition, ADH is inhibited by DMSO, one of the best co-solvents usually involved in biphasic reactions [37]. We will surely change the regeneration system in the future and explore new options [38].
We are continuing to expand the reaction substrate scope by investigating olefins containing ketone groups and unsaturated carboxylic acids. P450 BM3 mutants have been shown to react these substrates with high activities [10]. We hypothesize that finding substrates exhibiting both high isomerization and epoxidation rates will unlock a system we have been pursuing that undergoes dynamic isomerization-epoxidation. Since engineering of terminal selectivity for the P450 BM3 proved to be difficult, we plan to show that dynamic tandem reaction in the case of terminal-internal isomerization followed by selective epoxidation of the internal olefins formed or in the case of a cis-trans- isomerization followed by selective epoxidation of the cis- or trans-double bond.

2.4 Experimental procedures

2.4.1 Materials

The Escherichia coli cloning strain DH5α, expression strain BL21(DE3) and the expression vector pET28a were obtained from EMD Biosciences (San Diego, CA). Antibiotics and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Gold Biotechnology (St. Louis, MO) The plasmids pCWori+-bm3_RH47 and pCWori+-bm3_SH44 encoding P450 BM3 RH47 and SH44 respectively, were obtained from the Frances Arnold lab (Caltech, USA). Plasmid pET28a was obtained from Novagen (Madison WI). The T4MO plasmid was obtained from Haige Lu. All chemicals were obtained from Sigma-Aldrich (St. Louis) unless otherwise noted. NADPH tetrasodium salt was from Enzo Life Sciences (Ann Arbor, MI). NADP+ was purchased from Roche Diagnostics (Indianapolis, IN). 4-Methyl-cis-2-pentene was purchased from TCI America Fine Chemicals (Portland, OR). t-Butyl-trans-2-propene was from BOC Biosciences (Shirley, NJ). All PCR reagents and restriction enzymes were purchased from New
England Biolabs (Ipswich, MA), and DNA Miniprep and Gel Purification Kits were purchased from Qiagen (Valencia, CA).

2.4.2 DNA manipulations

P450 BM3 mutants were subcloned into the BamHI and EcoRI sites of pET28a for expression in BL21 and whole cells preparation. Primers used were BamHI-for-BM3 (GC`GGATCCATGACAATTAAAGAAATGCCTCAGC) and EcoRI-rev-BM3 (GC`GAATTC`TTACCCAGCCCACACGTCTTTTGC). Cut sites are underlined.

2.4.3 Protein expression and purification

The cytochrome P450 BM3 RH47 and SH44 were expressed according to literature [7]. Briefly, overnight cultures of DH5α-pCWori+ bm3 mutants were inoculated in 500 mL TB supplemented with 100 µg/mL ampicillin. After 24h of growth at 30°C, protein expression was induced with 0.5 mM ALA and 1 mM IPTG and allowed to grow for a further 24h at the same condition, after which the cells were harvested by centrifugation (6000 rpm, 4°C, 10 min). The cell pellets were resuspended in 20 ml of 0.1 M phosphate buffer pH 8.1 and 1 mg/ml of lysozyme was added. After a freeze-thaw cycle at -80°C, the cells were disrupted by sonication (5s on, 5 off, 40% amplitude) for 5 minutes, and the lysate was clarified multiple times by centrifugation (20,000 rpm, 4°C, 15 min). The clarified lysate was filtered through a 0.22 µM Amicon filter. The P450 concentration was measured by the carbon monoxide binding assay[39]. Typical concentrations of 10-20 µM of P450 BM3 mutants were readily obtained. This lysate was used as is in some tandem reactions.
For purification of the P450 BM3 mutants, the cell lysate was purified as described previously [40]. The lysate was loaded onto a column packed with DEAE 650-M resin (Toyopearl, Los Angeles, CA) coupled to a fast-performance liquid. A wash step of 15% NaCl in 25 mM phosphate buffer pH 8.1 was applied. The protein eluted at 25% NaCl in the phosphate buffer. Purity of the protein was assessed at around 70%. At this purity, the protein was judged to be pure enough for biocatalysis.

2.4.4 Preparation of whole cells of P450 BM3

Typically, a single colony carrying either gene cloned in pET28a was inoculated in 5 ml LB + 50 µg/ml ampicillin and let grow overnight (16h). This seed culture was used to inoculate 500 ml of TB media supplemented with 50 µg/ml kanamycin. The culture was grown at 37°C, 250 rpm until the OD had reached 0.8-1, upon which protein expression was induced with 0.5 mM 5-aminolevulinic and 0.5 mM IPTG. The shaking speed and temperature were decreased to 160 rpm and 30°C, and the cells were left to grow for 20h. The cells were harvested by centrifugation and resuspended in 0.1 M potassium phosphate buffer pH 7.5 containing 15% glycerol to a cell dry weight of 12 g/L.

2.4.5 General procedure for Isomerization of alkenes by sulfur activated RuHCl(CO)((4-nBu-Ph)₃)₃ in hexadecane

-A vial was prepared with 280 µl hexadecane, 40 µl 1.0 M alkene solution in hexadecane and in relevant cases 500 µL phosphate buffer (0.1 M). In a second vial, 37.7 mg sulfur on Celite (10 wt% sulfur) was weighed out and to this was added 784 µl 0.100 M RuHCl(CO)((4-nBu-Ph)₃)₃ in hexadecane and the mixture was stirred at room temperature for 5 min. Then the mixture was
filtered through cleanex paper in a Pasteur pipette and 40.0 μl of the filtrate was transferred to
the reaction vial that was then stirred at room temperature. Isomerization progress was followed
by GC after taking a 40.0 μl sample and diluting it in 340 μl toluene and 20.0 μl 0.10 M nonane
in toluene (Final conc. nonane 5.0 mM).

Notes

- In general at least 1.4 times the desired volume of activated catalyst needs to be treated
  with sulfur due to loss in transfer and filtration of the suspension. For low volumes (≤
  100 μl) larger volume excess is necessary.
- The activation can also be done in an Eppendorf or centrifuge tube, which avoids
  filtration by spinning down the Celite. Magnetic stirring is then difficult and the tube
  needs to be shaken frequently to keep the sulfur on Celite in suspension. Best done on a
  vortex shaker.
- When running pentanes contaminations by volatile solvents (acetone, diethyl ether etc)
  often mess up the analysis. Check which solvents are used in the washing container.
  Make sure to run one or more blanks before the samples to remove solvents in needle
  from previous samples.
- The stock of sulfur on celite was prepared by thoroughly grinding 10 wt% of sulfur with
  celite in a mortar to ensure high homogeneity

2.4.6 Synthesis of epoxide standards

1.28 g (18.2 mmol) cis-2-pentene was dissolved in 180 ml dichloromethane and cooled on an
ice-water bath. 4.62 g (55.0 mmol) NaHCO₃ and 9.02 g (36.6 mmol) m-chloro-parabenoic acid
(mCPBA) (70 wt%) was added and the solution was stirred intensely while slowly reaching room temperature. A lot of white precipitates were formed, which occasionally blocked the stirring. After 5h the reaction was complete according to a sample for GC (1 ml sample shaken thoroughly with 1 ml Na₂S₂O₅(aq) and 100 μl of the organic phase is diluted in 1.8 ml diethyl ether and 100 μl 0.1 M nonane in diethyl ether is added. RAL GC, method: MJ-hexene). The reaction was quenched by stirring intensely with 50 ml Na₂SO₄ until the two phases became clear. The two phases were separated and the aqueous phase was extracted with 3×20 ml dichloromethane. The combined organic phases were washed with 3×30 ml saturated NaHCO₃ (aq), dried over Na₂SO₄, filtered and carefully evaporated at 300 Torr and 15-20°C on rotavap to approximately 5ml. The crude product was chromatographed on silica eluting with first Pentane:DCM 9:1 to remove chlorobenzene formed and then 7:3 to elute the product. The fractions containing product according to TLC (SiO₂, Pentane:DCM 9:1, visualize with anisaldehyde) was evaporated to 5 ml and this crude was distilled on kugel rohr at 80°C 500 Torr after discarding fractions at 40, 50 and 60°C yielding 300 mg of cis-2,3-epoxypentane. Product is 77 wt% pure by ¹H-NMR and the only significant contamination is dichloromethane.

