Design and development of Baeyer-Villiger monooxygenases as reagents in organic synthesis

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In memory of Henri Barthkowiak, Sharon Clouthier, and Hectorine Lavigne

(three people who will always occupy a special place in my heart).

Preface

All chapters, with the exception of Chapters 1 and 3 have been published in peer reviewed journals. Portions of Chapter 2[1,2] have been published in either Angewandte Chemie International Edition or in the Journal of Organic Chemistry. Chapter 4[3] has been published in the Journal of Organic Chemistry, Chapter 5[4] Tetrahedron Asymmetry, and Chapter 6[5] in Journal of Molecular Catalysis B: Enyzmatic. Since each of the chapters contains its own introduction and reference section some repetition may result, but hopefully with negligible redundancy.

[1]. Reetz, M. T.; Brunner, B.; Schneider, T.; Schulz, F.; Clouthier, C. M.; Kayser , M. M. *Angew. Chem. Int. Ed. Engl.* **2004**, *43*, 4075-4078.

[2]. Kayser, M. M.; Clouthier, C. M. *J. Org. Chem.* **2006**, *71*, 8424-8430.

[3]. Clouthier, C. M.; Kayser, M. M. *J. Org. Chem.* **2006**, *71*, 8431-8437.

[4]. Clouthier, C. M.; Kayser, M. M. *Tetrahedron Asym.* **2006,** *17*, 2649-2653.

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Abstract

Directed evolution has been found to be a very useful tool for the targeted improvement of enzymatic properties. The application of directed evolution for the improvement of cyclohexanone monooxygenase (CHMO) selectivity for a specific substrate target (4-hydroxycyclohexanone) identified a number of mutant CHMO variants possessing enhanced enantioselectivity. Four of the best performing mutants of CHMO were investigated as catalysts for the oxidation of a variety of 4-substituted and 4, 4-disubstituted cyclohexanones. Overall several excellent catalyst matches (mutant/substrate) were identified. In a number of cases a mutant containing a single amino acid exchange (Phe432Ser) was found to be the most robust and selective enzyme catalyst. In several cases the Phe432Ser mutant was found to outperform wild type CHMO (WT-CHMO) in terms of conversion, efficiency and selectivity.

Based on the lessons learned from the directed evolutionary studies on CHMO, a rational design approach was used to improve the selectivity of another monooxygenase enzyme, cyclopentanone monooxygenase (CPMO). Through the application of a novel mutagenesis strategy, Complete Active Site Saturation Test (CAST), several successful mutants with enhanced enantioselectivities were identified. For example, the mutant catalyzed oxidation of 4-methylcyclohexanone gave the corresponding lactone with 92% enantiomeric excess (ee) compared to 46% ee achieved with wild type CPMO. This methodology provided a relatively inexpensive and rapid way to obtain mutant enzymes with the desired characteristics.

Six of the mutant CPMO variants obtained through the application of the CAST methodology were investigated further for the oxidation of a series of cyclohexanone

substrates substituted at the 4-position. Several mutant enzymes with improved enantioselectivities were identified. In general, the CPMO mutant variants involving mutation at the Phe156Gly157 position (library B) were found to be the most robust and selective of the CPMO clones. The successful improvement of CPMO illustrates how a family of cyclohexanone substrates may be used to explore the putative active sites of selected Baeyer-Villiger monooxygenases (BVMOs) and can aid in potential identification of mutational "hot-spots" within an active site.

To explore the effects of reaction conditions on the enzyme catalyzed oxidation of selected 4-substituted cyclohexanones, biooxidations using WT-CHMO, WT-CPMO, and selected CHMO and CPMO mutants under both growing and non-growing cell conditions were studied. The surprising result of this study was that several substrates that were known to give negative results (non-acceptance) under growing conditions afforded excellent conversions in the transformations under non-growing conditions.

Acknowledgements

I would like to express my deepest gratitude and appreciation to my advisor, Dr. Margaret M. Kayser, for her never-ending guidance and encouragement throughout my doctoral work. Her enthusiasm for chemical research and words of encouragement were a constant source of inspiration to me.

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(a.k.a. "Pub talks") cheering each other on and suggesting some useful ideas made my time here in Saint John more bearable.

On a more personal note, I am deeply thankful to my fiancée Heather M^{c}Grath and my daughter Megan Clouthier for all their loving support and encouragement throughout this entire work. Heather and Megan unselfishly provided never-ending patience and love. I am also deeply grateful to my loving parents, Michael and Evelyn, my sister Jennifer, and my extended family Gary, Rose, Ruth and Tom, for all their encouragement and support.

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List of Abbreviations

A	adenine
Ac	acetyl
Ar	aryl
BINOL	1,1'-bi-2-napthol
n-bu	(normal) butyl
t-Bu	*tert*-butyl
BV	Baeyer-Villiger
BVMO	Baeyer-Villiger monooxygenase
C	cytoseine
CALB	*Candida Antarctica* lipase
CAST	Complete Active site Saturation Test
CHMO	cyclohexanone monooxygenase
CPMO	cyclopentanone monooxygenase
d	days
DE	directed evolution
DFT	density functional theory
DNA	deoxyribonucleic acid
E	Enantiomeric ratio
E. coli	Escherichia coli
ee	enantiomeric excess
Et	ethyl
FAD	flavin adenine dinucleotide

FID	flame ionization detector
g	gram
G	guanine
GC	gas chromatography
h	hour(s)
HAPMO	Hydroxyacetophenone monooxygenase
HF	Hartree-Fock
HPLC	high performance liquid chromatography
Hz	hertz
IPTG	iso-propylthio-β-D-galactoside
IR	infrared spectroscopy, FT-IR Fourier transform-IR
J	coupling constant
L	liter
LB	Luria-Bertani
μ	micro
m-CPBA	*m*-chloroperoxybenzoic acid
Me	methyl
MMT	montmorillonite
mg	milligram
mM	millimolar
MOE	Molecular operating environment
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP

NMR	nuclear magnetic resonance
OD	optical density
PAMO	phenylacetone monooxygenase
ppm	parts per million
Ph	phenyl
PCR	polymerase chain reaction
n-Pr	(normal) propyl
i-Pr	*iso*-propyl
Py	pyridine
rpm	rotations per minute
T	thyamine
THF	tetrahydrofuran
TLC	thin layer chromotography
TFPA	trifluoroperacetic acid
UV	ultraviolet
UFF	united force field
WT	wild type

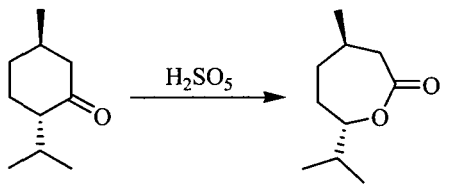
Chapter 1

Introduction

1-1 The Baeyer-Villiger reaction

The first reactions involving the conversion of acyclic or cyclic ketones into their corresponding esters or lactones were discovered by Adolf von Baeyer and Victor Villiger in 1899 while working on the ring cleavage of cyclic ketones.[1] Their early work involved the treatment of cyclic ketones such as menthone, carvomenthone, and camphor with monopersulfuric acid, the most powerful oxidizing agent known at the time (**Scheme 1-1**).[2]

Scheme 1-1



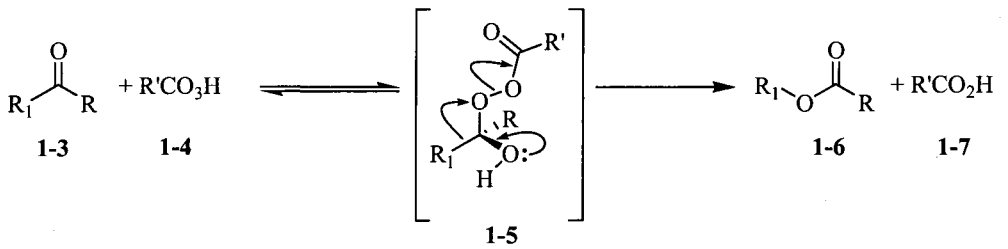
1-1 **1-2**

Since this seminal work, the Baeyer-Villiger (BV) oxidation has become a key component in the synthetic repertoire of organic chemists as it represents an important method for the cleavage of select carbon-carbon bonds in an oxygen insertion process. Some factors which have contributed to the overall success of the BV oxidation include its broad functional group tolerance and its generally predictable regiochemistry. Continued research on the BV oxidation has shown that a wide range of peroxy acids including 3-chloroperoxybenzoic acid, peroxytrifluoroacetic acid, and performic acid can also be utilized as the oxidant in this reaction.[2,3]

1-1-1 Mechanism of the Baeyer-Villiger oxidation

Mechanistically, the BV oxidation reaction is generally accepted to proceed *via* a simple two-step reaction. In the first step of the reaction a peroxy acid species attacks the carbonyl carbon atom of the ketone resulting in the formation of a tetrahedral intermediate termed the Criegee intermediate.[4] The second step involves intramolecular migration of the carbon-carbon bond which is adjacent to the perester oxygen of the intermediate resulting in the reformation of the carbonyl group and subsequent cleavage of the O-O bond (**Scheme 1-2**).[4] Generally, the most substituted (electron rich group) migrates with retention of configuration.

Scheme 1-2



1-3 **1-4** **1-5** **1-6** **1-7**

Two important pre-requisites for successful alkyl group migration and subsequent loss of the carboxylic acid by-product are: i) – the migrating carbon-carbon bond must be in an antiperiplanar position relative to the O-O bond of the peroxy acid; ii) – the same migrating carbon-carbon bond must also be antiperiplanar to the lone pair of the hydroxyl group. The aforementioned criteria for the BV oxidation reaction are known as the primary and secondary stereoelectronic effects, respectively (see **Figure 1-1**).[5] In fact,

the high degree of predictability in terms of the migratory aptitude which is associated with this reaction is heavily linked to these stereoelectronic effects.[6,7]

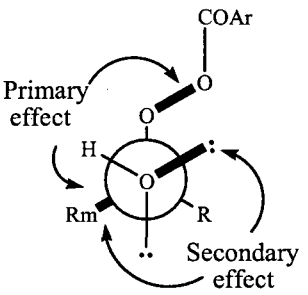


Figure 1-1: *The primary and secondary stereoelectronic effects associated with the Baeyer-Villiger reaction.*

A number of studies, both experimental and theoretical, have been performed to illustrate the primary stereoelectronic effect on migratory aptitude in the BV reaction. The most recent and straightforward experimental demonstration of stereoelectronic effects was performed by Crudden and coworkers based on the BV oxidation of α-fluoro-cyclohexanones.[8]

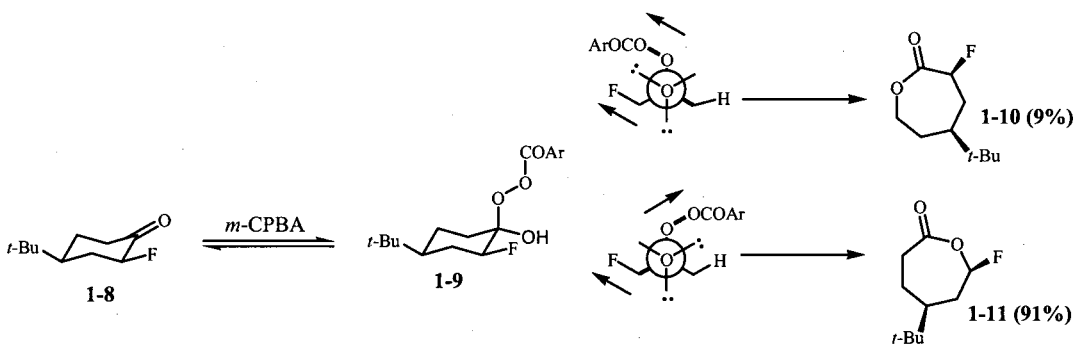


Figure 1-2: *Stereoelectronic analysis of Criegee intermediate* **1-9** *resulting from axial attack of a peracid on ketone* **1-8**.[8]

In order to explain the unusual behaviour (since the less electron-rich bond migrates preferentially) of ketone **1-8** when exposed to BV oxidation by peracid, the authors invoked stereoelectronic effects. As seen in **Figure 1-2**, migration occurs primarily from the conformation where the O-O bond of the peroxyester is oriented in such a way as to minimize the overall effects associated with dipoles. The authors therefore clearly illustrated that stereoelectronic effects are more important than intrinsic migratory aptitude.[8]

Despite the relative importance of the BV reaction, relatively few theoretical studies have been published on this topic compared to other reactions of similar importance. Many of the theoretical treatments which have appeared tend to focus their attention on modeling the second transition state relating to bond migration with little or no interest being paid to the first transition state associated with tetrahedral intermediate formation.[9-11]

Recently, the first comprehensive theoretical treatment of the BV oxidation reaction was published by Grein and co-workers in which the authors combine a detailed computational and experimental treatment for a series of model ketones oxidized with performic acid.[12] Extensive calculations were carried out using both *ab initio* and density functional theory (DFT) methods with extended basis sets to model the transition states associated with BV oxidation. Grein and co-workers identified the most plausible models for the first (**1-14**) and second transition (**1-16**) states for the reaction catalyzed with performic acid both with and without an acid catalyst (illustrated in **Figure 1-3**).[12]

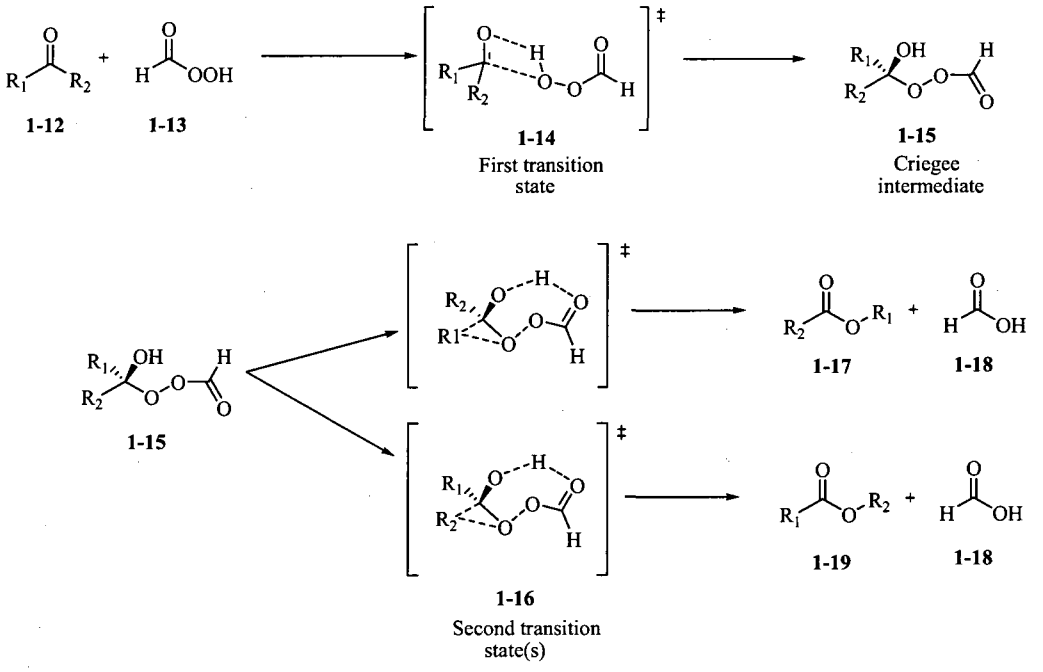


Figure 1-3: *Modeled transition states obtained for the uncatalyzed Baeyer-Villiger reactions of an acyclic ketone with performic acid* (R_1 and R_2 = methyl).[12]

Calculations showed that the first transition state relating to Criegee intermediate formation (**1-14**) was rate-determining for the model reactions in both the catalyzed and uncatalyzed reactions. In the case of fluoropentanone *versus* fluoroacetone, the authors were able to predict accurately the energetic differences (within 0.2 kcal/mol of experimental) for the second transition state analogues to **1-16** which lead to migration of the fluorinated subtituent *versus* the alkyl subtituent.[12] While this study filled a much needed gap relating to the comprehensive theoretical treatment of the BV reaction, the authors emphasized the fact that much work remained.

1-1-2 Chemical catalysis of the Baeyer-Villiger oxidation

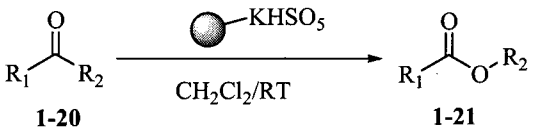
While the conventional method of performing the Baeyer-Villiger oxidation reaction involving the use of peracids such as *m*-chloroperoxybenzoic acid (*m*-CPBA) and trifluoroperacetic acid (TFPA) can be found throughout the literature the classical procedure suffers, from an environmental point of view, from two major drawbacks; the use of harmful peracids and the requirement for halogenated solvents. The ever increasing emphasis on the development of "green chemistry" methods has lead to the development of a number of novel methods to perform the BV oxidation reaction.[13]

The development of aqueous hydrogen peroxide (H_2O_2) as a green Baeyer-Villiger oxidant has been the focus of a significant amount of effort owing to the fact that H_2O_2 is a relatively safe, inexpensive, and relatively "clean" oxidant.[3,13] Unfortunately, because of the poor performance of hydrogen peroxide as a stand alone reagent for BV oxidation, development of a catalyst system capable of either activating H_2O_2 or the target ketone is necessary. Many recent studies have centered on the development of solid supported catalysts working in conjunction with H_2O_2 in order to catalyze the BV reaction.[3] To date many of the most successful approaches towards the application of aqueous peroxide entail the use of a solid catalyst, typically a modified clay or zeolite, coupled with H_2O_2.[14] This catalytic technique involves the initial activation of the carbonyl group by the solid catalyst followed by reaction with the H_2O_2 oxidant. Since the initial inspirational work of Corma and coworkers, many other studies employing modified clay and zeolitic materials have been reported.[15,16] Most recently, this idea was successfully applied using a tin modified montmorillonite (MMT) clay which when

coupled with H_2O_2 proved an excellent catalyst for the BV oxidation of a number of ketones with good overall yields.[16]

Also, work has been done in the area of silica supported BV oxidation catalysis where potassium peroxomonosulfate bound to acidic silica gel ($SiO_2 \cdot KHSO_5$) has been used in the BV oxidation of several ketones giving good to excellent yields (**Scheme 1-3**).[17,18] This catalyst system was used under mild conditions with dichloromethane as the solvent. In order to make the process "greener", Asenio and coworkers tested the same reaction, illustrated in **Scheme 1-3**, using supercritical CO_2 as the solvent under continuous-flow conditions with equal success thus eliminating the requirement for a chlorinated solvent.[18]

Scheme 1-3

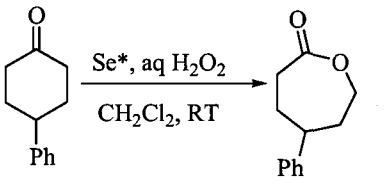


1-20 **1-21**

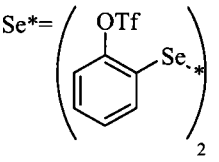
R_1 and R_2 = alkyl, cycloalkyl, and aryl

Recently, Ichikawa and coworkers have reported the application of organoselenium based compounds as catalysts for the BV oxidation reaction (**Scheme 1-4**).[19] The diselenide (**1-24**) reacts with aqueous H_2O_2 to generate selenic acid (not shown) which subsequently carries out the BV oxidation reaction.

Scheme 1-4



1-22 **1-23** (85% yield)

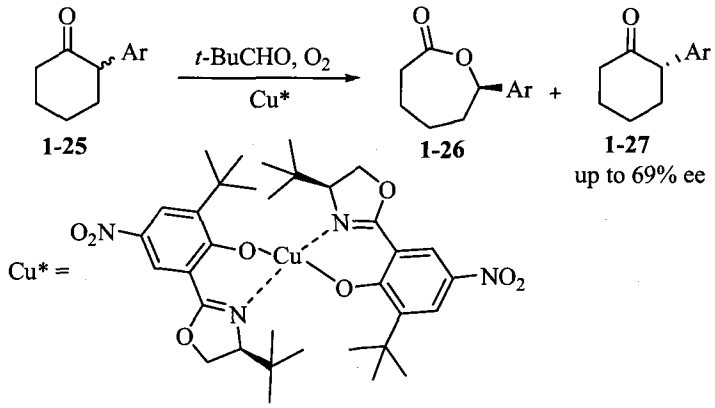


1-24

1-1-3 Asymmetric Baeyer-Villiger reactions

Because lactones, in particular γ-butyrolactones, have been identified as important components of the framework within the core structure of a number of biologically active molecules, much effort has been directed towards the development of an asymmetric variation of the BV reaction.[20-24] The initial attempts of metal catalyzed asymmetric BV oxidation dating back to the early 1990s used chiral copper or platinium catalysts with molecular oxygen or H_2O_2 as the oxidant, respectively, for the kinetic resolution of 2-substituted 5- and 6-membered cyclic ketones (**Scheme 1-5**).[20] Unfortunately, while both catalyst systems were quite active they were rather unselective, thus making them poor asymmetric catalysts.

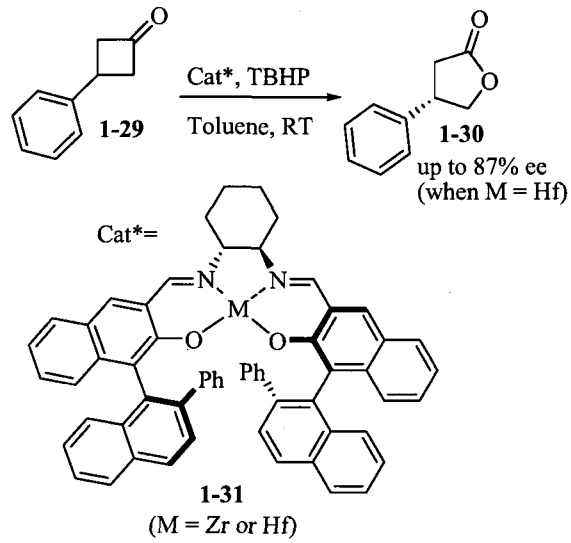
Scheme 1-5



1-28

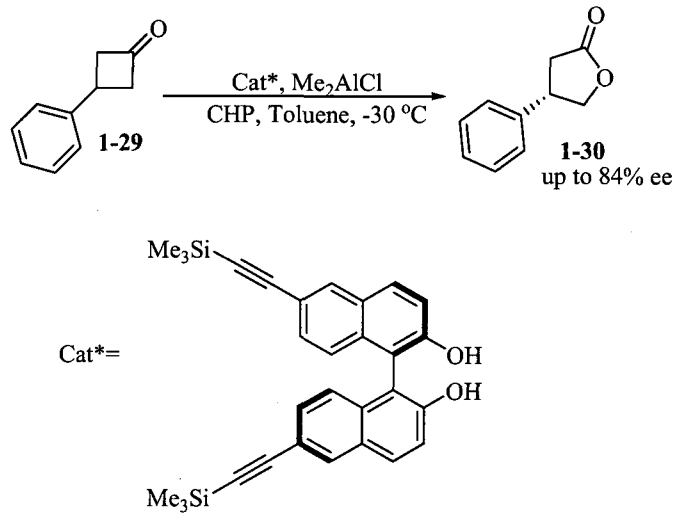
Recent efforts focusing on the development of chiral salen-metal complexes employing zirconium or hafnium metal centers have shown particular promise with enantioselectivities of up to 87% being reported for a model oxidation involving 1-phenylcyclobutantone (**Scheme 1-6**).[23] Bolm and coworkers have also reported the application of modified BINOL-type ligands in combination with aluminum reagents employing cumene hydroperoxide (CHP) as oxidant for the asymmetric BV oxidation (**Scheme 1-7**).[24] In particular the acetylenic substituted BINOL derivative **1-32** proved to be an excellent catalyst capable of converting **1-29** to **1-30** with good enantioselectivities and excellent yield.

Scheme 1-6



1-31
(M = Zr or Hf)

Scheme 1-7



1-32

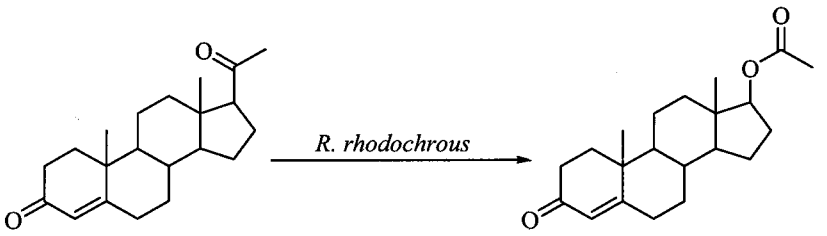
For both the salen derived and BINOL derived catalysts, however, the high selectivities observed were limited to substituted cyclobutanones and rather poor results were obtained for 5- and 6-membered cyclic ketones. Thus, the challenge to develop suitable catalysts with high chemo-, regio-, and enantioselectivities, remains. While chemists still struggle to develop an asymmetric version of the BV oxidation, nature has created a family of enzymes that possess a broad substrate base and which are frequently highly enantioselective catalysts for the "biological" BV reaction.

1-1-4 Enzymatic Baeyer-Villiger Reaction

Humankind has been employing enzymes to perform biocatalytic processes for thousands of years. From the conversion of ethanol to acetic acid using *Acetobacter* to the fermentation of sugars to produce ethanol, humans learned early on how to harness "Mother Nature's" chemical prowess. Given the vast array of organisms and fungi present in nature, it would seem quite possible that a natural biocatalytic option of performing the BV reaction would exist. The first real example of a biological BV oxidation came in the early 1950s when it was discovered that during the incubation of progesterone with *Penicillium* sp. or *Aspergillus flavus,* the corresponding testololactone was formed.[25,26] Later studies showed that this phenomenon was not restricted to steroids but occurred for a large variety of ketones. It was later shown that the BV oxidation reaction commonly occurs during the catabolic degradation of many microoganisms and fungi. Recently, an enzyme which is closely related to that first observed by Freid and Peterson was identified from *Rodococcus rhodochrous.* This

enzyme, called steroid monooxygenase, is able to convert a steroid (**1-33**) to the corresponding ester through BV oxidation (**Scheme 1-8**).[25, 26]

Scheme 1-8



1-33 **1-34**

Since the initial discovery, many microorganisms and fungi have been identified which harbor genes encoding for the expression of monooxygenases as part of their genome. Of all the known Baeyer-Villiger monooxygenase (BVMO) enzymes identified and isolated to date, none has received as much attention as cyclohexanone monooxygenase (CHMO) discovered in the 1970's by Trudgill and co-workers from *Acinetobacter* sp. NCIMB 9871 (E.C. 1.14.13.22).[27] Biochemical studies conducted later on *Acinetobacter* sp. by Trudgill *et al.* found that the CHMO enzyme plays a role in the metabolic pathway of cyclohexanol as outlined in **Figure 1-4**.[27]

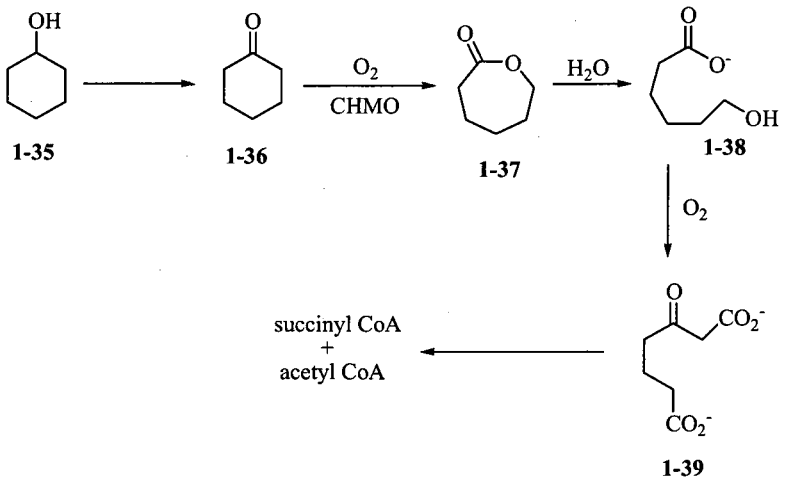


Figure 1-4: *Metabolism of cyclohexanol in* Acinetobacter sp. *NCIMB 9871.*

Owing to its importance as a BV oxidation catalyst, the CHMO enzyme was isolated and purified by Walsh and Chen for gene sequencing and biocatalytic evaluation.[28] Extensive substrate profiling studies have been conducted by a number of groups and it has been demonstrated that CHMO is able to oxidize cyclic ketones to lactones, aromatic ketones to esters, aldehydes to carboxylic acids, boronic acids to alcohols, sulfides into sulfoxides, and selenides into selenoxides (see **Figure 1-5**).[29-32]

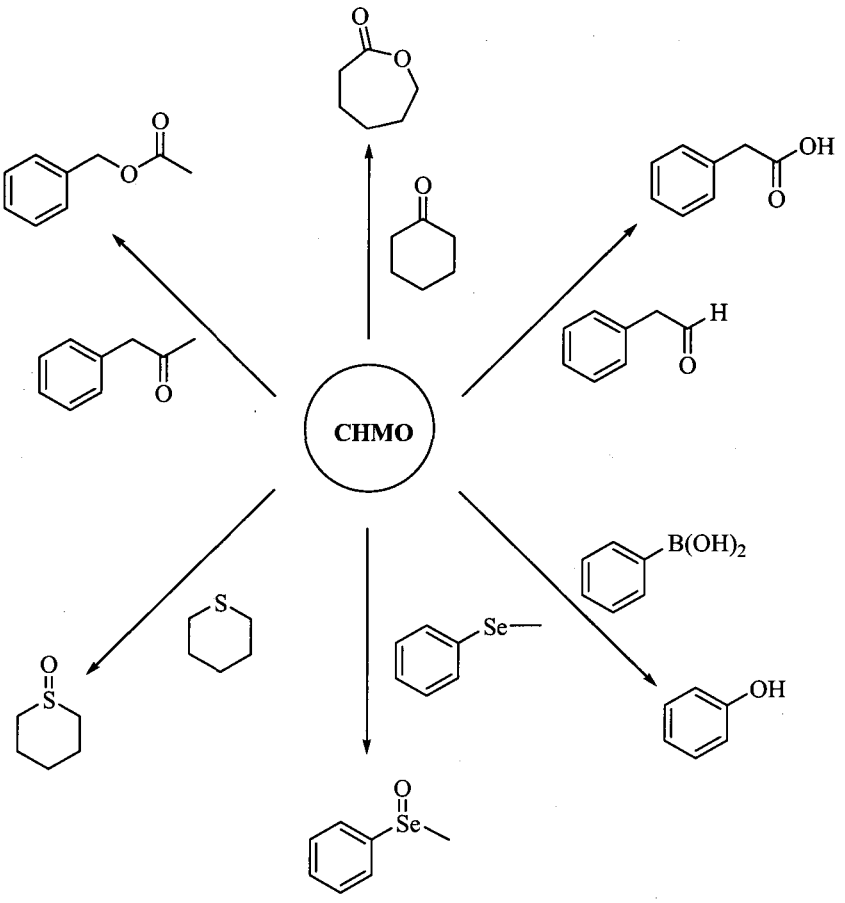


Figure 1-5: *Types of substrates which are accepted by wild type CHMO.*

As part of their studies on CHMO, Walsh and coworkers were the first to describe the mechanism of the BV reaction mediated by CHMO.[33] Through a series of kinetic studies, Walsh was able to determine that a C(4a)-hydroperoxyflavin species was involved in the catalysis and fulfilled the role of the peracid. The mechanism as proposed by Walsh, and subsequently refined by Sheng *et al.*, is illustrated in **Figure 1-6**.[33]

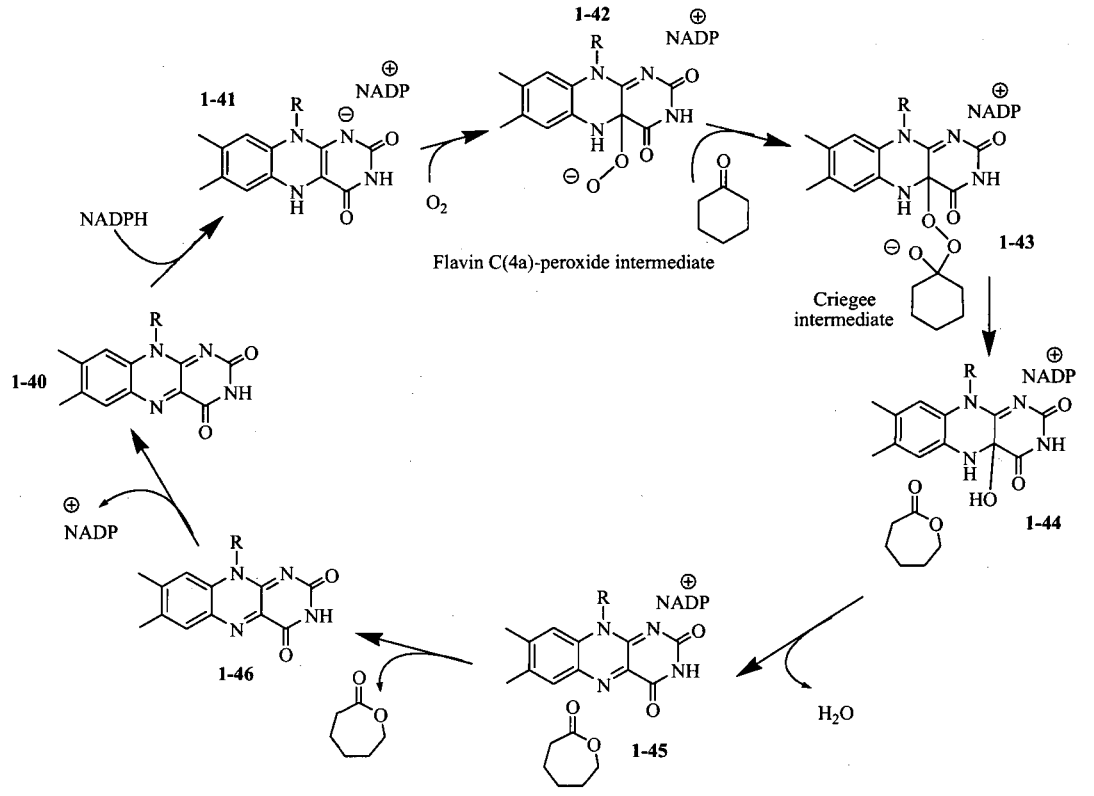


Figure 1-6: *Proposed mechanism of the Baeyer-Villiger oxidation mediated by CHMO* (as adapted from Sheng, *et al.*[33]).

In the initial steps of the catalytic cycle, the oxidized flavin **1-40** is reduced through a hydride transfer from a loosely bound nicotinamide adenine dinucleotide phosphate (NADPH) cofactor. The reduced form of flavin **1-41** is ready for subsequent oxygen binding which is closely followed by a rapid formation of the flavin C(4a)-peroxide **1-42**. Recently, the formation of the peroxyanion species **1-42** has been demonstrated to form upon addition of oxygen.[33] The terminal oxygen anion of **1-42** acts as the nucleophile and adds to the carbonyl group to form the tetrahedral Criegee intermediate **1-43** in a manner similar to chemical BV oxidation. The rearrangement of

the Criegee adduct results in the formation of the product lactone along with a C4a-hydroxy-flavin **1-44** species. Termination of the catalytic cycle occurs through the elimination of water, resulting in reformation of the oxidized flavin **1-40** and release of product as well as the oxidized $NADP^+$ species.[33]

From these mechanistic studies it was concluded that the biological BV reaction possesses the same characteristic requirements as the chemical version for the reaction to occur. Both Walsh and Chen, and Sheng *et al.* found that the flavin group remains tightly bound within the enzyme's active site throughout the entire catalytic cycle, which has important implications in terms of the development of the model(s) of the possible active site for CHMO.[33]

Because of the lack of an X-ray crystal structure for CHMO only indirect active site models based on extensive substrate profiles are currently available. Thus, apart from its natural role in the biodegradation and biosynthetic pathways of microbial systems, CHMO (from *Acinetobacter* sp. NCIMB 9871) has been found to oxidize well over 100 non-natural substrates. Data from these studies allowed for a number of predictions of the polar and spatial requirements of the active site. One of the most accurate of these tentative models is the "cubic space" model of Ottolina and coworkers.[34] This model, illustrated in **Figure 1-7,** applied molecular modeling software to minimize the total energy of a large series of acceptable substrates. These mimimized structures were then superimposed along the C-O peroxide bond to define various binding pockets that emerge from inserting the minimized structures inside a "box" comprised of several cubes.[34]

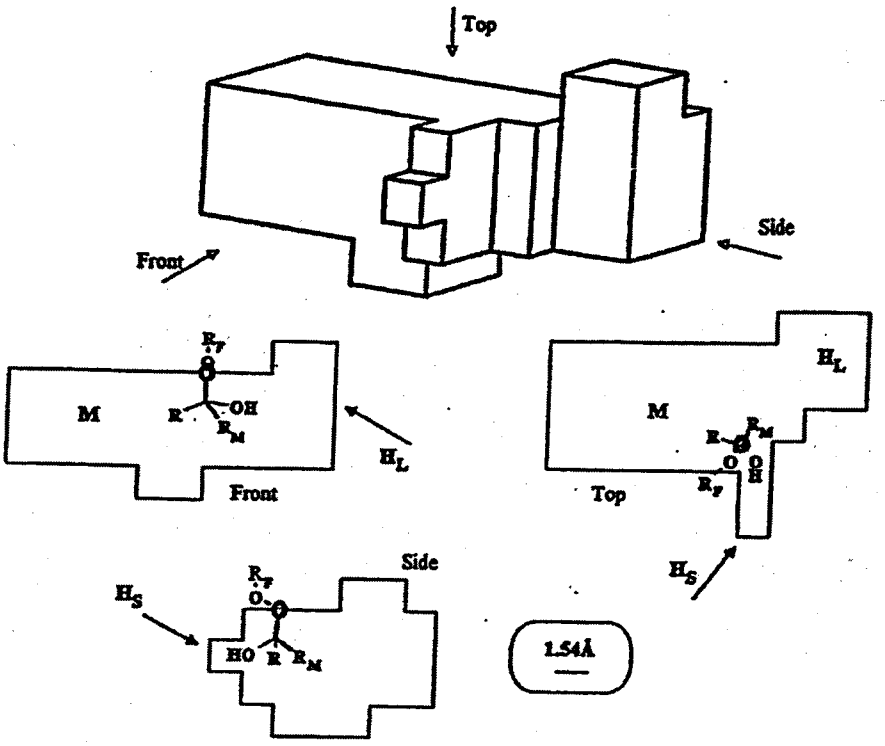


Figure 1-7: *Ottolina's box active site model of CHMO.*[34]

Another model which has been developed is the diamond lattice model.[35] Its principal limitation is that it can be rigorously applied only to systems containing solely sp^3 centres, particularly 6-membered ring ketone substrates. The basic concept of the model is shown in **Figure 1-8**; the carbon-carbon bond which is indicated by the darkened line migrates during fragmentation of the intermediate. The dotted lines represent allowable positions for substituents on the ring. Despite the limitations of the diamond lattice model, it has become a useful method for a rapid evaluation of proposed oxidations by CHMO.

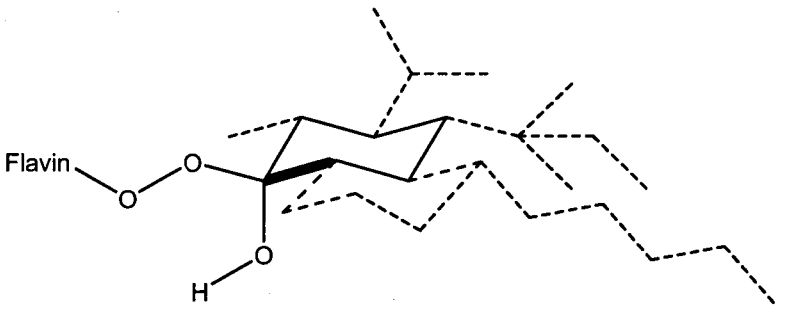


Figure 1-8: *The diamond lattice model of CHMO.*

1-2 Development of enzymes as bioreagents

1-2-1 Development of CHMO as a bioreagent

Although the use of biocatalysts in organic synthesis is increasing and inspite the obvious advantages (such as mild reaction conditions, energy savings, and the green nature of biotransformations) there are challenges in using enzymes as bioreagants in organic synthesis; they include the difficulties in isolation, low stability, cofactor requirements, and narrow substrate base.

While it has been recognized that BVMO enzymes represent a potentially useful route towards the enantiopure lactones, several challenges have hampered their widespread use by organic chemists. For instance, much of the early work involving CHMO was done with either the isolated enzyme or with whole cell parent microorganism. Working with the isolated CHMO enzyme required continuous addition of the expensive NADPH cofactor to maintain catalytic activity making the widespread use of this enzyme prohibitively expensive (see **Figure 1-6**). To complicate the matters,

CHMO is a relatively unstable enzyme and has short half life.[33] Initial attempts by several groups (Taschner, Furstoss, Carrea) involved the use of parent organisms in biotransformations to overcome the problems associated with both the stability of CHMO and its need for cofactors.[35, 36]

Unfortunately, whole-cell catalysis presents other problems which hinder its widespread use. These include the pathogenic nature of the enzyme-producing strain, potential competitive consumption of starting material *via* other expressed enzymes, or the metabolic degradation of product - any one of which can significantly complicate the process. A further point which can complicate the use of whole-cells as direct catalysts is that in some cases the bacterial strain requires the addition of a specific compound to induce the production of the enzyme, a fact which may complicate the final product isolation step. Eventually, to avoid the above problems in using CHMO as biocatalyst, the concept of a designer microorganism was developed.[37-39]

In the recombinant designer organism the production of the desired enzyme is controlled *via* a promoter which acts as an enzyme production switch. Under the influence of a strong promoter, such as IPTG, the production of only the desired enzyme by a microbial host can be increased substantially thus making the target enzyme the dominant protein produced by the organism. This technique has been successfully applied towards the overexpression of CHMO for biocatalysis through the cloning of the CHMO gene into a recombinant strain of *Saccharomyces cerevisiae* (yeast) and *Escherichia coli* (*E. coli*).[38,39] It has been shown that application of these recombinant organisms towards CHMO biocatalysis provides a way to maintain a constant supply of enzyme and cofactor while minimizing the possibility for competitive reactions including

metabolism of the product(s). Such a concept provides organic chemists with little training in biotechnology or microbiology with an access to powerful enzymatic reactions.

1-2-2 Improvement of enzymatic bioreagents

Given the major advances which have been made over the last several decades in the fields of biotechnology and microbiology it is no wonder that the number of enzymatic systems which are commercially available for use by chemists has dramatically increased. One of the key areas in which enzymes continue to play an important role is in the asymmetric synthesis of organic compounds, an area which has dominated a large part of basic organic research over the past three decades. For an enzymatic reaction to be of general use in asymmetric organic chemistry, the enzyme must possess the same general qualities as chemical catalysts. They must be able to transform a number of non-natural substrates in relatively good yield and with a high degree of enantioselectivity. Fortunately, just as is the case of chemical catalysis, biocatalysts can be modified through the application of various biotechnological strategies and procedures to improve certain desirable properties of an enzymatic system. Through the application of a given mutagenic strategy, one or more libraries of mutated enzymes are created and tested to identify those mutant enzyme variants which possess the desired property or function. The strategy which is employed for mutant library generation depends heavily on the knowledge one has about the enzymatic system, as illustrated in **Figure 1-9**.[40] The two main concepts which are currently being employed to improve enzymatic properties are the rational design and non-rational design approaches.

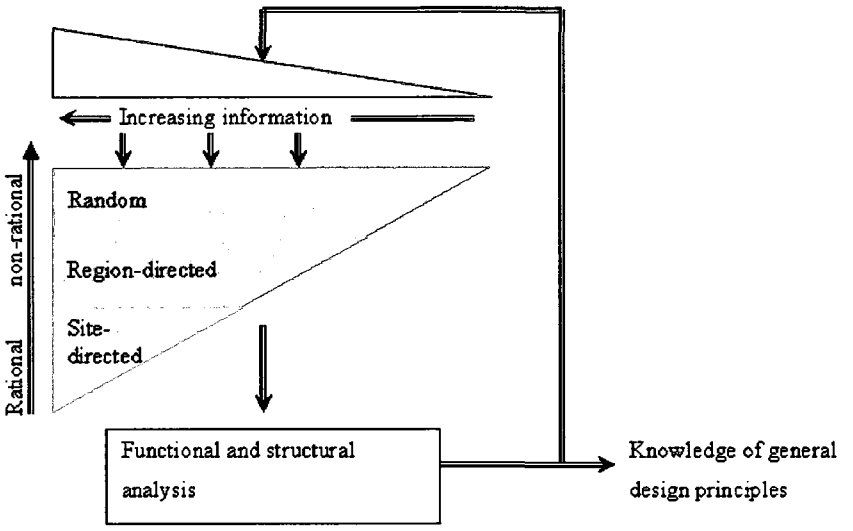


Figure 1-9: *A schematic diagram illustrating the approaches used to the design and engineering of functions into enzymes.*[40]

1-2-2a Rational design

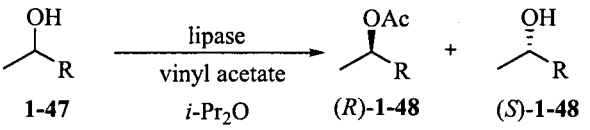
In order to use rational design mutagenesis strategy one must possess some knowledge of the structure of the enzymatic system of interest, either an experimentally determined X-ray or NMR structure or through the application of some homology-based modeling software.[41] This structural information is used in conjunction with existing experimental data to identify the area(s) within the enzyme which may directly influence a given property of interest (selectivity, substrate acceptance). Site-directed or specific mutagenesis can then be used to mutate single or mutiple amino acid positions or "hot regions" which have been identified by the researcher as being potentially significant.[42,43] These targeted amino acid positions can undergo either a specific

targeted amino acid (Phe→Gly) exchange or a diverse range of amino acids can be introduced at the specific position (Phe→19 other amino acids)

Typically to enhance the productivity of the mutagenic process, as well as increase the probability of finding positive mutations which increase the target property, a saturation mutagenesis approach is taken. What differentiates site-specific and saturation mutagenesis from each other is the use of specific versus non-specific degenerate oligonucleotide primers. In saturation mutagenesis, a codon of the form NNN or NNK where N=A, T, G, C and K=G, T is employed to introduce a full diversity (one amino acid is exchanged for the other nineteen possibilities) of mutations at a specific position.[43] In site-specific mutagenesis specific, oligonucleotide primers encoding for a single amino acid exchange are used.

Classically, the rational design approach is used to explain a known behaviour of an enzymatic system or family at the molecular level through the mutation of specific amino acid regions, or to aid in the mechanistic study of a family or class of enzyme. Rational design has also been used successfully to enhance or control the enantioselectivity of enzymes with excellent results, as summarized in a recent paper by Reetz and coworkers.[44] For example, Ema *et al.* reported the rational control of enantioselectivity for a *Burkholderia cepacia* lipase based on the mechanism of the enzyme.[45] Using focused mutagenesis the authors improved the enantioselectivity of the aforementioned lipase towards the kinetic resolution of secondary alcohols (**Scheme 1-9**).

Scheme 1-9



1-47 (*R*)-**1-48** (*S*)-**1-48**

a: R = Phenyl **b**: R = cyclohexyl

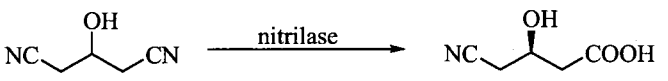
Thus, based on the known mechanism of action for *Burkholderia cepacia* lipase, a single site isoleucine 287 was targeted for exchange. One lipase variant, the Ile287Phe mutant, was found to result in a 2- to 3-fold increase in the E-value* for the the substrates **1-47a** and **1-47b**, respectively.[45]

The selectivity of *Candida Antarctica* lipase B (CALB), one of the most robust and efficient hydrolase catalysts showing high regio- and enantioselectivity, was also modified by a rational redesign strategy.[46] Typically, the CALB enzyme is strongly *R*-selective towards secondary alcohols, a fact which has been linked to the different binding modes of the alcohol enantiomers within the active site pocket.[47] By targeted mutation of a specific region in the stereospecificity pocket, the Trp104 residue, the authors were able to create an *S*-selective variant of the CALB enzyme. While the actual enantioselectivity of the Trp104Ala CALB mutant was poor (E=6.6 versus E>200 for WT) the fact that a single amino acid exchange resulted in a profound shift in selectivity is noteworthy.[46]

As part of a development strategy focused on finding an alternative synthesis for the cholesterol-lowering drug Lipitor®, DeSantis *et al.* examined the potential of employing nitrilase catalyzed desymmetrization of **1-49** to yield the (*R*)-4-cyano-3-

hydroxybutyric acid (*R*)-**1-50,** the ester of which is an intermediate in the synthesis of Lipitor® (**Scheme 1-10**).[48] Saturation mutagenesis at various positions in the nitrilase gene resulted in the identification of a mutant possessing high enantioselectivity, as well as high volumetric productivity (619 $g \cdot L^{-1} \cdot d^{-1}$).[48]

Scheme 1-10



1-49 (*R*)-**1-50**

1-2-2b Non-rational design: Directed evolution

Unlike the rational design approach discussed earlier the non-rational design, or directed evolutionary approach as it has become known, requires no knowledge of the structure or mechanism of the enzyme; only knowledge of the gene sequence is required. The directed evolution approach to enzyme engineering was conceived roughly two decades ago having been inspired by natural evolution.[51, 52] Through the application of a variety of genetic mutational techniques scientists have learned to accelerate the evolutionary process. What really gives the concept its evolutionary spin is the coupling gene mutation, enzyme expression and screening where the most promising mutants identified *via* screening are used as starting points for further rounds of genetic mutation, expression, and screening. Thus, in this way an evolutionary pressure is exerted on the enzymatic system until one mutant possessing all of the desired characteristics is identified.[49-54]

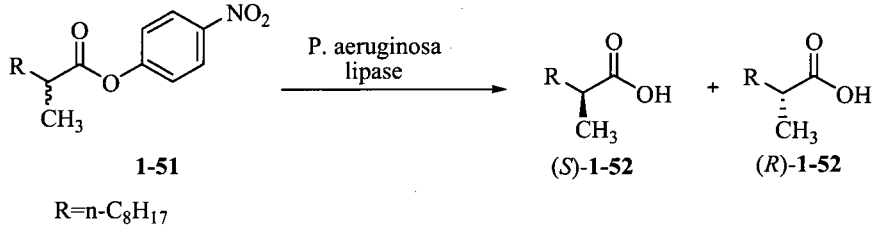
In order to improve an enzyme's target function through directed evolution, several requirements should be considered before the actual work it undertaken.[55] Initially the targeted enzyme function for improvement must be physically and biologically feasible or possible through the application of directed evolution. For example, a hydrolase enzyme cannot be converted into a reductase enzyme by subjecting it to random mutagenesis. The evolutionary libraries which are created must be complex enough to cover a broad region of the sequence space to capture any of the rare beneficial mutations. Lastly, a screening and/or selection strategy which is sensitive enough to capture even minute changes in the desired function of the enzyme must be developed.[55]

Many practical methods to accomplish directed evolution have been reported in the literature, some of which include error-prone polymerase chain reaction (ep-PCR), combinatorial site-saturation mutagenesis, and DNA shuffing.[56-61] The most widely used method for the enhancement of enzymatic enantioselectivities is the ep-PCR method.[56] The ep-PCR method is a modified standard PCR method where the standard PCR conditions have been altered to trigger the introduction of errors in the amplified gene.[57] Typically, the PCR reaction can be made more error-prone through a variety of techniques, such as increasing the concentration of DNA polyermase in the reaction mixture, increasing the concentration of $MgCl_2$ salt, adding a dilute concentration of $MnCl_2$ to the reaction mixture, or finally by increasing the concentration of the nucleotide bases dGTP, dCTP, and dTTP in the PCR reaction mixture.[56, 58]

Focusing on the application of directed evolution to improve the enantioselectivity of an enzyme, Reetz and coworkers were the first ones to report the application of a technique to enhance the enantioselectivity of a lipase from

Pseudomonas aeruginosa in the hydrolysis of racemic *p*-nitrophenol esters.[62] In this work the wild type enzyme which possessed an ee of 2% towards the (*S*)-enantiomer was subjected to successive rounds of ep-PCR. Within the first generation of lipase mutants a clone was identified with an improved ee of 31%. This mutant was used as a starting point for further rounds of mutagenesis, and after further rounds of mutation a mutant capable of resolving **1-51** to give (*S*)-**1-52** with an ee of 81% was identified (**Scheme 1-11**).

Scheme 1-11



1-51
R=n-C_8H_{17}

(*S*)-**1-52**

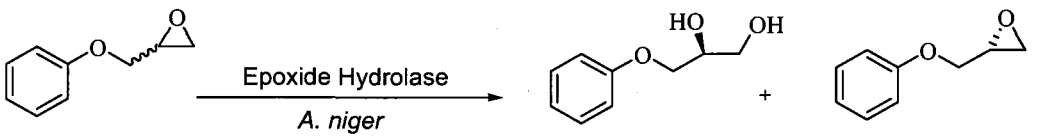
(*R*)-**1-52**

Interestingly, although the focus on the directed evolutionary study was the enhancement of the lipase for the kinetic resolution of (*S*)-**1-52** some of the mutant variants were found to be *R*-selective. Further rounds of mutagenesis resulted in the identification of an *R*-selective mutant possessing an E=30, thus providing access to either enantiomer.[63]

Directed evolution has also been applied to create a more enantioselective epoxide hydrolase from *Aspergillus niger* which has been shown to transform many epoxides into diols (**Scheme 1-12**).[64] In the kinetic resolution of the epoxide **1-53**, wild type hydrolase was found to form the (*S*)-**1-54** diol with an ee of 56% and a selectivity factor of E=4.6. Several libraries of epoxide hydrolase clones were generated in the first

round of ep-PCR, and of the approximately 20000 clones which resulted one was found to possess an enhanced enantioselectivity generating the (*S*)-**1-54** diol with with an ee of 74%. Sequencing of this clone revealed that three mutations, A217V, K332E, and A390E were responsible for this improvement in selectivity.

Scheme 1-12



1-53 (*S*)-**1-54** (*R*)-**1-54**

Analysis of a subsequent X-ray structure which was solved for the wild type epoxide hydrolase from *Aspergillus niger* revealed that two of the mutations (K332E and A390E) occurred at positions which were remote from the active site center. This fact adds to the value of conducting directed evolutionary experiments since such remote mutations would not have been considered in a more rational mutational study.

1-3 Goals of this work

The major goals which were proposed at the start of this work can be summarized in the following points;

1. As part of an ongoing project within the Kayser group focused on the development of better bioreagents suitable for use in organic synthesis, mutants resulting from the genetic enhancement of CHMO through the application of direct evolution were evaluated as bioreagents.

2. To apply a homology based model obtained for CPMO constructed from the closely related PAMO monooxygenase to rationally improved enantioselectivity of CPMO vis avis number of chosen substrates.
3. To apply a novel mutagenesis strategy known as Complete Active Site-Saturation Testing (CASTing) towards the rationalized improvement of the enantioselectivity of Baeyer-Villiger monooxygenase, namely specifically the CPMO enzyme.
4. To screen the mutant CPMO enzymes obtained from rational design with the broad goal of understanding factors controlling the enzymatic BV-oxidation.

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Chapter 2

Directed Evolution of Cyclohexanone Monooxygenase:

Screening of the CHMO mutants

2-1 Introduction

In 1899, Adolf Baeyer and Victor Villiger discovered that in the presence of an oxidant, such as monoperoxyacid, an acyclic ketone is transformed into an ester and a cyclic ketone into a lactone.[1] Since the seminal publication over 100 years ago, the Baeyer-Villiger reaction has become a powerful tool in the synthetic chemists' repertoire.[2,3,4] One of the major factors, which made the BV reaction such an important transformation, is that it represents one of the few synthetic methods allowing for the cleavage of a specific carbon-carbon bond.

The BV reaction, though powerful, is not without limitations - one of which is the requirement for harsh oxidizing agents. Classically, the reagents used for BV oxidations have included *m*-chloroperoxybenzoic acid (mCPBA), trifluoroperacetic acid (TFPA), and peroxyacetic acid all of which are strong oxidizing agents, explosive, and toxic.[2,5] A further drawback of these reagents is that there products are racemic. The latter point is a serious disadvantage since the enantiopure lactone and ester products of the BV oxidation are excellent building blocks for the synthesis of complex natural products, pharmaceuticals, and agrochemicals.[3] Because of the strict governmental requirements that all manufactured pharmaceuticals and agrochemicals (with chiral centres) are enantiopure, it is important that a large repertoire of enantiopure building blocks is available. This has resulted in much effort being devoted toward the development of catalysts capable of performing asymmetric BV oxidations.[6] Although some progress has been made towards the generation of such catalysts for the BV oxidation, the enantiopurity of the products is still not satisfactory.[6]

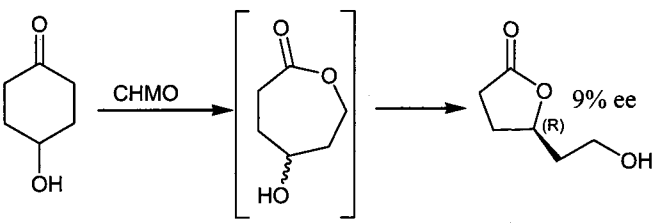
The need for more environmentally friendly, and enantioselective, reagents capable for the BV oxidations has led researchers to turn to the microbial world. In fact, many bacteria and fungi were found to possess enzymes which catalyze the BV oxidations. Why these Baeyer-Villigerases are present in the microorganisms is not clear but it has been speculated that they play a role in the oxidative degradation of toxins.[REF] Since the discovery of these Baeyer-Villigerases efforts were made to isolate, purify, and use them as catalysts. One such Baeyer-Villiger monooxygenase, cyclohexanone monooxygenase (CHMO), from *Acinetobacter* sp. NCIMB 9871 (E.C. 1.14.13.22) was isolated by Trudgill and co-workers in 1976[7] and later extensively studied by Walsh and Chen.[8] CHMO is a highly active catalyst for the BV reaction that selectively transforms a variety of ketonic substrates into the corresponding esters or lactones.[9,10] It is the most thoroughly investigated because of its extensive substrate base and high selectivity.[11]

Unfortunately, the isolated CHMO enzyme is rather unstable, with a half-life of 24 hours at 4°C and an expensive co-factor (NADPH) essential for its enzymatic activity. The direct use of parental organism *Acinetobacter* sp. is hindered by the fact that it is a class two pathogen and its use requires specialized training and properly equipped laboratory facilities. Use of the microbial host organism has the added disadvantage that several enzymes are often co-expressed as a consequence of the host's metabolic pathways. This situation can result in multiple products and/or the unwanted degradation of the desired product.

Fortunately, advancements made in the field of biotechnology have allowed for the development of CHMO as a useful bio-oxidant for the BV reaction. More

specifically, it was the pioneering work of Kayser and Stewart who were the first to sub-clone CHMO into a user friendly host organism, baker's yeast (*Sacharomyces cerevisae*), that allowed the extensive use of CHMO as a biocatalyst[12,13]. Since that time Baeyer-Villigerases from a variety of sources have been isolated, sub-cloned, and expressed in *E coli*.[14,15] The major reason for *E. coli* as a host is its well characterized metabolic pathways and easy of use.

Although an excellent biocatalyst, CHMO has its limitations. For biooxidations involving a number of bicyclic compounds, CHMO produces lactones in good to excellent yields but with only moderate enantioselectivity.[11] Also, although CHMO readily oxidizes several prochiral 4-allylcyclohexanones with high enantioselectivities, it converts 4-hydroxycyclohexanone to the corresponding lactone in good yield but with only a marginal enantiopreference (9% (*R*)).[16]



2-1a **2-2a**

Figure 2-1: *CHMO catalyzed oxidation of 4-hydroxycyclohexanone gives the unstable seven-membered lactone which rapidly rearranges to the more stable product* **[2-2a]**.

The fact that the γ-lactone **2-2a** is obtained with such a low enantiomeric excess is unfortunate since it is a versatile building block for several important macromolecules (**Figure 2-2**). For example, **2-2a** can be used as a synthetic intermediate in the total synthesis of decarestrictine D, which has been shown to inhibit cholesterol biosynthesis

with activity similar to that of the more complex mevinolin and compactin inhibitors.[17] The same lactone (**2-2a**) can also be used as a synthon in the total synthesis of the macrocyclic antibiotic vermiculine, a potent antibiotic possessing activity against a broad spectrum of organisms.[18]

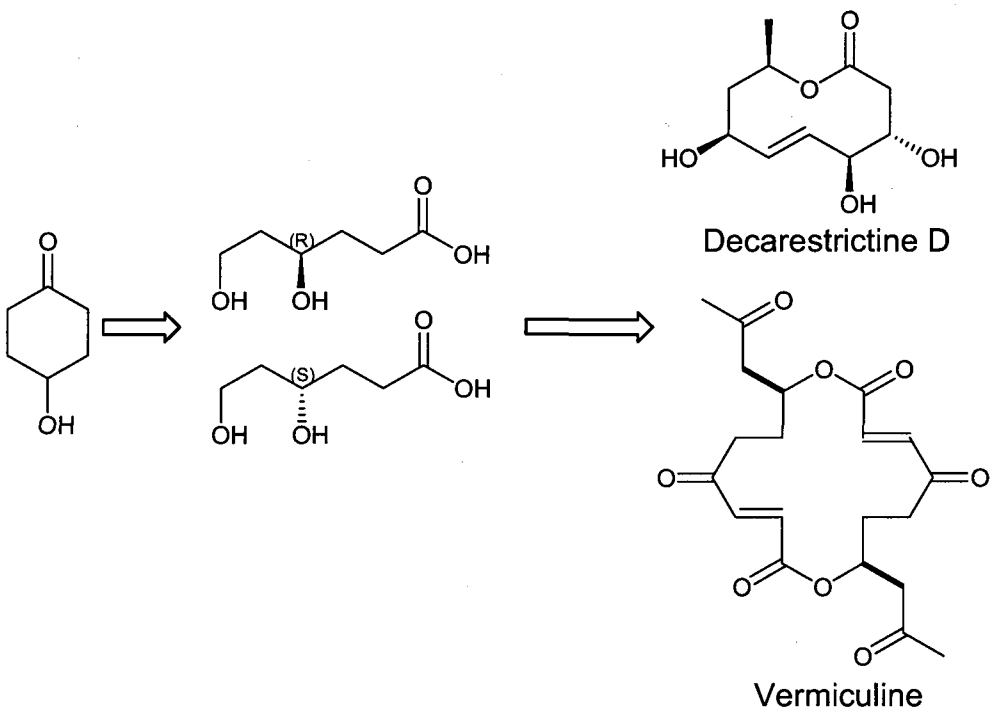


Figure 2-2: *Possible products that could result from the use of γ-lactone **2-2a** as a chiral synthon.*

A further point relating to 4-hydroxycyclohexanone is the fact the CHMO-catalyzed reaction gives **2-2a-(*R*)**, which is opposite to the usual ***S*** selectivity observed for other 4-substituted cyclohexanones. Two immediate questions come to mind. Firstly, why does an enzyme as selective as CHMO perform so poorly in the transformation of 4-hydroxycyclohexanone when it is highly selective for other prochiral six-membered

cyclic ketones? And secondly, can CHMO's selectivity be improved for 4-hydroxycyclohexanone, as well as for other substrates?

2-1-1 Directed evolution of CHMO

If one were dealing with a "classical" asymmetric catalysis, then you could simply modify the catalyst, either through exchange of the catalytically active metal centres or ligand tuning. To improve the catalystic performance of an enzyme modifications at the genetic level to produce new mutants with the desired properties.

Currently, two general strategies are employed to achieve the genetic modification of an enzyme, namely the rational and the non-rational design approaches.[19] In the rational design, knowledge of the proteins 3-D structure, from either X-ray or comparative modeling, is used in conjugation with experimental data and molecular modeling to identify regions of the sequence for targeted mutation. In the non-rational approach, little or no knowledge of the enzyme's active site structure or substrate scope are required. In the latter approach, the entire gene space is subjected to random mutagenesis allowing mutations to occur anywhere in the gene sequence. This can also allow for more than one mutation within a gene. Both of the above strategies have been successfully employed in the enhancement of stability, activity, and enantioselectivity of a variety of enzymatic systems.[19]

Directed evolution is a technique developed by researchers who have taken a lesson directly from nature.[24-26] In nature, spontaneous mutations occur in bacteria and viruses at a rate of roughly 10^{-5} to 10^{-6} per generation. Some mutations result in genes which yield unstable or misfolded proteins while others result in an enhanced genetic

product. The mutations in bacteria (for example) which yield poor genes do not survive while ones that generate enhancements in the gene are kept. The directed evolution of enzymatic properties is a simulation of a natural process.[27] One begins with a wild-type (WT) enzyme which catalyzes a reaction of interest but which possesses some undesired characteristic (low activity, poor selectivity, etc.). The gene encoding for the particular enzyme is subjected to genetic alteration, using either focused or random mutagenesis depending on the amount of information one has about the enzyme, resulting in a number of mutant enzyme libraries being created.

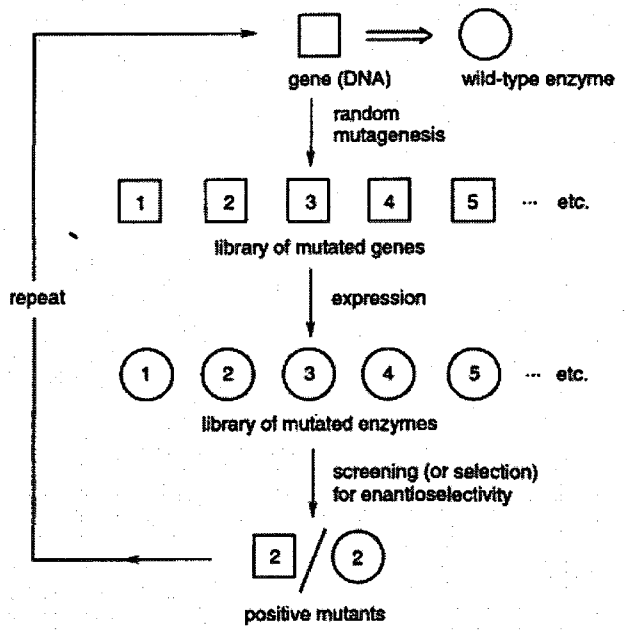


Figure 2-3: *General strategy for the directed evolution of an enantioselective enzyme (reproduced with permission from M. T. Reetz and Adv. Catalysis; Elsevier).*[19]

The mutant libraries are then inserted into an *E. coli* host, and plated on agar; the bacterial colonies are harvested and the whole cells are grown in deep-well plates. The whole cells grown in the deep-well plates are then used to inoculate deep-well reaction

plates which are used in the biocatalysis of the model reaction of interest. The reactions are screened to test for (mutant enzyme) variants possessing the enhanced quality of interest. The "best" mutants are then subjected to subsequent rounds of gene mutagenesis until the best possible enzyme results. This technique is given its evolutionary aspect *via* the fact that the inferior mutants are discarded and only the most successful are used as step-off points into the next round of mutagenesis.