An alternative work-up method is to avoid quenching with Na₂S₂O₅(aq) which forms significant amounts of chlorobenzene. Instead the reaction mixture can be treated with water and conc. NaOH until pH>12 and all non-reacted mCPBA is deprotonated and dissolve. The organic phase is then washed with water (pH>12) and dried and evaporated as usual. This yields less chlorobenzene and chromatography before distillation is not necessary, although chlorobenzene can still be detected but at very low concentrations.
2.4.7 Typical tandem isomerization-epoxidation of alkenes with cell lysate + ADH regeneration

A vial was prepared with 31.0 mg sulfur on Celite (10wt% S). Another vial was prepared with 310 μl 0.1 M KPi buffer pH 7.6, 10 μl alcohol dehydrogenase (1-2 U/ml), 5.0 μl 2-propanol (1% v/v), 100 μl lysate with 23.8 μM P450 BM3 RH47. The Ru-catalyst was then activated by addition of 644 μl 0.100 M RuHCl(CO)((4-nBu-Ph)3)3 stock solution in hexadecane to the vial with sulfur on Celite. While this mixture was stirring at room temperature 75.0 μl 4.0 mM NADP+ in 0.1 M KPi buffer was added to the vials with the enzyme. After stirring the Ru-catalyst with sulfur for 5 min the suspension was filtered through KimWipe paper in a Pasteur pipette and from the filtrate was taken 460 μl and diluted in 1.61 ml hexadecane to make a 0.0222M pre-mixed solution. From this was transferred 180 μl to the vials containing the enzyme and to start the reaction 20.0 μl 1.0 M 1-pentene in hexadecane was added. Reactions are stirred at room temperature and worked up by extraction with 300 μl hexanes and centrifugation to separate the phases. 198 μl of the organic phase is transferred to a GC vial inset together with 2.0 μl 25 mM nonane in ethanol. Samples analyzed by GC/MS at Metabolomics Centre. A typical oven program was as follows: 50°C for 5 min, 10°C/min to 120°C, 35°C to 310°C, hold at 310°C for 5 min. The program was slightly modified to accommodate various

2.4.8 Typical tandem isomerization-epoxidation of alkenes with whole cells of P450

The procedure was similar to 2.4.6, except that instead of the cell lysate plus ADH regeneration, 20 g/L of whole cells harboring P450 enzymes were used.

2.4.9 Typical tandem isomerization-epoxidation of alkenoic methyl esters with cell lysate + ADH regeneration
The aqueous phase contained: 20-40 µM P450, 2 U/ml ADH, 600 U/ml catalase, 0.4 mM NADP+, 1% 2-propanol, in phosphate buffer at a total volume of 500 µL. The ruthenium catalyst was added as 20 mol% in hexadecane. The reaction was started with the addition of substrate in the organic phase (0.25 M to 0.5 M). The volume ratios of hexadecane to buffer were varied in accordance to which experiment was ran. At the end of the reactions, dodecane internal standard was added, and the reaction was extracted with 300 µL ethyl acetate. The extraction was centrifuged to separate the two phases, and 200 µL of the organic phase was transferred to a GC vial for analysis. A typical oven program was as follows: 50°C for 5 min, 10°C/min to 200°C, 35°C to 310°C, hold 310°C for 5 min at a flow of 1 ml/min. A calibration curve built with the authentic terminal alkenoic acid methyl ester oxide was used to quantify the tandem reaction products.

2.4.10 Partition coefficient determination

The partition of organic substrates dissolved in organic solvent in aqueous buffers was determined as follows. In a 1:1 mixture of hexadecane: buffer was added various concentrations of alkene substrates. The mixture was vortexed for 10 seconds and let sit for 1 min. This process was repeated 5 times. The mixture was then centrifuged. With a Hamilton syringe, a 50 uL aliquot was carefully taken from the aqueous phase and extracted with 200 µL volumes toluene containing cyclohexane as the internal standard. One hundred µL of the top phase was transferred to a GC vial for analysis. A 60m HP-5-MS column (Agilent, CA) was used in these experiments. A typical GC program was as follows: 35°C for 5 min, 5°C/min to 80°C, 35°C/min to 310°C, hold 310°C for 5 minutes.
2.5 References

### 2.6 Tables

Table 2.1 List of requirements for metal catalyst and enzyme in tandem reaction

<table>
<thead>
<tr>
<th>Requirements for metal catalyst</th>
<th>Requirements for enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactivity at or below biological conditions</td>
<td>Compatible with metal catalyst</td>
</tr>
<tr>
<td>Air- and water-stable</td>
<td>Selectivity for one isomer</td>
</tr>
<tr>
<td>Solubility and reactivity in less polar solvents such as hydrocarbons</td>
<td>High yields for the selective epoxidation</td>
</tr>
</tbody>
</table>
Table 2.2 Regioselectivity of T4MO and P450 BM3 towards 1- and trans-2-hexene. a)
whole-cell assay 37 °C, 50 mM Tris-HNO3 buffer at pH=7.4 b) 0.2µM cell lysate, 0.75 mM NADPH in 100 mM phosphate buffer pH 8.1. Epoxide concentrations are in µM. Each substrate was added at a final concentration of 4 mM in ethanol (1% v/v). At least two independent experiments were performed. Errors are at most 15%. See experimental procedures for details.

<table>
<thead>
<tr>
<th>Enzyme/substrate</th>
<th>1-hexene</th>
<th>trans-2-hexene</th>
<th>1:1 mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4MOa</td>
<td>52c</td>
<td>46</td>
<td>33:33</td>
</tr>
<tr>
<td>T4MO (I100G)a</td>
<td>79</td>
<td>22</td>
<td>25:17</td>
</tr>
<tr>
<td>RH47b</td>
<td>233</td>
<td>100</td>
<td>140:4</td>
</tr>
<tr>
<td>SH44b</td>
<td>70</td>
<td>30</td>
<td>141:5</td>
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Table 2.3 Terminal regioselectivity of RH47 and SH44 in alkene mixtures. Experiments were set up as in b in Table 2.2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>RH47</th>
<th>SH44</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-octene/cis-2-octene</td>
<td>0.82 ± 0.02</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td>1-hexene/cis-2-hexene</td>
<td>1.20 ± 0.01</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>4-methyl-1-pentene/4-methyl-cis-2-pentene</td>
<td>8.30 ± 0.64</td>
<td>0.62 ± 0.10</td>
</tr>
<tr>
<td>t-butyl-1-propene/t-butyl-cis-2-propene</td>
<td>4.20 ± 0.29</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>1-pentene/cis-2-pentene</td>
<td>1.90 ± 0.21</td>
<td>1.40 ± 0.2</td>
</tr>
</tbody>
</table>
Table 2.4 Desirable solvent characteristics

<table>
<thead>
<tr>
<th>Feature</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low emulsion-forming tendency</td>
<td>Favorable properties for product recovery</td>
</tr>
<tr>
<td>Chemical and thermal stability</td>
<td>Nonbiodegradability</td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>Nonhazardous</td>
</tr>
<tr>
<td>Favorable distribution coefficient</td>
<td>Inexpensive</td>
</tr>
<tr>
<td>High selectivity</td>
<td>Available in bulk quantities</td>
</tr>
<tr>
<td>Low aqueous solubility</td>
<td>NADPH uncoupling of P450</td>
</tr>
</tbody>
</table>
Table 2.5 Activity of ruthenium catalyst in polar organic solvents

<table>
<thead>
<tr>
<th>entry</th>
<th>solvent</th>
<th>ratio (terminal:internal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EtOAc</td>
<td>22:78</td>
</tr>
<tr>
<td>2</td>
<td>Et₂O</td>
<td>78:22</td>
</tr>
<tr>
<td>3</td>
<td>THF</td>
<td>2:98</td>
</tr>
<tr>
<td>4</td>
<td>THF:H₂O (1:1)</td>
<td>14:86</td>
</tr>
</tbody>
</table>

³Reaction conditions: 1.0 mmol of 1-octene, 5 mol% catalyst, in 1.0 mL solvent at room temperature for 24 h. ⁴GC ratios.
Table 2.6 RuCl(CO)(PPh$_3$-tbutyl)$_3$ activity in organic solvents.

<table>
<thead>
<tr>
<th>entry</th>
<th>solvent</th>
<th>olefins (terminal:internal)$^b$</th>
<th>Rh-cat.$^c$</th>
<th>Ru-cat.$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pentane</td>
<td>98:2</td>
<td>1:99</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>pentane:phosphate buffer (1:1)</td>
<td>-</td>
<td>13:87</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>hexadecane</td>
<td>98:2</td>
<td>14:86</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>hexadecane:phosphate buffer (1:1)</td>
<td>98:2</td>
<td>48:52</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>phosphate buffer$^a$</td>
<td>98:2</td>
<td>29:71</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Reaction conditions: 0.25 mmol of 1-hexene, 1 mol% catalyst, in 0.25 mL solvent at rt for 24 h. $^b$GC ratios. $^c$Rh-cat.: RhHCO(L1)$_3$. $^d$Ru-cat.: RuHCl(CO)(L1)$_3$. $^a$I-Octene used as organic phase.
### Table 2.7 Tandem Isomerization-Epoxidation of various alkenes

<table>
<thead>
<tr>
<th>entry</th>
<th>alkene</th>
<th>P450</th>
<th>Total product (μM)</th>
<th>epoxide ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cis-2,3</td>
<td>trans-2,3</td>
</tr>
<tr>
<td>1</td>
<td>1-pentene</td>
<td>SH44</td>
<td>400</td>
<td>1.60</td>
</tr>
<tr>
<td>2</td>
<td>1-hexene</td>
<td>SH44</td>
<td>205</td>
<td>1.50</td>
</tr>
<tr>
<td>3</td>
<td>4-methyl-1-pentene</td>
<td>SH44</td>
<td>249</td>
<td>0.68</td>
</tr>
<tr>
<td>4</td>
<td>trans-2-hexene</td>
<td>SH44</td>
<td>238</td>
<td>2.40</td>
</tr>
<tr>
<td>5</td>
<td>1-pentene</td>
<td>RH47</td>
<td>1231</td>
<td>1.50</td>
</tr>
<tr>
<td>6</td>
<td>1-hexene&lt;sup&gt;1&lt;/sup&gt;</td>
<td>RH47</td>
<td>1097</td>
<td>1.80</td>
</tr>
<tr>
<td>7</td>
<td>4-methyl-1-pentene</td>
<td>RH47</td>
<td>787</td>
<td>0.05</td>
</tr>
<tr>
<td>8</td>
<td>trans-2-pentene</td>
<td>RH47</td>
<td>575</td>
<td>4.27</td>
</tr>
<tr>
<td>9</td>
<td>trans-2-hexene</td>
<td>RH47</td>
<td>238</td>
<td>3.60</td>
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<tr>
<td>10</td>
<td>4-methyl-trans-2-pentene</td>
<td>RH47</td>
<td>213</td>
<td>1.02</td>
</tr>
<tr>
<td>11</td>
<td>4-methyl-cis-2-pentene</td>
<td>RH47</td>
<td>245</td>
<td>1.13</td>
</tr>
<tr>
<td>12</td>
<td>t-butyl-trans-2-propene</td>
<td>RH47</td>
<td>87</td>
<td>7.70</td>
</tr>
</tbody>
</table>

<sup>1</sup>Small amounts of 3,4 epoxyhexanes formed

Aqueous phase: 1-10 μM P450, 1 U/ml ADH, 1% 2-propanol, 0.4 mM NADPH in phosphate buffer pH 8.1, 2:5 organic:aqueous v/v ratio
Table 2.8 Mutual solubilities of hexenes in hexadecane: buffer

<table>
<thead>
<tr>
<th>Top phase ratio</th>
<th>Aqueous phase ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>5:1</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>10:1</td>
<td>4.7 ± 1</td>
</tr>
</tbody>
</table>

Conditions: 1-hexene (100 mM) and cis-2-hexene (1 to 10 equiv.) mixed in the organic phase at various ratios. Bottom phase ratio determined by partition coefficient calculation.
Table 2.9 Tandem isomerization-epoxidation of 5-hexenoic acid methyl ester in different configurations

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate, concentration, catalyst loading&lt;sup&gt;b&lt;/sup&gt;</th>
<th>v/v organic: aqueous</th>
<th>P450 mutant, concentration</th>
<th>Epoxidized product distribution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total epoxide yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A, 0.5 M, 20 mol %</td>
<td>1:10</td>
<td>SH44, 20 μM</td>
<td><strong>0.38; 0.75; 1.00</strong></td>
<td>9.5</td>
</tr>
<tr>
<td>2</td>
<td>A, 0.5 M, 20 mol%</td>
<td>1:25</td>
<td>SH44, 20 μM</td>
<td>0.20; 0.50; 1.00</td>
<td>22.9</td>
</tr>
<tr>
<td>3</td>
<td>A, 0.25 M, 20 mol %</td>
<td>1:10</td>
<td>SH44, 20 μM</td>
<td>0.16; 0.46; 1.00</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>A, 0.25 M, 40 mol%</td>
<td>1:25</td>
<td>SH44, 20 μM</td>
<td>0.18; 0.53; 1.00</td>
<td><strong>35.4</strong></td>
</tr>
<tr>
<td>5</td>
<td>A, 0.5 M, 20 mol %</td>
<td>1:25</td>
<td>SH44, 40 μM</td>
<td><strong>0.22; 0.67; 1.00</strong></td>
<td><strong>20.3</strong></td>
</tr>
<tr>
<td>6</td>
<td>A, 0.5 M, 20 mol %</td>
<td>1:10</td>
<td>SH44, 40 μM</td>
<td><strong>0.44; 0.71; 1.00</strong></td>
<td>8.4</td>
</tr>
<tr>
<td>7</td>
<td>A, 0.25 M, 40 mol %&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1:25</td>
<td>SH44, 40 μM</td>
<td>0.25; <strong>0.92; 1</strong></td>
<td><strong>36.0</strong></td>
</tr>
<tr>
<td>8</td>
<td>A, 0.25 M, 20 mol %&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1:25</td>
<td>RH47, 40 μM</td>
<td>0.5; <strong>0.8; 1</strong></td>
<td><strong>32.2</strong></td>
</tr>
<tr>
<td>9</td>
<td>B, 0.5 M, 20 mol%</td>
<td>1:10</td>
<td>RH47, 20 μM</td>
<td>0.5; 1</td>
<td>15.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> all reactions ran at 37°C, with shaking (290 rpm), <sup>b</sup> only about half the catalyst is active, <sup>c</sup> terminal- cis+ trans 4, cis+trans-3, <sup>*</sup>cis-3, cis-4, terminal <sup>*</sup> Stirring instead of shaking
2.7 Figures

Figure 2.1 Reaction of nucleophiles with epoxides. Adapted from [1]
Figure 2.2 Schematic representation of a tandem isomerization-epoxidation for the epoxidation of terminal olefins
Figure 2.3 An alternative approach to study and engineer the tandem reaction. The reaction would start from thermodynamically downhill terminal-internal isomerization, followed by the selective epoxidation of one internal isomer.
Figure 2.4 Schematic representation of biphasic reaction
Figure 2.5 Growth of *E. coli* cells harboring pET28a-*bm3* RH47 under decane and dodecane containing increasing concentrations of 1-hexene.
Figure 2.6 Whole cell Biocatalysis in Biphasic Reaction. *E. coli* BL21 cells harboring pET28a-\textit{bm3\_RH47} in phosphate buffer 0.1 M, OD-20 ~ 4.8 g/L, 2\% glucose, 1M 1-hexene dissolved in organic solvent, 30°C, 40\% v/v organic phase volume ratio
Figure 2.7 P450 BM3 specific NADPH uncoupling/consumption rate in the presence of various solvents.
Figure 2.8 Formation of internal alkene by isomerization of 1-hexene catalyzed by RuHCl(CO)(P(PhnBu)3)3 activated by sulfur or dibenzothiophenes (DBTS) in hexadecane. Conditions: 0.1 M 1-hexene, 20 mol% cat., 0 or 1 equiv sulfur reagent, room temperature.
Figure 2.9 Formation of internal hexene isomers through isomerization of 1-hexene catalyzed by RuHCl(CO)(P(PhnBu)_3) pre-activated by 1.5 equiv elemental sulfur in the absence and presence of aqueous buffer and cell lysate without P450 (empty vector plasmid).
Figure 2.10 Isomerization of various alkenes with RuHCl(CO)(P(PhnBu)$_3$)$_3$, activated with 1.5 eq. elemental sulfur. Reactions were run in hexadecane: buffer 2:5 v/v.
Figure 2.11 Tandem isomerization-epoxidation with whole cells of P450 RH47 A) with sulfur activation of RuHCl(CO)(Ph₃P-n-buty)₃ B) no sulfur activation
Figure 2.12 Cell lysate tandem reaction. A) Representation of reaction scheme B) Time course of tandem reactions starting with 1-hexene and trans-2-hexene, respectively.
Figure 2.13 Verification of 4-methyl-1,2-epoxypentane production from tandem reaction of cis- or trans-4-methyl-2-pentene. Red and green traces: epoxidation of 4-methyl-trans-2- and 4-methyl-cis-2-pentene, respectively. Blue trace: tandem catalysis starting from 4-methyl-trans-2-pentene.
Figure 2.14 Influence of percentage of methyl-β-cyclodextrin on the partition of 1-hexene
Figure 2.15 Enhancement of epoxidation yields with the addition of methyl-\(\beta\)-cyclodextrin. A) Schematic representation of reaction with conditions. B) Core structure of methyl-\(\beta\)-cyclodextrin. C) Increase in epoxidation yields as a function of methyl-\(\beta\)-cyclodextrin concentration.
Figure 2.16 Time course of epoxidation of 1-hexene with the addition of methyl-β-cyclodextrin.