More specifically, the entire protein sequence space was exposed to randomization using the error-prone polymerase chain reaction (ep-PCR) mutagenesis method.[21] The ep-PCR process involves the application of standard PCR conditions[22] for sequence amplification coupled with reaction conditions which trigger the introduction of errors upon sequence elongation by the DNA polymerase. More specifically, the natural error rate of the polymerase enzyme is enhanced by increasing the salt concentrations used in the PCR reaction mixture. The excess of $MgCl_2$ aids in the stabilization of non-complementary base pairs which in turn enhances the probability of a mutation being found in the sequence of interest.[23] Currently there are no generally applicable rules for estimating the mutation frequency resulting from ep-PCR thus making it completely random. Application of this approach requires that each mutated enzyme be screened for enhancement of the desire enzymatic property. This presents a major obstacle to the wide spread application of this technique since a massive screening effort must be undertaken to ensure mutant enzymes which possess enhanced properties are identified. Unfortunately, even the extensive search for mutants with the highest increase in the property of interest (such as activity, thermostability, enantioselectivity) may not meet the desired level of improvement. This general approach illustrated above

has successfully been employed by Reetz and co-workers in the past for the enhancement of enantioselectivity of lipases from a number of bacterial sources.[28,29]

Recently the group of M. T. Reetz working in collaboration with the Kayser group at UNBSJ, took on a challenge of improving CHMO's selectivity towards 4-hydroxycyclohexanone **2-1a**.[20] Because of the lack of 3-D structural information or active site characteristics, the non-rational approach using complete gene randomized mutagenesis to enhance CHMO's enantioselectivity was chosen. Overall, in the directed evolution of CHMO, a total of 10 000 mutant enzymes were screened for their ability to catalyze the target reaction illustrated in **Figure 2-1**.

Table 2-1: *Screening results and sequences for the best CHMO mutants identified through directed evolution for* ***2-1a***.[20]

CHMO Variant	Amino acids exchanged	Enantiopreference	*ee* [%]
Wild-type	-	*R*	9
1-F1-F5[a]	Leu143Phe	*R*	40
1-E12-B5[a]	Phe432Ile	*R*	49
1-F4-B9[a]	Asp41Asn, Phe505Tyr	*S*	46
1-K2-F5[a]	Phe432Ser	*S*	79
2-D18-C8[b]	Leu143Phe, Glu292Gly, Thr433Ile, Leu435Gln, Thr464Ala	*R*	88

[a] Mutant obtained in the first round of ep-PCR. [b] Mutant obtained in the second round of ep-PCR (using 1-F1-F5 mutant as starting point).

In the end twelve mutants with high *S*-selectivity were identified in addition to several with enhanced *R*-selectivity. The results for the best four CHMO variants are given in Table 2-1.[20]

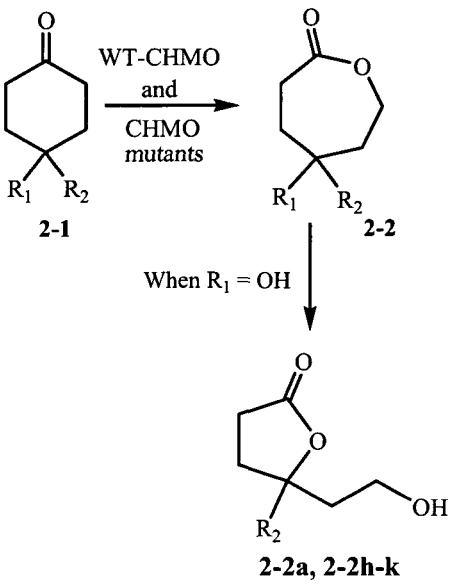
As **Table 2-1** shows, the first generation of mutants (indicated by the "1" at the beginning of the mutant designation) included several with improved enantioselectivity. While none of the CHMO variants identified in the first round of screening showed improvements of more than 49% ee in the *R* direction, one mutant, 1-K2-F5 resulting from a single Phe432Ser mutation, was found to yield **2-2a(*S*)** with a respectable 79% ee. To impart the directed evolutionary characteristic on the experiment, a second round of ep-PCR was implemented using the 1-F1-F5 mutant gene as the starting point for the next round of ep-PCR generated mutation. The second round yielded 2-D18-C8 which catalyzed the BV oxidation of 4-hydroxycyclohexanone with 88% ee in the ***R*** direction.[20]

To establish whether the enantioselective enhancement obtained using 1-K2-F5 was limited to just the 4-hydroxycyclohexanone case, this mutant was screened against the 4-methoxycyclohexanone substrate which is readily accepted by WT-CHMO with a 78% ee in favour of ***S***[30]. The S-selective mutant (1-K2-F5) was found to show essentially complete enantioselectivity (>98% S) when given 4-methoxycyclohexanone as a substrate[20]. These results suggested that while 1-K2-F5 was more enantioselective *vis-à-vis* **2-1a** than WT-CHMO, it still retained its ability to accept other substrates.

My part of the project on the evolution of CHMO involved the evaluation of the four first generation CHMO mutants shown in **Table 2-1** in the bio-oxidation of several prochiral 4-substituted cyclohexanones. Using the results obtained in the mutant evaluation study, the role which the Phe432Ser mutation plays in enhancing CHMO's selectivity will be addressed.

2-2 Evaluation of CHMO mutants

In order to evaluate the performance of the first generation mutants, a set of 15 substrates were chosen and are shown in **Figure 2-4**.



R_1 = OH	R_2 = H	**2-1a**	**2-2a**
OMe	H	**2-1b**	**2-2b**
OEt	H	**2-1c**	**2-2c**
OAllyl	H	**2-1d**	**2-2d**
Me	H	**2-1e**	**2-2e**
Et	H	**2-1f**	**2-2f**
n-Pr	H	**2-1g**	**2-2g**
OH	Me	**2-1h**	**2-2h**
OH	Et	**2-1i**	**2-2i**
OH	i-Pr	**2-1j**	**2-2j**
OH	Allyl	**2-1k**	**2-2k**
COOEt	H	**2-1l**	**2-2l**
Cl	H	**2-1m**	**2-2m**
Br	H	**2-1n**	**2-2n**
I	H	**2-1o**	**2-2o**

Figure 2-4: *Series of 4-substituted cyclohexanone substrates chosen to evaluate the first-generation CHM0 mutants.*

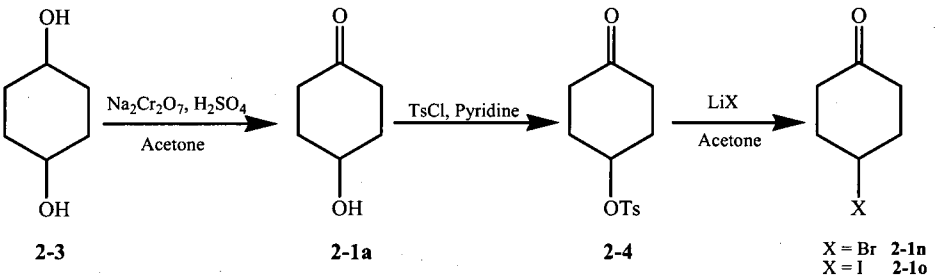
In order to explore mutational influences on the active site of CHMO, it was necessary to use a consistent set of substrates, which are readily accepted with high enantioselectivities by WT-CHMO. It was also desirable to work with a substrate set which would allow us to assess the scope of the newly constructed mutants. The prochiral 4-subsituted cyclohexanones shown in **Figure 2-4** meet these criteria. Many of the substrates shown in **Figure 2-4** are excellent substrates for WT-CHMO. Also, being six-membered rings, they are conformationally well understood and their substituents exist in a clearly defined orientation, either axial or equatorial. The latter point can be

exploited to gain a better understanding of the role the mutations have played on guiding enzymatic selectivity. The substrates shown in **Figure 2-4** possess a variety of substituents in the 4-position which could participate in hydrophobic, hydrophilic, as well as charged interactions with amino acid residues in the active site.

2-2-1 Substrate synthesis

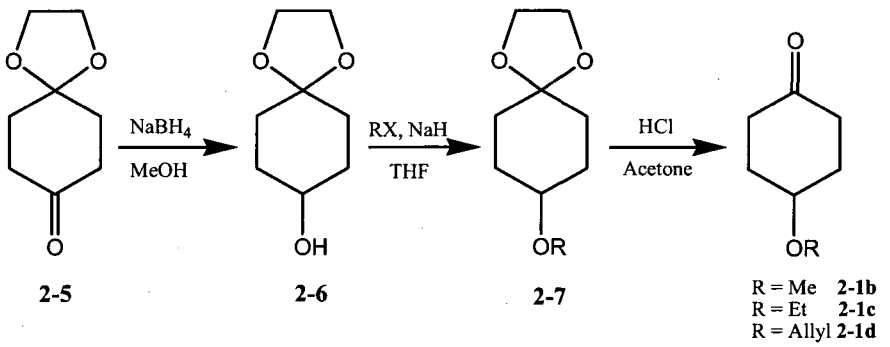
During earlier profiling studies of the WT-CHMO and WT-CPMO bioreagents conducted by the Kayser group, several of the substrates shown in **Figure 2-4** were synthesized.[30,31] The synthetic protocols for the preparation of substrates **2-1a**, **2-1n**, and **2-1o** are shown in **Scheme 2-1**.[32] Commercially available 1,4-cyclohexanediol [**2-3**] was oxidized using Jones' reagent to give 4-hydroxycyclohexanone [**2-1a**] in good yield. The resulting 4-hydroxyketone **2-1a** was converted to a tosylate with *p*-toluenesulfonyl chloride in pyridine to yield **2-4** in excellent yield (87%). The 4-halocyclohexane substrates were made from **2-4** by replacing the tosyl group with the desired nucleophiles (Br^- or I^-).

Scheme 2-1



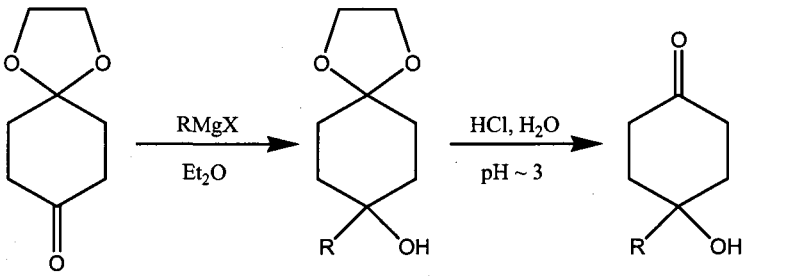
As a starting point in the synthesis of substrates **2-1b** through **2-1d** (see **Scheme 2-1**) commericially available 1,4-cyclohexanedione monoethylene ketal **2-5** was reduced using sodium borohydride in methanol to give the ketal alcohol **2-6** in quantitative yield. This ketal-alcohol was deprotonated with sodium hydride and treated with an alkyl halide in anhydrous tetrahydrofuran (THF) to give the alkoxy ketals **2-7** shown in **Scheme 2-2**. The ketal protecting group was then removed through hydrolysis with aqueous HCl in acetone to yield the target ketones **2-1b** to **2-1d** in varying yields.[32]

Scheme 2-2



A Grignard reaction was the route chosen for the synthesis of 4,4-disubsituted cyclohexanones **2-1h** to **2-1k**. Commercially available ketal **2-5** was treated with the desired alkyl Grignard in anhydrous ether to form the disubstituted ketal **2-8**, which was further subjected to acid hydrolysis to yield the target ketones (**Scheme 2-3**).

Scheme 2-3



2-5 **2-8**

R = Me **2-2h**
R = Et **2-2i**
R = i-Prop **2-2j**
R = Allyl **2-2k**

2-2-2 Bioreagents

For the bioreagents discussed here, the construction of the *E. coli* strain overexpressing the desired enzyme, CHMO (WT or mutants), has been described in detail previously[12,13,20]. For the reactions reported in this section the expression systems for WT or mutant CHMO are reported in the experimental section along with the general procedure for the biotransformations.

2-2-3 Baeyer-Villiger oxidations of 4-mono and 4,4-disubstituted cyclohexanones catalyzed by *E. coli*/CHMO (WT and mutants)

The results for the screening of a variety of 4-mono and 4,4-disubstituted cyclohexanones employing WT-CHMO and the first generation CHMO mutant variants are reported in Table 2-2.[33] All reactions were performed initially on a small scale (25 mL media in 250 mL baffled flask) at 24 °C and allowed to run for 24 hours to assess the activity of the mutants for a given substrate. Among the 4-alkoxycyclohexanone

substrates **2-1b**, **2-1c**, and **2-1d** under the screening conditions used, only 4-methoxycyclohexanone was found to be a substrate for CHMO (both WT and the mutants). In particular, the 1-K2-F5 and 1-E12-B5 mutants converted **2-1b** to the corresponding **2-2b(S)** lactone in excellent yield and high ee value. Thus, while the selectivity for 4-methoxycyclohexanone was enhanced for both of these mutants, their ability to accept larger 4-alkoxy substrates remained unchanged. For the 4-alkylcyclohexanones tested, the 4-methyl [**2-1e**] and 4-ethyl [**2-1f**] cyclohexanone substrates were found to be excellent substrates for both CHMO as well as the mutants tested. The 4-propylcyclohexanone [**2-1g**] on the other hand, which is known to be a good substrate for WT-CHMO, was accepted by 1-K2-F5 with only a slight increase in enantioselectivity.

Based on these results, it is speculative that WT-CHMO may possess a midsize hydrophobic pocket which is able to accommodate 4-alkyl substrates and that the size of the pocket is not significantly affected by the mutations. In fact, the mutational influence on the size of the active sites for most of the mutants was not felt until 4-propylcyclohexanone. The limited size active site is confirmed by the fact that bulky 4-*tert*-butylcyclohexanone is a poor substrate for either WT-CHMO or any of the mutants (results not shown in **Table 2-2**). Interestingly for carboethoxy substrate **2-1l**, while the enantioselectivity did not dramatically increase (93*S* → 99*S*) for the 1-K2-F5, a significant improvement in conversion was observed.

The carboethoxy substrate was also converted with high selectivity by 1-F4-B9 but with a conversion only slightly better (27% *vs.* 15%) than WT-CHMO. The 4-halocyclohexanones **2-1m** to **2-1o** were found to be good substrates for all the first

generation CHMO mutants similar to WT-CHMO in terms of enantioselectivities and conversions.

Table 2-2: *Baeyer-Villiger oxidation of 4-mono and 4,4-disubstitutted ketones by WT-CHMO and the first generation CHMO mutants*[33].

Ketone	% conv.[a] (%ee)[b]				
	WT CHMO	1-K2-F5 Phe432Ser	1-E12-B5 Phe432Ile	1-F1-F5 Leu143Phe	1-F4-B9 Asp41Asn/Phe505Tyr
2-1a OH/H	61 (9*R*)	100 (79S)	100 (49R)	100 (40R)	100 (46S)
2-1b OMe/H	84 (78S)	100 (99S)	100 (99S)	100 (99S)	100 (99S)
2-1c OEt/H	NR[c]	NR	NR	NR	NR
2-1d OAllyl/H	NR	NR	NR	NR	NR
2-1e Me/H	100 (95S)	100 (99S)	100 (99S)	100 (99S)	97 (99S)
2-1f Et/H	100 (95S)	100 (99S)	100 (99S)	96 (99S)	97 (99S)
2-1g n-Pr/H	100 (92S)	100 (99S)	NR	NR	NR
2-1h OH/Me	100 (96R)	100 (87R)	NR	20 (46R)	17 (63R)
2-1i OH/Et[d]	100 (94R)	100 (97R)	NR	NR	NR
2-1j OH/i-pr[d]	100 (97R)	100 (99R)	NR	NR	NR
2-1k OH/All[d]	100 (27R)	100 (97R)	NR	NR	NR
2-1l COOEt/H	15 (93S)	100 (99S)	NR	NR	27 (99S)
2-1m Cl/H	100 (95S)	100 (97S)	100 (97S)	100 (97S)	100 (97S)
2-1n Br/H	100 (97S)	100 (97S)	100 (97S)	100 (97S)	100 (97S)
2-1o I/H	100 (97S)	100 (97S)	100 (97S)	100 (97S)	100 (97S)

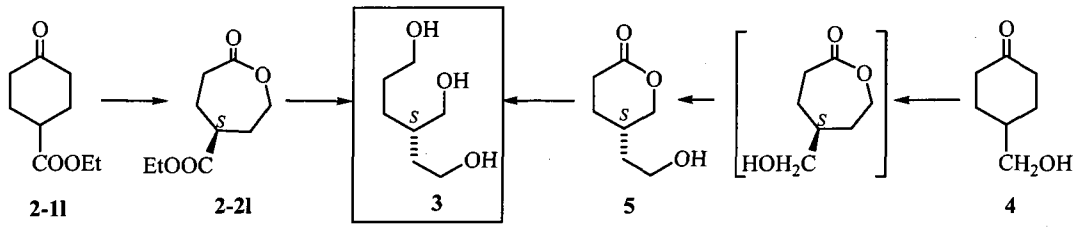
[a] Conversions based on GC analysis. [b] Values for enantiomeric excess from chiral-phase GC analysis. [c] NR= no reaction under established reaction conditions. [d] Absolute configuration assigned based on analogy with lactone **2-2h**.

The 4,4-disubstitued substrates [**2-1h** to **2-1k**] with combined hydroxyl and alkyl chains were screened in order to probe further the character of the active sites for WT-CHMO and the mutants. The results obtained were not disappointing with both WT-CHMO and 1-K2-F5 readily accepting all four substrates. In particular, the enantioselectivity for the 4-allyl-4-hydroxycyclohexanone **2-1k** was significantly enhanced from 27%*R* (WT-CHMO) to 97%*R* using the 1-K2-F5 mutant biocatalyst.

2-2-4 Determination of the absolute configuration of lactones

In order to understand the underlying factors which help to govern the enantioselectivity of CHMO, the absolute configurations of all the product lactones must be unambiguously established. For the substrates shown in **Table 2-2**, the chemical and optical purities of the lactonic products were determined by chiral-phase GC analysis. Althought absolute configurations of many lactones have been established earlier,[30,31] the conformation of the diagnostically important **2-2l** (COOEt) and 4,4-disubstituted group of substrates [**2-1h** to **2-1k**] were not known. The lactone **2-2l** was assigned through the route outlined in Scheme 5. Reduction of lactone **2-2l** with $LiAlH_4$ generated the triol (*S*)-**3**, which was found to be identical to that obtained by the reduction of a lactone previously established by Tachner *et al.* to be (*S*).[16, 34]

Scheme 2-5



For the 4,4-disubstituted ketones, comparison of the specific rotations of the lactonic products obtained from WT-CHMO and 1-K2-F5 indicated that the sign of the rotations did not change, and therefore the absolute configurations were probable the same for the series of biotransformations (see **Table 2-3**).

Table 2-3: *Enantiomeric excess (ee) and specific rotation values for 4,4-disubstituted lactones obtained through bioxidations with WT-CHMO and 1-K2-F5.*[33]

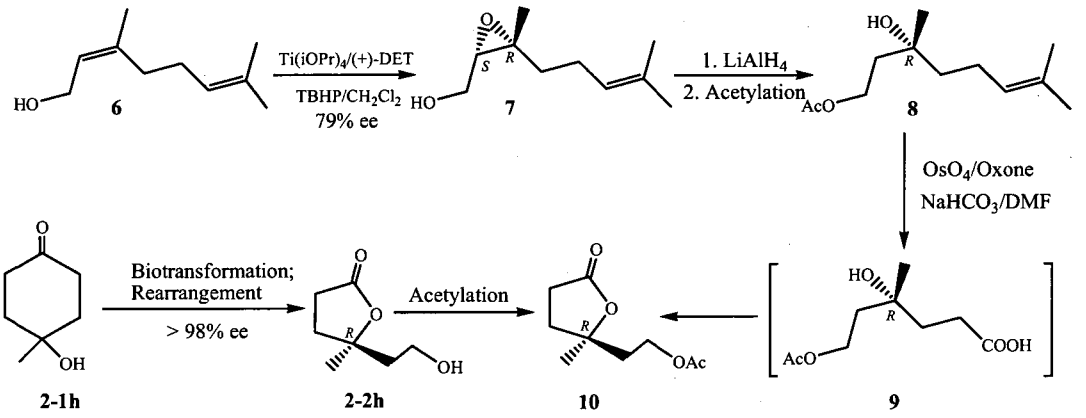
Lactone	R_1/R_2	WT-CHMO		1-K2-F5	
		ee	$[\alpha]^{25}_D$	ee	$[\alpha]^{25}_D$
2-2h	OH/Me	96 (*R*)[a]	-5.67 (*c*=1.53)	87	-4.64 (*c*=0.73)
2-2i	OH/Et	94 (*R*)[b]	-3.51 (*c*=0.73)	97	-3.68 (*c*=1.26)
2-2j	OH/i-Pr	97 (*R*)[b]	-5.21 (*c*=1.30)	99	-5.56 (*c*=1.10)
2-2k	OH/Allyl	27 (*R*)[b]	-3.07 (*c*=1.53)	98	-10.0 (*c*=3.00)

[a] Absolute configuration established experimentally. [b] Absolute configuration established by analogy to compound **2-2h**.

In order to establish the absolute configuration of **2-2h**, an asymmetric synthesis outlined in **Scheme 2-6** was undertaken. The synthesis of an enantioenriched **2-2h** was previously reported by Ichihara and co-workers who used this compound as an

intermediate towards the total synthesis of (-)-betaenone C.[35] Unfortunately the product was not fully characterized, in particular the rotations were not reported.

Scheme 2-6



In the scheme outlined above, commercially available natural product Nerol [**6**] was converted to the corresponding (*S*,*R*) epoxide *via* Sharpless epoxidation.[36] The resulting epoxide was then reduced by $LiAlH_4$ in THF at -40°C generating (*R*)-3,7-dimethyl-oct-6-ene-1,3-diol which was then acetylated to give **8**. The acetylated compound **8** was subjected to oxidative cleavage of the double bond[37] to give acid **9**. The resultant acid undergoes spontaneous ring closure to yield the five-member γ-lactone (*R*)-**10**. The lactone product **2-2h**, resulting from biooxidation, also forms a five-member γ-lactone, which when acetylated gives compound **10**. Direct comparison of the chiral phase GC plots for **10** which resulted from chemical synthesis (**Scheme 2-6**) and biooxidation established, unequivocally, that the absolute configuration of the product **2-**

2h from the biotransformations is (*R*). This was then taken as the benchmark for the analogous assignment of the other products of 4,4-disubsituted biooxidation (shown in **Table 2-3**) as (*R*).

2-3 Discussion

In order to gain a better understanding of the effects of the mutations on the selectivity of CHMO we must invoke one of the many models, published over the years, which attempted to explain the enantioselectivity of CHMO.[38-42] The model which can best rationalize the results obtained from CHMO-catalyzed oxidations is the diamond lattice model illustrated in **Figure 2-5**.[3,42] This model was formulated for a series of 2-, 3-, and 4-subsituted cyclohexanones and indicates the allowed positions for alkyl substituents. The model takes into consideration the stereoelectronic requirements of the non-enzymatic Baeyer-Villiger reaction, and takes advantage of the fact that the FAD group must be tightly bound and in a rigorously fixed position within the active site[3,42]

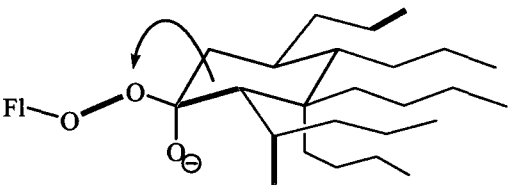
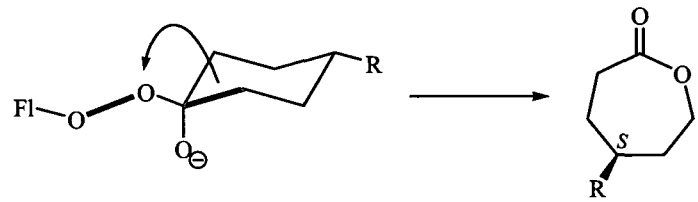


Figure 2-5. *A diamond lattice model of the allowed (green) and forbidden (red) positions for alkyl substituents.*[3,42]

An important assumption which was invoked when the model was developed was that the reactive conformation of the cyclohexanone substrate is formed prior to the

formation of the tetrahedral intermediate. Therefore, it is this preformed reactive conformation of the substrate which determines the eventual stereochemical outcome of the reaction. This assumption is in good agreement with recent mechanistic studies on cyclohexanone monooxygenase which indicates that the oxidation of the substrate rapidly followed the formation of the FAD peroxide.[43] Application of this model for the series of test substrates illustrated in **Figure 2-1** finds that this simple model accurately predicts the stereochemical outcome for the oxidations of 4-alkyl, 4-halo, and the 4-methoxy substituted cyclohexanones (see **Figure 2-6**).



R = alkyl, halo, methoxy

Figure 2-6: *Model-allowed lower energy conformation for 4-alkyl substituted cyclohexanone places the substituent in an equatorial position.*

The diamond lattice model can also be applied towards the rationalization of the stereochemical outcome of biooxidations involving 4,4-disubstituted cyclohexanones [**2-1h** to **2-1k**] as shown in **Figure 2-7**. In the case of 4,4-disubstiuted cyclohexanones, the alkyl substituent is expected to occupy the equatorial position, which leaves the hydroxyl in the axial position (see **Figure 2-7**). Based on the model assisted analysis, the corresponding (***R***) lactone resulting from the biooxidations is predicted, which is in agreement with experimental results.

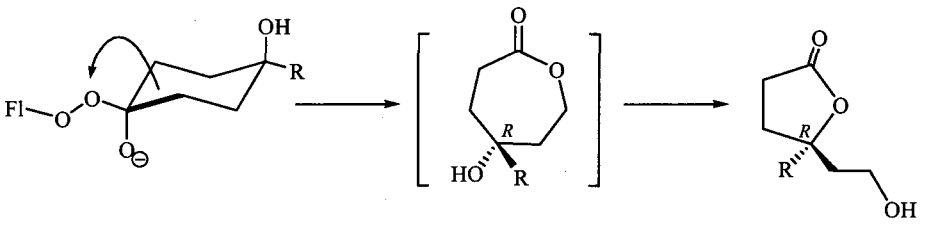


Figure 2-7: *Model-allowed lower energy conformation of the 4-alkyl-4-hydroxycyclohexanones* (**2-1h** to **2-1k**) *places the alkyl substituents in an equatorial position resulting in the R-lactone.*

This diamond lattice model also can be used to explain the low selectivities observed for CHMO for the 4-hydroxycyclohexanone substrate. In an early NMR study on the effects of transannular substituents in cyclohexanols, Stolow and co-workers[44] showed that electron-attracting substituent groups increased the proportion of molecules with the OH group in the axial conformation. In fact, for 4-hydroxycyclohexanone they determined a conformational distribution of 56:44 in favor of the axially oriented OH group.[44] If one now considers the diamond lattice model (see **Figure 2-7**, where R=H), the slightly higher proportion of substrate molecules in the axial orientation would lead to a higher proportion of (*R*) lactone, in agreement with the observed 9% ee for the biooxidation of 4-hydroxycyclohexanone by WT-CHMO (see **Table 2-1**).

In order to quantify the energetic differences in the relative stabilities of the axial and equatorial conformations, *Ab Initio* (gas phase) calculations were preformed using the Gaussian98[45] suite of programs for the 4-hydroxy [**2-1a**] as well as 4-hydroxy-4-methyl [**2-1h**] cyclohexanones shown in **Table 2-4**. Initially, geometry optimizations and frequency analysis were performed at the Hartree-Fock (HF) level of theory employing the modest 3-21G* split-valence basis set of Pople[46] for both conformations to ensure

only structures which are global "true" minima are used. Using these pre-optimized structures as a base, much more accurate HF and Density Functional Theory (DFT) calculations using the Becke three parameter Lee-Yang-Parr (B3LYP)[47,48] functional and the extended 6-311+G** basis of Pople[46] were used (see **Table 2-4**).

Table 2-4: *Calculated ground state energies for the axial versus equatorial conformations of 4-hydroxy (**2-1a**) and 4-hydroxy-4-methyl (**2-1h**) cyclohexanones.*[*]

Method	E_{HF} (au)	E_{B3LYP} (au)	E_{UFF} (au)
2-1a (R_2 axial)	-382.866922	-385.224106	-0.0150025
2-1a (R_2 equatorial)	-382.866132	-385.223366	-0.0140898
ΔE (in kcal/mol)	**0.50**	**0.46**	**0.57**
2-1h (R_2 axial)	-421.914609	-424.552449	-0.0218282
2-1h (R_2 equatorial)	-421.910885	-424.549428	-0.0193332
ΔE (in kcal/mol)	**2.34**	**1.90**	**1.57**

[*]Note: Optimization of the **1a** series in the ChemOffice suite of programs using the AM1 semi-empirical level of theory followed by calculation of the total energies of the systems resulted in a ΔE=0.39 kcal/mol in favour of the axial conformation. This result is in good agreement with the results reported in the NMR study of these compounds.

The theoretical energy difference (ΔE) between the optimized structures of **2-1a** (axial) and **2-1a** (equatorial) were found to be 0.46 kcal/mol (B3LYP) in favor of the axial conformation. This calculated result conforms to the NMR as well as the experimental results for the oxidation of **2-1a** with WT-CHMO. Calculation of the same axial *versus* equatorial energy difference for the 4,4-disubstuted **2-1h** showed a greater stability of the axially oriented OH conformation (ΔE=1.90 kcal/mol B3LYP results). Based on these results, we can conclude that when substrates (like **2-1a** or **2-1h**) can

participate in an interaction in which the energy of interaction ($E_{Interaction}$) is much greater than ΔE (see **Table 2-4**), then according to the diamond lattice model, the (*S*)-lactone would be the preferred product.

In the 1-K2-F5 CHMO mutant in which the aromatic phenylalanine residue has been replaced by a hydrophilic serine whose side chain can act as either a hydrogen bond donor or acceptor, such interactions stabilizing equatorial OH is a possibility. A recent calorimetric study by Letcher and Bricknell[49] reported the interaction energies for a variety of hydrogen-bonded systems at room temperature. They report the hydrogen-bond interaction energies for a series of OH···X type interactions (X=N, O, S or C=O). It was found that for OH···N the interaction energy is -5.89 kcal/mol, while OH···O involves -4.76 kcal/mol in energy. Although these values for the H-bond interaction energies involve close contacts and therefore strong H-bonds, it still gives an indication that even a weak (long distance) H-bond between an equatorially oriented 4-OH of **2-1a** with the 432 serine found in 1-K2-F5 would involve an interaction energy greater than ΔE favouring the axially oriented OH group. Using the same arguments for the 4,4-disubstuted **2-1h** it also shows that while a weak OH···O bond still may involve a significant $E_{Interaction}$ it is still not great enough to completely overcome the axial preference of the OH group. In addition, the experimental results show (**Table 2-2**) although a slight shift towards the S direction for the biooxidation of **2-1h** by 1-K2-F5 (87%*R*) compared to WT-CHMO (96%*R*) the overall effect is small. The fact that an increase in the size of the alkyl subsituent is accompanied by an increase in the ***R*** selectivity of the enzyme further bolsters this argument.

2-4 Conclusion

Application of directed evolution to improve the CHMO enzyme for the biooxidation of 4-hydroxycyclohexanone resulted in the production of a series of mutants with divergent selectivities. In particular, the 1-K2-F5 CHMO mutant, which contains a single amino acid exchange (Phe432Ser) was found to be the most selective and robust of all the first generation CHMO mutants evaluated in this study. This point is illustrated nicely for the 4-carboethoxycyclohexanone **2-1l** which was completely converted to lactone **2-2l** with 99% ee, and the 4-allyl-4-hydroxycyclohexanone **2-1k** which gave the lactone **2-2k** with complete convertion and a 97% ee.

Furthurmore, the diamond lattice model was successfully employed to explain the effects that the Phe432Ser mutation on the stereochemical outcome of the reactions involving the 4-hydroxy- and 4-hydroxy-4-methylcyclohexanone substrates. It is hoped that as more information about CHMO's structure and active site interactions become known, a more generalized model will be developed.

2-5 Experimental Section

All chemicals were purchased from commercial suppliers and were used as received. Thin layer chromatography was performed on 0.2 mm aluminum-backed silica gel plates. Flash chromatography was performed on 230 to 400 mesh silica gel. Chiral-phase capillary gas chromatography was performed on a BetaDex-325 (30 m x 0.25 mm x 0.25 μm) column. Capillary gas chromatography was performed on a DB-1301(15 m x 0.53 mm x 1.0 μm) column. Specific rotations $[\alpha]_D$ were measured at room temperature with energy source Na 589 and are given in 10^{-1}deg cm^2 g^{-1}. FTIR spectra were recorded as thin films. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution, except where otherwise specified, at room temperature using either a 250 MHz or 500 MHz FT-NMR spectrometer; chemical shifts (δ) are reported in ppm using Me_4Si as internal standard. *J* values are expressed in Hertz.