Data are from two independent reactions. Errors are at most 5%
Figure 2.17 Isomerization of various concentrations 5-hexenoic acid methyl ester at 37°C with 20 mol% RuHCl(CO)(PPh₃-nbutyl)₃
**Optimization of isomerization reaction of methyl-5-hexenoate**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate concentration (M)</th>
<th>RuHCl(CO)(PPh₃-nbutyl)₃ loading (mol%)</th>
<th>Volume hexadecane (µL)</th>
<th>Volume buffer (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25</td>
<td>40</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>40</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>40</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>20</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>20</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>40</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>80</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>8</td>
<td>0.50</td>
<td>20</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.50</td>
<td>40</td>
<td>300</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.18 Optimization of isomerization reaction. All reactions were run at 37°C. Reactions were extracted at 6h with 2 volumes of ethyl acetate and ran on GC-MS. Dodecane was used as the internal standard. The ratios of peak area of each isomer with the internal standard peak were used to quantify each isomer. A control with no isomerization catalyst was used as a negative control to correct for control area differences.
Figure 2.19 Isomerization of methyl-2,2-dimethylhex-5-enoate
Figure 2.20 $^1$H NMR of reaction in Figure 2.19
Figure 2.21 Tandem reaction of 5-hexenoic acid methyl ester. A. isomerization catalyst B. epoxidation enzyme
Figure 2.22 Determination of cis-selectivity of the tandem reaction. A) Isomerization in hexadecane: buffer with no P450 present. B) Trans-3-pentenoic acid methyl ester reaction. C) Trans-2-hexenoic acid methyl ester reaction.
CHAPTER 3. COOPERATIVE TANDEM METATHESIS-OXIDATION BY AN ORGANOMETALLIC COMPLEX AND A METALLOENZYME

3.1 Introduction

The olefin metathesis reaction, through which pairs of C=C bonds are reorganized, is one of the most remarkable and versatile metal-catalyzed reactions in synthetic chemistry (Figure 3.1). This is evidenced by the hundreds research articles and reviews involving olefin metathesis that are published every year [2]. Over the last two decades, the emergence and commercialization of highly selective ruthenium carbene and molybdenum catalysts have revolutionized synthetic methodologies by introducing this elegant way of creating double bonds. These catalysts have proven to be stable, easy to handle and modify, in particular the two structurally well-defined Grubbs and the Grubbs-Hoveyda catalysts (Figure 3.2). Due to its versatility and ever expanding substrate scope, olefin metathesis has found applications in the preparation of scarce molecules with highly desirable properties, including in alkane metathesis [3], asymmetric total synthesis [4], natural products synthesis (synthesis of diverse polycyclic compounds [5] or enantioselective synthesis [6, 7]), and in the synthesis or modification of steroids (reviewed by [2]) and medicinal agents [8], among others. Three advantageous features of metathesis catalysts are their wide solvent compatibility [9], functional group tolerance [10] and the ease with which they can be modified to achieve solubilities in various solvents. Through relatively simple modifications, olefin metathesis catalysts with solubility and activity in alkanes [11], ionic liquids [12], emulsions [13], and aqueous solvents [14-16] can be readily prepared as homogeneous or heterogeneous systems [17].
Although still in its infancy, there are several concurrent tandem reactions involving olefin metathesis reported, including both metathesis/metathesis [18, 19] and metathesis/non-metathesis tandem catalytic systems. The latter include metathesis/isomerization [20, 21], metathesis/cyclopropanation [22], and metathesis/Diels Alder cycloaddition [23]. However, these tandem reactions involve either only one metal center performing a single or both reactions, or two organometallic catalysts of the same discipline that are very similar (two ruthenium centers, for example).

In our work, we seek to engineer a platform for generation of one-pot reactions for the dynamic regio- and enantioselective enzymatic oxidation of olefins prepared by metathesis using metalloenzymes and metathesis catalysts. Namely, this would involve the tandem action of two metal catalysts from very different disciplines, working together to accomplish a synthetic goal not accessible by either catalyst alone. To this end, we conceived a study to assess whether metalloenzymes and organometallic catalysts could work cooperatively to catalyze a multistep process. We define cooperative reactivity in this context as two species catalyzing a reaction that would not occur with either catalyst alone and would occur in lower yield if conducted as two sequential reactions.

We chose to assess the potential cooperative reactivity of these catalysts with alkene reactants because alkenes are basic feedstocks and are often found as mixtures of cis and trans isomers, terminal and internal isomers, and as components of natural fatty acids. Thus, one could envision reactions catalyzed by organometallic complexes that lead to an equilibrating mixture of alkenes or the production of a mixture of alkenes in tandem with a selective reaction of one member of
the mixture catalyzed by a metalloenzyme. In the following work, we report this type of dynamic process and show that the combination of an olefin metathesis catalyst and a cytochrome P450 enzyme converts a mixture of alkenes to a single oxidation product. The metathesis catalyst leads to an equilibrating mixture of alkenes, and the P450 enzyme converts one member of the mixture of alkenes to the corresponding oxidation product.

One reaction with high synthetic value that one can envision would be the regioselective oxidation of medium chain alkenes from a dynamic mixture of shorter alkenes through a tandem metathesis-oxidation process. Beyond curiosity in science, our motivation also stemmed from the fact that there are very few metathesis/oxidations developed even in organic chemistry. Recently, three tandem metathesis/oxidation reactions that provide cis-diols or α-hydroxy ketones [24] and 2-quinolones [25] were published. However, these systems suffer from low regioselectivity, and no generation of a chiral center was achieved. In addition, the reactions were performed with one metal catalyst. Lastly, the ring closing metathesis step cannot establish a dynamic equilibrium since the equilibrium strongly favors the ring-closed form.

In order to prove that olefin metathesis catalysts and metalloenzymes could work cooperatively, we have to achieve three key steps. First, the metathesis catalyst must be stable in the presence of the enzyme, components of the buffer system and oxygen. Second, the enzyme system must be stable in the presence of the olefin metathesis catalyst. Third, one should choose reactions and enzymes that afford high yields and selectivity for the product(s) of the metathesis, while showing little to no activity for the metathesis substrate(s). In addition, the enzymatic oxidation reaction must exert a driving force on the equilibrium established by the olefin metathesis
catalyst, thus enable the tandem reaction to achieve a final yield higher or comparable to doing the reactions sequentially. In this Chapter, we show our results in establishing said tandem reactions with fatty acids and aromatic substrates using commercially available metathesis catalysts and the versatile cytochrome P450 BM3.

3.2 Results and discussion

3.2.1 Screening of metathesis catalysts

The make- or break condition for the tandem metathesis-oxidation to succeed is for the metathesis catalysts to be active in the presence of oxygen and enzymatic entities. The enzyme will use molecular oxygen to selectively oxidize C-C and C=C bonds. Generally, metathesis reactions are run using Schlenk tube techniques, away from moisture, oxygen and impurities, while using distilled substrates and solvents. After surveying the literature, it was found that commercially available metathesis catalysts can work in air, using non-distilled solvents and substrates [26-30]. For example, Connon and Blechert found that using the Grubbs 2\textsuperscript{nd} generation catalyst (or the Hoveyda-Grubbs 2\textsuperscript{nd} generation catalyst with an o-isopropyl group) could afford ring closing metathesis and even cross metathesis of challenging substrates with high yields. Another report by Lipshutz et al. showed that simply adding the amphiphile polyoxyethanyl \textit{a}-tocopheryl sebacate (PTS) allowed for unsymmetrical olefin cross-metathesis reactions in water, involving water-insoluble substrates [31]. This report also pointed out that metathesis reaction could happen “on” water, with the reaction occurring while the catalyst and the substrates remained insoluble.
We first tested five catalysts (Figure 3.2A) for their activity in air for the model cross metathesis reaction between methyl oleate and trans-3-hexene in dichloromethane. This reaction should produce methyl-dodecen-8-oate and 9-dodecene (cis + trans) as the desired cross metathesis products (Figure 3.2B). The results are listed in Table 3.1. All five catalysts showed activity in air in dichloromethane, although their selectivities were not as high as reactions performed in oxygen free conditions [32]. All reactions were completed within 30 min. To test the reversibility of the reactions, the vials were opened to allow trans-3-hexene to evaporate. We observed the formation of the methyl oleate starting material approximately 20 minutes after the opening of the vial, which suggested that the reactions were reversible. Therefore, a dynamic equilibrium of olefins could be maintained by the metathesis catalyst.

3.2.2 Finding the “right” reactions

Since the metathesis catalysts in air worked, we sought to find the right substrates for the olefin metathesis that would create the right selectivities for the P450 enzyme WT. In addition, we sought to find out whether the metathesis catalysts could remain stable under conditions of enzymatic oxidation (P450, NADPH, biphasic). We decided that to establish this tandem reaction, no P450 engineering should be required to afford either activity or stability for the enzyme, or stability for the metathesis catalysts. Therefore, we considered that the selectivity of certain P450 enzymes for the oxidation of fatty acids, alkenes and alkanes of certain chain lengths could be exploited for the selective reaction of one member of a mixture of alkenes. One such general scheme can be seen in Figure 3.4. The wild-type P450 BM3 enzyme hydroxylates C_{12-120} fatty acids selectively [33, 34]. We envisioned reactions in which a cross metathesis reaction between a shorter unsaturated fatty acid and a symmetrical alkene would elongate the
acid chain to create a substrate for P450 BM3. The P450 BM3 enzyme would oxidize the resulting longer-chain fatty acid (>C\textsubscript{12}). For example, we found that P450 BM3 shows 100% selectivity for dodecanoic acid hydroxylation at \(\omega\-1\), \(\omega\-2\) and \(\omega\-3\) (Figure 3.5) over hydroxylation of 5-hexenoic acid. In addition, the enzyme reacts with a more than 5-fold preference for oxidation of a \(\text{C}_{13}\) chain over a \(\text{C}_{11}\) chain with 82% selectivity for hydroxylation of the \(\omega\-2\) and \(\omega\-3\) carbons of tridecanoic acid (Table 3.2). With this system, we hypothesized that fatty acids of \(\text{C}_{13}\) length with an alkene at the \(\omega\-2\) position would undergo selective epoxidation. Based on the P450 WT selectivity, we therefore sought to design two tandem reactions (Figure 3.6).