2-5-1 Synthesis and characterization of substrates

4-Hydroxycyclohexanone ethylene ketal (2-6).[50] 1,4-Cyclohexanedione monoethylene ketal (5.0 g, 32 mmol) was added to 100 mL of methanol in a 250 mL round-bottom flask. The solution was cooled in an ice bath. $NaBH_4$ (3.75 g, 97 mmol) was added portion-wise over 30 minutes. After 3 minutes the ice bath was removed, and the mixture was stirred for an hour. The solvent was then removed by a rotatory evaporator and 35 mL of brine was added to the flask. The brine mixture was extracted with ethyl acetate (100 mL × 3), the organic layer was dried with anhydrous $MgSO_4$ and the solvent was removed by rotatory evaporation yielding the title compound as a colourless oil, (4.95 g, 98% yield). IR (in $CHCl_3$) υ_{max}: 3400 (s, br), 2934 (s), 2873 (m), 1433 (m), 1376 (m),

1127 (m), 1041 (m), 956 (m) cm^{-1}; ^1H NMR δ: 3.9 (4H, m), 3.64 (1H, heptet, *J*=4.6 Hz), 1.83 (5H, m), 1.56 (4H, m) ppm; ^{13}C NMR δ: 108.7 68.6, 64.2, 31.4, 31.0 ppm.

4-Methoxycyclohexanone (2-1b).[51,52] 4-Hydroxycyclohexanone ethylene ketal **3** (1.00 g, 6.3 mmol), NaH (0.30 g, 13 mmol), and methyl iodide (1.80 g, 7.50 mmol, 0.80 mL) were reacted according to the literature procedure to give 4-methoxycyclohexanone ethylene ketal **4a**. Deketalization[32] followed by flash chromatography with petroleum ether:ethyl acetate (20:1) as eluent gave compound **2-1b** as a colourless oil, (0.92 g, 68% yield); IR (in $CHCl_3$) υ_{max}: 2939(m), 2872(m), 2825(m), 1714(vs), 1441(m), 1350(m), 1248(m), 1099(s), 905(m) cm^{-1}; ^1H NMR δ: 3.61 (1H, heptet, *J*=2.9 Hz), 3.40 (3H, s), 2.53 (2H, m), 2.27 (2H, m), 2.03 (2H, m), 1.87 (2H, m) ppm; ^{13}C NMR δ: 210.3, 74.3, 58.6, 37.4, 30.0 ppm.

4-Ethoxycyclohexanone (2-1c).[52] 4-Hydroxycyclohexanone ethylene ketal (0.8 g, 5 mmol), NaH (2 g), and ethyl bromide (8 mL added in 4 portions) reacted according to the literature procedure to give 4-ethoxycyclohexanone ethylene ketal, (0.81 g). Deketalization[32] followed by flash chromatography with petroleum ether:ethyl acetate (20:1) as eluent gave **2-1c** as a pale yellow oil, (0.43 g, 61% yield); IR (in $CHCl_3$) υ_{max}: 2929(m), 2870(m), 1714(vs), 1442(m), 1345(m), 1233(m), 1105(s), 1089(m), 969(m) cm^{-1}; ^1H NMR δ: 3.61 (1H, heptet, *J*=2.9 Hz), 3.56 (2H, q, *J*=6.7 Hz), 2.56 (2H, m), 2.25 (2H, m), 2.10 (2H, m), 1.94 (2H, m), 1.19 (3H, t, *J*=7.0Hz) ppm; ^{13}C NMR δ: 211.1, 73.6, 63.2, 37.4, 31.0, 15.6 ppm.

General procedure for Grignard reactions

Magnesium metal (2.6 g, 110 mmol) and 50 mL of anhydrous ether were placed in a 250 mL flask equipped with a dropping funnel and a reflux condenser. Alkyl halide (80 mmol) dissolved in 40 mL of anhydrous ether was added dropwise at a rate sufficient to maintain a gentle reflux; this was continued for an additional 1.5 hours at room temperature. Ketal **2-3** (15 mmol) dissolved in 40 mL of anhydrous ether was added dropwise, then stirring was continued for an additional 3.5 hours at room temperature. The reaction was quenched with 70 mL of water. The organic layer was separated, and the aqueous layer was extracted with ether (150 mL × 3). The combined organic layers were dried with anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporation. The crude product was dissolved in 100 mL of aqueous HCl (pH 3.0) and stirred at room temperature for 24 hours. After extraction with EtOAc (150 mL × 3), the combined organic layers were dried with anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporation. The crude product was purified by chromatography on silica gel using petroleum ether:ethyl acetate (4:1).

4-Hydroxy-4-methylcyclohexanone (2-1h). Magnesium metal (2.6 g, 110 mmol), methyl iodide (5.7 g, 2.5 mL, 40 mmol) and ketal **3** (2.4 g, 15 mmol) reacted according to the general procedure to give a colourless oil (1.42 g, 75% yield); IR (in $CHCl_3$) υ_{max}: 3405(m), 2962(m), 2927(m), 2856(m), 1703(vs), 1416(m), 1375(m), 1251(m), 1133(s), 909(m) cm^{-1}; ^1H NMR δ: 2.76 (2H, ddd, *J*=14.1, 7.6, 2.1 Hz), 2.26 (2H, m), 1.97 (2H, m), 1.87 (2H, m), 1.38 (3H, s) ppm; ^{13}C NMR δ: 212.3, 68.4, 38.7, 37.1, 29.7 ppm.

4-Ethyl-4-hydroxycyclohexanone (2-1i).[53] Magnesium metal (2.6 g, 110 mmol), ethyl bromide (9.8 g, 90 mmol, 7.6 mL) and ketal **2-3a** (2.4 g, 15 mmol) reacted according to

the general procedure to yield colourless crystals (1.72g, 81% yield): mp 48-50 °C; IR (in $CHCl_3$) υ_{max}: 3440(m), 2964(m), 2936(m), 2881(m), 1709(vs), 1462(m), 1337(m), 1245(m), 1136(s), 1088(m), 941(m) cm^{-1}; ^1H NMR δ: 3.61 (1H, heptet, *J*=2.9 Hz), 3.56 (2H, q, *J*=6.7 Hz), 2.56 (2H, m), 2.25 (2H, m), 2.10 (2H, m), 1.94 (2H, m), 1.19 (3H, t, *J*=7.0Hz) ppm; ^{13}C NMR δ: 212.4, 70.3, 36.9, 36.4, 34.9, 7.6 ppm.

4-Hydroxy-4-isopropylcyclohexanone (2-1j). Magnesium metal (1.3 g, 55 mmol), isopropyl iodide (7.6 g, 45 mmol, 4.5 mL), and ketal **2-3** (1.2 g, 8 mmol) reacted according to the general procedure to give the title compound as a yellow oil (0.64 g, 56% yield); IR (in $CHCl_3$) υ_{max}: 3440(m), 2964(m), 2936(m), 2881(m), 1709(vs), 1462(m), 1337(m), 1245(m), 1136(s), 1088(m), 941(m) cm^{-1}; ^1H NMR δ: 2.72 (2H, ddd, *J*=14.1, 14.0, 7.9 Hz), 2.23 (2H, m), 1.89 (2H, m), 1.80 (2H, ddd, *J*=13.4, 12.8, 6.5 Hz), 0.94 (6H, d, *J*=6.8 Hz) ppm; ^{13}C NMR δ: 210.4, 73.3, 42.3, 38.9, 34.9, 14.2, 13.5 ppm.

4-Allyl-4-hydroxycyclohexanone (2-1k). Magnesium metal (1.3 g, 55 mmol), allyl bromide (7.6 g, 45 mmol, 4.5 mL), and ketal **2-3** (1.2 g, 8 mmol) reacted to give the title compound as a yellow oil (1.10g, 85% yield); IR (in $CHCl_3$) υ_{max}: 3440(m), 2964(m), 2936(m), 2881(m), 1709(vs), 1462(m), 1337(m), 1245(m), 1136(s), 1088(m), 941(m) cm^{-1}; ^1H NMR δ: 5.89 (1H, m), 5.23 (2H, dd, *J*=10.1 Hz), 2.72 (2H, ddd, *J*=14.1, 13.7, 6.4 Hz), 2.34 (2H, m), 2.27 (2H, m), 2.08 (2H, m), 1.95 (2H, t, *J*=9.8 Hz), 1.82 (2H, ddd, *J*=13.4, 13.1, 8.5 Hz) ppm; ^{13}C NMR δ: 209.7, 170.3, 68.6, 37.2, 30.4, 21.2 ppm.

4-Hydroxymethylcyclohexanone (4).[16, 54] 4-Ethylcyclohexanone carboxylate ester **2-1l** (1.5 g, 8.8 mmol), ethylene glycol (22 mL), and p-toluenesulfonic acid (0.200 g, 1.1 mmol) in 50 mL of benzene was reacted according to the literature procedure[5] to give 4-ethylcyclohexanone carboxylate ester monoethylene ketal, 1.35 g: ^1H NMR δ: 4.10 (2H,

q, *J*=6.7 Hz), 3.92 (4H, s), 2.31 (1H, m), 1.90-1.54 (8H, m), 1.23 (3H, t, *J*= 7.3 Hz); ^{13}C NMR δ: 175.2, 108.1, 64.3, 60.2, 41.6, 33.7, 26.3, 14.2 ppm. The resulting ketal was dissolved in 3 mL of anhydrous THF and added dropwise to a slurry of $LiAlH_4$ (0.200 g, 5.3 mmol) in 15 mL of anhydrous THF cooled in an ice bath. The reaction was monitored by TLC until complete and worked up according to the literature procedure, [5] to give 1.00 g of the reduced ketal: ^{1}H NMR δ: 3.93 (4H, s), 3.48 (2H, d, *J*=6.1 Hz), 1.77-1.50 (8H, m), 1.27 (1H, m) ppm; ^{13}C NMR δ: 109.8, 67.7, 64.2, 39.1, 34.1, 26.6 ppm. Subsequent deketalization[5] followed by flash chromatography using hexane:acetone (3:1) as the eluent gave **4** as a pale yellow oil, 0.40 g (36% overall yield). IR (in $CHCl_3$) υ_{max}: 3405(br), 2928(s), 2868(s), 1709(vs), 1422(m), 1335(m), 1168(m), 1086(m), 1037 (m) cm^{-1}; ^{1}H NMR δ: 3.57 (2H, d, *J*=6.7 Hz), 2.39 (4H, m), 2.05 (2H, m), 1.94 (1H, m), 1.66 (1H, m) 1.46 (2H, m) ppm; ^{13}C NMR δ: 211.8, 66.8, 64.2, 40.4, 38.6, 29.1, 26.6 ppm.

2-5-2 Biotransformations with *E. coli*/CHMO and *E. coli*/CHMO mutants

The *E. coli* strain BL21(DE3)(pMM4) (or JM109(DE3)(pET-22b) was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and was shaken overnight at 37 °C, 250 rpm. This culture was used at a 1:100 (v/v) ratio to inoculate an LB-Ampicillin medium supplemented with 2% glucose in a baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, until OD_{600} was approximately 0.3-0.4. IPTG stock solution (200 mg/mL) was added (0.1 μL per mL of medium) and the flask was shaken for another 30 minutes at 24 °C. The

substrate was then added; if cyclodextrin were necessary to alleviate solubility or toxicity problems, it was added at this stage. The culture was shaken at 24 °C, 250 rpm, and monitored by GC analysis until reaction was complete. The culture was then saturated with NaCl and extracted with ethyl acetate or dichloromethane. The combined extracts were washed once with brine and dried with anhydrous $MgSO_4$. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography on silica gel.

2-5-3 Characterization of biotransformation products reported in Table 2-2

5-Methoxyoxepan-2-one (2-2b). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1b** (100 mg, 0.78 mmol) was performed in the presence of 0.5 g β-cyclodextrin according to the general procedure. Chromatography on silica gel using petroleum ether: acetone (4:1) as eluent gave **2-2b** as a colourless oil (59 mg, 53% yield); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ = +17.14 (*c* 0.9, CH_2Cl_2); IR (neat) υ_{max}: 2938(s), 2854(m), 1743(vs), 1435(s), 1318(s), 1135(m), 1078(m) cm^{-1}; ^1H NMR δ: 4.42 (1H, ddd, *J*=13.0, 10.4, 2.5 Hz), 3.98 (1H, ddd, *J*=13.4, 6.7, 2.5 Hz), 3.50 (1H, heptet, *J*=3 Hz), 3.28 (3H, s), 2.89 (1H, m), 2.35 (2H, m), 1.98 (3H, m), 1.79 (1H, m) ppm; ^{13}C NMR δ: 175.9, 75.3, 63.1, 55.7, 33.2, 27.5, 26.6 ppm.

5-Methyloxepan-2-one (2-2e). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1e** (110 mg, 0.98 mmol) was performed in the presence of 0.5 g β-cyclodextrin according to the general procedure. Chromatography on silica gel using petroleum ether:ethyl acetate (4:1) as eluent gave lactone **2-2e** (80 mg, 64% yield); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ = -43.5 (*c* 1.43, CH_2Cl_2); IR (neat) υ_{max}: 2955(m), 2974(s), 2873(m) 1730(vs), 1449(s), 1338(s), 1164(m), 1078(m) cm^{-1}; ^1H NMR δ: 4.27 (2H, m), 2.65 (2H,

m), 1.88 (2H, m), 1.78 (2H, m), 1.52 (2H, m), 1.01 (3H, t, *J*=6.7 Hz) ppm; ^{13}C NMR δ:175.2, 68.2, 41.9, 35.0, 33.2, 29.1, 28.5 ppm.

5-Ethyloxepan-2-one (2-2f). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1f** (100 mg, 0.79 mmol) was performed in the presence of 0.5 g β-cyclodextrin according to the general procedure. Chromatography on silica gel using petroleum ether:ethyl acetate (4:1) as eluent yielded lactone **2-2f** (67 mg, 60%); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ = -27.7 (*c* 1.32, CH_2Cl_2); IR (in $CHCl_3$) υ_{max}: 2962(m), 2926(m), 2875(m), 1736(vs), 1440(m), 1337(s), 1175(s), 1079(s) cm^{-1}; ^{1}H NMR δ: 4.32 (2H, dt, *J*=13.5, 5.5 Hz), 2.67 (2H, m), 1.98 (2H, m), 1.49 (2H, m), 1.33 (2H, m), 0.92 (3H, t, *J*=7.3 Hz) ppm; ^{13}C NMR δ: 175.2, 68.2, 41.9, 35.0, 33.2, 29.1, 28.5, 11.3 ppm.

5-Propyloxepan-2-one (2-2g). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1g** (110 mg, 0.78 mmol) was performed in the presence of 0.5 g β-cyclodextrin according to the general procedure. Chromatography on silica gel using petroleum ether:acetone (5:1) as eluent gave lactone **2-2g** (61 mg, 50% yield); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ = -22.4 (*c* 1.71, CH_2Cl_2); IR (in $CHCl_3$) υ_{max}: 2957(m), 2927(m), 2871(m), 1736(vs), 1466(m), 1318(m), 1173(s), 1080(m) cm^{-1}; ^{1}H NMR δ: 4.30 (2H, dt, *J*=12.8, 6.7 Hz), 2.66 (2H, m), 1.95 (2H, m), 1.60 (2H, m), 1.49 (2H, m), 1.33 (5H, m), 1.01 (3H, t, *J*=6.7 Hz) ppm; ^{13}C NMR δ: 176.1, 68.2, 39.9, 38.7, 35.4, 33.2, 28.9, 19.9, 14.1 ppm.

5-(2-Hydroxyethyl)-5-methyldihydrofuran-2-one (2-2h). *E. coli* BL21(DE3)(pMM4)-mediated oxidation of ketone **2-1h** (100 mg, 0.78 mmol) was performed according to the general procedure. Chromatography on florisil using petroleum ether:acetone (2:1) as eluent gave **2-2h** as a colourless oil (46 mg, 41 % yield); 96% ee by chiral phase GC,

$[\alpha]^{25}_D$ = -5.67 (*c* 1.53, CH_2Cl_2) : IR (neat) υ_{max}: 3409(m), 2937(m), 2889(m), 1758(vs), 1460(m), 1383(m), 1269(m), 1188(m), 1091(m) cm^{-1}; ^1H NMR δ: 3.84 (2H, m), 2.64 (1H, dd, *J*=15, 6 Hz), 2.61 (1H, dd, *J*=15, 6 Hz), 2.22 (1H, s), 2.07 (2H, m), 1.98 (1H,dd, *J*=13, 6 Hz), 1.95 (1H, dd, *J*=13, 6 Hz), 1.45 (1H, s) ppm; ^{13}C NMR δ: 176.6, 86.0, 58.5, 42.8, 33.6, 28.8, 25.9 ppm.

5-(2-Hydroxyethyl)-5-ethyldihydrofuran-2-one (2-2i). *E. coli* BL21(DE3)(pMM4)-mediated oxidation of ketone **2-1i** (100 mg, 0.70 mmol) was performed according to the general procedure. Chromatography on florisil using petroleum ether:acetone (2:1) as eluent gave **2-2i** as a colourless oil (55 mg, 49% yield); 98% ee by chiral phase GC, $[\alpha]^{25}_D$ = -3.80 (*c* 1.0, CH_2Cl_2); IR (neat) υ_{max}: 3410(m), 2942(m), 2885(m), 1762(vs), 1422(m), 1245(m), 1198(m), 1065(m), 951(m) cm^{-1}; ^1H NMR δ 3.83 (2H, m), 2.62 (2H, m), 2.12 (2H, m), 1.98 (2H, m), 1.75 (2H, m), 0.96 (3H, t, *J*=7.6 Hz) ppm: ^{13}C NMR δ: 177.1, 88.5, 58.4, 40.3, 31.6, 30.7, 28.8, 7.9 ppm.

5-(2-Hydroxyethyl)-5-isopropyldihydrofuran-2-one(2-2j). *E. coli* BL21(DE3)(pMM4) mediated oxidation of ketone **2-1j** (100 mg, 0.64 mmol) was performed according to the general procedure. Chromatography on silica using petroleum ether:acetone (2:1) as eluent gave **2-2j** as a colourless oil (39 mg, 35%); 95% ee by chiral phase GC, $[\alpha]^{25}_D$ =-5.211 (*c* 1.30, CH_2Cl_2): IR (neat) υ_{max}: 3430(m), 2966(m), 2883(m), 1764(vs), 1470(m), 1255(m), 1204(m), 1052(s), 951(s) cm^{-1}; ^1H NMR δ: 3.85 (2H, m), 2.69-2.58 (2H, m), 2.19-2.06 (4H, m), 1.97 (1H, dd, *J*=13.4, 6.7 Hz), 1.00 (6H, q, *J*=6.7 Hz) ppm; ^{13}C NMR δ: 176.4, 90.59, 58.3, 38.9, 35.5, 29.2, 27.6, 16.9, 16.4 ppm.

5-(2-Hydroxyethyl)-5-allyldihydrofuran-2-one (2-2k). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1k** (90 mg, 0.58 mmol) was performed according to the

general procedure. Chromatography on florisil using petroleum ether:acetone (2:1) as eluent gave **2-2k** as a colourless oil (55 mg, 55%); 98% ee by chiral phase GC, $[\alpha]^{25}_D$ = -10.00 (*c* 3.00, CH_2Cl_2); IR (neat) υ_{max}: 3424(m), 3077(w), 2940(m), 2941(m), 1767(vs), 1434(m), 1278(m), 1190(m), 1048(m), 928(m) cm^{-1}; ^1H NMR δ: 5.77 (1H, m), 5.20 (2H, dd, *J*=10.4, 7.6 Hz), 3.83 (3H, m), 2.60 (2H, m), 2.47 (2H, m), 2.16 (3H, t, *J*=7.9 Hz), 1.99 (2H, m) ppm; ^{13}C NMR δ: 176.8, 131.5, 120.2, 87.3, 58.1, 43.5, 41.1, 30.5, 28.8 ppm.

7-Oxo-oxepan-4-carboxylic acid ethyl ester (2-2l). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **2-1l** (100 mg, 0.59 mmol) was performed according to the general procedure. Chromatography on silica gel using petroleum ether:acetone (4:1) as eluent gave **2-2l** as a colourless oil (69 mg, 63%); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ =-26.171 (*c* 2.8, CH_2Cl_2); IR (neat) υ_{max}: 2980(s), 2936(m), 1729(vs), 1443(m), 1255(m), 1188(m), 1072(m) cm^{-1}; ^1H NMR δ: 4.37 (1H, dd, *J*=13.4, 5.5 Hz), 4.21 (1H, m), 4.17 (2H, q, *J*=7.3 Hz), 2.68 (3H, m), 2.12 (4H, m), 1.27 (3H, t, *J*=7.3 Hz) ppm ; ^{13}C NMR δ: 175.0, 173.6, 66.7, 60.9, 44.1, 32.1, 31.4, 24.8, 14.2 ppm.

5-(2-Hydroxyethyl)-tetrahydropyran-2-one (5). *E. coli* BL21(DE3)(pMM4)-mediated oxidation of ketone **4** (100 mg, 0.59 mmol) was performed according to the general procedure. Chromatography on silica gel using petroleum ether:acetone (80:20) as eluent gave **5** as a colourless oil (49 mg, 48%); 99% ee by chiral phase GC, $[\alpha]^{25}_D$ = -35.234 (*c* 1.3, CH_2Cl_2). Spectral data are identical with those reported by Taschner et al.[6]

(*S*)-3-Hydroxymethyl)hexane-1,6-diol (3). $LiAlH_4$ was suspended in 30 mL of anhydrous THF and cooled in an ice-salt bath. 7-Oxo-oxepan-4-carboxylic acid ethyl ester **2-2l** (or (*S*)-tetrahydro-5-(2-hydroxyethyl)pyran-2-one **5**) (25 mg in 2 mL THF) was

added dropwise over 5 minutes and stirred at 0°C for 30 minutes, then at 25°C for 7 hours. When the reaction was completed (as determined by TLC) it was quenched with methanol, and filtered through celite. The filter cake was washed with methanol and the combined filtrate was concentrated by rotary evaporation and purified by flash chromatography on silica gel using ethyl acetate as eluent to give the title compound (-)-**3** as a thick oil (10 mg, 39% yield, $[\alpha]^{25}_{D}$ = -2.45 (*c* 0.611, MeOH); IR (neat) υ_{max}: 3326(s), 2938(s), 2870(m), 1445(m), 1051(s) cm^{-1}; ^{1}H NMR δ: 3.73 (6H, m), 2.26 (3H, s), 1.73 (6H. m), 1.29 (1H, t, *J*= 7.3 Hz) ppm.

(2*S*,3*R*)-3-Methyl-3-(4-methylpent-3-enyl)oxiran-2-yl)menthanol (7) Sharpless epoxidation was carried out on compound **6** as published[36] to give **7** (60% yield) as a colourless oil; $[\alpha]^{25}_{D}$ = -8.39 (*c* 1.90, CH_2Cl_2) IR (neat) υ_{max}: 3424(s), 2967(s), 2927(s), 2865(m), 1451(m), 1380(m), 1033(s) cm^{-1}; ^{1}H NMR δ: 5.08 (1H, t, *J*=7.3 Hz), 3.80 (1H, dd, *J*=7.1, 4.7 Hz), 3.65 (1H, dd, *J*=6.7, 4.3 Hz), 2.96 (1H, dd, *J*=7.3, 4.9 Hz), 2.10 (2H, m), 2.03 (1H, s), 1.50 (2H, t, *J*=7.3 Hz), 1.68 (6H, s), 1.33 (3H, s) ppm; ^{13}C NMR δ:132.5, 123.3, 64.2, 61.3, 33.2, 25.7, 24.2, 22.2, 17.6 ppm.

(*R*)-3-Hydroxy-3,7-dimethyloct-6-enyl acetate (8). Anhydrous THF (60 mL) under argon was cooled to -40 °C. $LiAlH_4$ (1.0 g, 26 mmol) was added slowly and stirred for 5 minutes. Compound **7** (1.9 g,11 mmol) dissolved in THF (3 mL) was added dropwise. The reaction mixture was maintained at -40 °C for 4 hours, then was allowed to warm to RT and was stirred overnight. The reaction was quenched with saturated tartaric acid (25 mL) and stirred for 1 hours. After suction filtration through celite, it was extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$. Chromatography on silica gel yielded (*R*)-3,7-dimethyloct-6-ene-1,3-diol (1.0 g, 53%) as a colourless oil;

$[\alpha]^{25}_D$ = 2.79 (*c* 1.50, CH_2Cl_2) IR (neat) υ_{max}: 3349(vs), 2968(s), 2926(s), 2854(m), 1450(m), 1376(m), 1118(m), 1060(s), 1028(w) cm^{-1}; ^1H NMR δ: 5.12 (1H, t, *J*=6.9 Hz), 3.87 (2H, m), 2.70 (2H, s), 2.05 (2H, dt, *J*=8.5, 7.3 Hz), 1.68 (6H, s), 1.56 (2H, t, *J*=7.3 Hz), 1.52-1.49 (4H, m),1.24 (3H, s) ppm; ^{13}C NMR δ:131.9, 124.2, 73.9, 59.8, 42.2, 41.7, 26.7, 25.7, 22.7, 17.7 ppm. The purified diol was dissolved in 22 mL of anhydrous CH_2Cl_2 and placed into a 50 mL round-bottom flask. The flask was cooled in an ice bath and the following reagents were added sequentially: 1.20 mL of acetic anhydride, 162 mg of 4,4-dimethylaminopyridine, and 1.70 mL of triethylamine. The reaction was maintained at 0°C until completed (by TLC). Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl acetate, washed once with $NaHCO_3$ and dried ($MgSO_4$). Purification on silica gel yielded the title compound **7**, 0.900 g (73%) as a pale yellow oil; 79% ee by chiral phase GC; IR (neat) υ_{max}: 3463(s), 2969(s), 2927(s), 2859(w), 1740(vs), 1452(m), 1368(m), 1245(s), 1121(m), 1034(m) cm^{-1}; ^1H NMR δ: 5.12 (1H, t, *J*=7.2 Hz), 4.24 (2H, t, *J*=6.7 Hz), 2.07 (2H, dt, *J*=8.5, 6.7 Hz), 2.04 (4H, s), 1.82 (2H, m), 1.68 (6H, s), 1.52 (2H, t, *J*=8.5 Hz), 1.21 (3H, s) ppm; ^{13}C NMR δ: 171.1, 132.1, 124.1, 71.9, 61.3, 42.2, 39.8, 26.9, 25.7, 22.7, 21.1, 17.7 ppm.

2-(*R*)-(Tetrahydro-2-methyl-5-oxofuran-2-yl)ethyl acetate (10).[37] 200 mg of olefin **8** was dissolved in 5 mL of DMF, and 100 μL of OsO_4 (2.5% in *t*-BuOH) was added and stirred for 5 min. Then solid oxone (2.25 g, 4 eq) and $NaHCO_3$ (315 mg, 4 eq) was added in one portion and the reaction was stirred at RT for 16 hours. Once reaction was complete (by TLC), Na_2SO_3 (740 mg, 6 eq) was added, to reduce any remaining Os(VIII), and stirred for 1.5 hours. Ethyl acetate (50 mL) was added to the reaction along with 5 mL of 1M HCl. The organic extract was washed with 1M HCl (5 mL × 3)

and brine, dried ($MgSO_4$) and the solvent removed under vacuum. The crude product was purified by chromatography on silica gel petroleum ether:ethyl acetate (3 : 1) yielding 65 mg (37%) of a colourless oil; 79% ee by chiral phase GC; ^{1}H NMR δ: 4.24 (2H, dt, *J*=14, 6.9 Hz), 2.63 (2H, m), 2.18 (3H, m), 2.07 (3H, s), 1.45 (3H, s), 1.27 (1H, s) ppm; ^{13}C NMR δ: 176.2, 170.8, 84.9, 60.1, 39.2, 33.4, 28.8, 25.9, 20.9 ppm.

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Chapter 3

Development of a new cyclopentanone monooxygenase expression system

3-1 Introduction

The biological Baeyer-Villiger (BV) reactions, catalyzed by the BV monooxygenase enzymes (BVMOs), offer eco-friendly and efficient routes towards the synthesis of a large number of enantiopure lactones (from ketones that are accepted by these enzyme).[1-3] The value of these enantiopure lactones as chiral building blocks in the synthesis of larger macromolecules and polymers has made the further development of BVMOs the focus of many groups. Early work on bio-oxidations using BVMOs involved the use of the host microorganism harbouring the BVMO as a catalyst. While some success was made *via* this route several problems were identified with using the bacterial host as catalyst. Some of the challenges faced when running bacterial reactions include the co-expression of more than one BVMO by the host as is the case for whole cells of *Pseudomonas putida* NCIMB 10007 which express two monooxygenases, namely MO1 and MO2.[2, 3] The pathogenic nature of bacteria must also be considered, along with the potential for further unwanted degradation of the desired product through further metabolism by the organism - an example of which is illustrated in **Figure 3-1**.

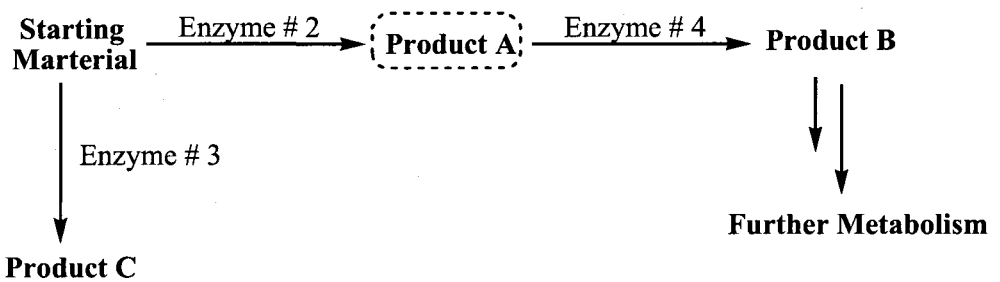


Figure 3-1: *Example of possible metabolic degradation of starting material as well as desired product A by whole cell bacteria.*

The limiting factor in the use of native (WT) Baeyer-Villigerases is the potential requirement for both enantiomers of a given lactone. In this case, one would need to find two BVMO enzymes accepting the same substrate with high but opposite enantioselectivities (divergent enantioselectivies). Finding enzymes with these characteristics can be a very difficult task given the large number of potential microbial hosts.

Recently, one such enzymatic couple was identified to exist between the well-known and characterized cyclohexanone monooxygenase (CHMO) enzyme from *Acinetobacter* sp. NCIB9871[5,6] and the lesser known cyclopentanone monooxygenase (CPMO) enzyme from *Comamonas* (formally *Pseudomonas*) sp. NCIB9872.[7]

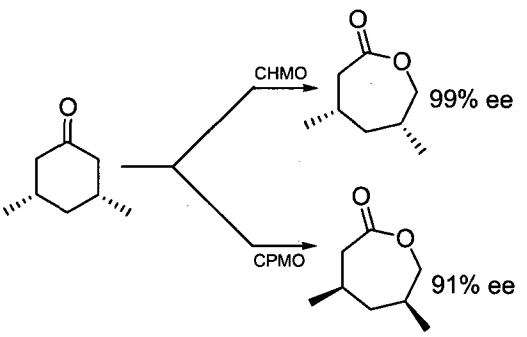


Figure 3-2: *Example of the bio-oxidation of cis-dimethylcyclohexanone illustrating the divergent enantioselectivities of CHMO and CPMO.*[22]

These two closely related enzymes were identified and isolated by Trudgill and co-workers about 25 years ago.[5,7] Initial studies found that CHMO and CPMO are related enzymes but of the two enzymes CHMO was shown to be highly enantioselective while CPMO was not, therefore further development of CPMO as a biocatalyst was not

undertaken. It was not until the work of Iwaki *et al.*, who characterized the gene cluster in *Comamonas* sp. NCIB9872 involved in cyclopentanol metabolism that interest in CPMO was rekindled (see **Figure 3-3**).[7,8]

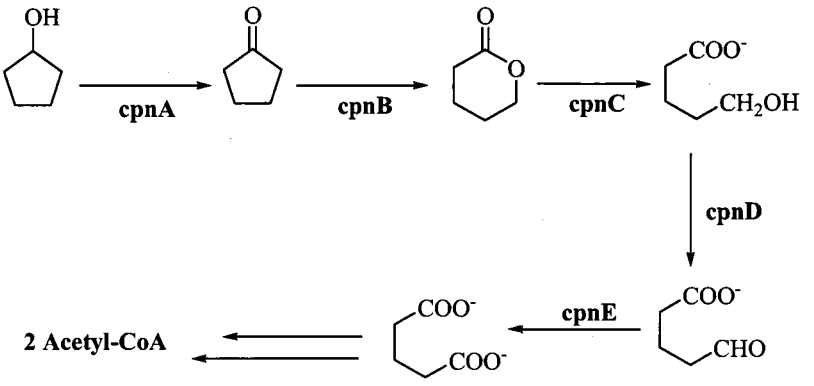
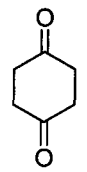
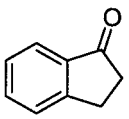
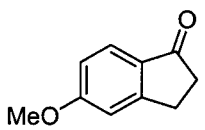


Figure 3-3: *Degradation pathway for cyclopentanol by* Comamonas *sp. NCIB9872, with possible β-oxidation leading to acetyl-coenzyme A.*[7]

Comparative studies of CHMO versus CPMO carried out in the Kayser lab verified that for several cyclohexanone and cyclopentanone substrates common to both CHMO and CPMO, the lactones obtained from CPMO oxidation possessed opposite but low enantioselectivity compared to CHMO.[8-10] While CPMO was found to have low enantioselectivities, it possesses a broad substrate base which may be even more extensive than that of CHMO making it a potentially valuable enzyme. **Table 3-1** shows three examples of reactions catalyzed by CPMO but not by CHMO. While it is desirable that an enzyme be highly selective, it is more important that it possesses a broad substrate base for a whole family of substrates. An enzyme which accepts a broad spectrum of

substrates, and which is robust, can be improved through enzyme modification and represents a good starting point for further development.

Table 3-1: *Biotransformations using CPMO and CHMO for three test substrates.*[8]

Ketone	*E. coli*/CHMO Yield %[a]	*E. coli*/CPMO Yield %[a]
 **3-1**	NR[a]	46
 **3-2**	NR[a]	98
 **3-3**	NR[a]	94

[a] Results for CHMO and CPMO catalyzed oxidation reported.[8]

In earlier work conducted by the Kayser group on CHMO, a more enantioselective CHMO mutant for a specific substrate was obtained using directed evolution (see Chapter 2).[11] In this case, the gene encoding for expression of the WT-CHMO was randomly mutated using error-prone PCR (ep-PCR). The resulting mutant enzymes (a total of 10 000) were screened for improvement in enantioselectivity for the desired target ketone and several mutants with enhanced enantioselectivity were identified.[11]

Since CPMO presents many of the same challenges as CHMO, i.e. a lack of X-ray crystal data and no knowledge of the actual active site make-up, it was felt that a similar strategy could be used to improve the enantioselectivity of CPMO. Since the gene (cpnB) encoding for the CPMO enzyme was initially cloned into a proprietary vector, the first step toward the development of a more enantioselective CPMO mutant was to place the CPMO gene into a known expression vector. This chapter describes the subcloning of the cpnB gene of CPMO into a known expression vector.

3-2 Sub-cloning of cyclopentanone monooxygenase

3-2-1 CpnB gene isolation and amplification

The gene encoding for CPMO (cpnB) was isolated from *Comamonas* sp. NCIB9872 and cloned by Iwaki *et al.*[8] during the study of the gene cluster involved in cyclopentanol metabolism. While this system was found to be quite good for general expression of CPMO in biocatalytic applications, little information was provided regarding the pSD80 vector used to carry and express the CPMO gene. We therefore decided to isolate the cpnB gene and clone it into a well characterized, commercially available plasmid vector, namely pET-22b(+).[12]

The choice of pET-22b(+) was based on its well established nature as a robust, and easy to handle expression system. This vector contains the pBR332 origin of replication[24] allowing for the autonomous capacity to make many copies of itself during the host's cellular replication cycle. To aid in colony selection, the expression vector (pET-22b(+)) contains an ampicillian resistance marker which ensures only bacteria containing plasmids will grow on agar plates which contain this family of antibiotics.

The vector also contains a broad array of restriction enzyme recognition sites giving pET-22b(+) the ability to accept genetic inserts of various sizes. A detailed vector map of the pET-22b(+) carrier containing the 1700 bp cpnB gene insert is shown in **Figure 3-4**.

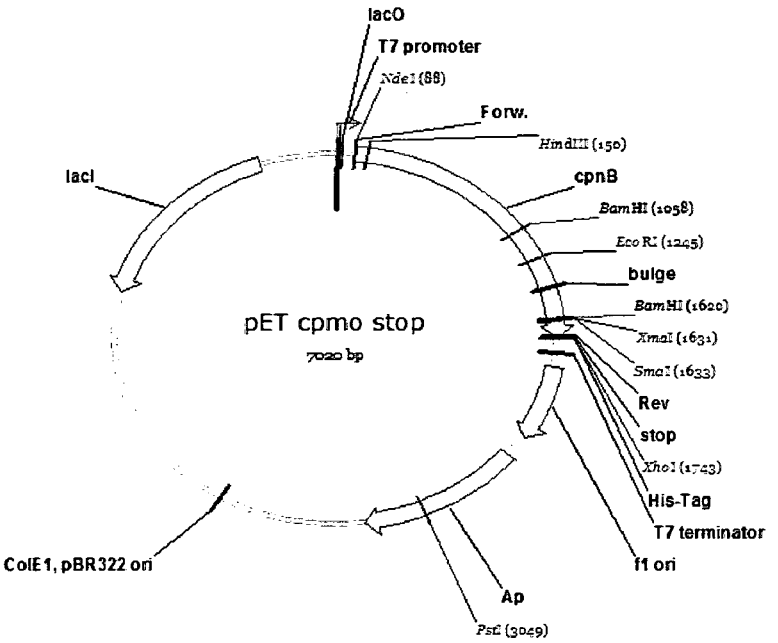


Figure 3-4: *Plasmid map showing the pET-22b(+) vector system containing the cpnB gene insert encoding for the CPMO enzyme.*

The pET-22b(+) vector system also contains an inset region which allows for the expression of a polyhistidine-tag protein, which is used in affinity purification of proteins. Like the earlier vector system, enzyme generation is under the control of a T_7 promoter, and upon induction with isopropyl-β-thio-D-galatoside (IPTG) it produces CPMO in large quantities.

To isolate the cpnB gene from the Iwaki plasmid[8] for further experimentation, the plasmid must first be isolated from the bacterial host. Therefore the Iwaki plasmid

was first isolated from *E. coli* using the Qiaprep spin miniprep kit® of QIAGEN. This method involves the initial isolation of the host bacterial cells from an overnight culture. The bacterial cells were harvested from the overnight culture *via* centrifugation, and the bacterial cell pellet was resuspended in a buffer. The cells were then broken using a chemical lysis agent in order to isolate the plasmid DNA from the cell. The crude plasmid DNA mixture was loaded on a small affinity column and after several wash steps the purified plasmid system was eluted from the column and the purity of the isolated plasmid was established by electrophoresis.

Once the plasmid DNA was isolated the cpnB gene was amplified using the polymerase chain reaction (PCR). In order to amplify only the cpnB gene, and not the entire plasmid vector, primers were designed to match the N-terminal (forward primer) and C-terminal (reverse complementary primer) regions of the cpnB gene. The primers used in this work are shown in **Table 3-2**.

Table 3-2: *Primers used for the PCR amplification of the cpnB gene encoding for CPMO.*

Primer designation	Primer nucleotide sequence[a,b]
CPMO-Forward	5′-GAGATATA**CATATG**ACCACCATGACCACC -3′
CPMO-Reverse complementary	5′-GTG**CTCGAG**TTAGCTGGCCAGCGAGAAG-3′
CPMO-Reverse complementary(Hist-tag)	5′-CAGTG**CTCGAG**GCTGGCCAGCGAGAAG -3′

[a] Primers used for PCR amplification were purified by HPLC prior to use by Invitrogen©.
[b] Restriction sites embedded in primers indicated by red, with the cleavage site underlined.

In designing the primers for sub-cloning into the pET-22b(+) vector system two points were considered. In the original cloning work[8] of Iwaki and co-workers a 1.7 Kb cpnB gene fragment was prepared which contained the Pst-I and Stu-I restriction sites. These sites were required in order to allow for correct overlap with the complementary restriction sites in their pSD80 vector located in the region after the strong T_7 promoter (Pst-I) and before the T_7 terminator (Stu-I) allowing for expression of the cpnB gene. The pET-22b(+) vector does not possess either of these restriction sites in the T_7 promoter regions, therefore new restriction sites had to be inserted into the cpnB gene to allow it to be inserted into pET-22b(+).

In this project, the Nde-I and Xho-I restriction endonuclease recognition sites, complementary to the T_7 promoter regions were inserted into the cpnB gene during PCR amplification.[12,13] It was also decided to take advantage of the ability of the pET-vector system to express enzyme variants which have a polyhistidine attached to the C-terminal region. Therefore primers that allow for the inclusion of this polyhistidine during protein expression were also designed (see **Table 3-2**). This hexa-histidine tagged protein could be purified which can usually be carried out using affinity chromatography and would allow for the use of the purified isolated enzyme, in either enzyme kinetics studies or for possible structural analysis.

To assess what are the optimal PCR amplification conditions are for annealing of the primers shown in **Table 3-2** to the cpnB gene, a PCR theromcycle program with a temperature gradient was used, and the results of the PCR amplification were analyzed by electrophoresis.

For the PCR amplification of the cpnB gene a 198 μL master mix of all the necessary reagents was prepared according to the protocol outlined in Table 3-3. A 30 μL aliquot of the PCR master mix was transferred into ten sterilized 200 μL PCR reaction tubes. The tubes were placed into a PCR thermocycler programmed to run the PCR reaction temperature profile shown in **Table 3-4**.

Table 3-3: *CPMO PCR master mix (master mix makes ten 30μL reactions).*

Reagent	Quantity (μL)
H_2O (sterile)	198
CPMO Forward Primer	6
CPMO Forward Reverse[(a)]	6
$MgSO_4$	12
CPMO template DNA	6
dNTPs[(b)]	30
KOD buffer (10H)	30
KOD Hotstart polymerase	6

[a] Refers to both regular and/or Hist-tagged primer. [b] Refers to a mixture of Deoxyribonucleoside Triphosphates.

Table 3-4: *Generalized PCR amplification thermocycler program implemented for the CPMO cloning project.*

Event	Temp. and time
1. Pre-heat thermocycler	94 °C and pause
2. KOD Hot-start Taq polymerase activation	94 °C for 2 minutes
3. DNA denaturation	94 °C for 20 seconds
4. Primer annealing	Temp gradient used (56.5 °C + n °C per well)[(a)] for 1 min
5. Elongation step	72 °C for 1 minute
Repeat steps 3-5 for 30 PCR cycles	
Final DNA elongation	72 °C for 30 minutes
Cool	4 °C and pause

The success of the PCR reaction was checked by agarose gel electrophoresis (Figure 3-5), and all successful PCR reactions were purfied by affinity chromatography (Qiagen QIAquick PCR purification method).

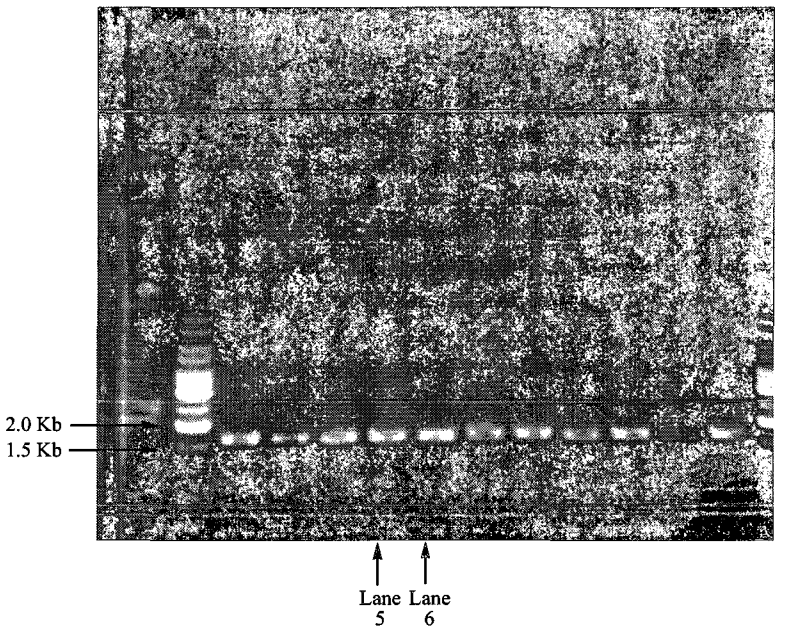


Figure 3-5: *Agarose gel electrophoresis picture of the PCR amplification of the cpnB gene using the primers shown in Table 3-1.*

As can be seen from **Figure 3-5**, the genes visualized in wells 2 to 12 are of the correct size (1.7 Kb) for the cpnB gene of CPMO. Using the intensity of the visualized bands as a qualitative guide, the most efficient PCR reactions were those visualized in wells 5 and 6. The annealing temperatures for the primers in those wells correspond to 57.5°C and 57.8°C, respectively. Based on this result, all further PCR amplifications

involving the primers given in **Table 3-1** were done at an annealing temperature of 57.8°C.

3-2-2 Preparation of cpnB insert and pET-22b(+) vector

Once the cpnB gene is successfully amplified by PCR, it must be inserted into the desired pET-22b(+) vector system. To do this both the cpnB gene and the pET-22b(+) must be readied to receive one another by subjecting them to a process called restriction digestion.[14] Restriction digestion involves cleavage of the genetic material in a very specific way using special enzymes called restriction endonucleases. More specifically, restriction endonucleases (also known as restriction enzymes) cleave a gene at specific positions in the nucleotide sequence based on the presence of specific recognition sites within the gene. Each restriction enzyme has a unique nucleotide sequence at which it cleaves the gene (see **Figure 3-6**).

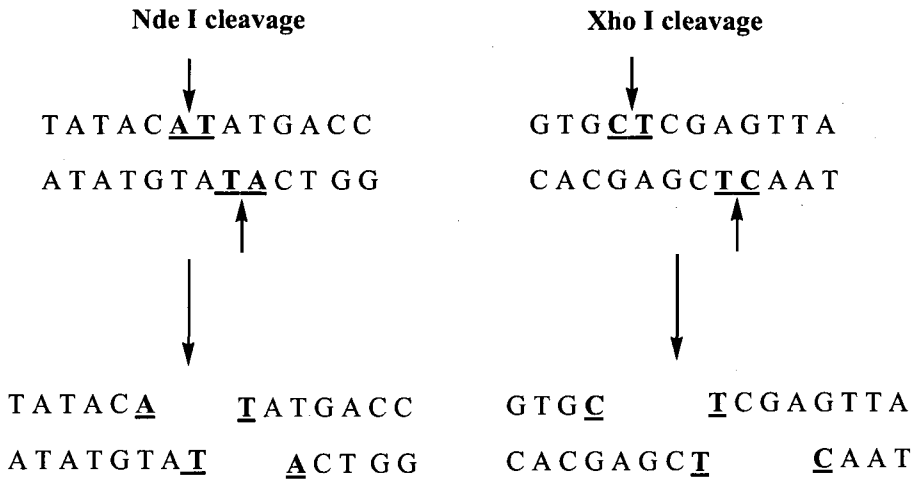


Figure 3-6: *Example of the restriction digestion of a nucleotide sequence for the NdeI and XhoI restriction endonucleases.*

When the restriction enzymes make "cuts" in the gene sequence, they do so in a staggered fashion (**Figure 3-6**) leaving behind a single strand of the nucleotide sequence to overhang at either the 5′ or 3′ end of the nucleotide sequence. These overhanging sequence regions are known as "sticky ends" of the digested gene, since they have the ability to bind a complementary overhanging region of another nucleotide sequence. It is this binding interaction which allows for a digested piece of a gene encoding for a given enzyme to be inserted into a host expression vector which has undergone a similar restriction digestion treatment. During the PCR amplification process of the cpnB gene, primers containing the Nde-I[15] (**CATATG**) and Xho-I[16] (**CTCGAG**) restriction sites were used, which were complementary to the restriction sites located in the pET-22b(+) vector system (see **Figures 3-4** and **3-6**).

Restriction digestion reactions for the pET-22b(+) vector and cpnB gene insert were performed using standard conditions[11,14] as shown in **Tables 3-5** and **3-6**.

Table 3-5: *Restriction digestion protocol for pET-22b(+).*

20 μL vector DNA (pET-22)
1.5 μL NdeI restriction enzyme
1.5 μL XhoI restriction enzyme
5 μL reaction buffer (Buffer#4)
22 μL H_2O (sterile)
Total volume= 50μL reaction

Table 3-6: *Restriction digestion protocol for the cpnB gene insert.*[a]

17 μL genomic DNA (CPMO)
3 μL NdeI restriction enzyme
3 μL XhoI restriction enzyme
5 μL reaction buffer (Buffer#4)
23 μL H_2O (sterile)
Total volume= 50μL reaction

[a] This digestion protocol was used for both the regular and Hist-Tagged CPMO gene.

The results of the digestion reactions were checked by electrophoresis. To remove any unwanted endonuclease and digested DNA by-products, which may inhibit subsequent microbiological steps, the digested product was purified by agarose gel electrophoresis.[17] The bands corresponding to the pET-22b(+) and cpnB gene were excised from the gel. The gel containing the DNA was frozen and the DNA separated from the gel using the freeze 'N squeeze® technique (Bio-Rad® Inc.).[17] The DNA isolated by gel extraction was further purified and concentrated by ethanol precipitation using the Pellet Paint® precipitation protocol of Novagen®.[18]

3-2-3 Ligation of cpnB with pET-22b(+) and transformation into *E. coli*

Once the cpnB gene and pET-22b(+) vector were digested and the resulting products purified, the two DNA fragments were ready to be coupled together. When the two DNA fragments (insert and vector) are mixed together, the complementary sticky-ends obtained from restriction digestion bind together held in place by hydrogen bonds between the nucleotide bases. This hydrogen-bonding is not strong enough to hold the two nucleotides together permanently. This problem can be overcome through the use of

an enzyme called T4 DNA ligase, which catalyzes the formation of phosphodiester bonds at the ends of DNA strands already being held together by base pairing.[14,19]

The reaction mixtures used for the ligation procedure is given in **Table 3-7**. To optimize the ligation procedure and as a control to ensure only colonies which have the pET-22b(+) vector containing the cpnB are obtained, six ligation reactions were done. The ligation reactions -2 and -1 were controls that do not contain the cpnB gene insert in the mixture. Reaction mixtures 0, 0A, +1, and +1A, contain both the cpnB gene and the pET-22b(+) for ligation. Each of the ligation reactions was done on a 10 μL scale in sterile microcentrifuge tube. Each ligation reaction was incubated at 16 °C for 16 hours without shaking.

Table 3-7: *Ligation reaction conditions for pET-22b(+) and CPMO gene ligation*[a,b].

-2: 1μL digested vector 0.5 μL ligation buffer (10H) 3.5 μL H_2O	-1: 1μL digested vector 0.5 μL ligation buffer (10H) 3.5 μL H_2O 0.5 μL T4 DNA ligase
0: 1μL digested vector 3 μL Insert (cpnB gene) 1 μL ligation buffer (10H) 4 μL H_2O 1 μL T4 DNA ligase	0A: 1μL digested vector 3 μL Insert (cpnB gene with Hist-Tag) 1 μL ligation buffer (10H) 4 μL H_2O 1 μL T4 DNA ligase
+1: 1μL digested vector 6 μL Insert (cpnB gene) 1 μL ligation buffer (10H) 1 μL H_2O 1 μL T4 DNA ligase	+1A: 1μL digested vector 6 μL Insert (cpnB gene with Hist-Tag) 1 μL ligation buffer (10H) 1 μL H_2O 1 μL T4 DNA ligase

[a] All reactions are 10μL total volume; b. Ligation reactions were incubated at 16 °C for approximately 16 hours.

Once the cpnB gene was successfully inserted into the vector it must then be uptaken by *E. coli* for replication of the vector and expression of the enzyme.[20,21] The ligation product obtained from the coupling of the cpnB gene target with pET-22b(+) was immediately transformed into the chemically competent *E. coli* BL21(DE3) host strain for expression.[14,19] For the transformation of plasmid DNA into chemically competent *E. coli* cells, approximately 300 μL of chemically competent BL21(DE3) which was stored at -80 °C was warmed to 0 °C in an ice bath. Six 1.5 mL sterile microcentrifuge tubes were pre-cooled in an ice bath. Then 50 μL of BL21(DE3) cells were pipetted gently into each 1.5 mL cooled microcentrifuge tube along with 2.5 μL of ligation product and were mixed together with a pipette tip. The transformation was incubated on ice for 90 minutes, then heat-shocked at 42 °C for 30 seconds. The heat-shocked cells were placed back in an ice bath for 1-2 minutes, and then 50 μL of LB medium (no antibiotic) was added to each tube. The transformations were incubated at 37 °C and shaken at 700 rpm for 45 minutes.

A 50 μL portion from each ligation reaction was plated on LB agar plates and incubated at 37 °C overnight. Colonies were obtained on plates from ligation reactions 0, 0A, +1, and +1A. No colonies were found on control plates –1 and –2. To confirm the colonies on plates 0, 0A, +1, and +1A actually contained the vector + insert five colonies on each of the plates were randomly picked. The colonies were then placed into approximately 5 mL of LB medium and the culture grown overnight. The plasmids from these overnight cultures were isolated, and subjected to PCR amplification reaction using the primers designed for the cpnB gene (see **Table 3-2**). Agarose gel analysis of the PCR product indicated the success of the ligation reaction which was confirmed by gene

sequencing. The newly transformed *E. coli* containing the newly cloned pET-22b(+) with the CPMO gene was streaked out on LB-Ampicillin agar plates and incubated at 37 °C until colonies were 1-2 mm in size.

Colonies for both the cpnB gene expression alone as well as the cpnB gene coexpressed with the Hist-tagged gene were used to make precultures according to general procedures outlined in the experimental section. The precultures for both of these CPMO expression systems were used to isolate plasmid DNA, which was sent for DNA sequencing. The integrity of both newly cloned systems was confirmed by DNA sequencing which verified the presence of the cpnB gene within the pET-22b(+) vector system.

3-3 General experimental Section

All chemicals were used as received from the suppliers. The KOD-Hot start polymerase, deoxyribonucleoside triphosphates (d-NTPs), and associated buffers were purchased from Novagen. Oligonucleotide primers used in this work were synthesized and purified by HPLC by Invitrogen. The restriction endonucleases Xho-I and Nde-I were purchased from Roche chemicals and New England Biolabs, respectively. The T4 DNA ligase enzyme was obtained from Novagen. All water added to any biological reactions was distilled, deionized, and autoclaved prior to use. All PCR reactions and electrophoresis experiments were done using equipment purchased from Biometra. The gels used in the qualitative analysis work contained a 0.7% agarose concentration with 1 μL of ethedium bromide added for UV visualization. The thermomixer and microcentrifuges used were from Eppendorf Co. All UV spectrophotometric work was done using an Eppendorf UV photometer at 260 nm.

3-3-1 Propagation of *E. coli* strains

The *E. coli* strain DH5α(pCPM201) or BL21(DE3)(pET-22b(+)) was streaked from frozen stock on to LB-Ampicillin plates and incubated at 37 °C until the colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and incubated at 37 °C and 250 rpm overnight. The culture was divided in half and an equal volume of sterile glycerol was added to one of them. The mixture was then divided into 0.5 mL aliquots and stored in a -80 °C freezer. All carrier *E. coli* host strains were propagated using this protocol.

3-3-2 Procedure to make chemically competent cells

From a single colony of BL21(DE3), a 5 mL overnight culture was inoculated and grown at 37 °C for 12-14 hours. Then 5 mL of pre-culture was used to inoculate a flask containing 300 mL of LB (no antibiotic). The cells were grown at room temperature until an OD_{600} = 0.6, then placed on ice for 15 minutes. The cells were then transferred to a precooled 50 mL centrifuge tube and spun at 4 °C; the supernatant liquid was discarded and the pellet was resuspended (**not pipetted**) in 35 mL of ice-cold 10 mM NaCl, and left on ice for 10 minutes. The cells were spun and then resuspended in 35 mL of ice-cold 75 mM $CaCl_2$ and kept on ice for 35 minutes. The cells were spun and then resuspended in 3 mL of ice-cold 75 mM $CaCl_2$ along with 0.5 mL of 100% glycerol and mixed gently by inversion and stored on ice for 1 hour. Then, 0.5 mL aliquots of the cells were transferred into sterile microcentrifuge tubes and shock frozen by placement in liquid nitrogen. The frozen cells were then stored at -80 °C freezer until needed.

3-4 References

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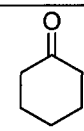
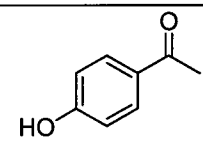
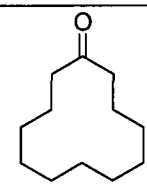
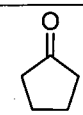
Chapter 4

Rational development of new Baeyer-Villigerases: Application of Combinatorial Active Site Saturation Testing (CAST).

4-1 Introduction

Over the last two decades developments in biotechnology have spawned a dramatic increase in the number of proteins which have been isolated, characterized, and subsequently developed as new biocatalysts. The Baeyer-Villigerase family of enzymes, capable of performing enzymatic Baeyer-Villiger oxidation, has also undergone significant growth and development. A recent review article reported that over the last 5 years, seven new BVMO enzymes have been characterized and their potential as bio-reagents tested.[1] Some of the most interesting are shown in **Table 4-1**.

Table 4-1: *Some recently identified and/or cloned BVMO enzymes along with the natural substrate which they accept.*[1]

BVMO (parent organism)	Date identified (cloned)	Native substrate
Cyclohexanone monooxygenase (*Anthrobacter BP2*)[2]	2003 (2003)	
Hydroxyacetophenone monooxygenase (*Pseudomonas fluorescence* ACB)[3]	2001 (2001)	
Cyclododecanone monooxygenase (*Rhodococcus* SC1)[4]	2001 (2001)	
Cyclopentanone monooxygenase (*Comamonas* NCIMB 9872)[5,6]	1976 (2002)	

Several of the new BVMOs were identified through the application of genome mining, a powerful tool which identifies characteristic sequence fingerprints or motifs for a target protein within a host microorganisms genome.[7, 8]

While the identification and cloning of new BVMO enzymes has an important role to play in the increasing pool of new biocatalysts, the further development of previously identified and existing BVMOs must be continued. One such BVMO, which was identified nearly 30 years ago, but was only recently cloned from its bacterial host organism, is cyclopentanone monooxygenase (CPMO) from *Comamonas sp.* (NCIMB 9872).[5,6] Screening studies using CPMO have found it to possess a generally broad substrate base (see Fig. 4-1), accepting a range of C_4 to C_6 cyclic ketones, norbornanone, and substituted indan-2-one and indan-1-one substrates.[6,9]

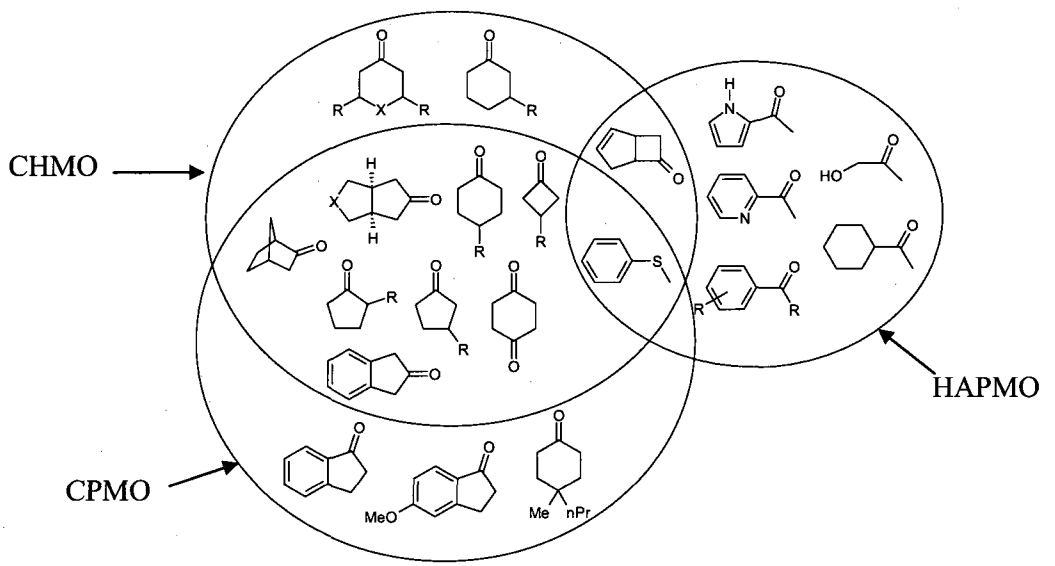
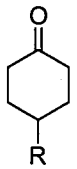


Figure 4-1: *Comparison of the substrate acceptance profiles for three BVMOs (CHMO, CPMO, and HAPMO).*

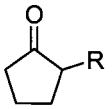
Unfortunately, CPMO has been found to be quite unselective for many of these substrates, a fact that was confirmed in recent studies published by Wang *et al.*, where CPMO was tested for its ability to accept a wide range of 2-substituted cyclopentanone and 4-substituted cyclohexanone rings (see **Tables 4-2** and **4-3**).[10, 11]

Table 4-2: *Bio-oxidation of selected 4-substituted cyclohexanones with CPMO to the corresponding caprolactones.*[10]

Substrate	Lactone		
	R	Yield (%)[a]	ee (%)
	Me	68	46 (***R***)
	n-Pr	68	36 (***S***)
	OH[b]	73	85 (***S***)
	OMe	71	28 (***S***)
	OEt	90	37 (***S***)
	OAllyl	80	45 (***S***)
	OAc	81	5 (***S***)

[a] All yields indicated are isolated yields. [b] The caprolactone product from the BVMO bio-oxidation of 4-hydroxycyclohexanone undergoes spontaneous rearrangement to give a five membered γ-butyrolactone.

Table 4-3: *Bio-oxidation of selected 2-substituted cyclopentanones with CPMO to the corresponding valerolactones.*[11]

Substrate	Ketone		Lactone	
	R	Yield (%)	ee (%)	Yield (%)	ee (%)
	allyl	5	-	95	-
	n-but	89	2 (***R***)	11	19 (***S***)
	cyclopentyl	54	-	46	16 (***?***)
	CH_2OH	95	12 (***S***)	5	43 (***R***)
	CH_2OCH_3	22	-	78	56 (***?***)
	$CH_2OAllyl$	70	-	30	81 (***R***)
	CH_2OAc	59	-	41	53 (***R***)

The problem of poor selectivity for a BVMO catalyzed reaction was recently addressed by the use of recombinant gene technology as descriped in chapter 2. Thus, WT-CHMO was evolved to improve its selectivity for a model reaction involving the bio-oxidation of 4-hydroxycyclohexanone (which WT-CHMO converts to the butyrolactone with a rather low selectivity (9% ee *R*)). After exposure of the WT gene to several rounds of error-prone PCR (ep-PCR), mutated enzymes showing enhanced selectivity towards 4-hydroxycyclohexanone were identified (**Table 4-4**). Sequencing showed that the most productive mutations enhancing the selectivity of the WT-CHMOs (for both the *S* and *R* directions) were clustered near the 432 or 143 positions, respectively. Further screening revealed that one mutant in particular, 1-K2-F5 (Phe432Ser), was a very active and highly selective bio-oxidation catalyst for several ketones.[14,15]

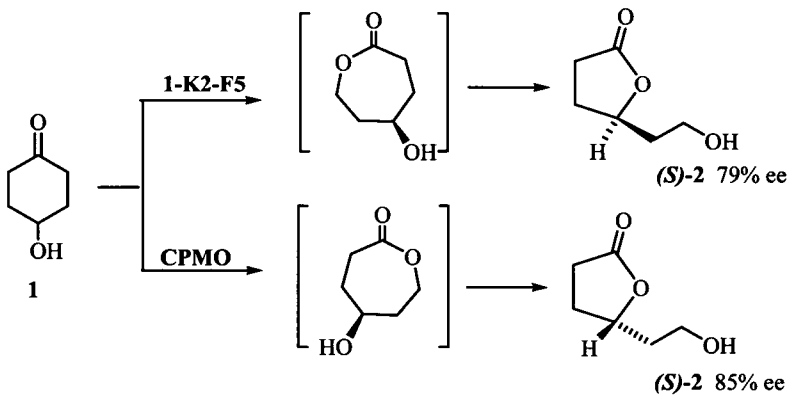
Table 4-4: *Conversion and selectivity for the best 1st generation CHMO mutant enzymes against 4-hydroxycyclohexanone.*[13]

CHMO	Result[a]
WT	100 (9% *R*)
I-K6-G2 (F432S)	100 (79% *S*)
I-F4-B9 D41N,F505Y	100 (46% *S*)
I-E12-B5 (F432I)	100 (49% *R*)
I-F1-F5 (L143F)	100 (40% *R*)

[a] Based on GC conversion, with selectivity given in parenthesis.

Comparison of the results obtained for the bio-oxidation of 4-hydroxycyclohexanone using CHMO (WT and mutants) and WT-CPMO showed a rather intriguing similarity in selectivities for 1-K2-F5 and WT-CPMO (see **Scheme 4-1**).

Scheme 4-1



Sequence alignments were done using the CLUSTAL-W sequence alignment server in order to compare WT-CHMO, WT-CPMO, and the 1-K2-F5 CHMO mutant, the results of which are shown in **Figure 4-2**.[17] The sequence alignment revealed that the serine residue introduced at position 432 in the the *S*-selective 1-K2-F5 mutant is in close proximity to a serine in the already *S*-selective WT-CPMO.

```
CPMO-WT_PNLLFGYGPQSPAGFCNGPSS
CHMO-WT_PNMFMVLGPNGP--FTNLPPS
CHMO-MU_PNMFMVLGPNGP--STNLPPS
```

Figure 4-2: *Truncated sequence alignment of the wild types CHMO, CPMO and the 1-K2-F5 CHMO mutant with a newly acquired S selectivity.*

Chapter 5 describes a rapid and inexpensive approach, which can be used to improve selectivity or extend substrate acceptability of BVMOs. Using the mutational information gained from the previous directed evolutionary study of CHMO, a semi-rational approach was employed to improve the enantioselectivity of CPMO. This semi-rational approach can be used when information about the residues in close proximity of an active site is available, either through structural or mutational information.

4-2 Rational improvement of CPMO

4-2-1 Homology modeling

Recently, through the application of genomic mining, an interesting development occurred in the field of BVMO research with the discovery of a novel, thermally stable BVMO.[16] The new Baeyer-Villigerase, phenylacetone monooxygenase (PAMO), isolated from the thermophilic bacteria *Thermobifida fusca* is thus far the only BVMO protein stable enough to produce crystals suitable for X-ray analysis. The X-ray structure of PAMO (PDB identification:1W4X) is shown in **Figure 4-3**.