In the first example reaction, the cross metathesis between 5-hexenoic acid and 7-tetradecene would yield 5-dodecenoic acids as the main cross metathesis product, while the P450 enzyme selectively hydroxylates the \(\text{C}_{12}\) cross metathesis product. Since the oxidation happens at carbons 2, 3 and 4, the double bond remains unreacted and can undergo further cross metathesis. An additional equilibrium could be established between the final product and the other metathesis components of the reaction. In reaction 2, the cross metathesis between 10-undecenoic acid and \textit{trans}-3-hexene would create a dynamic equilibrium mixture in which the cross metathesis product 10-tridecenoic acids would be selectively epoxidized by the wild type P450 BM3 enzyme. In both reactions, the self-metathesis of the starting acid will occur. A reduction of the self-metathesis of the starting acid can be used as an indication of the reaction being driven forward towards replacing the cross metathesis \(\text{C}_{12}\) intermediate.

3.2.3 Tandem metathesis-oxidation (5-hexenoic acid + 7-tetradecene)
In our first example reaction, the cross-metathesis of 5-hexenoic acid and \textit{trans}-7-tetradecene to afford 5-dodecenoic acids. This cross-metathesis reaction also yields the self-metathesis of the respective starting materials. Since one of the alkenes is symmetrical, its self-metathesis yields itself. We first attempted biphasic reaction systems. By this strategy, we hypothesize that the catalysts need to be stable only to the conditions under which its partner reacts. For the oxidation reaction, the WT P450 BM3 purified enzyme and an NADP+ alcohol dehydrogenase regeneration system were used. Catalase was added at 600 U/mL to disperse any formed hydrogen peroxide produced from the decoupling of NADPH from the oxidation of the fatty acid (Table 3.3).

Two biphasic configurations were chosen for the tandem reaction. First, we attempted an isooctane: buffer biphasic reaction system. Isooctane has been shown to be compatible with metathesis catalysts [9, 30] and has been used as a biocompatible solvent in biphasic biocatalysis involving P450 enzymes [35]. In this system, the metathesis catalyst is predissolved in dichloromethane, since the commercially available catalysts have very low solubility in this solvent. We find that the cross metathesis of 5-hexenoic acid with 1 equiv. of 7-tetradecene using 3-10 mol\% of C3 yields 33\% of the 5-dodecenoic acids (2) at equilibrium in a 1:2 v/v of isooctane: buffer with an \textit{E/Z} of 6:1. A 25\% yield of the self-metathesis of 5-hexenoic acid (3) was also isolated (Table 3.3, entry 1). Adding the P450 enzyme to this system gives an overall yield of 25\% for the hydroxylated products (4) (sum of \(\omega-1\), \(\omega-2\) and \(\omega-4\) products) after 48h, while 3\% of 2 remained, along with 12\% yield of 3. We also attempted a neat: buffer reaction (entry 3-6). In this case, 5 equiv. of \textit{trans}-7-tetradecene was used, and this alkene served as the organic phase as well, while the metathesis catalyst C3 was insoluble. After 5 hours, we found
that the equilibrium metathesis produced 60% of 2 and 5% of 3 (entry 3). Unfortunately, although the CM yields were significantly higher, yields of 4 remained around 25% (entry 4) in the tandem reaction. We noticed that the yields of 4 increased to 25%, which is the equilibrium achieved when 1 equiv. of tetradecene was used. This suggested that either secondary metathesis between the final hydroxylated products 4 and 2 continued to occur, or that the metathesis reaction itself was being driven towards making the self-metathesis product in the presence of enzyme components. When the enzymatic reaction was performed with the equilibrium concentration achieved in the metathesis reaction alone (entry 5), a yield of 20% of 4 was obtained.

In conclusion, for this reaction, we found that doing the two reactions in tandem did not significantly increase the yields of the final oxidation product compared to doing the two reactions sequentially, but only by a small margin. In addition, this result was not reproducible. We further found that the self-metathesis of 7-tetradecene was accompanied by isomerization. Many ruthenium olefin metathesis complexes are known to isomerize long chain alkenes, and self-metathesis of 7-tetradecene has been shown to produce a semi-symmetrical distribution of olefins C₉-C₂₁ [36], which could represent up to 70% of the starting tetradecene. In our system, after metathesis, we observed many new olefin peaks on GC-MS (Figure 3.6). Since we did not observe any additional cross metathesis product starting from the final product (polyols from C₁₂-5-OH (4)), we assumed that the olefin metathesis catalyst C₃ was not stable enough in the reaction. When we started the cross metathesis reaction with 4 and 7-tetradecene, we observed many diols, which corroborated our point of C₃ being unstable. More importantly, because the double bond does not react, the driving relies only on the solubility of the hydroxylated product.
compared to the starting materials and to the self-metathesis diacid. In effect, due to the higher solubility of the 3, a driving force cannot be implemented. At least when using olefinic fatty acids, we realized that reacting out the double bond is the only way to drive the reaction forward.

3.2.4 Tandem metathesis-oxidation (10-undecenoic acid + trans-3-hexene)

For this reaction, several components were optimized. First, we re-tested the catalysts stability in an isooctane: buffer biphasic reaction in the presence of enzymatic components (P450, NADPH). Second, we chose phosphite dehydrogenase as the NADPH regenerating enzyme, taking sodium phosphite to sodium phosphate, with concomitant oxidation of NADP+ to NADPH. Having this enzyme avoided the need to purchase the expensive NADP+-dependent T. brockii alcohol dehydrogenase. This enzyme has been previously engineered in our lab [37-39].

We investigated the reactivity of the five ruthenium carbene catalysts for the cross metathesis between 10-undecenoic acid and trans-3-hexene in this biphasic setting in the presence of P450 and NADP+. This reaction produces a dynamic equilibrium of the desired cross metathesis products cis and trans-10-undecenoic acids, as well as the self-metathesis product of 10-undecenoic acid. Complexes C4 and C5 did not catalyze the metathesis reaction under these conditions. The Hoveyda-Grubbs II C3 did convert 50% of the starting material after addition of the unsaturated fatty acid in two batches. In contrast, catalysts C1 and C2 continued to convert the unsaturated fatty acid even after addition of a third batch of acid (Figure 3.7). The subsequent experiments were conducted with catalyst C1.

3.2.4.1 Time course of 10-undecenoic acid and trans-3-hexene tandem reaction
Using catalyst 1, time course experiments were designed (Figure 3.8A). Since type I olefins such as terminal olefinic acids and esters have a high rate of homodimerization [40], the suppression of the self-metathesis of 10-undecenoic acid can be monitored as an indication that the enzyme reaction is exerting a driving force on the metathesis reaction. At a low equivalence of the alkene partner (1.5 equiv.), the self-metathesis of 1 was formed preferentially. When no P450 BM3 was present, a 95% conversion of the starting material was obtained, with only 18% yield of the cross metathesis product. A fed-batch tandem reaction time course showed a continuous production of 10,11-epoxytridecanoic acids over the 39h-reaction. The P450 BM3 WT reacted with the cross metathesis intermediate with 100% selectivity, while the intermediate was continuously replaced by the olefin cross metathesis, proving that indeed the epoxidation of the double bond of the cross metathesis product drove the cross metathesis reaction forward (Figure 3.9). \(^1\)H NMR and \(^{13}\)C NMR (Figures 3.13 and 3.14) and TMCES derivatization of the final products confirmed that both the cis- and trans-10-tridecenoic acids were epoxidized with >95% selectivity compared to respective hydroxylations at \(\omega-1\) or \(\omega-4\) (Figures 3.10). Concurrently, the concentration of self-metathesis was diminished by up to 50% over course of the reaction compared to the metathesis reaction alone (Figure 3.8B). Lastly, at every time point sampled, the concentration of starting material in the tandem reaction was always lower than in the metathesis alone (20 to 40%) as further evidence that more starting material was being funneled towards forming the cross metathesis products as a result of enzyme epoxidation (Figure 3.8C). A final yield of 27% of 10,11-epoxytridecanoic acids was obtained, which corresponds to a 1.5-fold improvement over doing both reactions sequentially, assuming that a 100% recovery can be achieved from the metathesis reaction, and a 100% yield can be obtained from the biotransformation.
3.2.4.2 One-pot tandem reactions with 10-undecenoic acid and trans-3-hexene