Figure 4-3: *X-Ray crystal structure of phenylacetone monoxygenase.*[16]

The PAMO structure shown in **Figure 4-3** was crystallized with only the FAD co-factor bound in the active site; unfortunately, attempts to co-crystallize the protein with the NADPH co-factor were unsuccessful. In order to assess the degree of sequence similarity between WT-PAMO, WT-CHMO, and WT-CPMO a full sequence alignment was conducted using the CLUSTAL-W program, the results of which are given in **Figure 4-4**.[17]

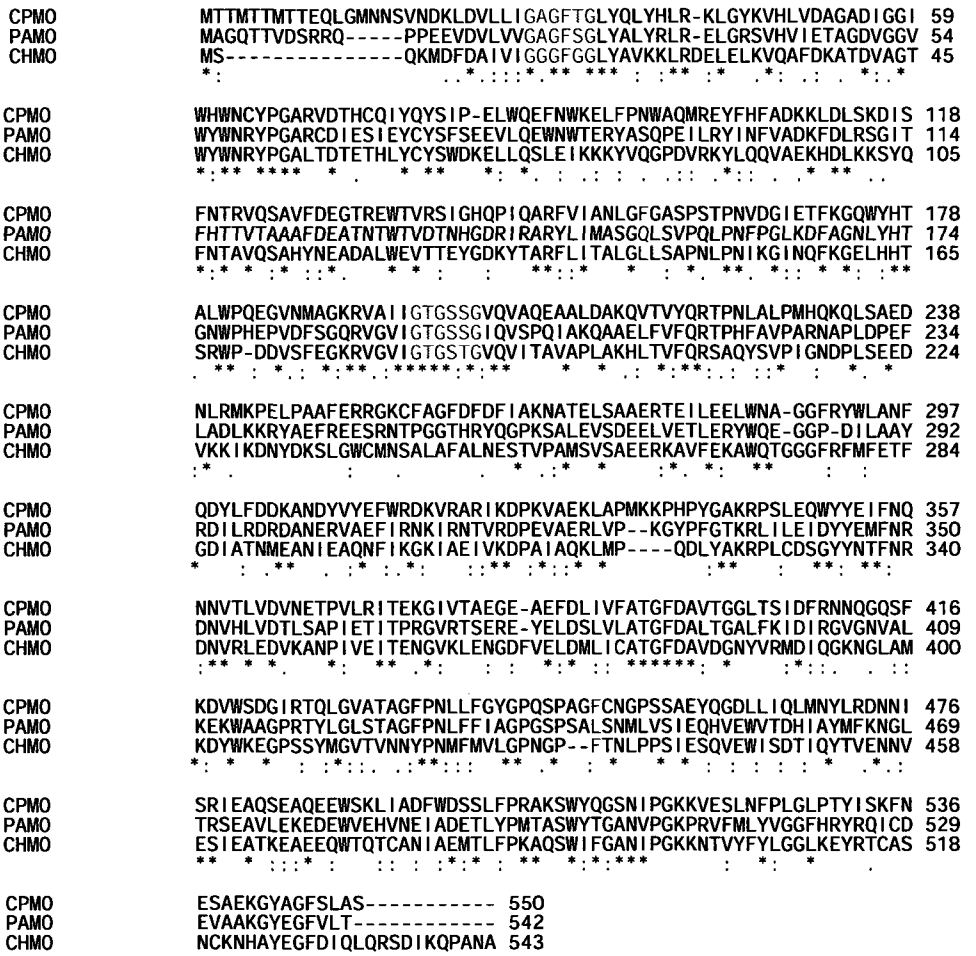


Figure 4-4: *Sequence alignment for PAMO, WT-CPMO, and WT- CHMO using ClustalW.*[17]

Overall CPMO and CHMO were found to possess 40% and 39% sequence homology, when compared to PAMO, respectively, versus a 36% homology compared with each other. Closer inspection of the sequences in **Figure 4-4** showed the Flavin

(FAD) and NADPH co-factor binding motifs, GXGXXG also known as the Rossmann fold motif (highlighted in green), were completely conserved.[18,19] The "hot-spots" identified at positions 143 and 432 (CHMO numbering) from the directed evolution of CHMO are highlighted in red.

Based on the high primary sequence homology that WT-CHMO and WT-CPMO have with PAMO, it is possible to make 3D structural predictions using the PAMO X-ray structure as a template. Homology modeling using the CPHmodel 2.0 program was performed for WT-CHMO and WT-CPMO with the results given in **Figure 4-5**.[20]

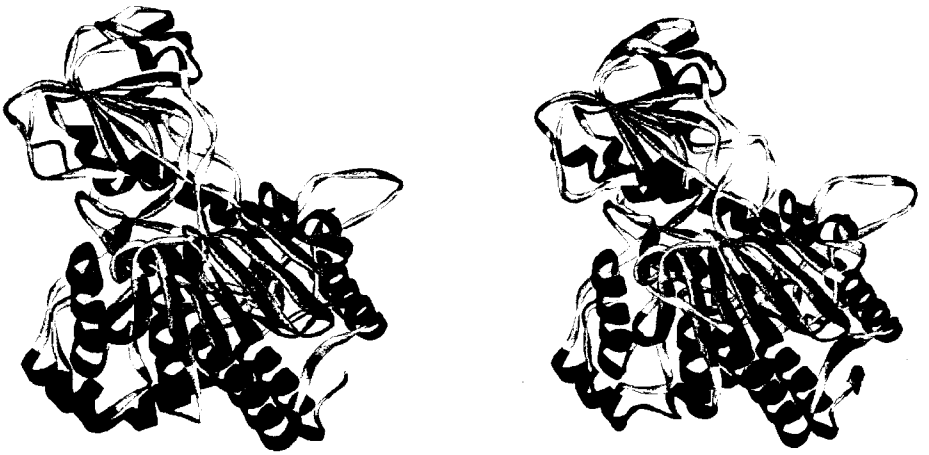


Figure 4-5: *Homology models for the CHMO (left) and CPMO (right) monooxygenase enzymes.*

Overlaying the PAMO X-ray structure with the homology models generated for WT-CHMO and WT-CPMO illustrate high conservation of the secondary structural elements within the family, with rms deviations in the homology models of 2.4 and 2.6

Å, respectively for WT-CHMO and WT-CPMO when compared to the PAMO X-ray structure.

Examination of the homology model of WT-CHMO allows pinpointing evolutionary "hot-spots" at the Leu143 and Phe432 positions both of which are found within the active site of CHMO. Overlap of the CHMO and CPMO models allowed for the identification of the analogous positions in CPMO. The Leu143 in CHMO was found to correspond to the Phe156 in CPMO while the Phe432 of CHMO corresponded to Phe450 in CPMO. Based on the influence these sites had on selectivity for CHMO the corresponding sites in CPMO represent attractive targets for mutation to improve selectivity of CPMO.

4-2-2 Rational mutation of CPMO

Once the sites in the WT-CPMO gene were identified for targeted (or focused) mutation, the next step was to decide on an overall mutational strategy. The strategy which was chosen combined the elements of rational design and random mutagenesis into a single step. This new mutational strategy, known as Complete Active Site Saturation Testing (CASTing), was recently developed by Reetz and co-workers to expand the substrate acceptance of an enzyme.[21] More specifically, Reetz *et al.* used CASTing to expand the substrate acceptance of a hydrolase enzyme (isolated from *Pseudomonas areuginosa*) to accept a variety of bulky carboxylic acid esters, which are poorly accepted by the wild type lipase. We felt this technique could also be applied towards the rational improvement of other enzymatic properties, such as enzymatic selectivity.

In principle CASTing is a very straightforward technique which requires the design and generation of small focused libraries of enzyme mutants produced by

simultaneous randomization of two sets of spatially-close amino acid positions around the active site (see **Figure 4-6**). The choice of spatially-close amino acids allows for potential synergistic conformational effects which could arise from the various side chain orientations. These synergistic effects are unpredictable and cannot be mimicked by single site saturation mutagenesis.

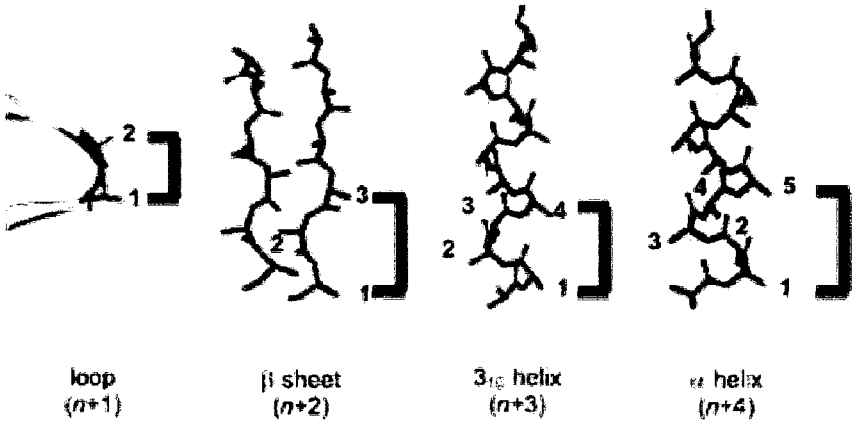


Figure 4-6: *Illustration of the basic secondary structural guidelines which are used when choosing mutational positions* (reproduced with the permission of M. T. Reetz).

To simplify the choice of amino acid positions required for inclusion in a CAST library, the choice of the CAST pair is governed by the side chain positions of the pair (they must point into the active site region of the enzyme), and by the secondary structure of the region to undergo modification (see **Figure 4-6**). Thus, if a member of

the pair occurs at position **n**, then the second position is found at the sequence position (**n+1**) for a loop, (**n+2**) for a sheet, and (**n+4**) for an α-helix.[22, 23]

Once the mutation positions are identified using the aforementioned secondary structural element guidelines, conventional saturation mutagenesis can used to induce the mutations. Saturation mutagenesis, also known as oligonucleotide directed mutagenesis, involves the design of oligonucleotide primers which bind to a specific region of the gene encoding sequence during a PCR applification. These oligonucleotide primers have regions which contain a random mix of incorrect nucleotide bases which can potentially encode for all 20 proteinogenic amino acids (**Figure 4-7**).

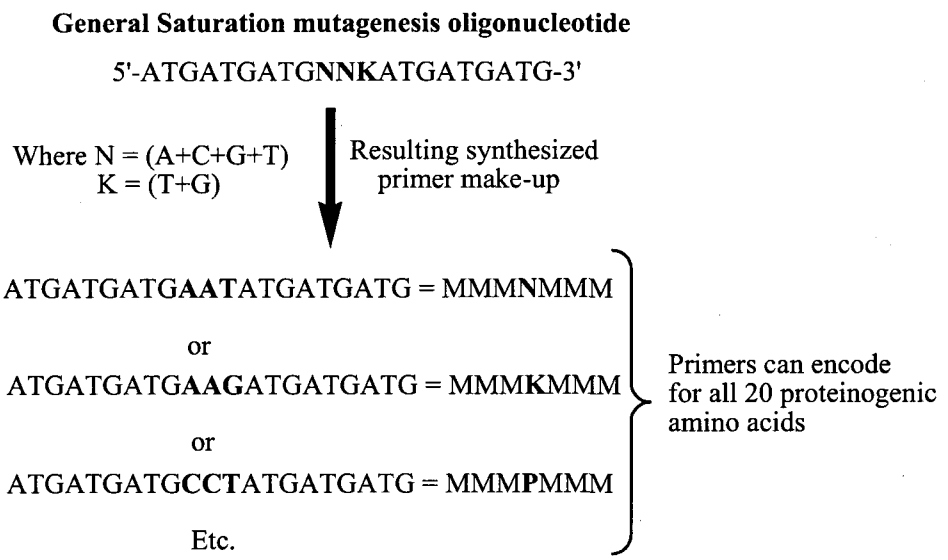


Figure 4-7: *General illustration of a saturation mutagenesis oligonucleotide primer set.*

Using the basic principles of CAST outlined above, a similar mutation strategy for CPMO was embarked upon, focusing on the Phe156 and Phe450 positions of CPMO as sites for the CAST libraries.[24] Based on the positions within the active site and the

CASTing rules for loop regions outlined above, the positions G449F450 (library A) and F156G157 (library B) were chosen as the sites to perform the CAST experiments. To further limit the amount of screening effort, a variation on the CAST theme involved the use of a reduced saturation oligonucleotide cassette (NDT codon) rather then a full saturation oligonucleotide cassette (NNK codon) was used. This variation on the CAST method has been termed restricted CAST. Restricted CAST simply means that rather then complete randomization at each position with the 20 possible amino acids, a randomization strategy which results in a limited number of amino acid exchanges, in this case 12 possible exchanges, of the WT amino acids can theoretically occur. This reduction in cassette size does not limit the impact of the technique since the reduced NDT codon cassette was designed to encode for a general sampling of amino acid types, as illustrated in **Table 4-5**. The advantage that arises from using a smaller set of amino acids is that the number of mutant clones which must be screened to ensure a 95% coverage of all possible amino acid combinations is significantly reduced.

Table 4-5: *Overview of the various reduced codon cassettes and the amino acids for which they encode.*

NDT[a] (General)	GBT (Hydrophobic)	NAK (polar)	YRT (small active)
Phe, Leu	Val	Tyr	Tyr
Ile, Val	Ala	His	Cys
Tyr, His	Gly	Gln	His
Asn, Asp	-	Asn, Asp	Arg
Cys, Arg	-	Lys	-
Ser, Gly	-	Glu	-

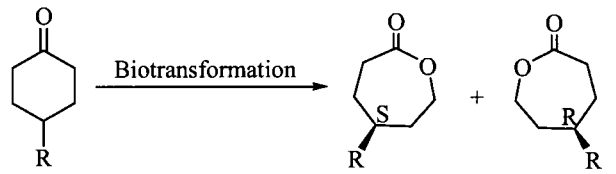
[a] Where N=A+T+C+G, D=A+T+G, and T=T.

When the NNK degenerate saturation codon is applied to randomize two positions simultaneously approximately 3000 clones need to be evaluated to ensure that 95% coverage was attained. For the NDT degenerate set, only about 430 clones need to be evaluated to ensure 95% coverage, a fact which significantly reduces the screening effort required, thus making reduced CASTing a more rapid and cost effective process.

4-3 Construction and evalution of CPMO mutants

For the evaluation of the CPMO mutants from libraries A and B three compounds, illustrated in **Scheme 4-2**, were selected as probe substrates. These substrates **4-1a** and **4-1c** were chosen based on their poor selectivities when subjected to WT-CPMO bio-oxidation or their lack of acceptance by WT-CPMO as in the case for **4-1b**.

Scheme 4-2



4-1a R=Me
4-1b R=*t*-Bu
4-1c R=OAc

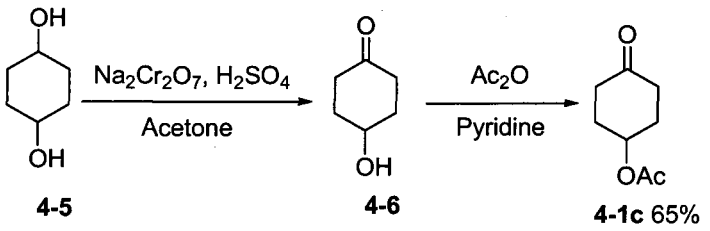
4-2a R=Me
4-2b R=*t*-Bu
4-2c R=OAc

4-3-1 Substrate synthesis

The synthetic protocol for the preparation of substrates **4-1c** is shown in Scheme 4-3.[25] Commercially available 1,4-cyclohexanediol **4-5** was oxidized using Jones' reagent to give 4-hydroxycyclohexanone **4-6** in good yield. The resulting 4-

hydroxyketone **6** was acetylated by exposure to acetic anhydride in pyridine to give the desired 4-acetoxycyclohexanone **4-1c** in 65% yield.

Scheme 4-3



4-5 **4-6** **4-1c** 65%

4-3-2 Construction of the CPMO mutants

The CPMO mutants used in this study were generated *via* the Quik-Change polymerase chain reaction (PCR) method using the oligonuleotide saturation mutagenesis primers given in Table 4-6.[26] These primers were designed to incorporate the desired mutations at the corresponding 156/157 and 449/450 positions of the CPMO gene. The resulting mutated CPMO plasmids were transformed directly into the *E. coli* BL21(DE3) host strain. The mutants grown on agar plates produced a large number of colonies of which 150 were harvested from each of two libraries for screening. The colonies were placed into deep-well culture plates containing LB grown media. Following the growth of the bacterial cultures, protein expression was induced by the addition of isopropyl-β-D-thioglacatopyranoside (IPTG), which was rapidly followed by substrate addition. All the reactions were allowed to proceed for 24 hours after which time the cultures were

extracted with ethyl acetate with the results of each reaction checked by chiral-phase GC analysis.[28]

Table 4-6: *Primers used for the generation of CPMO CAST libraries.*

Primer	Primer nucleotide sequence[a]
	Library A (449/450)
CPMO Forward CPMO Reverse	5′-GTCATCGCCAACCTTGGC**NDTNDT**GCCAGCCCCAGCACG-3′
	Library B (156/157)
CPMO Forward CPMO Reverse	5′-GGACCTCAATCGCCTGCG**NDTNDT**GCAACGGTCCGTCGAGC-3′

[a] Primers used for PCR amplification were purified by HPLC prior to use by Invitrogen©.

4-3-3 CPMO mutant evaluation

All mutant evaluation reactions, for the library A and B clones, were performed on a microscale (200 μL media in 96 deep-well plates) at room temperature and allowed to run for 24 hours to assess the activity and selectivity of the mutants. Unfortunately, no mutants capable of transforming 4-*tert*-butylcyclohexanone **4-1b** were identified in this study. Several mutants from both libraries did show enhanced selectivity for the 4-methylcyclohexanone **4-1a** and 4-acetoxycyclohexanone **4-1b** substrates. **Figure 4-8** illustrates the distribution of enantioselectivities for the bio-oxidation of 4-methylcyclohexanone **4-1a** using the CPMO mutants from both libraries (A and B). While several clones were identified which possessed enhanced *R* selectivies, none of the clones, from either library, yielded the corresponding *S* enantiomer.

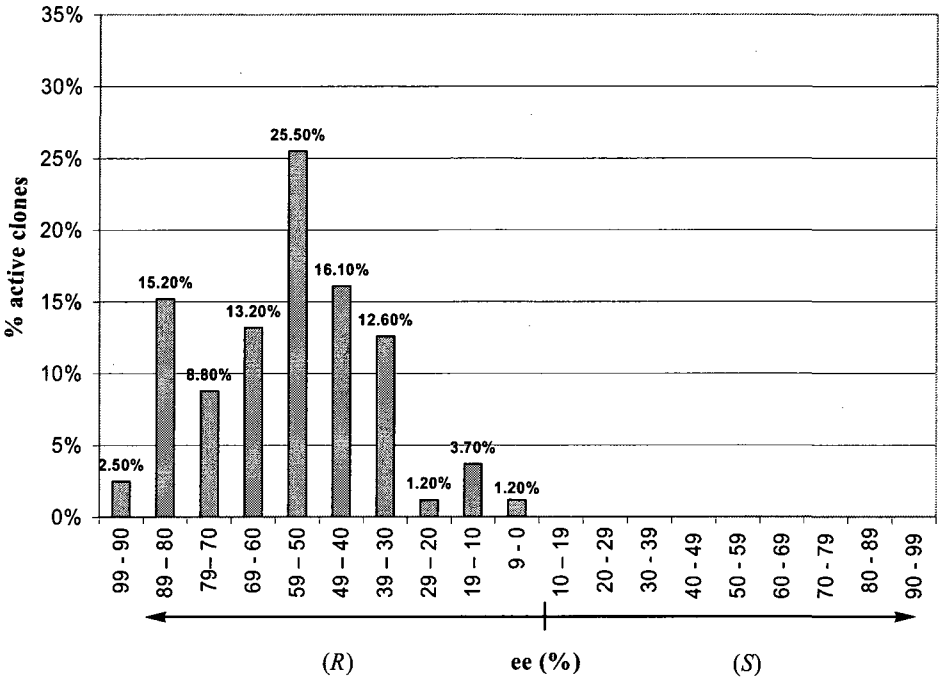


Figure 4-8: *Distribution of selectivities for the CPMO mutant bio-oxidation of the 4-methylcyclohexanone substrate.*

The results for select mutants which have been sequenced to determine their mutational characteristics are reported in **Tables 4-7** and **4-8** for the 4-methylcyclohexanone (**4-1a**) and 4-acetoxycyclohexanone (**4-1b**) substrates, respectively.

Table 4-7: *Select results obtained for the screening of the CPMO CASTing mutants for 4-methycyclohexanone to the corresponding caprolactone.*[24]

CPMO variant	Conversion (%)[a]	ee (%)[b]
Library A (449/450)		
WT-CPMO	100	46 *R*
A1-A3 (GlyPhe→SerTyr)	27	65 *R*
A1-A10 (GlyPhe→GlyIle)	74	92 *R*
A1-B5 (GlyPhe→AsnPhe)	65	31 *R*
A1-E1 (GlyPhe→ValTyr)	54	33 *R*
A1-F12 (GlyPhe→GlyCys)	**37**	68 *R*
Library B (156/157)		
B1-A3 (PheGly→LeuGly)	42	16 *R*
B1-A10 (PheGly→LeuPhe)	81	91 *R*
B1-A8 (PheGly→TyrTyr)	22	8 *R*
B1-C3 (PheGly→SerHis)	47	71 *R*
B1-C6 (PheGly→PheLeu)	39	71 *R*
B1-G4 (PheGly→AsnTyr)	67	88 *R*
B1-H7 (PheGly→HisLeu)	69	80 *R*

[a] Conversions reported are based on GC values. [b] Enantioselectivities were determined by chiral phase GC analysis.

Table 4-8: *Select results obtained for the screening of the CPMO CASTing mutants for 4-acetoxycyclohexanone to the corresponding caprolactone.*[24]

CPMO variant	Conversion (%)[a]	ee (%)[b]
	Library A (449/450)	
WT-CPMO	100	5 *S*
A1-A3 (GlyPhe→SerTyr)	20	59 *R*
A1-A10 (GlyPhe→GlyIle)	100	8 *R*
A1-B5 (GlyPhe→AsnPhe)	40	43 *R*
A1-E1 (GlyPhe→ValTyr)	NR[c]	NR
A1-F12 (GlyPhe→GlyCys)	89	13 *S*
	Library B (156/157)	
B1-A3 (PheGly→LeuGly)	100	33 *S*
B1-A10 (PheGly→LeuPhe)	9	ND[d]
B1-A8 (PheGly→TyrTyr)	NR	NR
B1-C3 (PheGly→SerHis)	NR	NR
B1-C6 (PheGly→PheLeu)	NR	NR
B1-G4 (PheGly→AsnTyr)	19	90 *R*
B1-H7 (PheGly→HisLeu)	55	74 *R*

[a] Conversions reported are based on GC values. [b] Enantioselectivities were determined by chiral phase GC analysis. [c] NR = no reaction. [d] ND = not determined.

For the 4-methylcyclohexanone substrate the most profitable mutation which occurred from the A library occurred in the A1-A10 clone and corresponded to a single amino acid exchange (GlyPhe→GlyIle) giving the highest increase in *R* enantioselectivity. Interestingly, this mutation involves the substitution of an aromatic amino acid with an amino acid possessing a long hydrophobic chain.

In the library B, the size of the amino acid plays an important role in influencing the selectivity since in all the mutants showing improved selectivity in **Table 4-7**, the small glycine residue was exchanged for an amino acid with a larger side chain. This suggests that the position PheGly156/157 in the active site of WT CPMO may not be in close contact with the methyl substituent of the substrate. This problem is resolved in the mutants by increasing the size of the amino acid residue, which creates a potentially smaller, and more stabilizing, hydrophobic pocket for the 4-methyl group.

For the CPMO mutant catalyzed bio-oxidation of 4-acetoxycyclohexanone substrate **4-1c** (see Table 4-8), the most dramatic enhancement in enantioselectivity (5% ee *S* to 90% ee *R*) is seen for the B1-G4 mutant in which 156/157 PheGly have been replaced with the hydrophilic asparagine and tyrosine respectively. This suggests that, unlike the 4-methylcyclohexanone, the more polar acetyl group was more directly influenced by the amino acid exchange suggesting a closer contact with the 156/157 residues.

These results lead us to think that the cyclohexanone substrates can assume two positions within the active site. In one case the reactive position of the substrate places the R group in contact with the 449/450 residue "space" leading to *S* selectivity, while the

other position places the substituent in proximity to the 156/157 residue "space" leading to *R* selectivity.

4-4 Conclusion

Overall, the application of CASTing towards the enhancement of enantioselectivity of CPMO has been achieved with the identification of several CPMO mutants possessing significantly enhanced enantioselectivities for the substrates chosen for this study. The modified CASTing procedure, termed restricted CASTing, which utilizes the NDT degenerate codon and the generation of modest mutant libraries allowed for the rapid generation and screening of the mutant libraries. Initial analysis of the screening results for the probe substrates coupled with the homology modeled derived active site for CPMO, offers some interesting clues into the possible mechanism of enantioselection in this family of BVMOs. The information obtained from this study can provide guidelines for the future modification of not only CPMO but also other related BVMOs to enhance their substrate base or to improve their enantioselectivities.

4-5 Experimental

4-5-1 Generation of Mutants

The isolated supercoiled double-stranded DNA (dsDNA) pET22b(+) vector containing the desired insert (expressing CPMO) was used as a template for Quik-Change PCR to generate the mutants.[29] Primers were designed which incorporate the desired mutational changes within a given region, namely the 142/143 and 435/436, regions of the CPMO gene. PCR reactions were performed using the established Stratagene Quik-Change-Kit protocol (Stratagene) with HotStart KOD-Polymerase (Novagen) using the primers reported in **Table 4-6**.

4-5-2 Characterization of new substrates and products

4-Acetoxycyclohexanone (4-1c). A mixture of 4-hydroxycyclohexanone (0.5 g, 4.4 mmol), acetic anhydride (3 mL), and pyridine (0.5 mL) was stirred at RT for 12 hours. The solution was vacuum distilled to half volume and showed 93% conversion. The concentrated solution was added to ethyl acetate and acidified to pH ~5 with concentrated HCl. The layers were separated and the aqueous layer was extracted with ethyl acetate (50 mL × 3). The combined organic layers was dried over anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporator. The crude product was purified by chromatography on silica gel using hexane : ethyl acetate (2:1) to give the title compound as a pale yellow oil (0.45 g, 65% yield); IR (in $CHCl_3$) υ_{max}: 2958(m), 2873(m), 1733(vs), 1437(m), 1376(m), 1239(s), 1127(m), 1041(m), 956(m) cm^{-1}; ^1H NMR δ: 3.84 (2H, m), 2.64 (1H, dd, *J*=15, 6 Hz), 2.61 (1H, dd, *J*=15, 6 Hz), 2.22 (1H, s), 2.07 (2H, m), 1.98 (1H,dd, *J*=13, 6 Hz), 1.95 (1H, dd, *J*=13, 6 Hz), 1.45 (1H, s) ppm; ^{13}C NMR δ: 209.7, 170.3, 68.6, 37.2, 30.4, 21.2 ppm.

5-Methyloxepan-2-one (4-2a). *E. coli* BL21(DE3)(pET22b(+) mediated oxidation of ketone **4-3a** (110 mg, 0.98 mmol) was performed in the presence of 0.5 g β-cyclodextrin according to the general procedure (see Chapter 2). Chromatography on silica gel using petroleum ether : ethyl acetate (4:1) as eluent afforded lactone **4-4a** (61 mg, 49% yield); 88% ee by chiral phase GC, $[\alpha]^{25}_{D}$ = +50.31 (*c* 2.3, CH_2Cl_2)**:** IR (neat) υ_{max}: 2955(m), 2974(s), 2873(m) 1730(s), 1449(s), 1338(s), 1164(m), 1078(m) cm^{-1}; ^1H NMR δ: 4.27 (2H,m), 2.65 (2H, m), 1.88 (2H, m), 1.78 (2H, m), 1.52 (2H, m), 1.01 (3H, t, *J*=6.7 Hz) ppm; ^{13}C NMR δ:175.2, 68.2, 41.9, 35.0, 33.2, 29.1, 28.5 ppm.

5-Acetoxyoxepan-2-one (4-2c). *E. coli* BL21(DE3)(pET22b(+) mediated oxidation of ketone **3c** (100 mg, 0.64 mmol) was performed according to the general procedure (see Chapter 2). Chromatography on silica gel using hexane : ethyl acetate (9:1) as eluent gave lactone **4c** (21 mg, 19% yield); 90% ee by chiral phase GC, $[\alpha]^{25}_{D}$ = +4.94 (*c* 2.2, CH_2Cl_2)**:** IR (neat) υ_{max}: 2955(m), 1737(vs), 1441(w), 1372(m), 1248 (m), 1151(m), 1061(m) cm^{-1}; ^1H NMR δ: 5.15 (1H, m), 4.47 (1H, ddd, *J*=13, 8.4, 4.9 Hz), 4.17 (1H, ddd, *J*=13, 6.7, 6.1 Hz), 2.94 (1H, m), 2.57 (1H, m), 2.14-2.01 (7H, m) ppm; ^{13}C NMR δ:174.7, 169.5, 69.2, 63.5, 34.0, 28.5, 27.6, 21.2 ppm.

4-6 References

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Chapter 5

Screening of the new cyclopentanone monooxygenase mutants.

5-1 Introduction

The ever increasing need for new enantiomerically pure compounds for use as building blocks in the synthesis of novel pharmaceuticals and agrochemicals has spurred the development of a large number of asymmetric catalytic techniques. One area of catalysis which has been particularly active is biocatalysis, mainly because of the intrinsically high activities and enantioselectivities which enzymes possess. These properties, coupled with their mild reaction conditions, and generally safe and environmentally compatible nature, make enzymes the ideal catalysts for large scale use.

One such biocatalytic reaction which has been the focus of a great deal of attention in recent years is the enzymatic Baeyer-Villiger (BV) oxidation reaction. The BV oxidation reaction is particularly important since it represents one of the few synthetic methods allowing for the cleavage of a specific carbon-carbon bond. In particular, the enzymatic BV oxidation of ketones into chiral lactones is very important because of the increased demand for optically pure building blocks. While some progress has been made in the development of a chemical oxidation method for performing the asymmetric BV oxidation reaction, most attempts to date have been only moderately successful.

Recent advances in molecular biological methods have allowed researchers access to an increasing number of novel BV enzymes from numerous bacterial and fungal sources which have been identified through genome mining to harbour enzymes capable of catalyzing the BV-oxidation reaction. Some of the BVMOs which have been identified and sub-cloned into suitable over-expression systems have been found to possess remarkably broad substrate tolerances.

Two such BVMOs which fall into this category are cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIB 9871 and cyclopentanone monooxygenase (CPMO) isolated from *Commamonas* sp. NCIB 9872. Both of these enzymes have been found to convert a broad range of ketone substrates into their corresponding lactones, but while wild type (WT) CHMO is generally quite enantioselective, WT-CPMO is rarely selective. As part of a continuing theme in the Kayser group which centers on the development of enantioselective bio-oxidation catalysts, the WT-CPMO enzyme became the focus of a rationalized genetic enhancement study to improve its enantioselectivity towards a select group of 4-substituted cyclohexanone substrates.

In the mutational study reported in chapter 2, information gained from the directed evolution of CHMO was used to identify key amino acid residues, namely the Phe156 and Phe450 in CPMO for targeted mutation. The mutational strategy which was used to construct CPMO mutant libraries was the Complete Active Site Saturation Testing (CAST) method of Reetz and co-workers. Using the CAST procedure, two small mutant libraries, library A (449/450) and library B (156/157) were constructed which contained a total of 300 mutant enzymes. These mutant libraries were tested against three probe substrates (two of which were readily oxidized by WT-CPMO but with low selectivities) - the non-polar 4-methylcyclohexanone (**5-1a**), the polar 4-acetoxycyclohexanone (**5-1i**), and the 4-*t*-butylcyclohexanone (**5-1d**) which is not accepted by the WT-CPMO enzyme.

In the initial mutational study (see Chapter 4), while no mutants capable of transforming the 4-*t*-butylcyclohexanone (**5-1d**) substrate were identified, mutants possessing enhanced enantioselectivities for the remaining two substrates (**5-1a** and **5-1i**)

were found. More specifically, three mutants from each library (A and B) were identified which showed the most dramatic increases in enantioselectivity, with the most profitable mutation in library A involving a possible enhancement in the hydrophobic nature of the active site with a single amino acid exchange of Phe to Ile. For library B, all three of the "best" mutants not only involved a double mutation within the active site, but also contained amino acid exchanges where a small residue (Gly) was exchanged for a bulkier one.

The focus of this chapter is the screening of the sequenced CPMO clones to test their potential as new BV oxidation catalysts for other prochiral 4-substituted cyclohexanones. The results of the screening experiments are analyzed in terms of the diamond lattice model to illustrate how well a chosen test set of cyclohexanone substrates may be used to explore the active site of a BVMO enzyme and aid in the design of productive mutations for specific substrates.

5-2 Screening results

The prochiral 4-subsituted cyclohexanones have previously been shown to be an excellent substrate-test set for CHMO. Several of these substrates illustrated in **Figure 5-1** are also good substrates for WT-CPMO. Altough readily accepted by CPMO, they are oxidized with only modest enantioselectivity making them ideal candidates for screening the newly generated CPMO mutants. Thus, to evaluate the influence of mutations from the CASTing strategy on the active site of CPMO, we chose a set of related 4-substituted ketones which, although readily accepted by WT-CPMO, give the corresponding lactones with low enatioselectivity excess values. Furthermore, to probe the effects of the CAST

mutations on the size and characteristics of the putative active site of CPMO, 4-substituents of varied hydrophobicity and size were included in this study.

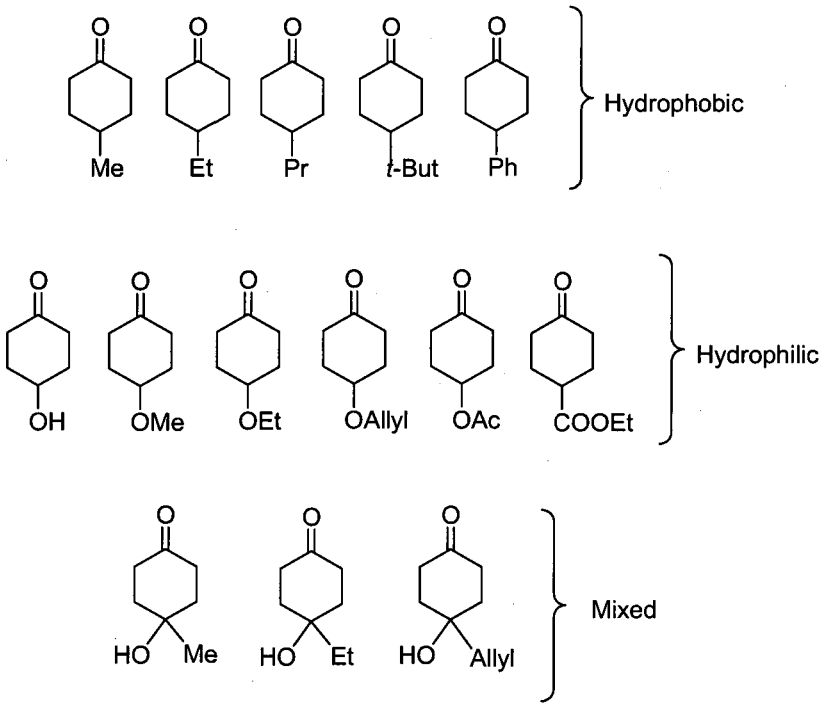
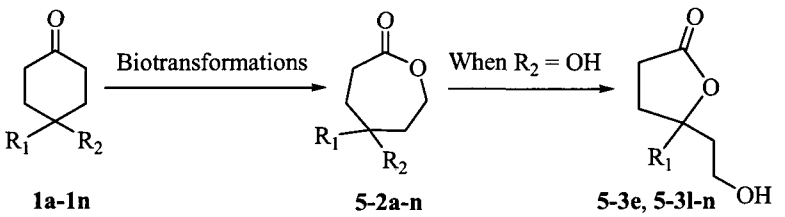


Figure 5-1: *Substrates chosen to test the mutational range of influence for the CPMO mutants generated through CASTing.*

The syntheses of the test substrates shown in **Figure 5-1** are described in Chapter 2 as was the characterization of the product lactones (see Chapter 2). All small scale screening and subsequent scale-up fermentation reactions shown in **Scheme 5-1** were performed under growing cell conditions. All reactions were monitored by achiral GC analysis while the enantiomeric composition of the lactones was established by chiral phase GC using previously established temperature programs that allow for resolution of the lactonic products (see appendix).

Scheme 5-1



1a-1n **5-2a-n** **5-3e, 5-3l-n**

5-1a	R_1 = H	R_2 = Me
5-1b	R_1 = H	R_2 = Et
5-1c	R_1 = H	R_2 = Pr
5-1d	R_1 = H	R_2 = *t*-Bu
5-1e	R_1 = H	R_2 = Ph
5-1f	R_1 = H	R_2 =OH
5-1g	R_1 = H	R_2 = OMe
5-1h	R_1 = H	R_2 = OEt
5-1i	R_1 = H	R_2 = OAllyl
5-1j	R_1 = H	R_2 = OAc
5-1k	R_1 = H	R_2 = COOEt
5-1l	R_1 = Me	R_2 = OH
5-1m	R_1 = Et	R_2 = OH
5-1n	R_1 = Allyl	R_2 = OH

In the case of the 4-hydroxy substituted lactones **5-1e**, **5-2l**, **5-2m**, **5-2n** spontaneous rearrangement to their corresponding γ-butyro-lactones **5-3** occurs, as illustrated in **Scheme 5-1**. The results of the extended screening of the CPMO mutants from library A and B are reported in **Tables 5-1** and **5-2**, respectively.

Table 5-1: *Baeyer-Villiger oxidation of 4-substituted cyclohexanones to the corresponding lactones catalyzed by WT and selected CPMO mutants from library A.*

Library A (449/450)	**Lactonic Products (2, 3)**[a]				
Substrates 1a-n (R_1/R_2)	WT-CPMO Conv.% (ee%)	GlyPhe→SerTyr	GlyPhe→GlyIle	GlyPhe→GlyCys	GlyPhe →ValTyr
Me/H	100 (46*R*)	27 (65*R*)	74 (92*R*)	37 (68*R*)	54 (33*R*)
Et/H	100 (32*S*)	32 (35*S*)	66 (22*S*)	79 (21*S*)	58 (51*S*)
Pr/H	100 (36*S*)	36 (5*S*)	81 (23*S*)	95 (24*S*)	69 (36*S*)
OH/H	100 (89*S*)	NR	100 (69*S*)	NR	NR
OMe/H	100 (28*S*)	91 (44*R*)	100 (43*S*)	96 (49*S*)	NR
OEt/H	100 (37*R*)	92 (77*R*)	94 (27*R*)	100 (25*R*)	NR
OAllyl/H	100 (53*S*)	8 (ND)	94 (50*S*)	20 (57*S*)	NR
OAc/H	81 (5*S*)	20 (59*R*)	100 (8*R*)	89 (13*S*)	NR
COOEt/H	100 (64*R*)	20 (90*R*)	76 (82*R*)	69 (78*R*)	NR
OH/Me	81 (85*S*)	NR	NR	NR	NR
OH/Et	92 (44*S*)[b]	NR	NR	NR	NR
OH/Allyl	63 (52*S*)[b]	NR	NR	NR	NR

[a] The absolute configurations of all lactonic products have been previously established (see ref.10, 14 and references therein);
[b] Absolute configuration was assigned by analogy to lactone **3k**; NR: no reaction; ND: not determined.

Table 5-2: *Baeyer-Villiger oxidation of 4-substituted cyclohexanones to the corresponding Lactones catalyzed by WT and selected CPMO mutants from library B.*

Library B (156/157)	**Lactonic Products (2, 3)**[a]				
Substrates 1a-m R_1/R_2	WT-CPMO Conv.% (ee%)	PheGly→LeuPhe	PheGly→AsnTyr	PheGly→HisLeu	PheGly→PheLeu
Me/H	100 (46*R*)	89 (91*R*)	67 (88*R*)	69 (80*R*)	39 (71*R*)
Et/H	100 (32*S*)	84 (90*R*)	55 (59*R*)	85 (88*R*)	46 (76*R*)
Pr/H	100 (36*S*)	94 (89*R*)	44 (58*R*)	22 (59*R*)	47 (86*R*)
OH/H	100 (89*S*)	23 (81*S*)	NR	NR	NR
OMe/H	100 (28*S*)	53 (75*R*)	39 (66R)	31 (70*R*)	51 (75*R*)
OEt/H	100 (37*R*)	77 (95*R*)	61 (92*R*)	56 (90*R*)	39 (88*R*)
OAllyl/H	100 (53*S*)	23 (>90*R*)	22 (52*R*)	14 (ND)	NR
OAc/H	81 (5*S*)	10 (ND)	19 (90*R*)	55 (74*R*)	20 (88*R*)
COOEt	100 (64*R*)	56 (72*R*)	39 (78*R*)	48 (79*R*)	50 (79*R*)
OH/Me	81 (85*S*)	NR	NR	NR	NR
OH/Et	92 (44*S*)[b]	NR	NR	NR	NR
OH/Allyl	63 (52*S*)[b]	NR	NR	NR	NR

[a] The absolute configurations of all lactonic products have been previously established (see ref.10, 14 and references therein);
[b] Absolute configuration was assigned by analogy to lactone **3k**; NR: no reaction; ND: not determined.

An examination of the screening results for the CPMO mutants given in **Tables 5-1** and **5-2** revealed several interesting points. Beginning with the 4-alkyl substituted ketones **5-1a-c**, the results for the modification at the 449/450 positions (library A) show that the only interesting result is the GlyPhe→GlyIle previously identified for 4-methylcyclohexanone (**5-1a**). A very modest increase in enantioselectivity was observed for the bioconversion of 4-ethylcyclohexanone (**5-1b**) when screened against the GlyPhe →ValTyr mutant, while no real enhancement in enantioselectivities were observed for the 4-propylcyclohexanone for any Library A clone.

The mutations at 156/157 position (library B) however, showed much more promise in terms of enhanced enantioselectivities for these substrates. In fact, the same mutant containing PheGly→LeuPhe double mutation, previously found to show enhancement in the earlier screening of the **5-1a,** was also found to be an excellent catalyst for the substrates **5-1b** (32% ee *S* to 90% ee *R*) and **5-1c** (36% ee *S* to 89% ee *R*). Most significantly, not only the enantioselectivity was enhanced by the PheGly→LeuPhe mutation but also the selectivity was also completely reversed for these substrates. For the 4-ethylcyclohexanone (**5-1b**) the mutant containing PheGly→AsnTyr also showed an excellent improvement in enantioselectivity (32% ee *S* to 88% ee *R*) with only a slight decrease in conversion with respect to the WT-CPMO result. Unfortunately, screening of the 4-*t*-butylcyclohexanone (**5-1d**) and 4-phenylcyclohexanone (**5-1e**) against the CPMO mutant libraries identified no mutants which would accept these substrates.

In the case of 4-alkoxycyclohexanone substrates **5-1g-i**, as with the 4-alkyl substrates, the library A position 449/450 couple seemed to exert only a modest influence on the selectivity of CPMO. The best library A result for this set of substrates was

observed 4-ethoxycyclohexanone (**5-1h**) in the GlyPhe→SerTyr mutant that showed a modest improvement in enantioselectivity (37% ee *R* to 77% ee *R*). The mutants from library B were more successful with the PheGly→LeuPhe mutant showing the most improved enantioselectivity. Improvements were observed for all the 4-alkoxy targets, with the best improvements being observed for the 4-ethoxycyclohexanone **5-1h** (37% ee *R* to 95% ee *R*) and 4-allyloxycyclohexanone **5-1i** (53% ee *S* to 90% ee *R*). Two other library B mutants were found to possess dramatically increased enantioselectivities for 4-ethoxycyclohexanone **5-1h**, the PheGly→AsnTyr (37% ee *R* to 92% ee *R*) and PheGly→HisLeu (37% ee *R* to 90% ee *R*). None of the CPMO mutants was able to transform the hydrophilic substrate **5-1f** or the mixed 4,4-alkylhydroxycyclohexanone substrates **5-1l-m**.

5-3 Discussion

To aid in the interpretation of the screening results obtained for the CPMO mutants, a three-dimensional homology model was constructed for both the CHMO and CPMO enzymes based on the known X-ray structure of PAMO. A primary sequence alignment showed a high sequence homology of 39% and 40% between CHMO, CPMO, and PAMO, respectively. This suggests that, the overall structures of these three monooxygenase enzymes should be highly conserved. The CHMO and CPMO models were constructed using the MOE homology modeling module based on the PAMO template structure. After initial primary sequence alignment, the MOE homology modeling module generated 25 templates for the given CPMO or CHMO protein. Each of the 25 models generated were subjected to a coarse (<10 kcal/mol) energy

minimization using the AMBER94 force field[21] to remove any possible Van der Waals clashes which may occur between atoms. A final overall model, based on an average of the 25 coarsely minimized model templates was then constructed for the CPMO and CHMO systems. The resulting intermediate models for CPMO and CHMO were then energy minimized assuring the final energy gradient was <0.01 kcal/mol.

Protein validation tools contained within the MOE program were used to further guide the protein model refinement process through identification of regions in the CPMO or CHMO models requiring further refinement *via* manual corrections. The final qualities of the CPMO and CHMO models were assessed by the protein report tool of MOE which indicated that each homology model was of good quality, with 98% and 99% of residues in the allowed zones of the Ramachandran plot, respectively. For CPMO and CHMO the protein report also showed that 86% and 87% of the residues were in the core of the plot, respectively. Placement of the FAD moiety within the CPMO and CHMO models was done by duplicating the coordinates of the FAD group from the PAMO X-ray structure, and the resulting structures where energy minimized. The refined CPMO homology model with the bound FAD group is shown in **Figure 5-2.**

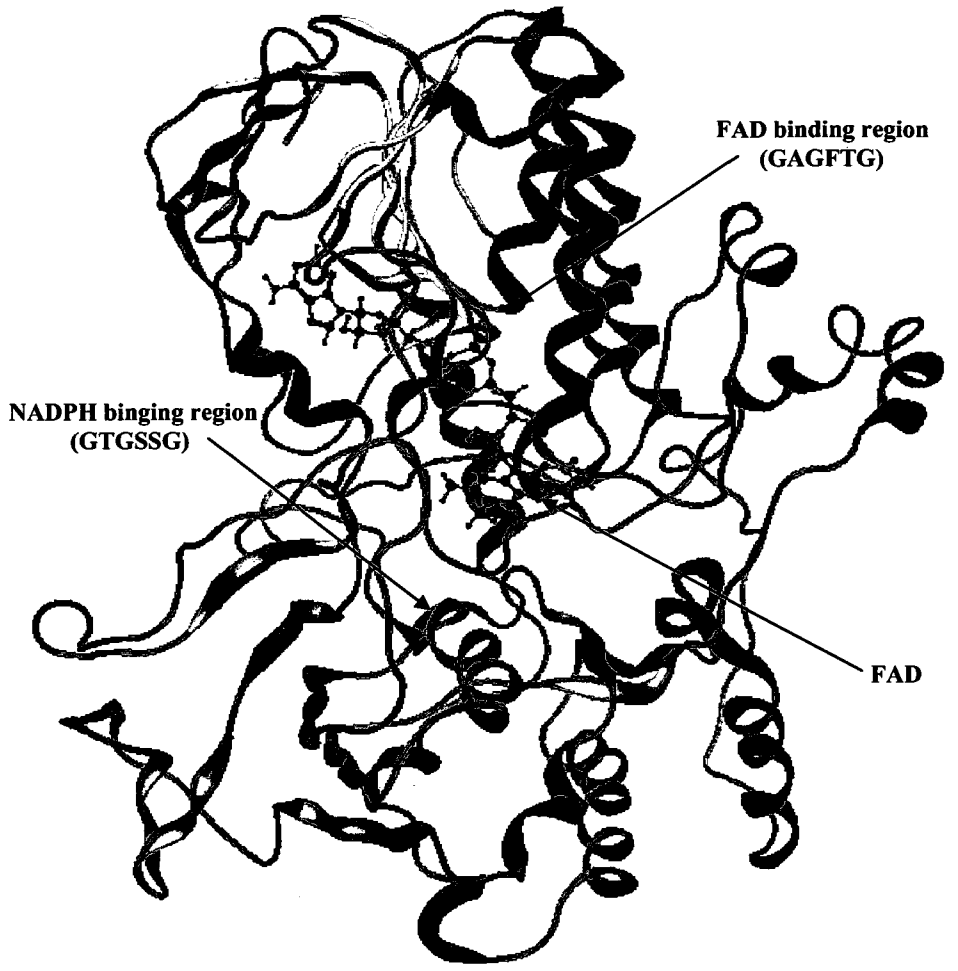


Figure 5-2: *Homology model for the CPMO protein illustrating the position of the FAD cofactor group* (shown in green)*, and locations of the nucleotide binding regions.*

Comparison of the CPMO and CHMO models with PAMO confirmed that the core regions of the three proteins are almost identical. In both the CHMO and CPMO models the catalytically essential arginine residues (Arg327 for CHMO and Arg344 for CPMO) are located on the *re*-side of the flavin ring (see **Figure 5-3**). The location of this arginine residue acted as a guide to help pinpoint the region within the putative CHMO and CPMO active sites where substrate binding takes place. To further confirm the

substrate binding pockets in CHMO and CPMO, the catalytic "hot-spot" residues identified in the directed evolutionary study on CHMO were located in the CHMO homology model. It turned out that the residues Leu143, Phe432, Pro426, and Phe505 in the CHMO homology model are all in proximity to the *re*-face of the FAD and the catalytically essential arginine.

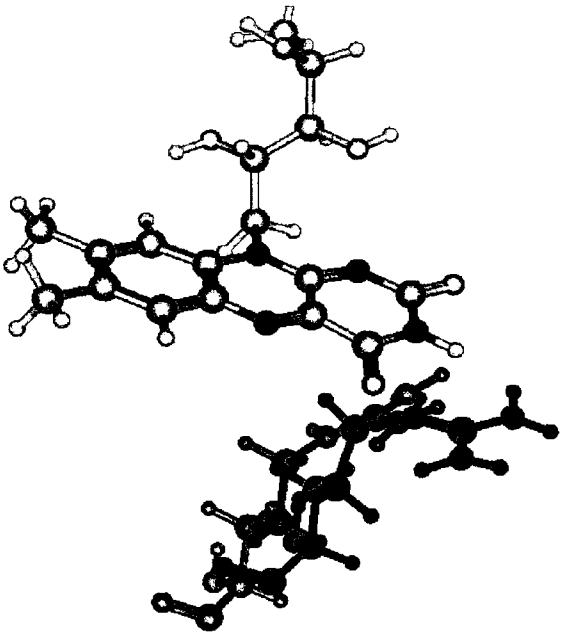


Figure 5-3: *Overlay of the CPMO and CHMO homology models, showing both the Arg327* (purple, CHMO) *and Arg344* (blue, CPMO) *on the re-face of the FAD.*

A comparison of the CHMO and CPMO models, and their mutants suggested that the most profitable mutations should be located at the 432 position in CHMO and at the 156/157 residues for CPMO. Analysis of the results obtained from the two studies indicates that the 432 position influence the *S*-selectivity in CHMO, while those at the 156/157 pair tend to improve the *R*-selectivity in CPMO. These residues appear to be

located on adjacent sites within the putative active sites of the CPMO and CHMO models, respectively (see **Figure 5-4**and **5-5**).

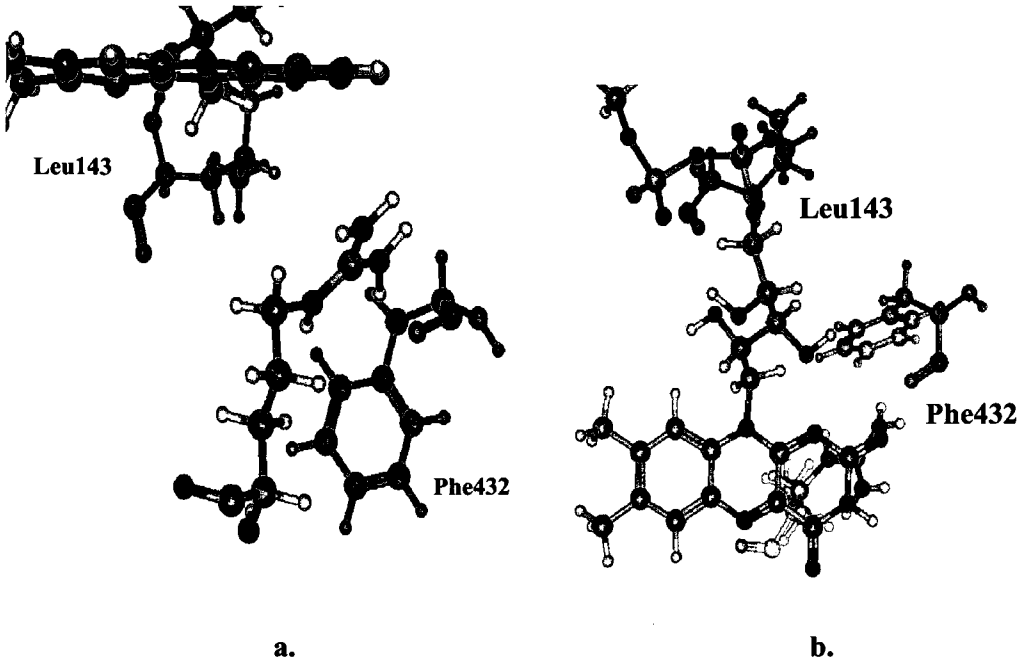


Figure 5-4: **a.** *Location of the key residues for CHMO Phe432 and Leu143* (green) *identified in the mutational studies on the CHMO*; **b.** *Overhead view of the two mutational locations.*

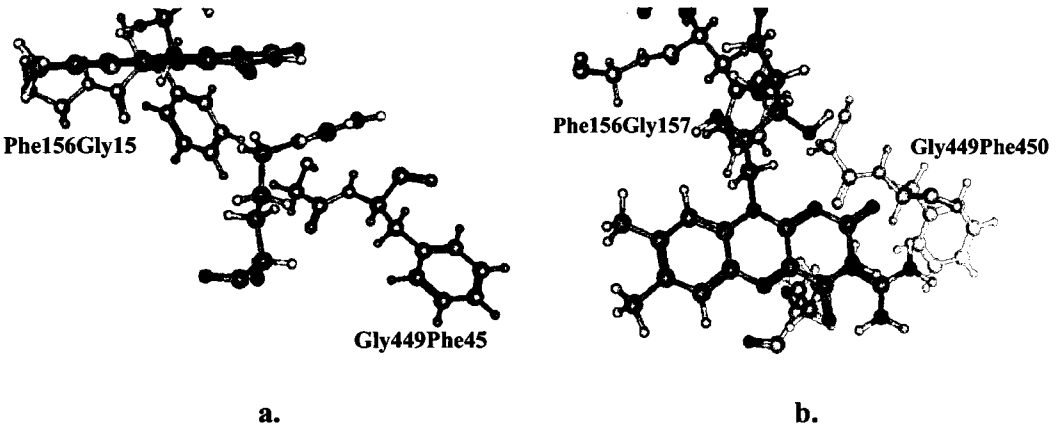


Figure 5-5: **a.** *Location of the CAST mutational positions Gly449Phe450 and Phe156Phe157* (orange) *for CPMO*; **b.** *Overhead view of the two mutational locations.*

Implementation of the diamond lattice model for predicting the stereochemical control in the CHMO catalyzed BV oxidations of cyclohexanones was shown to be in excellent agreement with the observed enantioselectivity changes observed in CHMO and CPMO (see **Figure 5-3**).[11] Application of the diamond model suggests that WT-CPMO has two pockets capable of accommodating the 4-equatorial substituent of the cyclohexanones. The 156/157 pocket that leads to *R*-lactones is marginally more accommodating, particularly for alkyl substituents. Therefore upon suitable amino acid exchanges in the 156/157 region, configuration A is favoured and the migrating bond leads to the higher ee value of (*R*)-lactone. In the case of the WT-CHMO enzyme, the pocket in 432 region is better developed resulting in frequently high *S*-selectivity. Successful mutations at the 432 residue improve that pocket and the configuration B is favored.

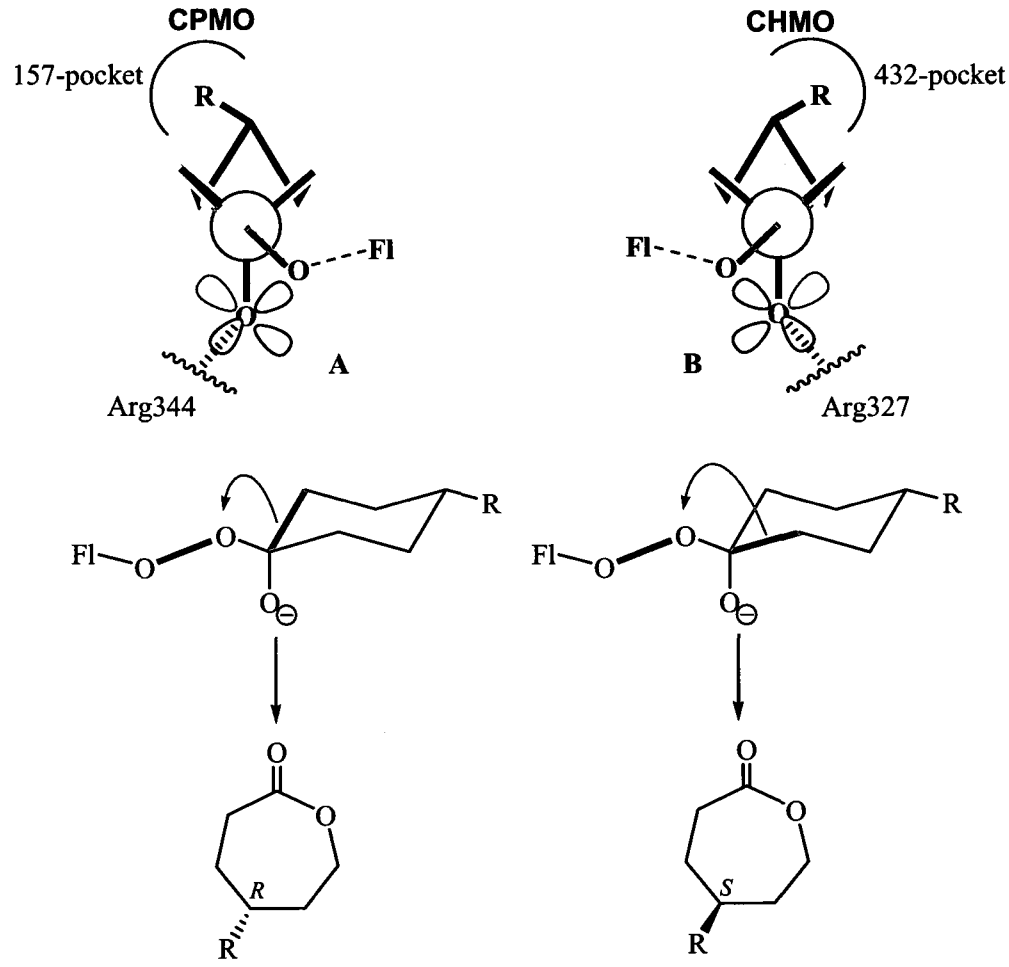


Figure 5-6: *The diamond lattice model predicts mutations of residues 156/157* (CPMO) *improve the interactions with the R substituent, therefore configuration A is favoured and the migrating bond leads to the* (R)*-lactone. The opposite is true when mutations occur at the 432 position* (CHMO).

A closer inspection of the homology models for CPMO and CHMO provides some insight into the poor performance of the CPMO library A mutants (Figure 5-7). Comparison of the sequences of amino acids in CPMO: Pro447/Ala448/Gly449/Phe450 with the corresponding regions in CHMO show that Pro447 overlaps with CHMO's Pro431 and Gly449 overlaps with Phe432 of CHMO. It appears (based on the generated CPMO homology model) that the Phe450 residue has its aromatic side chain pointing away from the putative active site (see **Figure 5-7**), thus limiting its overall influence on the active site character. Based on this finding, it appears that the positive Library A mutants which do influence the selectivity of CPMO and involve mutations at the Phe450 position may be exerting some form of indirect influence on the neighbouring amino acid residues.

A similar extended loop region is present in the PAMO when compared to the CHMO enzyme. Recently, Reetz *et al.* showed that this extended loop region or "bulge" was found to influence the substrate acceptance and enantioselectivity of the PAMO protein.[22] They found that upon deletion of the two residues which make up the bulge in PAMO, a new PAMO variant which possessed an enhanced substrate scope and enantioselectivity was produced.

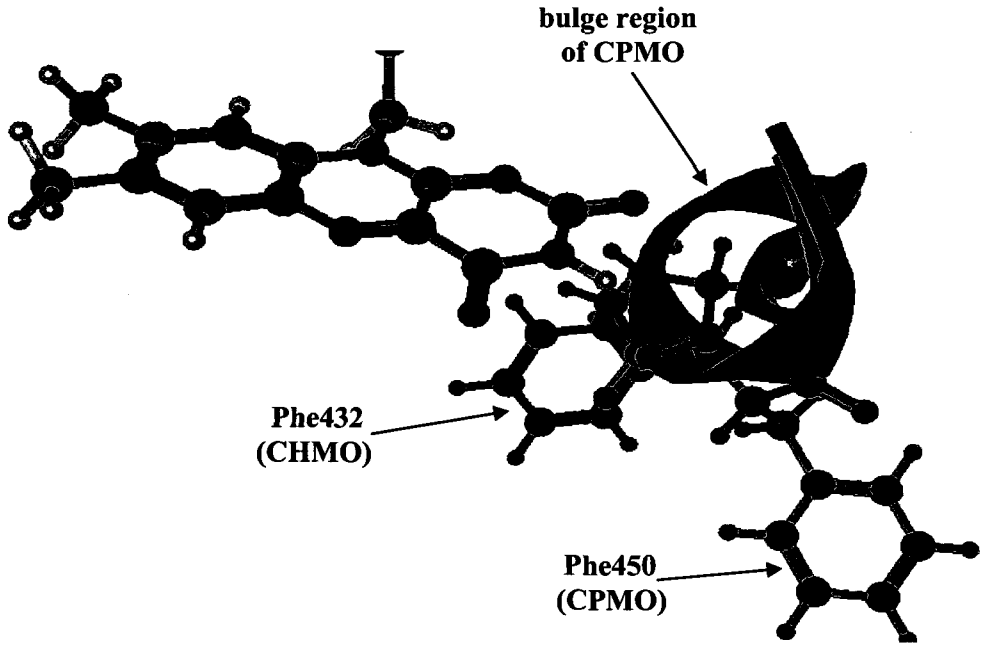


Figure 5-7: *Overall view of the CHMO and CPMO homology models generated using MOE. The positions of the key residues* (CHMO Phe432 green/CPMO Gly449 and Phe450 orange) *with the CPMO "bulge" clearly illustrated.*

5-4 Conclusion

In conclusion, the results listed in **Tables 5-1** and **5-2** show that a "mini evolution" such as that obtainable through the application of the CASTing strategy can yield mutants which provide improved enantioselectivities. In particular, an impressive increase in *R*-selectivities was observed for library B mutants that involved mutations in the 156/157 residues. The relatively small changes in enantioselectivity observed for the library A mutants suggest that mutations in positions 449/450 did not improve adequately the hydrophobic pocket possibly because this location was slightly off the principal interaction(s) between the enzyme's residue(s) and the R group of the substituent. In

general, the results reported here, reinforce the realization that mutations close to the active site have a higher impact on enantioselectivity, substrate selectivity, and "catalytic promiscuity", and that mutations at a residue that interacts directly with the substrate have the most profound impact.[23] It is believed that application of the diamond model in combination with modeling will provide useful blue-prints for improvements in selectivity for specific substrates.

5-5 Experimental characterisation of substrates and products

4-Allyloxycyclohexanone (5-1i). 4-Hydroxycyclohexanone ethylene ketal (0.8 g, 5 mmol), NaH (1 g), and allyl bromide (1 mL added in 4 portions) were reacted according to the literature procedure to give 4-allyloxycyclohexanone ethylene ketal, (0.5 g). Deketalization followed by flash chromatography with petroleum ether:ethyl acetate (20:1) as eluent gave **5-1i** as a yellow oil, (0.42 g, 58% yield); IR (in $CHCl_3$) υ_{max}: 3090(w), 3010(w), 2950(s), 2870(s), 1720(vs), 1650(m), 1420(m), 1345(m), 1105(s), 1060(s), 939(m) cm^{-1}; ^1H NMR δ: 5.97-5.91 (1H, m), 5.32 (1H, d, *J*=17.1 Hz), 5.17 (1H, d, *J*=13.4 Hz), 4.06 (2H, m), 3.76 (1H, m), 2.61-2.55 (2H, m), 2.28-2.23 (2H, m), 2.10-2.06 (2H, m), 1.94-1.91 (2H, m) ppm; ^{13}C NMR δ: 211.2, 135.0, 116.7, 72.2, 69.3. 37.2, 30.5 ppm.

5-Ethyoxyoxepan-2-one (5-2h): *E. coli* BL21(DE3)(pET22b(+) mediated oxidation of ketone **5-1h** (100 mg, 0.69 mmol) was performed in the presence of β-cyclodextrin according to the general procedure (see Chapter 2). Chromatography on silica gel using hexane: ethyl acetate (5:1) as eluent afforded lactone **5-2h** (66 mg, 61% yield); 92% ee by chiral phase GC; ^1H NMR δ: 4.47 (1H, ddd, *J*=13, 9.5, 1.2 Hz), 4.03 (1H, ddd, *J*=13, 6, 1.8 Hz), 3.63 (1H, m), 3.46 (2H, ddd, *J*=14, 12, 1.5 Hz), 2.94 (1H, m), 2.39 (2H, ddd, *J*=14, 8.5, 1.3 Hz), 2.00-1.76 (4H, m), 1.17 (3H, t, *J*=7 Hz) ppm; ^{13}C NMR δ: 176.4, 73.9, 63.8, 63.7, 34.4, 28.1, 27.7, 15.7 ppm.

5-Allyloxyoxepan-2-one (5-2i): *E. coli* BL21(DE3)(pET22b(+) mediated oxidation of ketone **5-1i** (104 mg, 0.67 mmol) was performed in the presence of β-cyclodextrin according to the general procedure (see Chapter 2). Chromatography on silica gel using

hexane:ethyl acetate (5:1) as eluent afforded lactone **5-2i** (25 mg, 22% yield); 90% ee by chiral phase GC; ^{1}H NMR δ: 5.91 (1H, m), 5.24 (2H, dd, *J*=17, 10 Hz), 4.52 (1H, dd, *J*=13, 10 Hz), 4.08 (1H, dd, *J*=6.5, 1.8 Hz), 4.00 (2H, m), 3.73 (1H, m), 3.00 (1H, t, *J*=13 Hz), 2.43 (1H, m), 2.00 (4H, m) ppm; ^{13}C NMR δ: 176.1, 135.0, 117.0, 73.6, 69.4, 63.6, 34.3, 28.1, 27.7 ppm.