One-pot batch reactions were set up and optimized by adjusting the NADPH regeneration rate, substrate loading and cross partner equivalence. With a fixed regeneration rate of 4 nmol/min, decreasing the substrate loading from 50 mM to 12.5 mM increased the yield of 4 from 35 to 70% (Table 3.4, entries 1a, 2a and 3a), which represents a respective 1.8, 2.0 and 3.1 fold improvement over doing the reactions sequentially (entries 1b, 2b and 3b). Being pseudo-first order with respect to substrate concentration, the lower starting acid concentration slowed down the metathesis reaction, allowing the enzyme more time to react a larger proportion of formed cross metathesis product. It is worth noticing that the cross metathesis product remaining in entries 1a, 2a and 3a was significantly lower than their equilibrium metathesis counterparts, while a large concentration of self-metathesis remained. While the metathesis catalyst remained active through that period, the self-metathesis product progressively precipitated out of solution, thus becoming less available for further metathesis to replenish the cross metathesis pool. In the case of entries 1a and 2a, addition of catalyst 1 did not significantly improve the yields. Using 3 to 10 equiv. of trans-3-hexene, the self-metathesis thermodynamic sink was almost completely avoided, as yields of up to 90% were obtained within 12h of reaction (entries 4a, 5a and 6a and Figure 3.15). Enzyme stability studies suggested that the enzyme was deactivated at the end of the reaction. However, we observed that in the presence of the pre-catalyst in PTS: buffer, the enzyme remained active. The enzyme deactivation may be caused by a toxic intermediate resulting from the olefin metathesis catalyst decomposition.

3.2.5 Tandem metathesis-epoxidation (4-butenyloxybenzoic acid + trans-3-hexene)
To assess whether the cooperative effect of the two catalysts extends beyond 10-undecenoic acid and \textit{trans}-3-hexene, we sought to identify a second combination of alkenes that would react in a similar fashion (Table 3.5). Studies on the reactions of unsaturated alkylbenzoic acid derivatives showed that the P450 BM3 enzyme epoxidized 4-hexenyloxybenzoic acid (6), an unnatural substrate, with a rate (judged by the consumption of NADPH) of 65 nmol NADPH/nmol P450 BM3·min⁻¹. This rate is 2.5-fold higher than that for reaction of 4-butenyloxybenzoic acid (5). Compound 6 formed in 58% yield from the cross-metathesis of 5 with 1.4 equivalents of \textit{trans}-3-hexene in a dioctyl phthalate: buffer biphasic system, with little formation of the product from self-metathesis of 5 (Table 3.5, entry 2, Figures 3.18 and 3.19). Moreover, P450 BM3 catalyzed the reaction of compound 6 to form epoxide 8 as a single product, as shown by NMR spectroscopy. Although the specific NADPH depletion rates of the enzyme for the epoxidation of 6 over 5 is only 2.5 and the rate of metathesis of 5 is slow, a final yield of 8 of 75% was obtained. A yield of 7 of 20% is also obtained. This yield is higher than the 58% yield for two reactions occurring sequentially in perfect yield (entry 1 and Figure 3.20). This example shows that the process can be conducted with several alkenes, including those that are unnatural classes of substrates for epoxidation by P450 BM3.

\textbf{3.3 Conclusions and outlook}

In summary, we have shown for the first time that an organometallic catalyst and a metalloenzyme can work in tandem for a highly selective and high yielding tandem cross-metathesis-epoxidation. The first reaction takes advantage of the remarkable selectivity of the P450 BM3 wild-type enzyme for \textgreater C_{12} fatty acid carbon chain lengths, while the second reaction takes advantage of the enzyme’s slightly higher selectivity for the cross metathesis product 4-
hexenyloxybenzoic acid. The two reactions work very efficiently, although it is known that exposed carboxylic acids are known to deactivate ruthenium metathesis catalysts [41]. In addition, the final product obtained represents an unnatural epoxy-fatty acid that could not be obtained through one catalytic reaction. So far, we notice that reaction of the double bond of the target substrate created by cross metathesis is vital to exert a driving force towards the final epoxidation product. Considering the shear immensity of substrate scope and versatility of both the olefin metathesis catalysts and P450 BM3, a platform of metathesis-oxidation tandem reactions could be, theoretically, a plug and play exercise, as long as the right selectivities and activities are satisfied. Moreover, since the P450 BM3 enzyme mutants obtained through directed evolution often show strict carbon chain length-dependent reactivity, these selective tandem metathesis-oxidations can be designed which could not be achievable using chemical catalysts alone. Tandem reactions of particular interest to us include the selective epoxidation of regio- and stereoisomers of simple linear olefins, or the oxidation of longer chain olefins generated from the metathesis of shorter olefins, two examples with potential resounding impact in the selective C-H bond functionalization for fine chemicals
3.4 Experimental procedures

3.4.1 Materials

All chemicals, solvents and catalysts were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and used without further purification. The Zannan catalyst was purchased from Strem Chemicals (Newburyport, MA). *E. coli* DH5α cells were purchased from the Cell Media Facility at the University of Illinois at Urbana-Champaign (Urbana, IL). Oligonucleotides for cloning were purchased from Integrated DNA Technologies (Coralville, IO.). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). NADPH was purchased from Enzo Life Sciences (Farmingdale, NY). NADP⁺ was purchased from Roche Diagnostics (Indianapolis, IN). 5-Aminolevulinic acid hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from GoldBio (St. Louis, MO).

3.4.2 Cloning, expression and purification of P450 BM3

The full gene encoding the wild-type P450 BM3 was amplified from the genomic DNA of *Bacillus megaterium* using primers *Bam*HI-forward (ATACCGGGATCCATGACAAATTAAGAAATGCTCAGCCAAAAACG) and *Eco*RI-reverse (ACGTGCGAATTCTTACGCCAGCCCCACGTCTTTTGCCTATC). Restriction sites are underlined. The gene was cloned into the vector pCWori⁺ digested with the same enzymes and transformed into *E. coli* DH5α. Expression and purification of P450 BM3 was done as follows. Briefly, 500 mL Terrific Broth (TB) medium supplemented with 100 mg/mL ampicillin, was inoculated with an overnight culture (5 mL) in a 2.8 L flask and incubated at 30°C and 250 rpm shaking. After 12 h of incubation, 5-aminolevulinic acid hydrochloride (ALA; 0.5 mM) was
added, expression was induced by addition of 1 mM IPTG; and the shaking speed was reduced to 180 rpm. Cells were harvested by centrifugation for 24 h after induction. The cell pellets were resuspended in 20 mL 100 mM potassium phosphate buffer, pH 8.1 and lysed by sonication. The enzyme was purified by FPLC using a DEAE-anion exchange column following published procedures [42, 43]. Enzyme concentration was measured in triplicates by the CO binding method [44].

3.4.3 Expression and purification of phosphite dehydrogenase (PTDH)

A thermostable phosphite dehydrogenase, previously engineered in Zhao laboratory, was purified according a previous protocol [39]. The buffers used for protein purification included start buffer A (SBA) (0.5 M NaCl, 20% glycerol, and 20 mM Tris-HCl, pH 7.6), start buffer B (SBB) (same as start buffer A but with 10 mM imidazole), and elution buffer (EB) (0.5 M imidazole, 0.5 M NaCl, 20% glycerol, and 20 mM Tris-HCl, pH 7.6). Transformants with pET15b-derived vectors were grown in Terrific Broth medium containing 100 μg/mL ampicillin at 37 °C with good aeration (shaking at 250 rpm). When the log phase was reached (OD\textsubscript{600} ~0.6), cells were induced with IPTG (final concentration 0.3 mM) and incubated at 25 °C with shaking at 250 rpm for 8 h. Cells were harvested by centrifugation at 5000 g, 4 °C, for 15 min, then resuspended in 3 mL/(g of cell pellet) start buffer A containing 0.6 mg/g of lysozyme, and stored at −80 °C. The frozen cell suspension was thawed at room temperature and lysed by sonication. Cells were centrifuged at 18000 g at 4 °C for 30 min, and the supernatant containing the crude extract was filtered through a 0.22 μm filter to remove any particles. Purification was done by affinity chromatography using FPLC. The targeted protein eluted at 100 mM of imidazole was buffer exchanged against 50 mM Tris-HCl, pH 7.4 and concentrated using an
Amicon-Ultra (10 kDa cut-off). The purified protein was stored in 15% glycerol, aliquoted and stored at -80 °C.