5-6 References

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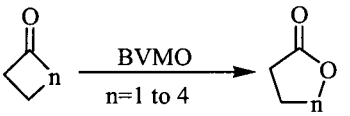
Chapter 6

Biotransformations using wild type and mutant Baeyer-Villiger monooxygenases: growing and non-growing conditions.

6-1 Introduction

The amount of research associated with the identification, isolation, and profiling of enzymes has increased dramatically over the last quarter century resulting in a significant number of enzymes which can be potentially applied as catalyst systems in the pharmaceutical and agrochemical industries. One such biocatalyst which has significant promise is the enzyme-mediated Baeyer-Villiger oxidation reaction which has been proven to be a viable route for the production of cyclic lactones (see **Scheme 6-1**).

Scheme 6-1



Enantio- and regioselective Baeyer-Villiger oxidations are important reactions in organic synthesis; development of safe, selective and environmentally friendly reagents[1,2] has been pursued through chemical and enzymatic methods.[3-5]

Baeyer-Villiger monooxygenases (BVMOs) from bacteria and fungi are particularly promising as catalysts as they possess remarkably flexible substrate tolerance often combined with high stereoselectivity.[6-8] Cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871[9] and cyclopentanone monooxygenase (CPMO) from *Comamona* sp. NCIMB 9872[10, 11] are two of the enzymes that transform a broad variety of ketones; however, while CHMO is often highly enantioselective,[5] CPMO rarely is.[12, 13] Recently, both enzymes were evolved to improve enantioselectivity and a number of mutants with desired characteristics were

identified.[14-17] The most successful mutant identified in the directed evolution of CHMO carried a single mutation F432S; its activity was comparable to that of the WT-CHMO and it showed a dramatic change of enantioselectivity in the oxidation of 4-hydroxycyclohexanone (from 9% *R* to 88% *S*).

Despite the fact that these enzymes are highly selective reagents for a broad array of substrates, they have not found widespread acceptance among synthetic chemists because of real and perceived difficulties.[18] It is important to point out, however, that many problems associated with the use of these biocatalysts, such as instability of the isolated enzymes and cofactor (NADPH) recycling, are solved when these enzymes are overexpressed in host organism such as baker's yeast or *E. coli*.[19, 20] The availability of overexpression systems for several BVMOs and their mutants allows organic chemists to treat them as reagents in organic synthesis.

A further obstacle which has limited the general application of these, and many other enzymes, in industry relates to the transition from the idealized small scale environment associated with a research laboratory to actual industrial production scale. Recently, several groups addressed the problem of making biocatalytic BV reactions practical and chemist "friendly".[19-24] The first attempt to enhance the scalability of the BVMO reaction was carried out by Walton and Stewart[22] who, utilizing recombinant *E. coli* overexpressing CHMO, performed a model BV oxidation by placing the cells under non-growing conditions.[22]

The application of non-growing or resting cells has been used in microbiology for decades, typically applied to biotransformations involving wild type microorganisms.[25, 26] In general, the non-growing cells biotransformations involve initial expression of the

bacteria or enzyme (in our case BVMO enzyme overexpression in *E. coli*) under classical growing conditions until the cells have reached their stationary phase or the point at which dwindling nutrient supplies dramatically reduce bacterial growth (bacterial numbers stabilize). The cells are harvested and re-suspended in a minimal salts medium lacking any nitrogen source, thus placing the cells in an environment which inhibits cell replication but still allows them to act as a catalyst. This final point is crucial in the case of the BVMO bio-oxidation since the cells biological "machinery" is required for co-factor recycling. The study of non-growing cells it was illustrated that volumetric productivity of CHMO catalyzed reactions was twenty-fold higher than the corresponding oxidations with growing cells.[22]

In a recent study, several mutants derived from the non-rational and rational modifications of CHMO[14] and CPMO[15] were screened against a set of 4-substituted cyclohexanones and it was found that although the enantioselectivities of the mutants were better than those of the wild-type enzymes, the conversions were usually low.[16] Mutant F432S, identified in the directed evolution of CHMO, was an exception; it not only showed a dramatic change in enantioselectivity in the oxidation of 4-hydroxycyclohexanone to the corresponding lactone (from 9% *R* to 79% *S*) but its activity was comparable to or better than that of the WT-CHMO.[14, 16]

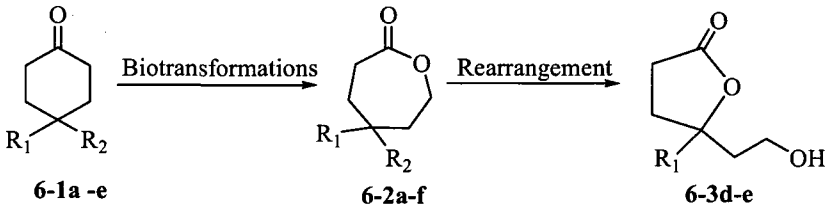
Having identified in the screening process the most enantio-improved mutants the production of selected lactones was scaled-up under non-growing conditions. The results were gratifying and interesting since not only was there a dramatic improvement in the conversions for several substrates, but also in a number of cases where no reaction was detected during the screening with growing cells, the transformation carried out with

resting cells gave good yields of highly enantioenriched lactones. The results obtained for the screening of six 4-substituted cyclohexanones using non-growing cells expressing WT-CHMO, WT-CPMO, and their mutants are reported here.

6-2 A comparison of Baeyer-Villiger oxidations performed under growing and non-growing conditions

The substrates investigated are shown in **Scheme 6-2**. The results of the screening under growing conditions[12,16] **(Table 6-1)** are compared with the transformations performed under non-growing conditions **(Table 6-2)**. The design and construction of CPMO mutants used in this study and the preparation of substrates and characterization of the lactonic products, including their absolute configuration, was previously described (see Chapter 2 and 4).[12,16] Under the non-growing conditions, biotransformations were carried out on 10 mg samples in 125 mL flasks as described in the Experimental Section. The initial scale-up reactions were performed on 100 mg samples of substrates in 500 mL baffled flasks. The reaction progress was monitored by chiral phase GC using the conditions that allowed baseline resolution for the enantiomeric lactones which have been established previously.[15, 16]

Scheme 6-2



6-1a -e **6-2a-f** **6-3d-e**

6-1a R_1 = OEt; R_2 = H
6-1b R_1 = OAllyl; R_2 = H
6-1c R_1 = OAc; R_2 = H
6-1d R_1 = Me; R_2 = OH
6-1e R_1 = Allyl; R_2 = OH
6-1f R_1 = *t*-Butyl; R_2 = H

Table 6-1. *Baeyer-Villiger oxidations of 4-substituted cyclohexanones to the corresponding lactones catalyzed by WT-CHMO, WT-CPMO and their mutants under growing conditions.*

No	Substrates R_1/R_2	WT-CHMO Conv.%[1] (ee%)[2]	I-K2-F5 Phe432Ser Conv.% (ee%)	WT-CPMO Conv.% (ee%)	B1-A10 Phe156Gly/Leu157Phe Conv.% (ee%)
6-1a	OEt/H	NR[3]	NR	100 (37 *R*) (24 h)	77 (95 R) (24 h)
6-1b	OAllyl/H	NR	NR	100 (53 *S*) (24 h)	23 (>90 *R*) (24 h)
6-1c	OAc/H	NR	NR	81 (5 *S*) (24 h)	10 (ND[4]) (24 h)
61d	OH/Me	100 (94 *R*) (24 h)	100 (97 *R*) (24 h)	81 (85 *S*) (24 h)	NR
6-1e	OH/Allyl	100 (27 *R*) (24 h)	100 (97 *R*) (24 h)	92 (44 *S*) (24 h)	NR
6-1f	*t*- Butyl	NR	NR	NR	NR

[1] Conversion monitored by GC; [2] enantiomeric excess (ee) monitored by chiral phase GC;[3] NR=no reaction; [4] ND=not determined

Table 6-2. *Baeyer-Villiger oxidations of 4-substituted cyclohexanones to the corresponding lactones catalyzed by WT-CHMO, WT-CPMO and their mutants under non-growing conditions.*

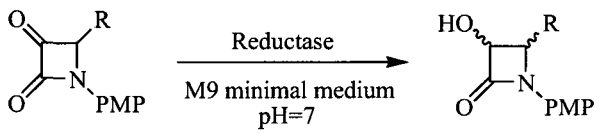
No	Substrates R_1/R_2	WT-CHMO Conv.%[1] (ee%)[2]	I-K2-F5 Phe432Ser Conv.% (ee%)	WT-CPMO Conv.% (ee%)	B1-A10 Phe156Gly/Leu157phe Conv.% (ee%)
6-1a	OEt/H	33 (50 *S*) (24 h)	77 (>99 *S*) (24 h)	100 (35 *R*) (4 h)	100 (**94** *R*) (9 h)
6-1b	OAllyl/H	24 (71 *S*) (24 h)	87 (>99 *S*) (24 h)	100 (52 *S*) (4 h)	100 (85 *R*) (9 h)
6-1c	OAc/H	32 (79 *S*) (24 h)	91 (>99 *S*) (24 h)	100 (8 *S*) (4 h)	100 (68 *R*) (11 h)
6-1d	OH/Me	100 (96 *R*) (8 h)	100 (86 *R*) (12 h)	100 (85 *S*) (10 h)	NR
6-1e	OH/Allyl	100 (28 *R*) (12 h)	100 (97 *R*) (8 h)	100 (55 *S*) (8 h)	NR
6-1f	*t*- Butyl	18 (98 *S*) (24 h)	NR[3]	NR	NR

[1] Conversion monitored by GC; [2] enantiomeric excess (ee) monitored by chiral phase GC;[3] NR=no reaction.

6-3 Discussion

A study[22] of the Baeyer-Villiger oxidation of cyclohexanone catalyzed by non-growing *E. coli* cells overexpressing CHMO demonstrated that the volumetric productivity of non-growing cells was an order of magnitude greater than that achieved with growing cells. Earlier work within the Kayser group[27] on the asymmetric reductions of β-lactams catalyzed by *E. coli* overexpressing yeast reductases showed that improved conversion and shorter reaction time were obtained with non-growing cells.

Scheme 6-3



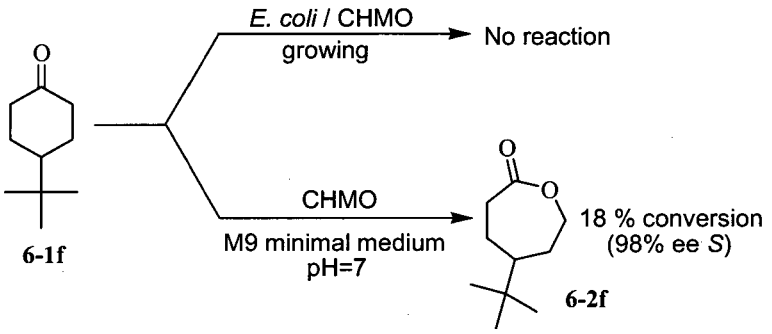
R = phenyl
thiophenyl
furyl

Based on this previous experience it was decided after preliminary screening with growing cells that identified the "useful" mutants, to carry out parallel reactions with non-growing cells.

An interesting development occurred during the re-run of the reaction of 4-*tert*-butylcyclohexanone **6-1f** with WT-CHMO under non-growing conditions. An earlier literature report[28] had indicated that **6-1f** was oxidized to the lactone in a reaction catalyzed by the isolated CHMO enzyme; while the conversion was only 17%, the enantiomeric excess was 98%. Several attempts to duplicate these results with whole-cell

catalyzed reactions under log-phase growth conditions failed. It was interesting to discover that in the course of the reaction with non-growing cells the substrate was accepted, and that the conversion and the enantiomeric excess were comparable to those obtained in the reaction catalyzed by the isolated enzyme (see **Table 6-2** and **Scheme 6-4**).

Scheme 6-4



To ensure that the results obtained using non-growing cells **6-1f** were not due to exposure of the substrate to a much greater cell density per volume of reaction, control experiments were conducted to compare growing and non-growing cell masses over a 24 h period. The control experiments revealed that the mass of growing cells produced over the duration of the biotransformation (24 h) was comparable with the mass of cells used under the non-growing conditions (see experimental sections). Based on this result, it is tempting to conclude that reactions performed with non-growing cells reflected the performance of the isolated enzyme better than the reactions with growing cells.

Studies attempting to understand and improve the CHMO non-growing cells reaction found that the frequently encountered problem of substrate acceptance and/or conversion may be, in many cases, linked to the sluggish transport of substrate into the cytosol where the reaction takes place.[22] The apparently more effective transport in the non-growing cells is likely related to the increased membrane permeability which is caused by a high concentration of hydrophobic substrate and product in the fermentation mixture.[29] A possible explanation for the increased membrane permeability of *E. coli* non-growing cells may be linked to increased osmolarity of M9 medium lacking reduced nitrogen used in the non-growing cells reaction compared to the LB-Miller broth used under growing cells conditions. While the exact nature or mechanism of the salt stress response that *E. coli* cells undergo in response to an increase in the osmolarity of the medium's osomotic pressure is not clear, however, it is clear that some osmoadaptation must take place.[30, 31] One can speculate that to avoid cell dehydration, the osmotic pressure inside the cells would increase which would allow certain solutes to accumulate within the cytosol. One of the osmoregulatory solutes which may in fact be taken up more readily into the cytosol of the *E. coli* cells could be the substrate added to the cells during the course of the reaction. [30, 31]

The results discussed above prompted the re-examination of several other "failed" reactions and it was discoverd that a number of other substrates, not accepted or poorly accepted during screening with growing cells, were converted to the corresponding lactones efficiently and with high enantiomeric excess (**Table 6-2**). The maximum conversion was achieved in several cases in a considerably shorter time. The most striking results were observed in oxidations of 4-ethoxy-, 4-allyloxy, and 4-acetoxy

cyclohexanones catalyzed by the CHMO mutant Phe432Ser which gave essentially enantiopure (>99% ee) (*S*)-**6-2a**, (*S*)-**6-2b**, and (*S*)-**6-2c** with excellent conversions within 24 hours. Under the same reaction conditions CPMO mutant Phe156Gly/Leu157Phe provided access to their (*R*) antipodes in good to excellent yields.

The oxidations of ketones with longer chains (**6-1a-c**) resemble the results obtained for the *tert*-butyl substrate **6-1f**. For example, 4-ethoxy cyclohexanone **6-1a** was not converted by growing cells, it was converted by the non-growing cells, but the conversion was only 33% after 24 hours. On the other hand, the same substrates were transformed by CPMO under both growing and non-growing conditions. In the latter case, however, the transformation under the non-growing conditions gave 100% conversion after only 4 hours. These results suggested that **6-1a** is a poor substrate for CHMO; in addition, its transport through the membrane is slow; the combined effect is that under growing conditions no lactone is formed within 24 hours. When the reaction is performed with non-growing cells, **6-1a** was transported through a more permeable membrane more efficiently, and the product accumulated slowly. These rationalizations were supported by several control experiments that monitor cell biomass (see the discussioj above). Ketone **6-1a** was a better substrate for the F432S mutant and therefore the conversion under non-growing conditions was higher; to make things more interesting, the mutant was also highly enantioselective. Since **6-1a** was an excellent substrate for CPMO, and therefore, even with growing cells complete conversion after 24 hours was obtained. With non-growing cells and the faster transport a complete conversion was achieved in 4 hours. The CPMO mutant F156G/L157F is fast and enantioselective.

6-4 Conclusions

In conclusion, the experiments described here emphasize the advantages of the biotransformations with the non-growing cells: the reactions are faster, cleaner (no metabolites), and better suited for scale-up work in fermenters in agreement with the results of Walton and Stewart.[22] Furthermore, they indicate that substrate-acceptance profiling frequently carried out with growing cells may fail to identify organisms and/or mutants that are suitable for the conversion of a substrate used in screening. Finally, overexpression systems for the mutants tested here are both highly active and enantioselective; overexpressed in easy to handle organisms they enrich a repertoire of divergent bioreagents for BV reactions.

6-6 Experimental

The synthesis and charaterization of all compounds used in this study have been reported previously.[12] The 4-*tert*-butylcyclohexanone **6-1f** substrate was commercially available and was purchased from Sigma-Aldrich. Chemical oxidations with *m*-chloroperbenzoic acid were performed on all substrates prior to biotransformations to establish appropriate conditions for the GC resolution of all lactones.

6-5-1 Biotransformations under growing conditions with *E. coli*/CHMO and *E. coli*/CHMO mutants

The *E. coli* strain BL21(DE3)(pMM4) (or JM109(DE3)(pET-22b) was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. A 250 μL aliquot of pre-culture was used to inoculate 25 mL of LB-Ampicillin medium supplemented with 20% glucose in a 125 mL baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, until OD_{600} was approximately 0.3-0.4. IPTG stock solution (200 mg/mL) was added (0.1 μL per mL of medium) and the flask was shaken for another 30 minutes at 24 °C. Then 10 mg of substrate was added along with cyclodextrin where necessary to alleviate solubility or toxicity problems. The culture was shaken at 24 °C, 250 rpm, and monitored by GC analysis until reaction was complete.

6-5-2 Biotransformations under non-growing conditions with *E. coli*/CHMO and *E. coli*/CHMO mutants

The *E-coli* strains BL21(DE3)(pMM4), JM109(DE3)(pET-22b(+)), or BL21(DE3)(pET-22b(+)) expressing cyclohexanone or cyclopentanone monooxygenase (WT or mutant) were streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. This culture was used to inoculate 500 mL of LB-Ampicillin supplemented with 4 g/L of glucose in a 1L baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm until OD_{600} was approximately 0.3-0.4. IPTG stock solution (200 mg/mL) was added to a concentration of 0.1 mM and the flask was shaken at 30 °C until the cells entered the stationary phase of growth (detectable when change in OD_{600} increased by less then 0.5 AU over a 30 minute time period). The cells were then harvested by centrifugation, and the cell pellet was re-suspended in 125 mL of a nitrogen-free minimal salts medium. For the biotransformation reactions, 25 mL of the re-suspended cell culture in M9 medium (lacking reduced nitrogen) was pipetted into a 125 mL baffled Erlenmeyer flask. To this was added 10 mg of substrate (1 equivalent of hydroxypropyl β-cyclodextrin was added in the cases of **6-1a**, **6-1b**, and **6-1f** to alleviate solubility problems) and the reaction was shaken at 24 °C, 250 rpm and monitored by GC analysis until reaction was complete, with 20 % glucose added as required.

6-5-3 Normalization experiments

6-5-3a Cell biomass formation under growing conditions with *E. coli*/CHMO, *E. coli*/CHMO mutant, *E. coli*/CPMO, and *E. coli*/CPMO mutant

The *E. coli* strain expressing one of the proteins listed in the title was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. A 250 μL aliquot of pre-culture was used to inoculate 25 mL of an LB-Ampicillin medium reaction culture supplemented with 20% glucose in a 125 mL baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, until OD_{600} was approximately 0.3-0.4. IPTG stock solution (200 mg/mL) was added (0.1 μL per mL of medium) and the flask was shaken for 24 hours. After 24 hours the cells were removed from the medium *via* centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 3 mL of water. The re-suspended cell biomass was transferred to pre-weighted 1.7 mL micro centrifuge tubes. The cells were then spun at 8500 rpm for 10 minutes, the supernatant was removed, and the tubes containing the isolated cell were weighed and their biomass recorded. The results obtained for the growing cells are given in **Table 6-3**.

Table 6-3: *Growing conditions.*[a]

BVMO variant	Biomass (mg)			Average biomass (mg)
	1	2	3	
WT-CHMO	192.9	191.9	192.5	192.4
1-K2-F5	213.8	203.6	208.4	208.6
WT-CPMO	188.3	185.1	186.0	186.5
B1-A10	169.1	165.6	168.3	167.7

[a] All normalization reactions carried out for 24 h.

6-5-3b Cell biomass formation under non-growing conditions with *E. coli*/CHMO, *E. coli*/CHMO mutant, *E. coli*/CPMO, and *E. coli*/CPMO mutant

The *E. coli* strain expressing one of the proteins listed in the title was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1-2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. This culture was used to inoculate 500 mL of LB-Ampicillin supplemented with 4 g/L of glucose in a 1L baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm until OD_{600} was approximately 0.3-0.4. IPTG stock solution (200 mg/mL) was added to a concentration of 0.1 mM and the flask was shaken at 30 °C until the cells entered the stationary phase of growth (detectable when change in OD_{600} increased by less then 0.5 AU over a 30 minute time period). The cells were then harvested by centrifugation, and the cell pellet was re-suspended in 125 mL of a nitrogen-free minimal salts medium. For the normalization experiments, 25 mL of the re-suspended cell culture in M9 minimal medium was pipetted into a 125 mL baffled Erlenmeyer flask, and the cells were shaken at 24 °C, 250 rpm until a predetermined length of time for each biotransformation had elapsed (indicated in Table 2). The cells were removed from the reaction culture *via* centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 3 mL of water. The re-suspended cell biomass was transferred to pre-weighed 1.7 mL micro centrifuge tubes. The cells were then spun at 8500 rpm for 10 minutes, the supernatant was removed, and the tubes containing the isolated cell were weighed and

their biomass recorded. The results obtained for the non-growing cells are shown in **Table 6-4**.

Table 6-4: *Non-growing conditions.*

BVMO variant	Biomass (mg)			Average biomass (mg)
	1	2	3	
WT-CHMO (24 h)	216.1	230.8	211.2	219.4
1-K2-F5 (24 h)	213.2	196.2	203.1	204.2
WT-CPMO (4 h)	172.4	172.6	171.9	172.3
B1-A10 (9 h)	167.0	164.6	165.4	165.7

The biomass of the cells used under non-growing conditions is very close to the biomass of the corresponding cells produced under growing conditions. The only exception is in the case of WT-CHMO where the quantity formed under growing conditions is consistently slightly smaller.

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Chapter 7

Conclusions and Future Work

As part of the principal goal involving the development of BVMO enzymes as useful biocatalysts in organic synthesis, the CHMO was subjected to genetic enhancement in a process known as directed evolution. A series of CHMO mutants derived from the directed evolutionary experiments were screened for their ability to oxidize a number of 4-mono- and 4,4-disubstituted cyclohexanone substrates with enhanced enantioselectivity. The screening of the libreries identified mutants with enhanced enantioselectivity and the braod substrate base of the parent CHMO. In particular, one CHMO mutant, the I-K2-F5 which carries a single amino acid exchange (Phe432Ser) was found to be more robust than wild type CHMO as well as generally more selective towards a number of substrate targets. It represents a promising candidate for further broad based substrate evolution as well as for development of large scale fermentation conditions.

The information which was gained from the initial directed evolution of CHMO has been applied towards the rational improvement of CPMO, which in early studies has been found to be a generally unselective oxidation catalyst. Homology based modeling was used to generate a structural model for the CHMO and CPMO enzymes based on a newly crystallized BVMO, namely PAMO. Superposing of the CHMO and CPMO enzymes was used to identify the location of the CHMO "hot-spots" in the closely related CPMO enzyme. Two positions, the Gly449 and Phe156 were identified as promising targets for focused mutagenesis. To generate the two mutagenic libraries, namely library A at positions Gly449 and Phe450 and library B corresponding to positions Phe156 and Gly157, a novel strategy known as CAST was applied. Generation and screening of the

two CPMO mutant libraries for improved selectivities towards the oxidation of the 4-acetoxycyclohexanone and 4-methylcyclohexanone identified several CPMO mutants possessing enhanced selectivities.

Further screening of selected CPMO variants from both libraries established that the enhancement in selectivity is not restricted to the test substrates. Mutants from the library B generally possessed better selectivities than those from the library A. In particular a CPMO mutant from the library B that carries a double mutation (PheGly→LeuPhe) was found to be an excellent biocatalyst. An analysis of the CPMO homology model coupled with the experimental information reveals that the location of the library A mutations (Gly449Phe450) was not the ideal choice. One of the residues of this mutational pair (the Phe450) has its side chain pointing away from the putative active site of CPMO which potentially limits the benefits of the mutation at this position. Based on the experience gained from this first round of mutations, a more promising position for mutation may be the Ala448Gly449 pair.

The results from the directed evolution of CHMO provided the base for a model that provides a plausible explanation for the enhanced enantioselectivity of the Phe432Ser mutation towards the 4-hydroxycyclohexanone substrate. This model, coupled with homology generated structures, was used to understand both the low enantioselectivity of WT-CPMO as well as the generally high *S*-selectivity observed for the CHMO. Overall, the results obtained from these biocatalyst engineering studies, when linked with the diamond lattice model, provide critical information about the key regions within the active sites of CHMO and CPMO, the regions that play a role in control of stereoselectivity.

Despite the CHMOs well studied characteristics many questions remain unanswered with respect to this enzyme's structure both in pre- and post-catalysis states. It has been suggested that BVMO enzymes undergo large conformational changes during the catalytic event in order to accommodate both the NADPH cofactor and the substrate within the active site.[1] One experimental method which offers the potential to study the dynamic nature of BVMOs is protein NMR.[2] Through the application of advanced Heteronuclear Multidimensional Quadrupole Coupling (HMQC) NMR experiments and the development of transverse relaxation optimized spectroscopy (TROSY) it is now possible to study larger proteins.[3] Using NMR, the dynamic behaviour of the protein's backbone can be observed allowing for the determination of dynamic events occuring during catalysis. Comparing the CHMO and CPMO mutants with their wild type analogues the influence of specific mutations, both near and remote, may be determined. Studies on the dynamic processes which occur in BVMOs would greatly improve our knowledge of not only BVMOs, but also help chemists and biochemists better understand some of the fundamental forces at working during enzyme catalysis.

Contining screening of the wild type CPMO towards the oxidation of 2- and 3-substituted cyclohexones, as well as substituted cyclopentanone ring systems, should be undertaken to assess the selectivities which the newly generated CHMO and CPMO mutants may possess towards these substrates. Any information gained in these experiments would help to refine the CHMO and CPMO models described in this thesis and help to increase understand of the influences of the mutations identified. More screening data will further aid modeling and molecular docking experiments and further refine our understanding of the orientation of the substrate in the active site of the these

and related enzymes. Overall the data collected in this work has significantly increased our understanding of the enantioselectivity of the CHMO and CPMO enzymes.

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Appendix I

General Experimental Conditions

General Instrumentation

Specific rotations $[\alpha]^{25}_{D}$ were measured on a Perkin-Elmer 241 polarimeter operating at room temperature with an Na 589 energy source. IR spectra were recorded from thin films on a Mattson Satellite FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution, at room temperature on either a Varian Unity 500 FT-NMR or Bruker AC-250 FT-NMR spectrometer. Chemical shifts (δ) are reported in ppm using TMS (Me_4Si) as internal standard. *J* values are expressed in Hz. Capillary gas chromatography was performed on a Hewlett Packard 5890 instrument employing a DB-1301 (15 m x 0.53 mm x 1.0 μm) column (J &W Scientific), and a Shimadzu GC-9A employing either a β-Dex225 (30 m x 0.25 mm x 0.25 μm) or β-Dex325 (30 m x 0.25 mm x 0.25 μm) chiral phase column (Supelco). Both GC instruments used a flame ionization detector (FID) and helium as the carrier gas. Thin-layer chromatography was performed on pre-coated aluminum backed silica gel 60 plates (SiliCycle). Flash chromatography was performed on silica gel (240-400 mesh, SiliCycle).

An MDF-U4086S ultra-low temperature freezer (SANYO Electric Co., Ltd) operating at -82°C was used to store *E. coli* strains. Cell culturing was performed on either an Innova 4000 incubator orbital shaker (New Brunswick Scientific) or a VWR orbital shaker (VWR Scientific). A Biofuge 13 microcentrifuge (Baxter CanLab) and a genie-2 Vortexer (VWR Scientific) were used in the preparation of samples for analysis. A CT 4 22 centrifuge (Jouan) employing 100 mL and 500 mL buckets was used to

remove cells after reactions and for the harvesting of cells. A Tgradient thermocycler (Biometra, Ltd) was used for all PCR reactions. All gel electrophoresis analysis was performed using a Minigel-Twin system (Biometra, Ltd) with a Power Pac 300 power supply (Bio-Rad Laboratories). The gels were visualized using a BioDocAnalysis chamber (Biometra, Ltd). Optical density was measured on either a Spectronic 20D spectrophotometer (Milton Roy Co.) or a UV-mini 1240 UV-Vis spectrophotometer (Shimazdu). A Büchi Rotavapor R-200 was used to evaporate the solvents.

Treatment of chemicals and solvents

All chemicals were purchased from commercial suppliers (Sigma-Aldrich, TCI America, Fisher Scientific, Fluka) and were used as received unless otherwise indicated. THF and ethyl ether were refluxed with and distilled over sodium metal in the presence of benzophenone under an argon atmosphere. Methylene chloride was distilled over calcium hydride and stored over molecular sieves (4 Å). Ethyl acetate and hexane were purified by fractional distillation.

Table I-1: *Typical operating parameters for GC HP-5890.*

Chromotograph:	
Carrier gas	UHP-helium 60mL/min
Oven temperature: program 1	initial temp.: 100°C initial time: 4 min final temp: 180 °C final time: 10 min rate: 10°C/min
Oven temperature: program 2	initial temp.: 200°C initial time: 4 min final temp: 220 °C final time: 15 min rate: 10°C/min
Injector temperature	250°C
Detector temperature	300°C
Range	2
Attenuation	0
Recorder:	
Attenuation	2
CHT SP	0.4
AR REJ	1000
Threshold	0
Peak Width	0.10

Table I-2: *Typical operating parameters for the Shimadzu GC-9A.*

Chromotograph:

Carrier gas	UHP-helium 50mL/min
Oven temperature: program 1	initial temp.: 100°C initial time: 2 min final temp: 180 °C final time: 15 min rate: 6°C/min
Oven temperature: program 2	initial temp.: 145°C (isotherm) time: 45 min
Injector temperature	225°C
Detector temperature	225°C
Range	10^2

Recorder:

Width	10	Slope	15
Drift	0	Min. Area	100
T. DBL	0	Stop.TM	32
Attenuation	2	Speed	2
Method	441	FORMAT	0
SPL. WT	100	IS.WT	1

Appendix II

Protocol for Maintenance of *E. coli* Strains

Standard LB media for routine growth and maintenance of *E. Coli* strains were used. Recipe per litre: Bacto Tyrptone : 10 g, Yeast Extract: 5 g, NaCl: 10 g along with 30 mg/L of ampicillin. For solid media, agar (15 g) was added to the aforementioned media. For short-term storage, the strains were kept on an agar plate sealed with parafilm at 4 °C (up to a maximum of 1 week). For long-term storage, the strains were stored at -82 °C in LB-ampicillin medium containing 15% glycerol.

Fresh plates of the engineered *E. coli* strains were streaked from frozen stock and incubated at 30 °C until the colonies ranged in size from 1-2 mm. A single colony was picked and used to inoculate 10 mL of LB-ampicillin medium in a 25 mL flask. The culture was incubated at 30 °C on an orbital shaker at 200 rpm overnight (14-16 h). 1.5 mL of sterile glycerol was added to the culture and the cells were frozen in aliquots at -82 °C for later use.

Appendix III

Procedures

$KMnO_4$ TLC dip reagent:

$KMnO_4$	1.5 g
K_2CO_3	10 g
NaOH (5%)	2.5 mL
H_2O	150 mL

Vanillin TLC dip reagent:

Vanillin	5 g
Methanol	100 mL
H_2O	200 mL
H_2SO_4 (conc.)	0.5 mL

***p*-anisaldehyde TLC dip reagent**

p-anisaldehyde	3.5 mL
H_2SO_4 (conc.)	5.0 mL
CH_3COOH (glacial)	1.5 mL
Ethanol	135 mL

Ampicillin stock solution (50 mg/mL)

Sodium ampicillin	2.5 g
Autoclaved H_2O	50 mL

Stored at 4 °C in an autoclaved bottle.

IPTG (1M)

Isopropylthio-β-D-galactoside	0.238 g
Autoclaved H_2O	1 mL

Sterile filtered through millipore filter and stored at -20 °C.

Luria-Bertani (LB) medium

Bacto Tyrptone	10 g
Bacto Yeast Extract	5 g
NaCl	10 g
Deionized H_2O	1 L

Autoclaved.

For LB-agar plates, 15 g/L of agar is added prior to autoclaving.

M9 minimal medium salts

Disodium Phosphate (Na_2HPO_4)	32 g
Monopotassium phosphate (KH_2PO_4)	7.5 g
NaCl	1.25 g
Ammonium Chloride (NH_4Cl)	2.25 g
Deionized H_2O	500 mL

Autoclave. Note: Ammonium chloride is omitted from media used for reactions under non-growing conditions.

M9 Minimal medium

M9 minimal medium salts	200 mL
Sterile $MgSO_4$ (1M)	2 mL
Sterile $CaCl_2$ (1M)	0.1 mL
Sterile Glucose (20%)	20 mL

Adjust volume to 1000 mL.

Appendix IV

E-value (enantiomeric ratio)

The E value reflects the relative rate at which the two enantiomers are converted to the products and may be calculated from the following equations:

$$E = \frac{\ln[(1-c)(1 - e.e.(S))]}{\ln[(1-c)(1 + e.e.(S))]} = \frac{\ln[(1-c)(1 + e.e.(P))]}{\ln[(1-c)(1 - e.e.(P))]}$$ ..... **Sih's equation**

e.e.(S) = enantiomeric excess of the substrate
e.e.(P) = enantiomeric excess of the product
c = the extent of conversion of the racemate

An easy to use computer program has been published on the internet (http://www.orgc.tu-graz.ac.at/) the allows one to calculate the E-values (for both Mac and PC platforms).

Appendix V :
Selected GC and NMR Spectra of Representative Compounds missing from thesis

Vita

Missing from thesis