3.4.4 General protocol for tandem reactions of 10-undecenoic acid

All reactions were set up and performed in air without prior purification of the solvents in 10-mL headspace crimp cap vials (Agilent Technologies). The aqueous phase (2 mL total volume), contained: 9 µM P450 BM3, 0.4-2 U/mL PTDH, 600 U/mL catalase, 0.2 mM NADP+, and 100 mM sodium phosphite, 50 mM Tris-HCl, pH 7.4. The metathesis catalyst was prepared as a 30 mg/mL stock in dichloromethane. The isooctane organic phase (500 µL total volume) contained 12.5 mM-100 mM 10-undecenoic acid, the appropriate equivalence of trans-3-hexene, and 20 mM eicosane as internal standard. The olefin metathesis catalyst was added to 2.5 mol% to start the reaction. Reactions were sealed to prevent the evaporation of trans-3-hexene and shaken at 180 rpm on a plate shaker (Tallboys, Thorofare, NJ). At the end of the reactions, the aqueous phase was acidified with 1N HCl, the metathesis catalyst was deactivated using 100-fold ethyl vinyl ether and extracted with 6 mL ethyl acetate. The organic phase was dried over magnesium sulfate and dried under vacuum. The residue was resuspended in 300 µL ethyl acetate. Finally, 100 µL of the mixture was derivatized with the addition of 50 µL methanol and 50 µL TMS-diazomethane and ran on GC-MS. The GC-MS program was as follows: 80 °C for 2 min, 20 °C/min to 310 °C, hold for 2 min.
3.4.5 Derivatization of 9-(3-ethyloxiran-2-yl) nonanoic acid methyl ester with BSTFA + 1% TMCS

The extracted tandem reaction previously derivatized with TMS-diazomethane was dried once more to evaporate hexane and methanol. To the residue was added 100 µL of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) containing 1% TMCS (trimethylchlorosilane), followed by incubation at 80 °C for 30 minutes.

3.4.6 Preparation of (E)-tridec-10-enoic acid

To a 10 mL glass vial: 184 mg (1.0 mmol) 10-undecenoic acid, 841 mg (10.0 mmol) trans-3-hexene, 18 mg Hoveyda-GrubbsII (3.0 mol %) were mixed in 4 mL dichloromethane. The solution was stirred at room temperature for 2 h, then the mixture was applied to silica gel column chromatography for separation directly with an eluent 4:1 hexane: ethyl acetate. The yield was 90%. ¹H NMR: 5.41-5.30 (m, 2H), 2.36-2.20 (t, 2H), 2.05-1.89 (m, 4H), 1.63-1.50 (m, 2H), 1.40-1.23 (m, 10H), 1.00-0.81 (t, 3H); ¹³C NMR: 205.9, 132.2, 129.7, 33.8, 32.8, 30.0, 29.9, 29.8, 29.0, 28.8, 25.8, 25.2, 14.0.

3.4.7 Preparation of 9-(3-ethyloxiran-2-yl) nonanoic acid

10 mL Tris-HCl buffer (50 mM, pH=7.5) containing 700 U/mL catalase, 3 µM P450 BM3 and 8 mg (0.038 mmole) (E)-tridec-10-enoic acid was added into a 10 mL glass vial, then 31 mg (0.038 mmole) NADPH was added into the mixture to initiate the reaction. After shaking at 200 rpm at room temperature for 12 h, the reaction was quenched by addition of 5 mL of 0.2N HCl aqueous solution. The mixture was extracted with ethyl acetate three times, and the combined organic phase was dried under anhydrous Na₂SO₄. The solvent was evaporated from the resulting
solution under vacuum. The crude oil was purified by silica gel column chromatography, eluting with hexane: ethyl acetate 4:1. The yield was 23%. $^1$H NMR: 3.97-3.74 (m, 1H), 3.65-3.48 (m, 1H), 2.34-2.22 (t, 2H), 2.11-1.85 (m, 2H), 1.78-1.51 (m, 4H), 1.50-1.20 (m, 10H), 1.08-0.87 (m, 3H); $^{13}$C NMR: 205.9, 68.4, 58.5, 33.8, 32.0, 30.1, 29.8, 29.5, 29.3, 29.1, 26.9, 25.3, 11.1.

3.4.8 General protocol for tandem reactions of 4-butylenoxybenzoic acid (5)

All reactions were set up and performed in air without prior purification of the solvents in 10-mL headspace crimp cap vials (Agilent Technologies). The aqueous phase (2 mL total volume), contained: 9 µM P450 BM3, 2 U/mL PTDH, 600 U/mL catalase, 0.2 mM NADP$, and 100 mM sodium phosphite, 50 mM Tris-HCl, pH 7.4. The metathesis catalyst was prepared as a 30 mg/mL stock in dichloromethane. 4-butylenoxybenzoic acid was dissolved in dioctyl phthalate with mild heating to 30 mM. In a separate vial, a 42 mM stock of trans-3-hexene was prepared in dioctyl phthalate. Equal volume of each stock solution was mixed to obtain 15 mM of 5 and 1.4 equiv. of trans-3-hexene to a final volume of 500 µL. The olefin metathesis catalyst was added to 5 mol% to start the reaction. Reactions were sealed to prevent the evaporation of trans-3-hexene and shaken at 180 rpm on a plate shaker (Tallboys, Thorofare, NJ). At the end of the reactions, the aqueous phase was acidified with 1N HCl, the metathesis catalyst was deactivated using 100-fold ethyl vinyl ether and extracted with 6 mL ethyl acetate. The organic phase was dried over magnesium sulfate and dried under vacuum. The residue was resuspended in 300 µL ethyl acetate. Finally, 100 µL of the mixture was derivatized with the addition of 50 µL methanol and 50 µL TMS-diazomethane. Prior to running on HPLC, the mixture was dissolved 10 fold in acetonitrile. A Zorbax C-18 column was used to run the samples. The HPLC program
was as follows: 40% acetonitrile (3 min), 40-100% acetonitrile (15 min), 100% acetonitrile (5 min), 40% acetonitrile (4 min).

3.4.9 Preparation of 4-hexenyloxybenzoic acid methyl ester

To a 10 mL glass vial: 100 mg (0.5 mmol) 4-(pent-4-enyloxy) benzoic acid, 125 mg (1.5 mmol) trans-3-hexene, 6.6 mg Hoveyda-GrubbsII (2.0 mol %) were mixed in 3 mL dichloromethane. The solution was stirred at room temperature for 4 h, then 1 mL of the mixture (the extra 2ml was used directly for the next step) was quenched by adding 100 µL MeOH and 600 µL (Trimethylsilyl) diazomethane (2N in hexane), After standing at room temperature for an hour, the mixture was evaporated and applied to silica gel column chromatography for separation directly with an eluent 6:1 hexane: ethyl acetate. The yield was 72%. ¹H NMR: 7.96 (d, 2H,), 6.89 (d, 2H), 5.63-5.56 (m, 1H), 5.48-5.41 (m, H),4.00 (t, 2H), 3.86 (s, 3H), 2.49-2.44 (m,2H), 2.03-1.99 (m, 2H), 0.98 (t, 3H); ¹³C NMR:167.18, 162.97,135.41, 131.71, 124.24, 122.59,114.31, 68.18,52.02, 32.56, 25.87, 13.96.

3.4.10 Preparation of 4-(3-(3-methyloxiran-2-yl) propoxyl) benzoic acid methyl ester (8)

2 mL of the reaction mixture prepared above was evaporated and re-suspended into 200 µL isopropanol for the enzyme reaction. To a 20 mL glass vial, 10 mL Tris-HCl buffer (50 mM, pH=7.5) containing 700 U/mL catalase, 3 μM P450 BM3 and 16 mg (0.075 mmole) 4-hexenyloxybenzoic acid was added, then 62  mg (0.076 mmole) NADPH was added into the mixture to initiate the reaction. After shaking at 200 rpm at room temperature for 12 h, the reaction was quenched by addition of 5 mL of 0.2N HCl aqueous solution. The mixture was extracted with ethyl acetate three times, and the combined organic phase was dried under
anhydrous $\text{Na}_2\text{SO}_4$. The solvent was evaporated from the resulting solution under vacuum. The crude oil was methylated by six equivalent of (trimethylsilyl) diazomethane in the presence of MeOH for an hour at room temperature, then the mixture was evaporated and purified by silica gel column chromatography, eluting with hexane: ethyl acetate 4:1. The yield was 16%. $^1$H NMR: 7.99 (d, 2H), 7.25(d, 2H), 4.15-4.12 (m, 2H), 3.87 (s, 1H), 2.91-2.88 (m, 1H), 2.76-2.74 (m, 1H), 2.16-2.08(m,1H),1.96-1.91 (m, 1H), 1.60-1.54(m, 2H), 1.00-0.84 (m, 3H); $^{13}$C NMR: 166.7, 162.4, 131.5, 122.6, 114.0, 64.8, 60.0, 55.4, 51.8, 31.9, 25.0, 9.8
3.5 References


### 3.6 Tables

Table 3.1 Cross metathesis of methyl oleate with *trans*-3-hexene in DCM in air

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst</th>
<th>8-dodecenoic acid methyl ester [%][a]</th>
<th>9-dodecane Conversion [%][a]</th>
<th>Selectivity[b]</th>
<th>Ester product (E/Z)[c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1</td>
<td>42.8</td>
<td>17.9</td>
<td>85.5</td>
<td>60.7</td>
</tr>
<tr>
<td>2</td>
<td>C2</td>
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<td>93.2</td>
<td>59.4</td>
</tr>
<tr>
<td>3</td>
<td>C3</td>
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<td>87.0</td>
<td>47.4</td>
</tr>
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<td>4</td>
<td>C4</td>
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<td>15.3</td>
<td>97.5</td>
<td>50.6</td>
</tr>
<tr>
<td>5</td>
<td>C5</td>
<td>19</td>
<td>11.0</td>
<td>90.1</td>
<td>30.1</td>
</tr>
</tbody>
</table>

[a] Estimated by GC-MS from area ratio with control reaction with no metathesis catalyst. Decane was added as internal standard.  
[b] Selectivity = [(mmoles 8-dodecenoic acid methyl ester + mmoles 9-dodecene)/mmoles of methyl oleate converted]  
[c] Estimated by GC-MS. All catalysts are known to be *E*-selective. *E/Z* ratio increases steadily.
Table 3.2 Kinetics and selectivity of P450 BM3 against 10-undecenoic acid and tridecanoic acid

<table>
<thead>
<tr>
<th></th>
<th>10-undecenoic acid</th>
<th>tridecanoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N) (nmol NADPH/nmolP450^{-1}.min^{-1^a})</td>
<td>580</td>
<td>1890</td>
</tr>
<tr>
<td>1:1 mixture selectivity (percent conversion){(^b,c})</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>Product</td>
<td>terminal epoxide</td>
<td>(\omega-1-4-OH (82% \sim 2,3))</td>
</tr>
</tbody>
</table>

\(^a\)0.5 mM substrate, 0.1 \(\mu\)M P450, 0.5 mM NADPH, 50 mM Tris-HCl pH 7.5, 1h
\(^b\)0.5 mM substrate, 0.2 \(\mu\)M P450, 1.5 mM NADPH, 50 mM Tris-HCl pH 7.5, 1h
\(^c\)GC-MS, substrate depletion compared to control with no enzyme
Table 3.3 Tandem metathesis-oxidation involving 5-hexenoic acid and *trans*-7-tetradecene

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acid substrate</th>
<th>Alkene Equiv</th>
<th>C12-5-ene[%]</th>
<th>Solvent</th>
<th>P450</th>
<th>C12-OH[%]</th>
<th>SM-C6[%]</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.210 mmol (0.10M)</td>
<td>1.0</td>
<td>33.0^a</td>
<td>isoctane: buffer (1:2)</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>5h</td>
</tr>
<tr>
<td>2</td>
<td>0.17 mmol (0.17 M)</td>
<td>1.0</td>
<td>3^a</td>
<td>isoctane: buffer (1:5)</td>
<td>10 nmol</td>
<td>24.7^a</td>
<td>11.7^a</td>
<td>48h</td>
</tr>
<tr>
<td>3</td>
<td>0.05 mmol</td>
<td>5.0</td>
<td>60</td>
<td>neat^b</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>5h</td>
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<td>4</td>
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<td>5.0</td>
<td>7^c</td>
<td>neat</td>
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<td>25.1^c</td>
<td>25.0^c</td>
<td>24h</td>
</tr>
<tr>
<td>5^d</td>
<td>0.05 mmol</td>
<td>5.0</td>
<td>0.03 mmol</td>
<td>neat</td>
<td>10 nmol</td>
<td>20.5</td>
<td>24h</td>
<td></td>
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</tbody>
</table>

^a All reactions performed in air. ^b Isolated by flash chromatography. ^c 7-tetradecene serves as organic phase, metathesis catalyst is insoluble. ^d GC-MS using authentic standards. ^dNo metathesis catalyst added.
Table 3.4 Tandem olefin cross metathesis-epoxidation of 10-undecenoic acid with trans-3-hexene.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reaction</th>
<th>Loading 1 (mM)</th>
<th>Equiv. 2</th>
<th>CM&lt;sup&gt;b&lt;/sup&gt; (mM)</th>
<th>SM&lt;sup&lt;b&gt; (mM)</th>
<th>3&lt;sup&gt;b (mM)</th>
<th>Yield of 3 [%]</th>
<th>Fold increase&lt;sup&gt;c</th>
<th>TTN&lt;sup&gt;d</th>
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<tr>
<td>1a</td>
<td>Tandem</td>
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<td>1.5</td>
<td>6.0</td>
<td>25.4</td>
<td>17.4</td>
<td>35.0</td>
<td>1.8</td>
<td>1930</td>
</tr>
<tr>
<td>1b</td>
<td>Metathesis</td>
<td>50</td>
<td>1.5</td>
<td>12.7</td>
<td>35.3</td>
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<td>3.3</td>
<td>8.6</td>
<td>12.0</td>
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<sup>a</sup>/2 v/v isooctane: buffer, 20 mM eicosane as internal standard, RT, 180 rpm, 12h. <sup>b</sup>CM = cross metathesis. Determined by GC-MS using synthesized authentic standard. <sup>c</sup>SM = self-metathesis. Determined by GC-MS. <sup>d</sup>[(CM prod. in tandem + 3)/CM prod. in metathesis]. <sup>e</sup>[mmol 3/mmol P450]
Table 3.5 Tandem olefin cross metathesis-epoxidation of 4-butyloxybenzoic acid with trans-3-hexene

![Chemical Structures]

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<th>Equiv.</th>
<th>CM&lt;sup&gt;b&lt;/sup&gt; (mM)</th>
<th>7&lt;sup&gt;c&lt;/sup&gt; (mM)</th>
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<sup>a</sup>1/2 v/v dioctylphthalate: buffer, RT, 180 rpm, 24h.  
<sup>b</sup>CM = cross metathesis.  
<sup>c</sup>Determined by HPLC at 254 nm using synthesized authentic standards.  
<sup>d</sup>[CM prod. in tandem + 8]/CM prod. in metathesis].  
<sup>e</sup>[mmol 8/mmol P450].  
<sup>f</sup>Self-metathesis of 5 minimal
3.7 Figures

Figure 3.1 Typical olefin metathesis reactions
Figure 3.2 Hexenolysis of methyl oleate A) Catalysts used in this work B) Cross-metathesis of methyl oleate and trans-3-hexene
Figure 3.3 General scheme of proof-concept for tandem metathesis-oxidation reactions

Tandem olefin chain elongation- fatty acid oxidation by cross metathesis of a carboxylic acid with an alkene

P450 BM3 WT is a natural C_{12}-C_{20} fatty acid ω-hydroxylase
Figure 3.4 Hydroxylation of 5-dodecenoic acids by P450 BM3 WT
Figure 3.5 Tandem metathesis-oxidation reaction schemes
Figure 3.6 GC-MS trace of the tandem reaction of 5-hexenoic acid with trans-7-tetradecene. The GC-MS signal was turned off between 8 and 9.5 min to hide trans-7-tetradecene.
Figure 3.7 Catalyst stability test in isooctane buffer (buffer contained 5 µM P450 BM3 lysate, 0.2 mM NADP⁺)
Figure 3.8 Fed-batch tandem cross-metathesis-epoxidation. A) Reaction scheme. B) Suppression of self-metathesis of 10-undecenoic acid by P450 BM3 in tandem with metathesis catalyst. C) Ratio of 10-undecenoic acid starting material present in the tandem compared to the metathesis alone.
Figure 3.9 Time course of fed-batch olefin metathesis-epoxidation
Figure 3.10 TMCES derivatization of 10,11-epoxytridecanoic acids Peaks at 10.28 and 10.32 min represent the cis isomers, while the ones at 10.6 and 10.68 min are the trans isomers.
Figure 3.11. $^1$H NMR spectrum of 10-tridecenoic acids
Figure 3.12 $^{13}$C NMR spectrum of 10-tridecenoic acids
Figure 3.13 $^1$H NMR spectrum of 10,11-epoxytridecanoic acid
Figure 3.14 $^{13}$C NMR spectrum of 10,11-epoxytridecanoic acid
Figure 3.15 Representative GC-MS traces of tandem reactions in comparison with the metathesis alone and with starting substrate control. It may be better to label those peaks with compound names.
Figure 3.16 $^{13}$C NMR spectrum of 4-hexyloxybenzoic acid
Figure 3.17 $^1$H NMR spectrum of 4-hexenylbenzoic acid
Figure 3.18 $^1$H NMR spectrum of 8
Figure 3.19 $^{13}$C NMR spectrum of 8
Figure 3.20 Representative HPLC traces of the tandem reaction involving 4-butyloxybenzoic acid and trans-3-hexene (red), substrate control (black) and metathesis alone (blue). Compounds were detected at 254 nm.